Interferon Stimulated Gene Expression in HIV/HCV Coinfected Patients Treated with Nitazoxanide/ Peginterferon-Alfa-2a and Ribavirin

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Abstract

A combination of nitazoxanide (NTZ), peginterferon (PegIFN), and ribavirin (RBV) may result in higher sustained virologic response (SVR) rates in hepatitis C virus (HCV) monoinfected patients. This study evaluated the effect of NTZ on interferon-stimulated gene (ISG) expression *in vitro* and *in vivo* among HIV/HCV genotype-1 (GT-1) treatment-naive patients. The ability of NTZ to enhance host response to interferon (IFN) signaling using the HCV cell culture system was initially evaluated. Second, ISG expression in 53 patients with treatment outcomes [21 SVR and 32 nonresponders (NR)] in the ACTG A5269 trial, a phase-II study (4-week lead in of NTZ 500 mg daily followed by 48 weeks of NTZ, PegIFN, and weight-based RBV), was assessed. The relative expression of 48 ISGs in peripheral blood mononuclear cells (PBMCs) was measured at baseline, week 4, and week 8 of treatment in a blinded manner. *In vitro* NTZ produced a direct and additive antiviral effect with IFN-alfa, with pretreatment of NTZ resulting in maximal HCV suppression. NTZ augmented IFN-mediated ISG induction in PBMCs from relapsers and SVRs ($p < 0.05$), but not NR. In ACTG A5269, baseline expression of most ISGs was similar between NR and SVR. NTZ minimally induced 17 genes in NR and 13 genes in SVR after 4 weeks of therapy. However, after initiation of PegIFN and RBV, ISG induction was predominantly observed in the SVR group and not NR group. NTZ treatment facilitates IFN-induced suppression of HCV replication. Inability to achieve SVR with IFN-based therapy in this clinical trial is associated with diminished ISG response to therapy that is refractory to NTZ.

Introduction

HEPATITIS C VIRUS (HCV) infection is a major public health problem with over 180 million people infected worldwide,[1] and due to shared routes of transmission, approximately one-third of all human immunodeficiency virus (HIV)-infected patients are coinfected with HCV in the United States.[2] While the successful implementation of antiretroviral therapy has dramatically decreased the number of AIDS-related opportunistic infections, morbidity and mortality rates related to HCV-related liver disease have increased in this group.[3–5] In comparison to HCV monoinfected patients, HIV/HCV coinfected patients have an accelerated progression of liver disease and only attain modest cure rates with interferon (IFN)-based therapies.[2,3] Along with significant associated toxicities, the standard treatment with IFN-based treatment yields high rates of relapse among HIV/HCV coinfected genotype-1 patients. This warrants the development of novel therapeutic regimens to improve sustained virologic response (SVR) in this population.

An adjunctive well-tolerated oral agent that had shown promising results in HCV monoinfected participants was

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nitazoxanide (NTZ). NTZ (2-acetyloxy-N-(5-nitro-2-thiazolyl) benzamide; Alinia), which is licensed in the United States for the treatment of *Cryptosporidium parvum* and *Giardia lamblia* in immunocompetent adults and children, has *in vitro* and clinical activity against a broad range of microorganisms, including protozoa, helminthes, bacteria, and viruses.[6,7] The initial reports on HCV antiviral activity were facilitated by studies for the treatment of infectious diarrhea in Egypt, in which activity against HCV genotype 4 was coincidentally noted and studied.[8,9] Other studies showed that the addition of NTZ to peginterferon (PegIFN) and ribavirin (RBV) increased SVR rates from 50% to 80% in genotype-4 HCV monoinfected Egyptian patients while reporting SVR proportions of 44% versus 32% with PegIFN/RBV alone in genotype-1 (GT-1) HCV treatment-naive subjects.[9,10] In HIV/HCV coinfected patients, the ACTG A5269 trial aimed to explore the efficacy of NTZ in HIV/HCV coinfected persons due to its suggested efficacy in HCV monoinfected patients, its minimal drug–drug interactions with antiretrovirals, and its known safety and tolerability in HIV-1-infected patients. This recent study showed a significant improvement in early virologic response (at least 2 $\log_{10}$ drop in HCV RNA from baseline) in patients treated with NTZ, however, SVR rates were similar to historic controls.[11]

Previously, we have shown that high baseline expression of interferon stimulated genes (ISGs) and lack of induction of ISGs with treatment is a strong negative predictor of achieving SVR among HIV/HCV coinfected subjects.[12] In this study, we performed an *in vitro* experimental study to determine the optimal efficacy of NTZ with IFN-alfa against HCV using a continuous infectious system. Furthermore, we evaluated the effect of NTZ on ISG expression in HIV/HCV coinfected, genotype-1 treatment-naive patients treated in the ACTG A5269 study, which used combination therapy of NTZ with PegIFN and RBV to treat HIV/HCV coinfected subjects.

Materials and Methods

Part 1: in vitro study

Study subjects. Peripheral blood mononuclear cells (PBMCs) were obtained from HIV- and HCV-negative healthy volunteers ($n = 10$) and HIV/HCV coinfected patients ($n = 30$) who were previously treated for HCV under IRB approved protocols by the National Institute of Allergy and Infectious Diseases (NIAID). All HIV/HCV coinfected patients were treated with either Pegylated IFN-alfa 2a or 2b with weight-based RBV. Treatment responses were defined as (1) SVR (no detectable HCV RNA 24 weeks after study treatment), (2) relapser (no detectable HCV RNA at the end treatment, but rebound of detectable virus after stopping therapy), and (3) nonresponder (NR) (detectable HCV RNA at the end of treatment).

Isolation of PBMCs and culture conditions. PBMCs were isolated after leukapheresis or from heparinized peripheral blood obtained by venipuncture by Ficoll Hypaque density gradient centrifugation (GE Healthcare). Isolated PBMCs were resuspended in RPMI 1640 medium (Invitrogen Corporation, Inc.) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, 2% glutamine and incubated at 37°C in a 5% CO_2 containing incubator for 24 h in the presence or absence of serial concentrations of IFN-alfa or

NTZ (Romark Laboratories, Inc.). Synergy between PegIFN and NTZ was assessed by comparing the effect of NTZ added simultaneously with PegIFN or 72 h post-PegIFN exposure.

Cell line and culture conditions. The Huh7.5 human hepatocellular carcinoma cell line was provided by Apath LLC. Cells were cultured in DMEM (Invitrogen Corporation, Inc.) supplemented with 10% FCS and 100 U/ml penicillin/100 μg/ml streptomycin at 37°C in a 5% CO_2 containing incubator.

HCV infectious clone. The infectious plasmid clone for the HCV J6/JFH1 infectious cell culture system was provided by Apath LLC. Transfection and subsequent infection of Huh 7.5 cells using this plasmid were performed as described elsewhere.[13]

HCV RNA quantitation. HCV RNA was determined using the Roche TaqMan HCV, v1.0 (Roche Molecular Systems), with LLQ of 43 IU/ml. Viral RNA was extracted using a viral RNA extraction Mini Kit (Qiagen, Inc.). HCV copy numbers were determined by quantitative real-time polymerase chain reaction (PCR). Results were analyzed using the comparative Ct method for the relative quantitation of gene expression.

Cell viability assay. Assessment of cytotoxicity in the presence or absence of NTZ and/or IFN-alfa was performed using the Calcein assay kit (Biotium, Inc.). To evaluate the cytotoxicity of NTZ on cells, NTZ was added to the HCV continuous culture system at various concentrations and evaluated at 48 h.

Quantitative PCR for ISGs. Validation of DNA microarray data was performed using a multiplex qPCR assay (Applied Biosystems) capable of detecting the expression of 20 ISGs and 3 housekeeping genes as previously described.[12] PBMCs in medium alone were used as a control to calculate the baseline expression of ISGs.

rs12979860 SNP genotyping. Genotyping of the ACTG A5269 and NTZ cohorts was performed by the Duke Institute for Human Genome Variation. Genotyping was conducted in a blinded manner on DNA specimens collected from each individual, using the 5′ nuclease assay with allele-specific TaqMan probes (ABI TaqMan allelic discrimination kit and the ABI7900HT Sequence Detection System (Applied Biosystems)). Genotyping calls were manually inspected and verified before release.

Part 2: ACTG A5269 study

Study design. ACTG A5269 trial was a single arm, phase 2 pilot study to evaluate the efficacy of NTZ added to standard of care therapy [peginterferon-alfa-2a (PegIFN) and RBV] in HIV/HCV coinfected, genotype-1, HCV treatment-naive patients. Subjects received NTZ 500 mg twice a day for 4 weeks, then the following regimen for 48 weeks: PegIFN 180 mcg subcutaneously once weekly, and weight-based RBV [≥75 kg (1,200 mg/day) or <75 kg (1,000 mg/day)]. Study criteria and details have been previously described.[11] The present analysis compares 21 SVR (no detectable HCV RNA 24 weeks after study treatment) and 32 NR (detectable HCV RNA at the end of treatment).
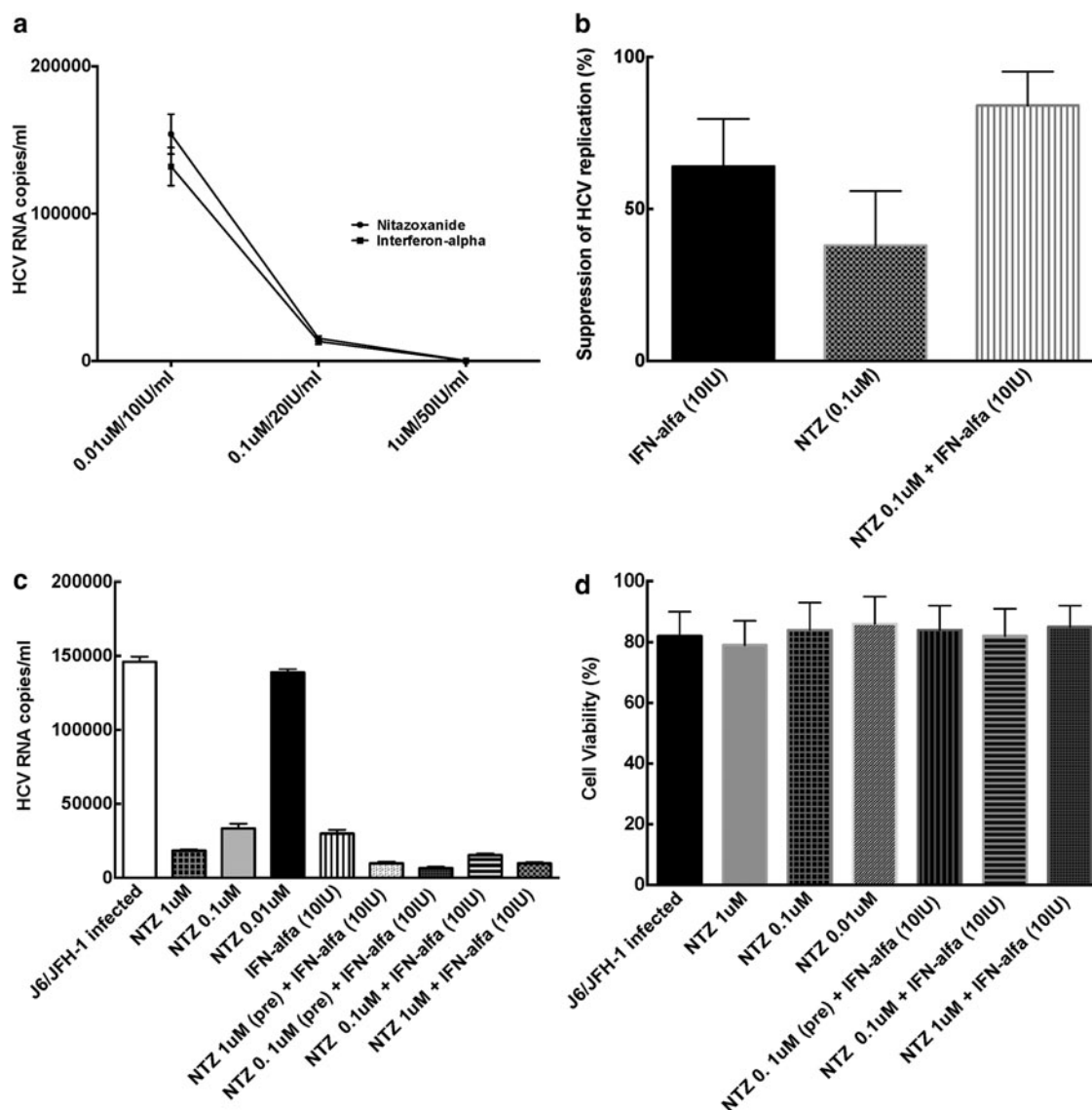
FIG. 1. Anti-HCV Effects of NTZ *in vitro.* **(a)** Comparable dose-dependent effects of NTZ and IFN-alfa on HCV replication. J6/JFH-1 HCVcc system was utilized to evaluate the effect of NTZ or interferon-alfa on HCV replication as described under the Materials and Methods. As shown here, both NTZ and IFN-alfa exerted a profound, dose dependent, and comparable effect on HCV replication *in vitro.* HCV RNA levels in the untreated medium only condition were $214{,}532 \pm 1{,}354$ IU/ml. All experiments were repeated four different times under identical conditions. **(b)** NTZ exhibits additive anti-HCV effect with IFN-alfa on HCV cc system J6/JFH-1. HCVcc system was utilized to evaluate the effect of NTZ or interferon-alfa on HCV replication as described under the Materials and Methods. As shown here, both NTZ and IFN-alfa exerted a profound effect on HCV replication *in vitro,* while when combined together demonstrated an additive effect. HCV RNA levels in the untreated medium only condition was 100%. All experiments were repeated four different times under identical conditions. **(c)** NTZ augments IFN-alfa-mediated suppression of HCV replication. J6/JFH-1 HCVcc system was utilized to evaluate the effect of pretreatment of NTZ on interferon-alfa effect on HCV replication as described under the Materials and Methods. As shown here, both pretreatment of Huh 7.5 cells with NTZ augmented the effect of IFN-alfa-based suppression of HCV in Huh 7.5 cells. HCV RNA levels in the untreated medium only condition were $187{,}451 \pm 1{,}987$ IU/ml. All experiments were repeated four different times under identical conditions. **(d)** NTZ does not affect cell viability of Huh7.5 cells. J6/JFH-1 HCVcc system was utilized to evaluate the effect of NTZ or interferon-alfa on cell viability as described under the Materials and Methods. As shown here, both NTZ and IFN-alfa alone or in combination had minimal effect on cell viability. Cell viability in the untreated medium only condition was $98.5\% \pm 3.4\%$. All experiments were repeated four different times under identical conditions. HCV, hepatitis C virus; IFN, interferon; NTZ, nitazoxanide.

Quantitative PCR for ISGs. Custom multiplex qPCR assay (Applied Biosystems) was used to measure expression of 48 ISGs (44 ISGs and 4 endogenous controls: 18S, GAPDH, GUSB, HPRT1) from PBMCs at baseline, week 4 (day 0 of PegIFN/RBV) and week 8 (week 4 of triple therapy).

Two-stage analysis. *Stage 1:* The analysis at the first stage was on samples at weeks 0 (baseline), 4, and 8 from 10 SVRs and 10 non-SVRs randomly selected from ACTG A5269. The results of the statistical tests from this stage were used to identify a subset of genes for the second stage of assays on the remaining ACTG A5269 subjects. The criterion was set at *p*-values <0.20 from two-sided exact Wilcoxon rank-sum tests that compared expressions from SVRs and non-SVRs. The testing laboratory was blinded to the SVR status and remained so for the remaining assays. There were 44 ISGs tested in Stage 1, plus the housekeeping genes HPRT1, GUSB, GAPDH, and 18S.

Stage 2: Based on the first stage analysis, 26 genes were selected. Twenty-two genes were selected from the baseline gene expression analysis, and an additional 4 genes from the analysis on changes in gene expression from baseline at week 4. The genes tested in the second stage were the 26 genes identified from the first stage, plus two genes (IFI27 and IFIT1) that were determined as significant genes from previous *in vitro* work on NTZ, and 4 housekeeping genes: 18s, GAPDH, HPRT1, and GUSB. Assays for the second stage were performed on the subjects who were not part of the first stage analysis.

Statistical methods

Part 1 (*in vitro*): Nonparametric tests were used for comparisons between groups.

Part 2 (ACTG A5269): The analyses were conducted using the combined data on the 28 selected genes from the two stages. Wilcoxon rank-sum tests were used for comparisons between groups using exact methods and stratified rank-sum tests for comparisons adjusting for a covariate. The Benjamini–Hochberg procedure (1995) was applied to account for multiple tests on 28 ISGs, with a false discovery rate at 10% for each study objective comparison. As an exploratory analysis, overall control of false positivity is set loosely at 10% so that promising genes are not missed.[14]

No additional adjustments were made to control for false positivity in analyzing multiple study endpoints (e.g., baseline, fold changes at weeks 4 and 8) and to address multiple study objectives. The analysis combined data from both stages without accounting for the two-stage approach. All analyses were conducted using SAS version 9.2.

Results

Part 1: in vitro *study*

NTZ exhibits dose-dependent suppression of HCV replication *in vitro.* NTZ demonstrated direct suppression of HCV replication in Huh7.5 cells in a dose-dependent manner (*p* = 0.002, compared to untreated cells) (Fig 1a). Escalating
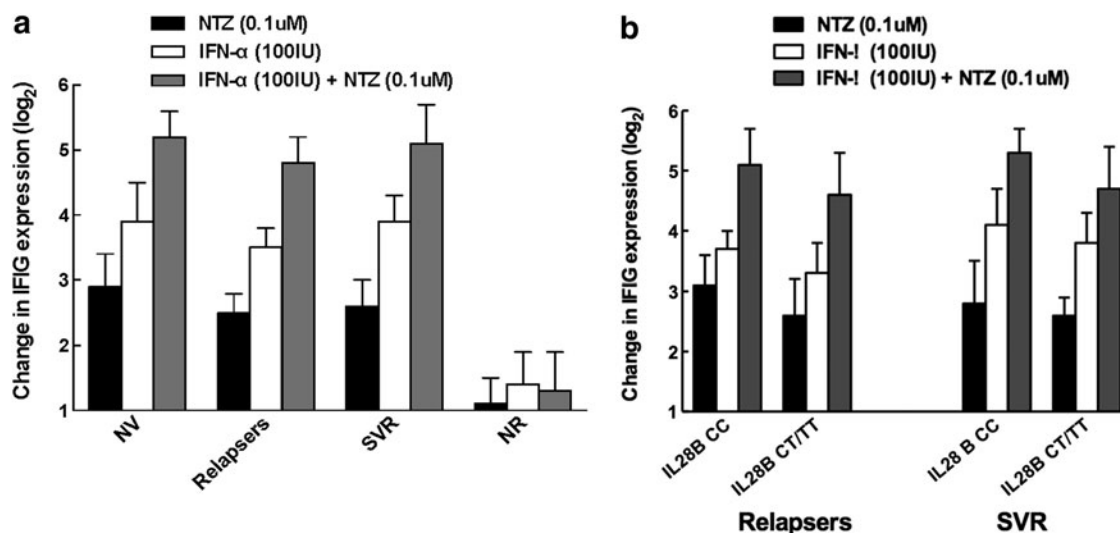

FIG. 2. Augmentation of IFN-inducible genes by NTZ. **(a)** NTZ augments IFN-inducible gene expression in PBMCs from HIV-negative normal volunteers, HIV/HCV-coinfected relapsers and SVRs, but not nonresponders. IFN-inducible gene expression was determined using PBMC (from normal volunteers, relapsers, those with SVR, and nonresponders) treated with either NTZ alone (0.1 μM), IFN-alfa alone (100 IU/ml), or both NTZ (0.1 μM) and IFN-alfa (100 IU/ml) as described in the Materials and Methods. PBMCs in medium alone were used as a control to calculate baseline expression of interferon-stimulated genes. As shown in the figure, addition of NTZ augmented the effect of IFN-alfa-mediated induction of IFN-inducible genes in all groups of patients except nonresponders. **(b)** NTZ augments IFN-inducible gene expression in PBMCs from HIV/HCV-coinfected relapsers and SVRs, irrespective of IL28B haplotype. IFN-inducible gene expression was determined using PBMC (from relapsers and those with SVR who were either IL28B cc or non-CC haplotypes) treated with either NTZ alone (0.1 μM), IFN-alfa alone (100 IU/ml), or both NTZ (0.1 μM) and IFN-alfa (100 IU/ml) as described in the Materials and Methods. IL28B CC haplotypes are associated with overall better response to interferon-based HCV therapy. Hence, IL28B non-CC haplotypes are less amenable to achieve SVR using IFN-based therapy. As shown in the figure, addition of NTZ augmented the effect of IFN-alfa-mediated induction of IFN-inducible genes in all groups of patients, regardless of IL28B haplotype suggesting that NTZ may be able to overcome genetic barriers of lack of response to IFN-alfa-based therapy. HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cell; SVR, sustained virologic response.

doses of NTZ were used from a range of 0.1 μM to 1 M, which showed maximal inhibition similar to doses used in other studies.[15,16] These doses were used in subsequent experiments with EC 50 and EC 90 of 0.33 and 1.1 μM, respectively.

NTZ has additive effect with IFN-alfa in suppressing HCV replication *in vitro.* We also treated Huh 7.5 cells with a combination of IFN-alfa and different doses of NTZ. We found that NTZ augmented the HCV antiviral effect of IFN-alfa leading to increased viral suppression (NTZ/IFN combination $p = 0.01$ vs. IFN alone and $p = 0.003$ vs. NTZ alone) (Fig 1b).

Pre-exposure to NTZ followed by IFN-alfa results in maximal suppression of HCV replication *in vitro.* It has been suggested that pretreatment with NTZ might lead to changes in the intracellular environment, thereby altering the treatment effect of subsequent therapies.[16] We found that pretreatment with NTZ resulted in greater *in vitro* HCV suppression of HCV replication when compared to combination therapy with IFN and NTZ without pretreatment *in vitro* ($p = 0.001$) (Fig 1c).

NTZ does not lead to cell apoptosis *in vitro.* NTZ and its active metabolite are highly bound (>99%) to plasma proteins in serum.[17,18] Higher concentrations of NTZ slightly lowered cell viability; however, over 85% of cells were still viable (Fig 1d).

NTZ induces ISG expression in PBMCs from normal volunteers, relapsers, and SVR, but not from NR. We have previously shown that induction of ISGs after initiation of therapy is associated with good treatment response[12] and

sought to evaluate the induction of the ISG pathway with NTZ. PBMCs in medium alone were used as a control to calculate baseline expression of ISGs. Treatment of PBMCs with NTZ resulted in a direct induction (fold change) of ISGs in PBMCs of relapsers ($p = 0.01$) and sustained responders ($p = 0.003$), but not NR ($p = 0.05$) (Fig 2a). NTZ treatment also significantly augmented IFN-mediated ISG expression in PBMCs from relapsers ($p = 0.002$) and SVR ($p = 0.001$), but not NR ($p = 0.3$). NTZ had similar effects on induction of ISG responses in both relapsers and SVR, irrespective of *IL28B* haplotype (Fig 2b). The distribution of IL28B CC haplotype was 0% for NR, 40% for relapsers, and 50% for those who achieved SVR. IL28B CC haplotype has been associated with SVR and induction of ISG response.[19]

Part 2: ACTG A5269 study

Study population. A subset of 53 patients (21 SVR, 32 NR) with confirmed outcomes was included in the final analysis (Table 1). Patients were predominantly male (83%), median age (49), IL28B CT/TT haplotype with genotype-1a infection (72%) largely representative of the HCV epidemic in the United States. About 70% was suppressed on current antiretroviral therapy. Non-nucleoside or protease inhibitor regimens comprised 75% of these regimens.

ISG expression

Baseline ISG expression as a predictor of SVR or nonresponse. High baseline ISG expression has been described as a negative predictor of achieving SVR in PegIFN- and RBV-based therapy for coinfected subjects. We examined whether there was a difference between baseline ISG expression between SVR and NR in the ACTG A5269 cohort. There were no

TABLE 1. ACTG A5269 BASELINE CHARACTERISTICS OF 21 SVR AND 32 NR

Baseline characteristics, n (%) or median (IQR)			
Host factors	*SVR (n = 21)*	*NR (n = 32)*	*Total (n = 53)*
Median age	46 (42.54)	50 (44.54)	49 (43.54)
Male gender	18 (85.7)	26 (81.3)	44 (83.0)
Black race	10 (47.6)	18 (56.3)	28 (52.8)
BMI <30	17 (80.9)	23 (71.9)	40 (75.5)
IL28B C/T OR T/T haplotype	13 (61.9)	25 (78.2)	38 (71.7)
HCV-related measures			
Genotype			
1a	14 (66.7)	24 (75.0)	38 (71.7)
1b	6 (28.6)	7 (21.9)	13 (24.5)
Log HCV RNA	6.29 (5.92, 6.64)	6.63 (6.16, 7.02)	6.41 (6.03, 6.73)
APRI >1.5	2 (9.5)	5 (15.6)	7 (13.2)
FIB-4 > 3.25	1 (4.8)	7 (21.9)	8 (15.1)
HIV-related measures			
CD4	446 (357, 748)	439.50 (306.5, 727)	445 (323, 738)
Proportion HIV VL suppressed	16 (76.2)	21 (65.6)	37 (69.8)
HIV ARV regimen			
Non-nuc	11 (52.4)	9 (28.1)	20 (37.7)
PI	6 (28.8)	14 (43.4)	20 (37.7)
Integrase inhibitor	1 (4.8)	0 (0)	1 (1.9)
Other	2 (9.6)	4 (12.4)	6 (11.3)
No ARVs	1 (4.8)	5 (15.6)	6 (11.3)

ARV, antiretroviral; BMI, body mass index; HCV, hepatitis C virus; HIV, human immunodeficiency virus; NR, nonresponder; SVR, sustained virologic response; VL, viral load.

statistically significant differences detected between SVR and nonresponse groups with respect to baseline gene expression. IFIT2 was expressed higher in SVR and ranked with the lowest p-value (p = 0.12, unadjusted for multiple tests) (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/aid).

Induction of ISG after 4 weeks of NTZ pretreatment.   One of the proposed mechanisms of action of NTZ is to induce ISG expression by enhancing protein kinase R (PKR) phosphorylation.[20] In this clinical trial, all subjects received 4 weeks of pretreatment with NTZ before initiation of PegIFN and RBV. We investigated whether there was a difference in ISG induction by NTZ between the two response groups (SVR and NR). There were 17 genes upregulated in NR and 13 genes upregulated in SVR, while other genes were unchanged. Hence, NTZ treatment was able to induce ISG expression in these subjects, regardless of therapeutic outcome of combination therapy (Table 2 and Supplementary Table S2).

At Week 8: (week 4 IFN, week 8 NTZ).   Next, we examined whether enhancing IFN responsiveness with NTZ pre-

treatment would result in an induction of ISG expression. There were 27 genes upregulated at week 8 from week 4. In this regard, those who achieved SVR had an increase in most ISGs compared to those in NR (Table 2 and Supplementary Table S2).

Discussion

Our results demonstrate that NTZ exhibits potent direct anti-HCV activity and is able to potentiate the anti-HCV effect of IFN-alfa in vitro. The additive effect of ISG induction, which is a surrogate marker of host response to PegIFN/RBV therapy, is not observed among NR, suggesting this group of individuals may not benefit from an IFN-sensitizing regimen. However, the addition of an agent that stimulates the IFN signaling cascade such as NTZ may be effective for IFN relapsers. In vitro studies performed in our laboratory and elsewhere suggest that NTZ has both (1) a direct suppressive effect on HCV replication and (2) a sensitizing effect on IFN gene signature and IFN-mediated suppression of HCV replication. Furthermore, NTZ pretreatment of HIV/HCV-coinfected subjects enhanced IFN signaling in PBMCs, regardless of the therapeutic outcome. However, NTZ resulted in lower induction of ISG by PegIFN

TABLE 2. ACTG A5269 GENE EXPRESSION AFTER 4 WEEKS OF NITAZOXANIDE PRETREATMENT; AFTER WEEK 4 INTERFERON, WEEK 8 NITAZOXANIDE

Week 4 change from baseline					Week 8 change from week 4				
Gene	p*	Higher in	SVR	NR	Gene	p*	Higher in	SVR	NR
IFI27	0.17	NR	Down	Up	IFI27	0.334	SVR	Up	Up
IFI44	0.093	NR	Down	Up	IFI44	0.065	SVR	Up	Up
IFIT1	0.595	SVR	Up	Down	IFIT1	0.649	NR	Up	Up
IFIT2	0.082	NR	Down	Down	IFIT2	0.268	SVR	Up	Up
IFIT3	0.522	NR	Up	Up	IFIT3	0.864	SVR	Up	Up
IFITM2	0.685	NR	Down	Up	IFITM2	0.134	SVR	Up	Up
IFITM3	0.097	NR	Up	Up	IFITM3	0.343	SVR	Up	Up
IFNA2	0.826	NR	Down	Down	IFNA2	0.782	SVR	Up	Up
IFNB1	0.685	SVR	Up	Down	IFNB1	0.878	SVR	Up	Down
IRF1	0.993	SVR	Up	Up	IRF1	0.561	SVR	Down	Down
IRF7	0.419	NR	Down	Down	IRF7	0.546	SVR	Up	Up
IRF9	0.893	NR	Down	Down	IRF9	0.443	SVR	Up	Up
ISG15	0.135	NR	Down	Up	ISG15	0.780	SVR	Up	Up
LY6E	0.044	NR	Down	Up	LY6E	0.167	SVR	Up	Up
MX1	0.249	NR	Down	Up	MX1	0.249	SVR	Up	Up
MX2	0.794	NR	Down	—	MX2	0.364	SVR	Up	Up
OAS1	0.371	NR	Up	Up	OAS1	0.907	NR	Up	Up
OAS2	0.473	SVR	Up	Up	OAS2	0.465	SVR	Up	Up
OASL	0.113	NR	Down	Up	OASL	0.479	SVR	Up	Up
PLSCR1	0.381	NR	Down	Up	PLSCR1	0.850	NR	Up	Up
RARRES3	0.177	NR	Up	Up	RARRES3	0.555	SVR	Up	Up
SP110	0.159	NR	Up	Up	SP110	0.222	SVR	Up	Up
STAT1	0.065	NR	Up	Up	STAT1	0.274	SVR	Up	Up
STAT2	0.822	NR	Down	—	STAT2	0.698	SVR	Up	Up
STAT6	0.893	SVR	Up	—	STAT6	0.763	SVR	Up	Up
TRIM14	0.646	NR	Up	Up	TRIM14	0.409	SVR	Up	Up
TRIM5	0.534	SVR	Up	—	TRIM5	0.558	SVR	Up	Up
TYK2	0.893	SVR	Down	Down	TYK2	0.907	SVR	Up	Up

The comparisons of gene expression at various times after enrollment is made to the baseline gene expression. Week 4 and 8 changes: gene expression = $2^{(\Delta Ct)}$, where $\Delta Ct = [(\text{target gene } Ct) - (\text{GAPDH } Ct)]$. Baseline, week 4 and week 8 gene expressions are the values of $2(\Delta Ct)$ at weeks 0, 4, and 8, respectively: $2^{(-\Delta 4 \Delta Ct)} > 1$ refers to upregulation at week 4 compared to baseline; $2^{(-\Delta 4 \Delta Ct)} < 1$ refers to downregulation at week 4 compared to baseline; Similar interpretation for $2^{(-\Delta 8 \Delta 4)}$ at week 8 compared to week 4.

None met the predetermined false discovery rate at 10% using the Benjamini–Hochberg procedure for multiple tests.

* Exact Wilcoxon test.

—, genes for which expression did not change.

and RBV in PBMCs from those who failed to achieve SVR than those who did.

These data are consistent with *in vitro* studies[9,16] that have suggested that NTZ has both direct and synergistic suppression of HCV replication. Phosphorylated eIF2alfa (p-eIF2α) and activated PKR are important mediators in the IFN-inducible antiviral pathways. Recent studies using Huh7 cells containing HCV genotype-1 replicons or Huh7.5 cells infected with HCV genotype-2a J6/JFH virus demonstrated that intracellular concentrations of p-eIF2α and the dsRNA-activated PKR were increased with NTZ treatment.[15] In addition, NTZ increased phosphorylation of PKR, which was more pronounced in the presence of HCV viral RNA. These findings suggest that NTZ likely induces PKR phosphorylation, resulting in an increased intracellular concentration of p-eIF2α, a key mediator of host defenses against viruses, and serves as a key explanation of NTZ's antiviral effects.[20] The addition of IFN to cell cultures further increased NTZ-induced p-eIF2α. *In vitro*, NTZ promoted PKR autophosphorylation, a key step in activating PKR's kinase activity for eIF2alfa. Finally, NTZ-induced p-eIF2α was reduced in the presence of specific inhibitors of PKR autophosphorylation, a key kinase that regulates the cell innate antiviral response. NTZ might represent a new class of small molecules capable of potentiating and recapitulating important antiviral effects of IFN.[17] In contrast to the available and upcoming directly acting anti-HCV agents, NTZ does not appear to induce direct antiviral resistance based on serial passage of HCV replicon-containing cell lines with increasing concentrations of NTZ and tizoxanide.[21] Our data demonstrating additive effect of NTZ on HCV replication with IFN-alfa are consistent with this proposed mode of action.

Our study demonstrates the activity of NTZ in augmenting IFN response in HIV/HCV coinfected patients. Previously, we have shown that high baseline expression of ISGs and lack of induction of ISGs with treatment are strong negative predictors of achieving SVR among HIV/HCV coinfected subjects.[12] In this regard, NTZ pretreatment resulted in enhanced IFN signaling in most study subjects. These data would suggest that NTZ is capable of inducing IFN signaling leading to ISG expression in HIV/HCV coinfected subjects, although the exact mechanism by which this happens is unclear. The basic assumption for the clinical trial design using a pretreatment phase of NTZ alone was to achieve sensitization of immune cells to exogenous IFN/RBV therapy similar to what we observed *in vitro*. However, PegIFN/RBV/NTZ therapy resulted in lower ISG induction in PBMCs from NR than that was observed in PBMCs of those who achieved SVR, suggesting a refractoriness in the IFN signaling pathway in nonresponders.

Future studies will focus on understanding the mechanisms of this refractoriness of IFN signaling observed in NR and development of modalities that may aid in its reversal. Such strategies may be vital in improving the response rates of HCV therapy in this difficult to treat patient population.

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Author Disclosure Statement

Anu Osinusi is an employee of Gilead Sciences. The other authors have no conflicts of interest.

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