SYNERGISM OF ANTIMALARIAL ANTIBIOTICS WITH HYDROGEN PEROXIDE IN INHIBITING *PLASMODIUM FALCIPARUM* GROWTH IN CULTURE

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Abstract. Although morbidity and mortality from malaria have steadily decreased worldwide, the ever present menace of the appearance of *Plasmodium falciparum* resistant to all antimalarials in current use, including most recently to artemisinin and its analogs, is of utmost concern, especially when development of new and affordable antimalarials has not kept abreast of this phenomenon. An alternative approach is to identify synergistic drug combinations, which would allow employment of otherwise non-efficacious antimalarial drugs. This study demonstrates that combinations of the chemical oxidant hydrogen hydroxide with antimalarial antibiotics targeting parasite mitochondrial and apicoplast ribosomes, which normally produce 'delayed-death' of parasites, act synergistically to inhibit *P. falciparum* growth in culture.

Keywords: *Plasmodium falciparum*, antibiotics, growth inhibition, hydrogen peroxide, synergism

INTRODUCTION

Some 219 million people living in tropical and sub-tropical regions of the world were estimated to have contracted malaria in 2010, with up to 800,000 deaths mainly in children living in sub-Saharan Africa (WHO, 2013). Treatment of this disease is compromised by the emergence of *Plasmodium falciparum* parasites resistant to all drugs in current clinical use, including, most worrisome of all, artemisinin and its analogs (Wongsrichanalai *et al*, 2002; Dorndorp *et al*, 2009), whilst development of new and affordable antimalarials has not kept pace with this predicament. One alternative strategy is to identify synergistic drug combinations, which either reverse antimalarial resistance [as in the case of reversal of chloroquine resistance with verapamil (Martin *et al*, 1987)] or broaden the window between a drug therapeutic and toxic level.

Although *Plasmodium* is a protozoan it contains an apicoplast organelle, a relict

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plastid of red algal origin (Lim and McFadden, 2010), allowing the development and employment of antibacterials as antimalarials (Goodman *et al*, 2007; Dahl and Rosenthal 2008). For examples, quinine in combination with tetracycline has been employed as a second-line therapy against *P. falciparum* malaria in Kampuchea (Denis, 1998), and doxycycline, a member of the tetracycline family of antibiotics, has been prescribed for treatment as well chemoprophylaxis of this type of malaria (Bradley and Bannister, 2001; WHO, 2010). Antibiotics that affect malaria parasite mitochondrial and apicoplast ribosome function give rise to the so-called 'delayed-death' effect in which drug treatment does not kill the intra-erythrocytic parasite during the first growth cycle but prevents schizogony in the subsequent cycle, even though the drug may have been removed after exposure of only 48 hours (Goodman *et al*, 2007).

During the malaria parasite intra-erythrocytic stages, ingestion of the host red cell cytosol by parasite acidic food vacuole results in conversion of oxyhemoglobin to methemoglobin and concomitant generation of superoxide anion and hydrogen peroxide (H_2O_2) (Atamna and Ginsburg, 1993). The malaria parasite lacks its own catalase and glutathione-dependent peroxidase with which to detoxify these reactive oxygen species (ROS), but instead relies on peroxiredoxins, glutathione and thioredoxin (Becker *et al*, 2004; Kawazu *et al*, 2008).

Although treatment *in vitro* of intra-erythrocytic *P. falciparum* produces lipid peroxidation of both parasite and host red cell membranes (Wozencraft, 1986), a recent report has demonstrated that exposure of isolated *P. falciparum* schizonts to 2-10 mM H_2O_2 results in a significant (but reversible under the experimental conditions used) drop in intracellular ATP content, presumably due to inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (and possibly other glycolytic enzymes), as well as alkalinization and acidification of the digestive food vacuole and cytosol, respectively, stemming from inhibition of the vacuolar(V)-type H^+-ATPase pump, but H_2O_2 treatment does not affect membrane integrity (van Schalkwyk *et al*, 2013).

In this study, we demonstrate that combinations of H_2O_2 with antibiotics that target malaria parasite intracellular organelle ribosomes produce a synergism in their ability to inhibit *P. falciparum* growth in culture.

MATERIALS AND METHODS

Chemicals

Chloramphenicol, chloroquine, cycloheximide and tetracycline were obtained from Sigma Chemical (St Louis, MO) and H_2O_2 and actinomycin D was from Riedel-Deltaen (Germany) and Merck, Sharp and Dohme (Whitehouse Station, NJ), respectively.

Parasite culture and determination of antiplasmodial activity

P. falciparum K1 strain (Thaithong *et al*, 1983) was grown in culture under 'candle jar' condition (Trager and Jensen,1976), and parasites were synchronized at the ring stage by sorbitol lysis (Lambros and Vanderberg, 1979). Parasite growth was determined by measuring [^{3}H]-hypoxanthine incorporation (Auparakkitanon *et al*, 2003). IC_{50} value (50% incorporation of radioactivity compared to no drug) is reported as mean ± SEM of 3 independent experiments conducted in triplicate.

Determination of drug combination inhibition

IC_{50} values of one drug (A) in the presence of a series of fixed concentrations

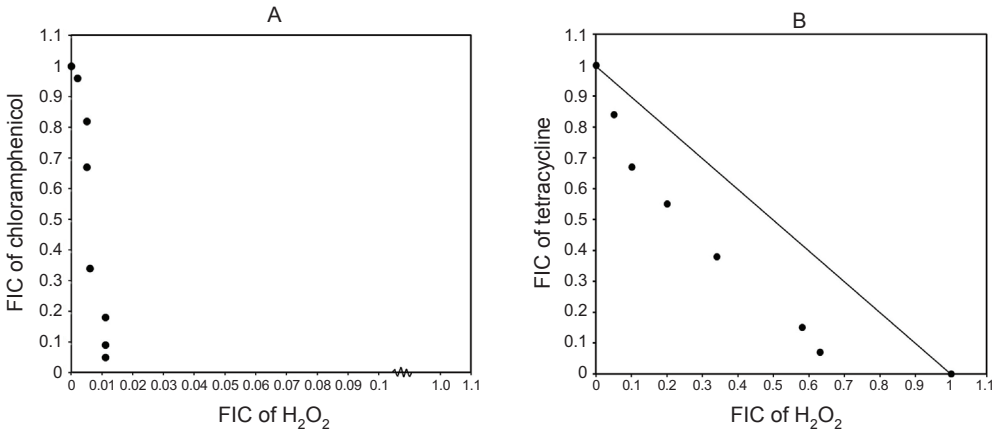

Fig 1–Isobologram of chloramphenicol (A) and tetracycline (B) versus H_2O_2. The solid line indicates an isobole where the two compounds act additively. FIC, fractional inhibitory concentration. Error bars have been omitted for sake of clarity. SEM is ≤ 0.01and 0.1 for (A) and (B), respectively.

of the other drug (B) were expressed as the mean sums of the fractional inhibitory concentrations (∑ FICs), defined as (IC_{50} of drug A in a mixture of drug B/ IC_{50} of drug A alone) + (IC_{50} of drug B in a mixture of drug A/ IC_{50} of drug B alone) for each fixed drug concentration. Three types of drug interaction are defined as follows: additive, ∑ FIC = 1; synergism, ∑ FIC < 0.25; and antagonism, ∑ FIC > 4.

RESULTS

Isobolograms generated from measuring FICs of the combination of chloramphenicol or tetracycline together with H_2O_2 against *P. falciparum* in culture revealed a marked synergism with the former compound pair (sum of FIC ranging from 0.05 to 0.96) and to a lesser extent with the latter combination (sum of FIC of 0.65 - 0.93) (Fig 1). The IC_{50} value of H_2O_2 was 27 ± 8 mM, while that of tetracycline and chloramphenicol was 59 ± 21 and 775 ± 32 M respectively, consistent with previous report (Ramya *et al*, 2007). It is worth noting that combinations of H_2O_2 with actinomycin D, chloroquine or cycloheximide resulted in additive effects (data not shown).

DISCUSSION

In bacteria tetracycline antibiotics inhibit 70S ribosome function by interfering with the ability of elongation factor EF-Tu, a GTP-binding factor required for location of the proper amino acyl-tRNA into the ribosome A site, and these drugs are thought to act similarly to inhibit protein synthesis in malaria parasite mitochondrion and apicoplast, both of which encode genes of their respective 70S ribosomes (Dahl and Rosenthal, 2008). *Plasmodium* apicoplast is apparently more sensitive to tetracycline and doxycycline than the mitochondrion as evidenced by the failure of apicoplast growth (elongation and segregation) during (second cycle) schizogony in contrast to the normal or subtle changes in mitochondrial appearance, resulting in failure to complete cytokinesis and progression into the third growth cycle, these events occurring even after only 48 hours of drug exposure (Dahl *et al*, 2006; Goodman *et al*, 2007).

A recent proteomics analysis of *P. falciparum* schizonts exposed to doxycycline (IC_{50} of 10 M for 24 hours at the ring stage and analysis at the second cycle of growth) demonstrated that a total of

64 proteins are differentially regulated, among which 10 and 2 apicoplast-located proteins are up- and -down regulated respectively, whereas only 2 mitochondrial-specific proteins are decreased relative to antibiotic-untreated control (Briolant *et al*, 2010). However, all of these proteins are nuclear encoded, and further transcriptional analysis revealed down-regulation of 3 apicoplast genes (*PftufA*, *PfsufB* and *PfclpC* encoding translation elongation factor, protein involved in iron metabolism and tRNA modification and protease required for apicoplast import of nuclear-encoded proteins, respectively), which are not seen at the protein level, whereas no changes in mitochondrial gene expression are observed. Some of the cytoplasmic proteins up-regulated in response to doxycycline, viz. 1- and 2-Cys peroxiredoxins and glyceraldehyde-3-phosphate dehydrogenase, comprise a group of parasite proteins expressed in general response to drug treatment. An earlier study employing microarray analysis had indicated that doxycycline (1 M at late ring/early trophozoite stage for 24 hours) causes apicoplast gene under-expression of no more than 1.6-fold in second cycle schizonts (Dahl *et al*, 2006). Thus it is not surprising that there was less synergistic response to tetracycline in the face of H_2O_2 exposure observed in this study, despite the reduction of ATP level and acidification of parasite cytoplasm induced by this oxidant (van Schalkwyk *et al*, 2013).

On the other hand, H_2O_2 exposure produced a marked synergistic inhibition of chloramphenicol on *P. falciparum* growth in culture. In bacteria, chloramphenicol inhibits peptidyl transfer reaction catalyzed by 23S rRNA located in the 50S large ribosomal subunit, and this is presumed to be its mode of action within malaria parasite mitochondrion and apicoplast. However, there is a paucity of studies on the exact molecular events caused by this antibiotic to the intra-erythrocytic malaria parasite, but whatever the plethora of gene transcriptional and protein translational consequences that ensue, they are exquisitely sensitive to H_2O_2-mediated insult, resulting in loss of delayed-death phenomenon, and presumably lethality during the first growth cycle.

The synergism in parasite growth inhibition reported here appears limited to combination of H_2O_2 and antibiotics targeting malaria parasite organelle ribosome, as an additive phenomenon was obtained with combination of the chemical oxidant and cycloheximide, an inhibitor of (eukaryote) 80S ribosome. This notion needs further verification as only two examples of antimalarial antibiotics were examined.

The results of this study demonstrate that by a judicious choice of drug combinations it should be possible to obtain beneficial anti-plasmodial drug partners of otherwise non-efficacious antimalarials. There are on-going efforts to develop novel antimalarials directed against enzymes of parasite glycolysis pathway and V-H^+-ATPase (van Schalkwyk *et al*, 2008, 2010).

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