A series of [(aryl)arylsulfanyl)methyl]pyridines (AASMP) have been synthesized. These compounds inhibited hemozoin formation, formed complexes ($K_d = 12$ to $20 \mu M$) with free heme (ferriprotoporphyrin IX) at a pH close to the pH of the parasite food vacuole, and exhibited antimalarial activity in vitro. The inhibition of hemozoin formation may develop oxidative stress in Plasmodium falciparum due to the accumulation of free heme. Interestingly, AASMP developed oxidative stress in the parasite, as evident from the decreased level of glutathione and increased formation of lipid peroxide, H$_2$O$_2$, and hydroxyl radical ($\cdot$OH) in P. falciparum. AASMP also caused mitochondrial dysfunction by decreasing mitochondrial potential ($\Delta \Psi_m$) in malaria parasite, as measured by both flow cytometry and fluorescence microscopy. Furthermore, the generation of $\cdot$OH may be mainly responsible for the antimalarial effect of AASMP since $\cdot$OH scavengers such as mannitol, as well as spin trap $\alpha$-phenyl-$n$-tertbutylnitrone, significantly protected P. falciparum from AASMP-mediated growth inhibition. Cytotoxicity testing of the active compounds showed selective activity against malaria parasite with selectivity indices greater than 100. AASMP also exhibited profound antimalarial activity in vivo against chloroquine resistant P. yoelii. Thus, AASMP represents a novel class of antimalarial.

Malaria affects 40% of global population and accounts annually for 300 to 500 million clinical cases with 1.5 to 2.7 million deaths (50, 72). The burden of malaria is increasing due to drug resistance, and there is an urgent need for new antimalarial drugs (25, 71). Intra-erythrocytic stages of malaria parasites consume and degrade huge quantities of hemoglobin in the food vacuole and release large quantities of redox active free heme as a by-product (49). Free heme (ferriprotoporphyrin IX) is very toxic (29, 30), and parasites detoxify free heme by forming hemozoin, mainly through the biocrystallization or biominalization process (18, 19, 41). Molecules that inhibit parasite growth through binding to heme are potential antimalarials, and the inhibition of hemozoin formation is considered a valid target for developing new antimalarials (16, 17). Again, the inhibition of hemozoin formation may develop oxidative stress due to the accumulation of free heme, which can generate highly reactive hydroxyl radical ($\cdot$OH), and the malaria parasite is susceptible to oxidative stress (29, 30). Therefore, the enhancement of oxidative stress to the parasite by any means is a promising strategy in developing new antimalarial agents.

Triarylmethanes represent an important class of medicinally important molecules (38) and are known to possess a wide variety of biological activities such as antitubercular (42–45, 54), anti-implantation (58), and anti-proliferative (1) activities and activity against breast cancer (55). The potent antimycotic drug clotrimazole, a member of the triarylmethanes, inhibits the in vitro parasite growth of different strains of chloroquine-sensitive and -resistant Plasmodium falciparum (51, 61, 65). Very recently, antimalarial agents based on the clotrimazole scaffold have been synthesized (23). Again, trisubstituted methanes (TRSMs) containing sulfide, sulfoxide, or sulfone spacers have also been reported to show various biological activities. A small set of $9$-(lupinylthio)xanthenes, $9$-(lupinylthio)dioxolanes, and $9$-(lupinylthio)diphenylmethanes was found to exhibit diverse biological activities (39). Arylsulfanyl and arylsulfonyl moieties are integral parts of many antimalarial agents. For example, the antimalarial activity of several arylacridinyl sulfoxides has been reported recently (52). Furoxan derivatives bearing a sulfone moiety were reported to have antimalarial activity (22). A series of imidazole-dioxolane compounds bind to the heme and showed promising anti-Plasmodium activity (68). These results prompted us to synthesize and evaluate a new series of TRSMs for antimalarial efficacy. Here we report the antimalarial activity of a series of [(aryl)arylsulfanyl)methyl]pyridines (AASMPs) that represents a new class of TRSMs. Our work focused on the evaluation of the antimalarial activity of these compounds, including the mechanistic details on the effect of heme interaction, hemozoin formation, and in vitro and in vivo antimalarial effect.
bromide (MTT), penicillin, streptomycin, and tetraethoxypropane were purchased from Sigma (St. Louis, MO). AlbuMax II was procured from Life Technologies, and Giemsa stain was purchased from Qualigens Fine Chemicals, India. [3H]hypoxanthine was purchased from Amersham Biosciences. All other chemicals were of analytical-grade purity.

Chemical synthesis. Chemical synthesis, as well as the characterization of all AASMP was provided separately (see additional information in the supplemental material).

Parasite culture (in vitro and in vivo). P. falciparum (clone NF54) was grown as described by Trager and Jensen (63) at a hematocrit level of 5% in complete RPMI medium (CPRMI; RPMI 1640 medium supplemented with 25 mM HEPES, 50 μg of gentamicin ml−1, 370 mM hypoxanthine, and 0.5% [w/v] [AlbuMaxII] in tissue-culture flasks (25 and 75 cm2) with loose screw caps. Used medium was changed with fresh medium once in 24 h, and the culture was routinely monitored through Giemsa-staining of thin smears. The growth of multidrug-resistant (MDR) P. yoelii in vivo was maintained in the Swiss mouse model as described previously (56).

Isolation of parasite from infected erythrocytes and preparation of parasite lysate. The parasite (P. falciparum and P. yoelii) was isolated as described previously (10). Briefly, erythrocytes with ~10% parasitemia (P. falciparum) or 50% parasitemia (P. yoelii) were centrifuged at 800 × g for 5 min, washed, and resuspended in cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 5.3 mM NaHPO4, and 1.8 mM KH2PO4). 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 centrifuged at 1,300 × g for 5 min to get a parasite pellet; finally, the pellet was washed with PBS 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 whole lysate was then stored at −20°C for future use. The protein content of the parasite lysate was estimated as described previously (33).

Hemozoin (β-hematin) formation. Hemozoin formation was assayed as described earlier (46, 59, 64). In brief, the assay mixture contained in a final volume of 1 ml: 100 mM sodium acetate buffer (pH 5.2), 100 μM hemin, parasite lysate (20 μl), and different concentrations of AASMPs. The reaction was initiated by the addition of AASMPs dissolved in DMSO at 1, 5, 10, 50, and 100 μM, and the amount of hemoglobin formed in the presence or absence of AASMPs was measured as described previously (5, 9, 26). Isolated parasite was sonicated in 200 μl of 10 mM ice-cold EDTA in a VCX 600 sonicator (by using a 9 son/10 s off cycle for 10 min) and then incubated at 37°C for 30 min to solubilize each pellet, and the hemoglobin concentration of the formic acid solution was determined in a Shimadzu UV/Vis1700 spectrophotometer at 540 nm (extinction coefficient = 1.56 × 104 M−1 cm−1). The hemoglobin content was expressed as nmol/mg of cell protein.

Measurement of antimalarial activity by monitoring [3H]hypoxanthine uptake. Inhibition of P. falciparum growth was studied by monitoring [3H]hypoxanthine uptake as described earlier (15). P. falciparum (NF-54 strain) was cultured in vitro as described earlier (63). Synchronization of the parasites to uniform ring stages was achieved by using 5% aqueous t-sorbitol as described earlier (31). In brief, P. falciparum culture was centrifuged at 2,000 rpm for 5 min to pellet the cells. The supernatant media was discarded, and packed cells were suspended in a five-times volume of 5% t-sorbitol and allowed to stand for 15 to 20 min at 37°C. The cells were washed twice with RPMI medium (without AlbuMax II) to remove sorbitol and cell debris. The pellet was suspended in complete RPMI 1640 medium to a final hematocrit of 2 to 3%. Synchronization of the culture was confirmed by microscopic examination of Giemsa-stained thin-smear slides. To see the effect of AASMP, the ring-synchronized P. falciparum (parasitemia, 0.5 to 1%) was cultured in multwell plates (200 μl/well) in the presence or absence of different concentrations of AASMPs. Chloroquine was used as a positive control. After 48 h [3H]hypoxanthine (0.7 μCi/well) was added in each well and further cultured for 48 h to monitor parasite viability by measuring the incorporation of [3H]hypoxanthine in parasite nucleic acids. P. falciparum was harvested and washed twice in PBS. These parasite pellets were dissolved in 100 μl of 10 mM NaOH by keeping at 37°C for 6 h. After incubation, it was placed in scintillation fluid and after 12 h, the [3H]hypoxanthine uptake was measured in a β-counter.

Measurement of hemozoin formation. The heme content in control and AASMP-treated P. falciparum was measured as described earlier (36). In brief, P. falciparum was cultured in the presence or absence of various concentrations of AASMP for 48 h. The culture was then centrifuged to pellet the cells, and the cell pellets were dissolved in formic acid (100°C, 30 min), and the whole lysate was then stored at −20°C for future use. The protein content of the parasite lysate was estimated as described previously (33).

Hemozoin formation was assayed as described earlier (46, 59, 64). In brief, the assay mixture contained in a final volume of 1 ml: 100 mM sodium acetate buffer (pH 5.2), 100 μM hemin, parasite lysate (20 μl), and different concentrations of AASMPs. The reaction was initiated by the addition of AASMPs dissolved in DMSO at 1, 5, 10, 50, and 100 μM, and the amount of hemozoin formed in the presence or absence of AASMPs was measured as described previously (5, 8, 26). In brief, parasite lysate (500 μl) was cultured in the presence or absence of various concentrations of AASMP for 48 h. After 48 h of treatment, the culture was washed twice with PBS, and the parasite was isolated.

The GSH contents from control and AASMP-treated parasites were determined as described previously (5, 9, 26). Isolated parasite was sonicated in 200 μl of 20 mM ice-cold EDTA in a VCX 600 sonicator (by using a 9 son/10 s off cycle for a period of 60 s) and centrifuged at 10,000 × g for 20 min to get clear lysate. The lysate (200 μl) was mixed with an equal volume of 10% trichloroacetic acid, and precipitated protein content was removed through centrifugation. The supernatant solution was taken to an equal volume of 0.8 M Tris-HCl (pH 9) containing 20 mM 5,5′-dithiobis-(2-nitrobenzoic acid) to yield the yellow chromophore of thionitrobenzoic acid, which was measured at 412 nm. GSH was used as a standard.

Measurement of lipid peroxide as an index of oxidative damage. P. falciparum culture (4% parasitemia, ring synchronized) was incubated with different concentrations of AASMPs for 48 h. After incubation, the parasite was isolated and mixed with PBS (500 μl) to prepare parasite lysate as described above, and the lipid peroxidation product from these lysates was measured as described earlier (5, 8, 26). In brief, parasite lysate (500 μl) was treated with 1 ml of trichloroacetic acid-thioarbituric acid mixture in 1 N HCl, followed by incubation for 15 min at 100°C. After incubation, the sample was cooled and centrifuged (4,000 rpm for 10 min). Supernatant was then collected, and the optical density was measured at 535 nm. The formation of lipid peroxide in parasite membrane was expressed as nmol/mg of cell protein.

Fluorometric measurement of intra-parasitic H2O2. P. falciparum (4% parasitemia) was cultured in the presence or absence of different concentrations of AASMP for a period of 48 h. The culture was then further incubated for 30 min in complete RPMI medium containing 10 μM 2′,7′-dichlorofluorescein diacetate. The culture was then washed twice with PBS, and the parasite was isolated from control and treated groups. Isolated parasites were lysed by mild sonication (5–5.5 pulses, bath-type sonicator) at 4°C. H2O2 was measured in control or AASMP-treated parasites by measuring the fluorescent dichlorofluorescein formed due to the oxidation of nonfluorescent probe dichlorofluorescein diacetate by H2O2 (37). Fluorescence intensities were recorded from the lysate in a Perkin-Elmer Life Sciences Lambda LS 50B spectrophotometer with a 5-mm
FIG. 1. Synthesis of AASMPs 4a to 4g. Reagents and conditions: dry tetrahydrofuran, room temperature, 1 h (a) and anhydrous AlCl₃, dry benzene, room temperature or reflux, 0.5 h (b).

path-length quartz cell in a total volume of 1 mL at wavelengths of 502 and 523 nm for excitation and emission, respectively. The H₂O₂ content was measured and is expressed as the fluorescence intensity/mg of parasite lysate protein.

Measurement of hydroxyl radical (·OH) generation. ·OH generated in the P. falciparum P. falciparum after AASMP treatment at different concentrations was measured by using DMSO as an ·OH scavenger (3, 5). In brief, P. falciparum culture (200 μL) (2% parasitemia, ring plus early trophozoite stage) was grown in a multwell plate in the presence or absence of different concentrations of AASMPs containing 20 μL of 25% DMSO for 48 h. DMSO (20 μL) was added in each time, along with the stipulated concentrations of AASMP when the medium was changed (once in 24 h). A negative control (parasite only) was made without DMSO and AASMP. After 48 h, the culture was centrifuged at 800 × g for 5 min, washed, and resuspended in cold PBS. The parasite was isolated as described above, and the isolated parasite was lysed in triple-distilled water and processed for the extraction of methanesulfinic acid formed by the reaction of ·OH with DMSO. Methanesulfinic acid formed was allowed to react with Fast Blue BB salt, and the intensity of the resulting yellow chromophore was measured at 425 nm for excitation and emission, respectively. The H₂O₂ content was measured by the addition of 0.7 μL of [³H]hypoxanthine per well, and the microplates were wrapped with aluminum foil and incubated for 4 h at 37°C. After incubation, all contents of the wells were again removed by using a pipette (cells had adhered to the walls of the wells), and 100 μL of DMSO was put into each well, followed by mixing, and the microplate was kept in the dark overnight. The next day, the absorbance for every well was measured at 570 nm by using a microplate reader.

In vivo antimalarial activity. The in vivo antimalarial activity of AASMP (4a, 4b, and 4e) was evaluated in a rodent model against MDR (chloroquine, mefloquine, and halofantrine) P. yoelii in BALB/c mice at three dose levels. In brief, five mice (22 ± 2 g) were inoculated intraperitoneally with 10⁵ parasitized RBC on day 0, and the compounds were administered after 6 h of parasite inoculation via the intraperitoneal route. The treatment was continued once daily at each dose level from days 0 to 3 via the intraperitoneal route. The compounds were diluted in groundnut oil so as to obtain the required drug dose per animal in 0.2 ml volume. The parasitemia levels from individual mice were recorded in Giemsa-stained thin blood smears on day 4. The mean value determined for a group of five mice was used to calculate the percent suppression in parasitemia with respect to the vehicle control group. α-β-Arctecher-treated mice were used as a positive control.

Statistical analysis. Data are shown as means ± the standard error of the mean (SEM). Statistical analysis was performed by using the Student t test or analysis of variance. Wherever applicable, a P value of <0.05 was considered as statistically significant.

RESULTS

Chemistry. Our method (14) for the synthesis of AASMPs involved S alkylation of different aryl or heteroaryl thios using carbinols 3a and 3b as the alkylation agents (Fig. 1). The formation of a sulfur link between a diarylmethane and an aryl or a heteroaryl ring can be achieved either by the nucleophilic attack of an aryl or heteroaryliolate ion on diarylmethyl halides and diarylmethyl-p-tolysulfonates or by protic or Lewis acid-catalyzed condensation of diarylcarbinols with different aryl or heteroaryliothols (6). Carbinols 3a and 3b were obtained by the Grignard reaction of 4-methoxyphenylmagnesium bromide with pyridine-3-carbaldehyde and pyridine-2-aldehyde, respectively. The S alkylation reactions on carbinols 3a and 3b were achieved in the presence of anhydrous AlCl₃ in dry benzene at room temperature. However, in the case of S alkylation of 2-mercaptopentheniazole on carbinol 3a the reaction was performed at reflux condition since 2-mercaptobenzeno-
zothiazole is insoluble in benzene at room temperature. Although in every case the reaction condition was like that of a typical Friedel-Crafts alkylation reaction due to the higher nucleophilicity of sulfur than that of aromatic ring carbon atoms, nucleophilic attack occurred through sulfur. All of the synthesized AASMPs (4a to 4g) are presented in Table 1.

**Inhibition of hemozoin (β-hematin) formation.** The synthesized compounds were tested regarding whether they can inhibit hemozoin formation in vitro. All AASMPs (4a to 4g) inhibited hemozoin formation mediated by both MDR *P. yoelii* whole-cell lysate and chloroquine-sensitive *P. falciparum* (NF-54) whole-cell lysate (Table 1). The 50% inhibitory concentration (IC₅₀) values for the inhibition of hemozoin formation were in the range of 11 to 40 μM. The LogP and pKₐ values of AASMPs were calculated for further characterization (Table 1).

**Interaction of AASMP with heme.** Drugs that inhibit hemozoin formation generally interact with free heme. Since AASMP inhibits hemozoin formation, we tested whether it could interact with free heme. Although it is more difficult to study the interaction of drugs with heme under the pH conditions of the food vacuole due to the poor solubility of heme at low pH, to get an accurate picture, the interaction of AASMPs (4a, 4b, 4e, and 4f) with heme was determined at pH 5.2, thus approximating the pH of the parasite food vacuole. A solution of heme at pH 5.2 showed a broad peak at 362 nm, indicating that dimers of the β-oxo type or the β-hematin type predominate under our in vitro conditions (7, 32). The addition of AASMPs clearly perturbed the heme spectrum (Fig. 2A, C, E, and G), a finding indicative of an interaction between the AASMP and the heme units. Titration with increasing amounts of AASMPs into the heme solution produced spectra with a well-defined isosbestic point in the Soret range, with reduction in the heme Soret molar absorptivity, and a shift of the Soret band to longer wavelengths. The binding of AASMPs to heme was studied by optical difference spectroscopy (Fig. 2B, D, F, and H). The apparent Kₛₒ values for the binding of AASMPs to heme, as calculated from the plot of 1/ΔA₃₆₂ against 1/[AASMPs] were shown in the insets (Fig. 2B, D, F, and H).

<table>
<thead>
<tr>
<th>Serial no.</th>
<th>Compound</th>
<th>Structure</th>
<th>CS LogP ± SD</th>
<th>CS pKₐ ± SD</th>
<th>Mean IC₅₀ for hemozoin formation (μM) ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4a</td>
<td><img src="image1" alt="structure" /></td>
<td>3.05 ± 0.79</td>
<td>5.11 ± 0.73</td>
<td>11 ± 4 18 ± 4</td>
</tr>
<tr>
<td>2</td>
<td>4b</td>
<td><img src="image2" alt="structure" /></td>
<td>3.30 ± 0.92</td>
<td>4.649 ± 0.713</td>
<td>16 ± 5 26 ± 5</td>
</tr>
<tr>
<td>3</td>
<td>4c</td>
<td><img src="image3" alt="structure" /></td>
<td>3.55 ± 0.95</td>
<td>5.09 ± 0.76</td>
<td>40 ± 10 20 ± 6</td>
</tr>
<tr>
<td>4</td>
<td>4d</td>
<td><img src="image4" alt="structure" /></td>
<td>3.59 ± 1.10</td>
<td>5.39 ± 0.67</td>
<td>40 ± 8 40 ± 6</td>
</tr>
<tr>
<td>5</td>
<td>4e</td>
<td><img src="image5" alt="structure" /></td>
<td>4.49 ± 1.09</td>
<td>5.71 ± 0.85</td>
<td>40 ± 5 20 ± 4</td>
</tr>
<tr>
<td>6</td>
<td>4f</td>
<td><img src="image6" alt="structure" /></td>
<td>3.82 ± 1.0</td>
<td>2.82 ± 0.78</td>
<td>35 ± 6 40 ± 5</td>
</tr>
<tr>
<td>7</td>
<td>4g</td>
<td><img src="image7" alt="structure" /></td>
<td>3.20 ± 0.87</td>
<td>3.47 ± 0.62</td>
<td>40 ± 8 40 ± 8</td>
</tr>
</tbody>
</table>

* CS LogP, ChemSilico LogP.
* CS pKₐ, ChemSilico pKₐ.
FIG. 2. Interaction of AASMPs with heme. (A, C, E, and G) Optical Soret spectroscopy for AASMP-hemin interaction at different concentrations of AASMP (5 to 40 μM) (i, 5 μM; f, 40 μM). (B, D, F, and H) Optical difference spectroscopy for AASMP-hemin complex formation (i, 5 μM; f, 40 μM). The inset shows the plot of 1/ΔA360 nm versus 1/[AASMP] to calculate the $K_D$. 

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TABLE 2. Effect of AASMPs on P. falciparum growth as measured by \[^{3}H\]hypoxanthine uptake

<table>
<thead>
<tr>
<th>Compound</th>
<th>[^{3}H]hypoxanthine uptake (mean IC50 [\mu M] ± SEM)*</th>
</tr>
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<tbody>
<tr>
<td>4a</td>
<td>5 ± 1*</td>
</tr>
<tr>
<td>4b</td>
<td>8 ± 2*</td>
</tr>
<tr>
<td>4c</td>
<td>12 ± 4†</td>
</tr>
<tr>
<td>4d</td>
<td>4 ± 0.5*</td>
</tr>
<tr>
<td>4e</td>
<td>12 ± 2*</td>
</tr>
<tr>
<td>4f</td>
<td>20 ± 7†</td>
</tr>
</tbody>
</table>

* *, P < 0.001; †, P < 0.002.

The mean binding affinities (± the SEM) of AASMPs with heme were determined to be as follows: 4a, 20 ± 4; 4b, 26 ± 6; 4c, 12 ± 3; and 4f, 14 ± 3. Of the \(K_D\) values compared to the AASMPs, 4e showed the highest affinity.

Inhibition of P. falciparum growth and development by AASMP. In view of the observations that AASMP associates with heme and inhibits hemozoin, it is expected that AASMPs may show some antiplasmodial activity. Interestingly, all AASMPs (4a to 4g) inhibited the growth of the malaria parasite concentration dependently, but 4a, 4b, and 4e were found to be very potent (IC50 \[\mu M\] = 5 ± 1*). Compound 4e effectively inhibited the growth of the parasite (Fig. 4A) and induced the formation of lipid peroxide (Fig. 4B) in the parasite in a concentration-dependent manner. \(H_2O_2\) and ·OH are very important members of reactive oxygen species, which cause oxidative stress under different situations. To determine whether AASMPs via stimulating the accumulation of intraparasitic \(H_2O_2\) reduced the GSH level and stimulated the lipid peroxidation, intraparasitic \(H_2O_2\) level was measured. Interestingly, these AASMPs also significantly (P < 0.001) increased the generation of intraparasitic \(H_2O_2\) (Fig. 5A) and stimulated the generation of highly reactive ·OH in a concentration-dependent manner (Fig. 5B).

AASMP reduces parasite-mitochondrial transmembrane potential (\(\Delta \psi_m\)). Generation of reactive oxygen species and associated oxidative stress causes mitochondrial dysfunction (4). In order to investigate whether oxidative stress induced by AASMP can lead to mitochondrial dysfunction in malaria parasite, alteration of mitochondrial membrane potential (\(\Delta \psi_m\)), a marker for dysfunction, was measured. The \(\Delta \psi_m\) was investigated by using membrane potential sensitive dye, JC1 (5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolocarbocyanine iodide). In intact healthy mitochondria with higher \(\Delta \psi_m\), JC1 would be accumulated in mitochondrial matrix to form J-aggregate, showing intense fluorescence at 590 nm. Mitochondria with open transition pores would be at low \(\Delta \psi_m\), and the accumulation of JC1 would be less in the matrix, leading to less availability of JC1 to form aggregates, showing weak fluorescence at 590 nm. Both fluorescence-activated cell sorting (Fig. 6A) and fluorescence microscopic (Fig. 6B) studies were done to measure the \(\Delta \psi_m\) in the presence or absence of the most effective AASMP (4e). Compound 4e effectively decreased the \(\Delta \psi_m\) (Fig. 6Ab) compared to the control (Fig. 6Aa). Fluorescence microscopic analysis clearly indicated that the formation of J-aggregate (red fluorescence, 590 nm) (Fig. 6Ba) or the ratio of 590 nm to 530 nm (Fig. 6Bc) in the trophozoite-infected red cell (control) was higher than that for 4e or antimycin A (positive control)-treated trophozoite-infected red cell (control).
fected red cells. In contrast, the green fluorescence (JC1 monomer, 530 nm) was much less in the control compared to 4e or antimycin A-treated cells (Fig. 6Bb). Thus, AASMP alters the mitochondrial potential of the malaria parasite.

Critical role of reactive oxygen species for AASMP-induced parasite growth inhibition. In order to assess whether the oxidative stress induced by AASMP is actually responsible for the inhibition of parasite growth, the effect of different antioxidant or ·OH scavengers, such as mannitol and spin traps like PBN, was studied on AASMP-induced P. falciparum death. The results indicated that ·OH scavengers significantly (P < 0.05) protected P. falciparum from AASMP-induced growth inhibition (Fig. 7).

In vitro cytotoxicity evaluation of selected active compounds. Thus far, we have demonstrated that AASMP offers an antimalarial effect by enhancing oxidative stress in P. falciparum. However, AASMP has a pyridine moiety and is highly hydrophobic, which may lead to damaging the RBC membrane. Thus, we investigated whether AASMP has any specificity toward the malaria parasite or whether it lyses any type of biological membrane, including the RBC membrane. The normal uninfected RBC were incubated with AASMP at different concentrations. After incubation, RBC were infected with P. falciparum-infected erythrocytes, and the uptake of [H3]hypoxanthine was monitored. The incorporation of [H3]hypoxanthine in P. falciparum cultured in RBC treated with different concentrations of AASMP (4b and 4e) was comparable to that of the control (Fig. 8). These results suggested that AASMPs at up to 40 μM did not affect the ability of RBC to support parasite invasion and growth. Therefore, it can be concluded that AASMP does not affect the viability of the RBC and that its antimalarial action is specific to the parasite only. We also evaluated the toxicity of AASMPs on nucleated proliferating mammalian MCF-7 cells. MCF-7 cells were incubated with 10 to 1,000 μM AASMPs, and MTT oxidation was monitored to determine the viability. Again, AASMP did not show any significant toxicity in mammalian nucleated cells also, and the selectivity index was greater than 100 (Table 3).

In vivo antimalarial activity against the malaria parasite MDR P. yoelii. In vitro antimalarial activity of AASMP encouraged us to evaluate the effect of AASMP against multidrug-resistant rodent malarial parasite MDR P. yoelii in vivo. Interestingly, AASMP showed antimalarial activity in vivo. Compound 4a suppressed the day 4 mean parasitemia by 20, 30, and 60% at doses of 20, 50, and 50 mg/kg, respectively (Table 4). Compound 4b suppressed the day 4 mean parasitemia by 30, 50, and 80% at doses of 25, 50, and 100 mg/kg, respectively (Table 4). Compound 4e suppressed the day 4 mean parasitemia by 40, 65, and 85% at dose levels of 10, 25, and 50 mg/kg, respectively (Table 4). Thus, 4a or 4b was effective, but 4e was found to be comparatively much more effective at low

![FIG. 4. Enhancement of oxidative stress in P. falciparum by AASMP. (A and B) The percent alteration of GSH (relative to the control) (A) and lipid peroxide (B) in P. falciparum was measured at different concentrations of AASMP as indicated. Values are means ± the SEM. *, P < 0.05; **, P < 0.002; ***, P < 0.001 (versus the control).](image1)

![FIG. 5. AASMP stimulates the generation of intraparasitic H2O2 and ·OH. The levels of H2O2 (A) and ·OH (B) in P. falciparum were measured at different concentrations of AASMP as indicated. Values are means ± the SEM. ***, P < 0.001 (versus the control).](image2)
concentrations in demonstrating an antimalarial effect in vivo in the rodent model.

**DISCUSSION**

Evidence has been presented to show that AASMP has antiplasmodial activity. The mechanistic studies reveal that it effectively inhibits hemozoin formation and induces oxidative stress in the malaria parasite to inhibit *P. falciparum* growth. The data indicate that this novel class of antimalarial shows selective activity against the malaria parasite with a selectivity index of greater than 100 and offers antimalarial activity in vivo in the rodent model.

Compounds that inhibit hemozoin formation usually interact with heme. The addition of AASMP clearly perturbed the heme spectrum, a finding indicative of an interaction between the AASMP and the heme units. The spectral changes of heme-AASMP mixtures were similar to those observed for molecular complex formation between metalloporphyrines and other aromatic molecules involving a cofacial π-π interaction (67).

Although all synthesized compounds showed antiplasmodial activity, compounds 4c, 4f, or 4g showed higher IC₅₀ values than compounds 4a, 4b, 4c, 4d, and 4e. The reason for the
higher IC\textsubscript{50} values for 4f or 4g is probably due to less accumulation in the parasite food vacuole by pH trapping for higher pK\textsubscript{a} values. LogP and pK\textsubscript{a} values of AASMPs (4a, 4b, 4c, 4d, and 4e) are suitable for the accumulation in the food vacuole of the parasite through pH trapping since the pK\textsubscript{a} values are close to or little greater than the pH (pH 4.5 to 5.2) of the parasite food vacuole. However, the pK\textsubscript{a} values of compounds 4f and 4g (Table 1) are not suitable for the accumulation in the food vacuole of the parasite through pH trapping, but we cannot exclude some other mechanism by which these two compounds may enter in the food vacuole.

The possible mechanism by which AASMP develops oxidative stress and parasite death is mediated through its inhibitory effect on hemozoin formation. The inhibition of hemozoin formation causes death of the parasite due to the accumulation of toxic free heme (28). Free heme can damage cellular metabolism of the malaria parasite by inhibiting enzymes, promoting the peroxidation of membranes and the production of reactive oxygen species in the acidic environment of the food vacuole (29, 30, 48). It also well known that the malaria parasite is very much susceptible to oxidative stress (11, 21, 73). Inhibition of heme detoxification function is known to kill the parasite through membrane lysis and the interference of other vital functions (20, 40, 60).

Our studies indicate that AASMP-induced oxidative stress is associated with the reduction of Δψ\textsubscript{m}. The loss of Δψ\textsubscript{m} is considered one of the most significant events in the oxidative stress-mediated mitochondrial pathway of cell death (27, 47, 74). It is already known that the alteration of Δψ\textsubscript{m} in the malaria parasite causes mitochondrial dysfunction, which may lead to parasite death. Atovaquone, a well-known antimalarial, inhibits parasite growth that reduces mitochondrial membrane potential (34, 57).

If AASMP inhibits \textit{P. falciparum} growth through the induction of oxidative stress, antioxidant treatment should protect the parasite from AASMP-induced parasite death. ·OH scavengers and spin traps remarkably protected \textit{P. falciparum} from growth inhibition. Since PBN or mannitol significantly protected (50 to 57%) \textit{P. falciparum} from AASMP-induced growth inhibition, it can be suggested that ·OH is the major reactive oxygen species generated by AASMP to offer antimalarial effect. Since PBN or mannitol can scavenge ·OH and did not offer 100% protection, we cannot rule out the role of H\textsubscript{2}O\textsubscript{2} or other reactive oxygen species for AASMP-induced parasite growth inhibition.

Interestingly, this class of compound exhibits acceptable selectivity against the malaria parasite and shows antimalarial activity.

### Table 3

In vitro cytotoxicity against nucleated proliferating mammalian cells (MCF-7) of selected active compounds 4a, 4b, and 4c and against \textit{P. falciparum} (NF-54)

<table>
<thead>
<tr>
<th>AASMP</th>
<th>MCF-7 (mean IC\textsubscript{50} [\textmu M] ± SEM)</th>
<th>MCF-7/NF-54 SI*</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td>1,000 ± 60</td>
<td>200</td>
</tr>
<tr>
<td>4b</td>
<td>1,000 ± 50</td>
<td>125</td>
</tr>
<tr>
<td>4c</td>
<td>650 ± 20</td>
<td>163</td>
</tr>
</tbody>
</table>

* SI, selectivity index, calculated as cytotoxicity IC\textsubscript{50}/antiplasmodial IC\textsubcript{50}.

### Table 4

Antiplasmodial activity of AASMPs (4a, 4b, and 4c) against MDR strain \textit{P. yoelii} in BALB/c mice

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Dose (mg/kg) daily for 4 days</th>
<th>Mean % suppression of parasitemia ± SEM on day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>4a</td>
<td><img src="image" alt="Structure" /></td>
<td>10</td>
<td>20 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>30 ± 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>60 ± 7</td>
</tr>
<tr>
<td>4b</td>
<td><img src="image" alt="Structure" /></td>
<td>25</td>
<td>30 ± 4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>50 ± 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>80 ± 6</td>
</tr>
<tr>
<td>4c</td>
<td><img src="image" alt="Structure" /></td>
<td>10</td>
<td>40 ± 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>65 ± 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>85 ± 5</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

* Percent suppression was calculated as [(C − T)/C] × 100, where C is the parasitemia in the control group, and T is the parasitemia in the treated group.
activity in vivo against the MDR rodent malaria parasite P. yoelii. In conclusion, this study has identified AASMP as a new class of TRSMs with antiplasmodial activity both in vitro and in vivo.

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