VIRUS-CELL INTERACTIONS



Characterization of Recombinant *Flaviviridae* Viruses Possessing a Small Reporter Tag



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Journal of

MICROBIOLOGY VICOLOGY

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ABSTRACT The family Flaviviridae consists of four genera, Flavivirus, Pestivirus, Pegivirus, and Hepacivirus, and comprises important pathogens of human and animals. Although the construction of recombinant viruses carrying reporter genes encoding fluorescent and bioluminescent proteins has been reported, the stable insertion of foreign genes into viral genomes retaining infectivity remains difficult. Here, we applied the 11-amino-acid subunit derived from NanoLuc luciferase to the engineering of the Flaviviridae viruses and then examined the biological characteristics of the viruses. We successfully generated recombinant viruses carrying the split-luciferase gene, including dengue virus, Japanese encephalitis virus, hepatitis C virus (HCV), and bovine viral diarrhea virus. The stability of the viruses was confirmed by five rounds of serial passages in the respective susceptible cell lines. The propagation of the recombinant luciferase viruses in each cell line was comparable to that of the parental viruses. By using a purified counterpart luciferase protein, this split-luciferase assay can be applicable in various cell lines, even when it is difficult to transduce the counterpart gene. The efficacy of antiviral reagents against the recombinant viruses could be monitored by the reduction of luciferase expression, which was correlated with that of viral RNA, and the recombinant HCV was also useful to examine viral dynamics in vivo. Taken together, our findings indicate that the recombinant Flaviviridae viruses possessing the split NanoLuc luciferase gene generated here provide powerful tools to understand viral life cycle and pathogenesis and a robust platform to develop novel antivirals against Flaviviridae viruses.

IMPORTANCE The construction of reporter viruses possessing a stable transgene capable of expressing specific signals is crucial to investigations of viral life cycle and pathogenesis and the development of antivirals. However, it is difficult to maintain the stability of a large foreign gene, such as those for fluorescence and bioluminescence, after insertion into a viral genome. Here, we successfully generated recombinant *Flaviviridae* viruses carrying the 11-amino-acid subunit derived from NanoLuc luciferase and demonstrated that these viruses are applicable to *in vitro* and *in vivo* experiments, suggesting that these recombinant *Flaviviridae* viruses are powerful tools for increasing our understanding of viral life cycle and pathogenesis and that these recombinant viruses will provide a robust platform to develop antivirals against *Flaviviridae* viruses.

Received 7 September 2017 Accepted 19 October 2017

Accepted manuscript posted online 1 November 2017

Citation Tamura T, Fukuhara T, Uchida T, Ono C, Mori H, Sato A, Fauzyah Y, Okamoto T, Kurosu T, Setoh YX, Imamura M, Tautz N, Sakoda Y, Khromykh AA, Chayama K, Matsuura Y. 2018. Characterization of recombinant *Flaviviridae* viruses possessing a small reporter Tag. J Virol 92:e01582-17. https://doi.org/10 .1128/JVI.01582-17.

Editor J.-H. James Ou, University of Southern California

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KEYWORDS Flaviviridae, reporter virus, antiviral screening, in vivo dynamics

The family *Flaviviridae* comprises single-stranded positive-sense RNA viruses and consists of four genera: *Flavivirus, Hepacivirus, Pegivirus,* and *Pestivirus.* All members encode 2 to 4 structural proteins followed by 7 to 8 nonstructural protein genes flanked by 5' and 3' untranslated regions (UTRs), with the genera *Hepacivirus, Pegivirus,* and *Pestivirus* sharing a much higher degree of similarity (1, 2). Although they share similar genome components, their host ranges and tissue tropisms differ strikingly. The viruses of genus *Flavivirus* can infect more than 50 species, with a wide host range from reptiles to mammals; these include dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus, tick-borne encephalitis virus, and Zika virus (3). The viruses of genus *Pestivirus*, including bovine viral diarrhea virus (BVDV), classical swine fever virus, and border disease virus, are causative agents for even-toed ungulate animals (4). Hepatitis C virus (HCV) from the genus *Hepacivirus* infects only humans and chimpanzees (5).

Since the first discovery of a green fluorescent protein (GFP) from the Aequorea victoria jellyfish in the 1960s (6), reporter proteins have been applied for an array of naturally occurring fluorescent proteins and genetically engineered derivatives for the monitoring and visualization of various intracellular events, including gene expression, protein localization, trafficking, interaction, and signaling pathways (7). In virus research, fluorescent proteins have been used to examine viral life cycles, tropism, and transmission (8–10). In addition to visualized fluorescent proteins, bioluminescence has become a powerful tool to investigate viral pathogenesis, immune responses to infection, and the efficacy of therapies in living animals (11). The bioimaging of viral infection has been achieved by using recombinant viruses possessing a reporter protein, allowing imaging to identify the specific sites of viral replication. Although recombinant viruses possessing a reporter protein have been demonstrated to be effective tools for the detection and quantification of viral replication both in vitro and in vivo, there is a size limitation regarding the accommodation of a foreign gene into a viral genome. In studies of Flaviviridae viruses, several groups have attempted to generate reporter-tagged viruses (12-14), but full-length infectious clones of viruses, especially of flavivirus, often are difficult to work with because many of the cDNA clones are deleterious for bacteria (15), and recombinant viruses carrying a large reporter gene are genetically unstable. In addition, the insertion of foreign genes into an irrelevant locus results in the disruption of the structural RNA elements required for viral replication.

To overcome these issues, we employed NanoLuc binary technology (NanoBiT) in this study (16). NanoBiT is a split reporter consisting of two subunits, high-affinity NanoBiT (HiBiT) (17) and large NanoBiT (LgBiT). The individual subunits do not possess enzymatic activity, but when HiBiT and LgBiT associate in cells or in vitro, the complex regains its NanoLuc enzymatic activity. We chose to insert smaller HiBiT subunits into selected viruses from the family Flaviviridae (DENV, JEV, HCV, and BVDV) and investigated optimal sites for the insertion to generate stable recombinant viruses. Our findings revealed that in susceptible cell lines, the propagation of the recombinant viruses possessing the split luciferase gene was comparable to that of parental viruses and significantly higher than that of recombinant viruses that had a full-length luciferase gene. The efficacy of antiviral reagents against the recombinant viruses could be monitored by the reduction of luciferase expression, which was correlated with that of viral RNA in vitro and in vivo. These results suggest that the recombinant Flaviviridae viruses generated in this study are powerful tools that can be used to increase our understanding of the viral life cycle and pathogenesis of Flaviviridae viruses. The recombinant viruses also will provide a robust platform for the development of therapeutic measures against infection with Flaviviridae viruses.

RESULTS

Determination of a suitable locus for insertion of the split-luciferase (HiBiT) gene into HCV genome and characterization of recombinant HCV. To determine an

ideal locus for the insertion of HiBiT into the HCV genome, we constructed 10 recombinant cDNA clones of HCV carrying the HiBiT gene (VSGWRLFKKIS) and a linker sequence (GS) in the N terminus of each viral protein (Fig. 1A). Infectious titers in the culture supernatants and intracellular luciferase activities in Huh7.5.1 cells lentivirally transduced with the other piece of the NanoLuc protein (LgBiT; Fig. 1B) then were determined at 72 h posttransfection with the recombinant HCV clones. Among the 10 HCV clones we examined, three viruses carrying HiBiT in the N terminus of E1, E2, or NS2 succeeded in the recovery of infectious viruses and luciferase expression (Fig. 1C). The highest viral titer and luciferase activity were obtained by the transfection of the HCV clone carrying HiBiT in the N terminus of NS2. In addition, we confirmed that the HiBiT tag was fused to viral NS2 (Fig. 1D). We therefore selected the recombinant virus for further characterization.

Earlier studies reported that the construction of the HCV recombinants incorporated a gene cassette encoding a full-length luciferase and 2A peptide of picornavirus in frame with the N terminus of the NS2 gene (18, 19). To examine the effects of HiBiT insertion on virus propagation, growth kinetics of the parental HCV JFH-1 strain (WT) and the recombinants carrying HiBiT (HBiT Luc), or a full-length NanoLuc luciferase gene (Nano Luc), was determined in Huh7.5.1 cells (Fig. 1E). The propagation of the HiBiT recombinant was slow but reached levels comparable to that of the parental virus at 72 h postinfection. Importantly, the propagation was significantly higher than that of the recombinant possessing a full-length luciferase, suggesting that the length of the insertion is critical for virus propagation.

To examine the stability of the reporter gene in the HiBiT recombinant, we serially passaged the virus in Huh7.5.1 cells for five rounds. The infectious titers were slightly elevated by the passages and almost reached plateau levels at around 10^{6.6} focus-forming units (FFU)/ml (Fig. 1F). Although two amino acid substitutions were observed (a threonine-to-isoleucine substitution at position 1496 in NS3 and a cysteine-to-serine substitution at position 2460 in NS5A) after the serial passage, the HiBiT luciferase gene was maintained (Fig. 1G). In addition, luciferase activities in cells infected with the recombinant viruses were similar even after five rounds of passage (Fig. 1H). These data suggest that the HiBiT gene in the N terminus of NS2 was stable in the HCV genome.

To evaluate the specificity of the luciferase activity of the recombinant HCV, the parental and recombinant viruses were inoculated into Huh7.5.1 cells, and intracellular HCV RNA and luciferase activity were determined (Fig. 1I). Although the increase of the intracellular HCV RNA levels of the recombinant HCV was comparable to that of the parental virus, the luciferase activity in the cells infected with the recombinant virus, but not with the parental virus, increased in accord with the increase of intracellular viral RNA.

Although only particular human hepatic cell lines are susceptible to the propagation of HCV, nonhepatic 293T cells that exogenously express Claudin1 (CLDN1), microRNA-122 (miR-122), and apolipoprotein E (ApoE) permit the complete propagation of HCV (20, 21). Expression of the LgBiT luciferase and these host factors was examined by immunoblotting in 293T cells upon transduction with lentiviral vectors (Fig. 2A). Although increases of intracellular luciferase activity and viral RNA upon infection with the recombinant HCV were observed in 293T cells expressing the LgBiT, CLDN1, and miR-122, an increase of those in the culture supernatants was only achieved when ApoE was additionally provided (Fig. 2B), suggesting that the HiBiT HCV recombinant can be used to monitor the replication of HCV.

Construction and characterization of the recombinant flaviviruses carrying the HiBiT gene. To construct the luciferase-tagged recombinant flaviviruses, we used the cDNA clones of JEV strain AT31 (22) from *Culex* mosquito-borne virus and the DENV-4 strain H241 from *Aedes* mosquito-borne virus as templates. The nonstructural protein NS1 of flavivirus is a tolerant protein for insertion of foreign peptide (23, 24), and secretory NS1 is utilized as a marker for diagnosis of infection (25). Therefore, we inserted the HiBiT luciferase gene in frame with the N terminus of the NS1 gene of JEV and DENV (Fig. 3A) and examined the biological characteristics of the recombinant



FIG 1 Determination of a suitable locus for insertion of HiBiT into the HCV genome and characterization of recombinant HCV. (A) A schematic representation of HCV and sequence of the HiBiT luciferase and the adjacent viral gene. Arrows indicate the insertion sites of the HiBiT luciferase gene. (B) Expression of the LgBiT protein was determined by immunoblotting at 48 h postransduction of lentiviruses into Huh7.5.1 cells. (C) Infectious titers and luciferase activity were determined upon infection with the recombinant HCV carrying the HiBiT luciferase at the respective N terminus of the viral protein gene. (D) Huh7.5.1 cells were infected with the parental and the recombinant HCV carrying HiBiT at the N terminus of NS2 at an MOI of 1. On day 3 postinfection, cells were lysed and subjected to immunoblotting. (E) Huh7.5.1 cells were inoculated with 100 µl of culture supernatants obtained from the transfected cells. The supernatants were collected at 24, 48, and 72 h postinfection. Virus titers were determined in duplicate in Huh7.5.1 cells. Asterisks indicate significant differences (*, P < 0.05) versus the results of the HiBiT recombinant virus. (F) The recombinant HCV carrying the tag in the N terminus of NS2 was passaged on Huh7.5.1 cells for five rounds, and 100 µl of culture supernatants was used to infect naive Huh7.5.1 cells. At 72 h postinfection, the culture supernatants were collected, virus titers were determined, and samples were subjected to passage. (G) Sequence analyses of the recombinant viruses before (P0) and after five rounds of passage (P5). (H) The luciferase activities in cells infected with the recombinant viruses before and after passage. (I) The intracellular RNA copies and luciferase activity were determined upon infection with the parental and recombinant HCV carrying the HiBiT luciferase at an MOI of 1. Asterisks indicate significant differences (*, P < 0.05) versus the results of the parental virus.



FIG 2 Evaluation of HiBiT recombinant HCV infection in nonhepatic cells. (A) The expression levels of CLDN1, ApoE, and LgBiT luciferase in 293T cells were determined by immunoblot analysis. (B) The cells were infected with the recombinant HCV at an MOI of 10, and the intracellular HCV RNA and luciferase activities at 72 h postinfection were determined by qRT-PCR and luciferase assay, respectively. The culture supernatants were harvested and infectious titers were determined by a focus-forming assay. The supernatants were also inoculated into the naive cells and the luciferase activity was determined by a luciferase assay.

viruses. The recombinant JEV was inoculated into LgBiT-expressing and nonexpressing Huh7 cells (Fig. 3B), and infectious titers and luciferase activities in the culture supernatants of the cells were determined (Fig. 3C). The infectious titers in the culture supernatants were slightly lower in the cells infected with the recombinant than in cells infected with the parental JEV (Fig. 3C, left). Luciferase activity was detected not only in the LgBiT-expressing Huh7 cells but also in nonexpressing cells upon infection with the recombinant JEV by addition of the recombinant LgBiT protein to infected cell lysates. This result suggests that expression of LgBiT in infected cells is not required for the utility of this reporter assay and extends the suitability of the assay for use in primary cell lines and/or invertebrate cell lines that may be difficult to transduce.

To evaluate the genetic stability of the recombinant JEV, we next passaged the virus in Huh7 cells five times, and infectious titers, viral gene sequence, and luciferase activities were determined (Fig. 3D, E, and F). Similar levels of infectious titers and luciferase activities were maintained, and no mutation was detected in the viral sequence of the recombinant, including the inserted gene, after five passages (Fig. 3D, E, and F), suggesting that recombinant JEV carrying the HiBiT gene is genetically stable. In addition, significant luciferase activities were detected in both Huh7 and C6/36 cells upon infection with recombinant DENV-4 possessing the HiBiT gene in the N-terminal region of NS1 (Fig. 3G), suggesting that the HiBiT luciferase-tagged system is applicable to flaviviruses.

Parental and recombinant JEV next were inoculated into Huh7 cells expressing the LgBiT and mosquito-derived C6/36 cells at a multiplicity of infection (MOI) of 0.1, and



FIG 3 Construction and characterization of the recombinant flavivirus carrying the HiBiT gene. (A) A schematic representation of flavivirus and sequence of the insertion site of HiBiT luciferase gene. The arrow indicates an insertion site of the luciferase gene. (B) The expression of the LgBiT protein was determined by immunoblotting at 48 h postransduction of lentiviruses into Huh7 cells. (C) The infectious titers and luciferase activity were evaluated in parental and LgBiT-expressing Huh7 cells upon infection with either wild-type or recombinant JEV. In the parental Huh7 cells, luciferase activity was determined by addition of the recombinant LgBiT protein. (D) The recombinant JEV was passaged on Huh7 cells for five rounds, and 100 μ l of culture supernatant was used to infect naive Huh7 cells. At 72 h postinfection, the culture supernatants were collected, virus titers before (P0) and after five rounds of passage (P5). (F) The luciferase activity and infectious titers were determined in Huh7 cells and mosquito-derived C6/36 cells upon infection with the recombinant DENV at 120 h postinfection.



FIG 4 *In vitro* growth kinetics of the HiBiT recombinant flavivirus and infectivity in susceptible cell lines. (A) Huh7 and C6/36 cells were infected with the parental and recombinant JEV. The intracellular JEV RNA level, virus titers, and luciferase activity were determined at the indicated time points by qRT-PCR, a focus-forming assay, and a luciferase assay, respectively. Asterisks indicate significant differences (*, P < 0.05) versus the results of the parental virus. (B) The indicated species of cells were inoculated with the recombinant JEV. At 72 h postinfection, the intracellular viral RNA levels, virus titers in supernatants, and luciferase signals were determined by qRT-PCR, a focus-forming assay, and a luciferase assay, respectively.

infectious titers in the culture supernatants, luciferase activities, and intracellular viral RNA levels were determined (Fig. 4A). In the C6/36 cells, the recombinant virus exhibited slow replication in the early phase but reached a level comparable to that of the parental virus in the late phase of infection. In contrast, the replication of the

recombinant JEV in the LgBiT-expressing Huh7 cells was lower than that of wild-type JEV. The luciferase activity in both cells infected with recombinant JEV, but not with parental JEV, increased in accord with the intracellular RNA.

Because flaviviruses, especially JEV, can infect various cell lines derived from invertebrate and vertebrate animals (1), we first examined the infectivity of the recombinant JEV to other cell lines derived from various mammals, including BHK-21 (hamster), HeLa (human), A549 (human), and Vero E6 (monkey) cells, in addition to the Huh7 and C6/36 cell lines (Fig. 4B). The recombinant virus exhibited a high infectivity to all of the cell lines examined, and the intracellular luciferase activity was correlated with the intracellular viral RNA. In addition, because the flavivirus NS1 is not only a component of viral replicase but also a secretory protein used as a marker for the diagnosis of flavivirus infection (25), we examined the luciferase activity in the culture supernatants of cells infected with recombinant virus. Luciferase activities in the supernatants were correlated with the intracellular viral RNA and extracellular infectious titers. These results indicate that recombinant JEV is infectious to various cell lines and that its infection can be monitored by the expression of both intracellular and extracellular luciferase.

Construction and characterization of the recombinant pestivirus carrying the HiBiT gene. The construction of a recombinant BVDV carrying a FLAG tag in the N terminus of the envelope proteins has been reported to exhibit characteristics similar to those of parental virus (26). Thus, we have generated a recombinant BVDV possessing HiBiT luciferase in the N terminus of the envelope protein E2 (Fig. 5A) and have determined its virological properties. Bovine MDBK cells were infected with parental and recombinant BVDV at an MOI of 0.1, and the infectious titers in the culture supernatants and the luciferase activities and viral RNA in the cells were determined. Because of the difficulty of achieving the exogenous expression of LgBiT protein in MDBK cells, luciferase activity was determined by the addition of recombinant LgBiT protein into cell lysates. The recombinant BVDV showed propagation that was comparable to that of the wild-type virus and produced increasing luciferase activity in cells upon infection (Fig. 5B).

Because HiBiT was inserted into the E2 envelope protein of BVDV, we examined the effect of insertion on physical properties of the viral particles. The parental and recombinant BVDV particles in the supernatants were analyzed by buoyant density ultracentrifugation, and infectious titers and viral RNA in each fraction were determined (Fig. 5C). The highest infectious titers and viral RNA of wild-type and recombinant BVDV were detected at densities of 1.07 and 1.08 g/ml, suggesting that the insertion of HiBiT in the N-terminal E2 region has no effect on particle formation of BVDV.

To evaluate the genetic stability of the recombinant BVDV, we next passaged the virus in MDBK cells five times, and infectious titers, luciferase activities, and viral genome sequences were determined. Similar levels of infectious titers at around 10^7 50% tissue culture infective doses (TCID₅₀/ml and luciferase activities were maintained, and no mutation was detected in the viral sequence of the recombinant, including the inserted gene after five passages (Fig. 5D, E, and F), suggesting that recombinant BVDV carrying the HiBiT gene is genetically stable.

Application of HiBiT *Flaviviridae* viruses for drug screening and investigation of *in vivo* viral dynamics. Direct-acting antiviral (DAA) agents have been applied in a clinical setting for chronic hepatitis C patients (27). In the present study, to determine the sensitivity of the HiBiT recombinant HCV to treatment with antiviral drugs, Huh7.5.1 cells were treated with various concentrations of DAAs (sofosbuvir [SOF], daclatasvir [DCV], and telaprevir [TVR]) or type I interferon (IFN- α) at 2 h postinfection with recombinant HCV, and intracellular HCV RNA and luciferase activities were determined at 48 h postinfection (Fig. 6A). The intracellular HCV RNA was reduced in a dosedependent manner for each reagent and was correlated with luciferase activity, suggesting that the sensitivity of luciferase expression upon infection with recombinant HCV is comparable to that of the viral RNA determined by quantitative reverse transcription-PCR (qRT-PCR).

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FIG 5 Construction and characterization of the recombinant pestivirus carrying the HiBiT gene. (A) Schematic representation of pestivirus and sequence of the insertion site of HiBiT luciferase gene. (B) MDBK cells were inoculated at an MOI of 0.1 of the parental and recombinant BVDV. The intracellular viral RNA, virus titers, and luciferase activity were determined at the indicated time points by qRT-PCR, TCID₅₀ determination, and luciferase assay, respectively. Asterisks indicate significant differences (*, P < 0.05) versus the results of the parental virus. (C) The culture supernatants of cells upon infection with the parental and recombinant BVDVs at an MOI of 1 were subjected to density gradient fractionation, and the infectious titers (upper) and viral RNA copies (lower) for each fraction were determined. (D) The recombinant BVDV was passaged on MDBK cells for five rounds, and 100 μ l of culture supernatant was used to infect naive cells. At 72 h postinfection, the culture (Continued on next page)

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To evaluate the sensitivity of recombinant JEV to treatment with antiviral reagents, Huh7 cells next were treated with mycophenolic acid (MPA) and IFN- α at 1 h postinfection with recombinant JEV, and intracellular viral RNA and luciferase activities were determined at 48 h postinfection (Fig. 6B). Intracellular viral RNA and luciferase activities were reduced in a dose-dependent manner for each reagent, as seen in the infection with recombinant HCV. MPA also exhibits antiviral activity against pestivirus (28); therefore, MDBK cells were treated with various concentrations of MPA at 1 h postinfection. Intracellular BVDV RNA and luciferase activities were determined at 48 h postinfection (Fig. 6C). In addition, recombinant BVDV was neutralized by various concentrations of polyclonal antibody against BVDV and inoculated into MDBK cells. Intracellular viral RNA and luciferase activities were reduced in a dose-dependent manner for each reagent. Collectively, these data indicate that the HiBiT recombinant *Flaviviridae* viruses generated in this study are useful for the screening of antiviral therapeutics.

To confirm the usefulness of the recombinant viruses for the screening of antiviral compounds, we next examined the commercially available protease inhibitor library (Table 1) by using recombinant HCV and JEV (Fig. 6D and E). Among the compounds we examined, three compounds (number 12, fluorouracil; number 20, pterostilbene; and number 39, DCV) exhibited more than 10-fold suppression of the luciferase expression in cells infected with recombinant HCV, comparable to suppression by IFN- α and the DAAs, including DCV and SOF. DCV (compound 39) is a well-known HCV inhibitor (29, 30). We observed here that compound 65 (DBeQ) was the only compound that could suppress luciferase expression by more than 10-fold in cells infected with recombinant JEV, i.e., comparably to IFN- α . DBeQ is a reversible inhibitor of p97/valosincontaining protein, a member of the ATPases associated with diverse cellular activities (31). Thus, we assessed the antiviral activity of DBeQ in cells infected with recombinant DENV-4 (Fig. 6F). DBeQ also showed suppression of luciferase expression comparable to that by IFN- α and MPA, suggesting that HiBiT recombinant flaviviruses are useful tools for the screening of antiviral reagents. We also confirmed that the compounds that inhibited viral replication exhibit no inhibitory effect on NanoBiT luciferase (Fig. 6G).

Moreover, to obtain the HiBiT recombinant virus without preparing cDNA clones, we used the circular polymerase extension reaction (CPER) method (32–35) for obtaining the recombinant flavivirus. The DENV-2 16681 strain in our laboratory was used to generate the HiBiT recombinant virus (Fig. 7). Following our published protocol (34, 35), we successfully obtained the infectious recombinant DENV-2, suggesting that this reporter system can be extensively applied to *Flaviviridae* viruses, including clinical isolates, without the construction of cDNA plasmids.

Finally, to evaluate the *in vivo* utility of recombinant HCV for monitoring viral dynamics and the efficacy of antiviral compounds, the HiBiT recombinant HCV was inoculated into human liver-transplanted chimeric mice (n = 15 mice total), and viral RNA and luciferase activity were determined after treatment with antivirals. Upon the inoculation of recombinant HCV into chimeric mice, the HiBiT luciferase gene was maintained in the virus and all mice became viremic (Fig. 8A), indicating that recombinant HCV can establish chronic infection *in vivo*. After treatment with a DAA (ombitasvir, or OBV) and pegylated IFN- α (PEG-IFN- α), HCV RNA was decreased in correlation with the luciferase activity (Fig. 8B), indicating that the *in vivo* viral dynamics and efficacy of antiviral reagents can be determined by using the recombinant HCV.

DISCUSSION

Reporter proteins, including fluorescent and bioluminescent proteins, have been utilized to monitor and visualize intracellular events. Due to the progress in the design

FIG 5 Legend (Continued)

supernatants were collected, virus titers were determined, and viruses were subjected to passaging. (E) The luciferase activities in cells infected with the recombinant viruses before (P0) and after five rounds of passage (P5). (F) Sequence analyses of the recombinant viruses before and after passage.



FIG 6 Application of the HiBiT Flaviviridae viruses for drug screening. (A) Huh7.5.1 cells infected with the recombinant HCV at an MOI of 1 were treated with SOF, DCV, TVR, and IFN- α at 2 h postinfection. The intracellular HCV RNA and luciferase activity levels were determined by qRT-PCR and a luciferase assay at

(Continued on next page)

of reverse genetic systems of viruses, various reporter proteins have been incorporated into many viral particles in order to elucidate viral dynamics and pathogenesis and to develop antiviral reagents. In this study, we developed the recombinant viruses of the family Flaviviridae possessing the HiBiT luciferase subunit. The most important issue for the construction of recombinant viruses is the identification of a suitable gene locus for the insertion of the foreign gene. To date, however, the protein structures of only a few viruses have been revealed, making the construction of the virus recombinants possible. Thus, we inserted the HiBiT luciferase in the N terminus of each viral protein of HCV without consideration of the structure, and we determined the infectivity of the recombinant viruses. Our findings revealed that the recombinants carrying HiBiT in the N terminus of E1, E2, and NS2, but not of other viral proteins, exhibited significant replication. Previous studies revealed crucial RNA sequence and secondary structure for viral propagation, such as cis-replication elements in HCV NS5B (36) and the conserved complementary cyclization sequence in the capsid and 3'-UTR of flaviviruses (37). It was also shown that insertions in the N terminus of E2 are tolerated by insertion of the HCV chimeric strain J6/JFH1 (38, 39). In the present study, we obtained the recombinant JFH1 strain tagged in the viral envelopes, but recombinants showed a lower replication rate than that of the parental virus, suggesting that subtle structural changes in association with host and/or other viral proteins participate in viral replication (40).

The reporter viruses constructed in previous studies used a full-length fluorescent or bioluminescent protein followed by an autoprotease, such as 2A of foot-and-mouth disease virus (18, 41, 42). The insertion of a large fragment encoding the reporter protein together with the cleavage sequences (reaching approximately 300 amino acids) into a viral genome presents the risk of impairment of viral growth and genetic stability. In this study, we observed that the recombinant HCV carrying the full-length luciferase gene exhibited a significant growth defect compared growth of the parental HCV (Fig. 1E). Recently, Eyre et al. showed that recombinant DENV carrying the full-length NanoLuc luciferase in NS1 impaired virus replication (23). Therefore, we chose to insert a smaller 11-amino-acid HiBiT peptide from the split NanoBiT luciferase system (16, 17) for generation of reporter viruses. The length of these genes is similar to that of commonly used tags, such as hemagglutinin (HA) and FLAG. We demonstrated that the NanoBiT luciferase system is applicable to *Flaviviridae* viruses, and the HiBiT recombinants were genetically stable and exhibited virological characteristics comparable to those of the parental viruses.

Although a split reporter assay is required in order to express a counterpart protein for the examination of the protein-protein interaction (43), it is generally difficult to express foreign proteins in primary and nonmammalian cells. In the present study, we showed that the NanoBiT luciferase assay can be used for cells that do not express LgBiT by the addition of the purified recombinant LgBiT protein to infected cell lysates, indicating that the NanoBiT luciferase assay also is applicable to primary and insect cells. In addition, the expression of LgBiT protein in cells showed no effect on virus replication *in vitro*. Although we have generated the recombinant *Flaviviridae* viruses carrying HiBiT in the N terminus of viral proteins, further studies are needed to determine the other suitable sites for the insertion of a foreign protein into viral protein genes. Indeed, Eyre et al. successfully generated the recombinant DENVs carrying the FLAG tag or split GFP tag within the middle site of NS1, showing high similarity to the parental DENV (23).

FIG 6 Legend (Continued)

48 h postinfection. (B) Huh7 cells infected with the recombinant JEV at an MOI of 0.1 were treated with MPA and IFN- α at 1 h postinfection. The intracellular viral RNA and luciferase signals were determined at 48 h postinfection. (C) MDBK cells infected with the recombinant BVDV at an MOI of 0.1 were treated with MPA at 1 h postinfection. The recombinant BVDV was treated with various concentrations of polyclonal antibodies at 1 h and inoculated with the complex into naive MDBK cells. The intracellular viral RNA and luciferase activity were determined by qRT-PCR and luciferase assay at 48 h postinfection. Recombinant HCV (D) and JEV (E) were subjected to a chemical library of 69 drugs to assess sensitivity. The relative luciferase activity compared with the activity of nontreated cells (M) is shown as bar graphs. (F) The sensitivity of IFN- α , MPA, and compound 65 to recombinant DENV-4 was determined as described above. (G) Huh7 cells and Huh7.5.1 cells cotransfected with the expression plasmids encoding HiBiT or LgBiT were treated with the compounds. The relative luciferase activity was determined as described above.

TABLE 1 Compour	d library of	protease	inhibitors	used i	in this	study
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1 1216 MK3102 1226714-47 C ₁ H ₂ C ₁ MO ₂ S 306.43 3 11581 Picolamine 373152.0 C ₁ H ₂ C ₁ MO ₂ S 306.14 4 T2893 Muscone 371152.0 C ₁ H ₂ M ₂ O 288.41 5 T0224 Glacotamine 31152.44 C ₁ H ₂ MO ₂ O 417.43 6 T0327 Glacotamine 9476.977.1 C ₁ H ₂ MO ₂ O 417.43 6 T0327 Glacotamine 9476.977.1 C ₁ H ₂ MO ₂ O 477.43 10 T0191 Linagliptin 48640.32.6 C ₁ H ₂ MO ₂ O 472.54 11 T0542 Stagliptin Hydrate 94567.22.1 C ₁ H ₂ M ₂ O 473.24 13 T1149 Parothytic sciul hydrochloride 9450.28.9 C ₂ H ₂ O, 443.2002 14 T149 Fandhatite 1952.18.9 C ₂ H ₂ O, 443.2002 15 T1731 Unorianti 1950.18.4 C ₂ H ₂ O, 443.2002 16 T2731 Unorianti 1980.74.8 C ₂ H	No.	Identity	Chemical name	CAS no.	Molecular formula	Molecular wt (g/mol)
2 T172 Apoptosis activator 2 79183-19-0 C ₁ ¹ / ₁ C ₁ ¹ / ₂ ¹ / ₂ ¹ / ₂ ¹ 108.14 4 T2893 Muscone 541-91-3 C ₁ / ₁ / ₁ / ₂ ,0 128.41 4 T0322 Glabeate mesylate 5674-61-9 C ₁ / ₁ / ₁ / ₂ / ₂ ,0 179.17 6 T0322 Glabeate mesylate 5674-61-9 C ₁ / ₁ / ₁ / ₂ / ₂ / ₂ ,0 28.41 7 T0087L Glimepinde 9479-77-1 C ₁ / ₁ / ₂ / ₂ / ₂ ,0 23.62 8 T0127 Stanglpin functor 668270-12-0 C ₁ / ₂ 40.02 10 T0191 Linoglpin functor 668270-12-0 C ₁ / ₂	1	T2316	MK3102	1226781-44-7	$C_{17}H_{20}F_{2}N_{4}O_{3}S$	398.43
3 T1581 Picolamine 3731-52-0 Cit Nu Cit Nu 23841 5 T0429 Glucosamine 3416-24-8 Cit Nu, No, T317 23841 6 T0372 Glucosamine 3416-24-8 Cit Nu, No, T317 477.48 7 T0087L Suffacetamide sodium 127.56.0 Cit Nu, No, S 480.22 9 T017 Sasaghtrin hydrate 94697012-0 Cit Nu, No, S 480.24 10 T0191 Lingipton 496827012-0 Cit Nu, No, S 472.34 11 T0242 Sisagliptin 49652-23-0 Cit Nu, No, S 380.83 13 T1140 Pennothrate 4952-23-0 Cit Nu, No, S 380.83 14 T1449 Fennthrate 4952-23-0 Cit Nu, No, S 380.83 15 T136 Jurit Advance 4952-23-0 Cit Nu, No, S 380.83 16 T737.30 Battinic and 172.44 49642-22 Cit Nu, No, S 380.33 17 T130 Lint and	2	T1772	Apoptosis activator 2	79183-19-0	C ₁₅ H ₀ Cl ₂ NO ₂	306.14
4 T2893 Muscone 541-91-33 C, H ₂ , G 238.41 6 10372 Gabexate megylate 5697461-9 C, H ₂ , N ₂ O, 237.47 6 10372 Gabexate megylate 5697461-9 C, H ₂ , N ₂ O, 237.62 8 10127 Glimepinde 94479-97-1 C, H ₂ , N ₂ O, 490.62 9 10173 Saxagliptin hydrate 94567-22.1 C, H ₂ , N ₂ O, 472.54 10 10191 Linagliptin 68267012.0 C, H ₂ , N ₂ O, 472.54 12 T0984 Pluorourall (5FU) 51.21.38 C, J ₄ , N ₂ O, 470.32 13 T1140 Poxycycline HCI 10592.13-9 C, J ₄ , N ₂ O, 480.896 14 T1149 Fenofibrata 49562.28-9 C, J ₄ , N ₂ O, 243.730.2 15 T1366 3-P, Pridylacetic acid hydrochlorid 125.46-2 C, H ₄ , O ₄ 470.38 16 T2731 Usria acid 1180.71-8 C, J ₄ , J ₄ , O ₄ 470.38 17 T284 Retuinin	3	T1581	Picolamine	3731-52-0	C _e H _o N ₂	108.14
5 T0429 Glucosamine 3416-24-8 C,H,NO,C 173.17 7 T0087L Suffacetamide sodium 127.56-0 C,H,N,NO,C 325.22 8 T0127 Glimepiirde 93479-97.1 C,H,N,NO,C 472.83 9 T0178 Saxagliptin hydrate 945667-22-1 C,H,F,N,O,C 407.32 11 T0242 Sitagliptin 46640-32-6 C,H,G,NO,C 407.32 12 T1140 Parother HC1 513.18 C,H,G,O,C 400.896 13 T1140 Parother HC1 49562-22-9 C,H,G,O, 440.396 14 T1149 Penofharic 49562-22-9 C,H,G,O, 440.3402 15 T1236 3-Pyridyloccit acid hydrochloride 4193-52-9 C,H,G,O, 440.3402 16 T2731 Unina acid 125-46-2 C,H,G,O, 440.25817 17258 Diymarine 16337-52-8 C,H,H,O, 470.5242 17274 Diymarine 16337-52-8 C,H,H,NO, 250.4437	4	T2893	Muscone	541-91-3	$C_{12}H_{20}O$	238.41
6 1032 Cabcate meylate 597/461-9 C, H-J, Ki, O, 417.48 7 T0087L Sulfactamide sodium 127.560 C, H, J, No, O, 420.62 8 T0127 Glimepiride 9479.97.1 C, H, J, No, O, 430.23 10 T0191 Linagliptin 466.463.2-6 C, H, J, Ko, O, 472.54 11 T0242 Sitagliptin 486.460.32-6 C, H, J, Ko, O, 480.886 13 T1140 Doxycycline HCI 10592.13-9 C, H, C, No, 480.886 14 T149 Fenofibrate 4956.226 C, H, L, No, 480.886 15 T1350 3-Pyrtobjacetic acid 477.51-1 C, H, L, No, 447.827.02 16 T2380 Bethinic acid 477.51-1 C, H, L, O, 245.30412 21 T0789 Phenylmethylsufforyl fluoride 32.99.8-6 C, H, C, O, 447.335.5 22 T0851 Hydroxychtoroquine suifite 32.99.8-6 C, H, L, O, 24.335.5 23 T1402 Fenof	5	T0429	Glucosamine	3416-24-8	C _c H ₁₀ NO _c	179.17
7 10087.1 Suffacetanide sodium 127.560 C, H, M, Mo, S. 226.22 9 10173 Saxagliptin hydrate 9479.97.1 C, H, M, O, S. 4906.2 9 10178 Saxagliptin hydrate 9476.97.1 C, H, M, O, S. 472.54 11 10242 Sitadjiptin 48640.32.6 C, M, F, NO, O. 470.32 12 10984 Fluoroural (5.4°U) 512.18 C, M, CNO, O. 480.896 13 11140 Doxycycline HCI 1952.13.9 C, H, CNO, O. 480.896 14 11149 Fenofbrate 4956.28.9 C, H, CNO, O. 480.896 15 11366 3-Pyridylacetic acid hydrochorde 1180.71.8 C, H, Q, O. 442.68817 172731 Usnic acid 472.46.2 C, H, Q, O. 442.68817 18 128.80 Betulinic acid 472.84 C, H, Q, O. 343.240.2 19 127.34 Oxymatrine 1583.75.28 C, H, Q, O. 343.240.2 19 127.48 Presymatryhiotoryhifunoracid 1	6	T0372	Gabexate mesulate	56974-61-9	$C_{4}=H_{2}=N_{2}O_{2}$	417 48
in in< in< </td <td>7</td> <td>T0087I</td> <td>Sulfacetamide sodium</td> <td>127-56-0</td> <td>C-H-N-NaO-S</td> <td>236.22</td>	7	T0087I	Sulfacetamide sodium	127-56-0	C-H-N-NaO-S	236.22
9 10178 Sawagiptin hydrate 94567-22-1 C ₁₄ , H ₄ , H ₅ 333.43 10 T0191 Linagliptin 668270-12-0 C ₄ , H ₄ , M ₅ O, 472.54 11 T0242 Sitagliptin 668270-12-0 C ₄ , H ₄ , M ₅ O, 472.54 12 T0984 Fluorourall (S-FU) 51-21-8 C ₄ , H ₄ , O ₅ , 400.95 13 T1140 Dosycycline HCI 10592-13-9 C ₄ , H ₄ , O ₅ , 360.83 14 T149 Fenofibrate 49552-28-9 C ₄ , H ₄ , O ₅ , 344.32402 17 T2728 Limonin 1180-71-8 C ₄ , H ₄ , O ₅ , 442.68817 19 T2754 Oxymattine 1063752-8 C ₄ , H ₄ , O ₅ , 77.19 19 T2754 Oxymattine 4237-86-2 C ₄ , H ₄ , O ₅ , 77.19 20 T0981 PrenyImethybulonyi fluoride 473-64 C ₄ , H ₄ , O ₅ , 27.19 21 T0789 PrenyImethybulonyi fluoride 473-64 C ₄ , H ₄ , O ₅ , 27.29 21 T128	8	T0127	Glimeniride	93479-97-1		490.62
Introduct Datagenerity in proting Description Description Description Description Description Carl Markov Description Description<	0	T0127	Savaglintin hydrate	045667-22-1	C H N	333 / 3
O O	10	T0170	Linadintin	668270-12-0		170 5 <i>1</i>
11 T0242 Sitegliptin 486460-32-6 C _k H _k E _k N ₀ 407.32 13 T1140 Dowycyline HCI 10592-13-9 C _k H _k C(N,0, 480.896 13 T1140 Dowycyline HCI 10592-13-9 C _k H _k C(N,0, 480.896 15 T1366 3-Prindlyacetic acid hydrochloride 6419-36-9 C _k H _k C(N,0, 480.896 16 T2731 Usinc acid 125.46-2 C _k H _k D,0, 442.68817 17 T2738 Limonin 1180-71-8 C _k H _k D,0, 264.3073 18 T2880 Percostilberne 537.42-8 C _k H _k D,0, 264.3073 19 T2754 Oymatrine 13621 420.1789-0 C _k H _k D,0, 338.9 21 T0402 Ferofibrica cid 420.1789-0 C _k H _k D,0, 318.7 22 T0451 Hydroxychloroquine sulfate 747.36-4 C _k H _k D,0, 216.3 21 T440 Captopril 653.27.1 H _k CD,0, 217.29 21 T244 Captopril	10		Enagiptin	000270 12 0	C ₂₅ H ₂₈ N ₈ O ₂	772.37
12 T0984 Fluorouracil (5-FU) 51-21-8 C,H ₂ FN ₂ O ₂ 130.08 14 T1149 Fenofibrate 49562-28-9 C,M ₂ H ₂ (D,O ₂ 300.83 14 T136 3-Pyridylacetic acid hydrochloride 619-39-9 C,H ₂ CNO ₂ 343.2402 16 T2731 Usnic acid 125.45-2 C,a ^H L ₂ O ₄ 470.5242 18 T2830 Betulinic acid 472.15-1 C,a ^H L ₄ O ₄ 264.37073 20 T2888 Pterostillene 537.42-8 C,a ^H L ₄ O ₄ 264.37073 21 T0789 Phenylmethylsulfonyl fluoride 329-96-6 C,H ₂ H ₂ O ₄ 33.95 23 T1462 Captopril 62571-86-2 C,H ₄ RO ₅ 33.95 24 T1462 Gaptopril 62571-86-2 C,H ₄ RO ₅ 70.96 24 T1462 Gaptopril 15521-367.5 C,G,H ₄ RO ₅ 70.96 25 Ritonavir 15521-367.5 C,H ₄ RO ₅ 70.96 71.97 24 T1462 Captopril <	11	T0242	Sitagliptin	486460-32-6	$C_{16}H_{15}F_6N_5O$	407.32
13 T1140 Doxycycline HCI 10592-13-9 C ₂₂ H ₂ C(NO ₄) 48086 15 T1366 3-Pyridylacetic acid hydrochloride 6419-36-9 C ₄ H ₄ C(O ₇) 134.34202 15 T271 Usnic acid 125.46-2 C ₄ H ₄ CO ₇ 344.32402 17 T2728 Limonin 1180-71-8 C ₃₀ H ₄ O ₆ 472.581 18 T280 Betulinic acid 472.151-1 G ₃₉ H ₄ O ₇ 265.30412 21 T0789 Phenylmethylsufforyl fluoride 329.96.6 C ₁₀ H ₇ CO ₅ 318.75 21 T0789 Phenylmethylsufforyl fluoride 420.96.8 418.75 433.95 23 T1402 Fenofbric acid 420.17.89-0 C ₁₀ H ₂₀ C(No ₅ S 318.75 24 T1462 Captopril 65571-86-2 C ₁₀ H ₂₀ C(No ₅ S 217.29 25 T1554 Ritonavir 15513.97.5 C ₁₀ H ₂₀ C,O ₂ 456.48.3 27 T243 Aloecenodin 48172-1 C ₁₀ H ₂₀ C,O ₂ 270.24 28 T2401 Alogitpitn benzoate 850649-62-6 C ₂₀ H ₂₀ NO ₄ 451.51 <	12	T0984	Fluorouracil (5-FU)	51-21-8	$C_4H_3FN_2O_2$	130.08
14 T1149 Fenofibrate 49562.28-9 C ₂₀ H ₂ (Do, T33.6 300.83 15 T1366 3-Prividyaccita cid hydrochloride 619-96-9 C ₁₀ H ₁₀ O ₁ 344.32402 16 T2731 Usnic acid 125-46-2 C ₁₀ H ₁₀ O ₁ 344.32402 18 T2830 Betulinic acid 472:15-1 C ₃₀ H ₄₀ O ₁ 426.68817 19 T2754 Oxymatrine 166375-28 C ₁₁ H ₂₁ O ₂ 264.37073 20 T2888 Pterostilbene 537.42-8 C ₁₁ H ₂₁ O ₄ 245.30412 21 T0789 Phenylmethylsulfonyl fluoride 329-96-6 C ₁₁ H ₂₁ O ₄ 33.95 23 T1402 Fenofibric acid 42017-89-0 C ₁₁ H ₂₁ O ₄ 318.75 24 T1402 Fenofibric acid 42017-89-0 C ₁₁ H ₂₁ O ₄ 318.75 25 T1554 Ritonavir 15521-86-7 C ₁₁ H ₂₁ O ₄ 340.42 25 T1554 Ritonavir 15221-86-7 C ₁₁ H ₂₁ O ₄ 344.24 26 T1554 <td< td=""><td>13</td><td>T1140</td><td>Doxycycline HCl</td><td>10592-13-9</td><td>$C_{22}H_{25}CIN_2O_8$</td><td>480.896</td></td<>	13	T1140	Doxycycline HCl	10592-13-9	$C_{22}H_{25}CIN_2O_8$	480.896
15 T1366 3-Pyridylacetic acid hydrochloride 6419-36-9 CyH ₄ Cl0, 1343 2420 17 T2721 Usnic acid 125-66-2 CyH ₄ Cl0, 442.68817 18 T280 Betulinic acid 472.151-1 Cy ₂ H ₄ O, 442.68817 19 T2754 Oxymatrine 16837-52-8 Cy ₁ H ₄ N,O ₂ 256.30412 21 T0789 Phenylmethylsufforyl fluoride 329-98-6 CyH ₇ CO ₂ 433.095 23 T1402 Fenofibric acid 42017.88-0 CyH ₈ CN,O ₂ 433.95 24 T1462 Captopril 65571-86-2 CyH ₈ NO ₂ S 212.72 25 T154 Cisplatin 15663-27-1 H ₆ ClN ₉ Pt 300.05 26 T1564 Cisplatin 1923-45-5 CyH ₈ NO ₄ S 432.42 20 T283 Aloe-emodin 4817-21 CyH ₈ NO ₄ S 384.24 27 T284 Aloe-fenodin 19272-17-0 CyH ₈ NO ₄ 451.51 21 T287 Acethydroxamic acid 546-88-3 CyH ₈ NO ₄ 452.42 28 T239 Bat	14	T1149	Fenofibrate	49562-28-9	C ₂₀ H ₂₁ ClO ₄	360.83
16 T2731 Usnic acid 125.46-2 C ₁₀ H ₁₀ O ₂ 344.32402 17 T278 Limonin 180.71.8 C ₂₀ H ₁₀ O ₂ 442.68817 18 T2830 Betulinic acid 472.15-1 C ₂₀ H ₁₀ O ₂ 264.37073 20 T2888 Pterostilbene 537.42-8 C ₁₀ H ₁₀ O ₂ 264.37073 21 T0789 Phenylmethylsulfonyl fluoride 329-98-6 C ₁₁ H ₁₀ O ₂ 174.19 21 T0789 Phenylmethylsulfonyl fluoride 327-96-0 C ₁₁ H ₁₀ O ₁₀ 3355 23 T1402 Fenofhric acid 42017-89-0 C ₁₁ H ₁₀ O ₁₀ 318.75 24 T1462 Captopril 65371-86-2 C ₁₁ H ₁₀ O ₁₀ 27.20.36 25 T1525 Ritonavir 155213-67-5 C ₁₂ H ₁₀ O ₁₀ 40151 28 T2401 Aloegiptin benzoate 850649-62-6 C ₁₄ H ₁₀ O ₁₀ 40151 29 T2399 Bortezomib (PS-341) 179324-69-7 C ₁₄ H ₂₀ N ₁₀ O ₁ 450.37 30 T1592 Acetohydroxamic acid 546-88-3 C ₁₄ H ₁₀ O ₁₀ 450.47 31 T1623 Lopinavir 19275-17-0 C ₂₄ H ₂₆ N ₁₀ O ₂ 451.51 31 T2296 SY472	15	T1366	3-Pyridylacetic acid hydrochloride	6419-36-9	C ₇ H ₈ CINO ₂	173.6
17 T228 Limonin 1180-71-8 $C_{pH}_{m}O_{p}$ 470.5242 18 T2800 Betulinic caidi 472.15-1 $C_{pH}_{m}O_{p}$ 442.08817 19 T2754 Oxymatrine 16337-52-8 $C_{nH}_{m}O_{p}$ 264.37073 20 T2888 Preostilbene 537-42-8 $C_{nH}_{m}O_{p}$ 183.55 21 T0789 Phenylmethylsulfonyl fluoride 329-98.6 $C_{nH}_{n}O_{p}O_{s}$ 318.75 23 T1402 Fenofibric aid 4207-78-0-0 $C_{nH}_{n}O_{p}O_{s}$ 318.75 24 T1462 Captopril 62371-86-2 $C_{nH}_{n}O_{n}O_{s}O_{s}$ 270.26 25 T154 Cisplatin 15643-27.1 $H_{n}C_{n}A_{n}P_{t}$ 300.05 26 T154 Aloeemodin 481-72.1 $C_{n}H_{n}N_{0}A_{s}$ 481.24 27 T2843 Aloeemodin 481-72.1 $C_{n}H_{n}N_{0}A_{s}$ 482.41 28 T2401 Alogiptin benzoate 80649-62-6 $C_{n}H_{n}N_{0}A_{s}$ 482.41 29 T239 Bortezonia (F5-341) 179224-697 $C_{n}H_{n}N_{0}A_{s}$	16	T2731	Usnic acid	125-46-2	$C_{18}H_{16}O_7$	344.32402
18 T2830 Betulinic acid 472-15-1 C _a H ₁₀ O ₂ Ad2.68817 20 T2888 Preostilbene 537-42-8 C ₁₀ H ₁₀ O ₂ 2630412 21 T0789 Phenylmethylsulforyl fluoride 329-98-6 C ₁₄ H ₁₀ O ₂ 43395 23 T1402 Fenofibric acid 42017-89-0 C ₁₀ H ₄₀ O ₁₀ 318.75 24 T1462 Captopril 62571-86-2 C ₄ H ₄₁ O ₁₀ O ₅ 43395 25 T1525 Ritonavir 155213-67.5 C ₁₀ H ₄₁ O ₁₀ O ₅ 27036 26 T1564 Cisplatin 15663-27.1 H ₄ CJN ₂ P ₄ 30005 27 T2843 Aloe-emotin 48172-1 C ₁₀ H ₄₀ O ₅ 27024 28 T2401 Alogiptin benzoate 850649-62-6 C ₂₀ H ₂₀ NO ₄ 384.24 30 T1592 Acetohydroxamic acid 192725-17-0 C ₁₀ H ₃₀ N ₄₀ O ₅ 325.162 31 T1623 Lopinavir 192725-17-0 C ₁₀ H ₃₀ N ₄₀ O ₅ 325.162 31 T2262 GH-50	17	T2728	Limonin	1180-71-8	$C_{26}H_{30}O_8$	470.5242
19 T2754 Oxymatrine 16837-52-8 C ₁₄ H ₁₀ O ₂ 254.37073 20 T2888 Pherostilbene 537.42-8 C ₁₄ H ₁₀ O ₂ 256.30412 21 T0789 Phenylmethylsulfonyl fluoride 329-98-6 C ₁₄ H ₁₀ O ₂ 133.75 22 T0951 Hydroxychloroquine sulfate 247-36-4 C ₁₄ H ₁₀ O ₂ 318.75 23 T1402 Fenofibric add 2017-89-0 C ₁₄ H ₁₀ O ₂ 217.29 24 T1462 Captopril 5563-27-1 C ₁₄ H ₁₀ O ₂ 270.26 25 T152 Ritonavir 15513-67-2 C ₁₄ H ₁₀ O ₂ 270.24 26 T1364 Aloe-emodin 481-72-1 C ₁₄ H ₁₀ O ₂ 270.24 26 T139 Bottezonib (F5-341) 179324-69-7 C ₁₄ H ₂₀ N ₂₀ 384.24 27 T2256 SYM472 1029877-94 C ₂₄ H ₂₀ N ₂₀ 475.47 38 T262 GHF-5074 74926983-8 C ₁₄ H ₁₀ N ₂₀ C ₅ 537.62 2117 P516206 S63329-66-2	18	T2830	Betulinic acid	472-15-1	$C_{29}H_{46}O_3$	442.68817
20 T2888 Pterostilbene 537-42-8 $C_{10}H_{10}O_{3}^{-1}$ 256.30412 21 T0789 Phenylmethylsulfour/l fluoride 329-98-6 $C_{11}H_{10}O_{15}$ 173.19 23 T1402 Fenofibric acid 42017.99-0 $C_{11}H_{10}O_{15}$ 318.75 24 T1462 Captopril 62571.86-2 $C_{11}H_{10}O_{5}$ 72.096 25 T1525 Ritonavir 155213.67.5 $C_{11}H_{10}O_{5}$ 270.96 26 T1564 Cisplatin 15663-27.1 $H_{C1}N_{P}P_{1}$ 300.05 27 T2843 Aloce-modin 48172-1 $C_{11}H_{10}O_{5}$ 270.24 29 T2399 Bortezomib (PS-341) 197324.69-7 $C_{12}H_{10}N_{10}O_{5}$ 251.62 31 T1623 Lopinavir 192725-17-0 $C_{12}H_{20}N_{10}O_{5}$ 251.62 32 T2266 SYR472 1029877-94 $C_{12}H_{20}N_{10}O_{5}$ 351.62 33 T2262 GH-5074 74926943.8 $C_{11}H_{10}N_{10}O_{5}$ 262.11 <	19	T2754	Oxymatrine	16837-52-8	$C_{15}H_{24}N_{2}O_{2}$	264.37073
21 T0789 Phenylmethylsulfonyl fluoride 329-98-6 Cul+FQ.5 174.19 22 T0951 Hydroxychioroquine sulfate 747-36-4 Cul+Hu_GDQ.5 433.95 23 T1402 Fenofibric acid 42017-89-0 Cul+Hu_GDQ.5 217.29 24 T1462 Captopril 62371-86-2 Cul+Hu_GDQ.5 217.29 25 T1524 Ritonavir 155213-67-5 Cul+Hu_GDQ.5 217.29 25 T1564 Cisplatin 15663-27-1 Hu_GDNQ.6 200.05 27 T2843 Aloe-emodin 48172-1 Cul+JuQ.6 270.24 28 T2401 Alogliptin berzoate 850649-62- Cul+JuQ.6 268.8 29 T1592 Acetohydroxamic acid 546-88-3 Cul+JuQ.6 75.07 31 T1623 Lopinavir 192725-17-0 Cul+JuQ.6 25.162 24 T2016 MLN9708 120902-80-8 Cul+JuQ.6 317.12 33 T2229 Raltegravir potassium 871038-72-1 <	20	T2888	Pterostilbene	537-42-8	C ₁₆ H ₁₆ O ₃	256.30412
21 T0789 Phenyimethylsufforvide 329-98.6 C ₁ H ₂ CIN ₂ O ₅ 174.19 22 T0951 Hydroxychioroquine suffate 747-36.4 C ₁ H ₂ CIN ₂ O ₅ 313.95 23 T1402 Fenofibric acid 42017.49-0 C ₁ H ₁ CIN ₂ O ₅ 217.29 23 T1525 Ritonavir 155213.67-5 C ₂ H ₁₄ N ₂ O ₅ 270.96 24 T1462 Captopril 62571.46-2 C ₁ H ₁₀ O ₅ 270.24 25 T154 Giplatin 15663.27-1 H ₂ (N ₁ P ₁ N ₂ O ₅ 270.24 28 T2401 Alocermodin 48172-1 C ₁ H ₁₀ O ₅ 270.24 29 T2399 Bortezomib (PS-341) 17924.69-7 C ₁ H ₂ N ₂ N ₂ O ₄ 486.24 30 T1592 Acetohydroxamic acid 546.88-3 C ₂ H ₂ N ₂ O ₄ 62.88 21 T2296 SYR472 1029877.94-8 C ₂ H ₂ N ₂ O ₄ 62.88 31 T1623 Lopinavir 192725.17-0 C ₂ H ₂ N ₂ O ₄ 6361.03 32 T2262 GHF-5074 74926983.8 C ₁ H ₁ N ₁ O ₄ 6361.03					10 10 5	
22 T0951 Hydroxychloroquine sulfate 747-36-4 $C_{17}H_{15}CO_{10}$ 318.75 23 T1402 Captopril 62571-86-2 $C_{17}H_{15}O_{15}$ 212.29 24 T1452 Ritonavir 155213-67-5 $C_{17}H_{15}O_{16}O_{5}$ 212.29 26 T1564 Cipatin 15633-77-1 H_{16}D_{16}O_{5} 270.24 28 T2401 Alogliptin benzoate 850649-62-6 $C_{28}H_{19}O_{4}$ 384.24 29 T2399 Bortezomb (PS-341) 179324-69-7 $C_{19}H_{28}N_{4}O_{4}$ 384.24 30 T1592 Acteohydroxamic acid 546-88-3 $C_{21}H_{28}N_{4}O_{5}$ 628.8 31 T1623 Lopinavir 192725-17-0 $C_{27}H_{48}N_{5}O_{6}$ 475.47 31 T262 GHF-5074 749269-83-8 $C_{10}H_{15}CP_{5}$ 325.162 34 T2016 MLN9708 1201902-80-8 $C_{29}H_{28}N_{10}O_{6}$ 483.2511 36 T2229 Nategravir potassium 870329-66-2 $C_{20}H_{20}FN_{10}O_{6}$ 480.2511 37 T217 PS162206 863329-96-2 C	21	T0789	Phenylmethylsulfonyl fluoride	329-98-6	C ₇ H ₇ FO ₂ S	174.19
23 T1402 Fendbric acid 42017-89-0 C ₁ H ₄ CO ₄ 318.75 24 T1462 Captopril 62571-86-2 C ₂ H ₄ NO ₅ 5 217.29 25 T1525 Ritonavir 155213-67-5 C ₂ H ₄ NO ₅ 5 217.29 26 T1564 Cisplatin 15663-27-1 H ₄ Cl ₄ N ₂ P ₄ P 30005 27 T2843 Aloe-emodin 481-72-1 C ₁₃ H ₁₀ O ₈ 270.24 28 T2401 Alogiptin benzoate 850649-62-6 C ₁₉ H ₂ N ₄ O ₄ 481.24 20 T2399 Bortezomib (PS-341) 179324-69-7 C ₁₉ H ₂ N ₄ O ₄ 384.24 30 T1592 Acetohydroxamic acid 546-88-3 C ₂ H ₂ N ₄ O ₄ 84.24 31 T1623 Lopinavir 192275-17-0 C ₂₁ H ₂ N ₄ O ₅ 628.8 32 T2266 SYR472 1029877-94-8 C ₂₀ H ₂ SC ₁ O ₄ 361.03 33 T2262 GHF-5074 749269-83-8 C ₆₄ H ₁₇ Cl ₄ O ₄ 361.03 34 T2016 MLN9708 1201902-80-82 C ₂₀ H ₂ SC ₁ O ₄ O ₄ 361.03 35	22	T0951	Hydroxychloroquine sulfate	747-36-4	$C_{18}H_{28}CIN_3O_5S$	433.95
24T1462Captopril62571-86-2C, H, NO, S217.2925T1525Ritonavir155213-67-5C, H, NO, S720.9626T1544Ciplatin15603-27-1H, LC, N, Pt300.0527T2843Aloe-emodin481-72-1C, H, NO, S270.2428T2401Alogliptin berzoate850649-62-6C, J, H, NO, A461.5129T2399Bortezomio (F5-341)179324-69-7C, H, NO, A461.5121T1623Lopinavir192725-17-0C, H, NO, A628.831T1623Lopinavir192725-17-0C, J, H, NO, A628.832T2266STR4721029877-94-8C, J, H, SCI, NO, A575.4733T2262GHF-5074749269-83-8C, J, H, SCI, NO, A510.1334T2016MLN9708120100-60-86C, J, H, SCI, NO, A361.0335T2122MLN2236 (hzzomib)1072833-77-2C, J, H, SCI, NO, A482.51136T229Nafamostar mesylate63329-66-2C, J, H, SO, SO, S33.73533.7337T2117P516206863329-66-2C, J, H, SO, SO, S33.73533.7338T2392Nafamostar mesylate635728-49-3C, SH, SN, NO, S537.5441T2743Ilomastat (GM6001, Galardin)14280-36-2C, SH, SN, NO, S377.2541T2743Ilomastat (GM6001, Galardin)14280-36-2C, SH, SN, NO, S377.2541T2743Ilomastar (GM6001, Galardin) <t< td=""><td>23</td><td>T1402</td><td>Fenofibric acid</td><td>42017-89-0</td><td>$C_{17}H_{15}CIO_4$</td><td>318.75</td></t<>	23	T1402	Fenofibric acid	42017-89-0	$C_{17}H_{15}CIO_4$	318.75
25T1525Ritonavir155213-67-5 $C_{23}H_{48}N_{20}S_{25}$ 720.9626T1564Cisplatin15663.27-1H ₄ Cl,N,Pt300.0527T2843Aloe-emodin481-72-1 $C_{12}H_{12}N_{0}O_{1}$ 270.2428T2401Alogliptin benzoate850649-62-6 $C_{23}H_{23}N_{0}O_{1}$ 384.2429T2399Bortezomib (PS-341)179324-69-7 $C_{12}H_{23}RN_{0}O_{2}$ 384.2430T1592Acetohydroxamic acid546-88-3 $C_{24}H_{23}N_{0}O_{2}$ 628.831T1623Lopinavir19225-17-0 $C_{23}H_{48}N_{0}O_{5}$ 628.833T2262GHF-5074749269-83-8 $C_{12}H_{12}RN_{0}O_{3}$ 475.4734T2016MLN72881201902-80-8 $C_{14}H_{11}C_{17}O_{2}$ 325.16235T2122MLN2238 (kazomib)1072833-77-2 $C_{14}H_{19}GL_{1N}O_{4}$ 361.0336T2239Raltegravir potassium871038-72-1 $C_{29}H_{27}RN_{0}O_{5}$ 422.51137T2117PSi62066633296-62-2 $C_{14}H_{15}N_{0}O_{5}$ 260.2238T2392Nafamostat mesylate82956-11-4 $C_{23}H_{23}N_{0}O_{5}S$ 593.7341T2743Ilomastat (GM6001, Galardin)142880-36-2 $C_{24}H_{26}N_{0}O_{6}$ 447.8842T2332Elvitegravir (GSX1349572)109119-65-6 $C_{24}H_{26}N_{0}O_{5}$ 593.7341T2743Ilomastat (GM6001, Galardin)142880-36-2 $C_{24}H_{26}N_{0}O_{5}$ 593.7341	24	T1462	Captopril	62571-86-2	$C_9H_{15}NO_3S$	217.29
26 T1564 Ciplatin 15663-27-1 H _c Cl,N,Pt 300.05 27 T2843 Aloe-emodin 481-72-1 C ₁ H ₁ O ₀ , 270.24 28 T2401 Alogliptin benzoate 850649-62-6 C ₂ H ₂ /N,O ₄ 384.24 29 T2399 Bortezomib (PS-341) 179324-69-7 C ₁ H ₂ BN,O ₄ 384.24 30 T1592 Acetohydroxamic acid 546-88-3 C ₁ H ₁ NO ₂ 75.07 31 T1623 Lopinavir 192725-17-0 C ₂ H ₄ BN,O ₅ 628.8 32 T2266 SYR472 1029877-94-8 C ₂ H ₁ Cl,PO ₂ 325.162 34 T2016 MLN9708 1201902-80-8 C ₂ H ₂ BCL,NO ₂ 310.13 35 T212 MLN2238 (kazomib) 1072833-77-2 C ₄ H ₆ BCL,NO ₂ 482.511 36 T239 Raltegravir potasium 871038-72-1 C ₄ H ₆ BCL,NO ₂ 593.58 37 T2117 PS16206 863329-66-2 C ₄ H ₁ S,O ₄ No ₅ 393.73 38 T2392 Nafamostat mes	25	T1525	Ritonavir	155213-67-5	C ₃₇ H ₄₈ N ₆ O ₅ S ₂	720.96
27T2843 (28Aloe-emodin481-72-1 $C_{12}H_{10}O_{5}$ 270.2428T2401Aloejitti benzoate850649-62-6 $C_{12}H_{23}D_{1}N_{10}O_{4}$ 384.2429T2399Bortezomib (PS-341)179324-69-7 $C_{10}H_{23}BN_{10}O_{4}$ 384.2430T1592Acetohydroxamic acid546-88-3 $C_{2}H_{20}N_{10}O_{4}$ 628.831T1623Lopinavir192725-17-0 $C_{21}H_{22}D_{10}N_{10}O_{5}$ 628.832T2266SYR47210298779-48-8 $C_{21}H_{22}D_{11}N_{0}O_{5}$ 517.1233T262GHF-5074749269-83-8 $C_{10}H_{11}C_{15}C_{2}$ 325.16234T2016MLN97081201902-80-8 $C_{20}H_{22}D_{11}N_{0}O_{5}$ 517.1235T212MLN2728(kazomib)1072833-77-2 $C_{4}H_{11}C_{15}C_{2}$ 361.0336T2239Raltegravir potasium871038-72-1 $C_{20}H_{22}D_{10}N_{0}O_{5}$ 482.51137T2117PSI6206863329-66-2 $C_{10}H_{12}N_{0}O_{6}$ 811.840T2324G1335)Daruanavir ethanolate635728-49-3 $C_{20}H_{23}N_{0}O_{6}$ 811.841T2743Ilomastat (GM6001, Galardin)14280-36-2 $C_{10}H_{21}N_{10}O_{6}$ 447.8842T332Elvitegravir (GSK1349572)1051375-19-9 $C_{20}H_{21}R_{2}N_{0}O_{5}$ 593.7341T2743Ilomastat (GM6001, Galardin)14280-36-2 $C_{9}H_{29}N_{0}O_{6}$ 447.8843T3232Elvitegravir (GSK1349572)1051	26	T1564	Cisplatin	15663-27-1	$H_6Cl_2N_2Pt$	300.05
28T2401Alogliptin benzoate850649-62-6 $C_{y_1}H_{y_2}N_iO_4$ 461.5129T2399Bortezomb (PS-341)179324-69-7 $C_{y_1}H_{y_2}N_iO_4$ 384.2421T1592Acteohydroxamic acid546.88-3 $C_{21}H_{y_2}N_iO_6$ 475.4731T1623Lopinavir192725-17-0 $C_{y_1}H_{y_2}N_iO_6$ 475.4733T2266SYR4721029877-94-8 $C_{10}H_{11}O_5O_6$ 325.16234T2016MLN97081021902-80-8 $C_{10}H_{12}BCI_NO_6$ 317.1235T2122MLN2238 (kazomib)1072833-77-2 $C_{10}H_{12}BCI_NO_6$ 361.0336T2239Rategoriv potassium871038-72-1 $C_{10}H_{12}SCN_60_6$ 813.2937T2117P56206663329-66-2 $C_{10}H_{13}FN_2O_5$ 260.2238T2392Nafamostat mesylate82956-11-4 $C_{29}H_{23}N_5O_6S_6$ 811.840T2324 (T3335)Darunavir ethanolate635728-49-3 $C_{29}H_{23}N_5O_6S_5$ 593.73411T2743Ilomastat (GM6001, Galardin)142880-36-2 $C_{20}H_{20}N_0A_4$ 388.4642T2332Elvitegravir (GSF9137, JTK-303)697761-98-1 $C_{21}H_{22}O_6N_0A_4$ 388.4644T2834Nobiletin478-01-3 $C_{2}H_{20}N_0A_5$ 277.2544T2834Nobiletin478-01-3 $C_{2}H_{20}N_0A_5$ 277.2545T3028Celastrol34157-83-0 $C_{2}H_{20}N_0A_5$ 205.6747T0100Atzanavir sulfate <td< td=""><td>27</td><td>T2843</td><td>Aloe-emodin</td><td>481-72-1</td><td>$C_{15}H_{10}O_5$</td><td>270.24</td></td<>	27	T2843	Aloe-emodin	481-72-1	$C_{15}H_{10}O_5$	270.24
29 T2399 Bortezomik (PS-341) 179324-69-7 C ₁₉ H ₂₅ N ₁ O ₄ 384.24 30 T1592 Acetohydroxamic acid 546-88-3 C ₂ H ₃ NO ₂ 75.07 31 T1623 Lopinavir 192725-17-0 C ₂₇ H ₄₈ N ₄ O ₅ 628.8 32 T2265 SYR472 1029877-94-8 C ₂₉ H ₂₆ RN ₄ O ₅ 675.47 33 T262 GHF-5074 749269-83-8 C ₁₀ H ₁₁ G1 ₂ FO ₂ 325.162 34 T2016 MLN9708 1201002-80-8 C ₁₀ H ₁₁ BN ₂ O ₂ 361.03 36 T2239 Raltegravir potassium 871038-72-1 C ₁₀ H ₁₁ BN ₂ O ₂ 361.03 36 T2239 Nalmostat mesylate 82956-11-4 C ₂₀ H ₂₅ RN ₄ O ₆ 482.511 37 T2117 PSI6206 63329-66-2 C ₁₀ H ₁₅ N ₁₂ O ₆ 861.33 39 T1786 Daclatasvir, BMS790052 109119-65-6 C ₄₀ H ₂₅ Q ₁₄ N ₄ O ₆ S 593.73 41 T2324 Itomastat (GM6001, Galardin) 14280-36-2 C ₂₀ H ₄₅ N ₄ O ₆ A 388.46 42 T3325 Dolutegravir (GSK 1349572) 105137-519-9 C ₂₀ H ₁₆	28	T2401	Alogliptin benzoate	850649-62-6	$C_{25}H_{27}N_5O_4$	461.51
30 T1592 Acetohydroxamic acid 546-88-3 C ₂ H ₃ NO ₂ 75.07 31 T1623 Lopinavir 192725-17-0 C ₃₇ H ₄₈ N ₄ O ₅ 628.8 32 T2296 SYR472 1029877-94-8 C ₂₂ H ₃₆ FN ₂ O ₆ 475.47 33 T2262 GH-F-5074 749269-83-8 C ₂₀ H ₁₁ CJ ₅ FO ₂ O 525.162 34 T2016 MLN9708 1201902-80-8 C ₂₀ H ₂₅ RJ ₂ O ₆ O 517.12 35 T2122 MLN2238 (kazomib) 1072833-77-1 C ₁₀ H ₁₀ FN ₁₀ O ₆ 482.511 36 T2239 Rategravir potassium 871332-66 C ₄₀ H ₂₅ CJ ₁₀ O ₆ 482.511 37 T2117 PSI6206 863329-66-2 C ₄₀ H ₁₂ CJ ₁₀ O ₆ 811.8 39 T1786 Daclatasvir, BMS790052 1009119-65-6 C ₄₀ H ₁₂ CJ ₁₀ O ₆ 811.8 40 T2324 (T3335) Darunavir ethanolate 635728-49-3 C ₂₉ H ₄₃ N ₄₀ O ₆ 88.46 41 T243 Ilomastat (GM6001, Galardin) 14280-36-2 C ₂₉ H ₄₃ N ₄₀ O ₆ 441.36 44 T2834 Nobiletin 478-01-3 C ₂₁ H ₄₅ N ₄₀ O ₆ </td <td>29</td> <td>T2399</td> <td>Bortezomib (PS-341)</td> <td>179324-69-7</td> <td>$C_{19}H_{25}BN_4O_4$</td> <td>384.24</td>	29	T2399	Bortezomib (PS-341)	179324-69-7	$C_{19}H_{25}BN_4O_4$	384.24
1T1623Lopinavir192725-17-0 $C_{3y}H_{48}N_{4}O_{5}$ 628.832T2296SYR4721029877-94-8 $C_{2y}H_{26}FN_{5}O_{6}$ 475.4733T262GHF-5074749269-83-8 $C_{1y}H_{25}FN_{5}O_{6}$ 325.16234T2016MLN97081201902-80-8 $C_{2y}H_{28}GI_{3N}O_{9}$ 517.1235T2122MLN238 (Ixazomib)1072833-77-2 $C_{1y}H_{23}BCI_{3N}O_{9}$ 361.0336T2239Raltegravir potassium871038-72-1 $C_{2y}H_{2y}EN_{4}O_{5}$ 360.2238T392Nafamostat mesylate82956-11-4 $C_{2y}H_{2y}N_{2}O_{5}$ 539.5839T1786Daclatsvir, BMS790521009119-65-6 $C_{4y}H_{2z}CI_{3N}O_{0}$ 811.840T2324(T3335)Darunavir ethanolate63728-49-3 $C_{2y}H_{2y}N_{2}O_{5}$ 539.7341T2743Ilomastat (GM6001, Galardin)142880-36-2 $C_{2y}H_{2y}N_{2}O_{5}$ 427.47843T2329Dolutegravir (GSK1349572)1051375-19-9 $C_{2y}H_{2y}N_{2}O_{5}$ 427.47844T2834Nobletin478-01-3 $C_{2y}H_{2y}O_{5}$ 420.4046945T3028Celastrol34157-83-0 $C_{2y}H_{2y}O_{5}$ 420.4046946T2792Glucosamine sulfate29031-19-4 $C_{1y}H_{2y}N_{2}O_{5}$ 77.2547T0100Atzanavir sulfate29975-97-7 $S_{4y}H_{3}N_{2}O_{5}$ 270.2550T1795Caflicomib (PR-171)86840-17-4 $C_{4y}H_{2y}N_{2}O_{5}$ 303.4<	30	T1592	Acetohydroxamic acid	546-88-3	$C_2H_5NO_2$	75.07
5117221521215212152121521215212152121521233T2262GHF-5074749269-83 $C_{12}H_{26}(F_1N,O_6)$ 325.16234T2016MLN97081201902-80-8 $C_{12}H_{26}(F_1N,O_6)$ 361.0335T2122MLN2238 (Ixazomib)1072833-77-2 $C_{12}H_{21}S(D_1N,O_6)$ 482.51136T2239Raltegravir potassium871038-72-1 $C_{20}H_{20}F(N,O_6)$ 482.51137T2117PSi620663329-66-2 $C_{12}H_{25}N_50_8S_2$ 539.5839T1786Daclatasvir, BMS7900521009119-65-6 $C_{40}H_{52}C_1N_40_6$ 811.840T2234(T3335)Darunavir ethanolate635728-49-3 $C_{20}H_{20}N_40_8$ 388.4641T2743Ilomastat (GM6001, Galardin)142880-36-2 $C_{20}H_{20}N_40_8$ 388.4642T2332Elvitegravir (GSK1349572)1051375-19-9 $C_{20}H_{20}N_40_8$ 388.4643T2329Dolutegravir (GSK1349572)1051375-19-9 $C_{20}H_{20}N_40_8$ 420.4046944T2834Nobletin478-01-3 $C_{21}H_{20}O_8$ 420.4046945T3028Celastrol34157-83-0 $C_{29}H_{20}N_80_5$ 277.2546T2792Glucosamine sulfate29031-19-4 $C_{40}H_{30}N_50_5$ 277.2547T0100Atazanavir sulfate29975-97-7 $C_{40}H_{50}N_50_5$ 270.2550T1292Clemizole442-52-4 $C_{19}H_{20}O_{10}S_5$ 303.451T	21	T1623	loninavir	102725-17-0	СНИО	678.8
J212230J10721022073070C22120105067137733T2262GHF-5074749269-83-8C_0H_10[C]FO2325.16234T2016MLN97081201902-80-8C_0H_3BCLN200517.1235T2122MLN2238 (ixazomib)1072833-77-2C_14H_18CLN200482.51136T2239Raitegravir potassium871038-72-1C_0H_3FNA05482.51137T2117PSi6206863329-66-2C_0H_3FNA05260.2238T2392Nafamostat mesylate82956-11-4C_2H_23N_20652539.5839T1786Daclatasvir, BMS7900521009119-65-6C_0H_3CIN060811.840T2324 (T3335)Darunavir ethanolate635728-49-3C_29H_28N_04388.4642T2332Elvitegravir (GS-9137, JTK-303)697761-98-1C_3H_3CIFN05447.8843T2329Dolutegravir (GSK1349572)1051375-19-9C_20H_18F_Naloa5441.3644T2834Nobiletin478.01-3C_1H_25_08402.4046945T3028Celastrol34157-83-0C_20H_18F_Naloa5277.2547T0100Atazanavir sulfate229975-97-7C_38H_30A_01-5802.9348T1853NMS 8731418013-75-8C_2H_2N_302303.450T1795Carfilzomib (PR-171)868540-17-4C_40H_35N_50-7704.8751T0100LAtazanavir198904-31-3C_38H_3C_N0_5325.8450T1795Carfilzomib (PR-171)868540-17-4<	30	T2206	SVD/72	1020877-04-8	C H EN O	475 47
3312202Ohr-30/4149209-380-8 $C_{16}P_{11}(U_2^{-1}O_2)$ 323.10234T2016MLN97081201902-80-8 $C_{00}P_{13}(EV_1O_2)$ 361.0335T2122MLN2238 (kazomib)1072833-77-2 $C_{14}H_{19}BC_{19}N_{10}O_{3}$ 482.51136T2239Raltegravir potassium871038-72-1 $C_{20}P_{32}(EV_1O_2)$ 260.2238T2392Nafamostat mesylate82956-11-4 $C_{21}H_{23}N_{2}O_{6}S_{2}$ 539.5839T1786Daclatasvir, BMS7900521009119-65-6 $C_{00}P_{13}(EV_1O_0)$ 884.640T2324 (T3335)Darunavir ethanolate635728-49-3 $C_{29}H_{43}N_3O_6$ 811.841T2743Ilomastat (GM6001, Galardin)142880-36-2 $C_{20}H_{28}N_4O_4$ 388.4642T2332Elvitegravir (GSK1349572)1051375-19-9 $C_{20}H_{18}F_{2}N_3NaO_5$ 441.3644T2834Nobiletin478-01-3 $C_{21}H_{20}O_8$ 402.4046945T3028Celastrol34157-83-0 $C_{20}H_{28}N_4O_1, S$ 802.9348T1853NMS 8731418013-75-8 $C_{27}H_{20}N_8O_7$ 704.8750T1795Carlizomib (PR-171)868540-17-4 $C_{40}H_{57}N_5O_7$ 704.8751T0100LAtazanavir198904-31-3 $C_{31}H_{10}O_5_2$ 303.453T2030Tiplaxtinin(PAI-039)393105-53-8 $C_{24}H_{16}F_{10}O_5_2$ 306.454T2009SB-3CT292605-14-2 $C_{10}H_{10}O_5_2$ 306.455T1	3Z 33	12290		740260 92 9	$C_{22}\Pi_{26}\Pi_{5}O_{6}$	473.47
3412010MLN9/08120192-00-5 $C_{20}h_{32}O_{19}V_{19}V_{50}$ 317.1235T2122MLN238 (Ixazomib)1072833-77-2 $C_{14}h_{13}BC_{15}V_{50}$ 361.0336T2239Raltegravir potassium871038-72-1 $C_{20}h_{20}FN_6O_5$ 482.51137T2117PSI6206863329-66-2 $C_{10}h_{13}FN_{20}S_5$ 539.5838T2392Nafamostat mesylate82956-11-4 $C_{14}h_{25}N_{20}S_5$ 539.5839T1786Daclatasvir, BMS7900521009119-65-6 $C_{40}h_{52}C_{12}N_6O_6$ 811.840T2324 (T3335)Darunavir ethanolate635728-49-3 $C_{29}h_{28}N_1O_4$ 388.4642T2332Elvitegravir (GSV137572)1051375-19-9 $C_{20}h_{16}F_{1N}NaO_5$ 447.8843T2329Dolutegravir (GSV1349572)1051375-19-9 $C_{20}h_{20}K_1O_5$ 447.8644T2834Nobiletin478-01-3 $C_{21}h_{20}O_6$ 402.4046945T3028Celastrol34157-83-0 $C_{29}h_{30}A_4$ 450.6238146T2792Glucosamine sulfate229975-97-7 $C_{28}h_{50}N_6O_5$ 277.2547T0100Atazanavir sulfate229975-97-7 $C_{28}h_{50}N_6O_5$ 200.6748T1853NMS 8731418013-75-8 $C_{29}h_{50}N_5O_7$ 719.9151T0100LAtazanavir198904-31-3 $C_{38}h_{52}N_6O_7$ 704.8752T1502Vildagliptin (LAF-237)274901-16-5 $C_{17}h_{25}N_0A_5$ 306.453T203	22	T2016		1201002 00 0	$C_{16}\Pi_{11}C_{12}FO_{2}$	525.102
351212MLR2.28 (M20110)107.283-77-2 $C_{14}H_{19}b_12N_0Q_4$ 501.0336T2239Raltegravir potassium871038-77-1 $C_{21}H_{25}N_0Q_5$ 260.2238T2392Nafamostat mesylate825611-4 $C_{21}H_{25}N_0Q_5$ 539.5839T1786Daclatasvir, BMS7900521009119-65-6 $C_{40}H_{32}C_{12}N_0Q_6$ 811.840T2324 (T3335)Darunavir ethanolate635728-49-3 $C_{29}H_{42}N_1Q_6$ 848.4642T2332Elvitegravir (GS-9137, JTK-303)697761-98-1 $C_{29}H_{28}N_1Q_4$ 388.4643T2329Dolutegravir (GSK1349572)1051375-19-9 $C_{20}H_{12}F_1N_3NaO_5$ 447.8844T2834Nobiletin478-01-3 $C_{21}H_{22}O_8$ 402.40469945T3028Celastrol34157-83-0 $C_{29}H_3O_4$ 450.62381146T2792Glucosamine sulfate29031-19-4 $C_{41}H_10O_5S$ 277.2547T0100Atazanavir sulfate29031-79-77 $C_{38}H_{52}N_6O_7$ 704.8748T1853NMS 87311418013-75-8 $C_{29}H_{20}N_0S_5$ 520.6749T1822Clemizole442-52-4 $C_{10}H_{20}CIN_3$ 325.8450T1795Carlizomib (PR-171)868540-17-4 $C_{40}H_{57}N_0A_7$ 704.8751T0100LAtazanavir198904-31-3 $C_{28}H_{20}N_0_5$ 303.453T2030Tiplaxtinin(PAI-039)393105-53-8 $C_{24}H_{25}N_0A_7$ 704.8754T2099SB-3CT <td>25</td> <td>T2010</td> <td>MLN2228 (hazomih)</td> <td>1201902-00-0</td> <td>$C_{20} \Pi_{23} D C I_2 N_2 O_9$</td> <td>261.02</td>	25	T2010	MLN2228 (hazomih)	1201902-00-0	$C_{20} \Pi_{23} D C I_2 N_2 O_9$	261.02
3012239Railegravit potassium67 (135-72-1) $C_{20}h_{20}h_{N0}O_{5}$ 442.51137T2117PSI6206863329-66-2 $C_{1}h_{15}R_{10}O_{5}$ 260.2238T2392Nafamostat mesylate82956-11-4 $C_{21}h_{25}N_{5}O_{6}S_{2}$ 539.5839T1786Daclatasvir, BMS7900521009119-65-6 $C_{40}h_{52}C_{10}h_{8}O_{6}$ 811.840T2324 (T3335)Darunavir ethanolate635728-49-3 $C_{20}h_{28}N_{4}O_{6}S$ 593.7341T2743Ilomastat (GM6001, Galardin)142880-36-2 $C_{20}h_{28}N_{4}O_{6}S$ 447.8842T2332Elvitegravir (GS-9137, JTK-303)697761-98-1 $C_{23}h_{25}CIFNO_5$ 447.8843T2329Dolutegravir (GS41349572)1051375-19-9 $C_{20}h_{16}F_{18}N_{40}O_{5}$ 441.3644T2834Nobiletin478-01-3 $C_{21}h_{25}O_{8}$ 402.4046945T3028Celastrol34157-83-0 $C_{9}h_{30}O_{4}$ 450.6238146T2792Glucosamine sulfate229975-97-7 $C_{38}h_{54}N_{6}O_{15}S$ 802.9348T1853NMS 8731418013-75-8 $C_{27}h_{28}N_{4}O_{3}S_{2}$ 520.6749T1822Clemizole442-52-4 $C_{40}h_{57}N_{5}O_{7}$ 719.9151T0100LAtazanavir sulfate19804-31-3 $C_{31}h_{16}S_{10}O_{2}$ 303.452T1502Vildagliptin (LAF-237)274091-16-5 $C_{17}h_{25}N_{5}O_{2}$ 303.453T2030Tiplaxtinin(PA1-039)333105-53-8	35	12122		10/2033-77-2	$C_{14}\Pi_{19}DC_{12}\Pi_{2}U_{4}$	301.05
3712117PSIb206863329-6b-2 $C_{10}T_{13}^{17}N_{2}O_{5}$ 200.2238T2392Nafamostat mesylate82956-11-4 $C_{21}H_{25}N_{5}O_{6}S_{2}$ 539.5839T1786Daclatasvir, BMS7900521009119-65-6 $C_{40}H_{52}C_{12}N_{8}O_{6}S$ 593.7341T2743Ilomastat (GM6001, Galardin)142880-36-2 $C_{20}H_{28}N_{4}O_{4}$ 388.4642T2332Elvitegravir (GS-9137, JTK-303)697761-98-1 $C_{20}H_{28}N_{4}O_{4}$ 388.4643T2329Dolutegravir (GSK1349572)1051375-19-9 $C_{20}H_{18}P_{13}N_{3}No_{5}$ 417.8644T2834Nobiletin478-01-3 $C_{1}H_{2}O_{5}$ 402.4046945T3028Celastrol34157-83-0 $C_{29}H_{38}O_{4}$ 450.6238146T2792Glucosamine sulfate229975-97-7 $C_{38}H_{54}N_{5}O_{15}$ 802.9347T0100Atazanavir sulfate229975-97-7 $C_{39}H_{50}N_{5}O_{5}$ 277.2549T1822Clemizole442-52-4 $C_{19}H_{20}ClN_{3}$ 325.8450T1795Carfilzomib (PR-171)98904-31-3 $C_{38}H_{5}N_{5}O_{7}$ 719.9151T0100LAtazanavir198904-31-3 $C_{34}H_{5}P_{5}N_{5}O_{2}$ 303.453T2030Tiplaxtinin(PAI-039)393105-53-8 $C_{24}H_{5}N_{5}N_{5}O_{2}$ 303.454T2099SB-3CT292605-14-2 $C_{15}H_{14}O_{5}S_{2}$ 306.455T1757ML3231572414-83-5 $C_{24}H_{6}F_{5}NO_{4}$ 439.3	36	T2239	Raitegravir potassium	8/1038-72-1	$C_{20}H_{20}FKN_6O_5$	482.511
3812592Natamostat mesylate8295c-11-4 $C_2H_{25}N_5Q_52_2$ 539.5839T1786Daclatasvir, BMS7900521009119-65-6 $C_{40}H_5Cl_2N_8O_6$ 811.872324 (T3335)Darunavir ethanolate635728-49-3 $C_{29}H_43N_5O_5$ 593.7341T2743Ilomastat (GM6001, Galardin)142880-36-2 $C_{20}H_{28}N_4O_4$ 388.4642T2332Elvitegravir (GS-9137, JTK-303)697761-98-1 $C_{22}H_{23}CIFNO_5$ 447.8843T2329Dolutegravir (GSK1349572)1051375-19-9 $C_{20}H_{18}F_{2N}NAO_5$ 441.3644T2834Nobiletin478-01-3 $C_{21}H_{22}O_8$ 402.4046945T3028Celastrol34157-83-0 $C_{29}H_{30}O_4$ 450.6238146T2792Glucosamine sulfate29031-19-4 $C_6H_1SNO_5$ 277.2547T0100Atazanavir sulfate22975-97-7 $C_{38}H_{54}N_6O_{11}S$ 802.9348T1853NMS 8731418013-75-8 $C_{27}H_{28}N_0S_5$ 220.6749T1822Clemizole442-52-4 $C_{19}H_{20}CIN_3$ 325.8450T1795Carfitzomib (PR-171)868540-17-4 $C_{30}H_{57}N_5O_7$ 719.9151T0100LAtazanavir198904-31-3 $C_{38}H_{51}N_6O_7$ 704.8752T1502Vildagliptin (LAF-237)274901-16-5 $C_{17}H_{25}N_0A_2$ 303.453T2030Tiplaxtinin(PAI-039)393105-53-8 $C_{24}H_16F_5NO_4$ 439.3854T2009SB-3CT	3/	1211/	PSI6206	863329-66-2	$C_{10}H_{13}FN_2O_5$	260.22
3911786Daclatsvir, BMS/900521009119-65-6 $C_{40}H_{52}C_{15}N_6O_6$ 811.840T2324 (T3335)Darunavir ethanolate635728-49-3 $C_{29}H_{43}N_3O_8$ 593.7341T2743Ilomastat (GM6001, Galardin)142880-36-2 $C_{20}H_{28}N_4O_4$ 388.4642T2332Elvitegravir (GS-137, JTK-303)697761-98-1 $C_{23}H_{23}ClFNO_5$ 447.8843T2329Dolutegravir (GSK1349572)1051375-19-9 $C_{20}H_{18}F_2N_3NaO_5$ 441.3644T2834Nobiletin478-01-3 $C_{21}H_{22}O_8$ 402.4046945T3028Celastrol34157-83-0 $C_{29}H_{38}O_4$ 450.6238146T2792Glucosamine sulfate29031-19-4 $C_{6}H_{15}NO_5$ 277.2547T0100Atazanavir sulfate29075-97-7 $C_{39}H_{54}N_6O_{11}S$ 802.9348T1853NMS 8731418013-75-8 $C_{27}H_{28}N_4O_5_5$ 520.6749T1822Clemizole442-52-4 $C_{19}H_{20}ClN_3$ 325.8450T1795Carfilzomib (PR-171)868540-17-4 $C_{40}H_{57}N_5O_7$ 719.9151T0100LAtazanavir198904-31-3 $C_{38}H_{28}N_6O_5$ 303.453T2030Tiplaxtinin(PAI-039)393105-53-8 $C_{24}H_{16}F_3NO_4$ 439.3854T2009SB-3CT292605-14-2 $C_{17}H_{25}N_0S_2$ 315.3255T1757ML3231572414-83-5 $C_{23}H_{24}N_6$ 384.4856T2424P220771247819	38	12392	Nafamostat mesylate	82956-11-4	$C_{21}H_{25}N_5O_8S_2$	539.58
4012324 (1335)Darunavir ethanolate $635728.49-3$ $C_{29}H_{43}N_3O_9S$ 593.73 41T2743Ilomastat (GM6001, Galardin)142880-36-2 $C_{20}H_{28}N_4O_4$ 388.46 42T2332Elvitegravir (GS-9137, JTK-303) $697761-98-1$ $C_{23}H_{23}CIFNO_5$ 447.88 43T2329Dolutegravir (GSK1349572)1051375-19-9 $C_{20}H_{18}F_3N_3NO_5$ 441.36 44T2834Nobiletin $478.01-3$ $C_{21}H_{22}O_8$ 402.40469 45T3028Celastrol34157.83-0 $C_{20}H_{30}O_4$ 450.62381 46T2792Glucosamine sulfate29031-19-4 $C_{6}H_{15}NO_5S$ 277.25 47T0100Atazanavir sulfate229975-97-7 $C_{38}H_{52}N_6O_1S$ 802.93 48T1853NMS 8731418013-75-8 $C_{27}H_{29}N_4O_3S_2$ 52.647 9T1822Clemizole $442-52-4$ $C_{10}H_{25}N_5O_7$ 719.9151T0100LAtazanavir198904-31-3 $C_{38}H_{52}N_6O_7$ 704.8752T1502Vildagliptin (LAF-237)274901-16-5 $C_{17}H_{25}N_3O_2$ 303.453T2030Tiplaxtinin(PAI-039)393105-53-8 $C_{24}H_{16}^3NO_4$ 439.3854T2049SB-3CT292605-14-2 $C_{18}H_{16}O_5S_2$ 306.455T1757ML3231572414-83-5 $C_{23}H_{28}N_6O_7$ 304.456T2424P220771247819-59-5 $C_{12}H_{17}F_{17}NO_4S_2$ 315.3257T2493PD 1517	39	11/86	Daclatasvir, BMS/90052	1009119-65-6	$C_{40}H_{52}CI_2N_8O_6$	811.8
41T2743Ilomastat (GM6001, Galardin)142880-36-2 $C_{20}H_{38}N_4O_4$ 388.4642T2332Elvitegravir (GS-9137, JTK-303)697761-98-1 $C_{23}H_{23}CIFNO_5$ 447.8843T2329Dolutegravir (GSK1349572)1051375-19-9 $C_{20}H_{18}F_2N_3NaO_5$ 441.3644T2834Nobiletin478-01-3 $C_{21}H_{22}O_8$ 402.4046945T3028Celastrol34157-83-0 $C_{29}H_{38}O_4$ 450.6238146T2792Glucosamine sulfate20931-19-4 $C_{1}H_{15}NO_5$ 277.2547T0100Atazanavir sulfate229975-97-7 $C_{38}H_{54}N_6O_{11}S$ 802.9348T1853NMS 8731418013-75-8 $C_{27}H_{28}N_4O_3S_2$ 520.6749T1822Clemizole442-52-4 $C_{19}H_{20}ClN_3$ 325.8450T0100LAtazanavir198904-31-3 $C_{38}H_{52}N_6O_7$ 704.8751T0100LAtazanavir198904-31-3 $C_{38}H_{52}N_6O_7$ 704.8752T1502Vildagliptin (LAF-237)274901-16-5 $C_{17}H_{25}N_5O_7$ 704.8753T2030Tiplaxtinin(PAI-039)393105-51-8 $C_{24}H_{16}F_3NO_4$ 439.3854T2099SB-3CT292605-14-2 $C_{15}H_{14}O_3S_2$ 306.455T1757ML3231572414-83-5 $C_{23}H_{24}N_6$ 384.4856T2424P220771247819-59-5 $C_{12}H_7F_3NO_3S_2$ 315.3257T2493PD 151746179461-52-0 $C_{1}H_8FNO_2S$	40	T2324 (T3335)	Darunavir ethanolate	635728-49-3	$C_{29}H_{43}N_3O_8S$	593.73
42T2332Elvitegravir (GS-9137, JTK-303)697761-98-1 $C_{23}^{2}H_{23}^{2}$ CFNO5447.8843T2329Dolutegravir (GSK1349572)1051375-19-9 $C_{20}H_{18}F_2N_3NaO_5$ 441.3644T2834Nobiletin478-01-3 $C_{21}H_2O_8$ 402.4046945T3028Celastrol34157-83-0 $C_{29}H_{38}O_4$ 450.6238146T2792Glucosamine sulfate29031-19-4 $C_{6}H_{15}NO_5$ 277.2547T0100Atazanavir sulfate229975-97-7 $C_{38}H_54N_6O_{11}S$ 802.9348T1853NMS 8731418013-75-8 $C_{27}H_{28}N_4O_35_2$ 520.6749T1822Clemizole442-52-4 $C_{19}H_{20}ClN_3$ 325.8450T0100LAtazanavir198904-31-3 $C_{38}H_52N_6O_7$ 704.8751T0100LAtazanavir198904-31-3 $C_{24}H_{16}^2NO_4$ 439.3854T2009SB-3CT292605-14-2 $C_{15}H_{16}O_5A_2$ 303.454T2009SB-3CT292605-14-2 $C_{15}H_{16}O_5A_2$ 306.455T1757ML3231572414-83-5 $C_{23}H_{28}N_6A_2$ 344.4856T2424P220771247819-59-5 $C_{12}H_7E_NO_3S_2$ 315.3257T2493PD 151746179461-52-0 $C_{11}H_6NO_2S$ 237.2558T2503PAC1315183-21-2 $C_{23}H_{28}N_0A_2$ 392.4959T2393Efavirenz154598-52-4 $C_{14}H_6CI_8NO_2$ 315.6860T1883 <td>41</td> <td>T2743</td> <td>llomastat (GM6001, Galardin)</td> <td>142880-36-2</td> <td>$C_{20}H_{20}N_4O_4$</td> <td>388.46</td>	41	T2743	llomastat (GM6001, Galardin)	142880-36-2	$C_{20}H_{20}N_4O_4$	388.46
43T2329Dolutegravir (GSK1349572)1051375-19-9 $C_20H_18F_2N_3NaO_5$ 441.3644T2834Nobiletin478-01-3 $C_{21}H_{22}O_8$ 402.4046945T3028Celastrol34157-83-0 $C_{29}H_{38}O_4$ 450.6238146T2792Glucosamine sulfate2091-19-4 $C_{6}H_{15}NO_9S$ 277.2547T0100Atazanavir sulfate229975-97-7 $C_{38}H_{54}N_6O_{11}S$ 802.9348T1853NMS 8731418013-75-8 $C_{27}H_{28}N_4O_5S_2$ 520.6749T1822Clemizole442-52-4 $C_{19}H_{20}ClN_3$ 325.8450T1795Carfilzomib (PR-171)868540-17-4 $C_{40}H_{57}N_5O_7$ 704.8751T0100LAtazanavir198904-31-3 $C_{38}H_{52}N_6O_7$ 704.8752T1502Vildagliptin (LAF-237)274901-16-5 $C_{17}H_{25}N_3O_2$ 303.453T2030Tiplaxtinin(PAI-039)393105-53-8 $C_{24}H_{16}F_3NO_4$ 439.3854T2009SB-3CT292605-14-2 $C_{15}H_{14}O_5S_2$ 306.455T1757ML3231572414-83-5 $C_{23}H_{24}N_6$ 384.4856T2424P220771247819-59-5 $C_{12}H_7E_2NO_3S_2$ 315.3257T2493PD 151746179461-52-0 $C_{11}H_6FNO_5$ 237.2558T2503PAC1315183-21-2 $C_{23}H_{28}N_6O_2$ 392.4959T2393Efavirenz154598-52-4 $C_{14}H_9CF_3NO_2$ 315.6860 <td< td=""><td>42</td><td>T2332</td><td>Elvitegravir (GS-9137, JTK-303)</td><td>697761-98-1</td><td>C₂₂H₂₂CIFNO₅</td><td>447.88</td></td<>	42	T2332	Elvitegravir (GS-9137, JTK-303)	697761-98-1	C ₂₂ H ₂₂ CIFNO ₅	447.88
44T2834Nobiletin478-01-3 $C_{29}H_{22}O_8$ 402.4046945T3028Celastrol34157-83-0 $C_{29}H_{38}O_4$ 450.6238146T2792Glucosamine sulfate29031-19-4 C_6H_1 , NO, S277.2547T0100Atazanavir sulfate229975-97-7 $C_{38}H_{54}N_6O_{11}S$ 802.9348T1853NMS 8731418013-75-8 $C_{27}H_{28}N_4O_3S_2$ 520.6749T1822Clemizole442-52-4 $C_{19}H_{20}ClN_3$ 325.8450T1795Carfilzomib (PR-171)868540-17-4 $C_{40}H_{57}N_5O_7$ 719.9151T0100LAtazanavir198904-31-3 $C_{38}H_{52}N_6O_7$ 704.8752T1502Vildagliptin (LAF-237)274901-16-5 $C_{17}H_{25}N_3O_2$ 303.453T2030Tiplaxtinin(PAI-039)393105-53-8 $C_{24}H_{16}F_3NO_4$ 439.3854T2009SB-3CT292050-14-2 $C_{19}H_{4}O_3S_2$ 306.455T1757ML3231572414-83-5 $C_{23}H_{24}N_6$ 384.4856T2424P220771247819-59-5 $C_{12}H_7F_2NO_3S_2$ 315.3257T2493PD 151746179461-52-0 $C_{11}H_8FNO_5$ 237.2558T2503PAC1315183-21-2 $C_{23}H_{28}N_{4}O_2$ 392.4959T2393Efavirenz154598-52-4 $C_{14}H_9CIF_3NO_2$ 315.6860T1883Des(benzylpyridyl) atazanavi1192224-24-0 $C_{7}H_4N_5O_7$ 537.65	43	T2329	Dolutegravir (GSK1349572)	1051375-19-9	$C_{20}H_{10}F_2N_3NaO_5$	441.36
45T3028Celastrol34157-83-0 $C_{29}H_{38}O_4$ 450.6238146T2792Glucosamine sulfate29031-19-4 $C_6H_{15}NO_9S$ 277.2547T0100Atazanavir sulfate229975-97-7 $C_{38}H_{54}N_6O_{11}S$ 802.9348T1853NMS 8731418013-75-8 $C_{27}H_{28}N_4O_3S_2$ 520.6749T1822Clemizole442-52-4 $C_{19}H_{20}ClN_3$ 325.8450T0100LAtazanavir198904-31-3 $C_{38}H_{52}N_6O_7$ 704.8751T0100LAtazanavir198904-31-3 $C_{38}H_{52}N_6O_7$ 704.8752T1502Vildagliptin (LAF-237)274901-16-5 $C_{17}H_{25}N_3O_2$ 303.453T2030Tiplaxtinin(PAI-039)393105-53-8 $C_{24}H_16F_3NO_4$ 439.3854T2009SB-3CT292605-14-2 $C_{15}H_{14}O_3S_2$ 306.455T1757ML3231572414-83-5 $C_{23}H_2N_6$ 384.4856T2424P220771247819-59-5 $C_{12}H_7ENO_3S_2$ 315.3257T2493PD 151746179461-52-0 $C_{11}H_8FNO_2S$ 237.2558T2503PAC1315183-21-2 $C_{23}H_2N_4O_2$ 392.4959T2393Efavirenz154598-52-4 $C_{14}H_9CIF_3NO_2$ 315.6860T1883Des(benzylpyridyl) atazanavi119224-24-0 $C_{26}H_{43}N_6O_7$ 537.65	44	T2834	Nobiletin	478-01-3	$C_{1}H_{2}O_{2}$	402.40469
46T2792Glucosamine sulfate29031-19-4 $C_6H_{15}NO_9S$ 277.2547T0100Atazanavir sulfate229975-97-7 $C_{38}H_{4}N_6O_{11}S$ 802.9348T1853NMS 8731418013-75-8 $C_{27}H_{28}N_4O_3S_2$ 520.6749T1822Clemizole442-52-4 $C_{19}H_{20}ClN_3$ 325.8450T1795Carfilzomib (PR-171)868540-17-4 $C_{40}H_{57}N_5O_7$ 719.9151T0100LAtazanavir198904-31-3 $C_{38}H_{52}N_6O_7$ 704.8752T1502Vildagliptin (LAF-237)274901-16-5 $C_{17}H_{25}N_3O_2$ 303.453T2030Tiplaxtinin(PAI-039)393105-53-8 $C_{24}H_{16}F_3NO_4$ 439.3854T2009SB-3CT292605-14-2 $C_{15}H_{14}O_3S_2$ 306.455T1757ML3231572414-83-5 $C_{23}H_{24}N_6$ 384.4856T2424P220771247819-59-5 $C_{12}H_{72}N_0S_2$ 315.3257T2493PD 151746179461-52-0 $C_{11}H_8FNO_2S$ 237.2558T2503PAC1315183-21-2 $C_{23}H_{28}N_4O_2$ 392.4959T2393Efavirenz154598-52-4 $C_{14}H_6ClF_3NO_2$ 315.6860T1883Des(benzylpyridyl) atazanavi1192224-24-0 $C_{28}H_4N_5O_7$ 537.65	45	T3028	Celastrol	34157-83-0	$C_{20}H_{20}O_4$	450.62381
1011/12District of the constraint of the constrai	46	T2792	Glucosamine sulfate	29031-19-4	C-HNO-S	277.25
1716100Addition builde122575 57 $C_{38}n_{54}n_{6}n_{11}$ $502,15$ 48T1853NMS 8731418013-75-8 $C_{27}H_{28}N_4O_3S_2$ 520.6749T1822Clemizole442-52-4 $C_{19}H_{20}ClN_3$ 325.8450T1795Carfilzomib (PR-171)868540-17-4 $C_{40}H_{57}N_5O_7$ 719.9151T0100LAtazanavir198904-31-3 $C_{38}H_{52}N_6O_7$ 704.8752T1502Vildagliptin (LAF-237)274901-16-5 $C_{17}H_{25}N_3O_2$ 303.453T2030Tiplaxtinin(PAI-039)393105-53-8 $C_{24}H_16F_3NO_4$ 439.3854T2009SB-3CT292605-14-2 $C_{15}H_{14}O_3S_2$ 306.455T1757ML3231572414-83-5 $C_{23}H_{24}N_6$ 384.4856T2424P220771247819-59-5 $C_{12}H_7F_2NO_3S_2$ 315.3257T2493PD 151746179461-52-0 $C_{11}H_8FNO_2S$ 237.2558T2503PAC1315183-21-2 $C_{23}H_{28}N_4O_2$ 392.4959T2393Efavirenz154598-52-4 $C_{14}H_9ClF_3NO_2$ 315.6860T1883Des(benzylpyridyl) atazanavi1192224-24-0 $C_{56}H_{43}N_5O_7$ 537.65	47	T0100	Atazanavir sulfate	220031 12 1		802.93
10011001111001110010 $C_{27}H_{28}H_{43}J_{22}$ 320.0749T1822Clemizole442-52-4 $C_{19}H_{20}ClN_3$ 325.8450T1795Carfilzomib (PR-171)868540-17-4 $C_{40}H_57N_5O_7$ 719.9151T0100LAtazanavir198904-31-3 $C_{38}H_{52}N_6O_7$ 704.8752T1502Vildagliptin (LAF-237)274901-16-5 $C_{17}H_{25}N_3O_2$ 303.453T2030Tiplaxtinin(PAI-039)393105-53-8 $C_{24}H_{16}F_3NO_4$ 439.3854T2009SB-3CT292605-14-2 $C_{15}H_{14}O_3S_2$ 306.455T1757ML3231572414-83-5 $C_{23}H_{24}N_6$ 384.4856T2424P220771247819-59-5 $C_{12}H_7E_2NO_3S_2$ 315.3257T2493PD 151746179461-52-0 $C_{11}H_8FNO_2S$ 237.2558T2503PAC1315183-21-2 $C_{23}H_{28}N_4O_2$ 392.4959T2393Efavirenz154598-52-4 $C_{14}H_9ClF_3NO_2$ 315.6860T1883Des(benzylpyridyl) atazanavi1192224-24-0 $C_{26}H_{4N}N_5O_7$ 537.65	48	T1853	NMS 873	1418013-75-8	C H N O S	520.67
47511022Clefinizoite442 52 4 $C_{19} I_{20} chr_3$ 525.0450T1795Carfilzomib (PR-171)868540-17-4 $C_{40} H_5 N_5 O_7$ 719.9151T0100LAtazanavir198904-31-3 $C_{38} H_{52} N_6 O_7$ 704.8752T1502Vildagliptin (LAF-237)274901-16-5 $C_{17} H_{25} N_3 O_2$ 303.453T2030Tiplaxtinin(PAI-039)393105-53-8 $C_{24} H_{16} F_3 NO_4$ 439.3854T2009SB-3CT292605-14-2 $C_{15} H_{14} O_3 S_2$ 306.455T1757ML3231572414-83-5 $C_{23} H_{24} N_6$ 384.4856T2424P220771247819-59-5 $C_{12} H_7 F_2 NO_3 S_2$ 315.3257T2493PD 151746179461-52-0 $C_{11} H_8 FNO_2 S$ 237.2558T2503PAC1315183-21-2 $C_{23} H_{28} N_4 O_2$ 392.4959T2393Efavirenz154598-52-4 $C_{14} H_9 ClF_3 NO_2$ 315.6860T1883Des(benzylpyridyl) atazanavi1192224-24-0 $C_{26} H_{43} N_5 O_7$ 537.65	40	T1822	Clemizole	442-52-4	C H CIN	325.84
5011753Cumizonin (1171)000040 174 $C_{40}n_{57}n_{5}0_{7}$ 715.5151T0100LAtazanavir198904-31-3 $C_{38}H_{52}N_6O_7$ 704.8752T1502Vildagliptin (LAF-237)274901-16-5 $C_{17}H_{25}N_3O_2$ 303.453T2030Tiplaxtinin(PAI-039)393105-53-8 $C_{24}H_{16}F_{3}NO_4$ 439.3854T2009SB-3CT292605-14-2 $C_{15}H_{14}O_3S_2$ 306.455T1757ML3231572414-83-5 $C_{23}H_{24}N_6$ 384.4856T2424P220771247819-59-5 $C_{12}H_7F_2NO_3S_2$ 315.3257T2493PD 151746179461-52-0 $C_{11}H_8FNO_2S$ 237.2558T2503PAC1315183-21-2 $C_{23}H_{28}N_4O_2$ 392.4959T2393Efavirenz154598-52-4 $C_{14}H_9CIF_3NO_2$ 315.6860T1883Des(benzylpyridyl) atazanavi1192224-24-0 $C_{26}H_{43}N_5O_7$ 537.65	50	T1705	Carfilzonih (PR-171)	868540-17-4	C H N O	710 01
51T0100LAtazanavir198904-31-3C_{38}H_{52}N_6O_7704.8752T1502Vildagliptin (LAF-237)274901-16-5C_17H_25N_3O_2303.453T2030Tiplaxtinin(PAI-039)393105-53-8C_24H_16F_3NO_4439.3854T2009SB-3CT292605-14-2C_15H_14O_3S_2306.455T1757ML3231572414-83-5C_23H_2AN_6384.4856T2424P220771247819-59-5C_12H_7F_2NO_3S_2315.3257T2493PD 151746179461-52-0C_11H_8FNO_2S237.2558T2503PAC1315183-21-2C_23H_28N_4O_2392.4959T2393Efavirenz154598-52-4C_14H_9CIF_3NO_2315.6860T1883Des(benzylpyridyl) atazanavi1192224-24-0C_26H_43N_5O_7537.65	50	11795		000040-17-4	C ₄₀ H ₅₇ N ₅ O ₇	715.51
52T1502Vildagliptin (LAF-237)274901-16-5 $C_{17}H_{25}N_3O_2$ 303.453T2030Tiplaxtinin(PAI-039)393105-53-8 $C_{24}H_{16}F_3NO_4$ 439.3854T2009SB-3CT292605-14-2 $C_{15}H_{14}O_3S_2$ 306.455T1757ML3231572414-83-5 $C_{23}H_{24}N_6$ 384.4856T2424P220771247819-59-5 $C_{12}H_7F_2NO_3S_2$ 315.3257T2493PD 151746179461-52-0 $C_{11}H_8FNO_2S$ 237.2558T2503PAC1315183-21-2 $C_{23}H_{28}N_4O_2$ 392.4959T2393Efavirenz154598-52-4 $C_{14}H_2CIF_3NO_2$ 315.6860T1883Des(benzylpyridyl) atazanavi1192224-24-0 $C_{26}H_{43}N_5O_7$ 537.65	51	T0100L	Atazanavir	198904-31-3	$C_{38}H_{52}N_6O_7$	704.87
53T2030Tiplaxtinin(PAI-039)393105-53-8 $C_{24}H_{16}F_3NO_4$ 439.3854T2009SB-3CT292605-14-2 $C_{15}H_{14}O_3S_2$ 306.455T1757ML3231572414-83-5 $C_{23}H_{24}N_6$ 384.4856T2424P220771247819-59-5 $C_{12}H_7F_2NO_3S_2$ 315.3257T2493PD 151746179461-52-0 $C_{11}H_8FNO_2S$ 237.2558T2503PAC1315183-21-2 $C_{23}H_{28}N_4O_2$ 392.4959T2393Efavirenz154598-52-4 $C_{14}H_9CIF_3NO_2$ 315.6860T1883Des(benzylpyridyl) atazanavi1192224-24-0 $C_{26}H_{43}N_5O_7$ 537.65	52	T1502	Vildagliptin (LAF-237)	274901-16-5	C ₁₇ H ₂₅ N ₃ O ₂	303.4
54T2009SB-3CT292605-14-2 $C_{15}H_{14}O_3S_2$ 306.455T1757ML3231572414-83-5 $C_{23}H_{24}N_6$ 384.4856T2424P220771247819-59-5 $C_{12}H_7F_2NO_3S_2$ 315.3257T2493PD 151746179461-52-0 $C_{11}H_8FNO_2S$ 237.2558T2503PAC1315183-21-2 $C_{23}H_{28}N_4O_2$ 392.4959T2393Efavirenz154598-52-4 $C_{14}H_9ClF_3NO_2$ 315.6860T1883Des(benzylpyridyl) atazanavi1192224-24-0 $C_{26}H_{43}N_5O_7$ 537.65	53	T2030	Tiplaxtinin(PAI-039)	393105-53-8	C ₂₄ H ₁₆ F ₃ NO ₄	439.38
55T1757ML3231572414-83-5C23H24N6384.4856T2424P220771247819-59-5C12H7F2NO3S2315.3257T2493PD 151746179461-52-0C11H8FNO2S237.2558T2503PAC1315183-21-2C23H28N4O2392.4959T2393Efavirenz154598-52-4C14H9CIF3NO2315.6860T1883Des(benzylpyridyl) atazanavi1192224-24-0C26H3NN5O7537.65	54	T2009	SB-3CT	292605-14-2	$C_{15}H_{14}O_{3}S_{2}$	306.4
56T2424P220771247819-59-5 $C_{12}^{2}H_{2}F_{2}NO_{3}S_{2}$ 315.3257T2493PD 151746179461-52-0 $C_{11}H_{8}FNO_{2}S$ 237.2558T2503PAC1315183-21-2 $C_{23}H_{28}N_{4}O_{2}$ 392.4959T2393Efavirenz154598-52-4 $C_{14}H_{9}CIF_{3}NO_{2}$ 315.6860T1883Des(benzylpyridyl) atazanavi1192224-24-0 $C_{26}H_{43}N_{5}O_{7}$ 537.65	55	T1757	ML323	1572414-83-5	$C_{23}H_{24}N_{6}$	384.48
57 T2493 PD 151746 179461-52-0 C11H ₈ FNO ₂ S 237.25 58 T2503 PAC1 315183-21-2 C2H ₂₈ N ₄ O ₂ 392.49 59 T2393 Efavirenz 154598-52-4 C14H ₉ ClF ₃ NO ₂ 315.68 60 T1883 Des(benzylpyridyl) atazanavi 1192224-24-0 C26H ₄₃ N ₅ O ₇ 537.65	56	T2424	P22077	1247819-59-5	$C_{12}H_{7}F_{2}NO_{3}S_{3}$	315.32
58 T2503 PAC1 315183-21-2 C ₂₃ H ₂₈ N ₄ O ₂ 392.49 59 T2393 Efavirenz 154598-52-4 C ₁₄ H ₉ ClF ₃ NO ₂ 315.68 60 T1883 Des(benzylpyridyl) atazanavi 1192224-24-0 C ₂₆ H ₄₃ N ₅ O ₇ 537.65	57	T2493	PD 151746	179461-52-0	C ₁₁ H ₈ FNO ₂ S	237.25
59T2393Efavirenz154598-52-4 $C_{14}^{-1}H_9CIF_3NO_2$ 315.6860T1883Des(benzylpyridyl) atazanavi1192224-24-0 $C_{26}H_{43}N_5O_7$ 537.65	58	T2503	PAC1	315183-21-2	C ₂₃ H ₂₈ N ₄ O ₂	392.49
60 T1883 Des(benzylpyridyl) atazanavi 1192224-24-0 $C_{26}H_{43}N_5O_7$ 537.65	59	T2393	Efavirenz	154598-52-4		315.68
	60	T1883	Des(benzylpyridyl) atazanavi	1192224-24-0	$C_{26}H_{43}N_5O_7$	537.65

(Continued on next page)

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No.	Identity	Chemical name	CAS no.	Molecular formula	Molecular wt (g/mol)
61	T1862	PR-619	2645-32-1	$C_7H_5N_5S_2$	223.328
62	T2625	MK0752	471905-41-6	C ₂₁ H ₂₁ ClF ₂ O ₄ S	442.9
63	T2639	LY2811376	1194044-20-6	$C_{15}H_{14}F_{2}N_{4}S$	320.36
64	T3075	FLI-06	313967-18-9	$C_{25}H_{30}N_2O_5$	438.52
65	T1969	DBeQ	177355-84-9	$C_{22}H_{20}N_4$	340.42
66	T1932	B-AP15	1009817-63-3	$C_{22}H_{17}N_{3}O_{6}$	419.39
67	T1924	LDN-57444	668467-91-2	C ₁₇ H ₁₁ Cl ₃ N ₂ O ₃	397.64
68	T1891	NSC 405020	7497-07-6	C ₁₂ H ₁₅ Cl ₂ NO	260.16
69	T2154	MG-132	133407-82-6	$C_{26}H_{41}N_3O_5$	475.62

TABLE 1 (Continued)

Reverse genetics techniques are necessary for virus research seeking to elucidate viral life cycle and pathogenesis and for the development of antivirals. To date, however, only a limited number of virus strains is available for reverse genetics. Moreover, it is generally difficult to construct cDNA clones of *Flaviviridae* viruses, especially flavivirus, and thereby to obtain the infectious viruses (15). In the present study, we initially generated the recombinant viruses using infectious full-length cDNA clones of HCV, JEV, DENV, and BVDV. A bacterium-free CPER method for the generation of infectious flaviviruses was developed recently (33–35). We therefore used the CPER method for the generation of an HiBiT recombinant DENV-2 16681 strain, and we successfully obtained a replication-competent DENV-2 virus possessing the HiBiT gene, suggesting that the recombinant reporter flaviviruses can be generated by CPER, and it is envisaged that this approach also can be applied to clinical isolates.

The development of effective prophylactics and therapeutics to control infectious diseases such as dengue and Zika is an urgent medical need, because no licensed reagents are clinically available at this time. We screened the commercially available compound library by using the recombinant *Flaviviridae* viruses described here, and we identified the compounds that significantly suppressed the viral replication and reported their antiviral activities (29, 30, 44), suggesting that the recombinant *Flaviviridae* viruses generated in the present study are applicable to the high-throughput screening of antiviral compounds against infection with flaviviruses. In addition, the viral dynamics and the sensitivity to the antivirals used in clinical settings were evaluated in human liver-transplanted chimeric mice by using recombinant HCV. Infection of recombinant HCV in mice could be monitored by luciferase activity, which was correlated with viral infectivity and sensitivity to the antivirals. Additional investigations of not only drug sensitivity but also tissue tropism by using animal models for other *Flaviviridae* viruses are necessary for further evaluation of the usefulness of the recombinant flaviviruses.

In summary, we constructed the recombinant viruses of the family *Flaviviridae*, possessing a small luciferase subunit in viral proteins, and our analyses indicated their usefulness for the screening of antiviral reagents and for investigations of viral dynamics *in vitro* and *in vivo*. Our findings will contribute to further studies, including those



FIG 7 Bacterium-free generation of an HiBiT recombinant flavivirus. Wild-type and HiBiT recombinant DENV-2 were generated by the CPER method. Luciferase activity and virus titer are shown as bar graphs.



FIG 8 Investigation of HiBiT *Flaviviridae in vivo* viral dynamics. Fifteen human liver-transplanted chimeric mice were injected intravenously with $10^{5.1}$ FFU (1 ml) of the reporter HCV. After the HCV RNA in blood reached a plateau, the chimeric mice were treated with the antiviral OBV and PEG-IFN- α , and mice were euthanized before and at 1 week posttreatment. Blood samples were collected for the detection of viral RNA (A), and the liver samples were subjected to determination of HCV RNA and luciferase signals (B).

of other RNA and DNA virus families, toward the engineering of recombinant viruses. The development of novel biological assays is required to improve our understanding of the molecular mechanisms of virus replication and pathogenesis and to continue advances in the discovery of antiviral reagents.

MATERIALS AND METHODS

Ethical statement. The animal experiments described here were approved by the Committee for Animal Experiment of Hiroshima University (A14-195) and were conducted in compliance with national and international animal experimentation guidelines.

Plasmids. The cDNA clones of CLDN1, miR-122, ApoE, LgBiT luciferase, and AcGFP were inserted between the Xhol and Xbal sites of the lentiviral vector pCSII-EF-RfA by using the infusion technique, and the resulting plasmids were designated pCSII-EF-CLDN1, pCSII-EF-miR-122, pCSII-EF-ApoE, pCSII-EF-LgBiT, and pCSII-EF-GFP, respectively. The plasmid pHH-JFH1 encodes the full-length cDNA of the JFH1 strain (GenBank accession number AB047639) (45). pHH-JFH1 encodes the full-length cDNA of the JFH1 strain (JenBank accession number AB047639) (45). pHH-JFH1-E2p7NS2mt contains three adaptive mutations in pHH-JFH1 (46). pJFH1-E2p7NS2mt-Nlucsec possesses full-length NanoLuc luciferase generated previously (19). BVDV was derived from full-length cDNA of BVDV strain NCP7 (GenBank accession number AF220247) (47). The plasmids pMW119-DV4 and pMWJEAT (22) encode a full-length infectious clone of the DENV serotype 4 H241 strain (GenBank accession number AY947539) and JEV AT31 strain, respectively. The cDNA clones encoding the viral sequence for transfection were flanked by a modified T7 promoter sequence at the 5' end and a Notl or KpnI restriction site at the 3' end. The cDNA clones encoding the liBiT luciferase gene were constructed by using a KOD-plus-mutagenesis kit (Toyobo) and the respective oligonucleotide primers. The plasmids used in this study were confirmed by sequencing with an ABI 3130 genetic analyzer (Thermo Fisher Scientific).

Cell lines. All of the mammalian cell lines were cultured at 37° C under the conditions of a humidified atmosphere and 5% CO₂. The human hepatocellular carcinoma-derived Huh7 cells, human embryonic kidney-derived 293T cells, human alveolar adenocarcinoma-derived A549 cells, human cervical cancerderived HeLa cells, baby hamster kidney fibroblast-derived BHK-21 cells, and African green monkey kidney-derived Vero E6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nakarai

Tesque) supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 10% fetal bovine serum (FBS). The Huh7-derived cell line Huh7.5.1 was kindly provided by Frank Chisari. The bovine kidneyderived MDBK cells were propagated in DMEM supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 5% BVDV antibody-free FBS (Japan Bio Serum), and 5% horse serum (Thermo Fisher Scientific). The *Aedes albopictus* mosquito-derived cell line C6/36 was grown in Leibovitz's L-15 medium (Thermo Fisher Scientific) with 10% tryptose phosphate broth and FBS at 28°C.

Antibodies and reagents. Mouse monoclonal antibodies to β -actin, double-stranded RNA (dsRNA), ApoE, and pestiviral NS3 were purchased from Sigma-Aldrich, English & Scientific Consulting Kft, Santa Cruz Biotechnology, and TropBio, respectively. Rabbit anti-CLDN1 and Alexa Fluor (AF) 488-conjugated anti-rabbit IgG antibodies were purchased from Thermo Fisher Scientific. Rabbit polyclonal antibody against HCV NS2 was obtained from Gene Tex. Rat anti-HA antibody was purchased from Roche Diagnostics. Rabbit anti-HCV NS5A antibody and anti-BVDV polyclonal antibody were generated previously (48, 49). The compounds DCV and SOF were purchased from Shanghai Haoyuan Chemexpress. OBV, PEG-IFN- α , TVR, IFN- α , and MPA were obtained from Chemscene, Roche, ChemStep, PBL Biomedical Laboratories, and Sigma-Aldrich, respectively. A chemical inhibitor library of 69 drugs (L1100; Protease Inhibitor Library) was purchased from TargetMol.

Immunoblotting. Cells lysed on ice in lysis buffer (20 mM Tris-HCI [pH 7.4], 135 mM NaCl, 1% Triton X-100, 10% glycerol) supplemented with a protease inhibitor cocktail, cOmplete Mini (Roche), were boiled in loading buffer and subjected to 5 to 20% gradient SDS-PAGE. The proteins were transferred to polyvinylidene difluoride membranes (Millipore) and incubated with the appropriate antibodies. The immune complexes were visualized with SuperSignal West Femto substrate (Thermo Fisher Scientific) and detected by use of an LAS-4000 image analyzer system (Fujifilm).

Preparation of viruses. All of the cDNA-derived JEV and BVDV were rescued as described previously (22, 50). The supernatants were collected from the electroplated cells, and the infectious titers were determined and expressed as focus-forming units (FFU) or expressed as 50% tissue culture infective doses (TCID_{so}) per milliliter. pHH-JFH1-E2p7NS2mt and mutants thereof were introduced into Huh7.5.1 cells. HCV in the supernatant was collected, and infectious titers were determined by a focus-forming assay and are expressed in FFU. The supernatants were collected and subjected to virus titration. The infectious DENV-4 H241 clone linearized with Notl was transcribed by using an mMESSAGE mMACHINE T7 Ultra kit (Thermo Fisher Scientific), and the *in vitro*-transcribed RNA (5 μ g) was electroporated into cells at 5 \times 10⁶ cells/0.5 ml under conditions of 190 V and 950 μ F using a Gene Pulser Xcell electroporation system (Bio-Rad) and then plated on DMEM containing 10% FBS. The supernatants were collected from the electroplated cells, and the infectious titers were determined by a focus-forming assay. JEV and DENV were propagated in C6/36 cells in order to reap sufficient virus yields.

Luciferase assay. Luciferase activity was measured by using a Bright-Glo luciferase assay system (Promega) and Nano-Glo HiBiT lytic detection system (Promega) according to the protocol provided by the manufacturer.

Virus replication kinetics. *In vitro* growth kinetics of the parental and recombinant viruses was evaluated in the susceptible cell lines. In the case of HCV, 100 μ l of the culture supernatants obtained from the transfected cells was inoculated with the naive Huh7.5.1 cells, and the cell culture supernatants were collected at 12, 24, 48, and 72 h postinoculation. Huh7 and C6/36 cells were inoculated with JEV at an MOI of 0.1. The supernatants of Huh7 cells were collected at 12, 24, 48, and 72 h postinfection, and the supernatants of C6/36 cells were collected at 24, 48, 72, 96, and 120 h postinfection. The replication kinetics of BVDV were determined in MDBK cells by inoculation at an MOI of 0.1 with the collection of cell culture supernatants at 12, 24, 48, and 72 h postinoculation. The virus titers were determined in duplicate using the respective cell lines.

Quantitative RT-PCR. For the quantification of viral RNA copies, total RNA was extracted from cells by using a PureLink RNA minikit (Thermo Fisher Scientific), and then first-strand cDNA synthesis and quantitative RT-PCR were performed by using a TaqMan RNA-to-Ct one-step kit and ViiA7 real-time PCR system (Thermo Fisher Scientific), respectively, according to the manufacturer's protocols. For quantification of viral RNA, the primer sets for the detection of the noncoding region reported in previous studies (51–53) were used. Fluorescent signals were determined by the ViiA7 system.

Neutralization assay. The polyclonal antibody against BVDV was 4-fold diluted and incubated with virus (8,000 TCID₅₀/ml) for 1 h and then inoculated into MDBK cells. Intracellular BVDV RNA levels and luciferase activity at 48 h postinfection were determined by qRT-PCR and a luminometer, respectively.

Buoyant density fractionation. The culture supernatants of cells infected with the parental and recombinant BVDV were concentrated with the use of Spin-X UF centrifugal concentrators (Corning), applied to the top of a linear gradient formed from 10% to 40% OptiPrep (Axis-Shield) in phosphatebuffered saline (PBS), and spun at 32,000 rpm for 16 h at 4°C by using an SW41 rotor (Beckman Coulter). Aliquots of 12 consecutive fractions were collected from top to bottom, and the density, infectious titer, and viral RNA level were determined for each fraction.

Generation of HiBiT recombinant flavivirus by CPER. The HiBiT recombinant flavivirus was generated by CPER as described previously (35), with some modifications. The viral RNA was obtained from the culture supernatants of the infected cells of the DENV-2 Thailand/16681/84 strain (GenBank accession number U87411) with the use of a PureLink RNA minikit. The viral RNA was reverse transcribed with a PrimeScript RT reagent kit (Perfect Real Time) (TaKaRa Bio) for cDNA. Seven fragments covering the entire gene were amplified by the respective primers and PrimeSTAR GXL DNA polymerase (TaKaRa Bio). The seven PCR products and UTR linker were cloned into pCR-Blunt II-TOPO vectors (Thermo Fisher Scientific). The plasmids were completely sequenced as described above. Eight PCR fragments next were generated with PrimeSTAR GXL DNA polymerase and primer pairs that have complementary ends with

a 24- to 30-nucleotide overlap. The resulting eight DNA fragments then were mixed in equimolar amounts (0.1 pmol each) and subjected to CPER with PrimeSTAR GXL DNA polymerase (an initial 2 min of denaturation at 98°C; 20 cycles of 10 s at 98°C, 15 s at 55°C, and 12 min at 68°C; and a final extension for 12 min at 68°C) to generate circular DNA. The CPER products were transfected into Huh7 cells with Trans IT LT-1 transfection reagent (Mirus). The culture supernatants were harvested 15 days posttransfection.

Animal experiments. For HCV infection, the generation of uPA+/+/SCID+/+ mice and the transplantation of human hepatocytes were performed as described previously (54, 55). All mice were transplanted with frozen human hepatocytes obtained from the same donor. The mice were anesthetized during infection, extraction of serum samples, and euthanasia. The concentration of human serum albumin was measured as described previously (54). Fifteen to 17 weeks after the hepatocyte transplantation, 15 mice were injected intravenously with 10^{5.1} FFU (1 ml) of the recombinant virus. Mouse serum samples were obtained at 1- or 2-week intervals after HCV infection, and the HCV RNA levels were measured. After HCV RNA levels in blood reached a plateau, OBV (5 mg/kg of body weight, per os) and pegylated IFN- α (30 μ g/kg, subcutaneously) were administered into the mice, and the liver samples were collected after euthanization for the detection of viral RNA and luciferase signals. The total RNA of the liver samples was purified with a PureLink RNA minikit and was subjected to gRT-PCR as described above. The remaining liver samples (150 mg) were homogenized by using an ultrasonic disintegrator in 1 ml of lysis buffer (100 mM Tris-HCl, 2 mM EDTA, 0.1% Triton X-100, pH 7.8), and the supernatants obtained after centrifugation at 15,000 \times g for 30 min at 4°C were used for luciferase assay as described above.

Statistical analysis. Results are expressed as the means \pm standard deviations or standard errors. The significance of differences in the means was determined by Student's t test.

ACKNOWLEDGMENTS

We thank M. Tomiyama and J. Higuchi for their secretarial work and O. Isken, M. Ishibashi, and Y. Sugiyama for their technical assistance.

This work was supported in part by grants-in-aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Science, Sports, and Technology (MEXT; http://www.mext.go.jp/) of Japan (16H06429, 16K21723, and 16H06432) and for Scientific Research (B) from MEXT of Japan (15H04736), from the Ministry of Health, Labor and Welfare of Japan and the Japan Agency for Medical Research and Development (AMED; http://www.amed.go.jp/) Research Program on Hepatitis, 17fk0210106h0002 and 16fk0310515h0105, and Research Program on Emerging and Reemerging Infectious Diseases, 17fk0108109h0001, and from the JSPS KAKENHI (https://www.jsps.go.jp/english/e-grants/), grant number 16J02628. T. Tamura is supported by a JSPS Research Fellowships for young scientists (https://www.jsps.go.jp/ english/e-grants/).

We have no conflicts of interest to declare.

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