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# Comparison of HCV NS3 protease and NS5B polymerase inhibitor activity in 1a, 1b and 2a replicons and 2a infectious virus

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ABSTRACT

The hepatitis C virus infection system represents an important new tool for drug discovery. In this study, we compared the in vitro antiviral efficacy of several NS3 and NS5B inhibitors in genotype 1a, 1b, and 2a replicons and in the 2a infectious virus system. The nucleoside inhibitor 2′-C-methyl adenosine showed similar efficacy in each system tested. Three non-nucleoside inhibitors had small differences in potency between genotype 1a and 1b. In contrast, there was a dramatic loss of potency for these non-nucleoside inhibitors in the genotype 2a replicon, 2a infectious virus, and 2a NS5B biochemical assays. The protease inhibitor BILN-2061 had similar efficacy against 1a and 1b replicons but was 61–109-fold less potent against the 2a replicon and virus, respectively. VX-950, a covalent protease inhibitor, had similar efficacy (<3-fold changes in $EC_{50}$) regardless of genotype or subtype. Importantly, we observed a significant correlation ($p < 0.0001$) in antiviral potency between the 2a replicon and 2a infectious virus for all classes of compounds tested.



## 1. Introduction

One hundred and thirty million people are chronically infected with hepatitis C virus (HCV) worldwide (Alter, 2007). Chronic HCV infection can lead to significant morbidity and mortality and is thus a major global health concern. HCV, a member of the Flaviviridae family, is the sole member of the *Hepacivirus* genus and is characterized by a high degree of genetic diversity. HCV is grouped into six major genotypes which can be further divided into multiple subtypes, and exists as a complex population of quasispecies within infected individuals (Simmonds, 2004; Simmonds et al., 1994).

The current standard of care for HCV patients is pegylated interferon (PEG-IFN) plus ribavirin (RBV) which leads to sustained virologic response (SVR) in approximately 50% of genotype 1 patients and approximately 70–80% of genotype 2 patients (Fried et al., 2002; Manns et al., 2001). The failure of many patients to achieve SVR, as well as the significant side-effect profile of PEG-IFN and RBV, highlight the need for new drugs with better efficacy and tolerability (Garber, 2007; Hofmann et al., 2007). To this end, intense efforts are underway to identify and develop novel small molecule inhibitors of HCV. Although no small molecule antivirals have been approved yet, the NS3 protease and NS5B polymerase are considered to be prime targets, and inhibitors of each enzyme have shown promising antiviral activity in clinical trials.

The development of robust genotype 1b and genotype 1a HCV replicon systems enabled the convenient *in vitro* study of antiviral compounds (Blight et al., 2000, 2003; Lohmann et al., 1999). A genotype 2a replicon and the HCV infectious clone based on the genotype 2a JFH-1 strain have been described recently (Kato et al., 2003; Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The genotype 2a replicon replicates to higher levels than genotype 1a and 1b replicons in vitro, and for this reason, it was hypothesized that it would be a good candidate virus to yield infectious particles (Kato et al., 2003). Indeed, the current robust HCV infectious systems are either chimeras based on JFH-1 or tissue culture adapted strains of JFH-1 (Gottwein et al., 2007, 2009; Jones et al., 2007; Koutsoudakis et al., 2006; Ma et al., 2008; Scheel et al., 2008; Tscherne et al., 2006, 2007; Zhong et al., 2006).

To explore the impact of genetic diversity (genotype and subtype) as well as the utility of the genotype 2a infectious system for antiviral screening, we evaluated a number of replicons and infectious virus for susceptibility to several classes of HCV inhibitors. Specifically, we performed a side-by-side comparison using three replicons (genotype 1a, 1b and 2a) and the genotype 2a infectious virus. Compounds assessed include four classes of NS5B polymerase inhibitors (those binding to the active site, and three distinct allosteric sites) and two active-site protease inhibitors (including a covalent, and non-covalent inhibitor).

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## 2. Materials and methods

### 2.1. Cell culture

Huh-Luc and Huh-Lunet cells were obtained from ReBLikon GmbH (Mainz, Germany). The genotype 1b-luciferase replicon Huh-Luc has been described previously (Vrolijk et al., 2003). All Huh-Lunet derived replicon cell lines (including those described below) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 10 mM nonessential amino acids. Replicon cell lines were selected and maintained in 0.5 mg/ml Geneticin® (Invitrogen, Madison, WI). Media and supplements were purchased from GIBCO-BRL, Life Technologies Ltd. (Madison, WI). All cell lines were maintained in humidified incubators at 37 °C and 5% $CO_2$.

### 2.2. Antiviral compounds

VX-950 (2-(2-{2-Cyclohexyl-2-[(pyrazine-2-carbonyl)-amino]-acetylamino}-3,3-dimethyl-butyryl)-octahydrocyclopenta[c]pyrrole-1-carboxylic acid (1-cyclopropylaminooxalyl-butyl)-amide), BILN-2061 (14-cyclopentyloxycarbonylamino-18[2-(2-isopropylamino-thiazol-4-yl)-7-methoxy-quniolin-4-yloxy]-2,15-dioxo-3,16-diaza-tricyclo[14.3.0.0]nonadec-7-ene-4-carboxylic acid) and 2′-C-methyl adenosine (2′CMeA) (2-(6-Amino-purin-9-yl)-5-hydroxymethyl-3-methyl-tetrahydro-furan-3,4-diol) were purchased from Acme Bioscience (Belmont, CA). The benzothiadiazine inhibitor (2-{3-[1-(Cyclopropylmethyl-amino)-4-hydroxy-2-oxo-1,2-dihydro-quinolin-3-yl]-1,1-dioxo-1,4-dihydro-1λ$^6$-benzo [1,2,4]thiadiazin-7-yloxy}-acetamide) was purchased from ChemALong (Lemont, IL). The thiophene (3-[Isopropyl-(4-methyl-cyclohexanecarbonyl)-amino]-5-phenyl-thiophene-2-carboxylic acid) and benzofuran (2-(4-Fluoro-phenyl)-5-isopropoxy-6-methanesulfonylamino-benzofuran-3-carboxylic acid methylamide) inhibitors were purchased from BioArc (Vadodara, India).

### 2.3. Subgenomic HCV replicon constructs

A plasmid encoding a genotype 1a (H77 strain) HCV subgenomic replicon, pH/SG-neo(L+I) was obtained from Apath, LLC (St. Louis, MO) (Fig. 1A). Plasmid p2aNeo which encodes a genotype 2a subgenomic replicon (Fig. 1C), was derived from pJFH-1, a plasmid containing the full-length genotype 2a (JFH-1, Toray Inc. Japan) genome as follows: The HCV non-structural genes and the plasmid backbone in p2aNeo were amplified by PCR from pJFH-1 using a primer set of HCV2aCoreAfeIrev and HCV2aNS3fw. HCV2aCoreAfeIrev has the sequence: 5′ TCTAGA AGCGCT tgggcg acggtt ggtgtt tctttt gg 3′ (HCV sequence in lower case) and encodes an Afe I site. HCV2aNS3fw has the sequence: 5′ GAGCTT ACGCGT atggct cccatc actgct tatg 3′ (HCV sequence in lower case) and encodes a Mlu I, site. The Afe I and Mlu I sites were introduced at the twentieth residue of core protein and upstream of NS3 to allow the insertion of the neomycin phosphotransferase II (neo) gene for selection of stable HCV replication in transfected cells and the EMCV IRES to enable the translation of the HCV non-structural proteins. The fragment encoding the neo gene and EMCV IRES was amplified by PCR from the pFKi389lucubineoNS3-3′_ET replicon (ReBLikon GmbH) (Fig. 1B). p2aNeo was generated by ligation of the two PCR fragments after digestion with Afe I and Mlu and the sequence was confirmed by DNA sequencing.

### 2.4. Infectious J6/JFH-1 virus construction

A J6/JFH-1 chimeric HCV reporter virus was constructed similar to that described by Jones et al. with the addition of a FLAG tag in the NS5A coding sequence (Jones et al., 2007) (Fig. 1D). The virus cDNA was assembled from two synthetic cDNAs (GeneOracle, Mountain View, CA). The first cDNA (pGO1) encoded the 5′UTR of JFH-1 and core–NS2 genes of the J6 strain of HCV and a portion of the NS3 gene from JFH-1. pGO1 included the *Renilla* luciferase reporter gene followed by the foot-and-mouth disease virus (FMDV) 2A peptide (Rluc2A) (Jones et al., 2007). This reporter cassette was included between the p7 and NS2 sequences and was flanked by two *MluI* restriction sites introduced into the sequences. The second cDNA (pGO2) encoded NS3-5B from the JFH-1 strain including the first 48 nucleotides of the 3′UTR with a *KpnI* site engineered at nucleotide 9920. pGO2 included an in-frame FLAG tag sequence inserted in the NS5A protein sequence at amino acid position 2390 where a GFP-FLAG insertion at this position has previously been shown to not affect replication (Moradpour et al., 2004). In addition, three silent

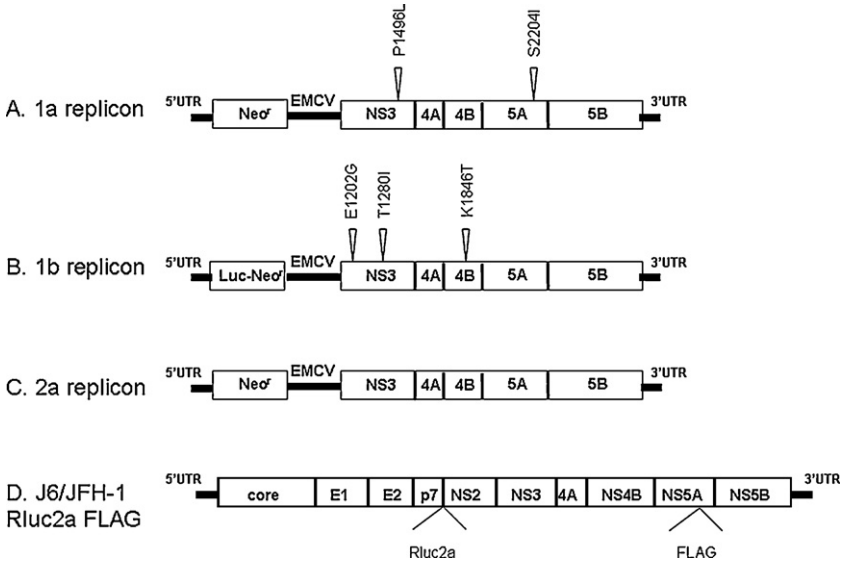


**Fig. 1.** Replicon and infectious virus constructs used in antiviral studies. Each construct contains genotype specific nontranslated regions at the 5′ and 3′ ends. For replicons, the HCV IRES drives the translation of the amino terminus of the core (19aa) and Neo$^r$ gene fusion, while the EMCV IRES drives the translation of the non-structural (NS) proteins NS3 to NS5B. (A) Genotype 1a replicon construct, (1a SG P+S). The adaptive changes in NS3 (P1496L) and NS5A (S2204I) are shown. (B) Genotype 1b replicon construct. Adaptive mutations in NS3 (E1202G, T1280I) and NS4B (K1846T) are indicated. (C) Genotype 2a JFH-1 replicon construct. (D) Genotype 2a J6/JFH-1 infectious virus construct. The J6 portion includes a Renilla luciferase (Rluc) cassette and the autoprotease FMDV 2a. The JFH-1 portion contains a FLAG insertion in NS5A.

mutations were included for subsequent cloning purposes to knock out restriction sites. The sites knocked out were: *BstEII* (nucleotide 5147), *NsiI* (nucleotide 5721), and *BamHI* (nucleotide 6430). The entire 3′UTR of JFH-1 was generated by the annealing of overlapping oligonucleotides and ten rounds of amplification with Pfu polymerase (Roche, Palo Alto, CA) followed by the addition of *Taq* polymerase (Invitrogen, Madison, WI) and 15 additional rounds of amplification. The extreme 5′ end of the oligonucleotide sequence contained an *EcoRI* restriction site before the *KpnI* site for cloning purposes. A single nucleotide change at nucleotide 40 (corresponding to nucleotide 9920, upstream of the polyT sequence) introduced a *KpnI* restriction site. An *XbaI* restriction site was added at the extreme 3′-terminus of the final oligonucleotide sequence. The PCR amplified 3′UTR was cloned into a Topo2.1 vector (Invitrogen) and sequenced. This 3′UTR sequence was subsequently subcloned into the *EcoRI*/*XbaI* sites of pUC19 to yield pUC19-3′UTR. The two synthesized cDNAs GO1 and GO2 then were inserted into pUC19-3′UTR by three-piece ligation after digestion with the appropriate enzymes, (*EcoRI*, *ClaI*, and *KpnI*). The GO1 sequence was completely digested with *EcoRI* then partially digested with *ClaI* to generate the appropriate piece. The GO2 sequence was cut with *ClaI* and *KpnI* and the pUC19-3′UTR was cut with *EcoRI* and *KpnI*. The correct sequence of the J6/JFH-1 Rluc2aFLAG construct was confirmed by DNA sequencing (Elim Biopharmaceuticals, Inc., Hayward, CA).

### 2.5. RNA transcription

In vitro transcripts were generated as previously described (Lindenbach et al., 2005). Briefly, plasmids were linearized by *HpaI* (genotype 1a), *ScaI*, (genotype 1b) or *XbaI* (genotype 2a) and purified using a Minelute column (Qiagen, Valencia, CA). RNA was transcribed from 10 μg of purified template by using the T7 Megascript™ kit (Ambion, La Jolla, CA). Reactions were incubated at 37 °C for 2 h, and then digested with 3 U of DNaseI (Ambion) for 15 min. RNA was purified using an RNAeasy kit (Qiagen) and then quantified by absorbance at 260 nm. RNA integrity was determined by agarose gel electrophoresis and ethidium bromide staining and was then stored frozen at −80 °C.

### 2.6. Generation of stable replicon cell lines

3 μg of each replicon RNA were transfected into Huh-Lunet cells using the DMRIE-C lipofection reagent (Invitrogen, San Diego, CA) in 6-well culture dishes. Two days post-transfection, cells were trypsinized and seeded in 10-cm culture dishes. Cells were selected in the presence of 1 mg/mL G-418 for three weeks. G-418-resistant colonies were isolated, expanded, and HCV replication was quantified using an endogenous NS3 assay described previously (Yang and Delaney, 2006). Clones positive for NS3 activity were further expanded.

### 2.7. Generation and titration of infectious viral particles

Stocks of infectious HCV J6/JFH-1 Rluc2aFLAG viral particles were generated as previously described (Lindenbach et al., 2005). Briefly, 10 μg of transcribed RNA was mixed with a suspension of trypsinized Huh-Lunet cells ($7.5 \times 10^6$ cells/ml in a volume of 400 μl). Cells were then electroporated at 260 V and 950 μF in a Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Electroporated cells were allowed to recover for 10 min at room temperature prior to addition of cell culture medium and plating in T75 flasks. Conditioned culture media containing virus was collected five to twenty-two days post-electroporation, filtered through a 0.22 micron filter (Millipore) and applied to naïve Huh-Lunet target cells to determine viral titer (see below). Virus titer was determined by calculating the 50% tissue culture infectious dose ($CCID_{50}$/ml) (Reed and Muench, 1938). Briefly, clarified cell culture supernatants were serially diluted and used to infect Huh-Lunet cells seeded in 96-well plates at 5000 cells/well 16 h earlier. Three days post-infection, cells were washed with PBS, fixed with ice-cold methanol/acetone (1:1), and stained for the presence of core expression using an anti-core antibody (C7-50, Abcam, Cambridge, MA), as described previously (Wakita et al., 2005).

### 2.8. Antiviral assays

Replicon cells were seeded at a density of 5000 cells per well in 96-well plates in 100 μl of cell culture medium and allowed to attach overnight. For viral infections, Huh-Lunet cells were seeded in 96-well plates (5000 cells/well) and allowed to attach overnight. Viral inocula were added at an MOI 0.05–0.1 for 6 h. Infected cells were then washed one time with cell media and refed 100 μl of culture media. HCV-infected Huh-Lunet cells were prepared as described above. Compounds were diluted in DMSO serially in 3-fold steps and then added to cell culture medium. Compound dilutions were then added to replicon or infected Huh-Lunet cell plates at final concentrations of 16,667–0.1 nM in a total volume of 200 μl of cell culture media and 0.5% DMSO. Following three days of incubation, media were removed, cells were washed once in PBS and lysed for RNA extraction and quantification by real time reverse transcription quantitative PCR (RT-qPCR) as described below. Alternatively, Fluc or Rluc was assayed according to the Manufacturer (Promega, Madison, WI). The percent inhibition of HCV replication for treated samples was calculated relative to the untreated controls (defined as 100%). Antiviral data were fit to the logistic dose response equation $y = a/(1 + (x/b)^c)$ using XLFit software (IDBS, Emmeryville, CA) and $EC_{50}$ values were calculated from the resulting equations as described previously (Delaney et al., 2001) Drug titration experiments were performed in duplicate within each experiment and reported $EC_{50}$ values represent averages from three or more independent experiments.

### 2.9. RNA extraction and RT-qPCR quantification

Total RNA was extracted from 96-well plates using the RNAeasy 96 kits (Qiagen) according to the manufacturer's instructions. RNA was eluted in 40 μl RNAse-free water. One-tenth of the reaction was used for the RT-qPCR reaction. HCV RNA copy number was determined by real time RT-qPCR using an ABI Prism 7300 (ABI, Foster City, CA) sequence detector system. TaqMan primers and probe (Invitrogen, Madison, WI) were Fam-labelled and directed against the viral 5′UTR and described previously (Hinrichsen et al., 2004; Lindenbach et al., 2005; Pause et al., 2003). In vitro transcribed HCV RNAs of the appropriate genotypes were used as standards during RT-qPCR analysis. RT-PCR was run as a multiplex assay with Joe-labelled 18S specific primers as a control (Invitrogen). Reaction conditions were 30 min at 44 °C; 10 min at 95 °C; followed by 38 cycles of 95 °C 15 s and 58 °C for 30 s. Data were determined as percent HCV RNA compared to an untreated control after 18S normalization.

### 2.10. NS5B enzymatic assay

NS5B-dependent RNA elongation activity was assayed using a heteropolymeric RNA template described previously (Hung et al., 2002). Reactions contained 50 mM Tris/HCl pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 40 ng/μl RNA template. The final nucleotides concentration in the reactions of non-nucleotide inhibitors were: 0.1 μCi of [α-$^{33}$P]CTP at 0.2 μM, and 500 μM ATP, GTP and UTP. Alternatively, the final nucleotides concentration in the reactions of 2′-C-methyl adenosine triphosphate were: 0.5 μCi of [α-$^{33}$P] ATP at 3 μM, and 500 μM CTP, GTP and UTP. Recombinant

genotype 1b Con-1 or genotype 2a JFH-1 NS5B with a C-terminus 21 residue deletion and a $His_6$-tag (NS5B Δ21 C-His) were used in the reactions at 75 nM for all reactions. NS5B enzymes were preincubated with RNA templates and compounds for 30 min at 34 °C, after which reactions were started by the addition of NTPs. Reactions were allowed to proceed for 90 min and then transferred onto 96-well DE81 filter membranes, washed three times with 100 mM $Na_2HPO_4$, once with ethanol, and dried. Scintillation fluid was added to the wells and counts per minute (cpm) were measured using a TopCount instrument (Perkin Elmer, Waltham, MA). $IC_{50}$ values were determined using Prism 4.0 software (GraphPad, San Diego, CA).

### 2.11. Statistical analysis

Statistical analysis was determined using Prism 4.0 software (GraphPad, LaJolla, CA). All comparisons were made with two-tailed T-tests. A $p$-value < 0.05 is reported as significant.

## 3. Results

### 3.1. Generation and characterization of stable replicon containing cell lines and infectious virus stocks

To compare the effects of antivirals in various replicons and the 2a infection system, we first obtained or generated replicon cell lines as well as infectious HCV particles. Huh-Luc cells that replicate the 1b replicon were obtained from ReBLikon. Stable cell lines replicating subgenomic 1a and 2a replicons were generated by transfection and G-418 selection. Clones 2aNeo-6 (genotype 2a) and HSG-57 (genotype 1a) were selected for antiviral studies based on their HCV replicon levels. Stable HCV replication in the cell lines was compared using RT-qPCR amplification of HCV RNA with genotype-specific HCV probes. RNA levels observed in replicon cell lines are shown in Fig. 2. The 2aNeo-6 cell line (genotype 2a) had the highest replication levels of the three replicon cell lines, although replication was not significantly higher in these cells compared to Huh-Luc cells (genotype 1b) ($2.4 \times 10^6$ vs $1.8 \times 10^6$ HCV RNA copies/ml; $p = 0.25$). The 2aNeo-6 cell line demonstrated consistently higher levels of replication compared to genotype 1a HSG-57 cells (213-fold HCV RNA copies/ml; $p < 0.0001$) (Fig. 2).

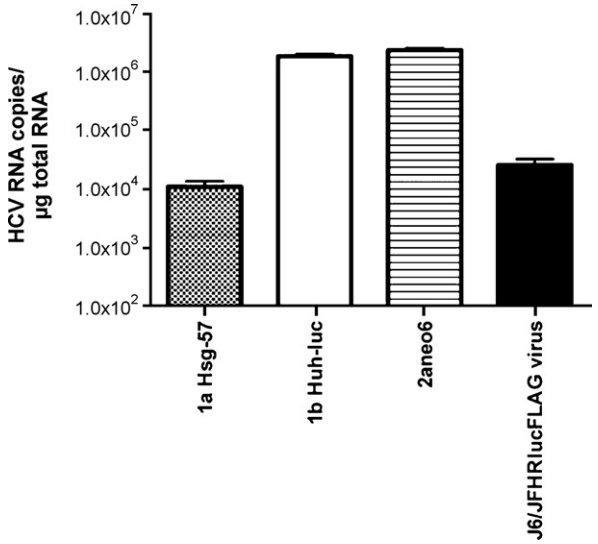


**Fig. 2.** HCV RNA levels in 1a, 1b and 2a replicon-harboring cells three days post-seed and in Huh-Lunet cells three days post-infection with J6/JFH-1 Rluc2aFLAG.

To generate an infectious virus construct, a chimeric J6/JFH-1 Rluc2aFLAG viral cDNA was synthesized and assembled by molecular cloning (Fig. 1). This construct was transfected into Huh-Lunet cells and supernatants containing infectious viral particles were collected several days post-transfection. HCV titers for the J6/JFH-1 Rluc2aFLAG virus were approximately $3 \times 10^3$–$1 \times 10^4$ $TCID_{50}$/ml, similar to those obtained with wild-type JFH-1 (data not shown). HCV RNA levels were determined by RT-qPCR three days post-infection using a genotype 2a specific RNA probe. 2a replicon cells contained 90-fold higher levels of HCV RNA compared to Huh-Lunet cells infected for three days with the J6/JFH-1 Rluc2aFLAG virus (Fig. 2) ($p = 0.0035$). RNA levels correlated with a low (<10%) level of infection with this system as measured by immunofluorescence (data not shown).

### 3.2. Antiviral studies of HCV replicons and infectious virus

To investigate the susceptibility of different HCV genotypes to antiviral compounds, we selected a panel of mechanistically distinct HCV inhibitors (Fig. 3). The NS3 protease inhibitors BILN-2061 and VX-950 are non-covalent and covalent active-site inhibitors of NS3 protease, respectively (Huang et al., 2006; Tong et al., 2006). The NS5B polymerase nucleoside inhibitor 2′CMeA and three types of allosteric non-nucleoside inhibitors (a thiophene reported by ViroChem Pharma, a benzofuran reported by ViroPharma, and a thiadiazine reported by Abbott) were also selected (Beaulieu, 2007; De Francesco and Carfí, 2007).

Table 1 shows the $EC_{50}$ values for the protease compounds, BILN-2061 and VX-950, for each replicon and the virus. BILN-2061 had an $EC_{50}$ of 1.1 nM against the 1b replicon but was 5.7-fold less active against the genotype 1a replicon ($p < 0.014$) and 61–109-fold less active against the 2a replicon and 2a infectious virus, respectively ($p < 0.0001$). The difference between the 2a replicon and virus was not significant ($p < 0.096$). VX-950 was much less potent than BILN-2061 against genotype 1 HCV with $EC_{50}$ values of 782 and 625 nM against 1a and 1b replicons, respectively. However, VX-950 retained activity against genotype 2a in both the replicon and infectious virus systems with $EC_{50}$ values < 2-fold different from those in genotype 1 ($p < 0.22$). These data were consistent and confirmed in independent studies using an NS3 substrate (Yang and Delaney, 2006) (data not shown) or the firefly luciferase or Renilla luciferase reagents in the case of the 1b replicon or 2a infectious virus, respectively (Table 1).

The NS5B polymerase inhibitors were also tested in each replicon and virus system (Table 1). The nucleoside NS5B inhibitor 2′CMeA had similar potency in all replicon cell lines and in cells infected with 2a HCV with $EC_{50}$ values ranging from 115 to 196 nM (Fig. 4). In contrast, the non-nucleoside NS5B inhibitors had variable activity against the different genotypes. The thiophene inhibitor had similar $EC_{50}$ values in 1a and 1b replicons (444 and 383 nM, respectively; $p > 0.08$) but had no activity in genotype 2a virus-infected and replicon cells at the highest tested concentration ($EC_{50}$ values > 16,000 nM). The benzothiadiazine NS5B inhibitor showed similar patterns of activity with $EC_{50}$ values of 347 and 54 nM against genotype 1a and 1b replicons ($p < 0.04$), respectively, but no activity in the genotype 2a systems ($EC_{50}$ values > 16,000 nM). The benzofuran NS5B inhibitor was the least potent inhibitor tested in genotype 1a replicon cells ($EC_{50}$ = 1227 nM) although it was 5.3-fold more potent against the genotype 1b replicon ($EC_{50}$ = 231 nM) ($p < 0.019$). Like the thiophene and benzothiadiazine inhibitors, the benzofuran inhibitor lost all measurable activity against genotype 2a HCV in both the replicon and the HCV infection systems ($EC_{50}$ values > 16,000 nM). Again, these data were confirmed in independent assays using the luciferase assays or NS3 substrate assay (Table 1 and data not shown).

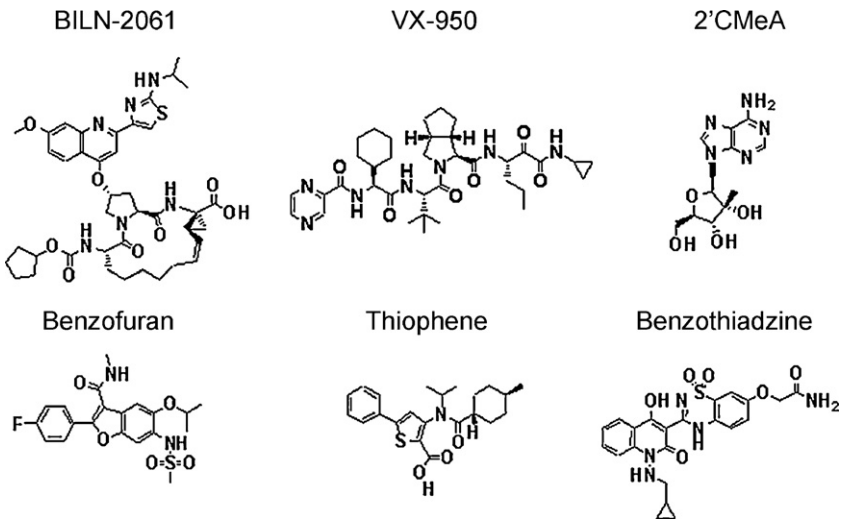


**Fig. 3.** Chemical structures of selected HCV inhibitors used for this study. Shown on the top are NS3 protease inhibitors BILN-2061 and VX-950 and the NS5B polymerase active-site inhibitor 2′-C-methyl adenosine. On the bottom are NS5B polymerase non-nucleoside benzofuran, thiophene, and benzothiadiazine inhibitors.

**Table 1**
$EC_{50}$ values for antiviral compounds against different HCV genotypes.

| Compound/assay | $EC_{50}$ (nM)[a] | | | | | |
|---|---|---|---|---|---|---|
| | 1a replicon RT-qPCR[b] | 1b replicon RT-qPCR[b] | Fluc[c] | 2a replicon RT-qPCR[b] | 2a virus RT-qPCR[b] | Rluc[d] |
| BILN-2061 | 6.3 ± 2.7 | 1.1 ± 0.5 | 0.6 ± 0.5 | 67 ± 13 | 120 ± 35 | 141 ± 37 |
| VX-950 | 782 ± 263 | 625 ± 182 | 570 ± 225 | 325 ± 125 | 392 ± 48 | 473 ± 279 |
| 2′CMeA | 178 ± 20 | 196 ± 42 | 210 ± 72 | 188 ± 78 | 115 ± 38 | 206 ± 102 |
| Benzofuran | 1227 ± 441 | 231 ± 76 | 223 ± 87 | >16,000 | >16,000 | >16,000 |
| Benzothiadiazine | 347 ± 91 | 54 ± 28 | 25 ± 14 | >16,000 | >16,000 | >16,000 |
| Thiophene | 444 ± 178 | 383 ± 121 | 204 ± 31 | >16,000 | >16,000 | >16,000 |

[a] Values represent the mean of triplicate experiments ± standard deviation.
[b] Reverse transcription quantitative PCR was used to quantify levels of viral RNA.
[c] Firefly luciferase was used as a reporter for viral replication.
[d] Renilla luciferase was used as a reporter for viral replication.

### 3.3. Correlation of compound inhibition of 2a replicon and 2a infectious virus

As described above, $EC_{50}$ values were determined for several compounds from several different classes of HCV inhibitors. To compare results obtained with the genotype 2a HCV infection system to those obtained with the genotype 2a replicon, we performed a Pearson correlation using the $EC_{50}$ data generated with both systems (Fig. 5). This analysis indicated that the data generated with these two genotype 2a systems are significantly correlated ($p < 0.0001$) with an $r^2$ value of 0.99. In contrast, there was no significant correlation between either genotype 1a or 1b replicons with the 2a virus.

### 3.4. Biochemical inhibition of genotype 1b and genotype 2a NS5B

To understand the loss of activity of the non-nucleoside inhibitors in the 2a replicon and infectious system, we tested the ability of these compounds to inhibit genotype 1b and genotype 2a NS5B polymerases in biochemical assays. Table 2 shows the $IC_{50}$ values of all three non-nucleoside inhibitors against purified NS5B from genotype 1b and genotype 2a. The benzothiadiazine compound was a potent inhibitor of genotype 1b NS5B enzymatic activity ($IC_{50}$ = 84 nM). The benzofuran compound and the thiophene compounds were 3.0-fold and 2.6-fold less potent in the same assay ($IC_{50}$ values were 256 nM and 215 nM, respectively). However, all three non-nucleosides were significantly less potent against genotype 2a NS5B ($IC_{50}$ values ranged from 6515 to > 10,000 nM). Only the benzofuran compound had measurable activity in the biochemical assay and was 25.4 times less active against the 2a enzyme compared to the 1b enzyme. As a control, 2′CMeA showed similar values of $IC_{50}$ against the 1b and 2a enzyme at 4900 and 6200 nM, respectively.

**Table 2**
Biochemical inhibition of NS5B polymerase inhibitors using genotype 1b and genotype 2a enzymes.

| Compound | $IC_{50}$ (nM)[a] | |
|---|---|---|
| | 1b | 2a |
| 2′CMeA | 4900 ± 1100 | 6200 ± 1200 |
| Benzofuran | 256 ± 64 | 6515 ± 589 |
| Benzothiadiazine | 85 ± 7 | >10,000 |
| Thiophene | 215 ± 62 | >10,000 |

[a] Values represent the mean of triplicate experiments ± standard deviation.

## 4. Discussion

Genotype 1b replicons have been a heavily used tool for HCV antiviral discovery efforts. The genotype 1a replicon, genotype 2a replicon and the genotype 2a infectious HCV system are more recent advances that provide additional tools for drug discovery and the investigation of HCV biology. Determining whether small molecule inhibitors are effective against multiple HCV genotypes will be crucial to ensure that agents can be used appropriately in the clinic. To characterize the activity profiles of six HCV antiviral compounds

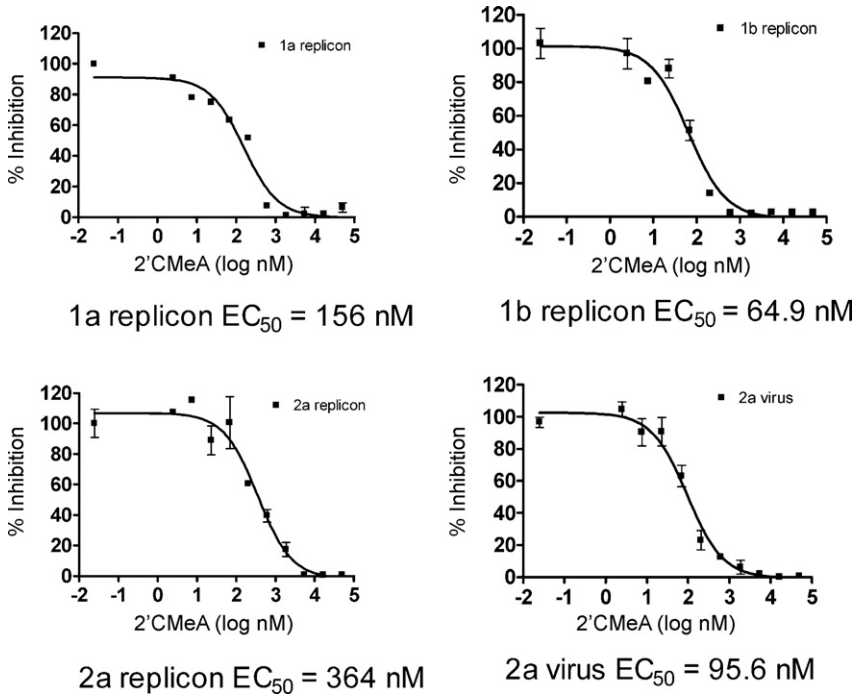


**Fig. 4.** Representative $EC_{50}$ curves for 2′CMeA from each of the four HCV experimental systems tested. RT-qPCR for HCV RNA was performed multiplexed with 18S as a control. Normalized HCV RNA is represented as 100% of untreated wells compared to 0% at the highest dose of compound. Error bars represent the range of duplicate wells for each condition.

that act through different modes or sites of action, we obtained a genotype 1b stable replicon cell line, generated genotype 1a and 2a stable replicon cell lines and an infectious genotype 2a virus. We then assessed the drug susceptibility for each of the replicon cell lines as well as virus-infected cells. In addition to providing a comparison of inhibitor activity against different genotypes, these studies also allowed us to compare HCV replication levels between systems and compare antiviral results between the replicon and the infectious HCV system.

The current system of J6/JFH-1 Rluc-Flag virus used to infect Lunet cells gives a low level (<10%) of infection as demonstrated by immunofluorescence studies (data not shown). Recent studies have demonstrated that the use of Huh-7 derived cells that express higher levels of the HCV entry factor CD-81 lead to more robust infection (Koutsoudakis et al., 2007). Furthermore, we observed that the addition of the Rluc reporter gene and flag tag slightly attenuates the virus. In a separate study, we demonstrated that the flag tag does not alter replication of a 2a replicon (data not shown). This agrees with previously reported data using a GFP insertion in the same position in NS5a in a 1b replicon (Moradpour et al., 2004). However, overall this reporter virus leads to levels of infection equivalent to wild-type JFH-1 virus (data not shown). The low level of infection is reflected in the 90-fold difference in RNA levels between the 2a replicon and this infectious virus. A more robust system is certainly desired for large scale drug discovery efforts. However, we are encouraged that the compounds tested in these studies show very similar activity in both 2a replicon and 2a infectious virus systems. In the future, studies involving a more robust infectious system will be pursued.

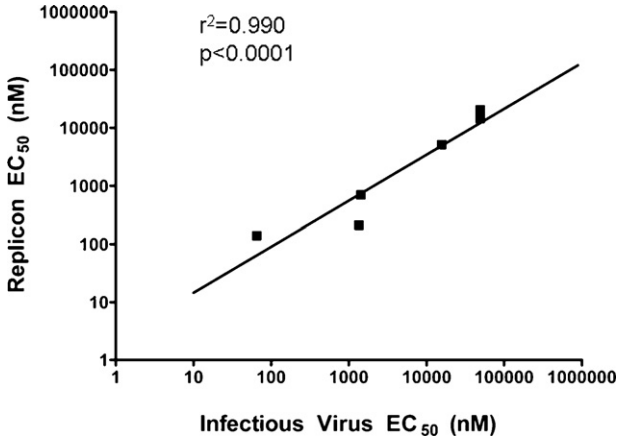


**Fig. 5.** Linear regression and Pearson correlation for antiviral activity data generated in the genotype 2a replicon and genotype 2a infectious virus systems.

We observed that BILN-2061 is a nanomolar inhibitor of genotype 1b replication but this activity is reduced 5.7-fold against genotype 1a. Genotype 2a replicon cells and genotype 2a HCV-infected cells were substantially less susceptible to inhibition by BILN-2061 (61–109-fold less). This change in potency has been reported previously and is likely to be due to polymorphisms in the genotype 2a protease (Lin et al., 2004; Reiser et al., 2005; Tong et al., 2006). VX-950, a covalent inhibitor of NS3 protease, was much less potent than BILN-2061. However, VX-950 did not show significant differences in potency between HCV genotypes. $EC_{50}$ values ranged from 325 nM to 782 nM and did not vary more than 2-fold from the 1b replicon results. These results are quite similar to those reported in a 1b luciferase replicon system (De Francesco and Carfí, 2007; Lin et al., 2004) and the 2a infectious virus system (Lindenbach et al., 2005).

The potency of the NS5B nucleoside inhibitor 2′CMeA was similar across HCV genotypes and in the 2a infectious virus system, similar to other reports (Beaulieu, 2007; Lindenbach et al., 2005). In contrast, the non-nucleoside inhibitors of NS5B had genotype-specific activity. There are several types of non-nucleoside NS5B inhibitors that bind to distinct allosteric sites on the NS5B enzyme. In this study we tested representatives of three types: benzofurans (which bind to a pocket in the NS5B "palm" domain), benzothiadiazines (which bind to a partially overlapping pocket in the NS5B "palm" domain), and thiophenes (which bind to a pocket in the

NS5B "thumb" domain). Our results for these molecules in a 1b replicon are comparable to those reported in a review of HCV polymerase inhibitors (Beaulieu, 2007). Using our 1b replicon data, our results identified 5.3-fold and 6.4-fold decreases in genotype 1a susceptibility compared to 1b for the benzofuran and benzothiadiazine inhibitors, but no change for the thiophene inhibitor. However, these non-nucleoside inhibitors had no activity against the 2a replicon or the 2a infectious virus at the highest tested inhibitor concentrations. In agreement with the cell based data, little or no inhibitory activity could be detected in biochemical studies using genotype 2a NS5B. These data suggest that the observed loss of activity between genotypes is dependent on the viral polymerase and is not due to cellular factors. Recently, Pauwels et al. reported an NS5B enzyme panel of multiple HCV genotypes. In their studies, the benzimidazole, benzothiadiazine, and thiophene inhibitors showed 1000-fold changes between the 1b and 2a NS5B enzymes due to polymorphisms in specific residues in the binding pocket of the antiviral compounds (Pauwels et al., 2007). In addition, Herlihy et al. recently reported intergenotypic HCV chimeric replicons using all six genotypes. Their results demonstrate that different classes of polymerase inhibitors that target different pockets of NS5B may have various levels of activity. They attribute the differences in activity to polymorphisms between the different genotypes (Herlihy et al., 2008).

We observed a significant correlation in $EC_{50}$ values obtained with the 2a replicon and the 2a infectious virus systems (Fig. 5). This correlation was significant ($p < 0.0001$) despite the 91-fold difference in replicating RNA levels between the replicon and infectious virus systems. This study indicates that at least for these inhibitor classes, the 2a replicon is highly predictive for results obtained with the infectious 2a virus. The infectious virus will likely be a highly useful tool to pursue antiviral targets that are not represented in the replicon system or to study aspects of the virus lifecycle other than replication, translation, and polyprotein processing. Our data demonstrate the differences in potency that can exist between viral genotypes, but also that these differences can be readily identified using available replicons or the genotype 2a infection system. Furthermore, in the absence of highly efficient genotype 1a and 1b infectious clones, it is reassuring to observe that antiviral potencies measured in replicons and infectious virus using the 2a system agree closely.

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