Inhibition of malaria parasite blood stages by tyrocidines, membrane-active cyclic peptide antibiotics from Bacillus brevis

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Abstract

Tyrothricin, a complex mixture of antibiotic peptides from Bacillus brevis, was reported in 1944 to have antimalarial activity rivaling that of quinine in chickens infected with Plasmodium gallinaceum. We have isolated the major components of tyrothricin, cyclic decapeptides collectively known as the tyrocidines, and tested them against the human malaria parasite Plasmodium falciparum using standard in vitro assays. Although the tyrocidines differ from each other by conservative amino acid substitutions in only three positions, their observed 50% parasite inhibitory concentrations (IC50) spanned three orders of magnitude (0.58 to 360 nM). Activity correlated strictly with increased apparent hydrophobicity and reduced total side-chain surface area and the presence of ornithine and phenylalanine in key positions. In contrast, mammalian cell toxicity and haemolytic activities of the respective peptides were considerably less variable (2.6 to 28 μM). Gramicidin S, a structurally analogous antimicrobial peptide, was less active (IC50 = 1.3 μM) and selective than the tyrocidines. It exerted its parasite inhibition by rapid and selective lysis of infected erythrocytes as judged by fluorescence and light microscopy. The tyrocidines, however, did not cause an overt lysis of infected erythrocytes, but an inhibition of parasite development and life-cycle progression.

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1. Introduction

Malaria is endemic in 107 third world countries, with an estimated 402 million clinical malaria cases in 2004 [http://www.rbm.who.int/wmr2005/; 1]. This level of incidence correlates with 0.7 to 3 million deaths annually [1], of which more than 70% are children under the age of 5 years. Of particular concern is the estimated 270–400 million malaria cases [1] caused by Plasmodium falciparum, of which a large number of strains are drug resistant, in particular towards chloroquine [2]. The global resistance of malaria parasites towards chloroquine is probably the consequence of its extensive use as antimalarial drug [2,3]. Reports of field strains of P. falciparum demonstrating in vivo resistance to the artemisinins, recently introduced as antimalarial drugs, are also disconcerting [4,5]. There is consequently an urgent need for new effective antimalarial agents.

Since the discovery of penicillin, a variety of secondary metabolites from fungi and bacteria (i.e. staphylococcus, streptomyces and bacilli species) have been identified and applied as antibiotics and chemotherapeutic agents [6]. An extremely active search was initiated after the Second World War for new antibiotics against a variety of pathogens, including Plasmodium species [7]. During this period many compounds, such as gramicidin D and tyrothricin from Bacillus brevis strains, were found to be active against malaria parasites [8]. The activity of tyrothricin was observed to rival that of quinine when tested in chickens infected with Plasmodium gallinaceum. However, neither tyrothricin nor gramicidin D reached the status of an antimalarial drug. The potent antimalarial activity of gramicidin D has been revisited recently.
The activity of the component in tyrothricin has, as yet, not been further investigated and thus warrants closer inspection, albeit more than 60 years later.

Tyrothricin is a complex mixture of *B. brevis* peptides and can contain the nine ribosomally produced linear gramicidins (gramicidin D) as well as up to 28 different non-ribosomally produced tyrocidines and tryptocidines [11]. The previously reported tyrothricin antimalarial activity [8] could be attributable to gramicidin D. However, the larger fraction of tyrothricin is composed of the tyrocidines [11] and it is therefore likely that these peptides also possess antiplasmodial activity. Furthermore, gramicidin S, a *Brevibacillus brevis* cyclic peptide antibiotic closely related to tyrocidines, has been shown to be antiplasmodial [9,12]. The tyrocidines also share with gramicidin S the features of broad-spectrum antibacterial and potent haemolytic activities [13–16]. It is accepted that the activity of most antimicrobial peptides is due to their membranolytic properties [17–19] and both tyrocidine A and gramicidin S have been shown to disrupt membrane permeability [20,21]. The membrane active *B. brevis* peptides may be an attractive group of antimalarial compounds, since their lytic mechanism of action conceivably makes it difficult for pathogens to develop resistance. This prompted us to isolate and investigate the parasite-inhibitory properties of the major individual tyrocidines in the tyrothricin complex and gramicidin S using standard dose–response assays and light microscopy.

### 2. Materials and methods

#### 2.1. Peptides

Gramicidin S from *Brevibacillus brevis* (Nagano) and tyrothricin from *Bacillus brevis* were purchased from Sigma-Aldrich (Steinheim, Germany). The tyrocidines were purified from tyrothricin by reverse phase HPLC and analysed for purity by electrospray mass spectrometry (ESMS) and ultra performance liquid chromatography linked to mass spectrometry (UPLC-MS), as described below.

Insoluble particulates in purified peptide preparations were removed beforehand by centrifugation (10 min at 8600 *g*). In order to ensure sterility, all the peptides were dissolved in 50% acetonitrile and freeze-dried in pyrolysed glassware (washed dry glassware was heated to 560 °C for 1 h). The peptides were weighed using an analytical protocol (drying of peptides under vacuum before duplicate/triplicate weighing on a 6-digit analytical scale). The scale chamber was kept free of water by placing dry phosphorpentoxide in a beaker inside the chamber. The analytically weighed peptide fractions were used to prepare 1.00–3.00 mg/mL stock solutions in 50% ethanol shortly before each assay. The concentration of each stock solution was confirmed by amino acid analysis. Dilutions of stock solutions were done with RPMI-1640 culture medium.

#### 2.2. HPLC fractionation of tyrocidines

The crude tyrocidine fraction was prepared by washing 50 mg of the commercially obtained tyrocidine three times with 5 mL aceton:diethyl ether (1:1, v/v) [22]. The undissolved peptide material was collected by centrifugation and dried under vacuum before further HPLC purification. Semi-preparative HPLC was used to purify tyrocidines A, A1, B, B1, C and C1 from the crude samples. Samples (20 mg/mL) were dissolved in 50% HPLC-grade acetonitrile, centrifuged for 10 min at 8600 *g* to remove particulates and subjected to purification on a semi-preparative Novapak HR C18 HPLC column (6 μm spherical particles, 300 mm × 7.8 mm; Millipore-Waters, La Jolla, USA). A linear gradient over 23 min at a flow rate of 3.0 mL/min was created from 50% eluant A (0.1% trifluoroacetic acid in analytical quality water) to 80% eluant B (90% acetonitrile and 10% A).

The tyrocidines A and A1 were further purified on an analytical C18 Novapak HPLC column (4 μm spherical particles, 60 Å pore size; 150 mm × 3.9 mm; Millipore-Waters, La Jolla, USA), using the same gradient with a 1.0 mL/min flow rate. The chromatography was monitored at 254 nm with a Waters Model 440 UV-detector. Peptide fractions were collected, lyophilised and analysed with ESMS and UPLC-MS. The same protocol was also used for analytical runs.

#### 2.3. ESMS and UPLC-MS of peptides

Electrospray ionisation mass spectrometry (ESMS) was performed on a Waters Q-ToF Ultima mass spectrometer fitted with a Z-spray electrospray ionisation source. Five or 10 μL of the sample solution (50 or 100 ng peptide in 50% acetonitrile) was introduced into the ESMS via Waters Acuity UPLC™. The carrier solvent was 50% acetonitrile in 0.1% formic acid, delivered at a flow rate of 300 μL/min during each analysis. A capillary voltage of 3.5 kV was applied, with the source temperature set at 100 °C and cone voltage at 35 V. Data acquisition was in the positive mode, scanning the second analyser (MS2), through *m/z* = 100–1999 (*m/z* is defined as the molecular mass to charge ratio). Representative scans were produced by combining the scans across the elution peak and subtracting the background.

UPLC-MS analysis was done on a Waters Acuity Ultra Performance Liquid Chromatograph connected to the above Waters Q-ToF Ultima mass spectrometer. Separation was achieved on a Waters UPLC BEH C18 column (2.1 × 50 mm, 1.7 μm spherical particles, Millipore-Waters, La Jolla, USA), using a 0.1% trifluoroacetic acid (A) to acetonitrile (B) gradient (100% A for 30 s, 0 to 30% B from 30 to 60 s, 30 to 60% B from 1 to 10 min, 60 to 80% B from 10 to 15 min at a flow rate 300 μL/min), followed by re-equilibration of the column to initial conditions.

#### 2.4. Parasite viability and haemolysis dose–response assays

*P. falciparum* (3D7 strain) was cultured under an atmosphere of 3% CO2, 4% O2 and 93% N2 in RPMI-1640 medium supplemented with 50 mM glucose, 0.65 mM hypoxanthine, 25 mM HEPES, 0.02% (w/v) NaHCO3, 0.048 mg/mL gentamicin, 0.5% (w/v) Albumax II and 2–4% (v/v) human O+ erythrocytes. Culture-derived parasitised erythrocytes were mixed with fresh culture medium and erythrocytes to yield a 2% parasitemia and 2% haematocrit suspension. Duplicate plates containing normal erythrocytes (2% haematocrit, 2.0 × 106 cells/mL), to determine haemolytic activity of the peptides, were also incubated for 48 h. After incubation the intact erythrocytes were sedimented in the microtitre plate wells by centrifugation at 200 g for 3 min in a swing-out rotor. The released haemoglobin was determined in the supernatants from their absorbance at 405 nm of a 1:8 dilution in water.

#### 2.5. Toxicity assays

HeLa cervical cancer cells and A549 human pulmonary epithelial cells were cultured in DMEM supplemented with 10% foetal calf serum and penicillin/streptomycin/fungizone. One day prior to drug pressure, cells were transferred to 96-well plates at a density of 1.5 × 104 cells/well. Serial dilutions of peptides in media was added to the parasite suspension. The plates were incubated at 37 °C for 48 h and parasite viability in each well was determined from the residual lactate dehydrogenase activity [23]. Duplicate plates containing normal erythrocytes (2% haematocrit, 2.0 × 105 cells/mL), to determine haemolytic activity of the peptides, were also incubated for 48 h. After incubation the intact erythrocytes were sedimented in the microtitre plate wells by centrifugation at 200 g for 3 min in a swing-out rotor. The released haemoglobin was determined in the supernatants from their absorbance at 585 nm in a microtitre plate reader.

#### 2.6. Dose–response data analysis

Tripletic or quadruplicate assays were performed for each peptide against each target cell. All dose–response data was analysed using Graphpad Prism version 3.01 for Windows (GraphPad Software, San Diego, California USA; www.graphpad.com). Absorbance values were converted to percentage parasite survival.
viability (in the case of the lactate dehydrogenase assays), percentage haemolysis or percentage HeLa and A549 cell death (CellTiter Blue™ assay) and non-linear regression (sigmoidal curve with variable slope) was performed on the dose–response data. Total growth was determined from control wells with no peptide added, while total haemolysis was determined by the addition of 200 μM gramicidin S or 250 μg/mL saponin.

The 50% P. falciparum inhibitory concentration (IC50), 50% haemolytic concentration (HC50) and 50% lethal concentration (LC50) for HeLa and A549 cells were calculated as described by Rautenbach et al. [24]. Apparent selectivity index.

2.7. Light and fluorescence microscopy

Peptides were added to P. falciparum cultures (2% parasitemia, 2% haematocrit) at concentrations equal to or above their IC50 values and Giemsa-stained blood smears prepared at various incubation time points for examination by light microscopy. Alternatively, aliquots were removed from the cultures, directly mounted on microscope slides under glass cover slips and viewed by phase-contrast light microscopy. For fluorescence microscopy analysis, aliquots removed from peptide-treated cultures were mixed with 4',6-diamidino-2-phenylindole (DAPI) and trypan blue in PBS (final concentrations 1 μM and 0.5%, respectively), mounted under coverslips on microscope slides and viewed by epifluorescence illumination using tetramethyl-rhodamine and ultraviolet filters. Microscopy assays were done on a Nikon Eclipse E600 fluorescence microscope fitted with a 100× Apochromat objective and images captured with a Media Cybernetics CoolSNAP-Pro monochrome cooled CCD camera.

3. Results and discussion

3.1. Peptide preparation

The tyrothricin complex from Sigma contained six major tyrocidines, with tyrocidine B the most abundant (33.8%) and tyrocidine C1 the least abundant, as calculated from UPLC peak areas (Fig. 1, Table 1). Tyrothricin was soluble in aqueous mixtures of organic solvents (acetonitrile or alcohol), but concentrations higher than 5 mg/mL resulted in aggregation and resultant loss of solubility within 30 min. Extraction of tyrocidines from tyrothricin, with ≥60% mass yield, led to improved solubility in 50% acetonitrile and we were able to dissolve up to 20 mg/mL for HPLC purification. We successfully purified the six major tyrocidines to >90%, as determined by analytical HPLC. “Impurities” was predominantly the result of a slight overlap in the collected peak fractions of the major tyrocidines. Purity and identity of the isolated peptides was confirmed by amino acid analysis (results not shown), ESMS and UPLC-MS (Table 1).

3.2. Activity of the purified peptides

The activity of the six tyrocidines and the related gramicidin S was evaluated against the blood stages of P. falciparum using a 48-h dose–response assay. The timing of the assay allows viable parasites to complete their full 48-h developmental cycle. Following erythrocyte invasion, the first stage of the parasite lasts approximately 16–20 h and is known as the ring stage due to the ring-like appearance of the parasites by light microscopy (refer to Fig. 6). The rings gradually enlarge and develop into trophozoites by 20–24 h after invasion. The trophozoites continue to expand and fill most of the infected erythrocyte volume. The schizont stage follows after approximately 36–40 h, when the nucleus undergoes multiple divisions. At the end of the schizont stage the plasma membrane invaginates around each nucleus to give rise to daughter parasites (merozoites) that are released when the infected erythrocyte ruptures. The merozoites rapidly invade fresh erythrocytes to re-initiate the 48-h cycle. For any particular peptide, dose–responses were indistinguishable when the 48 h incubations were initiated with synchronized cultures containing ring or the more mature trophozoite stages of the parasite. Gramicidin S was found to be the least active of all the peptides and only eight times more inhibitory towards parasite growth (IC50 at 1.3 μM) than lytic towards uninfected erythrocytes (Table 2). All six the tyrocidines had IC50 values in the nanomolar range (Figs. 2

![Fig. 1. UPLC™-MS chromatogram of tyrothricin. The top value given for each peak is the retention time and the value below is either the m/z of [M+H]⁺ or [M+2H]²⁺ molecular species of the major component in the eluting peak. The identities of the eluting compounds and chromatographic conditions are detailed in Table 1 and Materials and methods, respectively. Compounds in the peaks indicated with asterisks were investigated.](image-url)
The % abundance is derived from UPLC peak areas.

Table 1

<table>
<thead>
<tr>
<th>Peptide (Abbr.)</th>
<th>Structure</th>
<th>Mr exp (monoisotopic Mr)</th>
<th>Retention time (min): UPLC; HPLC</th>
<th>Abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrocidine A (O-FF) [25]</td>
<td>cyclo [VOLFPFNQY]</td>
<td>1269.68 (1269.7)</td>
<td>9.46; 11.54</td>
<td>23.6</td>
</tr>
<tr>
<td>Tyrocidine A1 (K-FF) [26]</td>
<td>cyclo [VKLPFNNQY]</td>
<td>1283.69 (1283.7)</td>
<td>9.29; 10.92</td>
<td>8.5</td>
</tr>
<tr>
<td>Tyrocidine B (O-WF) [25]</td>
<td>cyclo [VOLFPFWNQY]</td>
<td>1308.74 (1308.7)</td>
<td>8.32; 9.42</td>
<td>33.8</td>
</tr>
<tr>
<td>Tyrocidine B1 (K-WF) [26]</td>
<td>cyclo [VKLPFWNQY]</td>
<td>1322.78 (1322.7)</td>
<td>8.15; 8.84</td>
<td>12.8</td>
</tr>
<tr>
<td>Tyrocidine C (O-WW) [25]</td>
<td>cyclo [VOLFPVVNQY]</td>
<td>1347.76 (1347.7)</td>
<td>7.39; 7.75</td>
<td>16.5</td>
</tr>
<tr>
<td>Tyrocidine C1 (K-WW) [26]</td>
<td>cyclo [VKLPVVNQY]</td>
<td>1361.79 (1361.7)</td>
<td>7.27; 7.28</td>
<td>4.8</td>
</tr>
<tr>
<td>Gramicidin S [27]</td>
<td>cyclo [VOLFPOLLFP]</td>
<td>1140.73 (1140.7)</td>
<td>na; 1.41</td>
<td>–</td>
</tr>
</tbody>
</table>

Gramicidin S data are given for comparison. Abbreviations used for peptides indicate the identities of the amino acid residues in the three variable positions.

tyrocidines ranged between 2.6 and 8.6 μM, these values were 10 to $8 \times 10^3$ times higher than their corresponding IC$_{50}$ values, with tyrocidine A having the highest apparent selectivity index (Table 2, Figs. 2 and 3). A comparison of Figs. 2A and 3A clearly shows that the selectivity for the infected erythrocytes closely followed the activity-sequence trend.

The toxicity of the peptides was evaluated against HeLa and A549 cells (Table 2 and Fig. 2). The observed LC$_{50}$ s of the tyrocidines ranged between 9 and 28 μM. Gramicidin S showed identical toxicity towards the two eukaryotic cells, but all six the tyrocidine preparations were slightly less toxic towards the A549 cells. The LC$_{50}$ values were between 25 and $4 \times 10^3$ times higher than the corresponding IC$_{50}$ values, with tyrocidine A again having the highest apparent selectivity index (Table 2, Figs. 2 and 3).

3.3. Structure–activity relationships

We opted to use two parameters (apparent hydrophobicity and side chain surface area) to represent aspects of the overall chemical and structural character of the peptides. We used retention time (Rt) on a C$_{18}$ column as a measure of hydrophobicity and, although it is simplistic, we found it a useful parameter in our structure–activity studies. The two parameters were correlated with biological activity and selectivity, without an assumption of mechanism of action or specific target.

An increase in tyrocidine activity against P. falciparum correlated with an increase in retention on C$_{18}$-UPLC (and analytical C$_{18}$-HPLC), which is related to apparent hydrophobicity (Fig. 2). This suggests that overall hydrophobicity is a major determinant of the anti-parasitic activity of the peptides. The composition of the aromatic dipeptide unit, containing Phe and/or Trp, is the main distinguishing feature of the individual tyrocidine peptides (Table 1). Besides conferring differences in hydrophobicity, Phe and Trp also differ greatly in surface area [29,30]. We found an inverse linear correlation ($R^2=0.91$) between the Σ(side chain surface area) of the tyrocidines and antiplasmodial activity (Fig. 2B). This suggests that steric factors may influence antiplasmodial activity in addition to hydrophobicity. Interestingly, we also found a good linear inverse correlation ($R^2=0.95$) between retention time and side chain surface area, which suggests that the interaction of the larger Trp and Lys side chains with the C$_{18}$-acyl chains is weaker.

Although a number of studies on antimicrobial peptides proposed that hydrophobicity is directly related to haemolytic activity [28,29], we found no clear relationship between haemolytic activity or mammalian cell toxicity and retention on the hydrophobic C$_{18}$ columns (Table 2, Fig. 2A). Consequently, selectivity for the infected erythrocyte or parasite mirrored the antiplasmodial trends: selectivity depends on the apparent hydrophobicity of the isolated tyrocidine and the selectivity index increases with an increase in retention on the C$_{18}$ matrix (Fig. 3A). In addition, there is a good exponential correlation ($R^2=0.999$) between the Σ(side chain surface area) and selectivity for infected erythrocytes (Fig. 3B).

Further correlations were found between activity and the primary structure of the major tyrocidine in each preparation. The presence of Lys instead of Orn in the conserved VOLFP pentapeptide results in a marked decrease in antiplasmodial activity, which further correlates with a decrease in haemolytic activity and toxicity (compare K-FF, K-WF and K-WW with O-FF, O-WF and O-WW respectively in Table 2 and Fig. 3A). This suggests that lytic activity may contribute to tyrocidine action which may in turn depend on the chemical character of the Orn/Lys side chains in the cyclic peptide structure. The side chains of Lys and Orn differ due to the ε- and γ-amino groups ($pK_a=10.54$ vs. $pK_a=10.76$ respectively) [31] and in terms of hydrophathy ($-9.9$ and $-9.0$ respectively, according to Tossi’s
These differences can thus influence activity in a membrane environment, or binding to a different target.

Our data further indicate that the first Phe in the aromatic dipeptide unit is an important determinant of antiplasmodial activity and specificity. The presence of Trp instead of Phe in the first position of the FFNQY pentapeptide moiety produced a pronounced decrease in antiplasmodial activity (70 and 105 times for the Lys and Orn analogues respectively), but has little effect on haemolytic activity and cytotoxicity (Fig. 3, Table 2). Trp instead of Phe in the second position, however, does not markedly influence activity (1.5 and 4.8 times for the Lys and Orn analogues respectively).

Gramicidin S eluted much earlier from the C18 column than any of the tyrocidines and was much less active than the tyrocidines (Tables 1 and 2), however, it retained a comparable haemolytic activity and showed a higher toxicity. Gramicidin S lacks the FFNQY pentapeptide unit and has two VOLFP pentapeptide moieties, indicating the importance of the FFNQY sequence in antiplasmodial activity. It is possible that the cytolytic activity depends on the VOLFP sequence, while the FFNQY sequence might promote a non-lytic translocation which in turn depends on the binding mode, as explained below.

In the light that binding to the target cell membrane is possibly the first committed step in the tyrocidines mechanism of action, the above structure–activity correlations may also be

Fig. 2. Relationship between peptide activity and peptide hydrophobicity (A) or \( \Sigma \) (side chain volume) (B) (A) Peptide activity against mammalian cells (LC50), erythrocytes (HC50) and *P. falciparum* parasites (IC50) vs. apparent hydrophobicity, as determined by retention on a C18-UPLC column. (B) IC50 vs. peptide \( \Sigma \) (side chain volume). The linear fit has a \( R^2 = 0.91 \). Side chain surface areas are from [29], as calculated from Connolly surfaces [30]. Refer to Table 1 for an explanation of abbreviations and peptide identities.

Fig. 3. Relationship between the selectivity index and (A) apparent hydrophobicity, as determined by retention on a C18 column, and (B) \( \Sigma \) (side chain volume), the insert shows the exponential fit to the data, \( R^2 = 0.999 \). The selectivity index was calculated from IC50/LC50 and IC50/HC50 ratios. Side chain surface areas are from [29], as calculated from Connolly surfaces [30]. Refer to Table 1 for abbreviations and peptide identities.
explained by the binding mode of certain amino acid residues to the target membrane. Aromatic amino acid residues tend to prefer the membrane interface between head-groups and lipid layer—Trp has a shallow insertion and is inclined to form hydrogen bonds via its NH-group with fatty-acyl carbonyl groups [33], while Phe inserts deeper into the bilayer [34,35]. There are also differences between the binding of Orn and Lys, with the longer Lys side-chain binding stronger due to a “snorkel effect” (the longer butylene chain interacts with the acyl chains, while the amino group remains associated with the head groups in the aqueous phase) [36]. During binding, Phe and Lys would cause deeper insertion of the cyclic peptide into the membrane, while Trp and Orn would lead to shallower binding. It is therefore possible that two phenylalanines, rather than one (for example the first Phe), are necessary in the aromatic dipeptide unit to ensure deep penetration into the membrane during binding. On the other hand, the tighter binding of Lys may be detrimental to the tyrocidine mechanism of action, for example, by causing the peptide to be trapped in an unfavourable conformation. This differential binding may also explain the direct linear relationship ($R^2 = 0.91$) we observed between side chain surface area and anti-plasmodium activity. Alternatively, if membrane binding is not part of the tyrocidine mechanism of action, the FFNQY moiety may be specific for a separate sensitive target in the parasite.

Fig. 4. Fluorescence microscopy of infected erythrocytes incubated with trypan blue and DAPI. Left hand panels are fluorescence images, with trypan blue fluorescence in red and DAPI fluorescence in blue, while right hand panels are the corresponding phase-contrast images. (A) Control cultures after 30 min. Trypan blue fluorescence clearly delineates the infected erythrocyte membrane (arrowhead, top panel) and parasite plasma membrane (arrow, top panel) and is also associated with parasite-derived structures in the infected erythrocyte cytoplasm (arrow, bottom panel). The parasite nucleus (chromatin) is shown by the DAPI fluorescence. (B) Cultures treated with 8 μM gramicidin S for 30 min. Parasites are surrounded by a lysed erythrocyte ghost membrane, staining lightly with trypan blue (arrowhead, top panel). In addition, trypan blue has penetrated the parasite and accumulates intensely around the haemozoin crystal, which is found in the parasite food vacuole (arrows, both panels). (C) Control cultures after 6 h. Top panel shows two adjacent parasite-infected erythrocytes. The erythrocyte on the left is infected with a schizont-stage parasite containing multiple nuclei, as indicated by the DAPI staining. Bottom panel shows an erythrocyte infected with two trophozoites. (D) Cultures treated with 30 nM tyrocidine A for 6 h. Top panel shows an erythrocyte infected with two trophozoites. Bottom panel, an erythrocyte with a trophozoite containing two nuclei.
3.4. Light and fluorescence microscopy of peptide-treated 
*P. falciparum* cultures

We used light and fluorescence microscopy to gauge the 
effect of these membrane active cyclic peptides on *P. falciparum*
infected erythrocytes. In order to evaluate changes in parasite 
permeability, the cells were treated with the peptides below their 
respective haemolytic concentrations and visualised by fluores-
cence microscopy in the presence of an impermeant marker for 
cell leakage (trypan blue). We previously described this type of 
experiment on erythrocytes infected with mature stage 
*P. falciparum* and treated with lytic polyene antibiotics [12]. 
The plasma membrane of trophozoite-infected erythrocytes is 
more permeable to low molecular weight solutes than 
uninfected or ring-infected erythrocytes [37]. Consequently, in 
control trophozoite cultures, trypan blue was found not only 
associated with the erythrocyte plasma membrane (Fig. 4A top 
panel, arrowhead), but also inside the infected erythrocyte 
cytoplasm where it bound to parasite-derived structures (Fig. 4A 
bottom panel, arrow) and the plasma membrane of the parasite 
(Fig. 4A top panel, arrow), but with no staining inside the 
parasite itself. Uninfected erythrocytes appeared intact and 
impermeable to trypan, as expected. By contrast, in gramicidin 
S-treated trophozoite cultures, the parasites were surrounded by 
an erythrocyte ghost membrane bound to trypan blue (Fig. 4B, 
arrowhead), indicating lysis of the infected erythrocyte mem-
brane. Trypan blue fluorescence was also clearly visible inside 
the trophozoite, notably surrounding the hemozoin crystal which 
denotes the position of the parasite digestive food vacuole (Fig. 
4B, arrows), which suggests lysis of the parasite plasma 
membrane as well. The latter conclusion is supported by the 
results obtained with the membrane permeable DNA stain and 
nuclear marker, DAPI, which was included to evaluate changes 
in the chromatin packing (gramicidin S and the tyrocidines have 
been shown to interact with DNA [38–41]). Gramicidin S had a 
pronounced influence on the chromatin, as we observed diffuse 
DAPI staining spilling into the erythrocyte ghost after 
gramicidin S challenge (compare Figs. 4A and B). The presence 
of parasite DNA in the erythrocyte ghost confirms lysis of 
parasite nuclear and plasma membranes by the peptide. 
Gramicidin S-treated ring-infected erythrocytes and uninfected 
erythrocytes retained their native impermeability to trypan blue, 
however (results not shown). More importantly, in contrast to 
gramicidin S, the tyrocidines did not significantly affect DAPI 
staining of the parasite nuclei or the trypan blue fluorescence 
(compare Figs. 4C and D). This suggests that the parasites and 
infected erythrocytes maintain their integrity in the face of 
tyrocidine challenge and that the latter peptides act by a 
mechanism of action different from the rapid, overt lysis of 
trophozoites and trophozoite-infected erythrocytes caused by 
gramicidin S.

Real-time visualisation of the effect of the peptides was 
accomplished by monitoring trophozoite stage cultures chal-
enged with peptides at sub-haemolytic concentrations by phase-
contrast light microscopy. Treatment with 8 μM gramicidin S 
caused the formation >20% trophozoites in ghosts in less than a 
min and almost 70% after 8 min (Fig. 5). After 40 min only 1% 
infected erythrocytes survived and >30% free trophozoites were 
found. We also observed that gramicidin S rapidly lysed 
erythrocyte-free trophozoites isolated from infected cultures by
saponin treatment (results not shown). These results support the trypan blue and DAPI fluorescence experiments obtained with gramicidin S. In addition, they confirm the ability of some membrane active compounds to selectively lyse parasites and erythrocytes infected with mature parasites, as found for polyene antibiotics in our previous study [12] and highlight the infected erythrocyte membrane as a target for the development of potential antimalarial compounds. By contrast, as with the fluorescence microscopy experiments, the tyrocidines failed to produce visible changes to infected erythrocytes during incubations of up to 1 h.

Due to this lack of observable effects on infected erythrocytes during incubation with the tyrocidines, visualisation of tyrocidine action over a longer period was done by microscopic evaluation of Giemsa-stained smears. Smears of parasite cultures were prepared at various time-points following addition of sub-haemolytic tyrocidine A or C concentrations. After 6 h, there were no discernable morphological changes compared to controls (Fig. 6). However, after 21 h of incubation with the tyrocidines, a pronounced effect on parasite development was observed and progression of the life-cycle was inhibited or retarded. Addition of the tyrocidines to ring stage cultures (Fig. 6, see “ring starter cultures”) did not influence development into trophozoites or parasite numbers (expressed as % parasitemia in the bar graph), but the trophozoites were much smaller and more immature in appearance than those in the control cultures, suggesting a delay in development. In experiments initiated with trophozoite cultures (Fig. 6, see “trophozoite starter cultures”), control parasites had progressed normally, traversed the schizont stage during which cell division occurs and re-invaded erythrocytes to form rings, the predominant parasite stage in the culture after 21 h (Fig. 6, bar graph). By contrast, tyrocidine-treated cultures contained normal-looking schizonts and late trophozoites (Fig. 6), but comparatively few rings (Fig. 6, refer to bar graph, compare ring % parasitemia of control and treated cultures), again suggesting an inhibition of parasite maturation and progression through the life-cycle. This visual evaluation over 21 h suggests that the tyrocidines may have static activity towards the parasites. However, our dose response data over 48 h showed that virtually none on the parasites survived at the highest non-haemolytic concentrations we tested, indicating a “slow” cytocidal activity. In separate experiments, where tyrocidines were added to schizont-stage cultures for 6 h, invasion and ring formation per se was found to be insensitive to tyrocidines and was not responsible for the absence of rings in the 21 h experiments described above (results not shown).

4. Conclusions

We were able to isolate six tyrocidines, with nanomolar activity towards \textit{P. falciparum}, from a commercial tyrothricin complex. An increase in apparent hydrophobicity and decrease in side chain surface area of the major tyrocidine in each preparation correlated remarkably well with an increase the \textit{P. falciparum} selective activity. These correlations may be the consequence of differences in the binding of the peptides to the

Fig. 6. Giemsa-stained blood smears from \textit{P. falciparum} cultures treated with tyrocidine A. 30 nM tyrocidine A was added to synchronized cultures containing either ring or trophozoite stage parasites (ring and trophozoite starter cultures, respectively). At 0 h, trophozoite starter cultures contained mature trophozoites (thick arrow) in normal erythrocytes (arrowhead), as did control and tyrocidine A-treated cultures after 8 h. After 21 h, control cultures contained schizonts (thick arrow) and abundant rings (thin arrow). The bar graph depicts the distribution of parasite stages in the cultures after 21 h (including those obtained with cultures treated with 800 nM tyrocidine C\textsubscript{1}).
target cell membrane, possibly the first committed step in the tyrocidines mechanism of action, which in turn depends on the presence of Phe, Trp, Lys and Orn in key positions of the sequence. For example, the most active and hydrophobic peptide, tyrocidine A, has Orn and Phe–Phe–Phe in its primary structure, while Lys and Trp–Trp is found in the least active and most polar peptide, Tyrocidine C₁ (cyclo-VKLFPWWNQY).

The activity of the related, but more polar and smaller, gramicidin S differed significantly from the tyrocidines. Gramicidin S has a lower activity and specificity and its rapid lethal action is due to lysis of trophozoite-infected erythrocytes, as found previously for amphotericin B and related haemolytic polyene macrolides [12,42]. However, unlike amphotericin B, gramicidin S also causes the disruption of parasite membranes and chromatin. The antiplasmodial activity of the related tyrocidines does not depend on overt haemolysis, but an inhibition of parasite development. Current results do not allow firm conclusions to be drawn regarding the precise mode of action of the tyrocidines. The erythrocyte membrane is the first target encountered by these membrane-active peptides; they are haemolytic at micromolar concentrations and related structurally to gramicidin S, which selectively destroys infected erythrocyte membranes. Conceivably, the tyrocidines may disturb the permeability of infected erythrocyte membranes and affect the ability of the parasite to regulate small molecule homeostasis in its host cell, resulting in a gradual inhibition of parasite development. Alternatively, a tyrocidine sensitive molecular target is present in the infected cell or parasite itself, causing a delay in maturation. The tyrocidine activity towards this putative target is dependent on Phe in the aromatic dipeptide unit and Orn in the conserved pentapeptide moiety. Ongoing analysis of additional tyrocidines from the tyrothricin mixture and synthetic variants is important in estimating the optimal/minimal structural determinants of antiplasmodial activity and selectivity, which in turn will guide the design and synthesis of peptide derivatives and mimics for therapeutic evaluation. Specifically, Phe–Trp analogues will be included in future studies to clarify the role of the Phe in the first position of the aromatic dipeptide unit.

Although the haemolytic activity of most the tyrocidines in this study may be a concern, a number of tyrocidines and tyrocidine-like peptides remain to be evaluated for antiplasmodial activity. Due to the pronounced selective activity of tyrocidine A, this peptide and other tyrocidines with similar hydrophobic properties may be promising candidates for deriving lead compounds specific for *P. falciparum*-infected erythrocytes.

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References


