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Editors

Insecticides Design Using Advanced Technologies

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With 62 Figures, 9 in Color, and 32 Tables

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Preface

Insecticide development in recent years has been guided mostly by chemorational and biorational design based on understanding of the physiology and ecology of insects and crops. Among the recently developed groups of insecticides are the chitin synthesis inhibitors, benzoylphenyl urea's and buprofezin; the juvenile hormone mimics, fenoxycarb and pyriproxyfen; the ecdysone agonists, e.g. tebufenozide and methoxyfenozide; and the new neurotoxicants, the neonicotinoids and the avermectins. In addition, compounds affecting specific sites in insects have been developed; such as pymetrozine acting on the sucking pump of aphids and whiteflies, diafenthiuron affecting insect respiration and azadirachtin the hormonal balance of insects.

A limitation in each new class of compounds is the evolution of resistance in populations of key pests, which leads to control failures. This phenomenon and the desire to produce more selective and biorational compounds serve as the driving forces for insecticide design that ultimately lead to the development of new compounds.

Among the highlights of this book are the use of nanotechnology to increase potency of available insecticides, use of genetic engineering techniques for controlling insect pests, development of novel insecticides that bind to unique biochemical receptors, and exploration of natural products as a source for environmentally acceptable insecticides. In addition, screening for safe and potent insecticides using insect genomics and cell lines for determining biological and biochemical modes of action are discussed in detail in this book.

The authors of the various chapters are considered world leaders having a wealth of experience in pesticide chemistry and in advanced technologies for designing highly selective insecticides for controlling insect pests.

This book is intended to serve as a text for researchers, university professors, graduate students, and chemical industry personnel involved in developing new groups of insecticides to suit our future requirements.

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1 Nanosuspensions: Emerging Novel Agrochemical Formulations

YOEL SASSON, GANIT LEVY-RUSO, OFER TOLEDANO, ISAAC ISHAAYA

1 Introduction

Whereas nanotechnology and nanoparticle engineering has become ubiquitous in the pharmaceutical research and development arena starting as early as the mid-70s (Rosen and Abribat 2005), it has, quite surprisingly, only recently made its way to the field of agrochemical formulations and delivery systems. Contemporary reviews on pesticide formulations do not refer to dispersed systems in the submicron size range (Rodham 2000; Mulqueen 2003). The 24th Symposium on Pesticide Formulation and Delivery Systems, held in Tampa on October 2003 (Goss et al. 2005), did not have a single presentation on this topic. Even an updated cover story on agrochemical R&D in Chemical and Engineering News (Short 2005), which calls attention to the strong similarities between current agro and pharma research and development, also fails to indicate the great potential in nanoparticulate formulations as novel pesticide-delivery systems. In view of the prediction that "within 10 years nanomaterials will directly affect about half of all human health-related products" (Roco 2001), we strongly believe that a comparable transformation and paradigm shift will also take place in agricultural products and services.

The main drive for nanosizing of drugs, or drug carrier particles, stems from aqueous insolubility, which has become a widespread hurdle in pharmaceutical formulations (predominantly of biopharmaceuticals). It was estimated that close to 60% (Merisko-Liversidge 2002) of the new chemical entities currently in the discovery stage, primarily using high throughput screening practice, are poorly water soluble (solubility below 0.1 mg/l). The problem is far more severe for drugs that are insoluble both in aqueous and organic media (Lipinski 2002). No data exist for the new agrochemical molecules but we can safely assume that the same dilemma exists there. In general, molecules with a poor solubility show, concurrently, also a very slow dissolution rates. The performance of poorly soluble drugs is limited by the dissolution rate, and consequently they exhibit an erratic adsorption profile leading to poor and highly variable bioavailability, which is strongly dependant on experimental conditions (mainly fed-fasted state of the patient). Numerous drug candidates failed to reach commercialization due to solubility problems (Lipinski 2002) as it was realized that it is more expeditious and cost effective to redesign a molecular structure than to move a flawed material through

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the development process. Nanosizing of drug particles offers improved solubility but also has the potential for direct in vivo multiple absorption pathways including paracellular and transcellular activities (Kidane and Bhatt 2005). Nanoparticles were also assessed as potential targeted systems (Müller and Keck 2004), including in cancer therapy (Brannon-Peppas and Blanchette 2004; Jain 2005). An excellent demonstration for the advantageous efficacy of a nanoformulation in comparison to other delivery systems is given by Kayser et al. (2003) using the antiparasitic drug amphotericin B. In this study, the nanosuspended drug by far outperforms the liposomal and micronized formulas. A list of other hydrophobic drugs which have been successfully nanosized is given by Date and Patravale (2004) and by Patravale et al. (2004) (also see Rainbow 2004). Nanoparticles as dry powder or aqueous nanosuspension are also highly suitable for aerosol delivery to the lung (Sham et al. 2004) and for transdermal administration (Ceve 2004).

Nanosizing is not utterly new in medicinal science (see early review by Oppenheim 1980). Formulators of parenteral therapies have been preparing colloidal drug carriers since the 1970s. Typical examples are submicron emulsions and microemulsions, nanospheres, nanocapsules, liposomes and lipid or cyclodextrin complexes. The first report on synthetic nanoparticles was made by Speiser and coworkers from ETH in Zurich back in the mid-70s (Kopf et al. 1976). These authors described the preparation of polyacrylamide submicron beads via emulsion polymerization in hexane-water biphasic system initiated by gamma irradiation. The obtained particles were reportedly amorphous, nonhydrated and spheroidal with a diameter of approximately 100 nm and a surface area of 10 m^2/g. A 4% aqueous dispersion of the particles showed Newtonian flow properties with a viscosity of 3.52 centipoise at 20 °C. Surprisingly, this paper was cited only 12 times, and all prior to 1984.

Despite being a brilliant and innovative idea, the concept of polymeric nanoparticles as drug carriers has never made it to the pharmaceutical market, mainly due to regulatory limitations and some scale-up difficulties. Interestingly, unlike nanosized beads, microparticles of polylactic acid and of polylactic polyglycolic copolymer are accepted for parenteral administrations. It was argued that biodegradation of nanoparticles, which can freely circulate in the blood stream including the smallest capillaries (see Spenlehauer et al. 1997) and are internalized by cells, can cause cytotoxic effects. Another obstacle has been the low drug payload and the lack of cost-effective large-scale production methods. Research in this area still continues (Couvreur et al. 2002; Bialti et al. 2005). More advanced molecular scaffolds such as dendrimers (Aulenta et al. 2003; Gillies and Frechet 2005), fullerenes, nanotubes (Dennis et al. 2004) or nanowires, which are newcomers as drug carriers, are still in the research stage (Martin and Kohli 2004; Kaiser et al. 2005). Conversely, liposomal formulations did enter the market with several successful cosmetic and pharmaceutical controlled-release products, and several others are in the clinical phases. Nonetheless, major obstacles for faster market penetration of liposome-based formulations are limited physical

stability of the dispersions, drug leakage, low and non-specific activity, difficulties in upscaling and high production cost. The further fascinating potential of both polymeric nanospheres and liposomes for site specific delivery (drug targeting) was also never materialized in practice.

Another nanoformulation methodology (introduced in the mid-1990s) is the concept of solid lipid nanoparticles (SLN) including the improved versions of nanostructured lipid carriers (NLC) and lipid drug conjugate (LDC) (Müller et al. 2000). The three versions are all based on particles with a solid lipid matrix with an average diameter in the nanometer range. The particles are composed of lipids (typically triglycerides) that are solid at ambient up to the body's temperature and stabilized by surfactants. Various drugs can be trapped within the solid template. SLN exhibit excellent physical stability, protection of the incorporated labile drug from degradation, controlled drug release, good tolerability and potential site specific targeting. The main drawback of the SLN concept is that it is not fitting for organic non-soluble active ingredients. Other shortcomings are insubstantial loading capacity, potential drug expulsion after polymorphic transition during storage, and the relatively high water content of the dispersions. Despite numerous publications and patents issued for various SLN drug formulations (see a comprehensive list in Müller et al. 2000), no commercial product was yet announced (with the exception of the cosmetic formulation Nanobase by Yamanouchi introduced in Poland (see http://www.nanobase.pl/01_nanobase.html).

The state-of-the-art approach in drug delivery, which is also most pertinent for drugs that are poorly soluble both in aqueous and organic media, is based on the simple and straightforward concept of a carrier free nanosuspension of stabilized nanometer-sized drug particles. Typical trademarks in this field are Nanocrystal from Elan and Dissocubes from DDS. A comprehensive list of nanosuspension-based drug formulations in development and on the market is given by Rabinow (2004). We strongly believe that this methodology is the most significant to agrochemistry due to low production cost and minimal environmental effect. Nanosuspensions consisting of essentially pure active ingredients combined with minimal quantities of surface stabilizing agents may enable high payload with low toxicity and environmental impact (due to total exclusion of solvents). A comparison of technical and economical feasibility of various nanoscale delivery methods is presented by Date and Patravale (2004).

2 Solubility Enhancement Through Nanoization

As cited above, the factor of poor solubility is a major drawback for drugs and drug candidates. Downsizing of a drug particle, particularly to the submicron level, boosts bioavailability due to simultaneous enhancement of both the saturation solubility C_S and the dissolution rate dC/dt. This is described as follows:

The saturation solubility increases with decreasing particle size according to the Ostwald-Freundlich equation (Eq. 1) (also known as the Gibbs-Thomson and as the Kelvin equation):

$$\frac{S(d)}{S_0} = \exp\frac{\gamma V_m}{RTd} \quad (1)$$

where S(d) is the solubility (mol/kg H_2O) of crystals with inscribed diameter d(m) at temperature T(K), molar volume V_m (m^3/mol), surface free energy (surface tension) γ(mJ/m^2). R is the gas constant (8314.5 mJ/mol K). S_0 is the solubility of the bulk material ($d\rightarrow\infty$). With all other factors kept constant, the solubility increases with smaller particle size. However, for the solubility S(d) to differ significantly from the solubility S_0 of the bulk material (i.e., the ratio $S(d)/S_0 >> 1$), the exponential term needs to be much smaller than 1. This occurs only with a particle size in the nano range. This aforementioned phenomenon is another demonstration for the transformation of the physiochemical properties of materials on the nanoscale.

A nifty demonstration for the significance of the Ostwald-Freundlich equation is shown as follows. The relevant physical properties of three minerals (quartz, gypsum, and pyrite) are summarized in Table 1. In Fig. 1, the solubility as a function of the particle size is calculated. It is apparent that for materials with high surface energy (such as pyrite), the change in solubility for a particle size below 100 nm is three orders of magnitude (!).

A related graph was presented by Kipp (2004) who calculated the change in solubility of a hypothetical drug particle (molecular weight of 708, an interfacial surface tension of 50, 75, or 100 dyn/cm and density of 1 g/cm^3) as a function of the particle diameter.

Another reason for the increase in S(d) was postulated by Müller and Peters (1998) who suggested that upon nanoization, lipophilic surfaces from the inner part of the crystal are exposed to the aqueous dispersion medium. This will alter the surface tension γ consequently affecting the saturation solubility. The change in packing density (V_m) and differences in interfacial energy (γ) are the reasons for the variation in S(d) of different polymorphic forms. Methods of morphological manipulations to enhance a drug's solubility are reviewed by Shefter (1981) (see also the recent manuscript by Huang and Tong (2004)). Dissimilarity in solubility between pure enantiomeric crystals and racemic solids was also attributed to increased packing density of the latter

Table 1. Physical properties of minerals

Name	Formula	γ/mJ/m^2	M_w/g/mol	ρ/g/cm^3	V_m/m^3/mol
Quartz	SiO_2	350	60.085	2.649	22.68×10^{-6}
Gypsum	$CaSO_4 \times 2H_2O$	26	172.17	2.32	74.21×10^{-6}
Pyrite	FeS_2	4,733	119.98	5.02	23.90×10^{-6}

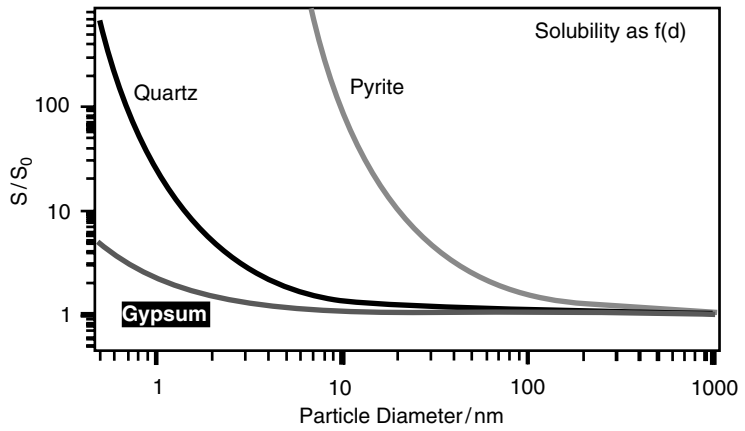

Fig. 1. Saturation solubility as function of particle size of minerals

("Wallach's rule", Brock et al. 1991). In general, amorphous particles are more soluble and encompass a higher dissolution rate (see later) in comparison with crystalline particles of the same size.

The Ostwald-Freundlich equation is also one of the main reasons for the phenomenon of crystal coarsening in suspension known the Ostwald ripening (see next chapter). The theory correlating particle size with saturation solubility has been challenged by several authors (Wu and Nancollas 1998; Tang and Nancollas 2002) but it is still widely cited in the pharmaceutical literature. In a unique experimental work, Müller and Peters (1998) have demonstrated that the saturation concentration of the drug RMPK 22 is indeed dependant on the particle size of the preparation. Thus, for suspension with a mean diameter of 2.4 μm, the saturation solubility was measured to be under 2 mg/ml. For nanosuspensions with a mean diameter of 800 and 300 nm, the measured saturation solubility was 3.2 and 3.6 mg/ml, respectively.

The nanoization process of a particle in a given dispersed system, until complete solubility, is realized in association with an increase in the positive surface energy in relation to negative volume energy, which makes the solution more energetically favorable (Van der Gun et al. 2001). The free energy change for dissolution (or nucleation) developed by Gibbs and Volmer is the sum of the free energy change for the formation of the nucleus surface ΔG_S and the free energy for the phase transition ΔG_V (a volume effect). For a spherical nucleus we have: (Eq. 2)

$$\Delta G = \Delta G_S + \Delta G_V = 4\pi r^2 \gamma + 4/3\pi r^3 \Delta G_V \quad (2)$$

where r is the nucleus radius. A free energy change as a function of r is shown in Fig. 2.

The two free energy terms ΔG_S and ΔG_V have opposite signs so that ΔG as a function of r passes through a maximum which corresponds to the critical

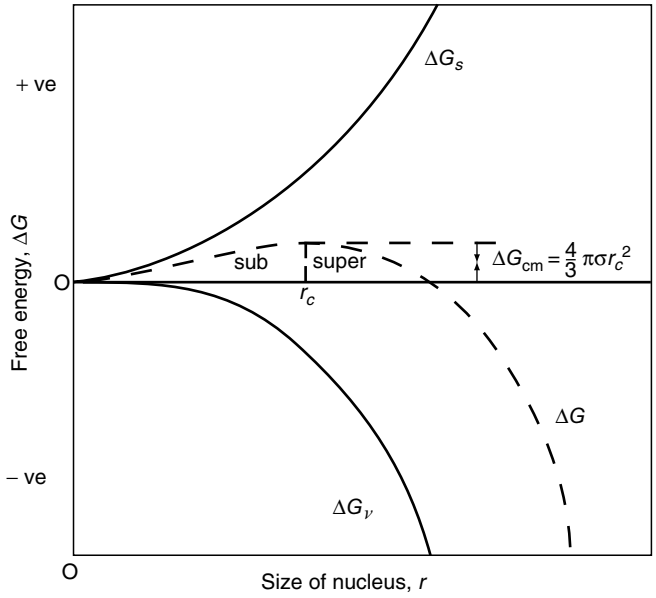

Fig. 2. Change of free energy with particle size

nucleus radius r_c. Particles with radius smaller than r_c dissolve while particles $r > r_c$ grow further.

The dissolution rate (dC/dt) is directly proportional to the surface area and to the concentration gradient. This is determined by the Noyes-Whitney equation (Eq. 3):

$$\frac{dC}{dt} = \frac{DA\left(C_S - C_B\right)}{h} \tag{3}$$

where C is concentration (mole/liter), D is the diffusion coefficient of the drug, h is the effective diffusion boundary layer, A is the effective surface area, C_S is the saturation solubility of the drug (equivalent to S in Eq. 1) and C_B is the bulk concentration of the drug. Since upon nanoization C_S and A mutually increase, the effect on the dissolution rate, and consequently on bioavailability, is substantial. Drug nanocrystals are therefore considered a smart delivery system, a universal unique principle which can be applied to any AI because any solid bioactive material can (in principle) be nanonized.

The effect of particle size on bioavailability of a bioactive molecule was demonstrated by Rieger and Horn (2001). Figure 3 presents the blood level of β carotene as a function of time after oral administration for uptake of doses with different particle size. It is apparent that the microparticles are practically inert.

An exciting development concerning the dissolution rate as a function of particle size was pioneered by Cooper and Ruddy (2005). These inventors

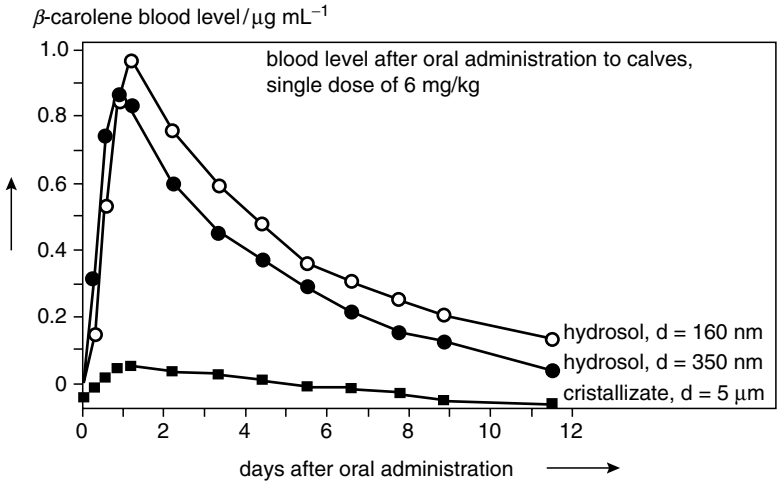

Fig. 3. Bioavailability of β-carotene as function of particle size (reproduced with permission from Rieger and Horn 2001, courtesy of Wiley Interscience)

have demonstrated that by controlling the particle size of a given drug and by prudently mixing particles of different sizes into one dose, one can rationally conceive the pharmacokinetic profiles of a given medication. Thus compositions having a combination of immediate-release and controlled-release characteristics could be designed.

The remarkable progress made in the solubility enhancement of poorly soluble drugs led Rabinow (2004) to forecast that "an era will emerge in which soluble drugs will be intentionally converted to insoluble complexes to take advantage of the benefits conferred by nanosuspension drug delivery".

3 Stabilization of Nanosuspensions

Formation of nanoparticles creates considerable potential energy due to the large new interface area between the solid particles and the contiguous medium. The Gibbs free energy change for suspension system at constant temperature and pressure is expressed by $dG = \gamma\, d\sigma$ where γ is the surface tension and $d\sigma$ is the change in surface area. dG is thus negative for $d\sigma < 0$. Consequently, suspensions are thermodynamically unstable and will eventually coalesce. It is the role of the formulator to find ways to control the macroscopic behavior of a given dispersed system by controlling its microscopic properties. Thus, the enhancement of the kinetic nonlability (or lability) of nanosuspensions is achieved by tailoring the particle–particle interactions via electrostatic or steric means. A major source of kinetic stability of colloids is the existence of an electric charge on the surface of the particles that

causes repulsion between them. One should discriminate between the immobile rigid layer of ions that stick tightly to the surface of the particle (and may include water molecules) and the outer layer of oppositely charged mobile ions. The combined system is called the electrical double layer. The electric potential on the particle, relative to its value at the medium, is called the electrokinetic potential or the zeta potential ζ. The repulsion forces between the nanoparticles thus inhibit the kinetics of the agglomeration process and, accordingly, increase the stability of the suspension. Aggregation or stabilization may also be tailored by adsorption of polymers on the particle surface. This may either influence the surface charge or function as a steric barrier avoiding close contact between the particles. The formulation developer explores and intensifies these repulsion forces to achieve a sustainable suspension. In practice, this is usually achieved empirically by the addition of surface active agents that are adsorbed to the solid–liquid interface and reduce the surface tension (and the driving force for coalescence). In addition, ionic amphiphiles provide an electrostatic barrier to particle agglomeration and non-ionic surfactants (particularly polymeric glycol ethers, e.g., poloxamers and other polymers) create a hydration zone—a protective layer of strongly bound water molecules around each particle (Kipp 2004). The latter were also claimed to render the nanoparticles more mucoadhesive or bioadhesive, particularly in the gastrointestinal tract (Liversidge et al. 2002). The synergic stabilizing effect of polymeric amphiphiles and ionic surface active agent such as dioctyl sodium sulfosuccinate (DSS) was demonstrated by Ryde and Ruddy (2002, 2003). Amphiphilic amino acid copolymers were recently proposed as novel stabilizers for nanocrystal dispersions (Lee et al. 2005).

The stabilizing effect of hydroxypropyl methylcellulose (HPMC) in 0.025% aqueous solution on nanoparticles of itraconazole was demonstrated by Rasenack and Müller (2002). Figure 4 shows the particle-size distribution of the drug (prepared by the solvent shift method, see below) in the presence and in the absence of HPMC stabilizer. The average particle size is 30 μm in the absence of the stabilizer and 600 nm in its presence.

The difference in dissolution rate of the nanosized itraconazole (after drying) in comparison with common crystals is evident (Fig. 5).

Rasenack and Müller (2002) have also demonstrated that the obtained average particle size strongly depends on the nature of the protective hydrophilic polymer with cellulose ethers performing the best.

It was argued (Müller and Keck 2004) that the stabilizing amphiphilic agents indeed establish another barrier to agglomeration but may also catalyze some degradation (hydrolysis and/or oxidation) of the outer monolayer of molecules thus protecting the inner part of the drug nanocrystal (similar to protective oxidized layer on top of aluminum).

Polymeric surfactants such as polyethylene glycol and polypropylene glycol or block copolymers thereof are effective stabilizers due to multiple attachments of hydrophobic sites on the particle surface. It was claimed that

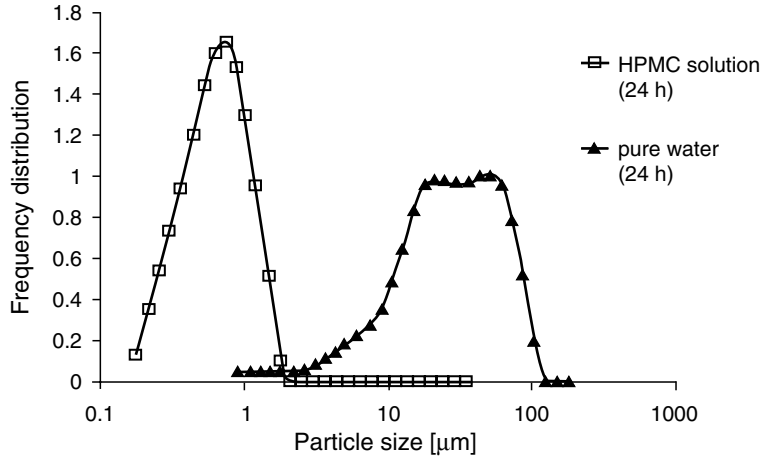

Fig. 4. Particle distribution of itraconazole 24 h after precipitation; pure water and HPMC solution (Rasenack and Müller 2002, reproduced with permission, courtesy of Springer Netherlands)

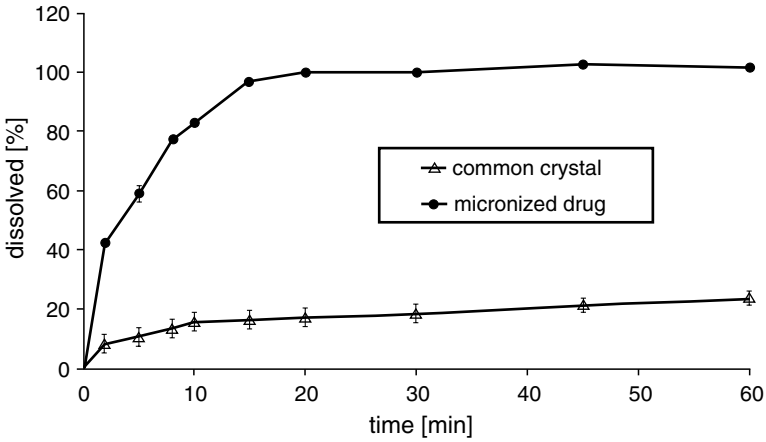

Fig. 5. Dissolution rate of itraconazole (Rasenack and Müller 2002, reproduced with permission, courtesy of Springer Netherlands)

detachment of these moieties at ambient temperature is highly unfavorable due to entropic reasons (Alexandridis and Hatton 1995). The stabilizing role of the latter is also steric: the pendant hydrophilic domains expand into the aqueous phase thus creating a barrier to aggregation. Chari et al. (1999) demonstrated that optimal stabilization of a nanodispersed organic dye could be attained only by a combination of additives: ionic

surfactant—sodium dodecyl sulfate (SDS) and an amphiphilic polymer—polyvinylpyrrolidone (PVP). None of the two functioned alone. These authors verified that the short-chain surfactants function as a filler to fill vacancies on the surface covered with the macromolecular surfactant and thus provide a defect-free monolayer that offers better protection against aggregation. The synergic effect between ionic and non-ionic polymeric surfactants stems also from the low solubility of polymers such as glycol copolymers at higher temperatures leading to particle aggregation. The ionic surfactants thus function also as cloud-point modifiers that raise the cloud-point temperature and improve stability at higher temperatures (Na and Rajagopalan 1994). Typical surfactant combinations are polysorbates (Tweens), poloxamines, poloxamers and PVP, as non-ionic and SDS, sodium dioctyl sulfosuccinate and sodium lauryl sulfate as ionic amphiphiles (Kipp 2004; Frank et al. 1998). A comprehensive list of surface modifiers is provided by Cooper and Ruddy (2005). The ability to control particle size by selecting the proper stabilizing agents led to the surprising discovery reported by Liversidge et al. (2001) that advocated an optimal particle size for highest stability. While the obvious anticipation is that the smaller the particle size the less stable the suspension would be, these authors found that nanoparticulate composition with a size range of 150–350 nm was much more resistant to crystal growth and particle aggregation than either compositions with particle size below 100 nm and compositions with particle size above 400 nm. The latter remarkable phenomenon was observed both under heating (sterilization) and during prolonged storage periods. This phenomenon has yet to be rationalized.

Figure 6 visualizes the nature of the stabilized suspension with the surface modified nanoparticles that are rapidly formed by nucleation but are resistant to crystal growth and agglomeration.

The balance between attractive and repulsive forces among particles in suspension has been quantitatively visualized by the classical DLVO (Derjaguin, Landau, Verwey, Overbeek) theory. This hypothesis assumes that there is a balance between the repulsive interaction between the electrically charged particles and the attractive interactions arising from van der Walls forces between the particles. The potential energy arising from the repulsion forces between the electric double layers on particles of radius r has the form (Eq. 4):

$$V_{\text{repulsion}} = \frac{Ar^2\xi^2}{R}\exp\left(s/r_D\right) \tag{4}$$

where A is a constant, ζ is zeta potential, R is the separation of the centers, s is the separation of surfaces (s=R – 2r for spherical particles of radius r) and r_D is the thickness of the double layer. This expression is applicable when the electric double layer is relatively thick ($r << r_D$). When the double layer is thin ($r_D << r$) the equation gets the form: (Eq. 5)

$$V_{\text{repulsion}} = 0.5\,Ar\xi^2\ln\left[1+\exp\left(-s/r_D\right)\right] \tag{5}$$

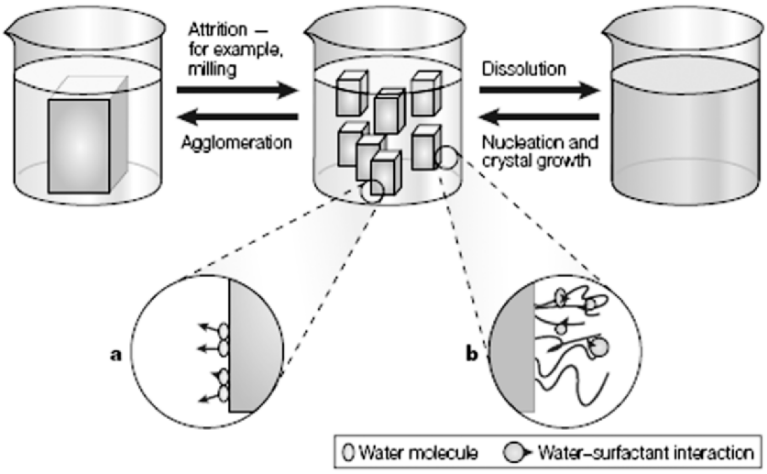

Fig. 6. Illustration of a stabilized (b) and non-stabilized nanosuspension (a) (reproduced with permission from Rabinow 2004, courtesy of Nature Publishing Group)

The potential energy resulting from attractive van der Walls interactions has the form (Eq. 6):

$$V_{\text{repulsion}} = \frac{A}{6}\left(\frac{2r^2}{s^2+4rs} + \frac{2r^2}{s^2+4rs+4r^2} + \ln\left(\frac{s^2+4rs}{s^2+4rs+4r^2}\right)\right) \qquad (6)$$

where A is the Hamaker constant. With superposition of the two opposing forces as a function of the interparticle distance, one obtains the net potential energy curve with the general form shown in Fig. 6.

The thickness of the double layer is a major component in strongly affecting the shape of the function in Fig. 7. The latter is dependant on the ionic strength of the system and on the presence of surface-modifying agents and/or adsorbed polymers. Under conditions where $r_D << r$ (thin double layer), typically obtained by increasing the ionic strength, the function shows a secondary minimum at (relatively) large separation, typical to particles in the micron size range. Aggregation of the particles arising from the stabilizing effect of this secondary minimum is termed "flocculation". Flocculated material can often be redispersed by simple agitation because the well (and the energy barrier) is normally very shallow. An irreversible aggregation of small particles into large aggregates (called coagulation) takes place when the distance between the particles is small and they enter the primary minimum of the potential energy curve where the van der Walls forces are governing. Discrepancies are occasionally observed between the classical DLVO theory and experimental results particularly at high ionic strength (Behrnes et al. 2000). The kinetics of the aggregation (and breakage) processes determining the stability of dispersed systems is a challenging theoretical and experimental problem and was treated using various mechanistic models (Schwarzer

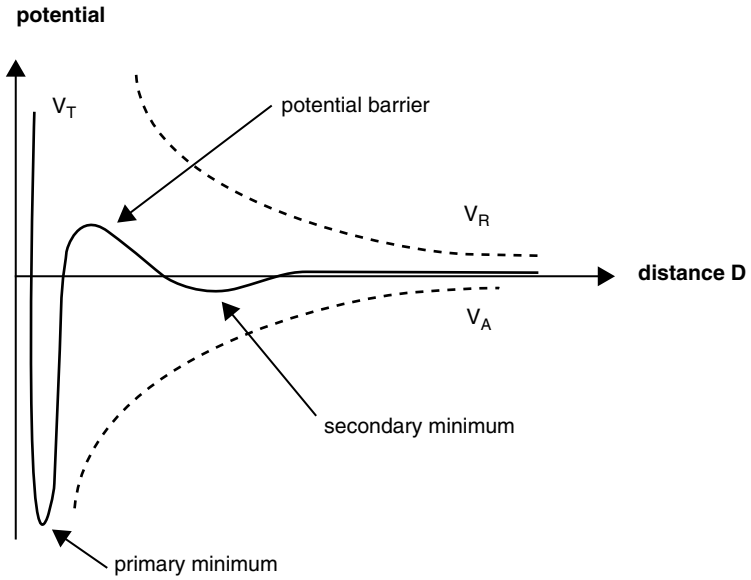

Fig. 7. DLVO theory

and Peukert 2005; Peukert et al. 2005; Biligily et al. 2005; Sommer et al. 2005). Of particular interest is the DLVO theory applied to living cells either between themselves (Chang and Chang 2002) or with relation to external surfaces (Sharp and Dickinson 2005).

An important phenomenon critical to the stability and shelf-life of nanosuspensions is the Ostwald ripening process. When crystals are dispersed in their own saturated solution, the smaller crystals tend to dissolve, and the resulting solute is then deposited on the larger crystals leading to growth of the latter. The driving force for ripening is the difference in solubility between small and large crystals as described above. Theoretically, the ripening process would not occur if all the particles were exactly the same size. As expected, the effect becomes significant only for crystals with a size <1 μm. The rate at which ripening occurs depends on the crystal size and the solubility and can be either diffusion-controlled or surface-integration-controlled (Madras and McCoy 2002; Gratz 1999 and 1997). Ripening changes the particle size distribution of a dispersed system over a period of time. Typically, nanosuspensions with homogeneous size distribution combined with low a priori solubility are less vulnerable to ripening (Müller et al. 2004). Drugs with solubility higher than 0.05 mg/l are susceptible to ripening (Lindfors 2004b). Since the ripening process requires mobility of the particles, one way to impede it is to increase the viscosity of the medium. Thickening additives such as alkyl polyglycosides can be used for that aim (Fowles et al. 1999). Researchers at Astra-Zeneca have recently introduced the concept of "Ostwald ripening inhibitors". Materials that are active in this capacity

should be totally non-soluble in water and are typically middle-chain triglycerides (Hedberg et al. 2003; Lindfors 2004a, 2005b). The Ostwald ripening also establishes a certain critical particle radius that determines which particles will grow and which will dissolve (Finsy 2004).

Figure 8 is a graph of the (mean particle diameter)^3 (nm^3) against time (minutes) for particles of felodipine prepared with and without the use of a ripening inhibitor (Miglyol 812 N) (Hedberg et al. 2003). The open circles in figure 8 represent the felodipine particles prepared with the inhibitor (Miglyol 812 N, a medium-chain triglyceride) and the solid circles felodipine particles prepared without an inhibitor. It is apparent that the presence of the inhibitor eliminated Ostwald ripening in the felodipine particles and the particle size remains constant, whereas the felodipine particles prepared without an inhibitor grew rapidly with time.

An additional method to improve stability of suspensions in prolonged storage is via complete removal of the aqueous medium by lyophilization and stocking the nanoparticles in dry lyophilized form. Dialysis is generally required before lyophilization (Kipp 2004). A more cost-effective method was recently proposed by Kipp et al. (2003) who advocated plain freezing of the nanoformulations. Other typical additives to solid or liquid nanoformulations are standard pharmaceutical excipients such as binding agents, filling agents, lubricating agents, suspending agents, sweeteners, flavoring agents, preservatives, buffers, wetting agents, disintegrants or effervescent agents (Cooper and Reddy 2005).

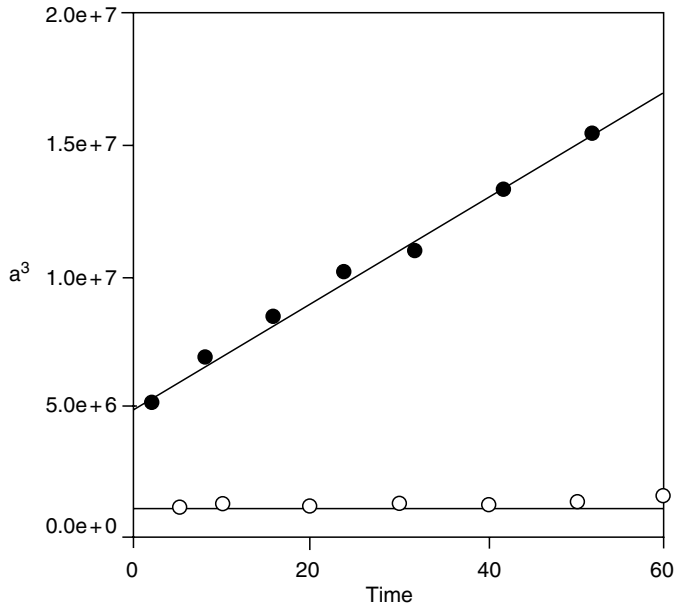

Fig. 8. Effect of Inhibitor on the ripening rate of felodipine nanoparticles (Hedberg et al. 2003)

4 Preparation of Nanosuspensions

In general, nanoparticles are prepared either by reducing larger particles (top-down) or by growing particles to a desired size (bottom-up or solution-based processes). These include dry and wet milling, supercritical fluids technologies, spray drying, different precipitations and recrystallization techniques and high-pressure homogenization. Comprehensive summary of the various nanoization methods can be found in Date and Patravale (2004), Patravale et al. (2004), Kipp (2004), Müller et al. (2004), Müller and Keck (2004), Rios (2004), Rabinow (2004) Merisko-Liversidge et al. (2003) and Rieger and Horn (2001); also see the patents survey by Cooper and Ruddy (2005).

4.1 Top-Down Methods

Top-down techniques are characterized by their dependence upon the input of mechanical energy and on the high shear forces required to overcome the adhesion power and to break down agglomerates. Müller et al. (2004) have recently reviewed the hardware used in industrial dispersion operations such as triple roller-, colloid-, and stirred-ball mills, kneaders, high-pressure homogenizers and the like. A novel (and quite uncommon) nanoparticle preparation using a membrane contactor where an organic phase is pressed through the membrane pores was very recently reported by Charcosset and Fessi (2005).

4.1.1 Nanoparticles by Milling Technologies

Liversidge et al. (1992) developed a media-milling technology called Nanocrystal (owned by Elan Drug Delivery) where ball meal with beads made of either glass, zirconium oxide, or other hard ceramics in a size of 0.4–3 mm are applied in water media containing the drug to be dispersed (see also Liversidge and Cundy 1995; Merisko-Liversidge et al. 1996). In 1994, a groundbreaking discovery was made simultaneously at Nano Systems (Bruno et al. 1996, filing date Jan 12 1994) and independently at Eastman Kodak (Czekai and Seaman 1996, filing date May 25 1994; Bishop and Czekai 1997; Romano et al. 2001) where researchers discovered that polymeric particles such as cross-linked polystyrene are the preferred milling media for nanosizing of organic materials, mainly drugs and pigments. The size of the milling media also has a direct effect on the efficiency of the grinding. Thus with media particles smaller than 75 μm, grinding below 100 nm was achieved (Czekai and Seaman 1999). Continuous grinding was also described (Czekai and Seaman 1998). This concept was adopted later into the milling equipment used by Elan and described in details in several publications (Merisko-Liversidge et al. 2003; Patravale et al. 2004; Date and Patravale 2004). In this technology, the milling chamber is charged with milling media (usually cross-linked polystyrene beads), water, drug and stabilizers. The drug concentration ranges

from 1 to 400 mg/ml. High-energy shear forces provide the energy input to fracture drug crystals into nanometer-size particles. The milling process is carried out under controlled temperature where the aqueous media effectively dissipates the heat generated during processing. The process can be performed in a batch or recycle mode. In batch mode, the time required to obtain dispersions with mean diameter <200 nm is 30–60 min. In a typical example, naproxen crystals with a mean particle size of 24.2 μm and D_{90} = 47.08 μm were milled for 30 min in a media mill to yield a nanodispersion with a mean particle size of 147 nm and D_{90} = 205 nm. The aqueous nanosized dispersions can be post-processed after drying into tablets, capsules, fast-melts and lyophilized for sterile product applications (Merisko-Liversidge et al. 2003). Two drugs using the Nanocrystal technology are already on the market. A tablet of the macrolide immunosuppressant sirolimus (Rapamune by Wyeth) and the antiemetic drug Emend by Merck (Kipp 2004). For small-scale milling operations, high-speed shaking at 4,800 rpm in the presence of 500-μm polystyrene beads with Mini Beadbeater (Biospec Products) was advocated (Lee et al. 2005). An exhaustive description and analysis of milling media is provided by Verhoff and coworkers (2003). The latter authors impart a pioneering idea where the milling media used is inert (such as silica) or biodegradable (e.g., polyglutaric-polylactic copolymer) and can be present in the final drug formulation.

Figure 9 shows the particle-size distribution of naproxen crystals before and after milling (Merisko-Liversidge et al. 2003).

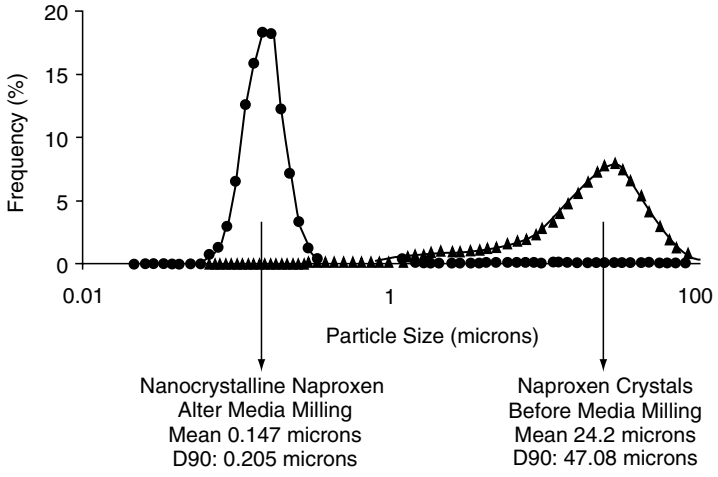

Fig. 9. The particle size distribution profile of naproxen crystals before (▲) and after milling (●). Before milling, the drug crystals had a mean particle size of 24.2 μm. After being processed for 30 min in a media mill, the mean particle size of the nanocrystalline dispersion was 0.147 μm with D_{90} = 0.205 μm. The particle size measurements were generated using laser light diffraction in a Horiba LA-910 using polystyrene nanospheres ranging in size from 0.1 to 10 μm as standards (reproduced from Merisko-Liversidge et al. 2003 with permission from Elsevier Publications)

The main advantage of the media-milling technology is its ability to handle poorly soluble drugs both in aqueous and organic media. In addition, nanosuspension with a large range of AI concentrations are feasible, batch-to-batch variation is minimal, scale-up is straightforward and the PSD obtained is fairly narrow.

The foremost drawbacks of milling technology as summarized by Rogers et al. (2004) are as follows: [a] Mechanical grinding between drug particles and milling media results in localized heat generation due to friction, which can lead to some drug decomposition. [b] Milling is typically non-homogeneous, and a portion of the particulates remained unmilled, resulting in broad PSD (contrary to other reports). This was determined mainly by SEM analysis where larger particles were observed for the milled samples. [c] The grinding media used in the milling process could serve as a source for contamination as was experimentally demonstrated (it was claimed by Merisco-Liversidge et al. (2003) that residual monomers in the final product are in the range of 50 ppb and residual insolubles generated from the media during processing are no more than 0.005% w/w based on the drug concentration in the dispersion or in the resulting solid form).

4.1.2 Nanoparticles via High-Pressure Homogenization

The technology is based on a piston-gap-type high-pressure homogenizer and was developed by Müller and coworkers in Berlin in two courses: the first was based on nanoization in aqueous media named Dissocubes (owned by Baxter) and the second was based on the same concept in non-aqueous media and termed Nanopure (owned by Pharmasol).

The principle behind the technology is the cavitation forces created in the high-pressure homogenizer (Müller et al. 1998, 2001; Krause and Müller 2001). Coarse suspension feed of the drug in aqueous surfactant solution passes through a high-pressure homogenizer applying around 1,500 bar of pressure. At this pressure, the suspension passes a tiny homogenization gap (typical width of 25 μm) in the homogenizer. Due to the miniature dimension of the gap, the flow rate of the suspension increases dramatically, resulting in intensification of the dynamic pressure (according to Bernoulli's equation). Simultaneously, the static pressure on the fluid decreases below the boiling point of water at room temperature. Consequently, water starts boiling at ambient temperature, leading to the formation of gas bubbles that implode (cavitate) when the suspension leaves the gap and normal pressure is reached again. The implosion shockwaves are sufficient to disintegrate the drug microparticles into nanoparticles. In addition, collisions between the particles also assist in the breakage of aggregates. The efficiency of the nanoization process viscosity enhancers are added to the feed suspension. The particle size of the final product inversely depends on the homogenization pressure. Another important operational parameter is the number of homogenization cycles. Evidently, the higher the number of cycles the smaller the particle size obtained.

In an extension of the above, the aqueous dispersion is replaced by a non-aqueous medium (e.g., polyethylene glycols or oil) or a water-reduced mixture (ethanol-water). This would allow working at below the freezing point of water. The obtained suspensions can be directly filled into soft gelatin capsules. In addition, drug nanocrystals in solid PEG can be used directly as powder for tablets fabrication. The latter is termed NanoPure (Müller 2002). A more advanced version (SolEmuls) was recently reported by Müller and coworkers (2004). In this modification, a hybrid formulation based on drug-loaded oil nanodroplets and drug nanocrystals of similar size (approximately 200 nm) is made again using a high-pressure homogenizer. The main advantage of the high-pressure homogenizer is that drugs that are poorly soluble both in aqueous and organic media can be expediently formulated into nanosuspensions. The process is suitable for handling varying amounts and concentrations of drug. Also, scale-up is straightforward and normally narrow size distribution of particles is obtained. Drawbacks of this method are the high cost of the equipment, the large amount of energy consumed, and the need for micronization of the feed stream prior to introduction into the homogenizer.

4.1.3 Sonication

Application of ultrasound irradiation for several hours was sufficient to break down the particles of cyclosporine, piroxicam, and other drugs to stable submicron level in the presence of phospholipids such as egg phosphatidylcholine combined with a second surface modifier such as block copolymers of ethylene oxide and propylene oxide (Parikh and Selvaraj 1999; Haynes 1992).

4.2 Bottom-Up Methods

4.2.1 Controlled Precipitation

This technology can be divided into two categories: reactive precipitation and liquid antisolvent precipitation (or solvent displacement).

4.2.1.1 Reactive Precipitation

The common approach is pH shifting in aqueous solution of an AI containing an acidic function (such as carboxylic acid) that can be deprotonated at high pH resulting in dissolution in water. Upon acidification, a large drop in the solubility takes place (frequently accompanied with a change in surface activity) and precipitation is formed. The particle size and morphology of the obtained precipitate is highly dependant on experimental conditions. The major factors are concentration of the neutralizing acid solution, precipitation

temperature, and the presence of stabilizers and crystallization inhibitors. Of particular interest is the report on 13 different polymorphs (!) of the drug phenobarbitone obtained by varying the experimental conditions using the pH shift technique. Submicron particle suspensions were attained for various pigments, drugs, and contrasting agents using this method.

In an remarkable augmentation of the reactive precipitation technique, Chen et al. (2004) fed the reacting solutions of sodium benzoate with HCl (to precipitate benzoic acid) into a high-gravity rotating pack bed apparatus (HGRP) and confirmed that as the rotating frequency of the packed bed was increased, the mean particle size decreased until a frequency of 25 Hz, above which no further change in particle size was observed. With the increase of the concentration and the volumetric flow rate of the feed, benzoic acid particles, as small as 10 nm (!) could be obtained. The formation of the ultra-fine particles was attributed to the intensified micromixing of reactants in the rotating bed, which enhances nucleation while suppressing crystal growth. This technique was applied for nanoization of the drug cephadrine (Zhong et al. 2005). Also see Bagchi et al. (1997).

4.2.1.2 Solvent Displacement Method

In this procedure, solution of the drug in a water-miscible organic solvent (methanol, acetone, isopropanol, DMF, DMSO, etc.) containing surface-active stabilizers is injected into a large excess of stirred aqueous media (Brick et al. 2003). The organic solvent rapidly dissipates into the aqueous phase, leaving behind stabilized nanoparticles dispersed in the predominantly water medium. The process can be carried out in batch or flow systems. The continuous flow systems are better suited for producing uniform particle size distribution (Texter 2001) and for scaleup (Rieger and Horn 2001). If required, the organic solvent can then be evaporated and the particles are isolated using e.g., lyophilization or spray-dry techniques. The same principle in reverse is used for nanoization of hydrophilic drugs. Thus nanoparticles of insulin were prepared by introducing aqueous solution of the latter into access of *tert*-butanol serving as antisolvent (Jacob et al. 2005).

The basic concept was pioneered as early as 1950 for the preparation of submicron sulfur particles. Water was slowly added to acetone or ethanol solution of sulfur to yield a monodispersed colloidal dispersion of sulfur in water (LeMer and Dingar 1950). It was introduced to pharmaceutics by Bruce and coworkers (1956) who crystallized penicillin G for parenteral administration by adding water to formamide solution of the latter. Solvent shift was later used by Fessi et al. (1989, 1992) for the fabrication of nanosized poly(D,L-lactide) particles that served as drug carriers. Interestingly, almost no mechanical energy is required for the formation of nanoparticles and only mild agitation is recommended for the mixing step. The method is still practiced for the fabrication of high-surface-area functional polymer colloids (Spenleuhauer et al. 1998; Clark et al. 2005).

The founders of the solvent-shift method for formation of drug nanoparticles are Violanto and Steigbigel (1988) and Violanto and Fischer (1989). These authors have added an antisolvent (water containing polyvinylpyrolidone) to DMSO-ethanol solution of a given antimicrobial drug (iodipamide ethyl ester) at a controlled rate and temperature. Consequently, spherical, amorphous particles were produced with extremely narrow size distribution. The average particle size and the particle-size distribution were found to strongly depend on the rate of addition of the antisolvent (aqueous surfactant solution) and temperature. Lower temperature and faster infusion rate resulted in smaller particles. The authors point out that the nanosuspension is a metastable state in which the free energy lies between that of the starting drug solution and the stable crystalline form. The joint synergic use of polyvinylpyrrolidone and poly(oxyethylene)-co-poly(oxypropylene) render the nanoparticles stable enough to be isolated by centrifugation, membrane filtration, or reverse osmosis.

Another early novel contribution was made by Horn from BASF in the development of carotenoid nanosuspensions (Horn et al. 1985; Horn 1989; Rieger and Horn 2001).

The principle in all of the above early examples is straightforward: nanoparticles are formed in supersaturated solutions that are generated by adding a miscible antisolvent to a solution of the active ingredient or, in the reverse process, injection of the solution into the antisolvent medium. There is an obvious difference in the timescale of the two protocols. In the former the phase transition is gradual, and in the latter, it is sudden and thus faster. Different factors are controlling the nature of the produced suspension in these two methods. In the slow version, the nature and lifetimes of the intermediate nucleation clusters will dictate the characteristics of the final stable particles formed. In the rapid injection method, the role of the initial nuclei is less important as the system is rapidly transformed into non-equilibrium distribution and the clusters formed are a priori unstable. Precipitation under high supersaturation conditions favors nucleation over growth and will result in smaller particles. The particle size is strongly dependant on the formed local instantaneous supersaturation, thus the key to producing submicron particles in solvent shifting is to create circumstances that favor rapid particle formation and very slow, or no, particle growth. These conditions are met in the rapid-injection technique. An illustration demonstrating the solvent-shift process mechanism for the formation of organic dye nanosuspension in water is shown in Fig. 10 (Brick et al. 2003).

It was experimentally confirmed that in the rapid-injection method the concentration of the solute is the critical parameter that controls the particle-size distribution (Van Keuren 2004). With a higher concentration of the solute, larger particles are obtained. A presence of the organic solvent in the receiving aqueous phase also resulted in larger particle size. The interfacial turbulence and enhanced mass transfer between the unequilibrated liquid phases (which shape the character of the precipitated particles) involving

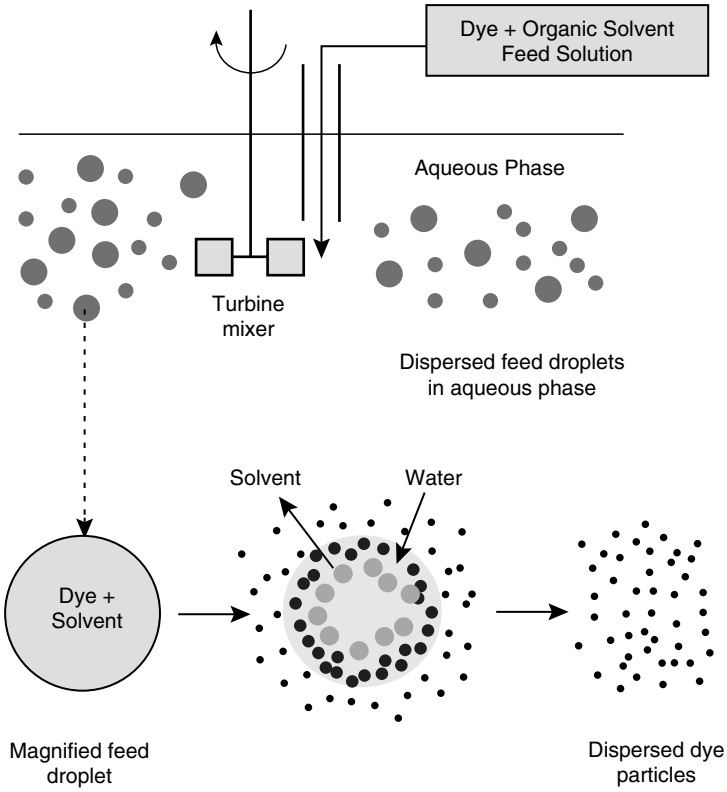

Fig. 10. Schematic description of preferred mechanism for particle formation. Droplets are formed by dispersion of the feed solution into the bulk. This process is followed by rapid counterdiffusion of solvent and water, leading to phase separation by spinodal decomposition. Within the droplet, particles begin to form as large-scale fluctuations in the concentration of solute, the amplitude of which is indicated by the intensity of *gray shading*. The size of the condensing region, because it includes more solvent, is larger than the particle that is finally formed, shown as the *black dots* around the outside. The size of the condensing region decreases and the concentration of the solute increases as the amount of water increases, which happens as the edge of the original droplet is approached. After most of the solvent is gone, the particles are dispersed into the bulk solution as a random spray, where they are stabilized by surfactant (reproduced with permission from Brick et al. 2003, courtesy of the American Chemical Society)

flow, diffusion, and surface tension is called the Marangoni effect (Fanton and Cazabat 1998; Mao and Chen 2004).

The most comprehensive study on the solvent-shift method was performed by Brick and coworkers (2003). These authors studied the formation of nanoparticles from organic solution of the organic dye yellow cyanophenyl furanone upon mixing in batch system with large excess of water containing surface active stabilizers. The solvents examined were acetonitrile, dimethylacetamide, dimethylformamide, dimethylsulfoxide, *N*-methylpyrrolidinone

and methanol. The dispersion stabilizers used were polyvinylpyrrolidinone (PVP) and sodium dodecyl sulfate (SDS) both added with the aqueous phase (0.5 and 0.1%, respectively).

In all cases, SEM (scanning electron microscopy) analysis revealed a precipitate comprised of spherical and smooth particles with morphology completely different from the irregularly shaped rough-surfaced particles produced by milling. TEM (transmission electron microscopy) and electron diffraction confirmed that the precipitate is amorphous in nature and it remained so after 1 week of aging. The particle size was strongly dependant upon the concentration of the dye, nature, and amount of the solvent and particularly the presence of organic solvent in the receiving aqueous phase. No effect on particle size was found to feed rate of the organic solution, amount of stabilizers, and stirring rate. Only upon the use of jet mixing with power several orders of magnitude stronger was there a change in the average particle size from 200 nm to 100 nm using the same feed. Good correlation was demonstrated between the particle-formation rate and the local supersaturation rate.

Kipp and coworkers (2004, 2005), who used light microscopy, confirmed that during the mixing of the two solvents, with agitation, a pre-suspension is formed. The nature of the latter depends on the conditions and the additives involved. Three categories were defined where the pre-suspension is composed of: (1) amorphous particles, semi-crystalline, or super-cooled liquid (2) crystalline particles and (3) crystalline friable particles that are fragile and easily broken down into smaller particles.

The pre-suspension is subjected to a subsequent energy addition step where impact, shear, or cavitation forces are applied on the system under controlled temperature, using sonication, homogenization, countercurrent flow homogenization or microfluidization methods. After the energy addition step is applied, pre-suspension (1) turns crystalline without a noticeable change in the average particle size (typically under 400 nm). Category (2) remains crystalline with the same average crystal size but the crystals obtained are less likely to aggregate (probably due to reordering of the surfactant molecules at the solid-liquid interface). Category (3) is the preferred form as the fragile crystals are effortlessly broken into the desired particle size. This methodology gives way to polymorph control in the submicron level (Werling et al. 2003).

Kipp et al. (2005) have also shown that smaller size particles are obtained if the polymeric surface active agent (e.g., poloxamer 188) is added to the organic solution of the drug and not to the water diluent.

A different conclusion was asserted by Kreitz et al. (2004) who claimed that for biomolecules an important feature of the solvent-shift method is that the nanoparticles obtained are typically amorphous. Note however that this method cannot be applied to drugs that are not soluble in organic solvents.

The solvent-shifting methodology was demonstrated on a laboratory gram scale and on a kilogram scale by Rogers et al. (2004) from Dow

Chemical Company using the drugs naproxen and danazol as model compounds. Methanol was applied as an organic solvent with poloxamer 407 (for danazol) and polyvinylpyrrolidone 55 k (for naproxen) as stabilizers. Solutions of the drugs in methanol (4.5% w/w for danazol and 6.7% w/w for naproxen) were injected into the mixing zone in a batch reactor (continuous system for kilogram scale) containing aqueous phase at different mixing zone temperatures (3, 25, and 50 °C). The slurry produced was consequently solvent-stripped under vacuum. The water was removed by lyophilization (lab scale) or spray dry (kilogram scale) to yield a stabilized dry powder. The materials obtained were characterized and compared with milled particles and with the physical blend of control particle using particle size analysis, XRD, SEM micrographs, and, most crucial, dissolution tests. The latter showed that the difference between the milled samples and the precipitated samples was not significant. However, both forms dissolved much faster than the physical blends or the bulk control samples. Small variations were measured for the dissolution rate of material precipitated at different temperatures. The presence of residual solvent (methanol) in the final product was confirmed to be at the level of 70 ppm for the kilogram-scale run.

It was concluded that the size and morphology of the stabilized drug nanoparticles can be manipulated by altering various operating parameters through the controlled precipitation process, thus allowing the potential for particle customization. The scalability of the process was also verified (see Kipp et al. 2002; Mathiowitz et al. 2003).

The pain relief effect of the COX-2 inhibitory drug celecoxib was enhanced upon nanoization using this methodology (Karim et al. 2003). Peptides such as cyclosporine were nanoized to size of 80–145 nm using the ethanol-water shift method (Gassmann and Sucker 2002).

In a modification of the solvent shifting mode ("evaporation technique"), Ruch and Matijevic (2000) created micron size of budesonide by the addition of water (as antisolvent) to an ethanol solution of the drug below its solubility limit. Only after partial evaporation of the ethanol was the dispersion formed. The morphology, size, and stability of the resulting particles were also sensitive to the evaporation technique. A technique where the solvent is continuously removed via evaporation from the mixture in parallel to the precipitation step to yield a solvent-free suspension was demonstrated by Johnston et al. (2002) and by Chaubal et al. (2005).

Although the solvent shift routine is widespread, very little fundamental information is available on the kinetics of nucleation and growth of organic compounds when precipitated under high supersaturation conditions produced by the addition of a non-solvent. See, however, the kinetic studies of Mahajan and Kirwan (1993, 1994), Brick et al. (2003) and the recent combined computational fluid dynamics and process simulation of Choi et al. (2005). Note also the summary by Rieger and Horn (2001).

4.2.2 Aerosol Procedures

In this technique, the drug is dissolved in a compatible volatile solvent (ethanol is preferred for biomaterials) and the solution is carried by pressurized inert gas through an atomizer followed by a heated tubular laminar flow reactor maintained at a temperature sufficient to evaporate the solvent (Eerikainen et al. 2003; Price and Kaerger 2004). The rapid evaporation of the solvent leads to supersaturation of the drug in the inert gas carrier followed by the formation of nanoparticulate material. The size and volume of the particles obtained is highly dependant on the nature of the atomizer, the temperature in the reactor, and the concentration of the feed solution. In general, narrow particle-size distribution is obtained. An advanced aerosol method is the electrohydrodynamic (EHD) spaying where the aerosol passes through an electric field (Coffee 2001). Another development is spray freezing of the drug solution into a cryogenic liquid (Williams et al. 2003). The main drawback of the aerosol method is that it is not suitable for poorly soluble drugs.

4.2.3 Microemulsion Template Methods

Microemulsions (ME) are transparent, well-characterized, thermodynamically stable, and easily manufactured systems composed of nanodispersed droplets of oil (in water) or of water (in oil) stabilized by surfactants and cosurfactants (usually a lipophilic alcohol). MEs were effectively applied for the syntheses of nanometric inorganic materials (Lopez-Quintela 2003). Trotta et al. (2003) have demonstrated the method for nanoization of the poorly soluble drugs mitotane (Trotta et al. 2001) and griseofulvin (Trotta et al. 2003). Addition of water to microemulsions of these drugs led to displacement of the organic phase from the internal phase into the external phase leading to formation of drug nanosuspensions with a particle size below 100 nm with low polydispersivity combined with increased dissolution rate. Progesterone with an average particle size of 12.6 nm was prepared this way by Hassan (2003).

The advantages of this technique are the simple equipment required, the good control of particle size, and the ease of scale up. There is no need in excessive kinetic energy input to achieve a nanosized drug. However, the method is not suitable for poorly soluble drugs and the large amount of surfactants required to create the microemulsion (and in some cases they have to be removed, e.g., by means of diultrafiltration) is also a drawback.

4.2.4 Supercritical Fluid Methods

Supercritical fluid-based technologies are a well-established nanoengineering practice. These methods benefit from the ability to control particle size and shape and from ease of scale-up. The known art distinguish between several

routines: RESS (rapid expansion from supercritical solutions), RESAS (rapid expansion from supercritical to aqueous solutions), SAS (supercritical anti-solvent methods) and SAS-EM (supercritical anti-solvent enhanced mass transfer method).

In the RESS process, the drug is dissolved in supercritical fluid (predominately CO_2), which is passed through a nozzle at supersonic speed, resulting in supersaturation and precipitation. The size distribution and morphology of the product is a function of the operational parameters of pressure, temperature, concentration, and structure of the nozzle. The method is suitable only for a drug with adequate solubility in the supercritical fluid. In addition, wide PSD is usually obtained mainly due to aggregation. In the RESAS (Young et al. 2000) version, the expansion of the supercritical solution is done through an orifice into aqueous solution containing surface active stabilizers. Here the nature of the particles obtained depends on the stabilizer nature and concentration, concentration of the AI and temperature of the solution, and the preheater. This method is considered more efficient than the RESS, however, it is also limited to drugs with some solubility in the supercritical fluid. A typical example of the latter is the nanoization of sterols and sterol esters reported by Kropf et al. (2001).

The SAS protocol is the opposite of the latter (Reverchon 1999). Here the drug is dissolved in a selected organic solvent and the solution is atomized into a flowing stream of $scCO_2$, which functions as an antisolvent. The main advantage of this practice is that it matches well with hydrophobic poorly soluble drugs. However, similar to RESS and RESAS, the obtained dispersed systems suffer from relatively broad PSD.

The SAS-MS method again uses the supercritical fluid as an antisolvent but the crystallization vessel is placed in an ultrasonic bath. The latter causes smaller droplet size and greatly enhances the mass transfer between the solution and the anti-solvent. This results in a generation of particles ten-fold smaller than those produced by the conventional SAS process (Chattropadhyay and Gupta 2000, 2001)

Another modification is the evaporative precipitation into an aqueous solution (EPAS), where the AI is dissolved in a normal hydrophobic volatile organic solvent (diethylether or dichloromethane) and the heated solution is sprayed through an atomizer into an aqueous phase containing stabilizers. Instant evaporation of the small solvent droplets results in high supersaturation followed by rapid nucleation of the drug and adsorption of stabilizers to the nanoparticles surface. Submicronic particles of the drug cyclosporine A were obtained by Chen et al. (2002) using this technique. Lyophilization was also used for the removal of the organic solvent (Brynjelsen et al. 2004).

5 Characterization of Nanoparticulate Systems

The significant characterization parameters and hardware for nanosuspensions are as follows:

5.1 Mean Particle Size and Particle-Size Distribution

Mean particle size and particle-size distribution are the most important parameters in designing a novel formulation and are routinely the first to be measured. The saturation solubility and the rate of dissolution are strongly dependant on particle size and, accordingly, are the biological performance, the physical stability, and the shelf life of the product. A complete picture of the population size and distribution can be obtained by analysis of data generated by photon correlation spectroscopy (PCS), laser diffractometry (LD) and Coulter counter.

Laser diffractometry is a fast and useful technique for the particle-size measurement in the range of 20 nm to 2,000 μm. The data obtained is based on volume size distribution. Typical LD output includes the D_{50} and D_{90}, which indicate that 50 or 90%, respectively, of the particles are below the indicated size. For mean diameters smaller than the above (in the range 3–3,000 nm) photon correlation spectroscopy (PCS) is used (Müller and Müller 1984). PCS can also be used for the determination of the polydispersity index (PI). The latter is an important parameter that controls the physical stability of nanosuspensions and should be maintained as low as possible for a robust suspension. A PI value of 0.1–0.25 indicates a narrow size distribution whereas PI value >0.5 indicates a very broad distribution. PCS measures light scattering at a fixed angle over time. Due to the Brownian motion of the particles, which depends on the particle size, the intensity will fluctuate as a function of the latter and will allow the size measurement. PCS fails in identifying contaminants with a particle size >3 μm, since such impurity might be critical for small blood capillaries with a diameter of 5–6 μm. For nanosuspensions intended for intravenous administration, particle-size analysis by Coulter counter methods is thus essential in addition to the PCS and LD analyses. The Coulter counter yields the absolute number of particles per unit volume for different size classes.

5.2 Surface Area

This essential parameter, which is critical to the rate of dissolution, is measured using the gas adsorption method (BET).

5.3 Particle Charge (Zeta Potential)

The zeta potential strongly affects the stability of nanosuspension. To obtain a stable nanosuspension, a zeta potential of ±20 to ±30 mV is considered necessary (depending on the nature of the stabilization energy, electrostatic only, or electrostatic combined with steric, Müller and Jacobs 2002). Zeta potential can be quantified by tracking the colloidal particles through a microscope as they migrate in a voltage field.

5.4 Contact Angle

This factor quantitatively signifies the hydrophilicity (wettability) of the particle surface. The contact angle is smaller for the dispersed drug stabilized with surfactants (Rasenack and Müller 2002). The contact angle is measured by the sessile drop technique using a goniometer.

5.5 Morphology and Crystalline State

Direct imaging of the dispersion using SEM yields real images and first-hand information about the nature of the particles independent of the particle size. Visualization of particle shape by atomic-force microscopy has also been reported (Shi et al. 2003; Garg and Kokkoli 2005). The changes in the morphology and the degree of the amorphous and crystalline fractions can be determined by X-ray diffraction analysis. DSC tests extend the scope of information related to polymorph transitions and energetics.

5.6 Saturation Solubility and Dissolution Rate

These are normally assessed using standard methods (USP) at controlled temperature, stirring rate, and dissolution medium (Rasenack and Müller 2002). The dissolved amount of the drug is determined spectrophotometrically, typically with a UV VIS spectrophotometer.

6 Nanoformulations of Crop-Protection Chemicals

The obstacle of water solubility is also a limiting factor in the development of crop-protection agents. The traditional solutions have been either to apply the insoluble pesticide as a dust or to spray it in organic solvent or in organic-based emulsion (EC). These methods are inefficient, costly, and inflict a profound environmental load (particularly the solvent-based systems).

The major potential benefits of the downsizing of agrochemical suspension formulation are [1] the improvement of efficacy due to higher surface area and higher solubility; [2] the induction of systemic activity due to smaller particle size and higher mobility and [3] the lower toxicity due to the elimination of organic solvents.

In an identical chronology to nanopharmacology, the first nanoparticles that made their way to crop-protection literature were nanosized macromolecules used as delivery vehicles. Thus polycaprolactone and polylactic acid nanospheres (NS) were examined for the nanoencapsulation of the insecticide ethiprole (Bohem et al. 2003). The authors concluded that NS did not provide a controlled release of the AI but, due to their small size, they

enhance the penetration through the leaf and consequently boost the systemic activity relative to the classical SC formulations. Conversely, controlled release was observed by Liu et al. (2001) who incorporated the fungicides tebuconazole and chlorothalonil into nanoparticles (100–250 nm) of polyvinylpyridine and of polyvinylpyridine-co-styrene. These formulations were successfully introduced into solid wood as preservatives. In recent Chinese patents, polymeric nanocapsules were suggested as vehicles for ivermectin (Shang et al. 2004) and for acetamiprid (Zheng and Shang 2004). In another Chinese patent, nanosized inorganic particles such as TiO_2, SiO_2, Fe_2O_3, or Al_2O_3 were advocated as carriers to pesticides (Wang et al. 2004). Also note the related interesting technology of photocatalytic decomposition of pesticide residues using titania doped with Fe^{+3} or other metals sprayed directly on crops (Gang 2004) or even incorporated into the pesticide formulation (Tang et al. 2003). Interestingly, with a few exceptions (see Tang et al. 2003), liposomal formulations were not hitherto reported as delivery systems to pesticides (evidently due to high cost, see Pons and Estelrich 1996).

Solid lipid micro- and nanoparticles were used by Frederiksen et al. (2003) to encapsulate gamma cyhalothrin with only limited success due to partial expulsion of the AI from some of the solid lipids used. However, this formulation exhibited 10 and 63 times the reduced toxicity towards fish and daphnia, respectively, (compared with a standard EC formulation) without an apparent loss in insecticidal activity. Surprisingly, the particle size of the solid lipid examined (between 0.3 and 100 μm) barely affected the biological activity.

Scientists from Dow Agroscience were the first to propose the use of nanosized aqueous dispersion formulation as a tool to enhance the bioavailability of pesticides (Storm et al. 2000, 2001). The authors pointed out the advantage of eliminating the need for organic solvents. Another interesting facet of the invention is that the stable nanosuspension also provides a mean for preparing a one part formulation of a plurality of pesticides which would be otherwise unstable in each other's presence. Storm and coworkers used milling technologies in presence of grinding media (including polymer beads) and surface active agent to obtain stable suspension of various herbicides, fungicides and insecticides with mean volume particle size in the 148–314 nm range (Storm et al. 2000, 2001). The efficacy of spinosad insecticide was demonstrated to strongly depend on particle size. Thus LC_{50} of the material when applied on spider mites was measured to be 15 mg/l for mean particle size of 404 nm, 11 mg/l for 372 nm, 7.6 mg/l for 332 nm and 4 mg/l for 163 nm.

Milling techniques do not suit active ingredients with low melting points. The obvious solution is the solvent shift method. This was shown in a recent patent by Crooks et al. (2003). The inventors describe the formation of atrazine nanoparticles upon the addition of 60 ml of 1% methanol solution of the herbicide containing 0.5% amphiphilic polymer (di-block polybutylacrylate-polyacrilic acid with xanthate end-chain) into 40 ml of water at 35 °C with gentle stirring. Once a suspension is formed, the mixture is diluted again 1:10 with water. The measured mass median diameter of the particles was 520 nm.

Evidently the PSD was not very sharp, as 25% 0f the particles had a diameter higher than 1,060 nm and 10% of the particles had a diameter higher then 6.17 μm.

The authors advocate that the obtained dispersion can be used as such (after further dilution with water) or the solvent can be removed by distillation. Another alternative is total drying using freeze drying or evaporation, which yields dry solid nanosized powder. The latter can be dispersed again upon the addition of water to regenerate the nanosized dispersions. Unfortunately, although the inventors claim the utility of this method to numerous types of pesticides, they illustrate and support the methodology with a very small number of experimental examples using only atrazine as the AI and methanol as a solvent.

Electrohydrodynamic (EHD) aerosol was used by Dvorsky and coworkers (2005) to produce sprayable and quick-dissolving agrochemical and veterinary formulations.

7 Nanoparticulate Formulation of Novaluron

7.1 Novaluron: A Novel IGR

Rimon (novaluron) is a benzoylurea insect-growth regulator introduced by Makhteshim Chemical Works in 1996 (Fig. 11). Rimon is a chitin synthesis inhibitor that acts by ingestion and contact and has translaminar activity (Ishaaya et al. 2002, 2003). It is highly effective in controlling lepidopteran larvae, whiteflies, and leaf miners (Ishaaya et al. 1996). Of particular interest is its considerable activity against all developmental stages of mosquitoes (Su et al. 2003; Mulla et al. 2003). In a recent report by the World Health Organization regarding Pesticide Evaluation Scheme (WHOPES 2004), it was concluded that novaluron is a safe IGR that is unlikely to present acute hazard in normal use. It was assessed as highly effective against mosquito larvae and pupae (particularly *Aedes aegypti* but also *Anopheles*, *Culex* and *C. Quinquefasciatus*) at low dosage (1–5 μg/liter AI). Novaluron has no cross-resistance with other leading compounds for controlling whiteflies such as buprofezin, pyriproxyfen, acetamiprid or imidacloprid (Ishaaya et al. 2005).

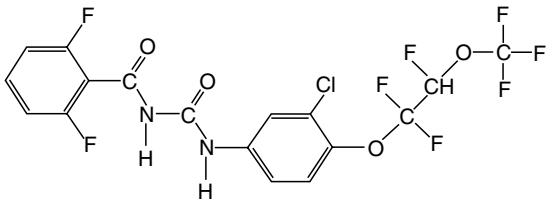

Fig. 11. Chemical structure of novaluron

Table 2. Physical properties of novaluron

MW	Melting point	Vapor pressure	Density	Water sol.	KOW log P
492.7	176–179 °C	<0.5 mPas	1.66	53 µg/l	5.27

Toxicity (rats) oral LD_{50} > 5,000 mg/kg, skin & eye LD_{50} > 2,000 mg/kg, inhalation LC_{50} (4h) > 5.15 mg/l air, NOEL (90d) 100 ppm

In a study on its effect on silverleaf whitefly reproduction, it was concluded that novaluron negatively affects female reproduction by reducing egg viability—an important long-term pest-management strategy that may reduce the number of insecticide applications (Cloyd et al. 2004). Novaluron is a rainfast compound suitable for the tropics and in rainy seasons (Ishaaya et al. 2001). It can be considered as a component for IPM programs as it has no appreciable effect on parasitoids and phytoseiids and a mild effect on other natural enemies (Ishaaya et al. 2001, 2002). Cabrera et al. (2005) have demonstrated that novaluron is compatible with different mite species. For example it has no harmful effects on the soil-dwelling predatory mite, *Stratiolaelaps scimitus*, which is used in North America for biological control of dark-winged fungus gnats (*Bradysia* spp.). The physical properties of novaluron are summarized in Table 2.

7.2 Preparation of Nanosuspensions of Novaluron

The preparation of the nanosized formulation is based on the solvent-displacement technique (Levy-Ruso and Toledano 2005). A concentrated DMSO solution of novaluron is added to water at ambient temperature in the presence of emulsifiers, polymeric dispersing agents, crystallization inhibitors, and stabilizers to yield a stable suspension with a mean particle size of 350 nm. In a typical procedure, 100 g of novaluron is dissolved in 150 g of DMSO at 40 °C and the solution is combined with 130 g of the surfactant TSP-54 (ethoxylated tristyryl phenol) and 65 g of the dispersing agent Witconol (ethylene oxide-propylene oxide block copolymer). The latter mixture is slowly added at 35–40 °C (while mixing) to 403 g of soft water containing the crystallization inhibitors PEG 200 (polyethylene glycol MW 200, 75 g) and PEG 1000 (polyethylene glycol MW 1000, 75 g). To the final suspension, 2 g of the stabilizer Kelzan (polysaccharide) is added while mixing. This concentrated suspension can be directly diluted with water and applied on selected targets. This procedure was scaled up to 100 l without any apparent difficulties.

7.3 Comparative Efficacy of Nanosuspension Formulations of Novaluron

In a series of assays we compared the toxicity of nanosized novaluron SC to that of the standard commercial EC and SC formulations on the cotton whitefly *Bemisia tabaci* and the Egyptian cotton leafworm *Spodoptera littoralis*,

both being important pests of cotton, vegetables, and ornamentals (Byrne et al. 1990; Horowitz and Ishaaya 1994; Horowitz et al. 1996).

In the first series of assays we determined the toxicity of the various formulations at a concentration of 0.3 mg AI/l on *B. tabaci* nymphs (Table 3). Cotton seedlings (with two true leaves) infested with first-instar nymphs were dipped in the test solution, air dried for 2 h and kept under standard room conditions of 26 ± 1°C, 65 ± 5 RH, and 14:10 h L:D until pupation. Larval mortality expressed in decreased pupation was then determined. The nanosized SC formulation resulted in identical potency to that of the standard EC formulation and was considerably more potent than the standard SC formulation (Table 3).

According to mortality curves (Fig. 12) and LC-50 and LC-90 values (Table 4), the nanosized SC formulation is 6–12-fold more potent than the standard commercial SC formulation. The increased toxicity of the nanosized formulation probably resulted from the increased penetration of novaluron through the cuticle into the larval body.

In another series of experiments, we determined the toxicity of nanosized novaluron SC formulation, as compared to the standard commercial EC and SC formulations, on *Spodoptera littoralis* larvae (Table 5, Fig. 13). Based on a previously published method (Ishaaya et al. 1995), castor been leaves treated with the test compound were exposed to early third instars (11 ± 1 mg) for 4-day of feeding; the larvae were then fed for an additional 4 days on untreated leaves. Larval mortality was determined at day 4 and day 8. Larval weight gain (LWG) was determined at day 4. According to mortality curves (Fig. 13) and LC-50 and LC-90 values (Table 4), the nanosized novaluron formulation was about 2.5-fold more potent than the commercial standard SC formulation and its toxicity approximates that of the EC formulation. As novaluron acts on *S. littoralis* larvae by ingestion (Ishaaya and Horowitz 1998), our results indicate that the nanosized SC formulation is able to penetrate the digestive tract and reach the biochemical site faster than the standard SC formulation. It can be safely concluded that the nanosized formulation acts more powerfully than the standard commercial formulation either by contact (faster penetration through the cuticle) as in the case of the whitefly *B. tabaci*, or by ingestion as in the case of *S. littoralis*.

Table 3. Effect of 0.3 mg AI/l nanosized novaluron formulation as compared with the standard EC and SC formulations on *Bemisia tabaci* first-instar nymphs

Formulation	No. of nymphs	Pupation % ± SEM
Untreated	113	98 ± 2a
SC-10 (commercial)	72	79 ± b
SC-10 (nanosized)	93	28 ± 5c
EC-10 (commercial)	156	28 ± 8c

Data are averages ± SEM of five replicates of 11–48 nymphs each. Means followed by the same letter do not differ significantly at $P = 0.05$
SC-10 (commercial): MVPS 1,500 nm, D90VPS = 3,500 nm
SC-10 (nanosized): MVPS = 350 nm, D90VPS = 520 nm

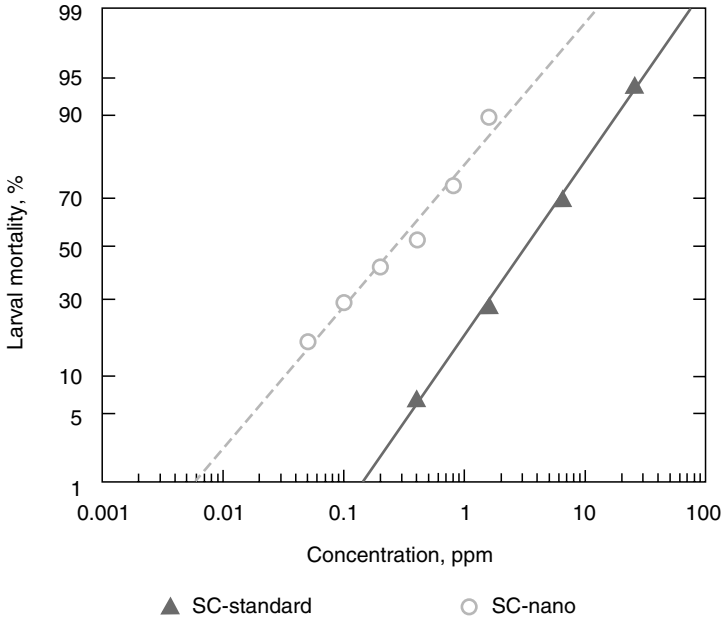

Fig. 12. Toxicity of the nanosized novaluron SC formulation as compared with the standard commercial SC formulation expressed in mortality curves on probit scales

Table 4. Toxicity of nanosized novaluron SC formulation as compared with the standard commercial SC formulation on *Bemisia tabaci* nymphs expressed in LC values

Formulation	*n*	Slope ± SEM	LC-50 (95% F.L.)	LC-90 (95% F.L.)
SC (commercial)	1,003	1.91 ± 0.18	3.6 (2.9–4.4)	17 (14–22)
SC (nanosized)	2,517	1.51 ± 0.08	0.3 (0.2–0.5)	2 (1–7)

Mortality curves were constructed from four concentrations each and untreated control. Each concentration was done with 5–10 replicates of 20–100 first-instar nymphs each. LC values were determined according to probit regression using POLO-PC analysis procedure

Table 5. Toxicity of nanosized novaluron SC formulation as compared with the standard commercial SC and EC formulations on third instars *Spodoptera littoralis*

Formulation	*n*	Slope ± SEM	LC-50 (95% F.L.)	LC-90 (95% F.L.)
SC (commercial)	730	2.97 ± 0.21	0.42 (0.36–0.49)	1.1 (0.9–1.6)
SC (nanosized)	200	3.64 ± 0.53	0.19 (0.15–0.23)	0.42 (0.34–0.57)
EC (commercial)	390	4.30 ± 0.46	0.15 (0.13–0.16)	0.30 (0.27–0.35)

Mortality curves were constructed from four concentrations and untreated control. Each concentration was done with 5–15 replicates of ten larvae each. LC values were determined according to probit regression, using POLO-PC analysis procedure

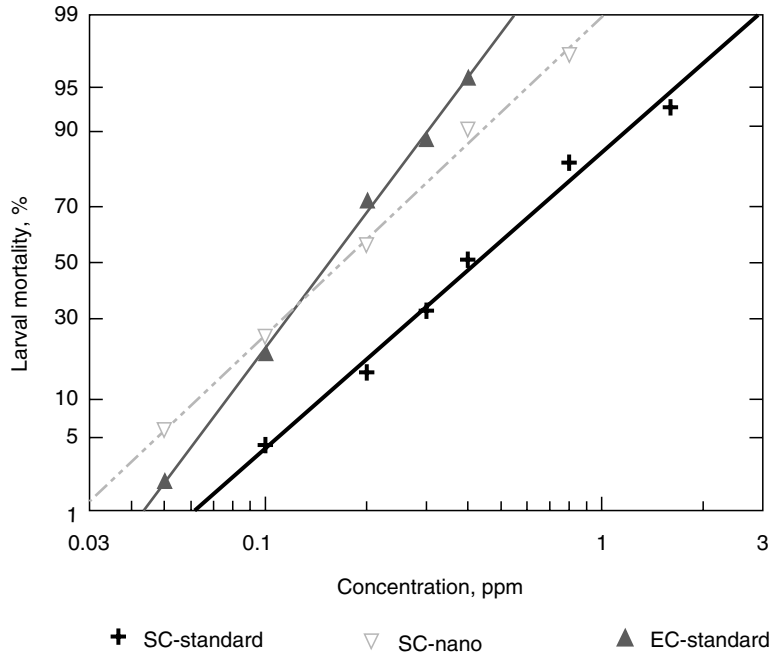

Fig. 13. Toxicity of the nanosized novaluron formulation as compared with the standard EC and SC formulations expressed in mortality curves

8 Conclusions

The field of aqueous nanosuspensions of crop-protection chemicals is an emerging technology with only inconsequential presence in the market. However, the great potential of formulations based on pure active ingredients dispersed in water, virtually free of organic additives or solvents, is extraordinarily appealing from both an economic (and particularly) an environmental perspective. We envisage numerous examples of nanoparticulate agro formulations with higher bioavailability, efficacy, and probably better selectivity in the very near future.

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2 Pharmacokinetics: Computational Versus Experimental Approaches to Optimize Insecticidal Chemistry

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1 Introduction

An ideal insecticide is a compound that is highly toxic to target insect pest species but has a low toxicity to non-target organisms, including beneficial species of insects, and with a high differential toxicity between insects and vertebrates. It should also have a low environmental impact, and be rapidly removed from the environment when adequate control of pest species has been achieved. Very few compounds approach this ideal, and this gives an indication of the difficulty of designing insecticides to meet these diverse (often conflicting) criteria. Toxicity, defined as *a capacity to cause injury in a living organism defined with reference to the quantity of substance administered or absorbed, the way in which the substance is administered and distributed in time (single or repeat doses), the type and severity of injury, the time needed to produce injury, the nature of the organism(s) affected, and other relevant conditions)* (Nordberg et al. 2004), is easy to achieve, especially on in vitro screens. However, the combination of high specific whole organism toxicity and low toxicity to non-target organisms is much more difficult to achieve even within an identified chemical class.

Currently, most of the commercially available insecticides act at a relatively small number of target sites, and most are nerve poisons causing disruption of nerve function by interfering with neurotransmitter action or by disrupting ion channel functioning. This limited number of target sites is associated with increasing problems of resistance, both through modified metabolic capabilities and modified target sites. There is an urgent need for insecticides with novel modes of action. However, the costs associated with the development of a new product are extremely high, and there is a need to provide useful information to aid the selection and modification of leads at an early stage. Some of the problems to be overcome are also encountered in the development of new pharmaceutical products. Useful biological activity at the site of action (adequate PD activity) is not necessarily reflected in high levels of activity when compounds are applied to whole organisms. In the case of insecticides, there is a further challenge not encountered in dosing subjects with therapeutic agents; the need to apply an appropriate dose on small animals, sometimes in flight, dispersed over a wide area such as a crop or an area of forest. In the case of both pharmaceuticals and insecticides, the

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delivery can be optimized by the combination of necessary physicochemical properties of the biologically active agent with an appropriate formulation.

1.1 Drug Design by the Pharmaceutical Industry

It is well recognized in the pharmaceutical industry that chemistry dictates biological activity through interaction with a site of action to produce biological responses. This involves the pharmacodynamic (PD) phase of poisoning. However, the pharmacokinetic (PK) phase that comprises absorption, distribution, metabolism, and excretion (ADME) also has an important impact on the poisoning process. Adjusting chemical structure to improve intrinsic biological activity (in the case of insecticides that is toxicity) usually has a deleterious effect on delivery to the target site. It is now widely held in the pharmaceutical industry that in silico prediction of ADMET (absorption, distribution, metabolism, excretion, and toxicity) is an essential element of the drug-design process, and its development is being given high priority. The urgency comes from the high costs of development and the associated need to eliminate candidate compounds with inappropriate ADMET properties at the earliest stage possible. ADMET models depend on the availability of appropriate physicochemical descriptors that can be rapidly and easily estimated for novel compounds using computational chemical methods without the need for experimental measurements. They also depend on sound physiologically based PK models. The latter can be based on a firm foundation of in vivo physiological measurements made over a long time scale on the main laboratory test species and on humans, and on correlated observations on in vitro systems.

1.2 Insecticide Design by the Crop-Protection Industry

Similar developments in the insecticide industry use the same computational chemical methods to generate the necessary descriptions of the physicochemical properties of insecticides as are used in the drug industry. However, useful in vivo physiological data in insects is very limited, in part because of the very small size of insects compared with mammals. Fortunately there are some excellent reviews on insecticide PKs in insects that cover a wealth of literature from the 1930s onwards. These include the reports of Ford (1972, 1988), Brooks (1976), Hartley and Graham-Bryce (1980), Welling and Paterson (1985), and Brealey (1988), that provide useful reading for those new to the field. The reader is referred to these publications for comprehensive accounts of insect PKs and detailed bibliographies of the published literature. The present review is based on a smaller subset of key references that have been used to extract the underlying principles that determine PK behavior in insects. This has been used to provide a synthesis of the available information that is pertinent to the problem of designing new and improved insecticides.

Much of the early work was based on studies of single insecticides acting on, for example, resistant strains of insects in order to determine whether penetration or metabolism provided explanations for changes in tolerance to insecticides following field use. These studies were often difficult to interpret, however, and broad generalizations impossible to infer. The reviews cited above provide syntheses of this material and lead to a more detailed understanding of this complex process. Physiologically based PK (PBPK) models of the type now used by the modern pharmaceutical industry are very rare in the area of insecticide research. In many cases, relatively simple compartmental models are used, and these reflect the experimental methods available for small animals. Often only the masses of material that can be recovered from the external surface of the insect and that recovered from the washed insect body at a range of times are known. Detection of the masses in the various body compartments at a range of elapsed times after dosing can be difficult to determine, and are available in only a limited number of published studies, and usually for larger insects. Radiolabeled compounds have been used in many studies in order to detect insecticides in insect extracts where realistic field doses are applied. When the radiolabel is measured following combustion of insect tissues or extracts, interpretation of PK profiles can be difficult because the total label may include metabolites as well as parent compound

1.3 Aims and Scope of This Review

This chapter will explore the available information on insecticide PKs in insects and assess the potential for developing quantitative predictive models. This should help the development teams working on new insecticides to prioritize their efforts in order to optimize the design of lead compounds.

2 Pharmacokinetic Modeling

Once an organism has contacted a biologically active material, a series of processes occurs. These include penetration of the integument, distribution throughout the body, metabolic activation, elimination as a result of detoxification and excretion (all PK processes that act to determine the level of bio-active material within the organism), and interaction with a site of action to elicit a response (PD processes). Most of these processes proceed simultaneously. A complex interaction between those PK processes will determine the levels of active ingredient in tissues or compartments of the body at any elapsed time following the initial contact. Detoxification, including metabolic activation, yields a series of metabolic products whose sequence can be inferred from a combination of their times to reach a maximum concentration in the insect, and their modified chemical functionality. The first PK phase is uptake from the point of contact followed by

distribution to the all parts of the body including the target tissue(s) that contains the site of action. As soon as the compound is within the body, the various processes involved in degradation and elimination will start to act on the material. What is required for insecticide design is an accurate description of how these processes determine the levels of the material at different sites within the organism and how the levels change with time after the initial contact.

2.1 Mathematical and Conceptual Pharmacokinetic Models

Models of this system can cover a range of complexity with varying degrees of simplification. Simple models are easier to parameterize but may be more difficult to interpret. Complex models aim to separate the various processes that govern the behavior of the materials, and are more difficult to parameterize but generally easier to interpret in terms of anatomy, physiology, and biochemistry. Profiles generated by simple models provide a summary of the overall behavior of the system. This behavior is generally retained as the complexity of the model increases in order to dissect the insect system into more compartments and to separate the individual processes. However, more complex models usually exhibit additional features or properties that are missed by the simpler representations.

2.2 Simple Experimental Approaches

It is difficult and time consuming to perform comprehensive PK experiments with sufficient time points to enable models to be fitted. Many PK studies have focused on individual processes such as penetration or metabolism. Often the aim has been to explain differences in tolerance of a single compound between groups of insects of the same species or between different species. Since only a limited range of compounds and insect species or strains is used, this inevitably limits the utility of the results and makes extracting a generalized understanding difficult. However, useful information concerning the properties of components of the PK phase of poisoning has been generated by these studies.

An understanding of the factors affecting the penetration process has been generated through studies using formulations intended to enhance the toxicity of existing active ingredients. Formulations are used to improve transfer and penetration of topically contacted materials. When applied to the insect surface, the formulation can modify the cuticle and, if it is stable (e.g., a nonvolatile oil) it may form part of the PK system. Possible effects include spreading to increase the surface area available for diffusion and modifying the relative affinities of the various surface components and underlying layers for the insecticide. The latter may impact the optimal route of penetration through the heterogeneous cuticle.

Early work by Webb and Green (1945) investigated the impact of carrier-solvent properties on the penetration of the insecticide diphenylamine through the cuticle of the sheep ked (*Melophagus ovinus*). The solvents were added to a powder formulation and produced a complex system. However, an enhanced movement of active ingredient was achieved, and the authors concluded that the effect was achieved through an increased movement through hydrophilic rather than lipophilic channels, emphasizing the heterogeneous nature of the cuticle and the multiplicity of routes through it. More recent work (Lankford and Dawson 1993) investigated the effect of another complex formulation on penetration. In this case, the formulation, a microemulsion, changed rapidly through loss of volatile components following application. The study of the transfer and penetration of cypermethrin to adult cockroaches (*Periplaneta americana*) also followed the penetration of formulation components, toluene and water. Not only did all three of the above compounds transfer rapidly to the cuticle, but also labeled water and toluene were found in hemolymph taken from the abdomen 5 min after topical application to the femur of the metathoracic leg. These results give evidence of the efficiency of the circulatory system. The rapid ingress of both toluene and water with contrasting physicochemical properties supports the idea of a number of routes, with different characteristics, through the cuticle.

A limitation of the simple experimental approach is that it is unable to take into the account the (sometimes complex) interactions between the various PK processes when interpreting the experimental data. In some cases this is addressed by combining whole animal and/or in vivo PD information with the PK data. Bioassays have proved particularly useful when available analytical methods have precluded measuring concentrations of a toxicant in the target tissue. The results of studies such as that of Hadaway et al. (1976) that measure only accumulation in the insect are difficult to interpret. They measured the accumulation of DDT in a range of insect species including the tsetse fly (*Glossina species*), other dipterans, locusts, the German cockroach (*Blatella germanica*), and the cotton stainer bug (*Dysdercus fasciatus*). A wide range of rates of accumulation was observed; the fastest in the tsetse fly, intermediate values for the locusts and the other dipterans, and the slowest for the bug and the cockroach. Whilst the information is useful in helping to explain some of the observed, interspecific differences in the toxicological behavior of DDT, the penetration curves in isolation are difficult to interpret since the effects of distribution and elimination are confounded. Interpretation is made easier when several PK processes are measured simultaneously and PD data are available, as in the studies reported by Burt and Lord (1968). These took into account penetration, sorption and detoxification of diazoxon, an organophosphate insecticide, applied to the American cockroach, and were easier to interpret. A combination of the PK measurements with neurophysiological measurements of symptoms of poisoning yielded a semi-quantitative account of the poisoning process. Exposure of exposed nerve cords to an in vitro concentration of 0.6 to 1 μM for 2 h produced neurotoxicological symptoms similar to those found in vivo 1–2 h after insects were dosed with

an LD95 of diazoxon. In these intact poisoned insects the concentrations in the hemolymph were in the range of 1 to 4 μM, indicating the importance of this tissue in transporting insecticide to the target organ, and the direct transfer of compound from the distribution phase to the target. Similar studies were undertaken by Corbitt et al. (1992) on the uptake of avermectins into the CNS of the American cockroach following injection of the insecticide into the body cavity. The concentrations of labeled avermectins in non-target and target tissues were found to be similar (in the range 3.6×10^{-8} to 5.5×10^{-8} M for testis and fat body, respectively). Increasing concentrations in the CNS were associated with increasing severity of symptoms, reflecting the findings of Leake and coworkers (1980) on the uptake of pyrethroids in the isolated CNS of the leech as discussed in more detail below. A further important finding was that for avermectins the nerve sheath played a significant role in delaying the penetration of avermectin to the site of action within the CNS.

PK approaches have been used to investigate the basis of resistance (identified by a failure to achieve control by normal field application rates of an insecticide) or enhanced tolerance that could lead to the development of resistance. Such studies can help to distinguish resistance due to PD mechanisms (e.g., site of action insensitivity to a toxicant) or PK mechanisms (e.g., enhanced detoxification, or impeded penetration), and to explain interspecific variation in tolerance. An early example of such a study is provided by that of Eldefrawi and Hoskins (1961) who investigated the association between rate of penetration, the rate of metabolism, and the toxicity of the carbamate insecticide Sevin in three insect species, the German cockroach, the milkweed bug, and the housefly. As part of the same study, they compared resistant and a susceptible strains of housefly. The pattern of interspecific differences in toxicity was associated with differences in penetration and detoxification rates. Sevin penetrates the cockroach slowly and is metabolized rapidly giving a low toxicity. In the milkweed bug it penetrates slowly but is poorly metabolized, thus producing high toxicity. Disappearance from the outer surface is rapid (75% of the applied dose in 4 h) in the housefly and this is associated with a high rate of detoxification and elimination, and a low toxicity. The major difference between the susceptible and resistant strains of housefly was the rate of detoxification, and this difference was reduced by the application of the synergist sesamex (a microsomal monooxygenase inhibitor) indicating a metabolic resistance factor.

A further example is provided by the comparison of the relative roles of penetration, metabolism and excretion of *trans*-cypermethrin in a resistant and a susceptible strain of the tobacco budworm (*Heliothis virescens*) undertaken by Little et al. (1989) to determine the nature of the resistance mechanism. There was a significantly slower penetration of pyrethroid into the resistant strain with the times for half of the topically applied material to penetrate being 11 and 30 h, respectively, in the susceptible and resistant strains, respectively. However, this was regarded as relatively unimportant compared with the large difference in detoxification rates and excretion rates. By 12 h after dosing, the resistant stain had excreted 20 times more pyrethroid in a

conjugated form than the susceptible strain. Treatment with the synergist piperonyl butoxide prevented the hydroxylation of the cypermethrin, reduced the excretion of conjugated material, and increased the level of parent compound in the resistant strain. This indicated that the resistance mechanism involved the microsomal monooxygenase system.

Similar approaches have been adopted in other studies. Evidence for a site of action insensitivity mechanism in a different resistant strain of *H. virescens* was found by Wilkinson and McCaffery (1991). Following topical application of *cis*-cypermethrin, (10 μg per insect) they found a higher exposure of the site of action in the CNS of resistant larvae, with a tissue concentration at 4 h after topical application of 46 μM; in the susceptible insects the equivalent concentration was 10 μM. Despite the larger exposure of the CNS of the resistant insects to *cis*-cypermethrin, all of the treated susceptible larvae died, whilst all of the resistant individuals survived the test, indicating a site of action insensitivity mechanism. Brealey et al. (1984) studied the PKs of DDT and transpermethrin in a number of strains of a mosquito, *Aedes aegypti*, which showed cross resistance to the two compounds. They showed that resistance to DDT was due to both a metabolic and a non-metabolic factor, but found no evidence of a metabolic factor in the resistance to the pyrethroid. No evidence of impeded cuticular penetration of insecticide was found in either species. In this study it was suggested that the non-metabolic factor was a nerve insensitivity mechanism such as the *kdr* type found in houseflies.

Whilst these studies indicate the utility of simple, focused PK experiments, and have provided answers to important questions concerning the role of PKs in determining toxicity, they have mostly concentrated on one or a small subset of the PK processes. In some cases the effects of a number of processes have been confounded. In order to avoid this problem, the PK phase has been further dissected in some studies where isolated tissues and organs have been used to investigate penetration and uptake of insecticides.

2.3 Penetration Through Isolated Cuticles

2.3.1 Rotating Diffusion Cell Studies

Cuticular penetration of a range of organic compounds through isolated cuticles of larvae of *Spodoptera littoralis* Boisd. has been investigated by Watson (1993) using a rotating diffusion cell (RDC). Cuticles prepared from final instar larvae were mounted in the RDC to investigate penetration from an infinitely large donor phase. Thirty-four compounds covering a range of molecular properties were studied. The form of the penetration curves (Fig. 1) suggested that, during the RDC assays, steady states arose within the cuticle. The steady-state flux and the time to establish steady state, the lag-time, were measured for each compound and relationships between chemical structure, molecular properties and the penetration parameters, flux and lag time, established. The study provided sufficient information to develop an

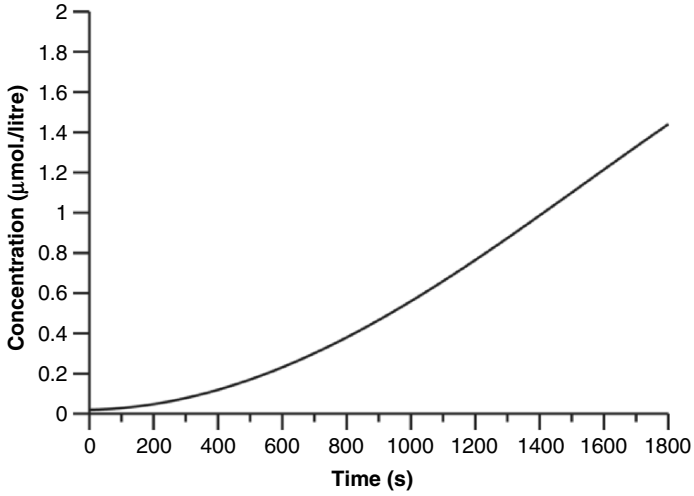

Fig. 1. Penetration in a rotating diffusion cell of a pyrethroid, permethrin, through the isolated cuticle of a sixth-instar larva of *Spodoptera littoralis*. The insecticide diffused from an ethanolic solution on the upper surface of the cuticle to an ethanolic receiving phase on the inner surface

elementary understanding of the process. The following features can be identified when the results obtained from the RDC assays are considered.

1. Penetration kinetics in the RDC are similar to those observed for diffusion across a polymer film (Crank 1975; Cussler 1984). Steady states are established within the cuticle once the epicuticle and endocuticle have retained sufficient penetrant. This process is known as "loading the cuticle".
2. Lag times vary with the polarity of a molecule, particularly its measured dipole. The size of the molecule is also a factor, long lag-times (and hence long times to establish steady state within the cuticle) being characteristic of small, polar molecules. Large hydrophobic molecules such as the pyrethroids generally have short lag-times, reflecting the speed with which a steady state is reached within the cuticle.
3. Flux is primarily determined by the molecular weight of the penetrant and its partition coefficient.
4. The properties of the cuticle may have some effect on penetration kinetics. In particular, increasing cuticle thickness is associated with longer lag times and possibly slower flux.
5. In this study, the degree of cuticle hydration, which is associated with cuticle thickness and larval weight, had little effect on penetration. This may be because, even after long periods of exposure to the dehydrating effects of ethanol, sufficient water remains within the endocuticle to maintain its hydrophilic properties.

The results are consistent with a penetration model that represents the cuticle as a bilayered membrane composed of a thin hydrophobic outer layer

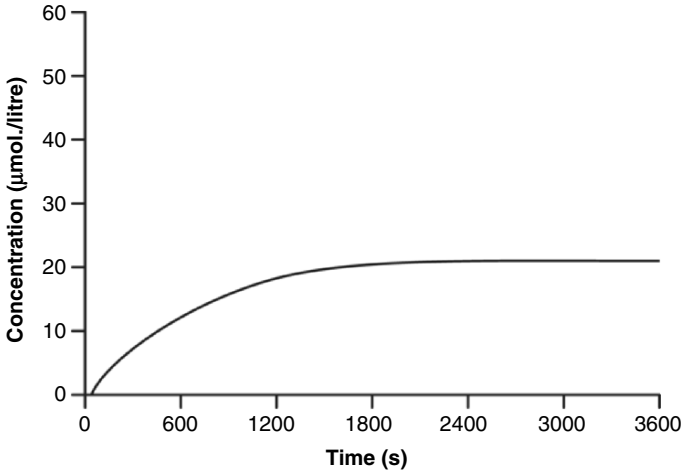

Fig. 2. Penetration in a static Ussing cell of a pyrethroid, permethrin, through the isolated cuticle of a sixth-instar larva of *Spodoptera littoralis*. The insecticide was applied as a discrete dose to the outer surface of the cuticle and accumulation in a collection medium (ethanol) in contact with the lower surface

(the epicuticle), which has a high lipid component, and a much thicker hydrophilic inner layer (the endocuticle), comprising a chitin/protein matrix and associated water. Flux appears to be controlled by partition across the epi/endo cuticular boundary and molecular size. The time taken to load the cuticle can be explained in terms of the capacitance of the wet endocuticle, which provides a large reservoir for small, polar materials.

Similar results (Fig. 2) were obtained for topical application studies obtained using small, static diffusion cells (Ussing cells). The most significant difference between these PK profiles and those obtained with the RDC is that for small, finite doses of material, an upper limit corresponding to a maximum amount penetrated is observed in the static system but not in the rotating system. By dividing this limit by the concentration calculated for the applied dose dissolved in the sink volume, an estimate can be made of the quantity retained within the cuticle. This can often represent a considerable source of loss of toxicant as discussed below.

2.3.2 Penetration of Imidacloprid (IMI) Across Isolated Gut and Cuticle

Scarr (1997) used an Ussing cell to study the penetration of IMI through the isolated cuticle of *S. littoralis* larvae. The protocol was based on the method of Watson (1993) using a simple diffusion cell containing 3 ml of receptor medium. For the isolated gut flow cell, a whole intact gut was removed by cuts at the oesophagus and the anus. At zero time, 1 µl of [^{14}C] IMI (390 ng) was injected into the crop, the oesophagus and anus sealed with silk thread and the

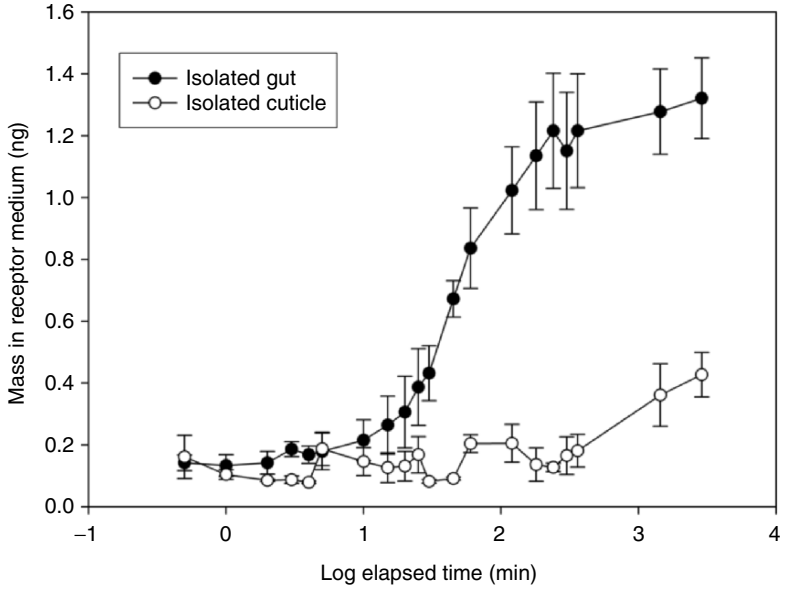

Fig. 3. Penetration in an Ussing cell of imidacloprid through isolated cuticle of a sixth-instar larva of *Spodoptera littoralis*, and through the wall of an isolated gut. The insecticide (390 ng) was applied to the external surface of the cuticle and into the crop lumen, respectively, in the two systems. In both preparations, accumulation in the receptor medium (hemolymph diluted with a trehalose solution) was measured with elapsed time

gut sac and suspended in the receptor medium. Both flow cells were stirred continuously. In both experiments, the receptor medium consisted of 1.5 ml of *S. littoralis* hemolymph and 1.5 ml of a solution containing trehalose (Sigma) (2% w/v) and (0.1% w/v) phenylthiourea (Sigma) to inhibit clotting and oxidation of the hemolymph. The results are shown in Fig. 3. Penetration through the gut wall shows a much smaller lag phase (around 10 min) than that (approximately 2 h) observed for penetration through the isolated cuticle. The overall rate of penetration through the gut is much more rapid than through the cuticle, and in this system, where there is no loss, the gut contents approach equilibrium with the receiving phase after 24 h. These observations are consistent with the observed difference in the speed of action of IMI in intact larvae of *S. littoralis* following oral and topical dosing (Scarr et al. 1994). The onset of vomiting, an early symptom of poisoning, was more rapid with oral dosing (after 3–6 min) than with topical application (onset within 1.5 h).

2.3.3 Retention of Applied IMI in the Integument of S. littoralis

An experiment was undertaken to investigate the capacity of the cuticle of *S. littoralis* to retain IMI. Two hours post-application of [^{14}C] IMI to the

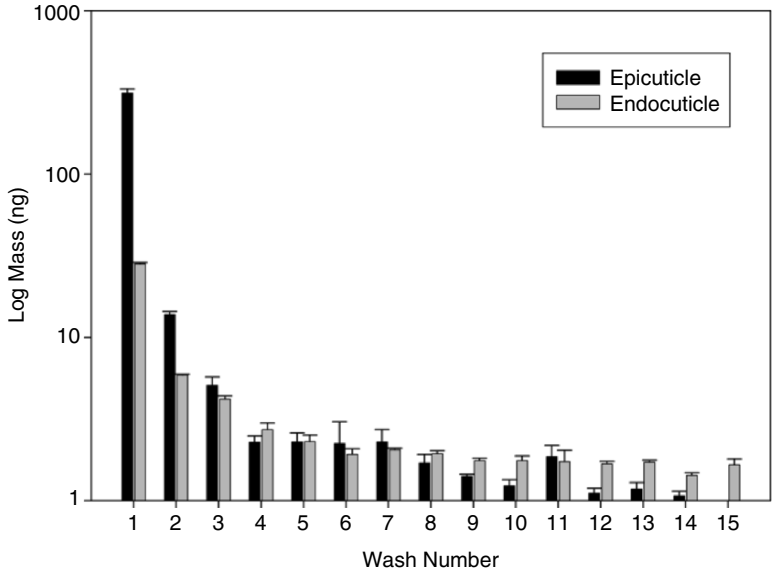

Fig. 4. Recovery at an elapsed time of 2 h of imidacloprid in 15 sequential washes (2 ml acetone) of cuticle isolated from sixth-instar larvae of *Spodoptera littoralis* and dosed (390 ng) on the epicuticular surface or the endocuticular surface

epicuticular face of an isolated rectangle of the cuticle of *S. littoralis* larvae, 83% of the applied dose was recovered in 15 acetone washes (Fig. 4). The largest fraction (73%) of the dose was recovered in the first wash and the remaining 10% in the subsequent 14 washes. This contrasts with the results obtained following application to the inner cuticle surface. Two hours post-application to the endocuticular face, only 20% of the applied dose was recovered in the washes. The balance (80%) of the mass applied to the endocuticle was subsequently recovered by grinding in liquid nitrogen and extraction of the resulting powder with acetone. Only a further 7% of the dose applied to the epicuticle was recovered by this treatment. Moreover, IMI in direct contact with the endocuticle is retained more extensively than that applied to the epicuticular surface and for which unfavorable partition across the epicuticular/ endocuticular interface will restrict access of IMI to the endocuticle. These intriguing results suggest that the capacity of the cuticle of lepidopteran larvae for small, polar compounds such as IMI is large, a result that is consistent with the conclusions of Watson (1993).

2.3.4 Uptake by Isolated Target Tissue

There are few in vitro studies of the uptake of insecticide into tissues and organs including the target tissue. An exception is the work of Oppenoorth and Van Der Plas (1986) who noted a steady rise in the level of toxicant in

a range of insect tissues bathed in aqueous solutions of insecticides. They proposed using the steady state tissue concentrations to obtain tissue partition coefficients with which to rank the affinities of compounds for particular tissues. Leake et al. (1980) performed similar experiments to follow uptake of two pyrethroids, namely NRDC157 (Mixed isomers of 3-phenoxybenzyl 3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate) and NRDC 161 (deltamethrin: (*S*)-α-cyano-3-phenoxybenzyl (1*R*,3*R*)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate) by isolated leech ganglia. Leake and her colleagues also noted an increase in tissue concentration with time (Phase 1) to approach a limiting value when steady state was presumably established (Phase 2). This uptake process was slow, however, and did not reach completion until 3 h after initial exposure to insecticide. The rates of uptake differed between the two compounds. These workers showed that at the times of onset of depolarization (13 min for NRDC161 and 194 min for NRDC157), very similar concentrations (400–800 pg per ganglion, 8–16 μM) were observed, suggesting that PD activity of these compounds is similar. This concentration of NRDC 157 in the leech target tissue is similar to that found in the central nervous systems of several insect species exposed to this pyrethroid insecticide (Brealey 1988) and to the concentration of cypermethrin found in the nervous system of larvae of *Spodoptera littoralis* (Ford 1988). However, the steady-state levels are much higher for NRDC161, an example of PK enhancement. Thus differences in the activity at the site of action can be attributed to the PK profiles, rather than PDs. Later studies by Leake et al. (1985) investigated tissue uptake in the isolated ganglia of *Hirudo medicinalis*. The movement of deltamethrin into ganglia perfused by an aqueous 'solution' of deltamethrin was investigated. Time-dose-response (tdr) data were observed for these perfused bathing assays. Assuming that movement of deltamethrin into these isolated ganglia followed the kinetics of diffusion into an infinite plane sheet, the various combinations of time and dose at which the onset of neurophysiological symptoms first appeared, corresponded to a fixed concentration (42 pM) that had accumulated at a site 75 μm into the ganglion. The boundary between the nerve cell bodies and the neuropile, where the nerve cell bodies attenuate to form the axon is found at this location. This site is known as the axon hillock, where there is a high density of cation channels, where pyrethroids are thought to act (Chalmers 1983).

3 Compartmental Modeling

General PK models are available in mammals where model organisms (particularly rats, mice, rabbits, and dogs) have been used over many years in the early stages of product development to assess the toxicological and PK properties of candidate compounds. These enable extrapolation to humans and the data obtained form the basis of in silico predictive models for drug

design. In contrast, for insects there are no such general models. This may, in part, be due to the small size of the animals that makes PK studies technically difficult and also to the diverse range of body form (from vermiform larvae to free flying adults) and physiological properties. The species used in PK studies have generally been chosen because of the availability of relevant toxicity data, or because of their pest status.

4 Simple Models

4.1 Two-Compartment PK Models

Where small insects such as mustard beetles, which weigh around 10 mg, have been used as the model species, then simple models based on the masses of insecticide remaining at the surface and the mass of penetrated material have been applied (Welling and Paterson 1985). An example of such a model is provided by the study of the PKs of pyrethroid insecticides following topical application to adult mustard beetles (Ford et al. 1981a and 1981b). The experimental method involved dosing the insecticide onto the external surface of the insects in a drop of acetone. The insect surface was then washed in methanol at a range of elapsed time. The washed insects were then ground in solvent to recover any material not recovered in the external rinses. The extracts were analyzed using GC to determine masses of the parent compound recovered. The mathematical representation used was based on a two-compartment model (Fig. 5), where material was considered to move from the external surface (compartment 1) into the body of the insect (compartment 2) from which elimination took place. Analytical solutions were available for two differential equations describing changes in the amount of insecticide on the surface and that in the insect body. There were three parameters (k_p, a first-order penetration rate constant, k_e, a first-order

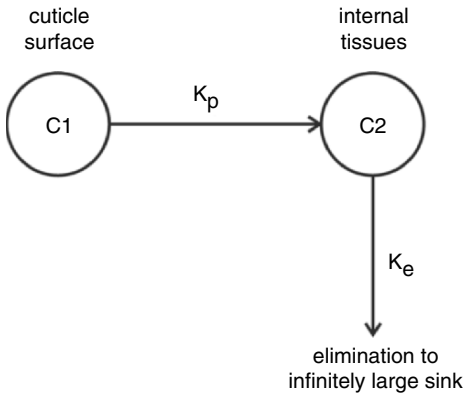

Fig. 5. A two-compartment model describing the penetration and elimination of topically applied insecticide. Movement from the insect surface to the internal tissues is determined by a first-order rate constant (k_p) for penetration, and a first order rate constant (k_e) for elimination from the internal compartment

elimination rate constant, and λ, a measure of the relative volumes and affinities of the two compartments) to be determined. The model gave acceptable fits for a set of 24 pyrethroid insecticides.

The rate of loss of insecticide from the external surface was determined by two processes; distribution to all internal tissues, and topping up the loss from the system. At any elapsed time, the level of toxicant in the internal compartment reflected the balance between the rate of penetration and the rate of elimination. The area under the PK profile in the internal compartment provides a measure of the exposure of the site of action (as well as the exposure of non-target tissues). A set of toxicity measurements was available for end-point mortality (occurring after days when all of the insecticide has been eliminated from the system), and knockdown (severe incoordination that occurs early, usually within minutes, in the poisoning process for many pyrethroids). For this series of compounds (methylbenzyl chrysanthemates), the authors found that 84% of the variation in endpoint toxicity between compounds could be explained by resistance to elimination ($1/k_e$), and that the remaining variation was attributable to PD activity. However, the authors could not be sure whether all of the residual variation that remained after accounting for $1/k_e$ was due to PDs, since the exposure of the site of action may have been modified by the properties of non-target tissues that form the bulk of the insect body. This could not be determined directly for these small insects.

PK profiles similar in shape to those found in the above study have been observed for a wide range of insect species (Ford 1972). The advantages of the simple modeling approach exemplified above are that the experiments are relatively easy to perform, analytical solutions are available, and unique solutions can be obtained. The quantitative estimates of the parameters can be used to gain understanding of the overall PK processes. For a series of closely related compounds, by using well-tried chemoinformatic methods, the PK parameter estimates can be used to generate predictive models to identify combinations of physicochemical properties that increase toxicity. The disadvantage is that there is some uncertainty associated with the interpretation of the PK data, since all of the organ systems of the animals are pooled. Thus the relative importance of these organs and their contributions to the overall intoxication processes are confounded.

5 Complex Models

Conceptual PK models that reduce the insect body and site(s) of application to a series of connected compartments can be translated into mathematical models comprising a series of linked, simultaneous equations, where typically exponential relationships are assumed. The number of equations necessary increases with the complexity of the model and the number of compartments considered. Analytical solutions are feasible for the simpler

systems, up to three compartments. However, for more complex models, it is usually necessary to use numerical methods to obtain solutions. Where the profiles of a number of compartments are similar, then those tissues can be combined for the purposes of the conceptual model, its formulation in terms of a reduced set of equations to be fitted, and of the collection of laboratory data.

Both formulation of the models and the associated experiments necessary to collect the PK data describing the PK profiles of the various tissues (compartments) become yet more challenging when the symptoms of poisoning impact on the insect's physiology and biochemistry. Under these circumstances, the PK parameters describing the processes may change as a function of exposure time, possibly in a non-linear manner. Despite these difficulties, physiologically based PK models are worth developing, since they offer insight into the processes that are important for the action of an insecticide or candidate compound.

5.1 The Complexity of the Insect Body Plan

In order to make use of the more complex models and to be able to interpret the results, it is necessary to measure some of the properties of the various tissue compartments. The insect body plan can take a wide variety of forms from vermiform larvae to flying adults. These have many features in common (Fig. 6). A central hemocoel divided by septa provides an efficient circulatory system that ensures that the hemolymph (equivalent to vertebrate blood) contacts all other tissues. The pump in this system is typically a pulsatile tube located dorsal to the gut and running the length of the animal. This pumps hemolymph in a posterior to anterior direction, and a non-contractile extension (the aorta) opens behind the brain to direct blood into the hemocoelic sinuses. The circulation times are variable depending on temperature and locomotory and ventilatory movements (both of the latter aid circulation). There is evidence from the dispersal of injected dyes and spores in infected insects that circulation times are in the order of 1–3 min (Beard 1953; Parker 1992). The heart rate in resting adult male American cockroaches is 130 beats per minute, and the stroke volume 1.9 µl (Tsai et al. 2004). This means that 247 µl of hemolymph are pumped per minute. Assuming that the insect

Fig. 6. A schematic diagram of the insect body indicating the anatomical relationships between the various organ systems: *1* the central nervous system (CNS); *2* the gut; *3* the heart; *4* the aorta; *5* the Malpighian tubules

weighs approximately 0.8 g, with a hemolymph volume of 10% bodyweight then the whole hemolymph volume would be pumped three times per minute. This provides for rapid transport of any insecticide from the point of contact, usually cuticle surface or gut, to all other tissues. There is a central nervous system typically comprising two large ganglia in the head (the "brain") between which the esophagus runs. These, like all of the smaller ganglia that are found along the length of the body, are joined by the double nerve cord. In contrast to vertebrates, the nerve cord in insects is located ventrally. The gut runs from an anterior mouth to a terminally located anus. The excretory system is made up of the Malpighian tubules that form the primary urine by secretion (as opposed to ultrafiltration as found in most vertebrates) and modify it as it moves along the tubules as occurs in the vertebrate nephron. The urine is discharged into the gut at the junction of the mid and hindguts. Further modification of the urine then takes place in the hindgut and rectum.

There are some major structural differences between insect forms, such as the vermiform larvae and the free-flying adults. One major difference is the use of a rigid cuticle as the skeleton in the latter, and a hydrostatic skeleton in the former where the cuticle is flexible. Rigid cuticles tend to contain considerably less water than flexible cuticles, for which imbibed water acts as a swelling agent and lubricant and which permits cuticular movement during, for example, locomotion. A major difference between adults and larvae is the state of development of the gonads and genitalia. Reproductive products form a large proportion of the body mass of some adult insects (16% in female locusts) and so may form a major sink for insecticide, and a route of loss of applied material from the body when eggs are laid. Depending on the location of the target site for an insecticide, some or most of the tissues can compete with the target tissue(s) for active material. For example, insects have fat bodies that are located throughout the body, are used to store lipids, and may act as a sink for lipophilic compounds. Knowledge of the sizes and properties of the tissues can therefore give guidance as to their importance in determining the PK phase of poisoning.

The various insect tissues can be dissected from individuals either as a separate exercise, or at a range of elapsed times after dosing with a compound in PK experiments. Once isolated, the tissues can be weighed, and in dosed insects extracted in a solvent for later analysis to determine the mass of parent compound accumulated, or the tissue can be placed on a cone for combustion where radio-labeled material has been used. In the latter case, total label will be measured, and this will include both parent compound and detoxification products. Separate experiments are then needed to determine the proportion of parent compound remaining as poisoning progresses. Values of tissue weights and the steady-state concentrations of some pesticides that have accumulated in a range of tissues are summarized in Table 1.

Table 1. Wet and dry weights of some tissues of larvae (200 mg) of *Spodoptera littoralis*, and steady-state tissue concentrations following topical application of a non-polar insecticide, cypermethrin (146 ng per insect), and a relatively polar compound, imidacloprid (390 ng)

Tissue	Tissue weight		Concentration (μM)	
	Wet weight (mg)	Dry weight (mg)	Cypermethrin	Imidacloprid
Hemolymph	57	1.5	0.0079	0.56
Gut wall	23	3	0.46	1.8
Gut contents	48	10	0.29	2.5
Fat body	21	2	0.033	2.1*
Cuticle	46	5	0.044	1.9
Nerve cord	1.4	0.5	1.7	0.86
Salivary gland	2.2	0.4	0.66	_

*Fat body and muscle combined

5.2 A Three-Compartment Model and Oscillatory Movement of Material Between Tissue Compartments

There are few published studies where complex PK models have been applied to insects, and insect anatomical compartments and physiological properties are poorly parameterized. In one study (Peace et al. 1987) of the toxicodynamics of pyrethroids in adult mustard beetles, a three-compartment model (Fig. 7)

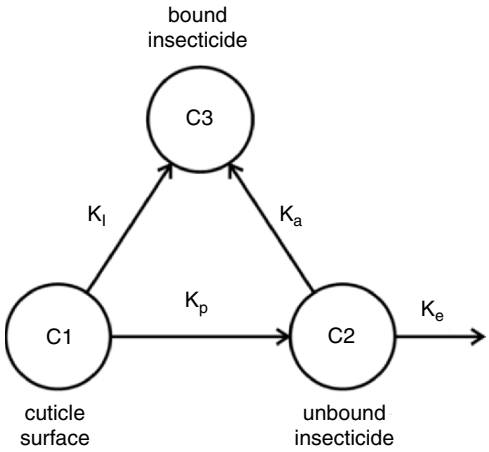

Fig. 7. A general three-compartment model describing the penetration, binding and elimination of topically applied insecticides in insects, where penetration from the surface of the cuticle to internal tissues (other than binding sites) is determined by a first-order rate constant (k_p) and movement to binding sites from the surface and from internal tissues, respectively, by first-order rate constants k_I and k_a, and elimination by k_e

was proposed to include an unidentified part of the internal tissues where non-specific binding could occur. These ideas were further developed (Greenwood et al. 1990) in a study using lepidopteran larvae to investigate the observed anomalies in fits obtained to wash off data for a two-compartment model. An analytical solution was obtained for the set of three linked differential equations that described this system. Solution of the equations required finding the three roots of a cubic equation. There were two sets of solutions to the auxiliary equations: one where all three roots were real, and one where one root was real, and the other two were complex conjugate. The second set of solutions was complex and had the form of a damped oscillation. This was interpreted as resulting from the linkage of the binding sites to site of application on the external surface, and generating recycling of material bound to the internal tissues. Oscillations in the levels of insecticide in the internal compartment have been observed in a number of studies but because of the large variation between individuals it has not been possible to demonstrate statistically that this is a real effect. However, supporting evidence can be found in the studies of Burt and Goodchild (1974) and Burt et al. (1977) who observed fluctuations in the values of ED50 for the pyrethroids (*Z*)-(*S*)-2-methyl-4-oxo-3-(penta-2,4-dienyl) cyclopent-2-enyl (1*R*,3*R*)-2,2-dimethyl-3-(2-methylprop-1-enyl) cyclopropanecarboxylate (pyrethrin I), and (*S*)-α-cyano-3-phenoxybenzyl (1*R*,3*R*)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate (deltamethrin or NRDC161) at long times after dosing adult *Periplaneta americana* and for pyrethrin I topically applied to adult *Musca domestica*. These fluctuations took the form of damped oscillations and may have been the result of oscillations in the level of toxicant in the target tissue containing the site of action.

5.3 Physiological Models

An early attempt to produce a physiological model for lepidopteran larvae is described in Greenwood et al. 1990. In this study, the system was divided into eight compartments (Fig. 8) and movement of material represented by a set of eight linked differential equations. In insects, the hemolymph provides the main mass transport system around the insect body and, since it contacts every other tissue, it formed the central compartment of this mamillary model. It was not possible to obtain an analytical solution for this model, and it was not fitted numerically. However, a model of this complexity does provide a conceptual framework for investigating the PKs of insecticides in insects and identifies parameters that could be estimated empirically from PK experiments.

An earlier study (McFarlane et al. 1977) showed how mathematical modeling based on a physiological representation of the housefly (*M. domestica*) could be used as a management tool to assist the development of novel insecticides. In this example, the model comprised four compartments and was

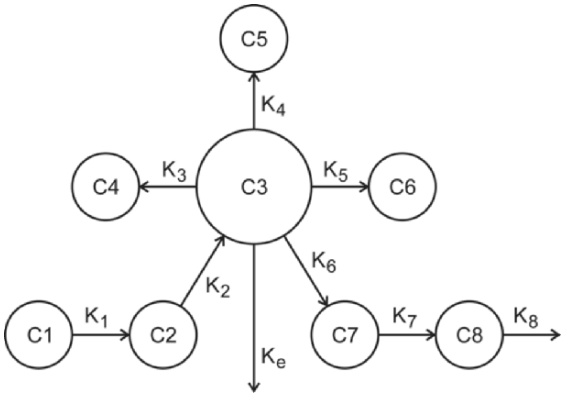

Fig. 8. A physiologically based eight-compartment physiological model describing insecticide pharmacokinetics in insects where the hemolymph (C3), the main distributive phase, forms the centre of a mamillary system: *C1* is the cuticular surface (the site of dosing), *C2* comprises the rest of the cuticle, *C4* the salivary glands, *C5* the fat body, *C6* the nervous system, *C7* the gut wall, and *C8* the gut contents, via which elimination from the body occurs

parameterized using a basis set of 12 rate constants, four compartment volumes and two concentrations Q_0 and E_0 representing the initial conditions with respect to the concentration of insecticide applied (at zero time) and the amount of free target receptor, the enzyme acetyl cholinesterase, respectively. The calibration for adult houseflies was estimated using *N*-methylcarbamoyl oxime. The authors concluded that *models of this form have considerable potential for establishing the important areas of study in an insecticide research programme by examining the sensitivity of the overall effect to changes in each parameter* and may *ultimately assist in the determination of correlations between laboratory and field data*.

5.3.1 Movement Between Tissues, Tissue Equilibria and Routes of Loss

When a compound comes into contact with the insect via the body surface, gut, or tracheal system, then it is available to a number of processes. Following diffusion from the site of contact, through the integument and into the hemolymph it is then potentially available to every tissue in the body. The rate of diffusion from the contact site to the hemolymph is maintained by the mass transport of compound from the inner surface, and the effective concentration in the hemolymph is kept low by binding to protein and lipid in the hemolymph and by movement to other tissues bathed by the hemolymph. Since the primary urine is formed by secretion from the hemolymph, unmodified compound can be eliminated directly in the urine. Alternatively, following diffusion into the gut, material can be lost in the feces, or in regurgitant where the poisoning process induces vomiting. Once in contact with the various

insect tissues, the parent compound can be detoxified, and end products of this metabolism can be eliminated in urine and feces. There is competition between the various tissues, both target and non-target, and the hemolymph. As steady state is achieved in the later stages of poisoning, when the distribution process is complete and penetration is matching the rate of loss, then the relative concentrations in the hemolymph and other tissues will reflect a partitioning between them.

5.3.2 Relative Tissue Affinities and Partitioning

The relative affinity of the tissues for a compound with a particular set of properties has been found to be related to the ratio of water to organic content of the tissues. An example of the relationship between the steady-state concentrations of the pyrethroid cypermethrin in various tissues of larvae of *Spodoptera littoralis* Boisd. and the water to organic content is shown in Fig. 9.

For this lipophilic compound ($\log K_{OW} = 6.6$), the affinity of the target tissue, the nervous system, relative to that of the distribution phase, the hemolymph, is high. This reflects its high ratio of organic material to water, which is measured as the ratio of its dry weight divided by its wet weight minus dry weight (this ratio may be regarded as a tissue partition coefficient). Other tissues fall in an intermediate region. There is a wide range (0.002–57 μM) of steady-state concentrations in the various tissues (Table 1). These observations are shown most clearly for *S. littoralis* larvae, for which seven tissues are plotted. The steady-state levels of cypermethrin that have accumulated in tissues of different species of insects produces a linear relationship when plotted (Fig. 9) against log (dry weight/wet weight–dry weight: log Kw/p). The same tissues sampled from different species cluster in similar regions of the two dimensional space of the graph. These results suggest that it should be possible to predict tissue affinities from knowledge of their wet and dry weights and the $\log K_{OW}$ of the compound. For more polar insecticides, the relative affinities of the tissues will be markedly different and such insecticides are expected to partition more favorably into wetter tissues such as the hemolymph to reach higher steady-state levels. This is confirmed by the results of Scarr (1997) for steady-state levels of the polar material imidacloprid (IMI), $\log K_{OW} = 0.57$, in a set of tissues of different water content (Table 1). In this example, we see the same division into wet and dry tissues and those of intermediate water content. Such partition behavior can lead to PK enhancement (amplification) of toxicity, where higher concentrations of insecticide accumulate preferentially in the target tissue (Greenwood et al. 1990). The distribution of the compound between tissues according to their organic content appears to be a general feature of insect PKs and is also likely to apply in vertebrates. This could have important implications for insecticide design and assessment of risk to non-target species during the registration of a novel insecticide.

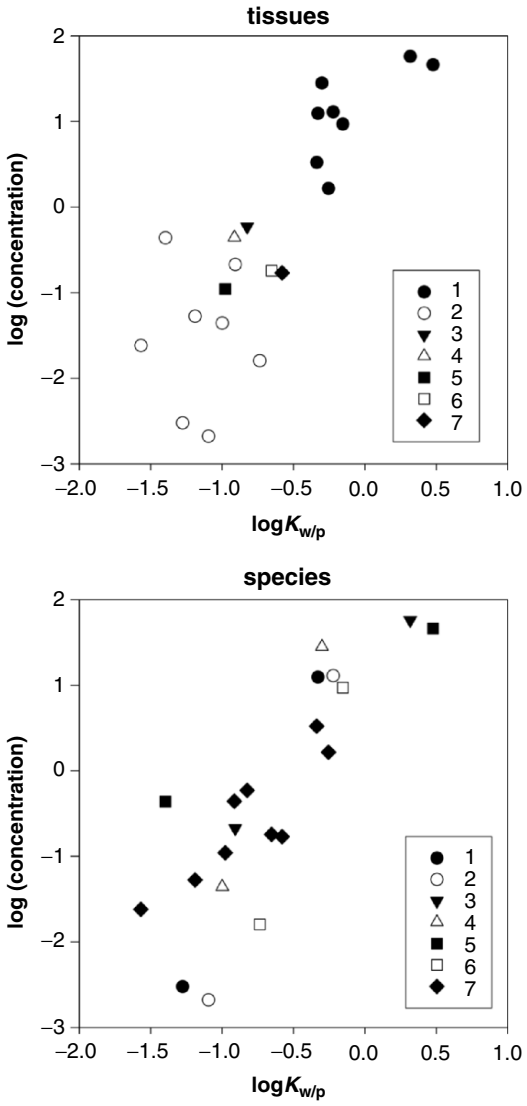

Fig. 9. The association between the concentration of cypermethrin in various tissues of a number of insect species and the ratio of organic matter to water content of the tissues. Key: tissue (*1*) the central nervous system, (*2*) hemolymph, (*3*) gut wall, (*4*) gut contents, 4 cuticle, (*5*) cuticle, (*6*) fat body and (*7*) salivary gland. Concentrations in central nervous system (higher value in every case) and haemolymph of the species (*1*) *Helicoverpa armigera* (larva), (*2*) *Agrotis segetum*, (*3*) *Diabrotica undecimpunctata* (nymph), (*4*) *Musca domestica* (adult), (*5*) *Musca domestica* (larva), (*6*) *Periplaneta americana* (nymph), and (*7*) *Spodoptera littoralis* (larva)

5.4 Potential Use of Pharmacokinetics in Insecticide Design—the Way Ahead

The studies reviewed in this short chapter have shown the requirement for PK processes to be taken full account of when designing novel candidate insecticides with improved environmental safety and toxicity profiles. The pharmaceutical industry already gives ADMET studies high priority during the development of improved drugs. Similar developments are also under investigation by the crop-protection industry. These methods are often based on empirical observation and employ inductive, deterministic equations for prediction of ADMET properties, often based on inductive QSAR regression models for which interpretation is difficult. What is required is a more deductive and analytical approach.

This review has highlighted the power of one such deductive approach based on the use of compartmental models for which linked differential equations can easily be formulated. A major problem with the methodology, however, arises from the difficulty and expense involved in obtaining estimates of the PK parameters that calibrate the PK profiles of a given biologically active material. This is traditionally achieved through the use of exhaustive extraction of the various tissue compartments, simple or complex, at different elapsed times using an organic solvent as extractant. The task is time-consuming and too costly to form part of a routine screening process. There is, however, an alternative approach that overcomes these problems.

5.4.1 Estimating Pharmacokinetic/Dynamic Parameters from Time/Dose/Response Data

Can the PK parameters be estimated directly from time/dose/response (tdr) data? Preliminary studies (Ford et al. 1985; Salt et al. 2003) suggest that this should in fact be possible. Consider the possibility of constructing a time-dose-response relationship (tdr) based on the notion that, for a response to occur, the internal exposure (K) of the organism to an insecticide must achieve some critical value. Ford et al. (1981a, 1981b) defined internal exposure of as the integral over time of the internal mass of insecticide. Using this representation, Salt and Ford (1984) showed that a given response, e.g., knockdown, occurred when this internal exposure reached a critical value K, where

$$K = \int_{t_L}^{t_{KD}} M_2(t)\,dt = C \int_{t_L}^{t_{KD}} \left(e^{\alpha_1 (t - t_L)} - e^{\alpha_2 (t - t_L)} \right) dt \qquad (1)$$

and where the exponents α_1 and α_2 are functions of the three PK parameters k_e, k_p and λ. C is also a function of these three parameters plus the initial dose applied M_o, and t_L is the asymptotic limiting value of the time to knockdown t_{KD} for large M_o. For given values of k_e, k_p, λ, Mo and K, Eq. (1) can be solved to obtain the predicted time to knockdown t_{KD}. Using the values of k_e, k_p and

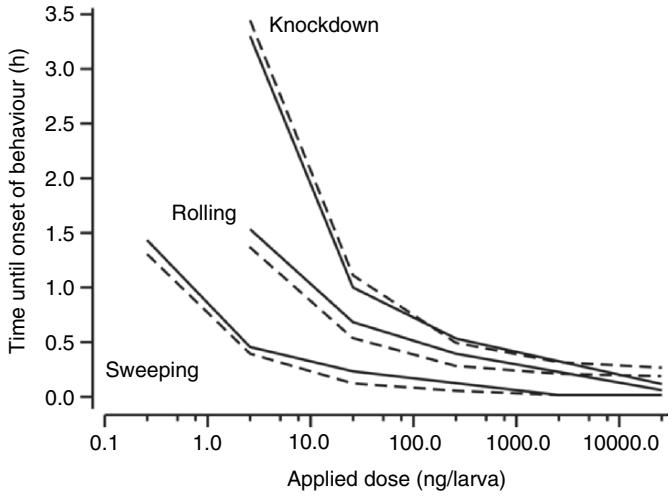

Fig. 10. Time-dose-response plots for the behavioral responses, sweeping, rolling and knockdown. *Solid line* observed, *dashed line* calculated using Eq. (1)

λ from Parker (1992) for Permethrin and the value of K found by Salt and Ford (1984), the predicted time to knockdown has been found for a range of applied doses M_o and are presented in Fig. 10 along with the observed knockdown times in sixth-instar larvae of *Spodoptera littoralis*. As can be seen, there is excellent agreement between the two sets of values. The concept of critical internal exposure has been extended to include other responses, sweeping and rolling (Fig. 10). These abnormal behavioral patterns are early symptoms of poisoning. Sweeping is a pronounced and rapid side-to-side movement of the anterior half of the body and rolling is an uncontrolled writhing over the surface of the substrate. Once again, there is good agreement between the observed times and those calculated from Eq. (1). From these results it can be seen that if we have estimates of k_e, k_p, λ, and K from the wash-off technique the calculated tdr closely follow that observed. The question arises; can the parameters of k_e, k_p, λ, and K be estimated directly from the observed time/dose/response (tdr) relationship? The answer to this is yes, but the non-linear regression approach requires good initial estimates of the parameters in order for the solution to converge.

In order to adopt this procedure for molecular design, it is necessary to obtain experimental data using insecticide topically applied over a large range of doses per insect. This range should be sufficient to establish the limiting time and dose for each compound. At each dose, the time after dosing required to attain each response is recorded to give combinations of time and dose that correspond to achieving the critical integral or internal exposure (K_{crit}) for that response. These data can then be analyzed for each response simultaneously to obtain unique estimates of the PK parameters (k_p, k_e, λ) for

the compound and the critical internal exposures associated with each toxicological response. In addition, two further parameters, the endpoint ED_{50} and the penetration lag time, can be estimated from the limiting values of dose and time that are observed experimentally. In crop-protection studies, the endpoint ED_{50} will correspond to the lowest median lethal dose (LD_{50}) that can be achieved; the lag time represents the time required to deliver a toxic concentration to the site of action within the insect. All six parameters provide important information that can be used for lead optimization and new compound registration. In this way, knowledge of the ADMET properties of a compound could be obtained from bioassay data that could be produced using routine screening procedures, at little cost and at a relatively early stage in the insecticide development process. The method is currently under submission for publication (Salt DW, Crichton R, Moyle J, Jepson P, and Ford MG).

6 Conclusions

There is a paucity of useful information available for insecticide kinetics from which broad generalizations can be inferred. Many of the publications describing the topic (going back to studies undertaken during the 1940s) provide useful data for validation of conceptual and mathematical models.

Definitive reviews have been published during this period and are valuable reading for those scientists who wish to understand the complexity of the PKs of insect species.

Mathematical models provide analytical descriptions of PKs that enable the investigator to separate the various PK processes deductively and provide a sounder basis for prediction of ADMET properties than using inductive QSAR models alone.

Such models now exist for penetration of the insect integument, tissue distribution, uptake by target tissues, and prediction of time/dose response (tdr) relationships of insecticides applied to insect species.

These tdr relationships can themselves be used to predict the ADMET properties of candidate insecticides by estimating their PK parameters based on a simple, two-compartmental model of insecticide PKs. This approach can be recommended for insecticide design and registration, provided further research is undertaken to establish and confirm the validity of the procedure and its generality of application.

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3 High-Throughput Screening and Insect Genomics for New Insecticide Leads

Paul Allenza, Russ Eldridge

1 Introduction

Feeding the world population has been a challenge in every century, and it is no different at the beginning of the new millennium. Insect damage worldwide is estimated to contribute up to 15% in pre-harvest loses to major crops (Oerke et al. 1995; Yudelman et al. 1998). The demands associated with feeding an ever-increasing human population make our ability to boost crop yields a continuing imperative. Insects cause significant damage and losses to human food, health, fiber, and structures. The cost to help control crop pests, turf and ornamental pests, house and garden insects, pests of forestry, livestock, and companion animals is approaching $10 billion per annum in conventional insecticide sales (PhilipsMcDougal 2004).

Even as the need for new insecticides increases, the discovery of new active molecules has become more challenging in our modern world; new products must meet a number of conflicting demands (Lund et al. 1989; Bloomquist 1996). New insecticides must be active on a broad range of insect pests; however, those same products have to be safe to beneficial insects, insect predators and parasites. They should also be fast-acting and highly toxic to insect pests at exceedingly low-use rates, but these same compounds must show little or no mammalian effects, even in acute and chronic toxicity studies done at high exposure rates. The insecticide should be long lasting so that it is highly effective between application intervals, but it should break down rapidly in the environment. A desirable property of new insecticides is to be internalized and circulated by target plants, but it should not be detected in groundwater or persist in the soil after application. In addition, a systemic insecticide should not accumulate to excessive amounts in harvestable portions of the plant. Growers want to be able to use an effective, safe insecticide (which passed this gauntlet of requirements) numerous times during the course of a growing season, and year after year; however, they do not want development of resistance in the targeted insect pests. A chemical control agent needs to be inexpensive, even though research and development for a new product takes more than a decade, and costs to bring a new insecticide to market is estimated to be greater than $200 million (Short 2005). Continue to find new insecticides with these demanding and conflicting characteristics, such as shown in Table 1, will require that the crop protection industry use every viable research approach at its disposal.

Insecticides Design Using Advanced Technologies (ed. by Isaac Ishaaya, Ralf Nauen and A. Rami Horowitz)

Table 1. Characteristics of an ideal insecticide

Highly efficacious
Use rates below 100 g ai/ha for field crops
Active on a wide range of insects including sucking and chewing pests, as well as ornamental, structural, and animal health pests
Active on all life stages of insect pests; egg, larvae, pupae, adult
Fast acting
Long lasting
Use on a broad range of crops under many conditions
Selective only for insect pests; no activity on beneficial arthropod predators and parasites
Safe for non target organisms
Low to no mammalian acute, oral and dermal toxicity
No carcinogenicity, mutagenicity
No developmental or reproductive toxicity
No neural toxicity
Environmentally benign; safe for birds, fish, bees
Breaks down rapidly in the environment
Inexpensive to manufacture
Easy to formulate
Easy to use
High mobility in plants
Limited mobility in soil and groundwater
Novel MOA for resistance management

2 Approaches for Insecticide Lead Identification

Traditionally, insecticides have been discovered using large-scale whole-organism screens. In this approach, chemists use standard techniques to synthesize new compounds, which are subsequently assayed for efficacy in the greenhouse on large numbers of plants using a range of insect pests. This has proven to be a highly effective method of insecticide discovery, and was responsible for the majority of insecticides that are currently on the market today, including the organophosphates, carbamates, pyrethroids, and others. Whole-organism screens have been fruitful because the insect pest contains the full range of potential target sites, both known and unknown. In addition, an insect active compound by definition contains favorable pharmacokinetic properties that can potentially be optimized further. The disadvantage to whole-insect testing is that it is also a slow and very resource-intensive approach. Greenhouse testing on an array of insect pests can require many grams of material, and thus chemists must focus on making large quantities

of compound for initial screening, rather than making new analogs that potentially could have better activity or properties. In addition, as the number of compounds to be tested increases, greenhouses, which are expensive to establish and maintain, became a limiting factor. Using this approach, maximum throughput tends to be in the hundreds of compounds per week, leading to a bottleneck in testing in the insecticide discovery process. However, a number of companies have applied industrial processes to whole organism screening to help alleviate some of these bottlenecks. Miniaturization of insect assays, artificial diets and utilization of sophisticated robotics have allowed testing of over 100,000 compounds per annum against a range of insect pests (Steinrucken and Hermann 2000). Although testing throughput has increased, the insect is necessarily viewed as a black box. Once an active compound is discovered, there may be little knowledge as to why or how compounds work against an insect pest, making optimization and registration more difficult.

Although these traditional methods have been responsible for our most effective and ubiquitous insecticides, few new modes of action have been identified over the past few decades, leading some to believe that that there might only be a finite number of effective insecticide target sites to exploit (Casida 1990; Casida and Quistad 1998). To date, traditional screening methods have produced commercial insecticides that act at fewer than 30 different target proteins or mechanisms of action (Insecticide Resistance Action Committee, MOA Classification version 5.1). Recently, genomics and high-throughput, in vitro, screening have expanded in the insecticide discovery arena and supply additional tools to allow us to find new and novel target sites, and new compounds targeting those sites. The recent sequencing of the *Drosophila* genome (Adams 2000) has revealed greater than 13,600 genes. It is unknown how many might be exploited as potential insecticide target sites, but as a point of reference, it was estimated that 177 genes are orthologs of human disease genes (Rubin et al. 2000). In addition, widespread availability of new technology in biochemical assays, combinatorial chemistry, genomics, automation, computational hardware, software, bioinformatics and high-throughput screening (HTS) all combine to allow a new approach to insecticide discovery.

As with new technologies in any discipline, applying new approaches is not easy, and can present extensive challenges. Presented here are four case studies in which high-throughput screening and insect genomics have been applied to an insecticide discovery program. In these studies, a number of different approaches to finding new insecticidal chemistry were pursued, based on established targets as well as novel targets or mechanisms of action. In the first case, high-throughput screening (HTS) methods were used to help find new chemistry scaffolds for an established target site. In the next three cases, different approaches to exploit new target sites are discussed: (1) assays developed and utilized to find potential insecticidal compounds against a novel "knowledge-based" target site, (2) screening of a large-scale

chemical library against an enzyme representing a new target site identified through a genomics approach with double stranded RNA interference, and (3) utilization of multiple biochemical assays as tools to help determine potential mode of action of insecticidal chemistries where the MOA is previously unknown.

2.1 HTS Using Established Agrochemical Targets

The capability to test large sets of compounds offers a number of advantages in the pursuit of new insecticides. It can be an effective approach for finding new compounds against proven target sites where synthesis of analogs around an active molecule has been investigated extensively, but has resulted in little progress in activity. With access to large chemical libraries of varying structures, the researcher can quickly test novel compounds and identify those few that interact with a proven target site, thus providing the scientist the potential to "jump" to new scaffolds. The new scaffolds can then be optimized for activity at the target site, and ultimately in the insect pests of interest. The new scaffolds may be more amenable to synthesis, be less expensive to manufacture, provide greater potency than the original analogs, change species spectrum, or possess new physical/chemical properties that could increase efficacy against insect pests.

Making or acquiring large numbers of compounds, and then rapidly testing them, may be the only practical route to finding those few chemistries that show activity at a target site. Of those few compounds that show activity at a target site, fewer still can be further optimized to high levels of activity in an insect pest. Depending on the screen, the compound library, and the "activity bar" that a company sets for what they define as a hit, most assays will yield a hit rate of less than 1%. It is not unusual to observe hit rates of less than 0.1% for screening of a random library of compounds against highly selective targets (Sills et al. 2002; Mercier et al. 2005). Using these methods, insecticide discovery truly is a numbers game.

Researchers at FMC (Eldridge 2002) developed an HTS assay against the insect ecdysone receptor to try to identify new, unique scaffolds at a highly researched target site. The development and use of the assay illustrates the benefits and the challenges of pursuing target site-based screening as an approach to insecticide discovery. It provides a good overview of the issues that can be encountered during the evolution of an HTS assay.

Ecdysone is the hormone responsible for molting in most insects. High hemolymph titers of 20-OH ecdysone triggers a molt, while a terpenoid compound called juvenile hormone determines the outcome of a molt. In the 1980s, researchers at Rohm and Haas discovered a new class of chemistry, the diacylhydrazines, which would mimic the effects of 20-OH ecdysone (Wing 1988; Wing and Aller 1990). The new compounds, represented by the commercial product tebufenozide (Mimic®), were the first non-steroidal

ecdysone agonists discovered. In many lepidopteran species, exposure to these compounds triggers a premature molt, and is characterized by the head capsule of the caterpillar slipping forward, occluding the mouthparts and mandibles, thus preventing the insect from feeding. These compounds were very attractive not only because they were efficacious but also because they acted at an insect-specific target site, and thus were safe for non-arthropods.

The screen to identify new molecules that acted at the ecdysone receptor was based on the ability of test compounds to compete at the ecdysone receptor with ^{3}H-Ponasterone A, a potent ecdysone analog. Initially, the receptor was obtained from a semi-purified preparation of *Drosophila* Schneider cells. In this binding assay, the receptor was incubated with the radioligand and the test compound of interest. Displacement of the ponasterone A by the test compound indicated affinity of the compound for the receptor, thereby indicating a hit. The active compound could then be titrated to determine the potency at the target site. In general, compounds were screened at a concentration of 10 μM, and any compound that reduced the ponasterone A signal by >50% was considered a hit. While the assay was relatively robust, the use of *Drosophila* Schneider cells as a source of ecdysone receptor was problematic for a number of reasons. To obtain enough receptor to run the assay at a medium throughput required large quantities of cells, material, and manpower. Cell culture reagents alone drove the cost to more than $10 per plate. Over 38 h of labor was necessary to provide enough cells to support the screening of approximately 40 microplates, representing a screening volume of little over 1,200 compounds. Screening of a large-scale compound library using this method would have been cost prohibitive.

To reduce the cost of obtaining sufficient quantities of ecdysone receptor for a large-scale screening effort, FMC researchers undertook a program to express the receptor gene from *Drosophila* and use the recombinant protein as the source of target site material. The gene was obtained through a licensing agreement with Stanford University (Koelle et al. 1991), and placed into a bacterial expression system. High levels of receptor production were achieved using this method; however, neither ponasterone A, nor 20-OH ecdysone binding was identical to that found with the native receptor. At the time, new literature references revealed that the functional ecdysone receptor was actually a heterodimeric complex consisting of the ecdysone receptor protein, and another protein from the steroid receptor superfamily called ultraspiracle (Yao et al. 1992, 1993). Both subunits are required to obtain high level binding of 20-OH ecdysone. This meant that it was necessary to clone and express multiple subunits in order to obtain a system that closely mimics that of the native receptor. Having to clone and express more than one subunit to obtain fully functional target sites is not unusual, and should be taken into account when calculating the amount of time and resources needed for developing a new HTS assay using recombinant target sites. Instead of the full-length clones, only the ligand binding domains of each

receptor were expressed in bacterial cells. When extracts of these receptor-expressing cells were mixed together, binding to ^{3}H ponasterone A and ecdysone were observed at levels reported for the native *Drosophila* receptor. The new recombinant protein preparation dropped the cost of the HTS assay significantly. The cost of reagents went from $10.00/plate to approximately $0.04/plate. In addition, labor time was reduced from 38 h to produce enough material for 40 plates, to 4 h to provide enough recombinant ecdysone receptor for 2,000 plates.

Once the assay was established, nearly 114,000 library compounds were screened against the *Drosophila* ecdysone receptor, at a rate of 500–1,000 compounds per day, with each compound tested in triplicate. The assay resulted in nearly 2,000 hits, giving a hit rate of 1.7%. The hits were grouped into 105 different scaffolds that showed not only binding activity, but some level of functional activity at the target site. Although the binding assay allowed for rapid screening of a large number of compounds, it did not reveal whether the hits are agonists or antagonists. Since the goal was to find agonists, i.e., compounds that would trigger a molt, a follow-up functional assay was required that would allow identification of agonist or antagonist activity for screening hits. A functional assay has the additional advantage that it can identify those screening hits that bind at a site on the receptor that is independent of the ligand site, but can still affect binding of the labeled ligand, and are thus false positives. The functional assay was based on a cell line that was engineered as a reporter system for ecdysteroid activity (Koelle et al. 1991). In response to ecdysone agonists, the engineered *Drosophila* Schneider cells produced high levels of β-galactosidase, which can be detected by incubating the cells with β-D-galactoside as a substrate. The assay provided a sensitive, rapid, function-based agonist system that could be used to further detail the activity of hits from the high-throughput screen. This provided another lesson: when working with binding assays, it is imperative to characterize the hits with follow-up functional assays. Development of a functional assay requires significant resources and time, but it is well worth the effort.

Of the original 105 scaffolds represented from the screening hits, compounds from 25 scaffolds showed agonist activity when tested in the functional assay. Generally, antagonists were found to greatly outnumber agonists in most assays, regardless of the target site. Although agonists numbered less than 25% of the total assay hits, many unique structures were obtained (Fig. 1). None of these structures resembled 20-OH ecdysone, or ponasterone A, the ligand used in the assay. In addition, most did not resemble the commercialized diacylhydrazine compounds, although there were a few scaffolds that were similar enough to the diacylhydrazines that this chemistry would have been detected in the assays.

Unfortunately, when the hits were tested against lepidopteran pests, they showed little whole-insect activity, regardless of whether they were an agonist or antagonist. This was also true even after optimizing for activity against the target site. A number of the optimized compounds bound to the recombinant

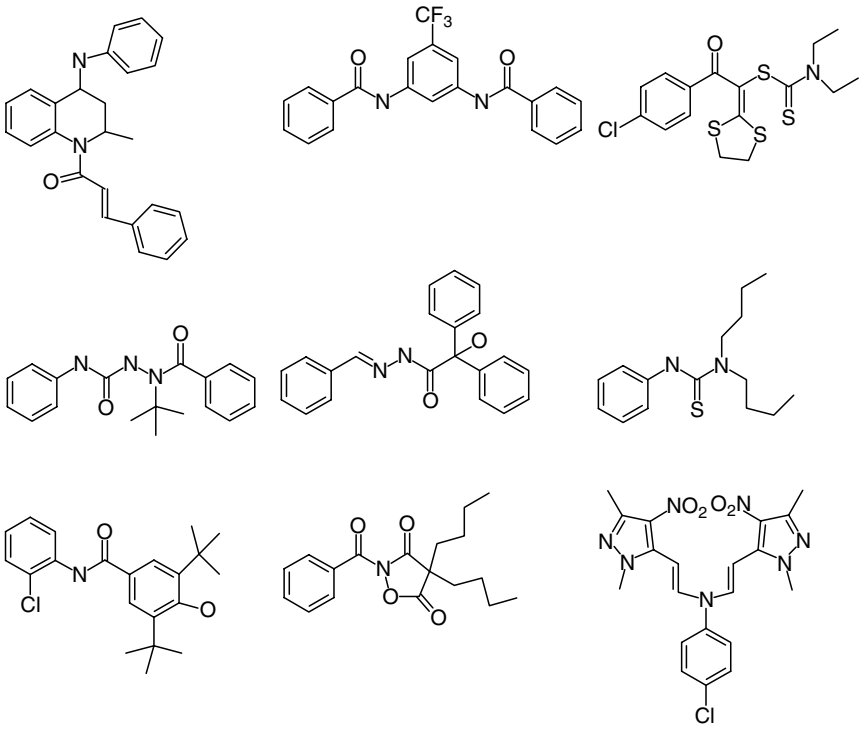

Fig. 1. Chemical scaffolds discovered in HTS assay with ecdysone receptor

Drosophila receptor with several orders of magnitude better affinity than that of 20-OH ecdysone itself, or of the commercial diacylhydrazine, tebufenozide (Mimic). It was theorized that the lack of efficacy of the compounds against lepidopteran insects was due to a fundamental difference between the *Drosophila* receptor and the receptor of the lepidopteran pest targets. To test this theory, the ecdysone and ultraspiracle genes from tobacco budworm (TBW), *Heliothis virescens,* were cloned and expressed. Some of the most potent *Drosophila* compounds were tested against the TBW receptor and many were found to have significant differences in affinity (Table 2). Based on these data, an additional 30,000-compound subset of the library was screened on the TBW receptor to uncover any new scaffolds. While many of the compounds that were hits in *Drosophila* were also found to be hits in the TBW screen, no new chemistries were identified that had not been identified in the *Drosophila* screen. In addition, there was a low correlation of binding affinity between compounds tested in the receptors from the two species (Fig. 2). While this difference can impact which compounds are found in the initial screen, it is even more important when optimizing hits on the target site. If possible, the researcher should always screen and optimize using the target site from the insect pest(s) of interest.

Table 2. Significant species-specific differences in ecdysone receptor binding affinities were observed for 20-OH ecdysone, tebufenozide (Mimic®), and one of the more potent scaffolds synthesized in the project

Structure	*Drosophila* IC50 (nM)	TBW IC50 (nM)
20-OH ecdysone	87 ± 5	404 ± 10
Tebufenozide (Mimic®)	334 ± 63	3.0 ± 0.2
FMC lead	<1	168 ± 6

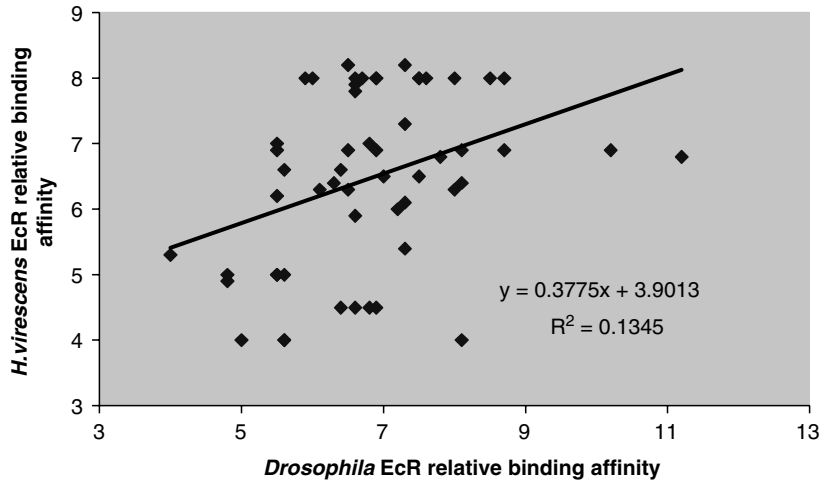

Fig. 2. Binding affinity at the *Drosophila* ecdysone receptor (EcR) does not predict affinity at the *Heliothis virescens* ecdysone receptor over a number of chemistry scaffolds

2.2 Insecticides Based on New Insect Targets

With the aforementioned characteristics for an ideal insecticide in mind (this may be used to define the insecticide of the future), it is reasonable to conclude that many of the successful new insecticides will interact with new targets. High-throughput screening with an established target may significantly improve insect spectrum, efficacy, safety, or change physical-chemical properties that affect environmental fate. Unless the assay allows for identification of compounds that interact at a new site on the target, it is unlikely to move one to a new mechanism of action and overcome insect resistance problems or cumulative toxicological concerns. Most agrochemical companies balance their research approach to actively seek chemistry affecting new targets and in doing this become irrevocably linked to genomics and modern insect biochemical methods, especially in the pursuit of validated insecticide targets. By definition, a truly new target cannot be linked to an effective commercial

insecticide and thus one can never be certain that a chemical that interacts with this target can have the characteristics of a "good" insecticide. A number of approaches are necessary to increase the probability of identifying a "valid" target to investigate and exploit.

Recognizing a new target site, and understanding MOA is of critical importance. Table 3 (from Insecticide Resistance Action Committee, MOA

Table 3. Grouping of MOAs for commercial insecticides according to IRAC (Insecticide Resistance Action Committee, MOA Classification version 5.1)

1. Acetylcholine esterase inhibitors
2. GABA-gated chloride channel antagonists
3. Sodium channel modulators
4. Nicotinic Acetylcholine receptor agonists / antagonists
5. Nicotinic acetylcholine receptor agonists (not in group 4)
6. Chloride channel activators
7. Juvenile hormone mimics
8. Unknown or non-specific mode of action (fumigants)
9. Unknown or non-specific mode of action (selective feeding blockers)
10. Unknown or non-specific mode of action (mite growth inhibitors)
11. Microbial disruptors of insect midgut membranes
12. Inhibitors of oxidative phosphorylation, disruptors of ATP formation
13. Uncoupler of oxidative phosphorylation via disruption of H proton gradient
14. [vacant]
15. Inhibitors of chitin biosynthesis, type 0, Lepidopteran
16. Inhibitors of chitin biosynthesis, type 1, Homopteran
17. Moulting disruptor, Dipteran
18. Ecdysone agonist / moulting disruptor
19. Octopaminergic agonist
20. Mitochondrial complex III electron transport inhibitors (coupling site II)
21. Mitochondrial complex I electron transport inhibitors
22. Voltage-dependent sodium channel blockers
23. Inhibitors of lipid synthesis
24. Mitochondrial complex IV electron transport inhibitors
25. Neuronal inhibitors (unknown MOA)
26. Aconitase inhibitors
27. Synergists
28. Ryanodine receptor modulator
29. Compounds with unknown mode of action
30. Miscellaneous non-specific (multi-site) inhibitors

Classification, version 5.1) shows the mechanism of action for commercial insecticides. Only those insecticides that have been developed commercially are relevant for insecticide resistance; a new target does not have to mean that it is new to science. Within a single class of insecticides, one finds multiple examples where the MOA is further broken down. An example is the sodium channel where pyrethroids (with over 40 active ingredients), pyrethrins and DDT all act. A separate classification as "voltage-dependent sodium channel blockers" is held alone by indoxacarb. Given only this information, it is unclear whether the insect sodium channel still presents new targets to exploit in the future. One can say that it is over-represented with close to 50 active ingredients, or it can be argued that only four classes of "chemical sub-groups" have been identified for such a critical MOA, so the complex sodium channel is a target worth further attention. The latter may indeed be the case, as recent work suggests that metaflumizone (Munger et al. 2006) is in fact an example of an insecticide that may act differently through blockage of the sodium channel causing a relaxed paralysis.

A review of known insecticide targets (Insecticide Resistance Action Committee, MOA Classification version 5.1) also reveals few modulators of the ryanodine receptor. A closer look at the receptor would make it difficult to conclude that new insecticide target sites might not be found. The *Drosophila* ryanodine receptor (Xu et al. 2000) is a large, complex (multi-domain) protein of 5,134 amino acids and shares about 45% identity with ryanodine receptors from mammalian cells. The protein has a large cytoplasmic domain at the amino-terminus and a small transmembrane domain at the carboxyl-terminal end. Clearly this could provide novel target sites (no resistance) and novel chemistry that affect this receptor and its critical cell function (calcium homeostasis). A newer class of chemistry, the anthranilic diamides have recently been described (Lahm et al. 2005) which act on the ryanodine receptor. This further supports the idea that this target may continue to be used to identify additional new commercial insecticides beyond the existing phthalamide flubendiamide or ryania extracts.

It is interesting to note that market and regulatory pressures could result in an increasingly important role for genomics in the insecticide discovery process. Through genomic processes there is an increased likelihood that specific target sites will be found that have lower levels of genetic homology to non-target species. While this may lead to safer insecticides, this target specificity could also result in a trend towards more "narrow spectrum" insecticides.

2.2.1 Knowledge-Based New Target Identification

Moving beyond exploitation of known modes of action for the identification of novel target sites, is the identification of targets representing a new mode of action altogether. A common method of such new MOA or target

identification can be referred to as knowledge-based. Here, one utilizes knowledge about insect physiology and neurobiology combined with knowledge about insecticide action to arrive at new potential targets. This approach could have taken one from knowledge of acetylcholinesterase inhibitors, exemplified by organophosphates and carbamates (which cause a sustained increase in acetylcholine neurotransmitter concentration), to a target-based search for agonists of the nicotinic acetylcholine receptor (to cause a acetylcholine-like stimulation). Obviously, the successful class of neonicotiniod insecticides provides 20/20 hindsight validation to this approach.

This logic can be applied to further examine the validated GABA-gated chloride channel and successful commercial antagonists (two classes; cyclodiene organochlorines and fipronil, with seven total active ingredients). Using a reasonable knowledge of biochemistry, one can define alternative methods to affect the same physiological process in an insect and hypothesize that inhibiting the function of glutamate decarboxylase (GAD) so as to prevent the synthesis of GABA could have a similar (inhibitory) effect, as does a receptor antagonist. However, FMC took a different approach than they had for the ecdysone receptor for development of the GAD assay and development and screening on this assay outlines some advantages and disadvantages of using genomics and HTS for discovery of new insecticides.

GABA is the major inhibitory neurotransmitter of the central nervous system and neuromuscular junction in insects (Sattelle 1990). Interference of GABA function is the mode of action for a number of prominent natural and synthetic insecticides, including avermetics, milbemycins and polychlorinated hydrocarbons such as chlordane, endosulfan, and dieldrin. While these compounds are thought to act at the GABA-gated chloride channel itself, any interference with the normal function or production of the GABAergic system should cause catastrophic effects in insects. The synthesis of GABA in insects is controlled by the enzyme glutamate decarboxylase (GAD) that catalyzes the formation of GABA from L-glutamate, and is the rate-limiting step of GABA metabolism in neurons (Roberts and Kuriyama 1968). Significantly inhibiting this enzyme should decrease the physiological concentration of GABA in the insect, potentially leading to insect death, thus making this a worthy target to clone, express and develop an assay for screening of potential insecticidal compounds.

The first step in developing a GAD assay was to clone the gene from a prominent insect pest, in this case the cotton aphid, *Aphis gossypii*. The published *Drosophila* gene sequence (Jackson et al. 1990) was used to design primers to isolate the full length cDNA clone from a proprietary cotton aphid gene library, and the gene was placed into a bacterial expression vector that allowed for production of relatively high levels of the GAD protein. The next step was to identify or create an assay method that would be amenable to HTS. Despite the importance of GAD in both insects and mammals, there was no published assay format that could easily be miniaturized to a HTS method. This is not unusual in development of assays for insect target sites. Generally,

there will be multiple published assay methods for a similar mammalian target site, but the protocols tend to be labor-intensive, and only suitable for low-throughput testing. However, each must be carefully reviewed to determine whether they could be modified to a high-throughput format, or if a new method must be developed. The published assays for GAD fell into three broad categories. The first was measurement of CO_2 formation by a radiometric method. This had the distinct disadvantage of requiring the trapping of 1-[^{14}C]-L-glutamate as a basis for the assay. Use of ^{14}C in an HTS assay could be a safety concern, and trapping and counting of the radioisotope in an automated format was problematic. The second, indirect spectrophotometric or spectrofluorometric measurement of GABA formation by a coupled enzyme assay, offered the most possibilities for developing a HTS assay. The methods in this category ranged from tracking the glutamate decarboxylation reaction by a change in light absorbance of an acid-base indicator to measuring NADPH formation in an enzyme-linked assay using GABAase and indirect measurement of GAD activity. This was rejected because of the potential complexity of enzyme-linked systems, and the fact that the compound being tested could be working on GAD, GABAase or a combination of the two. This would require extensive follow-up assays to sort out the site of action of the test compound. The third method was measurement of GABA through quantitative separation in an ion exchange column. In this method, rapid filtration with an anion exchange resin is used to separate glutamate from GABA. Development of an HTS assay based on a slow separation step on resin columns would be difficult, so this was rejected. Given the major drawbacks for each of these systems, and limited biochemical resources for the significant assay development effort expected, a decision was made to contact an outside vendor to help in the development of a new approach for the assay.

For this assay, PerkinElmer (Massachusetts, USA) provided experience, and comprehensive assay development services with their AlphaScreen® technology. AlphaScreen® is a bead-based, homogeneous, non-radioactive technology that was successfully applied to HTS assays for a number of different mammalian target sites including functional GPCRs (Elagoz et al. 2004; Noguchi et al. 2003), nuclear receptors (Bettoun et al. 2003; Rouleau et al. 2003), kinases (Gray et al. 2003; Warner et al. 2004), and protein-protein interactions (Hamilton et al. 2003; Zhang et al. 2003). With AlphaScreen® technology, a signal is generated when donor and acceptor beads are brought into close proximity through biological interactions. For the GAD assay application, PerkinElmer tethered an anti-GABA antibody to an acceptor bead, and GABA to a donor bead. Interaction between the antibody and GABA would bring the two beads together. When exposed to light at a specific wavelength, the complex emits light at a different, and characteristic wavelength, thus generating a distinct and easily detectable signal. The GAD assay required the mixing of donor and acceptor beads with the recombinant GAD protein, and glutamate substrate. Active GAD generates GABA from

glutamate. The GABA competes for the antibody recognition site and prevents formation of the donor/acceptor bead complex, thus reducing the signal. The presence of a GAD inhibitor reduces GABA production, increasing the signal.

Overall, development of the assay was straight forward, although it took longer than expected. As a rule, assay development timelines extend beyond what is predicted, whether one is contracting out, or establishing and optimizing an assay in-house. Constant contact with those at PerkinElmer who were administering the project as well as with scientists who were developing the assay was critical to success. When considering contracting for assay development, the projected oversight time, as well as all associated direct and indirect costs must be taken into account.

The key to this project was identifying a good source of antibody for use in the AlphaScreen® acceptor beads. The initial concern that the antibody specificity would be insufficient to recognize GABA molecules as compared to glutamate since the two compounds differ only in a carboxyl group, was unfounded. The expense associated with the custom AlphaScreen® beads coupled with limited quantities of recombinant protein, required that the assay be amenable to running in the smaller volumes allowed in 384-well plates. Development of the GAD assay took approximately 7 months, from the time of the initial contract with PerkinElmer, to the time screening of a 10,000 compound "reference" set had begun. The workflow, after initial assay development, involved the testing of a reference set of 10,000 compounds representing the testing library as a whole. Screening this set essentially acted as a "shake-down" run for the HTS campaign, making it possible to effectively adjust workflow and automate systems to run the assay. It also provided information on assay reproducibility from plate to plate, and day to day, as well as giving an initial indication of the hit rate when testing compounds at a given concentration.

The GAD assay was relatively robust, with a signal coefficient of variation of less than 9%. When compounds were run in duplicate, there was less than an 8% signal difference between the two replicates, whether the compounds were in the same test plate or in different plates. Based on these data, it was decided to screen each compound as a singlet rather than testing 2–3 replicates, as had been done for previous assays. The reduction in replicates significantly increased the number of compounds screened per unit time and significantly decreased the overall costs. The Z′-factor for the assay, which is a general measure of assay quality (Zhang et al. 1999) ranging from 0 (poor assay) to 1 (excellent assay), was determined to be 0.87. The assay cost was high but was still reasonable at $0.40/well including reagents, AlphaScreen® beads, and disposables such as robot pipette tips, and 384-well plates. To run a 100,000 compound library through the assay would cost approximately $40,000. Testing of the 10,000-compound set at a screening concentration of 10 μM revealed a hit rate of 0.49% (for this assay, a signal of greater than 35% of control was considered a hit). This number of actives was reasonable and appropriate based on the chemistry resources available to review and optimize hits.

In the screening campaign, a total of 116,000 compounds were run through the GAD assay. The screen provided 270 confirmed hits, giving a final hit rate of 0.23%, about half the hit rate observed in the representative set of 10,000 compounds. The assay identified diverse chemistry scaffolds. Although a number of these compounds were potent inhibitors of the recombinant GAD, most were not efficacious at this target site. For example, many of the hits provided pEC_{50} values in the sub-micromolar range, however, the assay revealed that these same compounds resulted in only partial inhibition of the GAD enzyme activity. In addition, upon testing of these weak hits against cotton aphid, few showed whole insect activity. The actual number of compounds that provided sufficient potency and efficacy, combined with whole insect activity, was a fraction of the total confirmed hits from the screen. However, translation to whole insect activity was achieved only after there was follow-up on the weak as well as strong hits through selection of additional analogs from archival holdings, or from purchases of analogs through outside vendors. In many cases, this can rapidly identify more potent analogs, without a significant commitment of chemistry resources.

2.2.2 Genomic Studies and Model Organisms

Recent literature is replete with target information derived from use of double-stranded RNA interference (Gonczy et al. 2000; Fraser et al. 2000; Manev et al. 2003). Analysis of 2,232 predicted genes on chromosome III of *C. elegans* showed that 133 gave discernable phenotypes in their assays with 78 associated with embryonic lethality. If one performs an online search using *C. elegans* as a model organism, for genes "knocked-out" by RNAi resulting in paralysis (a likely phenotype to be predictive of a good insecticide, see above), one gets 110 hits from chromosome III alone (there are five chromosomes, plus X). With this as a method of target identification we selected the matrix metalloprotease (MMP) (Hamacher et al. 2004; Leung et al. 2000) as a potential target for insecticides. MMP (H19M22.3) is annotated as a Gelatinase A type matrix metalloprotease in the public database, WormBase (Gene Summary for H19M22.3). Phenotypes associated with *C. elegans* RNAi experiments include Gro (slow growth), Ste (sterile) and adult Unc (uncoordinated), but more compelling are the effects associated with the fly homologs (genomic FBan0004859) CG4859 (Mmp1) and CG1794 (Mmp2). There are eight lethal and semi-lethal pupal and larval interruptions cited for this gene; e.g., 90% of Mmp1W439stop/$Mmp1^2$ animals survive to third instar, but only 10% pupate. Other phenotypes associated with this and other stop mutations make it clear that this protease is involved in critical developmental events. A search of insect gene sequences revealed close insect homologs, although the homology with mammalian species was not so great as to rule out a reasonable chance of safety through target selectivity (Brown et al. 2004). With this information at hand, the cloning and assay development were initiated.

The cotton aphid membrane-type matrix metalloproteinase is a 557-amino acid protein. Published information and bioinformatics led to the conclusion that the catalytic domain from approximately amino acid 20 to amino acid 180 (total of 161 amino acids) would provide the necessary functional protein for screening (Massova et al. 1998). Gene-specific antisense primers designed from FMC proprietary EST sequence information led to the cloning of the appropriate aphid functional domain. Following the addition of a polyHistidine 5′ tag with a cleavage site for subsequent purification, the recombinant protein was produced in *E. coli* and purified by affinity chromatography (Butler et al. 2001). Enzymatic activity of the purified His-MMP was detected using the synthetic fluorescent substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2TFA [Dpa = N-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl] (Holtz et al. 1999; Mucha et al. 1998). A robust, high-throughput assay was developed based on this activity with assay development parameters focusing on the enzyme's Zn++ dependency, pH, solvent (DMSO and methanol) effects, enzyme stability, consistency of signal to noise, consistency of IC50 values for standards and effective transition of assay to a 384-well microtiter plate format. Reproducible, facile screening was made possible through fluorometric measurements with a Fusion-μ® Universal Microplate Analyzer (available from Perkin-Elmer). For inhibition assays, the recombinant insect His-MMP and inhibitors (10 μM) were tested in a 384-well format. This made it possible to generate between 6,400 and 9,600 data points per day and complete screening of the designated compound library in a matter of a few weeks. The hit rate from the screened library varied from 0.18 to 1.18% depending on the level of inhibition used to define hits (range from >50 to >35%). Hits from the screening assay were confirmed at multiple concentrations and tested for activity in standard insect screens.

When screening for hits with a more speculative insect target such as MMP the cost of assay development and high-throughput screening inevitably play a more important role since one is adding target uncertainty to normal screening uncertainty as part of the risk component in the risk/benefit ratio. It was important from the outset that the MMP assay could be anticipated to move quickly and efficiently from concept to completion. Therefore, it was essential that appropriate sequence information was available from the pest cDNA library, that expression and enzyme purification costs were well understood, and that the screening costs could be minimized through miniaturization of the volume and efficient screening logistics (flow of materials, reagents and data capture). As an example for the latter, careful analysis of liquid-handling steps resulted in a 62% cost reduction over the original design for the high-throughput assay.

2.2.3 Identifying a New MOA from Active Chemistry

Tools of genomics and biochemistry can be applied to the determination of the MOA of existing active chemistry or chemistry identified directly from the screening of insects. To do this from the start is a daunting effort.

One must categorize leads in terms of even the most basic MOA classes. A battery of assays is necessary if one wants to further define the MOA with reference to known insecticides. As shown in Table 3, the current Insecticide Resistance Action Committee recognizes 27 MOA classes for commercial insecticides.

An extensive commitment of tools and expertise is required to evaluate a new compound and classify it as a known or novel MOA. This is clearly exemplified with MOA studies on a recently commercialized insecticide flonicamid, (ISK, FMC). MOA studies with this compound were done in order to try to uncover a novel MOA that could be used for future insecticide discovery research, as well as to support product understanding. Flonicamid has now been classified by IRAC in group #9, unknown or non-specific mode of action (selective feeding blocker). Reaching this conclusion required the evaluation of this chemistry in numerous biochemical assays. Specific assays performed included inhibition studies of acetylcholinesterase, nicotinic and muscarinic acetylcholine receptors, respiration (including Complex I, II, III, and IV inhibitors, phosphorylation inhibitors, uncouplers of oxidative phosphorylation, ionophores), GABA-receptor, octopamine receptor agonist, nitric oxide receptor agonist, Na-Channel, L-type Ca-channel, ryanodine Ca-channel, and calcium ATPase (body wall contraction assay). The fact that insects become intoxicated in less than 1 h also can serve to rule out chitin synthesis inhibition, juvenile hormone and ecdysone agonist activity and strongly suggests that flonicamid is not an insect-growth regulator. Each of these assays has been shown to respond as expected to reference commercial standards. The fact that flonicamid was not active in any of these assays, coupled with a lack of cross resistance to organophosphates, carbamates, pyrethroids or imidacloprid, strongly indicated an novel MOA.

Continued studies involving direct physiological measurement of flonicamid with cultured neurons derived from larval stage Lepidoptera, pupal stage *Drosophila*, and adult cockroach, *Periplaneta americana* confirmed that flonicamid, even at 10 μM did not disrupt the electrophysiological response to acetylcholine as did the neonicotinoids, imidacloprid and acetamiprid (Table 4). Flonicamid also failed to activate the inward current that was

Table 4. Inhibition of [^{3}H]-Imidacloprid binding using housefly head membranes

Compound	Class	Inhibition of [^{3}H]-Imidacloprid binding (IC_{50}, μM) using housefly head membranes
Imidacloprid	Agonist	0.00025
Nicotine	Agonist	0.1
Acetamiprid	Agonist	0.00004
Tubocurarine	Antagonist	0.8
MLA	Antagonist	0.01
Flonicamid	_	Not active (>>10)

observed when neurons were exposed to acetamiprid. In this way, direct measurements on the neuron were used to confirm the radioligand binding assay results (Staetz et al. 2006), which showed that flonicamid did not compete with imidacloprid or acetamiprid for binding to the nicotinic acetylcholine receptor. Recent evidence from a continuation of neurobiology studies (unpublished) has shown that flonicamid is indeed associated with a novel mode of action, blockade of the A-type potassium current in presynaptic nerve terminal. This finding is consistent with the sypmtomology of intoxicated aphids and points towards a new MOA to exploit.

3 Conclusion

Much new insecticide discovery is still driven by whole organism screening. The examples cited illustrate several effective methods for lead discovery using target based screening. The example of flonicamid illustrates how a set of target-based assays can also be used to evaluate new insect-active chemistries. In this context, the goal was to identify a potential new target for screening and better understand a commercial product. However, it is clear that some or all such mode of action studies may also be used in conjunction with random insect testing to "qualify" a lead for further evaluation, optimization or field testing. In this way, the tools and knowledge developed in an insect genomics and target-based screening effort function in concert with the parallel strategy for lead identification through insect testing.

This is a critical, indirect benefit from a Discovery target program. Essentially every research-based agrochemical company has experimented with target-based research. Clear, well-defined target-to-field success stories are missing, suggesting that a target-based approach is unjustified, but it is important to search below the surface and understand the interplay between target research and lead advancement in order to fully assess the value of an investment in target research. Based on recent evidence of potent insecticidal compounds associated with new insecticide targets such as the ryanodine calcium release channel (flubendiamide; Ebbinghaus-Kintscher 2005) and potassium channel (flonicamid) it is clear that target-based research will continue to play an important role in the discovery of important new insecticides.

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4 Transgenic and Paratransgenic Insects in Crop Protection

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1 Introduction

Broad-spectrum toxicity of insecticides is desired by the agrochemical industry to maximize market outlets and sales income. The more selective the insecticide, the smaller the market, and thus the lower the capability of recouping development investment. Nevertheless, selectivity is highly desirable from both environmental and pest-management standpoints, since nontarget and unintended impacts are reduced.

If an insecticide is selective and yet requires days to take effect, then it is undesirable for controlling pests that threaten immediate reductions in crop yield. Biological control agents are often selective with little side-effect and therefore suitable for niche markets, but have less broad-spectrum crop protection appeal compared to neurotoxic insecticides. Therefore, microbial pesticides and insect-specific viruses (so-called biologicals) do not provide the same broad income potential as neurotoxic insecticides and have not gained much more than a few percent of the total world crop protection market (Menn and Hall 1999). The dichotomy between chemical and biological control of pests is explained well in a recent review of migratory locust control programs (Lomer et al. 2001). The side-effects and problems with classical biological control were recently reviewed (Louda et al. 2003).

Products sold by the agrochemical industry are designed for crop protection in an individual field; however, federal and state governments view invasive exotic pests as regional problems, not on the smaller scale. The area-wide approach to pest control and therefore crop protection has always had a powerful tool in the sterile insect technique (SIT).

Biotechnology is providing modern improvements in SIT and a range of new tools for population control of insects and for crop protection. A new strategy called "symbiotic control" offers new tactics to disrupt the transmission of plant pathogens by insects and to disrupt reproduction in pest populations. Controversy is attracted to projects that employ recombinant organisms.

2 The Sterile Insect Technique

Since conception and development by Knipling (1955; LaChance et al. 1967; Klassen 2003), the sterile insect technique (SIT) has become entrenched as one possible organized response to insect pests with several examples.

Insecticides Design Using Advanced Technologies (ed. by Isaac Ishaaya, Ralf Nauen and A. Rami Horowitz)

SIT exposes target insects in a mass-rearing facility to a radiation dose sufficient to cause sterility, usually in the pupal stage (that is easiest to handle). The resulting adults are released daily over an infested area to swamp out reproduction. As applied to Lepidoptera (Knipling 1970), the technique had an added bonus called inherited sterility or F_1 sterility in which lower doses of radiation produce sterile effects that are passed on to offspring (LaChance et al. 1973, 1976; Tothová and Marec 2001). Lower radiation doses would improve the fitness of target insects compared to those exposed to full doses of radiation.

The pink bollworm has been controlled by various methods since its arrival in California in 1966. An SIT program was started 1968 as an area-wide suppression of pink bollworm, *Pectinophora gossypiella*, incursion into central California and the program remains active today (Walters et al. 2000). The costs of operating the mass-rearing facility in Phoenix and field operations are borne mostly by the California cotton industry in the form of a bale tax and directed by personnel from USDA-APHIS and CDFA. The California Cotton Pest Control Board oversees operations as the legal authority.

At its core, SIT is population control of insects by genetic methods, or genetic control of insects. Besides pink bollworm suppression, other SIT programs in California are aimed at controlling tephritids like the Mediterranean fruit fly, *Ceratitis capitata*. Both programs are justified by the economic threat to cotton and citrus, respectively (Mumford 2000).

3 Conditional Lethal Genes

The dream of genetic control of insects beyond the radiation-based SIT approach has been just that for many years; a dream. However, our imaginations took a giant leap with the report by Rubin and Spradling (1982) of genetic transformation of *Drosophila melanogaster*, the vinegar fly, using a transposable element as an insertion mechanism. The first non-*Drosophila* transformation of Mediterranean fruit fly (Loukeris et al. 1995) occurred over 10 years later and this was followed quickly by reports of other insect transformations. The key proved to be finding a transposable element and promoters that would work in the different insects being transformed. Radiation to produce sterility causes fitness loss and therefore mass-release numbers have to be large (100:1 in Mediterranean fruit fly; 60:1 in pink bollworm SIT programs) compared to wild strains in the area-wide SIT-like approach. Being able to insert single lethal genes allows back-crossing to greatly improve fitness and competitiveness in transgenic SIT-like strains (estimated to improve the release ratio to 5:1 for pink bollworm mass-reared to wild type strains in area-wide genetic control programs).

Our turn came in 1998 with pink bollworm (Miller 2001) using Mac Fraser's *piggyBac* transposable element. Steve Thibault designed a promoter based on

the *actin A3* promoter in *Bombyx mori* and used the *egfp* marker to achieve transformation. At the beginning, the pink bollworm project faced three major hurdles: to develop a transposable element and transformation protocol, to develop a marker gene and to develop a lethal gene that worked (Miller 2004a, 2004b). All of these steps had no precedent and were considered difficult, with the lethal gene identification perhaps being the most difficult.

A lethal gene system was actually discovered first when Karl Fryxell suggested using a *Notch* allele from *Drosophila melanogaster*, N^{60g11}. When the mutant *Notch* colony was added in equal numbers to wild types in bottles, the colony died out in three generations (Fryxell and Miller 1995). Five years later, the Luke Alphey group constructed a lethal gene from scratch using the *nipp* system from *Drosophila* and demonstrated population collapse in the same three generations (Thomas et al. 2000). The advantage of the Alphey lethal, which he called RIDL, (release of insects with dominant lethal genes), was that the gene was down-regulated by a tetracycline repressor element. In the presence of tetracycline, the lethal effect was not expressed and allowed mass-rearing.

Since chlorotetracycline is used in larval rearing diet to suppress bacterial growth, pink bollworm mass-rearing fits the RIDL construct particularly well. We dropped work on the *Notch* system in favor of this tet-repressor construct.

The pink bollworm transformation system was well established, but the first transformants with the RIDL gene cassette were not killed upon removal of tetracycline. This confirmed a general principle painfully learned from earlier work; a gene from one organism does not necessarily work all that well in another one. With no guide as to why the lethal effect was not expressed, Luke took several months to produce a version that did work. The lethal effects were demonstrated in the summer of 2004. Shortly after, a second-generation RIDL element was used to genetically transform Mediterranean fruit flies (Gong et al. 2005). This time the tet-repressor transactivator element (tTA) was itself used as the lethal product. The early results again show partial lethality, but the concept was established.

Looking to the future, other lethal gene constructs and strategies are beginning to appear (Miller 2004b). Park (et al. 2002) reported that deletion of ecdysis-triggering hormone gene in *Drosophila melanogaster* lead to unsuccessful molts from the first to the second larval instars in 98% individuals lacking the gene. Successful ecdysis was rescued by precisely timed injection of synthetic ecdysis-triggering hormone to demonstrate that this gene product itself was vital.

4 Regulatory Aspects

The transgenic pink bollworm project moved into the regulatory arena and quickly encountered resistance, mostly generated by scientific colleagues (Marshall 1998; Anonymous 2004a; Anonymous 2004b; Fox 2004; Miller

2004a). Despite the call for delay and more study, not one hazard connected to release of transgenic pink bollworm has been proposed.

From the issue of the first USDA permits for transgenic pink bollworm in 1999 until today, much has been discussed and debated because a permit is a definite act. A permit is either issued or it is not. Some people see this act of issuing a new permit as something of a watershed to be delayed as long as possible. The rationale for delay is to gather more information. In the end, however, no amount of lab data is going to substitute for data collected from field. Thus for transgenic pink bollworm the process was one of preparing the public for the inevitable day of release, rather than looking for any specific type of information that would describe an imagined result. Lack of an imagined hazard makes risk assessment an exercise in futility. You can't do a probability study of a hazard without defining a hazard or hazardous condition.

5 Symbiosis and Microbiology

Revolutions in microbiology and symbiosis are having ripple-effects on neighboring sciences. A new appreciation of bacterial diversity has emerged (Woese 2004), primarily based on the ease of amplifying conserved genes via the polymerase chain reaction (PCR).

This new ability to identify microbes has had an impact on the study of symbiosis (Xu and Gordon 2003; Woese 2004; Pennisi 2005). In entomology alone, the number of unexpected contributions of symbionts to the biology of hosts is sufficiently diverse (Bourtzis and Miller 2003) that training in symbiosis is now necessary to understand the biology of an insect to the same extent that training in molecular techniques is crucial to understanding modern biology.

5.1 Symbiotic Control of Disease Transmission

A new strategy (Beard et al. 1998, 2001, 2002; Durvasula et al. 1999; Rio et al. 2004) was developed to use symbionts to render insects vector-incompetent and thereby disrupt arthropod-borne pathogens of human diseases. The term "paratransgenesis" was coined to describe vector insects containing genetically modified symbiotic microbes. The approach is a variation on the theme of symbiotic therapy (Ahmed 2003) and is very similar to the recent innovative control of dental caries (Hillman 2002; Pennisi 2005), strategies for preventing HIV infections via symbiotic bacterial delivery (Chang et al. 2003), and delivery of anti-cancer therapies in humans via genetically altered or other symbiotic bacteria (Pilcher 2004).

The choice of symbiont selected for use in symbiotic control is critical to the success of the process. Only those symbiotic microbes that have established

a relationship with the host are suitable. Such symbionts are assumed to develop responsiveness to cues from the host plant or animal during co-evolution.

Symbiotic control strategy causes the least disruption to the system under consideration. Because it is biologically based, it is by nature a biological control approach more than a pesticidal approach even though it has elements of both. Classical biological control as used in entomology depends on a predator or parasite that attacks a pest insect. Usually, this requires discovery of the home origin of the pest organism, which is thought to contain the richest complex of parasites and predators that have had time to evolve with the pest organism over the longest period of time and in which the populations of any component are kept naturally to low numbers (Torchin et al. 2003).

The symbiotic control approach is unique in that it selects an organism that is established in the pest/host complex as it exists locally, rather than seeking a biological control organism such as a parasite or predator from the site of origin that might be in an exotic location abroad. Choosing a microbe that already exists in the pest/host complex eliminates many of the complexities of introducing a classical biocontrol agent from a foreign location and expecting it to adapt to local conditions with possible effects on other unintended hosts. A microbe selected locally would be pre-adapted to the local environment. If collected, cultured and re-released back into the environment, one would expect that it would be indistinguishable from the biota that already exists.

If the microbe is selected, cultured and genetically modified, and then reintroduced, we would expect that it would ultimately be out-competed in a certain amount of time by the native counterparts already present. In genetic manipulation, transgenic organisms commonly suffer a fitness cost (Purrington and Bergelson 1997; Silbermann and Tatar 2000; Catteruccia et al. 2003). All other aspects of the symbiotic control organism are the same; indeed, this approach would not function properly if the symbiont were not identical in all aspects so as to be recognized as virtually the same organism competing in the natural environment.

Thus it is a characteristic of this approach that the genetically altered organism is less fit than the unmodified counterpart. And indeed, we suggest this be the main parameter of the symbiotic organism that offers regulatory assurance that the life of the introduced organism is limited.

Unlike the lack of defined hazard mentioned above in release of recombinant pink bollworm, the choice of symbiont used for crop protection is entirely different. For example a permit request to do field testing of recombinant *Burkholderia cepacia* as a biopesticide to protect crops against fungal diseases was denied because literature clearly identifies this bacterium as a human pathogen (Holmes et al. 1998). Suggested use of *Alcaligenes* to protect grapevines against Pierce's disease faces the exact same well-defined hazard since *Alcaligenes* has been identified as forming nosocomial colonies in lungs of cystic fibrosis patients (discussion in second section below).

5.2 Dental Caries

The Oragenics company [www.oragenics.com] received a Food and Drug Administration permit for field safety trials of replacement therapy using recombinant *Streptococcus mutans*, the common mouth symbiotic bacterium of humans. Scientists at Oragenics selected a strain of *S. mutans* that produces a mild antibiotic that kills other members of the same species. Through recombinant methods they further removed or inactivated the gene responsible for converting glucose and other sugars in the human diet to lactic acid, the principle cause of tooth decay.

The product uses what Oragenics calls replacement therapy. It is at the practical leading edge of a new vision of oral biology (Pennisi 2005). As a commercial product using a recombinant bacterium for preventing tooth decay, it is pioneering symbiotic control of the modern type.

The adaptation of this new approach to protection of vineyards against Pierce's disease in California is described below.

5.3 Pierce's Disease

Pierce's disease (PD) in grapevines is caused by the bacterium, *Xylella fastidiosa*, which blocks the xylem flow of affected plants (Hackett et al. 2003; Hopkins and Purcell 2002). There are reportedly 145 strains of *X. fastidiosa* (*Xf*) each associated with particular host plants (Hendson et al. 2001; Rodrigues et al. 2003; Schaad et al. 2004; Hoddle 2004). Disease symptoms are produced in only a few of these (Purcell 1997). In transmission experiments a strain of *Xf* isolated from oleander was unable to cause disease in either grapes or almonds although the bacterium was present in all of these plants (Almeida and Purcell 2003).

The *Xf* strain causing PD in grapevines in California (PD-XF) probably came from native origins in the temperate band running from Florida through Texas to northeastern Mexico (Hoddle 2004). PD-XF has been in California for many decades (first outbreaks recorded in 1883), but previously appeared infrequently because native sharpshooters that act as vectors only occasionally attempt to feed on grapevines (Anonymous 2002).

This situation changed dramatically with the arrival of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* (Redak et al. 2004). The difference between GWSS and native leafhoppers is the difference between a minor nuisance and a major epidemic in terms of appearance of PD-XF and related diseases. Before the arrival of GWSS, PD-XF was handled as a short-term problem. With the arrival of GWSS, PD-XF became an emergency that threatens large areas wherever GWSS becomes established and is being addressed statewide with quarantine restrictions on movement of host plant materials from nurseries.

In addition to PD, other strains of *X. fastidiosa* have been transmitted to other host plants such as oleander, liquid amber, and crepe myrtle by the

GWSS. Oleanders are used as a screen along center dividers on California freeways or as ornamentals along the sides of right-of-ways. Requisition for removal and replacement of diseased oleanders in the green belt of the City of Tustin in Orange County, Southern California was $200,000 (Anonymous, no date posted). The City of Riverside, California requisitioned $175,000 for the same purpose in 2004. Loss and replacement cost of oleanders on highways in California was estimated at $125 million (Blua et al. 2002).

PD-XF outbreaks in vineyards in Temecula and Bakersfield have been checked recently by the systematic insecticide treatment of winter resting sites used by adults. This and spot treatments have decreased spring populations to levels that decrease the PD-XF threat, and slowed the spread of GWSS north through the San Joaquin Valley. Citrus is a preferred habitat for GWSS in California. Vineyards are at greatest risk when they are located near citrus because of movement by GWSS in the spring (Blua et al. 2001).

5.4 The Vector Insect

The glassy-winged sharpshooter (GWSS) is native to the south and eastern United States and northeast Mexico (Hoddle 2004). It appears to have co-evolved with strain of *X. fastidiosa* causing PD (PD-XF). We know little about the co-evolution of GWSS with other symbiotic bacteria in this native host range.

Xylella fastidiosa is transmitted by GWSS physically; that is, the PD-XF physically attaches to the cuticle inside the buccal (or precibarial) cavity of the insect in the foregut in a highly ordered way (Almeida and Purcell 2003; Brlansky and Timmer 1982; Brlansky et al. 1983; Purcell and Finlay 1979). From here it is presumably pulled into the xylem fluid of the host plant during the act of feeding. Exact details of transmission of the pathogen between insect and plant are not known, however, it was shown that nymphal GWSS that undergo a molt will lose the ability to infect plants unless they reacquire the pathogen. Since the cuticular lining of the foregut is cast off with each molt, we presume the biofilm of symbiotic bacterial colonies attached to the cuticle are also carried away.

Pymetrozine is an insecticide widely reported to prevent feeding by aphids and other plant-sucking insects (Bedford et al. 1998; Harrewjin and Kayser 1997; Wyss and Bolsinger 1997). Since pymetrozine is effective in preventing the transmission of viruses by aphids, we thought it might prevent GWSS from transmitting the pathogen causing Pierce's disease by inhibiting feeding. Although pymetrozine treatment drastically reduced processing of xylem and eliminated settled feeding, GWSS moved around on the host plants more and in fact the incidence of transmission of pathogen actually increased compared to controls (Bextine et al. 2004a) probably because GWSS made many more feeding attempts. This dramatically demonstrated the physical nature of the transmission of *Xf* by leafhoppers.

Attachment in the cibarium (precibarial cavity) suggests a very specialized function of these bacteria and an evolutionary adaptation to grow in this niche. The bacteria form biofilms in the buccal cavity (precibarium) and signaling between PD-XF (and other strains of *Xf*) and the host may trigger active attachment and biofilm formation. In the case of the pathogen, the signaling gene cassettes are known (Newman 2004). It seems likely that the same process supports attachment by other symbiotic bacteria.

Because the pathogen is physically attached to the insect, once acquired, it is ready for transmission to a new host plant relatively quickly. Given the good flying ability and wide host range of GWSS, the symbiotic bacteria they carry are exposed to a larger number of host plants than has been the case historically in California.

5.5 The Symbiont

All multicellular organisms naturally contain large numbers of symbiotic bacteria and plants are no exception (Araujo et al. 2002). Because the symbiont employed in symbiotic control must be chosen from those bacterial species already present, symbiotic control tactics are defined by the system under study, in this case the xylem of grapevine (and other host plants) and the precibarium of the foregut of the sharpshooter insect.

Three bacterial species were identified from the midguts of GWSS (in order of frequency): *Alcaligenes xylosoxidans* var. *denitrificans* (Axd), *Chryseomonas luteola*, and *Ralstonia pickettii*. Two *Bacillus* spp., *Bacillus coagulans* and *Bacillus brevis* were infrequently isolated from midgut samples. Axd and *R. pickettii* were also identified from precibarial (foregut) samples. Another isolate tentatively identified as a *Sporosarcina* sp. and a yeast-like organism were also infrequently isolated from precibarial samples.

We selected the Axd bacterium as an agent for symbiotic control of PD because it was found consistently in the xylem of host plants and in the precibarium of the vector insects and because this symbiotic bacterium and the pathogen occupied the same niche. Although there was a choice of more than one symbiont, the final selection of Axd involved other considerations such as ease of manipulation, abundance, vigor, practicality of delivery and absence of human pathogenicity. The literature reports Axd in nosocomial infections alongside known pathogens such as *Pseudomonas aeruginosa*, but never in colonies alone.

A completely different strategy in selecting the symbiont includes choosing bacteria that cannot be cultured or endosymbionts such as *Wolbachia* bacteria that cannot be cultured outside cells. These possible organisms as agents in symbiotic control present an entirely different complexity of approach, not least of which is lack of recombinant mechanism.

We genetically altered Axd to contain the DsRed fluorescent protein gene (RAxd) inserted into the chromosome. This marked symbiont was acquired

by GWSS and occupied the cibarial region for up to 5 weeks after clean GWSS were allowed to feed on stems that had been injected with the RAxd (Bextine et al. 2004b, 2005).

5.6 Genetic Engineering of GWSS/Grapevine Bacterial Symbionts

Bacteria used for practical symbiotic control are unlikely to be model bacteria like *E. coli*. This presents certain challenges for their genetic modification since, in many cases, there will have been little or no genetic work previously performed on species selected for development. Fortunately, generalized genetic tools based on *mariner* transposable elements are available that allow the stable insertion of genes via transposition into practically any prokaryote (e.g., Rubin et al. 1999). Suicide plasmids incapable of replicating in symbiotic bacteria have been made that contain all of the components needed to promote transposition into the chromosome and yet will not allow propagation of the plasmid. We have tested this system in *Alcaligenes xylosoxidans*, *Chryseomonas luteola*, and *Enterobacter agglomerans* with success. Furthermore, this system incorporates FLP (flippase) recombinase sites that allow later drug marker removal from the chromosomal constructs. Thus, strains of symbiotic bacteria can be made with a minimum of inserted DNA that contains no drug resistance (Lampe et al. 1998, 1999, 2000; Robertson et al. 1998).

5.7 Competitive Displacement

Since the niche described here (bacteria in the xylem of plants and vector insect foreguts) is already rich in microbiota, it is reasonable to assume there is a certain interaction between the microbial colonies. Quorum-sensing (a genetic reaction triggered by adjacent conspecific bacteria reaching a certain critical number) is an established form of communication between the genetic material in bacteria and the external environment. Not all bacteria have been shown to engage in quorum sensing. We assume that bacteria also interact with the host organism in a multitude of ways (see Newman et al. 2004). We assume that host interaction was a selection mechanism in establishing the microbial flora in this niche. Artificially removing normal gut symbionts can lead to fungal infections in insects (Dillon and Dillon 2004) and can disrupt metabolism in others (Lauzon 2003). Thus microbiota in the gut of insects plays a complex yet natural role in the biology of the host.

By selecting one of the components of the microbial floral from a niche, genetically altering it to produce a reagent, then reintroducing it to the same niche, we argue that the modified organism would have a limited lifetime determined by the fitness cost associated with the new chromosomal construct.

We tested this hypothesis by introducing RAxd into grapevines in commercial vineyards during the 2003 and 2004 growing seasons in California under the auspices of a permit from Environmental Protection Agency (permit TERA

R-03-01). RAxd was applied as a foliar mist, needle inoculation of the trunk, or soil drench. At various times following injection, samples were taken from petioles away from the site of injection. The purpose of these tests was to determine the behavior of RAxd in the grapevine only. RAxd and Axd do not appear to interact with the pathogen XF-PD in any way. Only after the RAxd is transformed with anti-XF-PD reagents would it be expected to have plant protection properties.

At a vineyard in Bakersfield, CA, RAxd was detected in grapevines 2 weeks after inoculation in all treatments, indicating that all application methods can successfully introduce *Axd* into plants. Only two grapevines that were treated with foliar application tested positive after 4 weeks (in both cases only 1/6 samples tested positive), indicating that populations declined over time (Table 1, left side). In Napa, fewer grapevines tested positive (Table 1, right side). No samples tested positive 6 weeks after inoculation.

These data suggested that all methods of introduction of RAxd were successful. The most compatible of these methods to vineyard operations would be soil drench simulating drip irrigation, or misting foliage. Delivery by drip irrigation should be compatible with irrigation plumbing and misting could be done by modifying farm machinery used to spraying herbicides or insecticides, or used in applying growth hormones.

The two sites mentioned above are in widely separated locations. Bakersfield is 35.4 °N × 119.0 °W and Napa is 38.3 °N × 122.3 °W. The driving distance between the two sites is 273 miles (437 km). The grapevines at the Bakersfield site were 8-year-old Red Globe table grapes and the Napa variety was a 12-year-old Merlot. We have no explanation for why the RAxd appeared to colonize the grapevines in Bakersfield more successfully than in Napa and these tests are being repeated in 2005.

The ability of RAxd to colonize plants in the laboratory was tested by inoculation of six host plants. Plants were grown from seed in 10-cm pots to about 40cm height. Stems were injected with RAxd or sterile water 5 cm above the

Table 1. Detection of RAxd from grapevines in two field tests

	Bakersfield					Napa				
	Plant Number									
Method	1	2	3	4	5	1	2	3	4	5
Foliar spray	+,0	0,0	+,■	0,0	+,■	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
Inoculate	0,0	+,0	0,0	0,0	+,0	0,0,0	+,0,0	0,0,0	0,0,0	0,0,0
Soil drench	+,0	+,0	0,0	+,0	0,0	0,0,0	0,0,0	0,0,0	0,0,0	+,■,0
Control	0,0	0,0	0,0	0,0	0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0

0 = No positive samples
+ = Positive sample after 2 weeks
■ = Positive sample after 4 weeks

Table 2. Size of colonies of the symbiont, RAxd, found by PCR analysis in young host plants 2 weeks following inoculation of stems

Plant type	Detected/tested: amount found
Lemon	25/25: 3,591,427 cells/2 cm
Chrysanthemum	19/20: 1,511,088 cells/2 cm
Orange	25/25: 943,305 cells/2 cm
Crepe Myrtle	8/25: 884,770 cells/2 cm
Periwinkle	10/25: 304,820 cells/2 cm
Grapevine	24/25: 18,225 cells/2 cm

(Taken from: Bextine, Lampe et al. 2004)

soil like. Two weeks later, a section of stem 2 cm long was taken 5 cm above the site of inoculation. The contents were examined by RT-PCR using primers designed to amplify the gene insert region and the size of colonies was determined (Table 2).

Citrus and chrysanthemums appeared to be preferred greenhouse hosts of RAxd and grapevine appeared to be the least acceptable host in terms of the size and movement of the RAxd. Based on these data, we thought it was important to repeat the trials in other than a greenhouse setting. Grapevines do not do well in greenhouses compared to the other plants. Note that crepe myrtle and periwinkle yielded colonies in less than half of the tested plants.

Plants from the test in Table 2 were kept in the greenhouse for 10 months and retested. Lemon and sweet orange plants survived best in the greenhouse and yielded 13/20 and 9/20 positive samples, respectively, for RAxd. RAxd was also present in a high proportion of the surviving periwinkles and chrysanthemums (5/5 and 6/7, respectively). Only one of two surviving grapevines tested positive and all eight surviving crepe myrtles were negative. These survival data are reflected in the 2-week sample data shown in Table 2 in which crepe myrtle was the least likely to host RAxd.

Analyses of the soil samples around the root ball of each plant showed no evidence of RAxd. When independent attempts were made at colonizing soil samples with RAxd, no samples yielded RAxd after any time interval. In other words, RAxd added to native soils did not survive. However, if soil was first sterilized, RAxd colonies were readily found after test inoculations. This suggests that RAxd was not capable of surviving in soil that had a pre-established native microbial community.

5.8 Quorum Sensing

The concept that dense cultures of bacteria are merely a collection of individuals has given way to the realization that individual bacteria are part of a community and behave akin to multicellular organisms. These community

members communicate with their own and other species by releasing and responding to the accumulation of signaling molecules known as auto-inducers. These auto-inducers, or signaling molecules, are assembled into a language that bacteria use to communicate. This language often represents the response of bacteria sensing other bacteria in their local environment. This phenomenon of cell-to-cell communication in bacteria, referred to as quorum sensing, is the mechanism behind the monitor and coordinated activities bacteria exhibit in response to cell density. Quorum sensing regulates many bacterial behaviors such as symbiosis, sporulation, conjugation, virulence, antibiotic production, and biofilm formation (Bassler 2002).

Multiple classes of chemical signals and signaling mechanisms are being identified and have been found responsible for quorum sensing behavior. To date, we know that Gram-negative and Gram-positive bacteria use different signaling molecules to regulate extracellular signaling mechanisms. It is becoming increasingly evident that bacterial cell–cell communication and community behavior are critical for successful interaction with other organisms, such as plants and animals (Parsek and Greenberg 2000). For example, bacterial cell–cell communication is involved in beneficial bacterial interactions with plants, such as legumes and nitrogen-fixing *Rhizobium* spp. *Rhizobium leguminosarum* uses quorum sensing signal-mediated gene regulation to maintain a mutualistic relationship with leguminous plants (Lithgow et al. 2000).

Numerous studies have demonstrated that quorum sensing also controls the production of virulence factors that enable bacteria to cause disease in their hosts (Passador et al. 1995; Winzer and Williams 2001; Donabedian 2003). The opportunistic pathogen *Pseudomonas aeruginosa* uses two separate quorum-sensing systems to regulate its growth and production of extracellular degradative enzymes (Whooley et al. 1983; Ochsner et al. 1994; Winson et al. 1995; Ochsner and Reiser 1995; Brint and Ohman 1995; Pearson et al. 1997). Interference with quorum sensing is seen as a promising, effective antibacterial strategy with synthetic anti-quorum sensing drugs in development.

Drugs are not the only means by which quorum-sensing systems can be disrupted. Bacteria themselves can also be used to scramble the language of other bacteria. Dong et al. (2000) found a *Bacillus* sp. from soil that was capable of enzymatic inactivation of the auto-inducing signal molecules produced by other bacteria in soil. Therefore, bacteria could be used to dampen or prevent the growth of other bacteria, or at least their virulence. This also can be considered as symbiotic control.

5.9 Practical Considerations

When applied to plants as a soil drench, mist or by physical injection, genetically modified Axd (GM-Axd) is found in the xylem fluid for a short period of time. Six weeks after application the GM-Axd is no longer found. We assume natural counterparts have displaced it.

When injected into growing grapevines, the GM-Axd does not invade the developing berry tissues, nor does it accumulate in the stems of the grape bunches. When exposed to ordinary vineyard soil, the GM-Axd does not survive. However, if the same soil is first sterilized, then the GM-Axd does colonize the soil. These results suggest that the organism would not persist in the environment. Axd, RAxd or GAxd (the egfp equivalent of RAxd) may be applied to grapevines in a spray, in drip irrigation, or by inoculation. Of these methods, misting is perhaps the most practical and compatible with farming operations. Inoculation is labor-intensive and not practical on a commercial scale. Soil drench or delivery in drip irrigation has some precedent, but the genetically modified organisms were not found to survive soil inoculation. For this reason, we assume movement from irrigation water through roots into the plant for uptake in the xylem fluid might be less efficient than foliar application. However, both of these methods need to be eventually tested on a commercial scale.

Use of recombinant Axd as described above faces severe regulatory scrutiny for reasons mentioned at the end of Sect. 6.1 above. At present, the Environmental Protection Agency will not issue permits for field testing of Axd genetically altered to contain a fluorescent dye marker gene because Axd is claimed to be a nosocomial organism, unless the target plants are burned at the end of the trial and the root ball soil is sterilized. Stated another way, EPA will not allow anyone to extract a symbiotic bacteria from nature and use it to treat the plants where the same organism was found. It is not clear why this is hazardous since the organism is already present there. This organism was chosen because it is present as an innocuous symbiont in the xylem fluid of host plants and because it resides along side the known plant pathogen causing Pierce's disease. Possibly, the regulatory concerns need to be re-examined. At present, there does not appear to be any way to do that.

6 Conclusions

Rachel Carson called for more sophisticated methods of pest control in agriculture some 40 years ago. Pesticides are the mainstay in crop protection because they can be applied quickly and are immediately effective. However, the application method is inefficient, with side-effects such as resistance development and actions on non-target organisms including disrupting biological control. Classical biological control takes time to develop, is often selective and is rarely immediately effective and can be incompatible with insecticide treatments. There are no current methods for controlling the insect transmission of pathogens causing plant disease other than reducing vector insect populations with insecticide treatments and removal of infected plants. A new method of symbiotic control offers the opportunity to deliver an anti-pest strategy very efficiently, but without the drawbacks of classical biological control and without interfering with other pest control practices.

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5 Future Insecticides Targeting Genes Involved in the Regulation of Molting and Metamorphosis

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1 Introduction

Because of their rigid exoskeleton, insects need to undergo periodic molts during which they shed their old cuticle and produce a new one to accommodate further growth. A number of hormones play critical roles in the initiation and coordination of the processes of molting and metamorphosis. Studies on several model insects such as *Bombyx mori, Hyalophora cecropia, Manduca sexta* and *Drosophila melanogaster*, have led to the identification of key hormones and the elucidation of their roles in molting and metamorphosis. Best known for their roles in these two processes are the steroid hormone ecdysone[1] and the sesquiterpenoid, juvenile hormone (JH). However, these developmental events also involve a number of relevant peptide hormones, including prothoracicotropic hormone (PTTH), eclosion hormone (EH), pre-ecdysis triggering hormone (PETH), ecdysis triggering hormone (ETH), crustacean cardioactive peptide (CCAP) and bursicon (BU).

When a larva reaches a critical weight, PTTH launches the molting process by inducing ecdysone secretion in prothoracic gland cells. Although ecdysone triggers and coordinates the molting process, the type of molt is determined by JH. In the presence of JH, "status quo" is maintained and ecdysone can only induce a larval-larval molt. In the absence of JH, however, ecdysone induces a metamorphic molt leading to the morphological changes seen in the transition from larva to pupa, and from pupa to adult (Riddiford 1994). With respect to the neuropeptides EH, PETH, ETH, and CCAP, they control the behavioral aspects of ecdysis whereas BU regulates tanning and sclerotization of the cuticle.

In this chapter we first summarize recent advances in our understanding of the biosynthesis and functions of key hormones involved in molting and metamorphosis. In the second half of the chapter, we discuss probable target sites for the rational design of insecticides, including biosynthetic enzymes, receptors and transcription factors, and propose various ways of exploiting these target sites for pest management. Our coverage will be restricted to the aspects of molting and metamorphosis that are relevant to finding target sites

[1] "Ecdysone" is used here as a generic term to refer to α-ecdysone, its metabolite 20-hydroxyecdysone (20E) and other natural ecdysteroids with similar biological activity.

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for insecticide discovery. For a comprehensive account of the regulation of molting and metamorphosis, we refer the reader to recent reviews published in Comprehensive Molecular Insect Science (Gilbert et al. 2005).

2 Hormonal Regulation of Molting and Metamorphosis

2.1 PTTH

2.1.1 Introduction

The first report of a factor from the brain regulating metamorphosis appeared in 1922, and was based on work conducted on the gypsy moth, *Lymantria dispar* (Kopec 1922). Subsequent research by numerous scientists led to the identification of this factor as prothoracicotrophic hormone [PTTH; see reviews by Nagata et al. (Nagata et al. 2005) and Rybczynski (Rybczynski 2005) for details on identification and characterization of PTTH]. The major function of PTTH is to stimulate ecdysteroid synthesis and secretion by prothoracic glands.

2.1.2 Biosynthesis

Most of what we know about PTTH biosynthesis is derived from work done on the Lepidoptera; comparatively little is known about non-lepidopteran PTTHs. The lepidopteran PTTH gene encodes a precursor protein (prepro-hormone) that is processed into an active peptide by proteolytic cleavage. For example, in *Bombyx mori*, the deduced amino acid sequence from the cloned cDNA contains an open reading frame encoding 224 amino acids (Kawakami et al. 1990). This precursor protein contains four peptides, including a signal peptide and the 109-amino acid PTTH. The mature PTTH is a homodimer, with the two subunits linked by disulfide bonds; there are also six cysteine residues involved in intramonomeric bonds (Nagasawa et al. 1984).

2.1.3 Mode of Action

The PTTH signal transduction pathway is complex, and very little is known about its action. PTTH most likely acts through a G-protein coupled (membrane) receptor by increasing calcium influx, leading to activation of calcium-calmodulin sensitive adenylyl cyclase, cAMP, protein kinase A, extracellular signal-regulated kinase, and protein-kinase-C phosphorylation of a series of proteins (Rybczynski 2005). Interestingly, the finding that very limited PTTH cross-reactivity can be elicited in assays of two different moth species [e.g., effect of *Antheraea pernyi* PTTH on *M. sexta* PTG activation;

(Rybczynski 2005)] suggests that there could exist significant differences among moth PTTH receptor binding/activation sites.

2.2 Ecdysone

2.2.1 Introduction

Studies in the 1920s and 1930s suggested the existence of a haemolymph factor involved in insect molting (Fraenkel 1935; Frew 1928; Koller 1929; Von Buddenbrock 1931). The steroidal "ecdysone", the immediate precursor of the active molting hormone, 20-hydroxyecdysone (20E), was isolated by Butenandt and Karlson in 1954 (Butenandt and Karlson 1954), and its structure identified 10 years later by Huber and Hoppe (Hoppe and Huber 1965; Huber and Hoppe 1965). Many "ecdysteroids" have since been isolated from various arthropods; these compounds feature several hydroxyl groups, in addition to the 3β-OH found on the cholesterol precursor. For a detailed account on ecdysteroid chemistry, synthesis and metabolism, see the recent review by Lafont et al. (2005).

2.2.2 Biosynthesis

During post-embryonic development, de novo biosynthesis of ecdysone takes place in the prothoracic glands (Hoffmann et al. 1977). Following degeneration of the latter, sometime before adult emergence, ecdysteroids are produced by the follicle cells that surround the terminal oocytes (Goltzene et al. 1978). The testes also contain ecdysteroids but whether they can synthesize them de novo remains uncertain (Lafont et al. 2005). Other tissues, including the epidermis, have also been shown to release ecdysteroids (Delbecque et al. 1990).

Ecdysone is synthesized from cholesterol, which is obtained either directly or indirectly (as phytosterols) from food sources. Phytosterols are first dealkylated to cholesterol through a series of enzymatic steps that take place in the insect gut and possibly other tissues (Lafont et al. 2005). Conversion of cholesterol to the prehormone ecdysone involves an initial dehydrogenation step and a series of final hydroxylation reactions, some of which are well characterized and are known to be effected by mitochondrial and/or microsomal P450 enzymes (Warren and Gilbert 1996; Warren et al. 2002, 2004). However, the intermediate enzymatic steps generating the Δ^4-diketol from 7-dehydrocholesterol remain, to this day, uncharacterized (Lafont et al. 2005). The conversion of ecdysone into the active 20E does not take place in the prothoracic gland (or the ovary), but in various peripheral tissues, including the epidermis, midgut, Malpighian tubules and fat body; this enzyme has recently been cloned from *D. melanogaster* (Petryk et al. 2003).

2.2.3 Mode of Action

A series of studies by Clever (1965) and Ashburner et al. (1974) led to the hypothesis that ecdysone functions through a receptor complex. Ecdysone and its receptor complex induce the transcription of a small set of early genes whose protein products, in turn, induce the transcription of a large set of late genes as well as suppress the expression of early genes. During the past two decades, Ashburner's model has become the central paradigm for explaining the mode of action of ecdysone. In 1991, the ecdysone receptor (EcR) cDNA was cloned from *D. melanogaster* (Koelle et al. 1991). EcR was found to be a member of the steroid hormone receptor superfamily. The members of this superfamily contain five domains: A/B (transactivation), C (DNA-binding), D (hinge), E (ligand-binding) and F (transactivation) domains. Soon it was discovered that the high affinity binding of EcR to both the DNA and the ligand (20E) depended on the heterodimerization of EcR with another nuclear receptor, the ultraspiracle (USP) protein (Thomas et al. 1993; Yao et al. 1993, 1992). The USP cDNA from *D. melanogaster* was simultaneously cloned by three groups (Henrich et al. 1990; Oro et al. 1992; Shea et al. 1990). The USP gene is also a member of the steroid hormone receptor superfamily and contains the A/B, C, D and E domains. Subsequently EcR and USP cDNAs were cloned from several insects and from a crab (Chung et al. 1998) and a tick (Guo et al. 1997). Comparison of the deduced amino acid sequences from these cDNAs showed that the 66 amino acid DNA binding domain and ligand-binding domain are well conserved among EcRs and USPs. However, the A/B, D and F domains are not very well conserved. Since the cloning of the EcR cDNA, much research has been conducted to understand the mode of action of ecdysone (Buszczak and Segraves 1998, 2000; Henrich and Brown 1995; Riddiford et al. 2000; Thummel 1995, 1997, 2002). The binding of ligand to EcR is greatly stimulated by the presence of USP (Yao et al. 1993). The binding of ligand to EcR also stabilizes the EcR/USP heterodimer and increases its affinity to ecdysone response elements (EcRE).

The crystal structure of USP has been elucidated by two groups (Billas et al. 2001; Clayton et al. 2001). The structure of USP is similar to that of its mammalian homologue, RXR, except that USP structures show a long H1-H3 loop and an insert between H5 and H6. These structures appear to lock USP in an inactive conformation by displacing helix 12 from agonist conformation. In addition, in both crystal structures, USP showed a large hydrophobic cavity, which contains phospholipid ligands. In subsequent studies, a homology model for USP was built based on the sequence comparison with RXR. Studies on interaction between the ligand and the receptor in this model showed that it is possible for JH and JH analogs to fit into the binding pocket of USP (Sasorith et al. 2002). Interestingly, bound lipids were absent in the recently solved crystal structure of USP from the hemipteran *Bemisia tabaci* (BtUSP) (Carmichael et al. 2005). The fact that a truncated version of BtUSP was used in these studies as well as expression of this protein in insect cells

[USPs from *Heliothis virescens* (HvUSP) and *Drosophila melanogaster* (DmUSP) that showed bound lipids were expressed in bacteria] and differences in the amino acid residues in the ligand binding pockets of BtUSP, DmUSP and HvUSP are among the factors that could explain the absence of ligand in the BtUSP pocket. In fact, the sequences of homologues of RXRs from insects belonging to the orders Hemiptera, Coleopera and Hymenoptera are closer to human RXRs than they are to USPs from Diptera and Lepidoptera.

The crystal structures of the lepidopteran (*Heliothis virescens*) EcR (HvEcR) protein were determined recently and showed highly flexible ligand-dependent binding pockets providing an explanation for the differential activity of steroidal and non-steroidal ligands (Billas et al. 2003). A *Choristoneura fumiferana* EcR (CfEcR) homology model was used to identify seventeen amino acid residues that are critical for 20E binding. Further analyses identified a mutant (A110P) that failed to respond to steroids, but whose response to non-steroidal ligands was unaffected (Kumar et al. 2002). Crystal structure of the EcR-USP heterodimer of the hemipteran, *Bemisia tabaci* (BtEcR), showed a ponasterone A binding pocket similar to that of EcR/USP from the lepidopteran *Heliothis virescens* (Carmichael et al. 2005). However, some differences in the ligand binding pockets, especially in those parts that are not in contact with ponasterone A, were observed between BtEcR and HvEcR (Carmichael et al. 2005).

2.3 Juvenile Hormones

2.3.1 Introduction

The sesquiterpenoid JH is secreted by the corpora allata (CA) and its synthesis/secretion is regulated by neuropeptides produced by neurosecretory cells of the brain. Allatostatins and allatotropins inhibit and stimulate, respectively, the synthesis of JH. JH is normally present during the rise in ecdysone titer in preparation for a larval molt, which ensures that the larva will molt into another larva. However, JH disappears shortly after the final larval molt, allowing ecdysone to induce metamorphosis (Riddiford 1994). JH is also involved in regulating various reproductive processes including oocyte growth (Wyatt and Davey 1996). Application of JH during the last larval instar results in extra larval molts or production of larval-pupal intermediates that seldom survive.

2.3.2 Biosynthesis

The most common form of JH, JH III, is derived from the C15 precursor farnesyl diphosphate (FPP), a critical intermediate of the mevalonate pathway that is elaborated from acetyl-CoA. In most organisms, the principal end

product of this pathway is cholesterol; however, insects do not produce cholesterol and, as a result, FPP is used for the production of alternative end products, including JH. The Lepidoptera produce four structural homologues of JH III—JH 0, JH I, JH II and 4-methyl-JH I–each bearing 1 to 3 ethyl branches derived from the substitution of propionyl-CoA for acetyl-CoA at a very early step of JH biosynthesis (Schooley et al. 1973). Up to the point of FPP formation, JH biosynthetic enzymes are homologous to those found in all living organisms that use the mevalonate pathway. The last steps of JH biosynthesis, however, appear to be specific to insects. These include the conversion of FPP to farnesol and farnesal, followed by oxidation to farnesoic acid (FA), esterification to methyl farnesoate (MF) and epoxidation to JH. In addition, at least one mevalonate pathway enzyme involved in the biosynthesis of the lepidopteran ethyl-substituted JHs shows substrate/product selectivity favoring ethyl-branched ligands (Sen and Ewing 1997; Sen and Roach 1996), thus conferring some lepidopteran specificity to this enzyme.

2.3.3 Mode of Action

Several hemolymph, cytosolic and nuclear JH binding proteins have been identified and characterized (de Kort and Granger 1996; Palli 1991). Based on the assumption that the JH receptor would be a member of the steroid/thyroid hormone superfamily, several groups used probes derived from various steroid/thyroid hormone family members and low stringency hybridization techniques. Palli et al. (1991) used human retinoic acid receptor cDNA as a probe and identified a steroid/thyroid superfamily member from *Manduca sexta*. Further characterization of this cDNA revealed that this is not a JH receptor but rather an ecdysone-induced transcription factor which plays a critical role in ecdysone signal transduction and is related *to Drosophila* hormone receptor 3 (Koelle et al. 1992) and hence named *Manduca* hormone receptor 3 (Palli et al. 1992). Unfortunately, a 29-kDa nuclear protein we identified in *M. sexta* epidermis turned out to be a low affinity JH binding protein (Palli et al. 1994; Xu et al. 2002a).

The mammalian retinoid X receptor (RXR) was shown to form heterodimers with several nuclear receptors including the farnesoid X-activated receptor (FXR). JH III, but not JH acid or methoprene, can bind/activate the FXR-RXR heterodimer (Yao et al. 1993). Methoprene and methoprene acid but not JHIII can activate RXR alone (Guo et al. 1997). These two studies suggested that RXR or its insect homologue *ultraspiracle* (USP) could play an important role in signal transduction of JH or JH-related compounds. Jones and Sharp (1997) showed that both JH III and JH III bisepoxide (JHB_3) bind to a *D. melanogaster* USP homodimer. Subsequent studies showed that *D. melanogaster* USP can bind to the direct repeat 12 (DR12) response element, and that JH III can induce a reporter gene placed under the control of the DR12 response element and JHE core promoter (Li et al. 2001; Xu et al. 2002b).

Progress in a totally different direction resulted from studies on *D. melanogaster* mutants that are resistant to methoprene [*Rst(1)JH*] (Wilson and Fabian 1986). An 85-kDa protein isolated from *Rst(1)JH* flies showed a 6-fold lower affinity than the wild-type protein for JHIII (Shemshedini et al. 1990). The *Rst(1)JH* gene was cloned and found to be a member of the basic helix-loop-helix (bHLH)-PAS family of transcriptional regulators (Ashok et al. 1998). The *Rst(1)JH* gene product is not vital, as shown by the production of null mutants that are viable (Restifo and Wilson 1998). The *Rst(1)JH* gene product was detected in several tissues including known JH-responsive tissues (Pursley et al. 2000). Recent studies showed that in vitro synthesized *Rst(1)JH* directly bound to JH III with high affinity (Miura et al. 2005). In addition, in transient transfection assays *Rst(1)JH* caused JH-dependent activation of a reporter gene suggesting that *Rst(1)JH* may function as JH-dependent transcription factor (Miura et al. 2005).

The action of JH on the uptake of the yolk protein vitellogenin (Vg) by developing oocytes appears to involve a membrane receptor at the surface of follicular cells. A 35-kDa JH binding protein was identified and proposed to be the critical protein involved in this action of JH in ovaries of *Locusta migratoria* (Sevela 1995).

The Broad complex of transcription factors (BR-C) count among the best-known models for studies on interaction of ecdysone and JH. In *D. melanogaster*, BR-C is required for metamorphosis; BR-C mutants can develop normally until the last larval stage but cannot undergo metamorphosis (Kiss et al. 1976, 1988, 1980). The BR-C mRNAs [four alternatively spliced isoforms (DiBello et al. 1991)] appear at the onset of metamorphosis in response to a rise in ecdysone titer and play a critical role in metamorphosis (Crossgrove et al. 1996; Hodgetts et al. 1995; Karim et al. 1993; Mugat et al. 2000; Renault et al. 2001; von Kalm et al. 1994). Working with both *D. melanogaster* and *Manduca sexta,* Zhou et al. (Zhou et al. 1998; Zhou and Riddiford 2002) showed that BR-C is an important specifier of pupal development. In the epidermis of *M. sexta* JH can prevent the ecdysone-induced expression of BR-C mRNAs (Zhou et al. 1998).

Identification of genes that are regulated by JH, followed by characterization of their promoter regions, may provide insights into the molecular basis of JH action. We used a differential display of mRNAs technique and found that the expression of the juvenile hormone esterase gene (*Cfjhe*) in CF-203 cells (a cell line from *Choristoneura fumiferana)* is induced by JH I and that the JH I induction is suppressed by 20E (Feng et al. 1999). To identify JH and 20E response regions in the *Cfjhe* promoter, we have screened a *C. fumiferana* genomic library with *Cfjhe* cDNA probe and identified genomic clones that contained a 1,270-bp promoter region. Analysis of this promoter region using reporter assays in CF-203 cells identified a 30-bp region that is sufficient for JH and 20E response observed for the *Cfjhe* gene. Mutational analysis on this 30-bp sequence identified a direct repeat 4 (DR4) element that mediates JH action (JHRE) (Kethidi et al. 2004). Nuclear proteins isolated

from CF-203 and *Drosophila melanogaster* L57 cells bind to this element. Protein kinase C-mediated phosphorylation and JH-mediated dephosphorylation play critical roles in modification of nuclear proteins that bind to this JHRE (Kethidi et al. 2006).

2.4 Ecdysis-Controlling Neuropeptides

2.4.1 Introduction

The pre-programmed behavior expressed at the time of ecdysis to help the insect shed its old cuticle is not directly elicited by ecdysone but by a small group of neuropeptides produced in various tissues. EH was the first one of these to be identified (Truman and Riddiford 1970), but at least three others are now known to be required for the full set of behaviors to be expressed: PETH, ETH and CCAP.

2.4.2 Biosynthesis

EH is produced by two ventromedian pairs of neurosecretory cells of the brain that project to neurohemal (i.e., peripheral release) areas of the hindgut, namely the proctodeal nerve (Copenhaver and Truman 1986; Truman and Copenhaver 1989). Following reconstruction of the nervous system in the pharate adult, however, the corpora cardiaca become the site for release of EH into the hemolymph (Horodyski 1996). Central release of EH also occurs, targeting neurons that contain CCAP (Morton and Simpson 2002). In *M. sexta*, EH is a 62-amino-acid neuropeptide (Table 1) encoded by a single copy gene; the prohormone contains a 26-amino-acid signal peptide which, once cleaved, releases the mature hormone (Horodyski et al. 1989; Marti et al. 1987).

PETH and ETH are synthesized by the large Inka cells of the epitracheal glands, which are attached to the underside of the tracheal trunk near the spiracles. cDNAs encoding ETH and PETH peptides have been cloned from *M. sexta* (Zitnan et al. 1999), *D. melanogaster* (Park et al. 1999) and *B. mori* (Zitnan et al. 2002). In all three species, the cDNA encodes a preprohormone which, following proteolitic cleavage, releases a signal peptide and three neuropeptides. In the two lepidopteran species, the first of these is PETH, a fully conserved 11-amino-acid peptide, followed by ETH and an ETH-associated peptide (ETH-AP). ETH is well conserved in these two species, with 23 and 26 amino acids in *B. mori* and *M. sexta*, respectively, and an identical NKNIPRM-NH_2 C-terminus. Whereas ETH and PETH are similar, amidated peptides, the ETH-APs show no sequence similarity to the former peptides. The first two neuropeptides contained in the *D. melanogaster* preprohormone are homologous to the lepidopteran PETH and ETH, and have been designated DmETH1 (18 amino acids) and DmETH2 (15 amino acids). The third one, an ETH-associated peptide (DmETH-AP), is strongly divergent (Table 1).

Table 1. Sequences of selected ecdysis-controlling neuropeptides: eclosion hormone (EH), pre-ecdysis-triggering hormone (PETH), ecdysis-triggering hormone (ETH), ETH-associated peptide (ETH-AP), crustacean cardioactive peptide (CCAP) and corazonin (Crz). Ms *Manduca sexta*; Bm *Bombyx mori*; Dm *Drosophila melanogaster*

MsEH	NPAIAT—GYDPM—EICIENCAQCKKMLGAWFEGPLCAESCIKFKGKLIPE-CEDFASIAPFLNKL-62
BmEH	SPAIAS—SYDAM—EICIENCAQCKKMFGPWFEGSLCAESCIKARGKDIPECE-SFASISPFLNKL-62
DmEH	LPAISHYTHKRFDSMGGIDFVQVCLNNCVQCKTMLGDYFQGQTCALSCL-KFKGKAIPDCEDIASIAPFLNALE-73
MsPETH	SFIKPNNVPRV-NH_2 11
BmPETH	SFIKPNNVPRV-NH_2 11
MsETH	SNEA—FDEDVMGYVIKSNKNIPRM-NH_2 23
BmETH	SNEAISPFDQGMMGYVIKTNKNIPRM-NH_2 26
DmETH1	DDSSPGFFLKITKNVPRL-NH_2 18
DmETH2	GENFAIKNLKTIPRI-NH_2 15
MsETH-AP	NYDSENRFDIPKLYPWRAENTELYEDDAQPTNGEEINGFYGKQRENM-OH 47
BmETH-AP	NYDSGNHFDIPKVYSLPFEFYGDNEKSLNNDDAEE—YYAKKMGSM-OH 44
MsCCAP	PFCNAFTGC-NH_2 9
DmCCAP	PFCNAFTGC-NH_2 9
DmCrz	SFNAASPLLANGHLHRASELGLTDLYDLQDWSSD-NH_2 34

CCAP was first discovered and identified by Stangier et al. (1988) as a cardioacceleratory factor in the pericardial organ of the crab *Carcinus maenas*. The peptide has since been isolated from several arthropod representatives, including insects. The CCAP gene is expressed in the central nervous system, and the corresponding cDNA has been cloned from *M. sexta* (Loi et al. 2001) and *D. melanogaster* (Park et al. 2003); in both species, conceptual translation of the coding sequence yielded a preprohormone containing a signal peptide as well as four putative neuropeptides, including the perfectly conserved PFCNAFTGC sequence of CCAP (Table 1), and three CCAP-associated peptides.

2.4.3 Modes of Action

Although the above-described peptides and associated cell signaling pathways appear well conserved among the insect species examined to date (Zitnan et al. 2003), the exact hierarchical sequence of their action has not yet been clearly established; however, models were proposed that include possible functional redundancy among these and other peptides (Kim et al. 2004; Morton and Simpson 2002).

In preparation for ecdysis, rising ecdysone levels induce the expression of the ETH gene (Zitnan et al. 1999), the promoter region of which contains an ecdysone response element (Park et al. 1999); the subsequent decline in ecdysone titer is required for peptide release (Zitnan et al. 1999). EH has been proposed to play an upstream role in coordinating the ecdysis behavioral sequence, evoking accumulation of cGMP in Inka cells and in neurons of the ventral ganglia. In Inka cells, the rise in cGMP levels is associated with ETH release, which then forms a positive feedback loop to cause the further release of EH by ventromedian brain cells; EH (and ETH) action on ventral ganglia causes the release of CCAP, which is believed to directly activate the ecdysis motor program [see (Morton and Simpson 2002)]. However, the cloning of the corazonin receptor in *M. sexta* (Kim et al. 2004), and recent observations made following the targeted ablation of EH and CCAP neurons in *D. melanogaster* (Clark et al. 2004; Park et al. 2003), call for a refinement of this general model. For example, an additional neuropeptide, corazonin (Table 1), appears to be the factor responsible for the *initial* release of PETH and ETH from Inka cells, with EH eliciting a *subsequent,* massive release: (i) injection of synthetic corazonin into *M. sexta* pharate larvae induces release of PETH and ETH as well as expression of preecdysis and ecdysis behaviors, (ii) corazonin hemolymph titers rise just before natural preecdysis, and (iii) corazonin receptor transcripts accumulate in epitracheal glands but not in epidermis, muscle and trachea (Iversen et al. 2002; Kim et al. 2004). With respect to ETH signaling, two splicing subtypes of the ETH receptor, a GPCR, were recently cloned from *D. melanogaster* (Iversen et al. 2002; Park et al. 2003); these respond preferentially to ETH but show differences in ligand sensitivity and specificity (Park et al. 2003).

2.5 Bursicon

2.5.1 Introduction

The peptide hormone bursicon causes the hardening and darkening of the new cuticle soon after molting. Although the biological activity of a factor present in central nervous system and in the hemolymph of insects that is capable of regulating hardening and darkening of the cuticle was discovered 43 years ago (Cottrell 1962; Fraenkel and Hsiao 1962, 1963; Fraenkel et al. 1966; Mills 1967), the molecular identity of bursicon hormone was revealed only recently (Dewey et al. 2004; Honegger et al. 2004; Luo et al. 2005; Mendive et al. 2005).

2.5.2 Biosynthesis

Bursicon activity was detected in the central nervous system and in the hemolymph (Cottrell 1962; Fraenkel and Hsiao 1962, 1963). Antibodies raised against the bursicon peptides detected bursicon-containing neurons in the

central nervous systems of cockroach, *Periplaneta. americana*, crickets, *Teleogryllus commodus*, *Gryllus bimaculatus*, the moth *Manduca sexta* and the fly, *Drososphila melanogaster* (Honegger et al. 2002). *Drosophila melanogaster* bursicon is a cystine knot heterodimeric protein consisting of two subunits encoded by the genes *burs* (*bursicon*) and *pburs* (*partner of bursicon*) and these proteins were detected in the nervous system suggesting that this tissue is the most likely source of bursicon hormone (Luo et al. 2005).

2.5.3 Mode of Action

Genetic analyses in *D. melanogaster* showed that bursicon acts through G-protein coupled receptor, LGR2 (leucine-rich repeat G-protein coupled receptor 2) encoded by rickets gene (Baker and Truman 2002). Mutation of rickets gene in *D. melanogaster* results in flies that fail to initiate tanning and wing expansion after adult emergence (Baker and Truman 2002). Some of the defects in these mutant flies can be rescued by injection of an analog of cAMP, suggesting that cAMP is involved in bursicon signal transduction. Identification of bursicon peptides and their receptor will definitely help in making progress on studies related to mode of action of this hormone.

3 Genes Involved in Molting and Metamorphosis as Target Sites for the Design of Biorational Insecticides

Both ecdysone and JH analogs have been successfully developed for the purpose of insect pest control. These are either known or hypothesized to interact with the receptors of the hormone they mimic, but their discovery or development was not based on prior knowledge of receptor structure. Future progress in this area will likely benefit from the cloning of the appropriate receptor proteins and the determination of their 3D structures. This type of data will not only facilitate analog design but should also make it possible to identify potential antagonists to block hormone-activated responses at the receptor level. Similarly, the cloning of enzymes involved in the biosynthesis of JH and edysone is expected to expedite the design of anti-JH and anti-ecdysone compounds. Here we first provide a brief overview of the successful hormone analogs, and then cover recent work on enzyme and receptor characterization.

3.1 Success Stories

3.1.1 Ecdysone Analogs

Over the years, several attempts have been made to use ecdysone analogs for insect control. The most dramatic discovery happened in the early 1980s when chemists at Rohm and Haas Company synthesized 1,2-diacyl-1-substituted

hydrazines that had potent insecticidal activity (see Fig. 1 for structures of four commercial insecticides and two compounds under development). This class of compounds induce a precocious larval molt in the susceptible insects, acting through the ecdysone receptor complex (Wing 1988), and has been the subject of a recent review (Dhadialla et al. 1998). Their narrow spectrum of activity makes them one of the best-fit compounds for use in integrated pest management programs. For example, tebufenozide is effective against many lepidopteran pests but has little or no effect on their hymenopteran parasites. Because of the safety profiles, ecdysone agonists are attractive for mosquito control in urban settings. Unfortunately, there are no ecdysone analogs currently available that can be used for mosquito control.

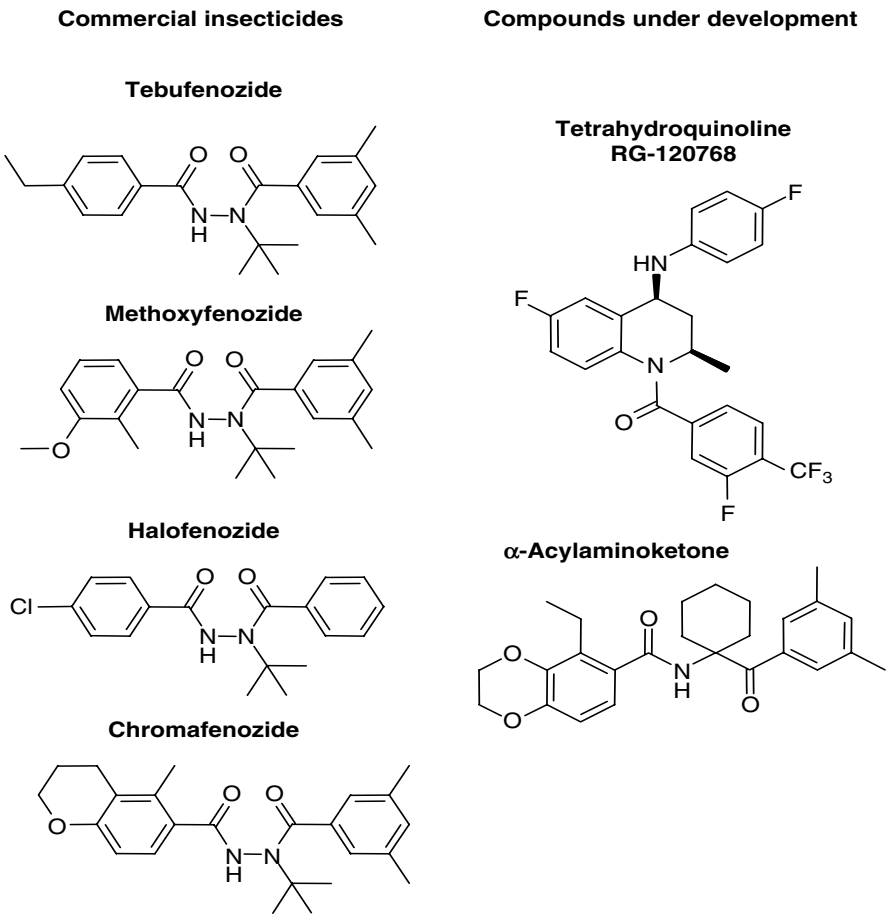

Fig. 1. Structures of stable nonsteroidal ecdysone agonists that are available commercially and those that are under development

As explained above, the ecdysone analogs work well on some species of Lepidoptera and Coleoptera but not on many of the species of insects tested, including aphids and mosquitoes. The binding affinities of these analogs to EcR/USP account for some of these differences (Dhadialla et al. 1998), but these differences alone do not account for all the species-specific actions. Our studies in insect cell lines (Sundaram et al. 1998) and yeast (Hu et al. 2001) suggested that ABC transporters are involved in efflux of these analogs from cells that are not susceptible.

3.1.2 JH Analogs

Numerous analogs of JH (JHAs) have been synthesized and tested for their pest control potential (see Fig. 2 for structures of commercial JH analogs). The principal effect of these compounds appears to be a disruption of embryogenesis and metamorphosis. Because of the latter effect, JHAs are most effective at controlling insects that are considered pests in the adult stage. For example, a terpenoidal JHA, methoprene, has been used in commercial products for mosquito control for the past 20 years. A number of formulations were found to be effective for the control of these insects (Akamatsu et al. 1975; Habets et al. 2001; Kline 1993; Laird 1985; McCarry 1996; Mulla and Darwazeh 1975; Prabhu and Nayar 1974; Ritchie and Broadsmith 1997; Wright et al. 1971). In these dipterans, methoprene induces various morphogenetic and developmental abnormalities resulting in their death during metamorphosis (Mulla 1995; Mulla and Darwazeh 1975, 1988; Mulla et al. 1985, 1986), but its exact mode of action at the molecular level is not well understood. Other JHAs such as the non-terpenoidal fenoxycarb and pyriproxyfen have been tested for mosquito control and showed various degrees of efficacy (Estrada and Mulla 1986; Mulla et al. 1986). There are recent reports that resistance to JHAs has developed in some species of mosquitoes [*Aedes nigromaculis* (Cornel et al. 2000) and *Ochlerotatus nigromaculis* (Cornel et al. 2002)].

Despite the fact that several JHAs are commercially used for the control of insect pests, including whiteflies, fleas and mosquitoes, we still do not fully understand how these analogs function, at the molecular level, to interfere with insect development and reproduction. Natural JHs and their analogs were shown to induce a rise in the mRNA levels of juvenile hormone esterase in lepidopteran insects such as *Trichoplusia ni* (Venkataraman et al. 1994), and of the jhI-1 and jh1-26 genes in *D. melanogaster* (Dubrovsky et al. 2000). Mosquitoes such as *Ae. aegypti* are highly sensitive to methoprene (LD_{50} is about 1 ppb or less), but it is still not clear whether this JHA causes mosquito mortality by mimicking JH action. Susceptibility of mosquitoes to methoprene gradually increases during larval development, and the larvae are more susceptible than pupae (Noguchi and Ohtaki 1974); the larvae, however, are most sensitive to methoprene at 10–30 h before the pupal molt (Georghiou and Lin 1974). When the last instar larvae of *Culex pipiens* were exposed to methoprene, the effects

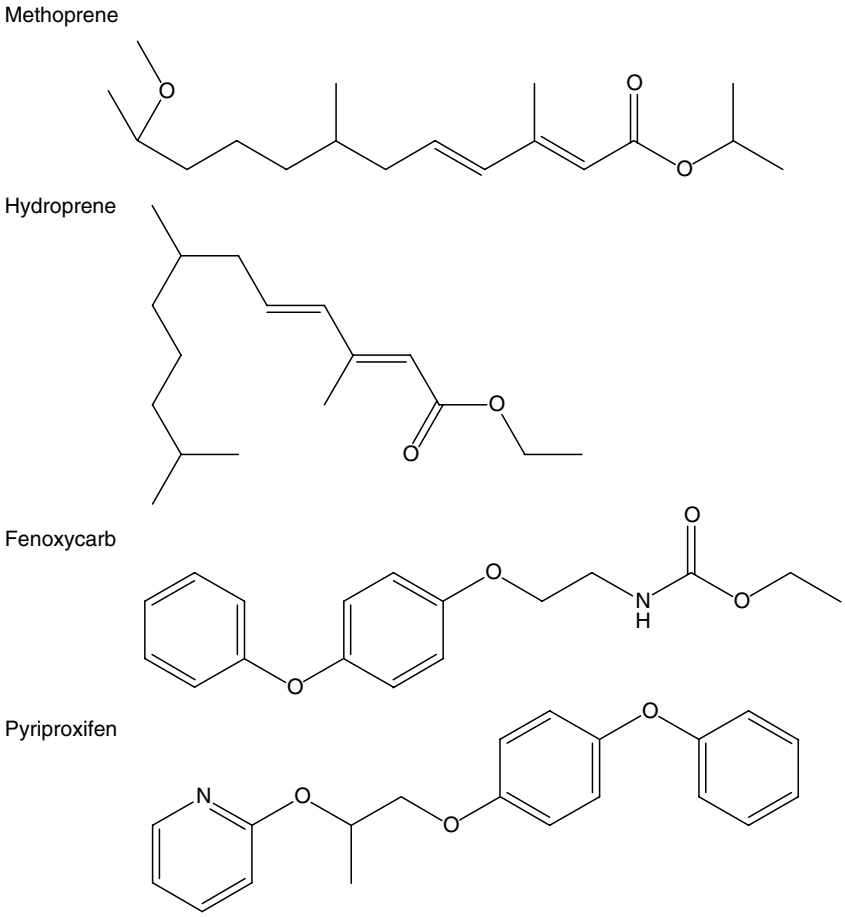

Fig. 2. Structures of four JH analogs commercially available

were observed in pupae and adults but not in larvae (Georghiou and Lin 1974). Similarly, in *An. Stephensi*, methoprene caused effects only in pupae and adults but not in larvae (Raj et al. 1978). However, methoprene was shown to be toxic to *Culex molestus* larvae (Farghal and Temerak 1981). It is not clear whether these contradictory results are due to the use of different species of mosquitoes or different doses of methoprene in these experiments. Methoprene was shown to inhibit follicle maturation (Judson and de Lumen 1976) and can rescue JH III activation of previtellogenic fat body nucleoli for ribosomal RNA production in allatectomized *Ae. aegypti* adults (Raikhel and Lea 1990). Although these studies point to a JH-mimicking activity of methoprene, its exact mode of action remains poorly understood. Recently, Nishiura et al. (Nishiura et al. 2003). showed that methoprene interferes with midgut remodeling during mosquito

metamorphosis. Methoprene-treated larvae of *Ae. aegypti* and *Culex quinquefasciatus* pupated, but the pupal midguts were morphologically similar to larval midguts.

3.2 Hormones, Receptors and Transcription Factors as Target Sites

Recent solving of crystal structures of the EcR proteins from *H. virescens* and *Bemisia tabaci* show different and only partially overlapping binding cavities for ecdysteroid and diacylhydrazine ligands, thereby providing a three-dimensional spatial explanation for the differential activity of these ligands in insects belonging to different orders (Carmichael et al. 2005). Our recent modeling and mutational studies on ecdysteroid, diacylhydrazine, and tetrahydroquinoline ligands also showed somewhat different and only partially overlapping binding cavities for these three groups of ligands (Kumar et al. 2002). In transactivation assays, tetrahydroquinoline compounds activate wild-type CfEcR poorly, but a change in a single amino acid in the ligand-binding domain (valine 128 to either phenylalanine or tyrosine) shifted the ligand specificity of CfEcR (Kumar et al. 2002) very much in favor of tetrahydroquinolines over diacylhydrazines. Similarly, mutation of alanine 110 to proline in the CfEcR completely eliminated ecdysteroid ligand binding while the binding of diacylhydrazine ligands to this mutant receptor remained unaffected (Kumar et al. 2002). In addition, mutating a single amino acid residue in the ligand-binding domain of EcR from *Aedes aegypti*, namely phenylalanine 529, resulted in altered ecdysteroid ligand specificity (Wang et al. 2000). These recent findings point to a highly flexible ligand-binding pocket of EcR and suggest opportunity in designing target-specific insecticides. As shown in Fig. 3, 18 amino acids are present in all lepidopteran EcRs but not in EcRs from other insects. Some of these residues are probably involved in diacylhydrazine biding and activity. Similarly, seven amino acid residues are present only in the ligand binding region of Dipteran EcRs (Fig. 3). In addition, four amino acid residues are specific to EcRs cloned from insects outside Lepidoptera and Diptera, as well as from crabs and tick (Fig. 3). As more EcRs are identified from pest insects and as efficient high-throughput screening assays are developed (Swevers et al. 2004; Tran et al. 2001), it should become increasingly feasible to identify ecdysone agonists that target various pest species (see Table 1 for details on positives and negative aspects of EcR as a target site).

3.3 Biosynthetic Enzymes as Target Sites

3.3.1 Juvenile Hormone

Given that JHAs are not very effective in controlling phytophagous insects that inflict damage to their host plants during their larval stages, several approaches are being explored for the development of control products

H1
H2
H3
H4
H5
s1
s2
s3
H6
H7
H8
H9
H10
H11
H12

aimed at lowering JH titers, the result of which would be the induction of precocious metamorphosis, thus reducing feeding damage. Compounds that can block or reduce JH biosynthesis through the inhibition of a JH biosynthetic enzyme have been reported from natural (e.g., the precocenes, brevioxime) and synthetic sources (e.g., fluoromevalonate), but none has yet been developed commercially for reasons of poor efficacy and/or high toxicity (Dhadialla et al. 2005). Some of these compounds are known to inhibit insect-specific JH biosynthetic enzymes such as the methyl farnesoate epoxidase, a P450 enzyme [for a list of known anti-JH compounds and associated references, see reference (Dhadialla et al. 2005)].

Current efforts aimed at developing inhibitors of JH biosynthesis include the design of protease-resistant mimetics of the allatostatins, the neuropeptides that have been isolated from several species of insects and which display varying degrees of inhibitory activity on JH biosynthesis. More relevant to this section, however, are the recent advances in the cloning and characterization of selected JH biosynthetic enzymes, as this new knowledge should permit the rational design of inhibitors showing greater effectiveness and selectivity than those tested in the past. Here we review recent work on four enzymes, including three affecting the late steps (i.e., post-FPP) of JH biosynthesis and one belonging to the lepidopteran mevalonate pathway.

Methyl farnesoate epoxidase (MFO). With the possible exception of what is observed in the Lepidoptera (Bhaskaran et al. 1986) the last step of JH biosynthesis is the epoxidation of MF. It has been known for some time that this epoxidase is a microsomal cytochrome P450 enzyme in cockroach and locust (Feyereisen et al. 1981; Hammock 1975) but only recently was its cDNA cloned from a cockroach, following the construction of a *Diploptera punctata* CA cDNA library and the 5′ end sequencing of 1,056 clones from it (Helvig et al.

Fig. 3. Alignment of amino acids present in the ligand binding domain of ecdysteroid receptors. The amino acid residues present only in EcRs from lepidopteran, dipteran or other insects, crabs and tick are *boxed*. The EcR sequences are from *Bicyclus anynana* (Bian, unpublished, gi:6580162), *Junonia coenia* (Juco, unpublished, gi:6580625), *Choristoneura fumiferana* [Chfu, (Kothapalli et al. 1995)], *Chilo suppressalis* [Chsu, (Minakuchi et al. 2002)], *Plodia interpunctella* [Plin, (Siaussat et al. 2004)], *Bombyx mori* [Bomo, (Kamimura et al. 1996; Swevers et al. 1995)], *Manduca sexta* [Mase, (Fujiwara et al. 1995)], *Heliothis virescens* [Hevi, (Billas et al. 2003; Martinez et al. 1999), *Calliphora vicina* (Cavi, unpublished, gi:12034940), *Lucilia cuprina* [Lucu,(Hannan and Hill 1997)], *Drosophila melanogaster* [Drme, (Koelle et al. 1991)], *Ceratitis capitata* [Ceca, Verras et al. 1999], *Aedes aegypti* [Aeae, (Cho et al. 1995)], *Aedes albopictus* [Aeal, (Jayachandran and Fallon 2001)], *Anopheles gambiae* (Anga, unpublished, gi:55234452), *Chironomus tentans* [Chte, (Imhof et al. 1993)], *Apis mellifera* (Apme, unpublished, XP_394760), *Locusta migratoria* [Lomi, (Saleh et al. 1998)], *Tenebrio molitor* [Temo, (Mouillet et al. 1997)], *Bamisia tobaci* [Bato, (Carmichael et al. 2005)], *Carcinus maenas* (Cama, unpublished, gi:40748295), *Celuca pugilator* [Cepu, (Chung et al. 1998)], and *Amblyomma americanum* [Amam, (Guo et al. 1997)]

2004). The heterologously expressed protein designated CYP15A1 showed high affinity for MF and converted MF to JH III. The enzyme could not use various MF-related compounds such as farnesol, farnesoic acid and farnesyl methyl ether as substrates, and it showed selectivity for the natural geometric isomer as well as for the 10*R* enantiomer. CYP15A1 is expressed only in the CA and only during peak JH production. Interestingly, selected 1,5-disubstituted imidazoles, known to inhibit JH biosynthesis and cause accumulation of MF in cockroach CA, displayed parallel effectiveness in inhibiting the activity of recombinant CYP15A1 and in vitro JH biosynthesis by isolated CA (Helvig et al. 2004). Surprisingly, no clear orthologs of CYP15A1 are found in *D. melanogaster*, perhaps because this fly produces a bisepoxide of JH III, the production of which may require a different epoxidase. Clearly, this research points to the high degree of insect and tissue (i.e., CA) specificity of this enzyme, which makes it an excellent target for the development of anti-JH compounds.

JH acid methyl transferase (JH acid MT). In the Lepidoptera, biogenesis of the methyl ester functional group of JHs is effected by an *O*-methyl transferase that uses JH acid as substrate; in other groups of insects, FA is the putative substrate of this enzyme. This enzyme has recently been cloned from *B. mori*, using a fluorescent differential display approach (Shinoda and Itoyama 2003). PCR amplicons whose abundance displayed developmental changes matching those expected for this enzyme were submitted to sequence analysis. One of the cDNAs was found to encode a 278 aa protein with no clear homolog but containing a conserved motif found on several *S*-adenosyl methionine (SAM)-dependent methyl transferases. Northern blot analysis showed that this transcript could be detected only in the CA, and a q-RT-PCR analysis indicated that its transcription starts declining at the spinning stage of last-instar larvae, concomitant with the documented loss of JH acid MT activity at this stage. The recombinant His-tagged enzyme could convert both JH acids and FA to the expected products, although it showed greater conversion rates with JH I acid and JH II acid as substrates as compared to JH III acid and FA (100 μM); no conversion was observed with several saturated and unsaturated fatty acids. Thus, JH acid MT displays insect and tissue selectivity in a manner similar to that described above for MFO. In addition, the JH acid MT of *B. mori*, shows substrate selectivity favoring the formation of ethyl-branched JHs.

Farnesol oxidase (FO). Farnesol oxidation in plants, vertebrates and non-JH producing tissues of insects is typically catalyzed by nicotinamide-dependent dehydrogenases (Sperry and Sen 2001). One such alcohol dehydrogenase, JGW, was recently cloned from two African *Drosophila* species (Zhang et al. 2004). It displays high substrate specificity for geraniol and farnesol, suggesting that it may be involved in JH biosynthesis. However, the JGW cDNA was obtained from total RNA extracted from whole-body adult flies (as opposed to ring glands, which contain the CA), and its expression, in *D. teissieri*, appears to be

confined to the testes (Zhang et al. 2004). Work on *M. sexta* strongly suggests that the enzyme responsible for the formation of farnesal from farnesol, within the CA, is not a dehydrogenase but a specific metal-dependent alcohol oxidase, given that the conversion is oxygen-dependent (Sperry and Sen 2001). Inhibition studies with CA homogenates indicate that this oxidase displays high specificity for alcohols with terpenol structures, but it does not seem to be involved in regulating the proportions of JH homologs produced (Sen et al. 2003). This enzyme has not yet been cloned from any insect species, but its substrate specificity suggests that research aimed at its molecular characterization would probably be rewarding.

Farnesyl diphosphate synthase (FPPS). FPPS is a pivotal mevalonate pathway enzyme that catalyses the sequential head-to-tail condensation of three isoprene (C5) units to generate the sesquiterpenoid (C15) FPP, a precursor to cholesterol and several other end products, including JH III in insects. Because of its ubiquity, FPPS has not been viewed as an ideal target for the design of insect-specific control products. In the Lepidoptera, however, this enzyme has been shown to display substrate selectivity for the ethyl-substituted precursors of ethyl-branched JHs (Sen and Ewing 1997; Sen et al. 1996). FPPS has now been cloned from several insects, including four species of moths (Kikuchi et al. 2001; Cusson et al. 2006). The lepidopteran genome contains at least two different FPPS genes, one of which encodes a protein featuring unique active-site substitutions that are predicted to favor the biogenesis of ethyl-substituted FPP, as suggested by the analysis of a homology model and docking simulations (Cusson et al. 2006). Although it is not yet clear whether this FPPS homolog plays a significant role in JH biosynthesis, the docking results suggest that it may be possible to block its activity in a Lepidoptera-specific manner.

3.3.2 Ecdysone

Inhibition of ecdysone synthesis, which is expected to prevent or delay the molt, may not be as effective a pest control strategy as the lethal induction of a precocious molt by an ecdysone agonist. Nonetheless, several studies have examined the efficacy of potential inhibitors of the P450 CYP enzymes involved in the final hydroxylation steps of ecdysteroid biosynthesis, both in vitro and in vivo [reviewed by (Hoffmann and Lorenz 1998)]. The compounds tested include acetylenic and allenic cholesteryl derivatives, which act as suicide substrates of the C-22 and C-25 hydroxylases (Burger et al. 1988, 1989; Mauvais et al. 1994; Roussel 1994), the imidazole fungicide ketoconazole, which is known to inhibit the ecdysone 20-monooxygenase (Jarvis et al. 1994; Lorenz et al. 1995), the neem tree compound azadirachtin (Lorenz et al. 1995) and the imidazole derivative KK-42 (Jarvis et al. 1994; Roussel et al. 1989). Although some of these compounds have shown promising inhibitory activity in vitro, none displayed degrees of in vivo developmental impacts that warranted their

commercial development, with the exception of azadirachtin, which appears to have multiple modes of action. However, the recent cloning of the P450 hydroxylases that are targeted by the above compounds (Petryk et al. 2003; Warren et al. 2004) should now make it possible to develop new inhibitors with greater potency and specificity. Interestingly, *D. melanogaster* individuals carrying mutations in these genes show severe developmental defects. The enzyme encoded by the *D. melanogaster* "*disembodied*" (*dib*) gene, which is responsible for ecdysteroid hydroxylation at C-22, shows strict substrate specificity (Warren et al. 2002) and, therefore, could prove to be a suitable insect-specific target site.

4 Utilization of Genes Involved in Molting and Metamorphosis for Development of Pest Management Tools

4.1 Small Molecules

Discovery, development, and registration of insecticides based on small chemicals continue to be important, especially for crops where transgenic pest-tolerant varieties are not yet available. As discussed in Palli and Retnakaran (Palli and Retnakaran 2001) the genes involved in molting and metamorphosis can be used for developing target-site-based high through put screening assays in cell lines and yeast (Table 2). The proteins expressed from these genes in insects or yeast or bacteria can also be used for identification of agonists and/or antagonists in high-throughput binding assays (Palli and Retnakaran 2001). With respect to identifying potential receptor agonist/antagonists as well as enzyme inhibitors, when the insect target proteins have mammalian homologues, one can take advantage of the available data on drugs that have been developed for therapeutic purposes. These could perhaps be used as starting points for in silico screening and design of insecticidal compounds. This type of broad-spectrum approach to chemical screening is illustrated in a recent review where a group of enzymes is examined for its potential as a target for both drug and herbicide development (Rohdich et al. 2004).

Similarly, when the target protein has no mammalian homolog but its structure may be determined or modeled, molecules that have shown some inhibitory activity in prior studies (e.g., imidazole derivatives in the case of methyl farnesoate epoxidase) may also be used as starting points for in silico design of more effective and more selective inhibitors.

4.2 Use of Genes Involved in Molting and Metamorphosis in Alternate Pest-Management Methods

Peptide hormone receptors may offer some interesting targets for the development of insect-selective control products in view of recent developments in the design of peptidomimetic analogs (Nachman et al. 1998). For example, limited cross-reactivity among PTTHs from different species suggests that the

Table 2. Positive and negative aspects of target sites involved in molting and metamorphosis

Target site	Positives	Negatives
Ecdysone receptor	Proven target site	Target site is not always present.
	Insecticides in market	Instant or quick mortality is not possible.
	Several classes of chemicals	Target site is not novel, Intellectual property issues.
	Under development	Insecticides based on this target site tend to have narrow spectrum of activity.
	Chemicals are safe	Development of resistance against insecticides was reported.
	Receptor genes from several insects are cloned	
	Target site amenable for high throughput screening	
	Design of species specific-insecticides possible.	
Juvenile hormone receptor	Proven target site	Target site was not identified.
	Insecticides in market	Target site is not always present.
	Chemicals are fairly safe	Instant or quick mortality is not possible.
	Target site is novel	Successful applications limited to a few adult pests.
	Insecticides based on this target site have broad spectrum of activity.	Development of resistance against insecticides was reported.
		Non target effects of insecticides are a concern.
Enzymes involved in ecdysteroid and JH biosynthesis	Several enzymes specific to insects were identified	Limited spatial and temporal expression of target site
	Target site is amenable to high throughput screening assays	Instant and quick kill may not be possible.
	Target site is novel	
	Broad spectrum insecticides possible	
PTTH and its receptor	Target site is novel	Limited spatial and temporal expression of target site
	Narrow spectrum insecticides possible	Instant and quick kill may not be possible
	Target site is amenable to high throughput screening assays	
Peptides involved in ecdysis and their receptors	Target site is novel	Limited spatial and temporal expression of target site
	Multiple target sites	Instant and quick kill may not be possible
	Amenable to high through-put screening assays	

structural analysis of the PTTH receptor may be a rewarding endeavor, inasmuch as it could pave the way to the design of species-specific PTTH agonists.

Very little work has been done with respect to using recently acquired knowledge on ecdysis-related neuropeptides (EH, PETH, ETH, CCAP) to develop novel insect control products, although some efforts are currently being made to develop ETH analogs and antagonists (R. Nachman, pers. comm.); the recent cloning of the ETH receptor (see ETH mode of action section) should facilitate this work.

Another potential route for utilization of genes involved in molting and metamorphosis in pest management is through over or under expression of these target genes by microorganism that specifically infect insects. Attempts have been made to use baculoviruses for this kind of approach with mixed results; see (Kamita et al. 2005) for detailed discussion on use of various genes including juvenile hormone esterase for improving baculoviruses. Similarly, genes involved in molting and metamorphosis can be used for pest management through transgenic crops and transgenic insects. Recent developments in these technologies should help in developing novel pest management methods that use genes involved in molting and metamorphosis.

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6 Trypsin Modulating Oostatic Factor for Developing Resistant Crops

Dov Borovsky

1 Introduction

Crop protection against agricultural pest insects is a major problem in growing and producing cultivated plants. Traditional control measures of breeding, chemical spraying, and integrated pest management have alleviated some of these problems. However, the rapid increase in insect resistance to chemical spraying and the slow pace of traditional plant breeding are posing a big challenge to agriculture in the 21st century. It can take 7–10 years and over US$50 million to develop and register a new insecticide (Zaim and Guillet 2002). Although biotechnological approaches offer an alternative to traditional agricultural control of pest insects, the effective genes that were expressed in agricultural crops (e.g., cotton and rice) to protect them against pest insects are mainly bacterial toxins of *Bacillus thuringiensis* (*Bt*). These toxins are effective against lepidopteran, dipteran, and coleopteran insects and most are still used as a microbial formulation (Metz 2003). While the use of *Bt* sprays and genetically modified (GMO) crops, especially cotton, seems to control agricultural pest insects, we cannot ignore the fact that insects are capable of rapidly developing resistance against *Bt* toxins (e.g., the diamondback moth *Plutella xylostella* and the tobacco budworm *Heliothis virescens*) and new approaches should continuously be developed to control insects effectively.

One approach is to utilize insect-specific peptide hormones to selectively control different insects; these hormones control diverse functions in insects such as digestion, reproduction, water balance, feeding behavior, metamorphosis, and sex attraction (Gäde and Goldworthy 2003; Menn et al. 1991). The advantage of this approach is that these hormones are insect-specific and they control vital functions in the life cycle of insects. Disruption of these processes causes irreversible damage and, eventually, death. Because these hormones are peptides, and are found naturally in insects, they are not xenobiotic and would not cause harm to the environment. One of the problems of using insect peptide hormones was that until recently not too many of them were fully characterized. Because of the peptidic nature of these compounds, they cannot be sprayed on an insect's cuticle unless they are attached to lipolitic moieties that will allow their transport through the cuticle into the insect's hemolymph. They also exhibit short residual activity, photolability of certain amino acids (e.g., tyrosine), pH sensitivity, and rapid degradation in

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the environment unless they are protected. Since many of these hormones are blocked either at the amino terminus as pyroglutamic acid derivatives or are amidated at the carboxylic terminus, the peptides cannot be expressed in baculovirus, bacterial, yeast, or plant cells, because molecular engineering of these cells to enable them to amidate or to block the amino terminus with pyroglutamic acid derivative has not been developed yet. However, several of these peptide hormones in the absence of the blocked carboxylic and amino termini have reduced activities that are still effective (e.g., diuretic hormone and ovarian ecdysiotropic hormone). Many of these peptide hormones have a high degree of sequence conservation among agricultural pest insects and economically may be good candidates for future plant protection. Most of these peptide hormones have not been cloned and expressed in plants or tested on agricultural pest insects except for trypsin modulating oostatic factor (TMOF) and pheromone biosynthesis activating neuropeptide (PBAN) from *Helicoverpa zea*, which was cloned and expressed in baculovirus and reduced neonate and 3rd instar survival time of *Trichoplusia ni* larvae by 26 and 19%, respectively (Ma et al. 1998). Thus, TMOF may offer an alternative approach in controlling agricultural pest insects.

2 Biochemical and Physiological Studies

2.1 The Discovery of Mosquito TMOF

Diverse antigonadotrophins or factors that inhibit egg development (oostatic hormones) have been demonstrated in the cockroach, *Blattela germanica* (Iwanov and Mescherskaya 1935), decapod crustaceans (Carlise and Knowles 1959), and the housefly, *Musca domestica*, (Adams et al. 1968; Kelly et al. 1984). In mosquitoes, Meola and Lea (1972) and Else and Judson (1972) similarly demonstrated an ovary-produced humoral factor secreted during oogenesis that inhibited yolk deposition in less developed follicles. In *Rhodnius prolixus*, oostatic hormone produced by the abdominal neurosecretory organs is a small peptide of M_r 1,411 that inhibits the action of Juvenile Hormone (JH) on vitellogenic follicle cells and prevents the ovary from accumulating vitellogenin from the hemolymph (Liu and Davey 1974; Davey 1978; Davey and Kunster 1981). In the house fly *M. domestica,* oostatic hormone seems to inhibit the release or synthesis of egg developmental neurosecretory hormone (EDNH) (Adams 1981), but in mosquito it was proposed that the hormone acts directly on the ovary (Meola and Lea 1972). Kelly et al. (1984) injected a crude extract of oostatic hormone from *M. domestica* into the autogenous mosquito *Aedes atropalpus*, and demonstrated inhibition of both egg development and ecdysteroid biosynthesis.

Borovsky (1985) reported that the mosquito ovary is a rich source for "oostatic hormone". Injections of the hormone into female mosquitoes inhibited yolk deposition and vitellogenin biosynthesis (Borovsky 1985).

However, when partially purified "oostatic hormone" was injected into female *A. aegypti*, both egg development and blood digestion were inhibited (Borovsky 1988). Injections of the hormone into decapitated and ovariectomized females (these females do not synthesize ecdysteroids and do not develop eggs but synthesize protease in their gut) inhibited trypsin-like enzyme biosynthesis and blood digestion in their midgut. These results suggested that "oostatic hormone" inhibits trypsin biosynthesis in cells of the midgut, and not the ovary or the endocrine system as was earlier suggested (Borovsky 1988). The hormone is not species specific, as injection of the hormone caused inhibition of egg development and trypsin biosynthesis in *Culex quinquefasciatus*, *Culex nigripalpus* and *Anopheles albimanus* (Borovsky 1988), and feeding of the hormone adsorbed to yeast particles to larval *Anopheles quadrimaculatus, Culex quiquefasciatus, Culex nigripalpus, Aedes aegypti* and *Aedes taeniorhynchus* caused trypsin inhibition, larval starvation and mortality (Table 1, Borovsky and Meola 2004). The hormone was named "trypsin modulating oostatic factor" (TMOF), and Borovsky and co-workers purified, sequenced and, using mass spectrometry, characterized the hormone as an unblocked decapeptide (NH_2-YDPAPPPPPP-COOH) (Borovsky et al. 1990). Several peptide analogues were synthesized and shown to possess TMOF activity (Borovsky et al. 1990, 1991, 1993, Borovsky and Meola 2004) (Table 2). The solution structure of the hormone was determined by NMR studies showed that the TMOF formed a rod-shaped left-handed helix about 30 angstrom long in solution due to the six-proline residues (Curto et al. 1993; Borovsky et al. 1990, 1993) (Fig. 1a).

2.2 Biological Activity and Mode of Action of TMOF

Because female mosquitoes take several blood meals, TMOF is rapidly metabolized in the thorax (half-life of 1.6 h) of intact mosquitoes after the blood meal has been digested, and trypsin biosynthesis has been terminated (Borovsky et al.

Table 1. Effect of *Aea*-TMOF on different mosquito species

Mosquito species	N	LC_{50} (mM ± S.E.M.)
Anopheles quadrimaculatus	3	0.383 ± 0.005[a]
Culex quinquefasciatus	3	0.458 ± 0.02[a]
Culex nigripalpus	3	1.056 ± 0.097[a]
Aedes aegypti	3	0.2 ± 0.015[b]
Aedes taeneorhynchus	3	0.483 ± 0.049[a]

Three groups of larvae (12 larvae per group) of first instar larvae were individually grown in microtiter plates containing different concentrations of TMOF (1.46–376 μg). Larval mortality was followed for 5–7 days. Mortality in control wells lacking TMOF was 5% or less. Larval mortality at lethal concentrations (LC_{50}) were determined using probit and two tailed Student's *t*-test.
[a]Significant difference from b, $p < 0.0148$. (With permission from Borovsky and Meola 2004)

Table 2. Activity profile of TMOF and its analogues on mosquito larvae

TMOF Analogues	LC_{50} (mM ± S.E.M.)	Activity (%)
YDPAPPPPPP	0.2 ± 0.015[a,b]	100
YDPAPPPPPPR	>71.6	0
$(H)_6$IEGRYDPAPPPPPP	0.34 ± 0.032[b]	59
YDPAPPPP	0.44 ± 0.05[b]	45
YDPAPP	0.64 ± 0.032[b]	31
YDPAP	0.64 ± 0.028[b]	31
YDPAPR	0.24 ± 0.01[a]	80
YDPAPK	>>2.9	0
YDPA	0.21 ± 0.01[a]	95
YDPAR	0.12 ± 0.017[a]	166
$(YDPAR)_4$	0.095 ± 0.007[b]	210
YDP	2.3 ± 0.36[b]	9
YDPR	0.24 ± 0.02[a]	80
YD	1.24 ± 0.05[b]	16
DPA	0.4 ± 0.03[b]	50
DPAR	0.46 ± 0.011[b]	43
$(DPAR)_4$	0.048 ± 0.002[b]	417
DPAP	0.98 ± 0.017[b]	20
DPAPPPPPP	0.44 ± 0.015[b]	45
PAPPPPPP	0.58 ± 0.029[b]	34
APPPPPP	1.18 ± 0.065[b]	17
PPPPPP	1.1 ± 0.025[b]	18
PPPP	1.5 ± 0.085[b]	13
PP	1.83 ± 0.07[b]	11
PAP	6.4 ± 0.23[b]	3
(d)YD	1.4 ± 0.017[b]	14
(d)YDP	0.51 ± 0.05[b]	39
Y(d)DP	0.28 ± 0.015[b]	71
(d)Y(d)DP	1.7 ± 0.029[b]	12
Y(d)DPAP	1.2 ± 0.26[b]	16

TMOF and its analogues were fed to first instar mosquito larvae (three groups of 12 larvae per group) in microtiter plates. Larval mortality was followed for 6 days. Lethal Concentrations (LC_{50}) at 50% mortality were obtained by Probit analyses. Statistical analyses were done using two tailed Student's *t*-test of paired samples. Mortality in control wells containing Brewer's yeast and without TMOF and its analogues was 0–5%.

[a]No significant difference from TMOF (LC_{50}) $p > 0.05$

[b]Significant difference from TMOF (LC_{50}) $0.001 < p < 0.05$. (With permission, adapted from Borovsky and Meola 2004)

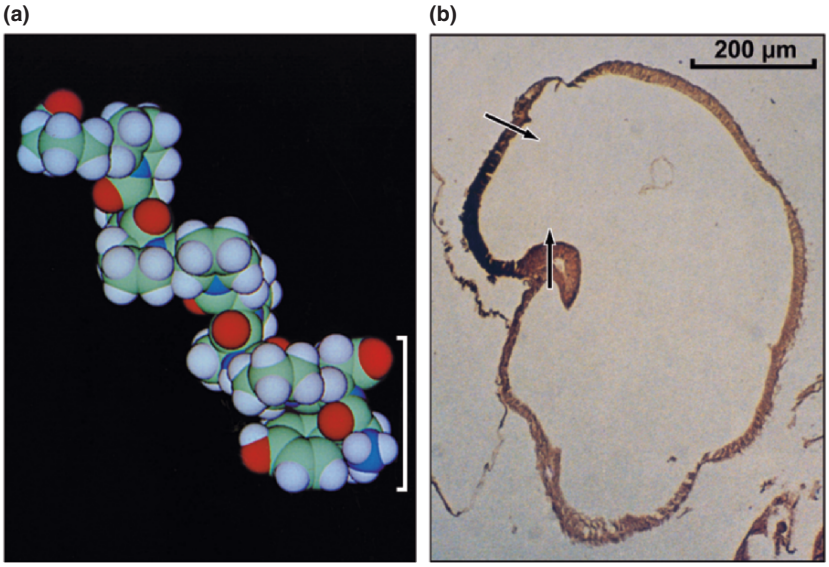

Fig. 1. **(a)** A 3-dimensional nuclear magnetic resonance (NMR) model of *Aea*-TMOF. A left-handed helix of six prolines can be observed at the C-terminus. The N-terminus with the first 4 amino acid sequence (YDPA), important in binding to the TMOF gut receptor is underlined. **(b)** Immunolocalization of TMOF binding to mosquito midgut receptor 72 h after the blood meal. Distinct binding of TMOF to its receptor was observed (*dark area between arrows*). With permission from Borovsky (2003) and Borovsky et al. (1994)

1993). Thus, inhibition of trypsin biosynthesis in the midgut was followed in ligated abdomens that synthesize trypsin but do not metabolize TMOF. At concentrations of 3×10^{-9} M and 6.8×10^{-6} M, TMOF inhibited 50 and 90% of trypsin-like enzyme biosynthesis in the midgut of *A. aegypti*, respectively (Borovsky et al. 1993). The amount of TMOF present in the hemolymph of control, untreated mosquitoes at 30 h and 38 h after the blood meal was determined by ELISA (Borovsky et al. 1992) to be between 33 and 37 ng, which is at least 30 fold higher than the amount that was found to cause 90% inhibition in the TMOF-treated mosquitoes (Borovsky et al. 1993). Similar results were obtained with hemolymph of female *C. quinquefasciatus* (Borovsky unpublished observations). TMOF does not act as a classical trypsin inhibitor (TLCK, TPCK and Soybean trypsin inhibitor) that binds to the active site of serine proteases and prevents protein hydrolysis. TMOF binds to a specific gut epithelial cell receptor (Fig. 1b) and then stops trypsin biosynthesis (Borovsky et al. 1990, 1994a).

2.3 Inhibition of Trypsin Biosynthesis by TMOF in Other Insects

Mosquito TMOF or its analogues inhibit trypsin biosynthesis in the cat flea, *Ctenocephalides felis*, in the stable fly, *Stomoxys calcitrans*, in the house fly, *Musca domestica*, in the sand fly, *Lutzomyia anthophora* and, the midge,

Culicoides variipennis (Borovsky et al. 1990, 1993). TMOF from the grey flesh fly *Neobellieria bullata* has been sequenced and characterized. The hormone is an unblocked hexapeptide (NH_2-NPTNLH-COOH), that like *Aedes* TMOF, stops trypsin biosynthesis and egg development in the flesh fly (Bylemans et al. 1994). The mosquito hormone did not affect trypsin biosynthesis in the flesh fly and the flesh fly's hormone did not affect trypsin biosynthesis in the mosquito. Both hormones specifically terminate trypsin biosynthesis in the gut of the mosquito or flesh fly, respectively, after the protein meal has been digested (Borovsky et al. 1990, 1992, 1993; De Loof et al. 1995; Bylemans et al. 1994).

TMOF also affects agricultural pest insects. Topical treatment of the citrus weevil *Diaprepes abbreviatus* larvae with TMOF that was dissolved in dimethyl sulfoxide (DMSO) caused 75 and 40% inhibition of weight gain and trypsin biosynthesis, respectively. Feeding TMOF (0.04%) in the diet to *D. abbreviatus* larvae caused significant decrease in the growth rate and trypsin biosynthesis (Yan et al. 1999). *Heliothis virescens* larvae synthesize both trypsin- and chymotrypsin-like enzymes in their guts (80 and 20% of gut proteases activity, respectively). Feeding or injections of *Aea*-TMOF into fourth instar *H. virescens* larvae caused 30 and 70% inhibition of trypsin biosynthesis in the larval gut, respectively (Nauen et al. 2001). Highly purified hemolymph fraction, crossreacted with *A. aegypti* TMOF antibodies indicating that *H. virescens* hemolymph contains a TMOF like peptide. Injecting *Aea*-TMOF and several analogues into second instar *H. virescens* stopped trypsin biosynthesis in the larvae (Table 3), and injecting a highly purified hemolymph fraction into *H. virescens* larvae caused 54% inhibition of trypsin biosynthesis in the larvae. *H. virescens* hemolymph when injected into *A. aegypti* females also inhibited trypsin biosynthesis in the female guts (45.6 to 100%) (Nauen et al. 2001). These results indicate that mosquitoes and *Lepidoptera* have close related TMOF like factor(s) that control trypsin biosynthesis.

Table 3. Effect of TMOF and its analogues on trypsin biosynthesis in *H. virescens*

Analogue	N	Inhibition of 50% trypsin biosynthesis (In_{50})
		(μM ± S.E.M.)
$YDPA(P)_6$	3	0.42 ± 0.026
YDPAPP	3	46 ± 2.8
FDPAP	3	145 ± 8
DPAP	3	190 ± 46
DPA	3	50 ± 2

Twelve groups of *H. virescens* (ten larvae per group) were injected with 0.5 μl of water containing TMOF and its analogues (1 μg to 1 ng). Twenty-four hours later, guts were removed and analyzed with BApNA for trypsin biosynthesis. Controls were injected with water and compared with non-injected controls. Results are expressed as 50% inhibition of trypsin biosynthesis and are average of three determinations ± S.E.M. (with permission, adapted from Nauen et al. 2001)

2.4 Genetic Characterization and Expression of TMOF

The effect of TMOF on the trypsin gene was first studied in *Neobellieria*. After injecting TMOF into these flies, the biosynthesis of trypsin mRNA was followed using Northern analysis (Borovsky et al. 1996). Feeding these flies a liver meal caused degradation of the endogenous trypsin early mRNA and synthesis of a new mRNA that corresponded with late trypsin biosynthesis associated with post meal digestion. In flesh flies that were injected with *Neobellieria* TMOF (10^{-9} M) the early mRNA did not disappear and the late mRNA that was synthesized was not translated. These results indicate that TMOF controls the translation of the late trypsin mRNA as would be expected for a hormone that is released after trypsin mRNA has already been transcribed (Borovsky et al. 1996). Injecting TMOF into female *A. aegypti* and *C. quinquefasciatus* following the late trypsin mRNA by RT-PCR and Northern blot analysis confirmed the observations that were reported for *Neobellieria* (Borovsky, unpublished observations). Similar results were obtained when 1–10 μg of TMOF and several analogues (DPA, FDPAP, YDPAP, YDPAPR) were injected into third instar *H. virescens* larvae (Borovsky and Butaye, unpublished observations); TMOF did not affect trypsin mRNA transcription but its translation, i.e., inhibition of trypsin biosynthesis, as was shown for *Neobellieria* (Borovsky et al. 1996).

2.5 The Effect of TMOF and Its Analogues on Insect Larvae

Feeding of [^{3}H]TMOF mixed with the blood meal to female *A. aegypti* stopped trypsin biosynthesis and inhibited egg development in the ovaries (Borovsky and Mahmood 1995). The [^{3}H]TMOF was also found circulating in the hemolymph indicating that TMOF traversed the mosquito gut into the hemolymph and bound a gut receptor on the hemolymph side of the gut (Borovsky et al. 1994, Fig. 1b). When *A. aegypti* and *C. quinquefasciatus* larvae were fed TMOF that was adsorbed onto yeast cells (188 μg TMOF/200 μg yeast cells), the larvae stopped synthesizing trypsin (88 and 91.7% inhibition, respectively, Table 4) (Borovsky and Meola 2004) and stopped growing. These results indicate that shutting off trypsin biosynthesis with TMOF can be used as a new approach to control larval growth and development, possibly leading to new biorational insecticides, which are desperately needed (Zaim and Guillet 2002). Nauen et al. (2001) have reported that *H. virescens* (Lepidoptera) larvae control their trypsin biosynthesis with a hormone that is similar to *Aea*-TMOF. Injections of *Aea*-TMOF or feeding it to fourth instar *H. virescens* larvae caused inhibition of trypsin biosynthesis and larval growth. TMOF also showed enhanced activity against the cotton boll weevil *Anthonomus grandis* by retarding its growth rate and enhanced its mortality and the black cutworm *Agrotis segetum* by retarding its growth rate. The hormone also affected ecdysteroid production in the prothoracic glands of the gypsy moth *Lymantria dispar*

Table 4. Effect of TMOF on trypsin biosynthesis in *Aedes aegypti* and *Culex quinquefasciatus* larvae

Species	Treatment	Trypsin (ng ± S.E.M./larva)	Inhibition (%)
A. aegypti	TMOF	1.17 ± 0.13[a]	88.0
	Yeast	9.87 ± 1.63[a]	
C. quiquefasciatus	TMOF	1.45 ± 0.36[b]	91.7
	Yeast	17.54 ± 2.68[b]	

Four groups of *Aedes aegypti* and *Culex quinquefasciatus* (24 larvae per group) 24 h after emergence were fed *Aea*-TMOF (188 µg) in microtiter plates containing 188 µl of water and 20 µg of Brewer's yeast. Forty-eight hours later, larvae were removed and analyzed for trypsin biosynthesis (Borovsky and Schlein 1988). Controls were fed only Brewer's yeast.

[a,b]Significant difference by two tailed Student's *t*-test $p < 0.01$ and $p < 0.0066$, respectively (adapted with permission from Borovsky and Meola 2004)

(Gelman and Borovsky, 2000). No effect was observed on *Spodoptera litura*, *Plutella xylostella* and *Cydia pomonella* indicating that these insects may have a different TMOF that regulates their digestion.

3 Molecular Biology Studies

3.1 Cloning and expression of *Aea*-TMOF by TMV, Chlorella, *Saccharomyces cerevisia,* Tobacco and Alfalfa plants

Although TMOF by itself is useful for establishing activity against insect larvae, the cost of chemical synthesis limits its usefulness as a commercial product. Consequently, several biological methods for producing TMOF were tried. TMOF was fused to the coat protein of tobacco mosaic virus (TMV) and the recombinant protein was fed to mosquito larvae causing inhibition of trypsin biosynthesis and larval mortality (Borovsky et al. 1998). When *H. virescens* larvae (fourth instars) were fed tobacco leaf discs infected with TMV-TMOF for 4 days a 2.3-fold decrease in weight, 2.2-fold decrease in trypsin and 2.6 decrease in chymotrypsin biosynthesis was observed (Borovsky D. results to be published elsewhere). These observations confirm that TMOF expressed on the coat protein of TMV infected tobacco plants can cause starvation of larval *H. virescens* after the larvae ate the recombinant tobacco leaves.

TMOF was also expressed in *Chlorella* sp. and the recombinant cells caused larval mortality within 72 h after feeding (Borovsky et al. 1998). Because *Chlorella* is a slow growing organism and the expression of the hormone was transient, TMOF and GFP-TMOF genes were cloned into a haploid strain of *S. cerevisiae* using homologous recombination and free plasmid expression (Nauwelaers and Borovsky 2002). Synthesis of TMOF and GFP-TMOF was followed by ELISA (Borovsky et al. 1992) and by fluorescence microscopy.

Mosquito larvae that were fed recombinant yeast cells that synthesized TMOF or GFP-TMOF in ng quantities did not digest the yeast cells and stopped growing and 38–83% of the larvae that were fed the recombinant yeast cells died. On the other hand, only 4–8% of larvae that were fed cells that were transformed with plasmids that were not carrying TMOF, or larvae that were fed Brewer's yeast died (Nauwelaers and Borovsky 2002).

Tortiglione et al. (2002) cloned and expressed six *Aea*-TMOF genes in transgenic tobacco plants and reported an increase in mortality of 20 to 33% in *H. virescens* that were fed on the transgenic plants. When TMOF was expressed in tobacco plants as a fusion with tomato prosystemin about 0.004% of the total soluble proteins was attributed to TMOF with low inhibition (4%) of *Heliothis virescens* larval growth (Tortiglione et al. 2003).

In collaboration with Professor Charles Powell (University of Florida, Indian River Research and Education Laboratory, Ft. Pierce, FL) and Dr. Robert Shatters (USDA, Ft. Pierce, FL) TMOF was cloned and expressed in alfalfa plants (results to be published elsewhere). To compare the potency of the transformed alfalfa plants, non-transformed alfalfa leaf discs were coated with recombinant yeast cells (*Pichia pastoris*) synthesizing TMOF and compared with non-transformed untreated wild type alfalfa. Larvae that fed on leaf discs that were treated with *Pichia*-TMOF didn't cause damage to the leaf discs (Table 5), and trypsin biosynthesis was inhibited by 84% (Table 6). From five recombinant plants that were tested only two plants C9 and B23 showed moderate leaf damage of 56 and 36%, respectively and inhibition of trypsin biosynthesis of 59 and 41.5%, respectively (Tables 5 and 6). Comparison between C9 that inhibited trypsin biosynthesis by 59% (Table 4) and C13 that inhibited the biosynthesis by 19.7% (results not shown) indicate that larvae cause less damage to C9 than to C13 leaf discs. These results indicate that for TMOF to be an effective insecticide and to fully protect plants the expression level will have to be increased.

Table 5. Leaf damage to recombinant alfalfa-TMOF by *H. virescens*

Larvae were fed:	N	Leaf damage (%)
TMOF (on leaf)	12	0
Recombinant plants		
C2	11	68
C9	12	56
C13	11	72
B5	12	78
B23	12	36

H. virescens larvae 24 h after emergence (first instar larvae) were fed for 3 days on alfalfa leaf discs that were transformed with pKylx *Aea*-TMOF plasmid using agrobacterium. Following the feeding, leaf discs were assessed for leaf damage. Control leaf discs with non-recombinant *E. coli* or yeast cells did not prevent leaf damage (results not shown) (unpublished observations from Borovsky et al.)

Table 6. The effect of feeding first instar *H. virescens* larvae on alfalfa TMOF

H. virescens larvae were fed	N	Trypsin (nmol/min/gut ± S.E.M.)	Inhibition (%)
TMOF (on leaf)	3	0.11 ± 0.001[a]	84
Recombinant plants			
C-2	8	0.72 ± 0.11	0
C-9	5	0.29 ± 0.05[b]	59
B-5	6	1.13 ± 0.07	0
B-23	4	0.34 ± 0.09[c]	41.5
Control (wild type)	6	0.79 ± 0.08[a,b,c]	0

H. virescens larvae 24 h after emergence (first-instar larvae) were fed for 3 days on alfalfa leaf discs that were transformed with pKylx *Aea*-TMOF plasmid using agrobacterium. Following the feeding, larvae were assayed for trypsin activity in their guts using BApNA (Borovsky and Schlein 1988). Control leaf discs with yeast cells without TMOF did not inhibit trypsin biosynthesis (results not shown). Same letters results indicate significant difference from control by Student's *t*-test

[a] $p < 0.0002$

[b] $p < 0.0003$

[c] $p < 0.003$ (Unpublished observations from Borovsky et al.)

4 Insect Resistance and Safety Issues

4.1 Potential Resistance Development to TMOF

A potential problem facing all insecticides is the development of resistance by the target insects by spontaneous mutation of the targeted gene and inactivation of the insecticide. The regulation of digestive proteases in the gut requires the synthesis and secretion of TMOF into the hemolymph, and binding to a midgut receptor. Without this mechanism, the insect's synthesis of digestive proteases will be unregulated, causing deleterious metabolic consequences for the insect. Because the regulation of trypsin requires both TMOF and its receptor to function, a point mutation in either of the TMOF or its receptor gene would also inactivate protease down regulation. For genetic mutations to effectively lead to insect resistance to TMOF, a simultaneous, complementary double mutation in both the TMOF and TMOF-receptor genes is required. The probability of a point mutation is estimated to occur at a frequency of 1 in 10^6 events. However, a simultaneous double mutation would occur with a frequency of 1 in 10^{12} events ($10^6 \times 10^6$). To maintain protease regulation the mutation to the TMOF gene and the TMOF receptor gene must be complementary with a frequency of 1 in 10^{14} based on a 1 in 400 probability that the mutations will yield complimentary amino acids in TMOF and the TMOF receptor (20×20 amino acids). Thus, resistance is unlikely to occur, even with the high reproductive capacity of insects and the selective pressure by exposure to TMOF.

4.2 Safety of TMOF

Safety, and the effect of TMOF and its analogues on non-target organisms, is the major concern of every new insecticide that is introduced to the environment. Thus, extensive testing has been initiated by the U.S. Environmental Protection Agency (Table 7) to determine if TMOF is suitable to be used in the environment (see Thompson et al. 2004 for details). The results of this testing indicates that TMOF can be degraded in vitro by leucine amino

Table 7. EPA Tier I safety tests requirements for the registration of TMOF in the USA

Test	OPPTS Protocol	Result
Health effects		
Acute oral toxicity	870.1100	$LD_{50} > 9$ g/kg
Acute dermal toxicity	870.1200	$LD_{50} > 5$ g/kg
Acute inhalation toxicity	870–1300	$LC_{50} > 2.4$ mg/L
Mutagenicity (1)		
Ames test	870–5100	NM to limit (5 mg/plate)
Mouse lymphoma	870.5300	NM to limit (5.36 mg/ml)
Chromosomal aberration	870.5375	NM to limit (5 mg/ml)
TMOF protease digestion (1)	IBI/NCSU	Degraded
Killed organism (yeast) testing	IBI/UF	100% killed
Ecological effects		
Acute avian oral toxicity (ducks)	885–4050	$LD_{50} > 1.25$gr/kg
Fish toxicity, feathered minnow	850–1400	$LD_{50} > 1 \times 10^6$ dead cells/ml
Fish toxicity, sheephead minnow	850–1400	$LD_{50} > 1 \times 10^6$ dead cells/ml
Mysid chronic toxicity	850–1350	$LD_{50} > 1 \times 10^6$ dead cells/ml
Daphnid chronic toxicity	850–1300	$LD_{50} > 1 \times 10^6$ dead cells/ml
Product performance testing:		
Mosquito, black fly and biting midge treatments	885.4050	Kills early instars of mosquito larvae
Additional Studies:		
Live TMOF-*Pichia* effect on *Daphnia magna*	850–1300	NNE > 10^6 cells/ml
Acute oral toxicity of live TMOF-*Pichia*	885–3050	$LD_{50} > 2 \times 10^8$ live cells/ml
Survival of TMOF-*Pichia* in a simulated environment	IBI/UF	No growth of cells

Pichia pastoris cells expressing TMOF were assayed using protocols developed by the Office of Prevention, Pesticide and Toxic Substances (OPPTS) to find out the safety of using recombinant *Pichia*-TMOF or TMOF alone (1). *NM* No mutagenic, *LD* Lethal dose, *LC* Lethal concentration, *NNE* No negative effect, *IBI* Insect BioTechnology Inc., *NCSU* North Carolina State University, *UF* University of Florida, Florida Medical Entomology Laboratory. Several tests, procedures and results have been described by Thompson et al. (2004)

peptidase, a pancreatic enzyme found in the pancrease of vertebrates into $DPAP_6$, PAP_6 and eventually AP_6 (Thompson et al. 2004), which have a 6-fold lower TMOF activity (Table 2). TMOF when administered by gavage to male and female mice at 2.0 g dry weight/kg body weight produced no negative effects, and when male and female mallard ducks were treated by gavage with 1.25 g dry weight of TMOF/kg body weight/day for 5 days no toxic effects were noted 35 days after the last treatment. Application of TMOF to the shaved skin of male and female rabbits at 2.0 g/kg for 1–2 days did not affect the rabbits. Additional studies using *Daphnia magna* with recombinant *Pichia*

Table 8. Effect of *Aedes aegypti* TMOF on different insects

Insect	Effect
Mosquito	
Aedes aegypti, Aedes taeniorhynchus, Culex quinquefasciatus, Culex nigripalpus, Anopheles albimanus, Aedes atropalpus	Larval starvation and death, inhibition of blood digestion and egg development in adults[b, c, d, e, f, g, h, i]
Stable fly, *Stomoxys calcitrans*	Inhibition of blood digestion and egg development in adults[e,h]
House Fly, *Musca domestica*	Inhibition of trypsin biosynthesis in adults[e]
Fleshfly, *Neobelliera bullata*	No effect on tryspin biosynthesis, the flesh fly has a unique TMOF[j]
Cat flea, *Ctenocehalides felis*	Inhibition of trypsin biosynthesis and blood digestion[e,h]
Midge, *Culicoides variipennis*	Inhibition of blood digestion in adults[e,h]
Sand fly, *Lutzomyia anthophora*	Inhibition of blood digestion[h]
Tobacco Budworm, *Heliothis virescens*	Larval starvation, inhibition of trypsin biosynthesis and food digestion[m]
Black Cutworm, *Agrotis segetum*	Larval growth retardation[a]
Cotton Boll Weevil, *Anthonomus grandis*	Larval growth retardation and enhanced[a] mortality
Citrus Weevil, *Diaprepes abbreviatus*	Larval growth retardation and inhibition of trypsin biosynthesis and food digestion[r]
Gypsy Moth, *Lymantria dispar*	Effect on ecdysteroid production in the prothoracic glands[k]
Diamondback Moth, *Plutella xylostella*	No apparent effect on larval growth[a]
Codling Moth, *Cydia pomonella*	No apparent effect on larval growth[a]
Colorado Potato Beetle, *Leptinotarsa decemlineata*	No apparent effect on growth[a]
Tobacco hornworm, *Manduca sexta*	No apparent effect on growth[q]

Based on unpublished observations from the authors's laboratory[a], and published observations: Borovsky 1988[b], Borovsky and Mahmood 1995[c], Borovsky and Meola 2004[d], Borovsky et al. 1990[e], 1991[f], 1992[g], 1993[h], 1998[i], Bylemans et al. 1994[j], Gelman and Borovsky 2000[k], Kelly et al. 1994[l], Nauen et al. 2001[m], Nauwelaers and Borovsky 2002[n], Tortiglione et al. 2002[o], 2003[p] Vanderherchen et al. 2005[q], Yan et al. 1999[r]

pastoris cells producing TMOF at a level of 10^6 yeast cells/ml (10 mg/ml) equivalent to TMOF (0.104 mg/ml) did not affect the mortality, growth, molting, time to first brood and production of viable neonates (Thompson et al. 2004) (Table 7). From these studies it appears that TMOF can be degraded by vertebrate digestive proteases and TMOF is not toxic to non-target organisms examined. Following these studies, the EPA issued a registration number 74411–1 for TMOF allowing it to be formulated into insecticides for the control of mosquito larvae. Since TMOF was shown to affect *H. virescens*, *Anthonomus grandis* and *Agrotis segetum* and not *Spodoptera litura*, *Plutella xylostella* and *Cydia pomonella*, we can assume that the hormone probably has a narrow species specificity (Table 8) and is safe to be used in the environment in recombinant plants against several agricultural pest insects.

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7 Nicotinic Acetylcholine Receptors as a Continuous Source for Rational Insecticides

P. JESCHKE

1 Introduction

The insect nicotinic acetylcholine receptor (nAChR) has become an important target in modern crop protection with the discovery of three classes of insecticides, the very small group of so-called nereistoxin analogues (1,2-dithiolanes) such as Takeda's cartap, bensultap and Sandoz' thiocyclam (Lee et al. 2003), the neonicotinoids (Yamamoto 1999) as the only major new class of ligands introduced in the past three decades (Kagabu 1997; Zhang et al. 2004a) and spinosyns (Dow AgroScience) (Table 1). The 1,2-dithiolanes based on insecticidally active natural origins like the insect-paralyzing factor nereistoxin isolated from a marine annelid, *Lumbriconercis heteropoda* Marenz (Okaichi and Hashimoto 1962) and the spinosyns which are directly used as naturally occurring insecticides. Both examples demonstrate that the influence of commercial nAChR inhibitors having a natural origin is remarkable and plays an important role in the discovery process for novel nAChR ligands. On the other hand, neonicotinoids resulted from the first lead structure nithiazine (Soloway et al. 1978, 1979; Schroeder and Flattum 1984; Kollmeyer et al. 1999) and are structurally distinct from all other synthetic and botanical active ingredients like nicotine and exhibit a favorable selectivity to insects vs. mammals (Tomizawa and Casida 2003; Casida and Quistad 2004).

The current market value of neonicotinoid insecticides (Kagabu 1997) is well over 600 million € per year, including imidacloprid as the most successful insecticide worldwide (Elbert et al. 1998; Nauen et al. 2001). Today, the seven commercial neonicotinoids like imidacloprid (Bayer CropScience), acetamiprid (Nippon Soda), nitenpyram (SumiTake), thiacloprid (Bayer CropScience), thiamethoxam (Syngenta), clothianidin (SumiTake/Bayer CropScience) and dinotefuran (Mitsui Chemicals) are the fastest growing and fourth major group of insecticides (behind organophosphates (OPs), methylcarbamates and pyrethroides), with widespread use against a broad spectrum of sucking and biting pest insects by several modes of application (Elbert et al. 1998; Nauen et al. 2001; Jeschke et al. 2002) in most countries in many agronomic cropping systems. Neonicotinoids show excellent activity against pest insects resistant to known classes of insecticides such as OPs, carbamates, pyrethroids, chlorinated hydrocarbons, and several other commercialized compounds (Denholm et al. 2002; Nauen and Denholm 2005).

Insecticides Design Using Advanced Technologies (ed. by Isaac Ishaaya, Ralf Nauen and A. Rami Horowitz)

Table 1. Evolution of the three important insecticidal classes (I–III) acting on nAChR

Insecticides (classes)	Launch	Remarks/Brand names*	Reference (selection)
Nicotine (NP)	1814	Nico Soap, Stalwart, XL All Insecticide (nicotine sulfate)	Schmeltz 1971
I. Nereistoxin (NP) and analogues		Marine annelid worm *Lumbriconereis heteropoda*	Nitta 1934; Okaichi and Hashimoto 1962; Sakai 1964
Cartap	1964	TI 1258[a], Padan, Cadan, Patap, Sanvex, Thiobel, Vegetox	Sakai 1969
Bensultap	1968	TI 68[a], Bancol, Malice, Ruban, Victenon, ZZ-Doricada	Sakai 1984; Menn et al. 1985
Thiocyclam hydrogen oxalate	1979	SAN 155 I[a], Evisekt, Evisect, Sultamine	Stevens and Berg 1977
II. Nithiazine and neonicotinoids	1978[b]	SD-03565, SKI-71 1. Lead structure	Soloway et al. 1978, 1979; Schroeder and Flattum 1984; Kollmeyer et al. 1999
Imidacloprid	1991	NTN 33893, Admire, Gaucho, Provado Confidor, Premise	Moriya et al. 1992, 1993; Elbert et al. 1998; Nauen et al. 2001
Acetamiprid	1995	NI-25, Mospilan	Takahashi et al. 1992; Matsuda and Takahashi 1996
Nitenpyram	1995	TI-304, Bestguard	Minamida et al. 1993; Akayama et al. 1999
Thiamethoxam	1998	CGA 293′343[c], Actara, Cruiser	Senn et al. 1998; Maienfisch et al. 1999a, 1999b
Thiacloprid	2000	YRC 2894, Calypso, Bariard, Alanto	Elbert et al. 2000; Yaguchi and Sato 2001; Jeschke et al. 2001
Clothianidin	2002	TI-435[b], Dantotsu, Fullswing, Poncho	Uneme et al. 1999; Ohkawara et al. 2002; Jeschke et al. 2003
Dinotefuran	2002	MTI-446, Starkle, Albarin	Kodaka et al. 1998; Zhang et al. 2000
III. Spinosyns (NPs) and spinosoids	1997	Tracer, Naturalyte, Conserve, Success *semi*-synthetic analogue	Salgado et al. 1997; Sparks et al. 2001

[a]Prodrug from nereistoxin
[b]Never commercialized for agricultural use
[c]Prodrug from clothianidin, *NP* natural product, * selection

Therefore they represent a welcome new class of chemistry and were grouped in the same mode-of-action (MoA) class by the Insecticide Resistance Action Committee (IRAC; an Expert Committee of Crop Life). In addition, the relatively low risk and target specificity of these products combined with their suitability for a range of application methods will maintain them as important insecticides also in Integrated Pest Management (IPM) strategies.

As known, only minor structural modifications of ligands confer selectivity among the mammalian nAChR subtypes (Holladay et al. 1997; Tomizawa and Casida 1999) and between insects and mammals (Liu and Casida 1993; Tomizawa et al. 1999). On the other hand, important parameters like electrostatic interaction, H-bonding, π,π-stacking interaction, dipole-dipole interaction and van der Waals contact all act closely together with the insecticide action (Kagabu 1997). Therefore, knowledge of the functional architecture and molecular aspects of insect and mammalian nAChRs (Gundelfinger and Schulz 2000; Tomizawa and Casida 2001) and their neonicotinoid-binding site is the basis for continued development of novel safe and effective active ingredients, which can have different pharmacokinetics from known insecticides such as imidacloprid and unique binding features with the insect nAChRs (Kagabu 1996). The past decade has witnessed broadly increasing number of publications in the field of nAChRs, which reflect the huge importance of these receptors as continuous source for rational design of novel insecticides as well as medicinal drugs.

2 Structure of the Nicotinic Acetylcholine Receptors (nAChRs)

The nAChRs are well-characterized pentameric transmembrane allosteric proteins involved in fast ionic responses to acetylcholine (ACh) at the vertebrate neuromuscular junction and in all animal central and peripheral nervous systems (Changeux and Edelstein 1998). They belong to the Cys-loop Ligand-Gated Ion Channel (LGIC) superfamily (Le Novère and Changeux 1999; Karlin 2002) and facilitated an impressive number of physiological, pharmacological and structural investigations (Corringer et al. 2000; Karlin 2002; Jensen et al. 2005).

The channel is formed in high concentrations at the vertebrate nerve-muscle synapse, where it mediates fast chemical transmission of electrical signals in response to ACh released from the nerve terminal into the synaptic cleft (Unwin 2005). As a large (290 kDa) glycoprotein, the muscular nAChR is assembled from a ring of five homologous subunits (α, γ, α, β, δ) each divided into three domains arranged around a central ion channel: *(i)* a large *N*-terminal extracellular ligand-binding domain, *(ii)* a membrane-spanning pore, and *(iii)* a smaller intracellular domain (Unwin 2005).

The human nAChR gene family consists of 16 subunits (9α and 7 non-α) (Millar 2003). The animal model, *Caenorhabditis elegans*, possesses the largest known nAChR gene family, consisting of at least 27 subunits (20α and

7 non-α) (Jones and Sattelle 2004; Jones et al. 2005). On the other hand, the smallest nAChR gene family to date is that of the fruitfly *Drosophila melanogaster*, consisting of 7α and 3 non-α-subunits (Littleton and Ganetzky 2000; Sattelle et al. 2005). Additional diversity of *Drosophila* nAChRs arises from alternative splicing (four of the ten subunits). Using *Drosophila*, A-to-I pre-mRNA editing has been demonstrated for the first time in nAChRs (Grauso et al. 2000; Lansdell and Millar 2000b; Sattelle et al. 2005).

These subunits combine to form multiple homo- or heteromeric penta nAChR subtypes (Holladay et al. 1997). The nAChR is composed of two ligand binding (α) and three non-α subunits (γ, δ or ε) or five α subunits (Arias 1997). The receptor ligand binding domain comprises seves loops (A–G) spaced on the protein chains of the α and non-α subunits (Changeux and Edelstein 1998; Le Novère and Changeux 1999). Residues within these loops have been investigated by using subunit chimeras and point mutations, combined with binding studies of substances, e.g., lophotoxins, α-conotoxins or α-neurotoxins that interact with structures in both subunits at the interface (Sine et al. 2004).

Sequences for each nAChR subunit predict hydrophilic extracellular domains containing a binding site for cholinergic ligands and four *trans*-membrane hydrophobic segments (TM1–4). The TM2 domain of the five subunits is considered to form the lumen of the cation-channel (Tomizawa and Casida 2001).

Insight into the structural mechanism of gating has been obtained by electron microscopical experiments on helical tubes grown from *Torpedo* postsynaptic membranes (Brisson and Unwin 1984; Toyoshima and Unwin 1990), using a rapid spray-freezing technique to mimic the synaptic release of ACh and trap the open-channel form (Unwin 1995, 2005). These experiments demonstrated that binding of ACh imitates two interconnected events in the ligand-binding domain (Unwin 2005).

2.1 Structure of the Insect nAChRs

Insect nAChRs are as diverse in structure, as are those from vertebrates. In general, the subunit composition of insect nAChRs resembles those of vertebrate neuronal nAChRs, because there are no neuromuscular nAChRs, respectively. Insects express in neuronal nAChRs, while, in vertebrates, endplate and neuronal nAChRs are distinguished. Although several genes encoding insect nAChR subunits have been cloned (Tomizawa et al. 1999; Grundelfinger and Schulz 2000; Tomizawa 2000) and the existence of further nAChRs subunits can be predicted from analysis of *Drosophila* genome (Littleton and Ganetzky 2000), the functional architecture, diversity and three-dimensional (3D) structure of the insect native AChRs still remains unknown (Tomizawa et al. 1999; Landsell and Millar 2000; Grundelfinger and Schulz 2000; Tomizawa 2000). As in vertebrates in insects, nAChRs mediate fast synaptic transmission as excitatory neurotransmitter-receptor complex widely distributed in the synaptic neutrophil regions of the central nervous system (CNS) in the insect brain (Breer and Sattelle 1987; Arias 1997; Corringer et al. 2000; Tomizawa et al. 2001, 2002; Tang 2002). The nAChRs are, besides to their

endogenous ligand ACh, responsive to chemically diverse substances and natural products such as nicotine and various toxins (Celie et al. 2004). Therefore insect nAChRs are major targets for several classes of chemicals with insecticidal activity.

Recently, identification of the second complete insect nAChR gene family from the genome of the malaria mosquito vector, *Anopheles gambiae* (Holt et al. 2002; genome available at http://www.ensembl.org/Anopheles_gambiae) was described (Jones et al. 2005). Like *Drosophila*, *Anopheles* possesses ten nAChR subunits with orthologous relationships evident the two insects. Interestingly, *Anopheles* orthologous of Dβ2 and Dβ3 possess the vicinal cysteines that define α-subunits. Whereas the fruit fly *Drosophila* nAChR gene family consists of seven α-subunits and three β-subunits, *Anopheles* possesses a nAChR gene family with an unusually high proportion of α-subunits, comprising nine α-subunits and only a single β-subunit. However, phylogenetic analysis reflects that Dβ2 and Dβ3 cluster with α-subunits of other insects (Jones et al. 2005).

It appears that insects have several types of nAChR subunits that could associate to form channels of disparate pharmacology, and this could explain some of the complex binding and electrophysiology seen with the insect cholinergic system. Seven nAChR subunits (four α-type, genomically nine α-types and three β-type, which exist only in *D. melanogaster*) have been cloned from fruitfly *D. melanogaster*, the insect model system. Three further putative nAChR α subunits (Dα5, Dα6 and Dα7) with sequence similarity to the vertebrate α7 subunit have been identified from *Drosophila* genome sequence data but there have been no reports, as yet, of their characterization by heterologous expression (Lansdell and Millar 2004).

Generally, it is clear that insect nAChRs vary with specificity of their interaction with neonicotinoid insecticides, however the appropriate subunit is unclear so far. A study which supports the hypothesis that there is a conserved neonicotinoid special sensitive subtype of the nAChR binding site in different insects like *Musca domestica*, *D. melanogaster*, *Aphis craccivora*, *Myzus persicae* is given (Zhang et al. 2000).

Unfortunately, as with many other integral membrane proteins, it has not been possible to obtain crystals of any nAChR of sufficient quality for high resolution X-ray crystallography. However, both the crystal structure of a soluble homopentameric acetylcholine-binding protein (AChBP) (Brejc et al. 2001) and the refined model of the membrane-associated *Torpedo* AChR (Unwin 2005), based on the crystal structure of AChBP, can support the understanding of the ligand-receptor interactions considerably (see Sect. 4.1).

3 Agonists vs. Antagonists

The advantage of electrophysiological measurements compared with biochemical assays is their ability to distinguish between agonists, causing channel opening, and antagonists, causing a desensitized block of the nAChR (Nauen

et al. 2001). Both functional differences in the MoA of ligands with high specificity for the nAChR is very important for insecticidal potency (Nauen et al. 2001). Electrophysiological measurements from isolated housefly neurons revealed that ligands acting agonistically on the nAChR were in general insecticidal active, whereas antagonistic ligands were mostly insecticidal nonactive (Wollweber and Tietjen 1999; Nauen et al. 1999a). All commercialized neonicotinoids are potent agonists on insect AChRs. This fact is supported by other studies which demonstrate a positive correlation between nerve activity induced in cockroach preparations and insecticidal activity against the green rice leafhopper for numerous neonicotinoids (Nauen et al. 2001; Nishimura et al. 1994, 1998). Agonistic action on nAChRs causes first hyperexcitation and then paralysis as shown in numerous symptomology studies with imidacloprid on different insect species (Sone et al. 1994; Nauen 1995; Mehlhorn et al. 1999). Naharashi et al. (1998) concluded that in general most insecticides are neurotoxicants and cause various forms of hyperexcitation and paralysis leading to death of animals (Nauen et al. 2001). Only minor structural modifications to nitromethylene compounds and other neonicotinoids have been shown to produce compounds with either or both of these properties.

3.1 Neonicotinoid Insecticides

The biochemical MoA of neonicotinoids has been investigated and characterized in the past decade. It has been demonstrated that they mimic the MoA of ACh (Liu and Casida 1993) or nicotine and bind agonistically to postsynaptic nAChRs in CNS of pest insects (Bai et al. 1991; Liu and Casida 1993; Nauen et al. 1996; Lind et al. 1999; Zhang et al. 2000; Nauen et al. 2001). Their ability to displace tritiated imidacloprid (or nicotine) from its binding site correlates well with their insecticidal efficacy (Tomizawa and Yamamoto 1993; Kagabu 1997; Tomizawa and Casida 2003).

Receptor-binding experiments in cockroach neurons indicate the existence of two α-Bgtx-sensitive nicotinic receptor subtypes. They are identified as desensitizing (nAChD), selectively inhibitable with 100 nM imidacloprid, and non-desensitizing (nAChN), inhibitable with 100 pM methyllycaconitine (Salgado and Saar 2004). However imidacloprid binds not competitive to α-Bgtx-sensitive nicotinic receptor subtypes. A direct evidence that there are distinct imidacloprid and α-Bgtx-sensitive sites or subtypes in *Drosophila* brain was given by Zhang et al. (2004b), respectively.

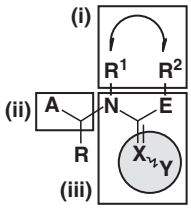
Agonist actions of imidacloprid on recombinant chicken α4/β2 and *Drosophila* SAD/chicken β2 hybrid nAChRs expressed in *Xenopus laevis* oocytes have been measured using a two-electrode voltage-clamp method (Matsuda et al. 1998). The binding activity measured using tritiated imidacloprid was found to be closely related to agonist activity against SAD/chicken β2 nAChR and to the insecticidal activity (Nishiwaki et al. 2003). High and positive correlations both between the agonist and the binding

activities and between the agonistic and insecticidal activities suggested that cation-channel opening of nAChRs induced by binding of the neonicotinoids to the receptor leads to the insecticidal effects (Nishiwaki et al. 2003).

One of the important features of neonicotinoid insecticides is their selectivity towards insect nAChRs; their affinity to interact with rat nAChRs was 1,000 times weaker than that with the insect receptor (Methfessel 1992; Zwart et al. 1994). Whereas the precursor molecule nithiazine acts only with micromolar affinity against housefly nAChRs (Schroeder and Flattum 1984), all neonicotinoid insecticides act with nanomolar affinity against housefly and other insect nAChRs (Bai et al. 1991; Cheung et al. 1992; Nauen et al. 2001), except thiamethoxam, which exhibits a comparatively low affinity for the [^{3}H]imidacloprid binding site (Wiesner and Kayser 2000). Therefore, neonicotinoids are highly toxic towards important insect species and less toxic to mammals, providing an excellent example of selective toxicity (see Sect. 4.4; Kagabu 1997; Yamamoto and Casida 1999).

3.1.1 Bioisosteric Segments of Neonicotinoids

Retrospective considerations regarding bioisosteric segments of all seven commercial neonicotinoids reflect general structural requirements (segments *i-iii*, Fig. 1) for the 5- and 6-ring systems and noncyclic structures (Jeschke et al. 2002; Jeschke and Nauen 2005), which can be important for further chemical investigations. Several common molecular features, when comparing compounds with ring systems, like imidazolidine (imidacloprid), and its isosteric alternatives, like 1,3-thiazolidine (thiacloprid), perhydro-1,3,5-oxadiazine



(i) Ring systems (R^1-R^2 , R^1-Z-R^2; Z = O, NMe) or noncyclic structures (R^1, R^2)

(ii) Heterocyclic or -cyclylic *N*-substituents [A-CHR-; R = H > alkyl]; *e.g.* CPM, CTM or TFM

(iii) Different types of pharmacophors [-N-C(E) = X-Y] *e.g.* nitroenamines [-N-C(E) = CH-NO_2] with E = S, N; *N*-nitro-guanidines [-N-C(N) = N-NO_2] and *N*-cyano-amidine [-N-C(E) = N-CN] with E = S, Me

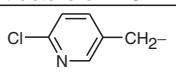
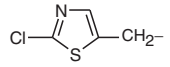
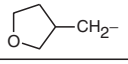
Structure of A-CHR-	Chemical name of this moiety	Abbreviation
	6-**c**hloro-**p**yrid-3-yl**m**ethyl	CPM
	2-**c**hloro-1,3-**t**hiazol-5-yl**m**ethyl	CTM
	*(±)*-**t**etrahydro-**f**ur-3-yl**m**ethyl	TFM

Fig. 1. Structural segments for neonicotinoids (data taken from Jeschke and Nauen 2005)

(thiamethoxam), or hexahydro-1,3,5-triazine (Agro Kanesho's AKD-1022) and the functional groups like *N*-nitroimino [=N-NO_2], *N*-cyanoimino [=N-CN], or nitromethylene [=CH-NO_2] of these insecticides, have been described (Tomizawa et al. 2000). After superposition of the most insecticidal active neonicotinoids, it was possible to state the molecular shape similarity of these compounds. It was found that electrostatic similarity of the most insecticidal active neonicotinoids correlates well with the nAChR binding affinity for the whole molecule (Nakayama and Sukekawa 1998; Sukekawa and Nakayama 1999), and similar results were obtained by Comparative Molecular Field Analysis (CoMFA) (Nakayama 1998, Okazawa et al. 1998). A hypothetical model of molecular recognition of neonicotinoid insecticides at the nAChR was described as well.

3.2 Natural Products Active on nAChR

Natural products can be an excellent source of lead structures for the discovery of simple synthetic active ingredients (Verpoorte 1998) also for nAChR ligands. In the area of insect control agents, numerous important insecticide classes have natural origins.

The main tobacco alkaloid nicotine, one of the oldest known nAChR agonists, is still used to control some homopteran pests such as aphids in greenhouse, e.g., in Japan (Nauen et al. 2001). The basis of nicotinoids is nicotine itself substituted in all possible positions (Lin et al. 1994). Due to the large number of nicotinoid analogues prepared, the structure activity relationship (SAR) of nicotine has been well established (Demaj et al. 1996; Cosford et al. 1996; Dukat et al. 1999; Tønder and Olesen 2001). Because of its high mammalian toxicity and relatively low level of insecticidal activity no major insecticidal class could be established through taking nicotine as a lead structure (Jeschke et al. 2002).

On the other hand, the 4-*N,N*-dimethylamino-1,2-dithiolane nereistoxin (Fig. 2), naturally occurring in the salivary glands of the marine annelid worm *Lumbriconereis heteropoda* Marenz (Polychaeta, Annelida), is active at cholinergic synapses (Narahashi 1973) and highly toxic to insects (Sakai 1964, 1969; Mitsudera and Konishi 1991). Registered compounds active on the insect nAChR include the very small group of so-called nereistoxin analogues (Fig. 2).

These products are in fact pro-insecticides that are converted metabolically (Sakai and Sato 1992) or (photo)chemically (Tsao and Eto 1989), e.g., by hydrolysis (cartap, bensultap) or by sulfur extrusion (thiocyclam), into the neurotoxic nereistoxin (Bettini et al. 1973). The site of action on nAChR of nereistoxin differs from that of the chloro-neonicotinoids such as imidacloprid, as shown by tests on recombinant nAChRs (chicken neuronal nAChRs $\alpha 7$ and hybrid *Drosophila*/chicken nAChRs, SAD/$\beta 2$ and ALS/$\beta 2$) expressed in *Xenopus laevis* oocytes (Delpech et al. 2003). Lee et al. (2003) described that nereistoxin and the bis(thiocarbamate) derivate cartap are equally

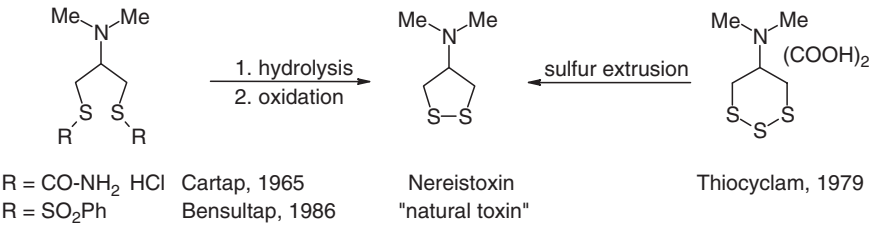


Fig. 2. Natural toxin nereistoxin and potential pro-insecticides cartap hydrochloride, bensultap (Takeda) and thiocyclam hydrogen oxalate (Sandoz)

potent, direct and selective-acting, and competitive displacers of [^{3}H]thienylcyclohexylpiperidine binding at the noncompetitive blocker site of insect nAChRs. However, nereistoxin also binds at the *Apis mellifera* [^{3}H]imidacloprid agonist site (Lee et al. 2003). As demonstrated, cartap acts without the requirements of metabolic activation, producing inhibitory neurotoxicity (Lee et al. 2003).

In this case, the knowledge of the molecular properties of the relevant insect nAChR proteins was not sufficient for a direct "biorational design" of new insecticidal molecules.

Studies on the vertebrate α4/β2 nAChR were based initially on *(S)*-nicotine (K_i = 1.0 nM), nornicotine and later on the potent bicyclic amphibian alkaloid, named *(–)*-epibatidine (K_i=0.045 nM) (Fig. 3).

The latter is a 2-(6-chloropyrid-3-yl)-azabicyclo[2.2.1]-heptane, which was isolated in 1992 from the skin of the Ecuadorian poison frog *Epipedobates tricolor*, containing the same 6-chloro-pyrid-3-yl moiety as unique structural element as imidacloprid, launched only one year before (Mencke and Jeschke 2002). It exhibits extremely high affinity for the α4β2 nAChR and was characterized as potent agonist at this subtype (Qian et al. 1993). Since that time, more than 50 total syntheses of racemic or enantiomerically pure materials have been published (Breining 2004). Its SAR has been studied intensively. Most analogues substituted in the pyridine 2- and 6-positions and on the bridgehead nitrogen atom are described (Damaj et al. 1996; Tønder and Olesen 2001). As for nicotinoids, bioisosteric replacement of the 6-chloro-pyrid-3-yl moiety for

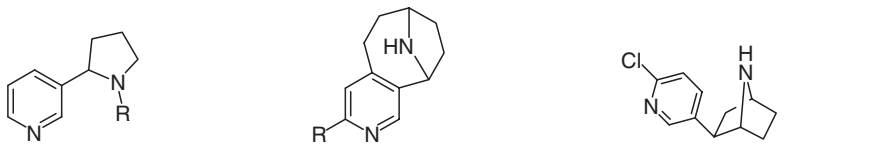


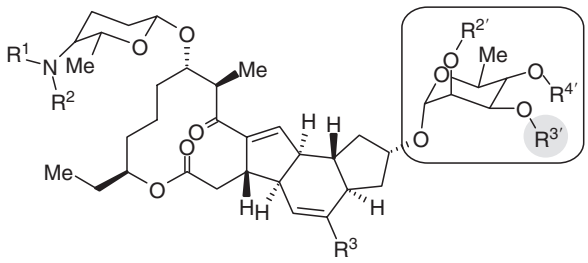
Fig. 3. Literature-known vertebrate α4/β2 nAChR inhibitors

a 3-methyl-isoxazol-5-yl moiety ($K_i = 0.6$ nM) is possible (Badio et al. 1997), however, the resulting epiboxidine has a 10-fold lower affinity than epibatidine.

The combination of subnanomolar affinity ($K_i = 0.39$ nM) and conformational rigidity makes the first bridged nicotinoid *(±)*-6′-pyrido[3,4-*b*]norhomotropane an excellent probe for further refinement of pharmacophore models, facilitate additional differentiation of the steric and electronic requirements of a α4/β2 nAChR target (Kanne et al. 2005). In this pharmacophore model, the 6′-methyl group may be equivalent to the acetyl methyl of ACh. Despite the fact that from the α4β2 nAChR agonists no real lead structure for insecticides could be identified, their SAR and MoA is important for the design of active ingredients in modern crop protection too.

The spinosyns (major factors spinosyn A and D), a family of insecticidal members of polyketide-derived macrocyclic lactones produced by the actinomycete fungus *Saccharopolyspora spinosa* (Mertz and Yao 1990) can be used directly in modern crop protection (Fig. 4).

Spinosad, a naturally occurring mixture of spinosyn A (primary component) and D, is marked by Dow AgroSciences as broad spectrum product for application in a variety of crops including cotton, vegetables and vine sectors.



Spinosyns

R^1, R^2 = Me; R^3 = H; $R^{2'}, R^{3'}, R^{4'}$ = Me	LC_{50} = 0.3 (Spinosyn A)
R^1 = H; R^2 = Me; R^3 = H; $R^{2'}, R^{3'}, R^{4'}$ = Me	LC_{50} = 0.4 (Spinosyn B)
R^1, R^2, R^3 = H; $R^{2'}, R^{3'}, R^{4'}$ = Me	LC_{50} = 0.8 (Spinosyn C)
R^1, R^2, R^3 = Me; $R^{2'}, R^{3'}, R^{4'}$ = Me	LC_{50} = 0.8 (Spinosyn D)

Spinosoids

R^1, R^2=Me; R^3=H; $R^{2'}, R^{4'}$ = Me, $R^{3'}$ = Et	LC_{50} = 0.03
R^1, R^2=Me; R^3=H; $R^{2'}, R^{4'}$ = Me, $R^{3'}$ = *n*Pr	LC_{50} = 0.05
R^1, R^2=Me; R^3=H; $R^{2'}, R^{4'}$ = Me, $R^{3'}$ = Allyl	LC_{50} = 0.06
R^1, R^2=Me; R^3=H; $R^{2'}, R^{3'}, R^{4'}$ = Et	LC_{50} = 0.02

Fig. 4. Structure and neonate *H. virescens* larval toxicity for the spinosyns and spinosoids, LC_{50} in mgl^{-1} (as selection adapted from Sparks et al. 2001)

The new family of fermentation-derived macrolides is highly active against thrips, fleas and primarily against a broad range of lepidopteran insect pests, but have no activity in vertebrates. The spinosyns also exhibit a very favorable environmental and toxicological profile.

Electrophysiological data revealed that spinosyn A, instead of depressing the ACh response, greatly prolongs its duration (Salgado 1997). The ability of spinosyn A to prolong the action of ACh indicates that both ligands can act simultaneously, and that they must act at separate and distinct binding site of the nAChR (Salgado 1997). Spinosyn A acts through a distinct site of the nAChR, where it can both activate the receptor alone and prolong the effect of ACh (Salgado 1997). However, spinosyns have no competition to tritiated imidacloprid, respectively. Up to now, more than 20 spinosyns and more than 800 spinosoids (*semi*-synthetic analogues) have been isolated or synthesized (Sparks et al. 2001).

Artificial neuronal network (ANN)-based quantitative structure activity relationship (QSAR) (Sparks et al. 2000) studies for the spinosyns suggested that modification of the 2′,3′,4′-tri-O-methyl-rhamnosyl moiety, e.g., extension of the alkyl chain $R^{2'}$-$R^{4'}$ from methyl (LC_{50} = 0.3 mg liter^{-1}) to ethyl (LC_{50} = 0.02 mgl^{-1}) could improve insecticidal activity by factor 15 against lepidopteran pests compared to spinosad (Creemer et al. 2000; Sparks et al. 2001). Analogues of spinosad that have been modified at the 3′-position ($R^{3'}$ = Et, nPr, Allyl; LC_{50} = 0.03–0.06 mgl^{-1}) show greater activity against some lepidopteran species under field conditions (Crouse et al. 2001). In addition, multiple linear regression (MLR)-based QSAR studies also suggest that whole-molecule properties such as ClogP and MOPAC dipole moment can help to explain their biological activity e.g. against the tobacco budworm *Heliothis virescens*. Among the spinosyns and spinosoids examined, it was found that the ones possessing a lower MOPAC dipole and a higher ClogP tend to be more active. However, although the ANN QSAR studies were able to provide very useful synthetic directions, due to the nature of ANNs is difficult to understand the physicochemical basis for modifications or improvements, respectively (Sparks et al. 2001).

Characterization of the clustered *S. spinosa* genes (Waldron et al. 2001) suggests that the spinosyns are synthesized largely by mechanism (polyketide synthase) similar to those used to assemble other polyketides like complex macrolides in other actinomycetes. However, there are several unusual genes in the spinosyn biosynthesis gene cluster that could encode enzymes which generate the tetracyclic polyketide aglycone nucleus (Sparks et al. 2001).

One additional example of a natural product is the potent agonist at the insect nAChR stemofoline (A-B = $-CH_2-CH_2-$), isolated from the leaves and stem of plant *Stemona japonica* (Ujváry 1999). The genus *Stemona* from the small monocotyledonous family *Stemonaceae* is known as a rich source of structurally complex alkaloids with mainly saturated ring systems (Pilli and Oliveira 2000; Seger et al. 2004). The pure alkaloid has fast-acting insecticidal activity against *M. persicae*, *H. virescens*, *Diabrotica balteata* (Lind et al. 2002;

Seger et al. 2004) and house fly larvae (*Culex* p. fatigans and *Aedes aegypty*), antifeedant and repellent activities (Kaltenegger et al. 2003; Mungkornasawakul et al. 2004). In addition, the stemofoline shows systemic (xylem mobility) and translaminar activity and acts as potent nAChR agonist (α-Bgtx displacement: EC_{50} = 1.7 nM; Lind et al. 2002). Stemofoline itself required a further increase in biological activity and therefore synthesis and screening began for substructures around this natural product lead structure that retain high potency against in vitro nAChR screens and which correlated with in vivo screen activity against insects (Lind et al. 2002). As result the tropane ether (EC_{50} = 310 nM) and pyridyl-cyanotropane chemistry (EC_{50} = 1 nM) was identified (Fig. 5).

Starting with 4-methoxy-phenyl-tropane ethers the *N*-substituted 3-chloro-pyrid-5-yl-cyanotropanes were found, which have an excitatory, nicotinic character, and act as agonists of the nAChR. The high in vivo activity of *N*-trifluoroethyl-3-chloro-pyrid-5-yl-cyanotropane correlates strongly with a propesticidal model that it is converted to the the *N*-unsubstituted 3-chloro-pyrid-5-yl-cyanotropane (R = H) with evidence stemming from metabolic, radioligand binding and electrophysiological experiments (Lind et al. 2002). Metabolic studies in larval *H. virescens* reflect a fast bioactivation of the cyanotropane by cleavage of the *N*-trifluoroethyl group within two hours forming the non-alkylated compounds. The pyridyl-cyanotropane pharmacology, determined in specific binding of [H^3]-3-chloro-pyrid-5-yl-cyanotropane on peach potato aphid (*M. persicae*) membranes (affinity K_d = 0.48 nM, binding site number B_{max} = 322 fmol/mg) and electrophysiological experiments, resembles that of the snake toxin α-Bgtx, but distinct from imidacloprid (K_d = 0.15 and 12.2 nM, B_{max} = 287 and 883 fmol/mg) and epibatidin (K_d = 1.9 and 11.25 nM, B_{max} = 344 and 904 fmol/mg) (Lind et al. 2002).

Pyridyl-cyanotropanes share high affinity binding site with α-Bgtx based on similarities in pharmacology but which are distinct from that of the high

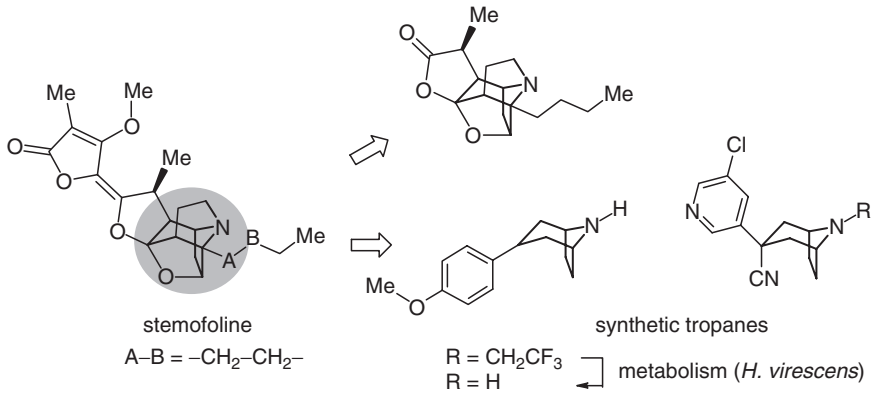


Fig. 5. Structure of natural product stemofoline containing the tropane substructure and the synthetic tropanes (Lind et al. 2002)

affinity imidacloprid and epibatidine sites (Lind et al. 2002). The heterogeneity observed in *M. persicae* is reflected in its genomic diversity of subunit genes for nAChR (Tomizawa and Casida 2001). This makes [H^3]-3-chloro-pyrid-5-yl-cyano-tropane to a specific biochemical tool for defining a sub-population of nAChR in insects, and for studying the binding behavior of ligands for the future discovery of novel neonicotinoids (Lind et al. 2002).

Recently, the occurrence of two related alkaloids, 16,17-didehydro-16(*E*)-stemofoline (A-B = –HC = CH–) and its isomer at C-4, the 16,17-didehydro-4(*E*)-16(*E*)-stemofoline (see isostemofoline), were found together with the known insecticidal compound, stemofoline (A-B = $–CH_2$-CH_2–), in *Stemona collinsae*. The 16,17-didehydro-16(E)-stemofoline (A-B = –HC = CH–, asparagamine A) was found to display a higher insecticidal activity against neonate larvae of *Spodoptera littoralis* (LC_{50} = 0.8 ppm; Kaltenegger et al. 2003) and antifeedant activities against the diamondback moth larvae than stemofoline (Jiwajinda et al. 2001).

4 Ligand Binding

In order to design novel active ingredients for crop protection or drugs, there is much interest in identifying ligand-binding sites at the nAChRs. The ACh-binding site for ligands of the nAChRs is located on the hydrophilic extracellular domain of the nAChR at the interface of two adjacent subunits (α-γ and α-β) and is formed by six distinct regions (loops A–F) in the N-terminal extracellular domain with each of the adjacent subunits contributing three loops (Cascio 2004; Celie et al. 2004).

In 2001 the crystal structure (resolution of 2.7 Å) of a soluble homopentameric AChBP secreted by the glial cells of the mollusc, snail *Lymnaea stagnalis*, has been reported (Brejc et al. 2001) and since this time, an increasing number of publications (from 8 to 69 in 2005) have been described in literature.

Recently, a refined structure of the membrane-associated *Torpedo* ACh receptor at 4 Å resolution was described (Unwin 2005). Herein Unwin confirmed that the two ligand-binding α-subunits have a different extended conformation from the three other subunits in the closed channel, and identified several interactions on both pairs of subunit interfaces, and within the α-subunits, which may be responsible for their *"distorted"* structures. The ACh-coordinating amino acid side-chains of the α-subunits are far apart in the closed channel, indicating that a localized rearrangement, involving closure of loops B and C (see Sect. 4.3.1; Fig. 6) around the bond ACh molecule, occurs upon activation (Unwin 2005).

Molecular dynamics simulations of a homology model of the ligand binding domain of the α7 nAChR were conducted with a range of bound ligands

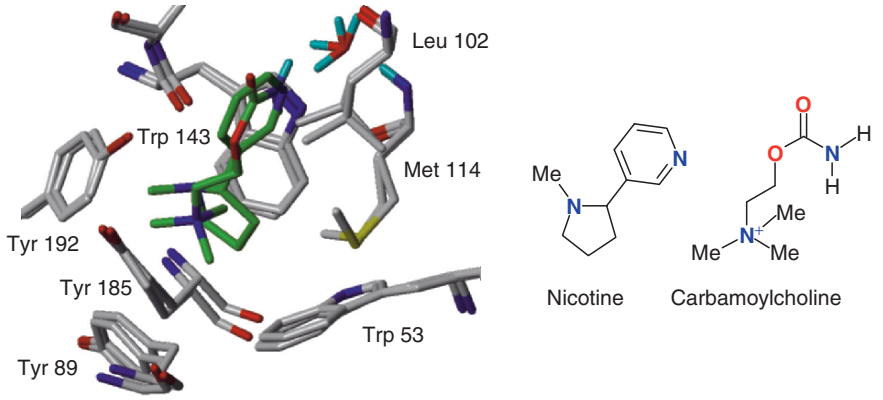


Fig. 6. Alignment of AChBP sites with bound nicotine and carbamoylcholine; both ligands are H-bonded to a NH protein backbone group via water molecule (data taken from Celie et al. 2004)

(e.g., antagonist *d*-tubocurarine (*d*TU), agonist ACh with/without potentiator Ca^{2+}) to induce different conformational states and to give insight into the conformations of the active and inactive states of the receptor as well (Henchman et al. 2005).

In CoMFA an 3D QSAR procedure (Cramer et al. 1988), the electrostatically and sterically favorable and/or unfavorable areas surrounding the ligands are determined, and may be helpful in further drug design of active ingredients (Kagabu et al. 2002a). Since few years the increasing use of computational tools in the field of nAChRs has led to the publication of several models of ligand-receptor interactions (Dutertre and Lewis 2004).

4.1 Acetylcholine Binding Protein (AChBP)

The AChBP modulates synaptic transmission in the mollusc's brain, binds ACh and other nAChR ligands, and resembles the *N*-terminus ligand binding domain of nAChRs. The structure of this 120-kD homopentamer reflects five identical subunits arranged in a cylinder of 80 Å in diameter with a central pore of 18 Å (Dutertre and Lewis 2004) in agreement with the dimension expected for the ligand domain of nAChRs from *Torpedo* electron microscopy data (Myazawa et al. 1999). The binding site is found in a cleft comprised mainly of aromatic residues from loops A–F (see Sect. 4.3.1; Fig. 7) and a series of β strands at the interface of two subunits, in accordance with the mutation experiments on AChRs (Dutertre and Lewis 2004). The loops A, B and C defining these ligand-binding, gating and cation-conducting properties have been extensively characterized by chemical labeling and by site-directed mutagenesis experiments combined with electrophysiological study of function (Leite and Cascio 2001).

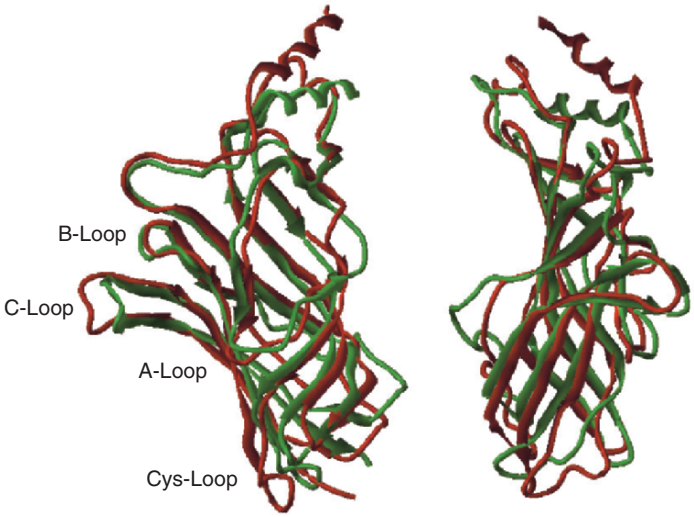


Fig. 7. nAChR structure–fold comparison AChBP color coded in green vs. nAChR α-subunit in red (data taken from Brejc et al. 2001; Unwin 2005; alignment by Gutbrod O)

The sequence of the AChBP protomer shows some homology (~24% sequence identity) to the *N*-terminal, extracellular region of the neuronal α7 subunit and forms a homopentamer (Smit et al. 2001).

The atomic determination of the AChBP, homolog to the amino-terminal extracellular domain of nAChRs, can be used to generate 3D models of the extracellular ligand-binding domain of ligand-gated ion channels and thus for screening and design at act on these channels and offers opportunities for the modeling of the ACh binding site (Grutter et al. 2004). Furthermore, chimeric proteins are provided that are capable of binding a ligand of a ligand-gated receptor, and comprising at least the amino acids of the AChBP in the same positions as in the AChBP (Smit and Sixma 2001). Since loops A–F are all seen in AChBP, amino acid residues contributing to nicotinoid binding may be identified by refering to the crystal structure (Shimomura et al. 2002). The 3D structure of AChBP demonstrated that glutamine (Gln) Q55 in the loop D is located close to tyrosine (Tyr) Y164, the residue corresponding to glycine (Gly) G189 of the α7 subunit (Shimomura et al. 2002).

The crystal structures of nicotine bound to AChBP ended a long debate confirming that the sp^2 pyridine nitrogen of nicotine is H-bonded to receptor residues through a bridging water molecule. The first direct experimental evaluation of the H-bond affinity of the nicotinium pyridine nitrogen was described (Arnaud et al. 2005).

Nicotine and carbamylcholine binding observed in AChBP, is most likely completely identical to that in the α-subunits of the nAChRs and helps to explain the modulatory roles of complementary subunits (Celie et al. 2004). As expected, the binding is characterized by substantial aromatic and hydrophobic

contributions, but additionally there are close contacts between protein oxygens and positively charged groups in these ligands (Celie et al. 2004).

It was found that the higher affinity of nicotine is due to main chain H-bond with the B loop and a closer packing of the aromatic groups. The first H-bond is between the pyridine N1 through a bridging water molecule to the main chain if residues leucine Leu102 and methionine Met114 (Fig. 6).

Based on this structure, it can be suggest that some ligands with the longer *N-N*-distance (optimal distances of 4.6 and 6.3 Å; Abreo et al. 1996) could position their second nitrogen in the place of the water molecule, to realize direct contact with the NH-protein backbone or main chain (Celie et al. 2004).

Recently, the crystal structure of the snake long α-neurotoxin, α-cobratoxin (α-Cbtx), bound to the AChBP was solved from good quality density maps despite a 4.2 Å overall resolution (Bourne et al. 2005). The structure exemplified the positions and orientations of all five three-fingered toxin molecules (e.g., long α-neurotoxin, α-Bgtx) inserted at the AChBP subunit interfaces and the conformational changes associated with toxin binding (cf. NMR investigations: Samson et al. 2002; Bourne et al. 2005).

Furthermore, the structure of a resolution of 2.4 Å of α-conotoxin (Ctx) PnIA (A10L D14 K) was presented as a potent blocker of the α-nAChR (Celie et al. 2005a), bound with high affinity to AChBP. The α-Ctx is buried deep within the ligand-binding site and interacts with residues on both faces of adjacent subunits. The toxin itself did not change conformation, but displaced the C loop of AChBP and induced a rigid-body subunit movement (Celie et al. 2005).

In addition, Celie et al. (2005b) described the crystal structure of a remote homolog, AChBP from *Bulinus truncatus*, which reveals both the conserved structural scaffold and the sites of variation in this receptor family. These include rigid body movements of loops that are close to the transmembrane interface in the receptors and changes in the intermonomer contacts, which alters the pentamer stability drastically (Celie et al. 2005b). Finally, structural, pharmacological and mutational analysis of both AChBPs shows how three amino acid changes in the binding site contribute to a 5 to 10-fold difference in affinity for nicotinic ligands (Cilie et al. 2005b).

On the other hand, the docking of 6-chloropyridazin-3-yl derivatives such as 3,8-diazabicyclo[3.2.1]octane and 2,5-diazabicyclo[2.2.1]heptane active on nAChRs into AChBP was investigated (Artali et al. 2005). The ligand-receptor complex has been analyzed by docking techniques using the binding site of HEPES complex with AChBP as template.

In summary, all these structures could be useful tools for understanding selectivity and for the development of new inhibitors of the nAChR.

4.1.1 Comparison AChBP vs. nAChR α-subunit

The AChBP has the same overall architecture as the extracellular portion of the nAChR (Unwin et al. 2002; Unwin 2005) and the presence of the vicinal cysteine pair characteristic of ligand-binding receptor subunits. Most of the

key residues that have been shown to contribute to the agonist binding domain of the nAChRs were also conserved in AChBP (Unwin 2005). AChBP is not an ion channel, but shows numerous nAChR properties, including binding of known nAChR agonists and competitive antagonists such as ACh, nicotine, *d*TC and α-Bgtx (Brejc et al. 2001; Smit et al. 2001; Karlin 2002). Therefore, most effort has concentrated on the ACh binding-site on the crystal structure of the AChBP and can be used as an example of the *N*-terminal domain of an α-subunit of nAChRs as template for docking simulations of competitive ACh ligands such as agonists and antagonists (Sine 2002) by so-called manual or automated modeling methods (Dutertre and Lewis 2004).

Each of the subunits in the ligand-binding domain has a hydrophobic core of the conserved residues that are grouped into three clusters, as in protomer AChBP (Brejc et al. 2001; Unwin 2005). Most of the surface loops correlate well between the two structures, even in regions where the amino acid sequences are not conserved (Unwin 2005). This begins to explain the modulatory roles of the complementary subunits. The AChBP (green) and AChR α-subunit (red) were found to posses identical folds and rather similar conformations (Fig. 7) (Brejc et al. 2001; Unwin 2005).

This means only minor differences near the ACh binding site are observable. The main ligand-binding nAChR α-subunits in the closed channel are in a "*distorted*" state, which is stabilized by inter- and intra-subunit interactions (Unwin 2005).

However, the binding site in the closed channel differs considerably from that in AChBP, where the ligand is present, indicating that the binding reaction is accompanied by a localized and concerted structural rearrangement in the membrane involving all the helices lining the pore (Unwin 2005; Brejc et al. 2001).

Recently, 3D models of the *N*-terminal part of nAChR were constructed and docked in the putative ligand-binding pocket. Ligand binding is driven by enthalpy and is accompanied by conformational changes in the ligand binding site. These hypothetical docking models offer a structural basis for rational design of drugs differentially binding to resting and active (or desensitized) conformations of the nAChR site. It was postulated that a glutamine (Gln) residue Q79 in loop D and glycin (Gln) G189 in the loop F of the chicken α7 subunit can interact with the nitro group of neonicotinoids like nitenpyram [=CH-NO_2], imidacloprid, thiamethoxam, clothianidin or dinotefuran [each with =N-NO_2], using a combination of site-directed mutagenesis and voltage-clamp electrophysiology (cf. Table 2; Shimomura et al. 2002, 2003).

Furthermore, in most of the insect non-α subunits, amino acid residues corresponding to Q79 of the α7 subunit are lysine (Lys) or arginine (Arg) moieties. These basic residues may interact with the nitro group of the abovementioned neonicotinoids through electrostatic force and, H-bonding (Fig. 8), strengthening the nicotinic receptor-insecticide interaction (Shimomura et al. 2002, 2003).

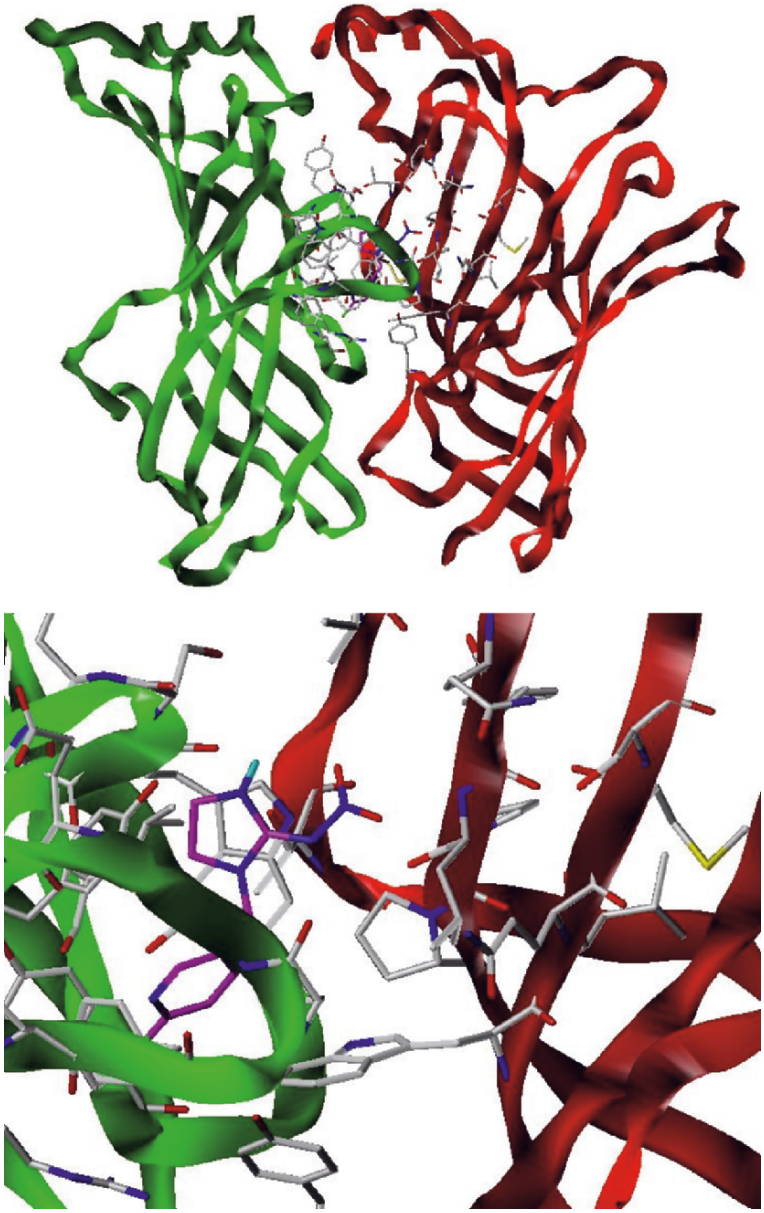


Fig. 8. Imidacloprid binding to AChBP homo dimer; identical subunits color coded in green and red, respectively (alignment by Gutbrod O)

Substitution of these basic residues was shown to result in reduction of the insecticide sensitivity of nAChRs (Shimomura et al. 2002, 2003), but not in reduction of affinity.

Table 2. Amino acid sequence in loop D (data taken from Shimomura et al. 2002)

Proteins	Sequences **79**
Chicken α7	TNIWL **Q** MYWTD
AChBP	VVFWQ **Q** TTWSD
Chicken β2	TNVWL **T** QEWED
Chicken β4	TNVWL **N** QEWID
Human β2	TNVWL **T** QEWED
Human β4	TNVWL **K** QEWTD
Torpedo γ	TNVWI **E** IQWND
Torpedo δ	SNVWM **D** HAWYD
Drosophila ARD	SNVWL **R** LVWYD
Drosophila SBD	TNLWV **K** QRWFD
Drosophila β3	THCWL **N** LRWRD
Locusta β	SNVWL **R** LVWND
Myzus β1	SNVWL **R** LVWRD

In loop F of insect non-α subunits, aromatic residues are present at the position corresponding to isoleucine (Ile) 191 of the chicken α7 subunit and tryptophan (Trp) residues are most frequently observed (Shimomura et al. 2004). Based on mutagenesis studies on the α7 receptor, it is assumed that a Trp-residue in loop F may contribute to strengthening neonicotinoid-insect nAChR interactions (Shimomura et al. 2004).

4.2 Nicotinic Pharmacophore Models

Before the X-ray of the AChBP was described, the structure of the nAChRs binding site(s), the rational design of novel potent and selective nAChR ligands has been facilitated by the identification of a specific 3D arrangement of essential chemical groups common to nAChR ligands, the so-called nicotinic pharmacophore. Traditionally, a pharmacophore is defined as the specific 3D arrangement of functional groups within a molecular framework that are necessary to bind to a macromolecule and/or an enzyme active site (Neamati and Barchi 2002). A pharmacophore is generally considered to represent a minimal set of essential structure-associated features common to a series of ligands with a given biological action (Glennon et al. 2004). Therefore, designation of the nicotinic pharmacophore is the first essential step towards understanding the interaction between nAChR and the class of neonicotinoids including the commercial products (Table 1). Several early "nicotinic pharmacophores" were described, but these either did not consider specific binding data or were derived on the basis of pharmacological data from peripheral nAChR assays (Glennon et al. 2004).

For example, a useful nicotinic pharmacophore model was already described in 1970 by Beers and Reich and subsequently improved by Sheridan et al. (1986). Beers and Reich (1970) suggested that the action of nicotinic agonists and antagonists were controlled by two factors: (1) a columbic interaction between a basic or quarternized amino nitrogen (e.g., an onium group) and the receptor in a certain distance, and (2) formation of an H-bond with the receptor involving an acceptor moiety on the nicotinic ligand. Starting from different models, a distance from the onium group to a point on the van der Waals surface of the H-bond acceptor of 5.9 Å was common to several ligands. Using a distance geometry approach, Sheridan and co-workers refined this model (Sheridan et al. 1986) and claimed that their model was consistent with the Beers and Reich model. Although these two models are not necessarily identical, both are derived from the concept that there exist for nicotinic ligands onium and H-bonding features that are located at a given distance from one another (Glennon et al. 2004). Nevertheless, both pharmacophore models have proven useful for the binding of numerous nicotinic agents at nAChRs and remarkable resilient (Glennon et al. 2004). The pharmacophore elements mimic the quaternary ammonium nitrogen and the ester H-bond acceptor group of the neurotransmitter ACh.

On the other hand, Tønder et al. (1999) suggested that vectors to a receptor-related feature and the distance between the termini of the vectors, not *N-N* distance per se, determines affinity. Ligands with *"long"* and *"short" N-N* distances are seemingly accounted for by this model (Glennon et al. 2004). However, the revised vector model (Tønder et al. 2001), in particular, may be unable to account for ligands with affinities greater than 10 nM (Tønder et al. 2001). Therefore, further revision is needed to also account for such ligands (Glennon et al. 2004).

But these models have considered the nAChR as a single complex. As subtype selective ligands are developed, more refined models will be forthcoming.

4.2.1 Binding Models of Neonicotinoids by 3D Pharmacophore Mapping

Until now, three binding models of neonicotinoids have been proposed to account for the high binding affinity of this important substance class (Zhang et al. 2004a). From SAR studies, the first two models I and II (Fig. 9b) suggest a primary role for the nitrogen at the 1-position of the neonicotinoid equating it to *N*-methyl-pyrrolidine nitrogen of nicotine.

Yamamoto et al. (1995) proposed that the nitrogen atom of the pyridine ring (CPM moiety) and the nitrogen atom at the 1-position of the imidazolidine ring in imidacloprid ($X = N$; $R^1\text{-}R^2 = CH_2CH_2$) interact with the H-donatating and electron rich sites of nAChR, respectively, because the distance between these two nitrogen atoms is very similar to the distance between the two nitrogen atoms on *(S)*-nicotine (Fig. 9a). In an early study, the sp^2 pyridine nitrogen of the nicotine neutral form has been identified as the only H-bond acceptor site (De Taeye and Zeegers-Huyskens 1987).

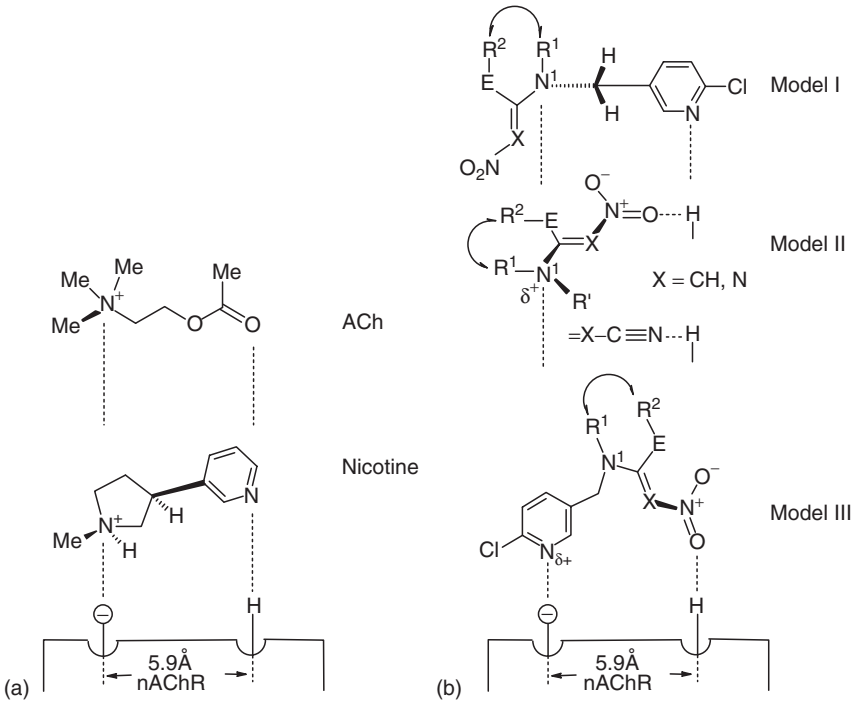


Fig. 9. Interaction of (**a**) ACh, nicotine and (**b**) neonicotinoids with the H-donatating and electron-rich sites of nAChR, exemplified by three models (I)-(III) (data taken from Yamamoto et al. 1995; Tomizawa et al. 2003)

Later it was found that in solution, both nitrogens of nicotine are involved in the H-bond interactions, 90% of these H-bonded complexes are being formed on the pyridine nitrogen (Graton et al. 2003). This result is in accordance with nicotine and carbamylcholine binding observed in AChBP (see Sect. 4.1, Fig. 6).

On the other hand, Kagabu (1996) proposed that the nitrogen atom at the 1-position of the imidazolidine ring of imidacloprid (X = N, R^1-R^2 = CH_2CH_2) and one of the oxygen atoms of the nitro group within the *N*-nitroimino pharmacophore [=N-NO_2] (at the van der Waals surface) play an important role in the interaction with the binding sites on nAChR (Fig. 9b, cf. Model II; Kagabu 1997). That means the oxygen of the nitro group and the cyano nitrogen are well situated as acceptors for H-bonding with the nAChR, in place of the ring nitrogen atoms in CPM and CTM. Thus the π-conjugated system composed of a *N*-nitroimino or *N*-cyanoimino group and the conjugated nitrogen in 1-position are considered essential moieties for the binding of neonicotinoids to the putative cationic subsite in insect nAChR.

In 1994, a CoMFA study of various protonated nicotinic ligands all containing a pyridine ring was published (Glennon et al. 1994). The CoMFA model clarified that the nitrogen of the CPM moiety interacts with an

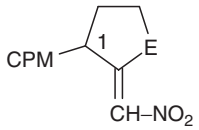
H-donating site of the nAChR, and that the nitrogen atom at the 1-position of the imidazolidine ring of imidacloprid (X = N, R^1-R^2 = CH_2CH_2) interacts with the negatively charged domain (Nakayama 1998; Okazawa et al. 1998).

That the nitro group of the neonicotinoid imidacloprid plays a key role in its selective actions on nAChRs as outlined in both model II and III was described by Shimomura et al. (2003) using combinatorial mutations in loops D and F of the chicken α7 nAChR (see Sect. 4.3.1; Shimomura et al. 2002, 2003).

The third and most recent model III (Fig. 9b) involves a crucial role for the nitro group within the 2-nitromethylene pharmacophore [=CH-NO_2] of the nitromethylene analogue of imidacloprid NTN 32692 (X = CH; R^1-R^2 = CH_2CH_2) an important contribution from the pyridine ring nitrogen (CPM moiety) and a supplemental role for the nitrogen in 1-position (Tomizawa et al. 2003). A first confirmation of this model III is exemplified by interaction of imidacloprid with the α7 nAChR, based on the AChBP as outlined in Fig. 8 (see Sect. 4.3.1; Shimomura et al. 2003).

In this connection, with 1-CH analogues of imidacloprid Zhang et al. (2004a) have shown that the nitrogen in 1-position of NTN 32692 is not absolutely essential for activity (Table 3). The nitromethylene analogue of imidacloprid is one of the most potent agonists known for *Drosophila* nAChR (K_i = 0.13 nM vs. K_i = 2.2 nM for imidacloprid, Zhang et al. 2003).

Table 3. Binding affinities of neonicotinoid derivates to the *Drosophila* nAChR



Compd. no.	E	K_i (nM ± SD, n = 3)[a]
1	NH	1.2 ± 0.3
2	S	180 ± 15
(Z)-3	O	24000 ± 1800[b]
(E)-3	O	48000 ± 3700[b]

[a]K_i values are calculated with the equation of Cheng and Prusoff (1973)
[b]Hydrolytically unstable so the shown activity is probably for the hydrolysis product, the lactone derivative (adapted from Zhang et al. 2004a)

However, the considerably reduced affinity at the *Drosophila* nAChR of the 1-CH-analogue (K_i = 1.2 nM) relative to that of NTN 32692 indicates that the nitrogen in 1-position distinctly enhances potency (Zhang et al. 2004a).

4.2.2 Ring Systems vs. Noncyclic Neonicotinoids

There are numerous examples of isosterism between ring and noncyclic systems among bioactive molecules (Koyanagi and Haga 1995). In the field of

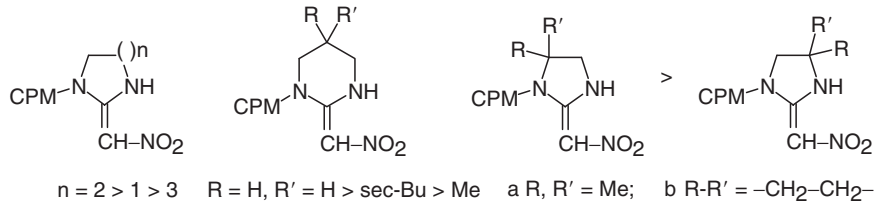


Fig. 10. Influence of ring size n and substituents R, R′ on binding activity of CPM-substituted 2-nitromethylene diazacyclyl insecticides

neonicotinoid chemistry very successful examples were already described (Jeschke et al. 2002). Based on the CoMFA maps of ring systems like imidacloprid (Okazawa et al. 1998, Okazawa et al. 2000), the steric field areas surrounding the pyridine ring and the C4-C5 and N3 positions of its imidazolidine ring were investigated (Kagabu et al. 2002a; Brackmann et al. 2005) (Fig. 10).

The slight enhancement of the binding activity of CPM-substituted 2-nitromethylene diazacyclyl insecticides by enlarging the ring size from five-membered ($n = 1$, binding $pK_i = 7.93$; Liu et al. 1993) to six-membered ($n = 2$, $pK_i = 8.28$; Kagabu et al. 2002a) is in accordance with the CoMFA results. The activity was decreased by further enlarging to a seven-membered ring system ($n = 3$, $pK_i = 6.01$; Kagabu et al. 2002a). On the other hand, introduction of substituents like methyl at the 5-position of the diazacyclohexane ($n = 2$, R = H, R′ = Me, $pK_i = 5.38$; Kagabu et al. 2002a; which is similar to imidacloprid: $n = 1$, R,R′ = H, $pK_i = 5.43$; Liu et al. 1993) as well as substituents at either the 5-position ($n = 1$, R,R′ = Me, $pK_i = 4.65$; Kagabu et al. 2002a) or 4-position ($n = 1$, R,R′ = Me, $pK_i = 3.48$; Kagabu et al. 2002a) of the imidazolidine ring were unfavorable to insecticidal activity, suggesting the existence of a sterically unfavorable area. A similar correlation was described for *mono*-hydroxylated imidacloprid derivatives ($n = 1$, R = H; R′ = OH: 5-position > 4-position; Nauen et al. 2000).

The 5-*sec*-butyl analogue ($n = 2$, R = H, R′ = *sec*-Bu, $pK_i = 6.04 \pm 0.12$; Kagabu et al. 2002a) shows the highest affinity among the diazacyclohexane derivatives.

The influence of substituents and heteroatoms in the ring system can be predicted by quantum chemical calculations (density functional theory (DFT)).

Figure 11a and b show the differences of molecular volumes after superposition of CTM-substituted 2-*N*-cyanoimino diazacyclyl compounds (ii) and (iii) as well as (i) and (iii), respectively. Whereas only minor differences are observed in Fig. 11a the effect of *N*-methyl in the triazacyclyl system seems to be more relevant in comparison to the appropriate diazacyclyl ring.

Recently, it has been demonstrated that the isomeric piperazine-3-one system (X = NCN, Y = O, Z = H_2, R = Me) is a suitable bioisosteric replacement for the imidazolidine ring system contained in chloro-neonicotinoid compounds such as 1-(6-chloro-pyrid-3-ylmethyl)-2-*N*-cyanoimino-imidazolidine (Samaritoni et al. 2003) (Fig. 12).

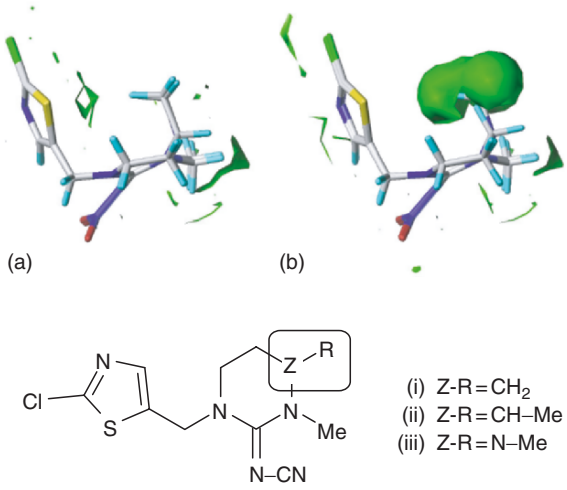


Fig. 11. Differences of molecular volumes. Geometry obtained from density functional theory. (**a**) Superposition between neonicotinoids (ii) and (iii), (**b**) Superposition between neonicotinoids (i) and (iii)

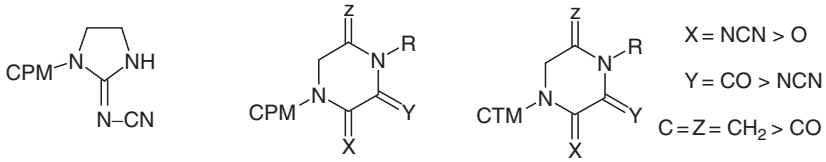


Fig. 12. Influence of functional groups on binding affinity of CPM- and CTM-substituted piperazine-3-ones

However, placement of the *N*-cyanoimino electron-withdrawing group in the 3-position (X = O, Y = NCN, C = Z = CH_2, R = H), or relocation of the carbonyl group in the 5-position (X = NCN, C = Y = CH_2, Z = O, R = Me), results in significantly decreased bioisosterism. A pharmacophore model describes the results on the basis of good overlap of the key pharmacophore elements of the isomeric piperazine-3-one system and imidacloprid; the less active regioisomers feature a smaller degree of overlap (Samaritoni et al. 2003). This pharmacophore model was shown to differ from the previous published model (Okazawa et al. 1998, 2000). The 6-chloro-pyrid-3-yl ring is in a folded conformation pointing toward the *N*-cyanoimino H-bond accepting groups (Samaritoni et al. 2003) (Fig. 13).

Both the CPM-substituted five- and six-ring systems (3-position: X = O, Y = NCN, C = Z = CH_2, R = H) show a high degree of overlap of their pharmacophores [= N-CN] and 6-chloro-pyrid-3-yl rings and a small degree of variation in the overlap of their ring systems, respectively. Furthermore, it seems that the

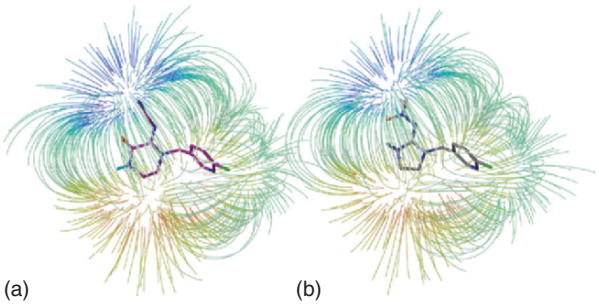
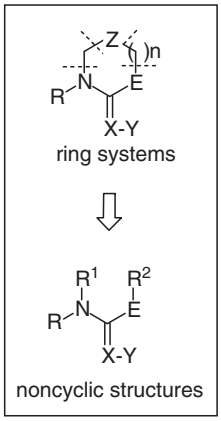


Fig. 13. Field lines are colour coded by the electrostatic potential (calculations at DFT level of theory by Beck ME); CPM-substituted 2-*N*-cyanoimino-piperazin-3-one (**a**) and imidacloprid (**b**)

NH/carbonyl position does not provide a significant degree of binding to the nAChR because opposite charges are represented by these two functionalities (Samaritoni et al. 2003).

In comparison to the corresponding ring systems (Fig. 14), the noncyclic neonicotinoids show similar broad insecticidal activity by forming a so-called *quasi*-cyclic conformation when binding to the insect nAChR (Kagabu 2003).

Therefore, the four commercial noncyclic neonicotinoids—nitenpyram, acetamiprid, clothianidin and dinotefuran—can be regarded as examples, if retrosynthetic considerations are carried out (Jeschke et al. 2002; Kagabu 2003). On the other hand, the partial cleavage of thiamethoxam or the Agro Kanesho's AKD 1022 compound into clothianidin in insects and plant tissues is already discussed (Nauen et al. 2003; Jeschke and Nauen 2004, 2005). It was found that thiamethoxam is an easy to cleave 6-ring neonicotinoid precursor of the noncyclic and highly active clothianidin which acts with high affinity on the same receptor site as imidacloprid and all other neonicotinoids. Based



Imidacloprid	R = CPM, n = 0, Z = CH_2, E = NH, X-Y = NNO_2
Thiacloprid	R = CPM, n = 0, Z = CH_2, E = S, X-Y = NCN
Thiamethoxam	R = CTM, n = 1, Z = O, E = NMe, X-Y = NNO_2
Agro Kanesho's AKD 1022	R = CTM, n = 1, Z = NMe, E = NMe, X-Y = NNO_2
Nitenpyram	R = CPM, R^1 = Et, E-R^2 = NHMe, X-Y = $CHNO_2$
Acetamiprid	R = CPM, R^1 = Me, E-R^2 = Me, X-Y = NCN
Clothianidin	R = CTM, R^1 = H, E-R^2 = NHMe, X-Y = NNO_2
Dinotefuran	R = TFM, R^1 = H, E-R^2 = NHMe, X-Y = NNO_2

Fig. 14. Commercial products: ring systems vs. noncyclic structures

on CoMFA results, a predicted binding site's characteristic for imidacloprid and the noncyclic neonicotinoids like nitenpyram, acetamiprid and related compounds based on affinity measurements to the nAChR of houseflies described.

Superposition of stable conformations of these commercial chloro-neonicotinoids showed that the preferred regions for negative electrostatic potentials near the oxygen atoms of the nitro group as well as the sterically forbidden regions beyond the imidazolidine 3-nitrogen atom of imidacloprid, were important for binding (Okazawa et al. 2000). The area around the 6-chloro atom of the CPM moiety was described as a sterically permissible region. Apparently the steric interactions were more important for noncyclic neonicotinoids than for the ring systems like imidacloprid.

Furthermore, it was suggested that the noncyclic neonicotinoids bind the nAChR recognition site in a manner similar to ring structures like imidacloprid, and that the electrostatic properties of the noncyclic amino and cyclic imidazolidine structures affected their binding affinity (Fig. 15).

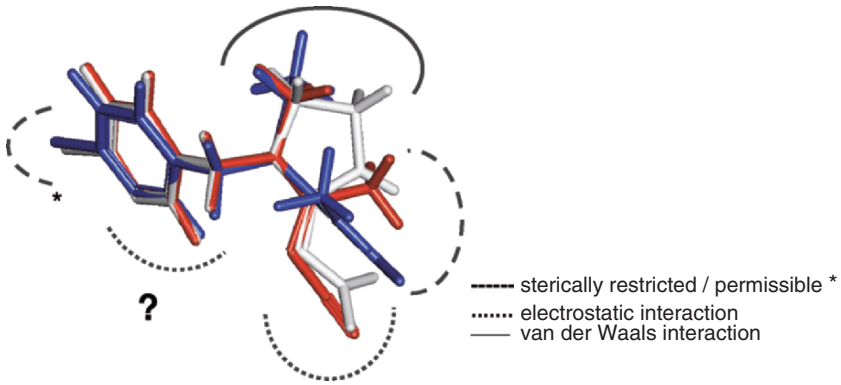


Fig. 15. Stable conformations of imidacloprid, nitenpyram and acetamiprid and predicted properties of their binding site (Okazawa et al. 2000)

In order to investigate the influence of noncyclic vs. cyclic *N*-nitro-guanidine moieties on agonistic activity, the noncyclic *(±)*-dinotefuran (R^1 = H, R^2 = Me) and the 2-*N*-nitroimino-imidazolidine related ring system (R^1-R^2 = $-CH_2CH_2-$) were tested on *D. melanogaster* SAD/chicken β2 hybrid receptors expressed in *Xenopus laevis* oocytes by electrophysiology (Kagabu et al. 2002b) (Fig. 16).

The 2-*N*-nitroimino-imidazolidine derivative was found to have the same pEC_{50} (= $-\log EC_{50}$) value (3.83) as *(±)*-dinotefuran (pEC_{50} = 3.65), therefore the TFM moiety can be taken as an isoster of the nitrogen-containing hetarylmethyl groups as *N*-substituent like CPM or CTM (Kagabu et al. 2002b), respectively (see Sect. 4.1.2.).

The small I_{max} value (0.12) of the 2-*N*-nitroimino-imidazolidine related ring system compared to *(±)*-dinotefuran (I_{max} = 1.04) is consistent with the

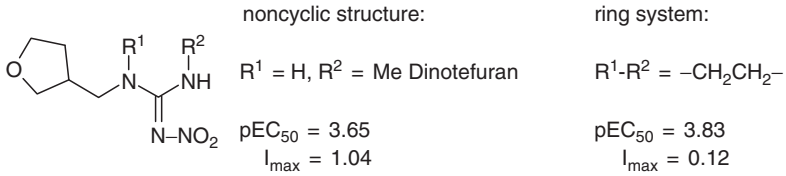


Fig. 16. pEC_{50} and I_{max} values derived from dose-response curves for the noncyclic dinotefuran and ring system analogue (adapted from Kagabu et al. 2002b); [pEC_{50} (= –log EC_{50}) is the concentration (M) that gives half of the maximum response; I_{max} is the maximum response of the normalized currents]

fact that the 2-*N*-nitroimino-imidazolidine moiety contributes to the partial agonistic action as found for imidacloprid (I_{max} = 0.50) (Kagabu et al. 2002a; Matsuda et al. 1998).

4.2.3 Isosteric Alternatives to the Heterocyclic N-Substituents

As known, the nitrogen-containing hetarylmethyl group as *N*-substituent like CPM or CTM has a remarkable strong influence on the insecticidal activity (cf. Fig. 9b, model III). X-ray crystal structure analysis of imidacloprid and related neonicotinoids indicated that the distances between the van der Waals surface of the CPM nitrogen and the atomic center of the pharmacophoric nitrogen are 5.45–6.06 Å (Tomizawa et al. 2000). This range coincides with the distance between ammonium nitrogen and carbonyl oxygen of ACh, and between the nitrogen atoms of *(S)*-nicotine (Kagabu 1997). Alternatively, the CPM and CTM moieties were assumed to be able to participate in H-bonding, like the pyridine ring of *(S)*-nicotine, and that this is important for the insecticidal activity.

The CTM substituent generally confers higher potency in the clothianidin and *N*-desmethyl-thiamethoxam series than the CPM moiety in imidacloprid, thiacloprid, acetamiprid, and nitenpyram series (Zhang et al. 2000). Finally, replacement of both CPM and CTM by the oxygen-containing five-membered carbocycle TFM resulted in the insecticide *(±)*-dinotefuran (cf. Table 1, Kodaka et al. 1998; Zhang et al. 2000). The TFM group can be taken as an isoster of the CPM and CTM moiety (Kagabu et al. 2002b). In an attempt to understand this, the H-bonding regions (Fig. 17, blue arrow) of CPM, CTM, and TFM were projected onto their respective Connolly surfaces (Jeschke et al. 2002).

The influence of fluorine in 2- and 5-position of the CPM group of imidacloprid on binding affinity at the nAChR was described (Jeschke 2004). It was found that the 6-chloro-5-fluoro-pyrid-3-yl moiety has the same pI_{50} value (9.1, tritiated imidacloprid) as imidacloprid, combined with a good activity against *M. persicae*, whereas the 6-chloro-2-fluoro-pyrid-3-yl moiety shows a somewhat lower pI_{50} value (8.3) than imidacloprid and is strongly active against

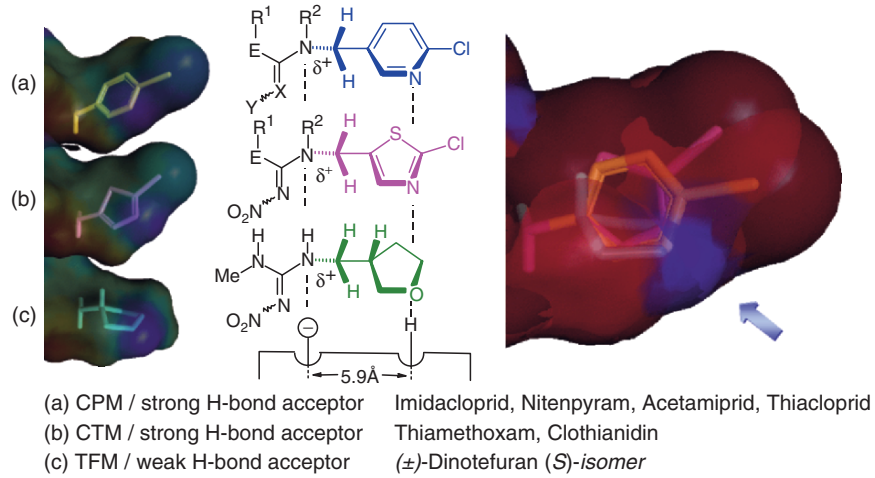


Fig. 17. Comparison of the isostere alternatives CPM, CTM and TFM (adapted from Jeschke and Nauen 2005)

Aphis fabae. Molecular modeling by first principles quantum chemical calculations for imidacloprid and all fluorinated imidacloprid derivatives optimized by high-level DFT have demonstrated that the geometries of imidacloprid and is 6-chloro-5-fluoro-analogue are somewhat different from that of the 6-chloro-2-fluoro-analogue (Jeschke 2004).

4.2.4 Bioisosteric Pharmacophors of Neonicotinoids

The particularly high potency of the neonicotinoids bearing *N*-nitroimino, *N*-cyanoimino, or nitromethylene moieties, which have a negative electrostatic potential, implies a positive electrostatic potential for the corresponding insect nAChR recognition site (Nakayama and Sukekawa 1998). Therefore, considerable attention has been given to the possible involvement of the pharmacophoric nitrogen in neonicotinoid action. In order to understand better the structural requirements, binding activity was analyzed using CoMFA (Akamatsu et al. 1997). SAR analyses have also been performed for in vitro activities (Nishimura et al. 1994). In particular, 3D QSAR procedures are helpful to predict the receptor-ligand interaction (Nakayama et al. 1998; Okazawa et al. 1998, 2000).

Recently, neonicotinoids with novel halogenated nitrobutadiene pharmacophores were patented (Fischer et al. 2003) (Fig. 18).

NMR-investigations found that the *trans*-isomer is stabilized by an intramolecular H-bond between the nitromethylene function and the NH of the diazacyclyl ring system. These derivatives reflect the space in surrounding of the nitromethylene pharmacophore with respect to the insect nAChR binding site.

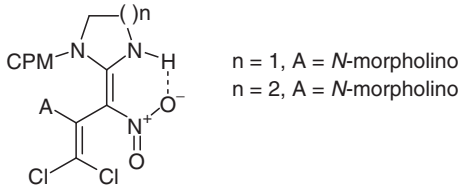
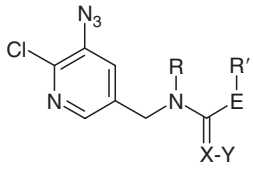


Fig. 18. Neonicotinoids with halogenated nitrobutadiene pharmacophore

4.3 Photoaffinity Probes for Insect nAChRs

A neonicotinoid affinity chromatography matrix has been used to separate the native *Drosophila* nAChR (Tomizawa et al. 1996). In order to investigate nAChR interactions and to identify the amino acids involved in imidacloprid binding, different azido-neonicotinoides containing a 5-azido-6-chloropyrid-3-ylmethyl moiety (Fig. 19) were prepared as useful photoaffinity probes (Kagabu et al. 2000; Tomizawa et al. 2001; Zhang et al. 2002; Maienfisch et al. 2003; Debnath et al. 2003).



(a) R-R′: –CH=CH–, E:NH, =X-Y:=N-NO_2
(b) R-R′: –CH=CH–, E:S, =X-Y:=N-NO_2
(c) R-R′: –CH=CH–, E:S, =X-Y:=N-CN
(d) R-R′: –CH_2–CH_2–, E:NH, =X-Y:=CH-NO_2
(e) R-R′: –CH_2–CH_2–, E:NH, =X-Y:=N-NO_2
(f) R-R′: –CH_2–CH_2–, E:S, =X-Y:=N-CN
(g) R: H, R′: Me, E:NH, =X-Y:=N-NO_2
(h) R: H, R′: CT_3, E:NH, =X-Y:=N-NO_2

Fig. 19. Structures of azido-neonicotinoid photoaffinity probes; azido-neonicotinoids (**a**–**d**) (see Kagabu et al. 2000; Zhang et al. 2002), (**e**–**g**) (see Maienfisch et al. 2003), (**h**) (see Tomizawa et al. 2001)

Furthermore, a tritium-radiolabeled noncyclic azido-neonicotinoid (Fig. 19, cf. h) with a specific activity was described (Tomizawa et al. 2001), having binding potency to a single high-affinity site in *Myzus* that is competively inhibited by imidacloprid. With the information available, imidacloprid analogues are selective for insect nAChRs (Tomizawa and Casida 1999; Tomizawa et al. 1999; Chao and Casida 1997).

Dα2 is the principal neonicotinoid-binding subunit identified by immunoblotting with a specific antibody (Schulz et al. 2000). Photoaffinity labeling suggests that the neonicotinoid binding site in *Drosophila* resides at the interface of a 66- and 61-kDa subunit (Tomizawa et al. 1996; Tomizawa and Casida 1997).

4.4 Selectivity for Insect vs. Mammalian nAChRs

Neonicotinoids more than 100-fold selective for insect nAChRs over vertebrate nAChRs (Methfessel 1992; Matsuda et al. 2001; Tomizawa and Casida

2001). Several research groups have published evidence related to the submolecular basis for this selectivity, based on the nAChR subunit composition and properties, as well as the steric and charge distribution characteristics of neonicotinoids (Yamamoto et al. 1995; Arias 1997; Yamamoto et al. 1998; Tomizawa and Casida 1999; Huang et al. 1999; Tomizawa et al. 2000; Lansdell and Millar 2000a; Zhang et al. 2000; Wiesner and Kayser 2000; Tomizawa and Casida 2001; Tomizawa and Casida 2003). Debnath (2003) and co-workers have demonstrated in a QSAR study performed using electrotopological state atom (ETSA) indices that *N*-nitro-imines, nitromethylenes and *N*-cyano-imines are more selective to *Drosophila* nAChR and safe for human beings, whereas *N*-substituted imines have affinity to the mammalian receptor. Selective toxicity involving low hazard for mammals and high potency to insect pests is an essential requirement for identifying safe and effective insecticides for the future (Tomizawa et al. 2000). Unlike nicotine, neonicotinoids exhibit selective toxicity towards insects based, at least in part, on their higher affinity for insect nicotinic AChRs (Liu and Casida 1993).

The positioning of *N*-desnitro-imidacloprid and the interacting amino acids in the mammalian site is based on superposition in molecular modeling with ACh and nicotinoids (Tomizawa et al. 2000; Corringer et al. 2000; Tomizawa et al. 2003). To elucidate the molecular basis of selectivity, amino acid residues influencing neonicotinoid sensitivity were investigated by site-directed mutagenesis of chicken α7 nicotinic AChR subunit, based on the crystal structure of AChBP (Shimomura et al. 2002) (Fig. 20).

The insect-selective neonicotinoids are not protonated with a *N*-nitroimine, *N*-cyanoimine, or nitromethylene group whereas the mammalian-selective compounds are ionized at physiological pH.

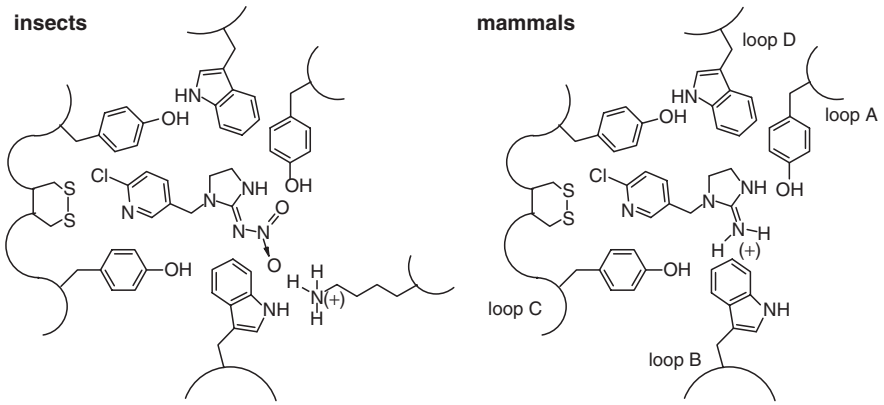


Fig. 20. Binding subsite specificity shown as hypothetical schematic models for neonicotinoid imidacloprid binding in the insect nAChR and nicotinoid *N*-desnitro-imidacloprid binding in the mammalian nAChR, each at the ACh agonist site (adapted from Tomizawa and Casida 2004)

5 nAChR-Based Screening Assays

Today, in vitro ultra high throughput screening (UHTS) bioassays with capacities of >100,000 compounds per day have been installed and a target-based approach using mechanism-based screens has replaced nonspecific assays. Receptor-based screens with a target of special interest for crop protection immobilized in microtiter well plates and affinity binding or enzyme activity are now used. This approach has been extensively reviewed (Wölcke and Ullmann 2001). The UHTS produces a great number of data (Wölcke and Ullmann 2001; Moaddel and Wainer 2003).

Affinity-binding probes are designed (see Sect. 4.3.) to identify competitively binding ligands; compounds that bind noncompetitively to the target are usually missed (Moaddel and Wainer 2003). As an alternative approach to the microtiter well plate format, the affinity chromatography is discussed (Moaddel and Wainer 2003; Moaddel et al. 2005). Up to now, the phases have included immobilized nAChRs like α3β4-nAChR and α4β2-nAChR as stationary phases alone (Zhang et al. 1998) or co-immobilized with the GABA-receptor and *N*-methyl-*(R)*-aspartate receptor (Moaddel et al. 2002). Results demonstrate for immobilized α3β4-nAChR-stationary phases that they can be used for online screening (lower HTS) for ligands both competitive and noncompetitive like nicotine, nornicotine, epibatidine and ACh. In addition, the results indicate that the immobilized nAChRs retained their ability to undergo agonist-induced conformational change from the resting to the desensitized states (Moaddel et al. 2005). The columns provide a unique ability to study the interactions of noncompetitive inhibitors with both of these conformational states (Moaddel et al. 2005).

In vitro hits can be selected from the test libraries, and are subsequently validated in microscreening assays in order to identify a real in vivo hit. These steps are followed by profiling of the candidate in greenhouse and field trials, and identification of a lead structure, which will be further optimized in research projects and later on in development projects. Results demonstrate that immobilized nAChR stationary phases can be used for the online screening for ligands to the nAChRs. However, this procedure is not yet usable for insect nAChRs.

6 Resistance

Although resistance is an important issue in modern applied entomology, it is far beyond the scope of this chapter to highlight in detail known cases and mechanisms of neonicotinoid resistance, and discuss management options for this important class of chemistry. For reasons of completeness, only a short chapter on neonicotinoid resistance issues is presented, and it is recommended that the reader to refer to an excellent review recently published

on this topic by Nauen and Denholm (2005). The review provides an up-to-date compilation of facts and references concerning neonicotinoid resistance in insect pest species. More information on general aspects of insecticide resistance can be found on the Web site of IRAC International (www.irac-online.org).

The important class of neonicotinoid insecticides has provided agricultural producers with invaluable new tools for managing some of the world's most destructive crop pests, primarily those of the order *Hemiptera* (aphids, whiteflies and planthoppers) and *Coleoptera* (beetles), including species with a long history of resistance to earlier-used classes of insecticides (Jeschke and Nauen 2005). Imidacloprid has proved remarkably resilient to resistance, and cases that have been reported are still relatively manageable and/or geographically localized. The existence of strong resistance in some species has nonetheless demonstrated the potential of pests to adapt and resist field applications of neonicotinoids. The ongoing introduction of further commercial neonicotinoids worldwide, unless carefully regulated and coordinated, seems bound to increase exposure to this substance class and to enhance conditions favoring resistant phenotypes (for review see Nauen and Denholm, 2005).

The incidence and management of insect resistance to neonicotinoid insecticides such as imidacloprid was recently reviewed by Denholm et al. (2002), and Nauen and Denholm (2005). Resistance to neonicotinoids is still rare under field conditions and baseline susceptibility data have been provided in the past and more recently especially for imidacloprid in order to monitor for early signs of resistance in some of the most destructive hemipteran pest insects such as *Bemisia tabaci*, *M. persicae* and *Phorodon humuli* (Cahill et al. 1996; Elbert et al. 1996; Elbert and Nauen 1996; Foster et al. 2003; Nauen and Elbert 2003; Rauch and Nauen 2003; Weichel and Nauen 2003).

In some cases, such as *Myzus* spp., the lower susceptibility to imidacloprid and other neonicotinoids was correlated with a decreased efficacy of nicotine (Nauen et al. 1996; Devine et al. 1996). More recently, Foster et al. (2003) demonstrated that tolerance to imidacloprid in *M. persicae* from different regions in Europe also provided cross-tolerance to acetamiprid. One species of major concern over the last decade is the tobacco or cotton whitefly, *B. tabaci* (Nauen et al. 2002; Rauch and Nauen, 2003; Nauen and Denholm, 2005). Resistance to imidacloprid conferring a high level of cross-resistance to thiamethoxam and acetamiprid was first demonstrated and studied in Q-type *B. tabaci* from greenhouses in the Almeria region of southern Spain, but was also detected in single populations from Italy and Germany as well (Nauen et al. 2002; Rauch and Nauen 2003). The first neonicotinoid, i.e. imidacloprid was introduced for controlling Colorado potato beetles *L. decemlineata* in North America in 1995. Concerns over resistance development were reinforced when extensive monitoring of populations from North America showed ca. 30-fold variation in LC_{50} values from ingestion and contact bioassays against neonates

(Olsen et al. 2000). Much of this variation appeared unconnected with imidacloprid use, and probably a consequence of cross-resistance from substance classes used earlier. The lowest levels of susceptibility occurred in populations from Long Island, New York, an area that has experienced the most severe resistance problems of all with *L. decemlineata*. Zhao et al. (2000) and Hollingworth et al. (2002). Other reports referring to resistance to neonicotinoid insecticides were species-wise of lesser importance and includes species either from field collected populations or artificially selected strains and included the small brown plant-hopper, *Laodelphax striatellus* (Sone et al. 1997), western flower thrips, *Franklienella occidentalis* (Zhao et al. 1995), houseflies and German cockroach, *Musca domestica* and *Blattella germanica*, respectively (Wen and Scott 1997), *D. melanogaster* (Daborn et al. 2001), *Lygus hesperus* (Dennehy and Russell 1996) and brown planthoppers, *Nilaparvata lugens* (Zewen et al. 2003).

Most comprehensive studies on the biochemical mechanisms of resistance to neonicotinoids using an agriculturally relevant pest species were performed in whiteflies, *B. tabaci* (Nauen et al. 1997; Nauen et al. 2002; Rauch and Nauen 2003; Byrne et al. 2003). Biochemical examinations revealed that neonicotinoid resistance in Q-type *B. tabaci* collected in 1999 was not associated with a lower affinity of imidacloprid to nAChRs in whitefly membrane preparations (Nauen et al. 2002). This was confirmed more recently by testing strains ESP-00, GER-01, ISR-02 obtained in the years 2000–2002 by Rauch and Nauen (2003) who biochemically showed that whiteflies resistant to neonicotinoid insecticides showed a high microsomal 7-ethoxycoumarin *O*-deethylase activity, i.e., up to 8-fold higher compared with neonicotinoid susceptible strains (see also Nauen and Denholm 2005). Metabolism studies in *B. tabaci* in vivo revealed that the main metabolite in neonicotinoid resistant strains is 5-hydroxy-imidacloprid. Conclusively, one can suggest that oxidative degradation is the main route of imidacloprid detoxification in neonicotinoid resistant Q-type whiteflies (Rauch and Nauen 2003). Cross-resistant studies with both acetamiprid and thiamethoxam recently revealed that the strain that had been selected with thiamethoxam for 12 generations demonstrated almost no cross-resistance to acetamiprid (cotton fields in Israel during the cotton-growing season 1999–2003), whereas the acetamiprid-selected strain exhibit high cross-resistance of >500-fold to thiamethoxam (Horowitz et al. 2004). A possible explanation of the reason for the lack of cross-resistance to acetamiprid in thiametoxam-selected whiteflies is that the two neonicotinoids have two different mechanisms (Horowitz et al. 2004). Resistance in thiamethoxam-selected whiteflies might be associated with the activation mechanism as pro-drug (see Sect. 6.3; Jeschke and Nauen 2004), whereas in acetamiprid-selected whiteflies, the compound itself is the primary target for detoxification and results in broad cross-resistance to all neonicotinoids (Horowitz et al. 2004).

General guidelines for the management of resistance to neonicotinoid insecticides were recently published by Elbert et al. (2005).

7 Concluding Remarks and Prospects

Up to now, it is still not possible to draw a perfect model of the insect native nAChR relative to structure and diversity. The identification and characterization of insect nAChR subtypes is still an important research field, and may open up a new era for subtype-selective insecticides (Tomizawa and Casida 2001). The sequencing of other genomes (http://hgsc.bcm.tmc.edu/projects; HGSC sequencing projects) will provide further insight into the diversity of insect nAChR gene families.

In this connection, the neonicotinoides are very effective probes for structural investigations of insect nAChRs (Tomizawa et al. 2003). The increase in knowledge of ligand binding to the pentameric nAChRs at high resolution will provide detailed information concerning rational insecticide design. The atomic determination of the AChBP, homolog to the amino-terminal extracellular domain of nAChRs, can be used to generate 3D models of the extracellular ligand-binding domain of ligand-gated ion channels and offer a structural basis for screening and rational design of novel insecticides acting on these channels. Therefore, the AChBP structure provides the theoretical foundation for designing homology models of the corresponding receptor ligand binding domains within the nAChRs (Sine et al. 2004). However, the models of nAChR extracellular domain represent so-called frozen *"snapshots"* of a particular state constrained by the crystal structure of AChBP (Grutter et al. 2004). Therefore, ongoing design of active ingredients and their optimization has to consider in its process conformational transitions of the nAChR, which allosteric modify and target site. Elucidation of the 3D structure of the ion channel in the closed and open conformations should thus dictate the orientation of the extracellular domain of nAChR relative to the membrane (Grutter et al. 2004). First insight gave the refined model of the membrane-associated *Torpedo* AChR at 4-Å resolution (Unwin 2005), which exemplified that all channels of the Cys-loop LGIC-superfamily are constructed around the same global principle.

Information about ligand-receptor interactions has to be deduced rather from the AChBP and not from the nAChR structure (Unwin 2005). The AChBP has resolved the various models of nicotine and ACh binding in the past and provides novel explanations for important issues on both the nAChR and the ligand site by addressing topics such as the charge compensation and the inter-nitrogen distance in the nicotinoid or neonicotinoid pharmacophore.

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8 Mitochondrial Electron Transport Complexes as Biochemical Target Sites for Insecticides and Acaricides

PETER LÜMMEN

1 The Mitochondrial Electron Transport Chain

1.1 Overview of Respiratory Electron Transport and Chemiosmotic Coupling

Almost 80 years ago Keilin's groundbreaking investigation of the iron-heme containing cytochromes led to the fundamental concept of the respiratory electron transport chain linking the dehydrogenation reactions studied earlier by Wieland and Thunberg to the oxygen-activating terminal oxidase (respiratory enzyme, "Atmungsferment") discovered by Warburg (Slater 2003). Shortly thereafter, evidence accumulated that the inner membrane of mitochondria was the site of respiratory electron transport.

Subsequently, additional redox-active prosthetic groups and electron carriers were discovered such as the flavin nucleotides, ubiquinone, the copper centers and the non-heme iron-sulfur clusters.

As soon as the central molecular components of the respiratory chain had been established, the next problem was to explain how the exergonic electron transfer reactions were mechanistically coupled to the highly endergonic synthesis of "high-energy phosphates", namely adenosine triphosphate (ATP)–a key question in bioenergetics until now.

In his chemiosmotic hypothesis, Mitchell postulated that the energy was stored as an electrochemical proton gradient across the inner mitochondrial membrane. The theory was based on the following assumptions:

(i) the mitochondrial membrane is almost impermeable for protons,
(ii) the redox reactions are tightly coupled to the vectorial translocation of protons across the membrane,
(iii) the proton electrochemical gradient $\Delta\mu_H^+$ across the membrane consists of a chemical potential difference (ΔpH) and an electrical potential difference, $\Delta\Psi$, as described by the equation

$$\Delta\mu_H^+ = F \cdot \Delta\psi - 2.3RT \cdot \Delta pH$$

(F, Faraday constant; R, gas constant, T, absolute temperature), often presented in a slightly different form as the "proton-motive force",

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$$\Delta_p = \Delta\mu_H^+ / F = \Delta\psi - \frac{2.3RT}{F} \cdot \Delta pH$$

(iv) the proton gradient is used by the inner membrane ATP synthase to drive ATP synthesis (Rich 2003).

The importance of mobile electron- as well as hydrogen-carriers connecting the protein complexes can be exemplified by ubiquinone, which is in stoichiometric excess over the protein-bound redox components, thereby emphasizing its substrate-like function. Full quinone reduction requires the addition of two protons. Consequently, the quinone reduction sites of proton-translocating dehydrogenases are located close to the matrix face of the membrane. In some cases, proton channels were suggested to allow access of matrix protons to the quinone sites.

In contrast, hydroquinone oxidation occurs close to the mitochondrial intermembrane face and the protons are concomitantly released to the intermembrane space. More sophisticated proton-pumping mechanisms have been proposed subsequently to account for the actual H^+/e^- stoichiometries determined experimentally.

1.2 Respiratory Electron Transport Complexes

The mitochondrial electron transport chain consists of four large transmembrane enzyme complexes (Fig. 1). Redox reactions are sequentially arranged according to the midpoint redox potential differences of the prosthetic groups and the mobile electron carriers involved.

The first enzyme, NADH:ubiquinone oxidoreductase (EC 1.6.5.3, complex I), oxidizes NADH and reduces the mobile electron/proton carrier ubiquinone (Grigorieff 1999; Friedrich and Böttcher 2004). As 4 $H^+/2e^-$ are translocated to the intermembrane space, complex I constitutes the first energy conservation site of the respiratory chain. Its structure and function will be discussed in more detail below.

Succinate:ubiquinone oxidoreductase (EC 1.3.5.1, SQR, complex II) directly links the respiratory chain to the citric acid cycle. Recently, the structure of the porcine SQR has been solved at 2.4 Å resolution (Sun et al. 2005). The key Krebs cycle intermediate succinate is oxidized to fumarate by protein-bound FAD as the immediate oxidant. From $FADH_2$ the electrons are transferred via three iron-sulfur clusters to ubiquinone. A proximal and a distal quinone binding site are present in the mitochondrial SQR enzymes. Finally, a b-type heme is probably involved in branching the flow of electrons from the last iron-sulfur cluster and the quinone reduction sites. Due to its low standard redox potential difference, succinate-dependent ubiquinone reduction does not provide sufficient free energy for ATP synthesis (Lancaster 2002).

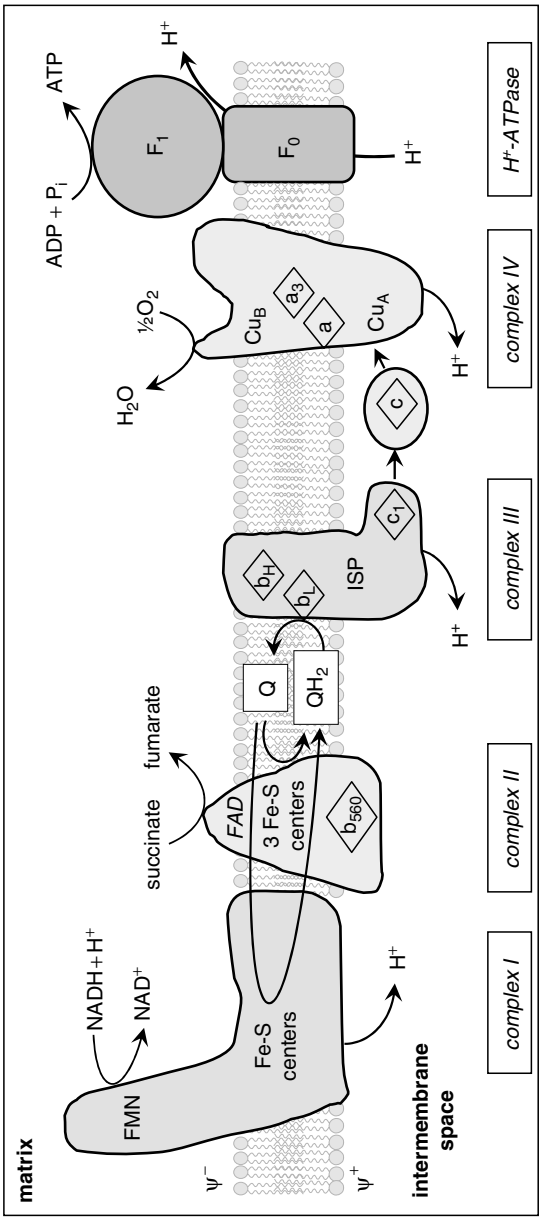

Fig. 1. Schematic representation of the mitochondrial electron transport chain. Complex I, NADH:ubiquinone oxidoreductase; complex II, succinate:ubiquinone oxidoreductase (SQR); complex III, ubihydroquinone:cytochrome *c* oxidoreductase; complex IV, cytochrome oxidase; H^+-ATPase/ATPsynthase. *Fe-S* non-heme iron-sulfur centers; *ISP* Rieske iron-sulfur protein; *FMN* flavin mononucleotide; *FAD* flavin-adenine dinucleotide; b_{560} cytochrome (heme) b_{560}; *Q* ubiquinone; QH_2 ubihydroquinone; b_L low-potential cytochrome b; b_H high-potential cytochrome b; c_1 *c*: cytochromes c_1 and *c*; Cu_A, Cu_B copper centers A and B; *a*, a_3 cytochromes a and a_3

Ubihydroquinone:cytochrome c oxidoreductase (EC 1.10.2.2, complex III, bc_1 complex) accounts for the second energy-conservation site. Ubihydroquinone donates its electrons to cytochrome c (Crofts 2004) via intermediate redox groups such as a binuclear iron-sulfur center and heme c_1. The reaction sequence is coupled to a proton-pumping mechanism involving two b-type hemes. The bc_1 complex is comparably well understood both in terms of structure and mechanism.

Cytochrome oxidase (EC 1.9.3.1, complex IV) finally reduces O_2 to water thereby re-oxidizing ferrocytochrome c. The catalytic core consisting of four subunits performs the redox reactions. Copper center Cu_A is the primary electron acceptor for the electron delivered by the reduced cytochrome c. From Cu_A the electron flows via heme a to heme a_3. At the second copper center, Cu_B, the terminal reduction of oxygen to water takes place, the necessary protons are taken up from the matrix ("scalar" protons). Additional protons are pumped across the inner mitochondrial membrane ("vectorial" protons, Michel et al. 1998; Tsukihara et al. 1996), so that cytochrome oxidase contributes to the proton-motive force as the third energy-conservation site.

Recent evidence suggests that respiratory chain complexes form supramolecular assemblies termed respirasomes in bacterial and mitochondrial systems (Schägger 2002). Stoichiometrical supercomplexes of complex I and the dimeric complex III constitute the core respirasome, detectable under mild isolation conditions (Schägger et al. 2004). Variable copy numbers of cytochrome oxidase are associated with the respirasome. Flux control analysis of respiratory NADH oxidase activity revealed that complexes I and III kinetically behaved like a single enzymatic entity confirming the conclusions from the protein-chemical data. However, as a wealth of kinetic data had suggested a mobile quinone pool in the inner mitochondrial membrane, the two concepts, seeming contradictory prima facie, had been reconciled by proposing that free and associated enzyme complexes are present in equilibrium which could be shifted, for example, by the phospholipid/protein ratio (Bianchi et al. 2004).

1.3 Respiratory Electron Transport Complexes as Target Sites for Insecticides

In view of the central role that the respiratory chain plays in energy metabolism, it is highly possible that it could be a target site for insecticides and acaricides. This was established, in fact, for the NADH:ubiquinone oxidoreductase (complex I) and the ubihydroquinone:cytochrome c oxidoreductase (complex III, bc_1 complex). Insecticides and acaricides specifically inhibiting complex I have been reviewed in the past (Hollingworth and Ahammadsahib 1995; Lümmen 1998; Friedrich et al. 1994a; Dekeyser 2005). Inhibition of complex III was described as the mode of action of insecticides and recently discovered acaricides (Hollingshaus 1987; Khambay et al. 2003; Koura et al. 1998).

Currently there are no reports of insecticides inhibiting succinate: ubiquinone oxidoreductase (SQR, complex II)*, although some complex II inhibitors show activity as fungicides (see for example: Phillips and Rejda-Heath 1993). Finally, no examples of insecticides acting on cytochrome oxidase (complex IV) have been reported.

During the least years we have witnessed tremendous developments in the field of structural biology devoted to respiratory chain enzymes. With the exception of complex I, high-resolution X-ray structures, some also with bound inhibitors, exist for the bc_1 complex, the cytochrome oxidase, and the mitochondrial succinate:ubiquinone oxidoreductase. The exquisite combination of kinetic, spectroscopic, and structural methodologies delivered new hypotheses and concepts of protein structures in relation to the catalytic mechanisms.

In the following, selected experimental approaches and recent results relevant for the molecular mode of action of insecticides and acaricides acting on respiration will be described.

2 NADH:ubiquinone Oxidoreductase (Complex I)

2.1 Structural Aspects Related to Mechanism

The proton-translocating NADH:ubiquinone oxidoreductase (complex I, EC 1.6.5.3) catalyses the NADH-dependent reduction of ubiquinone following the general reaction equation:

$$NADH + H^+ + UQ + 4H_n^+ \rightarrow NAD^+ + UQH_2 + 4H_p^+$$

the subscripts n and p designate the protons taken up at the negative (matrix) side and released at the positive (intermembrane) side of the inner mitochondrial membrane, respectively. As already mentioned, the proton-pumping activity of complex I contributes significantly to the total $\Delta\mu_H^+$.

Complex I discloses the most complicated structural organization of all respiratory chain enzymes with a total molecular mass of nearly 1,000 kDa. Plausibly, the high complexity is the main reason why important questions concerning the catalytic mechanism remain to be answered (Yagi and Matsuno-Yagi 2003; Friedrich and Böttcher 2004; Yano 2002). The bovine enzyme is the most thoroughly studied eukaryotic complex I in terms of subunit composition; 45 out of 46 subunits are known by sequence and their well-established nomenclature will be referred to in the following (Walker et al. 1992; Pilkington et al. 1993).

Electron microscopic analysis of two-dimensional crystals of the *Neurospora crassa* complex I revealed that the enzyme is arranged in a characteristic L-shaped assembly (Fig. 2) (Guenebaut et al. 1998). A peripheral, hydrophilic arm protrudes into the mitochondrial matrix almost perpendicular to the transmembrane arm.

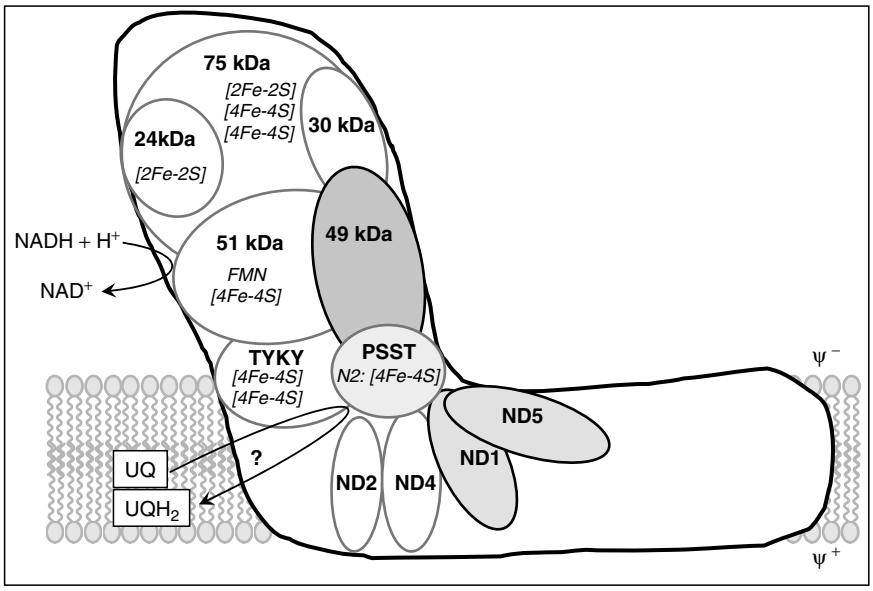

Fig. 2. Structural model of complex I. Only the subunits suggested to constitute the catalytic core and the transmembrane subunits found to be involved in quinone and/or inhibitor binding are depicted. The nomenclature follows the bovine enzyme. *FMN* flavin mononucleotide; *2Fe-2S* binuclear iron-sulfur cluster; *4Fe-4S* tetranuclear iron-sulfur cluster; *N2* iron-sulfur cluster with the highest midpoint potential, suggested as immediate electron donor to ubiquinone

Partial solubilization of the bovine complex I with the chaotropic detergent lauryldimethylamine oxide (LDAO) dissociated the enzyme into two subcomplexes, Iα and Iβ, which did not overlap with respect to their subunit composition as revealed by SDS polyacrylamide gel electrophoresis and HPLC analysis (Hirst et al. 2003). Subcomplex Iα was further split into subcomplex Iλ. The 15 hydrophilic subunits of Iλ included the catalytically active redox centers like the FMN cofactor (51-kDa subunit) and all EPR-visible iron-sulfur clusters: N1b, N4, N5 (75-kDa subunit), N3 (51-kDa subunit), N1a (24-kDa subunit), N6a, N6b (TYKY), and N2 (PSST).

The structural environment of cluster N2 is of particular importance for the catalytic mechanism. N2 has the highest midpoint potential ($E_{m,7}$ = −150 mV to − 50 mV, pH-dependent) and therefore serves as the immediate electron donor to ubiquinone. Assignment of N2 to the PSST subunit was suggested by the homology of PSST and 49kDa of complex I with bacterial [NiFe] hydrogenases (Albracht and Hedderich 2000; Hedderich 2004; Ahlers et al. 2000). Mutation of cysteines in PSST and its bacterial homologue Nqo6 resulted in the complete loss of the N2 EPR-signal (Duarte et al. 2002; Friedrich 1998). Additionally, mutations generated in the 49-kDa subunit also lacked the N2-related EPR signals indicating the location of N2 close to the interface of the two subunits (Kashani-Poor et al. 2001). Interestingly,

PSST has only three cysteine residues to ligate the iron-sulfur cluster, so that the fourth ligand, either a cysteine or another suitable residue, may be provided by the 49-kDa subunit.

A rotenone-sensitive, fast-relaxing ubisemiquinone radical, SQ_{Nf}, was detected during steady-state NADH oxidation. It was found to spin-couple to a nearby paramagnetic iron-sulfur center at a distance of 8–11 Å, which is assumed to be N2 (Yano et al. 2005; Brandt 1999).

The hydrophobic arm is embedded in the plane of the membrane. It is noteworthy that seven of its transmembrane subunits (ND1–6, ND4L) are encoded by the mitochondrial genome. Three of them, ND2, ND4, and ND5, are homologous to bacterial Na^+/H^+ antiporters (Steuber et al. 2000).

It is widely accepted that hydrophobic subunits are involved in quinone binding and proton translocation. For example, photoaffinity labeling studies revealed that NuoM, the *Escherichia coli* homologue of the mitochondrial ND4, was labeled with an azido-ubiquinone analogue. Additionally, ND4 was shown to contain a putative quinone binding motif (Gong et al. 2003).

However, the precise number of quinone binding sites (one, two or even three) is still a matter of debate (Holt et al. 2003).

2.2 Complex I Inhibitors as Insecticides and Acaricides

A plethora of structurally diverse complex I inhibitors, both from natural sources and chemical synthesis, is documented in the literature (Singer and Ramsay 1994; Friedrich et al. 1994a; Degli Esposti et al. 1993; Miyoshi 2001). The high-affinity inhibitors depicted in Fig. 3 have been either commercialized as acaricides or insecticides, or described as experimental insecticides (acaricides) such as the annonaceous acetogenins (e.g., rolliniastatin-2, Fig. 3, **4**), the miticidal chromone compound (Fig. 3, **10**), and the 2-decyl-4-quinazolinyl amine (Fig. 3, **11**).

Based on enzyme kinetic analysis of membrane-bound complex I activity, two inhibitor classes represented by piericidin A (Fig. 3, **2**) and rotenone (Fig. 3, **1**) had been distinguished. Class I compounds including piericidin A, fenazaquin (Fig. 3, **3**), fenpyroximate (Fig. 3, **7**), pyrimidifen (Fig. 3, **5**) and the related amino- and alkoxypyrimidines inhibited quinone reduction (partially) competitively (Friedrich et al. 1994b). In contrast, rotenone (class II) was characterized as a non-competitive inhibitor of ubiquinone reduction. Accordingly, two corresponding quinone binding sites were postulated (Degli Esposti 1998).

However, solubilized and partially purified housefly complex I was inhibited non-competitively by both aminopyrimidine (class I) and rotenone (class II) inhibitors (Lümmen 1999) confirming earlier results obtained with submitochondrial membranes (Jewess 1994).

To resolve these discrepancies of the enzyme kinetic data, independent methods were developed to directly measure inhibitor binding to complex I. The fluorescence quench titration procedure (Okun et al. 1999) took

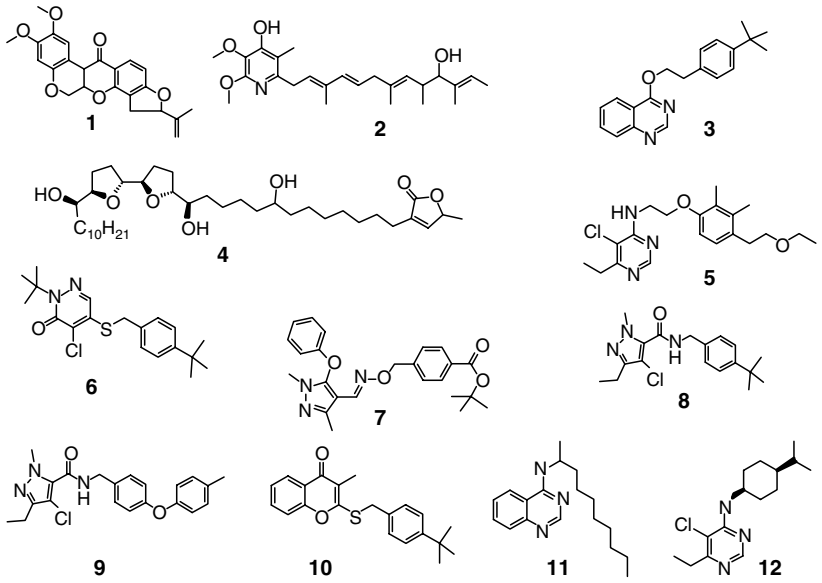

Fig. 3. Inhibitors of complex I with insecticidal/acaricidal activity. **1**, rotenone; **2,** piericidin A; **3**, fenazaquin; **4**, rolliniastatin-2; **5**, pyrimidifen; **6**, pyridaben; **7**, fenpyroximate; **8**, tebufenpyrad; **9**, tolfenpyrad; **10**, experimental chromone acaricide; **11**, 2-decyl-4-quinazolinyl amine (DQA), experimental insecticide; **12**, AE F119209, experimental aminopyrimidine insecticide

advantage of the fact that several aminoquinazoline insecticides, e.g., DQA (Fig. 2, **11**), exhibited suitable fluorescence properties to measure inhibitor binding: fluorescence was quenched in a concentration-dependent fashion and a nanomolar dissociation constant K_d = 17 nM for DQA was calculated. In addition, competition-type experiments showed that the insecticides rotenone, piericidin A, fenazaquin, fenpyroximate, pyrimidifen, and pyridaben displaced the fluorescent ligand from its binding site (Okun et al. 1999). In the same study, equilibrium saturation binding of the radiolabeled aminopyrimidine AE F119209 (Fig. 3, **12**) to partially purified housefly complex I revealed an apparent dissociation constant K_d = 9 nM, which fitted well to the inhibition constant (K_i) derived from kinetic analysis of the purified enzyme. In competition experiments, all insecticide/acaricide compounds mentioned above inhibited radioligand binding competitively, suggesting that their binding sites overlapped at least partially. By using the same radioligand, the mode of action of a novel acaricide structure, the chromone compound (Fig. 3, **10**), was elucidated (Lindell et al. 2004).

Genetic approaches using insecticidal complex I inhibitors as selective agents resulted in the isolation and characterization of inhibitor-resistant complex I mutants (Darrouzet and Dupuis 1997; Darrouzet et al. 1998). Surprisingly, mutation of a highly conserved valine (V407 M) in NuoD, the *Rhodobacter capsulatus* homologue of the mitochondrial 49 kDa subunit,

indicated that the peripheral subunit was involved in inhibitor binding. As suggested by the topology of the 49-kDa subunit, the inhibitor (quinone) binding site should be located close to the interface between the peripheral and the membrane arm. Subsequently, site-directed mutagenesis of the 49-kDa subunit from the yeast *Yarrowia lipolytica* corroborated these findings. For example, a D458A mutant showed significantly decreased inhibitor sensitivity (Kashani-Poor et al. 2001).

In addition, mutations in the PSST homologue from *Yarrowia lipolytica* (e.g., D136 N, E140Q) slightly decreased the sensitivity to DQA indicating that PSST, the subunit which most probably contains iron-sulfur cluster N2, contributed to the insecticide binding site (Ahlers et al. 2000).

Elegant photoaffinity labeling experiments with reactive insecticide analogues directly identified complex I subunits forming the inhibitor binding site.

Trifluoromethyl-diazirinyl[^{3}H]pyridaben ([^{3}H]TDP) bound with high affinity to mitochondrial electron transport particles and to the bacterial complex I homologue, NDH-1. Covalent labeling of a mitochondrial 23-kDa protein was found to be saturable reaching a maximum at 6 nM. This concentration correlated well with the inhibitory potency (K_i) in enzyme kinetic experiments. Interestingly, labeling was specifically inhibited by structurally diverse insecticides like piericidin A, pyridaben, fenpyroximate, and tebufenpyrad. The 23-kDa protein was purified and identified as PSST by sequence analysis. The data strongly suggested that PSST formed an integral part of the insecticide (inhibitor) binding site (Schuler and Casida 2001a; Schuler and Casida 2001b). Furthermore, the competition results reinforced the perception of a common binding domain for the insecticidal quinoid inhibitors.

In the same study, a second site was labeled by [^{3}H]TDP with lower affinity. Labeling of a 30-kDa protein did not reach saturation at concentrations up to 240 nM. The protein was shown to be ND1, a subunit encoded by the mitochondrial genome which had been affinity-labeled with a reactive rotenone analogue earlier (Earley et al. 1987). ND1-labeling was more or less unaffected by the insecticidal inhibitors (Schuler and Casida 2001b). Interestingly, NADH increased PSST labeling whereas ND1 labeling was reduced, indicating conformational changes of the catalytic core after reduction of N2.

Recently, a second photoaffinity label derived from an insecticidal compound, [^{3}H]trifluoromethyl-phenyldiazirinylfenpyroximate ([^{3}H]TDF), was reported to label a 50-kDa protein subsequently identified as ND5 (Nakamaru-Ogiso et al. 2003). Minor labeling of PSST was also detected. Rotenone, piericidin A, and pyridaben specifically inhibited ND5 labeling. As suggested by the sequence similarity between ND5, its bacterial homologues Nqo12/NuoL, and bacterial Na^+/H^+ antiporters, ND5 may be involved in proton translocation (Steuber 2003; Steuber et al. 2000). In accordance with the evolutionary relationship, [^{3}H]TDF labeling of ND5 was inhibited by two amiloride-type inhibitors of Na^+/H^+ antiporters.

The differential labeling of subunits by photoaffinity probes shows that the binding sites are not strictly identical, though the competition data suggest overlapping sites. In synopsis, the results from direct binding experiments agree well with the notion of a common inhibitor binding domain.

The opposite effect of NADH on binding of [^{3}H]TDP to PSST and ND1, respectively, might reflect conformational changes occurring during the catalytic cycle. This opens up the opportunity to apply high-affinity inhibitors as conformation-sensitive probes to investigate the proton-pumping mechanism in relation to redox catalysis which remains to be the most recalcitrant mechanistic problem of complex I.

In summary, a structural model of the catalytic core consisting of both hydrophilic and transmembrane subunits emerges. Plausibly, it is located close to the interface of the peripheral and the membrane subcomplex. The insecticides described above bind with high affinity to subunits of the catalytic core and block the electron transfer from N2 (PSST) to ubiquinone and the proton translocation, simultaneously. Hopefully, X-ray crystallographic data on complex I will be available in the near future to directly address the structural basis of inhibitor binding.

In either case, high-affinity inhibitors originating from agrochemical research efforts will remain valuable tools to develop our knowledge about the most complex respiratory enzyme.

3 Ubihydroquinone:cytochrome c Oxidoreductase

3.1 Structure and Enzymatic Mechanism

Ubihydroquinone:cytochrome c oxidoreductase (bc_1 complex, complex III) catalyses the electron transfer from reduced ubiquinone to cytochrome c which is coupled to the vectorial translocation of protons. The reaction is summarized as:

$$UQH_2 + 2cyt.c_{ox} + 2H^+_n \rightarrow UQ + 2cyt.c_{red} + 4H^+_p$$

the subscripts n and p denote the protons taken up at the negative side and released at the positive side of the membrane, respectively (Berry et al. 2000).

Mitochondrial bc_1 complexes are homodimers composed of 11 subunits per monomeric complex. Three subunits contain redox-active prosthetic groups and form the catalytic core: the diheme subunit cytochrome b containing the low-potential heme b_L and the high-potential heme b_H, the cytochrome c_1 subunit, and the 2Fe-2S (Rieske) iron-sulfur protein (Fig. 4). This most simple three-subunit assembly is essential and sufficient for electron transport and proton translocation, as it is realized in the bc_1 complex orthologs of bacteria.

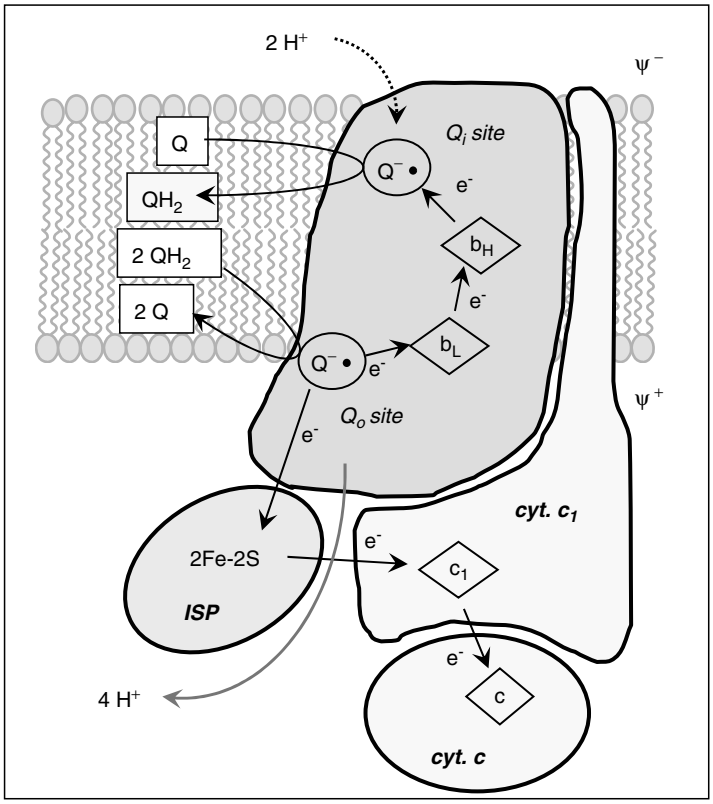

Fig. 4. Schematic representation of the bc_1 complex and the protonmotive Q-cycle. *Q* ubiquinone; QH_2 ubihydroquinone; b_L low-potential cytochrome b; b_H high-potential cytochrome b; *ISP* iron-sulfur protein (Rieske protein), membrane anchor subunits are omitted; The *arrows* denote the flow of electrons according to the protonmotive Q cycle. Electron bifurcation occurs at the Q_o site (see text)

Fortunately, X-ray structures of the bc_1 complexes from chicken, cow, and yeast have greatly enhanced our understanding of the structural requirements underlying the catalytic mechanism (Crofts and Berry 1998; Xia et al. 1997; Hunte 2001). Cytochrome b has eight transmembrane helices arranged in two helical bundles: helices A–E coordinate the two heme prosthetic groups through four conserved histidines. Cytochrome c_1 and the ISP each contain single transmembrane helices anchoring the extrinsic head domains to the membrane. The fundamental mechanism of complex III activity is the protonmotive Q cycle, which can be understood as an extension of Mitchell's original redox loop concept. The flow of two electrons from ubiquinol (UQH_2) is bifurcated at the UQH_2 oxidation site referred to as Q_o (Fig. 4). According to its high midpoint redox potential, the Rieske iron-sulfur protein is the initial acceptor for the first electron, which is then transferred via cytochrome c_1 to

the mobile electron carrier cytochrome c ("high-potential chain") (Hunte et al. 2003; Crofts 2004). Based on the principles of quinone redox chemistry it was proposed that a semiquinone intermediate is formed at Q_o during this process (Rich 2004), though direct experimental evidence is still lacking.

The second electron is transferred to the low-potential heme b_L and further to the high-potential heme b_H center of cytochrome b ("low-potential chain"). The electron is then used to reduce ubiquinone bound at the second quinone binding site, Q_i (quinone reduction site), located close to the matrix face. Since heme b_H transfers only one electron per quinol oxidized at Q_o, full reduction of ubiquinone requires two successive electron transfer steps. Two protons are taken up from the matrix side (Fig. 4).

Several models proposed to explain the electron channeling mechanism at center Q_o have been reviewed recently (Crofts 2004). An unexpected result from the X-ray crystallographic studies was the high degree of mobility of the ISP head group containing the iron-sulfur cluster: ISP was reduced by quinol while oriented towards cytochrome b (b-interface). The reduced ISP then performed a movement of about 25 Å to transmit the electron to cytochrome c_1 (c-interface). Intermediate positions of the iron-sulfur cluster could be distinguished in the structures (Esser et al. 2004). In the context of this discussion, the "moving Rieske" model is important to differentiate the various Q_o site inhibitors mechanistically.

3.2 Complex III Inhibitors with Insecticidal and Acaricidal Activity

High-affinity inhibitors of the bc_1 complex target either the quinol oxidation site, Q_o, or the quinone reduction site, Q_i. Q_i site inhibitors are represented by antimycin A.

Historically, Q_o inhibitors, also referred to as class I inhibitors, were further discriminated based on their effects on cytochrome b absorption spectra and ISP reduction: class Ia inhibitors including the β-methoxyacrylates and myxothiazol blocked electron transport between quinol and the ISP and caused a characteristic red-shift in the α- and β-bands of the reduced heme b_L spectrum. Class Ib inhibitors, represented by the chromone-type inhibitor stigmatellin, were believed to block the reduction of cytochrome c_1 by ISP and also to red-shift the heme b_L spectrum. Finally, class Ic compounds, e.g., the 2-hydroxy quinones, blocked electron transfer similar to stigmatellin, but had no effect on cytochrome b spectra. However, all Q_o inhibitors mentioned above bound in a mutually exclusive fashion suggesting that their binding sites overlapped to some extent.

Insecticides and acaricides known to inhibit complex III belong to the β-methoxyacrylates or the 2-hydroxynaphthoquinones (Fig. 5), known chemical families both acting on the quinol oxidation (Q_o) site.

In addition, the amidinohydrazone insecticide hydramethylnon originally introduced by American Cyanamid (now BASF; Fig. 5, **5**), has been described

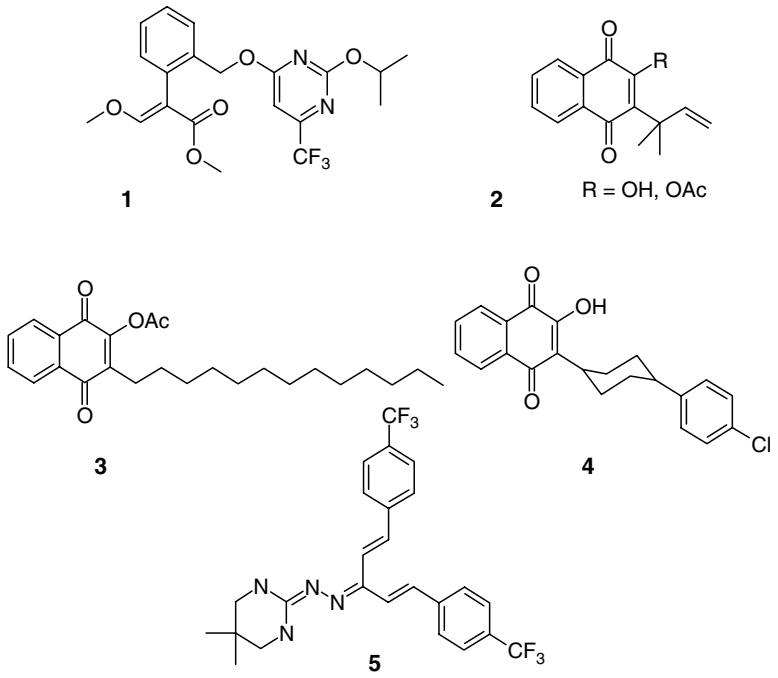

Fig. 5. Inhibitors of complex III (bc_1 complex) with insecticidal/acaricidal activity. **1**, fluacrypyrim; **2**, hydroxynaphthoquinones: BTG504 is the O-acetylated pro-insecticide, R = OAc, BTG505: R = OH; **3**, acequinocyl; **4**, atovaquone; **5**, hydramethylnon

as an inhibitor of complex III. However, its interaction with the quinone binding sites and the inhibitory mechanism were not investigated in detail (Hollingshaus 1987).

3.2.1 β-Methoxyacrylates

Synthetic β-methoxyacrylates (MOA) were developed from the strobilurins, antibiotics produced by certain species of basidiomycetes, into a commercially successful family of fungicides (Sauter et al. 1999; Bartlett et al. 2002). Although the fungicidal β-methoxyacrylates inhibit complex III enzymes from fungal, mammalian, and insect sources, target site selectivity, at least to some extent, between different bc_1 species had been reported (Roehl and Sauter 1994). Fluacrypyrim (Fig. 5, **1**) was the first miticidal methoxyacrylate compound developed by Nippon Soda and registered in Japan (Dekeyser 2005). Given the evolutionary conservation of the bc complexes it is clear that insect and fungal enzymes are inhibited in the same way by β-methoxyacrylates.

As already mentioned, early kinetic analysis revealed that β-MOA compounds inhibited the reduction of the Rieske-ISP by ubiquinol and caused

a red-shift of the dithionite-reduced heme b absorption spectra. Red-shift titrations in the presence of ubihydroquinone analogues indicated that the methoxyacrylates were non-competitive inhibitors with respect to the quinol substrate (Brandt et al. 1988). Remarkably, bc_1 complex depleted in iron-sulfur protein still bound methoxyacrylate inhibitors, suggesting that the ISP was not taking part in binding of class Ia compounds (Brandt et al. 1991). This view was later supported by the X-ray structure of bovine bc_1 in complex with the fungicidal β-methoxyacrylate azoxystrobin (PDB ID: 1SQB). The inhibitor did not restrict the mobility of the ISP catalytic head group thereby excluding tight contacts with the Rieske protein (Esser et al. 2004).

3.2.2 2-Hydroxynaphthoquinones

2-Acetoxy-1,4-naphthoquinones isolated from Chilean plants of the family *Scrophulariaceae* were shown to have biological activity against spider mites and the whitefly, *Bemisia tabaci* (Khambay et al. 1997). Inhibiton of bc_1 from the blowfly (*Calliphora erythrocephala*) and the whitefly was demonstrated to be the mode of action of the 3-hydroxy-naphthoquinone BTG505. Its O-acetylated derivative BTG504 was regarded as a pro-insecticide (Fig. 5, **2**) that was metabolically hydrolyzed to the active hydroxynaphthoquinone (Jewess et al. 2002).

A similar structure, acequinocyl (AKD-2023, Fig. 5, **3**), has been developed as an acaricide. Also in this case, hydrolysis of the acetic acid ester to the corresponding 2-hydroxyquinone was essential for respiration inhibition in housefly (*Musca domestica*) mitochondria. The bc_1 was concluded to be the site of action since ascorbate/N,N,N′,N′-tetramethyl-p-phenylendiamine (TMPD), but not succinate, restored oxygen consumption of the inhibited mitochondria (Kinoshita et al. 1999).

2-hydroxy-1,4-naphthoquinones represent a well-established bc_1 inhibitor class: more than 50 years ago, Ball et al. localized their site of respiration inhibition between cytochromes b and c (Ball et al. 1947). Later, 3-alkyl-2-hydroxy-1,4-naphthoquinones were used to treat infections with unicellular parasites like *Plasmodium* (malaria). Inhibition of complex III was described as the antiprotozoal mode of action (Fry and Pudney 1992).

The molecular mechanism of quinoid Q_o inhibitors was characterized by X-ray crystallography. The structures of the hydroxyquinone 5-n-heptyl-6-hydroxy-4,7-dioxobenzothiazole (HHDBT) bound to yeast bc_1 (Palsdottir et al. 2003), and the chromone compound stigmatellin bound to bovine complex III (Esser et al. 2004) revealed that the Rieske protein directly interacted with the inhibitors in both cases: hydrogen bonds were formed between oxygen atoms of the planar head groups and the protonated His161 (bovine nomenclature) of ISP. Consequently, ISP was fixed in the b position and its re-oxidation by cytochrome c_1 was prevented.

Recently, binding of the antimalarial 2-hydroxynaphthoquinone, atovaquone (Fig. 5, **4**), to the yeast bc_1 complex was analysed by enzyme kinetics and molecular modeling (Kessl et al. 2003). Atovaquone inhibited the enzyme competi-

tively with respect to quinol. The calculated, energy-minimized conformation of atovaquone resembled the stigmatellin conformation as detected in the yeast structure (Protein Data Bank entry code: 1EZV). It was then successfully docked into the Q_o site. The model predicted a hydrogen bond between the 2-hydroxy group of the inhibitor and a histidine residue of the Rieske protein that stabilized it in the b-position and thus prevented ISP movement.

As discussed for the methoxyacrylates, the binding site and the inhibitory mechanism of the hydroxyquinone-type bc_1 insecticides can be reasonably explained on the basis of the available structural information from vertebrate and yeast enzymes since bc_1 complexes are highly conserved.

4 The Respiratory Chain as Insecticide Target Site–Summary and Outlook

Several insecticides and acaricides have been found to effectively inhibit electron transport complexes of the mitochondrial respiratory chain. Concerning complex III, we now have a rather detailed picture how these compounds interfere with catalytic activity. Though much less is known about complex I, insights into the binding site and the inhibitory mechanism of quinoid inhibitors are continuously developing.

Noteworthy, all these compounds target ubiquinone/ubiquinol binding sites which may indicate privileged structural as well as physico-chemical properties of Q sites that make them particularly suitable for inhibitor design (favorable "druggability", Rich and Fisher 1999; Rich 1996).

One key feature of Q sites is the rather low affinity for the ubiquinone substrate which can be easily displaced by inhibitors (Rich and Fisher 1999).

The structural variety of known quinoid inhibitors suggests that they may bind in different spatial orientations and interact with different amino acid residues in the binding domains.

A drawback is seem in the evolutionary conservation of respiratory chain enzymes, so that toxicological problems of electron transport inhibitors are believed to be almost inevitable. However, with new Q site inhibitor structures occurring regularly in the literature, we should not a priori exclude the possibility of more selective (less toxic) compounds to be discovered. It is also conceivable that by exploiting the growing structural information, one may be able to rationally design less toxic electron transport inhibitors with insecticidal activity.

Note added in proof

At the 11th IUPAC Congress of Pesticide Chemistry, August 06–11, 2006, Kobe, Japan, the new acaricide cyenopyrafen, a compound from the acrylonitrile class of chemistry, was shown to inhibit complex II (SQR) at the same binding

site as the well established inhibitior, carboxin [Nakahira K, Takii S, Murakami H (2006) Mode of action of a novel miticide cyenopyrafen (ISO poposed, NC-512) – inhibitory characteristics of complex II of the respiratory chain. 11th IUPAC Congr Pestic Chem, Poster II-1-i-23B].

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9 Inhibition of Programmed Cell Death by Baculoviruses: Potential in Pest-Management Strategies

Rian Schwarz, Shannon Escasa, Basil Arif

1 Introduction

Programmed cell death, also called apoptosis, plays a key role in all organisms and ensures that embryonic development proceeds correctly, where unwanted tissues are removed or remodeled. For example, during mammalian embryo development, the digits are connected by webbing, but at the appropriate time, the webbing is removed via apoptosis revealing the individual fingers. Apoptosis also plays a critical role during viral infection, since cells infected by a virus may commit suicide rather than allowing the virus to spread to neighboring cells. Other classical examples of the important role of apoptosis involve the elimination of auto reactive and neoplastic cells (Haunstetter and Izumo 1998).

While apoptosis has been extensively studied with respect to human health, it is also clear that apoptosis plays a key role in the development of less complex organisms such as nematodes and insects. With respect to insects, the manipulation of apoptosis could be useful in pest control. For instance, tissue homeostasis, organ size, and tissue re-modeling are controlled by apoptosis to ensure the development of a healthy adult. If apoptosis is blocked, the insect will not develop correctly, offering an opportunity to control pest infestation. In addition, apoptosis triggered by virus infections could also be targeted. In this aspect, baculoviruses are of particular interest since they primarily infect insects, and their interference with apoptosis has been well documented. Baculoviruses offer an opportunity to develop environmentally benign insecticides. Some have also been genetically modified with the expression of toxins and hormones and have shown to be potentially effective as pest control agents (Bonning and Hammock 1996; Inceoglu et al. 2001).

For the sake of convenience, the process of apoptosis is often broken down into different stages. The activation stage is the phase that indicates that an event has taken place that may require the cell to undergo apoptosis. This signal can be external, such as the activation of a cellular surface receptor. The activation of the Toll pathway has previously been shown to be important for defense against bacterial and fungal infections (Medzhitov and Janeway 1997; Akira et al. 2001; Bischoff et al. 2004; Hoffman 2003; Zambon et al. 2005) and have also more recently been identified to play a role in protection against viral infection in Drosophila (Zambon et al. 2005). The activation of such pathways ultimately leads to transcriptional changes in the cell, allowing for the preparation and

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defense against invading microorganisms. Alternatively, the pro-apoptotic signal can be initiated internally in the cell, (e.g., by viral replication). Activation of these pathways can ultimately lead to the initiation of the execution phase of apoptosis, leading to caspase activation and cellular death. However, the ultimate decision of life or death at the cellular level is a result of the integration of many different signals. In particular, the outcome in the case of a viral infection will depend on the interplay between viral molecular mechanisms preventing apoptosis and cellular molecular mechanisms inducing apoptosis.

One of the major limitations of using viruses as insecticides is the ability of the insect cells to block virus replication by triggering apoptosis in the infected cells. Consequently, production of virus progeny is reduced, along with its spread to other cells and tissues. Interestingly, in response to such cellular defense mechanisms, baculoviruses have developed several strategies to block apoptosis in insect cells, promoting the replication of the virus and the spread of the infection. Figure 1 schematically illustrates the relationship between baculoviral infection and the host insect response. By understanding these viral anti-apoptotic strategies at a cellular level, it may be possible to manipulate and enhance the ability of the virus in terms of speed of kill and dissemination, allowing better control of the pest infestation. Of particular significance to baculovirus infections are two groups of anti-apoptotic proteins, P35/P49 and the inhibitors of apoptosis (IAPs) (Clem 2005), which are described in more detail later in this review. By understanding the biochemical and molecular mechanisms that regulate apoptosis in insect cells, unique avenues for pest control may be revealed.

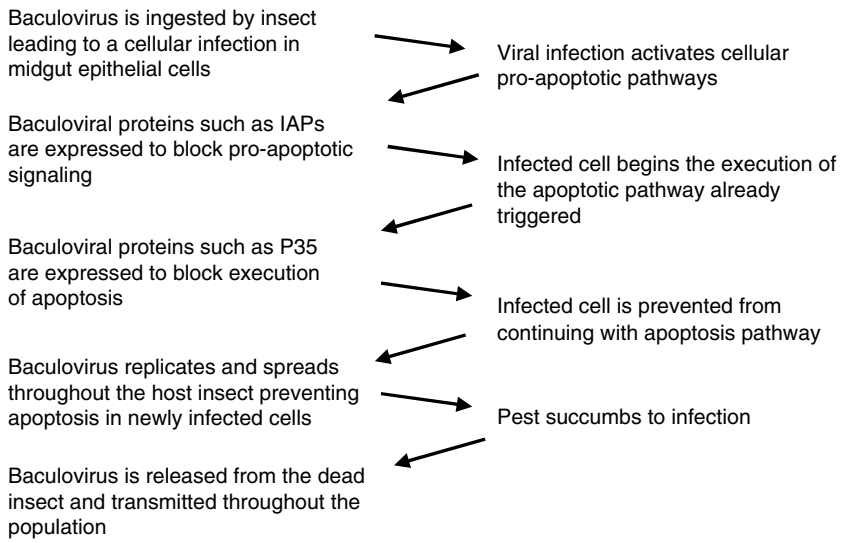

Fig. 1. Schematic diagram outlining the interaction between baculoviral infection and target pest cells. See text for details

The understanding of apoptotic pathways and their role in development and protection against toxic agents has grown immensely over the last decade. Fortunately for entomologists, many of the studies have to a large extent been based on the fruit fly, *Drosophila melanogaster,* and the nematode *Caenorhabditis elegans* (Kornbluth and White 2005). These invertebrate models provide a system of intermediate complexity, while allowing for powerful genetic studies by the generation of mutant lines that either remove a gene function or overexpress particular genes. In particular, cellular apoptotic regulators, which are enzymes responsible for the various phases of apoptosis (either pro- or anti-apoptotic in their activity), have been shown to have homologues in many animal species, such as mammals and *Drosophila*. For example, cellular apoptotic regulators such as IAPs, caspases and Bcl-2 family members are evolutionary conserved, thus results from these models can be applied to other organisms (Kornbluth and White 2005). In particular, some of the genes involved in apoptosis in *Drosophila* have also been identified in Lepidoptera. For example, an effector caspase related to DrICE has been identified in *S. frugiperda* (Ahmad et al. 1997; Seshagiri and Miller 1997) and several of the anti-apoptotic proteins such as DIAP1 in *Drosophila* have also been found to have homologues in *S. frugiperda* (Huang et al. 2001), *Trichoplusia ni* (Liao et al. 2002; Seshagiri and Miller 1997) and *Bombyx mori* (Huang et al. 2001).

Insects rely primarily on an innate immune system to protect from invading microorganisms, including viruses. However, our understanding of antiviral immune defenses in insects is far less advanced than the state of our knowledge on antifungal and antibacterial defenses. Recent studies in *Drosophila* indicate that survival of infection by the *Drosophila* C-virus requires a functional Jak-STAT and Toll signaling pathway in the fly (Dostert et al. 2005). These types of signaling pathways connect an apoptotic signal, such as cytokines or the binding of a viral particle to transcriptional responses of the cell. Indeed, at the level of the insect tissue, it appears that hemocytes have the ability to encapsulate and thereby limit viral infection (Franc 2002). In addition, a humoral mechanism of immune defense appears to be operating in insects. In this type of defense, bacteria and fungi are the main targets for antimicrobial lectins and peptides produced by the insect. These peptides bind to the foreign particle and either damage the membranes or target them for phagocytosis (Hoffmann et al. 2001). Lectins are carbohydrate-binding proteins that bind carbohydrate agglutinate cells (such as viruses and bacteria), or precipitate polysaccharides or glycoproteins (Brasseri 2002). They play a major role in nonself recognition, each with different carbohydrate-binding specificities (Wilson et al. 1999). Therefore, lectins with distinct sugar specificities are involved in recognition and protective roles in immune defense against microbial pathogens (Basseri 2002).

Once a cell is infected by a virus, it undergoes apoptosis to limit production of progeny and block the spread to other cells (Clem 2005). In this process, apoptotic bodies are formed that contain both cellular and viral

particles. The apoptotic bodies are surrounded by a membrane, preventing the release or spread from the initially infected cell as well as cellular components. In vivo, these apoptotic bodies are recognized by the hemocytes, ingested and removed from the system (Franc 2002). Apoptosis can also be observed as the cells undergo morphological and biochemical changes as well as molecular changes. These changes include plasma membrane blebbing, cell shrinkage, chromatin condensation, as well as the formation of apoptotic bodies. Two irreversible biochemical changes important to the onset of programmed cell death include: transglutaminase-mediated protein cross-linking and chromatin fragmentation (Schwartzman and Cidlowski 1993). One of the major molecular changes used to diagnose apoptosis is DNA fragmentation. However, it has been shown that caspase-3 is required for certain biochemical and morphological changes during apoptosis. For example, apoptosis in caspase-3-deficient mice may occur without DNA fragmentation (Janicke et al. 1998). In the final stages of cell death, cell remnants are engulfed by hemocytes, which express receptors capable of recognizing apoptotic surface markers. These receptors include the CD36 homologue of *Drosophila,* Croquemort (Franc et al. 1996, 1999) and Draper, a homologue of *C. elegans* CED-1 (Freeman et al. 2003). While these receptors have been well characterized during embryonic development, their role in clearing virally infected cells undergoing apoptosis remains speculative.

2 The Baculoviruses

Baculoviruses are a large family of invertebrate viruses with circular, supercoiled, double-stranded DNA genomes (Wood and Granados 1991). They are pathogenic to arthropods, particularly Lepidoptera, but have also been isolated from Diptera and Hymenoptera. Members of the *Baculoviridae* are currently divided into two genera: the nucleopolyhedroviruses (NPVs) and the granuloviruses (GVs) (Miller 1997). Nucleopolyhedroviruses contain many virions occluded in a protein that forms relatively large crystalline occlusion bodies (OBs). Granuloviruses contain a single virion in a small granular occlusion body (Miller 1997).

One of the earliest accounts of the benefits of baculoviruses took place in North America during the 1930s for protection of pine trees against the European spruce sawfly by using *Neodiprion hercyniae* nucleopolyhedrovirus (Bird 1953). However, the greatest success of baculoviruses as control agents was in Brazil against the velvet bean caterpillar infesting soybean plantations. One million hectares of soybean plants are protected annually with *Anticarsia gemmatalis* NPV (Moscardi 1999). Generally, baculoviruses have a limited host range and a slow killing rate, which has limited their use on wider scale. Therefore, strategies are being investigated to decrease these limitations for more effective results. One strategy to increase the efficiency of the virus is through genetic modification of the virus (Moscardi 1999).

There are certain limitations associated with the use of baculoviruses in pest control. They are generally slow acting and take a long time to kill the insect. Depending on temperature, dose, host, and virus species, in some insects there may be a time delay of a few days to several weeks between the time of ingestion of viral occlusion bodies and the time of death. During this time, the insect continues to feed and the damage to trees or crops by defoliation may be substantial (Feng et al. 2001). A second limitation is the strength of the virus itself. Viruses have evolved with the natural larval host and the tendency of this evolution is to produce a less effective viral pathogen. Therefore, virulence may be too low to cause a population decrease in the pests (Feng et al. 2001). Certain strategies have been developed to increase the efficacy of baculoviruses as biological pest control agents. The genetic modification of baculoviruses is one such strategy. Examples of genetic modification include: the expression of insect hormones, the expression of insect-selective toxins, and the deletion of endogenous baculovirus genes such as that encoding the ecdysteroid UDP-glucosyl transferase (O'Reilly and Miller 1989).

The first example of genetic modification in baculoviruses to enhance effectiveness against larval pests was the expression of insect hormones. If the hormones that regulate larval growth and molting are disrupted or interfered with, the insect's life cycle will be affected in a deleterious manner. For example, the functionally active diuretic hormone (DH) of *Manduca sexta* (the tobacco horn worm) was expressed in silkworm larvae by using a recombinant *Bombyx mori* nucleopolyhedrovirus (BmDH5). BmDH5 infection killed the larvae 20% faster than the wild-type BmNPV (Inceoglu et al. 2001). A second example is based on the juvenile hormone esterase (JHE), which is very important in the maintenance of the larval stage during metamorphosis, yolk synthesis, and in transport during ovarian development in adults (Feng et al. 2001). A modified JHE gene expressed in a recombinant AcMNPV virus significantly reduced the time of kill and damage caused by longer feeding times, compared to the wild type virus (Feng et al. 2001).

The expression of insect-selective toxins has proven to be efficient in pest control. An insect-specific toxin from the scorpion *Androctonus australis* (AaIT), expressed in baculoviruses has been promising to date. The neuronal sodium channel of larvae infected with AaIT is severely affected as the larvae stop feeding and display arching of the body (Bonning and Hammock 1996). Virulence is increased and time of death of the larvae has been decreased 25–40% compared to the wild-type. A second example of an effective insect toxin is TxP1, a toxin from *Pyemotes tritici* (straw itch mite). The mite can immobilize an insect 150,000 times larger than itself. Recombinant baculoviruses that express TxP1 killed the larvae 40% faster than the wild-type virus (Bonning and Hammock 1996). These, among other examples, illustrate the fact that the effectiveness of baculoviruses can be significantly enhanced by increasing virulence, decreasing time of death of the insect pests, and reducing damage to crops and forests.

A third method of increasing the efficiency of baculoviruses in pest control is the deletion of an endogenous gene. O'Reilly and Miller (1989) completed a study where an endogenous gene encoding the ecdysteroid UDP-glucosyltransferase (*egt*) was deleted from the genome of AcMNPV. This gene product is responsible for regulating the endocrine system of the host by conjugating ecdysone, the moulting hormone with sugars (O'Reilly and Miller 1989). In the wild-type virus, *egt* prolongs the larval stage of the insect, thus allowing it to forage for increased periods of time (Bonning and Hammock 1996). The deletion of *egt* from AcMNPV results in a 30% faster speed of kill and a 40% reduction in foliage feeding than the wild-type virus (O'Reilly and Miller 1989). Interruption in the regular development of the insect, such as gene deletion, gives the virus an advantage (Wood and Granados 1991). Many other genes, particularly those relating to apoptosis, may be used to genetically enhance baculoviruses to become more efficient and virulent pest control agents.

Baculoviruses carry genes that influence the apoptotic ability of their hosts (Miller and White 1998). Baculovirus infection triggers apoptosis in cells and tissues of a variety of hosts. Their ability to block apoptosis is a factor that helps define their host ranges because apoptosis functions as a host defense against virus infection (Thiem and Chejanovsky 2004). By controlling apoptosis, viruses can increase their influence over their developmental cycle. Inhibition of caspases allows greater viral production by an infected cell. Also, by blocking inflammatory caspases, viruses avoid immune response and counter-attack of the host (Cassens et al. 2003).

3 Molecular Basis of Apoptosis

In this section, three groups of proteins will be discussed as shown below:

Caspases:	Function to execute apoptosis and are found as cellular proteins.
IAPs:	Function to block apoptosis and are found both as viral and cellular proteins, however this review focuses on viral IAPs
P35/P49:	Function to block apoptosis and are found as viral proteins.

3.1 Caspases

The result of any successful pro-apoptotic signaling in the cell is the activation of caspases. Caspases are a family of enzymes that cleave their protein substrates following an aspartate residue. Their importance was first documented when CED-3 was found to play a key role in apoptosis in *C. elegans* (Yuan et al. 1993). Caspases can be divided into two groups, initiator caspases and effector caspases. Initiator caspases, such as Dronc in *Drosophila*, contain a large prodomain (> 90 amino acids) and are auto activated under apoptotic

conditions. The effector caspases, such as DrICE in *Drosophila*, are activated by initiator caspases and involve an internal cleavage to separate the large and the small subunits. Effector caspases have smaller prodomains of 20 to 30 amino acids. Following proteolytic cleavage, the active caspase (a tetramer containing two large and two small subunits) executes apoptosis by cleaving a large number of cellular proteins (Danial and Korsmeyer 2004). Proteins such as Dronc are ubiquitously expressed throughout development in insects and are controlled by the insect hormone ecdysone, which stimulates Dronc during metamorphosis (Dorstyn et al. 1999). This illustrates the importance of apoptosis in insect development. In total, there are seven identified genes encoding caspases in the *Drosophila* genome; Strica, DrICE, Decay, Damm, Dredd, Dcp-1 and Dronc (Dorstyn et al. 1999; Fraser and Evan 1997; Harvey et al. 2001; Song et al. 1997).

To date, Dronc is the best characterized of all the *Drosophila* caspases. Dronc has a caspase recruitment domain (CARD) at the N-terminus, which serves as a docking site for adaptor proteins. This domain promotes oligomerization and activation of bound caspases, and is usually referred to as the apoptosome (Zou et al. 1999). It has been shown that a protein in the apoptosome, dAPAF-1, is a necessary component for the activation of Dronc (Kanuka et al. 1999). It is thought that Dronc auto processes itself by cleaving at a glutamate residue, a process requiring dAPAF-1. While caspases in general are known to cleave after aspartate residues, the ability to cleave after a glutamate residue may be unique to Dronc (Muro et al. 2004). Inhibition of Dronc during fly development by RNA interference (RNAi) (Quinn et al. 2000) or by the genetic engineering of Dronc-null flies (Chew et al. 2004) indicates that Dronc is important for a significant amount of cell death. In the Dronc-null flies, stress induced apoptosis is almost completely abolished while developmental apoptosis is blocked only in certain tissues (Chew et al. 2004; Daish et al. 2004). This indicates that while Dronc plays a key role in regulating apoptosis in *Drosophila*, other mechanisms independent of Dronc must also be of importance.

The actions of Dredd, another *Drosophila* initiator caspase, are involved in the innate immune response (Georgel et al. 2001). In particular, it appears to be important for the activation of the NF-êB-like transcription factor Relish (Elrod-Erickson et al. 2000; Stoven et al. 2003). In mammals, this family of transcription factor is important in the activation of many anti-inflammatory genes, and supposedly acts in a similar manner in *Drosophila*. It is interesting to note that while most caspases are not transcriptionally regulated by cell stress, Dronc appears to be upregulated in response to pro-apoptotic signaling (Chen et al. 1998).

3.2 IAPs

A different group of proteins called inhibitors of apoptosis (IAPs), act by inhibiting both initiator and effector caspases (Deveraux et al. 1999; Seshagiri and Miller 1997). IAPs were first discovered in a granulovirus infecting the codling moth, *Cydia pomonella* (CpGV) as a molecule able to rescue the

annihilator strain of virus (see below)(Crook et al. 1993), as well as for protection against other pro-apoptotic signals (Birnbaum et al. 1994; Manji et al. 1997; Vucic et al. 1997). Although IAPs were first discovered as viral apoptosis inhibitors, they have also been found in almost all cell types, illustrating the universal importance of apoptosis regulation.

Strategies to block cellular apoptosis are present in many viruses other than baculoviruses. However, for entomologists the IAPs found in baculoviruses are of particular importance. IAPs are found in all baculovirus genomes sequenced to date and can be grouped into five members from IAP-1 to IAP-5, based on protein homology (Ikeda et al. 2004) (Table 1). Studies on IAPs have revealed that IAP-3 is found in both NPVs and GVs, while IAP-5 is found only in GVs (Ikeda et al. 2004). However, IAPs are more limited than P35 with respect to cell type and the nature of the apoptosis stimulus (Miller 1998). The functional unit of IAPs is the baculoviral IAP repeat (BIR) which consists of 80 amino acids folded around a zinc atom, and a C-terminal RING domain. The BIR domains bind the caspases and inhibit their activation by sequestering the caspase away from its substrates (Tenev et al. 2005; Zachariou et al. 2003). The RING domain encodes an ubiquitin ligase (Yang and Li, 2000) which transfers ubiquitin to target proteins to signal degradation by the 26S proteasome pathway (Joazeiro and Weissman 2000). However, not all IAPs discovered have anti-apoptotic functions (Bideshi et al. 1999; Clem and Miller 1994; Maguire et al. 2000). It is possible that these IAPs function in other cellular processes, such as removal of unwanted proteins by the ubiquitin-proteasomal pathway.

In *Drosophila*, the pro-apoptotic proteins Sickle, Reaper, Grim and Hid contain a short IAP binding motif (IBM) at their processed N-terminus, which allow them to bind IAPs (Chai et al. 2003; Yoo et al. 2002). By binding the IAPs at their BIR domain, the IBM proteins can sequester the IAPs from the caspases and allow for activation of the caspase cascade, thus allowing apoptosis to occur (Chai et al. 2003; Yoo et al. 2002). The IBM proteins are under tight transcriptional control regulation during *Drosophila* development. For example, Reaper is expressed only in cells destined to undergo apoptosis, not in cells that survive through development (White et al. 1994). The importance of the IAP proteins is also illustrated by studying the effect of knocking out DIAP1. In these cells, apoptosis proceeds unchecked (Goyal 2000; Yoo et al. 2002). This also suggests that the caspases in flies are in a basal-activated state ready to initiate apoptosis, and are blocked by binding to DIAP1. However, in studies where both DIAP1 and dAPAF-1 are absent, apoptosis can be limited, indicating that although the caspases are in a "ready to go state", the presence of dAPAF-1 is still required for full activation (Rodriguez et al. 2002).

3.3 P35

Apoptosis was first shown as an anti-viral mechanism in insects during infection by the baculovirus *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) (Clem et al. 1991). During AcMNPV infection, a viral protein P35

Table 1. Apoptotic inhibitors found in both granuloviruses (GVs) and nucleopolyhedroviruses (NPVs). Each apoptotic inhibitor is cross-referenced to its GenBank number as shown in the table. If more than one IAP is given, their GenBank numbers are separated by a '/' where only the last three digits differ

	Baculovirus	Abbreviation	Apoptosis inhibitor			
			IAPs	GenBank #	P35/P49	GenBank #
GVs	*Choristoneura occidentalis* GV	ChocGV	IAP-3, 5		P35/P49	
	Cydia pomonella GV	CpGV	IAP-3, 5 & IAP-3/1	NP_148801/878/900		
	Cryptophlebia leucotreta GV	CrleGV	IAP, IAP-3, 5	NP_891932/863/953		
	Adoxophyes orana GV	AdorGV	IAP-3, 5	NP_872543/553		
	Phthorimaea operculella GV	PhopGV	IAP-3/1,5	NP_663251/273		
	Plutella *xylostella* GV	PIxyGV	IAP-3	NP_068316		
	Xestia *c-nigrum* GV	XecnGV	IAP-3	NP_059285		
	Agrotis segetum GV	AgseGV	IAP-3/1, 5	YP_006291/238		
NPVs	*Autographa califomica* NPV	AcMNPV	IAP-1, 2	NP_054056/101	P35	NP_054165
	Bombyx mori NPV	BmNPV	IAP-1, 2	NP_047432/474	P35	NP_047533
	Lymantria dispar NPV	LdMNPV	IAP-2, 3	NP_047716/776		
	Choristoneura fumiferana NPV	CfMNPV	IAP-1, 2, 3	NP_848348/377/342		
	Choristoneura fumiferana defective NPV	CfDEFNPV	IAP-1, 2, 3, 4	NP_932645/675/639/796		
	Rachiplusia ou NPV	RoMNPV	IAP-1, 2	NP_703017/061	P35	NP_703122
	Neodiprion lecontei NPV	NeleNPV	IAP-3	YP_025208		
	Neodiprion sertifer NPV	NeseNPV	IAP-3	YP_025124		
	Spodoptera littoralis NPV	SpliNPV	IAP-3/2	AAO16211	P49	AJ006751

(*Continued*)

Table 1. Apoptotic inhibitors found in both granuloviruses (GVs) and nucleopolyhedroviruses (NPVs). Each apoptotic inhibitor is cross-referenced to its GenBank number as shown in the table. If more than one IAP is given, their GenBank numbers are separated by a '/' where only the last three digits differ—(Cont'd)

Baculovirus	Abbreviation	Apoptosis inhibitor			
		IAPs	GenBank #	P35/P49	GenBank #
Spodoptera litura NPV	SpltNPV	IAP-3/2	NP_258332	P49	NP_258323
Orgyia pseudotsugata NPV	OpMNPV	IAP-1, 2, 3, 4	NP_046197/230/191/262		
Helicoverpa armigera NPV C1	HearNPV C1	IAP-2, 3	NP_075131/172		
Helicoverpa armigera NPV G4	HearNPV G4	IAP-2, 3	NP_075131/172		
Mamestra configurata NPV A	MacoNPV A	IAP-2, 3	NP_613193/222		
Mamestra configurata NPV B	MacoNPV B	IAP-2, 3	NP_689283/312		
Epiphyas postvittana NPV	EppoNPV	IAP-1, 2, 3, 4	NP_203202/232/195/261		
Spodoptera exigua NPV	SeNPV	IAP-2, 3	NP_037848/870		
Adoxophyes honmai NPV	AdhoNPV	IAP-2, 3, 4	NP_818700/735/684		

is expressed early to prevent the host cell from undergoing apoptosis, allowing for viral replication and the spread of the virus to neighboring cells. P35 is a 35-kDa protein that has also been identified in other viruses such as BmNPV (Table 1). The protein blocks virus induced apoptosis by directly inhibiting the effector caspases (Clarke and Clem 2003; Fisher et al. 1999). The structure of P35 has a large loop domain called the reactive site loop (RSL), which is located above a central *B* sheet and contains the caspase recognition motif ^{84}DQMD^{87} (Pei et al. 2002).

In some studies, a spontaneous mutant form of the AcMNPV (which lacks a functional *p35* gene), called the annihilator (vAcAnh), has often been used (Clem 2005). It has been found that the mutant virus was unable to block apoptosis initiated by the cells, preventing a full-blown infection by the virus (Clem and Miller 1993; Hershberger et al. 1992). In numerous studies, P35 has proven to be a very strong cell-specific inhibitor of apoptosis. For example, while vAcAnh is unable to successfully replicate in *Spodoptera frugiperda* (SF21) cells it can in *Trichoplusia ni* (TN368) cells (Clem *et al.* 1991). Furthermore, the ability of the vAcAnh virus to replicate was restored when co-transfected with the wild-type virus expressing P35 in SF21 cells (Hershberger et al. 1994). It is also known that P35 is expressed early in viral infection and accumulates as viral replication progresses (Hershberger et al. 1994). Furthermore, expressing P35 under a strong but late promoter such as that of polyhedrin failed to prevent apoptosis clearly suggesting that early expression of the protein was essential in preventing programmed cell death (Hershberger et al. 1994). The budded viruses of both AcMNPV and vAcAnh have been used to study infection in *S. frugiperda* larvae. It was found that a 1,000-fold dose increase of the vAcAnh was required to reach a lethal dose of 50% (LD50) compared to the wild-type AcMNPV (Clem and Miller 1994). This is consistent with an important role for P35 not only in a cultured cell model, but also in the context of an organism. Further studies revealed that P35 is able to prevent cell death brought on by many other apoptotic inducers. For example, Actinomycin D, a strong transcriptional inhibitor rapidly induces apoptosis in wild-type SF21 cells. However, apoptosis was prevented in a mutant SF21 cell line, which stably expresses viral P35 (Cartier et al. 1994; Crook et al. 1993).

Expression of *p35* in mammalian cells was also shown to block apoptosis (Rabizadeh et al. 1993). However, at a genetic level, P35 appears to be unrelated to mammalian anti-apoptosis proteins, such as the mammalian proto-oncogene Bcl-2 (Friesen and Miller 1987). *p35* lacks significant homology to *bcl-2*, yet *bcl-2* acts to effectively prevent apoptosis induced by viral infection (Hinshaw et al. 1994; Levine et al. 1993). Furthermore, BCL2 is known to be associated with an adaptor protein Bax (Yin et al. 1994) in a membrane associated complex, while P35 is primarily found in the cytosol of infected cells (Hershberger et al. 1994). Consistent with a cytoplasmic localization, P35 can be detected in budded virus (Hershberger et al. 1994). When comparing the anti-apoptotic actions of P35 with the IAP family of proteins, it appears

that IAPs, such as one from the *Orgyia pseudotsugata* nucleopolyhedrovirus, are effective in blocking cellular apoptosis, however, they do not inhibit caspase activity in vitro. On the other hand, P35 is an effective inhibitor of cellular apoptosis as well as a stoichiometric inhibitor of in vitro caspase activity (Manji et al. 1997). This suggests that IAPs may act primarily at an early stage in the process of apoptosis, perhaps during the signaling events leading up to the activation of initiator caspases. Furthermore, unlike P35, the IAP proteins may require additional cellular proteins to carry out its function.

More recent studies have confirmed that P35 acts as a substrate for cellular caspases. Once the caspase cleaves P35, a conformational change results and a covalent bond is formed between the two molecules, blocking further activation by the caspase (Eddins et al. 2002; Xu et al. 2001). Structural studies of P35 complexed to human caspase 8 revealed that the inactivation of the caspase is a result of the formation of a covalent thioester bond. This bond between the caspase 8 catalytic Cys-360 residue and the Asp-87 residue is localized in a substrate-like reactive loop of the inhibitor. Once cleavage of the reactive site loop has occurred, P35 undergoes several conformational changes, which result in the insertion of the N-terminal portion into the enzyme's active site cleft. As a result, the catalytic His-317 residue is inaccessible, which prevents hydrolysis of the thioester bond (dela Cruz et al. 2001; Riedl et al. 2001; Xu et al. 2001). It appears that P35 inhibits mainly effector caspases based on its low affinity for Dronc (Meier et al. 2000).

P35 homologues have not been found in the genomes of organisms other than baculoviruses. Recently a homologue was discovered in the *Amsacta moorei* entomopoxvirus (R. Clem, pers. comm.). However, the P49 proteins from *Spodoptera litura* nucleopolyhedrovirus (SpltNPV) and *Spodoptera littoralis* NPV (SpliNPV) appear to act in a similar manner as P35, since it is able to block the actions of Dronc (Jabbour et al. 2002). P49 has a broader selectivity for caspase than P35. It is a 49 kDa protein with a protein structure homologous to P35 (SpliNPV P49 and AcMNPV P35 share a 48.8% aa identity) (Table 1). P49 contains the caspase recognition motif ^{91}TVTD^{94} (Du et al. 1999). It is a strong apoptotic suppressor and a substrate inhibitor of caspase. It appears to block activation of effector caspases at a step upstream from P35 but downstream from IAPs (Yu et al. 2005).

4 Conclusions

Baculoviruses have been shown to be an important tool in controlling pest infestations of economically important crops. However, one of the serious limitations of baculovirus as a pest control technique is the ability of the insect to rid itself of the virus, either by mechanical sloughing off of the epithelial midgut cells, or by insect cells undergoing apoptosis and thereby

preventing further infestation of the virus. Baculoviruses have in turn evolved molecular methods that aim to block the cellular-induced apoptosis. In this review, we focused on the IAPs and the P35/P49 family of viral proteins, which are good inhibitors of apoptosis. By understanding the molecular mechanism of how baculoviruses establish a productive infection in pests, more efficient pesticides can be developed by genetic engineering. For example, the affinity of the viral anti-apoptotic proteins could be increased by adding or removing domains, to create a more efficient block of cellular apoptosis. Furthermore, recombinant baculovirus could be engineered to be expressed at a higher concentration and/or optimal time point for blocking cellular apoptosis. Baculoviruses are an ideal target for such genetic engineering since the host specificity is very high only infecting insects, thus allowing for the development of an efficient environmental benign pesticide.

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10 Plant Natural Products as a Source for Developing Environmentally Acceptable Insecticides

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1 Introduction

Terrestrial plants produce a bewildering array of natural products—terpenoids, phenolics, alkaloids—likely exceeding 100,000 novel chemical structures. Many of these are thought to serve an ecological function for the plants producing them, serving to defend the plants from herbivores and pathogens. Such defensive chemistry is thought to be extremely widespread among the plant kingdom. This is the foundation upon which many scientists have viewed higher plants as a valuable resource that could be exploited for the discovery of new insecticides or for novel structures that could serve as lead compounds in insecticide development.

This rationale is based partly on the assumption that novel structures produced by plants may have novel modes-of-action, and that some or many of these may exhibit selectivity favoring mammals, given that insects are thought to be major agents of natural selection for plants and therefore defensive chemistry is more likely targeted towards insects than towards vertebrate herbivores. Several volumes have been published around the concept of natural insecticides from plants (Arnason et al. 1989; Hedin et al. 1997; Koul and Dhaliwal 2001; Regnault-Roger et al. 2005).

In reality, natural insecticides from plants have proven to be the exception rather than the rule, based on the conventional use of the term "insecticide"—a substance that kills insects. Although hundreds of compounds isolated from plants have demonstrated bioactivity to one or more insects based on bioassays in the laboratory, feeding deterrence or larval growth inhibition is far more commonly seen than death of the insects tested. This may partially explain why only a handful of botanical insecticides have been commercialized in industrial countries—in seeking bioactivity comparable to synthetic insecticides (i.e., acute toxicity to insects), very few plant natural products with satisfactory insecticidal activity have been identified from the thousands of plant extracts screened. In short, plant defensive chemistry has probably evolved to discourage insect (and mammalian) herbivory, rather than to kill the herbivores outright.

In this chapter we consider several plant natural products with insecticidal activity, review their structure–activity relations, and discuss efforts aimed at enhancing their bioactivity (efficacy and/or spectrum of action).

Insecticides Design Using Advanced Technologies (ed. by Isaac Ishaaya, Ralf Nauen and A. Rami Horowitz)

2 From Pyrethrum to Synthetic Pyrethroids

The development of the synthetic pyrethroids, arguably one of the most commercially successful classes of conventional insecticides, is often cited as an example of how a plant natural product can serve as a lead in the development of crop protection agents. However, it must be remembered that the first commercially significant pyrethroids—those used for crop protection in the field (e.g., permethrin and fenvalerate)—were not discovered until 1973. This was almost 25 years after the synthesis of the first synthetic pyrethroid, allethrin (in 1948), and nearly 60 years after the structural elucidation of the natural pyrethrins. What made the synthetic pyrethroids useful for agriculture was their reduced photolability in the presence of sunlight through halide substitution of the parent chrysanthemic acid and replacement of the butenolide ring with a more stable 3-phenoxyphenyl ring system (Fig. 1). As such, the modern pyrethroids bear little resemblance to their natural product progenitor (pyrethrin I), and even the mode-of-action of the later pyrethroid insecticides differs from the original natural product (Perry et al. 1998).

3 Azadirachtin and Related Limonoids from the Meliaceae

No insecticide of plant origin has been subjected to as much scrutiny in the past 20 years as that of neem, derived from the seeds of the Indian neem tree *Azadirachta indica* (Schmutterer 2002; Koul and Wahab 2004). Seeds of *A. indica* produce a suite of closely related limonoid triterpenes, the most important of which is azadirachtin (**1**, Fig. 2). In purity, this substance remains the most potent insect antifeedant discovered to date, at least in bioassays using the desert locust *Schistocerca gregaria* and some species of noctuid caterpillars. Antifeedant effects of azadirachtin (and other antifeedants of plant origin) vary widely in their potency to different insect species; some are behaviorally quite indifferent to it. Of greater value for insect control are the exceptional growth regulatory actions of this compound on most types of insect. Through interference with the insect neuroendocrine system, azadirachtin disrupts moulting and metamorphosis, blocks reproduction, and can cause anorexia in some species.

Azadirachtin was first isolated in 1968 (Butterworth and Morgan 1968), but its structure was not conclusively determined until several years later (Kraus et al. 1987). Rembold (1989) conducted one of the first structure–activity studies of the natural analogues of azadirachtin in neem seeds. Azadirachtin, relatively the most abundant among approximately one dozen closely related analogues, is also the most biologically active in most insect-based bioassays. Two very minor constituents (azadirachtins H and I) were found to be about twice as active as azadirachtin based on the *Epilachna* molting bioassay. Rembold (1989) also defined minimal structure requirements of the azadirachtin molecule for insect growth regulatory activity.

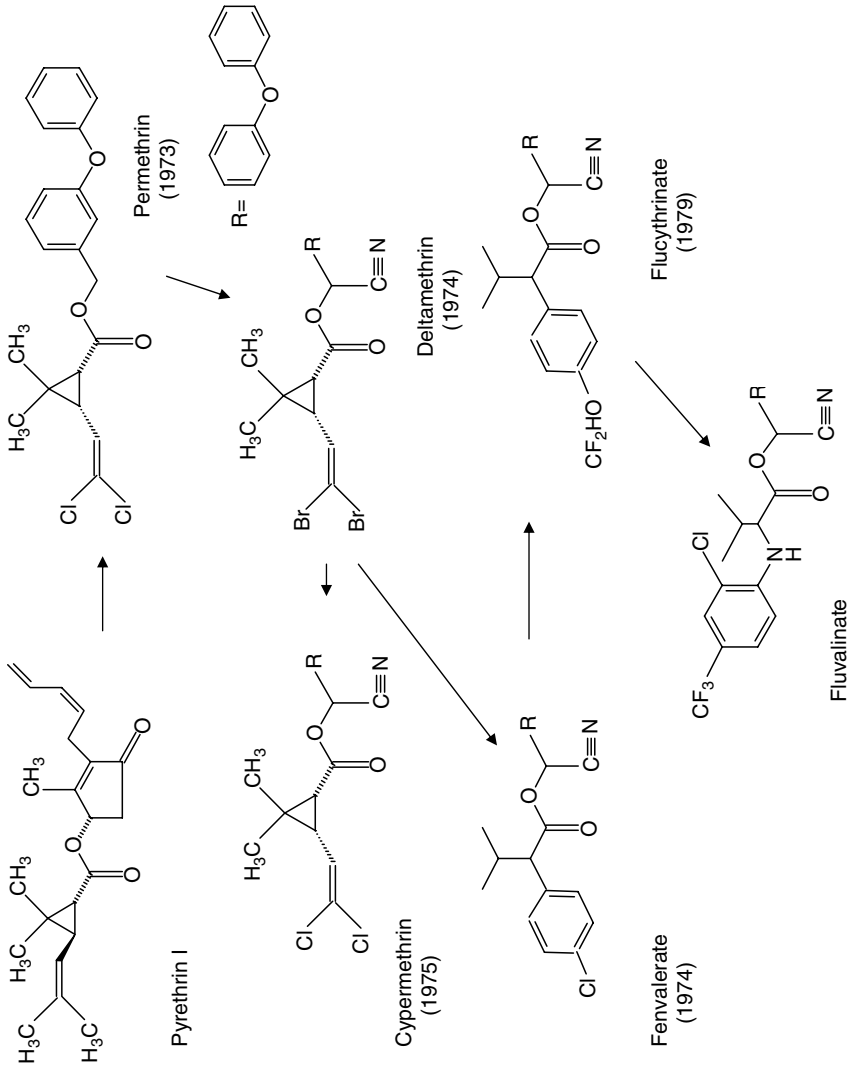

Fig. 1. "Evolution" of synthetic pyrethroids derived from the plant natural product pyrethrin I

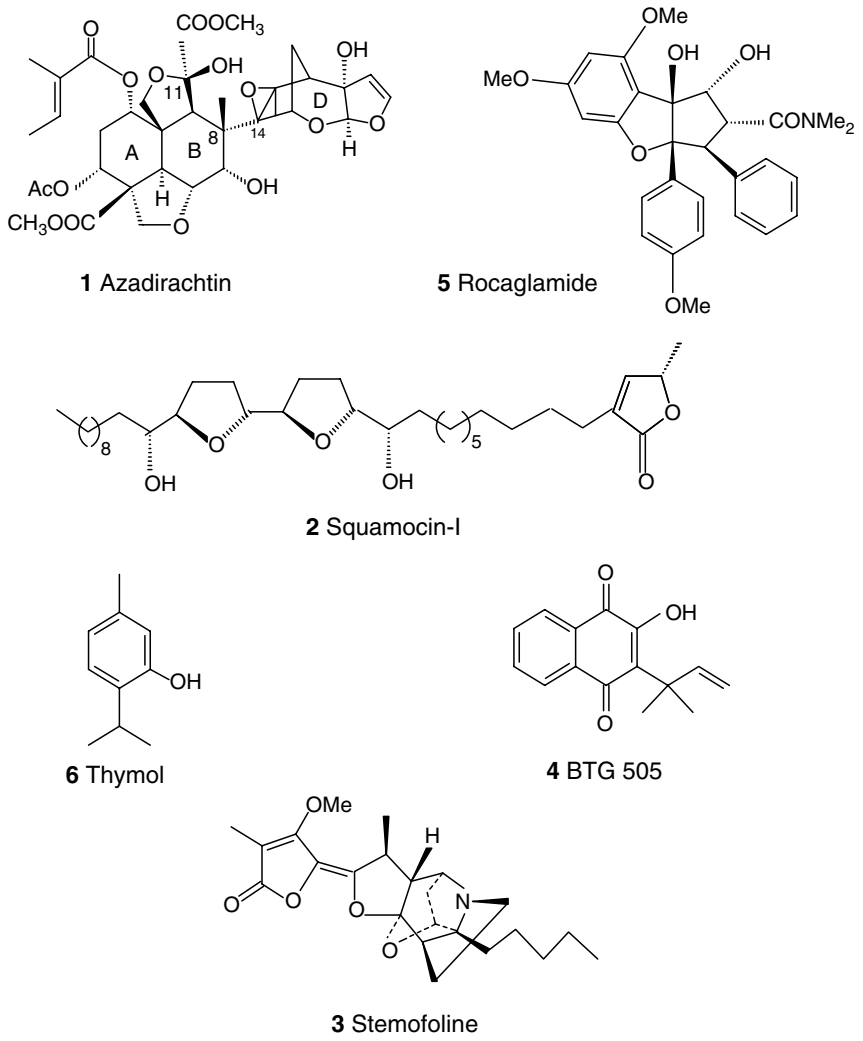

Fig. 2. Some insecticidal plant natural products discussed in this chapter

These observations were extended by Blaney and Simmonds, in collaboration with Ley (Blaney et al. 1990; Simmonds et al. 1995; Ley 1994). Almost 60 analogues, both natural and synthetic derivatives, have been investigated with respect to antifeedant and insecticidal actions on locusts and noctuid larvae. One important conclusion from this body of work is that almost all analogues are less potent than the parent compound azadirachtin, largely confirming Rembold's investigations. Another is that specific binding of azadirachtin analogues to membrane proteins, based on studies with insect cell cultures in vitro, correlates well with observations on insect behavior and

physiology in intact insects (Mordue [Luntz] et al. 1998). Of particular interest have been efforts by Ley and colleagues to synthesize azadirachtin de novo, a formidable undertaking given the large number of chiral centers and overall complexity of the molecule. Their strategy has been to synthesize the substituted decalin ("western") fragment and to synthesize the dihydrofuranacetal ("eastern") fragment, with the intent to link the two fragments through the crucial C8-C14 bond.

Antifeedant bioassay of the separate fragments in the cotton leafworm *Spodoptera littoralis* indicated that the decalin fragment possessed some bioactivity by itself (at 10^{-6} M), and that certain combinations of decalin and furanacetal fragments were synergistic (also at 10^{-6} M). However, in the same insect, azadirachtin is active as an antifeedant at 10^{-15} M; one could argue that compounds active only at concentrations nine orders of magnitude greater are hardly active at all.

To date, the total synthesis of azadirachtin has not been accomplished on any meaningful scale. Alternatives to synthesis include the production of this complex molecule through plant tissue culture, but to this point, extraction from neem seeds is the only commercially viable route to production of neem insecticides. Moreover, the use of neem or azadirachtin from natural sources has yet to achieve its' promise in the marketplace, even 15 years after its commercialization in the United States (Isman 2004). So while azadirachtin has proven to be a fascinating molecule for the study of insect feeding behavior and endocrine physiology, it has not been a fruitful lead for the production of synthetic insecticides to this point.

4 Acetogenins from the Annonaceae

Derivatives from species of the custard apple family have had a number of traditional uses for pest control and as vermifuges (reviewed in McLaughlin et al. 1997). These actions were erroneously attributed to a series of benzylisoquinoline alkaloids isolated from annonaceous plant extracts. Bioassay-driven fractionation employing a simple brine shrimp assay (*Artemia salina*) led to the isolation and elucidation of C-32 or C-34 long-chain fatty acids combined with a 2-propanol unit at C-2 to form an α-lactone, as the active principles (Mikolajczak et al. 1988, 1989). To date, more than 400 structures of this type have been isolated from about 40 species of the Annonaceae, particularly from the bark, fruit, and seeds. Species that have been more intensively examined include the temperate American paw paw (*Asimina triloba*) and the tropical sour sop (*Annona muricata*) and sweet sop (*Annona squamosa*). Each of these species contain complex mixtures of acetogenins comprising at least 30 compounds (e.g., squamocin, **2**, Fig. 2).

McLauglin and colleagues investigated structure-activity relations among 44 acetogenins isolated from *Asimina triloba, A. longifolia, Annona muricata,*

A. bullata, Goniothalamus giganeus and *Rollinia mucosa* (He et al. 1997), using yellow fever mosquito larvae (*Aedes aegypti*). Rotenone, a commercial botanical insecticide from *Derris elliptica* and related tropical legumes, was included as a positive control. The acetogenins, as inhibitors of mitochondrial NADH:ubiquinone oxidoreductase, have the same mechanism of action as rotenone, making the comparison particularly appropriate (Londershausen et al. 1991). Among the 44 acetogenins, two were significantly more toxic to mosquito larvae than rotenone, while a further 28 were statistically equitoxic to rotenone (LC_{50} = 1.2 ppm) (He et al. 1997). Compounds with adjacent bis-tetrahydrofuran rings and three hydroxyl groups were the most active; those with a mono-tetrahydrofuran ring and a single flanking hydroxyl group were the least active.

Although three patents based on the use of these substances for insect control were issued more than 15 years ago, no substantial commercialization has ensued. As mitochondrial poisons toxic to a wide range of organisms, including mammals, the acetogenins are not considered attractive lead molecules for synthetic efforts. As for the azadirachtins, the main use of the acetogenins for insect control will probably continue to be based on crude or partially refined extracts obtained from plant sources (Leatemia and Isman 2004).

5 Alkaloids from Stemonaceae

The family Stemonaceae, the only source of stemona alkaloids (Pilli and Ferreira de Oliviera 2000) is a small monocotyledonous family with three genera comprising about 30 species. *Stemona* is the largest genus with about 25 species occurring as subshrubs or twining herbs, mostly with perennial tuberous roots (Brem et al. 2002, 2004).

The stemona alkaloids include more than 70 different derivatives that have been classified into eight different groups by Ye et al. (1994) according to the sites of connection between the basic ring and side chain. Structurally, stemona alkaloids are characterized by a pyrollo(1,2-α)azepine (5,7 bicyclic AB-ring system) nucleus (Brem et al. 2002) common to all compounds in six of these groups or a pyrido(1,2-α)azepine nucleus (6,7-bicyclic AB-ring system) (Kaltenegger et al. 2003) in the more recently discovered stemoncurtisine group of stemona alkaloids.

Extracts from *Stemona* and *Croomia* have been used for centuries in Chinese and Japanese traditional medicines for the treatment of respiratory diseases and against enteric helminths and ectoparasites on humans and cattle (Xu et al. 1982; Sakata et al. 1978) and as insecticides (Xu, 2000; Brem et al. 2002; Kaltenegger et al. 2003).

Strong biological activity was exhibited by the crude extracts from the roots of *Stemona cochinchinensis* and *S. curtisii* against *Spodoptera littoralis* (Kaltenegger et al. 2003) and may be attributed to the presence of the alkaloid

stemofoline (**3**, Fig. 2). Moreover, crude extracts of *Stemona* species were almost as active as the isolated compounds. Crude extracts from *S. collinsae* displayed the highest insecticidal activity against *S. littorallis*. These were more active than extracts from two very active *Aglaia* species (Meliaceae) or a standard pyrethrum extract. The high activity of *S. collinsae* was attributed to the predominance of didehydrostemofoline accompanied by smaller amounts of stemofoline. In contact toxicity bioassays, the insect-toxic potencies of stemofoline and didehydrostemofoline exceeded even those of the pyrethrum extract. Stemofoline's mode of action is agonism of the insect nicotinic acetylcholine receptor (Godfrey et al. 2002).

Kaltenegger et al. (2003) investigated structure–activity relations of 13 stemona alkaloids based on insecticidal activity against neonates of *S. littoralis*. Modification or absence of the unsaturated lactonic 4-methoxy-3-methyl-2-furanone unit from stemocochinin and parvistemonine led to a strong decrease in toxicity. With regard to the five novel pyridoazepine derivatives, oxystemokerrin with an oxygen bridge between the C-1 and C-8 of the pyridoazepine nucleus displayed the strongest activity. Loss of the open butyl side chain resulted in the reduction of toxicity in pyridostemin. N-oxidation in oxystemokerrin-N-oxide or insertion of a double bond in the azepine ring of stemokerrin decreased activity. However, insertion of a double bond between C-7 and C-8 in dehydroprotostemofoline (pyrroloazepine derivative) increased toxicity. All these examples suggest that modification of the chemical structure of the parent compound leads to decreased biological activity of the analogues.

6 Napthoquinones from the Scrophulariaceae

A 5-year collaborative project led by researchers at IACR-Rothamsted (UK) resulted in the isolation of two active principles from the Chilean plant *Calceolaria andina* (Scrophulariaceae), related to the familiar garden "slipper" plants. These compounds (BTG 504 and BTG 505), identified as napthoquinones, are effective against a range of commercially important pests including the tobacco whitefly, *Bemisia tabaci,* aphids and the two-spotted spider mite, *Tetranychus urticae* (Khambay et al. 1995; 1999). They offer opportunities both as lead structures for analogue synthesis (Khambay et al. 1997a) and as new botanical pesticides (Khambay et al. 1997b) exhibiting low mammalian toxicity unlike other naphthoquinones. The use of the compounds as pesticides has been patented (Khambay et al. 1999) by BTG International Ltd.

Khambay et al. (2003) compared the insecticidal and fungicidal properties of dunnione (a known naphthoquinone) with BTG 505 (**4**, Fig. 2). Although, dunnione showed practically no activity against the house fly *Musca domestica,* the whitefly *B. tabaci*, the beetle *Phaedon cochleariae*, or the spider mite

T. urticae unlike BTG 504 and BTG 505, dunnione has an unusually broad spectrum of antifungal activity. The mode of action of dunnione is primarily through initiation of redox cycling, whereas, BTG 505 acts by inhibiting mitochondrial complex III (Khambay et al. 2003).

The biological activity of 2-hydoxy-3-subsituted-1,4-naphthoquinones was first reported by Fieser et al. (1948) showing that lapachol and hydrolapachol were active against *Plasmodium lophurae*, a malarial parasite of ducks. The recently developed antimalarial drug, atovaquone is a synthetic analogue of lapachol (Olliao and Trigg 1995). Antifungal activity of lapachol obtained from the wood extract of *Tabebuia serratifolia* (Bignoniaceae) was reported by Velasquez et al. (2004). Lapachol obtained from an ethanolic bark extract of *T. serratifolia* was more active (LC_{50} = 20.8 ppm), than the amine derivatives (LC_{50} values ranged from 242.6–899.4 ppm) against the larvae of *Aedes aegypti,* showing the importance of the hydroxyl group at the C-2 position for bioactivity (Oliviera et al. 2002). An outstanding property of these compounds is that they are effective against a range of resistant insect strains including the notorious B-biotype of the tobacco whitefly, *Bemisia tabaci*, which is devastating crops worldwide.

7 Rocaglamides from *Aglaia* (Meliaceae)

The genus *Aglaia* consisting of some 130 species widely distributed in the Indo-Malysian region (Nugroho et al. 1999) has attracted considerable attention in the past decade as a possible source of unique natural products. Phytochemical investigations of *Aglaia* have revealed the presence of a variety of compounds including rocaglamides (Ishibashi et al. 1993; Proksch et al. 2001), aglains (Bacher et al. 1999), bisamides (Brader et al. 1998), triterpenes (Weber et al. 2000) and lignans (Wang et al. 2002) with interesting biological activities.

There are more than 50 naturally occurring rocaglamide derivatives isolated to date (e.g., rocaglamide, **5**, Fig. 2) (Proksch et al. 2001). Rocaglamide derivatives are unusual aromatic compounds featuring a cyclopentatetrahydrobenzofuran skeleton and are strictly confined to members of *Aglaia*. Recently, several novel rocaglamide derivatives, isolated from different *Aglaia* species have been shown to have strong insecticidal activity (in some cases even comparable to azadirachtin), mostly against neonate larvae of *Spodoptera littoralis*, *Ostrinia* species and the gram pod borer, *Helicoverpa armigera* (Brader et al. 1998; Nugroho et al. 1997a, 1997b, 1999; Gussregan et al. 1999; Koul et al. 2004). The insecticidal mode-of-action as well as the potential anit-cancer activity of rocaglamides results from inhibition of protein synthesis, explaining the long time-to-death in treated insects (Satasook et al. 1993).

The insecticidal activity of rocaglamides can be attributed to the presence of the furan ring system since the closely related aglains, possessing a pyran ring, are devoid of insecticidal activity (Nugroho et al. 1999). The nature of

the substituents at C-1, C-2, C-3 and C-8b have also been suggested to be responsible for the bioactivity of the respective derivatives (Nugroho et al. 1997a, 1999; Schneider et al. 2000). Acylation of the OH group (with formic or acetic acid) at C-1 caused a reduction of insecticidal activity in neonate larvae of *S. littoralis* compared with other rocaglamide derivatives with a hydroxyl substituent isolated from the twigs of *A. duppereana* (Nugroho et al. 1997a). The reduction of insecticidal activity in the acetylated derivative indicates the first structure–activity relationship in this group of natural insecticides. There is a decline in insecticidal activity for rocaglamide derivatives featuring an unsubstituted C-2 in contrast with analogues possessing an amide or carboxylic substituent at this position. A similar trend has been noted in other rocaglamide derivatives isolated from *A. odorata* (Nugroho et al. 1999; Gussregan et al. 1997), and *A. elliptica* (Nugroho et al. 1997b). Substitution of a hydroxy group with the methoxy group at C-8b resulted in a complete loss of activity in compounds that were isolated from roots of *A. duperreana* (Chaidir et al. 1999) showing the importance of the OH group at C-8b. The strong bioactivity of rocaglamide derivatives against a number of insect pests suggests that they may serve as lead structures in the development of natural insecticides for plant protection.

8 Monoterpenoids from Plant Essential Oils

Plant essential oils, viz. steam distillates from certain aromatic and/or medicinal herbs or trees, have long been known to possess insecticidal or insect repellent properties, but only recently have some of the natural oils been commercialized for those uses. In broad terms, the monoterpenoids exhibit neurotoxicity to insects and mites on contact, but only at doses substantially greater than those obtained with natural pyrethrins or conventional insecticides. A more detailed examination of their efficacy and modes of action can be found elsewhere in this volume.

Coats and Rice explored structure–activity relations of naturally-occurring monoterpenoids based on topical, fumigant and ovicidal activities using the house fly (*Musca domestica*), red flour beetle (*Tribolium castaneum*) and southern corn rootworm (*Diabrotica undecimpunctata howardi*) as models (Rice and Coats 1994a). Activities varied with skeletal structure (cyclicity), amount of saturation and functional groups, but trends were somewhat inconsistent among the three species tested. In general, ketones tended to be more active than analogous aldehydes that were in turn more effective than the respective alcohols. Phenols were more active than saturated alcohols topically and as larvicides, whereas the saturated alcohols were more effective as fumigants.

This study was expanded to include a large number of synthetic derivatives obtained through acylation of alcohol substituents (Rice and Coats

1994b; Tsao et al. 1995). In most bioassays, pivalates were more active than acetates, although the latter were generally more effective as ovicides. Fluoroacetates were particularly effective as house fly fumigants. Derivitization resulted in 2–3 fold increases in toxicity in some bioassays, although among 55 compounds tested for topical toxicity to the house fly, the natural product, thymol (**6**, Fig. 2), was the most active; all seven thymol derivatives were significantly less toxic. Enhanced fumigant and ovicidal activities likely resulted from increased volatility and lipophilicity of the acylated derivatives.

Recent evidence for an octopaminergic mode-of-action for certain monoterpenoids (Enan 2001; Bischof and Enan 2004; Kostyukovsky et al. 2002), combined with their relative chemical simplicity may yet find these natural products useful as lead structures for the discovery of new neurotoxic insecticides with good mammalian selectivity.

9 Conclusions

In this chapter we have reviewed a number of plant natural products with insecticidal activities, some long known and others recently discovered. Only a handful of these have seen commercial use as botanical insecticides due to several limiting factors.

In the case of neem, high input cost (2.5–3 times more expensive than synthetic pyrethroids) helps explain why neem insecticides in the USA are currently positioned with an emphasis on high-value row crops (e.g., fresh market tomatoes) and greenhouse crops (Isman 2004). Although, neem-based insecticides enjoy a broad spectrum-of-action against pests (> 400 species), they lack efficacy against certain pests including some species of tephritid flies (apple maggot, cherry fruit fly). Owing to limited persistence on plants, multiple applications of neem may be necessary to achieve acceptable control against some important pests (e.g., codling moth on apple, bollworm on cotton), which may not be economically feasible (Isman 2004). Neem insecticides also lack contact action and work slowly, a situation often disheartening to growers used to synthetic pyrethroids or other contact toxins that kill pests in a matter of hours. Although the bioactivity of the dozen or so azadirachtin analogs isolated to date from neem is well documented, the contribution of the remaining limonoid constituents of neem kernels to overall efficacy of neem insecticides remains controversial (Isman 2002) and unfortunately has become a point of confusion and uncertainty for regulatory agencies charged with evaluating neem as a pesticide (Isman 2004). Regulatory approval has greatly limited the introduction of neem insecticides in Europe, particularly on food plants, owing to residue and environmental data requirements.

We have focused our review on structure–activity relations, both among naturally occurring analogues, and among synthetic derivatives, with a view

to the potential of these natural substances as leads for the development of synthetic insecticides, either with novel modes-of-action or with reduced environmental and human health impacts through other means.

In our opinion, the only successful example of this approach has been the development of the synthetic pyrethroids, an endeavor that took over 25 years to accomplish. A decade of research on total synthesis of azadirachtin failed to bear fruit; the chemical complexity of this molecule and the numerous structural requirements for insect-growth regulatory activity pose a formidable challenge to the synthetic chemist. Rocaglamide poses a similar challenge. However, less complex plant natural products with sufficient insecticidal action (naphthoquinones, monoterpenoids) may yet prove useful for the development of insecticides with novel modes-of-action.

Perhaps the strongest conclusion from our review is the optimization of plant chemical defenses on an evolutionary timescale—insecticidal active principles from plants are not easily improved upon! Furthermore, the insecticidal activity found in plants is often enhanced by the presence of suites of closely related compounds, acting synergistically or at the least diffusing selection by insect herbivores and thus forestalling the development of resistance in those insect populations.

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11 Essential Oils as Biorational Insecticides–Potency and Mode of Action

E. Shaaya, A. Rafaeli

1 Introduction

In the plant kingdom, some plant species are named aromatic on account of the volatile compounds that they contain, which confer an odor and a characteristic flavor to them. Aromatic plants have been mentioned in the Antiquity for their uses as medicinal plants, pot herb spices, and were also used to embalm the dead. Today, they are used in industry, particularly in cosmetics, perfumery, and as detergents as well as in the aromatic food industry. Besides these activities, a new field is developing: the use of these volatiles in insect pest management. In fact, one of the volatile fractions of aromatic plants, viz. the essential oils has a promising development in this field. These essential oils are also the most commonly used for industrial applications.

Essential oils are secondary metabolites abundant in some aromatic plant families, like *Conifers*, *Rutaceae, Umbelliferae, Myrtaceae* and *Labiatae*. The oils may be localized in different parts of the plants, such as hair, transport tubes, leaves, stems, flowers, or any other part, depending on the plant species. As essential oils are volatiles, they can be easily extracted by water vapor, contrary to the oleaoresins and resinoids, which are extracted by solvents and alcohols. They contain a large number of constituents, the majority of which possess an isoprenoid skeleton. The main compounds constituting the essential oils are monoterpenes, and to a lesser extent, sesquiterpenes, which contain ten or 15 atoms of carbon, respectively, more rarely they are diterpenes and also aliphatic or benzoic molecules. The monoterpenes can be divided into three groups: acyclic, monocyclic and bicyclic. The majority of essential oils contain a limited number of main constituents, but also minor compounds can play an important part in the fragrance and biological activity of the oil. The composition of essential oils in the same plant species can be varied, as a result of the physiological development of the plant, and its degree of maturity, climate, soil conditions, etc. Essential oils are secondary metabolites, which were first considered as phyto-metabolic waste products, but it soon became apparent that they have various important functions. They play a role at preventing excessive evaporation and they are donors of hydrogen in oxidation-reduction reactions. They also play a role in the defense of plants against insect- and microorganism attack. On the other hand, they may attract specific insects for pollination as well as specialist-phytophagous insects.

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The use of synthetic toxic insecticides has posed several problems to the environment and human health in their persistence and negative effects to non-target organisms. Thus, there is an urgent need to develop safe alternatives that will have the potential to replace the toxic chemicals, yet will be simple and convenient to use. Phytochemicals, such as essential oils and their constituents are known to possess insecticidal activities and most monoterpenes are pleasantly aromatic, exhibiting low toxicity to mammals, thereby posing them as good potential candidates for the use in pest management.

2 Essential Oils Activities on Insect Pests

2.1 Plant Resistance

Essential oils provide resistance to plants against phytophagous insect damage. The resistance of conifers such as the Scotch pine to the violet longhorned beetle (*Callidum violaceum*) and the flat bug (*Aradidae*) was found to be related to the high content of essential oils in the bark (Smelyanets and Khursin 1973; Karasev 1974). In wild tomato (*Lycopersicon hirsutum hirsutum*) sesquiterpene secretions, are associated with insect resistance (Gianfagna et al. 1992).

2.2 Insecticidal Activity

The toxic effect of essential oils against a large number of insect pests has been well documented. Some examples include, the essential oils from *Pinus* and *Mentha lavandula*, which were found to be toxic against the greenhouse white fly (*Trialeurodes vaporariorum*), the Colorado beetle (*Leptinotarza decemlineata*) the pear bug (*Stephanitis pyri*) and *Myzus persicae* (Mateeva and Kanov 1983). The essential oil extracted from the leaves of *Thujopsis dolabrata hondai* was found to be very toxic against the mite (*Dermatophagoides farinae*), the termite (*Coptotermes formosanus*) and the cockroach (*Periplaneta fuliginosa*) (Asada et al. 1989).

The toxicity of a large number of essential oils and their constituents has been also evaluated against a number of stored-product insects. Essential oils extracted from *Pogostemon hyneanus, Ocinman basilicum* and *Eucalyptus,* showed insecticidal activities against *Sitophilus oryzae, Stegobium paniceum, Tribolium castaneum,* and *Callosobruchus chinensis* (Desphande et al. 1974; Desphande and Tipnis 1977). Toxic effects of the terpenoids d-limonene, linalool and terpineol were observed on several Coleoptera damaging post-harvest products (Karr and Coats 1988: Coats et al. 1991; Weaver et al. 1991). Fumigant toxic activity and reproductive inhibition induced by a number of essential oils and their monoterpenoids were also evaluated against the bean weevil *Acanthoscelides obtectu*s (Say) and the moth

Sitotroga cerealella (Klingauf et al. 1983; Regnault-Roger and Hamraoni 1995). For a comprehensive review on the use of plant material for the protection of stored leguminous seeds against seed beetles see Boebe et al. (2001).

It should be mentioned that besides the phytochemical composition of the essential oils, other factors define the toxicity of the oil such as the degree of oil absorption in the treated commodity, the less absorption the higher the concentration of the oil in the inter-granular air. The entry of the oil either by inhalation, ingestion or skin absorption also plays an important role.

2.3 Repellency

A number of essential oils were found to exhibit repellent activity against various insects. The oils from *Ocimum sauve* and *Lippia sp*. indigenous in Kenya, repelled *Sitophilus zeamais* (Hassalani and Lwande 1989; Mwangi et al. 1992). Camphor, cineole, methyl eugenol, limonene, myrcene, thymol and the essential oil from berries of *Jupinerus communis* were all found to repel mosquitoes (Kalemba et al. 1991; Chokechaijaroenporn et al. 1994).

2.3.1 Anti-feedant

Acarus calamus oil was found to be a potent growth inhibitor and an anti-feedant when tested against the cutworm *Peridroma saucia* (Koul et al. 1990). The essential oils of *Artemisia tridentate* and *Chrysothamnus nauseosus* were found to exert anti-feeding effects against the Colorado beetle (Jermy et al. 1981). On the other hand, some essential oil components were found to be attractive to insects: α-ionone and ionol-related compounds can lure males of the fly *Bactrocera latifrons* (Flath et al. 1994). Some essential oils can affect insects in different ways: Tansy oil, *Tanacetum vulgare*, was toxic in *Sitophilus granarium*, repulsive in *Tribolium confusum* and attractive and paralyzing in *Rhyzopertha dominica* (Kurowska et al. 1993).

3 Efficacy of Essential Oils as Fumigants for the Control of Stored-Product Insects

Currently, the measures to control pest infestation in grain, dry stored food products, and cut flowers, rely heavily upon the use of gaseous and liquid insecticides, which pose a health hazard to warm blooded animals, and a risk of environmental contamination with serious ecological implications. Furthermore, the number of compounds available for use has declined in recent years as problems of insect resistance have progressed, and as mounting social pressures against the use of these materials have limited the introduction of new compounds. In addition, present trends in international food

legislation have placed greater and greater limitations on the use of toxic chemicals for food preservation. There is also an increasing consumer demand for wholesome natural foods free of chemical additive or toxicants. This situation has led to the search for natural compounds for the control of insects.

There are no established natural fumigants in use against pests attacking grain, dry stored food and other agricultural products. The two fumigants in use today are phosphine and methyl bromide. Lately, studies have revealed that insects are developing resistance to phosphine. Methyl bromide was classified as a compound with ozone-depleting potential. Also the 2005 ban of methyl bromide under the conditions of the Montreal Protocol in developed countries has a serious impact on industries and consumers who rely on it for structural fumigation and for post harvest commodity treatments. In addition, the U.S. Environmental Protection Agency has classified DDVP (2-2-dichlorovinyl dimethyl phosphate), which is in use for space treatment, as a possible human carcinogen and it is proposing to cancel most uses of this pesticide. Thus, there is an urgent need to develop safe alternatives that have the potential to replace the toxic fumigants, yet they should be simple and convenient to use.

Many aromatic plants have the capacity to synthesize chemicals which, when isolated, are deadly or repellent to many insect species but harmless to mammals. We have isolated many such compounds from the essential oils of several aromatic plants (Shaaya 1979; Shaaya et al. 1991, 1993, 1994, 1997). Their volatility and insecticidal efficacy make them good prospective fumigants, which represent ecologically safe alternatives to the toxic fumigants.

In order to isolate active essential oils, we screened, at first, a large number of essential oils extracted from aromatic plants and isolated their main constituents. Using space fumigation (see Shaaya et al. l997), two essential oils obtained from Labiatae plants, ZP51 and SEM76, were found as the most potent fumigants of all oils tested against the major stored-product insect pests. Moreover, they had a high activity against *Sitophilus oryzae* and *Tribolium castaneum,* which were found to be the most tolerant of all insects tested (Table l). The main constituent of each of these two oils, which account for approximately 80% of the oil, were found to also have a high activity, compared to a number of monoterpenes tested (Table 2).

Pilot tests in simulation glass columns filled to 70% volume with wheat, under conditions similar to those present in large grain bulks, showed that ZP51 at a concentration of 50 l/l air, equivalent to 50 g/t wheat and a 7-day exposure time were enough to obtain 94–100% mortality of all adult insects tested. It is noteworthy to mention that the recommended concentration of methyl bromide for wheat fumigation is 40 g/t. Larvae and pupae of *Tribolium castaneum* were found to be the most tolerant of all adults tested (Table 3). In addition, supplementation of 15% CO_2 = (200 g/t wheat) increased the activity of the oil against all insects tested (Table 3). The ZP51 oil was also found to cause sterility, reduction of fecundity, and egg emergence of F1 in the flour moth, *Ephestia cautella* (Table 4).

Table 1. Fumigant toxicity of essential oils against stored product insects in space test

Compound	*O. surinamensis*		*R. dominica*		*S. oryzae*		*T. castaneum*	
	LC-50	LC-90	LC-50	LC-90	LC-50	LC-90	LC-50	LC-90
Peppermint	8.2	19.4	9.6	16.0	7.5	14.9	11.1	15.0
Sage	7.6	12.7	6.7	10.8	11.7	23.1	>15	–
Oregano	4.3	8.1	8.4	>15	15.4	30.4	>15	–
Basil	6.7	11.7	10.0	16.7	>15	–	>15	–
Three-lobed sage	8.7	12.9	6.8	10.8	>15	–	>15	–
Bay laurel	13.4	32.0	7.0	10.5	>15	–	>15	–
Rosemary	8.3	13.4	9.2	11.6	>15	–	>15	–
Lavander	11.3	12.8	11.4	13.8	>15	–	>15	–
Anise	>15	–	8.8	21.3	>15	–	>15	–
ZP-51	1.7	2.8	2.8	4.5	0.7	1.4	2.5	3.2
SEM-76	–	<10	0.5	0.8	–	<10	0.9	1.4

Sixty adults in three replicates were used for each experiment. The data are the average of 5–8 experiments. Exposure time 24 h. The numbers are µl/L air

Table 2. Fumigant toxicity of essential oil constituents on stored product pests in space tests

Compounds	*O. surinamensis*		*R. dominica*		*S. oryzae*		*T. castaneum*	
	LC-50	LC-90	LC-50	LC-90	LC-50	LC-90	LC-50	LC-90
SEM -76	–	<1.0	0.4	0.6	–	<0.5	0.7	1.2
ZP -51	1.7	2.8	2.8	4.5	0.7	1.4	2.5	3.2
1,8-Cineol	3.1	7.3	2.5	4.0	7.2	14.2	7.5	8.5
Carvacrol	–	–	>15	–	–	–	>15	–
Limonene	>15	–	6.7	10.3	>15	–	7.6	8.6
Linalol	3.0	6.0	6.0	8.5	10.1	19.8	>15	–
Terpinen-4-ol	3.9	11.4	1.3	2.0	1.2	5.2	2.5	3.3
Terpineol	4.3	12.7	>15	–	>15	–	>15	–

Exposure time 24 h
LC-50 and LC-90 are expressed in µl oil/1 air

4 Insecticidal Mode of Action of Essential Oil-Toxicity

4.1 Introduction

The mechanisms of toxic action of essential oils have not been fully elucidated to date. Studies on the mode of action of essential oil treatments are of importance in order to establish the spectrum of activity and to determine

Table 3. Fumigant activity of ZP51, with and without CO_2, against five stored product insects on winter wheat, in columns 70% filling

Stage	Concentration (µl/l)	Insect mortality (%)				
		Sitophilus oryzae	*Tribolium castaneum*	*Oryzaephilus surinamensis*	*Rhizopertha dominica*	*Plodia interpunctella*
Adults	50	95	87	70	69	–
	50 (7 days)	100	100	100	100	–
	30	76	18	50	43	–
	15% CO_2	0	5	27	12	–
	30 +15% CO_2	100	83	85	93	–
Larvae	30	–	27	–	–	80
	15% CO_2	–	0	–	–	67
	30 +15% CO_2	–	34	–	–	90
Pupae	30	–	20	–	–	78
	15% CO_2	–	0	–	–	67
	30 +15% CO_2	–	13	–	–	100

Exposure time 3 days

Table 4. Late mortality during the development of F0 and F1 of *Ephestia cautella* larvae following treatment with the essential oil ZP-51

Eggs developed to adults (F_1) (%)	Eggs emerged (%)	Eggs layed from control (%)	Sterile females	Larvae developed to adults (F_0) (%)	Larval mortality (%)	Concentration µl/l
75	75	100	30	80	10	2
57	75	88	40	55	20	3
40	80	70	20	40	40	4
53	70	70	30	25	45	5
0	20	50	70	30	65	6

Third instar larvae were used; Exposure time 24 h in space fumigation

their full commercial potential, thereby providing useful information on the appropriate formulation types. Given the scant knowledge of the mode of action of individual essential oils as well as their mixtures, and of the lack of experimental protocols for these physiological tests, much more work has yet to be undertaken. Although many essential oil compounds have been shown to be toxic or repellent to a range of insects, structure–activity studies in individual

insect species provide few insights as to the exact mode of action of these compounds.

Many essential oils tested are effective without direct contact (Choi et al. 2003) indicating that delivery of these oils to target tissues occurs through a vapor phase, perhaps through penetration via the respiratory system. It is interesting to note that not all insects are affected by these terpenoids. For some insect species they act as allomones or they mimic insect pheromones representing semiochemical communication between insect and plant, where the evolution of chemical and physical defenses against a diverse insect group by the plant co-evolved with an ability of some insects to detoxify a wide range of plant chemicals. Thus the response to a given compound may be subject to considerable modification by other factors (both endogenous as well as exogenous) and there may be various mechanisms by which the target responses are modulated such as: the rate of excretion or inactivation of these compounds, changes in the target receptors for these compounds, or interactions with endogenous factors that may overcome the target responses.

Treatments with certain essential oils or their purified constituents have been reported to result in visible symptoms that point to a neurotoxic mode of action. These overt symptoms include hyperactivity, convulsions, and tremors followed by paralysis ('knockdown'), resembling those produced by organophosphate or carbamate insecticides. The onset of toxic signs is usually rapid, thereby also indicating a neurotoxic response. In the American cockroach, for example toxicity was revealed by hyperactivity followed by hyperextension of the legs and abdomen, then fast knockdown or quick immobilization followed by death (Enan 2001). Carpenter ants (*Camponotus pennsylvanicus* De Geer), and German cockroaches (*Blattella germanica*) that were included in this study showed fast immobilization/knockdown followed by mortality (Enan 2001). Such neurotoxic responses indicate that the target site of these essential oils may be the insect's neural system possibly affecting neural signaling.

4.2 Neurotransmitters in Insects

In insects, like vertebrates and other animals the nerve impulse conducted in the axon must cross a synapse in order to stimulate another neuron or an effector, e.g., muscle. In the synaptic connections transmission involves a chemical which is stored in the synaptic vesicles. The transmitter chemical attaches to a receptor site on the postsynaptic membrane thereby transferring a nerve impulse across the synaptic gap. The chemical transmitter may be of several types and is usually rapidly hydrolyzed and deactivated by appropriate enzymes. Within the insect systems these include nerve–nerve interfaces that are commonly cholinergic utilizing acetylcholine (Fig. 1) as neurotransmitter; this type of neurotransmitter is common with the vertebrate systems. Nerve–muscle and neurosecretory–blood interfaces are commonly

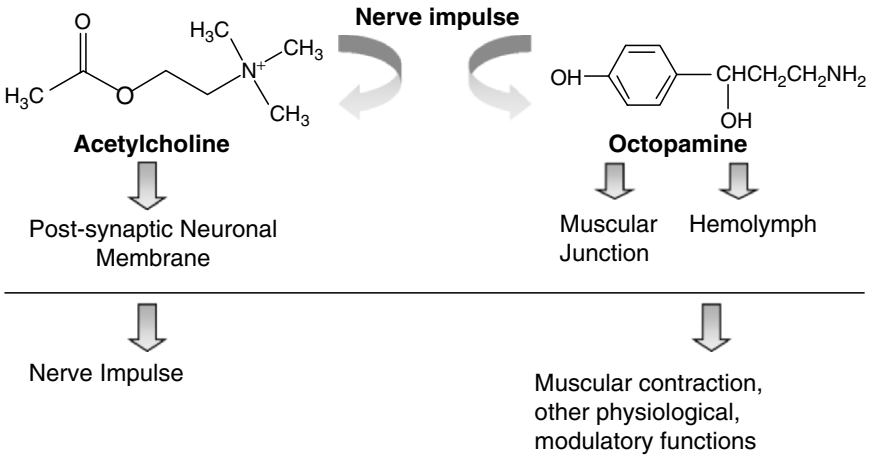

Fig. 1. Neurotransmitters in insect systems as possible sites of action of essential oil toxicity

octopaminergic (Fig. 1) utilizing octopamine as neurotransmitter or neurohormone. This type of transmitter is unique for invertebrate systems and has a physiological role analogous to norepinephrine and other catecholamines in vertebrates. Among some of its functions, octopamine can regulate an insect's heart rate, movement, behavior and metabolism.

4.3 Inhibitory Activity of Essential Oils on Acetylcholinesterase (AchE)

A common structural feature of terpenoids is their hydrocarbon skeleton, which in turn renders them hydrophobic. Many hydrophobic compounds are associated with protein or enzyme deactivation, where acetylcholinesterase is particularly sensitive. Compounds that inhibit or inactivate acetylcholinesterase cause acetylcholine to accumulate at synapses of cholinergic sites. This produces continuous stimulation of cholinergic fibers at neuromuscular junctions as well as throughout both the central and peripheral nervous systems causing overt neurotoxic responses leading to paralysis and death. Several essential oil monoterpenes demonstrated a competitive inhibition of acetylcholinesterase. Kinetic analyses of the hydrolysis of acetylthiocholine by eel acetylcholinesterase in the presence of pulegone-1, 2-epoxide, isolated from the medicinal plant, poleo (*Lippia stoechadifolia*), showed an irreversible inhibition of the enzyme in a manner similar to that shown for carbamates (Grundy and Still 1985). Five other monoterpenoids were similarly tested for their effects on acetylcholinesterase, and comparative results suggested that both the epoxy and keto groups are required for irreversible inhibition. However, in these studies, which demonstrated activity in vitro, no correlation was found between the onset of symptoms (preceding death) and inhibition of the roach acetylcholinesterases. In other words,

the symptomatics leading to death could not be attributed to the inhibitory actions on acetylcholinesterases.

In another study, five monoterpenes, representing characteristic constituents of secondary plant chemicals in leaves and a range of functional groups (e.g., citral, pulgenone, linalool, (–) bornyl acetate, cineole and gossypol) were tested on acetylcholinesterase activity from the eel in vitro and on toxicity, in vivo, in a stored product pest, *Tribolium castaneum*. All six terpenoids killed the insect in a dose-dependent manner and inhibited electric eel AChE. As these monoterpenes represent distinct functional groups, it may indicate that such an inhibition is widespread among the monoterpenes. However, these results also presented an anomaly in that the order of potency for the compounds differed according to whether in vivo or in vitro tests were performed. Thus, the most potent monoterpene in vivo was not necessarily the most potent in vitro (Ryan and Byrne 1988). It may be explained by the fact that a vertebrate AChE has different susceptibility to these monoterpenes than an insect's AChE. This is certainly true regarding the susceptibility of AChE to insecticides (Hollingworth et al. 1967). In our studies, using AChE extracted from the stored product insect, *Rhizopertha dominica* (Kostyukovsky et al. 2002) we showed that inhibition of AChE activity was obtained only when very high levels of the essential oils (10^{-3} M) were administered. These doses were at such high levels that they cannot account the toxic effects observed in vivo by the same compounds, and even on the same insect species (Shaaya et al. 2001, 2002), which were obtained at much lower concentrations of essential oils (10^{-6} M or 1.5 l/liter of air). The failure of these biologically active essential oils to produce a stronger enzyme inhibition indicated that acetylcholinesterase was probably not the main site of action for these essential oils.

4.4 Inhibitory Activity of Essential Oils on Octopaminergic Sites

Another possible target for essential oil neurotoxicity is the octopaminergic system in insects. Octopamine is a multifunctional, naturally occurring biogenic amine that plays a key role as a neurotransmitter, neurohormone and neuromodulator in invertebrate systems (Evans 1981). The acute and sublethal behavioral effects of essential oil compounds on insects, as well as their low toxicity in mammals and other vertebrates, are consistent with an octopaminergic target site in insects. The octopaminergic system in insects, represents a biorational target for insecticidal action and has been targeted by various insecticides in the past, e.g., formamidines (Haynes 1988; Perry et al. 1998). Various receptor subtypes exist that differ in their tissue and cellular localization, their affinity for octopamine and in their elicited second messenger pathways (Evans 1981). Many of the physiological functions of octopamine appear to be mediated by a class of octopamine receptors (type-2 receptors) specifically linked to a G_S-protein coupled to the enzyme

adenylate cyclase. These physiological actions have been shown to be associated with elevated levels of cyclic-AMP (Evans 1984).

In our studies, using an insect in vitro cuticular tissue preparation, the effect of octopamine on intracellular cyclic-AMP production was studied. In these studies, a significant increase in intracellular cyclic-AMP levels occurred in the presence of octopamine in cuticular sections of the 8th or 7th abdominal segments of the adult moth, *Helicoverpa armigera* (Rafaeli and Gileadi 1995). Although the functional significance of this response is as yet unknown, but may be related to cuticular plasticity for distension of the abdomen (Reynolds 1974; Orchard and Lange 1988), this bioassay was utilized to examine the effects of essential oils on octopamine-induced intracellular cyclic-AMP production. Intracellular levels of cyclic-AMP were observed to increase in relation to the exposure time to octopamine as compared to control tissue, which maintained a steady-state of basal levels of intracellular cyclic-AMP. On addition of various essential oil constituents (termed SEM-76 and ZP-51) extracted from aromatic plants endogenous to Israel, a significant increase in cyclic-AMP was observed. This increase did not differ from the increase as a result of octopamine. It is not clear whether the increase in cyclic-AMP levels was as a result of activation of adenylate cyclase or the inhibition of phosphodiesterase but, since these increases were observed in the presence of isobutylmethylxanthine (a phosphodiesterase inhibitor) there is a strong possibility that the latter may be the case. Moreover, on addition of the octopamine antagonist, phentolamine both the responses to octopamine as well as the essential oil constituents were strongly antagonized (Shaaya et al. 2001; Kostyukovsky et al. 2002). These effects were observed at low dilutions of the essential oils (estimated 10^{-7} and 10^{-8}M range), levels that induced the overt behavioral toxicity responses in vivo (Kostyukovsky et al. 2002).

Thus, consistent with the hypothesis that essential oils may affect octopaminergic target sites are the following facts, which reinforce this hypothesis: (1) the essential oil response is strongly insect-specific, as is octopamine neurotransmission; (2) their ability to mimic the action of octopamine at low doses; and (3) the inhibitory action on essential oil responses by phentolamine, an octopamine inhibitor. Similarly, significant increases of cyclic-AMP in brain tissues of the American cockroach and the carpenter ant were demonstrated, as was demonstrated on insect cuticle (Shaaya et al. 2001; Kostyukovsky et al. 2002), in response to low levels of essential oils (eugenol and ∞-terpineol) (Enan 2001). At higher levels of essential oils significant decreases in cyclic-AMP levels were reported. In addition, the octopaminergic increase in heart rates (Prier et al. 1994) was also mimicked by these essential oils and several essential oils were shown to block specific octopamine binding to receptor sites in the brains (Enan 2001). The effect of these oils on receptor binding activity, however, varied among test oils, which were subsequently classified as agonists, antagonists and non-octopamine receptor ligands (Enan 2001). There are still several issues to be

resolved concerning the mode of action of these compounds. It remains to be shown whether other biogenic amines are also possible targets for monoterpenoids and whether the toxic action of the monoterpenoids is mediated by other pathways viz., GABA receptors, adrenergic receptors, calcium channels and others.

5 Concluding Remarks

Insect control has an impact from both the agricultural and the public-health perspective. The use of toxic insecticides for pest control developed a number of environmental, economic and human health problems, besides the development of pest resistance, persistence of residues and negative effects on non-target organisms. For these reasons, the use of phytochemicals, as replacement for the synthetic insecticides, has generated considerable efforts. Aromatic plant volatiles have been known to affect behavioral responses of insect pests and have demonstrated biological activity against insects. Terpenoids, the major constituents of essential oils, are found to be most effective in pest control. In fact, terpenes and their derivatives have a major role in the insecticide industry. Today, the main use of essential oils is to protect domestic animals and clothes against moths, also to some extent, as a wood-preservative material. The oil and their constituents can be used in formulations with pyrethroids, additions to insect growth inhibitors, incorporation with polymers into sheets and as coating materials.

Our findings, as well as that of other researchers, suggest that certain essential oils particularly monoterpenoids, are highly selective to insects since they are probably targeted to the insect-selective octopaminergic receptor, a non-mammalian target. On the basis of this insect-selectivity a mixture of essential oils trademarked hexa-hydroxyl (EcoPCO Ecosmart Technologies, Franklin, Tennessee), and based on distinct combinations of different plant essential oils that significantly enhance the activity of these oils against insects was formulated. This patented technology demonstrates rapid insecticidal activity by combining oils with a common molecular structure (a six-membered carbon ring with an oxygenated functional group attached) and, since they are directed at octopaminergic sites, they are also classified as generally recognized as safe (GRAS) and/or have been approved for food and beverage consumption by the U.S. Food and Drug Administration (FDA).

In addition to the above, we showed that some essential oils and their main constituents have a great potential as fumigant alternatives to methyl bromide and phosphine for the control of stored-products insects. This makes the use of eco-chemical control the most promising method amongst the current alternative strategies aiming at decreasing the use of toxic insecticides.

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

GUY SMAGGHE

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

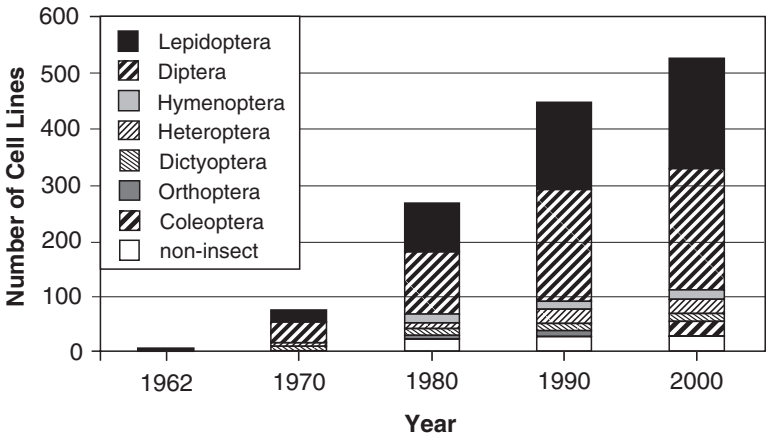


Fig. 1. The number of established invertebrate cell lines developed since 1962 categorized by insect orders. Each *bar* represents the total numbers of cell lines that have been reported up to the date listed (from Lynn 2001)

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,

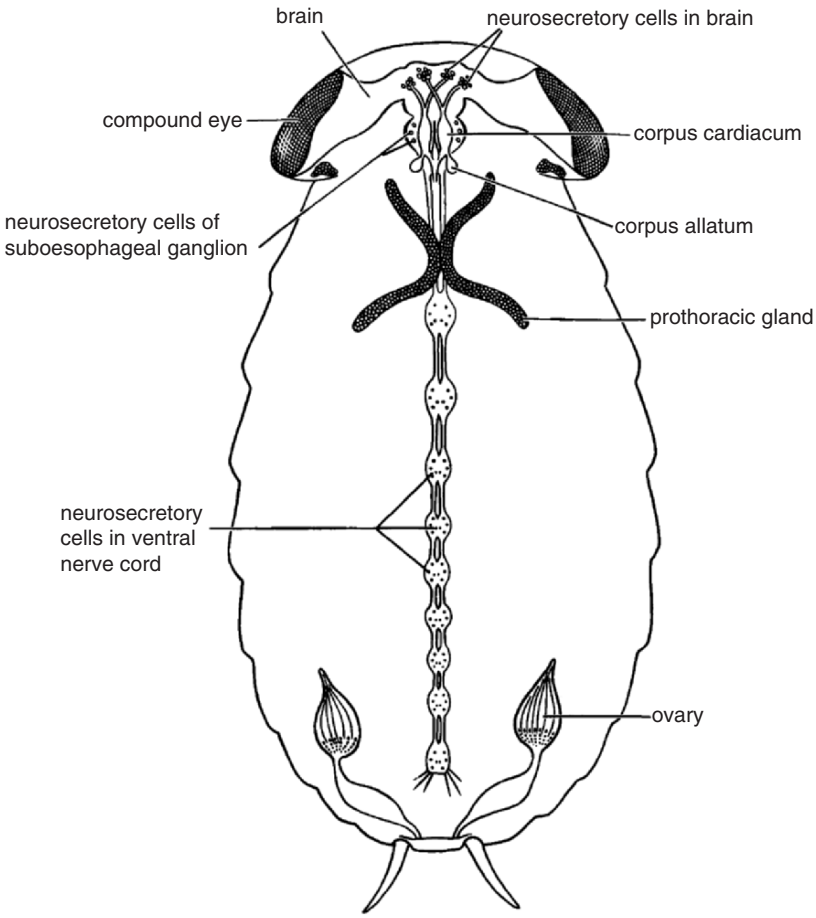
lactalbumin hydrolysates and fetal bovine serum (FBS); TC-100 (aka BML/TC-100), which is also a modification of Grace's medium, with the reduction of some amino acids, Krebs cycle intermediates and the addition of tryptose broth. Extensive optimization research by Goodwin and Adams (1980), Vaughn and 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*

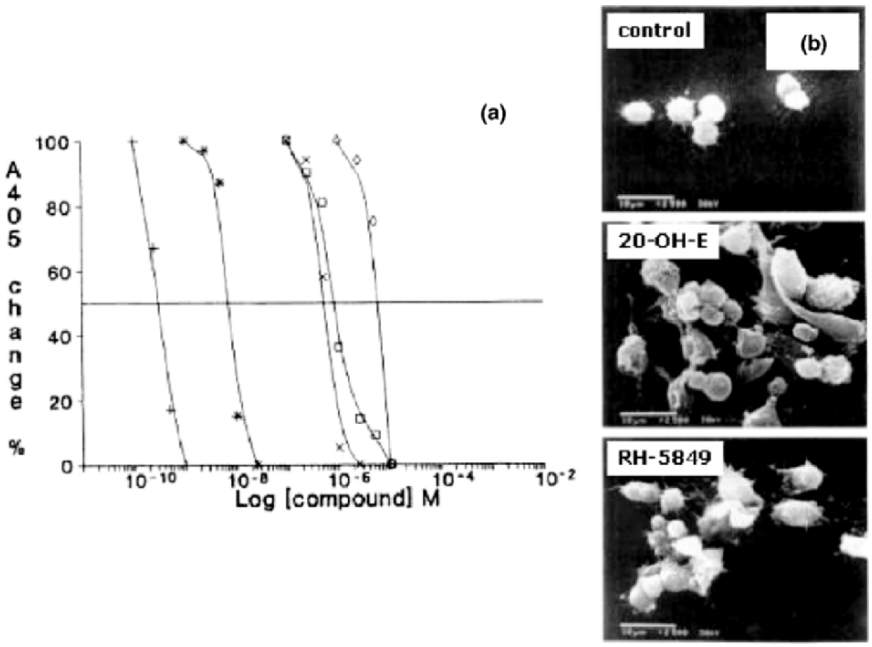


Fig. 3. Ecdysteroid-responsive *Drosophila* cells. (**a**) The B_{II} cells are grown in the wells of microtiter plates and respond to ecdysteroids with reduction in cell density and cell clumping that is reflected in an altered OD at 405 nm in the microtiter plate reader: *: 20-OH-E, □: ecdysone, +: 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

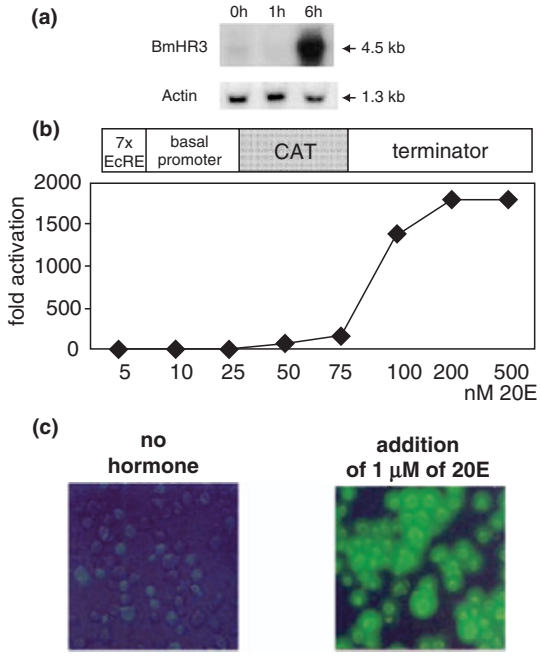


Fig. 4. Assessment of the primary response to 20-OH-E in silk moth-derived Bm5 cells, transformed with the pBmbA/ERE.gfp construct for induction of green fluorescence. (**a**) Northern blot analysis of 20-OH-E primary response gene BmHR3 expression at intervals of administration of 1 μM of 20-OH-E. Actin 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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