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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.