Thiazolides, a new class of antiviral agents effective against rotavirus infection, target viral morphogenesis inhibiting viroplasm formation

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ABSTRACT

Rotaviruses, nonenveloped viruses presenting a distinctive triple-layered particle architecture enclosing a segmented double-stranded RNA genome, exhibit a unique morphogenetic pathway, requiring the formation of cytoplasmic inclusion bodies called viroplasms, in a process involving the non-structural viral proteins NSP5 and NSP2. In these structures the concerted packaging and replication of the 11 positive-polarity ssRNAs to generate the viral dsRNA genomic segments occur. Rotavirus infection is a leading cause of gastroenteritis-associated severe morbidity and mortality in young children, but no effective antiviral therapy exists. Herein we investigate the anti-rotaviral activity of the thiazolidine anti-infective nitazoxanide, and reveal a novel mechanism by which thiazolides act against rotaviruses. Nitazoxanide, and its active circulating metabolite tizoxanide, inhibit simian A/SA11-G3P[2] and human Wa-G1P[8] rotavirus replication in different types of cells with EC50 ranging from 0.3 to 2 μg/ml, and CC50> 50 μg/ml. Thiazolides do not affect virus infectivity, binding or entry into target cells, and do not cause a general inhibition of viral protein expression, whereas they reduce the size and alter the architecture of viroplasms, decreasing rotavirus dsRNA formation. As revealed by protein/protein interaction analysis, confocal immunofluorescence-microscopy, and viroplasm-like structures formation analysis, thiazolides act by hindering the interaction between the non-structural proteins NSP5 and NSP2. Altogether the results indicate that thiazolides inhibit rotavirus replication by interfering with viral morphogenesis, and may represent a novel class of antiviral drugs effective against rotavirus gastroenteritis.
INTRODUCTION

Rotaviruses are complex nonenveloped viruses belonging to the Reoviridae family. The rotavirion has a distinctive triple-layered particle architecture that surrounds a genome composed of 11 segments of double-stranded RNA encoding six structural (VP) and six nonstructural (NSP) proteins (1, 2). The capsid structure comprises an inner-core shell of VP2 dimers, and an intermediate shell formed by trimers of the major structural protein VP6 which interacts with both the VP2 core protein and the outer shell constituted by the VP4 protein (the rotavirus spikes which express P-serotype epitopes) and VP7 glycoprotein trimers (which express G-serotype epitopes) (2). The P- and G-serotypes represent independently segregating neutralization epitopes imparting immunity to infection. VP7, which is the second most abundant protein in the virion, is co-translationally glycosylated as it is inserted into the endoplasmic reticulum (ER) membrane via a cleavable signal sequence found at the N-terminus of the protein (1, 2).

Rotaviruses exhibit a unique morphogenetic pathway. Double layered particles (DLPs) are assembled in the cytoplasm at special areas termed viroplasms and then bud into the ER forming a transiently enveloped viral particle. As particles move toward the ER interior, the acquired envelope is lost and replaced by the outer layer of VP7 and VP4 proteins (1, 2). Virus progeny is released by host cell lysis, and by a Golgi-independent raft-mediated secretory pathway (3). Only triple-layered particles containing VP4 and VP7 are able to infect host cells efficiently (2).

Rotaviruses represent a leading cause of severe diarrheal diseases, primarily in young children, worldwide (4, 5). No effective antiviral therapy for rotavirus infection exists. We have recently shown that nitazoxanide (NTZ), a thiazolide anti-infective licensed in the USA for treating diarrhea caused by Cryptosporidium parvum and Giardia lamblia in children and adults, is effective in reducing clinical symptoms associated with rotavirus (6) and norovirus (7) infection.

Herein we document the anti-rotaviral activity of nitazoxanide and its active circulating metabolite tizoxanide [2-hydroxy-N-(5-nitro-2-thiazolyl)benzamide] (TIZ), and investigate the mechanism of the antiviral action.
MATERIALS AND METHODS

Cell culture, treatment and transfections. Monkey kidney MA104, and human colorectal adenocarcinoma Caco-2 and HT-29 cells were grown at 37°C in 5% CO2 atmosphere in Medium 199 (MA104), DMEM (Caco-2) or RPMI-1640 (HT-29) (Lonza-Cambrex, Basel, CH) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and antibiotics. MA104 cells stably transfected with NSP5 fused to the enhanced green fluorescent protein (EGFP) (NSP5-EGFP/MA104 cells) obtained as described previously (8), and BSR-T7/5 cells (BHK-21 cells stably transfected with the T7 RNA polymerase gene), a kind gift of Karl Conzelmann (Ludwig-Maxilians Universität, Munich, Germany) were cultured in DMEM complete medium supplemented with 0.8 or 1 mg/ml geneticin (G-418, Invitrogen-Life Technologies, Carlsbad, CA) respectively. Nitazoxanide and tizoxanide (Romark Laboratories, Tampa, FL), dissolved in dimethylsulfoxide (DMSO) stock solution (25 mg/ml), were diluted in culture medium, added to infected cells immediately after the adsorption period, and kept for the entire time of the experiment, unless differently specified. Controls received equal amounts of DMSO that did not affect rotavirus replication. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to MTT-formazan conversion assay (Sigma-Aldrich) as described (9). For cell growth studies, cell numbers were counted in quadruplicate samples using a hemocytometer, and cell viability was determined by vital dye exclusion technique (0.1% trypan blue). For transfection experiments, the pT7-v-NSP5 and pT7-v-NSP2 plasmids were obtained as previously described (10-11). The NSP5 cDNA was derived from the OSU rotavirus strain and the NSP2 cDNA from the SA11 strain. NSP5-EGFP/MA104 and BSR-T7/5 cells were transiently transfected using Lipofectamine 2000 (Invitrogen-Life Technologies) according to the manufacturer’s instructions, or by standard calcium phosphate technique, as described by Sambrook et al. (12).

Virus infection and titration. Simian rotavirus A/SAl1-G3P[2] (SA11) and human rotavirus Wa-G1P[8] (Wa)(kindly provided by Franco Ruggeri, ISS Rome, Italy) were utilized for these studies. Confluent MA104, Caco-2 or HT-29 cell monolayers were inoculated with pre-activated [10 μg/ml trypsin-IX (Sigma-Aldrich), 1h at 37°C] SA11 or Wa rotaviruses for 1.5h at 37°C at a multiplicity of infection (m.o.i.) of 1 plaque-forming units (PFU)/cell, unless differently
specified. After the adsorption period, the viral inoculum was removed and cell monolayers were washed with phosphate-buffered saline (PBS) and maintained at 37°C in culture medium in the absence of serum and trypsin IX, unless differently specified. For multi-step virus growth curves, infected cells (0.01 PFU/cell) were incubated in the same medium containing 1 μg/ml trypsin-IX. Virus yield was determined after three cycles of freezing-thawing of infected cultures, by hemagglutinin titration (SA11) or infectivity assay (Wa), according to standard procedures (13,14). EC₅₀ (effective concentration 50%) and CC₅₀ (cytotoxic concentration 50%) were calculated using Prism 5.0 software (GraphPad Software Inc., San Diego, CA). In the case of SA11 virus, similar results (nitazoxanide EC₅₀= 1 μg/ml in MA104 cells) were obtained by using hemagglutinin titration or infectivity assay; hemagglutinin titrations were then used for this virus. Microscopical examination of rotavirus-induced cytopathic effect was performed using Leica DM-IL microscope. Images were captured on Leica DC-300 camera using Image-Manager500 software (Leica Microsystems-GmbH, Wetzlar, Germany).

**Metabolic labeling, analysis of protein synthesis and Western blot.** Cells were labeled with [³⁵S]-methionine-cysteine ([³⁵S]EasyTag™, Protein Labeling Mix, PerkinElmer, Waltham, MA) (10 μCi/10⁵ cells) at 3h p.i.. At 24h p.i., cells were lysed in L1-buffer (20 mM Tris-Cl pH 7.4, 0.1 M NaCl, 5 mM MgCl₂, 1% NP-40, 0.5% SDS) or Laemmli sample buffer, and the radioactivity incorporated into proteins was determined as described (14). Samples containing the same amount of radioactivity were separated by sodium dodecyl sulfate (SDS)-PAGE (3% stacking-gel, 10% resolving-gel) and processed for autoradiography (15). Autoradiographic patterns were analyzed in Typhoon-8600 Imager [Molecular Dynamics PhosphorImager™ (MDP), Amersham-Pharmacia Biotech, Piscataway, NJ] for quantitative evaluation of selected proteins. Images were acquired using ImageQuant software (Amersham-Pharmacia) (MDP analysis). For Western blot analysis, whole-cell extracts containing equal amounts (25 μg/sample) of protein (or equal volumes in the case of purified viral particles) were separated by SDS-PAGE, blotted to nitrocellulose, and incubated with antibodies against NSP5, NSP2, VP1, VP2 or VP4 produced in guinea pigs and against VP7 produced in rabbits (16), or polyclonal anti-tubulin antibodies (cloneH-235, Santacruz-Biotechnology, Santa-Cruz, CA) followed by decoration with peroxidase-labeled anti-guinea pig or anti-rabbit IgG (Jackson
ImmunoResearch, West-Grove, PA). Quantitative evaluation of selected proteins was determined by MDP analysis.

**Radiolabeling, isolation and analysis of viral particles.** For analysis of proteins incorporated into viral particles, SA11-infected (5 PFU/cell) MA104 cells were treated with TIZ (10 μg/ml) or vehicle after the virus adsorption period and, at 3h p.i., were labeled with \[^{35}\text{S}]-\text{methionine-cysteine (25 μCi/ml, 21h pulse)} in the presence of the drug. At 24h p.i., cell monolayers and supernatants of infected cultures were harvested and, after three cycles of freezing-thawing, lysates were subjected to centrifugation at 16,000 x g for 10 min to remove cellular debris. Supernatants were then subjected to ultracentrifugation at 180,000 x g (Beckman XL-100K Ultracentrifuge, rotor 70.1Ti; Beckman Coulter Inc.) for 2h (14). Pellets containing viral particles were resuspended in Laemmli sample buffer, and radiolabeled viral proteins were separated by 10% SDS-PAGE and examined by autoradiography, after exposure to AmplifyTM fluorographic reagent (GE Healthcare) (14). Autoradiographic patterns were visualized as described above.

For triple-layered particles (TLP) purification, MA104 cell monolayers (2x10^8 cells) were infected with SA11 rotavirus (5 PFU/cell) and treated with TIZ as indicated above. At 24h p.i., cells were frozen and thawed three times, and virus was pelleted by ultracentrifugation as described above. Pellets were extracted with Freon (trichlorotrifluoroethane; Sigma), and banded by equilibrium ultracentrifugation in CsCl gradient, as described (17). TLPs were collected, diluted in 20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) buffer (pH 6.6) containing 10 mM CaCl_2 and pelleted by ultracentrifugation at 110,000 x g for 1h in a Beckman ultracentrifuge using a SW55 rotor. TLP pellets were resuspended in 35 μl of water and used in SDS-PAGE and Western blot analysis.

**Immunoprecipitation.** Mock-infected or SA11-infected (5 PFU/cell) MA104 cells were utilized for co-immunoprecipitation experiments. Briefly, after lysis in TNN buffer (16) containing 1 mM phenylmethylsulphonyl-fluoride (PMSF) in the presence of protease-inhibitors cocktail (PIC, Roche-Diagnostics, Basel, CH), cell debris were removed by cold centrifugation (16,000 x g, 10 min). Lysates (100 μg) were incubated with anti-NSP5 guinea pig serum in RIPA-modified
buffer (18) containing protein-A-Sepharose (Sigma-Aldrich) at 4°C for 16h. After centrifugation pellets were processed for Western blot, as described above.

**Analysis of genomic double-stranded RNA.** Total RNA was extracted from mock-infected and SA11-infected cells (5 PFU/cell) at 10h p.i. as described previously (19). Extracts containing 25 μl of RNA were separated by Tris-Glycine non-denaturing PAGE (4% stacking-gel, 10% resolving-gel), transferred to a nylon membrane (Hybond N+; GE Healthcare), and incubated with anti-dsRNA antibodies (clone J2; English and Scientific Consulting Bt., Hungary)(20) followed by decoration with peroxidase-labeled anti-mouse IgG (Jackson ImmunoResearch, West-Grove, PA). In parallel, for loading control, equal volumes of total RNA for each sample were subjected to electrophoresis in 1% agarose gels and stained with ethidium-bromide.

**Immunofluorescence microscopy and viroplasm analysis.** Cells grown on coverslips were fixed with 4% paraformaldehyde (PFA) in PBS, permeabilized with 0.5% Nonidet P40-PBS, and then incubated with anti-NSP2 and anti-NSP5 guinea pig serum, followed by decoration with fluorescein and rhodamine isothiocyanate-conjugated goat anti-guinea pig IgG. Control incubations demonstrated non cross-reactivity between the anti-immunoglobulin conjugates, or between the anti-immunoglobulin conjugate and the irrelevant primary antibody. Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI) or Hoechst-33342 (Invitrogen). Images were captured and deconvolved with a DeltaVision microscope (Applied Precision) using the Soft-WoRx-2.50 software (Applied Precision). Alternatively, for confocal microscopy, images were captured using an Olympus Fluoview FV-1000 confocal laser scanning system (Olympus America Inc., Center Valley, PA) based on Olympus IX81 inverted microscope equipped with Olympus Plan-Apochromat 60× oil-immersion objective. Images (800 × 800 pixels resolution) were analyzed using Imaris 6.2 software (Bitplane, Zurich, CH). To determine the average size of viroplasms, the area (μm²) was measured on at least 400 viroplasms for each sample using FluoView-1000 software (Olympus). Three-dimensional (3D) isosurface reconstructions of confocal sections were obtained using Imaris 6.2 software. For viral RNA staining the Click-iT RNA-imaging kit (Invitrogen) was used according to the manufacturer's instructions. Briefly, mock-infected and SA11-infected (5 PFU/cell) MA104 cells were treated with 5 μg/ml actinomycin D. At 4h p.i., cells were labeled with 2 mM 5-ethynyluridine (EU) for 6h, then fixed
and permeabilized as described (21), and the Click-iT reaction cocktail containing the Alexa Fluor488-azide was added for 30 min. For quantitative determination of RNA fluorescent signal, at least 400 viroplasms for each condition were analyzed. After subtraction of background, ROI were selected in the cytoplasm of each cell and the average intensity was measured using FluoView software and expressed as Arbitrary Fluorescence Units (AFU). Images of a representative experiment of three with similar results are shown.

Automated analysis of viroplasm formation. For quantification of the number and area of viroplasms, the MA104/NSP5-EGFP cell line (8) expressing the fluorescent fusion protein NSP5-EGFP, which concentrates in viroplasms following rotavirus infection, was used. MA104/NSP5-EGFP cells were infected with SA11 rotavirus (5 PFU/cell) and, at 1h p.i., were treated with 10 μg/ml tizoxanide or vehicle. At 8h p.i. cells were fixed with 4% PFA and nuclei were stained with Hoechst 33342 (Life Technologies). The fluorescence images were acquired using an ImageXpress Micro automated high-content screening microscope (Molecular Devices) equipped with a 20x objective. Automated image analysis of viroplasm formation was performed by MetaXpress software (Molecular Devices) using the Transfluor application module, which identifies cell nuclei (blue channel) and quantifies the area and the number of fluorescent spots in each cell (green channel). A total of 9 fields were acquired, corresponding to approximately 1,000 cells analyzed per experimental condition and replicate.

Analysis of viroplasm-like structures (VLS) formation. NSP5-EGFP/MA104 cells (10⁵/well), plated in Lab-TekII coverglass chambers (Nunch-Thermo Fisher Scientific Inc. Waltham, MA), were transiently transfected with pT7v-NSP5 (800 ng) and pT7v-NSP2 (400 ng) plasmids, using Lipofectamine 2000. NSP5-EGFP/MA104 cells were treated with 25 ng/ml 12-O-tetradecanoylphorbol-13-acetate (TPA, Sigma-Aldrich) 1.5h before transfection. At 6h post-transfection, cell monolayers were treated with 10 μg/ml TIZ or vehicle and processed for immunofluorescence after 18h. Images were captured using an Olympus Fluoview FV-1000 confocal laser scanning system (Olympus America Inc.) based on Olympus Ix81 inverted microscope equipped with Olympus Plan-Apochromat 60x oil-immersion objective. Images (800 × 800 pixels resolution) were captured and analyzed on at least 100 cells for each sample using FluoView-1000 software (Olympus).
BSR-T7/5 cells (5x10^5/well) plated in 6-well plates were co-transfected with 6 μg/well of pT7v-NSP5 and pT7v-NSP2 plasmid DNA by standard calcium phosphate technique. At 4h post-transfection 10 μg/ml tizoxanide or vehicle was added. Cells were fixed and processed for immunofluorescence at 24h post-transfection. Samples were analyzed by confocal microscopy (Zeiss LSM510 Meta) equipped with a 63x NA 1.4 objective. Approximately 400 transfected cells were analyzed per experimental condition and replicate.

**Statistical analysis.** Statistical analysis was performed using the Student's *t*- test for unpaired data. Data were expressed as the mean±SD and *p* values of <0.05 were considered significant.
RESULTS

Cytoprotective and antiviral activity of thiazolides in in-vitro rotavirus infection.

The antiviral activity of nitazoxanide and tizoxanide was evaluated in vitro against two different rotaviruses, simian SA11-G3P[2] and human Wa-G1P[8] in cell culture. Confluent monolayers of MA104 cells were mock-infected or infected with SA11- or Wa-viruses (1 PFU/cell) for 1.5h at 37°C, and treated with different concentrations of the thiazolides after virus adsorption. At 24h p.i., both viruses caused a marked cytopathic effect, characterized by cell rounding and detachment (Figure 1A). At this time a remarkable cytoprotective effect of nitazoxanide was observed at concentrations above 1 μg/ml, as determined by microscopical examination (Figure 1A) or MTT assay (Figure 1B,C). Similar results were obtained with tizoxanide (Figure 1A).

Nitazoxanide treatment caused a dose-dependent inhibition of virus replication with EC\textsubscript{50} of 1 μg/ml and 2 μg/ml for SA11- and Wa-viruses respectively (Figure 1D,E). Tizoxanide was more active than NTZ against both the simian (EC\textsubscript{50}= 0.5 μg/ml) and human (EC\textsubscript{50}= 1 μg/ml) viruses (Figure 1D,E). Both drugs were effective at concentrations non-cytotoxic for uninfected cells (CC\textsubscript{50}> 50 μg/ml), as determined by MTT assay in mock-infected cells at 24h p.i..

Thiazolides antiviral activity was independent of the multiplicity of infection. SA11-virus replication was inhibited under conditions of both single-step virus growth (in the absence of trypsin, where no reinfection by viral progeny takes place) at high multiplicity (Figure 2A), and multi-step virus growth (in the presence of trypsin) at low multiplicity (Figure 2B,C). Under multi-step virus growth conditions, TIZ at 1 μg/ml, a concentration that did not affect cell proliferation under normal growth conditions or in mock-infected cells (Figure 2D), was found to completely prevent SA11-virus replication and partially protect host cells up to 48h p.i. (Figure 2C,E). Thiazolides antiviral activity at submicromolar concentrations, non-cytotoxic for uninfected cells (CC\textsubscript{50}> 50 μg/ml), was demonstrated also in human gut-derived Caco-2 and HT-29 cells infected with SA11- or Wa-viruses (Figure 3). In addition, similar level of antiviral activity was found on rotaviruses strains G1-G4 and G8-G9 after thiazolide treatment in vitro (J.F. Rossignol, unpublished results).

Thiazolides act at post-entry level.
Tizoxanide antiviral activity persisted for at least 48h after infection (Figures 2A,B). To investigate whether thiazolide-treatment before virus adsorption could protect host cells from viral infection, MA104 cells pretreated with 10 μg/ml TIZ for 9, 6 or 3h were infected with SA11-virus after removal of the drug. As shown in Figure 4A-Pre, TIZ-treatment up to 9h prior to viral infection had no effect on rotavirus replication. Moreover, treatment of the viral inoculum or treatment of cells only during the adsorption period (Figure 4A-Ad) did not inhibit virus replication, indicating that the drug is not directly affecting rotavirus particle stability, binding or entry into target cells. TIZ-treatment initiated between 0 and 3h p.i. was the most effective in inhibiting virus replication (Figure 4A-Post); treatment started at 6h p.i. was less effective, but still able to inhibit virus replication, whereas the drug lost its effect when administered at 12h p.i..

To investigate whether thiazolides affect virus protein synthesis, mock-infected or SA11-infected (5 PFU/cell) MA104 cells were treated with 10 μg/ml TIZ after virus adsorption and, at 3h p.i., were labeled with [35S]-methionine-cysteine. At 24h p.i., labeled proteins were analyzed by SDS-PAGE and autoradiography. As expected, rotavirus infection caused a dramatic shut-off of cellular protein synthesis. The main rotavirus structural proteins VP1, VP2, VP4, VP6 and VP7, and the nonstructural NSP4 and NSP2 proteins, were found to be synthesized in large amounts in untreated cells (Figure 4B). No major changes in rotavirus protein synthesis were detected in treated cells, with the exception of the reduction of a band, subsequently identified as the mature VP7 isoform (data not shown).

In parallel samples, mock-infected and SA11-infected (5 PFU/cell) cells were metabolically labeled with [35S]-methionine-cysteine at 3h p.i. for the next 21h, and radiolabeled virions were purified from the supernatant of infected cells, as described in Materials and Methods. Proteins incorporated into viral particles were analyzed by SDS-PAGE and autoradiography. As shown in Figure 4C, TIZ treatment caused a reduction of viral structural proteins in isolated viral particles; in particular levels of the outer-layer VP4 and VP7 proteins were found to be greatly decreased. A significant reduction in the yield of triple-layered particles (TLP) was confirmed following CsCl gradient purification of viral particles from TIZ-treated cells (Figure 4D).

These results suggest that thiazolides are not affecting viral protein synthesis, but may interfere with viral particles morphogenesis.
Thiazolides alter rotavirus viroplasm formation.

In order to investigate the effect of thiazolides on viroplasm formation, rotavirus-infected MA104 cells were treated with NTZ or TIZ, and viroplasms were visualized by immunofluorescence microscopy using antibodies against the two viroplasmic nonstructural proteins NSP5 and NSP2. Results shown in Figure 5A,B evidentiate the formation of large well-defined viroplasms in untreated cells, whereas in thiazolide-treated cells, despite the presence of high intracytoplasmic levels of NSP5 and NSP2, viroplasms are present as much smaller, punctated structures. Viroplasm size in control and TIZ-treated cells was then determined by confocal microscopy using FluoView-1000 software. The viroplasm average area was found to be highly reduced in TIZ-treated samples as compared to control (Figure 5C).

In order to determine the effect of thiazolides on the number of viroplasms per cell, and to confirm the confocal microscopy results, by quantifying the viroplasm area, MA104/NSP5-EGFP cells expressing the fluorescent fusion protein NSP5-EGFP, which concentrates in viroplasms following rotavirus infection, were infected with SA11 rotavirus (5 PFU/cell) and treated with 10 μg/ml tizoxanide or vehicle at 1h p.i.. At 8h p.i. cells were fixed, nuclei were stained with Hoechst 33342, and the fluorescence images were acquired and processed for automated analysis of viroplasm formation, as described in Material and Methods. The results, shown in Figure 6A,B, confirmed that thiazolide treatment caused a significant reduction in the number of viroplasms per cell, and greatly reduced the total viroplasm area in infected cells. Similar results were observed in human gut-derived Caco-2 cells infected with SA11- or Wa-rotaviruses (Figure 6C,D), indicating that thiazolide-induced alteration of viroplasm formation is independent of the cell type and virus strain.

Viroplasms represent the main site for generation of the rotavirus 11 dsRNA genomic segments and for the accumulation of transcriptionally active DLPs (2). To analyze the viroplasmic RNA content, mock-infected and SA11-infected MA104 cells were treated with 5 μg/ml actinomycin D immediately after infection to inhibit cellular mRNA synthesis, in the absence or presence of TIZ. At 4h p.i. cells were labeled with the uridine analog 5-ethynyluridine for the next 6h. 5-Ethynyluridine-labeled RNA was then detected by reaction with fluorescent azides as described in Materials and Methods. In the same samples viroplasms were detected by confocal immunofluorescence microscopy using antibodies against NSP5 and NSP2. Viroplasmic RNA levels, determined by FluoView-1000 software, were found to be reduced by more than 70% in
TIZ-treated samples (19.84±2.41 AFU) as compared to control (68.78±14.17 AFU), reflecting the decrease in viroplasm size (Figure 7A). A partial decrease in genomic dsRNA levels was confirmed in parallel samples, after RNA electrophoretic separation and detection by immunoblot analysis using anti-dsRNA antibodies (Fig. 7B), as described in Materials and Methods.

Thiazolides interfere with NSP5/NSP2 protein interaction.

In virus-infected cells, NSP5 interacts with NSP2 and the viral polymerase VP1 (10). Data shown in Figure 5A (insets) indicate that co-localization of NSP5 and NSP2 in viroplasms typical of rotavirus infection was less defined in tizoxanide-treated cells. In order to determine whether thiazolides may interfere with the NSP5/NSP2 interaction, SA11-infected MA104 cells were treated with NTZ or TIZ, and, at 6h p.i., whole-cell extracts were immunoprecipitated with anti-NSP5 antibodies and processed for SDS-PAGE and immunoblot analysis to visualize the co-precipitating NSP2 protein. Antibodies against the viral protein VP2 were used as a negative control (16). Data shown in Figure 8A indicate that, as previously reported (10,16), the non-structural protein NSP2, but not VP2, co-immunoprecipitated with NSP5 in rotavirus-infected cells. Treatment with both thiazolides, did not affect the total level of NSP2, whereas it reduced the ability of NSP2 to interact with NSP5. In a parallel experiment, the effect of thiazolide treatment on the accumulation of the two non-structural proteins was analyzed by Western blot at different times p.i. in TIZ-treated cells. The results, shown in Figure 8B, indicate that NSP5 and NSP2 cytoplasmic levels were not significantly different in control and TIZ-treated cells up to 8h p.i..

Since coexpression of NSP2 and NSP5 is known to lead to the formation of viroplasm-like structures (VLS) in uninfected cells (11), the effect of thiazolides on VLS formation was investigated in transient transfection experiments. MA104 cells stably transfected with NSP5 fused to the EGFP (NSP5-EGFP/MA104 cells) (8) were transiently cotransfected with NSP5 and NSP2 plasmids after 1.5h stimulation with TPA to increase transcription rates from the CMV promoter. After 6h, transfected cells were treated with 10 μg/ml TIZ or vehicle and, after 18h, VLS were visualized by confocal microscopy using FluoView-1000 software. As shown in Figure 9A (left panel), NSP5-EGFP appears to be uniformly diffused throughout the cytoplasm in mock-transfected cells. As expected, when NSP2 and NSP5 are coexpressed formation of
VLS becomes evident in untreated cells (Figure 9A, middle panel), whereas VLS could not be visualized in TIZ-treated cells under the same conditions (Figure 9A, right panel).

To further confirm the effect of thiazolides on NSP5/NSP2 interaction, a similar experiment was performed in BSR-T7/5 cells stably transfected with the T7 RNA polymerase gene. BSR-T7/5 cells were transiently co-transfected with the pT7-v-NSP5 and pT7-v-NSP2 plasmids, and treated with 10 μg/ml tizoxanide or vehicle at 4h post-transfection. Cells were processed for immunofluorescence at 24h after transfection using anti-NSP2 and anti-NSP5 antibodies, and VLS were visualized by confocal microscopy. As shown in Figure 9B, TIZ treatment resulted in a reduction of VLS size as compared to control.

All together these results indicate that thiazolides hamper the interaction between NSP5 and NSP2, thus affecting viroplasm formation.
DISCUSSION

Rotavirus gastroenteritis causes an estimated 500,000 deaths every year, most of which occur in poor countries. In developed nations, around 1 in 40 children younger than 5 years are hospitalized every year because of rotavirus diarrhea (4, 5). The mainstay of therapy is oral rehydration and maintenance of proper fluid and electrolyte balance, but there has been no effective treatment for rotavirus infection. In recent years, vaccines have been developed which are likely to reduce the burden associated with rotavirus disease, particularly the incidence of severe disease (22). There are inherent limitations to the vaccines, however, which include availability and incomplete protection. Effective antiviral drugs against rotaviruses are thus urgently needed, particularly for the treatment of acute phase infections in non-immune individuals.

The results described herein document the anti-rotaviral activity of nitazoxanide and its active circulating metabolite tizoxanide. The mechanism of antiviral action appears to be novel. Thiazolides do not affect virus infectivity, binding or entry into target cells, and do not cause a general inhibition of viral protein expression. Thiazolides instead reduced the size and altered the morphology of viroplasms, the cytoplasmic structures where replication of the virus genome takes place. Viroplasms contain the non-structural proteins NSP5 and NSP2 together with other structural proteins, including VP1, VP2, VP3 and VP6 (2,11,23). Rotavirus viroplasm formation is totally dependent on the interaction between NSP5 and NSP2 oligomers (24,25). In viroplasms the initial steps of viral morphogenesis occur, in a process involving the concerted packaging and replication of the 11 positive-polarity ssRNAs to generate the viral dsRNA genomic segments (25,26). Whereas thiazolides did not substantially affect NSP5 and NSP2 intracytoplasmic levels, they were found to hinder the interaction between NSP5 and NSP2. This was found both in infected cells by co-immunoprecipitation experiments with an anti-NSP5 antibody, and in uninfected cells where VLS formation, upon NSP5 and NSP2 co-expression, was impaired. Therefore, the observed reduction in viroplasm size under treatment with thiazolides may reflect a compromised viroplasm assembly.

The mechanism responsible for the impaired NSP2/NSP5 interaction remains to be established. A defect in NSP5 oligomerization was detected in thiazolide-treated cells (S. La Frazia and M.G. Santoro, unpublished observations). It could be hypothesized that post-translational
modifications of NSP5 (i.e. phosphorylation or glycosylation) may interfere with NSP5 oligomerization, and may weaken NSP2-binding leading to inhibition of viroplasm formation. This possibility is presently under investigation.

Thiazolide-induced viroplasm reduction may in turn impair core packaging and genome replication, before trafficking of double-layered particles (DLPs) into the ER. The level of viroplasm-associated rotavirus RNA was in fact found to be partially reduced by thiazolide treatment. It should be noted that the reduced amount of dsRNA genome segments observed in thiazolide-treated cells would predict lower levels of viral proteins as a consequence of reduced secondary transcription mediated by the newly assembled DLPs. However, as indicated above, no significant reduction of viral proteins was detected in treated cells. A similar observation was previously reported in a different context, showing unaltered amounts of viral proteins in infected cells when the viral polymerase VP1 was silenced (27). In the case of thiazolide treatment, it could be hypothesized that the impairment of viroplasm formation may result in a decreased recruitment of viral plus-strand RNAs for replication, thus increasing availability of mRNAs for translation.

As indicated in the Introduction, nitazoxanide is a safe, orally bioavailable anti-infective drug licensed in the USA for treating infections by Cryptosporidium parvum and Giardia intestinalis in children and adults (28). Recently nitazoxanide has been shown to also inhibit Mycobacterium tuberculosis proliferation in vitro (29). In addition to protozoal and bacterial infections, thiazolides have emerged as a new class of broad-spectrum antiviral drugs, and nitazoxanide is in late-stage clinical trials for treating chronic hepatitis C (30) and acute uncomplicated influenza (31). The molecular mechanism at the basis of the anti-infective activity of thiazolides is still not completely understood. In anaerobic organisms such as Giardia intestinalis or anaerobic bacteria, nitazoxanide acts by inhibiting pyruvate: ferredoxin oxidoreductase (PFOR), an enzyme essential for the pathogen energy metabolism, but that is not conserved in mammals (32). In the case of M. tuberculosis infection, inhibition of mTORC1 signaling and stimulation of autophagy was observed after thiazolide treatment, and the human quinone oxidoreductase NQO1 was proposed as a drug target (33).

In the case of viral infections, the fact that, in addition to rotavirus, nitazoxanide also inhibits the replication of several RNA and DNA viruses, including hepatitis B and C (30, 34), noroviruses (7) and influenza viruses (14), suggests that thiazolides, rather than directly affecting a viral
target, may act on host-regulated processes; however, the antiviral mechanism appears to be complex and has not been elucidated as yet. Thiazolides may act at multiple levels. The protein kinase PKR has been suggested as a possible nitazoxanide target during hepatitis C virus infection (35), whereas in the case of influenza viruses thiazolide treatment causes a post-translational modification of the viral hemagglutinin, impairing influenza virus assembly and maturation (14). In the case of rotaviruses, we now show that nitazoxanide and tizoxanide also impair virus morphogenesis, inhibiting viroplasm formation; however, we cannot exclude that thiazolides may inhibit multiple steps during rotavirus replication.

Finally, we have previously reported a study, which indicated that nitazoxanide could be effective in the treatment of rotavirus diarrhea (6). More recently, nitazoxanide was confirmed as a treatment option for rotavirus diarrhea in a randomized, single-blind, controlled trial in children aged from 28 days to 24 months (36). We now describe that thiazolides, at concentrations achieved in the blood and in the gut of humans after a standard oral dose (37), exert a potent antiviral activity against rotaviruses through a novel mechanism targeting rotavirus morphogenesis. Taken together, these observations indicate that nitazoxanide and its derivatives may represent a novel class of antiviral drugs effective against rotavirus gastroenteritis.
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FIGURE LEGENDS

Figure 1. Antiviral activity of thiazolides in rotavirus-infected cells.

(A) Cytoprotective effect of tizoxanide and nitazoxanide (10 μg/ml) in MA104 cells mock-infected (U) or infected with either SA11 or Wa rotaviruses (1 PFU/cell). Photographs (100X original magnification) at 24h p.i. from a representative experiment of three with similar results are shown. (B,C) MA104 cells infected with SA11 (B) or Wa (C) rotaviruses were treated with different concentrations of nitazoxanide or control diluent immediately after the virus adsorption period. The viability of infected cells was determined at 24h p.i. by MTT assay. Data are expressed as percentage of uninfected untreated control (U). (D,E) MA104 cells infected with SA11 (D) or Wa (E) rotaviruses were treated with different concentrations of NTZ ( ), TIZ ( ) or control diluent immediately after the virus adsorption period. Virus yield was determined at 24h p.i. by hemagglutination assay or infectivity assay for SA11- and Wa-rotaviruses respectively. Data in B-E represent the mean±SD of duplicate samples from a representative experiment of three with similar results. *=p<0.01; **=p<0.05.

Figure 2. The antiviral activity of tizoxanide against rotavirus is independent of the multiplicity of infection.

Single-step (A) and multi-step (B) SA11 virus growth curves were performed on MA104 cells infected at an m.o.i. of 10 or 0.01 PFU/cell and treated with 10 μg/ml tizoxanide ( ) or vehicle ( ) immediately after the virus adsorption period. Virus yield was determined at the indicated times p.i.. (C) MA104 cells infected at an m.o.i. of 0.01 PFU/cell were treated with different concentrations of TIZ or vehicle as in B and incubated in the presence of 1 μg/ml trypsin-IX. Virus yield was determined at 24h (Δ) and 48h ( ) p.i.. EC₅₀, identical at both times p.i., is indicated. In A-C virus yield, expressed as HAU/ml, represents the mean±SD of duplicate samples from a representative experiment of three with similar results. *=p<0.01; **=p<0.05.

(D) In a parallel experiment the effect of 1 μg/ml TIZ ( ) or vehicle ( ) on MA104 cell proliferation was determined under normal growth conditions or under mock-infection conditions (inset), as described in Materials and Methods. Data, expressed as number of cells x 10⁵/well represent the mean±SD of quadruplicate samples from a representative experiment of three with similar results. (E) Cytoprotective effect of TIZ (1 μg/ml) in MA104 cells mock-
infected (U) or infected with SA11 rotavirus (0.01 PFU/cell) and treated as described in C. Photographs (100X original magnification) at 24 and 48 h p.i. from a representative experiment of three with similar results are shown.

**Figure 3. Antiviral activity of nitazoxanide in rotavirus-infected human colon cells.**

Human gut-derived Caco-2 (A, B) and HT-29 (C, D) cells were infected with simian (SA11, A, C) or human (Wa, B, D) rotavirus at a multiplicity of infection of 1 PFU/cell. Immediately after virus adsorption, the viral inoculum was removed, and cell monolayers were treated with nitazoxanide at different concentrations (0.01 to 50 μg/ml). Virus yield, expressed as percentage of untreated control, represents the mean±SD of three independent experiments (n= 6).

* p<0.05; ** p<0.01

**Figure 4. Effect of tizoxanide on rotavirus protein synthesis and particle formation.**

(A) Tizoxanide acts at a post-entry level. MA104 cells were treated with TIZ (filled bars) at the indicated times before infection (Pre), immediately after adsorption (Post), or only during the adsorption period (Ad, dashed bar). Empty bar represents untreated control (C). Virus yield (24 h p.i.) is expressed as percent of control (Control=96.0±0 HAU/ml). Data represent the mean±SD of duplicate samples from a representative experiment of three with similar results. * p<0.01; ** p<0.05. (B) Autoradiography of [35S]-methionine-cysteine labeled proteins (21h-pulse, 3h p.i.) from mock-infected (U) or SA11-infected cells, treated with 10 μg/ml TIZ or vehicle. Major rotavirus structural (VP1, VP2, VP4, VP6, VP7) and nonstructural (NSP2, NSP4) proteins are indicated. Data show a representative experiment of three with similar results. (C) Autoradiography of [35S]-methionine-cysteine labeled proteins incorporated into viral particles purified at 24 h p.i. from SA11-infected lysates treated as in B. Viral proteins (VP1, VP2, VP4, VP6 and VP7) are indicated. (D) Immunoblot analysis of VP1, VP2, VP4 and VP7 levels of rotavirus TLPs purified at 24h p.i. from SA11-infected MA104 cells treated with 10 μg/ml TIZ (+) or vehicle (-). In C-D data from a representative experiment of two with similar results are shown.

**Figure 5. Effect of thiazolides on rotavirus viroplasm formation.**

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(A) Levels of NSP2 (red) and NSP5 (green) rotavirus proteins were detected in SA11-infected MA104 cells treated with 10 μg/ml NTZ, TIZ or vehicle (C) at 12h p.i. by indirect immunofluorescence. Nuclei are stained with DAPI (blue). The overlay of the fluorochromes is shown (MERGE). The enlarged areas (insets) highlight the localization of NSP5 and NSP2 in viroplasms. Images were captured and deconvolved with a DeltaVision microscope using SoftWoRx-2.50 software. Bar, 15 μm.

(B) Confocal 3D-reconstruction of SA11-infected MA104 cells (10h p.i.) treated with 10 μg/ml TIZ (left panel) or vehicle (right panel). Viroplasms are labeled with anti-NSP2 (red) and anti-NSP5 (green) antibodies. Nuclei are stained with Hoechst-33342 (blue). The overlay of the fluorochromes is shown. (C) The average viroplasm size was determined in SA11-infected MA104 cells treated with 10 μg/ml TIZ (filled bar) or vehicle (empty bar) as described in Materials and Methods. Data, expressed in μm², represent the mean + S.D. of at least 400 viroplasms for each condition. *=p<0.05.

Figure 6. Tizoxanide-induced inhibition of viroplasm formation is independent of the cell type and virus strain.

(A,B) NSP5-EGFP/MA104 cells were infected with SA11 rotavirus (5 PFU/cell) and, at 1h p.i., were treated with 10 μg/ml TIZ (+) or vehicle (-). At 8h p.i. the number (A) and the area (B) of viroplasms were evaluated as described in Materials and Methods. Data represents the mean±SD of duplicate samples from two independent experiments. *=p<0.01. (C,D) Human gut-derived Caco-2 cells grown on coverslips were infected with simian (SA11, C) or human (Wa, D) rotavirus at an m.o.i. of 3 PFU/cell. Immediately after virus adsorption, the viral inoculum was removed, and cell monolayers were treated with 10 μg/ml TIZ (+ TIZ) or vehicle. Levels of NSP5 (green) were detected at 24h p.i. by indirect immunofluorescence. The nuclei are stained with DAPI (blue). The overlay of the fluorochromes is shown. The images were captured and deconvolved with a DeltaVision microscope using SoftWoRx-2.50 software. Bar, 10 μm.

Figure 7. Tizoxanide reduces viroplasm-associated rotavirus RNA synthesis.

(A) SA11-infected MA104 cells were treated with 10 μg/ml TIZ (SA11+TIZ, g-i) or vehicle (SA11, d-f) in the presence of 5 μg/ml actinomycin D after virus adsorption. Mock-infected cells (U, a-c) are shown as control. Viral RNA synthesis was analyzed by 5-ethynyluridine incorporation into newly transcribed RNA (6h-pulse at 4h p.i.) followed by detection using...
Alexa Fluor488-azide (green), as described in *Materials and Methods*. Viroplasms and nuclei are stained with anti-NSP5 antibodies (red) and Hoechst-33342 (blue), respectively. The overlay of the three fluorochromes is shown (MERGE). Enlarged selected areas of panels d–i, indicated by white squares, are shown on the left in panels d’–i’. Images were captured and analyzed using an Olympus Fluoview FV-1000 confocal laser scanning system. Bar, 8 μm. (B) Total RNA from mock-infected (U) and SA11-infected MA104 cells treated with 10 μg/ml TIZ (+) or vehicle (−) were extracted at 10h p.i. Whole-cell extracts (25 μl) were processed by PAGE and Western-blot analysis using anti-dsRNA antibodies (top panel). Rotavirus dsRNA genomic segments (1-11) are indicated. Ribosomal RNA levels are shown as control (bottom panel). Positions of 28S and 18S ribosomal RNAs are indicated.

**Figure 8. Thiazolides interfere with NSP5/NSP2 protein interaction.**  
(A) Whole-cell extracts from mock-infected (U) and SA11-infected MA104 cells treated with 10 μg/ml NTZ, TIZ (+) or vehicle (−) were immunoprecipitated at 6h p.i. with anti-NSP5 antibodies and processed for SDS-PAGE and Western-blot analysis using anti-NSP2 antibodies. Antibodies against the viral protein VP2 were used as control. Immunoprecipitated (IP: αNSP5) and non-immunoprecipitated (INPUT) proteins from the same samples are shown (upper panels). VP2 and NSP2 levels, determined by MDA, are expressed as arbitrary units (bottom panels). Data show a representative experiment of three with similar results. (B) Immunoblot analysis of NSP2, NSP5 and α-tubulin levels at different times p.i. in SA11-infected and mock-infected (U) MA104 cells treated with 10 μg/ml TIZ (+) or vehicle (−). As expected, NSP5 glycoprotein is found as multiple 26-28kDa isoforms and a series of slower-migrating bands reflecting protein phosphorylation and glycosylation (10).

**Figure 9. Effect of tizoxanide on viroplasm-like structures formation.**  
(A) NSP5-EGFP/MA104 cells were stimulated with TPA (25 ng/ml) for 1.5h and transiently cotransfected with NSP5 and NSP2 plasmids (middle and right panels) as described in *Materials and Methods*. Mock-transfected cells are shown as control (left panel). At 6h post-transfection, cell monolayers were treated with 10 μg/ml TIZ (+TIZ) or vehicle (-TIZ) for the following 18h. Viroplasm-like structures (VLS), indicated by white arrows, are visualized by EGFP fluorescence (green). Nuclei are stained with Hoechst-33342 (blue). The overlay of the two
fluorochromes is shown (MERGE). Images were captured and analyzed using an Olympus Fluoview FV-1000. Bar, 8 μm. (B) BSR-T7/5 cells were transiently cotransfected with NSP5, and NSP2 plasmids, treated with TIZ (10 μg/ml) or vehicle (Control) for 24h, and labeled with anti-NSP5 (green) and anti-NSP2 (blue) antibodies. The overlay of the two fluorochromes is shown (MERGE). Images were captured and analyzed by confocal microscopy as described in Materials and Methods. Bar, 8 μm. In A-B data from a representative experiment of two with similar results are shown.