MAJOR ARTICLE

Killing of African Trypanosomes by Antimicrobial Peptides

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Antimicrobial peptides are components of the innate immune systems of a wide variety of eukaryotic organisms and are being developed as antibiotics in the fight against bacterial and fungal infections. We explored the potential activities of antimicrobial peptides against the African trypanosome *Trypanosoma brucei*, a vector-borne protozoan parasite that is responsible for significant morbidity and mortality in both humans and animals. Three classes of mammalian antimicrobial peptides were tested: α-defensins, β-defensins, and cathelicidins. Although members of all 3 classes of antimicrobial peptides showed activity, those derived from the cathelicidin class were most effective, killing both insect and bloodstream forms of the parasite. The mechanism of action of the cathelicidins against *T. brucei* involves disruption of surface membrane integrity. Administration of cathelicidin antimicrobial peptides to mice with late-stage *T. brucei* infection acutely decreased parasitemia and prolonged survival. These results highlight the potential use of antimicrobial peptides for the treatment of African trypanosomiasis.

Innate immunity is the first line of host defense against microbial infection. In mammals, this multifactorial defense system includes complement, lysozyme, phospholipases, and naturally occurring antibodies, as well as a diverse group of small peptides with antimicrobial activity [1, 2]. Antimicrobial peptides (AMPs) constitute a primitive immune defense mechanism and are found in a wide range of eukaryotic organisms, from humans to plants to insects [3]. In mammals, 2 broad classes of AMPs have been described—the defensins and the cathelicidins. The defensins comprise a group of structurally similar peptides containing conserved cysteine residues for intramolecular disulfide bonding. The α-defensins are peptides of 30–50 aa and are expressed mainly by neutrophils and epithelial cells in various anatomic locations. The β-defensins are similar in size but are phylogenetically older and vary in the placement of intramolecular bonding from the α-defensins [4]. The β-defensins also have a wide pattern of distribution, including the skin, respiratory tract and gastrointestinal tract. An additional group of mammalian AMPs are derived from the C-terminal domains of large preproteins called "cathelicidins." These structurally diverse microbicidal peptides are produced from proteolytic processing of the preprotein by a serine protease (e.g., elastase or proteinase 3) [5], which gives rise to the C-terminal AMP portion and a conserved larger N-terminal region component termed "cathelin," which has no known function [6]. Cathelicidins are generally expressed by neutrophils and epithelium [7]. Both defensins and cathelicidins kill a variety of bacteria and fungi by inserting into their cell membranes and disrupting membrane integrity [8]. The beneficial effects of some AMPs also may be related to their binding to lipopolysaccharide [6]. Importantly, AMPs are being explored as antimicrobial agents for the treatment of infections involving antibiotic-resistant bacteria and other pathogenic microbes.

African trypanosomes are parasitic protozoa that alternately infect their tsetse fly vectors and their mammalian hosts [9]. Infection by the African trypanosome *Trypanosoma brucei* subspecies causes devastating disease in both humans (sleeping sickness) and cattle (na-

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gana). The annual incidence of African trypanosomiasis in humans is estimated to be ~250–300,000 persons, with ~60 million at risk for infection [10]. Infection of livestock causes huge economic losses. In mammals, parasites replicate in the bloodstream, where they cause an undulating parasitemia [11]. Eventually, parasites cross the blood-brain barrier to give rise to the clinical manifestations of sleeping sickness and, ultimately, death [12]. The antiparasitic agents used for treatment of African trypanosomiasis are antiquated and have numerous toxic effects. Moreover, they can be inherently ineffective or become so by virtue of development of drug resistance by the parasite [13]. Thus, investigation of new chemotherapeutic agents is critical for the future control of this disease and others caused by similar protozoans.

MATERIALS AND METHODS

Parasite cultivation and viability assays. The 427 strain of *T. brucei* was used in this study. Procyclic (insect) forms were grown at 25°C in SDM-79 medium [14] supplemented with 10% fetal bovine serum. Bloodstream forms were cultivated at 37°C in HMI-9 medium [15]. AMP susceptibility assays were performed on stationary-phase parasites adjusted to a density of 10^8 cells/mL in conditioned medium. For AMP assays, 100 μL of adjusted cultures were incubated with AMPs in 96-well culture plates for up to 3 h. Parasite viability was assessed, as described elsewhere for *Leishmania* species [16]. In brief, 10 μL of a 5 mg/mL of stock substrate MTT was added to the cell cultures and incubated overnight at either 25°C or 37°C. Reduced substrate was solubilized by the addition of 100 μL of 10% SDS for at least 6 h. Absorbance was read spectrophotometrically at 550 nm. Assays were standardized using increasing numbers of cells from 5×10^5 to 1×10^7 cells. This demonstrated a linear relationship between cell number and absorbance.

Peptides. Cryptdin-4 was produced and purified essentially as described elsewhere [17] and was the kind gift of Dr. D. P. Satchell (University of California at Irvine). Human β-defensins 1 and 2 were purified from an insect cell/baculovirus expression system, as described elsewhere [18, 19]. Ovispirin and novispirin are 18-aa peptides generally resembling the N-terminus of SMAP-29 [20]. A non-AMP of 32 aa (KLHMAPEFSDQVRRKAQIGERRSDGDVSVRLD), based on the sequence of *T. cruzi* mitochondrial hsp40 [21], was used as a control. Peptides based on the sequence of the antimicrobial C-terminal regions of the cathelicidins (denoted "cathelicidins") were synthesized on an Applied Biosystems model 433A synthesizer at the 0.1 mmol/L scale, using solid-phase Fastmoc chemistry. Peptides were purified by reverse-phase high-performance liquid chromatography (HPLC) on a Vydac 218TP1022 column. Separations were performed at a flow rate of 10 mL/min, employing a linear gradient (0%–100%) of aqueous 0.1% trifluoroacetic acid (TFA) and acetonitrile containing 0.085% TFA (solvent B). Fractions were collected and subsequently monitored by analytical-scale reverse-phase HPLC on a 4.6 × 250-mm Vydac 218TP54 column employing isocratic elution conditions at a flow rate of 1 mL/min. Selected fractions were pooled and lyophilized for further characterization by mass spectrometry and capillary electrophoresis. Mass measurements were performed with a Hewlett-Packard model 1100 MSD equipped with an electrospray ionization source, using flow injection at 0.1 mL/min in 64% acetonitrile containing 0.05% TFA. Capillary electrophoresis was performed on a Hewlett-Packard instrument equipped with a 75 μmol/L (ID) × 80.5-cm fused-silicate extended light-path column. Experiments were conducted at 18°C in 100 mmol/L sodium phosphate (pH 2.9) at 20,000 V. Peptide concentrations were determined by quantitative amino acid analysis on a Beckman 6300 amino acid analyzer. All peptides were stored in 0.02% acetic acid at 4°C.

Electron microscopy. For electron microscopy, cells were fixed in 2.5% glutaraldehyde in 100 mmol/L sodium cacodylate buffer (pH 7.4), placed onto poly-L-lysine coated coverslips for at least 1 h, washed in buffer, and postfixed in 1% osmium tetroxide–1.5% potassium ferrocyanide for 30 min at room temperature. This was followed by 3 changes of buffer, en bloc staining with 2.5% uranyl acetate, and dehydration in ethanol. For transmission electron microscopy, the coverslips were embedded in Epon 812 resin and were sectioned on a Reichert Ultracut E ultramicrotome using a diamond knife.

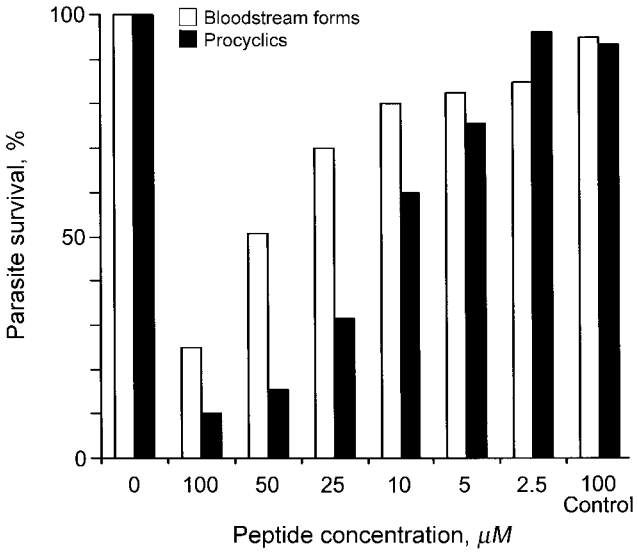

Figure 1. Cathelicidin-mediated killing of *Trypanosoma brucei*. Procyclic (insect) and bloodstream forms of *T. brucei* strain 429 were incubated with increasing concentrations of novispirin and were assayed by use of the MTT viability assay [16]. Both forms were susceptible to novispirin in a dose-dependent manner. Similar results were obtained with the other cathelicidins tested (see table 1). In all cases, SE was ±7%.

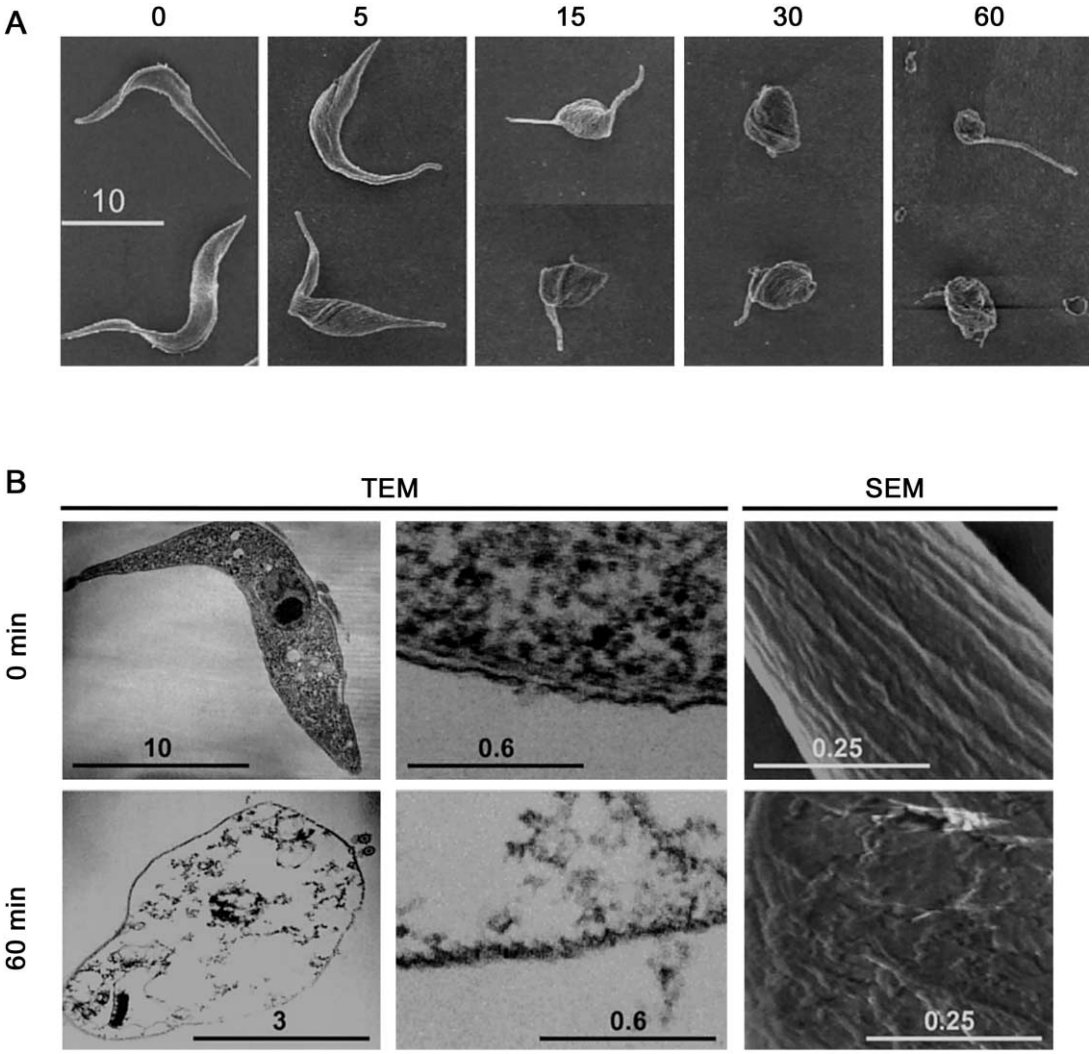

Figure 2. Electron microscopy of cathelicidin-treated parasites. Procyclic (insect) form parasites were treated with 25 μmol/L of various cathelicidin antimicrobial peptides (AMPs; shown here for protegrin-1) for the indicated times (h) and were processed for scanning and transmission electron microscopy (SEM and TEM, respectively), as described in Materials and Methods. *A,* Parasites assumed a more-rounded morphology with increasing times of treatment. *B,* Parasites treated with 25 μmol/L protegrin-1 for 60 min appeared "ghostlike," with absence of intracellular structures *(left panels)* and devoid of surface membranes *(center panels),* and the surfaces of treated parasites demonstrated a disrupted, distorted appearance *(right panels).* Treatment with other cathelicidins gave similar results (data not shown). *Bars,* μm.

Sections were collected onto grids and were stained with uranyl acetate and lead citrate for contrast. All grids were examined by use of a JEOL 100 CX transmission electron microscope. For scanning electron microscopy, coverslips were critical-point dried, placed onto specimen mounts using carbon tape, and coated with gold in a Bal-Tec MED 020 with single "cool" sputter device. All samples were examined by use of a Hitachi S4500-II cold-field emission scanning electron microscope.

Animal infections. For mouse infections, 6-week-old male A/J mice (Jackson Laboratories) were used. Intraperitoneal injections of 10^6 bloodstream-form parasites in 100 μL of medium were administered to each mouse. Cathelicidins were intraperitoneally administered daily at a concentration of 5 mg/kg (in 10 μL of peptide storage buffer), beginning 1 day after infection. Intraveneous administration via tail vein was performed at the indicated concentration in 2–5 μL of buffer. Parasitemias were measured daily in blood samples obtained by tail snip.

RESULTS

T. brucei *is susceptible to AMPs.* The susceptibility of *T. brucei* to AMPs was tested by incubation of 10^7 parasites with various peptides at a concentration of 50 μmol/L for 3 h at either 25°C for procyclic (insect) forms or 37°C for bloodstream forms. The peptides used were cryptdin-4, an α-defensin [22];

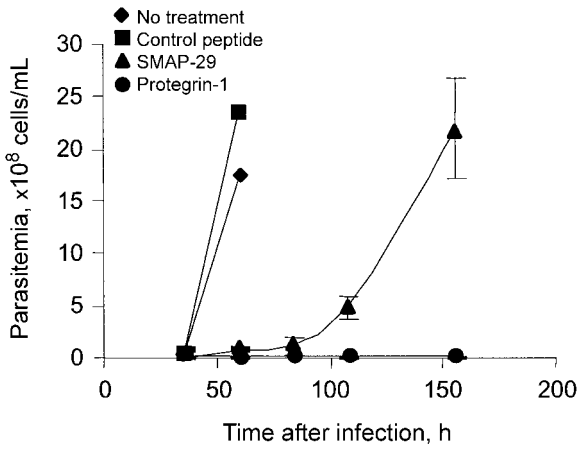

Figure 3. Pretreatment of bloodstream-form *Trypanosoma brucei* with cathelicidins reduces parasitemia in infected mice. Mice were injected intraperitoneally with 10^6 bloodstream-form parasites incubated with 25 μmol/L of either SMAP-29 or protegrin-1. Controls included parasites treated with either buffer alone (no treatment) or 100 μmol/L of a non–antimicrobial peptide (AMP). Parasitemias were measured at increasing times, as indicated. Data are mean ± SE.

human β-defensins 1 and 2 [18, 23]; and various cathelicidins, including SMAP-29 (a 29-aa arginine-rich peptide) [24], its analogs ovispirin and novispirin [20] and protegrin-1 [25, 26] (an 18-aa arginine and cysteine-rich peptide containing a β-hairpin). The effects of the peptides on *T. brucei* were assessed in 2 ways. First, cell viability was tested by measurement of the reduction of substrate MTT, which occurs only in living parasites. Second, parasites were examined microscopically during the course of incubation. Controls for both tests consisted of parasites incubated either with a non-AMP at the same concentration or with buffer alone. No significant effects were observed in these control cells. Only a minor reduction in survival (17%–33%) was found in parasites incubated with the α- or β-defensins (table 1). This contrasted greatly with the killing of parasites by the cathelicidins. The survival of parasites incubated with members of this AMP class ranged from 56% to 95%. Overall, the parasiticidal effect on procyclic (insect) forms was >80% for all cathelicidins tested. Since the activities of the defensins, particularly the β-defensins, can vary with salt concentration, the efficiency of parasite killing by the defensins and the cathelicidins may be related to the salt concentrations of the media used for incubation. Interestingly, procyclic (insect)– and bloodstream-form parasites showed different susceptibilities to many of the AMPs tested (table 1; figure 1). Although, in each case the parasiticidal effect was dose dependent, bloodstream forms were more resistant than procyclic (insect) forms under the same conditions. The effect is probably explained by the very different surface properties of the 2 life-cycle stages and was not seen only with the cathelicidins. Differential susceptibility also was consistently observed when the 2 forms of the parasite were incubated with members of the α- and β-defensin classes (table 1).

AMPs act on trypanosome membranes. Microscopic observation of parasites incubated with AMPs revealed dramatic changes in the structural integrity of the parasite, even after only 20 min of treatment (figure 2). Within 5 min of AMP incubation, 85%–90% of the parasites became immotile. Thereafter, cells lost their normal shape, developing a rounded morphology. The magnitude of the effect was dose dependent over a range of concentrations from 5 μmol/L to 100 μmol/L (data not shown). Transmission electron microscopy of treated cells revealed a nearly complete loss of surface membranes, as well as most intracellular contents. This is consistent with the loss of membrane integrity in bacteria incubated with AMPs and the postulated mode of action of these peptides on those organisms [8, 27, 28]. Scanning electron microscopy of parasites treated with much lower concentrations of protegrin-1 showed a studded appearance of the outer membrane (data not shown), similar to what has been seen on the surface of mycobacterial cells treated with this peptide [29].

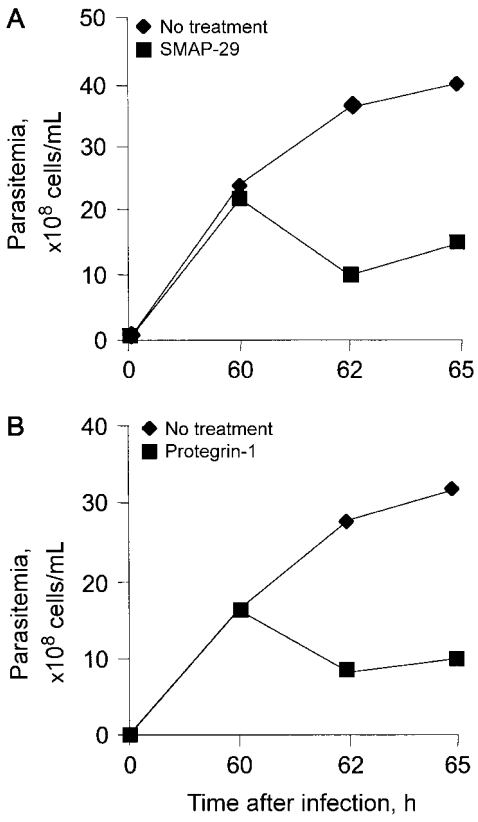

Figure 4. In vivo administration of cathelicidins acutely reduces parasitemia. Mice infected with bloodstream-form *Trypanosoma brucei* were treated intravenously at 60 h after infection with 1.5 mg/kg of the indicated peptide. Parasitemias were measured prior to and at the indicated times after peptide injection. Regardless of treatment, all mice died within 4–8 h after treatment because of already advanced parasitemia.

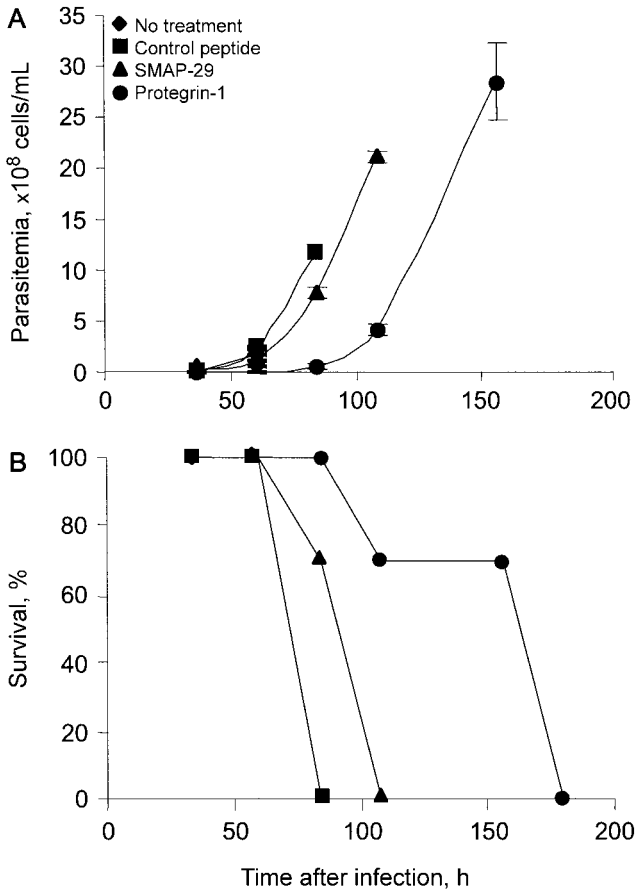

Figure 5. In vivo administration of cathelicidins increases survival of mice infected with *Trypanosoma brucei.* Cathelicidin peptides were administered intraperitoneally (5 mg/kg/day) to A/J mice infected with monomorphic 429 *T. brucei* bloodstream-form parasites. *A,* Parasitemias were measured at indicated times after infection. *B,* Mortality of infected mice. Three to 5 mice were used for each group. Data are mean ± SE.

Cathelicidins decrease in vivo viability of trypanosomes. Preincubation of bloodstream form parasites with cathelicidins (shown in figure 3 for SMAP-29 and protegrin-1) significantly reduced their capacity for growth in vivo. Incubation of the parasites with protegrin-1 for 30 min completely stopped parasite growth in mice. No parasites were detected in the blood of these mice over the course of the experiment. Mice inoculated with parasites preincubated with SMAP-29 developed parasitemia with delayed kinetics, reaching ~2×10^8/mL at 150 h after infection instead of at 50 h after infection. Mice infected with parasites incubated with buffer or control peptide developed increasing parasitemia and died within 4 days. These results corroborate the in vitro decrease in fitness of parasites after incubation with AMPs.

In vivo administration of cathelicidins to* T. brucei*–infected mice reduces parasitemia and delays mortality. To further test the effects of cathelicidin therapy on African trypanosomiasis in vivo, we administered protegrin-1 or SMAP-29 to mice with advanced parasitemia. These peptides were chosen because they are different structurally and have different spectra of antimicrobial activity [24, 30]. In addition, SMAP-29 showed minimal host toxicity in previous studies [24]. Infected mice developed parasitemias of ~$1.5–2.5 \times 10^9$/mL, at which time a single intravenous dose of 1.5 mg/kg of either SMAP-29 or protegrin-1 was given. Parasitemias were reassessed at 2 h and 5 h after treatment, revealing a reduction of ~50% in each case (figure 4). Mice treated in this manner eventually developed increasing parasitemia after the initial decrease and showed no difference in mortality from controls (data not shown). To test the effect of repeated administration of cathelicidins on the course of infection, we administered drugs with a daily intraperitoneal dosage (figure 5). Infections were established by intraperitoneal inoculation of 10^6 parasites/mouse. Parasitemias were measured daily, followed by intraperitoneal injection of 5 mg/kg of either SMAP-29 or protegrin-1. In each case, a significant delay in the onset of parasitemia was observed (figure 5*A*). This was most striking for the mice treated with protegrin-1. We also noted a significant delay in mortality in the mice treated with cathelicidins, which correlated directly with the delay in parasitemia. In protegrin-1–treated animals, mortality was delayed to twice the time of control animals.

DISCUSSION

These results highlight the in vitro and in vivo activities of AMPs against African trypanosomes. The cathelicidin class of AMPs was particularly effective at killing. It appears that these peptides exert this parasiticidal activity by disrupting cell wall/membrane integrity, as is the case with other microbes [8, 31].

Table 1. Antitrypanosomal activities of mammalian antimicrobial peptides (AMPs).

Peptide	Parasiticidal activity[a]	
	Procyclic (insect) form	Bloodstream form
α-Defensin		
Cryptdin-4	29.6	0
β-Defensins		
Human β-defensin 1	17.8	25.3
Human β-defensin 2	33.1	18.6
Cathelicidins		
Novispirin	81.3	56.4
Ovispirin	86.3	68.5
SMAP-29	95.0	71.5
Protegrin-1	95.2	39.4

[a] Percentage of parasites killed was calculated by determining the residual parasite viability in the MTT assay after incubation in 50 μmol/L of the indicated peptide for 3 h. No significant killing was detected in control incubations of parasites in buffer or non-AMP control. SE ±<6% in all cases. Data are representative of 3 independent experiments.

This disruption of membrane integrity probably leads to osmotic instability, causing cell swelling and eventual lysis. The loss of intracellular compartmentalization and contents also may reflect the ability of the peptides to gain entrance to the cell and directly disrupt internal membranes. The differences observed in the efficacy of AMPs against mammalian and procyclic (insect) forms of *T. brucei* probably reflect unique properties of the surface membranes of these 2 life-cycle stages of the parasite. The surface membranes of *T. brucei* contain an abundance of membrane proteins linked via glycosylphosphatidylinositol (GPI) anchors. The bloodstream form contains up to 10 million copies of the variant surface glycoprotein [32], which aids in resistance to complement-mediated lysis [33]. In the procyclic (insect) form, parasites express an altogether different surface coat, consisting of an abundant GPI-anchored protein called "procyclin" [34]. The abundance of these molecules may impart the parasite membrane with affinity for these peptides. Moreover, AMPs are highly cationic, which may relate to their general affinity for negatively charged cell surfaces [35]. Alternatively, the susceptibility of trypanosome membranes to AMP attack may be related to the abundance of GPIs. The GPIs of the 2 parasite forms have distinct structures [36–38], which also may account for differential susceptibility of the 2 life-cycle stages to AMPs.

The concentrations of cathelicidin peptides used in this study having significant in vitro trypanocidal activity were 2–10-fold higher than those sufficient to kill various bacteria, fungi and mycobacteria [29, 39, 40]. This may reflect differences in the assay conditions used in this study. In addition, these eukaryotic parasites may have developed natural resistance to the effects of endogenously produced AMPs that they may encounter during mammalian infection. The finding of significant in vivo activity of the peptides at the concentrations used suggests that, despite this natural resistance, the activities of AMPs might be exploited for use as antitrypanosomal drugs. Recent studies also have highlighted the activity of derivatives of the NK cell AMP, NK-lysin, against the related pathogen *Trypanosoma cruzi*. Interestingly, this peptide appears to exhibit reduced host toxicity and may kill intracellular parasites with minimal effect on host cells [41]. Nonmammalian AMPs, such as a recently described peptide, dermaseptin, purified from the skin of the tree frog of the genus *Phyllomedusa,* also appear to have significant activity against the trypanosomatids [42]. Our results provide the basis for further systematic study of the cathelicidin class of AMPs as potential antibiotics for treatment of African trypanosomiasis and diseases caused by related protozoan parasites.

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