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Mutations in HCV non-structural genes do not contribute to resistance to nitazoxanide in replicon-containing cells

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ABSTRACT

Nitazoxanide (NTZ) exhibits potent antiviral activity against hepatitis C virus (HCV) in cell culture. Previously, HCV replicon-containing cell lines resistant to NTZ were selected, but transfer the HCV NTZ-resistance phenotype was not observed following transfection of whole cell RNA. To further explore the nature of the resistance of HCV to NTZ, full length HCV replicon sequences were obtained from two NTZ-resistant (NTZ-11, TIZ-9), and the parental (RP7) cell lines. Numerous nucleotide changes were observed in individual HCV genomes relative to the RP7 HCV consensus sequence, but no common mutations in the HCV non-structural genes or 3′-UTR were detected. A cluster of single nucleotide mutations was found within a 5-base portion of the 5′-UTR in 20/21 HCV replicon sequences from both resistant cell lines. Three mutations (5′-UTR G17A, G18A, C20U) were individually inserted into CON1 ('wild-type') HCV replicons, showed reduced replication (5 to 50-fold), but none conferred resistance to NTZ. RP7, NTZ-11, and TIZ-9 were cured of HCV genomes by serial passage under interferon. Transfection of cured NTZ-11 and TIZ-9 with either whole cell RNAs from RP7, NTZ-11, or TIZ-9, 'wild-type' or the 5′-UTR mutation-containing replicon RNAs exhibited an NTZ-resistance phenotype. TIZ (the active metabolite of NTZ) was found to be inactive against the activity of HCV polymerase, protease, and helicase in enzymatic assays. These data confirm previous speculations that HCV resistance to NTZ is not due to mutations in the virus, and demonstrate that HCV resistance and most likely the antiviral activity of TIZ are due to interactions with cellular target(s).

1. Introduction

Nitazoxanide (NTZ, Alinia®, Romark Laboratories, LC), a broad spectrum, thiazolide anti-infective, is licensed in the United States for the treatment of diarrhea caused by *Cryptosporidium parvum* and *Giardia lamblia* and is currently in development for the treatment of chronic hepatitis C virus (HCV) infection (Rossignol, 2009). Phase 2 clinical trials of NTZ in patients with chronic hepatitis C have shown enhanced efficacy when administered in combination with peginterferon or peginterferon plus ribavirin (Rossignol et al., 2009, 2010). NTZ, and its active metabolite, tizoxanide (TIZ), exhibit potent antiviral activity against multiple genotypes of HCV, as well as replicons carrying representative mutations that confer resistance to protease inhibitors and nucleosides in cell culture. NTZ and TIZ have been shown to act in a synergistic manner with several clinically relevant direct acting antiviral agents (DAAs) (Korba et al., 2008b).

HCV replicon-containing cell lines resistant to the antiviral effects of NTZ/TIZ up to at least 50X the reported EC_{50} can be selected (Rossignol et al., 2010), but attempts to transfer NTZ-resistance by transfection of HCV genomes in whole cell RNA preparations from NTZ-resistant cells to naive cell cultures were unsuccessful (Korba et al., 2008a). These studies suggested, but did not conclusively demonstrate, that the NTZ resistance phenotype is most likely due to drug-induced changes in host factors. It is also possible that NTZ exposure induced specific mutations in the HCV genomes present in the resistant cell lines that are largely 'silent' with respect to a resistance phenotype in naive cells, but not so in drug-exposed cells. The aim of the current series of studies was to further explore the nature of the resistance of HCV to NTZ in replicon-containing cell cultures, with the primary focus being to determine if the observed resistance phenotype is a fundamental feature of the host, virus, or both.

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2. Experimental methods

2.1. Cell lines and culture conditions

The cell lines used in these studies have been previously described. RP7 was derived by transfection of Huh7 cells (ATCC) with a sub-genomic HCV replicon (genotype 1b, CON1) (Elazar et al., 2003). Two cell lines that confer resistance of HCV against 10 μM nitazoxanide (NTZ) or tizoxanide (TIZ), NTZ-11 and TIZ-9, were derived by serial passage of RP7 in increasing concentrations of either NTZ or TIZ (Korba et al., 2008a). Stock cultures were maintained in DMEM with 250 μg/ml G418 as previously described (Korba et al., 2008a). Medium for the two resistant cell lines also contained 10 μM NTZ. Stock solutions of NTZ or TIZ (supplied by Romark Laboratories, LC, Tampa, FL) were 10 mM in 100% tissue culture grade DMSO (Sigma–Aldrich, Inc., St. Louis, MO), and were stored for up to 2 weeks at 4 °C.

All three cell lines were 'cured' of resident HCV replicons by eight serial passages with 1000 IU/ml recombinant human interferon alfa-2b (PBL Laboratories, Inc., Piscataway, NJ). Medium for the two resistant cell lines also contained 10 μM NTZ during curing. Loss of HCV RNA was confirmed by polymerase chain reaction (PCR) analysis (data not shown). In addition, cultures of all three cured cell lines in 6-well culture plates failed to produce colonies when cultured in the presence of 500 μg/ml G418 (initial population of 1–2 $\times$ 10^6 cells, data not shown).

2.2. Transfection with HCV RNA

Cultures were transfected in 6-well culture dishes (Nunc, Inc.) using the Lipofectamine 2000™ reagent (Gibco, Inc., Gaithersburg, MD) following the Manufacturer's instructions and previously described procedures (Korba et al., 2008a). In brief, 24 h post-transfection, cultures were exposed to 500 μg/ml G418 and antiviral compounds for an additional 14–17 days. Media was replaced three times a week. Resultant macroscopic colonies were fixed in 7% formaldehyde/water and stained with 1% crystal violet dye (1:1 EtOH:water) and counted manually. Duplicate wells were used for each experimental condition in each experiment. The number of colonies in each drug-treated well was compared with the average number of colonies produced by each source RNA in the absence of drug treatment to express colony formation as a percent of untreated controls. Two to four independent experiments were conducted with two different preparations of each RNA source, resulting in 4–8 replicates for each treatment and source RNA. Whole cell RNA used for transfections was purified using Maxi or Midi columns (Qiagen, Inc., Germantown, MD) according to the Manufacturer's instructions. HCV replicon RNA from individual cloned genomes as prepared as described below.

2.3. Construction of HCV replicons containing point mutations

Three specific point mutations in the 5′-UTR were created in an HCV CON1 sub-genomic replicon background using site-directed mutagenesis (QuikChange II, Stratagene, Inc., La Jolla, California): G17A, G18A, C20T (C20U in HCV RNA). Sequencing of the complete 5′-UTR region in the resultant clones was performed to confirm that only a single mutation was present. HCV replicon RNA was transcribed and purified from *ScaI* linearized replicon source plasmid DNA using the MegaScript kit (Ambion, Inc., Austin, TX), and purified using MEGAclear kit (Ambion, Inc., Austin, TX).

2.4. Cloning and analysis of HCV sequences

Full length cDNA complementary to the HCV replicon genome was created with the SuperScript One-Step R/T-PCR for Long Template Kit (Invitrogen, Inc., Carlsbad, CA) following the Manufacturer's instructions using the corresponding primer pair: R/T-PCR P02 (5′-ACT TGA TCT GCA GAG AGG CCA GTA TC-3′) and PCR P01 (5′-GCA GCT GAG TGA TGG TAA GAC TAG AGA GG-3′).

Producing full genome length HCV replicon cDNA proved to be highly inefficient (approximately one of every 50–75 clones). As such, only a limited number of full length clones were produced. For efficiency, the 5′-UTR region was cloned separately and the HCV NS gene sequence region and 3′-UTR were cloned in two portions that overlapped by approximately 150 bases. The first sub-length HCV clones contained the NS3, NS4A and most of the NS4B coding regions, and the second sub-length clones contained the remainder of NS4B, the NS5A and NS5B coding regions, and the 3′UTR. Analysis of HCV sequences from the RP7 parental cell line did not reveal any significant sequence variances between the full length and sub-length clones. The intent of this practical approach was that, if any common mutations were observed to occur for both cell lines in each of the two sub-length clones, then full length sequences would be created to determine if these existed in the same HCV replicons.

The primer pair of R/T-PCR P01 (5′-GCA GCT GAG TGA TGG TAA GAC TAG AGA GG-3′) and PCR P01 (5′-GCC AGC CCC CGA TTG-3′) was used for reactions with the one-step R/T-PCR kit, to make the first sub-length DNAs. The primer pair of R/T-PCR P02 with PCR P02 (5′-GGA TGA ACC GGC TGA TAG CGT TCG-3′) was used to make the second sub-length DNAs. The DNA made by the R/T-PCR reactions was inserted into vectors using either the TOPO TA Cloning Kit (Invitrogen, Inc.) or the pGEM-T Vector System (Promega, Inc., Madison, WI), and then transformed into *Escherichia coli* Top 10 or *E. coli* Top 10F′ chemically competent cells (Invitrogen, Inc.). Positive clones were selected by colony PCR of the single colonies from the culture plates with same primers used to make the cDNAs. Plasmid DNA from the selected colonies was isolated using the QIAprep Spin Miniprep Kit (Qiagen, Inc.) and checked by restriction enzyme digestion as indicated below: Full length clones: *EcoR I* + *Hind III*; first sub-length clones: *EcoR I* or *Hind III* + *Sac II*; second sub-length-clones: *Nde I* + *Xho I* or *EcoR I* + *Kpn I*. Clones containing the proper restriction enzyme fragments were subjected to DNA sequencing (Macrogen, Inc., Rockville, MD).

The primers utilized for the sequencing were as follows. SP6 promoter: 5′-ATT TAG GTG ACA CTA TAG-3′, T7 Promoter: 5′-TAA TAC GAC TCA CTA TAG GG-3′, 1st sub-clone sequencing primers: SP P03: 5′-GGA ATG CAA GGT CTG TTG AAT GTC GTG AAG G-3′, SP P04: 5′-CAT CAT CAC TAG CCT CAC AGG CC-3′, SP P05: 5′-CGT ATG CAG CCC AAG GGT ATA AGG-3′, SP P06: 5′-GCC ATT CCA AGA AGA AAT GTG ATG AGC TCG-3′, SP P07: 5′-GTG TCT CAT ACG GCT AAA GCC-3′, SP P08: 5′-CAG GCT TGT CCA CTC TGC CTG-3′; 2nd sub-clone sequencing primers: SP P10: 5′-CGT AAA GTG CCC GTG TCA GG-3′, SP P11: 5′-CTC CAA GCG GAG GAG GAT GAG-3′, SP P12: 5′-CAG GCG CCC TGA TCA CGC CAT G-3′, SP P13: 5′-GGC CCT TTA CGA TGT GGT CTC-3′, SP P14: 5′-GAC AGC TAG ACA CAC TCC AGT CAA TTC CTG G-3′, SP P15: 5′-GGA GAC ATA TAT CAC AGC CTG TCT CG-3′.

2.5. Uptake of ^{14}C-TIZ

Cultures were grown to approximately 80% confluence on 12-well culture plates. ^{14}C-labeled TIZ (14.20 mCi/mmol, ABC Laboratories, Inc., Madison, FL) was added to the culture medium (*c.a.* 5 x 10^5 CPM/ml in a final TIZ concentration of 1.0 μM, 0.1% DMSO, 0.5 ml/well). Triplicate cultures were harvested at various time points post-exposure. For harvesting, culture medium was removed and the cultures were rinsed three times with PBS. Cells were then removed by trypsinization, centrifuged, the pellets lysed with RLT buffer (Qiagen, Inc.), and the lysates analyzed by scintillation counting.

2.6. In vitro analysis of HCV enzymatic activities

2.6.1. HCV NS3/4A protease

Full length NS3 protease/helicase and NS4A cofactor genes from HCV (CON1) were amplified using the primers NS3FLHis (5′-CAT ATG CAT CAC CAT CAC CAT CAC GCG CCT ATT ACG GCC TAC TCC-3′) with an *Nde I* restriction site, a hexa histidine tag, and NS4AFLR (5′-AAG CTT TCA GCA CTC TTC CAT CTC ATC G-3′), with a stop codon and *Hind III* restriction site at the end. Both the pET32a vector and the full length NS3/4A amplicon were processed with *Nde I* and *Hind III* enzymes and ligated to obtain the pET32NS3/4AFL expression construct.

E. coli BL21 Star (DE3) cells, transformed with pET32NS3/4AFL were grown at 37 °C in Luria broth containing 100 mg/mL ampicillin. Protein expression was induced with 0.5 mM IPTG at an OD600 of 0.6, and incubated overnight at 16 °C. The cells were harvested; lyzed by sonication in lysis buffer (50 mM HEPES; pH7.3, 20% glycerol, 0.5 M NaCl, 1 mM *b*-mercaptoethnol, 1% NP-40). The lysate was centrifuged at 18,000*g* for 1 h and the supernatant was incubated for 1 h with Talon metal affinity resin (Clontech Laboratories, Inc., Mountain View, CA) that was pre-equilibrated in the lysis buffer. After the incubation, the resin was washed with 20 column volumes of wash buffer (50 mM HEPES; pH7.3, 20% glycerol, 0.5 M NaCl, 20 mM imidazole, 1 mM *b*-mercaptoehanol, 0.1% NP-40). The fusion protein was then eluted from the resin using the elution buffer (50 mM HEPES; pH7.3, 20% glycerol, 0.5 M NaCl, 500 mM imidazole, 1 mM β-mercaptoehanol, 0.1% NP-40). The eluted fractions were checked for purity by SDS–PAGE and quantified spectrophotometrically. Fractions of highest concentrations were pooled and dialyzed against buffer containing 50 mM HEPES; pH7.3, 40% glycerol, 5 mM $MgCl_2$, 50 mM NaCl, 5 mM DTT and aliquots stored at −70 °C.

Purified full length recombinant NS3/4A protease was used in conjunction with the SensoLyte™ 490 HCV Protease Assay buffer and fluorogenic substrate components (Anaspec, Inc., San Francisco). The use of full length NS3/4A protein in the assay is prompted by the increasing evidence that the activities of NS3 protease and helicase domains are modulated by each other (Beran et al., 2007; Du et al., 2002; Lam et al., 2003; Kuang et al., 2004) and most likely more accurately models *in vivo* reactions. The reaction consisted of 20 nM purified enzyme and 1x HCV NS3/4A protease substrate, both diluted in 1× SensoLyte 490 HCV assay buffer. The HCV NS3/4A protease substrate was an EDANS/DABCYL FRET peptide which upon cleavage has excitation and emission maxima at 340 and 490 nm respectively. Fluorescence was measured after 30 min of incubation at room temperature. Analysis of inhibitors was performed by incubating test compounds with the enzyme for 15 min followed by 30 min incubation with the substrate. An established, clinically relevant, HCV protease inhibitor, VX-950 (telaprevir) (Perni et al., 2006) (purchased from Acme Bioscience, Inc., Palo Alto, CA) was used as an assay control.

2.6.2. HCV NS3 helicase

A double-stranded nucleic acid unwinding assay previously described (Kuang et al., 2004; Lam et al., 2003) was adopted with minor modifications. Full length NS3/NS4A protein was used to assay helicase activity. The helicase assay is dependent on the unwinding of a synthetic duplex DNA or RNA oligonucleotide substrate in which one of the strand is ^{32}P-labeled to enable radioactive detection after electrophoretic separation of the completed reactions.

The substrate DNA was generated by annealing an 18-base ^{32}P-labeled and an unlabeled 28-base oligonucleotide, creating a duplex substrate with a 3′-ssDNA overhang (required for helicase recognition and activity (Kuang et al., 2004; Lam et al., 2003; Rosales-Leon et al., 2007). The reaction consisted of 1 nM dsDNA substrate and 20 nM HCV helicase incubated in reaction buffer (25 mM MOPS, pH 6.5, 3 mM $MgCl_2$, 0.1% Tween 20) for 30 min at 37 °C. Unlabeled shorter oligonucleotides (250 nM) were used as trap DNA to prevent re-annealing of the unwound oligonucleotides (Kuang et al., 2004). The 10 μl reactions were initiated by the addition of 5 mM ATP and terminated by 2.5 μL of 5× stop buffer (250 nM Tris–HCl, pH 7.5, 20 mM EDTA, 0.5% SDS, 0.1% Nonidet p-40, 0.1% bromophenol blue, 0.1% Xylene cyanole FF, 50% glycerol). The reaction products were analyzed by non-denaturing 14% polyacrylamide gel electrophoresis. Helicase was measured by the relative quantitation of the radioactivity in the single-stranded reaction product and the duplex substrate using an Instant Imager™ Beta Scanner (PerkinElmer, Inc., Shelton, CT).

For the testing of inhibitors of HCV helicase, the compounds were diluted in dimethyl sulfoxide (DMSO), and pre-incubated with the enzyme for 15 min at 37 °C in the assay buffer. Activation of assay was initiated by the addition of substrate and ATP, followed by further incubation at 37 °C for 30 min. DMSO (0.1%) was added to the enzyme assay as a blank. Thioflavine S (Sigma–Aldrich, Inc.) was used as an inhibitor control. This dye is a non-proprietary compound that is reported to inhibit HCV helicase in a dose–response manner (Frick, 2010).

2.6.3. HCV NS5B polymerase

Several enzymatic assays for the HCV NS5B polymerase (RdRP) have been described (Behrens et al., 1996; Heck et al., 2008; Lohmann et al., 1997; Ludmerer et al., 2005). Most of these assays utilize either a non-specific RNA template, or a short template containing the 3′-UTR. The 5′-UTR also plays a pivotal role in the initiation of translation of the viral polyprotein (Brown et al., 1992; Bukh et al., 1992; Kolykhalov et al., 2000). A unique template for the HCV RdRP assay was created based on studies with *in vitro* Dengue Virus polymerase assays (You and Padmanabhan, 1999) that is believed to more closely model viral genome interactions between the 5′ and 3′ UTRs. This mini-genome (674 nucleotides) is comprised of the HCV 3′ and 5′-UTR's with small portions of the core (45 bp) and NS5B (28 bp) coding sequences as a linker. Particular advantages of this template scheme are that it allows the use of RNA templates with either genomic polarity, allows for genotype-specific matching of template and polymerase, and allows for the use of ^{32}P-labeled nucleotides other than UTP. NS5B was prepared and purified essentially as previously described (Behrens et al., 1996; Lohmann et al., 1997).

The *in vitro* RdRP assay was performed in 50-μl reaction mixtures using incorporation of [α-^{32}P]-UTP for 1 h at 30 °C into the RNA products. Reactions contained 300 ng of HCV RNA templates, 100 ng of NS5B proteins, 500 μM of each ATP, CTP and GTP, 10 μM of UTP, and 100 unit of RNase Out (Invitrogen, Inc., Carlsbad, CA) in 50 mM HEPES buffer (pH 7.3). The RNA products were isolated using the RNeasy Mini Kit (Qiagen Inc.), then ethanol precipitated, separated on 1.5% formaldehyde agarose gels (3 h at 100 V), and dried. Radioactivity was quantitated using an Instant Imager™ Beta Scanner. An established non-nucleoside NS5B inhibitor, HCV-796 (Howe et al., 2008) was used as an assay inhibitor control.

3. Results

3.1. HCV replicon sequence analysis

A total of 4 full length HCV clones and 10 individual clones of each of the two sub-length portions from the parental cell line, RP7, were examined, and a consensus sequence was established. The 5′-UTR was comprised of 341 nucleotides, the NS gene region contained 5958 nucleotides, and the 3′-UTR (consensus sequence) contained 237 nucleotides (6536 nucleotides total). Independent HCV clones from the RP7 cell line harbored an average of 1.7/1000 nucleotide differences (range of 0.3–5.2/1000) from the

consensus sequence scattered across the HCV genome, as is typically observed (Bradrick et al., 2006; Kieffer et al., 2010) for replicon HCV isolates due to the high error rate for the viral polymerase. Due to the variability known for specific regions of the 3′-UTR (Bradrick et al., 2006), an additional 10 clones from this region were sequenced for confirmation. Individual clones of the 3′-UTR contained numerous nucleotide differences in specific areas known for high variability, and the poly-U tracks within the 3′-UTR in individual clones exhibited length variability, again typically observed for HCV (Bradrick et al., 2006). The remainders of the 3′-UTR, as well as the complete 5′-UTR, were essentially completely conserved in the individual clones (Table 1). In no case did the full length HCV replicon clones display patterns of nucleotide variation that were different from the sub-length segments (data not shown).

A total of 10 (from TIZ-9) and 11 (from NTZ-11) individual HCV clones of each of the two sub-length portions and the 5′-UTR were sequenced. Individual clones contained numerous nucleotide differences from the HCV consensus sequence from the RP7 cell line, scattered along the genome: 5.5/1000 nucleotides (range 2.6–7.6/1000) for NTZ-11 and 3.6/1000 nucleotides (range 1.3–6.7/1000) for NTZ-11. Almost all of the nucleotide differences were present in only one or two independent clones. The variation observed in HCV clones from both NTZ-11 and TIZ-9 was significantly higher than that in HCV clones from the parental RP7 cell line ($p < 0.001$, one-tailed t test). With the exception of NS4A and the 3′UTR, variability in nucleotide sequence in individual HCV genes was also significantly greater among individual HCV clones from the NTZ-11 and TIZ-9 cell lines versus the parental RP7 cell line (Table 1). However, a high percentage of the nucleotide changes (in some instances more than 50%) were 'silent' (not resulting in an amino acid change) as reflected in the relatively lower number of amino acid changes (Table 1).

Table 2 presents nucleotide differences, and the resulting amino acid changes, in the HCV NS gene coding regions and the 3′-UTR, that were present in at least half of the individual HCV clones from the NTZ-11 and TIZ-9 cell lines. Several of these sequence changes (5 from NTZ-11, 4 from TIZ-9) were 'silent', resulting in no amino acid change. None of these amino acid changes appeared to have been previously associated with published mutations connected to HCV drug resistance to other investigative anti-HCV molecules (Fukuhara et al., 2010; Howe et al., 2008; Kieffer et al., 2010; Puyang et al., 2010; Sarrazin and Zeuzem, 2010; Tong et al., 2010). Most notably, none of the nucleotide differences or amino acid changes for the HCV NS coding regions or the 3′-UTR noted in Table 2 were common to HCV replicons in both the TIZ-9 and NTZ-11 cell lines. Essentially all of the nucleotide changes noted in Table 2 were absent from HCV clones from the parental RP7 cell line. The infrequent exceptions were G1823A (2 clones from RP7), T4404C (1 clone), A4507G (1 clone), and C4092T (1 clone).

Table 2
Frequent mutations observed in HCV replicon sequences in NTZ-resistant cell lines.

Protein	Nucleotide position	Amino acid change	Frequency[a]	Frequency in RP7 cell line[a]
NTZ-11 cell line				
NS3	T536C	–	6	0
	T680C	–	6	0
	T1071C	S243P	6	0
	C1181T	–	6	0
	G1823A	–	7	2
	T2203C	M620T	5	0
NS4A	T2347C	I37T	6	0
NS4B	G2957C	–	6	0
NS5A	T3291C	F35L	7	0
	T4143C	Y319H	7	0
	C4245T	P353S	9	0
	T4404C	S406P	9	1
	T4438C	L417P	7	0
	A4495G	E396G	8	0
	A4507G	E440G	8	1
NS5B	G5421A	A300T	7	0
	T6150G	S543A	8	0
TIZ-9 cell line				
NS3	A632G	–	5	0
	A2091G	K583E	7	0
NS4B	T3168A	C257S	8	0
NS5A	T3562C	F125S	8	0
	C4092T	R302W	7	1
	A4303G	E372G	6	0
NS5B	C5045T	–	5	0
	T5228A	–	5	0
	T5303C	–	5	0
	G5826A	A435T	6	0

Nucleotide differences from the RP7 consensus sequence observed in at least 50% of individual HCV replicon sequences isolated from NTZ-11 and TIZ-9 are listed. Nucleotides are numbered from the beginning of the 5′-UTR. The resultant amino acid changes in the indicated genes are also shown numbered for each individual coding sequence. Dashes indicate "silent' (no amino acid change) mutations.

[a] The numbers of individual clones carrying the indicated mutations are displayed. A total of 11 sets of sequences for each of NTZ-11 and RP7, and 10 for TIZ-9 were examined.

There was a cluster of nucleotide changes in a 5-base region (GGGGC) within the 5′-UTR observed in essentially all (20/21) of the individual clones from both the NTZ-11 (10/11 clones) and TIZ-9 (10/10 clones) cell lines (Fig. 1). The noted nucleotide changes present in the 5′-UTR were localized to one side of the stem of the 5′-proximal stem-loop (Luo et al., 2003) in this region (nucleotides 16–20) (Fig. 1).

3.2. Antiviral sensitivity of HCV replicons containing 5′-UTR mutations

The three most prevalent nucleotide changes noted in Fig. 1 were individually inserted into a 'wild-type' (CON1) HCV replicon background by site-directed mutagenesis and confirmed by sequence analysis of the entire 5′-UTR. The mutant replicons were

Table 1
Frequency of nucleotide and amino acid changes in HCV genes.

	Cell line source	Average difference (and range) from RP7 HCV consensus sequence observed in individual clones						
		5′-UTR	NS3	NS4A	NS4B	NS5A	NS5B	3′-UTR
Average number of Nucleotide changes Per clone (range)	RP7	0.3 (0–1)	5.7 (0–10)	0.3 (0–2)	1.7 (0–5)	4.0 (0–8)	4.5 (1–7)	0.8 (0–2)
	NTZ	1.8 (0–3)$^{\#}$	12 (6–16)$^{@}$	0.7 (0–2)	4.5 (2–8)$^{\#}$	11 (6–15)*	7.8 (5–12)$^{\#}$	0.4 (0–1)
	TIZ	1.1 (0–2)$^{\#}$	6.9 (3–12)	0.7 (0–2)	4.0 (1–6)$^{\$}$	8.2 (4–12)$^{\#}$	8.4 (3–11)$^{@}$	0.6 (0–2)
Average number of Amino acid changes Per clone (range)	RP7		3.2 (0–7)	0.2 (0–1)	0.8 (0–2)	2.8 (0–6)	2.2 (0–5)	
	NTZ		5.0 (1–8)	0.6 (0–2)	2.2 (1–4)$^{\#}$	8.3 (6–11) *	4.1 (0–8)$^{\#}$	
	TIZ		3.7 (1–7)	0.3 (0–2)	2.4 (1–3)$^{\$}$	6.0 (3–9)$^{\#}$	4.1 (1–7)$^{@}$	

The average number of nucleotide and amino acid changes in each HCV gene and the UTRs is indicated. Values in parentheses indicate the ranges of values among the clones analyzed. Significant differences from RP7 as determined by one-tailed t test: $^{@}p < 0.05$, $^{\#}p < 0.01$, $^{\$}p < 0.005$, $^{*}p < 0.001$. The number of nucleotides in the UTRs and genes are as follows: 5′UTR (341), NS3 (1896), NS4a (162), NS4B (783), NS5A (1341), NS5B (1776), 3′UTR (237).

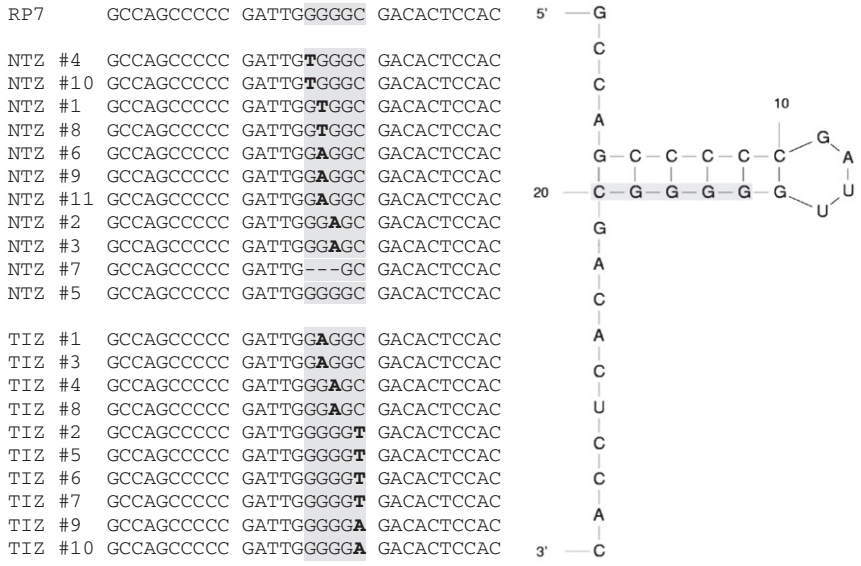

Fig. 1. Mutations observed in the 5′-UTR of individual HCV cDNAs isolated from NTZ-resistant cell lines NTZ-11 and TIZ-9. The top line displays the consensus RNA sequence of the first 30 nucleotides of the 5′-UTR for HCV replicons present in the parental cell line, RP7. This sequence was completely conserved in the 14 clones isolated from RP7 (data not shown). Differences from the RP7 consensus sequence are noted in bold. One clone (NTZ #7) harbored a small deletion in this region. The right side of the figure displays the predicted structure ('mfold' program, http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi) of the 5′-proximal stem-loop where the mutations are located (shaded regions).

analyzed for antiviral sensitivity in a transient transfection assay in naive Huh7.5 cells (Apath, LLC, Brooklyn, NY) (Korba et al., 2008a). Replicons containing each of the three 5′-UTR mutations appeared to be viable, but at highly different levels. Colony formation efficiency by replicons containing the G17A mutation appeared to be the most attenuated (approximately 50-fold less) with respect to the parental (wild-type) replicons (Table 2). Colony formation efficiency by replicons containing the other two mutations was approximately 8-fold (G18A) and 5-fold (C20U) lower than wild-type (Table 2). Previous studies examining deletions and multiple sequence alterations (but not single-base changes) of this stem-loop structure had demonstrated that it was essential for HCV viability in cell culture (Luo et al., 2003).

Colony formation by replicons containing any of the three 5′-UTR mutations appeared to be equally sensitive to both alpha interferon and NTZ as colony formation induced by the parental replicons (Table 3, Fig. 2). This is consistent with the previous observation that HCV replicons transfected in whole cell RNA from the two resistant cell lines also did not display an NTZ-resistant phenotype (Korba et al., 2008a). Of note is that 5.0 μM NTZ, a concentration that is one-half that used to culture the NTZ-11 and TIZ-9 cell lines, prevented all colony formation by the HCV constructs in naive cells.

3.3. Antiviral sensitivity of HCV replicons in cured cell lines

Data in this and a previous report (Korba et al., 2008a) indicate that the primary HCV NTZ-resistance phenotype of NTZ-11 and TIZ-9 is associated with host, not viral, factors. To directly test this hypothesis, both of these NTZ-resistant cell lines, as well as the parental RP7 cells, were 'cured' of HCV replicons by eight serial passages in the presence of 1000 IU/ml recombinant interferon alfa-2b (PBL Laboratories, Inc.). NTZ was also maintained at 10 μM for NTZ-11 and TIZ-9 cells during curing. Loss of HCV RNA was confirmed by PCR analysis (data not shown). In addition, culturing or mock transfections of all three cured cell lines in 6-well culture plates failed to produce G418-resistant colonies (initial population of 1–2 × 10^6 cells, data not shown).

G418-resistant colony formation efficiency of whole cell RNA from RP7, NTZ-11, or TIZ-9 cells displayed a pattern of resistance to NTZ, but not interferon, when transfected into cured versions of either NTZ-11 or TIZ-9 (Table 4, Fig. 2). The colony formation efficiency of these same RNA preparations was sensitive to both NTZ and interferon when transfected into the cured version of the parental cell line, RP7 (Table 4). Colony formation of 'wild-type' HCV replicon RNA was also resistant to NTZ following transfection into cured versions of NTZ-11 and TIZ-9, but sensitive to NTZ after

Table 3
Colony forming efficiency of HCV replicon RNA carrying 5′UTR mutations.

Replicon	Colony formation efficiency (% average of untreated control)													
	Total colonies (% WT)		Untreated		IFN-2.0 IU/ml		IFN-20 IU/ml		NTZ-0.2 μM		NTZ-1.0 μM		NTZ-5.0 μM	
	Avg.+S.D	Range	Avg.+S.D	Range	Avg.+S.D	Range	Avg.+S.D	Range	Avg.+S.D	Range	Avg.±S.D	Range	Avg.+S.D	Range
WT (CON1)	100 ± 8	(87–113)	100 ± 7	(87–113)	38 ± 9	(28–53)	10 ± 3	(7–13)	42 ± 9	(32–51)	10 ± 2	(8–14)	0	–
5′UTR G17A	2 ± 1	(1–3)	100 ± 12	(80–120)	41 ± 7	(32–48)	8 ± 4	(6–13)	45 ± 11	(32–54)	12 ± 8	(10–21)	0	–
5′UTR G18A	13 ± 3	(7–14)	100 ± 7	(88–113)	36 ± 9	(27–52)	6 ± 5	(3–13)	44 ± 7	(35–52)	16 ± 9	(9–22)	0	–
5′UTR C20U	24 ± 3	(18–27)	100 ± 6	(92–108)	31 ± 5	(28–38)	9 ± 4	(3–12)	42 ± 10	(31–54)	13 ± 4	(9–21)	0	–

Huh7.5 cells were transfected with the indicated HCV replicon RNAs (0.1–3.0 μg/well) as described in Section 2. Colony formation is expressed as a percentage of the average number of clones present in the untreated (control) transfections for each replicon RNA (independently set at 100%). The range of colony formation for each treatment condition in individual wells (4–8 replicates in 2–4 experiments) is also listed. The first entry lists the average colony formation efficiency (relative to the parental replicon, set at 100%) for each mutant construct.

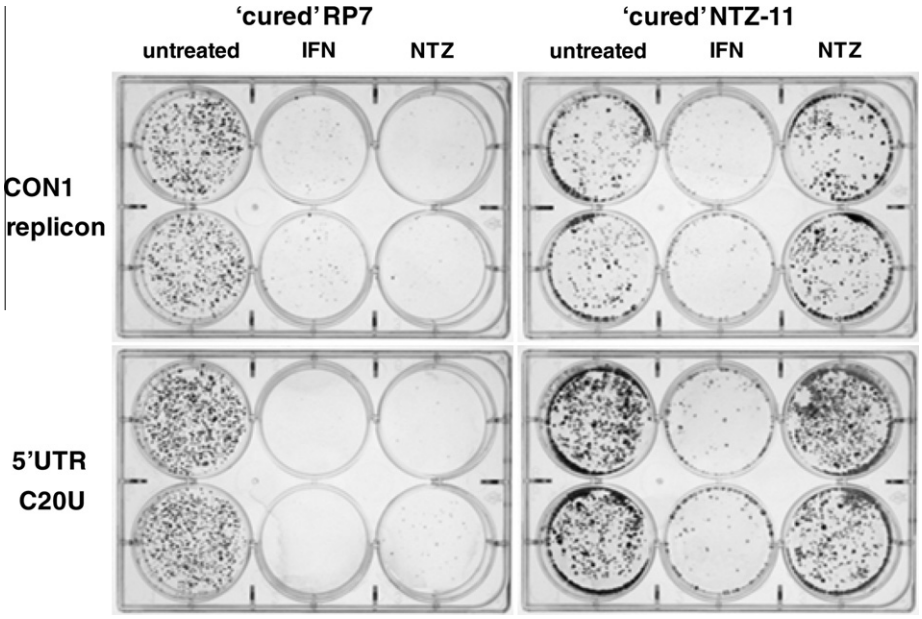

Fig. 2. Examples of HCV replicon-induced colony forming efficiency in replicon-cured cell lines. Transfections and treatments were performed as described in Section 2. IFN, interferon alpha (20 IU/ml), NTZ, nitazoxanide (2 μM).

Table 4
Colony forming efficiency of HCV replicon RNA in 'cured' cell lines.

Cell line	RNA	Untreated	Colony formation (% average untreated ± S.D.)				
			Interferon (IU/ml)		Nitazoxanide (μM)		
			2.0	20	1.0	2.0	5.0
Whole cell RNA							
Cured	RP7	100 ± 6	57 ± 5	9 ± 1	12 ± 1	6 ± 2	1
RP7	NTZ-11	100 ± 11	61 ± 1	8 ± 2	11 ± 2	8 ± 3	1 ± 1
	TIZ-9	100 ± 5	56 ± 6	8 ± 1	9 ± 1	7 ± 1	1 ± 1
Cured	RP7	100 ± 19	55 ± 4	8 ± 2	104 ± 18	94 ± 10	71 ± 5
NTZ-11	NTZ-11	100 ± 14	60 ± 2	8 ± 2	96 ± 13	93 ± 25	77 ± 8
	TIZ-9	100 ± 10	59 ± 4	8 ± 2	94 ± 6	90 ± 7	86 ± 5
Cured	RP7	100 ± 9	48 ± 3	8 ± 2	117 ± 11	107 ± 12	83 ± 2
TIZ-9	NTZ-11	100 ± 9	54 ± 3	9 ± 2	99 ± 5	91 ± 7	74 ± 4
	TIZ-9	100 ± 6	50 ± 5	7 ± 2	91 ± 10	89 ± 8	76 ± 5
Replicon RNA							
Cured	wt	100 ± 6	57 ± 5	9 ± 1	12 ± 1	6 ± 2	1
RP7	G18A	100 ± 9	48 ± 6	7 ± 2	10 ± 2	4 ± 1	1 ± 1
	C20U	100 ± 11	47 ± 2	7 ± 4	10 ± 1	7 ± 1	1 ± 1
Cured	wt	100 ± 7	55 ± 6	9 ± 1	93 ± 3	99 ± 8	81 ± 2
NTZ-11	G18A	100 ± 4	55 ± 5	10 ± 3	103 ± 8	102 ± 11	78 ± 7
	C20U	100 ± 7	28 ± 1	8 ± 2	91 ± 5	90 ± 4	73 ± 4
Cured	wt	100 ± 5	49 ± 6	10 ± 1	105 ± 14	91 ± 7	80 ± 6
TIZ-9	G18A	100 ± 5	47 ± 6	9 ± 1	105 ± 9	95 ± 8	83 ± 5
	C20U	100 ± 10	40 ± 3	9 ± 2	105 ± 7	92 ± 6	74 ± 8

transfection into Huh7 or the cured version of RP7 (Table 4). A similar pattern of resistance and sensitivity to NTZ was observed for colony formation following transfection of HCV replicons carrying the 5′UTR G18A or C20U mutations (Table 4). Due to the overall inefficiency of colony formation, HCV replicons containing the 5′UTR G17A mutation were not examined in these studies.

3.4. Sensitivity of HCV enzymatic activities to TIZ

To help to determine if tizoxanide (TIZ), the active metabolite of NTZ, directly targets HCV functions, the activity of TIZ in three HCV enzymatic assays was performed. TIZ was utilized in these enzymatic assays at concentrations (Korba et al., 2008b) that ranged from approximately the EC_{50} (0.2 μM) to 10× the EC_{90} (10 μM) concentrations in cell culture. TIZ did not appear to have any inhibitory activity against HCV NS3 protease or helicase activity (Table 4) in these studies. TIZ did appear to have a highly variable inhibitory effect on HCV NS5B polymerase activity at the highest concentrations used (Table 5. In 2 of 5 experiments TIZ inhibited HCV NS5B RdRp activity with an IC_{50} of 3.9 ± 0.4 μM, but in the remaining analyses, TIZ did not appear to have any inhibitory activity (data not shown). Using data combined from all 5 sets of reactions, the IC_{50} against NS5B polymerase activity was 8.8 ± 2.6 μM (Table 5).

Table 5
Effect of tizoxanide against HCV enzymatic activities.

Compound	Conc. (μM)	Relative HCV enzyme activity (% control ± S.D.)		
		NS3/4A protease	NS3 helicase	NS5B polymerase
None (0.1% DMSO)	10	100 ± 7	100 ± 2	100 ± 5
Tizoxanide	0.1	98 ± 7	99 ± 5	84 ± 2
	1.0	96 ± 6	105 ± 8	94 ± 10
	10	91 ± 10	104 ± 6	36 ± 33
VX-950	0.1	19 ± 5	–	–
Thioflavin-S	50	–	7.0 ± 0.9	–
HCV-796	1.0	–	–	8.0 ± 1.0

HCV enzymatic activities in *in vitro* assays were assessed as described in Section 2.

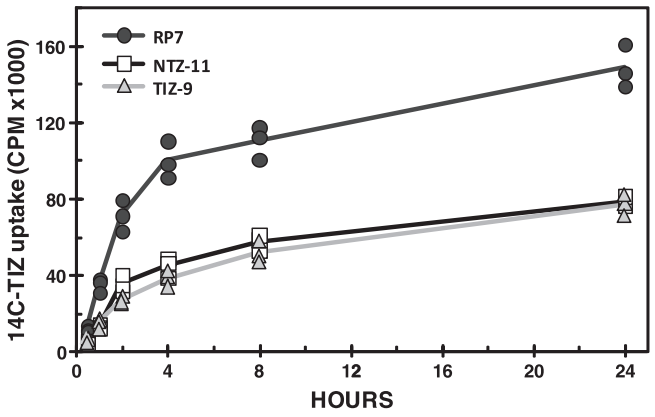

Fig. 3. Uptake/binding of ^{14}C-labeled in cell cultures. Cells were exposed to ^{14}C-TIZ for the indicated times and harvested as described in Section 2. ^{14}C levels for each of three replicate cultures at each time point are displayed. Lines denote average levels. Differences between RP7 and either NTZ-11 or TIZ-9 were significant at all time points ($p = 0.002$ to 0.015, two-tailed t test (MS Excell™) at various time points).

3.5. Cellular uptake/binding of TIZ

It is possible that the observed resistance of HCV to NTZ in NTZ-11 and TIZ-9 is due to a failure of cellular uptake/binding, as has been recently observed for ribavirin in certain resistant HCV replicon cell lines (Pfeiffer and Kirkegaard, 2005). To examine this possibility, cultures were exposed to ^{14}C-labeled TIZ for 30 min to 24 h, and levels of TIZ associated with the cells determined. At all time points examined, binding and/or uptake of ^{14}C-TIZ by the two TIZ-resistant HCV replicon cell lines (NTZ-11, TIZ-9) was approximately one-half of that observed for the parental HCV replicon cell line, RP7 (Fig. 3) ($p = 0.015$ to 0.02 (two-tailed t test (MS Excell™)) for either cell line at any time point). In these studies, it was not possible to distinguish between cellular binding and intracellular uptake.

4. Discussion

Previous studies demonstrated that HCV replicon-containing cell lines resistant to the antiviral effects of NTZ/TIZ can be selected, but attempts to transfer NTZ-resistance by transfection of HCV genomes in whole cell RNA preparations from NTZ-resistant cells to naive cell cultures were unsuccessful (Korba et al., 2008a). These studies suggested, but did not actually demonstrate, that the observed NTZ-resistance phenotype is most likely due to changes in host factors. However, it is possible that a combination of viral mutations that do not confer significant drug-resistance in naive cells and host factors within drug-treated cells could be responsible for the NTZ resistance phenotype. To resolve this issue, sequence analysis of complete HCV replicon sequences from two NTZ/TIZ-resistant cell lines was performed, and the HCV antiviral sensitivity patterns of isolates of the two resistant cell lines following 'curing' of HCV replicons with interferon was examined.

The data in the current series of investigations confirm previous speculations that HCV resistance to NTZ in replicon cell culture is not due to mutations in the virus. Together with the observation that TIZ does not directly affect HCV enzymatic activities and the broad spectrum of antiviral activity exhibited by NTZ (Korba et al., 2008b; Rossignol, 2009), these data are consistent with earlier hypotheses that the antiviral activity of this drug is due to its interactions with a cellular target(s) that are yet to be identified.

No nucleotide changes in the NS genes or the 3′-UTR relative to the consensus HCV sequence from the parental cell line common to both NTZ-resistant cell lines were observed. Sequence analysis of HCV genomes revealed a cluster of single base changes localized in a 5 base region in the 5′-UTR of essentially all HCV sequences from both NTZ-11 and TIZ-9. However, these mutations did not confer an NTZ-resistance phenotype when inserted into a 'wild-type' HCV background. This data is consistent with previous studies using HCV replicons in whole cell RNA preparations from these two cell lines (Korba et al., 2008a), and eliminates these mutations as candidates to explain the NTZ-resistant phenotype. Why these mutations were present in all HCV genomes examined is not known at the current time, but does lead to the speculation that TIZ somehow influences host/virus interactions at this site in the 5′-UTR.

The 5′-proximal stem-loop in the 5′-UTR where the mutations are located has previously been shown to be essential for HCV replication (Luo et al., 2003). Unlike our studies, the previous investigations involved deletions of the entire stem or multi-base substitutions of this 5-base region which most likely accounts for our ability to detect limited viability for the mutants. Analysis by the M-fold program (Mathews et al., 1999; Zuker, 2003; http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi) confirms that each of the observed single base changes would, in theory, disrupt the stem-loop structure to varying degrees depending on the location of the base changes (data not shown).

Interestingly, this same 5-base sequence in the 5′proximal stem loop has been identified as a binding site for NS5A (Huang et al., 2005), but the relevance of this finding to NTZ resistance, if any, is unclear at this time. There are several additional NS5A binding sites located in the 5′-UTR (Huang et al., 2005), but all of the remaining NS5A binding sequences in the 5′-UTR were conserved in all of the individual clones examined from all three cell lines. This result leads to speculation that TIZ may indirectly or directly, affect or interact with HCV NS5A under specific circumstances, but this remains to be demonstrated. It must be noted that, while several nucleotide changes were noted in NS5A in the two resistant cell lines, no changes common to both resistant cell lines were observed in this HCV coding sequence. It is possible to argue that one or more of the mutations observed in NS5A (or the other HCV genes) in the resistant cell lines, in combination with the observed changes in the 5′-UTR, could confer an NTZ resistance phenotype in naive cells. However, given the complexity and number of the potential combinations of mutations, this issue would be extremely difficult to address and not likely to be successful given that the original populations of HCV replicons in whole cell RNA did not confer NTZ resistance (Korba et al., 2008a).

The fact that HCV replication NTZ resistance following transfection of multiple sources of viral RNA into replicon-cured versions of NTZ-11 and TIZ-9 demonstrates this phenotype is a property of alterations in (as yet unidentified) host factor(s). This is consistent with the observations that TIZ did not have any apparent

activity against HCV protease, helicase, or polymerase in enzymatic assays. The inconsistent inhibition of NS5B RdRp activity at very high concentrations (20–40X the culture EC_{50}), would not reasonably be expected to be biologically relevant.

It is not yet determined if the modest impairment (2-fold) of the cellular uptake/binding of TIZ by NTZ-11 or TIZ-9 relative to the parental cell line RP7 can fully account for the observed HCV NTZ-resistance. Both NTZ-11 and TIZ-9 exhibit reduced sensitivity to the cytotoxic effects of NTZ and TIZ (3–5-fold elevations in CC50 compared to RP7; Korba et al., 2008a). Most likely, alterations in other cell processes are also involved. For example, NTZ has been shown to enhance the levels of eIF2 alpha, a principle element of the host UPR, in HCV replicon-containing cells, and to enhance auto-phosphorylation of PKR in enzymatic assays (Elazar et al., 2009). The status of the cellular pathways related to these regulatory factors in the resistant cell lines may prove fruitful in identifying the host factors involved in the TIZ-resistance phenotype. HCV resistance to NTZ is different from other host-targeting anti-HCV agents, such as the cyclophilin inhibitors, where resistance appears to be primarily due to mutations in viral sequences (Puyang et al., 2010).

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