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Chemical combinations elucidate pathway interactions and regulation relevant to Hepatitis C replication

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The search for effective Hepatitis C antiviral therapies has recently focused on host sterol metabolism and protein prenylation pathways that indirectly affect viral replication. However, inhibition of the sterol pathway with statin drugs has not yielded consistent results in patients. Here, we present a combination chemical genetic study to explore how the sterol and protein prenylation pathways work together to affect hepatitis C viral replication in a replicon assay. In addition to finding novel targets affecting viral replication, our data suggest that the viral replication is strongly affected by sterol pathway regulation. There is a marked transition from antagonistic to synergistic antiviral effects as the combination targets shift downstream along the sterol pathway. We also show how pathway regulation frustrates potential hepatitis C therapies based on the sterol pathway, and reveal novel synergies that selectively inhibit hepatitis C replication over host toxicity. In particular, combinations targeting the downstream sterol pathway enzymes produced robust and selective synergistic inhibition of hepatitis C replication. Our findings show how combination chemical genetics can reveal critical pathway connections relevant to viral replication, and can identify potential treatments with an increased therapeutic window.
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Introduction

The hepatitis C virus (HCV) is an enveloped single-stranded positive-sense RNA virus in the family flaviviridae (Lindenbach and Rice, 2001). The ~9.6 kb genome is translated into a single polyprotein that is subsequently processed into at least 10 structural and nonstructural proteins that are necessary for replication of viral RNA and assembly of new virions (Lindenbach and Rice, 2001). Historically, the study of the HCV life cycle has been difficult, given the inability of HCV to replicate *in vitro* in tissue-cultured cells (Lindenbach and Rice, 2005). However, the development of full length and subgenomic replicons, which express HCV proteins sufficient for replication of viral RNA in hepatoma (Huh-7) cells, has greatly improved our understanding of HCV biology and virus–host interactions (Lohmann *et al*, 1999; Blight *et al*, 2000).

A critical virus–host interaction required for HCV replication is the membrane-associated complex composed of viral and host proteins and altered cellular membranes, designated the membranous web (Egger *et al*, 2002; Gosert *et al*, 2003). This association with host membranes has proven to be a useful strategy for HCV as membranes can serve as a fixed object from which viral proteins can be tethered. FBL2 has been identified as a 50 kDa geranylgeranylated host protein that is necessary for localization of the HCV replication complex through its close association with the HCV protein NS5A and is critical for HCV replication (Wang *et al*, 2005). The extent of FBL2 geranylgeranylation is known to impact HCV replication. For example, inhibition of the protein geranylgeranyl transferase I (PGGT), an enzyme that transfers geranylgeranyl pyrophosphate (GGPP) to cellular proteins for the purpose of membrane anchoring, negatively impacts HCV replication (Ye *et al*, 2003). Conversely, chemical agents that increase intracellular GGPP concentrations promote viral replication (Kapadia and Chisari, 2005). Given the importance of host membranes to HCV replication, it is not surprising that metabolites from these pathways impact HCV RNA replication.

This interaction between HCV and host membranes provides the basis for current candidate therapies for treating HCV infections using statin drugs. Host cell membrane composition can be directly modified by products of the sterol pathway, which is vital for synthesis of cholesterol and isoprenoid intermediates, and the fatty acid biosynthetic pathway (Goldstein and Brown, 1990). Chemical inhibition of enzymes in either of these pathways has been shown to impact viral replication, both positively and negatively (Su *et al*, 2002; Ye *et al*, 2003; Kapadia and Chisari, 2005; Sagan *et al*, 2006; Amemiya *et al*, 2008). For example, statin compounds inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), the rate-limiting enzyme in the sterol pathway (Goldstein and Brown, 1990), and have been suggested to inhibit HCV replication through ultimately reducing the cellular pool of GGPP (Ye *et al*, 2003; Kapadia and Chisari, 2005; Ikeda *et al*, 2006).

However, clinical doses of statins currently used to treat hypercholesterolemia are not high enough to inhibit the synthesis of geranyl lipids. The use of statins for the treatment of HCV is likely to be further complicated by the reported compensatory increase in HMGCR expression *in vitro* and *in vivo* (Stone *et al*, 1989; Cohen *et al*, 1993) in response to treatment. The recent finding that HCV RNA replication increases with fluvastatin treatment in HIV/HCV coinfected patients (Milazzo *et al*, 2009) is consistent with an increase in HMGCR expression. Enzymes in the sterol pathway are regulated on a transcriptional level by sterol regulatory element-binding proteins (SREBPs), specifically SREBP-2, which is an ER membrane-bound transcription factor (Hua *et al*, 1993; Brown and Goldstein, 1997). When cholesterol stores in cells are depleted, SREBP-2 is escorted from the ER to the Golgi complex by SREBP cleavage-activating protein, a sterol-sensing escort protein (Hua *et al*, 1996; Brown and Goldstein, 1999). SREBP-2 is subsequently cleaved by the Golgi-localized proteases S1P and S2P, thereby releasing the N-terminal basic helix-loop-helix domain, which migrates to the nucleus and activates transcription of genes in the sterol pathway that contain sterol response elements in their enhancers (Smith *et al*, 1988, 1990; Sakai *et al*, 1996; Brown and Goldstein, 1999). Well-characterized target genes include HMGCR, HMG-CoA synthase, farnesyl pyrophosphate (FPP) synthase, squalene synthase (SQLS) and the LDL receptor (Horton *et al*, 2002). The requirement of additional downstream sterol pathway metabolites for HCV replication has not been completely elucidated.

Chemical genetics is an effective way of determining drug mechanisms (Stockwell, 2004) where, in the simplest form, single chemical perturbations can elucidate which components in a system are essential for a given phenotype. However, functional connections between system components are best identified either by direct interaction data or through measuring combination effects (Boone *et al*, 2007). One approach that has been successful is the use of chemical perturbagens applied in combination (Lehár *et al*, 2007, 2008). For such combination chemical genetic studies, the interaction needs to be compared to the individual single-agent effects to determine whether there is 'synergy,' where the agents cooperate toward a phenotype, or 'antagonism,' where they impede each other's activity (Greco *et al*, 1995). Thus, the mechanistic focus can be shifted from the individual targets to the interactions between them.

Here, we present a chemical genetic screen undertaken to further understand the impact of the sterol pathway and its regulation on HCV replicon replication using a combination high-throughput screening platform (cHTS) (Borisy *et al*, 2003). Using this approach, we identified several effective antiviral targets including SREBP-2 as well as targets downstream of HMGCR in the sterol pathway such as oxidosqualene cyclase (OSC) or lanosterol demethylase. Of note, combinations between probes targeting enzymes downstream of OSC produced robust synergies with each other or with a PGGT inhibitor. Furthermore, our data suggest that inhibition of the sterol pathway without inhibition of regulatory feedback mechanisms ultimately results in an increase in replicon replication. Consequently, combinations of inhibitors of targets upstream of OSC appeared antagonistic with dominant epistatic effects over inhibitors of downstream targets.

Results

To elucidate the detailed mechanism connecting the sterol biosynthesis pathway to HCV replication, we conducted a chemical genetic screen using our cHTS platform (Borisy *et al*, 2003; Lehár *et al*, 2009). We selected 16 chemical probes that are known to modulate the activity of target enzymes relating to the sterol biosynthesis pathway (Figure 1; Table I). These chemical probes provide dense sampling of the sterol pathway

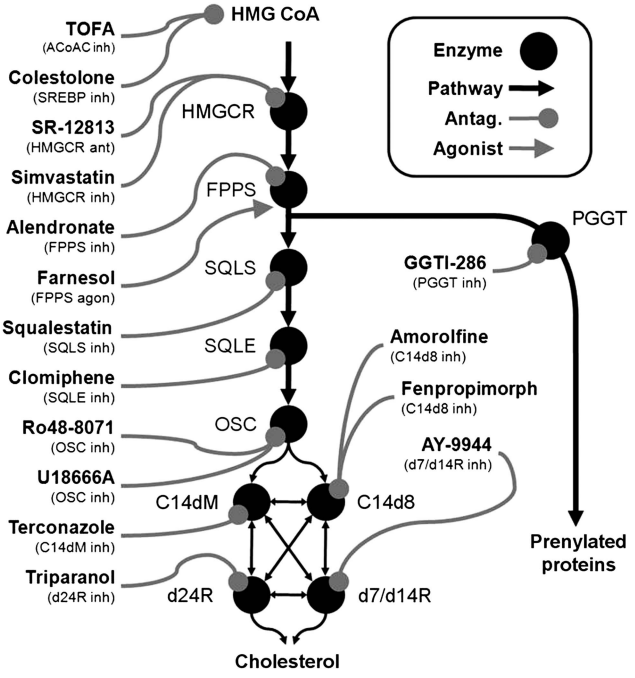

Figure 1 Chemical probes used to modulate targets relating to the sterol biosynthesis pathway, and also to target a proviral pathway mediated by host protein prenylation. The schematic diagram shows the sterol biosynthesis pathway, showing where it interacts with protein prenylation through geranylgeranyl transferase (PGGT). Each chemical probe is shown with a marker connecting it to an enzymatic target, either as an inhibitor/antagonist or as an agonist.

Table I Chemical probes that inhibit enzymes within and outside the sterol pathway

Drug name	CAS number	Conc. range (μM)	Target function	Target protein
TOFA	54857-86-2	0.02–26.5	Fatty acid synthesis	Acetyl-CoA carboxylase (ACoAC)
Colestolone	50673-97-7	0.03–29.2	Cholesterol metabolism	Sterol regulatory element binding protein (SREBP)
SR 12813	126411-39-0	0.03–29.4	Sterol synthesis	3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR)
Simvastatin	79902-63-9	0.03–29.4	Sterol synthesis	3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR)
Alendronate	121268-17-5	0.014–29.4	Sterol synthesis	Farnesyl pyrophosphate synthase (FPPS)
Farnesol	4602-84-0	0.03–29.4	Sterol synthesis	Squalene synthase (SQLS)
Squalestatin	142561-96-4	0.03–29.4	Sterol synthesis	Squalene synthase (SQLS)
Clomiphene	50-41-9	0.03–29.4	Sterol synthesis	Squalene epoxidase (SQLE)
Ro 48-8071	189197-69-1	0.03–29.4	Sterol synthesis	Oxidosqualene cyclase (OSC)
U18666A	3039-71-2	0.0007–10.5	Sterol synthesis	Oxidosqualene cyclase (OSC)
Terconazole	67915-31-5	0.03–29.4	Sterol synthesis	Lanosterol C14-demethylase (C14dM)
Amorolfine	78613-35-1	0.03–29.4	Sterol synthesis	Lanosterol C14-demethylase (C14dM)[a]
Fenpropimorph	67564-91-4	0.03–29.4	Sterol synthesis	Lanosterol C14-demethylase (C14dM)[a]
AY-9944	366-93-8	0.1–13.4	Sterol synthesis	Sterol delta-7 and delta-14 reductase (d7/d14R)
Triparanol	78-41-1	0.03–29.4	Sterol synthesis	Sterol delta-24 reductase (d24R)
GGTI-286	171744-11-9	0.07–17.9	Protein prenylation	Protein geranylgeranyl transferase I (PGGT)

[a]Target sterol delta-14 reductase and delta-8, delta-7 isomerase (C14d8) in plants and fungi.

in addition to exploring the protein prenylation pathway. This study involved comprehensively testing all the probes as single agents and in pairwise combinations, using assays that model both replicon replication and host viability. A comprehensive diagram of the cholesterol biosynthesis pathway is shown in Supplementary Figure S1.

Inhibition of the sterol pathway can inhibit or stimulate replicon replication

To confirm and extend previous studies that showed modulation of HCV replication through the sterol pathway, we added chemical probes of the sterol pathway to Huh-luc/neo-ET cells expressing an HCV genotype 1b subgenomic replicon and a luciferase reporter. The resulting impact on luciferase expression, a measure of replicon replication, was determined for all single agents after a 48 h incubation and the single-agent dose–response curves are reported in Figure 2A. A host proliferation counterscreen measuring cellular ATP levels after a 48 h incubation with compound was assessed in parallel (Figure 2A) in the parental Huh-7 cell line not containing a replicon and the data were used to evaluate the selectivity of each probe. We previously determined an acceptable selectivity index by microscopic visualization of cells treated with compounds. We found that Huh-7 cells could tolerate a measureable reduction in ATP levels of up to ∼30–40% without any visual impact on cell fitness (data not shown). All of the compounds tested produced either increases (proviral) or decreases (antiviral) in replicon replication. Measurements were obtained using serially diluted dose series (by a dilution factor of two), with at least four-fold replicates, and the inhibitions and standard error estimates were determined by calculating the mean change in response relative to untreated cell cultures on each experimental plate (see Materials and methods).

Several chemical probes with selective single-agent antiviral activity were identified. Colestolone, the OSC inhibitors Ro48-8071 and U18666A, as well as amorolfine and the related compound fenpropimorph, all inhibited HCV replication with marginal impact on host cell ATP levels. Colestolone is a potent inhibitor of cholesterol synthesis through many different mechanisms. Colestolone can be metabolized by the cell into polar sterols, which are known to inhibit HMGCR enzymatic activity (Swaminathan *et al*, 1995). In addition, colestolone inhibits SREBP2 activation (Schmidt *et al*, 2006), which ultimately reduces transcription of HMGCR and HMGCS, as well as many other SREBP2-dependent genes. Both U18666A and Ro48-8071 were highly potent and selective HCV replication inhibitors with inhibitory concentration at 50% effect (IC_{50}) values in the nM range. In the data presented in Figure 2A, Ro48-8071 treatment results in ∼50% inhibition of viral replication at the highest concentration shown in the figure. In other experiments not presented here, we observed up to 100% inhibition at higher concentrations. Although amorolfine and fenpropimorph mainly affect sterol isomerases and reductases in plants and fungi, their main target in mammalian cells appears to be the demethylation of lanosterol (Corio-Costet *et al*, 1988).

Many of our chemical probes, specifically SR-12813, farnesol and squalestatin, strongly promoted replicon replication. The actions of both farnesol and squalestatin ultimately result in an increase in the cellular pool of GGPP, which has been shown earlier to increase HCV replication (Ye *et al*, 2003; Kapadia and Chisari, 2005; Wang *et al*, 2005). SR-12813 is a reported HMGCR degradation enhancer (Berkhout *et al*, 1996) and was expected to inhibit HCV replication through a reduction in the cellular pool of GGPP. The proviral activity of this compound is not understood.

The selectivity of the remaining chemical probes tested in our screen is less clear, given the host cell ATP depletion observed at most antiviral concentrations. AY-9944 and triparanol, inhibitors of the downstream targets Δ7- and Δ24-reductase, respectively, exhibited marginal selectivity despite previous reports that inhibition of these sterol reductases is compatible with cell proliferation (Rujanavech and Silbert, 1986; Fernandez *et al*, 2005). Previous reports have suggested that viral replication is impacted by the products of the fatty acid biosynthesis pathway (Kapadia and Chisari, 2005; Amemiya *et al*, 2008). We found that the chemical probe

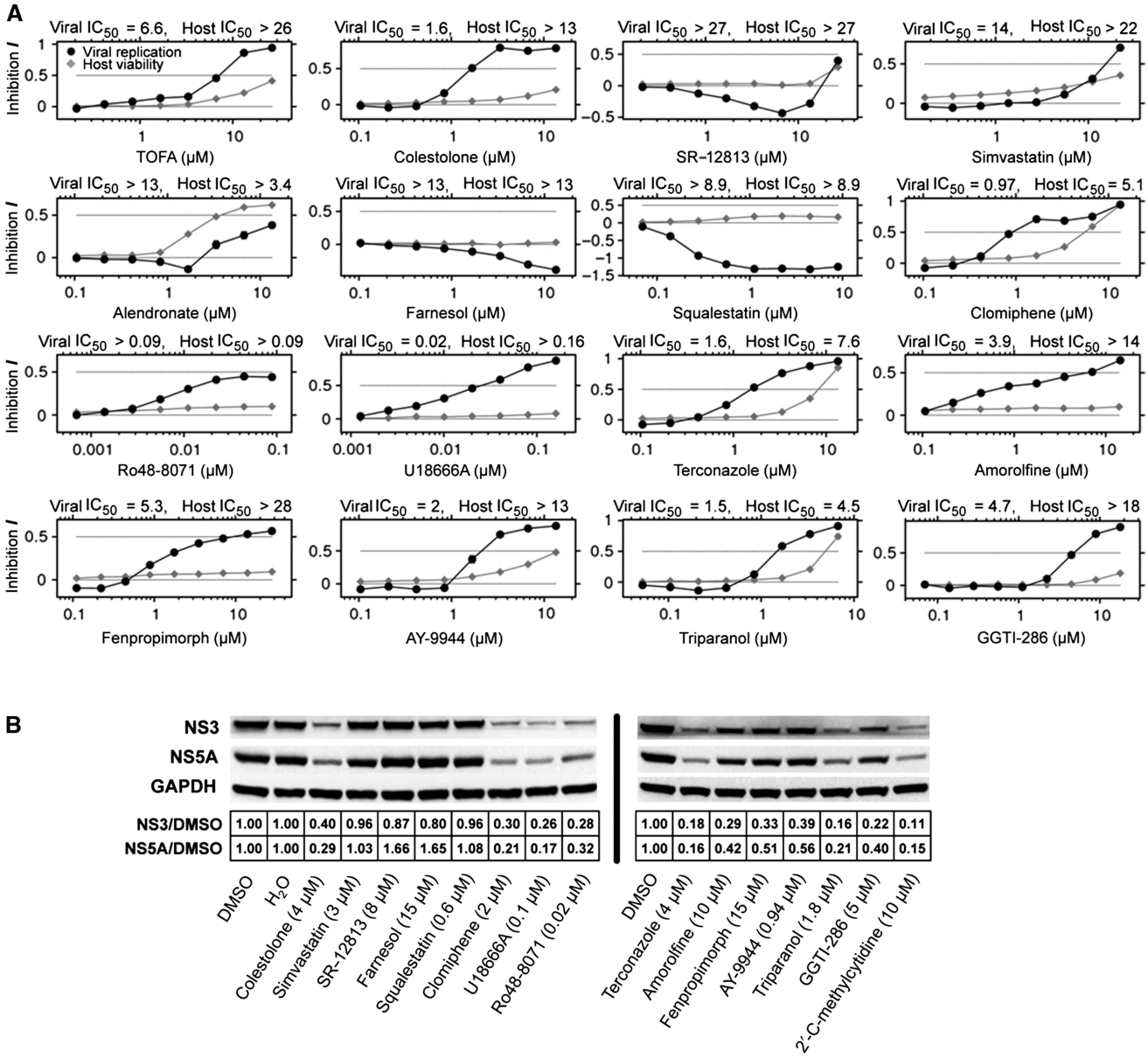

Figure 2 Single-agent activity for the chemical probes in this study. (**A**) In each panel we show the response curves for both the viral replicon and host viability assays after 48 h treatment with serial two-fold dilutions of compounds, with reference lines at 0 and 50% inhibition. IC_{50} values are given for each probe. Data represent means from at least three biological replicates of each drug at each concentration tested. Standard errors, estimated from the median absolute deviation (see Materials and methods), ranged between 3 and 20% at each tested dose, with the largest errors occurring where the response transitioned from inactive to inhibitory. (**B**) Effect on HCV protein synthesis by treatment of replicon cells for 96 h with the chemical probes used in this study. Protein bands from two western blots were quantified using densitometry. Expressed levels of HCV proteins NS3 and NS5A are shown as ratios normalized to GAPDH (see Materials and methods). Each blot is a representative chosen from three separate experiments.

TOFA, an inhibitor of acetyl-coenzyme A carboxylase (ACoAC), resulted in a modest depletion of ATP at most antiviral concentrations, making it difficult to determine whether the antiviral activity is specific or because of host toxicity. As ACoAC catalyzes the committed step in fatty acid synthesis, the resulting host toxicity is to be expected.

It has also been reported that statins inhibit HCV replication through a reduction in cellular GGPP levels (Ye *et al*, 2003; Kapadia and Chisari, 2005; Ikeda *et al*, 2006). However, in this study we found that all antiviral concentrations of simvastatin, in addition to many other statins we tested (data not shown), correlate with a reduction in host cell ATP levels after 48 h of treatment. Thus, there is a concern that some of the antiviral activity of statins may be nonspecific. Moreover, at statin concentrations not associated with ATP depletion, we consistently observed a proviral effect that appeared more robust for fluvastatin than for simvastatin (data not shown). Despite the questionable selectivity of statins in our screen, the antiviral activity of GGTI-286, an inhibitor of protein prenylation by PGGT that has been shown earlier to inhibit HCV replication, confirmed the reported importance of geranylgeranylation to HCV replicon replication (Ye *et al*,

2003; Wang *et al*, 2005). Alendronate, clomiphene and terconazole were also found to reduce host cell ATP levels to a great enough extent that the antiviral activity of these compounds cannot entirely be disassociated from their impact on host cell proliferation.

Given our use of luciferase activity as a model for HCV replication, we sought to validate the luciferase assay results by evaluating the impact of our chemical probes on HCV protein expression levels (Figure 2B). Chemical probes were added to Huh-luc/neo-ET cells as described in 'Materials and methods' at concentrations chosen for an absence of appreciable impact on host cell ATP levels to avoid nonspecific antiviral effects. In several cases, low doses of each compound were used, which necessitated a 96 h incubation in the presence of each probe to observe any impact on the expression of viral proteins NS3 and NS5A. These data confirm the antiviral activity of the OSC inhibitors Ro48-8071 and U18666A, colestolone, amorolfine, fenpropimorph and GGTI-286 as well as the proviral consequence of treating replicon cells with SR-12813 and farnesol. Squalestatin resulted in a greater increase in NS5A than in NS3 and was less proviral at the concentration chosen than in the luciferase assay. Of note, we consistently observed a greater level of enhancement with NS5A over NS3, which may be a result of the individual protein half-lives in the host cell. Chemical probes with questionable activity such as clomiphene, terconazole, AY-9944 and triparanol all appear to inhibit HCV protein levels at the nontoxic concentrations chosen without significant impact on GAPDH protein levels. Treatment of cells with simvastatin did not inhibit viral protein levels, an observation that is consistent with nontoxic concentrations in our luciferase assay data. These western blot results validate our luciferase assay-based screening approach.

Taken together, our data confirm the importance of geranylgeranylation to HCV replication and suggest that targeting SREBP-2 activation or select downstream sterol pathway enzymes such as OSC or lanosterol demethylase can reduce HCV replication. We therefore sought to identify synergistic combinations of inhibitors with the potential for greater control of HCV replication and to better understand the impact of sterol pathway regulation on replicon replication using our cHTS platform.

cHTS of the sterol pathway

After determining single-agent activities for all the chemical agents tested in this study, we designed dose-matrix experiments with concentrations centered around each drug's IC_{50} when possible. To evaluate the synergistic activity of compounds in combination, we compared the observed activity across each drug combination inhibition response surface to a superposition of effect (SPE) model that is derived from the single-agent activity curves (see Materials and methods). Unlike the standard reference models for combination effects (Greco *et al*, 1995) the SPE model smoothly interpolates between the activities of both inhibitory and stimulatory single agents, highlighting as synergies or antagonisms antiviral responses that fall above or below the SPE expectation. An overview of the replicon responses and the corresponding host combination activity is presented in Figure 3. The dose-matrix response surfaces for all the compound crosses performed in the HCV replicon assay and Huh-7 host cells are shown in Supplementary Figures S2 and S3.

In the host viability assay, many combinations are close to this SPE expectation, indicating the SPE model represents typical combination effects. The exceptions are the antiproliferation synergies between clomiphene and non-sterol targets, and to a lesser extent, its interactions with terconazole and triparanol, both of which showed additional antiproliferation synergies. There is also a curious antiproliferation antagonism between alendronate and probes targeting upstream enzymes. Both clomiphene and alendronate are used clinically for activities not related to the sterol pathway, which suggests these effects may be nonspecific. Host interactions are associated with the toxicity of particular compounds, and do not show pathway-dependent patterns similar to those in the antiviral screen, suggesting the antiviral interactions are not the result of a toxicity-dependent experimental artifact in most cases.

In the HCV replicon assay, however, strong mechanism-dependent patterns emerged, which are highlighted in Figure 4. In many cases, chemical combinations elicited Bateson-type epistatic responses, where one chemical agent's response predominates over the effect of the other chemical agent (Boone *et al*, 2007). Combinations targeting enzymes upstream of squalene epoxidase (SQLE) at the top of the sterol pathway elicited responses epistatic to the effect of targeting enzymes downstream of SQLE (Figure 4A). This was especially notable for combinations including simvastatin and either U18666A or squalestatin, and for squalestatin in combination with Ro48-8071. Treatment with squalestatin prevents the SQLS substrate, FPP from being further metabolized by the sterol pathway. As FPP concentrations increase, the metabolite can be shunted away from the sterol pathway toward farnesylation and GGPP synthetic pathways, resulting in an increase in host protein geranylgeranylation, including FBL2, and consequently replicon replication. This increase in replicon replication explains the source of the observed epistasis over Ro48-8071 treatment. However, less clearly understood are the epistatic effects observed between simvastatin and either squalestatin or U18666A. In these two conflicting examples, simvastatin is observed in one case to antagonize the proviral effect of squalestatin treatment and in another to antagonize the antiviral effect of U18666A. Confirmation of simvastatin's epistasis over U18666A was provided by western blot analysis (Figure 5A). The concentration of each compound was selected for an absence of impact on host proliferation. The results suggest that the observed contradictory effect of simvastatin is not an artifact. It is likely that several simultaneous and competing processes govern the outcome for these combinations, including HMGCR protein inhibition, unspecific cytotoxicity as measured by ATP depletion and HMGCR transcriptional regulation.

Combinations targeting the downstream end of the pathway led to inhibitory synergy in both the replicon and host viability assays, especially when both agents were downstream of OSC. In the lower part of the sterol pathway there exist parallel routes to the synthesis of cholesterol. Consequently, inhibition of one branch of sterol synthesis does not necessarily prevent

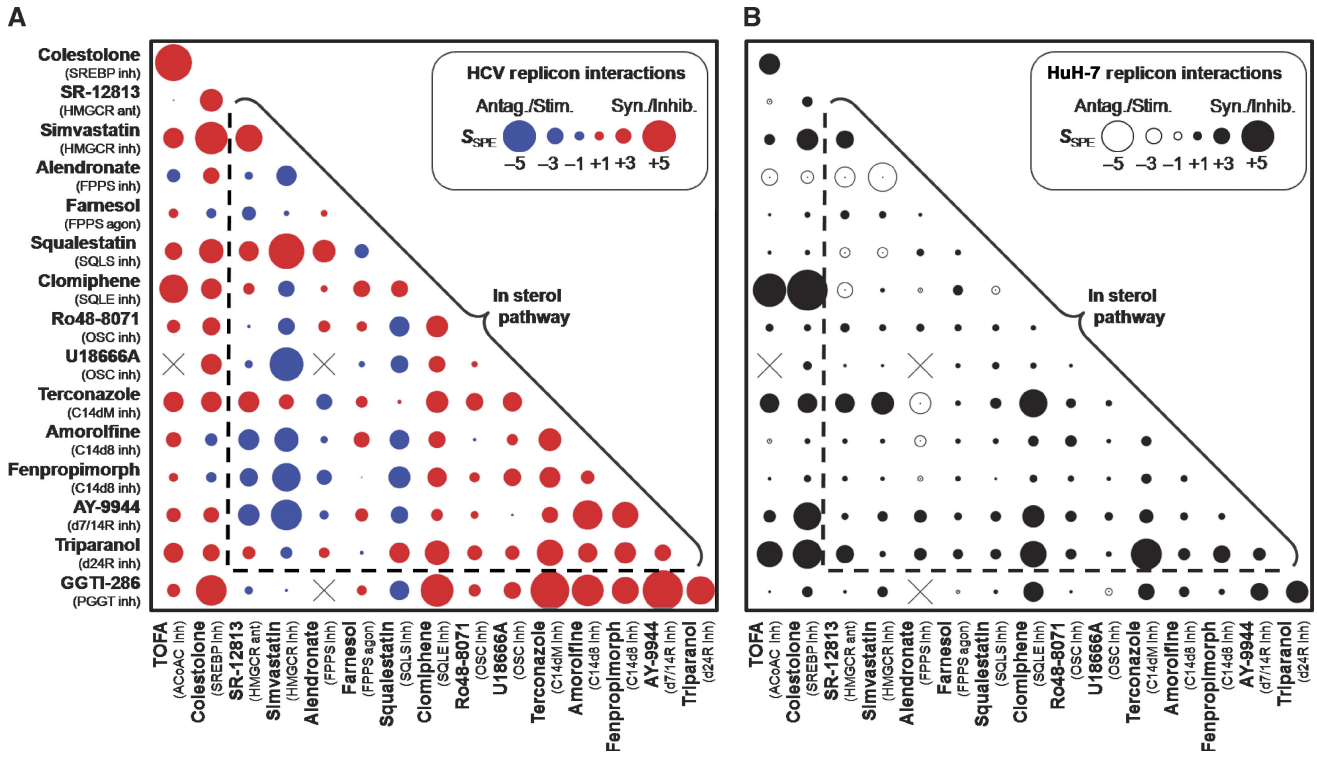

Figure 3 Overview of the HCV replicon and host responses showing the observed combination activity. Dose-matrix data were obtained by testing all pairs of serially diluted (two-fold) concentrations for each pair of probes (Supplementary Table S1). Activity values $A = -\log_{10}(T/U)$ were calculated at each dosing point by comparing the treated T viability level to untreated values V obtained from ~20 vehicle-treated wells arranged on each experimental plate (see Materials and methods). Dose matrices were obtained with at least four replicates. Uncertainties on the activity, estimated from the scatter between replicates (see Materials and methods), were typically ~0.1, averaged across each dosing matrix (Supplementary Table S1). For each pair of chemical probes, the level of interaction observed between the agents is shown as a circle scaled to the synergy score S, relative to a 'superposed effect' (SPE) model of non-interaction (see Materials and methods). The SPE model interpolates smoothly between single-agent effects that can be either inhibitory or stimulatory. Positive S (solid or red circles) correspond to synergistic interaction when both agents are inhibitory, and to the dominance at high combined concentrations of the inhibitory agent when one of the agents has stimulatory activity (e.g. squalestatin). Negative S corresponded to antagonism between inhibitors or dominance of the stimulatory agent. A synergy score of 1 means that there was a volume of 1 between the observed response and the SPE model surface, integrated over the dose matrix. The scale of these scores depends on the number and density of concentration points tested, and thus is mainly useful for comparisons between combinations, rather than providing an absolute reference level for synergy. Given that the individual activity measurements tended to have standard errors of $\sigma_a \sim 0.1$, only synergy scores outside of ± 3 can be considered significant, when integrated over the 64 combination dosing points. (**A**) In the replicon assay, combinations targeting sterol pathway enzymes downstream of OSC mostly appear to synergize more toward antiviral activity, and those upstream of OSC mostly appear to show 'epistasis,' where the effect of modulating an upstream target dominates over those of downstream targets at high combined concentrations. (**B**) Most combinations in the host toxicity assay produce responses that are close to the SPE expectation, indicating the model represents typical levels of interaction between compounds reasonably well. Where it does occur, strong synergistic viability inhibition ($S > 3$) is associated with compounds (e.g. clomiphene, terconazole and triparanol) that also show strong synergies outside of the sterol pathway (with TOFA and clomiphene, or GGTI-286), and host activity within the pathway obvious mechanism-dependent patterns.

synthesis of cholesterol nor does it result in host toxicity in every case. Figure 4B highlights examples of antiviral synergy resulting from treatment of cells with an OSC inhibitor in combination with an inhibitor of either an enzyme upstream or downstream of OSC. A combination of terconazole and U18666A is synergistic without similar combination effects in the host proliferation screen. Likewise, clomiphene was also synergistic when added to replicon cells in combination with U18666A. One of the greatest synergies observed in the downstream sterol pathway is a combination of amorolfine and AY 9944, suggesting that there is value in developing combinations of drugs that target enzymes in the sterol pathway, which are downstream of HMGCR.

Interactions with the protein prenylation pathway also showed strong mechanistic patterns (Figure 4C). GGTI-286 is a peptidomimetic compound resembling the CAAX domain of a protein to be geranylgeranylated and is a competitive inhibitor of protein geranylgeranylation. Most combinations of GGTI-286 with agents that target the pathway upstream of SQLE are consistent with SPE. For example, simvastatin impedes the antiviral effect of GGTI-286 at low concentrations but that antagonism is balanced by comparable synergy at higher concentrations. At the low simvastatin concentrations, it is possible that a compensatory increase in HMGCR expression leads to increased cellular levels of GGPP. Under normal conditions, GGPP binding to PGGT displaces a geranylgeranylated protein from the enzyme to recycle the catalytic pocket (Taylor *et al*, 2003). An increase in free GGPP could result in an increase in PGGT enzymatic turnover and decreased GGTI-286 efficacy, as is observed. The antiviral synergy observed at the higher inhibitor concentrations is likely nonspecific as synergy was also observed in the host viability assay. Further downstream, however, a competitive interaction was observed between GGTI-286 and squalestatin,

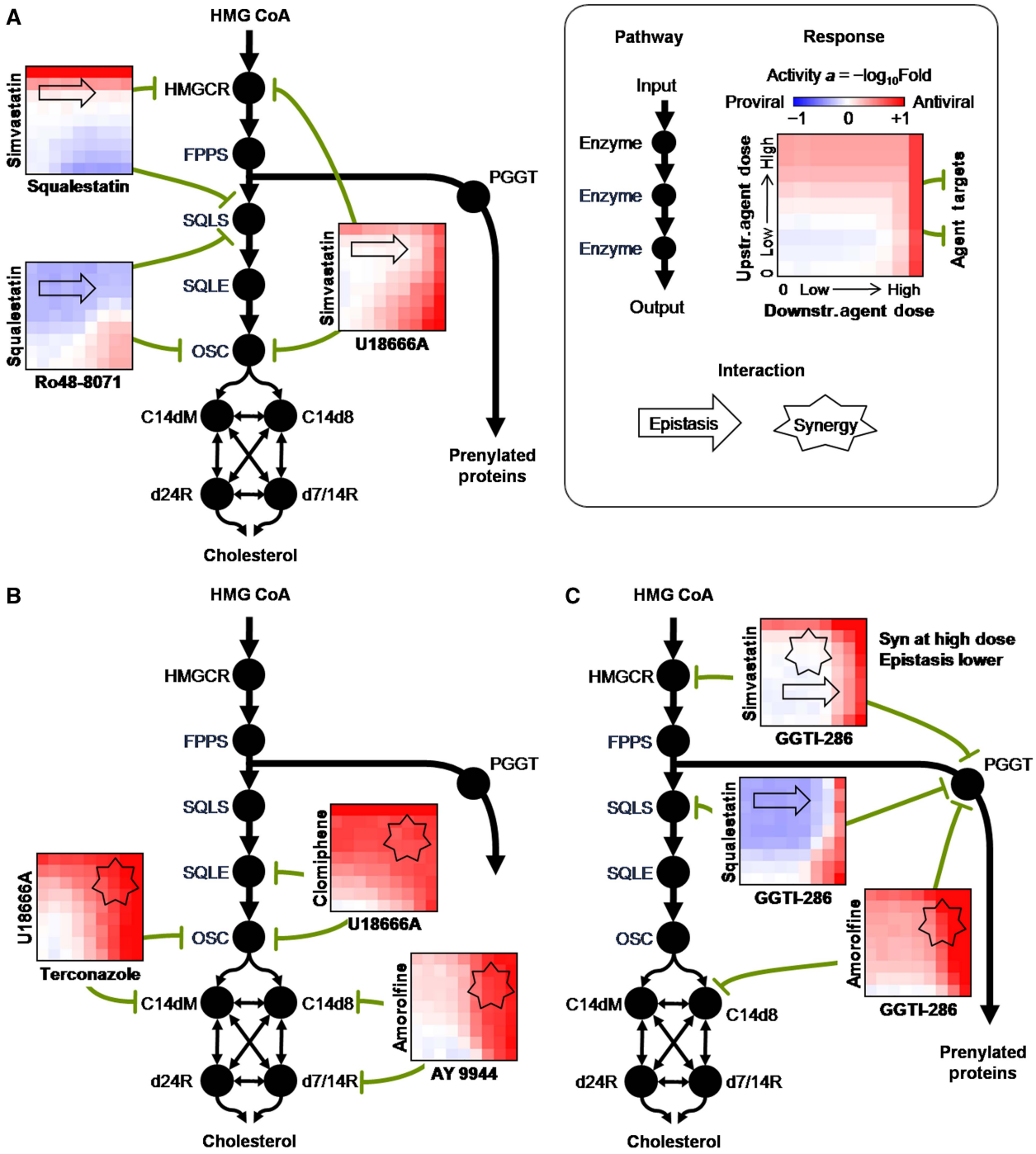

Figure 4 Multi-target interactions in the replicon assay. Each panel presents a schematic of the sterol pathway showing the connection to replicon replication mediated by protein prenylation, along with response matrices for combinations representing different kinds of mechanistic interactions. Dose matrices, colored to show the activity at each dose point, are shown with green markers indicating the targets of each combination, and with shape symbols to indicate the type of interaction seen (either synergy or epistasis with an arrow indicating the direction of dominance from single agent to combination activity). (**A**) Combinations targeting the top of the sterol pathway produced epistatic responses, where the upstream agent's response predominates at high concentrations, irrespective of the pro- or antiviral activity of the single agents. (**B**) Targeting the lower end of the pathway led to inhibitory synergy in both the replicon and host viability assays, especially when both agents were downstream of OSC. (**C**) Inhibitors of the prenylation pathway interacted weakly (close to the SPE expectation) with probes targeting enzymes at the upper end of the sterol pathway, but had significant interactions further downstream. The strongest synergies were produced in combination with agents targeting enzymes downstream of OSC.

where the opposing effect of one compound obscures the other compound's effect. This competitive relationship between GGTI and SQLE explains the epistatic response observed between those two agents. For inhibitors of targets downstream of OSC, such as amorolfine, there are strong antiviral synergies with GGTI-286. Notably, combinations with OSC inhibitors and GGTI-286 were selective, in that comparable synergy was not found in the host viability assay.

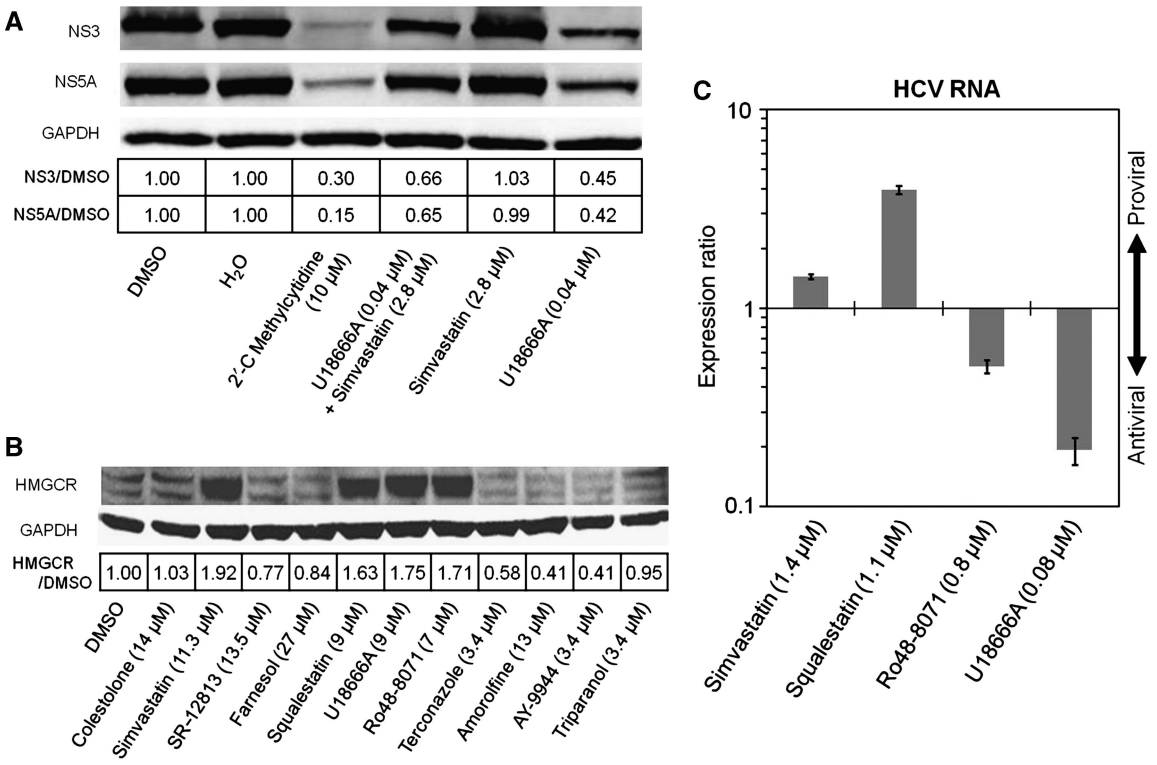

Figure 5 Validation experiments. (**A**) Evidence of epistasis at the level of HCV protein expression, after 96 h in Huh-luc/neo-ET replicon cells, between upstream-targeting simvastatin and downstream-targeting U18666A. Protein bands were quantified using densitometry and levels of expressed HCV proteins NS3 and NS5A are shown as ratios normalized to GAPDH. (**B**) Sterol pathway regulation revealed by HMGCR protein expression in HuH-7 cells 16 h after exposure to chemical inhibitors, showing feedback effects for probes targeting the upper end of the pathway. Antibodies specific for HMGCR and GAPDH were used to probe western blots of proteins separated by 10% Bis–Tris SDS/PAGE (see Materials and methods). (**C**) Confirmation that agents targeting the upper and lower pathway have respectively pro- and antiviral effects at 72 h in quantitative RT–PCR experiments on Huh-luc/neo-ET cells. Averaged expression levels from triplicate experiments were calculated after normalizing replicon copy number to total cellular RNA (see Materials and methods), and error bars show 95% confidence (two standard deviations).

This selectivity suggests that jointly targeting OSC and PGGT is a promising avenue for future HCV therapy development.

These data further support previous findings that inhibition of protein geranylgeranylation effectively inhibits HCV replication. We found combinations of inhibitors of the lower part of the sterol pathway that are effective and synergistic with each other when tested in combination. Furthermore, the combination effects observed with simvastatin suggest that, though statins inhibit HMGCR activity, the resulting regulatory consequences of such inhibition ultimately lead to undesirable epistatic effects.

Inhibition of sterol pathway enzymes directly impacts HMGCR protein expression and viral RNA replication

The above results suggest that HMGCR regulation may have a role in the epistasis of upstream sterol pathway chemical probes over downstream probes. To determine whether our chemical inhibitors result in an increase in HMGCR expression, we treated parental Huh-7 cells with the listed concentrations of each chemical for 16 h (Figure 5B). Cell lysates were extracted according to the methods and HMGCR protein expression was analyzed by SDS–PAGE separation and western blotting with antibodies specific for HMGCR and GAPDH. Consistent with previous studies (Stone *et al*, 1989; Cohen *et al*, 1993), treatment of cells with simvastatin resulted in an apparent overexpression of HMGCR. In addition, treatment with either OSC inhibitors (U18666A or Ro48-8071) resulted in overexpression of HMGCR, consistent with other studies showing that treating cells with OSC inhibitors increases HMGCR RNA levels and activity (Telford *et al*, 2005). Surprisingly, squalestatin also resulted in overexpression of HMGCR, suggesting additional regulation along the pathway. None of the other chemical probes tested produced increases in HMGCR protein expression. Significant reductions in HMGCR expression were not observed, which may be due to the half-life of the HMGCR protein, which can vary between >10 h and <20 min, depending on the cell type (Hampton, 2002).

Given the observed increases in HMGCR in response to simvastatin, squalestatin and the OSC inhibitors, we next sought to evaluate the consequences of such overexpression as they pertain to HCV replicon replication. Huh-luc/neo-ET cells were treated with each inhibitor in Figure 5C over 72 h in 96-well plates. Total cellular RNA was extracted from the treated cells and replicon RNA copy number in each treated population was quantified by RT–qPCR and normalized to total RNA. As expected, squalestatin and, to a lesser extent,

simvastatin mediated proviral effects, consistent with the observed coincident increases in HMGCR expression. In addition, these results are also in agreement with the luciferase expression data and the viral protein expression reported above in Figure 2, more so for squalestatin than for simvastatin. Small increases in replicon replication observed in both the luciferase and RT–qPCR assays were less often visualized in western blots for replicon protein expression (especially for simvastatin). It is possible that the greater dynamic range afforded by qPCR as well as the shorter half-life of luciferase protein in replicon cells contributes to some of the disparity. Counter intuitively, the observed increase in HMGCR protein expression in response to OSC inhibition did not translate to an increase in replicon replication, but instead inhibited HCV replicon replication. These results suggest that there are unique consequences associated with OSC inhibition that will require additional investigation to fully elucidate the impact on replicon replication.

Discussion

The work reported here represents the first large-scale systematic chemical genetic investigation of HCV replicon replication using a novel experimental design with both single and combined chemical probes at multiple doses. To identify selective antiviral effects, HCV activity and host cell viability were monitored using cellular phenotypes that integrate the functions of many pathways, providing insights into the regulation and interactions between pathways. This study extends the growing body of evidence connecting sterol pathway metabolism to HCV replication through protein prenylation, and identifies potential therapeutic treatments with potent and selective antiviral activity that warrant further development. Despite the limitations of the cell-adapted subgenomic replicon used in this study, the system has proven to be predictive of drug efficacy in clinical settings and we believe it to be a reasonable predictor of mechanistic interactions related to HCV replication. Further studies are warranted to investigate whether the host cell interactions observed here carry through to native HCV such as JFH1 (Wakita *et al*, 2005), a replication competent HCV strain.

The single-agent testing confirms the connection between sterol metabolism, protein prenylation and HCV replicon replication. For example, the strong proviral effect of squalestatin results from FPP being both a substrate of the sterol pathway and an intermediate toward protein prenylation. Farnesol, once pyrophosphorylated by the cell, is a substrate for the synthesis of GGPP by FPP synthase. FPP is also a substrate for the sterol pathway enzyme SQLS, which catalyzes the synthesis of squalene. The addition of farnesol increases both sterol synthesis and protein prenylation, explaining the observed proviral effect. Squalestatin prevents the further metabolism of FPP by SQLS, resulting in a buildup of FPP and, ultimately, a shunt toward the synthesis of GGPP, which is a substrate for geranylgeranylation of the required host protein FBL2 (Wang *et al*, 2005). This proviral activity is further accentuated by an increase in HMGCR expression seen here and elsewhere in response to either statins (Stone *et al*, 1989; Cohen *et al*, 1993) or squalestatin.

Further downstream, the OSC inhibitors Ro48-8071 and U18666A showed potent antiviral activity, despite the observation of a robust increase in HMGCR protein expression. Previous studies have shown that OSC inhibitors possess a concentration–effect relationship that is biphasic (Boogaard *et al*, 1987; Cohen and Griffioen, 1988; Mark *et al*, 1996; Telford *et al*, 2005). At lower concentrations of Ro48-8071 and U18666A, OSC is partially inhibited which favors the synthesis of 24(*S*),25-epoxycholesterol over the synthesis of cholesterol. 24(*S*),25-epoxycholesterol represses HMGCR activity (Peffley *et al*, 1998), enhances HMGCR protein degradation (Gardner *et al*, 2001) and blocks the activation of SREBP-2 (Janowski *et al*, 2001), which is consistent with the observed antiviral activity of these inhibitors at the concentrations tested in our screen. The consequence of partial OSC inhibition ultimately impacts intracellular GGPP levels, host protein geranylgeranylation and replicon replication. At higher concentrations, where OSC inhibition is complete, cholesterol synthesis is inhibited, resulting in activation of SREBP-2 and an increase in the expression of sterol pathway genes. This is consistent with the observed increase in HMGCR protein expression at concentrations in the μM range. Sterol pathway regulation, as mediated by SREBP-2 (Horton *et al*, 2002), was similarly impacted by the addition of colestolone or chemical probes targeting lanosterol demethylase, which lead to an accumulation of polar sterols (Corio-Costet *et al*, 1988; Schmidt *et al*, 2006), a down regulation of SREBP-2 activation and ultimately to a reduction in intracellular GGPP levels, host protein geranylgeranylation and replicon replication.

The combination effects we observed fall into consistent patterns that support this mechanistic understanding. Generally, there was upstream epistasis for combinations between one agent targeting the upper end of the sterol pathway (above SQLE) and another agent targeting the lower end of the pathway. However, for combinations inhibiting only lower sterol targets, below the protein prenylation shunt at SQLS, the response pattern shifts predominantly toward antiviral synergy. This is especially evident downstream of OSC where sterol metabolism becomes more complex. The cross-pathway combinations involving the PGGT inhibitor also showed upstream dominance for upper sterol targets. However, combinations that inhibited both PGGT and lower sterol targets consistently produced antiviral synergies, again especially downstream of OSC. The interaction discontinuity for viral replication between the upper and lower ends of the sterol pathway, and its absence in the host viability assay are consistent with the existence of a prenylation shunt at SQLS that predominantly enhances viral activity. Moreover, the epistasis is consistent with expectations for viral activity that does not directly affect upstream sterol metabolism, whereas the synergy responses are consistent with expectations for a metabolic pathway regulated by negative feedback from downstream pathway products (Lehár *et al*, 2007). This analysis demonstrates how a combination chemical genetic approach can accurately reveal the critical pathway connections relevant to different biological processes.

The comparison between viral replication and host viability also provides useful insights into potential antiviral therapies for HCV based on sterol pathway inhibition. Treatment with an HMGCR inhibitor (simvastatin) increased HCV replication at

concentrations not associated with host cell ATP depletion, a finding that may lend some insight into recent clinical studies of viral activity in patients treated with fluvastatin (Bader *et al*, 2008; Milazzo *et al*, 2009). Moreover, although HCV replication decreased under modulation of sterol-related targets such as SREBP-2, HMGCR, OSC, C14dM (also known as CYP51A1) or PGGT, all of the chemical probes we selected that target these enzymes are associated with clinically relevant toxicities that would prevent their direct translation to HCV clinical investigations (Polak, 1992; Haria and Bryson, 1995; Swaminathan *et al*, 1995; Lobell *et al*, 2001; Pyrah *et al*, 2001; Cenedella *et al*, 2004). These findings suggest that sterol pathway inhibitors administered alone may not be effective HCV therapeutics, given the compensatory overexpression of HMGCR. However, targeting the sterol pathway can be a viable antiviral strategy for treating HCV, if inhibitors of the pathway are combined. The antiviral synergies seen between the most downstream sterol targets and also their interactions with the prenylation pathway are generally not mirrored in the host viability response. As with single-agent OSC inhibition, the targeting of enzymes in the lower part of the sterol pathway leads to the accumulation of polar sterols (Corio-Costet *et al*, 1988), which inhibit HMGCR enzymatic activity (Cohen and Griffioen, 1988), so it is not surprising that simultaneous inhibition of the protein prenylation and lower sterol pathways produces synergistic antiviral effects. However, downstream OSC inhibitors such as Ro48-8071 and U18666A have more subtle side effects, such as the rapid induction of cataracts and irreversible lens damage in animals (Pyrah *et al*, 2001; Cenedella *et al*, 2004) that pose serious challenges for their therapeutic use. Limiting these associated toxicities is a worthwhile goal for further development of inhibitors targeting OSC.

In conclusion, this study provides a comprehensive and unique perspective into the impact of sterol pathway regulation on HCV replication and provides compelling insight into the use of chemical combinations to maximize antiviral effects while minimizing proviral consequences. Our results suggest that HCV therapeutics developed against sterol pathway targets must consider the impact on underlying sterol pathway regulation. Inhibitors that prevent SREBP-2 activation, inhibit PGGT or encourage the production of polar sterols have great potential as HCV therapeutics if associated toxicities can be reduced.

Materials and methods

Cell culture and HCV replicon

The human hepatoma cell line Huh-7 (Nakabayashi *et al*, 1982) and Huh-luc/neo-ET cells (ReBLikon, GmbH) were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Invitrogen) supplemented with 10% fetal bovine serum (Gibco, Invitrogen), 1% penicillin/streptomycin (Gibco, Invitrogen), 1% Gluta MAX-1 (Gibco, Invitrogen) and 1% non-essential amino acids solution (Gibco, Invitrogen) at 37°C, 5% CO_2. Huh-luc/neo-ET cells were grown in medium additionally supplemented with 250 μg/ml geneticin (G418, Gibco, Invitrogen). These cells stably express an HCV genotype 1b subgenomic replicon encoding firefly (*Photinus pyralis*) luciferase, the coding sequence for ubiquitin and neomycin phosphotransferase downstream of the HCV IRES and upstream of an EMCV IRES, which mediates translation of downstream viral nonstructural proteins NS3 to NS5B (Vrolijk *et al*, 2003). For all experimental procedures Huh-luc/neo-ET and Huh-7 parental cells were seeded in DMEM without phenol red in the absence of G418 and penicillin/streptomycin (screening medium).

cHTS luciferase assay and cell proliferation inhibition assay

The cHTS procedure including plate formats is described elsewhere (Lehár *et al*, 2009). Cells were seeded in 30 μl of screening medium at 4000 cells/well on white (Huh-luc/neo-ET cells for viral inhibition assay) or black (Huh-7 cells for proliferation inhibition assay) 384-well assay plates (Matrix) and incubated overnight for ~20 h. Using a MiniTrak Robotic Liquid Handling System (Perkin-Elmer) 1 μl of compound stock solutions (1000 × concentration in DMSO unless otherwise mentioned) in an X (two-fold dilutions of compound horizontally arrayed) or Y (two-fold dilutions of compound vertically arrayed) format was transferred from master plates into 384-well clear bottom plates containing 100 μl screening medium (dilution plates) and mixed thoroughly. From each X and Y dilution plates, 3.3 μl was subsequently transferred to the 384-well assay plates for a final compound dilution of 1:1000 generating a 9 × 9 (81 point) dose–response matrix. The cells were then incubated for 48 h before measuring luciferase activity (viral inhibition) or ATP depletion (proliferation inhibition). In all, 25 μl of SteadyLite (Perkin-Elmer) was added to the white 384-well assay plates and 15 μl of ATPLite (Perkin-Elmer) was added to the black 384-well plates, which were subsequently incubated at least 5 min before measuring the luminescent signal. All luminescence measurements were assayed for 0.1 s per well with an EnVision Xcite multilabel automatic plate reader with Enhanced Luminescence (Perkin-Elmer) and expressed as the number of relative light units detected. Compounds were assayed in duplicate 9 × 9 dose matrices on each plate and DMSO-only control wells were included as negative untreated controls.

Immunoblot analysis

For protein expression analysis, Huh-luc/neo-ET cells were seeded in 4 ml of medium at 250 000 cells per well on six-well plates and allowed to adhere for 6–8 h. Stock solutions of compound were added at a 1:1000 dilution and cells were incubated in the presence of compound over 96 h. Medium and compounds were refreshed once after an initial incubation of 48 h. Cells were washed in phosphate-buffered saline (Gibco,Invitrogen) and lysed by the addition of 1 × RIPA lysis buffer (0.5 M Tris–HCl, pH 7.4/1.5 M, NaCl/2.5%, deoxycholic acid/10%, NP-40/10 mM EDTA, purchased from Upstate) containing Complete, Mini Protease inhibitor cocktail and PhosSTOP phosphatase inhibitor cocktail tablets (Roche) according to the manufacturer's recommendations. Cell lysates were rocked for 30 min at 4°C and centrifuged at 10 000 *g* for 10 min at 4°C. The protein concentration of each extract was determined by BCA protein assay (Pierce) according to the manufacturer's protocol. Aliquots of extract containing 6, 8 or 10 μg of protein were heated at 70°C for 10 min (excluding lysates for HMGCR detection to minimize protein multimerization), separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis using NuPAGE Novex precast 10% Bis–Tris gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Invitrogen). Membranes were blocked in 1 × TBS/0.1% Tween-20 (TBS-T) containing 5% non-fat milk before probing with the following primary antibodies overnight at 4°C on a rocker: mouse monoclonal anti-HCV NS5A IgG1 (1:1000, Virogen), mouse monoclonal anti-HCV NS3 IgG (1:1000, Virogen), mouse monoclonal anti-GAPDH (1:10 000, Ambion) or mouse polyclonal anti-HMGCR (1:500, Novus). Membranes were washed 3 × 5 min in TBS-T before adding a peroxidase-conjugated Immuno-Pure rabbit anti-mouse IgG secondary antibody (Pierce) and incubating 1 h at room temperature. Protein bands were visualized using the chemiluminescence reagents SuperSignal West Femto Maximum Sensitivity Substrate or SuperSignal West Pico Chemiluminescent Substrate (Pierce) and an Alpha Imager digital imaging system (Alpha Innotech).

RNA preparation and quantitative RT–PCR

Measurements of HCV RNA levels in response to drug were carried out by first seeding Huh-luc/neo-ET cells in 100 μl of medium at 7500 cells per well for 72 h drug treatments and allowed to adhere overnight for ~20 h. Compounds were added at a 1:1000 dilution in duplicate and added to cells in three separate experiments. Total RNA was collected using an RNeasy 96-well kit (Qiagen) according to the manufacturer's protocol and quantified using the Quant-iT RiboGreen RNA Reagent (Invitrogen). Purified RNA (4 μl) was added to TaqMan reactions containing 10 μl of QuantiTect Probe RT–PCR Master Mix (Qiagen) and 0.2 μl of QuantiTect RT Mix. For each HCV-specific reaction, 1.7 μM of forward (5′-CCATAGATCACTCCCCTGTG-3′) and reverse (5′-CCGG TCGTCCTGGCAATTC-3′) primers and 0.85 μM of HCV-specific TaqMan probe (5′-FAM-CCTGGAGGCTGCACGACACTCA-3′-BHQ) were added. All 20 μl reactions were assayed in an Eppy Twin-Tec skirted PCR plate (Eppendorf) and subjected to quantitative one-step RT–PCR with an Eppendorf Realplex4 qPCR machine (Eppendorf) using the following program: 50°C for 30 min, 95°C for 15 min and 40 cycles of 95°C for 15 s followed by 60°C for 1 min 15 s. Absolute quantification of HCV RNA copy number was determined by comparing PCR signals to a standard curve generated from dilutions of a 160 bp PCR-amplified fragment of the 5′NTR of HCV. The 5′NTR fragment was generated by using the HCV-specific forward and reverse primers mentioned above and serial 10-fold dilutions were made in nuclease-free water containing yeast tRNA (25 μg/μl) as a carrier. Concentration of the 160 bp HCV standard was determined by optical density spectrophotometry at 260 nm and the corresponding copy number was determined using the following formula for double-stranded DNA molecules: (g of standard $\times 6.023 \times 10^{23}$ molecules/mol)/(660 g/mol/base $\times$ length of amplified product in bases) (Giulietti *et al*, 2001; Dorak, 2006). All qPCR samples quantified by comparison to the standard curve were subsequently normalized to total RNA per sample to account for variations in sample purification and preparation steps.

Chemical reagents

Small molecule enzyme inhibitors used in this study were TOFA (CAS# 54857-86-2), Colestolone (CAS# 50673-97-7), SR-12813 (CAS# 126411-39-0), Simvastatin (CAS# 79902-63-9), Alendronate (CAS# 121268-17-5), Farnesol (CAS# 4602-84-0), Squalestatin (CAS# 142561-96-4), Clomiphene (CAS# 50-41-9), Ro48-8071 (CAS# 189197-69-1), U18666A (CAS# 3039-71-2), Terconazole (CAS# 67915-31-5), Amorolfine (CAS# 78613-35-1), Fenpropimorph (CAS# 67564-91-4), AY-9944 (CAS# 366-93-8), Triparanol (CAS# 78-41-1) and GGTI-286 (CAS# 171744-11-9). DMSO was the solvent used for most chemical probes in this study. Dithiothreitol (DTT) at 100 mM in DMSO was used as a solvent for GGTI-286 whereas ddH2O was used as a solvent for squalestatin and U18666A.

Calculations

Dose matrices were assembled from replicate combination blocks on experimental 384-well plates. Each plate had two replicate dose matrices along with ~20 vehicle-treated wells, and at least two copies of each plate were obtained, resulting in 4–20 replicate matrices per combination (Supplementary Table S1). Single-agent responses were tested at 11 serially diluted doses and combination data as 9×9 dose matrices each testing all pairs of 8 serially diluted single-agent concentrations along with their single-agent doses as a control. Raw phenotype measurements T from each treated well were converted to normalized measures of inhibitory activity $a = -\log_{10}(T/V)$ or fractional inhibition $I = 1 - T/V$ relative to the median V of 20 vehicle-treated wells arranged around the plate (Lehár *et al*, 2009). The uncertainty for each activity measurement was estimated using the standard error σ_a or σ_I from the variance between replicates at the same treatment doses, propagated through the activity measurement expression. Thus, the standard error for activity $\sigma_a = \log_{10}(2.712)\ \mathrm{sqrt}(\sigma_T^2/T^2 + \sigma_V^2/V^2)$, and for inhibition $\sigma_I = \mathrm{sqrt}(\sigma_T^2/T^2 + (1-I)\ \sigma_V^2/V^2)$. The error estimates for each combination, averaged over all doses, are given in Supplementary Table S1.

The synergy for each combination was determined using a non-standard SPE model, to properly characterize interactions between single agents that can both inhibit and activate replicon expression. For the SPE model, the expected activity is derived as $a_{SPE} = \max(a_{min}, \min(a_{max}, a_{min} + a_{max}))$, if a_{min} and a_{max} are the lesser and greater single-agent activities at the same concentrations as in a tested combination point. SPE represents a model of expected response for non-interacting drug targets when each drug could be either inhibitory or stimulatory. When both drugs act in the same direction, a_{SPE} at any pair of concentrations is equal to the less extreme of the single drug activities at the component concentrations. When they act in opposing directions (one stimulatory and the other inhibitory), a_{SPE} is simply the sum of the drug activities. Overall, synergy for a combination was measured using a synergy score $S = \sum_{doses} (a_{data} - a_{SPE})$, which is the sum of the differences between the measured activity and the SPE expectation, overall combined concentrations tested. Combinations with $S > 0$ have response surfaces that are mostly more inhibited than the SPE expectation, resulting either from synergistic activity for inhibitory agents (both with $a > 0$), or from the inhibitor's activity dominating at high combined concentrations for drugs with opposing activities. Similarly, combinations with $S < 0$ represent either antagonism between inhibitory agents or dominance of the stimulatory agent. For most of our combinations, the activity measurements at each dose produced errors of $\sigma_a \sim 0.1$, so we would expect a volume calculated across 64 combination doses to have a corresponding $\sigma_S \sim 1.6$. Thus, only scores that are > 3 or < -3 can be considered significant at the ~95% confidence level.

Supplementary information

Supplementary information is available at the *Molecular Systems Biology* website (www.nature.com/msb).

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Conflict of interest

The authors declare that they have no conflict of interest.

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