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Lipid Metabolite Profiling Identifies Desmosterol Metabolism as a New Antiviral Target for Hepatitis C Virus

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Supporting Information

ABSTRACT: Hepatitis C virus (HCV) infection has been clinically associated with serum lipid abnormalities, yet our understanding of the effects of HCV on host lipid metabolism and conversely the function of individual lipids in HCV replication remains incomplete. Using liquid chromatography−mass spectrometry metabolite profiling of the HCV JFH1 cell culture infection model, we identified a significant steady-state accumulation of desmosterol, an immediate precursor to cholesterol. Pharmacological inhibition or RNAi-mediated depletion of DHCR7 significantly reduced steady-state HCV protein expression and viral genomic RNA. Moreover, this effect was reversed when cultures were supplemented with exogenous desmosterol. Together, these observations suggest an intimate connection between HCV replication and desmosterol homeostasis and that the enzymes responsible for synthesis of desmosterol may be novel targets for antiviral design.

Host-derived lipids are required for the replication of many viruses. For example, the genome replication and assembly of many viruses occur within subcellular compartments derived from host-derived membranes. Not surprisingly then, replication of flaviviruses, SARS, Semliki Forest virus, poliovirus, and vaccinia virus, among others, is associated with dramatic modification of host lipid membrane structures.[1] Furthermore, the lipid bilayer of enveloped viruses is composed entirely of host-generated lipid, and the cholesterol, sphingolipid, and glycerolipid classes of lipids play critical roles in the fusion step of viral entry for members of all three classes of viral fusion proteins.[2] Although the importance of host lipids has been widely recognized, our understanding has mostly been at the level of the function of broad lipid classes in viral processes. Viral selectivity for specific lipids within these general classes has not been carefully examined, and the effects of viruses on the functional output of host metabolic enzymes have received scant attention.

Hepatitis C virus (HCV) is an RNA virus in the *Flaviviridae* family that chronically infects 3% of the human population, predisposing these patients to liver fibrosis, steatosis, cirrhosis, and hepatocellular carcinoma. Chronic HCV infection is associated with hypolipidemia that is reversible upon antiviral treatment,[3−5] suggesting that HCV has unique effects on host lipid metabolism. Consistent with this, general inhibitors of sterol and sphingosine biosynthesis have demonstrated antiviral activity against HCV in cell culture.[6−9] The HCV core protein mediates the accumulation of cellular lipid droplets, and the lipid droplet storage organelle has been shown to play an essential role in HCV viral particle assembly.[10−12] In addition, analysis of the cellular transcriptome and proteome during HCV genome replication and infection has uncovered major changes in the expression of host metabolic enzymes, suggesting major changes in the homeostasis of these pathways.[8,13−15] Despite the accumulating data indicating that HCV manipulates host lipid metabolism specifically to promote its replication, efforts to interfere with this as an antiviral strategy have been limited. This is at least in part due to limited knowledge of the specific lipid metabolites required for HCV replication, an understanding of which might allow the design of strategies with potentially decreased host toxicity because they are targeted to homeostasis of specific lipid metabolites rather than to the blockade of entire metabolic pathways.

In an effort to improve our understanding of host lipid pathways that are manipulated by HCV and to identify potential host targets for new antiviral strategies, we performed untargeted and targeted analysis of changes in the steady-state abundance of lipids in the infectious HCV Japanese fulminant hepatitis strain 1 (JFH1) cell culture model. In this model, a full-length genotype 2a HCV genome supports all steps of the HCV life cycle, including viral entry, gene expression, genome replication, and assembly and secretion of infectious virions.[16−18] Untargeted analysis in positive and negative mode permitted detection of >10 000 lipid ions and revealed 26 metabolites whose abundance had a statistically significant change of 3-fold or more in JFH1-infected cells relative to the mock-infected control (Figure 1a and Supporting Information). The identity of the majority of these metabolites could not be readily assigned on the basis of retention time and mass-to-charge ratio (m/z).

An exception to this was metabolite M367T2277, which exhibited the greatest change in abundance in this analysis (~13-fold), with m/z and retention time similar to those of

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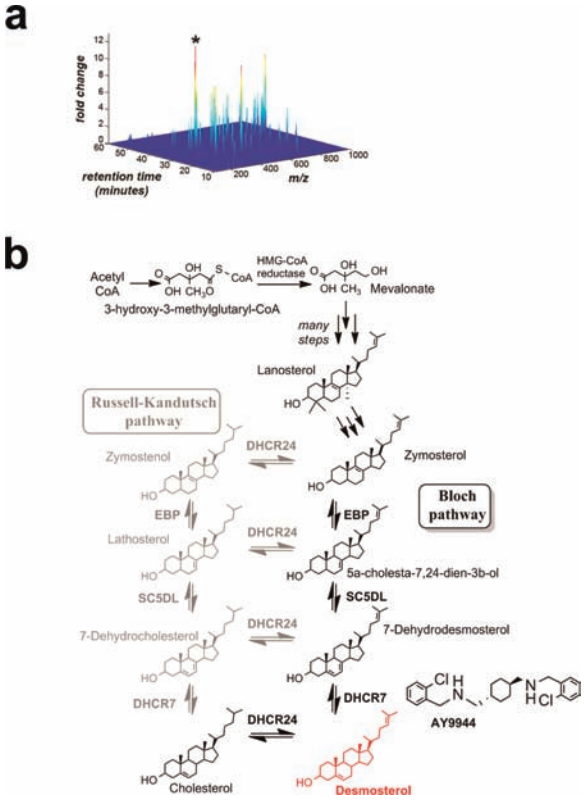

Figure 1. Untargeted lipid metabolite profiling identifies increased steady-state desmosterol in JFH1-infected cells. (a) Total lipid from mock- or JFH1-infected cells was quantified by LC/MS, and comparisons were made with XCMS software. * indicates ion M367T2277. (b) For reference, an abbreviated schematic of the late stages of cholesterol biosynthesis is shown.

isomers of dehydrogenated cholesterol. To unambiguously identify M367T2277, we compared the MS/MS spectrum for M367T2277 to spectra for desmosterol, zymosterol, and 7-dehydrocholesterol standards, which are isomers of dehydrogenated cholesterol with similar retention times and m/z parent ions (Supporting Information). This permitted unequivocal identification of M367T2277 as desmosterol, the immediate precursor to cholesterol in the Bloch branch of the biosynthetic pathway (Figure 1b). The assignment of M367T2277 as desmosterol and not 7-dehydrocholesterol was further supported by the observation that M367T2277 was not a reactive substrate in a Diels–Alder reaction[30] (Supporting Information). While efforts to identify and functionally characterize the additional metabolites enriched in the presence of JFH1 are ongoing, they will be described separately.

To additionally characterize the effects of JFH1 on major metabolic pathways, we also performed a parallel targeted analysis of the lipid metabolite ion data. This targeted lipid metabolite profiling analysis included 25 lipid metabolites whose retention times and m/z ratios were previously determined using authentic standards.[19] These metabolites represent all major cellular lipid classes, including mono-, di-, and triacylglycerols, free fatty acids, isoprenoids, phospholipids, sphingosines, and sterols (Supporting Information). Notably, the additional ions enriched in the presence of JFH1 in the untargeted profiling experiment did not correspond to any of the standards in the targeted profile. JFH1 was associated with relatively modest 1.5- to 2-fold changes in the steady-state abundance of free fatty acids, phospholipids, and both mono- and diacylglycerol species (Supporting Information). Although they are small in magnitude, these steady-state changes of highly abundant species may represent large changes in the total lipid content of the host cell as well as significant increases in the flux through these biosynthetic pathways. Moderate (>4-fold) perturbation of sphingosine metabolism was associated with JFH1, as expected,[7,15] and a 4-fold increase in retinol was detected in the JFH1 samples that has not previously been reported (Supporting Information), although retinol itself has been shown to enhance HCV replication in cell culture.[29] Consistent with the result from the untargeted profile, JFH1

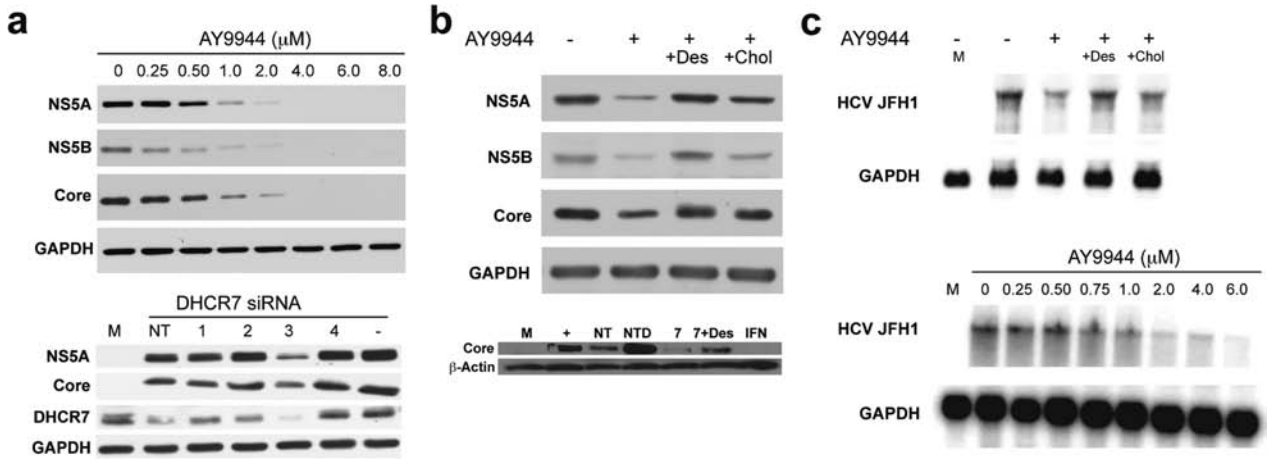

Figure 2. Inhibition of desmosterol synthesis reduces steady-state HCV JFH1 protein and RNA. (a) Steady-state viral protein levels determined by Western blot are shown for inhibition of DHCR7 by AY9944 (top) or RNAi-mediated depletion of DHCR7 (bottom). Four different, individual siRNAs (1–4) targeting DHCR7 were examined. (b) Western blot analysis of steady-state viral protein expression in the presence of AY9944 (top) or siRNAs targeting DHCR7 (bottom) illustrates rescue of viral protein expression in the presence of exogenous desmosterol. (c) Northern blot analysis of steady-state HCV RNA levels in the presence of AY9944 and AY9944 plus exogenous desmosterol (top) and increasing concentrations of AY9944 (bottom) show that viral RNA is also sensitive to desmosterol homeostasis. Des, desmosterol; Chol, cholesterol; M, mock infection; NT, non-targeting siRNA; NTD, non-targeting siRNA plus exogenous desmosterol; 7, DHCR7 siRNA pool; 1–4, individual DHCR7 siRNAs; 7-Des, DHCR7 siRNA pool plus exogenous desmosterol; IFN, interferon treatment control to eliminate viral replication.

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infection was associated with a large (~10-fold) increase in steady-state desmosterol that was not accompanied by a commensurate increase in steady-state cholesterol or 7-dehydrocholesterol. This was noteworthy because desmosterol metabolism has not previously been linked to HCV infection, despite wide appreciation for the importance of sterol metabolism for multiple aspects of HCV replication.[8,9,20−23] Since this phenomenon was specific to HCV JFH1 and was not observed in conjunction with dengue virus (DENV) (Supporting Information) or hepatitis B virus (HBV),[19] we deemed it unlikely to represent a general host response to viral infection. Rather, we favored the hypothesis that the accumulation of desmosterol reflects a specific perturbation of sterol metabolism that is induced by JFH1 and that has a beneficial effect on its replication. A corollary to this is that HCV should be inhibited under conditions that block desmosterol synthesis. To test these ideas, we used small-molecule and RNAi probes to perturb desmosterol homeostasis and evaluated the effects on HCV steady-state protein expression and replication.

Desmosterol is produced by the reduction of 7-dehydrodesmosterol catalyzed by Δ-7-sterol reductase (DHCR7) in the Bloch branch of cholesterol biosynthesis (Figure 1b). We inhibited the conversion of 7-dehydrodesmosterol to desmosterol with siRNAs targeting DHCR7 or with AY9944, a known small-molecule inhibitor of DHCR7.[24] We confirmed by mass spectrometry that treatment of cells with this compound resulted in the >500-fold increase in 7-dehydrodesmosterol and reduced desmosterol to undetectable levels, demonstrating that DHCR7 was inhibited by AY9944, as expected (Supporting Information). Inhibition of DHCR7 was associated with a dose-dependent decrease in steady-state expression of the HCV core, NS5A, and NS5B proteins in JFH1-infected cells with a measured IC_{50} = 0.63 ± 0.22 μM, whereas HBV and DENV were unaffected by AY9944 (Figure 2a and Supporting Information). Consistent with this, RNAi-mediated depletion of DHCR7 also reduced steady-state viral protein in the JFH1 infection model, whereas depletion of sterol-5-desaturase (SC5DL) and Δ-24-sterol reductase (DHCR24), proximal enzymes in the biosynthetic pathway, appeared to cause no detectable change in JFH1 replication (Figure 2a and Supporting Information). Together these data indicate that DHCR7 activity is important for HCV replication.

Importantly, the addition of exogenous desmosterol reversed the effect of RNAi-mediated depletion of DHCR7 or AY9944 treatment on HCV JFH1, resulting in restoration of core, NS5A, and NS5B to the levels observed in the absence of AY9944. We also found that exogenous cholesterol could partially rescue steady-state HCV protein expression, albeit to a lesser extent than exogenous desmosterol (Figure 2b and Supporting Information). In our analysis of DHCR7 inhibition, we discovered that, while conversion of deuterated desmosterol to deuterated cholesterol is the favored reaction catalyzed by DHCR24, the reverse reaction (conversion of deuterated cholesterol to deuterated desmosterol) can also occur in the presence of AY9944, albeit at a low level (Supporting Information). Consequently, we cannot exclude the possibility that the DHCR24-catalyzed conversion of cholesterol to desmosterol contributes to the rescue observed when exogenous cholesterol is added. Interestingly, the effects of AY9944 and AY9944 plus exogenous desmosterol on steady-state viral protein expression were also observed for steady-state viral RNA (Figure 2c), indicating that viral RNA replication is impacted by changes in desmosterol homeostasis. Additionally, although virion-associated cholesterol has previously been described as important for virion assembly, egress, and entry,[23,31] desmosterol homeostasis appears to affect HCV independently of these processes, since a HCV JFH1-derived subgenomic replicon (SGR) that lacks these steps of viral replication exhibited similar behavior in the presence of AY9944 and exogenous desmosterol and cholesterol (Supporting Information). Taken together, these results demonstrate that the DHCR7-catalyzed production of desmosterol is important for HCV replication and suggest that this specific step in cholesterol biosynthesis is a potential target for antiviral development.

Our discovery that HCV perturbs desmosterol homeostasis is especially interesting in light of the selective accumulation of 7-dehydrocholesterol, an alternative cholesterol precursor in cells replicating HBV.[19] HBV and HCV do not share sequence homology, genome organization, or replication strategies, but they do share tissue and host tropism and the propensity for long-term chronic infection that frequently leads to serum lipid abnormalities and development of steatosis, cirrhosis, and hepatocellular carcinoma.[3−5,25] Future investigation of the function and mechanism of DHCR7's role during HCV infection in the context of a whole organism will ultimately determine the therapeutic potential of targeting desmosterol metabolism as an anti-HCV strategy. More immediately, this work demonstrates the utility of lipid metabolite profiling in refining our knowledge of the lipids required for viral replication and the impact of viral pathogens on host lipid metabolism.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary figures, tables, and full methods can be found in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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