The effects of NS5A inhibitors on NS5A phosphorylation, polyprotein processing and localization

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Hepatitis C virus (HCV) non-structural protein 5A (NS5A) is a multi-functional protein that is expressed in basally phosphorylated (p56) and in hyperphosphorylated (p58) forms. NS5A phosphorylation has been implicated in regulating multiple aspects of HCV replication. We recently reported the identification of a class of compounds that potently inhibit HCV RNA replication by targeting NS5A. Although the precise mechanism of inhibition of these compounds is not well understood, one activity that has been described is their ability to block expression of the hyperphosphorylated form of NS5A. Here, we report that an NS5A inhibitor impaired hyperphosphorylation without affecting basal phosphorylation at the C-terminal region of NS5A. This inhibitor activity did not require NS5A domains II and III and was distinct from that of a cellular kinase inhibitor that also blocked NS5A hyperphosphorylation, results that are consistent with an inhibitor-binding site within the N-terminal region of NS5A. In addition, we observed that an NS5A inhibitor promoted the accumulation of an HCV polyprotein intermediate, suggesting that inhibitor binding to NS5A may occur prior to the completion of polyprotein processing. Finally, we observed that NS5A p56 and p58 separated into different membrane fractions during discontinuous sucrose gradient centrifugation, consistent with these NS5A phosphoforms performing distinct replication functions. The p58 localization pattern was disrupted by an NS5A inhibitor. Collectively, our results suggest that NS5A inhibitors probably impact several aspects of HCV expression and regulation. These findings may help to explain the exceptional potency of this class of HCV replication complex inhibitors.

INTRODUCTION

The ~9.6 kb hepatitis C virus (HCV) genome encodes a single polyprotein that is processed by cellular and viral proteases into structural proteins (core, E1 and E2), an ion channel protein (p7), and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (Poenisch & Bartenschlager, 2010). The non-structural proteins, excluding NS2, are necessary and sufficient for RNA replication in cell culture (Lohmann et al., 1999). NS5B is an RNA-dependent RNA polymerase and NS3 functions as both an RNA helicase and a serine protease. NS4A is an NS3 cofactor and the NS3–NS4A complex is responsible for cleaving the NS3–NS5B polyprotein into mature NS proteins. NS4B induces an endoplasmic reticulum (ER)-derived membrane web, the likely site of HCV replication (Egger et al., 2002). NS5A is essential for HCV RNA replication but its roles in this process are not well understood.

The ~447 aa NS5A protein interacts with other HCV NS replication proteins as well as with a wide variety of host proteins (Macdonald & Harris, 2004). NS5A has three distinct structural domains and an N-terminal amphipathic α-helix that functions in membrane localization (Tellingshuizen et al., 2004). NS5A domain I (aa 28–213) coordinates a single zinc atom, binds RNA in vitro and is essential for RNA replication (Huang et al., 2005;
In this report, we show that this class of inhibitors disrupts NS5A hyperphosphorylation without affecting basal phosphorylation, and we demonstrate that this activity is mediated by sequences within NS5A domain I. We also show that NS5A p56 and p58 segregate into distinct membrane fractions, and that an NS5A inhibitor alters this fractionation pattern. Finally, we show that an NS5A inhibitor promotes accumulation of an NS4B–NS5A precursor protein, suggesting that inhibitors may bind to NS5A prior to polyprotein processing.

RESULTS

NS5A inhibitors impair hyperphosphorylation without affecting basal phosphorylation within the C-terminal region of NS5A

The structures of three NS5A inhibitors (BMS-058, BMS-506 and BMS-790052) that are used in the current study are shown in Fig. 1. These compounds are all potent inhibitors of Con1 replicon replication and the effect of each is attenuated by the NS5A Y93H-resistance mutation (Table 1), suggesting that they probably inhibit HCV replication by a common mechanism. The mode of action of these inhibitors is not well understood, but one activity that has been ascribed to inhibitors of this class is the ability to block NS5A hyperphosphorylation (Lemm et al., 2010). To determine if BMS-058, BMS-506 and BMS-790052 also possess this activity, Con1 replicon plasmids were expressed in cell culture with a vaccinia virus-T7 expression system (MVA-T7) and NS5A was detected by Western analysis. As shown in Fig. 2(a), treatment with BMS-058 resulted in a dose-dependent decrease in the ratio of p58 to p56 with suppression of p58 expression evident at 0.8–20 pM of inhibitor (Fig. 2a). BMS-058 inhibited Con1 replicon replication with a mean EC50 of 2.8 pM (Table 1),

Table 1. EC50 values (nM) of inhibitors on Con1 subgenomic replicons

<table>
<thead>
<tr>
<th></th>
<th>BMS-058</th>
<th>BMS-506</th>
<th>BMS-790052</th>
<th>SB-220025</th>
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<tr>
<td>Wild-type</td>
<td>0.0028 ± 0.0019</td>
<td>0.0044 ± 0.0012</td>
<td>0.0024 ± 0.0009</td>
<td>48 ± 22</td>
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<tr>
<td>Y93H</td>
<td>0.67 ± 0.17</td>
<td>0.052 ± 0.020</td>
<td>0.037 ± 0.009</td>
<td>53 ± 44</td>
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Half-maximal effective concentration values are the mean ± SD from three or more transient replication assays. No cytotoxicity was observed at 1 μM. Replicons contain an S2204I REM.

We recently reported the identification of highly potent HCV replication complex inhibitors that target NS5A and are active both in vitro and in vivo (Gao et al., 2010). Mutations that confer resistance to these inhibitors map to the N-terminal region of NS5A (Fridell et al., 2010; Lemm et al., 2010). Elucidating the mechanism of action of these inhibitors will potentially increase our understanding of the essential functions of NS5A in the HCV life cycle. In this report, we show that this class of inhibitors disrupts NS5A hyperphosphorylation without affecting basal phosphorylation, and we demonstrate that this activity is mediated by sequences within NS5A domain I. We also show that NS5A p56 and p58 segregate into distinct membrane fractions, and that an NS5A inhibitor alters this fractionation pattern. Finally, we show that an NS5A inhibitor promotes accumulation of an NS4B–NS5A precursor protein, suggesting that inhibitors may bind to NS5A prior to polyprotein processing.
indicating that the effects of the inhibitor on hyperphosphorylation and replication occurred within similar concentration ranges. BMS-790052 and BMS-506 also inhibited p58 expression in the MVA-T7 assay (Fig. 2b). Furthermore, hyperphosphorylation was suppressed whether replicons expressed large amounts of p58 (no REM or an A2199S REM) or very little p58 (S2204I REM), indicating that the inhibitor activity was not appreciably affected by cell culture adaptive mutations. To determine if NS5A inhibitors also suppress basal phosphorylation, extracts were treated with \( \lambda \)-phosphatase prior to SDS-PAGE and Western analysis. Phosphatase treatment alters the gel migration of both p56 and p58, producing a single species that migrates slightly faster than p56 (Appel et al., 2005). As shown in Fig. 2(c), BMS-506 impaired p58 expression, but phosphatase treatment further increased the migration of NS5A in the gel (Fig. 2c, lanes 2 and 4), indicating that the inhibitor-treated NS5A was still basally phosphorylated. A residual amount of slower migrating NS5A that was visible on the Western blot was probably due to incomplete dephosphorylation (Fig. 2c, lanes 2 and 4). Deletion of aa 408–437 near the C-terminal region of NS5A has been shown to eliminate or greatly reduce NS5A basal phosphorylation (Huang et al., 2005). We observed that a replicon harbouring this deletion expressed two forms of NS5A, indicating that hyperphosphorylation of NS5A still occurred (Fig. 2c, lane 5). However, with this replicon, treatment with BMS-506 was indistinguishable from phosphatase treatment: the amount of the slower migrating NS5A species (corresponding to p58) was greatly reduced, but the faster migrating species was not affected (Fig. 2c, lanes 5–8), consistent with this species being deficient for basal phosphorylation. Collectively, these results suggest that the effect of NS5A inhibitors is specific for hyperphosphorylation, without affecting or requiring basal phosphorylation at the C-terminal region of NS5A.

**SB-220025 blocks NS5A hyperphosphorylation**

Kinase inhibitors that block NS5A hyperphosphorylation and inhibit HCV replicon replication by targeting CKI-\( \alpha \) have been described previously (Quintavalle et al., 2006). In the course of our investigations, we identified a commercially available inhibitor, SB-220025, that shares many of the properties of the CKI-\( \alpha \) inhibitors. SB-220025 effectively blocked NS5A hyperphosphorylation (Fig. 3a) and inhibited replication of an adapted Con1 replicon (EC\(_{50}\)=48 nM, Table 1). The potency of SB-220025, however, was not affected by the NS5A Y93H mutation (Table 1). While SB-220025 is marketed as a p38 mitogen-activated protein kinase (MAPK) inhibitor, a related MAPK inhibitor (SB-203580) did not block NS5A hyperphosphorylation (Fig. 3a). Quintavalle et al. (2006) demonstrated that the CKI-\( \alpha \) inhibitors that they identified inhibited the in vitro kinase activity of CKI-\( \delta \), a closely related CKI isoform. SB-220025 also potently inhibited the catalytic domain of CKI-\( \delta \) (Fig. 3b, c). In contrast, BMS-506 at a concentration of 1 \( \mu M \) had no effect on CKI-\( \delta \) activity (Fig. 3b), and neither SB-220025 nor the NS5A inhibitor substantially inhibited CKII in vitro (data not shown).

**Mapping sequences that mediate NS5A inhibitor activity**

The ability of NS5A inhibitors to block NS5A hyperphosphorylation provided a convenient assay to identify regions of the HCV polyprotein required for inhibitor activity. As shown in Fig. 4(a) (lanes 1 and 2), hyperphosphorylation of NS5A was suppressed by BMS-058 when NS5A was expressed as part of an NS3–NS5A polyprotein without NS5B. In fact, deletion of the entire region of NS5A downstream of residue 2209 (NS5A residue 237) did not eliminate hyperphosphorylation or inhibitor activity (Fig. 4a, lanes 3 and 4). For detection purposes, C-terminally truncated NS5A proteins were tagged with a V5 epitope. To confirm that hyperphosphorylation of V5-tagged NS5A was still dependent on the conserved serines in the central region of the protein, alanine was substituted for serine at four of these residues (2197, 2201, 2204 and 2207). These mutations resulted in expression of NS5A as a single species that was unaffected by the NS5A inhibitor (Fig. 4a, lanes 5 and 6). Similarly, deletion of sequences encoding the central serine residues also negated NS5A
hyperphosphorylation and inhibitor activity (Fig. 4a, lanes 7 and 8). These results indicate that the first 237 aa of NS5A, when expressed as part of an NS3–NS5A polyprotein, are sufficient to mediate the activity of NS5A inhibitors.
Hyperphosphorylation of NS5A from genotype 1b strains generally requires coexpression with other HCV NS proteins (Koch & Bartenschlager, 1999; Neddermann et al., 1999; Tanji et al., 1995). Asabe et al. (1997), however, demonstrated that NS5A from the HCV-J strain was hyperphosphorylated as a stand-alone polypeptide if 127–157 aa were deleted from the N terminus of the protein. We observed a similar result with Con1 NS5A (Fig. 4b). Full-length NS5A and NS5A with a deletion of the N-terminal 100 aa were each expressed as a single polypeptide in the MVA-T7 system, indicating that they were not hyperphosphorylated (Fig. 4b, lanes 1–4). However, when the N-terminal 157 aa of NS5A were deleted, two NS5A species were observed (Fig. 4b, lanes 5 and 6). SB-220025 blocked expression of the slower migrating species, consistent with this species corresponding to hyperphosphorylated NS5A (Fig. 4c, lane 3). In contrast, the NS5A inhibitor did not noticeably reduce hyperphosphorylation of the truncated NS5A polypeptide (Fig. 4b, lanes 5 and 6 and Fig. 4c, lanes 1 and 2), suggesting that the inhibitor did not bind to this NS5A species.

**BMS-790052 alters the membrane fractionation pattern of NS5A**

To examine the association of NS5A with intracellular membranes, lysates from replicon cells were fractionated by centrifugation on discontinuous sucrose gradients that were designed to separate ER and Golgi intracellular membranes (Bole et al., 1986; Choi et al., 2004). Cells harbouring a replicon with an R2884G REM in NS5B were chosen for these experiments because this replicon encodes wild-type NS5A and therefore abundantly expresses both p56 and p58 (Lohmann et al., 2001). After centrifugation, fractions were collected from the top to the bottom of the sucrose gradient and analysed by Western analysis (Fig. 5a). Golgin-97 was used as a marker for trans-Golgi membranes (Barr, 1999) and calnexin was used as a marker for ER membranes (Bergeron et al., 1994). These proteins were predominantly found in fractions 2 and 6, respectively (Fig. 5a). NS5A was observed in several fractions, but was most abundant in fractions 1, 3 and 6. Most of the NS5A protein present in fraction 6 was in the form of p56. In contrast, NS5A p58 was mostly located in fractions 1–3. NS3, NS5B and Rab5, an early endosome protein that co-localizes with the HCV replication complex (Stone et al., 2007), were all predominantly observed in fraction 6, suggesting that the majority of the HCV replication complex was concentrated in this fraction. To examine the potential effect of an NS5A inhibitor on the distribution of NS5A in the sucrose gradient, replicon cells were treated with BMS-790052 for 3 h prior to analysis. Inhibitor treatment reduced expression of NS5A p58 and concomitantly reduced the overall amount of NS5A in fractions 1–3 (Fig. 5b). Re-probing of the blots with a GRP78 antibody confirmed that the effect of the inhibitor was not due to differences in gel loading (Fig. 5b).

In the absence of BMS-790052, a substantial amount of NS5A p58 was detected in fraction 1 of the sucrose gradient (Fig. 5a, b). This fraction includes the input lysate and is predicted to contain non-membrane-associated cytosolic proteins and vesicle-associated proteins (Bole et al., 1986). A membrane-flotation assay similar to one described by Shi et al. (2003) was, therefore, used to determine if p58 was membrane associated. In this assay, cell lysates are layered...
beneath a sucrose gradient and during centrifugation intracellular membranes migrate into the gradient, while non-membrane-associated proteins remain in the input layer. As shown in Fig. 5(c), NS5A p56 and p58 both migrated to the upper layers of the gradient, suggesting that both isoforms were membrane associated. Some separation of p58 and p56 was observed in this assay, with a portion of p58 separating into a more buoyant fraction (Fig. 5c). Treatment of cell lysates with 1% Nonidet P-40 (NP-40) prior to centrifugation resulted in a shift of much of the NS5A to the non-membrane-associated fractions, although a proportion of NS5A remained in a detergent-resistant membrane fraction as described previously (Shi et al., 2003).

BMS-790052 promotes accumulation of the NS4B–NS5A precursor

While examining the effect of inhibitors on NS5A hyperphosphorylation, we observed that NS5A inhibitors also enhanced the accumulation of an NS5A antibody-reactive polypeptide of ~82 kDa. Examples of this activity with BMS-790052 are shown in Fig. 6(a), but similar results were also obtained with a related NS5A inhibitor (data not shown). For these experiments, Huh-7 cells were transfected with replicon RNA and NS5A was detected by Western analysis. An increase in the amount of the ~82 kDa protein was observed in BMS-790052-treated cells compared with DMSO-treated control cells with the accumulating polypeptide evident at BMS-790052 concentrations as low as 5–100 pM (Fig. 6a, lanes 2, 7, 9 and 10). A polypeptide migrating at a similar position in the gel was also detected in cells treated with an HCV NS3 protease inhibitor (Fig. 6a, lane 3), but was not observed in mock-transfected cells (lanes 4 and 5), suggesting that the accumulating polypeptide might be the NS4B–NS5A precursor (~85 kDa). To test this possibility, a cysteine to threonine substitution (C1972T) was introduced at the P1 position of the protease cleavage site at the NS4B–NS5A junction. This mutation reduces cleavage efficiency and results in the accumulation of both mature NS5A and NS4B–NS5A precursor (Yu et al., 2006). We reasoned that if the NS5A inhibitor also reduced cleavage efficiency between NS4B and NS5A, the effect would be more pronounced if the rate of cleavage was already compromised due to the mutation at the P1 position. In fact, this is what we observed. In the absence of inhibitor, the C1972T substitution resulted in an increase in the ratio of NS4B–NS5A precursor relative to mature NS5A (Fig. 6b, lane 3). Addition of BMS-790052 further increased the ratio of NS4B–NS5A precursor to mature NS5A, such that, in this case, very little mature NS5A was detected (Fig. 6b, lanes 4 and 6). The L31V and Y93H mutations that attenuate NS4B–NS5A cleavage (Fig. 6c), suggesting a correlation between these inhibitor activities.

**Fig. 6.** Accumulation of NS4B–NS5A precursor in inhibitor-treated cells. (a) In vitro-transcribed Con1 replicon RNA (no REMs) was transfected into Huh-7 cells that were treated with the indicated inhibitors. After ~16 h, NS5A expression was analysed by Western analysis. Lanes 1–5, 6–7 and 8–10 are from independent experiments. The positions of NS5A p56, p58 and a higher molecular mass polypeptide accumulating in inhibitor-treated cells are indicated with arrows. The approximate positions of molecular mass markers are indicated. (b) RNA transcribed from the indicated replicons was transfected into Huh-7 cells that were treated with DMSO (−) or 500 nM BMS-790052 (+). After 16 h, NS5A expression was analysed by Western analysis. C1972 indicates a Con1 replicon with a wild-type NS4B–NS5A cleavage site and no REMs. The C1972T replicon has a cysteine to threonine mutation at the P1 position of the NS4B–NS5A cleavage site. The C1972T-SI replicon also has an S2204I REM. (c) RNA from the C1972T-SI replicon with (LVYH=L31V+Y93H) and without (WT) substitutions conferring resistance to NS5A inhibitors was transfected into Huh-7 cells in the presence (+) or absence (−) of 1 nM BMS-790052. NS5A was detected by Western analysis.

**DISCUSSION**

Potent HCV replication inhibitors that are characterized by resistance mutations mapping within the N-terminal region of NS5A have recently been described (Fridell et al., 2010; Gao et al., 2010; Lemm et al., 2010). One activity attributed to these inhibitors is the ability to block NS5A hyperphosphorylation (Lemm et al., 2010). In this study, we showed that an NS5A inhibitor of this class impaired hyperphosphorylation without affecting the bulk of basal phosphorylation that occurs within the C-terminal region of NS5A. Moreover, we demonstrated that NS5A domains
II and III were not required for hyperphosphorylation or for the effect of inhibitors on hyperphosphorylation. While these results suggest that NS5A inhibitors specifically block NS5A hyperphosphorylation, our data do not exclude the possibility that basal phosphorylation at sites other than those within the C-terminal region of NS5A are also affected.

Hyperphosphorylation of Con1 NS5A is generally only observed if NS5A is expressed as part of a contiguous NS3–NS5A polyprotein (Koch & Bartenschlager, 1999; Neddermann et al., 1999). We found, however, that deleting the N-terminal 157 aa from Con1 NS5A enabled hyperphosphorylation even when the polypeptide was expressed in the absence of other HCV proteins. This result is very similar to results previously reported for NS5A derived from the HCV-J strain (Asabe et al., 1997). Hyperphosphorylation of the truncated Con1 NS5A polypeptide was not blocked by an NS5A inhibitor, but it was blocked by a cellular kinase inhibitor, SB-220025. SB-220025 also reduced hyperphosphorylation of replicon-expressed NS5A, and effectively inhibited replication of a replicon with an S2204I REM. The potency of SB-220025 was not affected by Y93H, a mutation which confers resistance to NS5A inhibitors. Overall, the properties of SB-220025 appear to be very similar to those of previously described inhibitors that target CKI-α, a kinase that plays a role in NS5A hyperphosphorylation and is required for HCV replication in cell culture (Quintavalle et al., 2006). At this time, however, we cannot be sure that the effects that we observed for SB-220025 on the HCV replicon were due to inhibition of CKI-α.

Collectively, our findings suggest that NS5A inhibitors prevent phosphorylation at one or more serine residues in the central region of NS5A by binding to the N-terminal region of the protein. In contrast, SB-220025 most probably directly inhibits the catalytic activity of a cellular kinase that is required for hyperphosphorylation. A role for the NS5A N-terminal region in regulating NS5A hyperphosphorylation was previously proposed by Asabe et al. (1997) who suggested that the binding of NS4A to the N-terminal region of NS5A induces a conformational change in NS5A that exposes the centrally located serine residues to a cellular kinase. In a similar vein, the binding of an inhibitor to the N-terminal region of NS5A could promote a protein conformation that shields the centrally located serine residues from phosphorylation.

Although several lines of evidence suggest that NS5A hyperphosphorylation plays an important role in controlling aspects of HCV replication (Huang et al., 2007), the actual physiological implications of blocking NS5A hyperphosphorylation remain unclear. In one hypothesis, NS5A hyperphosphorylation functions as a molecular switch, regulating a transition from replication to assembly (Evans et al., 2004). This hypothesis is supported by the finding that REMs that reduce the level of NS5A p58 expression also facilitate binding of NS5A to VAP-A, an intracellular vesicle trafficking protein that is important for HCV replication (Evans et al., 2004). According to this model, basally phosphorylated NS5A, p56, binds to VAP-A and performs a replication function, while hyperphosphorylated NS5A, p58, dissociates from VAP-A and triggers a switch to virion assembly. There is also evidence, however, that p58 itself may perform an essential role in RNA replication. For example, while mutations to individual serine residues implicated in NS5A hyperphosphorylation (S2197A, S2201A, S2204A and S2207A) yield a replication-enhancing phenotype, combining two or more of these substitutions completely blocks replication (Appel et al., 2005). Likewise, cellular kinase inhibitors that impair NS5A hyperphosphorylation also inhibit replication of adapted replicons (Table 1) (Neddermann et al., 2004). We recently reported genetic evidence suggesting that BMS-790052 blocks a cis-acting replication function of NS5A that is independent of hyperphosphorylation (Fridell et al., 2011). These same studies, however, also suggested the possibility that a hyperphosphorylated form of NS5A might provide a trans-acting function that is also required for RNA replication. The different subcellular fractionation patterns of p56 and p58 that we observed in this study support the hypothesis that these NS5A phosphoforms perform distinct functions. The majority of NS5A that co-localized with NS3 and NS5B in an ER-membrane containing fraction was p56, consistent with this NS5A phosphoform functioning as a component of the HCV RNA replication complex. In contrast p58, which also appeared to be membrane-associated, was mostly located in more buoyant fractions that were enriched for the trans-Golgi marker, golgin-97. Treatment with an NS5A inhibitor reduced the amount of p58 that was present in the more buoyant fractions. Whether or not this effect on localization was a direct consequence of the inhibitor blocking hyperphosphorylation is currently unclear. A trivial explanation for these results could be that NS5A that is dissociated from the replication complex is non-functional and it is this fraction of NS5A that is accessible to kinases that lead to hyperphosphorylation. Alternatively, hyperphosphorylation, or a conformational change associated with hyperphosphorylation, could provide a fraction of NS5A that fulfils distinct roles in RNA replication (e.g. modulating the cellular environment) and/or downstream processes (e.g. delivering viral RNA to the site of virion assembly). If the latter case, NS5A inhibitors would probably block these additional NS5A functions as a consequence of impairing hyperphosphorylation.

In a recently published report, BMS-790052 and a related NS5A inhibitor were each found to promote a redistribution of NS5A in replicon cells from the ER to lipid droplets (Targett-Adams et al., 2011). In another study, in which replicon-encoded NS5A was expressed using a vaccinia virus expression system, treatment with BMS-790052 shifted the localization pattern of NS5A from a punctate pattern to a more diffuse pattern (Lee et al., 2011). In the current study, the majority of NS5A p56 was found to fractionate with ER membranes with and without
Multiple effects of NS5A inhibitors on NS5A expression

BMS-790052 treatment. Differences in the experimental designs of these studies make it difficult to directly compare their results. Nevertheless, a common theme of the studies is an effect of NS5A inhibitors on NS5A localization. Future investigations will undoubtedly help to clarify the influence of inhibitors on NS5A localization and the implications of this on NS5A function.

In addition to the effects on hyperphosphorylation and NS5A localization, we also found that NS5A inhibitors are capable of promoting the accumulation of an NS4B–NS5A precursor in cells transiently expressing a Con1 replicon. Inhibitor-resistance mutations abrogated this effect, implying a correlation with anti-replication activity. This result suggests that NS5A inhibitors may interact with the HCV polyprotein before it is fully processed. One could imagine, for example, that NS5A inhibitors bind to the polyprotein precursor and hinder cleavage between NS4B and NS5A. It should also be noted that our data do not exclude the possibility that NS5A inhibitors influence processing at other HCV polyprotein cleavage sites. Given previously proposed connections between polyprotein processing and NS5A hyperphosphorylation (Neddermann et al., 1999), it seems possible that the effects of inhibitors on these processes are mechanistically linked. Moreover, the exceptional potency of this class of NS5A inhibitors might, in part, be explained if a polyprotein precursor represents a pharmacological target of these inhibitors. In this scenario, the inhibitor-binding site might be accessible only for a brief period during or prior to polyprotein processing and inhibitor binding would probably disrupt all downstream NS5A functions.

In conclusion, we have shown that NS5A inhibitors affect several facets of NS5A expression. Further investigations are needed to determine whether these inhibitor effects are a consequence or a cause of inhibition. At the same time, potent and specific NS5A inhibitors will continue to provide valuable tools to help dissect the functions of NS5A in the HCV life cycle.

METHODS

Cell culture, molecular clones and replicon assays. Cell cultures were maintained as described previously (Fridell et al., 2010). The R2884G replicon cell line (Huh-9-13) was obtained from R. Bartenschlager, Heidelberg University, Heidelberg, Germany. A Con1 replicon with a Renilla luciferase (luc) reporter has been described previously (Fridell et al., 2010). HCV replicon mutations were introduced with standard cloning techniques and were verified by sequence analysis. Con1 NS5A and NS3–NS5B expression clones were constructed with a pcDNA3.1 directional TOPO expression kit (pcDNA3.1D vector; Invitrogen). Inserts were generated by PCR amplification with platinum Pfx polymerase (Invitrogen). Translation initiation codons in the context of a CACCATG sequence were introduced immediately upstream of the HCV-coding region. Polyprotein clones with C-terminal truncations were made by replacing an EcoRI–XhoI fragment within the pcDNA3.1D–NS3–NS5A expression clone with PCR-generated fragments. A C-terminal V5 epitope was introduced by cloning inserts in-frame in the pcDNA3.1D vector. In other clones, a stop codon was included within the reverse primer, terminating translation immediately downstream of the HCV sequences. Inhibitor EC₅₀ values were calculated from transient replicon assays as described previously (Fridell et al., 2010).

Inhibitors. BMS-790052 (Gao et al., 2010), BMS-058, BMS-506 and a control HCV NS3 protease inhibitor (Lemm et al., 2010) were synthesized at Bristol-Myers Squibb (BMS). SB-220025 and SB-203580 were purchased from Cal-Biochem (EMD Biosciences). Inhibitor stocks were prepared in DMSO.

Transient-protein expression. HCV polypeptides were transiently expressed in BHK cells by using an attenuated vaccinia virus/bacteria phage T7 expression system (W Wyatt et al., 1995) as described previously (Fridell et al., 2011). Inhibitor or DMSO was added to cells at 3 h post-transfection and cells were harvested at 12–16 h for Western analysis. For phosphatase treatment, cell lysates were treated with β-phosphatase (26 U μl⁻¹; New England Biolabs) for 30 min at 30 °C prior to gel electrophoresis. To examine NS5A hyperphosphorylation and polyprotein processing from transiently expressed replicon RNA in Huh-7 cells, in vitro-transcribed replicon RNA (~20 μg per 100 mm dish) was transfected into Huh-7 cells with DMRIE-C transfection reagent (Invitrogen). Cells were treated with inhibitor or DMSO for 16–24 h prior to transfection and for 16 h after transfection, at which time cells were harvested for Western analysis.

Western analysis. Polycrylamide gel electrophoresis, immunoblotting and chemiluminescence detection were performed as described previously (Fridell et al., 2011). Antibodies included rabbit anti-NS5A (Lemm et al., 2010), anti-V5-HRP (1 : 5000; Invitrogen), mouse anti-NS3 (1 : 500; Virogen), rabbit anti-NS5B (1 : 1000; Virogen), goat anti-GRP78 (1 : 500; Santa Cruz Biotechnology), rabbit anti-Rab5 (1 : 500; Santa Cruz Biotechnology), mouse anti-golglin-97 (1 : 1000; Invitrogen) (1 : 500; Santa Cruz Biotechnology) and rabbit anti-calnexin (1 : 1000; Stressgen).

In vitro kinase assays. Assays were performed in 96- (40 μl per well) or 384-well (10 μl per well) formats with a Z’-Lyte kinase assay kit following the manufacturer’s instructions (Invitrogen). Kinase reactions containing 100 μM ATP, 2 μM Z’-Lyte 11 peptide substrate and 1.25 U recombinant enzyme (CKI-δ or CKII; New England Biolabs) μl⁻¹ were incubated for 1 h at room temperature. After addition of one-half volume of development solution (proteolytic cleavage), plates were incubated for 1 h and read with an excitation wavelength of 400 nm and emission wavelengths of 450 and 520 nm on a SpectraMax Gemini EM plate reader (Molecular Dynamics).

Subcellular membrane fractionation. Discontinuous sucrose gradient fractionation of ER and Golgi membranes was performed as described previously (Choi et al., 2004). Briefly, two 150 mm dishes of R2884G replicon cells (~80% confluent) were rinsed twice with PBS and collected by scraping in 0.5 ml hypotonic buffer (25 mM HEPES, pH 7.0, 0.2 M sucrose). Cells were incubated on ice for 10 min, lysed by passing 10 times through a 25-gauge needle and centrifuged (2 min, 16 000 g). Supernatant was loaded on a layered discontinuous sucrose gradient consisting of 2.0 (1.5 ml), 1.3 (3.4 ml), 1.0 (3.4 ml), 0.6 M sucrose (2.2 ml) in 25 mM HEPES and centrifuged (2 h, 40 000 r.p.m., 4 °C) in a Beckman SW41Ti rotor. Fractions (~1 ml) were collected from the top to the bottom of the tube and were analysed by Western analysis. For NS5A, golglin-97 and GRP78 detection, fractions were directly mixed with SDS-loading buffer and electrophoresed on 7.5 % polyacrylamide, Tris-HCl Criterion gels (Bio-Rad Laboratories). Other proteins were detected by Western analysis after proteins were concentrated by precipitation with 20 % trichloroacetic acid. Membrane-floatation assays were performed as described previously (Shi et al., 2003) with the following modifications. Cell lysates were prepared from two 150 mm dishes of R2884G replicon cells. Lysates (0.5 ml) were mixed with 4.5 ml 72 % sucrose, placed in the bottom of a centrifugation tube and layered with 5 ml 55 %
sucrose and 1.5 ml 10% sucrose. Following centrifugation (18 h, 38 000 r.p.m. Beckman SW41Ti rotor, 4 °C), fractions were prepared for immunoblotting without concentration as described above.

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REFERENCES


