Hypothesis

Does chloroquine really act through oxidative stress?

Diego Monti[a,*], Nicoletta Basilico[b], Silvia Parapini[b], Erica Pasini[a], Piero Olliaro[c], Donatella Taramelli[b]

[a] *Department of Organic Chemistry, University of Milan, CNR-ISTM, Via Venezian 21, 20133 Milan, Italy*
[b] *Institute of Microbiology, University of Milan, Via Pascal 36, 20133 Milan, Italy*
[c] *UNDP/World Bank/WHO-TDR, Geneva, Switzerland*

Received 19 April 2002; revised 24 May 2002; accepted 24 May 2002

First published online 3 June 2002

Edited by Felix Wieland

Abstract To assess whether molecular oxygen and oxidative stress contribute to chloroquine activity, we cultivated strains of *Plasmodium falciparum* in erythrocytes with carboxyhemoglobin and an atmosphere containing 2% CO, 5% CO_2 and 93% N_2. Results indicate that, contrary to common belief, oxygen is not involved in the activity of chloroquine. Reactive radicals formation is suggested.

Key words: Carboxyhemoglobin; Chloroquine; Carbon monoxide; Oxygen; Malaria; *Plasmodium falciparum*

Malaria continues to be a major health problem in tropical countries. Some 300–500 million clinical cases occur annually and result in 700 000–2.7 million deaths [1]. Chloroquine, a 4-amino 7-chloro quinoline, has been the mainstay of malaria control for decades. Despite years of use, its mode of action and the mechanism by which malaria parasites become resistant are only partly known. We know, however, that they are unrelated and independent [2]. Resistance of *Plasmodium falciparum* to chloroquine is multigenic and concerns drug accumulation in the parasite. In contrast, chloroquine and other quinoline drugs target a unique, essential metabolic pathway of the parasite, the ingestion and degradation of host cell hemoglobin (Hb) within the parasite's food vacuole. While globin-derived amino acids are used by the parasite, the remaining free heme moiety must be disposed: iron(II)protoporphyrin IX (Fe[II]PPIX) is oxidized to iron(III)protoporphyrin IX (Fe[III]PPIX) and, for the most part, converted into a crystalline pigment called hemozoin [3]. So, although chloroquine resistance is nowadays widely spread, knowing how chloroquine works is important in order to design newer and more effective drugs.

It is generally accepted that chloroquine prevents heme disposal through the formation of complexes with Fe[III]PPIX. Nuclear magnetic resonance, UV-visible and Mössbauer spectroscopy data concur to show that π–π interaction between the drug and the electronic system of hematin governs the formation of these adducts. The accumulation of free heme and/or of chloroquine–Fe(III)PPIX adducts is thought to generate oxidative stress, leading to peroxidation of parasite membrane lipids and parasitic death [3–5]. However, uncertainties still exist.

If the peroxidative damage hypothesis were correct, molecular oxygen would be important and play a key role in the initiation and amplification of the oxidative reactions. However, our findings are not supportive of this view. We found no significant variation in the activity of chloroquine when the oxygen tension in the culture was increased [6]. Also the presence of preformed peroxides was necessary for membranes to react with the drug–Fe[III]PPIX complexes and for lipid peroxidation to occur [7]. To further clarify if molecular oxygen and oxidative stress had a role in the mechanism of action of chloroquine, we compared *P. falciparum* (chloroquine resistant and susceptible clones) cultured in standard conditions (erythrocytes in an atmosphere of 1% O_2, 5% CO_2 and 94% N_2) and modified conditions (erythrocytes containing carboxy-Hb in 2% carbon monoxide (CO), 5% CO_2 and 93% N_2). The affinity of CO for Hb is 250 times greater than that of oxygen [8]. Parasites grew normally in the presence of carboxy-Hb; we observed no gross morphological changes after continuous culture under modified conditions for over a month and parasites showed microscopically birefringent crystals similar to native hemozoin. In vitro susceptibility to chloroquine remained unchanged, both for resistant and sensitive parasites. Verapamil, a chloroquine resistance modifier, maintained its effect on chloroquine resistant *P. falciparum* [9] (Fig. 1A).

Based on these findings we reached the following conclusions: (i) the metabolic pathways inhibited by CO are not essential for the survival and growth of the parasite. We refer in particular to the inhibition by CO of the respiratory chain (i.e. cytochrome a_3) [10], as well as to the findings of experiments where the oxygen consumption of Plasmodia was measured in the presence of cyanide. These data lead the authors to suggest the existence of a cyanide resistant, alternative respiratory pathway in *P. falciparum*, as described for plants and fungi [11]; (ii) parasites can grow in the conditions adopted in our experiment suggesting that they are capable of utilizing carboxy-Hb as well as oxy-Hb. This indirectly confirms that Plasmodia are microaerophilic organisms [12]; (iii) most importantly, the presence of oxygen appears to be irrelevant for chloroquine to act. These findings contrast with the common

*Corresponding author. Fax: (39)-02-50314072.
E-mail address: diego.monti@unimi.it (D. Monti).

Abbreviations: Hb, hemoglobin; CO, carbon monoxide; Fe[II]PPIX, iron(II)protoporphyrin IX; Fe[III]PPIX, iron(III)protoporphyrin IX

0014-5793/02/$22.00
PII: S0014-5793(02)02881-8

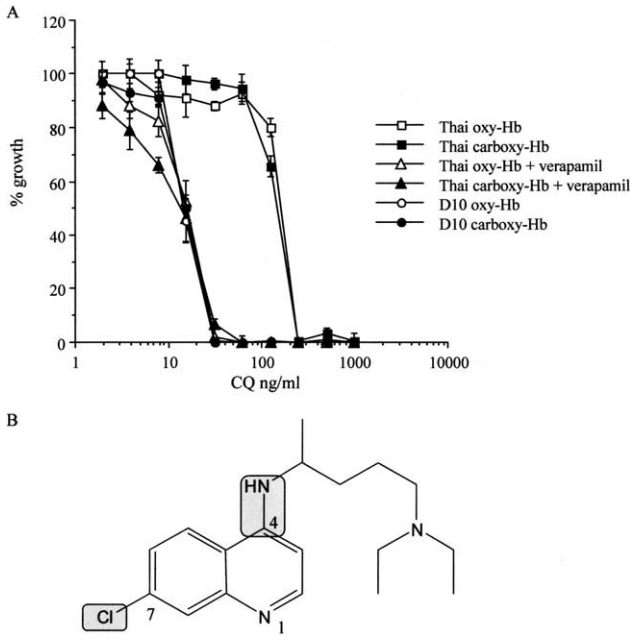

Fig. 1. A: Antimalarial activity of chloroquine against *P. falciparum* strains cultured in different atmospheres. *P. falciparum* chloroquine-sensitive clone D10 and multidrug resistant clone TM91C253 (Thai) were used in these experiments. Chloroquine susceptibility assays were performed on asynchronous parasites cultured in 96-well plates for 72 h at 37°C in standard (normal erythrocytes in an atmosphere of 1% O_2, 5% CO_2 and 94% N_2, empty symbols) and modified conditions (erythrocytes containing carboxy-Hb in 2% CO, 5% CO_2 and 93% N_2, solid symbols) in the presence of 5 μM verapamil. Parasite growth was determined by measuring the activity of the parasite lactate dehydrogenase as described [23]. All tests were performed at least three times in triplicate and results were analyzed by one-way analysis of variance. Giemsa-stained cultures were also observed microscopically. B: Structure of chloroquine.

belief that parasite death caused by chloroquine is mediated by toxic oxygen species derived from the accumulation of free heme or Fe[III]PPIX–chloroquine adducts [3,4]. The experimental conditions and practices adopted were designed to prevent oxygen contamination, so that activated oxygen species could not form. Therefore, we can conclude that in these conditions *Plasmodium* growth and chloroquine activity are unaltered. These observations allow us to propose alternative hypotheses on both the physiology of the parasite and the mode of action of chloroquine.

We submit that the food vacuole may represent an anaerobic compartment in a microaerophilic organism. The hypothesis that the conditions of the food vacuole are of functional anaerobiosis is plausible because at the acidic pH of this organelle, Hb saturation for oxygen is very low ($< 10\%$), and because the relative solubility of oxygen is significantly lower than that of CO_2 (Henry's coefficient) [13]. Our data do not allow further speculation on the parasite ability to carry on other oxygen-dependent metabolic pathways, such as de-novo pyrimidine synthesis, for which Plasmodia do not seem to have a salvage pathway [14].

Considering that the food vacuole is the main site of action of chloroquine, we would like to suggest the existence of an alternative mechanism of action of chloroquine, on the basis of both our own and published data. This hypothesis is supported by the fact that the formation of adducts via π–π bonds with the porphyrin ring is essential for the activity of quinoline antimalarials [3], and that the quinoline ring, as well as the side chain, of chloroquine can host radicals [15]. We thus propose the occurrence of an electron transfer between the redox couple Fe[II]PPIX/Fe[III]PPIX and the quinoline ring [16,17], to generate highly reactive radicals. That this transfer exists is corroborated by the observation that chloroquine protects Fe[II]PPIX/Fe[III]PPIX from oxidative degradation by reactive oxygen species (see [18] and Pasini et al., unpublished results). This hypothesis would explain the importance of stereoelectronic features for chloroquine antimalarial activity [19], since an electron transfer is linked to the electron density in position 4 and in the secondary nitrogen of the side chain, which in turn depends on the substituent in position 7 of the quinoline ring [3] (Fig. 1B). This theory also explains the importance of chlorine in position 7 and the fact that the antimalarial activity is lost when the nitrogen atom (N_4) is alkylated [3,20]. Investigations to further support this hypothesis are underway.

These results allow us to identify two characteristics to be sought in the design of newer antimalarials targeting processes in the food vacuole: the ability to act in the 'reducing' conditions of the food vacuole and to form radicals [21] in the presence of Fe(II)PPIX, a mechanism already proposed for artemisinin type compounds [22].

Acknowledgements: We are thankful to Prof. M. Ghione, presently retired, for invaluable advice and helpful discussion. We thank Dr. F. Ravagnani from the Blood Unit, National Cancer Institute, Milan, Italy, for providing fresh red blood cells for *P. falciparum* growth. This work was supported in part by the Ministero Italiano dell'Università e della Ricerca Scientifica e Tecnologica, Co-finanziamento 2001, #2001061849.

References

[1] Breman, J.G. (2001) Am. J. Trop. Med. Hyg. 64 (Suppl. 1–2), 1–11.
[2] Olliaro, P. (2001) Pharmacol. Ther. 89, 207–219.
[3] Egan, T.J. (2001) Mini Rev. Med. Chem. 1, 113–123.
[4] Loria, P., Miller, S., Foley, M. and Tilley, L. (1999) Biochem. J. 339, 363–370.
[5] Ginsburg, H., Famin, O., Zhang, J. and Krugliak, M. (1998) Biochem. Pharmacol. 56, 1305–1313.
[6] Monti, D., Omodeo-Salé, F., Basilico, N., Pasini, E., Parapini, S., Olliaro, P. and Taramelli, D. (2001) Am. J. Trop. Med. Hyg. (Suppl.) 65, 344.
[7] Omodeo-Salé, F., Monti, D., Olliaro, P. and Taramelli, D. (2001) Biochem. Pharmacol. 61, 999–1009.
[8] Spiro, T.G. and Kozlowski, P.M. (2001) Acc. Chem. Res. 34, 137–144.
[9] Martiney, J.A., Cerami, A. and Slater, A.F.G. (1995) J. Biol. Chem. 270, 22393.
[10] Babcock, G.T., Vichery, L.E. and Palemrs, G. (1978) J. Biol. Chem. 253, 2400–2411.
[11] Murphy, A.D., Doeller, J.E., Hearn, B. and Lang-Unnasch, N. (1997) Exp. Parasitol. 87, 112–120.
[12] Krieg, N.R. and Hoffman, P.S. (1986) Annu. Rev. Microbiol. 40, 107–130.
[13] Guzmàn-Casado, M. and Parody-Morreale, A. (2002) J. Chem. Educ. 79, 327–331.
[14] Gero, A.M. and O'Sullivan, W.J. (1990) Blood Cells 16, 467–484.
[15] Motten, A.G., Martinez, L., Holt, N. and Sik, R. et al. (1999) Photochem. Photobiol. 69, 282.
[16] Schellnut, J.A. (1983) Inorg. Chem. 22, 2535–2544.
[17] Wolf, B., Golenser, J., Chevion, M. and Saran, M. (1991) Int. Soc. Free Radical Research Bin. Meet., 5th 234.
[18] Papalexis, V., Siomos, M., Campanale, N., Guo, X., Kocak, G.,

Foley, M. and Tilley, L. (2001) Mol. Biochem. Parasitol. 115, 77–86.
[19] Karle, J.M. and Bhattacharjee, A.K. (1999) Bioorg. Med. Chem. 7, 1769–1774.
[20] Vippagunta, S.R., Dorn, A., Matile, H., Bhattacharjee, A.K., Karle, J.M., Ellis, W.Y., Ridley, R.G. and Vennerstrom, J.L. (1999) J. Med. Chem. 42, 4630–4639.
[21] Singer, J.A. and Purcell, W.P. (1967) J. Chem. Soc. 10, 754.
[22] Szpilman, A.M., Korshin, E.E., Hoos, R., Posner, G.H. and Bachi, M.D. (2001) J. Org. Chem. 66, 6531–6540.
[23] Makler, M. and Hinrichs, D. (1993) Am. J. Trop. Med. Hyg. 48, 205–210.