In Vitro Antimalarial Activity of a New Organometallic Analog, Ferrocene-Chloroquine

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Received 27 May 1997/Returned for modification 23 July 1997/Accepted 17 November 1997

The in vitro activities of new organometallic chloroquine analogs, based on 4-aminoquinoline compounds bound to a molecule of ferrocene, were evaluated against chloroquine-susceptible, chloroquine-intermediate, and chloroquine-resistant, culture-adapted Plasmodium falciparum lineages by a proliferation test. One of the ferrocene analogs totally restored the activity of chloroquine against chloroquine-resistant parasites. This compound, associated with tartaric acid for better solubility, was highly effective. The role of the ferrocene in reversing chloroquine resistance is discussed, as is its potential use for human therapy.

The ideal antimalarial drug is a cheap molecule that shows rapid curative activity in the absence of toxicity to the host. Except in regions where chloroquine-resistant isolates are endemic, chloroquine is still the favored treatment both for chemotherapies and for chemoprophylaxis because of its low cost, efficacy, and relative tolerance. Quinine is the drug of choice for severe chloroquine-resistant Plasmodium falciparum malaria. Sulfadoxine-pyrimethamine and halofantrine are both used as alternative antimalarial agents after treatment failure with chloroquine. Nevertheless, treatment against severe malaria still remains a problem because of drug resistance in many parts of the world. The search for new compounds for the treatment of malaria has been extensive, but the yield has been very low, as demonstrated by the program of the Walter Reed Army Institute of Research (less than 10 promising compounds among the 350,000 compounds tested) (35). There is still confusion and doubt about the mechanism of action of chloroquine (36).

Two approaches have been proposed for drug design. The first is the identification of drugs that do not possess any intrinsic antimalarial activity when used alone but that potentiate the effect of antimalarial drugs (e.g., verapamil and desferrioxamine). The second is the development of original antimalarial drugs with enhanced activity and low toxicity (e.g., halofantrine and mefloquine) (1).

We postulated that given the avidity of Plasmodium for free iron (20, 33), an effective way of removing the chloroquine resistance of parasites might be by the addition of iron to a chloroquine molecule. By following this hypothesis, some new organometallic compounds based on chloroquine with a ferrocene nucleus (dicyclopentadienyl iron) localized at different sites were synthesized (2, 4). The present work describes the investigation of the potential activities of these new compounds against P. falciparum parasites by using an in vitro model.

MATERIALS AND METHODS

Synthesis of ferrocene-chloroquine analogs. Ferrocene-chloroquine analogs were synthesized by the Laboratoire de Chimie Organométallique, J. Brocard, Université des Sciences et Technologies de Lille (2). The synthesis of SN1tar is illustrated in Fig. 1. The dimethylaminoethylferrocene (compound 2; 2.43 g; 10 mM) was metatalated with n-butylthiium (5 ml; 12.5 mM) in anhydrous ether under a nitrogen atmosphere. The lithium derivatives were condensed with N,N-dimethylformamide (0.5 ml; 12.5 mM) at room temperature to the resulting 2-(N,N-dimethylaminoethyl)ferrocene carboxaldehyde in respectable yields (70%). The aldehyde (compound 3; 1 g; 3.7 mM) was converted to the corresponding oxime by the addition of hydroxylamine hydrochloride (0.42 g; 6 mM) and sodium hydroxide (0.48 g; 12.2 mM) in ethanol under reflux. Condensation of compound 5 (0.55 g; 2 mM) with 4,7-dichlorochloroquine (1.98 g; 10 mM) in N-methyl-2-pyrrolidone (135°C; 4 h) under a nitrogen atmosphere gave 7-chloro-4-[[2-(N,N-dimethylaminomethyl)]-N-methylferrocenylamino]quinoline (compound 6) in respectable yields (60%) after purification by chromatography.

Preparation of drugs for in vitro tests. The control drug, chloroquine diphosphate, was supplied by Sigma (Strasbourg, France). Aqueous insoluble agents (SN1, SN2, and SN4) were dissolved in 20 μl of dimethyl sulfoxide (DMSO) and then in 80 μl of a 60% ethanol–40% water mixture with 1 mg of product. The aqueous soluble compounds (chloroquine diphosphate, SN1tar, and CICQ-2FcCOOH) were directly dissolved in 100 μl of 60% ethanol–40% water mixture with 1 mg of product. Stock solutions were diluted to 1 mg/ml in sterile water and were stored at 4°C in the dark. The final dilution in culture contained less than 0.07% ethanol and 0.02% DMSO, which had no measurable effect on the parasites in our system.

Culture-adapted strains of P. falciparum. Six culture-adapted lineages of P. falciparum were maintained in continuous culture by a method modified from that of Trager and Jensen (38). The chloroquine-resistant lineages were SGE2/Zaire (5), FG2, and FG4, the semi-chloroquine-resistant lineage was FG3, and the chloroquine-resistant clones were FCMI7/Thailand, FC6M7/Thailand (24), and FG1 (uncloned isolated lineage). FG1, FG2, FG3, and FG4 were isolated in our laboratory from Gabonese patients (10). Stock cultures were grown in 90-mm-diameter petri dishes with type O-positive European human erythrocytes (CNTS, Paris, France) at 5% hematocrit in RPMI 1640 medium (Sigma) with 25 mM HEPES buffer supplemented with 10% heat-inactivated type AB-positive serum (CNTS) and 0.2% glucose (RPMI-c). The cultures were incubated at 37°C in a candle jar and were monitored daily by counting the number of rings, old trophozoites, and schizonts on Giemsa-stained slides. The cultures were used in proliferation assays when less than 80% ring stages were obtained.

Proliferation tests. The drug susceptibilities of each lineage were evaluated by using a modification of the proliferation test described by Desjardins et al. (9), based on the level of hypoxanthine incorporation. A suspension of parasitized erythrocytes (PRBCs) in RPMI-c (100 μl/well, 20% hematocrit, 1% parasitemia) was distributed in 96 round-bottom wells preseeded with 100 μl of drugs in

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RPMI-c. The final concentrations of the compounds ranged from 0.001 to 10 μg/ml, with three or six replicates used for each concentration, after which the values were transformed into nanomoles for easy comparison. The results describing the activities of the compounds were compared to the results obtained for untreated parasitized erythrocytes (untreated PRBCs) and PRBCs treated with the solvents used for the drug (ethanol and DMSO). The plates were incubated at 37°C in a candle jar for 48 h. The \([\text{G}-3\text{H}\] hypoxanthine (Amersham, Little Chalfont, Buckinghamshire, United Kingdom) was added (2.5 μCi/well) for the final 18 h to assess parasite growth. At the end of the second incubation period, the contents of one well with untreated PRBCs were used to make a Giemsa-stained slide as a control for schizont development, and the plates were stored by freezing and thawing to lyse the erythrocytes. The contents of each well were collected with a cell harvester (Skatron Instruments). The amount of radioactivity incorporated by the schizonts was measured in a liquid scintillation counter (Beckman). For each experiment a series of controls were used: slides at the end of the culture, uptake of \([\text{G}-3\text{H}\] hypoxanthine by untreated PRBCs, solvent-treated PRBCs, and nonparasitized erythrocytes. With the aim of comparing the activities of the drugs, the IC50 was defined as the concentration of drug that resulted in 50% inhibition. The IC50 was calculated from extrapolation of the regression line by projection of a straight line through the mean disintegrations per minute (radioactivity level) between the maximum and the minimum values. In view of the results obtained under our experimental conditions, we designated the strains chloroquine susceptible if the IC50 was lower than 1,000 nM and chloroquine resistant if the average IC50 was greater than 1,000 nM. The high level of the IC50 is due to the elevation of the hematocrit to 10%.

RESULTS

Antimalarial activities of ferrocene-chloroquine compounds against reference strains. The activities of the organometallic analogs (SN1, SN2, and SN4) were evaluated by using the chloroquine-susceptible strain SGE2 and the chloroquine-resistant clones FC6 and FC17 (Table 1). Against chloroquine-resistant parasites, the new drugs were more effective than chloroquine. Their activities were the same against all parasite strains. However, SN1 was active against the three strains at a lower concentration.

Antimalarial activities of the tartaric acid forms of the chloroquine analogs against strains SGE2 and FC17. With the intention of comparing chloroquine activity with \(P. falciparum\) susceptibility to SN1tar, we classified the lineages according to their chloroquine resistance levels as described above. The standard deviation of the SN1tar IC50 was very low, indicating that this compound is equally active against susceptible and resistant strains (124 ± 74 nM without discrimination of the susceptibility or resistance of the lineage) (Table 2). It also has the same IC50 range as that of chloroquine for chloroquine-susceptible parasites (262 ± 235 nM). For chloroquine-resistant parasites, the IC50s of chloroquine were approximately 30 times greater than those of SN1tar, also without discrimination of the susceptibility or resistance of the lineage (3,774 ± 2,692 and 124 ± 74 nM, respectively).

DISCUSSION

Among the ferrocene analogs, SN1 and its tartaric acid form, SN1tar (which is soluble in aqueous solution), are effective against all parasites at a low concentration when compared to the efficacy of chloroquine tested against chloroquine-susceptible strains. Because the IC50 of these two compounds are similar, the aqueous solubility does not seem to have an influ-
ence on the in vitro antimalarial activity but makes its use easier. This aspect could be advantageous for in vivo use.

With the CICQ-2FcCOOH compound, we have demonstrated that the ferrocene molecule needs to be bound covalently to the chloroquine to inhibit the resistance of the parasites. Thus, the ferrocene by itself does not have an antimalarial activity but enhances the effectiveness of chloroquine when it is enclosed inside the molecule.

Previous studies have demonstrated the potential benefits of the chloroquine side chain modifications. Some compounds with different numbers of carbons (more than eight or less than eight carbon atoms) have been designed, with the aim of increasing the antimalarial activity. One of these compounds is CICQ-2FcCOOH, which contains a ferrocene moiety covalently attached to the chloroquine molecule.

TABLE 1. Susceptibility of resistant and nonresistant strains to ferrocene analogs compared to susceptibility to chloroquine

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC_{50} (nM)</th>
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<tbody>
<tr>
<td></td>
<td>SGE2</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>287 ± 264</td>
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<tr>
<td>SN1</td>
<td>196 ± 140</td>
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<tr>
<td>SN2</td>
<td>807 ± 310</td>
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<tr>
<td>SN4</td>
<td>554 ± 292</td>
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TABLE 2. IC_{50} of chloroquine for each chloroquine-resistant group confronted with SN1tar

<table>
<thead>
<tr>
<th>Strain</th>
<th>IC_{50} (nM)</th>
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<tbody>
<tr>
<td></td>
<td>Chloroquine</td>
</tr>
<tr>
<td>FCM6</td>
<td>3,774 ± 2,692 (17)</td>
</tr>
<tr>
<td>FCM17</td>
<td>971 ± 269 (3)</td>
</tr>
<tr>
<td>FG1</td>
<td>262 ± 235 (15)</td>
</tr>
<tr>
<td>SGE2</td>
<td>202 ± 194 (11)</td>
</tr>
<tr>
<td>FG2</td>
<td>180 ± 192 (10)</td>
</tr>
<tr>
<td>FG4</td>
<td>170 ± 182 (9)</td>
</tr>
</tbody>
</table>

* Values are the arithmetic mean IC_{50} ± standard deviation of the mean for the number of isolates (in parentheses). For chloroquine, values are the mean for each group (FCM6, FCM17, and FG1 for chloroquine-resistant strains; SGE2, FG2, and FG4 for chloroquine-susceptible strains; and FG3 for intermediate). For SN1tar, the value is the mean for all strains. Compounds were tested in triplicate at five concentrations ranging from 0.001 to 10 μg/ml, after which the results were transformed into nanomolar units.
At the intraerythrocytic stage, malaria parasites ingest the cytosol of their host cell and digest most of the hemoglobin inside the acid food vacuole of the host cell. Proteolysis of hemoglobin releases a toxic free heme (18, 39), the lethality of which is inhibited by the parasite by the formation of hemozoin, a polymerization product. The most convincing explanation of the activity of chloroquine lies in its capacity to inhibit the polymerization of the free heme by the formation of a toxic heme-chloroquine complex (12). In view of their closely related structures, the modes of action of ferrocene analogs and chloroquine could be identical. It could be possible that the affinity of SN1tar for free heme is greater than that of chloroquine, but this explanation is not completely satisfying. This theory of the mechanism of action of chloroquine has not been widely accepted, mainly because the affinities of both quinine and mefloquine for free heme are between 10^3- and 10^4-fold lower than that of chloroquine (6). An alternative theory has suggested that the release of iron from hemoglobin is essential for the supply of iron for the parasite’s anabolism (15); however, investigators have surmised that another possible function of chloroquine is its action of depriving the parasite of iron, since chloroquine inhibits the release of iron from hemoglobin (16). Desferrioxamine, an iron chelator, also has an antiplasmodial activity, but it seems that this is a direct effect against the parasite rather than a change in the body’s iron status (19, 21, 30). Because parasites must obtain iron from endogenous sources, it has been suggested that parasites obtain iron through transferrin receptors localized in the host cell membrane (20, 33), although the parasites grow normally in transferrin-depleted serum (34). Pollack (31) could not display a transferrin receptor in the host cell membrane, but he observed a nonspecific liaison between transferrin and the erythrocytic membrane. On the other hand, clinical reports suggest that iron supplementation is definitely contraindicated because it increase the susceptibilities of parasites by inhibition of the efflux as a result of the addition of iron to chloroquine. The tartaric acid form, being soluble in an aqueous solution, can favor the bioavailability of the drug. From an in vivo use perspective, it is possible that continuous or repeated drug pressure would decrease the susceptibilities of & P. falciparum & to SN1tar because parasites may develop a mechanism of resistance such as that which already exists against chloroquine. Some reports suggest caution with regard to iron refeeding, which could breed malaria in patients in areas where malaria is endemic (27, 29). Considering the weak contribution of iron in ferrocene-chloroquine, it is unlikely that this drug may favor a significant increase in blood iron levels and thus the likelihood of developing patent malaria. Furthermore, only 25% of the iron from ferrocene is usable in humans (37). On the other hand, if ferrocene-chloroquine is metabolized in the liver, as seen with TMH-ferrocene, the drug will probably lose its properties. Toxicological tests are evidently necessary to secure the low incidence and severity of adverse effects for therapy and prophylactic use. Leung et al. (25) have studied the acute toxicity of three substituted ferrocenes [acetylferrocene, ethylferrocene, and 2,2-bis(ethylferrocenyl)propane] in monkeys. Acetylferrocene was found to be the most toxic, with an oral lethal dose of between 10 and 100 mg/kg of body weight, which is
greater than the dose of SN1tar that would be used for normal treatment on the basis of chloroquine treatment (500 mg/day for an adult). The chronic toxicity of ferrocene in dogs was investigated by Yearly (43). No significant evidence of toxicity was detectable following oral administration of 30 mg/kg daily for a 6-month period.

Many antimalarial drugs have been synthesized and tested, but drug resistance is often associated with chloroquine resistance and their toxicities are sometimes greater than that of chloroquine (1). The concept behind ferrocene analogs is new, with the aim of improving the pharmacokinetics rather than activity. SN1tar could be a drug with real effectiveness for both therapy and prophylaxis against malaria at a low cost and would be of interest to countries where chloroquine-resistant \textit{P. falciparum} is widespread.

**ACKNOWLEDGMENTS**

We thank T. Williams for reading the manuscript and P. Deloron for the gifts of FC6 and FC57.

The Centre International de Recherches Médicales de Franceville is supported by the government of Gabon, Elf Gabon, and Ministère de la Coopération Française.

**ADDENDUM IN PROOF**

In all studies in which these molecules are to be used, the following nomenclature will apply: SN1, JB QN 4; SN2, JB CQ

**REFERENCES**


