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Retrocyclins neutralize bacterial toxins by potentiating their unfolding

Elena Kudryashova[*], **Stephanie Seveau**[†,‡], **Wuyuan Lu**[§], and **Dmitri S. Kudryashov**[*,‖,¶,1]

[*]Department of Chemistry and Biochemistry, The Ohio State University, Columbus, OH 43210, U.S.A.

[†]Department of Microbiology, The Ohio State University, Columbus, OH 43210, U.S.A.

[‡]Department of Microbial Infection and Immunity, The Ohio State University, Columbus, OH 43210, U.S.A.

[§]Institute of Human Virology and Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD 21201, U.S.A.

[‖]Center for Microbial Interface Biology, The Ohio State University, Columbus, OH 43210, U.S.A.

[¶]Public Health Preparedness for Infectious Diseases Program, The Ohio State University, Columbus, OH 43210, U.S.A.

Abstract

Defensins are a class of immune peptides with a broad range of activities against bacterial, fungal and viral pathogens. Besides exerting direct anti-microbial activity via dis-organization of bacterial membranes, defensins are also able to neutralize various unrelated bacterial toxins. Recently, we have demonstrated that in the case of human α- and β-defensins, this later ability is achieved through exploiting toxins' marginal thermodynamic stability, i.e. defensins act as molecular anti-chaperones unfolding toxin molecules and exposing their hydrophobic regions and thus promoting toxin precipitation and inactivation [Kudryashova et al. (2014) Immunity **41**, 709–721]. Retrocyclins (RCs) are humanized synthetic θ-defensin peptides that possess unique cyclic structure, differentiating them from α- and β-defensins. Importantly, RCs are more potent against some bacterial and viral pathogens and more stable than their linear counterparts. However, the mechanism of bacterial toxin inactivation by RCs is not known. In the present study, we demonstrate that RCs facilitate unfolding of bacterial toxins. Using differential scanning fluorimetry (DSF), limited proteolysis and collisional quenching of internal tryptophan fluorescence, we show that hydrophobic regions of toxins normally buried in the molecule interior become more exposed to solvents and accessible to proteolytic cleavage in the presence of RCs. The RC-induced unfolding of toxins led to their precipitation and abrogated activity. Toxin

[1] To whom correspondence should be addressed (kudryashov.1@osu.edu).

The structure for human α-defensin 1, human β-defensin 2 and retrocyclin-1 will appear in the PDB under accession codes 3GNY, 1FD3 and 1HVZ respectively.

AUTHOR CONTRIBUTION

Elena Kudryashova designed the experiments, produced proteins, acquired and analysed data and wrote the manuscript. Stephanie Seveau produced CDCs and Wuyuan Lu synthesized RC-101. Dmitri Kudryashov co-ordinated the project, designed the experiments, analysed data and wrote the manuscript.

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inactivation by RCs was strongly diminished under reducing conditions, but preserved at physiological salt and serum concentrations. Therefore, despite significant structural diversity, α-, β- and θ-defensins employ similar mechanisms of toxin inactivation, which may be shared by anti-microbial peptides from other families.

Keywords

bacterial toxins; defensins; retrocyclins; structural plasticity; thermodynamic instability; unfolding

INTRODUCTION

Defensins are small cysteine-rich peptides used throughout animal and plant kingdoms as a frontline innate immune defence against broad-spectrum bacterial, fungal and viral pathogens [1]. In primates, β- and θ-defensins are produced by various leucocytes and epithelial cells, whereas the production of α-defensins is limited to neutrophils and Paneth cells of the small intestine. In humans, production of α- and β-defensins can be either constitutive or inducible [2]; however, synthesis of human θ-defensin is blocked at the protein level due to the presence of a premature stop codon in the mRNA transcript of the θ-defensin pseudogene [3]. Given the anti-HIV-1 activity of θ-defensins [4], their evolutionary loss was suggested to be a contributing factor to HIV-1 susceptibility in humans [5].

All defensins share three characteristic intramolecular disulfide bonds and are divided into α-, β- and θ-subfamilies based on the pattern of these bonds, size of the molecule and the nature of the peptide (linear in α- and β-defensins compared with cyclic in θ-defensins; Figure 1). The secondary and tertiary structures of α- and β-defensins are conserved despite their variable primary sequence. Three disulfide bonds stabilize the three antiparallel β-sheets of α- and β-defensins (Figures 1A and 1B). The later primarily differ from each other by the length of the N-terminal α-helix segment (Figure 1B). Initially discovered in old-world primates [6], θ-defensins are the most recently evolved and the only cyclic peptides known to be produced by animals [7]. They are formed by head-to-tail ligation of the translation products from either one gene or two separate genes resulting in an 18-resudue cyclic θ-defensin peptide [6]. Intramolecular disulfide bonds of θ-defensins cross-linking the cyclic peptide backbone are arranged in a unique cyclic cystine ladder motif (Figure 1C) [8]. Products from several θ-defensin genes can be paired together in different combinations diversifying the subfamily [9].

With an anticipation of high therapeutic potential, human θ-defensin was synthesized based on the sequence encoded by the human θ-defensin pseudogenes and named retrocyclin-1 (RC-1) [10]. It was found that synthetic RCs share anti-bacterial activities with other natural defence peptides. Similar to α-defensins, they effectively kill bacteria by permeabilizing bacterial cell membranes [11]. Remarkable anti-viral properties of RCs were predicted based on their similarities to primate θ-defensins and attributed to their ability to bind both viral and host membrane glycoproteins involved in viral entry [4,12,13]. Finally, similar to α- and β-defensins, RCs were shown to inhibit several unrelated bacterial toxins, {e.g., *Bacillus anthracis* toxin [14], *Gardnerella vaginalis* toxin [15] and *Listeria monocytogenes*

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listeriolysin O (LLO) [16]), but the mechanisms of selective inhibition of toxins (without affecting host proteins) remained enigmatic.

Recently, we showed that human defensins from α- and β-subfamilies act as molecular anti-chaperones, i.e. take advantage of low thermodynamic stability of bacterial toxins to promote their unfolding [17]. Indispensable for pore forming or passing through the host membrane, the thermodynamic instability of toxins is efficiently exploited by defensins; destabilization by co-folding with defence peptides leads to toxin partial unfolding and precipitation via exposed hydrophobic regions. The defensin-promoted exposure of hydrophobic regions of toxins may increase their immunogenicity [18] and susceptibility to proteolytic cleavage and degradation [17].

Because the mechanisms of toxin inactivation by RCs remained unaddressed in previous studies, the present work is aimed to bridge this gap in our understanding of innate immune mechanisms and to investigate whether RCs neutralize bacterial effector proteins by mechanisms similar to those employed by other families of human defensins.

EXPERIMENTAL

Retrocyclins and proteins

Synthesis of RC-1 (a gift of Dr Robert Lehrer, UCLA) [10] and RC-101 [19] was described previously. Actin cross-linking domains (ACD) of MARTX (multi-functional auto-processing repeats-in-toxin) toxins from *Vibrio cholerae* and *Aeromonas hydrophila* (ACD_{Vc} and ACD_{Ah}) and a construct that spans all four effector domains of $MARTX_{Vc}$ in their natural orientation ($4dMARTX_{Vc}$), were expressed in *Escherichia coli* and purified as described previously [20]. *Clostridium difficile* toxin A and B glucosyltransferase domains (TcdA– and TcdB–GTD) were expressed in *Bacillus megaterium* cells (provided by Dr Lacy, Vanderbilt University) and purified as described [21]. Purification procedures for *Bacillus anthracis* protective antigen (PA) [22], LLO and *Streptococcus pneumoniae* pneumolysin (PLY) [23,24] were published previously. LFnACD, a fusion construct of the N-terminus of anthrax lethal factor (LF_n) and ACD, was purified as described [25]. Skeletal muscle actin preparation from rabbit skeletal muscle acetone powder [26] (Pel-Freez Biologicals) and human plastin isoform 3 (PLS3) [27] were described previously. Human IgG and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) were obtained from Sigma–Aldrich.

Limited proteolysis

Limited proteolysis procedure was conducted as described previously [17]. Briefly, 5 μM ACD_{Vc} protein was mixed with 25 μM RC-1 or RC-101 in 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$ buffer and cleaved by chymotrypsin (1:100 w/w ratio to protein) or thermolysin (1:200 w/w ratio) at 30 °C. Protein samples were resolved on SDS/PAGE. Total protein content (sum of all bands in a lane) was quantified using ImageJ software (http://rsb.info.nih.gov/ij/) [28].

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Acrylamide collisional quenching of tryptophan fluorescence

Tryptophan fluorescence quenching experiments were performed using a multifunctional plate reader (Tecan) with excitation and emission wavelengths 295 and 328 nm respectively. ACD_{Vc} was diluted in PBS (pH 7.4) to 2 μM with or without addition of5 fold mole excess of RC-101 and titrated with increasing amounts of freshly prepared acrylamide solution in PBS. Data were presented as Stern–Volmer plots, where the ratios of fluorescence intensities (F_0 /F) in the absence (F_0) and presence (F) of a given quencher (acrylamide) concentration were plotted against quencher concentration [29].

Differential scanning fluorimetry

Temperature melting profiles of 10 μM proteins in PBS (pH 7.4) in the presence of SYPRO Orange (SO) dye (Invitrogen) were obtained using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad) as described previously [20].

Precipitation assay

Precipitation of ACD_{Vc} and ACD_{Ah} was assessed by differential ultra-centrifugation. Prior to centrifugation, both ACD_{Vc} and ACD_{Ah} (5 μM final concentrations) were incubated in the absence or presence of RC-101 (at 1:1, 1:2, 1:3 or 1:5 molar ratios to proteins) for 30 min at 30 °C in a buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl. For reducing condition experiments, all the components (buffer solution, both proteins and RC-101) were supplemented with 10 mM tris(2-carboxyethyl)phosphine (TCEP) before mixing. Aliquots of the samples with and without 25 μM RC-101, in the presence or absence of TCEP, were withdrawn immediately prior to centrifugation for the ACD cross-linking activity assay (see below). All samples were centrifuged using a TLA-100 rotor in an Optima TL-100 ultracentrifuge (Beckman Coulter) at 280 000 ***g*** for 30 min at 4 °C. The supernatant and pellet fractions were resolved on SDS/PAGE. Gels were analysed using ImageJ software (http://rsb.info.nih.gov/ij/).

ACD cross-linking activity assay

ACD cross-linking activity assay was described in detail previously [30]. Briefly, cross-linking of 10 μM actin by ACD in a buffer containing 50 mM HEPES, pH 7.5, 0.2 mM $CaCl_2$, 0.5 mM ATP, 2 mM $MgCl_2$ was monitored by SDS/PAGE. To initiate the cross-linking, aliquots withdrawn from the pelleting assays were added to actin at a final ACD concentration of 25 nM. In a separate set of experiments, $4dMARTX_{Vc}$ (25 nM) was used to cross-link actin (5 μM) in the absence or presence of RC-101 (300 nM).

Cell culture

Cell culture experiments were conducted in triplicates essentially as described [17]. Briefly, LF_N ACD was mixed with PA (final concentrations 5 and 12.5 nM respectively) and added to complete Dulbecco's Modified Eagle's medium (DMEM) containing 10 % FBS. Then RC-101 (5 and 10 μM final concentrations) was added. The mixture was incubated for 20 min at 37 °C and used to replace the medium on the monolayers of rat small intestinal epithelial cell (IEC-18) line (A.T.C.C.). Phase contrast microphotographs were taken using a Nikon inverted microscope Eclipse Ti-E (Nikon).

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Statistical analysis

Data were analysed using Microsoft Excel and KaleidaGraph software. Error bars represent S.E.M. Statistical significance was determined by two-tailed Student's *t* test: *P*-values less than 0.05 were considered statistically significant.

RESULTS

Retrocyclins facilitate unfolding of ACD_{Vc}

RC-1 is a synthetic peptide corresponding to a putative ancestral human peptide encoded by *θ*-defensin pseudogene [10], whereas RC-101 is an RC-1 analogue that differs by a single amino acid substitution of arginine to lysine [19]. We assessed the effects of both RCs on unfolding of ACD, an actin cross-linking effector toxin, which is expressed as a part of larger toxins MARTX (*Vibrio* and *Aeromonas* spp.) [31] and valine-glycine repeat protein G1 (VgrG1) (*V. cholerae*) [32]. Previously we have demonstrated that similar to many other bacterial toxins, ACD from MARTX toxins of both *V. cholerae* and *A. hydrophila* (ACD_{Vc} and ACD_{Ah} respectively) have low thermodynamic stability and, at the physiological temperature of the human body, exist in equilibrium between fully folded and partially unfolded (molten globule) states [20]. Therefore, ACD can serve as a good model protein to study protein unfolding mediated by defensins [17].

Effects of RC-1 on ACD_{Vc} unfolding were assessed by differential scanning fluorimetry (DSF) [33] and limited proteolysis (Figure 2). In the presence of RC-1, hydrophobic regions of ACD_{Vc} got exposed to solution at lower temperatures, compared with ACD_{Vc} alone, as detected by an enhanced fluorescence of SO dye sensitive to a hydrophobic environment (Figure 2A). Next, the addition of RC-1 brought notable changes to the ACD_{Vc} cleavage pattern upon limited proteolysis of this toxin by chymotrypsin and thermolysin (Figure 2B). In the absence of RC-1, cleavage by both proteases resulted in formation of stable proteolytic products of ACD_{Vc} and the total protein content was not substantially reduced, suggesting that the cleavage occurred at a few places in the flexible loops of the toxin. In contrast, whereas moderately inhibiting the disappearance of the full size ACD_{Vc} toxin band, RC-1 caused dramatic reduction in the amount of total protein due to the almost complete lack of stable proteolytic fragments, which were highly pronounced in the absence of RC-1 (Figures 2B–2D). This suggests that similarly to *α*-defensin HNP1 (human neutrophil peptide 1), RC-1 promoted toxin unfolding exposing additional sites, proteolytic cleavage of which generates randomly fragmented polypeptides spread throughout the gel lane [17].

The effects of RC-101 on limited proteolysis of ACD_{Vc} were similar to those imposed by RC-1 (Figures 3A–3C). Namely, (i) high molecular mass ACD_{Vc} cleavage products were not accumulated (Figure 3A); (ii) the detectable amount of total protein in a lane was decreased, compared with ACD_{Vc} in the absence of RC-101 (Figures 3B and 3C); (iii) accumulation of low molecular mass products due to fragmentation of ACD_{Vc} at the additional sites was more prominent in the presence of RC-101, especially after increasing contrast of gel images (Figure 3A, boxed area).

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Collisional quenching of intrinsic tryptophan fluorescence by acrylamide showed higher accessibility of at least some of the eight tryptophan residues of ACD_{Vc} to quencher (acrylamide) in the presence of RC-101, which does not have any tryptophan residues and therefore does not contribute to intrinsic fluorescence of the analysed samples (Figure 3D). We have demonstrated previously that tryptophan quenching of the mammalian proteins and unaffected toxins is not increased in the presence of defensins [17]. Therefore, our data strongly suggest that both RC-1 and RC-101 promote unfolding of ACD_{Vc}.

RC-101 induces precipitation and abrogates activity of ACD_{Vc} and ACD_{Ah}

Precipitation of bacterial toxins in the presence of defensins is well recognized as one of the key mechanisms of toxin inactivation by these peptides [34]. Therefore, we tested whether the observed unfolding of ACD by RC-101 is accompanied by toxin precipitation by analysing toxin band redistribution between supernatant and pellet fractions upon ultra-centrifugation. We have demonstrated previously that both ACD orthologues from *V. cholerae* and *A. hydrophila* have low thermodynamic stability, with ACD_{Ah} being notably less stable than ACD_{Vc} [17,20]. Accordingly, under non-reducing conditions, addition of RC-101 induced precipitation of both toxins, although precipitation of ACD_{Ah} was more pronounced (Figures 4A and 4B, upper panels; Figures 4C and 4D). In the presence of reducing agent (TCEP), precipitation of both toxins was strongly reduced (Figures 4A and 4B, lower panels, Figures 4C and 4D), confirming that native conformation of the peptide, stabilized by three disulfide bonds, is essential for its specific activity.

Catalytic activity of ACD can be monitored *in vitro* by detecting the accumulation of covalently cross-linked actin species using SDS/PAGE [30]. We found that activity of both enzymes is severely abrogated (or even completely blocked in the case of ACD_{Ah}) following their incubation with RC-101 under non-reducing conditions, but nearly unaffected when ACD and RC-101 were incubated in the presence of TCEP (Figures 4E and 4F).

Bacterial toxins from several major families are destabilized by RC-101

Next, we utilized DSF to test whether the ability of RC-101 to promote toxin unfolding extends to other toxins from different, unrelated families: pore forming toxin (anthrax toxin PA), cholesterol-dependent cytolysins (CDCs) from *L. monocytogenesis* and *Streptococcus pneumoniae* (LLO and PLY), enzymatic *C. difficile* toxins (GTDs of TcdA and TcdB) and effector domains of the $MARTX_{Vc}$ and $MARTX_{Ah}$ toxins (ACD_{Vc}, ACD_{Ah} and $4dMARTX_{Vc}$ construct with all effector domains fused together). The DSF analysis demonstrated that similarly to other defensins, RC-101 is able to further destabilize and promote unfolding of thermodynamically marginally stable bacterial toxins (Figure 5). Toxins, which have been previously demonstrated to be less susceptible to inactivation by defensins (TcdA [35] and PLY [34]), were less affected by RC-101 (Figure 5) and HNP1 [17] alike. Importantly, none of the tested mammalian proteins was affected by RC-101 in a comparable manner (Figure 5).

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RC-101 inhibits ACD activity *in vitro* and in cell culture under physiologically relevant conditions

Next, we addressed the question whether the RC-101 effect on toxins would lead to toxins' inactivation under physiologically relevant conditions. To this end, the ability of ACD to cross-link actin *in vitro* and to cause cytotoxic cell rounding effects in cell culture were examined in the presence and absence of defensin [17]. For the cell culture experiments, the anthrax toxin delivery machinery (PA and LF_N) was employed to deliver the LF_N-fusion construct of ACD_{Vc} [25] into the cytoplasm of IEC-18 rat intestinal epithelial cells. We found that RC-101 reduced the rate of actin cross-linking imposed by the ACD domain of the $4dMARTX_{Vc}$ construct by 3.6-fold (Figures 6A and 6B). Moreover, addition of micromolar (5 and 10 μM) concentrations of RC-101 to nanomolar amounts of the LF_N ACD–PA complex in a serum-containing medium notably protected the cells from the cytotoxicity inflicted by LF_N ACD (Figure 6C). This confirms the potency of the RC under physiological salt and serum concentrations.

DISCUSSION

Defensins are a class of cysteine-rich anti-microbial peptides produced not only by fungi, plants and animals, but also by prokaryotes (e.g., laterosporulin class IId bacteriocin [36]) and, therefore represent some of the most ancient and most universal immune molecules on the planet. In higher eukaryotes, defensins are potent immuno-modulators [37]. A major anti-microbial mechanism of defensins is manifested via their direct interaction with the bacterial membrane, leading to its disorganization and compromised integrity [38]. Another major mechanism of the anti-microbial activity of defensins involves interaction with bacterial proteins, either cell-associated (e.g., secretion portals [39,40], fimbrial adhesins [41]) or secreted (e.g., toxins [34,35,42,43]). It is easy to recognize that both anti-microbial mechanisms are important and complementary. Many bacterial toxins are extremely potent and can be lethal to the host in small doses if not promptly neutralized. Therefore, inactivation of toxins in solution, before they have a chance to reach the cells, is essential, but not sufficient if bacteria remain active and produce more toxins. On the other hand, killing and eradicating bacteria from the body might not be sufficient if even trace amounts of toxins are released. Furthermore, toxins can remain active in the body for days hiding in intraluminal vesicles (ILV) and then can be released from initially affected cells in exosomes, even when the causative pathogen has been eradicated [44]. Therefore, both defensin mechanisms are essential.

The list of bacterial proteins neutralized by defensins is being updated every year since the first report on inactivation of a bacterial toxin by defensin in 2005 [45]. Therefore, it is increasingly clear that defensins can recognize and neutralize many unrelated proteins of bacterial nature, proteins with different properties, specific activities and structure. Recently we proposed that the key feature recognized by human α- and β-defensins is thermodynamic instability of the bacterial toxins essential for their ability to undergo dramatic conformational perturbations upon formation of membrane pores or upon unfolding for transition through such pores [17]. Now we demonstrate that retrocyclins, which belong to the family of θ-defensins, also exploit thermodynamic instability as a primary target to

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promote unfolding, susceptibility to proteolysis and precipitation of bacterial toxins. Future studies can help to uncover the detailed mechanisms of unfolding/co-folding of each individual toxin with the defensins. Given that toxin unfolding by defensins results in precipitation, which precludes the use of solution methods for structural studies, we anticipate that the magic angle spinning (MAS) solid-state NMR (MAS–NMR) technique [46] can be particularly helpful in future studies.

θ-defensins are likely to be more potent under *in vivo* conditions due to the overall higher stability and longer lifetime of cyclic peptides in serum and under condition of protease attack [47]. Therefore, initially introduced as promising anti-viral humanized synthetic peptides, analogous to those that are still active in old-world primates [6] and widely proposed as a topical microbicide [48–50], RCs should be seriously considered as first-line antidotes against a broad range of bacterial toxins. We predict that the newly discovered mechanism of RC anti-toxin activity will further promote their development as therapeutic agents.

The evolutionary loss of θ-defensins was suggested to be a contributing factor to HIV-1 susceptibility in humans [5]. This surprising evolutionary loss occurred after the divergence from orangutans, which have six intact and one defective θ-defensin genes [3]. Interestingly, all multiple human θ-defensin pseudogenes, as well as all the pseudogenes of chimpanzees, gorillas and bonobos, contain the identical non-sense mutation (the same as is in the defective θ-defensin gene from orangutans), which therefore is probably acquired from their common ancestor [3]. Since, the θ-defensin pseudogenes are genetically clustered with α-defensins, the apparently enigmatic duplication of these pseudogenes in humans is likely to be linked to expansion of the fully functional α-defensin genes [51]. In fact, one of the most credible explanations for the enigmatic evolutionary loss of functional and highly potent θ-defensins is that they have been replaced by somewhat less potent, but less energetically costly and more robustly produced α-defensins [7]. It is also plausible that human θ-defensins were traded for a yet unknown evolutionarily advantageous acquisition, with which they were in conflict, similarly to the pseudogeneization of uricase linked to improvements in fructose metabolism [52]. Finally, as a rather bold speculation one might suggest that a moderately increased vulnerability and exposure to viruses may benefit species via mechanisms of accelerated evolution. Even though viral infections are often devastating at the organismal level, by promoting horizontal gene transfer and provoking major genomic rearrangements viruses also play a critical and occasionally highly beneficial role in evolution of species. For example, the acquisition of genes encoding syncytins, proteins derived from the envelope protein of endogenous retroviral elements, enabled mammals to develop placenta and fetal-maternal tolerance and thereby fundamentally influenced the emergence of the entire Mammalia class [53,54]. Furthermore, loss of defensins could force our ancestors to develop alternative anti-viral strategies, some of which (such as inactivation of a gene responsible for synthesis of N-glycolylneuraminic acid, a common receptor for many pathogens) could beneficially affect brain development [55,56]. If the later scenarios are correct, than re-introduction of θ-defensins to the human population (either at the genetic or at the pharmaceutical levels) might bring all the benefits of increased resistance to viral and bacterial pathogens without negative side effects.

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Given that structurally diverse α-, β- and θ-defensins employ similar mechanisms of toxin inactivation, our findings suggest that defensins from other organisms may share the ability of human defensins to interfere with the folding-unfolding equilibrium of marginally stable bacterial toxins. In this regard, it will be very interesting and important to ascertain whether human and non-human anti-microbial peptides from other families can, at least partially, employ similar strategies in their anti-microbial defence mechanisms.

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Abbreviations

4dMARTX$_{Vc}$	four effector domains of MARTX$_{Vc}$ fused in their natural orientation
ACD	actin cross-linking domain of MARTX and VgrG1 toxins
CDC	cholesterol-dependent cytolysin
DSF	differential scanning fluorimetry
HNP1	human neutrophil peptide 1
LF$_N$	N-terminus of Bacillus anthracis lethal factor
LLO	Listeria monocytogenes lysteriolysin O
MARTX	multi-functional auto-processing repeats-in-toxin
MAS	magic angle spinning
PA	Bacillus anthracis protective antigen
PLY	Streptococcus pneumoniae pneumolysin
RC	retrocyclin
SO	SYPRO Orange
TcdA– and TcdB–GTD	Clostridium difficile toxin A and B glucosyltransferase domains
TCEP	tris(2-carboxyethyl)phosphine
VgrG1	valine-glycine repeat protein G1

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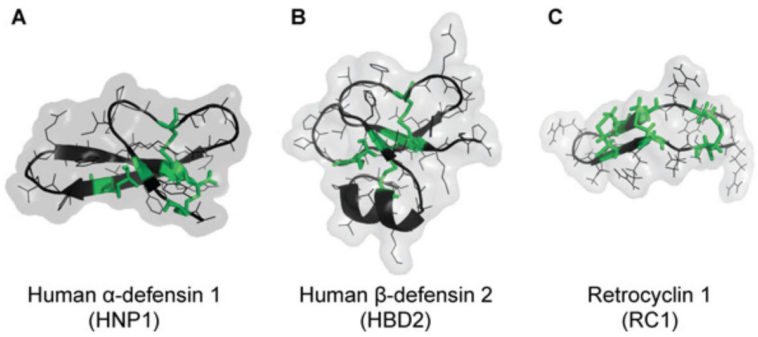

Figure 1. Structure of *α*-, *β*- and *θ*-defensins

(**A**) Human *α*-defensin 1. (**B**) Human *β*-defensin 2. (**C**) RC-1. Images were generated by PyMOL (http://www.pymol.org/) using PDB structures: 3GNY (**A**), 1FD3 (**B**), and 1HVZ (**C**). Characteristic cysteines connected via disulfide bonds are coloured in green. Note that even though structures of monomeric defensins are shown, in solution defensins have a tendency to form dimers or higher-order oligomers.

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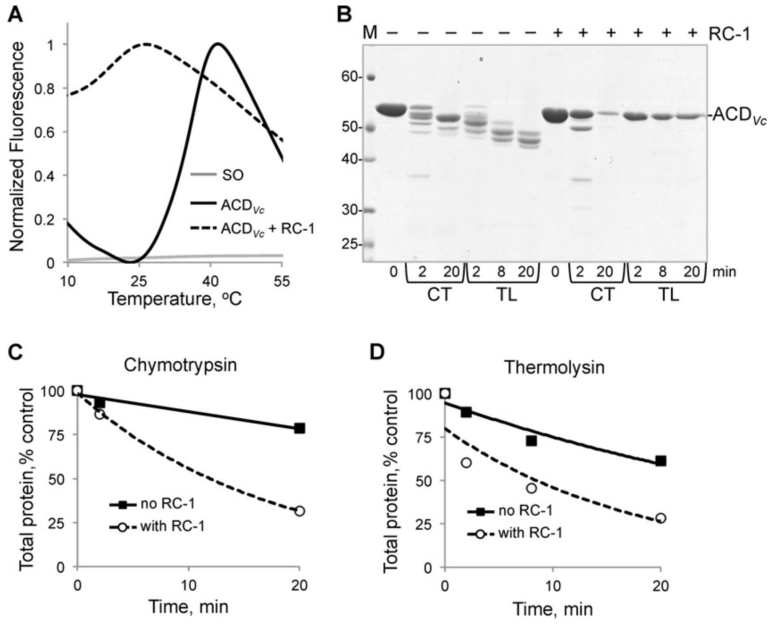

Figure 2. Effects of RC-1 on ACD_{Vc} toxin

(**A**) DSF of ACD_{Vc} (final concentration 10 μM) was carried out in the presence (dashed line) or absence (solid black line) of 5 fold mole excess of RC-1 (50 μM). SO dye alone (solid grey line). (**B**) Limited proteolysis of 8.7 μM ACD_{Vc} in the absence and presence of 5 fold mole excess RC-1 (44 μM). Samples were incubated at 30 °C in the presence of chymotrypsin (CT) or thermolysin (TL) for the indicated periods of time and subjected to SDS/PAGE. Position of the full-length ACD_{Vc} is indicated on the gel. M, molecular mass ladder (values in kDa). (**C** and **D**) Quantification of total protein content (sum of all bands in a lane) for CT (**C**) and TL (**D**) proteolysis.

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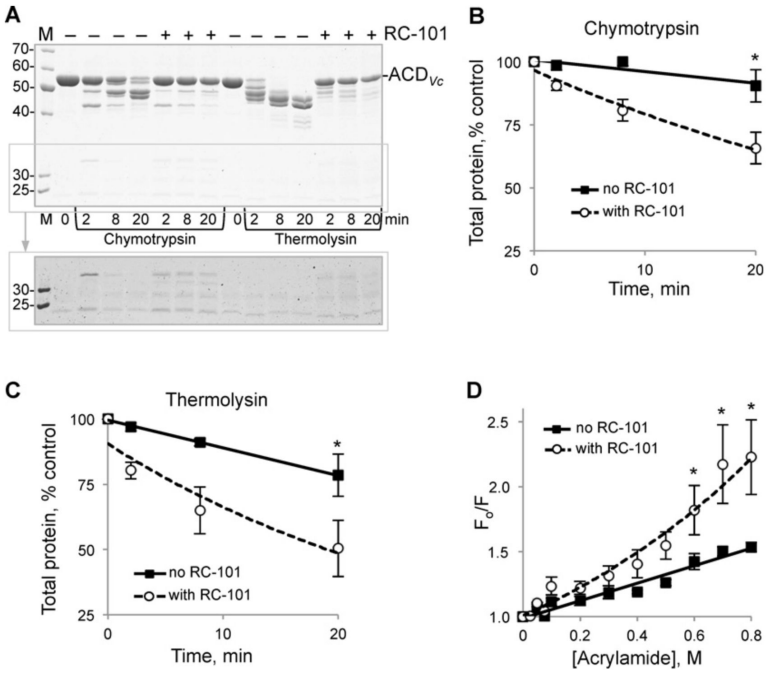

Figure 3. Effects of RC-101 on ACD_{Vc} toxin

(**A**) Limited proteolysis of 5 μM ACD_{Vc} in the absence and presence of 5 fold mole excess RC-101 (25 μM). Samples were incubated at 30 °C in the presence of chymotrypsin or thermolysin for the indicated periods of time and subjected to SDS/PAGE. Position of the full-length ACD_{Vc} is indicated on the gel. M, molecular mass ladder (values in kDa). Contrast was adjusted for the lower part of the gel (boxed) to reveal additional fragmentation of ACD_{Vc} in the presence of RC-101. (**B** and **C**) Amount of total protein (sum of all bands) ina lane was quantified and plotted against time for chymotrypsin (**B**) or thermolysin (**C**) cleavage. Error bars represent S.E.M., *P $<$0.05. (**D**) Stern–Volmer plots obtained for collisional acrylamide quenching of tryptophan fluorescence of 2 μM ACD_{Vc} in the absence (solid line) and presence (dotted line) of 5 fold mole excess of RC-101 (10 μM). Error bars represent S.E.M., *P $<$0.05.

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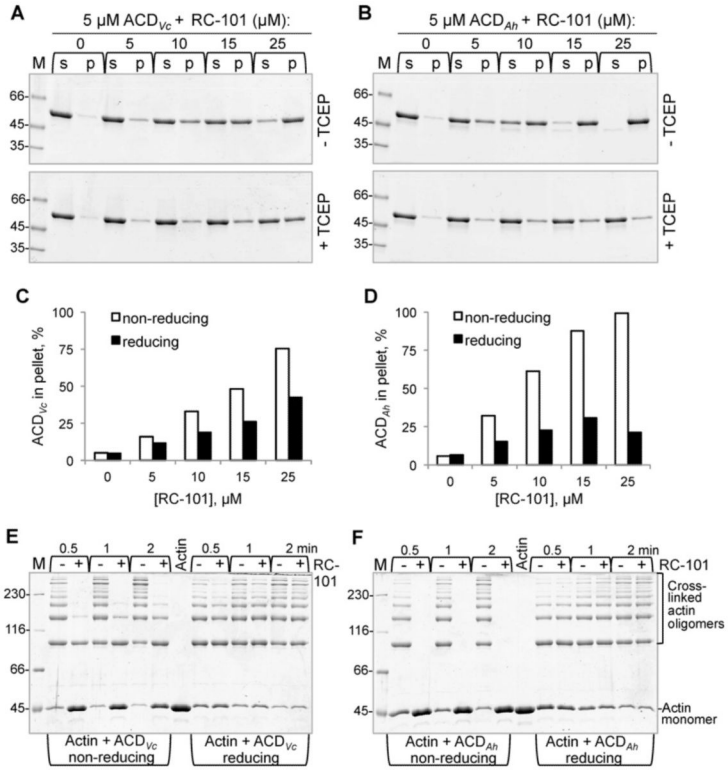

Figure 4. Effects of RC-101 on precipitation and activity of ACD toxins

(**A** and **B**) Precipitation of 5 μM ACD_{Vc} (**A**) and ACD_{Ah} (**B**) in the presence of increasing concentrations of RC-101 (0–25 μM) in the absence (upper panels) and presence (lower panels) of 10 mM TCEP. Following ultra-centrifugation, supernatant (s) and pellet (p) fractions were resolved on SDS/PAGE. M, molecular mass ladder (values in kDa). (**C** and **D**) Amount of protein in the supernatant and pellet fractions for ACD_{Vc} (**C**) and ACD_{Ah} (**D**) in the absence (non-reducing) or presence of TCEP (reducing) was quantified. (**E** and **F**) Actin cross-linking activity of ACD in the samples from (**A**) and (**B**) taken before ultra-centrifugation (as described in the 'Experimental' section) was analysed by SDS/PAGE. ACD_{Vc} (**E**) and ACD_{Ah} (**F**) incubated in the absence or presence of 25 μM RC-101 with or without of TCEP were added at a final concentration of ACD 25 nM to 10 μM actin and incubated for the indicated periods of time. Actin, actin alone; M, molecular mass ladder (values in kDa).

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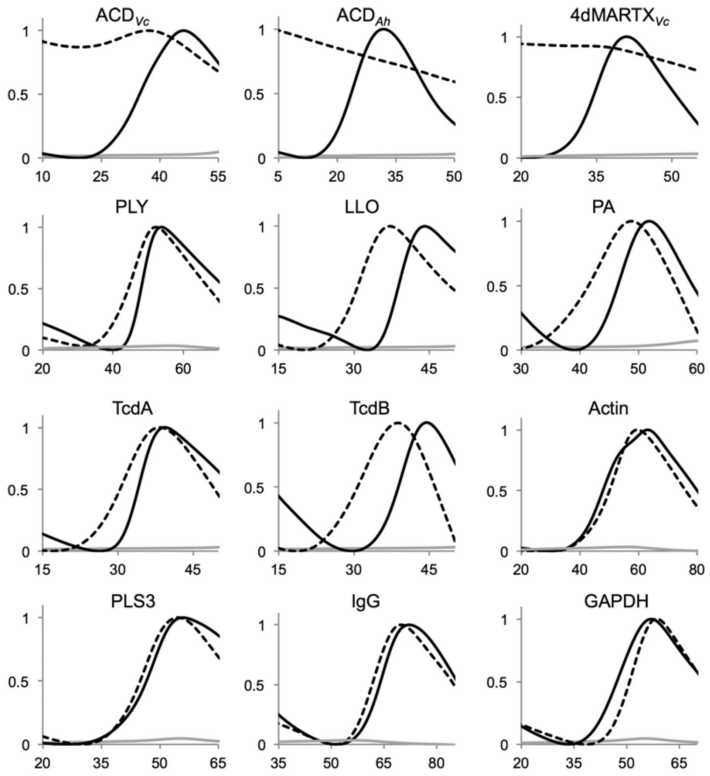

Figure 5. RC-101 potentiates thermal unfolding of toxins from several major families

DSF melting profiles of proteins (final concentration 10 μM) in 20 mM HEPES, pH 7.5, 150 mM NaCl in the presence (dashed line) or absence (solid black line) of 5 fold mole excess of RC-101. SO dye alone (solid grey line). Axes for all plots: x, temperature (°C); y, normalized fluorescence.

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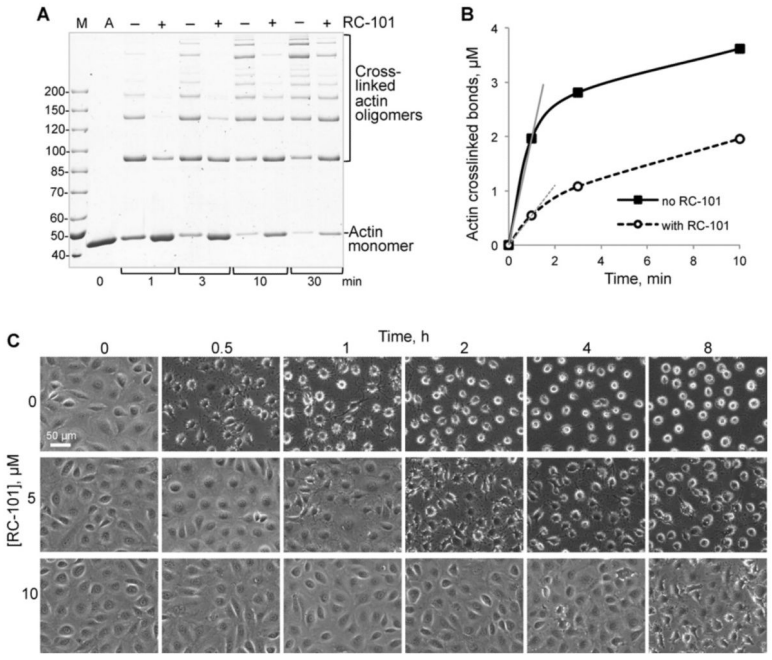

Figure 6. RC-101 inhibits ACD activity in vitro and protects cells from ACD toxicity

(A) Cross-linking of 5 μM actin by 25 nM 4dMARTX$_{Vc}$ in the absence or presence of 300 nM RC-101 was monitored by SDS/PAGE. A, actin alone; M, molecular mass ladder (values in kDa). (**B**) ACD cross-linking activity of 4dMARTX$_{Vc}$ was expressed as the concentration of formed isopeptide bonds and plotted against time. Initial reaction rates were calculated using linear slopes of the curves (grey lines) for the initial 1-min period. (**C**) IEC-18 intestinal epithelial cells were treated with a complex of LF$_N$ ACD and PA (5 and 12.5 nM final concentrations respectively). RC-101 was added at 5 or 10 μM to the mixture of LF$_N$ ACD and PA diluted in serum-containing medium and applied to the cells. Images were taken at the indicated time points. Scale bar is 50 μm.