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In Vitro Antiviral Activity and Preclinical and Clinical Resistance Profile of Miravirsen, a Novel Anti-Hepatitis C Virus Therapeutic Targeting the Human Factor miR-122

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Miravirsen is a β-D-oxy-locked nucleic acid-modified phosphorothioate antisense oligonucleotide targeting the liver-specific microRNA-122 (miR-122). Miravirsen demonstrated antiviral activity against hepatitis C virus (HCV) genotype 1b replicons with a mean 50% effective concentration (EC_{50}) of 0.67 μM. No cytotoxicity was observed up to the highest concentration tested (>320 μM) in different cell culture models, yielding a therapeutic index of ≥297. Combination studies of miravirsen with interferon α2b, ribavirin, and nonnucleoside (VX-222) and nucleoside (2′-methylcytidine) inhibitors of NS5B, NS5A (BMS-790052), or NS3 (telaprevir) indicated additive interactions. Miravirsen demonstrated broad antiviral activity when tested against HCV replicons resistant to NS3, NS5A, and NS5B inhibitors with less than 2-fold reductions in susceptibility. In serial passage studies, an A4C nucleotide change was observed in the HCV 5′ untranslated region (UTR) from cells passaged in the presence of up to 20 μM (40-fold the miravirsen EC_{50} concentration) at day 72 of passage but not at earlier time points (up to 39 days of passage). Likewise, a C3U nucleotide change was observed in the HCV 5′UTR from subjects with viral rebound after the completion of therapy in a miravirsen phase 2 clinical trial. An HCV variant constructed to contain the A4C change was fully susceptible to miravirsen. A C3U HCV variant demonstrated overall reductions in susceptibility to miravirsen but was fully susceptible to all other anti-HCV agents tested. In summary, miravirsen has demonstrated broad antiviral activity and a relatively high genetic barrier to resistance. The identification of nucleotide changes associated with miravirsen resistance should help further elucidate the biology of miR-122 interactions with HCV. (The clinical trial study has been registered at ClinicalTrials.gov under registration no. NCT01200420).

Hepatitis C virus (HCV) infects more than 170 million persons worldwide and is a major cause of chronic hepatitis, cirrhosis of the liver, and hepatocellular carcinoma (1). The current standard of care (SOC) for the treatment of chronic HCV infection includes either of the newly approved drugs sofosbuvir, a nonstructural 5B (NS5B) polymerase inhibitor, or simeprevir, an NS3/NS4A serine protease inhibitor (PI), combined with ribavirin (RBV) and, in many cases, pegylated alpha interferon (PEG-IFN-α). While regimens that include the PI telaprevir or boceprevir combined with PEG-IFN-α and RBV also are approved, they continue to be associated with significant side effects, drug resistance, and limited efficacy against certain HCV genotypes and in hard-to-treat patient populations (2). Current advances have led to the development of other direct-acting antivirals (DAAs) that target specific viral proteins of the HCV replication cycle, including the NS3/NS4A protease, NS5B polymerase, and NS4B and NS5A proteins (3–6). As with all infectious diseases, the potential for the pathogen to develop ways to circumvent treatment prompts the search for drugs with a novel mechanism of action. While the number of direct hepatitis C viral targets is limited, the virus depends on many host factors for successful propagation (7, 8). Some host-targeting agents (HTAs) have entered clinical development and include cyclophilin inhibitors (e.g., alisporovir and SCY-635) (9), entry inhibitors (e.g., ITX 5061) (10), and other inhibitors with unknown mechanisms of action (e.g., nitazoxanide) (11).

MicroRNAs (miRNAs) are regulators of a variety of biological processes (e.g., cell growth, development, and differentiation), and alterations in miRNA levels and function have been implicated in a range of human diseases (including cancer [12–14], infectious disease [15, 16], and cardiovascular disease [17, 18]). miRNAs act as posttranscriptional regulators of gene function by binding to complementary sequences on target mRNA transcripts (mRNAs). Interference with miRNA function represents a novel but rapidly emerging therapeutic approach to modifying gene function. Therapeutic oligonucleotides either mimicking or antisense to the target miRNA may be used to regulate gene expression by replacing (19) or alleviating (18) the repressive effects of the miRNA or by interfering with other noncanonical functions of the targeted miRNA.

miR-122 is a liver-specific miRNA that is abundantly expressed

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in hepatocytes and is known to be involved in fatty acid and cholesterol metabolism (20–22). The replication of HCV in hepatocytes has been shown to be critically dependent on the presence of miR-122 (23), with several proposed mechanisms of action. These include the protection of the 5′ untranslated region (UTR) from degradation by host nucleases (24–26), support of viral replication (24), or aid in translation of the viral polyprotein (27). In fact, miR-122 may participate in all of these aspects of viral proliferation. Important for all proposed mechanisms of action are the three miR-122 binding sites that have been identified in HCV; two in the 5′UTR (S1 and S2) and one in the 3′UTR (S3) (27, 28). In addition, there is strong evidence that interaction between miR-122 and the 5′UTR also occurs outside the canonical binding sites, including functional interactions within the first 5 nucleotides of the 5′ end of the viral genome (29). The binding site in the 3′UTR does not appear to play a role in the stimulation of HCV RNA replication. A study demonstrating that miR-122 is essential for HCV proliferation by binding to specific sites in the 5′UTR of HCV provided the rationale for the development of the anti-miR-122 therapeutic miravirsen (23, 28, 30). Miravirsen (formerly SPC3649) is a 15-base oligonucleotide that is complementary to part of miR-122 and is the first miRNA-targeting agent administered to patients.

Miravirsen has demonstrated *in vitro* antiviral activity against all HCV genotypes (31) and has produced long-lasting suppression of HCV RNA levels in an HCV-infected chimpanzee model, with no evidence of viral resistance (32). In a phase 2 proof-of-concept clinical trial in treatment-naive patients with chronic HCV infection, miravirsen monotherapy was associated with substantial, prolonged, and reversible pharmacodynamic responses (decreases in cholesterol levels) and demonstrated continuous and prolonged antiviral activity with no evidence of genetic resistance at the S1, S2, and S3 binding sites in subjects who experienced virologic rebound (33).

In the present *in vitro* study, we show that miravirsen is additive when combined with inhibitors of NS3, NS5B, and NS5A and is fully active against HCV replicons resistant to NS3, NS5A, and NS5B inhibitors. In addition, we identify nucleotide changes in the distal end of the 5′UTR in HCV isolated from *in vitro* serial passage studies and in subjects experiencing viral rebound in a miravirsen phase 2 clinical trial. The broad antiviral activity against wild-type and DAA-resistant HCV replicons, combined with a relatively high barrier to resistance due to interaction with a host function, highlight the advantages of miR-122 as a target. Furthermore, the identification of nucleotide changes associated with miravirsen resistance should help elucidate miR-122–HCV interactions.

MATERIALS AND METHODS

Compounds. Miravirsen sodium (miravirsen) and the control compound SPC4729 are 15-base oligonucleotides, comprised of 8 β-D-oxy-locked nucleic acid (LNA) and 7 DNA monomers and arranged in the sequences depicted in Fig. 1. The negative-control oligonucleotide SPC4729 is designed to have no homology to known microRNAs and to retain the same pattern of LNA and DNA moieties as miravirsen. Both oligonucleotides (miravirsen and SPC4729) were reconstituted in water to a concentration of 2 mM, as validated by absorption at 260 nm. IFN-α2b was purchased from R&D Systems (Minneapolis, MN). Ribavirin (RBV) was purchased from Sigma-Aldrich (St. Louis, MO). Telaprevir, VX-222, and BMS-790052 were purchased from Selleck Chemicals (Houston, TX). 2′-methyl cytidine (2′-MeC) was purchased from Toronto Research Chemicals (North York, Ontario, Canada).

FIG 1 Miravirsen sodium (miravirsen) and the control compound SPC4729 both are 15-base oligonucleotides, comprising β-D-oxy-LNA and DNA monomers, respectively. Both molecules contain 8 LNA and 7 DNA nucleotides arranged in the sequences depicted. Capital letters denote LNA-modified nucleotides (^{m}C is LNA-5-methyl-cytidine), and lowercase letters denote DNA nucleotides. All of the internucleotide linkages, 14 in total, are phosphorothioate linkages, as indicated by the subscript s, and all LNAs are β-D-oxy-LNA, as indicated by the superscript o.

Cell culture. The reporter cell line Huh-luc/neo-ET (termed ET) (34) was provided by Ralf Bartenschlager (University of Heidelberg, Germany). This cell line harbors the persistently replicating I389luc-ubi-neo/NS3-3′/ET replicon containing the firefly luciferase gene-ubiquitin-neomycin phosphotransferase fusion protein and encephalomyocarditis virus (EMCV) internal ribosomal entry site (IRES)-driven NS3-5B HCV coding sequences containing the ET tissue culture-adaptive substitutions (E1202G, T1208I, and K1846T). The cell line was cultured as described previously (34).

HCV antiviral and cytotoxicity assays. For *in vitro* antiviral and cytotoxicity assays, HCV replicon cells were seeded into 96-well tissue culture plates at 5.0×10^3 cells per well in a volume of 85 μl per well and incubated at 37°C in 5% CO_2 for 24 h. Following 24 h of incubation, serial dilutions of miravirsen or a negative-control oligonucleotide (SPC4729) were added directly to the cells in triplicate wells (gymnosis [35]). IFN-α2b was included as a positive control. Following 48 h of incubation, the plates were assessed for anti-HCV activity by measurement of luciferase reporter activity (Britelite plus luminescence reporter gene kit [PerkinElmer, Shelton, CT]) with a Wallac 1450 Microbeta Trilux liquid scintillation counter. Percent inhibition for each drug concentration relative to the untreated replicon cells was calculated from the mean relative light units (RLU) of the triplicate wells containing a given drug concentration as a percentage of the mean RLU from six replicate wells containing untreated replicon cells. The 50% and 90% effective concentrations (EC_{50} and EC_{90}) were calculated by linear regression. Data are expressed as the mean EC_{50} ± standard deviations together with the range representing the low to high experimental values. In parallel, separate plates were assessed for cytotoxicity by XTT dye reduction. The 50% cytotoxic concentration (CC_{50}) was calculated as the concentration of drug that decreased the percentage of formazan produced by infected, drug-treated replicon cells to 50% of that produced by drug-free replicon cells.

***In vitro* combination studies.** For *in vitro* combination studies, HCV replicon cells were cultured as described above. Antiviral activity (EC_{50}) and cytotoxicity (CC_{50}) were determined for each of the compounds alone or in combination in triplicate wells on 3 separate plates. Following the 24-h incubation, eight successive 2-fold dilutions of miravirsen in cell culture medium without G418 were evaluated alone or in all possible combinations with five successive 2- or 5-fold serial dilutions of a second compound. The second compound included BMS-790052 (NS5A protein inhibitor), VX-222 (nonnucleoside NS5B inhibitor), telaprevir (NS3 protease inhibitor), BILN-2061(NS3 protease inhibitor), and 2′-MeC (nucleoside NS5B inhibitor). Concentrations of each drug were selected that bracketed the EC_{50}, with high concentrations being 1.2 to 2.4 μM, 10 U/ml, 10 nM, 148 μM, 32 pM, 4.5 μM, and 1.0 μM for miravirsen, IFN-α2b, VX-222, RBV, BMS-790052, 2′-MeC, and telaprevir, respectively. Six wells in each plate also received medium alone as a no-treatment control. Following 48 h of incubation, the plates were assessed for anti-HCV activity and cellular cytotoxicity as described above. Data from each experiment were analyzed by the technique of Prichard and Shipman, Jr.

(36), using MacSynergy II, version 1.0 (36, 37). The difference between the observed antiviral effects from each combination and those expected if the interactions occurred independently (additive) are expressed as a volume ($\mu M^2\%$) above or below a plane that represents no interactive (additive) effects. Volumes with positive values at the 95% confidence interval ($P < 0.05$) indicate synergy, while volumes with negative values indicate antagonism. As a general guideline (37), volumes of between 50 and 100 $\mu M^2\%$ suggest moderate synergy or antagonism, and volumes of >100 $\mu M^2\%$ suggest strong synergy or antagonism.

***In vitro* antiviral activity against drug-resistant HCV replicons.** The *in vitro* antiviral activity of miravirsen was evaluated against HCV genotype 1b reporter replicons engineered to contain key amino acid substitutions in NS3 protease (A156T and R155K), NS5B polymerase (S282T and M423I), and NS5A protein (Y93H). Substitutions were constructed by site-directed mutagenesis of a bicistronic HCV replicon containing both a poliovirus IRES sequence at the 5′ end to increase luciferase translation and RNA replication and cell culture-adaptive substitutions in NS3 (E1202G and T1280I) and NS4B (K1846T) (38). *In vitro*-transcribed RNA from the mutated plasmids was used in transient-transfection assays of Huh 7 cells. Five reference compounds (BMS-790052 [NS5A], VX-222 [NS5B], telaprevir [NS3], BILN-2061[NS3], and 2′-MeC [NS5B]) also were included. Briefly, Huh7 cells were transfected with either the wild-type or the mutant RNA constructs by electroporation and subsequently seeded into 96-well black tissue culture plates at a cell culture density of 2.0×10^4 cells per well in a volume of 100 μl. Reference compounds and miravirsen were tested in 9-point, half-log (3.2-fold) serial dilutions. Luciferase activity was measured 72 h after compound treatment, and EC_{50}s were determined from dose-response curves. Fold resistance was expressed as the ratio of the EC_{50} for mutant HCV replicon to the EC_{50} for the wild-type HCV replicon.

***In vitro* selection studies.** The HCV reporter cell line Huh-luc/neo-ET was serially passaged in the presence of 750 μg/ml G418 alone or G418 with fixed and escalating concentrations of miravirsen, SPC4729, or telaprevir (positive control) for time periods of up to 148 days (see Fig. 3). Concentrations ranged from 1.00 to 80 μM (2- to 160-fold the mean miravirsen EC_{50} based on three independent determinations), 1.00 to 80 μM, and 0.60 to 6 μM (2- to 20-fold the telaprevir EC_{50}) for miravirsen, SPC4729, and telaprevir, respectively. Antiviral assays on passaged cultures were performed as described above using the luciferase assay or by quantitative reverse transcription-PCR (RT-PCR). Sequence analysis was performed on stored samples from G418 control-, miravirsen-, or SPC4729-passaged cells at days 39, 72, 117, 128, and 148 of the serial passage study. Amplification and sequence analysis of miR-122 binding sites (S1, S2, and S3) in the HCV 5′- and 3′UTRs was performed initially by site-specific primed RT-PCR followed by population-based sequencing. Briefly, total RNA was extracted and purified from the frozen cell pellets of cultured Huh-luc/neo-ET cells using RNeasy minikit spin columns and reagents (Qiagen, Valencia, CA) according to the manufacturer's recommended procedure. Following reverse transcription, binding sites (S1, S2, and S3, corresponding to HCV nucleotides 22 to 44 in the 5′UTR and 9387 to 9399 in the 3′UTR), were amplified by PCR (expand high fidelity; Roche, Almere, Netherlands), and S1, S2, and S3 nucleotide sequences were determined using the BigDye Terminator v1.1 cycle sequencing kit (ABI) using a cutoff of 25% for base calling of minority bases. 5′RACE (rapid amplification of cDNA ends) and sequencing analysis of the full-target HCV 5′UTR was performed by reverse transcription with a gene-specific primer located in the 5′UTR (*in vitro* samples) or in the HCV core region (clinical samples) (5′RACE system; Invitrogen). Sequence analysis was performed as described above. The LBCM4279 HCV isolate (genotype 1; GenBank accession number HM043170) was used as a reference sequence for 5′UTR analysis. Data from HCV nucleotides 1 to 69 (*in vitro* samples) or 1 to 341 (clinical samples) (the 5′UTR) are reported and comprise the S1 (nucleotides 23 to 28) and S2 (nucleotides 38 to 43) binding sites.

Sequence analysis of HCV clinical isolates. The selection and treatment of human subjects has been described previously (33). Briefly, a total of 36 subjects with chronic HCV genotype 1 infection were randomly assigned to receive five weekly subcutaneous injections of miravirsen at doses of 3 mg, 5 mg, or 7 mg per kilogram of body weight or placebo over a 29-day period. Subjects were monitored (HCV RNA levels and safety information) for 18 weeks after receiving the first dose of miravirsen. Amplification and sequence analysis of the entire 5′UTR (nucleotides 1 to 341) were performed by 5′ RACE as described above on representative samples from six subjects who had experienced virologic rebound (defined by an equal-to-or-greater-than 1 $\log_{10}$ increase in HCV RNA over the nadir) and from control samples derived from seven subjects who did not experience virologic rebound following a >1 $\log_{10}$ initial decrease in HCV RNA, had a <1 $\log_{10}$ initial decline in HCV RNA, or were treated with placebo. Sequence analysis for S1 and S2 binding sites has been reported previously (33). The study was approved by the institutional review board or ethics committee at each participating center and was conducted in accordance with the Declaration of Helsinki, good clinical practice guidelines, and local regulations. All patients provided written informed consent before enrollment into the study.

Construction and characterization of HCV mutant replicons. The single-site mutations C3U and A4C in the HCV 5′UTR were engineered into plasmid $pFKI_{341}PI$-luc_NS3-3′/ET (obtained from Ralf Bartenschlager) using a Stratagene QuikChange XL II mutagenesis kit (Agilent, La Jolla, CA) according to the manufacturer's recommended procedure. The bicistronic parental plasmid encodes the cell culture-adapted HCV replicon with the ET substitutions and contains the poliovirus IRES in the 5′ end to increase luciferase expression and the EMCV IRES-driven NS3-NS5B HCV coding sequences (38). The presence of the mutations in the plasmid was verified by DNA sequence analysis.

In vitro-transcribed replicon RNA was prepared by runoff transcription of *ScaI*-HF (New England BioLabs, Ipswich, MA) restriction enzyme-digested plasmids using MegaScript T7 RNA reagents (Life Technologies, Carlsbad, CA) according to the manufacturer's recommended procedure and quantified by absorbance at 260 nm.

In vitro-transcribed RNA was electroporated into Huh-cure cells (obtained from Ralf Bartenschlager) suspended to a concentration of 1×10^7 cells/ml in cytomix medium (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4-KH_2PO_4, 25 mM HEPES, 2 mM EGTA, 5 mM $MgCl_2$, and freshly added 2 mM ATP and 5 mM glutathione; pH 7.6). Four hundred μl of Huh-cure cells suspended in cytomix medium was mixed with 5 μg of *in vitro*-transcribed HCV replicon RNA and 5 μg of carrier RNA (Ambion [Life Technologies], Carlsbad, CA) and electroporated according to standard methods using a 0.4-mm-gap cuvette (Fisher Scientific, Pittsburgh, PA) and a Bio-Rad Gene Pulser II (Bio-Rad, Hercules, CA). The cells were adjusted to a cell density of 7.5×10^4 cells per ml culture medium and seeded in 96-well tissue culture plates at 7.5×10^3 cells per well in a volume of 100 μl for parallel determinations of EC_{50} and CC_{50} values. Following 24 h of incubation, the medium was replaced with 100 μl medium containing six or nine serial half-log dilutions of test or control compound in triplicate. Six additional wells in each plate received cell culture medium as a no-treatment control. Following 96 h of incubation, cells were assessed for cytotoxicity by XTT staining and anti-HCV activity by measurement of luciferase reporter activity. Data were transformed to present the HCV replicon levels in the treated samples as a percentage of the untreated samples, and EC_{50}s were determined by nonlinear regression from these dose-response curves. GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA) was used for the nonlinear regression analysis. Fold resistance was expressed as the ratio of the EC_{50} for mutant HCV replicon to the EC_{50} for the wild-type HCV replicon. Transfection efficiency was determined by luciferase activity in parallel wells 4 h after electroporation. The replication capacity (fitness) of the C3U and A4C HCV replicon sequences was evaluated by measuring the luciferase reporter signal at 4 h and 120 h after electroporation in cells cultured in the absence of compound (39). The 4-h time point represents the transfection efficiency

and was used as a normalization control for the calculation of viral replication capacity (RC) at 120 h. The relative replication capacity was calculated from the ratio of luciferase activity at 120 h to the 4-h luciferase activity using a formula where 120 and 4 h are the times at which the relative replication capacity is evaluated for mutant (mut) and wild-type viruses (wt): $RC = 100 \times [(luc_{120\ h\ mut}/luc_{4\ h\ mut})/(luc_{120\ h\ wt}/luc_{4\ h\ wt})]$.

RESULTS

***In vitro* antiviral activity and cytotoxicity of miravirsen.** Miravirsen sodium (miravirsen) is a novel 15-base oligonucleotide, comprised of 8 β-D-oxy-LNA and 7 DNA monomers and arranged in the sequence depicted in Fig. 1. All studies with miravirsen were performed without the use of a transfection agent (gymnosis) to simulate *in vivo* administration. Miravirsen demonstrated robust antiviral activity against HCV genotype 1b, with a mean EC_{50} of 0.67 μM (standard deviation, ±0.33; range, 0.40 to 1.08 μM) and mean EC_{90} of 5.40 μM (standard deviation, ±3.3; range, 3.71 to 9.15 μM). A negative-control oligonucleotide (SPC4729) (Fig. 1) was inactive up to the highest concentration tested (320 μM) under the same assay conditions. The cytotoxicity of miravirsen was determined by the XTT assay in the HCV replicon cells, as well as with various human cell lines, including Huh-7, TK 10, and HepG2 cell lines, and in primary hepatocytes, stimulated and unstimulated peripheral blood mononuclear cells (PBMCs), macrophages, and human bone marrow cells following 2 to 14 days of incubation. No cytotoxicity of miravirsen was observed up to the highest concentration tested (320 μM) in any cell culture, indicating an *in vitro* therapeutic index (TI) of >297, based on the high-end-of-the-range EC_{50} measurement of 1.08 μM. Measurements of the antiviral activity of miravirsen in the presence of human serum and serum proteins (40% human serum, 45 mg/ml human serum albumin, or 1 mg/ml alpha-1 acid glycoprotein) demonstrated no interference with the antiviral activity of miravirsen (data not shown).

***In vitro* antiviral activity of miravirsen in combination with other anti-HCV therapeutics.** Based on its unique mode of action, miravirsen was predicted not to interfere with the antiviral activity of other anti-HCV agents. To confirm this, the activity of miravirsen was evaluated when combined with other anti-HCV agents. Antiviral activity and cytotoxicity resulting from two-drug combinations of miravirsen with IFN-α2b, RBV, inhibitors of NS5B polymerase active and allosteric sites (2′-MeC and VX-222, respectively), NS3 protease (telaprevir), or NS5A (BMS-790052) were analyzed by the method of Prichard and Shipman, Jr. (36), as described in Materials and Methods. Briefly, the measured antiviral effects from each drug combination were subtracted from the antiviral effects expected if the drug interactions occurred independently (i.e., were additive). This difference then was portrayed as a three-dimensional surface-expressed dose response as a volume above (synergy) or below (antagonism) a horizontal plane which represented the level of inhibition if the drug combination was merely additive. As shown in Table 1, all two-drug combinations of miravirsen with other compounds produced total volumes calculated at the 95% confidence interval of less than 50 $\mu M^2\%$, indicating additive activity. The representation of these volumes as a three-dimensional dose-response surface above or below the horizontal plane representing the effects of no drug interaction is depicted in Fig. 2. In all experiments, no cytotoxicity was observed with any drug alone or in combination (data not shown).

TABLE 1 *In vitro* antiviral activity of miravirsen in combination with other HCV antivirals

Second drug[a]	Volume[b] ($\mu M^2\%$)		Interaction
	Synergy	Antagonism	
IFN-α2b	10.8; 10.3	−38.2; 0	Additive
Ribavirin	4.2 ± 11.7	−24.2 ± 138.2	Additive
VX-222	4.8; 0.8	−34.44; −4.27	Additive
BMS-790052	0; 18.5	−24.5; −0.1	Additive
Telaprevir	0 ± 3.1	−3.2 ± 6.9	Additive
2′-MeC	0 ± 1.3	−27.6 ± 38.0	Additive

[a] Five reference compounds, BMS-790052 (NS5A protein inhibitor), VX-222 (nonnucleoside NS5B inhibitor), telaprevir (NS3 protease inhibitor), BILN-2061(NS3 protease inhibitor), and 2′-MeC (nucleoside NS5B inhibitor), were studied.
[b] Volume of synergy or antagonism ($\mu M^2\%$) was calculated according to the method of Prichard and Shipman, Jr. (36), at the 95% confidence interval; results represent the medians (± standard deviations) (≥3 experiments) or individual values (1 or 2 experiments).

***In vitro* antiviral activity against drug-resistant HCV replicons.** *In vitro* experiments were conducted to evaluate the activity of miravirsen against HCV replicons constructed to contain substitutions that confer resistance to various DAA classes. Specifically, miravirsen was evaluated against HCV genotype 1b replicons containing key amino acid substitutions, rendering them resistant to the NS3 protease inhibitor telaprevir (A156T and R155K), the NS5B polymerase inhibitors 2′-MeC (S282T) and VX-222 (M423I), and the NS5A protein inhibitor BMS-790052 (Y93H). Each DAA was included as a positive control, and HCV genotype 1b replicon constructs were introduced into Huh-7 cells by transient transfection. In these assays, miravirsen demonstrated broad antiviral activity against all drug-resistant HCV variants tested with fold changes in susceptibility of less than 2-fold. In contrast, HCV replicons constructed to contain specific mutations demonstrated resistance (fold changes in susceptibility ranging from 4.4 to 45.8) distinct for each drug class tested (Table 2).

***In vitro* resistance studies. (i) Passage history, colony formation, and susceptibility testing.** To evaluate the ability of HCV to develop resistance to miravirsen when subjected to drug pressure, a series of *in vitro* serial passage studies was conducted. In this study, Huh-7 cells harboring HCV genotype 1b replicons were cultured in the presence of fixed or escalating concentrations of miravirsen for time periods of up to 148 days (Fig. 3). Concentrations ranged from 1.00 to 80 μM (approximately 2- to 160-fold the average miravirsen EC_{50} of 0.5 μM calculated at the onset of these experiments). The NS3/4a protease inhibitor telaprevir was used as a positive control. Negative controls included SPC4729 and medium containing G418 alone. Colony formation, susceptibility assays, and sequence analyses were performed on passaged cells throughout the study.

The presence of miravirsen resulted in a decrease in the rate of cell expansion and a reduction in HCV RNA without the emergence of distinct clonal populations following the initial 25 days of passage at concentrations of up to 20-fold the average EC_{50} (10 μM) (Fig. 4). In contrast, selection in the presence of telaprevir resulted in a decrease in the rate of cell expansion with the appearance of distinct individual resistant clonal populations (Fig. 4). Subsequently, miravirsen- and SPC4729-treated cells were cultured for an additional 10 days (35 cumulative days from the initiation of study), and antiviral activity was evaluated in each of these cell cultures. Miravirsen demonstrated comparable anti-

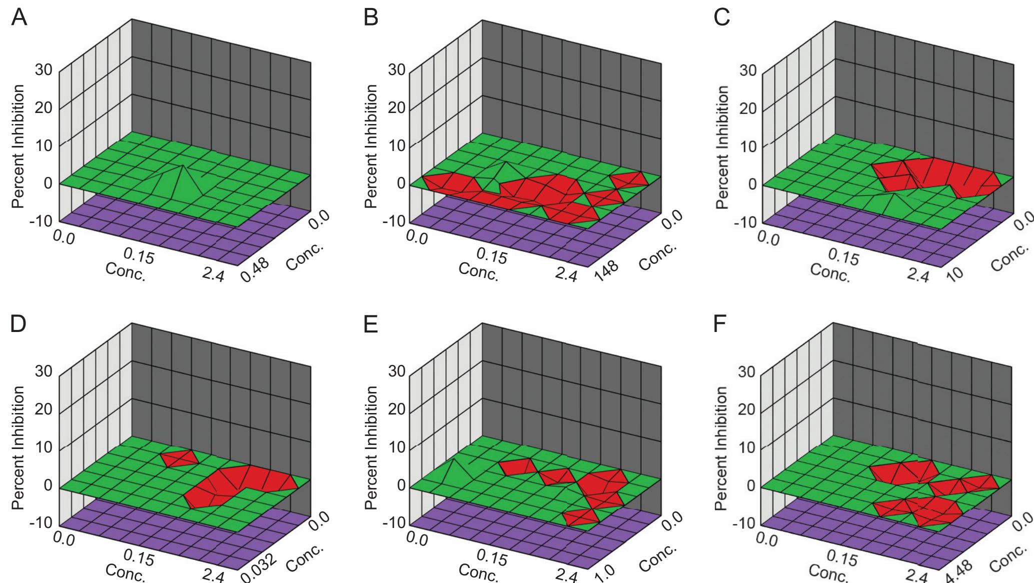

FIG 2 Analysis of the interaction of miravirsen with IFN-α2b (A), RBV (B), VX-222 (C), BMS-790052 (D), telaprevir (E), and 2′-MeC (F) by the method of Prichard and Shipman, Jr., using Macsynergy II, v1.0 (36). Eight successive 2-fold dilutions of miravirsen were evaluated alone or in all possible combinations with five successive 2- or 5-fold serial dilutions of a second compound. Calculated independent effects were subtracted from the observed combined effects. Volumes with positive values at the 95% confidence interval indicate synergy, while volumes with negative values indicate antagonism. Data are from one representative experiment. Conc., concentration.

HCV activity against cells passaged in the presence or absence of miravirsen, with EC_{50}s of 1.79 and 0.79 μM, respectively (data not shown), confirming the lack of measurable resistance and consistent with the lack of the emergence of distinct clonal populations seen at the earlier time point of 25 days of passage.

To continue to select for potential resistance, HCV replicon cells that had been treated with 10 μM miravirsen, 10 μM SPC4739, or G418 alone for 39 days were passaged for an additional 33 days (72 cumulative days from the initiation of study) in 2-fold increasing concentrations of miravirsen or SPC4729, up to a maximal concentration of 80 μM (160-fold the average miravirsen EC_{50}), and reevaluated for colony formation. During the conduct of these studies, cells passaged in increasing concentrations of miravirsen demonstrated a decrease in cell expansion but were never completely eliminated; the remaining cells existed as a thin patchy monolayer but not as distinct colonies (data not shown).

Since experimental conditions did not lead to the complete clearance of the HCV replicon from cells or result in the emergence of distinct resistant colonies, HCV replicon cell cultures were passaged with miravirsen at concentrations of 40 μM and 80 μM (80- and 160-fold the miravirsen EC_{50}, respectively) for an additional 27 days (99 cumulative days from the initiation of study). Distinct colonies still were not observed for cells passaged in 40 μM miravirsen, but small patches of viable cells were observed for cells passaged in 80 μM miravirsen (160-fold the EC_{50}) (data not shown). The latter cells were further cultured for 29 days in the presence of 80 μM miravirsen (128 cumulative days from the initiation of the study), and four individual HCV replicon cell clones were harvested and expanded for an additional 20 days (148 cumulative days).

TABLE 2 Antiviral susceptibilities of HCV genotype 1b replicons containing mutations in NS3, NS5B, and NS5A proteins

	Fold change[a]				
Compound	NS3 A156T	NS3 R155K	NS5B S282T	NS5B M423I	NS5A Y93H
Miravirsen	1.0	1.2	1.9	1.7	0.9
Telaprevir	36.1	4.6	1.0	NT	NT
2′-MeC	0.9	0.8	45.8	1.0	1.0
VX-222	NT	NT	NT	4.4	NT
BMS-790052	NT	NT	NT	NT	29.9

[a] Fold change was expressed as the ratio of the EC_{50} for the mutant HCV replicon to the EC_{50} for the wild-type HCV replicon. Ratios were derived from the mean EC_{50} from two experiments. NT, not tested.

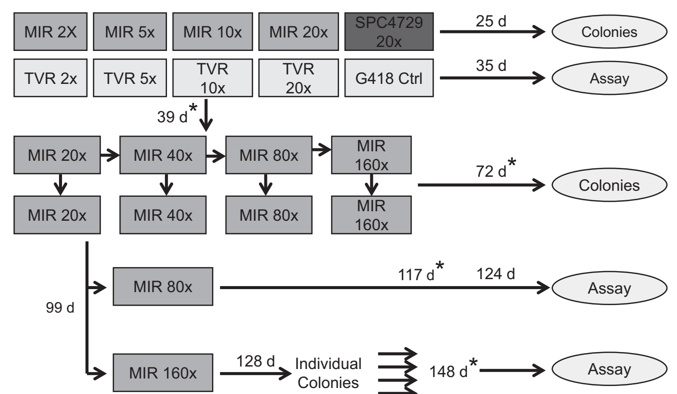

FIG 3 HCV replicon genotype 1b cells were serially passaged in the presence of G418 alone (ctrl) or G418 with miravirsen (MIR), SPC4729 (oligonucleotide negative control), or telaprevir (TVR) for time periods of up to 35 days (TVR) or 148 days (MIR, SPC4729, or G418 alone) in fixed or escalating concentrations at a multiple (x) of the EC_{50} concentrations of MIR (for MIR and SPC4729) or TVR. Colony formation, drug susceptibility assays, and nucleotide changes (*) were assessed throughout the study (d, days).

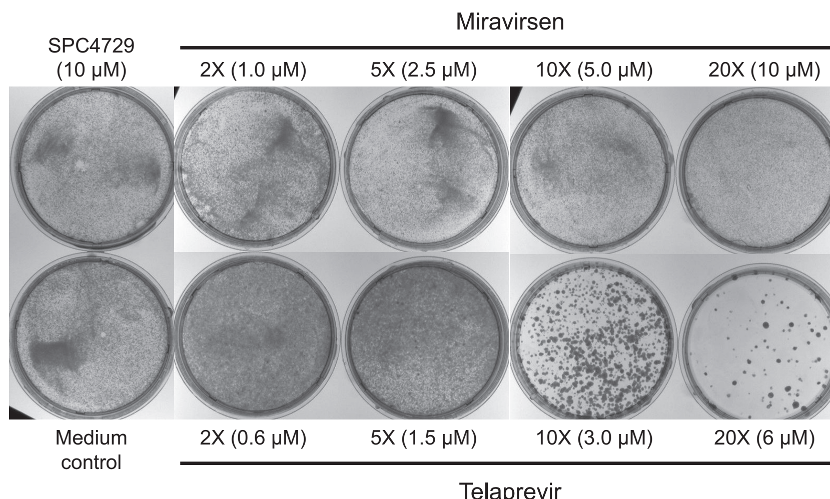

FIG 4 HCV replicon genotype 1b cells were passaged in the presence of G418 alone (medium control) or G418 with miravirsen, SPC4729 (oligonucleotide negative control), or telaprevir for 28 days in fixed concentrations at a multiple (X) of the EC_{50} of miravirsen or telaprevir. Colony formation was assessed by staining surviving cells with crystal violet.

Antiviral activity subsequently was evaluated for cells passaged in the presence of 40 μM miravirsen (80-fold the EC_{50}), 80 μM SPC4729, or G418 alone for 124 days and cells from clone 1 that had been passaged in the presence of 80 μM miravirsen (160-fold the EC_{50}) for 148 days in an assay using levels of HCV RNA as an endpoint (Fig. 3). In these assays, miravirsen demonstrated comparable anti-HCV activity in SPC4729- and G418-passaged cells, with EC_{50}s of 0.78 μM and 1.80 μM, respectively, but did not inhibit HCV in cells passaged in the presence of 40 μM miravirsen or clone 1 up to the highest concentration tested (320 μM; data not shown).

(ii) Sequence analysis. To evaluate genotypic changes that might be associated with reductions in susceptibility to miravirsen, we next performed sequence analysis on stored samples from G418 control-, miravirsen-, or SPC4729-passaged cells at 39, 72, 117, and 148 days of the serial passage study (Fig. 3 and Table 3). Initially, we amplified and performed population-based sequence analysis of miR-122 binding sites (S1, nucleotides 23 to 28; S2, nucleotides 38 to 43 [28]; and S3, nucleotides 9390 to 9396 [27]) in the HCV 5′- and 3′UTRs by site-specific primed endpoint RT-PCR, followed by population-based sequencing (Materials and Methods). No nucleotide changes of significance (e.g., changes that were observed after passage with miravirsen that had not been seen following passage with SPC4729 or G418 alone) were observed in S1, S2, or S3 binding sites from cells passaged in the presence of fixed or escalating concentrations of miravirsen (data not shown). Since miR-122 has been reported to bind to nucleotides at the extreme end of the 5′UTR (29), we then used 5′RACE to amplify and obtain sequence for the distal end of the 5′UTR, including the first 20 nucleotides that had been unavailable using the standard technique (Table 3). Nucleotide changes were identified by the comparison of each sequence to the original replicon cell line. All cell lines, including those passaged in G418 alone or the control oligonucleotide SPC4729, contained a change at position 1 from a G to an A (G1A). HCV replicon cells passaged in the presence of 20 μM (40-fold the EC_{50}) miravirsen for 72 days also contained a nucleotide change from an A to a C at position 4 that was maintained following additional passages but was not detected in cells passaged with SPC4729 or G418 alone. The A4C change was not observed in earlier cells passaged for up to 39 days of treatment with 20-fold the EC_{50} of miravirsen. This is in contrast to the levels observed following serial passage of telaprevir, where genotypic and phenotypic resistance emerged rapidly (Fig. 4 and data not shown) (40).

Clinical studies: sequence analysis. In the previously reported phase 2 proof-of-concept clinical trial of miravirsen monotherapy given to treatment-naive patients with chronic HCV infection, no nucleotide changes in S1, S2, or S3 binding sites were detected in any subject, including those who had experienced virologic rebound (defined by a ≥1 $\log_{10}$ increase in HCV RNA over the nadir) (33). To evaluate potential changes in the entire 5′UTR, including the first 21 nucleotides (nucleotides 1 to 341), we performed 5′RACE on representative samples from six subjects who

TABLE 3 Genotypic analysis of the 5′UTR from HCV replicon cells passaged in fixed and escalating concentrations of miravirsen[a]

Compound for cell passage	No. of days after selection	Concn (μM)	Nucleotide change
Replicon control	0	0	
Miravirsen	39	10	G1G/A
SPC4729	39	10	G1G/A
Miravirsen	72	20	G1A, A4C
Miravirsen	117	40	G1A, A4C
SPC4729	117	80	G1G/A
Miravirsen	148	40	G1A
Miravirsen (clone 1)	148	80	G1A, A4C
Miravirsen (clone 2)	148	80	G1A, A4C
Miravirsen (clone 3)	148	80	G1A, A4C
Miravirsen (clone 4)	148	80	G1A, A4C
SPC4729	148	80	G1A
G418 control	148	0	G1A

[a] Population-based sequence analysis of the 5′UTR (nucleotides 1 to 69) was performed following 5′ RACE of HCV RNA derived from HCV replicons from miravirsen-, G418 control-, or SPC4729-passaged cells at the indicated days after selection and concentrations. Nucleotide changes were identified by comparison of each sequence to a replicon control reference sequence.

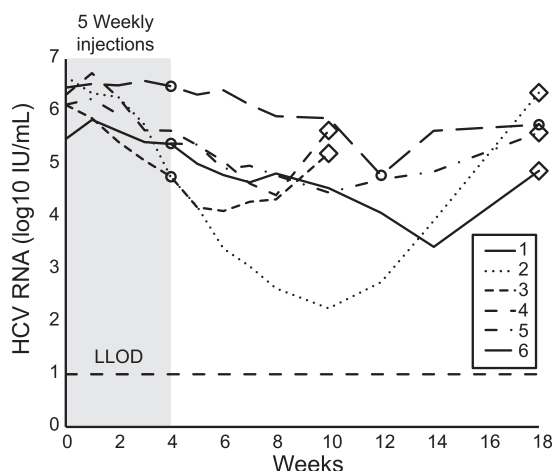

FIG 5 Subjects with chronic HCV genotype 1 infection were randomly assigned to receive five weekly subcutaneous injections of miravirsen at doses of 3 mg, 5 mg, or 7 mg per kilogram of body weight over a 29-day period (gray shading) (33). They were monitored for 18 weeks after randomization. Amplification and sequence analysis of the entire 5′UTR (nucleotides 1 to 341) were performed by 5′ RACE from six subjects (numbered 1 to 6) who experienced virologic rebound. Identification of the C3U nucleotide change (large open triangles) or wild-type sequences at position 3 (large open circles) are indicated. The lower limit of detection (LLOD) was 12 IU (1.08 $\log_{10}$ IU per ml).

had experienced virologic rebound (Fig. 5) and from control samples derived from seven subjects who did not experience virologic rebound following a $>1 \log_{10}$ initial decrease in HCV RNA, had a $<1 \log_{10}$ initial decline in HCV RNA, or were treated with placebo (data not shown). Nucleotide changes were identified by the comparison of each sequence to each subject's matched baseline control. Sequence analysis of the 5′ UTR from subjects who experienced virologic rebound indicated a nucleotide change from a C to a U at position 3 for 5 of the 6 subjects at the time of virologic rebound (Fig. 5). This mutation was not present at earlier time points during initial HCV RNA decline for two of these subjects (Fig. 5), nor was this mutation identified in control subjects (data not shown).

Phenotypic characterization of HCV replicons containing 5′UTR mutations. To characterize the effect of the mutations found either in the *in vitro* selection or in the patient population on the susceptibility of HCV to miravirsen, the single mutations C3U and A4C were engineered into HCV replicons, and *in vitro*-transcribed RNA subsequently was electroporated into Huh-cure cells (see Materials and Methods). We were unsuccessful in generating *in vitro*-transcribed RNA encoding the single mutation G1A or the double mutations G1A/A4C due to the necessity of a G nucleotide at position 1 for optimal transcription by T7 RNA polymerase. Thus, only data for C3U and A4C HCV variants are presented.

We initially evaluated the replication capacity (viral fitness) of HCV variants 120 h following transfection (see Materials and Methods). The relative replication capacity of replicon sequences containing C3U and A4C substitutions in the 5′UTR were 3.0% and 34% of the level of wild-type sequences, respectively, indicating a deleterious effect of the mutations on viral fitness.

We next evaluated the susceptibility of HCV mutant replicons to miravirsen and included telaprevir and SPC4729 as controls. Overall, there was a poor dose response when testing miravirsen against the C3U HCV variant that precluded high-fidelity curve fitting and definitive EC_{50} determinations for all but one of six experiments (Table 4 and data not shown). In that experiment, we measured an EC_{50} of 51 μM, yielding a fold change in susceptibility of 7 compared to the EC_{50} of wild-type virus. For some of the other experiments there was some indication of a dose response at higher concentrations of miravirsen, although in general the inhibition was incomplete, with an overall reduction in susceptibility of the virus to miravirsen. In contrast, the HCV C3U variant remained susceptible to the NS3 protease inhibitor telaprevir with a less than 1-fold change in susceptibility. We also evaluated the susceptibility of the HCV A4C variant to miravirsen; HCV replicons constructed to contain the single A4C mutation were fully susceptible to miravirsen as well as to telaprevir (Table 4).

Cross-resistance studies of HCV replicons containing 5′UTR mutations. As an extension of these studies, we evaluated the susceptibility of HCV C3U and A4C variants to a number of other HCV therapeutic classes, including DAAs, IFN-α2b, and ribavirin (Table 4). Data demonstrate that, as with telaprevir, the C3U replicon was fully susceptible to the NS5B polymerase inhibitors 2′-MeC and VX-222, the NS5A protein inhibitor BMS-790052, IFN-α2b, and RBV.

DISCUSSION

The HCV antiviral drug miravirsen is the first miRNA-targeting antisense agent administered to patients (33). Miravirsen interferes with the functions of miR-122 both in cholesterol homeostasis (20) and in viral proliferation (23). Miravirsen is an oligonucleotide drug, and as such it benefits from the increased nuclease resistance imparted by the LNA-modified sugar residues and the increased protein binding and cellular uptake resulting from the phosphorothioate backbone (41). Miravirsen may be dosed both *in vitro* and *in vivo* without the need for formulation; thus, robust antiviral activity can be achieved *in vitro* by simple supplementation into cells or *in vivo* and in human clinical trials by subcutaneous or intravenous delivery in saline solution. In the current study, we examined the *in vitro* efficacy of miravirsen against wild-type and mutant replicons with known resistance to other DAAs. Consistent with its novel mechanism of action, miravirsen was shown to be broadly active against HCV variants resistant to DAAs. We also demonstrated an additive response when combining miravirsen with these DAAs on wild-type replicons.

TABLE 4 Antiviral susceptibilities of HCV genotype 1b replicons containing mutations in the 5′UTR

	Fold change[a]	
Compound	A4C mutation	C3U mutation
Miravirsen	1	7
Telaprevir	2	1
2′-MeC	1	1
VX-222	NT	1
BMS-790052	NT	1
IFN-α2b	2	0.3
Ribavirin	NT	1

[a] Fold change was expressed as the ratio of the EC_{50} for the mutant HCV replicon to the EC_{50} for the wild-type HCV replicon. Ratios were derived from the individual EC_{50} determinations or from the mean EC_{50} from two or more experiments; the ranges, in fold changes, for telaprevir were 0.3 to 1.0 and for 2′-MeC were 0.4 to 0.8. NT, not tested.

In studies published to date on a total of 82 healthy volunteers and 37 patients with chronic HCV infection, with miravirsen dosing up to 12 weeks, there were no dose-limiting toxicities and subject discontinuations due to adverse events (33). Doses that are associated with beneficial effects (decreases in HCV RNA and decreases in alanine aminotransferase, or ALT) and pharmacodynamic effects (decreases in cholesterol) have been well tolerated. Consistent with these findings, we found little to no *in vitro* cytotoxicity up to the highest concentration tested (320 μM) when evaluated in a variety of different primary cells and cell lines.

We also examined the propensity of HCV to develop resistance to miravirsen *in vitro* following serial passage of HCV replicons in the presence of up to 80 μM (160-fold the EC_{50}) miravirsen for time periods of up to 148 days and compared our findings to that observed in subjects experiencing HCV RNA rebound in a phase 2 miravirsen clinical trial (33). We had originally performed RT-PCR analysis on S1, S2, and S3 binding sites in the HCV 5′- and 3′UTRs and found no genotypic changes in subjects who experienced viral rebound (33). We were unable, however, to derive sequence from the first 20 nucleotides of the 5′UTR, as the forward primer used in the PCR amplification annealed to the first 20 nucleotides. In this study, we used a specialized method (5′RACE) to derive sequence from the distal end of the 5′UTR, including these first 20 nucleotides. Using this method, we were able to verify that no nucleotide changes occurred in or around the miR-122 binding sites in the 5′UTR from nucleotide 1 to 69 up to 39 days of study (before 72 days of study). As would be expected, no changes in susceptibility to miravirsen or other signs of phenotypic resistance were observed during this period of time. This failure to generate a resistant cell population early in the study is in contrast to the genetic resistance to most DAAs that typically emerges rapidly (42), an observation confirmed in our *in vitro* study with the DAA telaprevir. The rapid emergence of resistance to DAAs that interact directly with a viral protein that is critical for viral replication can be explained by the fact that changes in a single amino acid residue often are sufficient to reduce the binding affinity of the DAA for its target significantly, thereby reducing the efficacy of these compounds. Therefore, the ease with which resistance occurs is due in part to the rapid replication rate of HCV combined with the low fidelity of its polymerase, which gives rise to mutations throughout the viral genome, resulting in extensive sequence variation in the HCV population (42). In contrast, the binding of miravirsen to miR-122 (a host factor) could not be directly affected by the genetic variability of the viral genomes. However, as discussed below, as with all HTAs, it is possible that other mechanisms for viral escape and/or viral resistance to miravirsen can be found (43, 44). Short-term exposure to miravirsen did not lead to any observed genetic or phenotypic changes in the replicon cells; however, a small fraction of the cell population proliferated slowly in the presence of the drug. When these cells were allowed to expand and the replicon contained therein was analyzed for sequence changes, a change at nucleotide 4 (A4C) was identified. In addition to the A4C change, we also detected a specific change at G1A in all isolates carrying the A4C change. However, this change also was observed in control samples in the absence of the A4C change, suggesting that this change did not necessarily occur as a result of selection in the presence of miravirsen.

Interestingly, although it could be expected from the observed changes in cell proliferation that the change in the replicon would reduce the susceptibility to miravirsen, when the A4C mutation was reintroduced by itself into a naive replicon and transfected into Huh-7-cells, there was no appreciable change in the susceptibility of the reengineered replicon to miravirsen relative to the wild-type replicon. Given that the A4C change was seen only in the presence of the G1A change, multiple attempts were made to recreate the double mutant but were unsuccessful. It is possible that resistance to miravirsen in the replicon cells is dependent on both mutations. Alternatively, it is possible that the resistance mechanism that acts through the A4C mutation also requires the selection of an altered interaction with a host factor in the original passaged replicon cell line. The alteration to the host cell factor would not be observed in the cell line into which the reengineered replicon was introduced.

The position of the mutation identified in clinical samples (C3U) is in a region of the 5′UTR that is not detected by the standard sequencing method initially used during the clinical trials of miravirsen. It is somewhat surprising that the genetic change observed (C3U) from the clinical trial was different from the change observed *in vitro* (A4C). The C3U change was observed with near-perfect correlation with the rebound of HCV RNA, and unlike the A4C change, when reengineered into the replicon, it was associated with a reduction in susceptibility to miravirsen.

Although the genetic changes identified differ between the *in vivo* and the *in vitro* samples, it is tempting to speculate that the underlying mechanism that led to their selection is the same, based on the near proximity of the C3U and A4C mutations. Given the requirement for the interaction between miR-122 and the HCV genome and the ability of miravirsen to interfere with this interaction, a number of models can be proposed to explain the apparent resistance. One possibility is that the genetic changes render the virus independent of miR-122 and insensitive to miravirsen. In this model, one could envision either a change of preference to a different miRNA or simply that the function of the interaction between miR-122 and the viral RNA has been replaced by an intramolecular interaction within the viral 5′UTR. This model seems unlikely, given that the selective pressure for this adaptation presumably also would be present in the absence of miravirsen. Structure modeling (data not shown) of the viral 5′UTR with or without these genetic changes also suggests that the structural changes that can be predicted would be minor and seemingly unlikely to explain resistance simply from an altered three-dimensional structure of the 5′UTR.

A different model suggests that the genetic changes observed allow for a functional interaction between miR-122 and the viral RNA, even in the presence of miravirsen, as has been proposed for maraviroc, an inhibitor of human immunodeficiency virus through its interaction with the host target receptor CCR5 (44). It remains difficult, however, to understand how the interaction between miR-122 and the replicon through simple Watson-Crick base pairing would be favored over the higher-affinity interaction between the LNA-based miravirsen and miR-122.

Ultimately, for replicons carrying either of these specific mutations, we observed a noticeable decrease in their fitness, demonstrating that these genetic changes interfere with a critical mechanism in the replication cycle in the replicon. These low fitness levels are consistent with the poor growth of the mutant replicons *in vitro* and may be associated with the late appearance of mutations in subjects that occur after the completion of therapy. In addition, there is little evidence that either of these variations are

common in naturally infected individuals, suggesting that the reduced fitness observed *in vitro* reflects a reduction in fitness in normally infected liver cells.

The mechanism of resistance remains to be determined. However, these data show that the resistance to miravirsen is late to emerge and generally occurs after the completion of therapy, reinforcing the observation that miravirsen, as a drug, has a relatively higher barrier to resistance than most DAAs.

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