Direct Interaction of Dermaseptin S4 Aminoheptanoyl Derivative with Intraerythrocytic Malaria Parasite Leading to Increased Specific Antiparasitic Activity in Culture*

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Antiplasmodial activity of the dermaseptin S4 derivative K₄S₄(1–13) (P) was shown to be mediated by lysis of the host cells. To identify antiplasmodial peptides with enhanced selectivity, we produced and screened new derivatives based on P and singled out the aminoheptanoylated peptide (NC7-P) for its improved antimalarial properties. Compared with P, NC7-P displayed both decreased antiparasitic efficiency and reduced hemolysis, including against infected cells. Antimalarial activity of P and its derivative was time-dependent and irreversible, implying a cytotoxic effect. But, whereas the dose dependence of growth inhibition and hemolysis of infected cells overlapped when treated with P, NC7-P exerted more than 50% growth inhibition at peptide concentrations that did not cause hemolysis. Noticeably, NC7-P but not P, dissipated the parasite plasma membrane potential and caused depletion of intraparasite potassium at nonhemolytic conditions. Confocal microscopy analysis of infected cells localized the rhodamine derivative in association with parasite membranes and intraerythrocytic tubulovesicular structures, whereas in normal cells, the peptide localized exclusively at the plasma membrane. Overall, the data demonstrate that antimicrobial peptides can be engineered to act specifically on the membrane of intra cellular parasites and support a mechanism whereby NC7-P crosses the host cell plasma membrane and disrupts the parasite membrane(s).

Malaria constitutes the most widespread infectious disease affecting hundreds of millions of people, causing the death of one million children every year in Africa alone (1). Because this dreadful situation could worsen because of the increasing resistance of parasites to available antimalarial drugs, new drugs must be developed.

Antimicrobial peptides have recently emerged as interesting tools for exploring new antimalarial targets (2–6). These ubiquitous peptides vary considerably in structure, size, amino acid sequence, and spectrum of action (7–11), but the most potent peptides always have a pronounced amphipathic and distinctly basic character (12–16). They are believed to exert cytolytic action through their effect on the membrane of target cells by a mechanism whose details remain to be fully understood. Antimicrobial action is not mediated by interaction with stereospecific targets such as receptors or enzymes (3, 17). Apparently, their charge and hydrophobicity are the main features affecting cytotoxicity (18–20). Some antimicrobial peptides were stipulated to form ion channels or pores (21, 22). Various basic models for a membranolytic mechanism were proposed ranging from pore formation to induction of structural defects (20–27) that lead to membrane permeabilization. Consequently, essential ions and metabolites are free to leak in and out and to dissipate the electric potential across the membrane, eventually leading to cell death.

Antimicrobial peptides often display a broad spectrum of activity affecting Gram-negative and Gram-positive bacteria, yeast and filamentous fungi, some enveloped viruses, and many types of cancer cells. Yet many are relatively inactive on normal eukaryotic cells (28–30). Although the basis for this discrimination is also unclear, it appears to be related to the lipid composition of the target membrane (i.e. fluidity, negative charge density, and the absence or presence of cholesterol) and the presence in the peptide-susceptible organisms of a large negative trans-membrane electrical potential (31–34). Such a peptide-based antimicrobial system has attractive advantages over classical antibiotics because it makes it extremely difficult for microbial targets to develop resistance (15, 35, 36). Nevertheless, a major drawback of such an antimicrobial system is reflected in its unsel ective activity over a wide range of cell types, which could be problematic, for instance, in systemic routes of administration (37).

Dermaseptin S4 is a 28-residue antimicrobial peptide isolated from frog skin (38). The native peptide was shown to exert antimalarial activity (4), whereas subsequent studies (17, 39) identified a 13-residue derivative, K₄S₄(1–13), displaying a considerable in vitro effectiveness on Plasmodium falciparum, the most lethal human parasite (5). The antimalarial action was rapid and was shown to be mediated by permeabilization of host cell plasma membrane. Although K₄S₄(1–13) was less hemolytic to normal erythrocytes, it was deemed necessary to develop additional derivatives that could affect the parasite with minimal threat to erythrocytes. Recently, acyl derivatives of K₄S₄(1–13) were shown to increase antimalarial activity, although the most potent antiparasitic peptides still displayed high hemolytic activity (6). In this study, a series of new dermaseptin S4 derivatives based on K₄S₄(1–13) were produced and investigated for antimalarial and hemolytic properties. After screening for the most selective compound, we investigated its detailed mechanism of action.

MATERIALS AND METHODS

Synthesis of Dermaseptin S4 Derivatives—The reference peptide K₄S₄(1–13) was synthesized by the solid phase method, applying the
Fmoc\(^1\) active ester chemistry on a fully automated, programmable peptide synthesizer (model 433A; Applied Biosystems) as described (17) with the following modifications. 4-Methylbenzhydrylamine resin (Novabiochem) was used to obtain amidated peptides. The various analogs were prepared by linking the N terminus of K-\(\text{S4(1-13)}\) to one of the comparison groups detailed in Table 1 as follows. After removing the Fmoc group (20% piperidine/N-methylpyrrolidone), the resin-bound peptide (20 mg) was suspended in 0.7 ml of dimethylformamide to which a 2-fold molar excess of the relevant tert-butylisoxyl-carbonyl protected amino-carboxylic acid was added followed by 3-fold molar excess of 1-ethyl-3- (dimethylaminopropyl)carbodiimide. In the specific case of t-BOC aminaldehyde-mediated depletion of acid, 1-ethyl-3-(dimethylaminopropyl)carbodiimide was used. In the case of K-\(\text{S4(1-13)}\), the reaction mixture was sonicated for 5 min and then agitated for 24 h at room temperature. The resin was washed with dimethylformamide and then with ether/dichloromethane (1:1) and dried for 4 h at 50°C. For visualization studies, rhodamine was covalently attached to the deprotected N terminus of NC6-P (Table I) while still linked to the resin. Peptide labeling, cleavage from the resin, and purification by HPLC were also performed as described (17). The purified peptides were subjected to amino acid analysis and mass spectrometry to confirm their composition. Peptides were stocked as lyophilized powder at -20°C. Prior to testing, fresh solutions were prepared in water, briefly vortexed, sonicated, centrifuged, and then diluted in the appropriate medium.

**Determination of Hemolytic Potential**—Human blood was rinsed three times in PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.3) by centrifugation for 1 min at 2700 \(\times g\) and then 2.5 \(\times 10^8\) red blood cells (RBC) suspended in 50 \(\mu\)l PBS were added to Eppendorf test tubes containing 200 \(\mu\)l of peptide solutions (serial 2-fold dilutions in PBS), PBS alone (for baseline values), or distilled water (for 100% hemolysis). After incubation (3 h under agitation, 37°C) the samples were centrifuged (3000 \(\text{rpm}\) for 5 min) and the supernatant (20 \(\mu\)l) was transferred to a 96-well plate (Serimax) and absorbance at 630 nm measured on a Wallac Envisage plate reader. The statistical data were obtained from at least three independent experiments performed in duplicate.

**Parasite Cultivation**—The W2 strain of \(P. falciparum\) was cultivated as described (40) using human RBC. The culture was synchronized by the sorbitol method (41) using the less toxic alanine, and infected cells as described (40) using human RBC. The culture was synchronized by the Percoll-alanine gradient (97% parasitemia, determined on Giemsa-strained thin blood smears) and incubated at 0.5% hematocrit in culture medium at 37°C, with or without 10 \(\mu\)M P or NC7-P. At time 0 and after 4 h, aliquots were taken, cells were washed in PBS, and parasites were freed by saponine (0.005% w/v in PBS)-induced lysis for several minutes at room temperature. The parasites were washed several times in PBS and finally washed with 110 mM MgCl\(_2\) buffered with 10 mM Heps. The parasites were disrupted by freezing and thawing, and potassium content in the supernatant was determined by induc-tive-coupled plasma atomic emission spectroscopy on an Optima 3300 Inductively coupled plasma atomic emission spectroscopy system (PerkinElmer Life Sciences).

**Intracellular Localization of Fluorescent Peptide by Confocal Microscopy**—Cultures (1% hematocrit) of trophozoites (~90% parasitemia) and uninfected human erythrocytes were incubated in the presence of the rhodaminated peptide at 1 and 10 \(\mu\)M. As shown in Fig. 1A, acylation of P resulted in increased hydrophobicity concomitant with increased acyl chain length. Comparatively, aminoacyl analogs had reduced hydrophobicity, presumably because of their increased polarity. Fig. 1B shows that increased hydrophobicity of acyl derivatives first counteracts the hemolytic activity of the parent peptide (as measured in PBS) and thereafter increases it. The aminoacyl derivatives display similar biphatic effect albeit at considerably higher concentrations. It is suggested that the biphatic effect results from the opposing forces of membrane solubilization (enhanced by acyl chain length) and surface aggregation (essential for hemolytic activity) (11).

**RESULTS**

To reduce hemolytic activity, acylated peptides (6) were converted to aminoacyl derivatives. Identity of the synthetic products (Table I) was confirmed by mass analysis of the HPLC-purified peptides (purity was > 95%). As shown in Fig. 1A, acylation of P resulted in increased hydrophobicity concomitant with increased acyl chain length. Comparatively, aminoacyl analogs had reduced hydrophobicity, presumably because of their increased polarity. Fig. 1B shows that increased hydrophobicity of acyl derivatives first counteracts the hemolytic activity of the parent peptide (as measured in PBS) and thereafter increases it. The aminoacyl derivatives display similar biphatic effect albeit at considerably higher concentrations. It is suggested that the biphatic effect results from the opposing forces of membrane solubilization (enhanced by acyl chain length) and surface aggregation (essential for hemolytic activity) (11).

**Selection of Antiplasmodial and Hemolytic Activities**—To identify peptides that will selectively kill the parasite without lysis of the host cell, the aminoacyl peptides were screened at a single dose of 10 \(\mu\)M for antiplasmodial and hemolytic activities. All of the peptides tested inhibited plasmodial growth to various extents. But, whereas derivatives with short hydrocarbon chains had either lower (e.g. NC2-P) or similar (e.g. NC4-P)
Antiplasmodial activity compared with the parent peptide P, the more hydrophobic peptides NC7-P and NC12-P were more active (Fig. 2a). Yet although derivatives with 2–7 hydrocarbons (NC2-P, NC4-P, and NC7-P) were less hemolytic, NC12-P displayed increased hemolytic action (Fig. 2b). To select for the most suitable derivative, we compared the ratio of relative inhibition to relative hemolysis. This analysis (Fig. 2c) revealed that NC4-P and NC7-P were the most selective, i.e. the antiplasmodial activity was superior to the hemolytic action.

Two additional branched derivatives (N2C6-P and N4C9-P) were prepared to assess the effect of modulating the charge and hydrophobicity. Compared with P, N2C6-P did not display improved antiplasmodial activity but resulted in increased hemolysis, whereas N4C9-P displayed reduced antiplasmodial and hemolytic activities (Fig. 2, a and b). Because NC7-P combined both increased antiplasmodial effect with lower hemolysis compared with P (as reflected by the increased lysis of the percentage of inhibition to the percentage of lysis in Fig. 2c), this derivative was chosen for further and more detailed investigations.

Detailed Determination of Antiplasmodial Activity and Stage Dependence of Selected Compounds—The dose response of NC7-P was investigated and compared with the parent peptide P using synchronized cultures of P. falciparum that were exposed to the peptides either at the ring or at the trophozoite stage. NC7-P was more effective than P at the ring stage (IC50 = 5.3 ± 0.7 and 7.7 ± 0.9 μM, respectively), but the opposite was observed for the trophozoite stage (IC50 = 6.2 ± 0.5 and 3.4 ± 0.3 μM, respectively). The stage dependence results indicated that ring stage parasites were less sensitive to P, as previously observed (5), than the more mature trophozoite stage. This was not observed with NC7-P. Moreover, the slopes of the dose-response curves were slightly higher for NC7-P (1.9 ± 0.5 and 2.3 ± 0.4, respectively) compared with P (1.5 ± 0.2 and 1.4 ± 0.1, respectively), indicating some differences in the stoichiometry of drug and target relations.

Time Dependence and Reversibility of Antiplasmodial Action—NC7-P (10 μM) was found to be similarly active, and its action was time-dependent both for ring and trophozoite stages, displaying maximal activity after 24 h of exposure (Fig. 3). Removal of the peptide from the culture after 5 h of incubation and measuring parasite viability 19 h later, revealed that the antiplasmodial effect proceeded further even in the absence of peptide in the medium. These results suggest that internalized peptide (see below) could not be removed from the cells and that the antiplasmodial effect was cytotoxic.

Hemolytic Activity versus Antiplasmodial Activity—To further understand the mechanism of antiplasmodial activity, P and NC7-P were tested simultaneously for their hemolytic and antiplasmodial activities. Infected cells at the young trophozoite stage were enriched (~90% parasitemia) from culture and exposed (0.5% hematocrit) to increasing peptide concentrations. Parasite viability was determined after 2 h of exposure to peptides followed by 4 h of exposure to hypoxanthine in culture conditions, whereas hemolysis was assayed on normal and infected erythrocytes after 6 h of exposure to the peptide. Under these conditions of short time exposure of trophozoite-enriched cultures, NC7-P was more inhibitory than P (IC50 = 14.2 ± 0.5 and 19.6 ± 1.6 μM, respectively). Yet NC7-P was much less hemolytic than P to infected erythrocytes (LC50 = >60 and 21.2 ± 0.6 μM, respectively), whereas this discrepancy was less pronounced for uninfected cells (Fig. 4).

Peptide-mediated Dissipation of the Parasite Plasma Membrane Potential and Induced Leak of Intraparasite Potassium—The fluorescent dye rhodamine 123 accumulates in parasites in correlation with the parasite membrane ΔΨ, and permeabili-
least twice in quadruplicate. The error bars of P and a marked reduction in the presence of NC7-P (Fig. 5).

A fluorescent peptide where rhodamine was covalently linked to the N terminus of the peptide was prepared and compared with NC7-P with respect to antimalarial activity and hemolytic activities as described above. The rhodamine-labeled peptide had similar antimalarial activity, but it was considerably more hemolytic than NC7-P (data not shown). Inspection by confocal microscopy (mid-depth Z slice) of uninfected human erythrocytes exposed to 1 or 10 μM labeled peptide for up to 2 h under culture conditions revealed intense labeling of many cells (Fig. 6a). The dye was seen associated exclusively with the erythrocyte membrane. Fluorescence intensity increased with concentration, but the number of labeled cells remained practically unchanged with time (not shown).

Similar exposure of trophozoite-infected human erythrocytes (~90% parasitemia) labeled many more cells with the same pattern of dose and time dependence seen with uninfected erythrocytes (Fig. 6b). Further magnifications disclosed that the label was specifically localized in the parasites and that labeling existed in the host compartment of inclusions that can be identified as tubulovesicular structures and Maurer’s clefts. In control experiments where the cells were incubated under similar conditions but in the presence of 10 μM free rhodamine and 10 μM unlabeled NC7-P, neither the infected nor the normal cells were labeled.

**DISCUSSION**

To design dermaseptin derivatives with greater selectivity, we have been guided by the following rationale. Selective activity of antimicrobial peptides demonstrably depends on the membrane lipid composition (17–27). The lipid composition of the infected cell is considerably different from that of uninfected erythrocytes and other somatic cells of the host in that it is devoid of cholesterol and it has considerably less sphingomyelin and phosphatidyl serine, larger concentrations of phospho-
phatidylycholine, phosphatidylyethanolamine, and phosphatidylinositol, and a decreased level of unsaturation of the fatty acids (43). Although the lipid compositions of host and parasite membrane are similar, the potential of the parasite membrane is considerably higher than that of the host cell membrane (42), and it is in the right polarity needed for enhancement of peptide incorporation (32–34). Thus, we hypothesized that the discriminating effect of the dermaseptin derivative could be exerted on two additional levels: (i) preference for infected cells because of lipid composition and (ii) the preference for the parasite membrane because of favorable ΔΨ once inside the cytosol of the infected cell. Increasing the lipophilicity of the peptide will render it more permeable through the host cell membrane and therefore more accessible to the parasite membrane. Because this feature also increases hemolytic activity (6, 17), we have used aminocycl moieties to reduce hydrophobicity and, hence, the risk for hemolysis.

This reasoning has been tested experimentally using seven peptides of varying N termini. For all derivatives the antiplasmodial and hemolytic activities were found to depend on the nature of the added moiety. Sorting the derivatives by their selectivity (ratio of the percentage of inhibition to the percentage of hemolysis), NC7-P was singled out as the most selective and was further used in parallel with the parent peptide P for detailed investigations. To acquire a deeper understanding of the selectivity effect, parallel determination of antiplasmodial activity and lysis of normal and infected erythrocytes were conducted for short incubation times. The IC50 values obtained in these experiments were understandably higher than those obtained in the standard dose-response test that lasted 24 h because of the time dependence effect and the increased number of infected cells (from 2 to ~90%, respectively). Outstandingly, whereas with P the dose dependence of growth inhibition and lysis of infected cells overlapped, with NC7-P more than 50% growth inhibition occurred at concentrations that did not cause lysis at all. This discrepancy is possibly even larger because both growth inhibition and lysis are time-dependent processes, and exposure to peptides was only 2 h in the first case and 6 h in the second. Noticeably, whereas P was more lytic to infected cells than to uninfected cells, such discrepancy was much less pronounced for NC7-P. This is a further demonstration of the lipid-dependent specificity of peptides.

Unlike P (5), NC7-P was not stage-selective, being equally inhibitory for both the young ring stage and the more mature trophozoites. We propose that P acts essentially by lysing the host cell membrane. It is more lytic to host cells harboring mature parasite stages, indicating dependence on changes induced by parasite in the host cell membrane. Because the latter evolves with parasite maturation, trophozoites are expected to be more sensitive than rings, as was found (5). In contrast, the selectivity of the NC7-P seems to depend on the differential ΔΨ of host and parasite membrane. Because this is established from the onset of parasite development, the permeable peptide is always sucked into the parasite membrane and affects it. For this reason stage dependence with NC7-P is neither expected nor observed.

The antiplasmodial effect of NC7-P was clearly time-dependent. It was found to have a stage-independent cytotoxic activity that persisted and accrued even when it was discarded from the culture. This indicates that the association of NC7-P with the parasite membrane is essentially irreversible and that even without saturation of the putative peptide binding sites, it results in the continuous loss of the parasites viability because of membrane permeabilization. The interaction of a fluorescent analog of NC7-P with uninfected erythrocytes was seen to be localized at the cell membrane. However, we cannot exclude the possibility that if the peptide was internalized, its fluorescence was quenched by hemoglobin. In infected cells, the fluorescent analog reached the parasite and labeled its plasma membrane and the tubulovesicular network that emerges from the parasitophorous vacuolar membrane and extends to the erythrocyte membrane (44, 45). The high resolution images obtained by fluorescence confocal microscopy recall the subcellular distribution of the fluorescent phospholipid NBD-PC (46), attesting to the association of the peptide with the membranes of the infected cell. Parenthetically, the staining of a subpopulation of infected cells conforms with partial inhibition of parasite growth as measured by the hypoxanthine viability assay that integrates the response of the entire parasite population.

To the best of our knowledge, this is the first demonstration ever of an antiplasmodial compound that acts on a subpopulation of cells in an all-or-none fashion rather than reducing vital processes in each cell.

The lipophilic and membrane-trophic character of NC7-P insinuates that its interaction with the parasite membrane would lead to nonselective permeabilization. The observed dissipation of ΔΨ could result from increased permeability to protons because the major generator of ΔΨ is presumably the V-type H+ pump (47, 48). Permeabilization to protons undercuts the function of the pump as the major regulator of cellular pH. Short circuiting of the electrogenic function of the pump presumably underlies the observed loss of cellular potassium, the maintenance of which seems to depend on ΔΨ. Although these presumptions could explain the gradual and irreversible loss of vital cellular functions, we cannot exclude at the present time the possibility that NC7-P acts on a different cellular target that mediates its cytotoxic action.

In conclusion, we demonstrate in this investigation that membrane active peptides can be engineered to act specifically on the membrane of the intracellular parasite to perturb its functions. This selective activity reduces the potential harm from inadvertent lysis of the erythrocytes of the host. This is a major achievement in the fine tuning of peptide composition toward its further development as a potential antimalarial drug. It has been shown previously that intravenous administration of P to rats was well tolerated at least up to 10 mg/kg (39) and that the LD50 of S4 derivatives (including P) administered intraperitoneally in mice was 25 mg/kg, whereas effectiveness against Pseudomonas aeruginosa-induced peritonitis...
was achieved at ≤4.5 mg/kg (49). Because NC7-P is less hemolytic than P, it can be assumed that it will be less toxic in vivo. Such concentrations are substantially higher than the IC50 of NC7-P against the malaria parasite, indicating that it could also be effective in vivo. It remains to be shown experimentally that NC7-P is not toxic to mammalian cells or to whole animals and that its antiplasmodial effect is maintained in vivo. Investigations of these aspects are underway in our laboratory.

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