Recombinant CYP6M2 Inhibition by Insecticides Recommended by WHO for Indoor Residual Spraying.

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Abstract

One of the key regimes in the prevention of malaria in developing countries is the indoor residual spraying of long lasting insecticides to try and eliminate the mosquitos that may be carrying malaria parasite. CYP enzymes have been implicated in the development of insecticide resistance in malaria-carrying mosquitos through their potential to metabolise insecticides.

Here we have developed a quick fluorometric screen for inhibitors of CYP6M2, one of the CYPs that may be involved in insecticide resistance. Recombinant CYP6M2 co-expressed with mosquito NADPH P450 reductase was shown to metabolise diethoxyfluorescein (K_m of 0.22 ± 0.03 µM) to a fluorescent metabolite and this reaction was used to screen currently used insecticides for inhibition of CYP6M2. Of the 15 compounds screened (12 from the WHO recommended list) for inhibition of CYP6M2, 12 inhibited CYP6M2, 5 of them with an IC_{50} of 1 µM or less.

We then looked for CYP6M2 mediated metabolism of some of the compounds that inhibited the enzyme using an LC-MS based assay. CYP6M2 was found to metabolise some insecticides suggesting that it may play a role in the development of insecticide resistance in malaria vectors. This quick inhibition screen could be used early on in the development of new insecticides to flag potential problems associated with the development of CYP mediated insecticide resistance.

Introduction

One of the major means of controlling malaria is through control of the principal vector of the malaria parasite, *Anopheles gambiae*. Vector control is achieved mainly through the use of insecticide impregnated bed nets and indoor residual spraying of insecticides. Strains of *A. gambiae* have been found in the field that have shown resistance to pyrethroids, the most commonly used insecticide in treated bed nets and indoor residual spraying. Although some resistance can be attributed to variations in the target site there is evidence that insecticide metabolism by cytochrome P450s may play a part[1,2]. It is important, therefore, to produce new insecticides that are not susceptible to metabolic resistance and early screening of new compounds would help to flag any issues associated with CYP mediated metabolism. We have used recombinant CYP6M2, a cytochrome P450 implicated in metabolic insecticide resistance[1,2], to look at the feasibility of screening novel insecticides for CYP mediated metabolism in a rapid fluorometric inhibition assay.

Methods

Recombinant CYP6M2 was co-expressed with mosquito NADPH P450 reductase in *E. coli*. Mosquito cytochrome b_5 was expressed in *E. coli* and partially purified over a Ni agarose column.

Bacterial membranes containing CYP6M2 were incubated with fluorogenic substrates in 50 mM potassium phosphate pH 7.4, 5 mM $MgCl_2$ at 37°C. The reaction was started by the addition of an NADPH generating system. Fluorescence was monitored continuously over a 30 minute time period after the addition of NADPH generating system.

The conditions above were used to determine CYP inhibition using a 3-fold serial dilution of inhibitor down a 96 well plate. The insecticide stock solutions (100 mM) were prepared in DMSO (present in the assay at 2.5% [v/v]). Wavelengths for detection of the fluorescent metabolites are shown in Table 1.

Incubations of insecticides with CYP6M2 (plus cytochrome b_5) were carried out in 100 mM potassium phosphate pH 7.4, 5 mM $MgCl_2$ at 37°C for 30 min. The reaction was stopped by the addition of acidified acetonitrile. Samples were analysed by LC-MS.

Results

Initial incubation of CYP6M2 with the six substrates listed in Table 1 highlighted MFC (7-methoxy-4-trifluoromethylcoumarin) and DEF (diethoxyfluorescein) as potential candidates for use in quick fluorometric screens for CYP6M2 inhibition. CYP6M2 was most active with DEF and the inclusion of cytochrome b_5 increased the activity significantly with both substrates (Fig 1). No activity was seen with 7-ER, MAMC, CEC or 7-EFC.

The Km for DEF metabolism by CYP6M2 with cytochrome b_5 was found to be 0.22 ± 0.03 µM.

Fig 1. Metabolism of MFC and DEF by CYP6M2 ± cytochrome b_5

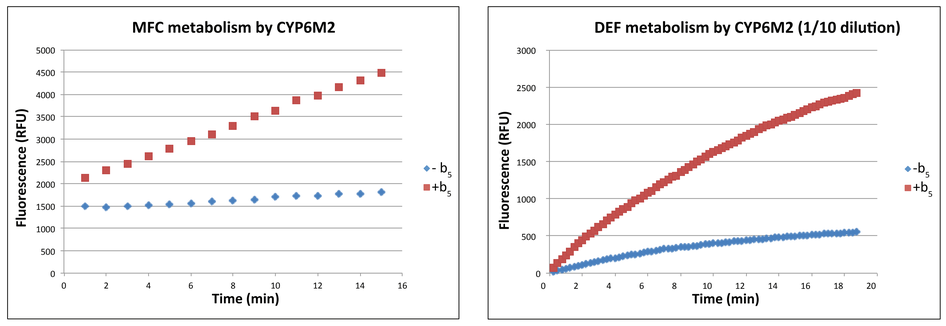


Table 1. Wavelengths used for the detection of fluorescent metabolites

Substrate	Wavelength (nm)	
	λ_{ex}	λ_{em}
7-EFC	431	535
7-ER	572	604
CEC	410	460
DEF	485	530
MAMC	429	470
MFC	431	535

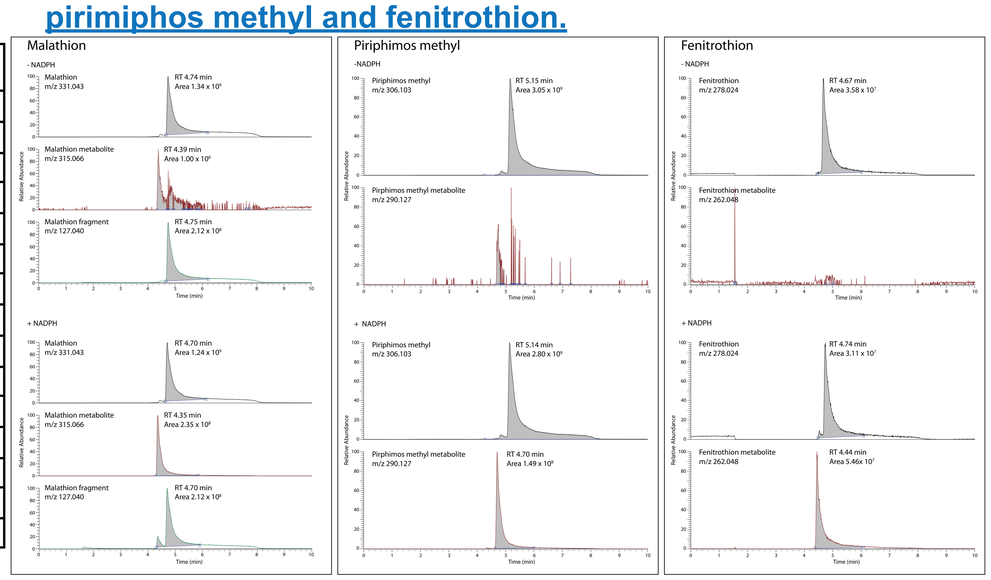
The ability of 15 insecticides (12 from the WHO recommended list) to inhibit CYP6M2 was assayed. Of these, 5 inhibited with an IC_{50} of < 1 µM and 7 with an IC_{50} of < 10 µM, summarised in Table 2. When comparing the IC_{50} at 2.5 min and 30 min, 3 compounds, malathion, pirimiphos methyl and fenitrothion appeared to show some time-dependent inhibition. These compounds were incubated with CYP6M2 ± NADPH generating system and samples were analysed by LC-MS. Chromatograms are shown in Fig. 2. In all three cases a metabolite was observed when NADPH was included in the reaction and in each case a mass loss of 16 was seen which is most likely a demethylation reaction. With malathion, when using the fragmentation chamber in the MS, a fragment with mass 127 was seen in both the substrate and metabolite peaks in the chromatogram giving a good indication of the demethylation site in the molecule.

Table 2. IC_{50} for CYP6M2 inhibition by 15 insecticides.

Compound	IC_{50} (µM)	Class
DDT	2.31 ± 0.56	OC
Chlorpyriphos	0.94 ± 0.19	OP
Diclorvos	> 10	OP
Fenitrothion	2.30 ± 0.72	OP
Malathion	0.14 ± 0.05	OP
Piriphimos methyl	1.22 ± 0.48	OP
Bendiocarb	> 100	C
Propoxur	> 100	C
Alpha-cypermethrin	0.32 ± 0.12	P
Bifenthrin	1.98 ± 0.37	P
Cyfluthrin	0.31 ± 0.08	P
Deltamethrin	0.51 ± 0.03	P
Etofenprox	1.84 ± 0.67	P
Lambda-cyhalothrin	1.04 ± 0.26	P
Permethrin	1.19 ± 0.25	P

n = 3 different CYP6M2 preps OC Organochlorine, OP, organophosphate, C, carbamate, P, pyrethroid

Fig 2. LC-MS analysis of CYP6M2 metabolism of malathion, pirimiphos methyl and fenitrothion.



Conclusion

We have used recombinant mosquito CYP6M2 to set up a rapid screen for insecticides' ability to inhibit CYP6M2. Some of the compounds that inhibited CYP6M2 were incubated with the enzyme and were shown to be metabolised by CYP6M2, indicating that inhibition may correlate with metabolism. Our data suggest that a rapid CYP inhibition screen using a panel of insect CYPs could be used to test for new insecticides' susceptibility to CYP mediated metabolic resistance. Additional work is being undertaken with other mosquito CYP isoforms to explore this further.

Refs. 1. Stevenson, B. J. *et al* (2011), Insect Biochem. Mol. Biol. 41, p492 - 502
2. Djouaka, R. F. *et al* (2008), BMC Genomics 9 p538 - 548.

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