Hepatitis C virus (HCV) infection is a major cause of chronic liver disease affecting 3% of the world’s population (WHO, 2012). HCV replication is prone to high error rates leading to a large diversity of genotypes and subtypes (Simmonds et al., 2005) with differences in susceptibility to current treatment and outcome of disease. The standard of care (SOC), a combination of pegylated interferon-alpha with ribavirin, and recently two direct acting antivirals have entered the clinics. However, because of side effects, drug resistance and viral genotype-specific differences in efficacy current and potentially also future therapies have their limitations. Here, we describe the development of a phenotypic high-throughput assay to identify new cross-genotype inhibitors with novel mechanism of action, by combining a genotype (gt) 1 replicon with the infectious HCV gt2 cell culture system. To develop this phenotypic multiplex assay, HCV reporter cells expressing RFP-NLS-IPS and gt1b replicon cells expressing NS5A-GFP were co-plated and treated with compounds followed by inoculation with gt2a HCV. At 72 h post treatment, RFP translocation as a marker for HCV infection and GFP fluorescence intensity as a marker for gt RNA replication were measured. Additionally, the total cell number, which serves as an indicator of cytotoxicity, was determined. This phenotypic strategy supports multi-parameter data acquisition from a single well to access cross-genotypic activity, provides an indication of the stage of the viral life cycle targeted, and also assesses compound cytotoxicity. Taken together, this multiplex phenotypic platform facilitates the identification of novel compounds for drug development and chemical probes for continuing efforts to understand the HCV life cycle.

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system for full length HCV gt1 was reported which is the most prevalent genotype worldwide (Li et al., 2012; Yi et al., 2006), however, screening involving cell-culture adapted HCV have only been performed for gt2 and gt1/2 chimeric viruses (Chockalingam et al., 2010; Gastaminza et al., 2010; Gentzsch et al., 2011; Wichroski et al., 2012).

In this context, by combining the gt1 replicon with the infectious HCV gt2 cell culture system our goal was to develop a high-throughput phenotypic assay to identify cross-genotype antivirals with a novel mechanism of action. Our devised strategy allows multiparameter data acquisition from a single well by a phenotypic approach by combining (i) the identification of novel HCV inhibitors with cross-genotypic activity, (ii) indication of the targeted stage of the virus life cycle, and (iii) early assessment of compound induced cytotoxicity.

Taking advantage of the observation that the mitochondrial antiviral signaling protein (MAVS/IPS-1), located in the outer mitochondrial membrane, is a cellular substrate for the HCV NS3-4A protease, Jones et al. developed a cell-based fluorescent reporter system allowing sensitive detection of HCV-infection in live cells (Jones et al., 2010; Loo et al., 2006). Overexpression of a fusion protein consisting of the membrane anchored C-terminal IPS-1 domain linked to a nuclear localization signal (NLS) and red fluorescent protein (RFP) (Fig. 1A), enables monitoring HCV infection events by measuring the translocation of cytoplasmic localized RFP into nuclei upon by NS3-4A protease mediated cleavage between RFP-NLS and IPS-1 (Fig. 1A and B).

To establish the phenotypic multiplex assay, Huh-7.5 derived RFP-NLS-IPS reporter cells were mixed at 1:2 ratio with Huh-7 gt1b replicon cells, expressing an HCV NS5A-GFP fusion protein.

**Fig. 1.** Constructs and concept of the phenotypic multiplex assay. (A) Protein structure of IPS-1 indicating the caspase recruitment (CARD) and proline-rich (PRO) domains, and HCV NS3-4A protease cleavage site at position C508 (arrow). Structure of the fusion reporter protein RFP-NLS-IPS with N-terminal TagRFP, followed by an SV40 nuclear localization signal (NLS, PKKKRKVG), and residues 462–540 of IPS-1. The C-terminal transmembrane domain (TM) anchors both IPS-1 and RFP-NLS-IPS in the outer mitochondrial membrane (Jones et al., 2010). Schematic of the HCV gt2a reporter-free chimeric Jc1 virus and the HCV gt1b replicon expressing an NS5A-GFP fusion protein as marker for viral replication. (B) Phenotype of RFP-NLS-IPS reporter cells showing RFP-NLS translocation into nuclei after HCVcc infection (white arrows). (C) Expected phenotypes of GFP- and RFP fluorescence distribution. Expression of NS5A-GFP (Huh-7 HCV gt1 replicon cells) and location of RFP-NLS-IPS (Huh-7.5 RFP-NLS-IPS cells) shown in green and red respectively is changed in response to the specificity of HCV inhibitors tested.
as a marker for viral replication (Moradpour et al., 2004), and co-plated into one well (Fig. 1A). The experimental protocol can be briefly described as follows: 2,400 cells per well were plated into 384-well assay plates, at 24 h post-plating compounds were added and after a 2 h incubation period at 37 °C, cells were inoculated with Jc1 (Lindenbach et al., 2006; Pietschmann et al., 2006), a reporter-free gt2a virus at a multiplicity of infection of 2 (Fig. 1A). At 72 h post-infection, plates were fixed with 2% paraformaldehyde, cell nuclei were stained with 10 μg/mL Hoechst-33342 and images were taken with an automated confocal microscope (ImageXpress Ultra, Molecular Device) at a magnification of 20×.

Utilizing this assay, various phenotypes, depending on the characteristics of HCV inhibitors, can be anticipated (Fig. 1C). In the absence of compound (Fig. 1C–r) or in presence of an inactive compound (Fig. 1C–s) infection with gt2a HCVcc induced RFP-NLS-IPS reporter cells to have red signal in the nuclei while the GFP replicon displays a green signal in the cytoplasm. Cross-genotypic inhibitors prevent both gt1 and gt2 HCV RNA replication, therefore the green signal from gt1 replicon would disappear and the red signal can be detected in the cytoplasm exclusively (Fig. 1C–t); whereas a gt1 specific inhibitor would lead to a decreased GFP expression in replicon cells and nuclear RFP

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**Fig. 2.** Image analysis. (A) Different parameters were extracted from the raw pictures by in-house image processing software; nucleus stained with Hoechst (blue channel), GFP expression (green channel) as a marker for HCV gt1 RNA replication and RFP signal (red channel) measured in the cytoplasm or inside the nucleus as a proxy for gt2 HCV infection. (B) Overview on data output from image analysis with corresponding information provided.
Fig. 3. Assay evaluation with reference compounds by dose response curve (DRC) analysis. (A) Different concentrations of NM-107 were tested (10 points 3-fold serial dilution from 292 μM) and analyzed according to the scheme shown in Fig. 2. Representative images and the respective concentrations are indicated. (B) Analyzed values generated by image processing software for EC100 (100 μM) and for the DMSO control. (C) DRCs of various HCV inhibitors. From each concentration of compounds treatment, gt1 RNA replication (green squares), gt2 HCVcc infection (red triangles), and cytotoxicity (black circles) were determined by percentage of GFP positive cells (AvgPercentCellGFP), Ratio (RFP translocated nucleus) and cell counting (SumCellNumber), respectively. Each value were normalized using DMSO/EC100 control into percentage of inhibition and shown in the graph with error bar of quadruplicate experiments. The table provides the EC50 and CC50 values calculated from sigmoidal fitting curves. (D) Early and late step inhibitors treatment. Representative images and the respective concentrations are indicated. Couple phenotype is highlighted by yellow circles.
localization in RFP-NLS-IPS reporter cells (Fig. 1C–4). Lack of RFP translocation but maintenance of GFP signal would either mean a gt2 specific inhibitor of replication (Fig. 1C–5) or a block at the viral entry level (Fig. 1C–6), which can be tested in a secondary assay by infection with a HCV gt1a/2a chimeras. If later steps in the viral life cycle are targeted preventing the release of infectious particles, primarily infected RFP-NLS-IPS cells would initially be infected and the translocation of RFP into the nucleus can be observed (Fig. 1C–7). Production of progeny virus and spread will, however, be inhibited resulting in a mixed pattern within the RFP-NLS-IPS cells with two translocation positive cells in close proximity (‘couple phenotype’) which is the result of primarily infection and cell division during the 72 h assay period (Fig. 1B).

To analyze the data, in-house image analysis algorithms were developed for the detection and quantification of certain cellular phenotypes (Fig. 2). Images from five fields per well were taken in three different channels. The algorithm identifies nuclei stained with Hoechst (blue channel) and determines the total number of cells, which along with nuclear size and intensity were used as indicators of compound induced cytotoxicity (Fig 2B). In the green channel, GFP fluorescence intensity, which is proportional to HCV gt1b RNA replication, is measured and expressed as percentage of GFP positive cells. In the red channel, the software determines the number of RFP-NLS-IPS expressing cells by RFP fluorescence intensity in either the nucleus or cytoplasm, and calculates the percentage of RFP translocation positive cells as a marker of HCVcc gt2 infection (Fig. 2).

The assay was validated by 10-points dose response curve (DRC) analysis using NM-107 (2′-C-methylcytidine) (Bassit et al., 2008), a nucleoside NS5B inhibitor with cross-genotypic activity, and A-837093 (Lu et al., 2007), a non-nucleoside NS5B inhibitor specific for gt1 (Fig. 3). As expected, increasing concentrations of NM-107, decreased both NS5A-GFP expression in the cytoplasm (gt1b replication) as well as RFP-NLS translocation into the nuclei (gt2a HCVcc infection) (Fig. 3A). This can be quantified by image processing as described in Fig. 2. A comparison of active concentrations of NM-107 treatment (effective concentration giving 100% inhibition; EC_{100}) with DMSO solvent control is shown in Fig. 3B. These data demonstrate that NM-107 efficiently inhibits both gt1b replication (reduction of GFP expression) as well as gt2 infection (reduction of translocated RFP) without affecting cell growth even at high concentrations (EC_{100}) (nuclear parameters measured in blue channel were unchanged). From these various outputs of total cell number (SumCellNumber), percent of GFP expressing cells (AvgPercentCellGFP), and RFP translocation cells (Ratio), DRCs can be derived to assess cytotoxicity, gt1b RNA replication and gt2 HCVcc infection, respectively as illustrated in Fig. 3C for NM-107 and A-837093. Both gt1 RNA replication and gt2 HCVcc infection were inhibited by NM-107 treatment in dose dependent manner as shown in green and red, respectively. This antiviral effect was not related to cytotoxicity that started to be detectable only at the highest compound concentrations (grey area in Fig. 3C). The EC_{50} of NM-107 was calculated from each DRC by non-linear regression analysis using Prism (GraphPad Software, Inc.) at 4.06 μM against gt1 RNA replication and 6.1 μM against gt2 HCVcc versus more than 300 μM for CC_{50} (cytotoxic concentration giving 50% cell death) (Fig. 3C). These values were comparable to published data (Bassit et al., 2008) and non-multiplexed assays using the gt1 replicon (4.46 ± 1.5 μM) or gt2 HCVcc (8.8 ± 2.2 μM). Likewise, a DRC analysis with A-837093 (Fig. 3C) resulted in dose dependent antiviral activity against gt1 replicons but not against gt2 HCVcc as shown by decreased GFP expression and unchanged RFP localization respectively (Fig. 3C lower chart). We tested several HCV inhibitors which have different mode of action to demonstrate that this assay is suitable to identify inhibitors targeting various steps in the viral life cycle (Fig. 3C table). Telaprevir, a NS3–4A protease inhibitor (Selleck Chemicals, USA) (Lin et al., 2006), GS-7977, a NS5B inhibitor (MedChem Express, China) (Murakami et al., 2010; Sofia et al., 2010), LY-411575, a late step inhibitor (BOC Science, USA) (Wichroski et al., 2012), and an antibody serving as an entry inhibitor by targeting CD81 (BD Biosciences, USA) were tested by 10-points DRC analysis as described above. EC_{50} values of each inhibitor are comparable with previously reported data. In addition, we observed couple phenotype which is the result of primarily infection and cell division during the 72 h assay period in late step inhibitor treatment (Fig. 3D).

The multiplex system presented here facilitates the simultaneous evaluation of not only antiviral activity and cytotoxicity but also provides basic mechanistic information. This strategy is time and cost effective, as more information can be acquired in comparison with classical assays using a single readout (e.g. luciferase values). Importantly, our multiplex assay is compatible with HTS. Furthermore, the spectrum of information gained in this one-step-approach narrows down which step in the life cycle the compound inhibits and facilitates a rapid inhibitor triage. Additionally, by combining different HCV genotypes, enables to identify drug candidates with cross-genotypic coverage and allowstranging of potentially genotype-specific compounds. Finally, the advantage of monitoring cytotoxic effects in parallel reduces the probability of selecting less favorable compounds. Taken together, the phenotypic assay described here facilitates the selection of antivirals with a novel mechanisms of action, which are potential new therapeutics and tools to elucidate the still poorly understood HCV life cycle.

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