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Prevention of renal damage caused by Shiga toxin type 2: Action of Miglustat on human endothelial and epithelial cells

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ABSTRACT

Typical hemolytic uremic syndrome (HUS) is responsible for acute and chronic renal failure in children younger than 5 years old in Argentina. Renal damages have been associated with Shiga toxin type 1 and/or 2 (Stx1, Stx2) produced by *Escherichia coli* O157:H7, although strains expressing Stx2 are highly prevalent in Argentina. Human glomerular endothelial cells (HGEC) and proximal tubule epithelial cells are very Stx-sensitive since they express high levels of Stx receptor (Gb3). Nowadays, there is no available therapy to protect patients from acute toxin-mediated cellular injury. New strategies have been developed based on the Gb3 biosynthesis inhibition through blocking the enzyme glucosylceramide (GL1) synthase. We assayed the action of a GL1 inhibitor (Miglustat: MG), on the prevention of the renal damage induced by Stx2. HGEC primary cultures and HK-2 cell line were pre-treated with MG and then incubated with Stx2. HK- 2 and HGEC express Gb3 and MG was able to decrease the levels of this receptor. As a consequence, both types of cells were protected from Stx2 cytotoxicity and morphology damage. MG was able to avoid Stx2 effects in human renal cells and could be a feasible strategy to protect kidney tissues from the cytotoxic effects of Stx2 *in vivo*.

1. Introduction

Typical hemolytic uremic syndrome (HUS) is characterized by nonimmune hemolytic anemia, thrombocytopenia and acute renal failure (Karpman, 2002). The classical form of HUS is a complication of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) infection, the most prevalent infectious agent responsible for the development of this pathology (Karmali et al., 1985). In Argentina, post-enteric HUS is endemic and approximately 400 new cases are reported annually. In the last decade, the estimated annual rate ranged from 10 to 17 cases per 100,000 children under 5 years old, 10 times the value reported by industrialized countries (Rivas et al., 2006b). It is the most common cause of acute renal failure in children and the second leading cause of chronic renal failure in children younger than 5 years old (Repetto, 1997). Five to 10% of children with STEC infection develop HUS and nowadays supportive care is the only available treatment. The mortality rate among children with HUS is 2.5–5% (Pianciola et al., 2014). STEC strains carry inducible lambda phages integrated into their genomes that encode Shiga toxin type 1 and/or 2 (Stx1, Stx2), with several allelic variants each one. The entire phage and specific regions within the phage can be gained or lost through horizontal gene transfer (Laing et al., 2012). Stx comprise a single 30 kDa A-subunit and a pentamer of non-covalently attached identical 7 kDa B-subunits. Enzymatic activity resides in the A subunit whereas the cell recognition receptor binding properties are in the B-subunits (Fraser et al., 2004). Clinical and histological renal damage has been strongly associated with Stx1 and/or Stx2 produced by *Escherichia coli* O157:H7 and other related bacterial strains frequently isolated from children with HUS, although strains expressing Stx2 are highly prevalent in Argentina (Rivas et al., 2006a). STEC are present in the intestinal tract of healthy cattle and disease outbreaks are frequently due to

Abbreviations: HUS, Hemolytic Uremic Syndrome; Stx2, Shiga toxin type 2; STEC, Shiga toxin (Stx)-producing *Escherichia coli*; MG, Miglustat; HGEC, human glomerular endothelial cells; HK-2, immortalized human proximal tubule epithelial cells; Gb3, globotriaosylceramide; GL1, enzyme glucosylceramide synthase.

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ingestion of undercooked ground beef, manure-contaminated water, vegetables, fruit or unpasteurized milk. After bacteria colonize the bowel, Stx is released into the gut lumen and then absorbed into the circulation, where the toxin reaches vascular endothelial cells and finally binds the glycolipid globotriaosylceramide (Gb3) on the plasma membrane of target cells (Jacewicz et al., 1986). Stx is internalized into the cell, and the A subunit inhibits protein synthesis by removing an adenine residue from 28S ribosomal RNA in the cytosol, causing apoptosis following protein synthesis inhibition which in turn leads to endoplasmic reticulum stress. In this regards, toxin activates multiple stress-associated signaling pathways in mammalian cells. The ribotoxic stress response is activated following the depurination reaction localized on eukaryotic ribosomes. The unfolded protein response may be initiated by toxin unfolding within the endoplasmic reticulum, and maintained by production of truncated, misfolded proteins following intoxication. Activation of the ribotoxic stress response leads to signaling through MAPK cascades, which appears to be critical for activation of innate immunity and regulation of apoptosis (Tesh, 2012).

The kidney is the most affected organ in diarrhea-associated HUS, where very Stx-sensitive cells express high amounts of biologically active Gb3 (Obrig, 2010). Gb3 receptor has been described in human glomerular microvascular endothelial cells (HGEC) (Amaral et al., 2013; Obrig et al., 1993), podocytes, mesangial cells, and proximal tubule epithelial cells of the kidney (Karpman et al., 1998; Lingwood, 1996). Recently, we developed primary cultures of HGEC and demonstrated that Stx2 decreased cell viability by endothelial injury similarly to that documented in biopsies of HUS patient kidneys. In addition, Gb3 mediates Stx2 cytotoxic effects in these cells (Amaral et al., 2013).

In recent years, new therapeutic strategies have been developed to achieve an effective treatment for Stx-mediated HUS, although so far none of them proved to be really effective. A novel therapeutic strategy was based on short-term inhibition of host Gb3 synthesis to reduce binding and uptake of Stx by host cells. The rate-limiting first step in the biosynthesis of Gb3 and others glycosphingolipids is the reaction catalyzed by the enzyme glucosylceramide (GL1) synthase (Kolter et al., 2002; Lee et al., 1999). Different inhibitors of GL1 synthase have been identified and could inhibit glycosphingolipid synthesis in cultured cells without inhibiting cell growth or raising intracellular ceramide levels (Lee et al., 1999). These inhibitors have been identified and assayed for substrate inhibition therapy for treating several glycosphingolipidoses such as Fabry, Gaucher, Sandhoff, and Tay-Sachs diseases (Abe et al., 2000; McEachern et al., 2007). In this regard, we demonstrated that C-9, also referred to as Genz-123346 (Genzyme Corporation, Waltham, MA) a specific inhibitor of GL1 synthase, decreased Gb3 expression levels and prevented the cytotoxic effects of Stx2 on primary cultures of human renal tubular epithelial cells (HRTEC) (Silbertsein C, 2008) and HGEC (Amaral et al., 2013). Recently, Miglustat: MG (Zavesca, Actelion Pharmaceuticals, Allschwil, Switzerland), a reversible inhibitor of GL1 synthase (Platt et al., 1994), was approved by the Food Drugs Administration (FDA) and the National Administration of Drugs, Food and Medical Technology (ANMAT-Argentina) for the treatment of Gaucher type 1 and Niemann-Pick C diseases. In this work, we assayed the action of MG on HGEC and immortalized human proximal tubule epithelial cells (HK-2) as an alternative compound that could prevent Stx2 cellular damage.

2. Material and methods

2.1. Toxin

Stx2 was provided by Phoenix Laboratory, USA. Miglustat (Zavesca) is an inhibitor of Gb3 synthesis: The chemical name for Miglustat is 1, 5-(Butylimino)-1, 5-dideoxy-D-glucitol or N-butyldeoxynojirimycin (NB-DNJ) ($C_{10}H_{21}NO_4$; MW: 219.28) and was provided by Actelion Pharmaceuticals, Allschwil, Switzerland.

2.2. Cell culture

HGEC were isolated from fragments of kidneys removed from pediatric patients undergoing nephrectomies performed at the Hospital Nacional “Alejandro Posadas”, Buenos Aires, Argentina (written informed consent was obtained from the next of kin, caretakers, or guardians on the behalf of the minors/children participants involved in our study). The Ethics Committee of the University of Buenos Aires approved the use of human renal tissues for research purposes.

The method used for primary culture of HGEC isolation was adapted from that described by McGinn et al. (2004). Briefly, segments of macroscopically normal human renal cortex were minced and digested using 0.1% collagenase type I in Hanks Balanced Salt Solution (HBSS, Sigma–Aldrich St. Louis, MO) with 0.1% fetal calf serum (FCS) at 37 °C for 30 min. Then, the glomeruli were collected after filtration through a 70 μm Nylon cell strainer and incubated with 0.6% collagenase type I (Sigma Aldrich, St. Louis, MO) in HBSS with 0.1% FCS (Internegocios SA, Buenos Aires, Argentina) at 37 °C for 30 min. Finally, cells were re-suspended in complete medium: M199 medium (Sigma Aldrich, St. Louis, MO) supplemented with 20% FCS, 3.2 mM L-glutamine (Life Sci, Invitrogen, Buenos Aires, Argentina), 100 U/ml penicillin/streptomycin (Life Sci, Invitrogen, Buenos Aires, Argentina) and 25 μg/ml endothelial cell growth supplement (ECGS, Sigma Aldrich, St. Louis, MO) and cultured in 0.2% gelatin (Sigma–Aldrich St. Louis, MO) coated flasks at 37 °C in a humidified 5% CO_2 incubator. For growth-arrested conditions, a medium with a half of FCS concentration (10%) and without ECGS was used. Cells were used between 2 and 7 passages, after characterization with VWF (DAKO, Tecnolab, Buenois Aires, Argentina) and PECAM-1 (DAKO, Tecnolab, Buenos Aires, Argentina) positive expression (Amaral et al., 2013).

HK-2, human proximal tubular epithelial cell line, was obtained from American Type Culture Collection (Manassas, VA) and grown in DMEM/F12 medium (Sigma Aldrich, St. Louis, MO) containing 10% FCS, 100 U/ml penicillin/streptomycin (Life Sci, Invitrogen, Buenos Aires, Argentina), 2 mM L-glutamine, 15 mM HEPES at 37 °C in a humidified 5% CO_2 incubator. For growth-arrested conditions, a medium without FCS was used. Cells were used between 3 and 20 passages.

2.3. Neutral red cytotoxicity assay

The neutral red cytotoxicity assay was adapted from previously described protocols (Creydt et al., 2006). HK-2 and HGEC cells were placed in 96-well plates and grown to confluence in complete medium. To evaluate the Stx2 effect on HK-2 viability, cells were incubated with Stx2 (0.001–100 ng/ml) in growth-arrested conditions for 24, 48 and 72 h. The 50% cytotoxic dose (CD_{50}) corresponds to the dilution required to kill 50% of cells.

To establish the MG non-toxic concentrations cells were treated with different doses of MG (from 200 to 1000 μM) every 24 h. Viability was measured after 24 h or 48 h of treatment.

To examine the effect of MG on Stx2 cytotoxicity, HGEC and HK-2 were pre treated as previously and then cells were incubated with or without 1 ng/ml of Stx2 concomitantly with MG. After Stx2 exposure, cells were treated with MG every 24 h.

After 72 h of Stx2 incubation, freshly diluted neutral red (Sigma Aldrich, St. Louis, MO) was added to the cells in a final concentration of 10 μg/ml and then were incubated for an additional 1 h at

37 °C in 5% CO_2. Cells were then washed and fixed with 200 μl 1% $CaCl_2$ + 1% formaldehyde and then lysated with 200 μl 1% acetic acid in 50% ethanol where neutral red was solubilized. Absorbance in each well was measure in an automated plate spectrophotometer at 540 nm. Results were expressed as percentage of viability, where 100% represents cells incubated under identical conditions but without toxin treatment. To study the action of MG on cells incubated with Stx2, results were expressed as percentage of cell death prevention, where 0% represents cells incubated under identical conditions but without MG treatment.

2.4. Gb3 expression on HGEC and HK-2

2.4.1. Microscopy

Gb3 expression was analyzed by confocal microscopy. HGEC and HK-2 were seeded on glass coverslips (12 mm), pre-treated or not with MG (200–1000 μM) for 24 or 48 h, then washed with PBS at pH 7.4 and fixed with 3% paraformaldehyde (10 min on ice). Fixed cells were first incubated overnight with a rat anti-human CD77 (AbD Serotec, Kidlington, Oxford,UK) and then with a goat IgM anti-rat conjugated with FITC, for 2 h. Coverslips were mounted on glass slides using Fluoromount G. Immunofluorescence images were acquired with a FluoView FV1000 confocal microscope (Olympus, Japan) using a Plapon × 60 1.42 NA oil immersion objective and images were analyzed using the Olympus FV10-ASW software.

2.4.2. Thin-layer chromatography

Gb3 levels were detected by thin-layer chromatography (TLC). HGEC and HK-2 were seeded in tissue culture flasks and grown at 37 °C in an atmosphere of 5% CO_2 until the cells were nearly confluent. Cells were treated or not with MG (750 μM) for 24 or 48 h and then washed with PBS at pH 7.4. Total cells glycolipids were extracted according to the method of modified Bligh and Dyer (Bligh and Dyer, 1959) and neutral glycolipids were subjected to TLC in a silica gel 60 aluminum plate. A purified glycosphingolipid standard (0.5–2.5 μg) (Matreya, Pleasant Gap, PA) was also added to the plate for comparison. After the solvent front reached the top of the plate, the gel matrix was air dried and treated with a solution of orcinol, water and sulfuric acid (Acros Organics, Belgium) to visualize the separated glycolipid components. The densitometric analysis of Gb3 bands from HGEC and HK-2 treated with MG were analyzed by Image Quant 5.0 software (Molecular Dynamics, General Electric Healthcare Bio-Sciences, Pittsburgh, PA).

2.5. Cell morphologic analysis by optic microscopy

HGEC and HK-2 cells were seeded on glass coverslips (12 mm), washed with PBS at pH 7.4 and pre-treated with MG (750 μM) as previously in growth-arrested conditions for 48 h. Then cells were exposed to Stx2 (1 ng/ml) in the presence of MG for 72 h. Finally, cells were fixed for 2 h at room temperature with alcohol 96°, stained with hematoxylin–eosin (H&E) and observed by light microscopy (×200 and ×400, Zeiss Axiophot, Germany). Cell counts were performed on four fields of ×200 magnifications each. Cell area values were obtained using Image J software (National Institutes of Health, USA).

2.6. Data analysis

Statistical analysis was performed using the Graph Pad Prism Software 5.0 (San Diego, CA). Data are presented as mean ± SEM. Mann Whitney-test was used for comparison between two groups. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Stx2 reduces HK-2 viability in a dose and time-dependent manner

The effect of Stx2 on HK-2 viability was evaluated at different concentrations and times. A significant decrease in the viability of cells treated with Stx2 was detected when HK-2 were incubated with increasing toxin concentrations for 24, 48 and 72 h (Fig. 1). Stx2 caused a significant reduction on HK-2 viability in a dose-dependent manner and the highest cytotoxicity was obtained at 72 h with a CD_{50} of 1 ng/ml Stx2, (Fig. 1, n = 3, $^*P < 0.05$).

3.2. Miglustat protects renal cells from Stx2 toxicity

We evaluated if MG, an inhibitor of GL1 synthase (Platt et al., 1994), could protect HGEC and HK-2 viability from Stx2 cytotoxicity.

When HK-2 and HGEC were treated with a range of 200–1000 μM of MG, these concentrations did not affect cell viability (Fig. 2A and B). Then, we evaluated the effect of Stx2 in HK-2 and HGEC pre-treated with MG. Cells were pre-incubated with different MG concentrations for 24 or 48 h and then treated with Stx2 (1 ng/ml) in the presence of MG for an additional of 72 h Stx2 effects on HGEC (Fig. 2A) and HK-2 (Fig. 2B) were significantly attenuated in a dose-dependent manner (n = 4, $^*P < 0.05$). The treatment with MG for 48 h caused a higher protection on the viability of HGEC and HK-2 compared to 24 h (Fig. 2C and D, n = 4, $^*P < 0.05$). The maximum protection of HGEC and HK-2 viability from Stx2 effects was 72% and 67%, respectively, obtained with 750 μM of MG after 48 h (Fig. 2C and D).

3.3. HK-2 cells express Gb3 receptor

Taking into account the sensitivity of HK-2 to Stx2 cytoxicity, the presence of Gb3 receptor was analyzed. Cells were cultured on glass coverslips and then Gb3 expression was study by fluorescence microscopy. A green (in the web version) halo revealed Gb3 localized on the cell surface membranes under optimal antibody concentrations (Fig. 3A). HGEC cells were used as positive control of Gb3 expression (Amaral et al., 2013).

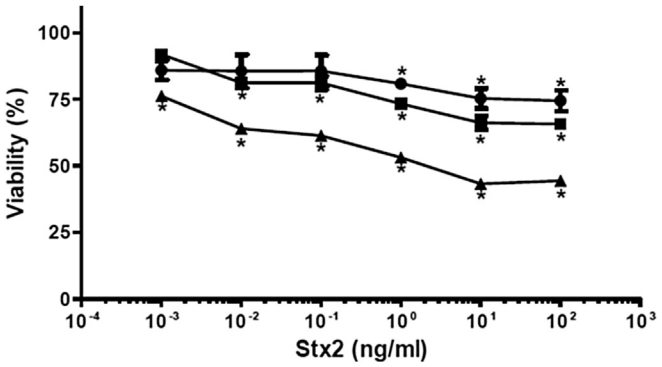

Fig. 1. Stx2 decrease HK-2 cells viability. Cells placed in 96-well plates were exposed to 0.001 ng/ml to 100 ng/ml Stx2 in growth-arrested conditions for 24, 48 and 72 h. Then, cells were incubated with neutral red for an additional 1 h at 37 °C in 5% CO_2. Absorbance of each well was read at 540 nm. One hundred percent represents cells incubated under identical conditions but without toxin treatment (Ctrl). Results are expressed as means ± SEM of three experiments, Stx2 *vs* Ctrl, or 24 h *vs* 48 h *vs* 72 h, $^*P < 0.05$. 24 h (—●—), 48 h (—■—) or 72 (—▲—).

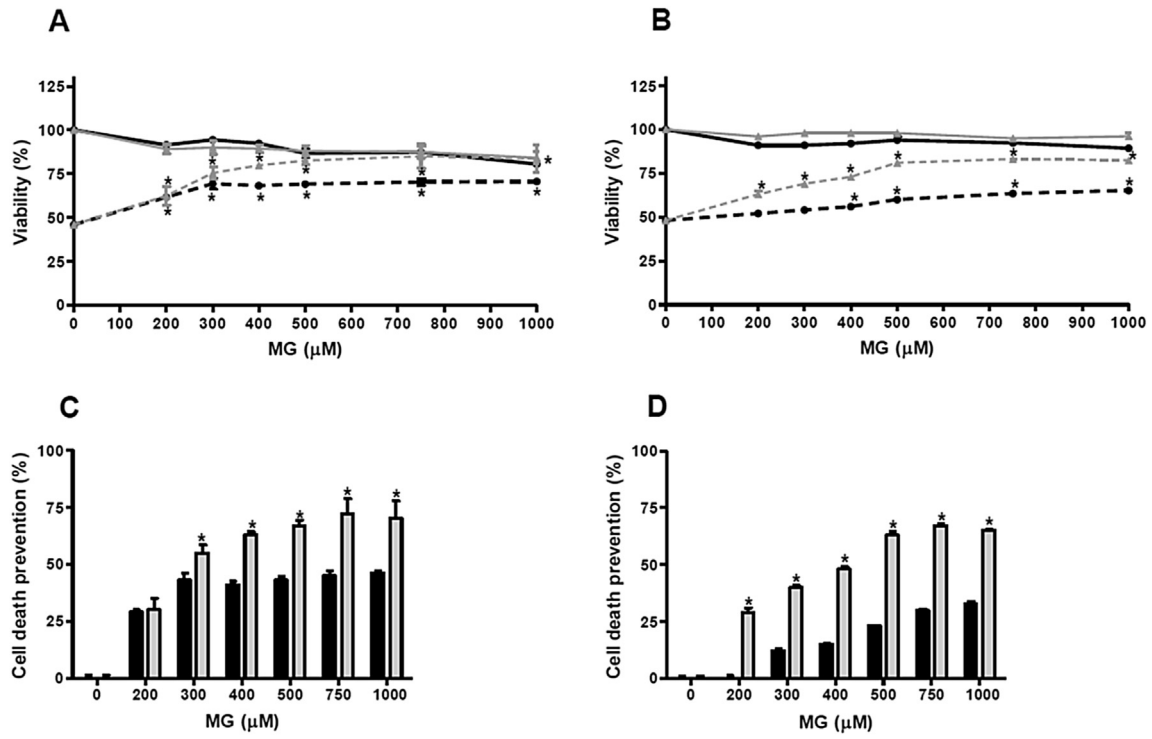

Fig. 2. Miglustat protection on HGEC and HK-2 exposed to Stx2. HGEC (**A** and **C**) and HK-2 (**B** and **D**) placed in 96-well plates were pre-treated with MG (200–1000 μM) for 24 or 48 h and then exposed to 1 ng/ml of Stx2 in the presence of MG for an additional of 72 h. Then, cells were incubated with neutral red for an additional 1 h at 37 °C in 5% CO_2. Absorbance of each well was read at 540 nm. One hundred percent represents cells incubated under identical conditions but without toxin treatment (Ctrl). Results are expressed as means ± SEM of four experiments. MG *vs* Ctrl ns. Stx2 + MG *vs* Stx2 and 48 h *vs* 24 h, $^*P < 0.05$. MG 24 h (—●—), MG 24 h + Stx2 (- -●- -), MG 48 h (—▲—) or MG 48 h + Stx2 (- -▲- -), MG 24 h + Stx2 (■) or MG 48 h + Stx2 (▭).

3.4. Miglustat decreases Gb3 expression on HK-2 and HGEC

In order to evaluate the mechanism involved in the protection of cell viability by MG, we analyzed the Gb3 expression, by fluorescence microscopy and TLC, on HK-2 and HGEC before and after treatment with MG (750 μM), (Fig. 3A and B). Neutral glycolipids extracted from control and treated cells with MG, for 24 or 48 h, were subjected to TLC and then visualized with orcinol staining (Fig. 3B). A Gb3 commercial standard was used as a positive control. The glycosphingolipids extracted from HGEC and HK-2 showed the same pattern of bands as Gb3 receptor standard independent of incubation with MG (Fig. 3B). However, the densitometric analysis

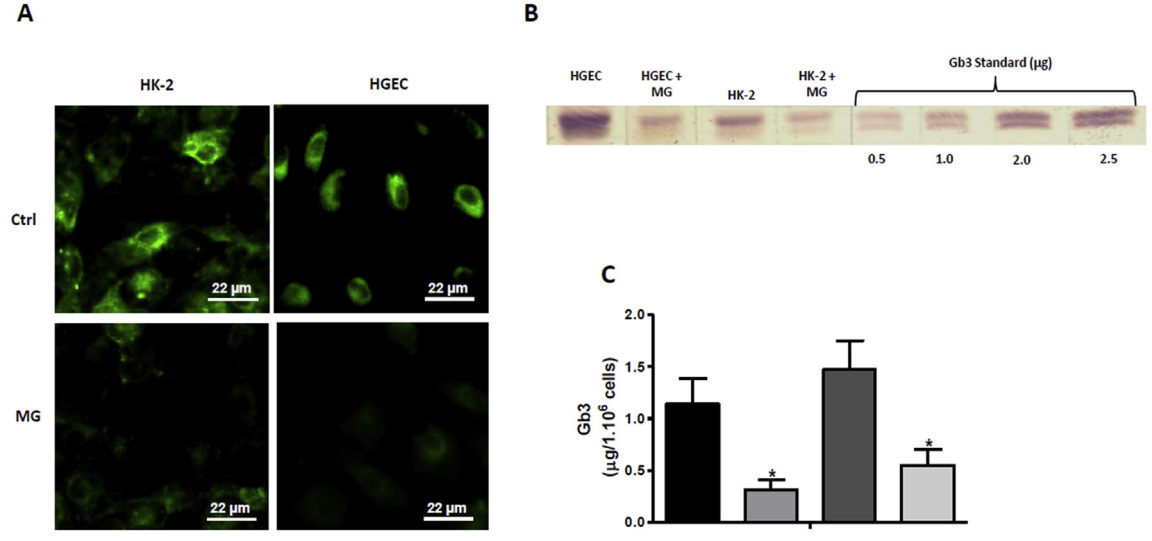

Fig. 3. Gb3 expression on HK-2 and HGEC treated with MG. HK-2 and HGEC seeded in glass coverslips were non-treated (Ctrl) or treated with MG (750 μM) for 48 h. Then cells were labeled with an anti-human CD77 conjugated with FITC and visualized by fluorescence microscopy (×400) (**A**). Neutral glycolipids extracted from HK-2 (0.8×10^6) and HGEC (2.5×10^6) non-treated (Ctrl) or treated with MG (750 μM) for 48 h were subjected to TLC and visualized with orcinol (**B**). The densitometric analysis of Gb3 bands was analyzed by Image Quant 5.0 software. Results are expressed as means ± SEM of three experiments, $^*P < 0.05$ (**C**). HGEC (■), HGEC + MG (▭), HK-2 (■) or HK-2 + MG (▭).

of Gb3 bands showed a decrease in Gb3 expression in both, HGEC and HK-2 cells after treatment with 750 μM MG for 48 h (Fig. 3B and C, n = 3, $^{*}P < 0.05$). This reduction was also observed by fluorescence microscopy (Fig. 3A). Twenty-four hours of MG (750 μM) also resulted in a reduction of Gb3 expression compared to controls but it was lower than that observed at 48 h (data not shown).

3.5. *Miglustat prevents morphologic alterations induced by Stx2 on HGEC and HK-2 cells*

Cells were stained with H&E and morphology was analyzed by light microscopy (Figs. 4A and 5A). HGEC and HK-2, previously incubated or not with MG (750 μM) for 48 h, were treated with Stx2 (1 ng/ml). Stx2 caused a significant reduction in the number of viable attached cells and in the intracellular edema of the remaining cells (Figs. 4B and C and 5B and C). In addition, MG protected HGEC and HK-2 cells from the detachment caused by Stx2 (Figs. 4B and 5B). Morphology of cells treated with MG was similar to controls.

4. Discussion

The kidney is the most affected organ in diarrhea-associated HUS, since cells express high amounts of biologically active Stx receptor, Gb3 (Obrig, 2010). Up to now, there are not effective therapies available for the management of HUS. In this sense, an alternative strategy for treating this disease is to block the anabolic pathways of sphingolipid metabolism to reduce binding and uptake of Stx by host cells. The inhibition of the first step in the biosynthesis of Gb3 and others glycosphingolipids is catalyzed by the enzyme GL1 synthase (Kolter et al., 2002; Lee et al., 1999). Concerning this issue, Moon et al. have found that epigallocatechin and curcumin, enzymes inhibitors of glycosphingolipids synthesis, were effective in decreasing Gb3 expression on intestinal epithelial cells (Moon et al., 2005, 2006). Recently, we have demonstrated that C-9, a specific inhibitor of GL1 synthase, decreased Gb3 expression levels and prevented the cytotoxic effects of Stx2 on HRTEC (Silbertsein C, 2008) and HGEC (Amaral et al., 2013). In this work, we have evaluated the action of Miglustat (MG), a new available commercial drug approved by ANMAT-Argentina. MG is an inhibitor of GL1 synthase (Platt et al., 1994), licensed for the treatment of type I Gaucher disease (C. Dulsat and Mealy, 2009) and is the only specific oral therapy approved to treat progressive neurological manifestation of Niemann-Pick disease type C (Alobaidy, 2015), both lyso-somal glycosphingolipids storage diseases. Thus, MG diminishes the abnormal lysosomal accumulation of glucosylceramide.

In order to analyze whether this drug can protect renal cells from Stx2 cytotoxicity, we studied the effects of MG on HGEC and HK-2 cell cultures. Firstly, we analyzed the susceptibility of HK-2 to Stx2 cytotoxicity to verify if this cell line is useful as a model of human renal tubular epithelial cells. HK-2 viability showed a significant decrease after exposure to increasing Stx2 concentrations over time. This HK-2 sensitivity to the toxin was associated with the expression of Gb3 receptor, analyzed by fluorescence microscopy and TLC. This fact was in agreement with previously results obtained in HRTEC (Creydt et al., 2006; Porubsky et al., 2014; Silbertsein C, 2008). Secondly, we assayed cytotoxicity of MG in both type of cells and we found that MG did not affect the viability of HK-2 and HGEC in the range of MG concentrations used in this work. These results are consistent with other studies using MG on other cell models (Bieberich et al., 1999; Platt et al., 1994).

In the present study, human renal cells were pre-incubated with MG and then treated with Stx2 in the presence of MG. This inhibitor

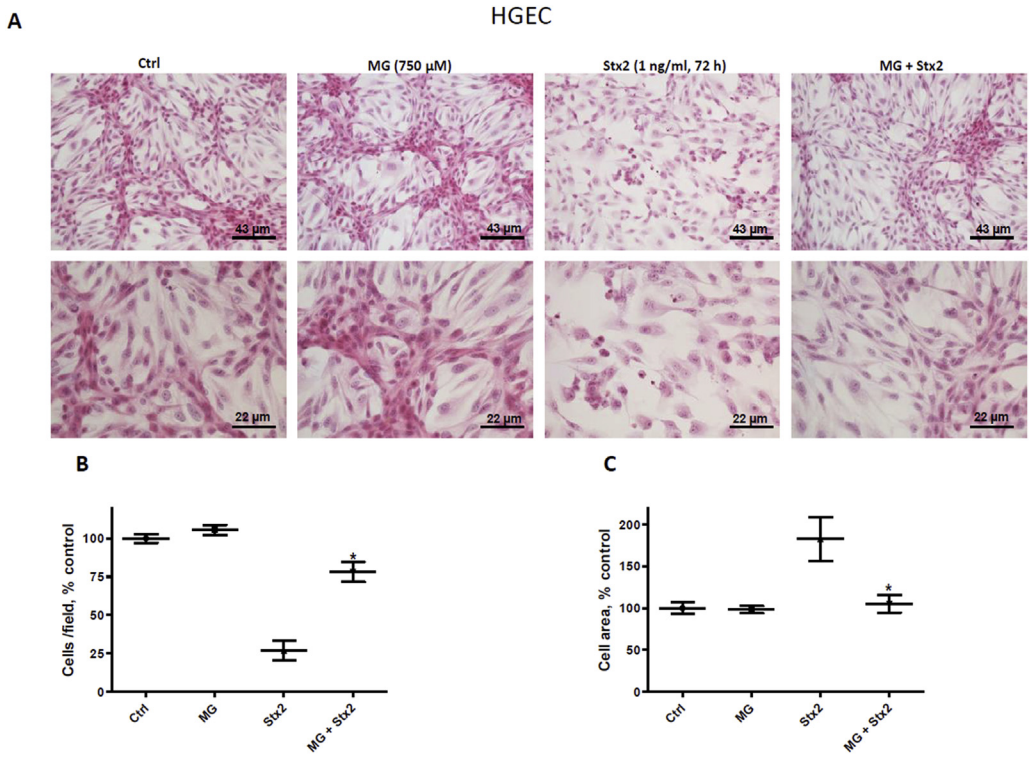

Fig. 4. HGEC are protected from morphologic changes induced by Stx2. HGEC seeded on gelatin coated glass coverslips were exposed to MG (750 μM) for 48 h and then to Stx2 (1 ng/ml) in the presence of MG for an additional of 72 h. Finally cells were stained with H&E and morphology (**A**) and number of HGEC (**B**) were analyzed by light microscopy (×200 and ×400). Cell area (**C**) was measured by using image J software. Results are expressed as means ± SEM of three experiments. One hundred percent represents values of cells control. MG vs Ctrl ns, MG + Stx2 *vs* Stx2, $^{*}P < 0.05$.

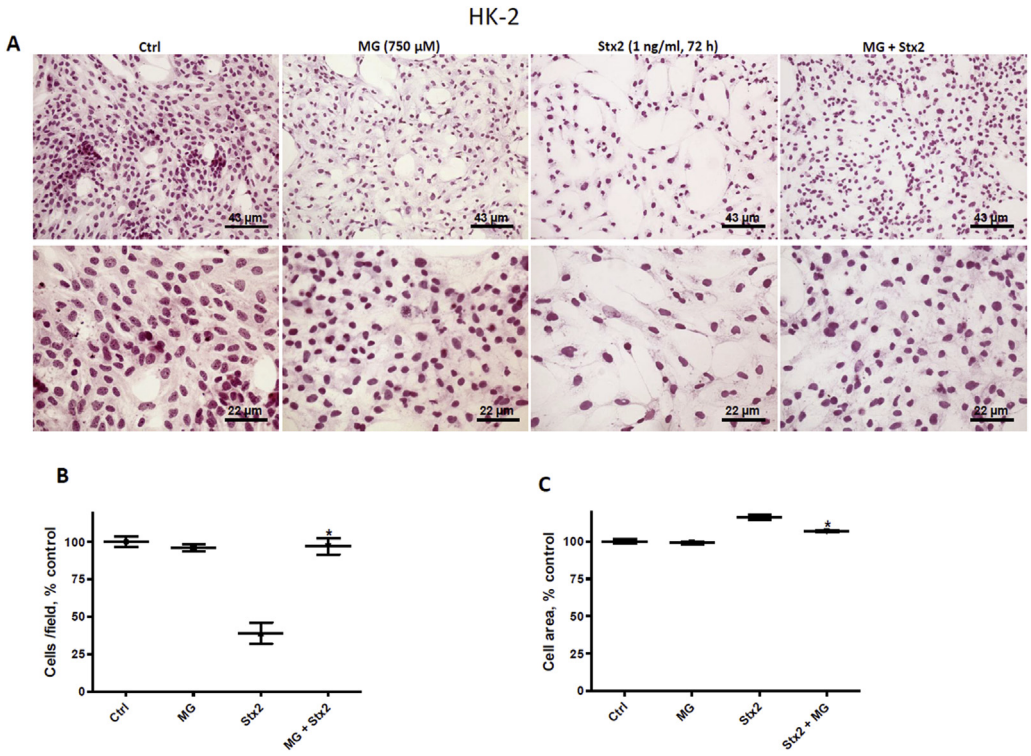

Fig. 5. HK-2 are protected from morphologic changes induced by Stx2. HK-2 seeded in glass coverslips were exposed to MG (750 μM) for 48 h and then to Stx2 (1 ng/ml) in the presence of MG for an additional of 72 h. Finally cells were stained with H&E and morphology (**A**) and number of HK-2 (**B**) were analyzed by light microscopy (×200 and ×400). Cell area (**C**) was measured by using image J software. Results are expressed as means ± SEM of three experiments. One hundred percent represents values of cells control. MG vs Ctrl ns, MG + Stx2 *vs* Stx2, $^{*}P < 0.05$.

protected the viability of human renal cells from Stx2 effects with an effectiveness of about 70%. The pre-treatment with MG for 48 h caused a significant protection compared to 24 h. In addition, MG reduced intracellular edema and cell detachment caused by the toxin in these cells.

These effects were associated to a marked decrease in the Gb3 expression on HK-2 and HGEC treated with μM concentrations of MG. In vitro, GL1 synthase is inhibited by MG at concentrations between 50 and 500 μM, preventing glucosylceramide accumulation in the lysosomes of macrophage-like myelomonocytic leukemia cell line (Platt et al., 1994). Although HK-2 cells showed a higher concentration of Gb3 receptor than HGEC cells, both exhibited the same sensitivity to Stx2, with a CD_{50} of 1 ng/ml after 72 h of treatment (Amaral et al., 2013). In addition, MG (750 μM) protected both cells in similar manner. Consequently, it has been shown that not only the density of Gb3 is important for Stx binding but also for the Gb3 isoforms. The heterogeneity of Gb3 ceramide and the variability in the carbon chain length of the fatty acid, and finally the Gb3 distribution and localization in the plasma membrane would be responsible for these differences (Pellizzari et al., 1992; Sandvig et al., 1994).

Taking into account this antecedent, although HK-2 showed a higher Gb3 concentration than HGEC, it is possible that the heterogeneity in Gb3 receptors between these different cells define the final susceptibility to Stx2.

A higher protection was previously observed with lower doses of C-9 (Amaral et al., 2013). In this regard, the effectiveness of C-9 seems could be related with the ability to inhibit the Gb3 synthesis (Zhao et al., 2007). However, C-9 is an experimental drug that has not yet been used in human.

In summary, we have demonstrated that HK-2 cell line expresses Gb3 receptor and is sensitive to the Stx2. According to these results, the HK-2 cell line may be used as a model for human renal tubular epithelial cells in order to study the effects of Stx2 in kidney related to HUS development. The treatment of human renal endothelial and epithelial cells with MG, at concentrations that reduce Gb3 expression, achieved positive results regarding protection from Stx2 cytotoxicity.

5. Conclusions

It is well documented that there is a 3–5 days period of time between the initial gastrointestinal symptoms by STEC infection and the development of HUS (Tarr et al., 2005). This time frame may be relevant for a future therapeutic use of MG to prevent renal failure as observed in patients with HUS.

It has been demonstrated that in animal models, MG is not metabolized and is concentrated and excreted intact via the kidney (Platt et al., 1997; Treiber et al., 2007). Further experiments are in progress in experimental animal models of HUS to prove if MG can neutralize Stx2 action *in vivo*.

Ethical statement

HGEC were isolated from fragments of kidneys removed from pediatric patients undergoing nephrectomies performed at the Hospital Nacional “Alejandro Posadas”, Buenos Aires, Argentina (written informed consent was obtained from the next of kin, caretakers, or guardians on the behalf of the minors/children participants involved in our study). The Ethics Committee of the University of Buenos Aires approved the use of human renal tissues for research purposes.

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Competing interests

The authors state no conflict of interest.

Author contributions

Participated in research design: Amaral, Ibarra, Repetto.
Conducted experiments: Girard, Sacerdoti, Rivera, Amaral.
Contributed new reagents or analytic tools: Girard, Sacerdoti.
Performed data analysis: Girard, Sacerdoti, Amaral.
Wrote or contributed to the writing of the manuscript: Amaral, Sacerdoti, Ibarra.

Transparency document

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