Characteristics of Novel Insect Defensin-Based Membrane-Disrupting Trypanocidal Peptides

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ity. In addition, t-peptide B also protected the mice from infection with antibiotic-resistant pathogenic *Escherichia coli*. Recently, we synthesized t-type and D-type peptides A, B, C, and D, and analyzed them for antibacterial activity. We found that pathogenic bacteria such as MRSA and *Pseudomonas aeruginosa* are more susceptible to D-9-mer peptides (MIC range, 17–84 μM) than to t-9-mer peptides, composed only of t-amino acids (MIC range, 347–669 μM). Moreover, the D-9-mer peptides exhibited synergic and additive effects in combination with antibiotics.

Considering the rapid resurgence of trypanosomiasis and the ever-increasing incidence of drug resistance against conventional drugs, antimicrobial cationic peptides might provide a solution, but the trypanocidal mechanisms of peptides have not been explored in detail. In this study, we focused on t-type peptide A as our model, the trypanocidal effects of synthetic D-type peptide A in *vitro*.

**Materials and Methods**

**Biochemical and cell culture reagents.** The microculture plates used were obtained from Bio-One CELLSTAR (Greiner, Krefsmunster, Germany). Heat-inactivated fetal bovine serum (FBS) was from Multiser Thermon Electron Corporation (Melbourne, Australia). Serum Plus was from JRH Biosciences (Kansas, USA), and bathocuproine was from Tokyo Chemical Industry (Tokyo). Isov’s modified Dulbecco’s medium (IMDM), diethyloxacarbocyanine (MitoProbe DiOC<sub>2</sub>(3) Assay Kit Component A), and carbonyl cyanide 3-chlorophenylhydrazone (MitoProbe DiOC<sub>2</sub>(3) Assay Kit Component B) were obtained from Invitrogen (Oregon, USA). Isoton II Coulter Isoton Diluent was from Beckman Coulter (California, USA), while all other reagents were from Sigma (St. Louis, MO, USA).

**Synthetic peptides.** Using t- and t-amino acids to produce t- and D-type peptides respectively, modified 9-mer peptides based on beetle defensins from *A. dichotoma* and *O. rhinoceros*, as well as control peptide, were synthesized by SIGMA GENOSYS (Sigma-Aldrich Japan, Life Science Division, Tokyo, Japan). The amino acid sequences and molecular weights (MWs) of the synthesized peptides are shown in Table 1. Carboxy amidated and arginine rich defensin-based peptides A, B, C, and D, which possess net positive charges, between +3 and +5, have been found to be effective against antibiotic activity. While control peptide E, with a net charge of +1, lacks such activity. These peptides were dissolved in 100% DMSO diluted to 2 mM in IMDM, sterilized by filtration (0.22 μm), and stored frozen at −20 °C when not used immediately. The final concentration of DMSO in the culture did not exceed 0.5% (v/v).

The trypanosomes. *Trypanosoma brucei* GUTat3.1 bloodstream form parasites were maintained in HMI-9 medium containing IMDM supplemented with 10% heat-inactivated fetal bovine serum, 10% Serum Plus, 2 mM pyruvate, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 39 μg/ml of thymidine, 183 μg/ml of cysteine, 136 μg/ml of hypoxanthine and 100 μg/ml of β-mercaptoethanol. To monitor cell numbers, the parasite cultures were diluted 100-fold in Isoton II and quantified with a Z1 Coulter Counter (Beckman Coulter, California, USA). Before the experiments, the parasites were monitored for viability by light microscopy and counted on a hemocytometer in order to calibrate the threshold setting of the Coulter counter to 3 μm.

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**Table 1. Peptide Sequences and Properties**

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Sequence&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Net charge</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>RLYLRIGRR-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>+4</td>
<td>1201.49</td>
</tr>
<tr>
<td>B</td>
<td>RLRKRIGRR-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>+5</td>
<td>1195.48</td>
</tr>
<tr>
<td>C</td>
<td>AYLAIRRR-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>+3</td>
<td>1130.41</td>
</tr>
<tr>
<td>D</td>
<td>DRLRIRGRR-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>+4</td>
<td>1151.48</td>
</tr>
<tr>
<td>E</td>
<td>AKGFAANHS-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>+1</td>
<td>901.00</td>
</tr>
</tbody>
</table>

<sup>a</sup> t-type and t-type peptides contained only t- and t-amino acids respectively.

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**Determination of the minimum inhibitory concentrations (MICs) of t- and D-type synthetic 9-mer peptides required to inhibit trypanosome proliferation.** To determine the MICs of the peptides required to inhibit trypanosome proliferation, *T. brucei* GUTat3.1 was incubated in 96-well plates containing 200 μl of HMI-9 medium (5 × 10<sup>5</sup> cells/ml) and 31.25, 62.5, 125, 250, or 500 μM of t- or D-type peptides (Table 1). The culture plates were incubated under 5% CO<sub>2</sub> at 37 °C for 48 h. Cell numbers were counted as described above.

**Time course and modified Time-Kill analysis of T. brucei GUTat3.1 growth.** *Trypanosoma b. brucei* GUTat3.1 was cultured in 200 μl as described above in the presence of 125, 250, and 500 μM t-peptide A. Cell numbers were determined every 12 h for 48 h. As a negative control, t-peptide E was used instead of t-peptide A. For modified Time-Kill analysis, the parasites (2 × 10<sup>5</sup> cells/ml) were incubated in the presence of various concentrations of t-peptide A, ranging from 0 to 800 μM, in HMI-9 medium, as described above. A small portion (10 μl) of culture was sampled at 0, 5, 30, 60, 180, and 360 min, transferred to a well containing 200 μl of fresh HMI-9 medium without peptide, and cultured for another 48 h.

The effects of t- and D-type synthetic 9-mer peptides on trypanosome plasma membrane potential. To evaluate the effects of the peptides on the membrane potential of trypanosome plasma membranes, flow cytometry was performed on *T. brucei* GUTat3.1 stained with a potential-sensitive dye, diethyloxacarbocyanine. Prior to flow cytometric analysis, 2 × 10<sup>5</sup> trypanosomes were cultured in 2 ml of HMI-9 medium in the presence of the indicated concentrations of t-type peptide A (t-peptide A) or control t-peptide E for 3 h in a 12-well culture plate. As a positive control, trypanosomes were incubated for 5 min with 100 μM carbonyl cyanide 3-chlorophenylhydrazone (CCCP), an uncoupling agent that abolishes membrane potential. The trypanosomes were then washed once and resuspended in IMDM medium at the original concentration. After staining with 75 nM diethyloxacarbocyanine for 30 min, the trypanosomes were washed in IMDM, resuspended at 4 × 10<sup>5</sup> cells/ml in IMDM, and analyzed on a flow cytometer (EPICS, Beckman Coulter, USA) at 488 nm excitation and 535 nm emission for green fluorescence.

**Trypanosome ultrastructure.** Transmission electron microscopy was used to assess ultrastructural changes in the parasites as a consequence of peptide exposure. The samples were fixed in 2.5% glutaraldehyde in phosphate buffer. They were then packed into plastic hematocrit tubes, centrifuged at 8,000 g for 3 min, and postfixed in 1% osmic acid. The fixed specimens were then dehydrated serially in ethanol and embedded in epoxy resin. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate prior to microscopic examination using a Hitachi 7500 transmission electron microscope (Hitachi, Tokyo).

**Trypanosome phospholipid analysis.** Trypanosomes (50 μg, dry weight) were resuspended in 1% NaCl, followed by the addition of 3.75 ml chloroform/methanol (1:2, v/v). After vigorous shaking, the sample was centrifuged at 2,000 g for 10 min at room temperature and the supernatant was collected. Chloroform/methanol/H<sub>2</sub>O (1:2:0.8, v/v/v) (4.75 ml) was added to the precipitate and the sample shaken vigorously. After centrifugation, chloroform and H<sub>2</sub>O (2.5 ml each) were added to the supernatant, and the lower layer was collected after vigorous shaking and centrifugation. The extracted phospholipids were evaporated, dissolved in chloroform/methanol (1:1 v/v), and kept...
frozen until use. The extracted phospholipids (1/50 volume) were used in analysis by thin-layer chromatography using HPTLC plates Silica gel 60 (Merck, Germany). The chromatography was conducted with chloroform/methanol/petroleum ether/acetic acid/H2O (100:75:50:6.5/5 v/v) at room temperature. Phosphatidycholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidyl-

lipid (PI) and phosphatidic acid (PA) served as standards. Phospholipid spots were detected by spraying the plate with reagent solution (10% CuSO4/85% H3PO4) and heating at 150 °C for 15 min. The thin-layer chromatography pattern of the separated phospholipid spots was analyzed by Image Master 1D Elite ver. 300 (Amersham Biosciences, Uppsala, Sweden). Phospholipid contents were calculated based on standard curves of each phospholipid.

Measurement of surface PS density. The presence of PS on the surface of T. b. brucei GUT Tat3.1 was evaluated by staining with Annexin V-FITC and analysis by flow cytometry. Parasites were collected by centrifugation at 2,000 g for 10 min and suspended in 500 μl of binding buffer (Annexin V-FITC Apoptosis Detection Kit). Annexin V-FITC (5 μl) was added to the parasites and this was incubated for 5 min at room temperature in the dark. The mode of PS fluorescence of 10^6 parasites was determined by flow cytometry. In calculation of the relative surface PS density, the mode of PS fluorescence was divided by the average surface area (μm^2) of a single African trypanosome or pig erythrocyte (the control) based on published data.21,22

Incubation of L- and D-type synthetic 9-mer peptides with human brain microvascular endothelial cells (HBMEC). To assess the biocompatibility and/or toxicity of the L- and D-type synthetic 9-mer peptides on host tissues, we used a well-described in vitro model of the human blood-brain barrier (BBB) composed of human brain microvascular endothelial cells (HBMEC).23–26 Electric cell-substrate impedance sensing (ECIS) (Applied Biophysics)27,28 was used to measure HBMEC transendothelial electrical resistance (TEER) change27 in real-time during peptide exposure. HBMECs were grown in 400 μl of Medium 199 supplemented with 10% FBS in 8-well gold electrode arrays, and after they reached maximal steady state TEER, corresponding to maximal confluence, changes in the resistance of the endothelial cell monolayers were monitored every 80 s in response to experimental variables.

Results

Determination of the MICs for L- and D-type synthetic 9-mer peptides

The L- and D-type modified 9-mer peptides designated A, B, C, and D were synthesized on the basis of the active sites of defensins, antimicrobial peptides from two different beetle species (Table 1).7,8,10 Peptide E was not related to the active sites of the beetle defensins, and was synthesized as a negative control that had a random sequence with no antimicrobial activity.19 The D-type peptides showed stronger inhibitory effects than the L-type peptides on trypanosome proliferation in vitro (Table 2). MICs of D-peptide A, B, C, and D ranged from 80–116 μM, whereas control peptide E revealed more than 500 μM (Table 2). The results indicated that our 9-mer peptides, especially D-types have the inhibitory effect on the growth of not only bacteria,10 but also trypanosomes.

Effect of D-peptide A on trypanosome growth

D-Peptide A, the most extensively studied antimicrobial synthetic peptide with regard to its strong effects on pathogenic bacterial multiplication10 and low antigenicity,19 was used to further assess its inhibitory effects on T. b. brucei GUT Tat3.1 growth. The parasites collected in log-phase growth were cultured in triplicate with 0–500 μM d-peptide A or D-peptide E as a negative control and monitored 12 h for growth. It was found that trypanosome proliferation was not strongly affected by D-peptide E (Fig. 1A). On the other hand, the growth of those cultured in the presence of D-peptide A was significantly retarded at all concentrations tested (Fig. 1A).

To determine whether the parasites would remain dormant during 48 h in cell culture or would be irreversibly damaged, a modified Time-Kill analysis was performed. When incubated with bloodstream-form T. b. brucei GUT Tat3.1, D-peptide A at concentrations of 400 μM and 200 μM irreversibly inhibited parasite proliferation, by 3 and 6 h respectively (Fig. 1B). D-Peptide A at 800 μM also induced an irreversible inhibition of growth within 5 min of exposure (Fig. 1B). On the other hand, the parasites exposed to 100 μM of D-peptide A for less than 360 min were still able to recover a normal level of cell proliferation, one identical to parasites cultured in medium without suppressive agents (Fig. 1B).

Table 2. MICs of L- and D-Type Peptides for Growth of T. b. brucei GUT Tat3.1

<table>
<thead>
<tr>
<th>Peptides</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E*</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-type</td>
<td>205 ± 22b</td>
<td>500 ± 0</td>
<td>125 ± 0</td>
<td>325 ± 75</td>
<td>N.D.*</td>
</tr>
<tr>
<td>D-type</td>
<td>116 ± 16</td>
<td>90 ± 31</td>
<td>95 ± 12</td>
<td>80 ± 17</td>
<td>&gt;500</td>
</tr>
</tbody>
</table>

*Control peptide.

bThe lowest concentrations from the dose-response curves yielding over 95% suppression of bloodstream-form T. b. brucei GUT Tat3.1 were considered as MIC. MIC values were based on the mean ± S.D. from three to 11 experiments. The difference between L and D-types for all peptides was significant (p < 0.05, paired Student’s t-test).

n.D., not determined.

Alteration of trypanosome membrane-potentials by d-peptide A correlated with the suppression of parasite growth

Alteration of trypanosome membrane-potentials by D-peptide A was analyzed by flow cytometry. Figure 2 shows the potential-dependent uptake of 3,3’-diethyloxacarbocyanine iodide [DiOC(3)] within trypanosomes as revealed by detection of 535 nm green fluorescence, which is associated with aggregated dye. Trypanosomes cultured for 3 h without peptide A showed features of hyperpolarization, as indicated by a high accumulation of green fluorescence (Fig. 2a). The same features were observed in parasites similarly treated with 200 μM D-peptide E (Fig. 2e). Features of depolarization were also evident in the disappearance of the fluorescent dye in the cells treated with 400 μM D-peptide A concomitant with an increased number of cells with low fluorescent dye intensities (Fig. 2d). These features were analogous to findings with trypanosomes that were treated with the depolarizing ionophore CCCP for 5 min (positive control) prior to staining with fluorescent dye (Fig. 2f). As found with CCCP, the lack of dye accumulation in the trypanosomes treated with 400 μM peptide A indicated a thorough dissipation of the membrane potentials of these cells. The intermediate states, showing partial depolarization of the cells exposed to 200 μM peptide A for 3 h coincided with the 50% inhibition of growth (Fig. 1B).
Alteration in trypanosome ultrastructure following D-peptide A exposure

Transmission electron microscopy was used to assess the way the trypanosomes are damaged by peptide treatment (Fig. 3). The ultrastructure of the untreated parasites showed normal intact plasmalemma, and the surface coat, kinetoplast, flagellar pocket, nucleus, and other membrane-encased organelles (Fig. 3a and b). However, trypanosomes treated with 400 μM D-peptide A for 3 h exhibited profound ultrastructural changes. A complete loss of the plasma membrane was observed (Fig. 3c). As a consequence, the cytoplasmic contents were largely missing, a typical feature of cell lysis. When the parasites were sectioned perpendicular to the longitudinal axis, the subpellicular microtubles, stripped of the plasma membrane, maintained a basket-like structure and string-of-beads characteristic with a diameter of 25.7 ± 6.5 nm (n = 4) (Fig. 3d). The profound disruption of the parasite plasma membranes that occurred at the concentration of D-peptide A used also coincided with a thorough collapse of the membrane potentials and irreversible inhibition of the cell growth.

Analysis of T.b. brucei GUTat3.1 phospholipid content and PS surface density

The phospholipid content of T.b. brucei GUTat3.1 was first analyzed to confirm that the parasites'
membrane contained PS. The PS content was 5.6% of total phospholipids, including PC, PI, PE, and PA (Table 3). Flow cytometric analysis indicated 3.7 × 10⁻² relative PS density/μm² on the surface of *T. brucei* GUTat3.1. Pig erythrocyte has 1.3 × 10⁻² relative PS density/μm² on the surface, as a background (Table 4).

### Table 3. Phospholipid Content of *T. brucei* GUTat3.1

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Relative phospholipid content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>49.1</td>
</tr>
<tr>
<td>PS</td>
<td>5.6</td>
</tr>
<tr>
<td>PI</td>
<td>18.0</td>
</tr>
<tr>
<td>PE</td>
<td>25.4</td>
</tr>
<tr>
<td>PA</td>
<td>1.9</td>
</tr>
</tbody>
</table>

### Table 4. Surface PS Density of *T. brucei* GUTat3.1 and Pig Erythrocyte

<table>
<thead>
<tr>
<th></th>
<th><em>T. brucei</em> GUTat3.1</th>
<th>Pig erythrocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mode fluorescence intensity (RU⁴)</td>
<td>3.8</td>
<td>1.4</td>
</tr>
<tr>
<td>Average surface area (μm²)</td>
<td>103</td>
<td>105</td>
</tr>
<tr>
<td>Relative surface PS density (RU/μm²)</td>
<td>0.037</td>
<td>0.013</td>
</tr>
</tbody>
</table>

⁴RU, relative unit

Effects of t- and d-type synthetic 9-mer peptides on HBMEC

To be selectively toxic against trypanosomes, d-peptide A should be compatible with the host cells, exhibiting minimum effects, if any, at concentrations below the minimum concentration toxic to trypanosomes. ECIS was used to assess the effects of d-peptide A on HBMEC monolayer integrity in real time. The HBMEC were incubated in the presence of 50, 100, 200, and 400 μM d-peptide A or 1,100 μM d-peptide E. Medium alone was the control. ECIS revealed a dose-dependent pattern with minor changes in HBMEC monolayer integrity when the endothelium was exposed to d-peptide A at concentrations, less than 200 μM (Fig. 4), conditions that cause irreversible suppression of trypanosome growth (Fig. 1). While a decrease in TEER due to d-peptide A was observed over a 20-h exposure, no suppression in TEER over the same time period was observed for the control d-peptide E, even at a 1,100 μM concentration. Taken together, the data suggest that the difference in the effects of d-peptide A between mammalian cells and trypanosomes provides evidence that d-peptide A does show selective toxicity against trypanosomes.

### Discussion

We examined the trypanocidal potential of beetle defensin-based cationic 9-mer peptides against bloodstream-form *T. brucei* GUTat3.1 as our parasite model. It is of interest that the significant antitrypanosomal effect observed with d-peptide A concentration (MIC = 116 μM) was lower than t-peptide A (MIC = 205 μM). For comparison, attacin derived from the tsetse fly, *Glossina morsitans morsitans*, shows potent antitrypanosomal activity at 10 μM. As with other pathogens, the resistance of d-type peptides to enzymatic degradation by the parasite probably contributes to their overall increased potency over their t-type counterparts.

The finding that the kinetic changes in the membrane potentials of the population of the trypanosomes exposed to 200 μM d-peptide A for 3 h were biphasic (Fig. 1B) indicates that a portion (more than half) of the parasites sustained a state of hyperpolarization, while...
a function of TEER in real time using ECIS. It was mammalian cells, we assessed monolayer integrity as an antimicrobial peptide derived from porcine leukocytes.5) that observed for parasites treated with protegrin-1, an a ghostlike appearance to the trypanosomes, similar to the loss of the plasma membrane (lysis) giving was complete dissipation of the membrane potential due to the rest of the parasites were capable of recovering. However, when the parasites were incubated for longer times or with higher D-peptide A concentrations, there was complete dissipation of the membrane potential due to the loss of the plasma membrane (lysis) giving a ghostlike appearance to the trypanosomes, similar to that observed for parasites treated with protegrin-1, an antimicrobial peptide derived from porcine leukocytes.5) Using a well-established model of human BBB composed of HBMEC27) to monitor potential toxic effects on the rest of the parasites were well tolerated by the cells.

Our membrane depolarization and electron microscopic studies suggest an interaction of D-peptide A and trypanosome membranes. It is generally thought that prokaryotes have negative charges on the surface of the cells, whereas eukaryotes have no charges. Because African trypanosomes are eukaryotes, this raised the question as to how D-peptide A is able to interact with the variable surface glycoprotein coated plasma mem-

brane of the parasite. This question prompted us to analyze the presence of negatively charged phospholipids on the surface of T. b. brucei GUTat3.1. We focused on the possible role of PS because of the presence of negatively charged phospholipid’s role in normal physiology, and pathology and because it was technically possible to detect its presence on the surface of trypanosomes using fluorescein-labeled annexin. Normally, PS is located totally in the inner leaflet of mammalian cells in an exclusively asymmetric distribution, although most membrane phospholipids indicate some preference for either leaflet.31) However, surface exposure of PS occurs in cancer cells,32) apoptotic cells,33) and sickle red blood cells,34) as a signal for recognition and removal of these cells by macrophages.35) Our results provide for the first time direct evidence of the presence of negatively charged phospholipids on the surface of trypanosomes, which might explain the principle mechanism by which peptides interact with trypanosomes. It is predicted that electrostatic interaction between cationic peptide drugs and the negatively charged plasma membrane components (e.g., PS) is required for trypanocidal activity.

In summary, at the moment safe drugs for the treatment of HAT are sadly lacking. Malarsoprol, given to patients when parasites are suspected to have entered the central nervous system, kills 5% of all patients receiving it, but left untreated, 100% of patients with HAT die.36) Furthermore, the incidence of Malarsoprol-refractory infections is increasing, and alternative treatment regimens are urgently required. We have found that D-peptides synthesized on the basis of insect defensins destroy trypanosome plasma membrane function, leading to the arrest of parasite proliferation. Since the physical properties of the structural membrane are unlikely to change readily, membrane-disrupting peptides represent a promising candidate when the parasites have developed a resistance to conventional drugs. Furthermore, using our present set of beetle-derived defensin-based peptides as a scaffold on which further improvements can be made by manipulating the residues, anti-trypanosome peptides of high therapeutic efficacy can be realized.

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