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Nitazoxanide, tizoxanide and other thiazolides are potent inhibitors of hepatitis B virus and hepatitis C virus replication

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Abstract

Nitazoxanide (NTZ), a thiazolide anti-infective, is active against anaerobic bacteria, protozoa, and a range of viruses in cell culture models, and is currently in phase II clinical development for treating chronic hepatitis C. In this report, we characterize the activities of NTZ and its active metabolite, tizoxanide (TIZ), along with other thiazolides against hepatitis B virus (HBV) and hepatitis C virus (HCV) replication in standard antiviral assays. NTZ and TIZ exhibited potent inhibition of both HBV and HCV replication. NTZ was equally effective at inhibiting replication of lamivudine (LMV) and adefovir dipovoxil (ADV)-resistant HBV mutants and against 2′-C-methyl cytidine (2′CmeC) and telaprevir (VX-950)-resistant HCV mutants. NTZ displayed synergistic interactions with LMV or ADV against HBV, and with recombinant interferon alpha-2b (IFN) or 2′CmeC against HCV. Pre-treatment of HCV replicon-containing cells with NTZ potentiated the effect of subsequent treatment with NTZ plus IFN, but not NTZ plus 2′CmeC. NTZ induced reductions in several HBV proteins (HBsAg, HBeAg, HBcAg) produced by 2.2.15 cells, but did not affect HBV RNA transcription. NTZ, TIZ, and other thiazolides are promising new antiviral agents that may enhance current or future anti-hepatitis therapies.

Keywords: Hepatitis B virus; Hepatitis C virus; Nitazoxanide; Thiazolides; Antiviral therapy

1. Introduction

Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) are major public health problems, causing more than an estimated 500 million chronic infections worldwide (Chen and Morgan, 2006; Lavanchy, 2004). Both viruses are a source of significant progressive liver disease, and are the major risk factors for nearly all cases of primary hepatocellular carcinoma (Chen and Morgan, 2006; Lavanchy, 2004; Wong and Lok, 2006). Licensed standards of care for both viral infections, while effective in many cases, are sub-optimal and do not result in virologic or clinical 'cures' in most individuals (Wong and Lok, 2006). The development of drug-resistance in HBV, including strains carrying resistance to multiple licensed agents is an emerging clinical problem, and drug-resistance for future HCV therapies is predicted to be a significant clinical issue (Tomei et al., 2005; Tong et al., 2006; Yim et al., 2006).

Nitazoxanide (NTZ) is a thiazolide anti-infective with activity against anaerobic bacteria, protozoa and viruses (Fox and Saravolatz, 2005; Pankuch and Appelbaum, 2006; Rossignol et al., 2006a; Rossignol and El-Gohary, 2006). Originally developed as a treatment of intestinal protozoan infections, the antiviral properties of NTZ were discovered during the course of its development for treating cryptosporidiosis in patients with acquired immune deficiency syndrome (AIDS). NTZ is marketed in the United States for treating diarrhea and enteritis caused by *Cryptosporidium* spp. or *Giardia lamblia* in adults and children down to 12 months of age (Alinia®, Romark Laboratories, Tampa, Florida USA). Clinical trials have demonstrated effectiveness of NTZ in treating diarrhea and enteritis associated with enteric protozoan infections caused by *Cryptosporidium* spp., *G. lamblia*, *Entamoeba histolytica* and *Blastocystis hominis* (Amadi et al., 2002; Ortiz et al., 2001; Rossignol et al., 2001, 2005, 2006b). Recent randomized double-blind clinical

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trials have demonstrated effectiveness of NTZ in treating *Clostridium difficile* colitis in adults, rotavirus gastroenteritis in young children, and rotavirus and norovirus gastroenteritis in adults (Musher et al., 2006; Rossignol et al., 2006a; Rossignol and El-Gohary, 2006). The mechanism of action of NTZ against anaerobic organisms is attributed to interference with pyruvate:ferredoxin oxidoreductase (PFOR) enzyme-dependent electron transfer reactions, which are essential for anaerobic energy metabolism (Hoffman et al., 2007). Its mechanism of antiviral activity has not been fully elucidated.

Following oral administration of a 500 mg tablet, NTZ is partially absorbed from the gastrointestinal tract and rapidly hydrolyzed in plasma to form its active circulating metabolite, tizoxanide (TIZ). NTZ is not detected in plasma. Maximum serum concentrations of TIZ, reach approximately 10 μg/mL (37 μM) (Stockis et al., 2002) following oral administration of one 500 mg NTZ tablet (Alinia®) with food. TIZ is glucurono-conjugated in the liver and excreted in urine and bile. Approximately, two-thirds of an oral dose pass through the intestinal tract and is excreted in feces as TIZ (Broekhuysen et al., 2000). The elimination half-life of TIZ from plasma is approximately 1.5 h. TIZ does not inhibit cytochrome P450 enzymes, and therefore, no drug–drug interactions are expected (Broekhuysen et al., 2000; Stockis et al., 2002). The most commonly reported side-effects in clinical trials include mild abdominal pain, headache, diarrhea and nausea, which occur at rates similar to those reported for patients receiving placebo. While most of the clinical experience with NTZ has involved 3–14 days of treatment, continual use of the drug for periods as long as 4 years has been evaluated in patients with AIDS-related cryptosporidiosis without any significant drug-related adverse events (Fox and Saravolatz, 2005; Rossignol, 2006).

In this report, we present results of *in vitro* studies characterizing the activities of NTZ, TIZ and other new thiazolides against hepatitis B and hepatitis C viruses.

2. Materials and methods

2.1. Materials

Lamivudine (LMV) and adefovir dipovoxil (ADV), and 2′-C-methyl cytidine were purchased from Moraveck Biochemicals, Inc. (La Brea, CA, USA). Recombinant human interferon alpha 2b (IFNα) was purchased from PBL Biomedical Laboratories (Piscataway, NJ USA). All other test compounds (Fig. 1) were provided by Romark Laboratories, L.C. (Tampa, FL, USA). Human serum (heat-inactivated, mixed gender, lot BRH125374) was purchased from Bioreclamation, Inc. (Hicksville, NY).

2.2. Antiviral assays

HBV antiviral assays were conducted as previous described (Korba and Gerin, 1992). Briefly, confluent cultures of 2.2.15 cells were maintained on 96-well flat-bottomed tissue culture plates (confluence in this culture system is required for active, high levels of HBV replication equivalent to that observed in chronically-infected individuals (Sells et al., 1988; Korba and Gerin, 1992). Cultures were treated with nine consecutive daily doses of the test compounds. HBV DNA levels were assessed by quantitative dot blot hybridization 24 h after the last treatment. Cytotoxicity was assessed by uptake of neutral red dye 24 h following the last treatment.

Activity against lamivudine-resistant (Allen et al., 1998) and adefovir dipovoxil (ADV)-resistant (Angus et al., 2003)

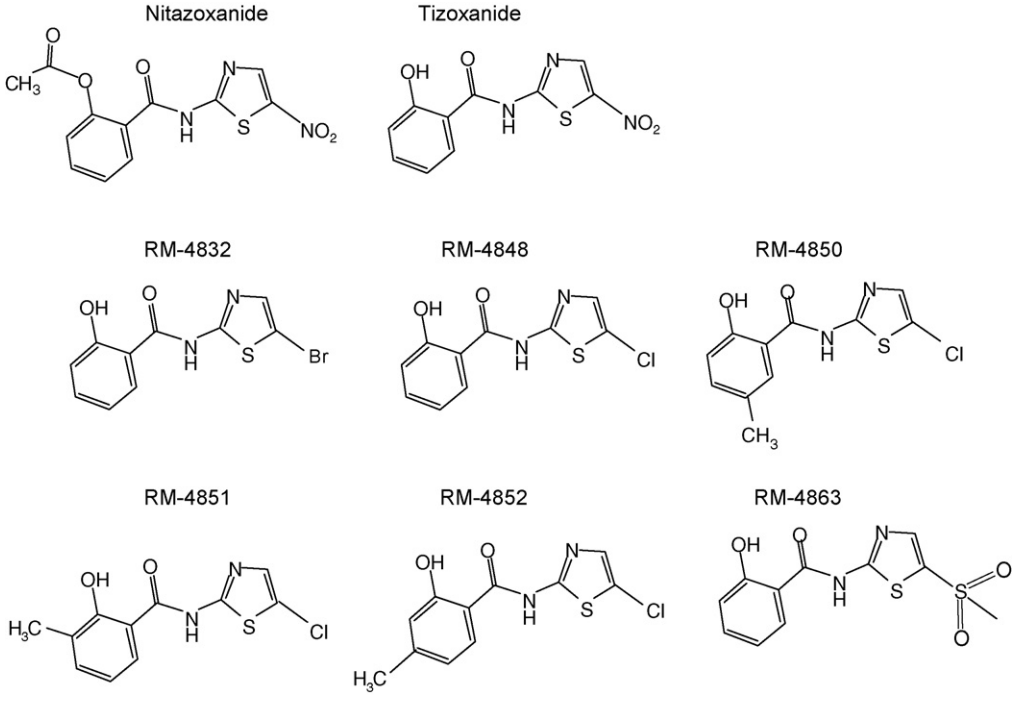

Fig. 1. Structures of thiazolides.

HBV mutants was performed in a 5-day assay using a transient transfection method as previously described (Iyer et al., 2004). Antiviral activity was determined by quantitative Southern blot hybridization of intracellular HBV DNA replication intermediates (HBV RI).

Semi-quantitative ELISA-based analysis of HBV proteins was performed as previously described (Korba and Gerin, 1995), except that HBeAg was analyzed using ETI-EBK Plus® ELISA (DiaSorin, Inc., Stillwater, MN USA). Samples were diluted (2–10-fold) to bring levels into the dynamic response ranges of the ELISA's. HBsAg, and HBeAg were analyzed from culture medium samples and HBcAg was analyzed from intracellular lysates (normalized for total cell protein content in each culture sample). Intracellular HBV RNA (normalized to the level of cellular B-actin RNA in each culture sample) was assessed by quantitative northern blot hybridization as previously described (Korba and Gerin, 1995).

Antiviral activity against HCV was assessed in a 3-day assay using the stably expressing HCV replicon cell line, AVA5 (subgenomic CON1, genotype 1b) (Blight et al., 2000) maintained as sub-confluent cultures on 96-well plates as previously described (Okuse et al., 2005). Antiviral activity was determined by blot hybridization analysis of intracellular HCV RNA (normalized to the level of cellular B-actin RNA in each culture sample) and cytotoxicity was assessed by neutral red dye uptake after 3 days of treatment. Additional studies were performed using Huh7 cells containing another HCV replicon, H/FL-Neo, a genotype 1a full-length construct (Blight et al., 2003). For studies involving added human serum, standard culture medium (which contains 10% fetal bovine serum) and assay conditions were maintained.

Activity against HCV NS5B S282T (Pierra et al., 2005) and NS3 A156S/T (Courcambeck et al., 2006) drug-resistant mutants was performed in stable replicon-containing cell lines in a 3-day assay performed as described above, except that cultures were maintained in 24-well culture plates. HCV mutants were constructed in the 1b genotype replicon BB7 present in AVA5 cells by site-directed mutagenesis (Stratagene QuickChange XL). HCV RNA's were transcribed (MEGAscript T7, Ambion, Inc.) following linearization of the plasmids with *Sca* I, and were then transfected into Huh7 cells (Lipofectamine™ 2000, Invitrogen, Inc.). Clonal cell lines were selected in the presence of G418 (Invitrogen, Inc.) and the appropriate challenge drug. Sequence analysis of the NS5B and NS3 genes from the clonal cell lines verified that only the introduced base changes were present.

Combination drug treatments for both HBV and HCV were performed as previously described (Korba, 1996; Iyer et al., 2004). Briefly, compounds were mixed at approximately equipotent concentrations and this molar ratio was maintained during serial dilution. Evaluation of drug interactions in the combination treatments was conducted against the corresponding monotherapies in the same experiments using Calcusyn® (Biosoft, Inc., Cambridge, UK).

Both the HBV and HCV antiviral studies utilized dilution steps ranging from as little as 2-fold to as much as 10-fold, comprised of 4–8 titration points, with 3–6 replicates each. Calculations of potency were based on data from two or more evaluations. EC_{50}, EC_{90} and CC_{50} values were calculated by linear regression analysis (MS EXCEL®, QuattroPro®) using data combined from all treated cultures (Korba and Gerin, 1992; Okuse et al., 2005). Standard deviations for EC_{50} and EC_{90} values were calculated from the standard errors generated by the regression analyses. EC_{50} and EC_{90} are drug concentrations at which a 2-fold, or a 10-fold depression of intracellular HBV DNA or HCV RNA (relative to the average levels in untreated cultures), respectively, was observed. CC_{50} is the drug concentration at which a two-fold lower level of neutral red dye uptake (relative to the average levels in untreated cultures) was observed. The Selectivity index (S.I.) was calculated as CC_{50}/EC_{90} for HBV assays and CC_{50}/EC_{50} for HCV assays. EC_{90} values were used for calculation of the S.I. in HBV assays since at least a three-fold depression of HBV DNA levels is typically required to achieve statistical significance in this assay system (Korba and Gerin, 1992). For combination treatments, EC_{50}, EC_{90}, CC_{50} and S.I. are presented for the first compound listed. The molar ratio of the compounds in each combination is also indicated.

3. Results

3.1. Hepatitis B virus (HBV)

3.1.1. Activities of compounds and combinations in 2.2.15 cell cultures

NTZ and its active metabolite, TIZ, exhibited selective inhibition of intracellular HBV replication and extracellular virus production by 2.2.15 cells (Table 1). Several other thiazolides (see Table 1) were also effective inhibitors of HBV replication in this assay system. Combinations of NTZ with either of two drugs licensed for anti-HBV therapy, lamivudine and adefovir dipovoxil, demonstrated synergistic interactions when used to treat 2.2.15 cells (Table 1, Fig. 2A and B). The anti-HBV assays were conducted under confluence as this provides the conditions for optimal HBV replication (Sells et al., 1988; Korba and Gerin, 1992). While under the conditions of the antiviral assay NTZ displayed minimal cytotoxicity (>100 μM, Table 1), cytotoxicity of NTZ in rapidly dividing cultures of 2.2.15 cells was higher (20 ± 1.3 μM).

NTZ and RM4850 were effective inhibitors of several HBV LMV-resistant and one ADV-resistant constructs in transient transfection assays in Huh7 cells (Table 2). No significant differences in potency of these thiazolides relative to that observed for wild-type HBV were observed for any of the drug-resistant viruses tested.

3.1.2. Effect of NTZ on production of HBV proteins

Unlike most viruses (including HCV), HBV RNA transcription and protein production are effectively separated from viral genome replication due to the presence of a long-lived population of covalently closed viral template genomes in the host cell nucleus (cccDNA) (see Locarnini, 2004 for a review). Intracellular HBV replication takes place in viral nucleocapsids located in the cytoplasm. As a result, most compounds that inhibit HBV

Table 1
Relative potency (μM) of test compounds against HBV replication

Compound		Extracellular virion DNA		Intracellular HBV RI[a]		Selectivity Index	
	CC_{50}	EC_{50}	EC_{90}	EC_{50}	EC_{90}	Virion	RI
LMV	2347 ± 88	0.05 ± 0.01	0.15 ± 0.02	0.16 ± 0.03	0.55 ± 0.06	15,646	4267
ADV	>300#	1.0 ± 0.2	3.0 ± 0.3			>100	
NTZ	>100	0.12 ± 0.02	0.83 ± 0.09	0.59 ± 0.07	2.1 ± 0.2	>121	>48
TIZ	>100	0.15 ± 0.02	0.58 ± 0.06	0.46 ± 0.05	1.2 ± 0.2	>172	>83
RM4832	>100	1.2 ± 0.1	4.0 ± 0.3	2.9 ± 0.3	8.7 ± 1.0	>25	>12
RM4848	>100	0.37 ± 0.07	1.7 ± 0.2			>58	
RM4850	>100	0.33 ± 0.07	0.83 ± 0.10	0.90 ± 0.10	2.0 ± 0.2	>120	>51
RM4851	>100	>10#	>10#	>10#	>10#	–	–
RM4852	>100	1.0 ± 0.1	3.3 ± 0.3	2.7 ± 0.3	6.3 ± 0.7	>30	>16
RM4863	>100	>10	>10	>10	>10	–	–
NTZ + LMV, 50:1	>100	0.06 ± 0.005	0.16 ± 0.02			>625	
NTZ + ADV, 3:1	>100	0.03 ± 0.004	0.11 ± 0.02			>909	

#, no significant cytotoxic or antiviral activity observed up to indicated concentration.
[a] HBV RI, intracellular HBV RNA replication intermediates.

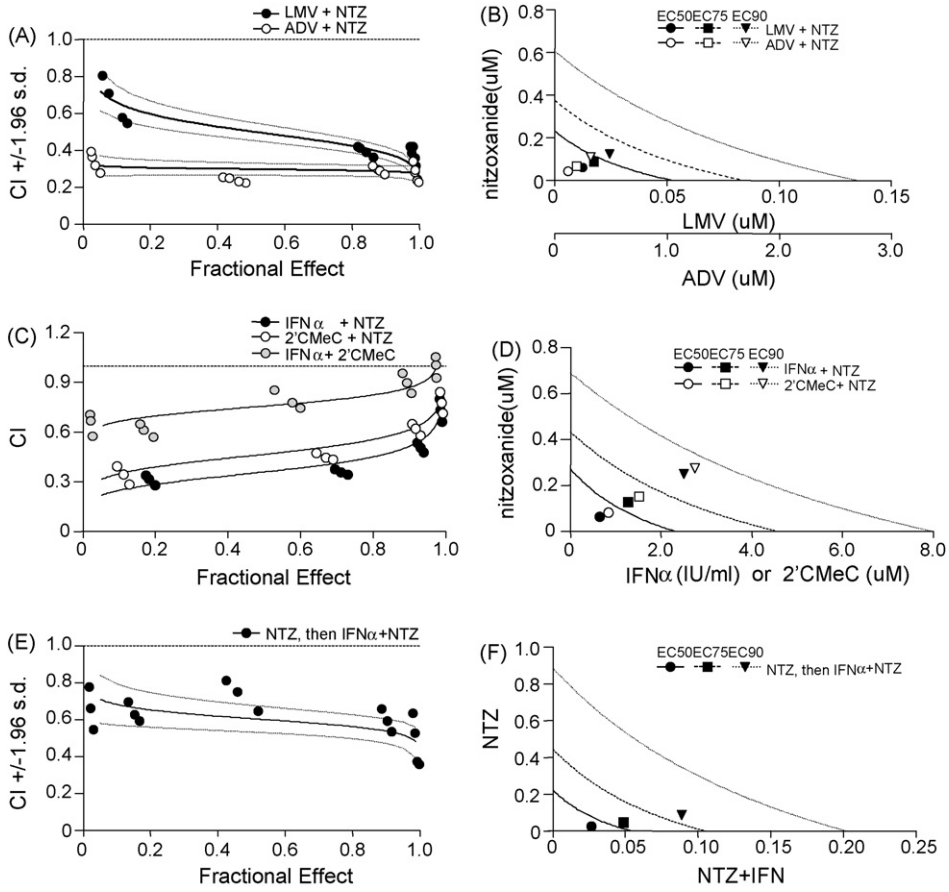

Fig. 2. Examples of the analysis of interactions between test compounds in combination treatments. Analysis of combination therapies was performed using Calcusyn™ software (Biosoft, Inc., Cambridge, UK). Panels A and B display anti-HBV treatments; panels C to F display anti-HCV treatments. Two types of evaluations are presented. Panels A, C, and E present Combination Index-Fraction (CI-Fa (of virus) affected) plots (Belen'kii and Schinazi, 1994). For these plots, a combination index [CI] greater than 1.0 indicates antagonism and a CI less than 1.0 indicates synergism. Evaluations of synergy, additivity (summation), or antagonism at different levels of virus inhibition (e.g. 5% (Fa = 0.5) to 99% (Fa = 0.99)) are provided by the plotted lines and points. Dotted lines in panel A indicate 1.96 standard deviations (not shown in panel C for clarity). Panels B, D, and F present conservative isobolograms. For these plots, EC_{50}, EC_{75}, and EC_{90} (50%, 75%, and 90% effective antiviral concentrations) values for the combination treatments are displayed as single points. Three lines radiating out from the axes denote the expected (e.g. additive) EC_{50}, EC_{75}, and EC_{90} values for drug combinations as calculated from the monotherapies. EC_{50}, EC_{75}, and EC_{90} values for the combinations that plot to the left (e.g. less than) of the corresponding lines indicate synergy, and values plotting to the right (e.g. greater than) of the corresponding lines indicate antagonism.

Table 2
Relative potency (μM) of test compounds against drug-resistant HBV mutants

HBV Mutant	Nitazoxanide		Lamivudine		Adefovir dipovoxil		RM4850	
	EC_{50}	EC_{90}	EC_{50}	EC_{90}	EC_{50}	EC_{90}	EC_{50}	EC_{90}
Wild-type	0.21 ± 0.03	0.77 ± 0.09	0.2 ± 0.1	0.9 ± 0.2	2.0 ± 0.2	7.0 ± 0.8	0.73 ± 0.08	2.0 ± 0.3
M204V	0.15 ± 0.02	0.70 ± 0.08	>100[#]	>100[#]	1.5 ± 0.2	7.2 ± 0.8	0.80 ± 0.10	2.1 ± 0.3
M204I	0.31 ± 0.05	1.0 ± 0.2	>100	>100	2.5 ± 0.3	8.5 ± 1.0	1.0 ± 0.2	2.4 ± 0.3
L180M	0.23 ± 0.03	0.80 ± 0.09	16 ± 2.0	46 ± 5.6	2.6 ± 0.3	7.3 ± 0.8	0.83 ± 0.09	2.2 ± 0.2
L180M/M204V	0.18 ± 0.02	0.72 ± 0.09	>100	>100	2.5 ± 0.3	7.6 ± 0.8	0.87 ± 0.11	2.1 ± 0.3
N236T	0.28 ± 0.03	0.85 ± 0.10	0.3 ± 0.1	1.2 ± 0.2	11 ± 1.3	32 ± 3.6	0.67 ± 0.08	2.2 ± 0.2

#, no significant antiviral activity observed up to indicated concentration.

Table 3
Relative potency (μM) of NTZ and lamivudine against HBV replication and HBV protein levels

	Nitazoxanide		Lamivudine	
	EC_{50}	EC_{90}	EC_{50}	EC_{90}
Virion production	0.19 ± 0.02	0.58 ± 0.04	0.05 ± 0.01	0.15 ± 0.02
HBV R.I.	0.73 ± 0.06	2.2 ± 0.3	0.16 ± 0.02	0.56 ± 0.07
HBV RNA	–	–	–	–
HBsAg	0.22 ± 0.03	1.0 ± 0.1	–	–
HBeAg	0.26 ± 0.02	1.3 ± 0.1	–	–
HBcAg	1.1 ± 0.1	3.0 ± 0.2	–	–

DNA replication (e.g. nucleoside analogues), do not typically alter HBV protein production, especially in cell culture.

Suspecting a novel mechanism of action of NTZ against HBV, we conducted studies to determine if the drug inhibited the production of major HBV proteins. As assessed by semi-quantitative EIA, NTZ reduced the levels of extracellular HBV surface and e antigens (HBsAg, HBeAg), as well as the levels of intracellular HBV nucleocapsid core antigen (HBcAg) in a dose-dependent manner (Table 3, Fig. 3). The potency of NTZ against HBsAg and HBeAg was similar to that observed against HBV virion production in the same experiment. The relative potency of NTZ against intracellular HBcAg was similar to that observed for the inhibition of intracellular HBV DNA replication. No quantitative interference with the ability of the EIAs to detect HBV proteins was observed in samples from control cultures to which 10 μM NTZ was added (data not shown).

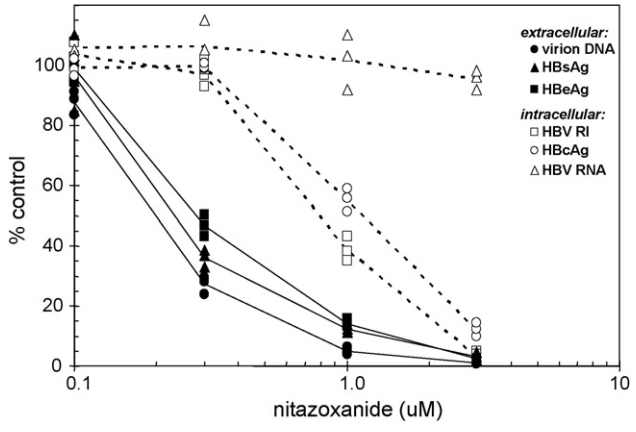

Fig. 3. Effect of NTZ on HBV nucleic acid and protein levels in 2.2.15 cells. Cultures of 2.2.15 cells were treated under standard procedures (Korba and Gerin, 1992, Antiviral Res. 19:55). HBV nucleic acids levels were determined by quantitative blot hybridization analysis (Korba and Gerin, 1992, Antiviral Res. 19:55; 1995, 28:225). HBV proteins levels were determined by semi-quantitative EIA (Korba and Gerin, 1992, Antiviral Res. 19:55; 1995, 28:225). Samples were diluted (2–10-fold) to bring levels into the dynamic response ranges of the EIA's. HBV virion DNA, HBsAg, and HBeAg were analyzed from culture medium samples. HBV RNA, HBV RI (HBV DNA replication intermediates), and HBcAg were analyzed from intracellular lysates.

NTZ did not induce a reduction in intracellular HBV RNA as assessed by Northern blot hybridization (Table 3, Fig. 3). In the same experiment, LMV did not affect the levels of HBV proteins or HBV RNA despite inducing significant reductions in HBV virion production and intracellular HBV DNA replication (Table 3).

3.2. Hepatitis C virus (HCV)

3.2.1. Activities of compounds and combinations in HCV replicon cell cultures

NTZ and TIZ selectively reduced intracellular HCV replication in AVA5 cells (Table 4). Combinations of NTZ or TIZ with either recombinant human interferon alpha 2b (IFNα), or an NS5B (HCV polymerase) inhibitor, 2′-C-methyl cytidine (2′CmeC, Pierra et al., 2005), exhibited synergistic interactions against HCV replication (Table 4, Fig. 2C and D).

Only two of the other thiazolides, RM4832 and RM4863, exhibited activity against HCV (Table 4). Antiviral activities of NTZ, TIZ and RM4832 against a full-length genotype 1a replicon (Blight et al., 2003) were equivalent to that observed for AVA5 cells (genotype 1b) (Table 4).

NTZ was an effective inhibitor of an NS5B and two NS3 drug-resistant mutants in Huh7 cells (Table 5). No significant differences in potency of NTZ relative to that observed for 'wild-

Table 4
Relative potency (μM) of test compounds against HCV replication

Compound	CC_{50}	EC_{50}	EC_{90}	Selectivity Index
Genotype 1b replicon				
IFNα	>10,000#[a]	1.9 ± 0.2[a]	8.9 ± 0.9[a]	>5263
2′CmeC	>300	1.6 ± 0.2	8.3 ± 0.7	>188
NTZ	38 ± 1.8	0.21 ± 0.03	0.93 ± 0.11	181
TIZ	15 ± 1.2	0.15 ± 0.02	0.81 ± 0.92	100
RM4832	98 ± 3.2	4.9 ± 0.5	20 ± 1.9	20
RM4848	15 ± 0.1	>20#	>20#	–
RM4850	2.3 ± 0.2	>20	>20	–
RM4851	5.6 ± 0.3	>20	>20	–
RM4852	6.7 ± 0.4	>20	>20	–
RM4863	2.8 ± 0.3	0.04 ± 0.005	0.59 ± 0.07	74
2′CmeC + IFNα, 1:1	>300	0.67 ± 0.007	2.3 ± 0.3	>448
NTZ + IFNα, 1:10	33 ± 1.3	0.06 ± 0.008	0.25 ± 0.03	550
NTZ + 2′CmeC, 1:10	35 ± 1.5	0.07 ± 0.005	0.28 ± 0.02	500
TIZ + IFNα, 1:10	17 ± 1.3	0.07 ± 0.01	0.22 ± 0.03	245
TIZ + 2′CmeC, 1:10	18 ± 1.1	0.06 ± 0.004	0.19 ± 0.02	300
Genotype 1a replicon				
IFNα	>10,000	2.1 ± 0.2	9.4 ± 0.9	>4762
2′CmeC	>300	1.8 ± 0.2	8.1 ± 0.8	>167
NTZ	49 ± 1.5	0.33 ± 0.05	1.1 ± 0.1	149
TIZ	14 ± 0.2	0.25 ± 0.03	1.0 ± 0.1	56
RM4832	88 ± 2.1	2.8 ± 0.3	9.4 ± 1.1	31

#, no significant cytotoxic or antiviral activity observed up to indicated concentration.
[a] Concentrations for interferon are expressed in IU/mL.

type' HCV (in AVA5 cells) was observed for any of the drug-resistant mutants tested.

Although the mechanism of action of NTZ is not known, the divergence of the intracellular protozoa and viruses inhibited suggests that a cellular, rather than common viral function is being affected. It is conceivable that changes in the intracellular environment induced by NTZ may alter the effect of subsequent treatment with other anti-HCV agents that also act though the induction of cellular pathways, particularly IFNα. As a preliminary investigation into this issue, we evaluated the effect of a regimen consisting of 3 days treatment with NTZ followed by 3 days of a combination of NTZ plus IFNα. Pre-treatment with NTZ monotherapy further improved the potency of combination treatment with NTZ plus IFNα by approximately three-fold ($p < 0.02$, Table 6). This staggered combination regimen was also modestly synergistic when compared to the simultaneous NTZ plus IFNα treatment combination (Fig. 2, panels E and F). Pre-treatment did not, however, affect the potency of combination treatment with 2′CmeC (Table 6).

3.2.2. *Effect of human serum on anti-HCV potency and cytotoxicity of TIZ in AVA5 cells*

NTZ and its circulating metabolite, TIZ, are highly bound (>99%) to plasma proteins in human serum (Broekhuysen et al., 2000; Stockis et al., 2002). To evaluate the effect of human serum on the anti-HCV potency and cytotoxicity of TIZ, human serum was added to the culture medium at various concentrations (Table 7). The CC_{50}, as well as the EC_{50}, and EC_{90} of TIZ increased with increasing concentrations of human serum up to 20%. The EC_{50} and EC_{90} in the presence of 30% human serum were similar to those at 20% human serum (a plateau effect) suggesting that maximum extent of protein binding had been reached. The levels of HCV and B-actin RNA in untreated cultures were similar at different concentrations of human serum up to 30% (data not shown). Higher concentrations of human serum significantly lowered cell viability (data not shown). The maximum EC_{50} and EC_{90} observed for NTZ in the presence of serum in these studies were at or below the average peak blood levels of 37 uM observed in patients given the prescribed dose of this drug (Stockis et al., 2002).

Table 5
Relative potency (μM) of test compounds against drug-resistant HCV mutants

HCV mutant	Nitazoxanide (μM)		IFNα (IU/mL)		2′CmeC (μM)		VX-950 (μM)	
	EC_{50}	EC_{90}	EC_{50}	EC_{90}	EC_{50}	EC_{90}	EC_{50}	EC_{90}
Wild-type	0.25 ± 0.03	0.97 ± 0.12	1.8 ± 0.2	9.0 ± 1.1	1.7 ± 0.2	7.0 ± 0.8	0.20 ± 0.04	2.0 ± 0.3
NS5B S282T	0.30 ± 0.05	1.1 ± 0.2	1.6 ± 0.2	8.7 ± 1.0	40 ± 5.0	>100#	0.25 ± 0.03	2.1 ± 0.2
NS3 A156T	0.28 ± 0.03	1.0 ± 0.2	1.7 ± 0.2	8.5 ± 0.9	1.5 ± 0.2	6.5 ± 0.8	>10#	>10#
NS3 A156S	0.23 ± 0.04	0.90 ± 0.13	2.0 ± 0.3	9.2 ± 1.1	1.8 ± 0.2	7.5 ± 0.9	>10#	>10#

#, no significant antiviral activity observed up to indicated concentration.

Table 6
Effect of NTZ montherapy pretreatment on combination therapy

Treatment (6 days total duration)	EC_{50}	EC_{90}
IFNα	1.7 ± 0.2	7.8 ± 0.8
2′CmeC	1.3 ± 0.2	5.8 ± 0.9
NTZ	0.20 ± 0.02	0.92 ± 0.10
NTZ + IFNα, 1:10	0.09 ± 0.010	0.24 ± 0.04
NTZ montherapy (3 days), then NTZ + IFNα (3 days)	0.03 ± 0.004#	0.09 ± 0.011$
NTZ + 2′CmeC, 1:10	0.05 ± 0.007	0.17 ± 0.03
NTZ montherapy (3 days), then NTZ + 2′CmeC (3 days)	0.06 ± 0.005	0.15 ± 0.02

Values are expressed as μM (TIZ, 2′CmeC) or IU/ml (IFN) of drug (first named drug in the case of combinations). #, $p = 0.0018$ vs. EC_{50} of 6 days treatment with NTZ + IFNα, 1:10 simultaneous combination. '$', $p = 0.0026$ vs. EC_{90} of 6 days treatment with NTZ + IFNα, 1:10 simultaneous combination. Values were analyzed using two-sample *t*-test of regression parameters.

Table 7
Relative potency (μM) of TIZ against HCV replication in the presence of human serum

Concentration of human serum (%)	EC_{50}	EC_{90}	CC_{50}
0	0.25 ± 0.01	0.98 ± 0.04	28 ± 0.9
2.5	0.48 ± 0.02	1.1 ± 0.1	65 ± 1.4
5	0.64 ± 0.05	2.3 ± 0.1	97 ± 3.9
10	1.1 ± 0.1	3.0 ± 0.2	>100
15	2.7 ± 0.3	15 ± 2.0	>100
20	9.4 ± 0.8	27 ± 2.2	>100
30	9.3 ± 0.9	32 ± 3.0	>100

4. Discussion

In this report, we present the first *in vitro* demonstrations of the activity of nitazoxanide and its active circulating metabolite, tizoxanide, against both HBV and HCV replication. Inhibition of both viruses was dose-dependent and selective relative to cytotoxicity under the conditions of the antiviral assays. Concentrations of tizoxanide required to inhibit viral replication, even those observed in the presence of additional human serum, are readily achieved in human plasma following oral administration of nitazoxanide (Broekhuysen et al., 2000; Stockis et al., 2002). Four of six other thiazolides tested exhibited selective activity against HBV replication, and two exhibited selective activity against HCV replication.

Nitazoxanide was active against both genotypes 1a and 1b HCV, commonly observed LMV and ADV-resistant HBV mutants, as well as representative HCV mutants resistant to telaprevir and 2′CmeC. Activity against genotype 2a HCV as well as additional drug-resistant HCV remains to be evaluated. If TIZ exerts its observed antiviral effects by targeting cellular processes, it is possible that resistance may not be as significant a problem for thiazolides as it is typically for direct-acting antiviral agents. The lack of diminished activity against several clinically relevant drug-resistant HBV and HCV variants presents the potential for use in rescue therapy or in combination with existing agents, and is consistent with an indirect mechanism of antiviral activity.

Nitazoxanide exhibited synergistic activity in combination with LMV or ADV against HBV, and with IFNα or 2′CmeC against HCV, consistent with a mechanism of action that is different from these antiviral agents. Pre-treatment of HCV replicon cells with nitazoxanide potentiated the effect of subsequent treatment with combinations containing IFNα, but not 2′CmeC, suggesting an interesting complementary activity with IFNα. Thiazolides may offer the possibility of new more effective combination treatments for chronic hepatitis C, whether used as additions to current standard-of-care, as replacements for ribavirin, or as part of novel combination regimens with other anti-HCV drugs in development.

The activity of nitazoxanide against HBV proteins could prove to be advantageous in treating chronic hepatitis B. Reductions in HBV protein levels, especially HBsAg and HBeAg, are of critical importance in elucidating long term antiviral responses *in vivo* (Menne et al., 2002; Korba et al., 2004; Wong and Lok, 2006).

While the mechanism of antiviral activity of nitazoxanide, tizoxanide, and the other thiazolides has not been fully elucidated, our findings are indicative of a mechanism that differs from direct-acting antiviral drugs and involves cellular processes. It is not at all apparent if the mechanisms of antiviral effectiveness and cytotoxicity involve similar cellular targets. The lack of an effect on levels of HBV RNA transcription indicates a post-transcriptional (possibly post-translational) mechanism. We propose that thiazolides may alter cellular processes required for virus protein production/maturation and/or assembly. Consistent with this hypothesis is the observation that the relative potencies of NTZ against secreted and intracellular HBV proteins are similar to that against secreted HBV virions and intracellular HBV RI, respectively. Given the substantial differences in the roles of these viral proteins, effects on more than one cell process are likely involved. Further studies are ongoing in our laboratories to more fully characterize the mechanism of activity of these drugs against HBV and HCV replication, and the potential for development of resistance.

The limited panel of thiazolides examined provides some initial clues into which components of TIZ are important for its anti-HBV and anti-HCV activities. The observation that the dual anti-hepatitis activity of TIZ can be separated into compounds that possess only anti-HBV or anti HCV activity has the potential to further elucidate the mechanism of action and identify the critical cellular targets involved. There will be a need to examine additional thiazolides for antiviral and cytotoxicity profiles.

Nitazoxanide, tizoxanide and other drugs from the thiazolide class are promising new antiviral agents that may enhance current or future therapies for viral hepatitis caused by HBV or HCV. NTZ is undergoing phase II clinical development for hepatitis C, and NTZ other thiazolides are also being developed for treating either hepatitis B or hepatitis C. The implications of a new class of antiviral drugs with the properties described herein could be significant from a clinical perspective.

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