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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

purification, and the viruses representing each plaque were propagated in BHK-21 cells in the presence of BRC. Plaque purification was performed as follows: BHK-21 cells were inoculated with bulk virus in EMEM containing 2% FBS, 10 μM BRC, and 1% low-melting agarose (Roche Diagnostics, Switzerland). After 5 days of cultivation, EMEM containing Neutral Red was added, and the observed plaque was picked up and transferred to naive BHK-21 cells. The isolated 12 independent viruses were propagated in BHK-21 cells. The bulk virus (after 16 consecutive passages) and 12 plaque-derived isolates were assessed for their sensitivity against 10 μM BRC.

2.10. Reverse transcription polymerase chain reaction and sequencing

The viral RNA was extracted and purified using a viral RNA extraction kit (Qiagen, Germany) following the manufacturer's instructions. cDNA was synthesized using SuperScript-III (Life Technologies, USA) and amplified using KOD-Plus Neo (TOYOBO, Japan) as 5 fragments. The amplified DNA was purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Germany), sequenced using Big-Dye Terminator v3.1, and analyzed on an ABI3130xl Genetic Analyzer (Applied Biosystems, USA).

2.11. Construction of N374H mutant virus and subgenomic replicon clone

A point mutation at the 374th amino acid of NS3 protein from asparagine (N) to histidine (H) was inserted into the infectious molecular clone DENV1 (02-20)/pMW119, designated N374H virus, and reporter subgenomic replicon DGL2, designated N374H replicon. Briefly, high-fidelity inverse PCR was performed using 5′-phosphorylated primers. The PCR product was ligated and transformed to Stbl2 competent cells (Life Technologies, USA). Sequences of mutant plasmids were verified as above described. These plasmids were transfected to 293T cells using Lipofectamine 2000 (Life Technologies, USA) and cultured for 3 days. The virus titer of cell culture supernatant was measured using plaque assay.

2.12. Statistical analysis

Data are expressed as means and standard deviations (SD). All statistical analyses were performed using Student's *t*-test; $P < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of compounds with anti-DENV activity from the chemical libraries

Novel anti-DENV compounds were identified by conducting focus reduction assay by using small molecule library consisting of 1280 pharmacologically active compounds. In the primary screening, BHK-21 cells infected with DENV-1 (02-20 strain) were cultured in the presence of each compound at a final concentration of 10 μM and reduction of viral focus formation was assessed. Next, compounds causing cell toxicity were excluded by evaluating cytotoxicity by using the MTT assay as the secondary screening (Fig. 1A). Through these screenings, 2-bromo-α-ergocriptine (bromocriptine: BRC) was identified as one of potent inhibitors of DENV-1, with an EC_{50} of 1.6 μM and a CC_{50} of 53.6 μM (Fig. 1B, C and Table 1). Among the hit compounds, we also found naltrindole, which has been reported as a potent DENV inhibitor that targets the NS4B protein (van Cleef et al., 2013). The inhibiting effect of BRC against the other DENV serotypes and other flaviviruses was also examined. The antiviral activity of BRC against DENV-2 to DENV-4 and tick-borne encephalitis virus (TBEV) was analyzed using plaque assay. BRC showed antiviral activity against not only DENV-1 but also other DENV serotypes and TBEV. The EC_{50} against DENV-2, 3, 4, and TBEV were 1.2, 0.8, 0.8, and 11.3 μM, respectively (Fig. S1 and Table 1). Since dengue fever is a human disease, we further investigated the anti-DENV-1 property of BRC in human A549, Huh7, and 293T cells and confirmed its antiviral activity in these cells without cytotoxicity (Fig. S2). Because BRC has been reported to be an agonist of dopamine receptors 2 and 3 (Geurts et al., 1999; Perachon et al., 1999; Velasco and Luchsinger, 1998), whether the agonistic activity was associated with the antiviral effect was then examined. Unexpectedly, quinpirole (mainly D2 receptor agonist (Fernandez-Duenas et al., 2012)) and rotigotine (mainly D3 receptor agonist (Scheller et al., 2009)) did not suppress DENV-1 replication (Fig. 1D). These results suggest that BRC inhibits flavivirus genus replication through a mechanism that is independent of the already-known pharmacological action.

3.2. BRC inhibits early stage of DENV life cycle

The stage of DENV life cycle that is affected by BRC was determined by performing time-of-drug-addition assay. During a single flaviviral life cycle, viral proteins are translated from genomic RNA in the first 1–5 h post infection (hpi); viral RNA synthesis occurs after 5 hpi, and progeny virions are assembled and released after 12 hpi (Chambers et al., 1990; Qing et al., 2009; Wang et al., 2011) (Fig. 2A). At 0, 2, 4, 6, 8, 12, and 18 hpi, culture medium was changed to 10 μM BRC-containing medium. At 24 hpi, the viral titer in the culture supernatant was quantified using plaque assay. The addition of BRC up to 6 hpi significantly reduced the virus yield (Fig. 2B). This result suggests that BRC might inhibit an early stage in the DENV life cycle. The target of BRC to DENV replication stage was also defined by performing time-of-drug-elimination assay. The data showed that BRC did not suppress the virus titer in the culture supernatant up to 2 hpi, although the virus titer was significantly suppressed when treatment commenced at 4 hpi (Fig. 2C). Thus, we concluded that viral attachment and entry stages were unlikely to be the points of action targeted by BRC.

3.3. BRC suppresses DENV RNA replication

To further clarify the mechanism of BRC-mediated DENV suppression, we used a reporter subgenomic replicon assay. The transient replicon system is a useful tool for estimating the stage of action (Fig. 3A). BRC remarkably reduced the level of luciferase activity 72 h post transfection with DGL2 in a dose-dependent manner (Fig. 3B); however, it did not affect the luciferase activity of non-replicative mutant DGL2-mut at any concentration tested (Fig. 3C). These results suggest that BRC inhibits DENV replication in the stages between post translation and viral RNA genome replication.

3.4. Isolation of BRC-resistant cloned virus

The responsible region in viral elements was identified by generating BRC-resistant virus variants by conducting 16 serial passages of DENV in the presence of 10 μM BRC. Resistance of these virus variants against 10 μM BRC was confirmed by conducting plaque reduction assay (data not shown). Next, BRC-resistant cloned viruses were isolated by plaque purification, and we obtained 12 clones. Among these clones, 4 were equally resistant to 10 μM BRC, as revealed by plaque assay. Viral RNA from the 4 resistant clones was purified and reverse transcribed to generate

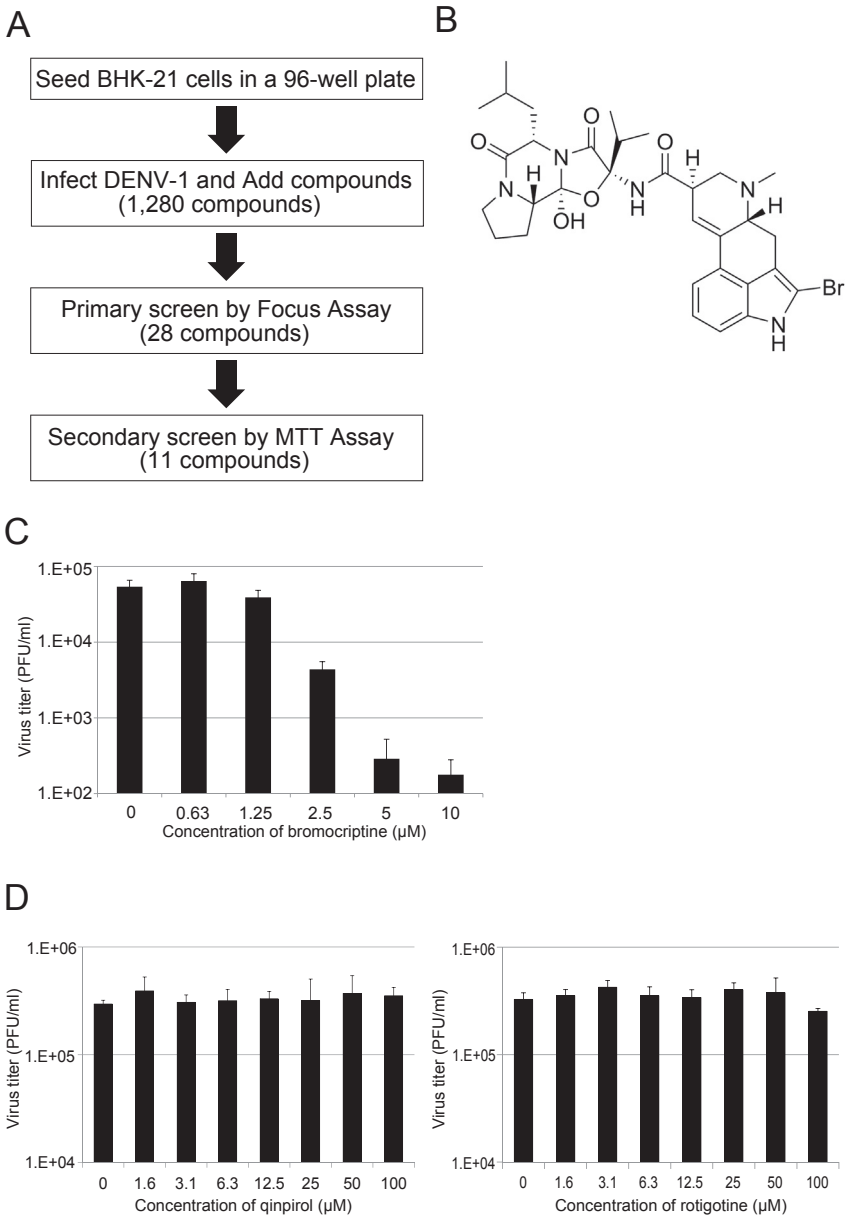

Fig. 1. BRC inhibits DENV replication. (A) Flow chart of the screen. (B) Structural formula of BRC. (C) BRC inhibits DENV replication in a dose-dependent manner. (D) Quinpirole and rotigotine do not suppress DENV replication. The viral titer of culture supernatant derived from DENV-1-infected BHK-21 cells at 72 hpi in the presence of increasing concentrations of BRC. Each data point represents the average (±standard deviation) from triplicate experiments.

Table 1
EC_{50} of BRC against DENV1-4 and TBEV.

EC_{50} (μM)					CC_{50} (μM)
DENV-1	DENV-2	DENV-3	DENV-4	TBEV	
1.6	1.2	0.8	0.8	11.3	53.6

cDNA. The change in viral genome that correlated with reduced susceptibility to BRC was mapped by determining the nucleotide sequences of the cloned viruses and comparing them to the parent DENV-1 (02-20). Although several mutations were found in the viral genome, the only common mutation among 4 viral clones was asparagine (N) to histidine (H) at the 374th amino acid in NS3 (N374H) (Table 2).

3.5. Efficacy of mutation in NS3 for conferring BRC resistance

Next, we examined whether N374H mutation is the responsible region for BRC resistance by inserting the mutation into the molecular clone of the parent strain and reporter replicon, DGL2. The mutant virus and replicon were cultured in the presence of increasing amount of BRC ranging from 0 to 10 μM, and the viral titer or luciferase activity was measured in the cell culture supernatant. The sensitivity curve of N374H mutant was shifted to a higher concentration of BRC compared with that in the parent virus, although the plaque size was smaller and the growth kinetics was lower than the parent virus. The EC_{50} of parent virus was 1.6 μM, whereas that of the N374H virus was slightly higher, 3.1 μM (Fig. 4A). In the replicon system, susceptibility to BRC showed similar trend. The EC_{50} of the parent replicon was 1.6 μM, and that of the mutant replicon was similarly as high (2.5 μM) as that of the

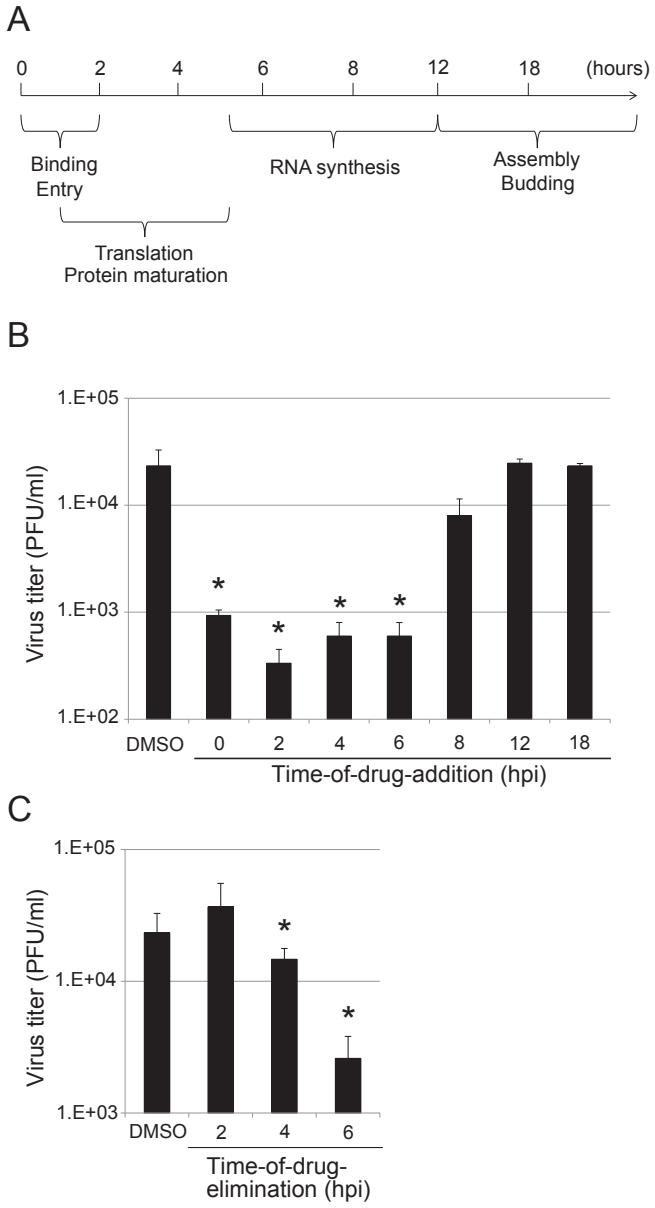

Fig. 2. BRC inhibits the early stage of DENV life cycle. (A) Time course of DENV propagation. Within 2 h, virus particles attach to host cell receptor and enter into the cell. At 5 h, viral genome RNA is released into the cytoplasm and translated to viral polyprotein. Subsequently, translated polyprotein is cleaved by host and viral proteases to mature proteins. From 5 h to 12 h, viral genome is replicated by viral RdRp and further proteins were translated from the amplified genome. After 12 h, amplified genome and translated structural proteins are assembled to progeny virions, and mature virions are released from the cells. (B) Time-of-drug-addition assay. 10 μM of BRC was added at 0, 2, 4, 6, 8, 12, and 18 hpi. At 24 hpi, released virus titer was analyzed by plaque assay. *; $P < 0.05$. (C) Time-of-drug-elimination assay. Cells were treated with BRC for 0–2 h, 0–4 h, and 0 to 6 hpi. At 24 hpi, released virus titer was analyzed by plaque assay. *; $P < 0.05$. Each data point represents the average (±standard deviation) from triplicate experiments.

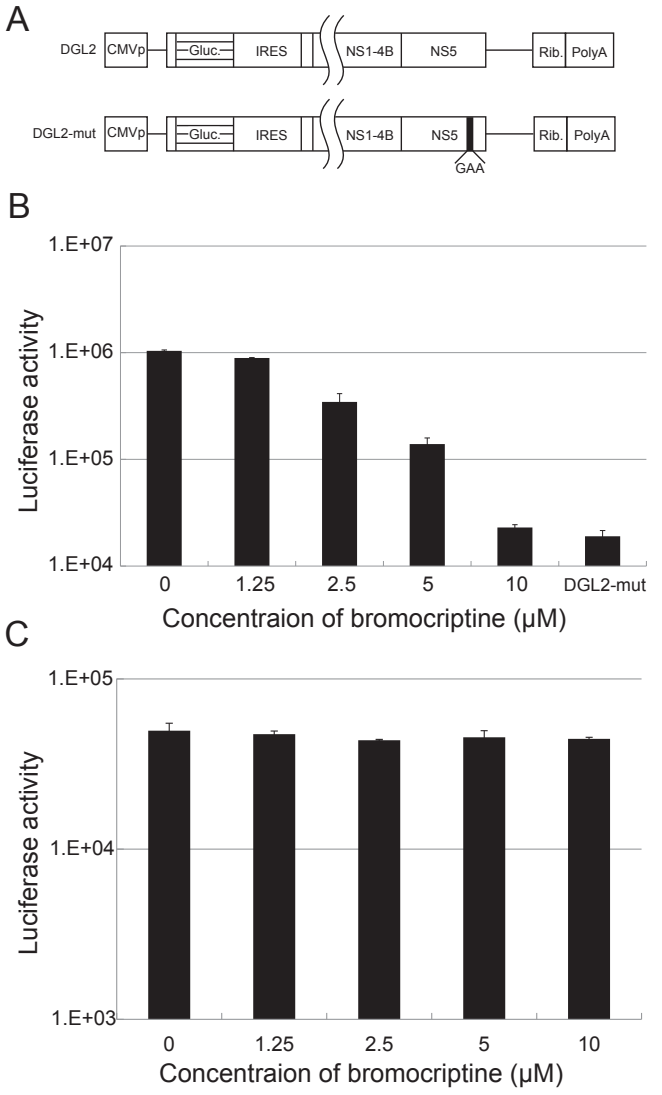

Fig. 3. BRC inhibits subgenomic reporter replicon. (A) Schematic representation of DGL2 and DGL2-mut. DGL2 does not produce infectious virus, since a large region of its genome is deleted, including the structural genes encoding the C, prM, and E proteins. It is self-replicating and its replication level is assessed by measuring luciferase activity, since the deleted sequence is replaced by a fusion construct encoding the secretory *Gaussia* luciferase (GLuc) gene and an internal ribosomal entry site (IRES). DGL2-mut contains an inactivating amino acid change (GDD to GAA) of *NS5*, which encodes an RNA-dependent RNA-polymerase. Luciferase activity of DGL2-mut is not derived from viral replication but is CMV promoter dependent. CMVp: Cytomegalovirus promoter. GLuc: secretory *Gaussia* luciferase. IRES: internal ribosome entry site. GAA: mutation in the active center for RNA-dependent RNA polymerase. Rib.: ribozyme sequence. (B and C) Luciferase activity of supernatant after 72 h of DNA-based replicon, DGL2 (B) or DGL2-mut (C), transfection in the presence of increasing concentrations of BRC. DGL2-mut was inserted in non-replicative mutation in RdRp. Each data point represents the average (±standard deviation) from triplicate experiments.

mutant virus (Fig. 4B). Although the N374H mutation does not confer complete resistance to BRC, it does produce a reduced susceptibility to BRC.

4. Discussion

DENV is the most common human arthropod-borne virus and causes a severe problem worldwide, mainly in tropical and subtropical regions. However, a licensed vaccines and specific chemical treatments against DENV infectious diseases are not available; hence, the development of antiviral compounds has been desired. We screened a chemical library by using viral focus reduction assay to identify novel anti-DENV compounds. Focus assay is a relatively low-throughput method but enables the evaluation of all the steps of viral life cycle. Therefore, we used DENV-1 02-20 strain and BHK-21 cells to perform HTS for clear focus formation and easy access to positive/negative signals in 96-well plates. Through the screening, we identified a novel DENV inhibitor, BRC, in a small compound

Table 2
Mutation sites in BRC resistant DENV genome.

Associated protein (Amino acid position)	NS2B (87)	NS3 (191)	NS3 (305)	NS3 (374)	NS3 (617)	NS4B (79)	NS4A (83)	NS5 (5)	NS5 (316)
WT	K	I	V	N	G	G	P	Q	A
clone1	T	I	A	H	A	W	A	E	A
clone2	K	R	A	H	A	W	P	Q	A
clone3	K	I	A	H	G	G	P	Q	A
clone4	K	I	V	H	G	G	P	Q	T

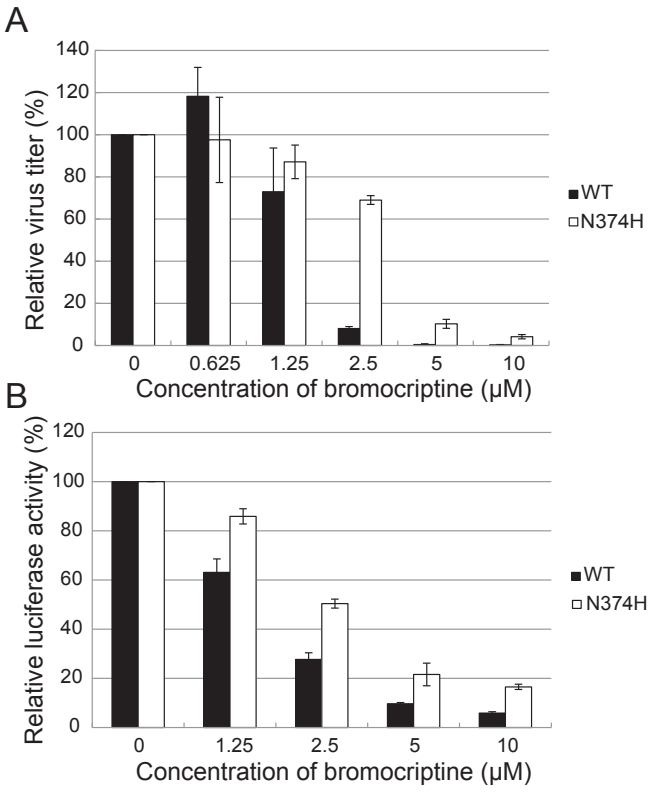

Fig. 4. Efficacy of mutation in NS3 for conferring BRC resistance. (A) Viral titer at 72 hpi in the culture supernatant of BHK-21 cells infected with wild-type DENV1 (WT) or N374H mutant (N374H) treated with 0–10 μM of BRC. (B) Luciferase activity in BHK-21 cells transfected with DGL2 (WT) or DGL2-N374H mutant (N374H) treated with 0–10 μM of BRC at 72 h post-transfection. All data were normalized to their control (0 μM of BRC). Each data point represents the average (±standard deviation) from triplicate experiments.

library.

BRC has been reported to be an agonist of dopamine receptors 2 and 3, and it is approved as a remedy for galactorrhea and Parkinson's disease (Ginther et al., 2012; Perachon et al., 1999; Zhang et al., 1999). In this study, the other dopamine receptor agonists, quinpirole and rotigotine, did not show anti-DENV activity, although BRC showed antiviral activity against all DENV serotypes 1–4 and TBEV. The inhibitory effect of BRC on TBEV replication was lower than its effect on DENV (Table 1). We assume that the viral replication rate is related to the efficacy of BRC. In fact, the propagation rate of TBEV was higher than that of DENV1–4. This finding could be explained by the two viruses differing in their level of dependence on the factor that BRC targets for their replication. These results suggest that BRC might inhibit the replication of a spectrum of *Flaviviridae* species, with varying efficacy.

Many antiviral compounds were selected as inhibitors of viral enzymes. During flavivirus drug development, some compounds targeted viral enzyme activities such as protease, helicase, MTase, and RdRp. We examined the influence of BRC on DENV NS2B3 protease, but no effect on viral protease activity was observed (Fig. S3). Furthermore, we assessed the effect of BRC on protease, NTPase, and RdRp activities using AlphaScreen assay (Takahashi et al., 2012), but no effect on viral enzyme activities was observed (data not shown). Thus, other viral proteins or host factors are likely to be involved in the antiviral mechanism exerted by BRC.

Time-of-drug-addition and time-of-drug-elimination assays suggested that BRC inhibited the translation or RNA synthesis steps in DENV life cycle. Furthermore, replicon assay showed that BRC restricts the same step of virus life cycle. Our subgenomic replicon system could specifically analyze translation and viral genome RNA replication steps, because DGL2 replicon deleted a large part of the genomic region containing structural genes in DENV. Consequently, the replicon system is a useful tool for not only screening the antiviral reagents but also evaluating the specific steps in virus life cycles.

The analysis of resistant mutants to BRC revealed that a single mutation (N374H) in NS3 protein could confer partial resistance to BRC. NS3 and NS5 are essential components of the replication complex consisting of several viral non-structural proteins as well as host proteins. Physical interaction of NS3 helicase domain region which includes N374 has been reported for NS4B and NS5 (Chen et al., 1997; Kapoor et al., 1995; Tay et al., 2015; Umareddy et al., 2006; Zou et al., 2011a, 2015) as well as several host proteins (Aguirre et al., 2012; Heaton et al., 2010; Khadka et al., 2011; Luo et al., 2015). These results suggested that the 374th amino acid is one of multiple regions targeted by BRC and other mutations or combinations might be required for complete resistance. We also note that N374H is a conservative change and BRC may still be able to bind to the target albeit less efficiently. We propose that BRC will serve as a useful tool compound to understand the details of the flaviviral RNA replication mechanism which can lead to new target identification and the design and development of specific antivirals.

Animal models are preclinical tools to evaluate the *in vivo* efficacy of antiviral compounds or vaccines (Chan et al., 2015; Omatsu et al., 2011). We also preliminary evaluated the *in vivo* efficacy of BRC using the AG129 mouse (deficient in interferon alpha/beta and gamma receptors) model (Watanabe et al., 2012), however, the compound was not beneficial to viremia reduction or mouse survival (Fig. S4). *In vivo* metabolism might influence its efficacy. Thus, further improvement of the compound and careful *in vivo* observation may be required if our finding is translated into clinical settings.

Taken together, BRC has novel antiviral activity against flavivirus, especially DENV, and it restricts the post-translation or early RNA synthesis steps. BRC is an approved agent for treatment of other diseases and can be used as an anti-DENV drug. However, the detailed mechanisms of its antiviral activity need to be identified for it to be developed as an anti-DENV drug. Such investigations should employ an appropriate model, such as cultured primary human cells. Moreover, *in vivo* studies using non-human primate models may be also beneficial to understanding the pharmacokinetics and antiviral activity of BRC for its application as a novel antiviral drug (Omatsu et al., 2011).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2016.04.014.

Glossary

2-Bromo-α-ergocriptine bromocriptine
Half maximal cytotoxicity concentration CC_{50}
Half maximal effective concentration EC_{50}

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