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The role of *Rdl* in resistance to phenylpyrazoles in *Drosophila melanogaster*

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ABSTRACT

Extensive use of older generation insecticides may result in pre-existing cross-resistance to new chemical classes acting at the same target site. Phenylpyrazole insecticides block inhibitory neurotransmission in insects via their action on ligand-gated chloride channels (LGCCs). Phenylpyrazoles are broad-spectrum insecticides widely used in agriculture and domestic pest control. So far, all identified cases of target site resistance to phenylpyrazoles are based on mutations in the *Rdl (Resistance to dieldrin)* LGCC subunit, the major target site for cyclodiene insecticides. We examined the role that mutations in *Rdl* have on phenylpyrazole resistance in *Drosophila melanogaster*, exploring naturally occurring variation, and generating predicted resistance mutations by mutagenesis. Natural variation at the *Rdl* locus in inbred strains of *D. melanogaster* included gene duplication, and a line containing two *Rdl* mutations found in a highly resistant line of *Drosophila simulans*. These mutations had a moderate impact on survival following exposure to two phenylpyrazoles, fipronil and pyriprole. Homology modelling suggested that the *Rdl* chloride channel pore contains key residues for binding fipronil and pyriprole. Mutagenesis of these sites and assessment of resistance *in vivo* in transgenic lines showed that amino acid identity at the Ala^{301} site influenced resistance levels, with glycine showing greater survival than serine replacement. We confirm that point mutations at the *Rdl* 301 site provide moderate resistance to phenylpyrazoles in *D. melanogaster*. We also emphasize the beneficial aspects of testing predicted mutations in a whole organism to validate a candidate gene approach.

1. Introduction

Insect pests are exposed to a diverse array of chemicals in their environment. It is necessary for insect populations to adapt to changes upon exposure to chemicals in order to survive. Thus there is a constant race between evolution of resistance to an insecticide and the discovery of new biologically active chemicals. One of the major challenges in the development of novel insecticides is cross-resistance due to exposure to older, obsolete chemicals. Cross-resistance may arise via a number of mechanisms, such as detoxification, where upregulation of a multi-substrate enzyme increases metabolism of a range of chemicals. For example, overexpression of the cytochrome P450 enzyme CYP6G1 provides resistance to multiple insecticide classes (Daborn et al., 2001, 2002). Alternatively, cross-resistance may occur due to mutations in insecticide target sites, where extensive use of an older insecticide may select for resistance mutations that persist in populations and contribute to allelic variation that can be selected by newer compounds acting at the same site. If the resistance allele is widespread or does not cause detrimental fitness effects to the organism it may linger at frequencies higher than could be maintained by mutation alone. Insecticides target a relatively small number of proteins (Raymond-Delpech et al., 2005), thus the occurrence of cross-resistance between insecticides is unsurprising. It is difficult to find new effective targets that are insect-specific, and to develop chemicals that are environmentally safe for widespread applications while being sufficiently profitable for commercialization (Casida and Quistad, 1998). The widespread use of DDT in the 1950s, and evidence of

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the emergence of multiple independent *kdr* alleles in many insect species, has been problematic for the control capabilities of new generation pyrethroid insecticides. Reduced neuronal sensitivity to the organochlorine dichlorodiphenyltrichloroethane (DDT) due to *kdr* mutations at the L1014 residue in the sodium receptor *para* provides broad-spectrum cross-resistance to pyrethrins and synthetic pyrethroids in a number of diverse insect taxa (Burton et al., 2011; Dong et al., 2014). A range of other substitutions in *para* enhance knockdown resistance to pyrethroids when present together with the original *kdr* change (Farnham et al., 1987; Dong et al., 2014).

One major class of insecticide target sites is the ligand gated chloride channels (LGCCs). In vertebrates LGCCs are pharmacologically significant receptors in the nervous system, providing binding sites for anaesthetics, barbiturates, steroids and benzodiazepines (Buckingham and Sattelle, 2005). In insects, members of the LGCC family have been the targets of lindane and cyclodiene insecticides for over 50 years. They are now the target of new generation insecticides including phenylpyrazoles, avermectins and isoxazolines (Cole et al., 1993; Cully et al., 1996; Ozoe et al., 2010). LGCCs mediate fast inhibitory neurotransmission, facilitating the influx of chloride into neurons upon binding of a neurotransmitter ligand such as GABA, glycine, histamine or glutamate. Insecticides act to antagonise or agonise this action, blocking or augmenting the response (Hosie et al., 1997). Insects contain 10-12 genes encoding LGCC receptor subunits, and so far in *Drosophila melanogaster,* four have been implicated in resistance to at least one insecticide class (Rdl, ffrench-Constant et al., 1993a,b; GluClα, Kane et al., 2000; ort, Iovchev et al., 2002; HisCl1, Yusein et al., 2008).

The most widespread case of resistance at a LGCC target site resulted from a single point mutation in the gene encoding the RDL subunit, causing an Ala^{301} to Ser replacement at the 2′ position of the chloride channel-lining M2 domain that provided 4000-fold resistance to the cyclodiene dieldrin in *D. melanogaster* (ffrench-Constant et al., 1993a,b). The identical mutation was found in multiple species at the equivalent site of RDL orthologues (Thompson et al., 1993; Andreev et al., 1999), as well as an Alanine to Glycine change in some strains of *Drosophila simulans* (ffrench-Constant et al., 1993a,b), and other insect species such as *Myzus persicae* (Anthony et al., 1998) and *Anopheles gambiae* (Du et al., 2005).

The first insecticide of the phenylpyrazole family, fipronil (Fig. 1a and b), was released commercially in 1993. Fipronil is a broad-spectrum chemical now used against a range of pests in agricultural and domestic settings. In 2007 a second phenylpyrazole, pyriprole, was released. Pyriprole is structurally similar to fipronil, with modifications including an additional pyridinylmethyl ring emerging from the 5′N of the pyrazole ring, and a $SCHF_2$ group replacing $S(O)CF_3$ (Fig. 1a–b). Little is currently known about the mode of action, toxicology or metabolism of pyriprole, and in most instances it is assumed to act similarly to fipronil (Prac-Tic 2006).

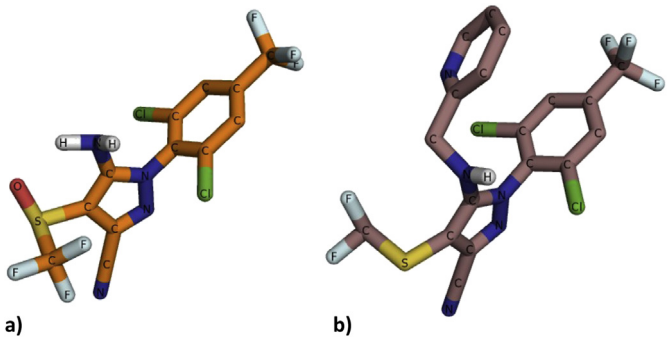

Fig. 1. Chemical structure of phenylpyrazoles. a) Fipronil: 5-amino-1-(2,6-dichloro-α,α,α-trifluoro-*p*-tolyl)-4-trifluoromethylsulfinylpyrazole-3-carbonitrile. b) Pyriprole: 1-(2,6-dichloro-α,α,α-trifluoro-*p*-tolyl)-4-(difluoromethylthio)-5-[(2-pyridylmethyl) amino]pyrazole-3-carbonitrile.

Similar poisoning symptoms to other GABA-gated chloride channel antagonists (endosulfan, dieldrin, lindane, picrotoxin and EBOB; Cole et al., 1993; Ci et al., 2007) and low level cross-resistance in cyclodiene *Rdl*-Ala^{301} to Ser resistant houseflies indicated that phenylpyrazoles were antagonists of the GABAergic system (Cole et al., 1993). However, observation of only low levels of cross-resistance to phenylpyrazoles in cyclodiene resistant strains suggested the two classes may have different binding sites in RDL, or interact with different receptor subunits. Fipronil was also found to block insect-specific GluCl receptors, contributing to the insect-selectivity of these phenylpyrazole insecticides and providing a second known target site (Naharashi et al., 2010). Expression of RDL subunits in *Xenopus laevis* oocyes showed that fipronil was a potent antagonist of wild-type RDL receptors, and this antagonism was reduced in dieldrin resistant Ser^{301} subunits (Hosie et al., 1995). More recently, a 20,000-fold fipronil resistant strain of *D. simulans* was isolated in which two mutations, Ala-to-Gly^{301} and Thr-to-Met^{350}, were identified in the *Rdl* gene of the resistant strain, *Eyguères 42* (Le Goff et al., 2005). Fipronil efficacy and physiological response to GABA were altered in double-mutant receptors in *Xenopus* oocyte expression lines. However, the authors suggested that other background factors such as metabolic detoxification or mutations in other target sites may contribute to the high level of resistance observed in this strain (Le Goff et al., 2005).

While there are many emerging reports of field and laboratory selected resistance to fipronil in a range of pest organisms (Kolaczinski and Curtis, 2001; Kristensen et al., 2004, 2005; Li et al., 2006; Wang et al., 2006; Nakao et al., 2010; Tang et al., 2010; Gondhalekar and Scharf, 2012; Miller et al., 2013), the majority of these are associated with mutations in *Rdl,* relating to cross-resistance to dieldrin. Some species show complete effectiveness of fipronil even when Ser^{301} alleles are present at high frequency, suggesting a negligible impact of this mutation on resistance (Bass et al., 2004). Other studies either show evidence of metabolic contributions, or are yet to confirm specific genetic mechanisms behind resistance (Wen and Scott, 1999; Wang et al., 2006). The widespread use of cyclodienes and the apparent persistence of *Rdl* mutations in the field may result in a skew towards assigning fipronil resistance mechanisms to the equivalent Ala-Ser mutation in pest insects, simply because it is present at a high underlying frequency. Also, because it contributes to a moderate improvement in survival in many species, it may resurface due to selection with phenylpyrazoles. But do mutations in *Rdl* contribute significantly to phenylpyrazole resistance?

In this study, we tested the importance of RDL as a phenylpyrazole target site by exploring the effects of naturally occurring and predicted polymorphisms in phenylpyrazole resistance. We used *D. melanogaster* inbred lines to identify naturally occurring mutations in *Rdl*, examined an RDL homology model to dock phenylpyrazoles and predict interacting residues, and generated transgenic lines to examine changes in the level of resistance provided by naturally occurring polymorphisms as well as artificial changes suggested by modelling in a controlled genetic background.

2. Methods

2.1. Toxicity of phenylpyrazoles to D. melanogaster

0.1% w/v fipronil and pyriprole stock solutions were prepared by dissolving 50 μg fipronil or pyriprole powder (Novartis Animal