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Abamectin resistance in *Drosophila* is related to increased expression of P-glycoprotein via the dEGFR and dAkt pathways

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ABSTRACT

Many insects have evolved resistance to abamectin but the mechanisms involved in this resistance have not been well characterized. P-glycoprotein (P-gp), an ATP-dependent drug-efflux pump transmembrane protein, may be involved in abamectin resistance. We investigated the role of P-gp in abamectin (ABM) resistance in *Drosophila* using an ABM-resistant strain developed in the laboratory. A toxicity assay, Western blotting analysis and a vanadate-sensitive ATPase activity assay all demonstrated the existence of a direct relationship between P-gp expression and ABM resistance in these flies. Our observations indicate that P-gp levels in flies' heads were higher than in their thorax and abdomen, and that both P-gp levels and LC_{50} values were higher in resistant than in susceptible and P-gp-deficient strains. In addition, P-gp levels in the blood–brain barrier (BBB) of resistant flies were higher than in susceptible and P-gp-deficient flies, which is further evidence that a high level of P-gp in the BBB is related to ABM resistance. Furthermore, we found greater expression of *Drosophila* EGFR (dEGFR) in the resistant strain than in the susceptible strain, and that the level of *Drosophila* Akt (dAkt) was much higher in resistant than in susceptible flies, whereas that in P-gp-deficient flies was very low. Compared to susceptible flies, P-gp levels in the resistant strain were markedly suppressed by the dEGFR and dAkt inhibitors lapatinib and wortmannin. These results suggest that the increased P-gp in resistant flies was regulated by the dEGFR and dAkt pathways and that increased expression of P-gp is an important component of ABM resistance in insects.

1. Introduction

The biggest challenge facing the agricultural industry is the growing resistance of invertebrate pests to pesticides. Abamectin (ABM) belongs to the family of avermectins (AVMs) which are fermented from a soil actinomycete microorganism, *Streptomyces avermitilis*. ABM is mainly used as an insecticide and miticide for crop protection and as a veterinary medicine (Clark et al., 1995). All AVMs share a similar mechanism of action; they interact with various ligand-gated ion chloride channels, such as glutamate-gated chloride channels (GluCls), γ-aminobutyric acid chloride channels and histamine-gated chloride channels (Sigel and Baur, 1987; Adelsberger et al., 1997, 2000; Shan et al., 2001; Zheng et al., 2002). AVMs can act directly on GluCls, a receptor found only in invertebrates, thereby causing an increase in chloride permeability that ultimately causes the paralysis of an insect's muscles and inhibition of chloride pumping (Cully et al., 1996). These unique properties have made ABM an effective insecticide and miticide (Geary, 2005). However, its extensive use over many years has led to the development of resistance in several insect pests (Rousw and Wright, 1986; Argentine and Clark, 1990; Scott et al., 1991; Liang et al., 2003; Pu et al., 2010). Some insects such as *Plutella xylostella, Liriomyza trifolii*, *Leptinotarsa decemlineata*, and *Frankliniella occidentalis* have developed a moderate (15–24-fold) level of resistance to ABM, but *Musca domestica* L. has a much higher (>60,000-fold) level of resistance (Scott et al., 1991). So far, most research on ABM resistance has focused on the biochemistry of metabolic enzymes (Clark et al., 1995) and the mutation of the related chloride channels (Kwon et al., 2010; Ghosh et al., 2012; Dermauw et al., 2012); however, the molecular mechanisms underlying this resistance have not been fully elucidated.

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ABM is a substrate and inhibitor for a membrane glycoprotein termed the P-glycoprotein (P-gp) (Lespine et al., 2009). P-gp belongs to a membrane-spanning protein superfamily with an ATP-binding cassette (ABC) that pumps xenobiotics out of cells via an ATP-dependent mechanism (Mizutani et al., 2008; Sui et al., 2012). Several homologs of P-gp have been identified with molecular cloning techniques in insect species such as *Drosophila melanogaster*, *Teleogryllus commodus*, and *Helicoverpa armigera* (Buss and Callaghan, 2008; Aurade et al., 2010). In addition, P-gp has been shown to be an indispensable structural component of the blood–brain barrier (BBB) in *Drosophila* (Mayer et al., 2009). P-gp is one of the most highly-conserved proteins (Buss and Callaghan, 2008) and may be widely distributed in insects.

Until recently there have been few reports in the literature linking ABM resistance to P-gp. Larvae of the tobacco budworm *Heliothis virescens* that are resistant to carbamate and thiodicarb pesticides were found to have 2–6-fold more P-gp than larvae of a susceptible strain (Lanning et al., 1996). Elevated P-gp protein expression was also found in a wild population of *Helicoverpa armigera* that is resistant to pyrethroids, organophosphates, and cyclodienes (Aurade et al., 2010; Srinivas et al., 2004). There is also evidence that the P-gp inhibitor verapamil significantly increases the toxicity of some insecticides (Buss et al., 2002). These findings suggest that insect P-gp homologues may play an important role in pesticide resistance.

It has also been reported that pyrethroids, cyclodienes, and organophosphates induce P-gp activity, or change in its expression levels, in both vertebrates and invertebrates (Buss and Callaghan, 2008; Buss et al., 2002; Srinivas et al., 2004). However, there have been few reports on the mechanism by which these chemicals up-regulate P-gp expression in insects. It has also been reported that P-gp expression was inhibited by the tyrosine kinase inhibitor of the epidermal growth factor receptor EGFR (Kitazaki et al., 2005), which plays crucial roles in cell migration, adhesion, apoptosis, cell cycle progression, growth, and angiogenesis (Hynes and Lane, 2005; Yarden and Sliwkowski, 2001). EGFR-downstream signaling pathways include the protein kinase B (Akt, PKB), extracellular-signal regulated protein kinase (ERK), and protein kinase C (PKC) relevant pathways (Li et al., 2011; Colabufo et al., 2011). Some drugs can down-regulate the *Mdr1b* (P-gp) gene by inhibiting the Akt or ERK pathway (Choi et al., 2008; Barancik et al., 2006; Chiarini et al., 2008). EGFR and Akt kinases and their pathways are highly conserved in eukaryotes (Shilo, 2005), their homologues in *Drosophila* being dEGFR and dAkt respectively. dEGFR is a member of the fly genome's EGFR ErbB family and is very similar to the human EGFR family member ErbB2 (Alvarado et al., 2009).

We here show that there is a direct relationship between P-gp and ABM resistance in *Drosophila*, and that increased P-gp expression in the heads of resistant *Drosophila* is mainly located in the BBB and highly correlates with ABM resistance. We also demonstrate that ABM induces P-gp expression through the activation of the dEGFR and dAkt pathways.

2. Materials and methods

2.1. Chemicals and antibodies

Abamectin (containing 93% avermectin B1a and 7% avermectin B1b) was obtained from the Beijing CAU Bio-technology Co., Ltd. (Beijing, China). $ATPNa_2$ was purchased from Sigma–Aldrich (St. Louis, MO, USA). Monoclonal anti-P-gp antibody (C219) was purchased from Abcam (Cambridge, UK). Anti-ErbB2 (EGFR) and anti-Akt antibodies were purchased from Cell Signaling Technology (Boston, MA, USA).

2.2. Drosophila stocks and culture

A P-gp-deficient *Drosophila* strain MDR65 (KG08723), referred to here as PMDR65, was obtained from Bloomington Drosophila Stock Center (Indiana University, Bloomington, IN, USA). All *Drosophila* were reared under standard conditions of 25 ± 0.5 °C, 65–75% RH and a 12:12 h dark/light cycle on standard cornmeal, yeast, sucrose and agar medium. The *Canton-S Drosophila* strain was used as the abamectin-sensitive strain; these flies were reared on a synthetic diet without ABM. An abamectin-resistant strain was derived from the *Canton-S* strain by feeding flies from that strain on medium containing progressively higher doses of ABM. ABM was dissolved in dimethyl sulfoxide (DMSO) and mixed with the standard *Drosophila* medium; the final DMSO concentration of the medium was below 0.5% (vol/vol).

2.3. Selection for abamectin resistant flies

Drosophila were selected for ABM resistance by gradually increasing the concentration of abamectin (ABM) in standard fly medium over more than 70 generations over 35 months. When more than 60% of flies could survive and reproduce in a medium containing a given concentration of ABM, living second-generation flies would then be fed medium containing a higher concentration of ABM. Whenever the fly population fell to <100 the level of ABM in the medium was held constant for a few generations before being increased. The medium was replaced monthly to maintain the potency of the ABM it contained.

Drosophila were initially fed medium containing 20 nM ABM for one month after which the ABM content was increased to 30 nM ABM. After 2 months at 30 nM ABM most flies were able to grow and reproduce and so were then transferred onto a medium containing 50 nM. In this way, flies were exposed to progressively higher doses of ABM. Finally, after 35 months of selection flies could survive on medium containing 900 nM ABM. This final resistant generation is hereafter referred to as the resistant strain.

2.4. Chemicals treatment, toxicity assay and larval LD_{100} bioassay

The flies were treated with different concentrations of ABM, verapamil, lapatinib, and wortmannin. 200 μL of different concentrations of the chemical prepared in acetone was transferred to individual glass scintillation vials. The vials were then rolled until the acetone had fully evaporated in order to coat the inside wall of the vials uniformly with the chemical. The vials were then left at room temperature for at least 4 h to dry completely. Mature flies (5–10 days old) were anesthetized with CO_2 and 25–35 flies per group were sorted into vials containing ordinary *Drosophila* medium. These flies were left overnight at room temperature to recuperate and the following day all live flies were transferred to the chemical-coated scintillation vials, which were then sealed with cotton plugs moistened with 5% sucrose solution. After the 48-h treatment, the flies were collected and samples were prepared to do the Western blotting analysis.

The toxicity of the flies to ABM was assessed by counting the number of dead and moribund (those that could not move or stand up) flies 24 h after exposure to ABM. Flies in vials that had been coated with acetone only served as controls. The data were analyzed using probit analysis in SPSS for Windows 13.0 (SPSS Inc., Chicago, IL, USA).

To assay larval LD_{100} to ABM, approximately 30 egg-laying adults were placed in a vial with food containing various doses of ABM and allowed to lay eggs for 5 days. After removal of the adults, the development of larvae, pupae, and adults was monitored for 3