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Soraphen A: A Probe for Investigating the Role of de Novo Lipogenesis during Viral Infection

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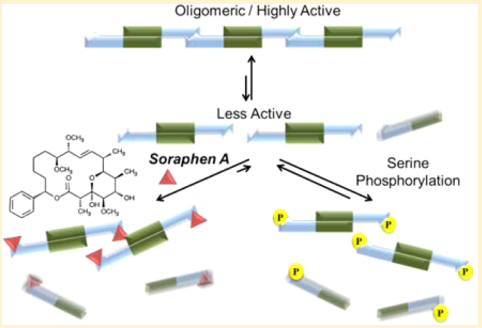
Supporting Information

ABSTRACT: Many viruses including the hepatitis C virus (HCV) induce changes to the infected host cell metabolism that include the up-regulation of lipogenesis to create a favorable environment for the virus to propagate. The enzyme acetyl-CoA carboxylase (ACC) polymerizes to form a supramolecular complex that catalyzes the rate-limiting step of de novo lipogenesis. The small molecule natural product Soraphen A (SorA) acts as a nanomolar inhibitor of acetyl-CoA carboxylase activity through disruption of the formation of long highly active ACC polymers from less active ACC dimers. We have shown that SorA inhibits HCV replication in HCV cell culture models expressing subgenomic and full-length replicons (IC_{50} = 5 nM) as well as a cell culture adapted virus. Using coherent anti-Stokes Raman scattering (CARS) microscopy, we have shown that SorA lowers the total cellular lipid volume in hepatoma cells, consistent with a reduction in de novo lipogenesis. Furthermore, SorA treatment was found to depolymerize the ACC complexes into less active dimers. Taken together, our results suggest that SorA treatment reverses HCV-induced lipid accumulation and demonstrate that SorA is a valuable probe to study the roles of ACC polymerization and enzymatic activity in viral pathogenesis.



KEYWORDS: *hepatitis C virus, coherent anti-Stokes Raman spectroscopy, CARS, HCV, lipids*

Viruses reorchestrate host primary and secondary metabolism to provide the necessary energy and building blocks for viruses to propagate.[1] For example, many viruses, including Dengue virus, hepatitis C virus (HCV), Usutu virus, and West Nile virus,[2−10] usurp control over cellular lipid biosynthesis to create the necessary membrane alterations that house viral replication complexes. In fact, HCV is an archetype in that virtually every step of its life cycle implicates cellular lipid pathways, ranging from entry through low-density lipoprotein (LDL) receptors to replication in lipid-rich endoplasmic reticulum (ER)-associated membranous webs to assembly and secretion mimicking the host cell's very low density lipoprotein (VLDL) secretion pathway.[6−10] HCV replication requires the formation of lipid-rich membranous webs composed of altered ER membranes and accumulated lipid droplets.[11−16] The formation of modified membranes referred to as the "membranous webs" is coincident with higher than normal cellular lipid levels.[8,11,17] To accomplish this metabolic change, the virus decreases catabolic activities such as lipid degradation and export and increases anabolic activities such as de novo lipogenesis,[12] by increasing cholesterol and fatty acid biosynthesis.[7,18,19] Although the broad requirement of lipids for viral propagation is clear, we still do not fully understand the roles of many of the specific lipid-synthesizing enzymes in viral infection.

Soraphen A (SorA) is a macrocyclic polyketide and was first discovered as an antifungal produced by the myxobacterium *Sorangium cellulosum*.[20] This compound allosterically inhibits acetyl-CoA carboxylase (ACC), an enzyme that catalyzes the rate-limiting step of de novo lipogenesis, the formation of malonyl-CoA from acetyl-CoA (Figure S1A). SorA inhibition of ACC has been found to repress elongation of the products of de novo lipogenesis and the formation of polyunsaturated fatty acids (PUFAs).[21] Fatty acid oxidation, which contributes to the lowering of lipid levels, increases during SorA treatment.[21] SorA inhibition of lipogenesis is mediated through interactions at the site of phosphorylation of ACC.[21] It binds to the biotin carboxylase domain near Ser222 of ACC2 with a K_d of 1 nM. Given its potent inhibition of fatty acid biosynthesis,[21,22] SorA

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has the potential to serve as a probe to investigate the role of fatty acid biosynthesis in viral pathogenesis. Because HCV activates ACC expression[23,24] through activation of the transcription factor sterol response element binding protein (SREBP),[24−26] we sought to establish SorA as a probe for ACC function in the virus life cycle and study its role.

RESULTS AND DISCUSSION

To assess the effect of SorA on HCV, virus levels were assayed using three different models, all consisting of human hepatoma cells stably expressing HCV genotype 1b replicons (Figure S1). These consisted of Huh7.5-FGR cells, containing the full genomic replicon (FGR); Huh7-SGR cells, containing the subgenomic replicon (SGR), which expresses only the proteins necessary to form the replication complex; and Huh7-SGR-Luc cells, which express a tricistronic replicon encoding a luciferase reporter enzyme in addition to the SGR. Huh7-SGR-Luc cells treated with SorA at various concentrations for 96 h showed a marked dose-dependent decrease in luciferase signal intensity yielding an IC_{50} value of 5 nM (Figure 1B). Similar treatment of Huh7-SGR cells produced analogous antiviral effects at both the protein and RNA levels via Western blot and qRT-PCR analyses, respectively (Figure S2).

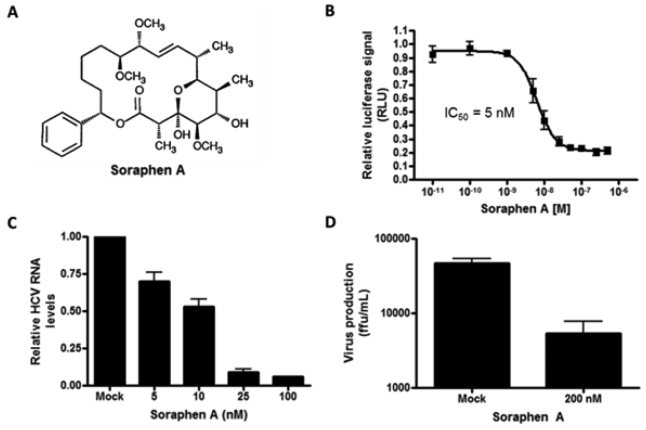

Figure 1. Effect of SorA treatment in Huh7 cells expressing HCV replicons: (A) chemical structure of Soraphen A (SorA); (B) luciferase assay analysis of HCV replication levels in Huh7-SGR-Luc cells treated with various dosages of SorA for 96 h; (C) qPCR analysis of HCV RNA levels in Huh7.5-FGR cells treated as in (B); (D) Huh7.5 cells infected with JFH-1T HCV (MOI = 0.1). Twenty-four hours post-infection, infected cells were treated with 200 nM SorA; 120 h post-treatment, viral titers were measured. Error bars represent the standard deviation of the mean ($N \geq 2$).

Huh7.5-FGR cells also displayed a decrease in viral protein levels via Western blot analyses after 96 h of SorA treatment. Immunoblotting of NS5A protein levels revealed that SorA induced down-regulation of NS5A levels with comparable IC_{50} values (Figure S2A,B). A parallel decrease in HCV RNA levels was observed by qRT-PCR (Figure 1C). It is important to note that in the IC_{50} range of SorA treatment (10−25 nM), no significant effect was observed on the rate of cellular proliferation (Figures S3).

The antiviral effect of SorA in the HCV replicon models could be attributed to inhibition of viral translation or replication. To delineate between the two stages, we performed SorA treatments for 96 h in Huh7.5 cells and then performed transient transfections of HCV FGR replicons expressing luciferase reporter. We examined luciferase activity at an early time point (6 h post-transfection) to determine whether SorA was mediating any effects at the translational level, similar to previous studies.[27] Interestingly, we observed an increase in luciferase activity upon 10 nM SorA treatment (Figure S4). This is likely attributed to increased IRES-mediated translation during SorA-mediated lipid depletion, similar to previous reports of increased IRES-mediated SREBP expression during serum starvation.[28] At longer time points, an antiviral effect was observed, consistent with our observations of SorA's effects on HCV replication. Overall, these results suggest that SorA's antiviral effect against HCV is due to inhibition of viral replication.

Next we examined the effects of SorA on HCV infection. We performed qRT-PCR analysis on Huh7.5 cells infected with JFH-1T, a cell-culture adapted high-titer strain of JFH-1 (genotype 2a).[29] With 200 nM treatments 24 h post-infection, we observed an 80% reduction in viral RNA 5 days after treatment (Figure S2C). No visible cell death was observed during these prolonged treatments, consistent with ACC inhibition being cytostatic rather than cytotoxic, as previously reported.[30,31] Viral titers were also determined, and we observed a >10-fold reduction in infectivity of the supernatants after 5 days of treatment (Figure 1D). These results confirm that SorA inhibits HCV infection and that this effect is conserved across at least two HCV genotypes.

Because SorA inhibition of ACC should result in an overall reduction of cellular lipids, we proceeded to investigate whether SorA reduced HCV levels through this mechanism in HCV cell culture models. Previous research shows that SorA reduces cellular lipids;[21] however, this phenomenon has not been correlated with reduced HCV replication. Cellular lipids were visualized and quantified by coherent anti-Stokes Raman scattering (CARS) microscopy[32,33] after SorA treatment. CARS microscopy is a label-free method that allows the direct visualization and quantification of cellular lipids by measuring the abundance of molecular vibrations of the C−H bond, which is more prevalent in lipids than other biomolecules.[33] Lipid levels decreased by 25 and 40% at 10 and 100 nM SorA concentrations, respectively, in Huh7 cells as measured by CARS microscopy (Figure 2A,B). Triglyceride (TG) assays revealed a parallel 50% reduction in cellular TG levels during 10 nM SorA treatment (Figure 2C). These findings are consistent with previous findings stating that lipogenesis is decreased and fatty acid oxidation increased as a result of SorA treatment.[21] We verified that a similar decrease in lipid synthesis was observed in HCV expressing cells through SorA treatment of Huh7-SGR and Huh7-FGR cells. Using CARS microscopy, we observed a >50% drop in cellular lipid levels in Huh7-SGR cells using only a 10 nM SorA treatment. Because we observed similar results in non-HCV-expressing cells (Figure 2B,C), the effect of SorA on hepatocyte lipid metabolism is therefore independent of the presence of HCV. Given the correlation between SorA's inhibition of lipogenesis and antiviral effect, our data suggest SorA impedes replication by reversing the up-regulation of host cell de novo lipid biogenesis by HCV.

Post-transcriptionally, ACC activity is regulated by post-translational modifications and protein interactions that govern the protein's ability to oligomerize. Oligomeric ACC exhibits much higher activity than ACC monomer or dimers. Thus, the regulation of polymerization directly affects enzymatic activity.[22] AMP-activated protein kinase (AMPK) mediated

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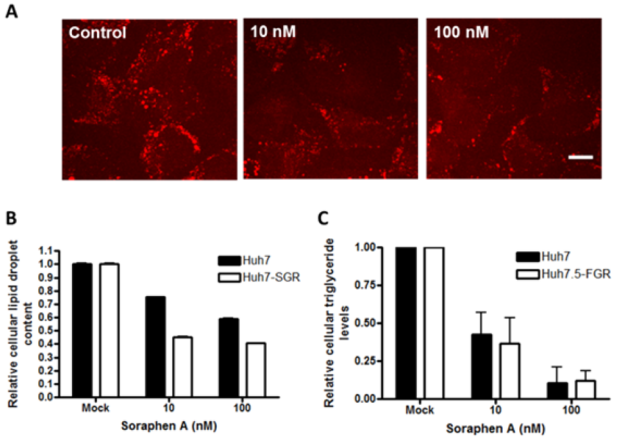

Figure 2. SorA inhibition of HCV replication correlates with decreased lipid storage. (A) Huh7 and Huh7-SGR cells were treated with a concentration range of SorA (Mock; 10−100 nM). Cells were fixed 96 h post-treatment and imaged by CARS microscopy. Representative images are shown for Huh7-SGR cells (n > 39 cells). Scale bar = 10 μm. (B) Average cellular lipid volume was measured by voxel analysis. Results shown are normalized to Mock. Error bars represent the standard error of the mean. (C) Triglyceride assays were performed on Huh7 and Huh7.5-FGR cells treated as in (A).

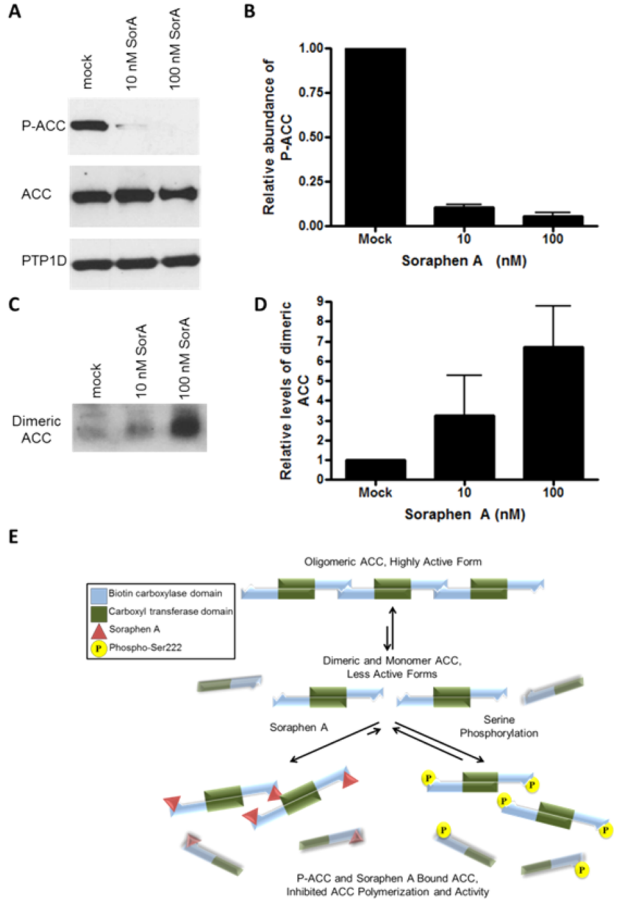

Figure 3. SorA-mediated inhibition of HCV correlates with decreased polymerization. (A) shows Western blot analyses of total ACC and phosphorylated ACC levels in Huh7.5-FGR cells treated with 10 or 100 nM SorA for 96 h. PTP1D was probed as a loading control. (B) Densitometry was performed on blots from (A), and the relative amount of phospho-ACC is shown normalized to total ACC levels. Error bars represent the standard deviation. (C) Native PAGE analysis was performed on cells treated as in (A). Western blotting was subsequently performed for measurement of ACC levels. Representative image of ACC dimer levels is shown. (D) Densitometry was performed on blots from (C), and the relative amount of dimeric ACC is shown. (E) Schematic showing SorA and AMPK phosphorylation's effects on ACC polymerization and activity.[22] SorA binds the biotin carboxylase domain of ACC, 25 Å from the active site,[36] and inhibits ACC activity by mimicking ACC phosphorylation and reducing polymerization.[21,22]

serine phosphorylation (Ser222 in ACC2 and Ser117 in ACC1) induces a conformational change, which allows the phosphorylated residue to form a salt bridge with a proximal arginine residue. This conformational change blocks the polymerization of ACC dimers and inhibits ACC enzymatic activity.[22]

Having used SorA to show that ACC activity is necessary for fully functional HCV replication (Figures 1 and 2; Figure S2), we next proceeded to investigate the influence of SorA on ACC phosphorylation and polymerization. Treatment of Huh7.5-FGR cells with 10 or 100 nM SorA results in a dose-dependent decrease in phosphorylation of ACC (Figure 3A,B), consistent with previous work suggesting SorA interacts with ACC near the same site at which it is phosphorylated by AMPK.[22] We then used Native Blue PAGE gels to determine protein−protein interactions and to detect protein complexes, as they do not require proteins to be denatured.[34] ACC1 monomers have a molecular weight of 265 kDa;[35] thus, it is expected that the dimeric form will be observed at approximately 530 kDa. As SorA inhibits the binding of the BC domains on high molecular weight ACC oligomers, an increase in the presence of dimers is expected. Treatment with SorA increases the dimer form of ACC1 and prevents oligomerization as observed by a concentration-dependent increase in the 530 kDa band (Figure 3C,D). These results are consistent with SorA's mechanism of action through inhibition of oligomerization of ACC and its resulting catalytic activity (Figure 3E).

Recent work has shown microRNA-27 (miR-27) as a key post-transcriptional regulator of lipid storage and triglyceride accumulation in human hepatocytes; furthermore, miR-27 expression is induced by HCV as part of its effects on host cell metabolic pathways.[37−39] In fact, HCV-induced activation of miR-27 expression contributes to lipid droplet accumulation and enlargement in the infected hepatocytes, and miR-27 appears to be a major determinant for hepatic steatosis.[37,38] Furthermore, miR-27 regulates a number of important mRNAs that regulate lipid metabolism and storage.[37−39] Here we tested the effects of SorA on miR-27 levels in Huh7.5-FGR cells using qRT-PCR (Figure S5). We observed a statistically significant 1.5-fold increase in miR-27a levels during SorA treatment. We interpret the increase in miR-27a levels as a homeostatic response to the inhibition of ACC activity to promote hepatic lipid storage to compensate for the SorA-mediated inhibition of de novo lipid biosynthesis.

Previous lipid profiling work revealed that SorA treatment results in a decrease in oleic acid (OA) and increases in intracellular cholesterol levels and linolenic acid levels.[21] Independently, each of these changes should influence SorA's effect on the virus. Because OA biosynthesis appears to be crucial to the formation of HCV replication complexes,[11] SorA-mediated decreases in OA should contribute directly to the drug's antiviral effects. Several studies have demonstrated an important role for cholesterol in replication[18,19,40] and cholesterol esters in HCV assembly.[41] Conversely, several polyunsaturated fatty acids, including linolenate, have been

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shown to have an antiviral effect against HCV through induction of lipid peroxidation.[42,43] Therefore, we expect the increases in cholesterol resulting from SorA treatment to dampen the small molecule's antiviral effect, whereas increases in linolenate likely enhance the antiviral effect of SorA.

SorA represents a unique inhibitor/probe in that it blocks ACC enzyme activity through inhibition of polymerization of ACC. It is these supramolecular complexes of ACC that retain high enzyme function. SorA shows potent inhibition of HCV replication in a number of cell models, including the infectious JFH-1 model, and across at least two genotypes (1 and 2). These data are consistent with previous work demonstrating that small molecule mediated perturbation of host fatty acid metabolism can be quite effective in inhibiting the virus.[14,18,19,32,44] Thus, the establishment of SorA as a potent probe for the role of de novo lipogenesis is likely to be relevant for a number of other viruses and pathogens that may be dependent on this key pathway.

In summary, we have shown that SorA significantly decreases levels of both HCV RNA and protein levels in HCV replication and infectious models. SorA treatment reduces ACC activity and lipid accumulation through inhibition of ACC polymerization, reversing virus-induced increases in de novo lipid biosynthesis. Collectively, these results establish SorA as an antiviral compound that functions through the inhibition of the formation of ACC supramolecular oligomeric complexes with high enzymatic activity. SorA should be broadly applicable to probe the role of ACC polymerization in the context of other viral infections.

METHODS

Experimental procedures are described in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

The following files are available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00019.

All methods and supplementary figures (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ACC, acetyl CoA carboxylase
CARS, coherent anti-Stokes Raman scattering
HCV, hepatitis C virus
LD, lipid droplet
OA, oleic acid
SorA, Soraphen A

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