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Inhibition of rubella virus replication by the broad-spectrum drug nitazoxanide in cell culture and in a patient with a primary immune deficiency

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ABSTRACT

Persistent rubella virus (RV) infection has been associated with various pathologies such as congenital rubella syndrome, Fuchs's uveitis, and cutaneous granulomas in patients with primary immune deficiencies (PID). Currently there are no drugs to treat RV infections. Nitazoxanide (NTZ) is an FDA-approved drug for parasitic infections, and has been recently shown to have broad-spectrum antiviral activities. Here we found that empiric 2-month therapy with oral NTZ was associated in the decline/elimination of RV antigen from lesions in a PID patient with RV positive granulomas, while peginterferon treatment had no effect. In addition, we characterized the effects of NTZ on cell culture models of persistent RV infection. NTZ significantly inhibited RV replication in a primary culture of human umbilical vein endothelial cells (HUVEC) and Vero and A549 epithelial cell lines in a dose dependent manner with an average 50% inhibitory concentration of 0.35 μg/ml (1.1 μM). RV strains representing currently circulating genotypes were inhibited to a similar extent. NTZ affected early and late stages of infection by inhibiting synthesis of cellular and RV RNA and interfering with intracellular trafficking of the RV surface glycoproteins, E1 and E2. These results suggest a potential application of NTZ for the treatment of persistent rubella infections, but more studies are required.

1. Introduction

Rubella virus (RV) is a small, enveloped virus with a positive single-stranded RNA genome (family *Togaviridae*, genus *Rubivirus*). RV is an important human pathogen because of its ability to cause multiple pathologies after establishing persistent infections in immune privileged body sites or in fetuses during the first trimester of pregnancy. Persistent infections in a fetus, which has an immature immune system, can result in multiple birth defects called congenital rubella syndrome (CRS) (Driscoll, 1969; Plotkin et al., 2011; Tondury and Smith, 1966). Post-rubella encephalitis, often fatal, has been documented following persistent infections in the brain with both wild type and vaccine viruses (Chaari et al., 2014; Guler et al., 2009; Wolinsky et al., 1982). Chronic recurrent rubella-associated arthritis can develop after immunization of adults (Fraser et al., 1983; Tingle et al., 1985). Granulomas in persons with primary immune deficiencies (PID) and Fuchs's uveitis are newly suggested diseases associated with decades-long persistent RV infections (Abernathy et al., 2015; Bodemer et al., 2014; Doan et al., 2016; Perelygina et al., 2016). Currently, there are no antiviral drugs to treat rubella infections and identification of treatments for chronic rubella diseases will be beneficial.

Nitazoxanide (NTZ) is an FDA-approved drug (licensed in US as Alinia®) for treatment of enteritides due to parasites, protozoa, and anaerobic bacteria (Cohen, 2005; Fox and Saravolatz, 2005). NTZ was also shown to have broad-spectrum antiviral activities and is currently in Phase II/III clinical trials for hepatitis C, influenza viruses, rotavirus and norovirus (Korba et al., 2008; Rossignol, 2014; Rossignol et al., 2009). No significant drug-related health issues have been reported; the drug is safe and is approved for pediatric use (Cohen, 2005; Fox and Saravolatz, 2005). NTZ targets host functions that are essential for viral replication, but are not pathogen-specific. Several anti-viral mechanisms have been proposed such as induction of innate immunity, downregulation of viral receptors or interference with maturation of viral structural proteins at the post-translational stage, probably due to

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Ca2+ depletion inducing chronic sub-lethal stress in the endoplasmic reticulum (ER) (Ashiru et al., 2014; Elazar et al., 2009; Gekonge et al., 2015; Korba et al., 2008; La Frazia et al., 2013; Rossignol et al., 2009; Trabattoni et al., 2016).

In this study, we investigated the ability of NTZ to control RV replication in cell culture and explored the mechanisms of the anti-RV activities of NTZ in this system. *In vitro* studies confirmed that NTZ inhibited RV replication and NTZ treatment of a PID patient showed therapeutic efficacy.

2. Material and methods

2.1. Cell cultures, viruses and treatments

Human umbilical vein endothelial cells (HUVEC) (Lonza) were cultured in Endothelial Growth Medium (Lonza). A549 (ATCC #CCL185) and Vero cells (ATCC #CCL CCL81) were maintained in Dulbecco's Modified Eagle Medium (Invitrogen) containing 5% FBS (Atlanta Biologicals) supplemented with 50 μg/ml gentamicin (Invitrogen). The vaccine strain RA27/3 and clinical isolates RVi/Dezhou. CHN/0.02 (RV-Dz), RVi/Seattle. WA.USA/16.00, RVi/Burlington. MA.USA/44.11, RVi/Yavapai. AZ.USA/4.10, and RVi/Redmond. WA.USA/18.11 were propagated in Vero cells. Nitazoxanide (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mg/ml. Medium containing 0.1% DMSO was used as a vehicle control in all experiments.

2.2. Case description

A female patient born in 1986 had received immunization against measles and rubella at ages 5 and 9 years. Later, she was diagnosed as having combined immunodeficiency involving defective B- and T-cell maturation and she had received immunoglobulin replacement treatments. Since 1999, the patient had suffered from chronic skin lesions of her right hand and arm. Bone and skin biopsies showed granulomatous histology but no causative infectious agent was identified (Fig. 1A–B). Frequent empiric treatments for bacteria, mycobacteria and fungi were given without any significant benefit. In 2015, she was one of the individuals (patient #1) with PID whose cutaneous granulomas were discovered to be positive for the vaccine RV strain, RA 27/3 (Perelygina et al., 2016). In 2015, the patient developed progressive multifocal leukoencephalopathy (PML) associated with polyomavirus JC (JCV). The effects of peginterferon alfa-2a and NTZ treatments on controlling JCV and modifying the PML course has been described in a separate publication, which also includes the detailed timeline of the treatments and the description of the patient's immune status (Hautala et al., 2017). In February 2016, the patient developed septic aspiration pneumonia resulting in death.

2.3. Ethics statement

Informed consent was obtained from the patient's caregiver by the attending physician accordingly to standards of practice in Finland. This project was determined by CDC to be Non-Human Research because CDC was restricted from access to personal identifying information.

2.4. Cytotoxicity and growth inhibition assay

Cells were seeded into 96-well plates at a density of 2×10^4 cells per well and treated with serially diluted NTZ for 48 h in quadruplicate. NTZ cytotoxicity and growth inhibition was assessed with a LDH-Based cytotoxicity detection kit (Roche) using the modified protocol (Smith et al., 2011).

2.5. Virus infection and titration

Confluent cell monolayers in 48-well plates were infected with RV at an MOI of 5 pfu/ml unless differently specified. After 1-h virus adsorption, the monolayers were washed three times with PBS and then treated with NTZ or DMSO. The supernatants were collected at 48 h post-infection (hpi) and titered on Vero cells by an immunocolorimetric

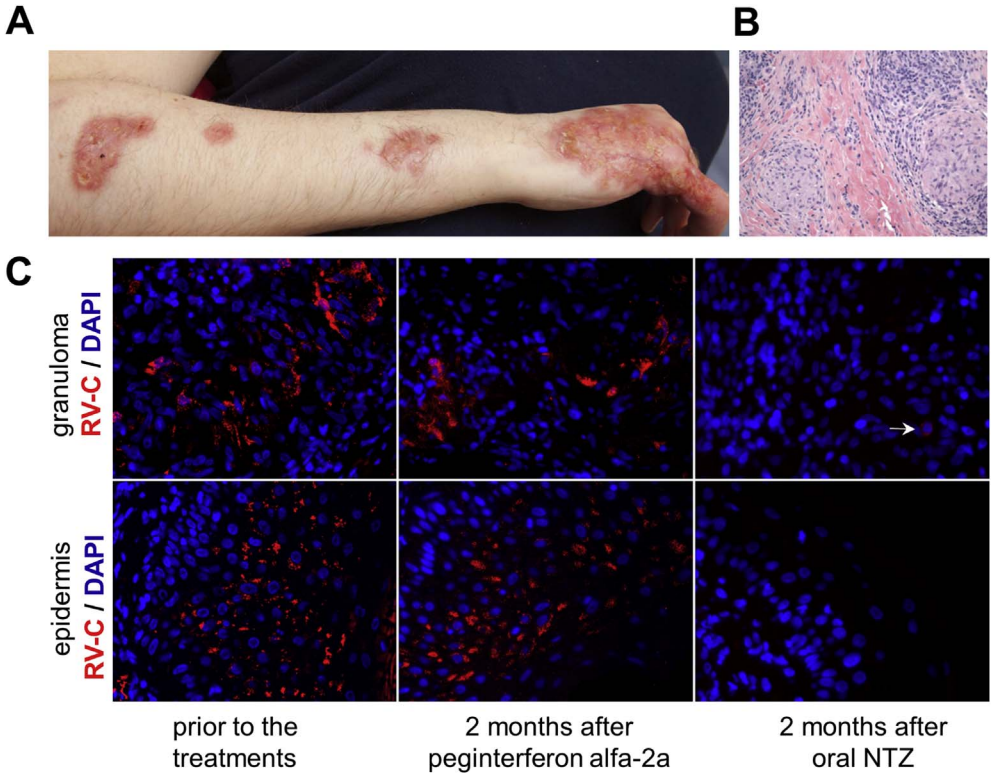

Fig. 1. RV antigen in lesions of a PID patient. (A) Cutaneous skin lesions. (B) Hematoxylin and eosin staining of a cutaneous granuloma. (C) Histological immunofluorescent staining showing distribution of RV capsid protein (red) in granulomas and epidermis of the same skin lesion before and after 2-month treatments with interferon and oral NTZ spaced one month apart. Nuclei were stained with DAPI. Multiple clusters of RV-positive cells were easily detected prior to the NTZ treatment. Note the lack of RV staining in the epidermis and a single weakly positive cell (the white arrow) in the granuloma after the NTZ treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)