Novel Cell-Based Assays Measuring Early and Late Effects of *Clostridium difficile* Toxins

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Abstract

Clostridium difficile infections are the leading cause of hospital-acquired infectious diarrhea and are caused primarily by two exotoxins, TcdA and TcdB. Both toxins have similar structural and functional characteristics: they bind to specific receptors on the surface of gut epithelial cells, are internalized through endocytosis, translocate to the cytoplasm, and inactivate rho-type GTPases via covalent glucosylation. This leads to downstream events that include morphological changes associated with disruption of the actin cytoskeleton and of epithelial tight junctions, release of pro-inflammatory mediators, and eventually cell death. Historically, assays used to assess the effects of toxins on cells have relied on evaluation of cell rounding or quantitation of ATP levels to estimate cell death, measurements that are either largely qualitative (cell rounding) or variable (ATP levels). In this study, several in vitro assays are described that robustly and quantitatively measure both early and late TcdA- and TcdB-dependent events in cells, including: (i) Rac1 glucosylation, (ii) changes in cellular morphology (measured as dynamic mass redistribution [DMR]), (iii) loss of epithelial integrity (measured by monitoring transepithelial electrical resistance [TER]), and (iv) cell death (measured as total cellular protein using a sulforhodamine B colorimetric assay). The assays were validated using the highly specific monoclonal antitoxin antibodies actoxumab and bezlotoxumab, which neutralize TcdA and TcdB, respectively. Differences in the potencies of the antibodies against toxins of different *C. difficile* ribotypes are consistent across various assay endpoints and different cell types, validating the pharmacological utility of the assays. The panel of assays described herein provides a useful toolset for characterizing the effects of existing and emerging toxin-targeted therapies on early- and late-stage cellular effects of the toxins.

Introduction

- *C. difficile* is a bacterial pathogen that colonizes and infects the intestines of patients whose gut microbiota has been disrupted following treatment with broad-spectrum antibiotics[1]
- *C. difficile* infections (CDI) are typified by severe diarrhea, pseudo-membranous colitis, and, in extreme cases, colonic rupture, sepsis, and death[2]
- The key pathogenic determinants of *C. difficile* are two exotoxins, toxin A (TcdA) and toxin B (TcdB), that target the epithelial cells that line the gut lumen[3]
- Assays used to assess toxin activity in mammalian cells are often largely qualitative and/or variable
- In this study, we utilized several in vitro assays that robustly and quantitatively measure both early and late TcdA- and TcdB-dependent events in cells, including toxin binding to mammalian cells, Rac1 glucosylation, changes in cellular morphology, loss of epithelial integrity, and cell death
- The neutralizing antibodies actoxumab and bezlotoxumab[5-8] were utilized to validate the assays; the antibodies prevented the various toxin-dependent effects measured in these assays

Materials and Methods

Binding of TcdA and TcdB to HT-29 and Vero Cells Assessed by Flow Cytometry (A): TcdA-Atto488 was preincubated with or without 200 µg/mL actoxumab or bezlotoxumab for 1 hour (RT), then added to HT-29 cells and further incubated in the dark for 30 minutes (4°C). Cells were washed and subjected to flow cytometry on an LSRII instrument.

Binding of TcdB to Vero Cells Assessed by Western Blotting (A): Toxins were preincubated with or without 200 µg/mL actoxumab or bezlotoxumab for 30 minutes and then allowed to bind to Vero or HT-29 cells for 30 minutes (4°C). Cells were washed and membranes isolated, solubilized, and resolved by SDS-PAGE. Western blotting was performed using actoxumab for TcdA or a combination of bezlotoxumab and antibody 2A11[4] for TcdB.

Rac1 Glucosylation In-cell Western Assay (B): Toxins were preincubated with or without actoxumab or bezlotoxumab and added to Vero cells previously grown overnight in collagen-coated 384-well plates. Following a 3-hour incubation, media were removed and cells were fixed, permeabilized, and blocked overnight. Total and nonglucosylated Rac1 was detected by in-cell Western using anti-total Rac1 or anti-nonglucosylated Rac1 as primary antibodies and quantifying the signal using the Li-Cor® Odyssey Classic instrument.

Dynamic Mass Redistribution (DMR) Assay – Epic® Assay (C): Toxins were preincubated with or without actoxumab or bezlotoxumab and added to Vero cells previously grown overnight in fibronectin-coated 384-well plates. Immediately following addition of toxin/antibody mixtures, DMR was monitored every 12 seconds for 200 minutes using the Epic BT-157900 instrument (Perkin Elmer). DMR values obtained at 180 minutes were compared across conditions.

Transepithelial Electrical Resistance (TEER) in 2-dimensional Caco-2 Cell Monolayer (D): Caco-2 cells were cultured for at least 14 days on well inserts in a 2-dimensional culture setup, in order to ensure full differentiation and confluency (confirmed by TEER readings plateauing at ≥600 $\Omega \cdot cm^2$). Toxins were preincubated with or without actoxumab or bezlotoxumab and added to the apical side of the well insert, following which TEER was monitored at specific times using the Epithelial Volt-Ohm Meter Millicell ERS-2.

Sulforhodamine B (SRB) Assay (E): Toxins were preincubated with or without actoxumab or bezlotoxumab and added to Vero cells previously grown overnight in 96-well plates. Following a 24-hour incubation (37°C), cells were washed and allowed to grow for a further 48 hours. Cells were fixed with 10% TCA followed by addition of the protein dye sulforhodamine B and further incubation for 10 minutes with shaking. Total cellular protein was quantitated using a SpectraMax (Molecular Biosystems) instrument at an absorbance wavelength of 570 nm.

References

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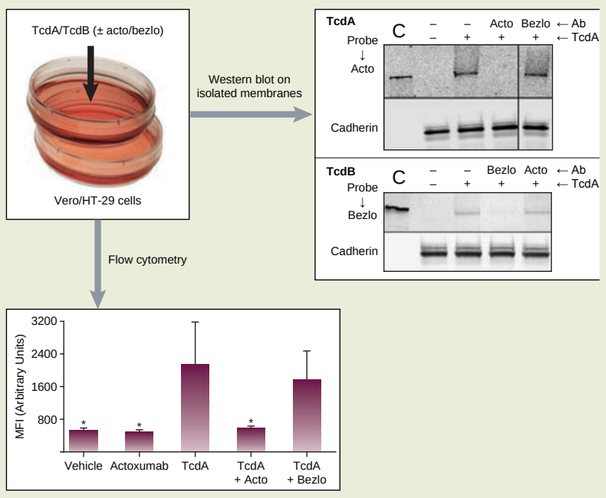
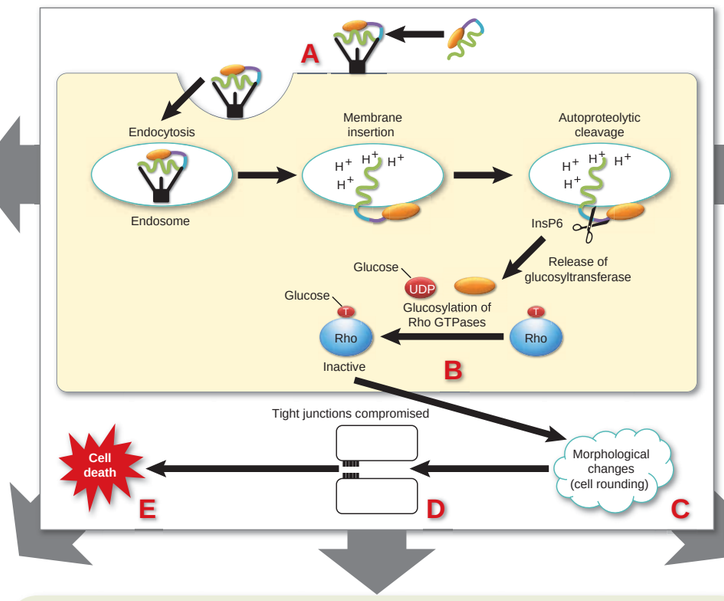
Results

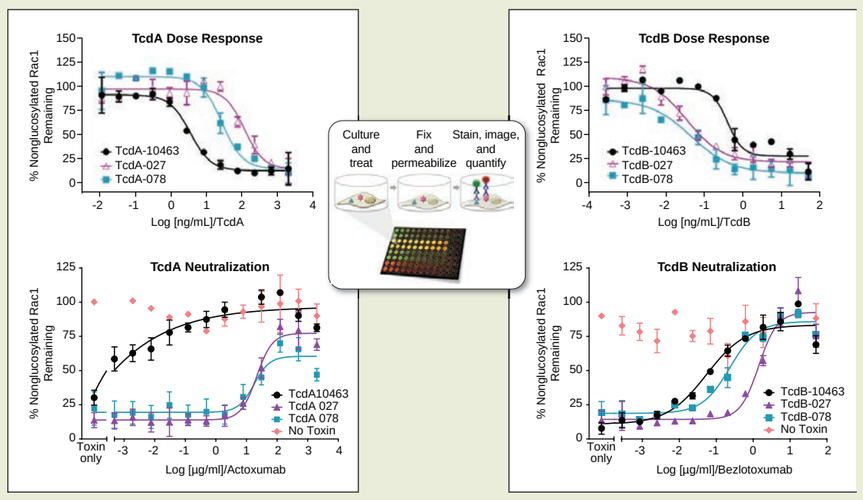
A. Assessment of toxin binding to mammalian cells using Western blotting and flow cytometry, and neutralization by actoxumab and bezlotoxumab



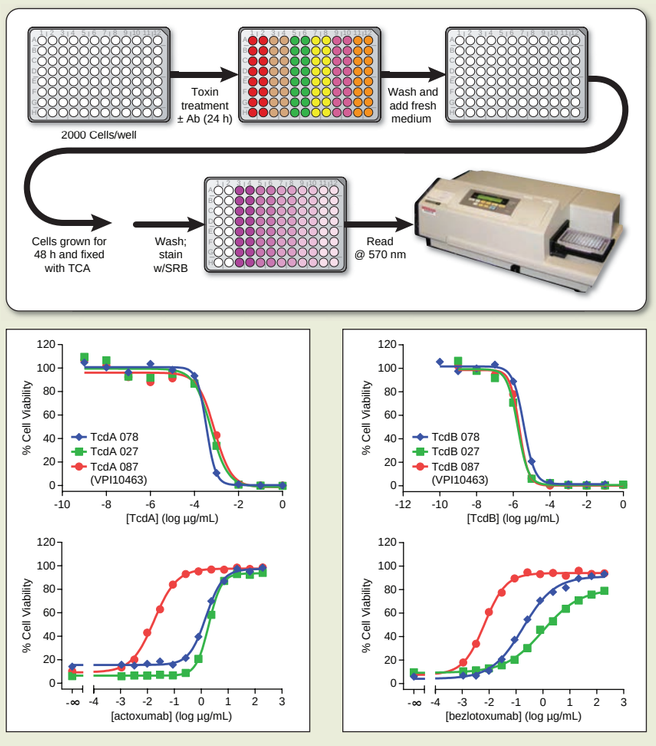
Intoxication of mammalian cells by *C. difficile* toxins: pathway and corresponding assays



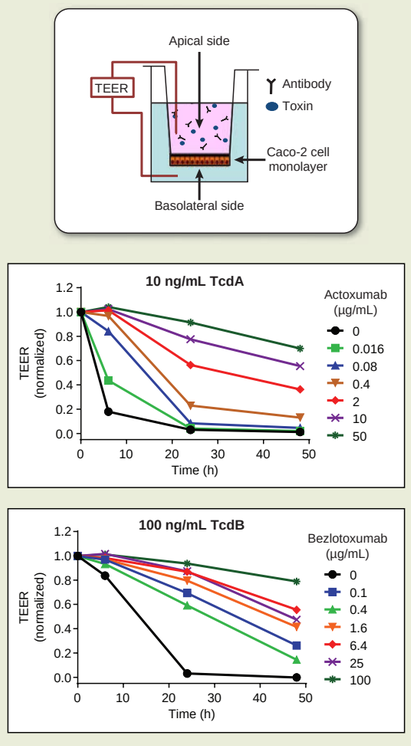
B. Effect of TcdA and TcdB from *C. difficile* ribotypes 027, 078, and 087 on Rac1 glucosylation in Vero cells using an in-cell Western assay, and neutralization by actoxumab and bezlotoxumab



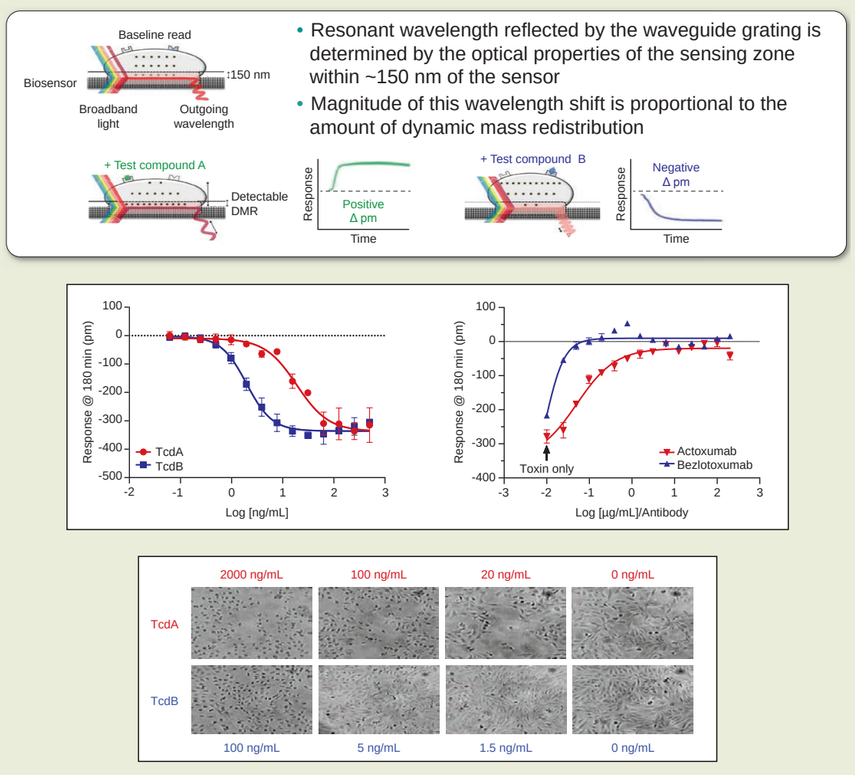
E. Effects of TcdA and TcdB from *C. difficile* ribotypes 027, 078, and 087 on Vero cell growth/survival assessed by Sulforhodamine B assay, and neutralization by actoxumab and bezlotoxumab[9]



D. Effects of TcdA and TcdB on the integrity of a gut epithelial cell monolayer using the trans-epithelial electrical resistance (TEER) assay, and neutralization by actoxumab and bezlotoxumab



C. Effects of TcdA and TcdB on cellular morphology assessed using the dynamic mass redistribution (Epic®) assay, and neutralization by actoxumab and bezlotoxumab



- Resonant wavelength reflected by the waveguide grating is determined by the optical properties of the sensing zone within ~150 nm of the sensor
- Magnitude of this wavelength shift is proportional to the amount of dynamic mass redistribution

Conclusions

- Various assays are described that quantitatively and robustly measure early and late effects of *C. difficile* toxins in mammalian cells
- The neutralizing antibodies actoxumab and bezlotoxumab, specific for TcdA and TcdB, respectively, prevent binding of the toxins to mammalian cells and thereby neutralize the various biological activities of the toxins