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ANTIMALARIAL ACTIVITY OF DIETHYLDITHIOCARBAMATE

POTENTIATION BY COPPER

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Abstract—The antimalarial activity of diethyldithiocarbamate (DDC) *in vitro* was potentiated by subtoxic concentrations of copper. DDC was also more potent in the presence of an intracellular source of copper, such as when parasites were grown in superoxide dismutase (SOD)-loaded erythrocytes. These data suggest that DDC forms a complex with copper, either intracellularly or extracellularly, which is toxic to malarial parasites. The exact cause of this toxicity is not known, but may be due to a membrane effect, since DDC and copper, in combination, exert a potent lytic effect on normal human erythrocytes.

Malaria, caused by intraerythrocytic protozoans of the genus *Plasmodium*, affects 800 million people per year [1]. This disease is an increasingly serious public health problem throughout the world, in part because drug-resistant strains of *P. falciparum*, the most lethal of the human malarias, are now quite prevalent. Thus, new antimalarial agents are greatly needed [2].

A number of chelating agents have been shown to exhibit potent antimalarial activity [3–8]. Chelators appear to exert their antimalarial effect by one of two different mechanisms. Some of these agents act by depriving the parasite of essential metal(s) [8]. Others, in contrast, appear to form toxic chelator–ligand complexes [9].

The antimalarial activity of the copper chelator diethyldithiocarbamate (DDC) was first noted by Scheibel and collaborators [4]. The potential usefulness of DDC as an antimalarial agent is suggested by the fact that disulfuram (Antabuse), a dimer of DDC, is used in the treatment of alcoholism, and is known to form DDC after administration to humans [10]. DDC is also a potent inhibitor of the copper,zinc-containing superoxide dismutase (SOD) [11]. Previously, we found that DDC-treated SOD was highly toxic to malarial parasites *in vitro* [12]. This effect may have been due to the formation of a DDC–enzyme complex [13] which slowly released a DDC–copper chelate.

Scheibel *et al.* [4] postulated that copper might potentiate the antimalarial activity of DDC. This suggestion was made on the basis of earlier studies of the antifungal activity of the closely related compound, dimethyldithiocarbamate. The antifungal activity of this agent was found to be potentiated by copper, particularly in a ratio of 2:1 (drug:metal) [10]. Other agents, such as 8-hydroxyquinoline (oxine), also have enhanced antibacterial and antifungal activity in the presence of copper or iron [10]. However, no one has yet determined experimentally whether there is indeed synergism between DDC and copper.

We have now demonstrated that the antimalarial activity of DDC is strongly potentiated by exogenous copper ions. In addition, an intraerythrocytic source of copper, such as the copper,zinc-containing SOD, also potentiates the antimalarial activity of DDC.

MATERIALS AND METHODS

Parasite cultivation and [^{3}H]*hypoxanthine incorporation. P. falciparum* was cultured by the method of Trager and Jensen [14]. The FCR3 strain was used for all experiments. To determine parasite viability, [^{3}H]hypoxanthine incorporation was used in a modification of the procedure of Desjardins *et al.* [15]. Starting parasitemias of 0.5 to 1.0% were prepared by diluting stock cultures with freshly washed blood. For experiments using resealed red cells, parasitized red cells were first gelatin-concentrated [16] to parasitemias of >25% and diluted to parasitemias of 0.5 to 1%. Infected red cells and various concentrations of drugs were aliquoted in triplicate wells of a 96-well microtiter plate. Each well contained 200 μL of RPMI 1640 medium (GIBCO, Grand Island, NY) and a final hematocrit of 5%.

The microtiter plates were incubated at 37° in candle jars. After 24 hr, 0.5 μCi of [^{3}H]hypoxanthine (Amersham, Arlington Heights, IL) was added to each well. After an additional 24 hr, the parasites were harvested using a PHD cell harvester (Cambridge Technologies, Cambridge, MA). Filters were immersed in Aquasol-2 (New England Nuclear, Boston, MA) and counted using a LKB Rackbeta scintillation counter.

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Preparation of SOD-loaded erythrocytes. Normal A− or A+ red blood cells (RBC) were obtained, following informed consent, from laboratory volunteers. Cells were washed three times in saline prior to red cell loading. Red cell loading was done by the method of Scott *et al.* [17]. Briefly, washed, packed RBC and bovine erythrocyte SOD (Sigma Chemical Co., St. Louis, MO) were mixed and sealed in dialysis tubing (mol. wt cutoff of 3500 daltons). A high surface to volume ratio was maintained to ensure ready lysis and resealing. The samples were lysed by dialysis against 5 mM potassium phosphate buffer (pH 7.4), containing 2 mM EDTA (lysing buffer), at 4° for 60 min. The dialysis tubing, containing lysed red cells, was transferred to a resealing buffer containing 0.16 M NaCl, 5 mM glucose and 5 mM potassium phosphate buffer (pH 7.4) and resealed for 30 min at 37°. Following resealing, cells were washed with saline until the supernatant fraction was clear (5–7 times). Resealed red cells were infected with malarial parasites within 24 hr of preparation. In all cases, parasites grown in resealed ghosts incorporated [^{3}H]hypoxanthine as well as or better than parasites grown in unlysed control cells. SOD was assayed by the method of McCord and Fridovich [18].

Lytic effects on red blood cells. Normal human blood was drawn in heparinized tubes and washed 3 times in isotonic Tris buffer (172 mM, pH 7.4). The washed erythrocytes were suspended to a hematocrit of 5% in the above buffer containing 50 μM DDC. Following a 5-min period of equilibration, various concentrations of copper sulfate were added slowly as the suspension was vortexed.

The cells were incubated at room temperature for 60 min. The suspensions were then gently mixed once again and spun at 1500 *g* for 5 min. The hemoglobin content of the supernatant fraction was estimated by measurement of the optical density at 542 nm. The value for 100% lysis was established by adding 100 μL of erythrocytes to 1.9 mL of water and incubating for 1 hr. Each point was determined in duplicate.

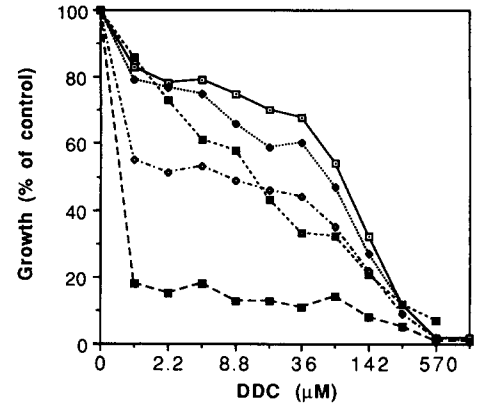

Fig. 1. Concentration–response curve for DDC in the presence of various concentrations of copper sulfate. Shown is the incorporation of [^{3}H]hypoxanthine as percent of control (absence of DDC, 10,000–60,000 cpm) for parasites grown in the presence of 0 μM (—⊡—), 0.2 μM (···◆···), 0.4 μM (---□---), 0.8 μM (-·-·◇·-·-), and 1.2 μM (——■——) copper sulfate.

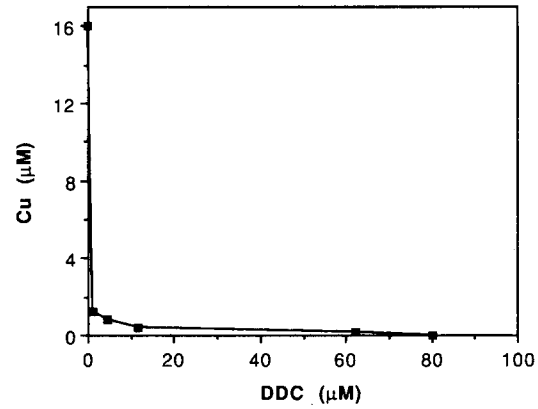

Fig. 2. Isobologram of EC_{50} values of various DDC and copper sulfate concentrations.

RESULTS

DDC and copper sulfate exhibited marked synergy in their *in vitro* antimalarial activities. The EC_{50} values of DDC alone and of copper sulfate alone were 105 and 16 μM respectively. In contrast, low concentrations of DDC had markedly enhanced antiparasitic activities when copper sulfate was present at concentrations ranging from 0.2 to 1.2 μM (Fig. 1). In the presence of 0.2, 0.4, 0.8 and 1.2 μM copper sulfate, the EC_{50} value of DDC was lowered to approximately 80, 15, 6 and 0.8 μM respectively. These concentrations of copper, which are only 2–8% of the copper sulfate EC_{50}, were not toxic to the parasites in the absence of DDC. This synergism between copper and DDC is particularly apparent when the EC_{50} values of DDC at various copper concentrations are plotted on an isobologram, yielding a concave-upward curve (Fig. 2).

To determine whether DDC has enhanced antimalarial activity in the presence of an intraerythrocytic source of copper, red cells were lysed and resealed with elevated amounts of copper,zinc-containing SOD. SOD-loaded red cells contained 17,300 ± 1,500 units/g Hb compared to lysed-resealed controls which contained 3,000 ± 100 units/g Hb. When SOD-loaded and control resealed cells were infected with *P. falciparum*, [^{3}H]hypoxanthine incorporations were 18,418 ± 3,843 and 22,422 ± 2,452 cpm respectively. Thus, SOD loading did not significantly affect parasite viability ($P > 0.10$).

The EC_{50} of DDC was found to be quite low when tested against parasites cultured in resealed control erythrocytes. When parasites were grown in control resealed RBCs, the EC_{50} of DDC was 6 μM, which is only about 5% of its EC_{50} against parasites grown in control unlysed cells.

Parasites were markedly more sensitive to DDC when grown in SOD-loaded red cells than when

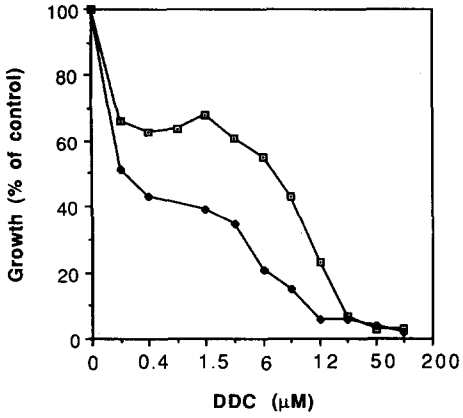

Fig. 3. Concentration–response curve for DDC against parasites grown in SOD-loaded (—◆—), and control lysed-resealed (—⊡—) red cells. Data are expressed as percent of control incorporation of [^{3}H]hypoxanthine (absence of DDC, 18,000–22,000 cpm).

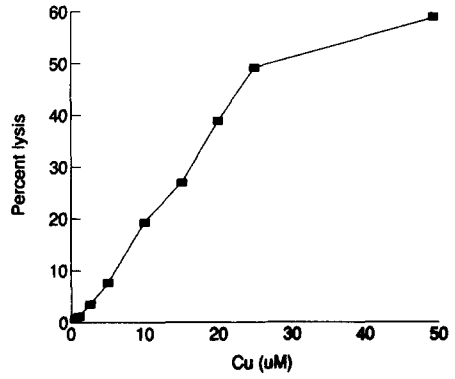

Fig. 4. Lysis of uninfected erythrocytes in the presence of 50 μM DDC and various concentrations of copper sulfate.

grown in control lysed-resealed red cells (Fig. 3). The EC_{50} of DDC in SOD-loaded red cells was 0.2 μM, which is only about 3% of the EC_{50} of DDC against parasites grown in control resealed cells.

To determine the mechanism of action of the DDC–copper chelate, the effects of the chelate on uninfected red cells were studied. DDC alone was not lytic for red cells nor was copper sulfate. However, lysis of up to 60% of cells occurred in the presence of both DDC and copper sulfate. The degree of lysis was roughly proportional to the amount of copper added (Fig. 4). In the absence of DDC, no lysis was caused by concentrations of copper as high as 100 μM.

DISCUSSION

In this paper, we report that the antimalarial activity of DDC was potentiated by both exogenous and endogenous copper. Furthermore, the DDC–copper complex was markedly lytic for uninfected red cells, suggesting that it may be toxic to membranes. These results raise the possibility that the antimalarial effects of DDC, even when used in the absence of added copper, may be mediated by the formation of a drug–metal complex within the infected erythrocyte.

The antimalarial activity of DDC was first reported by Scheibel and collaborators [4]. Extrapolating from the earlier studies of Alberts [9] on the antimicrobial activity of oxine, Scheibel postulated that the DDC–metal complex forms extracellularly, and, by virtue of its hydrophobicity, penetrates the erythrocyte membrane. Once in the membrane, the dissociation of the complex was thought to occur, leading to the release of DDC and the subsequent inhibition of a copper-dependent enzyme.

Our findings demonstrate that the antimalarial activity of DDC, as postulated, is indeed potentiated by extracellular copper. This synergism explains our earlier observation that the DDC-treated copper,zinc-containing SOD was toxic to malarial parasites. DDC, a potent inhibitor of this enzyme, forms a tight complex at its copper-containing active site [11]. The antimalarial activity of the inhibitor–enzyme complex may have been due to a gradual release of the DDC–copper chelate from the enzyme active site.

The synergy of DDC and copper does not appear to be due to copper enhancement of DDC uptake, since intracellular copper, such as that found in intra-erythrocytic copper,zinc-SOD, also augments the antimalarial activity of DDC. Further evidence for the ability of DDC to penetrate into red cells in the absence of copper can be found in the ability of exogenous DDC to inhibit SOD activity in intact erythrocytes [19]. If DDC does not have to form a complex with copper in order to penetrate the red cell, then how does copper potentiate DDC?

The lytic effects of the DDC–copper complex on uninfected erythrocytes suggests that the complex is directly toxic to membranes. This effect is consistent with the known solubility properties of the complex: whereas both copper sulfate and DDC are hydrophilic, the DDC–copper complex is highly hydrophobic [9]. The membrane toxicity of the DDC–copper chelate may also mediate its antimalarial activity. Since the antimalarial effect manifests at concentrations 10–100 lower than the hemolytic effect, the complex probably does not directly lyse parasitized red cells but may inhibit important membrane function(s). However, the mechanism by which the DDC–copper complex induces membrane damage is still unknown.

When SOD-loaded red cells are treated with DDC, the toxic DDC–copper complex may form intracellularly and then insert into a cell membrane. Accordingly, the antimalarial effect of DDC in the absence of added copper could be due to the chelation of copper from the endogenous erythrocytic SOD which is present both in the host cytoplasm and the parasite lysosomes [19–22]. Indeed, the possible accumulation, within lysed and resealed red cells, of exogenous metal may help explain why they were more sensitive to the antimalarial effect of DDC than parasites grown in normal erythrocytes (even though they were much less sensitive than parasites

grown in SOD-loaded lysed-resealed cells). Alternatively, the process of lysing and resealing may have resulted in membranes with enhanced fragility and susceptibility to damage by the DDC–copper chelate.

In summary, DDC appears to exert its antimalarial action by complexing with either extracellular or intracellular copper and then disrupting membranes. DDC, with its unique mode of action, might serve as a prototype for the development of new antimalarial agents.

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