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The cytochrome P450 *CYP6P4* is responsible for the high pyrethroid resistance in *knockdown resistance*-free *Anopheles arabiensis*

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ABSTRACT

Pyrethroid insecticides are the front line vector control tools used in bed nets to reduce malaria transmission and its burden. However, resistance in major vectors such as *Anopheles arabiensis* is posing a serious challenge to the success of malaria control.

Herein, we elucidated the molecular and biochemical basis of pyrethroid resistance in a *knockdown resistance*-free *Anopheles arabiensis* population from Chad, Central Africa. Using heterologous expression of P450s in *Escherichia coli* coupled with metabolism assays we established that the over-expressed P450 *CYP6P4*, located in the major pyrethroid resistance (*rp1*) quantitative trait locus (QTL), is responsible for resistance to Type I and Type II pyrethroid insecticides, with the exception of deltamethrin, in correlation with field resistance profile. However, *CYP6P4* exhibited no metabolic activity towards non-pyrethroid insecticides, including DDT, bendiocarb, propoxur and malathion. Combining fluorescent probes inhibition assays with molecular docking simulation, we established that *CYP6P4* can bind deltamethrin but cannot metabolise it. This is possibly due to steric hindrance because of the large vdW radius of bromine atoms of the dihalovinyl group of deltamethrin which docks into the heme catalytic centre.

The establishment of *CYP6P4* as a partial pyrethroid resistance gene explained the observed field resistance to permethrin, and its inability to metabolise deltamethrin probably explained the high mortality from deltamethrin exposure in the field populations of this Sudano-Sahelian *An. arabiensis*. These findings describe the heterogeneity in resistance towards insecticides, even from the same class, highlighting the need to thoroughly understand the molecular basis of resistance before implementing resistance management/control tools.

1. Introduction

Within the last ten years the burden of malaria has been greatly reduced in sub-Saharan Africa thanks to the scale-up of pyrethroid-impregnated long-lasting insecticidal treated nets (LLINs) (WHO, 2014) and indoor residual spraying (IRS) (WHO, 2006). By 2013, these interventions, in addition to chemotherapy, have helped reduce malaria-related mortality in the WHO African region by 54% (WHO, 2014). However, the disease still claimed 584,000 lives in 2013 alone, 90% of which occurred in the WHO African region (WHO, 2014). One of the challenges threatening these malaria intervention tools is the widespread resistance to the major insecticides used in LLINs and IRS, notably from the *Anopheles gambiae* complex and *Anopheles funestus* group (Coetzee and Koekemoer, 2013; Corbel and N'Guessan, 2013).

Across Africa, resistance to insecticides is heterogeneous even

Abbreviations: δ-ALA, δ-aminolevulinic acid; *An.*, Anopheles; cDNA, complementary DNA; CYPED, cytochrome P450 Engineering Database; DDT, dichlorodiphenyltrichloroethane; DDE, dichlorodiphenyldichloroethylene; IPTG, Isopropyl β-D-1-thiogalactopyranoside; NADP, nicotinamide adenine dinucleotide phosphate; ompA, outer membrane protein A; P450cam, P450 camphor hydroxylase; $PLANTS_{PLP}$, *Piece-wise linear potential* Protein-Ligand ANT System; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction; *Rdl*, resistance to dieldrin; *rp1*, resistance to pyrethroids 1.

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some times over relatively small distances (Ranson et al., 2009), thus implementation of any resistance management demands sound knowledge of dominant vector species distribution, behaviours, insecticide susceptibility/resistance status, and most importantly the molecular mechanisms of the resistance (Coetzee et al., 2000; Corbel and N'Guessan, 2013; Gatton et al., 2013).

In insects, two major mechanisms of resistance to insecticides have been described: (i) metabolic resistance due to overexpression and/or increase in the activity of the major enzymes involved in detoxification of insecticides (Hemingway and Ranson, 2000), and (ii) target-site insensitivity, which results in decreased sensitivity of the molecular target of the insecticide through point mutations, as in the voltage-gated sodium channel (*kdr* mutations), acetylcholinesterase (*ace-1* mutation) or the gamma-amino butyric acid mutation (*Rdl* mutation) (Du et al., 2005; Ffrench-Constant et al., 2000; Martinez-Torres et al., 1998; Ranson et al., 2000; Weill et al., 2004; Wondji et al., 2011). Recent evidence has stressed the preeminent role of metabolic resistance as the most important mechanism of resistance in the major Anopheline mosquito vectors (Hemingway, 2014) with cytochrome P450s especially from the CYP6 family taking the front seat in conferring resistance to the four major insecticides used for public health interventions (Duangkaew et al., 2011; Edi et al., 2014; Riveron et al., 2014, 2013).

Besides *An. gambiae*, *An. arabiensis* is the most efficient malarial parasite vector of the *An. gambiae* complex (Gilles and De Meillon, 1968) and in some places, especially the drier savannah, it remains the dominant vector species. Prospect of control of *An. arabiensis* through exploitation of indoor resting and feeding behaviours is confounded by its marked plastic behaviours, including marked zoophily, exophily and exophagy (Coetzee et al., 2000; Durnez and Coosemans, 2013; Sinka et al., 2011). There is also growing concern over the great role *An. arabiensis* is playing in residual malaria transmission even in settings where robust malaria control tools are effectively implemented (Durnez and Coosemans, 2013; Killeen, 2014). *An. arabiensis* is the dominant vector species in Chad, Central Africa where it's reported to be resistant to pyrethroids but susceptible to the carbamate bendiocarb and organophosphates, malathion and fenitrothion (Kerah-Hinzoumbe et al., 2008; Ranson et al., 2009; Witzig et al., 2013).

In 2009, the *An. arabiensis* populations from Ndjamena (*Ndja*), Chad were resistant to permethrin (Type I pyrethroid) and DDT (an organochlorine), only moderately resistant to a Type II pyrethroid deltamethrin (90% mortality rate), but susceptible to bendiocarb (a carbamate), malathion (an organophosphate) and dieldrin (an organochlorine) (Witzig et al., 2013). No 1014F or 1014S *kdr* mutations were detected in the *Ndja* population and the PBO synergist assay fully restored susceptibility to pyrethroids, suggesting metabolic resistance as the cause of pyrethroid resistance. Witzig and colleagues identified a major pyrethroid resistance QTL (*rp1*) in the 2R chromosomal arm which alone explained a quarter of the genetic variance to permethrin resistance. The QTL was enriched in P450s, some of which are orthologs of genes implicated in pyrethroid resistance in *An. gambiae* and *An. funestus* (Edi et al., 2014; Wondji et al., 2009). A qRT-PCR analysis revealed one of the CYP genes (*CYP6P4*) to be up-regulated 22-fold in the permethrin resistant populations compared with the susceptible populations. The role of this P450 in conferring metabolic resistance to *An. arabiensis* was also recently pointed out in a population from neighbouring Sudan, where microarray-based transcription profiling detected *CYP6P4* as one of the most over-expressed detoxification genes (Abdalla et al., 2014). However, there is so far no functional evidence that the *An. arabiensis CYP6P4* is responsible for the metabolic resistance toward the pyrethroids. In case *CYP6P4* is playing a role it remains unknown as to why the same population exhibited only a moderate resistance to deltamethrin. It also becomes imperative to establish whether *CYP6P4* is a cross-resistance gene, able to confer both Type I pyrethroid and DDT resistance.

To fill these gaps in knowledge, we performed a functional characterisation of the *CYP6P4*, establishing that it is the major P450 responsible for pyrethroid resistance in the *kdr*-free population of *An. arabiensis* from Chad. Using a combination of heterologous expression and *in vitro* characterisation we demonstrated the role of this P450 in metabolism of Type I and Type II pyrethroids with the exception of deltamethrin. Combining homology modelling and molecular docking simulations we established why this P450 could not metabolize deltamethrin, dissecting the molecular basis of deltamethrin susceptibility in these Chadian *An. arabiensis* populations.

2. Methods

2.1. Mosquito samples

The mosquitoes used in this research were adult, female *An. arabiensis*, field collected from Ndjamena (12° 6′ N, 15° 2′ E) by Witzig and colleagues (Witzig et al., 2013) and established as the *Ndja* colony in the Liverpool School of Tropical Medicine, UK. The populations were confirmed as *An. arabiensis* species using PCR (Scott et al., 1993). Susceptibility status, biochemical assays, QTL mapping and analysis of the expression pattern of the P450s spanning the *rp1* QTL of this population are given in detail in the above publication (Witzig et al., 2013).

2.2. Amplification and cloning of full length cDNA of An. arabiensis CYP6P4

RNA was extracted using the PicoPure RNA isolation Kit (Arcturus, Applied Biosystems, USA) from three pools of 10 permethrin-resistant female mosquitoes from *Ndja* as described in (Witzig et al., 2013). cDNA was synthesized from extracted RNA using SuperScript III (Invitrogen, USA) with oligo-dT20 and RNAse H (New England Biolabs, USA). Full length coding sequences of *CYP6P4* were amplified separately from cDNA of 10 mosquitoes using the Hot Start II Taq Polymerase (Thermo Fisher, UK) and the primers in Supplementary Table S1. To 14 μl PCR mix made up of 5× Phusion HF Buffer (with 1.5 mM $MgCl_2$ in final reaction), 85.7 μM dNTP mixes, 0.34 μM each of forward and reverse primers, 0.015U of Phusion High-Fidelity DNA Polymerase (Fermentas, Massachusetts, USA) and 10.71 μl of dH_20, 1 μl cDNA was added. Amplification was carried out using the following conditions: one cycle at 95 °C for 5 mins; 35 cycles of 94 °C for 20s (denaturation), 57 °C for 30s (annealing), and extension at 72 °C for 90s; and one cycle at 72 °C for 5 mins (final elongation).

PCR products were cleaned individually with QIAquick® PCR Purification Kit (QIAGEN, Hilden, Germany) and cloned into pJET1.2/blunt cloning vector using the CloneJET PCR Cloning Kit (Fermentas). These were then cloned into the *Escherichia coli DH5α,* plasmids miniprepped with the QIAprep® Spin Miniprep Kit (QIAGEN) and sequenced on both strands using the above primers.

2.3. Cloning and heterologous expression of recombinant An. arabiensis CYP6P4 in E. coli

The pJET1.2 plasmid bearing the full length coding sequence of *CYP6P4* was utilised to prepare the gene for expression. *CYP6P4* was prepared by fusing cDNA fragment from a bacterial *ompA+2* leader sequence with its downstream ala–pro linker to the NH_2-terminus of its cDNA, in frame with its initiation codon, as described (Pritchard et al., 1997). This is achieved in a PCR reaction using the