Antimalarial activity of isoquine against Kenyan
*Plasmodium falciparum* clinical isolates and association with polymorphisms in *pfcrt* and *pfmdr1* genes

John Okombo1,*, Steven M. Kiara1, Abdi Abdirahman1, Leah Mwai1, Eric Ohuma2, Steffen Borrmann1,3, Alexis Nzila4 and Steve Ward5

1Kenya Medical Research Institute (KEMRI)/Wellcome Trust Collaborative Research Program, PO Box 230, 80108 Kilifi, Kenya; 2Nuffield Department of Obstetrics & Gynaecology, University of Oxford, Women’s Centre, Level 3, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK; 3Department of Infectious Diseases, Heidelberg University School of Medicine, Heidelberg, Germany; 4King Fahd University of Petroleum and Minerals, Department of Chemistry, PO Box 468, Dhahran 31261, Saudi Arabia; 5Molecular and Biochemical Parasitology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK

*Corresponding author. Tel: +254-41-7522063; E-mail: jokombo@kemri-wellcome.org

Received 17 July 2012; returned 27 August 2012; revised 27 September 2012; accepted 30 October 2012

**Background:** The use of amodiaquine in prophylaxis is associated with serious toxicity, resulting from its metabolic conversion into a reactive quinone-imine metabolite by the hepatic cytochrome P450. To circumvent this toxicity, several amodiaquine analogues that lack the potential to form a quinone-imine derivative, while retaining antimalarial activity, have been designed. Isoquine is one of these promising molecules that has already reached Phase I clinical trials in humans.

**Methods:** We analysed the in vitro activity of isoquine against 62 *Plasmodium falciparum* isolates collected in Kenya and the association of this activity with polymorphisms in *pfcrt* and *pfmdr1* genes.

**Results:** The median concentration of isoquine that inhibited 50% of parasite growth (IC50) was 9 nM, compared with 56 nM chloroquine, 8 nM amodiaquine, 10 nM desethylamodiaquine, 69 nM lumefantrine and 1 nM dihydroartemisin. Isoquine activity was correlated with polymorphisms in *pfcrt* at codon 76, but not in *pfmdr1* at codon 86.

**Conclusions:** The high activity of isoquine against field isolates, including chloroquine-resistant isolates, with IC50 <10 nM, warrants its further development as an antimalarial.

**Keywords:** amodiaquine, cross-resistance, drug resistance

**Introduction**

The rapid selection and spread of antimalarial resistance remains a burgeoning problem that could compromise the control and management of malaria. Currently, malaria treatment hinges on the use of artemisinin-based combination therapy, with Coartem™ (lumefantrine/artemether) and Coarsucam™ (amodiaquine/artsunate) among the most efficacious and widely used combinations.1,2 However, compelling evidence now shows the emergence of resistance to artemisinin in South-East Asia and there is genuine concern that this could spread to Africa, as has been the case with other antimalarials. New drugs are therefore urgently needed.

One of the strategies to discover new drugs is to design novel and more potent molecules through structural modifications of existing ones. Amodiaquine, whose active metabolite is desethylamodiaquine (DEAQ), is a 4-aminooquinoline structurally similar to chloroquine, but with the N,N-diethyl-pentane-1,4-diamine chain substituted by 2-[(diethylamino)methyl]phenol. This drug is more potent than chloroquine due to its higher drug accumulation in the parasite digestive vacuole.3 However, its clinical use as a prophylactic agent was associated with agranulocytosis and life-threatening idiosyncratic hepatotoxicity.4 Toxicity is thought to result from the formation of reactive quinone-imine, the product of cytochrome P450-catalysed oxidation of the 1,4-aminophenol ring.5 To circumvent this toxicity, several amodiaquine analogues lacking the potential to form reactive metabolites via this quinone-imine pathway, while retaining potent antimalarial activity, have been designed. The most promising analogues are isoquine, an isomeric amodiaquine analogue...
(with the position of the 4-hydroxyl and Mannich side-chain interchanged), N-tert-butyl isoquine and des-ethyl isoquine. These molecules have proven promising in animal models and isoquine has reached Phase I trials in humans (S. W., personal communication). In this paper, we present the first report on the in vitro activity of isoquine against Plasmodium falciparum clinical isolates collected in Kenya, in relation to amino acid changes in pfcrt at codon 76 (pfcrt-76) and pfmdr1 at codon 86 (pfmdr1-86), the two genes associated with 4-aminoquinoline resistance. Using already published data on the activity of amodiaquine, DEAQ, lumefantrine, chloroquine and dihydroartemisinin, we also correlated the activity of isoquine with those aforementioned drugs.

Materials and methods

Chloroquine (diphosphate) and amodiaquine (dihydrochloride dihydrate) were purchased from Sigma Chemical Co. (Poole, Dorset, UK) while DEAQ (dihydrochloride) was purchased from LGC (Teddington, Middlesex, UK). Lumefantrine and dihydroartemisinin were gifts from Novartis Pharma AG (Basel, Switzerland). Isoquine (free base) was synthesized in-house. Samples were collected from malaria patients with ethical approval from the Kenya National Ethical Committee, between 2005 and 2008 in Kilifi District, Kenya. Parasites were adopted for long-term culture as previously described and antimalarial activity determined using radioisotopic (H)thiopoxanthine incorporation. Each experiment was carried out in duplicate and only those with a variation of <30% between them were considered. The results are summarized and presented as the median inhibitory concentration that kills 50% of parasites (IC50), with corresponding IQRs. The multidrug-resistant V1S and the drug-susceptible 3D7 parasite lines were used as reference strains. Samples (50 μL) from blood cultures infected with in vitro-adapted isolates were filter-paper spotted and single base changes at pfcrt-76 and pfmdr1-86 were detected using PCR-RFLP, as previously reported. In pfcrt, the amino acid lysine (K) at codon 76 represents the wild-type genotype while a substitution to threonine (T) represents the mutant. In pfmdr1, the wild-type genotype is represented by asparagine (N) at codon 86 while the mutant bears a substitution to tyrosine (Y) at this position. Differences in IC50 between the groups were compared using Wilcoxon signed-rank and Mann–Whitney tests, while correlations were measured using Spearman rank correlation analysis. All statistical analyses were conducted using STATA (College Station, TX, USA) and statistical significance was assessed at the 5% significance level.

Results and discussion

We tested the in vitro activity of isoquine against 62 P. falciparum isolates and also reanalyzed the already published data on amodiaquine, DEAQ, lumefantrine, chloroquine and dihydroartemisinin. Each in vitro test was carried out in duplicate and drug IC50s showed <30% variation against each isolate, except for isoquine and lumefantrine IC50s against two and three isolates, respectively. Thus, these three IC50s were not considered in further analyses. The median isoquine IC50s against V1S and 3D7 were 16.7 and 6.1 nM, respectively. Against the clinical isolates, isoquine was almost as active as amodiaquine, with IC50 of 9 nM (IQR 6–11) and 8 nM (IQR 6–9), respectively; the DEAQ IC50 was 10 nM. In comparison, the lumefantrine and chloroquine IC50s were 69 nM (IQR 48–119) and 56 nM (IQR 19–91), respectively; a ≥5-fold decrease in activity compared with isoquine. However, the dihydroartemisinin IC50 was significantly lower than that of isoquine (1 nM; IQR 0.9–2.0; P<0.001). Figure 1 shows the IC50 distribution of the drugs. Compared with the tested drugs, which are the most currently used antimalarials, isoquine is the second most active after the artemisinin derivatives and is highly active against the field isolates (median IC50 <10 nM). A similar activity range was observed with the two reference strains tested (V1S and 3D7), which is consistent with a previous report using the laboratory strains K1 and HB3. Its congomer, N-tert-butyl isoquine, showed a similar range of activity against field isolates (from Thailand, Rwanda and Kenya). Recently, a study has shown the in vitro potency of isoquine against P. falciparum gametocytes.

We also analysed pfcrt-76 and pfmdr1-86 genotypes in association with isoquine activity and extended this analysis to amodiaquine and DEAQ. Parasites harbouring pfcrt-76 mutation were significantly less susceptible to isoquine than wild-types [IC50 of 10 nM (IQR 6–22) versus IC50 of 6 nM (IQR 4–10); P=0.012]. On the other hand, mutations in pfmdr1-86 did not significantly affect isoquine activity: the isoquine IC50 was 10 nM (IQR 6–12) against mutants while it was 7 nM (IQR 6–10) against wild-types (P=0.45). In relation to amodiaquine, pfcrt-76 mutant isolates had a significantly higher IC50 than wild-types [8 nM (IQR 6–10) versus 7 nM (IQR 4–8); P=0.007]. The same trend was observed with pfmdr1-86. Indeed, the mutants had significantly higher amodiaquine IC50 than the wild-types [9 nM (IQR 8–10) versus 6 nM (IQR 4–8); P<0.05]. The activity of DEAQ was also influenced by polymorphism in pfcrt-76, with the mutants being less susceptible [IC50 of 13 nM (IQR 8–21)] than the wild-types [IC50 of 6 nM (IQR 8–9); P=0.0001]. The pfmdr1-86 mutant parasites had a significantly higher DEAQ IC50 [9 nM (IQR 8–20)] than the wild-types [IC50 of 8 nM (IQR 6–11); P=0.0146]. Thus, as with chloroquine, amodiaquine
and DEAQ (and discussed in this report for amodiaquine and DEAQ).\(^7,^8,^11\) PfCRT-76 has a bearing on isoquine activity. This, as discussed above, could also be explained by the correlation of these three drugs with isoquine activities. On the other hand, the effect of pfmdr1-86 mutations is more pronounced on the activities of amodiaquine and DEAQ than on that of isoquine.

In relation to chloroquine, mutant pfmdr1-86 alone plays only an ancillary role in resistance. Indeed, this mutation solely does not change chloroquine activity. However, its presence in a background of mutant pfcr7-76 significantly increases chloroquine resistance.\(^7,^8,^11\) Thus, we sought to investigate the relationship between the combined pfcr7-76 and pfmdr1-86 genotypes and isoquine activity. Mutant parasites at both codons had the highest isoquine IC\(_{50}\) [10 nM (IQR 6–20)] while wild-types were the most susceptible [4 nM (IQR 4–9)]; this activity against parasites with the other two combinations (mutant in one gene and wild-type in the other) fell between the two aforementioned extremes. However, none of these differences was statistically significant.

We further assessed the correlation between isoquine and chloroquine, amodiaquine, DEAQ, lumefantrine and dihydroartemisinin. Significant but moderately positive correlation was found with chloroquine (\(r=0.56, P<0.001\)), amodiaquine (\(r=0.6, P<0.001\)) and DEAQ (\(r=0.59, P<0.001\)), while no association was found with lumefantrine and dihydroartemisinin. This moderately positive relationship of isoquine (with chloroquine, DEAQ and amodiaquine) is in line with the fact that the activities of these drugs are decreased in pfcr7-76 mutants. However, it is noteworthy that isoquine is highly active against chloroquine-resistant parasites and, to some extent, DEAQ-resistant parasites. Indeed, this mutation solely does not change chloroquine activity. However, its presence in a background of mutant pfcr7-76 significantly increases chloroquine resistance.\(^7,^8,^11\) This, as discussed below, could also be explained by the correlation of pfcr7-76 and pfmdr1-86 genotypes with decreased susceptibility to lumefantrine and lacks cross-resistance with dihydroartemisinin. If this is confirmed, it could be a good alternative in a background of lumefantrine resistance in the Coartem\textsuperscript{TM} combination.

In summary, we have presented the first evidence of high in vitro activity of isoquine against \(P. falciparum\) field isolates, with IC\(_{50}\) <10 nM (the range of IC\(_{50}\)s that warrant further drug development). This drug also retains potency against chloroquine, DEAQ and lumefantrine reduced-susceptibility parasites. Isoquine has reached Phase I evaluation in humans (S. W., personal communication). Development is currently on hold awaiting the outcome of further toxicological evaluation. If this potential risk is resolved, isoquine could prove a useful addition to the antimalarial armamentarium.

**Acknowledgements**

We thank the Director of the Kenya Medical Research Institute for permission to publish this manuscript.

**Funding**

This study was supported by the European Developing Countries Clinical Trials Partnership (EDCTP). A. N. is thankful to King Fahd University of Petroleum and Minerals, Saudi Arabia (KFUPM) for financial support.

**Transparency declarations**

None to declare.

**References**