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In vitro efficacy of approved and experimental antivirals against novel genotype 3 hepatitis C virus subgenomic replicons

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ABSTRACT

Infection with genotype 3 hepatitis C virus (HCV) is common throughout the world, however no direct-acting antiviral (DAA) has been approved to treat this genotype. We therefore attempted to develop novel genotype 3 replicons to facilitate the discovery and development of new HCV therapies. A novel Huh-7-derived cell line 1C but not Lunet cells enabled the selection of a few stable colonies of a genotype 3a subgenomic replicon (strain S52). Genotypic analysis revealed a mutation of P89L in the viral NS3 protease domain, which was confirmed to enhance genotype 3a RNA replication and enable the establishment of highly replicating luciferase-encoding replicons. Secondary adaptive mutations that further enhanced RNA replication were identified in the viral NS3 and NS4A proteins. In addition, cell lines that were cured of genotype 3a replicons demonstrated higher permissiveness specifically to genotype 3a HCV replication. These novel replicons and cell lines were then used to study the activity of approved and experimental HCV inhibitors. NS3 protease and non-nucleoside NS5B polymerase inhibitors often demonstrated substantially less antiviral activity against genotype 3a compared to genotype 1b. In contrast, nucleoside analog NS5B inhibitors and host-targeting HCV inhibitors showed comparable antiviral activity between genotypes 3a and 1b. Overall, the establishment of this novel genotype 3a replicon system, in conjunction with those derived from other genotypes, will aid the development of treatment regimens for all genotypes of HCV.

1. Introduction

Chronic hepatitis C virus (HCV) infection represents a major unmet medical need with an estimated 170 million people infected worldwide (Lavanchy, 2011). Genotype 1 HCV is generally predominant throughout the world (Cornberg et al., 2011), but genotype 3 is also common in many regions. Genotype 3 accounts for more than 50% of HCV infections in India and Thailand, and approximately 30% in Australia and many European countries, including Great Britain, Germany, and Russia (Cornberg et al., 2011). Furthermore, genotype 3 HCV infection is associated with higher incidence of liver steatosis and hepatocellular carcinoma (Nkontchou et al., 2012).

Currently, the standard treatment for HCV genotype 3 patients is pegylated interferon and ribavirin for 24–48 weeks (Ghany et al., 2009), which has better efficacy in genotype 3 infection than genotype 1 but remains only partially efficacious with poor tolerability. Neither boceprevir nor telaprevir has been approved to treat genotype 3 HCV infection (Foster et al., 2011). Many HCV inhibitors are in advanced clinical development, however, the majority is being developed to treat genotype 1 infections. Thus, there is a clear need to develop novel therapeutic agents to treat chronic genotype 3 HCV infection, which also aligns with the increasing interest in developing next-generation "pan-genotypic" treatments for HCV (Gane et al., 2012).

The slow advances in treatment for genotype 3 HCV are, in part, due to lack of efficient in vitro tools. HCV replicons are self-replicating RNA sequences derived from the HCV genome (Bartenschlager, 2005). These replicons have been crucial in multiple aspects of drug discovery and development including the identification of novel classes of inhibitors and the characterization of clinical resistance. Despite successes in generating replicons derived from genotypes 1a, 1b, or 2a (Bartenschlager, 2005), generation of efficiently replicating RNAs from other genotypes has proven difficult. Here we report the isolation of novel genotype 3a replicons that efficiently replicate in cell culture. We demonstrate that robust replication requires the adaptive mutation

Abbreviations: HCV, hepatic C virus; GT, genotype; NS, non-structural; DAA, direct-acting antivirals; 2-CMeA, 2-*C*-methyl adenosine; CsA, cyclosporin A; Rluc, Renilla luciferase; RLU, relative light units; neo, neomycin transferase gene; G418, geneticin; EC_{50}, 50% effective inhibitory concentration.

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P89L in the NS3 protease domain, which can be further augmented by mutations in NS3 and NS4A. Combining selected adaptive mutations with a novel host cell line cured of genotype 3 replicons yielded highly efficient replication of genotype 3a HCV RNA in both transient-transfected cells and stably replicating luciferase-encoding replicon cell lines. These systems support efficient potency profiling of compounds in high-throughput antiviral assays. Notably, differential antiviral activity was observed with multiple classes of DAAs against genotype 3a compared to genotype 1b. The novel replicons and permissive cell lines described here provide valuable tools for drug development and molecular virology studies of genotype 3 HCV.

2. Materials and methods

2.1. Cell culture

Huh7 Lunet cells and 51C cells were described previously (Robinson et al., 2010). 1C cells were derived by curing an NS5A inhibitor GS-5885-resistant genotype 1a replicon clone derived from 51C cells, and showed much higher permissiveness to genotype 1a replicon replication (Peng et al., 2013). Replicon cells were cured to establish 1C cells and other cell lines as described in the Supplementary material. All cell lines were propagated in DMEM medium as described previously (Cheng et al., 2011).

2.2. Construction of plasmids encoding genotype 3a HCV subgenomic replicons

A plasmid (pGT3aS52NeoSG) encoding a subgenomic genotype 3a replicon based on the S52 infectious clone (GenBank accession #GU814263) (Gottwein et al., 2010) was prepared by DNA synthesis and cloning (GeneScript, Piscataway, NJ). Plasmids pGT3aS52-RlucNeoSG and Pi-GT3aS52RlucSG were generated as described in the Supplementary material. Adaptive mutations were introduced by site-directed mutagenesis as described previously (Cheng et al., 2011).

2.3. HCV replicon RNA transcription, genotypic analysis and NS5A staining

HCV replicon RNA in vitro transcription, isolation, RT-PCR, population sequencing and detection of NS5A protein by indirect immunofluorescence were performed as described previously (Cheng et al., 2011).

2.4. RNA transfection and isolation of stable replicon cell lines

10 μg of in vitro-transcribed RNA were transfected into the indicated permissive cell lines by electroporation as previously described (Robinson et al., 2010). 48 h after seeding cells, medium was replaced with complete DMEM supplemented with 0.25 mg/ml G418, which was refreshed twice per week. After 3 weeks, cell clones were isolated, expanded, and cryopreserved at early passages.

2.5. Replicon antiviral assays

1C cells were electroporated with replicon RNA and 90 μl was seeded in 384-well plates at a density of 2000 cells/well. The antiviral EC_{50} was determined as described previously (Cheng et al., 2011).

2.6. Antiviral compounds

Telaprevir, boceprevir, and 2-C-methyl adenosine (2-CMeA) were purchased from Acme Bioscience (Belmont, CA). Cyclosporine A was purchased from Sigma–Aldrich (St. Louis, MO). HCV-796 was synthesized by Curragh Chemistries (Cleveland, OH). Gilead compounds tegobuvir, GS-9451, GS-9669, and sofosbuvir were synthesized by Gilead Sciences. Filibuvir, MK-3281, MK-5172 and daclatasvir were made as described in the Supplementary material. All compounds had a purity of >95%, analyzed by HPLC.

3. Results

3.1. Construction and selection of a subgenomic genotype 3a replicon

A subgenomic genotype 3a replicon was constructed as previously described by Lohmann et al. (Lohmann et al., 1999) and based on the consensus sequence (GenBank accession #GU814263) of the genotype 3a S52 strain (Fig. 1A) (Gottwein et al., 2010). To enhance the basal level of replication, the NS5A mutation S232I (equivalent to the major adaptive mutation S2204I in genotype 1 polyprotein) was incorporated (Blight et al., 2003).

In vitro-transcribed replicon RNA was electroporated into Huh7 Lunet, 51C, or 1C cells. In Lunet cells, no stable replicon colonies emerged after six independent transfection experiments. Transfection into 51C cells also failed to yield any colonies with four independent transfections. In contrast, in 1C cells, a total of three colonies were selected from five independent transfections.

3.2. Characterization of stable genotype 3a replicon clones

To confirm that the selected colonies harbored replicating genotype 3a replicons, an immunohistochemical analysis using anti-NS5A antibody was performed. This analysis indicated high levels of NS5A expression, particularly in the perinuclear region (Fig. 2A). To determine if adaptive mutations were selected, genotypic analyses were performed for all three replicon clones. All clones had a mutation of leucine to proline at amino acid position 89 (P89L) within the viral NS3 protease domain. Clone GT3a#3 had an additional NS3 mutation of Q41R. Transfection of total cellular RNA extracted from each of these replicon clones resulted in large number of replicon colonies in Lunet cells (Fig. 2B). Individual clones or pools were isolated from these secondary transfections and were analyzed for additional genotypic changes. Although many new mutations were identified, they all appeared in conjunction with P89L (Supplementary Table 1). These results indicate that P89L serves as a key adaptive mutation for genotype 3 HCV replication in cell culture.

3.3. Generation of stably replicating luciferase-encoding genotype 3a replicons

We next sought to confirm that the NS3 mutation P89L enhanced replicon replication. To facilitate measurement of replication in cell culture, the Neo gene was replaced with a Renilla luciferase (Rluc)-neo fusion reporter as shown in Fig. 1B. Introduction of the mutation P89L dramatically improved genotype 3a Rluc-neo colony formation efficiency from an average of 0.6 colonies to 35 colonies per selection. Twelve individual Rluc-neo replicon colonies and a colony pool from Lunet and 1C cells were isolated and expanded. Essentially all the isolated clones and pools showed high replicon replication levels as demonstrated by high luciferase activity (>1×10^5 relative light units (RLU) with background of ~100 RLU), and many of the colonies were comparable

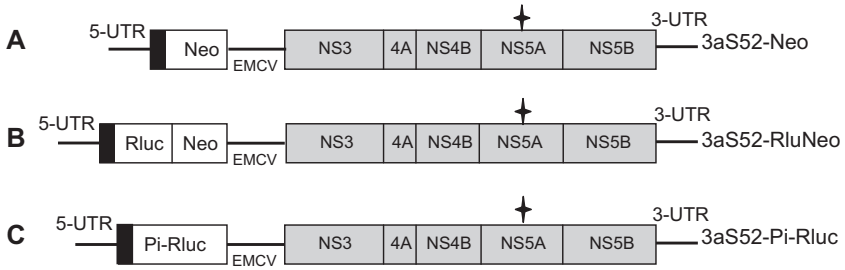

Fig. 1. Schematic diagram of genotype 3a replicon constructs. Genotype 3a S52 strain replicons encode a neomycin phosphotransferase II gene (neo) (A), a Renilla luciferase (Rluc)-neo fusion reporter (B) or a Poliovirus IRES-Renilla luciferase (Pi-Rluc) fusion reporter (C). The synthesized replicons incorporated the following elements from 5′ to 3′: the S52 5′ un-translated region (5′UTR); the Neo, Rluc-neo or Pi-Rluc reporter gene; the EMCV IRES; the NS3–NS5B polyprotein region of S52 including an NS5A adaptive mutation (S232I) and the 3′UTR of S52. ■ indicates HCV S52 core sequence. ▭ indicates HCV polyprotein sequence. "✦" indicates the S232I adaptive mutation. The 5′UTR, 3′UTR and EMCV IRES are shown as labeled.

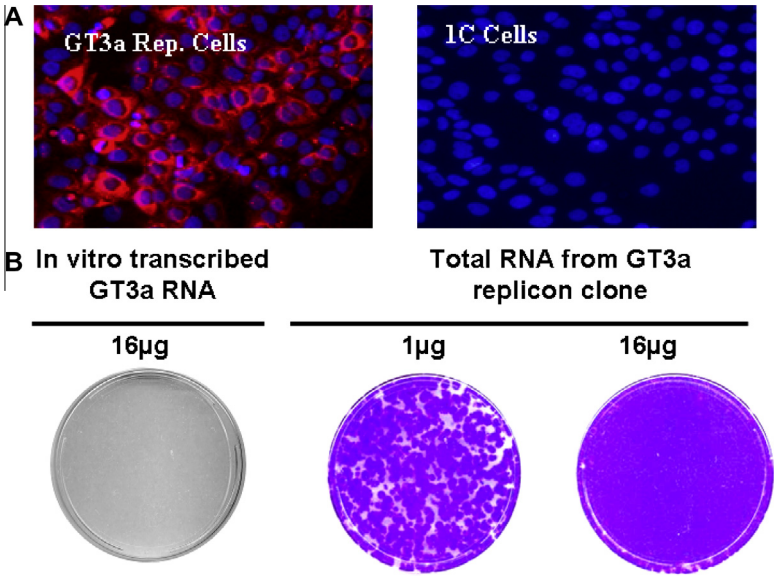

Fig. 2. Selection of stable genotype 3a neo replicon cell clones. (A) High NS5A expression in selected genotype 3a replicon cells. The genotype 3a replicon clone #1 was stained with anti-NS5A antibody (red) and Hoechst 33342 (blue, indicating nuclei). Parental 1C cells were stained as a negative control. (B) Total cellular RNA was extracted from the genotype 3a replicon clone #1 and then electroporated into Lunet cells at the indicated amounts. Transfected cells were resuspended in complete DMEM medium, plated in 100-mm diameter dishes, and cultured with 0.5 mg/ml G418. 3 weeks later, culture plates were fixed with 4% formaldehyde and stained with 0.05% crystal violet to visualize colonies. In vitro-transcribed GT3a replicon RNA was transfected in parallel as a control.

to highly adapted genotype 1a or 1b replicons in terms of luciferase activity (Supplementary Fig. 1). Genotypic analyses revealed the presence of P89L in all the replicon-containing cell clones. Additional mutations such as A166T or K583E in NS3 were also identified (Supplementary Table 2). Together, these data confirmed that the NS3 mutation P89L significantly enhances genotype 3a HCV replication and allows the establishment of stably replicating luciferase-encoding genotype 3a replicons.

3.4. Generation of luciferase-encoding genotype 3a replicons for transient transfection

To facilitate measurement of replication efficiency and kinetics of genotype 3a replicon in transient-transfection studies, the Rluc-Neo gene in the parental GT3a-Rluc-Neo replicon construct was replaced with an Rluc reporter gene downstream of the poliovirus IRES (Pi-Rluc-GT3a-P89L; Fig. 1C). As shown in Fig. 3, luciferase activity of the GT3a-Rluc-Neo-P89L replicon continuously decreased following transient transfection, likely due to insertion of the large Rluc-Neo gene. In contrast, Pi-Rluc-GT3a-P89L reached a replication level that was at least two orders of magnitude higher, and sufficient to measure the replication efficiency and kinetics of the genotype 3a replicon. Using this transient transfection construct, we found that absence of the NS5A mutation S232I abrogated sustainable replication (Fig. 3), indicating that S232I mutation is required in conjunction with P89L to support efficient genotype 3a replicon replication.

3.5. Establishment of permissive cell lines to improve genotype 3a HCV replication

Although replication of Pi-Rluc-GT3a-P89L could be readily measured in transient transfection assays, it was not robust enough for the high-throughput antiviral assays. Two approaches were taken to further improve replication efficiency. The first approach was to establish cell lines with enhanced permissiveness. To test the hypothesis that cured clonal cell lines might uniquely support genotype 3a replication, the original three stable genotype 3a replicon clones (described in Section 3.1) were cured. Importantly, two cured replicon clones, 3a-C2 and 3a-C3 but not 3a-C1, exhibited approximately 10-fold increase in permissiveness to genotype 3a RNA replication compared to the original 1C cells

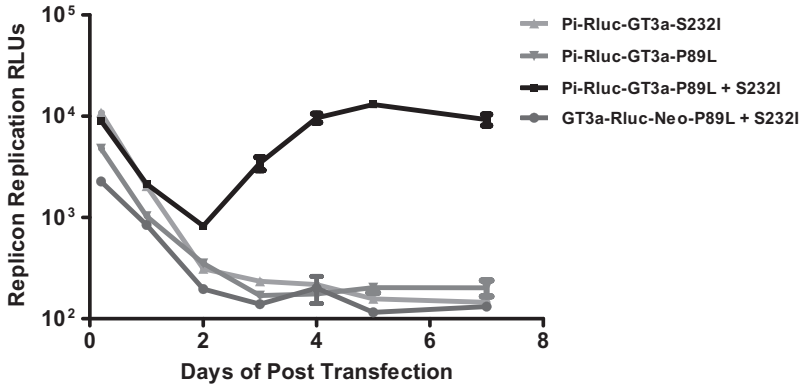

Fig. 3. Adaptive mutation P89L in NS3 requires S232I mutation in NS5A (S2210I) to enhance genotype 3a replicon replication. The mutation P89L in NS3 was introduced into the genotype 3a Pi-Rluc replicon construct in the presence and absence of S232I in NS5A by site-directed mutagenesis. All replicon RNAs were transfected into 1C cells individually and 1×10^4 transfected cells were plated into wells of a 96-well plate. At 4 h, and days 1–7 post transfection, cells were analyzed for Renilla luciferase activity. The background of the assay is ~100 RLUs. GT3a Pi-Rluc replicon without any adaptive mutation (wild type) was also tested and showed similar results as Pi-Rluc-GT3a-S232I or Pi-Rluc-GT3a-P89L replicons.

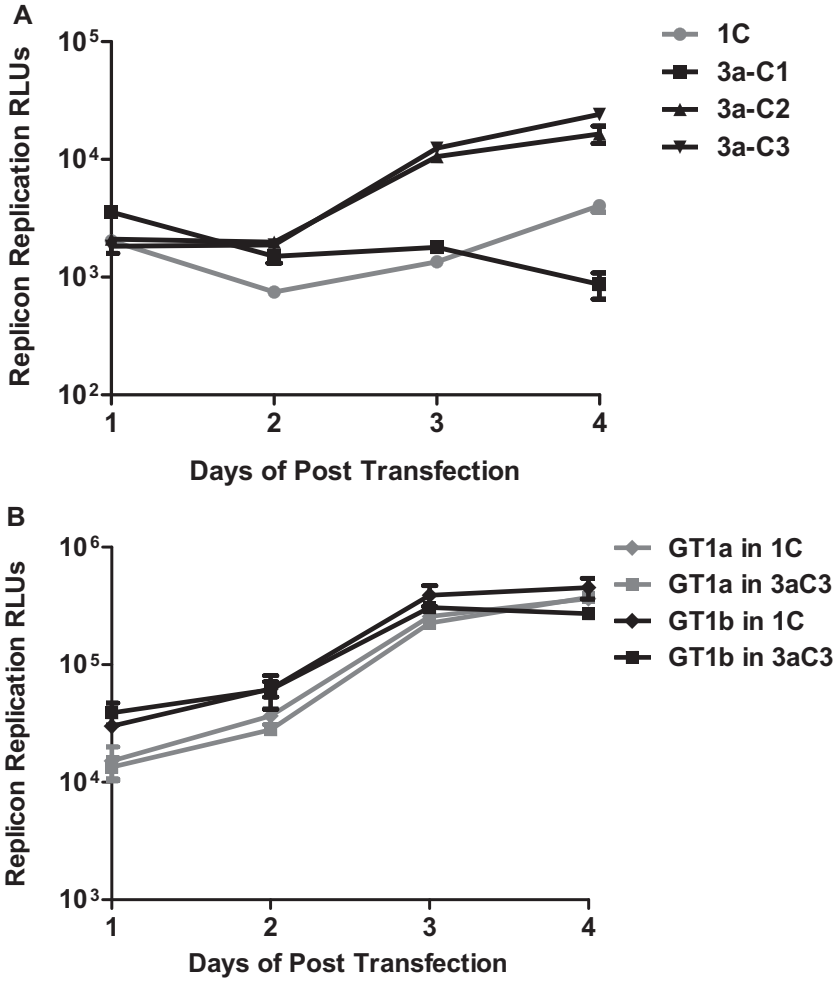

Fig. 4. Cured genotype 3a replicon cell line 3a-C3 has higher permissiveness to genotype 3a replicon replication. Pi-Rluc-GT3a-P89L with S232I in NS5A (A), or Pi-Rluc-GT1a and Pi-Rluc-GT1b (Robinson et al., 2010) (B) replicon RNAs were transfected into 1C or cured genotype 3a replicon cell lines. Luciferase activity was measured daily for 4 days post transfection. Two cured replicon cell lines, 3a-C2 and 3a-C3, had enhanced permissiveness to genotype 3a replication. The 3a-C3 cell line also supported genotype 1a and genotype 1b HCV replication with similar efficiency as in 1C cells.

(Fig. 4A). Interestingly, there were no notable differences for genotype 1a and 1b replication between 1C cells and these cured cell lines (Fig. 4B).

3.6. Characterization and use of secondary adaptive mutations to improve genotype 3a HCV replication

As a second approach to enhance replication of genotype 3a replicons, we examined if additional mutations selected in combination with P89L (Supplementary Tables 1 and 2) could further augment genotype 3a HCV replication. Six mutations that emerged in two or more independent clones were chosen and introduced into Pi-Rluc-GT3a-P89L individually (Table 1). All six mutations further augmented genotype 3a HCV replication (Fig. 5A). For instance, the P89L/K583E double mutation replicon replicated about 20-fold higher than the P89L single mutation replicon. Furthermore, when the P89L/K583E double mutation replicon was transfected into genotype 3a-permissive 3a-C3 cells, the replication efficiencies were further enhanced by approximately 5-fold; these levels were comparable to highly adapted genotype 1a or 1b replicons (Fig. 5B). These data indicate that robust genotype 3a HCV replication could be achieved by using either more permissive cell lines (3a-C3 cells) or secondary adaptive mutations (e.g. K583E in NS3).

3.7. Antiviral evaluation of HCV inhibitors against genotype 3a

Four classes of HCV DAAs (NS3 protease inhibitors, NS5A inhibitors, nucleos/tide NS5B polymerase inhibitors and allosteric NS5B polymerase inhibitors) as well as a host-targeted inhibitor were tested in the transient-transfection antiviral assay using the genotype 3a Pi-Rluc replicon with P89L/K583E double mutations in 1C cells. A standard genotype 1b Pi-Rluc replicon was assayed in parallel. EC_{50} values against both genotype 1b and 3a replicons were reproducibly generated for all inhibitors in a high-throughput 384-well assay by measuring luciferase activity (Table 2).

Cyclosporin A (CsA), which targets host cyclophilin A, had equivalent potency against genotype 1b and 3a. Inhibitors targeting the NS5B polymerase active site (2-CMeA or sofosbuvir) were 3-fold more active against genotype 3a compared to 1b. Interestingly, antivirals targeting palm site IV (HCV-796) on NS5B were also about 6-fold more active against genotype 3a. In contrast, the NS5B thumb site II inhibitors filibuvir and GS-9669 and the palm site III/IV inhibitor tegobuvir were approximately 13-, 63-, and 22-fold less active against genotype 3a compared to genotype 1b, respectively. The NS5A inhibitor daclatasvir was 13-fold less active against genotype 3a. The NS3 protease inhibitors boceprevir and telaprevir were slightly less potent (1.8- to 5.2-fold) against genotype 3a versus genotype 1b. However, the protease inhibitors BILN-2061, GS-9451, and MK-5172 were 64- to 378-fold less potent against genotype 3a. Overall, these results demonstrate that the genotype 3a transient transfection replicon system described above is robust and can serve as a valuable tool for drug discovery.

Table 1
Secondary mutations analyzed in GT3a-Pi-Rluc-P89L replicons.

Mutations	Number of appearances	Locations
A166T	4	NS3
K583E	4	NS3
Q41R	3	NS3
A379T	2	NS3
S534G	2	NS3
S1C	2	NS4A

Six mutations that emerged independently at least twice together with P89L were chosen and introduced into Pi-Rluc-GT3a-P89L (carrying S232I mutation in NS5A) individually by site-directed mutagenesis. All replicon RNAs were transfected into 1C cells and replication efficiency was determined by measuring luciferase activity.

4. Discussion

Infection with genotype 3 HCV is common throughout the world. Here we report the establishment of robust genotype 3a replicons to aid the discovery and development of new therapies for genotype 3 HCV infection. Using a novel permissive cell line (1C), stable genotype 3a colonies were identified, which revealed the key adaptive mutation, P89L in the NS3 protease. As a result, we were able to establish stable genotype 3a luciferase-encoding replicon cell lines, with persistent and robust HCV RNA replication comparable to standard genotype 1a and 1b stable replicon cell lines.

Cellular factors, e.g. the retinoic acid inducible gene-I (RIG-I) protein, can play a critical role in determining HCV replication in cell culture (Sumpter et al., 2005). Our study indicates that cellular factors also impact genotype 3a HCV replication, as observed in both the initial selection of stable replicon colonies in 1C cells, and the higher permissiveness of the cured 3a-C3 cell line (Fig. 2). Viral factors also play a critical role in establishing robust HCV replication (Lohmann et al., 2003). Indeed, the NS5A mutation S232I was essential for the isolation of P89L and genotype 3a replication in cell culture (Fig. 3).

Residue P89 is located on the surface of NS3 in close proximity to where the NS4A co-factor exits the serine protease domain. Interestingly, P89L was previously identified as a suppressor mutation during an alanine scan of the C-terminus of the NS4A protein (Lindenbach et al., 2007). It has been postulated that P89L may alter the interaction of NS3 protease with the NS4A co-factor. Further studies are needed to better understand the molecular role of P89L in genotype 3a HCV cell culture adaptation.

To facilitate measurement of replication efficiency and kinetics in transient transfection experiments, a Pi-Rluc-GT3a-P89L replicon was constructed (Lohmann et al., 2003). To further enhance replication efficiency of this construct, secondary adaptive mutations (e.g. K583E in NS3 protein) and more permissive cell lines (e.g. 3a-C3) were identified. These enhancements allowed the development of luciferase-encoding genotype 3a replicons for use in both stable replicon cells and transient-transfection experiments.

Five unique classes of HCV inhibitors were evaluated for antiviral potency against genotype 3a in parallel with genotype 1b in the same cellular background (1C). The compound genotype 3a antiviral activity is not expected to be affected by the presence of those adaptive mutations, because none of them has been previously associated with resistance phenotype to the known classes of HCV inhibitors. As expected, inhibitors targeting the NS5B active site and host factors had similar potencies against both genotype 1b and 3a. However, the antiviral results suggest that NS5B thumb site I or palm site IV might be more suitable pan-genotype targets than thumb site II and palm site III/IV (major potency differences between genotypes 3 and 1b) (Table 2). The NS5A inhibitor daclatasvir was 13-fold less potent against genotype 3a compared to genotype 1b, but remains a potent inhibitor. The protease inhibitor telaprevir was 5-fold less active against genotype 3a compared to genotype 1b. Nevertheless, this small change in susceptibility appears to be sufficient to drastically reduce the clinical activity of telaprevir in genotype 3 patients (Foster et al., 2011). GS-9451, BILN-2061, and MK-5172 had much more substantial changes in genotype 3a antiviral activity (64–378-fold less active compared to genotype 1b), likely resulting from the Q168 polymorphism in the genotype 3a NS3 protease domain (Liverton, 2010).

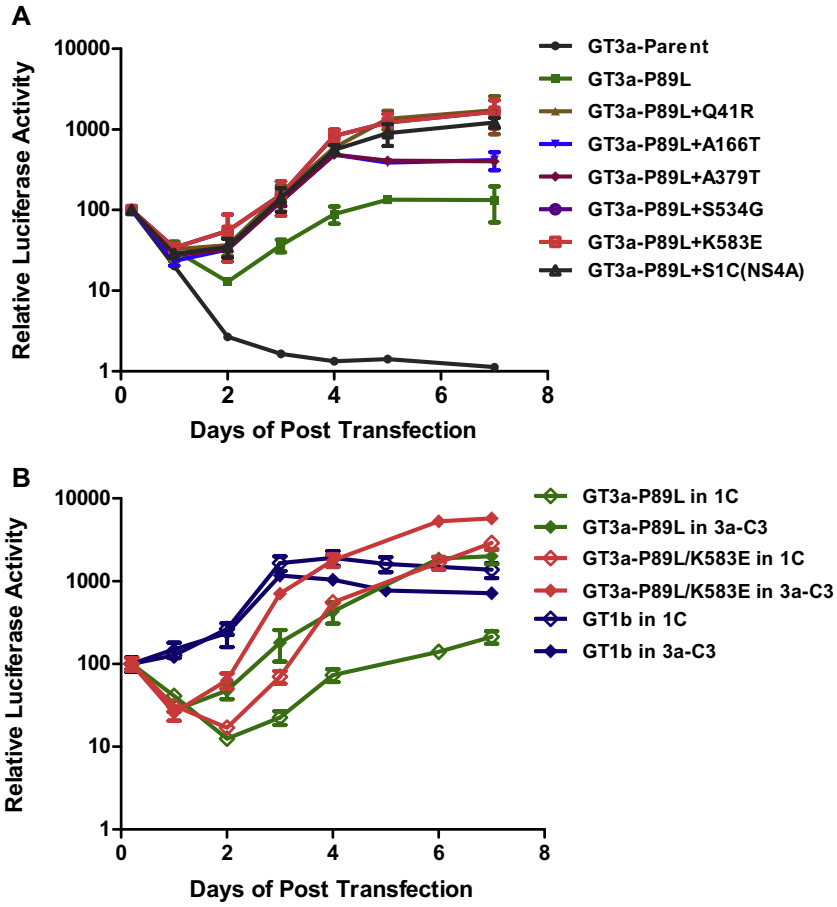

Fig. 5. Secondary mutations in NS3 or NS4A further enhanced genotype 3a replicon replication. Secondary mutations Q41R, A166T, A379T, S534G, and K583E in NS3, or S1C in NS4A were introduced into the GT3a-P89L Pi-Rluc replicon construct (carrying S232I in NS5A) by site-directed mutagenesis. Pi-Rluc-GT3a-parent replicon also carried the S232I mutation in NS5A. Replicon RNAs (Pi-Rluc-GT1b as a control) were individually transfected into 1C (A) or 3a-C3 (B) cells, and 1×10^4 transfected cells were plated per well in a 96-well plate. Cells were analyzed for Renilla luciferase activity at 4 h and daily for 7 days post transfection. Data presented in the figure are averages of at least two independent experiments. Similar results were seen with P89L/A166T double mutation replicon as P89L/K583E in 3a-C3 cells.

Table 2
Antiviral activities of HCV inhibitors against genotype 1b and 3a replicons.

Inhibitor classes	Compounds	GT3aRluc-P89L/K583E EC_{50} (nM)	GT1bRluc EC_{50} (nM)	GT3a/1b EC50 ratio
NS3 protease	Telaprevir	2217 ± 74	429 ± 31	5.2
	Boceprevir	368 ± 98	204 ± 22	1.8
	GS-9451	2039 ± 283	9.0 ± 0.3	220
	MK-5172	51 ± 15	0.80 ± 0.05	64
	BILN-2061	347 ± 156	0.91 ± 0.02	378
NS5A	Daclatasvir	0.59 ± 0.20	0.04 ± 0.02	13
NS5B nuc	Sofosbuvir	13 ± 2.2	40 ± 0.3	0.3
	2-CMeA	25 ± 7.1	86 ± 0.1	0.3
NS5B non-nuc	GS-9669	221 ± 39	3.5 ± 0.4	63
	MK-3281	537 ± 42	554 ± 46	1.0
	Tegobuvir	59 ± 19	2.7 ± 0.2	22
	Filibuvir	1474 ± 716	112 ± 13	13
	HCV-796	3.9 ± 0.7	23 ± 4.6	0.2
Host target	Cyclosporin A	128 ± 39	80 ± 1.1	1.6

Five unique classes of HCV inhibitors listed in the second columns were evaluated for antiviral activity against genotype 3a and genotype 1b Pi-Rluc replicons in 1C cells in a high-throughput 384-well transient transfection assay. Data in the table represent the mean of at least three independent experiments with standard deviations.

During the preparation of this manuscript, two genotype 3a replicons were reported (Saeed et al., 2013, 2012). Notably, those two replicons were established based on either a different genotype 3a strain (S310) or a different cellular background (Huh-7.5 cells) as compared to strain S52 in 1C cells in our study. As a result, we have identified a primary adaptive mutation (P89L) in NS3 that is distinct from the primary NS5A mutations or NS3 mutations observed in the two recent reports. Indeed, in contrast to P89L, none of the five major adaptive mutations from those reports conferred significant replication in our replicon system (Supplementary Fig. 2). Furthermore, broader cellular backgrounds (Lunet, 51C, 1C and 3a-C3 cells) were tested in our studies, and cured 3a-C3

was identified as a cell line with higher permissiveness specifically to genotype 3a RNA replication. With the optimized cell line and novel secondary adaptive mutations (e.g. K583E in NS3), we established a robust transient-transfection replicon system. Using this system, we were able to compare genotype 3a and genotype 1b antiviral efficacy for a broad group of approved and experimental HCV inhibitors spanning five classes of targets.

Overall, the robust and reproducible generation of antiviral data for genotype 3a in a novel cell line with unique adaptive mutations highlights the value of this replicon system. We anticipate that these new replicons, together with replicons derived from other HCV genotypes will aid the development of pan-genotypic HCV regimens for the treatment of HCV worldwide. These replicons also enable further molecular studies including possible investigation of the mechanisms underlying the liver steatosis associated with genotype 3.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.08.018.

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