Antiviral Research 172 (2019) 104643

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Identification of inhibitors of dengue viral replication using replicon cells expressing secretory luciferase

Fumihiro Kato[a,b,c,1], Yasunori Nio[d], Kazumi Yagasaki[c,e], Rieko Suzuki[f], Makoto Hijikata[g], Tomoyuki Miura[a], Isao Miyazaki[e], Shigeru Tajima[c], Chang-Kweng Lim[c], Masayuki Saijo[c], Tomohiko Takasaki[h], Takayuki Hishiki[a,f,i,*]

[a] Laboratory of Primate Model, Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto, Japan
[b] Department of Research Promotion, Japan Agency for Medical Research and Development, Tokyo, Japan
[c] Department of Virology I, National Institute of Infectious Diseases, Tokyo, Japan
[d] Regenerative Medical Unit, T-CiRA, Takeda Pharmaceutical Company, Tokyo, Japan
[e] Biomedical Research Institute Co, Ltd, Chiba, Japan
[f] Department of Microbiology, Kanagawa Prefectural Institute of Public Health, Kanagawa, Japan
[g] Laboratory of Tumor Viruses, Institute for Frontier Life and Medical Sciences, Kyoto University, Kyoto, Japan
[h] Kanagawa Prefectural Institute of Public Health, Kanagawa, Japan
[i] Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

ARTICLE INFO

Keywords:
Dengue virus
Replicon
Gaussia luciferase
High-throughput screening
Antiviral drug

ABSTRACT

Dengue virus (DENV) is the causative agent of dengue fever (DF), dengue haemorrhagic fever (DHF), and dengue shock syndrome (DSS) and continues to be a public health problem in the tropical and subtropical areas. However, there is currently no antiviral treatment for DENV infection. In this study, our aim was to develop a stable reporter replicon cell system that supports constant viral RNA replication in cultured cells. The isolated replicon cells exhibited high levels of luciferase activity in the culture supernatant concomitant with expression of virus-encoded NS1, NS3 and NS5 proteins in the cells. The NS1, NS3 proteins and dsRNA were detected in the replicon cells by immunofluorescence analysis. Furthermore, the anti-DENV inhibitors ribavirin and bromocriptine significantly reduced the luciferase activity in a dose-dependent manner. High-throughput screening with a compound library using the stably-transfected replicon cells showed a Z′ factor value of 0.57. Our screening yielded several candidates including one compound that has already shown anti-DENV activity. Taken together, our results demonstrate that this DENV subgenomic replicon cell system expressing a secretory luciferase gene can be useful for the high-throughput screening of anti-DENV compounds and the analysis of the replication mechanism of the DENV RNA.

Dengue virus (DENV), transmitted to humans by *Aedes* mosquitoes, is the aetiological agent of dengue fever (DF) and dengue haemorrhagic fever (DHF), which are self-limited febrile illnesses (Gubler, 1998). DF is relatively mild, but DHF can lead to the life-threatening dengue shock syndrome (DSS). The mortality rates of DHF and DSS in untreated cases exceed 20%, but decrease to less than 1% with proper medical care (WHO, 2018). DENV infects 390 million humans annually mainly in tropical and sub-tropical regions, posing a considerable public health problem in over 100 countries (Simmons et al., 2012; Bhatt et al., 2013). However, in spite of great concern over the world, there is still urgent need for the development of specific antiviral drugs for the prevention and treatment of the disease. (see Table)

High-throughput screening (HTS) system is a very important tool for the discovery of drug candidates. For example, hepatitis C virus replicon systems were developed and used for the discovery of several antiviral drug candidates (Lohmann et al., 1999; Ishii et al., 2006). Furthermore, some HTS systems have been already reported in flavivirus studies (Shi et al., 2002; Jones et al., 2005; Kato et al., 2016;

Abbreviations: CC_{50}, cytotoxicity concentration; DENV, dengue virus; EC_{50}, half-maximal effective concentration; Gluc, Gaussia luciferase; HTS, High-throughput screening; MPA, mycophenolic acid; NS, non-structural

* Corresponding author. Department of Microbiology, Kanagawa Prefectural Institute of Public Health, 1-3-1 Shimomachiya, Chigasaki, Kanagawa, 253-0087, Japan.
E-mail address: hishiki.4e6r@pref.kanagawa.jp (T. Hishiki).
[1] Present address: Department of Virology III, National Institute of Infectious Diseases, Tokyo, Japan

https://doi.org/10.1016/j.antiviral.2019.104643
Received 26 July 2019; Received in revised form 24 October 2019; Accepted 28 October 2019
Available online 31 October 2019
0166-3542/

Table 1
List of anti-DENV compounds discovered in this study.

Name	CCT018159	Mycophenolic acid (MPA)	Nocodazole	PD102807	Arcyriaflavin A	IMD0354
Function	Hsp90 inhibitor	GTP synthesis inhibitor	Microtubule inhibitor	Muscarinic receptor antagonist	CDK4/Cyclin D1 inhibitor	IκB kinase-β (IKKβ) Inhibitor
EC_{50} (μM)	1.1	0.3	0.3	4.7	1.0	1.2
CC_{50} (μM)	32.1	> 100	> 100	12.1	> 100	12.5
SI	29.2	> 333.3	> 333.3	2.7	> 100	10.4
Max Inhibition	84.3%	97.0%	72.7%	75.7%	69.1%	97.8%

EC_{50} and CC_{50} were calculated using Reed and Muench method.
Maximum inhibition rate was calculated the luciferase activity between 10 μM and negative control.

Patkar et al., 2009; Zhang et al., 2017). However, the drug discovery efforts have not been enough to develop antiviral drugs to the point of commercialization (Low et al., 2017).

DENV genome is approximately 11 kb consisting of a single positive strand RNA with an open reading frame that is translated into a large polyprotein that is cleaved to three structural proteins (C, prM, E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) by host and viral proteases (Lindenbach et al., 2007). For flaviviruses, since the structural proteins are not essential for genome replication in the cell, engineered sub-genome lacking the structural gene encoding region of the DENV genome can still be replicated in the form of a replicon (Chambers et al., 1990; Ng et al., 2007; Kato and Hishiki, 2016). Replicon does not produce any progeny viral particles and therefore is a valuable tool for the observation of the translation and genome synthesis steps in a wide range of laboratory setting fitted with sophisticated imaging equipment. Furthermore, if a reporter protein gene is inserted into the replicon genome this greatly facilitates the observation of the viral genome amplification. The inclusion of the reporter gene results in what is called a reporter replicon which is only capable of transient replication and suffers from the disadvantage that the cells need to be transfected for each assay (Kato et al., 2014). The addition of an antibiotic resistance gene makes it possible to select the cells where the replicon is functional and produce stable replication and reporter gene expression. Thus, the construction of a stable reporter replicon represents an invaluable tool that can be applied to a HTS and used for the discovery of antiviral drugs and the analysis of viral replication.

In this study, we aimed to develop a robust stable reporter replicon cell system that supports viral RNA replication continuously in cells derived from human hepatoma. We validated the robustness of the system by screening available chemical libraries as a tool for HTS and analysis of viral replication.

To develop a stable reporter subgenomic replicon cell system, RGL2, a DENV-1 molecular clone derived transient replicon which was reported previously (Kato et al., 2014; Tajima et al., 2006), was used as a template for the stable replicon construction (Fig. 1A). Major segments of the genes encoding the structural proteins were deleted. The sequences encoding the 34 N-terminal amino acids of the C protein and the 35 C-terminal amino acids of the E protein were preserved. The deleted sequences were replaced with a fragment containing a fusion of the gene encoding *Gaussia* luciferase (Gluc), the foot-and-mouth-disease virus 2A (FMDV2A) cleavage sequence, the neomycin-resistance gene, and the EMCV internal ribosome entry site (IRES). This replicon DNA was used as template for the in vitro production of transcribed replicon RNA (Fig. 1B). The subgenomic RNA was transfected into human hepatoma cells (Huh7), which were then cultured in the presence of G418. After G418 selection, 96 clones were picked up and expanded. Among the 96 selected clones, #68 exhibited the highest levels of luciferase activity in the culture supernatant. Therefore, we conducted further characterization of this clone.

The production of DENV proteins in the cell clone #68 was examined by western blotting. As shown in Fig. 2A, NS1, NS3, and NS5 proteins were detected in the cell lysate of the replicon cells and migrated in a manner similar to the cell lysate of DENV-infected cells. Furthermore, co-localization of NS3 and dsRNA, as well as NS1 and NS3 was detected by IFA (Fig. 2B). These results suggest that the produced replicon cells maintain the viral RNA replication and viral protein expression of the replicon in a stable manner.

To evaluate drug susceptibility of the replicon cells, ribavirin and bromocriptine which are compounds with already known anti-DENV properties were used (Kato et al., 2016). Replicon cells were cultured with 0–100 μM of ribavirin or 0–10 μM of bromocriptine for 72 h and then the luciferase expression was measured. The luciferase activity in the culture supernatant was significantly decreased in a dose dependent manner, the EC_{50} of ribavirin and bromocriptine were found to be 1.7 and 0.16 μM, and the CC_{50} were > 100 and > 10 μM, respectively (Fig. 3). Furthermore, intracellular luciferase activity and replicon RNA expression level were decreased similarly to extracellular luciferase activity by ribavirin (Figs. S1A–S1C). These results suggest that our replicon cells have susceptibility against anti-DENV compounds.

Next, to evaluate the assay quality of our replicon cells for HTS, the Z′ factor was calculated using the anti-DENV compound bromocriptine as the positive control and DMSO as the negative control. The average of luciferase activity for the positive control was 146,873, and for the negative control was 660,659 (Fig. 4). The calculated Z' factor was 0.57, suggesting that our replicon cells is a suitable method for HTS.

To identify novel anti-DENV compounds, a small molecule library, Tocriscreen compound libraries (#2890, R and D systems, USA), consisting of 1120 biologically active compounds was screened for inhibition of luciferase activity. In the first screening, the replicon cells were co-cultured with 10 μM of each compound for 3 days and the luciferase activity in the culture supernatant was measured in duplicate to eliminate false positives. Around 34 compounds were found to suppress the luciferase activity by more than 50%, and the cytotoxicity was less than 50% using Cell counting kit-8 (DOJINDO, Japan). In the second screening, the antiviral activity was evaluated using infectious virus. DENV-1 (02–20) infected-Huh7 cells were cultured in the presence of each compound at a final concentration of 10 μM, and the viral titer in the cell culture supernatant was measured by plaque assay. Among the 34 compounds identified in the first screening, 14 compounds suppressed the viral titer by more than 50%. Next, we investigated in more detail the efficacy of those compounds using our replicon cells, and found 6 compounds that exhibited significant antiviral effect without cytotoxicity using Cell counting kit-8 (Table). Among the 6 hit compounds, was mycophenolic acid (MPA), which has been reported as a potent DENV inhibitor (Diamond et al., 2002; Takhampunya et al., 2006). Taken together, these results suggest that the selected compounds are promising candidates for the development of anti-DENV agents, and also that our replicon cells are a useful tool for the HTS of anti-DENV compounds.

Development of HTS methods is one of the important factors necessary to reach break-throughs in drug discovery. In this study, we developed a HTS method using a reporter replicon stable expressing human hepatoma cell system. The replicon was constructed by

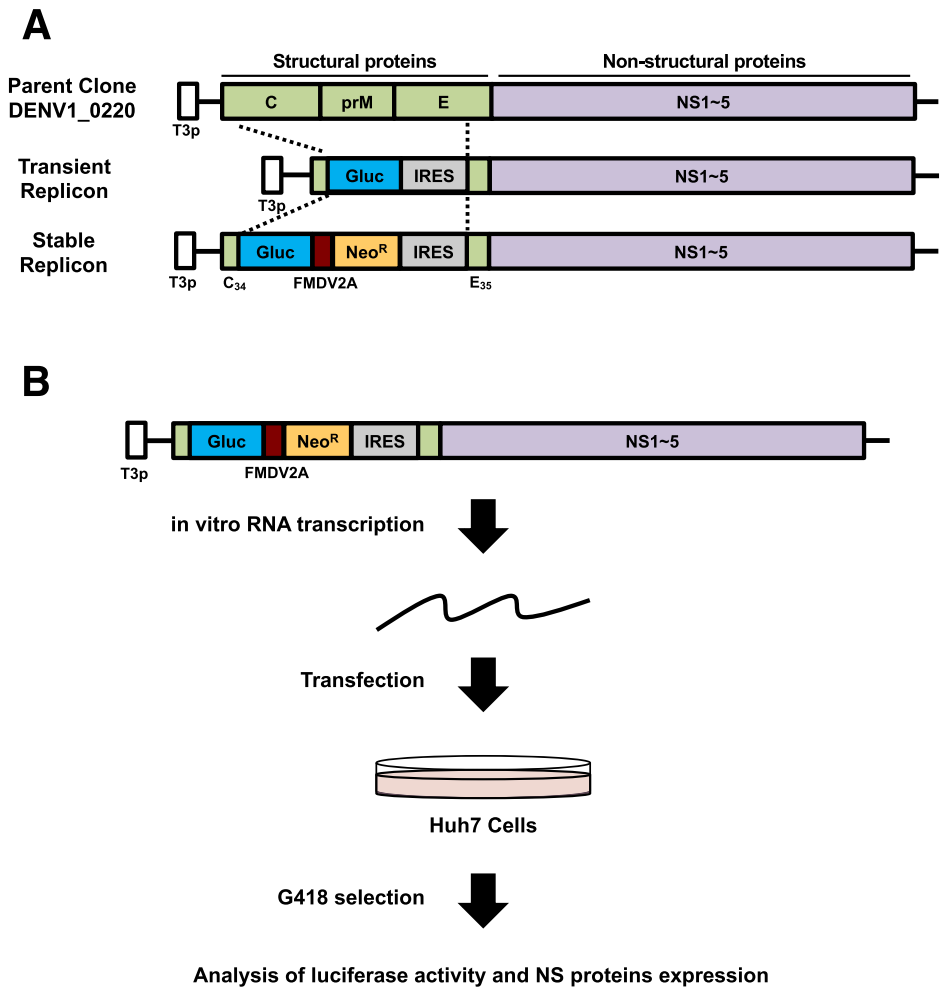


Fig. 1. Schematic outline of the construction of the replicon cells. T3p; T3 promoter, Gluc.; Gaussia luciferase gene, Neo^R; Neomycin resistant gene, and IRES; Internal ribosome entry site. The replicon RNA was synthesized using a mMESSAGE mMACHINE T3 Transcription kit (Thermo Fisher Scientific, USA) and purified using RNeasy Mini kit (QIAGEN, Germany). The RNA was transfected to Huh7 cells using the Lipofectamine MessengerMAX Reagent (Thermo Fisher Scientific, USA), which were then selected by using culture medium containing G418. Isolated cells were analyzed for luciferase activity and NS proteins expression.

modifications from a transient *Gaussia* luciferase (Gluc)-replicon previously reported (Kato et al., 2014). Gluc is smaller than firefly or *Renilla* luciferase, yields a stronger signal, and is secreted into culture medium (Tannous et al., 2005). Because of the use of a secretory reporter protein, it also eases importantly the measurement of the cells viability or cytotoxicity since it does not require cell lysis. These characteristics make our replicon cell system using Gluc a suitable method for HTS. However, in the first screening using this replicon cells, there is a possibility that false positive is caused by inhibition the enzymatic activity or the secretion of luciferase. To exclude it, another analysis using infectious virus or Gluc expression plasmid is important as the second screening. At least, we found that ribavirin and bromocriptine did not suppress the enzymatic activity and the secretion of luciferase using Gluc expression plasmid (Figs. S2A and S2B).

The Z′ factor is one of most important index to evaluate the efficiency of HTS. Generally, a screening method needs a Z′ factor value of more than 0.5 (Zhang et al., 1999). In this work, when using our replicon cells, the Z' factor was of 0.57 and it demonstrates that the replicon cell system is a suitable method for screening. We screened chemical libraries containing 1120 compounds using this replicon system and infectious DENV and identified 6 compounds that have a suppressive effect against the replicon. Among the 6 hit compounds it was reassuring to find mycophenolic acid (MPA), which is a well-known anti-DENV compound that has a defined antiviral mechanism that suppresses viral genome replication. This suggest that the replicon cell system described in this study is suitable for the discovery of anti-DENV drugs. This replicon can be used for the specific analysis of the RNA translation and synthesis steps during the DENV lifecycle, our results therefore suggest that the MPA and other identified compounds act by inhibiting the RNA translation or synthesis of the DENV genome. Several studies have reported that target factors of other hit compounds are associated with various viral lifecycle (Kalamvoki and Roizman, 2010; Ujino et al., 2012; Amaya et al., 2014; Cheng et al., 2015; Foo and Chee, 2015), therefore it is very interesting to clarify the molecular mechanism in DENV life cycle. Further studies are ongoing to elucidate the mechanism of antiviral activity of them.

In conclusion, we developed a DENV replicon cells expressing secretory luciferase and confirmed that it is a useful tool for drug discovery and for the analysis of virus replication, and allowed the identification of 5 novel compounds with antiviral activity against DENV.

Acknowledgments

We are grateful to Subhash Vasudevan and Satoru Watanabe (Duke-NUS Medical School) for insightful comments and suggestions as well as the gift of anti-NS1, -NS3, and -NS5 antibodies. We also thank the members of the Laboratory of Primate Model (Institute for Frontier Life and Medical Sciences, Kyoto University) and the Department of Microbiology and Cell Biology (Tokyo Metropolitan Institute of Medical Science) for helpful discussions. This work was supported by JSPS

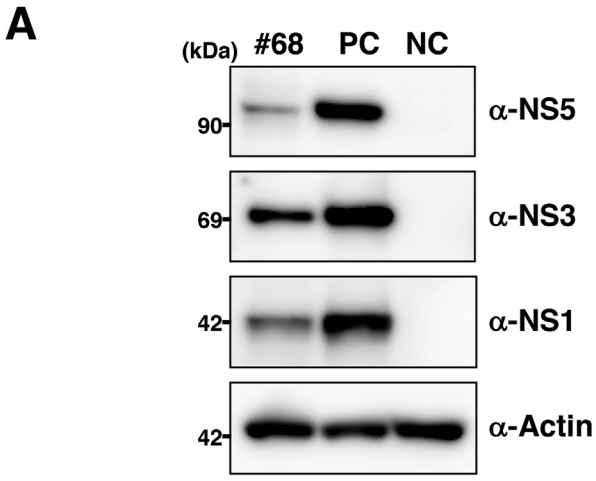
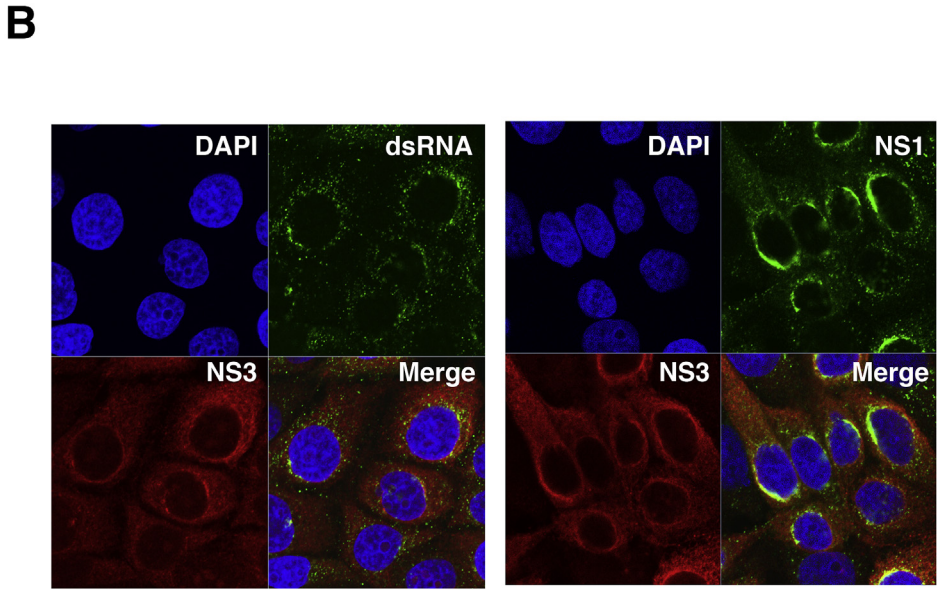


Fig. 2. Expression of viral proteins and genome in replicon cells. (A) Western blot analysis of NS1, NS3, and NS5 protein expression in replicon cells #68 (lanes 1), DENV infected Huh7 cells (lane 2; PC), and naive Huh7 cells (lanes 3; NC). Mouse anti-DENV NS1 antibody (#165, Biomedical Research Institute, Japan), human anti-DENV NS3 antibody (3F8, (Moreland et al., 2010)), and human anti-DENV NS5 antibody (Zhao et al., 2014) were used as primary antibody. (B) Indirect immunofluorescence analysis of dsRNA (green) and NS3 (red), as well as NS1 (green) and NS3 (red) in replicon cells. Mouse anti-DENV NS1 antibody (56.2, (Watanabe et al., 2012)), a human anti-DENV NS3 antibody (3F8, (Moreland et al., 2010)), a rabbit anti-DENV NS3 antibody (GTX124252, Genetex, USA), and a mouse anti-dsRNA antibody (J2; English and Scientific Consulting, Hungary), were used as primary antibody.



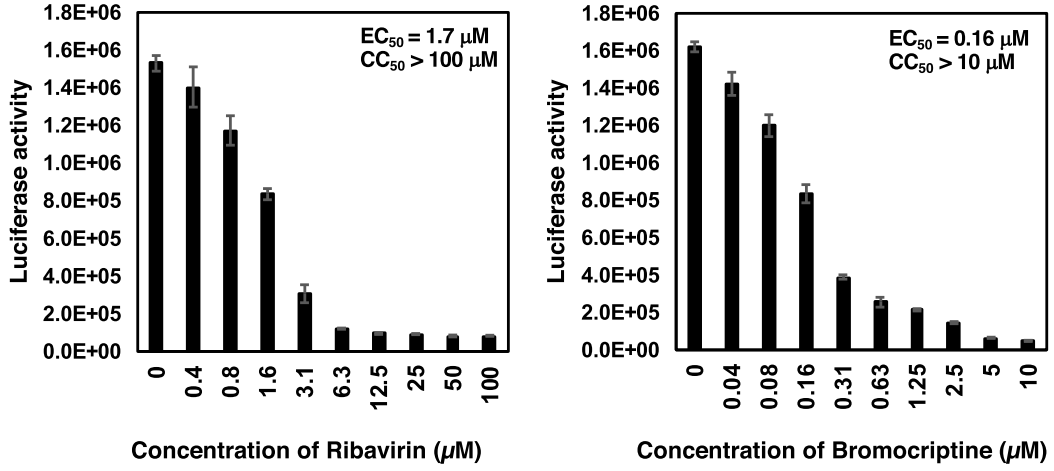


Fig. 3. Susceptibility of replicon cells to the action of anti-DENV compounds. Replicon cells were plated into 96 well and cultured overnight. Medium was changed to different concentrations of anti-DENV inhibitors (ribavirin and bromocriptine). After 72 h of incubation, the cells were washed by PBS, and fresh medium was added. After 4 h from the medium changing, luciferase activity in the culture supernatant was measured. The y-axis represents raw luciferase data. The EC_{50} of each antiviral compound was calculated using Reed and Muench method.

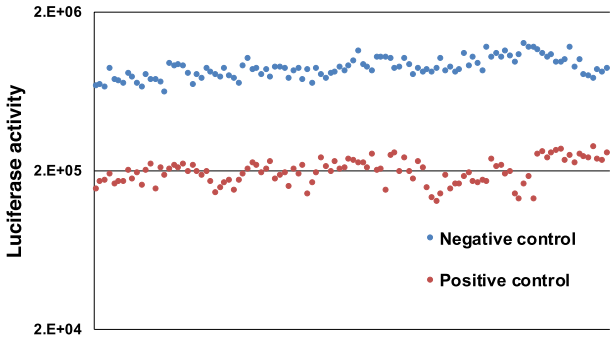


Fig. 4. Evaluation for HTS method using Z′ factor. The Z′ factor determination for replicon cells was done using bromocriptine as positive control and DMSO as negative control. 8 wells of a 96-well plate were treated for 72 h with 10 μM bromocriptine or 0.1% DMSO in each control and the assay was repeated 14 times. The y-axis represents raw luciferase data. The Z′ factor was calculated using following formula; Z = 1-(3 × SD (pos.) - 3 × SD (neg.))/(Ave. (Pos.) - Ave. (Neg.)). A value of more than 0.5 is recognized as applicable in HTS.

KAKENHI under Grant Number JP15K19109 and JP17K08870, and by the Research Program on Emerging and Re-emerging Infectious Diseases of the Japan Agency for Medical Research and Development (AMED) under Grant Number JP18fk0108035.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2019.104643.

References

Amaya, M., Voss, K., Sampey, G., Senina, S., de la Fuente, C., Mueller, C., Calvert, V., Kehn-Hall, K., Carpenter, C., Kashanchi, F., Bailey, C., Mogelsvang, S., Petricoin, E., Narayanan, A., 2014. The role of IKKβ in Venezuelan equine encephalitis virus infection. PLoS One 9 (2), e86745.

Bhatt, S., Gething, P.W., Brady, O.J., Messina, J.P., Farlow, A.W., Moyes, C.L., Drake, J.M., Brownstein, J.S., Hoen, A.G., Sankoh, O., Myers, M.F., George, D.B., Jaenisch, T., Wint, G.R., Simmons, C.P., Scott, T.W., Farrar, J.J., Hay, S.I., 2013. The global distribution and burden of dengue. Nature 496, 504–507.

Chambers, T.J., Hahn, C.S., Galler, R., Rice, C.M., 1990. Flavivirus genome organization, expression, and replication. Annu. Rev. Microbiol. 44, 649–688.

Cheng, H., Lear-Rooney, C.M., Johansen, L., Varhegyi, E., Chen, Z.W., Olinger, G.G., Rong, L., 2015. Inhibition of Ebola and Marburg virus entry by G protein-coupled receptor antagonists. J. Virol. 89 (19), 9932–9938.

Diamond, M.S., Zachariah, M., Harris, E., 2002. Mycophenolic acid inhibits dengue virus infection by preventing replication of viral RNA. Virology 304, 211–221.

Foo, K.Y., Chee, H.Y., 2015. Interaction between flavivirus and cytoskeleton during virus replication. BioMed Res. Int. 427814.

Gubler, D.J., 1998. Dengue and dengue hemorrhagic fever. Clin. Microbiol. Rev. 11, 480–496.

Ishii, N., Watashi, K., Hishiki, T., Goto, K., Inoue, D., Hijikata, M., Wakita, T., Kato, N., Shimotohno, K., 2006. Diverse effects of cyclosporine on hepatitis C virus strain replication. J. Virol. 80, 4510–4520.

Jones, C.T., Patkar, C.G., Kuhn, R.J., 2005. Construction and applications of yellow fever virus replicons. Virology 331, 247–259.

Kalamvoki, M., Roizman, B., 2010. Interwoven roles of cyclin D3 and cdk4 recruited by ICP0 and ICP4 in the expression of herpes simplex virus genes. J. Virol. 84 (19), 9709–9717.

Kato, F., Hishiki, T., 2016. Dengue Virus Reporter Replicon Is a Valuable Tool for Antiviral Drug Discovery and Analysis of Virus Replication Mechanisms. Viruses 8.

Kato, F., Ishida, Y., Oishi, S., Fujii, N., Watanabe, S., Vasudevan, S.G., Tajima, S., Takasaki, T., Suzuki, Y., Ichiyama, K., Yamamoto, N., Yoshii, K., Takashima, I., Kobayashi, T., Miura, T., Igarashi, T., Hishiki, T., 2016. Novel antiviral activity of bromocriptine against dengue virus replication. Antivir. Res. 131, 141–147.

Kato, F., Kobayashi, T., Tajima, S., Takasaki, T., Miura, T., Igarashi, T., Hishiki, T., 2014. Development of a novel Dengue-1 virus replicon system expressing secretory Gaussia luciferase for analysis of viral replication and discovery of antiviral drugs. Jpn. J. Infect. Dis. 67, 209–212.

Lindenbach, B.D., Thiel, H.-J., Rice, C.M., 2007. *Flaviviridae*: the Viruses and Their Replication, fifth ed. Lippincott-Raven Publishers.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., Bartenschlager, R., 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285, 110–113.

Low, J.G., Ooi, E.E., Vasudevan, S.G., 2017. Current status of dengue therapeutics Research and development. J. Infect. Dis. 215, S96–s102.

Moreland, N.J., Tay, M.Y., Lim, E., Paradkar, P.N., Doan, D.N., Yau, Y.H., Geifman Shochat, S., Vasudevan, S.G., 2010. High affinity human antibody fragments to dengue virus non-structural protein 3. PLoS Neglected Trop. Dis. 4, e881.

Ng, C.Y., Gu, F., Phong, W.Y., Chen, Y.L., Lim, S.P., Davidson, A., Vasudevan, S.G., 2007. Construction and characterization of a stable subgenomic dengue virus type 2 replicon system for antiviral compound and siRNA testing. Antivir. Res. 76, 222–231.

Patkar, C.G., Larsen, M., Owston, M., Smith, J.L., Kuhn, R.J., 2009. Identification of inhibitors of yellow fever virus replication using a replicon-based high-throughput assay. Antimicrob. Agents Chemother. 53, 4103–4114.

Shi, P.Y., Tilgner, M., Lo, M.K., 2002. Construction and characterization of subgenomic replicons of New York strain of West Nile virus. Virology 296, 219–233.

Simmons, C.P., Farrar, J.J., Nguyen v, V., Wills, B., 2012. Dengue. N. Engl. J. Med. 366, 1423–1432.

Tajima, S., Nukui, Y., Ito, M., Takasaki, T., Kurane, I., 2006. Nineteen nucleotides in the variable region of 3' non-translated region are dispensable for the replication of dengue type 1 virus in vitro. Virus Res. 116, 38–44.

Takhampunya, R., Ubol, S., Houng, H.S., Cameron, C.E., Padmanabhan, R., 2006. Inhibition of dengue virus replication by mycophenolic acid and ribavirin. J. Gen. Virol. 87, 1947–1952.

Tannous, B.A., Kim, D.E., Fernandez, J.L., Weissleder, R., Breakefield, X.O., 2005. Codon-optimized Gaussia luciferase cDNA for mammalian gene expression in culture and in vivo. Mol. Ther. 11, 435–443.

Ujino, S., Nishitsuji, H., Sugiyama, R., Suzuki, H., Hishiki, T., Sugiyama, K., Shimotohno, K., Takaku, H., 2012. The interaction between human initiation factor eIF3 subunit c and heat-shock protein 90: a necessary factor for translation mediated by the hepatitis C virus internal ribosome entry site. Virus Res. 163 (1), 390–395.

Watanabe, S., Tan, K.H., Rathore, A.P., Rozen-Gagnon, K., Shuai, W., Ruedl, C., Vasudevan, S.G., 2012. The magnitude of dengue virus NS1 protein secretion is strain dependent and does not correlate with severe pathologies in the mouse infection model. J. Virol. 86, 5508–5514.

WHO, 2018. Dengue and Severe Dengue.

Zhang, J.H., Chung, T.D., Oldenburg, K.R., 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. J. Biomol. Screen 4, 67–73.

Zhang, Q.Y., Li, X.D., Liu, S.Q., Deng, C.L., Zhang, B., Ye, H.Q., 2017. Development of a stable Japanese encephalitis virus replicon cell line for antiviral screening. Arch. Virol. 162, 3417–3423.

Zhao, Y., Moreland, N.J., Tay, M.Y., Lee, C.C., Swaminathan, K., Vasudevan, S.G., 2014. Identification and molecular characterization of human antibody fragments specific for dengue NS5 protein. Virus Res. 179, 225–230.