Virology 444 (2013) 343–354

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Characterizations of HCV NS5A replication complex inhibitors

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ARTICLE INFO

Article history:
Received 2 April 2013
Returned to author for revisions
2 May 2013
Accepted 28 June 2013
Available online 27 July 2013

Keywords:
NS5A replication complex inhibitor
Antiviral
Covalent attachment
HCV
NS5A dimer
Daclatasvir

ABSTRACT

The hepatitis C virus NS5A protein is an established and clinically validated target for antiviral intervention by small molecules. Characterizations are presented of compounds identified as potent inhibitors of HCV replication to provide insight into structural elements that interact with the NS5A protein. UV-activated cross linking and affinity isolation was performed with one series to probe the physical interaction between the inhibitors and the NS5A protein expressed in HCV replicon cells. Resistance mapping with the second series was used to determine the functional impact of specific inhibitor subdomains on the interaction with NS5A. The data provide evidence for a direct high-affinity interaction between these inhibitors and the NS5A protein, with the interaction dependent on inhibitor stereochemistry. The functional data supports a model of inhibition that implicates inhibitor binding by covalently combining distinct pharmacophores across an NS5A dimer interface to achieve maximal inhibition of HCV replication.

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Introduction

Chronic hepatitis C virus infection remains an important area of unmet medical need requiring the continued search for assays to find specific and effective inhibitors. Cell-based hepatitis C virus (HCV) replication assays provide many advantages that traditional enzymatic assays lack or have difficulty duplicating. Capturing the entire replication cycle using cells based assays can probe for inhibitors that can target enzymatic function or mechanisms more elusive in nature such as transient associations of biomolecules or the capturing of unique conformations. These transient functions can represent essential processes involved in virus replication that cannot readily be duplicated in vitro (Sun et al., 2003; Sheaffer et al., 2008; Holler et al., 2009). The use of cell-based HCV replicon screens has allowed the discovery of a number of small molecule inhibitors that implicate viral proteins with no known enzymatic function as targets, including NS4B and NS5A (MacDonald and Harris, 2004; Conte et al., 2009; Green et al., 2008; Einav et al., 2008; Shotwell et al., 2012). Problematically, the replication of viruses can be quite sensitive to effects that are off-target from a specific virus function possibly affecting a cell process(es) required for replication or normal cell maintenance; thus a screening "hit" could simply reflect cell toxicity. The multiplexed design for antiviral screening represents a more ideal situation in that multiple readouts can be used to prioritize inhibitors with higher confidence of specificity. This type of cell-based approach using a cost effective multiplexed assay has been used to identify a series of compounds at Bristol-Myers Squibb with modest initial potency whose resistance changes were mapped to the NS5A protein of HCV (O'Boyle et al., 2005; Lemm et al., 2010, 2011; Gao et al., 2009; Romine et al., 2011). The amino acid changes resulting in compound resistance were demonstrated to be caused by substitutions at amino acids L31, Q54 and Y93 in NS5A. Using a model of the NS5A Domain 1 crystal structure (Love et al., 2009; Tellinghuisen et al., 2005), these changes have been determined to lie at a dimer interface of NS5A. The inhibitors are hypothesized to disrupt an essential NS5A function(s) that relies upon dimer formation required for replication. This suggests that the function(s) of NS5A, beyond its known involvement in packaging of the viral genome (Tellinghuisen et al., 2008a; Lindenbach et al., 2005; Appel et al., 2005a), are likely to be more diverse than originally contemplated and that different forms of NS5A, i.e. -phosphorylated and/or conformationally changed; may perform functions not readily duplicated in vitro (Huang et al., 2005, 2007; Appel

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http://dx.doi.org/10.1016/j.virol.2013.06.032

Compound	Structure	EC_{50} (uM) / CC_{50} (uM)
BMS-346		0.0001 / >10.0
BMS-671 BMS-690		0.15 / >10.0 >3.3 / >10.0
BMS-194		0.007 / ~6.0
BMS-350		0.4 / ~3.0
BMS-351		0.09 / >10.0

Fig. 1. Stilbene based compounds used in this study for affinity purification and UV activated crosslinking in HCV replicon cells. BMS-346 represents the active lead compound chosen for use as an affinity reagent in the series due to the potency of the un-modified compound. BMS-671 and BMS-690 represent the biotinylated 5A compounds used in affinity purification that differ only in stereochemistry. BMS-194 and BMS-350 contain UV reactive azide groups, while BMS-350 also includes a biotin moiety. BMS-351 contains 2 UV reactive (Bis-azide) groups. The associated EC_{50} and CC_{50} values are listed for each compound. EC_{50}=concentration of compound resulting in 50% HCV FRET activity when compared to DMSO control. CC_{50}=concentration of compound resulting in 50% reduction of Alamar blue conversion when compared to DMSO control.

et al., 2005b; Evans et al., 2004; Kim et al., 1999; Reed et al., 1997; Tellinghuisen et al., 2007, 2008b; Fridell et al., 2011b).

Recent publications clearly demonstrate the utility of HCV NS5A inhibitors, validating this approach as a clinically-relevant target, proven effective and potent in suppressing viral replication in HCV-infected subjects (Nettles et al., 2008; Gao et al., 2008, 2010; Asselah, 2010). The development of HCV NS5A inhibitors required extensive optimization of the original lead structure which proved to be cryptic in nature (Lemm et al., 2011 and reviewed in Belda and Targett-Adams, 2012). This enterprise resulted in the synthesis of a wide range of analogs designed to probe different aspects of the pharmacophore, optimize for broad genotype inhibition and build in properties commensurate with oral bioavailability (O'Boyle et al., 2005; Nettles et al., 2008; Gao et al., 2008, 2010; Bristol-Myers Squibb, 2004, 2007, 2008a, 2008b, 2008c; Gao et al.; Lemm et al., 2011; Romine et al., 2011). This compound estate provided molecules with a wide range of virological properties, several of which presented as useful tools with which to probe aspects of NS5A function and drug–target interactions. This report will focus on a small subset of compounds which were chosen for exploration as pharmacophore probes and for use as affinity reagents to characterize interactions with the HCV NS5A protein. The compounds are demonstrated to bind to NS5A with high affinity and specificity with evidence supporting binding *via* NS5A protein dimers. The use of compounds representing truncated versions of the larger, symmetrical inhibitor pharmacophore, characterized by daclatasvir, provides further evidence of direct and specific interactions occurring between the NS5A protein and this compound class. These compounds demonstrate an ability to separate dual-linked NS5A resistance changes (L31V and Y93H) into individual changes using substructures of the pharmacophores present within the symmetrical inhibitor chemotype. The results suggest that a specific interaction occurs between the NS5A protein and discrete structural elements of the individual compounds which, when united into a single, symmetrical molecule, can greatly enhance antiviral potency.

Results

Previous resistance mapping of HCV replication inhibitors discovered at Bristol-Myers-Squibb indicated that the compounds target the HCV NS5A protein as indicated by resistance substitutions at amino acid leucine 31 to valine (L31V) and, tyrosine 93 to histidine (Y93H) as well as others (Lemm et al., 2010; Gao et al., 2008, 2010). Medicinal chemistry efforts to optimize the potency and in vitro profile of the inhibitors generated multiple chemotypes, a subset of which have been used to test for direct interactions with the NS5A protein. The parent compound selected for modification, BMS-346 (EC_{50}~100 pM), is a potent inhibitor of HCV genotype 1b (gt1b) replication. The BMS-346 scaffold was modified to introduce affinity or cross-linking groups to determine if the modified compounds would: (i) retain inhibitory activity and (ii) be useful as probes for NS5A protein isolation. This compound and related stilbene-based NS5A inhibitors used in these studies are shown in Fig. 1 along with their HCV replicon inhibitory activities. The changes required to generate affinity probes reduced the activity by ~28–1600-fold but, because of the high inherent potency of the prototype molecule, still yielded active compounds with EC_{50} values ranging from 7 to 400 nM. As shown in Fig. 1, the BMS-346 scaffold was modified to introduce biotin,

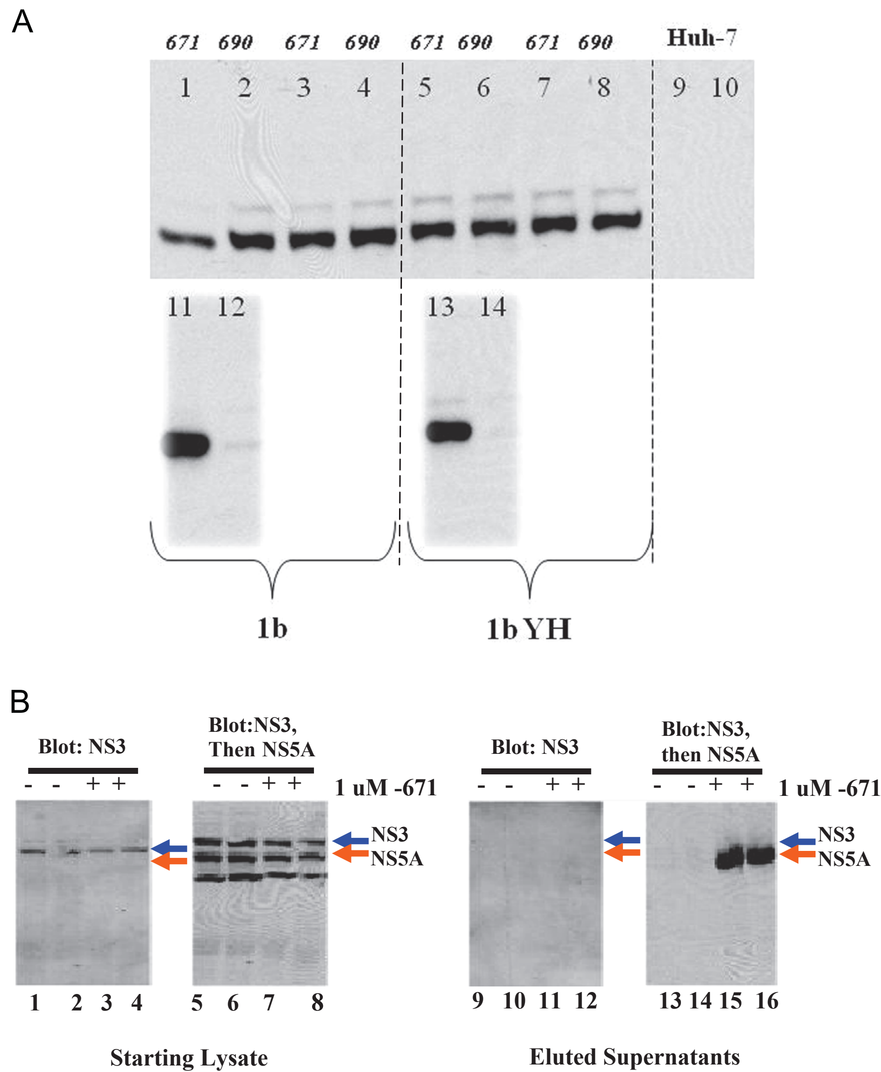

Fig. 2. (A) Detection of compound binding to NS5A protein in both wild-type and resistant cells. 1b cells or 1b NS5A Y93H cells exposed to either BMS-671 (active) or BMS-690 (inactive) compounds followed by streptavidin affinity purification and probing with anti-NS5A antibody suggest at least two modes of binding are possible: productive (inhibitory) or non-productive (non-inhibitory). Lanes 1 & 2+5 & 6 – Starting fractions, Lanes 3 & 4+7 & 8 – Not bound fractions, Lanes 11 & 12+13 & 14 – Bound Fractions. 671 or 690 above the lanes refers to the compound added to the cells. The bound fractions are the proteins that remained bound to the streptavidin beads following washing and were eluted using sample buffer at 100 °C. As seen, BMS-671 results in a similar enrichment compared to BMS-690 from both cell types. Huh-7 lysate was run as a control with only the eluted material shown for both 671 (lane 9) and 690 (lane 10). (B) Starting lysates and bound proteins following exposure of cells to DMSO (−Lanes) or BMS-671 compound (+Lanes) bind to and enrich for NS5A but not NS3. The panel on the left shows the composition of the starting lysate: Rows 1 thru 4 show blotting of the HCV replicon cell lysate samples and probing with anti-NS3 antibody; the same blot was then stripped and re-probed with anti-NS5A anti-body in lanes 5 thru 8. The arrows indicate the position where NS3 or NS5A would be expected to migrate through the gel. The proteins are present in both untreated and compound treated cells, with a slight reduction due to antiviral activity seen in the +columns, indicating BMS-671 added. The lower mw band in lanes 5–8 is due to NS5A proteolysis in the starting samples. Following binding, the samples were applied to streptavidin beads, followed by extensive washing then eluted with SDS–sample buffer with heating to 100 °C: Rows 9 thru 12 show blotting of the streptavidin beads samples and probing with anti-NS3 antibody; the same blot was then stripped and re-probed with anti-NS5A anti-body as indicated in lanes 13 thru 16.

either with or without an azide group cross-linking moiety. The biotin group was considered to be useful as an affinity tag for isolation of interacting proteins, while the cross-linking functionality was anticipated as useful for peptide isolation *via* covalent linkage following UV activation. The introduction of biotin to the stilbene chemotype yielded compound BMS-671 which had somewhat reduced replicon activity (EC_{50}~100 nM). A diastereomer of BMS-671, BMS-690 showed greatly reduced activity (>3 μM) when tested on HCV replicon cells. These two compounds are identical except for the absolute configuration at the proline moiety and this structure–activity relationship mirrors that observed with dimeric inhibitors of HCV NS5A. Preliminary experiments using these affinity probes (BMS-671 and BMS-690) and NS5A protein expressed in *E. coli* revealed no evidence of specific binding; nor did additional experiments with protein expressed in rabbit reticulocyte lysates (data not shown). These results are in agreement with other studies and suggest an absolute requirement for compounds to be preincubated with replicon cells in order to detect specific compound association with the NS5A protein (Gao et al., 2010; Targett-Adams et al., 2011; Lim et al., 2012). Experiments using BMS-671 and BMS-690 in HCV replicon cells determined previously that an interaction with NS5A could be detected (Gao et al., 2010; Targett-Adams et al., 2011). To extend and confirm these results, HCV replicon cells consisting of either wild-type gt1b (Con-1), a mutant containing the Y93H in NS5A or Huh-7 cells devoid of replicon were incubated for ~14 h at 37 °C with the affinity compound probes. Following treatment, the cells were washed with PBS, and NP-40 buffer was added to produce a cell lysate. Cell lysates (starting fractions) were incubated with streptavidin beads for 2 h at 4 °C. The beads were

pelleted and washed extensively with NP-40 buffer (not bound fractions), while proteins bound to the washed beads were eluted with SDS-PAGE buffer (100 °C). All samples were then fractionated on a 10% SDS-PAGE gel and the fractionated proteins probed with an NS5A antibody to reveal specific enrichment of NS5A for both wild-type and mutant Y93H NS5A (Fig. 2A). This pattern indicated that compound BMS-671 is capable of efficiently binding to and enriching HCV NS5A protein from both cell lines with an estimated ~25–50% efficiency, while the diastereomer BMS-690 was less efficiently pulled down. Furthermore, this result suggests that binding is necessary but not sufficient for inhibition and requires additional modifications for inhibitory competency. This is reminiscent of modifications required to impart gt1a activity using gt1b only inhibitors or to provide additional mutant coverage (Gao et al., 2011; St. Laurent et al., 2013). Additional experimental controls, including Huh-7 cells not expressing replicon gave no enrichment signal as seen in lanes 9 and 10 Fig. 2A; similarly the non-biotin-containing parent compound, BMS-346, also failed to enrich NS5A (data not shown).

To further evaluate the specificity of the affinity selection of NS5A, DMSO or BMS-671 was added to an equal number of replicon cells, and a western blot of starting lysates and lysates eluted from streptavidin beads was probed sequentially with NS3 followed by NS5A antibody. This sequential method ensures that identical lysates were evaluated for NS3 and NS5A interactions following affinity enrichment with the biotin arm of the compound. As shown in Fig. 2B, NS5A (lanes 15 and 16) was significantly enriched compared to either the bound fraction of NS3 protein (lanes 11 and 12) or the NS5A proteins present in the starting material (lanes 7 and 8). This enrichment pattern indicates that the active compound BMS-671 specifically interacts with NS5A protein and can remain associated with the biotin moiety during washing of the affinity matrix. The pattern for the inactive diastereomer BMS-690 was similar to the DMSO treated cells, with no significant binding to NS3, NS5A, or NS5B (data not shown).

Since the presence of the biotinylated inhibitor BMS-671 specifically enriched for NS5A, a strong association appeared to exist between the NS5A protein and this compound. However, it was possible that a reactive compound derivative, not readily detectable in this system, formed a covalent linkage with NS5A since specific binding could only be detected if the compound was incubated with replicon cells. Therefore, it was considered possible that incubation at 37 °C in the cellular milieu provided an "activation" step necessary for binding. Although preliminary experiments suggested that the observed inhibition was reversible (data not shown), additional experiments were conducted to determine if covalent linkage occurred. Since the interaction of biotin with streptavidin is one of the tightest non-covalent interactions known (Kd~10^{-15} mol/L), it was anticipated that if the binding of inhibitor to NS5A is high-affinity but non-covalent, it should be possible to identify conditions that leave biotin–streptavidin associated while releasing NS5A protein from the compound. To determine if NS5A binding to BMS-671 is non-covalent, lysates from HCV replicon cells exposed to BMS-671 were incubated with streptavidin beads, and washed beads were exposed to conditions that were contemplated to cause protein unfolding. Multiple conditions were tested for release of the NS5A protein from the biotin–streptavidin bead complex, with biotinylated flourescein used as a control to monitor the retention of biotin by the streptavidin beads. The release of NS5A from the bead-linked compound was monitored by western analysis with densitometry, while the control biotinylated-flourescein was monitored by fluorescence. Conditions tested included washes with: (1) Glycine–HCl, pH=3.5; (2) 8 M guanidine HCl; (3) 8 M urea; (4) 30% acetonitrile; (5) 2 M NaCl with 0.5% NP-40; (6) 5% SDS with 25% glycerol; (7) 5% β-mercapto ethanol; (8) 50 mM Tris–HCl, pH 3 and 10; or (9) SDS–sample buffer at 25 °C and 70 °C (Biorad #161-0737: 62.5 mM Tris–HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol Blue). The elution data indicated that SDS–sample buffer at 70 °C rather than 25 °C would efficiently release the NS5A protein while leaving the biotinylated flourescein bound to the streptavidin beads. The data is shown for 20 fractions (10 fractions at 70 °C, 10 fractions at 100 °C) in Fig. 3. The two measurements, one for flourescein and one for NS5A protein, are shown as bar graphs from individual fractions in Fig. 3. As shown, the NS5A protein mostly eluted in fractions 70-1 to 70-4 while the biotinylated flourescein remained firmly associated with the beads until heating to 100 °C (fraction 100-1). Quantification of the amount of NS5A retained on the beads versus amount of starting NS5A protein was estimated to be ~35%, suggesting that the stoichiometry for the NS5A-compound complex, even at saturating concentrations of 1 μM used in the experiments, reflected an abundance of non-compound-bound NS5A protein despite 100% replication inhibition. The small amount of NS5A protein detected in the 100 °C wash likely represents material trapped in the beads and was estimated to be $<4\%$ of the sample. These data strongly suggested that the NS5A protein is tightly associated with but not covalently bound to the compound.

Since the interaction between NS5A and BMS-671 appeared to be stable under acidic conditions and up to 5% SDS at room temperature, the samples were subjected to classical immunoprecipitation to determine if the interaction between the inhibitor and NS5A is direct or possibly through a known or unknown auxiliary protein(s) (Seng-Li, 2006; Tellinghuisen and Rice, 2002). HCV replicon cells were incubated with BMS-671 or DMSO for 16 h. Lysates from the treated cells were prepared in 2% SDS and immunoprecipitated with antibodies directed against either NS3 or NS5A. The antibody–antigen interaction was then disrupted with acidic conditions that were shown previously not to disrupt the NS5A/inhibitor interaction (condition #1 from elution studies) and the antibody-conjugated beads were removed by centrifugation, and the buffer neutralized. The neutralized supernatants, spiked with SDS-PAGE sample buffer (but not heated to retain bound compound (condition #9 from elution studies)), were

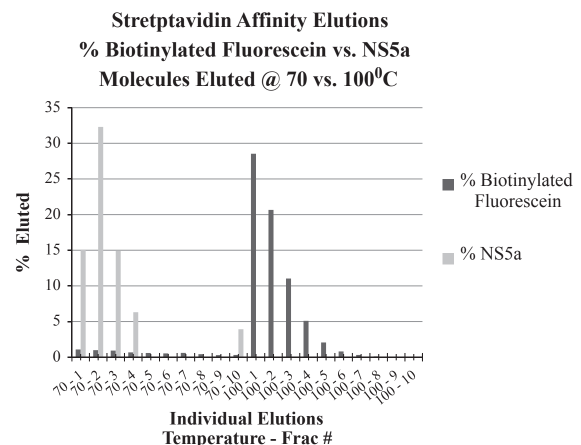

Fig. 3. NS5A replication complex inhibitors bind non-covalently with high-affinity to the NS5A protein as shown for the differential release of NS5A protein bound to biotinylated compound versus biotinylated flourescein following exposure of streptavidin beads to SDS. The first 10 fractions following exposure to SDS buffer at either 70 °C or 100 °C are shown (10 fractions eluted at 70 °C and 10 fractions from 100 °C are indicated). The relative amounts of NS5A present in each fraction was determined by densitometry scanning of a NS5a specific western (lighter bars) while Flourescein labeled biotin was monitored via fluorescence (darker bars) as described in materials and methods. A separation of NS5A protein from streptavidin tagged with flourescein is clearly shown indicating a release of protein from compound but not release of biotin from streptavidin.

fractionated by SDS-PAGE. Gels were loaded with duplicate alternating samples from cells treated with either DMSO or BMS-671, transferred to nitrocellulose membranes and then probed with either NS3 or NS5A antibody, as shown in Fig. 4. The bands corresponding to NS3 or NS5A were then marked and the membranes washed free of signal. The same membranes were then re-probed with biotin antibody. The data indicates that only the NS5A band co-migrates with a significant biotin signal seen in the compound treated lanes (Fig. 4). This co-migration of protein and biotin was not detected in samples from DMSO-treated cells or samples from inhibitor-treated cells immunoprecipitated with NS3 antibody. These data strongly suggest that the biotin containing compound co-migrates to this position due to an association with the NS5A protein, providing evidence for a direct and specific interaction between the inhibitor and the HCV NS5A protein.

The affinity selection and immunoprecipitation data suggested that there is a specific and direct high-affinity interaction between NS5A and the inhibitor BMS-671. To address the possibility that the binding interaction is potentiated by biotin, a related compound, lacking a biotin moiety, was used to test the interaction. BMS-194 (EC_{50}~7 nM) is a potent analog of BMS-671 which lacks biotin but contains an azide group that can be photo-activated by UV irradiation to afford a reactive nitrene (Fig. 1). A tritium radiolabel was incorporated into BMS-194 (~24,000 cpm/pmole) as a method for detection. HCV replicon cells were exposed to BMS-194, rinsed in cold PBS and exposed to UV light to enable activation of the azide cross-linker prior to preparation of cell lysates. NS5A and NS3 were immunoprecipitated from the lysates, fractionated by SDS-PAGE under conditions that were shown not to disrupt the protein–compound interaction, and transferred to nitrocellulose in duplicate for detection using either chemiluminescence (for protein) or autoradiography (for compound). As shown in Fig. 5, the arrows indicate the position of the protein bands corresponding to NS3 or NS5A which were marked on the membrane after chemiluminescence exposure for HCV protein detection. The area indicated by arrows show the positions where

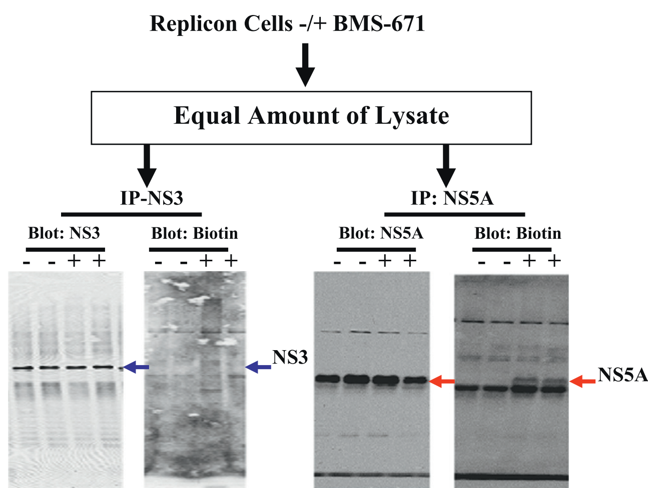

Fig. 4. Immuno-precipitation and detection of NS3 and NS5A followed by detection of biotin indicates that biotinylated compound is associated with NS5A protein but not NS3. The biotin containing active compound, BMS-671 (+ Lanes), or DMSO (− Lanes) was added to HCV replicon cells; soluble lysates made, divided in half and used for either NS3 or NS5A immunoprecipitation. The arrows indicate the position of the protein bands corresponding to NS3 or NS5A which were marked after chemiluminescence exposure of lanes cut from both sides of the membrane. Following HCV protein detection, the membranes were aligned and marked at areas corresponding to the HCV proteins and probed for biotin. Biotin-specific bands are seen (arrow) at the same position marked by comparison to the membranes at the NS5A position while the corresponding area for NS3 detects no significant signal for biotin at the position that NS3 was detected.

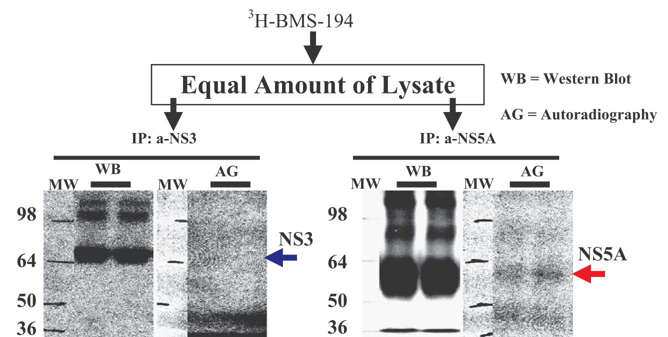

Fig. 5. Immuno-precipitation and detection of NS3 and NS5A by chemiluminescence and autoradiography using the radiolabeled NS5A inhibitor BMS-194 demonstrates compound NS5A binding is not dependent upon biotin. The arrows indicate where NS3 (left panel) or NS5A (right panel) proteins migrated following compound exposure as shown in the WB (western blot) lanes versus the AG (autoradiography) lanes.

NS5A or NS3 protein would be located. The radio-labeled compound, if present, would be detected by placement of a dried nitrocellulose membrane in direct contact with photographic film. As shown in Fig. 5, the autoradiogram detects no significant signal for radioactivity at the position that NS3 was detected but contains a weakly detectable signal at a position where NS5A migrates. This signal is not dependent upon IP efficiency since the amount of NS3 protein present on the membrane (~313 ng) versus NS5A (~20 ng) was determined to be ~16-fold greater by comparison to a western standard (data not shown). These data again suggest that the inhibitor interacts specifically with the NS5A protein and that the interaction is not dependent upon biotin.

As depicted in Fig. 1, BMS-350 incorporates both a biotin tag and a UV activateable azide moiety. Although BMS-350 has reduced potency and increased cytotoxicity in HCV replicon assays, specific inhibition was confirmed by the sensitivity of this compound to resistance mutations (data not shown). This compound was used to prepare and isolate an NS5A peptide to which the compound was presumed to interact with since the complex could be captured by photoactivation of the azide cross-linking moiety followed by dual affinity selection of first the FLAG epitope for NS5a protein followed by monomeric avidin to capture the biotin group on the compound. Huh-7 cells expressing a replication-competent FLAG-tagged NS5A replicon (Gao et al., 2004) were exposed to BMS-350 overnight followed by washing with PBS and exposure to UV light. Lysates were prepared and NS5A protein was enriched as described in the Methods section. Peptides released from the monomeric avidin beads were identified by mass spectroscopy to yield two overlapping peptide fragments consistent with amino acids 21–30 of NS5A (TWLQSKLLPR). The assignment, based on molecular weight and not amino acid sequence, identifies a putative binding region for BMS-350 in domain 1 of NS5A. These data are consistent with the affinity selection, providing additional evidence that the series of compounds shown in Fig. 1 interact directly with NS5A. The binding results are also consistent with previously published genetic studies that show NS5A inhibitors target the N-terminus of the protein (Lemm et al., 2010). Furthermore, the peptide cross-linked to inhibitor is adjacent to leucine 31; substitutions at L31 are associated directly with resistance to compounds in this series (Lemm et al., 2010; Gao et al., 2008). These data strongly suggest that the symmetrical compounds shown in Fig. 1 bind avidly, reversibly and specifically to the amino terminus of the NS5A protein.

NS5A has recently been demonstrated to form dimers in vivo (Lim et al., 2012; Bhattacharya et al., 2012) complementing the

initial dimeric crystal structure of NS5A Domain 1 revealed by Tellinghuisen and Love (Love et al., 2009; Tellinghuisen et al., 2005). It has been hypothesized that the symmetrical NS5A replication complex inhibitors would likely bind to a dimeric form of the NS5A protein such that a single compound would interact with 2 NS5A proteins across the dimer interface. To explore this possibility, a bis-azido analog of the mono-azide was synthesized in order to examine if the compound would be capable of covalently linking the NS5A protein dimers. The joining of 2 NS5A proteins would be detected as a multiple of the monomeric NS5A protein with the molecular weight shifted from 56 kDa ($N=1$) to ~112 kDa ($N=2$) by western immuno-analysis. To test this, the bis-azide along with DMSO and control compounds consisting of: BMS-346 (parental compound), BMS-350 (mono-azide), BMS-052 (daclatasvir, DCV) or a NS3 protease inhibitor asunaprevir (ASV) were added to replicon cells. As shown in the top of Fig. 6, lane 6, a band corresponding to a dimer ($N=2$) of NS5A is clearly present only in the bis-azide sample lane and is not detected in any other compound lane containing the crude-cell lysates. To exclude the formation of NS5A–NS5B and NS5A–NS3 heterodimers, antisera against either NS3 or NS5B was used on replicate blots that contained samples from either DMSO-treated cells or BMS-351-treated cells that were processed for enrichment via the FLAG epitope in the NS5A protein. As seen at the bottom of Fig. 6, additional higher molecular weight NS5A reactive bands are present in the bis-azide-treated samples following enrichment with FLAG beads but are not detected in the DMSO-treated cells. The NS3 and NS5B antisera can detect their respective HCV proteins but do not show any specific reactivity in the NS5A dimer region even following enrichment or longer exposure (data not shown). This result demonstrates that the compound can bind and crosslink NS5A from at least 2 separate NS5A proteins resulting in the detection of a dimeric NS5A species. The presence of higher molecular weight species detected with the NS5A antisera in both the crude cell lysate and enriched lysates may suggest additional multimer complexes are possible or that the protein is aggregating non-specifically. Further experiments are planned to address these possibilities.

The previous experiments demonstrated that a specific physical interaction between NS5A proteins and small molecule inhibitors could be detected in replicon cells. To further delineate the interaction between these inhibitors and HCV NS5A in replicon cells, a genetic approach utilizing resistance characterization was used to relate changes in inhibitor structure to the interaction with NS5A. To accomplish this, a select group of compounds on which

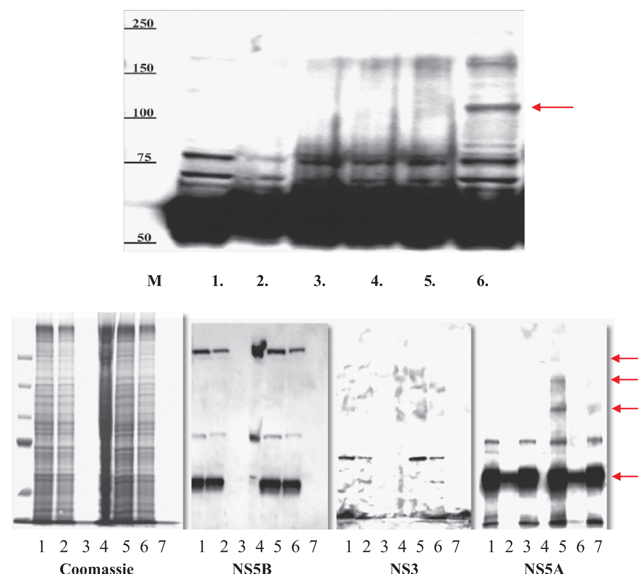

Fig. 6. TOP – NS5A specific western on total cell lysates from cells exposed to HCV inhibitors provide evidence that NS5A replication complex inhibitors interact with NS5A protein dimers and possibly higher-order multimers: (1) DMSO, (2) NS3 Inhibitor, (3) BMS-052 (Daclatasvir, DCV), (4) BMS-346 (Parent), (5) BMS-350 (Mono-azide), and (6) BMS-351 (Bis-azide). Lane M are markers with sizes (Kd) indicated on the gel. The band just above the 100 kDa marker in lane 6 is specific for compound BMS-351 only with the red arrow indicating the area expected for an NS5A dimer. Bottom – Comparison of coomassie stained gel of enriched gt1b FLAG extracts treated with either DMSO or BMS-351 to westerns for NS5B, NS3 or NS5A indicating that the affinity tag is accessible following cross-linking of NS5A dimers. Gels are replicates and consist of Markers (present only in 1st gel),then 7 lanes consisting of: (1) start – DMSO, (2) not bound-DMSO, (3) eluted –DMSO, (4) Huh-7 cells (non-replicon), (5) Start+351, (6) not bound+351, (7) eluted+351. Red arrows indicate approximate positions expected for NS5A of $N=1$–4.

Compound	Structure	WT EC_{50} / CC_{50}	EC_{50} LVYH	Fold-Resistance
BMS-411		0.042nM / 5.4uM	230nM	~5500 X
BMS-407 *"CORE"*		100nM / >10.0uM	>3.3uM	> 33 X
BMS-556 *"CAP"*		60nM / >10.0uM	>10uM	> 170 X
DCV		0.006nM / >10uM	237nM	~39,500 X

Fig. 7. Bi-benzyl compound series used for resistance selection with the EC_{50} and CC_{50} values listed on HCV replicon cells demonstrate the ability to retain inhibitory activity from replication complex inhibitor compound fragments. The fully symmetrical compound, BMS-411 is used for comparison to the less potent compounds listed underneath. BMS-407 represents the bi-benzyl series "core" region while BMS-556 was chosen to represent the "cap" region of the fully symmetrical series. The regions in common with BMS-411 are highlighted in blue for each compound. Note the loss in potency compared to BMS-411 for each compound. Resistance represents the fold-change seen with the compounds compared to non-selected replicon cells. EC_{50}=concentration of compound resulting in 50% HCV FRET activity when compared to DMSO control. CC_{50}=concentration of compound resulting in 50% reduction of Alamar blue conversion when compared to DMSO control.

Table 1
A. EC_{50} values of compounds tested on cells selected with either BMS-556, representing the cap region, or on BMS-407 representing the core region suggest distinct differences. The individual values indicate all selections resulted in resistance with the most resistance noted for the cap region and the least fold-resistant with the core region. The observed inhibitory activity for BMS-411 (symmetrical) suggests that the cap and core selected cells contain different mutations since the activities vary on these selected cells.

Compound type	Potency on resistant cells EC_{50} selected (μM)	Wild type potency EC_{50} WT (μM)	FOLD vs. WT
CAP selected (556)			
CAP (556)	23.7	0.04	655
Core (407)	2.6	0.15	18
Symmetrical (411)	2.43E−03	1.77E−05	136.9
Core selected (407)			
Core (407)	5.2	0.15	35.2
CAP (556)	1.6	0.04	44.8
Symmetrical (411)	7.05E−05	1.77E−05	~4.0

B. Sequencing results following RT-PCR and subsequent cloning of PCR products reveal different substitutions: V (6/6)=Amino acid seen (number of clones with mutation/total clones sequenced). The cap is shown to be more affected by Y93 changes while the core is more affected by L31 changes. The Q54 amino acid indicated 2 different mutations present as well 1 un-changed (wt) sequence.

Compound selection	L31	Q54	Y93
BMS-407 (core)	V (6/6)	R (3/6), L (2/6)	Not detected
BMS-556 (cap)	Not detected	Not detected	H (8/8)

BMS-790052 (daclatasvir, DCV) is based was selected for study. Fig. 7 shows the replicon activity and resistance profile of 3 NS5A inhibitors used for analysis on both wild-type and a double resistant cell line containing the linked substitutions at amino acids L31 and Y93 of NS5A. DCV is listed for comparison at the bottom of Fig. 7. Inspection of the potencies on either the wild-type or mutant cells indicates that BMS-411, like DCV, retains nanomolar activity toward the LVYH replicon cells. The less potent inhibitors (BMS-407 and 556), which incorporate pharmacophoric elements present in all of the compounds (highlighted in blue in Fig. 7), are inactive on the LVYH cells at the concentrations tested. These data indicate that the replicon inhibition observed is NS5A-specific for each compound, although the potency varies over 17,000-fold for these inhibitors.

The less potent compounds can be viewed as forming 2 classes of NS5A inhibitors with BMS-407 referred to as the CORE, derived from the biphenyl series on which DCV is based and BMS-556 referred to as the CAP representing a “half” of NS5A symmetrical inhibitors. These compound elements are active and independent pharmacophores present in the fully symmetrical compounds, with the covalent linkage in BMS-411 and related compounds such as DCV, cementing both elements together. To probe each pharmacophore's interaction with the NS5A protein, BMS-556 (cap) or BMS-407(core) were used to select resistance at ~5-X EC_{50} in the presence of G418. Following plating of wild-type replicon cells in the presence of inhibitors with G418, colonies of resistant cells emerged which were treated subsequently with increasing concentrations of the respective inhibitors, up to 10 μM. The population of selected cells was characterized for inhibitor sensitivity (Table 1, section A). The selected cells demonstrated resistance to the analogs used for selection as well as to the fully symmetrical inhibitor. The specificity of resistance toward NS5A was demonstrated by titration with NS3 and NS5B inhibitors, which retained similar potency on both the wild-type and selected cells (data not shown). As shown in Table 1 section A, replicon cells selected with the cap-containing molecule BMS-556 show greater resistance to the cap-selected cells while resistance is also detected for the other inhibitors. Replicon cells selected with the core pharmacophore-containing inhibitor show similar levels of resistance to the truncated inhibitors, suggesting there is a less well-defined binding interaction between the core compound and the NS5A protein. The potency data indicates that the resistance mutations selected by the core and cap analogs are distinct from each other, as revealed by the resistance levels of 137-fold for the cap selections versus ~4-fold for the core selected cells when tested versus the fully symmetrical compound. Sequencing of the 5A region for replicon clones revealed a difference in amino acid substitutions (Table 1, section B) that accounted for the resistance patterns. The core compound genotype results indicate a preference for interactions with both the L31 and the Q54 region. The cap compound resistance data suggests that this pharmacophoric element is interacting and affected more with the Y93 region of the NS5A protein under the conditions used for selections. This pattern is consistent with the hypothesis that fully symmetrical inhibitors make contact with the NS5A protein in close proximity to all of the amino acids L31, Q54 and Y93 within the NS5A binding region. DCV resistance on these substitutions have been reported to be similar (~20-fold) confirming the ability of both regions to be capable of interaction within compounds containing both elements (Fridell et al., 2010).

Discussion

The NS5A inhibitors described in this report were used in a series of experiments to probe and characterize the manner by which these compounds interact with the NS5A protein. The study of the binding and interactions of the inhibitors could only be efficiently accomplished by the application of these inhibitors to HCV replicon cells, since numerous attempts using purified NS5A proteins were unsuccessful at detecting binding with the same level of specificity. The use of HCV replicon cells to characterize compound binding provided biochemical data indicating that NS5A inhibitors are tightly and specifically bound non-covalently to the NS5A protein. The use of SDS and heating was required to preferentially remove the NS5A protein from a mono-biotinylated compound compared to a flourescein-streptavidin control emphasizing the high binding affinity possible with this compound series. The stability of the 5A-compound complex in the numerous elution conditions allowed additional manipulations and experiments to be performed probing the interactions of inhibitors with the 5A protein that would have been much more difficult to ascertain otherwise. The specificity of these inhibitors was

further confirmed by the successful cross-linking and isolation of a compound-peptide fragment corresponding to the amino-terminal region of the NS5A protein using intact HCV replicon cells. Complementing and extending the compound binding studies was the detection of an NS5A dimer using the bis-azide derivative. These results demonstrated that cross-linking of NS5A proteins is compatible with a stoichiometry of at least 1 compound to 2 NS5A proteins and is in agreement with other studies looking at the propensity of NS5A to form dimers in intact cells (Bhattacharya et al., 2012). Additional binding modes are possible, as reflected by the 2 crystal forms of NS5A (Love et al., 2009; Tellinghuisen et al., 2005) and likely given the potential for NS5A to be conformationally flexible to allow each domain of the protein to fulfill its multifaceted role. Based upon the available data from our group as well as others, direct binding across a dimer interface is the preferred hypothesized modality and the current model used for explaining the binding results with symmetrical compounds. The detection of higher molecular weight NS5A cross-linked multimers may suggest that additional compound–protein interactions are occurring, although the amount of monomeric 5 A species present indicates that, similar to the dimer, these are in a low minority. The bis-azide data suggests specific interactions since the application of cell-permeable chemical cross-linkers does not yield precise NS5A reactive bands indicative of discrete $N=2$, 3 etc. but a smear of reactivity indicating non-specific cross-linking is occurring (unpublished observations). The NS5B polymerase and the NS3 protease protein comparators used in several of the binding studies did not reveal interactions that were detected with NS5A in this compound series, even when present in excess, further confirming the specificities of the inhibitors for NS5A.

HCV replicon resistance selection used to complement the compound binding studies demonstrated the ability to separate mutations in the NS5A protein to areas between amino acids 30 and 93 while the resistant replicons remained sensitive to inhibitors targeting the NS3 protease or the NS5B polymerase. Further genetic evidence implicating NS5A domain1 protein interactions with NS5A replication complex inhibitors has been demonstrated for different HCV genotypes, confirming the specificity of these inhibitors for the NS5A protein (Lemm et al., 2010; Gao et al., 2008; Scheel et al., 2011). Additionally, resistance selection and characterization of resistance substitutions have been reported for analogs of the inhibitors used in this study including DCV (Lemm et al., 2010; Fridell et al., 2010) with similar results. Although the selection using less potent inhibitors readily generated separate areas of changes, the conditions used for selection as well as polymorphisms present in the replicon cell population may influence which mutations arise (Sun et al., 2012). The presence of low levels (~1%) of known 5A resistance mutations have been observed (unpublished observations) and may also influence mutation generation, especially with less potent inhibitors. The most potent compound characterized in this series was a symmetrical inhibitor similar to DCV. The stereochemistry of this compound and the interactions with the NS5A dimer are hypothesized to represent distinct inhibitory pharmacophoric elements designated as the cap and core moieties. The inherent inhibitory potency of the fully symmetrical compound allowed for significant antiviral activity to be retained by pharmacophore fragments resulting in inhibitory activity being readily discerned. Fragmentation of small molecules into more basic binding elements has been demonstrated as a useful method to screen for compounds that bind to targets of interest (Erlanson, 2012; Everts, 2008) and may be able to provide a starting point for additional NS5A compound optimizations. The use of genotype 1b cells likely assisted in this endeavor since BMS-671 can bind to not only the mutant Y93H gt1b cell line, as demonstrated in Fig. 1 but also to the gt1a NS5A protein but, like the Y93H mutant, is not inhibitory (unpublished observations). Thus, as stated previously, binding appears necessary but not sufficient for inhibition, requiring additional function(s) that further structural modifications can overcome, exemplified by those present in symmetrical and non-symmetrical higher molecular weight compounds (Fridell et al., 2010; Gao et al., 2011; Lawitz et al., 2012; Christopher O'Brien and Agresti, 2012). The ability of small molecules to bind specifically to NS5A with no detectable consequence has utility in combination with other NS5A targeting compounds and will be the focus of additional disclosure in the future (Bristol-Myers Squibb, 2012b; Gao et al., 2013).

The use of in vitro NS5A assays have recently reported negative results using a dimer interference assay with BMS-052 (DCV, Lim et al., 2012). The absence of demonstrable specific binding of the compounds to purified protein or crude cell extracts negated our ability for direct biochemical characterizations performed with defined components, such as RNA binding interactions, as demonstrated by Huang et al. and others (Huang et al., 2005; Lim et al., 2012). Disruptions of replication complexes in association with membranes as evidenced with immunostaining by DCV in intact cells have been reported, providing the first evidence of a mechanism of action related to compound addition to intact cells (Targett-Adams et al., 2011; Lee et al., 2011). This may suggest, and is reasonable with the model to be presented, that the compounds initially interact with a NS5A protein form that is only present transiently, requiring the compound to be present prior to translation/folding and with subsequent binding to an intermediate (s) prior to polyprotein processing. This is reminiscent of the requirement for an intact polyprotein consisting of NS3 to 5A for 5A hyperphosphorylation to occur (Neddermann et al., 1999) and is in agreement with studies performed by Fridell et al. who hypothesize that hyperphosphorylation is possibly attributable to unique conformations of NS5A (Fridell et al., 2011a). Other possibilities that might explain the lack of in vitro compound binding could be technical in nature, with the protein requiring cofactors or conditions that are not duplicated in our attempts to assess drug–target interactions. These possibilities remain under active investigation.

The initial thiazolidinones lead series of NS5A inhibitors were the basis for the synthesis of the compounds presented here (Romine et al., 2011). Using a multiplexed cell-based screen, the initial lead BMS-858 (Lemm et al., 2010) was used as a platform for further modification and SAR studies which resulted in the evolution of NS5A inhibitors to compounds described in this report and elsewhere (Belda and Targett-Adams, 2012). The use of the thiazolidinone lead compounds to generate resistant replicons has been shown to result in the appearance of mutations located at amino acids 31, 54 and 93 of the NS5A protein (Lemm et al., 2010), indicating the relatedness to the more sophisticated and potent symmetrical compounds. However, it has since been established that select thiazolidinones have the potential to undergo oxidation in DMSO solution and replicon media forming a reactive radical intermediate that can either react with molecular oxygen or dimerize to a more potent symmetrical compound (Lemm et al., 2010, 2011; Gao et al., 2009; Romine et al., 2011). The compounds presented in this report are devoid of the thiazolidinone ring and are known to be chemically stable. Therefore the resistance data generated by the cap and core components presented herein reflect the activity of the parent molecules.

The smaller pharmacophoric elements embedded in the larger symmetrical molecules can be inhibitory by themselves although at a significant cost in potency, suggesting the importance of uniting the elements into a single molecule for optimal inhibition of NS5A activity. The region of interaction corresponds to the area implicated in dimer formation of NS5A documented in crystal structures (Love et al., 2009; Tellinghuisen et al., 2005) and recently demonstrated in vitro *(*Lim et al., 2012*)*. A symmetrical molecule, or one that has the necessary functionality to complement the target, can be envisaged to interact with a region between the monomers, binding/interacting with a stoichiometry

of one inhibitor to one NS5A dimer or one compound per two NS5A monomers with at least 6 amino acids in the protein (~3 per dimer) and likely others forming an interaction network (Fridell et al., 2010). This suggests a possible mechanism by which the compounds modulate a function(s) of the dimer necessary for replication but which does not necessarily rely upon preventing protein association into a dimeric species; indeed, the inhibitors may promote association of NS5A into dimers (Huang et al., 2005). This scenario can be depicted as a model of compound bound to the NS5A protein dimer while the protein complex interacts with the lipid monolayer (Appel et al., 2005a, 2005b; Elazar et al., 2003). The hypothetical model presents one of the 2 solved NS5A domain 1 crystal dimers binding to BMS-411. The compound–protein structure containing more authentic NS5A amino acids (aa's 25–215, Tellinghuisen et al., 2005) was used but both dimeric forms could be biologically relevant with higher-order polymeric structures possible by combining the two conformations (Love et al., 2009). The model is captured in Fig. 8 from the perspective of looking upward from the membrane into the 5A protein and through to the NS5A putative RNA binding site. This model, which best agrees with the data, was developed using QUANTA (Quanta Modeling Environment, 2006) and the coordinates of the Domain 1 dimer from the Tellinghuisen et al. X-ray structure (Tellinghuisen et al., 2005) and the NMR structure of the amino terminal alpha helix by Penin et al. (2004). The model depicts the region(s) of interaction with the fully symmetrical compound with the amino acids implicated in compound resistance highlighted in yellow. The model shows BMS-411 bound across the dimer interface with the *CAPS* proximal to Y93 and the *CORE* located under L31. Q54 sets near the ends of BMS-411 and is in contact with L28 of the amino terminal alpha helices. The Q54 interaction with the N-terminal helix may assist in positioning L31 over the CORE. While this model is compatible with the observed potency differences between genotypes and the isolated resistance mutations, a more definitive understanding of the drug–target binding mode will require solution of NS5A or NS5A dimer with compound bound.

NS5A has been demonstrated to have essential functions in replication, viral nucleic acid encapsidation and host cell modulation,

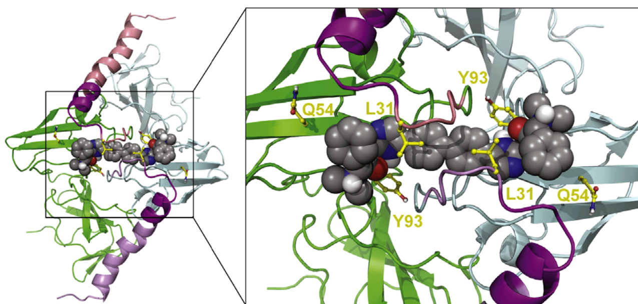

Fig. 8. Hypothetical model of BMS-411 interaction with NS5A protein dimer presents one possible NS5A replication complex inhibitor binding mode. The model was constructed using the NS5A Domain 1 crystal structure (1ZH1) and amino terminal alpha helical NMR structure (1R7C). The coordinates were incorporated into the model and the BMS-411 compound. The residues 32–35 were modeled in by hand to enforce symmetry using QUANTA (Quanta Modeling Environment, 2006). The smaller model depicts the region(s) of interaction with the fully symmetrical compound with the amino acids implicated in compound resistance highlighted with yellow carbon atoms (ball and stick). The larger model shows BMS-411 bound across the dimer interface with the *CAPS* proximal to Y93 amino acids and the *CORE* lying under the L31 residues. The Q54 residues set near the ends of BMS-411 and are in contact with L28 of the amino terminal alpha helices. The Q54 interaction with the N-terminal helix may assist in positioning L31 over the *CORE*. Colors in smaller model: NS5A domain I residues 36–198 are blue and green and respective N-terminal regions 1–20 and 31–35 are mauve and bisque; The photoaffinity labeled fragment, residues 21–30, are purple; the carbon atoms of BMS-411 are gray, all other atoms are oxygen: red, nitrogen: dark blue, and polar hydrogen: white. The image was generated using PYMOL (PyMOL).

all of which are important in the viral replication cycle (Scheel et al., 2011; Tellinghuisen et al., 2007, 2008b; Huang et al., 2007; Fridell et al., 2011b). Thus, the NS5A inhibitors may perturb multiple interactions, including membrane associations (Targett-Adams et al., 2011; Lee et al., 2011; Fridell et al., 2013) or egress from the cells (Guedj et al., 2013),whose sum reflects the high level of potency achieved with this chemotype. Compound binding to the NS5A dimer could cause disruption of the protein–lipid complex resulting in dominant and non-permissive replication. The model suggests that interactions may be transmitted *via* the lipid bilayer by modulation of the alpha-helical interactions that have been shown to be essential for NS5A function (Elazar et al., 2003). The recent description of the association of NS5A with TBC1D20 and Rab1 provides membrane-associated proteins whose functions may also be modulated by the NS5A compound series (Nevo-Yassaf et al., 2012). The abundance of cellular proteins interacting with the NS5A protein could also be attenuated by interaction with this class of inhibitor, providing opportunity for further study.

The NS5A protein has been estimated to be present at a concentration of ~70 nM in the HCV replicon cell line that was used for these studies (data not shown) and has been estimated by other groups to be present at 20,000–40,000 copies per replication complex (Quinkert et al., 2005). The ability to inhibit the replicon with picomolar amounts of compound suggests that an inhibitory mechanism is amplified in the cells since the ratio of protein to compound is estimated to be >1000 fold. This has been documented for other inhibitors such as cyclosporine that target a minority of cyclophilin proteins while exerting potent biological effects due to the protein–inhibitor complex being a dominant and active inhibitor of calcineurin (Halloran, 2001). This suggests that the NS5A protein, either as a dimer or higher polymeric form, may bind compound in a fashion that allows communication with other HCV replication centers in the cell, either directly or indirectly, to cause replication incompetency. Further work is required to more fully define the mechanism(s) by which these unique compounds function.

Precisely how the compounds inhibit HCV replication remains a complex question to answer but it is clear that the HCV NS5A protein represents a crucial link to many required viral functions and presents an excellent clinical target for small molecule antiviral intervention. The design of daclatasvir was guided, in part, by the results obtained with the compounds reported herein. Daclatasvir is currently undergoing Phase 3 clinical testing (Lok et al., 2012; Chayama et al., 2012; Sun et al., 2012), providing impetus for further mechanism of action studies. The complex interactions that occur between symmetrical inhibitors and the amino-terminal of NS5A generate specific and tight binding interactions that lead to potent inhibition of HCV replicon replication.

The characterizations and model presented here based on the compound classes described in this report provide a number of tools and observations to use in further defining a mechanism (s) amenable to future experimentation as well as possible application to other related viruses that contain an NS5A analog.

Experimental procedures

Cell culture and compounds

Huh-7 cells were grown in Dulbecco's modified Eagle medium (DMEM) with 100 U/ml penicillin/streptomycin and 10% fetal bovine serum (FBS). HCV replicon cell lines were isolated as previously described (Lemm et al., 2005, 2010; Krieger et al., 2001) and maintained in media that also contained 0.3–1.0 mg/ml Geneticin (G418). Huh-7 cells cured of a Con1 replicon were generated as previously described (Lemm et al., 2005) and propagated in DMEM with penicillin/streptomycin and 10% FBS.

Compound synthesis

The compounds used in this study were prepared at Bristol-Myers Squibb. Detailed synthesis of the compound class has been presented and will be the subject of future publications (Bristol-Myers Squibb, 2004, 2007, 2008a, 2008b, 2008c, 2012a; Gao et al., 2010; Romine et al., 2011; St. Laurent et al., 2012, 2013; Belda and Targett-Adams, 2012).

Affinity purification of NS5A protein

For use as affinity reagents, compounds (typically 6 μM, unless noted differently) were added to separate T-175 flasks of HCV gt1b (Con-1) or NS5A gt1b mutant (Y93H) replicon cells (~80% confluent, previously described (Fridell et al., 2010) and incubated under normal growth conditions for ~18 h. Cells were removed with EDTA-free Cell Dissociation Buffer (Invitrogen Corporation, Carlsbad, CA), centrifuged (4000*g*, room temperature) and resuspended in ice cold NP-40 lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, 1 × Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN)]. After 15 min at 4 °C, non-soluble cell particulates were removed by centrifugation (1500*g*, 10 min, 4 °C). A portion of the supernatant was saved as an input control for immunoblot analysis (see below) and the remainder was mixed with streptavidin-agarose beads (typically 30–100 μl, Sigma Corporation, St. Louis, MO) and incubated with gentle rocking at 4 °C. Beads were pelleted by brief centrifugation, washed 5 times (500 μl NP-40 lysis buffer), resuspended in SDS gel loading buffer, and heated for 3 min at 100 °C. (Heating and elution from affinity matrices was varied for some experiments as described in the text.) After a brief spin (14,000*g* 5 min) supernatants were electrophoresed on 7.5% Criterion gels (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's recommended procedure.

To compare the binding affinity of the 5A-protein/compound complex to the Streptavidin–Biotin interaction, biotinylated flourescein (Sigma #B9431) was used with detection of fluorescence in a Cytoflour 4000 instrument (Excite=485, Emission=530). Beads (streptavidin beads mixed with lysates or mixed with biotinylated flourescein) were washed extensively (~10 times) with binding buffer at room temperature prior to elution to reduce non-specifically bound materials. After the washes, SDS–sample buffer was added to an equal volume of beads (~100 μL) which were then incubated at temperatures of 70 °C for several elutions followed by 100 °C for several elutions (5 min each). Following incubation the beads/SDS–sample buffer was mixed, briefly centrifuged and supernatants removed for analysis. For immunoblot analysis, proteins were transferred to nitrocellulose (Towbin et al., 1979), blocked in 5% fat free milk in Tris buffered saline (Biorad#170-6435) and analyzed by probing with combinations of primary polyclonal rabbit antibodies directed at HCV NS5A, NS5B or NS3 (used/made/purified in-house) or anti-NS3 #ab18664 (monoclonal, Abcam), anti-NS5B #ab35586 (rabbit polyclonal, Abcam), rabbit anti-mouse HRP-conjugated secondary #ab6728-1 (Abcam), goat anti-rabbit HRP-conjugated secondary antibody (Biorad#170-6515) or with a biotin-HRP antibody (Sigma#BN-34). Antibody complexes were detected by chemiluminescence with a Western Lightning Chemiluminescence Reagent Plus kit (PerkinElmer, Waltham, MA) following manufacturer's instructions. Densitometry of NS5A specific bands was performed using exposed film and a Molecular Devices Storm detector (#860) with comparison to protein standards according to manufacturer's directions.

FLAG tagging of HCV replicon

A FLAG epitope tag was introduced into NS5A in the Con1 1b replicon clone with the Quick Change Mutagenesis kit (Stratagene Corporation, La Jolla, CA) following manufacturer's instructions. The mutagenic oligos were (+) 5′-ACTACCCGTCATGACTACAAG-GACGATGACGACA AAGAGGCCAACCT-3′ and (−) 5′-AGGTTGG-CCTGTTTGTCGTCATCGTCCTTGTA GTCATGACGGGTAGT-3′. The sequence of the modified clone was verified by sequence analysis. A replicon cell line containing a FLAG-tagged NS5A protein was selected as described above (O'Boyle et al., 2005; Lemm et al., 2010; Gao et al., 2004).

Selection of resistant replicons

Selection of resistant replicon cells was performed by growing HCV replicon cells in media containing compounds at increasing concentrations up to 10 μM final. Briefly, media containing compound (~5X – EC_{50}) was added to monolayers of HCV 1b-377-neo replicon cells at ~25% confluence in the presence of 0.5 mg/mL G418 in a T25 tissue culture flask. The cells were split 1:10 upon confluency; selection of cells with BMS-556 and BMS-407 was changed after passage 6 to 10 μM until passage 17. Replicon cells maintained in the presence of DMSO were used as a control. After 5–6 weeks, control DMSO-selected replicon cells and compound-selected cells were expanded for testing for compound sensitivity using the HCV replicon FRET assay and processed for RT-PCR and topo cloning.

RT-PCR and sequencing

Total RNA was isolated from gt1b (Con 1) HCV replicon cells using the Qiagen RNAeasy kit (#74104) as described by the manufacturer. Typically, a T25 flask at ~80% confluency was used for total RNA isolation. Total RNA was processed into cDNA using random hexamers followed by HCV specific forward primer (5′-CCAGGGGAG-GGGGCTGTGCAGTGGATG) and reverse primer (5′-CATTGATGGG-CAGCTTGGTTTCCTC). The resulting PCR product containing the NS5A region was processed using Qiagen PCR clean-up (#28104) kit. The DNA product band was gel purified and the DNA bands Topo cloned (pCR 2.1 TOPO vector, Invitrogen) according to manufacturer's directions. Individual DNA clones were picked, purified (Qiaprep Spin Miniprep kit #27106) and sent for automated fluorescence based sequencing. Sequencing data was processed and assembled using Sequencher (Gene Codes Corp., Ann Arbor, MI) DNA analysis program.

Fluorescent resonance energy transfer assay for HCV inhibitors (HCV FRET-assay)

The fluorescence resonance energy transfer assay (FRET) assay was performed as previously described (O'Boyle et al., 2005) for compound evaluations. Briefly, following titration of compounds and 72 h incubation in assay plates at 37 °C, the plates were washed with phosphate-buffered saline and then used for FRET assay by the addition of 30 μL of the FRET peptide assay reagent per well. The assay reagent consisted of 1 × -luciferase cell culture lysis buffer (Promega #E153A), 150 mM NaCl and 20 μM FRET peptide. The plate containing assay reagent was then read in a kinetic mode in a Cytoflour 4000 instrument which had been set to 340 nm excite/490 nm emission, automatic mode for 20 cycles. EC_{50} values were calculated as the concentration of compound which caused a 50% reduction in HCV FRET activity versus a DMSO control.

Crosslinking, Immunoprecipitations and peptide identification

To detect binding using the radiolabeled inhibitor, BMS-194, the protocol for use of BMS-671 as an affinity ligand was adapted with the following modifications: (i) HCV 1b replicon cells expressing FLAG-tagged NS5A were used. (ii) During the crosslinking step, cells were overlaid with 1.5 mL of cold PBS, and exposed to UV light in a UV 2400 Stratalinker (Stratagene Corporation, La

Jolla, CA) for 5 min. (iii) FLAG antibody-conjugated beads (#F2426, Sigma Corporation, St. Louis, MO) were incubated with the cell lysates for NS5A isolation, or immobilized protein-G beads (IP-50, Sigma Corporation, St. Louis, MO) pre-absorbed with HCV NS3 antibody were used for NS3 isolation. (iv) After detection of proteins, the membrane blot was sprayed with EN3HANCE (PerkinElmer, Waltham, MA) and exposed to Kodak BioMax MS film. A Kodak BioMax TranScreen was used according to manufacturer's instruction to improve the exposure efficiency.

An inhibitor containing both a biotin and a photo-reactive aryl azide moiety BMS-350 was used to determine the NS5A interaction site while a bis-azide homolog (BMS-351) was used to determine the ability to detect cross-linked NS5A dimers. The compound addition, UV crosslinking, and cell lysate preparation were carried out as described above for NS5A interaction site determination. To isolate NS5A at μg levels for NS5A interaction site determination, 2 mL of anti-FLAG M2 beads (Sigma Corporation, St. Louis, MO) were incubated with cell lysates collected from 98 culture dishes (175 cm^2), and the lysate bead mixture was loaded on a column (1 × 10 cm). After washing with 20 column volumes of wash buffer, the NS5A was eluted with 6 mL of elution buffer (100 mM Glycine [pH 2.0], 2 mM PMSF, 0.1% NP-40). NS5A-containing fractions were concentrated by centrifugation using an Amico Ultra-4 microconcentrator, and fractionated by SDS-PAGE. Proteins were stained with Coomassie Blue (BioRad Laboratories, Hercules, CA), and the NS5A band (identified by alignment to recombinant NS5A) was sliced from the gel. Trypsin digestion, accompanied by TCEP and IAA modification of cysteine residues, was performed with an In-Gel Tryptic Digestion Kit (Pierce, Rockford, IL) following the manufacturer's instructions. The digested peptide mixture was incubated with monomeric avidin agarose beads (#20227, Pierce, Rockford, IL) according to manufacturer's instructions to purify BMS-350-bound peptide fragment(s). The bound peptide fragments were subsequently eluted with biotin (1 mg/mL) and four fractions (25 μL each fraction) were collected. To increase the elution efficiency, beads were incubated in elution buffer for 20 min at room temperature prior to collecting each fraction. The eluted peptide fragment(s) were analyzed for mass by Matrix Assisted Laser Desorption Ionization–Mass Spectrometry (MALDI/MS). Prior to MALDI/MS analysis, samples (20 μL) were further purified using a P10 ZipTip (Millipore, Milford, MA), and mixed with an equal volume of the matrix α-cyano-4-hydroxycinnamic acid (10 mg/mL in 50% acetonitrile/0.1% TFA). MALDI/MS was performed on a vMALDI-LTQ mass spectrometer (Thermo-Fischer, San-Jose, CA) with the operating laser power at 30 arbitrary units. Data were acquired in profile mode across the 400–4000 m/z scan range. All solvents used were HPLC grade or higher.

To investigate the potential of NS5A to form dimers, BMS-351 or control compounds were added to 10 confluent T-175 flasks at 6 μM final and allowed to incubate for ~18 h. The cells were then washed with cold PBS and the cells collected after exposure to cell dissociation buffer (Gibco #13151-014) pelleted and resuspended in PBS with protease inhibitors (Roche Applied Science, Indianapolis, IN). The resuspended cells (5 mL in cold PBS) were placed onto a 100 mm dish and cross-linked as described above using the Stratalinker. The cells were then pelleted and resuspended in 4 mL of cold NP-40 lysis buffer with 0.1% Deoxycholate (NP-40-deoxy) for 10 min, spun at 3000 rpm for 10 min, and the supernatants collected for either additional FLAG enrichment or used for SDS-PAGE with coomassie staining and western analysis. FLAG enrichment relied upon batch binding to M2 beads followed by extensive washing with NP-40-deoxy buffer with Protease Inhibitors, with a final wash in PBS followed by elution with 3X-FLAG peptide (0.15 mg/mL, Sigma#F4799) in PBS.

Acknowledgments

We thank Xin Huang and Bernadette Kunzle for DNA sequencing, Susan Roberts for critical reading of the manuscript and Mark Cockett for continued support.

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