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G.知的所有権の出願・取得状況

1.特許取得

出願日：平成22年9月9日、出願番号:「特願2010-202355」、発明の名称：「難治性ウイルス感染症の治療剤」、発明者：小原道法、中川慎一郎、出願人：東京都医学研究機構

2.実用新案登録

なし

3.その他

なし

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
研究分担報告書

C型肝炎の治療とキャリアからの発症予防に関する基盤研究

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研究要旨　HCV JFH1 株を Huh7.5.1 細胞に感染させる系を用いて、HCV の全生活環を標的とした抗 HCV 薬のスクリーニングを行った。細胞内 core 蛋白質の cell-based ELISA による測定、および HCV による細胞変性効果の解除を指標としたアッセイを用いて、既存薬ライブラリー、試薬ライブラリーの約 2000 化合物を評価し、抗 HCV 治療薬候補を得た。

A．研究目的

変異を起こして耐性になりやすいウイルスの治療には多様な標的を持つ抗ウイルス剤が必要とされる。C 型肝炎ウイルス（HCV）の侵入過程や粒子の放出過程を標的とする阻害剤のスクリーニングは、レプリコン細胞では不可能であるため、Huh7.5.1 細胞-JFH1 の感染系を用いて、治療薬開発のための探索系を確立し、HCV の全ライフサイクルを標的とするスクリーニングを行い、その作用を解析する。

B．研究方法

Huh7.5.1 細胞-JFH1 の感染系を用いて種々化合物の HCV 阻害活性の評価と作用点解析を行った。阻害活性は JFH1 感染による Huh7.5.1 細胞の細胞変性効果の解除、cell-based ELISA による細胞内の Core 蛋白質の測定を指標とした。試薬として用いられている種々の化合物、既存薬を収集した化合物ライブラリーをスクリーニングした。アッセイの感度を上げるため、無血清培地を使用した。

（倫理面への配慮）

培養細胞を用いた研究であり、倫理面での問題はない。

C．研究結果

既存薬ライブラリー、試薬ライブラリーの約 2000 化合物を 2 種類のアッセイ系で評価した。HMG-CoA 還元酵素阻害剤、SERMs など既に HCV 阻害活性が報告されている薬剤以外に、imatinib、gefitinib などのチロシンキナーゼ阻害剤、orlistat などの脂肪酸合成酵素阻害剤、非ステロイド性抗炎症薬、核内レセプターアゴニストなどに活性が見いだされた。チロシンキナーゼ阻害剤は HCV 感染後に添加すると、HCV 阻害作用が弱くなることから、少なくとも一部は侵入過程を標的としていると考えられた。

D．考察

既存薬のライブラリー、試薬ライブラリーを 2 種類のアッセイ系で評価した。既に医薬品として使用されている薬剤のいくつかに抗 HCV 作用が確認された。これらは安全性、体内動態が十分に調べられている薬剤であり、非臨床試験から始める新規開発よりも有利な点が多い。今後さらに多くの既存薬を評価する予定である。imatinib、gefitinib を含むチロシンキナーゼ

阻害剤が抗 HCV 作用を示したが、imatinib (ABL、PDGFR、KIT 阻害)と gefitinib (EGFR 阻害)は阻害スペクトラムが異なっている。実際にチロシンキナーゼの阻害が抗HCV作用に関与しているかどうかは不明であり、その作用機序の解析は今後の課題である。2 種類のアッセイ系は再現性があり、抗HCV薬のスクリーニング系として十分信頼できると考えられたが、ヒットする物質は重なり合うものの完全には一致しなかった。複数のアッセイ系を用いることで、より網羅的なHCV阻害物質の検出が可能となると考えられる。

E．結論

HCV の全ライフサイクルを標的とする抗 HCV 薬スクリーニングを行った。2 種類のアッセイ系を用いて既存薬ライブラリー、試薬ライブラリーの約 2000 化合物を評価した。今まで HCV 阻害作用の報告のない薬剤に活性を見いだした。

F．健康危険情報

なし

G．研究発表

学会発表

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H．知的財産権の出願・登録状況

なし

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
研究分担報告書

抗C型肝炎ウィルス(HCV)活性を有する化合物の探索・創製研究

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研究要旨　FH-1株由来のC型肝炎ウィルス(HCV)を持続感染させたヒト肝癌細胞株Huh-7細胞株を用いた評価系において顕著な抗HCV活性を有する化合物（ヒット化合物）を見出し、構造活性相関研究に適した化合物群のデザイン・創製・評価を行った。その結果、構造活性相関に関して、宿主細胞への細胞障害活性と抗HCV活性を分離可能であることを示唆する重要な知見が得られた。本研究成果から、今後のさらなる論理的な抗HCV剤の設計・創製が期待できる。

A．研究目的

C型肝炎ウイルス（HCV)は持続感染を引き起こし、慢性肝炎、肝硬変、肝癌といった重篤な疾病をもたらす。現在、臨床ではペグインターフェロンa(PEG-IFN)とリバビリン(RBV)の併用療法などが用いられているが、奏効率、副作用などの観点から、さらなる新規治療薬の開発が希求されている。

そこで、本研究ではJFH-1株由来のHCVを持続感染させたヒト肝癌細胞株Huh-7細胞株を用いた評価系において抗HCV活性を有する化合物の探索・同定を行い、ヒット化合物を起点とした創薬化学的研究を行うことを目的とした。本研究成果は、新しいファーマコホアを有する新規HCV治療薬の開発に有用な知見をもたらすことが期待される。

B．研究方法

(i)抗HCV活性評価

JFH-1株由来のHCVを持続感染させたヒト肝癌細胞株Huh-7細胞株へ様々な小分子化合物を種々の濃度で添加し、3日間培養後、試験化合物の抗HCV活性を評価した。HCVレベルの指標として細胞外のHCV core蛋白質をELISA法により、また、細胞障害性の指標として細胞内の全RNA量あるいは細胞内ATP含量をそれぞれ測定した。

(ii)化学合成

3成分one-pot reactionにおける、反応試薬、反応溶媒、反応温度等、さまざまな反応条件検討を行った。反応経過、ならびに反応生成物の追跡は、シリカゲル薄層クロマトグラフィー(TLC)を用いて行った。さらに、反応生生物の同定・解析は、核磁気共鳴スペクトル解析(NMR)、質量分析スペクトル(MS)解析等を用いて行った。

C．研究結果

(i)ヒット化合物の同定

培養細胞系を用いた抗HCV活性を有する化合物スクリーニングの結果、KUSC-M001を含むキノリン骨格を有する化合物群をヒット化合物と同定した。KUSC-M001は、10-50□Mの濃度域で顕著な抗HCV活性を示したが、強い細胞障害性も観察された。

(ii)構造活性相関研究

まず、はじめにKUSC-M001の効率的な合成経路の検討を行った。その結果、多少、収率は劣るけれども(収率：15-25%)、シクロペンタジエン、3－ピリジンカルボアルデヒド、2，4－ジ

フルオロアニリンの3成分 one-pot reaction、すなわち、酸で活性化されたアルデヒドとアニリンが縮合して生じるイミンとシクロペンタジエンの逆電子要請型 Diels-Alder 反応で合成可能であった。興味深いことに、生成物 KUSC-M001 の立体化学は単一のジアステレオマーであった。続いて、様々なアルデヒド誘導体およびアニリン誘導体を反応に供することで、KUSC-M001 の各種類縁化合物(KUSC-M002～KUSC-M016)を効率よく合成することができた。

KUSC-M001 は 50mM で HCVcore 蛋白質量を 44%程度抑制したが、強い細胞障害性を示した。各種類縁化合物の抗 HCV 作用を検討した結果、なかでもアニリン由来部分を変換した化合物群に興味深い構造活性相関に関する知見が得られ、特に、KUSC-M016 は細胞障害性を示さない濃度域において抗 HCV 活性を示し、IC50 値は約 3 mM であった。

D. 考察

ヒット化合物として見出した KUSC-M001 はジアステレオマー混合物として報告されている goldicide A と平面構造は同一であるが、本研究において 3 成分 one-pot reaction により合成された KUSC-M001 は単一のジアステレオマーであった。したがって、KUSC-M001 が有する抗 HCV 活性は単一化合物に由来する生物活性であることが示された。しかし、KUSC-M001 が有する抗 HCV 活性は細胞障害性に起因する可能性が示唆された。

そこで、KUSC-M001 の各種類縁化合物 KUSC-M002～KUSC-M016 を 3 成分 one-pot reaction により合成し、抗 HCV 活性を測定したところ、KUSC-M016 は細胞障害性を示さない濃度域において顕著な抗 HCV 活性を示した。KUSC-MM016 は KUSC-001 のアニリン由来芳香環上の置換基を変換した化合物であるので、キノリン骨格上の芳香族置換基が活性発現に重要であることが示唆されるとともに、これら化合物群における宿主細胞への細胞障害活性と抗 HCV 活性の 2 つの生物活性を分離可能であることが示唆された。さらに、これらの結果は、KUSC-016 の作用点が必ずしも goldicide A と同じであるとは限らず、今後、KUSC-016 の詳細な薬理活性評価を行うことで、抗 HCV 剤開発のための新しい標的が明らかになる可能性も期待できる。

E. 結論

JFH-1 株由来の C 型肝炎ウィルス(HCV)を持続感染させたヒト肝癌細胞株 Huh-7 細胞株を用いた細胞培養系において、抗 HCV 活性を有する化合物を見出し、構造活性相関研究に適した化合物群のデザイン・創製・評価を行った結果、今後の展開が期待される KUSC-016 を見出した。

F. 研究発表

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G. 知的所有権の出願・登録状況

1. 特許取得

なし。

2. 実用新案登録

なし。

3. その他

なし。

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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Ⅳ．研究成果の刊行物・別冊

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Production of Infectious Hepatitis C Virus by Using RNA Polymerase I-Mediated Transcription[▽]

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In this study, we used an RNA polymerase I (Pol I) transcription system for development of a reverse genetics protocol to produce hepatitis C virus (HCV), which is an uncapped positive-strand RNA virus. Transfection with a plasmid harboring HCV JFH-1 full-length cDNA flanked by a Pol I promoter and Pol I terminator yielded an unspliced RNA with no additional sequences at either end, resulting in efficient RNA replication within the cytoplasm and subsequent production of infectious virions. Using this technology, we developed a simple replicon *trans*-packaging system, in which transient transfection of two plasmids enables examination of viral genome replication and virion assembly as two separate steps. In addition, we established a stable cell line that constitutively produces HCV with a low mutation frequency of the viral genome. The effects of inhibitors of N-linked glycosylation on HCV production were evaluated using this cell line, and the results suggest that certain step(s), such as virion assembly, intracellular trafficking, and secretion, are potentially up- and downregulated according to modifications of HCV envelope protein glycans. This Pol I-based HCV expression system will be beneficial for a high-throughput antiviral screening and vaccine discovery programs.

Over 170 million people worldwide have been infected with hepatitis C virus (HCV) (22, 33, 37), and persistence of HCV infection is one of the leading causes of liver diseases, such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma (16, 25, 38). The HCV genome is an uncapped 9.6-kb positive-strand RNA sequence consisting of a 5′ untranslated region (UTR), an open reading frame encoding at least 10 viral proteins (Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and a 3′UTR (46). The structural proteins (Core, E1, and E2) reside in the N-terminal region.

The best available treatment for HCV infection, which is pegylated alpha interferon (IFN-α) combined with ribavirin, is effective in only about half of patients and is often difficult to tolerate (25). To date, a prophylactic or therapeutic vaccine is not available. There is an urgent need to develop more effective and better tolerated therapies for HCV infection. Recently, a robust system for HCV production and infection in cultured cells has been developed. The discovery that some HCV isolates can replicate in cell cultures and release infectious particles has allowed the complete viral life cycle to be studied (23, 49, 53). The most robust system for HCV production involves transfection of Huh-7 cells with genomic HCV RNA of the JFH-1 strain by electroporation. However, using this RNA transfection system, the amount of secreted infectious viruses often fluctuate and mutations emerge in HCV genome with multiple passages for an extended period of time (54), which limits its usefulness for antiviral screening and vaccine development.

DNA-based expression systems for HCV replication and virion production have also been examined (5, 15, 21). With DNA-based expression systems, transcriptional expression of functional full-length HCV RNA is controlled by an RNA polymerase II (Pol II) promoter and a self-cleaving ribozyme(s). DNA expression systems using RNA polymerase I (Pol I) have been utilized in reverse genetics approaches to replicate negative-strand RNA viruses, including influenza virus (12, 29), Uukuniemi virus (11), Crimean-Congo hemorrhagic fever virus (10), and Ebola virus (13). Pol I is a cellular enzyme that is abundantly expressed in growing cells and transcribes rRNA lacking both a 5′ cap and a 3′ poly(A) tail. Thus, viral RNA synthesized in cells transfected with Pol I-driven plasmids containing viral genomic cDNA has no additional sequences at the 5′- or 3′ end even in the absence of a ribozyme sequence (28). The advantages of DNA-based expression systems are that DNA expression plasmids are easier to manipulate and generate stable cell lines that constitutively express the viral genome.

We developed here a new HCV expression system based on transfection of an expression plasmid containing a JFH-1 cDNA clone flanked by Pol I promoter and terminator sequences to generate infectious HCV particles from transfected cells. The technology presented here has strong potential to be the basis for *trans*-encapsidation system by transient transfection of two plasmids and for the establishment of an efficient and reliable screening system for potential antivirals.

MATERIALS AND METHODS

DNA construction. To generate HCV-expressing plasmids containing full-length JFH1 cDNA embedded between Pol I promoter and terminator se-

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quences, part of the 5′UTR region and part of the NS5B to the 3′UTR region of full-length JFH-1 cDNA were amplified by PCR using primers containing BsmBI sites. Each amplification product was then cloned into a pGEM-T Easy vector (Promega, Madison, WI) and verified by DNA sequencing. Both fragments were excised by digestion with NotI and BsmBI, after which they were cloned into the BsmBI site of the pHH21 vector (a gift from Yoshihiro Kawaoka, School of Veterinary Medicine, University of Wisconsin-Madison [29]), which contains a human Pol I promoter and a mouse Pol I terminator. The resultant plasmid was digested by AgeI and EcoRV and ligated to JFH-1 cDNA digested by AgeI and EcoRV to produce pHHJFH1. pHHJFH1/GND having a point mutation at the GDD motif in NS5B to abolish RNA-dependent RNA polymerase activity and pHHJFH1/R783A/R785A carrying double Arg-to-Ala substitutions in the cytoplasmic loop of p7 were constructed by oligonucleotide-directed mutagenesis. To generate pHHJFH1/ΔE carrying in-frame deletions of parts of the E1 and E2 regions (amino acids [aa] 256 to 567), pHHJFH1 was digested with NcoI and AscI, followed by Klenow enzyme treatment and self-ligation. To generate pHH/SGR-Luc carrying the bicistronic subgenomic HCV reporter replicon and its replication-defective mutant, pHH/SGR-Luc/GND, AgeI-SpeI fragments of pHHJFH1 and pHHJFH1/GND were replaced with an AgeI-SpeI fragment of pSGR-JFH1/Luc (20). In order to construct pCAG/C-NS2 and pCAG/C-p7, PCR-amplified cDNA for C-NS2 and C-p7 regions of the JFH-1 strain were inserted into the EcoRI sites of pCAGGS (30). In order to construct stable cell lines, a DNA fragment containing a Zeocin resistance gene excised from pSV2/Zeo2 (Invitrogen, Carlsbad, CA) was inserted into pHH21 (pHHZeo). Full-length JFH-1 cDNA was then inserted into the BsmBI sites of pHHZeo. The resultant construct was designated pHHJFH1/Zeo.

Cells and compounds. The human hepatoma cell line, Huh-7, and its derivative cell line, Huh7.5.1 (a gift from Francis V. Chisari, The Scripps Research Institute), were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with nonessential amino acids, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. *N*-Nonyl-deoxynojirimycin (*N*N-DNJ) and kifunensine (KIF) were purchased from Toronto Research Chemicals (Ontario, Canada), castanospermine (CST) and 1,4-dideoxy-1,4-imino-D-mannitol hydrochloride (DIM) were from Sigma-Aldrich (St. Louis, MO), 1-deoxymannojirimycin (DMJ) and swainsonine (SWN) were from Alexis Corp. (Lausen, Switzerland), and *N*-butyl-deoxynojirimycin (*N*B-DNJ) was purchased from Wako Chemicals (Osaka, Japan). BILN 2061 was a gift from Boehringer Ingelheim (Canada), Ltd. These compounds were dissolved in dimethyl sulfoxide and used for the experiments. IFN-α was purchased from Dainippon-Sumitomo (Osaka, Japan).

DNA transfection and selection of stable cell lines. DNA transfection was performed by using FuGENE 6 transfection reagent (Roche, Mannheim, German) in accordance with the manufacturer's instructions. To establish stable cell lines constitutively producing HCV particles, pHHJFH1/Zeo was transfected into Huh7.5.1 cells within 35-mm dishes. At 24 h posttransfection (p.t.), the cells were then divided into 100-mm dishes at various cell densities and incubated with DMEM containing 0.4 mg of zeocin/ml for approximately 3 weeks. Selected cell colonies were picked up and amplified. The expression of HCV proteins was confirmed by measuring secreted core proteins. The stable cell line established was designated H751JFH1/Zeo.

***In vitro* synthesis of HCV RNA and RNA transfection.** RNA synthesis and transfection were performed as previously described (26, 49).

RNA preparation, Northern blotting, and RNase protection assay (RPA). Total cellular RNA was extracted with a TRIzol reagent (Invitrogen), and HCV RNA was isolated from filtered culture supernatant by using the QIAamp viral RNA minikit (Qiagen, Valencia, CA). Extracted cellular RNA was treated with DNase (TURBO DNase; Ambion, Austin, TX) and cleaned up by using an RNeasy minikit, which includes another step of RNase-free DNase digestion (Qiagen). The cellular RNA (4 μg) was separated on 1% agarose gels containing formaldehyde and transferred to a positively charged nylon membrane (GE Healthcare, Piscataway, NJ). After drying and cross-linking by UV irradiation, hybridization was performed with [α-^{32}P]dCTP-labeled DNA using Rapid-Hyb buffer (GE Healthcare). The DNA probe was synthesized from full-length JFH-1 cDNA using the Megaprime DNA labeling system (GE Healthcare). Quantification of positive- and negative-strand HCV RNA was performed using the RPA with biotin-16-uridine-5′-triphosphate (UTP)-labeled HCV-specific RNA probes, which contain 265 nucleotides (nt) complementary to the positive-strand (+) 5′UTR and 248 nt complementary to the negative-strand (−) 3′UTR. Human β-actin RNA probes labeled with biotin-16-UTP were used as a control to normalize the amount of total RNA in each sample. The RPA was carried out using an RPA III kit (Ambion) according to the manufacturer's procedures. Briefly, 15 μg of total cellular RNA was used for hybridization with 0.3 ng of the β-actin probe and 0.6 ng of either the HCV (+) 5′UTR or (−) 3′UTR RNA probe. After digestion with RNase A/T1, the RNA products were analyzed by electrophoresis in a 6% polyacrylamide–8 M urea gel and visualized by using a chemiluminescent nucleic acid detection module (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions.

Reverse transcriptase PCR (RT-PCR), sequencing, and rapid amplification of cDNA ends (RACE). Aliquots (5 μl) of RNA solution extracted from filtered culture supernatant were subjected to reverse transcription with random hexamer and Superscript II reverse transcriptase (Invitrogen). Four fragments of HCV cDNA (nt 129 to 2367, nt 2285 to 4665, nt 4574 to 7002, and nt 6949 to 9634), which covers most of the HCV genome, were amplified by nested PCR. Portions (1 or 2 μl) of each cDNA sample were subjected to PCR with TaKaRa LA *Taq* polymerase (Takara, Shiga, Japan). The PCR conditions consisted of an initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 3 min. The amplified products were separated by agarose gel electrophoresis and used for direct DNA sequencing. To establish the 5′ ends of the HCV transcripts from pHHJFH1, a synthetic 45-nt RNA adapter (Table 1) was ligated to RNA extracted from the transfected cells 1 day p.t. using T4 RNA ligase (Takara). The viral RNA sequences were then reverse transcribed using SuperScript III reverse transcriptase (Invitrogen) with a primer, RT (Table 1). The resultant cDNA sequences were subsequently amplified by PCR with 5′RACEouter-S and 5′RACEouter-R primers, followed by a second cycle of PCR using 5′RACEinner-S and 5′RACEinner-R primers (Table 1). To establish the terminal 3′-end sequences, extracted RNA sequences were polyadenylated using a poly(A) polymerase (Takara), reverse transcribed with CAC-T35 primer (Table 1), and amplified with the primers 3X-10S (Table 1) and CAC-T35. The amplified 5′ and 3′ cDNA sequences were then separated by agarose gel electrophoresis, cloned into the pGEM-T Easy vector (Promega), and sequenced.

Western blotting. The proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA) after separation by SDS-PAGE. After blocking, the membranes were probed with a mouse monoclonal anti-HCV core antibody (2H9) (49), a rabbit polyclonal anti-NS5B antibody, or a mouse monoclonal GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (Chemicon, Temecula, CA), followed by incubation with a peroxidase-conjugated secondary antibody and visualization with an ECL Plus Western blotting detection system (Amersham, Buckinghamshire, United Kingdom).

Quantification of HCV core protein. HCV core protein was quantified by using a highly sensitive enzyme immunoassay (Ortho HCV antigen ELISA kit; Ortho Clinical Diagnostics, Tokyo, Japan) in accordance with the manufacturer's instructions.

Sucrose density gradient analysis. Samples of cell culture supernatant were processed by low-speed centrifugation and passage through a 0.45-μm-pore-size filter. The filtrated supernatant was then concentrated ~30-fold by ultrafiltration by using an Amicon Ultra-15 filter device with a cutoff molecular mass of 100,000 kDa (Millipore), after which it was layered on top of a continuous 10 to 60% (wt/vol) sucrose gradient, followed by centrifugation at 35,000 rpm at 4°C for 14 h with an SW41 rotor (Beckman Coulter, Fullerton, CA). Fractions of 1 ml were collected from the bottom of the gradient. The core level and infectivity of HCV in each fraction were determined.

Quantification of HCV infectivity. Infectious virus titration was performed by a 50% tissue culture infectious dose ($TCID_{50}$) assay, as previously described (23, 26). Briefly, naive Huh7.5.1 cells were seeded at a density of 10^4 cells/well in a 96-well flat-bottom plate 24 h prior to infection. Five serial dilutions were performed, and the samples were used to infect the seeded cells (six wells per dilution). At 72 h after infection, the inoculated cells were fixed and immunostained with a rabbit polyclonal anti-NS5A antibody (14), followed by an Alexa Fluor 488-conjugated anti-rabbit secondary antibody (Invitrogen).

Labeling of *de novo*-synthesized viral RNA and immunofluorescence staining. Labeling of *de novo*-synthesized viral RNA was performed as previously described with some modifications (40). Briefly, cells were plated onto an eight-well chamber slide at a density of 5×10^4 cells/well. One day later, the cells were incubated with actinomycin D at a final concentration of 10 μg/ml for 1 h and washed twice with HEPES-saline buffer. Bromouridine triphosphate (BrUTP) at 2 mM was subsequently transfected into the cells using FuGENE 6 transfection reagent, after which the cells were incubated for 15 min on ice. After the cells were washed twice with phosphate-buffered saline (PBS), they were incubated in fresh DMEM supplemented with 10% FBS at 37°C for 4 h. The cells were then fixed with 4% paraformaldehyde for 20 min and permeabilized with PBS containing 0.1% Triton X-100 for 15 min at room temperature. Immunofluorescence staining of NS5A and *de novo*-synthesized HCV RNA was performed as previously described (26, 40). The nuclei were stained with DAPI (4′,6′-diamidino-2-phenylindole) solution (Sigma-Aldrich). Confocal microscopy was performed