Modification of oxidative status in *Plasmodium berghei*-infected erythrocytes by E-2-chloro-8-methyl-3-[(4′-methoxy-1′-indanoyl)-2′-methyliden]-quinoline compared to chloroquine

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E-2-chloro-8-methyl-3-[(4′-methoxy-1′-indanoyl)-2′-methyliden]-quinoline (IQ) is a new quinoline derivative which has been reported as a haemoglobin degradation and ß-haematin formation inhibitor. The haemoglobin proteolysis induced by Plasmodium parasites represents a source of amino acids and haeme, leading to oxidative stress in infected cells. In this paper, we evaluated oxidative status in Plasmodium berghei-infected erythrocytes in the presence of IQ using chloroquine (CQ) as a control. After haemolysis, superoxide dismutase (SOD), catalase, glutathione cycle and NADPH + H+ -dependent dehydrogenase enzyme activities were investigated. Lipid peroxidation was also assayed to evaluate lipid damage. The results showed that the overall activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were significantly diminished by IQ (by 53.5% and 100%, respectively). Glutathione peroxidase activity was also lowered (31%) in conjunction with a higher GSSG/GSH ratio. As a compensatory response, overall SOD activity increased and lipid peroxidation decreased, protecting the cells from the haemolysis caused by the infection. CQ shared most of the effects showed by IQ; however it was able to inhibit the activity of isocitrate dehydrogenase and glutathione-S-transferase. In conclusion, IQ could be a candidate for further studies in malaria research interfering with the oxidative status in Plasmodium berghei infection.

Key words: malaria - quinoline - oxidative stress - haemoglobin degradation

Haemoglobin proteolysis in intraerythrocytic malarial parasites is a biochemical event which occurs in the acidic digestive vacuole and is mediated by a group of proteases in order to provide free amino acids necessary for protein synthesis (Goldberg et al. 1990, Gamboa & Rosenthal 1996). In this process, the oxidant haeme group is separated from the globin chains, a process in which Fe2+ is oxidised to Fe3+ and the electrons produced react with molecular oxygen to form oxygen radicals (Mohan et al. 1992a, Atamna & Ginsburg 1993, Ginsburg & Atamna 1994, Postma et al. 1996, Francis et al. 1997) Parasites try to protect themselves from the deleterious effect of these free haeme groups by crystallising part of the haeme moieties into an insoluble non-toxic pigment called haemozoin or ß-haematin (Bendrat et al. 1995, Francis et al. 1997). The remaining haeme is detoxified by GSH-dependent degradation. In this context, intraerythrocytic glutathione plays a major role in detoxifying free haeme moieties together with antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidise (GPs) (Atamna & Ginsburg 2005). An alternative mechanism of haeme detoxification was proposed by Loria et al. (1999), who showed that haemoglobin oxidises spontaneously due to the acidic environment of the digestive vacuole, producing superoxide anions. This oxygen radical formation follows the production of H2O2 and the destruction of the porphyrin ring of the toxic haeme molecule. Plasmodium parasites utilise both the host’s as well as their own antioxidant systems to avoid a creation of a pro-oxidative environment and the development of oxidative stress (Ginsburg & Atamna 1994, Mylonas & Kouretas 1999).

A series of E-2-quinolinylbenzocycloalkanes have demonstrated antimalarial activity in vivo and in vitro, inhibiting haemoglobin degradation and ß-haematin synthesis. E-2-chloro-8-methyl-3-[(4′-methoxy-1′-indanoyl)-2′-methyliden]-quinoline (IQ) proved to be the best compound tested. This compound was able to decrease parasitaemia levels and increase post-infection survival in *Plasmodium berghei*-infected mice (Charris et al. 2005). On the other hand, some quinoline compounds currently in use exert their activities by increasing oxidative stress in the parasitised erythrocyte. For example, chloroquine (CQ) functions by preventing haeme crystallisation. Additionally, the activity of this drug can be enhanced by depletion of reduced glutathione. Primaquine metabolites induce substantial oxidative stress; the endoperoxide artemisinin is thought to react with haeme moieties, forming cytotoxic radicals (Becker et al. 2004).

In this context, taking into consideration the relationship between haemoglobin degradation and oxidative stress in infected cells as well as the search for potential
antimalarial compounds with new pharmacological targets, we have evaluated the ability of IQ to modify the oxidative status of *P. berghei*-infected red blood cells.

**MATERIALS AND METHODS**

*Synthesis of IQ* - The compound was synthesised by a base-catalysed Claisen-Schmidt condensation according to Charris et al. (2005). In short, a mixture of the 2-chloro-3-formylquinoline (1 mmol), 4-methoxy-1-indanone (1 mmol) and sodium methoxide (catalytic) in methanol (8 mL) was stirred at RT overnight. Water was added and the resulting precipitate was collected via filtration, washed with water and recrystallised using ethyl acetate. It is important to mention that 1H NMR spectra of compounds belonging to this family showed that the protons of the β and 3′ positions absorbed as a triplet around 8.1 ppm and a doublet around 3.8 ppm, respectively, with coupling constants J ranging 0.9-1.2 Hz, clearly indicating the appearance of allenic coupling among these positions. Additional support for these structures was obtained from 13C NMR. The main shift of carbonyl carbons has a slight variation at 192 ppm in the indanone structure. The enhancement in the CO bond in the non planar enone moiety.

*Experimental host and strain maintenance* - Male albino mice (BALB/c, 18-22 g) received a commercial pellet diet and were housed under conditions approved by the Ethical Committee, School of Pharmacy, Central University of Venezuela. *P. berghei* was used for infections. Mice were infected intraperitoneally with 107 infected erythrocytes. Blood samples were removed when the animals reached 25% parasitaemia and they were diluted in phosphate buffer solution (PBS 10 mM, pH 7.4, 0.1 mL). Parasitaemia was monitored by microscopic examination of Giemsa stained smears.

*Incubation of erythrocytes with IQ* - 1 x 10⁶ non-infected erythrocytes and *P. berghei*-infected erythrocytes from mice (2-3% parasitaemia and 4% haematocrit) were incubated in RPMI 1640 medium (pH 7.3) supplemented with 20% foetal calf serum in presence of IQ or CQ (large-scale cultures) (10 µM, 24 h x 37°C, 10% O₂, 5% CO₂, 85% N₂ gas mixture) (Janse & Waters 1995). After 24 h of culture, non-treated infected erythrocytes mostly developed to the schizont stage, while the growth of treated (IQ or CQ), infected erythrocytes was arrested. Samples were centrifuged (5000 g x 10 min x 4°C), washed with saline solution and haemolysed.

*Activities of SOD and catalase (CAT)* - For SOD activity determination, samples were haemolysed with 0.4 vol of chloroform/ethanol (1:5:1) and activity was measured according to McCord and Fridovich (1969), which tracks the reduction of cytochrome C at 550 nm. For CAT activity determination, samples were haemolysed with 4 vol of chilled water and diluted 1:500 with phosphate buffer (pH 7.0) and activity was measured according to Aebi (1984), which tracks the reduction of H₂O₂ (30 mM) at 240 nm. The results were expressed as the percentage decrease in activity compared to non-infected and non-treated erythrocytes (control cells).

*Activities of dehydrogenase enzymes* - To determine the activities of glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD) and isocitrate dehydrogenase (ID), samples were lysed (1:10) with stabilised solution (5 µL of β-mercaptoethanol, 1 mL of EDTA 10% and water to 100 mL, pH 7.0). Measurements were determined according to Bergmeyer et al. (1974), Bernt and Bergmeyer (1974) and King (1974), respectively, which track the oxidation of NADPH + H⁺ at 340 nm. The results were expressed as percentage of activity compared to non-infected and non-treated erythrocytes (control cells).

*Glutathione cycle determinations* - Reduced and oxidised glutathione (GSH, GSSG) from erythrocytes were detected according to a modification of Tietze (1969). In short, samples were lysed with stabilised solution (1:10) and 1 vol was added to 7 vol of a mixture which contained phosphate buffer, 10 mM/NADPH + H⁺ (0.2 mM pH 7.5) and 25 µL of dinitro-benzene (DTNB, 0.6 mM). The mixture was completed with 30 µL of buffer phosphate 10 mM, pH 7.0. To measure GSH levels, the increase in optical density at 412 nm was recorded, representing the formation of the reduced product, thionitrobenzene. GSSG levels were determined after the addition of glutathione reductase (10 µg/mL) to the samples. To determine GPx activity, samples were lysed with stabilised solution (1:10) and measurements were taken according to Paglia and Valentine (1967) with a modification made by Flohé and Günzler (1984), which registers NADPH + H⁺ oxidation at 340 nm. For glutathione-S-transferase (GST) activity, samples were haemolysed with stabilised solution (1:10) and measurements were taken according to Habib et al. (1974), which tracks the conjugation of 1-Cl-2,4-dinitro benzene with reduced glutathione at 340 nm. The content of reduced and oxidised glutathione was expressed as the ratio of GSSG/GSH. The activities of GPx and GST were expressed as units of enzyme/mg.

*Determination of erythrocyte lipid peroxidation* - To obtain membrane samples, erythrocytes were lysed by treatment with phosphate buffer (5 mM, pH = 7.4) at 4°C (1:10). The haemolysate obtained was centrifuged (20000 g x 10 min at 4°C). Membrane pellets were washed three times with saline solution.

Erythrocyte lipid peroxidation was determined by measuring thiobarbituric acid (TBA) reactivity according to Buege and Aust (1978). In short, membrane samples were mixed with phosphoric acid 10% P/V in 0.1N HCl (1:6) and TBA (0.6% P/V in 0.1N HCl) (1.2). Samples were incubated in boiling water for 45 min. Malondialdehyde (MDA), an end product of fatty acid peroxidation, can react with TBA to form a coloured complex that has a maximum absorbance at 532 nm. Results were expressed as µmol of MDA/mg.

Protein levels were determined according to Lowry et al. (1951). Data was presented as the average of four independent experiments (n = 4) and tested for statistical significance using unpaired t-tests for specific group comparisons assuming 95% confidence limits using GraphPad Prism 4.02 software.
Ethics - Mice were housed under conditions approved by the Ethical Committee, School of Pharmacy, Central University of Venezuela.

RESULTS

The parameters of IQ purity and structure confirmation were as follows: yield 90%; m.p. 208-210°C; IR (KBr) cm⁻¹: 1705 (CO). 1H NMR CDCl₃: δ 2.75 (s, 3H, CH₃), 3.88 (d, 2H, H₃, J: 1.92 Hz), 3.93 (s, 3H, OCH₃), 7.07 (d, 1H, H₅, J:7.92 Hz), 7.39 (t, 1H, H₆, J: 7.67 Hz), 7.47 (m, 1H, H₆'), 7.51 (m, 1H, H₇), 7.59 (d, 1H, H₇, J:6.68 Hz), 7.74 (d, 1H, H₅, J: 8.16 Hz), 8.03 (t, 1H, Hv, J: 1.90 Hz), 8.39 (s, 1H, H₄). 13C NMR: 17.42, 28.74, 55.63, 115.69, 116.39, 126.09, 126.95, 127.75, 127.38, 128.63, 128.71, 131.61, 136.81, 138.14, 139.30, 146.46, 150.29, 156.70, 193.40. Anal: C, 72.10; H, 4.39; N, 3.89%.

Defence against oxidative stress represents a very important route for the detoxification of free radicals in infected erythrocytes. Results of experiments on the main antioxidant systems in presence of IQ (Fig. 1) and control CQ are expressed in Figs 2, 3 and Table.

Infection of erythrocytes with *P. berghei* leads to important decreases in the first line of oxidative defence. SOD and CAT activities diminished due to the infection, which could be responsible for the oxidative stress observed in these cells, while treatment with IQ or CQ prevented some loss of activity compared to untreated, infected cells (Fig. 2).

The activities of NADPH + H⁺-dependent dehydrogenases were also affected, showing significant increases in G6PD, 6PGD and ID in infected, untreated erythrocytes (Fig. 3). Treatment with IQ resulted in a decrease in overall activity for the first two enzymes, which are responsible for the proper functioning of the hexose-monophosphate shunt. CQ-treated, infected erythrocytes also showed significant inhibition in the overall activity of these enzymes as well as in ID activity (Fig. 3).

The GSSG/GSH ratio was increased due to infection (Table), which indicates that the oxidised form is prevalent. In presence of IQ and CQ, this ratio increased significantly compared to untreated and infected erythrocytes. Infection did not change the activity of GPx significantly. However, it increased the overall activity of GST, while CQ decreased the activity of this enzyme.

*P. berghei* infection promotes oxidative stress in infected cells. This was corroborated by higher levels of lipid peroxidation. IQ and CQ were able to decrease lipid damage in infected cells, protecting the host from the deleterious effects of oxidative burst on membranes (Fig. 4).

DISCUSSION

Haemoglobin degradation by *Plasmodium* parasites produces active redox products, free haeme and H₂O₂, leading to oxidative stress in infected cells. However, the parasite utilises an efficient enzymatic antioxidant system including SOD, CAT, glutathione cycle and dehydrogenase enzymes to avoid the deleterious effects of these redox products. Elements of this antioxidant system could constitute potential targets in the search of new antimalarial compounds.

Several antimalarial drugs, such as the endoperoxide antimalarials, seem to act by interfering with the oxidative status of erythrocytes during infection (Ittarat et al. 2003). Indeed, part of the biological activity of CQ results from the augmentation of oxidative stress within the parasites (Toler et al. 2006). This drug leads to increases in the number of oxidised proteins as the erythrocytic cycle progresses to mature stages (Radfar et al. 2008). Additionally, CQ treatment mediated oxidative stress in the host; this effect was exacerbated in *Plasmodium falciparum* infected patients administered with the drug (Farombi et al. 2003).

A group of E-2-quinolinylbenzocycloalcanones have demonstrated antimalarial activity in vivo. Particular attention has been paid to the compound IQ, which was shown to be able to reduce and delay the progression of malaria, decreasing the parasitaemia level by 24% compared to non-treated mice (control) and increasing survival rate up to 12 days over controls. This compound inhibited haemoglobin proteolysis and ß-haematin formation in vitro, suggesting a possible mechanism similar to that of CQ for its antimalarial activity (Charris et al. 2005).

In this paper, we demonstrated that this compound, which is a quinoline-related structure like CQ, was able...
to decrease the hexose-monophosphate shunt in infected erythrocytes, because the overall activity of G6PD and 6PGD were diminished. These results should be added to the known antimalarial properties of this compound and show that these enzymes could represent new targets for antimalarial chemotherapy, as has previously been demonstrated for some antimalarials against purified G6PD (Cotton & Sutorious 1971). The disruption in this system could produce lower levels of NADPH + H⁺, which would make the cells more sensitive to oxidative stress because the glutathione cycle would be compromised due to its dependence on NADPH + H⁺ produced by the pentose-phosphate pathway. CQ shared these effects on the dehydrogenase enzymes and also inhibited the overall ID activity in infected cells; these results are in agreement with previous studies which demonstrated inhibitory actions of this quinoline derivative on some of these enzymes (Jarzyna et al. 2001, Sahni et al. 2007) to prevent a general production of NADPH + H⁺ in infected erythrocytes.

On the other hand, the GSSG/GSH ratio was higher in quinoline-treated infected cells compared to untreated infected erythrocytes, which could be a consequence of the rise in oxidant species in infected cells treated with the compounds. Indeed, the overall activity of GPx was decreased in infected cells treated with each of the quinolines tested. This was also confirmed in malaria patients treated with CQ (Farombi et al. 2003), an effect that could be attributable to the GSSG/GSH increase and to compromised activity of the hexose-monophosphate shunt. The activity of GST increased due to the infection. However, in contrast to a previous report which showed that another cycloalcanone inhibited erythrocyte GST, no changes were observed when the cells were incubated with IQ (Palmen & Evelo 1998). Our results showed that the effects of IQ on the glutathione system were shared by CQ. However, this antimalarial drug also inhibited the overall activity of GST, which could be due to inhibition of the enzyme by CQ-haeme complex formation (Srivastava et al. 1999). In this context, quinoline compounds act on erythrocytes parasitised by a sensitive *P. berghei* strain, inducing a dramatic decrease in

### Table

Effect of 2-chloro-8-methyl-3-[4'-methoxy-1'-indanoyl]-2’-methylidene]-quinoline (IQ) and chloroquine (CQ) on the glutathione system

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GSSG/GSH</th>
<th>GPx (U/mg prot x 10⁻⁶)</th>
<th>GST (U/mg prot x 10⁻⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NINT</td>
<td>23.83 ± 0.93</td>
<td>21.08 ± 0.52</td>
<td>40.37 ± 2.76</td>
</tr>
<tr>
<td>I</td>
<td>33.19 ± 0.51a</td>
<td>18.39 ± 1.6</td>
<td>59.39 ± 6.1a</td>
</tr>
<tr>
<td>NI + IQ</td>
<td>21.48 ± 0.94</td>
<td>22.86 ± 0.96</td>
<td>42.14 ± 4.26</td>
</tr>
<tr>
<td>I + IQ</td>
<td>61.15 ± 0.29b</td>
<td>12.69 ± 0.67b</td>
<td>58.05 ± 6.7b</td>
</tr>
<tr>
<td>NI + CQ</td>
<td>24.08 ± 0.67</td>
<td>22.74 ± 0.67</td>
<td>44.65 ± 5.36</td>
</tr>
<tr>
<td>I + CQ</td>
<td>62.81 ± 0.82c</td>
<td>14.32 ± 0.54c</td>
<td>23.36 ± 0.17b</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SEM. *a*: p < 0.05 compared to non-infected and non-treated erythrocytes (NINT); *b*: p < 0.01; *c*: p < 0.05 compared to infected erythrocytes (I) (n=4); GPx: glutathione peroxidase; GST: glutathione-S-transferase; GSH: reduced glutathione; GSSG: oxidized glutathione; NI: non-infected erythrocytes.
intraerythrocytic reduced glutathione (Bhatia & Charot 1984). This reduction and the decrease in the overall activity of the NADPH + H⁺-dependent dehydrogenases would follow a decrease in the overall GPx activity, which, in turn, exacerbates the oxidative stress.

We have shown that SOD activity decreased markedly during infection, which is in agreement with results demonstrated in malaria patients and in P. berghei-infected erythrocytes (Farombi et al. 2003, Rodrigues & Gamboa 2009); however, treatment with IQ and CQ prevented some loss of the activity of this enzyme compared to infected, untreated cells. This event could be produced in response to a compensatory effect due to modification of the glutathione cycle and hexose-monophosphate activity, results that were also reported for the bis-quinoline compound dequalinium (Rodrigues & Gamboa 2009). Overall CAT activity was also diminished due to the infection, which could also be responsible for the oxidative stress observed in red blood cells infected with Plasmodium (Mohan et al. 1992b). While IQ treatment did not modify the activity of this enzyme in the infected cell population, CQ could reduce the decrease in enzyme activity in infected cells.

Lipid peroxidation was also a consequence of the infection. Oxidative stress promotes lipid damage and, consequently, it may affect the cell membrane (Dondorp et al. 1999). Infected cells treated with IQ or CQ showed lower levels of MDA, a lipid peroxidation marker. This effect could be beneficial, protecting the cells from haemolysis, which results in the development of the anaemia typically observed in malaria patients. This is in agreement with data published on another cycloalcanone, which did not cause lipid peroxidation in erythrocytes (Palmen & Evelo 1998) as well as data on another quinoline derivative, which also diminished malondialdehyde levels in infected cells (Rodrigues & Gamboa 2009). Nevertheless, this result is contradictory because both quinoline compounds seem to promote oxidative stress in infected cells. This protective quinoline-mediated decrease in lipid peroxidation effect may be a consequence of other effects in infected cells instead of an effect resulting from oxidative stress. A previous study has demonstrated the ability of CQ to inhibit lipid peroxidation in cell samples (Jackson et al. 1984), possibly due to its effects as a phospholipase-A2 inhibitor. Additionally, Nosál et al. (1995) reported that this compound inhibited arachidonic acid liberation from membrane phospholipids.

On the other hand, both quinolines have reported to inhibit haeme crystallisation (Chong & Sullivan 2003, Charris et al. 2005). This porphyrin is toxic to cells by way of free radical-dependent mechanisms (Kumar & Bandyopadhyay 2005). In this context, there is a strong correlation between the effects of these compounds on β-haematin formation and the inhibition of different enzymes involved in oxidative stress. Free haeme catalyses oxidation, covalent cross-linking and formation of protein aggregates as well as the degradation of small peptides. There is also the possibility of formation of IQ-haeme complexes, as it occurs with CQ in the food vacuole of the parasites (Sullivan et al. 1996), which, by mimicking haeme, stalls all mechanisms of haeme crystallisation, resulting in the death of the malaria parasite. It is likely that the plasmoidal activity of these compounds results from augmentation of parasite oxidative stress.

In conclusion, we have investigated different effects caused by IQ in P. berghei-infected erythrocytes to evaluate the mechanism of action of this possible antimalarial agent. In the presence of this compound, the overall activity of the hexose-monophosphate shunt in infected erythrocytes was diminished, which could also lead to a decrease in glutathione cycle activity due to compromised levels of NADPH + H⁺. This effect could prevent some loss of SOD activity related to infection. Lipid peroxidation was also decreased by this compound, which would protect the host from the severe anaemia caused by the infection. Although more molecular studies of drug-receptor interactions are needed, our results suggest that IQ has antimalarial properties with a mechanism of action which involves not just the inhibition of haemoglobin proteolysis and haeme crystallisation but results in a modification of the oxidative status of infected cells; these results were generally recapitulated by CQ. In this context, this compound could be a candidate for further studies as a possible treatment for malaria.

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