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Detoxification of ivermectin by ATP binding cassette transporter C4 and cytochrome P450 monooxygenase 6CJ1 in the human body louse, *Pediculus humanus humanus*

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Abstract

We previously observed that ivermectin-induced detoxification genes, including *ATP binding cassette transporter C4* (*PhABCC4*) and *cytochrome P450 6CJ1* (*CYP6CJ1*) were identified from body lice following a brief exposure to a sublethal dose of ivermectin using a non-invasive induction assay. In this current study, the functional properties of PhABCC4 and CYP6CJ1 were investigated after expression in either *X. laevis* oocytes or using a baculovirus expression system, respectively. Efflux of [^{3}H]-9-(2-phosphonomethoxyethyl) adenine ([^{3}H]-PMEA), a known ABCC4 substrate in humans, was detected from *PhABCC4* cRNA-injected oocytes by liquid scintillation spectrophotometric analysis and PhABCC4 expression in oocytes was confirmed using ABC transporter inhibitors. Efflux was also determined to be ATP-dependent. Using a variety of insecticides in a competition assay, only co-injection of ivermectin and dichlorodiphenyltrichloroethane led to decreased efflux of [^{3}H]-PMEA. PhABCC4-expressing oocytes also directly effluxed [^{3}H]-ivermectin, which increased over time. In addition, ivermectin appeared to be oxidatively metabolized and/or sequestered, although at low levels, following functional expression of CYP6CJ1 along with cytochrome P450 reductase in Sf9 cells. Our study suggests that PhABCC4 and perhaps CYP6CJ1 are involved in the Phase III and Phase I xenobiotic metabolism of ivermectin, respectively, and may play an important role in the evolution of ivermectin resistance in lice and other insects as field selection occurs.

Keywords: *Pediculus humanus humanus*, ivermectin, tolerance, detoxification, ATP binding cassette protein (ABC) transporters, P450.

Introduction

Ivermectin is the first commercially available macrocyclic lactone endectocide derived from the natural avermectins that are produced by a soil microorganism, *Streptomyces avermitilis* (Geary, 2005). It has been widely used to control endo- and ectoparasites, including nematodes and pest arthropods, such as fleas, ticks, scabies, lice, mites and flies, infesting both humans and companion animals (Campbell, 1985; Woodruff & Burg, 1986). Ivermectin is an allosteric modulator of glutamate-gated chloride channels (Sparks & Nauen, 2015), which are only found in invertebrates (Kehoe *et al.*, 2009), leading to enhanced chloride permeability in the nervous and muscle systems of insects (Duce & Scott, 1985) and nematodes (Dent *et al.*, 1997). The ivermectin-induced hypopolarization in target cells eventually causes paralysis and death of intoxicated invertebrate organisms.

As ivermectin has a completely different mode of action [Insecticide Resistance Action Committee (IRAC) Mode of Action (MoA) Class 6] and structure (macrocyclic lactone) when compared to the most widely used

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pyrethroid-based pediculicides (eg permethrin; IRAC MoA Class 3A; pyrethroid) (Sparks & Nauen, 2015), it is an effective alternative pediculicide for the control of pyrethroid-resistant louse populations without the possibility of cross-resistance. A new topically applied ivermectin formulation, which is less invasive than oral formulations, killed permethrin-resistant human head lice (Strycharz *et al*., 2008), and the effectiveness of an oral treatment for difficult-to-control head louse infestations has also been reported (Chosidow *et al*., 2010).

In order to maximize the time over which ivermectin remains efficacious as a new pediculicide, it is essential to understand possible resistance mechanisms, particularly metabolic detoxification, given its well-researched oxidative detoxification (Yoon *et al*., 2002) and ATP-dependent efflux (James & Davey, 2009). In our previous study, ivermectin-induced detoxification genes, including ATP binding cassette (ABC) transporter and cytochrome P450 monooxygenase (P450) genes, were identified from body lice following contact or immersion exposure to ivermectin using a non-invasive induction assay (Yoon *et al*., 2011).

ABC transporters comprise a large family of membrane-integrated proteins found in large numbers in all organisms. They are involved in several physiological processes, including the translocation of a wide variety of substances across biological membranes, including metabolites and xenobiotics (Holland & Blight, 1999), and are also responsible for the phenotype of multi-drug resistance, which has been identified in cancer cells and pathogens (Lage, 2003; Higgins, 2007). Most ABC transporters contain two multiple membrane-spanning domains and two cytosolic ATP-binding domains (ABDs), and are classified based on the sequence, organization of ABDs and transport activity. In body lice, a total of 36 functional ABC transporters have been identified, including ABC-A to ABC-H types (Lee *et al*., 2010), which is substantially fewer than that in *Drosophila melanogaster* (65 ABC transporters) (Roth *et al*., 2003). Numerous studies have also shown that ivermectin can be transported and excreted by ABC transporters (Pouliot *et al*., 1997; Xu *et al*., 1998; Lespine *et al*., 2007; Jani *et al*., 2011). In our previous study, *ABC transporter C4* (*PhABCC4*) from body lice was significantly overexpressed following a brief, non-invasive, treatment of a sublethal dose of ivermectin (Yoon *et al*., 2011). The injection of double-stranded RNAs (dsRNAs) against *PhABCC4* into body lice reduced the *PhABCC4* transcript and increased the sensitivity of injected lice to ivermectin, indicating that this gene is probably involved in Phase III xenobiotic metabolism of ivermectin, thereby providing tolerance (Yoon *et al*., 2011). In addition, a similar study using *Rhipicephalus microplus* has shown that ivermectin efflux is sensitive to MK571 (ABC-C type blocker), further implicating a C-type ABC transporter in ivermectin transport (Pohl *et al*., 2011).

P450s are an important metabolic system mediating the oxidation of endogenous and xenobiotic compounds and are commonly involved in pesticide resistance in various arthropod species (Scott, 1999). Along with ABC transporters, P450s have been implicated as a principal resistance mechanism for the avermectins (ie ivermectin and abamectin) in a variety of arthropods, including the Colorado potato beetle (Yoon *et al*., 2002), the diamond back moth (Qian *et al*., 2008) and two spotted spider mite (Stumpf & Nauen, 2002). Thirty-seven functional P450 genes were annotated in the body louse genome, which is the smallest number of P450s for any insect genome currently sequenced with the greatest reduction seen in the CYP3 clade (Lee *et al*., 2010). Amongst these P450 genes, *CYP6CJ1*, *CYP9AG1* and *CYP9AG2* were found to be significantly overexpressed when lice were treated with a sublethal dose of ivermectin (Yoon *et al*., 2011). In addition, the injection of dsRNA of *CYP9AG2* into body lice resulted in increased sensitivity to ivermectin as judged by median lethal time values, suggesting that P450s are involved in the oxidative xenobiotic metabolism of ivermectin along with PhABCC4 as described above (Yoon *et al*., 2011).

In order to validate the roles of ABC transporters and P450s in the metabolic detoxification of ivermectin in body lice, we functionally expressed and characterized PhABCC4 and CYP6CJ1 using either the *X. laevis* oocyte or baculovirus expression systems, respectively. The translocation activity and substrate specificity of PhABCC4 and the catalytic properties and ivermectin metabolizing potential of CYP6CJ1 were examined to investigate their roles in ivermectin detoxification.

Results

Functional characterization of expressed PhABCC4

PhABCC4 was expressed in *X. laevis* oocytes by injection of *PhABCC4* capped cRNA. To confirm the functional expression of PhABCC4, oocytes were injected with [^{3}H]-9-(2-phosphonomethoxyethyl) adenine ([^{3}H]-PMEA, commonly known as Adefovir), which is a well-established substrate for ABCC4 (Schuetz *et al*., 1999; Imaoka *et al*., 2007; Pohl *et al*., 2011). The efflux of [^{3}H]-PMEA was significantly increased in *PhABCC4* cRNA-injected oocytes over time whereas double-distilled H_2O (ddH_2O)-injected oocytes, not expressing PhABCC4, did not efflux [^{3}H]-PMEA to any appreciable extent [Fig. 1; two-way analysis of variance (ANOVA), $P < 0.01$]. The efflux of [^{3}H]-PMEA as judged by counts per min (CPMs) in the bathing solution was significantly higher (7.3-fold) in PhABCC4-expressing oocytes at 30 min following [^{3}H]-PMEA injection when compared with

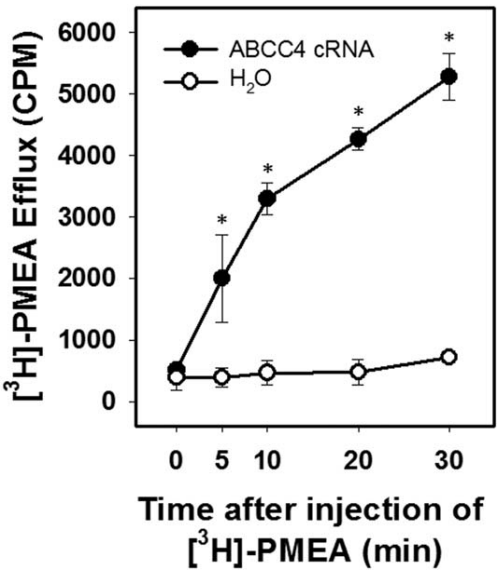

Figure 1. Efflux of [^{3}H]-9-(2-phosphonomethoxyethyl) adenine ([^{3}H]-PMEA) over time. The total count per minute (CPM) value was significantly greater for *Pediculus humanus humanus ATP binding cassette transporter C4* (*PhABCC4*) cRNA-injected compared to H_2O-injected oocytes. Symbols with an asterisk (*) indicate a statistically significant difference between ABCC4 cRNA- and H_2O-injected oocytes ($P < 0.05$).

ddH_2O-injected oocytes ($P < 0.001$), indicating that the efflux was not a result of leaky/damaged oocytes. In addition, the efflux of [^{3}H]-PMEA was decreased in a concentration-dependent manner following co-injection with increasing concentrations of unlabelled PMEA as a competitive ligand (one-way ANOVA, $P < 0.0001$), resulting in a mean effective concentration (EC_{50}) value for PMEA of 74.6 μM (Fig. 2A). [^{3}H]-PMEA efflux was also increased by injecting increasing amounts of Mg-ATP, a hydrolysable form of ATP (one-way ANOVA, $P < 0.05$), but was decreased in a concentration-dependent manner when either the nonhydrolysable ATP analogue, adenylyl-imidodiphosphate (AMP-PNP) [mean inhibition concentration (IC_{50}) = 0.59 mM; one-way ANOVA, $P < 0.001$] or the ABCC-specific inhibitor, MK571 (IC_{50} = 26.7 μM, one-way ANOVA, $P < 0.005$), were injected in increasing concentrations, confirming the functional expression of PhABCC4 in oocytes (Fig. 2B–D). To further characterize PhABCC4, the effect of either quercetin (Brand *et al*., 2006) or verapamil (Bai *et al*., 2004; Prehm & Schumacher, 2004), both well-established inhibitors of PMEA efflux, were investigated. [^{3}H]-PMEA efflux was inhibited by both quercetin (IC_{50} = 62.3 μM; one-way ANOVA, $P < 0.0001$) and verapamil (IC_{50} = 28.9 μM; one-way ANOVA, $P < 0.0001$) in a concentration-dependent manner (Fig. 2E–F).

Characterization of ivermectin efflux by PhABCC4

In our previous study, PhABCC4 was suggested to be involved in the Phase III xenobiotic metabolism of ivermectin as its knockdown by RNA interference increased the toxicity of ivermectin to dsRNA-injected lice (Yoon *et al*., 2011). To confirm it could directly compete with [^{3}H]-PMEA efflux, a competition assay was conducted with ivermectin. Co-injection of increasing concentrations of unlabelled ivermectin led to a significant decreased efflux of [^{3}H]-PMEA with an EC_{50} value of 4.6 μM (Fig. 3A; one-way ANOVA, $P < 0.0001$).

[^{3}H]-ivermectin was also injected into oocytes previously injected with PhABCC4 cRNA and the efflux of [^{3}H]-ivermectin itself determined over time to investigate the direct efflux of ivermectin by PhABCC4. As shown in Fig. 3B, the efflux of [^{3}H]-ivermectin increased over time and the amount of efflux was significantly greater (1.5-fold at 240 min) in the oocytes expressing PhABCC4 when compared with control oocytes (two-way ANOVA, $P < 0.001$ from 60 to 250 min). This result indicates that PhABCC4 is capable of directly effluxing ivermectin and that Phase III xenobiotic metabolism of ivermectin in body lice is, in part, responsible for the increased tolerance to ivermectin apparent following the induction of PhABCC4 by sublethal concentrations of ivermectin using the non-invasive induction assay.

Insecticide substrate preference of PhABCC4

To confirm that ivermectin is a preferred substrate of PhABCC4, efflux competition assays with three representative insecticides of different structural classes and mode of actions were conducted. The efflux of [^{3}H]-PMEA was not affected by either dichlorvos (DDVP), a relatively more water-soluble organophosphate insecticide (Fig. 4A), or imidacloprid, a systemic neonicotinoid insecticide (Fig. 4B), indicating that ivermectin, a hydrophobic insecticide, was selectively competing at the same receptor with PMEA. Dichlorodiphenyltrichloroethane (DDT), a highly lipophilic insecticide, also selectively inhibited the efflux of [^{3}H]-PMEA in a concentration-dependent manner similar to ivermectin (IC_{50} = 10.0 μM; one-way ANOVA, $P < 0.0001$; Fig. 4C).

Functional expression of CYP6CJ1 and ivermectin metabolism

Recombinant CYP6CJ1 was co-expressed with NADPH cytochrome P450 reductase (CPR) in Sf9 cells using a baculovirus expression system. The functional expression of CYP6CJ1 was confirmed by a CO difference spectrum (data not shown) and by using a range of fluorescent model substrates, which showed that the expressed P450 has catalytic activity when co-expressed with CPR in the presence of NADPH (Fig. 5A). The enzymatic activity of each of the

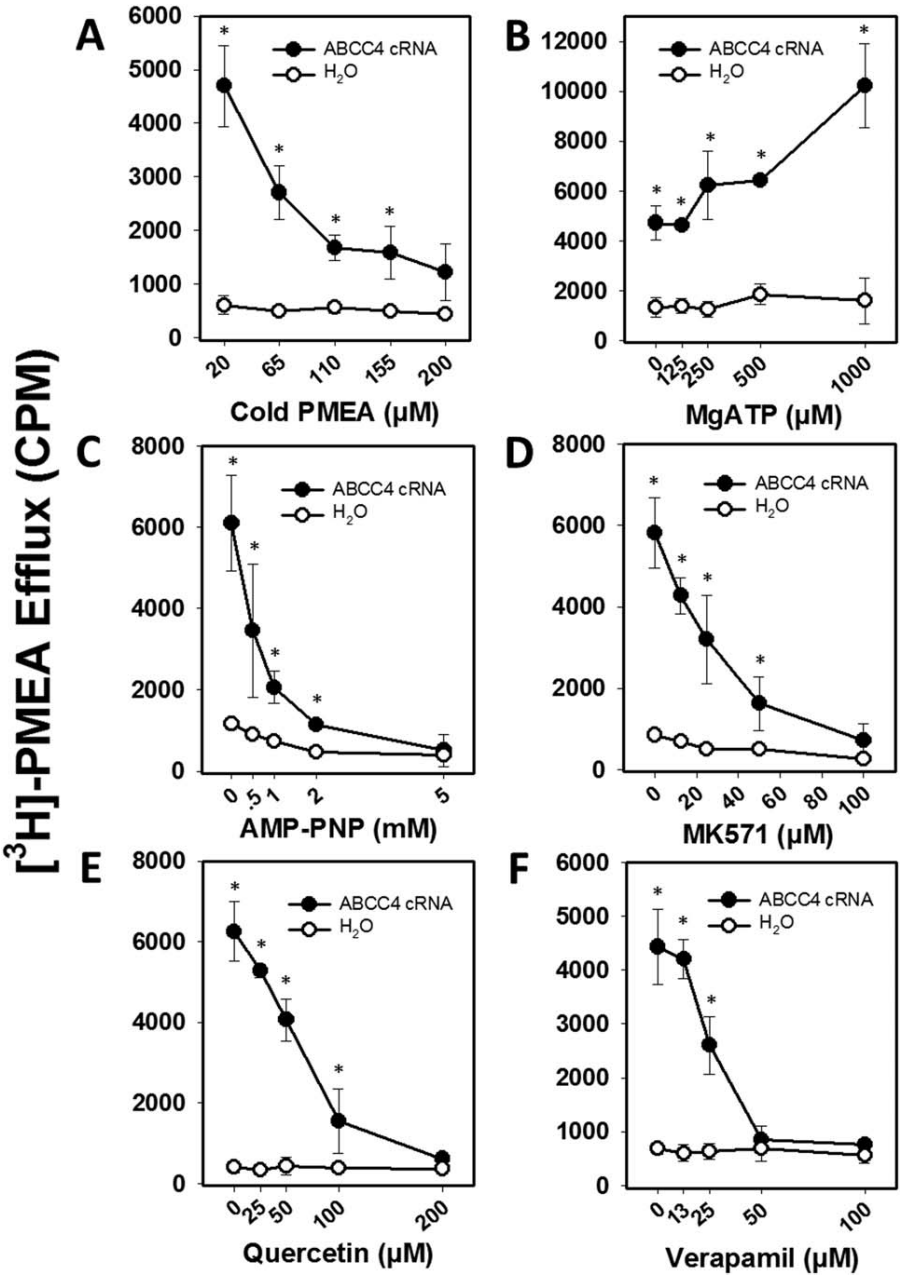

Figure 2. Efflux of [^{3}H]-9-(2-phosphonomethoxyethyl) adenine ([^{3}H]-PMEA) following co-injection with competitor, inducer or inhibitor compounds. The count per minute (CPM) values were determined in the presence of the indicated concentrations of (A) the competitor, unlabelled PMEA (mean effective concentration = 74.6 μM), (B) the inducer, MgATP, (C) Adenylyl-imidodiphosphate (AMP-PNP) [nonhydrolysable ATP analogue, mean inhibition concentration (IC_{50}) = 0.59 mM], (D) MK571 (ABCC-specific inhibitor, IC_{50} = 26.7 μM), (E) quercetin (inhibitor of PMEA efflux, IC_{50} = 62.3 μM) or (F) verapamil (inhibitor of PMEA efflux, IC_{50} = 28.9 μM). Symbols with an asterisk (*) indicate a statistically significant difference between ATP binding cassette transporter C4 (ABCC4) cRNA- and H_2O-injected oocytes ($P < 0.05$).

three substrates, 7-benzyloxy-4-trifluoromethyl coumarin (BFC), 7-ethoxy-4-trifluoromethyl coumarin (7-EC) and 7-benzyloxymethoxy resorufin (BOMR), was significantly higher compared with the control (Fig. 5A).

The metabolism of ivermectin by microsomes co-expressing CYP6CJ1/CPR was measured directly by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis. The recombinant CYP6CJ1/CPR microsomes incubated in the absence of NADPH did not show any reduction of ivermectin. In the presence of NADPH, however, a 5.4 ± 1.2% depletion of ivermectin was observed after a 2 h incubation (Fig. 5B). Although not statistically different ($P > 0.05$) from Sf9 control microsomes in the presence of NADPH, there is a small but noticeable reduction of ivermectin when incubated with CYP6CJ1 expressing microsomes in the presence of NADPH. This result suggests that CYP6CJ1 may be involved in the oxidative Phase 1 xenobiotic metabolism of ivermectin or more likely in its sequestration in body lice and could be, in part, responsible for the increased tolerance to ivermectin apparent following the induction of CYP6CJ1 by sublethal concentrations of ivermectin using the non-invasive induction assay.

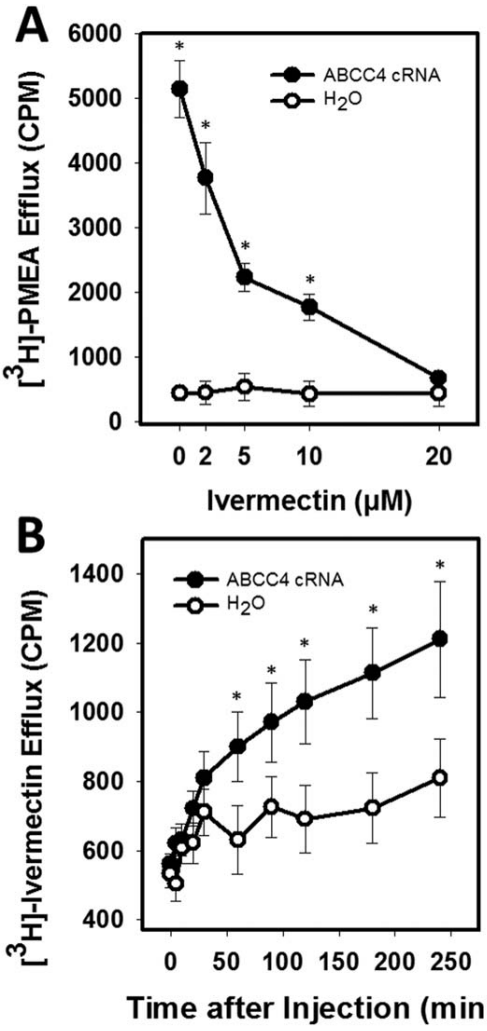

Figure 3. (A) Efflux of [^{3}H]-9-(2-phosphonomethoxyethyl) adenine ([^{3}H]-PMEA) following co-injection with increasing concentrations of ivermectin. Efflux was decreased by increasing concentrations of nonlabelled ivermectin (mean inhibition concentration = 4.6 μM). (B) Efflux of [^{3}H]-ivermectin (9 μM) over time. The count per minute (CPM) value was significantly greater for *Pediculus humanus humanus* ATP binding cassette transporter C4 (PhABCC4) mRNA-injected compared with H_2O-injected oocytes. Symbols with an asterisk (*) indicate a statistically significant difference between ABCC4 cRNA- and H_2O-injected oocytes ($P < 0.05$).

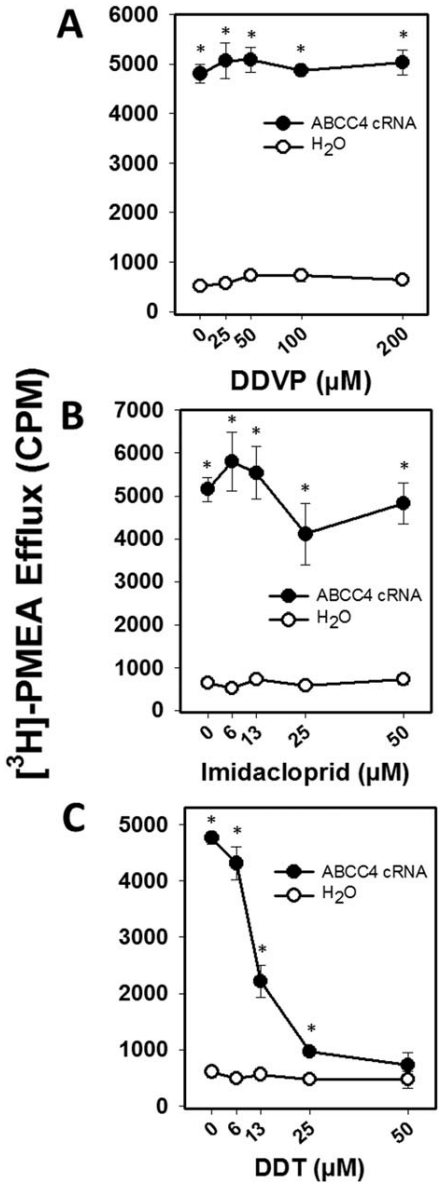

Figure 4. Efflux of [^{3}H]-9-(2-phosphonomethoxyethyl) adenine ([^{3}H]-PMEA) following co-injection with increasing concentration of insecticides. The count per minute (CPM) values were determined in the presence of the indicated concentrations of (A) dichlorvos (DDVP), (B) imidacloprid or (C) dichlorodiphenyltrichloroethane (DDT) (mean inhibition concentration = 10.0 μM) at 30 min post injection of [^{3}H]-PMEA. Symbols with an asterisk (*) indicate a statistically significant difference between ATP binding cassette transporter C4 (ABCC4) cRNA- and H_2O-injected oocytes ($P < 0.05$).

Discussion

In this study, the roles of PhABCC4 and CYP6CJ1, which were the most significantly induced genes in body lice following a non-invasive induction assay in our previous study (Yoon *et al.*, 2011), were investigated in the xenobiotic metabolism of ivermectin using either the *X. laevis* oocyte or Sf9 cell-based baculovirus expression systems, respectively. We showed that PhABCC4 was capable of directly detoxifying ivermectin by Phase III (ABC transport-dependent efflux) xenobiotic metabolism. The data on the oxidative metabolism and/or sequestration of ivermectin by CYP6CJ1 are less convincing owing to the small reduction of ivermectin in the microsomal-based *in vitro* system used.

The *X. laevis* oocyte expression system has been used as a model system for examining the functional characteristics of the various types of ABC transporters

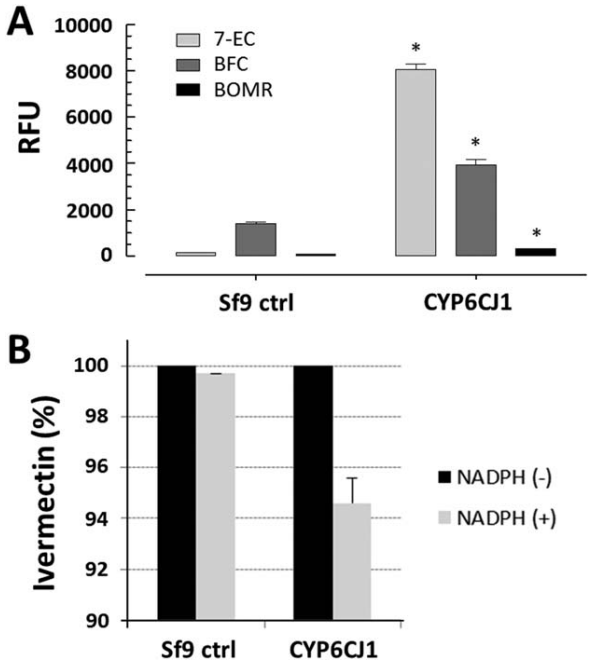

Figure 5. (A) Metabolism (O-dealkylation and O-dearylation) of different artificial coumarin and resorufin substrates by microsomal preparations of Sf9 cells functionally co-expressing cytochrome P450 6CJ1 monooxygenase (CYP6CJ1) with NADPH cytochrome P450 reductase (CPR). 7-EC, 7-ethoxy-4-trifluoromethyl coumarin; BFC, 7-benzyloxy-4-trifluoromethyl coumarin; BOMR, 7-benzyloxymethoxy resorufin; RFU, Relative Fluorescence Unit. Data are mean values ± SD (n = 4). Symbols with an asterisk (*) indicate a statistically significant difference between control and expressed CYP6CJ1 ($P < 0.05$). (B) Depletion of ivermectin incubated for 2 h with microsomal preparations of Sf9 cells functionally expressing either CYP6CJ1/CPR or control cells (Sf9 ctrl) in the presence or absence of NADPH. Data are mean values ± SD (n = 3).

(Tsuruoka *et al*. 2002; Nakanishi *et al*., 2003; Jutabha *et al*., 2010). Specifically, Janke *et al*. (2008) first demonstrated that *X. laevis* oocytes represented a valid system for the characterization of the human multidrug resistance protein 4 (MRP4), the ortholog of PhABCC4, and its variants by confirming its proper expression and insertion into the plasma membrane. This previously published study also validated that the *X. laevis* oocyte expression system is a useful method with which to conduct *in vitro* transport studies. The efflux of PMEA, a well-known ABCC4 substrate in humans, was likewise observed in MRP4-expressing oocytes, indicating that the activity of the transporter is similar to that found in body lice as reported in the current study.

Inhibitors of ABC transporters have been used to study the involvement of ABC transporters in the evolution of ivermectin resistance (Buss *et al*., 2002; Bartley *et al*., 2009; Pohl *et al*., 2011). In the cattle tick, *Rhipicephalus microplus*, a significant increase in ivermectin toxicity was observed when ivermectin-resistant larvae were pre-exposed to sublethal concentrations of MK571, suggesting the importance of ABC C-type transporters in larval defence against ivermectin (Pohl *et al*., 2011). We likewise observed that PhABCC4 expressed in *Xenopus* oocytes was able to efflux PMEA, efflux was competitively blocked by increasing concentrations of unlabelled PMEA ($IC_{50} = 75$ μM) and MK571 ($IC_{50} = 27$ μM), efflux increased in the presence of Mg-ATP and a hydrolysable ATP analogue and decreased in the presence of a nonhydrolysable ATP analogue. Furthermore, increasing concentrations of ivermectin effectively blocked PMEA efflux ($IC_{50} = 4.6$ μM) and ivermectin itself was directly effluxed with efflux increasing over time. These results are consistent with our initial findings that: (1) *PhABCC4* transcripts were significantly increased following brief exposure to sublethal amounts of ivermectin using the non-invasive induction assay that produced tolerance in susceptible lice; and (2) lice injected with *PhABCC4* dsRNA became more sensitive to ivermectin toxicity (Yoon *et al*., 2011). These findings and the findings reported in the current study confirmed that PhABCC4 is directly involved in the detoxification of ivermectin by Phase III xenobiotic metabolism and overexpression of this transporter could function as a mechanism for resistance once field selection occurs.

Recently, an overexpressed P450 (CYP392A16), which is associated with abamectin resistance in a resistant strain of *Tetranychus urticae*, the two-spotted spider mite, was found to oxidatively hydroxylate abamectin, resulting in a substantially less toxic metabolite and resistance (Riga *et al*., 2014). Although the CYP392A16 of *T. urticae* belongs to a different CYP family to CYP6CJ1 (clades 2 and 3, respectively), it is possible that their substrate specificities overlap as many P450s have relatively broad substrate specificities. In addition to oxidative hydroxylation at C_{24} (24-OH) and C_{26} (26-OH), ivermectin is also oxidatively detoxified by O-demethylation at the 3′ carbon in the distal sugar moiety (3′-OH). Although CYP6CJ1 was capable of both O-dealkylation and O-dearylation reactions, the O-dealkylation of 7-EC was significantly favoured, suggesting that the ivermectin metabolite formed may be the 3′-OH metabolite formed by the O-demethylation of the distal sugar moiety. CYP6CJ1 has likewise been determined to be closely related to human CYP3A4 (Yoon *et al*., 2011), a P450 known to metabolize a diversity of insecticides, including ivermectin, and erythromycin, a close structural analogue of ivermectin (Wacher *et al*., 1995). Additionally, *CYP6CJ1* showed the greatest level of ivermectin induction by quantitative PCR analysis and is most closely related to *Anopheles gambiae* CYP6Z2, which binds a broad range of xenobiotics, including ivermectin (Mclaughlin *et al*., 2008). In the present study, however, the low level of oxidative metabolism by CYP6CJ1 did not allow the identification of the

ivermectin metabolite formed *in vitro* and may be more suggestive of an additional role in ivermectin sequestration as mentioned above. It is possible, however, that the low level of ivermectin metabolism, as evidenced by the reduction of ivermectin over time in the *in vitro* microsomal assay following expression in Sf9 cells, may have occurred for a number of other reasons. It is well known that ivermectin possesses unique solubility properties when compared to many other insecticides in that it is not water soluble or lipophilic but is hydrophobic, being soluble only in solvents such as dimethyl sulfoxide. This property results in relatively slow toxicokinetics and its relatively slow lethal action on many insects. In the *in vitro* assay used, there may have been substantial non-specific binding of ivermectin by the microsomes, reducing its ability to be metabolized efficiently by CYP6CJ1. Furthermore, the standard conditions used in the *in vitro* assay (a single protein concentration, a single ivermectin concentration and only a 2 h incubation time) probably were not optimal for the oxidative metabolism of ivermectin. Lastly, in addition to CYP6CJ1, two other P450s were identified using the non-invasive induction assay, CYP9AG1 and 9AG2, both of which need to be examined in terms of their role in the oxidative metabolism of ivermectin.

Nevertheless, the induction of both *ABCC4* and *CYP6CJ1* has been shown to occur during ivermectin-induced tolerance and the knockdown of these two genes resulted in increased louse sensitivity to ivermectin, indicating a possible interaction between them. Our current finding that ABCC4 directly effluxes ivermectin in an ATP-dependent fashion could allow for substantial protection against the toxic action of ivermectin even in the presence of a low level of oxidative ivermectin metabolism or sequestration by CYP6CJ1. As it is not known if this ABC transporter and P450 are co-localized in lice, further tissue distribution studies would be necessary to prove such an interaction.

In summary, PhABCC4 clearly metabolizes ivermectin by way of Phase III xenobiotic metabolism. The role of CYP6CJ1 is less clear but it appears to reduce the amount of ivermectin either by Phase 1 oxidative xenobiotic or sequestration. However, additional experiments are necessary to confirm this contention.

If the activities of these two proteins are found to be enhanced in louse populations following field selection, they would probably result in metabolic resistance to ivermectin. Monitoring of the expression profiles of these putative metabolic resistance genes in field louse populations on a regular basis should provide crucial information on the early stages of ivermectin resistance development, which then could be used in a proactive resistance management programme for this valuable new pediculicide.

Experimental procedures

Ethical statement

All experiments in this study were carried out based on ethical procedures and scientific care according to the animal use protocol that had been reviewed and approved by the Institutional Animal Care and Use Committee of University of Massachusetts Amherst (protocol number: 2013-0026).

cRNA preparation

The *PhABCC4* cDNA sequence (4416 bp) was obtained from the National Center for Biotechnology Information website (GenBank accession number, XM_002427843). To increase efficiency and accuracy of the PhABCC4 translation in *X. laevis* oocytes, codon usage optimization was performed using the GeneOpimizer Algorithm™ by Life Technologies (Carlsbad, CA, USA) as a part of the gene synthesis service (GeneArt™). The optimized *PhABCC4* gene was synthesized and inserted into the pcDNA_3.1 plasmid using *Kpn*I and *Xho*I cloning sites. The plasmid DNA containing the optimized *PhABCC4* was purified from transformed *Escherichia coli* K12 cells (dam^+, dcm^+, rec^-). DNA sequencing was performed to verify the sequence of the optimized *PhABCC4*. Template DNA for cRNA synthesis was generated by PCR using the plasmid as a template with a primer set containing T7 promoter sequence (forward, 5'-TAATACGACTCACTATAGGGGCTTGCTTGTTCTTTTTGCAG-3' and reverse, 5'-GCCACTGTGCTGGATATCTG-3') in a Mastercycler pro thermal cycler (Eppendorf AG, Hamburg, Germany). The PCR was conducted using the following cycling conditions: a single denaturation cycle at 98°C for 2 min and 30 cycles of 98°C for 10 s, 63°C for 15 s and 72°C for 4 min. The PCR product was purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA), and then used to synthesize capped cRNA using a mMessage mMachine kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. The cRNA was purified with lithium chloride precipitation, diluted to 1 μg/μl and run on a denaturing formaldehyde agarose gel to confirm size and purity.

Expression of PhABCC4 *in X. laevis oocytes*

Adult *X. laevis* frogs (Nasco, Fort Atkinson, WI, USA) were anaesthetized with 0.2% Tricaine (Sigma, St Louis, MO, USA) and the oocytes surgically extracted. The oocyte follicles were treated with Type IA Collagenase (160 μg/ml; Sigma) in OR2 buffer (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) at room temperature for 45 min, washed and then manually defollicated using fine forceps. Stage V and VI oocytes were isolated, and injected with 25 nl of capped PhABCC4 cRNA at a concentration of 1 μg/μl or injected with ddH_2O for control oocytes. Oocytes were incubated for 3 days at 18°C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5) supplemented with 5% horse serum, 1% sodium pyruvate and antibiotics (penicillin and streptomycin) prior to experimental use.

Efflux assays using radiolabelled substrates

For efflux assays, tritium-labelled ([^{3}H])-PMEA (97.3% pure, Moravek Biochemicals, Brea, CA, USA) and [^{3}H]-ivermectin (Ivermectin-B1a [22,23-^{3}H], 98% pure, American Radiolabeled Chemicals, St Louis, MO, USA) were used as substrates of expressed PhABCC4. Oocytes were injected with 25 nl of either a 20 μM [^{3}H]-PMEA solution containing 10 000 disintegrations per minute (DPM) or with a 9 μM [^{3}H]-ivermectin solution containing 20 000 DPM. Oocytes were washed twice with ND96 buffer before being transferred individually into a single well of a 96-well plate with 100 μl ND96 buffer in each well. At predefined time points, 85-μl aliquots were removed from each well and the aliquots pooled for eight to 10 oocytes. 500 μl of the resulting pooled sample was mixed with 5 ml of BioFluor liquid scintillation fluid (New England Nuclear, Boston, MA, USA) and CPM determined using a liquid scintillation counter (1209 Rackbeta, Wallac Inc., Gaithersburg, MD, USA).

For competition or inhibition assays, a range of nonradiolabelled PMEA, AMP-PNP, MK571, quercetin, verapamil, DDVP (> 95% pure), imidacloprid (99% pure), DDT (98% pure) (all from Sigma) and ivermectin (98%, Chem Service, Inc., West Chester, PA, USA) concentrations were co-injected into ABCC4-expressing oocytes along with 25 nl of a 20 μM [^{3}H]-PMEA solution. After 30 min incubation, [^{3}H]-PMEA efflux was measured using the same method described above. Probit analysis was conducted to determine the mean effective concentration (EC_{50}) or mean inhibition concentration (IC_{50}) value using PoloPlus software (LeOra Software, Petaluma, CA, USA). All experiments were replicated three times with independent injection of cRNA using oocytes from different frogs.

Baculovirus-mediated expression of CYP6CJ1

The *P. humanus humanus* coding sequence of CYP6CJ1 (XP_002432833) was obtained by gene synthesis (Geneart, Burlingame, CA, USA). Sf9 cells (Invitrogen, Carlsbad, CA, USA) were maintained under serum-free conditions at 27°C with Sf-900 II SFM according to the manufacturer's manual. The respective P450 and a CPR from *D. melanogaster* (accession number Q27597) was inserted into the pDEST8 expression vector (Invitrogen). The PFastbac1 vector containing no foreign DNA was used to produce a control virus. The recombinant baculovirus DNA was constructed and transfected to Sf9 insect cells (Gibco, Rockville, MD, USA) using the Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer's instructions. The titre of the recombinant viruses was determined following the standard protocols provided by the supplier. For expression, Sf9 cells were co-infected with baculovirus of the respective P450 and CPR with a multiplicity of infection (MOI) of 3:0.5. Hemin chloride (2.5 μg/ml) was added to the culture media to compensate for the low levels of endogenous heme in insect cells. Control cells were co-infected with the baculovirus containing vector with no insert (Sf9 control) and the recombinant baculovirus only expressing CPR using the same MOI ratios as described above. After 48 h, cells were harvested, washed with phosphate-buffered saline and microsomes of the membrane fraction prepared according to standard procedures and stored at −80°C (Phillips & Shephard, 2006). P450 expression and functionality were estimated by measuring CO-difference spectra in reduced samples (Oumura & Sato, 1964).

Enzyme activity measurements

P450 enzymatic activity was confirmed by measuring the O-dealkylation and O-dearylation activity on a range of fluorescent model substrates using microsomal preparations in a 96-well plate as described earlier (Zimmer *et al*., 2014). The following analytical grade substrates were used: 7-EC; BFC; BOMR (all purchased from Thermo Fisher Scientific, San Jose, CA, USA). The fluorescence was measured with a Spectra Max M2 Spectrofluorimeter plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) at the appropriate excitation/emission wavelength settings according to the manufacturer's instructions. The activities of CYP6CJ1-expressing microsomes were compared to control microsomes obtained from Sf9 cells infected with recombinant baculovirus containing only CPR. All experiments were replicated four times.

Ivermectin depletion assay

Ivermectin depletion was assayed by incubation of the recombinant CYP6CJ1/CPR microsomes (0.4 mg/ml total protein content) in 0.1 M potassium phosphate buffer with an NADPH-regenerating system (Promega, Madison, WI, USA; 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$, 0.4 U/ml glucose-6-phosphate dehydrogenase) and ivermectin (1 μM) at 27°C for 2 h. The total assay volume was 200 μl, with three replicates for each data point. Microsomes without NADPH served as controls. The assay was stopped by the addition of 800 μl of ice-cold acetonitrile and centrifuged for 10 min at 18 000 *g* after 1 h on ice. The supernatant was subsequently analysed by tandem mass spectrometry. Recovery rates of ivermectin using microsomal fractions without NADPH were normally close to 100%.

UPLC-MS/MS analysis

All samples obtained from ivermectin metabolism/depletion assays were analyzed using an Ultra Performance LC (Waters Acquity UPLC system) equipped with online degasser, column oven, autosampler and Acquity C18 RP column, 50 × 2.1 mm i.d., 1.7 μm particle size (Waters, Eschborn, Germany). Samples were separated by gradient elution using a mobile phase consisting of HPLC-grade water [+0.1% (v/v) formic acid] and acetonitrile [+0.1% (v/v) formic acid] with a constant flow rate of 0.6 ml/min. The gradient elution conditions were as follows: 0 min acetonitrile : water 10:90, 1.5 min 95:5, 3 min 95:5, 3.1 min 10:90 and 4 min 10:90. The mass spectrometer used in this study was a TSQ Vantage instrument equipped HESI II source (Thermo Scientific, Bremen, Germany) operating in positive ion mode at an ionization voltage of 3000 volts. High-purity nitrogen at 500°C was used as the sheath/auxiliary gas as well as collision gas. Ivermectin depletion was detected by single-reaction monitoring. All experiments were replicated three times.

Statistical analysis

All statistical analyses were performed using GraphPad Prism (v. 5, GraphPad Inc., San Diego, CA, USA) software. Mean and

SD were calculated for each data set and then statistical differences were determined by ANOVA followed by Bonferroni post hoc tests and Student's *t*-tests.

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References

Bai, J., Lai, L.Q., Yeo, H.C., Goh, B.C. and Tan, T.M.C. (2004) Multidrug resistance protein 4 (MRP4/ABCC4) mediates efflux of bimane-glutathione. *Int J Biochem Cell Biol* **36**: 247–257.

Bartley, D., McAllister, H., Bartley, Y., Dupuy, J., Menez, C., Alvinerie, M. *et al*. (2009) P-glycoprotein interfering agents potentiate ivermectin susceptibility in ivermectin sensitive and resistant isolates of *Teladorsagia circumcincta* and *Haemonchus contortus*. *Parasitology* **136**: 1081–1088.

Brand, W., Schutte, M.E., Williamson, G., van Zanden, J.J., Cnubben, N.H.P., Groten, J.P. *et al*. (2006) Flavonoid-mediated inhibition of intestinal ABC transporters may affect the oral bioavailability of drugs, food-borne toxic compounds and bioactive ingredients. *Biomed Pharmacother* **60**: 508–519.

Buss, D., McCaffery, A. and Callaghan, A. (2002) Evidence for p-glycoprotein modification of insecticide toxicity in mosquitoes of the *Culex pipiens* complex. *Med Vet Entomol* **16**: 218–222.

Campbell, W. (1985) Ivermectin: an update. *Parasitol Today* **1**: 10–16.

Chosidow, O., Giraudeau, B., Cottrell, J., Izri, A., Hofmann, R., Mann, S.G. *et al*. (2010) Oral ivermectin versus malathion lotion for difficult-to-treat head lice. *N Engl J Med* **362**: 896–905.

Dent, J.A., Davis, M.W. and Avery, L. (1997) avr-15 encodes a chloride channel subunit that mediates inhibitory glutamatergic neurotransmission and ivermectin sensitivity in *Caenorhabditis elegans*. *EMBO J* **16**: 5867–5879.

Duce, I. and Scott, R. (1985) Actions of dihydroavermectin B1α on insect muscle. *Br J Pharmacol* **85**: 395–401.

Geary, T.G. (2005) Ivermectin 20 years on: maturation of a wonder drug. *Trends Parasitol* **21**: 530–532.

Higgins, C.F. (2007) Multiple molecular mechanisms for multidrug resistance transporters. *Nature* **446**: 749–757.

Holland, I.B. and Blight, M.A. (1999) ABC-ATPases, adaptable energy generators fuelling transmembrane movement of a variety of molecules organisms from bacteria to humans. *J Mol Biol* **293**: 381–399.

Imaoka, T., Kusuhara, H., Adachi, M., Schuetz, J.D., Takeuchi, K. and Sugiyama, Y. (2007) Functional involvement of multidrug resistance-associated protein 4 (MRP4/ABCC4) in the renal elimination of the antiviral drugs adefovir and tenofovir. *Mol Pharmacol* **71**: 619–627.

James, C.E. and Davey, M.W. (2009) Increased expression of ABC transport proteins is associated with ivermectin resistance in the model nematode *Caenorhabditis elegans*. *Int J Parasitol* **39**: 213–220.

Jani, M., Makai, I., Kis, E., Szabo, P., Nagy, T., Krajcsi, P. and Lespine, A. (2011) Ivermectin interacts with human ABCG2. *J Pharm Sci* **100**: 94–97.

Janke, D., Mehralivand, S., Strand, D., Gotel-Armbrust, U., Habermeier, A., Gradhand, U. *et al*. (2008) 6-mercaptopurine and 9-(2-phosphonyl-methoxyethyl) adenine (PMEA) transport altered by two missense mutations in the drug transporter gene ABCC4. *Hum Mutat* **29**: 659–669.

Jutabha, P., Wempe, M.F., Anzai, N., Otomo, J., Kadota, T. and Endou, H. (2010) *Xenopus laevis* oocytes expressing human P-glycoprotein: probing trans-and cis-inhibitory effects on [^{3}H] vinblastine and [^{3}H] digoxin efflux. *Pharm Res* **61**: 76–84.

Kehoe, J., Buldakova, S., Acher, F., Dent, J., Bregestovski, P. and Bradley, J. (2009) Aplysia cys-loop glutamate-gated chloride channels reveal convergent evolution of ligand specificity. *J Mol Evol* **69**: 125–141.

Lage, H. (2003) ABC-transporters: implications on drug resistance from microorganisms to human cancers. *Int J Antimicrob Agents* **22**: 188–199.

Lee, S.H., Kang, J.S., Min, J.S., Yoon, K.S., Strycharz, J.P., Johnson, R. *et al*. (2010) Decreased detoxification genes and genome size make the human body louse an efficient model to study xenobiotic metabolism. *Insect Mol Biol* **19**: 599–615.

Lespine, A., Martin, S., Dupuy, J., Roulet, A., Pineau, T., Orlowski, S. *et al*. (2007) Interaction of macrocyclic lactones with P-glycoprotein: structure-affinity relationship. *Eur J Pharm Sci* **30**: 84–94.

Mclaughlin, L.A., Niazi, U., Bibby, J., David, J.P., Vontas, J., Hemingway, J. *et al*. (2008) Characterization of inhibitors and substrates of *Anopheles gambiae* CYP6Z2. *Insect Mol Biol* **17**: 125–135.

Nakanishi, T., Doyle, L.A., Hassel, B., Wei, Y., Bauer, K.S., Wu, S. *et al*. (2003) Functional characterization of human breast cancer resistance protein (BCRP, ABCG2) expressed in the oocytes of *Xenopus laevis*. *Mol Pharmacol* **64**: 1452–1462.

Oumura, T. and Sato, R. (1964) The carbon monoxide-binding pigment of liver microsomes. *J Biol Chem* **239**: 2370–2378.

Phillips, I.R. and Shephard, E.A. (2006) *Cytochrome P450 Protocols*, 2nd edn. Humana Press Inc., Totowa, NJ.

Pohl, P.C., Klafke, G.M., Carvalho, D.D., Martins, J.R., Daffre, S., da Silva Vaz, I. *et al*. (2011) ABC transporter efflux pumps: a defense mechanism against ivermectin in *Rhipicephalus* (*Boophilus*) *microplus*. *Int J Parasitol* **41**: 1323–1333.

Pouliot, J.F., L'Heureux, F., Liu, Z., Prichard, R.K. and Georges, E. (1997) Reversal of P-glycoprotein-associated multidrug resistance by ivermectin. *Biochem Pharmacol* **53**: 17–25.

Prehm, P. and Schumacher, U. (2004) Inhibition of hyaluronan export from human fibroblasts by inhibitors of multidrug resistance transporters. *Biochem Pharmacol* **68**: 1401–1410.

Qian, L., Cao, G.C., Song, J.X., Yin, Q. and Han, Z.J. (2008) Biochemical mechanisms conferring cross-resistance between tebufenozide and abamectin in Plutella xylostella. *Pestic Biochem Physiol* **91**: 175–179.

Riga, M., Tsakireli, D., Ilias, A., Morou, E., Myridakis, A., Stephanou, E.G. *et al*. (2014) Abamectin is metabolized by CYP392A16, a cytochrome P450 associated with high levels of acaricide resistance in *Tetranychus urticae*. *Insect Biochem Mol Biol* **46**: 43–53.

Roth, C.W., Holm, I., Graille, M., Dehoux, P., Rzhetsky, A., Wincker, P. *et al*. (2003) Identification of the *Anopheles gambiae* ATP-binding cassette transporter superfamily genes. *Mol Cells* **15**: 150–158.

Schuetz, J.D., Connelly, M.C., Sun, D., Paibir, S.G., Flynn, P.M., Srinivas, R. *et al*. (1999) MRP4: a previously unidentified factor in resistance to nucleoside-based antiviral drugs. *Nat Med* **5**: 1048–1051.

Scott, J.G. (1999) Cytochromes P450 and insecticide resistance. *Insect Biochem Mol Biol* **29**: 757–777.

Sparks, T.C. and Nauen, R. (2015) IRAC: mode of action classification and insecticide resistance management. *Pestic Biochem Physiol* **121**: 122–128.

Strycharz, J.P., Yoon, K.S. and Clark, J.M. (2008) A new ivermectin formulation topically kills permethrin-resistant human head lice (Anoplura: Pediculidae). *J Med Entomol* **45**: 75–81.

Stumpf, N. and Nauen, R. (2002) Biochemical markers linked to abamectin resistance in *Tetranychus urticae* (Acari: Tetranychidae). *Pestic Biochem Physiol* **72**: 111–121.

Tsuruoka, S., Ishibashi, K., Yamamoto, H., Wakaumi, M., Suzuki, M., Schwartz, G.J. *et al.* (2002) Functional analysis of ABCA8, a new drug transporter. *Biochem Biophys Res Commun* **298**: 41–45.

Wacher, V.J., Wu, C.Y. and Benet, L.Z. (1995) Overlapping substrate specificities and tissue distribution of cytochrome-P450 3a and P-Glycoprotein - implications for drug-delivery and activity in cancer-chemotherapy. *Mol Carcinog* **13**: 129–134.

Woodruff, H.B. and Burg, R.W. (1986) The antibiotics explosion. *Discov Pharm* **3**: 338–341.

Xu, M., Molento, M., Blackhall, W., Ribeiro, P., Beech, R. and Prichard, R. (1998) Ivermectin resistance in nematodes may be caused by alteration of P-glycoprotein homolog. *Mol Biochem Parasitol* **91**: 327–335.

Yoon, K.S., Nelson, J.O. and Clark, J.M. (2002) Selective induction of abamectin metabolism by dexamethasone, 3-methylcholanthrene, and phenobarbital in Colorado potato beetle, *Leptinotarsa decemlineata* (Say). *Pestic Biochem Physiol* **73**: 74–86.

Yoon, K.S., Strycharz, J.P., Baek, J.H., Sun, W., Kim, J.H., Kang, J.S. *et al.* (2011) Brief exposures of human body lice to sublethal amounts of ivermectin over-transcribes detoxification genes involved in tolerance. *Insect Mol Biol* **20**: 687–699.

Zimmer, C.T., Bass, C., Williamson, M.S., Kaussmann, M., Wölfel, K., Gutbrod, O. *et al.* (2014) Molecular and functional characterization of CYP6BQ23, a cytochrome P450 conferring resistance to pyrethroids in European populations of pollen beetle, *Meligethes aeneus*. *Insect Biochem Mol Biol* **45**: 18–29.