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Selection and Characterization of Replicon Variants Dually Resistant to Thumb- and Palm-Binding Nonnucleoside Polymerase Inhibitors of the Hepatitis C Virus

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Multiple nonnucleoside inhibitor binding sites have been identified within the hepatitis C virus (HCV) polymerase, including in the palm and thumb domains. After a single treatment with a thumb site inhibitor (thiophene-2-carboxylic acid NNI-1), resistant HCV replicon variants emerged that contained mutations at residues Leu419, Met423, and Ile482 in the polymerase thumb domain. Binding studies using wild-type (WT) and mutant enzymes and structure-based modeling showed that the mechanism of resistance is through the reduced binding of the inhibitor to the mutant enzymes. Combined treatment with a thumb- and a palm-binding polymerase inhibitor had a dramatic impact on the number of replicon colonies able to replicate in the presence of both inhibitors. A more exact characterization through molecular cloning showed that 97.7% of replicons contained amino acid substitutions that conferred resistance to either of the inhibitors. Of those, 65% contained simultaneously multiple amino acid substitutions that conferred resistance to both inhibitors. Double-mutant replicons Met414Leu and Met423Thr were predominantly selected, which showed reduced replication capacity compared to the WT replicon. These findings demonstrate the selection of replicon variants dually resistant to two NS5B polymerase inhibitors binding to different sites of the enzyme. Additionally, these findings provide initial insights into the in vitro mutational threshold of the HCV NS5B polymerase and the potential impact of viral fitness on the selection of multiple-resistant mutants.

Hepatitis C virus (HCV), a positive-strand RNA virus, is a member of the genus *Hepacivirus* in the *Flaviviridae* family and is the leading cause of liver disease worldwide. It is estimated that over 170 million individuals are infected with HCV (43). The current standard of care provides good clinical efficacy for patients infected with genotype 2 and 3 but is less efficacious for patients infected with the most prevalent genotype, genotype 1, thereby emphasizing the urgent need for more effective HCV-specific antiviral therapies (15, 27).

The HCV RNA-dependent RNA polymerase is an essential enzyme for viral RNA replication and represents an attractive therapeutic target. HCV polymerase has the "right-hand" polymerase fold with finger, thumb, and palm domains (22). As with other RNA-dependent RNA polymerases, the extended "fingertips" contact a thicker thumb domain to create an encircled active site constituting the closed, active conformation of the enzyme (7, 16, 22, 32). With the advent of the HCV replicon system there have been extensive developments supporting the discovery of new HCV polymerase nonnucleoside inhibitors (1–3, 5, 6, 11, 36). Several chemical classes of nonnucleoside inhibitors that inhibit the isolated enzyme and replication in the replicon system have been shown to bind at distinct sites on HCV polymerase. These polymerase inhibitors include benzothiadiazines, binding to the palm domain near the active site (38, 40), thiophene carboxylic acids which bind at the outer surface of the thumb domain (thumb I site), and benzimidazoles and indoles which bind to the thumb domain near the fingertips (thumb II site) (12, 20, 39).

A limiting factor for the efficacy of antiviral therapies targeted against retroviruses and RNA viruses is the emergence of resistance, as has been extensively described for human immunodeficiency virus (9). HCV is an RNA virus and as such, replicates as a quasispecies, a population of genetically heterogeneous and monophyletic variants (13, 17). This high genetic heterogeneity, due to the error-prone nature of its RNA-dependent RNA polymerase, represents an opportunity for the virus to evade antiviral treatment. The development of successful therapies based on inhibitors targeted against viral enzymes requires an understanding of the nature of resistant HCV variants likely to emerge upon treatment and their fitness. Understanding the structural basis for inhibitor resistance will help in the design of more-efficacious therapies that may present a greater challenge to the virus.

Using the HCV subgenomic replicon system, we report here the selection and characterization of HCV variants resistant to a thiophene-2-carboxylic acid (NNI-1) which binds to the thumb I site. Through combination studies of NNI-1 with a potent polymerase inhibitor which binds to the palm domain (NNI-3), we have studied the effect of targeting simultaneously different sites of the NS5B polymerase. Upon long-term treatment with both inhibitors, a small number of replicon colonies were isolated. Only 65% of the replicon variants from those replicon colonies contained multiple mutations on the same

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genome conferring dual resistance to both classes of inhibitors. Further characterization provided initial insights into the potential mutational threshold of the HCV NS5B, with important implications for combination drug therapy for the treatment of HCV infection. The identification of mutations in the HCV polymerase gene responsible for resistance to these structurally different HCV inhibitors alone or in combination is important for the design of future combination therapies.

MATERIALS AND METHODS

Plasmid construction. The Con1 HCV subgenomic replicon used in this study is based on a adapted dicistronic HCV subgenomic replicon construct previously described (19), and it contains the *Renilla* luciferase gene as a reporter gene. The Con1-adapted transient replicon (rep PI-luc/ET) and cured Huh-7 cells were obtained from R. Bartenschlager (23). Resistance mutations were introduced into this construct by PCR-based site-directed mutagenesis using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs were confirmed by double-stranded DNA sequencing.

The NS5B570 strain BK (NS5B570-BK) expression vector containing an N-terminal hexahistidine tag and a 21-amino-acid deletion in the NS5B C terminus was kindly provided by Hilary Overton, Roche Discovery Welwyn (25).

Compounds. Compounds 2′-C-methyl cytidine (2′-C-Me-C), thiophene-2-carboxylic acid (NNI-1), and benzothiadiazine (NNI-2) were synthesized at Roche Palo Alto LLC. NNI-3 (35a) was synthesized at Array BioPharma. Stocks of 10 mM were prepared in 100% dimethyl sulfoxide (DMSO) and stored at −20°C.

IC_{50} determinations using the replicon system. Briefly, 5,000 Huh-7 cells containing Con1 HCV subgenomic replicon (2209-23 cells) were plated in 96-well plates in a total volume of 100 μl of growth medium in Dulbecco's modified Eagle medium containing 5% (vol/vol) fetal bovine serum without G418. Inhibitors were added 24 h postplating in threefold dilutions at a final DMSO concentration of 1% (vol/vol). After 3 days, cells were harvested and the *Renilla* luciferase signal was quantified using a *Renilla* luciferase assay system (Promega, Madison, Wis.). The 50% inhibitory concentration (IC_{50}) values were calculated as the concentration of inhibitor at which a 50% reduction in the levels of *Renilla* luciferase signal was observed compared to untreated control sample results.

Transient transfections were performed as previously described (23). Inhibitors were added 24 h posttransfection in threefold dilutions at a final DMSO concentration of 1% (vol/vol), and firefly luciferase reporter signals were read 72 h after addition of inhibitors by use of a luciferase assay system (Promega, Madison, Wis.).

Selection of replicon cells resistant to polymerase inhibitors. Resistant replicons were selected using methodologies described previously (33, 41). For the selection of dual resistance 1.5×10^5 replicon cells were plated in 6-cm dishes in the presence of 0.5 mg/ml of G418 and in the absence or presence of inhibitors NNI-1 and NNI-3 in a checkerboard fashion at concentrations of 1×, 5×, or 10× the IC_{50}. Culture medium, including fresh inhibitors or DMSO dilution, was changed once a week. Cells were passaged at a 1-to-2 or 1-to-4 dilution when ~95% confluence was reached. After 4 weeks in culture, cells were harvested and total RNA was extracted for further characterization through direct sequencing of PCR products spanning the entire NS5B region.

Identification of resistance mutations. Total RNA from replicon cell lines was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturers' instructions. Reverse transcription-PCR was carried out in the NS5B regions, and PCR amplicons were sequenced on both strands by use of an ABI 3730 xl DNA analyzer. Sequences were analyzed using Sequencher and VNTI software. For DNA sequencing of molecular clones, NS5B amplicons were cloned into the TOPO cloning vector (Invitrogen, Carlsbad, CA).

Replication capacity of HCV NS5B inhibitor-resistant variants. Cells were lysed using a luciferase assay system (Promega, Madison, Wis.) following the manufacturer's instructions. Replication levels of either wild-type (WT) or resistant mutant transient replicons were determined as the ratio of the firefly luciferase signal at 4 days postelectroporation divided by the luciferase signal at 4 h posttransfection to normalize for the transfection efficiency. The replication capacity of the mutants was expressed as their normalized replication efficiency compared to that of the wild type, set at a value of 1.

Protein expression and purification of the HCV NS5B. Protein expression was induced in *Escherichia coli* strain M15 harboring an inducible NS5B expression vector by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), when optical densities at 600 nm between 1.5 and 3.5 had been reached, followed by a 16- to 18-h incubation at 22°C. NS5B570-BK and Met423Thr mutant proteins were purified to homogeneity using a three-step protocol including subsequent column chromatography on nickel-nitrilotriacetic acid (QIAGEN), SP-Sepharose HP (GE Healthcare), and Superdex 75 resins (GE Healthcare). For the Leu419Met mutant protein, the nickel-nitrilotriacetic acid column was replaced by a Talon Superflow column (BD Biosciences).

In vitro HCV polymerase enzymatic assay. The enzymatic activity of WT and mutant NS5B570-BK polymerases was determined by measuring the incorporation of radiolabeled nucleotide monophosphates into acid-insoluble RNA products. IC_{50} values were measured in 50-μl reaction mixtures containing either an 8 μg/ml:4 μg/ml ratio of poly(A)/oligo(U_{16}) (template/primer) or 12 μg/ml of in vitro-transcribed complementary internal ribosome entry site (cIRES) RNA template (25), 1 μCi of tritiated UTP (42 Ci/mmol), 1 μM ATP, CTP, and GTP (with cIRES RNA template only), 40 mM Tris-HCl (pH 8.0), 40 mM NaCl, 4 mM dithiothreitol, 4 mM $MgCl_2$, 5 μl of inhibitor serially diluted in DMSO, and 200 nM enzyme. Kinetic parameters for each enzyme were measured as previously described (18).

Protein crystallography. Crystals of WT NS5B570-BK were grown by hanging drop vapor diffusion using 7 mg/ml protein over a reservoir containing 50 mM sodium citrate (pH 4.9), 24% polyethylene glycol 4000, and 7.5% glycerol. A 5 mM concentration of compound was added for cocrystallization (NNI-2) or in mother liquor for ~22-h soaks of apoprotein (apo) crystals (NNI-1). Crystals were transferred to cryoprotectant (mother liquor plus 20% glycerol) and flash frozen in liquid nitrogen. Data were collected at beamline 5.0.2 of the advanced light source at 1-Å wavelength. Data were reduced with HKL2000 software (34). The molecular replacement solution calculated using Phaser (29) was refined by cycles of Refmac (8, 31), with manual inspection and rebuilding using PyMOL (10) and Coot (14). (For data reduction and refinement statistics, see Table 4.) Molecular images were generated with PyMOL (10).

Binding experiments. Equilibrium binding constants were determined using a Biacore S51 biosensor (BIACORE Life Sciences, Uppsala, Sweden). Interactions between protein immobilized on the sensor chip and compound injected over the surface are monitored in real time as a change in surface plasmon resonance as measured in resonance units (RU) (26). Sensor chips were prepared using standard primary amine chemistry as described in the BIACORE manual to attach 8,000 RUs of neutravidin (Pierce) to the surface of a CM5 chip. WT or mutant NS5B570-BK polymerases were minimally biotinylated by incubation for 3 h on ice with NHS-LC-LC-biotin (Pierce) in a ratio of 0.8:1 protein: biotin and desalted on a PD-10 column (Amersham Biosciences). Approximately 4,500 RUs of biotinylated protein were captured to the neutravidin surface. Binding was monitored at 20°C in 50 mM HEPES (pH 8.0)–600 mM NaCl–10 mM $MgCl_2$–5 mM dithiothreitol–5% DMSO at a flow rate of 90 μl/min. Raw data were processed, solvent corrected, double referenced, and fit to a Langmuir binding model using the Scrubber II software package (BioLogic Software, Campbell, Australia).

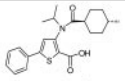
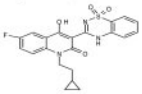
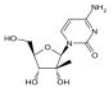
Protein structure accession numbers. Coordinates for the crystal structures are deposited with the Protein Data Bank under accession codes 2GIR for NNI-1 and 2GIQ for NNI-2.

RESULTS

Potent nonnucleoside inhibitors of HCV polymerase include thiophene-2-carboxylic acid derivatives (e.g., NNI-1), which bind to the thumb I site, benzo-1,2,4-thiadiazine derivatives (NNI-2), which bind to the palm site (5, 39), and NNI-3, a potent NS5B polymerase inhibitor that also binds to the palm site. To confirm the inhibitory activity of these structurally diverse compounds, we used a genotype 1b recombinant HCV polymerase (NS5B570-BK) with two different RNA templates, polyadenine and HCV cIRES, as well as a genotype 1b HCV subgenomic replicon (Table 1). All compounds showed high levels of inhibition of the recombinant enzyme and replicon replication.

Selection of variants resistant to NNI-1. Replicon clones resistant to NNI-1 were selected by two methods: (i) clonal selection and (ii) sequential passaging. Resistant replicon cellular clones were selected in the presence of 2 μM of NNI-1 and showed greater than 60-fold-reduced susceptibility to the inhibitor compared to the untreated cell line controls. No

TABLE 1. Activity of different structural classes of HCV inhibitors in the GT-1b replicon and recombinant NS5B enzyme

Compound Structural class	Binding site	Structure	HCV Con1 Replicon IC_{50} (nM)[a]	HCV NS5B570-BK IC_{50} (nM)[b]	
				Poly A RNA template	cIRES RNA template
NNI-1 Thiophene	Thumb I		150 ± 30	290 ± 40	140 ± 40
NNI-2 Benzothiadiazine	Palm		260 ± 10	100[c]	131 ± 40
NNI-3	Palm		2 ± 0.26	170 ± 40	120 ± 40
2′-C-Me-C[d]			1100 ± 30	40 ± 10[e]	150 ± 40

[a] The IC_{50} is the average ± standard error of mean of the results from at least three independent experiments.
[b] The IC_{50} is the average ± standard error of mean of the results from at least two and up to six independent experiments.
[c] Single IC_{50} determination.
[d] Data for 2′-CMe-C previously described (21).
[e] 2′-C-Mc-TP tested using Poly 1 RNA template.

TABLE 2. Selection of resistance to NNI-1 through sequential passaging

Passage[a]	NNI-1 IC_{50}[b] (μM)	NS5B mutations[c]	No. of molecular clones with indicated resistance mutation/ total no. of clones[e]					
			WT[d]	419M	423T	423I	423V	482L
Untreated P9	0.2	WT	10/10					
Treated P3	0.2	WT	17/18					1/18
Treated P5	2	L419M, M423T		7/19	9/19	3/19		
Treated P9	9	L419M, M423T	1/28	8/28	14/28		3/28	2/28

[a] P, passage number. All passages were carried out either in the absence (untreated) or in the presence of 4 μM of NNI-1.
[b] The IC_{50} values represent the averages of duplicate results from one single experiment.
[c] Direct sequencing from PCR products, representing the major population (in one-letter amino acid code).
[d] WT indicates no mutation or no known mutation related to HCV polymerase drug resistance.
[e] Number of molecular clones spanning the entire NS5B coding region showing a particular mutation at any of the NNI-1 identified resistance amino acid residues compared to the total number of molecular clones analyzed for each sample.

cross-resistance to 2′-C-Me-adenosine or to a benzo-1,2,4-thiadiazine derivative was observed (data not shown). Sequencing analysis of the NS5B polymerase gene showed common mutations at residues 419 (Leu419Met) and/or 423 (Met423Thr) in all replicons isolated from the NNI-1-resistant cellular clones. These mutations were not present in replicons isolated from untreated cells.

To minimize the possibility that the phenotypic resistance observed in the resistant cellular clones was due to cell adaptation and to confirm the relevance of the selected mutations, sequential passaging of replicon cells in the presence of inhibitor and G418 was carried out. After nine sequential passages in the presence of NNI-1, a dramatic reduction in sensitivity to NNI-1 was observed compared to the untreated cell results (Table 2). Sequencing of the polymerase coding region derived from the NNI-1-resistant pools (passages 5 and 9) confirmed the presence of common mutations at residues Leu419Met and Met423Thr, as observed in the NNI-1-resistant replicon cellular clones. By molecular cloning and sequencing of single HCV RNA genomes we confirmed that mutant replicons contained either Leu419Met or Met423Thr but not the double mutant Leu419Met/Met423Thr (Table 2). One clone from passage 3 revealed substitution Ile482Leu, whereas the remaining seventeen were WT. Among samples from passages 5 and 9, Leu419Met or Met423Thr were the most frequently observed substitutions, although other (minor) variants Met423Ile, Met423Val, and Ile482Leu were also identified. Mutant replicons containing Met423Ile were observed at low frequency after five passages but were no longer detected in the quasispecies by passage 9.

Characterization of mutations conferring resistance to NNI-1. Major mutant variants Leu419Met and Met423Thr, the minor variants Met423Ile and Ile482Leu, and the double mutant Leu419Met/Met423Thr were engineered into the wild-type transient replicon for further characterization of their replication capacities and drug sensitivities (Table 3). All mutant replicons conferred resistance to NNI-1, with double mutant Leu419Met/Met423Thr showing additive levels of resistance compared to the individual mutants (Table 3). No cross-resistance was observed with NNI-2 (Table 3) or with 2′-C-Me-C (data not shown). The replication capacity of single mutant Met423Thr was similar to that of the wild-type replicon (100%). However, single mutants Leu419Met, Met423Ile, and Ile482Leu and the double mutant Leu419Met/Met423Thr showed reduced replication capacity (80%, 25%, 45%, and 19%, respectively) compared to the WT capacity (Table 3). Amino acid substitutions Leu419Met and Met423Thr were further characterized by introduction into the wild-type recombinant NS5B570-BK protein for the determination of steady-state kinetic parameters using the Michaelis-Menten kinetic model. Either amino acid substitution had a negligible effect on the kinetic parameters of the recombinant HCV polymerase, with V_{max}^{app}/K_m^{app} values for both mutant enzymes similar to the WT values (data not shown). Importantly, both mutant enzymes conferred specific resistance to NNI-1 inde-

TABLE 3. Characterization of mutations in the thumb domain

Polymerase(s)	Replication capacity[a]	Transient replicon IC_{50} (μM)		HCV NS5B570-BK IC_{50} (μM)[c]	NNI-1 K_D (μM)[d]
		NNI-1[b]	NNI-2[b]	NNI-1	
WT	1	0.3 ± 0.03	0.4 ± 0.06	0.14 ± 0.04	0.02
L419M	0.8 ± 0.13	15 ± 2.4	0.4 ± 0.05	11.0 ± 4.4	0.23
M423T	1.0 ± 0.21	8.4 ± 0.9	0.3 ± 0.04	15.0 ± 2.1	0.44
M423I	0.25 ± 0.02	4.4 ± 0.8	0.3 ± 0.07	ND[e]	ND
I482L	0.45 ± 0.16	16.6 ± 3.5	ND	ND	ND
L419M/M423T	0.19 ± 0.05	25 ± 2.4	ND	ND	ND

[a] Replication capacity compared to the WT in the absence of inhibitor.
[b] IC_{50} values represent the averages ± standard errors of means of the results from at least three independent experiments.
[c] IC_{50} values represent the averages ± standard errors of means of the results from at least three independent experiments using the cIRES template.
[d] K_D values were determined using Biacore technology as detailed in Materials and Methods.
[e] ND, not determined.

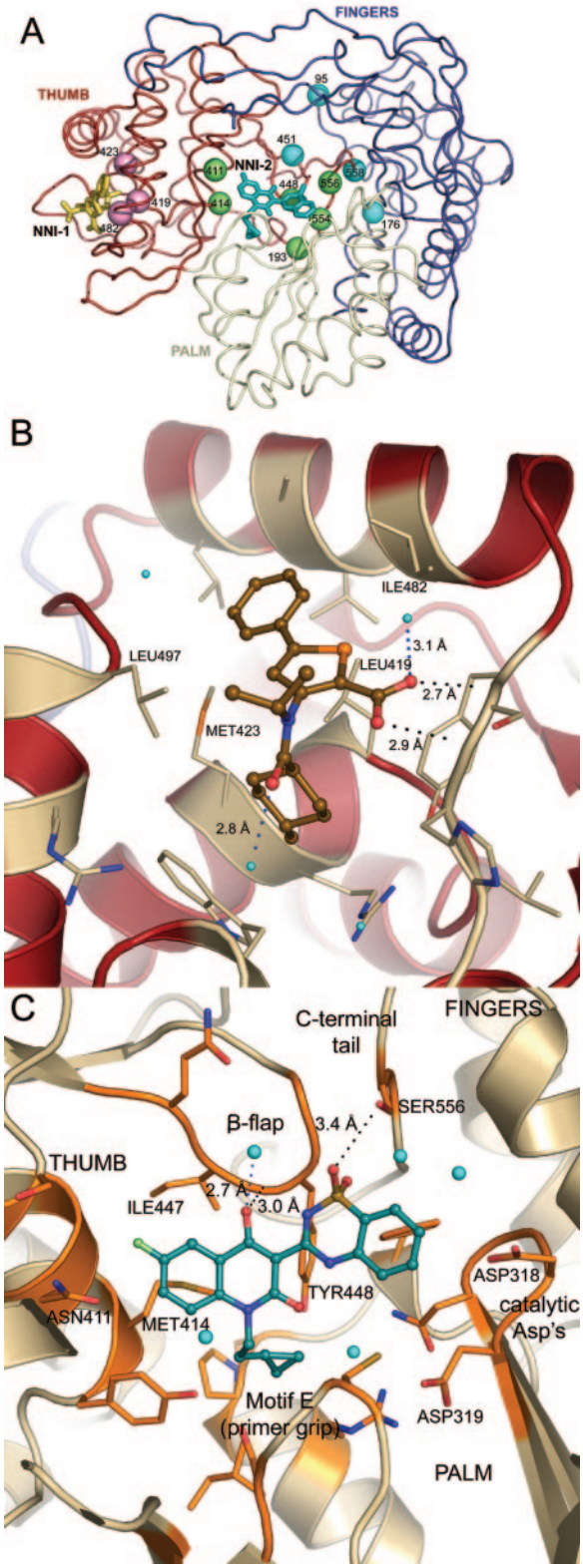


FIG. 1. (A) The overall structure of HCV polymerase. The structure of the NS5B highlights the finger domain (blue), palm domain (pale green), and thumb domain (red). Inhibitor binding positions are shown for NNI-1 (gold) and NNI-2 (cyan). The locations of primary resistance mutations are indicated by spheres for thumb-site resistance (pink) and palm site resistance (green and cyan). Of the palm-site resistance mutations, those not in direct contact with the inhibitor are colored cyan. (B) Details of NNI-1 (gold) binding to the HCV polymerase thumb domain (red). Amino acids with any atom within 5 Å of the inhibitor are colored tan. Hydrogen bonds are shown in dashed lines. The bulkier unsaturated group of NNI-1 that is directed down into the protein positions the inhibitor such that the distal saturated ring lies in a deep surface groove formed by adjustment of residue 497 relative to the apo structure. Resistance residues 419 and 423 are centrally located under the inhibitor, and residue 482 is on the helix at upper right. (C) Details of NNI-2 (teal) binding to the HCV polymerase palm domain. Amino acids within 5 Å of the inhibitor are colored orange. Salient functionally-important structural features of the polymerase encircle the binding site. Predominant resistance residues 411 and 414 are on the thumb domain helix that forms the binding site wall at the left of the figure.

TABLE 4. Crystallography data for NS5B polymerase bound to NNI-1 or NNI-2

Data set (inhibitor)	NNI-1	NNI-2
No. of molecules per asymmetric unit	2	2
Spacegroup	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions *a*, *b*, *c* (Å)	85.15, 105.75, 126.20	85.87, 105.42, 126.10
Resolution range (Å)[a]	50–1.90 (1.93–1.90)	50–1.65 (1.71–1.65)
% Completeness[a]	99.1 (100)	94.2 (63.9)
$I/\sigma(I)$[a]	22.8 (1.67)	13.13 (1.09)
R_{sym} (%)[a]	8.8 (71.5)	11.8 (54.9)
No. of observations[a]	1,817,398	1,188,578
No. of unique reflections[a]	89,694 (4,477)	128,433 (8,599)
R_{cryst}[b]	19.93	20.35
R_{free}[c]	23.81	22.84
Avg B factor (Å^2)		
Protein (chains A/B)	33.4	25.0
Water molecules	45.8	35.0
RMSD[d] bond length (Å)	0.010	0.008
RMSD bond angle (degrees)	1.288	1.154
Ramachandran plot (% amino acids in region)		
Favored	93.4	92.7
Allowed	6.6	7.3
Generous	0	0
Disallowed	0	0

[a] Numbers in parentheses are for the highest-resolution shell.

[b] $R_{cryst} = \frac{\Sigma_{hkl}||F_{obs}| - |(F_{calc})||}{\Sigma_{hkl}|F_{obs}|}$.

[c] R_{free}, R_{cryst} with 5% of F_{obs} excluded from refinement.

[d] RMSD, root mean square deviation.

pendent of the RNA template used or the presence of absence of cIRES (Table 3) or poly-adenosine (data not shown).

Mechanism of resistance to NNI-1. Inhibitor NNI-1 binds to the previously described thumb I site (4, 24, 42) (Fig. 1A). Here, we describe the cocrystallographic structure of the WT polymerase and NNI-1 (Fig. 1B and Table 4). The acid group of NNI-1 forms hydrogen bond interactions with the backbone amides of Ser476 and Tyr477. There are extensive hydrophobic interactions with both the phenyl and the cyclohexyl groups of the inhibitor, as the compound assumes a bent conformation around residues Leu419 and Met423. The saturated cyclohexyl group of NNI-1 perturbs the binding pocket, and we observed a slight shift of residue Leu419 and a significant rotamer change for Met423 relative to the apo-enzyme conformation. Furthermore, Leu497 at the base of a long helix adopts both an alternate rotamer as well as a new backbone position, thus creating a deep hydrophobic groove for the compound's phenyl group (formed by residues Leu419, Met423, Ile482, Val485,

Ala486, Leu489, and Leu497) (Fig. 1B). Indeed, the entire helix from residues 497 to 513 shows a significant (~1 Å) shift to accommodate the compound, while the rest of the thumb domain remains well aligned compared to the apo-enzyme structure. A similar conformation of the thumb binding site, also for a bound inhibitor with a saturated moiety at a position analogous to that of the cyclohexyl group of NNI-1, was reported previously (24). Other thumb-binding compounds without the saturated ring system generally do not show the same structural changes (4, 42). We have shown that resistance to a thiophene-2-carboxylic acid derivative is conferred by a mutation at amino acid residue 419, 423, or 482 of the NS5B and that mutant transient replicons bearing residue 423Thr have a replication capacity similar to that of the WT. Modeling of these substitutions shows a significant alteration of the binding pocket such that inhibitor affinity would be expected to be greatly reduced, constituting a plausible mechanism of resistance. Ile482Leu is a relatively conservative substitution; however, the outer branch point of leucine at this position creates direct steric clash with the inhibitor sulfur atom. Mutations Leu419Met, Met423Ile, and Met423Thr change both the volume and the morphology of the pocket. Mutation Met423Thr also increases the protein surface polarity in a location where the inhibitor presents a hydrophobic interface. Equilibrium dissociation constants were determined using an optical biosensor to monitor the real-time association and dissociation of compounds to wild-type or mutant Leu419Met and Met423Thr HCV NS5B polymerases captured on the surface of a sensor chip as described in Materials and Methods. Table 3 shows equilibrium disassociation constant (K_D) values of 0.23 and 0.44 μM for Leu419Met and Met423Thr, respectively, compared to 0.02 μM for the WT (10- to 20-fold reduction), consistent with a mechanism of resistance involving a reduction in affinity of NNI-1 for the mutant enzymes.

Selection of replicons dually resistant to combination treatment with thumb and palm site inhibitors. We have solved the cocrystal structure of the HCV NS5B polymerase bound to NNI-2 (Table 4) and show that the compound binds to HCV polymerase in the palm domain at a site located at the base of the relatively large internal polymerase cavity, in agreement with a recent report (38). The bound conformation of NNI-2 shows an approximately 22° bend between the thiadiazine and quinolinedione ring systems. The inhibitor lies open-faced on the palm surface abutting the thumb domain, and its perimeter is surrounded by several sequentially disparate and important features of the polymerase (Fig. 1C). Proceeding clockwise as portrayed in Fig. 1C, these include the catalytic GDD motif, motif E (or "primer grip") that links the palm and thumb I domains, the inner base of the thumb I domain itself, and, finally, the β-flap motif and C-terminal tail that protrude toward and into the catalytic core. Specifically, the inhibitor core contacts residues Phe193, Asn316, Gly410, Asn411, Met414, Gln446, Ile447, Tyr448, Gly449, and Ser556. In addition, the cyclopropyl group extends deeper into a lipophilic pocket (formed by residues Pro197, Arg200, Cys366, Ser368, Leu384, Met414, Tyr415, and Tyr448) (Fig. 1C). A heteroaryl sulfonyl oxygen forms a hydrogen bond to Ser556, notably positioned on the NS5B C-terminal tail, and the neighboring NNI-2 hydroxyl group forms a hydrogen bond to an ordered water molecule and the backbone amide of Tyr448 of the NS5B β-flap. As expected, the residues with direct contact to the NNI-2 inhibitor include many of the mutations shown to confer resistance to this class of inhibitors, such as residues 411 and 414.

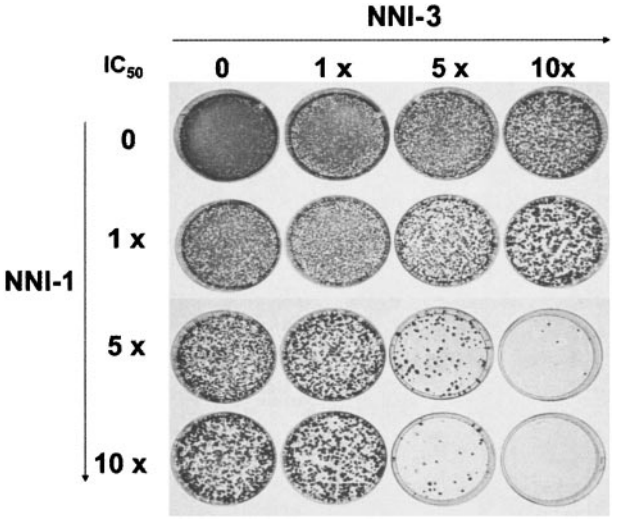


FIG. 2. Increased inhibition of replicon colony formation units in the presence of increasing concentrations of a thumb- and a palm-binding polymerase inhibitor. The results of a representative colony formation assay experiment representing three experiments are shown. Inhibitors concentrations were added as a multiple of their IC_{50}. Colonies were stained with crystal violet.

To study the inhibitory effect of two NS5B polymerase inhibitors targeting different sites within the enzyme, as well as to assess the feasibility of selecting dual escape mutants with respect to both inhibitors, replicon cells were treated simultaneously with NNI-1 and NNI-3, a potent compound proposed to bind to the palm site and to make many contacts with the polymerase similar to those seen with NNI-2 (Table 1, Fig. 1A). In the presence of either compound alone, large numbers of replicon colonies (approximately 2,000 colonies) were observed, albeit the numbers were significantly reduced compared to the untreated control results (Fig. 2). In the presence of 5 × IC_{50} of both compounds, there was a dramatic decrease in the number of replicon colonies (approximately 100 colonies), showing a more-than-additive inhibitory effect by two inhibitors targeting different sites of the HCV polymerase. This is likely to reflect the increased difficulty in the selection of variants resistant simultaneously to two or more compounds (Fig. 2, Table 5). When both compounds were present at 10 × IC_{50} (approximately their IC_{90}), the ability to establish replicon colonies was almost completely abolished (10 colonies). These results suggest an increased challenge to both viral replication and to the selection of replicons resistant simultaneously to both classes of compounds (in Fig. 2, compare the number of colonies obtained with NNI-1 or NNI-3 alone at 10 × IC_{50} to the number obtained with the combination treatments at 5× the IC_{50} of NNI-1 and 5× the IC_{50} of NNI-3).

To characterize the replicons selected in the dual combination selection experiment, colonies obtained either in the absence of inhibitors or in the presence of only one or both inhibitors were harvested and the NS5B coding region was

TABLE 5. Frequency of resistant colony formation in the presence of single or combined NS5B polymerase inhibitors

Parameter	NS5B polymerase inhibitors[a]			
	10× NNI-1	10× NNI-3	5× NNI-1, 5× NNI-3	10× NNI-1, 10× NNI-3
No. of colonies[b]	2,000[c]	2,000[c]	100	10
Frequency (%)[d]	1.3	1.3	0.06	0.006

[a] Multiple of IC_{50} values.
[b] Values represent the average results of three independent experiments.
[c] Approximate number.
[d] Frequency determined as (number of colonies/initial number of cells) × 100.

sequenced (Table 6). As observed in the previous experiments performed with NNI-1 alone, resistance mutations at residues Leu419Met and Met423Thr were identified. In the presence of NNI-3 alone, substitutions Asn411Ser, Met414Thr, and Ser556Asn were selected. Mutations at residues 411 and 414 after prolonged treatment of replicon cells with benzothiadiazine derivatives have been previously described (30, 40). We also observed that mutation Ser556Asn confers resistance to NNI-3 (S. Le Pogam, H. Kang, and I. Najera, unpublished results). In the presence of 10× the IC_{50} for NNI-1 and 5× the IC_{50} for NNI-3, a further reduction in the number of replicon colonies was observed, coincident with the accumulation of amino acid substitutions conferring resistance to both classes of inhibitors: Met414Leu, Leu419Met, Met423Thr, Tyr448His, Cys451Arg, and Ile482Leu (Table 6). Substitutions Met414Leu (30), Cys451Arg (40), and Tyr448His (30) confer resistance to palm-site inhibitors.

In order to investigate whether the selected replicons were dually resistant to both inhibitors and whether the observed resistance mutations were present on the same strand of HCV replicon RNA, PCR products from replicon colonies obtained in the presence of 10× the IC_{50} for NNI-1 and 5× the IC_{50} for NNI-3 were cloned and sequenced (Table 7). Analysis of 44 molecular clones showed that 43 of them (97.7%) contained amino acid substitutions that conferred resistance to either class of inhibitors and that only 1 clone (0.3%) had a wild-type genotype, with only one amino acid substitution at residue 37 (Val to Ile) not known to confer resistance to HCV polymerase inhibitors. Of the 43 resistant replicons, 28 (65%) of them contained amino acid substitutions that conferred simultaneous resistance to both classes of inhibitors (predominantly at residues 414 and 423). Substitution Ile482Leu was found in 32 of the 44 clones (74%), in combination with palm-resistance mutations in 17 of the 32 clones (53%), and in 2 clones (6%) with palm and other thumb resistance mutations (Table 7). In eight clones (25%) Ile482Leu was present in combination with mutations not yet characterized, whereas five clones (16%) contained mutation Ile482Leu only. Nine dual resistance clones contained mutations Met414Thr (palm) and Met423Thr (thumb) (Table 7). Two clones revealed a single mutation each, at either position 419 or 423. No clones containing resistance mutations in the palm site alone were observed. Phenotypic characterization of the most frequent variant, double-mutant Met414Leu/Met423Thr, and of the triple mutant containing replication-impaired Ile482Leu (Met414Leu/Met423Thr/Ile482Leu) was performed. Double-mutant Met414Leu/Met423Thr showed a slightly reduced replication capacity (70%) compared to WT or to the single mutant capacities. The presence of mutations Met414Leu and Met423Thr on the same genome conferred a dual-resistance phenotype on NNI-1 and NNI-3 (Table 8). Triple-mutant Met414Leu/Met423Thr/Ile482Leu showed 70% replication capacity and high-level resistance to both inhibitors, demonstrating an increased resistance to NNI-1 in the presence of two resistance mutations in the inhibitor's binding site (Table 8).

DISCUSSION

Due to the high genetic heterogeneity of HCV in vivo (17, 28, 37), the rapid selection of drug-resistant HCV variants upon treatment with antivirals is to be expected when viral suppression is incomplete. Using the replicon system we have selected mutant replicons with reduced susceptibility to a thumb I-binding inhibitor (but not to other nonnucleoside NS5B polymerase inhibitors binding to the palm domain or to nucleoside analogs such as 2′-C-Me-C) under different experimental conditions, supporting the conclusion that these resistant variants were selected due to the direct antiviral pressure of the compound on the HCV replicon population. All thumb I inhibitor resistance mutations selected in our studies are directly involved in the inhibitor binding site. The most frequently observed mutations, Leu419Met and Met423Thr, are well tolerated, as they did not have a significant effect on replicon RNA replication. Furthermore, the catalytic efficiency (V_{max}^{app}/K_m^{app}) of the recombinant enzyme bearing either substitution Leu419Met or substitution Met423Thr was similar to that of the wild type, consistent with the hypothesis that these positions are not directly involved in the catalytic steps of RNA synthesis. The Ile482Leu and Met423Ile mutant replicons were observed only as minor variants, possibly due to

TABLE 6. Genotypic characterization of NS5B coding region obtained in dual combination selection experiments

Compound(s)[a]	NS5B known resistant mutations[b]
Untreated	**WT**
10 X NNI-1	**L419M, M423T**
10 X NNI-3	**N411S, M414T, S556N**
10 X NNI-1, 5 X NNI-3	**M414L, L419M, M423T, C451R, I482L, Y448H**

[a] Compound(s) concentration used for each selection plate expsressed as multiple of the inhibitor's IC_{50}.
[b] Amino acid substitutions observed in the polymerase coding region through direct sequencing of PCR products (in one-letter amino acid code). Red, resistance mutations to thumb binding inhibitors. Blue, resistance mutations to palm binding inhibitors.

TABLE 7. Mutational patterns of dual resistant replicons selected in the presence of both $10\times$ IC_{50} of NNI-1 and $5\times IC_{50}$ of NNI-3

No. of clones[a]	NS5B sequence at resistance amino acid residues[b]								
	H95R	*F193S*	M414L	L419M	M423T	Y448H	C451R	I482L	Others[c]
1									x
1				x					
1					x				
5								x	
8								x	x
7						x		x	
1	x							x	
1		x						x	
2			x		x			x	
9			x		x				
8							x	x	

[a] From a total of 44, number of molecular clones carrying the specified mutations; in black, number of clones carrying mutation(s) not known to be related to HCV polymerase drug resistance; in red related to resistance to thumb binding inhibitors and in **purple and bold** carrying mutations related to resistance to thumb and to palm binding inhibitors.

[b] Amino acid substitutions observed in the polymerase coding region through DNA sequencing of molecular clones (in one-leter amino acid code). **Blue and bold**, resistance mutations to palm binding inhibitors. *Blue not bold and italic*, mutation likely to confer resistance to palm binding inhibitors as it is in the inhibitor's binding site. **Red in bold**, resistance mutations to thumb binding inhibitors.

[c] Mutations not described to be related to resistance to thumb o to palm HCV polymerase inhibitors.

their reduced replication capacity (45% and 25% compared to the WT, respectively). Mutants Ile482Leu, Met423Ile, and Met423Val were observed at different sequential passages, illustrating the plasticity and evolution of the quasispecies population in the presence of inhibitor's selective pressure (Table 2). The double-mutant Leu419Met/Met423Thr was never isolated in our studies, suggesting that under our experimental conditions its replication capacity (19% of the WT capacity) is below the "fitness threshold" required for detection despite its highly resistant phenotype.

Modeling of these substitutions showed a significant alteration of the binding pocket such that inhibitor affinity would be expected to be reduced, constituting a plausible mechanism of resistance. The results of both our biosensor- and structure-based modeling experiments are consistent with reduced inhibitor affinity, constituting a mechanism of resistance for mutants Leu419Met and Met423Thr. Ile482Leu and Met423Ile are also interesting, as Leu482 is the wild-type consensus amino acid for HCV genotype 2a and Ile423 is the wild-type consensus amino acid for genotype 5, suggesting that genotypes 2a and 5 may be less sensitive to this class of NS5B polymerase thumb inhibitors.

TABLE 8. Phenotypic characterization of dual-resistance replicons

Polymerase(s)	Replication capacity[a]	Transient replicon assay IC_{50}[b]	
		NNI-1(μM)	NNI-3 (nM)
WT	1	0.3 ± 0.03	2.6 ± 0.3
M423T	1 ± 0.21	8.4 ± 0.9	1.6 ± 0.2
I482L	0.45 ± 0.16	16.6 ± 3.5	1.7 ± 0.4
M414L	1.24 ± 0.23	0.3 ± 0.03	50 ± 9
M414L/M423T	0.7 ± 0.12	7.5 ± 0.9	44 ± 7.5
M414L/M423T/I482L	0.7 ± 0.13	>33	40 ± 10

[a] Replication capacity compared to the WT in the absence of inhibitor.

[b] The IC_{50} values for NNI-1 and NNI-3 represent the averages ± standard errors of the means of the results from three independent experiments.

The selection of drug-resistant virus requires a balance between its ability to escape the inhibitor's selective pressure while preserving its replication competence; clearly, variants with higher replication capacity have a higher probability of selection. Indeed, our in vitro data show that highly resistant (major) single mutants Met423Thr or Met414Leu can replicate in vitro to levels similar to WT levels, whereas (minor) single-mutant Ile482Leu only replicates in vitro to 45% of the levels of WT replicon. The effective treatment of HCV infection will likely require multiple antiviral drugs with different resistance profiles to delay the emergence of resistance, as has been shown in human immunodeficiency virus (35). In the present study, treatment with either a thumb or a palm inhibitor alone rendered large numbers of resistant replicon colonies in vitro, a potential indication of the likely rapid emergence of HCV-resistant variants upon initiation of monotherapy. Importantly, by combining two inhibitors binding to the thumb and to the palm sites of the HCV polymerase we observed a greater-than-additive inhibitory effect of replicon RNA replication. After treatment with concentrations approximating the IC_{90} of each compound in combination, only a small number of resistant replicon colonies was obtained, possibly a reflection of the increased complexity of selecting replicon variants bearing multiple resistance mutations and/or of their impaired replication capability (Table 5). Our data showed that dual resistance to thumb I- and to palm-site inhibitors is mediated by mutations Met423Thr and/or Ile482Leu in the thumb domain and mutations in the palm and finger domains (related to palm-site inhibitors), in agreement with previous reports (30, 33, 40).

Mutation Ile482Leu, located within the thumb inhibitor-binding pocket, was only observed as a minor variant after sequential passages in the presence of the thumb I-site inhib-

itor alone (in approximately 6% of mutant variants), possibly due to its low replication capacity (Table 2). Interestingly, it was the most abundant among replicons selected in the presence of both inhibitors (Table 6), likely a reflection of the increased replication capacity when present in combination with substitutions Met414Leu and Met 423Thr (Table 8).

Our results are in agreement with recent studies on the combined effects of NS3 protease inhibitor BILN 2061 and NS5B inhibitors, A-782759 (30), or benzimidazol 5-carboxamide derivatives (20, 30), which also show an enhanced inhibitory effect on replicon replication compared to treatment with a single inhibitor.

In summary, we have described for the first time HCV replicons resistant to a thiophene-2-carboxylic acid polymerase inhibitor and shown that the mechanism of resistance is due to the reduced binding affinity of the inhibitor to the resistant mutant enzymes. We have also demonstrated the selection of replicons with dual resistance to inhibitors binding to the thumb and palm sites. Perhaps most importantly, the combination experiments show that dual-resistance replicons have amino acid substitutions that map directly to both the thumb and palm sites. Thus, combination therapy apparently does not significantly complicate the resistance profiles observed with single-compound treatments. While our findings demonstrate the theoretical possibility of selecting HCV viruses resistant to multiple inhibitors targeting different sites of the polymerase, the frequency of such mutants was calculated to be approximately 0.006% compared to the frequency of 1.3% of selecting resistant mutants under the selective pressure of a single inhibitor. Importantly, although 97.7% of the replicon variants were isolated from colonies obtained in the presence of both inhibitors, only 65% of them contained resistance mutations to both inhibitors on the same genome and were, therefore, dually resistant. This suggests that only a small percentage of replicon variants that showed an impaired replication capacity compared to the WT replicon capacity would be able to replicate for a longer term in the presence of inhibitors targeting these two sites of the NS5B polymerase. These experiments with dual-resistance mutations indicate the potential to dramatically reduce the ability of the HCV virus to replicate in the presence of therapies targeting these two distinct sites on the polymerase. Further investigations are required to determine whether this in vitro reduction in viral replication will be observed in patients and whether it will be sufficient to reduce virus replication to levels that allow the immune system to control and perhaps clear the infection.

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