Pharmacological disruption of hepatitis C NS5A protein intra- and intermolecular conformations

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Non-structural 5A protein (NS5A) has emerged as an important pharmacological target for hepatitis C virus (HCV). However, little is known about the conformation of NS5A intracellularly or how NS5A inhibitors achieve the picomolar (pM) inhibition of virus replication. Here, we have presented two structurally related small molecules, one that potently inhibits HCV replication and selects for resistance in NS5A, and another that is inactive. Resistance to this antiviral was greater in genotype 1a than in genotype 1b replicons and mapped to domain 1 of NS5A. Using a novel cell-based assay that measures the intracellular proximity of fluorescent tags covalently attached to NS5A, we showed that only the active antiviral specifically disrupted the close proximity of inter- and intramolecular positions of NS5A. The active antiviral, termed compound 1, caused a repositioning of both the N and C termini of NS5A, including disruption of the close approximation of the N termini of two different NS5A molecules in a multimolecular complex. These data provide the first study of how antivirals that select resistance in domain 1 of NS5A alter the cellular conformation of NS5A. This class of antiviral disrupts the close proximity of the N termini of domain 1 in a NS5A complex but also alters the conformation of domain 3, and leads to large aggregates of NS5A. Current models predict that a multicomponent cocktail of antivirals is needed to treat HCV infection, so a mechanistic understanding of what each component does to the viral machinery will be important.

INTRODUCTION

Hepatitis C virus (HCV), a member of the family Flaviviridae of positive-strand RNA viruses, chronically infects approximately 170 million people worldwide (WHO, 2011). Over time, ongoing virus replication within the liver often leads to severe clinical manifestations such as fibrosis, cirrhosis and hepatocellular carcinoma. Medical treatments for HCV are limited by the lack of a vaccine and generally require a cocktail of multiple antiviral medications. Currently, patients undergo treatment with a combination of pegylated alpha interferon (IFN-α) and ribavirin in addition to an HCV protease inhibitor if the patient is infected with genotype 1. Even with all three drugs, the therapy is prolonged, viral escape can still occur and severe side effects occur, highlighting a need for improved antiviral cocktails with better efficacy and safety profiles (Ghaniy et al., 2011).

HCV encodes at least 10 proteins that are potential targets of antiviral therapy. Most of the viral proteins associate with other viral and cellular proteins to form a nucleic acid replication complex and/or a lipid droplet-associated assembly complex. Therefore, the effect of antiviral compounds on isolated proteins in biochemical assays may differ from those on proteins in vivo. One viral target, non-structural 5A protein (NS5A), is known to interact with a large number of viral and cellular proteins (Schmitz & Tan, 2008). NS5A contains an N-terminal amphipathic α-helix followed by three domains separated by two low-complexity sequences (LCS I and II). Whilst two related crystal structures have been determined with different dimeric interfaces for the N terminus of NS5A (Tellinghuisen et al., 2005; Love et al., 2009), the protein is also likely to have regions of disorder, and the overall tertiary and quaternary structure of NS5A in the cell is unknown. There has been a focus on NS5A for some time as a possible mediator of IFN resistance (Enomoto et al., 1996) as the target of host-cell cyclophilin inhibitors (Ansari & Striker, 2012; Ansari et al., 2013; Membreno et al., 2013) and more selective antivirals (Lok et al., 2012),

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One supplementary figure is available with the online version of this paper.
but a detailed mechanistic understanding of NS5A inhibitors is still missing. Here, we have presented an analysis of the genotype 1 activity of a lead compound, termed compound 1, and showed that, in cell culture, it alters the conformation of multimeric NS5A without dissociating NS5A into monomers.

RESULTS

Compound 1, but not compound 2, potently inhibits HCV replication

Lead compounds that inhibited HCV genotype 1a and 1b replicons were identified through small-molecule screening (Chen et al., 2010) and the structure–activity relationship was explored via the synthesis and analysis of more than 100 derivatives. As a result, compounds were identified that had various levels of antiviral activity. To better understand the antiviral effect of one of these, termed here compound 1, a truncated molecule with identical core but without both valine-carbamate moieties, termed compound 2, was also synthesized (Fig. 1). Both activity and cytotoxicity effects were determined for Huh7 derivative cells containing either a genotype 1a or genotype 1b subgenomic replicon. Compound 1 showed potent activity against both genotype 1a and 1b replicons, with 50% effective concentration \( EC_{50} \) values of 23.1 and 9.8 pM, respectively. Compound 2 exhibited activity that was >10000-fold less than that observed for compound 1, with \( EC_{50} \) values on both replicons of >1 μM, demonstrating the importance of the substituted valines for activity (Table 1). Cytotoxicity assays were run in parallel for genotype 1b, and both compounds had 50% cytotoxicity concentration \( CC_{50} \) values of 35 μM (\( CC_{50} \) of 38.0 μM for compound 1 and 46.8 μM for compound 2).

Table 1. Antiviral activity of compounds on stable and transient HCV replicons.

<table>
<thead>
<tr>
<th>Compound/ NS5A mutant</th>
<th>( EC_{50} ) (pM)</th>
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<tbody>
<tr>
<td>Compound</td>
<td>Genotype 1a</td>
</tr>
<tr>
<td>1</td>
<td>23.1</td>
</tr>
<tr>
<td>2</td>
<td>6500 000</td>
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<table>
<thead>
<tr>
<th>NS5A mutant</th>
<th>Compound 1</th>
<th>Daclatasvir</th>
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<tbody>
<tr>
<td>1a WT</td>
<td>10.7</td>
<td>19</td>
</tr>
<tr>
<td>1a L31M</td>
<td>1020</td>
<td>3600</td>
</tr>
<tr>
<td>1a L31V</td>
<td>3500</td>
<td>18 000</td>
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<tr>
<td>1a M28T</td>
<td>2500</td>
<td>7100</td>
</tr>
<tr>
<td>1a M28V</td>
<td>25.1</td>
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</tr>
<tr>
<td>1a Q30H</td>
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<td>8900</td>
</tr>
<tr>
<td>1a Q30K</td>
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<td>1a Q30R</td>
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<td>1a Y93C</td>
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</tr>
<tr>
<td>1b D320E</td>
<td>6.2</td>
<td>3.8</td>
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<tr>
<td>1b L28V</td>
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<td>2.6</td>
</tr>
<tr>
<td>1b L31F</td>
<td>12</td>
<td>22</td>
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<tr>
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<tr>
<td>1b L31W</td>
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<tr>
<td>1b P32L</td>
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<td>27</td>
</tr>
<tr>
<td>1b Y93H</td>
<td>48</td>
<td>440</td>
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<tr>
<td>1b Y93N</td>
<td>130</td>
<td>509</td>
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Compound 1 antiviral activity is compromised by mutations in domain 1

As part of the characterization of this series, replicon cells were selected for resistance to initial lead compounds in the series (unpublished data). In both genotype 1a and 1b replicon cells, resistance mapped to domain 1 of NS5A. The level of resistance varied by mutation and compound tested. Several of these mutations in NS5A have also been shown to compromise the cell-culture activity of daclatasvir, an HCV inhibitor currently in late-stage clinical trials (Fridell et al., 2011). Therefore, we made point mutations in domain 1 of NS5A replicons, including known sites of resistance for daclatasvir in the genotype 1a and 1b replicons, to determine whether domain 1 NS5A mutations also conferred resistance to compound 1. Similar to the results with other members of the NS5A inhibitor class including daclatasvir, mutations at positions L28, L31, Q30 and Y93 in genotype 1a decreased the antiviral activity of compound 1 by 70–700-fold, with the exception of the M28V mutation, which had activity similar to the WT. Little to no impact on activity was observed for the mutations in genotype 1b, with several

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Fig. 1. Chemical structure of compounds 1 and 2. Structures of the synthesized active (compound 1) and inactive (compound 2) compounds (Chen et al., 2010) used in this study are shown.
mutations (L28V, L31F, L31V and P32L) in domain 1 causing no impact on activity, whilst others (L31W, Y93H and Y93N) modestly reduced activity by 6-16 fold. Interestingly, a mutation in genotype 1b associated with cyclophilin resistance, D320E (Puyang et al., 2010), had no effect on the antiviral activity of compound 1. Although mutations in domain 1 of the genotype 1b replicon had much smaller effects on the antiviral activity than genotype 1a mutations (Table 1), these results indicated that compound 1 targets NS5A for its antiviral activity and show that the activity of compound 1 compares to that of daclatasvir against various mutants (Bechtel et al., 2011).

Only antiviral compound 1 alters the conformation of NS5A domain 1

In order to determine how compound 1 produces such a potent antiviral effect, unsuccessful attempts were made to demonstrate direct binding between several members of this series and recombinant NS5A produced from bacteria, using standard binding assays including Biacore and isothermal titration calorimetry (data not shown). Given the lack of positive results with purified NS5A, cellular expression constructs of NS5A with two fluorescent tags [cyt fluorescent protein (CFP) and the affinity tag CCPGCC, which is capable of binding to the fluorescence resonance energy transfer (FRET) acceptor labelling compound FlAsH; see Methods] were engineered to probe interactions occurring in the intracellular environment (Figs 2-4). Briefly when the CFP and CCPGCC motifs were fused in sequence [Fig. 2a, constructs (i) and (ii); details in Bhattacharya et al., 2012], there was both a FlAsH load and a FRET signal seen. As progressively larger portions of NS5A were inserted between the two FRET tags [Fig. 2a, constructs (iii)–(vi)], FRET but not FlAsH load declined, indicating that the CCPGCC motif was still accessible but that the fluorescent tags were no longer in close proximity (<10 nm). Once these data established that portions of NS5A could disrupt the FRET signal between CFP and FlAsH all expressed in the same polypeptide chain, these constructs were utilized to determine whether compound 1, which putatively worked via NS5A, could alter the FRET of specific constructs. Cells transfected with each construct individually [Fig. 2a, constructs (i)–(vi)] were exposed to compound 1 and the FRET signal was measured. With increasing concentrations of compound 1, the FRET signal of CFP–NS5A(Thr213–CCPGCC) was lowered [Fig. 2a, construct (iii)], but there was no effect on the FRET of constructs (i), (ii), (iv) and (v), suggesting that compound 1 alters the proximity of tags surrounding domain 1 of NS5A. There appeared to be a bimodal response to compound 1, as the FRET signal decreased to a plateau between 2 and 40 pM and then decreased to another plateau between 100 and 200 pM. Compound 2 had no effect on the FRET of CFP–NS5A(Thr213–CCPGCC) (Fig. 2b). This suggested that, in cells exposed to compound 2, the N terminus of NS5A is still in close proximity to the CCPGCC tag at aa 213, in contrast to the situation when there is antiviral activity. Furthermore, the expression level of NS5A [Fig. 2a, construct (iii)] protein was not changed when exposed to up to 200 pM concentration of compound 1 (Fig. 2c).

Only antiviral compound 1 alters the conformation of NS5A domain 3

In addition to placing the CFP at the N terminus of NS5A [Fig. 2a, constructs (i)–(vi)], constructs were also made that placed CFP at the C terminus [Fig. 3, constructs (vi)–(ix)]. As was the case when CFP is placed at the N terminus, CFP at the C terminus displayed a FlAsH load in all constructs but only displayed FRET when the tags were fused sequentially [Fig. 3a, construct (i), or with only domain 3 separating the tags (construct (viii)]. Each construct was transfected and exposed to the indicated concentrations of compound 1, and both FRET and FlAsH loads were determined. Whilst the FlAsH load did not change with increasing concentrations of compound 1, NS5A(Cys342–CCPGCC)–CFP [Fig. 3a, construct (viii)] lost its FRET signal when exposed to micromolar concentrations of compound 1. Interestingly, at concentrations of compound 1 greater than 40 pM there was no increase in FRET (Fig. 3b), suggesting that the two tags moved back closer together but not as close as when the cells were unexposed. This bimodal response is similar to what was seen with CFP–NS5A (Thr213–CCPGCC) (Fig. 2b) in occurring after 40 pM, but resulted in an increase in FRET signal rather than a decrease. Compound 2 had no effect on NS5A(Cys342–CCPGCC)–CFP (Fig. 3b). Although the NS5A to actin expression level was not entirely uniform at every concentration of compound 1, the FRET efficiency did not reflect protein expression levels and clearly changed at 40 pM before the increased ratio of NS5A to actin was observed (Fig. 3c).

Only antiviral compound 1 alters intermolecular FRET of NS5A domain 1

A double transfection experiment was then performed with the CFP and CCPGCC encoded by two separate plasmids each at the N terminus of NS5A [Fig. 4a, constructs (x) and (xi)]. FRET was observed when these two constructs were co-transfected in HuH7.5 cells (Fig. 4a). Similar constructs with CFP and CCPGCC at the C terminus, or with one at the N terminus and the other at the C terminus, showed no FRET signal (Bhattacharya et al., 2012), indicating only the N termini are in close proximity. When cells co-transfected with the N-terminal tagged NS5A FRET constructs were exposed to compound 1, FRET between the tags was reduced, indicating that the close proximity of the N termini was disrupted; however, compound 2 had no effect on FRET in this experiment (Fig. 4b).

Compound 1 affects the intracellular localization of NS5A

To analyse further whether the NS5A-targeting compound caused apparent changes in NS5A distribution in cells, we
performed confocal microscopy to detect the distribution of NS5A, with a CFP C-terminal tag [construct (xii); see Fig. 6a] following compound 1 treatment. In parallel experiments, the transfected cells were also treated with DMSO and compound 2 to serve as a negative control. In DMSO- and compound 2-treated cells, the NS5A appeared mostly cytoplasmic and associated with protein disulfide isomerase (PDI), an endoplasmic reticulum (ER)-resident protein (Fig. 5). However, NS5A distribution was changed dramatically when treated with compound 1 at 40 pM, a concentration that had the maximal effect on the conformation of domain 3 (Fig. 5) and reduced HCV replicon activity (Table 1). The treated cells contained large foci of NS5A, the majority of which were not ER associated as they did not co-localize with PDI. A similar change in NS5A distribution pattern has been reported for other NS5A inhibitors (Lee et al., 2011; Qiu et al., 2011; Targett-Adams et al., 2011).

**Compound 1 increases NS5A aggregation**

As compound 1 both relocated NS5A and increased the distance between the N termini (Fig. 4b), a co-immunoprecipitation experiment was performed to determine whether NS5A was still in a multimeric complex in the presence of compound 1 (Fig. 6). Our previous work had demonstrated that transient co-expression of CFP-tagged NS5A and untagged NS5A [constructs (xii) and (xiii) in Fig. 6a] allowed the co-immunoprecipitation of both forms in the detergent- and RNA-free environment by a CFP antibody (Bhattacharya et al., 2012). When the same experiment was performed in the presence of either 2 or
60 pM of compound 1, increased rather than decreased co-immunoprecipitation was seen (Fig. 6c).

**DISCUSSION**

Recently, there have been exciting data suggesting that small-molecule inhibitors that select for resistance in NS5A are active in patients and have picomolar potency in cell culture (Gao et al., 2010; Lok et al., 2012; Suk-Fong Lok, 2013). Compound 1 (Fig. 1) has been shown to be an inhibitor of NS5A at picomolar levels by its loss of potency on replicons containing resistance mutations in domain 1 of NS5A associated with NS5A inhibitors. We used compound 1 to probe the intracellular conformation of NS5A and demonstrated conformational changes in domains 1 and 3 in the presence of compound 1 using a recently developed method for examining both intra- and intermolecular proximity relationships between fluorescent tags fused to one or more molecules of NS5A (Bhattacharya et al., 2012): FRET analysis in that study suggested that the intermolecular distance between the N termini of NS5A in a multimeric complex, between the N terminus and a tag at aa 213, and between the C terminus and a tag at aa 342 are in close proximity (<10 nm). Compound 1 disrupted all three conformation-dependent FRET signals, whilst compound 2, a related analogue with no antiviral activity, had no effect on any of the FRET signals. This is consistent with compound 1 binding NS5A in a manner similar to the inhibitors proposed by others (Targett-Adams et al., 2011; O’Boyle et al., 2013), but our assay did not discriminate between a direct binding and some indirect alteration in the environment of NS5A caused by the compound.

Quercetin, a natural product that has only modest antiviral activity and does not relocalize NS5A, disrupted two of these three FRET signals (Bhattacharya et al., 2012). Unlike compound 1, quercetin did not disrupt the domain 1 FRET between the N-terminal tag and a tag at aa 213. The change in FRET signal induced by quercetin in domain 3 also differed in not having a bimodal response, as was seen with
Fig. 4. Intracellular proximity of the N termini of different NS5A molecules is decreased by compound 1. (a) Schematic of N-terminally tagged CFP and CCPG GCC NS5A constructs used for co-transfection. FRET efficiency and FIAsh load ratio indicated that the N termini of the NS5A molecules were in close proximity. (b) The N-terminal proximities of NS5A were disrupted when exposed to the antiviral compound 1.

Compound 1. Domain 1 of NS5A, containing the determinants for resistance to NS5A inhibitors, is critical for RNA binding, although there are also contributions to RNA binding from both C-terminal regions (Foster et al., 2010; Hwang et al., 2010). Domain 3 is dispensable for replication but has an essential role in viral assembly (Tellingshuisen, 2008). Our observation that compound 1 can induce conformational changes in both domain 1 and domain 3 is consistent with the inhibitory action on both viral RNA replication and viral assembly proposed for daclatasvir (Guedj et al., 2013).

Interestingly, supplementary data suggest the effect on the FRET signal when the FRET tags flank domain 1 [Fig. 2a, construct (iii)] is similar between daclatasvir and compound 1, but not when the FRET tags surround domain 3 [Fig. 3a; construct (viii)] (Fig. S1, available in JGV Online). Whilst clearly the resistance mutations are common between these two examples of NS5A inhibitors, there are differences in how these changes impact the activity of the compounds, suggesting similar yet distinct binding modes. For example, compound 1 and daclatasvir show similar reductions in EC50 values compared with WT on nine out of 15 NS5A mutant replicons tested. However, reductions in EC50 values compared with WT for the two compounds differed by 5–21-fold on the genotype 1a NS5A Q30H and Q30K replicons, and genotype 1b NS5A L31V, L31W, P32L, Y93H and Y93N replicons. The most dramatic difference was observed on the genotype 1a NS5A Q30K replicon where the EC50 value for compound 1 was reduced by 467-fold, whilst the value for daclatasvir was reduced by 10,000-fold. The differences in activity against the resistant mutants suggest that there could be slight differences in binding between the two compounds and may explain the differences in how compounds alter NS5A relevant to domain 3.

We also showed that there is a redistribution of the intracellular localization of NS5A from the ER to non-ER compartments, as measured by immunofluorescence of CFP-tagged NS5A. A similar relocation induced by NS5A inhibitors has also been observed by Targett-Adams et al. (2011) and Lee et al. (2011) in the context of the HCV replicon. The GFP-tagged NS5A used by Targett-Adams et al. (2011) localized to lipid droplets, so the different sites of localization between the two NS5A constructs in the absence of NS5A inhibitors may have affected the ability to observe any relocation. The ability of NS5A inhibitors to interact with NS5A in the absence of other HCV non-structural proteins is supported by the observation of Targett-Adams et al. (2011) that binding to a biotinylated NS5A inhibitor is reduced but not completely eliminated when NS5A is expressed by itself. Negative data by us and others (O’Boyle et al., 2013) in which, for example, NS5A expressed in Escherichia coli was unable to be shown to bind to NS5A inhibitors, does not exclude the possibility that NS5A, when expressed in mammalian cells by itself, binds at least compound 1 as we have shown here.

When NS5A is in the presence of compound 1, the intermolecular distance between NS5A N termini is
Fig. 5. Formation of larger foci of NS5A in the presence of compound 1. Huh7.5 cells were transfected with CFP-tagged NS5A and the cells were untreated (0 pM) or treated with 40 pM compound 1 or 2 for 48 h. The cells were examined for nuclei staining with DAPI, the distribution of NS5A by detecting CFP and staining of the ER by detecting an ER-resident protein, PDI. The merged images are also presented.

Increased (Fig. 4b). This does not result in the disruption of NS5A multimers, however. In fact, in the presence of compound 1, large aggregates of NS5A form (Figs 5 and 6).

Fig. 6. NS5A exists in multimeric aggregates in the presence of compound 1. (a) Schematic of constructs (xii) and (xiii). (b) NS5A and NS5A–CFP were expressed in Huh7.5 cells in the presence of the indicated amounts of compound 1. Expression of each protein in the total cell lysate was confirmed by Western blot with anti-NS5A antibody. (c) Immunoprecipitation of the total cell lysate was carried out with anti-CFP antibody and co-precipitated proteins were detected by Western blot with anti-NS5A antibody.
inhibitors, as perhaps a single molecule of compound 1 can inactivate multiple NS5A molecules bound together in a complex as has been proposed for daclatasvir (Lernmark et al., 2010; O’Boyle et al., 2013). We speculate that additional NS5A molecules in these aggregates may bind additional compound 1 in a manner that induces the bimodal response in domains 1 and 3.

In conclusion, a novel approach for probing the effect of NS5A inhibitors on intracellular NS5A protein conformation expressed without viral interacting partners is presented. In cells, compound 1 increases the distance between the N terminus and aa 213, and also causes a rearrangement of domain 3. These conformational changes coincide with a redistribution and aggregation of NS5A. These observations are consistent with the current understanding of the mechanism of action of NS5A inhibitors and provide a platform for exploring conformational changes occurring within the cell.

METHODS

Synthesis of compounds. Synthesis of compounds 1 and 2 (Fig. 1) is detailed in Chen et al. (2010). Daclatasvir was synthesized and characterized at GlaxoSmithKline as part of the development programme for GSK2336805, an NS5A inhibitor in clinical development.

Replicons and cells. Genotype 1a replicon cells, also called LucA 1.19 cells, are a Huh-7-derived cell line bearing the genotype 1a H77 NS3–5B bicistronic subgenomic replicon (Blight et al., 2003) constructed at GlaxoSmithKline. It contains several adaptive mutations (NS5B Q51H, NS5A K66R, NS5A and S323I) and the luciferase gene, and encodes neomycin resistance. Genotype 1b Con1 replicon cells, referred to as ET cells, were licensed from ReBLikon (Lohmann et al., 2003). Stable replicon assays were performed in 384-well assay plates containing DMSO or dilutions of compound. Stable replicon cells (5 × 10⁴) were added to each well. Cells were incubated at 37 °C and 5% CO₂. Plates were removed from the incubator at 48 h after treatment and allowed to equilibrate to room temperature. HCV replication was monitored by measuring firefly luciferase activity using Steady-Glo (Promega) and measurement of luminescence in an EnVision 2103 Multilabel Reader (Perkin Elmer). Cytotoxicity was measured on parallel plates using CelTiter-Glo (Promega). Replicon EC₅₀ and CC₅₀ values were calculated by curve fitting the data to the Hill equation, using a non-linear least-squares curve-fitting program. Transient transfections used plasmid constructs containing WT or mutant replicons as templates for in vitro transcription reactions using a T7 Express kit (Promega). The in vitro transcriptions were aliquoted and stored at 2 °C before use. For genotype 1a constructs, 5 × 10⁶ Huh7/7 Lunct cells (ReBLikon) were electroporated with 15 μg RNA in Cytosol [prepared at the GSK laboratories according to van den Hoff (1992), supplemented with 2 mM ATP and 5 mM glutathione (van den Hoff et al., 1992)]. Electroporation was performed in 0.4 cm cuvettes using a Bio-Rad Gene Pulser II at 270 V, 950 μF and infinite resistance. Transient transfections with genotype 1b constructs were performed similarly except that 5 × 10⁶ ET cells were incubated with 5 μg RNA in PBS. ET C8T cells are a cell line cured of replicon RNA by serial treatment of ET replicon cells with IPN-α. Electroporated cells were resuspended in complete medium, and 26 10⁴ cells were transferred to wells of a 96-well plate containing compounds or DMSO. Cells were incubated at 37 °C and 5% CO₂ for 3 days, and inhibition of HCV replication was measured as for the stable replicon cells using Bright-Glo (Promega). Mutations in the NS5A genotype C2 or 1a were made using standard molecular biology techniques. Replicons were sequenced after mutagenesis.

Vectors and chemical compounds. The FRET donor ECFP-N1 and ECFP-C1 vector were purchased from Clontech; FRET acceptor labelling compound FLASh-EDT (Lumio Green; in this study referred to as FLASh), Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG were from Invitrogen; Hank’s balanced salt solution (HBSS) was from Cellgro; 1,2-ethanediol (EDT) was from Fluka Analytical; FLASh removal compound 2,3-dimercapto-1-propanol (British anti-jeutan or BAL), NP-40, sodium deoxycholate, SDS and fluoromount were from Sigma; anti-CFP polyclonal antibody was from BioVision; anti-NS5A mAb was from Meridian Life Science; anti-PDI mAb was from Santa Cruz Biotechnology; EDTA-free Halt Protease Inhibitor Cocktail and the detergent-removal spin column were from Thermo Scientific; and the RNase cocktail was from Ambion.

Genetic constructs. The 17 aa CCPGCC FLASh-binding motif AEAARAREACPGGCCARA (Adams et al., 2002), in this study referred to as the CCPGCC motif, was fused in-frame to the C terminus of ECFP by PCR [Fig. 2a, construct (i)]. The full-length NS5A sequence of Con1 (aa 1–447, GenBank accession no. AJ238799) was cloned in-frame in either the ECFP-N1 or ECFP-C1 vector, and the CCPGCC motif was introduced separately in four critical positions (S1, T213, C342 and C447) of these N-terminal [Fig. 2a, constructs (i)–(v)] and/or C-terminal tagged NS5A [Fig. 3a, constructs (vi)–(x)]. We also made the NS5A N-terminal fused CFP and CCPGCC motif separately [Fig. 4a, constructs (x) and (xi)]. For the co-immunoprecipitation study, we made NS5A C-terminally fused CFP, and NS5A without any tag constructs [Fig. 6, constructs (xii) and (xiii)]. For the confocal study, we used the NS5A C-terminally fused CFP construct [Fig. 6, construct (xii)]. Details of these constructs have been given previously (Bhattacharya et al., 2012).

FLASh binding and acquisition of cellular FRET data. For experiments including inhibitors, cells were incubated with the indicated amount of compounds 1 and 2 at 8 h post-transfection, and the FRET assay was carried out after 40 h (48 h post-transfection).

Intramolecular NS5A FRET. After 48 h of transfection with the indicated FRET construct in Huh7.5 cells (Apati Llc), the cells were washed with HBSS and labelling was carried out with 1 μM FLASh in loading buffer at 37 °C in 5% CO₂ for 1 h. After clearance of non-specific binding of FLASh with 250 μM EDT, the cells were covered with 1 ml loading solution before imaging. The epifluorescence microscopy images were captured with a cooled CCD camera using a 10 objective in an inverted Nikon microscope by standard band-pass excitation/emission filter cubes (excitation 436/20 nm, emission 480/30 nm, dichroic 455 nm for CFP; excitation 500/20 nm, emission 535/30 nm, dichroic 515 nm for fluorescein) as described previously (Bhattacharya et al., 2012). After image acquisitions (emission of both CFP and FLASh before and after FLASh clearance), the mean pixel intensity was analysed using the ImageJ program (http://imagej.nih.gov/ij/download.html) at a scale of 0–255 by selecting low to medium expression of cells. The FRET ratio was calculated by CFP emission after FLASh loading, after the BAL wash/CFP emission after FLASh loading, and before the BAL wash, and from the FRET ratio we calculated the FRET efficiency as 1−[I₁/I(1/FRET ratio)] (Miyawaki & Tsien, 2000) from 30-50 individual cells to calculate the mean signal intensity ± SE, details of which have been described previously (Bhattacharya et al., 2012).

Intermolecular FRET. Co-transfection in Huh7.5 cells was carried out with NS5A N-terminally fused CFP [Fig. 4a, construct (x)] and NS5A N-terminally fused CCPGCC [Fig. 4a, construct (xi)]. After
48 h of transfection, the FlAsH labelling and FRET measurement were carried out as described previously (Bhattacharya et al., 2012).

**Confocal microscopy.** The confocal microscopy was performed as described previously (Das et al., 2006). In brief, HuH7.5 cells were grown on 12 mm glass coverslips and transfected with NS5A C-terminally fused CFP-expressing plasmid [Fig. 6a, construct (xii)]. The cells were treated with 40 PM compounds 1 and 2 for 48 h before they were fixed and processed for confocal studies. The staining was performed with anti-CFP polyclonal antibody and anti-PI-PI mAb for 1 h at room temperature. The cells were washed and further incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated goat anti-mouse IgG for another 1 h at room temperature. Following the wash, the cells were stained with 0.5 mg DAPI ml⁻¹ to stain the nuclei. The coverslips were mounted upside down on a glass cover slide in the presence of Fluoromount (Sigma Aldrich, St Louis), and images were captured with a motorized Zeiss Axioplan II equipped with a rear-mounted excitation filter wheel (0.2 μm Z-increment. 40 Plan Apo, oil-immersion lens), a triple-pass (DAPI/HTC/Texas Red) emission cube and an Orca AG CCD camera (Hamamatsu). The images were collected and analysed using the OpenLabs 5.0 and Velocity 5.0 programs (Improvision).

**NS5A multimerization study.** The NS5A multimerization study was carried out as described previously (Bhattacharya et al., 2012). Briefly, the co-transfection of NS5A-CFP and NS5A (with no tag) plasmids [constructs (xii) and (xiii)] were carried out in HuH7.5 cells. After 8 h of transfection, the cells were exposed to compound 1 for 40 h before harvesting (48 h post-transfection). For background control, a mock transfection was carried out in parallel. After 48 h of transfection, the cells were lysed in lysis buffer (25 mM Tris/150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% NP-40, 10 mM EDTA-free protease inhibitor cocktail). The confirmation of expression of both tagged (NS5A-CFP) and untagged (NS5A) protein was carried out by Western blotting using anti-NS5A mAb. The detergents were removed from the cell lysate by a detergent-removing spin column and the lysates were treated with 5 μl RNase cocktail enzyme mix ml⁻¹ overnight at 4°C. Co-immunoprecipitation was carried out with anti-CFP polyclonal antibody and Western blotting was performed with anti-NS5A mAb.

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