Progress Report: Development a fluorescent 96-well assay for dual detection of esterase- and GST-mediated pyrethroid resistance in mosquito

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Reporting Period: July 1, 2012 to December 31, 2012

Abstract

Resistance to pyrethroid insecticides involves two primary mechanisms of action: (*i*) target site insensitivity and (*ii*) elevation in the activity of detoxification enzymes such as esterases, glutathione *S*-transferases (GSTs) and cytochrome P450s. Rapid, population-level detection of these resistance mechanisms is critical for maintaining the effectiveness of pyrethroid insecticides. We have developed a series of pyrethroid-like, fluorescent substrates for the detection of esterase and GST activities. During the reporting period, we have tested these fluorescent substrates with recombinant mosquito and authentic murine GSTs. We are currently developing two of these fluorescent substrates for use in a rapid and sensitive, 96-well assay for the simultaneous detection of elevated GST and esterase activities in pyrethroid resistance in mosquitoes.

Objectives

I. Test the efficacy of pyrethroid-like, ester-containing, fluorescent compounds as potential substrates for mosquito glutathione *S*-transferases
II. Develop a high throughput fluorescent assay for the dual detection of elevated esterase and GST activities in pyrethroid resistant mosquito

Introduction

Synthetic pyrethroids and natural pyrethrins are commonly used to control agricultural pests and occasionally adult mosquitoes in California. Unfortunately, pyrethroid resistance has been detected in numerous insects including *Culex pipiens* complex mosquitoes in California (McAbee et al., 2003) and elsewhere in the US (Liu et al., 2004; Liu et al., 2006). The two primary mechanisms of pyrethroid resistance involve (*i*) target site insensitivity and (*ii*) the elevated activity of detoxification enzymes such as esterases, cytochrome P450s, and GSTs (Vontas et al., 2001). Recent evidence from field-collected mosquitoes indicates that operational resistance to pyrethroids commonly involves both target site and enzyme-mediated mechanisms (A. J. Cornel, unpublished).

The rapid detection of insecticide resistance is crucial for maintaining the effectiveness of chemical insecticides. In mosquitoes, biological assays such as the bottle bioassay (Brogdon and McAllister, 1998) are commonly used to detect insecticide resistance. These bioassays are relatively inexpensive and easy to bring online, however, they are slow, require relatively large numbers of insects, and the interpretation of their end points can be subjective. PCR-based assays using fluorescent primers are also available for the rapid and sensitive detection of target site mutations involved in insecticide resistance (e.g., Tripet et al., 2006). A robust PCR-based assay for the detection of elevated gene expression of detoxification genes, however, has not been developed.

The pyrethroid-like fluorescent substrates (Fig. 1) developed in this project will potentially lead to a new generation of rapid and highly sensitive assays for the detection of elevated GST activity associated with resistance to pyrethroid insecticides. These fluorescent substrates are esters composed of an acid moiety that is found in some commonly used pyrethroids and an alcohol moiety consisting of 7-hydroxy-4-methylcoumarin. The acid moiety of four of our substrates (*cis*-/*trans*-DCVC and *cis*-/1*R trans*-TFMCVC) mimic the commonly used type I pyrethroids permethrin and bifenthrin, respectively, and the type II pyrethroids cypermethrin and cyhalothrin, respectively. Because these pyrethroid-like compounds are esters, they have the potential to function as dual esterase/GST substrates in the presence of an appropriate GST or esterase inhibitor. The ultimate goal of this project is to develop a simple 96-well format assay that MVCDs can use for the detection of elevated GST and/or esterase activity from the homogenate of a single mosquito.

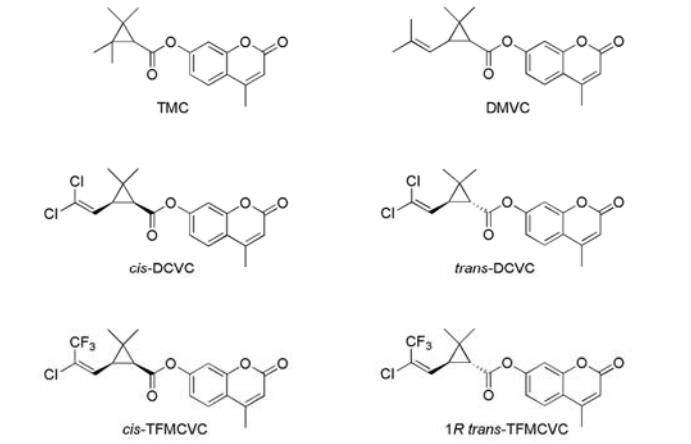

Fig. 1. Chemical structures of the pyrethroid-like ester compounds used in this study

Results and Discussion

Specific activity of recombinant mosquito and authentic mouse GSTs toward the pyrethroid-like fluorescent substrates

The general procedures for the preparation of the pyrethroid-like fluorescent substrates, preparation of the recombinant mosquito and authentic murine GSTs fluorescent assay conditions, etc. are given in our recent publication (Huang et al., 2012).

The recombinant mosquito GST, CpGSTD1, selectively metabolized all 6 of the pyrethroid-like esters with specific activities that ranged from approximately 10 to 230 nmol min^{-1} mg^{-1} (Table 1). CpGSTD1 metabolized the *cis*-isomers of DCVC and TFMCVC at approximately 2- to 5-fold faster rates than their corresponding *trans*-isomers. CpGSTD1 showed up to 20-fold lower specific activity for TMC and DMVC in comparison to DCVC and TMCVC. The murine GST preparation appeared to show significantly lower specific activity (1 to 4 nmol min^{-1} mg^{-1}) with both the *cis*- and *trans*-isomers of DCVC and TFMCVC, and no detectable metabolism of TMC and DMVC (Table 1). On the basis of specific activity values, CpGSTD1 showed the following preference for our pyrethroid-like substrates: *cis*-TFMCVC > *cis*-DCVC > *trans*-DCVC > 1*R trans*-TFMCVC > DMVC > TMC.

Enzyme kinetic analysis of recombinant mosquito GST with the pyrethroid-like fluorescent substrates

CpGSTD1 metabolized the *cis*- and *trans*-isomers of DCVC and TFMCVC with maximum velocity (V_{max}) values in the range of 50 to 490 nmol min^{-1} mg^{-1} (Table 2). The V_{max} of CpGSTD1 for the *cis*-isomers of both DCVC and TFMCVC were 3- and 9-fold higher, respectively, than the corresponding *trans*-isomer. In contrast to the pyrethroid-like fluorescent substrates, CpGSTD1 metabolized the general GST substrates CDNB and DCNB with

significantly higher (209- and 5-fold, respectively) higher V_{max} values of 102,000 and 2,320 nmol min^{-1} mg^{-1}, respectively (Table 2). These higher rates, however, required relatively high substrate concentrations as indicated by significantly higher (up to 140-fold) K_M values (Table 2). The turnover (k_{cat}) of all of the pyrethroid-like substrates was very slow (Table 2). In a manner consistent with the specific activity data of CpGSTD1 (Table 1), the specific activity constants (k_{cat}/K_M ratio) of CpGSTD1 for *cis*-DCVC and *cis*-TFMCVC were 2- and 5-fold higher, respectively, in comparison to the corresponding *trans* isomer.

The ability to quickly, accurately, and quantitatively detect both target site mutations and elevated levels of pyrethroid detoxification enzymes is a critical component of the continued effective use of pyrethroids. We are currently developing *cis*-TFMCVC and *cis*-DCVC for use in a simple fluorescent assay to detect elevated levels of GST and esterase activities that may be associated with mosquito resistance to commonly used synthetic pyrethroids.

References

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Table 1. Specific activity of CpGSTD1 and mouse GSTs toward pyrethroid-like substrates

Substrate	Specific Activity[a] (nmol min^{-1} mg^{-1})	
	CpGSTD1	murine GSTs
TMC	10.5 ± 1.0	n.d.
DMVC	37.7 ± 7.6	n.d.
cis-DCVC	134 ± 12	3.5 ± 0.3
trans-DCVC	79.5 ±5.2	3.9 ± 0.4
cis-TFMCVC	233 ±9.9	1.4 ± 0.2
1*R trans*-TFMCVC	44.3 ± 5.6	4.0 ± 0.6
CDNB	98,000 ± 4,000	33,700 ± 2,000

[a]The enzyme assays were performed in 90 mM sodium phosphate, pH 7.4, buffer containing 720 ng of CpGSTD1 or 1,400 ng of mouse GSTs, 50 µM substrate, 750 µM GSH, and 0.5% (v/v) DEGEE at 34ºC. The values are corrected for background hydrolysis. The results shown are the mean ± standard deviation of four replicates. The "n.d." indicates that no activity was detected above background hydrolysis.

Table 2. Enzyme kinetic properties of CpGSTD1 with pyrethroid-like fluorescent and spectrophotometric substrates[a]

Substrate	V_{max} (nmol min^{-1} mg^{-1})	K_M (µM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
cis-DCVC	298 ± 13.6	9.0 ± 0.8	0.12	1.3×10^4
trans-DCVC	107 ± 4.5	5.9 ± 0.5	0.04	6.8×10^3
cis-TFMCVC	487 ± 14.0	10.0 ± 0.7	0.19	1.9×10^4
1*R trans*-TFMCVC	52.5 ± 1.5	5.0 ± 0.4	0.02	4.0×10^3
CDNB	102,000 ± 2,500	240 ± 18	40.4	1.7×10^5
DCNB	2,316 ± 152	691 ± 106	0.92	1.3×10^3

[a]The fluorescent enzyme assays were performed in 90 mM sodium phosphate, pH 7.4, buffer containing 184 ng of CpGSTD1, pyrethroid-like substrate (1 to 31.2 µM), 750 µM GSH, and 2% (v:v) DEGEE. The spectrophotometric assays were performed in 100 mM sodium phosphate, pH 6.5, buffer containing 100 ng of CpGSTD1, 5 mM substrate (CDNB or DCNB), 5 mM GSH, and 1.67% ethanol at 30ºC. All of the values are corrected for background hydrolysis. The results shown are the mean ± standard deviation of four replicates.