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A 96-well based analysis of replicon elimination with the HCV NS5A replication complex inhibitor daclatasvir

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A 96-well based replicon elimination and colony formation assay is presented for comparing the resistance barrier of the hepatitis C virus (HCV) NS5A replication complex inhibitor daclatasvir (DCV, BMS-790052) on three HCV genotypes (gts) in a proof of concept experimental protocol. The 96-well assay format provides both individual colony as well as population characterization and is readily applicable to other HCV direct-acting antiviral agents (DAAs). The assay provides an assessment of HCV replication levels over a 5 $\log_{10}$ range by measuring a luciferase reporter resident in the HCV replicons. Individual colony status can be measured with a separate and compatible resazurin assay to assess relative host cell fitness following inhibitor treatments. The methods employed are non-toxic and leave intact isolatable colonies that can be used for phenotyping and genotyping. The utility of the assay is demonstrated by the identification and isolation of resistant variants as well as in the ranking of the relative resistance barrier for the replication complex inhibitor DCV for gts 1a, 1b and 2a. The format provides a quantitative ranking based upon luciferase activity and has the ability to monitor DAA resistance development over time for large numbers of compounds.

1. Introduction

Hepatitis C virus (HCV) is a positive stranded RNA virus which presents a major challenge for treatment of a chronic infection that is one of the leading causes of liver failure resulting in the need for transplantation. The current standard of care for HCV has evolved from an pegylated-interferon-α + ribavirin regimen to inclusion of the small molecule direct acting antiviral agents (DAAs) telaprevir or boceprevir, which target the viral NS3 protease and lead to significantly improved cure rates (Jacobson et al., 2011; Kwo et al., 2010). However, treatment of HCV using DAAs presents a challenge due to the high replication capacity of the virus and the error-prone RNA dependent RNA polymerase resulting in the generation of numerous viral quasispecies that can escape inhibition (Fanning, 2008). The ability of the viral – quasispecies to escape the inhibitory activity of DAAs due to the selection of resistant variants, defined as the resistance barrier, is dependent upon a complex interaction of a large number of variables and subtleties which includes the mechanism of action of the inhibitor being used, the pharmacokinetics of the inhibitor, the fitness of mutations affecting the inhibitor potency and the abundance of these mutations. The sum of these variables can be estimated and quantified using in vitro methods such as replicon elimination assays that can be correlated to efficacy seen clinically (Pelosi et al., 2012; Wang et al., 2012). The ability to assess the resistance barrier therefore, becomes an important measure of an inhibitor's potential to prevent resistant variants from arising due to selective pressure. Methods to examine the resistance barrier for HCV inhibitors in a pre-clinical setting are valuable in providing a method to prioritize compounds based upon the quantitation of an inhibitor's resistance barrier. Treatment of HCV replicons with combinations of DAAs, much like those explored during the evolution of HIV highly active anti-retroviral therapy (HAART), if compared pre-clinically, may enable a more cost-effective assessment for potential clinical efficacy (Shafer and Vuitton, 1999). The ability to assess treatments in vitro is therefore especially relevant when comparing multiple combinations of inhibitors such as those needed for HCV.

Many of the currently described HCV replicons incorporate the *Renilla* luciferase gene which provides a sensitive and robust luminescence signal to monitor HCV replication (Blight et al., 2000). Assays to assess antiviral activities routinely use the luciferase read-out for dose-response curves that yield reliable reproducibility, increased sensitivity, and convenience relative to other assays interrogating HCV replicon inhibition (Hao et al., 2007;

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O'Boyle et al., 2005; Robinson et al., 2011a). In this report, the luciferase-based assay protocol was further adapted in pursuit of providing an assay method to pre-clinically rank the ability of HCV inhibitors to eliminate the replicon from the host cell background. Comparisons using this assay method were made to the more traditional method of staining cell-culture dishes by monitoring the effect of a potent NS5A HCV replication complex inhibitor daclatasvir (DCV) versus three viral genotypes (gts). The use of a 96-well multi-plate format is presented that allows for more samplings of the population during compound treatment. The HCV replicons can be observed and monitored multiple times and isolated for further characterizations if desired to look at phenotype and gt. Importantly, the assay method can be applied to provide an estimated ranking of the HCV DAA resistance barrier based upon the residual amount of luciferase activity remaining following replicon elimination. The ability to quantify and compare the amount of resistant virus remaining for different gts defines a relative resistance barrier. The assay allows a gt sensitivity ranking toward the NS5A replication complex inhibitor DCV to be estimated and compared establishing a convenient method for HCV resistance barrier DAA measurements.

2. Materials and methods

2.1. Cell culture and replicon elimination assays

The HCV replicons have been previously described (Fridell et al., 2010, 2011) and consisted of: JFH1 (gt 2a) replicon cell line, the Con1 replicon clone (gt 1b) and the H77C (gt 1a) replicon cell lines which contain a *Renilla* luciferase (luc) reporter. The cells were maintained in complete DMEM (Life Technologies, Grand Island, NY, USA) supplemented with 1.0 mg/mL G418 (Geneticin, Life Technologies, Grand Island, NY, USA), 10% FBS (Sigma, St. Louis, MO, USA) and Pen/Strep (Life Technologies, Grand Island, NY, USA) Inhibitor half-maximal effective concentrations (EC_{50}'s) were calculated from replicon assays as described (Fridell et al., 2010). HCV replicon elimination assays were performed by diluting cells to ~2400 cells per mL in DMEM, 10% FBS + 1.0 mg/mL G418, Pen/Strep. The diluted cells were pipetted into either 100 mm tissue culture treated dishes (10 mL per plate, 24,000 cells) or into 96-well plates (0.1 mL per well, 240 cells) followed by placement into a humidified incubator for overnight attachment (typically ~12 h). The next day media was removed, treatments placed onto cells and the plates returned to the incubator for various time periods. Medias were removed after 7 days, the cells rinsed 1× and then replaced with media containing DMEM + 1.0 mg/mL G418 and allowed to incubate for additional periods of time before being assayed for either luciferase activity or resazurin conversion.

2.2. Daclatasvir (DCV)

DCV was synthesized at Bristol-Myers Squibb with >99% purity.

2.3. Luciferase assays

The *Renilla* luciferase assay was performed as recommended by the manufacturer and relied upon the live-cell substrate Enduren (Promega, Madison, WI, USA). This pro-luciferase substrate is only efficiently activated in live cells and was shown to be relatively non-toxic for the host Huh-7 cells (data not shown) when the cells are briefly exposed (~15 min) and rinsed 3× with media following data collection. To determine luciferase activity, Enduren substrate was diluted from a 60 mM stock in 100% DMSO by 1–1000 into DMEM media with 10% FBS, Pen/Strep that had been pre-warmed to 37 °C. Media was removed and 50 μL of DMEM/Enduren substrate added to each well. The plates were placed back into an incubator for 15 min before being read on a TopCount monitor (Perkin Elmer, Waltham, MA, USA). Plates saved for subsequent measurements had the Enduren/DMEM media removed and were rinsed 3× with 100 μL of DMEM, 10%FBS, 1.0 mg/mL G418 before having 100 μL of either media with compounds or only media placed back onto cells. Plates were then returned to the incubator. Luciferase measurements of all HCV replicon cell lines were capable of reaching maximum levels of ~10^5 (>100,000) luciferase signal from background levels of ≤10^2 (~100). Colonies were characterized by rinsing with PBS and trypsinized followed by placement into individual tissue culture containers for additional growth; cells were amplified and used to confirm phenotype/gt.

2.4. Cell viability resazurin assay

Cell-Titer-Blue cell viability assay was performed according to the manufacturers (Promega, Madison, WI, USA) recommendations. Briefly, the cell titer blue reagent (resazurin) was added to individual cell wells (10 μL/well containing 100 μL media) and assay reagent allowed to remain for up to 4 h or longer. Plates were removed and processed for fluorescence detection on a Gemini Fluorescence reader set at 560 excite, 590 emission; (maximum signal ~ 25,000). Cell plates were then either rinsed with media 3× and Enduren assay was performed in tandem or the plates were rinsed and returned to the incubator for additional time.

2.5. Colony staining

At indicated exposure times, cell plates (100 mm dishes or 96-well plates) had media removed, were rinsed with PBS and allowed to air dry completely. A crystal violet solution (Sigma) diluted to 0.2% in a 20% methanol/water (v/v) solution was briefly placed onto the plates followed by rinsing in water. The plates were allowed to air-dry before stained images were digitally captured (Syngene, Frederick, MD, USA).

2.6. Calculations and graphing of data

Luciferase activity from each well of the 96-well plates was placed into an Excel spreadsheet (Microsoft Office, Redmond, WA, USA) and either averaged or screened to include only wells with luciferase activity ≥500 units for colony counts. 500 U were chosen as a cutoff (~5× above background) since colonies with under 500 counts were generally not viable following picking (data not shown). All numbers are presented for three independent experiments (Table 2) for the residual luciferase at 1000× EC_{50} and for the number of colonies seen with luciferase >500. The gt1b replicon values were normalized to a value of 1 and used as the comparator versus gt1a and gt 2a for both luciferase counts and colony counts since gt 1b had the highest barrier to DCV resistance (most easily eliminated). The relative resistance barrier was additionally calculated by setting the luciferase activity in DMSO control plates to 100% activity and dividing the luciferase activity from each DCV titration (from 0.3× EC_{50} to 1000× EC_{50}) to obtain a percentage of the control for each gt.

2.7. RT-PCR and genotyping of colony isolates

To provide HCV replicon cells for phenotyping and genotyping, individual luciferase positive wells from 96-well plates exposed to either DMSO or 100× EC_{50} DCV were trypsinized and place into a 48-well tissue culture plate containing media + G418 for growth. This process was repeated with movement to a 24-well, 6-well and finally to a T-75 flask. Cells from the T-75 were then used for