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Short communication

Development of a cell-based assay for high-throughput screening of inhibitors against HCV genotypes 1a and 1b in a single well

Rubina Mondal*, Gennadiy Koev, Tami Pilot-Matias, Yupeng He, Teresa Ng, Warren Kati, Akhteruzzaman Molla

Antiviral Research, Global Pharmaceutical Research and Development, Abbott Laboratories, Abbott Park, IL 60064, USA

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ABSTRACT

The Hepatitis C (HCV) replicon system is a useful tool for the high-volume screening of inhibitors of HCV replication. In this report, a cell-based assay has been described, which monitors the inhibition of HCV genotypes 1a and 1b as well as cytotoxicity, from a single well of a 96-well plate. A mixture of two stable replicon cell lines was used: one containing a 1a-H77 replicon expressing a firefly luciferase reporter, and the other one containing a 1b-N replicon with a secreted alkaline phosphatase reporter, thus allowing us to monitor replication of two HCV genotypes in the same well. Cytotoxicity was measured using the Resazurin cytotoxicity assay. The assay was validated with known HCV inhibitors and showed that the antiviral activity and cytotoxicity of compounds were reproducibly measured under screening conditions. It was also showed that the assay's signal-to-noise ratio and Z' coefficient were suitable for high-throughput screening. A panel of HCV inhibitors showed a good correlation between EC_{50} and TD_{50} values for 1a and 1b replicon activity and cytotoxicity measured using either a single replicon format or mixed replicon format. Thus, the use of this mixed replicon format provides an economical method for simultaneous measurement of compound activity against two HCV genotypes as well as cytotoxicity, thereby reducing cost of reagents and labor as well as improving throughput.

1. Article outline

Hepatitis C (HCV) is a global health problem and the WHO estimates that about 3% of the world's population has been infected, of which 170 million are HCV carriers at risk of developing liver cirrhosis and/or liver cancer (WHO, 1997). There is no vaccine available for HCV and the current standard of care consisting of a pegylated interferon alpha/ribavirin (PEG-IFN/RBV) combination is effective in only a subset of infected patients. HCV subtypes 1a (52%) and 1b (25%) are the most common genotypes in the United States. These subtypes also are predominant in Europe (50% 1a, 20% 1b), and >50% in both South America and Australia. In Japan and Russia, subtype 1b is responsible for up to 73% and 69% respectively of cases of HCV infection (Zein, 2000). HCV patients infected with genotype 1 are particularly difficult to treat. Sustained virological response rate to PEG-IFN/RBV therapy in these patients is only 40–50% compared to the approximately 80% response rate in patients infected with HCV genotypes 2 and 3 (Fried et al., 2002; McHutchison et al., 1998). The absence of alternative therapy for non-responders or those ineligible for IFN-based therapy due to severe side effects has generated a patient population in urgent need of new and more effective drugs to treat HCV infection.

HCV is an enveloped virus that contains a single stranded, positive-sense RNA genome of about 9.6 kilobases. A subgenomic replicon system that recapitulates the replication cycle of HCV has been established in a human hepatoma cell line (Blight et al., 2000; Lohmann et al., 1999).

Levels of HCV subgenomic replicon RNA replication can be determined by quantitative RT-PCR (Stuyver et al., 2003). However, replicon constructs have been developed that contain reporter genes allowing a simplified and more economical way of monitoring replication such as firefly luciferase (Krieger et al., 2001), secreted alkaline phosphatase (SEAP), (Yi et al., 2002), chloramphenicol acetyltransferase (Hirowatari et al., 1995), beta-lactamase (Murray et al., 2003), or beta-galactosidase (Goergen et al., 1994).

High-throughput compound testing assays have been developed and are used to find inhibitor leads for further optimization. Compound screens based on HCV enzyme inhibition (NS5B polymerase or NS3 protease) are very efficient, allowing for a high-throughput format. But replicon cell-based assays, although limited to a lower throughput level, have the advantage of identifying inhibitors of all targets encoded by the non-structural genes in the replicon, and

* Corresponding author. Tel.: +1 847 9359154; fax: +1 857 9382756.
E-mail addresses: rubina.mondal@abbott.com, rubinamondal@yahoo.com (R. Mondal).

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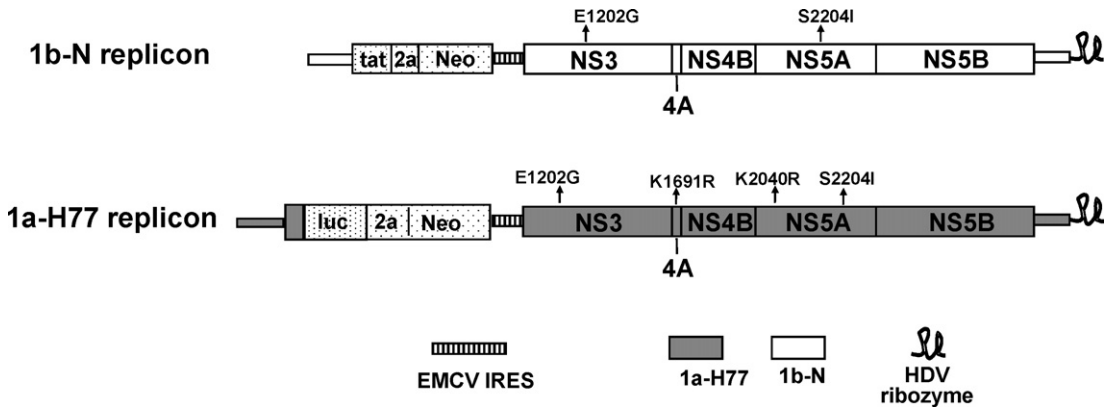


Fig. 1. Organization of subgenomic HCV replicons. Open-reading frames are shown as boxes and non-translated regions as bars. Replicons contain HCV non-structural proteins from genotype 1b (strain N) or genotype 1a (strain H77), as well as HIV tat-FMDV 2a protease-neomycin phosphotransferase cassette (tat-2A-neo), and luc-2a-luciferase cassette. Positions of amino acid changes introduced to enhance HCV replication are marked (FMDV, foot-and-mouth disease virus; HDV, hepatitis delta virus; EMCV IRES, encephalomyocarditis virus internal ribosome entry site).

can exclude impermeable and cytotoxic compounds. Biochemical assays are used when specific targets are studied and involve binding or enzyme inhibition mechanisms. But, if the target requires other cellular/viral proteins to function (as in the replicase), cell-based assays would provide the full complement of proteins and factors necessary for function of those targets. It presents the targets in a more physiological context; i.e. in complexes with other HCV non-structural proteins and host cofactors. For example, GS-9190 is a compound discovered by Gilead, which is reportedly a polymerase inhibitor, but does not inhibit HCV polymerase in a biochemical enzyme assay (Shih et al., 2007). Compounds like this would be missed in a conventional enzyme-based screen. Also since

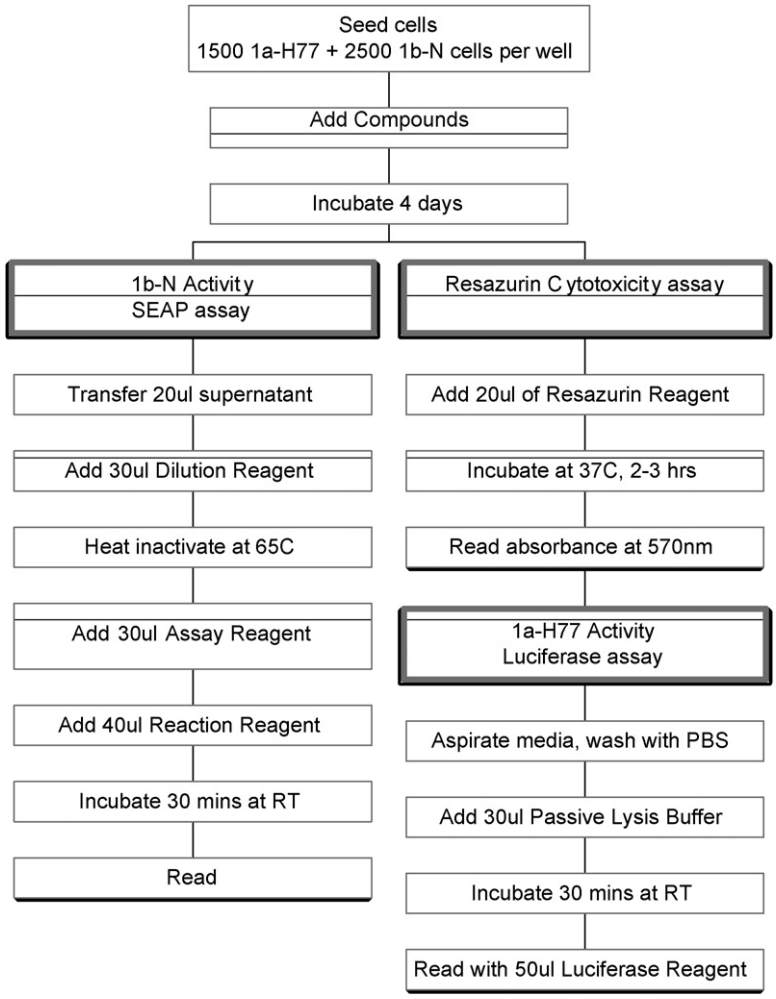


Fig. 2. Flowchart for the HCV replicon screen. 1a-H77 and 1b-N replicon cells are mixed and seeded into 96-well plate and compounds added the following day. After 4 days of incubation, supernatant from wells is transferred and SEAP assay performed for 1b replicon activity. Then Resazurin-based assay is performed to assess the cytotoxicity. Finally cells are lysed and luciferase signal read for 1a activity.

cell-based assays do not target a specific protein or enzyme, they can be used to identify novel targets.

In this report, a cell-based high-throughput screening (HTS) assay has been described, which measures three parameters from a single well of a 96-well plate: cytotoxicity as well as inhibition of HCV genotype 1a and 1b subgenomic replicons. The Resazurin-based cytoxicity assay was used to monitor cytotoxicity. Resazurin is an active component of the commercially available Alamar Blue reagent and can be used directly to determine cytotoxicity (McMillian et al., 2002; Nakayama et al., 1997; O'Boyle et al., 2005). To monitor the effect of compounds on HCV genotypes 1a and 1b, a mixture of two stable replicon cell lines was used (Fig. 1). One is the 1b-N replicon cell line that contains the non-structural genes from the HCV genotype 1b N strain (1b-N) along with a SEAP reporter (Yi et al., 2002). The first cistron of the bicistronic construct encodes a fusion of the human immunodeficiency virus (HIV) tat gene followed by the gene encoding the 2a protease of foot-and-mouth disease virus (FMDV) and the neomycin phosphotransferase gene. The second cistron contains the non-structural genes NS3-NS5B from HCV 1b-N with the adaptive mutations E1202G and S2204I (numbered relative to the amino acid position in the viral open reading frame). The hepatitis delta virus ribozyme was inserted after the 3′ NTR so that the exact 3′ end of the HCV sequence will be generated after ribozyme cleavage (Chung et al., 2001). The host cell line En5-3 is derived from Huh7 cells and is stably transfected with the SEAP gene under control of the HIV LTR promoter. Replication of the replicon results in the expression of the Tat protein, which transactivates the HIV LTR resulting in the expression of SEAP, which is secreted into the cell culture medium. This system allows detection of SEAP in the supernatant of the cells for the measurement of anti-HCV activity of inhibitors. The other stable replicon cell line was derived from HCV genotype 1a strain H77 (1a-H77) and contains the firefly luciferase reporter followed by the gene encoding the 2a protease of FMDV and the neomycin phosphotransferase gene in the first cistron. The NS3-NS5B sequence in the second cistron and the 5′- and 3′-non-translated regions (NTRs) were derived from genotype 1a-H77. Amino acid changes E1202G, K1691R, K2040R and S2204I were introduced to enhance replication (Blight et al., 2003; Grobler et al., 2003). The replicon contains the hepatitis delta virus ribozyme following the 3′ NTR as described above.

The 1a-H77 and 1b-N replicon cell lines were maintained as previously described (Mo et al., 2005). A panel of 12 compounds, all of which have been reported to have antiviral activity and which target different non-structural genes (NS5B polymerase or NS3 protease) were tested for activity and toxicity in both the single replicon and mixed replicon formats. The details of the synthesis of the Abbott benzothiadiazine NS5B polymerase inhibitors have been described elsewhere (Pratt et al., 2005; Randolph et al., unpublished results; Wagner et al., unpublished results). Other polymerase and protease inhibitors were synthesized according to procedures described previously: Wyeth HCV-796 (Burns et al., 2004), Gilead NS5B polymerase inhibitor (Bondy et al., 2007), Shire NS5B polymerase inhibitor (Corfield et al., 2007), Intermune ITMN-191 NS3 protease inhibitor (Blatt et al., 2005) and Boehringer Ingelheim BILN-2061 NS3 protease inhibitor (Faucher et al., 2004; Lamarre et al., 2003). Human leukocyte Interferon (IFN-α) was purchased from PBL Biomedical Laboratories, Piscataway, NJ. The experiment was performed twice in triplicate ($n = 6$). The cells were incubated with compounds, four-fold serial dilutions, from 100 μM to 0.4 μM, in order to encompass a wide concentration range and to obtain accurate EC_{50} and TD_{50} measurements. Replicon inhibition data were analyzed using the GraphPad Prism software (version 4, GraphPad Software, San Diego, CA, USA).

The use of different reporters for HCV genotypes 1a and 1b for replicon activity, as well as the Resazurin assay for cytotoxicity has allowed three different measurements from a single well of a cell culture plate. A flowchart describing this process is shown in Fig. 2. First, after 4 days of incubation with or without compounds, a small fraction of the supernatant is taken from the test plate to detect 1b-N replicon inhibition by measuring the SEAP activity. Second, the test plate is incubated with the Resazurin reagent, and a cytotoxicity measurement is taken. And, finally, the cells are lysed and 1a-H77 replicon inhibition is determined by measuring the activity of luciferase. In a typical experiment, 1a-H77 and 1b-N replicon cells were trypsinized, counted, mixed and seeded into each well of a 96-well white plate (1500 + 2500 respectively per well). After incubation at 37 °C overnight, the replicon cells were treated with serial dilutions of the compounds. The compounds were initially diluted in dimethyl sulfoxide (DMSO) to generate a 200X stock for each dilution, while the human IFN-α was diluted in cell culture medium. The medium from overnight cell culture plates was removed and replaced with fresh medium containing a 0.5% DMSO solution of inhibitor in a series of four-fold dilutions in medium. After 4 days a fraction of the supernatant from the wells was collected and the SEAP activity was measured

Table 1
Comparison of TD_{50}'s obtained from Resazurin cytotoxicity assay and MTT assay.

Compound	Resazurin cytotoxicity assay TD_{50} (μM) ± S.D.	MTT assay TD_{50} (μM) ± S.D.
	Single replicon format	Single replicon format
A-848837.5	32.3 ± 0.5	31.3 ± 1.8
A-782759.0	85.0 ± 19.8	79.9 ± 12.6
A-837093.5	38.0 ± 10.5	34.6 ± 5.8
A-802581.5	99.2 ± 1.2	98.8 ± 0.7
A-876828.0	75.7 ± 10.9	59.8 ± 8.0
A-924825.0	11.6 ± 1.1	13.6 ± 5.6
HCV-796	>100	>100
Gilead	>3[a]	>3[a]
Shire	53.0 ± 4.7	73.2 ± 37.9
ITMN-191	81.2 ± 24.6	91.4 ± 10.2
BILN-2061	19.8 ± 2.4	21.3 ± 4.5
IFN	>1000 IU/ml	>1000 IU/ml

No significant difference in toxicity was observed between the two assays.
[a] Precipitation of the compound was observed at 10 μM concentration, so toxicity was reported as greater than the next lower concentration, >3 μM.

Table 2
Z′-factor coefficients and signal-noise ratios for single and mixed replicon format with different combination of cells for Luciferase, SEAP and Resazurin cytotoxicity assays.

		Z factor	Signal–noise ratio
Single replicon format			
Resazurin cytotoxicity assay	1A-H77$_{(4000\ cells/well)}$	0.63	10
Luciferase assay	1A-H77$_{(4000\ cells/well)}$	0.99	1051
SEAP assay	1B-N$_{(4000\ cells/well)}$	0.73	6
Mixed replicon format			
1a-H77$_{(2000\ cells/well)}$ + 1B-N$_{(2000\ cells/well)}$			
Resazurin cytotoxicity assay		0.62	18
Luciferase assay		0.57	1014
SEAP assay		0.62	5
1a-H77$_{(1500\ cells/well)}$ + 1B-N$_{(2500\ cells/well)}$			
Resazurin cytotoxicity assay		0.86	17
Luciferase assay		0.70	1553
SEAP assay		0.72	6
1a-H77$_{(2500\ cells/well)}$ + 1B-N$_{(1500\ cells/well)}$			
Resazurin cytotoxicity assay		0.74	16
Luciferase assay		0.56	1816
SEAP assay		0.67	6

Note that no difference in signal-to-noise ratio was observed between either single replicon or mixed replicon format or between three combination of cells. Experiments were performed with 1500 and 2500 cells/well of 1a-H77 and 1b-N cells respectively because the *Z′*-factor obtained was >0.7 for all the three assays with this combination of cells.

Table 3
Comparison of EC_{50} and TD_{50} for twelve compounds using both single replicon and mixed replicon formats.

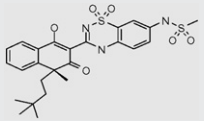
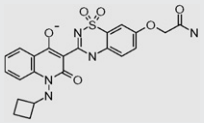
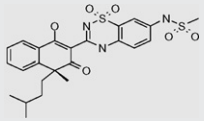
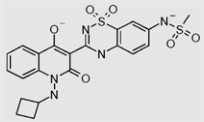
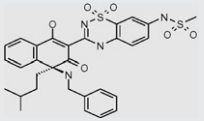
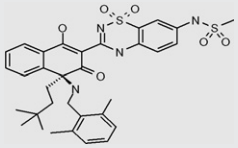
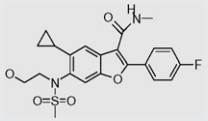
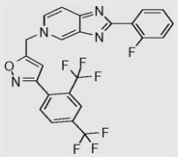
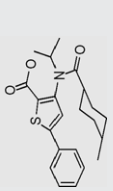
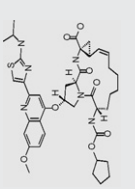
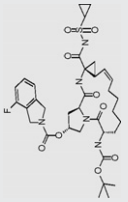
Compound	Structure	1a-H77 replicon			1b-N replicon			1a-H77 replicon		
		EC_{50} (μM) ± S.D.		Single/ mixed ratio	EC_{50} (μM) ± S.D.		Single/ mixed ratio	TD_{50} (μM) ± S.D.		Single/ mixed ratio
		Single replicon	Mixed replicon		Single replicon	Mixed replicon		Single replicon	Mixed replicon	
Polymerase inhibitors										
A-848837		0.004 ± 0.001	0.006 ± 0.0003	0.7	0.008 ± 0.002	0.008 ± 0.002	1.0	32.27 ± 0.50	38.69 ± 0.88	0.8
A-782759		0.083 ± 0.017	0.083 ± 0.024	1.0	0.204 ± 0.004	0.167 ± 0.012	1.2	85.03 ± 19.83	94.82 ± 7.33	0.9
A-837093		0.009 ± 0.002	0.011 ± 0.001	0.8	0.012 ± 0.005	0.013 ± 0.0002	0.9	37.99 ± 10.51	57.82 ± 22.01	0.7
A-802581		0.007 ± 0.001	0.009 ± 0.001	0.8	0.009 ± 0.001	0.013 ± 0.004	0.7	99.17 ± 1.17	91.15 ± 12.52	1.1
A-876828		0.058 ± 0.024	0.098 ± 0.013	0.6	0.092 ± 0.012	0.130 ± 0.010	0.7	75.65 ± 10.90	86.43 ± 0.95	0.9
A-924825		0.011 ± 0.006	0.007 ± 0.0003	1.5	0.018 ± 0.002	0.025 ± 0.002	0.7	11.62 ± 1.10	21.44 ± 0.63	0.5
Wyeth HCV-796		0.025 ± 0.002	0.022 ± 0.003	1.1	2.515 ± 0.159	2.809 ± 0.643	0.9	>100	>100	
Gilead NS5B polymerase inhibitor		0.002 ± 0.000	0.005 ± 0.002	0.5	0.018 ± 0.001	0.012 ± 0.006	1.5	> 3	> 3	

Table 3 (*Continued*)

Compound	Structure	1a-H77 replicon			1b-N replicon			1a-H77 replicon		
		EC_{50} (μM) ± S.D.		Single/ mixed ratio	EC_{50} (μM) ± S.D.		Single/ mixed ratio	TD_{50} (μM) ± S.D.		Single/ mixed ratio
		Single replicon	Mixed replicon		Single replicon	Mixed replicon		Single replicon	Mixed replicon	
Shire NS5B polymerase inhibitor		0.269 ± 0.025	0.460 ± 0.026	0.6	1.452 ± 0.130	1.455 ± 0.236	1.0	53.00 ± 4.68	71.42 ± 16.59	0.7
Protease inhibitors										
BILN-2061		0.007 ± 0.000	0.009 ± 0.002	0.8	0.005 ± 0.002	0.005 ± 0.003	1.0	19.78 ± 2.36	24.45 ± 0.01	0.8
Intermune ITMN-191		0.002 ± 0.001	0.002 ± 0.001	0.9	0.005 ± 0.001	0.004 ± 0.001	1.2	81.20 ± 24.63	>100	
Others										
IFN		0.343 ± 0.092 IU/ml	0.398 ± 0.008 IU/ml	0.9	2.162 ± 0.143 IU/ml	2.043 ± 0.887 IU/ml	1.1	>1000 IU/ml	>1000 IU/ml	

No significant difference observed for 1a and 1b activity and cytotoxicity between single replicon and mixed replicon format.

using the Phospha-Light Chemiluminescent Reporter Gene Assay System (Applied Biosystems), according to the manufacturer's protocol. In brief, 20 μl of supernatant is transferred into a 96-well white plate and 30 μl of 1X Dilution buffer added and heated for 30 min at 65 °C. Plates are cooled on ice to room temperature. Assay buffer (40 μl) is added and incubated for 5 min. Finally 40 μl of 1X Reaction buffer is added, plates incubated for 30 min and read for 1b SEAP signal. Then the toxicity of the compounds was measured by Resazurin cytotoxicity assay, which incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity resulting from cell growth. Reduction related to cell growth causes the indicator to change from an oxidized/non-fluorescent/blue form to a reduced/fluorescent/red form. Resazurin (1 mg/ml in PBS, Sigma–Aldrich) was added to the assay plates to a final concentration of 0.1 mg/ml. Plates were gently shaken to mix the reagent with the culture medium and then incubated at 37 °C for 2–3 h. Fluorescence was monitored at 530–560 nm excitation wavelength and 590 nm emission wavelength. This assay was selected due to the fact that the reagent is non-lytic and non-toxic to living cells. The cells were fully viable for the duration of the assay. Results were comparable to the conventional MTT assay (Table 1). Finally the medium was removed from the wells, cells washed twice with 1X PBS and lysed with 30 μl of passive lysis buffer (Promega). After 30 min of incubation at room temperature, luciferase signal from the plates was read using a Firefly luciferase kit (Promega) and a PerkinElmer Wallac EnVision Victor.

Table 2 shows the optimization of 1a-H77 and 1b-N replicon cell line cell numbers when used as a mixture in a single well. To choose the optimal assay format, we considered a signal to noise ratio and a *Z′*-factor for each variation of the assay. The signal to noise ratio, also known as the window, is the mean of the positive signal over the mean of the background. The background is determined by measuring replicon levels in wells treated with the NS3 protease inhibitor BILN-2061 at 0.1 μM. At this concentration the compound results in 99% inhibition of HCV replicon replication. The *Z′*-factor, also known as the screening window coefficient is a measure of the robustness of the assay (Zhang et al., 1999). To determine the *Z′*-factor, replicon cells were plated, either in the single or the mixed format, and one half of the plate (48 wells) was treated with 0.5% DMSO in cell culture medium (positive control) and the other half with 0.1 μM BILN-2061 in cell culture medium (negative control). The cells were incubated for 4 days and then luciferase, SEAP, and Resazurin cytotoxicity assays were performed as described above. The *Z′*-factor was calculated for the antiviral and cytotoxic endpoints from the no drug control wells using the equation: $1 - [(3 \times \text{S.D. positive control}) - (3 \times \text{S.D. negative control})/(\text{mean positive control} - \text{mean negative control})]$ where S.D. is the standard deviation (Zhang et al., 1999). *Z′*-factor values between 0.5 and 1 indicate a well-performing assay. Three combinations of cells were used: 2000 cells per well each of 1a-H77 and 1b-N, or 1500 1a-H77 + 2500 1b-N cells/well or 2500 1a-H77 + 1500 1b-N cells/well. The signal-to-noise ratio was unaffected when the assay utilized any of the three combinations of cells/well. Luciferase, SEAP and Resazurin cytotoxicity signal to noise ratio of >1000, ~6 and ~16, respectively were obtained with any of the three combinations of cell numbers used. Also, there is no significant difference in the signal to noise ratios between the single replicon and mixed replicon format for any of the three mentioned assays. A mixture of 1500 and 2500 cells/well of 1a-H77 and 1b-N, respectively, was chosen to perform our experiments because the *Z′*-factor obtained was >0.7 for all three assays with this combination of cells (Table 2). Both EC_{50} and TD_{50} values were found to be very similar for the 12 compounds irrespective of the assay format used (Table 3). There was no significant difference in the EC_{50} and TD_{50} values between single replicon format and mixed replicon format. Correlation factors of 0.96, 0.99 and 0.98 were obtained for 1a-H77 replicon activity, 1b-N

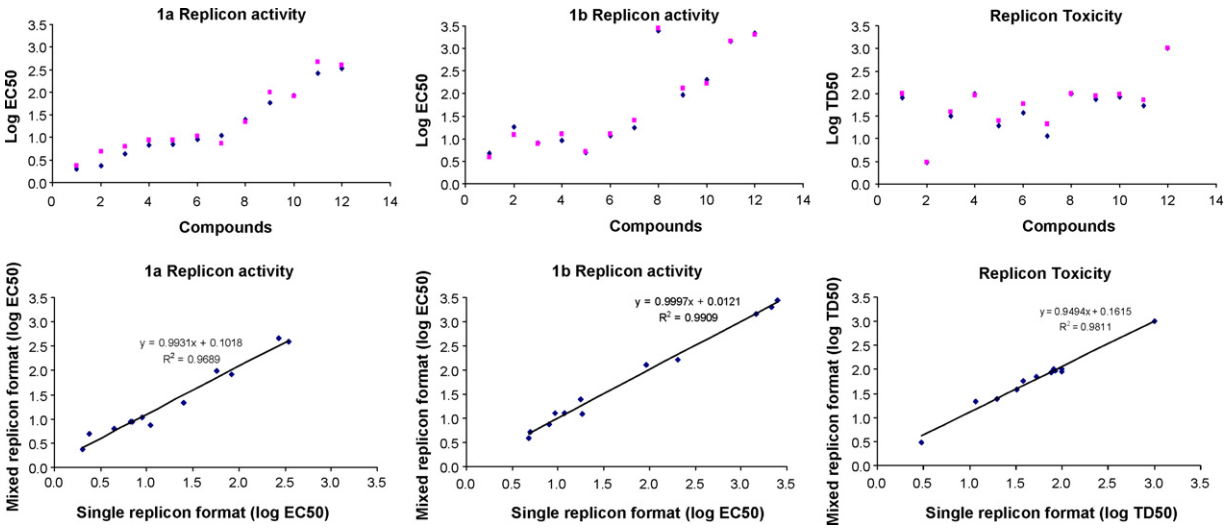


Fig. 3. No significant difference was seen between single replicon (blue diamond) and mixed replicon format (red square) when EC_{50} and TD_{50} of a set of twelve compounds were compared (upper panel). Correlation factors of 0.96, 0.99 and 0.98 were obtained for 1a replicon activity, 1b replicon activity and cytotoxicity, respectively, for the single replicon format vs. the mixed replicon format (lower panel).

replicon activity and cytotoxicity, respectively, for the single replicon format vs. the mixed replicon format (Fig. 3). It is noteworthy that the Wyeth polymerase inhibitor HCV-796 showed a relatively high EC_{50} of greater than 2 micromolar in our 1b replicon assay. The genotype 1b replicon used in this assay is based on the N-strain of HCV (Yi et al., 2002) which has an Asparagine (N) at position 316 of the NS5B. It was previously reported that HCV-796 is less active against isolates with a 316N (Howe et al., 2006).

The high-throughput replicon assay described here has the potential to be a highly useful and efficient tool for identification of compounds with antiviral activity against HCV genotypes 1a and 1b. Both of these genotypes are difficult to treat with the IFN/RBV-based standard of care, therefore the current HCV drug discovery and development is mostly focused on covering them as the first priority. Due to significant differences in the amino acid sequence between the two genotypes, some inhibitors show difference in potency against the two. As a practical matter, a drug must be sufficiently active against both genotypes 1a and 1b. However, this requirement does not extend to early screening hits. The use of our screening assay will allow identification of compounds with inhibitory activity against either genotype. Once such hits are identified, their further optimization will ensure adequate activity against both genotypes 1a and 1b. It is likely that compounds active against both genotypes 1a and 1b will have a higher resistance barrier. Broad HCV isolate coverage results from targeting more phylogenetically conserved binding sites on viral enzymes. These sites therefore, are less likely to easily allow development of resistance mutations without a significant fitness cost.

In summary, a convenient cell-based assay that measures inhibition of HCV genotypes 1a-H77 and 1b-N replicons and cytotoxicity, all from a single well by utilizing two reporters, luciferase and SEAP, and a non-lytic cytotoxicity assay has been established. Our results show excellent correlation between the values obtained using the conventional single readout assays and mixed replicon format. The Z'-factor assessment of assay quality suggests that this method could be used for medium to high-throughput screening applications. This assay can be used to determine compounds' in vitro activity against two different HCV genotypes and cytotoxicity at early stages of drug candidate selection. Since Resazurin was not destructive to the cells and did not cause any effect on the HCV replicon luciferase signal, it is possible to multiplex these assays. Because of the low variability, high signal-to-noise ratio, and the limited number of manipulations required, this assay could potentially be adapted to the 384-well format. By using this mixed-replicons format assay to determine compound activity against two HCV genotypes and cytotoxicity, we can reduce cost of reagents and labor and improve throughput.

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