12 Insect Cell Lines as Tools in Insecticide Mode of Action Research

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1 Introduction

Herbivorous insects are a major threat in the continuous supply of food and fibres for human consumption. Additionally, parasitic insects and arthropod vectors of important diseases must be controlled. Synthetic insecticides play a major role in pest insect control, with chemical, environmental and toxicological properties having been improved considerably over the last six decades. In 2001, a total of 7.56 billion USD was spent to protect crops from damage by pest insect species (Nauen and Bretschneider 2002; Beckmann and Haack 2003). The world market for insecticides is still dominated by compounds irreversibly inhibiting acetylcholinesterases (AChE). Together, these AChE inhibitors (organophosphates [OPs] and carbamates) and the insecticides acting on the voltage-gated sodium channel (in particular the pyrethroids) account for approximately 70% of the world market. However, due to similarities in the nervous system of insects and vertebrates, these agents can exhibit considerable toxicity towards higher organisms and, therefore, their non-selective mode of action may cause devastating environmental problems. The extensive use of such compounds has caused the development of resistance to these agents in many pest insects. In the end, high resistance levels lead to the fact that effective concentrations must exceed the legally recommended concentrations, thus making the compounds useless. Therefore, the need to search for novel insecticides with a better efficacy or a new mode of action is obvious and involves a race against time. An intense search for alternatives less harmful to the environment has been initiated in laboratories around the world. Since then, there is a steady progression towards the development of narrow-spectrum insecticides that act on insect specific targets. Together there has been a surge of interest in biological control agents (BCAs), led by *Bacillus thuringiensis* (*Bt*). The underlying theme has been to search for unique sites in insect pest that can be selectively targeted. Various approaches are currently being examined, including the hormonal regulation of key developmental processes, and the biosynthesis of chitin in insects.

The considerable knowledge that has been gained in insect physiology and endocrinology has encouraged the development of insecticide screening procedures that specifically interfere with insect-specific metabolic pathways and endocrine mechanisms. The suitability of such a strategy is demonstrated

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by various insect growth regulators (IGRs) that impair insect endocrine regulation of moulting and metamorphosis processes, such as juvenile hormone analogues (JHAs), ecdysteroid receptor agonists or moulting accelerating compounds (MACs), and chitin synthesis inhibitors (e.g., benzoylphenyl urea, BPU) Additionally, there are newer insecticides/acaricides that act specifically on insect neurological/nerve, energy metabolism and muscle targets, and the insect midgut structures.

For screening purposes, over the past decades different industries have demonstrated an increasing interest in the development of in vitro methods to replace conventional animal toxicity tests. The ultimate goal is to achieve an alternative system that allows for the testing of candidate compounds, formulations and finished products and enables the accurate prediction of toxic efficacy for the whole animal. This being said, there are a certain number of key requirements that need thorough consideration before developing an alternative cell-based testing procedure. In particular the following points need to be addressed:

- dependable intra- and inter-laboratory reproducibility
- high predictive power to guarantee correct toxicity assessment decisions
- relevance to the type of compounds that are intended to be tested
- simplicity
- possibility for high throughput screening (HTS) with automation/robots
- low cost/benefit ratio

Established insect cell lines fulfil these criteria and therefore should be useful tools for screening with enough homogeneous materials. Furthermore, tests employing cell cultures can be readily automated. Additionally, cell-based assays can be developed that enable the elucidation of new modes of action for insecticide candidates. Insect cell cultures that have retained their arthropod specific metabolic pathways or hormonal regulation will also allow the development of screening procedures using insect specific targets.

In this paper, the advantages of insect cell lines for biorational insecticide screening will be demonstrated for different targets, namely the interference with insect endocrine systems, such as those involving the ecdysteroids and juvenile hormones (JHs), and with insect specific metabolic pathways like chitin biosynthesis. Also attention will be given to the novel insecticide chemistries that act on insect ligand and voltage-gated ion channels. The numerous whole insect genome projects in the past decade (e.g., those involving the fruit fly *Drosophila melanogaster*, the malaria mosquito *Anopheles gambiae*, the honeybee *Apis mellifera*, the tobacco budworm *Heliothis virescens*, the silk moth, *Bombyx mori* and several other insects/invertebrates; see http://www.ncbi.nlm.nih.gov/) provide important comparative genetic information for the development of such screening cell systems. At the end of this chapter the possible use of insect cell culture is discussed to screen for insecticidal proteins (such as *Bacillus thuringiensis*) will also be discussed. Based on plant-insect relationships, such insecticidal proteins provide biotechnological

opportunities with transgenic plant to combat pest insects. This chapter will be of interest to biocontrol and insecticide specialists, biomedical researchers, virologists, parasitologists, entomologists, microbiologists, geneticists, and also graduate students in related fields of insecticide, biomedical and biotechnology research.

2 Insect Cell Cultures

To date, the success of insect cell cultures has been demonstrated by reports of over 500 established cell lines (Lynn 2001; Lynn et al. 2005). Continuous insect cell lines have been an important research tool since Thomas Grace and Shangyin Gao (Z-Y Gaw)—two major authorities and pioneers of insect cell culture—developed the first insect cell lines from the emperor moth *Atheraea eucalypti* at CSIRO in Canberra, Australia (Grace 1962) and the silk moth *B. mori* (Wuhan, China 1958), respectively. The two had never met and they were unaware of each other's work, but they shared outstanding talents as creative inventors. In the 45 years since then the field has been quite productive. Figure 1 shows a current list that was assembled from compilations by Hink and co-workers (Hink 1972, 1976, 1980; Hink and Bezanson 1985; Hink and Hall 1989) and approximately 30 other published reports over the past decade indicate that there are now over 500 established lines (Lynn 2001; Lynn et al. 2005). Figure 1 also shows that most of the lines have been derived from Lepidoptera (moths and butterflies) and Diptera (flies and mosquitoes). Only

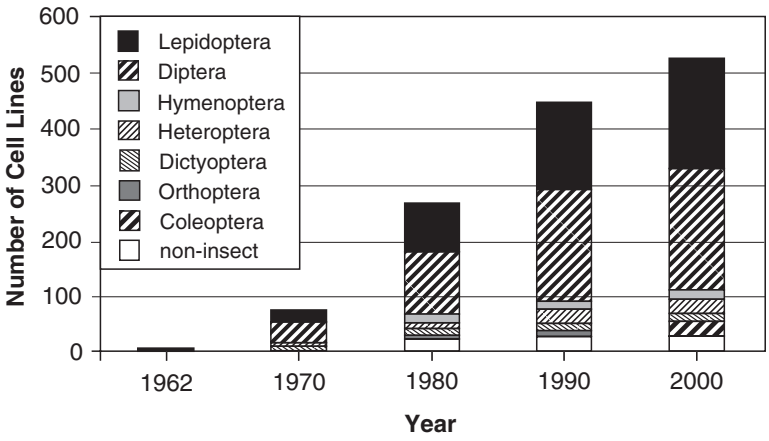


Fig. 1. The number of established invertebrate cell lines developed since 1962 categorized by insect orders. Each *bar* represents the total numbers of cell lines that have been reported up to the date listed (from Lynn 2001)

just over 20% originate from other invertebrates. The different sources that have been used successfully for developing established insect cell cultures are:

- Ovaries were the first tissues employed and were commonly used throughout the 1960s and 1970s, especially with Lepidoptera.
- Embryos are commonly used sources of cell for cultures. Lynn (2001) indicates that nearly half of all the insect cell lines were initiated from embryos. Since embryos contain all the cell types that will eventually differentiate into larval and adult tissue, cell lines from these tissues can contain a wide diversity of cell morphologies.
- Hemocytes are readily obtainable but not as amenable to growth in culture. Melanization is a common problem in hemocyte cultures; the use of phenyloxidase inhibitors (e.g. reduced gluthione, cysteine or phenylthiourea) is indicated to help overcome this problem.
- Imaginal discs (i.e., aggregates of progenitor cells for adult tissues) are important tissues in the insect's development because they are developmentally determined to become specific structures after metamorphosis; yet they consist of undifferentiated cells. Researchers are faced with two problems: imaginal discs are difficult to find, and similar to hemocytes, they may have significant levels of phenyloxidase activity requiring the use of inhibitors.
- Fat body is an important physiological tissue that has many functions equivalent to the mammalian liver and fat. It is also a target tissue of many insect pathogens. Fat body cells are a likely source of growth factors so coculture with other tissues can improve cell growth, particularly of primary cultures.
- Midgut is a very important tissue and relevant for pest control and pathology, as will be discussed later.
- Neonate larvae are newly hatched larvae in which tissues are generally more developed than those in embryos, although significant amounts of undifferentiated cells remain present.

On culture media to use for insect cell cultures, many formulations exist in addition to the "old standard media" of Grace's, Schneider's, and Mitsuhashi and Maramorosch. In the earliest days in insect cell culture, insect hemolymph was frequently used as a supplement that may provide necessary factors but it can also be problematic by introducing detrimental substances. FBS was an effective substitute, but insect-specific factors are likely to be quite different from those for mammals. In addition to FBS, other undefined additives have been used including protein hydrolysates, serum albumin, tryptose broth, and conditioned medium. Insect hormones, especially ecdysteroids, are growth promoting at low concentrations and cause differentiation at higher (moulting-inducing) levels. Antibiotics are essential for primary cultures but should be discontinued as soon as possible after consistent cell growth is achieved. Examples of second generation media include: Hink's TNM-FH, which is Grace's medium modified by the addition of yeastolate,

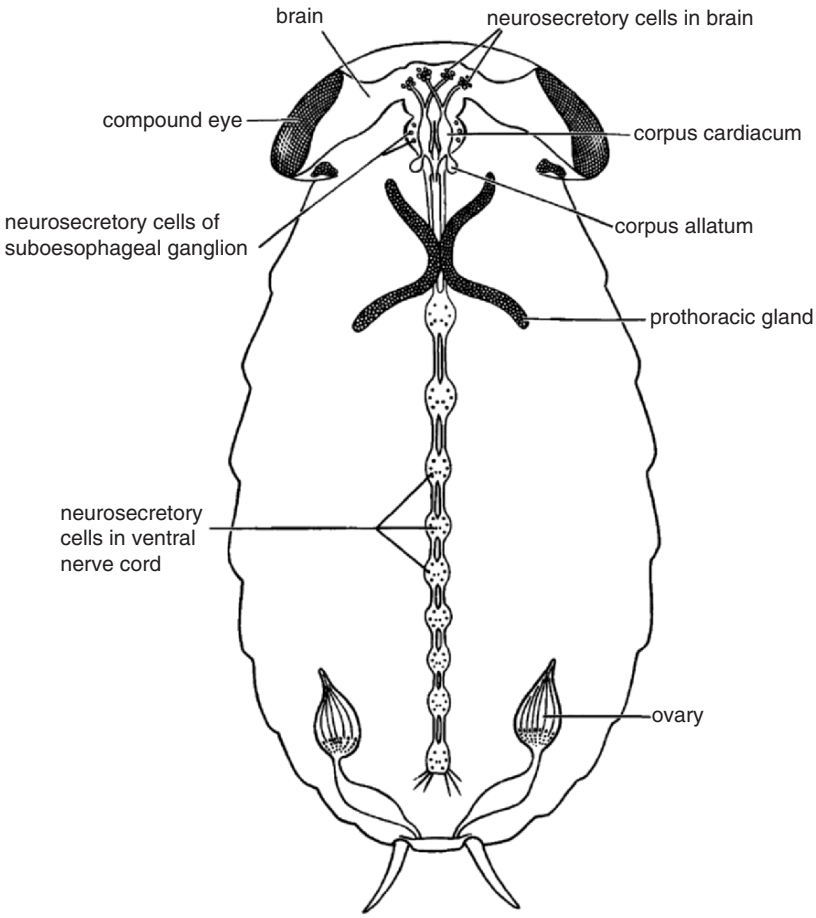
lactalbumin hydrolysates and fetal bovine serum (FBS); TC-100 (aka BML/TC-100), which is also a modification of Grace's medium, with the reduction of some amino acids, Krebs cycle intermediates and the addition of tryptose broth. Extensive optimization research by Goodwin and Adams (1980), Vaughn and Fan (1989) and Vaughn and Weiss (1991) resulted in the development of several serum-free culture media, with ILP-41 being the most commonly used basal medium. Currently, a variety of serum-free media is commercially available and is widely used throughout the world, with some examples being: the Ex-Cell 400 series, insect-EXPRESS, Sf-9000 II, SFX-Insect, Drosophila-SFM. With regards to cell culture media and related techniques, the Society for In Vitro Biology or "SIVB" (formerly the Tissue Culture Association; www.sivb.org), headquartered in Baltimore (MD, USA), plays an important role in training and updating cell and tissue culturists around the world. For example, the SIVB offers training in important areas such as quality assurance guidelines, detection methods for maintaining cultures free of contaminants, and techniques involved in the inventorying and maintenance of cell lines. This society also is involved in the dissemination of information on current knowledge and novel achievements in the field of insect cell and tissue culture.

As reviewed by Sohi et al. (1995), insect cell lines have been important tools in a variety of disciplines, ranging from basic biological research on developmental and physiological processes, to highly practical uses in the production of biopesticides and vaccines. Insect cells grown in vitro have provided a major impetus towards the commercial application of insect cell technology in several areas of biotechnology. In particular, the baculovirus expression vector system has become a major tool in the production of recombinant proteins for research and medical uses. Although most of the protein expression work has been performed using three or four cell lines, evidence suggests that new lines can be beneficial for specific proteins. Many researchers also have an interest in particular insect species or tissues that may not be represented among the currently available cell lines. While the small size of most insects and the specificity of insect tissues (for instance endocrine and nerve tissues) can make developing new cell lines challenging, past successes by many researchers have shown that they are attainable.

3 Endocrine Strategies

3.1 Screening for Ecdysteroid and Juvenile Hormone Activities

Agrochemical research over the last 30 years has resulted in the discovery of chemically novel insecticides that mimic the action of insect hormones. This concept of interfering with the insect hormones as a selective mechanism to control pest insects has been introduced already in 1967 by Carrol Williams (1967) when announcing "third generation pesticides". Figure 2 shows, in a



SITES OF PRODUCTION	HORMONES	FUNCTIONS
Neurosecretory cells (NSC) in brain and ventral nerve cord	Various neuropeptide hormones	Many functions, regulation of other endocrine glands, homeostasis, growth, development, behaviour, reproduction
Corpora cardiaca	Various peptide hormones, e.g. AKH	Regulation metabolism, heart beat and others
Corpora allata	Juvenile hormones	Regulation (status quo) of moulting/metamorposis, reproduction
Prothoracic glands (in larval stages); ovaries/testis (in adults)	Ecdysteroids	Regulation (trigger) of moulting/metamorphosis, reproduction

Fig. 2. Simplified scheme of the different sites of production in insects, the insect hormones and their function (redrafted after Chapman 1969; Spindler et al. 1993)

simplified manner, the different sites of hormone production in insects, as well as the function of each hormone. The two non-peptide hormones that are known to regulate insect growth, metamorphosis and reproduction are the terpenoid juvenile hormones (JHs) with a C18, C17 or C16 structure, and the steroid moulting hormones or ecdysteroids. The JHs are produced by the corpora allata (CA), with JH III being the most prevalent (Lafont, 2000). The ecdysteroid group includes more than 250 polyhydroxysterols that occur in plants and animals, with 20-hydroxyecdysone (20-OH-E) being the physiologically active moulting hormone in most insects (Lafont 2000). Ecdysteroids are produced in the prothoracic glands in larvae and in the reproductive organs in adults (ovaries and testis), and in the abdominal integument of some insects (Hoffmann and Gerstenlauer 1997). In the larval stages, 20-OH-E initiates the moulting and metamorphosis process in larvae, and JH (as the "status quo hormone") regulates these changes during the commitment period. In adults, the orchestration of both hormones controls oocyte development, maturation and release, as well as yolk deposition (or "vitellogenesis") and accessory gland development.

3.2 Ecdysteroid

The concentration of insect hormones can be modified by interference with the hormone biosynthesis, processing and degradation. Furthermore, the biological activity of the hormone can be either mimicked or interrupted by hormone analogs/mimics/agonists or antagonists to the respective hormone receptor.

The biosynthesis of ecdysteroids can be inhibited by interaction with the prothoracicotropic hormone (PTTH), a polypeptide produced by neurosecretory cells from the insect brain that is responsible for the production of ecdysone at the prothoracic glands, the primary source of ecdysteroids in developing insects (Nijhout 1994). Azadirachtin, the active compound of the neem tree (*Azadirachta indica*), is an example of the latter activity; however, it should be said that this plant extract has multiple actions (Schmutterer 1995). Specific inhibition of ecdysteroid synthesis has been achieved by acetylenic steroids that irreversibly inhibit the C22-hydroxylase; thus preventing the formation of biologically active ecdysteroids. Also imidazole compounds, KK-42 (1-benzyl-5-[(E)-2,6-dimethyl-1, 5-heptadienyl] imidazole) and KS-175 (4-phenoxyphenoxypropyl imidazole), have been reported to inhibit ecdysteroid synthesis, leading to disrupted development as seen mainly in Lepidoptera. Several insect cell cultures have been used to test the effect of ecdysteroid synthesis inhibitors on in vitro hormonal production. Recently, Amrani et al. (2004) reported that the amounts of ecdysteroids released into the culture medium by ovary cultures of *Tenebrio molitor* were significantly reduced by treatment with 10 μM of KK-42. The inhibitory action of KK-42, as well as that of KS-159, on ecdysteroid production in prothoracic glands and ovaries from other insects was also shown in whole animal

experiments and in tissue cultures (Kiuchi et al. 1985; Kiuchi and Akai 1988; Kadano-Okuda et al. 1987, 1994; Shiotsuki et al. 1999). Jarvis et al. (1994) screened three groups of putative ecdysteroid synthesis inhibitors using follicle cell cultures of *L. migratoria*, and found that KK-42 and ICI-L-635, as well as buprofezin, inhibited biosynthesis. In addition, Kuwano et al. (1983, 1992) and Akai and Mauchamp (1989) reported that KK-42 and terpenoid imidazoles suppress JH levels and can be exploited as insect growth regulatory insecticides. Interestingly, Lorenz et al. (1995) reported that the non-steroidal ecdysone agonist RH-5849 enhanced ecdysteroid synthesis in ovary cultures, but inhibited hormone production in abdominal integument cultures; however, this discrepancy in tissue response cannot be explained at present. In contrast to azadirachtin that is commercially used in pest control, the latter compounds have only been used in tissue culture or primary cell culture assays and are limited to laboratory insects. Other antagonists being considered for anti-ecdysteroid effects include the brassinosteroids, curcubitacins, limonoids and triterpenes (Richter and Koolman 1991; Decombel et al. 2005; Dinan and Hormann 2005; Van Loocke et al. 2006b).

To date, the advantage of established cell lines for mode of action studies and screening assays for endocrine systems has already been demonstrated several times (Peronnet et al. 1989; Spindler et al. 1993). However, it should be noted that insect cell lines are not suitable to study the activation or degradation of ecdysteroids and juvenile hormones. Reasons for this are that there are no continuous cell lines available from hormone producing glands from insects. In contrast, there exist different reports of tissue cultures using prothoracic glands, ovaries and testis to evaluate the effects of modulators; however this work is limited to short-term primary tissue cultures of prothoracic glands, ovaries, testis and abdominal integument epidermal cells (Kelly et al. 1992; Jarvis et al. 1994; Loeb et al. 1998, 2001a; Soltani et al. 1998; Gilbert et al. 2002; Gu and Chow 2005). From the vertebrate field, Hornsby and McAllister (1991) reported on their progress in culturing stereogenic cells. Although these efforts may have stimulated corresponding work in insects over the years, however, since then no reports on the success on stereogenic cell cultures from insects have been made.

In the last 30 years, a number of insect cell lines from different insect species and tissue origins have been tested and shown to respond to ecdysteroids. It is of course possible to develop cell lines for specific purposes without knowing their actual cell type. This concept has been proven frequently with research on ecdysteroids in which many *D. melanogaster* cell cultures were developed from embryos, and a *Manduca sexta* cell line, the first lepidopteran cell line developed for ecdysteroid research (Eide et al. 1975). Typical cellular responses are as reported in dipteran cells, i.e., they elongate and aggregate in response to ecdysteroids (*D. melanogaster* cells; Courgeon 1972; Cherbas et al. 1980), and undergo morphological effect that are specific for ecdysteroids (*Chironomus tentans* cells; Wyss 1982; Spindler et al. 1991; Spindler-Barth et al. 1992). In addition, Dinan and co-workers (2001a) used

D. melanogaster B_{II} tumorous blood cells to test different ecdysteroids and extracts with ecdysteroid agonistic or antagonistic activities. This B_{II} cell line did not metabolize ecdysone, 20-OH-E or ponasterone A (25-deoxy-20-hydroxyecdysone) (Dinan et al. 1985). Cultures in the presence of these new ecdysteroids and/or extracts lead to changes in cell morphology and a reduction in cell density that was assessed turbidometrically by monitoring the absorbance of individual wells at 405 nm in a microtiter plate reader, with the decline being expressed as a percentage of untreated controls (Fig. 3) (Clément et al. 1993; Dinan et al. 2001a). In addition to *D. melanogaster* and *C. tentans* cell lines, cell lines from a wide range of species belonging to different insect orders, have been reported to respond to ecdysteroids with similar characteristic changes in morphology and cell proliferation dynamics. Table 1 provides a survey of the different ecdysteroid-responsive cell lines from a variety of insect orders, including Diptera, Lepidoptera, Coleoptera and Hymenoptera. Over the years, other cell lines from numerous economically important species have been developed [e.g., thrips *Frankliniella*

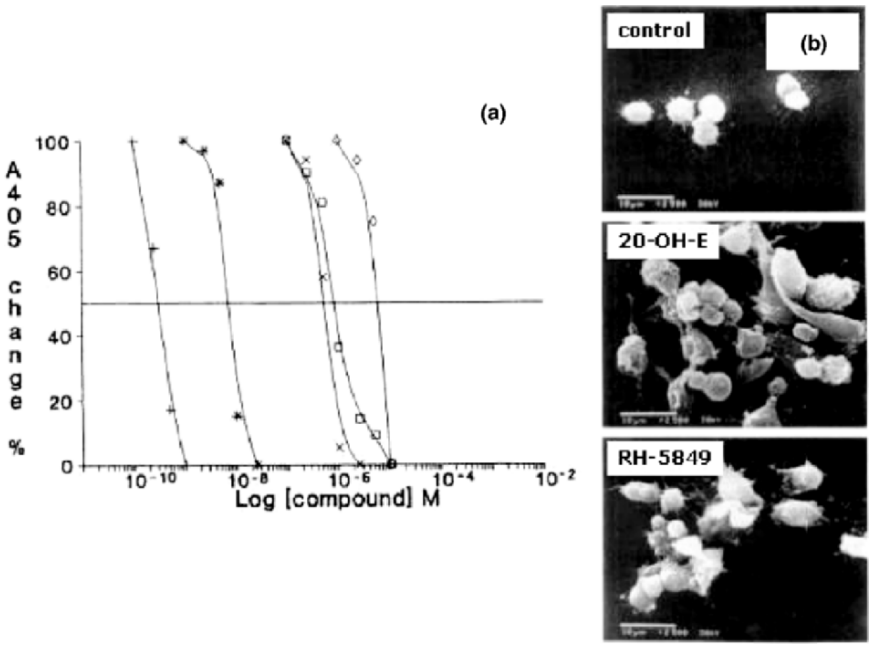


Fig. 3. Ecdysteroid-responsive *Drosophila* cells. (**a**) The B_{II} cells are grown in the wells of microtiter plates and respond to ecdysteroids with reduction in cell density and cell clumping that is reflected in an altered OD at 405 nm in the microtiter plate reader: *: 20-OH-E, □: ecdysone, +: ponasterone A, △: RH-5849, and ×: tebufenozide (RH-5992) (redrafted after Dinan et al. 2001b). (**b**) Scanning electron micrograph of Kc cells showing cell aggregates and irregular shapes with long, spindly processes in response to 1 μM 20-OH-E and 100 μM of the non-steroidal ecdysteroid agonist RH-5849 (× 2500) (redrafted from Wing 1988)

Table 1. A list of ecdysteroid-responsive cell lines derived from different insect/invertebrate species and tissue origin

Species	Origin	Name	Reference
Drosophila melanogaster	Embryo	Kc	Courgeon et al. (1972)
	Embryo	S3	Berger et al. (1978)
	Embryo	67j25D	Metakovskii et al. (1977)
	Embryo	GM1	Courgeon et al. (1972)
	Embryo	GM3	Courgeon et al. (1972)
	Embryo	MDR	Berger and Wyss (1980)
	Hemocytes	B_{II}	Dinan (1985)
	Hemocytes	L(2)mbn	Ress et al. (1997)
	Hemocytes	S2	own unpublished results
	Wing disc	C1,8+	Peel and Milner (1992)
Aedes albopictus	–	C7/10	Lan et al. (1993)
	Hemocytes	C6/36	Smagghe et al. (2003)
Chironomus tentans	Embryo	–	Wyss (1982)
Manduca sexta	Embryo	–	Eide et al. (1975)
	Embryo	MRRL-CH	Marks and Holman (1979)
Plodia interpunctella	Pupal imaginal wing discs	IAL-PID2	Lynn and Oberlander (1983)
Choristoneura fumiferana	Pupal ovaries	FPMI-CF-70	Palli et al. (1997b)
	Midgut	FPMI-CF-203	Palli et al. (1997a)
Malacosoma disstria	Hemocytes	IPRI-MD-66	Sohi et al. (1995)
Spodoptera frugiperda	Imaginal wing discs	IAL-SFD1	Lynn and Oberlander (1983)
	Pupal ovaries	IPLB-SF-9AE	Vaughn et al. (1977)
		IPLB-SF-21AE	
Ostrinia nubilalis	Embryo	UMC-OnE	Trisoyono et al. (2000)
Spodoptera exigua	Embryo	BCIRL-SeE-CLG4	Grasela et al. (2000), Decombel et al. (2005)
Trichoplusia ni	Imaginal discs	IAL-TND1	Lynn and Oberlander (1983)
Bombyx mori	Ovaries	BmN-4	Maeda (1989), Belloncik et al. (1991)
	Ovaries	Bm5	Grace (1967), Swevers et al. (2004)
Leptinotarsa decemlineata	Embryo	ZIZ-LD-1	Dübendorfer and Liebig (1992)
	Pupal fat body	LD	Long et al. (2002), own unpublished results
Anthonomus grandis	–	BRL-AG-2	Stiles and Newman (1992)

Table 1. A list of ecdysteroid-responsive cell lines derived from different insect/invertebrate species and tissue origin—(Cont'd)

Species	Origin	Name	Reference
Blatella germanica	Embryo	UM-BGE4	Ward et al. (1988)
Trichogramma exiguum	Muscle-like	IPBL-Tex2	Lynn et al. (1991)
Homarus americanus	Primary culture of testis	–	Chang (1997)
Pacifasticus leniusculus	Primary culture of hematopoietic tissue	–	Chang (1997)

IPBL Insect Biocontrol Laboratory, USDA-ARS, Beltsville, MD, USA
IAL Insect Attractants, Behavior, and Basic Biology Research Laboratory, USDA-ARs, Gainesville, FL, USA
BCIRL Biol Control Insects Research Laboratory, USDA-ARS, Columbia, MO, USA
ZIZ Zoological Institute Zürich, Zürich, Switzerland

occidentalis and *Thrips tabaci* (Nagata et al. 1997), aphids *Acyrthosiphon pisum* (Peters and Black 1971), Mediterranean fruit flies *Ceratitis capitata* (Cavalloro 1981), leafhoppers/cicades *Nephotettix cincticeps* (Mitsuhashi 1965), flesh flies *Sarcophaga peregrine* (Homma and Natori 1997)]. However, the responsiveness of these lines to ecdysteroids has not been tested. Furthermore, numerous attempts have been made to establish long-term cultures from other arthropod/crustacean tissues over the past 10 years. Next to pest insects, several arthropods are vectors of important diseases. In addition, arthropods/crustaceans can be regarded as important sensors of pollution by insecticides and the availability of crustacean cell lines is relevant for the development of cell-based detection systems that are pollution sensors (as reported later in this chapter). Primary cell cultures of arthropods/crustaceans have been developed and these respond to 20-OH-E by reducing contact inhibition and increasing invasive behaviour 7 days after hormone treatment (Chang 1997). Additionally, in this study, death of mesodermal cells and the proliferation of spermatogonia in lobster testicular cell cultures were observed. It is promising that these observations mimic pre-moulting events as seen in vivo, i.e., at the moment of pre-moulting, the circulating 20-OH-E concentrations rise and specific cell populations die, while other populations increase. Similarly, primary lymphoid cell cultures could be prepared from hemolymph of *Penaeus monodon* shrimps (Loh et al. 1997). As reviewed by Mulford and Villena (2000), primary cultures from about ten crustacean species (fresh and sea water) and different tissue origin survive for a relatively long time (ranging from 1 week to 4 months), however, the establishment of a continuously dividing crustacean cell line in culture has not been successful so far.

With the current knowledge on cell biochemistry, hormone-responsive effects on cell proliferation can be readily quantified using automated colorimetric techniques. These methods are based on the measurement of cell components and are relatively simple and suitable for multiple samples. A good example of such indirect methods of cell determination is for instance the tetrazolium assay. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] is a colorimetric technique performed in microtiter plates as alternative for the time-consuming direct cell counting Bürker chamber method (Wilson 1986). This technique was originally developed to substitute for the [^{3}H]thymidine uptake assay in T-cell lines (Mosmann 1983) and to provide a rapid and simple way of detecting T-cell growth factors and lymphotoxins (Heeg et al. 1985). This assay depends on the reduction by living cells of tetrazolium salt, MTT, to a purple formazan product. The cleavage of MTT is accomplished by NADPH or NADH that results from dehydrogenase enzymes in metabolically active cells (Berridge and Tan 1993), and the amounts of formazan are proportional to the cell numbers in the culture medium. However this original technique endured several limitations. Specifically, a less than optimal sensitivity, a variable background due to protein participation on adding an organic solvent to dissolve the purple formazan, and a low solubility of the product. Some of the problems were inherent to the use of vertebrate cells. Denizoit and Lang (1986) and Decombel et al. (2004) reported on some modifications to overcome some of these problems.

In addition, other good and commercially available cell viability/proliferation/toxicity assays are the lactate dehydrogenase (LDH) assay, the luciferase assay from Promega (Madison, WI, USA) and assays using fluorophores from Invitrogen-Molecular Probes (Carlsbad, CA, USA) such as FITC (fluoresceine isothiocyanate) in a flowcytometer.

For screening assays a number of biochemical parameters that are connected/involved with the morphological responses of insect cells after addition of ecdysteroids, can be used as molecular tracers of ecdysteroid activity. For example, Dinan et al. (1990) reported on an induced AChE activity in different cell lines (*D. melanogaster*, *C. tentans*) using a microfluorometric assay. In these studies, AChE is considered to be an enzyme that is a good indicator of cell differentiation. In addition, the ability to produce ecdysteroid-specific (cuticle) proteins by insect cell lines has been used to provide a framework for biochemical investigation of ecdysteroid action. Lan et al. (1993) reported on the effect of 20-OH-E in *A. albopictus* mosquito cells, specifically on their growth, morphology, synthesis of ecdysteroid-inducible proteins (EIPs), and expression of a transfected gene regulated by a synthetic ecdysteroid-responsive element. Furthermore, Stiles and Newman (1992) established two insect cell lines, UM-BGE4 from the cockroach and BRL-AG-2 from the boll weevil, and, using antibodies specific for EIPs, found that the proteins from these lines corresponded in size to specific proteins observed in abdominal cuticle extracts. Additionally, Fretz et al. (1993) reported on

pronounced changes in the pattern of newly synthesized proteins using two-dimension polyacrylamide gel electrophoresis (PAGE). However, it should be remarked that only a few of such biochemical responses are suitable for screening purposes. For more information on these and other studies, please see Table 1, which presents an overview of the cell lines, including their sources of origin, their morphological and biochemical responses, and relevant references. Efforts made towards the isolation of ecdysteroid-responsive cell lines and of resistant clones have also stimulated the study of the mechanism of action of ecdysteroid hormones at the molecular level in the past decade.

Using some of the methods described above, namely observations on AChE induction and cell morphology changes with *Drosophila* Kc cells, Wing and his co-workers at Rohm and Haas (Spring House, PA, USA; now Dow AgroSciences, Indianapolis, IN, USA) (Wing 1988; Wing et al. 1988) reported in 1988 on their discovery of the first nonsteroidal ecdysteroid receptor agonist, dibenzoylhydrazine RH-5849. In their experiments, the Kc cells responded to RH-5849 in a similar manner as they did to 20-OH-E, with the typical formation of cellular processes, the inhibition of cell proliferation (Fig. 3), and the induction of AChE. Additionally, similar to 20-OH-E, RH-5849 was able to compete with ^{3}H-ponasterone A for binding to a high-affinity ecdysteroid receptor sites from Kc cell extracts. The induction of AChE enzyme activity correlated well with RH-5849's affinity for the ecdysteroid receptor. Similar tests were also performed with cells of other dipterans, namely *C. tentans* (Spindler-Barth et al. 1991), *A. albopictus* (Smagghe et al. 2003) and *D. melanogaster* Kc cells (Minakuchi et al. 2003) and S2 (unpublished results). Lepidopteran cells from different species and tissue of origin have also been subjected to these assays with positive results: the chitin-producing epithelial imaginal disc PID2 cells (Silhacek et al. 1990), the ovarian Sf-9 cells (Nakagawa et al. 2000, 2002; Minakuchi et al. 2003; Nakagawa 2005), the CF-203 midgut cells (Palli et al. 1997a, 1997b), and the embryonic beet armyworm *Spodoptera exigua* cell line SeE-CLG4 (Decombel et al. 2004). In all these studies, the responses varied in respect to the formation of cellular extensions and arborisations, the induction of cell aggregations, the inhibition of cell proliferation, the induction of AChE activity or that of apoptosis. Interestingly, in all assays, there was a good correlation between the extent of the cellular responses (e.g., in morphology, cell proliferation, induction of the AChE enzyme) and the affinity of the corresponding substance to the ecdysteroid receptor. From these cell assays and others with whole insects in the laboratory and the field, the compounds of this new class of dibenzoylhydrazines were found to be particularly toxic to Lepidoptera.

When searching for ecdysteroid receptor agonists, a first approach should be to determine the binding affinity with the targeted receptor, in this case the nuclear ecdysteroid receptor. However, the labeled compound (^{3}H-ponasterone A) is not available in large quantities nor at reasonable prices for HTS systems, and the ligand binding assay itself is time-consuming and

requires much material. As an alternative for labeled ponasterone A, Dhadialla and co-workers reported at the 14th Ecdysone workshop in 2000 on the use of a photoaffinity analog of RH-5849; however, the affinity is found too low to eliminate false positives. Therefore, it is more convenient to screen for ecdysteroid receptor interacting compounds based on another criterion and then afterwards determine if the biological effect is mediated by the ecdysteroid receptor.

Given the need for screening systems that efficiently recognize compounds which interact with the ecdysteroid receptor, several researchers have made efforts to transfect insect cell lines with reporter constructs in order to develop the needed assays. Until these assays were developed, screening systems were commonly based on morphological observations and/or growth responses of insect cell lines. These latter tests were slow and required careful monitoring and, therefore, had limited applicability to analysis in HTS formats. Ecdysteroid-responsive cells have since been developed that contain the ecdysteroid receptor, its partner protein Ultraspiracle (USP) and the necessary activators and repressors (Henrich 2005). For example, reporter gene assays have been used to measure hormonal activity (Mikitani 1995b; Toya et al. 2002; Swevers et al. 2004). In these studies, both ponasterone A and 20-OH-E bound to the ecdysteroid receptors and activated the luciferase gene contained in the ecdysteroid-responsive reporter plasmid in a dose-response manner in both Sf-9 and Kc cells. Based on the high lepidopteran specificity of the dibenzoylhydrazine-based compounds, the same technique was performed for an ovarian cell line from the silk moth, *B. mori*. Earlier assays had demonstrated that these cells were sensitive to ecdysteroids, however, conditions to quantify the response of the cells to ecdysteroids were laborious and time-consuming as the cell numbers needed to be counted after a 3-day culture period. Swevers et al. (2004) constructed a cell-based HTS system with these *B. mori* Bm5 cells with a reporter gene construct encoding the green fluorescent protein (GFP) that was able to measure the hormone activity of ecdysone agonists. Bm5 cells grown in IPL-41 medium with 10% FBS were seeded in microtiter culture plates and transfected with a mixture of plasmids pBmbA/ERE.gfp and pBmA.hmB containing Lipofectin. Semiclonal cell populations with intense fluorescence induced by 20-OH-E were selected by antibiotic (hygromycin B) selection (Fig. 4). The test to screen ecdysteroid-active substances consisted of three simple steps that can be readily adapted to an HTS format: (1) the distribution of transformed cells in microtiter plates, (2) the addition of compounds/extracts at selected concentrations, and (3) the quantification of fluorescence intensity by a fluorescence plate reader. With the transformed Bm5 cells, the median effect concentration for 20-OH-E was 75–100 nM (Fig. 4). A similar assay was developed for substituted dibenzoylhydrazines and a good correlation between cell responses and insecticidal toxicity was reported. Interestingly this assay is also suitable for the screening of ecdysteroid antagonistic activities (Swevers et al. 2004). With the development of transformed cells, a large chemical

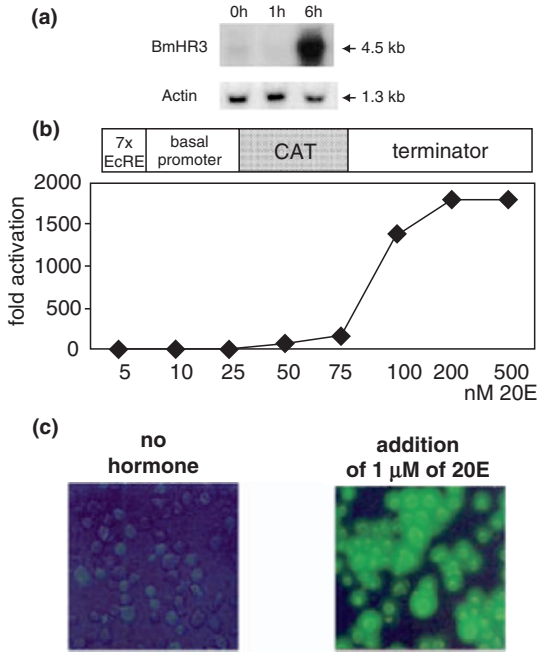


Fig. 4. Assessment of the primary response to 20-OH-E in silk moth-derived Bm5 cells, transformed with the pBmbA/ERE.gfp construct for induction of green fluorescence. (**a**) Northern blot analysis of 20-OH-E primary response gene BmHR3 expression at intervals of administration of 1 μM of 20-OH-E. Actin hybridizations were carried out as control. Molecular weight of hybridizing mRNAs is shown on the *right*. (**b**) Induction of CAT activity from reporter construct pBmbA/ERE.cat after treatment with different concentrations of 20-OH-E (5–500 nM). Expression levels of induced relative to non-induced cells are indicated. Drawing of the reporter construct is at the *top*. (**c**) Observation of induction of green fluorescence by 20-OH-E by fluorescence microscopy. Untreated and treated transformed Bm5 cells are shown on the *left* and *right*, respectively. (×40) (redrafted from Swevers et al. 2004)

library of 180 dibenzoylhydrazine-like compounds was tested and the ecdysteroid receptor interactions were calculated using three-dimensional quantitative structure-activity relationship (3D-QSAR) tools (Weelock et al. 2006). Currently, these results obtained with in silico comparative molecular field analysis (CoMFA) and superimposition models help to provide better fundamental insights in ligand-ecdysteroid receptor interactions, and they also allow the formulation of predictions for enhanced activities and/or new lead molecules.

In the last decade, intensive research performed by different groups using insect cell lines have lead to the elucidation of QSARs of numerous compounds. These findings will lead to a better understanding of the mechanism of the ecdysteroid receptor and the action of dibenzoylhydrazine-like insecticides. The most recent achievements, together with the currently available

information on the ecdysteroid receptor crystalline structure, the QSAR results and molecular modelling, are available in Billas and Moras (2003), Billas et al. (2005) and Dinan and Hormann (2005).

Since 1988, different ecdysteroid receptor agonists having a dibenzoylhydrazine-based structure have been synthesized and tested for an enhanced activity and species specificity. This work has lead to the commercialization of 4 ecdysone agonists or MACs: tebufenozide, methoxyfenozide, helofenozide and chromafenozide. Additionally, other new leads and classes of non-steroidal ecdysone agonists have since been identified with the use of ecdysteroid receptors from *D. melanogaster*, *H. virescens* and *P. interpunctella* in cell-based assays: 3,5-di-*tert*-butyl-4hydroxy-N-*iso*-butyl-4-hydroxybenzamide (DTBHIB) by Mikitani (1996a), and tetrahydroquinoline [1-aryl-4-(arylamino)-1,2,3,4-tetrahydroquinoline] and α-acylaminoketone by RHeoGene and FMC Co. (Spring House, Philadelphia, PA, USA) (Smith et al. 2003; Tice et al. 2003; Palli et al. 2005b). 8-O-Acetylharpagide, isolated from *Ajuga reptans* by Merck Co. (Rahway, NJ, USA) scientists, was misinterpreted as a new agonist lead as the extract contained a small amount of 20-OH-E (Elbrecht et al. 1996; Dinan et al. 2001c). As part of an extensive survey of natural products to detect those showing ecdysteroid agonist or antagonist activities in the B_{II} bioassay (Dinan et al. 2001a), the agonist maocrystal E was isolated from *Isodon* spp. (Labiatae). Ecdysone agonists cannot only be exploited as selective insecticides, but also as pharmaceuticals for gene therapy and inducers of virus resistance in plants (Palli et al. 2005a).

Concerning the ecdysteroid receptor antagonists, previous assays demonstrated that cell cultures can be used to detect brassinosteroids (Hetru et al. 1986; Spindler et al. 1991; Richter and Koolman, 1991). Brassinosteroids are endogenous plant growth regulators with a steroidal structure; the structural similarity between brassinosteroids and ecdysteroids has given rise to several studies to discover an ecdysteroid-interfering activity in insects. In one of the earliest reports on brassinosteroids in arthropods, it was stated that these plant hormones competed with 20-OH-E to bind to the ecdysteroid receptors. This conclusion was reached because of experiments using isolated imaginal discs of *Phormia terra-novae*, where very weak agonist effects were displayed (Hetru et al. 1986). An anti-ecdysteroid activity was also reported by Lehmann et al. (1988) demonstrating that two brassinosteroids had a weak affinity for the partially purified ecdysteroid receptors from *Calliphora vicina*. Unfortunately, as Spindler et al. (1991) stated, the biological effect of brassinosteroids and their binding capacity to ecdysteroid receptors was never tested in the same manner. Similarly, as shown by Decombel et al. (2004) for 24-epibrassinolide the lepidopteran cell line SeE-CLG4 allows the simultaneous detection of different cytotoxic effects that is especially important for possible hormone antagonists. Very recent experiments demonstrated that, in the transformed Bm5/ERE.gfp cells, this brassinosteroid had an antagonistic effect against 500 nM 20-OH-E without altering the cell viability (Van Loocke et al. 2006a; unpublished results).

3.3 Juvenile Hormones

Considerable progress has been made in the last 30 years concerning compounds having JH activities. However, to date, JH's mechanism of action is not well understood also at the molecular level. JH appears to have a dual receptor mechanism, with both membrane and intracellular receptors (Dhadialla et al. 2005; Goodman and Granger 2005). Nonetheless, it is known that the morphological responses of several insect cell lines to ecdysteroids can at least be partially antagonized by JH and JH analogs (JHAs) (Retnakaran et al. 1985). Studies by Lezzi and Wyss (1976), Wyss (1976), Cherbas et al. (1989), and Ress et al. (1997) showed the modulation of ecdysteroid-induced effects by JH and JHAs in dipteran cells. Easton and Horwath (1991) also reported that JH and the JHA methoprene (at 3 μg/ml) had a direct effect on primary cells derived from fat body of *T. molitor* larvae. In this study, these compounds produced an elevated level of antifreeze proteins. However, the mechanism by which these protein levels were altered (i.e., elevated synthesis and/or release), requires further study. In the early 1990s, Spindler et al. (1993) also found that the formation of multicellular vesicles in *C. tentans* epithelial cells is prevented by JH in the presence of 20-OH-E. Similarly, the ecdysteroid-induced AChE activity was reduced by JH and JHAs in IAL-PID2 cells (Oberlander et al. 2000). In this study, methoprene, fenoxycarb and farnesol inhibited cell proliferation while linoleic acid had no effect. The concentrations at which JH compounds were effective ($\geq$50 μg/ml) were in the same range of concentrations that were effective in inhibiting ecdysteroid-induced development in intact *P. interpunctella* imaginal discs in vitro. However, it should be noted that the suitability of this cell line as a routine JH mimic assay was constrained by the lack of a strong correspondence between the degree of inhibition of cell proliferation in vitro and the relative activity of the JH compounds in vivo. Decombel et al. (2004) also reported on a high cell proliferation inhibitory action of the JHA pyriproxyfen in SeE-CLG4 and PID2 cells, with EC_{50}'s of approximately 2.5 mg/l. In continuation of our study with SeE-CLG4 cells, it was noted that ecdysteroids inhibited cell proliferation but when JH was added to the culture medium then the effect was reversed (unpublished results). However, it should be remarked that the modulatory effects of JH and JHA can also be provoked by a cytotoxic effect, and so cell viability should be evaluated. As an alternative, as it is known that JH has an ecdysteroid receptor-antagonizing effect, the same approach can be used for JHAs in the presence of 20-OH-E. The three best known JHAs, methoprene, fenoxycarb and pyriproxyfen, were tested with the transformed Bm5/ERE.gfp cells and they all showed an antagonistic activity against 500 nM 20-OH-E and 100 nM ponasterone A; however, this was at the relatively high concentrations of >10 μM. An additional experiment to test the viability of Bm5 cells with similar concentrations demonstrated a reduced cell viability that can account for the antagonistic responses by the JHA (Van Loocke et al. 2006b; unpublished results). With

abdominal integument cell cultures of *T. molitor*, Aribi et al. (2006) reported that the JHA pyriproxyfen inhibited the ecdysteroids' activities in the culture medium, suggesting that this was its mode of toxicity.

It is clear that interference with ecdysteroid and juvenile hormone activities is an effective means of controlling insects. However, for the different JHA and MACs tested so far, there exists considerable differences in susceptibility. In this respect the various insect cell cultures are useful as they allow researchers to evaluate the biological activity and insect specificity of different compounds. The compounds can be applied at known concentrations and can be targeted towards known effector sites (hormone receptor), and these tests can be performed for a multitude of different insect species and tissues. As documented by several authors, JHAs have not proven to be as effective as control agents as originally expected. However, they have advantages for controlling pests, namely in the area of public heath as many of them are environmentally friendly. The anti-JH compounds have, for the most part, remained at the experimental stage. Interfering with JH action will become an attractive option, once the JH receptor is characterized and cDNA clones become available. When this occurs, insect cell-based HTS assays will open many possibilities.

4 Insect-Specific Metabolic Pathways with Chitin and Cuticle Synthesis

Chitin is a β-1,4-linked amino polysaccharide homopolymer of N-acetyl-D-glucosamine (GlcNAc) and one of the most copious polysaccharides in the biosphere (as reviewed by Palli and Retnakaran 1999; Cohen 2001). With its fibrillar form it contributes strength and rigidity to structural elements and serves as a mechanical support and a protective barrier in exoskeletons and in midgut peritrophic matrices of arthropods or cell walls in fungi. Chitin is produced in abundance by invertebrates, notably arthropods (insects, crustaceans) and to a minor extent in mollusc, annelids and nematodes (eggs). It is an integral component in cell walls of fungi (except in Oomycetes) but it is absent in plants and vertebrates. This taxonomic difference provides the rationale for considering chitin as a safe and largely selective insecticide target. The classic approach in this area is to interfere with the periodic formation and degradation of the insect exoskeleton. In insects, chitin is synthesized by the chitin synthase enzyme that is membrane-bound and catalyzes the addition of GlcNAc units to a dolichol carrier. Chitin is degraded by three different chitinases: endochitinase, exochitinase and chitobiase. In the exoskeleton and peritrophic matrix, chitin is covalently linked to a matrix of proteins and glycoproteins. The proteins can further be cross-linked by sclerotization and the cuticle may be tanned to various degrees, with 3,4-dihydroxyphenylalanine (DOPA) decarboxylase (DDC) being the key enzyme

in the formation of the sclerotizing agent N-acetyl dopamine. Any interference in this process of cuticle biosynthesis can be exploited to lethally inhibit growth and development of pest insects.

In the 1970s researchers at Philips-Solvay-Duphar (Weesp, the Netherlands) discovered diflubenzuron and its chitin inhibiting properties. Since then, a multitude of derivatives of similar IGRs with different biological activities and species selectivities have been synthesized (Retnakaran et al. 1985; Retnakaran and MacDonald 1988; Palli and Retnakaran 1999; Dhadialla et al. 2005). To date, the BPUs are an important group of IGRs that are known to interfere with chitin synthesis, however, their exact mode of action is not discovered yet. The BPUs are often species-specific insecticides. In general they are not active as aphicides but are active against other major pest insects such those found in the following insect orders: Lepidoptera (most BPUs), Coleoptera (most BPUs), Diptera (cyromazine), and Homoptera (specifically whiteflies; buprofezin). In addition to the BPUs, there exist several other compounds that inhibit chitin synthesis. A wide variety of chitin synthesis inhibitors belonging to different chemical groups have been reported (Londershausen et al. 1993, Palli and Retnakaran, 1999; Cohen, 2001) and some of the more common ones are listed in Table 2. Several types of bacterial and fungal antibiotics have been shown to block chitin synthesis at the cellular level through their inhibition of specific enzymes or of intracellular functions of the Golgi apparatus and the endoplasmic reticulum. Certain metabolic inhibitors that block dihydrofolate reductase have been shown to inhibit chitin formation by interfering with nucleic acid synthesis. Therefore, it appears that since the synthesis of chitin occurs in the epidermal cell, insect cell cultures may serve as tools to study the site of action for most of these inhibitors.

Given the potential of chemistries with an insect-specific target, large investments have been made in Research and Development divisions of companies within the agrochemical industry to determine the genetic basis of chitin metabolism inhibition in order to develop effective molecular screening tools. It is rather surprising that, although the BPUs were introduced almost three decades ago, their exact mechanism of biochemical lesion has not yet been elucidated. Conceivably, this failure resides in a lack of in-depth knowledge about chitin synthesis and deposition. With this in mind, Cohen (2001) reviewed the recent cloning and sequencing work of the insect genes that encodes the chitin producing proteins. This concurs with the reports of several authors that screening for interference with chitin biosynthesis of insects is possible. With the advent of biotechnology and the availability of both cDNA and antibody probes in combination with insect cell cultures, it is possible to develop HTS assays for discovering new chemicals that can block chitin formation (Oberlander, 1989; Londershausen et al. 1993; Spindler et al. 1993; Palli and Retnakaran, 1999).

Over the last decades, several continuous insect cell lines have been established that are able to synthesize or degrade at least parts of the cuticle. Major

Table 2. List of inhibitors of chitin biosynthesis (from Palli and Retnakaran 1999)

Inhibitor	Mode of Action	Effect
A. Antibiotics		
1. Puromycin	A nucleoside antibiotic that interferes with t-RNA function and inhibits protein synthesis.	Inhibits chitin/protein synthesis in the blue crab. Indicates that concurrent protein synthesis is essential for chitin formation.
2. Cycloheximide	Binds to sub unit of ribosomes and prevents protein synthesis.	Inhibits GlcNAc uptake in *Plodia* cells indicating protein synthesis is essential.
3. Tunicamycin	Prevents transfer of GlcNAc to dolichol phosphate and prevents glycosylation.	Blocks chitin synthesis in *Triatoma*. Dolichol pathway is blocked.
4. Polyoxin-D	Structural analog of UDP-GlcNAc and competitively inhibits chitin synthase.	Inhibits chitin biosynthesis in *Chilo*.
5. Nikkomycin	Structural analog of UDP-GlcNAc and is a more powerful chitin synthase inhibitor than polyoxin-D.	Inhibits GlcNAc incorporation into chitin in *Tribolium*.
6. Brefeldin	Disrupts the glycosylation function of Golgi	Chitin-protein complex not formed in the blue crab.
7. Monensin	Inhibits glycosylation in the endoplasmic reticulum during the process of extra-cellular secretion (Palade pathway is blocked)	Prevents chitin complexing with protein for vesicle formation enroute to cuticle in the blue crab.
8. Avermectin	Anti-parasitic drug that prevents chitin synthesis; effective on helminth parasites.	Inhibits chitin formation in *Artemia*.
B. Metabolic Inhibitors		
9. Aminopterin	Inhibits dihydrofolate reductase and interferes with nucleic acid synthesis in the epidermis.	Indirectly inhibits normal chitin deposition in *Musca*.
10. Cyromazine	An S-triazine inhibitor of dihydrofolate reductase.	Abnormal chitin formation in *Lucilia*.

C. Insect Growth Regulators		
11. Buprofezin	Interferes with the mitotic apparatus.	Inhibits cuticle formation and chitin biosynthesis in *Nilaparvata*.
12. Diflubenzuron (and analogs)	It is the harbinger of all BPUs; widely considered to be an inhibitor of the arthropod chitin synthase.	Inhibits chitin synthesis in numerous insects.
D. Alkaloids		
13. Vinblastine	Combines with tubulin and inhibits the assembly of microtubules resulting in blocking mitosis.	Inhibits cuticle formation in *Plodia* wing disks.
14. Colcemid	Binds to tubulin and prevents tubulin assembly into microtubules and blocks mitosis.	Cuticle is not formed in *Plodia* wing discs.
E. Hormones/Analogs		
15. 20-OH-E	Moulting hormone expresses and represses moulting cycle genes and is released at a precise time frame.	Chitin is synthesized in its absence during the intermoult stage in *Manduca*.
16. Tebufenozide (RH-5992) and analogs	Dibenzoylhydrazines that are agonists of 20-OH-E; stable and persist in epidermis cells.	Persistence during intermoult stage prevents chitin synthesis.

advancements in this area have been contributed primarily by the research groups of Herbert Oberlander (USDA-ARS, Gainesville, FL, USA), Klaus-Dieter Spindler (University of Düsseldorf; now University of Ulm, Ulm, Germany), and Arthur Retnakaran (Great Lakes Forestry Centre, Sault Ste. Marie, ON, Canada). The successful stimulation of chitin synthesis by ecdysteroids in tissue culture provided favourable prospects for investigating the action of chitin synthesis inhibitors in vitro. Organ cultures of imaginal discs and integument epidermis of different insects including Lepidoptera, Coleoptera and Orthoptera, have been used a models (Oberlander and Fulco 1967; Ferkovich et al. 1981; Soltani et al. 1987; Nakagawa et al. 1992, 1993; Mikólajczyk et al. 1994; Oberlander and Silhacek 1998; Oberlander and Smagghe 2001).

The feasibility of using insect cell lines to study the mode of action of inhibitors of chitin synthesis was suggested at the end of the 1980s by Londershausen et al. (1987) and Oberlander (1989). Ward et al. (1988) showed that the cockroach (*Blatella germanica*) cell line UMBGE-4 synthesized a chitin-like material that was stimulated by 20-OH-E and inhibited by diflubenzuron. In another example chitin synthesis in the epithelial cell line of *C. tentans* was inhibited by 20-OH-E and SIR-8514 (triflumuron) (Spindler-Barth et al. 1989; Londershausen et al. 1993; Spindler-Barth 1993). In these assays it was observed that the chitin synthesis inhibitory effects could only be measured in cultured cells, but not in cell-free homogenates. Chitin synthesis inhibitors were also tested in the IAL-PID2 cell line derived from imaginal discs of the Indian meal moth, *P. interpunctella*, to determine if they would inhibit uptake of chitin precursors in the target cells (Porcheron et al. 1988). The PID2 cells responded to treatment with 20-OH-E with increased uptake of GlcNAc, N-acetylgalactosamine and D-glucosamine, precursor of chitin, but not D-glucose or D-mannose (Porcheron et al. 1988). Teflubenzuron (an analog of diflubenzuron) did not reduce GlcNAc uptake by the PID2 cells, while diflubenzuron had a small inhibitory effect (Oberlander et al. 1991). Although these effects cannot be explained mechanistically, they are consistent with those obtained with intact imaginal discs (Oberlander and Silhacek 1998).

More recent studies using cell lines have been performed for the screening of chitin synthesis inhibitors. Decombel et al. (2004) reported on an extensive survey on the biological response of lepidopteran cells to different groups of insecticides (*S. exigua* and *P. interpunctella*). In this study, a comparison of diflubenzuron and lufenuron showed that the former had no or a limited effect on insect cell cultures. However, the high toxicity against third-instars (LC_{50} 0.098 mg/l; using a diet overlay assay) was reflected in an EC_{50} of around 10 mg/l (in the culture medium) for the inhibition of the proliferation of SeE-CLG4 cells. In contrast the chitin-producing PID2 cells exhibited a strong decrease in cell proliferation of more than 90% when incubated with 10 mg/l lufenuron.

Interference with chitin synthesis and chitin degradation can be measured conveniently using insect cell lines as was shown previously (Londershausen

et al. 1987; Oberlander 1989; Spindler et al. 1990, 1993; Palli and Retnakaran 1999). The use of whole cell assays for screening purposes can be supplemented with tests using homogenates of the same tissue. Chitin degradation by chitinases and hexosaminidases can then be measured with high sensitivity using GlcNAc and its oligomers coupled to a fluorogenic dye as was developed by McCreath and Gooday (1992).

The feasibility of cell cultures allows investigators to determine the structure-activity relationships of BPUs at cellular level. This ability aids researchers in the design and synthesis of inhibitory compounds. Extensive work of Nakagawa et al. (1987, 1989, 1993) demonstrated that the introduction of electron-withdrawing and hydrophobic substituents at the para-position of the phenyl (aniline) moiety enhanced activity, whereas larger groups were unfavourable. Interestingly, a linear relation was calculated between in vitro activities and in vivo larvicidal toxicities after separate consideration of the hydrophobic factors participating in absorption and transport in the insect body.

More recently, Abo-Elghar et al. (2004) investigated the mode of action of diflubenzuron by using of integument cell cultures and cellular vesicles of German cockroach *B. germanica* and *D. melanogaster*. Based on previous work, diflubenzuron affects the ã-thioGTP-stimulated Ca^{2+} transport in vitro in intracellular vesicles from the integument (Nakagawa et al. 2004). The newest results corroborated that the site of action of diflubenzuron, and BPUs in general, is an ABC (ATP binding cassette) transporter, i.e., the sulfonylurea receptor (SUR). These results explain this compound's inhibitory action on chitin synthesis in these two species. Here, glibenclamide, one of the most commonly used sulfonylureas for type II diabetes treatment, was the positive control.

The ability to culture chitin-producing cells can also help to improve the understanding of the mechanism of newer compounds. In the chemical class of 2,4-diphenyl-1,3-oxazolines, the mode of action of etoxazole (2,4-diphenyl-1,3-oxazoline) has been argued to be moulting inhibition during development with a mechanism suggested to be similar to that of chitin inhibitory BPUs. However, experimental results supporting this hypothesis are lacking (Ishida et al. 1994; Suzuki et al. 2002). With this in mind, the effect of the acaricide/insecticide etoxazole on chitin biosynthesis in the fall armyworm, *S. frugiperda*, was investigated using epidermal cell cultures. In this study, the incorporation of [^{14}C]N-acetyl-D-glucosamine was used to quantify the chitin inhibitory action. The IC_{50}-values generated were 2.95 µM and 0.071 µM for etoxazole and triflumuron, respectively (Nauen and Smagghe 2006). These values were in keeping with the lower chitin scores observed in the insect cuticle after etoxazole treatment of whole *Spodoptera* caterpillars. The results obtained in this latter study supported the hypotheses that the strong moulting inhibition effects of 2,4-diphenyl-1,3-oxazolines, such as etoxazole, lead to their larvicidal activity (similar to the BPUs) by their inhibition of chitin biosynthesis.

The exploitation of the sclerotization process in insects as target for insect pest control is more difficult than that of the inhibition of chitin synthesis. Any inhibition in the sclerotization process would block the successful growth and development of pest insects. The properties and the hormonal regulation of DDC, one of the major enzymes, have been extensively studied in insects by different laboratories (Hopkins and Kramer 1992; Riddiford et al. 1999). The two key enzymes involved, DDC and phenoloxidase, seem to be rather similar in insects and vertebrates (Londershausen et al. 1993). Therefore, although there are cell lines available with DDC activity, this property has not been yet used for screening purposes.

Several cell lines synthesize glycoproteins that are secreted into the culture medium in a hormonally controlled manner, as reported by Spindler and coworkers (1993). However, the biological function of these proteins is not yet clear. One hypothesis on the role of these glycoproteins is that they may participate in the ecdysteroid-induced aggregation of cells. Another suggested role of these proteins is that they function as chitin precursors. In the case of GlcNAc-rich glycoproteins, this latter function may be possible. Additionally, glycoproteins may be components of the extracellular matrix involved in the hormonally controlled differentiation processes. Stiles and Newman (1992) reported on the ecdysteroid dependent secretion of several cuticular proteins in lepidopteran cell lines. Interference with the synthesis and secretion of these proteins as a novel insecticide target may be possible, however, this approach is not yet been used for screening purposes.

For chitin synthesis, it has been well documented in insects that this is closely associated with specific glycoproteins and the covalent binding of chitin to proteins. In this context, insecticidal lectins can be of great interest to control pest insects. Lectins are proteins of non-immune origin that are able to interact with cells through sugar-specific binding sites (Gatehouse et al. 1995; Peumans and Van Damme 1995; Carlini and Grossi-de-Sa 2002). Using intact insects, toxicity assays have shown that plant lectins can provoke toxic effects or disturb insect development and fecundity, making them putative candidates as insecticidal proteins, however, the mode of action of lectins on insects is still poorly understood. But there is good evidence that plant lectins can interact with the insect midgut, and by doing so, interfere with insect growth. Here, the peritrophic matrix in the insect midgut, in addition to the insect cuticle, is a major target that consists of chitin imbedded in a matrix of (glyco)proteins (Wang and Granados 2001). With use of lepidopteran midgut cell cultures (CF-203), Smagghe et al. (2005) reported on a series of plant lectins with specificity for mannose, galactose and GlcNAc oligomers and tested for their activity on cell growth. Our data showed that the effect of lectins on cells is not correlated with the carbohydrate-binding activity of the lectin. This concurs with previous tests wherein lectins can elicit a variety of biological activities such as mitosis, toxicity, cell-growth inhibition and apoptosis. However, it should be emphasized that fine specificity of different lectins towards oligosaccharides and glycans can be very different even though they interact with the same monosaccharide. To complete these

studies, Vandenborre et al. (2006) used His-tagged lectin under the fluorescence and confocal microscopy for evaluating lectin binding to membrane receptor(s) and/or cellular internalization. The use here of insect midgut cells allows investigating the interaction of lectin with receptor proteins in relation to possible signal transduction pathways that can lead to inhibition of the growth of insect midgut cells.

In summary, several permanent insect cell lines, which are able to synthesize or degrade at least parts of the cuticle are available and may be used for screening purposes. The report of the production of chitin-like material by a continuous insect cell line opened new possibilities for the research on the cuticle system because the preservation of one or more differentiated pathways in continuous cell lines provides an opportunity to analyse and manipulate these pathways (Marks and Ward 1987). This is not possible with living insects. Therefore, continuous insect cell lines in which differentiated functions have been preserved are useful for physiological and biochemical work and become a powerful tool for the study of cuticle formation at the cellular level.

5 Other Insect Targets Related to the Insect Neurological/ Nerve, Energy Metabolism and Muscle System

Cell culture approaches have been made to better understanding the activity of the insect nervous system. For instance Levine and Weeks (1996) investigated the regulation of the dendritic remodelling and programmed death of identified motoneurons in *M. sexta* as a step towards elucidating cellular and molecular mechanisms of effectors of neuronal phenotype. These changes contribute to the dramatic reorganization of behaviour that accompanies metamorphosis. These studies provide strong evidence that *Manduca* motoneurons are direct targets of potential hormone and novel insecticide action, and set the stage for further studies of the specific mechanisms involved.

Decombel et al. (2004) reported on an extensive survey on the biological response of different groups of insecticides towards lepidopteran cell cultures (SeE-CLG4) in vitro. Representative products of different neuroinsecticide classes: AChE inhibitors (parathion, chlorpyrifos), acetylcholine receptor agonists (imidacloprid, spinosad), inhibitors and uncouplers of the oxidative phosphorylation (chlorfenapyr, fenbutatin oxide), site I electron transport inhibitors (fenazaquin, pyridaben), GABA receptor inhibitors (abamectin) and voltage-gated channels affectors (endosulfan, bifenthrin) were used, and cell proliferation as criterion of activity. In few cases a strong insecticidal toxicity was reflected in a strong effect on cell proliferation. Interestingly, a very high activity was scored for chlorfenapyr with an EC_{50} of 0.014 mg/l. Chlorfenapyr, belonging to the group of inhibitors of phosphorylation to disrupt energy transport in the mitochondria, is formulated as a pro-insecticide that needs (in vivo) activation by the oxidative removal of the N-ethylmethyl

group. This activation is mediated by a P450-dependent mono-oxygenase, an enzyme that is necessary for the functioning of the insecticide. The results obtained by Decombel et al. (2004) indicated that this enzyme system is present in the lepidopteran cell line, and therefore the cell cultures can be exploited as a useful model system. In the current experiments with lepidopteran SeE-CLG4 cells (Decombel et al. 2004), the high sensitivity for the acaricide fenazaquin (EC_{50} 0.014 mg/l) and pyridaben (EC_{50} 0.0083 mg/l) was also of great interest for screening and further fundamental research on complex specific enzyme systems. Fenazaquin and pyridaben are member of the new group of METI (mitochondrial energy transport inhibitor) insecticides/acaricides that are reported to act by inhibiting NADH-ubiquinone oxidoreductase (complex 1), and this enzyme is one of the most complicated known with 43 subunits catalyzing electron transfer from NADH to ubiquinone through flavin mononucleotide (FMN) and up to eight iron-sulphur clusters. However, there also existed great variations between in vivo toxicity against intact insects and the in vitro activity against SeE-CLG4 cells for the neurotoxic compounds tested. For instance, the OPs that inhibit AChE, appeared to have only a slight biological activity with SeE-CLG4 cells. However, the low activity in the cell bioassay does not correspond with the highly neurotoxin nature of OPs. This discrepancy can result from the lack of specific (neuro)receptors required for insecticide action in the cell line, but present in the whole organism. However, even when receptors are present, it is not guaranteed to score an effect on cell proliferation. Therefore, if specifically neurotoxins are pursued, electrophysiological and neurochemical approaches should be attained (Zlotkin 1999). For example voltage-clamp analysis reveal delayed and prolonged opening of the voltage-gated channel where radioligand binding assays exposure the affinity of potential insecticides for specific neuroreceptors (Narahashi 1996).

On insecticidal muscle effects, Lynn et al. (1991) reported an a continuous cell line from the hymenopteran egg parasitoid *Trichogramma exiguum* that forms highly contractile muscle-like cells following three days exposure to the insect hormone 20-OH-E. Optimum response results from continuous treatment with 0.1 μg/ml hormone. PAGE analysis indicated that two major proteins were induced: myosin and actin, suggesting the cells to be myoblasts. These observations indicate these cells may provide a significant tool in the study of insect muscle morphogenesis.

6 Insect Cell Lines as Proxies for *Bacillus thuringiensis* Insecticidal Proteins

In order to evaluate the effect of *B. thuringiensis* toxins midgut epithelium has to be cultured. The midgut epithelium of lepidopteran larvae is a complex and dynamic tissue composed of a monolayer of columnar and goblet cells with

stem cells lying along the base of the epithelial cells (Billingsly and Lehane 1996; Smagghe and Tirry 2001). The midgut epithelium is responsible for the digestion, absorption and transport of nutrients and inorganic ions. In addition, the larval midgut epithelium is the target site for the initiation of most viral infections as well as for *B. thuringiensis* insecticidal toxins (Federici 1993). Studies on the development and physiology of the midgut epithelium and on the mode of action of pathogens or biopesticides like *B. thuringiensis* ä-endotoxins are difficult to carry out in vivo. However, significant progress has been made in the preparation of primary cultures of midgut insect stem cells from Lepidoptera in vitro. Such cultures of midgut epithelial cells have been established and maintained in vitro for periods of up to 3–6 months while preserving their differentiated characteristics (Sadrud-Din et al. 1996; Loeb et al. 2000, 2003). The successful application of these primary midgut cell cultures for the study of *Bt* endotoxin binding to the microvilli of intact epithelial cells from different lepidopteran species has been reported (Baines et al. 1997; Wang and McCarthy 1997). In order to pursue interest in insect midgut interaction also with pathogens, continuous cultures of midgut epithelial cells from fifth-instar lepidopteran *Pseudoletia unipuncta* larvae were developed (Garcia et al. 2001). Long term culture and maintenance of the midgut cells were achieved in TNM-FH medium. The columnar cells were round to rectangular in shape and possess a brush border, while the goblet cells have a classic flask-like shape with a central cavity. Attempts were also made with coleopteran cell cultures. Dübendorfer and Liebig (1991) report on primary cells and permanent line from midgut of *L. decemlineata* embryos in view to test the specific toxicity of *B. thuringiensis* var. *tenebrionis* against chrysomelid beetles. In continuation of the latter study, Bellonick et al. (1997) developed four continuous beetle cell lines (Colorado potato beetle; DA1 and DA2 from adult hemolymph, DL1 from larval hemolymph, DO1 from eggs) and screened for toxic effects due to different Bt commercial formulations and mycotoxins such as destruxins by *Metarhizium anisopliae*.

Microscopic observations and electrophysiological studies involving the patch clamp technique and fluorescent probes have been used to investigate the action of ä-endotoxins on insect cells derived from different species and tissues. However as discussed by Gringorten (2001) it should be said that there exists an inherent risk of over-interpreting results from experiments with insect cells and caution should be used in drawing conclusions from them regarding insecticidal mode of action. Continuous cell lines assume morphological and physiological characteristics that can be very different from those of the progenitor tissue from which the primary cultures were prepared, and their response to Bt toxins often does not correlate with that of the insects from which the cells were derived. Even established midgut cell lines bear little resemblance to midgut cells in vivo, and their susceptibility to activate toxins correlates poorly with that of the host insect. Generally they are sensitive to fewer toxins than the host insect. But on the other hand primary midgut cell cultures appear to be susceptible to a broader spectrum of

ä-endotoxins than the host insect, a feature that, as with midgut preparations and brush border membrane vesicles (BBMVs), creates a bias towards overrating insecticide activity from in vitro assays.

An obvious drawback to use insect cell lines for studying toxin effects in vitro and attempting to draw conclusions about mode of action in vivo, has been the inability to reproduce the asymmetric environmental conditions of the midgut cells are exposed to. Namely the steep pH gradient across the epithelium, with the apical surface of the plasma membrane exposed to a highly alkaline medium and the basal surface exposed to a neutral or slightly acidic medium. All experiments with cultured insect cells, including midgut cells, have to be performed at neutral pH conditions to avoid alkaline injury from the solvent alone. Under such conditions, ion channel activity may be quite different than at alkaline pH.

Nonetheless insect cell cultures may be poor indicators of insecticide activity, they have been proven to be useful for the characterisation of the toxin activity spectra and investigation of the membrane permeabilizing effects, particularly in determining pore size (Knowles and Ellar 1987; Potvin et al. 1998; Villanlon et al. 1998). As with the columnar cells in vivo, cultured insect cells respond to toxin injury by swelling and lysis (Himeno 1987; Loeb et al. 2001b). In a pivotal study with CF-1, cells from neonates of the spruce budworm *C. fumiferana*, the cytolytic effect of toxin was studied in the presence of neutral solutes with different hydrodynamic radii and a model for membrane pore formation and colloid osmotic lysis was developed to describe the toxin mechanism of action (Knowles and Ellar 1987). But in some cases the ion channel activity induced in cell lines appears to be less selective than in midgut epithelial cells in vivo. This was exemplified for the permeability to both cations (K^+, Na^+, H^+) and anions (Cl^-) in the fall armyworm Sf-9 cell line (Vachon et al. 1995; Villalon et al. 1998).

7 Suitability of Insect Cell Lines as Sentinels for Environmental Toxicity and Chemistry

Dinan et al. (2001b) reported on an extensive survey with use of the B_{II} bioassay with ecdysteroid-responsive cell line from *D. melanogaster*, for screening of about 80 environmental contaminants including industrial chemicals, pesticides, pharmaceuticals, endocrine disrupting compounds, phyto-estrogens and vertebrate steroids. Xenobiotics possessing an ecdysteroids/hormone agonistic activity bring about a reduction in absorbance relative to control cells, whereas antagonists in the presence of 20-OH-E can be expected to increase the absorbance when compared with wells containing cells treated with the same concentration of 20-OH-E alone. Cytotoxic or cytostatic agents (which would also lower cell density) may be differentiated from ecdysone agonists by examination of the cells in situ by

phase-contrast microscopy; ecdysone agonists specifically induce cellular elongations followed by cell clumping. It should be remarked that also other compounds can induce cellular elongations. Braeckman et al. (1997, 1999) also observed cell elongations after treatment/uptake of cadmium in *Aedes* mosquito cells. Apart from androst-4-ene-3,17-dione, vertebrate steroids were inactive at concentrations up to 1 mM. The vast majority of xenobiotics did not possess an (ant)agonistic activity. Among the industrial chemicals, antagonistic activity was observed for bisphenol A (BPA) (EC_{50} 0.1 mM) and diethylphthalate (DEP) (EC_{50} 2 mM). BPA is an intermediate in the production of polycarbonate and epoxy resins (Staples et al. 1998). DEP is used in pharmaceutical coatings, as a fixative in cosmetics, manufacture of celluloid, solvent for cellulose acetate in the manufacture of varnishes and ropes, denaturation of alcohol, plastic films and as a vehicle for pesticide sprays (Okita and Okita, 1992). Some organochlorine compounds were also characterized by a weak antagonistic activity, including *o,p-′* dichlorodiphenyldichloroethylene (DDE), *p,p′*-DDE, and the commercial insecticides dieldrin and lindane (EC_{50} 30 μM). The only pharmaceutical showing any detectable antagonist activity was 17α-ethynylestradiol (EE), a synthetic estrogen used as a female contraceptive. In the context of recent publications on potential endocrine disruption in marine and freshwater arthropods, these findings suggest that for some compounds (e.g., diethylstilbestrol), ecdysteroid receptor-mediated responses are unlikely to be involved in producing chronic effects. After previous successful experiences with transformed lepidopteran *B. mori* cells (Swevers et al. 2004), we also developed recently in our laboratory a cell based bioassay using *D. melanogaster* S2 cells transfected with a plasmid with an ecdysone-responsive construct (Soin and Smagghe 2006; own unpublished results). The plasmid contains ecdysone-responsive elements followed by an actin promoter and a luciferase reporter gene to quantify the ligand binding by luminescence.

8 Elucidation of Insecticide Resistance Mechanisms Using Insect Cell Lines

As reported above and in literature, the selective toxicity of dibenzoylhydrazine-type ecdysone agonist insecticides is primarily determined by the different binding affinity of ligands to the ecdysteroid receptors, which in turn is due to the difference of the primary sequence of the target receptor site of receptors. The difference in detoxifying ability between species is another factor responsible for the selective toxicity. However, at present any research group has documented on a modified insect ecdysteroid hormone receptor complex in pest insects as resistance process for ecdysone agonists. In the laboratory with in vitro cultured cell lines, Wing (1988) reported that

when *Drosophila* Kc cells were incubated continuously for 4 weeks in either 1 μM 20-OH-E or 100 μM RH-5849, the surviving cell did not respond to either compound by elaborating processes or slowing their proliferation. Both of these resistant populations also showed a dramatically reduced capacity to bind ponasterone A relative to untreated cells. This cross-resistance is compelling evidence that 20-OH-E and RH-5849 act through the ecdysteroid receptor. Also Cherbas and co-workers reported such effects of resistance in *D. melanogaster* cells after continuous exposure (L. Cherbas, 2001, pers. comm.). Similarly, Spindler-Barth and Spindler (1998) reported with cells of another dipteran *Chironomus tentans* after a continuous presence for a period of about 2 years with gradually increasing concentrations of 20E or tebufenozide, that a loss of activity was seen for both compounds. In these resistant subclones tested so far, all hormonally regulated responses that are known from sensitive cells were no longer detectable, assuming that the hormone-signalling pathway itself is interrupted. In the resistant subclones, the ligand binding to the ecdysteroid receptor was clearly affected. In addition, an increase in 20-OH-E metabolism and a reduction in receptor concentration were noted in some clones, which effect was also seen later in another study using imaginal discs of selected cotton leafworm, *Spodoptera littoralis* (Smagghe et al. 2001).

In continuation, Sundaram et al. (1998) reported that accumulation and active exclusion of tebufenozide account resistance in dipteran cells compared to lepidopteran *C. fumiferana* cells. In a further study, the resistant cells excluded actively tebufenozide by ATP-binding cassette (ABC) transporters (Retnakaran et al. 2001). Among various transporters, Pdr5p was responsible for the active exclusion of tebufenozide in yeast. Mutants with the pleiotropic drug resistance (PDR5) deletion can also selectively accumulate halofenozide and methoxyfenozide. Also Grebe et al. (2000) successfully selected clones with defects in ecdysteroid receptor function by treating an epithelial cell line from *C. tentans* with tebufenozide at 0.1 to 0.1 μM. With the use of these insect cell cultures, several types of hormone resistance were distinguished with regard to hormone binding. Also enhanced metabolism of 20-OH-E was associated with hormone resistance in clones of the epithelial cell line from *C. tentans* selected under the continuous pressure of 20-OH-E (Spindler-Barth and Spindler 1998). Recent assays in our own laboratory use ecdysteroid-responsive cells of the beet armyworm (SeE-CLG4) to help in better explaining the mechanisms behind the occurrence of insecticide resistance (Smagghe et al. 1998, 2003; Moulton et al. 2002; Osorio et al. 2006). *S. exigua* is an important lepidopteran pest in agriculture and horticulture causing high damage in vegetables, cotton and ornamentals in the world (CABI, 1972). After culture during 6–9 months under continuous pressure of hormone (20-OH-E) and insecticide (methoxyfenozide), these SeE-CLG4 cells lost their sensitivity against both compounds. Nine clones that showed resistance levels of >500 fold towards 20-OH-E and methoxyfenozide were selected. Current investigations focus on the impact of ecdysteroid receptor

modifications to explain the loss of response to 20-OH-E and methoxyfenozide (Mosallanejad et al. 2006; own unpublished experiments).

Within the IRAC (Insecticide Resistance Action Committee) mode of action classification scheme, one of the major issues in resistance management strategies is the rotation of compounds with different modes of action in order to prevent or delay the rapid development of resistance. Therefore, it is highly desirable to elucidate the mode of action of newer compounds more detailed. In this frame, the effect of the acaricide/insecticide etoxazole was investigated on chitin biosynthesis with epidermal cell cultures of *S. frugiperda* and the incorporation of [^{14}C]N-acetyl-D-glucosamine (Nauen and Smagghe 2006). Based on these results, it could be concluded that etoxazole is an acaricide with a mode of action similar to BPUs, i.e., inhibiting the incorporation of chitin precursor. This supports that no cross-resistance was reported between hexythiazox and etoxazole (Ishida et al. 1994).

In the group of neurotoxic insecticides, the capacity to develop resistance against malathion was investigated with the use of insect cell cultures of the CCE/CC128 cell line, derived from fertilized eggs of the Mediterranean fruit fly, *Ceratitis capitata*, (Rossi and Rainaldi 1997). After 20 cycles of pulse-chase treatment of malathion (48 exposure followed by 48 h recovery), a Mal 90-selected cell population was obtained. DNA content and different enzyme activities were evaluated. The results document that the carboxyesterase activity is involved in Mal 90 cell line resistance and support the idea that the medfly cell line and, more generally, insect cell cultures, could represent a promising system to investigate insecticide resistance mechanisms.

9 Conclusions

This chapter reviewed the utilization of insect cell lines derived from a multitude of insect species. Primarily, the use of insect cell cultures allows determining the biological efficacy and mode of action of insecticides and novel candidates at the cellular level. In addition, cell can be engineered such that they respond to a biological stimulus by the generation of an easily detectable signal in a HTS system for novel bio-active insecticides.

In second, in whole animal tests, the biological activity is a much more complex and other black box situation. The efficacy is modified by uptake, metabolism and excretion. Often differences in the concentration of the insecticide at the target site due to variations in metabolism cause species specific effects, although the action site at the cellular level is the same. Cell lines also provide homogenous material in relatively large amounts and where the selected target sites are directly present for the candidate insecticides. The cell culture tests can be performed independent of the insect developmental stages, its cuticle formation and degradation, and the presence of hormones in the insect body.

In third, compared to whole animal and organ cultures, the use of insect cell cultures makes rapid screening for putative novel insecticides simplified and less time consuming. Another advantage is that permanent cell cultures are easy to discriminate between general cytotoxic effects and interactions with specific target sites. It should however be said here that there exists an inherent risk of over-interpreting results from experiments with insect cells and caution should be used in drawing conclusions from them regarding insecticidal mode of action.

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References

Abo-Elghar GE, Fujiyoshi P, Matsumura F (2004) Significance of the sulfonylurea receptor (SUR) as the target of diflubenzuron in chitin synthesis inhibition in *Drosophila melanogaster* and *Blattella germanica*. Insect Biochem Mol Biol 34:743–752

Akai H, Mauchamp B (1989) Suppressive effects of an imidazole derivative, KK-42 on JH levels in hemolymph of *Bombyx mori* larvae. J Seric Sci Tokyo 58:73–78

Amrani L, Zerguine K, Farine J-P, Smagghe G, Soltani-Mazouni N (2004) Imidazole derivative KK-42 reduces ecdysteroid titers and interferes with reproductive processes in adult females of *Tenebrio molitor*. Pestic Biochem Physiol 80:163–172

Aribi N, Smagghe G, Lakbar S, Soltani-Mazouni N, Soltani N (2006) Effects of pyriproxyfen, a juvenile hormone analog, on development of the mealworm, *Tenebrio molitor*. Pestic Biochem Physiol 84:55–62

Beckmann M, Haack KJ (2003) Chemical pest control – insecticides for agriculture. Chemie in Unserer Zeit 37:88–97

Baines D, Schwartz JL, Sohi SS, Dedes J, Pang A (1997) Comparison of the response of midgut epithelial cells and cell lines from lepidopteran larvae to CryIA toxins from *Bacillus thuringiensis*. J Insect Physiol 43:823–831

Beckers C, Maroy P, Dennis R, O'Connor J, Emmerich H (1980) The uptake and release of ponasterone A by the Kc cell line of *Drosophila melanogaster*. Mol Cell Endocrin 17:51–59

Belloncik S, Allard C, Rouleau D (1991) Adaptation of a *Bombyx mori* cell line to serum-free media. Proc 8th Intl Conf Invert Fish Tissue Culture, Anaheim, CA, USA, 178–180

Belloncik S, Charpentier G, Tian L (1997) Development of four cell lines from the Colorado potato beetle (*Leptinotarsa decemlineata*). In: Maramorosch K, Mitsuhashi J (eds) Invertebrate cell culture. Novel directions and biotechnology applications. Science Publishers, Enfield, NH, USA 3–17

Berger E, Ringler R, Alahiotis S, Frank M (1978) Ecdysone-induced changes in morphology and protein synthesis in *Drosophila* cell cultures. Dev Biol 62:498–511

Berger E, Wyss C (1980) Acetylcholinesterase induction by β-ecdysone in *Drosophila* cell lines and their hybrids. Somatic Cell Genet 6:631–640

Berger EM, Cox G, Ireland R, Weber L (1981) Actin content and synthesis in differentiating *Drosophila* cells in culture. J Insect Physiol 27:129–137

Berridge MV, Tan AS (1993) Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch Biochem Biophys 303:474–478

Billas IML, Moras D (2003) Structural studies of nuclear receptors. In: Voss G, Ramos G (eds) Chemistry of crop protection. Progress and prospects in science and regulation. Wiley-VCH, Weinheim, pp 177–192

Billas IML, Iwema T, Garnier JM, Mitschler A, Rochel N, Moras D (2003) Structural adaptability in the ligand-binding pocket of the ecdysone hormone receptor. Nature 426:91–96

Billingsly PF, Lehane MJ (1996) Structure and ultrastructure of the insect midgut. In: Lehane MJ, Billingsly PF (eds) Biology of the insect midgut. Chapman and Hall, London, UK 31–54

Braeckman B, Simoens C, Rzeznik U, Raes H (1997) Effect of sublethal doses of cadmium, inorganic mercury and methylmercury on the cell morphology of an insect cell line (*Aedes albopictus*, C3/36). Cell Biol Int 12:823–832

Braeckman B, Smagghe G, Brutsaert N, Cornelis R, Raes H (1997) Cadmium uptake and defense mechanism in insect cells. Environ Res 80A:231–243

CABI (1972) Distribution map of pests No 302: *Spodoptera exigua*. CABI, Wallingford, UK

Carlini CR, Grossi-de-Sa MF (2002) Review plant toxic proteins with insecticidal properties. A review on their potential as bioinsecticides. Toxin 40:1515–1539

Cavalloro R (1981) Establishment of continuously in vitro growing cell lines of med fly (*Ceratitis capitata* Wied.). Rev Can Biol 40:181–186

Chang ES (1997) In vitro applications for research in crustacean endocrinology. In: Maramorosch K, Mitsuhashi J (eds) Invertebrate cell culture. Novel directions and biotechnology applications. Science Publishers, Enfield, NH, USA, pp 245–251

Chapman RF (1969) The insects, structure and function. The English Universities Press, London

Cherbas P, Cherbas L, Williams CM (1977) Induction of acetylcholinesterase activity by β-ecdysone in a *Drosophila* cell line. Science 197:275–277

Cherbas L, Yonge CD, Cherbas P, Williams CM (1980) The morphological response of Kc-H cells to ecdysteroids: hormonal specificity. Wilhelm Roux's Arch 189:1–15

Cherbas L, Koehler MD, Cherbas P (1989) Effects of juvenile hormone on the ecdysone response of *Drosophila* Kc cells. Dev Genet 10:177–188

Clément CY, Bradbrook DA, Lafont R (1993) Assessment of a microplate-based bioassay for the detection of ecdysteroid-like or antiecdysteroid activities. Insect Biochem Mol Biol 23:187–193

Cohen E (1981) Acetylcholinesterase activity in an *Aedes aegypti* cell line. Experientia 37:429–431

Cohen E (2001) Chitin synthesis and inhibition: a revisit. Pest Manage Sci 57:946–950

Couderc JL, Cadic AL, Sorbier ML, Dastugue B (1982) Ecdysterone induction of actin synthesis and polymerization in a *Drosophila melanogaster* cultured cell line. Biochem Biophys Res Commun 97:173–181

Courgeon AM (1972) Action of insect hormones at the cellular level. Morphological changes of a diploid cell line of *Drosophila melanogaster*. Exp Cell Res 74:327–336

Decombel L, Smagghe G, Tirry L (2004) Action of major insecticide groups on insect cell lines of the beet armyworm, *Spodoptera exigua*, compared with larvicidal toxicity. In Vitro Cell Dev Biol 40:43–51

Decombel L, Tirry L, Smagghe G (2005) Action of 24-epibrassinolide on cell line of the beet armyworm, *Spodoptera exigua*. Arch Insect Biochem Physiol 58:145–156

Denizoit F, Lang R (1986) Rapid colorimetric assay for cell growth and survival. J Immunol Meth 89:271–277

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

Dhadialla TS, Retnakaran A, Smagghe G (2005) Insect growth and development disrupting insecticides. In: Gilbert LI, Kostas I, Gill S (eds) Comprehensive insect molecular science, vol. 6. Pergamon Press, New York, pp 55–116

Dinan L (1985) Ecdysteroid receptors in a tumorous blood cell line of *Drosophila melanogaster*. Arch Insect Biochem Physiol 2:295–317

Dinan L, Spindler-Barth M, Spiondler K-D (1990) Insect cell lines as tools for studying ecdysteroid action. Invert Reprod Dev 18:45–53

Dinan L, Bourne PC, Meng Y, Sarker SD, Tolentino RB, Whithing P (2001a) Assessment of natural products in the *Drosophila melanogaster* BII cell bioassay for ecdysteroid agonist and antagonist activities. Cell Mol Life Sci 58:321–342

Dinan L, Bourne P, Whiting P, Dhadialla TS, Hutchinson TH (2001b) Screening of environmental contaminants for ecdysteroid agonist and antagonist activity using the *Drosophila melanogaster* B-II cell in vitro assay. Environ Toxicol Chem 20:2038–2046

Dinan L, Whiting P, Bourne P, Coll J (2001c) 8-O-Acetylharpagide is not an ecdysteroid agonist. Insect Biochem Mol Biol 31:1077–1082

Dinan L, Hormann RE (2005) Ecdysteroid agonists and antagonists. In: Gilbert LI, Kostas I, Gill S (eds) Comprehensive insect molecular science, vol. 3. Pergamon Press, New York, pp 197–242

Dübendorfer A, Liebig B (1991) Primary culture of embryonic cells of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae): cell differentiation in vitro and establishment of permanent cell lines. Proc 8th Intl Conf Invert Fish Tissue Culture, Anaheim, CA, pp 178–180

Dübendorfer A, Liebig B (1992) Cell differentiation in vitro and establishment of permanent ecdysone-responsive cell lines from embryonic tissues of the Colorado potato beetle, *Leptinotarsa decemlineata*. J Insect Physiol 38:397–407

Easton CM, Horwath KL (1991) Constitutive and induced antifreeze protein production in fat body cultures derived from the beetle, *Tenebrio molitor*. Proc 8th Intl Conf Invert Fish Tissue Culture, Anaheim, CA, pp 178–180

Eide PE, Caldwell JM, Marks EP (1975) Establishment of two cell lines from embryonic tissue of the tobacco hornworm, *Manduca sexta* (L.). In Vitro 11:395–399

Elbrecht A, Chen Y, Jurgens T, Hensens OD, Zink DL, Beck HT, Balick MJ, Borris R (1996) 8-O-acetyl-harpagide is a nonsteroidal ecdysteroid agonist. Insect Biochem Mol Biol 26:519–523

Ellman G, Courtney KD, Andres V, Featherstone RM (1961) A new a rapid colometric determination of acetylcholinesterase activity. Biochem Pharma 7:88–95

Federici BA (1993) Insecticidal bacterial proteins identify the midgut epithelium as a source of novel target sites for insect control. Arch Insect Biochem Physiol 22:367–371

Ferkovich SM, Oberlander H, Leach CE (1981) Chitin synthesis in larval and pupal epidermis of the Indian mealmoth, *Plodia interpunctella* (Hübner), and the greater wax moth, *Galleria mellonella* (L.). J Insect Physiol 27:509–514

Fretz A, Lübbert A, Markmann U, Spindler-Barth M, Spindler K-D (1993) Effects of 20-hydroxyecdysone on the protein synthesis in the epithelial cell line from *Chironomus tentans*. Insect Biochem Mol Biol 23:159–164

Garcia JJ, Li G, Wang P, Zhong J, Granados RR (2001) Primary and continuous midgut cell cultures from *Pseudaletia unipuncta* (Lepidoptera: Noctuidae). In Vitro Cell Dev Biol 37:353–359

Gatehouse AMR, Powell KS, Van Damme EJM, Peumans WJ (1995) Insecticidal properties of plant lectins their potential inplant protection. In: Pustzai A, Bardocz S (eds) Lectins. Biomedical perspectives. Taylor and Francis, London, pp 35–58

Gilbert LI, Rybczynski R, Warren JT (2002) Control and biochemical nature of the ecdysteroidogenic pathway. Annu Rev Entomol 47:883–916

Goodman WG, Granger NA (2005) The juvenile hormone. In: Gilbert LI, Kostas I, Gill S (eds) Comprehensive insect molecular science, vol. 3. Pergamon Press, New York, pp 319–408

Goodwin RH, Adams JR (1980) Nutrient factors influencing viral replication in serum-free insect cell culture. In: Kurstak E, Maramorosch K, Dübendorfer A (eds) Invertebrate systems in vitro. Elsevier, Amsterdam, pp 493–509

Grace TDC (1962) Establishment of four strains of cells from insect tissues grown in vitro. Nature 195:788–789

Grace TDC (1967) Establishment of a line of cells from the silkworm *Bombyx mori*. Nature 216:613

Grebe M, Rauch P, Spindler-barth M (2000) Characterization of subclones of the epithelial cell line from *Chironomus tentans* resistant to the insecticide RH 5992, a non-steroidal moulting hormone agonist. Insect Biochem Mol Biol 30:591–600

Gringorten JL (2001) Ion balance in the lepidopteran midgut and insecticidal action of *Bacillus thuringiensis*. In: Ishaaya I (ed) Biochemical sites of insecticide action and resistance. Springer, Berlin Heidelberg New York, pp 167–207

Gu SH, Chow Y-S (2005) Analysis of ecdysteroidogenic activity of the prothoracic glands during the last larval instar of the silkworm, *Bombyx mori*. Arch Insect Biochem Physiol 58:17–26

Heeg K, Reimann J, Kabelitz D, Hardt C, Wagner H (1985) A rapid colorimetric assay for the determination of IL-2-producing helper T-cell frequencies. J Immunol Meth 77:237–246

Henrich V (2005) The ecdysteroid receptor. In: Gilbert LI, Kostas I, Gill S (eds) Comprehensive insect molecular science, vol. 3. Pergamon Press, New York, pp 243–285

Hetru C, Roussel J-P, Mori K, Nakatani Y (1986) Activité antiecdystéroïde de brassinostéroïdes. CR Acad Sci Paris 302:417–420

Hink WF (1972) A catalog of invertebrate cell lines. In: Vago C (ed) Invertebrate tissue culture. Academic Press, New York, pp 363–387

Hink WF (1976) The second compilation of insect cell lines and culture media. In: Maramorosch K (ed) Invertebrate tissue culture research applications. Academic Press, New York, pp 19–369

Hink WF (1980) The 1979 compilations of invertebrate cell lines and culture media. In: Kurstak E, Maramorosch K, Dübendorfer A (eds) Invertebrate systems in vitro. Elsevier, Amsterdam, pp 533–578

Hink WF, Bezanson DR (1985) Invertebrate cell culture media and cell lines. In: Kurstak E (ed) Techniques in the life science, vol. C1. Elsevier, County Clare, Ireland, 1–30

Hink WF, Hall RI (1989) Recently established invertebrate cell lines. In: Mitsuhashi J (ed) Invertebrate cell system applications, vol. 2. CRC Press, Boca Raton, FL, pp 269–293

Hoffmann KH, Gerstenlauer B (1997) Effects of ovariectomy and allatectomy on ecdysteroid synthesis and ecdysteroid titers during larval-adult development of *Gryllus bimaculatus* de Geer (Ensifera: Gryllidae). Arch Insect Biochem Physiol 35:149–158

Homma K-I, Natori S (1997) Growth factors of *Sarcophaga peregrine*. In: Maramorosch K, Mitsuhashi J (eds) Invertebrate cell culture. Novel directions and biotechnology applications. Science Publishers, Enfield, NH, USA, pp 69–76

Hopkins TL, Kramer KJ (1992) Insect cuticle sclerotization. Annu Rev Entomol 37:273–302

Hornsby PJ, McAllister JM (1991) Culturing steroidogenic cells. Meth Enzymol 206:371–380

Hsu AC-T, Fujimoto T, Dhadialla TS (1997) Structure activity study and conformational analysis of tebufenozide, the first commercialized non-steroidal ecdysone agonist. In: Hedin P, Hollingworth R, Miyamoto J, Aster E, Thompson D (eds) Phytochemical pest control agents. ACS Symp Ser, Washington, DC, 658 pp 206–219

Ishida T, Suzuki J, Tsukidate Y, Mori Y (1994) YI-5301, a novel oxazoline acaricide. Proc Brighton Crop Prot Conf: Pest Diseases 1:37–44

Jarvis TD, Early FGP, Rees HH (1994) Inhibition of the ecdysteroid biosynthetic pathway in ovarian follicle cells of *Locusta migratoria*. Pestic Biochem Physiol 48:153–162

Kadano-Okuda K, Amornsak W, Yamashita O (1994) Controlled ecdysteroid accumulation in eggs of silkworm, *Bombyx mori*, by an imidazole compound (KK-42), and embryogenesis in these eggs. Arch Insect Biochem Physiol 25:121–135

Kadano-Okuda K, Kuwano E, Eto M, Yamashita O (1987) Inhibitory action of an imidazole compound on ecdysone synthesis in prothoracic glands of the silkworm, *Bombyx mori*. Dev Growth Differ 29:527–533

Kelly TJ, Masler EP, Thyagaraja BS, Bell RA, Imberski RB (1992) Development of an in vitro assay for prothoracicotropic hormone of the gypsy moth, *Lymantria dispar* (L.) following studies on identification, titers and synthesis of ecdysteroids in last-instar females. J Comp Physiol 162B:581–587

Kiuchi M, Kimura K, Akai H (1985) Induction of trimolters from a tetramolter strain of *Bombyx mori* by anti-juvenoid treatment. J Seric Sci Jpn 54:77–80

Kiuchi M, Akai H (1988) In vivo and in vitro inhibition of prothoracic gland activity by 1-benzyl-5-[(E)-2,6-dimethyl-1,5-heptadienyl] imidazole (KK-42) in *Bombyx mori*. In: Kuroda Y, Kurstak E, Maramorosch K (eds) Invertebrate and fish tissue culture. Japan Sci Soc Press, Tokyo, Japan, 60–63

Knowles BH, Ellar DJ (1987) Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* α-endotoxin with different insect specificity. Biochim Biophys Acta 924:509–518

Kuwano E, Takeya R, Eto M (1983) Terpenoid imidazoles: new anti-juvenile hormones. Agric Biol Chem 47:921–924

Kuwano E, Hisano T, Eto M, Suzuki K, Unnithan GC, Bowers WS (1992) Insect growth regulating activity of 1,5-disubstituted imidazole against *Bombyx mori* and *Oncopeltus fasciatus*. Pestic Sci 34:263–268

Lafont R (2000) Understanding insect endocrine systems: molecular approaches. Entomol Exp Appl 97:123–136

Lan Q, Gerenday A, Fallon AM (1993) Cultured *Aedes albopictus* mosquito cells synthesize hormone-inducible proteins. In Vitro Cell Dev Biol 29A:813–818

Lehmann M, Vorbrodt H-M, Adam G, Koolman J (1988) Antiecdysteroid activity of brassinosteroids. Experientia 44:355–356

Lenoir-Rousseaux JJ, Gautron J (1987) Activity, localization and molecular-form of acetylcholinesterase in the male accessory glands of metamorphosing of *Tenebrio molitor* L. Insect Biochem 17:739–750

Lezzi M, Wyss C (1976) The antagonism between juvenile hormone and ecdysone. In: Gilbert LI (ed) The juvenile hormones. Plenum Publishing, New York, pp 252–269

Levine RB, Weeks JC (1996) Cell culture approaches to understanding the actions of steroid hormones on the insect nervous system. Dev Neurosci 18:73–86

Loeb MJ, Kochansky J, Wagner RM, Woods CW (1998) Structure-function analysis of *Lymantria* testis ecdysiotropin: a search for the active core. Arch Insect Biochem Physiol 38:11–18

Loeb MJ, Vaughn JL, Clark EA (2000) Primary cultures of midgut cells from *Heliothis virescens* can be frozen and stored. In Vitro Cell Dev Biol Anim 36:7–10

Loeb MJ, De Loof A, Gelman DB, Hakim RS, Jaffe H, Kochansky JP, Meola SM, Schoofs L, Steel C, Vafopoulou, Wagner RM, Woods CW (2001a) Testis ecdysiotropin, an insect gonadotropin that induces synthesis of ecdysteroid. Arch Insect Biochem Physiol 47:181–188

Loeb MJ, Phyllis AW, Narang N, Hakim RS, Goto S, Takeda M (2001b) Control of life, death, and differentiation in cultured midgut cells of the lepidopteran, *Heliothis virescens*. In Vitro Cell Dev Biol 37:348–352

Loeb MJ, Clack EA, Blackburn M, Hakim RS, Elsen K, Smagghe G (2003) Stem cells from midgut of lepidopteran larvae: clues to the regulation of stem cell fate. Arch Insect Biochem Physiol 53:186–198

Loh PC, Tapay LM, Lu Y (1997) Quantal assay of shrimp viruses in primary shrimp lymphoid cell cultures. In: Maramorosch K, Mitsuhashi J (eds) Invertebrate cell culture. Novel directions and biotechnology applications. Science Publishers, Enfield, NH, USA, pp 253–259

Londershausen M, Spindler-Barth M, Kammann V, Spindler K-D, Thomas H (1987) Insect cell lines as models for study of insecticides interfering with cuticle formation. Zentralbl Bakteriol Mikro Hyg A 267:302

Londershausen M, Turberg A, Buss U, Spindler-Barth M, Spindler K-D (1993) Comparison of chitin synthesis from an insect cell line and embryonic tick tissues. In: Muzarelli RAA (ed) Chitin enzymology. European Chitin Society, Acona, Italy 75–82

Long SH, McIntosh AH, Grasela JJ, Goodman CL (2002) The establishment of a Colorado potato beetle (Coleoptera: Chrysomelidae) pupal cell line. Appl Entomol Zool 37:447–450

Lorenz J, Lent M, Hoffmann KH (1985) Effects of pharmacological agents on ecdysteroid synthesis in vitro in ovaries and abdominal integument from female adult crickets, *Gryllus bimaculatus*. Z Naturforsch 50C:286–293

Lynn DE, Oberlander H (1983) The establishment of cell lines from imaginal wing discs of *Spodoptera frugiperda* and *Plodia interpunctella*. J Insect Physiol 29:591–596
Lynn DE, Guzo D, Guthrie K, Dougherty EM (1999) Morphogenesis of a hymenopteran cell line into highly contractile cells in response to 20-hydroxyecdysone. Proc 8th Intl Conf Invert Fish Tissue Culture, Anaheim, CA, USA, pp 178–180
Lynn DE (2001) Novel techniques to establish new insect cell lines. In Vitro Cell Dev Biol Anim 37:319–321
Lynn DE, Goodman C, Caputo G (2005) Techniques for the development of new insect cell lines. Proc 2005 Annual Meet Soc In Vitro Biology, Baltimore, MD, USA
Maeda S (1989) *Bombyx mori* nuclear polyhedrosis virus and their use for expression of foreign genes in insect cells. In: Mitsuhashi J (ed) Invertebrate cell system applications, vol. 2. CRC Press, Boca Raton, FL, USA, pp 167–181
Marks EP, Ward GP (1987) Cell culture techniques for studying insect cuticle. Arch Insect Biochem Physiol 6:217–225
Marks EP, Holman GM (1979) Ecdysone action on insect cell lines. In Vitro 15:300–307
McMorris TC, Patil PA (1993) Improved synthesis of 24-epibrassinolide from ergosterol. J Org Chem 58:2338–2339
McCearth KJ, Gooday GW (1992) A rapid and sensitive microassay for determination of chitinolytic activity. J Microbiol Meth 14:229–237
Metakovskii EV, Cherdantseva EM, Gvozdev VA (1977) Action of ecdysterone on surface membrane glycoproteins of *Drosophila melanogaster* cells in culture. Mol Biol 11:158–170
Mikitani K (1995) Sensitive, rapid and simple method for evaluation of ecdysteroid agonist activity based on the mode of action of the hormone. J Seric Sci Jpn 64:534–539
Mikitani K (1996a) A new nonsteroidal chemical class of ligand for the ecdysteroid receptor 3,5-di-tert-butyl-4hydroxy-N-isobutyl-benzamide shows apparent insect molting hormone activities at molecular and cellular levels. Biochem Biophys Res Commun 227:427–432
Mikitani K (1996b) Ecdysteroid receptor binding activity and ecdysteroid agonist activity at the level of gene expression are correlated with the activity of dibenzoylhydrazines in larvae of *Bombyx mori*. J Insect Physiol 42:937–941
Mikólajczyk P, Oberlander H, Silhacek DL, Ishaaya I, Shaaya E (1994) Chitin synthesis in *Spodoptera frugiperda* wing imaginal discs. I. Chlorfluazuron, diflubenzuron, and teflubenzuron inhibit incorporation but not uptake of [^{14}C]-N-acetyl-D-glucosamine. Arch Insect Biochem Physiol 25:245–258
Minakuchi C, Nakagawa Y, Miyagawa H (2003) Validity analysis of a receptor binding assay for ecdysone agonists using cultured intact insect cells. J Pestic Sci 28:55–57
Mitushashi J (1965) In vitro cultivation of the embryonic tissues of the green rice leafhopper, *Nephotettix cincticeps* Uhler (Homoptera: Cicadelidae). Jpn J Appl Entomol Zool 9:107–118
Morganelli CM, Berger EM (1986) Effects of 20-ecdysone on *Drosophila* cells: regulation of endogenous and transfected genes. Insect Biochem 16:233–240
Mosallanejad H, Soin T, Smagghe G (2006) In vitro effects of 20-hydroxyecdysone and ecdysone agonists on *Spodoptera exigua* cell line. Abstracts 58th Intern Symp Crop Protect, Ghent, Belgium
Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival. J Immunol Meth 65:55–63
Mulford AL, Villena AJ (2000) Cell cultures from crustaceans: shrimps, crabs and crayfish. In: Mothersill C, Austin B (eds) Aquatic invertebrate cell culture. Springer-Praxis, Chichester, UK, pp 63–134
Moulton JK, Pepper DA, Jansson RK, Dennehy TJ (2002) Pro-active management of beet armyworm (Lepidoptera: Noctuidae) resistance to tebufenozide and methoxyfenozide: baseline monitoring, risk assessment, and isolation of resistance. J Econ Entomol 95:414–424
Nagata T, Goldbach R, Peters D (1997) Towards the establishment of a semi-continuous cell line of thrips and application of primary cell cultures to study tomato spotted wilt virus replication. In: Maramorosch K, Mitsuhashi J (eds) Invertebrate cell culture. Novel directions and biotechnology applications. Science Publishers, Enfield, NH, USA, pp 25–31

Nakagawa Y, Sotomatsu T, Irie K, Kitahara K, Iwamura H, Fujita T (1987) Quantitative structure-activity studies of benzoylphenylurea larvicides. III. Effects of substituents at the benzoyl moiety. Pestic Biochem Physiol 27:143–155

Nakagawa Y, Matsutani M, Kurihara N, Nishimura K, Fujita T (1989) Quantitative structure-activity studies of benzoylphenylurea larvicides. VIII. Inhibition of N-acetylglucosamine incorporation into the cultured integument of *Chilo suppressalis* Walker. Pestic Biochem Physiol 43:141–151

Nakagawa Y, Matsutani M, Kurihara N, Nishimura K, Fujita T (1992) Inhibition of N-acetylglucosamine incorporation into the cultured integument of *Chilo suppressalis* by diflubenzuron. Pestic Biochem Physiol 42:242–247

Nakagawa Y, Matsumura F, Hashino Y (1993) Effect of diflubenzuron on incorporation of [^{3}H]-N-acetylglucosamine ([^{3}H]-NAGA]) into chitin in the intact integument from the newly molted American cockroach, *Periplaneta americana*. Comp Biochem Physiol 106C:711–415

Nakagawa Y, Minakuchi C, Ueno T (2000) Inhibition of [^{3}H]ponasterone A binding by ecdysone agonists in the intact Sf-9 cell line. Steroids 65:537–542

Nakagawa Y, Minakuchi C, Takahashi K, Ueno T (2002) Inhibition of [^{3}H]ponasterone A binding by ecdysone agonists in the intact Kc cell line. Insect Biochem Mol Biol 32:175–180

Nakagawa Y (2005) Nonsteroidal ecdysone agonists. Vitam Horm 73:131–173

Narahashi T (1996) Neuronal ion channels as target sites of insecticides. Pharmacol Toxicol 78:1–14

Nauen R, Bretschneider T (2002) New modes of action of insecticides. Pestic Outlook 241:241–245

Nauen R, Smagghe G (2006) Mode of action of etoxazole. Pest Manage Sci 62:379–382

Nijhout HF (1994) Insect hormones. Princeton University Press, Princeton, NJ, USA

Oberlander H, Fulco L (1967) Growth and partial metamorphosis of imaginal disks of the greater wax moth, *Galleria mellonella*, in vitro. Nature 216:1140–1141

Oberlander H (1989) Insect cell lines as models for the study of biorational pesticides. In Vitro Cell Dev Biol Anim 25:18A

Oberlander H, Silhacek DL, Leach CE, Ishaaya I, Shaaya E (1991) Benzoylphenylureas inhibit chitin synthesis without interfering with amino sugar uptake in imaginal wing discs of *Plodia interpunctella*. Arch Insect Biochem Physiol 18:219–227

Oberlander H, Silhacek DL (1998) New perspectives on the mode of action of benzoylphenylurea insecticides. In: Ishaaya I, Degheele D (eds) Insecticides with novel modes of action. Springer, Berlin Heidelberg New York, pp 92–105

Oberlander H, Smagghe G (2001) Imaginal discs and tissue cultures as targets for insecticidal action. In: Ishaaya I (ed) Biochemical sites of insecticide action and resistance. Springer, Berlin Heidelberg New York, pp 133–150

Oettling G, Schmidt H, Drews U (1985) The muscarine receptor of chick-embryo cells, correlation between ligand binding and calcium mobilization. J Cell Biol 100:1073–1081

Ogura T, Minakuchi C, Nakagawa Y, Smagghe G, Miyagawa H (2005) Molecular cloning, expression analysis and functional confirmation of ecdysone receptor and ultraspiracle from the Colorado potato beetle *Leptinotarsa decemlineata*. FEBS J 272:4114–4128

Okita RT, Okita JR (1992) Effects of diethylphthalate and other plasticizers on laurate hydroxylation in rat-liver microsomes. Pharm Res 9:1648–1653

Osorio A, Schneider MI, Martínez AM, Smagghe G, Díaz O, Corrales JL, Avilés M, Pineda S (2006) Selection for resistance to spinosad and methoxyfenozide in the beet armyworm, *Spodoptera exigua*. Abstracts 58th Intern Symp Crop Protect, Ghent, Belgium

Palli SR, Sohi SS, Cook BJ, Lambert D, Ladd TR, Retnakaran A (1995) Analysis of ecdysteroid action in *Malacosoma disstria* cells: cloning selected regions of E75- and MHR3-like genes. Insect Biochem Molec Biol 25:697–707

Palli SR, Caputo GF, Brownwright AJ, Sofi SS (1997a) Studies on apoptosis in a continuous midgut cell line, CF-203, of the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae). In: Maramorosch K, Mitsuhashi J (eds) Invertebrate cell culture. Novel directions and biotechnology applications. Science Publishers, Enfield, NH, USA, pp 43–51

Palli SR, Sohi SS, Cook BJ, Primavera M, Retnakaran A (1997b) Screening of 12 continuous cell lines for apoptosis. In: Maramorosch K, Mitsuhashi J (eds) Invertebrate cell culture. Novel directions and biotechnology applications. Science Publishers, Enfield, NH, USA, pp 53–60

Palli SR, Retnakaran A (1999) Molecular and biochemical aspects of chitin synthesis inhibition. In: Jollés P, Muzzarelli RAA (eds) Chitin and chitinases. Birkhäuser Publishing Ltd, Basel, pp 85–98

Palli SR, Retnakaran A (2001) Ecdysteroid and juvenile hormone receptors: properties and importance in developing novel insecticides. In: Ishaaya I (ed) Biochemical sites of insecticide action and resistance. Springer, Berlin Heidelberg New York, pp 107–132

Palli SR, Hormann RE, Schlattner U, Lezzi M (2005a) Ecdysteroid receptors and their applications in agriculture and medicine. Vitam Horm 73:59–100

Palli SR, Tice CM, Margam VM, Clark AM (2005b) Biochemical mode of action and differential activity of new ecdysone agonists against mosquitoes and moths. Arch Insect Biochem Physiol 58:234–242

Peel DJ, Milner MJ (1992) The response of *Drosophila* imaginal disc cell lines to ecdysteroids. Roux's Arch Dev Biol 202:23–35

Peronnet F, Ziarczyk P, Rollet E, Courgeon AM, Becker J, Maisonhaute C, Echalier G, Best-Belpomme M (1989) *Drosophila* cell lines as a model for studying the mechanisms of ecdysteroid action. In: Koolman J (ed) Ecdysone: from chemistry to mode of action. Georg Thieme-Verlag, Stutgart, pp 378–383

Peters D, Black LM (1971) Techniques for the cultivation of cells of the aphid *Acyrthosiphon pisum* in primary cell cultures. Tag Ber Dt Akad Landwirtsch Wiss, Berlin 115:129–139

Peumans WJ, Van Damme EJM (1995) Lectins as plant defence proteins. Plant Physiol 109:347–352

Porcheron P, Oberlander H, Leach CE (1988) Ecdysteroid regulation of amino sugar uptake in a lepidopteran cell line derived from imaginal discs. Arch Insect Bicohem Physiol 7:145–155

Potvin L, Laprade R, Schwartz JL (1998) Cry1Ac, a *Bacillus thuringiensis* toxin, triggers extracellular Ca^{2+} influx ad Ca^{2+} release from intracellular stores in Cf1 cells (*Choristoneura fumiferana*, Lepidoptera). J Exp Biol 201:1851–1858

Quack S, Fretz A, Spindler-Barth M, Spindler K-D (1995) Receptor affinities and biological responses of non-steroidal ecdysteroid agonists on the epithelial cell line from *Chironomus tentans* (Diptera: Chironomidae). Eur J Entomol 92:341–347

Ress C, Maas U, Dorn A (1997) The *Drosophila* tumorous blood cell line l(2)mbn and its response to insect hormones, hormone agonists, and the natural growth regulators azadirachton and plumbaginoids. In: Maramorosch K, Mitsuhashi J (eds) Invertebrate cell culture. Novel directions and biotechnology applications. Science Publishers, Enfield, NH, USA, pp 93–103

Retnakaran A, Granett J, Ennis T (1985) Insect growth regulators. In: Kerkut GA, Gilbert LI (eds) Comprehensive insect physiology, biochemistry and pharmacology, vol. 12. Pergamon Press, New York, pp 529–601

Retnakaran A, MacDonald A (1988) Biosynthesis and deposition of chitin in insects and interference with this system as a means of control. In: Sehnal F, Zabza A, Denlinger DL (eds) Endocrinological frontiers on physiological insect ecology. Wroclaw Technical University Press, Wroclaw, Poland, pp 547–565

Retnakaran A, Palli SR, Tomkins WL, Primavera M, Brownwright A, Gill SK (1997) Chitin-protein complex system in insects. In: Domard A, Roberts GAF, Varum KM (eds) Advances in chitin science, vol. 2. Jacques Andres Publisher, Lyon, France, pp 110–118

Retnakaran A, Gelbic I, Sundaram M, Tomkins W, Ladd T, Primavera M, Feng Q, Arif B, Palli SR, Krell P (2001) Mode of action of the ecdysone agonist tebufenozide (RH-5992), and an exclusion mechanism to explain resistance to it. Pest Manage Sci 57:951–957

Richter K, Koolman J (1991) Antiecdysteroid effects of brassinosteroids in insects. In: Cultler HG, Yokota T, Adam G (eds) Brassinosteroids: chemistry, bioactivity and applications. ACS Symp Ser, Washington, DC, 474:231–245

Riddiford LM, Hiruma K, Lan Q, Zhou BH (1999) Regulation and role of nuclear receptors during larval molting and metamorphosis of Lepidoptera. Am Zool 39:736–746

Rosenberry TL, Scoggin DM (1984) Structure of human erythrocyte acetylcholinesterase characterization of intersubunit disulfide bonding and detergent interaction. J Biol Chem 259:5643–5652

Rossi E, Rainaldi G (1997) Malathion resistance in CCE/CC128 cell line of Mediterranean fruit fly *Ceratitis capitata* (Diptera: Tephritidae). In: Maramorosch K, Mitsuhashi J (eds) Invertebrate cell culture. Novel directions and biotechnology applications. Science Publishers, Enfield, NH, USA, pp 61–66

Sadrud-Din SY, Loeb MJ, Hakim RS (1996) In vitro differentiation of isolated stem cells from the midgut of *Manduca sexta* larvae. J Exp Biol 199:319–325

Schmutterer H (ed) (1995) The neem tree. Wiley VCH, Weinheim, Germany

Shiotsuki T, Yukuhiro F, Kiuchi M, Kuwano E (1999) Effect of 1-(4-phenoxyphenoxypropyl) imidazole (KS-175) on larval growth in the silkworm *Bombyx mori*. J Insect Physiol 45:1049–1055

Sikarov JL, Duval N, Anselmet A, Bon S, Krejci E, Legay C, Osterlund M, Reimund B, Massoulie J (1988) Complex alternative splicing of acetylcholinesterase transcripts in Torpedo electric organ; primary structure of the precursor of the glycolipid-anchored dimeric form. EMBO J 7:2983–2993

Silhacek DL, Oberlander H, Porcheron P (1990) Action of RH-5849, a non-steroidal ecdysteroid mimic, on *Plodia interpunctella* (Hübner) in vivo and in vitro. Arch Insect Biochem Physiol 15:201–212

Smagghe G, Eelen H, Verschelde E, Richter K, Degheele D (1996) Differential effects of nonsteroidal ecdysteroid agonists in Coleoptera and Lepidoptera: analysis of evagination and receptor binding in imaginal discs. Insect Biochem Mol Biol 26:687–695

Smagghe G, Dhadialla TS, Derycke S, Tirry L, Degheele D (1998) Action of the ecdysteroid agonist tebufenozide in susceptible and artificially selected beet armyworm. Pestic Sci 54:27–34

Smagghe G, Nakagawa Y, Carton B, Mourad AK, Fujita T, Tirry L (1999) Comparative ecdysteroid action of ring-substituted dibenzoylhydrazines in *Spodoptera exigua*. Arch Insect Biochem Physiol 41:42–53

Smagghe G, Tirry L (2001) Insect midgut as a site for insecticide detoxification and resistance. In: Ishaaya I (ed) Biochemical sites of insecticide action and resistance. Springer, Berlin Heidelberg New York, pp 293–321

Smagghe G, Carton B, Decombel L, Tirry L (2001) Significance of absorption, oxidation, and binding to toxicity of four ecdysone agonists in multi-resistant cotton leafworm. Arch Insect Biochem Physiol 46:127–139

Smagghe G, Decombel L, Carton B, Voigt B, Adam G, Tirry L (2002) Action of brassinosteroids in the cotton leafworm *Spodoptera littoralis*. Insect Biochem Mol Biol 32:199–204

Smagghe G, Dhadialla TS, Lezzi M (2002) Comparative toxicity and ecdysone receptor affinity of non-steroidal ecdysone agonists and 20-hydroxyecdysone in *Chironomus tentans*. Insect Biochem Mol Biol 32:187–192

Smagghe G, Braeckman BP, Huys N, Raes H (2003) Cultured mosquito cells *Aedes albopictus* C6/36 (Dip., Culicidae) responsive to 20-hydroxyecdysone and non-steroidal ecdysone agonist. J Appl Ent 127:167–173

Smagghe G, Pineda S, Carton B, Del Estal P, Budia F, Viñuela E (2003) Toxicity and kinetics for methoxyfenozide in greenhouse-selected *Spodoptera exigua* (Lepidoptera: Noctuidae). Pest Manage Sci 59:1203–1209

Smagghe G, Ryckaert J, Soin T, Caputo G, Van Damme EJM (2005) Effect of plant lectins on growth of insect midgut cells. In Vitro cell Dev Bio 41:34A

Smith HC, Cavanaugh CK, Friz JL, Thompson CS, Saggers JA, Michelotti EI, Garcia J, Tice CM (2003) Synthesis and SAR of cis-1-benzoyl-1,2,3,4-tetrahydroquinoline ligands for control of gene expression in ecdysone responsive systems. Bioorg Med Chem Lett 13:1943–1946

Sobek L, Böhm GA, Penzlin H (1993) Ecdysteroid receptors in last instar larvae of the wax moth *Galleria mellonella* L. Insect Biochem Mol Biol 23:125–129

Sohi SS (1995) Development of lepidopteran cell lines. In: Richardson CD (ed) Methods in molecular biology: baculovirus expression protocols. Humana Press Inc., Totowa, NY, USA, pp 397–411

Sohi SS, Palli SR, Cook BJ, Retnakaran A (1995) Forest insect cell lines responsive to 20-hydroxyecdysone and two nonsteroidal ecdysone agonists, RH-5849 and RH-5992. J Insect Physiol 41:457–464

Soltani N, Quennedey A, Delbecque JP, Delachambre J (1987) Diflubenzuron-induced alterations during in vitro development of *Tenebrio molitor* pupal development. Arch Insect Biochem Physiol 5:201–209

Soltani N, Smagghe G, Soltani-Mazouni N (1998) Evaluation of the ecdysteroids agonist RH-0345 on the hormonal production by integument explants and ovaries in mealworms. Med Landbouww Univ Gent 63(2):547–554

Spindler-Barth M, Schmidt H, Drews U, Spindler K-D (1988) Increase in activity of acetylcholinesterase by 20E-ecdysone in a *Chrinomus tentans* cell line. Roux's Arch Dev Biol 197:366–369

Spindler-Barth M, Spindler K-D, Londershausen M, Thomas H (1989) Inhibition of chitin synthesis in an insect cell line. Pestic Sci 25:115–121

Spindler-Barth M (1991) Hormonal regulation of acetylcholinesterase in an epithelial cell line from *Chironomus tentans*. Z Naturforsch 46c:1089–1093

Spindler-Barth M, Turberg A, Spindler K-D (1991) On the action of RH-5849, a nonsteroidal ecdysteroid agonist, on a cell line from *Chironomus tentans*. Arch Insect Biochem Physiol 16:11–18

Spindler-Barth M (1993) Hormonal regulation of chitin metabolism in insect cell lines. In: Muzarelli RAA (ed) Chitin enzymology. European Chitin Society, Acona, Italy, pp 75–82

Spindler-Barth M, Spindler K-D (1998) Ecdysteroid resistant subclones of the epithelial cell line from *Chironomus tentans* (Insecta, Diptera). I. Selection and characterization of resistant clones. In Vitro Cell Dev Biol Anim 34:116–122

Spindler K-D, Spindler-Barth M, Londershausen M (1990) Chitin metabolism: a target for drugs against parasites. Parasitol Res 76:283–288

Spindler K-D, Spindler-Barth M, Turberg A (1991) Action of brassinosteroids on the epithelial cell line from *Chironomus tentans*. Z Naturforsch 47c:280–284

Spindler K-D, Quack S, Spindler-Barth M (1993) Insect cell lines as tools for insecticide screening. Trends in Comparat Biochem Physiol 1:1045–1056

Stiles B, Newman SM (1992) Evidence for the induction of cuticle proteins by 20-hydroxyecdysone in 2 established insect cell lines. Arch Insect Biochem Physiol 21:23–40

Soin T, Smagghe G (2006) Endocrine disruption in aquatic insects: a review. Ecotoxicology (in press)

Staples CA, Dorn PB, Klecka GM, O'Block ST, Harris LR (1998) A review of the environmental fate, effects and exposures of bispenol A. Chemosphere 36:2149–2173

Sundaram M, Palli SR, Krell PJ, Sohi SS, Dhadialla TS, Retnakaran A (1998) Basis for selective action of a synthetic moulting hormone agonist, RH-5992 on lepidopteran insects. Insect Biochem Mol Biol 28:693–704

Suzuki J, Ishida T, Kikuchi Y, Ito Y, Morikawa C, Tsukidate Y, Tanji I, Ota Y, Toda K (2002) Synthesis and activity of novel acaricidal/insecticidal 2,4-diphenyl-1,3-oxazolines. J Pestic Sci 27:1–8

Swevers L, Kravariti L, Ciolfi S, Xenou-Kokoletsi M, Wong G, Ragousis N, Smagghe G, Nakagawa Y, Mazomenos V, Iatrou K (2004) A high-throughput screening system for fast detection of ecdysteroid mimetic and antagonistic substances using transformed *Bombyx mori*-derived cell lines. FASEB J 18:134–136

Tice CM, Hormann RE, Thompson CS, Fritz JL, Cavanaugh CK, Michelotti EL, Garcia J, Nicolas Z, Alberico F (2003) Synthesis and SAR of alpha-acylaminoletone ligands for control of gene expression. Bioorg Med Chem Lett 13:475–478

Toya T, Fukasawa H, Masui A, Endo Y (2002) Potent and selective partial ecdysone agonist activity of chromafenozide in Sf9 cells. Biochem Biophys Res Commun 292:1087–1091
Trisyono A, Goodman CL, Grasela JJ, McIntosh AH, Chippendale GM (2000) Establishment and characterization of an *Ostrinia nubilalis* cell line, and its response to ecdysone agonists. In Vitro Cell Dev Biol 36:400–404
Vandenborre G, Soin T, Jacobsen L, Caputo G, Van Damme EJM, Smagghe G (2006) Analysis of Nictaba in insect midgut cells. In Vitro Cell Dev Biol 42A:33
Vaughn JL, Goodwin RH, Tompkins GJ, McCawley P (1977) The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera: Noctuidae). In Vitro 13:213–217
Vaughn JL, Fan F (1989) Use of commercial serum replacements for the culture of insect cells. In Vitro Cell Dev Biol Anim 25:143–145
Vaughn JL, Weiss SA (1991) Formulating media for the culture of insect cells. BioPharm 4:16–19
Voight B, Whiting P, Dinan L (2001) The ecdysteroid agonist/antagonist and brassinosteroid-like activities of synthetic brassinosteroid/ecdysteroid hybrid molecules. Cell Mol Life Sci 58:1133–1140
Vachon V, Paradis MJ, Marsolais M, Schwartz, Laprade R (1995) Ionic permeabilities induced by insecticidal toxins of *Bacillus thuringiensis* in Sf9 cells. J Membr Biol 148:57–63
Wang P, McCarthy WJ (1997) Cytolytic activity of *Bacillus thuringiensis* Cry1C and Cry1AC toxins to *Spodoptera* sp. midgut epithelial cells in vitro. In Vitro cell Dev Biol 33:315–323
Wang P, Granados RR (2001) Molecular structure of the peritrophic membrane (PM): identification of potential PM target sites for insect control. Arch Insect Biochem Physiol 47:110–118
Ward GB, Newman SM, Klosterman HJ, Marks EP (1988) Effect of 20-hydroxyecdysone and diflubenzuron on chitin production by a cockroach cell line. In Vitro cell dev Biol Anim 24:326–332
Wheelock CE, Nakagawa Y, Harada T, Oikawa N, Akamatsu M, Smagghe G, Stefanou D, Iatrou K, Swevers L (2006) High throughput screening of ecdysone agonists using a reporter gene assay followed by 3-D QSAR analysis of the molting hormonal activity. Bioorg Med Chem 14: 1143–1159
Williams CM (1967) Third generation pesticides. Sci Am 217:13–17
Van Loocke K, Nakagawa Y, Watanabe B, Swevers L, Iatrou K, Geelen D, Reheul D, Smagghe G (2006a) Structure-activity relationship of brassinosteroids (BRs) and BR-hybrids in ecdysteroid signaling in a lepidopteran (Bm5) and dipteran (S2) cell line. Abstracts 16th Intern Ecdysone Workshop, Ghent, Belgium
Van Loocke K, Swevers L, Iatrou K, Smagghe G (2006b) Inhibitory effect of the ecdysteroid signaling by the juvenile hormone analogues, pryriproxifen, kinoprene and fenoxycarb, in a dipteran (S2) and lepidopteran (Bm5) cell line. Abstracts 16th Intern Ecdysone Workshop, Ghent, Belgium
Villanlon M, Vachon V, Brosseau R, Schwartz JL, Laprade R (1998) Video imaging analysis of the plasma membrane permeabilizing effects of *Bacillus thuringiensis* insecticidal toxins in Sf9 cells. Biochim Biophys Acta 1368:27–34
Wilson AP (1986) Cytotoxicity and viability assays. In: Freshney RI (ed) Animal cell culture: a practical approach. IRL Press, Washington, DC, pp 183–216
Wing KD (1988) RH 5849, a non-steroidal ecdysone agonist: effects on a *Drosophila* cell line. Science 241:467–469
Wing KD, Slawecki R, Carlson GR (1988) RH-5849, a nonsteroidal ecdysone agonist: effects on larval Lepidoptera. Science 241:470–472
Wyss C (1976) Juvenile hormone analog counteracts growth stimulation and inhibition by ecdysones in clonal *Drosophila* cell line. Experientia 32:1272–1274
Wyss C (1982) *Chironomus tentans* epithelial cell lines sensitive to ecdysteroids, juvenile hormone, insulin and heat shock. Exp Cell Res 139:309–319
Zlotkin E (1999) The insect voltage-gated sodium channel as target of insecticides. Annu Rev Entomol 44:429–455