Human peptide α-defensin-1 interferes with Clostridioides difficile toxins TcdA, TcdB, and CDT

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Abstract

The human pathogenic bacterium Clostridioides difficile produces two exotoxins TcdA and TcdB, which inactivate Rho GTPases thereby causing C. difficile-associated diseases (CDAD) including life-threatening pseudomembranous colitis. Hypervirulent strains produce additionally the binary actin ADP-ribosylating toxin CDT. These strains are hallmarkmed by more severe forms of CDAD and increased frequency and severity. Once in the cytosol, the toxins act as enzymes resulting in the typical clinical symptoms. Therefore, targeting and inactivation of the released toxins are of peculiar interest. Prompted by earlier findings that human α-defensin-1 neutralizes TcdB, we investigated the effects of the defensin on all three C. difficile toxins. Inhibition of TcdA, TcdB, and CDT was demonstrated by analyzing toxin-induced changes in cell morphology, substrate modification, and decrease in transepithelial electrical resistance. Application of α-defensin-1 protected cells and human intestinal organoids from the cytotoxic effects of TcdA, TcdB, CDT, and their combination which is attributed to a direct interaction between the toxins and α-defensin-1. In mice, the application of α-defensin-1 reduced the TcdA-induced damage of intestinal loops in vivo. In conclusion, human α-defensin-1 is a specific and potent inhibitor of the C. difficile toxins and a promising agent to develop novel therapeutic options against C. difficile infections.

KEYWORDS

bacterial AB-type toxins, CDAD, clostridial actin ADP-ribosylating toxin, clostridial Rho-glucosylating toxin, defensins, toxin inhibitor

Abbreviations: A subunit, active subunit; AB, active/binding; AFM, atomic force microscopy; B subunit, binding and transport subunit; C. Clostridioides; CDAD, C. difficile-associated diseases; CDI, C. difficile infections; CDT, C. difficile transferase; CDTa/b, CDT A/B subunit; DPhPC/n-decane, diphanytanyl phosphatidyicholine in n-decane; DMEM, Dulbecco’s Modified Eagle’s Medium; FCS, fetal calf serum; iHIO, induced human intestinal minigut organoid; MEM, modified Eagle’s medium; PBS-T, PBS containing 0.1% Tween20; TcdA, C. difficile toxin A; TcdB, C. difficile toxin B; TEER, transepithelial electrical resistance.
1 | INTRODUCTION

The human intestinal pathogen *Clostridioidees* (*C.* formerly *Clostridium* *difficile*) is capable of producing three different protein toxins: toxin A (TcdA), toxin B (TcdB), and *C. difficile* transferase toxin CDT. *C. difficile* infections (CDI) may lead to *C. difficile*-associated diseases (CDAD) that are characterized by clinical symptoms like watery diarrhea, abdominal pain, and fever. Severe forms of CDAD range from pseudo-membranous colitis to toxic megacolon or sepsis. The clinical symptoms are strictly related to the toxins that are produced by *C. difficile* because they are responsible for the destruction of the gut barrier leading to severe enterotoxicity in humans. The major virulence factors are the toxins TcdA and TcdB. They are alone sufficient to fully develop the disease pattern. TcdA and TcdB are single-chain toxins and members of the family of large clostridial glucosylating toxins. Once in the cytosol of their host target cells, TcdA and TcdB mono-glucosylate the GTP-binding proteins of the Rho family, including Rho, Rac, and Cdc42. Glucosylation inactivates Rho proteins resulting in disturbed Rho-dependent intracellular processes. However, various human epidemic *C. difficile* strains produce in addition CDT, which is a member of the binary actin ADP-ribosylating toxins. CDT-induced ADP-ribosylation of actin causes depolymerization of the cytoskeleton and the formation of microtubule-based cell protrusions that most likely increase the adherence of the bacteria in the human colon. Thus, CDT might act as a colonizing factor but its role in disease is not fully understood. Nevertheless, the presence of CDT increases the severity of CDAD. During the last 15 years, an increase in hypervirulent and antibiotics-resistant *C. difficile* strains, associated with high mortality, was reported. Outbreaks of highly toxic strains resistant to ciprofloxacin and levofloxacin were observed in North America and later also in Europe. Nowadays, CDIs are among the most common healthcare-associated infections, leading to an increased financial burden on public healthcare systems. Due to the fact that hypervirulent strains come along with increased resistance against antibiotics, novel pharmacological strategies to neutralize the toxins are urgently needed and human defensins are particularly interesting in this context. The naturally occurring human defensins are small, cysteine-rich cationic peptides. Defensins are generally classified in α- and β-defensins. The α-defensins are produced in human neutrophils and in intestinal Paneth cells whereas β-defensins are mainly expressed in epithelial tissue and secretory glands. They represent an important part of the innate immune system, because they inactivate invasive pathogenic bacteria. However, there is increasing evidence that α-defensin-1 also neutralizes bacterial protein toxins including the *C. difficile* toxin TcdB. Here, we investigated the effect of human α-defensin-1 on TcdA and CDT. We report that α-defensin-1, but not the closely related β-defensin-1, protects cultured cells as well as clinically relevant human intestinal organoids (miniguts) from intoxication with TcdA and CDT. In addition, we confirm the inhibition of TcdA by α-defensin-1 in a mouse model in vivo. Most importantly, this peptide neutralizes the cytotoxic effects of the medically relevant combination of TcdA, TcdB, and CDT, which mimics the situation after infection with hypervirulent *C. difficile* strains.

2 | MATERIALS AND METHODS

2.1 | Protein expression, purification, and acquisition of inhibitors

The used recombinant toxins were expressed and purified as described before. α-Defensin-1 and β-defensin-1 were purchased from PeptaNova (Sandhausen, Germany).

2.2 | Cell culture and intoxication experiments

Cells were cultured in 10 cm dishes and reseeded at least three times per week. Vero cells (African green monkey kidney cells, DSMZ, Braunschweig, Germany) were cultured in MEM plus 10% fetal calf serum (FCS; both GIBCO life technologies, Carlsbad, CA, USA), 1 mM sodium pyruvate, 2 mM L-glutamine, 0.1 mM non-essential amino acids, and 100 U/mL of penicillin/100 µg/mL of streptomycin. Caco-2 cells (human epithelial colorectal adenocarcinoma cells, ATCC HTB-37, Manassas, VA, USA) were cultured in DMEM (GIBCO life technologies, Carlsbad, CA, USA) plus 10% FCS, 1 mM sodium-pyruvate, 0.1 mM non-essential amino acids, and 100 U/mL of penicillin/100 µg/mL of streptomycin. For experiments, cells were seeded in various plastic dishes and incubated with the respective compounds in the individual media under humidified conditions at 37°C and with 5% CO2. Inhibitors were added to the cells in FCS-free media except for transepithelial electrical resistance (TEER) measurements, were complete cell culture medium was used. After defined time points, pictures were taken using an Axiovert 40CFI microscope from Zeiss (Oberkochen, Germany) connected to a ProgRes C10 CCD camera from Jenoptik (Jena, Germany). Cell pictures were processed using the ImageJ software (NIH, Bethesda, MD, USA). Original magnification ×200.

2.3 | Identification of the glucosylation status of Rac1 in toxin-treated cells

Cells were grown to confluence and intoxicated with TcdA or TcdB either in the presence or in the absence of α- or β-defensin-1. For negative controls, cells were treated with medium only. After specified incubation times, the cells were
washed and lysed by freezing to −20°C and subsequent thawing. Cell lysates were transferred to SDS-PAGE followed by Western Blotting. Non-glucosylated Rac1 was detected by immunodetection with a monoclonal mouse anti-Rac1-antibody (1:1000, BD Biosciences, 610650, Franklin Lakes, NJ, USA) in combination with the specific polyclonal horseradish peroxidase (HRP)-conjugated secondary antibody (chicken anti-mouse IgG-HRP, 1:2500, Santa Cruz Biotechnology, Dallas TX, USA). Comparable protein loading was confirmed via immunodetection of β-actin (mouse anti-actin, 1:10 000, Thermo Fisher Scientific, Waltham, MA, USA). To visualize the proteins of interest, the enhanced chemiluminescence (ECL) system (Millipore Corporation, Burlington, MA, USA) was used according to the manufacturer’s instruction.

2.4 | Sequential in vitro ADP-ribosylation of actin in CDT-treated cells

Cells were grown to confluence in well plates. CDT and respective peptides α- or β-defensin-1 were added simultaneously to the cells or the cells were treated with medium only (control). After 6 hours, the treated cells and corresponding controls were washed with PBS and scraped off in 50 µL of ADP-ribosylation-buffer containing 1 mM DTT, 5 mM MgCl₂ and 1 mM EDTA, 20 mM Tris-HCl pH 7.5 plus complete protease inhibitor (Roche, Mannheim, Germany). The cells were lysed by freezing to −20°C and subsequent thawing and equal amounts of lysate protein were incubated with 10 µM biotin-labeled NAD⁺ (Treven, Gaithersburg, MD, USA) in the presence of 300 ng CDTα for 30 minutes at 37°C. By the addition of SDS sample buffer and subsequent heating at 95°C for 10 minutes, the enzyme reaction was stopped. Afterward, SDS-PAGE and Western Blot transfer onto a nitrocellulose membrane were performed. The biotin-labeled ADP-ribosylated actin proteins were detected with peroxidase-coupled streptavidin and subsequent chemiluminescence reaction. Equal protein loading was confirmed by immunodetection of Hsp90 (mouse anti-Hsp90, 1:500, Santa Cruz Biotechnology, Dallas TX, USA).

2.5 | In vitro ADP-ribosylation of actin in CDTα-treated Vero cell lysate

CDTα (200 ng) was pre-incubated with either α- or β-defensin-1 (6 µM) for 20 minutes at 37°C to elucidate effects of the defensins toward the enzyme component CDTα in the absence of cell lysate. Afterward, Vero cell lysate (40 µg) in ADP-ribosylation buffer was added in the presence of 10 µM biotin-labeled NAD⁺ and incubated for an additional 30 minutes at 37°C. In parallel, CDTα was subjected directly with both α- or β-defensin-1 and cell lysate for 30 minutes at 37°C to exclude any pre-incubation step. In vitro enzyme reaction was stopped by adding SDS sample buffer, and heating and SDS-PAGE and Western Blot were performed. Biotin-labeled actin was detected with peroxidase-coupled streptavidin and subsequent chemiluminescence reaction. Equal protein loading was confirmed by immunodetection of Hsp90.

2.6 | TEER measurements

TEER was measured using the EVOMX apparatus (WPI, Sarasota, FL, USA) provided with the STX2 electrode. To achieve a confluent Caco-2 monolayer, 0.7 × 10⁵ cells were seeded on 24-well Hanging Inserts (Millipore Cell Culture Inserts, EMD Millipore Corporation, Burlington, MA, USA) and incubated for 3 days at 37°C. The toxins, as well as α-defensin-1, were added apically. Raw resistance data were transformed into unit area resistance by the subtraction of the blank resistance and by multiplying the resulting data by the effective surface area of the used filter membrane (here 0.3 cm²).

2.7 | Immunofluorescence microscopy

Cells were seeded overnight with a density of 30 000 cells per well in 8-well plates (ibidi GmbH, Gräfeling, Germany). Cells were intoxicated with either TcdA, TcdB, CDT or the mixture of all toxins. After indicated time points, the cells were fixated with 4% PFA for 20 minutes, permeabilized with Triton-X 100 (0.4% in PBS) for 5 minutes, treated with 100 mM glycine in PBS-T (PBS with 0.1% Tween) for 2 minutes and blocked with 5% skim milk in PBS-T for 30 minutes at 37°C. For immunostaining against non-glucosylated Rac1, cells were incubated with a monoclonal mouse anti-Rac1-antibody (BD Biosciences, 610650, Franklin Lakes, NJ, USA, 1:100 diluted in 5% skim milk in PBS-T) for 30 minutes at 37°C. After washing, a goat anti-mouse IgG cross-adsorbed secondary antibody Alexa Fluor 568 (Invitrogen, A11004, Carlsbad, CA, USA) was added (1:750 in 5% skim milk in PBS-T) for 30 min at 37°C. For F-actin staining, samples were incubated with phalloidin-FITC (1:100 in 5% skim milk in PBS-T) for 30 minutes at 37°C. After washing, a goat anti-mouse IgG cross-adsorbed secondary antibody Alexa Fluor 568 (Invitrogen, A11004, Carlsbad, CA, USA) was added (1:750 in 5% skim milk in PBS-T) for 30 min at 37°C. For F-actin staining, samples were incubated with phalloidin-FITC (1:100 in 5% skim milk in PBS-T) for 30 minutes. Nuclei were stained with Hoechst 33342 (1:10 000 in 5% skim milk in PBS-T) for 5 minutes. Immunofluorescence microscopy images were obtained using the iMic Digital Microscope (40× magnification, oil) and the Live Acquisition 2.6 software (both FEI, Thermo Fisher Scientific, Waltham, MA, USA). Raw images were processed with ImageJ software.

2.8 | Toxin–α-defensin-1 interaction analysis using the Dot Blot system

Toxins were spotted in duplicates onto a nitrocellulose membrane in decreasing concentrations (serial dilution,
starting at 0.75 μg for TcdB/CDTa and 0.25 μg for TcdA/CDTb) via vacuum aspiration. The membrane was blocked with 5% skim milk in PBS-T and cut in half. One half of the membrane was incubated with 400 ng/mL α-defensin-1 in PBS-T for 1 hour at RT and the other half was incubated only in PBS-T. Unbound α-defensin-1 was removed by washing with PBS-T. Membranes were incubated with the primary goat anti-α-defensin-1-antibody (1:1000 in PBS-T, Thermo Fisher Scientific, Waltham, MA, USA) followed by incubation with a secondary antibody (human anti-goat IgG-HRP, 1:2500, Santa Cruz Biotechnology, Dallas TX, USA). Bound α-defensin-1 was detected using the ECL-system.

2.9 Precipitation studies

At first, possible protein-aggregates were removed by centrifugation of the toxin stock solution (10 000 rcf at 4°C for 20 min). Then, 1 μg of the respective toxin was diluted in MEM to a total volume of 30 μL. α-Defensin-1 was added to the indicated samples at a final concentration of 6 μM. The samples were incubated at 37°C for 15 minutes to allow the formation of precipitates. Afterward, the samples were centrifuged (14 000 rcf at 4°C for 20 min) to collect the formed precipitates. Unbound α-defensin-1 was removed by centrifugation (10 000 rcf for 20 min). It was reconstituted into lipid bilayer membranes by adding small amounts of a concentrated stock solution (0.2 mg/mL; 3 μM in PBS) to the aqueous phase to one side (the cis-side) of a membrane in the black state. A voltage of 20 mV was applied through silver/silver chloride electrodes with salt bridges to the cis-side of the lipid bilayer membranes. The current through the membranes was measured with a home-built current amplifier based on a Burr-Brown operational amplifier. The amplified signal was recorded with a strip chart recorder (Rikadenki, Freiburg, Germany).

2.10 Atomic force microscopy measurements

Before starting the experiment, TcdA was centrifuged (10 000 rcf for 20 min at 4°C) to remove possible protein self-aggregates. Afterward, 500 ng TcdA was subjected either to 6 μM α-defensin-1 in water or water alone. The samples were incubated for 15 minutes at 37°C and subjected to drop-casting on freshly cleaved mica followed by drying in air. Atomic force microscopy (AFM) measurements were performed on a Vecco Multimode IIIa atomic force microscope using cantilevers (Olympus, Shinjuku, Tokyo, Japan) with a spring constant of about 1.7 N/m. The raw data were processed (plane correction only) and analyzed with NanoScope software (Bruker, Billerica, MA, USA).

2.11 Black lipid bilayer experiments with CDTb in the absence of CDTa

Black lipid bilayer membranes to study the interaction between channels formed by CDTb and human α-defensin-1 were formed from a 1% solution of diphytanoyl phosphatidylcholine (DiPhPC; Avanti Polar Lipids, Alabaster, AL, USA) in n-decane as described previously in detail. The membranes were formed from the lipid solution across small circular holes with a surface area of about 0.4 mm². The hole connected two aqueous compartments of 5 mL volume each in a Teflon cell. The aqueous KCl solutions (Merck, Darmstadt, Germany, analytical grade) were buffered with 10 mM MES to pH 6. CDTb (from C difficile strain 196) was produced as his-tagged protein in the Bacillus megaterium expression system. It was reconstituted into lipid bilayer membranes by adding small amounts of a concentrated stock solution (0.2 mg/mL; 3 μM in PBS) to the aqueous phase to one side (the cis-side) of a membrane in the black state. A voltage of 20 mV was applied through silver/silver chloride electrodes with salt bridges to the cis-side of the lipid bilayer membranes. The current through the membranes was measured with a home-built current amplifier based on a Burr-Brown operational amplifier. The amplified signal was recorded with a strip chart recorder (Rikadenki, Freiburg, Germany).

2.12 Calcium (Ca²⁺)-imaging

Caco-2 cells were seeded with a density of 150 000 cells per well in 8-well plates (ibidi GmbH, Gräfeling, Germany) for 2 days. Afterward, the cells were kept in bath solution (containing 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM Glucose, 10 mM HEPES, pH 7.4). The cells were loaded for 45 minutes at 37°C with 3 μM of Fura-2AM and were then washed three times with bath solution. After measuring the baseline for 2 minutes, the respective treatments were performed and calcium flow was recorded for 20 minutes using the iMic Digital Microscope (40x magnification, oil) and the Live Acquisition 2.6 software (both FEI, Thermo Fisher Scientific, Waltham, MA, USA). Resulting ratio images were created with excitation light pulses at 340 and 380 nm followed by subsequent ratio calculations (340/380).

2.13 Intoxication of induced human intestinal minigut organoids

The ethical committee of Ulm University and Tübingen University agreed with the use of human material in the present study (Nr 0148/2009, 638/2013BO1). The performance of the experiments ensued in compliance with the guidelines of the Federal Government of Germany and the Declaration of Helsinki concerning Ethical Principles for Medical Research Involving Human Subjects. Induced human intestinal minigut organoids (iHIOs) were generated in a stepwise differentiation protocol from induced pluripotent stem cells as described previously. iHIOs
were grown in Matrigel (Corning Inc., Corning, NY, USA) in 24 well plates and intoxicated with a mixture of TcdA (20 pM), TcdB (20 pM) and CDT (4.2/5.3 nM) in the presence or absence of α-defensin-1 (12 µM) or were left untreated. After 5 hours, iHIOs were washed with PBS, the Matrigel was removed and the organoids were fixated with 4% PFA in 10% sucrose solution for 20 minutes at RT and incubated in 25% sucrose solution overnight at 4°C. Next, iHIOs were placed in plastic Cryomold molds (Sakura Finetek, Staufen im Breisgau, Germany), embedded in Tissue-Tek OCT Compound (Sakura Finetek, Staufen im Breisgau, Germany) and frozen in liquid nitrogen. Afterward, the frozen iHIOs were cut into sections of 8 µm and dried overnight at RT. Next, the iHIOs were rehydrated, blocked in 10% goat serum, permeabilized with 0.2% Triton X-100 in PBS, treated with a mouse anti-E-cadherin-antibody (610182, BD Bioscience, Franklin Lakes, NJ, USA) overnight at 4°C and subsequently incubated with a fluorescence-labeled secondary goat anti-mouse-568 antibody (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) in 5% goat serum and 0.1% Triton-X 100 in PBS for 1 hour at RT. F-actin was stained with phalloidin-FITC and nuclei were stained with DAPI. Images were acquired using the ApoTome Axio Z1 imager microscope (Carl Zeiss, Oberkochen, Germany) and processed with ImageJ.

2.14 | In vivo intestinal ligated loop experiment

All experiments were performed in accordance with French and European Community guidelines for laboratory animal handling. The protocols of experiments were approved by Institut Pasteur CETEA (Comité d’Ethique en Expérimentation Animale) with the agreement of laboratory use (N° 2013-0118) and with the N°02027.02 from the Ministère de l’Éducation Nationale, de l’Enseignement Supérieur et de la Recherche. Adult male Swiss mice from Charles River were fasted for 18 hours before surgery. Mice were deeply anesthetized with ketamine (50 mg/kg body weight; Imalgene 1000; Merial, Lyon, France) and xylazine (10 mg/kg body weight; Rompun, Med’Vet, France). A mouse laparotomy was performed and two ileal loops (approximately 4 cm long) were isolated as previously described. Two hundred microliters of PBS containing 3 × 10^7 cytotoxic units of TcdA as tested in Vero cells grown in 96 well plate, or TcdA and α-defensin-1 (10 µM) were injected. TcdA was purified from C. difficile VPI10463 as previously described. After 4 hours incubation, mice were killed via cervical dislocation and the intestinal loops were harvested, fixed in 4% formaldehyde, sliced, and stained with hemalum and eosine. Four mice were used with two ileal loops each (TcdA and TcdA + α-defensin-1).

3 | RESULTS

3.1 | α-Defensin-1 protected cultured cells from intoxication with TcdA, TcdB, and the combination of both toxins

Prompted by the previous results that α-defensin-1 neutralizes the cytotoxic effects of TcdB in vitro, we investigated whether α-defensin-1 protects cultured cells from intoxication with TcdA. Rounding of adherent cells as a specific and highly sensitive response to the TcdA-catalyzed glucosylation of Rac1 is an established endpoint to monitor intoxication. In the first set of experiments, we treated Vero cells with TcdA in the absence or presence of α-defensin-1 and determined the number of rounded cells over time. The amount of non-round cells clearly indicated the protective role of α-defensin-1 on the intoxication process (Figure 1A). To better mimic the physiological situation in the infected gut, we next combined TcdA and TcdB in a 1:1 ratio. Also for this combination, a significant inhibition of toxin-induced cell rounding by α-defensin-1 was observed (Figure 1A). The protective effect of α-defensin-1 on TcdA and the combination TcdA/TcdB was also obvious for human colon Caco-2 cells by analyzing the cell morphology (Figure 1B, left panel) and the glucosylation status of Rac1 from lysates of those cells (Figure 1B, upper right panel). Unmodified Rac1 from untreated cells is recognized by a specific antibody, which does not detect glucosylated Rac1. By this antibody, the inhibitory effect of α-defensin-1 on TcdA and the combination of TcdA and TcdB were confirmed in Caco-2 cells (Figure 1B, right upper panel). In contrast to α-defensin-1, the closely related β-defensin-1 had no inhibitory effect (Figure 1B, lower right panel), which demonstrated a specific mode of action of α-defensin-1. To measure the integrity of the epithelial barrier function of confluenously growing Caco-2 cells after treatment with TcdA in the presence or absence of α-defensin-1, the TEER was analyzed (Figure 1C). TcdA-treatment strongly reduced TEER in Caco-2 monolayers and α-defensin-1 delayed this effect. As reported earlier, no change in TEER was observed, when Caco-2 cells were incubated with α-defensin-1 alone.

In addition, the glucosylation status of Rac1 was analyzed immunohistochemically by the specific anti-Rac1 antibody that detects only non-glucosylated, but not glucosylated Rac1. Caco-2 cells were treated with either TcdB, TcdA or the combination TcdA/TcdB in the presence or absence of α-defensin-1, followed by Rac1 immunostaining and fluorescence microscopy. Upon intoxication of Caco-2 cells with TcdA, an impact on the cortical actin was detected (Figure 2). The presence of α-defensin-1 decreased the TcdA-mediated effect on the cortical actin and less Rac1 was glucosylated in these cells. A comparable result was obtained for the combination of TcdA/TcdB. After the treatment of cells with TcdA/TcdB and α-defensin-1, F-actin and the distribution of...
FIGURE 1  α-Defensin-1 protects cells from intoxication with TcdA and the combination of TcdA and TcdB. A, Vero cells were intoxicated for 4 h with either TcdA (10 pM), TcdB (10 pM), the combination of both toxins (each 10 pM) and α-defensin-1 (6 µM) or were left untreated for negative control. Representative images, as well as time-dependent inhibition of TcdA and TcdA/TcdB, are depicted. Values are given as mean ± SD (n = 3). Scale bar: 50 µm. B, Caco-2 cells were treated with α-defensin-1 and either TcdA (10 pM), TcdB (10 pM) or the combination of both toxins (each 10 pM) for 7 h. Scale bar: 50 µm. Non-glucosylated Rac1 was detected by immunoblotting with an anti-Rac1 antibody. Comparable protein loading was confirmed by actin staining with a specific antibody. No inhibition of TcdA was observed for β-defensin-1. (C) To verify the inhibitory effects of α-defensin-1, TEER measurements were performed with TcdA (30 pM) in the presence or absence of α-defensin-1 (6 µM). Values are given as mean ± SD (n = 2). Significance was tested by the Student’s t test (ns = not significant, *P < .05, **P < .01, ***P < .001)
α-Defensin-1 prevents glucosylation of Rac1 after intoxication with TcdB, TcdA and the combination TcdA/TcdB. Caco-2 cells were incubated with 6 µM α-defensin-1 and either TcdB (10 pM), TcdA (10 pM) or the combination of both toxins (each 10 pM) for different time points (TcdB: 6 h, TcdA: 8.5 h, TcdA/TcdB: 6 h). For negative control, cells were left untreated. Afterward, cells were fixed, permeabilized, and non-glucosylated Rac1 was detected by a specific antibody in combination with a fluorescent-labeled secondary antibody. Nuclei were stained with Hoechst 33342 and F-actin was stained with phalloidin-FITC. Scale bar: 20 µm
non-glucosylated Rac1 were more similar to untreated control cells.

3.2 | α-Defensin-1 protected cells from intoxication with the binary toxin CDT

To test whether α-defensin-1 also affects the intoxication with CDT, Vero cells were treated with the binary toxin CDT in the presence or absence of α-defensin-1 and the number of round cells was determined. Here, cell rounding is a consequence of the CDTα-catalyzed ADP-ribosylation of G-actin followed by F-actin depolymerization and serves as a specific and sensitive endpoint to monitor intoxication with CDT.38

The CDT-induced cell rounding was decreased in the presence of α-defensin-1 in a time-dependent manner (Figure 3A). A comparable result was obtained for Caco-2 cells (Figure 3B, upper panel). Consistently, the CDTα-catalyzed ADP-ribosylation of actin, detected in the cytosol of living cells, was prevented when cells were treated with CDT in the presence of α-defensin-1 as analyzed by

**FIGURE 3** α-Defensin-1 protects cells from intoxication with CDT. A, Vero cells were intoxicated with CDT (0.8/1.1 nM) and α-defensin-1 (6 µM) or were left untreated for control. Representative images after 4 h, as well as time-dependent inhibition of CDT, are shown and values are given as mean ± SD (n = 3). Scale bar: 50 µm. B, Caco-2 cells were treated with α-defensin-1 or β-defensin-1 (each 6 µM) and CDT (2.1/2.7 nM) for 6 h. For α-defensin-1 and CDT, representative images are shown. Scale bar: 50 µm. ADP-ribosylated and biotin-labeled actin was detected by immunoblotting using the ECL system. Comparable amounts of loaded proteins were confirmed by Hsp90-staining with specific antibodies. For β-defensin-1, no inhibition was observed. C, TEER measurements were performed using Caco-2 cells with CDT (0.7/0.9 nM) in the presence or absence of α-defensin-1. Values are given as mean ± SD (n = 2). D, Caco-2 cells were incubated with 6 µM α-defensin-1 and the binary toxin CDT (2.1/2.7 nM) or were left untreated as a negative control. After 5 h, the cells were fixed, permeabilized and F-actin was stained with phalloidin-FITC. In addition, nuclei were stained with Hoechst 33342. Scale bar: 20 µm. Significance was tested by the Student’s t test (ns = not significant, *P < .05, **P < .01, ***P < .001)
sequential ADP-ribosylation (Figure 3B, lower panel). In this Western blot analysis, a strong signal of biotin-actin indicates no modification of G-actin in the living cells. As observed for TcdA/TcdB before, β-defensin-1 showed no comparable protective effect toward CDT (Figure 3B, lower panel). To further analyze this effect, TEER measurements with Caco-2 cells were performed. Treatment of Caco-2 cells with CDT strongly decreased the TEER, indicating the disruption of the epithelial barrier function. The presence of α-defensin-1 significantly reduced this effect, indicating its protective effect toward intoxication with CDT (Figure 3C). Moreover, α-defensin-1 prevented the CDT-induced depolymerization of F-actin in Caco-2 cells as analyzed by fluorescence microscopy after phalloidin-FITC staining of F-actin. In the presence of α-defensin-1, the overall structure of the actin cytoskeleton was mainly preserved (Figure 3D), confirming the results obtained in the previous experiments.

### 3.3 | α-Defensin-1 directly and specifically bound to TcdA, TcdB, and CDTb in vitro

To investigate the molecular mechanisms underlying the protective effect of α-defensin-1, a direct interaction between the toxins and α-defensin-1 was analyzed. For TcdB, a direct interaction was already shown and it was demonstrated that human defensins facilitate the local unfolding of thermodynamically unstable regions of TcdB and TcdA in vitro. The direct binding to α-defensin-1 to TcdA and CDT was analyzed by dot blotting. The toxins were spotted onto a nitrocellulose membrane, incubated with α-defensin-1, and the bound defensin was detected with a specific antibody. The results are shown in Figure 4.

In line with previous results, the defensin bound to TcdB but also to TcdA. For the binary toxin CDT, binding of the defensin was observed to the transport component CDTb but not to the enzyme component CDTa (Figure 4A).

![Figure 4](image-url)
3.4 Interaction between TcdA and α-defensin-1 resulted in aggregation

Because previous work suggested that defensin/toxin-interactions and subsequent complex formation might be a common feature, the aggregation of TcdA with α-defensin-1 was investigated. When TcdA was incubated together with α-defensin-1, precipitation was detected. As shown in Coomassie blue-stained gels, TcdA shifted from the supernatant- to the pellet-fraction only when the toxin was incubated with α-defensin-1 (Figure 4B, upper panel). In contrast, incubation of TcdA with β-defensin-1 did not lead to any observable precipitation, again indicating the specificity of α-defensin-1 (Figure 4B, lower panel). To further characterize the aggregation, we investigated the interaction between α-defensin-1 and TcdA by atomic force microscopy (AFM). Only the combination of TcdA with α-defensin-1, but not with β-defensin-1, resulted in the formation of large structures (Figure 4C), likely representing TcdA/α-defensin-1-aggregates.

**Figure 5** α-Defensin-1 does not influence the enzymatic activity of CDTa in vitro, but protects cells from cytotoxic CDTb-effect. A, CDTa (200 ng) was pre-incubated with either α- or β-defensin-1 at 37°C for 20 min (left panel) or not (right panel). For control, CDTa was incubated alone. Next, 40 μg Vero cell lysate was added in the presence of 10 μM biotin-NAD⁺ and in vitro enzymatic reaction took place for 30 min at 37°C. ADP-ribosylated and thereby biotin-labeled actin was detected by immunoblotting using the ECL system. Comparable amounts of loaded proteins were confirmed by Hsp90-staining with specific antibodies. B, Vero cells were incubated with CDTb (4 nM) and with either α- or β-defensin-1 (each 6 μM). For control, Vero cells were left untreated. After 5 h, pictures were taken and cell viability was measured. Values are given as mean ± SD (n = 3). Scale bar: 50 μm. Significance was tested by the Student’s t test (ns = not significant, *P < .05, **P < .01, ***P < .001)
3.5 | Influence of α-defensin-1 on the individual components of CDT

Driven by the results from the dot blot experiment, where α-defensin-1 interacted with CDTb but not CDTa, the effects of α-defensin-1 on the single components of CDT were investigated. The α-defensin-1 had no effect on the ADP-ribosylation of actin by CDTa, independently whether CDTa was pre-incubated with α-defensin-1 or not (Figure 5A).

In contrast, α-defensin-1 neutralized the biological activity of CDTb, which was analyzed by monitoring the cytotoxic effects caused by very high concentrations of CDTb in the absence of CDTa (Figure 5B). In this case, CDTb forms pores in the cytoplasmic membrane of Vero cells, which result in a change in cell morphology (Figure 5B, upper panel) and cell death (Figure 5B, lower panel), similar to the B-component of Clostridium perfringens iota toxin.40 Again, in contrast to α-defensin-1, β-defensin-1 did not show any protective effects (Figure 5B), indicating the specific interaction between α-defensin-1 and CDTb.

3.6 | Effect of α-defensin-1 on the formation of the CDTb-pore in the absence of CDTa

The results obtained so far showed that α-defensin-1 directly interacts with CDTb and most likely blocks the uptake of CDTa by the CDTb channels. One possible mechanism could be the blockage of the CDTb channels since it was already described that heterocyclic compounds or cyclo-dextrane derivatives can act as pore blockers for other bacterial toxins.41-44 To check this possibility in the case of α-defensin-1 bilayer experiments with reconstituted CDTb channels were performed. The α-defensin-1 was added to one or both sides of the reconstituted channels to see if the channels responded to the addition of α-defensin-1. Figure 6A shows the current recording of a typical experiment of this type. CDTb was added in a final concentration of about 200 ng/mL to the cis-side of a black lipid bilayer membrane made of DiPhPC/n-decane. About 2 minutes after the addition of CDTb to the cis-side of the black lipid bilayer membrane, the membrane current started to increase. About 12 minutes after the onset of the conductance, human α-defensin-1 was added to both sides of the membrane in a concentration of 80 nM (Figure 6A, left black arrow). The current through the CDTb channels did not show any substantial decrease that would be expected if the channels were blocked by α-defensin-1. Instead, the current recording showed only an insignificant increase. The addition of α-defensin-1 was repeated two times: first after five and then after about 10 minutes (Figure 6A, middle and right black arrows), which resulted in its final concentration in the aqueous phase of 240 nM. These additions did not influence the CDTb-induced membrane conductance, indicating that α-defensin-1 was not able to block the conductance of already existing CDTb channels. However, the formation of additional CDTb-channels was no longer observable, which suggested that α-defensin-1 inhibits the formation of CDTb-channels.

To address this question, CDTb was mixed with human α-defensin-1 and the mixture was added to the cis-side of a black lipid bilayer membrane in the same concentration as in the previous experiment (CDTb: 200 ng/mL, α-defensin-1:80 nM). Here, the current recording showed only a minor increase (Figure 6B) compared with the current increase when CDTb was added prior to α-defensin-1. In line with these experiments, no substantial current increase could be observed, when α-defensin-1 was added first to the aqueous phase of one side of a black lipid bilayer in a concentration of 80 nM followed by the addition of CDTb in a final concentration of 200 ng/mL to the same side (data not shown). The results obtained in the black lipid bilayer experiments were confirmed using the calcium imaging method on living Caco-2 cells. When CDTb was added to intact cells, a pore was formed resulting in an increase in the Fura-2 AM-ratio, indicating an increase in the intracellular calcium concentration (Figure 6C, upper part). When α-defensin-1 was added at the same time as CDTb, no CDTb-pores were formed at all (Figure 6C, lower left part). When CDTb was added 5 minutes prior α-defensin-1, CDTb-pores were formed until α-defensin-1 was added. Afterward, no additional pores were formed (Figure 6C, lower right part).

3.7 | Effect of α-defensin-1 on the intoxication of cells with the combination of TcdA, TcdB, and CDT

To better mimic the medically relevant situation in the gut after infection with hypervirulent strains of C. difficile, the effect of α-defensin-1 on the intoxication of Caco-2 cells with the combination of all three toxins was investigated. Caco-2 cells were incubated with a mixture of TcdA, TcdB, and CDT in the presence or absence of α-defensin-1. After defined time points, cells were analyzed for the number of rounded cells.

A time-dependent inhibition of all toxins became evident (Figure 7A). Furthermore, co-incubation of α-defensin-1 also protected the structure of the actin cytoskeleton of Caco-2 cells challenged with the three toxins. Untreated Caco-2 cells possessed a distinct actin ring at the cell cortex as well as thin fibers within the cell body whereas non-glucosylated Rac1 was widely distributed throughout
**FIGURE 6** α-Defensin-1 does not influence existing CDTb-pores; however, it blocks the formation of new CDTb-channels. A, Current increase of a DiPhPC/n-decane membrane after the addition of about 200 ng/mL CDTb to the cis-side of the black membrane. The aqueous phase contained 1 M KCl, 10 mM MES, pH 6. The applied potential was 20 mV at the cis-side of the membrane. The three arrows indicate the subsequent addition of increasing concentrations of α-defensin-1 to both sides of the membrane, T = 20°C. The addition of α-defensin-1 did not lead to a decrease in the current through the CDTb channels. B, Current recording of a black DiPhPC/n-decane membrane when 1 µg CDTb dissolved in PBS was mixed with 1 µg α-defensin-1 dissolved in 10 µL ultrapure water. The mixture was added afterward to 5 mL aqueous phase on the cis-side of the membrane about 3 minutes before the start of the recording. The aqueous phase contained 1 M KCl, 10 mM MES, pH 6; Vm = 20 mV; T = 20°C. The addition of the CDTb-α-defensin-1 mixture to the aqueous phase had only a minor effect on the conductance of the membrane, indicating that only a minor number of CDTb channels were formed in the membrane. C, Caco-2 cells were seeded in an 8-well ibidi plate for 2 days. Afterward, the cells were pretreated for 45 min with 3 µM Fura-2AM. After measuring the baseline for 2 minutes, different treatments were performed. CDTb (1 µg/mL) in the absence of α-defensin-1 was able to form pores in the membrane of Caco-2 cells (upper part). When added simultaneously (CDTb: 1 µg/mL, α-defensin-1:6 µM), no CDTb-pores were formed (lower left part). When CDTb (1 µg/mL) was added prior to α-defensin-1, pores began to form what could be stopped after addition of α-defensin-1 (6 µM, lower right part).
the cytosol. These characteristics were destroyed by the addition of the toxins; however, the typical distribution of actin and Rac1 was largely preserved in the presence of α-defensin-1 or were left untreated for control. Representative images are shown and for quantitative analysis, the percentage of Caco-2 cells displaying an obvious intoxication morphology was determined. Values are given as mean ± SD (n = 3). Scale bar: 50 µm. B, For visualization of the actin cytoskeleton, Caco-2 cells were incubated with the mixture of all three toxins (TcdA: 10 pM; TcdB, 10 pM, CDT 2.1/2.7 nM) and α-defensin-1 (6 µM). After 6 h, F-actin and non-glucosylated Rac1 were stained. Nuclei were additionally stained with Hoechst 33342. Scale bar: 20 µm. (C) Protective effect of α-defensin-1 in TEER-measurements performed with the mixture of all toxins (TcdA: 30 pM; TcdB; 10 pM, CDT: 2.1/2.7 nM). Values are given as mean ± SD (n = 3). Significance was tested by the Student’s t test (ns = not significant, *P < .05, **P < .01, and ***P < .001).

FIGURE 7  α-Defensin-1 protects Caco-2 cells from intoxication with the combination of all three C. difficile toxins. A, Caco-2 cells were incubated at 37°C for 6 h with a mixture of TcdA (10 pM), TcdB (10 pM), and CDT (2.1/2.7 nM) in the presence or absence of α-defensin-1 or were left untreated for control. Representative images are shown and for quantitative analysis, the percentage of Caco-2 cells displaying an obvious intoxication morphology was determined. Values are given as mean ± SD (n = 3). Scale bar: 50 µm. B, For visualization of the actin cytoskeleton, Caco-2 cells were incubated with the mixture of all three toxins (TcdA: 10 pM; TcdB, 10 pM, CDT 2.1/2.7 nM) and α-defensin-1 (6 µM). After 6 h, F-actin and non-glucosylated Rac1 were stained. Nuclei were additionally stained with Hoechst 33342. Scale bar: 20 µm. (C) Protective effect of α-defensin-1 in TEER-measurements performed with the mixture of all toxins (TcdA: 30 pM; TcdB; 10 pM, CDT: 2.1/2.7 nM). Values are given as mean ± SD (n = 3). Significance was tested by the Student’s t test (ns = not significant, *P < .05, **P < .01, and ***P < .001).

3.8  α-Defensin-1 protected human intestinal organoids from intoxication with a mixture of TcdA, TcdB, and CDT

Next, the findings were transferred to a human intestinal organoid model (iHIOs, miniguts), which represents a highly complex human intestinal model system. iHIOs comprise the ability to expand in three-dimensional culture conditions with potential for unlimited self-renewal. They display key features of gut tissue including crypt-like structures, tight
junctions, adhesion molecules, and epithelial and even non-epithelial intestine compartments. Upon incubation of the iHIOs with the mixture of TcdA, TcdB, and CDT, a disruption of cortical E-Cadherin and actin-cytoskeleton was observed. The presence of α-defensin-1 decreased the CDT-mediated F-actin destruction and maintained the cortical localization of E-Cadherin (Figure 8). Thus, α-defensin-1 protected the miniguts from intoxication with the combination of all C. difficile toxins. Noteworthy, no side-effects induced by α-defensin-1 were observed.

3.9 | α-Defensin-1 protected intestinal mice loops from TcdA-induced damages

As proof of concept and to investigate the inhibitory capacity of α-defensin-1 in vivo, an ileal loop model was used. It was already reported, that ileal loop models were well suited to visualize the effects of TcdA on guts. After 4 h incubation time, Swiss mice intestinal loops injected with TcdA displayed clear toxin-induced tissue damage including the beginning breakdown of the epithelial barrier integrity compared to PBS-treated control loops (Figure 9A,B). In contrast, TcdA plus α-defensin-1 injected intestinal loops showed a lower level of histologic damage as depicted in Figure 9C.

In conclusion, the human peptide α-defensin-1 specifically protected Vero and Caco-2 cells as well as human intestinal organoids not only from TcdB, but also from TcdA and CDT, and most importantly, from the mixture of all three toxins by directly binding to the toxins which likely neutralizes their cytotoxic activities. Moreover, the potency of α-defensin-1 as an inhibitor of bacterial toxins could be shown exemplarily for TcdA in an in vivo mouse model.
4 | DISCUSSION

The protein toxins TcdA and TcdB are the major virulence factors of *C. difficile*. In addition, the binary toxin CDT is produced by hypervirulent and partially antibiotic-resistant *C. difficile* strains and may act as a colonization factor, resulting in an increase in morbidity and mortality of CDAD. The secreted toxins are responsible for the clinical symptoms and therefore, represent the central drug targets to treat/prevent CDAD. It is highly necessary to identify and to develop alternatives to mere antibiotic treatment, like antibodies against TcdA and TcdB, which are able to decrease the severity of CDI.

The inhibitory role of α-defensin-1 was already reported for TcdB and other bacterial toxins including anthrax lethal toxin and ADP-ribosylating toxins where the peptide inhibited the enzyme activities. Here, we demonstrate that the human peptide α-defensin-1 inhibits the cytotoxic mode of action of TcdA, TcdB, and CDT in a time- and concentration-dependent manner. We observed a direct interaction between TcdA and α-defensin-1 which resulted in the formation of biologically inactive TcdA/α-defensin-1-aggregates in vitro what was directly demonstrated by AFM. The toxin/α-defensin-1 aggregate formation might trap TcdA and TcdB and prevent binding and/or uptake of the toxins into their host cells. However, unspecified interactions between cationic defensins and the toxins could be excluded, since, in all experiments, the closely related β-defensin-1 did not inhibit or influence the intoxication of cultured cells.

For the third toxin CDT, also a time- and concentration-dependent inhibition by α-defensin-1 could be observed. However, the underlying mode of action seems to be different and is most likely based on the direct interaction between the binding and transport component CDTb and α-defensin-1. These findings are somehow contradictory to results found in the past, where an influence of α-defensin-1 was linked to the neutralization of the enzymatic activity of ADP-ribosylating toxins like DT or ETA. In our experiments, a direct interaction was only observable between α-defensin-1 and CDTb. Furthermore, our hypothesis was strengthened by the fact, that in our hands α-defensin-1 had no influence on the ADP-ribosyltransferase activity of CDTa in vitro, regardless of whether CDTa was pre-incubated with α-defensin-1 or not. This is in line with recent results obtained for the closely related iota toxin from *C. perfringens*, where α-defensin-1 interacts with and inhibits the biological activity of the transport subunit iota b but has no effect on iota a, which is the actin ADP-ribosylating enzyme subunit. A further clear evidence that the mode of inhibition is based on the interaction between α-defensin-1 and CDTb was found when CDTb alone (in the absence of CDTa) was applied to cells. Recently, it was shown that at high concentrations, CDTb alone is sufficient to induce morphological changes and cell death. The presence of α-defensin-1 protected cultured cells almost completely from the cytotoxic CDTb-effect. In artificial lipid bilayer experiments, this protection effect was examined in more detail. Planar lipid bilayer membranes have widely been used for the study of reconstituted binding components of different AB-type toxins. Bacterial binding components form heptameric channels that function as protein conducting nanopores for the transport of the enzymatic subunits into the target cells via the early or late endosomal pathway. Small amounts of the binding components added to one side of the membranes allow the formation of many asymmetric transmembrane channels without the need of the native...
host cell receptors. The results presented here suggest that the interaction of human α-defensin-1 with CDTb did not lead to a direct block of the CDTb-channels as it was observed for other typical pore blockers like chloroquine and its related compounds or derivatives. In contrast, the data suggest that α-defensin-1 either inhibits oligomer formation of CDTb or blocks the reconstitution of CDTb into lipid bilayer membranes. Furthermore, these results could be confirmed in living Caco-2-cells using the calcium imaging method.

Notably, the inhibitory capacity of α-defensin-1 was confirmed by a human intestinal organoid system. Human miniguts are derived from plucked hair form a healthy donor and contain crucial characteristics of human guts. For the transmembrane adhesion protein E-Cadherin as well as for F-actin, clear cytotoxic effects caused by bacterial toxins were reported. We observed that α-defensin-1 was able to protect the organoids from intoxication with the medically relevant combination of TcdA, TcdB, and CDT and prevented the tissue damage caused by the bacterial toxins. Former studies reported on cytotoxic side effects of α-defensin-1 starting at concentrations of 14 µM. However, the concentrations used here are well below the concentrations reported by Nishimura and coworkers and both defensins (α- and β-defensin-1) did not show any adverse effects on cells. In addition, α-defensin-1 does not cause any visible cytotoxic effects in the human minigut model system. Moreover, as proof of concept, the inhibitory capacity of α-defensin-1 was investigated in an ileal loop assay in the presence of TcdA. We could show that mice treated with α-defensin-1 were protected from the cytotoxic effects of TcdA.

In conclusion, our results suggest that α-defensin-1 might serve as an attractive pharmacological compound to prevent the clinical symptoms caused by the C. difficile toxins TcdA, TcdB, and CDT and, therefore, to treat patients suffering from CDAD. This therapeutic strategy would benefit from the native role of α-defensin-1 as a defense mechanism battling antibiotic-resistant bacteria as well as their potent protein toxins.

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AUTHOR CONTRIBUTIONS
S. Fischer, P. Papatheodorou and H. Barth designed the study, interpreted the results, and wrote the manuscript. S. Fischer, A.-K. Ückert, A.-K. Mittler, M. Landenberger, C. Hoffmann-Richter, M. Hägele, M. R. Popoff, and R. Benz performed the research. M. Müller and A. Kleger supervised the minigut studies. U. Ziener supervised the AFM measurements. C. Schwan, P. Papatheodorou, and K. Aktories provided toxins. H. Barth supervised the study.

CONFLICT OF INTEREST
The authors declare no conflicts of interest.

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