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Basal Cytotoxicity of Four Insect Sex Pheromones in CHO-K1 Cells

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Integrated Pest Management (IPM) techniques constitute one of the most successful methods for controlling agricultural pests. Since there is no single environmentally sustainable approach to pest control, IPM entails a combination of various strategies and practices, including the use of natural products, natural enemies or cultural methods to maintain pests at acceptable levels, the application research of relevant research and the implementation of farmer training programs to increase the sustainability of farming systems by avoiding the use of conventional toxic pesticides (Dent, 1993). However, effective non-chemical alternatives to chemical methods are not always available, and in such cases harmless products, sampling techniques or action thresholds, should be deployed to both reduce toxic side effects or the presence of undesirable residues in food, and maintain environmental sustainability (Bloomers, 1994). The mode of action of IPM chemicals is based on insect biology and physiology, thus providing for greater target pest specificity, lower non-target organism toxicity, rapid environmental degradation and low persistence, among others. These agents include natural and synthetic compounds that either modify sex-coupling behavior (semiochemicals; including allelochemicals and natural insect pheromones) or regulate insect growth inhibiting the formation of new chitin in the insect cuticle during the molting process or mimicking juvenile hormones, leading to insect hormone imbalance and concomitant failure of the molting process (Okazawa et al, 1991; Takagi et al, 1995; Dhadialla et al, 1998; Cardé and Minks, 1995).

Sex pheromones disrupt effective insect mating. A sufficiently high concentration of these compounds in the air, blankets the trails of female insects, confusing the males who are unable to trace the pheromone source and thereby diminishing insect propagation. Since a specific pheromone is required for each pest, the application of these substances does not kill beneficial insects or natural enemies, avoiding ecological imbalance (Gaston et al., 1977). Many insect pheromones initially marketed as insect attractants, are currently used against specific pest in IPM programs. (Z,E)-7,11-hexadecenylacetate (7,11-HDDA) -gossyplure - is successfully used against *Pectinophora gossypiella* (pink bollworn) in cotton fields, and Z-11-hexadecenal (11-HDAL) - virelure - is effective against *Platypillia candidactyla* (artichoke plume moth). Manufacturers claim that both pheromones are totally safe and international organizations consider them to be non toxic agents. In fact, acute toxicity of most of these compounds in experimental animals

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at exposure levels, excess of 5 mg/kg (p.o.), 2 mg/ml (dermal) and 5 mg/l (inhalation), figures that would indicate that these substances can be safely managed (EPA registration numbers 53575-15 and 50675-8, for 7,11-HDDA and 11-HDAL, respectively). Nevertheless studies on the basal cytotoxicity of these compounds and analogues are lacking in the scientific literature. In the research discussed here under, the authors evaluated the cytotoxicologic effects of four natural insect pheromones on mammalian CHO-K1 cells to compare the *in vitro* toxic effects under different cell culture conditions after 24 to 72 hr exposure. All the compounds tested were evaluated in serum-containing medium, serum-free medium and medium containing 1% bovine serum albumin. Finally, the survey also addressed the effect of the rat liver submitochondrial S9 fraction on the cytotoxicity of the four pheromones.

MATERIALS AND METHODS

The reagent grade chemicals and cell culture components used, namely culture medium HAM F-12, antibiotics, trypsin/EDTA solution, HEPES, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (neutral red NR dye), bovine serum albumin (BSA), NADP, glucose-6-phosphate and dimethyl sulfoxide (DMSO), were obtained from Sigma Chemical Co., (St. Louis, MO. USA). Fetal calf serum (FCS) was obtained from Boehringer Ingelheim GmbH, (Germany). The insect pheromones ZZ/ZE-7,11-hexadecadienyl acetate (7,11-HDDA, gossyplure, pheromone from *Pectinophora gossypiella*), Z-11-hexadecenal (11-HDAL, pheromone from *Platypillia candidactyla*), Z-9-hexadecenal (9-HDAL, pheromone for *Heliothis armigera*) and Z-11-hexadecen-1-ol (11-HDOL, pheromone for *Laconobia oleracea*), were obtained from Sigma Chemical Co., (St. Louis, MO. USA). To prepare the primary stock solutions, each compound was completely dissolved in sterilized DMSO, whereafter the stock solutions were dissolved in the culture media to reach the desired dilutions. DMSO in the cultures never exceed of 0.2 % (v/v).

CHO-K1 cells were obtained from the American Type Culture Collection (ATCC CCL 61). 10^4 cells/cm^2 were plated in monolayers in 22 cm^2 polystyrene tissue culture dishes with HAM F12 medium (Sigma Chemical Co.) supplemented with 25 m*M* HEPES buffer (pH 7.4) and 10 % heat-inactivated FCS and gentamicin (30 μg/mL). Cells were counted on an improved Neubauer haemocytometer and cell viability was determined by exclusion intake of Trypan Blue dye. Cells were allowed to reach 65 % confluence (day 3, mid log-phase) and then pulsed with different concentrations of the insect pheromones. They were subsequently washed and the medium was replaced after 24, 48 and 72 hr, determining cell viability by the method mentioned below.

The NRI assay method described by Borenfreund and Puerner (1985) was used to estimate insect pheromone cytotoxicity in CHO-K1 cells. Cells were allowed to attain 65 % confluence and then pulsed with different concentrations of the insecticides tested. After the culture was exposed to the tested agents for 24 hr, 48 hr and 72 hr, it was transferred to a fresh medium containing NR dye at a concentration of 40 μg/mL, prepared and preincubated overnight at 37 °C. The

plates were then reincubated at 37 °C for a further 3 hr for lysosome uptake of the supravital NR dye in viable cells. The medium was then removed and the cells fixed with 0.5 % formaldehyde in 1 % $CaCl_2$; 200 µL of a solution of 1 % acetic acid in 50 % ethanol were then added to extract the dye from the cells. After rapid agitation of the plate for 20 min on a microtiter plate shaker, absorbance was measured at 540 nm using an ELISA reader (Merck/Mios). Curves were fitted using Sigma Plot Scientific Graphing Software (Microsoft) for non-linear regression using probit/log concentration graphs to obtain NRI_{50} values.

Rat liver submitochondrial S-9 mix was freshly prepared as described by Venitt et al. (1984). 30 % of S9 mix was used in this study. Final mixture contained 1 M KCl, 0.25 *M* $MgCl_2$, 0.2 *M* glucose-6-phosphate, 0.04 *M* NADP, 0.2 *M* $NaHPO_4$, deionised water and the S9 fraction derived from homogenized rat livers (Sprague-Dawley rats, 6-8 wk old weighing 200 g ± 15) after administration a 500 mg/kg (body weight) dose of Aroclor 1254 (Sigma Chemical Co.), using peanut oil as a solvent. 1 mL of freshly prepared S9 mix was incubated with the stock concentrations of each insect pheromone for 24 hr at 37 °C before starting the NRI assays.

RESULTS AND DISCUSSION

Lepidopteran pheromones consist of unbranched aliphatic chains with alcohol, aldehyde or acetate functions at the end of the molecule which interact with male receptors and disrupt effective mating. As acute toxicity of these substances for non-target organisms, i.e. reptilians, birds or mammals, has been considered to be non-relevant under field use conditions, their use is felt to constitute good agricultural practice. Consisting as they do of environment-friendly compounds, pheromones contribute to enhance ecological sustainability. The cytotoxic effects, however, of these compounds has been not yet described.

The cytotoxicity of four insect pheromones was determined using the NRI assay, which monitors the lysosomal function in viable cells. Each pheromone was added to CHO-K1 cultures during the mid-log phase (day 3) and incubated for 24 (figure 1) , 48 and 72 hr, after which cell viability was analyzed by the above technique in the absence of FCS in the culture medium. Dose/response curves show that the toxic effect of each compound increases significantly with time. Probit/log graphs yielded the NRI_{50} values shown in table 1. The first row for each insect pheromone in table 1, shows the mid-point cytotoxic value (NRI_{50}) after three exposure times, in absence of FCS. After 24 h, the cytotoxicity of these compounds ranked as follows: 7,11-HDDA > 11-HDOL > 11-HDAL = 9-HDAL; at longer exposure durations the variations proved to be negligible.

The presence of proteins in the incubation medium, may change cytotoxicity parameters due to unspecific binding or degradational enzymatic reactions. Previous experiments showed that 10 % FCS protected CHO-K1 cells from pheromone exposure. To analyze this question, cultures that has attained 65 % confluence were incubated in the presence of different concentrations of each pheromone, for 24, 48 and 72 hr in media containing 10 % FCS or 1 % BSA.

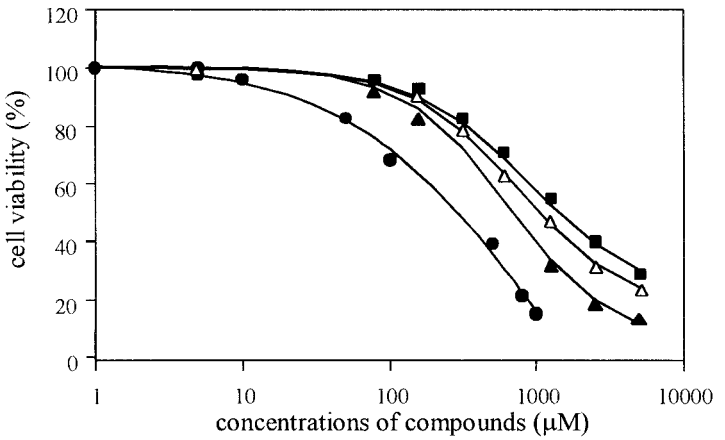

Figure 1. Cytotoxicity of lepidopteran pheromones on CHO-K1 cells. Cells were incubated for 24 hr in presence of different concentrations of (●) 7,11-HDDA, (■) 11-HDAL, (Δ) 9-HDAL and (▲) 11-HDOL. Cytotoxic effect was assessed by the NRI assay, representing the average of NRI_{50} values from three determinations.

Table 1. Effect of FCS and 1 % BSA on the cytotoxicity (NRI_{50}) of insect sex pheromone insecticides.

		Cytotoxicity values (NRI_{50}) μM		
compound	additions	24 hr	48 hr	72 hr
7,11-HDDA	no	290 ± 20	40 ± 1	26 ± 6
	10% FCS	704 ± 81	225 ± 11*	137 ± 10*
	1% BSA	1795 ± 97*	1392 ± 26*	893 ± 50*
11-HDAL	no	1575 ± 98	117 ± 21	62 ± 15
	10 % FCS	3271 ± 153*	1910 ± 15*	1050 ± 10*
	1 % BSA	3182 ± 116*	1235 ± 225*	1136 ± 137*
9-HDAL	no	1276 ± 94	81 ± 21	80 ± 26
	10% FCS	1492 ± 56	1447 ± 8*	1034 ± 25*
	1% BSA	1199 ± 72*	353 ± 63	92 ± 4
11-HDOL	no	582 ± 23	38 ± 1	32 ± 2
	10 % FCS	3042 ± 98*	2905 ± 65*	2672 ± 54*
	1 % BSA	3444 ± 55*	2751 ± 114*	2251 ± 71*

(*) $P < 0.001$ vs. control. Cells were incubated with different concentrations of each pheromone in presence and absence of 10 % FCS or 1 % BSA, for 24, 48 and 72 hr. After these times, NRI assay was performed in order to evaluate the NR_{50} values by the probit/log approach. NR_{50} values are average ± SE of four experiments by triplicate.

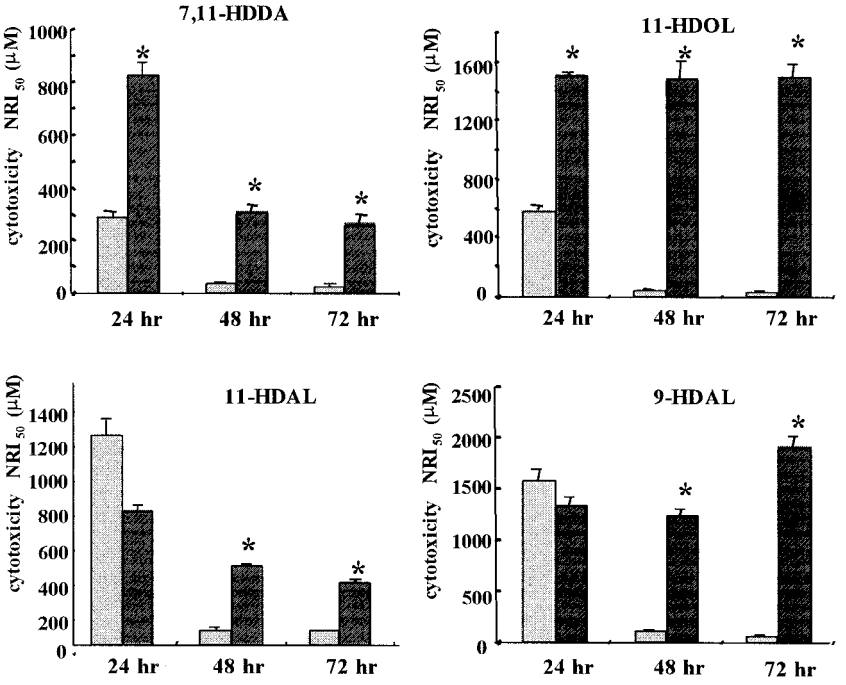

Figure 2. Effect of rat liver microsomal S9 fraction (hatched columns) on cytotoxicity of lepidopteran pheromones. 5 mM stock of each compound dissolved in DMSO, was incubated with 10 % S9 fraction , containing NADP and glucose 6-phosphate dehydrogense in phosphate saline buffer. After 24 hr, mixtures were placed onto 65 % confluence CHO-K1 cultures and exposed for an extent of 24 hr, 48hr and 72 hr. Cytotoxic effect was assessed by the NRI assay, representing the average of NRI_{50} values ± SE of four independent determinations.

After exposure, NRI assays were conducted and viability data was fitted pursuant to a probit/log graph (table 1). Cytotoxicity was found to drop significantly ($P < 0.001$) either with 10 % FCS or 1 % BSA after exposures to lepidopteran pheromones for 24, 48 and 72 hr. The protection conferred by soluble proteins after 72 hr was found to be more effective against 11-HDAL and 11-HDOL than 7,11-HDDA and 9-HDAL.

In addition to assessing the toxicity of insect pheromones, an attempt was made to determine the cytotoxic effect of these compounds if previously exposed to the submitochondrial fraction (S9-fraction) for 24 hr. Each agent was incubated in 30 % of rat S9 fraction mix for 24 h in the presence of an NADPH-regenerating system to generate phase I metabolites. After incubation, protein was acid-precipitated and prior to cell exposure, neutralized with NaOH. Probit-log likelihood was used to determine the NRI_{50} values of the metabolites shown in figure 2. Protection against the parent compound was found to be high for all the lepidopteran pheromones tested, with the NRI_{50} values higher, in most cases, than

where untreated compounds were used. However, whereas no significant change was observed in the level of protection afforded the CHO-K1 cultures against 11-HDOL and 9-HDAL by the S9 fraction after 72 h of exposure, the NRI_{50} values of cells exposed to S9 fraction-treated 7,11-HDDA and 11-HDAL exhibited a time-dependent pattern of decline.

It has been described that the high lipophylicity of their chemical structure, can produce a thin oily film onto water surface which may, in turn, have a toxic effect on aquatic invertebrates. Accidental spillage of insect pheromones into aquatic environments is unlikely, however, due to the particular way such substances are handled. Indeed, pheromones are used to bait traps of many designs, often consisting of open-ended plastic hollows or plastic bags, that emit the volatile product through both sides or the walls, respectively, thus reducing liquid spillage or undesirable contamination to these compounds.

In addition to the resulting unlikely availability to non-target animals, both acute or chronic toxicity have been reported to be low. However, the results obtained using the standard CHO-K1 cell line show that under laboratory conditions, cytotoxicity levels are relevant. As might be expected, the cytotoxic effect is both dose- and time-dependent and the presence of proteins (10 % FCS or 1 % BSA) in the culture medium significantly reduces the lethal effects of the four pheromones studied. No toxicokinetic studies have been performed in experimental animals to date, but binding to serum proteins after absorption may be one of the reasons for their low toxicity after acute or chronic exposure. Due to their long hydrophobic aliphatic chain, resembling normobiotic fatty acids, these compounds can be carried and/or distributed by serum albumin, reducing cellular bioavailability and undesirable effects. In addition, the preincubation of pheromones with microsomal enriched rat liver S9 fraction, originated non-cytotoxic metabolites, thus suggesting hepatic degradation of these substances, which would explain their organic safety. Hydrolysis and oxidation of functional groups, i.e. acetate or aldehyde, were assayed *in vitro*, incubating 7,11-HDDA and 9-HDAL, with a carboxylesterase (Vogt et al., 1985) and a yeast aldehyde dehydrogenase (Blatter et al., 1990) respectively, 30 min before incubation with the cells (data not shown). After effective hydrolysis or oxidation of these functional groups the cultures suffered no-lethal consequences, indicating that the pheromones in these form, have less adverse effects on the cells.

Lepidopteran pheromones produced significant cytotoxicity in CHO-K1 cells that can be mitigated with 10 % FCS or 1 % BSA. The phase I metabolites produced in oxidative reactions, contribute to attenuation of these negative effects, suggesting that protein binding and oxidative metabolism may be responsible for the low toxic effects observed on non-target animals.

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REFERENCES

Blatter EE, Tasayco ML, Prestwich GD, Pietruszko R (1990) Chemical modification of aldehyde dehydrogenase by a vinyl ketone analogue of an insect pheromone. Biochem J 272: 351-358

Bloomers LHM (1994) Integrated pest management in European apple orchards. Ann Rev Entomol 39: 213-241

Borenfreund E, Puerner JA (1985) Toxicity determined in vitro by morphological alteration and neutral red absorption. Toxicol Lett 24: 119-124

Cardé RT, Minks AK (1995) Control of moth pests by mating disruption. Ann Rev Entomol 40: 559-585

Dhadialla TS, Carlson GR, Le DP (1998) New insecticides with ecdysteroidal and juvenile hormone activity. Ann Rev Entomol 43: 545-569.

Dent D (1993) Integrated insect pest management. In: Dent D (ed) Insect Pest Management, CAB International, Wallingford, UK, pp. 439-533

Daughtrey WC, Smith JH, Biles RW (1990) Subchronic toxicity evaluation of tridecyl acetate in rats. Fundam Appl Toxicol 14: 104-112

Gaston LK, Kaae RS, Shorey HH, Sellers D (1977) Controlling the pink bollworm by disrupting sex pheromone communication between adult moths. Science 196: 904-905

Okazawa T, Barkote'e B, Suzuki H, Kawada H, Kere N (1991) Field evaluation of an insect growth regulator, pyriproxifen, against Anopheles punctulatus on north Guadalcanal, Solomon Islands. J American Mosq Control Assoc 7: 604-607

Takagi M, Tsuda Y, Wada Y (1995) Laboratory evaluation of a juvenile hormone mimic, pyriproxyfen, against Chironomus fuscicieps (diptera: chironomidae). J American Mosq Control Assoc 11: 474-475

Tasayco ML, Prestwich GD (1990) Aldehyde oxidizing enzymes in an adult moth: in vitro study of aldehyde metabolism in Heliothis virescens. Arch Biochem Biophys 278: 444-451

Vogt RG, Riddiford LM, Prestwich GD (1985) Kinetic properties of a sex pheromone-degrading enzyme: the sensillar esterase of Antheraea polyphemus. Proc Natl Acad Sci USA 82: 8827-8831

Venitt S, Crofton-Sleigh C, Froster R (1984) Bacterial mutation assays using reverse mutation. In: Venitt S, Parry JM (eds) Mutagenicity Testing a practical approach, IRL press, Oxford, Washington DC, pp 45-98