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Antimalarial activity of azadipeptide nitriles

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ABSTRACT

Azadipeptide nitriles—novel cysteine protease inhibitors—display structure-dependent antimalarial activity against both chloroquine-sensitive and chloroquine-resistant lines of cultured *Plasmodium falciparum* malaria parasites. Inhibition of parasite's hemoglobin-degrading cysteine proteases was also investigated, revealing the azadipeptide nitriles as potent inhibitors of falcipain-2 and -3. A correlation between the cysteine protease-inhibiting activity and the antimalarial potential of the compounds was observed. These first generation azadipeptide nitriles represent a promising new class of compounds for antimalarial drug development.

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Malaria remains one of the world's most devastating infectious diseases, with around 500 million clinical cases and 1–2 million deaths annually.[1] Malaria is caused by infection with protozoan parasites of the genus *Plasmodium*, with the four main species that infect humans being *Plasmodium falciparum,* which is responsible for most morbidity and mortality, *Plasmodium vivax, Plasmodium malariae,* and *Plasmodium ovale.* More than 40% of the global population is at risk of malaria, particularly those in sub-Saharan Africa and regions of Asia. There is currently no licensed malaria vaccine and although there are several antimalarial drugs that are used clinically for chemoprophylaxis and treatment, the usefulness of most of these therapies is under threat due to drug resistance. Chloroquine was once the mainstay of antimalarial therapy. However, this drug is now ineffective against *P. falciparum* in most regions of the world,[2] and clinical treatment failures have recently been reported for other antimalarial drugs, including artemisinin-based combination therapies (ACTs).[3–5] To address this enormous public health issue, there is an urgent need to identify and develop new antimalarial drugs.

Essential processes within the parasite, particularly those that are unique to *Plasmodium* and not the host, represent good targets for new antimalarial drugs.[6] One of the best studied pathways is the process of hemoglobin degradation whereby, as it grows and matures, the intra-erythrocytic form of the parasite imports red cell cytosol into a compartment called the food vacuole.[7] Within the food vacuole hemoglobin is proteolytically degraded by the partially redundant activity of papain-like cysteine proteases (falcipains) and aspartic proteases (plasmepsins) to ultimately form amino acids that are utilized by the parasite to grow and replicate.[8,9] The *P. falciparum* falcipains involved in hemoglobin catabolism (falcipain-2, falcipain-2′, and falcipain-3) are recognized as promising new antimalarial drug targets.[10]

Inhibitors for papain-like cysteine proteases are mainly derived from peptides with electrophilic entities that are susceptible to covalent interaction with the thiol at the active site of the cysteine protease.[11,12] Peptide-derived vinylsulfones have been developed as irreversible inhibitors of cysteine proteases[13] and their ability to impair parasite development in vivo has been shown.[14] However, vinyl sulfones are also known to interact with the active site of the proteasome.[15] Peptide nitriles have received increasing attention in recent years as the cyano group gives rise to a preferred interaction with the active-site thiol by the formation of an enzyme-bound thioimidate that can result in potent enzyme inhibition.[16–18] The isoelectronic replacement of the C_αH-unit by a nitrogen atom in the P^1 position of dipeptide nitriles leads to a dramatic increase in the inhibitory potency, and the concurrent methylation of the P^2–P^1 peptide bond to better stability against proteolytic degradation.[19] Such improvements are encouraging research towards the medical application of these peptide analogues. Herein, we report on the antimalarial potential of a panel of these azadipeptide nitriles.

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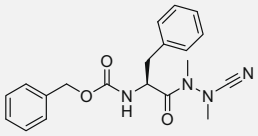
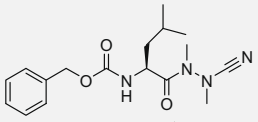
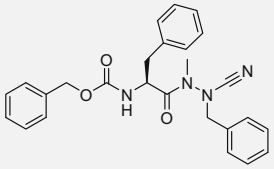
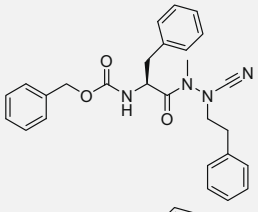
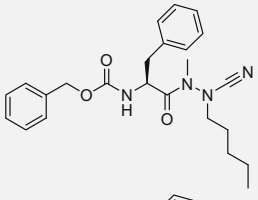
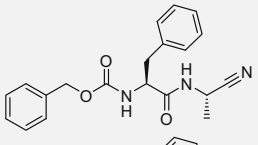
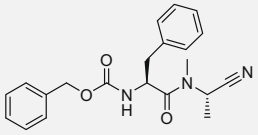
The inhibitors used in this study have been synthesized as described[19] and their antimalarial effect was determined against cultured *P. falciparum* infected erythrocytes using standard isotopic growth-inhibition assays. Assays were carried out using a chloroquine-sensitive (3D7)[20] and a chloroquine-resistant (Dd2)[21] *P. falciparum* line, essentially as previously described.[22–24]

Compounds **4** and **5** (Table 1), both bearing phenylalanine in P^2 and phenylethyl and pentyl in P^1, respectively, displayed the best activity against *P. falciparum* parasites, with IC_{50} values of ~4 and ~3 μM, respectively. The length of the substituent in the P^1 position may be important, as shortening the phenylethyl moiety in **4** to benzyl in **3** resulted in slightly reduced activity (IC_{50} ~4 μM vs ~7 μM). Reduction of the P^1 substituent of the derivatives **5**, **4**, and **3** to a simple methyl group (compound **1**) led to decreased activity. This suggests the P^1 residue to be important for the antimalarial activity of the azadipeptide nitriles. When comparing the analogues **1** and **2**, differing in the Phe and Leu moiety in P^2, no significant difference was obvious. The isoelectronic replacement of the nitrogen adjacent to the cyano group by a CH unit resulted in a loss of activity. This applies to compound **6**—the direct carbon analogue of **1**—as well as to the carba-desmethyl analogue **7**, demonstrating the importance of the azadipeptide nitrile scaffold for the antimalarial properties of these dipeptide derivatives. For all compounds tested, there was no difference in IC_{50} values for the chloroquine-sensitive versus the chloroquine-resistant *P. falciparum* lines.

We next examined the ability of the azadipeptide nitriles to inhibit the papain-like proteases of *P. falciparum* using recombinant falcipain-2 and -3.[25] For falcipain-2 the best inhibitor was **2**, bearing a Leu side chain in the P^2 position and exhibiting an IC_{50} value of 59 nM. In contrast, the corresponding Phe-derived analogue **1** inhibited the same enzyme with ~10-fold less activity (IC_{50} 570 nM). Replacing the methyl substituent in the P^1 position of that derivative by benzyl (compound **3**) nearly doubled the IC_{50} value. Extension of the benzyl residue to phenylethyl as well as its replacement by a pentyl moiety increased the affinity to falcipain-2 (compounds **4** and **5** vs compound **3**). Compared to the parent compound **1**, the carbon analogues **6** and **7** were considerably less potent. Similar to falcipain-2, falcipain-3 was better

Table 1
In vitro inhibitory effects of dipeptide nitriles **1**–**7** against *P. falciparum* infected erythrocytes and recombinant *P. falciparum* falcipain enzymes

Compd	Structure	*P. falciparum* IC_{50} (μM)		r*Pf* cysteine proteases IC_{50} (nM)		Papain[a] K_i (nM)	*C* log *D* (pH 7.4)
		Dd2	3D7	Falcipain-2	Falcipain-3		
1		15 ± 7	9.8 ± 6.5	570 ± 70	18,000 ± 9000	3.9	2.84
2		18 ± 6	16 ± 3	59 ± 1	1900 ± 100	8.2	2.35
3		7.5 ± 1.6	7.1 ± 1.6	1000 ± 100	1600 ± 100	42	4.62
4		3.6 ± 2.2	5.3 ± 2.8	430 ± 20	300 ± 30	6.3	4.87
5		2.1 ± 1.3	2.9 ± 1.0	310 ± 10	110 ± 10	10	4.45
6		>25	>25	28,000 ± 4000	16,000 ± 2000	2200	3.18
7		>25	>25	>50,000	>50,000	750,000	3.42

[a] Data taken from Ref. 19.

inhibited by the Leu-derived compound **2** than by the Phe derivative **1**. The inhibition of this enzyme by **1** was poor with an IC_{50} of only 18 μM. Interestingly, the affinity of **1** could be sequentially increased by extending its P^1 methyl group over benzyl and phenylethyl to pentyl, with the latter compound **5** exhibiting an IC_{50} of 110 nM against falcipain-3. Such a trend could not be observed for the related enzyme papain. When comparing compounds **1** and **6** at falcipain-3, it becomes obvious that the potency of the azadipeptide nitrile **1** is similar to that of its carba-desmethyl analogue **6**. This did not apply to falcipain-2 and papain or to thiol-dependent human cathepsins[19] and may indicate a stronger hydrogen bond interaction of falcipain-3 to the P^2–P^1 amide bond. However, the direct carbon analogue of **1**, that is, compound **7**, was an even weaker inhibitor of falcipain-3. The substrate specificity of the two *P. falciparum* cysteine proteases has been extensively studied with regard to the non-primed binding sites.[26–29] For small substrates, a pronounced specificity for Leu in the P^2 position could be confirmed for both enzymes, while more promiscuity was observed for the processing of bigger substrates and hemoglobin.[29] Concerning the S^2 preference, the results obtained with falcipain-2 and -3 are in accordance with their known substrate specificity, as the Leu derivative **2** shows considerably stronger inhibition than **1**, bearing a Phe side chain in P^2. As for other papain-like proteases, the P^1 specificity is less strict. For falcipain-2 and -3, Arg and Lys and Arg and Gln were best accepted, respectively,[29] suggesting the S^1 pocket of the enzyme to be deep but rather shallow. This might be reflected by the observation that compound **5** with a pentyl moiety in P^1 is fairly potent against both enzymes. However, it remains unclear whether the S^1 pocket can be addressed by the azadipeptide nitriles, as their backbone geometry is slightly different compared to their carbon analogues.

The finding that the growth of *P. falciparum* was more potently inhibited by the azadipeptide nitriles (**1–5**) than by the carbon analogues **6** and **7** is similar to the inhibition observed for the isolated cysteine proteases. This suggests a correlation of the enzyme inhibitory and the anti-malarial activity in this series of compounds. However, there are differences in the structure–activity relationships when considering the isolated enzymes, particularly falcipain-2, and the parasite. On the one hand, compounds **4** and **5**, which inhibited the parasites' growth with IC_{50} values equal to or less than 5 μM, also showed a pronounced enzyme inhibitory activity (IC_{50} <500 nM). On the other hand, with the Leu derivative **2**, exhibiting an IC_{50} value of 59 nM against falcipain-2, a weaker growth inhibition (IC_{50} ~17 μM) was achieved. Thus, the physicochemical properties of the inhibitors may influence the anti-malarial properties. Calculation of the log *D* values (using the program Marvin 3.0, Chemaxon; Table 1) indicated the azadipeptide nitriles to be slightly less lipophilic than their carbon-based counterparts (**1** and **2** vs **6** and **7**). Furthermore, extension of the residue in P^1 led to a significant increase in the lipophilicity as the compounds **3–5** exhibit log *D* values greater than 4. Generally, the lipophilicity of an antimalarial agent is important, as three different membranes need to be passed before the compounds can enter the parasite.[30]

The effects of the compounds on the recombinant enzymes exceeded those on the parasites' growth by one order of magnitude. This might reflect the redundancy of enzymes involved in hemoglobin degradation in the malaria parasite. As already-mentioned, besides the falcipain-2 and -3, four different pepsin-like aspartic proteases, the plasmepsins, participate in the protein digestion inside the food vacuole.[31,32] Such a difference in the activities towards the single enzymes and the parasite in the intra-erythrocytic stage was also observed with cysteine protease inhibitors of other chemotypes[33,34] as well as with diamine-based plasmepsin inhibitors.[35] The synergistic action of a combination of cysteine and aspartic protease inhibitors on the inhibition of parasites' growth has been shown.[36]

In summary, these data indicate that azadipeptide nitriles are a new class of antimalarial compounds with in vitro whole cell activity of the compounds correlating with the inhibitory activity against the trophozoite cysteine proteases. These results provide a good starting point for further work towards the development of this class of compounds for antimalarial use. This should be achievable by introducing a greater structural variety into the P^1 position of the azadipeptide nitriles, which is quite feasible from a synthetic point of view.

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24. Briefly, compounds were incubated in triplicate wells of 96 well culture dishes with synchronous ring-stage infected erythrocytes (0.25% parasitemia and 2.5% hematocrit) at different concentrations for 48 h before adding 0.5 μCi [^{3}H]hypoxanthine and then incubating for a further 24 h. Following harvesting onto 1450 MicroBeta filter mats (Wallac) and determining ^{3}H incorporation using a 1450 MicroBeta liquid scintillation counter, the percentage inhibition was calculated compared to matched controls that received DMSO vehicle alone (0.05%). The 50% inhibitory concentration values (IC_{50}) were determined using linear interpolation of inhibition curves.[23]
25. Both enzymes were assayed at 25 °C using Z-Leu-Arg-AMC as substrate at a concentration of 25 μM in 100 mM sodium acetate pH 6.0, 5 mM DTT, 0.75% DMSO. One microliter of the compound dissolved in DMSO was diluted into 100 μL of assay buffer. Fifty microliters of the enzyme in assay buffer were added and the resulting mixture was incubated for 10 min. The reaction was initiated by addition of 50 μL of assay buffer containing the substrate and read immediately in a Fluoroskan Ascent microplate spectrofluorometer. Every compound was assayed at eight different concentrations, with 50 μM being the

highest inhibitor concentration; all other concentrations were created by exponential fivefold dilution. The IC_{50} values were determined by plotting the percentage of inhibition relative to the control reaction in the absence of inhibitor over the logarithmic compound concentration by using the equation $v/v_0 = 1/(1 + 10^{\log[I]}/IC_{50})$. Data analysis was done with the program GraphPad Prism 4 (GraphPad Software).

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