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Potentiation of the Antimalarial Agent Rufigallol†

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We have discovered a remarkable synergistic antimalarial interaction between rufigallol and the structurally similar compound exifone. The synergistic effects were produced in chloroquine-susceptible and chloroquine-resistant clones of *Plasmodium falciparum*. The degree of potentiation as estimated by standard isobolar analysis was ~60-fold for experiments initiated with asynchronous parasites. The most pronounced synergism was observed in experiments with synchronized trophozoite-infected erythrocytes, in which the degree of synergy was at least 300-fold. While the mechanism underlying this drug potentiation remains unresolved, it is hypothesized that rufigallol acts in pro-oxidant fashion to produce oxygen radicals inside parasitized erythrocytes. These radicals would attack exifone, thereby initiating its transformation into a more potent compound, a xanthone.

Malaria remains a global health problem. Each year this disease infects up to half a billion people and exacts a toll of roughly 2 million deaths, primarily of children under 5 years of age (25). Treatment of malaria is becoming more complicated in part because of the emergence of drug-resistant strains of *Plasmodium falciparum*, which causes the most deadly form of the disease. As a result, there is a great need for improved chemotherapy and prophylaxis for malaria. A structurally diverse group of therapeutic agents collectively referred to as oxidant drugs holds the promise of effective treatment of multidrug-resistant *Plasmodium* parasites (30). These drugs act to render parasites (or their host cells) more susceptible to attack by oxygen radicals or cause enhanced production of oxygen radicals inside parasitized erythrocytes (13). Some compounds in this class are currently in use (e.g., primaquine and artemisinin) (6, 17, 30) or have previously been employed to treat malaria (e.g., methylene blue) (2, 16, 30, 31). To improve the therapeutic efficacy of these agents and to counter the development of drug resistance, it is important to identify drugs which act in combination and in synergy with the oxidant antimalarial agents.

We have recently identified 1,2,3,5,6,7-hexahydroxy-9,10-anthraquinone (rufigallol) as a particularly active antimalarial compound (32). On the basis of its structural resemblance to known antimalarial hydroxynaphthoquinones and its ability to bind iron, we speculated that rufigallol functions as a redox-active iron-chelating agent. To further investigate the mode of action of rufigallol, we set out to compare the antimalarial activity of rufigallol with that of 2,3,4,3′,4′,5′-hexahydroxybenzophenone (also known as exifone or Adlone). We believed that while exifone is structurally related to rufigallol, the absence of one of the carbonyl groups would diminish both its redox-cycling capacity and its iron-chelating ability, resulting in a less active compound. Here we describe the in vitro inhibitory activity of exifone together with the surprising discovery of a potent antimalarial synergistic interaction between rufigallol and its chemical cousin.

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† We dedicate this study to the memory of Barbara C. Lockwood of Stirling, Scotland. She was a highly talented biochemist and an emerging bright star in the field of parasitology when she passed away in the Fall of 1993. We will long miss her youthful enthusiasm and creative approach to scientific endeavors.

MATERIALS AND METHODS

Chemicals and reagents. Rufigallol was prepared by the method of Azim and Ayaz (5), while the synthesis of exifone followed procedures outlined by Bleuler and Perkins (7). 3,3′-Dihydroxybenzophenone and 3,3′,4,4′-tetrahydroxybenzophenone were synthesized by the methods of Klemm et al. (20) and Ayres and Denney (4), respectively. Chemical characterization and confirmation of purity to at least 99% were obtained by elemental analysis, proton nuclear magnetic resonance, and gas chromatography-mass spectrometry (i.e., of the corresponding methylether derivatives), each achieving values consistent with the structure. All other benzophenones were purchased from Aldrich Chemical Company (Milwaukee, Wis.); maclurin (2,3′,4,4′,6-pentahydroxybenzophenone) was obtained from the Sigma-Aldrich rare chemical collection. Benzophenones obtained from commercial vendors were used as received. [^{3}H]ethanolamine was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, Mo.).

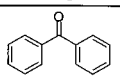
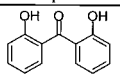
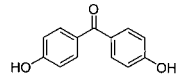
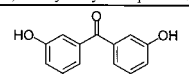
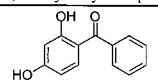
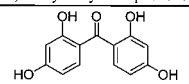
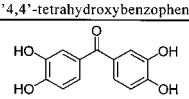
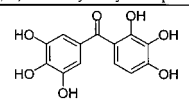
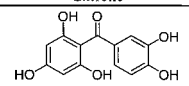
Culture of *P. falciparum*. The chloroquine-susceptible West African clone D6 and the chloroquine-, quinine-, and pyrimethamine-resistant Indochina clone W2 were obtained from the Walter Reed Army Institute for Research (23, 24). Human serum and erythrocytes were obtained from local volunteers and used within 14 days. Erythrocyte preparations were depleted of leukocytes by density gradient centrifugation over layers of Ficoll Histopaque with densities of 1.077 and 1.119, respectively (Sigma Chemical Company, St. Louis, Mo.). Erythrocyte pellets were washed twice in phosphate-buffered saline, resuspended in complete medium at a hematocrit of 33%, and stored at 4°C until use.

Parasites were cultured in group A^+ human erythrocytes and suspended at a 3.3% hematocrit in RPMI 1640 which contained 4 g of glucose per liter; 50 mg of gentamicin per liter, and 10% group A^+ human serum and was buffered with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and 25 mM $NaHCO_3$ (29). Cultures were maintained at 37°C in a gas mixture of 5% O_2, 5% CO_2, and 90% N_2. Synchronization to ring forms was achieved by using the technique of sorbitol lysis (21). Incubation of ring forms for an additional 20 to 24 h in complete medium provided a population enriched for mature trophozoites (≥95%).

Drug testing. Synergy between rufigallol and the various benzophenones was measured geometrically by construction of isobolograms (with a minimum of six coordinates). The antimalarial activities of drug combinations were determined by monitoring the incorporation of [^{3}H]ethanolamine into parasite lipids (9). (Parallel studies employing [^{3}H]hypoxanthine yielded equivalent results, as described below.) Stock solutions of test compounds were made in dimethyl sulfoxide at 10 mM. Dilutions were made into medium containing 10% serum (complete medium). The final concentration of dimethyl sulfoxide never exceeded 1% (vol/vol), which control experiments demonstrated was not sufficient to influence the rate of radiolabel incorporation into parasitized erythrocytes.

Experiments were conducted with 96-well plates in a checkerboard format. A 160-μl volume of complete medium containing parasitized erythrocytes (0.2% parasitemia, yielding a final hematocrit of 3.3%) were placed into all test wells

TABLE 1. Antimalarial activity of benzophenones in the presence and absence of rufigallol

Drug	IC_{50}	Degree of potentiation
 benzophenone	~100μM	none
 2,2'-dihydroxybenzophenone	2.5μM	none
 4,4'-dihydroxybenzophenone	>50μM	none
 3,3'-dihydroxybenzophenone	10μM	none
 2,4-dihydroxybenzophenone	~75μM	none
 2,2'4,4'-tetrahydroxybenzophenone	37.5μM	none
 3,4,3',4'-tetrahydroxybenzophenone	10μM	none
 Exifone	4.1μM n=33	50-60 fold[a] 350 to 500-fold[b]
 2,3',4,4',6-pentahydroxybenzophenone	50μM	~2-fold

[a] Derived from experiments with asynchronous parasite-infected erythrocytes.
[b] Derived from experiments with synchronous trophozoite-infected erythrocytes.

except three, to which was added medium containing unparasitized erythrocytes (background control). Twenty-microliter dilutions (10×) of exifone were added down the plate (columns 1 to 10) to achieve final concentrations in the range of 0 to 10 μM. (Note that screening of other benzophenone derivatives required adjustment of the concentration range to 100 μM.) Twenty-microliter dilutions of rufigallol (10×) were then added across the plate (rows A to H) to achieve final concentrations in the range of 0 to 1 μM. Radiolabelled ethanolamine was added after 48 h of incubation, and the experiment was terminated after 72 h by collecting the cells onto glass fiber filters with an automated Skatron multiwell harvester. The concentration of a drug or drug mixture giving 50% inhibition of label incorporation (IC_{50}) was calculated by nonlinear regression analysis of the semilogarithmic dose-response curve.

RESULTS

Comparison of the antimalarial activities of exifone and related benzophenones. Asynchronous cultures of *P. falciparum* were exposed to concentrations of exifone and other benzophenones to determine IC_{50}s (Table 1). The values shown are averages of at least three experiments each performed in duplicate (all values were within 10% of the mean). The IC_{50} of exifone for the chloroquine-sensitive D6 clone of *P. falciparum* was roughly 20 times weaker than that of the structurally similar compound rufigallol (IC_{50}s, 4.1 μM and 226 nM, respectively). Collectively, the benzophenones were unremarkable antimalarial agents with IC_{50}s in the range of 2.5 to 100 μM, comparatively weak considering that most of the antimalarial agents in use today demonstrate in vitro activity in the submicromolar range.

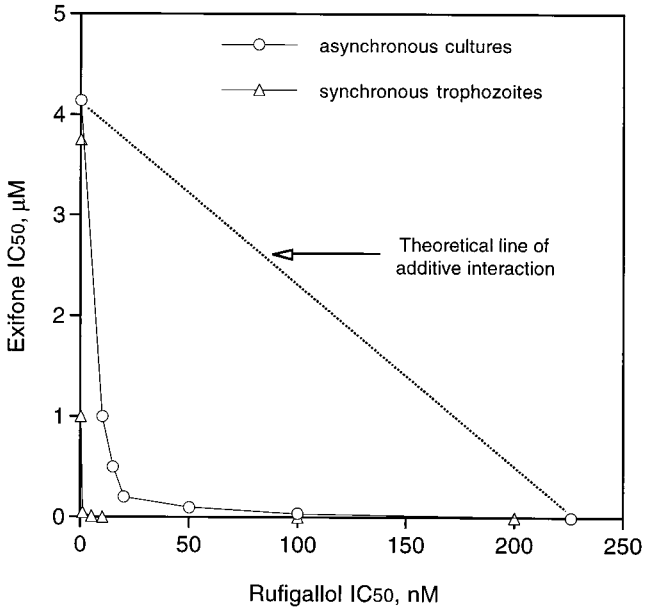


FIG. 1. Isobolograms showing synergistic antimalarial activity between rufigallol and exifone against *P. falciparum* clone D6 in experiments initiated with synchronized trophozoites (△) and asynchronous parasites (○).

Synergistic antimalarial activity of rufigallol with exifone. Standard isobolar analysis was employed for evaluating synergism between the various combinations of rufigallol and exifone. The experiment was set up in 96-well plates with decreasing concentrations of exifone down the plates and of rufigallol across the plates. In combination, striking synergy was observed, as indicated by the downward deviation of the values relative to the theoretical line of addition (Fig. 1). For example, the combination of 5 nM rufigallol with 0.22 μM exifone delivered the same growth-inhibitory effect as either drug alone at its respective IC_{50}. Calculating the degree of potentiation by geometric means of an isobole yielded a value of ~60-fold.

The other benzophenones listed in Table 1 were also tested for the ability to interact synergistically with rufigallol. Compared with the profound effect of combining exifone with rufigallol, none of the compounds tested produced significant enhancement of the antiplasmodial response of rufigallol. Conversion of exifone to its corresponding hexamethylether rendered the compound ineffective as an antimalarial agent (IC_{50}, ≥100 μM) and also extinguished its ability to influence the activity of rufigallol. From our brief structure versus activity analysis, it seems apparent that positioning of free hydroxy groups around the benzophenone ring system, especially the presence of an *ortho*-hydroxy group, is an important structural feature affecting the observed drug potentiation.

Effect of developmental stage on synergistic drug interaction. We also conducted isobolar analysis of the rufigallol-exifone drug pair on cultures containing synchronized parasites. Little or no augmentation of antimalarial activity was evident between the two drugs in experiments initiated with ring forms. By contrast, the inhibitory effect of the drug combination was most pronounced when the experiment was initiated with trophozoite-infected erythrocytes, in which case a

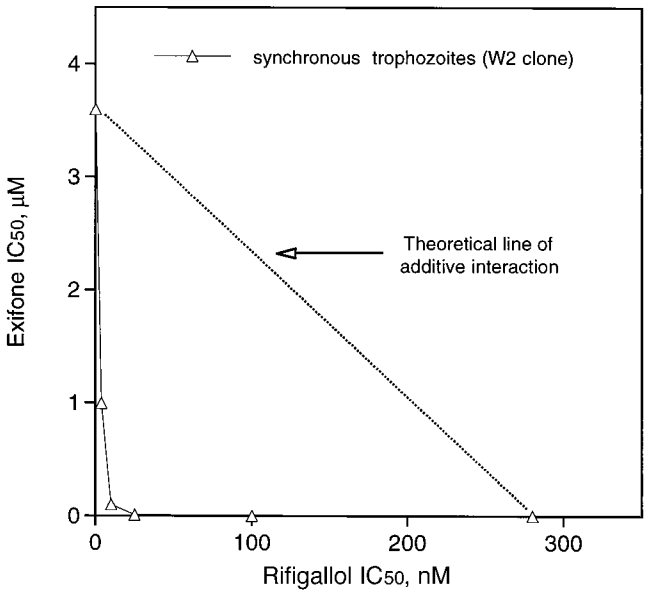


FIG. 2. Isobolar analysis of rufigallol and exifone against multidrug-resistant *P. falciparum* clone W2. Experiments were initiated with synchronized trophozoite-infected erythrocytes.

combination of 1 nM rufigallol with 10 nM exifone produced an effective 50% inhibitory response. Figure 1 shows the accentuated concave curve of the isobole obtained with the trophozoite stage cultures. While the curve is difficult to represent graphically, as it is so close to the *x* and *y* axes (for presentation, the *y* axis is drawn in micromolar increments while the *x* axis is in nanomolar increments), we estimated the degree of potentiation to be at least 300-fold.

Effectiveness of rufigallol-exifone against chloroquine-resistant parasites. We also tested the combination of rufigallol and exifone against the multidrug-resistant W2 strain. As before, the 72-h [^{3}H]ethanolamine incorporation method was used to assess the effects of both drugs on parasite growth. Presented in Fig. 2 is a typical isobologram derived from experiments initiated with trophozoite-synchronized infected erythrocytes. The IC_{50}s of both rufigallol and exifone for the W2 clone were similar to those for the D6 clone, and there was strong potentiation between the two drugs.

DISCUSSION

The widespread resistance of malarial parasites to chloroquine and the emerging problem of multidrug resistance indicate that new strategies for treatment of malaria must employ combinations of chemotherapeutic agents that have different or complementary mechanisms of action. Ideally, such combinations would consist of drugs that interact synergistically toward elimination of the parasite.

The data presented in this report demonstrate that rufigallol and exifone interact synergistically to inhibit the growth of drug-susceptible and multidrug-resistant strains of *P. falciparum* in vitro, suggesting that the underlying mechanism of drug potentiation circumvents existing multidrug resistance mechanisms. It is noteworthy that the synergistic response was most pronounced in experiments initiated with synchronized mature trophozoites, while only moderate effects were observed for the immature ring forms. These observations led to speculation that later stages of parasite development are most susceptible to the drug combination—a phenomenon that is well established for the 4-aminoquinolines (10). A stage-specific action by rufigallol-exifone may relate to a number of factors, including (i) the relative instability of rufigallol in the culture medium, (ii) the stage-specific transport of either drug by mature parasites, and (iii) the selective exploitation or targeting of a metabolic process specific to mature trophozoites.

While the biochemical mechanism underlying this synergistic drug interaction has yet to be elucidated, we can put forth a hypothesis. We believe that increased susceptibility of *P. falciparum* to rufigallol in the presence of exifone could be explained as follows. Rufigallol enters parasitized erythrocytes leading to the formation of hydrogen peroxide in a fashion similar to the well-documented redox-cycling behavior of hydroxynaphthoquinones. In the presence of large quantities of adventitious iron (or iron chelates such as heme) (1), the hydrogen peroxide produced is readily decomposed to hydroxyl radicals, as formulated by Haber and Weiss (3, 12). These highly reactive radicals attack exifone, forming an intermediate that undergoes cyclodehydration to become a potent tricyclic antiparasitic agent which we predict to be 2,3,4,5,6-pentahydroxyxanthone (Fig. 3).

The basis for this "xanthone hypothesis" is the fact that a similar condensation reaction leads to formation of xanthones from *ortho*-hydroxybenzophenones in both chemical and biochemical systems (14, 15, 28). In this regard, it is to be pointed out that there are at least 300 naturally occurring xanthones, almost exclusively derived from members of two families of higher plants (i.e., *Guttiferae* and *Gentianaceae*), while certain fungi (*Aspergillus* and *Actinoplanes* spp.), ferns, and lichens are also known to produce them (18, 19). The pharmacologic significance of xanthones as secondary plant metabolites has not been fully explored, although it has been found that some of these chemicals exhibit both antibacterial and antifungal activities (28). While we are unaware of any reports citing the antimalarial activity of naturally occurring xanthones, xanthone-containing plants are described in folk remedies for treatment of fevers (11).

It seems appropriate to note that exifone has had a storied past. In the 1970s and throughout the 1980s, the drug was used in the treatment of cognitive decline associated with age in geriatric and Parkinsonian patients (26, 27). Oral doses as high as 1 g/kg were administered for therapy and did not produce measurable short-term toxicity in geriatric patients. In 1989, it was discovered that continued administration of high doses of the drug to elderly patients (i.e., 600 mg/day for 2 to 4 months) caused reversible liver damage in about 1 in 15,000 patients (8, 22). In at least one case, exifone was believed to be so hepatotoxic that the patient died and the drug was removed from human trials. We are unaware of any clinical studies involving rufigallol; however, our own in vitro studies indicate that the compound only modestly suppresses colony formation by human bone marrow stem cells (32). Observable deleterious effects occurred at concentrations of $\geq$10 μM, which is slightly greater toxicity than that of chloroquine in parallel experiments.

In light of the growing problem of multidrug-resistant malarial parasites, the development of new drugs and useful drug combinations is of primary importance. The results presented here may lead to advances on both issues of concern. Insight into the mechanisms underlying the observed drug synergy between exifone and rufigallol can aid in the design of new and more effective derivatives and may highlight a rational basis for design of combination chemotherapy for malaria. If the xanthone hypothesis is correct, it might be possible to replace rufigallol with an existing antimalarial agent that acts through

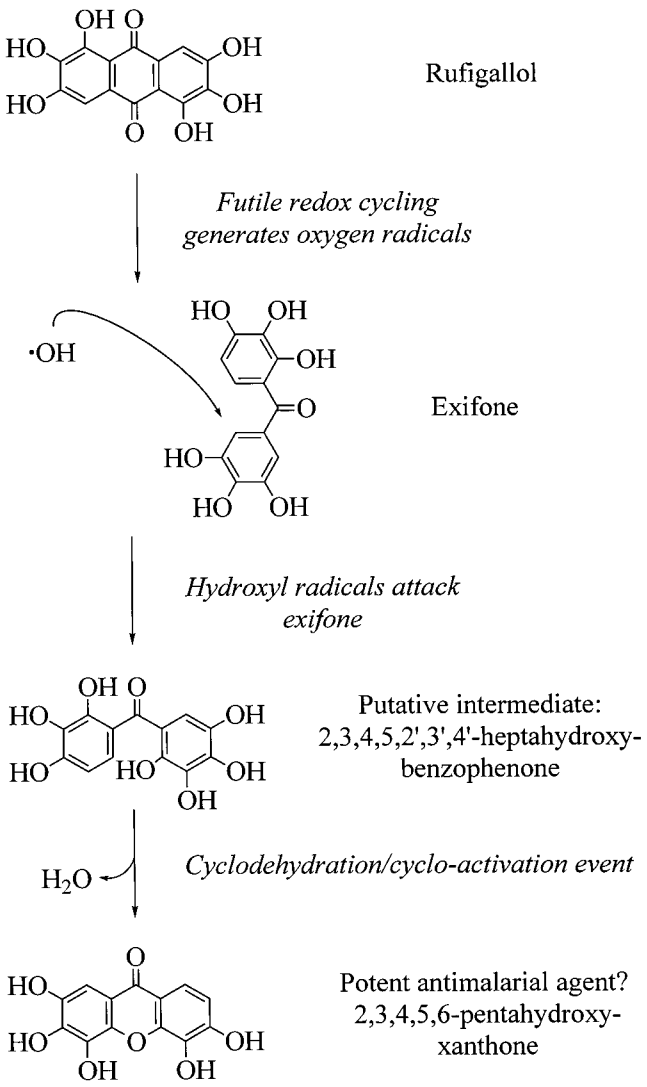


FIG. 3. Hypothetical scheme for the synergistic antimalarial interaction between rufigallol and exifone.

a pro-oxidant mechanism (e.g., primaquine). And while we believe that short-term administration of exifone, as part of such a drug combination, may be safe for treatment of life-threatening cases of acute malaria, chemical synthesis of analogs will be pursued in hopes of identifying a compound with superior antimalarial properties without the attendant concerns of liver toxicity.

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