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# Letters

## Synthesis and Antimalarial Property of Orally Active Phenoxazinium Salts

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**Abstract:** Phenoxazinium salts were found to display good antimalarial efficacy in vivo against *Plasmodium berghei*. Several compounds provided 100% parasitemia clearance at a dose of 20−30 mg $kg^{-1}$ × 4 days (ip) and good survival effects without obvious acute toxicity. They also showed excellent potency by oral administration. A preliminary pharmacokinetic study revealed that the oral availability of **1a** was excellent.

Malaria is one of the most important infectious diseases in the world, as it remains a major health problem. It infects more than 300 million people per year and causes more than one million deaths annually, mostly among young children in sub-Saharan Africa. Malaria is re-emerging as the biggest infectious killer and is currently a first priority tropical disease of the World Health Organization.[1,2] A major contributor to malarial morbidity and mortality is almost certainly the increasing resistance of malaria parasites to available chemotherapeutics, such as chloroquine (CQ[a]) and pyrimethamine.[3,4] Therefore, strong interest has been directed at the search for new antimalarial agents that can be dosed orally.[5−7] As part of our studies to develop synthetic antimalarial compounds, we have earlier reported that rhodacyanines, having a $\pi$-delocalized lipophilic cationic (DLC) structure,[8] exhibit strong antimalarial efficacies in vitro against the *Plasmodium falciparum* parasite.[9] In the course of our investigation, we were striving for the design and synthesis of new antimalarial candidates possessing the DLC structure.[10−12] Recently, we have found several compounds that show excellent in vivo potency against *P. bergei* in the mouse model when administered intraperitoneally (ip).[10] However, further development is still required, because these compounds showed poor to moderate efficacy as oral treatments. We describe here the biological properties and partial structure−activity relationship study of a class of phenoxazinium salts **1** as orally active antimalarial candidates.

Several research groups have sporadically reported on cationic dyes with a heteroaromatic cationic skeleton that demonstrate antimalarial properties[13−15] since Guttmann and Ehrich reported the antimalarial efficacy of methylene blue (**2**).[16,17] Vennerstrom and co-workers re-examined the in vitro antimalarial potencies of xanthene, azine, oxazine, and thiazine dyes and reported that several of them displayed strong in vitro activity against drug-resistant *P. falciparum*.[13] In connection with our working hypothesis, the above dyes intrigued us as DLC compounds. At the outset of our program, the in vivo evaluation of the antimalarial potency of commercially available dyes (Figure 1)[18] was carried out using rhodent malaria *P. berghei* NK-65 (drug-sensitive strain) in mice. The assay was performed according to Peters' 4-day suppressive test protocol.[19] The suppression (%) of malaria protozoa on day 4 is determined by comparing the parasitemia of infected mice, which are injected with the test compounds for 4 days with that of untreated controls. Table 1 summarizes parasitemia suppression data at a dose of 5.0 mg $kg^{-1}$ × 4 days (ip), along with reported $EC_{50}$ values in vitro against *P. falciparum* from the literature.[13] Phenoxazinium salt **1a** (basic blue 3), whose purity was ∼60% in the commercial source, showed 50% parasitemia suppression without several apparent symptoms of acute toxicity, such as diarrhea and body weight loss (entry 1). Phenothiazinium **2** (methylene blue) and xanthenium **3** (pyronin Y) displayed less of a suppression effect than **1a**, although they showed no apparent acute toxicity during the medication period (entries 2 and 3). However, phenazinium **4** (Janus green B) resulted in animal death before day 4 (entry 4). Further in vivo screening of commercial phenoxazinium salts **5**−**7** was carried out, but all the compounds showed weak suppression effects (entries 5−7). The results indicated that phenoxazinium salt would be more promising as a lead compound compared to the other heteroaromatic cations, and

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[a] Abbreviations: CQ, chloroquine; DLC, $\pi$-delocalized lipophilic cations; Piv, pivaloyl; MSD, mean survival days.

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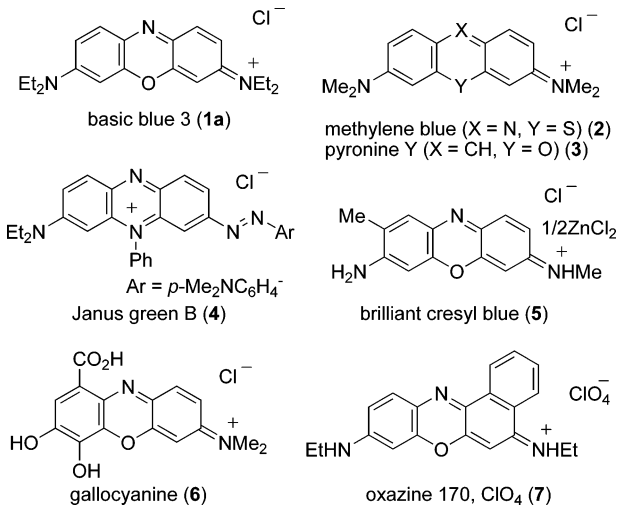



**Figure 1.** Heteroaromatic cations tested for in vivo antimalarial activity.

**Table 1.** Antimalarial Activity of **1a** and **2**−**7**

| | | in vitro[a] | in vivo[b] |
|---|---|---|---|
| entry | cmpd | $EC_{50}$ (nM) | suppression (%) |
| 1 | **1a**[c] | NT[i] | 50.6 |
| 2 | **2**[d] | 4.0 | 15.9 |
| 3 | **3**[e] | 450 | 20.6 |
| 4 | **4**[f] | 4.9 | toxic |
| 5 | **5**[f] | 5.5 | 11.0 |
| 6 | **6**[g] | 670 | 15.6 |
| 7 | **7**[h] | NT[i] | 21.8 |

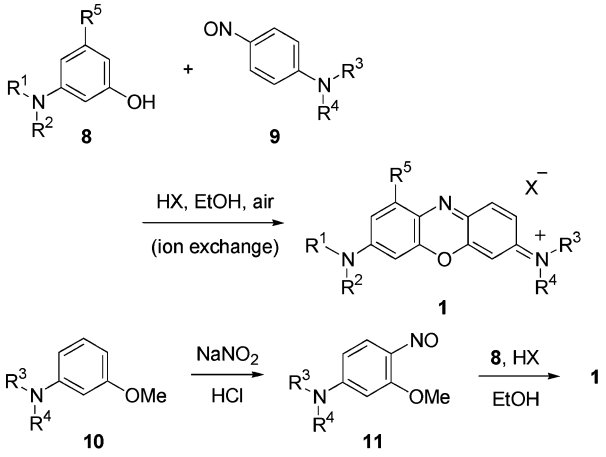
[a] Reported data in ref 13 (against *P. falciparum* W2 strain). [b] Reported data in ref 13 (against *P. berghei* NK-65 at a dose of 5.0 mg $kg^{-1}$ × 4 days). [c] Approximately 60% dye content. [d] Approximately 80% dye content. [e] Approximately 50% dye content. [f] Approximately 65% dye content. [g] Approximately 90% dye content. [h] Greater than 95% dye content. [i] Not tested.

that fully substituted nitrogen atoms on both ends of the phenoxazinium framework would be crucial for good in vivo antimalarial efficacy. Additionally, we observed that there was no good correlation between in vitro and in vivo efficacies (entries 2 and 5 vs entries 3 and 6).

Next we synthesized several phenoxazinium salts, including **1a**, with high purity.[20] Compounds **1a**−**g** were prepared by the reaction of *m*-aminophenol **8** with *p*-nitrosoaniline **9** in the presence of acid, followed by auto-oxidation by air.[21] Each compound was purified by column chromatography on silica gel and, if necessary, through an ion-exchange process. As an alternative route, **1** can be prepared by a two step sequence from *m*-anisidine **10**.[22] Namely, **1a** was synthesized from 3-methoxy-4-nitrosoaniline **11**, which was readily obtained from **10** (Scheme 1). The overall chemical yield of **1a** was higher in the latter method.

With several synthetic phenoxazinium salts in hand, selected compounds were assessed for their in vitro antimalarial activity against *P. falciparum* K1 (CQ-resistant strain) according to the procedures described by Desjardins and co-workers.[23] Their cytotoxicities were determined using a rat skeletal myoblast cell line, L-6. Selectivity, defined by the ratio $EC_{50}$ (L-6)/$EC_{50}$ (*P. falciparum*), was also determined. Both the commercially available (∼60% purity) and synthetic (>95% purity) **1a** exhibited very strong antimalarial activity in proportion to the purity (Table 2, entries 1 and 2). The selective toxicity of **1a** was excellent as well. The semi-zinc salt of **1b**, which can be obtained as a stable crystalline form of **1b**, also showed good activity (entry 3). Furthermore, the dibutyl analog **1g** also shows strong activity (entry 4). On the contrary, the activity of a series

**Scheme 1.** Synthesis of Phenoxazinium Salts




| cmpd | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ | X |
|---|---|---|---|---|---|---|
| **1a** | Et | Et | Et | Et | H | Cl |
| **1b** | Me | Me | Me | Me | H | $ClO_4$ |
| **1c** | Et | Me | Me | Me | H | $ClO_4$ |
| **1d** | Et | Et | Me | Me | H | $ClO_4$ |
| **1e** | Et | Et | Me | Me | H | Cl |
| **1f** | Bu | Bu | Me | Me | H | $ClO_4$ |
| **1g** | Bu | Bu | Et | Et | H | Cl |
| **1h** | -$CH_2CH_2NHCH_2CH_2$- | | Me | Me | H | Cl |
| **1i** | Me | Me | Me | Me | OH | Cl |
| **1j** | -$CH_2CH_2NMeCH_2CH_2$- | | Me | Me | OH | Cl |
| **1k** | -$CH_2CH_2NMeCH_2CH_2$- | | Me | Me | OPiv | Cl |

**Table 2.** In Vitro Antimalarial Activity against CQ-Resistant *P. falciparum* K1

| entry | cmpd | $EC_{50}$ (nM) | selectivity |
|---|---|---|---|
| 1 | **1a**[a] | 4.7 | 830 |
| 2 | **1a**[b] | 2.8 | 2400 |
| 3 | **1b**·$^1/_2ZnCl_2$ | 12.6 | 700 |
| 4 | **1g** | 2.4 | 350 |
| 5 | **1h**·HCl | 28 | 2300 |
| 6 | **1j**·HCl | 200 | 440 |
| 7 | **1k**·HCl | 270 | 160 |
| 8 | CQ | 1500 | 32 |

[a] Purchased from MP biochemicals (∼60% purity). [b] Synthetic compound (>95% purity).

of piperazine-containing phenoxaziniums, **1h**, **1j**, and **1l**, was not so strong (entries 5−7). Namely, it was made clear that the introduction of additional hydrophilic groups into **1** resulted in a decrease in antimalarial efficacy.

The in vivo potency of synthetic **1a**−**l** was also evaluated using the above-mentioned procedure (5.0 mg $kg^{-1}$ × 4 days, ip), and observation of malaria-infected mice after the end of treatment was continued in order to record their mean survival days. The results are summarized in Table 3. Synthetic basic blue 3 (**1a**), as the pure form, provided 81% suppression of parasitemia on day 4 (Table 3, entry 1) without obvious signs of acute toxicity. Namely, the in vivo activity of **1a** is also increased in proportion to its purity (see Table 1, entry 1). The tetramethyl analog **1b** showed a 40% suppressive effect (entry 2), whereas the activity of its semi-$ZnCl_2$ salt markedly decreased (entry 3). The phenoxazinium salts **1c**−**g** and **1h**·HCl, whose $R^5$ substituent is a hydrogen atom, provided good to excellent parasitemia suppression (60−94%) at the same dose (entries 4−9). Notably, the mean survival days of the mice treated with **1d**−**g** are significantly prolonged as compared with

**Table 3.** In Vivo Antimalarial Activity against *P. berghei* at a Dose of 5.0 mg kg$^{-1}$ (ip)[a]

| entry | cmpd | % supp. on day 4 | MSD[b] (d) |
|---|---|---|---|
| 1 | **1a**[c] | 80.6 | ND[e] |
| 2 | **1b** | 45.9 | 6.7 |
| 3 | **1b**·$^1/_2ZnCl_2$ | 17.1 | 5.6 |
| 4 | **1c** | 71.7 | 6.7 |
| 5 | **1d** | 77.0 | 9.4 |
| 6 | **1e** | 85.3 | 10.0 |
| 7 | **1f** | 90.3 | 11.4 |
| 8 | **1g** | 83.1 | 16.0 |
| 9 | **1h**·HCl | 60.4 | 8.6 |
| 10 | **1i** | 4.2 | 5.3 |
| 11 | **1j**·HCl | 17.4 | 5.3 |
| 12 | **1k**·HCl | 19.9 | 5.4 |
| 13 | CQ[d] | 90.6 | 22.7 |

[a] In vivo evaluation was carried out according to Peters' four-day suppressive protocol using five ICR-mice. [b] MSD = mean survival days. MSD for untreated mice (control) is 5.0−5.3 days. [c] Synthetic **1a** (>95% purity). [d] 10 mg kg$^{-1}$ × 4 days were dosed. [e] Not determined.

**Table 4.** Dose-Efficacy Response of **1a**, **1e**, and **1h**·HCl

| entry | cmpd | dose (mg kg$^{-1}$) | % supp. on day 4 | MSD[b] (d) |
|---|---|---|---|---|
| 1 | **1a**[a] | 2.5 | 30.5 | ND[c] |
| 2 | | 5.0 | 46.3 | ND[c] |
| 3 | | 10 | 80.4 | >11 |
| 4 | | 20 | 95.8 | >14 |
| 5 | **1e** | 2.5 | 58.7 | 7.8 |
| 6 | | 5.0 | 85.3 | 10 |
| 7 | | 10 | 99.9 | 13 |
| 8 | | 20 | 100 | >17[d] |
| 9 | **1h**·HCl | 5.0 | 54.8 | 6 |
| 10 | | 10 | 78.5 | 7.4 |
| 11 | | 20 | 98.1 | 17.6 |
| 12 | | 30 | 100 | 16.4 |

[a] Puchased **1a** (~60% purity). [b] MSD = mean survival days. MSD for untreated mice (control) is 5.0−5.2 days. [c] Not determined. [d] Two of five mice treated survived on day 30.

untreated mice. The in vivo antimalarial potency of **1f** (5.0 mg kg$^{-1}$ day$^{-1}$) is comparable to that of CQ at a dose of 10 mg kg$^{-1}$ day$^{-1}$ (entry 13). On the contrary, the introduction of a hydroxy or acyloxy group as an $R^5$ substituent lead to significant loss of suppression and survival effects (entries 10−12). The results indicated that the hydrogen atom as the $R^5$ substituent would be essential for the antimalarial property of phenoxazinium salts.

Compounds **1a** (purchased), **1e**, and **1h**·HCl, which showed no apparent body weight loss of the tested mice during the medication period (5.0 mg kg$^{-1}$ day$^{-1}$), were selected to investigate their dose-efficacy response via ip administration. As shown in Table 4, significant improvements in the suppression of parasitemia were observed in accordance with the amounts of drugs in all cases. Interestingly, 40% of mice (among the five tested mice) treated with **1e** at a dose of 20 mg kg$^{-1}$ × 4 days survive beyond day 30. Nevertheless, **1e** and **1h**·HCl showed apparent acute toxicity, including body weight loss and diarrhea, at doses of more than 20 or 30 mg kg$^{-1}$ × 4 days, although the growth of malaria parasites was almost perfectly suppressed at these doses. It was evident that ip administration of the tested compounds at a dose of less than 20 mg kg$^{-1}$ day$^{-1}$ would afford the best effects.

As a next stage, the antimalarial efficacy of **1a** (purchased) and **1h**·HCl by oral administration (po) was assessed. Preliminary toxicity tests indicated that the 50% lethal dose ($LD_{50}$) of both compounds was more than 200 mg kg$^{-1}$ (po). As can be seen from Table 5, both compounds showed 100% clearance of parasitemia on day 4 at a dose of 90 or 100 mg kg$^{-1}$ × 4 days (entries 1, 3, and 8). Beyond our expectation, phenoxazimium salts showed good oral bioavailability. Moreover, 60% of mice (3/5) treated with **1a** at a dose of more than 90 mg kg$^{-1}$ day$^{-1}$ survive beyond day 30; however, for **1h**·HCl, none of the treated mice was alive on day 30. The antimalarial effect of **1a** given by the oral route was also dose-dependent between 20 and 100 mg kg$^{-1}$ × 4 days (entries 1−4). When the medication program was changed to short-span dosing (three times a day), the total treatment dose could be reduced to 45 mg kg$^{-1}$ day$^{-1}$ for complete parasitemia clearance. However, the survival effect was not prolonged (entries 5 and 6). A single dosing on day 1 at 100 mg kg$^{-1}$ provided 100% parasitemia suppression, but none of the treated mice survived beyond day 30 (entry 7).

**Table 5.** In Vivo Antimalarial Activity by Oral Route (po)

| entry | cmpd | medication program | % supp. on day 4 | MSD[b] (d) | mice alive on day 30 |
|---|---|---|---|---|---|
| 1 | **1a**[a] | 25 mg kg$^{-1}$ day$^{-1}$ × 4 days | 82.3 | 7.6 | 0/5 |
| 2 | | 50 mg kg$^{-1}$ day$^{-1}$ × 4 days | 98.6 | 14 | 0/5 |
| 3 | | 90 mg kg$^{-1}$ day$^{-1}$ × 4 days | 100 | >24 | 3/5 |
| 4 | | 100 mg kg$^{-1}$ day$^{-1}$ × 4 days | 100 | >28 | 3/5 |
| 5 | | 30 mg kg$^{-1}$/8 h × 12 times | 100 | >13 | 2/5 |
| 6 | | 15 mg kg$^{-1}$ / 8 h × 12 times | 100 | 14 | 0/5 |
| 7 | | 100 mg kg$^{-1}$ day$^{-1}$ × 1 day | 100 | 13 | 0/5 |
| 8 | **1h**·HCl | 100 mg kg$^{-1}$ day$^{-1}$ × 4 days | 100 | 17 | 0/5 |

[a] Puchased **1a** (~60% purity). [b] MSD = mean survival days. MSD for untreated mice (control) is 5.6 days.

**Table 6.** Pharmacokinetic Property of **1a**[a]

| route | $AUC_{0-480\ min}$ (mg mL min$^{-1}$) | $t_{1/2}$ (miin) | $C_{max}$ (min) | BA (%) |
|---|---|---|---|---|
| iv[b] | 43.0 | 5.8 | | |
| po[b] | 41.4 | 59.2 | 10 | 96.3 |

[a] Puchased **1a** (~60% purity). [b] Average of three animals administered at 10 mg kg$^{-1}$ as a saline solution.

A preliminary pharmacokinetic study using Wister rats was performed (Table 6). The half-life values of **1a** (purchased) by intravenous (iv) and po administration were determined as 5.8 and 59 min, respectively. The $C_{max}$ by the oral route was reached within 10 min. The oral bioavailability of **1a** (10 mg kg$^{-1}$) was determined to be excellent (96% BA). These results clearly show that phenoxazinium salts, such as **1a**, would be a new and promising class of candidate orally available antimalarials, but it will be necessary to improve the plasma clearance.

In conclusion, we have chosen phenoxazinium salts **1** as a class of antimalarial active compounds among the several types of heteroaromatic cations and have evaluated the in vitro and in vivo antimalarial potency against *P. falciparum* (CQ-resistant K1 strain) and *P. berghei* (NK-65 strain), respectively. Some compounds were found to possess promising in vitro and in vivo efficacy. Compounds **1a** and **1h**·HCl are also orally bioavailable. Currently, further pharmacodynamic and toxicological studies of **1** are under consideration, and the synthesis of phenoxazinium salts possessing improved pharmacokinetic properties are now progressing.

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**Supporting Information Available:** Experimental procedures and characterization data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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