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Hepatitis C virus complete life cycle screen for identification of small molecules with pro- or antiviral activity

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ABSTRACT

Infection with the hepatitis C virus represents a global public health threat given that an estimated 170 million individuals are chronically infected and thus at risk for cirrhosis and hepatocellular carcinoma. A number of direct antiviral molecules are in clinical development. However, side effects, drug resistance and viral genotype-specific differences in efficacy may limit these novel therapeutics. Therefore, a combination of well tolerated drugs with distinct mechanisms of action targeting different steps of the viral replication cycle will likely improve viral response rates and therapy success.

To identify small molecules that interfere with different steps of the HCV replication cycle, we developed a novel dual reporter gene assay of the complete HCV life cycle and adapted it to 384-well high-throughput format. The system is based on a highly permissive Huh-7 cell line stably expressing a secreted luciferase. Using these cells and an efficient HCV luciferase reporter virus, perturbations of each step of the viral replication cycle as well as cell viability can be easily and quantitatively determined. The system was validated with a selected set of known HCV entry, replication and assembly inhibitors and then utilized to screen a library of small molecules derived from myxobacteria. Using this approach we identified a number of molecules that specifically inhibit HCV cell entry, or primarily virus assembly and release. Moreover, we also identified molecules that increase viral propagation. These compounds may be useful leads for development of novel HCV inhibitors and could be instrumental for the identification of as yet unknown host-derived viral resistance and dependency factors.

1. Introduction

HCV is a positive-sense, hepatotropic RNA virus of the family *Flaviviridae* (Lemon et al., 2007). The virus is transmitted parenterally and new infections are primarily acquired by sharing contaminated needles, in healthcare settings and – probably to a lesser extent – through vertical and sexual transmission (Maheshwari et al., 2008; Martinez-Bauer et al., 2008; Deterding et al., 2008). While acute HCV infection is mostly asymptomatic, in 55–85% of cases virus infection is not eliminated (Hoofnagle, 2002) and within 20 years about 20% of chronically infected adults will develop cirrhosis (Seeff, 2002). In fact, chronic HCV infection is one of the most common indications for orthotopic liver transplantation (Brown, 2005). HCV is highly variable and viral strains are classified into at least seven different genetic groups (genotypes, GTs) which differ from each other by ca. 31–33% at the nucleotide level (Simmonds et al., 2005; Gottwein et al., 2009). This enormous variability is a key mechanism that permits continuous viral immune evasion and a substantial challenge for development of antiviral therapy. Chronic HCV infection is treated with pegylated interferon alpha (peg-IFNα) and ribavirin curing 80% of genotype 2 and 3 and approximately 50% of genotype 1-infected individuals (Manns et al., 2006). Given the suboptimal response rates and substantial side effects of this treatment, new direct antiviral inhibitors are being developed and clinically tested (for a recent review, see Schinazi et al., 2010).

The HCV genome is about 9.6 kb in length and encodes a polyprotein of about 3000 amino acids in a single open reading frame. Co- and post-translational cleavages mediated by both cellular and

Abbreviations: ApoB/E, apolipoprotein B/E; ConA, concanamycin A; HCV, Hepatitis C virus; HCVcc, cell culture-derived HCV; HCVpp, HCV pseudoparticles; HCV_{TCP}, HCV trans-complemented particles; MLV, murine leukemia virus; peg-IFNα, pegylated interferon alpha; VSV, vesicular stomatitis virus.

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viral proteases liberate at least 10 viral proteins, of which at least the N-terminal three (core, E1 and E2) are major constituents of the extracellular virion (Moradpour et al., 2007), whereas the non-structural proteins NS3 to NS5B are necessary and sufficient for viral RNA replication (Lohmann et al., 1999). The NS2 protease mediates an essential cleavage of the polyprotein at the NS2–NS3 junction, and the p7 protein forms cation-selective ion channels in vitro (Grakoui et al., 1993; Hijikata et al., 1993; Premkumar et al., 2004; Griffin et al., 2003; Pavlović et al., 2003). Both proteins are essential co-factors for the assembly and release of infectious HCV particles (Steinmann et al., 2007a,b; Jones et al., 2007).

Upon attachment to the cell surface and utilization of the four minimal cellular entry factors, CD81, scavenger receptor class B type I (SR-BI), claudin-1 (CLDN1) and occludin (OCLN) (Pileri et al., 1998; Scarselli et al., 2002; Evans et al., 2007; Ploss et al., 2009; Liu et al., 2009), the virus is taken up by the cell via clathrin-mediated endocytosis (Blanchard et al., 2006). Subsequently, the RNA genome is released into the cytoplasm in a pH-dependent fusion step that occurs in early endosomes (Tscherne et al., 2006; Koutsoudakis et al., 2006; Meertens et al., 2006) that are acidified by vacuolar ATPases (V-ATPases) (Casey et al., 2010). Important post-fusion steps of the HCV replication cycle are mediated by the cellular microtubule network (Roohvand et al., 2009), which is also involved in the formation and transport of membrane-associated replication complexes designated as membranous webs where RNA replication takes place (Gosert et al., 2003; Wölk et al., 2008). Ultimately, newly synthesized viral RNA is packaged into progeny virus particles, which are then liberated from the infected cell.

Use of HCV cell culture systems including subgenomic replicons (Lohmann et al., 1999), retroviral HCV pseudoparticles (HCVpp) (Bartosch et al., 2003; Hsu et al., 2003) and the JFH1-based infection system (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005) has revealed details of the molecular mechanisms that govern HCV replication. Together with recent genome-wide host factor screenings based on RNA-interference, application of these models has identified multiple HCV dependency factors (Li et al., 2009; Tai et al., 2009). Among these, particularly cellular proteins involved in lipid metabolism participate in multiple steps of the viral life cycle (summarized in (Ye, 2007; Popescu and Dubuisson, 2010)): For instance, lipoproteins like HDL and oxidized LDL modulate the efficiency of HCV cell entry (Bartosch et al., 2005; Voisset et al., 2005; Meunier et al., 2005; von Hahn et al., 2006). Moreover, geranylgeranylated lipids provided by the mevalonate pathway are essential for HCV RNA replication (Ye et al., 2003; Kapadia and Chisari, 2005), probably via mediating membrane association of FBL2 and its interaction with HCV NS5A (Wang et al., 2005). Finally, cellular factors involved in the secretion of lipoproteins like the microsomal triglyceride transfer protein (MTP), apolipoprotein B and E (ApoB, ApoE) have been recognized as essential co-factors of HCV particle production (Huang et al., 2007; Chang et al., 2007; Gastaminza et al., 2008). Together these studies highlight a complex and intricate network of HCV dependence on host-derived factors and pathways.

Large-scale screenings for identification of new HCV inhibitors and essential pro- or antiviral host factors have been hampered by the lack of efficient high-throughput assays that encompass the complete viral life cycle. Although a number of models based on subgenomic HCV replicons are available to identify inhibitors of HCV RNA replication (Bourne et al., 2005; Ng et al., 2007; Peng et al., 2007; Mondal et al., 2009), only very recently first assays have been established that monitor interference with other steps of the viral life cycle (Li et al., 2009; Gastaminza et al., 2010; Chockalingam et al., 2010). Here we describe an unbiased cell-based screening system encompassing the entire viral replication cycle that discriminates between antiviral activity and cytotoxicity and at the same time distinguishes between inhibitory influence on RNA translation and replication, and other steps of the viral life cycle. This novel model was used to screen a unique library of natural compounds from myxobacteria thus identifying a number of bioactive compounds with pro- or anti-viral activity and distinct mode of action.

2. Materials and methods

2.1. Plasmids

The plasmids pFK-Luc-Jc1 and pFK-Jc1, encoding the genotype 2a/2a chimera Jc1 with or without the firefly luciferase reporter gene, as well as the reporter replicon pFKi389Luc-EI/NS3-3′_JFH1_dg have been described recently (Pietschmann et al., 2006; Koutsoudakis et al., 2006). The plasmids pHIT60 (Cannon et al., 1996), a MLV Gag-Pol expression construct, pRV-F-Luc, a firefly luciferase transducing vector, as well as pczVSV-G (Kalajzic et al., 2001) or pcDNA3ΔcE1E2-J6 or pHIT456 (Cannon et al., 1996) encoding MLV Env were used for pseudoparticle production. For lentiviral gene transfer the three plasmids pCMVΔR8.74 (Dull et al., 1998), pczVSV-G (Kalajzic et al., 2001) and pWPI-G-Luc were used. To generate the lentiviral vector pWPI-G-Luc-BSD a PCR was performed for amplification of the G-Luc gene from the plasmid pFKi389G-Luc-EI-JFH1/Con1/C-842_dg using the oligonucleotides S-BamH1-G-Luc (5′-GCCGGATCCCATGGGAGTCAAAGTTCTGTTTG-3′) and A-Mlu-G-Luc (5′-AGTACGCGTTTAGTCACCACCGGCCCCCT-3′). The amplicon was digested with *Bam*HI and *Mlu*I and inserted into pWPI_BSD (Steinmann et al., 2008) restricted with these enzymes.

2.2. Cell culture and cell lines

Huh-7.5 (Blight et al., 2002), Huh7-Lunet (Friebe et al., 2005) and 293T (DuBridge et al., 1987) cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 2 mM L-glutamine, non-essential amino acids, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal calf serum at 37 °C and 5% CO_2. Huh7-Lunet-hCD81-Gluc cells were generated from Huh7-Lunet-hCD81 (Bitzegeio et al., 2010) via lentiviral gene transfer, as described recently (Haid et al., 2010) using the three plasmids pCMVΔR8.74, pWPI-G-Luc-BSD, and pczVSV-G in a ratio of 3:3:1. Selection was carried out in the presence of 5 μg/ml blasticidin.

2.3. Compound library

The collection of natural products used for screening consists of 114 compounds that had been islotated at the Helmholtz Centre for Infection Research from cultures of myxobacteria during the past 30 years (Reichenbach and Höfle, 1999). The compounds were checked for integrity and purity (>95%) by LC–MS and dispensed into a 96-well format at a concentration of 0.5 mg/ml in DMSO. Nine compounds that had been shown to be highly effective in cell culture systems were also provided at a concentration of 10 μg/ml.

2.4. Complete life cycle dual luciferase screen

Huh7-Lunet-hCD81-Gluc cells were electroporated with Luc-Jc1 RNA and seeded on 96- or 384-well plates at a density of 2×10^4 cells per well or 7×10^3 cells per well, respectively, as described above. Four to five hours after transfection compounds were added at the appropriate concentration at a final concentration of 1% or 0.32% of solvent (DMSO or methanol). At 48 h 150 μl (96-well format) or 18 μl (384-well format) supernatant of the transfected cells was used to infect naïve Huh7-Lunet-hCD81-Gluc

cells that had been seeded the day before at a density of 7×10^3 cells per well or 2.4×10^3 cells per well, respectively. The transfected cells were washed once with PBS, lysed directly on the plate with 35 μl or 8 μl of ice-cold lysis buffer (0.1% Triton X-100, 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT), and frozen. After thawing 20 μl or 8 μl of lysate were transferred on white luminometer plates and supplemented with 72 μl or 20 μl of assay buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT, 2 mM ATP, 15 mM K_2PO_4) and 40 μl or 12 μl of luciferine solution (200 μM luciferine, 25 mM glycylglycine). Firefly luciferase activity was measured for 1 s using a plate luminometer (LB 960 Centro XS3; Berthold, Freiburg, Germany). After another 48 h, 20 μl or 12 μl supernatant of the infected cells was inactivated with 0.1% Triton X-100 and gaussia luciferase activity was measured for 0.1 s after adding 60 μl or 45 μl of 1:2000 or 1:4400 diluted coelenterazine (P.J.K., Kleinblittersdorf, Germany) using the same luminometer. Firefly luciferase activity of the infected cells was determined as described above. For liquid handling in 384-well plates an Evolution P3 robotic device from PerkinElmer was used. Approximately 80 nl of the natural compounds from myxobacteria were transferred by a pin-tool (V&P Scientific, San Diego, CA) to the cell cultures to give a concentration of ~1.6 μg/ml (or ~32 ng/ml in case of highly effective compounds).

2.5. Freeze and thaw lysates of HCV-transfected cells and virus titration

Huh7-Lunet-hCD81-Gluc cells were transfected with Jc1 genomes and seeded on 6-well plates. Compounds were added 4–5 h later. At 48 h post-transfection cell culture supernatants were harvested and virus titers determined by $CCID_{50}$ as described elsewhere (Haid et al., 2010). Cell-associated infectivity was prepared essentially as described (Gastaminza et al., 2006). Briefly, cells were extensively washed with PBS, scraped and centrifuged for 5 min at $1000 \times g$. Cell pellets were resuspended in 500 μl of DMEM containing 10% FCS and subjected to three cycles of freeze and thaw using liquid nitrogen and a thermo block set to 37 °C. Samples were then centrifuged at $10{,}000 \times g$ for 10 min at 4 °C to remove cell debris, and cell-associated infectivity was determined by the CCID50 assay.

2.6. Quantitative detection of HCV core protein by ELISA

HCV core protein was quantified using an enzyme-linked immunosorbent assay (ELISA) according to the instructions of the Manufacturer (Wako Chemicals, Neuss, Germany).

2.7. Quantification of ApoB, ApoE and albumin by ELISA

Secretion of ApoB, ApoE and albumin was quantified by harvesting the supernatant of cells treated with the appropriate drugs for 48 h and using an ELISA according to the Manufacturer's instructions (ApoB and ApoE: Mabtech, Nacka Strand, Sweden; albumin: Bethyl, Montgomery, TX).

2.8. Statistical analysis

Screening data were analyzed in the statistical environment R (http://www.r-project.org) using the Bioconductor package RNAither (Rieber et al., 2009). In brief, raw F-Luc intensity values were first log-transformed, and manually checked for quality problems. Both F-Luc and G-Luc measurements were then normalized plate-wise to the negative controls by subtracting the median of the negative controls and dividing by the median absolute deviation (MAD) of the negative controls. Resulting *z*-scores express how many standard deviations a particular measurement is away from the negative controls, and is a direct measurement of the strength of an effect. Replicate *z*-scores per compound were summarized using the mean, and statistical significance assessed by a two-sided Student's *t*-test. Due to the low number of independent measurements per compound ($n = 4$), a nonparametric test is not feasible, and *p*-values have to be interpreted with caution and may be overly optimistic. Percent viability and percent F-Luc inhibition were computed from the normalized data relative to the negative controls.

3. Results

3.1. Establishment and validation of a cell-based assay for screening of the complete HCV replication cycle

To facilitate identification of novel bioactive compounds with HCV-specific antiviral activity, we developed a robust and sensitive cell based assay based on a cell line derived from Huh-7 human hepatocarcinoma cells. The cells used in this study originated from Huh7-Lunet cells that are highly permissive to HCV RNA replication (Friebe et al., 2005), but which express only limited quantities of CD81, an essential HCV entry factor (Koutsoudakis et al., 2006). Robust expression of human CD81 was established using lentiviral gene transfer thus rendering these cells also highly susceptible to HCV cell entry (Bitzegeio et al., 2010). Besides this, we employed a lentiviral vector (Fig. 1A) to introduce a constitutively expressed transgene for a secreted gaussia luciferase (G-Luc) from the marine copepod *Gaussia princeps*. The resulting cell line was designated Huh7-Lunet-hCD81-Gluc and is highly permissive for HCV propagation. In addition, it expresses a simple biomarker (G-Luc) which reflects both cell number and cell viability and that can be monitored non-invasively by sampling the culture fluid. Comparison with a commercial cytotoxicity assay based on membrane integrity revealed similar sensitivity and reproducibility between the commercial assay and our cytotoxicity measurement based on secretion of G-Luc (Supplementary Fig. 1), indicating its usefulness for a first judgment of cytotoxicity of a given compound. To quantify HCV replication in these cells, we utilized the highly efficient HCV reporter virus Luc-Jc1 which expresses the firefly luciferase derived from *Photinus pyralis* (Koutsoudakis et al., 2006). As a consequence, cell viability and HCV replication can be measured simultaneously using a dual luciferase assay determining G-Luc and F-Luc activities in culture fluids and cell lysates, respectively.

To permit simple detection of influences on RNA replication, virus production and also cell entry, we devised a screening protocol that encompasses the entire HCV replication cycle and that is based on two measurements of HCV-dependent F-Luc activity (Fig. 1B): First, Huh7-Lunet-hCD81-Gluc cells were transfected with Luc-Jc1 RNA and seeded into culture plates. Four hours later, individual compounds were added to the culture fluid and co-cultured with the HCV-replicating cells throughout the entire assay. A first reading of F-Luc activity was conducted 48 h post-transfection and reflects HCV RNA translation and replication efficiency in the presence of compounds since at this time point secondary rounds of infection do not significantly contribute to the HCV-specific F-Luc signal (Koutsoudakis et al., 2006). At this time point (48 h post-transfection), culture fluid of transfected cells is collected and used to inoculate naïve Huh7-Lunet-hCD81-Gluc cells. Another 48 h later, both G-Luc and F-Luc activities produced in the inoculated cells were determined. These readings reflect cell viability and the efficiency of HCV RNA replication, virus production and cell entry, thus encompassing at least one entire replication cycle of HCV in the presence of the compounds.

To validate the assay we assessed the influence of a set of well known HCV inhibitors with specific and distinct mechanism of action on cell viability and HCV propagation in our cell based

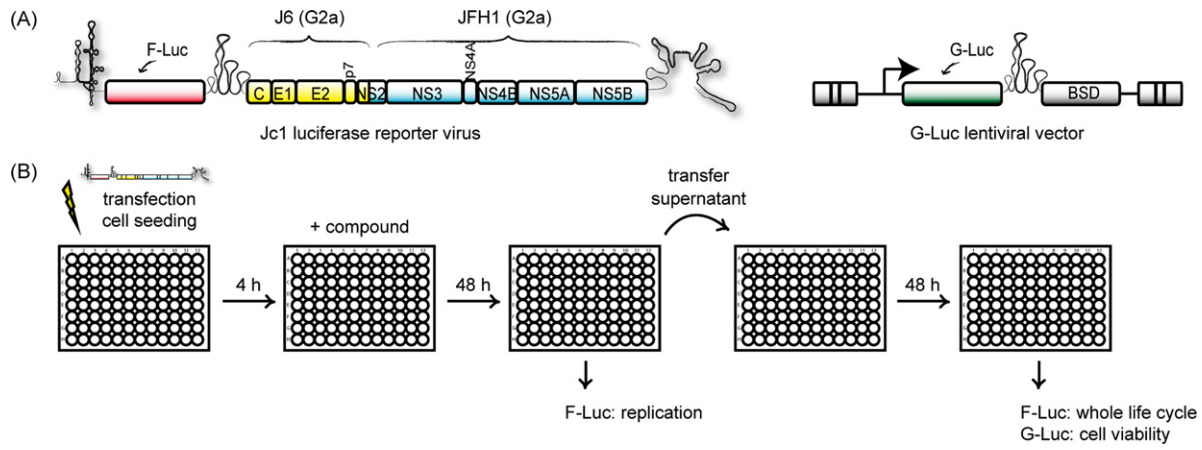


Fig. 1. Constructs and set up of the complete replication system. (A) Schematic representation of the lentiviral vector encoding the gene for the secreted luciferase of *Gaussia princeps* (G-Luc) and the bicistronic HCV Luc-Jc1 reporter virus encoding for the luciferase of *Photinus pyralis* (F-Luc). (B) Huh7-Lunet-hCD81-Gluc cells were transiently transfected with Luc-Jc1 RNA and seeded into 96- or 384-well plates, respectively. After 4 h compounds were added. Cells were lysed 48 h later and firefly luciferase activity was measured to detect HCV replication. The supernatant was used to infect naïve Huh7-Lunet-hCD81-Gluc cells whose firefly signal after another 48 h reflects the efficiency of the complete viral replication cycle in the presence of the drugs. A portion of the culture fluid of these cells was used to determine G-Luc activity to monitor cell viability.

assay (Fig. 2). Among these controls, we used concanamycin A (ConA) which inhibits cellular V-ATPases, thus preventing acidification of intracellular compartments as HCV cell entry inhibitor (Koutsoudakis et al., 2006; Tscherne et al., 2006). Pegylated interferon alpha (peg-IFNα) was employed as potent inhibitor of HCV RNA translation and replication, and the iminosugar derivative N-nonyl deoxygalactonojirimycin (NN-DGJ) that may act via inhibiting p7 (Pavlović et al., 2003; Steinmann et al., 2007a,b) was utilized as an HCV assembly inhibitor. Additionally, puromycin, an antibiotic that inhibits cellular protein translation, was used as a control that inhibits HCV propagation indirectly via cellular toxicity.

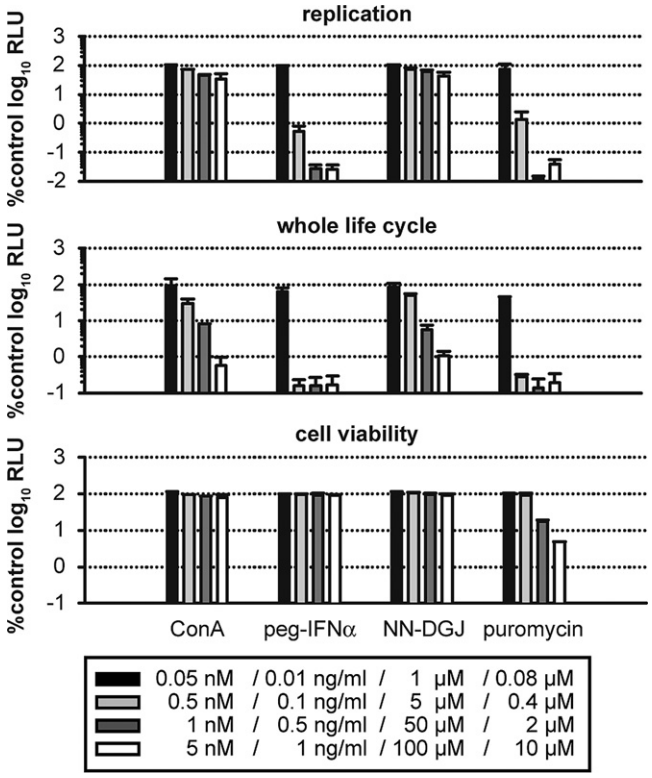


Fig. 2. Validation of the screening system using established HCV inhibitors with distinct mode of action. The assay was conducted as described in the legend of Fig. 1. Drugs interfering with different stages of the HCV life cycle were used at the indicated concentrations. ConA impedes HCV cell entry, peg-IFNα interferes with translation/RNA replication, NN-DGJ with HCV assembly, and puromycin blocks cellular translation and is therefore cytotoxic. Depending on the solvent of the compound, results were normalized to DMSO or untreated controls. Mean values and standard deviations of at least three independent experiments are given.

As expected, peg-IFNα strongly decreased F-Luc activity already in the first reading taken 48 h post-transfection. The reduction of F-Luc activity which reflects the strong inhibition of HCV translation and/or RNA replication by IFNα was dose-dependent and lowered F-Luc activity by more than 1000-fold at a concentration of 0.5 ng/ml (Fig. 2). Similarly, peg-IFNα strongly reduced F-Luc signals also in the second reading which reflects the entire HCV replication cycle with 0.1 ng/ml of peg-INFα already reducing HCV-specific reporter activity to background levels of the assay. In contrast, both the entry inhibitor ConA and the assembly inhibitor NN-DGJ, only reduced F-Luc activity in the second reading whereas at the first measurement time point, reporter activity was essentially not changed by these drugs. Finally, addition of puromcyin diminished F-Luc activity at both readings and at the same time G-Luc activity in a dose-dependent fashion. These profiles reflect the specific mechanisms of action of these drugs and establish that our assay distinguishes between inhibitors that primarily affect HCV RNA translation and replication and those that preferentially interfere with other steps of the HCV life cycle including cell entry and virus production. Importantly, at the same time we can exclude compounds that prevent HCV propagation merely due to cytotoxicity by analyzing the influence on G-Luc activity.

3.2. Screening of natural compounds derived from myxobacteria

Having established this novel assay we screened a library of 123 natural compounds that had been isolated from myxobacteria at the Helmholtz Centre for Infection Research over a period of 30 years (Reichenbach and Höfle, 1999). The screen was performed in duplicates and repeated twice. Based on these data we calculated *z*-scores to measure the strength of effects for each individual compound relative to the negative controls, and corresponding *p*-values (Student's *t*-test) to assess statistical significance. These computations were repeated for the first and second readings of F-Luc reflecting RNA replication and the complete replication cycle, respectively. In addition, these values were also determined for the G-Luc activity (Fig. 3, Supplementary Table). Approximately 76% of compounds had little influence on cell viability, reducing the G-Luc reading by less than 50% compared to the mean value of the

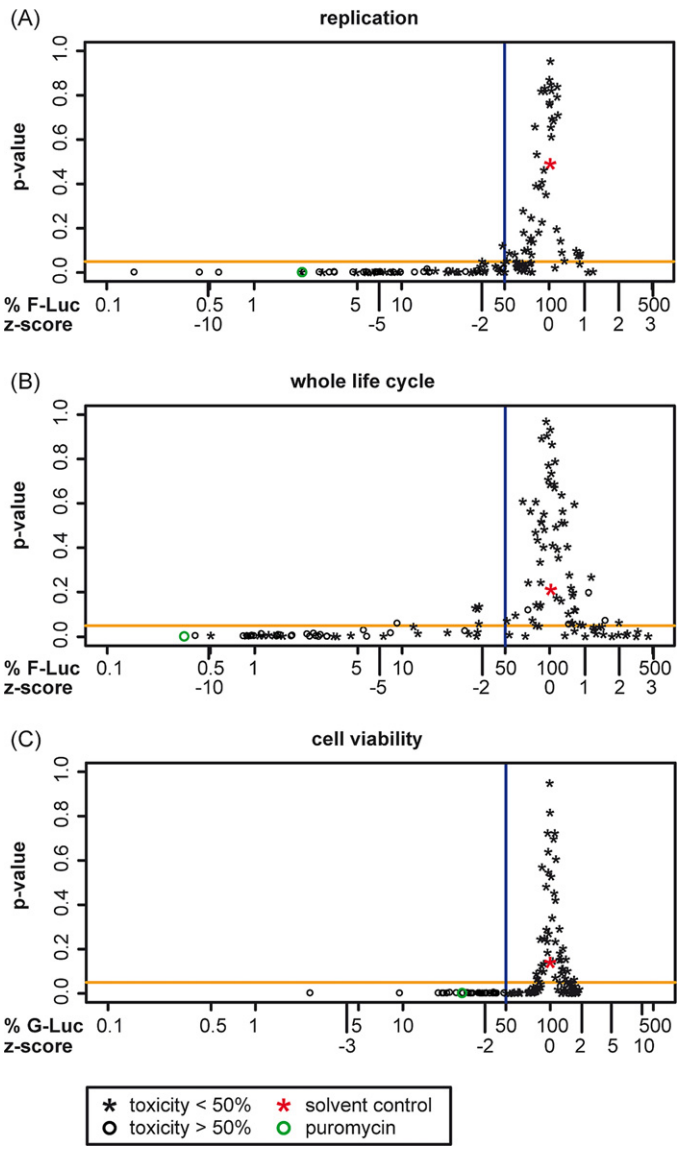


Fig. 3. Screening of 123 natural compounds isolated from myxobacteria. The screen was performed at a concentration of 3.2 μM in duplicates and repeated twice. Based on these data *z*-scores with corresponding *p*-values were calculated for the first F-Luc reading reflecting HCV translation/RNA replication (A), the second F-Luc reading representing the complete viral life cycle (B) and the G-Luc measurement which mirrors cell density and cell viability (C). Green: puromycin as positive control, red: DMSO as negative control, open circle: compound with cytotoxicity >50%. Asterisk: compound with cytotoxicity <50%. Red asterisk: solvent control. The blue line indicates a luciferase reading of 50% of the negative controls, the orange line indicates a *p*-value of 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

negative controls. Corresponding compounds reducing viability by more than 50% were excluded from further analysis. Interestingly, we identified 24 compounds with a *z*-score >2 or <−2, significantly modulating RNA replication, and 23 compounds which affected the complete replication cycle with a *z*-score >2 or <−2, and at the same time not decreasing cell viability by more than 50% (Fig. 3).

To get a first indication by which mechanism these compounds interfere with HCV propagation, we plotted the mean values of the replication signal against the mean values of the complete replication cycle reading (Fig. 4B). In this representation of our data it can be deduced if compounds primarily affect HCV translation/RNA-replication or alternatively other replication cycle steps like assembly or virus entry. Translation/replication inhibitors will reduce the number of viral proteins and RNA in the transfected

Table 1
(A) Ranking of strongest inhibitors of replication (F-Luc signal normalized to DMSO control) with corresponding *z*-scores and *p*-values. (B) Ranking of compounds with highest I_{HLC} (HCV-life cycle selectivity index) and associated F-Luc signals for replication and whole life cycle (normalized to DMSO control). (C) Enhancers of the whole life cycle signal that in parallel inhibit replication about 2-fold or more.

(A)	Replication (% control)	*z*-Score	*p*-Value
Myxothiazole A	1.4	−9.4	2E−03
Crocapeptin B	2.0	−8.6	5E−04
Disorazole A1	4.6	−6.9	3E−04
Noricumazole A	5.3	−6.3	2E−03
Disorazole B1	6.0	−6.7	6E−06
Vioprolid C	9.8	−4.7	6E−03

(B)	Replication (% control)	Whole life cycle (% control)	I_{HLC}
Apicularen A	25.8	0.5	51.0
Apicularen B	43.8	1.7	26.5
Archazolid A	23.0	1.0	23.8
Disorazole E1	10.1	1.6	6.4
Tartrolon	24.4	4.0	6.1
Disorazole B1	6.0	1.3	4.5
Disorazole A1	4.6	1.3	3.6
Myxalamide S	59.2	17.3	3.4

(C)	Replication (% control)	Whole life cycle (% control)	I_{HLC}
Chondramide B	23.3	224.8	0.1
Vioprolid D	49.0	284.3	0.2
Vioprolid B	22.1	365.5	0.1
Chondramide A	33.4	366.9	0.1
Chondramide C	54.3	482.2	0.1

cells and thus also de novo virus production which depends on efficient translation and replication. Consequently, such inhibitors (e.g. peg-IFNα, protease inhibitor boceprevir, polymerase inhibitor 2′-*C*-methyladenosine [2′CMA]) will proportionally reduce both the replication and complete life cycle reading (Fig. 4A). In contrast, entry or assembly inhibitors like concanamycin A (ConA) or N-nonyl deoxygalactonojirimycin (NN-DGJ) and quinidine, respectively will not affect the replication signal and only reduce the value reflecting the complete replication cycle. Therefore, these hits will appear in the upper left half of the panel with the most potent and specific entry/assembly inhibitors in the top left corner (e.g. ConA and NN-DGJ, Fig. 4A). Notably, toxic compounds diminish both F-Luc readings due to compromising cell viability and in turn virus propagation. As a consequence, these toxic compounds (e.g. puromycin) will be indistinguishable from translation/replication inhibitors (e.g. peg-IFNα) (Fig. 4B). Thus, we excluded all compounds which modulate cell viability more than 2-fold from our analysis to identify specific HCV inhibitors (Fig. 4C).

Of the 123 substances 30 displayed toxicity greater than 50% and were therefore excluded from further analysis. Among the 93 remaining compounds 24 inhibited replication by more than 50% and with a *z*-score below −2. In total 6 compounds inhibited replication by more than 90% (Table 1A). Interestingly, this analysis revealed 8 compounds which displayed a 3-fold or stronger influence on the complete life cycle reading compared to the replication signal, indicating that these compounds may inhibit HCV primarily via blocking life cycle steps distinct from translation and RNA replication. To separate these hits from compounds that primarily act via reducing translation or RNA replication we introduced an HCV-life cycle selectivity index (I_{HLC}) which is based on our experiments depicted in Fig. 4A and that this correlates inhibition of the complete replication cycle to the influence on RNA-replication through dividing the RNA-replication signal by the value of the complete replication cycle (I_{HLC} = % of control RNA-replication/% of control complete replication cycle). This index is a convenient measure that

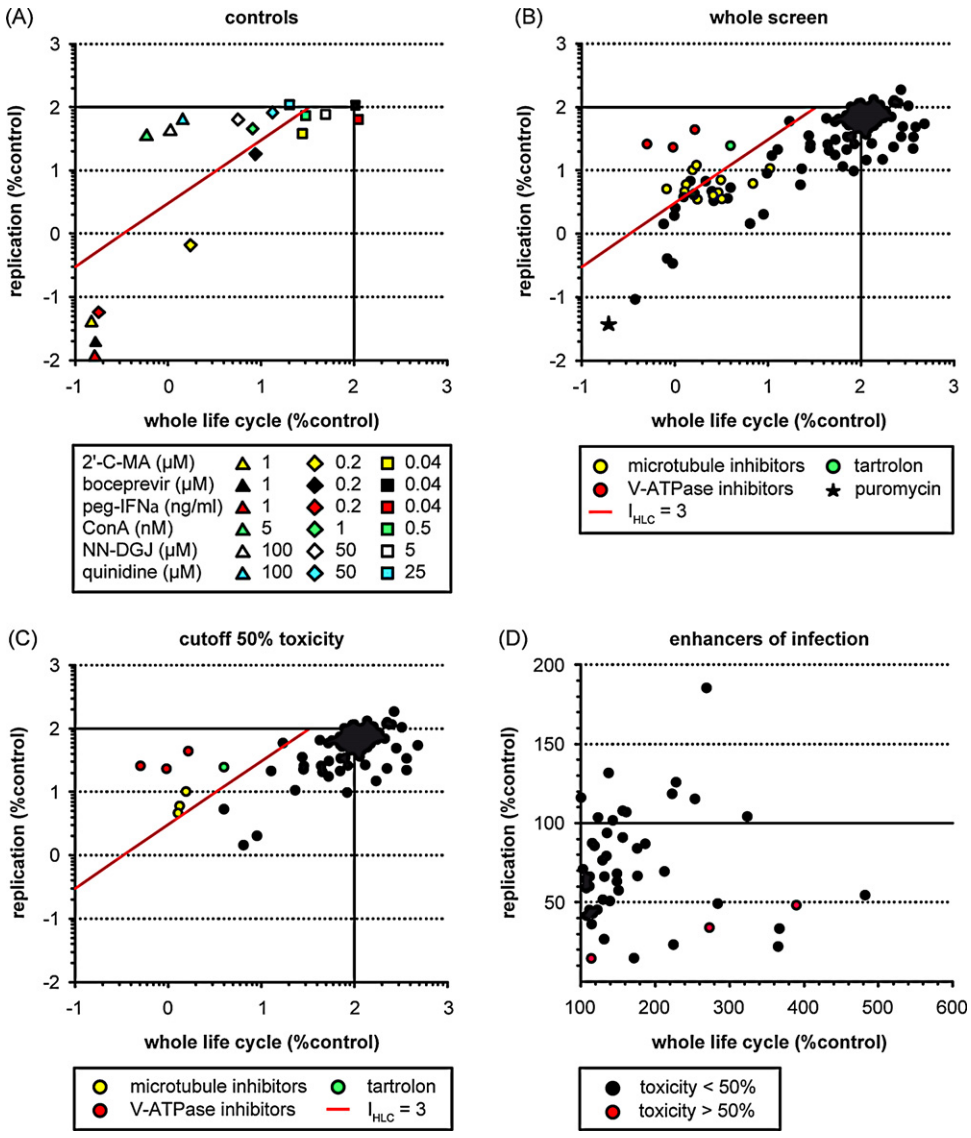
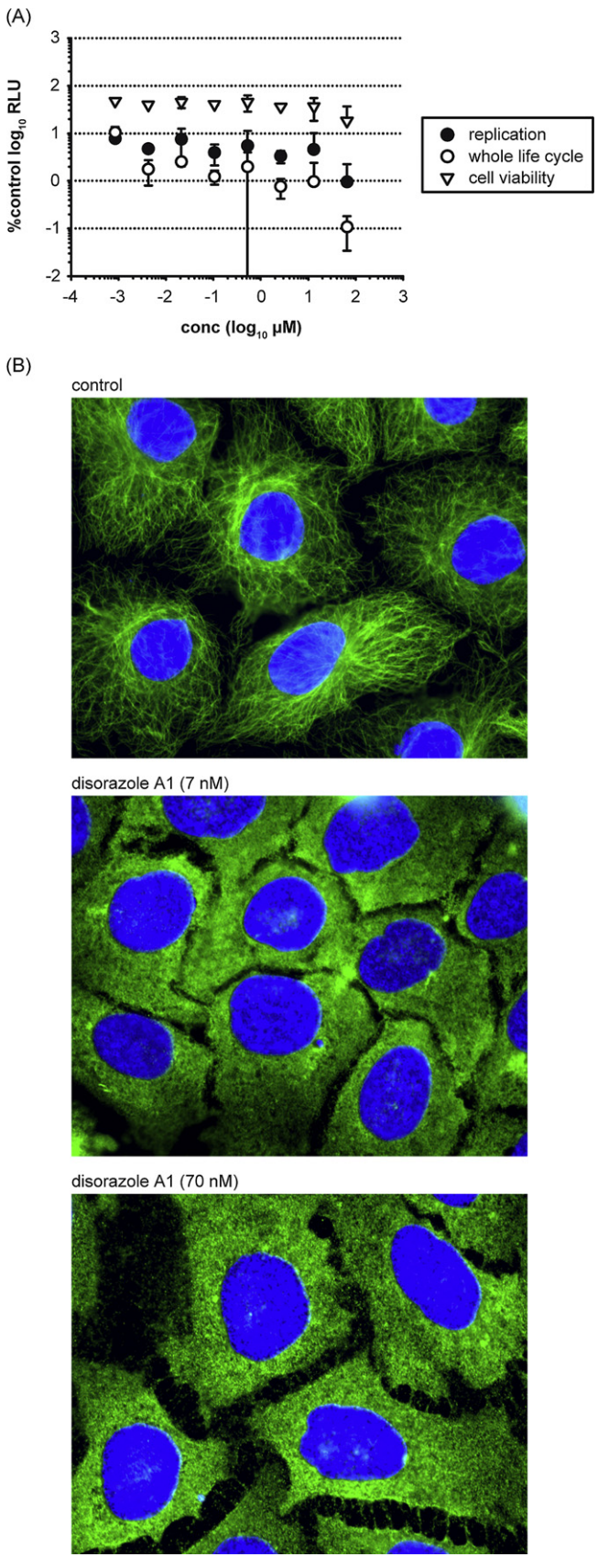


Fig. 4. Relation between HCV RNA replication and complete life cycle signals. (A) Different doses of replication inhibitors 2′CMA (yellow) and boceprevir (black), assembly inhibitors NN-DGJ (white) and quinidine (blue), entry inhibitor ConA (green) and peg-IFN were analyzed in our screening assay taking a replication and whole life cycle reading. Different doses are indicated by quadrangles, diamonds and triangles with increasing dose, respectively. Mean values of the replication signal were plotted against the whole life cycle signal to characterize compound-dependent influences on HCV propagation. Based on these data we introduced the HCV-life cycle selectivity index (I_{HLC}) which relates the first F-Luc measurement to the second and identifies compounds that preferentially inhibit the complete replication cycle. A threshold with an I_{HLC} index equivalent to 3 (3-fold stronger influence on the whole life cycle than on the replication signal) was marked with a red line, identifying compounds with a significant selectivity for the complete replication cycle. Note that increasing doses of replication inhibitors 2′CMA and boceprevir enhanced the antiviral activity in both readings approximately proportionally displaying an I_{HLC} index smaller than 3. (B) All myxobacterial compounds tested (each at a single dose) are depicted. Known inhibitors of V-ATPases or microtubule formation are shown in red or yellow, respectively. Tartrolon is represented as green circle; puromycin is given as black asterisk. (C) Data pairs with corresponding toxicity values above 50% were eliminated. (D) Enhancers of infection. Data pairs with a whole life cycle signal above 100% were depicted with their corresponding replication values. Compounds showing toxicity greater than 50% are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

indicates to which degree a given molecule preferentially inhibits HCV life cycle steps distinct from translation/RNA replication thus facilitating rapid identification of compounds that may have a novel and unique mode of action compared to replication inhibitors currently in clinical testing. A threshold level equivalent to an I_{HLC} value equal to three was chosen as cut off for a significant selectivity toward non-translation/replication life cycle steps since all tested doses of the replication inhibitors 2′-C-methyladenosine (2′CMA) and boceprevir resulted in an I_{HLC} value smaller than three (Fig. 4A). The red line depicted in Fig. 4 indicates this cut off value and denotes and I_{HLC} index of 3. Interestingly we also observed 5 compounds that increased the signal of the complete replication cycle by more than 2-fold while at the same time inhibiting replication by ~50% or even more (Fig. 4D, Table 1C) suggesting two different mechanisms of action opposing each other in the HCV life cycle.

3.3. *Microtubule inhibitors interfere with viral replication.*

The library analyzed by us contained 12 microtubule inhibitors of the disorazole, tubulysin, and epothilone families. Each of these molecules diminished signals of the HCV replication and, proportionally, also the complete life cycle reading (Fig. 4A and B). However, most of these molecules resulted in toxicity exceeding the threshold level of a 2-fold reduced G-Luc signal compared to the control. Among the tested microbutule inhibitors disorazole A1 displayed moderate toxicity, and was therefore chosen



for validation experiments on a 96-well format (Fig. 5A). These assays confirmed an IC_{90} in the low nanomolar range for both the replication and complete life cycle assay. Furthermore, indirect immunofluorescence microscopy was performed to confirm the influence of this compound on microtubule formation within cells (Fig. 5B). In contrast to control treated cells that contained a network of microtubules extending towards the cell periphery, these structures were dissolved after 18 h treatment with the inhibitor at two different concentrations. These results are in agreement with the observation of disorazol A1-induced inhibition of tubulin polymerization and complete depolymerization of microtubules in vitro (Elnakady et al., 2004). In conclusion, all microtubule inhibitors comprised in the library were identified in our screening confirming the great importance of the microtubule system for HCV replication (Roohvand et al., 2009; Wölk et al., 2008) and reinforcing the robustness of the screening system.

3.4. V-ATPase inhibitors impede viral cell entry.

Our library also comprised three inhibitors of V-ATPases, apicularen A and B and archazolid A (Huss et al., 2005). In contrast to the microtubule inhibitors theses molecules showed nearly no influence on viral replication but a very pronounced effect on the whole life cycle signal (Fig. 4A and B). In fact, the most potent substance, apicularen A, reduced the replication signal to 25.8% of the control while at the same time diminishing the value for the complete replication cycle to 0.5% resulting in an I_{HLC} index of 51. Therefore, this drug has as high selectivity for inhibition of HCV assembly/release or cell entry and was chosen as representative for validation experiments and dose–response determination on 96-well plates (Fig. 6A), where its prominent antiviral effect specifically on the complete life cycle reading was confirmed. To further investigate the influence of this drug on HCV cell entry, we used retroviral pseudoparticles carrying HCV glycoproteins E1 and E2. V-ATPases are essential for acidification of intracellular compartments and consequently V-ATPase-specific inhibitors are expected to primarily inhibit viruses that enter cells via a low pH-dependent fusion mechanism. Therefore, besides HCV pseudoparticles we used retroviral pseudotypes carrying vesicular stomatitis virus glycoproteins (VSV-G) and murine leukemia virus (MLV) envelope proteins which enter cells by low pH-dependent and -independent routes, respectively (Fig. 6B). As expected, apicularen A as well as concanamycin A, a well established inhibitor of V-ATPases, inhibited cell entry of HCV or VSV pseudoparticles to background levels, respectively, whereas entry of MLV peudoparticles was only moderately affected by these drugs. This indicates that the effect of apicularen A on the whole life cycle signal is likely primarily due to inhibition of HCV cell entry mediated by blockade of cellular V-ATPases. In addition these results confirm that the assay and the I_{HLC} index reliably distinguish between inhibitors of translation/replication and alternative steps of the viral life cycle.

Fig. 5. Antiviral activity of microtubule inhibitors. (A) All 12 microtubule inhibitors derived from three different chemical families comprised in the library displayed strong antiviral activity. The precise dose–response relationship was established for disorazole A1 which among these inhibitors showed the highest inhibitory activity in the screen with toxicity values below 50%. Measurements were conducted in duplicates for replication (black circle), whole life cycle (open circle) and cell viability (open triangle). Results were normalized to the methanol solvent control (MeOH) and mean values and standard deviations of at least three independent experiments are given. (B) Cells were treated with two different concentrations of disorazole A1 or solvent control. After 18 h microtubules were visualized using indirect immunofluorescence microscopy employing an α-tubulin-specific monoclonal antibody. Cell nuclei were stained using 4′-6-diamidino-2-phenylindole (DAPI).

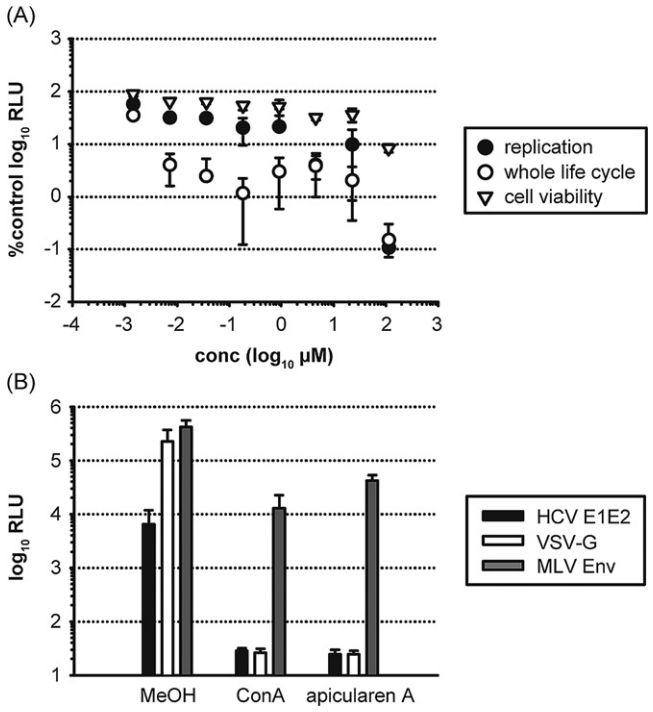


Fig. 6. Antiviral effect of V-ATPase inhibitors. (A) Among the three V-ATPase inhibitors, Apicularen A, which displayed highest potency in the screening was chosen as representative for this group of compounds and used for further analysis. The dose–response relationship was determined in duplicates for replication (black circle), whole life cycle (open circle) and cell viability (open triangle) signals. Results were normalized to the methanol solvent control and mean values and standard deviations of at least three independent experiments are shown. (B) Cells were inoculated with retroviral particles pseudotyped with the glycoproteins of HCV (black bars), VSV (white bars) or MLV (gray bars) in the presence of 3.6 μM apicularen A or 5 nM ConA. After 48 h the cells were lysed and firefly luciferase activity was measured. Experiments were performed in duplicate and mean values and standard deviations of at least three independent experiments are displayed.

3.5. Tartrolon inhibits virus entry and release

Among all compounds tested, tartrolon, a boron-containing antibiotic from myxobacteria (Irschik et al., 1995; Schummer et al., 1996), was the strongest hit in the whole life cycle assay that was not toxic at the tested dose, and that does not act via inhibition of V-ATPases or microtubules. Tartrolon was offered as the boron free tartrolon A which easily binds available boron to form tartrolon B. At a concentration of ~3.2 μM used in the 384-well screen this compound inhibited the complete replication assay to 4% of the control and displayed a clear selectivity for non-translation/replication life cycle steps with an I_{HLC} value of 6.1. Confirmation experiments on 96-well plates revealed a dose-dependent decrease of the whole life cycle signal with an IC_{90} in the nanomolar range. To distinguish influences of the drug on cell entry and RNA replication we compared inhibition of HCV by tartrolon between cells transfected with a subgenomic replicon and cells inoculated with HCV-transcomplemented particles (HCV_{TCP}). These HCV_{TCP} particles are created by transfecting a subgenomic HCV replicon into HCV packaging cells which constitutively express core, E1, E2, p7 and NS2 thus resulting in the production of infectious virions encasing the replicon via trans-complementation (Steinmann et al., 2008). Since the replicon RNA lacks viral structural proteins, p7 and NS2, HCV_{TCP} are strictly single round infectious thus permitting analysis of an influence of the drug on HCV cell entry and RNA replication. Interestingly, the inhibition of HCV by tartrolon was much more pronounced in the HCV_{TCP} infection assay compared to the replication assay based on cells transfected with the subgenomic replicon RNA displaying an IC_{90} of approximately 1 μM and 50 nM, respectively (Fig. 7B). This result suggested that tartrolon has a moderate influence on RNA replication but primarily impedes HCV cell entry steps. To directly asses the influence on HCV cell entry we used HCV pseudoparticles and pseudotypes carrying VSV- or MLV-derived glycoproteins as controls (Fig. 7C). Strikingly, tartrolon at a dose of 0.35 μM reduced infection by HCV, VSV and MLV pseudotypes to background levels indicating that this drug strongly interferes with cell entry steps shared between these different particle types and thus arguing for a broader mechanism of action against viral cell entry. To distinguish if the drug impedes viral cell entry by acting on the host cells or the virus particle itself, we incubated either the cells or the virus with the drug prior to inoculation (Fig. 7D). Pre-incubation of the cells with the drug reduced infectivity of HCV_{TCP} by about hundred-fold, a reduction that was comparable to the degree of inhibition when the drug was present during inoculation. In contrast, pre-incubation of the virus particle and subsequent separation of the drug from the particle by ultrafiltration caused only an about 5-fold decrease of reporter activity. Together, these data suggest that the drug impedes cell entry primarily via targeting host cell components.

Next we analyzed the influence of tartrolon on assembly and release of infectious HCV particles. To this end, we transfected the cells with Jc1 RNA without any reporter and added the compound 4 h later. After 48 h the supernatant was harvested to measure extracellular amounts of core protein (Fig. 8A) and infectivity (Fig. 8B). Furthermore, intracellular viruses were extracted from the cells via repetitive cycles of freeze and thaw for determination of intracellular core amount and infectivity (Fig. 8A and B). To gain first mechanistic information and to reveal distinct (or comparable) mechanisms of action between different HCV assembly inhibiting compounds, we compared tartrolon with additional assembly inhibitors that have recently been described. More specifically, we used N-butyl-deoxynojirimycin (NB-DNJ), an inhibitor of cellular a-glucosidases which prevents proper folding of HCV glycoproteins and thus assembly of infectious particles (Steinmann et al., 2007a,b). In addition, we used naringenin, a grapefruit flavonoid which inhibits secretion of apolipoprotein B (ApoB) and infectious HCV (Nahmias et al., 2008). Finally, we also included quinidin which disturbs HCV assembly/release by a so far unknown mechanism (Chockalingam et al., 2010). Recent evidence has well established that HCV particle production depends on host cell factors needed for assembly and release of lipoproteins including MTP, ApoB and ApoE (Popescu and Dubuisson, 2010). Therefore, we also monitored the influence of these drugs on secretion of apoB and apoE. Moreover, extracellular albumin levels were quantified to reveal a possible impact of these compounds on the general secretory capacity of treated cells. All four compounds did not reduce intracellular core protein levels, thus excluding a significant influence on cell viability, HCV RNA translation and replication at the chosen drug dose (Fig. 8A). However, release of core from cells was substantially decreased (ca. 10-fold) by tartrolon, NB-DNJ and naringenin and approximately 100-fold in case of quinidine. Extracellular infectivity was reduced approximately commensurately for all four drugs (Fig. 8B). In turn the specific infectivity of extracellular virions (i.e. the infectivity associated with a given quantity of core protein) was unchanged or circa only two-fold reduced (Fig. 8C). Interestingly, selectively quinidine and naringenin reduced the quantity of intracellular infectious particles, suggesting that they may prevent virus production by additional mechanisms thus causing a lower abundance of intracellular infectious viruses. Notably, among all tested compounds, only naringenin specifically interfered with ApoB release in a dose dependent fashion (Fig. 8D). These results confirm previous findings by Nahmias et al. (2008) who noted decreased ApoB secretion in the presence of naringenin and suggest that quinidine, NB-DNJ and tartrolon inhibit HCV virus produc-

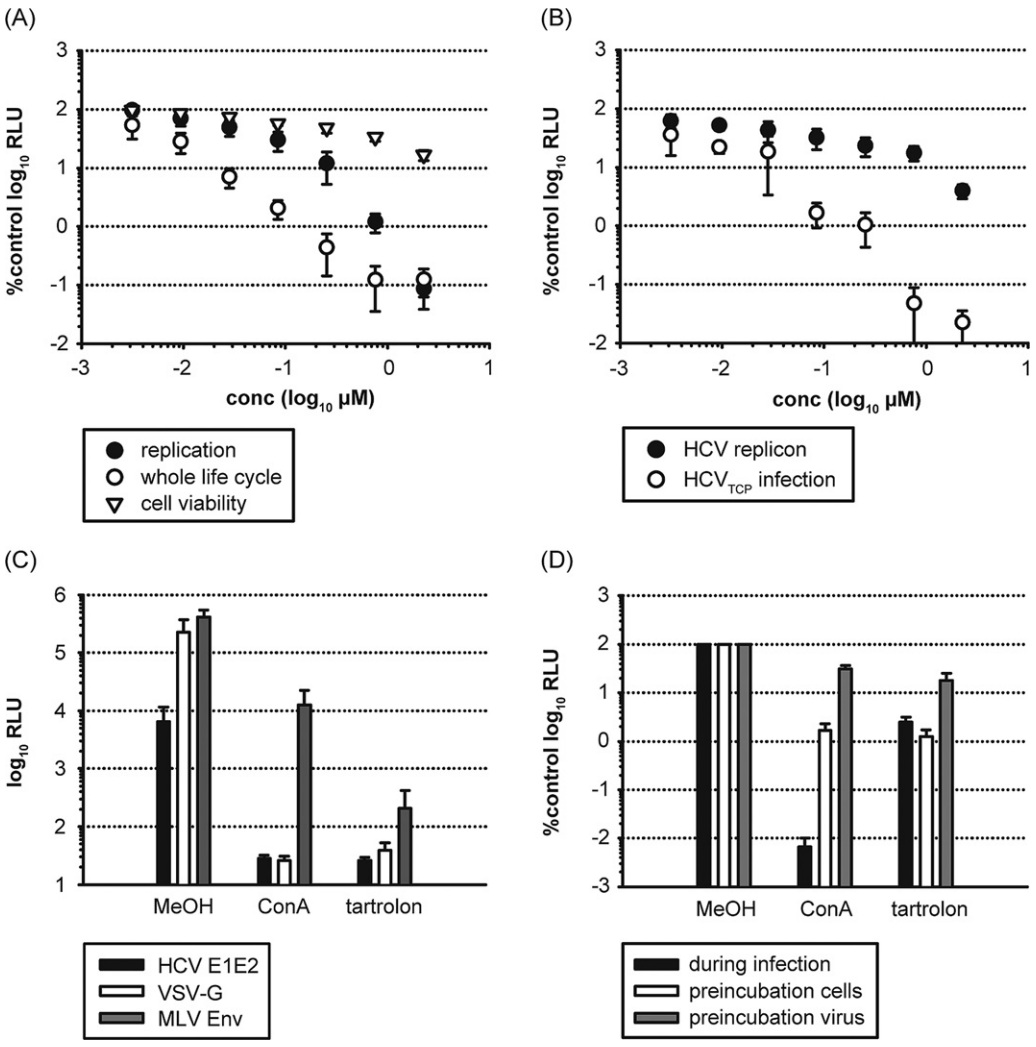


Fig. 7. Influence of tatrolon on HCV RNA replication and cell entry. (A) The dose–response relationship was determined for replication (black circle) and whole life cycle (open circle) signal of Luc-Jc1 as well as cell viability (open triangle). Results were normalized to the methanol solvent control. Mean values and standard deviations of at least three independent experiments are given. (B) Cells were transfected with subgenomic replicon RNA (black circle) or inoculated with HCV_{TCP} carrying the corresponding subgenomic RNA (open circle). Tartrolon was added at a concentration of 0.35 μM during inoculation or 4 h post-transfection and was present until harvesting and determination of F-Luc activity 48 h later. Results were normalized to the methanol solvent control and mean values and standard deviations of at least three independent experiments are given. (C) Cells were inoculated with retroviral particles pseudotyped with the glycoproteins of HCV (black bars), VSV (white bars) or MLV (gray bars) during treatment with tartrolon at a concentration of 0.35 μM. After 48 h the cells were lysed and firefly luciferase activity was measured. Experiments were performed in duplicates and mean values and standard deviations of at least three independent experiments are shown. (D) Cells were inoculated with HCV_{TCP}. The drugs were present during inoculation (black bars). Alternatively, cells (white bars) or virus particles (gray bars) were pretreated with the compounds and the drugs were washed away from the cell surface or they were separated from the particles by ultrafiltration prior to inoculation. Results were normalized to the methanol solvent control. Mean values and standard deviations of at least three independent experiments are displayed.

tion by mechanisms independent from ApoE and ApoB lipoprotein assembly and secretion.

4. Discussion

Only very few cell-based screening systems at high-throughput level are available that permit identification of HCV-specific inhibitors interfering with any step of the viral life cycle. Here we describe the establishment of a 384-well based screening set up that encompasses the complete HCV life cycle. Using a two-step procedure consisting of initial transfection and subsequent inoculation of naïve cells, the assay not only identifies HCV-specific inhibitors but also distinguishes between inhibitors of viral RNA translation/replication and compounds interfering with other steps of the viral life cycle. At the same time, false positive screening hits due to cytotoxicity are easily detected based on monitoring the constitutively expressed secreted gaussia luciferase as convenient biomarker for cell viability and cell density. Finally, with a dynamic range of approximately 1000 and 100 for the replication or whole life cycle assay, respectively, the simple and cost efficient enzymatic tests permit robust and highly reproducible quantification of HCV propagation.

At present, two other reports about whole life cycle screening approaches of compound libraries have been published (Chockalingam et al., 2010; Gastaminza et al., 2010). However, neither of these assays has been adapted to 384-well high-throughput format or is able to differentiate mechanisms of action already during the primary screening process. Recently, a genetic screen at 384-well format, based on RNA interference and interrogating the entire HCV life cycle has been reported (Li et al., 2009). This assay is based on two consecutive rounds of HCV infection and uses a microscopic readout quantifying the relative abundance of HCV core in the infected cells using indirect immunofluorescence. However, the dynamic range of this system is comparatively narrow.

Using our cell based assay we screened a library of 123 small natural compounds isolated from myxobacteria. Toxicity cut off

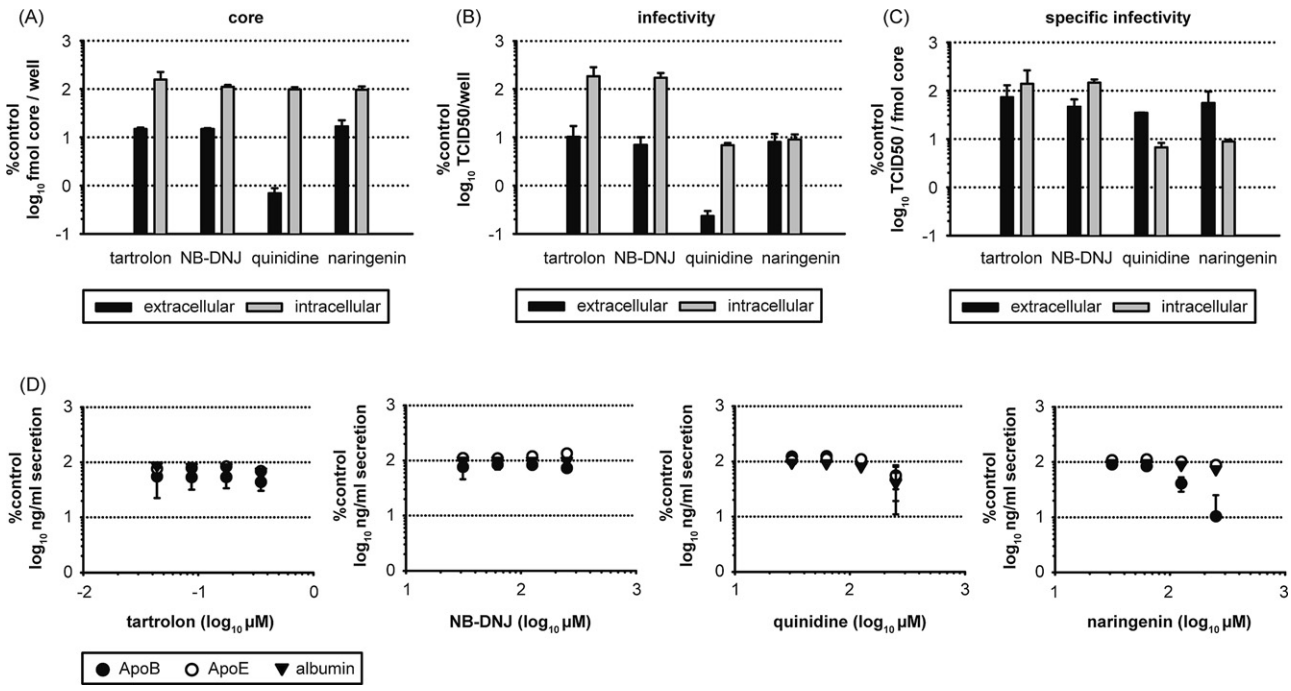


Fig. 8. Influence of tartrolon, NB-DNJ, quinidine and naringenin on HCV particle production, and release of lipoproteins and albumin. Cells were transfected with Jc1 RNA, and 4 h later the inhibitors were added. Tartrolon was used at a dose of 0.35 μM, NB-DNJ at 250 μM and quinidine and naringinine both at 100 μM, respectively. After 48 h the supernatant was harvested and intracellular lysates were prepared by repetitive cycles of freeze and thaw. Intra- and extracellular amount of core protein was measured by a core-specific ELISA (A), infectivity was determined with a limiting dilution assay (B) and specific infectivity was calculated (C). Mean values and standard deviations of at least three independent experiments are given. In each case values were normalized to the respective solvent control. (D) Secretion of albumin (black tringle), ApoB (black circle) and ApoE (white circle) in the presence of increasing doses of the drugswere monitored using ELISA assays specific for human albumin, ApoB and ApoE, respectively.

was set to 50% and of the remaining 93 compounds 24 showed an inhibitory effect on translation/replication of at least 50% with a *z*-score below −2. Among these, 6 compounds inhibited HCV replication by more than 90%. Furthermore, we identified 8 compounds that displayed a 3–50-times stronger influence on the whole life cycle signal than on replication, indicating that these molecules preferentially target other steps like virus entry, assembly, or release. Interestingly, we also identified 5 molecules that increased viral propagation by more than 2-fold; although at the same time inhibiting HCV RNA translation/replication by 50% or even more. These compounds may have differential effects on virus propagation opposing each other. Notably, we have recently observed that glucocorticosteroids moderately reduce HCV RNA replication but at the same time facilitate HCV cell entry via mediating the upregulation of essential viral entry factors (Ciesek et al., 2010). In this case these opposing effects also caused a net gain in the efficiency of the complete replication cycle although RNA replication was reduced. It will be interesting to find out the mechanism by which these novel compounds indentified here facilitate virus propagation. Future studies in this direction may reveal unknown host-derived viral resistance and dependency factors which may also be targets for viral therapy.

The high number of bioactive compounds included in our library was not expected. However, myxobacteria possess the largest genomes among bacteria and have an extraordinary potential to produce secondary metabolites with unique properties that are potential leads for drug discovery (Wenzel and Müller, 2009). In fact, molecules of our test library have already been the source for novel antibiotics and anti-cancer drugs. For instance, etnangien, an inhibitor of bacterial RNA polymerase as well as retroviral DNA polymerase (Irschik et al., 2007), argyrin A, a compound with broad antitumoral activity (Nickeleit et al., 2008), and epothilone, which is already used in the clinics for treatment of breast cancer (Atzori and Fornier, 2008) are all derived from myxobacterial metabolites.

Our library encompassed 12 inhibitors of microtubules of the families disorazoles (Hopkins and Wipf, 2009), epothilones (Reichenbach and Höfle, 2008) and tubulysins (Sasse et al., 2000), which were all recognized in the screen as hits with a matching inhibitory profile again confirming the accuracy and reliability of the assay. All of them strongly inhibited both the replication and whole life cycle signal. Since microtubules are crucial for cellular transport processes it is likely that blockade of these cellular structures interferes with viral propagation in multiple ways. In fact, an involvement of microtubules in the formation and transport of the membranous webs, the site of HCV RNA replication, has been reported (Gosert et al., 2003; Lai et al., 2008; Wölk et al., 2008). Additionally, a dynamic microtubule network is essential for HCV cell entry and post-fusion steps (Roohvand et al., 2009). Finally, it was suggested that microtubules play a role in the core-mediated redistribution of lipid droplets (Boulant et al., 2008) which are important organelles for virus assembly (Miyanari et al., 2007). However, despite of the high degree of viral dependence on a functional microtubule system, the central importance of these structures for cell viability clearly limits options to develop inhibitors with therapeutic potential.

As a second class of inhibitors, our screening identified all inhibitors of V-ATPases present in the library (apicularen A and B as well as archazolid A (Huss et al., 2005)). These molecules displayed a common inhibitory profile in our assay with negligible effect on viral replication but a very strong decrease of the whole life cycle signal. In case of apicularen A we used HCV pseudoparticles to confirm inhibition of HCV cell entry.

Among all tested molecules, tartrolon, a boron-containing macrodiolide antibiotic (Irschik et al., 1995; Mulzer and Berger, 2004), displayed the most interesting phenotype in our assays and exerted a clear preference for inhibition of non-translation/replication steps of the viral life cycle with an I_{HLC} index of 6.1. Our data indicated that tartrolon impedes HCV cell

entry as shown by strong inhibition of HCV_{TCP} and HCV pseudoparticle infection. Interestingly, not only HCV cell entry, but also infection by retroviral pseudotypes carrying VSV-G or MLV envelope proteins was inhibited by the drug. This result suggests a broader influence on viral entry at a step shared at least by these three viruses carrying different viral envelope proteins. Consequently, it is unlikely that the drug interferes with specific virus-receptor interactions. Since also MLV pseudoparticles which fuse at the plasma membrane in a pH-independent fashion were inhibited, tartrolon probably inhibits viral cell entry independently of blocking cellular V-ATPases. Our experiments incubating the drug either with virus particles or the target cells suggest that tartrolon primarily impedes viral cell entry via acting on cellular components. This conclusion is based on our finding that pre-incubation of the cells inhibited cell entry as efficiently as presence of the drug during inoculation, whereas pre-incubation of the particle itself and subsequent separation of the compound from the particle resulted in a moderate inhibition only. Although based on these data we cannot rule out that the virus particle itself may be affected as well. It is also possible that separation of tartrolon from the virus particle by ultrafiltration was not complete and that residual compound inhibited cellular components in the latter assay thus resulting in partial inhibition of cell entry.

On the other hand, our results indicate that tartrolon not only inhibits viral cell entry but also reduces the amount of extracellular HCV particles without affecting their specific infectivity. The latter finding lends further support to the notion that tatrolon does not act directly on the virus particle. Importantly, the effect on virus release was not caused due to a general disruption of cellular secretion processes, since extracellular amounts of albumin remained unchanged under drug treatment in non-toxic concentrations. Likewise, inhibition of HCV assembly/release was not due to a blockade of the secretion process of lipoproteins which is tightly linked to that of HCV (Huang et al., 2007; Gastaminza et al., 2008), since extracellular amounts of ApoB and ApoE were not affected either. In summary, our data indicate that tartrolon targets different steps of the HCV life cycle. In case of cell entry, this compound also impairs infection by retroviral pseudotypes independently of the viral glycoprotein used, probably by interfering with a cellular factor(s) or processes shared between these viruses. These findings are suggestive of a relatively broad antiviral mechanism exerted by tartrolon which may be due to inhibition of a cellular target that may be crucial for the propagation of different viruses. In fact, it is well known that viruses from diverse families usurp conserved cellular pathways and machineries for cell entry (e.g. clathrin mediated endocytosis) and virus release (e.g. endosomal sorting complex required for transport; ESCRT). Thus, it is conceivable that molecules which interfere with such cellular processes exert broad antiviral activity against multiple viruses. In addition, interference with for instance cellular vesicle transport may cause viral defects during cell penetration as well as during virus assembly and release. Further mechanistic studies investigating the cellular response to tartrolon should help to more precisely define the mechanism(s) of its antiviral activity. Of note, antiviral activity has been ascribed to a number of different types of antibiotics including boromycin a boron-containing macroloid antibiotic derived from *Streptomyces* which is structurally related to tartrolon (Kaptein et al., 2010; Balzarini et al., 2003; Zhang et al., 2009; Birk et al., 2008; Kohno et al., 1996). It will therefore be interesting to address in future research if other members of this group of antibiotics have antiviral activity as well.

Targeting host factors instead of viral proteins is an attractive yet controversial strategy for antiviral intervention. Clearly, the benefits of a broad spectrum antiviral activity – for instance against all HCV genotypes which share the dependence on the specific host factor – have to be carefully weighed against problems arising from cytotoxicity due to inhibition of a possibly essential cellular factor. Feasibility of such antiviral strategies will likely heavily depend on which host factor is targeted and how the virus and the cell depend on its function(s). Nevertheless, successful clinical application of chemokine receptor antagonists in HIV-1 therapy (Westby and van der Ryst, 2005, 2010) and encouraging clinical data for cyclophilin inhibitors in HCV therapy (Watashi, 2010) encourage further efforts to target host factors for antiviral therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2010.12.005.

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