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Antiplasmodial and Prehemolytic Activities of α-Peptide–β-Peptoid Chimeras

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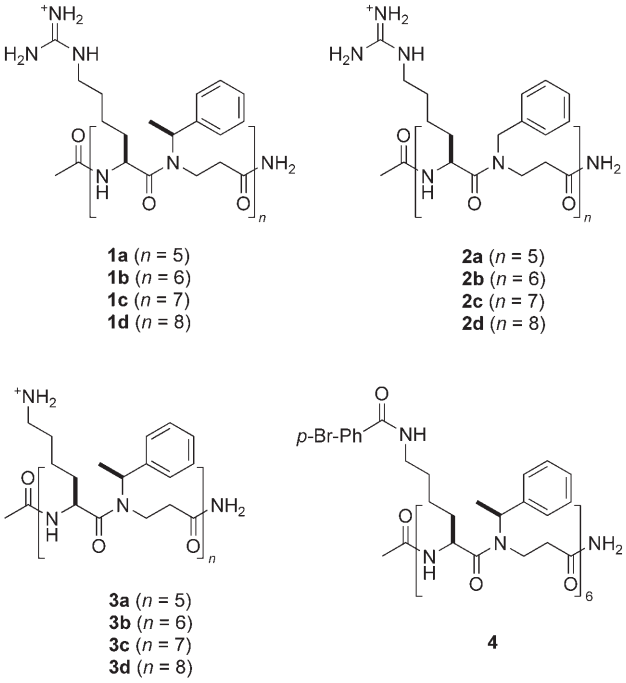
Malaria, especially the type caused by *Plasmodium falciparum* parasite strains, is a major tropical disease, estimated by the World Health Organization (WHO) to infect 300 to 500 million people per year, with a mortality rate of 1.1 to 2.7 million deaths per year.[1] The advancement of resistance towards known drugs calls for the discovery of novel classes of antiparasitic compounds.[2] Recently, the antiplasmodial activity of antimicrobial peptides of fungal and amphibian origin[3] as well as of peptides from a phage-display library[4] has been reported. Although some of these peptides contain nonproteinogenic amino acids, they are all α-peptides and are as such susceptible to proteolytic degradation. However, natural products that contain multiple *N*-methylated amino-acid residues have also been shown to have antiplasmodial activity.[5,6] Hence, we found it would be interesting to test whether the recently reported antimicrobial α-peptide–β-peptoid chimeras[7] would display antiparasitic activity.

Novel peptidomimetic backbone designs are of interest as a means of expanding the diversity of biomimetic polymers[8,9] with respect to folding and biological properties. The chimeric α-peptide–β-peptoid constructs (Scheme 1), which have been found to possess antimicrobial activity without lysing human red blood cells in the active concentration range, are resistant against proteolysis. These chimeras are readily assembled by solid-phase peptide synthesis protocols by using building blocks that are efficiently prepared on the gram scale.[7,10] Here, we describe the discovery of promising biomimetic antiplasmodial oligomers with this backbone as well as the profiling of their membrane activity.



Scheme 1. Chemical structures of α-peptide–β-peptoid chimerasused in this study.

The molecular design chosen here is based on alternating repeats of *N*-alkylated β-alanine (β-peptoid) units[11,12] and α-amino acids. The rationale behind this design was to gain structure-promoting effects and lipophilicity from the unnatural chiral β-peptoid residues (for circular dichroism studies, see refs. [7], [11], and [12]), while the α-amino acids provide the side-chain functionality and intramolecular hydrogen bonding

Table 1. Antiplasmodial, hemolytic, and prehemolytic activities of the α-peptide–β-peptoid chimeras as well as magainin 1, magainin 2, and the nonpeptidic reference chloroquine.

Compound	Inhibition of *P. falciparum* IC_{50} [μM]	Conc. at which hemolysis was observed [μM]	Erythrocyte alterations at IC_{50}
1a	12.6 ± 1.8	> 70[b]	~50%[c]
1b	6.0 ± 0.7	> 70[b]	~25%[c]
1c	4.5 ± 1.0	~1	~70%[c]
1d	3.6 ± 0.4	~5	~50%[c]
2a	44% at ~70 μM[a]	> 70[b]	–
2b	23.6 ± 7.2	> 70[b]	> 99%[c]
2c	13.6 ± 3.4	> 70[b]	< 75%[c]
2d	8.5 ± 1.4	~6	~50%[c]
3a	0%[a]	–	–
3b	24% at ~90 μM[a]	> 90[b]	–
3c	45% at ~90 μM[a]	> 90[b]	–
3d	21 ± 9	> 290[b]	~10%[c]
magainin 1	5% at ~80 μM[a]	> 80[b]	none[d]
magainin 2	47% at ~80 μM[a]	> 80[b]	none[d]
chloroquine	0.055 ± 0.025	none	none

[a] Less than 50% inhibition at 200 μg mL^{-1}. [b] No hemolysis observed in the tested concentration range. [c] Stomatocytogenic; [d] At 200 μg mL^{-1} (80 μM).

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capability. Initially, the guanidinylated chimeras (**1a**–**2d**) were tested for their in vitro inhibition of *P. falciparum* parasites; IC_{50} values as low as 4–6 μM were observed (Table 1). These activities are comparable to those observed for other antiplasmodial peptides,[3] albeit considerably lower than the antimalarial drug chloroquine (IC_{50} 0.055 μM, under the same assay conditions). Interestingly, our simple compound design, based on repeats of two different types of residues, inhibited *P. falciparum* growth in a concentration range in which the natural host-defense peptides, magainin 1 and 2, showed no significant activity (Table 1).

Previous studies of α-peptides have addressed their hemolytic properties in relation to the antiplasmodial effect.[3] However, studies of other natural products have demonstrated that prehemolytic effects—manifested as alterations of erythrocyte membrane morphology due to incorporation of the compounds in the lipid bilayer—could correlate with antiplasmodial activity; this suggests that the antiplasmodial effect is mediated by host–cell effects.[13,14] Therefore, human red blood cells were incubated with various antiplasmodial chimeras and assayed for membrane alterations by using microscopy, as previously described.[13,14] These investigations revealed that all guanidinylated chimeras, despite their low hemolytic activity at the concentrations that corresponded to the observed IC_{50} values, invoked severe membrane alterations that were observable at low concentrations (Table 1). Thus, chimeras **1a**–**d** and **2a**–**d** cannot be considered promising lead structures despite their high antiplasmodial activity. Nevertheless, the lysine-containing hexadecamer **3d**[10] also showed significant antiplasmodial activity (Table 1), but most importantly it was not hemolytic at concentrations up to tenfold the IC_{50} value (>290 μM). In addition, this compound did not alter the morphology of healthy erythrocyte membranes significantly at the IC_{50} concentration (only 10% stomatocyte formation). By contrast, 3 μM of the guanidinylated compound **1b**, which was the most potent nonhemolytic derivative, gave rise to prehemolytic transformation of about 20% of the native discocytes into stomatocytes (Figure 1A and B). Severe damage to the erythrocyte membranes was observed at higher concentration (18 μM; Figure 1C). Thus, we suggest that the assessment of hemolytic activity, which is often used as a guiding test for possible eukaryotic toxicity, might be inadequate as it only detects advanced membrane disruption, whereas effects at subhemolytic concentrations can reveal adverse interactions.

Erythrocytes treated with 18 μM of the less active, nonguanidinylated compound **3b** did not show alterations in membrane morphology (Figure 1D). Nonetheless, we observed echinocytic shapes invoked by higher concentrations of this compound (Figure 1E). The echinocytic shape is believed to arise from amphiphile incorporation into the outer leaflet of the membrane bilayer, whereas stomatocytogenesis arises from amphiphile incorporation into the inner leaflet.[15] Thus, while the guanidinylated compounds were all stomatocytogenic, the lysine-containing compounds gave rise to both stomatocytes and echinocytes depending on the oligomer length; this suggests that different modes of membrane interaction could be at play. This finding supports the conclusion from previously reported investigations with membrane-active β-peptides and synthetic model vesicles, in which differences in vesicle leakage kinetics as a function of β-peptide length was observed.[16]

Compounds **1b** and **3b** were also able to induce vesicle disruption, as assessed by dye (calcein) release experiments with liposomes composed of a binary mixture of neutral dipalmitoylphosphatidylcholine (DPPC) and anionic dipalmitoylphosphatidylglycerol (DPPG) phospholipids in a 4:1 molar ratio. The results paralleled the biomembrane activities as well as our previously reported antimicrobial activities;[7] the guanidine-functionalized chimera **1b** was the most potent (Figure 2). This is in agreement with previous observations that arginine-rich

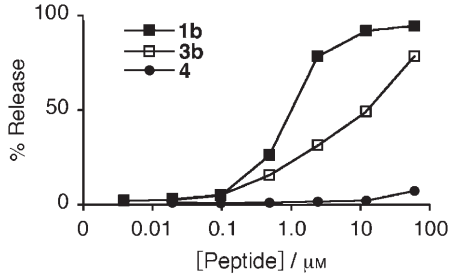

Figure 2. Results of calcein-release experiments; the noncationic analogue **4** was also tested, but as expected it did not cause significant leakage; this shows that amphiphilicity is a requirement for vesicle disruption.

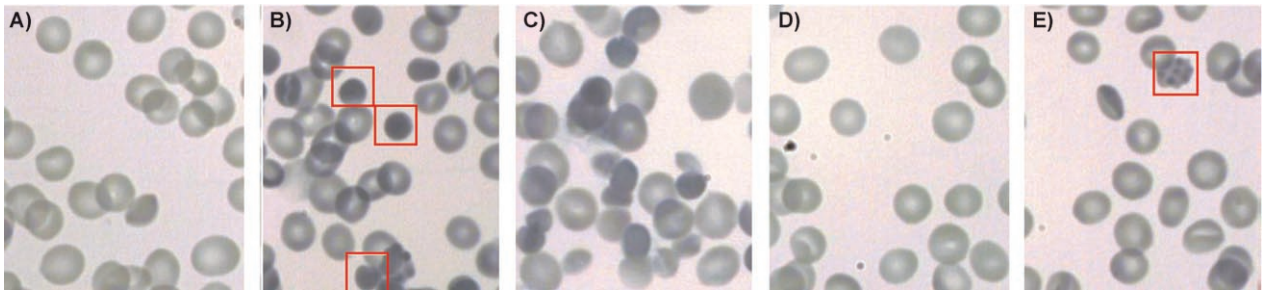

Figure 1. Results of erythrocyte membrane-activity experiments. A) Untreated control red blood cells (RBCs). B) RBCs after incubation with compound **1b** (3 μM); 20% stomatocytogenesis (typical examples are highlighted with red boxes). C) RBCs after incubation with compound **1b** (18 μM); 75% stomatocytogenesis. D) RBCs after incubation with compound **3b** (18 μM); significant change in cell shape as compared to the control sample was not observed. E) RBCs after incubation with compound **3b** (150 μM); <5% echinocytogenesis (a typical example is highlighted with the red box).

peptides bind membrane lipids stronger than their lysine analogues due to their ability to form bidentate hydrogen bonds to the phospholipid head groups.[17]

The lysine-containing analogues clearly interacted with a variety of synthetic and biological membranes, but were nonhemolytic and considerably less toxic to erythrocytes than their guanidinylated counterparts. We visualized the interaction with HeLa cells by monitoring the uptake of a 5(6)-carboxyfluorescein-labeled analogue (**5**) with confocal laser scanning microscopy (Figure 3). This preliminary experiment showed that the

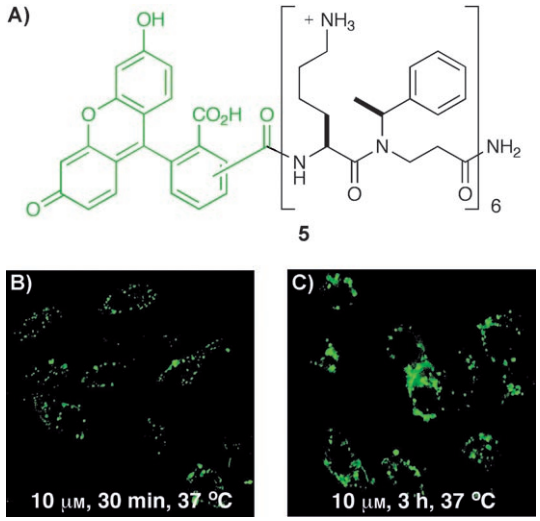

Figure 3. A) Structure of 5(6)-carboxyfluorescein-labeled chimera **5**. Confocal laser scanning microscopy images of **5** (10 μM) incubated with HeLa cells for B) 30 min and C) 3 h. The images were recorded with live cells to avoid artifacts caused by fixation.

lysine analogues rapidly entered HeLa cells at low μM concentrations. When tested in the erythrocyte-membrane assay, compound **5** was not hemolytic and did not alter the membrane shape at concentrations of up to 100 μM. Thus, our lysine-containing compounds could also have potential as nontoxic delivery vehicles for fluorophores or small-molecule drugs. Further exploration of these properties is in progress and these results will be reported in due course.

In summary, the first peptidomimetics with an unnatural backbone construct that show antiplasmodial activity in vitro are described. The lysine analogue **3d** had an IC_{50} of 21 μM and was neither hemolytic nor did it alter the membrane morphology of healthy erythrocytes significantly at this concentration. Together with the results of the cellular-uptake experiments with fluorescent analogue **5** this suggests that the antiplasmodial activity might be due to interaction with an intracellular target rather than to impairment of the host cell membrane. Thus, although the in vitro potency of the currently described peptidomimetics cannot compete with that of the drug chloroquine, the significance of our findings lies in the identification of a novel, nonhemolytic, and proteolytically stable class of antiplasmodial agents. These data show that the antiplasmodial potency increases with the length of the ligand, and, furthermore, that oligomer length can affect the mechanism of the interaction with membranes. The presence of α chirality in the β-peptoid residues generally causes an increase in potency, which is in contrast to the observed antibacterial activity towards *E. coli*.[7] Also, the chiral compounds appear to exhibit better membrane selectivity (**1b** vs. **2b**); however, this presumption needs further validation.

We believe that the discovery of the antiplasmodial activity of α-peptide–β-peptoid chimeras, and the possibility of affecting their hemolytic/prehemolytic activity by structure manipulation is of general interest in the field of peptidomimetic research.

Experimental Section

In vitro antiplasmodial activity: Antiplasmodial activities were determined by using chloroquine-senstitive *P. falciparum* strain 3D7, based on three or more individual compound samples each tested in triplicate, as previously described.[14]

Calcein release: The calcein-release assay was performed essentially as described previously[18] by using unilamellar liposomes that encapsulated calcein and were prepared from a mixture of DPPC and DPPG (4:1 molar ratio, 25 μM final lipid concentration).

Chemical synthesis of 5: Oligomerization was performed by using solid-phase peptide synthesis as previously described,[7] and the N terminus was acylated with 5(6)-carboxyfluorescein (5 equiv), diisopropylcarbodiimide (5 equiv), and hydroxybenzotriazol (5 equiv) in DMF (4 mL) for 16 h, under N_2. The resin was drained and washed with DMF (3×), treated with 20% piperidine–DMF (4 mL, 2×10 min), and washed extensively with DMF, MeOH, and CH_2Cl_2 (3× each). Crude **5** was cleaved from the support with TFA/CH_2Cl_2 (95:5, 3 mL, 1 h), and purified to homogeneity by preparative RP-HPLC (250×20 mm, Phenomenex C18 column, 5 μm, 100 Å) by using an Agilent 1100 LC system with a multiple-wavelength UV detector. A gradient with eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile), which was increased linearly from 5% to 40% of B during 30 min, was applied. The lyophilized product was analyzed by using RP-$HPLC_{254nm}$ > 95% and ESI-MS (*m/z*): $[M+H]^+$ calcd for $C_{123}H_{164}N_{19}O_{18}$ 2195.3 Da; found 2195.6 Da (Esquire LC mass spectrometer); lyophilized product was stored at −20 °C.

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