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 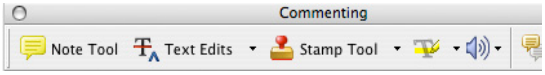

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 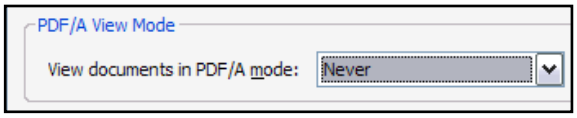

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 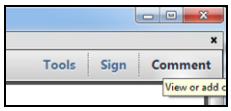

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 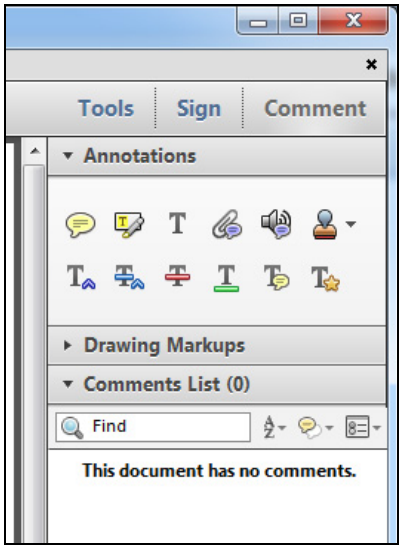
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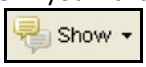
Review	To review your changes, click on the 'Show' button  on the Commenting tool bar. Choose 'Show Comments List'. Navigate by clicking on a correction in the list. Alternatively, double click on any mark-up to open the commenting box.	Your changes will appear automatically in a list below the Comment tool bar. Navigate by clicking on a correction in the list. Alternatively, double click on any mark-up to open the commenting box.
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HCV NS5A Inhibitors Disrupt Replication Factory Formation: A Novel Mechanism of Antiviral Action

See "Daclatasvir-like inhibitors block early biogenesis of hepatitis C virus-induced membranous replication factories independent of RNA replication," by Berger C, Romero-Brey I, Radujkovic D, et al, on page 000.

In recent years, progress in the development of effective antivirals to treat chronic hepatitis C virus (HCV) infection has accelerated enormously. For more than a decade the standard of care therapy for HCV was a combination of pegylated interferon-α and ribavirin (pegIFN-α/RBV) for 24-48 weeks. Unfortunately this treatment regimen is associated with only moderate efficacy (50%-80% sustained virologic response rates) and severe side effects. However, as a result of the efforts of academic and industry research groups and advances in our understanding of the HCV life cycle, made possible by reliable cell culture systems, numerous promising direct-acting antivirals are in advanced stages of clinical development or have already been approved. The addition of first-generation NS3/4A protease inhibitors, telaprevir and boceprevir, to pegIFN-α/RBV therapy significantly improved sustained virologic response rates for genotype 1 infections.[1,2] Likewise, the recent approvals of the second-wave NS3/4A protease inhibitor simeprevir,[3] and the highly effective nucleotide analog inhibitor of the viral NS5B polymerase, sofosbuvir,[4] brings closer the goal of a safe, effective, all-oral, and IFN-free direct-acting antiviral combination therapy in the near future. Along with molecules that target NS3/4A and NS5B, potent inhibitors of the viral NS5A phosphoprotein will likely be important components of future direct-acting antiviral combination therapies. Indeed, the first-in-class NS5A inhibitor daclatasvir (DCV) and structurally related NS5A inhibitors ledipasvir and ombitasvir are in the final stages of clinical development for use in various combinations, and a number of second-generation NS5A inhibitors with higher genetic barriers to resistance (eg, ACH-3102, MK-5172, and GS-5816) are in earlier stages of clinical development. NS5A has no known enzymatic activity and to date the exact mechanism(s) of action of these inhibitors and indeed the exact functions of NS5A remain unclear. In this issue of *Gastroenterology*, Berger et al[5] report that NS5A inhibitors interact with NS5A and block formation of the "membranous web" (MW) that houses HCV RNA replication, independent of effects on HCV RNA replication. Furthermore, the authors present evidence that DCV derivatives interact with NS5A dimers and moderately impair functional interaction of NS5A with phosphatidylinositol-4 kinase IIIα (PI4KIIIα) that stimulates local accumulation of PI4-phosphate (PI4P) at sites of HCV RNA replication. Together this study sheds new light on the mechanisms of action of this unique and extraordinarily potent class of antivirals.

Given its essential roles in multiple aspects of the HCV life cycle, NS5A is an attractive and unique target of antiviral therapy for chronic HCV infection. Since the identification of DCV (formerly BMS-790052) as a potent, pangenotypic inhibitor of HCV RNA replication,[6] a number of studies have investigated the potential mechanism(s) of action of DCV and structurally related NS5A inhibitors. Collectively, these studies have identified several inhibitor properties that may explain their efficacy (Figure 1*B*; reviewed in[7–9]). First, their remarkable potency (picomolar to low nanomolar median effective concentration values) suggests that these inhibitors may synergistically disrupt multiple functions of NS5A in the HCV life cycle and/or target essential events in establishment of replication sites that in time will prevent continued HCV RNA replication. Second, the location of resistance mutations in domain I of NS5A (namely substitutions at L31 and Y93; Figure 1*A*) indicate that domain I-associated functions are specifically targeted. In this context, the class-defining resistance site Y93 is located at opposing, membrane-proximal surfaces of the dimer interface for both "back-to-back" and "clam-like" alternative domain I (genotype 1b) crystal structures.[10,11] Third, biotin-tagged DCV derivatives enable precipitation of NS5A from pretreated HCV replicon-harboring cells,[6,12] although interestingly fail to precipitate NS5A from replicon lysates or pretreated NS5A-overexpressing cells.[12] In this context, it is noteworthy that preformed replication complexes (RCs) are refractory to inhibition of HCV RNA replication by NS5A inhibitors.[12–14] Furthermore, NS5A inhibitors have been shown to induce redistribution of NS5A from endoplasmic reticulum-derived foci,[12–14] possibly to lipid droplets,[12] and limit hyperphosphorylation of NS5A,[14–16] although these effects may be indirect. Finally, it has been suggested that NS5A inhibitors may disrupt interactions of NS5A with HCV RNA, other viral proteins, and/or host factors that are coopted by NS5A during the HCV life cycle. The study of Berger et al[5] comprehensively addresses many of these properties of NS5A inhibitors and uniquely addresses their effects on NS5A-induced PI4P accumulation and HCV-induced membrane rearrangements.

Initially, Berger et al[5] demonstrate that DCV derivatives display potent antiviral activity against established HCV RNA replication and particularly pronounced inhibitory effects on the early establishment of HCV RNA replication and production of infectious virus. This is consistent with the recent, elegant kinetic studies from McGivern et al,[17] who provided evidence that NS5A inhibitors target newly forming RCs and early events in virus assembly. Next, the authors use NS3-5B protein expression systems, that mirror

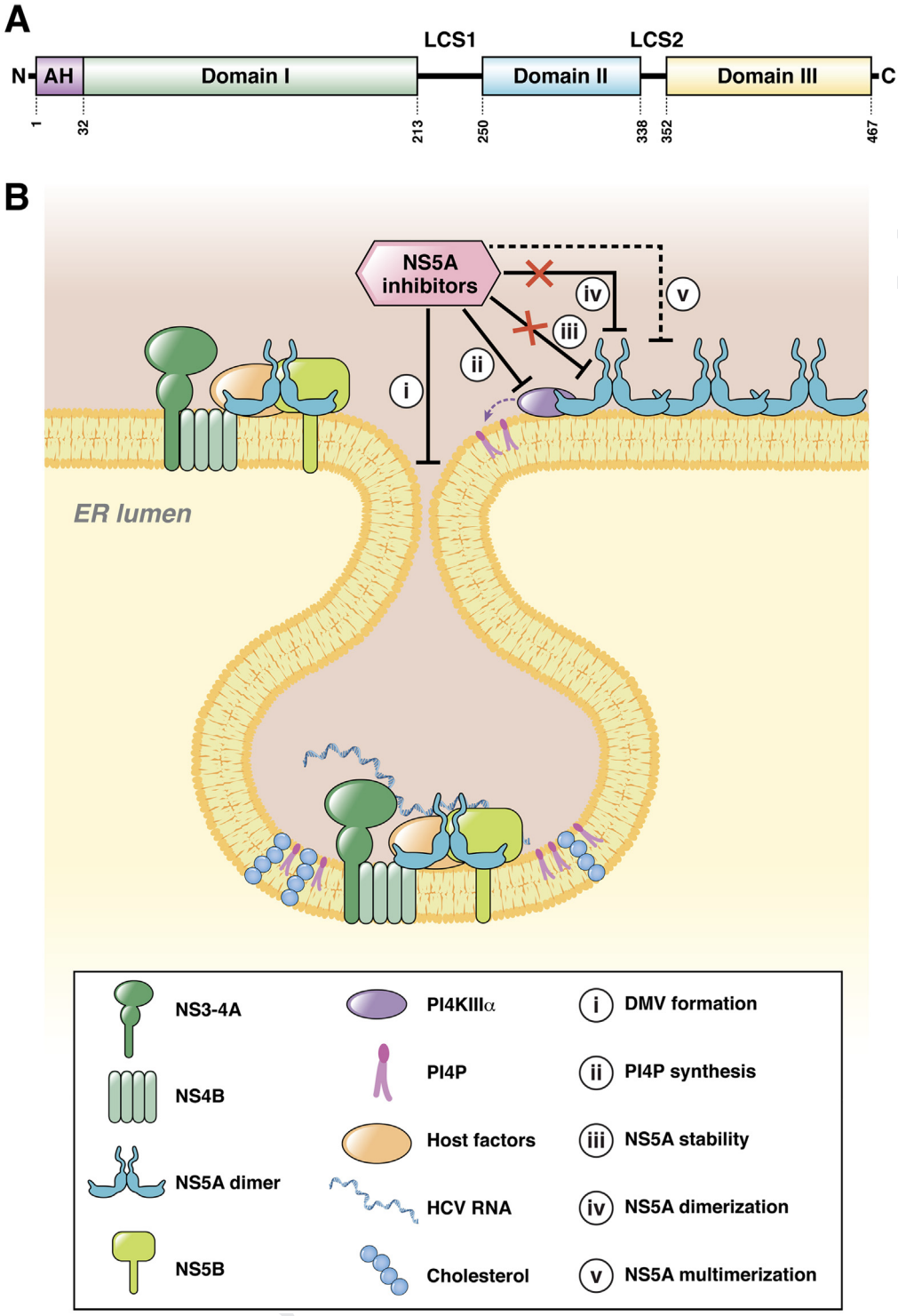

Figure 1. Disruption of virus-induced replication compartment formation by NS5A inhibitors. (*A*) NS5A is composed of 3 domains (domains I, II, and III) separated by low-complexity sequences (LCS). An amphipathic helix (AH) at the amino (N) terminus within Domain I dictates anchorage of the protein to endoplasmic reticulum-derived membranes. Numbers refer to amino acid positions in NS5A from the JFH-1 isolate. (*B*) Sites of NS5A inhibitor action. NS5A inhibitors appear to (i) block double membrane vesicle (DMV) biogenesis and (ii) inhibit functional interaction of NS5A with PI4KIIIα that otherwise stimulates local production of PI4P. However, NS5A inhibitors have no significant effect on (iii) NS5A stability or (iv) NS5A dimerization, but (v) may disrupt formation of higher order NS5A multimers. Other consequences of inhibitor binding include disruption of virus particle assembly, inhibition of NS5A hyperphosphorylation (potentially indirect), and likely impairment of the interactions of NS5A with viral and host replication complex components (not shown).

virus-encoded NS protein expression uncoupled from HCV RNA replication, to demonstrate that neither NS5A stability nor NS5A dimerization are altered by DCV derivatives. Interestingly, analysis of NS5A dimerization in the context of NS3-5B polyprotein expression required the generation of a viable "tandem NS5A" cassette that encoded differently tagged NS5A proteins, because dimerization "in trans" was not detectable. This suggests that NS5A dimerization may be compartmentalized and/or tightly coupled to polyprotein translation/cleavage.

Next, the authors demonstrate that a biotin-tagged NS5A inhibitor enables precipitation of NS5A from cells that express the NS3-5B polyprotein and that inhibitor-mediated NS5A precipitation is impaired (~30%) in the context of

the Y93H resistance mutation. This reduced binding is suggested to contribute to the 750- to 1,000-fold increase in NS5A inhibitor resistance associated with the Y93H substitution. As described, molecular docking studies show that DCV likely binds to membrane-proximal surfaces of both "back-to-back" and "clam-like" dimer structures, to potentially perturb the folding and/or flexibility of the linker region between the *N*-terminal amphipathic helix and domain I and in turn alter NS5A multimerization, membrane association, and/or interaction with viral or host RC components. In this context, Berger et al[5] demonstrate that DCV derivatives moderately impair interaction of NS5A with PI4KIIIα at very high inhibitor concentrations, but significantly inhibit HCV-induced PI4P accumulation in a dose-dependent manner. Importantly, this effect was not observed in the context of the Y93H resistance mutation. This suggests that NS5A inhibitors disturb the functional interaction of NS5A with PI4KIIIα in a similar manner to a class of experimental mutations in NS5A that also limit PI4P accumulation and inhibit HCV RNA replication and NS5A hyperphosphorylation.[18] Although inhibitor-mediated disruption of functional NS5A-PI4KIIIα interaction may contribute to the anti-HCV properties of these inhibitors, it is possible that this effect is one of several consequences of inhibitor-dependent disruption of the overall structure and/or flexibility of NS5A. In line with this, other important interactions of NS5A with host factors (VAP-A, VAP-B, ANXA2, oxysterol binding protein, etc) may also be directly or indirectly disrupted by inhibitor binding. Furthermore, because NS5A hyperphosphorylation is regulated directly or indirectly by PI4KIIIα,[18] inhibitor binding may alter the accessibility of NS5A phosphoacceptor sites to this and other kinases and shift the balance between alternative NS5A phosphoforms (and possibly dimer conformations) that are otherwise regulated by a cascade of phosphorylation events.[19]

Arguably, the most intriguing findings of this study are the striking effects of DCV derivatives on the HCV-induced MW and, specifically, the biogenesis of double membrane vesicles (DMVs) that are the presumed sites of efficient HCV RNA replication.[20–22] Ultrastructural studies revealed that inhibitor treatment significantly reduces the size and frequency of DMVs resulting from NS3-5B polyprotein expression or productive HCV infection and completely blocks the biogenesis of DMVs in the context of early inhibitor treatment and NS3-5B polyprotein expression. Importantly, these effects were absent or limited in the context of the Y93H NS5A inhibitor resistance mutation.

Although it was initially considered that oligomerization of NS4B was largely responsible for HCV-induced membrane rearrangements, a recent study has revealed that a concerted action of the nonstructural proteins (NS3-5B) is required for normal MW formation and that NS5A, in particular, is responsible for DMV formation.[22] How might NS5A mediate DMV formation and how might NS5A inhibitors disrupt this function? Although a number of models for formation of HCV DMVs from endoplasmic reticulum membranes have been proposed,[22] it is not clear exactly how NS5A participates. Strong evidence indicates that recruitment and activation of PI4KIIIα by NS5A and subsequent PI4P enrichment contributes to morphologically normal MWs.[23] Similarly, a recent study revealed that enrichment of cholesterol in these membrane microdomains by the PI4KIIIα effector oxysterol binding protein is also essential to MW integrity.[24] As NS5A expression alone induces DMV formation,[22] it may also participate more directly in membrane rearrangements beyond roles in enrichment of PI4P and cholesterol. It is possible that the alternative dimer forms of NS5A coexist and form a superhelical polymer, as suggested by Love et al,[10] such that membrane bending during DMV formation is the result of membrane anchorage of NS5A by its *N*-terminal amphipathic helix and NS5A oligomerization. The presence of inhibitor-bound NS5A molecules could "lock" a particular dimer form, prevent NS5A oligomerization, and/or distort NS5A oligomers to disrupt DMV formation and ultimately prevent de novo formation of functional RCs.

Taken together, this work from Berger et al[5] provides a significant advance in our understanding of the mechanisms of action of NS5A inhibitors and a new paradigm in antiviral therapy. With NS5A inhibitors as an example, therapeutic targeting of virus-induced membrane rearrangements for other positive-strand RNA viruses could provide an additional, potent approach to antiviral drug development that complements traditional targeting of viral enzymes.

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