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Effect of Phenothiazines on the Development of the Cotton Leaf Worm, *Spodoptera littoralis* (Lepidoptera: Noctuidae)

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Calmodulin is a ubiquitous Ca^{2+}-binding protein, which has numerous functions in cell biology, including cAMP-dependent signal transduction and cell division. Drugs with a phenothiazine ring system have been used for decades in the treatment of schizophrenia and other psychoses, and their anticancer potential has been reported. Here we present the first evidence for the disruption of normal insect development by low doses of two typical phenothiazines, chlorpromazine and trifluoperazine, administered via a semi-synthetic diet to larvae of the cotton leaf worm (*Spodoptera littoralis;* Lepidoptera, Noctuidae), a polyphagous pest of various crops. At 0.3 percent trifluoperazine in the diet the development of the larvae to the adult stage was completely prevented. In view of their moderate toxicity to vertebrates, including humans, and in view of the availability of numerous phenothiazine drugs at reasonable costs, agricultural applications of phenothiazines appear possible.

Keywords: phenothiazines, chlorpromazine, trifluoperazine, insect development inhibitor, *Spodoptera littoralis*.

Phenothiazines and related derivatives have for several decades been the most important antipsychotics, and are widely used in the treatment of schizophrenia and other mental illnesses (Forrest et al., 1974; Shen, 1999). These compounds were originally synthesized as potential antimalarial agents, and are also known to have anthelmintic properties (Dominguez et al., 1997; Lyons et al., 1993). A generally less well-recognised aspect of these drugs is their cytotoxicity, which seems to be mainly based on their ability to inhibit the effects of calmodulin, by Ca^{2+}-dependent binding to the hydrophobic domain of this protein. Phenothiazines and some related structures belong among the most potent calmodulin antagonists (Weiss et al., 1980; Roufogalis et al., 1983). Calmodulin is an ubiquitous Ca^{2+}-binding protein that has pivotal roles in many aspects of cellular regulation (Cheung, 1982; Klee et al., 1980; Johnson and Mills, 1986), among which its role in cell cycle progression at G1/S and G2/M transitions is of especial importance (Rasmussen and Means, 1989). Calmodulin antagonists, including trifluoperazine (TFP) *(Fig. 1)* affect cAMP metabolism in different organs (MacNeil et al., 1985), and they impair, among others, protein (Kumar et al., 1991) and DNA synthesis (Tomita et al., 1987), and DNA repair (Charp and Reagan, 1985), to name only a few effects on basic cell functions. Thus it may not surprise that TFP prevents liver regeneration (Alexander et al., 1988), and this and

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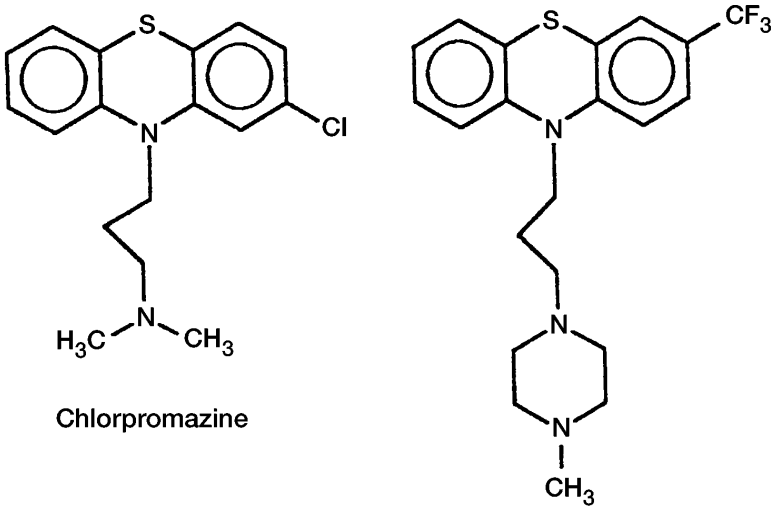

Fig. 1. Structural formulae of chlorpromazine (3-chloro-10[3'-dimethylaminopropyl]phenothiazine) and trifluoperazine (2-trifluoromethyl-10[3'-(4-methylpiperazin-1-propyl)]phenothiazine

other phenothiazines inhibit the growth of a variety of mammalian cells (Hait and Lee, 1985; Grief et al., 1989; Nordenberg et al., 1999), as well as plant cells (Szabó et al., 1997). Their cytotoxicity suggested the introduction of calmodulin antagonists, including phenothiazines, as potential cancer chemotherapeutic agents (Jones, 1985; Hait, 1987; Nagy et al., 1996). The ability of phenothiazines to form charge-transfer complexes (mostly by acting as electron donors) (Gutmann et al., 1974) is another property, which contributes to their manifold biological activities, among which interactions with a variety of neurotransmitter receptors are of importance (Motohashi et al., 2000). Among the more recent discoveries (Kristiansen, 1989; Molnár, 1997; Molnár et al., 1993; Motohashi et al., 1992a, b; Page and Lagnado, 1995) the antiplasmid effect of phenothiazines will presumably become of especial importance. Furthermore, phenothiazines have potentials as antiparasitic agents, as well as in the prevention of drug resistance, and the potentiation of the immune system (Tanaka et al., 1997; Molnár et al., 1998).

In view of this wide range of actions, surprisingly few toxicological reports on insects have been published. These are maily focusing on insects of veterinary importance, or studying infuence on a great variety of biochemical and physiological processes (Blenau et al., 1998; Coats et al., 1976; Degen et al., 2000; Khabour and Sadiq, 1999; Lees-Miller and Caveney, 1982; Man'ko et al., 2000; Marjamaki et al., 1994; Pendleton et al., 1996; Quraishi, 1967; Schlinke and Palmer, 1973; van Schaik and Graf, 1993). However, no information appears to exist on their effects on insect development. In pursuing the idea of potential insecticide properties of the phenothiazines, we chose as a first model a lepidop-

terous species, the cotton leaf worm *Spodoptera littoralis* Boisd. (Lepidoptera, Noctuidae), a well-known polyphagous pest of various crops (e.g. cotton, soybeans, alfalfa), with great economic importance in the Mediterranean, and the tropical and subtropical regions of the Old-World (Cayrol, 1972). Out of the numerous phenothiazines chlorpromazine (CPZ) (3-chloro-10[3'-dimethylaminopropyl] phenothiazine) *(Fig. 1)* was used because it is the best known phenothiazine drug, and trifluoperazine (TFP) (2-trifluoromethyl-10[3'-(4-methyl-piperazin-1-propyl)]phenothiazine *(Fig. 1),* because much work on biological effects of calmodulin antagonists were carried out with this drug.

Materials and Methods

Chemicals

Laboratory chemicals were from Merck (Darmstadt, Germany); chlorpromazine and trifluoperazine were from Sigma Chemical Co. (St. Louis, MO).

Insects

Larvae of cotton leaf worm (*Spodoptera littoralis* Boisd) (Lepidoptera, Noctuidae) were obtained from the Laboratory of Chemical Ecology, Department of Plant Protection Science, Swedish Agricultural University, Alnarp. Third instar larvae were randomly selected from the synchronised laboratory culture, reared continuously on a semi-synthetic diet during many generations.

Diets

The diet was prepared according to Hinks and Byers (1976) containing, however, mashed potatoes instead of beans. It was the same as that used for rearing. The drugs were dissolved in tap water (50 mg per ml) and were added during continuous stirring, before the diet cooled below 60 °C. Final concentration of the drugs: 1 g per kg (3.1 mmol/kg CPZ ; 2.5 mmol/kg TFP (experiment 1), and 3 g per kg TFP (7.5 mmol/kg) (experiment 2). The control diet contained the same amount of tap water as the drug-containing diets. Portions of the diets were transferred into rearing boxes, where they cooled to room temperature and became solid.

Testing procedure

Groups of 20 third instar larvae were placed on each diet, respectively, to which they had access during their entire development. Cultures were kept in environmental chambers at 24 °C and a 16 h light, 8 h dark photoregime. The weight of each larva was determined before placing it on the diet, and again before pupation. One week after pupation, pupae were carefully removed from their pupation chamber, the number of living pupae were recorded, then these pupae were individually weighed. Their sex was determined visually based on external appearance. The pupae were then placed on moistened filter paper, and the number of emerging adults was recorded daily.

Statistics

Larval/pupal weights, and length of developmental time were analysed by one way ANOVA. If the F value was significant, differences between means were tested for significance by the Bonferroni/Dunn test using SuperANOVA® software (Abacus Concepts, 1989, Berkeley, California). Survival percentages were analysed by Fisher's exact test (InStat).

Results

Effects of 0.1 percent chlorpromazine (CPZ) and trifluoperazine (TFP) in the diet on development

At an approximate consumption of 3–5 g of the diet the total drug intake during the developmental period corresponded to 9–15 μmol CPZ and 7.5–12.5 μmol TFP per larva. These amounts of the drugs drastically reduced the survival of the insects. Only 35 and 40 percent, respectively, of adults emerged from pupae, in comparison with 95 percent of controls. Differences with similar tendencies were evident already in earlier stages of the development *(Table 1),* and larval survival was only 95 percent, compared with 100 percent in the case of controls.

Table 1

Effect of 0.1 percent chlorpromazine (CPZ) and trifluoperazine (TFP) in the diet on survival rate of *Spodoptera littoralis*

Stage of insect development	Survival of insects (percent)		
	CPZ	TFP	Control
Larva (before pupation)	95	95	100
Pupa (one week after pupation)	85	90	100
Adult	35*	40*	95

The asterisk indicates a statistically significant difference ($p < 0.05$) between a treated and the corresponding control group (Fischer's exact test)

The duration of development of both male and female cotton leaf worms from 3rd instar larva to the adult stage was retarded in the surviving insects following treatment with both CPZ and TFP, however, the differences were significant only for TFP *(Table 2).* The difference was greater in TFP-treated males than in females. Behavioural changes (e.g. reduced motor activity or changes in feeding habits), which are indicative of sedative effects of the drugs, were not observed, but may nevertheless have occurred.

Surviving larvae of both sexes treated with CPZ or TFP had nearly the same weight as controls (not shown), but female pupae of the treated groups (CPZ: 331 ± 17 mg; TFP 338 ± 13 mg) exhibited a significantly lower weight than controls (406 ± 15 mg).

Table 2

Effect of 0.1 percent chlorpromazine (CPZ) and trifluoperazine (TFP) in the diet on the duration of development of *Spodoptera littoralis*

Sex	Duration of development# (Days)		
	CPZ	TFP	Control
Male	22.0 ± 0.6	23.8 ± 0.5*	20.7 ± 0.4
Female	20.0 ± 0.4	20.3 ± 0.3*	18.8 ± 0.3

#Duration of development was determined from the time when a third instar larva was exposed to the test medium until emergence of the adult.
The values are means ± S.E.; the asterisk indicates a statistically significant difference ($p < 0.05$) between a treated and the corresponding control group (ANOVA followed by Bonferroni / Dunn test).

Effects of 0.3 percent trifluoperazine (TFP) in the diet on development

Being somewhat more potent than CPZ, the experiments were continued with 0.3 percent (7.5 mmol/kg) TFP in the diet. Under these conditions not a single treated larva developed to the adult stage, and only 30 percent pupated successfully, whereas 95 percent of the controls pupated, and 85 percent developed to adults *(Table 3).* Larval survival was under these conditions 75 percent in the treatment group, and 100 percent in the control group.

Table 3

Effect of 0.3 percent trifluoperazine (TFP) in the diet on survival rate of *Spodoptera littoralis*

Stage of insect development	Survival of insects (percent)	
	TFP	Control
Larva before pupation	75*	100
Pupa one week after pupation	30*	95
Adult	0*	85

The asterisk indicates a statistically significant difference ($p < 0.05$) between a treated and the corresponding control group (Fischer's exact test)

The duration of development until pupation was not exactly determined, because it would have required the disturbance of the larvae inside the pupation chamber. However, larvae of the treated group started to prepare their pupation chamber several days later than the controls, and they had a markedly prolonged developmental phase.

The weight of both the fully developed larvae (60 percent) and the pupae (50 percent) was considerably lower in the TFP treatment group, as compared with untreated controls *(Table 4).*

Table 4

Effect of 0.3 percent trifluoperazine (TFP) in the diet on the weight of *Spodoptera littoralis*

Stage of insect development	Sex	Weight (mg)	
		TFP	Control
Larva at start (mid stage)	Unknown	170 ± 9	158 ± 9
Larva (before pupation)	Unknown	283 ± 26*	487 ± 29
Pupa (one week after pupation)	Male	178 ± 13*	360 ± 10
	Female	176#	373 ± 10

Data are mean values ± S.E., the asterisk indicates a statistically significant difference ($p < 0.05$) between a treated and the corresponding control group (ANOVA followed by Bonferroni/Dunn test).
#determination of SE was not possible, as only one female pupa survived.

Discussion

To our knowledge this is the first report on the effect of phenothiazine drugs on the development of an insect species. It was shown that low concentrations of the drugs added to a semi-synthetic diet markedly interfered with the development of cotton leaf worm: CPZ or TFP at 0.1 percent in the diet retarded larval development, caused larvae moulting to smaller pupae, and reduced development to adult stage by more than 50 percent. TFP at 0.3 percent caused 100 percent mortality. Only 75 percent of the treated larvae were able to complete their larval development, and merely 30 percent of the treated larvae produced pupae.

The experimental design did not allow one to determine exactly the amount of diet consumed by the insects. However, it is evident from the results that a total ingestion of 20–40 µmol (9–15 mg) of TFP during the larval stage completely prevented the development of *S. littoralis* larvae into adults.

As was mentioned in the introduction, phenothiazines have multiple molecular targets and pharmacological actions. It is, therefore, not possible to discuss at present mechanisms of action that may underlie our observation. However, there is no doubt that the calmodulin antagonism of the phenothiazines could be of especial importance in our context, because of its known multiple functions, and the divergent actions of phenothiazines on higher animals (see Introduction). Moreover, it is known that signal transduction pathways involved in insect moulting and pheromone synthesis involve Ca^{2+} -calmodulin regulation of adenylate cyclase (Bondaryk, 1983; Granger et al., 1995; Gilbert et al., 1988; Matsumoto, 1997). Most probably a multitude of processes of importance for normal growth and development of insects are impaired by phenothiazine-type calmodulin antagonists. The known chlolinesterase inhibitory properties of phenothiazines may be of importance as well (Legheand et al., 1975; Fernandez et al., 1975).

It may appear somewhat premature to discuss the potentials of practical applications of phenothiazines in the control of agriculturally important pests. However, in view of their moderate toxicity to vertebrates, and in view of the fact that phenothiazines with diverse

biological and physicochemical properties are readily available at relatively low cost, this novel possibility of fighting pests should be considered and these type of compounds could be used as novel leads for the development of pest control agents.

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