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Cross-resistance profiles of malaria mosquito P450s associated with pyrethroid resistance against WHO insecticides

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ABSTRACT

Extensive use of pyrethroids for malaria control in Africa has led to widespread pyrethroid resistance in the two major African vectors of malaria *An. gambiae* and *An. funestus*. This is often associated with constitutively elevated levels of cytochrome P450s involved with pyrethroid metabolism and detoxification. P450s have the capacity to metabolise diverse substrates, which raises concerns about their potential to cause cross-resistance. A bank of seven recombinant P450s from *An. gambiae* (CYPs 6M2, 6P2, 6P3, 6P4, 6P5, 9J5) and *An. funestus* (CYP6P9a) commonly associated with pyrethroid resistance were screened against twelve insecticides representing the five major classes of insecticides recommended by WHO for malaria control; permethrin, etofenprox and bifenthrin (type I pyrethroids), deltamethrin, lambda cyhalothrin and cypermethrin (type II pyrethroids), DDT (organochlorine), bendiocarb (carbamate), malathion, pirimiphos methyl and fenitrothion (organophosphates) and pyriproxyfen (juvenile hormone analogue). DDT was not metabolised by the P450 panel, while bendiocarb was only metabolised by CYP6P3. Pyrethroids and pyriproxyfen were largely susceptible to metabolism by the P450 panel, as were organophosphates, which are activated by P450s. Primiphos-methyl is increasingly used for malaria control. Examination of the pirimiphos-methyl metabolites generated by CYP6P3 revealed both the active pirimiphos-methyl-oxon form and the inactive oxidative cleavage product 2-diethylamino-6-hydroxy-4-methylpyrimidine. The inhibition profile of CYPs 6M2, 6P2, 6P3, 6P9a and 9J5 was also examined using diethoxyfluorescein (DEF) as the probe substrate. Bendiocarb was the weakest inhibitor with IC_{50} > 100 μM across the P450 panel, while CYP6M2 showed strongest inhibition by malathion (IC_{50} 0.7 μM). The results suggest that P450s present at elevated levels in two major Anopheline vectors of malaria in Africa have the capacity to metabolise a diverse range of pyrethroid and organophosphate insecticides as well as pyriproxyfen that could impact vector control.

1. Introduction

Cases of malaria transmitted by Anopheline mosquitoes in Africa have halved since the millennium due to the use of long-lasting insecticide treated bednets (LLINs) as well as indoor residual spraying (IRS) of houses and improved drug therapy (Bhatt et al., 2015). Since 2000 over a billion LLINS have been distributed across the continent, however, with pyrethroids being the predominant insecticide class approved for use with LLINs, pyrethroid resistance is now widespread in *An. gambiae* and *An. funestus* (J Hemingway et al., 2016a) the two major vectors of malaria. Two alternative classes of insecticide, organochlorines and carbamates, recommended for IRS by the World Health Organization are increasingly being used for IRS. However, mosquito populations are already showing resistance to these as well (Ranson and Lissenden, 2016), most notably in Cote d'Ivoire and Mali with resistance to all four classes (Cisse et al., 2015; Ranson et al., 2012). Pyriproxyfen, a juvenile hormone analogue, and chlorfenapyr, a pyrrole insecticide, are being trialled for use with bednets (Bayili et al., 2017; Tiono et al.,

Abbreviations: b_5, cytochrome b_5; CPR, cytochrome P450 reductase; CYP, cytochrome P450; DEF, diethoxyfluorescein; IRS, indoor residual spray; LLIN, long-lasting insecticide treated bednets

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2015), while novel chemistries are due to enter the market in the next few years (Hemingway et al., 2016b). However, with resistance mechanisms in African mosquitoes primed by exposure to pyrethroids, early identification of potential cross-resistance issues is an important consideration in the delivery of insecticides for vector control.

A number of different mechanisms can lead to insecticide resistance including target site insensitivity, cuticular thickening, behavioural avoidance and biodegradation (Hemingway et al., 2004; Li et al., 2006; Wood et al., 2010). The latter is driven by the constitutive overexpression of detoxifying enzymes (metabolic resistance) and frequently associated with the overexpression of cytochrome P450 enzymes (CYPs) (David et al., 2013). In the past decade transcriptome studies have shown CYPs 6P3 and 6M2 to be commonly over expressed in pyrethroid resistant populations of *An. gambiae* (David et al., 2013; Paine and Brooke, 2016), while CYPs 6P9a, 6P9b and 6M7 in *An. funestus* are frequently associated with pyrethroid resistance (Coetzee and Koekemoer, 2013; Riveron et al., 2013; Wondji et al., 2009). Since P450s can metabolise structurally diverse substrates, populations of pyrethroid resistant mosquitoes with constitutively elevated P450 levels may have the capacity to metabolise other insecticide classes, potentially leading to cross-resistance. Indeed, CYP6P3 has been shown to metabolise bendiocarb (Edi et al., 2014) as well as pyriproxyfen (Yunta et al., 2016) along with six other P450s associated with pyrethroid resistance (CYPs 6M2, 6P2, 6P3, 6P4, 6P5 and 6Z2) (Yunta et al., 2016). This highlights the potential for pyrethroid metabolising P450s to impact vector control using other classes of insecticides. Conversely, elevated levels of P450 activity could promote negative cross-resistance in metabolically activated organophosphates such as malathion, fenitrothion and pirimiphos-methyl. This is particularly relevant for pirimiphos-methyl, which has been reformulated as Actellic® 300CS to increase longevity on sprayed walls (Kanyangarara et al., 2016; Oxborough et al., 2014; Rowland et al., 2013) and is now widely used for IRS operations to control pyrethroid resistant mosquito strains in Africa (Oxborough, 2016).

In vitro screening for drugs metabolised or inhibited by recombinant P450 enzymes is well established as a means of early identification of potential P450-mediated drug interactions *in vivo* (Wienkers and Heath, 2005). Here, we have established a bank of six recombinant P450s that have been found overexpressed in pyrethroid resistant populations of *An. gambiae* (CYPs 6M2, 6P2, 6P3, 6P4, 6P5, 9J5) and one that is frequently associated with pyrethroid resistance in *An. funestus* (CYP6P9a). They were used to examine the metabolism and inhibition of compounds representing five major classes of insecticides recommended by WHO for adult mosquito control (Fig. 1). The results provide a profile of P450-mediated insecticide interactions *in vitro* that may help inform the prediction of potential resistance liabilities in the field.

2. Materials and methods

2.1. Reagents

Oligonucleotides were synthesized by Eurofins genomics and enzymes for DNA manipulation were supplied by Thermo Scientific. Isopropyl-ß-D-thio-galactopyranoside (IPTG), 5-aminolevulinic acid (ALA), and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) were supplied by Melford (UK). Insecticides were supplied by ChemService: 1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane (DDT), (*S*)-α-cyano-3-phenoxybenzyl (1*R*,3*R*)-*cis*-2,2-dimethyl-3-(2,2-dibromovinyl)-cyclopropanecarboxylate (deltamethrin) and 3-phenoxybenzyl (1*R,S*)-*cis,trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate (permethrin, mixture of isomers). HPLC solvents were supplied by Fisher Scientific. Other chemicals were obtained from Sigma-Aldrich unless indicated otherwise.

2.2. Gene cloning

The pCWori + expression vector was used for the production of *An. gambiae* CYPs 6M2 and 6P3, 6P4, 6P5, 9J5 as previously described (Yunta et al., 2016). The cDNA sequence encoding CYP6P9a (GenBank ID: AY729661, previously named CYP6P9) was isolated by RT-PCR using RNA purified from *An. funestus* FUMOZ as described (Matambo et al., 2010). Our first attempt to express CYP6P9a used the *E. coli* ompA leader sequence and pCW-ori + as previously described for CYP6Z2 (Mclaughlin et al., 2008) were unsuccessful. Therefore we used another common strategy for P450 expression, which is to replace the natural P450 amino-terminus with a sequence (MALLLAVF) derived from the bovine steroid 17 α-hydroxylase (Barnes et al., 1991). To introduce the amino-terminal 17α modification the 5′-end of CYP6P9a cDNA was amplified using KOD DNA polymerase (Novagen) with the forward primer: 5′-TTCATATGG**CT**CT**GT**T**ATTAGCA**GTTTTT**G**CCGCG TTCATCTTCGTAG-3′ (*Nde*I restriction site at the initiation codon, underlined), and the reverse primer 5′- CGCTCTAGACTACAACTTTTCCA CCTTC-3′ (with an *Xba*I restriction site, underlined). The resulting 17α-CYP6P9 was ligated into pCWmod1 *via* *Nde*I and *Xba*I to create pCW::17α-CYP6P9a. The construct was sequenced and the amino acid sequence matches CYP6P9a (GenBank: ACG68818.1) (Wondji et al., 2009). A single plasmid expression system for the P450 and CPR was constructed by ligating the expression cassette (cDNA and *tactac* promotor) containing the *An. gambiae* NADPH P450 reductase (CPR) cDNA from the pACYC:AgCPR plasmid described above into the pCW:P450 expression plasmid. Briefly, the expression cassette containing the promoter and Ag CPR sequence was cut from a pCW based expression vector with BamHI and ligated into BamHI digested pCW::17α-CYP6P9a. This yielded a plasmid with the Ag CPR and CYP6P9a expression cassettes in a 'head to tail' orientation with the CYP6P9a cassette following the Ag CPR cassette. In the new construct, although the expression of each of Ag CPR and CYP6P9a is under the control of its own promoter, the addition of IPTG to the culture induces the expression of both as the control in each case is a *tactac* promoter.

2.3. Production of E. coli membranes co-expressing P450 and AgCPR

CYPs 6M2, 6P3 and 6P9a were co-expressed with AgCPR from a single plasmid, while CYPs 6P4, 6P5, 9J5 were co-expressed with AgCPR following co-transformation of competent *E. coli* DH5α cells with pCW:P450 plasmid and pACYC:*AgCPR*. Cultures were supplemented with 1.0 mM 5-aminolevulinic acid (heme precursor) and incubated at 23 °C for 18–24 h after 1 mM IPTG induction. P450 expression, *E. coli* membrane isolation and determination of P450 and AgCPR content was performed as previously described (Yunta et al., 2016). Samples were stored in aliquots at −80 °C. *An. gambiae* cytochrome b_5 (b_5) was prepared as described previously to supplement enzyme reactions at a 10:1 M ratio, b_5:P450 (Stevenson et al., 2011).

2.4. Insecticide metabolism

P450s were tested against the insecticides from the WHO recommended list of insecticides for public health use. Catalytic activity was assessed by measuring substrate turnover (disappearance of substrate with time). 10 mM stock concentrations of insecticides were prepared in methanol or acetonitrile and diluted to 10× the assay concentration in 20% (*v*/v) methanol or acetonitrile immediately before each experiment to minimise precipitation of insecticide. Standard reactions contained a final organic solvent concentration of 2% (v/v) with 20 μM insecticide, 0.1 μM P450, 1.0 μM cyt b5 in 200 mM Tris-HCl for pH 7.4, and NADPH regeneration components (1 mM glucose-6-phosphate (G6P), 0.25 mM $MgCl_2$, 0.1 mM $NADP^+$, and 1 U/mL Glucose-6-phosphate dehydrogenase (G6PDH)). These were incubated for a specified time at 30 °C with 1200 rpm orbital shaking and quenched by adding 0.2 mL of acetonitrile or methanol. Samples were then

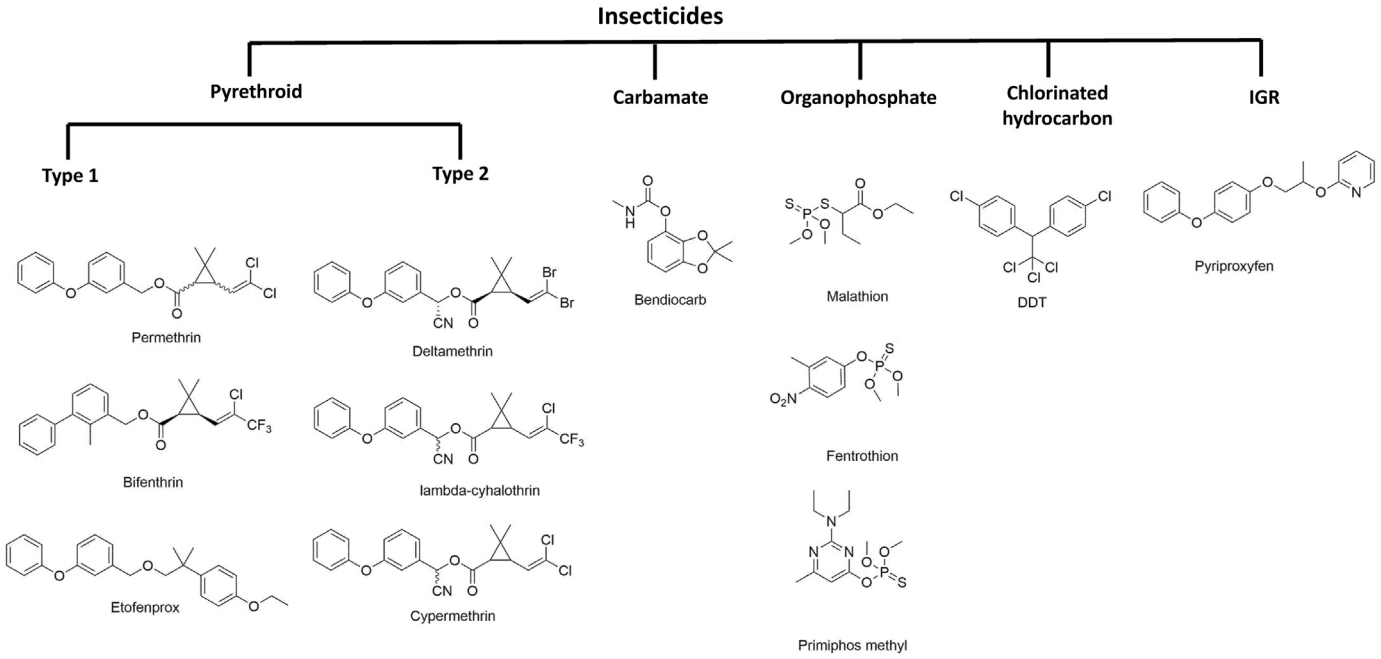

Fig. 1. Chemical structures of WHO insecticides used in this study.

incubated with shaking as before for an additional 10 min before centrifuging at 20,000 × *g* for 5 min. 0.15 mL of the supernatant was then transferred to HPLC vials, stored at room temperature and analyzed within 24 h. Reactions were performed in triplicate and compared against a negative control with no NADPH regenerating system to calculate substrate depletion.

2.5. HPLC analysis

100 µL of organic solvent-quenched reaction supernatant was analyzed by reverse-phase HPLC with a 250 mm C18 column (Acclaim 120, Thermo Scientific) and a mobile phase optimized for each insecticide (Table S1). The system was set at 23 °C with 1 mL/min flow rate except for bendiocarb it was performed at 40 °C. Reactions with permethrin, deltamethrin, etofenprox, bifenthrin or bendiocarb were monitored by absorbance at 226 nm whereas DDT, malathion, pyriproxyfen, pirimiphos-methyl and fenitrothion were monitored at 232 nm. The insecticide was quantified by peak integration (OpenLAB Chromatography Data System). Elution times for all insecticides used are summarized in Table S1. In the case of stereoisomers in the insecticide mix, the insecticide concentrations were measured as the total area under the two peaks.

2.6. Diethoxyfluorescein metabolism and IC_{50} analysis

Variable ligand concentrations were used for IC_{50} calculations with DEF used at ~ K_M for each P450 (*i.e.* 0.5, 1.4, 0.7, 1.0, 3.5 and 0.5 µM for CYP6M2, CYP6P2, CYP6P3, CYP6P4 and CYP9J5 respectively) and 0.1 µM P450. For calculation of the kinetic parameters (K_M and V_{max}), each P450 was used at a final concentration of 10 nM (1 pmol/reaction) and DEF concentrations in the range: 0, 0.31, 0.63, 1.25, 2.5, 5, 10 and 20 µM. DEF reactions were carried out at 25 °C in 50 mM KPi at pH 7.4 containing 1 mM glucose-6-phosphate (G6P), 0.1 mM $NADP^+$, 0.25 mM $MgCl_2$, and cytochrome b_5 at a 10:1 M ratio, b_5:P450. $NADP^+$ and G6P were excluded from the minus NADPH controls. For IC_{50} analysis, three replicates of positive and negative control reactions were run for each P450/substrate combination in opaque white 96-well (flat-based) plates in triplicate. The Michaelis-Menten and IC_{50} fitting calculations were performed using Graphpad Prism 6. Data were fitted to the dose-response model and plots with $R^2 < 0.95$ were rejected.

2.7. Mass spectrometry analysis for pirimiphos-methyl metabolism

The chromatographic-mass spectrometric analysis was performed on a high resolution Thermo Q-Exactive mass spectrometer (MS) which was coupled to a 1290 series Agilent LC system. Separation of pirimiphos-methyl and its metabolites was carried out on a Waters Acquity BEH C18 (2.1 × 50 mm; 1.7 µm) analytical column using water (0.1% formic acid) (A) and acetonitrile (0.1% formic acid) (B) as mobile phases in a 12-min gradient program: 0–1 min (5% B); 1–8 min (5% B to 100% B); 8–10 min (100% B); 10–10.1 min (100% B to 5% B); 10.1–12 min (5% B). A sample volume of 5 ul was injected. Throughout the chromatographic run time (12 min) a mass range of 100–1000 *m/z* at 35 K resolution was monitored in full scan positive ion mode. The MS conditions for the analysis (heated electrospray capillary temperature, sheath gas, auxiliary gas flow rate, spray voltage) were constantly maintained at 320 °C, 55 (arbitrary units), 10 (arbitrary units), 3500 V, respectively.

3. Results

3.1. Profiling insecticide metabolism

CYPs 6P2, 6P4, 6P5 and 9J5 were previously cloned into pCW-ori + and co-transformed with AgCPR-pACYC for small-scale (0.1–0.2 L) *E. coli* expression (Yunta et al., 2016); co-expression of *An. gambiae* NADPH-cytochrome P450 oxidoreductase (AgCPR) being required for coupled electron transfer from NADPH to P450 for catalysis. CYPs 6 M2, 6P3 and 6P9a were expressed in tandem with AgCPR from a single pCW-ori + plasmid to facilitate routine larger scale enzyme production (10–20 L fermentor); these P450s were amongst the earliest pyrethroid resistance markers to be functionally validated and most heavily used for *in-vitro* screening. The relative P450 contents and CPR activities are provided in Table S2.

The catalytic activity of the P450s were assessed by measuring insecticide turnover against twelve WHO recommended insecticides in the presence and absence of NADPH. The insecticide classes and individual active ingredients tested included type I pyrethroids

Table 1
Pyrethroid metabolism by mosquito P450s.

P450	% Insecticide depletion					
	Pyrethroids type I			Pyrethroids type II		
	Permethrin	Etofenprox	Bifenthrin	Deltamethrin	λ-cyhalothrin	Cypermethrin
Single plasmid						
CYP6M2[a]	58.5 ± 2.2	68.8 ± 1.1	38.9 ± 1.6	55.4 ± 1.4	49.4 ± 0.5	36.8 ± 1.8
CYP6P3[a]	100.0 ± 0.0	99.8 ± 0.3	76.7 ± 0.3	98.2 ± 0.2	83.3 ± 15.4	98.4 ± 0.1
CYP6P9a[a]	87.8 ± 0.7	98.5 ± 0.2	53.4 ± 1.6	97.0 ± 0.2	67.1 ± 3.1	89.5 ± 2.7
Dual plasmid						
CYP6P2	53.8 ± 1.6	75.5 ± 4.2	nd	68.4 ± 4.8	nd	nd
CYP6P4	88.5 ± 0.4	94.0 ± 0.9	nd	88.5 ± 1.4	nd	nd
CYP6P5	56.8 ± 5.9	24.4 ± 5.5	nd	47.0 ± 1.9	nd	nd
CYP9J5	76.9 ± 1.2	98.9 ± 0.2	nd	58.1 ± 4.0	nd	nd

nd, not determined.
[a] Incubation time 2 h instead 90 min.

(permethrin, etofenprox and bifenthrin), type II pyrethroids (deltamethrin, lambda cyhalothrin and cypermethrin), an organochlorine (DDT), a carbamate (bendiocarb), organophosphates (malathion, pirimiphos methyl and fenitrothion) and a juvenile hormone analogue (pyriproxyfen). A cut-off value of 20% substrate depletion was used to distinguish true substrate turnover from baseline variability (Jones and Houston, 2004).

Results of pyrethroid metabolism are presented in Table 1. All pyrethroid compounds were metabolised to some degree by the P450 panel. *Anopheles gambiae* CYP6P3 produced > 75% substrate depletion for all pyrethroid substrates. *Anopheles funestus* CYP6P9a was similar, apart from lower levels of bifenthrin and lambda-cyhalothrin turnover (53.4 and 67.1% respectively). CYP6P4 demonstrated strong activity against deltamethrin, while CYPs 6P2, 6P5 and 9J5 produced lower, but reproducible deltamethrin turnover (68.4, 47.0 and 58.1% respectively). Permethrin produced similar results except for CYP9J5, which displayed high activity comparable to CYP6P4. Etofenprox was strongly metabolised by all except for CYP6P5.

When tested against the other classes of insecticide (Table 2) none of the P450s showed significant activity against DDT (organochlorine) while only CYP6P3 metabolised bendiocarb (35% substrate depletion). *An. gambiae* CYPs 6M2, 6P2, 6P3, 6P4, 6P5, and 9J5 have previously been shown to metabolise pyriproxyfen (Yunta et al., 2016). *An. funestus* CYP6P9a also metabolised the juvenile growth hormone (84.34 ± 0.77% depletion) (Table 2). Except for CYP6M2 and pirimiphos-methyl (8.5 ± 1.4% depletion) the three organophosphates malathion, pirimiphos-methyl and fenitrothion were metabolised by all the P450s tested.

Pirimiphos-methyl is becoming widely used for IRS operations. It requires P450 activation into the insecticidal pirimiphos-methyl oxon form to inhibit acetylcholinesterase, but can also undergo P450 mediated detoxifying dearylation reactions (Fig. 2). In order to investigate this further, LC-MS analysis was performed on the products of pirimiphos-methyl metabolism generated by *An. gambiae* CYP6P3 (Fig. 3), which represents one of the P450s most commonly overexpressed in pyrethroid resistant populations of *An. gambiae* (Ingham et al., 2018). Known metabolites 2-diethylamino-6-hydroxy-4-methylpyrimidine and its des-N-ethyl analogue, pirimiphos-methyl oxon and des-N-ethylpirimiphos-methyl were targeted using accurate mass technology (Roberts and Hutson, 1999). Extracted ion chromatograms of $[M + H]^+$ indicated the presence of the insecticidal pirimiphos-methyl oxon (*m/z* 290.1264) and the inactive 2-diethylamino-6-hydroxy-4-methylpyrimidine (*m/z* 182.1287) although confirmation of metabolite identification for the latter was limited by a lack of analytical reference standards in our study (Fig. 3).

3.2. Inhibition screening of WHO insecticide panel

Fluorescent substrate based inhibition screening of P450s is routinely used in the pharmaceutical industry for investigating drug-drug interactions with P450s. As well as being a rapid method of screening for insecticide interactions with mosquito P450s, the relative strengths of inhibition of insecticides can indicate potential synergistic effects. The fluorogenic probe DEF was chosen as a general probe as it is metabolised by all of the P450s apart from CYP6P4 and CYP6P5. (Yunta et al., 2016). The ability of the panel of insecticides to inhibit the mosquito P450s was assayed and compared with piperonyl butoxide (PBO), a P450 inhibitor widely used in insecticide formulations as a synergist (Table 3). The compounds were categorized according to their activity as P450 inhibitors as potent (IC_{50} < 1 μM), moderate (IC_{50} 1–10 μM) and weak inhibitors (IC_{50} > 10 μM) (Krippendorff et al., 2007). PBO was the strongest inhibitor with IC_{50} values ranging from

Table 2
Non pyrethroid metabolism by P450s.

P450	% Insecticide depletion					
	DDT	Bendiocarb	Malathion	Pirimiphos-methyl	Fenithrothion	Pyriproxyfen
Single plasmid						
CYP6M2	2.9 ± 2.1	0 ± 11.2	26.8 ± 8.8	8.5 ± 1.4	35.4 ± 10.4	30.93 ± 4.7[a]
CYP6P3	4.1 ± 5.6	35.0 ± 2.5	68.8 ± 8.4	100.0 ± 0.0	58.2 ± 11.7	100.0 ± 0.0[a]
CYP6P9a	3.9 ± 2.3	10.7 ± 23.7	31.0 ± 4.1	42.0 ± 7.0	41.9 ± 10.3	84.34 ± 0.77
Dual plasmid						
CYP6P2	5.1 ± 3.7	5.2 ± 1.6	30.3 ± 9.7	50.6 ± 10.1	21.9 ± 9.7	58.03 ± 1.4[a]
CYP6P4	1.4 ± 1.5	6.8 ± 4.2	44.4 ± 10.1	47.0 ± 16.2	42.8 ± 14.9	81.63 ± 0.6[a]
CYP6P5	0.6 ± 0.3	7.9 ± 2.9	24.2 ± 3.6	23.6 ± 7.5	33.1 ± 12.1	39.96 ± 1.0[a]
CYP9J5	2.6 ± 2.5	8.1 ± 1.4	66.7 ± 9.2	95.7 ± 2.5	50.7 ± 6.5	24.78 ± 2.1[a]

[a] Published in Yunta et al. (2016).

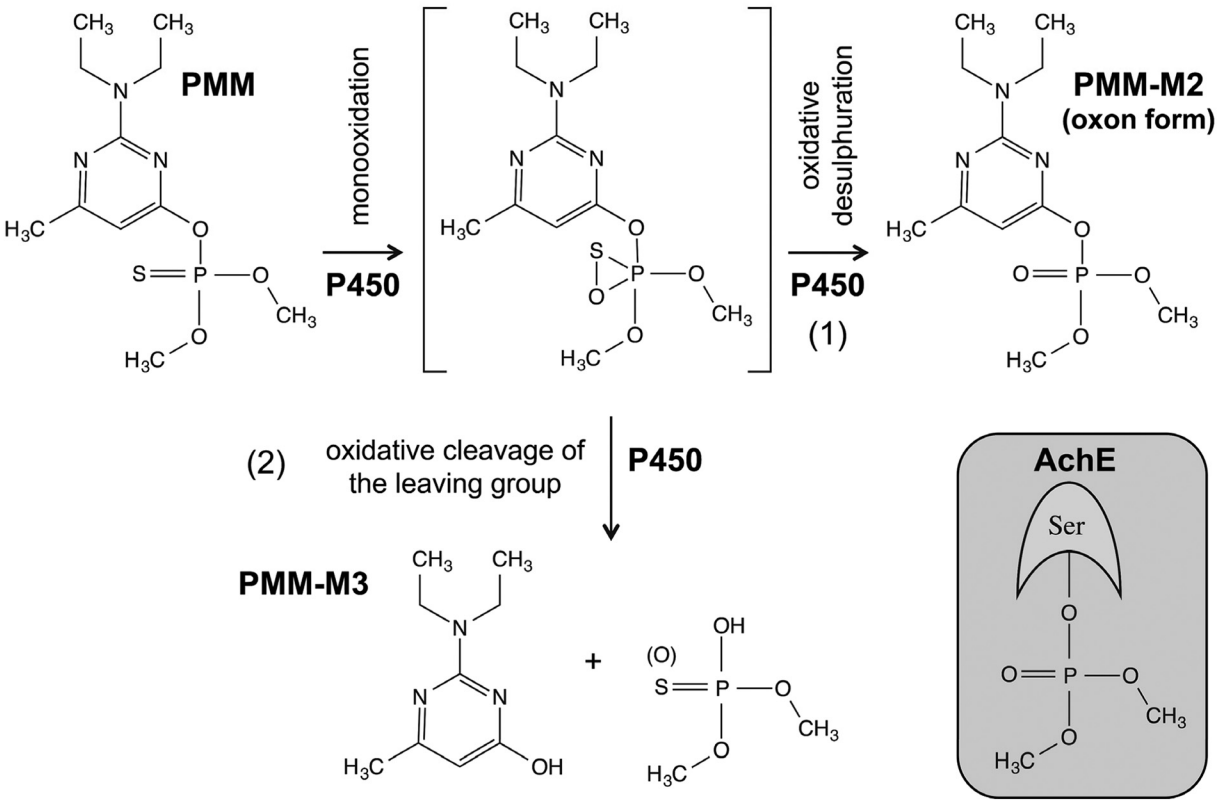

Fig. 2. Metabolism pathway of pirimiphos-methyl. Following an insertion of oxygen into the pirimiphos-methyl molecule, a reactive intermediate collapses (1) by desulfuration, or (2) by cleavage of the ester linkage leading to PMM-M2 and PMM-M3 respectively. Alternative routes of P450 metabolism of pirimiphos-methyl that can produce other metabolites are not shown. The oxon form inhibits the AchE by forming covalent bound with a serine residue at the active site. PMM, pirimiphos-methyl; PMM-M2, pirimiphos-methyl; PMM-M3, 2-diethylamino-6-hydroxyl-4-methylpyrimidine and AchE, acetilholinesterase.

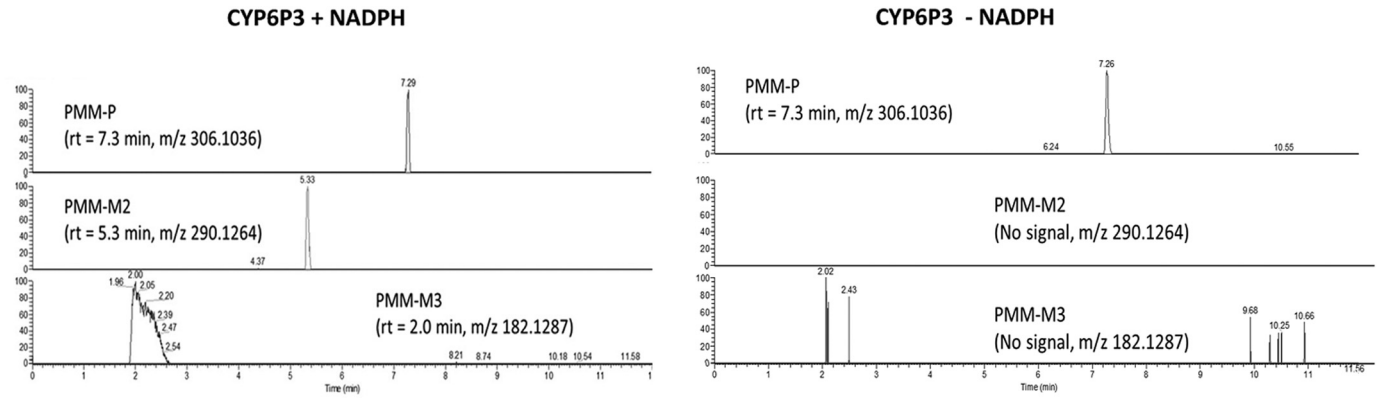

Fig. 3. Extracted ion chromatograms $[M + H]^+$ from CYP6P3 membranes. Pirimiphos-methyl parent (PMM—P) 7.3 min (*m/z* 306.1036) observed in the presence (+) and absence (−) of NADPH. Metabolites pirimiphos-methyl oxon (PMM-M2) 5.26 min (290.1264) and 2-diethylamino-6methy-4-hydroxy-pyrimidine (PMM-3) 2.0 min (*m/z* 182.1287) observed in the presence of NADPH. Signals within the extracted ion chromatograms at different retention times correspond to low level background ions and/or electronic spikes.

0.3 μM with CYP6M2 to 3.9 μM for CYP9J5. Pyrethroids and DDT were moderate to weak inhibitors in the range 3–17 μM. The inhibition profile of OP insecticides varied from potent inhibition of CYP6M2 by malathion (IC_{50} = 0.7 μM) to weak inhibition by fenitrothion of CYP9J5 (IC_{50} = 48.9 μM). Bendiocarb was the weakest inhibitor producing IC_{50} values in the range 100 to > 1000 μM.

4. Discussion

P450s play a key role in the metabolism and disposition of insecticides in mosquito vectors of malaria. Since P450s are capable of metabolising structurally diverse substrates, P450 mediated cross resistance between insecticide classes is a major concern (Edi et al., 2014). Having recently demonstrated that six *An. gambiae* P450s (CYPs 6M2, 6P2, 6P3, 6P4, 6P5, and 9J5) associated with pyrethroid resistance can metabolise pyriproxyfen (Yunta et al., 2016), we have further examined their ability to metabolise a wider range of insecticides representing five major classes of insecticide recommended by WHO for vector control. We have also extended the panel of recombinant enzymes to include CYP6P9a from *An. funestus* (Matambo et al., 2010; Riveron et al., 2013; Wondji et al., 2009). Overall, there was limited activity against DDT and carbamate compounds but extensive enzyme cross-reactivity against all the pyrethroid and organophosphate compounds tested and the juvenile hormone inhibitor

Table 3
IC_{50} values (μM) of WHO insecticides.

Insecticide	IC_{50} values (μM)				
	CYP6M2	CYP6P3	CYP6P9a	CYP6P2	CYP9J5
Deltamethrin	4.2[a]	3.2[a]	2.6	5.0[a]	6.1[a]
Permethrin	8.1[a]	6.8[a]	5.7	8.6[a]	6.5[a]
λ-Cyhalothrin	6.6	9.6	3.7	4.8	4.2
Cypermethrin	1.5	2.6	4.2	9.8	13.5
Bifenthrin	5.6	3.5	8.5	10.7	2.5
Etofenprox	12.0	8.3	8.1	4.7	8.0
Pirimiphos	4.3	3.0	1.7	14.9	5.3
Malathion	0.7	8.0	1.9	17.6	26.9
Bendiocarb	> 1000	185.2	102.9	> 1000	> 1000
DTT	8.7	3.1	3.1	13.4	13.3
Fenitrothion	11.5	22.5	13.0	26.7	48.9
PBO	0.3	2.0	1.2	2.7	3.9
Pyriproxyfen	14.1[a]	15.8[a]	9.9	9.9[a]	19.9[a]

[a] Published in Yunta et al. (2016).

pyriproxyfen (Yunta et al., 2016).

Deltamethrin and permethrin are the most commonly used pyrethroids for LLINs. Both were metabolised by *An gambiae* CYP6M2, CYP6P3, and *An funestus* CYP6P9a, consistent with a role in detoxification as previously reported (Muller et al., 2008; Riveron et al., 2013; Stevenson et al., 2011). Furthermore, these P450s were also capable of metabolising etofenprox, bifenthrin λ-cyhalothrin and cypermethrin to varying degrees. The orthologues *An. gambiae* CYP6P3 and *An. funestus* CYP6P9a, produced particularly high levels of depletion across the pyrethroid range, with lowest substrate depletion levels observed for bifenthrin (77 and 53% respectively), although this may be explained by the high levels of CPR contained in these membranes. A further four *An gambiae* P450s that are overexpressed in pyrethroid resistant populations (CYP6P2, CYP6P4, CYP6P5 and CYP9J5) (Edi et al., 2014; Hemingway et al., 2013; Toé et al., 2015) were confirmed to metabolise deltamethrin, permethrin and etofenprox, with highest levels of activity observed with *An. gambiae* CYP6P4 and CYP9J5. *An. arabiensis* CYP6P4 has previously been reported to selectively metabolise permethrin but not deltamethrin (Ibrahim et al., 2016). Since *An. gambiae* and *An. arabiensis* CYP6P4 are identical in amino acid sequence, differences in substrate metabolism might reflect different cytochrome *b*5 ratios used and/or reaction times as we used a high P450: b5 ratio (1:10), and an extended reaction time (90 min). Bifenthrin and λ-cyhalothrin tended to produce lower turnover values. Both are fluorinated molecules, which limits sites of P450 metabolism, while bifenthrin is structurally constrained by a rigid bi-phenyl alcohol moiety that might limit binding in a catalytically productive orientation in the P450 active site.

The metabolism of the non-pyrethroid insecticides was varied. None of the P450s metabolised DDT, while CYP6P3 was the only P450 to metabolise bendiocarb, as previously demonstrated (Edi et al., 2014). Most striking was the broad cross-reactivity with organophosphate molecules. Pirimiphos-methyl, which is becoming widely used for IRS for malaria control in Africa (Oxborough, 2016), was highly metabolised by *An. gambiae* CYP6P3 (100% depletion) and CYP9J5 (96% depletion) as well as *An. funestus* CYP6P9a (42% depletion). Mass spectrometry analysis of the products of CYP6P3 metabolism indicated that both the active pirimiphos-methyl-oxon form may be formed along with the inactive oxidative cleavage product 2-diethylamino-6-hydroxy-4-methylpyrimidine. Thus, elevated levels of expression of CYP6P3 and other cross-reacting P450s could feasibly increase or decrease pirimiphos-methyl insecticidal activity depending on the relative rates of production of the active pirimiphos-methyl-oxon and inactive oxidative cleavage products. At present, it is unclear if CYP6P3 is a positive marker for enhanced pirimiphos-methyl activity and mosquito susceptibility or if it is linked with resistance. This recommends further investigations to characterize the pharmacokinetic profile of pirimiphos-methyl metabolism in relation to mosquito control. It is notable that pyrethroid resistant populations of *An. gambiae* in western Kenya remain susceptible to an organophosphate (malathion) (Wanjala et al., 2015), while pirimiphos-methyl (Actellic® 300CS) is effective against pyrethroid resistant Anopheles populations (Kanyangarara et al., 2016; Rowland et al., 2013). Conversely, resistance to pyrethroids and pirimiphos-methyl, as well as bendiocarb and DDT, has recently been detected in Anopheline mosquito populations in Tanzania (Kisinza et al., 2017).

While pyrethroid based LLINs have been integral to malaria control operations in Africa, the rapid increase in pyrethroid resistance is driving the use of alternative vector control strategies. This includes combining active ingredients with different modes of action. Pirimiphos-methyl for example is used for IRS alongside deltamethrin and permethrin impregnated nets (Oxborough, 2016), while new generation LLINs have been developed that incorporate both permethrin and pyriproxyfen (Tiono et al., 2018). The majority of the pyrethroid compounds produced moderate inhibition across the P450 range (IC50's 1–10 μM), suggesting that pyrethroid-P450 interactions could affect other active-ingredient biotransformation and potency that merits further research.

5. Conclusions

Overall, the *in vitro* profiling indicates that several Anopheles P450s associated with elevated levels of pyrethroid metabolism in African mosquito populations can metabolise at least 4 different insecticide classes recommended by WHO for malaria control. However, complex pharmacokinetic factors need to be taken into consideration and further research is recommended to identify the factors that influence insecticide metabolism and disposition *in vivo*. Nevertheless, with new active ingredients being developed for mosquito control in Africa (J Hemingway et al., 2016b), rapid *in vitro* profiling could be usefully applied to flag potential metabolic cross resistance liabilities. Furthermore, it would be advisable to continue to expand the bank of P450s to include other metabolic resistance candidates including CYP9K1, a P450 that is evolving strong association with deltamethrin resistance in *An. coluzzii* populations in Bioko Island, Equatorial Guinea (Vontas et al., 2018), *An gambiae* and *An. coluzzii* in Cameroon (Fossog Tene et al., 2013) and *An. parensis* in Uganda (Mulamba et al., 2014).

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Appendix A. Supplementary data

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