Fast Hepatitis C Virus RNA Elimination and NS5A Redistribution by NS5A Inhibitors Studied by a Multiplex Assay Approach

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Hepatitis C virus (HCV) infects approximately 3% of the world’s population, which accounts for about 170 million chronically infected individuals. Annually, there are more than 350,000 deaths from HCV-related cirrhosis and hepatocellular carcinoma (1). In the United States, there are more than 3 million people with chronic HCV infection, and about 15,000 die from HCV-related liver disease each year.

HCV is a positive-strand RNA virus grouped in the genus Hepacivirus within the family Flaviviridae (2). It is classified into at least 6 genotypes (gt), and its error-prone polymerase leads to more than 50 subtypes (3). The long open reading frame, which encodes the HCV polyprotein, is processed by host and viral proteases and gives rise to three structural proteins (the capsid protein core and envelope glycoproteins E1 and E2) and seven nonstructural (NS) proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (4). NS2 and p7 are essential for virus assembly but not structural (NS) proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (4). NS2 and p7 are essential for virus assembly but not RNA replication, whereas NS3 to NS5B are involved in a membrane-associated RNA replicase complex (RC) (5). The NS5 protein is composed of a serine protease and an RNA helicase/nucleoside triphosphatase (NTTase), NS4A serves as a cofactor for NS3 serine protease (6), NS5B is the RNA-dependent RNA polymerase (7), and NS5A is considered to play key roles in multiple steps of the HCV life cycle.

NS5A is an ~450 amino acid phosphoprotein composed of an N-terminal amphipathic α-helix and three domains (domain I to domain III), each of which is able to bind independently to the 3′ untranslated region (UTR) of the viral positive-strand genomic RNA. Domain I of NS5A is required for RNA replication and modulates the interaction between NS5A and the endoplasmic reticulum (ER) membrane (8, 9). Domains II and III bind the peptidyl-prolyl isomerase cyclophilin A to support HCV replication (10). Domain III interacts with the HCV core protein at lipid droplets (LDs) and plays a major role in the assembly of infectious virus particles (11–13).

In the past, the standard treatment of HCV-infected patients involved weekly injections of pegylated alpha interferon (IFN-α) in combination with oral administration of RBV and one HCV NS3/4A protease inhibitor, boceprevir or telaprevir (14). The side effects from IFN-α treatment can be severe, including depression, flu-like symptoms, and anemia (15–17). Boceprevir and telaprevir are the first direct-acting antiviral agents (DAAs) approved for anti-HCV treatment, suggesting that an IFN-sparing treatment regimen is feasible. In fact, the Food and Drug Administration (FDA) approved an interferon-free combination for safe and very effective treatment of patients with HCV gt1: the protease inhibitor ABT-450 with ritonavir and the NS5A inhibitor ombitasvir (18, 19). The newer NS3/4A protease inhibitor danoprevir (DNV) was shown to be highly selective and potent against gt1 HCV (18, 19). DNV also was shown to be safe and well tolerated with few side effects, while earlier therapeutic strategies for the treatment of hepatitis C virus (HCV) infection relied exclusively on interferon (IFN) and ribavirin (RBV), four direct-acting antiviral agents (DAAs) have now been approved, aiming for an interferon-free strategy with a short treatment duration and fewer side effects. To facilitate studies on the mechanism of action (MOA) and efficacy of DAAs, we established a multiplex assay approach, which employs flow cytometry, a Gaussia luciferase reporter system, Western blot analysis, reverse transcription-quantitative PCR (RT-qPCR), a limited dilution assay (50% tissue culture infectious dose [TCID50]), and an image profiling assay that follows the NS5A redistribution in response to drug treatment. We used this approach to compare the relative potency of various DAAs and the kinetics of their antiviral effects as a potential preclinical measure of their potential clinical utility. We evaluated the NS5A inhibitors ledipasvir (LDV) and daclatasvir (DCV), the NS3/4A inhibitor danoprevir (DNV), and the NS5B inhibitor sofosbuvir (SOF). In terms of kinetics, our data demonstrate that the NS5A inhibitor LDV, followed closely by DCV, has the fastest effect on suppression of viral proteins and RNA and on redistribution of NS5A. In terms of MOA, LDV has a more pronounced effect than DCV on the viral replication, assembly, and infectivity of released virus. Our approach can be used to facilitate the study of the biological processes involved in HCV replication and help identify optimal drug combinations.

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effects as monotherapy in treatment-naïve patients and nonresponder. A third protease inhibitor, sitaprevir, was recently approved by the FDA, whereas it was announced that telaprevir is discontinued. Sofosbuvir (SOF) is a nucleotide analog inhibitor of HCV NS5B polymerase that acts as a chain terminator to inhibit viral genome replication (20). SOF exhibits pan-genotypic antiviral activity against all HCV genotypes and has a high barrier to resistance due to its targeting of the highly conserved NS5B active site (21). On 6 December 2013, the FDA approved SOF as a component of a combination antiviral treatment regimen for chronic HCV infection. Daclatasvir (DCV) is an early NS5A-targeting antiviral with broad in vitro HCV genotypic coverage and a 50% effective concentration (EC_{50}) in the low picomolar range (22). It has been proposed to block and suppress both viral genome replication and secretion by altering the proper localization of NS5A into functional RCs (23–25). Ledipasvir (LDV) is a highly active newer NS5A-targeting antiviral that retains full activity against NS3/4A and NS5B inhibitor-resistant HCV strains (26). LDV has shown additive to moderately synergistic antiviral activity when combined with other classes of HCV DAAs (26). A combination of SOF with LDV achieved a high rate of sustained virologic response (SVR) for previously treated and untreated patients with HCV gt1 infection (27, 28) and has gained FDA approval for the treatment of gt1 HCV infection. The precise mechanism of action (MOA) of these compounds is under investigation.

To facilitate characterization of the kinetic profiles of DAAs, we used a multiplex assay system that included flow cytometry, a Gaussia luciferase reporter system, Western blot analysis, reverse transcription-quantitative PCR (RT-qPCR), a limited dilution assay, and an image profiling assay. We used this approach to demonstrate that only NS5A inhibitors can have statistically reliable dose responses for drug effects at early time points. NS5A inhibitors were shown to have early effects on replication, assembly, and infectivity of released virus.

MATERIALS AND METHODS

Cells and viruses. Huh-7.5.1 cells have been previously described (29). Cells were propagated in Dulbecco’s modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Jcl-378-1-NS5A-Ypet (Jcl1/Ypet) and Jcl1-FLAG2(p7-nsGluc2A) (Jcl1/Gluc2A) were obtained from Charles Rice’s laboratory. Briefly, Jcl1/Ypet was engineered to encode yellow fluorescent protein (YFP) inserted at codon 378 of NS5A (domain III). To improve the titer of the resulting virus, residues 262 to 295 were deleted. Jc1/Gluc2A was engineered to carry a Gaussia luciferase reporter gene, which was inserted in tandem with the foot and mouth disease autoprotoelectic peptide sequence 2A, between p7 and NS2, as previously described (30).

Compounds and antibodies. DCV (RMS-790052) and DNV (RG7277) were purchased from Selleckchem. LDV (GS-5885) was purchased from MedChem Express. SOF (GS-7977) was purchased from Acme Bioscience. Mouse monoclonal primary antibody 9E10 specific for NS5A was produced as described by Lindenbach et al. (31). Mouse monoclonal antibodies specific for HCV core (C7-50) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (G-9) were purchased from Abcam and Santa Cruz Biotechnology, respectively. Secondary antibodies Alexa 488 and horseradish peroxidase (HRP)-conjugated IgG were purchased from Invitrogen and Santa Cruz Biotechnology, respectively. DRAQ5 for nuclei staining was purchased from Thermo Scientific.

In vitro transcription and electroporation of HCV RNAs. Jcl1/Ypet and Jcl1/Gluc2A plasmids were linearized by XbaI and purified with the Wizard SV gel and PCR clean-up system (Promega). Purified template DNA (1 μg) was subsequently transcribed using the MEGAScript T7 RNA production system (Ambion). Template DNA was removed by treatment with Turbo DNase (Ambion) at 37°C for 15 min. RNA was cleaned up by an RNeasy minikit (Qiagen), and RNA quality was monitored by agarose gel electrophoresis. RNA (10 μg) was electroporated into 5 × 10^5 Huh-7.5.1 cells using 4-mm gap electroporation cuvettets (Fisher Scientific). After one pulse at 950 μF and 270 V with a Gene Pulser system II (Bio-Rad), cells were suspended in DMEM plus 10% FBS and plated in a T175 flask.

Polyethylene glycol (PEG) precipitation of extracellular HCV particles. Virus-containing culture supernatants were clarified by centrifugation (3,000 × g) and transferred to 15-ml disposable conical centrifuge tubes. Viruses were precipitated by adding one-fourth volume sterile-filtered 40% (wt/vol) PEG-8000 in phosphate-buffered saline (PBS) (final concentration of 8% [wt/vol]) and overnight incubation at 4°C. Viral precipitates were collected by centrifugation (4,000 × g, 30 min) and washed twice with PBS. Supernatants were removed, and pellets were resuspended in 1 ml of DMEM containing 10% FBS.

Limited dilution assay (TCID_{50}). A total of 6 × 10^3 cells/well were plated onto a 96-well plate. The cells were infected with 50 μl of serial dilutions ranging from undiluted to 10^{-3}, 72-h postinfection (hpi) cells were fixed with 100% methanol for 30 min at -20°C and then washed with PBS followed by 0.1% Tween 20 in PBS (PBS-T). The cells were permeabilized with PBS-T and blocked with 1% bovine serum albumin (BSA)-0.2% skim milk in PBS-T. Hydrogen peroxide (3%) was added to block the endogenous peroxidase activity. The cells were stained with mouse monoclonal primary NS5A antibody 9E10 (1:25,000, ImmPRESS anti-mouse IgG (1:3) (Vector Laboratories), and 3,3′-diaminobenzidine (DAB) substrate (1 drop/ml) (Invitrogen), respectively. The NS5A-positive wells were counted and recorded by using a light microscope. The 50% tissue culture infectious dose (TCID_{50}) was calculated by a Reed-Muench calculator as previously described (32).

Flow cytometry. A total of 2 × 10^5 cells/well were plated in a 12-well plate. On the following day, the cells were infected with Jcl1/Ypet virus at a multiplicity of infection (MOI) of 0.3 for 48 h. The cells were washed with PBS and then detached with 0.25% trypsin-EDTA. The cells were washed by ice-cold PBS and then fixed with 2% paraformaldehyde and washed twice with ice-cold PBS. After centrifugation, the cell pellets were resuspended in 200 μl of PBS and analyzed by a BD Accuri C6 flow sampler.

Gaussia luciferase reporter system. A total of 2 × 10^5 cells/well were plated in a 96-well plate or 2 × 10^3 cells/well were plated in a 12-well plate. On the following day, the cells were infected with Jcl1/Gluc2A virus at an MOI of 0.5 for 48 h (a different MOI was used here because of different virus fitness). Then 30 μl of supernatant was added to 30 μl of lysis buffer from the Pierce Gaussia luciferase glow assay kit (Thermo Scientific) in black opaque 96-well microplates and incubated at room temperature for 20 min. Coelenterazine (50 μl of 1X) was added according to the manufacturer’s instructions, and luciferase activity (relative light units [RLU]) was measured with an EnSpire 2300 multilabel plate reader (PerkinElmer).

Western blot analysis. Whole-cell extracts were prepared in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris HCl [pH 7.5], 1 mM EGTA, 1 mM EDTA, 0.1% SDS, and 1% Triton X-100) containing a cocktail of protease inhibitors (Sigma) and quantitated by the Bradford assay (Bio-Rad). Then 50 μg of protein was subjected to electrophoresis on an SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride Immobilon-P membrane (Millipore). Membranes were probed with anti-NS5A (1:14,000), anti-core (1:8,000), or anti-GAPDH (1:5,000) antibody, followed by an HRP-conjugated secondary antibody. Bound antibodies were visualized by adding Lumina Forte Western HRP substrate (Millipore) to the membrane and imaged with a Fuji camera system.

Image profiling. A total of 2 × 10^4 cells were seeded in a 96-well 0.1% gelatin-coated image plate (BD Falcon). On the following day, the cells were infected with Jcl1/Gluc2A virus at an MOI of 0.5. Antiviral compounds were added 48 hpi. The cells were fixed at various time points with
3.7% phosphate-buffered formalin, permeabilized with PBS-T for 15 min, blocked with 1% BSA-0.2% skim milk in PBS-T for 30 min, and stained with anti-NS5A antibody (1:2,500) for 1 h. The secondary antibody Alexa Fluor 488 goat anti-mouse IgG (H + L) (Invitrogen) (1:2,000) was used to label the anti-NS5A antibody. DRAQ5 (Thermo Scientific) was used to stain the nuclei. The protein redistribution was analyzed from confocal images taken with a Zeiss LSM 510 Meta confocal microscope with Autostage, Multitile, and MultiTime series 4.0.31 beta software. Imaging was carried out using a 40× objective, capturing 9 images per well, using 1 well/drug dose level (9 multichannel image sets [or tiles] per dose level) and spanning through 10 drug dose levels. Images were processed using CellProfiler 2.0 (r11052) software. CellProfiler is open source software designed for the analysis of cell-based images, for quantifying cell characteristics of HCV inhibition by DCV, LDV, DNV, and SOF by flow cytometry. It was possible to detect significant changes in viral translation at 24 h following the addition of DCV, DNV, and SOF (Fig. 2B; see also Fig. S1 and Table S1 in the supplemental material). Collectively, these data suggest that flow cytometry is not sensitive enough for detection of early dose-dependent effects but yields statistically reliable dose-response data at later time points.

Effect of various DAAs on Jc1/Gluc2A viral translation measured by a released luciferase reporter system. The Jc1/Gluc2A reporter virus was previously engineered to secrete Gaussia luciferase in the cell culture medium, allowing for noninvasive monitoring of viral translation kinetics by measuring secreted luciferase activity without having to lyse the infected cells (36) (Fig. 1A). Similar to the flow cytometry assay, we evaluated the kinetic characteristics of HCV inhibition by DCV, LDV, DNV, and SOF by following dose-response curves at four time points after initiation of treatment by addition of the individual inhibitors to preinfected cells (8, 24, 48, or 72 hpt) (Fig. 3). Our data with this luciferase reporter system show that consistent dose-response curves can be obtained at 8 hpt only for LDV (Fig. 3B; see also Fig. S2 in the supplemental material). Hence, the Gaussia luciferase-based reporter system provides statistically reliable and facile assessment of drug treatment effects on viral translation at 24 hpt and later
time points and only in the case of LDV at 8 hpt (see Fig. S2 and Table S2 in the supplemental material).

**Effect of various DAAs on viral translation assessed by Western blot analysis of HCV core and NS5A proteins from whole-cell lysates.** Since the luciferase-based assays were able to detect changes in viral translation at 8 hpt, we decided to also try a Western blot analysis that informs the presence of two viral proteins, HCV core and NS5A. We conducted these experiments using a high concentration of DCV, DNV, SOF, or LDV (100\(\times\) EC\(_{50}\) of the respective inhibitors) where any reduction in viral protein on Western blotting would be pronounced and unambiguous. Indeed, at 24 hpt there was a significant decrease in the amounts of the viral proteins NS5A (Fig. 4A) and HCV core (Fig. 4B). There were smaller changes with respect to no drug added in the levels of NS5A and core proteins at 8 hpt except for LDV treatment (Fig. 4C and D). Specifically, HCV protein levels in whole-cell extracts treated with DCV, DNV, or SOF were 80 to 92% of those in DMSO-treated cells (Fig. 4C and D). Moreover, these differences were not statistically significant. However, the levels of NS5A and core proteins in whole-cell lysates were significantly decreased for LDV treatment (Fig. 4C and D). Hence, Western blot analysis can generally be used to measure drug effects on the levels of NS5A and core proteins at later time points.

**Image profiling method for monitoring changes in NS5A distribution of HCV upon treatment with DAAs.** While enzymatic or virological assays have been useful for HCV inhibitor screening, cell-based image profiling assays are very informative for monitoring protein redistribution or other phenotypic changes and drug cytotoxicity. We developed an automated image profiling assay that allows quantification of changes in the amount and distribution of NS5A and other viral or host proteins in response to treatment with DAAs from various families. This assay is based on multiparameter data acquisition from thousands of individual HCV-infected cells and employs CellProfiler for data analysis. We take advantage of the observation that upon DAA treatment, NS5A redistributes in the cell, changing its localization from RCs to LDs (25, 37). We monitored NS5A intracellular distribution either by staining it with anti-NS5A antibody when we use virus that does not have NS5A fused to Ypet or by following YFP in cells infected with virus that encodes NS5A fused to Ypet. The kinetics of NS5A redistribution and HCV virus suppression in response to drug treatment were almost identical when different viruses and staining methods were used. A large number of parameters were probed to optimize the experimental conditions and allow automated data acquisition, including the type of plates, microscope, objective, cell line, number of cells per well, type and amount of virus, timing and sequence of addition, transfection or infection, type of antibody used, concentration range of inhibitors, hardware modifications/addition of compatible microscope stage, number of cells needed per field for reliable analysis, choice of software for analysis, and optimization of software parameters for optimal alignments.

In the optimized protocol, we collected at least nine multi-channel image sets (1 image set/tile) per well in 96-well plates by...
automated confocal microscopy (specific details for hardware are described in Materials and Methods). Typically, we automatically collected images for hundreds of cells per single condition that corresponded to a single drug concentration at a specific time point posttreatment. Acquired images were further converted to a format compatible with the CellProfiler software using the LSM Toolbox functions in the ImageJ program (38) (Fig. 5A). With CellProfiler, individual nuclei and cell objects were segmented and counted in every image set (Fig. 5B). Cytoplasm objects were defined by subtraction of parent nucleus objects from child cell objects. Multiple parameters were measured, including the size, shape, intensity, and texture of each of these cytoplasmic objects. Specifically, an average of more than 100 cells was identified per tile (1 image set), and conditions were calibrated to have an average of 40 of these cells infected with HCV. Ultimately, we collected data sets for an average of 750 infected cells per condition in two independent experiments. The relevant information was archived for each single cell of the hundreds of cells per condition and analyzed in response to various drug concentrations. We found that “Gabor texture” (39) was the CellProfiler parameter that correlated best with the drug concentration. Gabor texture is evaluated by applying mathematical filters to the images to measure the frequency content in different orientations (40). Application of the Gabor filter to the pixel matrix of cytoplasm objects within the image detects correlated bands or wavelets of intensities within the objects. In the present case, the “bands” of intensity are defined by the size and spacing of the fluorescent NS5A speckles. If NS5A fluorescence is diffuse, the cytoplasm has a low Gabor texture; when NS5A redistributes into larger bright speckles that have dim spaces in between, the Gabor texture increases (40). An example of this analysis is shown in Fig. 5B where we compare the changes in Gabor textures of NS5A speckles in the presence of increasing amounts of DCV (Fig. 5B, left panel) or DNV (Fig. 5B, right panel). At an early time point of 8 h, the left panel shows that DCV causes a dose-dependent redistribution of NS5A, whereas the right panel shows that DNV does not cause a dose-dependent redistribution of NS5A. Extending this type of analysis for all four drugs at multiple concentrations and for various treatment time points (Fig. 6), we found that all drugs altered the intracellular distribution of NS5A but with different kinetics. Specifically, whereas at 24 hpt all drugs redistributed NS5A (Fig. 6), at 8 hpt only NS5A inhibitors (Fig. 6A and B) altered the distribution of NS5A in a dose-dependent manner. Taken together, these data demonstrate that image profiling is a technique that allows quantification of NS5A redistribution at early time points.

Effect of various DAAs on HCV RNA levels measured by RT-qPCR. In the above assays we followed the effect of DAAs on the kinetics of HCV replication by monitoring the fraction of infected cells (flow cytometry), the levels of expressed protein from viral RNA (luciferase assay), or the morphological changes that accompany NS5A redistribution during drug treatment. However, in order to obtain direct information regarding the decline in the relative levels of viral RNA following the administration of the inhibitors, we used an RT-qPCR method. Similar to the results with the other methods, we observed dose-dependent responses at the later points (24, 48, and 72 hpt) for treatment with any of the four drugs. Interestingly, this approach revealed a detectable reduction in RNA copy numbers at the 8-h early time point for all four treatments, but to a different extent (Fig. 7; see also Table S3 in the supplemental material). Specifically, the largest decrease was observed during treatment with NS5A inhibitors, where there was a 50% or 75% decrease in the HCV RNA copy number at 8 h following administration of DCV and LDV, respectively (Fig. 7A and B), but there was also an ~35% reduction in the HCV RNA copy number at 8 h following the addition of DNV (Fig. 7C) or SOF (Fig. 7D).
Effect of DAA treatment on Jc1/Gluc2A virus infectivity measured by limited dilution and RT-qPCR assays on released virus.

The significant changes in viral RNA and NS5A relocalization detected in HCV-infected cells treated with NS5A inhibitors might also affect events downstream of the virus assembly, such as virus release and virus infectivity. We performed an extracellular virus RT-qPCR to assess virus copy numbers released in the cell culture medium after an 8-h exposure to high concentrations of DAAs (100 EC50). Here, we are using high concentrations of DAAs because any reduction would be more pronounced and also be cause high drug concentrations are relevant in a therapeutic context where treatment is carried out at concentrations much higher than EC50s. Similar to the intracellular virus RT-qPCR data, we observed a significant reduction in RNA copy numbers for cells treated with DCV, LDV, or DNV (Fig. 8A). Specifically, the strongest reduction was observed in the NS5A inhibitor-treated cells (Fig. 8A). Next, to test whether extracellular virus infectivity was also reduced along with the amount of released virus, we determined virus infectivity by using a limited dilution assay. We observed a pronounced reduction in virus infectivity, suggesting that NS5A inhibitors significantly affect both virus release and infectivity (Fig. 8B). Treatment with DNV or SOF had smaller or insignificant effects, respectively, on extracellular virus infectivity (Fig. 8B). Collectively, these data demonstrate that the limited dilution assay is a useful tool for the characterization of kinetic profiles of distinct classes of DAAs. Furthermore, NS5A inhibitors exhibit a rapid inhibition of virus infectivity shortly after administration to HCV-infected cells.

DISCUSSION

The time of infection before treatment and the duration of treatment play essential roles in HCV drug development. Patients whose viral loads drop quickly and who show rapid virological responses (RVR) following treatment are more likely to be cured and thus RVR can be a useful predictor of the SVR (41–44). Although a direct link between rapid viral load suppression and RVR is not known, fast suppression of viral replication may be a useful measure of potential therapeutic benefit, as it decreases the chances for the appearance of drug-resistant variants. That said, other factors also affect emergence of drug resistance, including
the genetic barrier to drug resistance. This may be the reason why SOF, a compound with a high genetic barrier to resistance but relatively low rate of virus decline, is a very effective antiviral, especially when used in combinations. Nonetheless, fast kinetics of antiviral action by DAAs might also be a useful attribute and of potential interest during the process of preclinical evaluation of the clinical utility of compounds. Here, we established an assay system that can be used to inform which antiviral or combination of antivirals exerts a more potent effect and which exerts it the fastest in a cell culture–derived HCV system. The assays include flow cytometry, a *Gaussia* luciferase reporter system, Western blot analysis, RT-qPCR, a limited dilution assay, and an image profiling assay that is used for the first time with HCV infectious virus. The first five assays can be used in a multiplex 12-well plate format, whereas the image profiling assay can be used efficiently in a 96-well plate format. We used these assays to systematically compare LDV, DCV, DNV, and SOF and obtain diverse mechanistic information. The flow cytometry assay is not as sensitive as the other assays and requires more cells and virus. However, it provides unique information because it detects the percentage of infected cells at various time points postinfection. The luciferase assay provides a facile and sensitive method to follow changes in viral protein translation and can be combined with the less sensitive Western blot analysis that can probe changes in the translation of specific viral proteins. The image profiling assay provides unique information on the morphological changes in NS5A aggregates during the course of treatment. It has the advantage of allowing early detection of a phenotypic change (NS5A redistribution) but does not provide information on the infectivity or nucleic acid of the virus. The RT-qPCR assay quantifies intracellular and extracellular viral RNA following addition of DAAs. While it is sensitive and can be modified to provide information about replication intermediates such as the negative-sense HCV RNA, it cannot differentiate between infectious or noninfectious virus. In contrast, the limited dilution assay can provide information on the infectivity of released virus. In the time frame examined (up to 72 h), we expect that new infections have been occurring. Hence, we anticipate that under these conditions all assays measure a cumulative effect on viral replication and new infection. While all assays provided statistically significant estimations of HCV inhibition at later time points (24 hpt or more), the image profiling, RT-qPCR, and limited dilution assays were able to un-
ambiguously quantify the effects of drug treatment at early time points (8 h). Our data demonstrate that the NS5A inhibitor LDV, followed closely by DCV, has the fastest effect on redistribution of NS5A, on suppression of viral RNA, and on infectivity of released virus.

Other studies have also reported on the kinetics of inhibition by DAAs. Targett-Adams et al. compared the kinetic profiles of DCV, the NS3/4A inhibitor BILN 2061, and the NS5B inhibitor HCV-796. They used a luciferase-based reporter system and RT-qPCR to measure viral protein translation and viral RNA copy numbers in replicon-harboring cells. They reported that DCV treatment leads to NS5A redistribution from the ER to LDs (25, 37, 45) and it is temporally correlated with suppression of viral replication (25). These results are consistent with our data in Fig. 7 showing that LDV and DCV treatments have a rapid effect on suppression of viral RNA (as early as 8 hpt), which also temporally correlates to early NS5A redistribution observed at 8 hpt. However, in contrast to our data, Targett-Adams et al. reported that an NS5B inhibitor, HCV-796, and a protease inhibitor, BILN 2061, exhibited more rapid onset of HCV inhibition than DCV. This discrepancy might be attributed to variations in experimental conditions. Specifically, Targett-Adams et al. did not use an infectious HCV system, and it is possible that the effects of blocking DCV may have been underestimated because the replication system that was used did not account for the role of NS5A in virus assembly. In addition, the previous study evaluated different protease and polymerase inhibitors than this study and at lower concentrations (up to 15× their EC_{50}s versus up to 100× EC_{50}s in this study). Further evidence for the rapid onset of the NS5A inhibitor antiviral effects comes from the kinetic imaging experiments presented in Fig. 6, where NS5A redistribution in response to LDV or DCV treatment is apparent as early as 8 hpt, compared to at least 24 hpt for DNV and SOF. Similarly, the most significant suppression of viral RNA (Fig. 7) and viral proteins (Fig. 4C and D) at early time points (8 hpt) is observed in cells treated with LDV, followed by those treated with DCV. Our data are also consistent with the recent study by Guedj et al. (46), who reported a very rapid decline in HCV RNA during DCV-based treatment of HCV-infected patients (a decline in serum HCV RNA as early as 6 h after administration). Guedj et al. (46) used mathematical modeling to propose that DCV suppresses extracellular HCV RNA faster than the NS5B inhibitor NM107 because it blocks both viral RNA synthesis and virion assembly/export.

More recently, using the HCV gt1a cell culture-infectious virus H77S.3, McGivern et al. compared the kinetics of antiviral activity for several DAAs (47). They employed an assay that measures infectious focus-forming units and determined the quantity of infectious virus released from drug-treated H77S.3-infected cells. Consistent with the data of McGivern et al., we also found that LDV rapidly suppresses the release of infectious virus (Fig. 8). However, in contrast to our data, McGivern et al. observed a more rapid onset of HCV inhibition using protease and polymerase inhibitors, rather than NS5A inhibitors (47). This difference might be due to the fact that McGivern et al. used the H77S.3 gt1a virus that has been reported to replicate less efficiently than the gt2a Jc1 virus used in the present study (48, 49). In addition, different protease and polymerase inhibitors were used in the McGivern et al. study (47).
Using the luciferase reporter system and a gt2a replicon system, Bartenschlager and colleagues found no significant differences between HCV-infected cells that were either untreated or treated with high concentrations of the DCV analog BMS-553 (50× EC90) for 8 h (50). This is consistent with our data that showed no significant effect of DCV treatment at 8 hpt when we also used a luciferase reporter system (Fig. 3A). Based on electron microscopy data, Bartenschlager and colleagues proposed that BMS-553 acts by abrogating early formation of the membranous web where RNA replication occurs in HCV-infected cells. Hence, DCV may have a more rapid antiviral effect than DNV and SOF not only because its target is involved in multiple steps of the virus life cycle but also because, unlike the other drugs, DCV can disrupt the sites of HCV replication at the membranous web.

In summary, this study presents a comprehensive approach for the characterization of the kinetic profile of existing and new antivirals toward the identification of optimized combinations that might lead to shorter treatment durations, without compromising SVR, and result in cost savings for treatment. Moreover, such systematic analysis can be useful for the dissection of the MOA of antivirals and the role of viral or host proteins in the HCV life cycle.

**FIG 7** Assessment of HCV inhibition by DAAs from 3 classes using the RT-qPCR assay. Dose-response curves are shown for DCV (A), LDV (B), DNV (C), and SOF (D). Jc1/Gluc2A virus-infected Huh-7.5.1 cells were treated with DMSO or different concentrations of the HCV inhibitors. The total cellular RNA was extracted by TRIzol reagent and analyzed for HCV 5′ UTR RNA by RT-qPCR at the indicated time points as described in Materials and Methods. Cycle threshold \( (C_T) \) values were converted to copy numbers based on the standard curve of pure Jc1/Gluc2A genome-encoded plasmid. Data were normalized to data for DMSO controls. Each data point represents the average value from 2 individual experiments. Error bars represent SEM.

**FIG 8** Assessment of HCV inhibition by DAAs from 3 classes using the infectivity assay. Jc1/Gluc2A virus-infected Huh-7.5.1 cells were treated with DMSO or 100× EC50 of the HCV inhibitors. Extracellular virus was precipitated by PEG and analyzed for viral titer by a TCID50 assay, and extracellular viral RNA was detected by RT-qPCR at 8 hpt as described in Materials and Methods. Data were normalized to data for DMSO controls. Each data point represents the average value from two individual experiments. Error bars represent SEM. The statistical analysis was performed by using Student’s \( t \) test \((^* P < 0.05)\). (A) Extracellular RNA copy number per milliliter; (B) extracellular viral infectivity per milliliter.
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