DEVELOPMENT OF A FAST, FLUORESCENCE BASED SCREEN FOR INHIBITION OF *B. TABACI*, AND *T. URTICAE* CYTOCHROME P450S.

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Abstract

Insecticide resistance is becoming increasingly problematic in commercially important pest species and insect cytochrome P450s have been implicated in conferring resistance by metabolizing active compounds [1, 2, 3]. A means of determining whether novel compounds interact with the cytochrome P450s thought to be involved in resistance would, therefore, be a useful tool to look for resistance liabilities in compounds early in development. Here we have used three CYP isoforms from two pest species, CYP6CM1 (*B. tabaci*) and CYP392A11 and CYP392E10 (*T. urticae*) to set up a simple screen for inhibition of enzyme activity using fluorogenic substrates. The CYPs were coexpressed with NADPH P450 reductase (from *A. gambiae* with CYP6CM1 and from *T. urticae* with CYP392A11 and CYP392E10) in *E. coli*. Bacterial membranes containing the recombinant CYPs were incubated with a range of fluorogenic substrates to determine which, if any, would be suitable to take further. CYP6CM1 showed activity with diethoxyfluorescein (DEF), 7-ethoxyresorufin (7-ER), 3-cyano-7-ethoxycoumarin (CEC), 7-methoxy-4-trifluoromethylcoumarin (MFC) and 7-ethoxy-4-trifluoromethylcoumarin (7-EFC) however DEF gave the best combination of activity and Km. DEF was the only compound tested that was metabolized by CYP392A11 and CYP392E10. The Km for DEF turnover was as follows; CYP6CM1 1.1 µM, CYP392A11 10.6 nM and CYP392E10 3.8 µM. Both CYP6CM1 and CYP392E10 were activated by the inclusion of *A. gambiae* cytochrome b5 at a 10 fold excess over the CYP and further assay development work was carried out using enzymes supplemented with cytochrome b5 for both of those isoforms. A fluorescence plate reader assay was set up using DEF as the substrate at the following concentrations; 1 µM for CYP6CM1, 4 µM for CYP392E10 and 50 nM and 10 nM for CYP392A11. 15 insecticides were screened for their ability to inhibit the insect CYPs to demonstrate that the assay could pick up potential CYP interactions. For each CYP, at least two of the insecticides inhibited DEF turnover with an IC50 <10 µM. The IC50s tended to increase with incubation time suggesting that the inhibitor (insecticide) may be being metabolized by the CYP. This simple, fast, *in vitro* assay could, therefore be incorporated early on in insecticide development programmes to flag potential CYP based resistance liabilities in target pest species that may arise.

Methods

Recombinant CYP6CM1, CYP392A11 and CYP392E10 were co-expressed with mosquito (CYP6CM1) or *T. urticae* (CYP392A11 and CYP392E10) NADPH P450 reductase in *E. coli* in 10 litre fermenters. Mosquito cytochrome b_5 was expressed in *E. coli* and partially purified over a Ni agarose column. Expression constructs for the insect CYPs were kindly provided by Prof. J. Vontas, Associate Professor Biotechnology & Applied Biology, University of Crete.

Bacterial membranes containing CYPs 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 and fluorescence was monitored continuously over 30 minutes. CYP inhibition was determined using a 3-fold serial dilution (from 50 µM to 0.07 µM final concentration) of inhibitor down a 96 well plate. The insecticide stock solutions (5 mM) were prepared in DMSO (present in the assay at 1.5% or 2.5% [v/v]). Wavelengths for detection of the

Results

Initial incubation of CYPs with the six substrates listed in Table 1 highlighted DEF as the most promising substrate to use in an inhibition screen. CYP6CM1 also showed activity with 7-ER, CEC while CYP392A11 and CYP392E10 were only active with DEF. The activity of CYP6CM1 and CYP392E10 increased 2.5 and 10 fold repsectively with the inclusion of *A. gambiae* cytochrome b_5 in the assay at a 10 fold excess over the CYP. The Km for each enzyme with DEF was determined in order that an appropriate concentration could be selected for the inhibition screen (Table 2).

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
BFC	431	535
7-BQ	430	530

7-EFC 7-ethoxy-4-trifluoromethylcoumarin,
7-ER 7-ethoxyresorufin,
CEC 3-cyano-7-ethoxycoumarin,
DEF diethoxyfluorescein,
MAMC 7-methoxy-4-aminomethylcoumarin,
MFC 7-methoxy-4-trifluoromethylcoumarin,
BFC 7-benzyloxy-4-trifluoromethylcoumarin,
7-BQ 7-benzyloxyquinoline

Table 2. Km for DEF and concentration used for inhibition.

Isoform	DEF Km (µM)	DEF concentration in assay (µM)
CYP6CM1 + b5	2.2	1
CYP392E10 + b5	3.8	4
CYP392A11	0.007	0.01 and 0.05

Fig. 1. Typical IC_{50} curve

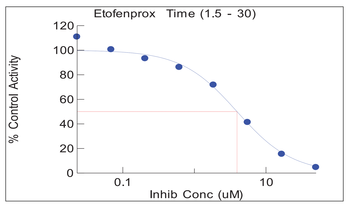
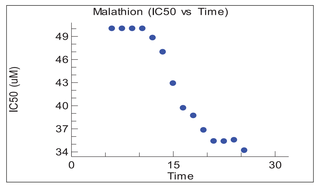
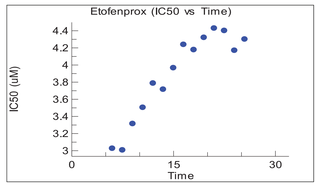


Fig. 2. IC_{50} changes with time for CYP6CM1





The ability of 15 insecticides covering 4 compound classes to inhibit the CYPs was assayed. The IC_{50} values were determined with two concentrations of DMSO present, 1.5% v/v and 2.5% v/v in the assay. CYPs are susceptible to inhibition by DMSO however, at the lower DMSO concentration, many of the insecticides appeared to be insoluble at the top concentration. In general, at the higher DMSO concentration, the IC_{50} values were lower indicating that solubility may indeed be an issue. The results are summarised in Table 3 (2.5% v/v DMSO assays only). Two DEF concentrations were used for CYP392A11 due to the low Km. At a DEF concentration of 10 nM (close to the Km) the fluorescent signal was very low leading to variable reaction rates. The assay was, therefore, run with a DEF concentration of 50 nM.

Because this a continuously monitored assay, the IC_{50} at the start can be compared with that after 30 min which gives addtional insight into the interaction of the compound with the CYP. For instance, with CYP6CM1, the IC_{50} for etofenprox at 30 min is 1.4 fold higher than that at 2.5 min, implying that the concentration of the compound in the assay is reducing with time, possibly as a result of metabolism by the CYP. In other cases, e.g. CYP6CM1 with malathion, the IC_{50} falls with time indicating that time dependent inactivation of the enzyme is occurring (Fig. 2).

Table 3. IC_{50} for CYP inhibition by 15 insecticides.

Insecticide	Class	CYP6CM1		CYP392A11		CYP392E10	
		IC_{50} (µM)	IC_{50} start / IC_{50} end	IC_{50} (µM)	IC_{50} start / IC_{50} end	IC_{50} (µM)	IC_{50} end / IC_{50} start
DDT	OC	5.2	1.4	11.4	1.3	42	1
Cyfluthrin	P	1.3	1.6	14.2	1.3	>50	1
Cyhalothrin	P	4.1	1.7	23.6	1.2	>50	1
Dichlorvos	OP	>50	1	18	0.9	>50	1
Fenitrothion	OP	2.2	1.6	>50	1.1	>50	0.6
Piriphemos methyl	OP	0.6	1.6	19	1.4	5.2	1.4
Etofenprox	P	3.9	1.4	6.6	1.9	28	1
Malathion	OP	41	0.7	>50	1	>50	1
Propoxur	C	>50	1	>50	1	>50	1
Permethrin	P	2.7	1.6	19	1.4	28	1.3
Cypermethrin	P	2.9	1.6	16	1.5	45	1.3
Bendiocarb	C	>50	1	>50	1	>50	1
Deltamethrin	P	1.1	2.0	18	1.4	39	1.1
Chlorpyriphos	OP	0.3	0.7	6.5	1.1	3.1	0.9
Bifenthrin	P	9.4	0.8	44	1.2	17	0.8

OC Organochlorine, OP, organophosphate, C, carbamate, P, pyrethroid

Conclusion

We have used recombinant insect pest CYPs that have been implicated in conferring resistance to set up a rapid screen for their inhibition by insecticides, demonstrating potential interactions of the compounds with the enzymes. In many cases, the IC_{50} for the compound increased with time implying that it may be a substrate for the enzymes and thus would be broken down *in vivo*. This novel, rapid CYP inhibition screen, using a panel of insect CYPs could be used to test for new insecticides' susceptibility to CYP mediated metabolic resistance early on during the development process.

Refs. 1 Demaeght, P. et al. (2013) Insect Biochem. Mol. Biol. 43 544 – 554. Molecular analysis of resistance to acaricidal spirocyclic tetronic acids in *Tetranychus urticae*: CYP392E10 metabolises spirodiclofen, but not its corresponding enol.

2 Riga, M. et. al. (2015) Insect Biochem. Mol. Biol. 65 91 – 99. Functional characterization of the *Tetranychus urticae* CYP392A11, a cytochrome P450 that hydroxylates the METI acaricides cyenopyrafen and fenpyroximate.

3 Karunker, I. et. al. (2009) Insect Biochem. Mol. Biol. 39 697 – 706. Structural model and functional characterization of the *Bemisia tabaci* CYP6CM1vQ a cytochrome P450 associated with high levels of imidacloprid resistance.