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# Amodiaquine, an antimalarial drug, inhibits dengue virus type 2 replication and infectivity

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ABSTRACT

Dengue virus serotypes 1–4 (DENV1–4) are transmitted by mosquitoes which cause most frequent arboviral infections in the world resulting in ~390 million cases with ~25,000 deaths annually. There is no vaccine or antiviral drug currently available for human use. Compounds containing quinoline scaffold were shown to inhibit flavivirus NS2B–NS3 protease (NS2B–NS3pro) with good potencies. In this study, we screened quinoline derivatives, which are known antimalarial drugs for inhibition of DENV2 and West Nile virus (WNV) replication using the corresponding replicon expressing cell-based assays. Amodiaquine (AQ), one of the 4-aminoquinoline drugs, inhibited DENV2 infectivity measured by plaque assays, with $EC_{50}$ and $EC_{90}$ values of 1.08 ± 0.09 μM and 2.69 ± 0.47 μM, respectively, and DENV2 RNA replication measured by *Renilla luciferase* reporter assay, with $EC_{50}$ value of 7.41 ± 1.09 μM in the replicon expressing cells. Cytotoxic concentration ($CC_{50}$) in BHK-21 cells was 52.09 ± 4.25 μM. The replication inhibition was confirmed by plaque assay of the extracellular virions as well as by qRT-PCR of the intracellular and extracellular viral RNA levels. AQ was stable for at least 96 h and had minor inhibitory effect on entry, translation, and post-replication stages in the viral life cycle. DENV protease, 5′-methyltransferase, and RNA-dependent RNA polymerase do not seem to be targets of AQ. Both p-hydroxyanilino and diethylaminomethyl moieties are important for AQ to inhibit DENV2 replication and infectivity. Our results support AQ as a promising candidate for anti-flaviviral therapy.



## 1. Introduction

Dengue virus (DENV), mosquito-borne member of flavivirus genus of *Flaviviridae* family has a positive-strand RNA genome. The four serotypes of DENV cause 390 million infections annually (Bhatt et al., 2013; Mitka, 2013). Secondary infections by a different DENV serotype could lead to severe clinical manifestations due to antibody dependent enhancement. Currently, there is no vaccine or antiviral drug available to combat dengue diseases.

DENV RNA encodes a single polyprotein which is processed to yield 3 structural proteins, capsid (C), precursor membrane (prM), and envelope (E), and 7 nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Lindenbach et al., 2007). For viral RNA replication, *cis*-acting RNA elements encoded within the 5′- and 3′-untranslated regions (UTR), coding regions within the N-terminal capsid protein and the C-terminal envelope as well as the 7 NS proteins are sufficient (for excellent reviews, see Lindenbach et al., 2007; Paranjape and Harris, 2010; Villordo and Gamarnik, 2009; Westaway et al., 2003). To study the *cis*-acting RNA elements and trans-acting viral and host factors required for replication, subgenomic replicons have been constructed for several flaviviruses, Kunjin (Khromykh and Westaway, 1997), WNV (Alcaraz-Estrada et al., 2013; Shi et al., 2002), YFV (Jones et al., 2005a), DENV2 (Alvarez et al., 2005; Jones et al., 2005b;

*Abbreviations:* DENV, dengue virus; AQ, amodiaquine; CQ, chloroquine; AQD, amodiaquine derivative; NS, nonstructural protein; WNV, West Nile virus; RdRP, RNA-dependent RNA polymerase.

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Manzano et al., 2011; Ng et al., 2007), and DENV4 (Alcaraz-Estrada et al., 2010). Stable replicon expressing cell lines have been used in screening antiviral compounds as potential inhibitors of viral replication (for reviews, see Noble et al., 2010; Sampath and Padmanabhan, 2009). Since flavivirus RNA encodes several enzymes that play important roles in the virus life cycle, *in vitro* assays have been developed for screening antiviral compounds. For example, *in vitro* assays for the two-component viral protease, NS2B–NS3pro (Lai et al., 2013; Mueller et al., 2008; Yusof et al., 2000), 5′-RNA methyltransferase catalyzed by the N-terminal domain of NS5 (Dong et al., 2008b; Egloff et al., 2002; Ray et al., 2006) and RNA-dependent RNA polymerase (RdRp) catalyzed by the C-terminal domain of NS5 (Ackermann and Padmanabhan, 2001; Niyomrattanakit et al., 2010, 2011). These assays have been useful to identify inhibitors of protease (Ezgimen et al., 2012; Johnston et al., 2007; Lai et al., 2013), 5′-RNA methyltransferase (Lim et al., 2011; reviewed in Dong et al., 2008b), and RdRp (Niyomrattanakit et al., 2010; Wu et al., 2011; Zou et al., 2011) by small molecule compounds.

Using *in vitro* protease and reporter replication-based assays, we identified compounds containing the 8-hydroxyquinoline (8-OHQ) scaffold as the potent inhibitors of both DENV2 and WNV proteases (Ezgimen et al., 2012; Lai et al., 2013; Mueller et al., 2008). In this study, we sought to investigate whether quinoline derivatives that are already known FDA-approved antimalarial drugs such as chloroquine, amodiaquine, and primaquine could inhibit viral proteases as well as replication of DENV and WNV.

## 2. Materials and methods

### 2.1. Compounds

Amodiaquine dihydrochloride dihydrate (4-[(7-chloroquinolin-4-yl)amino]-2(diethylamino methyl)phenol) (AQ), (Catalog # A2799-5g) was obtained from Sigma Aldrich (St. Louis, MO). Quinoline derivatives were obtained from National Cancer Institute/Developmental Therapeutics Program (NCI/DTP) in 10 mg quantities. The compounds were dissolved in DMSO unless otherwise stated to make up 50 mM stock solutions, and were stored as aliquots at −20 °C. For some experiments, an aqueous solution of AQ was used as indicated.

### 2.2. Construction of stable DENV2 replicon expressing BHK-21 cell line

The construction of DENV2 *Renilla luciferase* reporter replicon has been described previously (Manzano et al., 2011). The construction of an expression plasmid encoding DENV2 *Renilla luciferase* (*Rluc*) reporter replicon containing the selectable marker, neomycin resistance gene (*Neo*$^r$) and the stable BHK-21 cell line are described under Supplementary Methods (Fig. S1 and Table S1).

### 2.3. Replicon inhibition assay

BHK-21 cells expressing DENV2 replicon (BHK-21/DENV2), Vero cells expressing DENV4 (Vero/DENV4) (Alcaraz-Estrada et al., 2014), and WNV (Vero/WNV) (Alcaraz-Estrada et al., 2013) replicons were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), nonessential amino acids (Mediatech, Manassas, VA), 100 I.U./ml penicillin, 100 μg/ml streptomycin (penicillin–streptomycin), and 300 μg/ml G418 (Fisher Scientific, Pittsburgh, PA). Cells (~$10^4$/well) were seeded into 96-well μClear black microtiter plate (Greiner Bio-One, Monroe, NC) and were incubated for 6 h at 37 °C under $CO_2$ (5%) followed by addition of the compounds in 1% DMSO. at final concentrations as indicated. DMSO (1%) alone was used as the no-inhibitor control (100% luciferase activity or 0% inhibition). Cells were incubated at 37 °C for indicated time points (for example, 24 h for experiments shown in Table 1; 48 h for experiments shown in Fig. 2). Cells were lysed and *Renilla luciferase* (*Rluc*) activities were measured according to manufacturer's protocol (Promega, Madison, WI, USA) using a Centro LB 960 luminometer (Berthold Technologies, Oak Ridge, TN). Data were reported as percent inhibition relative to 1% DMSO (0% inhibition) and mycophenolic acid (100% inhibition) as controls. Selected compounds showing greater than 80% inhibition were further analyzed to determine the effective concentration at which 50% inhibition was obtained ($EC_{50}$). To calculate the $EC_{50}$ values compounds were serially diluted to final indicated concentrations (0, 0.1, 1.0, 5.0, 10, 25, 50, 100 μM for experiments shown in Table 1, or expanded serial dilutions described in Fig. 2 legend), and the % activity values at various concentrations of the compound were plotted in nonlinear regression using GraphPad Prism v5 software (La Jolla, CA).

### 2.4. Cytotoxicity assay

Cytotoxicity of AQ was evaluated by two methods. First, naïve BHK-21 or Vero cells were treated with compounds in parallel to the replicon cells. This method was used in evaluating $CC_{50}$ of 24 h treatment of the 4 selected compounds. The cell viability was assessed by measuring the ATP level using CellTiter-Glo® luminescent cell viability assay kit (Promega) according to manufacturer's protocol. Briefly, naïve BHK-21 or Vero cells (~$10^4$ - cells/well) were seeded in 96-well plates. Cells were incubated for 6 h at 37 °C. Compounds were added at the same concentrations as in the replicon assays as described in Table or figure legends. CellTiter-Glo® substrate was added according to manufacturer's protocol and the plate was read in a luminometer. Data were analyzed to determine the 50% cell viability ($CC_{50}$) value using GraphPad Prism v5 software.

In the second method, the viability of replicon expressing cells to various concentrations of each compound was measured in the same sample used for luciferase activity measurements using Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, MD) at 2 h before lysis and *Rluc* activity measurements. This colorimetric assay utilized highly soluble and non-cytotoxic tetrazolium salt (WST-8), added to the experimental cultures, and incubated at 37 °C for 2 h. The plate was read at $A_{585nm}$ using the Concert TRIAD spectrophotometer (Dynex, Chantilly, VA). Cells were washed, lysed, and the *Rluc* activities were measured as described above. $CC_{50}$ values were calculated using the GraphPad Prism v5 software.

### 2.5. Inhibition of DENV2 RNA replication and infectivity in BHK-21 cells

BHK-21 cells were seeded into 12-well plate ($10^5$ cells/well) and incubated overnight at 37 °C. Cells were infected with DENV2 at a multiplicity of infection (MOI) of 0.01 or 1 as indicated. After infection, cells were washed with PBS and incubated with 1.5 ml of MEM supplemented with 2% FBS, 100 I.U./ml penicillin/100 μg/ml streptomycin (referred as maintenance medium). AQ at indicated concentrations was added and cells were incubated at 37 °C for various time points as indicated. DMSO (1%), as a no-compound control (100% infection), and mock-infected control using medium alone (0% infection) were included. Supernatants were collected from the time point experiments and the virus titers determined by plaque assay.

### 2.6. Plaque assay

BHK-21 cells were seeded at ~$10^5$ cells/well (12-well plate) or ~$5 \times 10^4$ cells/well (24-well plate) and then incubated at 37 °C

**Table 1**
$EC_{50}$, $CC_{50}$, and TIs of selected compounds.

| Compd. # | Drugs/compounds | DENV2 replicon | | | WNV replicon | | |
|---|---|---|---|---|---|---|---|
| | | $EC_{50}$ | $CC_{50}$ | TI | EC50 | CC50 | TI |
| **1** | AQ | 10.81 ± 1.43 | 80.01 ± 6.27 | 7.4 | 14.63 ± 2.21 | 24.40 ± 2.49 | 1.66 |
| **9** | Quinacrine mustard | 0.39 ± 0.10 | 2.40 ± 0.63 | 6.15 | 0.36 ± 0.09 | 0.97 ± 0.11 | 2.69 |
| **5** | Chloroquine mustard | 2.90 ± 0.78 | 30.47 ± 9.77 | 10.5 | 2.95 ± 0.77 | 3.64 ± 0.93 | 1.23 |
| **4** | Chloroquine ethyl phenyl mustard | 3.60 ± 0.83 | 74.13 ± 21.50 | 20.57 | 1.55 ± 0.40 | 5.02 ± 1.23 | 3.24 |
| **12** | AQD1 | 21.09 ± 1.22 | 44.57 ± 2.98 | 2.11 | 14.85 ± 0.48 | 66.37 ± 4.64 | 4.47 |
| **13** | AQD2 | 29.68 ± 2.26 | 35.97 ± 5.04 | 1.21 | 30.15 ± 6.05 | 23.15 ± 10.83 | 0.76 |
| **14** | AQD3 | 4.76 ± 0.63 | 14.05 ± 2.57 | 2.95 | 8.00 ± 1.27 | 9.36 ± 1.08 | 1.17 |
| **15** | AQD4 | 15.36 ± 1.31 | 16.88 ± 2.06 | 1.1 | 3.31 ± 0.73 | 14.58 ± 0.51 | 4.4 |
| **16** | AQD5 | 88.71 ± 3.88 | >100 | N/A | 33.40 ± 3.09 | >100 | N/A |
| **17** | AQD6 | 12.49 ± 2.19 | 16.39 ± 0.46 | 1.31 | 6.29 ± 0.52 | 18.20 ± 1.05 | 2.9 |
| **18** | AQD7 | 18.97 ± 1.73 | 17.73 ± 1.96 | 0.93 | 14.84 ± 1.14 | 34.77 ± 1.78 | 2.34 |
| **19** | AQD8 | >100 | >100 | N/A | >100 | >100 | N/A |

The efficacy ($EC_{50}$), cytotoxicity ($CC_{50}$), and TIs of selected antimalarial compounds for inhibition of DENV2 and WNV Rluc reporter replicon replication are shown. This initial screening of compounds was done by addition of serially diluted compounds at 0, 0.1, 1.0, 5.0, 10, 25, 50, 100 μM to DENV2 and WNV replicon-expressing cells and incubation for 24 h as described under Section 2. The structures and the properties including % inhibition compound numbers, their structures are shown in Table S2 and Fig. S2.

until reaching 90% confluence. Cells were infected with the supernatants collected from experiments of AQ treatments. Cells were washed with PBS, and incubated with 1.5 ml of overlay medium (maintenance medium containing 1% methylcellulose). The plates were incubated for 3–4 days at 37 °C under 5% $CO_2$. After plaques became visually apparent by microscopy, cells were fixed and stained with 11.1% formaldehyde, 4.75% isopropanol, and 1% crystal violet for 30 min. The number of plaque forming units (PFU) per ml was determined.

### 2.7. $EC_{50}$ and $EC_{90}$ measurements by plaque assay

BHK-21 cells were seeded as described above. AQ at indicated concentrations in 1% DMSO was added to DENV2 (MOI of 0.01 or 1)-infected cells during adsorption and/or post-infection. Supernatants were collected at indicated time points post-infection for plaque assays.

### 2.8. Quantitative RT-PCR (qPCR)

Intracellular RNAs were extracted from the infected cells by treatment with TRIzol reagent (Invitrogen, Life, Grand Island, NY) according to the manufacturer's protocol. Total RNAs were quantified using Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA) and adjusted to 1 μg/μl for reverse transcription (iScript cDNA synthesis, BioRad, Hercules, CA). Quantitative RT-PCR (qPCR) was performed as previously described (Manzano et al., 2011). Briefly, the region of viral RNA encoding DENV2 NS1 gene was amplified using the forward and reverse primers (DENV2 NS1-F and DENV2 NS1-R; Table S1). The glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) was chosen as the housekeeping reference RNA and amplified by PCR using the forward and reverse primers (Table S1). The viral RNA copy numbers were calculated in AQ-treated cells relative to the DENV2-infected and AQ-untreated cells (1% DMSO alone). Supernatants from the experiment were also collected and stored as aliquots (1 ml) at −70 °C until use. Supernatant were concentrated by Amicon-15 (Millipore, Billerica, MA). Viral RNAs were extracted by QIAamp viral RNA mini kit (QIAgen, Valencia, CA) prior to cDNA synthesis as described above.

### 2.9. Time-course analysis on DENV2 infectivity

The protocol was adapted from Stahla-Beek et al. (2012). BHK-21 cells were grown in 6-well plates (~$2.5 \times 10^5$ cells/well) and were infected with DENV2 at a MOI of 0.01 in the maintenance medium. Cells were washed with PBS and incubated with 3 ml of maintenance medium. AQ at indicated concentrations was added and cells were incubated at 37 °C. Supernatants were sampled as indicated and stored at −70 °C for plaque assays.

### 2.10. Order of addition assay

The protocol was adapted from Schmidt et al. (2012). BHK-21 cells were grown in 12-well plates and were treated with AQ in one of three ways. In (1), AQ (5 μM) and DENV2 (MOI of 1) were diluted in maintenance medium and incubated for 15 min at 37 °C before adding to BHK-21 cells (pre-incubation). In (2), the mixture of AQ and DENV2 was added to BHK-21 cells directly (co-infection). In (3), BHK-21 cells were infected with DENV2, or mock-infected with 1% DMSO containing medium (1 h at 37 °C), washed, and incubated with medium containing AQ (post-infection). Supernatants were collected at 24, 48, 72 h post-infection for plaque assays.

### 2.11. Time of addition assay

The protocol was adapted from Wang et al. (2011). BHK-21 cells were grown in 12-well plates and infected with DENV2 (MOI of 1). AQ (5 μM) or DMSO alone (1%) was added to DENV2-infected cells at different times post-infection as indicated. Supernatants were collected at 72 h post-infection to determine the titer by plaque assay.

In addition, we performed a time of addition assay with a slight modification to study the effect of AQ (5 μM) on viral translation by collecting more samples as indicated within the first 12 h after addition of AQ or DMSO (1%) (MOI of 1). Supernatants were collected at 48 h post-infection for plaque assay.

## 3. Results

### 3.1. Screening of antimalarial compounds for flaviviral replication inhibition

Antimalarial quinoline compounds were screened using BHK-21/DENV2 and Vero/WNV replicon cells (Table S2 and Fig. S2). Antimalarial compounds that were tested at 50 μM final concentration include 4-aminoquinoline derivatives such as chloroquine (CQ; 3), chloroquine ethyl phenyl mustard (4), chloroquine mustard (5), chloroquine pamoate (6), chloroquine sulfate (7), amodiaquine (AQ; 1), 6-methoxyquinoline derivatives such as apoquinine

(2), primaquine (8), and quinine (11), quinine hydrobromide hydrate (10) and an acridine derivative, quinacrine mustard (9). AQ (1), having a diethylaminomethyl group showed 76.31 ± 1.60% and 96.30 ± 0.39% inhibition of DENV2 and WNV replicon replication, respectively. Two chloroquine derivatives and one acridine derivative (compounds **4**, **5**, and **9** in Table 1) having a mustard group showed >99% inhibition of both BHK-21/DENV2 and Vero/WNV replicon replication (Fig. 1A). These derivatives contain a common side chain, a dichloroethylamino group, also known as a mustard group. However, the compounds, **4**, **5**, and **9**, had low TI values in the range of 2–3 in inhibition of Vero/WNV replicon replication due to their cytotoxicity to Vero cells (Table 1). However, compounds with a dichloroethylamino side chain have known cytotoxicity and potential to form skin-blisters (Keyes, 2004; Welsh et al., 2004). Therefore, compounds **4**, **5**, and **9** were not pursued further.

Based on the results of the primary screening (Fig. 1A), eight AQ derivatives (AQD1-8) (Table S2) were obtained for further analysis. AQD1, AQD3, AQD4, AQD6, and AQD7 showed strong inhibition of DENV2 and WNV replicon replication (Fig. 1B). However, none of AQDs showed higher TIs than AQ due to their cytotoxicity (low $CC_{50}$ values; Table 1). Our results further showed that compounds lacking a diethylaminomethyl group adjacent to phenolic OH moiety (AQD5 and AQD8) failed to inhibit the replicon replication with $EC_{50}$ values of ~100 μM (described in Section 3.4. below).

$EC_{50}$ and $CC_{50}$ of the compounds were tested in BHK-21/DENV2, Vero/DENV4, and Vero/WNV replicon cells and their therapeutic indices (TIs) were calculated. TI value of AQ was 7.03 in BHK-21/DENV2 replicon cells, with the $EC_{50}$ of 7.41 ± 1.09 μM (Fig. 2A) whereas in Vero/DENV4 cells, the TI of AQ was only 1.14 (Fig. 2B), and that in Vero/WNV cells it was 4.98 (Fig. 2C) due to the cytotoxicity of AQ to Vero cells. We verified that AQ did not interfere with the *Rluc* enzyme activity when added to the BHK-21/DENV2 replicon cell lysate (Fig. S3).

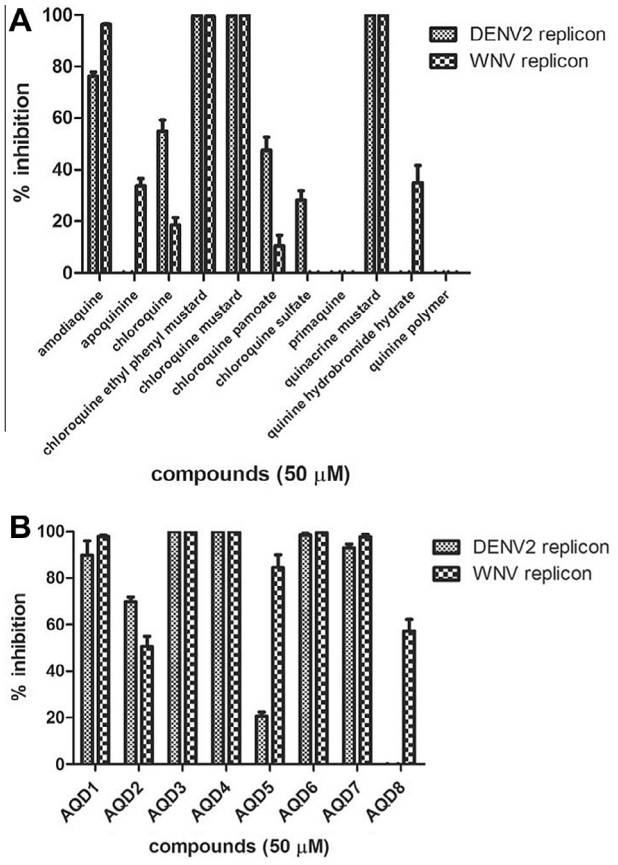


**Fig. 1.** Screening of quinoline derivatives for inhibition of replicon replication (A & B). BHK-21/DENV2 and Vero/WNV replicon cells (~$10^4$ in 100 μl) were seeded and incubated for 6 h. Compounds were added to the final concentrations of 50 μM in 1% DMSO, the incubation continued for 24 h, and the *Rluc* activities were measured. MPA (5 μM in 1% DMSO) and 1% DMSO were used as 100% and 0% inhibition controls. Error bars represent the standard error of the mean from triplicates. Experiments were repeated to confirm the findings.

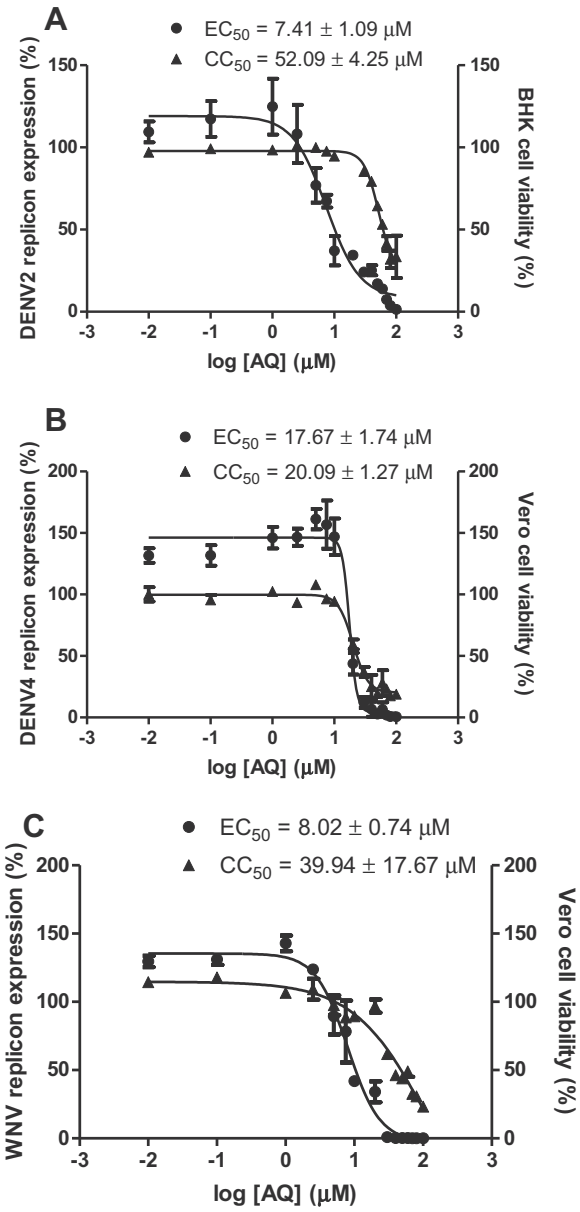


**Fig. 2.** $EC_{50}$ and $CC_{50}$ values of AQ for replicon inhibition and viability of replicon expressing cells. (A) BHK-21/DENV2 replicon, (B) Vero/DENV4 replicon, and (C) Vero/WNV replicon cells (~$10^4$ in 100 μl) were seeded and incubated for 24 h. AQ at 0, 0.01, 0.1, 1, 2.5, 5, 7.5, 10, 20, 30, 40, 50, 60, 70, 80, or 100 μM in 1% DMSO were added in triplicate wells. Cells were incubated at 37 °C for 48 h, washed, lysed, and *Rluc* activities were measured. Cytotoxicity was measured in the same replicon expressing cells treated with the various concentrations of AQ as described under Section 2. The results were confirmed by two independent experiments.

### 3.2. AQ inhibits DENV2 viral RNA levels and infectivity

Next, we analyzed the effect of AQ on the intracellular and extracellular DENV2 RNA levels as well as the virus infectivity in

BHK-21 cells. The viral replication was quantified by qRT-PCR and the virus infectivity by plaque assay. Cells were treated with a fixed concentration of AQ (5 μM) and the infected cells were incubated for 72 h (Fig. 3A). Results showed a significant difference ($p < 0.001$) between the AQ-treated and untreated groups. The results indicate that AQ effectively inhibited DENV2 replication with the reducing levels of intracellular and extracellular RNAs. The virus infectivity measured as PFU/ml of supernatant from the AQ-untreated and AQ (5 μM)-treated cells by plaque assay also showed a significant reduction upon treatment with the drug (Fig. 3A). Taken together, these results support the conclusions of results using BHK-21/DENV2 replicon cells (Figs. 1 and 2).

Next, we sought to determine the $EC_{50}$ and $EC_{90}$ values for AQ-mediated inhibition of extracellular release of DENV2 from the infected BHK-21 cells. To this end, BHK-21 cells were infected with DENV2 and treated with AQ at various concentrations as indicated. The supernatants collected at 72 h (MOI of 1) or 96 h (MOI of 0.01) post-infection were analyzed by plaque assay. The virus titers at various concentrations of AQ were plotted using GraphPad Prism v5 software. The $EC_{50}$ value of 1.08 ± 0.09 μM (MOI of 1) (GraphPad plot not shown) and $EC_{90}$ values of 2.69 ± 0.40 μM (MOI of 1) as well as $EC_{90}$ of 2.71 ± 0.85 (MOI of 0.01) from an independent experiment are shown (Fig. 3B).

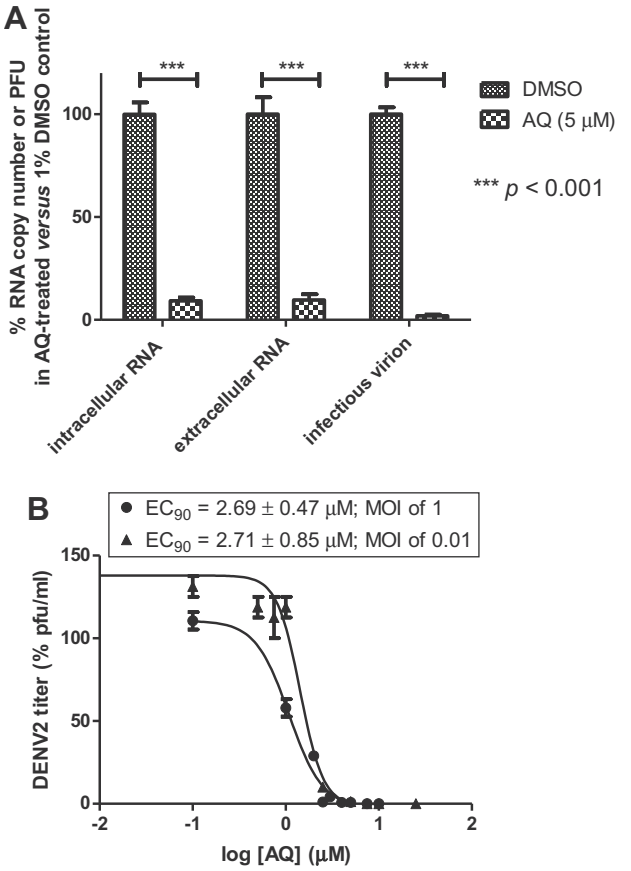


**Fig. 3.** Inhibition by AQ of DENV2 replication analyzed by qPCR and infectivity (plaque) assay. (A) qPCR and infectivity. BHK-21 cells were infected with DENV2 (MOI of 1) and treated with AQ (5 μM) or no-AQ control (1% DMSO) during infection and post-infection. Cells were incubated for 72 h at 37 °C and intracellular and extracellular RNAs were analyzed by qPCR or by plaque assay as described under Section 2. The relative amounts of viral RNA copy number or PFU in AQ-treated cells were calculated as % of the amounts in AQ-untreated cells. Each sample was tested in duplicate and results were confirmed by three independent experiments. Difference between AQ treated and 1% DMSO-treated groups derived from each approach was evaluated by paired *t*-test, two tailed. (B) $EC_{90}$ values for inhibition of DENV2 infectivity by AQ. BHK-21 cells were infected with DENV2 (MOI of 1) and treated with AQ at 0.1, 0.5, 1, 2.5, 5, or 10 μM, or infected with DENV2 (MOI of 0.01) and treated with 0.01, 0.1, 0.5, 0.75, 1, 2.5, 5, 7.5, 10, or 25 μM, during infection and post-infection. Incubation was done at 37 °C and the supernatants were collected at 72 h (MOI of 1) or 96 h (MOI of 0.01). The virus titers were determined by plaque assay. $EC_{90}$ values were calculated using GraphPad Prism v5 software. Data represented duplicates of two independent experiments.

### 3.3. Mode of inhibition of DENV2 infection by AQ

The results thus far indicated that AQ inhibited DENV2 replication in the stable replicon-expressing cells as well as the virus titers and RNA copy numbers in DENV2 infected BHK-21 cells as shown by plaque assays and qRT-PCR. All subsequent experiments were performed in BHK-21 cells infected with DENV2 to determine the stage of the virus life cycle targeted by AQ.

First, a time course of DENV2 infectivity in the absence and presence of different concentrations of AQ (Fig. 4A and B). AQ was added to the DENV2 infected cells during adsorption and the compound was present throughout the duration of the experiment (96 h) post-infection. As shown in Fig. 4, in mock-infected control cells (1% DMSO alone), DENV2 infectivity titer gradually increased over time. However, in the AQ-treated cells, the virus titers (PFU/ml) were reduced significantly in a dose-dependent manner ($p < 0.05$) (Fig. 4A and B). At 5 μM, the plaque formation was reduced ⩾90%. The observation that the AQ-mediated inhibition increased over time indicating that AQ was stable at least up to 96 h post-infection.

To analyze AQ's effect on viral entry, AQ at various concentrations was incubated with DENV2 (MOI of 0.01) on the BHK-21 monolayer for 1 h adsorption period, and then overlay medium in the absence of drug, was added and incubated at 37 °C for 3–4 days as described under Section 2. Data obtained from this experiment is referred to as direct plaque assay (Fig. 4C, stippled bars). Concurrently, virus supernatants collected from cells infected with DENV2 in the presence 1% DMSO or AQ present during and up to 96 h post-infection were also used for plaque assay (referred as indirect plaque assay). The virus titers were determined as above (Fig. 4C, open bars). The virus titers from indirect plaque assays (Fig. 4C, open bars) showed significant inhibition of DENV2 infectivity by AQ at 5 μM similar to the results shown in Fig. 4, A and B. However, in the direct plaque assay, since AQ was present only during adsorption, the plaque titer at 5 μM AQ was not significantly different from the titer at 1 μM and its DMSO control (Fig. 4C, stippled bars). These results indicate that AQ at 5 μM does not inhibit DENV2 adsorption. However, the virus titer was reduced by 69.23 ± 1.57% and 82.70 ± 2.48% when treated with AQ at 10 and 25 μM, respectively, during adsorption (Fig. 4C, stippled bars). The results indicate that the inhibition of DENV2 entry requires a higher concentration of AQ (10 or 25 μM) than that is required to inhibit infectivity when added post-infection (5 μM).

To confirm that AQ had the optimal inhibitory effect on DENV2 replication when added during post-infection period, we expanded the order of addition of AQ at various time points in three different ways as follows. In (1), a mixture of AQ and DENV2 (MOI of 1) were incubated for 15 min at 37 °C prior to addition to BHK-21 cells. In (2), AQ and DENV2 (MOI of 1) were simultaneously added to BHK-21 cells. In both cases, after adsorption, the cells were washed and incubated with maintenance medium in the absence of AQ. In (3), AQ was added to BHK-21 cells at post-adsorption with DENV2 and it was present throughout post-infection period up to 72 h. Supernatants were collected at 24, 48, and 72 h post-infection for plaque assay (Fig. 4D). Results indicated that the inhibition of DENV2 infectivity by AQ was maximum (⩾90% reduction of plaque titer from its DMSO control) at 48 and 72 h post-infection (Fig. 4D, striped bar). These results indicated that the major step for inhibition of DENV2 infectivity by AQ is post-entry and fusion events, possibly at viral translation, replication, or assembly of the virus.

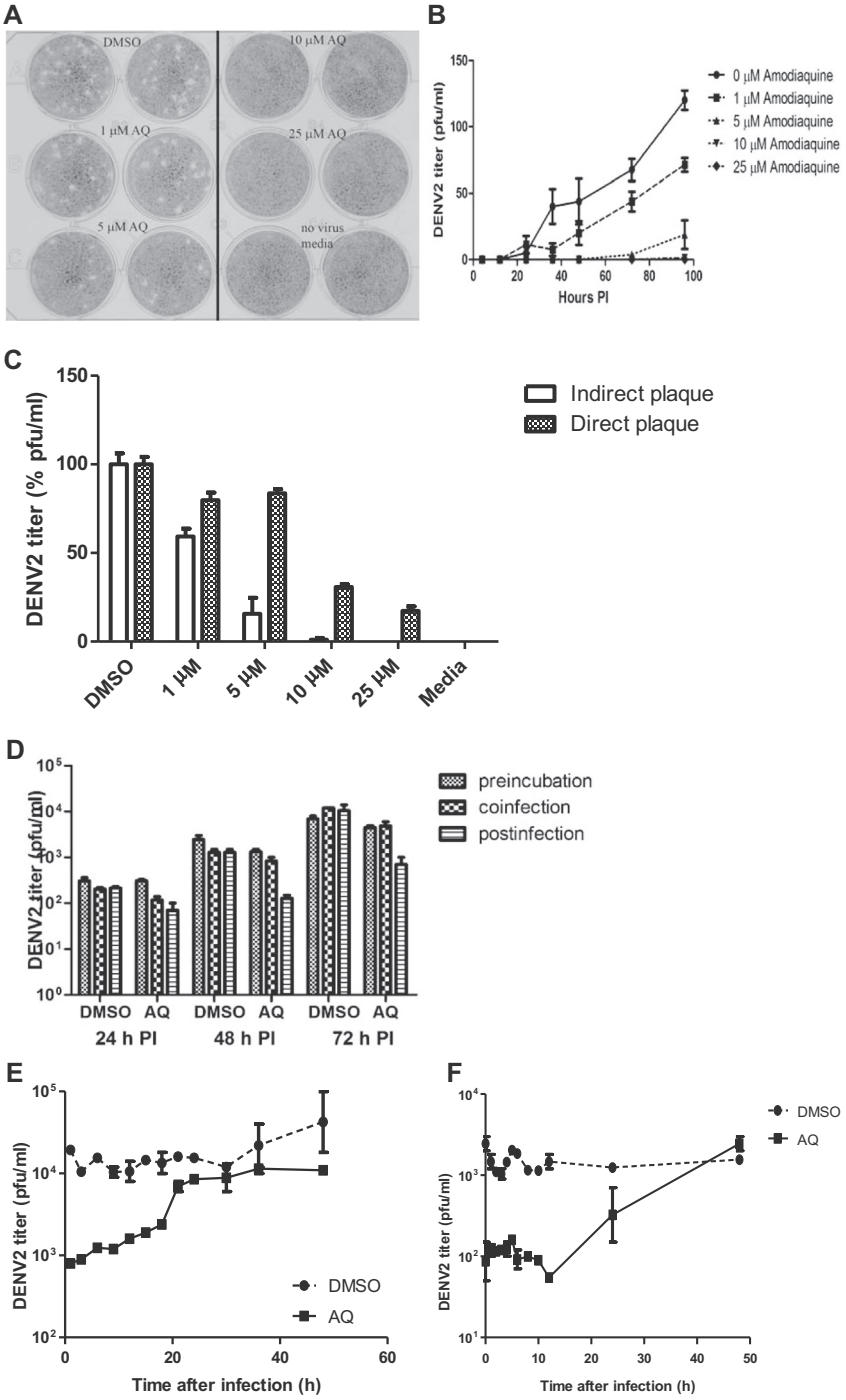


**Fig. 4.** Time-course analysis of AQ inhibition of DENV2 infectivity. (A and B) BHK-21 cells were infected with DENV2 (MOI of 0.01). Cells were washed with PBS and incubated with 3 ml of maintenance medium. AQ at 0, 1, 5, 10, or 25 μM in 1% DMSO or 1% DMSO alone, was added and the incubation continued at 37 °C. Supernatants were sampled at 4, 12, 24, 36, 48, 72, and 96 h post-infection. DENV2 infectivity was analyzed by plaque assay. (C) Measurement of AQ inhibition of DENV2 infectivity by direct plaque assay. BHK-21 cells (~$10^5$ cells in 1 ml) were seeded into a 12-well plate. AQ at final concentrations of 0, 1, 5, 10, or 25 μM in 1% DMSO were added to DENV2 (MOI of 0.01) during adsorption for 1 h at 37 °C. Cells were washed with PBS and maintained with overlay medium containing 1% methylcellulose without AQ during post-infection as described in Section 2. Plaques (pfu/ml) were counted after 3–4 days of incubation and the titers were normalized using no-AQ control as 100%. Plaque assay was also performed by incubation with AQ at 0, 1, 5, 10, 25 μM in 1% DMSO added during DENV2 infection (MOI of 0.01). AQ was present during post-infection period for 96 h. Supernatants were collected at 96 h post-infection for plaque assay. Titers (PFU/ml) were normalized to those obtained with no-AQ control taken as 100%. Data represented means and SEM of two independent experiments. (D) Order of addition assay. This experiment was performed as described under Section 2. AQ (5 μM in 1% DMSO) and DENV2 (MOI of 1) were treated in one of 3 ways. In (1), AQ and the virus were pre-incubated at 37 °C for 15 min before adsorption to BHK-21 cells for 1 h; in (2), AQ and the virus were added to BHK-21 cells together and incubated for 1 h; in both cases, cells were washed and incubated with the maintenance medium without AQ. In (3), AQ was added after virus adsorption and wash with PBS (post-infection). AQ was present throughout the duration of the experiment. Supernatants were collected at 24, 48, and 72 h post-infection for plaque assay. DMSO (1%) alone was used as mock-infection control. Error bars indicated the standard error of the means of experiments done in duplicate. The results were confirmed by an independent set of experiments. (E) Time of addition assay. This experiment was performed as described under Section 2. AQ (5 μM in 1% DMSO) was added to DENV2 infected BHK-21 cells (MOI of 1) at 1, 3, 6, 9, 12, 15, 18, 21, 24, 30, 36, or 48 h post-infection. Supernatants were collected at 72 h post-infection for analysis of the viral titers. Error bars indicated the standard error of the means of experiments done in duplicate. Results were confirmed by an independent set of experiments. (F) Time of addition assay to probe the AQ effect on viral translation. AQ (5 μM in 1% DMSO) or 1% DMSO alone was added to DENV2-infected BHK-21 cells (MOI of 1) at 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, or 48 h post-infection. Supernatants were collected at 48 h post-infection for plaque assay. Error bars indicated the standard error of the means of experiments done in duplicate.

Next, to pinpoint the post-infection stage of inhibition more precisely, we added AQ at 5 μM to DENV2 infected BHK-21 cells (MOI of 1) at various time points post-infection as indicated. Supernatants were collected at 72 h post-infection and were analyzed for time-dependent plaque reduction. The plaque reduction by ⩾90% was found even when the drug was added as late as 15 h post-infection (Fig. 4E). At 21 h post-infection and later time points, the drug lost its inhibitory effect.

We investigated the effect of AQ within the first 12 h post-infection to examine whether AQ is a translational inhibitor similar to a compound with benzomorphan core structure (Wang et al., 2011). Results indicated that addition of AQ up to 12 h inhibited DENV2 infection steadily (Fig. 4F) and even added at 15 h post-infection (Fig. 4E) beyond which there was a steady increase in titer (loss of inhibition), suggesting that AQ could inhibit early events including translation of viral RNA templates released from the endosomes and events prior to assembly of viral RNA replicase complex and on-set of viral RNA replication (Fig. 4E and F).

### 3.4. Diethylaminomethyl group adjacent to phenolic OH of AQ is crucial for inhibition of DENV2 replication and infection

From the analysis of AQ and its derivatives using BHK-21/DENV2 replicon cells, compounds lacking a diethylaminomethyl group, AQD5 and AQD8, failed to inhibit replicon replication. The $EC_{50}$ values were ~100 μM (Table 1 and Table S2), at least 10-fold higher than that of AQ. We chose AQD8 for further study using the infectivity assay. AQD8 at various concentrations was added to DENV2 infected BHK-21 cells (MOI of 1). After 72 h incubation, supernatants were collected and the virus titers were determined by plaque assay. Results showed an $EC_{90}$ of AQD8 at 31.20 ± 5.15 μM (Fig. 5), which is more than 10-fold higher compared to that of AQ ($EC_{90}$ of 2.69 ± 0.47 μM). Thus, AQD8 has a modest potency in inhibition of replicon replication and viral infectivity. These results indicate that the diethylaminomethyl group is a key chemical structural moiety of AQ contributing to inhibition of DENV2 replication and infectivity.

Next, we sought to investigate the effect of CQ, another FDA-approved antimalarial drug and a 4-aminoquinoline derivative, (Fig. S2) on the replication of DENV2. BHK-21 cells were infected with DENV2 at a MOI of 1 and treated with CQ at various concentrations. The virus titers of the supernatants were determined by plaque assay. As shown in Fig. 5, CQ inhibited DENV2 replication in BHK-21 cells in a dose-dependent manner ($EC_{90}$ = 5.04 ± 0.72 - μM) although it did not inhibit DENV2 replicon replication (Fig. 1). These results suggested that the inhibition of CQ is at either an early stage such as virus entry or at a late stage such as virus assembly into infectious particles.

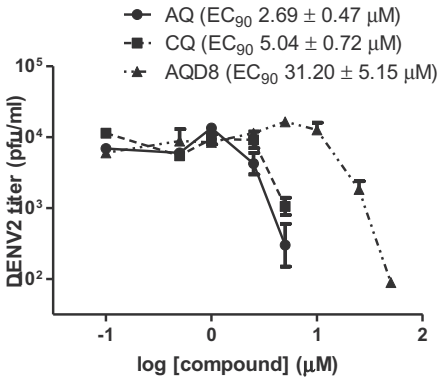


**Fig. 5.** Structural determinants of AQ for inhibition of DENV2 infectivity. BHK-21 cells were infected with DENV2 in duplicate wells at a MOI of 1. AQ at 0.1, 0.5, 1, 2.5, 5, or 10 μM as well as CQ or AQD8 at 0.1, 0.5, 1, 2.5, 5, 10, 25, or 50 μM in 1% DMSO were added to the virus-infected cells. Cells were incubated at 37 °C for 72 h. Supernatants were collected and the virus titers were determined by plaque assay. The $EC_{90}$ values were determined as described under Section 2. The results were confirmed by an independent set of experiments. Structures of AQ, CQ, and AQD8 are shown in Fig. S2.

### 3.5. In vitro viral enzyme assays to probe the mechanism of action of AQ

The above findings supported the notion that AQ targets a factor functioning in viral replication. The factor could be of either viral or host origin. We first investigated whether AQ could be an inhibitor of the viral protease activity of NS3, or the 5′-RNA methyltransferase and RNA-dependent RNA polymerase activities of NS5 using established *in vitro* biochemical assays.

We first tested the effect of AQ on DENV2 NS3 protease using the fluorescence-based *in vitro* protease assay (Lai et al., 2013; Li et al., 2005; Yusof et al., 2000). The $IC_{50}$ value of AQ for inhibition of DENV2 protease was ⩾250 μM (Fig. S4A) and AQ up to 500 μM had no effect (data not shown). Hence, the viral protease is not the likely target of AQ.

Next, we investigated whether the 5′-RNA methyltransferase activity of the N-terminal domain of DENV2 NS5 could be the target of AQ. The N-terminal domain of NS5 has activities for both N-7 and 2′-O methylations to form the type I cap (Dong et al., 2008a; Ray et al., 2006) and the full-length NS5 ($NS5_{FL}$) was twofold more efficient in the formation of type I cap (data not shown). Therefore, we used the $NS5_{FL}$ for the *in vitro* assay to test the effect of AQ. The results indicated that AQ did not inhibit the formation of type I cap by $NS5_{FL}$ up to 1 mM (Fig. S4B).

Next, we examined whether RNA synthesis catalyzed by the NS5 RdRP enzyme might be the target of AQ. Both positive (+) and negative (−) strand RNAs were used as templates in an *in vitro* RdRP assay in the presence of 50 μM AQ for 60 min. Inhibition of RdRP activity by AQ was only 10.58 ± 0.94% for positive-strand RNA synthesis. No inhibition (−17.12 ± 4.37%) was observed for the negative-strand synthesis (Fig. S4C), suggesting that DENV2 RdRP is not a likely target of AQ.

NS3 is endowed with ATP-dependent RNA helicase activity (Benarroch et al., 2004; Li et al., 1999; Shiryaev et al., 2009; Yon et al., 2005). We attempted to use a fluorescence-based assay (Boguszewska-Chachulska et al., 2004; Frick et al., 2004; Lam et al., 2004) for NS3 RNA helicase in which a partially double-stranded oligodeoxynucleotide was used as a substrate for NS3 RNA helicase to study the efficacy of amodiaquine. One strand of the substrate was labeled with the fluorophore, fluorescein, and the other with the black hole quencher such that there is no fluorescence in the absence of NS3. The incubation with the NS3 helicase released the fluorescein-labeled strand in a time-dependent manner. Although the assay was successful in measurement of the helicase activity of NS3, amodiaquine interfered with the assay due its binding to the substrate as reported previously (O'Neill et al., 1998) (data not shown).

## 4. Discussion

Amodiaquine (AQ) was synthesized in 1980s by addition of 4-hydroxyanilino group to chloroquine (CQ). AQ was proven effective against chloroquine-resistant strain (reviewed in O'Neill et al., 1998). The drug was later withdrawn from long-term antimalarial prophylaxis due to its rare but serious side effects, agranulocytosis and hepatotoxicity (WHO 1990, reviewed by Olliaro and Mussano, 2003). Cochrane systematic review in 1996, however, supported the use of AQ for treatment of uncomplicated malaria. Besides, an adverse drug reaction study found no significant difference

among CQ, AQ, and sulfadoxine/pyrimethamine antimalarials (reviewed by Olliaro and Mussano, 2003). This impact enforced WHO 1997 to modify its recommendation such that AQ became an optional treatment of uncomplicated malaria (reviewed by Olliaro and Mussano, 2003). AQ regimen for antimalarial treatment is 3 days of 600–800 mg/day in an adult. Pharmacogenetic studies have discovered a genetic trait in patient population who are susceptible to an adverse effect of hepatotoxicity due to AQ linked to polymorphisms of CYP2C8 gene, one of the cytochrome P450s. The genetic variants of CYP2C8 gene, CYP2C8*2 and CYP2C8*3, were reported to be responsible for defective metabolism resulting in slow clearance of AQ (reviewed by Kerb et al., 2009). The genotypes, CYP2C8*2 and CYP2C8*3, were recorded in African and Caucasian population with the frequency of 11–17%, and 15%, respectively. Dose-adjustments of AQ and increased safety precautions are required for treatment of patients with these polymorphisms in order to prevent the intoxication. Given the anti-dengue efficacy shown in this study, AQ is likely to become a powerful candidate for treatment of DENV infected patients.

We studied the mode of inhibition of DENV2 replication and infectivity by AQ and investigated the potential targets of AQ using *in vitro* enzyme assays. Time-course analysis suggested that the drug was stable and reproducibly inhibited DENV2 infectivity up to 96 h post-infection. We also learned that the viral entry and internalization was partially inhibited by the drug but the major effect of drug occurred at a later stage of the viral life cycle up to 15 h post-infection. This effect of AQ at early stages (adsorption/entry) would be absent in replicon-expressing cells and hence the $EC_{50}$ values are higher (Table 1 and Fig. 2). AQ was also ineffective to inhibit replication that is already established as seen in the time of addition assays. A previous study showed that DENV2 replication starts 12 h post-infection as suggested by rapid increase of intracellular RNA levels in BHK-21 cells quantified by qRT-PCR (Shrivastava et al., 2011). In this study, we have shown that the drug was less effective when added later than 15 h post-infection as measured by viral infectivity (Fig. 4E–F). Based on our results, we conclude that early stages of virus life cycle including translation, assembly of viral replicase complex and initiation of viral RNA replication could be targets of AQ.

It has been reported that AQ is a potent inhibitor of histamine-N-methyltransferase (E.C. 2.1.1.8) with a Ki of 18.6 nM (Horton et al., 2005). The crystal structure of two molecules of AQ in complex with histamine-N-methyltransferase at the SAM binding pocket, and the nearby outer pocket were solved (Horton et al., 2005). Another crystal structure of AQ in complex with phosphoethanolamine methyltransferase from *Plasmodium falciparum* at SAM binding pocket was also reported (Lee et al., 2012). However, our results excluded the 5′-RNA methyltransferase and the RdRP activities of NS5 as well as the protease activity of NS3 as the targets of AQ.

Viral replication requires both viral and host factors (reviewed in Bollati et al., 2010; Ivanyi-Nagy and Darlix, 2012; Sampath and Padmanabhan, 2009). It is possible that AQ targets a nonstructural protein without an enzymatic function or an unknown host factor(s) required for replication. Isolation and characterization of AQ-resistant mutants and mapping these mutations by sequence analysis might reveal the identity of the putative viral target. Although most of the DENV2 infected BHK-21 cells die within 12 days in the presence of 5 μM AQ (unpublished results), a longer incubation period, up to 30 days, might yield escape mutants (Teramoto et al., 2008). An *in vitro* RdRP system (Ackermann and Padmanabhan, 2001; Nomaguchi et al., 2003) could provide an insight into whether the replication initiation factors of viral replication are targets of AQ.

In this study, we provide evidence that another antimalarial compound, CQ, inhibited DENV2 infectivity with an $EC_{90}$ of 5.04 ± 0.72 μM consistent with the results reported by a previous study (Farias et al., 2013). In the study by Farias et al. (2013), the maximum inhibitory effect was seen between 6 and 24 h of incubation whether the CQ was added at 1 h after infection with no further addition, or CQ replenished after 12 and 24 h after initial addition at 1 h. This inhibitory effect of CQ is past the entry and fusion events, well into translation and initiation of replication phases. Moreover, in our study, we provide evidence showing that the profile of inhibition of DENV by CQ is similar to that by AQ (Fig. 5) as the effect of AQ was lost when added after ~15–20 h post-infection (Fig. 4E and F).

CQ seems to have diverse antiviral effects depending on the virus. For example, in the HIV-1 infection of primary, or established T or monocytes, CQ did not seem to inhibit entry or production of steady state levels of HIV-1 mRNA but did affect the production of HIV-1 virions (Sperber et al., 1993). CQ has been reported to increase the intracellular pH and interferes with prM to M cleavage at the virion maturation step in DENV2 life cycle but 100 μM concentration was used in that study (Randolph et al., 1990). CQ has been used in other therapeutic applications. For example, CQ is being used in a randomized controlled clinical study for treatment of dengue in Vietnamese adults (Tricou et al., 2010). CQ has also been to have antiviral activities against HIV-1 (Solomon and Lee, 2009; Sperber et al., 1993) and SARS Coronavirus (De Clercq, 2006; Vincent et al., 2005) as well as for treatment of cancer and arthritic diseases. Moreover, ferroquine (FQ), a CQ derivative structurally similar to AQ, inhibited HCV infection at an early step by interfering with fusion (Vausselin et al., 2013) and had minor inhibitory effects on viral replication and assembly. From the currently available data, we conclude that each quinoline compound interferes at a different stage in flaviviral life cycle. Further studies are required to identify the target and precise step that is targeted by AQ as well as derivatives of the drug that are least cytotoxic for treatment of DENV infected patient population.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.03.014.

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