Nitazoxanide and its primary metabolite, tizoxanide, inhibit hepatitis C virus (HCV) replication in HCV replicon systems. To study the potential for resistance, we subjected Huh7 cells harboring HCV replicons to serial passage in 250 μM G418 and increasing concentrations of nitazoxanide or tizoxanide. Passage of the replicon-containing cell lines in either compound resulted in increases in the 50% effective concentrations (EC_{50}) (7- to 13-fold), EC_{90} (14- to 36-fold), and 50% cytotoxic concentrations (2- to 4-fold) of both compounds. Serial passage in either compound did not alter the susceptibility of HCV replicons to ribavirin or 2'-C-methylcytidine. Interestingly, serial passage in nitazoxanide or tizoxanide resulted in increased sensitivity to alpha interferon 2b: EC_{90} and EC_{90} were reduced three- and eightfold, respectively. Replicons isolated from these cell lines had no greater ability to confer tizoxanide resistance, or increased susceptibility to alpha interferon, than replicons isolated from the parental cell line that had not previously been exposed to nitazoxanide or tizoxanide. These findings are indicative of a cell-mediated activity differing from that of other anti-HCV drugs but complementary with interferon and are consistent with the enhanced response rates observed clinically when nitazoxanide is combined with pegylated interferon therapy. Finally, unlike data for other compounds in advanced clinical development for HCV, these data are consistent with resistance in HCV replicon-containing cell lines conferred by changes in the host and not by mutations in the virus.

Nitazoxanide (NTZ) is a thiazolide antiinfective with activity against a broad range of anaerobic bacteria, protozoa, and viruses (3, 6, 9, 10). While NTZ was originally developed as a treatment for intestinal protozoan infections, its antiviral properties were discovered during the course of its development for treating cryptosporidiosis in patients with AIDS. NTZ is undergoing clinical development for the treatment of chronic hepatitis C and has shown promising results as adjunct therapy with peginterferon or peginterferon plus ribavirin (RBV) in treating patients with chronic infections with hepatitis C virus (HCV) genotype 4 (8).

NTZ and its circulating metabolite, tizoxanide (TIZ), inhibit the replication of HCV in HCV genotype 1a- and 1b-derived replicon cells. Median 50% effective concentrations (EC_{50}) of approximately 0.13 and 0.15 μM for NTZ and TIZ, respectively, were found in these replicon systems (4). NTZ and TIZ exhibit synergistic activity with alpha interferon and 2'-C-methylcytidine (2'C-MeC) and are effective against HCV replicons that are resistant to 2'C-MeC and telaprevir (VX-950) (4). Furthermore, lead-in treatment of replicon-containing cells with NTZ potentiates the effect of subsequent combination treatment with NTZ plus alpha interferon (4). Antiviral mechanisms are under investigation, but the broad antiviral spectrum of TIZ is consistent with activity on host processes.

In this report, we present results of in vitro studies conducted to evaluate the activities of NTZ, TIZ, alpha interferon, RBV, and 2'C-MeC against HCV replication in HCV replicon-containing cell lines after serial passage in increasing concentrations of NTZ or TIZ. To determine whether the observed phenotypic TIZ resistance, and the associated increased interferon sensitivity, results from mutations within the HCV genome or from cellular adaptations, we transfected whole-cell RNA from TIZ-sensitive and TIZ-resistant HCV replicon-containing cells into naïve Huh7.5 cells and measured the efficiency of HCV colony formation in the presence of TIZ or alpha interferon.

MATERIALS AND METHODS

Compounds. NTZ and TIZ were provided by Romark Laboratories, L.C. (Tampa, FL). Recombinant interferon alpha 2b (IFN) was purchased from PBL Biomedical Laboratories (Piscataway, NJ). RBV was purchased from Sigma-Aldrich (St. Louis, MO). 2'C-MeC, a nucleoside inhibitor of the HCV NS5B polymerase (7), was purchased from Moravek Biochemicals, Inc. (La Brea, CA).

Cell lines. A parental replicon-containing cell line was established by electroporation of RNA transcribed in vitro from the Sca-I-linearized Bart79I plasmid into Huh7 cells (2). Bart79I encodes a second-generation high-efficiency bicistronic subgenomic replicon of genotype 1b containing a single adaptive mutation (S1791) in the NS5A gene and the neomycin phosphotransferase gene in the first cistron. The electroporated cells were plated along with naïve Huh7 feeder cells and grown in a medium consisting of Dulbecco’s modified Eagle’s medium (4.5 g/liter glucose, 1-glutamine, and sodium pyruvate) (Mediatech 10-013-CV), 10% fetal bovine serum, 1% penicillin-streptomycin, 1% l-glutamine (final concentration, 2 mM), and 1% minimal essential medium nonessential amino acids (100X) (Invitrogen), to which 1 mg/ml of G418 was added. After 3 weeks, G418-resistant colonies appeared. One of the resulting colonies was isolated, expanded, passaged in 700 μg/ml G418, and termed RP7.

Serial passage in NTZ or TIZ. In an effort to produce NTZ- or TIZ-resistant replicons, RP7 cells were subjected to a resistance-promoting regimen as follows. The cells were grown in the medium described above containing 700 μg/ml G418 (Invitrogen), 1% tissue culture-grade dimethyl sulfoxide (Sigma), and an initially low concentration of the drug (NTZ or TIZ), which was then steadily increased every week. On days 1 through 5 of each week, the medium was changed daily to provide a source of fresh drug. No medium changes were performed on days 6 and 7. The initial drug concentration was 0.02 μM, followed by 0.05, 0.1, 0.5, and 1 μM, and subsequent weekly increases of 1 μM until a final concentration of 11 μM was reached.
μM for NTZ or 9 μM for TIZ was reached. During selection, individual cultures were passaged upon reaching confluence. Two representative cell lines were chosen from several isolates for detailed analyses. The resulting cells were subsequently passaged at the final concentrations for at least 2 months.

**Determination of anti-HCV activity and cytotoxicity.** Antiviral activity for each test compound and for combination treatments was determined as previously described (4, 5). Briefly, replicon cell lines were maintained as subconfluent cultures on 96-well plates. Compounds were added daily for 3 days in fresh medium. Twenty-four hours after the last dose of compound, antiviral activity was determined by blot hybridization analysis of intracellular HCV RNA, and cytotoxicity was assessed by neutral red dye uptake. EC_{50}, EC_{90}, and 50% cytotoxicity concentrations (CC_{50}) for each test compound were calculated by linear regression analysis using data combined for all treated cultures. Antiviral and toxicity assays utilized triplicate cultures for each drug concentration; 12 untreated cultures were included in each assay. HCV and β-actin RNA quantitation standards were included on each individual hybridization blot.

EC_{50} and EC_{90} are defined as the drug concentrations producing 50% and 90% reductions, respectively, in the levels of intracellular HCV RNA relative to the average levels in untreated cultures. CC_{50} is defined as the drug concentration producing a 50% reduction in neutral red dye uptake relative to the average levels in untreated cultures. The selectivity index is calculated as the CC_{50} divided by the EC_{50}.

**Transfection assays.** Whole-cell RNAs were prepared from the RP7 parental cell line and from cell lines passaged and maintained in either 11 μM NTZ or 9 μM TIZ (the NTZ-11 and TIZ-9 cell lines, respectively) by using Qiagen RNA preparation columns. Huh7.5 cells maintained on 60-mm-diameter culture dishes were transfected with 1.0 to 2.0 μg whole-cell RNA by using the Lipofectamine 2000 reagent according to the manufacturer’s instructions. Four days posttransfection, cells were exposed to 125 μg/ml G418 and the indicated concentrations of TIZ, IFN, or 2'C-MeC for an additional 10 to 14 days. Media containing the test compounds were replaced at 48- to 72-h intervals. Colonies were stained using crystal violet dye and were counted manually. Three plates were used for each experimental point. Despite the observation that basal HCV replicon levels were equivalent in all three cell lines, the G418 concentration was reduced from 250 μg/ml to 125 μg/ml for the transfection studies to accommodate potential reductions in fitness for HCV replicons from the resistant cell lines, such as have been observed for other drug-resistant variants (11). This lower level of G418 is sufficient to completely suppress Huh7.5 colony growth following either mock transfection or control transfection with RNA derived from Huh7.5 cells (data not shown). The number of colonies in each drug-treated dish was compared with the mean number of colonies obtained for each source RNA in the absence of drug treatment in order to express colony formation as a percentage of that in untreated controls.

**Solubilization and handling of test compounds.** TIZ and 2'C-MeC were solubilized in 100% tissue culture-grade dimethyl sulfoxide (Sigma). IFN was solubilized and/or diluted in sterile phosphate-buffered saline–1% bovine serum albumin according to the manufacturer’s instructions. Stock solutions of each compound (0.2 mM for TIZ, 10 mM for 2'C-MeC, and 10,000 IU/ml for IFN) were stored (at −70°C for IFN, 4°C for TIZ, and −20°C for 2'C-MeC) in quantities sufficient for a single experiment and were used only once. Aliquots of test compounds were made from the stock solutions in individual tubes and stored at the appropriate temperatures. On each day of treatment, aliquots of the test compounds were suspended in the culture medium at room temperature and immediately added to the cell cultures, thereby subjecting each aliquot of test compound to the same, limited number of freeze-thaw cycles.

**RESULTS**

The antiviral activities and cytotoxicities of NTZ, TIZ, IFN, RBV, and 2'C-MeC in the RP7, NTZ-11, and TIZ-9 cell lines are summarized in Table 1. The basal levels of HCV RNA in all three cell lines were equivalent (Table 1). Both of the resistant replicon cell lines (NTZ-11 and TIZ-9) exhibited lower sensitivities to the cytotoxic (2- to 4-fold) and antiviral (7- to 34-fold) effects of NTZ or TIZ than the parental replicon cell line, RP7. The relative sensitivities of HCV replication to either RBV or 2'C-MeC in NTZ-11 and TIZ-9 were equivalent to that observed for RP7. However, the potency of IFN against HCV replication in the NTZ-11 and TIZ-9 cell lines was enhanced three- to eightfold over that observed in RP7 cells.

To test the hypothesis that the decreased sensitivity to TIZ and the increased sensitivity to IFN in the NTZ-11 and TIZ-9 cell lines resulted from genetic mutations within the HCV replicons harbored in those cells, we transfected naïve Huh7.5 cells with total RNAs extracted from the NTZ-11, TIZ-9, and RP7 cell lines. HCV-induced colony formation efficiencies were then determined in the presence of G418 and TIZ or IFN. As a control, the colony formation efficiencies of HCV replicon RNAs from the various sources in the presence of 2'C-MeC, which had equal potency in the parental and TIZ-resistant cell lines, were also determined.

As shown in Table 2, there were no significant differences in the sensitivity of colony formation to TIZ, IFN, or 2'C-MeC based on the source of transfected RNA. Levels of 100 IU/ml IFN or 10 μM TIZ completely abolished colony formation regardless of the RNA source used for transfections. No significant differences were noted in the relative numbers of colonies produced by RNA extracted from the resistant versus parental cell lines (Table 2).

**DISCUSSION**

After serial passage of the genotype 1b replicon-containing cell line RP7 in either NTZ or TIZ, the relative sensitivities of
TABLE 2. HCV-induced colony formation efficiencies in Huh7.5 cells transfected with RNA from parental or thiazolide-resistant HCV replicon-containing cell lines

<table>
<thead>
<tr>
<th>RNA source</th>
<th>TIZ 1 µM</th>
<th>TIZ 10 µM</th>
<th>IFN 10 IU/ml</th>
<th>IFN 100 IU/ml</th>
<th>2°C-MeC (10 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP7</td>
<td>7 ± 4</td>
<td>0</td>
<td>4 ± 2</td>
<td>0</td>
<td>10 ± 2</td>
</tr>
<tr>
<td>NTZ-11</td>
<td>8 ± 3</td>
<td>0</td>
<td>4 ± 2</td>
<td>13 ± 5</td>
<td>9 ± 2</td>
</tr>
<tr>
<td>TIZ-9</td>
<td>12 ± 4</td>
<td>0</td>
<td>7 ± 1</td>
<td>9 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

*Expressed as the mean percentage of colony formation in untreated cells ± standard deviation. Results were compiled from five independent experiments. The relative levels of colonies produced in these experiments in the absence of treatment were as follows: with RP7 RNA, 100% (control level); with NTZ-11 RNA, 103% (range, 73 to 143% of control); with TIZ-9 RNA, 95% (range, 82 to 130% of control).

the resultant cell lines to the cytotoxic and anti-HCV effects of NTZ or TIZ decreased severalfold. The susceptibilities of HCV replicons in the resistant cell lines to RBV or 2′-MeC did not change. However, HCV replication in these cell lines was severalfold more sensitive to alpha interferon than that in the parental cell line. The latter finding was consistent with the results of earlier studies showing that lead-in treatment of HCV replicon-containing cell lines with NTZ potentiated the activity of subsequent treatment with NTZ plus IFN (4). These results are also consistent with recent clinical data where increased virologic response rates were observed when NTZ was administered before and during treatment with pegylated interferon (with or without RBV) (8). The mechanisms of the interactions between TIZ and IFN with respect to HCV inhibition are currently under investigation.

Significantly, while we were able to generate HCV replicon-containing cell lines with phenotypic resistance to NTZ and TIZ by serial passage in increasing concentrations of NTZ or TIZ, we were unable to transfer the resistance by transfection of viral RNAs isolated from the TIZ-resistant replicon-containing cell lines. Thus, although sequence changes might be expected to arise during long-term cell passaging due to the error-prone HCV polymerase (extensive sequence analysis is ongoing), it is unlikely that introduction of any of these mutations into the original wild-type replicons will confer phenotypic resistance to NTZ upon introduction into naïve Huh7 cells. Rather, consistent with the increase in CC50 observed in cells after passage in NTZ or TIZ (Table 1), current data support the interpretation that the observed resistance phenotype is most likely primarily related to (as yet unidentified) changes in the host. The apparent absence of acquired resistance within HCV genomes exposed to NTZ or TIZ is in marked contrast to the rapid emergence of resistance upon treatment with other anti-HCV agents in advanced clinical development, such as inhibitors of NS3 protease or of NS5B polymerase, where specific mutations within the HCV genome arise and are known to be responsible for mediating the observed resistance phenotype following transfection into HCV-naïve Huh7 cell cultures.

Overall, our findings suggest that NTZ and TIZ inhibit HCV replication by a cell-mediated mechanism that differs from that of classical virus-specific drugs but is complementary to that of alpha interferon. The potential for use of this class of drugs in treating chronic hepatitis C is promising and supports further clinical investigation.

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REFERENCES

1. Reference deleted.