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ANTIMALARIAL AGENTS, 2.[1,2] ARTESUNATE, AN INHIBITOR OF CYTOCHROME OXIDASE ACTIVITY IN *PLASMODIUM BERGHEI*

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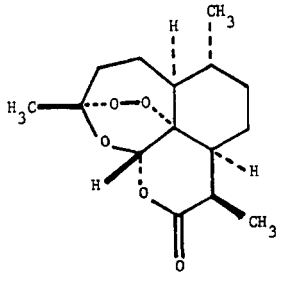
ABSTRACT.—The activity of the cytochrome oxidase, which is located in the plasma and the nuclear and the food-vacuole-limiting membranes as well as in the mitochondria of the trophozoites of *Plasmodium berghei*, was inhibited completely by sodium artesunate, an antimalarial drug, in vitro at 1 mM and in vivo at 100 mg/kg iv. This enzyme appears to be a target for the antimalarial mechanism of action of artesunate and qinghaosu.

Qinghaosu (**1**, artemisinin, QHS), a novel sesquiterpene lactone endoperoxide antimalarial constituent isolated from the traditional Chinese herbal remedy "Qinghao" (*Artemisia annua*), and sodium artesunate (**2**),[5] the sodium succinate derivative of a dihydroqinghaosu, are effective antimalaria drugs with rapid action and low toxicity. The water soluble **2** is more active than **1** and is used more often clinically (1). Pharmacologic studies and clinical observations in every type of malarial infection show that **1** has direct parasiticidal action on *Plasmodium* in the erythrocytic stage and is not effective in the tissue stage (2-9). Studies on the initial mechanism of action have established that **1** causes primarily membranous structural changes that take place at the food-vacuole-limiting membrane and nuclear membrane as well as the mitochondria, endoplasmic reticulum, and nucleoplasm, leading to the formation of autophagic vacuoles and the loss of a lot of cytoplasm to kill the parasites (3). Compounds **1** and **2** have also been shown to have a different mechanism of action from the other class of antimalarial agents, such as sulfonamides, trimethoprim, and pyrimethamine, which are inhibitors of folic acid metabolism (8). The exact mechanism of antimalarial action of **1** and **2** at the enzymatic level has not been explored.



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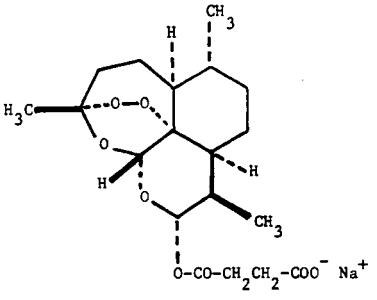

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[1]For part 1 of this series, see S. Tani, N. Fukamiya, H. Kiyokawa, H.A. Musallam, R.O. Pick and K.H. Lee, *J. Med. Chem.* (in press).

[2]A preliminary report of this work was presented at the International Research Congress on Natural Products at the University of North Carolina, Chapel Hill on July 10, 1985, Abstract No. 153.

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[5]The structure and stereochemistry of artesunate has recently been determined unequivocally by a single-crystal X-ray analysis (A.T. McPhail, S. Tani, Y. Zhao, and K.H. Lee, to be published).

Herein we report the inhibition by **2** of cytochrome oxidase activity within the intraerythrocytic trophozoite of mice infected with *Plasmodium berghie*.

That the direct parasiticidal action of **1** occurs in the erythrocytic trophozoite of *Plasmodium* as mentioned above, coupled with the fact that the multilamellate membrane systems found in the cytoplasm of the trophozoites of *P. berghei* is associated with strong cytochrome oxidase activity as reported by Theakston *et al.* (9), prompted our examination of the inhibition of cytochrome oxidase activity as a possible target for drug action.

The subcellular and cytochemical demonstration of cytochrome oxidase was based upon a modified Nadi reaction using osmiophilic reagents, such as an indoaniline derived from cytochrome oxidase oxidation of *N*-benzyl-*p*-phenylenediamine (BPDA) in the presence of 1-naphthol, according to Seligman *et al.* (10). The highly colored indoaniline is then converted, by treatment with osmium tetroxide, to an osmium black, which is an electron-opaque coordination polymer insoluble in H_2O and organic solvents. Cytochrome oxidase activity was located in the form of small numerous black deposits as seen in Figure 1. These black deposits disappear when cytochrome oxidase activity is inhibited by an inhibitor such as **2**, as shown in Figure 2 as the osmiophilic indoaniline will no longer be produced from BPDA and 1-naphthol due to the lack of cytochrome oxidase.

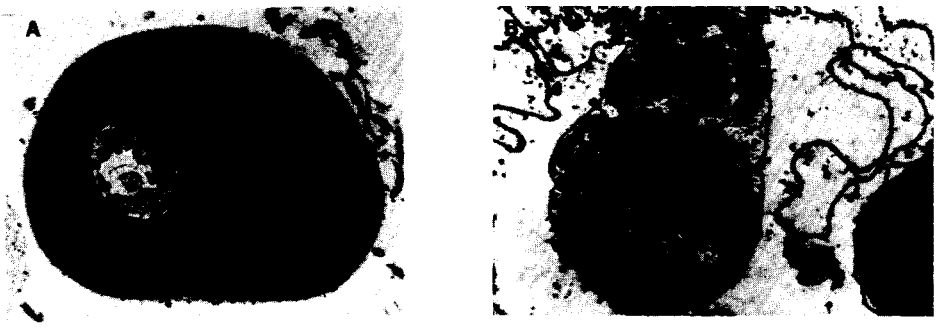

FIGURE 1. Electron micrograph of the infected red blood cells of a trophozoite of *Plasmodium berghei* after incubation at 37° with 2.5% $NaHCO_3$ solution (1 ml added with 19 ml distilled H_2O). Osmium black deposits indicate cytochrome oxidase activity in the parasite (× 12,600). A and B were taken from numerous separate experiments.
E: erythrocyte; P: *Plasmodium berghei*;
M: osmium black deposits which indicate enzyme activity in the whorled membranes.

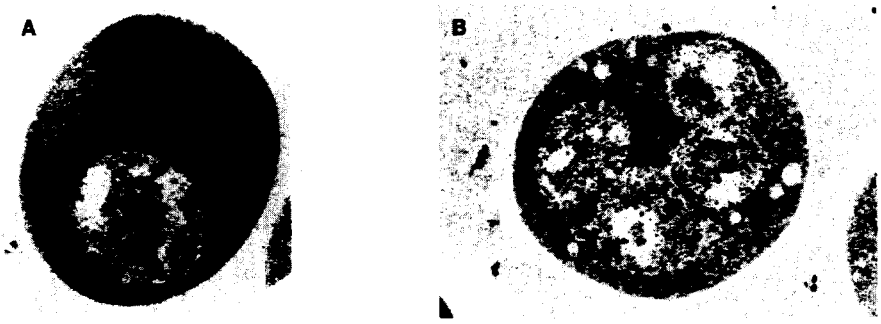

FIGURE 2. Electron micrograph of the infected red blood cells of a trophozoite of *Plasmodium berghei* after incubation at 37° with 10^{-3}M of sodium artesunate. The cytochrome oxidase activity of the parasite was inhibited completely as osmium black deposits were no longer found. A and B were taken from numerous separate experiments.
E: erythrocyte; P: *Plasmodium berghei*.

EXPERIMENTAL

The procedure for the preparation of tissue before electron microscopic examination was carried out as follows. The CF_1 male mice (approximately 30 g body weight, each) were divided into three groups with six mice per group. Each group was injected intraperitoneally 0.2 ml saline suspension of ca. 5×10^6 *P. berghei* red blood cells per mouse. After 3 days, the blood samples were collected when parasitemia reached above 10%. The blood samples were then mixed with various concentrations of sodium artesunate at 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} M in 0.22 M sucrose solution and incubated at 37° for 30 min. The resulting blood blocks were fixed in cold 4% HCHO for 30 min. It was then washed for 15 min in 0.22 M buffered sucrose followed with 0.1 M phosphate buffer at PH 7.4, and incubated in a medium (BPDA, 2 mg/ml, 2 ml; 1-naphthol, 1 mg/ml, 2 ml; 0.1 M sodium phosphate buffer, pH=7.4, and 0.44 M in sucrose, 3 ml; catalase, 0.03 mg/ml, 0.5 ml; cytochrome c, 7 mg)(11) at 37° for 30 min. At the end of the incubation period, the blocks of tissue were washed three times for 5 min each in 0.22 M buffered sucrose and then 0.1 M phosphate buffer (pH 7.4) at room temperature. The blocks were then exposed to osmium tetroxide by immersion of blocks in 1% aqueous, unbuffered osmium tetroxide at 50° for 60 min followed by dehydration and embedment in Epop-Araldite. Ultrathin sections were cut on a Porter-Blum microtome (MT2-B) equipped with a Dupont diamond knife. Care was taken to ensure the inclusion of tissue from the reaction surfaces of the blocks. Sections, mounted on copper grids, were stained first with 5% aqueous uranyl acetate at room temperature for 20 min and then rinsed and stained again with instant lead citrate for 7 min. Finally, it was further rinsed with 0.02 M NaOH and then H_2O. The sections were studied with a Zeiss EM 10 CA electron microscope at 80 KV with a 60 μ aperture.

RESULTS AND DISCUSSION

The result of the foregoing in vitro study is shown in Figure 2, in which it clearly shows that the conspicuous deposits of osmium black, which is the indication of the sites of cytochrome oxidase activity within the trophozoites of *P. berghei* as reported by Theakston *et al.* (9), disappeared completely upon administration of sodium artesunate (**2**) at 10^{-3}M when compared to those of the control (Figure 1) without drug treatment. It is to be noted that there is a dose dependent response to **2**; as the dose of **2** increased above 10^{-3}M, the inhibition of cytochrome oxidase activity decreased. It showed no inhibition at 10^{-7}M.

An in vivo study carried out by a single dose intravenous infection of sodium artesunate at 100 mg/kg, which is equivalent to the human clinical use dosage to CF_1 male mice infected with *P. berghei*, followed by incubation of the blood samples taken after 1 week of infection (infected rate over 20%) with the same medium described above, also led to a complete inhibition of the cytochrome oxidase activity.

As shown in Figure 1, the cytochrome oxidase activity was located mostly in the plasma membrane, the nuclear membrane, and the limiting membrane of the food vacuole, instead of in the endoplasmic reticulum or in the cytoplasmic stroma, found in the cytoplasm of the trophozoites of *P. berghei*, as indicated by Theakston *et al.* (9). The cytochrome oxidase was also found to be the only enzyme possessed by the mitochrondria of the trophozoites of *P. berghei* during the course of the parasites' life cycle (11). These observations led to the conclusion that one of the mechanisms of action of artesunate is due to its inhibition of cytochrome oxidase which occurs at the plasma, the nuclear and the food vacuole limiting membranes as well as in the mitochondria of the trophozoites of *P. berghei*. These results support and extend the previous conclusion with respect to membranous structural change caused by **1** and **2** as described above and further indicate that cytochrome oxidase in the membranes and mitochondria is a target for drug action.

ACKNOWLEDGMENTS

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