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Resistance Profile of a Hepatitis C Virus RNA-Dependent RNA Polymerase Benzothiadiazine Inhibitor

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Recently, a benzo-1,2,4-thiadiazine antiviral agent ($C_{21}H_{21}N_3O_4S$; compound 4) was shown to be a potent, highly specific inhibitor of the primary catalytic enzyme of the hepatitis C virus (HCV) replicase complex. In this study, we selected for resistance to confirm the mechanism of action for compound 4 in HCV replicon cells. As expected, spontaneous mutations or fluidity in the HCV polymerase (NS5B) coding sequence occurred upon routine passage of the HCV replicon cells in the absence of compound 4. After 1 month of culture in the presence of 10 μM compound 4, or 20 times the 50% inhibitory concentration of the replicon, replicon cells were almost 20-fold less susceptible to compound 4. Twenty-one NS5B cDNA clones were generated from the resistant replicon cells. Five mutations in the 21 NS5B clones were present at frequencies higher than that of control replicon cells, and no clone contained more than a single mutation within the polymerase gene. RNA-dependent RNA polymerase studies using purified recombinant NS5B containing these single point mutations allowed the identification of residue 414 as sufficient for biochemical resistance to compound 4. Further, the contribution of this residue to confer cell-based resistance to compound 4 was validated using a stable recombinant mutant replicon cell line which harbors a methionine-to-threonine change at residue 414. The potential for additional mutations in other nonstructural genes of HCV to contribute to the resistance profile of compound 4 is discussed.

Hepatitis C virus (HCV), a positive-strand RNA virus of the *Flaviviridae* family, represents the major etiological agent of posttransfusion and sporadic non-A, non-B hepatitis (4). The nonstructural protein 5B (NS5B) encodes the catalytic polymerase which is responsible for RNA-dependent RNA polymerase (RdRp) activity (1, 12) and terminal transferase activity (15). *Flaviviridae* RdRps have been shown to initiate RNA synthesis via a de novo mechanism based on in vitro assays (10, 11, 14, 16, 18), a process that is also presumed to occur in HCV-infected cells (9).

Recent structural analysis (3) and cell-based replicon systems have advanced our understanding of HCV replication. These systems rely upon the nonstructural proteins to stably replicate subgenomic viral RNA in Huh-7 cells (2, 13), and they have been used to evaluate antiviral agents. Recently, a benzothiadiazine compound was shown to have highly specific antiviral activity against the HCV RdRp, inhibiting viral RNA synthesis in both biochemical and cell-based replicon systems (5). Further, this agent shows synergy with interferon-alpha (IFN-α) in the replicon system, confirming a distinct mode of action (V. K. Johnston, D. Maley, R. Gagnon, C. W. Grassmann, S.-E. Behrens, and R. T. Sarisky, unpublished data). Although HCV replication is sensitive to IFN-α with a 50% inhibitory concentration (IC_{50}) of less than 10 U/ml, long-term treatment with up to 1,000 U/ml in the presence of G418 to maintain the replicon RNA did not yield IFN-resistant variants (7). Viral RNA continued to replicate in IFN-α-treated cells, albeit at levels 10-fold lower than in untreated cells.

In this study, we selected for replicon cell resistance to a benzothiadiazine inhibitor of NS5B by long-term passage in the presence of G418 to maintain the replicon RNA and a concentration of compound 4 sufficiently above the cell-based IC_{50}. Insight into the mechanism of inhibition and active-site binding of this agent is presented.

MATERIALS AND METHODS

Cell culture. Huh-7, a human transformed hepatocyte line, and clone A, a Huh-7 replicon-expressing cell line (kind gift of C. Rice, Rockefeller University, New York, N.Y.), were maintained in Dulbecco's modified Eagle medium (Invitrogen) containing 10% fetal calf serum (FCS; JRH Biosciences), 1% nonessential amino acids (Invitrogen), 1% penicillin-streptomycin (Invitrogen), and 1 mg of Geneticin (Invitrogen) per ml. The cells were passaged (1:3) approximately three times per week once 70% confluent growth was reached.

Biochemical RdRp assay. The biochemical RdRp assay was performed essentially as described previously (5). Briefly, 50 nM enzyme, 0.2 μCi of [α-^{33}P]GTP, 0.6 μM GTP, 250 nM 5′-biotinylated oligo(rG_{13})-poly(rC) in 20 mM Tris-Cl (pH 7.5), 5 mM $MgCl_2$, 25 mM KCl, and 3 mM dithiothreitol. The 25-μl reaction mixture was terminated after 2 h at 25°C upon the addition of an equal volume of 100 mM EDTA and transferred to a streptavidin-coated FlashPlate. After incubation at 25°C for 30 min, the plate was washed extensively and counted using a Packard TopCount microplate reader.

Resistance selection. Approximately 10^6 clone A cells were seeded in a T75 flask for subconfluent conditions (approximately 50% confluent growth). Ten milliliters of Dulbecco's modified Eagle medium containing a final concentration of 10 μM compound 4 and 1 mg of G418 per ml was added to the flask. When the cells in the flask reached approximately 80% confluent growth (every 3 days) during the first 2 weeks, the cells were split, and fresh medium containing the compound was added at each passage. The doubling time of the cells remained constant at around 40 h. During week 3, fresh medium containing both compound 4 and G418 was added, but cell growth had slowed down and the flask did not require splitting. During week 4, the cells resumed growing at their original growth rate, and cells were split as indicated above for weeks 1 and 2. After 4

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weeks of culture with compound 4, cells were cultured for two passages in the absence of compound 4 to ensure removal of antiviral agent and allow for susceptibility testing.

cDNA generation. All medium from the flask of clone A cells passaged with compound 4 was removed. Approximately 5 ml of Trizol was added to the flask and rocked for 10 to 15 min at 25°C. The supernatant was transferred to a 15-ml conical tube, 1 ml of chloroform was added, and the sample was shaken vigorously for 30 s prior to centrifugation at 2,000 × *g* for 30 min. The top layer of supernatant was transferred to a new 15-ml conical tube, and 2.5 ml of isopropanol was added to that supernatant to precipitate the RNA at 25°C for 20 min. After precipitation, the sample was centrifuged at 3,000 × *g* at 4°C for 45 min to pellet the RNA. The RNA pellet was washed with 70% ethanol and allowed to air dry for 15 min before resuspending the RNA in water containing diethyl pyrocarbonate. RNA was stored at −80°C. Using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen), first-strand cDNA was synthesized using 2 μl of RNA at 42°C for 60 min with an NS5B-specific reverse transcriptase (RT) primer (5′-TTGAGTCGTACTCCTCCATGCC-3′). NS5B primers were designed to add an *Eco*RI restriction site to both ends. The forward NS5B primer (5′-CGGAATTCCGTCGATGTCCTACACATGGACAGGC-3′) and reverse NS5B primer (5′-GCGAATTCGCTCATCGGTTGGGGAGTAGATAGATCG-3′) were used to amplify the NS5B gene (~1.8 kb). The *Eco*RI site was used to ligate the PCR fragment into pET21 (Novagen). Transformation was performed using DH5-α competent cells, and the transformants were plated on plates containing Luria-Bertani (LB) medium and ampicillin (100 μg/ml). Colonies were selected and grown in 2.5 ml of culture overnight in a 37°C shaker. PCR was performed using the forward and reverse NS5B primers described above with 2 μl of bacterial culture in 50-μl reaction mixture volume using Platinum *Taq* polymerase (Gibco/BRL). The reaction mixtures were placed in an Eppendoff thermal cycler set at the following cycling parameters: 35 cycles, with 1 cycle consisting of 1 min at 94°C, 2 min at 55°C, and 2 min at 72°C. Positive PCR products were purified by using Qiaquick PCR purification kit (Qiagen) and resuspended in 50 μl of sterile water. The NS5B clones ($n = 21$) were sequenced and aligned to the sequences of BB7 NS5B replicon. The consensus mutations were compiled, since the culture represented a pooled population of drug-selected colonies. Key mutations were then generated by site-directed mutagenesis into the BB7 NS5B replicon.

Site-directed mutagenesis. The HCV NS5B coding region of type 1b (pBBVII) was cloned into pET21b as follows. The *Bam*HI fragment of pBBVII, encoding part of NS5A and all of NS5B was cloned into pET21b for modification. PCR mutagenesis was used to knock out an internal *Nde*I site and add a new *Nde*I site encoding the initiating methionine for NS5B. A *Sma*I site was introduced by mutagenesis into NS5B at the 3′ Δ21 amino acid position. The following linker (5′-CGTCACCACCACCACCACCACCACTGATCTAGAGG) encoding a sequence with a six-His tag, stop codon, and *Xho*I and *Asc*I restriction sites was inserted into the above construct cut with *Sma*I/*Asc*I. The resulting vector was digested with *Nde*I/*Xho*I and reintroduced into pET21b, resulting in the NS5B expression vector pETBBVIINS5Bdelta21-6xHis. This construct was used as a template for mutagenesis by the Stratagene QuikChange site-directed mutagenesis protocol. Briefly, 200 ng of template and 300 ng of each mutagenesis primer were added to 5 μl of 10× reaction buffer, 100 μM mix of all four deoxynucleoside triphosphates, 1 μl of *Pfu* Turbo polymerase in a final volume of 50 μl. The following mutagenesis primers were used to engineer the following mutations in the Δ21 NS5B coding sequence: Lys50Arg, 5′-GCGCAAGCCTGCGGCAGAg GAAGGTCACCTTTGACA-3′; Met71Val, 5′-GACGTGCTCAAGGAGGTG AAGGCGAAGGCGTCCAC-3′; Asn411Ser, 5′-TCAATTCCTGGCTAGGCA GCATCATCATGTATGCGC-3′; and Met414Thr, 5′-GGCTAGGCAACATC ATCACGTATGCGCCCACCTTG-3′. The Val581Ala mutation was engineered within replicon RNA using the primer 5′-TGCCTACTCCTACTTTCTGCAGG GGTAGGCATCTAT-3′. Cycling parameters were as defined in the Stratagene manual for single-amino-acid changes. *Dpn*I (20 U) (Invitrogen) was added to each reaction mixture and incubated at 37°C overnight. Reactions were ethanol precipitated and used to transform supercompetent XL1 cells (Stratagene).

Protein expression and purification. XL1 transformed colonies were screened by sequencing. DNA from clones containing the appropriate mutation were transformed into *Escherichia coli* strain BL21Gold(DE3)pLysS cells (Stratagene). Several clones were screened for optimal protein expression by inoculating cultures grown overnight in LB medium supplemented with 50 μg of ampicillin per ml, 35 μg of chloramphenicol (Sigma) per ml, and 1% glucose. Three-milliliter portions of cultures grown overnight were used to inoculate 47 ml of LB medium supplemented with 50 μg of ampicillin per ml, 35 μg of chloramphenicol per ml, and 1% glucose. Cultures were incubated at 30°C until the optical density at 600 nm was between 0.8 and 1.0. The culture temperature was lowered to 25°C, and protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Invitrogen) for 4 h. Optimal expression was identified by Western blotting with antipeptide antibodies to NS5B, and the clone was scaled up in 500 ml of culture using the protocol described above.

NS5B protein was purified essentially as described earlier (5). Briefly, *E. coli* cell lysate was applied to Talon metal affinity resin (Clontech), and the bound proteins were eluted with 30 mM and 200 mM imidazole in steps. The 200 mM Talon eluate was dialyzed against buffer A containing 20 mM Tris-Cl (pH 8.0), 3 mM dithiothreitol, 150 mM NaCl, and 10% glycerol and applied to a poly(U) Sepharose 4B (Pharmacia) column. After the column was washed with 10 column volumes with buffer A, the bound protein was eluted with buffer A plus 1 M NaCl. Fractions containing NS5B were pooled and concentrated to 30 mg/ml using a Centricon 10 concentrator (Amicon). The purity of NS5B was >95% as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

TaqMan quantitation of viral RNA. Viral RNA was quantified using TaqMan analyses exactly as described previously (5). Specifically, replicon cells were plated at 3×10^3 cells per well in a 96-well plate and placed at 37°C and 5% CO_2 in Dulbecco's modified essential medium containing 10% fetal calf serum, 1% nonessential amino acids, and 1 mg of Geneticin per ml. After 4 h to allow the cells to attach, 1 μl of compound dilution was added to the medium (eight wells per dilution). Briefly, 11 dilutions (2.5-fold dilutions) of 1 mM stock test compound in dimethyl sulfoxide (DMSO) were prepared with final concentrations ranging from 10,000 to 1.0 nM for most assays unless indicated otherwise. Plates were incubated for 40 h, until the cells in the plate reached approximately 80% confluent growth. After removal of medium, 150 μl of guanidine salt-based RLT buffer (catalog no. 79216; Qiagen) was added to each well, and RNA was purified according to the manufacturer's recommendations (Qiagen RNeasy) and eluted twice in 45 μl of distilled H_2O prior to RT-PCR. Approximately 40 μl of TaqMan EZ RT-PCR (Applied Biosystems) master mix (1× TaqMan EZ buffer, 3 mM manganese acetate, 0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP, 0.6 mM dUTP, 0.2 mM neo-forward primer, 0.2 mM neo-reverse primer, 0.1 mM neo-probe, 1× cyclophilin mix, 0.1 U of rTth DNA polymerase per μl, 0.01 U of AmpErase UNG per μl, and distilled H_2O to 40 μl) was added to each tube of a 96-tube optical plate along with 10 μl of RNA elution. The primers and probes specific for HCV RNA detection of the neomycin gene were as follows: neo-forward, 5′-CCGGCTACCTGCCCATTC-3′; neo-reverse, 5′-CCAGATCATCC TGATCGACAAG-3′; neo-probe, 5′-FAM (6-carboxyfluorescine)-ACATCGC ATCGAGCGAGCACGTAC-TAMRA (tetrachloro-6-carboxyfluorescine)-3′. Additionally, the predeveoped assay reagent (control reagent) human cyclophilin was used for normalization. Samples were mixed briefly and placed in an ABI7700 cycler (Applied Biosystems) set at the following cycling parameters: (i) 2 min at 50°C; (ii) 30 min at 60°C; (iii) 5 min at 95°C; (iv) 40 cycles, with 1 cycle consisting of 20 s at 94°C and 1 min at 55°C. The reaction is monitored in real time over 40 cycles to generate a raw amplification plot on a logarithmic scale. The plot is analyzed during the exponential phase, at ~25 cycles, to determine the quantities of cDNA. The relative cDNA levels for the neomycin gene and cyclophilin were determined compared with controls treated only with DMSO, and the ratio of the neomycin gene/cyclophilin was used for IC_{50} calculation ($n = 8$). The exponential phase provides very consistent data and a theoretical sensitivity range of 7 log units.

RESULTS

Resistance selection. Compound 4 ($C_{21}H_{21}N_3O_4S$; Fig. 1A) was reported previously to have an IC_{50} around 500 nM in a wild-type HCV cell-based replicon system (5). In this study, we utilized the same wild-type replicon (clone A) cells, which harbor around 1,200 copies of subgenomic RNA per cell, to initiate resistance selection. Prior to continuous culture in the presence of compound 4 for selection of resistance, the baseline level of viral RNA after treatment with 10 μM compound 4 (approximately 20 times the IC_{50}) was assessed. After 40 h of treatment with 10 μM compound 4, the level of viral RNA (subgenomic replicon RNA normalized to cyclophilin RNA) was reduced by 1.5 log units (approximately 95%) compared to the control sample (Fig. 1B). The reduction in viral RNA is shown as a percentage of control (replicon RNA from DMSO-treated cells).

Since the resistance breakpoints for the replicon system have yet to be defined and a twofold change in viral RNA as mea-

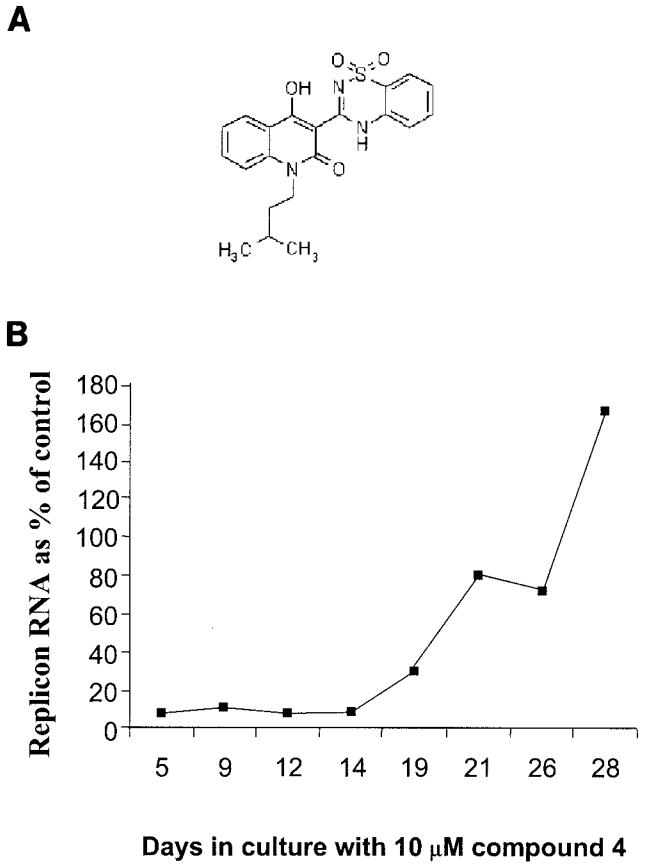

FIG. 1. Selection of resistance to compound 4 in HCV replicon cells. (A) Chemical structure of compound 4. (B) Replicon RNA levels after treatment with compound 4 was measured by TaqMan analysis and plotted as a percentage of control RNA (DMSO-treated cells) on the *y* axis. The number of days in culture with 0 μM compound 4 was plotted on the *x* axis.

sured by real-time PCR is within the error range, a minimum 10-fold increase in viral RNA (during extended culture with a single concentration of compound 4) compared to the 40-h treatment baseline level with 10 μM compound 4 was presumed to represent a significant increase in viral RNA. Our hypothesis was that a 10-fold increase in viral RNA from the baseline level in the presence of compound 4 may be associated with genetic changes in replicon RNA. Such amplification of replicon RNA may potentially indicate the establishment of an antiviral-resistant state.

Even with continual treatment of the replicon cells with 10 μM compound 4 and 500 mg of G418 per ml beyond 40 h, this reduction in replicon RNA levels remained constant for the first 2 weeks of culture in the presence of 10 μM compound 4, as determined by analysis of duplicate cultures. However, by week 4, replicon RNA levels increased more than 1 log unit above the 40-h treatment baseline level. Total cell RNA from this preparation was used for sequence determinations.

To confirm that the week 4 cultures showed a reduction in susceptibility to compound 4, these cells were passaged twice thereafter in the absence of compound 4 (and presence of 500 mg of G418 per ml) to ensure complete removal of compound 4 from the cell cultures prior to antiviral testing. Next, a dose-response curve of compound 4 was analyzed with dilutions ranging from 2 nM to 20 μM. Unlike wild-type replicon cells (IC_{50} = 550 nM) (Fig. 2A), the resistant replicon culture obtained after 28 days of selection with compound 4 showed a decrease in susceptibility to compound 4 with an IC_{50} of >8 μM (Fig. 2B). Interestingly, replicon RNA levels were similar for both 10 and 20 μM concentrations of compound 4. Although no precipitation of compound was apparent upon microscopic analysis, this does not preclude solubility limitations as an explanation. Testing of the compound 4-selected resistant replicon with IFN-α resulted in an IC_{50} of less than 10 U/ml (data not shown), similar to that of the wild-type control replicon.

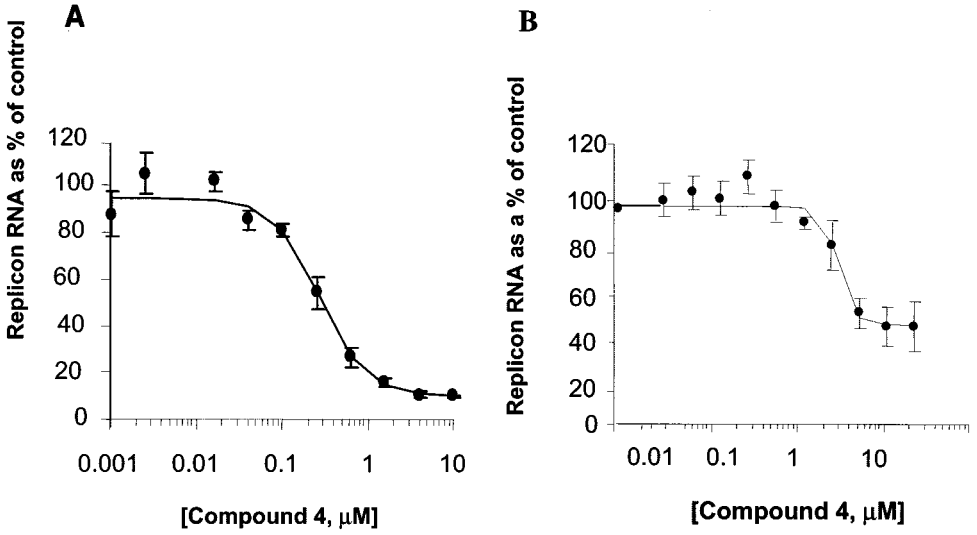

FIG. 2. Susceptibility of wild-type replicon (A) and resistant replicons (B) to compound 4. Replicon RNA levels after treatment with compound 4 was measured by TaqMan analysis and plotted as a percentage of control RNA (DMSO-treated cells) on the *y* axis, and the concentration of compound 4 (in micromolar) was plotted on the *x* axis.

TABLE 1. Frequency of NS5B mutations in compound 4-selected cells

NS5B residue change	No. of clones (n = 21)
Lys50Arg	8
Met71Val	2
Asn411Ser	4
Met414Thr	4
Val581Ala	3

Determination of resistance mutations. A total of 21 independent NS5B clones were generated from the replicon culture obtained after selection with compound 4 for 4 weeks. The DNA sequence for all clones was determined. In parallel, cDNA clones from wild-type replicon cells passaged in parallel but treated only with G418 were also examined for genetic drift in NS5B sequence (data not shown). Similar mutations in the compound 4-selected cells and the control replicon cells were found, although with reduced frequency in the control cells (no mutations were found with a frequency greater than 1 of 21 clones). The NS5B residue changes and frequencies are presented in Table 1.

Residues conferring biochemical resistance. The relative contributions of the polymerase mutations were evaluated for their contribution to biochemical resistance using the in vitro RdRp assay. Recombinant polymerases containing the single point mutations (Lys50Arg, Met71Val, Asn411Ser, or Met414Thr) were expressed, purified, and evaluated for catalytic activity. Only four of the five mutants were examined, since the biochemical assay utilized a polymerase containing a 21-residue carboxy-terminal truncation. Unlike the BK strain of HCV, purification of the full-length replicon BB7 strain polymerase resulted in poor enzyme yield and quality (data not shown), which was insufficient to allow a robust comparison between mutant RdRps. Since the Val581Ala mutation resided in the C-terminal tail region of NS5B, it was omitted from this initial study for comparison purposes, and all biochemical analyses were performed using an RdRp with a truncation of the C-terminal 21 residues. The replication kinetics for wild-type NS5B and the Lys50Arg, Met71Val, Asn411Ser, and Met414Thr mutant polymerases are shown in Fig. 3. All polymerases except for the Asn411Ser mutant polymerase exhibited linear kinetics, a characteristic which is critical when comparing the activity of various antiviral agents across polymerase clones. Testing with the 3′ dGTP chain terminator showed similar IC_{50}s (all less than 100 nM), except for the Asn411Ser mutant polymerase, which had a fivefold-higher IC_{50} (Table 2). Susceptibility profiling with compound 4 indicated that only residue 414 indeed impacted potency, with a biochemical IC_{50} increasing from 80 nM for wild-type RdRp to >10,000 nM when the methionine at residue 414 was changed to threonine (Table 2).

Transfer of biochemical resistance to replicon. Further, we assessed whether the methionine-to-threonine modification at residue 414 which indeed conferred biochemical resistance could partially account for the resistance profile in compound 4-selected cells. To that end, a stable Huh7- replicon cell line encoding the modified polymerase was generated. A dose-response titration with compound 4 (Fig. 4A) demonstrated that the M414T cell line had an IC_{50} of >10,000 nM, a susceptibility profile similar to that shown with compound 4-selected resistant cells. Further, both cell lines (compound 4 selected and the M414T replicon cells) demonstrated incomplete inhibition, with no apparent further reductions in viral load when between 10 and 20 μM compound 4. Interestingly, a difference in average copy number of viral RNA per cell was apparent between the compound 4-selected cells (~50 copies per cell) and the M414T replicon cells (~500 copies per cell) (data not shown). It is unclear how this difference contributes to compound 4 susceptibility. Susceptibility to IFN-α remained unchanged in the M414T replicon cells (Fig. 4B), compared to wild-type replicon cells or to compound 4-selected replicon cells.

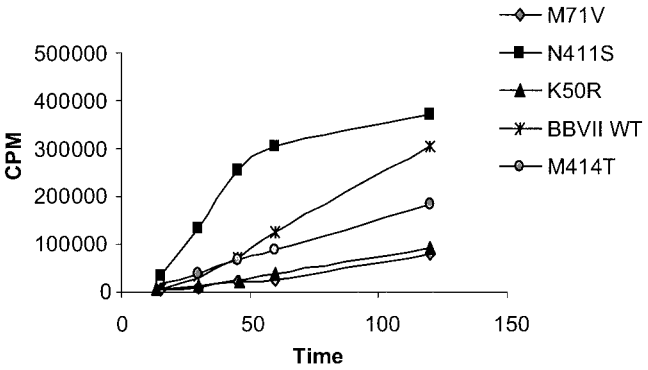

FIG. 3. Activity of recombinant RdRps. Using the standard RdRp assay, total incorporated radiolabeled product is indicated on the *y* axis and time (in minutes) is shown on the *x* axis. The RdRp assay used 10 nM HCV polymerase and 250 nM poly(rC)-oligo(G) RNA substrate. BBVII WT is the wild-type polymerase from replicon clone A as a control (2).

Additional replicons were generated expressing either Lys50Arg, Met71Val, or Asn411Ser mutations, and none were found to have an impact on cell-based IC_{50} for compound 4 (data not shown); these data are in agreement with the biochemical IC_{50}s for the respective recombinant RdRps. Surprisingly, we were unable to generate a stable cell line containing the Val581Ala mutation to allow investigation into the potential contribution of this mutation on resistance. It is unclear whether this particular mutation directly impacts replicon viability or whether technological limitations were responsible.

Clonal selection of resistance. Selection for resistance to compound 4 after 35 days of continuous culture resulted in the selective amplification of a mutation at residue 414 in the

TABLE 2. Inhibition profile of recombinant HCV polymerases in a biochemical RdRp assay

NS5B residue change	IC_{50} (nM)	
	3′ dGTP	Compound 4
Lys50Arg	100	90
Met71Val	95	110
Asn411Ser	520	480
Met414Thr	90	>10,000
None (wild type)	100	80

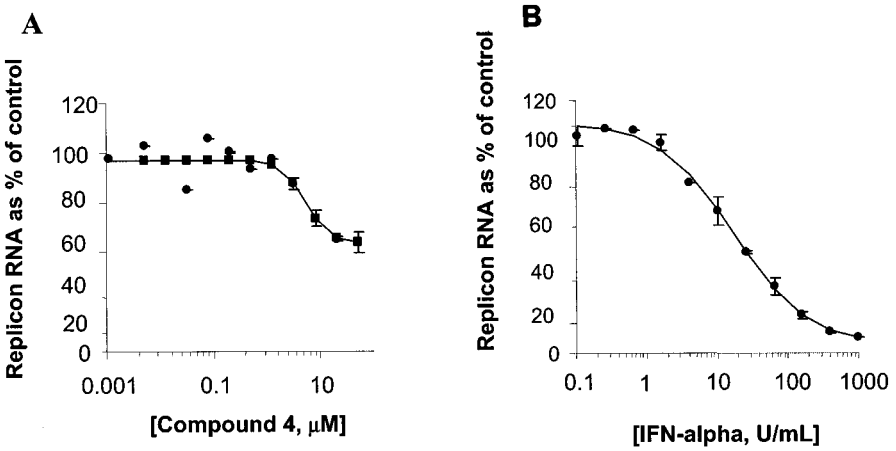

FIG. 4. Inhibition profiles for compound 4 (A) and IFN-α (B) in Met414Thr HCV replicon cells. Replicon RNA levels after treatment with compound 4 was measured by TaqMan analysis and plotted as a percentage of control RNA (DMSO-treated cells) on the *y* axis, and serial dilutions of compound were titrated in either micromolar concentration (compound 4) or units per milliliter (IFN-α) on the *x* axis.

RdRp coding region. Other mutations may exist within the nonstructural genes and may contribute individually or in cooperation with the mutation at residue 414 to the resistance phenotype. Individual point mutations identified across the entire nonstructural coding region were not present in more than one cDNA clone containing the M414T mutation. Thus, the ancillary contributions of other nonstructural gene mutations, excluding the M414T mutation in NS5B, has not been assessed. To that end, selection for clonal resistant colonies, compared to a pooled population, will help provide such insights.

DISCUSSION

The HCV RdRp, a central catalytic enzyme of replication, represents a viable target for identification of antiviral agents to treat chronic HCV infections. In this study, we report that the benzothiadiazine compound 4 directly inhibits the viral RdRp in the cell-based replicon system, as evidenced by the selection of antiviral resistance. Selection with compound 4, at a concentration 20 times the replicon IC_{50}, resulted in the identification of several clones containing a mutation at residue 414 in the polymerase coding region. The change from methionine to threonine at residue 414 abolished the inhibition activity of compound 4 in a biochemical RdRp assay. It is not absolutely clear why the population of replicon sequences analyzed did not contain a greater frequency of Met414Thr clones. Perhaps the selective pressure was insufficient to result in preferential amplification of a subset of clones. Nonetheless, a recombinant replicon was engineered to contain the Met414Thr mutation, and this stable replicon cell line was indeed impaired for susceptibility to compound 4.

Although compound 4 is clearly an inhibitor of the viral RNA polymerase (5, 6), mutations within the replicase complex could also be expected upon passage with antiviral agent. However, this does not preclude the possibility that other cellular changes may have occurred as a result of drug treatment.

Overall, this work clearly demonstrates that the replicon system can be utilized to select for antiviral resistance to HCV enzyme inhibitors. Consistent with this observation, Trozzi et al. (17) were able to select replicons with decreased sensitivity to serine protease inhibitors upon culturing in the presence of antiviral agent with neomycin. Herein, a single point mutation within the viral polymerase was shown to confer nonresponsiveness to the benzothiadiazine agent, compound 4. Other mutations in the replicase complex coding region (NS3-NS5) or the Val581Ala mutation within NS5B may also have contributed to the cell-based resistance. Other such mutations may individually or in cooperation with the mutation at threonine 414 further impact the resistance phenotype.

Interestingly, similar to studies with IFN-α (7), replicon RNA levels were about 10-fold lower upon selection with compound 4 and continued to remain at this reduced level even after removal of the antiviral agent (e.g., washout period). The resistant replicon cells showed the lower susceptibility to compound 4 even after 2 months culture in the absence of compound 4. However, the long-term stability of such mutations is unclear.

In this study, a panel of point mutations in the viral RdRp were analyzed. One recombinant polymerase with an Asn411Ser mutation showed nonlinear kinetics of incorporation and increased polymerase activity. This mutant enzyme may have a defect in the beta-hairpin loop, detailed by Hong et al. (8). As such, binding to RNA may no longer be a rate-limiting step compared to wild-type RdRp and may provide this polymerase with more replication product. However, this study did not examine for differences in replication rate, using conditions for single cycle synthesis, although this is the focus on an ongoing study.

Consistent with the data reported herein, susceptibility testing of a related, less potent, compound from the same chemical class (compound 1 [5]) in the Met414Thr replicon cells had an IC_{50} of >50 μM, which is at the limit of solubility testing (data not shown). Together, these data allow us to substantiate that the Met414Thr mutation may have an impact on the benzothiadiazine class of antiviral agents.

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