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 (FBS, 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% CO₂. Huh-luc.neo-ET cells were grown in medium additionally supplemented with 250μg/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 combination high throughput screening procedure including plate formats is described elsewhere⁵. 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 (Huh7 cells for proliferation inhibition assay) 384-well assay plates (Matrix) and incubated overnight for approximately 20 hours. Using a MiniTrak™ Robotic Liquid Handling System (Perkin-Elmer) 1 μl of compound stock solutions (1000X concentration in DMSO unless otherwise mentioned) in an X (2-fold dilutions of compound horizontally arrayed) or Y (2-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 plate, 3.3 μl was subsequently transferred to the 384-well assay plates for a final compound dilution of 1:1000 generating a 9 x 9 (81-point) dose response matrix. The cells were then incubated for 48 hours prior to measuring luciferase activity (viral inhibition) or ATP depletion (proliferation inhibition). 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 minutes before measuring the luminescent signal. All luminescence measurements were assayed for 0.1 sec per well with an EnVision™ Xcite multilabel automatic plate reader with Enhanced Luminescence (Perkin-Elmer) and expressed as the number of relative light units (RLU) detected. Compounds were assayed in duplicate 9x9 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 6-well plates and allowed to adhere for 6 – 8 hours. Stock solutions of compound were added at a 1:1000 dilution and cells were incubated in the presence of compound over 96 hours. Medium and compounds were refreshed once after an initial incubation of 48 hours. Cells were washed in phosphate-buffered saline (PBS, Invitrogen-Gibco) and lysed by the addition of 1X 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 minutes at 4°C and centrifuged at 10,000xg for 10 minutes 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 minutes (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 (PVDF, Invitrogen). Membranes were blocked in 1X TBS/0.1% Tween-20 (TBS-T) containing 5% non-fat milk prior to 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 3X 5 min in TBS-T prior to adding a peroxidase-conjugated ImmunoPure 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**

Measurement 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 7,500 cells per well for 72 hour drug treatments and allowed to adhere overnight for approximately 20 hours. Compounds were added at a 1:1000 dilution in duplicate and added to cells in 3 separate experiments. Total RNA was harvested 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'-CCATAGATCACTCCCTGTG-3') and reverse (5'-CCGGTGTCTCCTGCAATTC-3') primers and 0.85 μM of HCV-specific TaqMan probe (5'-FAM-CCTGGAGGCTGCACGACACTCA-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 x 6.023 x 10^{23} molecules/mole) / (660 g/mol/base x length of amplified product in bases) (Dorak, 2006; (Giulietti et al, 2001). 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.

**Validation of chemical effects on viral protein expression**

To confirm effects on viral activity due to single agent treatments (Fig. 1b), Huh-luc/neo-ET replicon cells were treated with chemical probes for 96 hours. Cell lysates were harvested and antibodies for NS3, NS5A, and GAPDH were used to probe western transfers of proteins separated by 10% Bis-Tris SDS/PAGE. Protein bands were quantified using densitometry and amounts of HCV proteins NS3 and NS5A are shown as percentages normalized to GAPDH. Drug Concentrations (in μM): Colestolone (3.75), Simvastatin (3.25), SR 12813 (7.5), Farnesol (15.0), GGTTI-286 (5.0), Squalastatin (0.63), Clomiphene (1.87), U18666A (0.1), Ro 48-8071 (0.02), Terconazole (3.75), Amorolfin (10.0), Fenpropimorph (15.0), AY-9944 (0.94), Triparanol (1.87), 2’-C-methylcytidine (10.0).
To validate the epistasis on HCV protein expression levels, S Cell lysates were harvested and antibodies for NS3, NS5A, and GAPDH were used to probe western transfers of proteins separated by 10% Bis-Tris SDS/PAGE. Protein bands were quantified using densitometry and amounts of HCV proteins NS3 and NS5A are shown as percentages normalized to GAPDH. Drug Concentrations (in μM): 2′-C-methylcytidine (10.0), U18666A x Simvastatin (0.04 x 2.8), Simvastatin (2.8), U18666A (0.04)

Impact of sterol pathway chemical probes on HMGCR protein expression. Huh7 cells were treated for 16 hours with each indicated chemical probe and total cell lysates were harvested. Antibodies specific for HMGCR and GAPDH were used to probe western transfers of proteins separated by 10% Bis-Tris SDS/PAGE. Drug concentrations (in μM): Colestolone (13.5), Simvastatin (11.3), SR 12813 (13.5), Farnesol (27.0), Squalestatin (9.0), U18666A (9.0), Ro 48-8071 (6.8), Terconazole (3.4), Amorolfine (13.5), AY-9944 (3.4) and Triparanol.

Impact on HCV RNA replication by chemical probes which stimulate HMGCR expression. Huh-luc.neo-ET cells were treated with each indicated chemical probe for 72 hr. Values represent averages of the % inhibition of HCV RNA from 3 separate RT-qPCR experiments ± standard deviations after normalizing viral copy number to total cellular RNA.

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), Ro 48-8071 (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 while ddH2O was used as a solvent for squalestatin and U18666A.

Calculations

Dose matrices were assembled from replicate combination blocks on experimental 384-well pates (Lehár et al, 2009). 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. After normalization, we calculated average activity values between replicate measurements at the same treatment doses, along with \( \sigma_a \) or \( \sigma_i \), the accompanying standard error estimates. Single agent responses were tested at eleven serially-diluted doses and combination data as 9x9 dose matrices each testing all pairs of 8 serially-diluted single agent concentrations along with their single agent doses as a control.

The synergy for each combination was determined using a superposition of effect model (SPE), where \( a_{\text{SPE}} = \max(a_{\min}, \min(a_{\max}, a_{\min} + a_{\max})) \), 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, \( a_{\text{SPE}} \) 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), \( a_{\text{SPE}} \) is simply the sum of the drug activities. Overall synergy for a combination was measured using a synergy score \( S = \sum_{\text{doses}} (a_{\text{data}} - a_{\text{SPE}}) \), which is the sum of the differences between the measured activity and the SPE expectation, over all 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.

**Supplementary References**


Supplementary Figures

**Figure S1a.** Diagram of the cholesterol biosynthetic pathway (upper section). Shown are the major intermediates and enzymes for cholesterol biosynthesis. The chemical probes used in this study are shown along with the enzymatic targets that they modulate.
Figure S1b. Diagram of the cholesterol biosynthetic pathway (lower section). Shown are the major intermediates and enzymes for cholesterol biosynthesis. The chemical probes used in this study are shown along with the enzymatic targets that they modulate.
Figure S2. Dose matrices in the HCV replicon expression assay. Overview of the replicon responses showing combination activity. Corresponding host activity as displayed in Sup. Fig. S3. Chemical probes were chosen that modulate targets relating to the sterol biosynthesis pathway, and dose matrix data were obtained testing all pairs of serially-diluted concentrations for each pair of probes. The response matrices displayed show the activity $a$ across all dose pairs in a combination, in terms of the fold increase over vehicle-treated wells ($a = +1$ corresponds to a tenfold decrease in replicon activity).
Figure S3. Overview of the host activity responses, to be compared with Fig. S2. Chemical probes were chosen that modulate targets relating to the sterol biosynthesis pathway, and dose matrix data were obtained testing all pairs of serially-diluted concentrations for each pair of probes. The response matrices displayed on the lower-left hand side of the grid show the activity $a$ across all dose pairs in a combination, in terms of the fold increase over vehicle-treated wells ($a = +1$ corresponds to a tenfold decrease in cell proliferation activity).