MAJOR ARTICLE

Evaluation of ITX 5061, a Scavenger Receptor B1 Antagonist: Resistance Selection and Activity in Combination With Other Hepatitis C Virus Antivirals

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ITX 5061 is a scavenger receptor B1 antagonist that has entered phase 1 clinical trials in hepatitis C virus (HCV)–infected humans. We evaluated ITX 5061 in combination with interferon-α, ribavirin, and HCV protease and polymerase inhibitors in a genotype 2a infectious virus system. ITX 5061 is a potent inhibitor of HCV replication and is additive to synergistic with interferon-α, ribavirin, BILN2061, VX950, VX1, and 2′-C-methyladenosine. Resistance selection experiments were performed using a Jc1-FEO virus co-culture system and intermittent ITX 5061 exposure under neomycin selection. We identified a mutant virus with a substitution of aspartic acid for asparagine at the highly conserved position 415 in E2 (N415D). Introduction of this mutation into wild-type virus conferred high-level resistance to ITX 5061. There was no cross-resistance between ITX 5061 and HCV protease inhibitors or interferon-α. These results suggest that ITX 5061 is a promising compound for study in combination with other HCV inhibitors.

Hepatitis C virus (HCV) infects >170 million persons and is a major cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Current therapy for HCV, a combination of pegylated interferon, ribavirin, and an HCV protease inhibitor, results in sustained responses in 60%–80% of those treated and is accompanied by significant toxicity. These limitations have spurred intense drug discovery activities to increase response rates and to reduce toxicity. This has resulted in the emergence of a number of promising compounds [1].

For unknown reasons, HCV derived from the plasma of infected individuals replicates poorly in cultured cells. Subgenomic HCV replicons have supported the study of nonstructural elements of the HCV replication complex but not cellular entry. In a significant breakthrough, the HCV isolate termed Japanese fulminant hepatitis 1 (JFH-1), genotype 2a, was found to replicate efficiently in cell culture. This allows the study of viral entry after the generation of high-titer infectious virus stocks when introduced into highly permissive cells, such as the human hepatoma cell line Huh7.5.1 [2–5].

Currently, 4 host proteins, CD81 [6], scavenger receptor B1 (SR-B1) [7], claudin-1 (CLDN1) [8], and occludin [9, 10], have been found to be essential receptors for HCV entry. SR-B1 is a 509–amino acid protein with 2 transmembrane domains and intracellular N and C termini. It was initially identified as the major physiological receptor for high-density lipoprotein (HDL) in the liver [11, 12]. Numerous studies have established its role in HCV entry [13, 14]. ITX 5061 is a clinical-stage, small-molecule compound that has been shown in animal and human studies to increase

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HDL levels by inhibiting the SR-B1 protein pathway [15]. It has also been shown to inhibit HCV entry into hepatocytes in vitro [16]. Resistance selection with ITX 5061 has not been reported to date. The previously described cell culture–associated variant in E2, G451R, is known to possess an increased affinity for CD81 and has been shown to confer resistance to ITX 5061 in vitro, with an approximate 50-fold increase in the 50% effective concentration (EC_{50}) [17, 18].

Most small-molecule HCV inhibitors currently in development target nonstructural proteins essential for viral replication (eg, NS3 protease and NS5B polymerase). Error-prone replication and high rates of viral turnover in infected individuals will necessitate multitarget therapy using compounds with discrete resistance profiles [19]. Compounds targeting HCV entry offer a unique mechanism of action and would not be expected to have cross-resistance with other inhibitor classes. This approach has been validated for other chronic viral infections with high rates of resistance development, such as the human immunodeficiency virus type 1 (HIV-1) CCR5 antagonist maraviroc [20].

Although the addition of HCV protease inhibitors to pegylated interferon and ribavirin has increased the response rate and decreased the treatment duration for patients with genotypes 1a and 1b HCV infection, further improvement in the response rate and the ability to treat the substantial fraction of patients with contraindications to interferon is dependent on the development of regimens that contain multiple directly acting antiviral agents. Because an entry inhibitor would best be used as a component of a drug cocktail, it is important to determine how a potential entry inhibitor would interact with the other antivirals. In this study, we evaluated the activity of ITX 5061 in combination with the standard of care compounds and HCV polymerase inhibitors. We also identified a mutant Jc1/N415D under ITX 5061 selection and evaluated the entry activity of the mutant Jc1/N415D and another mutant Jc1/G451R reported in previous articles [17, 18].

MATERIALS AND METHODS

Compounds

ITX 5061 is an arylketoamide for which the structure has been described elsewhere [15]. Additional compounds tested include interferon-α (Sigma-Aldrich); ribavirin; the peptidomimetic HCV protease inhibitors BILN 2061, VX-950, and VX1, which is a close structural analogue of VX-950 (Vicki Sato, Vertex Pharmaceuticals); and a nucleoside analogue HCV RNA–dependent RNA polymerase inhibitor (RdRpI), 2′-C-methyladenosine (William Lee, Gilead Sciences) [21].

Cell Culture

Human hepatoma Huh-7.5.1 cells (kindly donated by Francis Chisari, Scripps Research Institute) or Huh7 cells were grown at 37°C and 5% carbon dioxide in DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1 × nonessential amino acid mix, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum.

Virus Plasmids

The plasmid pFL-Jc1 was a kind gift from Dr Charles Rice (The Rockefeller University). The chimeric full-length construct pFL-Jc1 has been described elsewhere [3, 22–24]. The firefly luciferase gene has been inserted to pFL-Jc1 to develop a plasmid pFL-Jc1-luc for use as a reporter of viral replication [25]. Similarly, the pFL-Jc1-FEO plasmid, expressing a luciferase-neomycin phosphotransferase fusion protein, was constructed for use in resistance selection experiments. HCV2aChluc, also a HCV GT2a/2a chimera reporter virus, has been described elsewhere [16, 26]. A single nucleotide change was made in the HCV2aChluc construct to generate the A156S amino acid substitution in the NS3 protein. A 2-step polymerase chain reaction (PCR) mutagenesis was performed using the following mutation primers: 5′-CTCTTCCGAtCAGCTGTGTGCTCTCG-3′ (sense) and 5′-GCACACAGCTGaTCGGAAGAGCCCA-3′ (antisense). Amplification primers designed within NS3 are as follows: 5′-CTTATGCCCAGCAAACACGAGG-3′ (sense) and 5′-ACGACCACCACATCTCCCTG-3′ (antisense). The final approximately 1.2-kb amplification product was cut using *AvrII* and *SpeI* restriction endonucleases, and the desired 239–base pair fragment was ligated into a similarly digested HCV2aChluc plasmid construct. All cloning constructs were confirmed by DNA sequence analysis.

Preparation of Infectious HCV

Wild-type or mutant RNA was transcribed in vitro using the T7 RiboMAX Express Large Scale RNA Production System (Promega). After RNA cleanup (Qiagen RNeasy Mini kit), transfection was performed as described elsewhere [27]. Four hundred microliters of a Huh-7.5.1 or Huh7 cell suspension (10^7 cells/mL) was placed in a 0.4-cm cuvette with 10 μg of RNA and electroporated (Bio-Rad Gene Pulser System) using a single square wave at 260 V and 25-millisecond pulse length. The cells were plated in 15-cm tissue culture dishes (Corning), and supernatant was harvested and concentrated using a centrifugal filter (Amicon 100K; Millipore).

Titration of Infectious HCV

Naive Huh-7.5.1 cells plated into a 96-well plate were infected with serial 10-fold dilutions of the infectious supernatants. The inoculum was incubated with cells overnight at 37°C and replaced with fresh complete media and cultured for an additional 72 hours. The cells were stained using a monoclonal mouse anti-CORE immunoglobulin G (IgG) antibody (MA1-080; Thermo Scientific) at a dilution of 1:300, followed by incubation with a 1:100 dilution of horseradish peroxidase–conjugated polyclonal goat antimouse IgG (12–349; Millipore) for 1 hour at room

temperature. The infectivity titer was calculated from the mean number of foci counted under a fluorescence microscope in the last and second-to-last well of the dilution series that yielded CORE-positive foci [2].

Luciferase Compound Activity Assay

Cell culture and luciferase compound assays were performed as described in detail elsewhere [21, 28–30]. In brief, Huh7.5.1 cells were seeded into 96-well plates at a density of 10 000 cells per well in 100 μL medium. After incubating overnight for attachment, Jc1-luc wild-type or N415D or G451R virus at a multiplicity of infection (MOI) of 0.01 was added to wells with or without compounds at the specified concentrations. All conditions were run in triplicate. After 24 hours, medium was aspirated and replaced with 100 μL of complete medium containing an identical concentration of compound(s), followed by an additional 48-hour incubation. The luciferase assay (Bright-Glo; Promega) was performed according to the manufacturer's instructions, using a microplate luminometer (Veritas microplate luminometer; Turner Biosystems). The relative light units for each condition were reported as the mean plus or minus the standard error of the mean for the 3 wells.

Synergy Testing

The EC_{50} of ITX 5061 and compounds was determined independently and used to set the range of concentrations for synergy experiments.

ITX 5061 was tested in combination with each of the compounds listed above at two 2-fold serial dilutions above and below the EC_{50}. The ratio of the 2 compounds tested remained fixed across the dosing range.

ITX 5061 With Ribavirin or VX950 in the HCV2aChluc system

One hundred microliters of serially diluted compound solutions (100, 30, 10, 3, 1, 0.3, and 0.1 nM) was inoculated onto Huh-7 cells in a 96-well plate. Subsequently, 100 μL of HCV2aChluc virus was added onto the cells (MOI, 0.6). After coincubation for 72 hours, Renilla luciferase expression was determined (Renilla Luciferase Assay; Promega) and reported as relative light units.

In Vitro Selection of ITX 5061-Resistant Mutant

Four hundred microliters of a Huh-7.5.1 cell suspension (10^7 cells/mL) was electroporated with 10 μg of Jc1-FEO RNA. After 4 days, supernatants were collected and the cells were treated with trypsin and counted. Three million transfected cells and 3×10^6 naive cells, pretreated with ITX 5061 at 200 nM ($10 \times EC_{50}$), were seeded into a 15-cm dish, followed by the addition of 7 mL of infectious supernatant. Every 3–4 days the cells were split 1:2 with the addition of naive Huh-5.1 cells and one-third volume of the supernatant from the previous selection dish. At day 21, 0.5 mg/mL of G418 was added to the cell culture dish. After the addition of G418, the cells were split 1:4 after they were 80%–90% confluent. At each split, additional naive Huh7.5.1 cells (3×10^6) were added and co-culture continued for another 21 days with ITX 5061 (200 nM). As previously, at each split, approximately 7 mL of supernatant from the prior selection dish was added to the new co-culture. At each passage and after completion of selection (6 weeks total), aliquots of cells and supernatant were stored at −80°C.

Naive Huh7.5.1 cells were infected with day 42 supernatant, as described elsewhere. After 48 hours, the cells were harvested to extract total RNA using the RNeasy miniKit (Qiagen). The E2 envelope protein coding sequence was amplified by reverse-transcription PCR, using the primers Jc1-E2-1 and Jc1-E2-2 and the LA Taq DNA polymerase (Takara Laboratories). The nucleotide sequences were determined using automated sequencing with the following primers: Jc1-E2-1, 5′-TGCGTGTCCCCGAGGTCATTATAG-3′; Jc1-E2-2, 5′-TCGCAGCGTGCAAGATGACCAG-3′; Jc1-E2-3, 5′-GACATCATTAGCGGGGCTCATTGGG-3′; and Jc1-E2-4, 5′-GCTTCTCTAGTGCTGCTTCGGC-3′.

In Vitro Site-Directed Mutagenesis

Candidate resistance mutations identified on sequencing were introduced into the Jc1-Luc plasmid using in vitro site directed mutagenesis (Quickchange XL; Stratagene). The forward primer 5'-aggcagaaaatccagctcgttgacaccaatggcag-3' and reverse primer 5'-ctgccattggtgtcaacgagctggattttctgcct-3' were used to construct the N415D mutant. The forward primer 5'-cagcttcaactcgtcaagatgtcccgaacgcat-3' and reverse primer 5'-atgcgttcgggacatcttgacgagttgaagctg-3' were used to construct the G451R mutant.

Data Analysis

Determinations of compound interactions were quantified on the basis of the median-effect principle described by Chou and Talalay [31]. Combination indices (CIs) were determined using Calcusyn (Biosoft) for each experiment at the EC_{50}, 75% effective concentration (EC_{75}), and 90% effective concentration (EC_{90}) levels. Five replicates per condition were evaluated. A CI < 0.9 was considered synergistic, CI ≥ 0.9 and ≤ 1.1 was considered additive, and a CI > 1.1 was deemed antagonistic.

RESULTS

Inhibitory Activity of the Compounds Using Jc1-Luc Virus

Huh-7.5.1 cells infected with Jc1-luc virus at an MOI of 0.01 yielded 20 000–40 000 relative light units per 10 000 cells at 72 hours (data not shown). ITX 5061 is a potent inhibitor of Jc1-Luc replication in this system, with an EC_{50} of 20.2 nM. The EC_{50} values for ITX 5061 and each of the companion compounds studied in the Jc1-Luc assay system are listed in Table 1. These results confirm that ITX 5061 is a potent anti-HCV inhibitor, although the absolute EC_{50} values differ depending on assay conditions.

Table 1. Inhibitory Activity and Cytotoxicity of the Compounds on Wild-Type and Mutant Jc1-luc Virus In Vitro

Compounds	EC_{50} ± SEM (nM) WT	N415D	G451R	CC_{50} (μM)
ITX 5061	20.2 ± 1.37	>20 000 (>1000×)	190 ± 44.8 (9.4×)	>100
IFN-α	3.01 ± 0.39 IU/mL	1.62 ± 0.34 IU/mL (0.54×)	2.76 ± 0.50 IU/mL (0.92×)	>100 IU/mL
BILN2061	492 ± 56.7	301 ± 19.6 (0.61×)	427 ± 113 (0.88×)	35
Gilead	252 ± 33.5	232 ± 36.3 (0.92×)	125 ± 30.4 (0.50×)	>100
VX1	88.1 ± 12.7	65.2 ± 2.16 (0.74×)	143 ± 42.9 (1.62×)	100
Anti-CD81	38.0 ± 6.09 ng/mL	27.2 ± 2.90 ng/mL (0.72×)	53.1 ± 1.48 ng/mL (1.40×)	>5 μg/mL

Fold-change compared with wild-type is indicated in parentheses for each compound tested in mutant viruses. EC_{50} is shown as nM, and CC_{50} concentration is shown as μM except where noted.

Abbreviations: CC_{50}, 50% cytotoxicity concentration; EC_{50}, 50% effective concentration; IFN, interferon; SEM, standard error of the mean; WT, wild type.

ITX 5061 Was Additive or Synergistic With Interferon-α, BILN2061, 2′-C- Methyladenosine, and VX1

Results for the synergy experiments are shown in Figure 1. ITX 5061 was not antagonistic with any compound studied. Specifically, ITX 5061 was additive with interferon-α, BILN2061, and 2′-C- methyladenosine at 50% effective dose (ED_{50}), the CIs were 1.00, 0.98, and 1.09, respectively. At 75% effective dose (ED_{75}) and 90% effective dose (ED_{90}), synergy was seen with all compounds tested. Combinations of ITX 5061 and VX1 showed consistent synergy at ED_{50}, ED_{75}, and ED_{90}. Similar results were obtained in the HCV2aChLuc system, in which ITX 5061 also showed consistent synergy with VX-950 (Figure 2). No compound combinations showed cytotoxicity at the highest concentrations used in the synergy studies (data not shown).

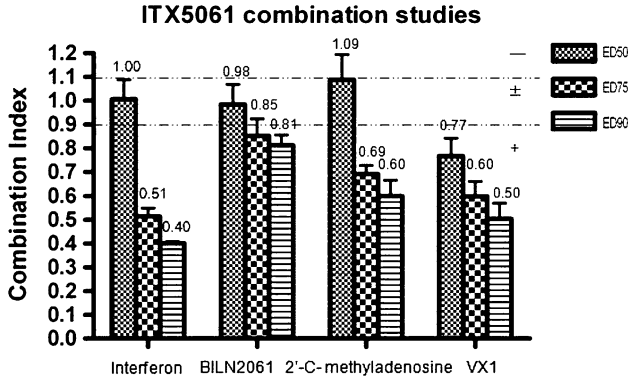

Figure 1. Combination index (CI) of ITX 5061 in combination with various anti–hepatitis C virus (HCV) compounds. Numerical values above the bars are the mean CI. Error bars represent the standard error of the mean of the CI. The 2 lines at 0.9 and 1.1 represent the bounds of an additive interaction. +, synergy; ±, additivity; −, antagonism. The 50% effective dose (ED_{50}), 75% effective dose (ED_{75}), and 90% effective dose (ED_{90}) refer to the combination index at the 50% effective concentration (EC_{50}), 75% effective concentration (EC_{75}), and 90% effective concentration (EC_{90}), respectively, of each compound.

Effects of Combination of ITX 5061 With Ribavirin Is Additive to Moderately Synergistic

Although ribavirin alone does not exhibit strong antiviral activities in vitro, it is a key component of the standard of care in patients. Varying amounts of ITX 5061 were coincubated with a high concentration of ribavirin (15 μM); ribavirin alone did not have significant inhibitory effect on the infectivity of 2a chimeric virus (Figure 3). However, no antagonistic effect was discerned in the combination of ITX 5061 and ribavirin.

E2 Mutant N415D Confers High-Level Resistance to ITX 5061

After a total of 6 weeks of ITX 5061 selection, population sequencing identified a single nucleotide change of A to G at position 1583 of Jc1 in E2 that resulted in an amino acid change of asparagine to aspartic acid at position 415 (N415D; submission ID: 1469400; accession number: JN578258). To verify that the N415D mutation resulted in a resistant phenotype, it was introduced into wild-type Jc1-Luc virus. We then evaluated the ability of Jc1/N415D virus to infect Huh7.5.1 cells in the presence of ITX 5061. The previously described mutant G451R is less dependent on SR-B1 during the entry process and was evaluated in parallel with the N415D mutant [17, 18]. The results showed that Jc1/N415D infection is resistant to SR-B1 blockade by ITX 5061 (Table 1 and Figure 5*A*), with an EC_{50} (>20 μM) that is >1000-fold higher than that for wild-type Jc1 (20 nM). The EC_{50} for ITX 5061 with the G451R virus was 190 nM, representing an approximate 10-fold increase over that for wild-type virus (Table 1 and Figure 5*B*). Inhibitory concentrations for all other compounds tested were similar to those for wild-type virus with the N415D and G451R mutants (fold-change, 0.5–2.0) (Table 1).

N415D Mutant Shows an Increased Sensitivity to CD81 Blockade, Compared With the G451R Mutant

Inhibition of viral infection by anti-CD81 antibody was evaluated for all 3 viruses: wild-type, N415D, and G451R (Table 1 and Figure 5*B*). The EC_{50} and EC_{90} for anti-CD81 with the wild-type and N415D virus were 38.0 ng/mL and 156 ng/mL and

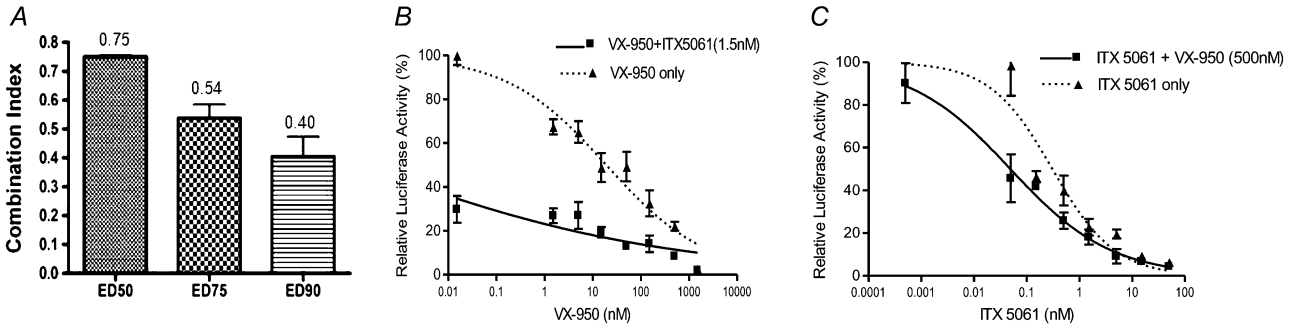

Figure 2. Combination treatment of ITX 5061 with VX-950 (HCV NS3 protease inhibitor). Huh7 cells were infected with HCV2aChluc. *A,* Combination index (CI) of ITX 5061 in combination with VX950. Numerical values above the bars are the mean CI. Error bars represent the standard error of the mean of the CI. The 50% effective dose (ED_{50}), 75% effective dose (ED_{75}), and 90% effective dose (ED_{90}) refer to the combination index at the 50% effective concentration (EC_{50}), 75% effective concentration (EC_{75}), and 90% effective concentration (EC_{90}), respectively. *B,* With various concentrations of VX-950 (indicated on the x-axis) with (*square*) or without ITX 5061 (*triangle*). *C,* With various concentrations of ITX 5061 (indicated on the x-axis) with (*square*) or without VX-950 (*triangle*). Viral replication was measured by luciferase assay 72 hours after infection.

27.2 ng/mL and 170 ng/mL, respectively. Consistent with previous reports [18], the G451R virus was less susceptible to inhibition by anti-CD81 antibody, with an EC_{50} and EC_{90} of 53.1 and 312 ng/mL, respectively.

NS3 Protease Mutant (A156S) Showed High Resistance Level to VX-950 but Not to ITX 5061

Mutations in the alanine at position 156 of the NS3 coding region have been shown to confer resistance to many HCV protease inhibitors, such as SCH-503034 (boceprevir; Schering-Plough), SCH-6 (Schering-Plough), BILN-2061 (Boehringer Ingelheim), and VX-950 (Vertex). Therefore, we introduced A156S mutation in the backbone of HCV2aChLuc genome and investigated the infectivity of the mutant virus in the presence of compounds. As expected, HCV2aChLuc (A156S) showed high-level resistance to VX-950, compared with wild-type HCV2aChLuc. In contrast, no significant difference was observed in the inhibition of infection for both wild-type and the mutant virus by ITX 5061 (see Figure 4), indicating the expected lack of cross-resistance between a protease inhibitor and a viral entry inhibitor.

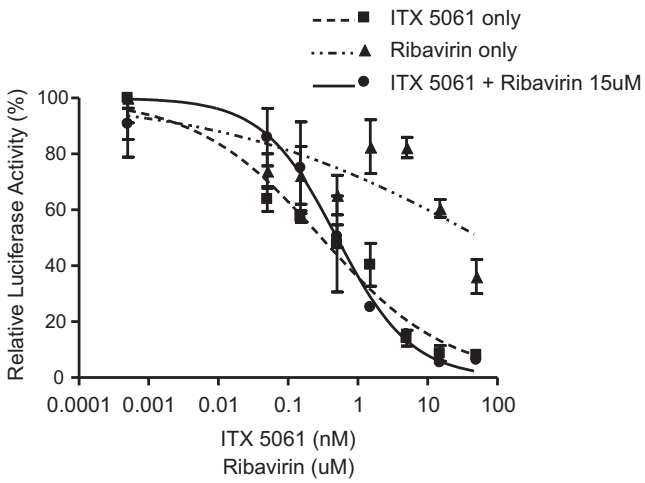

Figure 3. Combination of ITX 5061 with ribavirin. Huh7 cells were infected with HCV2aChluc with various concentrations of ITX 5061 only (*square*) or ribavirin only (*triangle*) and in combination (*circle*). Viral replication was measured by luciferase assay 72 hours after infection.

DISCUSSION

HCV entry is an essential step in the viral life cycle and, thus, a promising target for both preventive and therapeutic chemotherapy. Virus-neutralizing antibodies inhibiting HCV entry have been reported to be associated with viral clearance [32]. Therefore, HCV entry inhibitors have potential clinical applications for both the treatment of chronic hepatitis C and in the prevention of graft reinfection after liver transplantation.

SR-B1 has been found to be an essential receptor for HCV entry. ITX 5061 is a clinical-stage, small-molecule compound that increases HDL levels in animals and patients by inhibiting the SR-B1 protein pathway [15]. We have shown that this compound is also a potent inhibitor of HCV replication in vitro ([16] and this study). In addition, we observed that ITX 5061 was additive to synergistic with several HCV inhibitors representing the standard of care and 2 of the major classes of novel HCV inhibitors currently being explored clinically.

HCV replicates to levels 10–100-fold higher than HIV-1 in chronically infected individuals; combined with an equally error-prone polymerase, the result is the preexistence of drug-resistance mutations in a chronically infected individual [19]. Resistance to most compounds or compound classes has already been described in vitro, and rapid resistance has been seen with monotherapy in vivo [33]. To move beyond interferon-based therapy of HCV infection, combinations of direct acting viral inhibitors with unique resistance profiles will be required.

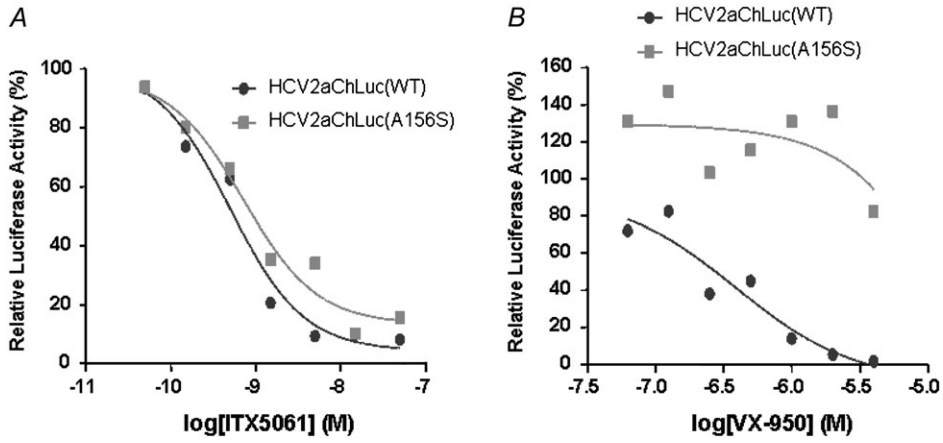

Figure 4. HCV2aChLuc (A156S) protease mutant is resistant to VX-950 but not to ITX 5061. HCV2aChLuc (A156S) mutant virus (*square*) was inoculated onto Huh7 cells in the presence of either ITX 5061(*A*) or VX-950 (*B*) and incubated for 3 days. At 3 days after infection, the cells were lysed and measured by luciferase assay. The same assay was done with the wild type of HCV2aChLuc (*circle*) in parallel.

Combinations of HCV inhibitors that are synergistic or, at a minimum, not antagonistic may produce greater viral load decreases in vivo and could potentially prevent the appearance of multidrug-resistant virus [21]. Although during initial proof-of-concept studies ITX 5061 is being evaluated as monotherapy, its maximal therapeutic value will be in combination with other antivirals.

In resistance selection studies, we identified the N415D mutant, which confers high-level resistance to ITX 5061 in cell culture. Of interest, this mutant was previously described as emerging during long-term passage of HCV strain JFH1-infected cells and lies in a highly conserved region (412–423) of E2 just downstream of HVR1 [34]. Characterization of this mutant was notable for (1) increased affinity for CD81, (2) decreased sensitivity to neutralization by anti–SR-B1, and (3) increased susceptibility to neutralization by serum IgG antibodies from chronically HCV-infected patients [34]. A search of sequences deposited in the Los Alamos National Laboratories HCV database shows high conservation at this position, with only 1 of 1300 HCV sequences having an aspartic acid at position 415. The high conservation of this region of E2, combined with an increased sensitivity to neutralization by antibodies found in persons with chronic HCV infection, suggests that there may be a high barrier to selection of this mutant in vivo. Ongoing studies of ITX 5061 in HCV-infected persons may shed further light on whether this mutant is selected for in vivo with ITX 5061 exposure.

As expected, the N415D mutant remains sensitive to interferon-α and protease and polymerase inhibitors. The G451R mutant also has a reduced sensitivity to ITX 5061 but

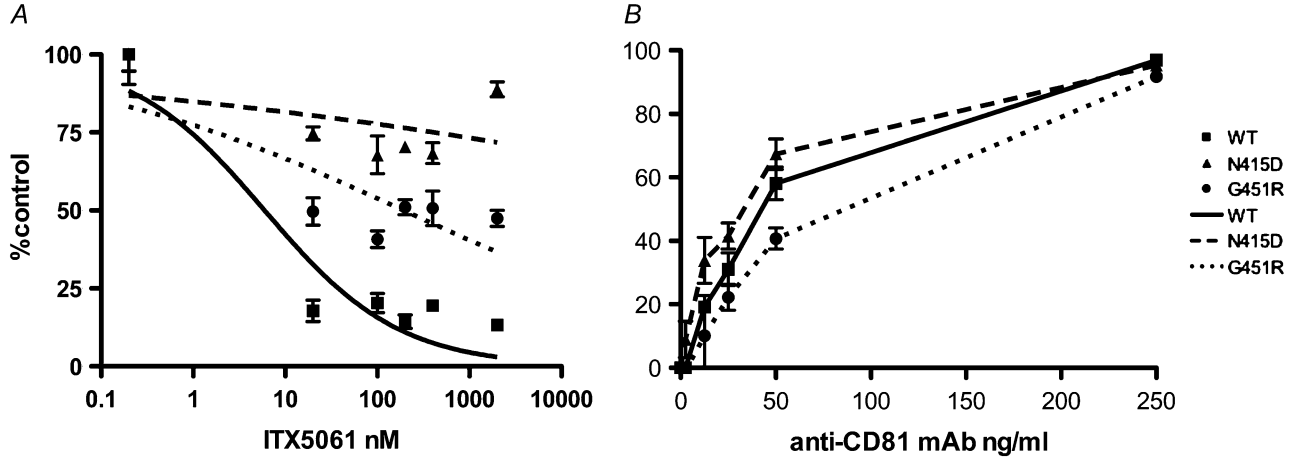

Figure 5. Activity of ITX 5061 and anti-CD81 antibody on wild-type and mutant Jc1-luc in vitro. Wild-type Jc1-luc (*square*), Jc1/N415D mutant virus (*triangle*), and Jc1/G451R mutant virus (*circle*) were inoculated onto Huh7.5.1 cells in the presence of ITX 5061 or anti-CD81 antibody and incubated for 3 days. Luciferase expression was assessed 3 days after infection.

displays a decreased sensitivity to inhibition by anti-CD81 antibody. The differing sensitivity of these 2 mutants to inhibition by anti-CD81 suggests that a different mechanism of resistance may be present. On the basis of these results, a clinical assessment of ITX 5061 in combination with other directly acting HCV inhibitors is warranted, provided that initial clinical trials validate its antiviral activity in humans.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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