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Inhibition of vaccinia virus replication by nitazoxanide

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Abstract

Nitazoxanide (NTZ) is an FDA-approved anti-protozoal drug that inhibits several bacteria and viruses as well. However, its effect on poxviruses is unknown. Therefore, we investigated the impact of NTZ on vaccinia virus (VACV). We found that NTZ inhibits VACV production with an EC_{50} of ~2μM, a potency comparable to that reported for several other viruses. The inhibitory block occurs early during the viral life cycle, prior to viral DNA replication. The mechanism of viral inhibition is likely not due to activation of intracellular innate immune pathways, such as protein kinase R (PKR) or interferon signaling, contrary to what has been suggested to mediate the effects of NTZ against some other viruses. Rather, our finding that addition of exogenous palmitate partially rescues VACV production from the inhibitory effect of NTZ suggests that NTZ impedes adaptations in cellular metabolism that are needed for efficient completion of the VACV replication cycle.

Keywords

Vaccinia virus; nitazoxanide; protein kinase R; metabolism; palmitate

Introduction

The thiazolide nitazoxanide (NTZ) has extraordinarily broad antimicrobial activity (Shakya et al., 2017). In the United States, it is FDA-approved for the treatment of cryptosporidiosis and giardiasis and it has been used extensively throughout the world for these and other parasitic infections. Because it appears to be quite safe and also has activity against bacteria and viruses, it is being evaluated in multiple clinical trials for a variety of other infectious and even some noninfectious diseases (clinicaltrials.gov, 2017; Koszalka et al., 2017;

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Nikolova et al., 2014). Understanding how NTZ functions will be important for optimizing these efforts to repurpose it for new clinical applications.

NTZ has been reported to have activity in cell culture against many medically important viruses. In addition to a RNA viruses such as influenza virus, hepatitis C virus, and human immunodeficiency virus type I (Rossignol and Keeffe, 2008; Rossignol et al., 2009; Trabattoni et al., 2016), it is known to inhibit a few DNA viruses including human cytomegalovirus (HCMV) (Mercorelli et al., 2016). This breadth of activity is striking since these viruses utilize a wide range of replication strategies. Various studies aiming to identify the NTZ mechanism(s) have identified effects on innate immune signaling, which might explain its broad antiviral effects (Petersen et al., 2016b; Trabattoni et al., 2016). However, other studies have implicated virus-specific effects of NTZ, such as its ability to inhibit transcriptional activation by the HCMV IE2 protein (Mercorelli et al., 2016) and to block the maturation of the influenza A hemagglutinin protein (Rossignol et al., 2009). Thus, whether NTZ acts through multiple different virus-specific mechanisms or one common underlying one remains unclear.

The impact of NTZ on poxviruses has not been reported. Poxviruses are important as pathogens, as vaccine vectors, and as models of host-virus interactions. Variola, the cause of smallpox, has been eradicated from circulation in humans, but the potential for intentional or inadvertent re-introduction remains a concern (Ferguson et al., 2003; Koblentz, 2017). Other poxviruses, including monkeypox, are relatively uncommon but occasionally cause life-threatening disease in humans (McCollum and Damon, 2014). Some others, such as molluscum contagiosum virus, are very common but cause only mild disease. In addition to its value in the smallpox elimination campaign, vaccinia virus (VACV) has been used experimentally as a vector to deliver other antigens, as well as for oncolytic therapy (Chan and McFadden, 2014). However, VACV can cause severe disease in immunocompromised patients and even in individuals with the common skin condition eczema (Bray and Wright, 2003; Petersen et al., 2016a). Although vaccinia immune globulin, cidofovir and a few experimental compounds have activity against poxviruses, development or identification of new safe drugs that inhibit poxviruses could have clinical applications and facilitate development and testing of new applications of VACV.

Here we report our investigations of the impact of NTZ on the VACV replicative cycle. We found that NTZ inhibits VACV production, further extending its spectrum activity to *Poxviridae* family. We show that several factors and pathways that have been suggested to mediate the antiviral effects of NTZ appear not to mediate its impact on VACV. Rather, our finding that addition of palmitate but not several other metabolites, partially reverses the antiviral effects of NTZ suggests that the drug blocks specific metabolic adaptations needed for efficient viral reproduction.

Methods

Cells, viruses

Primary human foreskin fibroblasts (HF) and BSC40 cells were propagated and maintained at 37°C in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10%

NuSerum (Corning) and penicillin-streptomycin (100 U/ml). HF with CRISPR/Cas9-mediated knock out of STING (HF STING$^{-/-}$) or PKR (PKR$^{-/-}$) have been described (Child et al., 2018; Gray et al., 2016).

WT VACV, VC2 containing a lacZ cassette (VC2+lacZ) and VACV lacking its PKR antagonist E3L (VACVΔE3L, kindly provided by Bertram Jacobs, Arizona State University) have been described (Beattie et al., 1995; Child et al., 2012). HF were infected at a high (3 pfu/cell) or low (0.1 pfu/cell) MOI for 1 hour, after which the inoculum was removed and replaced with medium containing vehicle alone (0.1% DMSO) or NTZ immediately or at later times (Fig. 1D), diluted from a 10 mM stock made in DMSO. Viral production was measured 24–48 h post infection (hpi) either by measuring β-galactosidase (β-gal) activity using a fluorometric substrate cleavage assay (Child et al., 2006), or by titering virus in cell lysates, made by three freeze-thaw cycles, on BSC40 cells. Other drugs and metabolites were added at 1 hpi along with 0.1% DMSO or NTZ.

For glutamine starvation experiments, monolayers were infected with VACV (MOI = 0.1) in glutamine-free DMEM (Gibco) containing 2% NuSerum or in normal DMEM for 1h. After removing the inoculum, the monolayers were refed with the appropriate medium containing either 0.1% DMSO, 10 μM NTZ, 7mM dimethyl-α-ketoglutarate (αKG, Aldrich Chemistry), or a combination of NTZ and αKG. Viral production was measured at 48 hpi by β-gal activity assays.

Chemical reagents

NTZ, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), antimycin A, 2,4-DNP, oligomycin, and sodium palmitate were obtained from Sigma. Z-VAD-FMK was obtained from Selleckchem. Tizoxanide (TZ) was obtained from MedChem Express. Cidofovir was obtained from Gilead Sciences Inc. DL-mevalonolactone was obtained from TCI Chemicals. Sodium palmitate was conjugated to fatty acid free BSA (Sigma) according to the Seahorse Bioscience protocol.

Immunoblot analyses

Cell lysates were made by washing the monolayers with phosphate-buffered saline (PBS) followed by incubation in 2% sodium dodecyl sulfate (SDS). Following sonication, the proteins were separated by SDS-PAGE, transferred to a membrane (Immobilon®-P), and then probed with antibodies using the Western Star chemiluminescent detection system (Applied Biosystems) using the following antibodies: VACV D8 and A14 (obtained from Yan Xiang, University of Texas, San Antonio (Meng et al., 2011)), PKR D7F7 (#12297, Cell Signaling Technology), P-PKR E120 (ab32036, Abcam) actin (A2066, Sigma-Aldrich), IRF3 (D83B9, Cell Signaling Technology), H3 (ab1791, Abcam), STING (ab92605, Abcam).

Viral genome replication and transcription

VACV DNA replication was assessed by real-time PCR analyses of DNA purified from HF infected with VACV (MOI = 3) and treated with 0.1% DMSO, 10 μM cidofovir (CDV), or 10 μM NTZ. The cells were washed once with PBS, and lysed in 100 μl TE buffer

containing 1% SDS, then incubated in 150 μg/ml proteinase K, 1% SDS, 200 mM NaCl and 0.3 μL β-mercaptoethanol at 55°C for 30 min. Following phenol:chloroform extraction, the DNA was ethanol precipitated and resuspended in TE. 10 ng of each sample was analyzed by real-time PCR with primers that bind to the E3L gene (forward - 5′-CGTCAGCCATAGCATCAGCA-3′, and reverse 5′-TGACAACGGAGGCGGATAAG-3). The relative genome replication was calculated as the fold change between samples collected at 1hpi and 24hpi.

The same primers were used to analyze expression of E3L RNA. Whole cell RNA was purified from mock infected and infected cells (MOI = 3) using TRiZOL (Ambion, Inc.), then reverse transcribed using the Applied Biosystems High Capacity cDNA Reverse Transcription kit, and quantified by real time PCR. The relative abundance of E3L RNA, normalized to cellular GAPDH (primers: forward 5′-gaaggtcggagtcaacggattt -3′ and reverse 5′-gaatttgccatgggtggaat -3′) was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Nuclear-cytoplasmic fractionation

HF in 6 well plates were mock infected or infected with VACV (MOI = 3) and treated with 0.1% DMSO or 10μM NTZ 1 hpi. At 4 hpi, the monolayers were washed twice with PBS, and lysed by incubation in 300 μl of PBS containing 1mM dithiothreitol and 1% NP-40 for 10 min at room temperature. The cytoplasmic fractions were separated by pelleting of the nuclei at 4,000×g for 10 min at 4°C. The nuclear pellets were washed once with PBS, and then lysed in 35μl 2% SDS and sonicated. HCMV that had been UV irradiated in a Stratalinker 1800 (Stratagene) for 6 auto-cross-link cycles, was used as a control for IRF3 translocation to the nucleus. Equal volumes were analyzed by immunoblot assays as described above.

Radiolabeling

HF were infected with VACV (MOI = 3) and then treated with 0.1% DMSO or 10 μM NTZ. 1 hour prior to harvest, the medium was replaced with methionine-free DMEM (Gibco) supplemented with 2% NuSerum and 100 μCi/ml ^{35}S-methionine (Perkin Elmer Health Sciences, Inc.). After washing the monolayers with PBS, the cells were lysed in 2% SDS. Following sonication of the lysates, equal volumes were separated by SDS-PAGE. The gels were stained with Coomassie blue, which showed similar total protein loading in each lane, dried, and analyzed by autoradiography.

Statistical analysis

Unpaired two-tailed t tests were performed using Prism 7 software (GraphPad).

Results

NTZ inhibits vaccinia virus production

To determine whether NTZ inhibits VACV, we used a recombinant virus that expresses *lacZ*, under control of the P11 late promoter, which allows β-galactosidase (β-gal) activity assays to measure viral production. We first infected human fibroblasts (HF) at a low MOI (0.1 pfu/

cell), and at 1 hpi, removed the inoculum and added medium with varying concentrations of NTZ (Fig. 1A). NTZ inhibited production of new virus in a dose-dependent manner, increasing from a ~1 log reduction at a concentration of 5 μM, to more than 2 logs at 20 μM (Fig. 1A). The active metabolite of NTZ, tizoxanide (TZ) (Rossignol, 2014), resulted in a comparable level of inhibition. We detected a similar dose-response curve by measuring viral titers (Fig. 1B), confirming that β-gal activity measurements provide a reliable measure of viral production as we have shown in several previous studies (Carpentier et al., 2016; Child et al., 2004; Child et al., 2002) (Fig. 1B). NTZ also inhibited VACV production in HeLa cells, BSC40 cells, and guinea pig fibroblasts (data not shown), suggesting that its activity is not cell type- or host species-specific. NTZ inhibited VACV production after a high MOI (3 pfu/cell) infection of HF (Fig. 1C) and even when added as late as 6 h post infection (Fig. 1D) supporting the conclusion that it acts after viral entry. Dose-response curves made from 3 independent experiments, revealed EC_{50} values ranging from 0.7μM to 2.6μM with a median of 1.95μM (Fig. 1E). Thus, NTZ is a potent inhibitor of VACV replicative cycle.

NTZ inhibits the VACV replicative cycle at early times post infection

To investigate the mechanism by which NTZ inhibits VACV, we first explored when during the viral lifecycle NTZ acts. By qRT-PCR, we detected transcription of the E3L early gene at 1 hpi and it increased ~20-fold by 4 hpi in the absence of NTZ (Fig. 2A). We observed a small and nonsignificant decrease in E3L transcript accumulation by 4 hpi when NTZ was added at 1 hpi. These result support the conclusion that NTZ functions after VACV has entered the cells and has started to express its genes.

We monitored *de novo* protein synthesis by metabolic labeling with [^{35}S]-methionine at 8 and 24 hpi. After viral entry, VACV shuts off host protein synthesis as early as 6 hpi in cells such as BS-C-1, but progression through the replicative cycles appears to be slower in HF (Burgess and Mohr, 2015; Liu et al., 2014). This shutoff is mediated in part by the action of the late protein D10 and to a lesser extent by the early protein D9, both of which increase the degradation of host mRNAs, effectively prioritizing the synthesis of its own viral proteins (Parrish and Moss, 2006; Shors et al., 1999). In untreated infected HF, we detected shut off of host protein synthesis at 24 hpi, but not at 8 hpi. Treatment with NTZ prevented the shut off of host protein synthesis (Fig. 2B). NTZ did not diminish protein synthesis in uninfected cells at either time point.

We also evaluated the impact of NTZ on late viral protein synthesis by examining infected cell lysates for representative viral proteins (Fig. 2C). Immunoblot assays revealed that NTZ markedly reduced accumulation of the VACV envelope proteins D8 and A14 at 24 hpi. Together with the noted decrease in expression of β-gal, which is regulated in this virus by the P11 late promoter (Fig. 1), these data suggest that NTZ blocks VACV prior to the late phase of the replication cycle.

As viral DNA replication is necessary for the expression of intermediate and late VACV genes, inhibition of viral DNA replication may explain the inhibitory effects of NTZ on late viral protein production. Therefore, we assessed the impact of NTZ on viral genome replication by real time PCR (Fig. 2D). In untreated cells, we detected a ~1000-fold increase

in genome copy number at 24 hpi compared to 1 hpi. NTZ reduced this genome amplification by more than 10-fold, even more than the control VACV inhibitor cidofovir. These results provide additional support for the conclusion that NTZ acts at an early phase of the replication cycle.

NTZ does not function by activating protein kinase R (PKR)

Previous reports have shown that NTZ can activate PKR, leading to eIF2α phosphorylation and inhibition of translation, potentially explaining its broad antiviral effects (Ashiru et al., 2014; Elazar et al., 2009). However, our radiolabeling results did not show any evidence that NTZ reduced protein synthesis in either uninfected or infected cells (Fig. 2B), suggesting that PKR was not activated under these conditions.

To investigate the interaction of NTZ and the PKR pathway more directly, we evaluated the total abundance and phosphorylation state of both PKR and eIF2α, in the presence or absence of NTZ (Fig. 3A). NTZ did not alter the level of PKR phosphorylation in mock infected cells or in VACV infected cells at 8 or 24 hpi, compared to the untreated controls (Fig. 3A). Phosphorylation of eIF2α was likewise unaffected. As a control, we included cells infected with VACVΔE3L, a mutant lacking the PKR antagonist E3L, which is well known to activate the PKR pathway and thus increase the levels of phospho-PKR and phospho-eIF2α (Child et al., 2002; Langland and Jacobs, 2002), as was evident in our blots.

We also evaluated the antiviral activity of NTZ in $PKR^{-/-}$ HF in which PKR expression was eliminated by CRISPR/Cas9 genome editing. NTZ was as effective at inhibiting VACV production in $PKR^{-/-}$ as in wild-type HF (Fig 3B). We also found the NTZ was similarly effective at inhibiting VACV in $PKR^{-/-}$ HeLa cells (data not shown). Thus, NTZ does not affect the PKR pathway with or without VACV infection and PKR is not necessary for NTZ's VACV-inhibitory activity.

NTZ does not act by STING or IRF3 signaling

Although NTZ did not increase PKR expression in our cells (Fig. 3), it has been reported to induce IFN expression and a subset of other interferon-regulated genes *in vivo* and in isolated PBMCs (Petersen et al., 2016a; Trabattoni et al., 2016). NTZ has also been shown to cause mitochondrial stress by uncoupling the inner mitochondrial membrane (Amireddy et al., 2017). Therefore, we considered the possibility that NTZ might act by causing leakage of mitochondrial DNA (mtDNA) into the cytosol, where it could activate the cGAS/STING pathway (Liu et al., 2016; Rongvaux et al., 2014). Moreover, since VACV is a DNA virus that replicates in the cytoplasm, its genome could potentially act synergistically with cytosolic mtDNA leakage to activate cGAS/STING. We therefore compared the antiviral impact of NTZ in wild type vs. $STING^{-/-}$ HF (Fig. 4A). NTZ inhibited VACV to a similar extent in both cells, leading us to conclude that this pathway is not necessary for NTZ function.

In addition to cGAS/STING, several other cellular sensors signal through pathways that converge on IRF3 (Chen and Jiang, 2013). Phosphorylation and dimerization of IRF3, followed by translocation to the nucleus leads to an upregulation of type I IFN and interferon-stimulated genes (ISGs). To investigate whether IRF3 activation mediates the

NTZ effects, we determined the localization of IRF3 in NTZ treated HF, with and without infection (Fig. 4B). We did not detect any IRF3 relocalization to the nucleus in NTZ treated cells regardless of VACV infection, while a substantial portion of the cellular IRF3 did relocalize to the nuclear fraction after infection with UV-inactivated HCMV, as has been reported (DeFilippis et al., 2010). The distribution of STING in the cytoplasmic fraction and histone H3 in the nucleus confirmed the success of the fractionation protocol. Thus, NTZ appears not to exert its anti-VACV effects through IRF3.

Role of mitochondrial functions in the NTZ anti-VACV mechanism

VACV alters cellular metabolism during infection. For example, the VACV replicative cycle requires exogenous glutamine, whereas glucose deficiency has no impact (Fontaine et al., 2014; Greseth and Traktman, 2014). The impaired VACV production in glutamine-deficient medium can be rescued by supplementation with alpha-ketoglutarate (α-KG). Therefore, we hypothesized that inhibition of glutamine metabolism by NTZ during infection might play a role in its ability to suppress VACV production. We explored this possibility by infecting HF with VACV in glutamine-free or normal medium, with no drug or NTZ, and in the presence or absence of α-KG (Fig. 5A). In glutamine-free medium, α-KG partially rescued VACV infection, as has been reported (Fontaine et al., 2014; Greseth and Traktman, 2014). However, α-KG did not rescue of viral production from inhibition by NTZ. In the presence of glutamine, α-KG alone inhibited viral production and caused complete inhibition when used in combination with NTZ. Although we do not know why α-KG inhibited VACV when glutamine was present, its failure to reverse the antiviral effect of NTZ suggests that the drug does not act by blocking glutamine utilization.

Because NTZ is a mitochondrial uncoupler (Amireddy et al., 2017), we next considered whether energy depletion would mimic the antiviral effects of NTZ. Oligomycin (OM), which inhibits the mitochondrial F_1F_O-ATP synthase, had no effect on VACV production (Fig. 5B). Similarly, antimycin A, which inhibits cytochrome c reductase in the electron transport chain, only slightly reduced VACV production. Since both drugs inhibit mitochondrial ATP production, it seems unlikely that ATP depletion by mitochondrial uncoupling explains the NTZ effect. Additionally, although treatment with the protonophore FCCP inhibited VACV production, another OXPHOS uncoupler, 2,4-DNP did not have a large effect, suggesting that loss of inner mitochondrial membrane potential is not sufficient to account of the NTZ antiviral effects.

Cell death by apoptosis and pyroptosis can be triggered by mitochondrial factors. Although NTZ does not cause any clear cytopathology suggestive of apoptosis or pyroptosis in uninfected HF, it is possible that these pathways are being triggered by NTZ in infected cells and lead to an antiviral state. However, our finding that the pan-caspase inhibitor zVAD did not reduce the antiviral effect of NTZ argues against these mechanisms (Fig. 5B).

Palmitate partially rescues anti-viral effect of NTZ on VACV

In bacteria and parasites, NTZ has been shown to inhibit pyruvate:ferredoxin oxidoreductase (PFOR), a thiamine-dependent enzyme that produces acetyl-CoA from pyruvate (Kennedy et al., 2016). Although vertebrate cells do not have PFOR, they have a functionally similar

enzyme, pyruvate dehydrogenase (PDH) that also uses thiamine pyrophosphate (TPP) as a cofactor to produce acetyl-CoA, which is needed in a variety of metabolic pathways including fatty acid and cholesterol synthesis. Since palmitate is a precursor for complex fatty acid synthesis, and can also be converted to acetyl-CoA via β-oxidation and thereby potentially obviate the dependence on PDH, we tested whether palmitate could suppress the NTZ anti-viral effect.

Interestingly, we found that addition of palmitate significantly lessened inhibition of VACV production by NTZ (Fig. 6). Although we cannot exclude the possibility that palmitate alters the pharmacology of NTZ, these results suggest that NTZ may be interfering with fatty acid metabolism in a way that is circumvented in part by adding palmitate. Moreover, addition of mevalonate, an intermediate in the cholesterol biosynthesis pathway, further rescued viral production.

Discussion

To our knowledge, this is the first report demonstrating that NTZ inhibits a member of the *Poxviridae* family. The potency of inhibition of VACV by NTZ (EC_{50} = ~2 μM) is similar to previously reported values for other viruses including as HCMV (EC_{50} = 3.2 μM) (Mercorelli et al., 2016), influenza A (1.0 – 3.3 μM) (Rossignol et al., 2009) and HCV (EC_{50} = 0.25 μM) (Korba et al., 2008). In fact, the potency of NTZ is even quite similar to what has been reported for bacteria and parasites (Lam et al., 2012; Laura et al., 2015). We found that TZ, the active metabolite of NTZ that is detected in plasma (Rossignol, 2014), is comparably active against VACV (Fig. 1A–C). Pharmacologic data from humans suggest that standard dosing with 500 mg of NTZ is sufficient to achieve TZ plasma levels above 10 μM within 1 hour after treatment, and maintain levels above that threshold until approximately 5 hours post treatment, with a peak concentration of ~35 μM (Balderas-Acata, 2011). Moreover, TZ plasma levels only dropped below the EC_{50} 10 hours post treatment, suggesting that standard dosing should be sufficient to sustain an effective antiviral concentration during treatment. Another study reported a lower peak level, 6.4 μM, after a single 500 mg dose (Stockis et al., 1996). However, treatment with a higher single dose, 4 g, resulted in peak plasma concentrations close to 200 μM in patients and appeared to be safe (Stockis et al., 2002). The concentration remained above 30 μM for over 12 hours, further supporting the possibility of treating viral infections with NTZ. Thus, NTZ has potential utility in controlling poxviral infections.

Although the similarity of the dose responses across multiple infection systems seems most consistent with NTZ acting on a single cellular process, there are publications suggesting it targets specific virus factors. In the case of HCMV, Mercorelli *et al.* found that NTZ inhibited viral production and we have confirmed this effect (data not shown) (Mercorelli et al., 2016). These authors went on to provide evidence that NTZ inhibited the transcriptional activation properties of the HCMV IE2 protein. However, those experiments relied on IE2 expression by transduction of adenovirus vectors, followed by NTZ treatment for 72 hours before assaying the effect on an IE2 target gene. The reduced expression of IE2-target genes under these conditions could be due to NTZ inhibiting IE2 protein function as the authors suggest, but the experiments did not rule out the possibility that NTZ might be inhibiting IE2

activity by inhibiting its expression from the adenoviral vector. Further studies will be needed to distinguish between these possibilities.

Another example of NTZ targeting specific viral targets has been reported for influenza A. Unlike during HCMV infection, TZ was shown to act at a post-translational step, selectively inhibiting the maturation of the viral hemagglutinin by blocking the terminal glycosylation of HA after synthesis in the ER, subsequently hindering its progression to the Golgi and further insertion into the plasma membrane (Rossignol et al., 2009). Although these results suggested that NTZ inhibited a specific step in the viral life cycle of influenza, the dependency of glycosylation on metabolic intermediates including acetyl-CoA (Wellen and Thompson, 2012) may indicate that a more general effect of NTZ on the host cell metabolism may be the cause of the defect in HA maturation. NTZ has also been shown to cause the mobilization of Ca^{2+} from the ER into the cytosol, perturbing N-linked glycosylation and trafficking of the BVDV E2 glycoprotein from the ER to the Golgi (Ashiru et al., 2014), supporting the notion that NTZ may not be inhibiting influenza by a virus-specific mechanism.

The more parsimonious explanation for the broad antiviral effects of NTZ is that it alters a host cell pathway that is required by many viruses. The viruses that are now known to be sensitive to NTZ include RNA and DNA viruses, ones that replicate in the nucleus and in the cytoplasm, and ones that are enveloped as well as non-enveloped. All viruses share a reliance on the host cell protein synthetic machinery. Thus, the idea that NTZ might activate PKR in infected cells was an appealing one. However, we found that the PKR pathway was not activated by NTZ (Fig. 3A). Consistent with our results, Rossignol et al. found that TZ did not inhibit translation or affect eIF2α phosphorylation in uninfected or influenza A infected cells (Rossignol et al., 2009). Moreover, we found that NTZ still exerts its antiviral effects on VACV (Fig. 3B) and on HCMV (data not shown) in $PKR^{-/-}$ cells. Thus, at least in several viral systems, PKR does not mediate the NTZ effects.

Another possibility we considered was that NTZ might activate the interferon system and thereby inhibit multiple different viruses through various ISGs. VACV is unusual among DNA viruses in that it replicates in the cytoplasm. Coupled with the observation that NTZ causes mitochondrial stress, we considered the hypothesis that the combined effects of VACV DNA with mitochondrial DNA that could leak out of the mitochondria might activate the cGAS–STING pathway. However, NTZ remained effective even in $STING^{-/-}$ cells, ruling out that possibility. While others have detected an effect of NTZ on expression of a subset of ISGs (Trabattoni et al., 2016), we found no evidence that a major mediator of the IFN pathway, IRF3, is activated by NTZ (Fig. 4). Together with the fact that NTZ inhibits bacteria and protozoa in various culture systems in which there is no interferon, these results suggest that the interferon pathway activation cannot explain the broad antimicrobial effects of NTZ.

Since NTZ uncouples mitochondrial electron transport, we considered the possibility that mitochondrial effects, aside from DNA leakage, might mediate the antiviral responses. The failure of the pan-caspase inhibitor z-VAD to block the NTZ antiviral effects argues against pyroptosis or apoptosis, both of which can be mediated by mitochondrial factors, being a

key element of the NTZ mechanism. Since NTZ uncouples mitochondrial electron transport from ATP production, we also investigated whether energy depletion might be important. However, another OXPHOS uncoupler, dinitrophenol (DNP), the F_1F_o-ATP synthase inhibitor, oligomycin, and a respiratory chain complex III inhibitor, antimycin A, (Fig. 6) did not inhibit viral production to a large extent, thus arguing that energy limitation is not critical for the NTZ antiviral effect.

Vaccinia virus production is dependent on the metabolic switch of host cells from glucose oxidation to glutamine anaplerosis to support biosynthetic activities (Fontaine et al., 2014). Uncoupled respiration can likely lead to a relative depletion of acetyl-CoA and other TCA cycle intermediates resulting in insufficient substrates necessary to support added demands imposed by viral replicative cycle (Balcke et al., 2011) Consistent with this idea, we found that addition of palmitate, which can produce acetyl-CoA by β-oxidation, but not alpha-ketoglutarate did partially reverse the inhibitory effect of NTZ (Fig. 6).

Interestingly, Greseth and Trakman discovered that palmitate also partially rescued the VACV production from the effects of several fatty acid biosynthesis pathway inhibitors (Greseth and Traktman, 2014). Blocking mitochondrial import of palmitate with the CPT1 inhibitor, etomoxir, maintained the negative effects on viral production and this result was interpreted as a requirement for palmitate in energy production. This mechanism might also explain the antiviral activity of NTZ on other viruses, including HCMV and HCV, that alter fatty acid metabolism (Huang et al., 2013; Nasheri et al., 2013; Oem et al., 2008; Park et al., 2009; Spencer et al., 2011; Yang et al., 2008). On the other hand, our finding that other mitochondrial inhibitors had little or no effect on VACV production argues against the notion that NTZ acts by depleting energy production and suggests a possible imbalance in TCA cycle metabolites. As well, we observed that NTZ blocked the VACV-induced shut off of protein synthesis and markedly inhibited viral DNA and protein synthesis, which was not the case in studies using fatty acid synthesis and CPT1 inhibitors. Variation in the experimental conditions used, including cell types, might account for some of the differences. Regardless, the common observation in both studies that palmitate partially rescues VACV production is intriguing.

Our results add VACV to the list of viruses that are susceptible to NTZ. VACV is unusual in that it is a DNA virus that replicates in the cytoplasm and thus has quite different host cell requirements and potentially different susceptibilities to host defenses than other viruses that have been studied. Our investigation of the mechanism of anti-VACV action differ from some reports in other systems that have suggested that NTZ acts by triggering one or more host defense system, especially the PKR pathway. Although the mechanism or mechanisms by which NTZ exerts such a broad antiviral effect remains unclear, the partial rescue of its inhibitory effects by palmitate suggests an effect on the production of acetyl-CoA. Further investigation of the metabolic impact of NTZ in uninfected and infected cells should help to more clearly elucidate the mechanism.

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Highlights

- Nitazoxanide inhibits vaccinia virus replication.
- The inhibition occurs after entry but before late gene expression.
- The mechanism is likely due to nitazoxanide interfering with metabolic adaptions needed for efficient virus replication

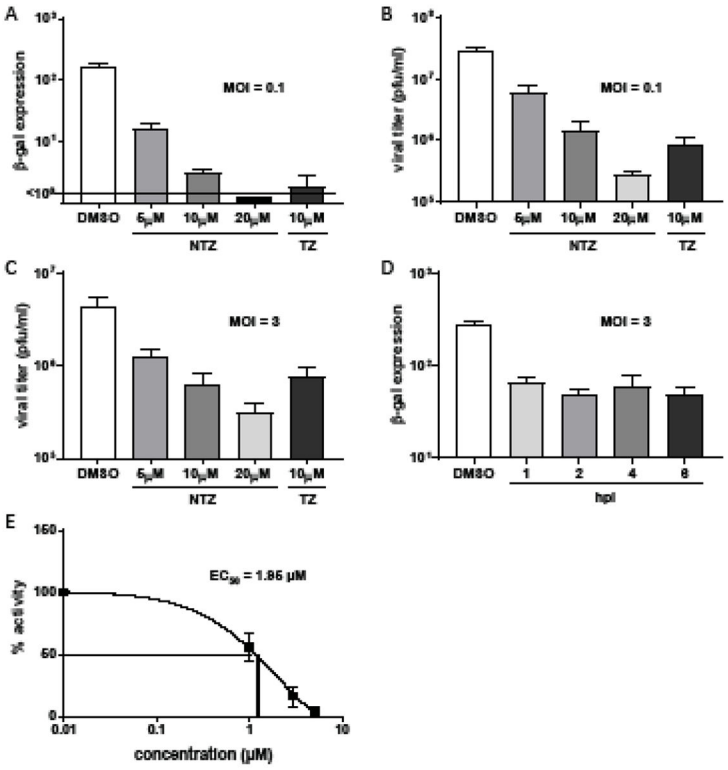

Figure 1. NTZ inhibits VACV production

HF were infected with VACV at the indicated MOIs and treated with varying concentrations of NTZ or 10 μM TZ at 1 hpi (A–C) or with 10 μM NTZ added at the times indicated in (D). Viral production was quantified by measuring β-gal activity at (A) 48 hpi or (D) 24 hpi or by titering cell-associated virus at (B) 48 hpi and (C) 24 hpi (mean ± SD, N=3). (E) The EC_{50} was calculated using Prism 7 (MOI 0.1). Depicted is the median EC_{50} from 3 independent experiments.

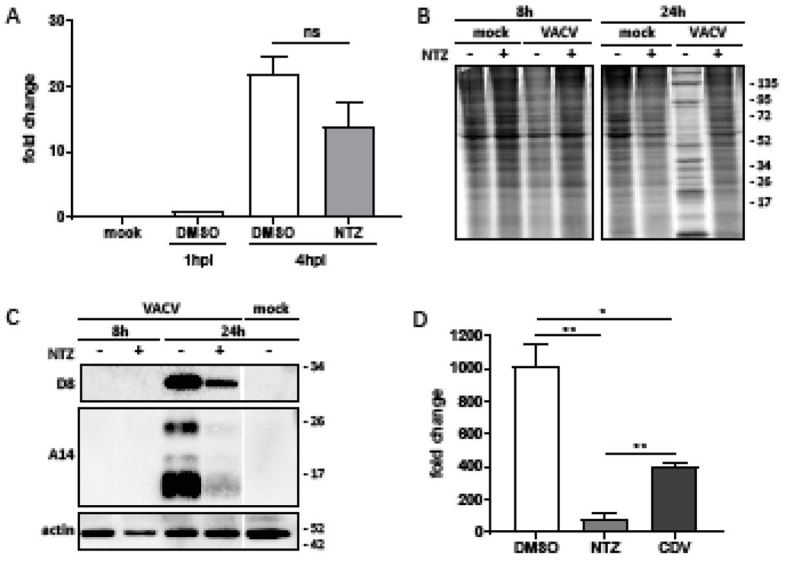

Figure 2. NTZ inhibits the VACV replicative cycle after entry

HF were infected with VACV at an MOI of 3 and mock treated or treated with 10 μM NTZ at 1h pi. (A) E3L transcript abundance, relative the amount present at 1 hpi, was quantified by qRT-PCR as described in the Methods. (B) Proteins were labelled by incubating cells with 100 μCi/ml of S^{35}-methionine for 1h before collecting cell lysates and were analysed by SDS-PAGE and autoradiography. (C) Cell lysates were collected at the indicate times timepoints and the accumulation of late VACV proteins D8 and A14 was monitored via immunoblot assay. (D) The fold increase in VACV DNA at 24 hpi, compared to amount present at 1 hpi, was measured by qPCR. (* $p < 0.05$; ** $p < 0.005$; ns, not significant)

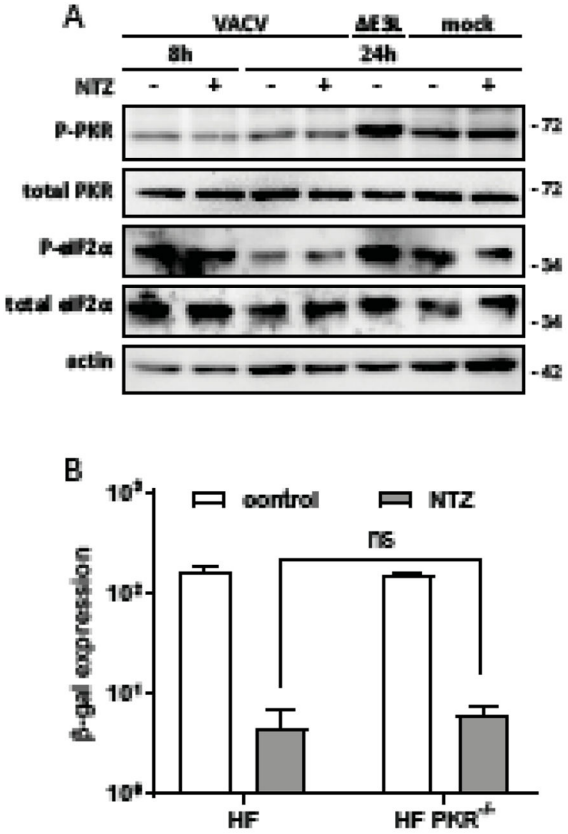

Figure 3. NTZ does not act via PKR

(A) Lysates of HF infected with VACV or VACVΔE3Lat an MOI of 3 were analysed by immunoblots for PKR, P-PKR, eIF2α, P-eIF2α, and actin accumulation. (B) Viral production was monitored at 48 hpi of HF or HF-PKR$^{-/-}$ (MOI = 0.1) with VACV after addition of no drug (0.1% DMSO) or 10 μM NTZ at 1 hpi (mean ± SD, N=3; ns, not significant)

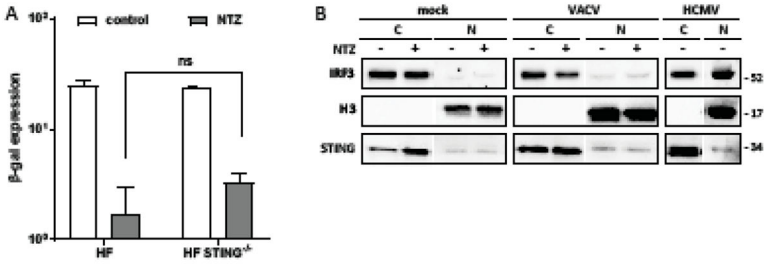

Figure 4. NTZ does not act by cGAS/STING or IRF3 signaling

(A) HF or HF STING$^{-/-}$ were infected with VACV (MOI = 0.1) and mock treated or treated with 10 μM NTZ at 1 hpi. Viral production was determined at 48 hpi by β-gal expression (ns, not significant). (B) HF were mock infected or infected with VACV (MOI = 3) and mock treated or treated with 10 μM NTZ at 1 hpi. UV-inactivated HCMV was used as a control for IRF3 translocation to the nucleus. Nuclear-cytosolic fractionation was performed at 4 hpi, and IRF3 distribution was determined by immunoblot assays. H3 was used as a control for the nuclear fraction, and STING expression for the cytosolic fraction.

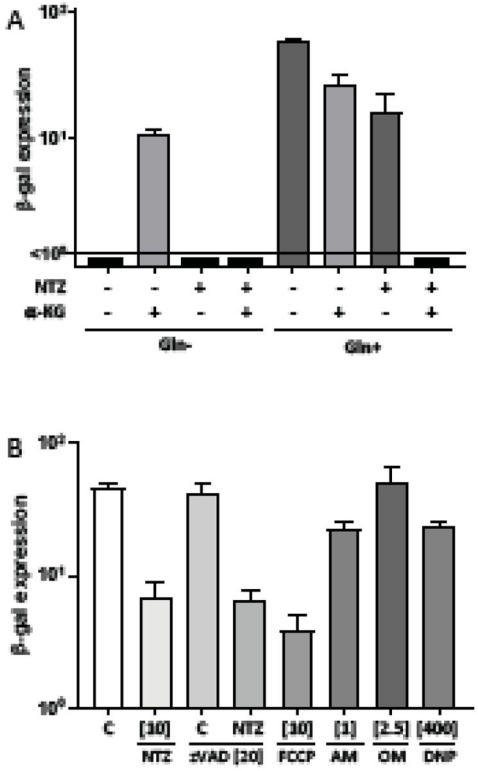

Figure 5. α-KG supplementation does not rescue VACV production after treatment with NTZ

(A) HF were infected with VACV (MOI = 0.1) in DMEM either containing or lacking glutamine (Gln), and treated at 1hpi with either 10 μM NTZ or 7mM α-KG, or a combination of both in the respective media. Viral production was determined at 48 hpi by β-gal expression. (B) HF were infected with VACV (MOI = 0.1) and treated with 10 μM NTZ, 20 μM Z-VAD-FMK (zVAD), 10 μM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), 1 μM antimycin A (AM), 2.5 μM oligomycin (OM), 400 μM 2,4-dinitrophenol (DNP), or a combination of 10 μM NTZ and 20 μM zVAD. Viral production was measured at 48 hpi by β-gal expression.

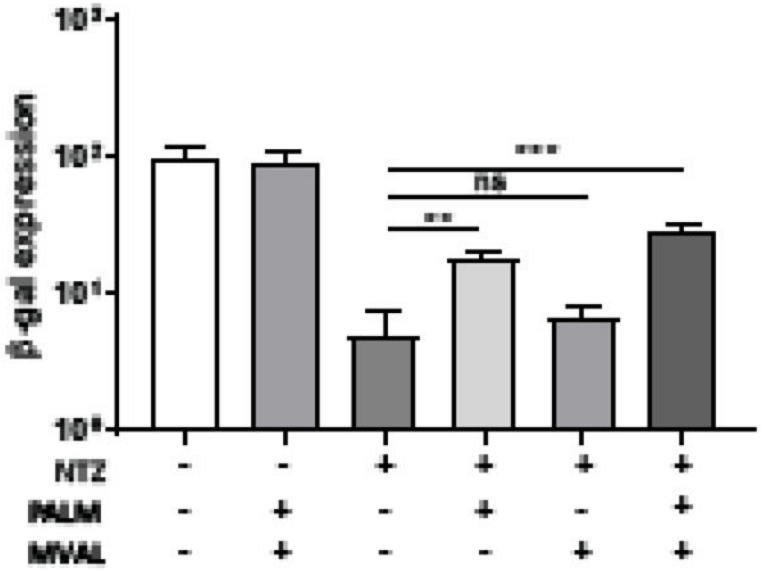

Figure 6. Palmitate rescues viral production after NTZ treatment

HF were infected with VACV (MOI = 0.1) and mock treated or treated with 10 μM NTZ at 1 hpi along with 100 μM palmitate (PALM) or mevalonate (MVAL) or both. Viral production was measured at 48 hpi by β-gal expression. (**$p < 0.005$, ***$p < 0.001$)