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

Siwaporn Boonyasuppayakorn [a,1], Erin D. Reichert [a,2], Mark Manzano [a,3], Kuppuswamy Nagarajan [b], Radhakrishnan Padmanabhan [a,*]

[a] Department of Microbiology & Immunology, Georgetown University, USA
[b] Alkem Laboratories Ltd, Bangalore 560 076, India

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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.

* Corresponding author. Address: Department of Microbiology & Immunology, Georgetown University, Med-Dent Bldg., Room SW309, 3900 Reservoir Road, Washington DC 20057, USA. Tel.: +1 202 687 2092; fax: +1 202 687 1800.

E-mail addresses: sb563@georgetown.edu, tarnsiwaporn@gmail.com (S. Boonyasuppayakorn), erin.reichert@dtra.mil (E.D. Reichert), mark.manzano@northwestern.edu (M. Manzano), rp55@georgetown.edu (R. Padmanabhan).

[1] Current address: Department of Microbiology and Immunology, Faculty of Medicine, Chulalongkorn University, 1873 Rama 4 Road, Pathumwan, Bangkok 10330, Thailand. Tel.: +66 2 256 4132.

[2] Current address: Biological Therapeutics Medical S&T Division (CBM), Joint Science and Technology Office, Chemical and Biological Defense Directorate, Defense Threat Reduction Agency, Fort Belvoir, VA, USA.

[3] Current address: Department of Microbiology-Immunology, Feinberg School of Medicine, Northwestern University, Morton 6-639, 303 E. Chicago Ave, Chicago, IL 60611, USA.

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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 ~5×10^4 cells/well (24-well plate) and then incubated at 37 °C