Inhibitory effect of flavonoids against NS2B-NS3 protease of ZIKA virus and their structure activity relationship

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Abstract

Objectives To determine the inhibitory activities of flavonoids against NS2B-NS3 protease of ZIKA virus (ZIKV NS2B-NS3^{pro}) expressed in *Escherichia coli* BL21 (DE3) and their structure activity relationship. **Results** ZIKV NS2B-NS3^{pro} was expressed in *E. coli* BL21(DE3) as a 35 kDa protein. It had a K_m of 26 μ M with the fluorogenic peptide Dabcyl-KTSAVLQS GFRKME-Edan. The purified ZIKV NS2B-NS3^{pro} was used for inhibition and kinetic assays to determine the activities of 22 polyphenol compounds. These

polyphenol compounds at 100 μ M inhibited the activity of ZIKV NS2B-NS3^{pro} by 6.2–88%. Seven polyphenol compounds had IC_{50} ranging from 22 ± 0.2 to 112 ± 5.5 μ M. Myricetin showed a mixed type inhibitory pattern against ZIKV NS2B-NS3^{pro} protease. Its IC_{50} value was 22 ± 0.2 μ M with a K_i value of 8.9 ± 1.9 μ M.

Conclusion The chemical structure of a polyphenol compound and its inhibitory activity against ZIKV NS2B-NS3^{pro} can be explored to develop highly selective inhibitors against ZIKV NS2B-NS3^{pro}.

Hee-jung Lim and Thi Thanh Hanh Nguyen contributed equally to this work.

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Introduction

Zika virus (ZIKV) belongs to family Flaviviridae. Dengue, West Nile, yellow fever, and Japanese encephalitis viruses transmitted to humans by mosquitoes in genus *Aedes* all belong to family Flaviviridae. ZIKV was first isolated from a sentinel monkey in 1947 (Faye et al. 2014). It is responsible for an unprecedented epidemic currently occurring in Brazil and America (Campos et al. 2015). Before this outbreak, ZIKV was not viewed as an important pathogen because the majority of its infections are asymptomatic (Duffy et al. 2009). However, there is now growing evidence showing that ZIKV infections

might be linked to fetal and newborn microcephaly (ECDC 2016; WHO 2016) with serious neurological complications such as Guillain–Barré syndrome (GBS) (Petersen et al. 2016). Moreover, ZIKV intersects with the placenta and causes microcephaly by targeting cortical progenitor cells, inducing cell death caused by apoptosis and autophagy and impairing neurodevelopment (Cugola et al. 2016; Li et al. 2016). The increases of Guillain–Barré syndrome and microcephaly associated with ZIKA infection have led the World Health Organization (WHO) to declare ZIKV infection as a global public health emergency in February of 2016 (ECDC 2016; WHO 2016). To date, no vaccine or therapeutic has been clinically approved for preventing or controlling ZIKV infection.

ZIKV has a single positive sense RNA genome of approx. 11 kb. It is initially translated as a single polyprotein (Kuno and Chang 2007) and then post-translationally cleaved into three structural proteins: capsid (C), premembrane/membrane (prM), and envelope (E) as well as nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Baronti et al. 2014). NS3 protein of ZIKV possesses putative protease activity at its N-terminus and putative ATPase/helicase, nucleoside triphosphatase, and 5'-triphosphatase activities at its C-terminus (Zhu et al. 2016). The polyprotein is cleaved co-translationally and post-translationally by cellular proteases of furin-type or Golgi-localized proteases. The viral serine protease is embedded in the N-terminal domain of NS3 (NS3^{pro}) (Bollati et al. 2010). Since NS3 is essential to life cycle of ZIKV, it is an attractive target for the development of antiviral drugs (Lei et al. 2016).

Polyphenols are secondary metabolites found abundantly in a wide variety of food such as fruits, vegetables, herbs, seeds, cereals, and beverages such as coffee, tea, cocoa, and wine (Vinson et al. 2001). Polyphenols possess antiviral activities against influenza virus (A/H1N1, A/H3N2 and B virus) (Liu et al. 2008), severe acute respiratory syndrome coronavirus (SARS-CoV) (Nguyen et al. 2012), and dengue fever virus (Zandi et al. 2011). However, there has been no report on the inhibitory activity or structure–activity relationship of polyphenols against NS2B-NS3^{pro} of ZIKV. Therefore, we expressed NS2B-NS3^{pro} from ZIKV in *Escherichia coli* and studied the inhibitory activities of 22 polyphenol compounds belonging to four groups of flavonols, flavanols, flavones, and

flavanones against ZIKV NS2B-NS3^{pro} and their structure–activity relationship.

Materials and methods

Preparation of ZIKV NS2B-NS3^{pro}

ZIKV NS2B-NS3^{pro} gene was synthesized after codon optimization (Genscript, Piscataway, NJ, USA) for expression in *E. coli* based on amino acid sequence of NS2B-NS3^{pro} (GenBank Accession No. ALU33341.1) (Cunha et al. 2016). It was inserted into pET28a vector (Novagen, Darmstadt, Germany) (pET28a-NS2B-NS3^{pro}) for the expression of NS2B-NS3^{pro} enzyme with poly-histidine tags in both N-terminal and C-terminal ends. The protein encoding NS2B-NS3^{pro} comprised of 49 NS2B amino acid residues (amino acid residues 1421–1469) were linked by a flexible GGGSGGGG linker with 186 NS3^{pro} amino acid residues (amino acid residues 1503–1688) (Supplementary Fig. 1). pET28a-NS2B-NS3^{pro} was transformed into *E. coli* BL21(DE3) which was then grown in LB supplemented with kanamycin (50 µg ml⁻¹) at 37 °C. Cells were induced with 0.5 mM IPTG when the OD₆₀₀ reached 0.5. Induced cells were kept at 16 °C for 12 h at 200 rpm. Cells were collected by centrifugation (8000×g for 30 min at 4 °C), resuspended in 50 mM Tris/HCl (pH 7.0), and lysed via sonication. After centrifugation (12,000×g for 30 min), the cell lysate was loaded onto 8 ml Ni-agarose resin. Proteins were eluted from the column with elution buffer (50 mM Tris/HCl, 30 mM NaCl, 500 mM imidazole, pH 8). Fractions containing pure protein were pooled, concentrated, and dialyzed against 50 mM Tris/HCl (pH 7). The concentration of protein was determined using the Bio-Rad protein assay. Purified protein was confirmed by 12% SDS-PAGE.

Enzyme activity

Proteolytic activity of NS2B-NS3^{pro} was measured using a fluorescence resonance energy transfer (FRET)-based assay. Substrate was labeled by 5-[(2'-aminoethyl)-amino]naphthalenesulfonic acid (Edans) and 4-[[4-(dimethylamino)phenyl]azo] benzoic acid (Dabcyl) as energy transfer pair (Bachem, Switzerland).

Fig. 1 SDS-PAGE and Lineweaver–Burk plot of the purified ZIKV NS2B-NS3^{pro} expressed in *E. coli* BL21(DE3). **a** SDS-PAGE. Lane M the molecular mass marker; lane 1 cell lysate with 0.5 mM IPTG induction to overexpress of ZIKV NS2B-

NS3^{pro}; lane 2 purified ZIKV NS2B-NS3^{pro} after Ni-NTA column chromatography. **b** Lineweaver–Burk plot of ZIKV NS2B-NS3^{pro} with substrate from 2.5 to 40 μ M after 11 min of incubation

Fluorogenic peptide Dabcyl-KTSAVLQSGFRKME-Edans was used as substrate. Enhanced fluorescence due to cleavage of this substrate catalyzed by protease was monitored at 538 nm with excitation wavelength at 355 nm using a fluorescence plate reader (Nguyen et al. 2012). The reaction mixture containing 26 μ M FRET substrate and 8.7 μ g enzyme in 50 mM Tris/HCl (pH 7) was incubated at 25 °C with continuous monitoring of fluorescence for 11 min. Relative fluorescence units (RFUs) were recorded with SpectraMax M3 (Molecular Devices, USA) with excitation wavelength at 355 nm and fluorescence emission wavelength at 538 nm.

The effect of pH on ZIKV NS2B-NS3^{pro} enzyme activity was studied from (pH 3–11) using 50 mM of the following buffers: glycine/HCl (pH 3), sodium acetate buffer (pH 5–6.5), Tris/buffer (pH 7–9), and glycine/NaOH buffer (pH 9.5–11). The optimum pH was determined by incubating ZIKV NS2B-NS3^{pro} enzyme in each buffer for 15 min using 26 μ M FRET as substrate.

Kinetic parameter of NS2B-NS3^{pro} was obtained using substrate from 2.5 to 40 μ M in the fluorescent assay after 11 min incubation. Reaction responses were linear within this time. Michaelis–Menten constant (K_m) was calculated from the Lineweaver–Burk plot using Sigmaplot program (Systat Software, USA).

Inhibition assay

Epigallocatechin gallate (EGCG) and astragalin were obtained from Amore Pacific (Yongin, South Korea). EGCG glucosides were prepared as described

previously (Moon et al. 2006). Rutin and ampelopsin were purchased from Acros Organics (NJ, USA) and ZR Chemicals (Shanghai, China), respectively. Others chemicals were purchased from Sigma. For initial screening, each compound was either dissolved in dimethyl sulfoxide (DMSO) or distilled water to obtain 10 mM of stock solution. The reaction mixture contained 26 μ M FRET substrate, 8.7 μ g enzyme, and 100 μ M inhibitor in 50 mM Tris/HCl (pH 7.0). The reaction was run at 25 °C for 11 min. Inhibition activity was calculated using the following Eq. (1):

$$\text{Inhibition activity (\%)} = 100 - \left[\frac{(S - S_0)}{(C - C_0)} \right] \times 100 \quad (1)$$

where C is the RFUs of the control (enzyme, buffer, and substrate) after 11 min of reaction, C_0 is the RFUs of the control at time zero, S is the RFUs of the test sample (enzyme, inhibitor, buffer, and substrate) after 11 min of reaction, and S_0 is the RFUs of the tested samples at time zero.

The 50% inhibitory concentration (IC_{50}) was defined as the concentration of NS2B-NS3^{pro} inhibitor necessary to reduce NS2B-NS3^{pro} activity by 50% relative to a reaction mixture containing NS2B-NS3^{pro} enzyme without any inhibitor. Inhibitor kinetic studies were performed for myricetin at various inhibitor concentrations (0–50 μ M) with various substrate concentrations (10–50 μ M). The inhibition type was determined using Lineweaver–Burk and Dixon plot (1/v as a function of inhibitor concentration [I]). Inhibition constant (K_i) was calculated using Sigmaplot program.

Results and discussion

Expression of active ZIKV NS2B-NS3^{pro}

Similar to other flavivirus proteases such as those of dengue virus (DENV) and West Nile virus (WNV), the mature form of ZIKV protease consists of the N-terminal domain of NS3 which carries the catalytic triad Ser¹³⁵-His⁵¹-Asp⁷⁵. It requires the central hydrophilic domain of NS2B as a co-factor for enzymatic activity (Falgout et al. 1991; Lei et al. 2016). Its cleavage sites have common Lys-Arg, Arg-Arg, Arg-Lys, or Gln-Arg motifs (Bessaud et al. 2006; Lei et al. 2016). ZIKV NS2B-NS3^{pro} was designed to compose 49 NS2B amino acid residues (amino acid residues 1421–1469) linked by a flexible GGGGSGGGG linker with 186 NS3^{pro} amino acid residues (amino acid residues 1503–1688) (Supplementary Fig. 1). It was cloned and expressed in *E. coli* BL21(DE3). The purification of recombinant ZIKV NS2B-NS3^{pro} was achieved in a single Ni-agarose chromatography with 21-fold purification. SDS-PAGE analyses revealed a protein band of approximately 35 kDa (Fig. 1a) with over 80% in purity.

ZIKV NS2B-NS3^{pro} proteins cleaved the fluorogenic peptide Dabcyl-KTSAVLQSGFRKME-Edans with $\lambda_{\text{ex}} = 355$ nm and $\lambda_{\text{em}} = 538$ nm at 25 °C in 50 mM Tris/HCl (pH 7) (Supplementary Fig. 2). The maximum activity of the purified ZIKV NS2B-NS3^{pro} was obtained at pH 7 (Supplementary Fig. 2). ZIKV NS2B-NS3^{pro} showed over 80% activity at pH of 6.5–9.5 (Supplementary Fig. 2).

Michaelis–Menten kinetic and Lineweaver–Burk plot for the determination of K_m are shown in Fig. 1b. The K_m of ZIKV NS2B-NS3^{pro} was 26 μM .

Inhibitory activities of polyphenol compounds against ZIKV NS2B-NS3^{pro}

Polyphenols are natural compounds ubiquitously found in plants (Vinson et al. 2001). Polyphenols fisetin, quercetin, anringtonin, and rutin can inhibit dengue virus, a member of the family Flaviviridae that also contains ZIKV (Zandi et al. 2011). Therefore, in this study, 18 flavonoid compounds belonging to four groups of flavonoids (flavonol, 6 compounds; flavanol, 8 compounds; flavone, 2 compounds; flavanone, 2 compounds) and four non-flavonoid type phenolic compounds (pyrogallol, pyrocatechol, caffeic acid,

Fig. 2 Structures of flavonoid inhibitors and others. 1 Luteolin; 2 chrysin; 3 myricetin; 4 quercetin; 5 ampelopsin; 6 astragalgin; 7 rutin; 8 icaritin; 9 hesperidin; 10 naringin; 11 epigallocatechin gallate (EGCG); 12 epicatechin gallate (ECG); 13 gallicocatechin gallate (GCG); 14 catechin gallate (CG); epicatechin (EC); 15: EGCG-7-*O*- α -glucopyranoside; 16 EGCG-4'-*O*- α -glucopyranoside; 17 epigallocatechin (EGC); 18 catechin; 19 pyrogallol; 20 pyrocatechol; 21 caffeine; 22 gallic acid

and gallic acid) were selected to test their inhibitory activities against ZIKV NS2B-NS3^{pro}. The structures of these compounds are shown in Fig. 2. Results on the inhibitory activities of these polyphenol compounds at 100 μM against NS2B-NS3^{pro} are shown in Table 1. Among these tested compounds, myricetin, astragalgin, rutin, epigallocatechin gallate, epicatechin gallate, gallicocatechin gallate, and luteolin at 100 μM inhibited more than 40% of the activities of ZIKV NS2B-NS3^{pro}. Therefore, they were selected for the determination of IC₅₀ values. Results are shown in Table 1. The IC₅₀ of myricetin, astragalgin, rutin, EGCG, epicatechin gallate (ECG), gallicocatechin gallate (GCG), and luteolin were 22 ± 0.2 , 112 ± 5.5 , 105 ± 2.9 , 87 ± 1.2 , 89 ± 1.6 , 99 ± 1.8 , and 53 ± 1.3 μM , respectively (Table 1).

Among the tested compounds, myricetin showed the strongest inhibitory activity against ZIKV NS2B-NS3^{pro}. Therefore, it was subjected to kinetic characterization. Inhibitory kinetic experiments were performed with myricetin from 0 to 50 μM with the substrate from 10 to 50 μM . Lineweaver–Burk and Dixon plots were used to analyze the inhibition modes of these compounds. Results are shown in Supplementary Fig. 3. The slopes of these lines confirmed that myricetin was a mixed type of inhibitor against NS2B-NS3^{pro} (Supplementary Fig. 3). Based on linear regression analysis of the Dixon plot (Supplementary Fig. 3), the inhibitor constant (K_i) of myricetin was 8.9 ± 1.9 μM .

Relationship between chemical structures of inhibitor and their activities against ZIKV NS2B-NS3^{pro}

In flavone class of compounds, luteolin showed 2.8 times higher inhibition activity compared to chrysin presumably due to the presence of two OH groups at C3' and C5' in its B-ring. The order of inhibitory activities of the flavonol class of compounds at 100 μM was: myricetin < rutin < astragalgin < quercetin < ampelopsin < icaritin. Icaritin had lower inhibitory

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Table 1 Inhibitory activities of the 22 polyphenol compounds against NS2B-NS3^{pro}

Group	No.	Compound	Inhibition (%) ^c	IC ₅₀ (μM)
Flavone	1	Luteolin (D) ^a	54	53 ± 1.3
	2	Chrysin (D)	19	–
Flavonol	3	Myricetin (D)	88	22 ± 0.2
	4	Quercetin (D)	37	–
	5	Ampelopsin (D)	23	–
	6	Astragalin (D)	48	112 ± 5.5
	7	Rutin (D)	49	104 ± 2.9
	8	Icaritin (D)	19	–
Flavanone	9	Hesperidin (D)	7	–
	10	Naringin (D)	12.5	–
Flavanol	11	Epigallocatechin gallate (W) ^b	51.5	87 ± 1.2
	12	Epicatechin gallate (D)	52	89 ± 1.6
	13	Gallocatechin gallate(D)	52	99 ± 1.8
	14	Catechin gallate (D)	32	–
	15	EGCG-7- <i>O</i> -α-glucopyranoside (W)	25	–
	16	EGCG-4'- <i>O</i> -α-glucopyranoside (W)	23	–
	17	Epigallocatechin (D)	7	–
	18	Catechin (D)	7	–
	19	Pyrogallol (W)	11	–
	20	Pyrocatechol (W)	8	–
Other	21	Caffeine (W)	6	–
	22	Gallic acid (W)	15	–

The reaction mixture contained 26 μM FRET substrate, 8.7 μg of enzyme, and 100 μM inhibitor in 50 mM Tris/HCl (pH 7.0). The reaction was run at 25 °C for 11 min

– Not determined

^a Dissolved in DMSO

^b Dissolved in distilled water

^c Inhibition at 100 μM

activity compared to other compounds in this class. The methoxy group at C4' in the B-ring and the prenyl group at C8 position in the A-ring of icaritin might have contributed to its lower inhibitory activity against ZIKV NS2B-NS3^{pro}. Myricetin contains three OH groups on its B-ring. Ampelopsin has the same three OH groups at its B-ring but lacks the double bond between the C2 and C3 positions in its B-ring. The absence of the double bond in ampelopsin might have decreased its inhibitory activity against ZIKV NS2B-NS3^{pro}. This may be the primarily reason why ampelopsin had lower inhibitory activity than myricetin. Quercetin had lower inhibitory activity compared to myricetin, luteolin, and astragalin. Rutin having glycosylation at C3 position in the C-ring but no OH group at C3' in the B-ring had higher inhibitory activity than quercetin, although their inhibitory activities were lower than those of myricetin and luteolin.

These results suggest that the presence of OH group at C3', C4', and C5' in the B-ring is very important for the inhibitory activity of flavonols against ZIKV NS2B-NS3^{pro} while the absence of OH group or

glycosylation at C3 position in the C-ring of flavonols only has slight effect on its inhibitory activity against ZIKV NS2B-NS3^{pro}. Hesperidin and naringin belong to flavanones. They have a single bond between the C2 and C3 positions in the C-ring. They showed weak inhibitory activities against ZIKV NS2B-NS3^{pro}, indicating that bulky glycosylation at C7 in the A-ring can decrease their inhibition activities against ZIKV NS2B-NS3^{pro}. EGCG, EGCG-7-*O*-α-glucopyranoside, EGCG-4'-*O*-α-glucopyranoside, epigallocatechin, catechin gallate, catechin, ECG, and GCG belong to flavanol class of compounds. They lack the double bond (between the C2 and C3 positions) and C4=O in the C-ring. EGCG, EGCG-7-*O*-α-glucopyranoside, EGCG-4'-*O*-α-glucopyranoside, catechin gallate, ECG, and GCG contain galloyl moiety at the 3-OH position in the C-ring. They showed higher inhibitory activities against ZIKV NS2B-NS3^{pro} than epigallocatechin and catechin. EGCG, ECG, and GCG (epimer of EGCG) showed similar inhibition activities. There was no significant difference in inhibitory activity against ZIKV NS2B-NS3^{pro} between

epigallocatechin and catechin, indicating that the OH group at C5' in the B-ring does not affect their inhibitory activities. EGCG-7-*O*- α -glucopyranoside and EGCG-4'-*O*- α -glucopyranoside showed lower inhibitory activities against ZIKV NS2B-NS3^{pro} than EGCG due to glycosylation at C7 position in A-ring and C4' position in B-ring, respectively.

In summary, Of 22 polyphenol compounds tested against ZIKV NS2B-NS3^{pro}, seven had IC₅₀ values of 22 ± 0.2 to 112 µM. Based on structure–activity relationships of these compounds against ZIKV NS2B-NS3^{pro}, the double bonds between C2 and C3 in the B-ring, OH groups at C7 in the A-ring, C2 in the C-ring, C3', C4', and C5' in the B-ring, and galloyl moiety at C3 in the C-ring all played important roles in the inhibition activities against ZIKV NS2B-NS3^{pro}. These relationships between chemical structures and inhibitory activities against ZIKV NS2B-NS3^{pro} can be further explored to develop highly selective inhibitors against ZIKV NS2B-NS3^{pro}.

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Supporting information Supplementary Fig. 1—Nucleotide sequence of the ZIKV NS2B-NS3^{pro} gene. The codon optimized gene was synthesized based on amino acid sequence deposited at GenBank (accession No. ALU33341.1).

Supplementary Fig. 2—Effect of pH on ZIKV NS2B-NS3^{pro} activity.

Supplementary Fig. 3—Dixon plot analysis of the inhibition of NS2B-NS3^{pro} by myricetin (●: 10 µM; ○: 15 µM; ▼: 25 µM; △: 50 µM).

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