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Plasmodium berghei: *In vitro* and *in vivo* activity of dequalinium

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Abstract

Bisquinoline compounds have exhibited remarkable activity *in vitro* and *in vivo* against *Plasmodium* parasites by inhibition of heme detoxification. We have tested the ability of dequalinium 1,1′-(1,10-decanediyl)bis(4-amino-2-methylquinoline), a known antimicrobial agent, to inhibit β-hematin synthesis using a non-emzymatic colorimetric assay and globin proteolysis by electrophoretic analysis (SDS–PAGE-15%). Dequalinium was able to inhibit both processes *in vitro* with close correlation to a murine malaria model, reducing parasitemia levels, prolonging the survival time post-infection and curing 40% of infected mice using a combination therapy with a loading dose of chloroquine. These results confirm that dequalinium is a promising lead for antimalarial drug development.

Index Descriptors and Abbreviations: β-Hematin; Bisquinolines; CQ, chloroquine; DQ, dequalinium; Globin; Heme; LEP, leupeptin; Malaria; *P. berghei*, *Plasmodium berghei*; DMSO, dimethyl sulfoxide; Globin; GSH, glutathione; Heme; PEP, pepstatin; PBS, phosphate-buffered saline; RBC, red blood cells; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

1. Introduction

Malaria remains one of the main infectious diseases in the tropical world, with 1.5–3 million of deaths per year, mainly of children under 5 years of age (Weekly Epidemiological Record, 1997; Greenwod and Mutabingwa, 2002). *Plasmodium* parasites have been effectively treated with quinoline compounds over the past 50 years; however, the rise in resistant strains is becoming an increasing problem in tropical areas (Peters, 1998).

During the erythrocytic stages, *Plasmodium* parasites degrade host hemoglobin through a group of proteases (aspartic, cysteine and metalloproteases) to provide free amino acids, which are necessary for protein synthesis (Goldberg et al., 1990; Goldberg, 1992). In this process, free toxic heme is liberated from the globin chains, and *Plasmodium* species have developed an unique mechanism to detoxify these heme moieties through the formation of an unsoluble pigment called hemozoin or β-hematin (Fitch et al., 1982).

Dequalinium (DQ) is a bisquinoline compound widely used in infectious diseases such as trichomoniasis (Levinson, 1959), superficial mycosis (Viglioglia, 1962), vaginal infections caused by a range of bacteria and fungi (Della Casa et al., 2002), and oral and skin infections (Kaufman, 1981; Coles et al., 1958). DQ has also shown anaesthetic properties (Guruswami and Thampai, 1967) and antitumor activity (Weiss et al., 1987; Galeano et al., 2005).

Dequalinum salts have been investigated as a safe and effective approach for the treatment of malaria. The salts are highly effective *in vitro* against both chloroquine sensitive and resistant strains of *P. falciparum* at low concentrations (Makler, 1990).

Other bisquinoline derivatives (especially piperaquine) have also shown activity as potential malaria chemotherapeutic (Raynes, 1999), some of them being active against chloroquine-resistant strains (Li et al., 1981; Lin et al., 1982; Lin, 1991; Vennerstrom et al., 1992). In this context we have evaluated the ability of dequalinium to inhibit β-hematin synthesis and globin proteolysis *in vitro* together with its effects on a *Plasmodium berghei*-murine model.

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2. Materials and methods

2.1. Reagents

Dequalinium (Fig. 1) was a generous gift given by Meyer Productos Terapéuticos, S.A. Caracas, Venezuela. For *in vitro* assays, it was prepared as 100 mM stock solution in DMSO and stored at 4 °C. The stock solution was diluted before the assays to reach final concentrations. For *in vivo* experiments, the dissolved drug was diluted in Saline–Tween 20 (1%) and adjusted to a final concentration of 20 mg/kg. Leupeptin, pepstatin, chloroquine, and saponin were commercially available from Sigma Co.

2.2. Inhibition of β-hematin synthesis

Inhibition of β-hematin synthesis was performed according to a previous procedure (Baelmans et al., 2000). Briefly, a solution of hemin chloride in DMSO (50 μL, 5.2 mg/mL) was distributed in 96-well microplates. Different concentrations of dequalinium dissolved in DMSO (100–5 mM) were added in triplicate in test wells (50 μL) at final concentrations between 0.25 and 25 mM. Controls contained either water (50 μL), DMSO (50 μL) or chloroquine (50 μL, 2.5 mM). β-Hematin synthesis was initiated by the addition of buffer acetate (100 μL, 0.2 M, pH 4.4). Plates were incubated at 37 °C for 48 h to allow completion of the reaction and centrifuged (4000 RPM × 15 min, IEC-CENTRA, MP4R). After discarding the supernatant, the pellet was washed three times with DMSO (200 μL) and finally dissolved in NaOH solution (200 μL, 0.2 N). The solubilized aggregates were further diluted 1:2 with NaOH solution (0.1 N) and absorbance recorded at 405 nm (Microplate Reader, BIORAD-550). The results were expressed as percentage inhibition of β-hematin synthesis compared to control.

2.3. Experimental host and strain maintenance

Male albino mice (National Institute of Hygiene strain), weighing 18–22 g, were maintained on a commercial pellet diet and housed under conditions approved by the Ethics Committee, Faculty of Pharmacy, Central University of Venezuela. *P. berghei* (ANKA strain), a rodent malarial parasite, was used for infection. Mice were infected by *i.p* passage of 10^7 infected erythrocytes diluted in phosphate-buffered saline solution (PBS 10 mM, pH 7.4, 0.1 mL). Parasitemia was monitored by microscopic examination of Giemsa stained smears.

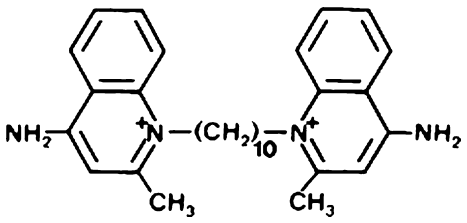

Fig. 1. Dequalinium structure.

2.4. Parasite extracts

Blood of infected animals with high levels of parasitemia (30–50%) was collected by cardiac puncture into a heparinized syringe, and centrifuged (500*g* × 10 min, 4 °C). Plasma and buffy coat were removed, and the red blood cell (RBC) pellet was washed twice with chilled PBS-glucose (5.4%). The washed RBC pellet was centrifuged on a discontinuous percoll gradient (80–70% percoll in PBS-glucose, 20,000*g* × 30 min, 4 °C) (Deharo et al., 1994). The upper band (mature forms) was removed by aspiration, collected in Eppendorf tubes and washed twice with chilled PBS-glucose, and the infected RBC was lysed with the non-ionic detergent, saponin (0.1% in PBS × 10 min). Cold PBS (1 mL) was added, and the samples were centrifuged (13,000*g* × 5 min, 4 °C) to remove erythrocyte cytoplasmic material (including erythrocyte hemoglobin). The free parasites were mixed with PBS-glucose (5.4%) and subjected to three freeze–thaw cycles (−70 °C/+37 °C). The final homogenate was used in the hemoglobin proteolysis assay (Rosenthal, 1995).

2.5. Mouse native hemoglobin

Native hemoglobin from non-infected mice was obtained by treating one volume of pellet erythrocytes with two volumes of water. The resulting lysate was used as the substrate in the hemoglobin proteolysis assay.

2.6. Analysis of globin proteolysis

The proteolytic effect of parasite extract on the native mouse hemoglobin was assayed using 96-well tissue culture plate (Greiner Bio-One). The assay mixture contained: mouse native hemoglobin (10 μL), parasite extract (50 μL), GSH (10 μL, 10 μM), and buffer acetate (0.2 M, pH 5.4) to a final volume of 100 μL. The dissolved compounds (5 mM) were incorporated into the incubation mixture. The incubations were carried out at 37 °C for 18 h and the reaction was stopped by addition of buffer (upper Buffer 25%, SDS 2%, β-mercaptoethanol 5%, glycerol 20%, Bromophenol Blue 0.0025%). The degree of digestion was evaluated electrophoretically by SDS–PAGE-15% (Rosenthal, 1995) by visual comparison of the globin bands (14.4 kDa) followed by densitometric analysis.

2.7. 4-day suppressive test (Peters' test)

Dequalinium was also tested in a malaria murine model according to Peters and Robinson (1999). Briefly, NIH male albino mice (18–23 g) were inoculated *i.p* with 10^7 RBC infected with *P. berghei* ($n = 5$). The drug was dissolved in DMSO (0.1 M) and diluted with Saline–Tween 20 solution (2%). Two hours after infection, the compound

was administered once *i.p* (20 mg/kg) for 4 consecutive days. At day four post-infection, the parasitemia was counted by examination of Giemsa stained smears. Chloroquine (25 mg/kg) was used as a positive control. The results were expressed as percentage of parasitemia at fourth day post-infection and percentage of surviving mice.

Data were statistically analyzed with GraphPad Prism 3.02 using unpaired *t*-tests for specific group comparisons, assuming 95% confidence limits.

3. Results

Dequalinium exhibited a marked inhibition of β-hematin synthesis which did not differ significantly from chloroquine (Fig. 2). The ability of DQ to inhibit this synthetic process might be due its capacity to complex to free heme moieties as seen with other antimalarials (Raynes, 1999).

Electrophoretic analysis showed that dequalinium inhibits globin proteolysis (intact band at 14.4 kDa, Fig. 3). This may be due to the inhibition of the cysteine and/or aspartic proteases of parasites, since it is also inhibited by leupeptin and pepstatin. Chloroquine exhibited low level of inhibition (Fig. 3). Densitometric analysis showed more than 50% inhibition of globin proteolysis, which is useful considering that both quinoline or bisquinoline compounds mainly act

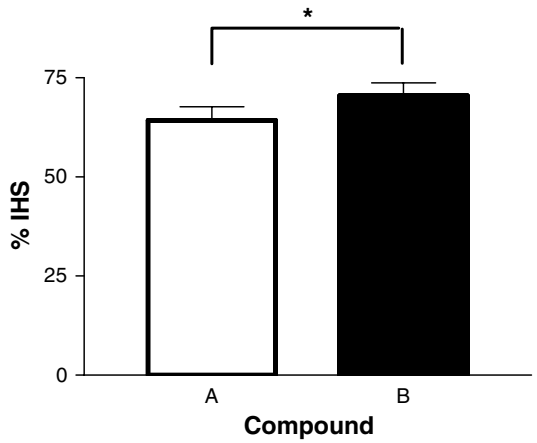

Fig. 2. Inhibition of β-hematin synthesis (%IHS). The results are expressed as the mean ± standard error of the mean. A = Dequalinium; B = Chloroquine; $n = 3$; $^{*}p > 0{,}05$.

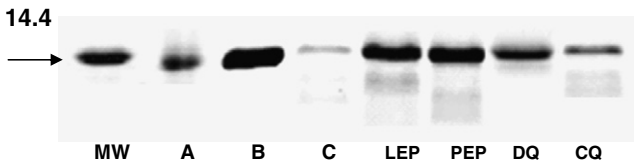

Fig. 3. Effects on globin proteolysis by dequalinium. Standard Molecular Weight (MW) is expressed in kilodaltons (14.4 kDa). A: control human hemoglobin; B: undegraded globin (control hemoglobin without trophozoites); C: control hemoglobin with trophozoites of *P. berghei*; LEP: hemoglobin with trophozoites and leupeptin. PEP: pepstatin. DQ: dequalinium. CQ: chloroquine.

Table 1
Inhibition of globin proteolysis (%IGP)

Compound (5 mM)	% IGP
Leupeptin	86.25 ± 0.69
Pepstatin	88.79 ± 0.66
Dequalinium	56.76 ± 3.96*
Chloroquine	23.82 ± 1.85

The results are expressed by the mean ± standard error of the mean.
* $p < 0.05$ compared to chloroquine.

Table 2
Effects of dequalinium (20 mg/kg) on parasitemias at fourth day post-infection (%*P*)

Treatment	% *P*
Saline	26.00 ± 4.64
Dequalinium	4.33 ± 1.67**,†
CQ	1.91 ± 0.66

The results are expressed by the mean ± standard error of the mean. CQ: chloroquine (25 mg/kg).
** $p < 0.01$ compared to saline.
† $p > 0.05$ compared to CQ. $n = 5$.

Table 3
Effects of dequalinium with a loading dose of chloroquine on parasitemias at 4 day post-infection with *Plasmodium berghei*

Treatment	% *P*
Saline	22.4 ± 1.83
CQ-60	18.8 ± 1.82
CQ-60/dequalinium	12.4 ± 1.6**,†
CQ-25	3.2 ± 0.58
Dequalinium	14.39 ± 0.75**

CQ-60: mice treated with a loading dose of chloroquine (60 mg/kg at day 0 post-infection).
** $p < 0.05$ compared to saline-group mice.
† $p < 0.05$ compared to the mice treated only with a loading dose of chloroquine (60 mg/kg) at day 0 post-infection. $n = 5$.

by inhibiting the β-hematin synthesis; DQ exhibits good activity which is substantially better than CQ (Table 1).

In *P. berghei*-infected mice, DQ was able to reduce parasitemia at 4 days post-infection to a level which was not significantly different from chloroquine-treated mice (Table 2). This *in vivo* result confirms the *in vitro* properties of dequalinium against *P. berghei*.

Because of these promising results, we further investigated its activity with a different dosing protocol (20 mg/kg, days 1, 2, 3, 4 post-infection) in combination with a loading dose of CQ (60 mg/kg, day 0). Dequalinium was able to lower the parasitemia levels significantly compared to non-treated mice (Table 3) and prolonged the survival time post-infection, curing 40% of infected mice followed to 30 days post-infection (Fig. 4). CQ-treated mice (25 mg/kg/day for 4 days) were all cured over the same follow up period.

4. Discussion

Plasmodium parasites proteases degrade the host hemoglobin in order to provide amino acids for protein

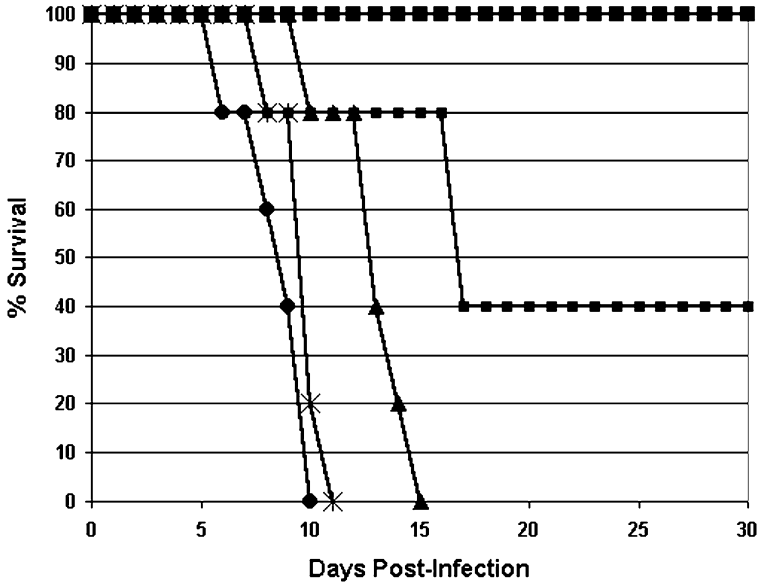

Fig. 4. Survival days post-infection of *P. berghei*-infected mice. (■) Chloroquine 25 mg/kg; (●) Saline; (▲) Chloroquine loading dose (60 mg/kg); (■) Chloroquine loading dose + dequalinium; (X) Dequalinium 20 mg/kg.

synthesis (Goldberg, 1992). The free heme moieties produced crystallize spontaneously in the acid environment of the digestive vacuole, in the absence of proteins and enzymes (Egan et al., 1994). In this way, *Plasmodium* species, which lack heme oxygenases, detoxify heme by sequestering the toxic moiety into an inert crystal, known as hemozoin or β-hematin (malaria pigment). This mechanism guarantees parasite survival since free heme is highly toxic because of its oxidant properties (Orijh and Fitch, 1993; Bohle et al., 1997). The heme crystal consists of a non-covalent coordination complex, with the ferric iron of each heme moiety being chelated onto the propionic acid chain of the adjacent moiety (Slater et al., 1991; Blauer and Akkawi, 1997; Basco et al., 1994). Compounds which inhibit β-hematin synthesis and hemoglobin degradation might be potential antimalarials.

Bisquinolines are compounds which contain two quinoline nuclei combined through an aliphatic or aromatic linker. Examples include bis(quinolyl) piperazines, such as piperaquine, hydroxypiperaquine, dichloroquinazine and 1,4-bis(7-chloro-4-quinolylamino) piperazine (Li et al., 1981; Lin et al., 1982; Lin, 1991; Raynes et al., 1995) and other diamine-linked analogs such as *N*,*N*′-bis(quinolin-4-yl) alkane-α,ω-diamine derivatives (Vennerstrom et al., 1992; Ridley et al., 1996; Hofheinz and Masciadri, 1996; Ridley et al., 1997). Our results suggest that the bisquinoline derivative dequalinium, which is already used as an antimicrobial agent, could also be an antimalarial. Moreover, DQ was able to inhibit β-hematin synthesis, as do other quinoline and bisquinolines (Adams et al., 1996; Raynes et al., 1996). In this context, it would be interesting to investigate the binding affinity of this drug to free heme, in order to clarify the effects on β-hematin synthesis more exactly in future investigations.

DQ also blocked globin proteolysis as a secondary mechanism with almost 60% inhibition. As we determined by SDS–PAGE-15%, CQ-treatment of trophozoites cultured with hemoglobin partially inhibited globin proteolysis; however, DQ was more than twice as active as CQ.

Our results suggest that the decrease in globin proteolysis caused by the inhibition of *P. berghei*-cysteine and aspartic proteinases may contribute to the antimalarial action of this bisquinoline. This compound was not tested as a falcipain or plasmepsin inhibitor *in vitro*; however, the mechanism of action on hemoglobin degradation could be related to the inhibition of one or both of these proteases.

Piperaquine is a known bisquinoline compound used as antimalarial agent (Zhu et al., 1982; Davis et al., 2005); however there are no data available to support its effects against globin proteolysis but some investigations have shown that bisquinoline compounds inhibit the β-hematin synthesis as a mechanism of action (Ridley et al., 1997). We can confirm that DQ has both mechanisms and its dual actions would be necessary for its *in vivo* effects. These results corroborate the findings *in vitro*, since dequalinium was able to reduce parasitemia levels at 4 days post-infection and, in combination with a loading dose of chloroquine, 40% of treated mice were cured. This result is promising because DQ was able to increase survival time, and cured some infected animals.

On the other hand, *in vivo* antimalarial activity of CQ is much stronger than that of DQ and this could be due to dosage or bioavailability problems, since it is more effective than chloroquine *in vitro* according to our results. It would therefore be interesting to assay some more water-soluble derivatives in different dose regimen. In addition, it is important to note that bisquinoline compounds, although sometimes active against CQ resistant parasites (Vennerstrom et al., 1992), have also shown significant resistance

(Fan et al., 1998). Moreover, it would be necessary to test other bisquinolines, since comparative data would be useful. In spite of the possible resistance to these compounds, these drugs could be used in combination therapies for the treatment of uncomplicated multidrug resistant falciparum malaria (Ashley et al., 2005; Denis et al., 2002).

In conclusion, our results suggest that dequalinium could be a potential drug for the treatment of malaria, inhibiting both β-hematin synthesis and globin proteolysis. It will be necessary to test this bisquinoline compound against chloroquine-resistant strains in future to determine its true utility.

Acknowledgments

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