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Antimalarial Activity of Selected Aromatic Chelators

II. Substituted Quinolines and Quinoline-*N*-oxides

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SUMMARY

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The human malaria parasite, *Plasmodium falciparum*, has been shown *in vitro* to be sensitive to lipophilic chelators with high metal-binding constants. Growth inhibition of the parasite correlates directly with the ability of the compound to chelate, and favorable activity has been demonstrated among the alkyl thiocarbamates, pyridine-*N*-oxides and quinolines. The 5-substituted 8-hydroxyquinolines and 2-mercaptoquinoline-*N*-oxide, which have had some use in medicine and cosmetics, possess these characteristics. They also exhibit active antimalarial effects *in vitro* at concentrations as low as 5×10^{-8} M, making them more potent than quinine sulfate in the same system. Antimalarial effects are not antagonized by cobalt or potentiated by iron, but toxicity of the 5-substituted oxines to the parasite is increased by increasing oxygen levels *in vitro*.

INTRODUCTION

Plasmodium falciparum, a microaerophile (1), degrades glucose via the Embden-Myerhof scheme to organic end products rather than CO_2 and H_2O (2, 3), and 16-hr growth studies result in homolactate fermentations (4, 5). There is no difference in glucose utilization when the parasite is incubated either aerobically or anaerobically, suggesting the absence of a classical Pasteur effect. It would therefore appear that the terminal respiratory pathway for energy generation, present in most tissues of the mammalian host, is functionally different from the oxidative reactions in the parasite.

We postulated that, in the malaria parasite, oxygen might be used largely through the action of biosynthetic metallo-protein oxidase enzymes rather than for the generation of energy, and that chelators inhibitory to these enzymes might exert selective inhibition on the growth of *P. falciparum*. These agents must be lipophilic to ensure penetration of both the red blood cell and the parasite. Permanent inhibition is best achieved by agents with high metal-binding constants. In agreement with this hypothesis, agents endowed with these properties, e.g., diethyldithiocarbamate (and its oxidation product, tetraethylthiuram disulfide), oxine,[1] and PNO, inhibit growth and the rate of glycolysis of malaria parasitized red cells (lactate production being inhibited prior to glucose utilization) without affecting normal cells (4, 5). The antimalarial activity of these compounds correlates with their ability to chelate metals.

As a class, the 5-substituted oxines chelate metal ions well and are known to be markedly bactericidal (6). One member of this group, 5-methyl oxine (Intetrix) (Fig. 1), has low mammalian toxicity (7) and has been used safely in humans for the control of cholera (8, 9). Therefore, it was decided to test the sensitivity of the human malaria parasite, *P. falciparum*, to 5-methyl oxine and other quinolines. The studies were undertaken to suggest structural characteristics of the drug which might prove fruitful in future antimalarial chemotherapy.

MATERIALS AND METHODS

P. falciparum strain FCR-3/Gambia, subline D (10), was grown *in vitro* (11) in 6% red cell suspensions exactly as described in our previous studies (4, 5). 5-Methyl oxine (Fig. 1), 5-nitro oxine (Fig. 2), and PNO were dissolved in

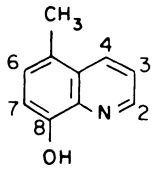

FIG. 1. *Structure of 5-methyl-8-hydroxyquinoline*

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[1] The abbreviations used are: oxine, 8-hydroxyquinoline; PNO, 2-mercaptopyridine-*N*-oxide; DMSO, dimethyl sulfoxide; QNO, 2-mercaptoquinoline-*N*-oxide; $Zn(QNO_2)$, bis-2-mercaptoquinoline-*N*-oxide, zinc salt; Na(QNO), 2-mercaptoquinoline-*N*-oxide, sodium salt.

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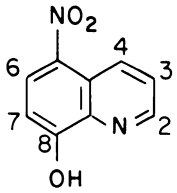

FIG. 2. *Structure of 5-nitro-8-hydroxyquinoline*

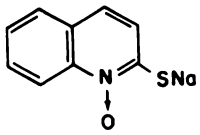

FIG. 3. *Structure of 2-mercaptoquinoline-N-oxide, sodium salt*

DMSO, certified spectroanalyzed (Fisher Scientific Company, Pittsburgh, Pa.), to give a concentration of 80 mg/ml. $Zn(QNO)_2$ was dissolved to give a concentration of 40 mg/ml. Fifty microliters of this solution were added to 40 ml of medium. Controls contained equal quantities of DMSO only. Na(QNO) (Fig. 3) was dissolved directly in 40 ml of medium. All experiments were accompanied by a normal medium control without DMSO. Stock solutions were sterilized by filtration and appropriately diluted with sterile medium to achieve desired concentrations of drug. 5-Nitro oxine and PNO were purchased from Aldrich Chemical Company, Inc. (Milwaukee, Wisc.), and recrystallized. Samples of 5-methyl oxine (Intetrix) were obtained as gifts from Dr. Pierre Huve (Paris, France) and Sigma-Tau (Rome, Italy). The two salts of 2-mercaptoquinoline-*N*-oxide were gifts from Dr. D. H. Steinberg (Ciba-Geigy Company, Ardsley, N. Y.). Statistical significance was assessed by Student's *t*-test at the 0.05 level in all cases. Quinine equivalent is defined as the ratio (by weight) of the concentration of quinine to that of the test drug required to achieve the same inhibition.

RESULTS

Effect of 5-methyl oxine and 5-nitro oxine at two different O_2 tensions. The conventional candle jar technique (12) of growing malaria was used in determining the growth effects of *P. falciparum* in 5-methyl oxine at concentrations of 6.28×10^{-4} to 6.28×10^{-8} M, and results are shown in Table 1. The standard candle jar used throughout the world in studies such as this varies in gas concentration between 14.5 and 17.8% oxygen, and 1.1 and 3.3% CO_2 (1).

As is evident in Table 1, essentially all parasites were dead 24 hr after exposure to 6.28×10^{-4} M drug; there was approximately 48% inhibition at 6.28×10^{-5} M and 27% inhibition at 6.28×10^{-6} M. Incubations of *P. falciparum* 48 hr after exposure to 6.28×10^{-6} M drug resulted in 77% inhibition. Concentrations of 6.28×10^{-7} M and 6.28×10^{-8} M inhibited 69 and 54%, respectively. On the 4th experimental day, 72 hr after exposure to 6.28×10^{-7} M drug, there was 94% inhibition of growth, and, at 6.28×10^{-8} M, a 90% inhibition resulted. This *in vitro* antimalarial effect is significantly greater than the inhibition resulting from either Antabuse or its reduction product,

TABLE 1

Growth of Plasmodium falciparum in 5-methyl-8-quinolinol

Parasites were grown 24 hr in 1.5-ml Petri dishes in a candle jar before exposure to 5-methyl oxine.

	% Parasitemia[a] at		
	2 Days	3 Days	4 Days
5-CH_3 oxine, 100 μg/ml (6.28×10^{-4} M)	1.5 (1.3–1.8)[b]	0.9 (0.4–1.5)[b]	0
DMSO control	2.6 (2.1–3.0)	5.5 (4.9–7.0)	9.6 (8.1–10.4)
5-CH_3 oxine, 10 μg/ml (6.28×10^{-5} M)	1.4 (1.1–1.8)[c]	0.6 (0.4–1.3)[d]	0
DMSO control	2.7 (2.2–3.3)	6.0 (5.0–7.2)	10.0 (9.0–10.9)
5-CH_3 oxine, 1 μg/ml (6.28×10^{-6} M)	1.6 (1.4–2.2)	1.3 (0.9–1.8)[d]	0
DMSO control	2.2 (2.0–2.4)	5.7 (4.7–6.8)	9.0 (7.0–11.2)
5-CH_3 oxine, 0.1 μg/ml (6.28×10^{-7} M)	2.7 (2.1–3.3)	1.6 (0.9–1.9)[c]	0.5 (0.1–0.7)[b]
DMSO control	2.1 (1.9–2.2)	5.1 (4.8–5.4)	8.9 (7.7–10.7)
5-CH_3 oxine, 0.01 μg/ml (6.28×10^{-8} M)	1.9 (1.5–2.8)	2.9 (2.1–4.5)[c]	1.1 (0.4–2.0)[c]
DMSO control	2.9 (2.2–3.5)	6.3 (4.7–9.1)	11.0 (9.3–11.7)

[a] % Parasitemia is expressed as infected erythrocytes per 100 erythrocytes. The initial parasitemia was 0.38% (±0.03% SD). The average and range (in parentheses) are given from counts on four dishes. This is the result of one of four similar experiments.

[b] Essentially all dead.

[c] Few abnormal.

[d] Many abnormal.

TABLE 2

Growth of Plasmodium falciparum in Na(QNO)

Parasites were grown 24 hr in 1.5-ml Petri dishes in a candle jar before exposure to Na(QNO).

	% Parasitemia[a] at		
	2 Days	3 Days	4 Days
Na(QNO), 10 μg/ml (5.02×10^{-5} M)	0.8 (0.8–0.9)[b]	0.5 (0.2–1.0)[c]	0
Na(QNO), 1 μg/ml (5.02×10^{-6} M)	0.7 (0.4–1.4)	0.5 (0.3–0.7)[c]	0
Na(QNO), 0.1 μg/ml (5.02×10^{-7} M)	1.4 (1.2–1.6)	1.7 (1.4–2.0)[c]	0.6 (0.4–0.7)[d]
Na(QNO), 0.01 μg/ml (5.02×10^{-8} M)	1.6 (1.3–2.0)	2.9 (2.6–3.3)	4.9 (4.6–5.2)
Na(QNO), 0.001 μg/ml (5.02×10^{-9} M)	1.8 (1.2–2.3)	3.4 (3.1–3.9)	6.8 (6.3–7.5)
Na(QNO), 0.0001 μg/ml (5.02×10^{-10} M)	1.6 (1.2–2.1)	3.6 (3.2–4.1)	5.6 (5.1–6.3)
Normal control	1.2 (0.8–1.6)	3.5 (2.8–4.2)	6.7 (5.8–7.6)

[a] % Parasitemia is expressed as infected erythrocytes per 100 erythrocytes. The initial parasitemia was 0.16% (±0.02% SD). The average and range (in parentheses) is given from counts on three dishes. This is the result of one of two similar experiments.
[b] Few abnormal.
[c] Many abnormal.
[d] Essentially all dead.

diethyldithiocarbamate (4). Growth rates in DMSO controls were the same as those seen in the absence of DMSO.

The same inoculum was also grown at decreased oxygen tension in a modular incubator chamber (Billups Rothenberg, Del Mar, Calif.). Conditions were identical with those of the previous study, except that O_2 and CO_2 concentrations were 2.58 and 1.74% (±0.02%), respectively, determined by us in an earlier study (1) to be optimal for growth. The results in these microaerophilic studies are in contrast to those seen under the higher oxygen titer of a candle jar. although continuous culti-

TABLE 3

Growth of Plasmodium falciparum in PNO

Parasites were grown 24 hr in 1.5-ml Petri dishes in a candle jar before exposure to PNO.

	% Parasitemia[a] at		
	2 Days	3 Days	4 Days
PNO, 10 μg/ml, (7.86×10^{-5} M)	0.8 (0.5–1.1)[b]	0	0
DMSO control	1.5 (1.3–1.7)	4.1 (3.3–4.6)	7.4 (6.0–8.2)
PNO, 1 μg/ml, (7.86×10^{-6} M)	0.8 (0.6–1.1)[c]	0	0
DMSO control	1.4 (1.3–1.5)	2.5 (1.7–3.3)	6.0 (4.8–6.6)
PNO, 0.1 μg/ml, (7.86×10^{-7} M)	1.2 (0.9–1.6)	0	0
DMSO control	1.2 (1.0–1.5)	2.7 (2.1–3.2)	6.8 (5.9–7.6)
PNO, 0.01 μg/ml, (7.86×10^{-8} M)	1.5 (1.3–1.7)	1.4 (0.9–2.1)[c]	1.4 (1.0–1.7)[d]
DMSO control	1.5 (1.0–2.0)	3.2 (2.4–4.4)	7.0 (6.1–7.8)
PNO, 0.001 μg/ml, (7.86×10^{-9} M)	1.5 (1.0–1.8)	3.7 (3.2–4.3)	6.3 (5.9–7.1)[c]
DMSO control	1.6 (1.4–1.9)	3.9 (3.6–4.4)	7.1 (5.6–8.3)
PNO, 0.0001 μg/ml, (7.86×10^{-10} M)	1.6 (0.9–2.0)	2.7 (2.1–3.2)	5.2 (5.0–5.6)
DMSO control	1.1 (0.6–1.4)	3.7 (2.9–4.5)	6.1 (4.7–7.2)

[a] % Parasitemia is expressed as infected erythrocytes per 100 erythrocytes. The initial parasitemia was 0.16% (±0.02% SD). The average and range (in parentheses) is given from counts on three dishes. This is the result of one of six similar experiments.
[b] Essentially all dead.
[c] Few abnormal.
[d] Many abnormal.

vation can be maintained under either set of conditions. At 6.28×10^{-6} M 5-methyl oxine, inhibition was the same at either oxygen concentration, but at 6.28×10^{-7} M, the toxicity for *P. falciparum* was abolished at the lower oxygen level, growth being equal to the control level in 72 hr. Similar results of decreasing drug toxicity with decreasing oxygen level were observed in three other experiments. This contrasts with observations of the unsubstituted oxine and tetraethyl thiuram disulfide (5), where oxygen tension did not alter sensitivity of the parasite to the chelator. It was noted, in agreement with earlier findings (1), that lower O_2 levels resulted in controls which grew 10–25% better than in the candle jar. Growth effects of 5-nitro oxine at low O_2 versus candle jar are identical with those of 5-methyl oxine. Repetition of these experiments failed to reveal any advantage in antimalarial activity *in vitro* which could be attributed to the 5-nitro group. The quinine equivalent of these compounds ranges from approximately 1.0 to 10.2.

Growth at different concentrations of QNO. PNO has been shown to have potent *in vitro* antiplasmodial activity (5). However, PNO has lower stability constants and lower partition coefficients than oxine (13) and belongs to a different series of compounds. Provided that the stability constants do not change appreciably and that steric hindrance is not introduced, raising the partition coefficient might be expected to improve the antimalarial activity of PNO. These considerations led to the testing of QNO.

Multiplication of *P. falciparum in vitro* in the presence of Na(QNO) at 5.02×10^{-5} to 5.02×10^{-10} M is shown in Table 2. One day after the addition of 5.02×10^{-5} M QNO, the multiplication of parasites was inhibited by 33%. Within 48 hr after addition of 5.02×10^{-5} and 5.02×10^{-6} M QNO, an 86% inhibition resulted, and at 5.02×10^{-7} M a 51% inhibition occurred. On the 4th experimental day (72 hr after drug was added to the system), a 91% inhibition resulted at 5.02×10^{-7} M and a 27% inhibition at 5.02×10^{-8} M. Equimolar concentrations of $Zn(QNO_2)$ resulted in similar rates of growth inhibition.

Under identical conditions and with the same initial inoculum, growth inhibition was determined in PNO (Table 3). In contrast to QNO, there was complete inhibition 48 hr after addition of doses as low as 7.86×10^{-7} M PNO, and 56% inhibition at 7.86×10^{-8} M PNO. The following day, this concentration produced 80% inhibition. This agrees in general with our results reported earlier using PNO (5), although small differences in potency did occur that could be attributed to differences in red cells (13, 14). Albert *et al.* (13, 14) reported that erythrocytes contain a diffusible antagonist to these agents, and these studies used blood obtained from multiple normal donors. These experiments suggest that PNO is slightly more potent than QNO in inhibiting the *in vitro* growth of *P. falciparum*.

DISCUSSION

Years ago, Albert and his co-workers demonstrated that oxine exerts its antifungal and antibacterial action by forming a metal chelate. They showed that the oxine alone can enter the cells without apparent injury to the organism (14, 15). Concentrations of oxine or metal ions, innocuous when added separately, became strongly antibacterial when added together in molar ratios of 1:1 or 2:1 (16). Albert (16) therefore reasoned that a toxic complex of ligand and metal (presumably copper or iron) forms extracellularly and enters the cell. Albert (16) postulated that, once inside the cell, this complex is capable of catalyzing the oxidation of labile —SH groups at the enzyme level, in turn inhibiting metabolism. This is due to chelated metals being chemically more active than the metals themselves. In support of this theory, Albert (16) demonstrated that cobalt, which is capable of blocking these oxidative reactions, is antagonistic to the antimicrobial action of oxine/iron or oxine/copper in a variety of systems (14, 16).

Ligands which grip the metal between two oxygen atoms (instead of between two nitrogen atoms or a nitrogen atom and an oxygen atom) might be expected to have the greatest affinity for calcium or magnesium (both of which are present in the media) and therefore exclude the chelation of a heavier metal (14). In our studies at 72 hr, both agents of this type, 8-hydroxyquinoline-*N*-oxide, with an ED_{50} of 3.10×10^{-5} M, and 2-hydroxypyridine-*N*-oxide, with an ED_{50} of 4.50×10^{-5} M, are markedly less inhibitory than PNO or oxine. This diminished antiplasmodial activity may be due to (a) formation of a ligand/metal complex which does not kill the parasite, (b) a decreased tendency to break down inside the cell to the active 1:1 complex and free chelator, or (c) decreased partition coefficients. We determined the partition coefficient of 8-hydroxyquinoline-*N*-oxide to be 32.3, as compared with 67 for oxine, 1.07 for PNO, and 0.03 for 2-hydroxypyridine-*N*-oxide (13). Therefore, the diminished activity *in vitro* of these agents does not correlate with decreasing partition coefficients. Instead, activity appears to correlate with preference for copper, an ion these agents grip less well. The copper chelators 5-methyl oxine and 5-nitro oxine show potent antimalarial activity *in vitro*, exhibiting an ED_{50} of 6.28×10^{-8} M at 48 hr. This is more active than quinine sulfate, which has an ED_{50} of 6.39×10^{-7} M under identical conditions.[2] Equimolar concentrations of cobalt acetate did not antagonize 6.28×10^{-5} or 6.28×10^{-6} M 5-methyl oxine. Higher concentrations of cobalt were toxic to controls. In addition, equimolar concentrations of iron salts did not potentiate 6.28×10^{-6} or 6.28×10^{-7} M 5-methyl oxine.

A higher oxygen level did, however, potentiate the inhibitory effects of 5-substituted oxines. This contrasts with observations of the unsubstituted oxine and tetraethylthiuram disulfide (5). No significant difference in the drug potency of these two compounds has been noted at relatively low and high oxygen tensions.

In the absence of drug, parasites grow 10–25% better at lower O_2 levels than they do in the candle jar (1). This is presumably due to oxidant stress by the malaria parasite upon the host cells (17) at the higher oxygen level. The parasite itself may generate oxidants in the erythrocyte in the face of a progressive decrease in intracellular catalase activity (18, 19). It is known that the malaria parasite has dihydroorotate dehydrogenase activity (20, 21); this enzyme is known to reductively change oxygen to superoxides and hydrogen peroxide (22, 23).

The increase in antimalarial activity of 5-substituted

[2] L. W. Scheibel and A. Adler, unpublished results.

oxines at the higher oxygen tensions may be caused by inhibition of the activity of enzymes which partially protect the cells from oxidative damage. For example, superoxide dismutase and catalase activities have been reported to be inhibited by chelators of this type (24–27). 5-Substituted oxines may indeed reduce the natural resistance to oxidative damage of the parasite-red cell complex, but this is not the primary antimalarial effect for two reasons. First, this was not observed in the other closely related chelators studied so far. Second, low doses of these compounds inhibit glycolysis before they exhibit these effects (4, 5), inhibiting lactate production some 30% in 6 hr, at a time glucose utilization is still maximal.

It remains to be seen whether or not 5-methyl oxine is an effective antimalarial in human medicine. 5-Methyl oxine has been safely used against cholera (8, 9) and the halogenated oxines, i.e., 7-iodo-5-chloro-oxine (Vioform); 7-iodo-oxine-5-sulfonic acid (chiniofon, Ferron, Yatren); 5:7-di-iodo-oxine (Diodoquin), have been successfully employed in treating amoebic dysentry. It has been suggested, however, that chelation, which is essential for the antibacterial action of oxine drugs, may not be the complete source of efficacy of the halogenated oxines as amoebicides. Their activity appears to be due in part to the weakness of the carbon-iodine bond in position 7 of the oxine nucleus and to the steady evolution of inorganic iodine that ensues (7).

5-Methyl oxine is one of the more mutagenic antimalarials. Its mutagenic potency as measured in forward mutation assays in *Salmonella typhimurium* was nearly 30 times that of chloroquine and nearly twice that of quinacrine.[3] Although the use of 5-methyl oxine represents a potential genetic hazard, it may be possible to reduce the genetic toxicity of this compound by suitable structural alterations or through the use of antimutagenic agents (28, 29). In this connection, it is of considerable interest that a similar antimalarial agent, PNO (5), is not mutagenic.[3] This provides another example for the possibility of dissociating chemotherapeutic from mutagenic effects (29).

Albert (16) has shown that, in general, the antimicrobial activity of these compounds correlates with their partition coefficient. Small changes in molecular structure which raise partition coefficients improve antibacterial action and *vice versa*. Introducing an additional ring to the pyridine nucleus of PNO results in a quinoline nucleus. This would be expected to raise the partition coefficient of the compound and, provided that the 2:3 face is avoided, one might expect QNO to be more antiplasmodial than PNO. Instead, there was a slight reduction in potency, with a 48-hr ED_{50} of 5.02×10^{-7} M for QNO as compared with 7.86×10^{-8} M for PNO. However, antimalarial activity was exhibited in Na(QNO) as low as 5.02×10^{-8} M in 72 hr. Following this line of reasoning in view of the favorable results with oxine, it might be fruitful to add a benzene at the 5:6 or 6:7 face, since substitution adjacent to the hydroxyl group has little detrimental effect on chelating (30) and might facilitate entry of the cell. Addition of an aromatic ring to the 2:3 face of oxine may also result in an effective chemotherapeutic agent based on the favorable partitioning inside the cell of the resulting acridine or phenazine, but this would undoubtedly be antagonized to some degree by steric factors introduced by nitrogen-adjacent substitution (5, 7), which would strain the metal-nitrogen bonds.

In any event, attention to these details of molecular architecture may not only lead to new chemotherapeutic approaches to malaria low in mutagenic potential, but may also shed new light on the metabolism of the parasite. It is of considerable interest that the organisms studied until now have lacked the ability to develop more than minimal resistance to these highly active metal chelates (15). This is an important consideration at a time of world-wide resurgence of malaria and proliferation of drug-resistant strains.

[3] J. Seed, personal communication of unpublished observations.

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