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Pesticide Biochemistry and Physiology 80 (2004) 106–112
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Biochemistry & Physiology
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Indoxacarb resistance in the house fly, *Musca domestica*

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Received 24 November 2003; accepted 11 June 2004
Available online 3 August 2004

Abstract

Indoxacarb (DPX-MP062) is a recently introduced oxadiazine insecticide with activity against a wide range of pests, including house flies. It is metabolically decarbomethoxylated to DCJW. Selection of field collected house flies with indoxacarb produced a New York indoxacarb-resistant (NYINDR) strain with >118-fold resistance after three generations. Resistance in NYINDR could be partially overcome with the P450 inhibitor piperonyl butoxide (PBO), but the synergists diethyl maleate and *S*,*S*,*S*-tributyl phosphorothioate did not alter expression of the resistance, suggesting P450 monooxygenases, but not esterases or glutathione *S*-transferases are involved in the indoxacarb resistance. Conversely, the NYINDR strain showed only 3.2-fold resistance to DCJW, and this resistance could be suppressed with PBO. Only limited levels of cross-resistance were detected to pyrethroid, organophosphate, carbamate or chlorinated hydrocarbon insecticides in NYINDR. Indoxacarb resistance in the NYINDR strain was inherited primarily as a completely recessive trait. Analysis of the phenotypes vs. mortality data revealed that the major factor for indoxacarb resistance is located on autosome 4 with a minor factor on autosome 3. It appears these genes have not previously been associated with insecticide resistance.

Keywords: Insecticide resistance; Genetic analysis; Cross-resistance; *Musca domestica*

1. Introduction

Indoxacarb (DPX-MP062) is a recently introduced oxadiazine insecticide with activity against a wide range of pests [1], including house flies [2]. In insects, indoxacarb appears to be decarbomethoxylated to DCJW by an esterase/amidase [3]. Several studies have demonstrated that DCJW is effective at blocking sodium channels [3–8], and it is more effective than indoxacarb at this target site [5,6,8,9]. However, indoxacarb and DCJW have also been shown to affect mammalian nicotinic acetylcholine receptors [10] and have a weak effect on mammalian $GABA_A$ receptors [9]. Unfortunately a comparative study of the effects of indoxacarb and DCJW at these target sites in insects has not been reported.

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doi:10.1016/j.pestbp.2004.06.004

With any new insecticide there are several questions to be addressed. Is there cross-resistance conferred by any other known resistance mechanisms? How rapidly could resistance develop and to what level? How many genes are involved? Is the resistance gene(s) dominant or recessive? Is there cross-resistance to other insecticides? To provide some answers to these questions we selected field collected house flies for resistance to indoxacarb. We also examined the linkage and inheritance of indoxacarb resistance, as well as the cross-resistance patterns and effects of synergists at overcoming the resistance. Our results indicate that resistance can be readily selected in house flies. The mechanisms and genetic linkage of the resistance are discussed.

2. Materials and methods

2.1. Chemicals

Methomyl, indoxacarb (methyl (*S*)-*N*-[7-chloro-2,3,4a,5-tetrahydro-4a(methoxycarbonyl)indeno[1,2-*e*][1,3,4]oxadiazin-2-ylcarbonyl]-4′-(trifluoromethoxy) carbanilate) and its *N*-decarboxomethoxylated metabolite DCJW were from DuPont (Wilmington, DE). Spinosad was from Dow AgroSciences (Indianapolis, IN). Fipronil was from Aventis (Research Triangle Park, NC). Dimethoate and chlorfenapyr were from American Cyanamid (Princeton, NJ). Dieldrin, piperonyl butoxide (PBO) and diethyl maleate (DEM) were from Aldrich (Milwaukee, WI). Tetrachlorvinphos and *S*,*S*,*S*-tributylphosphorotrithioate (DEF) were from Chem Service (West Chester, PA) and cyfluthrin was from Bayer (Kansas City, MO). Abamectin was from Merck Sharp and Dohme (Rahway, NJ). Toxaphene was from Hercules (Wilmington, DE). γ-Chlordane was from Velsicol Chem. (Chicago, IL). Lindane was from Dr. C.F. Wilkinson.

2.2. House fly strains

Two susceptible strains were used: CS (wild type) [11]; and aabys, a strain with the recessive morphological markers *ali-curve* (*ac*), *aristapedia* (*ar*), *brown body* (*bwb*), *yellow eyes* (*ye*), and *snipped wings* (*snp*) on autosomes 1, 2, 3, 4, and 5, respectively. Three insecticide-resistant colonies were used: LPR, YPER, and OCR. LPR is a multi-resistant strain having high levels of resistance to pyrethroid insecticides (e.g., 5900-fold resistance to permethrin) due to increased oxidative metabolism mediated by cytochrome P450 CYP6D1 [12–14]. Two other mechanisms of resistance to pyrethroid insecticides in the LPR strain are insensitivity of the nervous system (*kdr*) and decreased cuticular penetration (*pen*) [12,15]. YPER is a multiple resistant strain with more than 18,000-fold resistance to permethrin due to *super-kdr* and monooxygenase-mediated detoxification [16]. OCR is a cyclodiene-resistant strain (presumably due to an altered GABA receptor, *Rdl*). Additionally, house flies were collected by sweep net from within caged-layer poultry facilities in seven different counties across New York state during the summer of 1999 [17]. House flies were reared as described previously [16].

2.3. Selection of the New York indoxacarb-resistant strain and inheritance of resistance

Indoxacarb selections were done by topical application (see below) to 1-day-old house flies (Table 1). The inheritance of resistance was examined by mass crossing unmated CS females to New York indoxacarb-resistant (NYINDR) males. The F_1 progeny were bioassayed as described below.

2.4. Bioassay

Bioassays were carried out by topical application of a 0.5-μl drop of insecticide in acetone solution to the thoracic notum of 4- to 6-day-old female flies. Each of the three replicates consisted of 20 flies per dose and at least three doses, giving greater than 0 and less than 100% kill. All tests were run at 25 °C and were replicated four times. Mortality was assessed after 48 h, except for indoxacarb and spinosad, which were assayed after 72 h due to the slower acting nature of these insecticides [18]. Bioassay data were pooled and analyzed by standard probit analysis [19], as adapted to personal computer use by Raymond [20] using Abbott's [21] correction for control mortality. PBO, DEF or DEM were applied at a dose of 10 μg/fly in a

Table 1
Selection of the NYINDR strain with indoxacarb

Generation	Females			Males		
	Dose (μg/fly)	Number	% Mortality	Dose (μg/fly)	Number	% Mortality
Parental	1.0	659	88	0.5	596	91
F_1	1.0	186	25	0.5	206	38
F_2	20	320	26	10	360	48
F_3 (=NYINDR)	20		0			

0.5 μl acetone solution to the thoracic notum 1 h prior to dosing with insecticide.

2.5. Selection of the rind strain

Due to the highly recessive nature of indoxacarb resistance in NYINDR strain (see below) it was necessary to establish a resistant strain in which each autosome was marked with a visible mutant marker. To facilitate this, the NYINDR strain was crossed to aabys as outlined in Table 2. This resulted in a strain highly resistant to indoxacarb and having recessive mutant markers on each of the autosomes (i.e., *ac;ar;bwb;ye;snp* phenotype). This strain was named *rind* (resistance to indoxacarb).

2.6. Linkage analysis

The chromosomes involved in the resistance to indoxacarb in the *rind* strain were evaluated using a modified F_1 male backcross method based on the scheme developed by Tsukamoto [22], using a total of 11,823 female and male flies at a diagnostic dose of 3 μg indoxacarb/fly applied as described above. This method involves crossing susceptible wild type females (CS) with resistant marker strain males (*rind*), backcrossing the F_1 males to resistant females (i.e., *rind* female × F_1 (CS female × *rind* male) male) and testing separately each phenotypic class of the progeny with a diagnostic dose of the insecticide. Since crossing over is very rare in male house flies, this method permits the detection and measurement of the "recessive" effect of each chromosome (i.e., the resistance contributed by individual autosomes).

3. Results and discussion

The LD_{50} values of DCJW to the susceptible CS, and three insecticide-resistant strains of house

Table 2
Procedure used to isolate the *rind* strain

Generation	Females		Males	
	Dose (μg/fly)	% Mortality	Dose (μg/fly)	% Mortality
aabys (female) × NYINDR (male)				
F_3	1.0	80	0.3	80
F_6	3.0	88	0.3	85
F_7	3.0	28	0.5	45
F_8	10	74	1.0	64
F_9	10	31	1.0	63
F_{10}	10	13	1.0	14
F_{15}	10	17	1.0	28
F_{17}	10	13	1.0	40
F_{18}	20	64	1.5	74
F_{19}	20	43		
F_{20} (=*rind*)	20	25		

In addition to the selection with indoxacarb the F_4, F_5, and F_8 were sorted and only flies that had a minimum of 2, 3 or 4, of the aabys markers, respectively, were saved. Starting with F_{16}, only flies having all of the aabys markers were used. Thus, the F_{17} and all subsequent generations had the *aabys* phenotype.

flies are shown in Table 3. There was no significant cross-resistance to DCJW in either the LPR or YPER strains indicating that neither the *kdr* nor *super-kdr* mutation provided protection to DCJW. The OCR strain was slightly (2-fold) more sensitive to DCJW compared to CS.

Indoxacarb was moderately toxic to house flies (Table 4) in agreement with previous reports [2,8]. DCJW was 5-fold more active than indoxacarb against the CS strain, similar to a previous report [2] and consistent with the idea that DCJW is the bioactivated form of indoxacarb [3].

Field collected house flies were already 13-fold cross-resistant to indoxacarb (LD_{50} = 2.2 μg/fly; 95% CI: 1.6–3.4; n = 925, slope = 0.7), and three generations of selection resulted in a strain having >118-fold resistance to indoxacarb (Table 4). This strain was named NYINDR. Conversely, the NYINDR strain showed only 3.2-fold resistance to DCJW (Table 4). Thus, it appears the indoxacarb resistance in NYINDR could be due, at least in part, to a change in the amount of DCJW that is produced (i.e., metabolism of indoxacarb into a non-toxic material, or a decreased rate of indoxacarb activation), or to an altered target site (if indoxacarb acts at a different target site than DCJW).

The NYINDR strain was resistant to abamectin (2.1-fold), cyfluthrin (4.4-fold), dieldrin (2.5-fold), γ-chlordane (1.3-fold), lindane (9.4-fold), spinosad (4.1-fold), tetrachlorvinphos (10-fold), and toxaphene (2.3-fold) (Table 4). NYINDR was more sensitive to fipronil (2.2-fold) and chlorfenapyr (1.4-fold) compared to CS. However, since the NYINDR strain was established from multi-

Table 3
Toxicity of DCJW to one insecticide susceptible (CS), and three insecticide-resistant strains of house fly

Strain	LD_{50}[a] (95% CI)	Slope (SE)	n	RR[b]
CS	34.5 (31.1–38.2)	4.3 (0.4)	440	—
LPR	43.2 (38.1–49.1)	3.6 (0.4)	360	1.3
YPER	32.1 (28.1–36.3)	4.1 (0.5)	360	0.9
OCR	17.7 (14.5–21.6)	3.6 (0.6)	580	0.5

[a] LD_{50} in units of ng/fly.
[b] Resistance ratio = LD_{50} of resistant strain/LD_{50} of susceptible strain.

Table 4
Toxicity of selected insecticides to the CS and NYINDR strains of house fly

Insecticide	CS	NYINDR			
	LD_{50}[a] (95% CI)	LD_{50}[a] (95% CI)	n	Slope (SE)	RR[b]
Indoxacarb[d]	0.17 (0.15–0.19)	>20 (6.7[c])	200		>118
DCJW	0.035 (0.031–0.038)	0.082 (0.074–0.090)	300	3.6 (0.3)	3.2
Spinosad[d]	0.054 (0.049–0.058)	0.22 (0.20–0.24)	420	3.9 (0.4)	4.1
Fipronil[d]	0.011 (0.010–0.012)	0.0050 (0.0025–0.0084)	300	4.3 (0.4)	0.45
Cyfluthrin[d]	0.011 (0.008–0.016)	0.048 (0.043–0.054)	360	3.3 (0.4)	4.4
Chlorfenapyr[d]	0.13 (0.11–0.15)	0.095 (0.089–0.100)	300	8.8 (1.0)	0.73
Dimethoate[d]	0.070 (0.064–0.076)	0.071 (0.064–0.077)	480	4.0 (0.4)	1.0
Tetrachlorvinphos[d]	0.085 (0.078–0.092)	0.86 (0.77–0.96)	420	3.1 (0.3)	10
Methomyl[d]	0.28 (0.25–0.30)	0.28 (0.26-0.31)	360	4.0 (0.4)	1.0
Abamectin[d]	0.0021 (0.0019–0.0022)	0.0044 (0.0038–0.0050)	420	2.5 (0.3)	2.1
Dieldrin	0.017 (0.015–0.018)	0.043 (0.039–0.046)	240	6.7 (0.8)	2.5
γ-Chlordane	0.13 (0.12–0.14)	0.17 (0.16–0.18)	300	5.2 (0.6)	1.3
Lindane	0.017 (0.015–0.022)	0.16 (0.14–0.18)	240	4.0 (0.6)	9.4
Toxaphene	0.62 (0.56–0.66)	1.4 (1.3–1.5)	360	6.1 (0.6)	2.3

[a] LD_{50} in units of μg/female fly.
[b] Resistance ratio = LD_{50} of resistant strain/LD_{50} of susceptible strain.
[c] Percent mortality at 20 μg/female fly.
[d] CS values from Shono and Scott [27].

resistant field collected flies [17] it is not clear if these differences represent resistance (due to previous use of the insecticide) or cross-resistance (or negative cross-resistance) due to selection with indoxacarb or another insecticide.

Treatment of CS flies with PBO resulted in a 7.7-fold synergism of indoxacarb toxicity (LD_{50} = 0.022 μg/fly (0.020–0.025), n = 420, slope = 2.9 (0.07)) suggesting significant P450 monooxygenase-mediated detoxification in this susceptible strain. The effect of PBO on the NYINDR strain was even more substantial, although the resulting bioassay line was not straight which is indicative of a heterogeneous population (data not shown). This suggests variability within the NYINDR strain to the synergistic effect of PBO. These bioassay results indicate that in about 40% of the NYINDR flies PBO reduced the resistance ratio about 7-fold, while the other 60% are much less (at least 10-fold) affected. Therefore, it appears that P450 monooxygenases are one of the mechanisms of indoxacarb resistance in this strain, but that the gene(s) responsible for this mechanism is heterozygous.

The indoxacarb LD_{50} for the NYINDR strain was >20 μg/fly following pretreatment with either DEF or DEM indicating that neither synergist substantially altered the toxicity of indoxacarb to the NYINDR strain. Thus, it appears that the mechanism of resistance is not due to metabolic detoxification by hydrolases or glutathione *S*-transferases. Treatment of CS flies with DEF resulted in a 2.2-fold synergism of indoxacarb toxicity suggesting a small, but significant hydrolase-mediated detoxification in this susceptible strain (data not shown).

The toxicity of DCJW was evaluated against the aabys and *rind* strains of house fly (Table 5). The *rind* strain was 4.2-fold resistant. However, the monooxygenase inhibitor PBO could overcome this resistance. Thus, resistance to DCJW appears to be mediated by P450 monooxygenases.

Given the 9.4-fold resistance to lindane in the NYINDR strain we determined the toxicity of lindane to the aabys (LD_{50} = 0.033 μg/fly) and *rind* (LD_{50} = 0.094 μg/fly) strains. The low (2.9-fold) resistance to lindane in the *rind* strain suggests no (or very limited) cross-resistance between indoxacarb and lindane in these strains.

Indoxacarb resistance in the NYINDR strain was inherited primarily as a completely recessive trait, although the presence of a plateau in the dose response line of the F_1 suggests there was a non-recessive resistance gene that was present in NYINDR, but that it was not homozygous. The LD_{50} of the F_1 (CS female × NYINDR male) was 0.18 μg/fly (95% CI: 0.15–0.21) giving a degree of dominance [23] of <-0.98. Similarly, indoxacarb resistance in *rind* was inherited as a recessive trait (LD_{50}) of the F_1 (CS female × *rind* male) was 0.37 μg/fly (95% CI: 0.33–0.42) giving a degree of dominance of <-0.52.

Analysis of the phenotypes vs. mortality data in the backcross generation reveals that the major factor for indoxacarb resistance is located on autosome 4 and a minor factor is associated with autosome 3 (Table 6). The only other known resistance gene on autosome 4 in house fly is *Rdl* (target site insensitivity to cyclodienes). Indoxacarb resistance does not appear to be caused by *Rdl* because there were only very low levels of resistance to dieldrin, toxaphene and γ-chlordane in NYINDR (Table 4). There are three resistance mechanisms associated with autosome 3 of the house fly: *kdr*

Table 5
Toxicity of DCJW with and without PBO to two near isogenic strains: susceptible (aabys) and indoxacarb resistant (*rind*)

Strain	PBO[a]	LD50[b] (95% CI)	Slope (SE)	*n*	SR[c]	RR[d]
aabys	–	14.5 (12.8-16.2)	4.5 (0.6)	520	—	—
aabys	+	10.2 (9.1–11.2)	6.1 (0.9)	240	1.4	—
rind	–	60.3 (53.4–67.8)	2.8 (0.3)	660	—	4.2
rind	+	13.5 (10.9–16.7)	3.6 (0.7)	640	4.5	1.3

[a] PBO applied at 2 μg/fly 1 h prior to treatment with DCJW.
[b] In units of ng/fly.
[c] Synergism ratio = LD_{50} of DCJW/LD_{50} of DCJW + PBO.
[d] Resistance ratio = susceptible (aabys) strain LD_{50}/resistant (*rind*) strain LD_{50}.

Table 6
Factorial analysis of indoxacarb resistance in the *rind* strain of house fly

Autosome(s)	Effect	Mean square	*F* value
5	−58.30	70.82	2.42
4	666.77	9262.02	316.83**
4 + 5	−1.46	0.04	0.00
3	86.98	157.63	5.39*
3 + 5	−10.92	2.49	0.09
3 + 4	−50.82	53.81	1.84
3 + 4 + 5	10.89	2.47	0.08
2	−53.91	60.55	2.07
2 + 5	10.54	2.31	0.08
2 + 4	−13.23	3.65	0.12
2 + 4 + 5	2.06	0.09	0.00
2 + 3	−4.16	0.36	0.01
2 + 3 + 5	−1.17	0.03	0.00
2 + 3 + 4	−5.91	0.73	0.02
2 + 3 + 4 +	−0.34	0.00	0.00
1	11.36	2.69	0.09
1 + 5	−12.06	3.03	0.10
1 + 4	−60.82	77.06	2.64
1 + 4 + 5	−5.83	0.71	0.02
1 + 3	25.37	13.41	0.46
1 + 3 + 5	6.86	0.98	0.46
1 + 3 + 4	2.39	0.12	0.00
1 + 3 + 4 + 5	12.06	3.03	0.10
1 + 2	−6.59	0.91	0.03
1 + 2 + 5	7.14	1.06	0.04
1 + 2 + 4	0.10	0.00	0.00
1 + 2 + 4 + 5	−1.84	0.07	0.00
1 + 2 + 3	−15.62	5.08	0.17
1 + 2 + 3 + 5	−4.82	0.48	0.02
1 + 2 + 3 + 4	−7.10	1.05	0.04
1 + 2 + 3 + 4 + 5	−12.81	3.42	0.12
Error		29.23	

* Statistically significant at the 0.05 level.
** Statistically significant at the 0.01 level.

(and *super-kdr*), *pen* (decreased penetration) and esterase-mediated detoxification. It does not appear that *kdr* or *super-kdr* is involved in indoxacarb resistance because the level of resistance to the pyrethroid insecticide cyfluthrin is low (Table 4) and there was no cross-resistance in the LPR or YPER strains (Table 3). In addition, the sequence of the *para*-homologous voltage sensitive sodium channel (VSSC) in the *rind* strain does not have the *kdr* (L1014F) mutation (Rinkevich and Scott, unpublished). It also does not appear that *pen* is involved because there is no cross-resistance in the LPR strain (Table 3) even though this strain has *pen* [12]. Furthermore, there was no cross-resistance to dimethoate, an insecticide for which delayed penetration would likely give a low level of protection [24]. Decreased penetration has, however, been associated with 2.5-fold cross-resistance to indoxacarb in the R-OP strain of house flies [2]. It also does not appear that esterase (or glutathione *S*-transferase)-mediated detoxification is involved because there was no reduction in resistance when indoxacarb was applied with DEF (or DEM). Thus, it appears that the mechanisms of resistance to indoxacarb on autosomes 3 and 4 are previously undescribed.

It is generally believed that resistance genes are extremely rare, sometimes having evolved only once and spread throughout the world [25]. Why then were we able to select for indoxacarb resistance so easily (three generations of selection) in a species which had never been exposed to the insecticide in the field (especially with one of the resistance genes being highly recessive)? Obviously the frequency of the indoxacarb resistance genes must be fairly high in field populations of house flies. One possible way this could happen would be if there was cross-resistance to indoxacarb because of selection with a previous insecticide. But what previous insecticide use might be responsible? Exact spray histories with synthetic insecticides at any specific dairy are difficult to obtain. By examining recommendations made by Cornell University for fly control since 1954, and considering published accounts of fly control methods [26] an overall picture of the synthetic chemicals used for fly control can be ascertained.

Generally, house fly control at dairies in NY involved DDT and methoxychlor (1945–1955), other chlorinated hydrocarbons such as lindane, and chlordane (1950–1955) organophosphates including malathion, diazinon, dimethoate, etc. (1954-present), pyrethrins (usually with PBO) (1950-present), permethrin (1982-present) and cyfluthrin (1994-present), although there was (and is) considerable variation in insecticide use between dairies. We did not find any cases of >10-fold cross-resistance in NYINDR to any other insecticide, thus we are unable to postulate a link between any specific previously used insecticide and the high

frequency of indoxacarb resistance genes in house fly populations.

In conclusion, we selected a strain of house fly that was >118-fold resistant to indoxacarb following three generations of selection. Resistance was associated with a major factor on autosome 4 and a minor factor on autosome 3.

Acknowledgments

We thank A. Paul and C. Leichter for technical assistance, and K. Wing for the samples of DCJW and indoxacarb. This work was supported by Hatch project 414 and the S. Daljit and Elaine Sarkaria Professorship.

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