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Original Article

Advancing high-throughput anti-HCV drug screening: a novel dual-reporter HCV replicon model with real-time monitoring

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Abstract

Background and purpose: Hepatitis C virus (HCV) infection is a global health concern due to its substantial impact on morbidity and mortality. The burden of diseases related to HCV continues to escalate, particularly as infections progress to late-stage liver conditions, resulting in hepatocellular carcinoma on a global scale. Direct-acting antivirals effectively target HCV replication; however, their unreasonable costs and adverse effects emphasize the need for accessible and efficient therapeutic alternatives with minimal side effects. The primary aim of this study was to devise an HCV replicon system featuring a dual-reporter mechanism to facilitate high-throughput screening of potential novel antiviral agents.
Experimental approach: The full-length HCV genome (pJFH1) was used to construct an HCV replicon system. The glycoprotein regions (E1 and E2) were substituted with a red fluorescent reporter, mCherry, enabling visualization of protein synthesis within the replicon. In addition, an adjacent green fluorescent reporter, dBroccoli, was strategically introduced in proximity to the NS5B stop codon to serve as a reliable indicator of HCV replication activity by monitoring the fluorescence signals.
Findings/Results: The findings of this study unequivocally validated the effectiveness of the novel HCV replicon system for transfecting Huh-7 cells. Furthermore, the replicon system demonstrated a concentration-dependent response to anti-HCV pharmaceutical agents including telaprevir and sofosbuvir.
Conclusion and implications: These compelling results underscored the potential utility of the proposed HCV replicon system as an innovative model for the expeditious high-throughput screening of prospective anti-HCV agents within a short timeframe.

Keywords: Anti-HCV agents; Hepatitis C virus; High throughput screening; RNA aptamer.

INTRODUCTION

Hepatitis C virus (HCV) is a bloodborne pathogen responsible for liver inflammation that affects more than 58 million individuals worldwide (1). HCV infection can result in persistent hepatitis, cirrhosis, hepatocellular carcinoma, and an elevated susceptibility to severe liver complications, potentially leading to grave health outcomes. There is currently a lack of vaccines available to prevent HCV infections. In response to this public health challenge, the World Health Organization (WHO) has established an ambitious target to eliminate viral hepatitis by 2030, with the development of effective pharmaceutical interventions forming a pivotal component of this overarching strategy.

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A groundbreaking advancement in this regard was the introduction of direct-acting antivirals (DAAs), a novel class of drugs introduced in 2011. These agents exhibit a commendable safety profile, are conveniently administered through an oral once-daily regimen, and have efficacy rates surpassing 95% (2). Despite these promising attributes, the global adoption of DAAs has been hampered by concerns regarding their adverse effects, high economic costs, and suboptimal patient compliance. Consequently, the quest for the identification and development of novel therapeutics or anti-HCV agents persists with an emphasis on formulations that are not only cost-effective but also demonstrate enhanced efficacy and safety profiles compared with existing DAAs (3). This pursuit aligns with the broader imperative of meeting the WHO's elimination goals for viral hepatitis, necessitating a comprehensive and nuanced approach to drug development and therapeutic intervention in HCV.

HCV is taxonomically classified within the Flaviviridae family, specifically under the genus Hepacivirus, with its genomic diversity encompassing 8 genotypes (GT1-8) and 86 subtypes displaying nucleotide distinctions of 30% and 15%, respectively (4,5). Structurally, HCV manifests as a positive-sense single-stranded RNA virus with a diameter ranging from 55 to 65 nm. The HCV genome encodes a polyprotein that is processed to yield 10 viral proteins, including core proteins, envelope proteins E1 and E2, ion channel protein p7, and non-structural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). NS proteins primarily participate in the replication processes of HCV, with NS2-NS3 and NS3-NS4 acting as viral proteases, NS5B serving as a viral RNA-dependent RNA polymerase, and NS5A playing a pivotal role in the assembly of newly synthesized RNA and structural HCV proteins (6,7).

The intricacies of studying the replication cycle of HCV in cell culture systems have prompted the development of various models, among which subgenomic replicons and HCV generated from cell culture (HCVcc) stand out. These models are invaluable for comprehensively investigating the HCV replication cycle and evaluating the efficacy of anti-HCV interventions (5). The particular remark is HCVcc derived from a wild-type HCV genotype 2a originally isolated from a Japanese patient with fulminant HCV-associated hepatitis (JFH1). This specific HCVcc model has garnered widespread utilization in scientific inquiry, emerging as a cornerstone for studying the complete HCV life cycle and conducting screening to assess the effectiveness of anti-HCV agents (8-10). The use of this cell-derived HCV system has proven to be instrumental in unraveling the molecular intricacies governing the HCV life cycle. Its significance extends beyond basic research, positioning it as a valuable tool for developing specific antiviral drugs (9). The HCV replicon is a sub-genomic RNA that can replicate independently in hepatoma cells. The replicons are composed of antibiotic-resistance genes for selection and non-structural proteins NS3 to NS5B, which encode essential enzymes for viral replication (11). HCV replicon systems have become crucial in preclinical research and the development of efficient DAAs. HCV replicons can be divided into 2 elements depending on the cistron used to construct the replicon: a bicistronic replicon and a monocistronic replicon (5). Bicistronic replicons were a replicon that was constructed with 2 gene clusters: (i) the HCV 5’NTR and the first 16 codons of the core gene fused in-frame with the selectable neomycin phosphotransferase gene (*neo*), and (ii) the internal ribosomal entry site (IRES) of the encephalomyocarditis virus (ECMV) that directs the translation of replicon (5). The other type of replicon is the monocistronic replicon, which lacks the EMCV and IRES. They consist of only HCV 5'NTR, which translates only non-structural coding region, NS3 to NS5B. Therefore, they are structurally similar to the viral genomes. However, the model lacks a detection system. Monocistronic replicon replication can be investigated by detecting viral proteins using specific antibodies and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR), which requires skill and expensive equipment. Although the full-length HCV genome can perform a replication process similar to natural

HCV, selection markers still need to be considered. The G418 selection marker is widely used and requires approximately 3 weeks for replicon library preparation (5). Within the array of HCV proteins, NS5B plays a distinctive role as an RNA-dependent RNA polymerase, orchestrating the intricate replication of the viral RNA genome throughout the viral replication cycle. Notably, the catalytic site of NS5B shows remarkable conservation across diverse HCV genotypes, making this enzyme an appealing and promising target for developing anti-HCV drugs (12-14).

Although the study of infectious HCVcc systems has markedly enriched our understanding of HCV biology including identifying variants resistant to DAAs within the context of infected viral life cycles (15-17), the indispensability of HCV replicon technology persists. Characterized by its user-friendly nature, this technology plays a pivotal role in HCV research by serving as a cornerstone for identifying candidate DAAs and assessing DAA resistance. Its easy usage facilitates streamlined experimentation, contributing to the ongoing quest for therapeutic modalities that enhance the overall efficacy of HCV treatment. The interplay between HCV replicon technology and infectious HCVcc systems is characterized by synergistic collaboration. This collaboration significantly advances our understanding of HCV pathogenesis, shedding light on the intricacies of viral replication and assembly, and the development of resistance to antiviral agents. The combined use of these technologies contributes synergistically to the refinement of therapeutic strategies against HCV infection, providing a comprehensive approach to tackling the challenges posed by this viral infection. As research in this field progresses, integrating insights from both HCV replicon technology and infectious HCVcc systems will continue to be instrumental in shaping the landscape of HCV treatment modalities, paving the way for more effective and targeted interventions in the ongoing battle against hepatitis C.

Aptamers characterized by their single-stranded folded nucleic acid structures have emerged as powerful molecular tools capable of recognizing specific molecules with high affinity. An aptamer was used to construct a biosensor using a fluorescent dye (cyanine3) RNA aptamer directed against HCV (18). Notably, recent advancements have been made in methods for assaying RNA-modifying enzymes using light-up RNA aptamers (19-21). These assays leverage RNA aptamers that activate a fluorometric substrate upon binding, resulting in fluorescence proportional to enzymatic activity (19). This approach offers distinct advantages: it is straightforward, precise, and sensitive. Thus, it is highly effective for analyzing enzyme activity. Furthermore, RNA aptamer assays can be adapted to high-throughput screening platforms, facilitating the identification of inhibitors from compound libraries and the discovery of novel inhibitors targeting HCV-NS5B. This study aimed to develop a replication-deficient HCV replicon system tailored for high-throughput anti-HCV screening. Thus, we genetically modified pJFH1, a plasmid encoding the full-length HCV RNA genome. This modification involved the introduction of a fluorescent RNA aptamer designed to monitor the real-time activity of HCV NS5B during viral RNA replication within cells. This innovation laid the integration of the aptamer biosensor, which allowed for the dynamic observation of NS5B activity through fluorescence signals. This novel HCV replicon model represented a promising advancement in anti-HCV screening.

MATERIALS AND METHODS

Cell culture, reagents, and viruses

HepaRG and Huh-7 cell lines were cultivated in Dulbecco's modified eagle's medium mixture nutrient F-12 (DMEM/F12) with 10% fetal bovine serum (FBS) containing 100 IU/mL penicillin and 100 µg/mL streptomycin (Invitrogen, USA). The cells were maintained at 37 $^{\circ}$C with 5% CO_2 and subcultured every 2-3 days. The pJFH-1 plasmid carrying the full-length cDNA of HCV genotype 2a was kindly provided by Prof. Wakita Takaji (National Institute of Infectious Diseases, Tokyo, Japan). Anti-HCV agents,

sofosbuvir, and telaprevir were purchased from Apex Bio, Taiwan. The Lipofectamine® 3000 transfection kit was purchased from Invitrogen, USA. (5Z)-5-[(3,5-difluoro-4-hydroxyphenyl) methylidene] -2, 3-dimethylimidazol-4-one (DFHBI) was purchased from Sigma-Aldrich, USA. The restriction endonucleases such as *Kpn*I, *Hin*dIII, *Xba*I, and *Not*I were purchased from New England BioLabs, UK.

Construction of the HCV replicon

The full length of HCV RNA was amplified using specific primers HCV-F (5'- CCCCCTGCAGG TTAATACGACTCAC TATTACCTGCCCCTAATAGGGGC-3') and HCV-R (5'-GGGTCTAGAACATGATCTG CAGAGAGACCAGT-3') under the following conditions: initial denaturation at 98 °C for 30 s, denaturation at 98 °C for 10 s, annealing at 68.7 °C for 15 s, and extension at 72 °C for 4 min for 35 cycles. After confirming the size of the amplicon by agarose gel electrophoresis, the amplicon was ligated into the pUC19 plasmid vector.

The DNA sequences of 2 reporter aptamers, mCherry and dBroccoli, along with a fragment of the HCV genome were amplified by PCR using the following primers: mCherry F (5'-CTTCTTAAGCTTGCGGG AGACGTCGAGTCCACCCCCGGGCCCCG GGTGGTCCCCTTGACCA-3') and mCherry R (5'- GGACTCGACGTCTCCCGCAAGC TTAAGAAGGTCAAAATTCAACAGCTTG TACAGCTCGTCCATGCCGCC-3'). The PCR conditions included denaturation at 98 °C for 10 s, annealing at 68 °C for 15 s, and extension at 72 °C for 15 s for 35 cycles. The primer for dBroccoli was dBroccoli F (5'-CTCCCCGCTCGGTAGTTGCCATGTGTAT GTGGGAGACGGTC-3') and dBroccoli R (5'-CTAGTGTGTGCCGCTTTGCCATGAATGA TCCCGAAGGATCATCAG-3'). The PCR conditions were an initial denaturation at 98 °C for 30 s, denaturation at 98 °C for 10 s, annealing at 66 °C for 15 s, and extension at 72 °C for 15 s for 35 cycles.

Four fragments of the HCV genome were amplified by PCR using 4 sets of primers. Fragment 3F (5'-GATCA TTCATGGCAAAGCGGCACACACTAGGT ACACTCC-3') and fragment 3R (5'-CTCCTCGACGTCCCCGGCTTGCTTGAGG AGAGAGAAATTTGTGGCAAAGGGGAA ACCGGGTAGGTTCC-3') were used for the 3rd fragment. The PCR conditions were an initial denaturation at 98 °C for 30 s, denaturation at 98 °C for 10 s, annealing at 68 °C for 15 s, and extension at 72 °C for 3 min for 35 cycles. Fragments 4 and 5 were amplified using fragment 4F (5'-CTTCTTAAGCTTGCGGGAGACGTCGAG TCCACCCCCGGGCCCCGGGTGGTCCCC TTGACCA-3') and fragment 4R (5'-ACATACACATGGCAACTACCGAGCGGG GAGTAGGAAGAG-3') for the 4th fragment, and fragment 5F (5'-TCCATGTCATA CTCCTGGACCGG -3') and fragment 5R (5'-CTCCTCGACGTCCCCGGCTTGCTTGAGG AGAGAGAAATTTGTGGCAAAGGGGAA ACCGGGTAGGTTCC-3') for the 5th fragment. These 2 fragments were amplified using a 2-step PCR protocol with an initial denaturation at 98 °C for 30 s, denaturation at 98 °C for 10 s, and extension at 72 °C for 4 min for 35 cycles.

The last fragment of the HCV genome (fragment 6) was amplified using fragment 6F (5'-CTTCTTAAGCTTGCGGGAGACGTCGAG TCCACCCCCGGGCCCCGGGTGGTCCCC TTGACCA-3') and fragment 6R (5'-GGAGTATGACATGGAGCAGCACACGGT GGTATCG-3') under the following conditions: initial denaturation at 98 °C for 30 s, denaturation at 98 °C for 10 s, annealing at 68.7 °C for 15 s, and extension at 72 °C for 15 s for 35 cycles. The sizes of all amplicons were confirmed by agarose gel electrophoresis. All fragments, including mCherry and dBroccoli, were assembled to construct the replication-deficient HCV replicon using the Gibson Assembly® Master Mix (New England Bio Labs, USA) according to the manufacturer's protocol.

Replicon transfection

The HCV replicon was digested with the FastDigest *Xba*I restriction enzyme for 10 min, and subsequently *in vitro* transcription was performed. The DNA template was mixed with *Xba*I and 5X buffer according to the manufacturer's protocol in a 1.5-mL microcentrifuge tube and incubated for

10 min at room temperature. The reaction was stopped by heating at 65 °C for 10 min. The digested-replicon was purified, and all reagents were added according to the T7 *in vitro* transcription manufacturer's protocol before incubating at -20 °C for 4-5 h. After that, the HCV RNA replicon solution was purified and dissolved in diethyl pyrocarbonate-treated water.

Approximately 6×10^4 Huh-7 cells were seeded in a 96-well plate and cultured in DMEM media with 10% FBS 2 days before the experiment. On the experiment day, the culture media was replaced by DMEM media without FBS. The transcribed HCV RNA replicon solution was mixed with DMEM media without FBS and Lipofectamine® 3000 reagent according to the manufacturer's protocol and incubated the mixture for 15 min at room temperature. After incubation, the mixture was added to each well. The 96-well plate was incubated for 40 h at 37 °C with 5% CO_2.

Anti-HCV activity assay

Huh-7 cells were transfected with HCV replicons using Lipofectamine® 3000 in a 96-well plate. After incubating at 37 °C with 5% CO_2 for 2 h, the various concentrations of anti-HCV agents, sofosbuvir and telaprevir, were added to each well and further incubated for 40 h. After incubation, the transfection cells were fixed with 4% formaldehyde for 10 min at room temperature before washing wells twice with PBS. Then, 300 nM 4',6-diamidino-2-phenylindole (DAPI) solution was added to each well and incubated for 5 min at room temperature. After washing with PBS, the solution of DFHBI at a concentration of 100 µM was added and incubated for 15 min. The fluorescence signals were observed by confocal microscopy (AX/AX R Confocal Microscope System, Nikon, Japan). The fluorescence signals were determined by a cell imaging system (EVOS Cell Imaging Systems, Thermo Fisher Scientific, USA). Anti-HCV activity was determined *via* the fluorescence expression level compared with the untreated cells.

The quantitative real-time RT-PCR

Huh-7 cells were transfected with HCV replicon using Lipofectamine® 3000 and incubated for 40 h. After incubation, transfected cells were harvested, and HCV RNA was extracted using a QIAamp® Viral RNA mini kit (QIAamp, Germany). Real-time RT-PCR was performed by mixing total RNA with KAPA SYBR FAST One-Step qRT-PCR Kits (Kapa Biosystems, USA). The reaction was performed by using the Mx3000P qPCR System (Agilent Technologies, Inc., USA) with specific primers for JFH-1 HCV (forward primer: 5'-TCTGCGGAACCGGTGAGTA-3' and reverse primer: 3'-TCAGGCAGTACCAC AAGGC-5'). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as the internal control.

Statistical analysis

All experiments were performed in triplicate. The data were presented as mean ± SD and analyzed using one-way ANOVA followed by Tukey multiple comparison test *via* GraphPad Prism 8.0 software.

RESULTS

Construction of the HCV replicon

A novel HCV replicon was constructed in the present study by using a series of genetic manipulations. Initially, the HCV genome was transferred from plasmid pJFH1 to pUC19, a common vector used in molecular cloning. Subsequently, the fragments encoding mCherry and dBroccoli were added to the pUC19-HCV construct. The resulting plasmid, pUC19-HCV, was thoroughly validated by sequencing and demonstrated 100% similarity to the full-length HCV genome. Refinement of the HCV replicon involved the replacement of the E1 and E2 regions of the HCV genome with fragments encoding mCherry. The green fluorescent reporter dBroccoli was strategically inserted after the stop codon of NS5B. A schematic representation of the newly constructed HCV replicon is shown in Fig. 1.

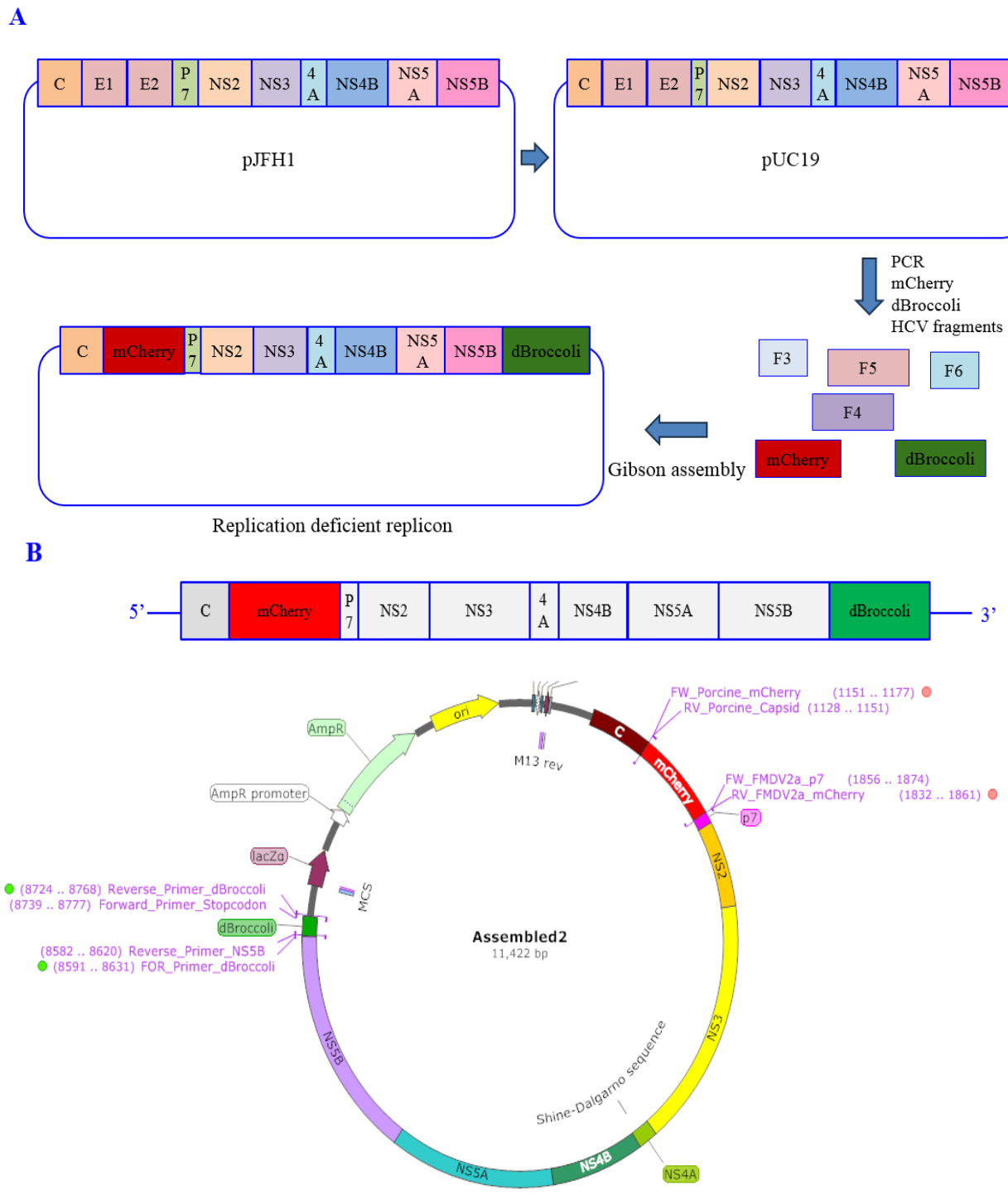

Fig 1. Schematic diagram of the constructed HCV replicon. (A) Construction of replication deficient HCV replicon and (B) the order of genetic codon of replicon from 5' to 3' as C-mCherry-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-dBroccoli. HCV, Hepatitis C virus; PCR, polymerase chain reaction.

To further validate the successful construction of the replicon, a series of restriction digests was performed using 3 distinct endonuclease sets: *Not*I and *Hin*dIII (set 1), *Hin*dIII and *Kpn*I (set 2), and *Xba*I (set 3). These results confirmed the integrity and structure of the replicon. Specifically, *Not*I and *Hin*dIII digestion yielded 3 fragments measuring 5.2, 4.1, and 1.78 Kb, indicating successful cleavage at the corresponding recognition sites. Similarly, *Hin*dIII and *Kpn*I digestion produced 4 fragments of 5.2, 2.5, 1.8, and 1.2 Kb, corroborating the expected cleavage pattern. Notably, the *Xba*I digestion, which targeted a single site, resulted in a single DNA fragment measuring 11 kb (Fig. 2).

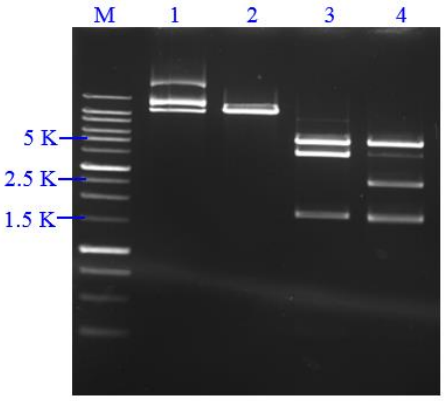

Fig. 2. The HCV replicon digested with restriction endonucleases. Lane M, XLarge DNA Ladder ((GeneDireX, Inc., Germany) Cat. No. DM013-R500); lanes 1 and 2, HCV replicon digested with *Xba*I; lane 3, HCV replicon digested with *Hin*dIII and *Not*I; lane 4, HCV replicon digested with *Hin*dIII and *Kpn*I. HCV, Hepatitis C virus.

The restriction digestion results provided compelling evidence for the accurate assembly and structural integrity of the newly constructed HCV replicon. This meticulous validation ensured replicon faithfully represented the intended genetic modifications, paving the way for its subsequent use in anti-HCV high-throughput screening studies.

Expression of the HCV replicon

To investigate the optimal expression time for the newly constructed HCV replicon, a series of transfections into the Huh-7 cell line was conducted, followed by incubation for varying durations between 24 and 48 h. The outcomes revealed that green and red fluorescence signals were prominently observed in Huh-7 cells transfected with the HCV replicon. In contrast, pJFH-1 cells lacking mCherry and dBroccoli exhibited no discernible fluorescence signals. The emergence of red fluorescent signals from mCherry confirmed the ongoing translation process. Additionally, the appearance of green fluorescence signals was contingent on the binding and activation of the fluorometric substrate (DFHBI) by dBroccoli during RNA synthesis. Importantly, the intensity and quantity of fluorescence signals were directly proportional to the enzymatic activity. Figure 3A and 3B visually depict cells exhibiting the fluorescence intensity of dBroccoli and mCherry at specific time points post-transfection. Notably, both red and green signals were observed near the nucleus, which appeared blue after staining with DAPI (Fig. 3A). Quantification of the fluorescence signals at different time points revealed that Huh-7 cells transfected with the HCV replicon exhibited the highest fluorescence signal at 40 h post-transfection (Fig. 3C). Therefore, this time point was identified as the optimal for achieving the highest level of fluorescence.

The decision to select the 40-h post-transfection time frame for further experiments was substantiated by the demonstrable peak in fluorescence expression under these conditions. This optimal time point served as a critical parameter for subsequent investigations, ensuring that the experiments were conducted under conditions that maximized the expression of fluorescent signals. This thorough optimization process, enhanced the precision and reliability of the experimental setup, providing a solid foundation for subsequent studies involving the HCV replicon system.

Evaluation of the generated HCV replicon model

The established anti-HCV agents including telaprevir and sofosbuvir were employed to assess the constructed HCV replicon. The fluorescence signals in drug-treated groups were evaluated at the concentrations of 0.1, 1, and 10 µM compared to untreated cells. Fluorescence signals from the drug-treated groups were consistently lower than those from the untreated group (Fig. 4). Across all HCV drug-treated groups, a decrease in fluorescence signals was observed at the 0.1, 1, and 10 µM concentrations of sofosbuvir or telaprevir, except dBroccoli and mCherry fluorescence signals in the 0.1 µM telaprevir-treated groups (Fig. 4). The significant differences were observed in the sofosbuvir-treated groups at the concentrations of 0.1, 1, and 10 µM compared with untreated group (Fig. 4A). Upon comparing the fluorescence signals of the sofosbuvir-treated groups with untreated cells, statistically significant differences were identified at the concentrations of 0.1, 1, and 10 µM (Fig. 5). Moreover, the telaprevir-treated groups exhibited fluorescence signals compared to untreated cells at the concentrations of 0.1, 1, and 10 µM, which was not significant at the concentration of 0.1 µM (Fig. 4B).

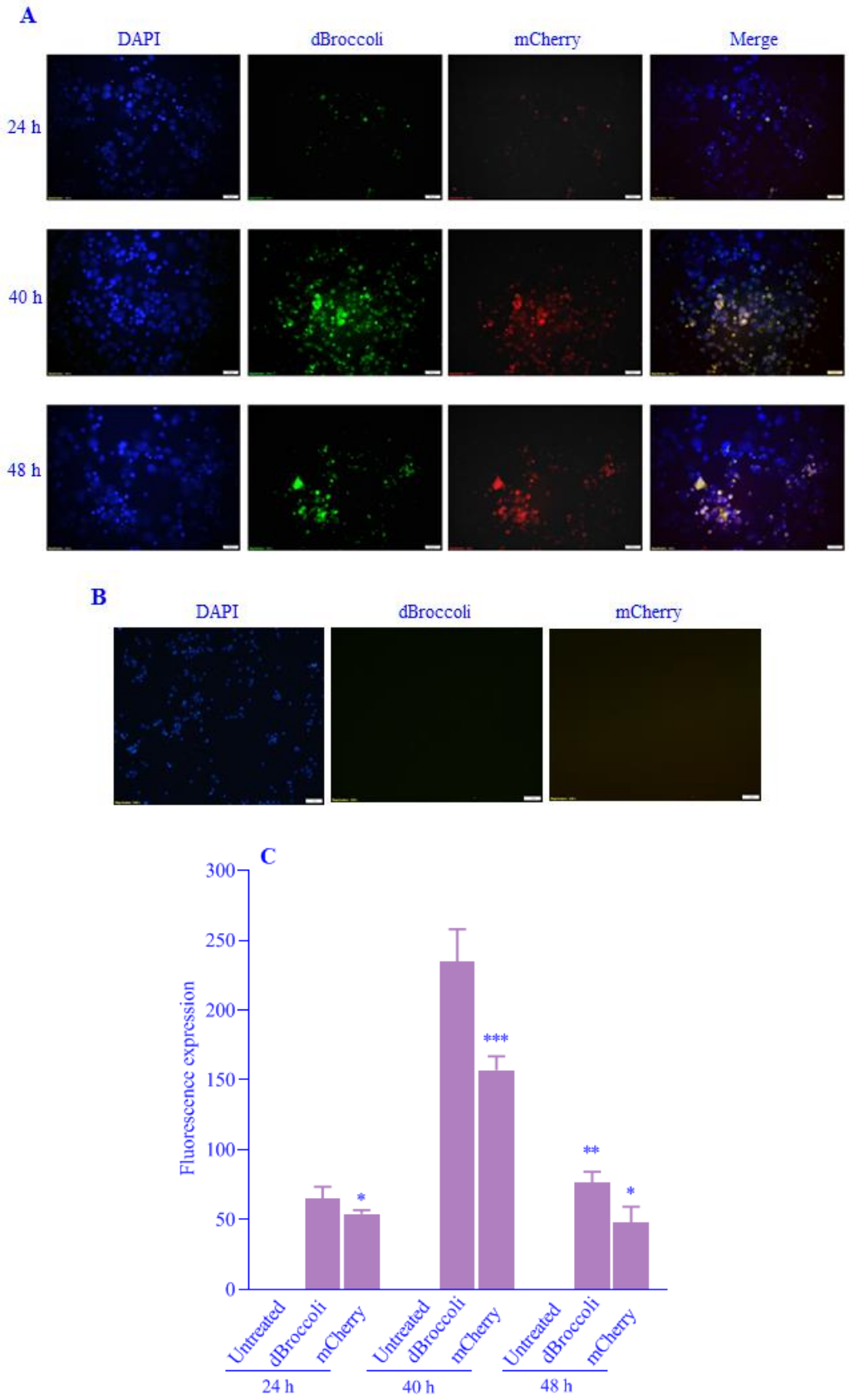

Fig. 3. The fluorescence signals of HCV replicon in Huh-7 cells. The green and red (A) fluorescence signal from HCV replicon transfected Huh-7 compared with (B) untransfected cells. The expressions of reporter systems were observed under a confocal microscope at 24, 40, and 48 h post-transfection. The photo of untransfected cells was taken at 40 h; (C) the expression of fluorescence reporters at 24, 40, and 48 h post-transfection. Data were expressed as mean ± SD, n = 3. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ demonstrated significant differences compared with untreated in respective time points. HCV, Hepatitis C virus; DAPI, 4',6-diamidino-2-phenylindole.

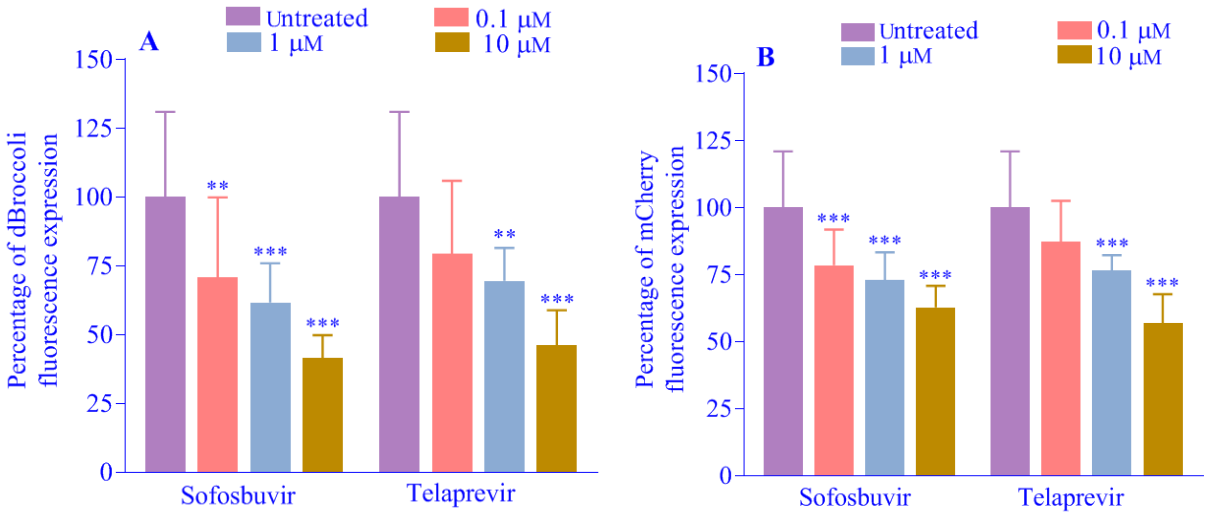

Fig. 4. Fluorescence signals detected from cells transfected with the HCV replicon in HCV drug-treated groups at the concentrations of 0.1, 1, and 10 µM. (A and B) fluorescence signals from dBroccoli and mCherry expressions, respectively. Data were expressed as mean ± SD. $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ demonstrated significant differences compared with untreated cells in the respective drugs. HCV, Hepatitis C virus.

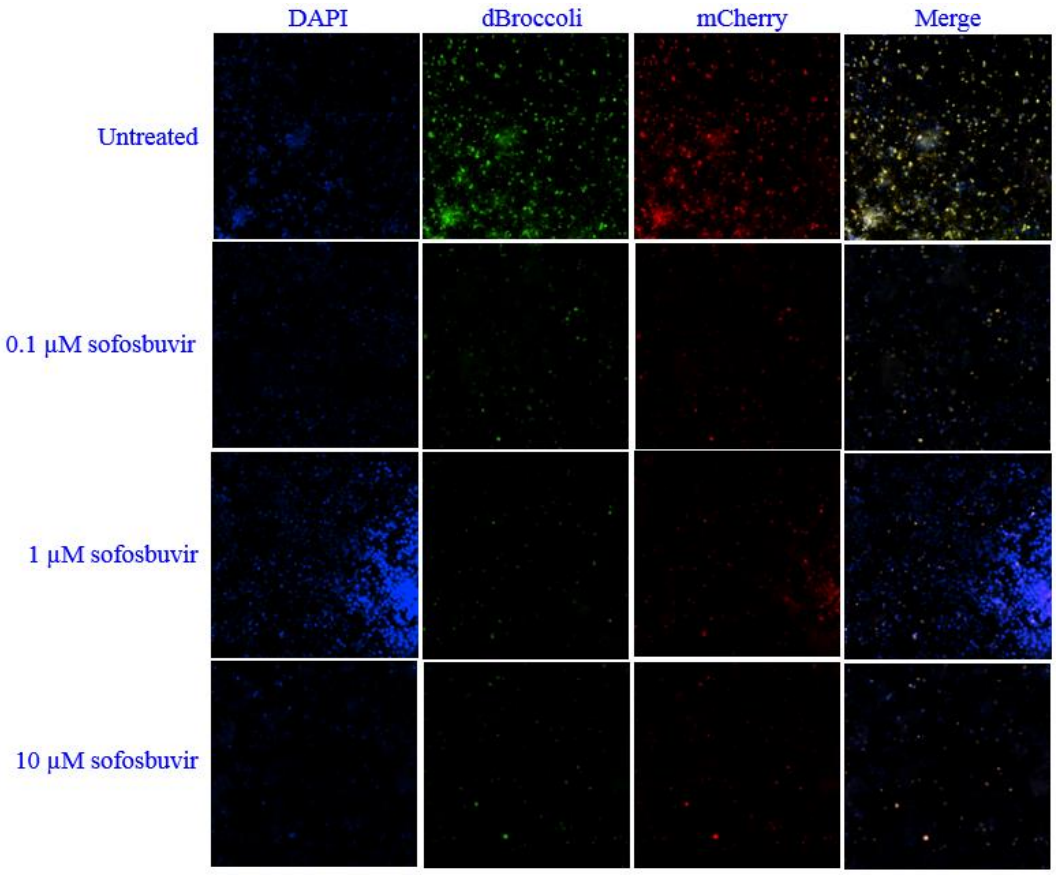

Fig. 5. The fluorescence signals between untreated-Huh-7 cells compared with sofosbuvir-treated HCV replicon-transfected Huh-7 cells. Huh-7 cells were transfected with the HCV replicon and incubated with various concentrations of sofosbuvir. The transfected cells were observed under the confocal microscope at 40 h post-infection. HCV, Hepatitis C virus; DAPI, 4',6-diamidino-2-phenylindole.

The further analysis of dBroccoli fluorescence signals within each concentration from the telaprevir-treated groups revealed a significant decrease at the drug concentration of 10 µM. Moreover, the assessment of mCherry fluorescence signals within each concentration from the telaprevir-treated groups showed a significant reduction at 1 and 10 µM. However, no significant difference was observed in mCherry fluorescence signals from telaprevir-treated groups at the concentration of 0.1 µM (Figs. 4 and 6).

Validation of the generated HCV replicon model

To confirm the successful generation of the HCV replicon model, a comprehensive investigation of its mechanism of action was conducted using real-time RT-PCR. The relationship between fluorescence drift correction and the cycle in real-time RT-PCR of the sofosbuvir- or telaprevir-treated groups was shown in Fig. 7. This study aimed to elucidate the dynamics of HCV RNA expression under different conditions. The results revealed that the HCV RNA levels were detectable in varying cycles for each condition. HCV RNA was detected later in the drug-treated groups than in the negative group (untreated). This observation aligned with findings from previous experiments, where the amount and fluorescence intensity were reduced in the presence of higher concentrations of both sofosbuvir and telaprevir (Figs. 5 and 6). The delayed detection of HCV RNA in the drug-treated groups further supported the antiviral action of sofosbuvir and telaprevir on the HCV replicon.

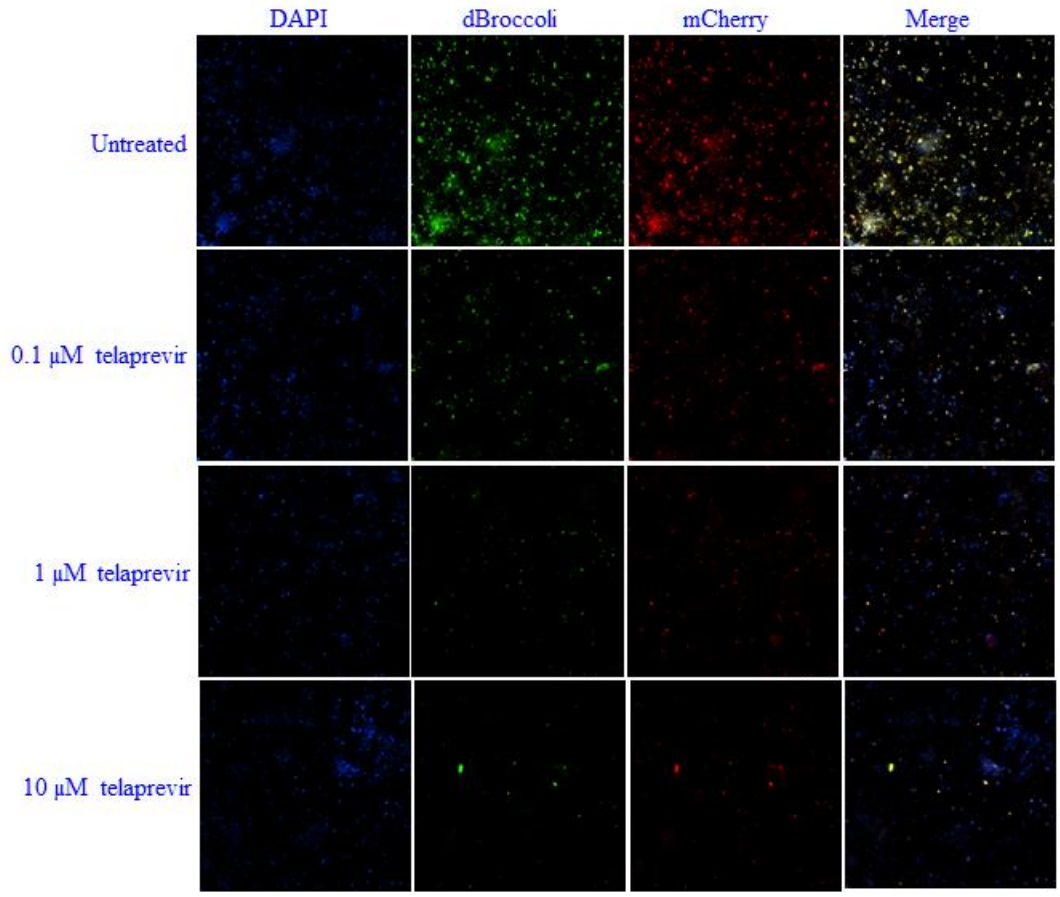

Fig. 6. The fluorescence signals of untreated-Huh-7 cells compared with telaprevir-treated HCV replicon-transfected Huh-7 cell lines. Huh-7 cells were transfected with the HCV replicon and incubated with various concentrations of telaprevir. The transfected cells were observed under the confocal microscope at 40 h post-infection. HCV, Hepatitis C virus; DAPI, 4',6-diamidino-2-phenylindole.

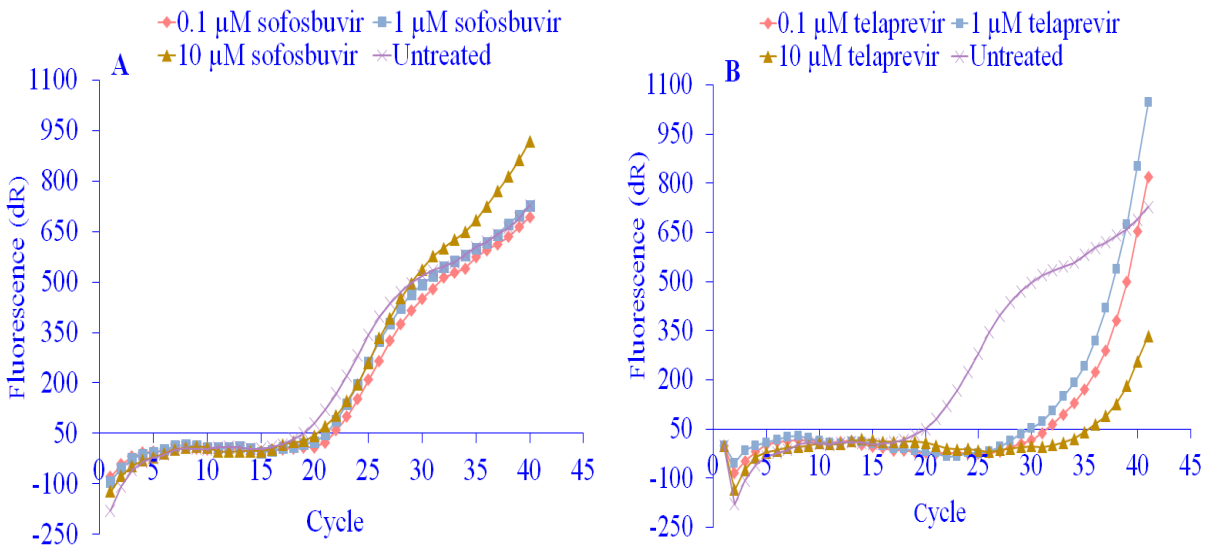

Fig. 7. The relationship between the fluorescence dR and the cycle of real-time reverse transcription polymerase chain reaction of (A) sofosbuvir- or (B) telaprevir-treated groups. The glyceraldehyde-3-phosphate dehydrogenase RNA was used as the internal control. dR, drift correction.

DISCUSSION

Studies on HCV replication models have evolved significantly since the discovery of the virus in 1989, marking the inception of research models for understanding the HCV life cycle and exploring new antiviral agents and vaccines (22). Various *in vitro* and *in vivo* methods have been proposed for generating HCV particles, including subgenomic replicons, HCV pseudo-particles (HCVpps), HCVcc, HCV-derived serum, and animal models (10). Among these, the HCV clone JFH-1, isolated from a patient with fulminant hepatitis, demonstrates efficient replication in cell culture systems, producing infectious HCV particles in Huh-7 cells (9). This cell-derived HCV system has proven valuable for studying the molecular mechanisms of HCV replication and investigating specific antiviral drugs. However, screening for anti-HCV compounds using this method is labor-intensive and time-consuming (23). Therefore, a rapid and simple method for anti-HCV screening was needed. In this study, a novel HCV replicon was successfully developed and expressed in Huh-7 cells. Moreover, this replicon responded to available anti-HCV agents in a concentration-dependent manner and could be applied for high-throughput screening of new anti-HCV agents.

This replication-deficient replicon used 2 fluorescent aptamers as reporter systems: mCherry for observing protein synthesis and dBroccoli, a green fluorescent reporter, for monitoring RNA replication (Figs. 1 and 2). Notably, this replicon did not contain an IRES found in other viruses, which is typically a strong promoter for transcription and, consequently, protein translation, resulting in lower expression of the HCV polyprotein compared to other replicons. Some studies reported a full-length genomic HCV replicon with adaptive mutations using the neomycin phosphotransferase gene (neo) as a selective marker (24,25). However, the replication efficiency of full-length replicons was approximately 5-fold lower than that of their similarly mutated subgenomic counterparts, and no evidence of infectious progeny release from hepatoma cells was observed (5). In this study, polyprotein translation occurred efficiently in the cell and HCV machinery, allowing for effective observation using fluorescent reporters (Fig. 3).

Comparisons with other replicon models highlighted the advantages of the proposed system. Traditional bicistronic replicons using neo as a selectable marker typically require approximately 3-4 weeks to generate G418-resistant cell lines (5). More recent models using firefly luciferase (FLuc), Renilla luciferase (RLuc), and green fluorescent protein (GFP) as reporter systems have reduced the screening period to 72 h post-transfection (26-29). Another fluorescent reporter system that utilizes GFP inserted within the NS5A

region was developed (30). However, this GFP insertion reduces the replication capacity of replicons by approximately 100 folds compared with the parental replicon without GFP (31). In this study, the time course of fluorescence reporter expression was observed within 24 h post-transfection, with optimal expression at 40 h post-transfection and reduced fluorescence at 48 h (Fig. 3). This replicon model demonstrated the efficient replication and synthesis of both HCV RNA and proteins. Importantly, this system reduced the screening period from 5 days to 40 h (23,26).

The HCV replicon was further examined using the currently available DAAs sofosbuvir and telaprevir. Treatment with each drug resulted in decreased fluorescence signals from the replicon in a concentration-dependent manner (Fig. 4). Sofosbuvir treatment significantly decreased fluorescence signals at the concentrations of 0.1, 1, and 10 μM (Fig. 5). Similar results were observed with telaprevir treatment, in which the fluorescence signals from replicon-transfected cells were significantly different from those of the untreated group at all concentrations tested (Fig. 6). Peng *et al*. established a stable cell line transfected with bicistronic HCV replicon genotype 4 containing the neo gene and RLuc as a model for anti-HCV screening; however, the generation of cell lines was time-consuming, as mentioned previously (32). Han *et al*. developed a chimeric replicon carrying the full-length NS5B and an RLuc reporter system to evaluate the antiviral activity of sofosbuvir (33). The chimeric replicon responded to sofosbuvir in a concentration-dependent manner but still required 3 days post-transfection. The replicon model also showed a concentration-dependent effect when tested with telaprevir and sofosbuvir within a shorter period. Elbadawy *et al*. established a JFH1-based HCV subgenomic replicon with a double-reporter system. This replicon also used G418 resistance as a selectable marker to produce a cell line with a reporter system; however, FLuc-JFH1/RLuc was used in an end-point assay (34). Reporter cells were lysed in lysis buffer before fluorescence measurements. The novel HCV replicon constructed in this study could be observed simply by expressing a dual reporter system under a fluorescence microscope. Therefore, the effect of each tested compound was detected in real-time.

In our study, the effect of DAAs on HCV replicons was evaluated using real-time RT-PCR. All drug-treated groups showed delayed threshold cycle (Ct) values compared with the untreated group. The results in Fig. 7 revealed that this replicon could synthesize HCV protein and RNA and respond efficiently to available DAAs. However, the effect of sofosbuvir differed slightly between the HCVcc- and replicon-transfected groups. One possible reason for this is that NS5B functions in the late stages of the HCV replication cycle (35). It is time-consuming to express and function effectively. Since the transfected cells were harvested 40 h post-infection, the difference in Ct values might not be observed. These explanations were in accordance with the fluorescence signals obtained in our experiments. Because this replicon did not have the E1 and E2 regions, it was not infectious, which is another considerable advantage. The experiment could be performed in a biosafety level 1 facility, which is more convenient than the HCVcc model, where a biosafety level of 2 or above is required.

CONCLUSION

In this study, we developed a replication-deficient HCV replicon using dual-fluorescence reporters. This replicon expression was observed within 40 h and responded to the DAAs sofosbuvir and telaprevir in a concentration-dependent manner. The developed HCV replicon proved to be a promising and efficient platform for high-throughput screening to identify new inhibitors from compound libraries or to discover novel anti-HCV agents. Its rapidity, simplicity, and biosafety make it a valuable tool for advancing HCV research and drug discovery.

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Conflict of interest statement

The authors declare no conflict of interest.

Authors' contributions

K. Chitsombat contributed to the methodology, investigation, software, and data curator; K. Sa-ngiamsuntorn contributed to the study design, methodology-supported reagents and materials, data analysis, and data discussion; S. Chimnaronk participated in conceptualization, study design, methodology, and data interpretation and supported reagents and materials, data discussion, and supervision; M. Traidej Chomnawang was involved in conceptualization, study design, methodology, data interpretation, data discussion, supervision, and writing-review and editing; K. Thirapanmethee contributed to conceptualization, methodology, data interpretation, data discussion, supported reagents and materials, supervision, writing-original draft, review and editing, and funding acquisition. All authors approved the final version of the manuscript.

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