Supporting Information

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SI Materials and Methods

Anti-HCV Molecules and Reagents. The LOPAC^{1280} small-scale, small-molecule library containing 1280 compounds in 96-well format, each concentrated at 10 mM in DMSO solution, was purchased from Sigma-Aldrich. 2′-C-methyladenosine (2′CMA), VX-950, human anti-CD81 JS-81 mAb, and human IFN α-2a were obtained from Carbosynth (Berkshire, UK), Stereo Chem Research (Hyderabad, India), BD Biosciences, and PBL, respectively. For characterization of the effect of drug hits on HCV replication, assembly/release, and entry, small molecules were purchased separately from Sigma-Aldrich and dissolved in DMSO to a 10-mM stock concentration. The CellTiter-Glo Luminescent Cell Viability Assay kit for cell viability measurements was purchased from Promega. The BioLux *Gaussia* Luciferase Assay Kit for *Gaussia* luciferase (Gluc) activity assays was purchased from New England Biolabs.

Cell Culture Reagents. Unless otherwise specified, growth medium for all cell culture work was DMEM containing 4500 mg/L glucose, 4.0 mM L-glutamine, and 110 mg/L sodium pyruvate (Thermo Scientific HyClone) and supplemented with 10% FBS (Atlanta Biologicals) and 1× nonessential amino acids (Thermo Scientific HyClone).

Generation and Titering of Infectious HCVcc. The chimeric genotype 2a HCV, Jc1, used for the n4mBid cell-protection small-molecule screen and for quantitative RT–PCR and infectious virus production studies of leading HCV replication/infectious virus production inhibitor candidates (Fig. 3*A*) was previously described (1). The *Gaussia* luciferase (Gluc)-expressing Jc1FLAG(p7-nsGluc2A) HCV used for probing the effect of inhibitors on HCV replication and infectious virus production has been reported elsewhere (2). HCVcc was generated from Huh-7.5 hepatoma cells as previously described (3). Briefly, using the Ampliscribe T7 high-yield transcription kit (Epicentre), RNA was transcribed in vitro from the full-length HCV genome and electroporated into Huh-7.5 cells using a ECM 830 electroporator (Harvard Apparatus) at the following settings: 650 V, 99-μs pulse length, 5 pulses, 1.1-s interval. Infectious supernatants were collected at 96, 120, 144, and 168 h post electroporation and frozen at −80 °C until later use. Infectious titers were quantified by limiting dilution titration on naive Huh-7.5 cells (4). Briefly, Huh-7.5 cells were seeded in 96-well plates at 6400 cells/well. The next day, HCVcc-containing supernatant was applied at serial 10-fold dilutions ranging from 10^{-1} to 10^{-6} and, after 72 h, infection was detected by staining for NS5A with the 9E10 anti-NS5A primary antibody and PE-conjugated goat anti-mouse secondary antibody. Median tissue culture infectious doses ($TCID_{50}$)/mL were calculated using the method of Reed and Muench (5).

Gluc Activity Assay. Gluc assays for both HCV replication/infectious virus production and entry studies were carried out as follows: 8 μL of Gluc-containing supernatant was dispensed into white 96-well plates (Corning). Gluc activities were determined using a Berthold Centro LB 960 luminometer using the following sequence of events: inject 20 μL Gluc substrate, shake for 2 s, delay for 5 s, and measure for 5 s.

Production of HCV Pseudoparticles and Entry Inhibition Assay. To create the Gluc-expressing lentiviral provirus pTRIP-Gluc, the Gluc gene was amplified from pCMV-Gluc (New England Biolabs) using the primers 5′-ATCTTAGGATCCCCACCGGTCGCCACCATGGGAGTCAAAGTTCTGTTTGCCC and 5′-ATCTTACTCGAGTTAGTCACCACCGGCCCC and cloned into the BamHI and XhoI restriction sites of the pTRIP vector (6, 7). The vector expressing the HCV H77 E1E2 envelope glycoprotein sequence (residues 170–746) that was used to generate HCVpp and was based on the pcDNA3.1(−)neo vector (Invitrogen) has been previously described (8). To generate HCVpp and controls, 293T cells were transfected with a 1:1:4 weight ratio of pTRIP-Gluc, HIV gag-pol (9), and one of HCV H77 E1E2, VSV-G (9), or empty pcDNA3.1(−)neo vector, giving rise to HCVpp, VSV-Gpp, and envelope-deficient pseudoparticles (Env^{-}pp), respectively. On the day before transfection, 8×10^5 293T cells were seeded in a 35-mm well. The following day a total of 1.5 μg DNA was transfected using 7.5 μL TransIT-LT1 (Mirus). Media was replaced after 6 h. Supernatants were harvested 48 h after transfection, pooled, filtered (0.45-μm pore size), and stored at 4 °C for up to 1 week.

For entry inhibition studies, Huh-7.5 cells were seeded in 96-well plates at 1.8×10^4 cells/well. Inhibitors were added to cells 6–8 h post seeding, followed by incubation at 37 °C in a 5% CO_2 atmosphere for an additional hour. HCVpp, Env^{-}pp, and VSV-Gpp were subsequently added to cells at 1:5, 1:5, and 1:500 dilutions, respectively, in the continued presence of drugs. After 12–14 h, the pseudoparticle/inhibitor-containing media was aspirated, and cells were washed 5 times with growth media and incubated at 37 °C/5% CO_2. Forty-eight hours after washing, Gluc-containing supernatants were collected for determination of Gluc activities as described in *Gluc Activity Assay*. A neutral red uptake assay for the determination of viable cell counts was carried out on the remaining cells immediately after collection of Gluc supernatants, as previously described (10). Raw Gluc data were normalized to viable cell levels from the relevant wells, as determined from the neutral red cell viability assay. All of the presented HCVpp entry data represent the difference in viable-cell-level–adjusted supernatant Gluc activities between HCVpp- and Env^{-}pp-transduced cells for a given small-molecule treatment condition.

Small-Molecule Cytotoxicity Determination for Entry Inhibition Dose–Response Assay. Dose-dependent cytotoxicity of entry inhibitors (Fig. 2) was determined in separate plates but in parallel with entry inhibition dose–response assays. Huh-7.5 cells were seeded at the same density as for the entry inhibition assay in 96-well plates. Inhibitors were added 6–8 h after seeding at the same time as inhibitor addition to the entry inhibition assay plates, followed by incubation at 37 °C/5% CO_2. After 12–14 h, at the same time as the inhibitor removal/washing step in the entry inhibition assay, inhibitor-containing media was aspirated and replaced with fresh growth media. Six hours later, cell viability was determined using the CellTiter-Glo assay kit, as described in *Materials and Methods* (main text).

Gluc-Based Determination of HCV Replication and Infectious Virus Production Inhibition. For a preliminary determination of the effect of drug hits on HCV replication and infectious virus assembly and release (Table 1), a Gluc reporter HCV system was used. Briefly, Huh-7.5 cells electroporated with Jc1FLAG(p7-nsGluc2A) RNA (see *Generation and Titering of Infectious HCVcc*) were seeded in 24-well plates, and small molecules were added to the cells 6 h post seeding. Forty-eight hours post electroporation, Gluc-containing infectious supernatants were collected, and Gluc activities were measured to give replication inhibition data. On the same day as the supernatant collection, 10 μL of supernatant was used to infect freshly seeded Huh-7.5 cells in 24-well plates containing 1 mL of growth media per well, resulting in a 100-fold dilution of the original infectious supernatant in inhibitor-free media. Twelve to 14 h post

infection, cells were washed twice with 1 mL complete growth media to remove preexisting Gluc, and 0.5 mL growth media was added to each well before incubation at 37 °C in a 5% CO_2 atmosphere. Forty-eight hours post washing, supernatants were collected, and Gluc activities were measured to give supernatant infectivity data. Gluc-containing supernatants were stored for up to a week at 4 °C before measuring Gluc activities.

Quantitative RT–PCR and Determination of Supernatant HCV Infectivity. For determination of the inhibitory effect of leading drug hits on HCV replication and infectious virus production, Huh-7.5 cells were electroporated with Jc1 HCV genomic RNA as described in *Generation and Titering of Infectious HCVcc* and seeded in 24-well plates in duplicates for each drug. Six hours post electroporation, appropriately concentrated small molecules were added to cells. Forty-eight hours post electroporation, media was replaced with fresh small-molecule–containing media. Ninety-six hours post electroporation, the supernatant from one small-molecule–treated cell replicate was collected and frozen at −80 °C for determination of infectious HCV levels by $TCID_{50}$ assay (see *Generation and Titering of Infectious HCVcc*), and the remaining cells were washed four times with Dulbecco's phosphate-buffered saline and then frozen for subsequent extraction of total cellular RNA using the RNeasy Mini kit (Qiagen) and quantitative reverse transcriptase real-time PCR determination of intracellular HCV RNA levels. Supernatant from the second small-molecule–treated cell replicate was aspirated, and cell viability was determined using the CellTiter-Glo assay as described in *Materials and Methods* (main text). Supernatant infectious virus levels in Fig. 3*A* are HCVcc $TCID_{50}$/mL values normalized to viable cell levels determined as just described. For the quantitative RT–PCR, primers targeting the HCV genome were 5′-CGGGAGAGCCATAGTGG and 5′-AGTACCACAAGGCCTTTCG. As an internal control, mRNA levels of phosphoglycerate kinase 1 (PGK1) were determined for each RNA template using the primers 5′-TGAAGAGGGAGCCAAGATTGTC and 5′-CAGTGACAAAGTCAACAGGCAAGG. Real-time measurements were carried out in a CFX96 Real-Time PCR Detection System (Bio-Rad), using the iScript One-Step RT–PCR kit with SYBR Green (Bio-Rad). Quantitative RT–PCR reactions were carried out in duplicate and normalized to mRNA levels of the PGK1 gene. Reaction efficiencies for each primer set were determined from the slope of a standard curve, and were used to adjust relative expression levels according to the method of Pfaffl (11).

Cytotoxicity of Inhibitors on Electroporated Huh-7.5 Cells. For determination of inhibitor cytotoxicities on Huh-7.5 cells electroporated without HCV RNA (Fig. 3), Huh-7.5 cells were electroporated as described in *Generation and Titering of Infectious HCVcc*, but with only sterile water instead of HCV RNA. Cells were seeded in 24-well plates, and small-molecule–containing media was added 6 h post electroporation. Small-molecule-containing media was refreshed 48 h post electroporation. Ninety-six hours post electroporation, supernatants were aspirated and cell viabilities were determined using the CellTiter-Glo Luminescent Cell Viability Assay kit. Briefly, after aspiration of media from wells, 200 μL of CellTiter-Glo reagent diluted fivefold in water was added to cells, followed by gentle vortexing for 2 min and incubation at room temperature for an additional 8 min. A total of 10 μL of sample from each well was transferred to a white 96-well plate, and luminescence was measured in a Berthold Centro LB 960 luminometer for 0.1 s.

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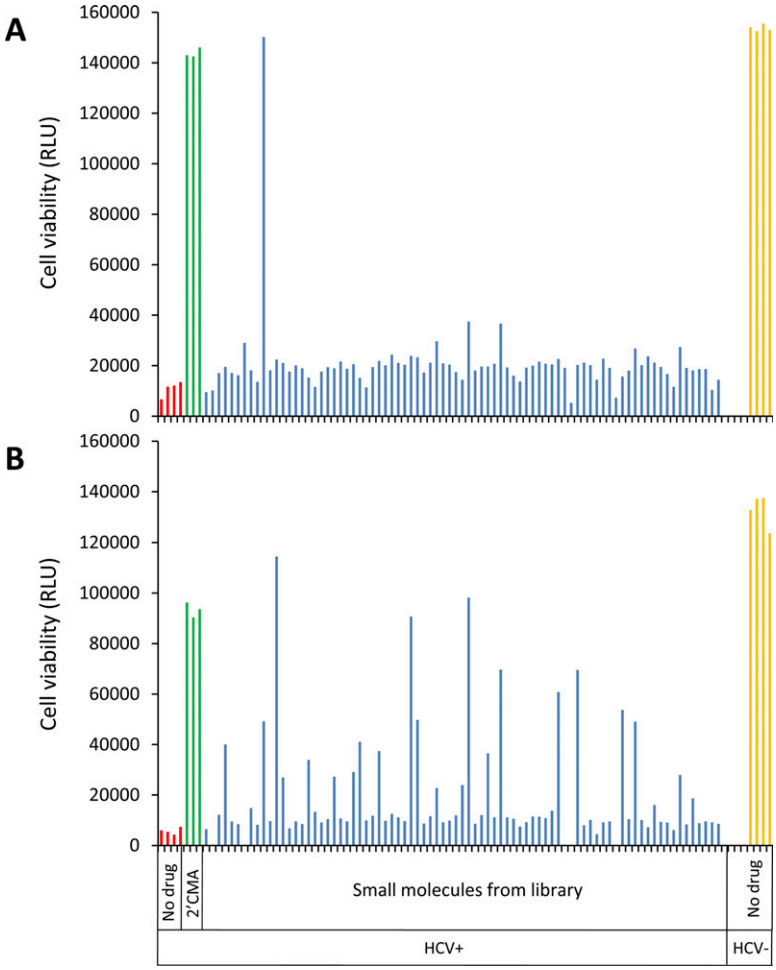
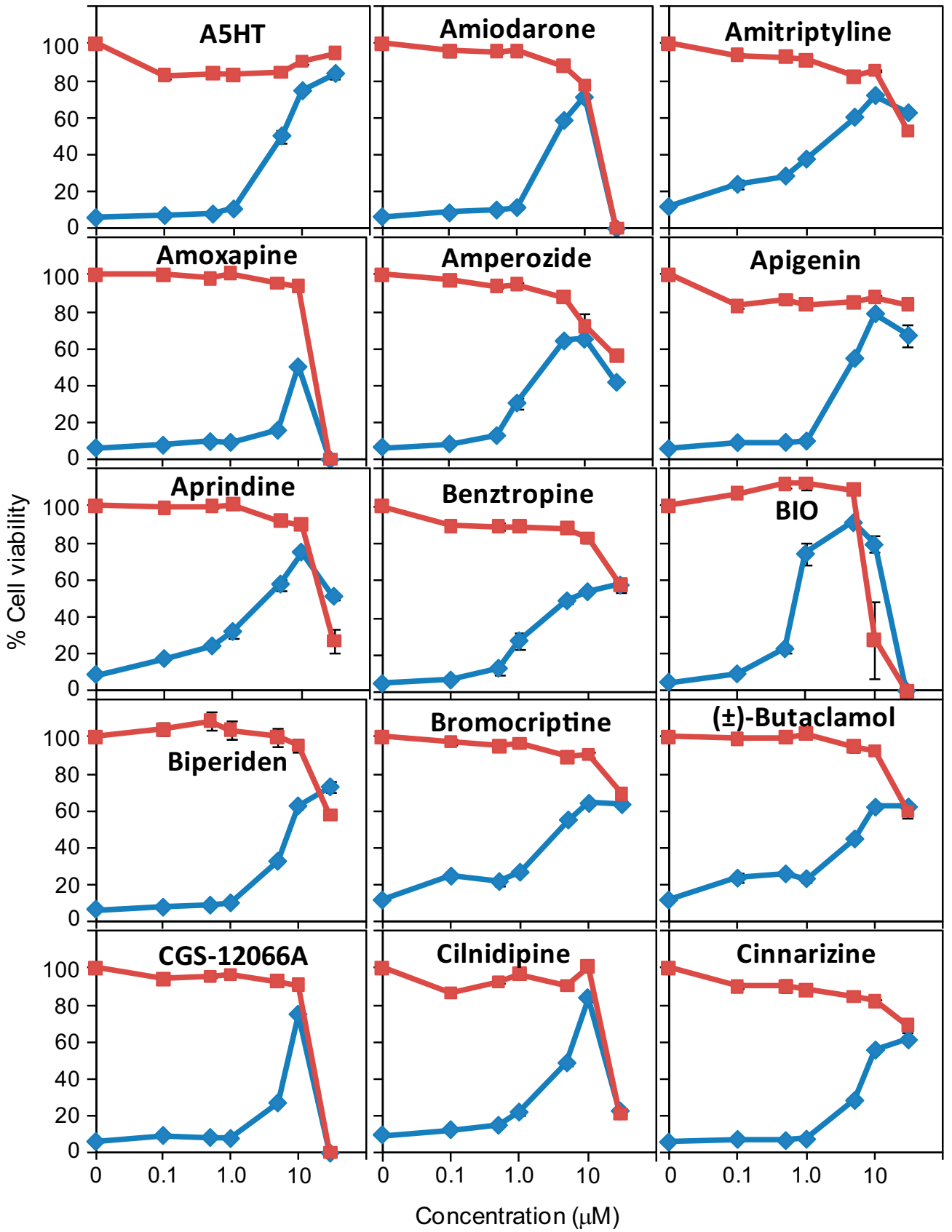


Fig. S1. Sample results from n4mBid cell protection screen for anti-HCV molecules. N4mBid cells, HCVcc (MOI 0.5–1.5), and drugs concentrated at 1 μM (*A*) and 10 μM (*B*) were added to 96-well plates, and plates were incubated at 37 °C and 5% CO_2 for 4 days before cell viability was measured using CellTiter-Glo assay. For "No drug" controls, growth media containing 0.1% DMSO was used in place of small-molecule–containing media. For "HCV−" control, HCVcc was replaced with UV-inactivated HCVcc. RLU, relative luciferase units. 2′CMA, 2′-C-methyladenosine.



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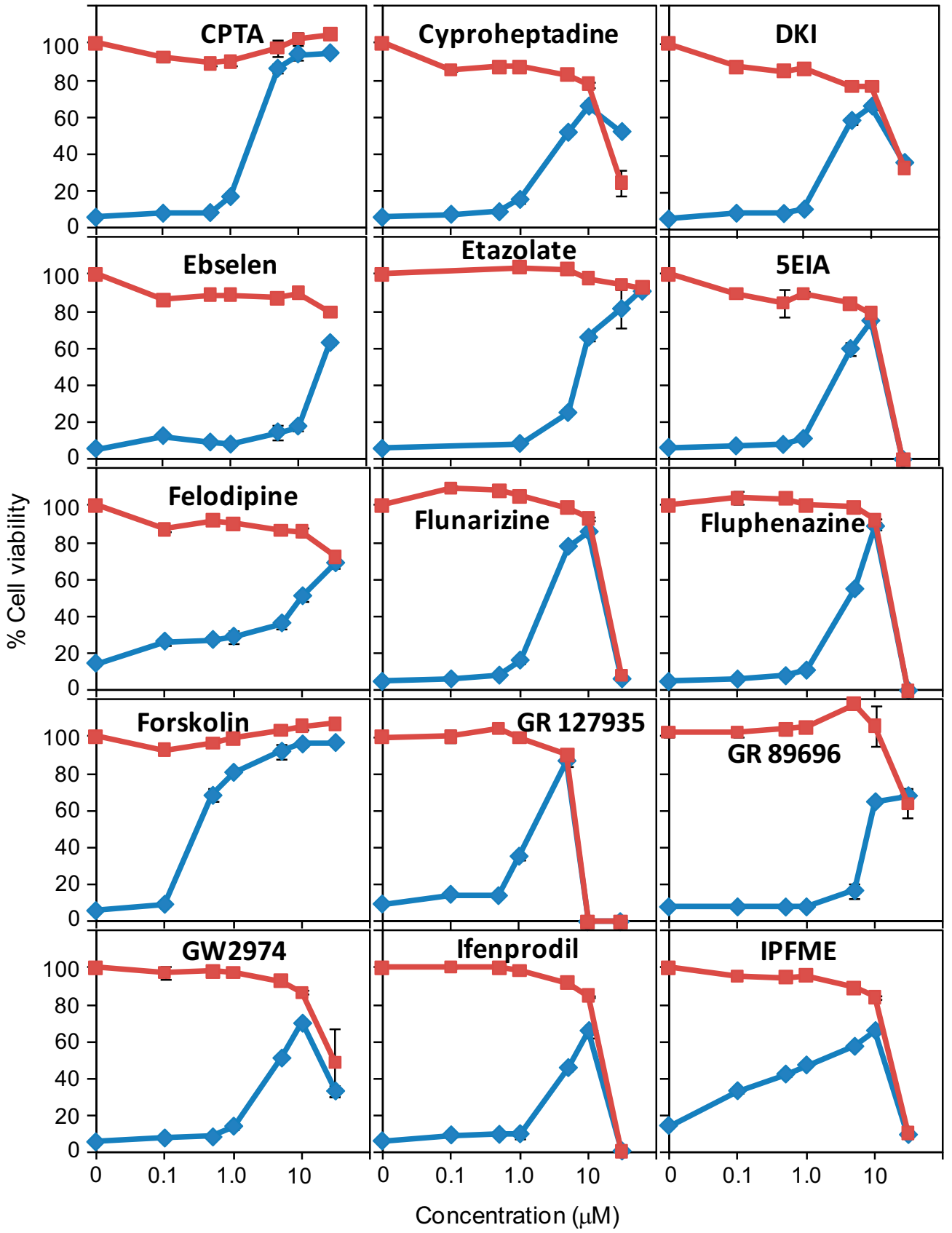

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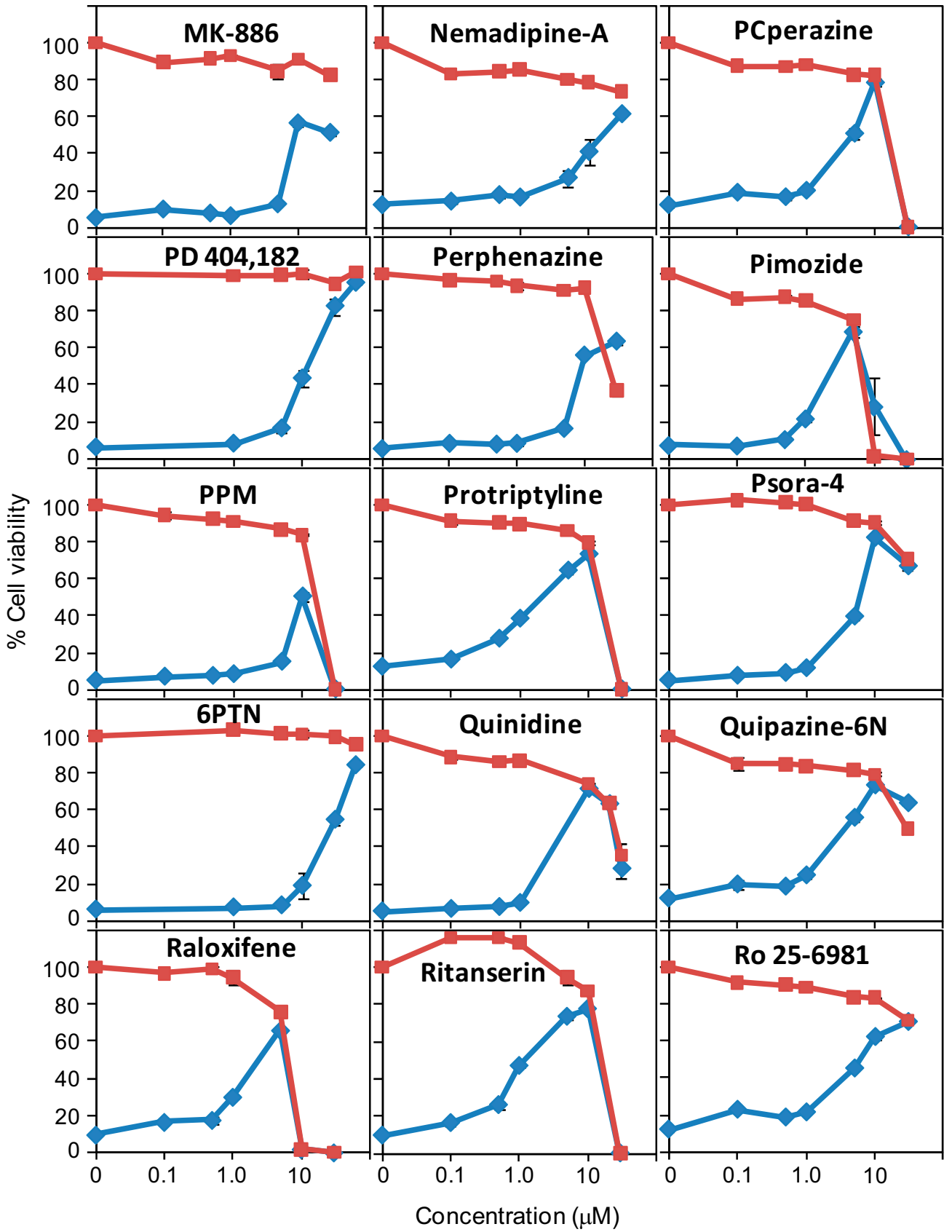

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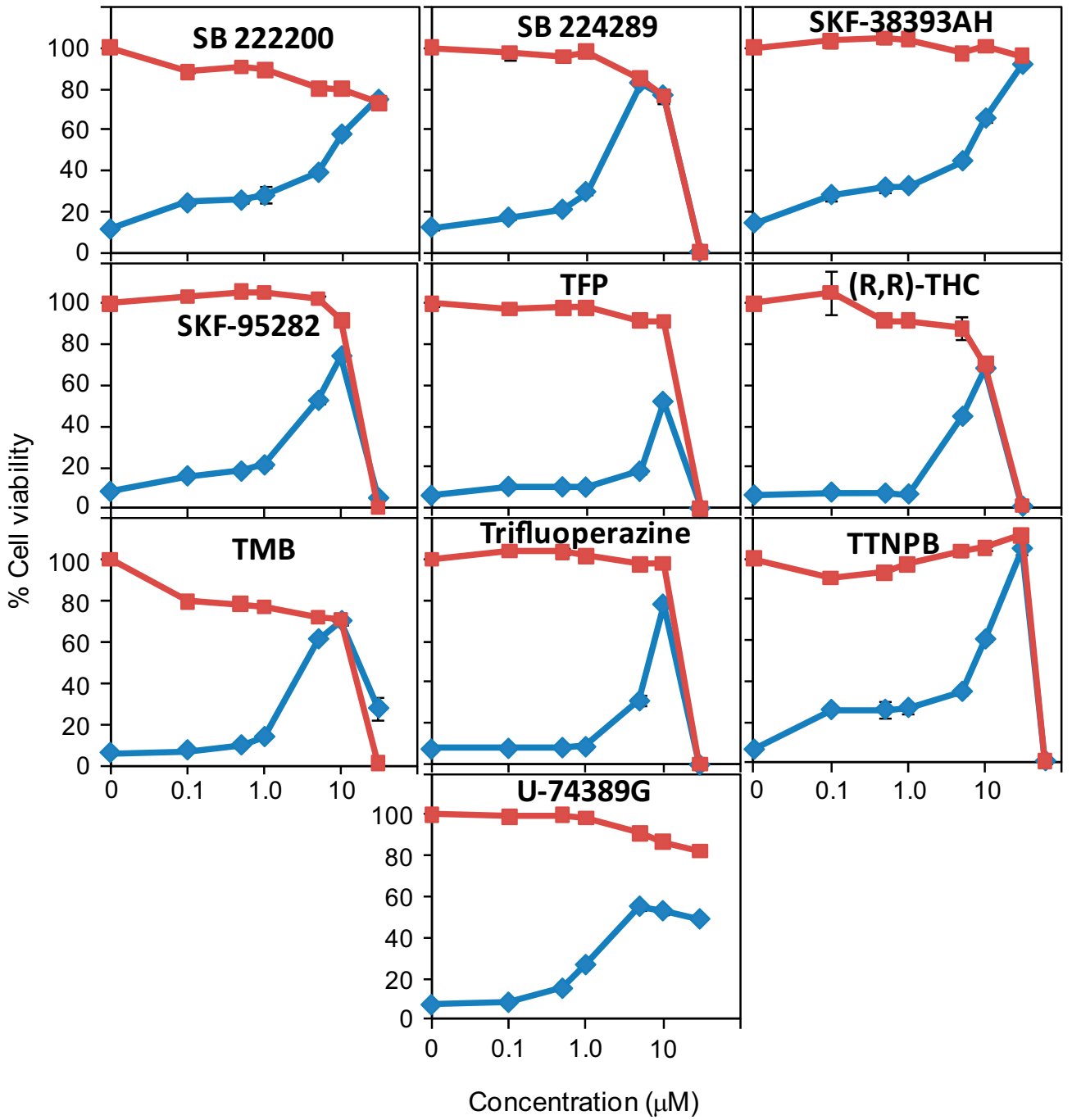

Fig. S2. Dose–response curves of leading anti-HCV drug hits from small-molecule screen reporting n4mBid cell viability in the presence (blue) and absence (red) of Jc1 HCVcc infection. Cell viability data are presented as a percentage of mock-treated cells in the absence of both HCVcc infection and small molecules. Values are the mean ± SD of duplicate measurements. Data shown are representative of two independent experiments.

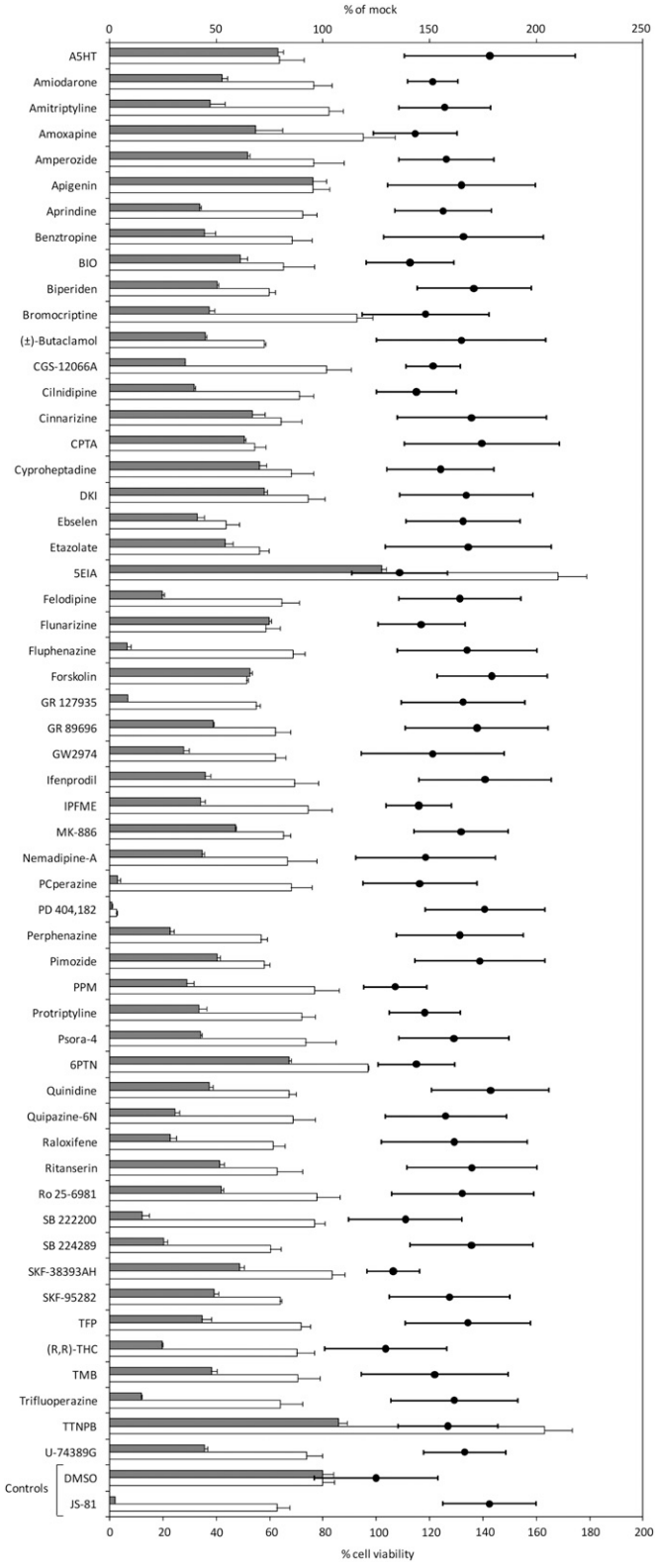

Fig. S3. Gluc data reporting the effect of 55 leading anti-HCV drug hits on the entry of HCVpp (shaded bars) and VSV-Gpp (open bars) into Huh-7.5 cells. Entry levels are based on the viable cell level-adjusted supernatant Gluc activity of Huh-7.5 cells 48 h after transduction with Gluc reporter lentivirus pseudotyped with H77 HCV E1E2, VSV-G, or no envelope (control). Gluc activities were adjusted for viable cell levels as determined by a neutral red uptake assay, and the background reading, namely the viable-cell-level-adjusted supernatant Gluc activity of Env$^-$pp-transduced cells, was subtracted for each small-molecule treatment condition. Concentrations of small molecules used are specified in Table 1 (SDC). Anti-CD81 antibody JS-81 (1 μg/mL) is included as a positive control. Cell viability of pseudoparticle-transduced cells (solid circles) was determined by a neutral red uptake assay 48 h post transduction (mean of $n = 6$, error bars, SD). Gluc activities are the mean ± SD of duplicate measurements. All data are expressed as a percentage of 0.1% DMSO-treated cells and are representative of at least two independent experiments.

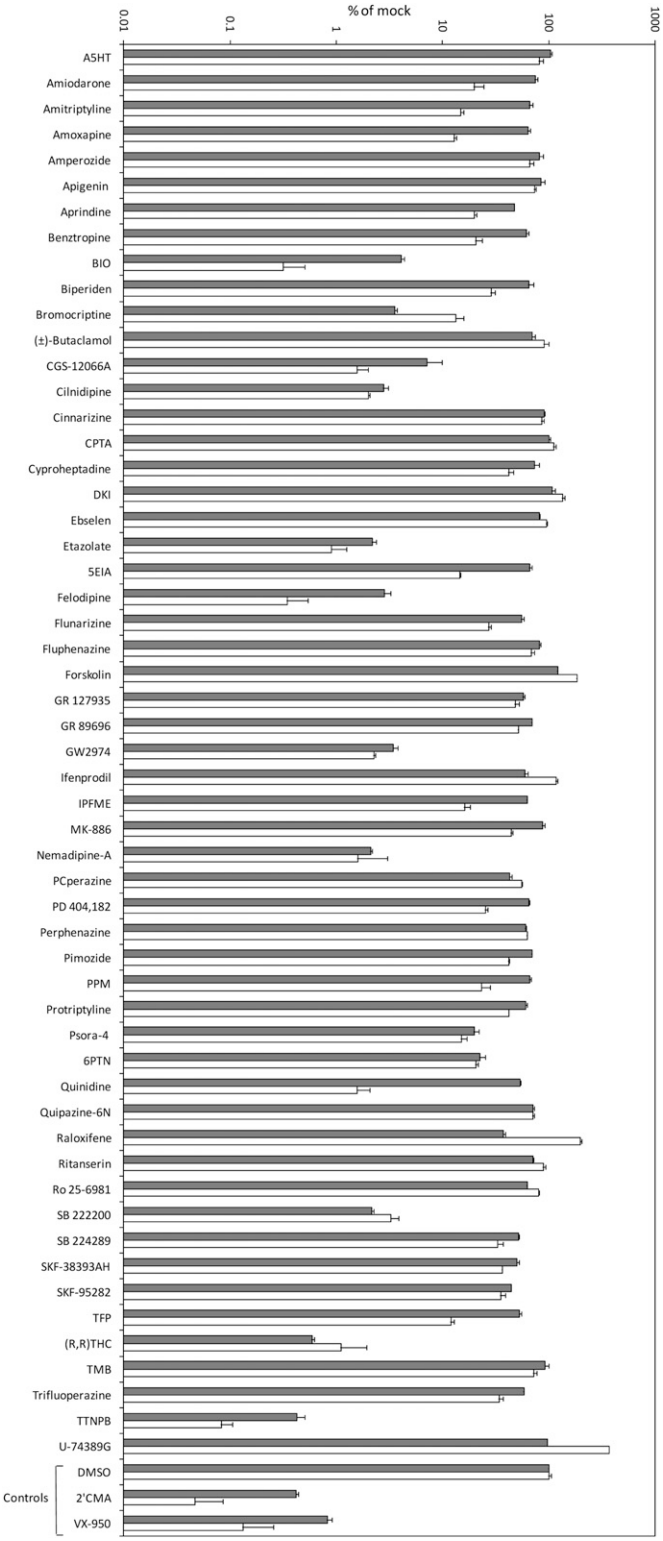

Fig. S4. Gluc data reporting the effect of 55 leading anti-HCV drug hits on replication (shaded bars) and infectious virus production (IVP, open bars) of a Gluc reporter strain of HCV. Virus replication levels were determined by measuring supernatant Gluc activities of Huh-7.5 cells 48 h after electroporation with genomic RNA of a Gluc reporter HCV and subsequent treatment with small molecules. IVP levels represent supernatant Gluc activities of Huh-7.5 cells 48 h after infection with 100-fold dilutions of the aforementioned 48-h post-electroporation supernatants and subsequent washing to remove preexisting Gluc. Concentrations of small molecules used are specified in Table 1 (SDC). The viability of all small-molecule–treated cells 48 h after electroporation with HCV genomic RNA was determined by visual inspection to be at least ~60% that of the 0.1% DMSO-treated control cells. Gluc activities are expressed as a percentage of 0.1% DMSO-treated cells (mean of $n = 2$; error bars, SD). Data are representative of at least three independent experiments.

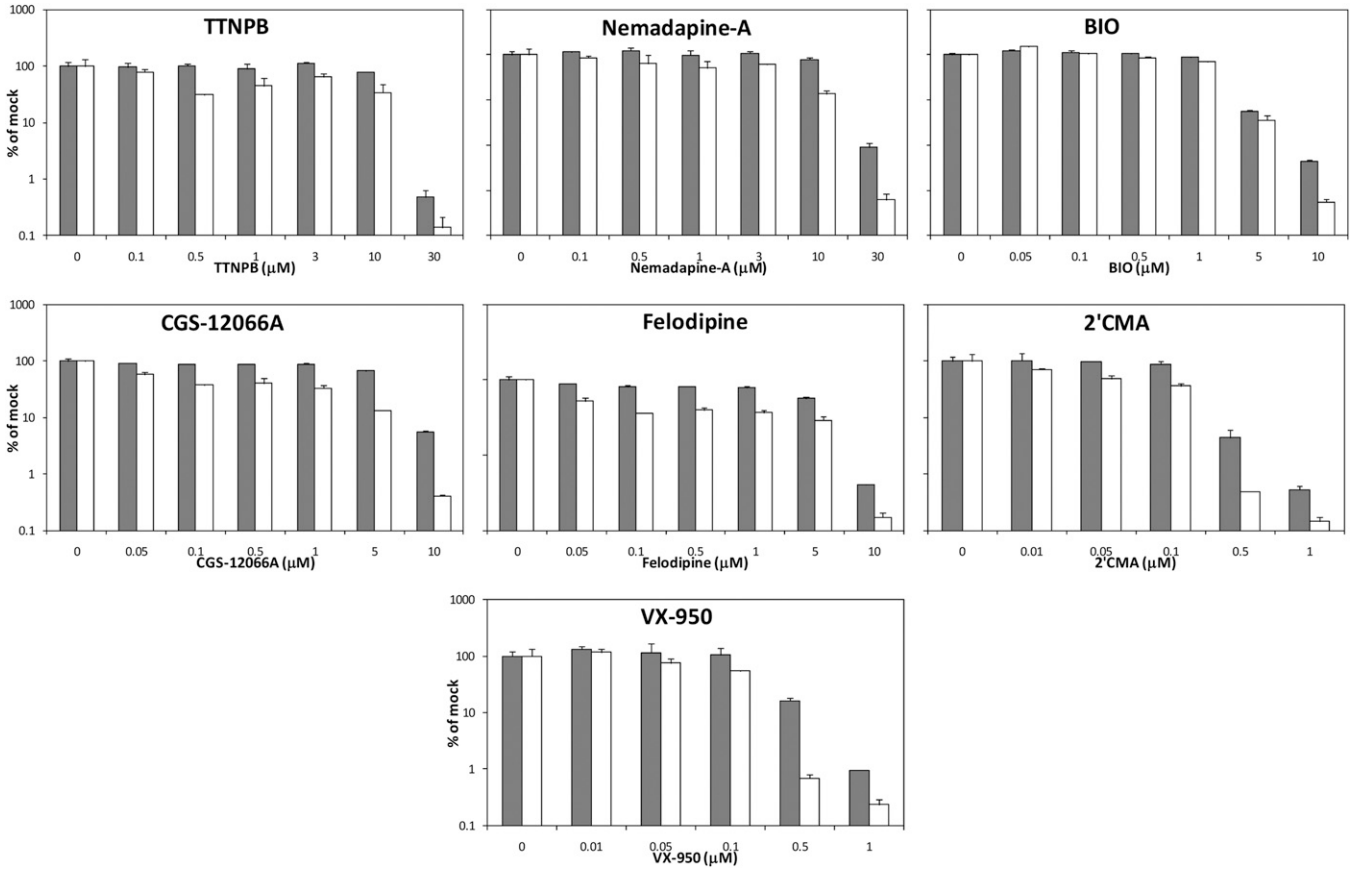

Fig. S5. Dose-dependent inhibition of replication (shaded bars) and infectious virus production (open bars) of a Gluc reporter strain of HCV by selected drug hits, carried out according to the same procedure as for Fig. S4 (mean of $n = 2$; error bars, SD).

Table S1. Abbreviations and full names of major hits from anti-HCV small-molecule screen

Shortened name	Full name	Sigma catalog no.
A5HT	*N*-Acetyl-5-hydroxytryptamine	A 1824
Amiodarone	Amiodarone hydrochloride	A 8423
Amitriptyline	Amitriptyline hydrochloride	A 8404
Amoxapine	Amoxapine	A-129
Amperozide	Amperozide hydrochloride	A 6976
Apigenin	Apigenin	A 3145
Aprindine	Aprindine hydrochloride	A 7606
Benztropine	Benztropine mesylate	B 8262
BIO	(2′Z,3′E)-6-Bromoindirubin-3′-oxime	B 1686
Biperiden	Biperiden hydrochloride	B 5311
Bromocriptine	(+)-Bromocriptine methanesulfonate	B 2134
(±)-Butaclamol	(±)-Butaclamol hydrochloride	B-168
CGS-12066A	CGS-12066A maleate	C-106
Cilnidipine	Cilnidipine	C 1493
Cinnarizine	Cinnarizine	C 5270
CPTA	8-(4-Chlorophenylthio)-cAMP sodium	C 3912
Cyproheptadine	Cyproheptadine hydrochloride	C 6022
DKI	Diacylglycerol kinase inhibitor I	D 5919
Ebselen	Ebselen	E 3520
Etazolate	Etazolate hydrochloride	E 1896
5EIA	5-(N-Ethyl-N-isopropyl)amiloride	A 3085
Felodipine	Felodipine	F 9677
Flunarizine	Flunarizine dihydrochloride	F 8257
Fluphenazine	Fluphenazine dihydrochloride	F 4765
Forskolin	Forskolin	F 6886
GR 127935	GR 127935 hydrochloride hydrate	G 5793
GR 89696	GR 89696 fumarate salt	G-133
GW2974	GW2974	G 0668
Ifenprodil	Ifenprodil tartrate	I 2892
IPFME	3-(^{1}H-Imidazol-4-yl)propyl di(p-fluorophenyl)methyl ether hydrochloride	I-160
MK-886	MK-886	M 2692
Nemadipine-A	Nemadipine-A	N 4163
PCperazine	Prochlorperazine dimaleate	P 9178
PD 404,182	PD 404,182	P 2742
Perphenazine	Perphenazine	P 6402
Pimozide	Pimozide	P 1793
PPM	Propionylpromazine hydrochloride	P 7780
Protriptyline	Protriptyline hydrochloride	P 8813
Psora-4	Psora-4	P 9872
6PTN	6(5H)-Phenanthridinone	P 8852
Quinidine	Quinidine sulfate	Q 0875
Quipazine-6N	Quipazine, 6-nitro-, maleate	Q-109
Raloxifene	Raloxifene hydrochloride	R 1402
Ritanserin	Ritanserin	R-103
Ro 25–6981	Ro 25–6981 hydrochloride	R 7150
SB 222200	SB 222200	S 5192
SB 224289	SB 224289 hydrochloride	S-201
SKF-38393AH	(±)-SKF-38393, N-allyl-, hydrobromide	S-168
SKF-95282	SKF-95282 dimaleate salt	S 5317
TFP	Triflupromazine hydrochloride	T 2896
(R,R)-THC	(R,R)-cis-Diethyl tetrahydro-2,8-chrysenediol	D 8690
TMB	TMB-8 hydrochloride	861804
Trifluoperazine	Trifluoperazine dihydrochloride	T 8516
TTNPB	Arotinoid acid	T 3757
U-74389G	U-74389G maleate	U 5882