Tetraethylthiuram disulfide (Antabuse) inhibits the human malaria parasite Plasmodium falciparum
(malaria/in vitro culture/disulfiram)

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Contributed by William Trager, May 30, 1979

ABSTRACT Plasmodium falciparum in culture grows optimally at 3% oxygen. Oxygen levels down to 0.5% still support growth, but anaerobic conditions do not. These findings, and the absence of the Krebs cycle in Plasmodium, suggested that in this organism oxygen may not function in electron transport but rather may act through metalloprotein oxygenases. Tetraethylthiuram disulfide (Antabuse, disulfiram) and its reduction product diethylidithiocarbamate inhibit many metalloprotein oxygenases and have a lipid/H2O partition coefficient and high binding constant for metal ions, favoring selective toxicity to the malaria parasite. These compounds exhibited active antimalarial effects in vitro in concentrations down to 0.1 µg/ml, the lowest level tested. Tetraethylthiuram disulfide at a level as low as 1 µg/ml inhibited parasite glycolysis with no effect on glycolysis of normal erythrocytes. Erythrocytes pretreated with this drug at 10 µg/ml did not support growth of the parasite.

Plasmodium falciparum, when cultured in vitro (1), has been shown to be a microaerophile, growing best at 3% O2 but tolerating O2 levels as low as 0.5% without appreciable reduction in multiplication rates (2). However, there does appear to be a critical level of O2 below which the parasite will not survive, in contrast to true anaerobic forms of life. It has been known for some time that the malaria parasite does not use the Krebs cycle to degrade its glucose completely to CO2 and H2O but instead excretes organic end products (3, 4). This suggests that the parasite may depend on reactions involving oxygenases and hydroxylases in which O2 is a chemical reactant (5). These oxidations are catalyzed by oxidase enzymes or enzyme systems that activate the O2 and oxidize the substrate. Oxidases are often metalloproteins, containing metals such as iron, copper, or molybdenum, and are present in microorganisms, plants, and animals (6). Therefore, we felt, first, that a chelating agent with activity against such metalloproteins might have selective toxicity to malaria parasites without interference with the cellular metabolism of the host tissues. One type of agent emerges from the rest with high probability of achieving this objective, the dialkylidithiocarbamates and their dimers, the thiamur disulfides. Diethylidithiocarbamate (DDC) is a chelating agent with proven inhibitory activity against many of these enzymes (7). There is no sharp distinction among the alkyl dithiocarbamoyl compounds, and they can be transformed into each other in nutrient solutions (8). Dimethylidithiocarbamate is strongly fungitoxic, particularly to fungi with obligatory aerobic metabolism. Fortunately, higher plants and vertebrate animal possessing an obligatory aerobic type of metabolism show little or no response to these substances (7, 9, 10). The diethyl derivative (Antabuse, disulfiram) is even less toxic to mammals (11), and, in clinical trials, single doses of up to 6 g and daily doses of 0.25–0.60 g for several months produced no effects apart from those following ingestion of alcohol. Therefore it has received attention in aversion therapy in the treatment of alcoholics (12), as well as heavy metal poisoning (7). Second, these compounds exhibit lipid/H2O partition coefficients favoring penetration (7). This is an important consideration because the compound must penetrate the erythrocyte membrane, equilibrate with the erythrocyte cytoplasm, then traverse the parasitophorous vacuole membrane and space, and finally penetrate the limiting membranes of the intracellular parasite and possibly even subcellular organelles. Increasing the hydrocarbon chain of the alkyl groups or introducing aryl groups increases the lipophilic characteristics of the drug molecule but unfortunately decreases activity, probably due to steric hindrance (9). Last, these compounds have favorable binding constants enabling them to compete effectively with naturally occurring chelators in the cell for metal ions (13–15). Therefore, on the basis of these favorable parameters one would expect DDC and its dimeric oxidation product tetraethylthiuram disulfide (TETD) to exhibit highly selective inhibitory activity toward the malaria parasite P. falciparum.

MATERIALS AND METHODS

P. falciparum, strain FCR3/FGM (Gambian strain) (16) was grown in vitro in 8% erythrocyte suspensions by using the petri dish–candle jar method as described by Jensen and Trager (17). Type A erythrocytes and sera were used throughout the study. Four petri dishes of parasites were grown at each drug concentration unless otherwise specified. The media and parasitized erythrocytes were dispensed into petri dishes and parasites were allowed to multiply in the absence of drug for 24 hr. Smears were made of all cultures daily and stained with Giemsa's stain. A minimum of 1000 erythrocytes were counted on each slide and growth was expressed each day in percentage erythrocytes infected with P. falciparum. Initial parasitemia was determined by counting parasites in 40,000 erythrocytes. TETD was dissolved in dimethyl sulfoxide (Me2SO), certified Spectranalyzed (Fishier), to give a concentration of 80 µg/ml. Fifty microliters of this solution was added to 40 ml of medium. Controls contained equal quantities of Me2SO only. DDC, sodium salt, was dissolved directly in 40 ml of medium. Stock solutions were sterilized by filtration and appropriately diluted with sterile medium to achieve desired concentrations of drug. TETD and DDC were recrystallized reagents.

For the determination of glucose, lactate, pyruvate, and protein, perchloric acid was added to the cultures to a final concentration of 3%. The protein was removed by centrifugation at 27,000 X g for 15 min and was determined in the pellet by the procedure of Lowry et al. (18). The supernatant was

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neutralized with KOH. Lactate was quantified enzymatically by a modification of the method of Lowry et al. (19). Lactate dehydrogenase (Worthington) used in this assay was diluted with 0.9% NaCl rather than albumin and the buffer used was 2-amino-4-methyl-1-propanol, pH 9.7. Pyruvate was determined according to the method of Bücher et al. (20) except for the substitution of 0.5 M glycylglycine buffer in place of 0.4 M triethanolamine buffer. Glucose was assayed spectrophotometrically at 340 nm by a modified method of Stein (21). The reaction mixture contained 40 μmol of glycylglycine buffer (pH 7.4), 0.8 μmol of MgCl₂, 3.75 μmol of ATP and 0.3 μmol of NADP⁺, 0.56 unit of hexokinase and 0.28 unit of glucose-6-phosphate dehydrogenase (Boehringer and Soehne) in 0.05 M glycylglycine buffer (pH 7.4). The total volume was 1.0 ml.

Studies involving "pretreated" erythrocytes utilized uninfected erythrocytes stored in the refrigerator in TETD at a total concentration of 10 μg/ml for 24 hr. These were then washed three times in a 2-fold excess of drug-free medium and mixed with concentrated samples of predominantly schizonts separated in gelatin (22) at time zero. This mixture was added to 3-cm diameter petri dishes in normal medium to make a final 8% cell suspension and incubated in a candle jar at 38°C. Daily changes were done with drug-free complete medium and slides were made to assess parasitemia.

RESULTS

Growth at Different Concentrations of DDC and TETD. Typical effects of growth of *P. falciparum* in DDC or its oxidation product TETD at concentrations of 100–0.1 μg/ml are shown in Tables 1 and 2. It can be seen that there was no multiplication of the parasites as early as 24 hr after the addition of concentrations of these compounds greater than 1 μg/ml. This suggests an immediate effect on the development of malaria by DDC or TETD. Concentrations as low as 0.1 μg/ml exhibited an 82% reduction in growth 72 hr after the addition of DDC to the culture (96 hr after the start of the experiment) and no survivors at the completion of the experiment. Similar results were seen with TETD: a 50% reduction in growth 72 hr after administration of the drug and a 74% reduction at the end of the experiment. Thus both TETD and its physiological reduction product, DDC, showed an equivalent effect. This inhibition appeared to be directly proportional to the concentration of the compound used.

Effect of TETD on Glycolysis of *P. falciparum*-Infected and Normal Erythrocytes. Evidence has been reported that these compounds exhibit their toxic effects on sensitive fungi by inhibition of the paired mercapto groups of the lipoic acid/dehydrogenase system (23). Therefore, assessment of glycolysis by measuring glucose utilization and lactate/pyruvate production in parasitized and normal erythrocytes was done in TETD and Me₂SO controls (Table 3). Examination of slides made at the start of incubation with the drug and 22 hr later, at termination, showed no change in parasite numbers or morphology except that the 100 μg/ml dose appeared to kill the plasmodia. In conformity with this finding, there was 100% inhibition of glucose utilization and lactate production over the last 16 hr at this high drug dose.

With TETD at 10 μg/ml, there was only a 44% inhibition of glucose utilization, significantly less than the 70% inhibition achieved with TETD at 1 μg/ml. This is termed "concentration quenching" or "biphasic response" (15) and in general agrees with the results reported by others on pyruvate production in fungi in the presence of these compounds (24, 25). This is determined by the type of complex formed between chelator and

### Table 1. Growth of *P. falciparum* in DDC

<table>
<thead>
<tr>
<th>DDC, μg/ml</th>
<th>2 days</th>
<th>3 days</th>
<th>4 days</th>
<th>5 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0.6 (0.4-0.8)</td>
<td>0.5 (0.4-0.6)</td>
<td>0.5 (0.4-0.7)</td>
<td>0.5 (0.3-0.7)</td>
</tr>
<tr>
<td>0.1</td>
<td>0.8 (0.5-1.0)</td>
<td>1.4 (0.8-1.8)</td>
<td>1.2 (0.9-1.7)</td>
<td>0.4 (0.1-0.8)</td>
</tr>
<tr>
<td>0</td>
<td>1.1 (1.0-1.3)</td>
<td>1.5 (1.2-1.9)</td>
<td>6.5 (4.1-8.0)</td>
<td>8.0 (6.3-9.6)</td>
</tr>
</tbody>
</table>

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

* % parasitemia is expressed as infected erythrocytes per 100 erythrocytes. The initial parasitemia was 0.75%. The average and range (in parentheses) are given from counts on four dishes. This is the result of one of three similar experiments.

† Grossly abnormal or dead.

### Table 2. Growth of *P. falciparum* in TETD

<table>
<thead>
<tr>
<th>TETD, μg/ml</th>
<th>2 days</th>
<th>3 days</th>
<th>4 days</th>
<th>5 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.2 (0.1-0.2)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0.7 (0.4-1.0)</td>
<td>1.4 (1.1-1.6)</td>
<td>5.8 (5.4-6.8)</td>
<td>9.7 (8.2-11.1)</td>
</tr>
<tr>
<td>10</td>
<td>0.2 (0.2-0.2)</td>
<td>0.2 (0.1-0.2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>1.2 (0.9-1.4)</td>
<td>1.6 (1.3-1.9)</td>
<td>5.5 (4.4-7.0)</td>
<td>7.5 (6.5-8.0)</td>
</tr>
<tr>
<td>1</td>
<td>0.8 (0.6-1.1)</td>
<td>0.6 (0.3-0.9)</td>
<td>0.5 (0.4-0.7)</td>
<td>0.3 (0.2-0.6)</td>
</tr>
<tr>
<td>0</td>
<td>1.0 (0.8-1.2)</td>
<td>1.5 (1.2-1.8)</td>
<td>6.5 (4.8-7.4)</td>
<td>9.5 (8.2-10.8)</td>
</tr>
<tr>
<td>0.1</td>
<td>1.2 (0.8-1.5)</td>
<td>1.3 (0.8-2.1)</td>
<td>3.2 (2.5-4.5)</td>
<td>2.4 (1.5-3.2)</td>
</tr>
<tr>
<td>0</td>
<td>1.0 (0.7-1.5)</td>
<td>1.4 (1.2-1.6)</td>
<td>6.2 (4.9-7.1)</td>
<td>9.1 (8.0-11.4)</td>
</tr>
</tbody>
</table>

Parasites were grown 24 hr if 1.5-ml petri dishes in a candle jar before exposure to TETD.

* In all cases 0 indicates the Me₂SO control.

† % parasitemia is expressed as infected erythrocytes per 100 erythrocytes. The initial parasitemia was 0.75%. The average and range (in parentheses) are given from counts on four dishes. This is the result of one of six similar experiments.

‡ Grossly abnormal or dead.
metal ion. DDC can form two complexes with divalent cations [metal-DDC and metal-(DDC)₂]. Presumably, the 1:1 complex predominates at low drug levels and is maximally inhibitory to glycolysis. This is termed "first zone of inhibition." Higher drug concentrations favor formation of the 1:2 complex, which is less inhibitory (7). As the concentration of drug is raised still further, free dithiocarbamate predominates, resulting in a rebound effect, termed a "second zone of inhibition" (24, 26). It is interesting to note that this concentration quenching effect was not seen by us in the growth studies of P. falciparum (Tables 1 and 2), suggesting that growth inhibition by these compounds in malaria may be the result of another biochemical mechanism. It is known that EDTA is able to compete effectively with DDC for divalent cations extracellularly, reducing toxicity at low concentrations of the thiocarbamate (26). In our studies, however, 0.5 mM EDTA had no antagonistic effect on growth inhibition by DDC or TETD. This further indicates growth inhibition by these compounds may not be related to the decrease in glycolysis in TETD. In contrast, the inhibition of glucose utilization by TETD in the first 6 hr was low. The effect at doses of 100 and 1 μg/ml was almost insignificant compared to the effect seen at 10 μg/ml. Replica experiments at 6 hr resulted in similar low utilisations with small quantitative variations. This suggests that the primary inhibitory effect of TETD on P. falciparum may not be through glucose utilization. Similar findings were reported in fungus cultures treated with the closely related compound dimethylthiocarbamate (25). On the other hand, the biphasic response is seen in lactate production at both 6 hr and after an additional 6 hr of incubation. Both 100 and 1 μg/ml concentrations of TETD gave greater inhibition than the 10 μg/ml dose. The effect on lactate production in the first 6 hr of incubation paralleled the 16-hr results. In contrast to data reported on fungi, pyruvate did not accumulate to any significant extent in our experiments on P. falciparum. Levels of pyruvate were low, none being detected in 6 hr and only 0.01–0.02 μmol/mg of protein accumulating in 16 hr. The concentration quenching results were also evident here and reflect those seen in lactate production. No pyruvate accumulated in uninfected cells.

It is significant, however, that TETD at 100, 10, or 1 μg/ml had no effect on glycolysis of the uninfected erythrocytes. This suggests that inhibition of glycolysis by TETD is specific for parasitized erythrocytes even though both the malaria parasite and the mature erythrocytes rely on glycolysis for their metabolism. It is also significant to note there is a marked effect on glycolysis in the infected cells at doses 1/10th those reported as normal serum levels (27, 28).

**Growth of P. falciparum in Sera from Volunteers on TETD.** In an effort to test whether, in fact, the effect is on recipient erythrocytes, two volunteers with type A blood were placed on therapeutic doses of TETD (500 mg orally once a day) for 4 days. Blood was drawn from them 4 hr and 4 days after the start of the experiment. The serum was separated from the erythrocytes and frozen for use in the in vitro cultivation system. This in vitro system for growing P. falciparum normally employs only a 10% concentration of serum in RPMI 1640 medium (17). To approach the pharmacologic levels of drug circulating in the volunteers, it was necessary to raise the proportion of serum used in the test system. Our studies indicated that normal serum could be added to the in vitro cultivation system as high as 50% without deleterious effects on the growth of the parasite, provided the final glucose concentration was 2 mg/ml.

Sera from volunteers taking TETD were then added to the culture system at a 50% concentration and supplemented with glucose (2 mg/ml). This serum did not inhibit the growth of P. falciparum, suggesting either that the drug is partitioned rapidly in the erythrocytes in vivo and actually is not in the serum, or that it is bound too tightly to pass into recipient erythrocytes to be inhibitory to the parasite.

**P. falciparum Infections of Erythrocytes Treated with TETD.** Linderholm and Berg (29) reported that, after administration of TETD to humans, appreciable amounts of DDC can be found in erythrocytes as long as 30 hr after ingestion of the drug. Therefore, erythrocytes were pretreated with TETD at 10 μg/ml (equivalent to serum levels reported in humans taking therapeutic doses of this drug) for 24 hr in the refrigerator and then thoroughly washed. Such cells did not support

### Table 3. Effects of TETD on glucose utilization and the formation of lactate in normal and infected erythrocytes (RBCs) by P. falciparum

<table>
<thead>
<tr>
<th>Incubation, hr</th>
<th>TETD, μg/ml</th>
<th>Glucose utilized</th>
<th>Lactate produced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Infected RBCs</td>
<td>Normal RBCs</td>
</tr>
<tr>
<td></td>
<td></td>
<td>μmol/mg protein</td>
<td>% inhibition</td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>0.268</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.288</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.259</td>
<td>27.7</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.358</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.328</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.318</td>
<td>0</td>
</tr>
<tr>
<td>Additional</td>
<td>16</td>
<td>0.573</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.342</td>
<td>43.9</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.610</td>
<td>43.9</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.207</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.608</td>
<td>70</td>
</tr>
</tbody>
</table>

Parasites were grown in 4-ml petri dishes to a 7% parasitemia. Slides were made to assess parasitemia. TETD was added in specified concentrations, each accompanied by a Me₂SO control. Zero time dishes were immediately harvested. Growth in the remaining dishes was allowed to continue for 6 hr, and the supernatants were removed for analysis. Fresh media containing TETD or Me₂SO were added and parasites were allowed to grow an additional 16 hr. Slides were made for all petri dishes to assess growth and morphology. All reactions were stopped by the addition of perchloric acid to a final concentration of 3%. This is the result of one of two similar experiments.

* Controls contained the same amount of Me₂SO as was introduced with the stated concentration of TETD.
Table 4. Effect of pretreatment of erythrocytes with TETD on subsequent growth of *P. falciparum*

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>% parasitemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal medium</td>
<td>0.7</td>
</tr>
<tr>
<td>Normal medium + MesSO</td>
<td>1.0</td>
</tr>
<tr>
<td>TETD</td>
<td>0.6</td>
</tr>
<tr>
<td>TETD + serum</td>
<td>0.5</td>
</tr>
</tbody>
</table>

This is the result of one of three similar experiments. The average and range (in parentheses) are given from counts on four dishes.

growth of *P. falciparum* (Table 4). This suggests the lipophilic drug may accumulate on or in the erythrocytes, preventing them from supporting growth of the malaria parasite. When the experiment was done with erythrocytes incubated in TETD for 24 hr at 38°C and subsequently treated as were those pretreated in the refrigerator, the inhibition was less dramatic. This apparent discrepancy is not yet explained.

**DISCUSSION**

TETD is a relatively safe compound used clinically to treat alcohol abuse patients by aversion therapy (30). It is thought that this drug sensitizes them to alcohol presumably through inhibition of aldehyde oxidase. Failure to abstain from ethanolic beverages results in a rise in serum acetaldehyde and a wide variety of unpleasant systemic manifestations (31), which encourage abstinence from spirits. It is interesting to note that inordinately high doses (500–1000 mg/kg) DDC injected intravenously have been reported to damage islets of Langerhans in experimental animals, thereby causing diabetes (32), probably by reacting with the Zn++ in the islet cells. However, doses of sodium DDC required to produce hyperglycemia were 500–1000 mg/kg, and the hyperglycemia was only temporary in 11 out of 15 animals. The lethal dose of TETD is 3 g/kg body weight (12, 33), but lower doses are relatively nontoxic apart from those symptoms seen after ingestion of alcohol.

Our studies demonstrate that low doses of TETD and its reduction product, DDC, are highly inhibitory to growth of the human malaria parasite *P. falciparum*. This is consistent with the finding that the closely related compound tetramethylthiuram disulfide and its carbamate reduction product are more toxic to parasitic molds than to saprophytic fungi (10). In addition, the glycolysis of the malaria parasite appears specifically sensitive to low doses of TETD, in contrast to glycolysis in the host erythrocyte. This may result from differential sensitivity of host and parasite glycolytic enzymes, or less directly as a consequence of higher than usual methemoglobin concentration, which is known to be present in malarial erythrocytes (34). TETD is rapidly and almost quantitatively reduced to DDC (35–37), presumably through the glutathione/glutathione reductase system of erythrocytes (37, 38), in many in vitro systems and in vivo. The reduction can be demonstrated spectrophotometrically at 450 nm (26, 39) and occurs in our in vitro cultivation system, indicating that the chemical moiety DDC is in fact generated in our system. It is known that methemoglobin promotes oxidation of DDC to TETD (35, 36) and TETD but not DDC is a potent inhibitor of hexokinase (35). Therefore, cells high in methemoglobin such as the *P. falciparum*-infected cells in culture would be sensitive to TETD inhibition of glycolysis, presumably at hexokinase. In any case, it seems likely that the growth inhibition exerted by DDC and TETD does not stem mainly from its effect on glycolysis.

Malaria parasites free from host cell contamination have been shown to be rich in cytochrome oxidase, a metalloprotein (3, 40, 41). Our unpublished studies also show that *P. falciparum* is extremely sensitive to KCN at concentrations to 5 μM in vitro. It is known that many metalloprotein oxidases in addition to cytochrome oxidase are inactivated by cyanide (42). In contrast, the dithiocarbamates show no effect on the cytochrome system (43) and affect O2 consumption only in concentrations that far exceed those that inhibit growth of organisms in test systems (8). Therefore the inhibition of the growth of malaria parasites observed in this study is not likely due to inhibition of terminal electron transport reactions in this organism.

In contrast to the potent in vitro activity of this drug in our studies, serum drawn from volunteers on therapeutic doses of TETD was not inhibitory to *P. falciparum* in culture at a 50% serum/medium mixture. Drug concentrations reported to be circulating in patients taking TETD fall within the "parasiticidal" limits seen in our growth studies. It has been reported that the therapeutic dose of 250–500 mg per day in humans results in a "plasma" concentration of 0.5–1.0 mg/100 ml, and the drug persists in pharmacologically active levels for about 5 days (28). Because TETD in concentrations as little as 1:1,000,000 (10⁻⁷ M) in vitro totally inhibits acetaldehyde oxidase of rabbit and rat liver (44, 45), the unusually high plasma concentration of the drug reported must be reexamined. Our studies suggest that human erythrocytes treated with levels of TETD reported as circulating in patients taking this drug did not support growth of parasites as long as 6 days later. This indicates that the erythrocytes are changed in some way by TETD, making them inhospitable to the parasites. It remains to be seen if erythrocytes taken from patients receiving the drug would also have resisted infection, because the work of Lindholm and Berg (29) suggests that all of the DDC is found in erythrocytes of humans taking TETD.

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