

Synthetic nonamer peptides derived from insect defensin mediate the killing of African trypanosomes in axenic culture

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Abstract Synthetic antimicrobial 9-mer peptides (designated as peptides A and B) designed on the basis of insect defensins and their effects on the growth of African trypanosomes were examined using two isolates of *Trypanosoma congolense*, IL1180 and IL3338, and two isolates of *Trypanosoma brucei*, ILTat1.1 and GUTat 3.1, under axenic culture conditions. Both peptides inhibited the growth of all bloodstream form (BSF) trypanosomes at 200–400 µg/mL in the complete growth medium, with peptide A being more potent than peptide B. In addition, these peptides exhibited efficient killing at 5–20 µg/mL on BSF trypanosomes

suspended in phosphate-buffered saline, whereas procyclic insect forms in the same medium were more refractory to the killing. Electron microscopy revealed that the peptides induced severe defects in the cell membrane integrity of the parasites. The insect defensin-based peptides up to either 200 or 400 µg/mL showed no cell killing or growth inhibition on NIH3T3 murine fibroblasts. The results suggest that the design of suitable synthetic insect defensin-based 9-mer peptides might provide potential novel trypanocidal drugs.

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Introduction

African trypanosomes are protozoan parasites that cause sleeping sickness in human and nagana in cattle in large areas of sub-Saharan Africa. Currently, about 60 million people are at risk of human African trypanosomiasis and the annual incidence of the disease is approximately 300,000 cases (Kennedy 2004). As for livestock production, the Food and Agriculture Organization of the United Nations estimates an annual loss of US\$ 4.5 billion due to animal trypanosomiasis. Despite numerous efforts such as the elaboration of chemotherapeutic drugs (Anene et al. 2001; Barrett et al. 2007; Keiser et al. 2001), tsetse fly vector control schemes (Aksoy 2003; Vreysen 2001), and vaccine development (Lalmanach et al. 2002), African trypanosomiasis still brings a substantial negative impact on human life (Kuzoe 1993; Stich et al. 2003) as well as livestock productivity (McDermott and Coleman 2001; Naessens et al. 2002) in the tsetse-infected areas where poverty and malnutrition are prevalent. So, further investigation on the search for a new class of chemotherapeutic drugs is necessary for better control of African trypanosomiasis.

Antimicrobial peptides (AMPs) have been isolated and characterized from a wide range of organisms in invertebrates, vertebrates, plants, and even bacteria (Boman 1995; Hoffmann et al. 1999; Jack et al. 1995; Lehrer and Ganz 1999). AMPs are relatively short polypeptides (10–50 amino acids) with an overall positive charge and substantial proportion of hydrophobic residues (Hancock and Sahl 2006). These AMPs are produced in various cells and organs and have important roles in the host's innate immune defense system (Brown and Hancock 2006; Hancock and Sahl 2006; Yang et al. 2002). One of the well-characterized families of AMPs is the defensin (Ganz 2003; Ganz and Lehrer 1994). Defensins have characteristic six disulfide-paired cysteines and are found in both vertebrates and invertebrates. Invertebrate defensins have been identified from insects, scorpions, mussels, and ticks, and these are effective against gram-positive as well as some gram-negative bacteria, fungi, yeast, and protozoa (Bulet et al. 1999). Most of the defensins are amphipathic molecules that have clusters of positively charged as well as hydrophobic amino acid residues (Hancock and Sahl 2006). Bacterial cell membranes are consisted of a lipid bilayer that is covered by negatively charged phospholipid head groups and hydrophobic fatty acids. So, the mechanisms of defensin molecules to kill the bacteria are currently hypothesized to be a direct electrostatic interaction to the bacterial cell membranes through their positively charged domains. As defensin molecules accumulate in microbial membranes, they rearrange their location and result in the formation of pores in the microbial membrane (Chen et al. 2006; Ganz 2003; Yeaman and Yount 2003). As bacterial cell membranes are the primary target of defensin molecules, the occurrence of resistance mutations to the microbicide will be unlikely.

Previously, we designed synthetic oligopeptides based on the amino acid sequence of the active site of insect defensins (Ishibashi et al. 1999; Saido-Sakanaka et al. 1999). These oligopeptides were as effective as the original defensin molecule in killing gram-positive and gram-negative bacteria including methicillin-resistant *Staphylococcus aureus*. Further modification of the amino acid sequence of these defensin peptides yielded novel 9-mer peptides that possess stronger antimicrobial activity and have a broader pathogen spectrum than the previous oligopeptides (Saido-Sakanaka et al. 2004). Thus, these 9-mer peptides are good candidate microbicides against bacteria, fungi, and possibly protozoan parasites. Recent reports have shown that several AMPs that originated from vertebrates and invertebrates were effective in killing several species of trypanosomes (Boulanger et al. 2002; Brand et al. 2002; Haines et al. 2003; Hu and Aksoy 2005; Madison et al. 2007; McGwire et al. 2003; Nok et al. 2002). The trypanocidal mechanism of the AMPs was suggested to

be the disruption of parasite's membrane integrity in the case of mammalian cathelicidin (McGwire et al. 2003), or pore formation in the case human defensin α -1 (Madison et al. 2007). However, the trypanocidal mechanism of other classes and types of AMPs, including our insect defensin-derived peptides, should further be explored in detail. In this study, to expand our knowledge on trypanocidal effects of AMPs and their perspectives for therapeutic application, we examined the in vitro effects of synthetic 9-mer AMPs derived from beetle defensin on several strains of salivarian trypanosomes, both bloodstream and procyclic insect forms (PCFs), under axenic culture conditions. As we demonstrate in this study, the efficient killing activities of these 9-mer peptides on different species and strains of African trypanosomes show the potential of these peptides as a novel trypanocidal drug.

Materials and methods

Reagents

All the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

Peptide synthesis

Modified 9-mer peptides composed of L-type amino acids were synthesized according to the information of the amino acid sequence of the active site of insect defensins, as described (Ishibashi et al. 1999; Saido-Sakanaka et al. 1999). The amino acid sequences of 9-mer peptides were as follows: RLYLRIGRR-NH₂ [peptide A; molecular weight (M.W.) 1,201.49], RLRLRIGRR-NH₂ (peptide B; M.W. 1,195.48), and AKGFAANHS-NH₂ (control peptide; M.W. 900.98). The control peptide has no sequence homology to insect defensins and was used as a negative control. Peptides A and B were demonstrated to have antimicrobial activity (Iwasaki et al. 2007), whereas the control peptide had no activity. Amino acid sequence and molecular mass of the peptides were confirmed by protein sequencer (Procise™ cLC, PE Biosystems) and matrix-assisted laser desorption ionization mass spectrometry (Voyager, PerSeptive Biosystems), respectively. These peptides were dissolved in phosphate-buffered saline (PBS) at 1 mg/mL, sterilized by 0.22 μ m Millipore membrane filter, and stored frozen at -20°C until use.

Axenic culture of African trypanosomes

The bloodstream forms (BSFs) *Trypanosoma congolense* (IL 1180 and IL3338) and *Trypanosoma brucei brucei* (GUT at 3.1 and ILTat1.1) were passaged at 3-day intervals

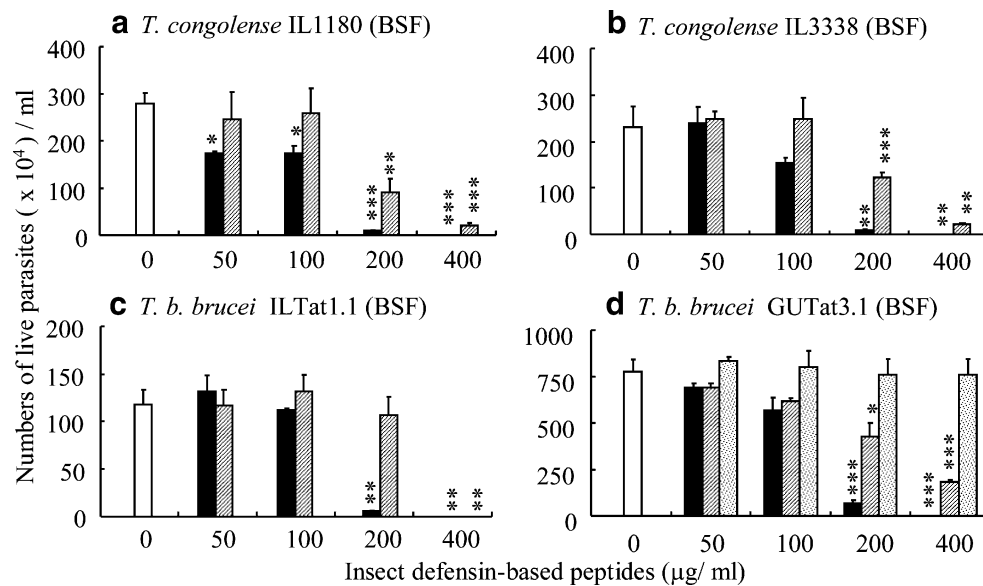


Fig. 1 Effects of insect defensin-based peptides on the growth of BSF *T. congolense*, IL 1180 (a) and IL3338 (b), and *T. brucei brucei*, ILTat 1.1 (c) and GUT at 3.1(d), under in vitro culture. The parasites were suspended in the growth medium at 1×10^4 parasites per milliliter and seeded into a 96-well plate (100 μ L per well in triplicates) with or without varying concentrations of the peptides. After culture at 34°C

in humidified 4.5% CO₂–95.5% air for 3 to 5 days, numbers of live parasites per milliliter were counted by hemocytometer. Data represent the mean values \pm SEM from three culture wells. * P <0.05, ** P <0.01, *** P <0.001 between the peptide-treated and nontreated groups (after Student's *t* test). Open bars none, closed bars peptide A, hatched bars peptide B, and dotted bars control peptide

in vitro according to the culture protocols (Hirumi and Hirumi 1991, 1994) at 34°C in humidified 4.5% CO₂–95.5% air. Details of the culture medium for *T. congolense* (BSF) and *T. brucei brucei* (BSF) were also described previously (Kitani et al. 2002).

The PCFs *T. congolense* IL 1180 and *T. brucei brucei* (GUT at 3.1 and ILTat1.1) were prepared by transforming BSFs of the parasites and were passaged in SDM-79 medium (Brun and Schonenberger 1979) at 27°C in humidified 4.5% CO₂–95.5% air.

Growth inhibition and trypanocidal assay

BSFs trypanosomes were suspended in the appropriate culture medium at 1×10^4 parasites per milliliter, seeded into a 96-well plate in triplicates (100 μ L per well) with or without varying concentrations of the 9-mer peptides, and cultured at 34°C in humidified 4.5% CO₂–95.5% air. After 3 to 5 days of culture, numbers of live parasites in the well were enumerated by hemocytometer counts, as described previously (Kitani et al. 2002). For direct cytotoxic assay, trypanosomes were harvested from stock culture and washed twice by PBS supplemented with 10 mg/mL glucose (PSG). Trypanosomes were suspended in PSG at 4×10^6 parasites per milliliter, seeded into a 96-well plate in triplicates (100 μ L per well) with or without varying concentrations of the 9-mer peptides, and cultured either at 34°C for BSFs or at 27°C for PCFs in humidified 4.5%

CO₂–95.5% air. After 3 h of incubation, numbers of live (with vigorous movement) and dead (with no movement and ghost-like appearance) parasites in the well were enumerated by hemocytometer counts.

Electron microscopy

BSFs *T. brucei brucei* GUT at 3.1 were collected from stock culture, washed twice with PSG, and treated with insect defensin-based peptides A and B and control peptide (at 50 μ g/mL) at 34°C for 3 h. The samples were fixed in 2.5% glutaraldehyde in phosphate buffer for 2 h, packed in plastic hematocrit tube, and centrifuged at 8,000 \times g for 3 min. The pellet was then treated with 1% osmium tetroxide for 2 h. The fixed samples were dehydrated in a series of ethanol and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a transmission electron microscope (Hitachi 7500, Hitachi, Japan).

Cytotoxic assay with murine fibroblasts

The murine fibroblast cell line, NIH3T3 (RCB150, RIKEN Cell Bank, Japan) was used to assess the cytotoxic activity of the 9-mer peptides on mammalian cells. For growth inhibition assay, NIH3T3 cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum at 2×10^4 cells per milliliter and seeded

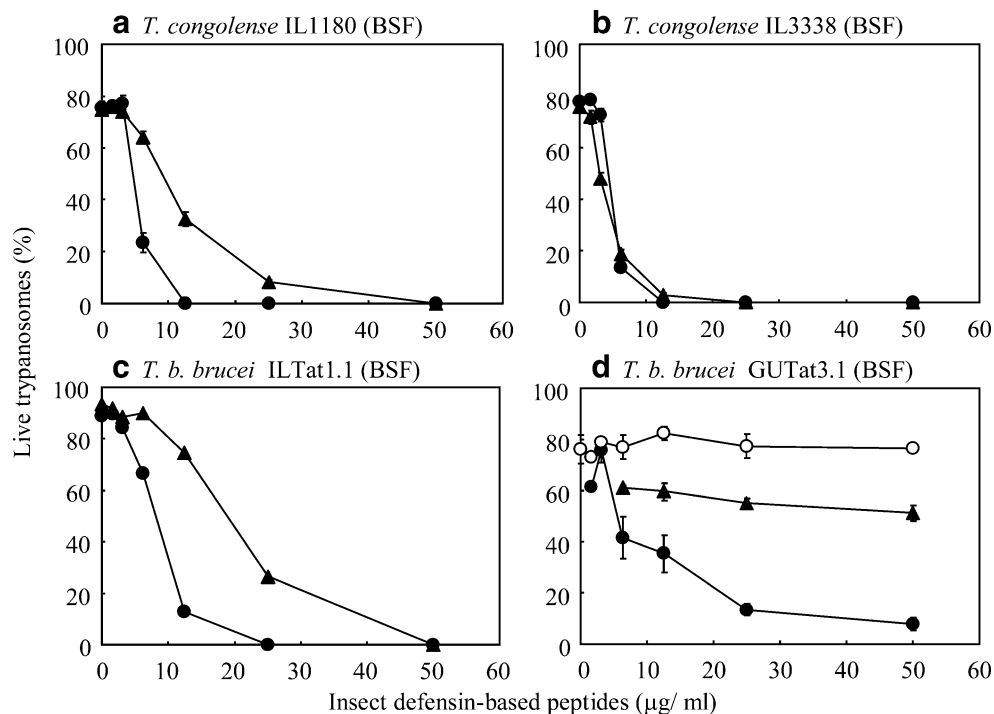


Fig. 2 Effects of insect defensin-based peptides on viability of BSF *T. congolense*, IL 1180 (a) and IL3338 (b), and *T. brucei brucei*, ILTat 1.1 (c) and GUT at 3.1(d), suspended in PSG. The parasites were suspended in PSG at 4×10^6 parasites per milliliter, seeded into a 96-well plate in triplicates (100 µL per well) with or without varying concentrations of the 9-mer peptides, and cultured at 34°C in humidified 4.5% CO₂–95.5% air. After 3 h of incubation, numbers

of live (with vigorous movement) and dead (with no movement and ghost-like appearance) parasites in the well were enumerated by hemocytometer counts. Data represent the mean values \pm SEM from three culture wells. The viability of the parasites in the nontreated control groups at the end of incubation ranged from 80% to 90%. Closed circles peptide A, closed triangles peptide B, and open circles control peptide

into a 96-well plate in triplicates (100 µL per well) with or without varying concentrations of the 9-mer peptides. After incubation for 48 h at 37°C in humidified 5% CO₂–95% air, cell growth was quantified by a cell viability test based on cleavage of tetrazolium salt (Cell Proliferation Reagent WST-1, Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instruction. For direct lytic assay, NIH3T3 cells were harvested from stock culture and washed twice with PBS. The cells were suspended in PBS at 7.5×10^5 cells per milliliter, seeded into a 96-well plate in duplicate with or without varying concentrations of the 9-mer peptides, and incubated at 37°C in humidified 5% CO₂–95% air for 3 h. Cell viability was assessed by trypan blue dye exclusion test, and numbers of live and dead cells in the well were enumerated by hemocytometer counts.

Statistical analyses

P values were calculated using the Student's *t* test for the growth inhibition, and *P* values of <0.05 were considered statistically significant.

Results

Effects of insect defensin-based peptides on the growth of BSF trypanosomes in culture medium

The impact of insect defensin-based peptides A and B on the growth of BSFs *T. congolense* IL1180 and IL3338 is shown in Fig. 1a, b, respectively. Both peptides A and B inhibited the growth of both strains of *T. congolense* in a dose-dependent manner. Peptide A strongly inhibited the growth of the parasite at 200 and 400 µg/mL (165 and 330 µM, respectively), whereas peptide B showed similar but less potent growth inhibition than peptide A. Observation of the parasites using an inverted phase contrast microscope showed that most of the tested parasites were vigorously motile at lower peptide concentrations (50 and 100 µg/mL), but very few or no live trypanosomes were present in the cultures treated with higher concentrations (200 and 400 µg/mL). In addition, the parasites became immobile within a few minutes after the addition of peptide A at 400 µg/mL and the affected parasites seemed to suffer severe cell membrane damage with ghost-like morpholog-

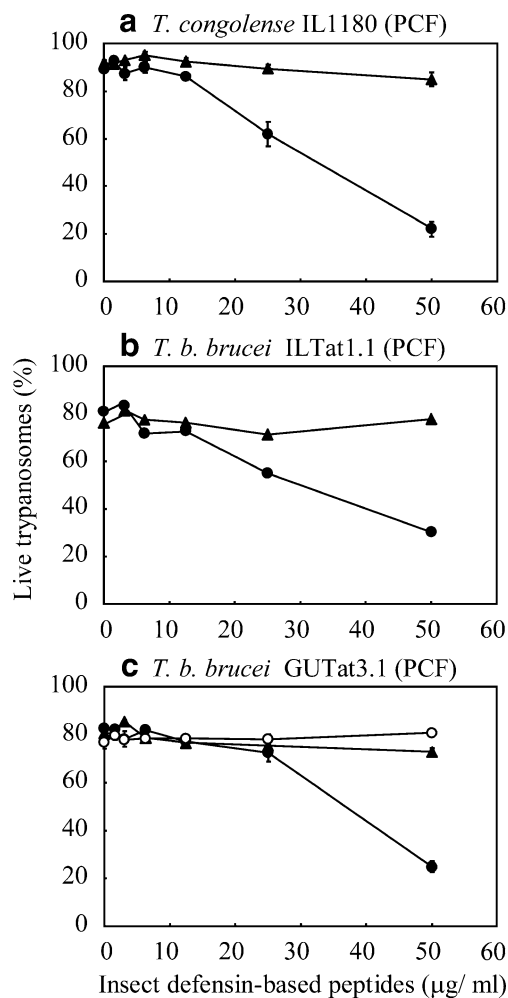


Fig. 3 Effects of insect defensin-based peptides on PCF *T. congolense* IL 1180 (a) and *T. brucei brucei* ILTat 1.1 (b) and GUT at 3.1 (c) suspended in PSG. The parasites were suspended in PSG at 4×10^6 parasites per milliliter, seeded into a 96-well plate (100 μL per well) in triplicates (a and c) or duplicates (b) with or without varying concentrations of the 9-mer peptides, and cultured at 27°C in humidified 4.5% CO_2 –95.5% air. After 3 h of incubation, numbers of live (with vigorous movement) and dead (with no movement and ghost-like appearance) parasites in the well were enumerated by hemocytometer counts. Data represent the mean values \pm SEM from three culture wells. The viabilities of the parasites in the nontreated control groups at the end of incubation were approximately 80% to 90%. Closed circles peptide A, closed triangles peptide B, and open circles control peptide

ical appearance (data not shown). These results suggested that the insect defensin-based peptides act quickly and strongly and alter cell membrane integrity of these strains of trypanosomes. Similarly, as shown in Fig. 1c, d, the growth of BSFs of *T. brucei brucei* ILTat1.1 and GUTat 3.1 was significantly affected by these insect defensin-based peptides. Again, peptide A was more potent in inhibiting the growth of both strains of *T. brucei brucei*, and similar cell membrane damage and morphological alterations were

induced by the peptide in these strains of trypanosomes. In sharp contrast, the control peptide, which has no sequence homology to any insect defensins and was used as a negative control, did not affect the growth of *T. brucei brucei* GUTat 3.1 at any of the concentrations tested (Fig. 1d). So, these results indicate that the inhibitory effect is associated with specific activity of insect defensin-based peptides A and B, but not with nonspecific factors, such as increased protein concentration or potential residual toxic molecules that remained after the peptide preparation process.

Effects of insect defensin-based peptides on trypanosomes suspended in PSG

The potency of AMPs to kill microorganisms is antagonized by the presence of divalent cations, monovalent cations, and polyanions such as heparin and mucins (Hancock and Sahl 2006). In addition, the presence of serum possibly lowers the titer of the insect defensin-based peptides. Therefore, the direct cytotoxic effect of these insect defensin-based peptides was assessed on trypanosomes suspended in PSG. As shown in Fig. 2a, b, both peptides A and B dose-dependently killed both strains of BSFs *T. congolense* after 3 h of incubation. In contrast to the complete trypanosome growth medium (Fig. 1), the trypanocidal effect of these peptides was more evident when assayed in PSG. Peptide A completely killed *T. congolense* parasite at 12.5 $\mu\text{g/mL}$ (10 μM), whereas peptide B did so at similar or slightly higher concentrations than peptide A (Fig. 2a, b). In addition, these insect defensin-based peptides similarly killed *T. brucei brucei* ILTat1.1 BSFs (Fig. 2c). BSFs of *T. brucei brucei* GUTat 3.1 were also killed by peptide A, but its efficacy was less than that observed in the other strains (Fig. 2d). Furthermore, peptide B was not so effective in killing this strain than the other *T. brucei brucei* strain (Fig. 2d). The reason for this variability is not clear at this moment, but it can be concluded that peptide A was more potent in killing both strains of *T. brucei brucei*. The control peptide did not exhibit any trypanocidal effects on BSFs of *T. brucei brucei* GUTat 3.1 at any of the concentrations tested (Fig. 2d).

Next, we examined the trypanocidal activity of the insect defensin-based peptides on PCFs of *T. congolense* IL1180 as well as *T. brucei brucei* ILTat1.1 and GUTat 3.1 suspended in PSG. In contrast to BSF trypanosomes, PCF trypanosomes were more refractory to killing by the insect defensin-based peptides. Even after treatment with peptide A at 50 $\mu\text{g/mL}$ (40 μM) for 3 h, approximately 20–30% of the PCFs *T. congolense* as well as *T. brucei brucei* parasites were still motile and, therefore, considered alive (Fig. 3a–c). Furthermore, peptide B was totally ineffective in killing all strains of PCF trypanosomes at the doses tested (Fig. 3a–c).

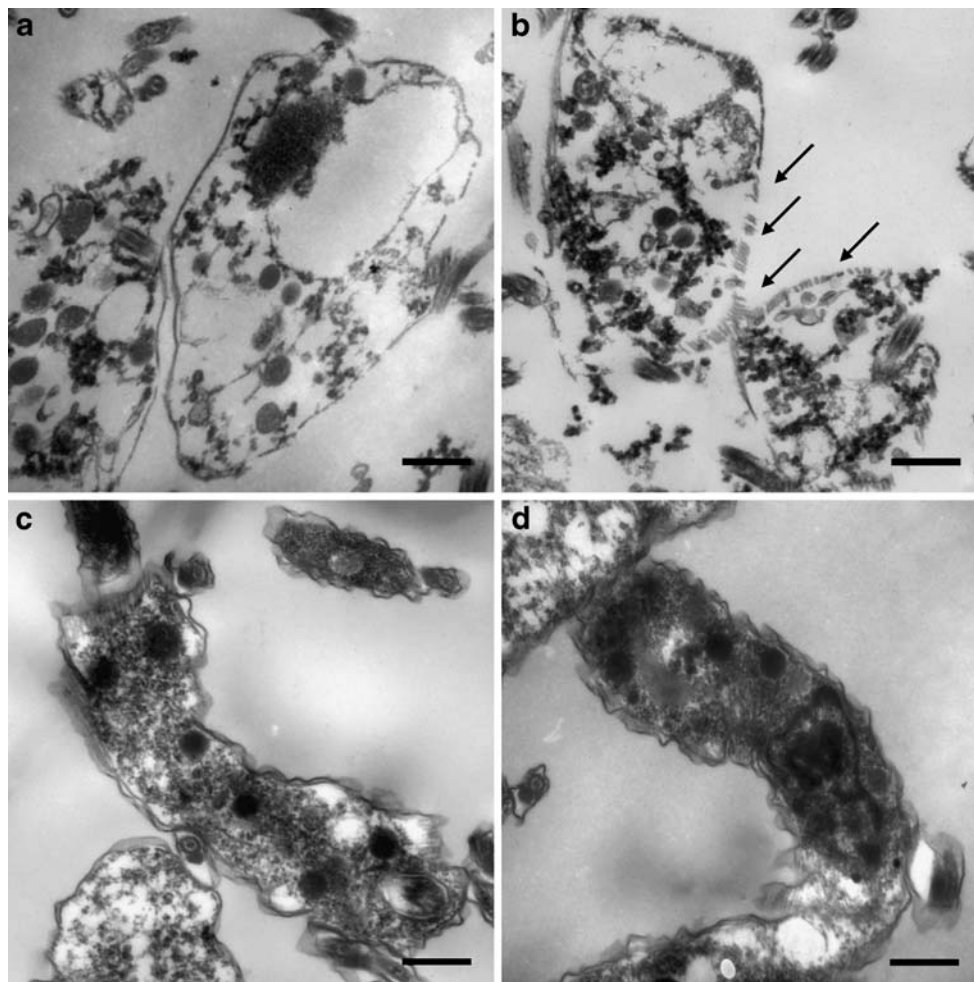


Fig. 4 Electron micrographs of insect defensin-based peptide-treated parasites. BSF *T. brucei brucei* GUT at 3.1 suspended in PSG were treated with insect defensin-based peptides at 50 $\mu\text{g}/\text{mL}$, incubated at 34°C for 3 h, and processed for transmission electron microscopy, as described in the “Materials and methods” section. **a** Peptide A severely affected the plasma membrane integrity of the parasites. The parasites exhibited ghost-like and swollen appearance and the cytoplasmic organelles were also disrupted. The leakage of the cytoplasmic content as well as the loss of VSG coat was apparent.

b Peptide B induced very similar morphological alterations as peptide A. Plasma membrane integrity was lost, and the arrays of subpellicular microtubules were left (*arrows*), forming a cage-like skeletal delineation of the affected parasites. **c** Control peptide did not affect the ultrastructures of the parasite, showing intact plasma membrane and surface glycoprotein coat, as well as dense cytoplasm containing intact organelles, which were quite similar to those observed in the untreated healthy parasites (**d**). Scale bars=0.5 μm

The control peptide showed no killing effect on *T. brucei brucei* GUTat 3.1 PCFs (Fig. 3c). These results suggest that the efficacy of the insect defensin-based peptides to kill trypanosomes differs depending on the life stages of trypanosome. Insect forms are more refractory to the insect-derived defensin peptides than BSFs which inhabit in the mammalian hosts.

Disruption of membrane integrity of trypanosomes by insect defensin-based peptides

Transmission electron microscopy revealed severe damage in plasma membrane integrity in *T. brucei brucei* GUTat 3.1

BSF parasites after treatment with defensin peptides A and B at 50 $\mu\text{g}/\text{mL}$ (40 μM) for 3 h (Fig. 4a, b). The cytoplasmic organelles were disrupted and the leakage of the cytoplasmic content was apparent. In addition, the variable surface glycoprotein (VSG) coat was completely stripped off. The parasites exhibited a ghost-like and swollen appearance, quite similar to the microscopical observation reported previously (McGwire et al. 2003). Despite the loss of membrane integrity, the arrays of subpellicular microtubules (Hemphill et al. 1991) were clearly observed (Fig. 4b). However, these structures were no longer associated with the plasma membrane and formed a cage-like skeletal delineation of the affected parasites. In

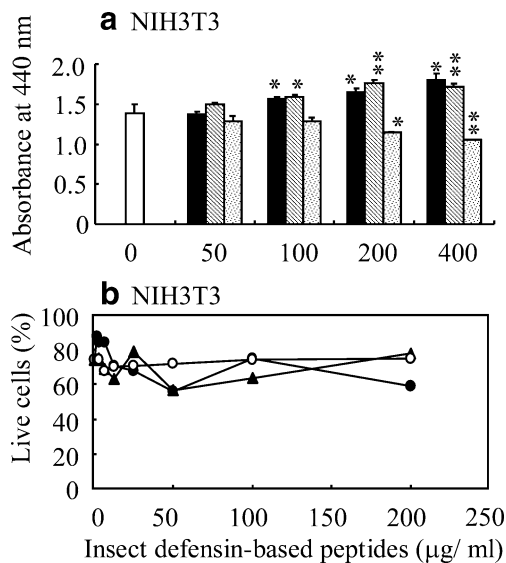


Fig. 5 Effects of insect defensin-based peptides on mouse NIH3T3 fibroblasts. **a** The growth analysis of NIH3T3 cells in the presence of insect defensin-based peptides. The cells were suspended in the growth medium at 2×10^4 cells per milliliter and seeded into a 96-well plate in triplicates (100 µL per well) with or without varying concentrations of the 9-mer peptides. After incubation for 48 h at 37°C in humidified 5% CO₂–95% air, cell growth was quantified by WST-1 colorimetric assay, as described in the “Materials and methods” section. Data represent the mean values \pm SEM from three culture wells. * $P < 0.05$, ** $P < 0.01$ between the peptide-treated and nontreated groups (after Student's *t* test). *Open bars* none, *closed bars* peptide A, *hatched bars* peptide B, and *dotted bars* control peptide. **b** Direct lytic effects of insect defensin-based peptides on NIH3T3 cells. The cells were suspended in PBS at 7.5×10^5 cells per milliliter, seeded into a 96-well plate in duplicates with or without varying concentrations of the 9-mer peptides, and incubated at 37°C in humidified 5% CO₂–95% air for 3 h. Cell viability was assessed by trypan blue dye exclusion test, and numbers of live and dead cells in the well were enumerated by hemocytometer counts. *Closed circles* peptide A, *closed triangles* peptide B, and *open circles* control peptide

comparison, the ultrastructures of the parasites treated with the control peptide at the same concentration were not significantly affected, showing intact plasma membrane and surface glycoprotein coat, as well as dense cytoplasm containing intact organelles (Fig. 4c). These morphological hallmarks were similar to those observed in the untreated healthy parasites (Fig. 4d).

Effects of insect defensin-based peptides on murine NIH3T3 fibroblasts

The growth inhibitory and cytolytic effects of insect defensin-based peptides on mammalian cells were examined on mouse NIH3T3 fibroblasts, using a WST-1 cell proliferation assay and trypan blue dye exclusion assay. As shown in Fig. 5a, the growth of NIH3T3 cells was not

significantly inhibited by peptides A and B at concentrations up to 400 µg/mL (330 µM). On the contrary, we observed some small growth-promoting effect at higher concentrations, which is statistically significant but not functionally significant. The control peptide did not show much growth inhibition on NIH3T3 cells, although a slight decrease in cell proliferation was observed between 200 and 400 µg/mL. As for the cytolytic effect of insect defensin-based peptides on NIH3T3 cells, none of the peptides showed significant cell killing at concentrations up to 200 µg/mL (165 µM) after exposure for 3 h (Fig. 5b). These results indicate that insect defensin-based peptides, which are effective in killing trypanosomes, do not affect the growth and plasma membrane integrity of mammalian cells.

Discussion

To expand our knowledge on trypanocidal effects of AMPs and their perspectives for therapeutic application, we compared the effects of synthetic 9-mer peptides designed on the basis of beetle defensin on several strains of African trypanosomes cultured in vitro as well as different life cycle stages. We observed moderate but clear growth inhibitory effects by the insect defensin-based peptides in different strains of BSF *T. congolense* as well as *T. brucei brucei* parasites in the complete growth medium. These insect defensin-based peptides exhibited more potency when parasites were suspended in PSG. The PCFs were more refractory to the peptides than the BSFs. Electron microscopy demonstrated that the primary targets affected by the peptides were parasite plasma membrane and surface coat. So, as reported previously (Madison et al. 2007; McGwire et al. 2003), our synthetic 9-mer peptides have trypanocidal activity primarily through its action on cell membrane integrity.

The BSF and PCF trypanosomes express different surface glycoproteins, and this might explain the differences in susceptibility to the insect defensin-based peptides between the two life cycle stages. Plasma membrane of the BSF parasites is heavily covered with VSG, and these coat proteins have protective roles in evading from the attack by the host's antibodies or complement (Davitz et al. 1987). On the other hand, the surface of PCF parasites is covered by another type of glycoprotein, consisting of procyclic acidic repetitive proteins (Treumann et al. 1997). So, VSG expressed on BSF parasite might have higher affinity to the insect defensin-based peptides due to its overall negative charges on the surface, as bacterial cell membrane does so. To support this hypothesis, we have recently found the presence of negatively charged phospholipids on the

surface of BSF *T. brucei brucei* GUT at 3.1 (Yamaga et al., submitted for publication). The content of phosphatidylserine in trypanosomes was approximately three times higher than that in pig erythrocytes. Therefore, the affinity of our insect defensin-based peptides for trypanosomes is likely to be the electrostatic interaction between the positively charged cationic peptides and the negatively charged parasite surface. Differences in charge and phospholipids composition between BSF and PCF parasites could explain why PCF are refractory to the insect defensin-based peptides, but additional studies would be needed to confirm this. In addition to the difference in the surface coat proteins, BSF parasites solely use glucose for energy production through glycolysis, whereas PCF parasites have more elaborated systems, the tricarboxylic acid cycle and the respiratory chain in the mitochondrion after glycolysis (Clayton and Michels 1996). So, as well as affecting the cell membrane integrity, the insect defensin-based peptides might interact with the glycolysis-related proteins in BSF parasites and exhibit cytotoxicity.

Our insect defensin-based peptides exhibited significant killing on BSF trypanosomes, whereas PCF trypanosomes were more resistant to the peptides. Several researchers reported similar observations. Insect-derived stomoxyn (Boulanger et al. 2002) was more effective in killing BSF than PCF parasites, as revealed in the in vitro assays with *Trypanosoma brucei rhodesiense*. In contrast, mammalian defensins and cathelicidins showed stronger parasitocidal activity on PCF rather than BSF *T. brucei* (McGwire et al. 2003). So, it may be speculated that BSF parasites have acquired some resistance to mammalian-derived AMPs, and on the other hand, PCF parasites do so against insect-derived AMPs through the evolutionary process. If so, our insect defensin-based peptides suggest the possibility for designing a new class of trypanocidal drugs against BSF African trypanosomes. Recently, antitrypanosomal activity of particular liposomes against African trypanosomes, including *T. congolense*, *T. brucei rhodesiense*, and *T. brucei brucei*, was reported (Kuboki et al. 2006). In this report, BSFs were more susceptible than PCFs to the liposome. There may be some similarity between the mechanisms of insect defensin-derived peptides and these liposomes, and understanding one may help understanding the trypanocidal mechanism of the other.

Compared to attacin derived from the tsetse fly (Hu and Aksoy 2005), which was effective at 10 μM , our insect defensin-based peptides need about 15 times more concentration (200 $\mu\text{g}/\text{mL}$ or 165 μM assayed in the growth medium) to exert the effect. In addition, the presence of divalent and monovalent cations, polyanions, sera, and proteases in the host's bloodstream may possibly antagonize the effect of the peptides. Nevertheless, neither cell viability nor growth of NIH3T3 cells was compromised by the insect

defensin-based peptides at 200 or 400 $\mu\text{g}/\text{mL}$, respectively. In addition, intraperitoneal injection of these peptides into mice revealed no significant toxicity (Koyama et al. 2006). As for the obstacle of proteolytic degradation, we have recently generated similar 9-mer peptides composed of only D-type amino acids. Interestingly, the D-type peptides showed stronger inhibitory effect than L-type peptides on the BSF *T. brucei brucei* (Yamaga et al., submitted for publication). Unlike L-type peptides, D-type peptides would be resistant to enzymatic degradation, such as reported in the case of AMP evasion by *Leishmania* (Kulkarni et al. 2006). D-type peptides also provoke little or negligible immune response in the host (Koyama et al. 2006). Taken together, synthetic insect defensin-based peptides promise to contribute to the development of a new class of membrane-disrupting trypanocidal drugs. The efficacy of these peptides, especially the D-types with further improvements and modifications, should be evaluated in trypanosome-infected animals.

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