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Original Contribution

Aryl aryl methyl thio arenes prevent multidrug-resistant malaria in mouse by promoting oxidative stress in parasites

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ABSTRACT

We have synthesized a new series of aryl aryl methyl thio arenes (AAMTAs) and evaluated antimalarial activity *in vitro* and *in vivo* against drug-resistant malaria. These compounds interact with free heme, inhibit hemozoin formation, and prevent *Plasmodium falciparum* growth *in vitro* in a concentration-dependent manner. These compounds concentration dependently promote oxidative stress in *Plasmodium falciparum* as evident from the generation of intraparasitic oxidants, protein carbonyls, and lipid peroxidation products. Furthermore, AAMTAs deplete intraparasite GSH levels, which is essential for antioxidant defense and survival during intraerythrocytic stages. These compounds displayed potent antimalarial activity not only *in vitro* but also *in vivo* against multidrug-resistant *Plasmodium yoelii* dose dependently in a mouse model. The mixtures of enantiomers of AAMTAs containing 3-pyridyl rings were found to be more efficient in providing antimalarial activity. Efforts have been made to synthesize achiral AAMTAs **17**–**23** and among them, compound **18** showed significant antimalarial activity *in vivo*

Introduction

Malaria is a major health problem in developing countries. According to the World Health Organization (WHO), it infects more than 300 million people per year and causes more than one million deaths annually, mostly among young children in the age group of less than 5 years [1]. Malaria is reemerging as the biggest infectious killer and is currently a first-priority tropical disease [2,3]. The management of malaria is becoming extremely difficult due to limitations associated with vaccine development [3,4], vector control, and increasing spread of resistance against established antimalarial drugs [5,6]. Therefore, it is necessary to search for new antimalarial drug, which will be effective against multidrug-resistant parasites *in vivo*.

The intraerythrocytic stages of malaria parasites are mainly responsible for clinical manifestations associated with the malaria [7]. During intraerythrocytic stages, malaria parasites degrade vast amounts of hemoglobin inside food vacuoles and release globin along with large quantities of toxic pro-oxidant-free heme [8–10]. During the process of oxidation of oxyhemoglobin [oxyHb (Fe^{+2})] to methemoglobin [met-Hb (Fe^{+3})] electrons are liberated, resulting in the formation of superoxide anions ($O_2^{\bullet -}$). $O_2^{\bullet -}$ is either dismutated by *P. falciparum* cytosolic superoxide dismutase (PfSOD1) to yield hydrogen peroxide (H_2O_2) or can react with H_2O_2 to form hydroxyl radicals ($^{\bullet}OH$). Furthermore, the interaction between heme and intracellular H_2O_2 releases iron (Fe^{+3}), which can generate oxidants via the Fenton reaction [8,9]. Therefore, free heme (Fe^{+3}) offers a major toxic insult to the parasite, which if allowed to accumulate may reach to 300–500 mM [2,11,12] and lead to the development of oxidative stress [13,14]. Free heme (Fe^{+3}) can intercalate in the membrane causing changes in membrane permeability and lipid organization and induces lipid peroxidation of parasite membrane [15,16], which ultimately leads to parasite death. The malaria parasite is very much susceptible to oxidative stress during intraerythrocytic stages [8,9,17]. This free heme not only causes oxidative stress to parasites but the host cell is also equally susceptible, as observed from changes in RBC membrane fluidity, possibly due to changes of membrane lipid composition, lysing erythrocytes and inducing cell death [16,18–23]. It also inhibits key metabolic enzymes as well as redox-active molecules (glutathione) and perturbs cellular redox balance [24,25]. However, parasites have unique rescue mechanisms against free heme

Abbreviations: AAMTAs, aryl aryl methyl thio arenes; H_2O_2, hydrogen peroxide; $^{\bullet}OH$, hydroxyl radical; PBN, phenyl-a-*tert*-butyl-nitrone; *P. falciparum*, *Plasmodium falciparum*; IC_{50}, Inhibitory concentration required to inhibit 50% hemozoin formation; EC_{50}, Inhibitory concentration required to inhibit 50% *P. falciparum* growth; K_D, dissociation constant

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toxicity by converting it into less toxic hemozoin [8,9,26]. Therefore, molecules which interact with heme and interfere in its conversion into hemozoin are effective antimalarials [8,9,14,26]. Apart from hemozoin formation, to endure oxidative burden, malaria parasites also contain diverse antioxidants such as glutathione and thioredoxin-dependent proteins, superoxide dismutase, γ-glutamyl-cysteine synthetase, glutathione reductase, glutathione-*S*-transferase, thioredoxin reductase (TrxR), thioredoxins thioredoxin-dependent peroxidases (TPx), and peroxiredoxin [8,17]. Apart from these antioxidant proteins, *P. falciparum* macrophage migration inhibitory factor (PfMIF) also exhibits thioredoxin (Trx)-like oxidoreductase activity and may also contribute to reduce the oxidative burden [27]. Numerous drugs exhibit potent antimalarial activity by targeting these crucial enzymes or preventing hemozoin formation and thereby promoting oxidative stress [9,28–30]. Trisubstituted methane (TRSM) has the capability to interact with heme and this class of molecules also inhibits hemozoin formation and offers antimalarial activity [28,30–32]. Here, we report the synthesis of a large number of TRSM derivatives, aryl aryl methyl thio arenes (AAMTAs) and evaluation of their antimalarial activities *in vitro* and *in vivo*. The data indicate that these molecules interact with free heme, inhibit hemozoin formation, promote oxidative stress in parasites, and prevent *P. falciparum* growth. These AAMTAs also show antimalarial activity *in vivo* against a multidrug-resistant strain of parasite (MDR strain, *P. yoelli*) in a BALB/c mouse model.

Materials and methods

Materials

Hemin, RPMI 1640, saponin, SDS, chloroquine, glutathione (GSH), thiobarbituric acid (TBA), trichloroacetic acid (TCA), α-phenyl-*n*-*tert*-butyl-nitrone (PBN), dichlorofluorescein diacetate (DCF-DA), fetal calf serum (FCS), manitol, dimethyl sulfoxide (DMSO), penicillin, streptomycin, actinomycin D, and 2,4-dinitrophenylhydrazine were purchased from Sigma (St. Louis, MO, USA). Albumax II was procured from Life Technologies (USA). Giemsa stain was purchased from Qualigens Fine Chemicals (India). [^{3}H]Hypoxanthine was purchased from Amersham Biosciences (USA). Cell Proliferation Reagent WST-1 was procured from Roche Applied Science. All other chemicals were of analytical grade purity.

Chemical synthesis

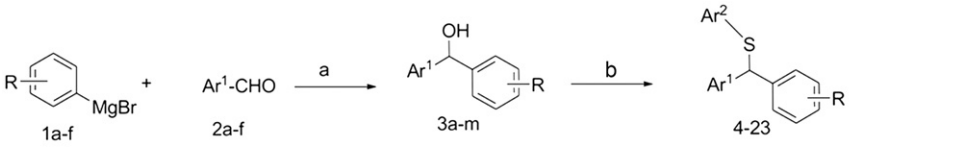
Detailed methods for synthesis and characterization of all aryl aryl methyl thio arenes derivatives are provided separately (Fig. 1 and see the additional information in the supplementary data).

Parasite culture

P. falciparum was cultured according to the method of Trager and Jensen [33,34]. In brief, parasite culture was maintained at a hematocrit level of 5% in complete RPMI 1640 medium supplemented with 25 mM Hepes, 50 μg ml^{-1} gentamycin, 370 μM hypoxanthine, and 0.5% (w/v) AlbuMaxII in tissue culture flasks with loose screw caps. The medium was regularly changed with fresh medium once a day. The growth of *P. falciparum* was regularly checked after Giemsa staining of thin blood smears. *In vivo* growth of *P. yoelii* was maintained in BALB/c mice. Male BALB/c mice (20–25 g) were inoculated intraperitoneally with *P. yoelii* (MDR strain) as described earlier [18–20]. Parasitemia was monitored by preparing thin smears of blood and subsequent Giemsa staining. Experiments on animals were conducted after obtaining permission from the animal ethics committee and in accordance with the institutional guidelines for the care and the use of laboratory animal.

Preparation of parasite lysate

Parasites (*P. falciparum* and *P. yoelii*) were isolated as described [33,35]. In brief, erythrocytes with either ~10% parasitemia (*P. falciparum*) or 50% parasitemia (*P. yoelii,* from infected mice blood) were centrifuged at 800 *g* for 5 min, washed twice, and resuspended in cold phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 5.3 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). For erythrocytes lysis, an equal volume of 0.5% saponin in PBS (final concentration 0.25%) was added to the erythrocyte suspension and kept on ice for 15 min. It was further centrifuged at 1300*g* for 5 min to obtain parasite pellet. The pellet was subsequently washed with PBS thrice and either used immediately or kept at −80 °C. The isolated parasite was lysed in PBS by mild sonication (30 s pulse, bath-type sonicator) at 4 °C and the lysate was then stored at −20 °C for further use. Protein content of the parasite lysate was estimated by the method of Lowry et al. [36].



1a, R = *m*-OCH$_3$
1b, R = *p*-OCH$_3$
1c, R = *o*-OCH$_3$
1d, R = *p*-SCH$_3$
1e, R = H
1f, R = *o*-SCH$_3$

2a, Ar^1 = 2-Pyridyl
2b, Ar^1 = 3-Pyridyl
2c, Ar^1 = 4-Quinoliryl
2d, Ar^1 = Phenyl
2e, Ar^1 = 2-thiomethyl phenyl
2f, Ar^1= 4-methoxyphenyl

3a, R = *p*-OCH$_3$, Ar^1 = 2-Pyridyl
3b, R = *p*-OCH$_3$, Ar^1 = 3-Pyridyl
3c, R = *p*-OCH$_3$, Ar^1 = 4-Quirolyl
3d, R = *o*-OCH$_3$, Ar^1 = 3-Pyridyl
3e, R = *o*-OCH$_3$, Ar^1 = 2-Pyridyl
3f, R = *m*-OCH$_3$, Ar^1 = 3-Pyridyl
3g, R = *m*OCH$_3$, Ar^1 = 2-Pyridyl
3h, R = *p*-SCH$_3$, Ar^1 = 3-Pyridyl
3i, R = *p*-SCH$_3$, Ar^1 = 2-Pyridyl
3j, R = H Ar^1 = 3-Pyridyl
3k, R = H, Ar^1 = Phenyl
3l, R = *o*-SCH$_3$, Ar^1 = 2-thiomethyl phenyl
3m, R= *p*-OCH$_3$, Ar^1 = 4-methoxy phenyl

4, R = *p*-OCH$_3$, Ar^1= 2-Pyridyl, Ar^2= Phenyl
5, R = *p*-OCH$_3$, Ar^1= 2-Pyridyl, Ar^2= 2-methyl-phenyl
6, R = *p*-OCH$_3$, Ar^1= 3-Pyridyl, Ar^2= 4-flouro-phenyl
7, R = *p*-OCH$_3$, Ar^1= 3-Pyridyl, Ar^2= 2-benzoxazolyl
8, R = *p*-OCH$_3$, Ar^1= 4-quinolyl, Ar^2 = phenyl
9, R = *o*-OCH$_3$, Ar^1= 3-Pyridyl, Ar^2= Naphthalenyl
10, R = *m*-OCH$_3$, Ar^1= 3-Pyridyl, Ar^2 = Naphthalenyl
11, R = H, Ar^1 = 3-Pyridyl, Ar^2 = Naphthalenyl
12, R = *p*-SCH$_3$, Ar^1 = 3-Pyridyl, Ar^2 = Naphthalenyl
13, R = *o*-OCH$_3$, Ar^1= 2-Pyridyl, Ar^2 = Naphthalenyl
14, R = *m*-OCH$_3$, Ar^1= 2-Pyridyl, Ar^2 = Naphthalenyl
15, R = *p*-SCH$_3$, Ar^1 = 2-Pyridyl, Ar^2 = Naphthalenyl
16, R = *p*-OCH$_3$, Ar^1= 3-Pyridyl, Ar^2 = 2-Pyridyl
17, R = H, Ar^1 = Phenyl, Ar^2= 2-Pyridyl
18, R = H, Ar^1 = Phenyl, Ar^2= 2-benzoxazolyl
19, R = *o*-SCH$_3$, Ar^1 = 2-thiomethylphenyl, Ar^2= 2-benzoxazolyl
20, R = *o*-SCH$_3$, Ar^1 = 2-thiomethylphenyl, Ar^2= 2-Pyridyl
21, R = *o*-SCH$_3$, Ar^1 = 2-thiomethylphenyl, Ar^2= 2-benzothiazolyl
22, R= *p*-OCH$_3$ Ar^1= 4-methoxyphenyl Ar^2 = 2-Pyridyl
23, R= *p*-OCH$_3$ Ar^1= 4-methoxyphenyl Ar^2 = 2-benzoxazolyl

Fig. 1. Scheme of synthesis of aryl aryl methyl thio arenes (**4**–**16**) along with achiral AAMTAs (**17**–**23**). Reagents and conditions are as follows: (a) dry THF, rt, 1 h; (b) conc H_2SO_4, dry benzene, reflux, 0.5 h or anhydrous $AlCl_3$, dry benzene, rt, 0.5 h.