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ANGIE 5: A NOVEL ANTIMICROBIAL PEPTIDE WITH POTENT INHIBITORY ACTIVITY AGAINST CLOSTRIDIOIDES DIFFICILE TOXINS

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ABSTRACT

Clostridioides difficile infection (CDI) remains a significant global health concern, primarily driven by the action of large clostridial toxins, TcdA and TcdB. These toxins are responsible for the severe gastrointestinal symptoms and systemic complications associated with CDI. Current therapeutic approaches face challenges, including the rise of antibiotic resistance and recurrent infections. This study investigates the potential of Angie 5, a novel antimicrobial peptide (AMP) derived from the angiogenin family, as a therapeutic agent against C. difficile toxins. Our findings demonstrate that Angie 5 effectively inhibits both TcdA- and TcdB-induced cytotoxicity in cellular models and delays intracellular Rac1 glucosylation. Furthermore, computational and biochemical analyses suggest a direct interaction between Angie 5 and the toxins, potentially interfering with their cellular uptake or subsequent intracellular processing, rather than direct in vitro enzymatic inhibition. These results highlight Angie 5 as a promising candidate for the development of novel anti-toxin strategies against CDI, offering a potential alternative or adjunct to conventional antibiotic treatments.

Keywords: Clostridioides difficile, TcdA, TcdB, Antimicrobial Peptide, Angie 5, Toxin Inhibition, Glucosyltransferase, CDI Therapy.

INTRODUCTION

Clostridioides difficile infection (CDI) stands as a formidable global health challenge, consistently ranking among the leading causes of healthcare-associated diarrhea and colitis. This infection imposes a substantial burden on healthcare systems worldwide, both in terms of patient morbidity and mortality, as well as significant economic costs [31, 42]. The clinical spectrum of CDI ranges from mild to severe diarrhea, with critical cases escalating to life-threatening conditions such as pseudomembranous colitis, colonic perforation, and toxic megacolon [31]. The primary drivers of CDI pathogenesis are two large, potent AB-type protein toxins secreted by C. difficile: toxin A (TcdA) and toxin B (TcdB) [3, 28, 53].

TcdA (approximately 310 kDa) and TcdB (approximately 270 kDa) are classified as clostridial glucosylating toxins (CGTs) and are the main virulence factors responsible for the myriad symptoms associated with CDI [3, 26, 40, 53]. These toxins are sophisticated, single-chain proteins comprising multiple functional domains essential for their uptake and action within host cells. According to the ABCD model, they possess at least four distinct domains:

- A-activity: The enzymatically active N-terminal glucosyltransferase domain (GTD).
- C-cleavage: The cysteine protease domain (CPD).
- D-delivery: The delivery domain.
- B-binding: The combined repetitive oligopeptides (CROPs) domain at the C-terminus [26, 40].

The intricate mechanism of toxin action begins with their entry into host cells. A two-receptor model has been postulated for TcdA and TcdB, where both toxins bind to specific receptors on the cell surface via their CROPs domain or a preceding second receptor-binding domain [17, 45]. Following receptor binding, the toxins undergo endocytosis. TcdB primarily utilizes clathrin-mediated endocytosis, while TcdA has been shown to undergo clathrin-independent, PACSIN2-dependent endocytosis [11, 18, 41]. Once internalized within endosomes, acidification of these vesicles, facilitated by vesicular adenosine triphosphatases (V-ATPases), triggers a critical conformational change in the toxins. This change leads to membrane insertion and pore formation in the endosomal membrane [4, 39]. Through these pores, the GTD and CPD translocate into the host cell cytosol [26]. In the cytosol, the molecule inositol hexakisphosphate (InsP6) binds to and activates the CPD, leading to autocatalytic cleavage and the release of the GTD [13, 19]. The released GTD then acts as a glucosyltransferase, covalently attaching a glucose moiety from UDP-glucose to small GTPases of the

Rho and/or Ras family, including Cdc42, RhoA, and Rac1 [2, 30]. This mono-O-glucosylation inactivates these GTPases, which are crucial regulators of the actin cytoskeleton [2, 21, 30]. The inactivation of Rho GTPases results in the collapse of the actin cytoskeleton, leading to characteristic cytopathic cell rounding, disruption of the intestinal barrier, intestinal damage, and ultimately, cell death, contributing to the severe clinical symptoms of CDI [2, 21, 30]. Beyond TcdA and TcdB, hypervirulent C. difficile strains may also produce a third toxin, the binary Clostridium difficile transferase toxin (CDT), which possesses ADP-ribosyltransferase activity [16].

Despite ongoing advancements, the management of CDI remains a significant challenge. Current guideline-recommended treatment options primarily involve antibiotics such as oral vancomycin or fidaxomicin as first-line drugs [42]. However, these antibiotic therapies often disrupt the delicate balance of the gut microbiota, which can predispose patients to recurrent CDI episodes and contribute to the alarming rise of antibiotic-resistant C. difficile strains [35, 42]. The increasing incidence of severe, refractory, and recurrent CDI underscores an urgent and critical need for novel therapeutic strategies. These new approaches should ideally target the toxins directly, thereby mitigating toxin-mediated damage without indiscriminately eradicating the beneficial gut flora, which is a major drawback of conventional antibiotic treatments [42, 43].

Antimicrobial peptides (AMPs) represent a diverse and ancient class of host defense molecules, forming an integral part of the innate immune system across various life forms [35]. These peptides typically exhibit broad-spectrum antimicrobial activity, often by disrupting bacterial membranes, but many also possess immunomodulatory or anti-toxin properties [35, 43]. Their distinct mechanisms of action, which often differ from those of conventional antibiotics, make them highly attractive candidates for the development of new anti-infective agents, potentially offering a means to circumvent the escalating issue of antibiotic resistance [43, 58].

Angiogenins, a family of ribonucleases, are well-known for their roles in angiogenesis, the formation of new blood vessels. However, recent research has unveiled their substantial and multifaceted antimicrobial functions [25]. For instance, human angiogenin 4 has been demonstrated to exert direct antimicrobial activity against a variety of pathogens, including bacteria and fungi, and contributes to the modulation of the gut microbiota [25, 38, 48]. Beyond angiogenins, fragments derived from other endogenous proteins, such as hemoglobin and β-2-microglobulin, have also been shown to possess antimicrobial properties, with some exhibiting pH-dependent activity [15, 20, 24]. This accumulating evidence strongly suggests that endogenous peptides, beyond their primary physiological functions, can serve as crucial host defense molecules, offering a rich source for identifying novel therapeutic agents.

Given the pivotal role of TcdA and TcdB in CDI pathogenesis and the burgeoning therapeutic potential of AMPs, this study was designed to investigate whether Angie 5, a novel antimicrobial peptide derived from the human endogenous protein angiogenin, could inhibit the activity of C. difficile toxins. We hypothesized that Angie 5 might interfere with toxin function, either by directly binding to the toxins, disrupting their cellular uptake, or interfering with their subsequent intracellular processing, thereby mitigating their cytotoxic effects. This research aims to pave the way for the development of antimicrobial peptide-based anti-toxin strategies to address C. difficile-associated diseases (CDADs).

MATERIALS AND METHODS

Peptide Synthesis and Characterization

Angie 5, along with Angie 1, 3, 6, and 7, were obtained from PSL Heidelberg (PSL, Heidelberg, Germany), synthesized using standard Fmoc solid-phase peptide synthesis (SPPS) chemistry. The reference Angie peptide, corresponding to the naturally occurring 17-amino acid sequence of angiogenin (positions 64-80), was synthesized in-house at the Core Facility Functional Peptidomics (CFP, Ulm, Germany), following methods previously described by Harms et al. [22].

Briefly, peptide synthesis involved the use of a Liberty Blue microwave synthesizer (CEM Corporation, Matthews, NC, USA). Following synthesis, the peptides underwent purification via reversed-phase high-performance liquid chromatography (HPLC) using a Waters system (Waters, Milford, MA, USA). A Phenomenex C18 Luna column (particle size 5 µm, pore size 100 Å) was employed with an acetonitrile/water gradient under acidic conditions to achieve a purity greater than 95%. The purified peptides were then lyophilized using a freeze-dryer (Labconco, Kansas City, MI, USA). The identity and molecular mass of each peptide were verified by liquid chromatography-mass spectrometry (LC-MS) using a Waters system. For experimental use, the peptides were dissolved in sterile deionized water to prepare stock solutions, which were stored at -20°C. The terminal groups of the Angie peptides were NH2– at the N-terminus (lysine, K) and -COOH at the C-terminus (isoleucine, I), indicating no additional modifications.

Toxin Preparation

The native Clostridioides difficile toxins TcdA and TcdB, derived from C. difficile VPI 10,463, were generously provided by Klaus Aktories (University of Freiburg, Germany). These toxins were purified according to established protocols [29] to ensure homogeneity. Toxin concentrations were determined spectrophotometrically. The toxins were stored under conditions recommended by the supplier to maintain their activity and stability.

Cell Culture

For cytotoxicity and cellular activity assays, three distinct cell lines were utilized:

- Vero cells: African green monkey kidney epithelial cells (DSMZ, Braunschweig, Germany).
- HeLa cells: Human cervical carcinoma cells (DSMZ, Braunschweig, Germany).
- CaCo-2 cells: Human epithelial colorectal adenocarcinoma cells (ATCC HTB-37, Manassas, VA, USA).

All cell culture materials were purchased from Gibco (Thermo Fisher Scientific, Waltham, MA, USA) unless otherwise specified. Cells were maintained under humidified conditions at 37°C in a 5% CO$_2$ atmosphere.

Vero and HeLa cells were cultivated in Minimum Essential Medium (MEM), supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin. CaCo-2 cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% FCS, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were routinely trypsinized and reseeded every two to three days, maintaining a maximum passage number of 25 to ensure consistent cell behavior. Prior to intoxication experiments, cells were seeded in appropriate culture dishes one or two days in advance and treated in FCS-free media with toxins and the respective compounds to avoid interference from serum components. Regular checks for mycoplasma contamination were performed to ensure the integrity of cell cultures.

Cytopathic Cell Rounding Assay

The inhibitory effect of Angie 5 on TcdA- and TcdB-induced cytotoxicity was primarily assessed using a morphological cell rounding assay. This assay leverages the characteristic cellular morphology changes (cell rounding) induced by C. difficile toxins as a reliable readout.

Cells (Vero, HeLa, or CaCo-2, as specified for each experiment) were seeded in 96-well plates one or two days prior to the assay to allow for proper adherence and monolayer formation. On the day of the experiment, cells were pre-incubated with various concentrations of Angie peptides (typically 100 µM, with concentration series down to 1 µM for Angie 5) or an equivalent volume of sterile water (solvent control) in FCS-free medium for 30 minutes. Following this pre-incubation, a fixed concentration of TcdA or TcdB (e.g., 10 pM for TcdB, 180 pM for TcdA, or a combination thereof, depending on the toxin's potency). The treated cells were then incubated under humidified conditions at 37°C with 5% CO$_2$. Cell morphology was monitored hourly for at least 6 to 7 hours using an inverted light microscope (Leica DMil microscope connected to a Leica MC170 HD camera, both Leica Microsystems GmbH, Wetzlar, Germany). For quantitative analysis, rounded and non-rounded cells were counted from at least 200 cells per well in three random fields using the online software Neuralab (https://neuralab.de). The percentage of rounded cells from the total cell count was calculated for each time point and concentration.

Toxin Glucosylation Status of Intracellular Rac1

To analyze the effect of Angie peptides on the glucosylation status of intracellular Rac1, a key substrate of TcdB, Vero cells were seeded in 24-well plates one day before treatment. Cells were treated with varying concentrations of Angie peptides or solvent control and TcdB (typically 50 pM) in FCS-free medium. Incubation times varied, ranging from 2 hours for standard assays to increasing intervals (30 min, 60 min, 90 min, 120 min, 150 min, 180 min) for time-course experiments.

At the end of the intoxication period, whole-cell lysates were prepared by harvesting cells directly in 2.5x Laemmli buffer (0.3 M Tris-HCl, 10% SDS, 37.5% glycerol, 0.4 mM bromophenol blue, 100 mM DTT). Samples were then heat-denatured at 95°C for 10 minutes prior to SDS-PAGE and immunoblotting.

Gel Electrophoresis and Immunoblotting

Protein separation was performed using SDS-PAGE with either 8% or 12.5% acrylamide gels, chosen based on the molecular weight of the target protein. Following electrophoresis, proteins were transferred from the gels onto nitrocellulose membranes via semi-dry Western blotting. Transfer efficiency was monitored by staining the membranes with Ponceau-S (AppliChem GmbH, Darmstadt, Germany).

Membranes were then blocked at room temperature for at least 30 minutes using 5% skim milk powder in PBS-T (PBS containing 0.1% Tween 20). After blocking, membranes were subjected to several washing steps in PBS-T before incubation with primary antibodies.

Primary antibodies used included:

- Mouse anti-Racl antibody (1:1000, #610651; clone 102; BD Biosciences, Heidelberg, Germany) for detection of non-glucosylated Rac1.
- Mouse anti-Hsp90 antibody (1:1000, Santa Cruz Biotechnology, Heidelberg, Germany) as a loading control.
- Mouse anti-Racl antibody (Clone 23A8, Sigma-Aldrich Chemie GmbH, Germany) for detection of total Rac1.

Following primary antibody incubation and washing steps, membranes were incubated with horseradish peroxidase (HRP)-coupled secondary antibodies:

- Goat anti-mouse IgG (H+L) secondary antibody (1:5000, #31430; Thermo Fisher Scientific, Waltham, USA).

- Mouse IgG kappa-binding protein (m-IgGK BP-HRP; 1:5000, Santa Cruz Biotechnology, Dallas, USA; sc-516,102).

Signals were detected using Pierce ECL Western blotting substrate (Thermo Fisher Scientific, Waltham, MA, USA) and visualized with the iBright 1500 system (Thermo Fisher Scientific). Signal quantification was performed using ImageJ software v.1.52.a (NIH). Signal intensity for non-glucosylated Rac1 was normalized to the loading control Hsp90 or Ponceau-S staining, and relative values were calculated against appropriate controls (e.g., TcdB 0 min or non-treated control).

Cellular Binding Assay

To evaluate the effect of Angie peptides on TcdB binding to target cells, an immunoblot-based cellular binding assay was performed. Vero cells were seeded in 24-well plates one day prior to the experiment. On the day of the assay, cells were pre-cooled on ice for 30 minutes to inhibit endocytosis. Subsequently, Angie peptides (100 µM) or water (solvent control), along with TcdB (500 pM), were added to the pre-cooled cells and incubated for 1 hour on ice. This temperature allows toxin binding to cell surface receptors but prevents internalization.

After incubation, cells were harvested, and whole-cell lysates were prepared in 2.5x Laemmli buffer and heat-denatured at 95°C for 10 minutes. Samples were then subjected to SDS-PAGE and immunoblotting. TcdB was detected using an anti-TcdB antibody (1:1000, Anti-Clostridium difficile Toxin B antibody, Abcam, Cambridge, UK). Hsp90 served as a loading control. Signal quantification was performed using ImageJ software v.1.52.a (NIH), with TcdB signals normalized to Hsp90 or Ponceau-S staining and expressed relative to the TcdB-treated control.

In Vitro Precipitation Assay

To determine if Angie peptides form precipitates with TcdB, potentially entrapping the toxin and preventing its cellular entry, an in vitro precipitation assay was conducted. TcdB (50 ng, equivalent to 2 ng/µL) was incubated with Angie peptides (100 µM) or water (solvent control) in a total volume of 25 µL PBS for 30 minutes at 37°C. As a positive control for precipitation, α-defensin-6 (6 µM), previously shown to precipitate TcdB, was included.

Following incubation, samples were centrifuged at 14,000 rpm for 20 minutes at 4°C to separate the supernatant (S) and pellet (P) fractions. The supernatant was carefully transferred to a new tube, and the pellet was resuspended in an equal volume of PBS. Both fractions were then prepared for SDS-PAGE and immunoblotting. TcdB was detected using an anti-TcdB antibody (1:1000, Abcam, Cambridge, UK). Signal intensity for TcdB in the supernatant and pellet fractions was quantified using ImageJ software and normalized to the supernatant fraction of the TcdB-alone control.

In Vitro Glucosylation Status of Rac1 from Whole-Cell Lysates

To assess the direct effect of Angie peptides on the intrinsic glucosyltransferase activity of TcdB in vitro, an enzyme activity assay was performed using CaCo-2 cell lysate as a source of Rac1 substrate.

CaCo-2 cells were seeded in 10 cm culture dishes and grown for two to three days. Cells were then washed and frozen before lysis. Cell lysates were prepared in either glucosylation buffer (50 mM HEPES, 100 mM KCl, 2 mM MgCl2, 1 mM MnCl2, 100 mg/L BSA, pH 7.5) or glucosylation buffer without BSA (50 mM HEPES, 100 mM KCl, 2 mM MgCl2, 1 mM MnCl2, pH 7.5). Lysates were centrifuged at 10,000 x g for 1 minute, and the supernatant was collected. Protein concentration was determined using a Nanodrop spectrophotometer.

Angie peptides (100 µM) or water (solvent control) and TcdB (10 nM) were mixed in the respective glucosylation buffer and directly added to 40 µg of CaCo-2 cell lysate. Samples were incubated for 2 hours at 37°C. Following incubation, samples were subjected to SDS-PAGE and immunoblotting. Non-glucosylated Rac1, total Rac1, and Hsp90 (as loading control) were detected as described previously. Signal quantification was performed using ImageJ software, with non-glucosylated Rac1 signals normalized to Hsp90 or Ponceau-S staining.

Actin-Staining and Fluorescence Microscopy

To visualize the integrity of the actin cytoskeleton, a key target of C. difficile toxins, fluorescence microscopy was performed. Vero cells were seeded and grown for one day in 18-well µ-slides (ibidi GmbH, Gräfelfing, Germany). Cells were treated with Angie peptides (100 µM) or water (solvent control) and intoxicated with TcdB (50 pM) in FCS-free medium for 2 hours at 37°C.

After treatment, cells were washed with PBS, fixed with 4% paraformaldehyde for 20 minutes, and permeabilized using 0.4% (v/v) Triton X-100 in PBS for 5 minutes (if required). Quenching was performed for 2 minutes in 100 mM glycine in PBS. This was followed by a blocking step for 1 hour at 37°C in PBS-T (PBS containing 0.1% Tween 20) containing 10% normal goat serum (Jackson ImmunoResearch, West Grove, PA, USA) and 1% BSA.

For visualization of the cytoskeleton, F-actin was stained for 1 hour at 37°C using the membrane-permeant SiR-actin (SiR-actin kit, Spirochrome, Stein am Rhein, Switzerland). Finally, cell nuclei were stained for 5 minutes using Hoechst 33,342 (1:10,000, Thermo Fisher Scientific, Waltham, MA, USA). After completing the staining procedure, the slides were examined via fluorescence microscopy using a BZ-X810 Keyence fluorescence microscope equipped with a Plan Apochromat 40X objective and BZ-X filters (Keyence Deutschland GmbH, Neu-Isenburg, Germany). Image acquisition and analysis were performed using BZ-X800Viewer v1.3.0.

In Silico Prediction of the Complex TcdB-Angie 5

The structural prediction of the TcdB-Angie 5 complex was performed using a multi-tool approach to enhance prediction accuracy and explore the conformational space. Three web-based protein-peptide docking tools were employed:

- AlphaFold3: (https://alphafoldserver.com/) [1]
- HPEPDOCK: (http://huanglab.phys.hust.edu.cn/hpepdock/) [57]
- PEP-SiteFinder: (https://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-SiteFinder/) [44]

In all simulations, the TcdB protein was designated as the receptor, and the Angie 5 peptide served as the ligand. The amino acid sequence and three-dimensional (3D) structure of TcdB were retrieved from the Protein Data Bank [8] under the accession code 6OQ5 [12].

For AlphaFold3, the receptor and peptide sequences were provided as input, whereas for HPEPDOCK and PEP-SiteFinder the 3D structure of TcdB and the peptide sequence were used. All docking simulations were performed in a blind manner using the default parameters of each server. Since AlphaFold3 generates only five docking models per run, the docking procedure was repeated three times with this tool. The resulting models from each server were clustered based on a root-mean-square deviation (RMSD) threshold of 20 Å, yielding the ten most representative clusters per server. The best structure from each cluster was selected based on the scoring function of the respective server.

To facilitate comparison and identify the most stable complex, the binding energy of the selected structures was re-evaluated using the Prodigy server (https://rascar.science.uu.nl/prodigy/) [56]. The TcdB-Angie 5 complex with the lowest predicted binding energy was considered the most probable conformation. Interaction analysis of the selected complex and the identification of key binding residues (hot spots) in the peptide were performed using PPCheck (https://caps.ncbs.res.in/ppcheck/) [47].

To validate these findings, in silico mutagenesis was conducted by manually modifying the Angie 5 peptide within the same structural framework, transforming it into the other peptides included in this study. The binding energy of these modified complexes was then recalculated using the same scoring approach.

In Vitro Autoprocessing Assay of TcdB

To investigate the effects of the Angie peptides on the intrinsic cysteine protease activity of TcdB, an in vitro autoprocessing assay of TcdB was conducted. TcdB with or without the different Angie peptides was incubated for 1 hour at 37°C in a 20 mM Tris-HCl buffer containing 150 mM NaCl at pH 7.4. The autoprocessing activity was induced by the addition of 1 mM inositol hexakisphosphate (InsP6) (Santa Cruz Biotechnology). To inhibit the autoprocessing of TcdB, a positive control containing 1 mM N-ethylmaleimide (NEM) (Sigma Aldrich by Merck) was added. The reaction was stopped by the addition of Laemmli buffer. The samples were incubated for 10 minutes at 95°C and subjected to SDS-PAGE and immunoblotting, while TcdB was detected, using an anti-TcdB antibody (1:1000, Anti-Clostridium difficile Toxin B antibody, Abcam, Cambridge, UK).

Determination of the Stability of Angie 5 in Human Plasma

For the application and development of Angie 5 as a therapeutic agent for the treatment of CDIs in the future, the stability of the peptide is of importance and major concern. For the determination of the stability of Angie 5, its half-life was determined in human plasma using MALDI-TOF, according to a work by Freisem et al. [15], with minor modifications. A sample (0.5 mL) of human plasma was spiked with 20 µM Angie 5 and incubated at 37°C. Aliquots (50 µL) were separated at 0, 15, 30, 60, and 120 minutes, respectively, and mixed with 250 µL 0.1% TFA in acetonitrile at -20°C. The mixture was centrifuged at 13,000 rpm for 30 seconds, and 150 µL of the supernatant was mixed with 150 µL 20% acetic acid in ice. The samples were analyzed with an Axima Confidence MALDI-TOF MS (Shimadzu) in linear mode using exactly the same measurement conditions for all samples spotted on a 384-well plate. Wells were coated with 0.5 µL of 10 mg/mL CHCA previously dissolved in TFA/water/acetonitrile/2-propanol (2.5/47.5/25/25, v/v), and the solvent was allowed to evaporate. Then, each sample (0.5 µL), previously mixed with matrix (0.5 µL), was applied onto the dry pre-coated well, and the solvent was allowed to evaporate. Laser shots were automatically done following a regular circular raster of a diameter of 2000 µm and spacing of 200 µm on each well; 100 profiles were acquired per sample, and 20 shots were accumulated per profile. An accelerating voltage of 20 kV was applied to the ion source. Measurements of each sample were done in triplicate. The measurement and MS data processing (peak area calculation) were controlled with MALDI-MS Application Shimadzu Biotech Launchpad 2.9.8.1 (Shimadzu). The half-life was calculated using GraphPad Prism version 10.3.1 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com. Data (signal area vs. time) was fitted to a one-phase decay curve.

Bacterial Culturing

All bacterial strains used for the susceptibility testing are listed in Supplementary Table 1. The C. difficile strain used is the strain VPI 11186 and PCR negative for cdtB, tcdA, and tcdB genes. All bacteria were cultivated on Tryptone Soya Agar with Sheep Blood (Thermo Scientific, Waltham, MA, USA) at 37°C and 5% CO$_2$. C. difficile was cultivated under anaerobic conditions, created by a GENbag anaer bag (bioMérieux, Marcy-l'Étoile, France). Liquid cultivation of C. difficile was performed in Brain-Heart infusion medium (Oxoid, Dardilly, France) supplemented with 0.5% yeast (Gibco) and 0.4 g/L L-cysteine (Fluka-

Honeywell Research Chemicals, Morris Plains, NJ, USA) (BHI). After inoculation, the liquid culture was overlayed with sterile liquid Vaseline (VWR, Radnor, PA, USA) to achieve anaerobic conditions. For liquid cultivation of P. aeruginosa, A. baumannii, and E. coli, bacteria were inoculated in lysogeny broth (LB-Miller) and incubated at 37°C with shaking at 160 rpm. E. faecium, S. aureus, and K. pneumoniae were grown in Todd-Hewitt Broth (Oxoid, Dardilly, France) supplemented with 5% yeast at 37°C and 5% CO$_2$.

Radial Diffusion Assay

To investigate antimicrobial activity of the Angie peptides, an overlay-assay was performed, as previously described [52]. In short, bacteria were inoculated in liquid agarose with a density of 2×107 cells per plate. Wells were put into the solidified 1% agarose and filled with the different Angie peptides (100 µM). After 3 hours of incubation at 37°C, an overlay with nutrient agar was performed. Plates were incubated at 37°C and 5% CO$_2$ and inhibition zones were measured after overnight incubation. For C. difficile, some modifications were made to account for the anaerobic conditions. In detail, 1 mL of a C. difficile overnight culture was directly added to the agarose, mixed and a plate was poured. After drying for 5 minutes at 4°C, wells were put in the agarose and filled with 10 µL of the Angie peptides 1, 3, and 5 in concentrations ranging from 100 µM to 1 mM or the various Angie peptides (5 mM and 100 µM for the reference Angie). The plate was incubated at 37°C in a GENbag anaer bag for 3 hours, then an overlay with 10 mL BHI-Agar was conducted. After overnight incubation, inhibition zones were measured. As a positive control, LL-37 (Anaspec, Fremont, CA, USA) was used at a concentration of 1 mg/mL for C. difficile and 100 µg/mL for all other bacteria.

Transmission Electron Microscopy of C. difficile

To investigate effects of Angie 5 on C. difficile, transmission electron microscopy was performed, as previously described [20]. Shortly, C. difficile was grown for 3 hours and cells were harvested by centrifugation (2 min, 8800 xg). The pellet was reconstituted in 10 mM phosphate solution and either Angie 5 or water was added. For the incubation period (37°C, 1 h), an overlay with sterile liquid Vaseline was performed to ensure anaerobic conditions. The samples were subsequently fixed using 3.5% glutaraldehyde, 1% saccharose in phosphate buffer. Bacterial cells were postfixed in osmium tetroxide and dehydrated in a graded series of propanol. Finally, cells were stained with uranyl acetate, embedded in Epon and ultrathin sections were prepared using standard procedures. A Jeol 1400 Transmission Electron Microscope was used to analyze the samples and at least 25 pictures per sample were taken. The experiment was conducted once.

Reproducibility of Experiments and Statistics

All performed experiments were conducted independently from each other at least three times. The number of replicates (n) for experiments or tested conditions is given in the figure legends, while representative results are shown in the figures. If not stated otherwise in the figure legends, the statistical analysis performed was a one-way ANOVA in combination with Dunnett's multiple comparison test using GraphPad Prism Version 9 (GraphPad Software Inc., San Diego, CA, USA). The obtained p values are depicted as follows: ns= not significant $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

RESULTS

Angie 5 Possesses Antimicrobial Activity Against C. difficile

A radial diffusion assay was performed to assess the antimicrobial activity of the Angie peptides against C. difficile. Initial testing at higher concentrations revealed distinct inhibition zones around certain Angie peptides and the positive control, LL-37, which is a known antimicrobial peptide with activity against C. difficile [36]. As depicted in Supplementary Fig. 1b, Angie 5 consistently produced the largest inhibition zone, followed by Angie 3 and Angie 1. In contrast, Angie 6, Angie 7, and the reference Angie peptide showed minimal or no inhibition of C. difficile growth, evidenced by the absence or very small size of their inhibition zones. These initial findings indicated that Angie 1, 3, and 5 possessed antimicrobial activity, while the others did not.

To further characterize the active peptides, a concentration series including lower concentrations of Angie 1, 3, and 5 was tested using the radial diffusion assay (Fig. 2a, b). All three peptides demonstrated a comparable and dose-dependent inhibition of C. difficile growth. Notably, at the lowest tested concentration of 100 µM, Angie 1 exhibited the largest inhibition zone among the active peptides.

To gain insight into the mechanism of C. difficile inhibition by Angie 5, transmission electron microscopy (TEM) was employed (Fig. 2c, d). TEM images of C. difficile treated with Angie 5 revealed significant cellular damage, characterized by disrupted cell membranes and evident cytoplasmic leakage. This contrasted sharply with the control sample, where C. difficile cells treated only with solvent (water) predominantly maintained intact cellular membranes and appeared viable. Although some damaged bacterial cells were observed in the control due to sample preparation under non-ideal anaerobic conditions, the overwhelming evidence pointed to membrane disruption as a primary mode of action for Angie 5. This mechanism is consistent with the known actions of many cationic antimicrobial peptides, which often target and destabilize bacterial cell membranes [24, 35, 58].

Furthermore, to evaluate the broader antimicrobial spectrum of the Angie peptides, we tested their activity against a panel of ESKAPE pathogens (Pseudomonas aeruginosa, Acinetobacter baumannii, Escherichia coli,

Enterococcus faecium, Staphylococcus aureus, and Klebsiella pneumoniae) using the radial diffusion assay (Supplementary Table 1, Supplementary Fig. 2). Similar to the C. difficile results, LL-37 served as a positive control. Among the tested ESKAPE pathogens, only P. aeruginosa and A. baumannii were inhibited by the Angie peptides. P. aeruginosa showed comparable inhibition by Angie 1, 3, and 5. Interestingly, A. baumannii was inhibited exclusively by Angie 5, highlighting its unique potency. Angie 6, 7, and the reference Angie again showed no inhibitory effect on any of the ESKAPE pathogens. These findings suggest that the antimicrobial activity of Angie peptides, particularly Angie 5, extends beyond C. difficile to other clinically relevant bacterial species.

Angie 5 Protects HeLa, Vero, and CaCo-2 Cells from Intoxication with TcdB

To investigate the antitoxin activity of the Angie peptides against TcdB, the historical C. difficile strain VPI 10,463 was utilized. TcdB's glucosyltransferase domain (GTD) modifies small GTPases of the Rho and Ras-family (e.g., Cdc42, RhoA, Rac1) within host cells, leading to the collapse of the cytoskeleton and characteristic cell rounding. This cytopathic effect provides a reliable and quantifiable readout for assessing the protective effects of potential inhibitors.

HeLa, Vero, and CaCo-2 cell monolayers were simultaneously treated with various Angie peptides and TcdB in serum-free medium to exclude interference from serum components. Cell morphology was monitored hourly for 7 hours using light microscopy. The percentage of rounded cells from the total cell count was quantified for each time point.

The results demonstrated that Angie 5 provided robust protection against TcdB-induced cell rounding across all three cell lines. In HeLa cells (Fig. 3b, c) and CaCo-2 cells (Fig. 3f, g), only Angie 5 significantly inhibited TcdB-mediated cell rounding. In contrast, Vero cells (Fig. 3d, e) showed additional protection by Angie 1 and Angie 3, albeit to a lesser extent than Angie 5. Angie 6, Angie 7, and the reference Angie peptide did not inhibit TcdB-mediated cell rounding in any of the tested cell lines (Supplementary Fig. 3, Supplementary Fig. 4). These findings highlight Angie 5 as the most potent inhibitor among the tested Angie peptides, with some cell line-dependent variations in the efficacy of other peptides.

Given the robust inhibition observed with Angie 5, a more detailed dose-response analysis was performed in Vero cells (Fig. 4). In addition to 100 μM Angie 5, concentrations of 50 μM and 40 μM Angie 5 also significantly inhibited TcdB-mediated cell rounding. However, a concentration of 30 μM Angie 5 showed no significant protective effect. For the 3-hour time point after toxin addition, the half-maximal inhibitory concentration (IC50) for Angie 5 was calculated to be 40.94±1.08μM (mean ± SEM) (Fig. 4d-e).

To investigate whether pre-incubation of TcdB with Angie 5 could enhance its inhibitory effect, a separate experiment was conducted where TcdB and Angie 5 were pre-incubated for 15 minutes before addition to Vero cells (Supplementary Fig. 5). The IC50 value for Angie 5 after 3 hours of TcdB intoxication in this pre-incubation setup was 46.34±1.14μM (mean ± SEM) (Supplementary Fig. 5d-e). A comparison of normalized rounded cell percentages after 3 hours of intoxication revealed no significant difference in inhibition between simultaneous addition and pre-incubation for most tested concentrations, except for 10 μM Angie 5 (Supplementary Fig. 6). This suggests that pre-incubation does not substantially enhance the inhibitory effect of Angie 5 on TcdB-mediated cell rounding.

Angie 5 Protects Cells from Intoxication of Vero Cells with TcdA and the Medically Relevant Combination of TcdA and TcdB

Beyond TcdB, we also investigated the protective capabilities of Angie peptides against TcdA, another clinically relevant toxin produced by C. difficile. Vero cells were treated with Angie peptides and TcdA, and TcdA-induced cell rounding was assessed microscopically. As shown in Fig. 5, Angie 5 demonstrated the strongest inhibition of TcdA-induced cell rounding, followed by Angie 1 and Angie 3. Representative cell images are provided in Supplementary Fig. 7.

Furthermore, the inhibitory effect of Angie peptides on the medically relevant combination of TcdA and TcdB was examined in Vero cells (Fig. 5, Supplementary Fig. 7). The combination of TcdA and TcdB also showed significant inhibition by Angie 5, and to a lesser extent, by Angie 1 and Angie 3. Supplementary Fig. 7 displays representative images of Vero cells treated with Angie peptides and either TcdA alone or the TcdA/TcdB combination after 7 hours. Given that TcdB is generally considered more cytotoxic than TcdA and is regarded as the major virulence factor of C. difficile [9, 28], subsequent mechanistic experiments focused primarily on TcdB.

Angie 5 Delays TcdB-Mediated Glucosylation of Rac1 in Vero Cells in a Time- and Concentration-Dependent Manner

To further understand how Angie peptides inhibit toxin activity, we analyzed their effect on the glucosylation of intracellular Rac1, a key substrate of TcdB, upon toxin uptake into Vero cells. This was assessed by immunoblotting for unmodified, non-glucosylated Rac1, as well as total Rac1 and Hsp90 as loading controls (Fig. 6).

Whole-cell lysates from non-treated control cells exhibited a strong signal for non-glucosylated Rac1, as expected. However, in cells treated with TcdB for 2 hours, the signal for non-glucosylated Rac1 was very weak or almost absent, indicating extensive glucosylation and inactivation of intracellular Rac1 by the toxin. As a control, cell lysates generated immediately after TcdB addition (time point 0 min) showed no decrease in the Rac1 signal, confirming

that residual TcdB during in vitro sample preparation did not cause Rac1 glucosylation.

For Angie 1, 3, 6, 7, and the reference Angie, no signal for non-glucosylated Rac1 was obtained in the immunoblot, indicating that these peptides did not inhibit TcdB-induced Rac1 glucosylation (Fig. 6b-c, e-f). In stark contrast, Angie 5 showed a clear concentration-dependent increase in the signal for non-glucosylated Rac1 (Fig. 6d-e). This suggests that Angie 5 effectively inhibited TcdB-mediated Rac1 glucosylation.

A time-lapse intoxication experiment was performed to investigate the kinetics of TcdB inhibition by Angie 5 (Fig. 7). Vero cells were treated with 100 μM Angie 5 and TcdB, or with TcdB alone, for increasing time intervals up to 3 hours. The results clearly demonstrated that Angie 5 delayed TcdB-mediated Rac1 glucosylation. While TcdB alone rapidly led to complete Rac1 glucosylation, the presence of Angie 5 significantly prolonged the time before Rac1 was fully glucosylated. This indicates that Angie 5 does not completely prevent glucosylation but rather slows down the process, suggesting an interference with an earlier step in the toxin's intracellular pathway or a reduction in the rate of active toxin delivery to the cytosol.

Angie 5 Inhibits TcdB-Induced Collapse of the Actin Cytoskeleton in Vero Cells

To visually confirm the protective effects of Angie 5 on cellular integrity, we performed fluorescence microscopy to analyze the status of the actin cytoskeleton after TcdB intoxication (Fig. 8). The actin cytoskeleton is a primary target of TcdB, and its disruption leads to the characteristic cell rounding observed in cytotoxicity assays.

In untreated control cells, F-actin filaments appeared organized in a regular, well-structured network. Upon intoxication with TcdB alone, F-actin filaments became condensed and disorganized, consistent with cytoskeletal collapse and cell rounding. When Vero cells were co-treated with Angie 1 or Angie 5 and TcdB, the F-actin structure appeared significantly more comparable to that of untreated control cells, indicating a preserved actin cytoskeleton. In contrast, Angie 3, 6, 7, and the reference peptide did not effectively inhibit TcdB-mediated collapse of the actin cytoskeleton, aligning with their limited protective effects in the cell rounding assays. These visual observations further support the protective role of Angie 5 against TcdB-induced cellular damage.

Angie 5 Does Not Lead to Precipitation of TcdB in vitro

To investigate a potential mechanism of inhibition, we tested whether Angie 5 or other Angie peptides form precipitates with TcdB, which could physically entrap the toxin and prevent its entry into cells. This mechanism has been previously demonstrated for other anti-toxin peptides, such as human α-defensin-6 against TcdB [6].

TcdB was co-incubated with Angie peptides or, as a positive control, α-defensin-6. After incubation, samples were centrifuged to separate the supernatant (S) and pellet (P) fractions, and TcdB was detected by immunoblotting (Fig. 9). In the positive control sample with α-defensin-6, a significant portion of the TcdB signal was found in the pellet fraction, clearly indicating precipitate formation between α-defensin-6 and TcdB. In the negative control (TcdB alone), most of the TcdB signal remained in the supernatant fraction, as expected. Importantly, for all Angie peptides, the strongest TcdB signal was consistently observed in the supernatant fraction, demonstrating that the Angie peptides do not form precipitates with TcdB in vitro. This suggests that their inhibitory mechanism is not based on direct physical sequestration of the toxin in solution.

Angie 5 Does Not Prevent Binding of TcdB to Vero Cells

Since no precipitation of TcdB by Angie peptides was observed, we further investigated whether the inhibitory mechanism might involve interference with the initial step of intoxication: the binding of TcdB to the cell surface. An immunoblot-based binding assay was performed where Vero cells were pre-cooled to 4°C to allow toxin binding to surface receptors while preventing subsequent endocytosis (Fig. 10). Cell surface-bound TcdB was then analyzed by immunoblotting of whole-cell lysates.

Our results showed that all Angie peptides had no negative influence on TcdB binding to Vero cells. In fact, Angie 5 appeared to cause a slight, though not statistically significant, enhancement in toxin binding to cells. This finding indicates that the protective effect of Angie 5 is not mediated by preventing the initial binding of TcdB to its cellular receptors. This suggests that Angie 5 likely interferes with a step subsequent to cell surface binding, such as cellular uptake or intracellular processing of the toxin.

Angie 5 Does Not Inhibit Glucosyltransferase Activity of TcdB in vitro

Next, we investigated whether the glucosyltransferase activity of TcdB itself is directly inhibited by the Angie peptides in vitro. An enzyme activity assay was performed using CaCo-2 cell lysate as a source of the Rac1 substrate (Fig. 11).

As expected, in the absence of TcdB, non-glucosylated Rac1 was readily detected by the specific antibody. However, when TcdB was added to the cell lysate, the glucosylation of Rac1 by TcdB strongly reduced the antibody detection of non-glucosylated Rac1, indicating successful enzymatic modification of the substrate. When the Angie peptides were co-added with TcdB to the cell lysate, the Rac1 antibody signals remained reduced, comparable to the TcdB-alone samples. This crucial finding indicates that the Angie peptides, including Angie 5, do not directly inhibit the glucosyltransferase activity of TcdB in vitro (Fig. 11c-d).

Since the glucosylation buffer used for this assay typically contains BSA, which could potentially bind to Angie peptides and interfere with their activity, we repeated the experiment using glucosylation buffer without BSA. The results were comparable, with no inhibition of TcdB's enzyme activity by the Angie peptides observed (Supplementary Fig. 8). Additionally, the effect of Angie peptides on TcdB-mediated cell rounding in Vero cells was assessed under serum-containing conditions using the cytopathic cell rounding assay (Supplementary Fig. 9). Under these conditions, Angie peptides 1, 3, and 5 still demonstrated inhibition of TcdB-mediated cell rounding. Taken together, these findings confirm that BSA and/or other serum proteins do not appear to sequester Angie peptides or interfere with their anti-toxin effects, and that the protective mechanism of Angie 5 is not due to direct enzymatic inhibition of TcdB's glucosyltransferase activity.

In silico Prediction of the Complex Between TcdB and Angie 5

Despite the rapid advancement of bioinformatics tools for predicting peptide-protein complexes, this task remains highly challenging today [55]. This is especially true for long peptides, as their structural flexibility and complex interactions with proteins make accurate modeling more difficult. Additionally, the use of large proteins as receptors significantly increases the search space for the peptide, further complicating efficient prediction. To address these challenges, we employed three different search algorithms implemented in the servers AlphaFold3 [1], HPEPDOCK [57], and PEP-SiteFinder [44]. This multi-tool approach enabled us to explore the full conformational space of Angie 5 while ensuring a thorough search of its interaction region with the TcdB receptor.

A total of 315 TcdB-Angie 5 complexes were obtained from the simulations: 15 using AlphaFold3, 100 with HPEPDOCK, and 200 with PEP-SiteFinder. These raw predictions were then clustered into 23 representative structures based on a root-mean-square deviation (RMSD) threshold of 20 Å: 3 distinct clusters emerged from AlphaFold3, and 10 from each of the other two methods. These clusters showed a high representativity in the regions encompassing the N-terminal glucosyltransferase domain (GTD) and the cysteine protease domain (CPD), followed by the C-terminal combined repetitive oligopeptides (CROPs). However, no significant number of clusters were found in the central delivery and receptor-binding domain (DRBD), indicating a low probability of interaction by this specific region (Fig. 12).

Each docking software employs its own scoring function to rank predicted binding poses. However, these scoring functions are not directly comparable across different software. Therefore, to select the most stable and probable complex, it was necessary to normalize the binding energy using an additional server that recalculates the binding energy for all structures using a unified scoring function. Accordingly, after selecting the representative structure from each cluster based on the scoring function of its respective server, the binding energy of each structure was recalculated using the Prodigy server [56]. This comprehensive analysis revealed that the most stable TcdB-Angie 5 complex corresponded to cluster 1 from AlphaFold3 (Supplementary Fig. 10).

The optimal binding pose predicted that the Angie 5 peptide is located in a region where all functional domains of TcdB structurally converge, despite being separated in the primary sequence (Fig. 13a). Specifically, the N-terminus of Angie 5 (residues LYS1-GLY9) adopts an extended conformation that deeply buries itself into a hydrophobic pocket. This pocket is formed at the intricate interface between the GTD, CPD, and DRBD domains. Due to the predominantly hydrophobic nature of this N-terminal region of Angie 5, the entire system is significantly stabilized by a high number of hydrophobic interactions (Fig. 13b), while simultaneously remaining shielded from the polar aqueous solvent. Conversely, the C-terminus of Angie 5 (residues ASN10-ILE17) exhibits a more polar character and is strategically positioned at the interface between the solvent and the protein surface. Correspondingly, this region of the peptide adopts an α-helical secondary structure, which enables it to expose its polar residues to the solvent while maintaining crucial non-polar contacts with the protein. In this C-terminal region, the peptide establishes both electrostatic and hydrophobic interactions. Notably, ARG12 plays a particularly important role by forming strong hydrogen bonds and salt bridges, which are highly stabilizing interactions. Furthermore, Angie 5 forms extensive interactions with the three-helical bundle (3-HB, residues 766-841) and the hinge region (residues 1792-1834) of TcdB. The hinge region is known to directly interact with a three-stranded β-sheet in the CPD (referred to as the β-flap, residues 742-765), an interaction that is crucial for CPD activation [12]. These results suggest that Angie 5 may interfere with CPD activation, potentially inhibiting the autocatalytic cleavage of TcdB and thus preventing the release of the active GTD into the cytosol, which would explain the observed delay in intoxication. However, an in vitro autoprocessing assay of TcdB showed that the auto-processing of TcdB was not directly influenced by the Angie peptides (Supplementary Fig. 11), further supporting the hypothesis that Angie 5 acts on a step prior to or during the intracellular processing after endocytosis, but not on the intrinsic enzymatic activity or autoprocessing.

To further validate our in silico findings and correlate them with experimental results, we generated in silico mutants by modifying the Angie 5 peptide within the same structural framework to mimic the sequences of the other Angie peptides included in this study. The binding energy of these modified complexes was then recalculated using the same unified scoring approach (Prodigy). Consistent with the experimental results from the cytotoxicity assays,

Angie 5 and Angie 3 exhibited similar, strong binding energies, while the other peptide variants (Angie 1, 6, 7, and reference Angie) showed lower affinities for the TcdB receptor (Supplementary Table 2, Supplementary Fig. 12). The primary structural difference between Angie 5 and Angie 3 is the presence of isoleucine (ILE) at position 5 in Angie 5, whereas Angie 3 has threonine (THR) in this position (Table 1). This substitution, particularly the presence of the aromatic phenylalanine (Phe) at position 2 in Angie 5 (compared to leucine in Angie 3), appears to play a key role in stabilizing the interaction. Aromatic rings are frequently involved in π-π, cation-π, and CH-π interactions, which are often crucial for protein structure and protein-ligand binding [23, 46]. This fact indicates that the aromatic Phe residue is more relevant than the aliphatic Leu residue in these peptides for optimal binding and inhibitory potential. These findings suggest that the specific amino acid composition, particularly the hydrophobic and aromatic residues at key positions, is critical for the high binding affinity and inhibitory potential observed for Angie 5.

Determination of the Stability of Angie 5 in Human Plasma

For the successful development of Angie 5 as a future therapeutic agent for CDI, its stability within a biological matrix, particularly human plasma, is a critical pharmacokinetic parameter. The half-life of Angie 5 in human plasma was determined using MALDI-TOF mass spectrometry. The calculated half-life (t1/2) of Angie 5 in human plasma was found to be 5.441 minutes (Fig. 14). This relatively short half-life suggests that while Angie 5 is potent in vitro, strategies to enhance its in vivo stability will be crucial for its clinical application.

Discussion

The increasing global burden of Clostridioides difficile infection (CDI), exacerbated by the rise of antimicrobial resistance and high rates of recurrent infections, underscores an urgent need for novel therapeutic strategies [31, 42]. Current antibiotic-centric treatments, while effective in eradicating the bacterium, often disrupt the delicate gut microbiome, creating a vicious cycle of recurrence [35, 42]. This study presents compelling evidence that Angie 5, a novel antimicrobial peptide derived from human angiogenin, acts as a potent inhibitor of C. difficile toxins TcdA and TcdB, offering a promising anti-toxin approach that could complement or even provide an alternative to conventional antibiotic therapies.

Our findings demonstrate that Angie 5 consistently and robustly protects various cell lines, including human colon carcinoma CaCo-2 cells, from TcdA- and TcdB-induced cytotoxicity. This protective effect is characterized by the prevention of cytopathic cell rounding and the maintenance of actin cytoskeleton integrity, which are direct consequences of toxin action. Importantly, our time-course experiments revealed that Angie 5 delays the intracellular glucosylation of Rac1 by TcdB, a critical step in the toxin's pathogenic mechanism. This observed delay, rather than a complete blockade of glucosylation, suggests that Angie 5 interferes with an upstream event in the toxin's cellular pathway, such as its entry into the host cell or its subsequent intracellular trafficking and processing, rather than directly inhibiting the glucosyltransferase enzyme itself. This hypothesis is strongly supported by our in vitro enzyme activity assays, which showed no direct inhibition of TcdB's glucosyltransferase activity by Angie peptides in cell lysates.

The lack of in vitro enzymatic inhibition by Angie 5, coupled with its observed cellular protection, points towards a mechanism that involves interference with the toxin's journey within the host cell. Our cellular binding assays further refined this understanding, demonstrating that Angie 5 does not prevent the initial binding of TcdB to the cell surface receptors. This rules out a simple competitive binding mechanism at the receptor level. Similarly, the in vitro precipitation assays confirmed that Angie peptides do not form insoluble complexes with TcdB in solution, meaning their protective effect is not due to physical sequestration of the toxin before it reaches the cell. Taken together, these results strongly suggest that Angie 5's anti-toxin activity is mediated by interfering with the cellular uptake of TcdB or its subsequent intracellular processing steps, such as endosomal escape or autocatalytic cleavage, which are crucial for the delivery of the active GTD to the cytosol. While our in vitro autoprocessing assay showed no direct influence on TcdB's autocatalytic cleavage, the in silico docking results provide a compelling structural basis for this proposed mechanism.

The multi-tool in silico docking simulations predicted a highly stable interaction between Angie 5 and TcdB, with the peptide binding to a converged region at the interface of the GTD, CPD, and DRBD domains. This region is critical for the toxin's function, as it involves the interplay between the catalytic domain, the cleavage domain, and the delivery/receptor-binding domains. The detailed analysis of the most stable complex revealed that the hydrophobic N-terminus of Angie 5 buries itself into a hydrophobic pocket formed by these domains, establishing numerous stabilizing hydrophobic interactions. Concurrently, the more polar C-terminus of Angie 5 adopts an α-helical structure at the solvent-protein interface, forming electrostatic and hydrophobic interactions, with ARG12 playing a significant role. Crucially, Angie 5 was predicted to interact extensively with the three-helical bundle (3-HB) and the hinge region of TcdB, which are known to be involved in CPD activation and the overall conformational changes required for toxin translocation and activity [12]. While the in vitro autoprocessing assay did not show direct inhibition of cleavage, the in silico data suggest that binding to this critical interface could subtly alter the toxin's conformation, delaying the sequence of events necessary

for efficient delivery of the active GTD into the cytosol. This could explain the observed delay in Rac1 glucosylation rather than complete inhibition.

Our results on the importance of specific amino acid residues for Angie 5's activity are also noteworthy. The comparison of Angie 5 with other Angie peptide variants, both experimentally and in silico, highlighted the critical role of hydrophobic and aromatic residues, particularly phenylalanine (Phe) at position 2 and isoleucine (Ile) at position 5. Peptides with higher Gravy scores (indicating greater hydrophobicity) generally showed stronger inhibitory activity. This aligns with the in silico prediction that the hydrophobic N-terminus of Angie 5 is crucial for its stable interaction within a hydrophobic pocket on TcdB. Aromatic residues, such as Phe, are known to participate in various stabilizing interactions like π-π stacking, which could contribute significantly to the binding affinity and overall stability of the peptide-toxin complex [23, 46]. These insights provide a rational basis for future optimization of Angie 5, potentially by incorporating more aromatic or hydrophobic residues to enhance its potency and specificity against C. difficile toxins.

The anti-toxin properties of Angie 5 are consistent with a growing body of research demonstrating the multifaceted roles of endogenous peptides in host defense. Human α-defensins, for instance, have been shown to directly neutralize C. difficile toxins by binding to them, preventing their activity [6, 14, 19]. Specifically, α-defensin-6 and α-defensin-1 interfere with TcdA and TcdB, suggesting a conserved strategy of the innate immune system against these potent bacterial toxins [6, 14]. The discovery of Angie 5's inhibitory activity further expands this repertoire of natural anti-CDI agents, validating the concept of peptide-based therapeutics for toxin-mediated diseases [35, 43]. The angiogenin family, from which Angie 5 is derived, has already been recognized for its broad antimicrobial properties, including activity against Mycobacterium tuberculosis and Salmonella typhimurium [25, 38, 48, 52].

Beyond its antitoxin activity, our study also confirmed the antimicrobial properties of Angie 5 against a non-toxin producing C. difficile strain, as well as against P. aeruginosa and A. baumannii, two significant ESKAPE pathogens. The transmission electron microscopy images clearly showed that Angie 5 disrupts bacterial cell membranes, leading to cytoplasmic leakage and cell death. This membrane-disrupting mechanism is common among cationic and hydrophobic AMPs [24, 35, 58]. The dual capacity of Angie 5 to both inhibit bacterial toxins and directly kill the bacteria that produce them is a highly attractive feature for a therapeutic agent. Such a dual-action compound could offer a significant advantage in a clinical setting by simultaneously addressing both the infection and its pathogenic consequences, potentially reducing bacterial load and mitigating toxin-mediated damage.

A critical consideration for the clinical translation of any peptide therapeutic is its in vivo stability and pharmacokinetics. Our determination of Angie 5's half-life in human plasma revealed a relatively short duration of 5.441 minutes. While comparable to other natural peptides like Angie 1 [38], this short half-life necessitates further optimization for systemic in vivo application. Strategies to enhance peptide stability are well-established and include chemical modifications such as D-amino acid substitutions, N/C-terminal capping, cyclization, or conjugation to larger macromolecules [10, 22]. Encapsulation in delivery systems, such as liposomes or dendritic mesoporous silica nanoparticles, could also significantly improve its bioavailability and half-life, as demonstrated for other peptides [7, 22, 58]. Future pharmacological studies will focus on these aspects to develop Angie 5 into a clinically viable therapeutic.

While our in vitro and in silico findings are highly encouraging, several limitations and future directions warrant consideration. The current study is primarily based on cellular models and computational predictions. Therefore, in vivo validation in animal models of CDI is an essential next step to confirm the efficacy, safety, and pharmacodynamics of Angie 5 in a complex biological system. Furthermore, high-resolution structural biology techniques, such as cryo-electron microscopy or X-ray crystallography, would be invaluable to precisely map the interaction interface between Angie 5 and the toxins. Such detailed structural information could inform the rational design of more potent and specific peptide inhibitors. Investigating the potential for combination therapy, where Angie 5 is used alongside existing antibiotics or other anti-toxin agents, could also yield synergistic benefits and potentially reduce the emergence of resistance. Finally, assessing Angie 5's protective effect against hypervirulent C. difficile strains (e.g., BI/NAP1/027), which may express slightly modified toxins, would be crucial for its broad clinical applicability.

CONCLUSION

In summary, this study provides robust evidence that the antimicrobial peptide Angie 5 is a potent inhibitor of Clostridioides difficile toxins TcdA and TcdB. Its mechanism of action involves interference with the toxin's intracellular pathway, likely at the stage of cellular uptake or subsequent processing, leading to a delay in Rac1 glucosylation and protection against cytotoxicity. This anti-toxin activity is complemented by its direct antimicrobial properties against C. difficile and other bacterial pathogens. The detailed in silico analysis provides a structural basis for the interaction between Angie 5 and TcdB, highlighting the importance of hydrophobic and aromatic residues for its inhibitory potential. Despite a relatively short half-life in plasma, which can be addressed through future pharmaceutical optimizations, Angie 5 represents a promising candidate for the development of novel anti-toxin strategies. These findings pave the way for further investigation into Angie

5's therapeutic potential, offering a new hope in the fight against this challenging and recurrent infection.

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