

Study the antiviral activity of some derivatives of tetracycline and non-steroid anti inflammatory drugs towards dengue virus

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Abstract. Various clinical symptoms are caused by dengue virus ranging from mild fever to severe hemorrhagic fever while there is no successful anti-dengue therapeutics available. Among different strategies towards identifying and developing anti-dengue therapeutics, testing anti-dengue properties of known drugs could represent an efficient strategy for which information of its medical approval, toxicity and side effects is readily available. In this study, we evaluated the antiviral activity of some medical compounds towards dengue NS2B-NS3 protease (DENV2 NS2B-NS3pro) as a target to inhibit dengue virus replication. Mefenamic acid, a non-steroid anti inflammatory drug and doxycycline, a derivative antibiotic of tetracycline both showed significant inhibition potential against DENV2 NS2B-NS3pro *K_i* values $32 \pm 2 \mu\text{M}$ and $55 \pm 5 \mu\text{M}$ respectively. The effective cytotoxic concentrations of 50% (CC₅₀) against Vero cells were evaluated for mefenamic acid ($150 \pm 5 \mu\text{M}$) and doxycycline ($125 \pm 4 \mu\text{M}$). Concentrations lower than CC₅₀ were used to test the inhibition potential of these compounds against DENV2 replication in Vero cells. The results showed significant reduction in viral load after applying mefenamic acid and doxycycline in concentration-dependent manner. Mefenamic acid reduced viral RNA at EC₅₀ of $32 \pm 4 \mu\text{M}$ whilst doxycycline EC₅₀ was $40 \pm 3 \mu\text{M}$. Mefenamic acid showed higher selectivity against dengue virus replication *in vitro* compared to doxycycline. These findings underline the need for further experimental and clinical studies on these drugs utilizing its anti-dengue and anti-inflammatory activities to attenuate the clinical symptoms of dengue infection.

INTRODUCTION

Dengue virus is a member of the family of flaviviridae which includes West Nile virus (WNV), yellow fever virus (YFV) and Japanese encephalitis virus (JEV), Kunjin virus (KUNV), and tick-born encephalitis virus (TBEV) (Botting & Kuhan, 2012). The four serotypes of dengue virus (DENV1-4) infect millions of people each year worldwide and cause dengue fever (DF), severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Botting & Kuhan, 2012). Annually, there are approximately 0.5 million cases of DHF and DSS that lead to more than

20,000 deaths worldwide (Gubler, 2002). In Malaysia, a recent study showed that from 1000 subjects about 91.6% were found to be DENV1-4 seropositive indicating that DENV1-4 might be endemic in Malaysia for long time into the future (Muhammad *et al.*, 2011).

Dengue virus is a positive single-stranded RNA that is translated to polyprotein by the host cell's ribosome. Viral polyprotein is cleaved by viral and cellular proteases to 10 structural and non-structural proteins. Dengue NS3 protein possesses trypsin-like serine protease activity in its 180 amino acid N-terminal residues (Falgout *et al.*, 1991)

while the C-terminal region is responsible for RNA-helicase and RNA-stimulated NTPase activities (Wengler, 1993). The protease catalytic activity of NS3 protein depends on the interaction with its cofactor NS2B (47 amino acids) to form NS2B-NS3 protease (NS2B-NS3pro) (Yusof *et al.*, 2000). With the exception of C-prM which is a structural protein, NS2B-NS3pro functions by cleaving viral non-structural proteins at the protein junctions of NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 (Stocks & Lobigs, 1998). The disruption of NS2B-NS3pro function is lethal to viral replication (Geiss *et al.*, 2009). As such, this protein is considered as an effective target for antiviral drugs (Tomilnson *et al.*, 2009).

At present, there are no clinically available anti-dengue drugs or vaccines (Botting & Kuhan, 2012). Patients with dengue infection receive only medical care that is targeted towards reduction of symptoms. Given that developing and obtaining approval for the use of new medicinal agents is slow and expensive process, a more time- and cost- effective approach could be to investigate known drugs whose ADMET properties are already available. Antibiotics and anti-inflammatory drugs are commonly used to reduction the symptoms caused by microbial infections and the antiviral activity of drugs such as tetracycline derivatives (Yang *et al.*, 2007) and non-steroidal anti-inflammatory drugs (NSAIDs) (Inglot, 1969, 1983; Okamoto *et al.*, 2009) has been reported previously. Therefore, in this study the inhibitory effects of these derivatives were investigated *in vitro* using a dengue NS2B-NS3 protease assay and a virus inhibition assay.

MATERIALS AND METHODS

***In vitro* dengue protease assay (NS2B-NS3pro)**

Recombinant dengue-2 NS2B-NS3pro was produced in *Escherichia coli* as a single chain protein which contains 48 amino acids of NS2B and 185 amino acids of NS3 (Yusof *et al.*, 2000; Yon *et al.*, 2005). This assay was optimized using a fluorogenic peptide

substrate ((Boc-Gly-Arg-Arg-AMC) Osaka, Japan, Lot no: 580907) as previously described (Rothan *et al.*, 2012a,b). Three types of experiments were carried out simultaneously: enzyme and substrate using different combinations of the components; enzyme, different concentrations of inhibitor and substrate; and different concentrations inhibitor and substrate to eliminate the effect of the compound and DMSO on substrate cleavage.

The standard reaction mixture (200 μ l) was prepared in black 96-well plates containing 200 mM Tris-HCl (pH 8.5), 2 μ M recombinant NS2B-NS3pro, 5 mM NaCl, 15% glycerol, and the test compound (0.0, 50, 100, 200 μ M) prepared in DMSO. Incubation for 30 min at 37°C was performed before starting the reactions by adding 100 μ M of fluorogenic peptide substrate. Further incubation was performed for 30 min at 37°C and the release of free AMC was measured using an Infinite M200 Pro fluorescence spectrophotometer (Tecan Group Ltd., Switzerland). Substrate cleavage was normalized against buffer only (control) at the emission of 440 nm upon excitation at 350 nm. Fluorescence values obtained with the non-inhibitor control (0.0 μ M of the test compound) were taken as 100%, and those in the presence of inhibitors were calculated as the percentage of inhibition of the control using GraphPad Prism (version 5.01) software under non-linear regression. All assays were performed in triplicate and repeated twice. The inhibition percentage was calculated using the following formula:

$$\% \text{Inhibition} = (\text{Intensity of enzyme activity} - \text{intensity left after inhibition}) / \text{intensity of enzyme activity} \times 100.$$

Cell Viability

The MTT assay was used in this study to determine the maximal non-toxic dose (MNTD) and cytotoxic concentration showing 50% toxic effect (CC₅₀) of mefenamic acid, doxycycline and ribovirin as a positive control. Vero cells were cultured for 24 hrs on 96-well plates. Then, Dulbecco's modified Eagle's medium (DMEM) medium containing the compounds (0.0, 25, 50, 100, 200, 300, 400 μ M) was added and incubated for 24, 48 and 72 hrs. The compounds were dissolved in

DMSO and the final concentration of DMSO was less than 1% of the total volume in each well. Ten microlitre solutions of freshly prepared 5 mg/ml MTT in PBS was added to each well and allowed to incubate for an additional 2 hrs. Then, the media was removed and DMSO was added (100 µl/well). Plates were then swirled gently to facilitate formazan crystal solubilization. The absorbance was measured at 570 nm using a microplate reader (Tecan Infinite M200 Pro, Switzerland). Triplicate wells were analyzed for each concentration. Percentage of cell viability was calculated as follows:

$$100 - \left(\frac{\text{Absorbance of peptide treated cells}}{\text{Absorbance of untreated cells}} \right) \times 100.$$

Virus inhibition assay

Local DENV2 strain were propagated in C6/36 HT cells and titrated by plaque formation in Vero cells. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) was used as a growth medium or 2% FBS as a maintenance medium in the virus assays.

To investigate the inhibitory effect of the test compounds on DENV2 yield in Vero cells, the cells were grown in a 24-well plate (1.5×10^5 cells/well) and incubated for 24 hrs under optimal conditions (37°C and 5% CO₂). Virus supernatant was added to the wells (MOI of 2) followed by incubation for 1 hr with gentle shaking every 15 min for optimal virus to cell contact. Virus supernatant was removed, and the cells were washed twice with fresh serum free DMEM media to remove residual virus. New complete DMEM media was added without compounds and DENV2-infected cells were incubated at standard conditions for 24 hrs. Then, the DMEM media were removed and new media without compound (control) or containing different concentrations of mefenamic acid, doxycycline or ribovirin as a positive control was added and the infected cells were incubated for 72 hrs. To determine intracellular copy number of viral RNA after treatment with the compounds, Vero cells were collected and stored at -80°C for viral quantification using Real time-PCR.

Dengue virus quantification by Real time-PCR

The intracellular DENV2 RNA was quantified using RT-PCR. A standard curve was generated by 10-fold serial dilution of known copies of DENV2 RNA. Total RNA was extracted from the DENV2-infected cells using RNeasy H Plus Micro Kit (Qiagen, Germany) according to the manufacturer's instructions. A region of 100 bp located at the 5'UTR region of the virus genome was used as the target for PCR primer design. One-step RT-PCR using SyBr Green Master Kit (Qiagen, Germany) was used to conduct absolute quantification using an ABI7500 machine (Applied Biosystems, Foster City, CA). The PCR profile was 1 cycle of 50°C for 30 min, 1 cycle of 95°C for 15 min, 40 cycles of 0.15 min denaturation at 95°C, and 1 min annealing at 55°C with a single fluorescence emission measurement. Dissociation curve analysis was performed at the end of each run to confirm PCR primer specificity. Results were analyzed using the company's Sequence Detection Software Version 1.3.

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). *p* values < 0.05 were considered significant. Error bars are expressed as ± SD.

RESULTS

Inhibition of dengue NS2B-NS3 serine protease

The recombinant dengue protease (NS2B-NS3pro) consisted of 48 amino acids of NS2B, a 9 amino acid linker and 180 amino acids of NS3 (Fig. 1A) was produced as a recombinant enzyme in *E. coli* (Fig. 1B). The NS2B-NS3pro enzyme was produced as a soluble single chain peptide with molecular weight of 37 kDa (Fig. 1C). The activity of the recombinant NS2B-NS3pro was evaluated and optimized by measuring the fluorescence emission from cleaved fluorescence substrate using increased concentrations of the enzyme (Fig. 1D).

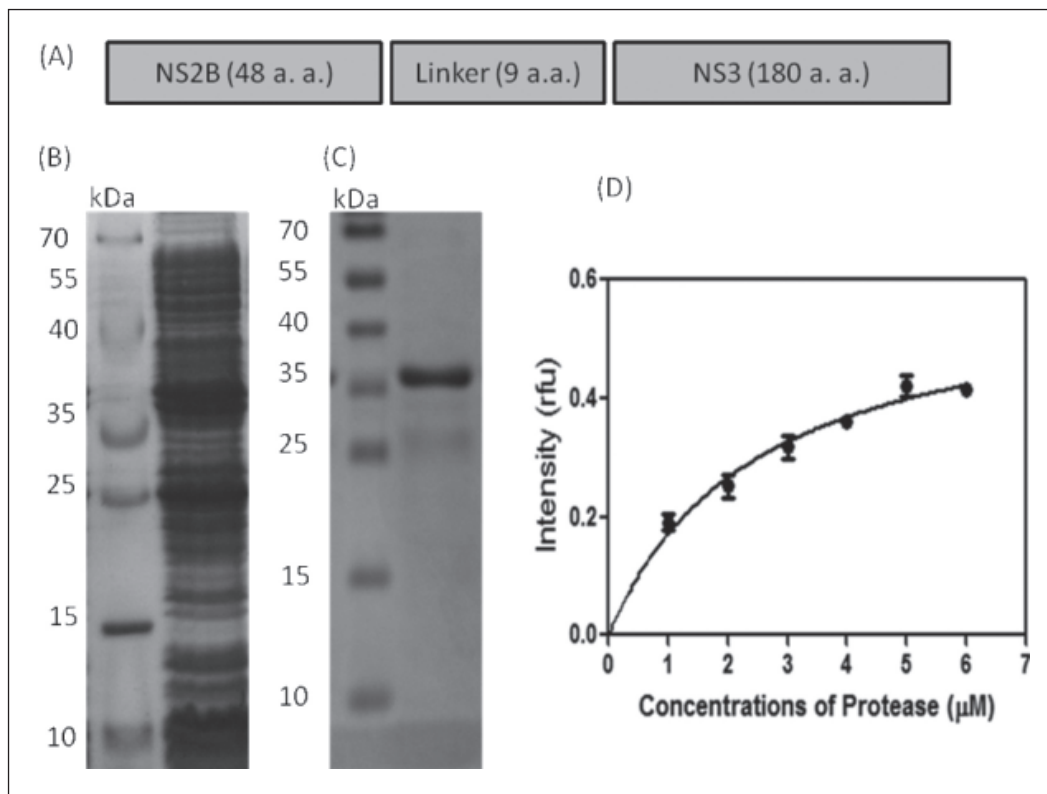


Figure 1. Production of dengue NS2B-NS3pro as a single peptide in *E. coli* (A) Construction of dengue NS2B-NS3pro by joining 48 amino acids of NS2B with 180 amino acids of NS3 by 9 amino acids. (B) *E. coli* proteins profile showing expression of NS2B-NS3pro at approximately 37 kDa. (C) Purified NS2B-NS3pro by Nickel column chromatograph. (D) Measuring and optimizing the activity of the recombinant NS2B-NS3pro in catalyzing the fluorescence substrate using increased concentrations of the enzyme

The data represented in table 1 showed that the test compounds inhibited dengue NS2B-NS3pro at different levels. Among the eight compounds, mefenamic acid and doxycycline showed the highest inhibitory effects against NS2B-NS3pro at 25 µM (37.4 ± 2.1 and 35.6 ± 2.6) and 100 µM (78.3 ± 5.1 and 53.8 ± 2.8) respectively.

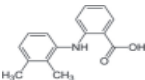
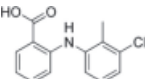
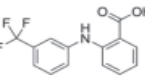
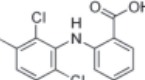
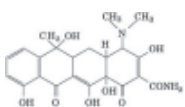
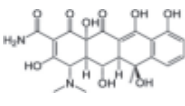
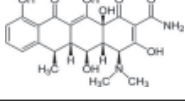
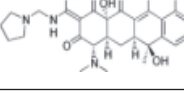
Further kinetic analysis was performed to confirm the ability of mefenamic acid and doxycycline to inhibit the NS2B-NS3pro enzyme at increased concentrations of the substrate. The values of K_m and V_{max} were determined in the presence and absence of the inhibitors at four different concentrations (0.0-200 µM). The results showed that increasing concentrations of mefenamic acid and doxycycline reduced the maximum reaction velocity.

A marked reduction in the reaction velocity was observed at 50 µM of mefenamic acid compared to other concentrations (Fig. 2A) while doxycycline reduced the reaction velocity to a lesser extent at the same concentration (Fig. 2B). The results showed constant a K_m value with increased concentration of both inhibitors. These observations suggest that the compounds are non-competitive inhibitors of dengue NS2B-NS3pro since increased concentrations were not able to affect the affinity between the enzyme and substrate.

Compounds cytotoxicity

The 50% cytotoxic concentration (CC_{50}) of mefenamic acid and doxycycline was determined against the Vero cells that were used for testing the inhibition potential of

Table 1. The inhibitory effect of the medical compounds against dengue NS2B-NS3pro

Compound	Chemical structure	% Inhibition at 25 μM	% Inhibition at 100 μM
Mefenamic acid		37.4 \pm 2.1	78.3 \pm 5.1
Tolfenamic acid		27.6 \pm 3.7	34.9 \pm 4.1
Flufenamic acid		15.2 \pm 1.1	35.9 \pm 3.1
Meclofenamic acid		19.4 \pm 2.2	43.0 \pm 1.4
Tetracycline		23.6 \pm 2.1	33.5 \pm 4.8
Oxytetracycline		18.8 \pm 2.1	39 \pm 4.1
Doxycycline		35.6 \pm 3.2	53.8 \pm 2.8
Rolitetracycline		32.8 \pm 2.6	38.9 \pm 2.9

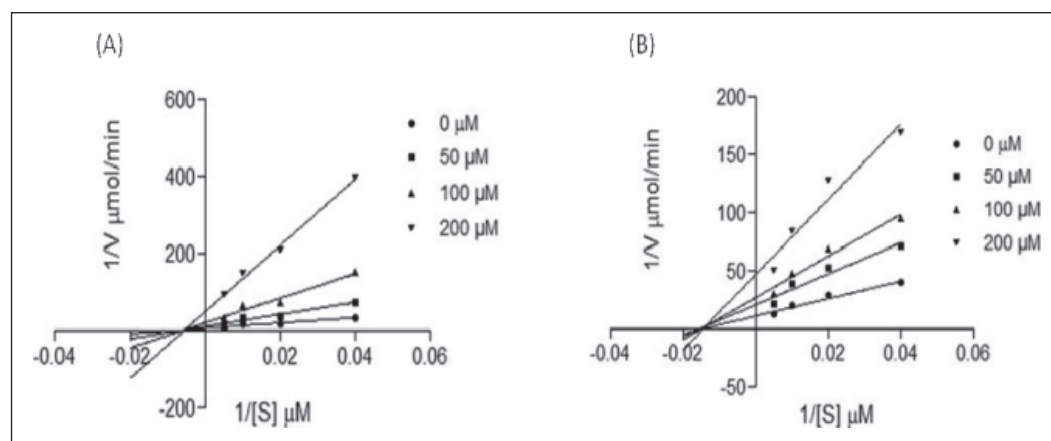


Figure 2. Lineweaver-Burk plot of mefenamic acid and doxycycline

(A) mefenamic acid non-competitively inhibited NS2B-NS3pro ($K_i = 32.0 \pm 2.9 \mu\text{M}$, $K_m = 152.0 \mu\text{M}$, $V_{\text{max}} = 0.19 \mu\text{M}/\text{min}$ and $\alpha = 1.6$). (B) Doxycycline non-competitively inhibited NS2B-NS3pro ($K_i = 55.6 \pm 5.7 \mu\text{M}$, $K_m = 151.0 \mu\text{M}$, $V_{\text{max}} = 0.14 \mu\text{M}/\text{min}$ and $\alpha = 1.6$)

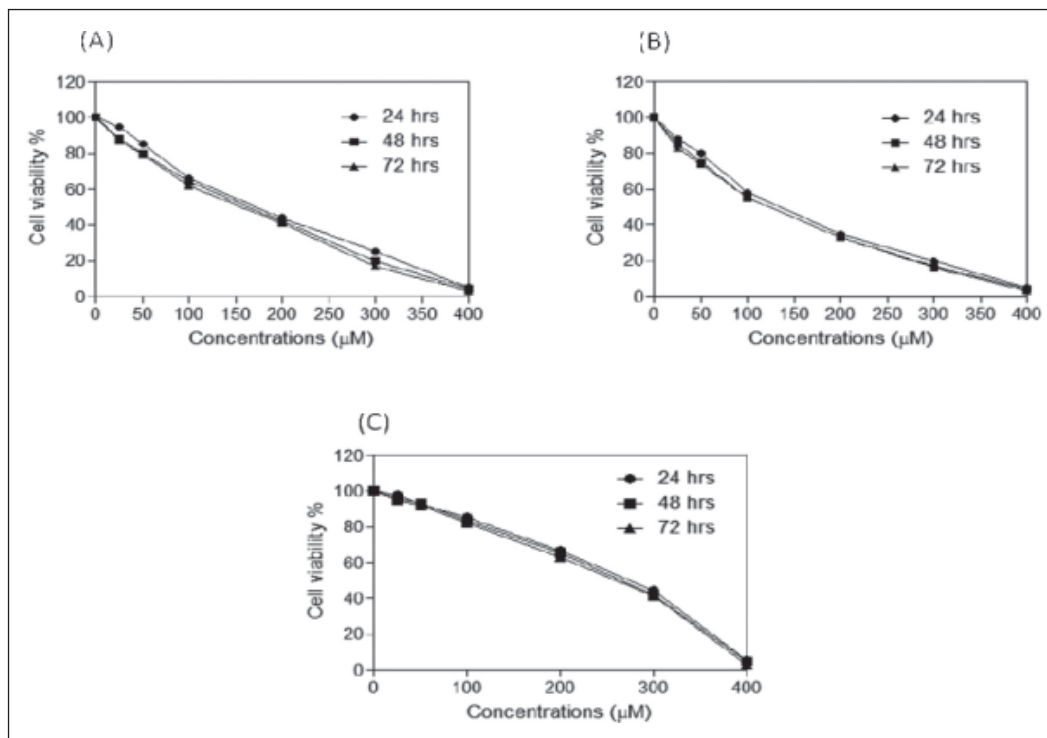


Figure 3. The effects of mefenamic acid and doxycycline on the cells viability (A) Mefenamic acid cytotoxic concentration 50% (CC₅₀) was approximately $150 \pm 5 \mu\text{M}$. (B) Doxycycline CC₅₀ was approximately $125 \pm 4 \mu\text{M}$. (C) Ribavirin CC₅₀ was approximately $280 \pm 7 \mu\text{M}$

these compounds against DENV2-infected cells. Serial concentrations of both compounds were added to a Vero cell monolayer and incubated for 24, 48 and 72 hrs. From the graphs represented in figure 3 (A and B), the CC₅₀ values were estimated to be $150 \pm 5 \mu\text{M}$ for mefenamic acid and $125 \pm 4 \mu\text{M}$ for doxycycline. In order to use as a positive control in the viral inhibition assay, ribovirin cytotoxicity was also estimated in this study to be $280 \pm 7 \mu\text{M}$. Hence the doses of the compounds that were used in the viral assays were less than the CC₅₀ values.

Reduction of dengue virus yield

Vero cells were infected with DENV2 and incubated for 24 hrs then, separately treated with mefenamic acid or doxycycline for 72 hrs to reduce the possible anti-metabolic effects of the drugs on DENV2 infected-cells. The intracellular viral RNA was quantified using qPCR and normalized with GAPDH as a reference gene to eliminate the possible anti-metabolic effect of these compounds.

Both mefenamic acid and doxycycline showed significant reduction in viral RNA ($p < 0.001$) at concentrations of $30 \mu\text{M}$ and $60 \mu\text{M}$ compared to untreated cells (Fig. 4A). Mefenamic acid reduced viral RNA at EC₅₀ of $32 \pm 4 \mu\text{M}$ whilst doxycycline EC₅₀ was $40 \pm 3 \mu\text{M}$. Significant differences in the percentage of viral RNA was observed between mefenamic acid and doxycycline at $30 \mu\text{M}$ ($p < 0.001$) and $60 \mu\text{M}$ ($p < 0.01$). Mefenamic acid showed a higher selectivity index (4.7) against dengue virus replication *in vitro* compared to doxycycline (3.1). (Fig. 4).

DISCUSSION

We conducted this study to evaluate the anti-dengue properties of some compounds that are used as antibiotics (tetracycline derivatives) and anti-inflammatory agents (mefenamic acid derivatives). It was found that mefenamic acid and doxycycline

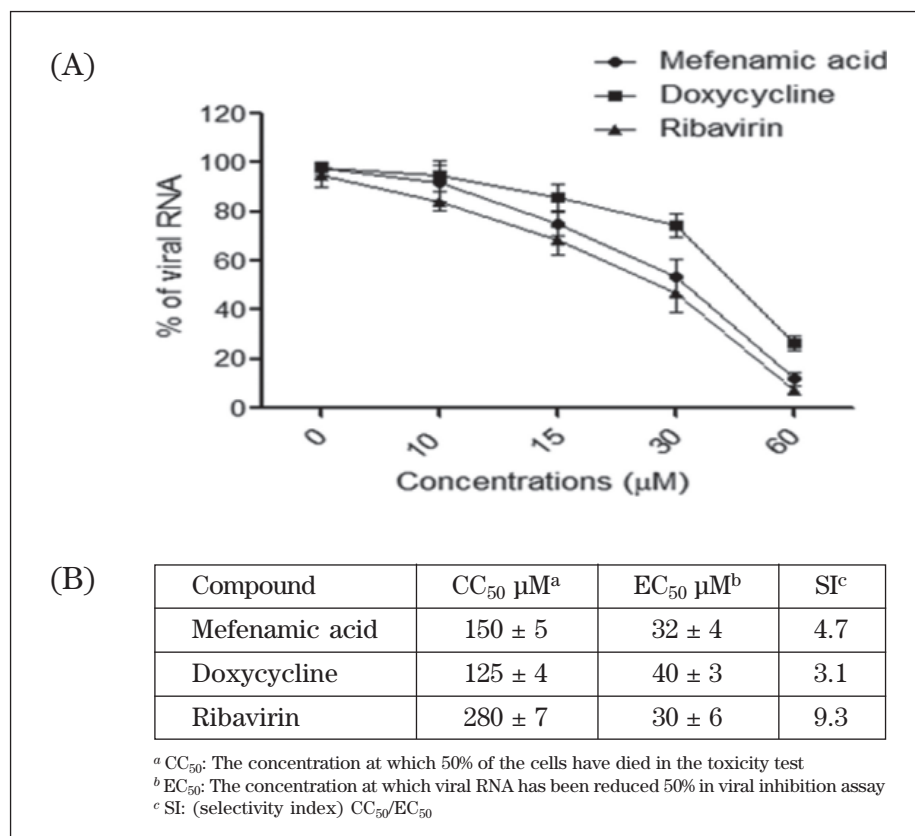


Figure 4. Percentage of viral RNA reduction after treatment with the test compounds (A) Mefenamic acid and doxycycline significantly reduced viral RNA ($p < 0.001$) at concentrations of 30 μM and 60 μM compared to untreated cells. (B) Mefenamic acid reduced viral RNA at EC₅₀ of 32 μM whilst doxycycline EC₅₀ was 40 μM . Significant differences in the percentage of viral RNA was observed between mefenamic acid and doxycycline at 30 μM ($p < 0.001$) and 60 μM ($p < 0.01$). Mefenamic acid showed higher selectivity index (4.7) against dengue virus replication *in vitro* compared to doxycycline (3.1). Both of the compounds showed lower selectivity index against dengue compared to ribavirin (9.3) as a positive control (2-way ANOVA with Bonferroni post test)

exhibited the highest inhibitory effect against dengue NS2B-NS3pro. Viral replication was significantly reduced in DENV-infected cells after applying these compounds at 50% effective concentrations (EC₅₀), which were considerably less than the 50% cytotoxic concentrations (CC₅₀) values. The results confirmed the higher inhibition potential of mefenamic acid compared to doxycycline towards viral replication *in vitro*.

Dengue virus has positive single-stranded RNA that is translated to polyprotein by the host cells' ribosome. Virus polyprotein is cleaved by viral NS2B-NS3 serine protease and cellular proteases to 10 structural and non-structural proteins (Clum *et al.*, 1997).

Disruption of viral NS2B-NS3pro would lead to inhibition of viral replication in host cells (Geiss *et al.*, 2009). A variety of different flaviviruses protease inhibitors have been previously reported. Most of these inhibitors have charged moieties such as short cationic peptides inhibitors (Knox *et al.*, 2006), peptide-aldehyde inhibitors (Stoermer *et al.*, 2008) and non-peptidic guanidine compounds (Ganesh *et al.*, 2005). On the other hand, series of non-charged 8-hydroxyquinoline compounds has also been identified (Mueller *et al.*, 2008). Both the compounds reported in this study, mefenamic acid and doxycycline, contain negatively charged moieties and hydrophobic groups.

These compounds were found to non-competitively inhibit dengue NS2B-NS3pro with K_i values of 32.0 μM and 55.6 μM , respectively. It is known that the binding between the dengue protease subunits depends on the interaction between negatively charged amino acids in NS2B and positively charged amino acids in NS3 (Erbel *et al.*, 2006). Therefore, these compounds may inhibit the activity of dengue protease by disturbing the binding between enzyme subunits that lead to significant reduction in its activity.

Previous studies have reported the anti-viral activity of mefenamic acid and doxycycline. The inhibitory effect of mefenamic acid against RNA viruses has been estimated as 90% at a concentration of 30 μM (Inglot, 1969). Furthermore, the EC_{50} of mefenamic acid against bovine viral diarrhoea virus (BVDV) as a model of hepatitis C virus (HPC) has been found to be 62.6 μM with CC_{50} less than 100 μM against Madin-darby bovine kidney (MDBK) cells (Okamoto *et al.*, 2009). Doxycycline also shows antiviral activities and is used efficiently to treat tick-borne infections that transmit bacterial, protozoal, and viral infections to humans (Buckingham, 2005). Its antiviral activity has been reported against herpes simplex virus (Kirchner & Emmert, 2000), retrovirus (Sturtz, 1998) and dengue virus (Inglot, 1969). This compound inhibited dengue virus replication with EC_{50} value estimated to be 55.6 μM (Yang *et al.*, 2007). In this study, mefenamic acid inhibited dengue replication in Vero cells with EC_{50} value estimated to be 32 μM and its CC_{50} value against Vero cells was approximately 150 μM whilst doxycycline showed EC_{50} of 40 μM at CC_{50} value of 125 μM . The values of selectivity index of mefenamic acid and doxycycline were lower than the selectivity index of ribavirin a nucleoside analogue that has demonstrated inhibition of the DENV methyltransferase (Benarroch *et al.*, 2004). However, the low side effects of these drugs besides its positive effects on the cytokine levels in patients with dengue infection (Castro *et al.*, 2011) may lead to consider these drugs as candidates for dengue fever treatments.

Because there is no actual animal model available for dengue infection, clinical studies would be required to obtain conclusive evidence of anti-dengue properties of mefenamic acid and doxycycline. The anti-dengue activity of these compounds combined with their anti-inflammatory effects may assist in attenuating dengue clinical symptoms like dengue fever and severe dengue hemorrhagic fever and dengue shock syndrome.

In conclusion, mefenamic acid and doxycycline showed significant anti-dengue activity through their ability to inhibit viral protease activity. Further experimental and clinical studies should be carried out to investigate their potential utilization for the attenuation of the clinical symptoms of dengue infection.

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Abbreviations.

(DENV2) dengue virus serotypes 2; (NS2B) NS2B cofactor amino acids sequence 49-95 in DENV2; (NS3pro) NS3 protease amino acids sequence 1-185 in NS3 protease; (NS2B-NS3pro) NS2B fused to NS3pro via 9 amino acids (G4-T-G4); (AMC) fluorogenic peptide substrate (Boc-Gly-Arg-Arg-AMC).

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