The baseline in vitro activity of the antimalarials pyronaridine and methylene blue against Kenyan *Plasmodium falciparum* isolates

John Okombo*1, Steven M. Kiara1, Leah Mwai1, Lewa Pole1, Eric Ohuma1, Lynette Isabella Ochola1, Alexis Nzila**1

1Kenya Medical Research Institute (KEMRI)/Wellcome Trust Collaborative Research Program, PO Box 230, 80108, Kilifi, Kenya;

**Current address: King Fahd University of Petroleum and Minerals, Biological studies, Department of Chemistry, PO Box 468, 31261 Dhahran, Saudi Arabia.

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Key words: malaria, plasmodium, drug resistance, pyronaridine, methylene blue, *Pfcrt*, *Pfmdr1* and *Pfnhe*, in vitro activity

*Corresponding author: John Okombo, Kenya Medical Research Institute (KEMRI)/Wellcome Trust Collaborative Research Program, PO Box 230, 80108, Kilifi, Kenya; Email: jokombo@kilifi.kemri-wellcome.org
Abstract

We have analysed the in-vitro activities of pyronaridine and methylene-blue against 59 Kenyan *Plasmodium falciparum* isolates in association with polymorphisms in *Pfcrt* (codon 76), *Pfdmdr1* (codon 86) and *Pfnehe* full-sequence. The median inhibitory concentration that kills 50% of parasites for pyronaridine and methylene-blue were 13.5 and 3.3 nM respectively. Their activities were not associated with polymorphisms in these genes. The drugs’ high in vitro activities indicate that they would be efficacious against Kenyan isolates in vivo.
Introduction

Coartem® (lumefantrine [LM] and artemether) and amodiaquine (AQ)/artesunate (ART) are currently the first lines of treatment of uncomplicated malaria (8, 29). However, reports indicate that resistance to LM may arise relatively quickly (30). Likewise, evidence suggests that the efficacy of AQ, whose active in vivo metabolite is desethylamodiaquine (DEAQ), is reduced in areas of high CQ resistance (12).

The combinations of piperaquine (PIQ)/dihydroartemisinin (DHA), and of pyronaridine (PRN)/ART, are being developed as alternative antimalarials (2). In spite of these alternatives, the search for new active compounds is being pursued. Methylene-blue (MB) is an old antimalarial that was abandoned because of its side effect of turning urine blue. However, this drug has been extensively used for the treatment of met-haemoglobinaemia (5). The burgeoning problem of drug resistance has led to a renewed interest in this drug (28). In this paper, we report on the in vitro activity of PRN and MB against Kenyan Plasmodium falciparum field isolates and on the change in their activities in relation with polymorphisms in Pfcr7 at codon 76 (Pfcr7-76), Pfmdr1 codon 86 (Pfmdr1-86) and in Pfnhe.

We analysed Plasmodium falciparum fresh isolates collected in the Kenyan district of Kilifi and adapted for long-term cultures as detailed previously (13, 15) Antimalarial activity was measured in the presence of varying concentrations of each compound, and results were expressed as the drug concentration required for 50% inhibition of [3H]hypoxanthine incorporation into parasite nucleic acid (IC50) (15). We employed two reference strains: V1S, the multidrug-resistant strain, and 3D7, the drug-sensitive strain. We analysed the antimalarials of chloroquine (CQ), MB, AQ and quinine (QN) [purchased from Sigma Chemical Co. Poole, Dorset, UK], and LM, PIQ, DEAQ and DHA (gifts from Professor Steve Ward, Liverpool School of Tropical Medicine, Liverpool, UK).
Blood samples (50 µl) of in-vitro adapted isolates were spotted onto filter paper, and single-base changes at Pfcr-76 and Pfmdr1-86 were detected as reported elsewhere (13). In this paper, we re-analysed the sequencing of Pfnhe published previously (15).

Statistical analyses were carried out using the Stata programme (Stata version 11, College Station, Texas). We compared differences between groups using the Wilcoxon rank-sum test and measured correlations using the non-parametric Spearman pairwise analysis. All statistical analysis was assessed at the 5% significance level.

We have analysed the chemo-sensitivity profiles of 59 P. falciparum field isolates against PRN and MB. As comparators, we have included the already published data on same isolates against CQ, LM, PIQ and QN, AQ, DEAQ and DHA (13-15, 27). Median IC50s for PRN and MB against the multidrug resistant strain V1S were 12 and 1 nM respectively, and values pertaining to full sensitive strain 3D7 were 8 and 2 nM, respectively.

Against field isolates, median IC50s for PRN and MB were 13.5nM, IQR [4.6-31.5], 3.3nM, IQR [1.7-8.0], respectively. Values pertaining to CQ, LM, PIQ, and DHA are presented in Figure 1, and those of QN, AQ and DEAQ were 141.1nM, IQR [50.9-268.2]; 7.8nM, IQR [5.4-8.6], 8.5nM, IQR [7.4-16.9]; PRN was more active than CQ, LM, PIQ; MB was more active than all these aforementioned drugs, except DHA. Our data are line with previous reports showing the high potency of PRN in vitro against isolates from Cameroon (26), Senegal (22) and Gabon (9).

PRN was used up to the 1990s, as a monotherapy, for the treatment of malaria in China (4), and was tested in Cameroon, with encouraging results (25). Its combination with ART (Pyramax®) has proven efficacious in many African countries, including in Kenya (24, 31), in line with our in vitro data. PRN has also been proven potent in vitro against P. falciparum isolates from South East Asia, a known area of multidrug resistance (23). Interestingly, PRN is also active against Plasmodium vivax in vitro (23), and recently, the in vivo efficacy of Pyramax®
against \textit{P. vivax} has been shown, making this combination a potential drug for the treatment of \textit{P. vivax} infection as well (18).

MB is has proven efficacious in African, mainly in Burkina Faso (3, 10, 11, 32), and has gametocytocidal properties, thus could be part of treatment combinations to reduce transmission of \textit{P. falciparum} (6). Our data show that MB is active \textit{in vitro}, in line with 2 previous studies using African parasites (1, 17). Thus, this drug would also be efficacious against the Kenyan parasite population.

We also investigated the role of polymorphisms within \textit{Pcrt}-76, \textit{Pfmdr1}-86 and \textit{Pfnhe}. PRN is more active against parasites harbouring wild type than mutant at \textit{Pcrt}-76 codon (IC\textsubscript{50}s of 6 versus 20 nM) and at \textit{Pfmdr1}-86 codon (IC\textsubscript{50}s of 7 versus 19 nM), however these differences were not significant (p>0.05). This lack of association has also been reported elsewhere (19).

Data pertaining to MB showed that its activity was not affected by polymorphisms in \textit{Pcrt}-76 and \textit{Pfmdr1}-86. Likewise, no change was observed between the activity of PRN and MB and polymorphisms in \textit{Pfnhe}, a gene associated with QN resistance (16), in line with a previous report (17).

We also analysed the correlation between PRN and MB \textit{in vitro} activities with those of CQ, LM, PIQ, AQ, DEAQ, QN and DHA. PRN activity was significantly correlated with those of the quinolone based drugs PIQ, AQ, DEAQ and QN, but not with CQ’s (Table 1). PRN \textit{in vitro} activity was found to correlate with CQ or AQ or QN in some studies (20, 21, 23), but not in others (9, 19). We did not find a correlation between PRN and DHA activities, as reported previously (9).

However, a significant correlation between PRN and ART was found (19, 23). Since artemisinin resistance is now emerging (7), it is important to establish the extent to which this resistance may affect PRN activity. We observed a significant correlation between MB and CQ, LM, DEAQ, QN and DHA, while no association was found between MB and PIQ, and QN and PRN. To the best of our knowledge, only one study addressed this correlation: MB activity was not correlated with CQ, QN, DEAQ, LM and DHA (17). Clearly, further investigations are needed to define the relationship between MB and other antimalarial’s \textit{in vitro} activity.
We have provided the first evidence on the *in vitro* activity of PRN and MB against isolates from Kenya. The high *in vitro* activity of these 2 drugs are in line with data reported in other parts of Africa, and also confirm (at least in the case of PRN), the reported efficacy of Pyramax® in Kenya.
Acknowledgment

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References


Figure 1: Median inhibitory concentrations that kill 50% of parasites (IC_{50}s) of chloroquine (CQ), lumefantrine (LM), piperaquine (PIQ), pyronaridine (PRN), methylene blue (MB) and dihydroartemisinin (DHA). Values are in nM, and in bold are represented median IC_{50}s. Parasites were adapted in vitro for long term culture prior to assessing IC_{50}s.
Table 1: Correlation coefficient (r) between the in vitro activity of pyronaridine (PRN) and methylene blue (MB) with chlorquine (CQ), lumefantrine (LM), piperaquine (PIQ), amodiaquine (AQ), desethylamodiaquine (DEAQ), quinine (QN), dihydroartemisin (DHA). The non-parametric Spearman statistical test was used, and values in bold are those that are statistically significant.

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