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Antimalarial Activity of Selected Aromatic Chelators

III. 8-Hydroxyquinolines (Oxines) Substituted in Positions 5 and 7, and Oxines Annelated in Position 5,6 by an Aromatic Ring

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SUMMARY

The growth of *Plasmodium falciparum*, a human malaria parasite, has been shown to be inhibited *in vitro* by low concentrations of lipophilic chelators with high metal-binding constants. There is a direct correlation between the inhibition of parasite multiplication and the ability of a given compound to chelate. A high degree of potency is achieved in the case of 8-hydroxyquinoline (oxine). Use of this compound *in vivo* is limited by toxicity, but small substitutions made to this nucleus dramatically lower toxicity to higher animals. 2-Mercaptoquinoline-*N*-oxide, useful in the cosmetics industry; 2-mercaptopyridine-*N*-oxide (i.e., pyrithione), a standard drug in dermatology; 5-methyl oxine, an oral cholera prophylactic; and diethyldithiocarbamate (and tetraethylthiuram disulfide), used in the treatment of alcoholism, exhibit at least as much plasmodiacidal activity as quinine sulfate *in vitro*. It appears that increasing lipophilicity and persistence may increase the utility *in vivo* of these compounds as potential antimalarial agents. Although antiplasmodial activity of the more lipophilic 3-ring chelators is achieved at 1.0×10^{-6} M to 2.5×10^{-7} M within 72 hr (making them equivalent to quinine *in vitro*), other factors must influence this activity. Likewise, substitution of oxine with chloro groups at C-5 or C-5,C-7 also increases lipid solubility. In addition, these changes improve ionization of the phenol and thereby improve chelation, but there is no resulting improvement of antiplasmodial activity from these modifications. A 7-alkyl-substituted, 5-chloro-8-quinolinol, known to exhibit little toxicity for higher animals, also demonstrates plasmodiacidal activity at concentrations as low as 3.1×10^{-7} M, similar to the potency of quinine. This compound also contains additional basic nitrogen atoms in the C-7 alkyl groups. 6-Hydroxy-1,7-phenathroline also contains an extra ring nitrogen. Both changes do not reduce biological activity.

INTRODUCTION

The growing malaria parasite degrades glucose almost quantitatively to lactate over 16 hr (1, 2). There is no evidence of a classical Pasteur effect in either the primate malaria parasite, *Plasmodium knowlesi* (3), or the human parasite, *P. falciparum*.[1] In addition, there is no evidence of the existence of a tricarboxylic acid cycle in *P. knowlesi* (3, 4), and optimal growth in *P. falciparum* occurs *in vitro* at 3% O_2 with good growth resulting at 0.5% O_2 but not below (5). This would suggest that oxygen does not mediate to any significant degree an ATP-generating electron transport system, but instead participates in biosynthetic reactions through metalloprotein oxidases. These would presumably be oxygen transferases and mixed-function oxidases. This is a significant departure from the metabolism of mammalian host cells.

The rationale of our approach in choosing compounds selectively toxic to the intracellular malaria parasite involves lipophilic chelators known to inhibit these oxidase enzymes in cell-free systems. Permanent inhibition is achieved by utilizing agents with high metal-binding constants. Tetraethylthiuram disulfide (Antabuse) and its reduction product, diethyldithiocarbamate, are agents of this type. They inhibit *in vitro* growth of the human malaria parasite, *P. falciparum* (1) at levels below those reported as peak serum levels in patients receiving this drug (6).

Antabuse also inhibits glycolysis in parasitized red cells with no effect on normal red cells. Unfortunately, these relatively nontoxic compounds sensitize the recipient to

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

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alcohol and have serum levels of relatively short duration. This severely limits their usefulness as potential antimalarial agents. Turning from the alkylthiocarbamates to aromatic chelators, we demonstrated that 2-mercaptopyridine-*N*-oxide and 2-mercaptoquinoline-*N*-oxide are as potent as quinine at inhibiting the *in vitro* growth of malaria (7). Both of these agents are used by the cosmetics industry (8) and exhibit low mammalian toxicity.

Oxine,[2] a lipophilic, aromatic chelator with high metal-binding constants, is also known to inhibit a variety of metalloprotein oxidase enzymes. It rapidly inhibits growth and glycolysis of parasitized red cells, and these effects correlate directly with chelation (2). Unfortunately, unsubstituted oxine has been shown to be diabetogenic *in vivo*, but minor substitutions to the oxine nucleus result in compunds with low toxicity to animals. 5-Methyl oxine has been safely used in humans for the control of cholera. In addition, our studies have demonstrated it to be more plasmodiacidal than quinine *in vitro* (7). However, literature reports have suggested that compounds similar to 5-methyl or 5-nitro oxine might exhibit poor activity *in vivo* resulting from rapid excretion or degradation (9, 10). In an attempt to develop an agent rapidly absorbed through membranes and slow to be excreted *in vivo*, consideration was given to choosing substituted derivatives with especially high lipid:water partition coefficients (11). In addition, some time ago, Albert and co-workers (12, 13) demonstrated that antibacterial effects rise and fall directly with the increase and decrease of the partition coefficient. Therefore, several substituted oxines were investigated to delineate structural characteristics which might contribute to prolonged activity in the host, maximal inhibition to the malaria parasite, and minimal toxicity to higher animals.

METHODS

P. falciparum strain FCR-3/Gambia, Subline D was grown in a candle jar exactly as described in our previous studies (1, 2, 7). 5,6-Benzo oxine, 6-HP, and 5-chloro oxine (Table 1) were dissolved in Me_2SO, certified spectroanalyzed (Fisher Scientific Company, Pittsburgh, Pa.), to give a concentration of 80 mg/ml. A portion of this solution (50 μl) was added to 40 ml of medium. 5:7-Dichloro oxine (Table 1) was dissolved in Me_2SO to give a concentration of 40 mg/ml. A portion (100 μl) of this solution was added to 40 ml of medium. Controls contained equal quantities of Me_2SO only. KAN-322 (Table 1) was dissolved directly in 40 ml of medium and determined to be neutral. All experiments were accompanied by a normal medium control without Me_2SO. Stock solutions were sterilized by filtration and appropriately diluted with sterile medium to achieve desired concentrations of drug. 5,6-Benzo oxine, 6-HP, 5-chloro oxine, and 5:7-dichloro oxine were obtained as gifts from Dr. Adrien Albert (Canberra, Australia). KAN-322 was obtained from Dr. Leslie M. Werbel, Warner Lambert Company (Ann Arbor, Mich.). 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 dose of quinine to the test drug required to achieve the same inhibition. The dose required to reduce parasitemia to 50% of the control level is defined as ED_{50}. These ED_{50} values were determined graphically on semilog paper from drug concentrations and corresponding inhibitions. Parasites were grown for 24 hr (1 day) in 1.5-ml Petri dishes in a candle jar before exposure to 8-quinolinol. The ED_{50} on the 2nd experimental day corresponds to inhibition following 1 day of contact with "drug"; inhibition on day 4 of study results from 3 contact days of "drug."

[2] The abbreviations used are: oxine, 8-hydroxyquinoline; 6-HP, 6-hydroxy-1,7-phenathroline (6-hydroxy-*m*-phenanthroline); Me_2SO, dimethyl sulfoxide; KAN-322, 5-chloro-7-(3-diethylaminopropylaminomethyl)-8-quinolinol dihydrochloride.

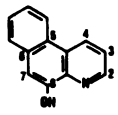
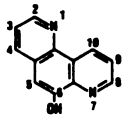
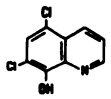
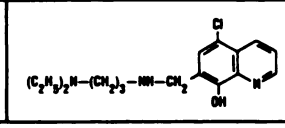
RESULTS

Growth at different concentrations of 5,6-benzo oxine and 6-HP. Introducing a benzene at the 5:6 face of oxine to produce 5,6-benzo oxine (Table 1) increases the oleyl alcohol:water partition coefficient from 67, for unsubstituted oxine, to 234 for 5,6-benzo oxine (14). 6-HP (Table 1) (15) is a similar compound with an additional heterocyclic nitrogen atom. Typical growth effects of *P. falciparum* in 5,6-benzo oxine and 6-HP are shown in Table 1. There was statistically significant inhibition of growth as early as 24 hrs after the addition of 5×10^{-4}, 5×10^{-5}, and 5×10^{-6} M 5,6-benzo oxine and 6-HP, as evidenced by Student's *t*-test at the 0.05 level. This suggests a rapid

TABLE 1

8-Quinolinols

Concentration required to reduce *in vitro* growth of *Plasmodium falciparum* 50% (ED_{50}) after exposure for 1 day and 3 days.

No.	Structure	1 Day	3 Days
I[a]		1.8×10^{-4}M	1.0×10^{-6}M
II[b]		5.0×10^{-4}M	2.5×10^{-7}M
III[c]		2.8×10^{-5}M	9.7×10^{-7}M
IV[d]		3.4×10^{-5}M	4.7×10^{-6}M
V[e]		1.5×10^{-6}M	3.1×10^{-7}M

[a] 5,6-Benzo oxine.
[b] 6-HP.
[c] 5-Chloro oxine.
[d] 5:7-Dichloro oxine.
[e] KAN-322.

onset of inhibition by these compounds. Within 48 hr of contact with the test substance, 5.1×10^{-7} M concentrations of 5,6-benzo oxine inhibited growth by 40%. Concentrations of 5.0×10^{-7} M 6-HP resulted in approximately 89% inhibition. Incubations of *P. falciparum* for 72 hr in 5.1×10^{-7} M 5,6-benzo oxine resulted in 38% inhibition, and 5.0×10^{-7} M 6-HP resulted in 88% inhibition. Growth rates in Me_2SO controls were the same as those seen in the absence of Me_2SO. The growth inhibition of 5,6-benzo oxine closely approximates that resulting from quinine sulfate *in vitro*,[1] resulting in a quinine equivalent of 1.

Growth at different concentrations of 5-chloro oxine and 5:7-dichloro oxine. Introducing chloro-substituents to oxine increases the partition coefficient from 67 to 294 in the case of 5:7-dichloro oxine (14). Also, chlorine exhibits a relatively strong negatively inductive effect, attracting electrons more strongly than hydrogen (16). Growth studies of *P. falciparum in vitro* were conducted at concentrations of 5.6×10^{-5} to 5.6×10^{-8} M 5-chloro oxine and 4.7×10^{-5} to 4.7×10^{-7} M 5:7 dichloro oxine. The onset of action was relatively slow, as inhibition within 24 hr in 5-chloro oxine and 5:7-dichloro oxine occurred only at 5.6×10^{-5} and 4.7×10^{-5} M concentrations of the mono- and dichloro oxines, respectively. Within 48 hr of contact with the chelator (3rd day of experiment), 5.6×10^{-6} M concentrations of 5-chloro oxine completely inhibited growth (as all parasites appeared to be dead). Death in all instances was assessed by failure to grow in subculture following the repeated washing of parasites in fresh medium. In contrast, there was no statistically significant difference between growth in 4.7×10^{-6} M 5:7-dichloro oxine and its control. Following growth for 72 hr in 5.6×10^{-7} M 5-chloro oxine, there was a 25% inhibition in multiplication, in contrast to that seen with 4.7×10^{-7} M 5:7-dichloro oxine, which resulted in no inhibition to parasite development. However, there was a 50% inhibition by 4.7×10^{-6} M 5:7-dichloro oxine, less than that observed in 5,6-benzo oxine.

Growth at different concentrations of KAN-322. KAN-322 is an alkyl-substituted oxine chelator with an aliphatic group at C-7 optimized for maximal amebicidal activity *in vitro* and *in vivo* (17). The mechanisms of action for KAN-322, as well as that of other compounds studied, results from chelation.

Typical growth effects of KAN-322 on *P. falciparum* are shown in Table 1. There was significant growth inhibition 24 hr after the addition of 2.5×10^{-6} M drug, suggesting a rapid onset of action. This compares favorably with 5,6-benzo oxine and 6-HP. After 48 hr of contact with the drug, there was 32% inhibition in growth at a dose of 2.5×10^{-7} M, and a 91% inhibition at 2.5×10^{-6} M. On the 4th day of the experiment (72-hr contact with the drug), there was a 39% inhibition at 2.5×10^{-7} M, approximating the inhibition seen by equal *in vitro* concentrations of quinine.

DISCUSSION

Scheibel *et al.* (1, 2, 7) have demonstrated a marked sensitivity of the human malaria parasite to a variety of chelators with high lipid:water partition coefficients and metal-binding constants. Chelators of this type are known to participate in at least three different types of interactions with enzymes, all of which are potentially important to living systems. These are (a) interaction with sulfhydryl groups, (b) interaction with amino groups, and (c) interaction with certain metal atoms of enzymes (18). In addition, chelators used in this study have been shown to be inhibitory to many metalloprotein oxidase enzymes in cell-free systems (19, 20), lending further support to the proposed mechanism of action against the malaria parasite.

The antimalarial effects on growth of the parasite appear to correlate with the ability of these compounds to chelate metals (2). The metal-binding effect in this class of heterocyclic compounds depends on a hydroxyl group so situated that it can form a 5-membered ring that includes the ring-nitrogen atom and the cation of a metal (15). Of the seven isomeric monohydroxyquinolines, only the oxine is capable of forming complexes with divalent metallic ions through chelation (21), and both 5-hydroxyquinoline (2) and 2,3-dihydroxyquinoxaline[1] are inactive at inhibiting growth of malaria. Both 5,6-benzo oxine and 6-HP have the optimal arrangement of hydroxyl- to ring-nitrogen for chelation to occur. In addition, there are no bulky groups adjacent to the ring-nitrogen which would strain the metal-nitrogen bonds and diminish antimalarial activity (2, 15). Substitution adjacent to the hydroxyl group has little detrimental effect on chelating (22). Therefore, one would expect 5,6-benzo oxine and 6-HP, with an additional aromatic ring as compared with oxine, to be more lipophilic and therefore more plasmodiacidal. Surprisingly, this was not the case. Instead, 5,6-benzo oxine and 6-HP were less active than oxine, which approaches a 72-hr ED_{50} of 6.9×10^{-9} M in a large number of experiments (2). This was most evident by the last day of the experiment.

In general, these results agree with those of 2-mercaptopyridine-*N*-oxide compared with 2-mercaptoquinoline-*N*-oxide (7). The addition of the extra aromatic ring does not endow the compound with more antimalarial potency and, in fact, decreases it slightly. This may indicate that there is steric hindrance to the bigger molecules on the plasmodial enzyme or receptor site, in contrast to what has been reported in bacterial systems, or would at least suggest that other factors contribute to the antimalarial activity of these compounds which are not observed in bacterial systems. Therefore, we undertook further studies to delineate structural characteristics of the drug which might contribute to the chemotherapeutic activity of these agents.

The halogenated oxines, i.e., 7-iodo-5-chloro-oxine (Vioform, Entero-Vioform), 7-iodo-oxine-5-sulfonic acid (chinofon, Ferron, Yatren) and 5:7-diiodo-oxine (Diodoquin), have been used in human medicine for years, especially for the treatment of amebic dysentery. However, their activity appears to be due in part to the weakness of the carbon-iodine bond in the position 7 of the oxine nucleus and to the steady evolution of inorganic iodine that ensues (15). Several studies have demonstrated that plasma protein-bound iodine is increased after treatment with iodochlorhydroxyquin (23). Without a doubt, a significant portion of the ingested drug is absorbed, and steady-state plasma levels of at least 5 μg/ml can be maintained (24, 25). This serum level is many times higher than the *in vitro* concentration of noniodi-

nated oxines, mercapto-*N*-oxides, or alkyl thiocarbamates used in our studies to achieve plasmodiacidal effects (1, 2, 7). Unfortunately, however, chronic use of iodochlorhydroxyquin has been linked to subacute myelo-optic neuropathy (24, 26). Therefore, to avoid these potentially serious side effects, we directed our attention to noniodinated analogues of oxine, which act principally through chelation.

The addition of an electron-withdrawing nitro or chloro group in position C-5 of oxine might be expected to increase the ionization of the phenolic group and improve chelation. Our previous studies showed no such improvement in plasmodiacidal activity of 5-nitro oxine over that of 5-methyl oxine (7). It can be argued that the nitro group is too rapidly changed in the biological system to endow it with any advantage. Therefore, our attention was directed to chloro-substituted oxines, which would have the additional advantage of increased lipophilicity. The presence of electron-withdrawing groups in either the *o*- or *p*-position from the hydroxyl would be expected to increase the acidity of the phenol groups more than substituents added to the *m* position. The effects of di-substitution would be expected to be cumulative (27).

Our results suggest that neither 5-chloro oxine nor 5:7-dichloro oxine exceeds the activity of unsubstituted oxine or 5-nitro oxine against *P. falciparum in vitro*, and the *o-p* di-substituted form is slightly less active than the *p*-substituted form (5-chloro oxine). Apparently, little can be gained in activity (*in vitro*) by increasing the acidity of the 8-hydroxy group under these conditions. However, drug localization within the parasite or persistence in the host based on increased lipophilicity may be improved by these changes.

Maximal lipid solubility alone does not in itself determine the utility of the agent since the presence of an extra polar, heterocyclic ring-nitrogen at position 1 of 6-HP does not reduce the plasmodiacidal potency to any extent. Instead, activity appears to exceed slightly that observed with either the mono- or dichloro oxines or 5,6-benzo oxine. In fact, even though 5:7-dichloro oxine has a partition coefficient of 294, a value 20% higher than that of 5,6-benzo oxine, it is less plasmodiacidal than the aromatic-substituted forms. This contrasts somewhat with results seen in other microorganisms. Some time ago, Albert *et al.* (14, 15) demonstrated that antibacterial effects rise and fall directly with the increase and decrease of the partition coefficient. They presented evidence that an increase in partition coefficient resulted in almost a commensurate increase in bacteriostasis; conversely, as lipophilic properties are progressively lowered by the insertion of extra heterocyclic nitrogen atoms into oxine, bacterostasis decreases (12, 13). On the other hand, the addition of a side chain of only three carbon atoms restores the partition coefficient to the same value it was before the extra ring-nitrogen was inserted.

Therefore, combining the characteristics which would be well-tolerated by the host but yet maximally active and persistent, a compound with a lipophilic halogen at C-5 and an alkyl group at C-7, preferably with at least one basic group, was chosen for evaluation.

KAN-322 contains these structural prerequisites. In addition, it has been found to be active *in vitro* against *Entamoeba histolytica* and is one of the most potent of a series of analoques tested against experimental intestinal amebiasis in rats and dogs (10, 17). It is well-tolerated in higher animals, presumably acting through chelation instead of through the liberation of iodine as do the more traditional quinolinol amebicides. It has been reported that increased systemic activity of this compound also is favored by the long side-chain with additional amino grouping as well as the halogen at C-5 (10, 17, 28). In addition to proving to be one of the most effective luminal amebicides, it was as active as chloroquine when administered orally against hepatic amebiasis in hamsters. It remains to be seen whether or not this *in vivo* activity can be also directed against the primate malaria parasite. In our studies, KAN-322 approximates the activity of 5,6-benzo oxine against *P. falciparum in vitro*, giving it a quinine equivalent of 1.

In any event, studies to elucidate the factors required to aid in the delivery of the chelator to the primary site of action within the parasite and exclude those known to potentiate systemic toxicity appears to be the most rational approach to take (29) in the development of these compounds as possible antimalarials. Although the parent compound, oxine, is diabetogenic to animals (30), substituents may be added to it to lower dramatically mammalian toxicity without eliminating antiplasmodial activity (7). It should be emphasized that even though chloroquine has a quinoline nucleus there is no appreciable decrease in sensitivity by the chloroquine-resistant FVO strain *in vitro*[1] to either oxine or the 5-substituted oxines. Therefore, this targeted approach in a well-controlled system may allow us to exploit the idosyncrasies peculiar to the parasite, but still remain within host tolerance.

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