Cross-Genotypic Examination of Hepatitis C Virus Polymerase Inhibitors Reveals a Novel Mechanism of Action for Thumb Binders

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Direct-acting antivirals (DAAs) targeting proteins encoded by the hepatitis C virus (HCV) genome have great potential for the treatment of HCV infections. However, the efficacy of DAAs designed to target genotype 1 (G1) HCV against non-G1 viruses has not been characterized fully. In this study, we investigated the inhibitory activities of nonnucleoside inhibitors (NNIs) against the HCV RNA-dependent RNA polymerase (RdRp). We examined the ability of six NNIs to inhibit G1b, G2a, and G3a subgenomic replicons in cell culture, as well as *in vitro* transcription by G1b and G3a recombinant RdRps. Of the six G1 NNIs, only the palm II binder nesbuvir demonstrated activity against G1, G2, and G3 HCV, in both replicon and recombinant enzyme models. The thumb I binder JTK-109 also inhibited G1b and G3a replicons and recombinant enzymes but was 41-fold less active against the G2a replicon. The four other NNIs, which included a palm I binder (setrobuvir), two thumb II binders (lomibuvir and filibuvir), and a palm β-hairpin binder (tegobuvir), all showed at least 40-fold decreases in potency against G2a and G3a replicons and the G3a enzyme. This antiviral resistance was largely conferred by naturally occurring amino acid residues in the G2a and G3a RdRps that are associated with G1 resistance. Lomibuvir and filibuvir (thumb II binders) inhibited primer-dependent but not *de novo* activity of the G1b polymerase. Surprisingly, these compounds instead specifically enhanced the *de novo* activity at concentrations of ≥100 nM. These findings highlight a potential differential mode of RdRp inhibition for HCV NNIs, depending on their prospective binding pockets, and also demonstrate a surprising enhancement of *de novo* activity for thumb RdRp binders. These results also provide a better understanding of the antiviral coverage for these polymerase inhibitors, which will likely be used in future combinational interferon-free therapies.

Nearly 3% of the world's population is infected with hepatitis C virus (HCV), a leading cause of chronic liver disease and hepatocellular carcinoma (1). A member of the *Flaviviridae* family, HCV is an enveloped virus which has a positive-sense, single-stranded RNA (ssRNA) genome of 9.6 kb. Upon infection, the genome is translated into a single polyprotein that is then processed into structural and nonstructural proteins. The genome shows substantial heterogeneity, and therefore HCV has been classified into six different genotypes (G1 to G6), which are approximately 35% divergent at the nucleotide level (2). Genotypes are further classified into subtypes (1a, 1b, 1c, etc.), with about 20% intersubtype nucleotide divergence (2).

Until recently, treatment of HCV infections involved a combination of pegylated interferon and ribavirin (PEG-IFN/RBV), a regimen that is lengthy and poorly tolerated and has various response rates among the HCV genotypes. Among patients infected with the most prevalent HCV genotype, G1, around 50% achieve a sustained virological response (SVR) with PEG-IFN/RBV therapy, compared to ~80% of those infected with G2 or G3 viruses (3). For more than a decade, extensive efforts have been devoted to the development of direct-acting antivirals (DAAs), compounds which specifically inhibit HCV replication by targeting viral nonstructural proteins. Three protease inhibitors have so far been approved for treatment of HCV G1, in combination with PEG-IFN/RBV, and have increased SVR rates by nearly 30% compared to those with PEG-IFN/RBV therapy alone for that particular genotype (4–7). The first HCV nucleoside inhibitor (NI), sofosbuvir, was also recently approved for HCV treatment in combination with PEG-IFN/RBV, with SVR rates of around 90% in HCV patients, although the drug is less effective against G3a viruses in IFN-free regimens (8–10). These four approved HCV DAAs rep-

resent the forerunners of a group of around 30 DAAs in phase 2 or 3 clinical trials (11).

The HCV RNA-dependent RNA polymerase (RdRp) has long been a prime target for antiviral development because of its critical role in viral replication and the absence of a mammalian homologous enzyme. The RdRp has a "right-hand" structure with finger and thumb domains that encircle the active site, located in the palm domain (12–14). Current DAAs targeting the HCV RdRp are classified into two groups. Nucleoside inhibitors, such as sofosbuvir, are substrate analogues that cause termination during synthesis of new RNA molecules. In contrast, the binding of nonnucleoside inhibitors (NNIs) to the RdRp inhibits conformational changes essential for polymerase activity (15). HCV NNIs have been identified as encompassing a diverse range of chemical scaffolds (16). However, these have all been found to bind the RdRp at one of five NNI sites (reviewed in reference 17). Two binding sites lie within the thumb subdomain: thumb I (T1), to which compounds such as benzimidazole and indole derivatives (e.g., deleobuvir, BMS-791325, and TMC647055) bind, and thumb II (T2), which is the target site for thiophene-2-carboxylic acids (18), such as VX-222 (lomibuvir) and GS-9669, as well as dihydropyranones, such as PF-00868554 (filibuvir) (19). Two other NNI

sites have been characterized within the palm domain, distinct from but in close proximity to the RdRp active site. The palm I site (P1) has been targeted by proline (20), benzodiazepine (21), and benzothiadiazine (22) derivatives, such as RG7790 (setrobuvir), ABT-333, and ABT-072, whereas compounds that bind to the palm II site (P2) include benzofurans, such as HCV-796 (nesbuvir) (23). Imidazopyridines, including the NNI GS-9190 (tegobuvir), are another class of compounds which bind to the palm site of the polymerase; however, unlike other palm binders, this binding uniquely involves an interaction with the β-hairpin which extends from the thumb into the palm domain (site P-β) (24, 25).

The lack of a cell culture model for HCV genotypes other than G1 and G2 (26) has significantly hindered the development of antivirals specifically targeting HCV genotypes G3 to G6. Studies to determine the efficacy of HCV G1-developed NNIs on G3 to G6 viruses have therefore largely involved recombinant enzymes made in *Escherichia coli* (27, 28) or the use of chimeric subgenomic replicons containing the NS5B coding region on a G1b/G2a backbone (29–32). However, chimeric replicons are often prone to replication fitness losses, and the contribution of the backbone RNA/proteins to the differential antiviral effects is poorly characterized (29). Recently, replicons of HCV G3a and G4a were described (33–35), therefore allowing a closer examination of the efficacies of HCV G1 DAAs against G2 to G6 viruses, which represent between 35% and 90% of all HCV infections in some countries (36–40).

The RdRp of HCV, like those of other members of the *Flaviviridae* family, can catalyze transcription through primed elongation as well as through a primer-independent (*de novo*) mechanism (41–43). *De novo* polymerase activity is believed to be the mechanism by which viral genome replication is initiated *in vivo*, and it is critical for the preservation of the terminal ends of the ssRNA genome during replication (42). The two modes of RdRp activity are believed to correspond to different conformations of the enzyme. A "closed" conformation, facilitated by the interactions between the finger and thumb domains, allows the *de novo* formation of a dinucleotide for replication initiation (44). The more relaxed "open" conformation of the HCV RdRp is formed by displacement of the β-hairpin loop as well as a C-terminal segment just upstream of the transmembrane domain, called the linker (45, 46). This open conformational change allows room for the nascent double-stranded RNA (dsRNA) and is thought to be responsible for the primer-extension activity of the RdRp (45, 46). Studies involving NNIs and recombinant RdRps have focused primarily on the ability of the NNIs to inhibit the primer-dependent activity of the HCV RdRp, and little is currently known on what effect NNIs have on *de novo* RdRp activity. Yi et al. (47) recently demonstrated that T2-binding HCV NNIs inhibited the primed activity but had little effect on the *de novo* activity of the HCV G1b RdRp. Conversely, compounds which bound to the P1 and P2 sites inhibited both modes of RdRp activity (47, 48). These compounds, however, were examined using only a narrow range of concentrations (0 to 200 nM), and the effects of NNI binding to the T1 and P-β sites remain to be examined (47). Furthermore, it is unclear how the various NNIs affect both modes of activity, i.e., *de novo* and primed elongation, across RdRps of different HCV genotypes.

In this study, RdRp inhibitors representing molecules that bind to all known sites on HCV polymerase were screened for the ability to inhibit G1b, G2a, and G3a replicons and G1b and G3a

recombinant enzymes. Furthermore, the inhibitory profiles of HCV NNIs against *de novo* and primed RdRp activity were analyzed, revealing a novel mechanism of action for T2 and P-β binders.

MATERIALS AND METHODS

Compounds. All compounds were purchased from commercial vendors, dissolved in 100% dimethyl sulfoxide (DMSO), and freshly diluted to the desired concentration on the day of the experiment. These compounds included 2′-C-methylcytidine (2CM) (Sigma-Aldrich, St. Louis, MO), JTK-109 (Dalton Pharma Services, Toronto, Canada), tegobuvir (GS-9190) and nesbuvir (HCV-796) (Haoyuan Chemexpress, Shanghai, China), filibuvir (PF-00868554) and setrobuvir (ANA-598) (Acme Biosciences, Palo Alto, CA), and lomibuvir (VX-222) (Selleckchem, Houston, TX). The structures of molecules examined in this study are shown in Fig. 1.

Quantitative RdRp assays. Recombinant RdRps were expressed in *E. coli* and purified by affinity chromatography as described previously (49, 50). Recombinant RdRps used in this study were those from HCV G1b strain Con1 (GenBank accession number AJ238799) and G3a strain VRL69b (accession number EF189901) (50). The *de novo* activity of RdRps was measured by monitoring the formation of double-stranded RNA from a single-stranded homopolymeric template, poly(C), by using the fluorescent dye PicoGreen (Invitrogen, Carlsbad, CA) as described previously (49). Alternatively, radioactive-nucleotide incorporation assays (1 μCi [^{3}H]rGTP/reaction mix) were also used to measure enzyme activity (50). Half-maximal inhibitory concentration (IC_{50}) values were calculated for each compound by nonlinear regression in GraphPad Prism, version 6.02.

Gel-based RdRp assays. Polyacrylamide gel-based assays were used to examine the two mechanisms of RdRp activity, i.e., primer extension and primer-independent (*de novo*) activities, using the method described by Yi et al. (47). The RNA template PE46 was designed to direct primer extension activity through a stable hairpin at the 3′ end, whereas the template LE19p can direct only *de novo* RdRp activity due to the addition of puromycin at the 3′ end of the RNA (47). Reactions were performed using either 1 μM PE46 or 0.5 μM LE19p. Each reaction mixture contained 240 nM RdRp, 0.2 mM rGTP, 0.1 mM (each) rCTP, rATP, and rUTP, 5 mM dithiothreitol (DTT), 2.5 mM MnCl$_2$, and 20 mM Tris-HCl in a 25-μl final volume. Reaction mixtures were incubated for 5 h at 30°C in the presence of test compounds or the compound vehicle DMSO (0.5% [vol/vol]). PE46 products were run in 15% denaturing polyacrylamide gels containing 7 M urea (Bio-Rad, Hercules, CA), whereas LE19p products were run in urea-free 15% polyacrylamide gels (Bio-Rad). Gels were stained with SYBR green II (Invitrogen) and visualized on a Gel Doc molecular imager (Bio-Rad). RNA band intensities were quantified by densitometry using ImageJ (version 1.46r).

Cells, replicons, and plasmids. A bicistronic HCV G1b replicon containing a luciferase reporter gene (51) was kindly provided by Ralf Bartenschlager (University of Heidelberg, Germany). The bicistronic G3a replicon S52/SG-Feo, containing a chimeric gene encoding firefly luciferase fused with neomycin phosphotransferase (33), was kindly provided by Charlie Rice (The Rockefeller University, New York). The plasmid for a tricistronic HCV G2a replicon (tri-JFH1) (52) was kindly provided by John McLauchlan (The University of Glasgow, Scotland). For the G2a replicon, RNA transcripts were generated and electroporated into Huh-7 cells for clonal selection. Cells were grown in the presence of 750 μg/ml of Geneticin for 3 weeks, and surviving colonies were isolated and characterized for replicon RNA levels and HCV NS5a expression as described previously (53).

HCV replicon assays. The antiviral activity of HCV NNIs was examined using G1b, G2a, and G3a replicon-bearing cells and increasing concentrations of each test compound (0.01 nM to 100 μM). The nucleoside inhibitor 2CM was used as a positive control for replicon inhibition. Huh-7 (G1b and G2a) or Huh-7.5 cells (G3a) bearing the HCV sub-
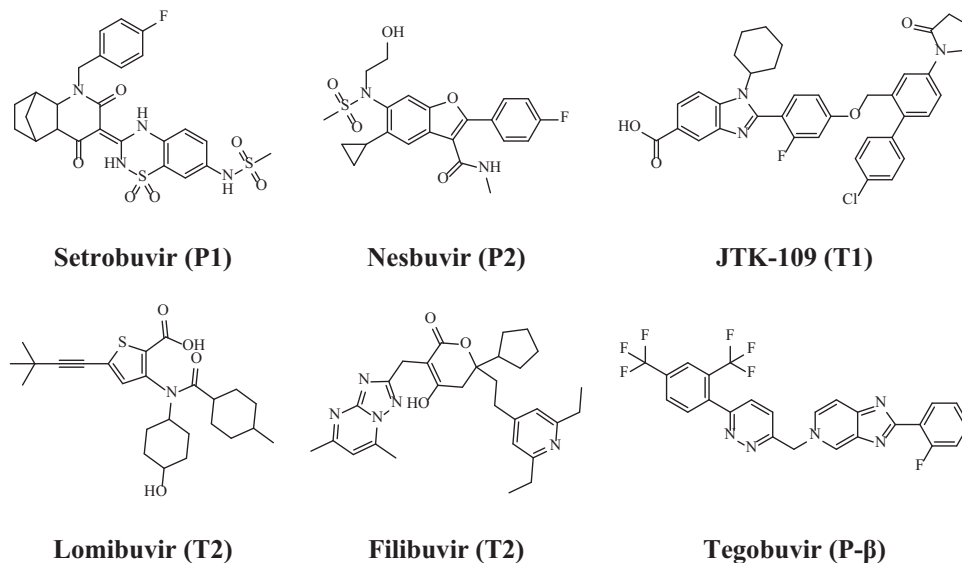
FIG 1 Chemical structures of HCV NNIs examined in this study. Compounds were selected which bind to the palm (P1 and P2), thumb (T1 and T2), or palm β-hairpin interaction site (P-β) of RdRp. The binding site for each NNI is indicated in parentheses.

genomic replicons were seeded into 96-well plates at a density of 5,000 cells/well in antibiotic-free Dulbecco's modified Eagle's medium (DMEM). On the next day, compounds were freshly diluted in complete medium, added to the cells, and incubated for 72 h. Untreated cells were incubated with 0.5% DMSO, the compound vehicle. Replication of the HCV replicons was determined by luciferase activity, measured using a luciferase assay system kit (Promega, Madison, WI) following the manufacturer's instructions. Luminescence was measured on a FLUOstar Optima microplate reader (BMG Labtech, Ortenberg, Germany). Replication of HCV replicons in treated cells was compared to that in untreated cells to calculate percent inhibition. Half-maximal effective concentration (EC_{50}) values were determined by nonlinear regression in GraphPad Prism, version 6.02.

RESULTS

Inhibitory activity of NNIs against HCV replicons. In order to examine the cross-genotype activity of HCV NNIs, replicons of HCV G1b, G2a, and G3a were used. Compounds were selected which bind to each of the five known NNI sites, and their ability to inhibit HCV replicon replication was assessed at increasing concentrations (0.01 nM to 100 μM). As a positive control, 2CM, an NI known to be an HCV cross-genotypic inhibitor, was also used. All three HCV replicons were susceptible to inhibition by 2CM. Specifically, the EC_{50} for the G1b replicon was 767.1 nM; however, 5-fold (3.8 μM) and 3-fold (2.2 μM) increases in EC_{50} were observed with the G2a and G3a replicons, respectively (Fig. 2; Table 1). In contrast, the five NNIs demonstrated differential inhibitory efficacies across replicons of genotypes 1b, 2a, and 3a. First, the P1 inhibitor setrobuvir demonstrated a significant loss of inhibitory activity against HCV G2a and G3a replicons in comparison to the EC_{50} of 8.1 nM for the G1b replicon. Only 44% ± 14.6% inhibition of G2a replicon replication was observed with the maximal concentration of 100 μM (>12,000-fold increase in EC_{50}). Similarly, when examined using the G3a replicon, the EC_{50} was 26.1 μM, or >3,200-fold higher than that for the G1b replicon (Fig. 2; Table 1). The P2 inhibitor nesbuvir had an EC_{50} of 16.6 μM when examined using the G1b replicon, and it showed comparable activities against all three genotypes, with only 3-fold and 2-fold

reductions in potency against G2a (EC_{50} = 43.3 μM) and G3a (EC_{50} = 39.9 μM) replicons, respectively, compared to the G1b replicon (Fig. 2; Table 1). Overall, these results show that the P2 binder nesbuvir is a better cross-genotype inhibitor than the NNI setrobuvir, which binds to the P1 site.

Among the thumb binders, JTK-109, which binds to the T1 site, inhibited the replication of the G1b replicon with an EC_{50} of 257.1 nM, while a 6-fold increase in EC_{50} was observed for the G3a replicon (EC_{50} = 1.5 μM) and a 41-fold increase in EC_{50} was observed for the G2a replicon (EC_{50} = 10.6 μM) (Fig. 2; Table 1). Both T2 binders, lomibuvir and filibuvir, inhibited the G1b replicon, with EC_{50}s of 5.9 nM and 79.2 nM, respectively. These two T2 inhibitors appeared to be specific G1b inhibitors, with a significant loss of inhibitory activity observed with lomibuvir against the G2a and G3a replicons (678-fold and >2,000-fold increases, respectively, in the EC_{50}), while filibuvir demonstrated 40-fold and 59-fold increases in the EC_{50} for the G2a and G3a replicons, respectively (Table 1). Lastly, tegobuvir, a P-β binder, inhibited the replication of the G1b replicon with an EC_{50} of 3.2 nM, but its inhibitory activity was significantly reduced against G2a (>3,400-fold) and G3a (822-fold) replicons compared to the G1b replicon (Fig. 2; Table 1).

Inhibitory activity of HCV NNIs against recombinant RdRps. The effects of HCV NNIs on the *de novo* activity of G1b and G3a RdRps were analyzed using an RdRp fluorescence assay. Inhibition of recombinant RdRp activity was measured by the reduction of dsRNA produced from a poly(C) template, as determined by PicoGreen fluorescence, and compared to control reactions (49). Consistent with replicon results, the P1 binder setrobuvir inhibited the G1b RdRp with an IC_{50} of 157.5 nM and was 17-fold less potent when examined using the G3a RdRp (IC_{50} = 2.6 μM) (Fig. 3; Table 2). In contrast to setrobuvir, and also consistent with the replicon results, the P2 RdRp inhibitor nesbuvir demonstrated similar efficacies against both G1b and G3a RdRps, with IC_{50}s of 76.7 nM and 75.0 nM, respectively (Fig. 3; Table 2). In a similar pattern, JTK-109 (T1 binder) showed comparable
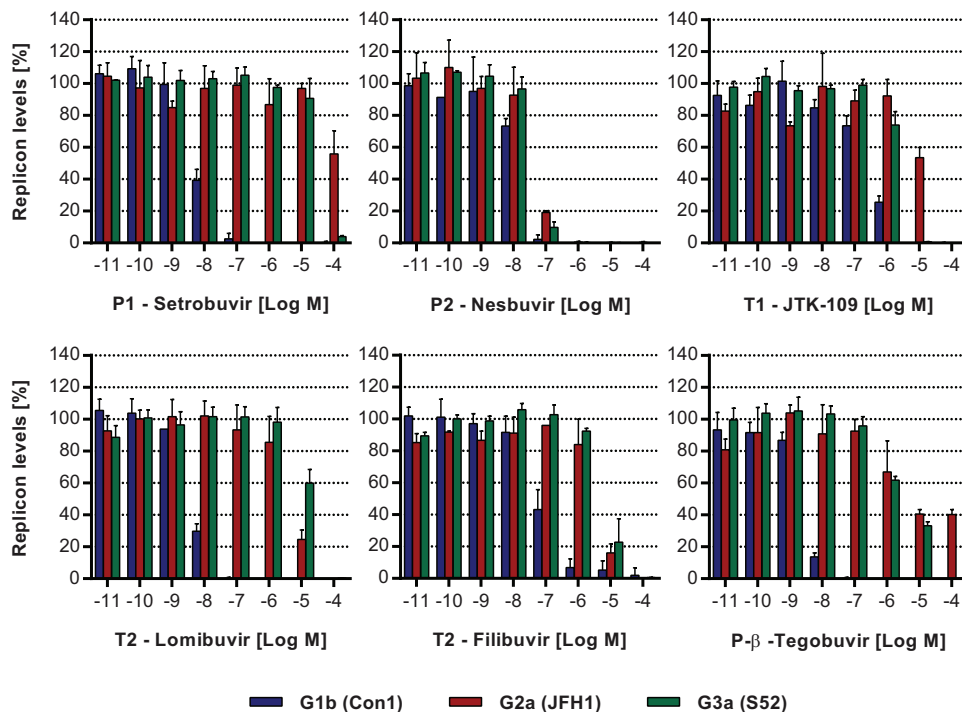
FIG 2 Inhibitory activities of HCV NNIs against subgenomic replicons. The activities of HCV NNIs were examined using subgenomic replicons of HCV G1b (blue bars), G2a (red bars), and G3a (green bars), which all contained luciferase reporter genes. Cells harboring HCV replicons were treated with the compounds, and luciferase activity was measured 72 h later. Results are shown as percentages of the mock (0.5% [vol/vol] DMSO) treatment level. Data are the averages and standard deviations of results from triplicate experiments.

inhibitory activities against both G1b and G3a RdRps, with IC_{50}s of 35.4 nM (G3a) and 107.6 nM (G1b) (Table 2).

Surprisingly, when the T2 binders were examined using the RdRp fluorescence assay, an increase in the *de novo* activity of the HCV G1b RdRp was observed as measured by increased dsRNA formation (Fig. 3). Upon the addition of both lomibuvir and fili-buvir, which inhibited the G1 replicon, the G1b RdRp *de novo* activity appeared to increase 50% at 100 nM and >100% at 1 μM (Fig. 3). This effect was not observed for the G3a RdRp, i.e., the *de novo* activity for the G3a enzyme was not induced by lomibuvir or filibuvir (Fig. 3). In fact, lomibuvir inhibited the G3a *de novo* RdRp activity, but only at higher concentrations (45.5% ± 6.6% inhibition at 100 μM), whereas filibuvir did not inhibit the G3a RdRp at any of the examined concentrations (Fig. 3). Induction of the *de novo* activity of the G1b RdRp was not limited to T2 binders, as tegobuvir, which binds to the P-β site, also specifically en-

hanced G1b RdRp activity, although this effect was not as potent as that of lomibuvir or filibuvir, with a 75.5% ± 7.5% increase in G1b RdRp activity at 100 μM (Fig. 3). Tegobuvir had no observ-able effect on the activity (enhancement or inhibition) of the G3a RdRp (Fig. 3).

In order to eliminate the possibility that the enhancement of G1b *de novo* RdRp activity by T2- and P-β-binding NNIs was an artifact of the PicoGreen assay system, an alternative radioactive-nucleotide incorporation assay was used, with lomibuvir as a rep-resentative of the three *de novo* activity-enhancing NNIs. A marked increase in the *de novo* activity of the G1b enzyme by lomibuvir was again seen with the radioactive assay; *de novo* activ-ity increased by 39.1% ± 10.6% at 100 nM and by 115% ± 29.7% at 10 μM. Lomibuvir did not enhance the *de novo* activity of the G3a RdRp (data not shown). In the radioactive assay, lomibuvir also inhibited both G1b and G3a RdRps at very high concentra-

TABLE 1 Cross-genotype activities of HCV inhibitors against subgenomic replicons

Compound	Binding site	G1b replicon		G2a replicon			G3a replicon		
		EC_{50} (nM)	95% CI[a]	EC_{50} (nM)	95% CI[a]	Fold change[b]	EC_{50} (nM)	95% CI[a]	Fold change[b]
2CM	NI	767.1	520.5–1,130	3,765	1,548–9,156	5	2,249	1,437–3,517	3
Setrobuvir	P1	8.1	6.1–10.6	>100,000		>12,000	26,060	17,690–38,390	3,217
Nesbuvir	P2	16.6	10.9–25.4	43.3	27.3–68.8	3	39.9	27.4–58.1	2
JTK-109	T1	257.1	153.6–430.2	10,620	6,434–17,520	41	1,508	944–2,409	6
Lomibuvir	T2	5.9	4.4–7.9	3,998	2,406–6,643	678	12,030	8,744–16,540	2,039
Filibuvir	T2	79.2	55.8–112.6	3,176	1,904–5,296	40	4,698	3,448–6,401	59
Tegobuvir	P-β	3.2	2.3–4.3	10,960	3,207–37,440	3,425	2,629	1,845–3,746	822

[a] CI, confidence interval.
[b] Fold change in EC_{50} compared to that obtained using the G1b replicon.
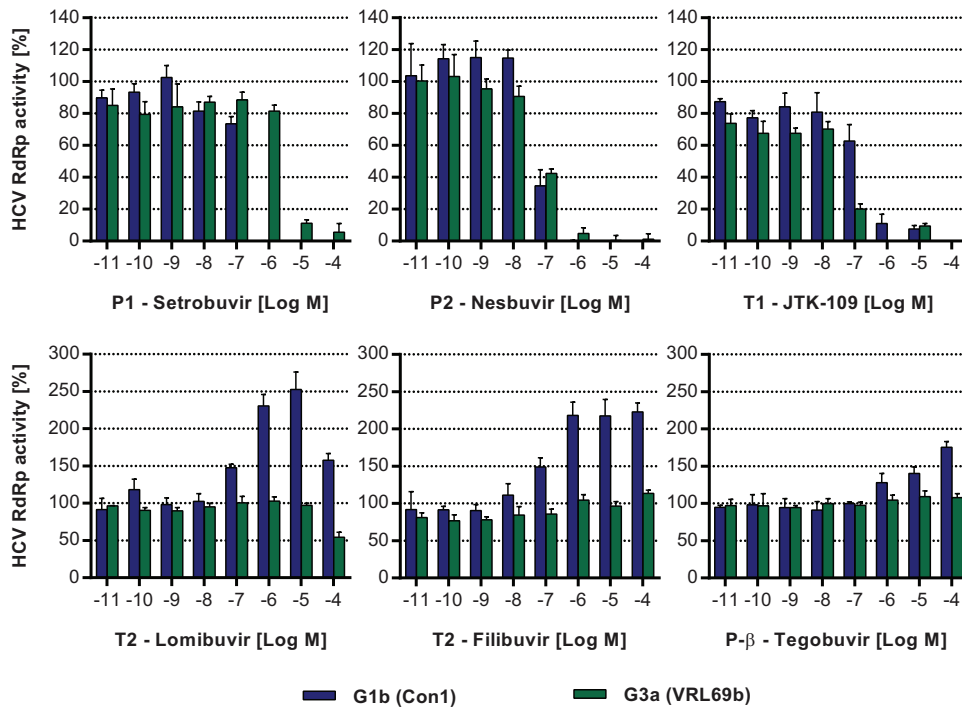
FIG 3 Effects of HCV NNIs on *de novo* activity of recombinant G1b and G3a RdRps. The inhibitory activities of HCV NNIs were examined using G1b (blue bars) and G3a (green bars) recombinant RdRps. Assays were performed using a homopolymeric poly(C) RNA template, and RdRp activity was measured via the quantitation of dsRNA formation using the fluorescent dye PicoGreen. RdRp activity is shown as a percentage of control reactions incubated with the compound vehicle DMSO (0.5% [vol/vol]). Data are the averages and standard deviations of results from triplicate experiments.

tions, and it was more potent against the G3a RdRp (81% ± 1.5% inhibition) than against the G1b RdRp (41% ± 3.3% inhibition) at 100 µM (data not shown).

Gel-based examination of RdRp inhibition. The mechanism of G1b RdRp inhibition by the different NNIs was further analyzed using two gel-based assays described by Yi et al. (47). Only the G1b RdRp was used for this analysis, as it is the target enzyme for all six NNIs used in this study. The PE46 template is designed to form a hairpin to initiate primed transcription (47), while the LE19p template has a puromycin modification of the 3′ terminus, allowing only *de novo* dsRNA synthesis (47). NNIs that bind to the palm and thumb sites (T1, T2, P1, and P2) inhibited the primer-dependent activity of the G1b RdRp, as assessed using the PE46 template (Fig. 4A). These inhibitors exerted inhibitory activity at concentrations between 10 nM and 100 nM, with >82% inhibition at 1 µM, with the exception of JTK-109, which required concentrations of 1 µM and 10 µM for 45% and 84% inhibitions of G1b RdRp activity, respectively (Fig. 4A).

Gel-based analysis of the *de novo* G1b RdRp activity was performed to confirm observations of enhanced RdRp activity by T2- and P-β-binding NNIs in the quantitative fluorescence and radioactive *in vitro* assays (Fig. 3). In the gel-based assay, inhibitors which bound to the P1, P2, and T1 sites abolished the *de novo* activity of the HCV G1b RdRp when examined at concentrations of >100 nM (Fig. 4B). In contrast, an increase in *de novo* RdRp activity was again observed with the T2 inhibitors lomibuvir and filibuvir. The addition of lomibuvir resulted in an 80% increase in the *de novo* activity at 100 nM and a 180% increase at 1 µM (Fig. 4B), as quantified by densitometry (data not shown), but at a higher concentration (100 µM), a modest (4.5%) reduction in the activity was observed (Fig. 4B). In the gel-based assays, filibuvir also enhanced *de novo* transcription of the G1b RdRp, with a 27% increase at 100 nM, a 76% increase at 1 µM, a 144% increase at 10 µM, and a 240% increase at 100 µM, as quantified by densitometry (Fig. 4B). Overall, these results are consistent with the pattern observed using the fluorescence and radioactive RdRp assays, where T2 binders enhanced the *de novo* activity but inhibited primed elongation of the HCV G1b RdRp. The P-β binder tegobuvir had no observable effect on G1b RdRp activity when examined using either the primed or *de novo* gel-based assays (data not shown).

Analysis of resistance mutations in HCV RdRp sequences. In order to gain insights into the differential efficacy of HCV inhibitors, the amino acid sequences for the G1, G2, and G3 RdRps were analyzed for known substitutions conferring resistance to all compounds tested in this study (reviewed in reference 54). A summary

TABLE 2 Inhibitory activities of HCV NNIs against recombinant RdRps

Compound	Site	G1b RdRp		G3a RdRp		Fold change[b]
		IC$_{50}$ (nM)	95% CI[a]	IC$_{50}$ (nM)	95% CI[a]	
Setrobuvir	P1	157.5	82.8–299.5	2,605.0	1,392–4,873	17
Nesbuvir	P2	76.7	21.6–271.9	75.0	57.6–97.7	1
JTK-109	T1	107.6	45.6–253.8	35.4	15.0–83.6	0.3
Lomibuvir	T2	Enhanced		>100,000		
Filibuvir	T2	Enhanced		>100,000		
Tegobuvir	P-β	Enhanced		>100,000		

[a] CI, confidence interval.
[b] Fold change in IC$_{50}$ compared to that obtained with the G1b RdRp.
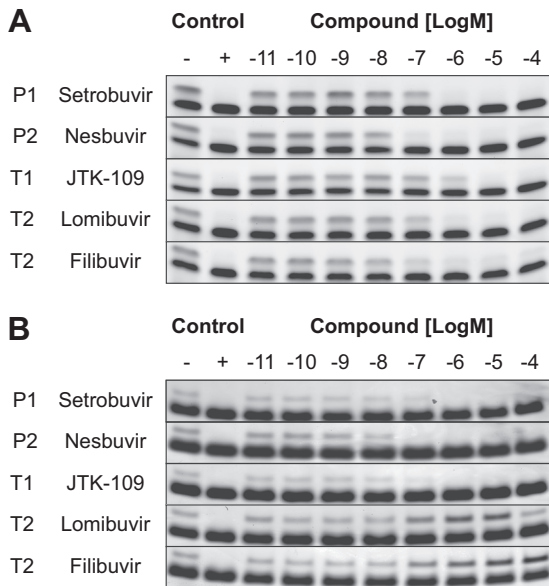
FIG 4 Effects of HCV G1 NNIs on primed and *de novo* RdRp activities. The effects of HCV NNIs on primed and *de novo* activities of the HCV G1b RdRp were analyzed using gel-based assays. Reaction mixtures were run in the absence of compound (negative control), in the absence of RdRp (positive control), and in the presence of increasing compound concentrations (0.01 nM to 100 μM). (A) Primer-dependent activity was examined using the PE46 RNA template, and reaction products were analyzed in a 15% denaturing gel. (B) Primer-independent or *de novo* RdRp activity was analyzed using LE19p, and products were run in a 15% nondenaturing gel. The RNA templates PE46 and LE19p are the bottom bands in both panels, and the RdRp products are the top bands. P1, P2, and T1 binders inhibited both modes of G1b RdRp activity at concentrations of >100 nM. At the same concentrations, however, T2 binders inhibited primed transcription (A) while unexpectedly enhancing *de novo* activity, by 80% (lomibuvir) and 27% (filibuvir) (B). The two T2 binders also enhanced G1b *de novo* RdRp activity at 1 μM, by 180% (lomibuvir) and 76% (filibuvir), as quantified by densitometry.

of amino acid substitutions associated with G1b resistance to the NNIs in this study is shown in Fig. 5. Residues are also shown for the RdRps from the G2a (JFH-1) and G3a (S52) replicon strains and for the G3a strain VRL69b. HCV G3a VRL69b was used for recombinant enzyme experiments in this study and differed from the S52 G3a RdRp by only 6 amino acids, none of which are known to confer resistance to HCV RdRp inhibitors (Fig. 5).

Examination of HCV G2a and G3a RdRp sequences revealed multiple naturally occurring resistance residues in both G2a and G3a RdRps, which likely confer resistance to HCV NNIs that bind to the P1, T2, and P-β sites but not to NNIs which bind to P2 or T1. Specifically, residues G556 and F445, implicated in G1 RdRp resistance to setrobuvir (P1) (55, 56) and tegobuvir (P-β) (25), respectively, were found in both G2a and G3a RdRp sequences (Fig. 5). Both G2a and G3a RdRps contained residues I419 and L482 (27, 29) (Fig. 5), which are associated with resistance to the T2 binders lomibuvir and filibuvir (47, 57, 58). Furthermore, the amino acid residue A494, also associated with T2 NNI resistance in G1b, was present in the G2a RdRp sequence but not in the G3a RdRp sequence (Fig. 5). The amino acid substitution V494A has been selected *in vivo* after treatment of G1 HCV-infected patients with lomibuvir (72), and also by us *in vitro*, using the G1b replicon (unpublished data). In contrast, none of the substitutions previously known to confer resistance to JTK-109 (T1) and nesbuvir (P2) were present in G2a or G3a RdRp (29) (Fig. 5).

DISCUSSION

The standard of care for treatment of HCV infection is currently undergoing significant changes, moving from traditional interferon-based regimens to shorter, interferon-free combination DAA regimens. Several dozen DAAs are in different stages of clinical trials (11), and four have so far been approved for use in combination with PEG-IFN/RBV. With all-oral, interferon-free combination therapies now on the horizon, a better understanding of the cross-genotypic specificities and modes of action for the different G1 NNIs is warranted, particularly as replicons are now

RdRp site	Compound	S282T	C316Y/N	S365T/A	M414T/L	L419M/V/I	M423T/I/V	M426T/V	C445F	Y448C/H	Y452H	I482L/V/T	A486V	V494I/A	P495S/L/A/T	G554D	S556G	D559G
NI	2CM	▩																
P1	Setrobuvir				▩											▩	▩	▩
P2	Nesbuvir		▩	▩														
P-β	Tegobuvir		▩	▩					▩	▩								
T1	JTK-109																	
T2	Filibuvir						▩					▩						
T2	Lomibuvir					▩	▩					▩		▩				
HCV G2a (JFH-1)					Q	I			F			L		A		G		
HCV G3a (S52)						I			F			L		C		G		
HCV G3a (VRL69b)						I			F			L		C		G		

FIG 5 Amino acid substitutions which confer resistance to HCV NNIs used in this study. Previously characterized substitutions in the HCV G1 RdRp which confer resistance to compounds analyzed in this study are shown. The corresponding amino acid residues for non-G1 RdRps analyzed in this study are shown along the bottom, and those that confer resistance to HCV NNIs are highlighted for G2a and G3a RdRps.

available for other HCV genotypes. In this study, we examined six representative NNIs, which between them bind to all five RdRp binding pockets, for the ability to inhibit G1, G2, and G3 HCV replicons. Furthermore, the ability of the NNIs to inhibit both modes of transcription, i.e., *de novo* and primed elongation, was also assessed. Of the NNIs selected for this study, lomibuvir, setrobuvir, and tegobuvir are currently in phase II clinical trials, whereas clinical development has been halted for JTK-109, nesbuvir, and, more recently, filibuvir (59).

Examination of the cross-genotypic efficacy of the NNI setrobuvir (P1 binder) revealed that it had little effect on the replication of the G2a (EC$_{50}$ > 100 μM) and G3a (EC$_{50}$ = 26 μM) replicons compared to that of the G1b replicon (EC$_{50}$ = 8.1 nM) (Table 1). Similarly, recombinant G3a RdRp was far less susceptible to inhibition by setrobuvir (IC$_{50}$ = 2.6 μM) than G1b RdRp was (IC$_{50}$ = 157.5 nM) (Table 2). The loss of activity of benzothiadiazines, such as setrobuvir, against G2a and G3a viruses is consistent with previous reports on chimeric replicons (29, 30, 32) and recombinant G2a and G3a RdRps (27, 28). Resistance to benzothiadiazines in G2a and G3a viruses appears to be due to the glycine residue at position 556 (55, 56) of the RdRp, an amino acid substitution which was detected in benzothiadiazine-resistant G1 replicons (60) and in G1 HCV-infected patients after treatment (61). Surprisingly, mutating G556 in the G3a RdRp to a serine, the residue naturally occurring in the G1b enzyme, did not confer susceptibility to benzothiadiazines, at least in recombinant RdRp studies (27). Thus, the precise mechanism of G3a resistance to this class of molecules remains to be resolved.

Of the six NNIs examined, only nesbuvir (site P2) demonstrated cross-genotypic inhibitory activity against G1b, G2a, and G3a replicons, with EC$_{50}$s between 16.6 nM and 43.3 nM (Table 1). Nesbuvir also inhibited the *de novo* activity of the G3a RdRp, with an IC$_{50}$ of 75 nM, compared to 76.7 nM for the G1b RdRp (Table 2). These findings are consistent with previous studies using HCV replicons and RdRps, where nesbuvir demonstrated equivalent potencies against all HCV genotypes (28–30, 32, 62), and correspond with the absence of naturally occurring substitutions in the P2 binding region of G2a and G3a RdRps.

In addition to inhibiting the G1b replicon (EC$_{50}$ = 257.1 nM) and G1b RdRp (EC$_{50}$ = 107.6 nM), the benzimidazole JTK-109 (site T1) demonstrated similar inhibitory activities against the G3a replicon (EC$_{50}$ = 1.5 μM) and enzyme (IC$_{50}$ = 35.4 nM). However, JTK-109 was 41-fold less potent against the G2a replicon (Fig. 2; Table 1). The reasons for the reduced efficacy of T1 binders, such as JTK-109, against G2a viruses are not fully understood (29). For instance, although substitutions at the P495 residue are known to confer resistance to JTK-109 in both G1b replicon and recombinant enzyme models (63), these residues are not observed in the G2a RdRp (Fig. 5). However, a naturally occurring alanine at position 494 in the G2a RdRp, which is not present in the susceptible G3a RdRp, may explain the reduced inhibitory activity of T1 binders against the G2a replicon (Fig. 5). Indeed, the V494A substitution has been associated with resistance of recombinant G1b RdRp to other thumb I binders, such as indole-*N*-acetamides (64), and further work is needed to confirm if this substitution confers JTK-109 resistance.

Resistance to the T2 inhibitors lomibuvir and filibuvir has been attributed to L419I and I482L substitutions in the HCV RdRp (47, 57, 58), both of which are present in G2a and G3a viruses (27, 29). This is consistent with our finding of a lack of activity for these

drugs against G2a and G3a replicons (Fig. 5), against which T2 binders were much less potent than the case against the G1b replicon (Table 1). Tegobuvir (P-β binder) was less potent against the G2a (>3,400-fold) and G3a (822-fold) replicons than against the G1b replicon (Table 1). Resistance to tegobuvir in G2a and G3a replicons is likely due to the C445F substitution (Fig. 5), which lies in the β-hairpin and has been shown to confer resistance to this class of compounds in replicon studies (25). It is worth noting that the reduced potencies of lomibuvir, filibuvir, and tegobuvir against HCV G3a observed in this study are higher than those recently reported for a different G3a replicon based on the same HCV G3a strain, S52 (63-fold, 13-fold, and 22-fold, respectively) (62), but more consistent with levels reported previously using chimeric HCV replicons (29, 30, 32).

To our surprise, *de novo* but not primed RdRp activity of the recombinant G1b RdRp was enhanced approximately 2-fold by a 1 μM concentration of the T2 binders lomibuvir and filibuvir and, to a lesser degree, by the P-β binder tegobuvir (Fig. 3 and 4). The increased RdRp activity was detected using *de novo* RdRp assays with both fluorescent and radioactive outputs and was further confirmed by gel-based assays (Fig. 4), indicating that it was a true effect and not an artifact of the assay used. Furthermore, no increase in *de novo* activity was detected when these compounds were examined using the G3a RdRp. When tegobuvir was examined using the gel-based RdRp assay, no effect on G1b RdRp activity was detected, in contrast to the enhancement of G1b RdRp activity seen using the RdRp fluorescence assay. This may reflect a reduced assay sensitivity which meant that the lower level of RdRp enhancement seen with tegobuvir than with lomibuvir and filibuvir (Fig. 3) could not be detected in the gel-based assay.

Most studies to date that have analyzed NNI RdRp inhibition have used primer-dependent assays, so it is likely that the enhancement of *de novo* RdRp activity has gone unnoticed. In fact, both thiophene-2-carboxylic acids (18) and dihydropyranones (19, 65), the scaffolds from which lomibuvir and filibuvir, respectively, were developed, were identified from high-throughput screening studies using primed RdRp assays, and further evaluation was achieved using the replicon model. In a recent study, Yi et al. reported no observable effect on *de novo* activity of the G1b RdRp by lomibuvir or filibuvir (47). However, lomibuvir and filibuvir were examined over a relatively narrow range of concentrations (50 to 200 nM), and indeed, a small increase in RdRp activity can be observed in Fig. 3 from that paper for lomibuvir and filibuvir at a concentration of 200 nM (47). In contrast to lomibuvir and filibuvir, tegobuvir was identified using HCV replicon and infectious (JFH-1) cell culture systems (24). Interestingly, in another recent study, tegobuvir was shown to increase the G1b primed RdRp activity by 50% at a concentration of 3.7 μM but resulted in a 40% inhibition at 100 μM, an observation that the authors were unable to explain (66). Although the RdRp assay used in the latter study uses a self-primed RNA template, no measures were taken to block the *de novo* RdRp activity; therefore, both modes of activity may be in operation in that assay system. The effect was not observed with the T2 binders lomibuvir and filibuvir in the same study (66). In summary, the enhancement of the HCV RdRp activity by T2 and P-β NNIs reported in this study can be observed in two previous reports (47, 66); however, the effect has so far gone unnoticed or unexplained.

The mechanism of action for tegobuvir and T2 inhibitors is poorly understood. Tegobuvir requires intracellular activation to

form a covalent inhibitor of the HCV RdRp (67) and is not thought to interact with the enzyme before activation (66), although, interestingly, no activation was needed to enhance *de novo* RNA synthesis in the present study. In contrast, recent evidence suggests that filibuvir and lomibuvir reduce RdRp binding to RNA but do not block the interaction completely (66). Structural analysis of the RdRp in complex with thiophene-2-carboxylic acids indicated that these molecules bind to the closed conformation of the enzyme (68) and induce conformational changes that may interfere with enzymatic activity (68, 69). Although the authors of that study suggested that this resulted in an initiation-incompetent enzyme, our results indicate that filibuvir and lomibuvir in fact increase the initiation (*de novo*) but inhibit the elongation activity of the G1b RdRp. Recently, recombinant HCV RdRp was proposed to exist as a mixture of conformations which are in dynamic equilibrium, with stabilization of one conformation occurring at the expense of the other in solution (45). Given that formation of the first few phosphodiester bonds and the transition to elongation are two known rate-limiting steps of the HCV RdRp reaction (44, 70, 71), it is likely that T2 and P-β enhance the initiation efficiency (*de novo*) of the HCV RdRp by stabilizing the RdRp conformations required for these rate-limiting steps. However, further studies are needed to validate such effects and to detail the mechanism of action for the thumb-interacting subset of HCV NNIs.

In summary, we have analyzed the inhibitory activities of six representative HCV inhibitors that bind to the five NNI sites across three genotypes: G1b, G2a, and G3a. Our data indicate that only the P2 inhibitor nesbuvir is cross-genotypic, as naturally occurring amino acid residues in non-G1 RdRps largely confer resistance against other NNIs. We also report a previously uncharacterized enhancement of *de novo* RdRp activity by the T2 binders lomibuvir and filibuvir, as well as the imidazopyridine tegobuvir (P-β), which improves our understanding of the mechanisms by which these compounds exert antiviral activity against HCV.

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