Antiviral Research 99 (2013) 6–11

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Short Communication

Development of a multiplex phenotypic cell-based high throughput screening assay to identify novel hepatitis C virus antivirals ☆

Hee-Young Kim [a], Xiaolan Li [b], Christopher T. Jones [c], Charles M. Rice [c], Jean-Michel Garcia [a], Auguste Genovesio [b], Michael A.E. Hansen [b], Marc P. Windisch [a,*]

[a] Applied Molecular Virology, Institut Pasteur Korea, 696 Sampyeong-dong, Bundang-gu, Seongnam-si, Gyeonggi-do, South Korea
[b] Image Mining, Institut Pasteur Korea, 696 Sampyeong-dong, Bundang-gu, Seongnam-si, Gyeonggi-do, South Korea
[c] Center for the Study of Hepatitis C, Laboratory of Virology and Infectious Disease, Rockefeller University, NY, USA

ARTICLE INFO

Article history:
Received 5 February 2013
Revised 24 April 2013
Accepted 27 April 2013
Available online 6 May 2013

Keywords:
HCV
multiplex
phenotypic
high throughput screening assay

ABSTRACT

Hepatitis C virus (HCV) infection is a global health concern with chronic liver damage threatening 3% of the world's population. To date, the standard of care is 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 gt1 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.

© 2013 The Authors. Published by Elsevier B.V. All rights reserved.

Hepatitis C virus (HCV) 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 (PEG-IFNα) and ribavirin (RBV), results in unsatisfactory rates of sustained virologic response (SVR) of 40–50% for patients infected with HCV genotype (gt) 1 and about 80% for those infected with gt 2 or 3. Furthermore, this treatment regimen is associated with severe side effects often responsible for low adherence to treatment (Chevaliez and Pawlotsky, 2007; Hayashi and Takehara, 2006; Manns et al., 2006; Shepherd et al., 2007). Recently, the addition of expensive direct-acting antiviral agents (DAAs) to the previous SOC has improved the SVR rates in HCV gt1 infected patients, but unfortunately accompanied by additional side effects (Ghany et al., 2011). This emerging clinical data prompted us to develop a high-throughput screen (HTS) assay to identify novel antiviral targets.

Various strategies have been applied to screen compound libraries to identify new HCV antivirals; e.g. target-based enzymatic assays using the viral protease, helicase or polymerase. Potentially promising compounds require secondary profiling in more complex cell-based assays to assess membrane permeability, protein interactions, and toxicity, all of which will be used to improve the physiochemical, pharmacokinetic, and pharmacodynamic properties during compound optimization. The development of the HCV subgenomic replicon system (Lohmann et al., 1999) enabled screening of small molecule libraries targeting both viral and cellular targets in a more physiological context (Lemm et al., 2010; Mondal et al., 2009). In 2005, an HCV gt2 infectious clone was described supporting the production of infectious HCV particles in cell culture (HCVcc), enabling for the first time the investigation of the full viral life cycle (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). An infectious cell culture

☆ This is an open-access article distributed under the terms of the Creative Commons Attribution-NonCommercial-No Derivative Works License, which permits non-commercial use, distribution, and reproduction in any medium, provided the original author and source are credited.

* Corresponding author. Tel.: +82 31 8018 8180; fax: +82 31 8018 8014.
E-mail address: mpwindisch@ip-korea.org (M.P. Windisch).

0166-3542/$ - see front matter © 2013 The Authors. Published by Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2013.04.020

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 nucleus 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

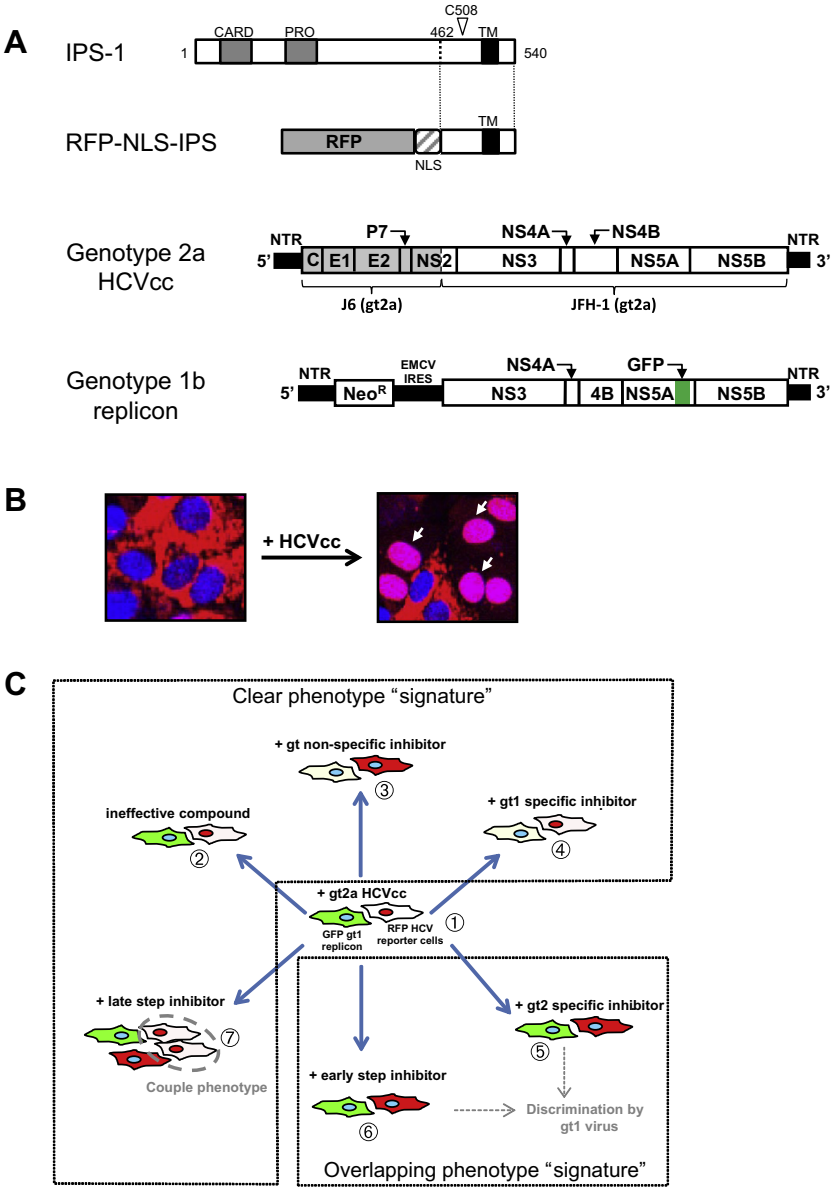
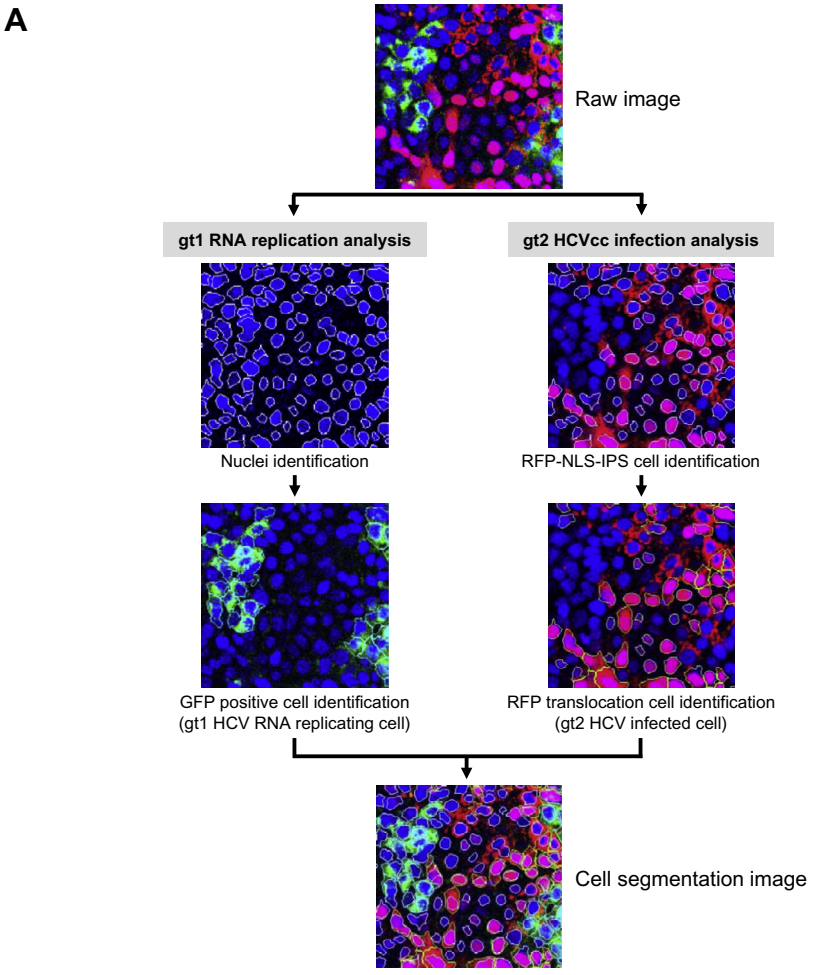


Fig. 1. Constructs and concept of the phenotypic multiplex assay. (A) Protein structure of IPS-1 indicating the caspase recruitment (CARD) and proline-rich (PRO) domains, and HCV NS3-4A protease cleavage site at position C508 (arrow). Structure of the fusion reporter protein RFP-NLS-IPS with N-terminal 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-①) or in presence of an inactive compound (Fig. 1C-②) 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-③); whereas a gt1 specific inhibitor would lead to a decreased GFP expression in replicon cells and nuclear RFP

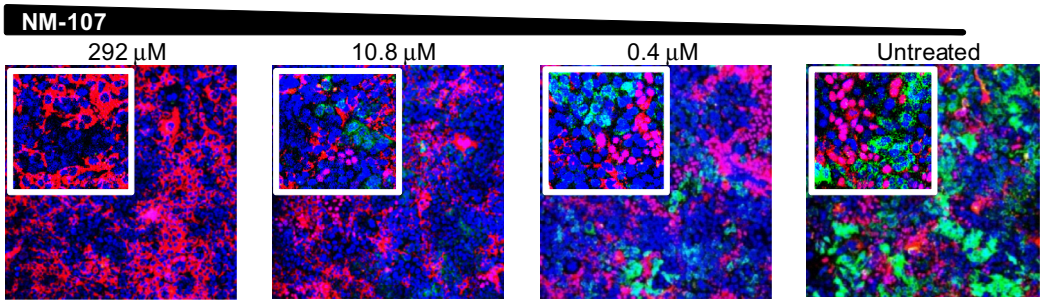


B

	Output	Information
Blue Channel Hoechst label Nucleus staining	SumCellNumber	Total cell number
	AvgNucleiIntensity	Mean nuclei intensity
	AvgNucleiVolume	Mean nuclei size
	AvgCellDistance	Mean cell distance
Green Channel GFP label gt1b RNA replication	SumCellGFPNumber	GFP positive cell number
	AvgCytoplasmGFPIntensity	Mean cytoplasmic GFP intensity
	AvgPercentCellGFP	Percentage of GFP positive cell
		(GFP positive cell number/Total cell number)*100
Red Channel RFP label gt2 HCVcc infection	NumCellsRFP	RFP-NLS-IPS expressing cell number
	Ratio	Percentage of RFP translocation cell
		(RFP translocation positive cell number/RFP-NLS-IPS expressing cell number)*100

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.

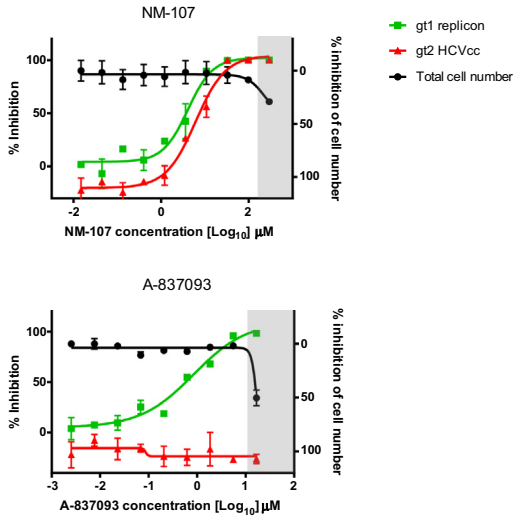
A



B

		NM-107 EC_{100} (100 μM)	DMSO control (No treatment)
Blue Channel Hoechst label Nucleus staining	SumCellNumber	10931	9769
	AvgNucleusIntensity	1532.5	1883.6
	AvgNucleusVolume	254.4	250.7
	AvgCellDistance	24.7	26.2
Green Channel GFP label gt1b RNA replication	SumCellGFPNumber	377	4227
	AvgCytoplasmGFPIntensity	138.3	1404.8
	AvgPercentCellGFP	3.4	43.3
Red Channel RFP label gt2 HCVcc infection	NumCellsRFP	6487	5168
	Ratio	5.3	41.7

C



		EC_{50}	CC_{50}
NM-107 gt non-specific NS5B inhibitor	gt1 RNA replication (GFP analysis)	4.06 μM	>300 μM
	gt2 HCVcc infection (RFP translocation analysis)	6.1 μM	
A-837093 gt1 specific NS5B inhibitor	gt1 RNA replication	0.84 μM	16.5 μM
	gt2 HCVcc infection	>16.7 μM	
Telaprevir gt non-specific protease inhibitor	gt1 RNA replication	0.59 μM	>30 μM
	gt2 HCVcc infection	0.20 μM	
GS-7977 gt non-specific NS5B inhibitor	gt1 RNA replication	0.034 μM	>10 μM
	gt2 HCVcc infection	0.070 μM	
LY-411575 Late step inhibitor	gt1 RNA replication	>10 μM	>10 μM
	gt2 HCVcc infection	0.09 μM	
Anti-CD81 antibody Entry inhibitor	gt1 RNA replication	>4 μg/mL	>4 μg/mL
	gt2 HCVcc infection	0.17 μg/mL	

D

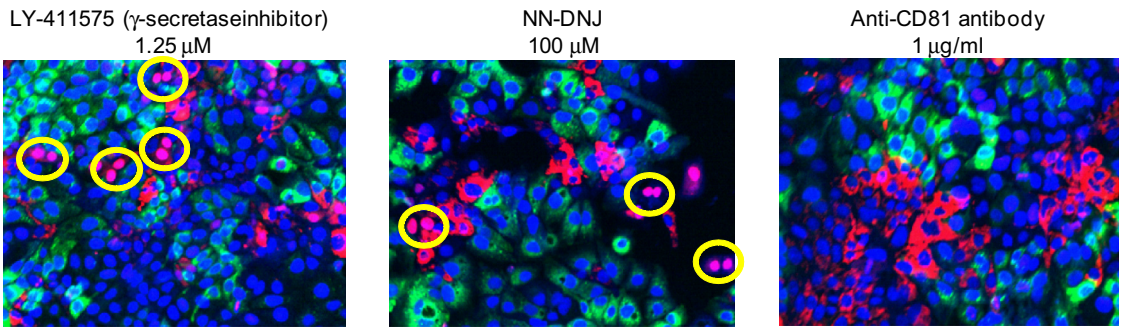


Fig. 3. Assay evaluation with reference compounds by dose response curve (DRC) analysis. (A) Different concentrations of NM-107 were tested (10 points 3-fold serial dilution from 292 μM) and analyzed according to the scheme shown in Fig. 2. Representative images and the respective concentrations are indicated. (B) Analyzed values generated by image processing software for EC_{100} (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/EC_{100} control into percentage of inhibition and shown in the graph with error bar of quadruplicate experiments. The table provides the EC_{50} and CC_{50} 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 gt 1a/2a chimera. 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 replicon) 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 Bioscience, 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 allowstriaging 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.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST/No. 2011-00244), Gyeonggi-do and KISTI. C.T.J. and C.M.R. were supported by grants from the NIH (CA057973 and DK085713), the Starr Foundation and the Greenberg Medical Research Institute.

References

Bassit, L., Grier, J., Bennett, M., Schinazi, R.F., 2008. Combinations of 2′-C-methylcytidine analogues with interferon-alpha2b and triple combination with ribavirin in the hepatitis C virus replicon system. Antivir. Chem. Chemother. 19, 25–31.

Chevaliez, S., Pawlotsky, J.M., 2007. Interferon-based therapy of hepatitis C. Adv. Drug Deliv. Rev. 59, 1222–1241.

Chockalingam, K., Simeon, R.L., Rice, C.M., Chen, Z., 2010. A cell protection screen reveals potent inhibitors of multiple stages of the hepatitis C virus life cycle. Proc. Natl. Acad. Sci. USA 107, 3764–3769.

Gastaminza, P., Whitten-Bauer, C., Chisari, F.V., 2010. Unbiased probing of the entire hepatitis C virus life cycle identifies clinical compounds that target multiple aspects of the infection. Proc. Natl. Acad. Sci. USA 107, 291–296.

Gentzsch, J., Hinkelmann, B., Kaderali, L., Irschik, H., Jansen, R., Sasse, F., Frank, R., Pietschmann, T., 2011. Hepatitis C virus complete life cycle screen for identification of small molecules with pro- or antiviral activity. Antiviral Res. 89, 136–148.

Ghany, M.G., Nelson, D.R., Strader, D.B., Thomas, D.L., Seeff, L.B., American Association for Study of Liver, D., 2011. An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practice guideline by the American Association for the Study of Liver Diseases. Hepatology 54, 1433–1444.

Hayashi, N., Takehara, T., 2006. Antiviral therapy for chronic hepatitis C: past, present, and future. J. Gastroenterol. 41, 17–27.

Jones, C.T., Catanese, M.T., Law, L.M., Khetani, S.R., Syder, A.J., Ploss, A., Oh, T.S., Schoggins, J.W., MacDonald, M.R., Bhatia, S.N., Rice, C.M., 2010. Real-time imaging of hepatitis C virus infection using a fluorescent cell-based reporter system. Nat. Biotechnol. 28, 167–171.

Lemm, J.A., O'Boyle 2nd, D., Liu, M., Nower, P.T., Colonno, R., Deshpande, M.S., Snyder, L.B., Martin, S.W., St Laurent, D.R., Serrano-Wu, M.H., Romine, J.L., Meanwell, N.A., Gao, M., 2010. Identification of hepatitis C virus NS5A inhibitors. J. Virol. 84, 482–491.

Li, Y.P., Ramirez, S., Jensen, S.B., Purcell, R.H., Gottwein, J.M., Bukh, J., 2012. Highly efficient full-length hepatitis C virus genotype 1 (strain TN) infectious culture system. Proc. Natl. Acad. Sci. USA 109, 19757–19762.

Lin, K., Perni, R.B., Kwong, A.D., Lin, C., 2006. VX-950, a novel hepatitis C virus (HCV) NS3-4A protease inhibitor, exhibits potent antiviral activities in HCv replicon cells. Antimicrob. Agents Chemother. 50, 1813–1822.

Lindenbach, B.D., Evans, M.J., Syder, A.J., Wolk, B., Tellinghuisen, T.L., Liu, C.C., Maruyama, T., Hynes, R.O., Burton, D.R., McKeating, J.A., Rice, C.M., 2005. Complete replication of hepatitis C virus in cell culture. Science 309, 623–626.

Lindenbach, B.D., Meuleman, P., Ploss, A., Vanwolleghem, T., Syder, A.J., McKeating, J.A., Lanford, R.E., Feinstone, S.M., Major, M.E., Leroux-Roels, G., Rice, C.M., 2006. Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. Proc. Nat. Acad. Sci. USA 103, 3805–3809.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., Bartenschlager, R., 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 285, 110–113.

Loo, Y.M., Owen, D.M., Li, K., Erickson, A.K., Johnson, C.L., Fish, P.M., Carney, D.S., Wang, T., Ishida, H., Yoneyama, M., Fujita, T., Saito, T., Lee, W.M., Hagedorn, C.H., Lau, D.T., Weinman, S.A., Lemon, S.M., Gale Jr., M., 2006. Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. Proc. Nat. Acad. Sci. USA 103, 6001–6006.

Lu, L., Dekhtyar, T., Masse, S., Pithawalla, R., Krishnan, P., He, W., Ng, T., Koev, G., Stewart, K., Larson, D., Bosse, T., Wagner, R., Pilot-Matias, T., Mo, H., Molla, A., 2007. Identification and characterization of mutations conferring resistance to an HCV RNA-dependent RNA polymerase inhibitor in vitro. Antiviral Res. 76, 93–97.

Manns, M.P., Wedemeyer, H., Cornberg, M., 2006. Treating viral hepatitis C: efficacy, side effects, and complications. Gut 55, 1350–1359.

Mondal, R., Koev, G., Pilot-Matias, T., He, Y., Ng, T., Kati, W., Molla, A., 2009. Development of a cell-based assay for high-throughput screening of inhibitors against HCV genotypes 1a and 1b in a single well. Antiviral Res. 82, 82–88.

Moradpour, D., Evans, M.J., Gosert, R., Yuan, Z., Blum, H.E., Goff, S.P., Lindenbach, B.D., Rice, C.M., 2004. Insertion of green fluorescent protein into nonstructural protein 5A allows direct visualization of functional hepatitis C virus replication complexes. J. Virol. 78, 7400–7409.

Murakami, E., Tolstykh, T., Bao, H., Niu, C., Steuer, H.M., Bao, D., Chang, W., Espiritu, C., Bansal, S., Lam, A.M., Otto, M.J., Sofia, M.J., Furman, P.A., 2010. Mechanism of activation of PSI-7851 and its diastereoisomer PSI-7977. J. Biol. Chem 285, 34337–34347.

Pietschmann, T., Kaul, A., Koutsoudakis, G., Shavinskaya, A., Kallis, S., Steinmann, E., Abid, K., Negro, F., Dreux, M., Cosset, F.L., Bartenschlager, R., 2006. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. Proc. Nat. Acad. Sci. USA 103, 7408–7413.

Shepherd, J., Jones, J., Hartwell, D., Davidson, P., Price, A., Waugh, N., 2007. Interferon alpha (pegylated and non-pegylated) and ribavirin for the treatment of mild chronic hepatitis C: a systematic review and economic evaluation. Health Technol. Assess. 11, 1–205, iii.

Simmonds, P., Bukh, J., Combet, C., Deleage, G., Enomoto, N., Feinstone, S., Halfon, P., Inchauspe, G., Kuiken, C., Maertens, G., Mizokami, M., Murphy, D.G., Okamoto, H., Pawlotsky, J.M., Penin, F., Sablon, E., Shin, I.T., Stuyver, L.J., Thiel, H.J., Viazov, S., Weiner, A.J., Widell, A., 2005. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology 42, 962–973.

Sofia, M.J., Bao, D., Chang, W., Du, J., Nagarathnam, D., Rachakonda, S., Reddy, P.G., Ross, B.S., Wang, P., Zhang, H.R., Bansal, S., Espiritu, C., Keilman, M., Lam, A.M., Steuer, H.M., Niu, C., Otto, M.J., Furman, P.A., 2010. Discovery of a beta-d-2′-deoxy-2′-alpha-fluoro-2′-beta-C-methyluridine nucleotide prodrug (PSI-7977) for the treatment of hepatitis C virus. J. Med. Chem. 53, 7202–7218.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11, 791–796.

WHO, 2012. <http://www.who.int/csr/disease/hepatitis/whocdscsrlyo2003/en/index4.html>.

Wichroski, M.J., Fang, J., Eggers, B.J., Rose, R.E., Mazzucco, C.E., Pokornowski, K.A., Baldick, C.J., Anthony, M.N., Dowling, C.J., Barber, L.E., Leet, J.E., Beno, B.R., Gerritz, S.W., Agler, M.L., Cockett, M.I., Tenney, D.J., 2012. High-throughput screening and rapid inhibitor triage using an infectious chimeric Hepatitis C virus. PLoS ONE 7, e42609.

Yi, M., Villanueva, R.A., Thomas, D.L., Wakita, T., Lemon, S.M., 2006. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc. Natl. Acad. Sci. USA 103, 2310–2315.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D.R., Wieland, S.F., Uprichard, S.L., Wakita, T., Chisari, F.V., 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. USA 102, 9294–9299.