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Multiple synergistic interactions between atovaquone and antifolates against *Plasmodium falciparum* in vitro: A rational basis for combination therapy

Eunice Wambui Nduati, Edward Mberu Kamau *

Kenya Medical Research Institute/Wellcome Trust Collaborative Research Program, Wellcome Trust Research Laboratories, Nairobi, Kenya

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Abstract

The use of synergistic drug combinations for the treatment of drug-resistant malaria is a major strategy to slow the selection and spread of *Plasmodium falciparum* resistant strains. In order to investigate synergistic compounds, with different modes of action, as alternative candidates for combination therapy, we used standard in vitro *P. falciparum* cultures and an established synergy testing method to define interactions among dapsone (DDS), atovaquone (ATQ), chlorproguanil (CPG) and its triazine metabolite chlorcycloguanil (CCG). Strong synergy was observed in the combinations DDS/CCG and ATQ/CPG. Multiple combination of these drugs, DDS/CCG/CPG/ATQ also exhibited high synergy although not higher than that of either of the two drug combinations separately. The use of this triple combination DDS/CPG/ATQ, even without an increase in synergy over their double combinations, ATQ/CPG and DDS/CCG, would contribute towards slowing the selection pressure since these drugs act against different targets and would delay the selection of parasites resistant to the three drugs, extending the useful therapeutic life of these valuable compounds.

Keywords: Synergy; Malaria; *Plasmodium falciparum*; Antifolates; Combination therapy

1. Introduction

Combination therapy is increasingly being used as the standard method in treatment of both tuberculosis and HIV/AIDS. This approach has been advocated for the treatment of malaria and its therapeutic potential demonstrated (White et al., 1999). Following the 2001 recommendations by the WHO on Antimalarial Drug Combination Therapy, countries in the region have been urged to adopt artemisinin-based combination therapies (ACTs) for treatment of uncomplicated malaria. Despite the adoption of these therapies by several African countries, WHO and the pharmaceutical companies have warned of a pending supply shortfall for ACTs.

The scientific basis for combination therapy is simple. Drug resistance, more often than not results from mutations in the genes that encode the drug targets. As long as the mutations to resistance are in unlinked genes, the probability that a single parasite simultaneously carries mutations that confer resistance to each of the drugs is the product of the mutation rates to resistance for each individual drug in the combination (White and Olliaro, 1996).

* Corresponding author at: WHO/AFRO Malaria Unit Box BE 773 Belvedere, Harare, Zimbabwe. Tel.: +263 47 241 38054; fax: +263 4 746127.
E-mail addresses: kamaue@afro.who.int, emberu@hotmail.com (E.M. Kamau).

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A major advantage of combination therapy is that it may enable mutual protection of one inhibition mechanism by the other thereby extending the useful therapeutic life of the components involved (White et al., 1999). In addition to providing multiple sites of attack on the parasite, drug combinations may also produce a quantitative anti parasitic effect far greater than the additive effect of individual drugs (Dinah and Gillies, 1985; Yeo et al., 1997). Although the combination of sulfadoxine and pyrimethamine (SP) is still being used in Africa as first line treatment for non-severe *falciparum* malaria and for intermittent preventive treatment (IPT) for pregnant women and lately for intermittent preventive treatment in infants (IPTi) on trials basis, recent studies have demonstrated increasing resistance to this combination in parts of Africa (Mutabingwa et al., 2001; Landgraf et al., 1994). Furthermore SP resistance has reached dimensions that have prompted some countries to adopt combination therapies that do not contain SP, rising fears that the dreaded moment of running out of economically sustainable therapies has indeed come. Widespread SP failure will be devastating in Africa because of its enormous caseload (Amukoye et al., 1997; White et al., 1999) and delay in making affordable replacements available at community level.

Like SP, the combination DDS and CPG, recently launched by GlaxoSmithKline (GSK) is clinically effective (Mutabingwa et al., 2001; Watkins et al., 1988) and shows synergistic interaction in vitro (Winstanley et al., 1995). However, DDS/CPG is more potent in vitro than SP (Winstanley et al., 1995) and is eliminated rapidly (Watkins et al., 1987; Winstanley et al., 1997). Because of these parameters, DDS/CPG may provide a stopgap replacement for SP. However there is need to "protect" DDS/CPG from the onset rather than wait for resistance to emerge and then trying to slow its spread. Efforts to combine DDS/CPG with artesunate are being pursued by GSK.

In addition, the combination of atovaquone (ATQ) and proguanil (PG) [Malarone^{TM}] has recently become available for the treatment of falciparum malaria, particularly in areas of multi-drug resistance (de Alencar et al., 1997) and is not antagonized by folate derivatives (Yeo and Rieckmann, 1997). This combination is synergistic, but in this case it is the pro-drug PG, rather than the triazine metabolite cycloguanil (CG), that interacts with ATQ (Canfield et al., 1995). Despite the occasional breakthrough, public health authorities in some developed countries are recommending ATQ/PG for malaria prevention for travelers to endemic countries, an indication that this drug combination may serve a useful role as a chemoprophylactic agent.

An informed consideration of the tested combinations suggests that the combination DDS/CPG/ATQ may possess superior characteristics since it consists of two synergistic systems, ATQ/CPG and DDS/CCG, targeting different pathways. Further, the multiple combinations enable mutual protection of one system by the other thereby extending useful therapeutic life.

The objective of this study was to investigate the potential use of synergistic compounds as alternative candidates for combination therapy using DDS, CCG, CPG and ATQ against two laboratory reference isolates, W282 and V1/S. These compounds were chosen on the basis of their availability, potency and safety.

2. Materials and methods

The standard method for continuous cultivation of *P. falciparum* developed by Trager and Jensen (1976) and subsequent application (Desjardins et al., 1979) was used. The two laboratory strains, W282 and V1/S, were chosen because; W282 represents a high proportion of isolates currently found in parts of Africa in respect to their *dihydrofolate reductase* (*dhfr*) genotype (Basco et al., 1995; Plowe et al., 1996; Nzila et al., 1998; Mutabingwa et al., 2001; Kublin et al., 2002). This strain carries a *dhfr* allele with three mutations, at codons 108, 51 and 59. Based on a predictive drug resistance model, parasites that carry this genotype are likely to be of borderline sensitivity to treatment with SP (Watkins et al., 1997). If further mutations were to be selected in these triple mutant isolates, one likely position would be a mutation at codon 164, a change found in some Southeast Asia and South American isolates, highly resistant to SP. This genotype is represented in this study by the laboratory reference strain V1/S, which carries mutations at codons 108, 51, 59, 164 in the *dhfr* gene, although some recent data reveal rare resistant alleles (Mookherjee et al., 1999; Hastings et al., 2002; Bates et al., 2004).

2.1. Culturing procedure

The culture medium was composed of RPM1 1640 (special batch - no. PABA or folic acid [Life Technologies, Scotland]), with 25 mmol/l HEPES buffer [Gibco BRL, Scotland], 25 mmol/l $NaHCO_3$ [BDH, UK] and 10% (v/v) normal human serum [National Blood Service, Bristol, UK]. Parasites were maintained