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Aryl-alkyl-lysines: small molecular membrane-active antiplasmodial agents†‡

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Due to emerging resistance there is a steady need for new antimalarial drugs. Here, we report a new class of water soluble, non-toxic compounds, aryl-alkyl-lysines, with promising activity against the ring stage of *Plasmodium falciparum*. The optimal compound perturbed the plasma membrane potential and the digestive vacuole of parasites. In the murine model of malaria (*Plasmodium berghei* ANKA) the compound was able to increase the survival of mice by at least 5 days by an intra-peritoneal route. Further, the compounds showed no apparent toxicity to mice at the concentration tested.

Although the World Health report on malaria has documented a substantial decrease in mortality due to malaria, the disease still poses a huge threat considering the rapid rate at which resistance is being developed against frontline antimalarial drugs.[1] The WHO recommended artemisinin based combination therapies only in 2005, but resistance against artemisinin has been already reported in South-east Asia.[1] Since vaccines against malaria parasites are yet to reach the clinics, much emphasis is still stressed on the use of drugs.[2] Thus, there is a constant need for drugs with a novel mechanism of action.

The first "soldiers" that an invading pathogen encounters upon infecting a host are antimicrobial peptides (AMPs).[3,4] These are small protein molecules, mostly cationic in nature, bearing facial amphiphilicity which helps them to interact with the membranes of microorganisms.[5] AMPs have been found to possess activity against bacteria, fungi, viruses, parasites in addition to having immunomodulatory properties.[3,4,6]

Several natural AMPs, such as magainin,[7] dermaseptin derivatives,[8] NK-2,[9] scorpine,[10] cecropin–melittin[11] and gramicidin[12] possess potent activity against *Plasmodium falciparum*.[13,14] Although the mechanism of action is not clearly understood, it is believed that membrane activity remains the primary feature of such antiplasmodial peptides.[14] It has been established that the membranes of parasite-infected erythrocytes (RBCs) differ from their uninfected counterparts, as there are increased levels of phosphatidylinositol and phosphatidic acid, and decreased contents of sphingomyelin.[15] Moreover, translocation of anionic phosphatidylserine happens from the inner leaflet to the outer leaflet of the infected RBCs.[16] These two phenomena, make the infected RBCs more anionic compared to their uninfected counterparts, which explains the initial interaction between cationic peptides and such cells.[13] Several other peptides and synthetic mimics of AMPs have been prepared which show potent antiplasmodial activity.[17] Oligoacyl lysines, which possess a variety of biological functions also showed potent antiplasmodial activity *in vitro* and *in vivo*.[18] Some small non-peptidic mimics of host-defense peptides showed excellent activity both *in vitro* and in murine models.[19]

In this report, we describe the antiplasmodial properties of aryl-alkyl-lysines. We have investigated the activity of the compounds against 3D7 strains of *P. falciparum* and the activity of the best compound against a chloroquine-resistant strain *in vitro*. The stage of parasite development, at which the compound was most active was also studied. The plausible mechanism of action has also been investigated. *In vivo* studies of the best compound revealed good activity in the murine model of cerebral malaria with negligible toxicity.

The synthesis of the aryl-alkyl-lysines (compounds **1–4**) was carried out following our previously published protocol.[20] Compounds **5–7** are being reported for the first time here. The synthetic scheme for the preparation of the compounds is presented in Scheme 1. In the first step, aromatic aldehydes (benzaldehyde, 1-naphthaldehyde, 4-quinolinecarboxaldehyde, 2-naphthaldehyde and biphenyl-4-carboxaldehyde) were reacted with aminoalkanes (aminohexane or aminooctane) to obtain Schiff's bases. Reduction of the Schiff's bases with sodium borohydride yielded secondary amines (**1a–7a**), which were precipitated out as their HCl

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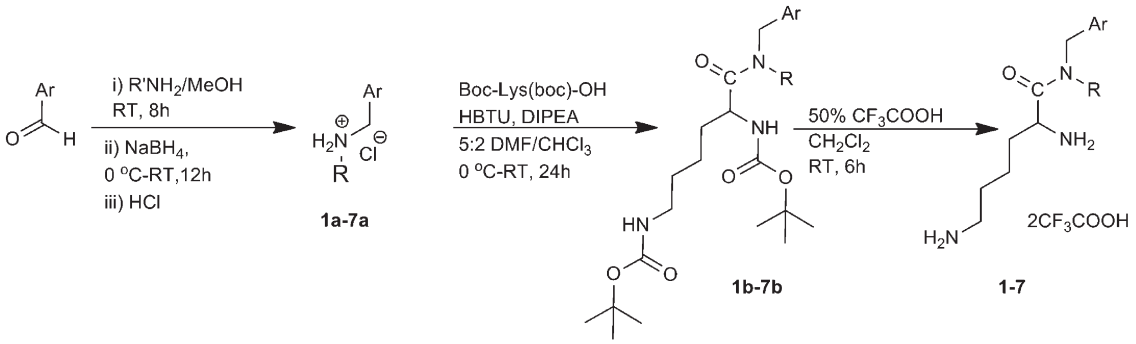
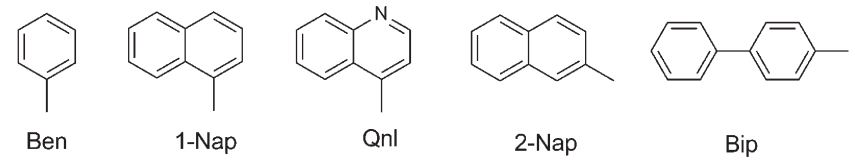


where Ar can be,



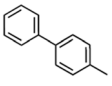
1a, 1b and 1: Ar = Ben, R = C_6H_{13}; **2a, 2b and 2:** Ar = Ben, R = C_8H_{17}; **3a, 3b and 3:** Ar = 1-Nap, R = C_6H_{13}; **4a, 4b** and **4**: Ar = 1-Nap, R=C_8H_{17}; **5a, 5b and 5:** Ar = Qnl, R = C_6H_{13}; **6a, 6b and 6:** Ar = 2-Nap, R = C_6H_{13}; **7a, 7b and 7:** Ar = Bip, R = C_6H_{13};

Scheme 1 General synthetic scheme for the preparation of the compounds (**1**–**7**).

salts. These compounds were then coupled to Boc-Lys(Boc)-OH using HBTU coupling chemistry which were purified using column chromatography. Finally, deprotection of the Boc groups yielded the final compounds (**1**–**7**). All the compounds were purified to more than 95% purity using reverse phase HPLC. The compounds were subsequently characterized using NMR, IR and mass spectrometry. The details of the synthesis and characterization of the compounds are given in the ESI.‡

Previously, we had reported the efficacy of aryl-alkyllysines as topical antibacterial agents.[20] For an effective antiplasmodial candidate, solubility in water and toxicity are important criteria. In this preliminary study, we chose the relatively non-toxic but highly water soluble compounds. Thus, we chose to study the antiplasmodial properties of the benzene and naphthalene based compounds which bear hexyl and octyl chains. The IC_{50} of the compounds, which is defined as the concentration that inhibits the growth of 50% of

Table 1 *In vitro* antimalarial activity and haemolytic values of the compounds

Compd. no.	Ar	R	R′	IC_{50} (μM)	HC_{50} (μM)	HC_{50}/IC_{50}
1		C_6H_{13}	H	>5	>1000	—
2		C_8H_{17}	H	4	564	141
3		C_6H_{13}	H	1	850	850
4		C_8H_{17}	H	3	96	32
5		C_6H_{13}	H	2.3	>1000	>400
6		C_6H_{13}	H	2	620	310
7		C_6H_{13}	H	2	144	57

the total number of parasites, was determined by the SYBR green assay and also validated microscopically.[21] The antiplasmodial activities of the compounds are reported in Table 1. Compound **1**, which contained a benzene core and a hexyl chain was not active till 5 μM (in order to select a promising candidate, the IC_{50} of the compound should be less than 5 μM). **2**, containing a benzyl core and an octyl chain, was active at an IC_{50} value of 4 μM. The compounds of the 1-substituted naphthalene series (compounds **3** and **4**) exhibited promising antimalarial activity. Compound **3** displayed an IC_{50} of 1 μM while compound **4** was active at 3 μM. From this initial screening, compound **3** emerged as the best compound.

Most of the 4-aminoquinoline and 8-aminoquinoline drugs possess some structural similarities to compound **3**. The naphthalene core closely resembles the quinoline moiety. This observation led us to substitute the naphthalene moiety with a quinoline moiety. Thus, we carried out the synthesis of compound **5** with 4-quinolinecarboxaldehyde. In order to identify a more potent compound we carried out a structure–activity relationship study wherein we varied the aromatic core keeping the chain length and lysine moiety constant. In order to understand if the configuration was important for the study, we used 2-naphthaldehyde for the synthesis instead of 1-naphthaldehyde to obtain compound **6**. Subsequently, we replaced the naphthaldehyde with biphenyl-4-carboxaldehyde to obtain compound **7**. However, in this structure–activity relationship study, no significant increase in activity was achieved. For example, compound **6** was as active as compound **3** with an IC_{50} value of 1.2 μM whereas the compounds **5** and **7** had IC_{50} values of 2.5 μM each.

The hemolytic property of an antimalarial drug is an important parameter that needs to be considered while employing the compounds in murine models. It is imperative to have drugs which are non-hemolytic at the concentration at which they are active on parasites. The haemolytic activity of the compounds has been represented as their HC_{50} value, which is the concentration at which they show 50% haemolysis. The HC_{50} values of the compounds are furnished in Table 1. The HC_{50} values of the compounds varied from 96 μM to >1000 μM. The HC_{50} value of compound **3** was 850 μM, which was excellent while its IC_{50} value was 1 μM. In order to gauge the toxicity of the compounds towards plasmodial cells over mammalian cells, we had looked at the selectivity ratio of all the compounds. The selectivity ratio (S R.) is defined as the ratio between HC_{50}/IC_{50}, which emphasizes the ability of the compounds to act specifically on the plasmodial cells. The selectivity of compound **3** was the best in the series with a S.R. of 850. Most of the other compounds containing a hexyl chain exhibited impressive selectivity, for example compound **5** (S.R. >400) and compound **6** (S.R. of 516). Although the 4-quinoline derivative (compound **5**) exhibited HC_{50} values much higher than 1000 μM, it was less potent than the naphthalene derivative (compound **3**). Even the biphenyl derivative (compound **7**) although potent was significantly more toxic. All the other studies were thus conducted with compound **3**.

Since, compound **3** was the most active compound in the study, we screened the antimalarial activity of **3** against two other strains of *P. falciparum*, RKL-9, a chloroquine sensitive strain and MRC-2, a chloroquine resistant strain. Against RKL-9, compound **3** had an IC_{50} of 1.4 μM and against the MRC-2 strain, which was resistant to chloroquine, compound **3** displayed an IC_{50} of 5 μM. Although the activity of **3** was somewhat marginal, the activity against a drug-resistant strain indicated that this class of compound could be developed further as drugs against strains resistant to chloroquine.

In order to ascertain the mechanism of action of the compounds, we performed several assays using the most active compound **3**. We first wanted to determine in what stage the compound acted on the parasites. The malarial parasites have three distinct stages in their asexual life cycle, rings, trophozoites and schizonts. In order to understand the effect of the compound on the different stages, first, the parasites were tightly synchronized, grown to their respective stages and then the compound was added. The compound was found to be most active on the ring stage. In 48 h, in the untreated cases, the rings had grown to late trophozoites or schizonts of the same cycle. Upon treatment with compound **3** at a concentration of 2 × IC_{50}, the growth of the rings was found to be arrested and the few parasites that remained were either in the ring stage or early trophozoite stage (Fig. 1). The parasites were also observed to be severely stressed. The compound did not seem to have significant activity on the trophozoite stage or the schizont stage since in both the control as well as the compound treated case, the parasites had developed normally with hardly any change in parasitemia (Fig. S1‡). Thus, it was concluded that the compound specifically stalled the development of the parasites at the ring stage.

Furthermore, we conducted fluorescent dye based experiments to understand whether the compound had any effect on the parasite plasma membrane or the digestive vacuole of the parasite. The assay to probe if the compound perturbs the plasma membrane potential of the parasite was done using rhodamine 123. This dye is known to fluoresce when the membrane potential is maintained, however, if the membrane potential is perturbed, the dye loses intensity. When the dye is used at a concentration of 1 μM, the effect on the membrane potential of the plasma membrane of the parasite

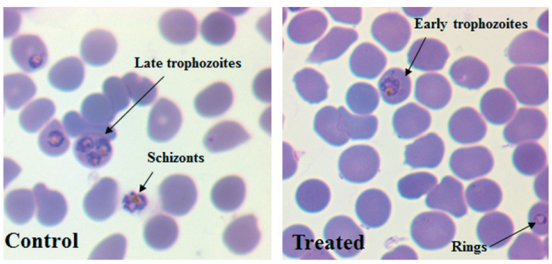


Fig. 1 Compound **3** can stall the development of the parasites in ring stages.

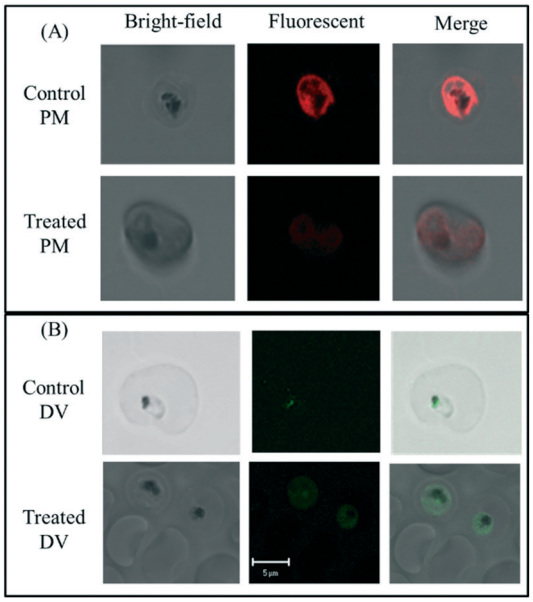


Fig. 2 A) Perturbation of the membrane potential of the parasite membrane (PM) and that of the digestive vacuole (DV). Compound **3** (treated PM) causes greater perturbation of the membrane potential in comparison to the untreated (control PM). B) The intensity of the fluorescence exhibited by the dye Lysotracker green is localized in the DV while in the treated case the dye has diffused all over the cytoplasm.

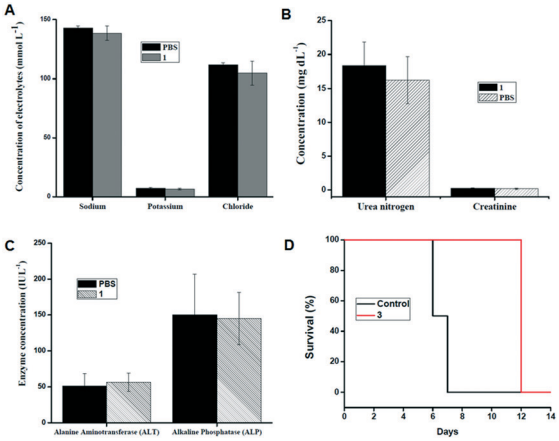


Fig. 3 *In vivo* studies with 20 mg kg^{-1} of compound **3**. (A–C) Acute toxicity of compound **3** in comparison to the vehicle (PBS). No observed toxicity with respect to A) electrochemical balance, B) kidney functions and C) liver functions. D) Survival of mice treated with compound **3** in comparison to the vehicle.

can be monitored.[19] From Fig. 2A it is clear that upon treatment with compound 3, the plasma membrane of the parasite is perturbed. There is significant loss in fluorescence compared to the control.

The digestive vacuole of the parasite is acidic in nature, and the acid sensitive dye, Lysotracker green, stains the intact digestive vacuole green. However, if the potential of the digestive vacuole is perturbed, the dye stains the cytoplasm and there is a significant decrease in fluorescence.[19] Compound 3 was found to act on the parasite digestive vacuole and cause significant perturbation of its potential. There was a decrease in fluorescence and the dye stained the cytoplasmic components of the parasites (Fig. 2B).

Next we investigated the profile of compound 3 in mice (experimental studies with mice were conducted adhering to the institution's guidelines for animal husbandry at the Indian Institute of Science). Initially, we determined the LD_{50} of the compound in mice and determined the sub-chronic toxicity of the compound. For determining the LD_{50} of the compound, we chose to inject different concentrations of the compound, intravenously into mice *via* the tail-vein. The compound was injected at concentrations of 175 mg kg^{-1}, 55 mg kg^{-1}, 17.5 mg kg^{-1} and 5.5 mg kg^{-1} and checked the survival of the mice for fourteen days. We followed the Spearman–Karber method to determine the LD_{50} of the compound.[22] The LD_{50} of the compound was determined to be 98 mg kg^{-1}. When injected with 20 mg kg^{-1}, the mice exhibited no significant toxicity to any of the biochemical parameters of the blood, 48 h after injection (Fig. 3). Specifically, we checked the change in sodium, potassium, chloride (measure of electrochemical balance of the blood), alanine transferase (ALT, biomarker for liver health), aspartate aminotransferase (AST, biomarker for liver and heart health), urea nitrogen (biomarker for kidney and liver health) and creatinine (biomarker for kidney health). From the data presented in Fig. 3(A–C), there is no statistically significant imbalance in any of the parameters.

We, then investigated the ability of compound 3 to reduce parasitemia and increase the survival of mice afflicted with malaria. We introduced cerebral malaria in Swiss-Harlan mice by injecting them with *P. berghei* ANKA strain. Peter's four day test was followed for determining the *in vivo* efficacy of the compound. Specifically, we treated the mice for four consecutive days with compound 3 at a concentration of 20 mg kg^{-1} intraperitoneally (I.P.). The compound was able to reduce parasitemia in all the mice significantly, as observed from Giemsa stained blood smears from the mice five days after injection of the parasites. Although the mice were not completely cured of parasites, the percentage of parasitemia was lowered by more than 60% in the treated case. The survival of the infected mice was also increased due to the treatment of the compound. While all the mice in the control succumbed to death within 7 days of injection, the mice treated with the compounds expired on the 12th day.

Their broad range of activities against different microorganisms coupled with their immunomodulatory properties has made AMPs an attractive scaffold for drug design.[23] Although there are many reports of their antibacterial properties, the field of antimicrobial peptides/peptidomimetics has few reports on their antiplasmodial properties.[17–19,24] Some other peptides have also been designed for antimalarial activity.[25–27] Previously, amino acids have been conjugated to well-known malarial drugs for superior activity, albeit, aryl-alkyl-lysines represent simple designs with no malarial scaffold incorporated.[28–32] This study reports the activity of aryl-

alkyl-lysines against malarial parasites. The most active antiplasmodial agent, compound **3**, was effective in stalling the development of the parasites inside the RBCs and was more active on the ring stage of the parasite. This phenomenon has also been reported in other examples of synthetic membrane active peptides or their mimics. Although studies conducted with fluorescent dyes Rhodamine 123 and Lysotracker green emphasize the membrane damaging nature of the compound, alternative mechanisms of action might play a role. Well tolerated in mice, the compound exhibited a significant therapeutic efficacy at 20 mg kg^{-1} in the murine model of cerebral malaria. Experiments conducted with higher dosages (30 mg kg^{-1} and higher) showed that despite better effective clearance of malarial parasite, multiple dosages of high concentration seemed to have chronic toxicity as the mice indicated prolonged malaise and succumbed to death earlier than expected (data not shown). Although, these compounds are not ideal drug candidates for treating malaria, they do represent a starting point for development of more effective antiplasmodial agents. We plan to synthesize and test several membrane-active compounds based on lysines and amino acids, to elucidate the structural parameters required for obtaining potent antimalarial activity. Further, we also plan to impart membrane-active properties to known antimalarial drugs to obtain enhanced activity.

Conclusions

The goal of this initial study was to understand if the compounds possessed any antimalarial activity, and the study is, by no means, comprehensive. Given the selective potency shown by the compounds, it is imperative to design further structure–activity relationship studies. Future studies will be directed towards the evaluation of the antimalarial activity against more strains. Further, the ability of this class of compound against *Plasmodium vivax* would be determined. Determination of activity in the other stages of the parasite life-cycle, such as the liver stage, would also need to be studied. Overall, aryl-alkyl-lysines are interesting candidates which warrant further investigation for the design of new generation of antimalarial compounds that act by perturbing the membrane of the parasites.

Ethical statement

Experimental studies with mice were conducted adhering to the institution's guidelines for animal husbandry at the Indian Institute of Science.

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