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Novel antiviral activity of bromocriptine against dengue virus replication

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ABSTRACT

Dengue virus (DENV) infectious disease is a major public health problem worldwide; however, licensed vaccines or specific antiviral drugs against this infection are not available. To identify novel anti-DENV compounds, we screened 1280 pharmacologically active compounds using focus reduction assay. Bromocriptine (BRC) was found to have potent anti-DENV activity and low cytotoxicity (half maximal effective concentration [EC_{50}], 0.8–1.6 μM; and half maximal cytotoxicity concentration [CC_{50}], 53.6 μM). Time-of-drug-addition and time-of-drug-elimination assays suggested that BRC inhibits translation and/or replication steps in the DENV life cycle. A subgenomic replicon system was used to verify that BRC restricts RNA replication step. Furthermore, a single amino acid substitution (N374H) was detected in the NS3 protein that conferred resistance to BRC. In summary, BRC was found to be a novel DENV inhibitor and a potential candidate for the treatment of DENV infectious disease.

1. Introduction

Dengue virus (DENV) is transmitted to humans by *Aedes* mosquitoes; it causes dengue fever (DF) and dengue hemorrhagic fever (DHF), which is a self-limiting febrile illness (Gubler, 1998). DF is relatively mild, but DHF leads to the life-threatening dengue shock syndrome (DSS); mortality in patients diagnosed with DHF and DSS is 1–5%. DENV annually infects 50–100 million humans in tropical and sub-tropical regions, posing a considerable public health problem in over 100 countries (Simmons et al., 2012; WHO, 2013).

DENV belongs to the family *Flaviviridae* and consists of four serotypes (DENV1–4). These viruses are enveloped and have a single-stranded positive-sense RNA genome of approximately 11 kb (Lindenbach et al., 2007). A single long open reading frame of the viral RNA encodes a polyprotein that is processed by cellular and viral proteases into three structural (C, prM, and E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5). The structural proteins form the virus particles and play roles in receptor binding, virus fusion, and virion assembly. NS proteins are responsible for the replication of the viral genome and evasion from host immunity.

Many antiviral compounds have been reported to inhibit DENV replication *in vitro* and *in vivo* (Lim et al., 2013). High-throughput screening (HTS) using viral-enzyme assay, and subgenomic

Abbreviations: DENV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; NS, non-structural; BRC, bromocriptine; HTS, high-throughput screening; MOI, multiplicity of infection; hpi, hours post infection.

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replicon cells assay have been used for developing antiviral drugs by screening chemical libraries (Lim et al., 2013; Noble et al., 2010; Wang et al., 2015; Yang et al., 2014; Zou et al., 2011b). However, no approved antiviral drug is yet available for the treatment of DENV infectious disease.

In this study, we screened small-molecule chemical library, LOPAC®1280, for agents with antiviral activity against infectious DENV-1 by using focus reduction assay and found that 2-bromo-α-ergocriptine (bromocriptine: BRC) could inhibit DENV replication. BRC interfered with DENV post-translation and/or RNA synthesis steps. Furthermore, a single amino acid substitution in the NS3 protein (N374H) was involved in resistance to BRC. These findings suggest that BRC inhibits DENV replication by targeting the NS3 protein.

2. Materials and methods

2.1. Cell culture

Baby hamster kidney cells (BHK-21) and African green monkey kidney cells (Vero) were maintained in Eagle's minimum essential medium (EMEM; Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS; JR Scientific), penicillin, and streptomycin sulfate (Nacalai Tesque, Japan) under 5% CO_2 at 37 °C. Human hepatoma Huh7 cells and human embryonic kidney (293T) cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, USA) supplemented with 10% FBS and antibiotics under 5% CO_2 at 37 °C. Hybridoma-producing anti-E antibody (HB-112) was maintained in Roswell Park Memorial Institute 1640 medium (RPMI1640; Life Technologies, USA) supplemented with 10% FBS and antibiotics under 5% CO_2 at 37 °C, and its supernatant was centrifuged at 1000 × *g* for 5 min.

2.2. Viruses

DENV-1 02-20 strain was derived from a full-length infectious cDNA clone, D1 (02-20)/pMW119 (Tajima et al., 2006). DENV-2 16681 strain was kindly provided by Dr. Takeshi Kurosu and Dr. Kazuyoshi Ikuta (Research Institute for Microbial Diseases, Osaka University, Japan). DENV-3 00-40 (Ito et al., 2007) and DENV-4 09-48 strains (unpublished) were isolated in 2000 from a Japanese traveler returning from Thailand and in 2009 from a Japanese traveler returning from Indonesia, respectively. These DENV1-4 strains were cultured in Vero cells. TBEV Oshima strain was propagated in BHK-21 cells (Takashima et al., 1997). These viruses were stored at −80 °C until use.

2.3. Focus reduction assay and chemical compounds

BHK-21 cells were seeded in a 96-well plate (5×10^4 cells per well). At 1 day after seeding, DENV-1 (02-20 strain) was infected at 50 focus forming units per well. After 1 h of incubation, culture supernatant was replaced with EMEM containing 2% FBS, 1% methyl cellulose, and 10 μM of the test compound. After additional 3 days of cultivation, cells were fixed with 4% paraformaldehyde and permeabilized with 0.01% Triton X-100. The permeabilized cells were incubated with 4G2, which was produced from HB112 hybridoma, as the primary antibody. Next, Dako Envision kit/HRP was used as the secondary antibody, and di-amino benzidine tetra hydroxyl carbonate was used for staining.

The LOPAC®1280 library (Sigma–Aldrich, USA) was used for screening in this study. Additional bromocriptine (BRC; bromocriptine mesylate) and other dopamine agonists quinpirole and rotigotine were purchased from Sigma–Aldrich.

2.4. MTT assay

BHK-21 cells were seeded in a 96-well plate (5×10^4 cells per well). After 3 days of cultivation with the compound (0–10 μM), 30 μL of 0.5% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well. After 3 h of incubation, the supernatant was replaced with 100 μL of methanol with 1% Triton-X100, and then vortexed vigorously to dissolve the formazan. Absorbance at 565 nm and reference at 655 nm were measured using a microplate reader (Berthold Technologies, Germany). Half maximal cytotoxicity concentration (CC_{50}) was calculated using the Reed and Muench method (Reed and Muench, 1938).

2.5. Plaque assay

BHK-21 cells were seeded in 12-well plates (5×10^5 cells per well). After 1 h of DENV inoculation, EMEM supplemented with 2% FBS and 1% methyl cellulose was added, and the cells were incubated for additional 5–6 days. Next, the cells were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) and stained with methylene blue. The half maximal effective concentration (EC_{50}) against each virus was calculated as described above.

2.6. Time-of-drug-addition assay

BHK-21 cells were seeded in 12-well plates (5×10^5 cells per well). The cells were infected with DENV-1 (multiple of infection [MOI] = 1) for 1 h. Next, the viral inoculum was removed, and the cells were washed twice with PBS. At 0, 2, 4, 6, 8, 12, 18, and 24 h post-infection, 10 μM of BRC was added to the infected cells. After 24 h of infection, cell culture supernatant was collected, and viral titer was measured using the plaque assay.

2.7. Time-of-drug-elimination assay

BHK-21 cells were seeded in 12-well plates (5×10^5 cells per well). The cells were infected with DENV-1 at MOI of 1 for 1 h. Next, the cells were washed with PBS twice, and fresh medium containing 10 μM BRC was added. After 2, 4, and 6 h of infection, the cells were washed with PBS twice and were cultivated in fresh medium. After 24 h of infection, cell culture supernatant was collected, and viral titer was measured by plaque assay.

2.8. Transient DNA-based subgenomic replicon assay

A transient replicon assay was conducted using DNA-based *Gaussia* luciferase expressed replicon (DGL2) and DGL2-mut as described previously (Kato et al., 2014). BHK-21 cells were seeded in 24-well plates (2.5×10^5 cells per well) and transfected with 100 ng of DGL2 or DGL2-mut by using X-treamGENE HP DNA transfection reagent (Roche Diagnostics, Schweiz), and 0–10 μM of each compound was added at time zero. The cell culture medium was collected and replaced with fresh medium containing 10 μM of each compound every 24 h. The luciferase activity of the culture medium was measured using a microplate reader.

2.9. Production and isolation of mutant DENV-1 resistant to BRC

BHK-21 cells (5×10^5) were seeded in 6-well plates and infected with DENV-1 at MOI of 0.01 for 1 h. After infection, culture medium was replaced with fresh medium containing 10 μM BRC. At 3 or 4 days after transfection, the cell culture supernatant was collected and inoculated to naive BHK-21 cells containing 10 μM BRC for 16 times. The supernatant was subjected to plaque