Short Communication: Nitazoxanide Inhibits HIV Viral Replication in Monocyte-Derived Macrophages

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Abstract

We document the anti-HIV activity of nitazoxanide (NTZ), the first member of the thiazolide class of anti-infective drugs, originally effective against enteritis caused by *Cryptosporidium parvum* and *Giardia lamblia*. NTZ has been administered extensively worldwide, with no severe toxicities associated with its use. Here, we show for the first time that NTZ decreases HIV-1 replication in monocyte-derived macrophages (MDM) if present before or during HIV-1 infection. This NTZ effect is associated with downregulation of HIV-1 receptors CD4 and CCR5, and increasing gene expression of host cell anti-HIV resistance factors APOBEC3A/3G and tetherin. As NTZ is already in clinical use for other conditions, this newly described anti-HIV activity in MDM may facilitate innovative intensification strategies against HIV-1 when combined with current antiretroviral drug regimens.

ALTHOUGH GREAT STRIDES have been made in the fight against HIV/AIDS, more than 33 million people are infected with HIV-1, and infection results in the need for lifelong treatment with antiretroviral therapy.¹ The two main HIV-1 cellular targets are CD4⁺ T lymphocytes and macrophages. Loss of CD4⁺ T cells by apoptosis as a result of both HIV-1 and bystander mechanisms² is one of the hallmarks of HIV. In contrast, there is no strong evidence to support the decline of resident tissue macrophages during chronic infection.^{3–5} HIV-1 infection of macrophages contributes to viral pathogenesis, immune deregulation (activation), and viremia during late stage disease.^{3,4}

Nitazoxanide (NTZ, marketed in the United States as Alinia, Romark Laboratories, L.C.) is the first of a new class of small molecules, the thiazolides, licensed in 2002 and originally developed for the treatment of diarrhea in patients with *Cryptosporidium parvum* and *Giardia lamblia*.⁶ NTZ has also recently shown antiviral activity against influenza,⁷ rotavirus,^{8,9} and hepatitis B virus (HBV) and hepatitis C virus (HCV)¹⁰ in cell culture and in patients.^{11,12} NTZ was shown to have activity against HCV and HBV infection through suppressing viral replication¹⁰ by increasing the phosphorylation of eIF2 α , which plays a role in the interferon (IFN)-induced antiviral response.¹³ Furthermore, in a cohort of patients infected with HCV Genotype 4, NTZ was added to standard of care (SOC) with Peg-IFN- α -2a and compared against SOC. The addition of NTZ resulted in both a more rapid virologic response and higher rates of sustained viro-

logic response (79% vs. 50%, $p=0.023$)^{11,12,14} supporting the potential for NTZ to intensify antiviral regimens. NTZ activity against HCV Genotype 1 has been more limited.^{15,16} Here, we tested the hypothesis that NTZ can control HIV-1 replication in monocyte-derived-macrophages infected *in vitro* with CCR5-using strains of virus, and we investigate the mechanisms that might facilitate the anti-HIV-1 effect.

To first establish the biological activity of NTZ on monocyte-derived macrophage (MDM) viability and modulation, we incubated freshly elutriated monocytes (5×10^5 cells/condition obtained from the University of Pennsylvania Human Immunology Core facility) in sterile Teflon pots in complete culture medium (RPMI 1640, Sigma) supplemented with 5% human pooled sera, 10 ng/ml MCSF (PeproTech, Rocky Hill, NJ), and increasing concentrations of NTZ (0 μ g/ml to 30 μ g/ml) (Romark Laboratories, Tampa, FL). After 48 h, whole cell lysates were harvested and the viability and activity of NTZ were evaluated by cell dye-exclusion and Caspase 3 activation, while assaying for the expression of phosphorylated eIF2 α . To first test viability, cells were harvested every 2 days for 12 days and assayed for active Caspase 3 (BD Biosciences, San Jose, CA) induction to detect apoptosis. MDM cultured in the presence of NTZ showed no significant change in survival as compared to cells cultured in the absence of NTZ (Fig. 1A). As described in cell lines but not yet in MDM,¹³ Western blot analysis of MDM at 48 h showed that NTZ exhibited a dose-dependent increase in phospho-eIF2 α (Cell Signaling Technology, Inc.,

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FIG. 1. Antiviral activity of nitazoxanide (NTZ) in HIV-infected human monocyte-derived macrophages (MDM). (A) Evidence of NTZ activity as measured by eIF2 α phosphorylation in freshly isolated elutriated monocytes incubated for 48 h in the presence of 1 (μ g/ml), 3 (μ g/ml), and 10 (μ g/ml) of NTZ. (B) MDM cultured in the presence of 10 (μ g/ml) NTZ showed no significant decrease in cell viability compared with cells cultured in the absence of NTZ. (C) MDM incubated with 10 (μ g/ml) NTZ before/during or (D) after (right) viral adsorption via spinoculation with 50 ng/ml p24 equivalent of HIV-1 Bal. Virus output in both (C) and (D) on top is shown every 2 days until 12 days postinfection by p24 ELISA whereas the means $\pm$ standard errors of three independent experiments at day 12 postinfection are shown at the bottom. (E) The expression of HIV-1 receptors CD4 (left) and CCR5 (right) in MDMs incubated in the absence (top) or presence (bottom) of 10 (μ g/ml) NTZ for 72 h. Shaded histogram represents the isotype control. Bottom panel shows a composite of CD4 and CCR5 staining from four different donors. (F) NTZ negatively regulates early and late stages in the HIV-1 lifecycle. MDMs were treated with 10 (μ g/ml) NTZ (filled bars) and assayed for the expression of APOBEC3A/3G and tetherin. The solid bar represents the untreated control. The bar graph represents the mean of three independent experiments $\pm$ standard error. In all panels, an unpaired *t*-test was used to test differences with an asterisk representing a *p* value less than 0.05.

Danvers, MA) peaking at 10 μ g/ml NTZ. β -Actin (Cell Signaling Technology, Inc.) was used for protein loading control (Fig. 1B).

Having established a bioactive NTZ concentration in MDM via changes in eIF2 α phosphorylation without impairing MDM viability, we next investigated whether NTZ could block HIV-1 infection and replication in MDM. We tested the ability of NTZ to regulate HIV-1 infection and replication in MDM by the addition of NTZ before/during (pre) or after (post) HIV-1 adsorption. Five $\times 10^6$ cells/ml elutriated monocytes were differentiated into MDM for 4 days in 24-well plates using media as above. At day 5 of culture, 10 μ g/ml NTZ was added and the cells were pre-incubated for an additional 3 days (72 h) before the addition of HIV-1. HIV-1_{Bal} (equivalent to 50 ng p24, University of Pennsylvania Center for AIDS Research), a CCR5 using laboratory-adapted strain of HIV-1, was added in the presence of NTZ and the cells were spinoculated at 1,800 rpm for 3 h to maximize viral binding. The virus-containing supernatant was removed and the virus exposed cells incubated for an additional 12 days with NTZ replenished every 2 days. A sample of the culture supernatant was harvested on day 0 and every 2 days thereafter at the time of media change. To test the effects of NTZ if added after (post) adsorption, MDMs were cultured and spinoculated as above with HIV exposure, to be immediately followed by three warm 1 $\times$ phosphate-buffered saline (PBS) washes prior to the addition of 10 μ g/ml NTZ and subsequent culture as above. Using HIV p24 antigen levels as a measure of viral replication (ELISAs measured at the University of Pennsylvania CFAR) in supernatant, we observed that NTZ treatment initiated before/during (pre) or after (post) infection resulted in a 2- to 10-fold decrease in viral replication when compared to infected untreated control MDMs. These observations were reproduced in three independent experiments (Fig. 1C and D).

We next investigated the mechanism of action by which NTZ may be reducing HIV replication in MDM. First, to determine whether NTZ affects HIV-1 binding or entry, we analyzed the modulation of cell-surface HIV-1 receptor and coreceptor (CD4 and CCR5, respectively) expression on MDMs after a 72-h exposure to 10 μ g/ml NTZ. Cells incubated in the presence of NTZ showed a significant decrease in cell surface expression of both CD4 and CCR5 (Fig. 1E), which is consistent with a lower infection and subsequent viral replication outcome as shown above.

Second, we tested whether regulation of interferon-stimulated genes associated with inhibition of HIV-1^{17–20} was induced with NTZ as thiazolides have been shown to interfere with viral infection through activation of type I interferon-associated mechanisms.^{15,21,22} We analyzed the modulation of two known type I interferon-induced negative regulators of viral replication (APOBEC 3G and tetherin^{23–28}) following 48-h incubation of MDM with 10 μ g/ml NTZ. We utilized the following real-time reverse transcriptase polymerase chain reaction (RT-PCR) primers to characterize the expression of IFN-response genes following NTZ exposure, i.e., tetherin, primer sequence: 5'-AAG AAA GTG GAG GAG CTT GAG G-3' (forward); 5'-CCT GGT TTT CTC TTC TCA GTC G-3' (reverse), APOBEC3A, primer sequence: 5'-TTC TTT GCA GTT GGA CCC GG-3' (forward); 5'-CTC ATC TAG TCC ATC CCA GG-3' (reverse), and APOBEC3G, primer sequence: 5'-TTA CCT GCT TCA CCT

CCT GG-3' (forward); 5'-TCA TCT AGT CCA TCC CAG GG-3' (reverse). β -Actin was used as an internal control; primer sequence: 5'-TTC CTG GGG ATG GAG TC-3' (forward); 5'-CAG GTC TTT GCG GAT GTC-3' (reverse). Our data showed that both APOBEC3A/G, and tetherin, potent anti-HIV host factors,^{17,24–26} were significantly up-regulated in MDMs treated exogenously with NTZ (Fig. 1F), supporting the belief that NTZ may be acting by limiting infection at early and late stages of the viral life cycle. As NTZ is metabolized *in vivo* to tizoxanide,²⁹ we independently confirmed that the latter metabolite (having better bioavailability in the blood) if added *in vitro*, as described for NTZ above, resulted in similar suppressive activity against HIV-1 replication via the upregulation of tetherin and APOBEC 3A/G (data not shown).

Taken together, these data indicate that NTZ can negatively regulate early and late stages in the infection cycle including (1) decreased binding and fusion via down-regulation of CD4 and CCR5, (2) impairment of particle infectivity by competing against viral DNA synthesis through an APOBEC-dependent mechanism,^{23,28,30} and (3) inhibition of viral particle release via upregulation of tetherin.^{27,31} Our data do not exclude a role for added interferon-mediated antiviral mechanisms associated with interferon signaling, e.g., MX2.^{19,32,33}

NTZ anti-HIV activity has also been suggested by a recent report by Tan *et al.*³⁴ that describes a novel drug screening method used to identify strongly efficacious and synergistic drug pairs, with the overall goal of expanding current antiretroviral therapy (ART) repertoires. This study found that in a T cell line (Sup T1), NTZ synergizes with known HIV drugs (integrase inhibitors, nucleoside and nonnucleoside reverse transcription inhibitors), exerting its antiviral effect post HIV-1 entry, before or at reverse transcription. Although the SupT1 data support an antiviral activity for NTZ in T cells, future experiments will need to directly establish NTZ's anti-HIV activity in primary CD4 T cells.

The recent demonstration that type-I interferons, when added to ART, can mediate HIV-1 suppression in the subsequent absence of ART and result in a decrease of total HIV DNA, raises the priority for strategies that may induce similar effects.^{35,36} Our study supports the premise that NTZ may provide an interferon-like effect on monocyte-derived macrophages, thereby acting to decrease the HIV load within this cell subset *in vivo*. Taken together, our present data support the hypothesis that NTZ could intensify ART-based strategies by activation of anti-HIV host mechanisms in MDM otherwise not targeted by current ART regimens. Future experiments will need to quantify the HIV cellular infection rate or expression in tissue macrophages of subjects receiving NTZ therapy in conjunction with ART to explore this hypothesis further.

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Author Disclosure Statement

No competing financial interests exist.

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