A Glucosylceramide Synthase Inhibitor Prevents the Cytotoxic Effects of Shiga Toxin-2 on Human Renal Tubular Epithelial Cells

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Abstract: Shiga toxin-2 binds to the globotriaosyl-ceramide receptor on the plasma membrane of target cells. The high level expression of this receptor in renal epithelial cells may account, at least in part, for acute renal failure observed in children with hemolytic uremic syndrome. The cytotoxic effect of Shiga toxin-2 was assayed on primary cultures of human renal tubular epithelial cells treated with a new specific inhibitor of glucosylceramide synthase (C-9), the rate-limiting first step in the glycosphingolipid biosynthetic pathway. The treatment of the cells with 1-5 μM C-9 for at least 24 h significantly neutralized the action of 1 ng/ml Shiga toxin-2 on cell viability. The expression levels of globotriaosyl-ceramide significantly decreased when cells were incubated with 1 μM C-9 for 48 h. We propose here that prevention of globotriaosyl-ceramide synthesis by the C-9 could be a novel substrate inhibition therapy to neutralize Shiga toxin-2 action in renal epithelial cells.

Keywords: Shiga toxin, proximal tubule epithelial cells, globotriaosyl-ceramide, glucosylceramide synthase, glycosphingolipid, hemolytic uremic syndrome.

1. INTRODUCTION

Post-diarrhea hemolytic-uremic syndrome (HUS) is the most common cause of acute renal failure in children in Argentina and the second cause of chronic renal failure in children under 5 years of age (data from the registry of the Nephrology Committee of the Argentine Society of Pediatrics). The acute stage of the disease is marked by hypertension, edema, hematological abnormalities, and anuria [1, 2]. Clinical and histological renal damages have been strongly associated with Shiga toxin type 1 and 2 (Stx1, Stx2) produced by *Escherichia coli* O157:H7 and other related bacterial strains frequently isolated from children with HUS [3]. These strains express Stx1 and/or Stx2 although those that express only Stx2 are highly prevalent in Argentina [3]. Stx contains an A subunit monomer (32 kDa) bound non-covalently to five B subunits (7.7 kDa) [4]. The B subunit pentamer binds to the glycolipid globotriaosylceramide (Gb3) on the plasma membrane of target cells. Then, Stx is internalized into the cell, and the A subunit exhibits RNA N-glycohydrolase activity and cleaves a specific adenine residue on the 28S ribosomal RNA in the cytosol, thereby inhibiting protein synthesis. Therefore, Gb3 is the functional receptor for Stx found on the surface of target cells in the kidney producing Stx-mediated diseases. Gb3 is expressed in glomerular endothelial cells, podocytes, mesangial cells, and proximal tubule epithelial cells of the kidney [4-5]. Proximal tubular damage is evident in renal tissue during the early stages of HUS, raising the possibility that the proximal tubule may be an important early target of Stx action. The binding of Stxs to renal tubular epithelial cells expressing Gb3 *in vitro* [5, 6] and *in vivo* [7-9] can inhibit protein synthesis and induce apoptosis and necrosis. Recently, we have demonstrated that Stx2 is able to inhibit water absorption on human renal tubular epithelial cells (HRTEC) [10].

We propose here that the inhibition of Gb3 synthesis in target cells would be a new strategy to prevent the binding of Stx to the receptor. The rate-limiting first step in the biosynthesis of Gb3 and others glycosphingolipids is the conversion of ceramide to glucosylceramide [11]. This reaction is catalyzed by the enzyme glucosylceramide synthase (GL1 synthase) which glycosylates the ceramide using uridine diphospho-glucose [12]. Subsequently, galactose is added to produce galactosyl-glucosyl-ceramide, following which additional sugars are added in the biosynthetic pathway to produce Gb3 and other glycosphingolipids. Different inhibitors of glucosylceramide synthase have been identified and assayed for substrate inhibition therapy for treating several glycosphingolipidoses such as Fabry, Gaucher, Sandhoff and Tay-Sachs disease [13, 14]. These compounds can inhibit glycosphingolipid synthesis in cultured cells without inhibiting cell growth or raising intracellular ceramide levels [11].

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1875-0443/08

In the present work we have assayed a new potent inhibitor of GL1 synthase (C-9) in a primary culture of HRTEC. C-9 significantly decreased Gb3 expression levels and prevented the cytotoxic effects of Stx2. The inhibition of Gb3 synthesis may be used as a potential treatment for protection against the pathological effects of Shiga toxin producing HUS.

2. MATERIALS AND METHODS

2.1. Reagents

Reagents were obtained from Sigma Chemical Co (St. Louis, MO, USA). Tissue culture flasks, dishes, and multiwell plates were from Falcon (Orange Sci, Graignette Business Park, Belgium). The C-9 [(1R, 2R)-nonanoic acid [2-(2',3'-dihydro-benzo [1-4]dioxin-6'-yl)-2-hydroxy-1-pyrrolidin-1-ylmethyl-ethyl]-amide-L-tartaric acid salt] is a proprietary compound supplied by Genzyme Corporation.

2.2. Stx2 Purification

Stx2 was purified by affinity chromatography under native conditions, as for the protocol [15]. Briefly, recombinant *E. coli* DH5α containing the Stx2 cloned into pGEM-T-Easy were cultured in LB supplemented with 100 μg/ml ampicillin until an OD_{600} of 0.6. The bacterial pellet was resuspended in 50 mM phosphate buffer saline (PBS, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8). The suspension was then sonicated, centrifuged, and the supernatant was precipitated with a saturated solution of SO_4 $(NH_4)_2$ following centrifugation at 12,000 rpm for 20 min. The pellet containing the Stx2 was resuspended in PBS and dialyzed against 10 mM PBS. The Stx2 was then affinity purified using an agarose-galabiose resin (Calbiochem, La Jolla, CA). After washing with 10 mM PBS containing 1 M NaCl, the Stx2 holotoxin was eluted with 50 mM PBS containing 0.5 M melibiose (Sigma, St Louis, MO, USA). To assay the purity of the bands fractions, samples were run on a 12.5% SDS polyacrylamide gel and electrotransferred to a nitrocellulose membrane. Immunoblot analysis using the monoclonal antibodies against the A and B subunits (Biodesign International, ME, USA) of Stx2, showed two bands of 32 kDa and 7.7 kDa that corresponds to Stx2A and Stx2B respectively [15]. The purification procedure resulted in approximately 1 ng/μl Stx2. Cytotoxic activity of purified Stx2 was also measured by neutral red uptake in Vero cells treated with the toxin for 72h. The 50% cytotoxic dose (CD_{50}) for Vero cells and HRTEC of Stx2 (1 ng/ml) incubated during 72 h was chosen to use on HRTEC experiments.

2.3. Cell Culture

Human renal tubular epithelial cells (HRTEC) were isolated from kidneys removed from different patients undergoing nephrectomies for renal cell carcinoma from the "Unidad de Urología, Hospital Posadas", Buenos Aires, Argentina. The Ethics Committee of the Universidad de Buenos Aires approved the use of human renal tissues for research purposes. The cortex was dissected from the renal medulla and the primary culture of the HRTEC was performed according to the methods described previously [6]. Briefly, the cortical fragments were incubated for 1 h at 37ºC in a buffer containing 0.1% collagenase type I. Then, they were washed, centrifuged and resuspended in RPMI 1640 (Hy-Clone) medium supplemented with 5% fetal calf serum, 2 mM L-glutamine and 100 U/ml penicillin/streptomycin (all from GIBCO BRL, Grand Island, NY, USA). Cells were incubated in 5% CO_2 atmosphere at 37ºC and grown in flasks up to confluence. The cells were then trypsinized, concentrated in fetal calf serum containing 5% dimethyl sulfoxide and stored in liquid nitrogen for subsequent use. Cells were cultured in T-25 flasks in RPMI medium with supplements and 1% endothelial cell growth factor and used between 3-5 passages. By light microscopy, more than 95% of the cells had similar morphologies. These cells were confirmed as epithelial cells by positive staining for cytokeratins. Less than 10% of cells were positive with the antibody against an epithelial membrane antigen (EMA, Dako, Glostrup, Denmark) present in the distal tubular epithelial cells. The presence of fibroblasts was ruled out by lack of reactivity with an antibody directed to human fibroblast common antigen (Dako). Furthermore, the cells were also negative for the endothelial cell antibody PECAM CD31 (Dako). All samples were developed by immunoperoxidase by using RTU Vectastain Kit (Vector, Burlingame, USA).

HRTEC proliferated rapidly and formed a completely confluent monolayer after 3-5 days of culture. For cell viability, the cells were grown on 96-well plates up to 90% confluence. All HRTEC were studied under growth-arrested conditions (with serum free-medium).

2.4. Neutral Red Assay

Cell viability was assayed by the measurement of neutral red uptake on HRTEC, as the protocol described previously [6]. HRTEC were seeded in 96-well plates and grown to confluence in complete RPMI medium. The cells were then exposed to 1 ng/ml Stx2 in growth-arrested conditions for 24 h. For studies examining the protective effect of Gb3 inhibition, HRTEC were incubated in the presence of different concentrations of C-9 (from 0.05 to 50 μM) per 24 or 48 h in growth-arrested conditions before the addition of the toxin. The cells were then incubated with or without 1ng/ml Stx2 for additional 24 h. Two hundred microliters of freshly diluted neutral red in PBS was then added per well to a final concentration of 50 μg/ml and cells were incubated for an additional 3 h at 37ºC in a 5% CO_2 incubator. Cells were then washed with 1% $CaCl_2$ and 4% formaldehyde and solubilized in 1% acetic acid and 50% ethanol. Absorption in each well was read in an automated plate spectrophotometer at 546 nm. Results are expressed as neutral red uptake percent, and 100% represents cells incubated under identical conditions but without toxin treatment.

2.5. Quantization of HRTEC Globotriaosyl-Ceramide Levels by Mass Spectrometry

Confluent HRTEC were incubated with 5 μM C-9 for 48 h in growth-arrested conditions before the addition of Stx2. The cells were then incubated with or without 1ng/ml Stx2 for an additional 24 h. Control cells were incubated without the addition of C-9 and Stx2. For mass spectrometry analysis, cell samples at -80°C were allowed to thaw. An internal standard cocktail containing N-heptadecanoyl ceramide trihexoside (C17-Gb3) was added to microcentrifuge tubes and dried under nitrogen gas. Cells were added and vortexed to help incorporate the internal standards into the cell solution. An organic solution was then used to precipitate proteins. The resulting solution was sonicated and centrifuged. An

aliquot of the supernatant was then transferred to an auto-sampler vial prior to analysis. Samples were analyzed on a system consisting of an HTC PAL autosampler, Agilent 1200 HPLC, and API-4000 mass spectrometer. Positive-ion ESI-LC/MS/MS methods were used for the determination of Gb3 concentration in cell samples.

3. RESULTS

3.1. C-9 Neutralized the Cytotoxic Effects of Stx-2 on HRTEC

Cell viability was assayed to evaluate whether either Gb3 synthesis or decrease in Gb3 levels in Gb3 levels protects HRTEC from Stx2 cytotoxicity. For this purpose, HRTEC were treated with different concentrations of C-9 for 24 h and 48 h followed by the incubation with Stx2 at a concentration of 1 ng/ml for 24 h. We have used 1 ng/ml of Stx2 because it corresponds to the dilution of Stx2 required to decrease at least 50% the viability of HRTEC after 72 h of exposure [6]. The incubation with this dose of Stx2 for 24 h inhibited the cell viability to about 60-70%. Fig. (**1**) shows that C-9 significantly neutralized, in a dose-dependent way, the inhibition in cell viability produced by Stx2. When HRTEC were pre-incubated with 5 μM C-9 for 24 h and 48 h, followed by Stx2 for 24 h, inhibition of Stx2 effects on the cell viability was almost totally reverted (89 ± 1% and 93 ± 2 %, respectively). This dose of C-9 was the optimum to prevent Stx2 effects. There were not significant differences in the cell viability between the pre-incubation for 24 h and 48 h at different concentrations of C-9 (Fig. **1**). The co-incubation with C-9 plus 1 ng/ml Stx2 for 24 h did not neutralize the cytotoxic effects of Stx2 on HRTEC (Fig. **1**). On the other hand, concentrations of C-9 up to 5 μM for 72 h did not affect the cell viability while doses higher than 50 μM for 48 h were toxic for HRTEC (data not shown). As the treatments of the HRTEC were performed in arrest of growth, it was not possible to assay the drug and the toxin for more than 72 h. HRTEC did not survive more than 3 days without growth factors and bovine fetal serum.

3.2. Inhibition of Glucosylceramide Synthesis by C-9

Mass spectrometry was used to quantify the C-9 inhibitory activity for GL1 synthase in HRTEC. The levels of Gb3 in cells incubated in growth-arrested conditions with and without the addition of this compound were quantified. An inhibition of approximately 85% in Gb3 levels was observed after incubation with 5 μM of C-9 for 48h compared with non-treated cells (Fig. **2**; 0.37 ± 0.04 μg *vs* 2.39 ± 0.26 μg Gb3 per 60,000 cells, respectively). HRTEC Gb3 levels were also significantly decreased (77%) after treatment with C-9 for 48h followed by 1ng/ml Stx2 for 24h (0.56 ± 0.07 μg per 60,000 cells) showing that Stx2 did not change significantly the effect of C-9 on Gb3 levels. On the contrary, Gb3 levels were not altered when HRTEC were treated with the toxin alone (Fig. **2**).

4. DISCUSSION

Although many laboratories have made efforts to develop an effective treatment for Stx-mediated HUS, a specific therapy has not been found yet. The syntheses of compounds mimicking the Gb3 receptor [16, 17], antibodies against Gb3, or vaccines against Stx subunits [18] are some of the different neutralizing agents that have been assayed to prevent Stx1 and/or Stx2-mediated diseases. It has been demonstrated that Gb3 and/or its derivatives are exclusive receptors *in vivo* and that they mediate the tissue damage and pathological features caused by Stx1 and Stx2 [19].

In the present study, we found that the pre-incubation of HRTEC with various concentrations of C-9 for at least 24h prevented the cytotoxic effect of Stx2 on HRTEC. We have also observed a significant decrease of Gb3 levels in HRTEC

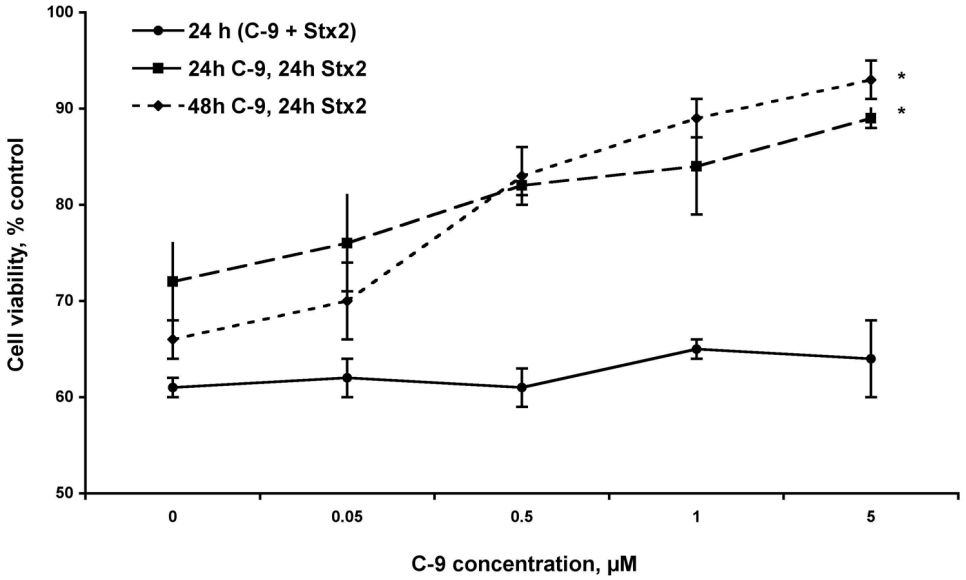

Fig. (1). Effects of C-9 on cell viability in human renal tubular epithelial cells (HRTEC) treated with Shiga toxin type 2 (Stx2). HRTEC were co-incubated with 0, 0.05, 0.5, 1 and 5 μM of C-9 and 1 ng/ml Stx2 for 24 h (-●-), or pre-incubated with same concentrations of C-9 for 24 h (-■-) and 48 h (-◆-) followed by 1 ng/ml Stx2 for 24 h. After incubation, cell viability was assayed by neutral red uptake. Results are expressed as percentage of cell viability. One hundred percent represents cells incubated under identical conditions without treatments. Data are shown as means ± standard error of the mean (SEM) (n=3). **P* < 0.05 for curves of pre-incubation *vs* co-incubation with C-9, using one-way analysis of variance (ANOVA).

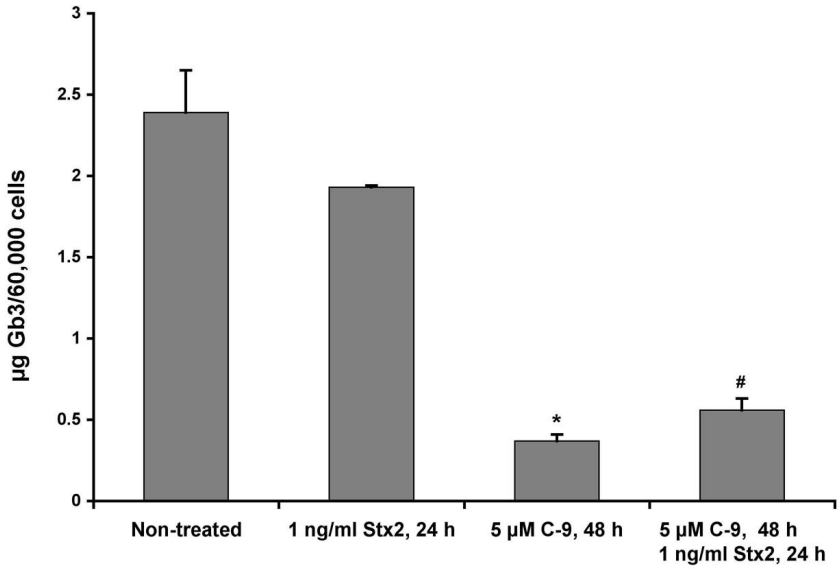

Fig. (2). Effect of C-9 on Gb3 expression in human renal epithelial cells (HRTEC). HRTEC were pre-incubated with or without 5 µM C-9 for 48h before the incubation with or without 1ng/ml Stx2 for 24h. After treatments, globotrioasyl-ceramide (Gb3) levels were measured by mass spectrometry analysis. Each bar represents the mean ± SEM of three experiments. Student's t-tests indicate significant differences (*****$P < 0.001$) for C-9 treated *vs* untreated cells, and ($^{\#}P < 0.001$) for C-9 and Stx2 *vs* Stx2 treated cells.

treated for 48 h with this compound. Moreover, our results demonstrate that the C-9 treatment that produced the maximum reduction in Gb3 levels (less than 20%), also produced almost a total neutralization of Stx2 effects on cell viability (93 ± 2%).

C-9 has been previously characterized as a potent and specific inhibitor of GL1 synthase that blocks the conversion of ceramide to glucosylceramide [11]. The drug was proposed as a novel therapeutic approach for the treatment of type 2 diabetes [20]. These studies showed the drug to be well tolerated and effective in mice and rats after multiple weeks of oral treatments. Other inhibitors for GL1 synthase that were highly effective to deplete Gb3 have been proposed for substrate inhibition therapy of Fabry disease [13], an inherited X-linked disorder that is manifested by the accumulation of glycosphingolipids including the Gb3. Additionally, another potent GL1 inhibitor has been assayed in a murine model of Gaucher disease to decrease the aberrant lysosomal accumulation of glucosylceramide [14]. On the other hand, the treatment of α-GalA-knockout mice, a murine Fabry model, with recombinant human α-GalA (Fabrazyme) has also been shown to reduce levels of Gb3 in multiple tissues [21]. However, α-GalA-knockout mice were significantly protected against lethal intraperitoneal doses of Stx2 or oral doses of Stx2-expressing bacteria, while the treatment with Fabrazyme restored the susceptibility of knockout mice to lethal doses of Stx2 [22].

It has been demonstrated that Gb3 synthase null mutant mice showed no reaction to Stx1and Stx2 at doses as much as 100 times that those administered to wildtype [19]. We have shown that Stx2 caused a reduction of HRTEC viability in a dose- and time-dependent manner obtaining a CD_{50} of 1 ng/ml after 72 h. The incubation of HRTEC with the same dose of Stx2 for 24 h inhibited the protein synthesis to more than 80% [6]. Inflammatory factors that increase cell surface Gb3 expression, like IL-1β and LPS increase the cytotoxic effects of Stx2 on the cells [6].

The kidney expresses relatively high levels of Gb3 compared with other organs, which may account, at least in part, for renal targeting in HUS [23, 24]. There is evidence that renal tubular injury observed in the HUS [25] is not only secondary to glomerular and arteriolar injury induced by Stx2 but also due to the direct action of the toxin on tubular epithelial cells [26]. Proximal tubular damage is evident in renal tissue during the early stages of HUS [27], raising the possibility that the proximal tubule may be an important early target of Stx action. In the present work, we have used primary cultures of proximal tubular epithelial cells obtained from human kidneys. We have recently published the biophysical and functional characterization of HRTEC monolayers growing on permeable supports. Our results validated these cells as a useful experimental model of human renal proximal tubule [10].

In summary, we propose here that prevention of Gb3 synthesis by GL1 synthase inhibitors such as C-9 could be a novel substrate inhibition therapy to prevent the binding of Stx2 to the receptor and consequently, neutralize the cytotoxic activity of the Stx2 in kidney cells. Further experiments are in progress in an experimental model of HUS in rats to find out if C-9 may neutralize the Stx2 action *in vivo*.

5. ACKNOWLEDGEMENTS

We would like to thank Jonathan Rogers at Genzyme Corp. for his assistance with the mass spectrometry analysis. We thank Dr Juan Ignacio Bortagaray who provided us the material of nephrectomies from the "Sección de Cirugía Pediátrica, Hospital Nacional Prof. A. Posadas", Buenos Aires, Argentina. We also thank Rosa Scuteri, MD and Judith Peterschmitt, MD for their help to get the C-9. This work was supported by grants from Universidad de Buenos Aires (CI: UBACYT M030 and CS: UBACYT M440).

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Received: October 07, 2008 Revised: November 20, 2008 Accepted: November 25, 2008