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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

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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

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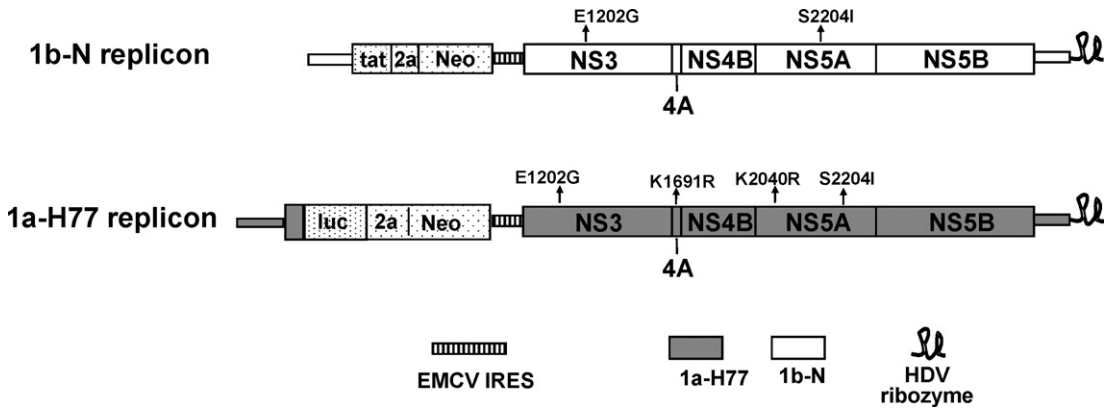

Fig. 1. Organization of subgenomic HCV replicons. Open-reading frames are shown as boxes and non-translated regions as bars. Replicons contain HCV non-structural proteins from genotype 1b (strain N) or genotype 1a (strain H77), as well as HIV tat-FMDV 2a protease-neomycin phosphotransferase cassette (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

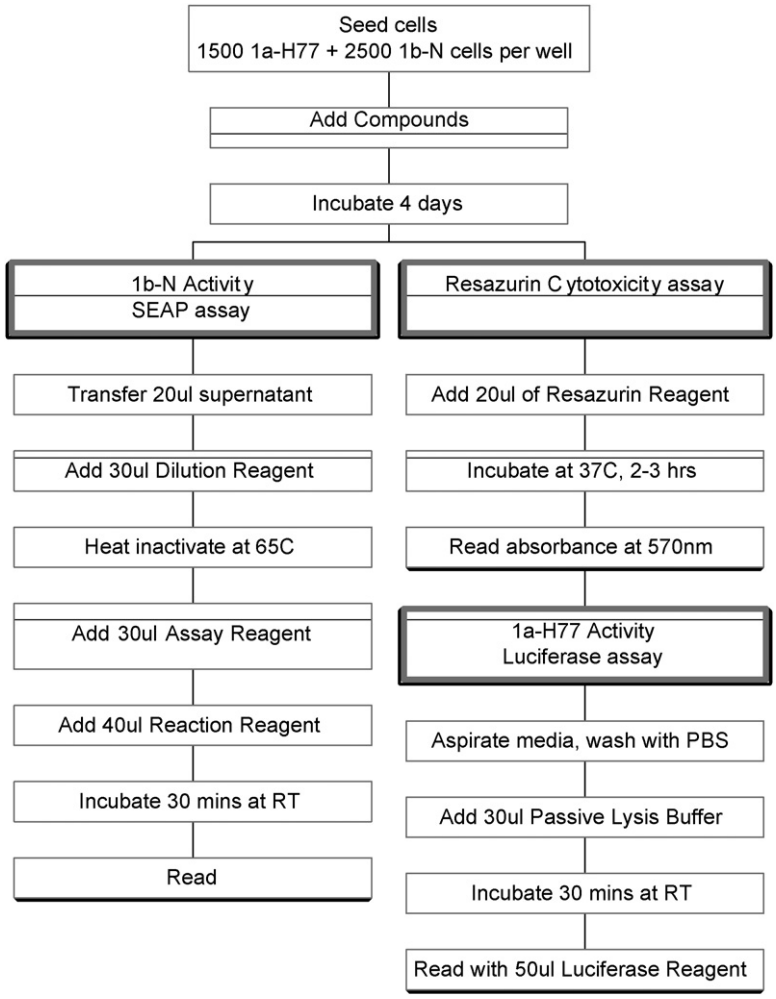

Fig. 2. Flowchart for the HCV replicon screen. 1a-H77 and 1b-N replicon cells are mixed and seeded into 96-well plate and compounds added the following day. After 4 days of incubation, supernatant from wells is transferred and SEAP assay performed for 1b replicon activity. Then Resazurin-based assay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