Benchmarks

Hepatitis C genotype 1a replicon improved through introduction of fitness mutations

Christian Voitenleitner¹, Jill Bechtel¹, Ann Arfsten², and Robert Hamatake¹

¹GlaxoSmithKline, Research Triangle Park, NC, USA, ²InterMune, Brisbane, CA, USA

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The use of subgenomic replicon systems has long been a valuable screening tool for the discovery of small molecule antivirals against Hepatitis C virus. While genotype 1a replicon systems have been widely used in stable systems, use in transient assays has been hampered by low signal. Here we describe the generation of a more robust genotype 1a (H77) replicon through the introduction of two fitness mutations, NS4A-K1691R and NS4B-E1726G, for use in transient transfections. While these mutations significantly improved the signal to noise ratio, leading to more robust data, they have no effect on the potency of tool compounds against various targets of HCV, thereby making this new system a powerful tool for screening of compounds against the genotype 1a replicon.

Hepatitis C virus (HCV) is a plus-strand RNA virus in the *Flaviviridae* family with replication restricted to hepatocytes of humans and chimpanzees. HCV infects more than 3% of the world population, leading to increased risk of liver cirrhosis and hepatocellular carcinoma. The search for HCV model systems has been hampered by the fact that HCV isolates taken from patients replicate poorly, if at all, in in vitro cell culture systems. The first selectable subgenomic replicon capable of replicating autonomously—generated in the laboratory of Ralf Bartenschlager (1)—revealed a series of so called adaptive mutations that allow this construct to replicate in tissue culture (2–4). These subgenomic HCV replicons have proven to be powerful tools for studying viral replication or performing drug screening, and additional mutations have been reported that enhance the fitness of stable genotype 1a systems (5). Given the error prone nature of the RNA-dependent RNA polymerase NS5B, resistance to direct acting antivirals

develops rapidly necessitating the ability to screen a compound against a panel of resistant replicons during drug development. Since keeping stable replicon cell lines of all these mutant replicon systems is prohibitive, mutant replicons are often used in transient assays rather than in stable cell lines. In transient assays, the hepatoma cell line Huh7-Lunet (6) is electroporated with in vitro transcribed HCV RNA that contains a luciferase reporter. The transfected cells are transferred onto 96-well plates containing compounds of interest in a dilution series. Luciferase activity is measured after 72 h to establish EC₅₀ values of compounds against certain replicons (Figure 1A). The genotype 1a-H77 subgenomic replicon (7), which contains a luciferase/neomycin (neo) reporter cassette as well as the structural proteins NS3 through NS5B and two adaptive mutations in NS3/4A and NS5A, P1496L and S2204I, respectively, has very low luciferase levels after 72 h resulting in a low signal to noise ratio (Figure 2A).

This low signal makes it difficult to obtain robust data on the potency of compounds against the 1a-H77 replicons, especially those resistance mutant replicons exhibiting even further diminished replication capacity.

In order to quickly evaluate lead compounds against our large panel of resistance mutations, we needed to create a system with a quick luciferase reporter readout that had a robust signal to noise ratio at the assay readout time of 72 h. An additional advantage of a transient system is that there is no additional accumulation of mutations as can happen in a stable cell line propagated for several cell division cycles. In order to achieve this goal, we started with a replicon system that originated from a resistance screen with an HCV inhibitor targeting NS4B and revealed a mutation in NS4B, E1726G, which conferred fitness to the 1a-H77 replicon. Replicons carrying this mutation manifested higher luciferase units at the 72 h time point of a transient transfection (Figure 2A), yet did not impact the activity of HCV inhibitors to various targets (Figure 2C). Furthermore, a publication from Stan Lemon's laboratory reported several mutations that enhanced replication of the 1a-H77 replicon (8). We chose the mutation in the NS4A gene, K1691R, for minimal interference with our compound targets, while still displaying increased fitness in the 1a replicon. In an effort to further improve the luciferase signal resulting from transient transfection of the 1a-H77 replicon, we introduced both mutations into the 1a-H77 subgenomic replicon. Site-directed mutagenesis using the Quikchange Kit from Stratagene resulted in the 1a-fit (1a-H77-K1691R/E1726G) replicon (Figure 1B).

In order to assess and compare the robustness of these replicons, we performed a transient transfection, followed by a time course up to the assay readout time of 72 h (Figure 2A). After the initial burst of luminescence at 4 h, which stems from translation of the luciferase gene from the transfected RNA rather than replicated RNA, we saw an initial decline in all samples, which indicated the lag until proteins from the replicated RNA begin to be made. At 48 h, 1a-E1726G showed higher signal than 1a-H77; this difference continued through the 72 h time point. The 1a-fit vector consistently showed the highest luminescence, with a 10-fold higher readout than the 1a-E1726G and a hundred-fold higher readout than 1a-wt at 72 h. Some mutations obtained from resistance screens and introduced into the replicon system are known to reduce replicon fitness significantly. One example

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is the NS5B site IV mutation in 1a, C316Y, which has only 7% replication capacity compared with 1a-wildtype (9). We tested this mutant replicon and showed that, although replication fitness is still impaired relative to wildtype, the luciferase units of 1a-fit-C316Y after 72 h are high enough (>300,000 RLU) to give a robust signal to noise ratio (Figure 2B). This signal represents a vast improvement when compared

with the signal of C316Y in the original 1a-H77, which ranged from about 1500 to 3000 RLU.

To verify that the two newly introduced mutations had no effect on compound potency of HCV inhibitors hitting various targets, we tested and compared a series of inhibitors against the 1a-H77, the 1a-E1726G and the 1a-fit replicons (Figure 2C). When tested against a variety

Figure 1. Schematic of transient transfections. (A) Transient transfection. Five μ g of in vitro translated RNA was electroporated into 1.25×10^7 Huh-7-Lunet cells suspended in 400 μ L cytomix (10) supplemented with 2 mM ATP and 5 mM glutathione. Cells were electroporated at 270 V, 950 μ F and infinite resistance and immediately transferred to a conical tube containing 40 mL of DMEM. 199 μ L of the cell suspension was plated in 96-well plates containing 1 μ L compound in DMSO at an appropriate concentration and dilution series across each row. Plates were incubated at 37°C and 5% CO₂ for 3 days. For the luciferase assay, the supernatant was replaced by 25 μ L BrightGlo reagent (Promega) and read in an Envision 2103 Multilabel reader. The IC₅₀ values were determined with appropriate programs. (B) The 1a-fit replicon consists of a luciferase/neomycin (Neo) reporter/selection cassette, preceded by the HCV5'-UTR and the nonstructural proteins from NS3 to NS5B. It also carries the H77 adaptive mutations P1496L in NS3/4A and S2204I in NS5A. Additionally, the vector contains two additional mutations, the K1691R mutation in NS4A and the E1726G mutation in NS4B.

Figure 2. The 1a-fit replicon shows enhanced fitness in transient assays. (A) Time-course of luminescence with 1a-wt, 1a-E1726G, 1a-fit and the replication deficient negative control GND. After a transient transfection with RNA of various vectors, Luciferase reads were taken after 4h, 24h, 48h and 72h. The standard deviation of each time point is shown by error bars. The P values of 1a-wt/1a-E1726G and of 1a-E1726G/1a-fit were significant with 3e-23 and 2e-27, respectively. (B) Transient transfection of Huh7-Lunet cells with either the 1a-fit or the 1a-fit-C316Y RNA. The luminescence of 2 independent experiments is shown in a linear scale in relative luciferase units. (C) Comparison of EC₅₀ values of 1a-H77 and 1a-fit replicon systems with HCV inhibitors against different targets.

of compounds targeting protease, NS4B, NS5A, and various sites of NS5B, the 1a-fit replicon resulted in the same compound potency as the 1a-H77 or the 1a-E1726G replicon, suggesting that neither of these two newly introduced mutations altered compound potencies, but were merely conferring fitness to the replicon.

In summary, we have shown that introducing two mutations in NS4A and NS5B, respectively, could enhance replication fitness and increased the signal to noise ratio about 100-fold compared with the 1a-H77 replicon, resulting in more robust data for HCV inhibitors. This newly described replicon construct provides a quick reporter gene response through its Luciferase readout, as well as a robust signal for reliable determination of compound potencies. Because of the relative ease of introduction of mutations into the replicon, this system can be used to quickly profile a large number of compounds on a panel of resistance mutations against various HCV targets.

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Competing interests

The authors declare no competing interests.

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Address correspondence to Christian Voitenleitner, GlaxoSmithKline, 5 Moore Drive HCV DPU, N3.3235C, Research Triangle Park, NC, USA. Email: christian.a.voitenleitner@gsk.com

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