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Inhibition of rotavirus infection in cultured cells by *N*-acetyl-cysteine, PPARγ agonists and NSAIDs

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ABSTRACT

Although the current rotavirus vaccines have shown good tolerance and significant efficacy, it would be useful to develop alternative or complementary strategies aimed at preventing or treating acute diarrhoeal disease caused by this viral agent. A variety of antiviral strategies other than vaccines have been assayed for rotavirus infection management. The recently demonstrated sensitivity of rotavirus infectivity to thiol/disulfide reagents prompted assays for screening drugs that potentially affect cellular redox reactions. MA104 or Caco-2 cells were inoculated with the rotavirus strains RRV, Wa, Wi or M69 and then incubated with different concentrations of drugs belonging to a selected group of 60 drugs that are currently used in humans for purposes other than rotavirus infection treatment. Eighteen of these drugs were able to inhibit rotavirus infectivity to different extents. A more systematic evaluation was performed with drugs that could be used in children such as *N*-acetylcysteine and ascorbic acid, in addition to ibuprofen, pioglitazone and rosiglitazone, all of which affecting cellular pathways potentially needed by the rotavirus infection process. Evidence is provided here that rotavirus infectivity is significantly inhibited by NAC in different cell-culture systems. These findings suggest that NAC has the potential to be used as a therapeutic tool for treatment and prevention of rotavirus disease in children.

1. Introduction

Group A rotaviruses are known as being the leading cause of acute severe diarrhoea affecting children under 5 years of age worldwide (Parashar et al., 2006). This viral agent causes an estimated half a million deaths in children each year, predominantly in developing countries (Parashar et al., 2006; Danchin and Bines, 2009). Currently, two rotavirus vaccines (RotaTeq, Merck, and Rotarix, GlaxoSmithKline Biologicals) are being used in developed and developing countries, resulting in a substantial reduction in deaths from rotavirus infection (Santosham, 2010; Nelson and Glass, 2010). However, the logistic problems associated with vaccination programs in the poorest countries are challenging the potential of rotavirus vaccines for reducing the risk of death from diarrhoea (Santosham, 2010). Moreover, the age-restricted recommendation for vaccine administration has been considered as a serious impediment for the widespread use of these vaccines in developing countries where the lowest vaccine coverage and the lowest on-time immunisation occur (Santosham, 2010; Clark and Sanderson, 2009) . Although these current rotavirus vaccines have shown good tolerance and significant efficacy, their attenuated live nature has raised some concerns related to viral shedding and the risk of transmission (Anderson, 2008) as well as their costs, efficacy and safety (Parez, 2008). The above considerations support the need for developing alternative or complementary strategies aimed at either preventing or treating the acute diarrhoeal disease caused by rotaviruses.

Rotaviruses belong to the family *Reoviridae,* and their infectious virions have a triple-layered protein capsid composed of an outermost layer (VP7 and VP4), an intermediate layer (VP6), and an inner core layer (VP2) that contains VP1 and VP3, as well as 11 double-stranded RNA genome segments (Estes and Kapikian, 2007). Trypsin cleavage of VP4 into VP5* and VP8* products activates rotavirus particles for cell entry (Benureau et al., 2005). Rotavirus entry into cells appears to be a multistep process in which viral structural proteins VP7 and VP4 (VP5* and VP8*) interact with several cell surface molecules (Lopez and Arias, 2006) including sialic acid (Haselhorst et al., 2009), integrins (Zarate et al., 2004; Graham et al., 2003) and Hsc70 (Guerrero et al., 2002; Zarate et al., 2003). It has been recently shown that rotavirus infectivity is inhibited by the treatment of cells with membrane impermeant thiol/disulfide exchange inhibitors and antibodies against protein disulphide isomerase (PDI), which suggests that infectivity was dependent on cell surface redox activity (Calderon et al., 2012).

Maintaining cell functions requires an intracellular redox balance being more reducing than oxidant (Schafera et al., 2001). This

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balance is regulated by the four main inter-related redox couples GSH/GSSG (the glutathione system), NADH/NAD^+, NADPH/$NADP^+$ and Trx(SH)2/Trx(S-S) (the thioredoxin system), from which GSH is the most abundant intracellular free thiol (Auroma et al., 1989). The oxidative stress of cells is mainly due to either a decrease in GSH or an increase in GSSG levels. GSH plays an important role in the proper functioning of the immune system (Arranz et al., 2008) which is crucial in preventing bacterial and viral infections. Many human diseases have been found to be associated with cell oxidising environments, including bacterial (Oberley-Deegan et al., 2010) and viral infections (Schwarz, 1996; Stephensen et al., 2007), as well as some post-surgery conditions (Baker et al., 2009). It has been found that some viral infections cause a pro-oxidant state in cells and body fluids leading to a decrease in GSH concentrations (Nencioni et al., 2003; Schwarz, 1996). Previous studies have provided *in vitro* evidence that the rabies virus causes axonal injury through oxidative stress, which suggests that such redox imbalances may be involved in the degeneration of neuronal processes observed *in vivo* (Jackson et al., 2010). Virus infection-associated oxidative stress has also been observed for the herpes simplex virus type 1 (Kavouras et al., 2007), hepatitis C virus (Clément et al., 2009), hepatitis B virus (Severi et al., 2006), influenza virus (Buffinton et al., 1992; Technau-Ihling et al., 2001) and HIV/AIDS (Israël and Gougerot-Pocidalo, 1997).

N-Acetylcysteine (NAC) is an amino acid that functions as a free radical scavenger antioxidant agent that is able to replenish glutathione (GSH), the most powerful cellular antioxidant (Atkuri et al., 2007). NAC achieves this replenishment by supplying cysteine for GSH synthesis in an *in vivo* reaction, which takes place primarily in the liver (Cotgreave, 1997). Although NAC did not prevent A/H1N1 virus influenza infection, it did significantly reduce the incidence of clinical symptoms (De Flora et al., 1997), whereas GSH has been reported to inhibit infection by the influenza virus in both cultured cells and mice (Cai et al., 2003). High doses of NAC have proven to be synergistic with oseltamivir treatment in protecting mice from fatal influenza infection (Garozzo et al., 2007). A synergistic combination of NAC and ribavirin was also effective in preventing mice from lethal influenza virus infection (Ghezzi and Ungheri, 2004). A long-term NAC administration attenuated influenza symptoms in elderly patients with chronic degenerative disease (De Flora et al., 1997). In addition, a patient infected with the A/H1N1 influenza virus improved rapidly after treatment with a high-dose NAC therapy in combination with antiviral medication (Lai et al., 2010). In addition, GSH and NAC have been shown to inhibit the induction of HIV-1 expression in a chronically HIV-1-infected pro-monocytic cell line and blood mononuclear cells (Ho and Dougla, 1992).

The anti-tumour activity of nonsteroidal anti-inflammatory drugs (NSAIDs) has been partially attributed to the inhibition of cyclooxygenase-2 (COX-2), which is responsible for the increased synthesis of prostaglandins (Marnett and Kalgutkar, 1999). COX-2 expression appears to be regulated by various mitogen-activated protein kinases (MAPKs) and transcription factors, such as NF-kB (Bartlett et al., 1999; Newton et al., 1997; Subbaramaiah et al., 2000). Moreover, PKA-mediated ERK1/2 and NF-kB pathways have been shown to be involved in the COX activity induction during rotavirus infection (Rossen et al., 2004).

Peroxisome proliferator-activated receptor gamma (PPARγ) ligands have been found to down-regulate the transcriptional activation of COX-2 through multiple mechanisms (Subbaramaiah et al., 2001), including the inhibition of multiple steps of the NF-kB pathway (Straus et al., 2000). Macrophages and endothelial cells have been found to express comparables levels of PPARγ, COX-2 and p-IkBα, with COX-2 expression being primarily induced by NF-kB (Vandoros et al., 2006). NF-kB activation has been shown to occur during infection by several viruses such as HIV, herpes viruses, the encephalomyocarditis virus, and rotaviruses (Roulston et al., 1999; Rossen et al., 2004). It has also been reported that MAPK pathways seem to contribute to the replication of some viruses, including the herpes simplex virus type 2 (Smith et al., 2000), the influenza virus (Pleschka et al., 2001), the encephalomyocarditis virus (Hirasawa et al., 2003), and rotaviruses (Rossen et al., 2004). An exacerbated inflammatory response to some respiratory viruses has been attributed to immune dysregulation characterised by pro-inflammatory cytokine secretion (Peiris et al., 2010). PPARs participate in the antagonism of central inflammatory pathways such as NF-kB, AP1, and STAT, whereas down-regulation of these signalling pathways by thiazolidine-2-4-diones (TZDs), including pioglitazone and rosiglitazone, has been shown to lead to reduced levels of oxidative products in monocyte–macrophages (Jiang et al., 1998). The potential use of PPARγ agonists for down-regulating the inflammatory response to virus-induced pulmonary inflammation has been recently highlighted (Bassaganya-Riera et al., 2010).

Understanding the mechanisms of molecular and cellular disturbances caused by rotaviral infection, including infection-associated oxidative stress, may enable advances in therapeutic strategies using antioxidant agents for inhibiting virus replication or preventing the pro-oxidant-associated cell injury. In the present study, we have provided evidence that rotavirus infectivity is inhibited by NAC, pioglitazone and rosiglitazone, which are drugs that affect the NF-kB pathway involved in the COX-2 transcriptional activation that has been reported to mediate post-binding rotavirus infectivity. Our findings also suggest that NAC has the potential to be used as a therapeutic tool for treatment and prevention of rotavirus disease in children.

2. Materials and methods

2.1. Cells, viruses and reagents

MA104 and Caco-2 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% foetal calf serum (FCS, Eurobio), 100 U/ml penicillin, 100 μg/ml streptomycin at 37 °C and 5% CO_2. Intestine 407 (CLL-6™) cells were obtained from ATCC. Rotavirus strains Wa (sialic acid (SA)-independent), Wi (SA-independent), M69 (SA-independent) (human) and RRV (SA-dependent) (simian) were kindly provided by Dr. C.F. Arias (Instituto de Biotecnología, UNAM, Cuernavaca, Mexico). These rotavirus and reovirus strains were propagated in MA104 cells and cesium chloride-purified as previously described (Gualtero et al., 2007). Rotaviruses were activated by treatment with 10 μg/ml trypsin at 37 °C for 30 min. All drugs used were of USP (U.S. Pharmacopeia) grade and consisted of pharmaceutical active ingredients without excipients. Drugs were dissolved in ethanol, MEM or dimethyl sulfoxide (DMSO), diluted in MEM and then sterilised through 0.22 μm membranes (Millipore, Bedfore, MA, USA). Drugs used and their sources are summarised in Table 1. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and rabbit anti-goat IgGs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal rabbit sera against rotavirus structural proteins or rotavirus non-structural proteins NSP4 and NSP5 were generated in our animal facilities.

2.2. Determination of the maximum non-toxic concentration

The cytotoxicity of drugs used was determined by adding different concentrations of each drug to MA104 or Caco-2 cell monolayers for 12 h at 37 °C. After this time, the viability of treated cells were determined using the Trypan blue exclusion assay, and the apoptotic effect was determined by observing the chromatin

Table 1
Drugs used.

Active ingredient	Concentration range examined (μM)	Active ingredient	Concentration range examined (μM)	Active ingredient	Concentration range examined (μM)
Acetaminophen	0.5–200	Gentamicin sulfate	0.5–100	Metoclopramide hydrochloride	0.5–100
Acetylsalicylic acid	0.1–100	Glibenclamide	0.5–100	Metronidazole	0.5–100
Amiodarone hydrochloride	0.5–150	Gliclazide	0.5–100	*N*-Acetylcysteine	0.73–150[a]
Ascorbid acid	0.004–4	Glimepiride	0.5–100	Naproxen sodium	0.5–150
Captopril	0.5–150	Hydrochlorothiazide	0.1–100	Hyoscine butylbromide	0.5–150
Cephalexin	0.1–100	Ibuprofen	0.5–184	Nitrofurantoin	0.1–100
Celecoxib	0.5–150	Indomethacin	0.08–67	Oxytetracicline hydrochloride	0.1–100
Clotrimazole	0.5–100	Isosorbide dinitrate	0.5–100	Pioglitazone	0.04–30[a]
Diclofenac sodium	0.1–78	Ketoprofen	0.2–196	Piracetam	0.5–150
Diphenoxylate	0.5–150	Ketorolac tromethamine	0.5–150	Piroxicam	0.03–12
Diltiazem hydrochloride	0.5–100	Lansoprazole	0.1–100	Propanolol Hydrochloride	0.5–150
Dimenhydrinate	0.5–100	Levothyroxine sodium	0.1–100	Quinapril hydrochloride	0.5–100
Dipyrone	0.1–200	Lincomycin hydrochloride	0.5–100	Ranitidine	0.5–150
Enalapril maleate	0.5–100	Loperamide hydrochloride	0.5–150	Rosiglitazone	0.4–15[a]
Erythromycin	0.1–100	Loratadine	0.5–100	Sibutramine hydrochloride	0.5–100
Esomeprazole magnesium	0.5–150	Losartan potassium	0.1–100	Sucralfate	0.5–150
Phenytoin	0.5–100	Lovastatin	0.1–100	Ticlopidine hydrochloride	0.5–100
Flunarizine	0.5–100	Mebendazole	0.5–100	Trimebutine	0.5–100
Furosemide	0.5–100	Ivlefenamic acid	3.3–1037	Sulfamethoxasole	0.5–100
Gabapentin	0.5–100	Metformin hydrochloride	0.5–100	Valdecoxib	0.5–150
Genfibrozil	0.5–100	Methocarbamol	0.5–100	Verapamil hydrochloride	0.5–100

[a] Drug concentration expressed in μM units.

condensation and nuclear fragmentation following Hoechst 33258 staining (Mosman, 1983; Guerrero et al., 2010).

2.3. Antiviral assay

The potential antiviral activity of each drug was assayed as follows: (1) MA104 or Caco-2 cell monolayers in 96-well culture plates were separately incubated with rotavirus strains (RRV, Wa, Wi or M69) for 1 h at 37 °C to allow for attachment to cell surface. After incubation the unbound virus was washed off with DMEM, cells were treated with different concentrations of drugs for 1 h, and were incubated for 12 h at 37 °C after washing off the drug. (2) Cell monolayers were incubated with different drug concentrations for 1 h at 37 °C followed by washing with DMEM and incubation with the virus for 1 h at 37 °C. After washing off the virus, cells were incubated for a further 12 h at 37 °C. (3) Cell monolayers were incubated with the virus for 1 h at 37 °C, then the virus was washed off and different drug concentrations were added. Incubation was continued for 12 h at 37 °C. In all cases, after the final incubation period the drug-treated and untreated control cells were submitted to cold methanol fixation [Guerrero et al., 2010], reaction with rabbit polyclonal anti-rotavirus antibodies (1:3000) and treatment with HRP-conjugated goat anti-rabbit secondary antibodies (0.133 μg/ml, Santa Cruz Biotechnology Inc). HRP activity was determined with amino-ethyl-carbazole (Sigma) in 50 mM Na-acetate buffer, pH 5.0, and 0.04% H_2O_2, and infectivity was assessed using a focus-forming unit (FFU) assay (Arias et al., 1987).

ELISA was used to test for the effect of the drugs on rotavirus structural protein accumulation. MA104 cell monolayers in 96-well plates were inoculated with rotavirus RRV (0.5 moi) for 1 h at 37 °C. After washing the inoculum, cells were overlaid with a medium containing NAC or ibuprofen at different concentrations, and incubated for 11 h at 37 °C. Cells were lysed with RIPA buffer and the lysates were applied to 96-well ELISA plates coated with rabbit anti-rotavirus polyclonal antibodies (1:1000). Following washing with PBS–Tween, goat anti-rotavirus polyclonal antibodies were added to wells, and the HRP-conjugated rabbit anti-goat antibody was used as a secondary antibody (1:3000). The reaction was developed using the OPD system. The effect of NAC on virus structural proteins was also determined by SDS–PAGE/Western blotting. Cells were infected and incubated with NAC as described above and then were lysed via two cycles of freeze–thawing. Lysates were treated with Laemmli's buffer and analysed by SDS–PAGE/Western blotting. The rotavirus structural proteins were revealed using rabbit anti-rotavirus polyclonal antibodies and HRP-conjugated goat anti-rabbit antibodies (0.4 μg/ml, Santa Cruz Biotechnology Inc.). The antigen–antibody reaction was visualised using using SuperSignal West Pico Trial Kit (Thermo Scientific).

The effect of drugs on the formation of viral infectious virions was determined by incubating MA104 cell monolayers with RRV (MOI 0.02) for 1 h at 37 °C. After incubation for this period, virus was washed off with DMEM and different drug concentrations added for 12 h at 37 °C. Following this incubation time, drugs were washed and cells incubated for a further 12 h at 37 °C. Cells were lysed by submitting them to two cycles of freeze–thawing before treating the lysates with trypsin (10 μg/ml) and testing their infectivity on MA104 cell monolayers in 96-well culture plates. After 12 h incubation at 37 °C, cells were submitted to the immunochemistry FFU assay described above.

2.3.1. Analysis of Hsc70 and PDI expression

Immunofluorescence analysis of Hsc70 and PDI expression during rotavirus infection and drug treatment of MA104 cells was performed by seeding cells on glass coverslips and growing them until they reached 80% confluence. Cells were inoculated with MEM or rotavirus RRV (0.5 moi) for 1 h at 37 °C and the unbound virus was removed by washing with MEM. Cells were cultured at 37 °C and fixed at different times post infection (p.i.) with ice cold methanol. Viral infection was assessed using the immunochemistry assay as indicated above. Following this the same fixed cells were washed with PBS and incubated with 50 mM NH_4Cl for 30 min at room temperature before the addition of goat polyclonal anti-Hsc70 or -PDI antibodies (2 μg/ml, Santa Cruz Biotechnology Inc) for 1 h at 37 °C. After three washes with PBS, an FITC-labelled anti-goat polyclonal secondary antibody (0.88 μg/ml, Santa Cruz Biotechnology Inc) diluted in PBS containing 0.1% Tween-20 and 1% BSA was added to cells for 30 min at 4 °C. Coverslips were air dried and then mounted in 70% glycerol in PBS on slides before examination under a fluorescent microscope (Nikon). Ten randomly selected representative fields for the different p.i. times were observed and the images were photographed. The total area of the FITC-stained Hsc70 or PDI was quantitated using the ImageJ software version 1.33a (NIH, http://rsb.info.nih.gov/ij) and the results were expressed as pixels per cell.

Hsc70 and PDI levels in rotavirus infected cells were also evaluated in cell lysates using capture ELISA. Briefly, 96-well immunoassay plates were coated with goat polyclonal anti-Hsc70 or -PDI (0.4 μg/ml, Santa Cruz Biotechnology Inc.), -PDI (0.4 μg/ml, Santa Cruz Biotechnology Inc.) or -rotavirus (1:1000, generated in our animal facilities) antibodies. After washing with PBS–Tween-20 (0.05%) and blocking with PBS–Tween containing 3% skimmed milk and 2% ovalbumin, plates were incubated with cell lysate samples. After incubation overnight at 4 °C and washing with PBS–Tween, rabbit anti-Hsc70, -PDI or -rotavirus antibodies (1:1000, generated in our animal facilities) were added and incubated for 1 h at 37 °C. Following washing with PBS–Tween, plate wells were incubated with HRP-conjugated goat anti-rabbit antibody (0.08 μg/ml, Santa Cruz Biotechnology Inc.) for 1 h at 37 °C. Detection was performed with OPD substrate (Pierce), and PBS–Tween and cell lysates from non-infected cells were used as controls.

Alternatively, Hsc70 and PDI levels were assessed using Western blotting analysis of samples from rotavirus-infected cells treated or not with NAC, pioglitazone or rosiglitazone. Rotavirus infected cells were detached from 75 cm^2 tissue culture flasks with PBS–EDTA, collected by centrifugation and lysed in RIPA buffer containing PMSF (50 μg/ml). Proteins from lysates were quantified using the NanoDrop 1000c Spectrophotometer (Thermo Scientific) and then separated by SDS–PAGE before being transferred to PVDF membranes. The Western blots were blocked by 5% skimmed milk in PBS–Tween and then probed with either goat anti-Hsc70 or -PDI antibodies (0.2 μg/ml, Santa Cruz Biotechnology Inc.). Goat anti-vimentin antibodies were used as a control. The blots were incubated with HRP-conjugated rabbit anti-goat antibody (0.4 μg/ml, Santacruz Biotechnology Inc.) and visualised using SuperSignal West Pico Trial Kit (Thermo Scientific) or AEC.

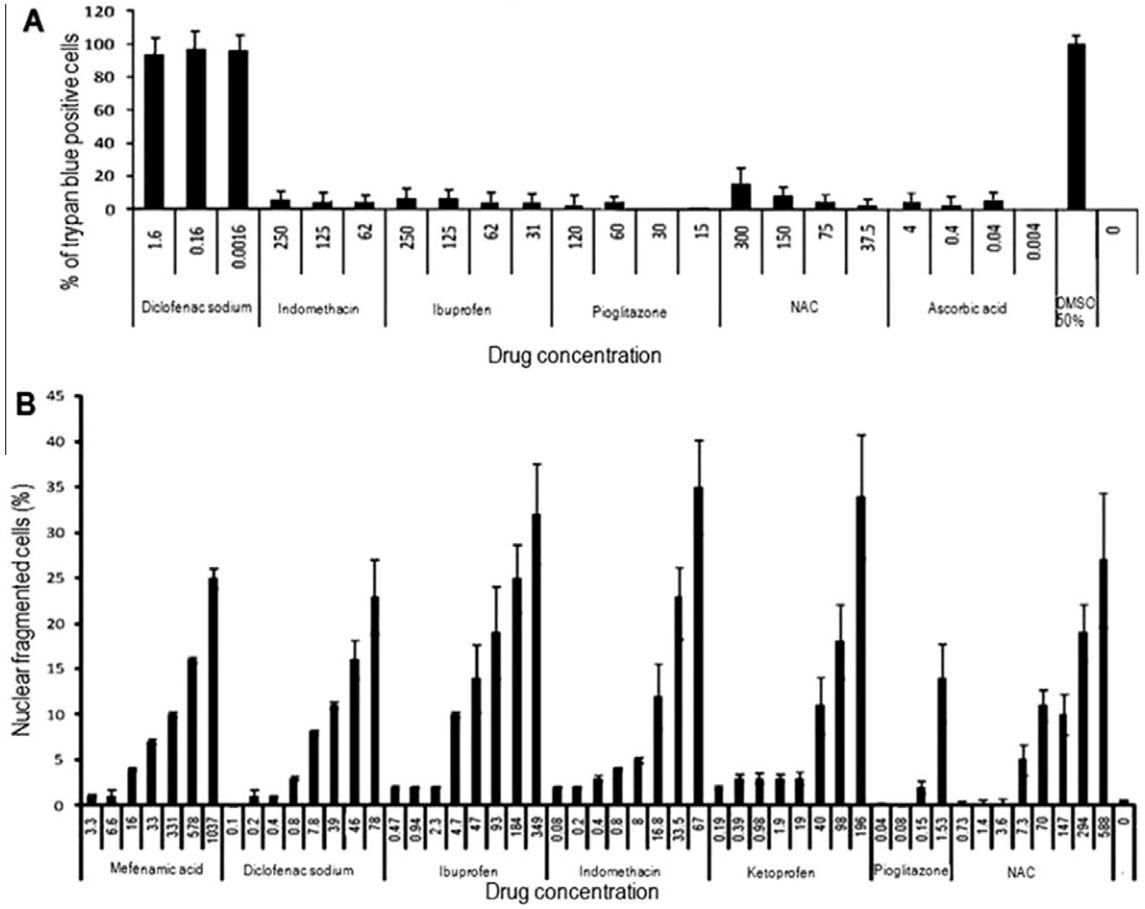

Fig. 1. The effect of selected drugs on the percentage of viable and apoptotic cells. (A) MA104 cell monolayers in 96-well plates were treated for 12 h at 37 °C with selected drugs at the indicated concentrations (mM for NAC and μM for the other drugs). Cell viability was determined using the Trypan blue exclusion assay. (B) Cells were treated as indicated in (A). The rate of apoptosis was determined by analysing the nuclear fragmentation after Hoechst 33258 staining. Values are presented as percentage relative to those obtained for untreated control cells. Error bars represent the SD from two independent assays performed in duplicate.

2.3.2. Flow cytometric analysis

MA104 cell monolayers in 96-well culture plates were inoculated with rotavirus RRV (0.5 moi) for 1 h at 37 °C before washing with PBS and incubation with 30 mM NAC for 11 h at 37 °C. Cells were harvested with PBS–EDTA for intracellular Hsc70 and PDI staining, and were then fixed in ice cold methanol for 30 min. The fixed cells were washed with PBS and incubated with 50 mM NH_4Cl for 30 min at room temperature before the addition of goat polyclonal anti-Hsc70 or -PDI antibodies (2 μg/ml, Santa Cruz Biotechnology Inc.) in 1% BSA. Following incubation for 1 h at 37 °C, cells were washed three times with PBS and then treated with FITC-conjugated rabbit anti-goat secondary antibody (0.88 μg/ml, Santa Cruz Biotechnology) diluted in 1% BSA for 20 min at 4 °C. After two additional PBS washes, rotavirus- or mock-infected cells were analysed using a Dako Cyan ADP flow cytometer (Dako, Glostrup, Denmark).

2.4. Statistical analysis

Data were expressed as mean ± SEM from at least three independent experiments carried out in duplicate for each experimental condition. Group differences were analysed by ANOVA using a significance level of 95% ($\alpha = 0.05$).

3. Results

3.1. Cell viability

To assess the possible cytotoxic effects of the different drug treatments on MA104 and Caco2 cell viability, cells were incubated with different concentrations of the respective drug. As shown in Fig. 1A, for the Trypan blue assay, there was no change in the cell viability for cells treated with ibuprofen, indometacin, NAC, pioglitazone or ascorbic acid at the indicated concentrations when compared to the control untreated cells. However, diclofenac (Fig. 1A) and piroxicam (Data not shown) treatment showed a high cytotoxic effect, especially at concentrations higher than 1.6 μM. Therefore, these drugs were excluded from the study. Similarly, for the drugs producing infection inhibition greater than 50% (Fig. 2A), the cell percentage showing nuclear fragmentation did not exceed 20% (Fig. 1B) when using Hoechst 33258 staining. The MTT assay for cell viability was discarded because of the false positive reactions in response to several drug treatments may be due to the presence of residual drugs assayed (Hamid et al., 2004).

3.2. Antiviral activity

In order to assess the potential antiviral activity of the drugs, three different strategies were followed to test the inhibitory activity of these drugs on the rotavirus infectivity in MA104 and Caco-2 cells, as described in Section 2.3. The experimental approach which retained the drug after virus attachment for 1 h at 37 °C proved to be the most effective way to affect rotavirus infectivity. A first screening was conducted which involved selecting 18 drugs (Table 1) from a group of 60 based in their ability to produce an infectivity inhibition higher than 50% in comparison to the infectivity observed in untreated rotavirus infected cells. As the purpose of the present study was to identify drugs that could be used in children, the evaluation was only continued with NAC and ascorbic acid. In addition, some NSAIDs, such as ibuprofen, pioglitazone and rosig-

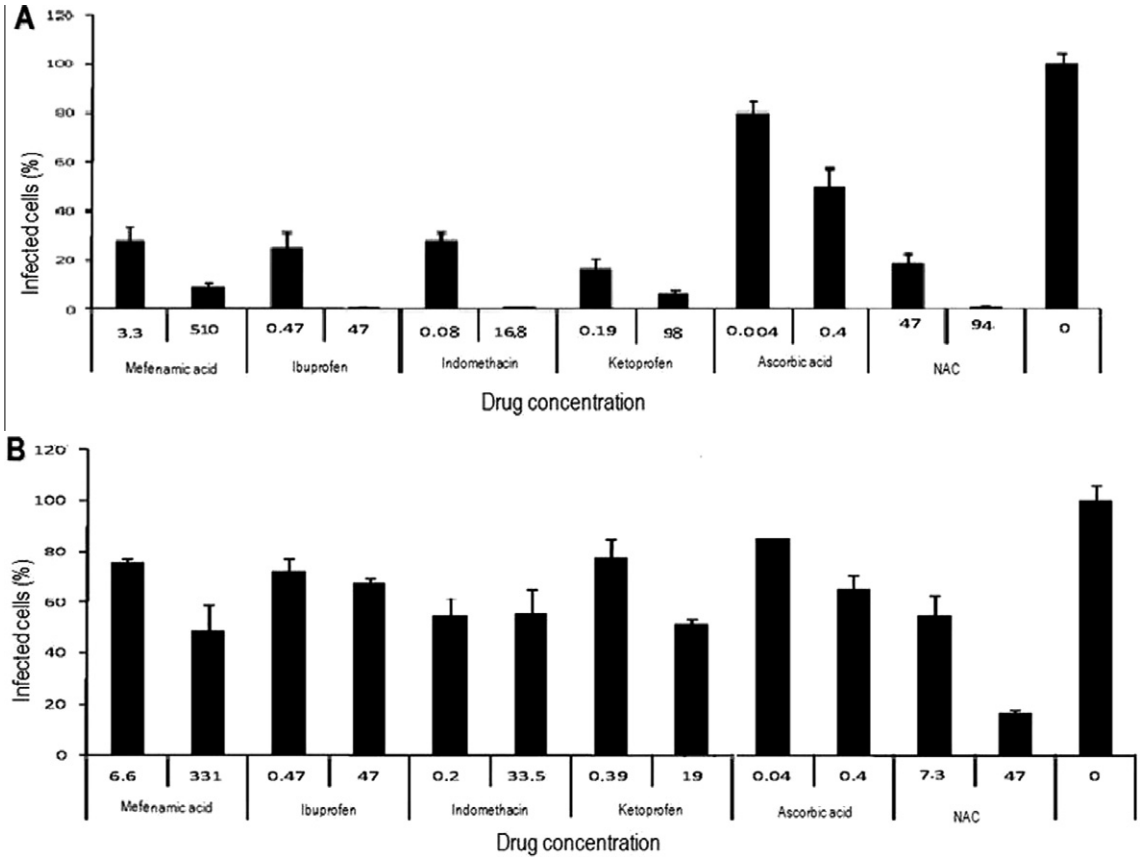

Fig. 2. Rotavirus infectivity decrease after treatment with NSAIDs and NAC. MA104 (A) and Caco2 (B) cell monolayers in 96-well plates were inoculated with RRV (0.02 moi) for 1 h at 37 °C, washed with MEM and then incubated with each drug at the indicated concentrations (mM for NAC and μM for the other drugs) during 11 h at 37 °C. Infectivity was determined by immunocytochemistry assay and values expressed as mean percentage regarding untreated control cells. The error bars represent the SD from three replicates for each set of values.

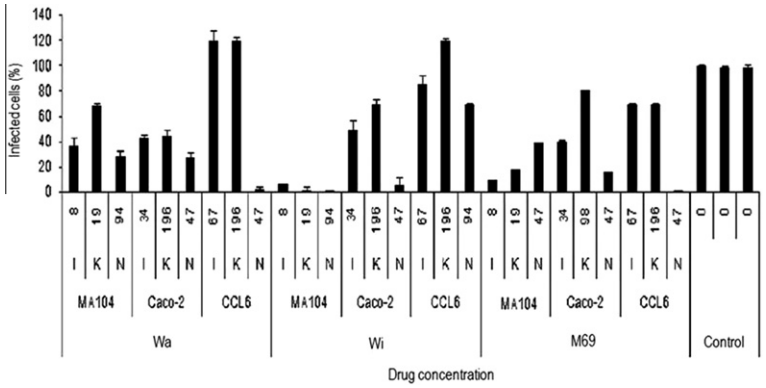

Fig. 3. Rotavirus strain- and cell line-dependent inhibition of rotavirus infectivity by NSAID and NAC treatment. MA104, Caco2 or CCL6 cell monolayers in 96-well plates were inoculated with rotavirus Wa, Wi or M69 for 1 h at 37 °C. After removing the inoculum, MA104 cells were overlaid with MEM containing indomethacin (I), ketoprofen (K) or NAC (N) at the indicated concentrations (mM for N and μM for I and K). After 11 h-incubation at 37 °C, the number of infected cells was determined by immunochemistry assay. Results are expressed as the mean percentage of infected cells compared with the mean percentage of infected control cells without drug treatment. Error bars represent SD for three independent experiments performed in duplicate.

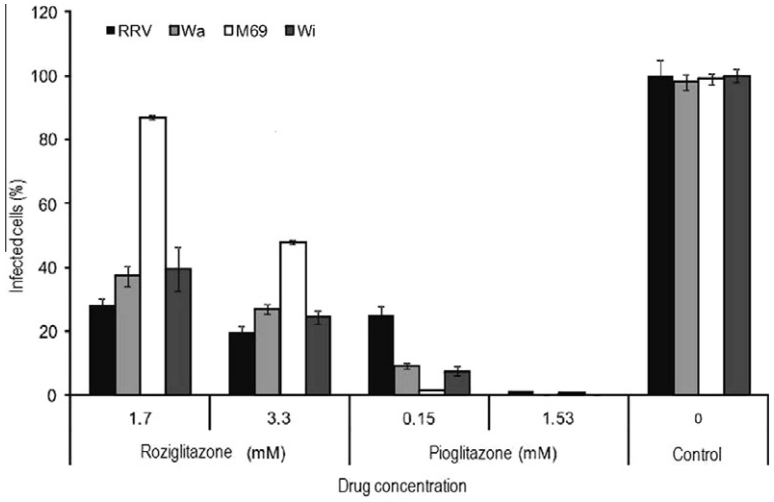

Fig. 4. The effect of PPARγ ligands on rotavirus infectivity. MA104 cell monolayers were inoculated with rotavirus RRV, Wa, Wi or M69 for 1 h at 37 °C. Following virus removal by washing, cells were incubated with the indicated pioglitazone or rosiglitazone concentrations. After 11 h-incubation for at 37 °C, the number of infected cells were counted following immunochemical staining. Data are presented as the mean percentage of infected cells regarding the infected cells without drug treatment. Error bars represent SD from three independent experiments performed in duplicate.

litazone, were further investigated as they produced a significant inhibition of rotavirus infection and there is information available regarding their action mechanisms.

To compare the relative inhibitory effects of mefenamic acid, ibuprofen, indomethacin, ketoprofen, NAC, pioglitazone and ascorbic acid on rotavirus infection, MA104 cell monolayers in 96-well culture plates were incubated with the rotavirus RRV at 0.02 moi for 1 h at 37 °C. After washing the viral inoculum with DMEM, drugs were added and maintained until 12 h p.i. Fig. 2A shows that, with the exception of ascorbic acid which inhibited rotavirus infection by about 50%, mefenamic acid, ibuprofen, indomethacin and NAC produced mean percentage inhibition values ranging between 79% and 95% at the highest concentrations tested (Fig. 2A). To determine whether these drugs have a differential effect on rotavirus infectivity depending on the type of cell line being used Caco-2 cell monolayers were infected with rotavirus RRV and treated with drugs as indicated above. Except for NAC, which produced an inhibitory effect of 93%, the remaining drugs produced much lower infectivity inhibition, which ranged from 35% to 53.6% (Fig. 2B). These findings suggest that some of the inhibitors tested produce differential inhibitory effects depending on the cell line used.

To determine whether the inhibitory effect on virus infectivity produced by the drugs indicated above was dependent on the rotavirus strain used, MA104, Caco-2 and intestine 407 (CCL-6) cell lines were inoculated with rotavirus strains Wa, Wi or M69. Their infectivity was assayed in the presence of each drug using the same protocol indicated above for rotavirus RRV. It was found that the infectivity inhibition produced by the drugs was different for each rotavirus strain when the assay was conducted on a particular cell line (Fig 3). Similarly, the infectivity inhibition produced by each drug was different when one particular rotavirus strain was assayed in different cell lines (Fig. 3). Ketoprofen showed the lowest inhibitory effect (25%) when tested on Caco2 cells infected with rotavirus strain M69, whereas its highest inhibitory effects (98% and 90%) were observed for the rotavirus strains Wi and M69, respectively, when infecting MA104 cells (Fig. 3). Rotavirus Wa and Wi infection was insensitive to ketoprofen in CCL-6 cells. Indomethacin-inhibited infection by rotavirus Wa (62% and 52%), Wi (95% and 50%) and M69 (93% and 60%) was seen when the assay was conducted in MA104 and Caco2 cells, respectively, whereas inhibition values for the these rotavirus strains were 0%, 10% and 30%, respectively, in CCL-6 cells (Fig. 3). NAC showed less dispersed

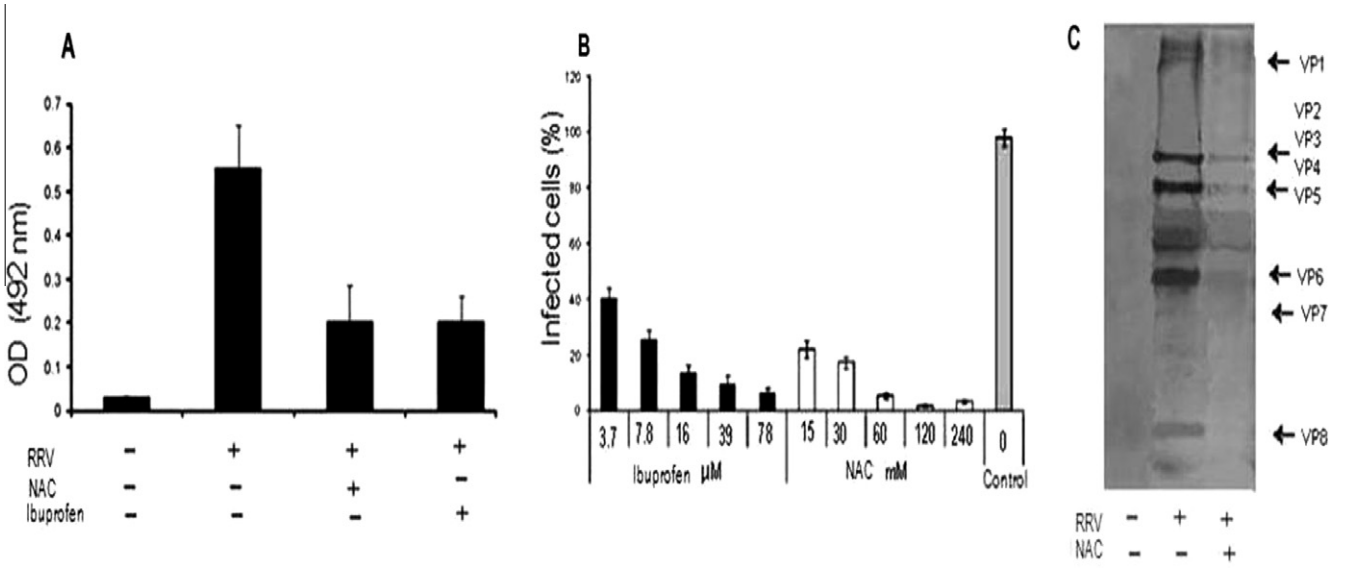

Fig. 5. The effect of ibuprofen and NAC on rotavirus structural protein and infectious particle accumulation. MA104 cell monolayers in 96-well plates were inoculated with rotavirus RRV (0.2 moi) for 1 h at 37 °C. After washing the inoculum, cells were incubated for 11 h at 37 °C in medium containing 30 mM NAC (A and C) and ibuprofen or NAC (B) at the indicated concentrations. (A) RIPA lysates from NAC-treated cells were analysed in ELISA plates coated with rabbit anti-rotavirus polyclonal antibodies. Rotavirus antigen was detected using goat anti-rotavirus polyclonal antibodies and the HRP-conjugated rabbit anti-goat antibody/OPD system. Results are presented as mean OD values ± SD from triplicate wells. (B) Cells infected as described in A were treated or not with NAC or ibuprofen at the indicated concentrations and incubated for 11 h at 37 °C. Cells were subjected to two cycles of freeze–thawing and the lysates assayed for infectivity on MA104 cell monolayers. Infected cells were determined by immunochemistry assay and the results expressed as mean percentage ± SD infectivity relative to untreated cells. (C) Cells were infected and incubated with NAC as described in (A). Cells were lysed by two cycles of freeze–thawing and treated with Laemmli's buffer. The lysates were analysed by SDS–PAGE/Western blotting and the rotavirus structural proteins revealed using rabbit anti-rotavirus polyclonal antibodies and HRP-conjugated goat anti-rabbit antibodies.

infectivity inhibition values (62–90%) in MA104 and Caco-2 cells infected with the rotavirus strains Wa, Wi and M69 (Fig. 3). These results indicate that NAC, and indomethacin to a lesser extent, showed an infection inhibitory effect that was the least rotavirus strain- and cell line-dependent.

To determine whether PPARγ, an antagonist of inflammatory pathways such as NF-kB, plays a role in rotavirus infectivity, MA104 cell monolayers were incubated with rotavirus RRV, Wa, Wi or M69 for 1 h at 37 °C. After washing the viral inoculum, cells were treated with rosiglitazone or pioglitazone and incubated for 12 h at 37 °C before analysis by the FFU assay. Fig. 4 shows that rosiglitazone was able to reduce infectivity by about 80% (RRV), 73% (Wa), 50% (M69) and 75.6% (Wi), whereas pioglitazone produced infectivity inhibition of about 98.8% (RRV), 100% (Wa), 99.2 (M69) and 100% (Wi).

3.3. Expression of rotavirus structural proteins is inhibited by ibuprofen and NAC

To assess whether the inhibitory effect of ibuprofen and NAC on rotavirus infectivity affected the synthesis of its structural proteins, MA104 cells were inoculated with RRV for 1 h at 37 °C, and the cells were further incubated for 11 h at 37 °C after PBS washing in the presence or absence of 39 μM ibuprofen or 30 mM NAC. Following this, the cells were lysed in RIPA or Laemmli's buffer for ELISA and Western blotting analysis, respectively. As shown in Fig. 5A, ibuprofen or NAC treatment reduced the ELISA absorbance due to the rotavirus structural antigen by approximately 2.5 times in comparison to that observed in untreated cell lysates. The ability of ibuprofen or NAC to inhibit rotavirus infectivity was further examined in terms of their effects on the infectious particle assembly. This was performed by virus infection assays using lysates from RRV-infected MA104 cells which had been treated with ibuprofen or NAC, in addition to lysates from untreated cells. Infectivity of dilutions from cell lysates was measured by inoculating them into MA104 cells and revealing their infectivity using the immunocytochemistry assay described above. It was found that both ibuprofen and NAC appeared to inhibit the number of rotavirus infectious particles in a dose-dependent manner, as the percentage of FFU was reduced by increasing drug concentrations (Fig. 5B). In agreement with ELISA results, the Western blotting analysis showed the same trend for the rotavirus structural protein accumulation after NAC treatment (Fig. 5C).

3.4. Ibuprofen, NAC and pioglitazone return to basal levels the rotavirus-induced increased expression of Hsc70 and PDI

To test for the effect of rotavirus infection on Hsc70 and PDI expression during the progress of rotavirus infection, MA104 cells were infected with RRV and harvested every 2 h until 16 h.p.i. Cells were processed for rotavirus antigen detection using the immucytochemistry assay described above, while Hsc70 and PDI expression was followed by immunofluorescence. The rotavirus infection progress correlated with the growing expression levels of Hsc70 and PDI, while the presence of 30 mM NAC abolished the virus antigen signal and returned both cellular proteins to their basal immunofluorescence (Figs. 6A and 7A). The semi-quantification of the immunofluorescence signals indicated that intracellular expression levels for both cellular proteins increased 2 h.p.i. when comparison was made with non-infected control cells (Figs. 6B and 7B). ELISA analysis of RIPA cell lysates for the rotavirus antigen and Hsc70 and PDI expression showed that the increased profile over time was similar for both virus antigens and cellular proteins studied (Figs. 6C and 7C). The relative intensity for Hsc70 and PDI bands on a Western blot was found to be increased in samples from rotavirus-infected cells in comparison to samples from non-infected cell lysates (Figs. 6D and 7D). In contrast, the presence of NAC, ibuprofen or pioglitazone significantly decreased the Hsc70 or PDI expression in RRV-infected cells. Similar results were observed

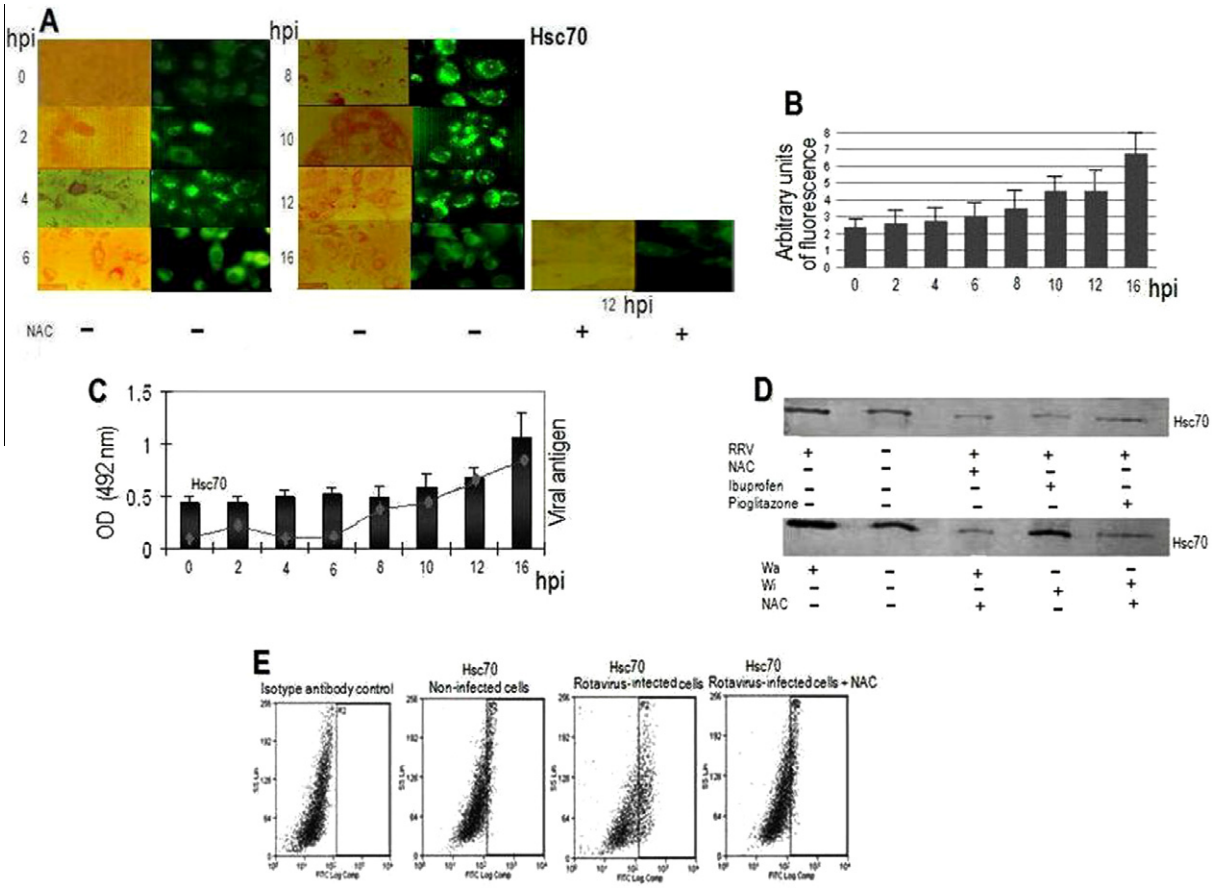

Fig. 6. Effect of ibuprofen, NAC and pioglitazone on rotavirus-induced expression of Hsc70. (A) MA104 cells were infected with RRV and harvested every 2 h until 16 h.p.i. Rotavirus antigen was detected by immucytochemistry assay, while Hsc70 expression was visualised by immunofluorescence. NAC effect was determined at 12 h.p.i. (B) Quantification of the FITC-stained Hsc70 using the ImageJ software version 1.33a at the indicated p.i. times. (C) ELISA analysis of the rotavirus infected cell lysates at different p.i. times. Rotavirus (continuous line) and Hsc70 (bars) are shown. (D) SDS–PAGE/Western blotting analysis of samples from rotavirus-infected MA104 cells collected at the indicated h.p.i. PVDF membranes were probed with goat anti-Hsc70 antibodies. Goat anti-vimentin antibodies were used as a control. The reaction was revealed using HRP-conjugated rabbit anti-goat antibody and the SuperSignal West Pico Trial Kit. (E) Flow cytometry analysis of rotavirus-infected MA104 cells which had been treated or not with 30 mM NAC. Cells harvested at 12 h.p.i. were methanol-fixed and incubated with goat anti-Hsc70 antibodies at 12 h.p.i. Cells were treated with FITC-conjugated rabbit anti-goat secondary antibody and analysed with a Dako Cyan ADP flow cytometer. Isotype antibodies were used as a control.

for cells infected with rotavirus strains Wa or Wi when the culture was performed in the presence of NAC (Figs. 6D and 7D).

In order to study whether the antiviral effect caused by NAC is related to the Hsc70 and PDI expression levels, MA104 cells were inoculated with RRV and submitted to FACS analysis 12 h.p.i. This analysis indicated that there is an enhanced Hsc70 and PDI expression in rotavirus-infected cells and that that expression was returned to the basal levels found in non-infected control cells when the infection occurred in the presence of 30 mM NAC (Figs. 6E and 7E). However, there was no evidence that the NAC inhibitory effect is primarily exerted on Hsc70 and PDI expression or, alternatively, on a different pathway involved in rotavirus life cycle.

To comparatively examine the effects of ibuprofen, NAC and pioglitazone on Hsc70 and PDI expression during rotavirus infection, MA104 cells were inoculated with RRV for 1 h at 37 °C before washing. Cells were further incubated for 11 h at 37 °C with or without these rotavirus infectivity inhibitors. A Western blot analysis showed that rotavirus infection in the absence of inhibitors led to an increased intensity of the Hsc70 and PDI bands when compared to non-infected control cells. Conversely, the incubation of infected cells in the presence of either inhibitor resulted in a band intensity for these two cellular proteins similar to that found in non-infected control cells (Figs. 6D and 7D). In addition, a similar analysis for Wa and Wi infection of MA104 cells showed that this infection led to enhanced intensity for Hsc70 and PDI bands, which was returned to basal levels when the infection took place in the presence of NAC.

4. Discussion

As a result of searching for antiviral agents against rotavirus infection, a broad spectrum of widely prescribed drugs used for purposes other than virus infections were tested. Among 60 tested drugs an initial group of 18 drugs was identified, including NSAIDs and PPARγ agonists, that was able to produce an inhibitory activity on rotavirus infectivity of at least 50% in cell culture systems. Because the main purpose of the present study was to find drugs that might be used in children, work focused on establishing the sensitivity profile of rotavirus infectivity to NAC and ascorbic acid. However, ibuprofen, pioglitazone and rosiglitazone, which showed significant inhibitory activity, were also included in the study as their known action mechanisms could contribute to the understanding of basic cellular mechanisms involved in rotavirus infection.

Basic rotavirus infection studies have mainly used cultured cell lines, such as MA104 and Caco2 (Isa et al., 2008). This study attempted to determine whether the inhibitory effect produced by

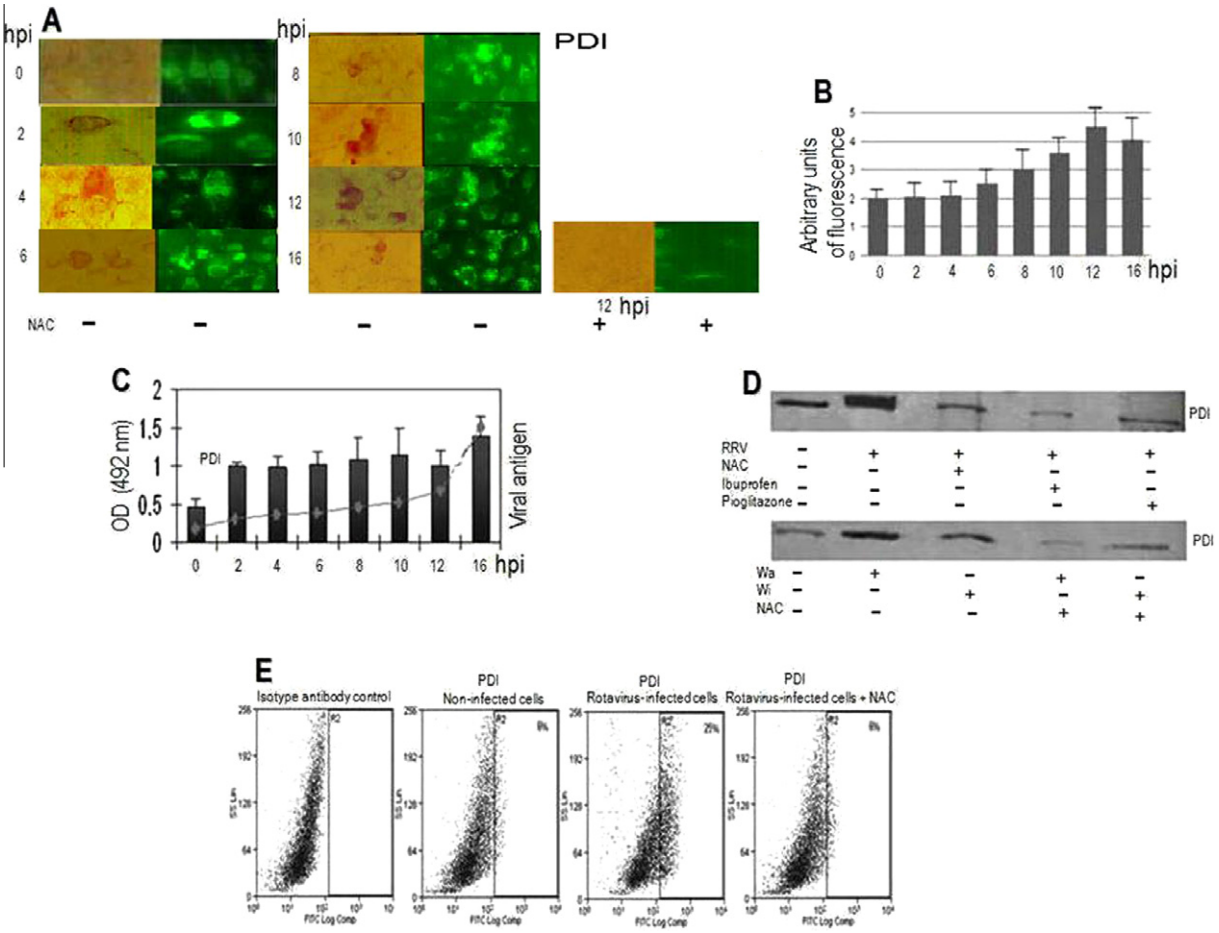

Fig. 7. Effect of ibuprofen, NAC and pioglitazone on rotavirus-induced expression of PDI. (A) MA104 cells were infected with RRV and harvested every 2 h until 16 h.p.i. Rotavirus antigen was detected by immucytochemistry assay, while PDI expression was visualised by immunofluorescence. NAC effect was determined at 12 h.p.i. (B) Quantification of the FITC-stained PDI using the ImageJ software version 1.33a at the indicated p.i. times. (C) ELISA analysis of the rotavirus infected cell lysates at different p.i. times. Rotavirus (continuous line) and PDI (bars) are shown. (D) SDS–PAGE/Western blotting analysis of samples from rotavirus-infected MA104 cells collected at the indicated h.p.i. PVDF membranes were probed with goat anti-PDI antibodies. Goat anti-vimentin antibodies were used as a control. The reaction was revealed using HRP-conjugated rabbit anti-goat antibody and the SuperSignal West Pico Trial Kit. (E) Flow cytometry analysis of rotavirus-infected MA104 cells which had been treated or not with 30 mM NAC. Cells harvested at 12 h.p.i. were methanol-fixed and incubated with goat anti-PDI antibodies at 12 h.p.i. Cells were treated with FITC-conjugated rabbit anti-goat secondary antibody and analysed with a Dako Cyan ADP flow cytometer. Isotype antibodies were used as a control.

the selected drugs were to some extent dependent on the rotavirus strains and cell lines used. The results showed that, except for NAC, the drugs assayed were less effective at inhibiting rotavirus RRV infection when assayed on Caco2 cells in comparison to MA104 cell results. This differential inhibitory behaviour suggests that RRVs could differentially use existing cellular pathways depending on the target cell, or that these cellular pathways could exhibit differential sensitivities to the drugs assayed. Moreover, the assay using different rotavirus strains to infect three different cell lines indicated that the infectivity inhibition produced by each drug was different for each rotavirus strain when the inhibition assay was performed on a particular cell line. Differential infectivity sensitivity to each drug assayed for one particular rotavirus strain was also observed when the infectivity assay was performed in other cell lines. Taken together, these findings also suggest that rotavirus strains probably use differentially some cellular pathways without excluding differential cellular sensitivities to the drugs tested. Interestingly, the NAC inhibitory activity was found to be the least rotavirus strain- and cell line-dependent.

Using *in vitro* results from the NAC inhibitory effect on rotavirus infection for estimating the appropriate NAC doses to be used in clinical trials deserve some considerations involving bioavailability. Since NAC (i.e. 30 mM) is in direct contact with cultured cells and these are being infected with culture-adapted rotavirus strains, the potential inhibitory effect of NAC on virus infection *in vivo* would need additional considerations. After oral administration of NAC it should be noted that initially the drug would be in direct contact with the cells (enterocytes) being infected. It would remain to be determined whether NAC would have any inhibitory effect by directly contacting the enterocyte luminal surface following oral drug delivery or whether its inhibitory effect would be more related with the amount of drug absorbed into the enterocytes from the lumen. Determining the half maximal effective concentration (EC_{50}) for NAC in rotavirus-infected enterocytes *in vivo* would require knowledge about its return kinetics from the systemic circulation back to the small intestinal cells either as NAC itself or cysteine. We have found that three unvaccinated children being positive to rotavirus infection drastically reduced their diarrhoeal episodes and rotavirus antigen following NAC treatment (60 mg/kg/day) for 4 days (Guerrero and Acosta, 2012). NAC could have an advantage over nitazoxanide (a drug used for treating rotaviral diarrhoea in children) as the former can be used even in pre-term new-born infants (Ahola et al., 1999) whilst the latter may only be administered to children older than 1 year of age (Rossignol et al., 2006; Bailey and Erramouspe, 2004).

In searching for a basic explanation for the NAC antiviral activity, it must be mentioned that NF-kB is an oxidative responsive

transcription factor that can be activated by ROS, cytokines, or viruses (Garcia et al., 2009; Brigelius-Flohe et al., 2004). It has also been found that rotaviruses can activate NF-kB-dependent gene expression and induce the production of IL-8 and other cytokines (Casola et al., 2002; Sheth et al., 1996; Rollo et al., 1999). However, recent results have suggested that although rotaviruses can strongly activate NF-kB during infection, this activated transcription factor is prevented from inducing gene expression in MA104 cells (Holloway et al., 2009). Many viral infections have been reported to cause a redox imbalance in host cells. However, rotavirus infection has been recently reported to lack induction of oxidative stress in Caco2 cells, while showing a concomitant increase of manganese superoxide dismutase (MnSOD). However, the possibility that a short time increase in ROS levels was responsible for MnSOD increase during rotavirus infection was not disproved (Gac et al., 2010). Increased production of ROS has been shown to be responsible for the MnSOD overexpression through the activation of NF-kB factor by ROS (Jones et al., 1997).

Although the present work did not directly address the mechanism of NAC-mediated inhibition of rotavirus infectivity, NF-κB inhibition could be a possible mechanism based on previously published results (Schubert et al., 2002; Bagchi et al., 2010; Gupta et al., 2010). More specifically, inhibition of rotavirus infectivity produced by NAC could be due to the suppression of the NF-kB DNA binding activity (Lappas et al., 2003), which is required for COX-2 expression (Newton et al., 1997). This is consistent with the fact that COXs and PG2 have been identified as important mediators of rotavirus infection acting at a post-binding step (Rossen et al., 2004). NAC antioxidant activity has been shown to block the activation of the nuclear transcription factor NF-kB, which is the transcription element that activates HIV from its latent state (Nabel and Baltimore, 1987). Blocking NF-kB activation has explained the inhibition of HIV expression in various cultured cells when treated with NAC (Raju et al., 1994).

On the other hand, PPARγ is a nuclear receptor that is able to suppress inflammatory responses mainly via trans-repression mechanisms (Ricote and Glass, 2007). It has been found that pioglitazone and other thiazolidinediones (TZDs) inhibit hyperglycemia-induced intracellular ROS production and mitochondrial reactive oxygen species (mtROS) production in endothelial cells, an effect that is accompanied by MnSOD induction and mitochondrial biogenesis (Fujisawa et al., 2009). It is therefore plausible to suggest that the inhibition of rotavirus infectivity by pioglitazone and rosiglitazone, which are two PPARγ agonists, could take place through the ability to inhibit some steps of the NF-kB pathway. The result of this would be the down-regulation of COX-2 transcriptional activation (Subbaramaiah et al., 2001; Straus et al., 2000).

Rotavirus infection was able to induce an increase in Hsc70 and PDI expression levels in infected cells. Hsc70 has been identified as a cell surface receptor for rotaviruses during its entry to MA104 cells (Guerrero et al., 2002; Zarate et al., 2003; Gualtero et al., 2007), and VP7 has been suggested as an endoplasmic reticulum (ER) PDI substrate during rotavirus assembly (Svensson et al., 1994; Mirazimi and Svensson, 1998; Maruri-Avidal et al., 2008). In addition, MA104 cell surface PDI has been found to contribute to rotavirus entry (Calderon et al., 2012). It is therefore not surprising that these cellular proteins are increased in rotavirus infected cells. On the other hand, the inhibitory effects of ibuprofen, NAC or pioglitazone were associated with the return of Hsc70 and PDI to expression levels, which were indistinguishable from those observed in non-infected cells. However, the assay described in this study did not provide evidence concerning the primary mechanisms involved in returning Hsc70 or PDI expression to basal levels after treating rotavirus infected cells with these inhibitor drugs.

A database of drug-associated gene expression profiles has been built in order to identify molecules that induce cellular gene expression changes following cell treatment (Lamb et al., 2006; Lamb, 2007). This approach has been used for identifying molecules that induce the expression of cellular functions acting in opposition to those induced by virus infection (Josset et al., 2010). The data generated in this study suggest that NAC and pioglitazone provide an unfavourable cellular environment for rotavirus multiplication. It appears that cellular gene expression could be modified upon virus infection and that some of the cellular proteins expressed could be involved in the rotavirus cell cycle. These results also suggest that some of the drugs tested had a different degree of inhibitory activity against virus strains and/or the cell line being infected. The antiviral approach presented in this study is based more on targeting the cellular factors needed for virus infection rather than inhibiting virus encoded functions. The effective inhibition of cellular pathways could circumvent the emergence of drug-resistant viruses. The results shown here regarding the targeting of cellular pathways may provide therapeutic tools that may be useful for treating rotavirus infections and other viral infections.

In conclusion, in the present study evidence was provided suggesting that rotavirus infectivity is inhibited in cultured cells by NAC, pioglitazone and rosiglitazone, which are drugs affecting the NF-kB pathway. This pathway is involved in the global control of the pro-inflammatory response of endothelial cells, including the COX-2 transcriptional activation that mediates post-binding rotavirus infectivity. (Rossen et al., 2004). Our results are extending the knowledge about the implication of NF-κB activation and COX-2 activity in rotavirus infection cycle as inhibition of these cellular functions has been reported to affect rotavirus infection at a step following virus binding to cell surface receptors (Rossen et al., 2004). As a whole, our findings suggest that NAC has the potential to be used as a therapeutic tool for treatment and prevention of rotavirus disease in children.

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