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Antimalarial activity of phenazines from lapachol, β-lapachone and its derivatives against *Plasmodium falciparum* in vitro and *Plasmodium berghei* in vivo

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Abstract—The antimalarial activity of benzo[*a*]phenazines synthesized from 1,2-naphthoquinone, lapachol, β-lapachone and several derivatives have been tested against *Plasmodium falciparum* in vitro using isolates of parasites with various susceptibilities to chloroquine and/or mefloquine. Parasite growth in the presence of the test drugs was measured by incorporation of [^{3}H]-hipoxanthine in comparison to controls with no drugs, always testing in parallel chloroquine, a standard antimalarial. Among seven benzophenazines tested, four had significant in vitro activities; important, the parasites resistant to chloroquine were more susceptible to the active phenazines in vitro. The doses of phenazines causing 50% inhibition of parasite growth varied from 1.67 to 9.44 μM. The two most active ones were also tested in vivo against *Plasmodium berghei* in mice, in parallel with lapachol and β-lapachone. The 3-sulfonic acid-β-lapachone-derived phenazine was the most active causing up to 98% inhibition of parasitaemia in long term treatment (7 doses) subcutaneouly, whereas the phenazine from 3-bromo-β-lapachone was inactive. Thus, these simple phenazines, containing polar (–Br,–I) and ionizable (–SO_3H, –OH) groups, easily synthesized from cheap, natural or synthetic precursors (lapachol and β-lapachone), at rather low cost, provide prototypes for development of new antimalarials aiming the chloroquine resistant parasites.

1. Introduction

Malaria is one of the most important parasitic infections of humans due to its high morbidity and mortality, a threat to over 2 billion people living in areas of high incidence. *Plasmodium falciparum*, the causative agent of the malignant form of malaria, has high adaptability by mutation and is resistant to various types of antimalarial drugs, a serious setback to antimalarial programs, since it precludes the use of cheap and previously effective drugs like chloroquine. New families of active compounds are needed as well as poly chemotherapy associating molecules with independent mechanism of action, in order to decrease the risk of resistance.[1] We have previously screened the antimalarial activity of medicinal plants[2,3] and natural products,[4] measuring their blood schizontocidal effects against *Plasmodium berghei* and *P. falciparum*.

Lapachol (**1**), a prenyl naphthoquinone isolated from plants of the Bignoniaceae family, is structurally related to atovaquone, which, together with proguanil, is highly effective in the prevention of *P. falciparum* malaria.[5] Curiously, lapachol, considered an antimalarial agent,[6] showed only borderline activity against *P. berghei* in mice[7] or against *P. falciparum* in vitro.[7,8]

β-lapachone (**2**), another naturally occurring quinone, is easily synthesized by sulfuric acid treatment of lapachol or lomatiol[9] or other methods[10] and has a wide range of

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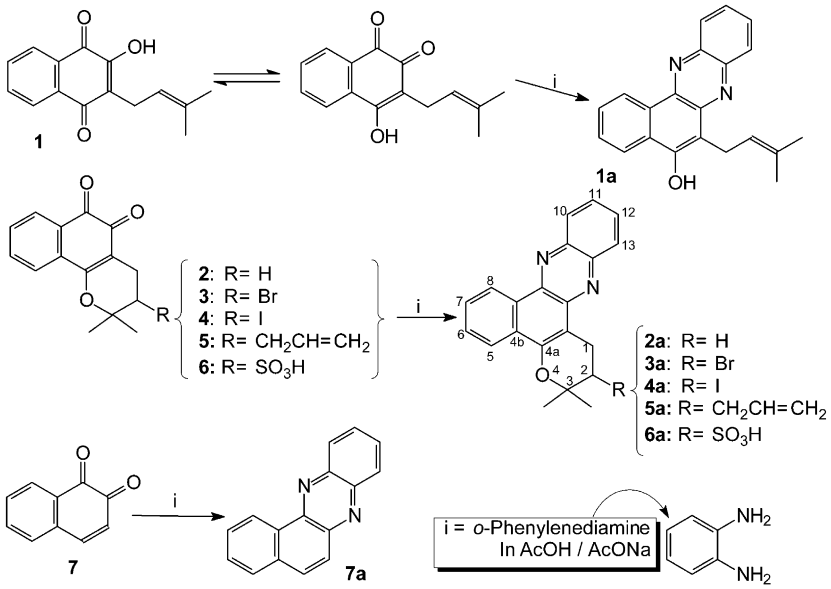

Figure 1. Structures and synthesis of the studied phenazines.

biological activities, including antibacterial, antifungal, trypanocidal, antipsoriatic, antiinflammatory and anticancer.[11] The synthesis of heterocycles derived from β-lapachone and their activities against *Trypanosoma cruzi* have been reported.[12] One of the possible heterocycles is the phenazinic derivative. In general, natural and synthetic phenazines have attracted considerable attention because of their interesting biological activities,[13] including antimalarial[14] and antihepatitis C viral replication.[15] Benzo[*a*]phenazines are efficient DNA intercalating ligands with antitumor activity in leukaemia and solid tumors.[16] The β-lapachone phenazine (3,3 - dimethyl - 2,3 - dihydro - 3*H* - benzo[*c*]pyran[3,2 - *a*]-phenazine) (**2a**), known for a considerable time,[17] had been assayed, in vitro, against human tumor cell lines (NCI anticancer screen) and was considered inactive.[18] That is, to our knowledge, the only report concerning its biological activity.

In the present work, we performed chemical reactions with the quinones lapachol (**1**), represented by a tautomeric mixture (Fig. 1),[19] β-lapachone (**2**)[20] and its derivatives (**3–6**)[20–22] and 1,2-naphthoquinone (**7**) with phenylenediamine[23] (Fig. 1), following previously described procedures,[17] and tested their antimalarial activities in vitro[27] and in vivo.[31]

2. Synthesis

Phenazines were synthesized[23] from lapachol, easily obtained and commercially available (PVP, Piauí, Brazil) at low cost, giving **1a**, from β-lapachone, giving **2a**, from 3-bromo-β-lapachone to **3a**, from 3-iodo-β-lapachone to **4a**, from 3-allyl-β-lapachone to **5a** and from 3-sulfonic acid-β-lapachone to **6a** (Fig. 1), using a simpler phenazine (**7a**), synthesized from 1,2-naphthoquinone (**7**), as a pattern. The phenazines **4a**, **5a**, and **6a** are new compounds. Their analytical data are in full agreement with the proposed structure.[24–26]

3. Biological activity

All the synthesized phenazines were tested for antimalarial activity in vitro.[27] The compounds were dissolved in water + DMSO 0.02% (v/v) and administered orally, by gavage, to the mice. For the subcutaneous treatment, instead of water, saline solution was used and the same amount of DMSO. For the bioassays in vitro, the amount of DMSO in water was 0.002% (v/v). The results are in Table 1 and Figure 2, which illustrate the dose–response curves of one inactive (**2a**) and one active (**3a**) phenazine and their IC_{50}. For chloroquine (**CQ**) the IC_{50} values were 0.2 to 0.4 μM with the drug-resistant isolate BHz 26/86 and W2; and 0.02 to 0.08 μM with isolates HB3 and D6, chloroquine sensitive. The phenazines containing polar [–Br (**3a**), –I (**4a**)] or

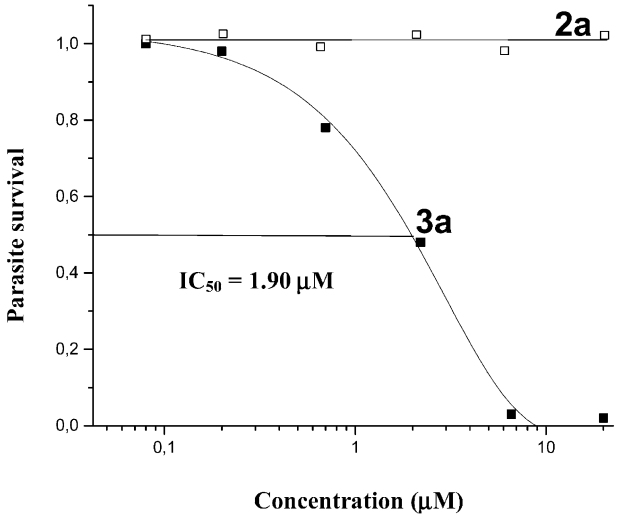

Figure 2. Dose-response curve from [^{3}H]-hipoxanthine incorporation by the *P. falciparum* BHz 26/86 parasites (chloroquine partially resistant) in the presence of various concentrations of two phenazines tested, one active, **3a** (■) in which the value of the IC_{50} is illustrated and one inactive **2a** (□) up to the dose of 20 μM.

Table 1. Susceptibility of four different *Plasmodium falciparum* strains to benzo[α]phenazines and to chloroquine (**CQ**), an antimalarial reference drug, in vitro, measured by the inhibitory concentration (IC_{50}) dose of the parasite growth in relation to control cultures with no drugs

Parasite strains	IC_{50} values (μM)							
	3a	**6a**	**4a**	**1a**	**2a**	**5a**	**7a**	**CQ**
BHz26/86[a]	1.90±0.05	1.75±0.02	2.30±0.13	3.14±0.04	>20	>20	>20	0.2±0.03
HB3[b]	8.75±0.14	6.14±0.18	6.65±0.24	9.44±0.16	>20	>20	>20	0.02±0.01
D6[c]	4.07±0.03	2.43±0.02	4.26±0.15	3.72±0.025	>20	>20	>20	0.08±0.05
W2[d]	3.04±0.1	1.67±0.05	3.22±0.09	3.54±0.03	>20	>20	>20	0.4±0.04

[a] CQ partially resistant.
[b] CQ and mefloquine sensitive.
[c] CQ sensitive and mefloquine resistant.
[d] CQ resistant and mefloquine sensitive. Values are mean±standard deviation in one representative experiment in triplicate.

Table 2. Antimalarial activity of lapachol (**1**), β-lapachone (**2**) and two benzo[α]phenazines (**3a**, **6a**) against *Plasmodium berghei* in mice infected with blood parasites, treated by oral or subcutaneous routes, during four consecutive days

Compd	Route	Dose (mg/kg)	% Inhibition of parasite growth[a]		
			Exp 1	Exp 2	Exp3[b]
1	Oral	200	0	0	—
2		200	28	34	—
3a		200	11	10	—
6a		200	33	36	—
CQ		25	99	98	—
3a	Subcutaneous	100	0	0	0
3a		200	0	0	0
6a		100	34	31	—
6a		200	50	40	98
CQ		15	99	99	99

[a] Calculated in relation to the control non-treated mice, at day 5 after parasite inoculation ($n=3$–5 animals per group).
[b] The same, except by the 7-day treatment schedule.

ionizable groups [–SO_3H (**6a**), –OH (**1a**)] had the highest antimalarial activities. The IC_{50} value for each drug was determined using a non-linear curve fitting program. The IC_{50} were confined to the following ranges for each drug: **3a**, 1.90–8.75 μM; **6a**, 1.67–6.14 μM; **4a**, 2.3 to 6.65 μM and, **1a**, 3.14–9.44 μM (Table 1). For **2a**, **5a** and **7a**, the IC_{50} values were higher than 20 μM, thus considered inactive. In conclusion, three degrees of susceptibility to the phenazines in vitro were observed, namely the highly susceptible group (BHz 26/86 and W2 parasites), the partly susceptible group (D6 parasites), and the less susceptible group (HB3 parasites) with IC_{50} values 3 up to 4.6-times higher than the values detected for BHz 26/86 and W2 parasites (Table 1). Important, the chloroquine-resistant *P. falciparum* strains (W2 and BHz) were more sensitive to the phenazines than the chloroquine-sensitive strains (HB3 and D6) and these differences were statistically significant (values of $p<0.01$ for HB3 and <0.05 for the D6 isolate) within each tested molecule (Mann–Whitney U test, NCSS2000/PASS 2000 Statistical package, Kaysville, UT, USA). Despite the fact that the more active compounds in vitro are at least 5 to 10-times less active than chloroquine, their IC_{50} values are in the micromolar concentration range (Table 1), comparable to recently reported results.[8,34]

Some available molecules were also tested in *P. berghei*-infected mice[31] (Table 2) against the NK65 strain, as described.[33] Our data show that lapachol (**1**) was inactive and β-lapachone (**2**) had a borderline activity (up to 200 mg/kg) given orally, by gavage, in 4 doses. The two most active phenazines in vitro were either inactive (**3a**) or had a borderline activity (**6a**) in vivo at the doses of 200 mg/kg by oral route. Given by subcutaneous route **3a** was inactive; **6a** caused up to 50% inhibition of

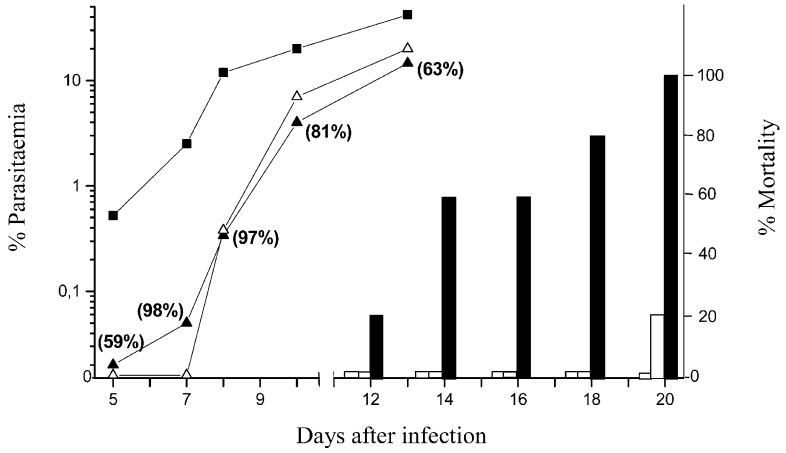

Figure 3. Course of parasitaemia by *P. berghei* in mice at different days after infection and treatment with: saline (control) [■], **6a** [▲], and chloroquine [△], by subcutaneous route for 7 consecutive days. The values in parenthesis show parasite inhibition growth by **6a** in relation to control groups. Cumulative mortality rates are shown for controls (black bars), **6a** (white bars) and chloroquine (gray bars) treated mice.

parasite growth (100 and 200 mg/kg), in four doses (Table 2). Neither of them increased mice survival in relation to the control non-treated groups (data not shown). Since these results may reflect low bioavailability of the two drugs in rodents, we next performed two additional experiments, treating the mice for 7 days with compounds **3a** and **6a** (200 mg/kg, subcutaneous). Whereas **3a** remained inactive (data not shown), **6a** showed up to 98% inhibition of parasite growth (Fig. 3) and a significant reduction in mortality ($p \leq 0.05$) in relation to non-treated control groups by day 20. Chloroquine used in parallel (15–25 mg/kg) caused intense reduction of parasitaemia and of mortality in all experiments (Table 2, Fig. 3).

The in vivo low antimalarial activity of such phenazines, by oral route, may be a result of slow uptake or rapid elimination of the active metabolites to an intracellular compartmentalization, or inactivation of the compound in vivo as shown for other molecules.[35] Indeed, this seems to be the case of **6a** if used in longer term treatment (7 doses) instead of the 4-day suppressive routine test.

Assayed as antimalarial for the first time, these phenazines, some of them new and rather active, should provide leads for development of antiparasitic drugs since they are easily synthesized at low cost, and have shown activities in the micromolar ranges, especially the sulfonide derivative ($R = SO_3H$), active in vivo and in vitro against malaria.

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23. Analytical grade reagents were used throughout the experiments. All the phenazines (**1a**–**7a**) were prepared according to the classical procedure described by Hooker,[17] from the reaction of the appropriated quinones (**1**–**7**) with *o*-phenylenediamine (Scheme 1). General procedure[17]: a mixture of the quinone (20.6 mmol), 158.5 mmol of crystalline sodium acetate, 44.5 mmol of phenylenediamine hydrochloride and 90 mL of glacial acetic acid were heated on steam-bath for a short time and monitored by silica gel TLC. After reaction, the mixture was diluted with water and allowed to stay overnight. The resulting oil was dissolved in diethyl ether and the organic solution washed with distilled water, dried over anhydrous sodium sulphate, followed by concentration under vacuum to an amorphous residue from where the phenazines were easily isolated in crystalline forms after silica gel chromatography, using a mixture of hexane–ethyl acetate of increasing polarity. The chemical structures were checked by ^1H, ^{13}C NMR, elemental analysis (C, H, N), IR and UV–vis spectroscopy and are in full accordance with the assigned structures. The new compounds are **4a**, **5a** and **6a**. The main data for the compounds are presented: (**1a**) purple needles, 161.5–162.5 °C [161.5–162.5 °C, from ETOH[24]]; (**2a**) yellow needles, 130 °C [lit. 130.5–133.5 °C[24,25]]; (**3a**)[26] dark yellow needles, 189 °C with decomposition, ^1H NMR: δ H [200 MHz, $CDCl_3$; *J* (Hz)] 9.33–9.27 (m, 1H, H-8), 8.32–8.16 (m, 3H, H-10, H-13, H-5), 7.84–7.70 (m, 4H, H-6, H-7, H-11, H-12), 4.50 (dd, $J = 9$ and 6 Hz, H-2); 4.00 (dd, $J = 18$ and 6 Hz, H-1), 3.70 (dd, $J = 18$ and 6 Hz, H-1), 1.73 (s, 3H, $-CH_3$), 1.62 (s, 3H, $-CH_3$) ppm. ^{13}C NMR (50.32 MHz, $CDCl_3$): δ C 22.3 (CH_3), 26.7 (CH_3), 30.2 (CH_2-1), 52.5 (CH-2), 78.6 (C-3), 108.6 (C-14b), 122.2, 125.1, 128.1 (C), 128.5 (C),

128.8 (C), 129.5, 129.6, 129.7, 130.7, 140.0, 140.4, 142.5, 143.5, 150.5 ppm; (**4a**), dark yellow needles, 185.5 °C. IR (KBr, cm^{-1}): 3057, 2973, 2932, 1625, 1597, 1530, 1494, 1414, 1383, 1348, 1333, 1318, 1236, 1158, 1111, 1100, 1053, 1031, 958, 768, 755, 654, 604, 557. UV ($\lambda_{max.}$, nm, log °): 424 (4.13), 407 (4.13), 359 (3.88), 301 (4.71), 291 (4.71), 263 (4.65), 275 (4.66), 237 (4.72), 224 (4.68). ^1H NMR (200 MHz, $CDCl_3$, δ): 9.27 (m, 1H, H-8); 8.34–8.10 (m, 3H); 7.78–7.68 (m, 4H); 4.58 (dd, $J = 9$ and 6 Hz, H-2); 4.1–3.66 (m, 2H); 1.78 (s, 3H); 1.63 (s, 3H); ^{13}C NMR (50.32 MHz, $CDCl_3$): δ 150.5 (C), 142.4 (C), 139.9 (C), 139.7 (s); 130.3(C), 129.5 (CH), 129.4 (CH), 129.3 (CH), 129.1 (C), 128.4 (CH), 128.3 (CH), 127.7 (CH), 125.0 (CH), 121.8 (CH), 109.3 (C), 78.4 (C), 32.6 (CH_2), 31.5 (CH_3), 27.5 (CH_3), 23.6 (CH); (**5a**), yellow needles, 149–150 °C. IR (KBr, cm^{-1}): 3055, 2980, 2935, 2840, 1624, 1597, 1528, 1494, 1413, 1386, 1347, 1334, 1294, 1159, 1126, 1035, 987, 913, 859, 755, 659, 605, 560. UV ($\lambda_{max.}$, nm, log °): 429 (3.98), 410 (3.99), 359 (3.72), 342 (3.66), 302 (4.60), 283 (1.86), 276 (4.50), 237 (4.59), 224 (4.54). ^1H NMR (200 MHz, $CDCl_3$) δ H: 9.16 (m, 1H, H-8), 8.14 (m, 3H), 7.78 (m, 4H), 6.00 (m, 1H), 5.10 (m, 2H), 3.6 (dd, 1H), 2.82 (dd, 1H), 2.58 (m, 1H), 2.05 (m, 2H), 1.62 (s, 3H), 1.18 (s, 3H); (**6a**) orange needles, 245 °C with decomposition. IR (KBr, cm^{-1}): 3539, 3477, 3337, 1688, 1629, 1596, 1567, 1488, 1452, 1408, 1337, 1240, 1217, 1175, 1115, 1094, 1055, 1042, 994, 936, 857, 782, 729, 628, 535, 452. ^1H NMR (200 MHz, $CDCl_3$) δ H: 9.2 (m, 1H, H-8); 8.32–8.18 (m, 1H); 7.96–7.76 (m, 2H); 7.04–6.92 (m, 2H); 6.90–6.80 (m, 2H); 3.28 (d, 1H); 3.18–3.02 (m, 1H); 1.87 (s, 3H); 1.47 (s, 3H) and (**7a**), yellow needles, 128 °C,[25] ^1H NMR (200 MHz, $CDCl_3$) δ H 9.42–9.39 (m, ArH); 8.38–8.26 (m, 2 ArH); 8.03–7.75 (m, 7 ArH).

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27. The phenazines (**1a**–**7a**, Fig. 1) were maintained at −20 °C as 10 mg/mL stock solutions in dimethylsulfoxide (Sigma, St. Louis, MO, USA) and used as a solution with final concentration of DMSO as 0.002%. *P. falciparum* culture and antimalarial tests in vitro: four *P. falciparum* isolates with different susceptibility to chloroquine and mefloquine were used: clone W2 (chloroquine-resistant, mefloquine-sensitive); clone D6 (chloroquine-sensitive, mefloquine-resistant); clone HB3 (chloroquine and mefloquine-sensitive); and isolate BHz 26/86 (chloroquine partially resistant), previously isolated from an imported case of malaria from the Amazon region.[7] All parasites were maintained in continuous culture on human erythrocytes as described.[28] Briefly, trophozoites synchronized in sorbitol[29] were cultured at 1–2% parasitaemia, incubated with phenazines previously diluted in medium without hypoxanthine in 96-well culture plates. After 24 h of incubation parasite-drugs, 25 μL/well of medium containing [^3H]-hypoxanthine (0.5 μCi/well) were added followed by 18 h at 37 °C.[30] The plate was frozen (6–18 h) and thawed, the cells were harvested [Tomtec 96-Harvester (Tomtec Inc., Handen, CT, USA)] in glass fiber filters (Wallac Oy, Turku, Finland), which were placed in sample bags (Wallac) and immersed in scintillation fluid (Optiphase super mix, Wallac), followed by radioactive emissions counted in a 1205 Betaplate reader (Wallac). The reduction in hypoxanthine uptake, a marker of inhibition of parasite growth, was calculated in relation to control samples with no drugs, then plot as function of drug concentrations. The 50% inhibitory concentration (IC_{50}) values were determined by linear regression analyses of the linear segments of the curves.
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31. Antimalarial tests in vivo: the suppressive Peters test[32] as modified[2] was used. Briefly, adult Swiss outbread mice, 20 ± 2 g weight, inoculated by intraperitoneal route with *P. berghei*-infected red blood cells (1×10^5), were daily treated by oral or subcutaneous route, during 4 days. The samples were dissolved in an aqueous solution of DMSO 0.02% (v:v) and this solution was, later, diluted to the final volume with saline and given in a 200 μL volume per animal. Two control groups (*n*5) were used each time, one treated with non-curative doses of chloroquine (≤25 mg/kg), one untreated or treated with saline, as specified in the results. On day 5 after parasite inoculation blood smears were prepared, methanol fixed, stained with Giemsa, and microscopically examined by counting parasitaemia in 1000 up to 6000 erythrocytes. Overall mortality was daily monitored until all controls died. The inhibition of parasite growth in the drug-treated groups was calculated in relation to non-treated controls.[33] All phenazines were tested in 3 independent experiments. For **6a**, an additional experiment was performed, using a 7-day schedule, by subcutaneous route, with parasitaemia monitored at several days after inoculation.
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