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Toxic Mechanisms of Pyrethroids Studied
in Vitro

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To Katriina, Kristiina and Kimmo

CONTENTS

List of original publications

This thesis is based on the following original communications referred to in the text by their Roman numerals I-IV:

I   Kakko I, Toimela T, Tähti H (2000): Piperonyl butoxide potentiates the synaptosome ATPase inhibiting effect of pyrethrin. Chemosphere 40: 301-305.

II Kakko I, Toimela T, Tähti H (2003): The synaptosomal membrane bound ATPase as a target for neurotoxic effects of pyrethroids, permethrin and cypermethrin. Chemosphere 51:474-480.

III Kakko I, Toimela T, Tähti H (2004): The toxicity of pyrethroid compounds in neural cell cultures studied with total ATP, mitochondrional enzyme activity and microscopic photographing. Environmental Toxicology and Pharmacology 15:95-102.

IV Kakko I, Toimela T, Tähti H (2004): Estradiol potentiates the effect of certain pyrethroid compounds in the MCF-7 human breast carcinoma cell line. ATLA 32, in press.

Abbreviations

AD	Anno Domini
ATCC	American type culture collection
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BBB	blood brain barrier
BCA	bicinchoninic acid
CNS	central nervous system
DCC	dextran-coated charcoal
DDT	dichlorodiphenyltrichloroethane
DEET	N,N-diethyl-m-toluamide
DMEM	Dulbecco´s Modified Eagle Medium
DMSO	dimethylsulfoxide
EDC	endocrine disrupting chemicals
EDTA	ethylenediaminetetra-acetate
F12K	Kaighn´s Modification (of Hams F12) L-glutamine medium
GABA	γ-aminobutyric acid
IARC	International Agency for Research on Cancer
LD_{50}	lethal dose
MCF-7	human breast carcinoma cell line
MEM	minimum essential medium
Mg^{2+} ATPase	magnesium-activated adenosine triphosphatase
Na^+, K^+ ATPase	sodium-potassium-activated adenosine triphosphatase
NIE-115	neuroblastoma cell line
PB	pyridostigmine bromide
PBO	piperonyl butoxide
SEM	standard error of mean
SH-SY5Y	human neuroblastoma cell line
TCA	trichloroacetic acid
Tris	tris (hydroxymethyl) aminomethane
WST-1	cell proliferation reagent (tetrazolium salt) WST-1
WHO	World Health Organization

Abstract

Pyrethroids are insecticides widely used in agriculture and gardening. They form a group of chemically different compounds with a high degree of efficacy and low human toxicity. Pyrethroids are often regarded as a modern generation of 'safe' insecticides, because they are rapidly metabolised in mammals and ecosystems, which explains their low toxicity. The natural pyrethroid compounds, pyrethrins, were already known in China in the first century AD. In the 1940s the producing of synthetic pyrethroids began, and pyrethroid compounds are now routinely synthesised by the agrochemical industry. The neurotoxicity of pyrethroids is based on their effects on the neuronal cell membrane. Previously, the voltage-sensitive sodium channel has been studied as a principal molecular target site for pyrethroids. The non-neurotoxic effects referred to are e.g. allergenic effects, oestrogenic effects and interaction with the cell cycle.

In the present *in vitro* study, three commercial pyrethroid products (Biospray, Biokill and Ripcord) were studied. In Biospray S, pyrethrin is the active compound, while Biokill contains permethrin and Ripcord cypermethrin. The neurotoxicity was investigated in studies of neural membrane effects in isolated rat brain synaptosomes, and the acute cell toxic effects were followed in neuronal cell cultures. The hormone-like effects were evaluated in a human breast carcinoma cell line.

In synaptosomal membranes, the adenosine triphosphatase (ATPase) was shown to be a target site for the membrane effect of pyrethroids. Piperonyl butoxide, a well-known synergist of pyrethrin, potentiated the synaptosome ATPase -inhibiting effect of pyrethrin. In the SH-SY5Y neuroblastoma cultures, the viability of the cells decreased in acute exposures. The toxic effect was dependent on the chemical structure of the respective pyrethroid compounds. Pyrethroids without an alpha cyano group (pyrethrin and permethrin) were less toxic than cypermethrin with the same group. Microscopic photographs confirmed the result of the biochemical viability tests. The morphological changes were seen in the exposed cultures even at concentrations lower than those obtained with the viability tests. In human breast carcinoma cell line (MCF-7), pyrethroid compounds had a clearly synergistic effect with oestradiol. At low concentrations, pyrethroids increased markedly the cell proliferative effect of oestradiol, but at high concentrations, their effect was cell toxic. The cell toxicity was studied with two biochemical viability tests: WST-1 test based on the mitochondrial metabolic enzyme activity and, ATP test based on the total cellular ATP content.

The present study showed that the neural membrane integral protein ATPase is a target for the effects of pyrethroids. The different acute toxicities of the test compounds in the neuroblastoma cell line confirmed the toxicity-increasing effect of the alpha cyano group in the pyrethroid structure. The *in vitro* study on breast carcinoma cell line showed the oestrogen-like effect of pyrethroids, which should receive special attention when the toxic risks of pyrethroids are further evaluated.

Introduction

Pyrethroid insecticides are used on a worldwide scale. It has been calculated that pyrethroids accounted for 25% of the insecticide market in the industrial countries in the 90s (Williamson et al. 1996). Pyrethroids are the most common insecticides for both indoor and agricultural purposes. Their chief advantages are high insecticidal potency and low mammalian toxicity with rapid metabolism and lack of terrestrial accumulation.

The history of these compounds is very old. Natural pyrethrin was already known in China in the first century AD. The first written accounts of these compounds date from the 17th century literature. Pyrethroids, synthetic analogues of pyrethrin, have been produced since 1940 (Elliott et al. 1973, Elliot 1980). At first, they were not stable enough for extensive use in agriculture. The replacement of light-unstable centres in the molecule by light-stable groups permitted large-scale use of pyrethroids. An ever increasing demand for pyrethroid compounds has resulted in the synthesis of new analogues with better stability in the light and air, better persistence, more selectivity in target species, low mammalian toxicity, and better biodegradability (Neumann and Peter 1987).

Pyrethroids are considered the safest of highly potent insecticides (He et al. 1989, Chen et al. 1991, Pauluhn 1999, Mertz 1999, Ray and Forshaw 2000, Bateman 2000). Higher rates of enzymatic biotransformation, detoxification and excretion are responsible for their lower toxicity for mammals. Compared to mammals, the potency in insects is five-fold e.g. for tetramethrin. This is partly due to the lower body temperature and partly slower enzymatic detoxification in insects. Their smaller body size gives less time for the compound to be detoxified before reaching the target site. However, the major mechanism of the selective toxicity of pyrethroids is obviously the high sensitivity of the neural sodium channels of insects to pyrethroids (Narahashi et al. 1995, Song and Narahashi 1996). When all the differences in the various factors are multiplied, the overall difference in the sensitivity to pyrethroids between mammals and insects is estimated to be 2250-fold. This is in the same order of magnitude as the difference in the acute lethal dose, (LD_{50}) of tetramethrin, which ranges from 500 to 4500- fold in mammals compared to the LD_{50} in insects (Narahashi 2000).

Severe pyrethroid insecticide poisonings are uncommon in the developed world, but common in developing countries, because of the wide use of pyrethroids in agriculture (Bateman 2000). There is evidence of central nervous system (CNS) toxicity caused by pyrethroids (Rosenberg et al. 1999, Muller-Mohnssen 1999). Toxicity has been reported in farmers who are involved in

spraying insecticides indoors in closed and poorly ventilated areas (Chen et al. 1991).

Due to the lipophilic nature of pyrethroids, biological membranes and tissues take up pyrethroids. At the cellular level, pyrethroids act on a variety of biochemical and physiological target sites. The neural membrane sodium channel is the main target of pyrethroids in insects, and the insecticidal efficiency is based on this effect. However, the mammalian sodium channel is much less sensitive to pyrethroid (Narahashi et al. 1995, Narahashi 1996). Also ATP-utilising enzymes (ATPases) and ion pumps may be involved in the neurotoxic actions of pyrethroids (Litchfield 1985). In addition, pyrethroids can disturb hormone-related functions (Go et al. 1999). Exposure to an endocrine disrupter can alter the endocrine system by increasing the risk of endocrine diseases and disorders (Colborn et al. 1993, Kavlock et al. 1996, Ankley et al. 1997).

Neurotoxic and non-neurotoxic effects of pyrethroids are similar to those of DDT, which was banned in the late 60s. DDT is now very rarely used except in very special cases where, its benefits far outweigh its risk to environment, e.g. in the eradication of malaria mosquitoes in developing countries, especially in Africa (Walker 1996, Walker 2000, Barlow et al. 2001, Brölsch et al. 2001).

The present study was set up to investigate the neurotoxic potential of different pyrethroid compounds by using rat brain synaptosomes and human neuroblastoma cell line cultures. The hormonal interactions were evaluated in human breast carcinoma cell line.

Review of literature

1. Chemical structure and biotransformation of pyrethroids

Initially, pyrethroids were extracted from the dried and powdered flower heads of *Chrysanthemum cinerariaefolium*. The extract contains chrysanthemic (pyrethrin I) and pyrethric acid esters (pyrethrin II) in about equal quantities (Figure 1). Natural pyrethrin is a mixture (1:1) of pyrethrin I and pyrethrin II (Davies 1985).
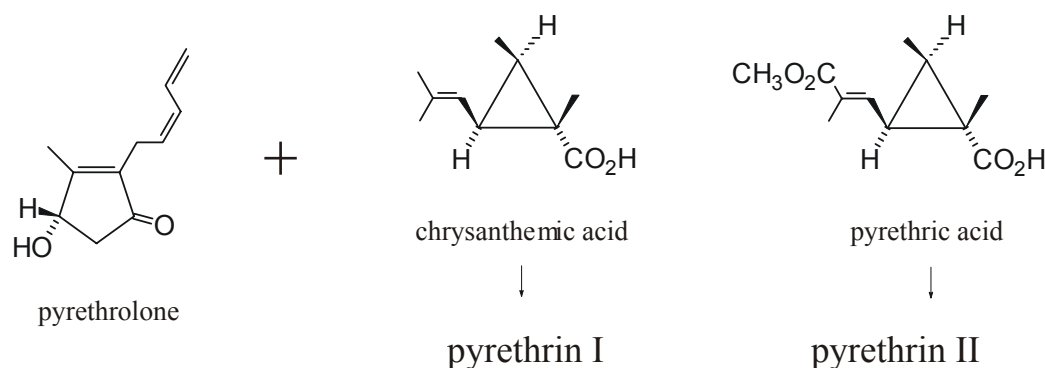
Figure 1. *Pyrethrin I is an ester of pyrethrolone and chrysanthemic acid. Pyrethrin II is an ester of pyrethrolone and pyrethric acid.*

Pyrethroids, the synthetic analogues of pyrethrin, fall into two distinct categories (Type I and Type II) based on the symptoms in experimental animals receiving acute toxic doses (Verschoyle and Aldridge 1980). Type I pyrethroids, e.g. pyrethrin and permethrin, do not have an alpha cyano group while Type II pyrethroids such as cypermethrin and fenvalerate, contain an alpha cyano group (Figure 2). It is generally agreed that Type II pyrethroids are more potent insecticides due to the alpha cyano group in their structure (Vijverberg et al. 1982, Glickman and Casida 1982, Holloway et al. 1989, Tabarean and Narahashi 1998).

In addition, the stereochemistry of these compounds has an effect on efficacy (Narahashi 1985). Several pyrethroids have isomeric forms, which show distinct different toxicities and insecticidal potencies. Generally, the *cis* -isomers are more toxic than *trans* -isomers. Cypermethrin occurs as both *cis* - and *trans* - isomers, the former being a more potent insecticide (Narahashi 1996).
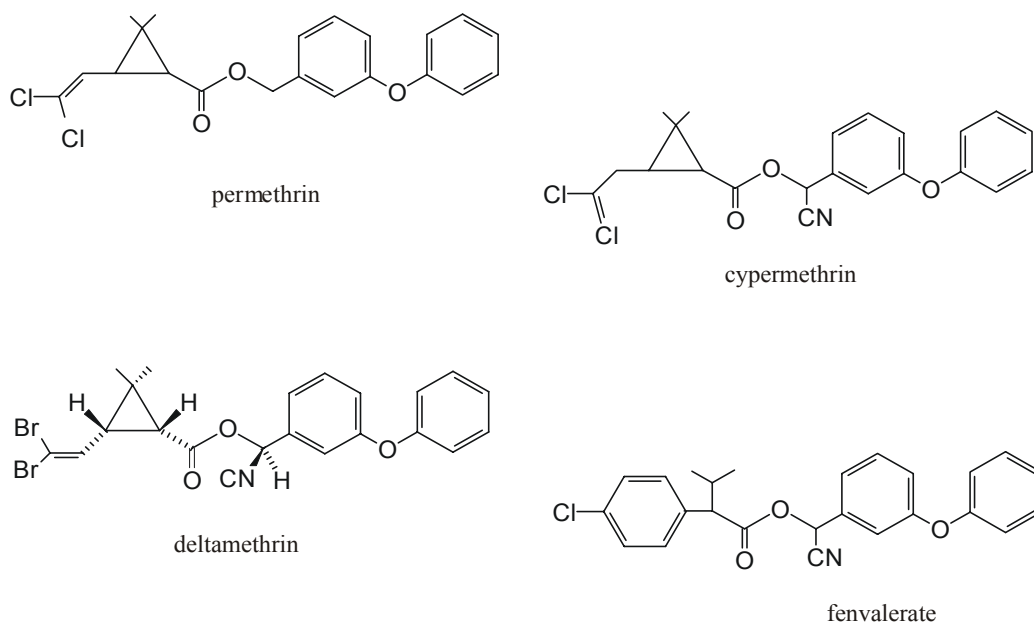
Figure 2. *Chemical structures of pyrethroids with and without an alpha cyano group.*

Thus it is important to define the ratio of isomers when evaluating the toxicological properties of pyrethroid compounds (Shono et al. 1979, Soderlund et al. 1983, Ecobichon 1996). It is, however, difficult to determine the importance of the ratio of isomers in the acute toxicity of the pyrethroids, because the vehicle has a marked influence on the LD_{50} values (Table 1).

Table 1. *Influences of the vehicle and the ratio of cis:trans -isomers on the acute oral LD_{50} of pyrethroids (FAO according by Litchfield 1985)*

Pyrethroid	Isomer ratio cis:trans	Species	Vehicle	LD_{50} (mg/kg)
Permethrin	(40:60)	Rat	Water	2949
Permethrin	(40:60)	Rat	DMSO	1500
Permethrin	(40:60)	Rat	Corn oil	500
Permethrin	(40:60)	Mouse	Water	4000
Permethrin	(40:60)	Mouse	Corn oil	540
Cypermethrin	(40:60)	Mouse	Aq. Susp	779
Cypermethrin	(50:50)	Mouse	DMSO	138
Cypermethrin	(50:50)	Mouse	Corn oil	82
Permethrin	(80:20)	Rat	Corn oil	224
Permethrin	(60:40)	Rat	Corn oil	445
Cypermethrin	(90:10)	Rat	Corn oil	367
Cypermethrin	(40:60)	Rat	Corn oil	891

Pyrethroids are very rapidly metabolised in mammals. Commercial pyrethroids have an ester group that seems to be a very important structure in the molecule. *In vivo* in mammals the primary detoxification step is ester cleavage, possibly by non-specific carboxylases, followed by hydroxylation reactions in cytochrome-P450 system and by several conjugation reactions (Kaneko et al. 1981, Heder et al. 2001). The low toxicity in mammals suggests that the metabolites are not significantly toxic. Only lipophilic unmetabolised pyrethroids are active in having their effects on neural membranes (Shaw and Chadwick 1998). The metabolic pathway results in a significant increase in the water solubility of pyrethroids, thus facilitating their rapid excretion in the urine. The biotransformation of pyrethroids mainly occurs in the liver. Major metabolic reactions, such as ester cleavage, hydroxylation, and formation of hydrophilic conjugates, have also been detected in mesenteric lymph nodes in e.g. rats and mice. The metabolic activity in new-born and young animals is not so effective as in adults, being significantly lower just after the birth (Miyamoto et al. 1995, Kale et al. 1999).

Although the pyrethroids are lipophilic, no bioaccumulation has been observed after subacute dosing to mammals (Miyamoto et al. 1995). According to Litchfield about 90% of the permethrin dose in rats is eliminated in the urine and faeces within three days after administration. A large proportion of the total elimination occurs during the first 24 hours, and nothing could be detected after 7 weeks in the adipose tissues. Absorption of cypermethrin from the gastrointestinal tract and its final elimination are quite rapid. E.g. cyclopropane moiety in the rat over a 7-day period ranged about 60% in the urine and 50% in the faeces. An alpha cyano group was, however, eliminated more slowly (Litchfield 1985).

The ambient temperature has a profound effect on the insecticidal activity of pyrethroids, an effect that is augmented by lowering the temperature. This negative temperature dependence has been ascribed to the slow metabolism of pyrethroids in insects at low temperature. However, recent studies have shown that the metabolic factor is not so important as the increased sensitivity of the nerve cell membrane to the insecticide at low temperatures (Salgado et al. 1989, Narahashi 1996, Ginsburg and Narahashi 1999, Motomura and Narahashi 2000).

Synergists, e.g. piperonyl butoxide (PBO), are commonly used as constituents of pyrethrin and pyrethroid products in the control of insects. PBO increases the toxicity of all pyrethroids to houseflies and some other insects. The effect is based on the PBO-induced inhibition of the oxidative degradation of pyrethroids in insects. PBO enhances the potency of pyrethroid esters in the 10 to 300-fold range (Casida et al. 1983, Matsumura 1985).

2. Acute toxicity of pyrethroids

Compared to many other insecticides pyrethroids are considered safe because they do not accumulate in the environment and have low mammalian toxicity. However, there is evidence that pyrethroid compounds may not be as safe as claimed previously (Leng et al. 2003). Case reports of systemic poisonings of humans have shown that the pharmacotherapy of pyrethroids of pyrethroids is difficult, and the duration of poisoning can be unexpectedly long (Leake et al. 1985, Miyamoto 1993, Peter et al. 1996, Brölsch et al. 2001, Cameron and Hill 2002). In connection with systemic poisoning, anticonvulsants have been used, but their efficacy is low (Wilks 2000). Pyrethroids belong to a class of compounds in which the rate of absorption is a crucial factor in determining their toxicity. There is a huge difference between the oral and the intravenous toxicities of pyrethroids. The steady-state *in vivo* concentration determined by the absorption and detoxification rates determines whether any symptoms occur. The lipophilicity of pyrethroids enables rapid access to tissues, including the central nervous system. Intraperitoneal dosing (5-8 mg/kg) of deltamethrin containing an alpha cyano group, has caused concentrations of 0.12-0.45 nMol/g in rat brain. These concentrations have caused the first neural symptoms in acute exposure (Aldridge 1990).

Pyrethroids can develop a variety of toxic symptoms in mammals. Traditionally, these symptoms are classified into Type I and Type II (Table 2). The toxic symptoms of pyrethroids detected in animal experiments are often considered to be similar to those in human intoxication (Miyamoto 1993).

Table 2. *Toxic symptoms (Type I and Type II) and chemical properties of technical pyrethroids in experimental rats (Ecobichon 1996)*

Type	Pyrethroid	*cis:trans*	LD_{50} mg/kg oral	Water solubility mg/l	Symptoms
I: no alpha cyano group	Pyrethrin Permethrin	20:80	584-900 >6000	Insoluble 0.2	"T-syndrome" Aggressive sparring Increased sensitivity to external stimuli Prostration Tremor
	Allethrin Tetramethrin				
II: with alpha cyano group	Cypermethrin Deltamethrin Fenvalerate	47:44	200-800	1.0	"CNC- syndrome": Salivation Choreoathetosis Pawning and burrowing

Acute toxicity associated with the pyrethrins seems to be based on the allergenic properties of pyrethroids. Human exposure to the natural pyrethrins

causes contact dermatitis (from local erythrema to severe vesicular eruption) asthma-like attacks, and anaphylactic reactions (Mccord et al. 1921, Mitchell et al. 1972). The dermal absorption of pyrethrins is slow and usually prevents systemic poisoning, although a significant reservoir of pyrethrin may remain bound to the epidermis. Dermal absorption and poisoning can be avoided by decontamination of the skin (Ray and Forshaw 2000).

There has been little evidence of the allergic-type reactions in human subjects exposed to synthetic pyrethroids. Immunologic and allergic properties of synthetic pyrethroids in combination with the synergist PBO have been detected in human lymphocytes and basophiles *in vitro*. Immunosuppressive effects have been found with deltamethrin, cypermethrin and permethrin (Kowalczyk-Bronisz et al. 1990, Madsen et al. 1996, Diel et al. 1998, Diel et al. 1999). A notable form of toxicity with synthetic pyrethroids has been cutaneous paresthesia observed in workers spraying pyrethroids containing an alpha cyano group (He et al. 1989, Chen et al. 1991, Altenkirch et al. 1996, O'Malley 1997).The symptoms were impairment of facial sensation and dizziness combined with a burning, itching or tingling sensation of the exposed skin. The signs and symptoms disappeared in 24 hours after exposure. The paresthesia developed several hours after the exposure and continued for 12-18 hours (Tucker and Flannigan 1983). Paresthesia occurs because of a direct effect of pyrethroids on intracutaneous nerve endings. The induction of paresthesia depends on the neurotoxic potency of the pyrethroid compounds. Doses sufficient to cause paresthesia are far lower than those causing systemic toxicity. Skin paresthesia is a unique toxic symptom caused by pyrethroids, and no detailed molecular mechanism has been proposed yet. Repetitive firing in peripheral skin nerves has been postulated to be a possible mechanism (Miyamoto 1993).

3. Chronic toxicity of pyrethroids

There is evidence of chronic consequences and irreversible injuries in patients with acute pyrethroid intoxication even when the metabolism of the pyrethroids is effective. Complications frequently observed after more than 2 years have been: cerebro-organic disorders (personality disorders), visual disturbances, tinnitus, sensomotor polyneuropathy in the lower legs, and vegetative nervous disorders (paroxysmal tachycardia, increased heat sensitivity). Many of these patients exhibit pathological autoimmune findings and develop autoimmune diseases, e.g. autoimmune haemolysis (Muller-Mohnssen 1999).

Non-specific neuronal symptoms appeared in the Persian Gulf War veterans exposed to a mixture of insecticides used to protect their health e.g. against malaria. The emergence of such symptoms may be due to simultaneous exposure to multiple agents, e.g. the insecticide permethrin, the anti-nerve agent PB (pyridostigmine bromide), and the insect repellent DEET (N, N-diethyl-m-toluamide). Combinations of these agents produced more neurotoxic symptoms

than those caused by individual agents. The combination of three agents caused neuropathological lesions. After the Persian Gulf War about 5% of the veterans have complained of neurological symptoms of unknown ethiology (Abou-Donia et al. 1996).

Paroxysmal epileptic activity has been found to appear after chronic exposure to cypermethrin (Condes-Lara et al. 1999). Although actual Parkinsonism has not been observed, there are findings which confirm the fact that dopaminergic neurotransmission is affected by exposure to pyrethroid. This may contribute to the overall spectrum of the neurotoxicity of pyrethroids (Karen et al. 2001).

The developing blood brain barrier (BBB) is highly vulnerable to a single exposure (1/50th of LD50) or repeated exposures to cypermethrin. During brain development in rats, even after withdrawal of treatment, some neurological dysfunctions have appeared later in the life. In adult rats, no effect on BBB permeability was seen even at high dose levels of pyrethroids (1/25th of LD50) and in combination with PBO (600 mg/kg, i.p.) (Gupta et al. 1999).

Inhibition of cell cycle progress during mitosis has been found in human lymphocyte cultures. The induction of abnormal cell divisions may be due to a mechanism similar to that of the well-known spindle poison colchicine. Pyrethroids can induce chromosomal aberrations, sister chromatid exchanges and micronuclei in mammalian cell cultures, including human cells (Agarwal et al. 1994). An increasing amount of micronuclei in the bone marrow of the rat and the mouse has also been found after oral or intravenous application of deltamethrin (Bolognesi et al. 1993, Barrueco et al. 1994, Surralles et al. 1995).

With respect to their carcinogenic risk, most pyrethroids are placed into category 3 (enough evidence for being classified as carcinogenic) by the International Agency for Research on Cancer (IARC 1991). Due to the genotoxic potential of pyrethroids, induction of abnormal cell division may have to be considered (Gandhi et al. 1995).

4. Neurotoxic mechanisms of pyrethroids

4.1. Neural membrane as a target of pyrethroids

A fluid-mosaic model has been generally accepted as the basic structure of the cell membrane (Singer and Nicolson 1972). The membrane is a bilayer made up of lipids (mostly phospholipids and cholesterol) and proteins embedded in the membrane. Proteins exist as separate globular units. Many proteins extend through the membrane (integral proteins), whereas others (peripheral proteins) are more loosely bound to the membrane. The membrane proteins carry out many functions, e.g. as carriers, receptors and ion channels.

The fluidity of the membrane has been shown to be dependent on the cholesterol content and on the orientation of phospholipid molecules in the

membrane (Macdonald et al. 1988). Pyrethroid insecticide Type I (allethrin) modifies the bilayer phospholipid order. This may be due to a possible aggregation of the insecticide in the lipid bilayer, creating special domains with a consequent increase in membrane disorder and instability. Experiments with permethrin demonstrate similar results and show that it increases membrane fluidity by its preferential localisation in the hydrophobic core of the membrane (Moya-Quiles et al. 1995, Moya-Quiles et al. 1996). The lipophilicity of pyrethroids allows rapid access to tissues, including the nervous system (Verschoyle and Aldridge 1980, Aldridge 1990), and incorporation into the cell membranes of the nervous tissue.

Differences in the permeability of the cell membrane for certain ions are due to specialised integral proteins. Integral proteins act e.g. as ion channels, some of which are continuously open, whereas others have gates that are opened and closed by alterations in the membrane potential. A voltage-gated channel is the Na^+ channel. Some integral proteins are carrier proteins that bind ions and other molecules and then change their configuration, moving the bound molecule from one side of the cell membrane to the other. When carrier proteins move substances, e.g. ions, against their electric and chemical gradients, the transport requires energy, which is provided by the hydrolysis of ATP catalysed by ATPases. Carrier proteins are e.g. Na^+, K^+ ATPase and Mg^{2+} ATPase.

Na^+, K^+ ATPase consists of an α subunit and a β subunit. Both of them extend through the cell membrane (Figure 3).
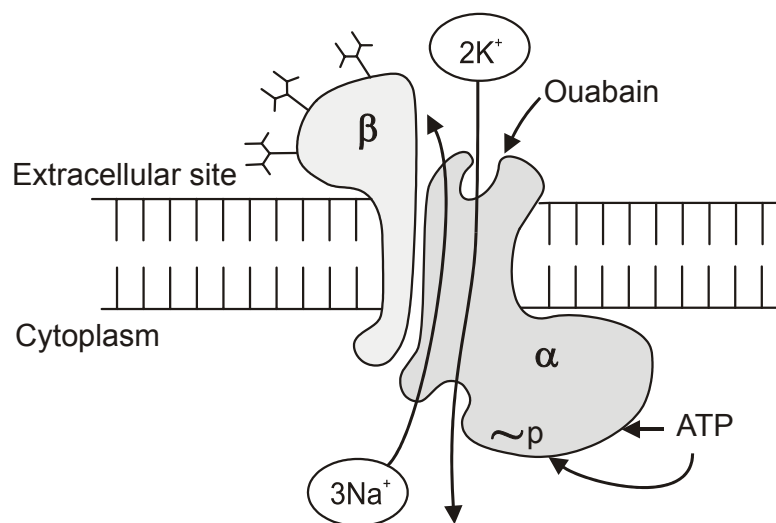
Figure 3. *The structure of Na^+, K^+ ATPase*

Na^+ and K^+ transport occurs through the α subunit. The β subunit is a glycoprotein. Only the α subunit has been shown to contain the binding sites for ATP and ouabain. Ouabain is known to bind specifically to the catalytic subunit of the enzyme Na^+, K^+ ATPase (Herrera et al. 1987). It is required to maintain the osmotic balance across cell membranes by keeping the intracellular

concentration of Na^+ low. Na^+, K^+ ATPase moves three Na^+ ions out of the cell in exchange for two K^+ ions, which it pumps into the cell. It tends to cause an electric potential across the membrane, with the inside negative compared to the outside. The balancing role is largely by K^+, which is actively pumped into the cell by Na^+, K^+ ATPase but can also move freely. It has been suggested that, in neural cells. Na^+, K^+ ATPase activity may also be a subject to modulation by receptor-mediated messenger pathways (Bertorello and Aperia 1989).

ATP-hydrolysing enzymes have been found to be sensitive to the actions of pyrethroid insecticides (Dorman and Beasley 1991). The neural membrane ATPase was target for pyrethroids in the invertebrate cockroach (Clark and Matsumura 1987), in rat synaptosomes (Rao et al. 1984), and in the developing rat brain (Malaviya et al. 1993, Husain et al. 1994). As lipophilic compounds, pyrethroids can incoporate into the lipid bilayer in the neural tissue. This can disturb the phospholipid orientation and cause changes in the fluidity of the membrane. Disturbances in Na^+, K^+ ATPase activity and changes in the membrane fluidity have been found to be the mechanism which mediate the neurotoxicity of many lipid soluble compounds such as organic solvents (Korpela and Tähti 1988, Engelke et al. 1992, Vaalavirta and Tähti 1995, Tähti et al. 1999).

4.2. Effects of pyrethroids on sodium channel

A variety of chemicals and toxins has been found to affect the ionic permeability. Many toxic agents are known to bind to sodium channels and to alter their properties. They can block the channels or change the channel activity. The main target of pyrethroids in invertebrates as well as in vertebrates has been shown to be sodium channels (Narahashi 1962, Jacques et al. 1980, Narahashi 1985, Chinn and Narahashi 1986, Narahashi 1996, Zlotkin 1999). Because of the highly hydrophilic nature of pyrethroids, the subunit of the sodium channel to which pyrethroids may bind is uncertain (Soderlund et al. 1983, Narahashi 2000). As a consequence of the complexity of the mammalian nervous system, studies on intact animals have not yielded conclusive fundamental information, concerning the mechanism of action of pyrethroids (Ecobichon 1996).

Pyrethroids display high affinity to Na^+ channels and evoke their toxic effects through changes in the functions of these channels. It is probable that the large variety of signs and symptoms as well as the biochemical and physiological changes are a consequence of the primary interaction of pyrethroids with the sodium channel (Aldridge 1990).The binding of pyrethroids to Na^+ channels causes a prolonged channel opening. Prolonged sodium current causes a depolarising after-potential. When the depolarising after-potential reaches the excitation threshold, repetitive activity can be initiated in various regions of the nervous system. Neuromuscular disorders characterised by hyper-excitation, ataxia, convulsions and eventually paralysis can be induced by pyrethroid exposure. There is evidence that an alteration of only 1% of the total number of

Na$^+$ channels is sufficient to induce repetitive discharges and hence the symptoms of poisoning in animals (Cooper et al. 1984, Narahashi et al. 1995, Narahashi 1996, Narahashi 2000). A single-channel analysis using neuroblastoma cells (N1E-115) clearly indicated that sodium channels in the presence of pyrethroid deltamethrin or fenvalerate were kept open for several seconds, compared to the normal opening of a few milliseconds (Holloway et al. 1989). Type I and Type II pyrethroids have shown several important features of their different mechanisms of action on the sodium channel (Vijverberg and van den Bercken 1990, Narahashi 1996). Type I pyrethroids affect sodium channels, causing repetitive neuronal (sensory, motor) discharges and a prolonged negative after-potential, as well as slight increases in the time constant for sodium current in activation (Joy 1994). Type II pyrethroids are more potent but slower in action than Type I pyrethroids. The prolongation of single sodium channel currents is more pronounced in the exposure of Type II pyrethroids than in those of Type I. Type II pyrethroids slow the deactivation (the tail currents upon repolarisation) of sodium channels to a greater extent than those of Type I, leading to a persistent depolarisation of the nerve membrane, which leads to conduction block. (Narahashi et al. 1998). This depolarising action can have a dramatic effect on the sensory nervous system, which results in an increase in the number of repeated discharges (Vijverberg et al. 1982, Holloway et al. 1989, Vijverberg and van den Bercken 1990). This alone could account for the tickling and burning sensation felt on the exposed skin. In addition, a slight depolarisation at presynaptic nerve terminals results in an increased release of the neurotransmitter, serious disturbance of synaptic transmission and generation of symptoms associated with Type II pyrethroids (Norton 1986, Ecobichon 1996).

Some authors assert that this difference in behaviour between the two groups of pyrethroids can be linked not only to the different binding affinity to the site of sodium channels, but also to the physicochemical properties, e.g. lipophilicity, which permit these compounds to remain in the membrane (Motomura and Narahashi 2001).

5. Oxidative stress as a toxic mechanism of pyrethroids

Some studies of pyrethroids indicate cell stress that alters antioxidant enzymes. Alteration in antioxidant enzymes suggests involvement of free radical intermediates in pyrethroid toxicity. In addition, evidence of cell stress was observed in reduced mitochondrial function in mouse brain tissue after permethrin exposure *in vivo* (Kale et al. 1999, Karen et al. 2001). The cleavage of cypermethrin, fenvalerate and the ester metabolites of fenvalerate releases cyanohydrins, which are unstable in physiological conditions and decompose to cyanides and aldehydes. These may produce free radicals and thus cause oxidative stress. The toxic effects of oxygen free radicals and reactive oxygen compounds can lead to a wide variety of harmful conditions, including aging,

cancer, atherosclerosis and stroke (Kale et al. 1999, Grajeda-Cota et al. 2004). Cypermethrin and fenvalerate have been shown to induce oxidative stress and alterations in the antioxidant enzymes in erythrocytes. Erythrocytes may be susceptible to oxidative damage due to the presence of polyunsaturated fatty acids, heme iron and oxygen. Fenvalerate-treated mice showed thyroid dysfunction and an increase in liver and kidney lipid peroxidation due to the generation of free radicals (Maiti et al. 1995, Giray et al. 2001).

Some attempts have been made to use antioxidants as antidotes in pyrethroid intoxication. Vitamin E oils have been used for the prophylactic treatment of paresthesia caused by pyrethroids. Vitamin E is a biological antioxidant which prevents accumulation of peroxides and protects cells from the damaging effects of free radicals (Tucker and Flannigan 1983, Eitenmiller 1997, Kimmick et al. 1997). It prevents pyrethroid-induced oxidative stress, even through the mechanism has not been elucidated yet (Kale et al. 1999, Giray et al. 2001). There is evidence that vitamin E can e.g. block the pyrethroid-modified portion of sodium current without any effect on the normal sodium channel currents (Narahashi 2000). The role of vitamin C in the prevention of disease and malignancy has been studied over several decades (Halliwell 2001, Song et al. 2001). It has been suggested that ascorbic acid protects against gastric cancer (Feiz and Morbarhan 2002). The presence of vitamin C reduces lipid peroxidation produced by cypermethrin, increases the stability of the membrane, and decreases the liberation of cytoplasmic enzymes (Grajeda-Cota et al. 2004).

6. Endocrine disrupting effects

Endocrine disruption has emerged as an environmental issue, based on the hypothesis that exposure to certain environmental chemicals alters the endocrine system, increases the incidence of endocrine diseases and disorders, and affects adversely the development in both human and wildlife. Colborn and coworkers have listed up pyrethroids as endocrine-disrupting chemicals (EDC) (Colborn et al. 1993, Ankley et al. 1997).

Endocrine disrupters form a group of compounds which may alter the amount of hormones inappropriately and mimic or block their action. Research on the effects of these chemicals has not been conclusive, but mounting evidence links endocrine disrupters to a range of reproductive problems, including e.g. testicular and prostate cancer of the male, and miscarriage, as well as breast, ovarian and uterine cancer of the female. Reproductive dysfunction involving exposure to hormonally activity has been implicated in human breast cancer (Wolff et al. 1993, Hunter et al. 1997). However, there is considerable uncertainty regarding the relationship between adverse health outcomes and exposure to environmental endocrine disrupters (Kavlock 1999).

The endocrine-disrupting chemicals include numerous pesticides. The most common are DDT, lindane, malathion and some pyrethroids. Especially

synthetic pyrethroids are suspected of disrupting endocrine functions. Pyrethroid exposures have been associated with acute reproductive effects and may produce chronic and developmental impairments. There is evidence a strong association between foetal death due to congenital anomalies and residential exposure to pyrethroids (5 fold risk during the 3rd-8th week of pregnancy (Bell et al. 2001a, Bell et al. 2001b). Recently, several *in vitro* studies have evaluated the interaction of pyrethroids with the oestrogen/androgen receptor. However, the findings in these studies are inconsistent. Some results are positive, showing hormone-like effects (Garey and Wolff 1998, Go et al. 1999, Kasat et al. 2002, Chen et al. 2002), while others were negative (Gaido et al. 1997, Nishihara et al. 2000, Saito et al. 2000a, Saito et al. 2000b, Sumida et al. 2001). Pyrethroid compounds may possess critical structural properties similar to oestrogen metabolites. Oestrogen and its metabolites are thought to affect DNA and to alter the synthesis of metabolic enzymes (Service 1998).

The WHO has reported that fenvalerate and permethrin did not cause any impairment in the reproductive performance of rats in a two-generation study (WHO 1999a, WHO 1999b). Fenvalerate and permethrin showed neither antagonist nor agonist effects on the androgen receptor or the oestrogen receptor. These negative results are consistent with some of the above *in vitro* studies. On the other hand, there are very few *in vivo* studies focusing on the endocrine-disrupting ability of pyrethroids (Narahashi et al. 1998, Lambright et al. 2000, O'Connor et al. 2000, Kunimatsu et al. 2002).

The aims of the present study

Epidemiological accounts, clinical reports, and experimental studies indicate that pyrethroids can elicit neurotoxic effects in humans. Their insecticidal efficacy is based on their action on the sodium channel in the neural membrane. In mammals, the ion channel is much less sensitive, and the neurotoxicity may also depend on other membrane effects.

Pyrethroid exposures have also been associated with hormone-like effects, but the mechanism has not been elucidated yet.

The purpose of the present study was

1. to investigate the effects of pyrethrin and PBO (a well-known synergistic compound to pyrethrin) on the rat brain synaptosomal integral enzyme ATPase

2. to investigate the effects of the chemically different synthetic pyrethroids permethrin and cypermethrin on the rat brain synaptosomal integral enzyme ATPase

3. to evaluate the toxicity of pyrethrin, permethrin and cypermethrin in a human neural cell culture (SH-SY5Y neuroblastoma cell line)

4. to evaluate the hormone-like effects of pyrethroids in a human breast carcinoma cell culture (MCF-7 cell line)

Materials and methods

1. Pyrethroid compounds (I, II, III, IV)

Four commercial pyrethroid products were studied: Biospray S, Biospray, Biokill, and Ripcord. Biospray S (Kemira) contained pyrethrin 100 g/l as an active compound, Biospray (Kemira) contained pyrethrin and piperonyl butoxide (PBO) 1:4, Biokill (Jesmond Ltd, England) contained permethrin 2.5 g/l, and Ripcord (American Cyanamid Company, USA) cypermethrin 100g /l and a mixture of xylene and petrol 820 g/l. (Table 3)

Table 3. *The pyrethroids used in the present study according to certificate analyses by Kemira and Berner*

Commercial compound	Active ingredients	Alpha-cyano group -C=N	LD_{50} mg/kg rat dermal	LD_{50} mg/kg rat oral
Biospray	pyrethrin +PBO**(1:4)	no	>1500	584-900 7500
Biospray S	pyrethrin	no	>2000	>2000
Biokill	permethrin*	no	>5000	>2000
Ripcord	cypermethrin*	yes	>1734	>2000

** piperonyl butoxide (synergist)
* synthetic pyrethroid

2. Experimental animals and synaptosome preparation (I, II)

Sprague Dawley rats (males, body weight 200-300g) were used for isolation of the synaptosomal membranes of the brain. The rats were grown in standardised conditions in the animal laboratory of the Medical School, University of Tampere. Constant humidity, constant ambient temperature and a regular light /dark cycle were maintained. The rats were given standard food and tap water ad libitum. After the rats were decapitated, the cerebrum was gently separated from the cerebellum, brain stem and meninges. The cells of the cerebrum were dissociated, and the synaptosomal membranes were isolated in a non-toxic isoosmotic Percoll gradient system (Nagy and Delgado-Escueta 1984).

3. Protein determinations (I,II)

The total protein contents of the synaptosome preparations were determined by a commercial method of Pierce (BCA* Protein Assay). It is a highly sensitive method for spectrophotometric determinations of protein concentration, based on a bicinchoninic acid (BCA) reagent. The protein contents in individual synaptosome preparations were 0.35 ± 0.05 mg/ml on an average.

4. ATPase measurements in the exposure of synaptosomes to pyrethroids (I, II)

In the determination of the ATPase activities, a modification of the discontinuous method based on the determination of inorganic phosphate was used (Phillips and Hayes 1989). Different concentrations of the test substances were added in to the 2 ml reaction mixture: 50 mM Tris-HCl- buffer pH 7.6, 1.5 mM ATP (Sigma Chemical Co), 5 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, and 0.1 ml synaptosome suspension. The tubes were tightly stoppered, mixed and incubated for 1 h at 37°C with continuous shaking. The total ATPase activity was measured with sodium (Na^+), potassium (K^+) and magnesium (Mg^{2+}) present in the reaction mixture. The activity of Mg^{2+} ATPase was only determined in the presence of Mg^{2+}ions in the reaction mixture (50 mM Tris-HCl-buffer pH 7.6, 1.5 mM ATP, 15 mM $MgCl_2$ and 0.1 ml synaptosome suspension). The difference between the total and the Mg^{2+} -activated ATPase gave the activity of the Na^+, K^+ ATPase. After 1-h incubation, 2 ml ice-cold 10% TCA was added, followed by centrifugation at 800 x g for 10 min. Into 1 ml supernatant, 0.2 ml of the reagent (1% $(NH_4)_{6Mo7O24}$ 4 H_2O/ 0.5 N H_2SO_4 solution), and 2 ml of 1% ascorbic acid were added. After a 25 min reaction time, the absorbances of the samples were read at 770 nm. The enzyme activities were expressed as moles of inorganic phosphate formed (µmol Pi/hr/mg prot). To compare the effects of the test compounds, the enzyme activities were calculated as % of control, and the ATPase activities of the samples treated were given as percentages of the activities of the control samples.

5. Cell cultures and exposure to pyrethroids (III, IV)

5.1. SH-SY5Y cell cultures (III)

SH-SY5Y Neuroblastoma Cell Line (ATCC Catalogue No. CRL-2266) was used. SH-SY5Y is a thrice-cloned subline with a relatively stable neuroblastic phenotype.

Nunc 75 cm^2 flasks (Cat. No.156472) and Nunclon 96-MicroWell Plates (Cat. No. 167008) were used for the cell cultures. The cell culture reagents and solutions came from Gibco, Paisley: Minimum Essential Medium with Earle's salts, w/o L-Glutamine (Gibco 21090-022), Nutrient Mixture F-12K Kaighn's Modification L-Glutamine (F-12K) (Gibco 21127-022), Nonessential amino acids (Gibco 25030-024), Penicillium G, Streptomycin, Amphotericin B as Fungizone® (Gibco 15240-035), Foetal Bovine Serum (Gibco 10106-169), and L-glutamine (Gibco 25030-024).

For exposure to pyrethroids and viability measurement, passages 32-33 of SH-SY5Y cells were seeded at a density of 40000 cells /well to 96- microwell plates, 100 µl / well, and grown in 1:1 MEM /Ham's F12K medium with 0.1 mM nonessential amino acids and 2 mM L-glutamine, 100 U/ml Penicillium G, 25 µg/ml Amphotericin B, and 10% foetal bovine serum. The cells were grown for 24 h at 37°C, and, before exposure, the medium was changed in to a serum-free one and incubated for 30 min at 37°C. The different test substances were added into the wells, and the cells were grown for further 24 h. The molar concentration means the concentration of the component of commercial product in the culture medium. The exposure was stopped by adding 10% TCA into the wells. The plates were frozen at -75°C.

5.2. MCF-7 cell cultures (IV)

The cell line was a human breast carcinoma cell line. MCF-7 was purchased from American Type Culture Collection (ATCC Catalogue No. HTB-22).

Nunc 75 cm^2 flasks (Cat. No. 156472) and Nunclon 96- MicroWell Plates (Cat. No. 167008) were used for the cell cultures. The cell culture reagents were as follows: Dulbecco´s modified Eagle's medium/ Nutrient mixture F-12 Ham (DMEM/F 12) medium (high glucose with 2,5 mM L-glutamine) with phenol red (Sigma D 2906), Antibiotic/antimycotic solution (Gibco 15240-035), Foetal Bovine Serum (Gibco 10106-169), Dextran-Coated Charcoal Serum (DCC, Gibco 10106-169), Insulin (Sigma I-4011), and 17-β-Oestradiol (Sigma E-2758).

MCF-7 cells were cultured according to the instructions of ATCC in high-glucose DMEM/F 12 medium with 10% foetal bovine serum and 10 ng/ml insulin. The cells were split 1:4 after 80% confluence. MCF-7 cells were seeded at a density of 5000 cells /well to 96- microwell plates, 100 µl medium well, and they were grown in DMEM / F-12 medium with 100 U/ml penicillin, 100 µg/ml streptomycin, 10 ng/ml insulin, and 5% DCC. The cells were then grown for 24 h at 37°C with 5% CO_2 in a humidified incubator.

The test compounds were suspended in the growth medium. Before exposure, the medium in cell culture wells was partly removed, and the different test substances at final concentrations with 0,1 nM oestradiol were added in to the wells. The cells were then grown for further 7 days. The exposure was stopped by adding 10% TCA into the wells (0,5% final concentration). The plates were deep-frozen at -75°C.

6. Cell viability tests

6.1. The bioluminescent assay for ATP determinations (III, IV)

ATP measurement is based on oxidation of the light-emitting luciferin in the ATP-dependent reaction. The bioluminescent method utilises the enzyme luciferase, which catalyses the formation of light from ATP and luciferin (DeLuca and McElroy 1974, Gould and Subramani 1988, Baldwin 1996). The efficiency of the reaction reflects the cellular ATP levels measured with luminescence.

   After 24-h exposure (SH-SY5Y cell line) and 7-d exposure (MCF-7 cell line), 100 µl of a mixture of Tris-Acetate buffer and ATP monitoring reagent (1:5) was added into the wells. The plates were shaken and measured with ThermoLabsystems Luminoskan Ascent using a 1000 ms integration time. Black microwell plates for luminometric measurements came from ThermoLabsystems (Black Cliniplate No. 950 2867). The bioluminescent Assay of ATP by ThermoLabsystems (6415000 ATP Monitoring Reagent) was used.

6.2. Mitochondrial metabolic enzyme activity measurement (WST-1 test)(III, IV)

The WST-1 cell test is a colorimetric assay for the quantification of cell proliferation and cell viability. The evaluation of cell proliferation is based on the cleavage of tetrazolium salt WST-1 to formazan by various mitochondrial dehydrogenases in viable cells. The number of viable cells is proportional to the absorbance detected at the wavelength of 450 nm. The cytotoxicity test was based on the ready-to-use cell proliferation reagent WST-1 (Roche, Cat. No.1644 807) and an electronic coupling reagent, diluted in phosphate-buffered saline (Cook and Mitchell 1989, Berridge 1996).

   After 24-h exposure (SH-SY5Y cell line) and the 7-d exposure (MCF-7 cell line), 10 µl of WST-1 cell proliferation reagent was added to 100 µl of the medium in each well, and the plate was shaken for 1 min. The plates were incubated for 50 min in a humidified atmosphere, at 37° C, and the absorbances were measured at 450 nm wavelength with Multiskan MS (Labsystems).

7. Microscopic photographing of cell cultures

The cell cultures were microscopically examined and photographed at each exposure level. For digital photographing, an inverted phase-contrast microscope (Olympus CK40) was used. The digital camera was Olympus DP 10 (with 200x magnification.)

8. Statistical calculations

Three to five independent experiments at each dose level were performed with three parallels in each determination. The results were calculated as % of control so that the control values were 100%. The mean and standard errors of Mean (Mean ± S.E.M.) in independent experiments were calculated at each concentration level. In the statistical analyses, the unpaired Student `s t-test and one-way ANOVA with Dunnet´s post test (GraphPad Prism) were used.

9. Ethical considerations

All animal procedures (rat decapitation and isolation of synaptosomes) were approved by the ethical committee for animal experiments in the Tampere Medical School.

Results

1. Effects of pyrethroids and piperonyl butoxide (PBO) on synaptosomal ATPase activity (I, II)

The activity of the membrane-bound integral protein ATPase was determined at different concentrations of pyrethroids. The ATPase activities of the samples treated were given as percentages of the activities of control samples.
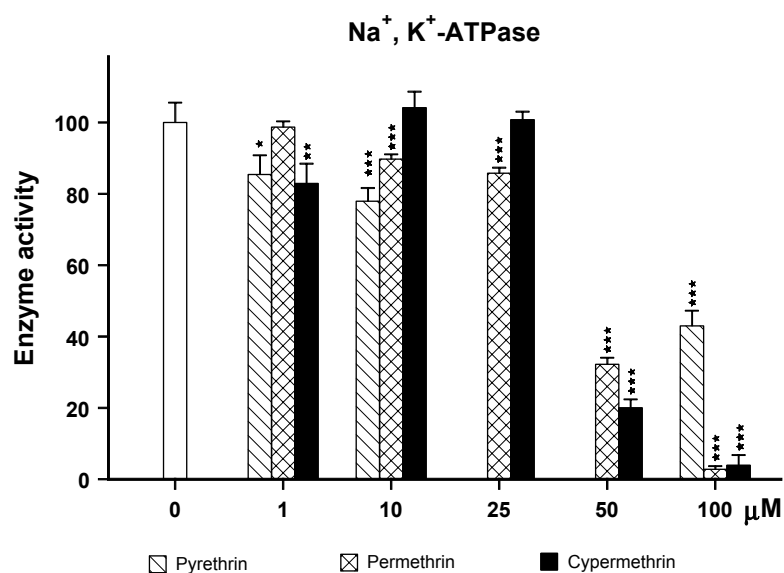
Na$^+$, K$^+$-ATPase

Figure 4. *The effects of pyrethrin, permethrin and cypermethrin on the activity of Na$^+$, K$^+$ ATPase. The enzyme activity is expressed as percentage of the control value (100% activity).*

At low concentrations (1-10 μM), pyrethrin (Biospray S) decreased dose-dependently the activity of Na$^+$, K$^+$ ATPase to 77%, and at high concentrations (100 μM) to 43% (Figure 4). Permethrin (Biokill) also decreased dose-dependently the activity of Na$^+$, K$^+$ ATPase similarly to pyrethrin, but more slightly. However, at high concentrations permethrin was very toxic (Figure 4). Pyrethrin did not affect Mg^{2+}-activated ATPase in concentrations of 1-10 μM, but the activity increased by 100% in high pyrethrin concentration (I). Exposure to different permethrin concentrations had a different effect on Mg^{2+}-activated

ATPase. At low concentrations, Mg^{2+}-activated ATPase increased in contrast to pyrethrin exposure (II).

Cypermethrin (Ripcord) did not cause any dose-dependent effect on the activity of Na^+, K^+ ATPase at lower concentrations, unlike pyrethrin and permethrin. In $1\,\mu M$ cypermethrin concentrations, the activity of Na^+, K^+ ATPase decreased, whereas the activity increased in $10\,\mu M$ concentrations. In high concentrations, cypermethrin was as toxic as permethrin (Figure 4). Exposure to different cypermethrin concentrations had an increasing effect on Mg^{2+}-activated ATPase, similarly to permethrin (II).

PBO potentiated effectively the effect of pyrethrin (Biospray S) in low concentrations, while PBO alone had no effect on the activity of total ATPase. The mixture of PBO and pyrethrin (Biospray) had no effect on the activity of Mg^{2+} ATPase at low concentrations, but over $10\,\mu M$ concentrations of pyrethrin combined with PBO were toxic (I).

2. Effects of pyrethroids on the viability of cultured neural SH-SY5Y cells, evaluated with ATP and WST-1 tests (III)

SH-SY5Y neuroblastoma cell cultures were used to study cell toxicity, which was evaluated by determining the total ATP content of the cells (Figure 5) and the activity of the mitochondrial metabolic enzymes (WST-1 test).
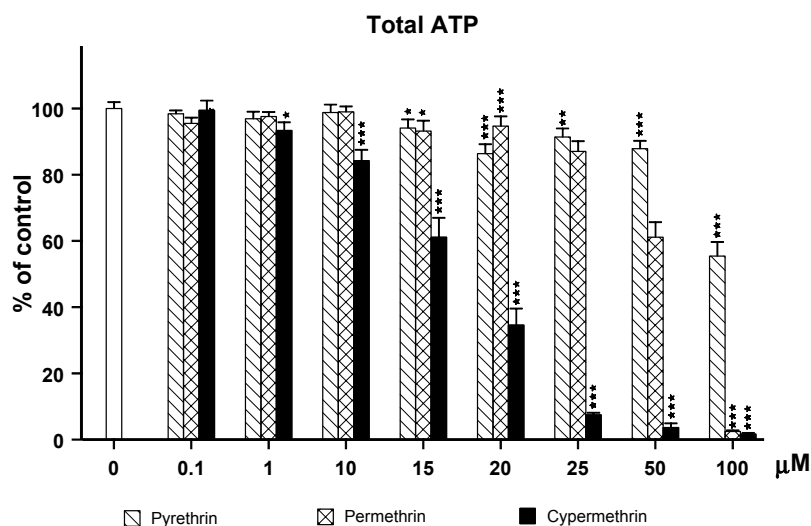
Figure 5. *The effect of pyrethrin, permethrin and cypermethrin on the total ATP in SH-SY5Y cell cultures. The enzyme activity is expressed as percentage of the control value (100 %).*

30

At low concentrations (0.1-10 μM), pyrethrin and permethrin had only a slightly decreasing effect on the total ATP. At high concentrations (50-100 μM), permethrin was more toxic than pyrethrin. However, the most toxic substance was cypermethrin (with an alpha cyano group). The toxicity of cypermethrin could be seen already in 1 μM concentration, and it was dose-dependent (Figure 5). The results obtained with WST -1 test cytotoxicity were quite different, compared to ATP test. At low concentrations, all pyrethroid compounds increased the mitochondrial metabolic enzyme activity, whereas at high concentrations, all pyrethroid compounds were toxic, as in the ATP test.

## 3. The cytotoxicity of pyrethroid compounds in SH-SY5Y cell cultures, evaluated with microscopic photographs (III)

Exposure to 0.1-100 μM of pyrethrin, permethrin and cypermethrin showed dose-dependent cytotoxicity in microscopic photographs. The cell toxicity was detected at lower concentrations than in the total ATP measurement. In visual examination of the cell cultures, the toxicity was first seen as appearance of intracellular vacuoles and aggregates of cells, later by the disappearance of neuritis, and eventually by rounding-up of cells. At the highest concentrations, only indefinite aggregates of damaged and dying cells were seen.

The most toxic pyrethroid was cypermethrin, followed by permethrin and pyrethrin. At 10 μM cypermethrin concentration, only vague aggregations of cells were seen. Interestingly, the total ATP was still about 84% of control at this concentration. At 15 μM permethrin concentration, the photographs showed clear changes in cell morphology. However, the amount of the total ATP at the same concentration was about 92%, compared with the controls. In pyrethrin-exposed cultures, signs of toxicity were also clearly seen at 15 μM concentration in microscopic photographs. In this concentration, the total ATP was 94% of control levels.

4. Comparison of the toxicities of pyrethroid compounds in neural (SH-SY5Y) and non-neural (MCF-7) cell lines (III, IV)

In the cell cultures of SH-SY5Y neuroblastoma cells and MCF-7 human breast carcinoma cells, the most toxic pyrethroid was cypermethrin with both methods (the cellular ATP content and WST-1). All pyrethroid compounds decreased the cellular ATP in SH-SY5Y cell cultures and increased it in MCF-7 cell cultures at low concentrations (0.1-1 μM). The pyrethroid compounds were not toxic to

SH-SY5Y cells at low concentrations, but at high concentrations (50-100 μM) they had a clearly decreasing effect on the total ATP (III)(Figure 6).
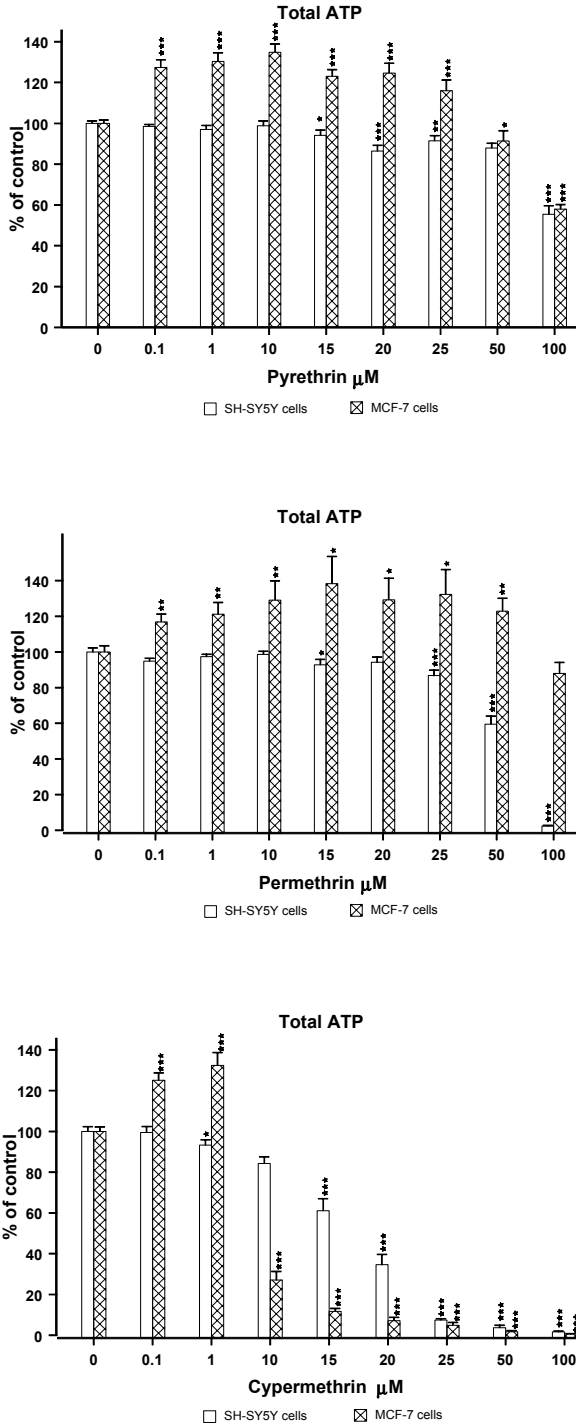
Figure 6. *The effect of pyrethrin, permethrin and cypermethrin on the total ATP in SH-SY5Y cell cultures and MCF-7 cell cultures.*

In the MCF-7 cell cultures at low concentrations all pyrethroid compounds increased the total ATP. Pyrethrin and cypermethrin increased the activation of the total ATP clearly, permethrin only slightly. At high concentrations, a decreasing effect was seen on the total ATP, similar to that in SH-SH5Y cell cultures. Cypermethrin decreased the total ATP clearly in MCF-7 cell cultures. At 10 μM concentration of cypermethrin the total ATP decreased 70% in MCF-7 cell line and only 10% in SH-SY5Y cell line (Figure 6).

The total ATP test revealed cytotoxicity more clearly than the WST test. Compared to ATP test, the results detected with WST-1 test were different in SH-SH5Y cell. At low concentrations, all pyrethroid compounds increased the mitochondrial metabolic enzyme activity (WST-1 test). At high concentrations, all pyrethroid compounds were toxic, like in the ATP test. In MCF-7 cell cultures, the WST-1 test did not show any significant changes of cell proliferation at < 50 μM permethrin and pyrethrin concentrations (IV).

5. The hormone-like effects of pyrethroid compounds in MCF-7 cell cultures (IV).

The combined effect of pyrethroid compounds and oestradiol on the cell proliferation was studied in the MCF-7 human breast carcinoma cell cultures. Cell proliferation was evaluated by determining the total ATP and the activity of mitochondrial metabolic enzymes (WST-1 test). Oestradiol concentration was 0.1 nM at every dose level of pyrethroid compounds.

In the coexposure of oestradiol with different concentrations of pyrethroids, there was a clear increase in the MCF-7 cell proliferation. The 0.1 μM concentration of pyrethrin with 0,1nM oestradiol increased the total ATP to 388% compared to the control (no pyrethroid compounds, no oestradiol). Without oestradiol, the ATP content increased to 127% of control. The corresponding values for cypermethrin were 373% (with oestradiol) and 125% (without oestradiol), and for permethrin 346% and 116%, respectively. Compared to the control (no oestradiol, no pyrethroids), oestradiol alone increased the cell proliferation by 280% in the ATP test and by 130% in the WST-1 test. Without oestradiol, the pyrethroids increased the proliferation slightly. High concentrations were cytotoxic, and the greatest cell toxicity was that of cypermethrin in both tests (IV).
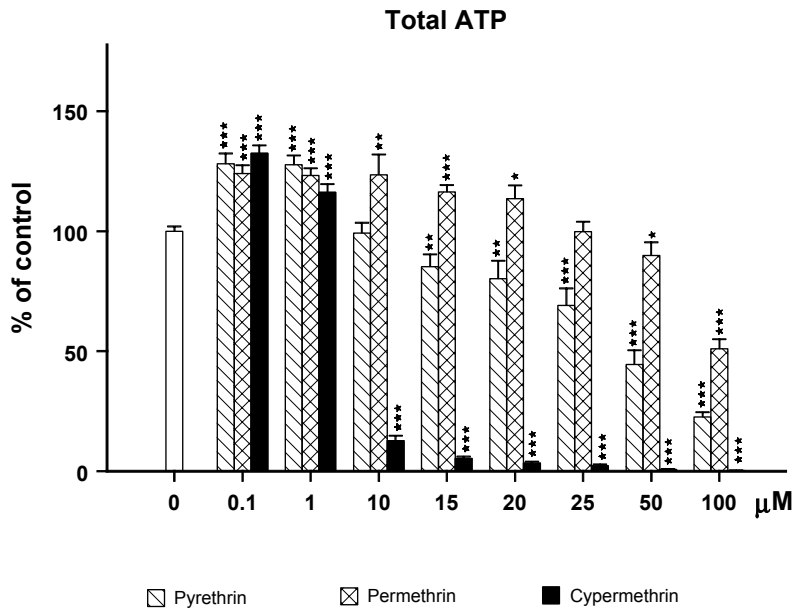
Figure 7. *The effect of pyrethroid compounds at different dose levels on the oestradiol induced proliferative effect in MCF-7 cell line. The effect is expressed as percentage of the control (containing 0.1 nM oestradiol).*

To describe the effect of pyrethroid on the cell proliferation caused by oestradiol, the ATP and WST-1 results of the coexposure were compared to the effect of oestradiol only (Figure 7). At low dose-levels, pyrethroid compounds increased significantly the proliferation of MCF-7 cells. At 0.1-1 μM concentrations pyrethrin and cypermethrin caused a significant increase in the ATP. Permethrin had the same effect at 0,1-20 μM concentrations. In WST-1 test no corresponding effect was seen. At high concentrations pyrethroid compounds were toxic.

Discussion

1. Neurotoxicity of pyrethroid compounds *in vitro*

In the present study, the effects of pyrethroid compounds on neural membrane structural proteins and on neural cell toxicity were evaluated *in vitro*. The plasma membrane is a selective barrier containing specific canals and pumps. The modifications of the physico-chemical state of phospholipids can modulate the activity of these proteins (Damjanovich et al. 2002). Changes in phospholipids modulate the membrane fluidity, which in turn influences the enzymatic activity and the functionality of receptors and channels in the cell membrane. Increase of fluidity in the hydrophilic-hydrophobic region of the bilayer can influence the activity of Na^+, K^+ ATPase activity (Vaalavirta and Tähti 1995, Engelke et al. 1996, Nasuti et al. 2003). The activity of this enzyme is modulated by membrane lipids (Cornelius 1991).

In this study pyrethroid compounds had a decreasing effect on the activity of Na^+, K^+ ATPases in rat brain synaptosomal membranes. Pyrethrin and permethrin had a clear dose-dependent inhibiting effect on the activity of Na^+, K^+ ATPase. At lower concentrations pyrethrin was more toxic than permethrin. The effect of cypermethrin was quite different. It had a slightly decreasing effect on the activity of Na^+, K^+ ATPase at the lowest concentrations, no effect at the moderate concentrations and clear toxic effect at higher concentrations. The different results of cypermethrin compared to those of permethrin and pyrethrin may be due to the smaller lipid solubility of cypermethrin (Table 2). This could be related to the presence of an alpha cyano group, which limits the incorporation of cypermethrin into the membrane. There is evidence that cypermethrin, administered by gavage for two months in rats, induces alterations in erythrocytes, increasing the reduction of plasma membrane fluidity in the hydrophobic region of the bilayer where cypermethrin is preferentially localised (Gabbianelli et al. 2002). The decrease of membrane fluidity could induce changes of activities and conformation of membrane proteins, e.g. sodium channels (Nasuti et al. 2003). Tähti et al. (1999) have shown that the inhibition of ATPase activity by lipophilic compounds correlates with the changes in membrane fluidity.

Pyrethroids without an alpha cyano group could enter the membrane more easily than cypermethrin. They are incorporated among the polar heads of phospholipids and may reduce their orientation, thus making the membrane more fluid in this region. As a consequence of plasma membrane perturbation induced

by permethrin water permeability may increase (Nasuti et al. 2003). *In vivo* studies on deltamethrin have shown a decrease in Na^+, K^+ ATPase activity in rat brain (Husain et al. 1994).

The effect of pyrethroids on the activity of Mg^{2+} ATPase was quite different; our results showed an increasing effect with pyrethrin, permethrin and cypermethrin. Also *in vivo* in the study of Bhatnagar and Kataria (1997), Mg^{2+} ATPase was found to increase due to oral permethrin exposure in rats. However, the physiological significance of the increased Mg^{2+} ATPase activity requires further investigation. Magnesium is especially required as a catalyst for many intracellular enzymatic reactions, particularly those relating to carbohydrate metabolism. Mitochondria are known to be responsible for cellular energy production by catabolising carbohydrates. It was interesting that in this study also mitochondrial dehydrogenases were activated by pyrethroids (Publication III).

PBO, which is commonly used as a synergist with pyrethrin, increased significantly the toxicity of pyrethrin. A mixture of pyrethrin and PBO decreased the activity of Na^+, K^+ ATPase significantly more than pyrethrin alone even at low concentrations, while PBO alone had no effect on the activity of ATPase. The reason for this may be that the target of PBO is not the neural membrane but the molecule of pyrethrin itself. PBO may change the ability of pyrethrin molecules to incorporate in the membrane and consequently potentiate the effect of pyrethrin.

In the neuroblastoma (SH-SY5Y) cell cultures our results on the toxicity of pyrethroid compounds correlated well with the results on synaptosomal ATPases. At low pyrethroid concentrations only slight effects were seen in both measurements, whereas at higher concentrations the pyrethroids were clearly toxic. An exception was cypermethrin, which showed strong toxicity in SH-SY5Y cells being clearly toxic already at lower concentration than pyrethrin and permethrin. The dose-dependent cell toxicity of pyrethroids was clear in the total ATP test. The results obtained with WST-1 test were not as consistent. This may be due to the different endpoints of the tests. The WST-1 test is based on a specific metabolic process, and toxins disturbing mitochondrial consistency may affect the results. In this study, the WST-1 test showed a considerable reduction of mitochondrial metabolic enzyme activities at high concentrations. At low concentrations, however, a clear activation was evident with all pyrethroid compounds studied. Especially cypermethrin increased clearly the activity of mitochondrial dehydrogenases in WST-1 test. Similar results in WST-1 test at low concentrations of toxic substances has also been observed previously in other studies (Toimela and Tähti 2004), but the reason for this has not been clarified yet. In pyrethroid metabolism, reactive oxygen species are formed and pyrethroids may produce oxidative stress and alteration in the antioxidant enzymes (Kale et al. 1999). Cypermethrin may induce oxidative stress by forming aldehydes and other lipophilic conjugates (Grajeda-Cota et al. 2004). Evidence of cell stress has also been found in mice treated with high doses of permethrin: their mitochondrial functions were reduced (Karen et al. 2001).

Hence, increased activity of WST-1 may indicate oxidative stress and so be an early marker of toxicity.

In the MCF-7 cell cultures the toxic effects of pyrethroid compounds were different compared to SH-SY5Y cell cultures. At low concentrations all pyrethroid compounds increased the total ATP contrary to that in the SH-SH5Y cell line. At high concentrations, there was a decreasing effect on the total ATP similar to that in the SH-SY5Y cell cultures. However, cypermethrin was more toxic in MCF-7 cell cultures than in SH-SY5Y cell cultures, and the decreasing effect of cypermethrin on the total ATP was clearer. Differences in the results may be due to the different origin of cell lines and to different exposure time. SH-SY5Y cells are neurones, which are very sensitive and form the most important target of pyrethroids. The exposure time was longer in MCF-7 cell culture (7d) than SH-SY5Y cell cultures (24 h). In the longer exposures presumably some cells divide and some die in the same culture. The cytotoxicity seen in longer exposure cultures may also originate from the inhibition of growth, not from actual cell death. One explanation for the cell proliferation in MCF-7 cell cultures after pyrethroid exposure may be an oestrogen-like effect of these compounds. Pyrethroids have been reported to have an oestrogenic properties in MCF-7 cells, especially pyrethroids with an alpha cyano group. Hence, they may act as xenoestrogens (Go et al. 1999). Also in the present study, cypermethrin with an alpha cyano group had the strongest proliferating effect on MCF-7 cells.

In MCF-7 cell cultures, the WST-1 test did not show any significant changes in cell proliferation, contrary to SH-SY5Y cell cultures. It may be due to the weak effect of pyrethroids on MCF-7 cells in general. There is evidence that pyrethroids have structural components similar to oestrogen metabolites which are thought to affect cell DNA (Service 1998, Kasat et al. 2002).

In the present studies on the acute cell toxicity of pyrethroids, cypermethrin was the most toxic one. The alpha cyano group is usually connected to the toxicity of the pyrethroid in question, especially as regards the neurotoxicity of pyrethroids (Soderlund et al. 2002). The alpha cyano group -containing pyrethroids develop severe symptoms of skin paresthesia. However, free cyanide does not seem to be involved in the toxicity manifestation. Overall successive underlying events are apparently more complicated in Type II than in Type I compounds, although no detailed molecular mechanisms have been found yet (Kaneko et al. 1981, Miyamoto 1993, Miyamoto et al. 1995). Some authors assert that differences in behaviour between the two groups of pyrethroids can be linked to their different binding affinity to the site of sodium channel and also to their the physicochemical properties, e.g. lipophilicity, which is a consequence of increased lipid peroxidation (Motomura and Narahashi 2000). In general, pyrethroids with an alpha cyano group are more toxic, but even the isomeric form has an effect on the toxic potency of the pyrethroid compounds (Hill 1985, Walker 1996, Wilks 2000). In this study, an alpha cyano group had no important effect on the function of synaptosomal ATPase, but in the cell cultures

cypermethrin was the most toxic pyrethroid compound evaluated with mitochondrial metabolic activity and ATP content.

The microscopic photographs of the cell cultures of the neuroblastoma cell line (SH-SY5Y) exposed to the study compounds correlated well with the results obtained in WST-1 test and ATP test. In addition, photographs confirmed that the most toxic pyrethroid compound was the pyrethroid with an alpha cyano group, i.e. cypermethrin. In microscopic photographs of cell cultures, its cell toxicity was seen at lower concentrations than in the total ATP test and WST-1 test. It especially changed the morphology of neurons and of the whole neuron culture already at very low concentrations. This is an interesting new observation, suggesting that digital photographing of the exposed cell cultures shows alterations highly comparable with those in the biochemical cell toxicity tests.

2. Hormone-like effects of pyrethroids *in vitro*

Many pesticides show hormonal activity, and they have thus been classified as endocrine disruptors. The best known compound in this respect is DDT (Solomon and Schettler 2000). Pyrethroids are commonly used insecticides, but the mechanisms of their hormone-like effects are still unclarified. However, there is some evidence that pyrethroids have an endocrine-disrupting effect. Recently, it has been suggested that they have an oestrogenic potential (Garey and Wolff 1998, Go et al. 1999, Solomon and Schettler 2000). Pyrethroids show a similarity to polyphenoles and oestrogen metabolites, which may explain their oestrogenic potential (Service 1998). The neurotoxic potency of pyrethroids is based on the interaction with the neural membrane, which can lead to increased depolarisation and transmitter release. In general, chemicals that interfere with neurotransmitters have often similarities to hormones (Go et al. 1999, Solomon and Schettler 2000). Oestrogen and its metabolites have been thought to affect DNA binding, to induce DNA damage and DNA repair, and to alter the biotransformation enzymes (Service 1998, Kasat et al. 2002). In previous studies some pyrethroids, e.g. fenvalerate, have been shown to mimic oestrogenic activity in MCF-7 human breast carcinoma cells (Go et al. 1999). In this study also the potentiating effect of pyrethroids on MCF-7 cells may be oestrogen-like. Exposure of MCF-7 cells to pyrethroids has also been shown to induce oncogenic expression (Kasat et al. 2002).

In the present study, pyrethroids (pyrethrin, permethrin and cypermethrin) at low concentrations increased the cell proliferative effect of the 0.1 nM oestradiol. The combined effect of oestradiol and pyrethroid compounds was significantly greater on the total ATP than the effect of pyrethroid compounds only or of oestradiol only. This confirms the hormone-like effects of pyrethroids. The most effective synergists at the lowest concentrations were pyrethrin and

cypermethrin. At high concentrations all pyrethroids were cytotoxic. This is in accordance with the results of Go et al. (1999). The synergist effect may be due to the interaction of pyrethroids with the hormonal regulation of the cell cycle. As in previous studies, pyrethroids can affect the regulation of the cell cycle and, consequently, the mitotic cell cycle progression. Induction of chromosomal aberrations has been show *in vitro* (Surralles et al. 1995, Hadnagy et al. 1999), but not in human *in vivo* studies (Paldy et al. 1987, Bolognesi et al. 1993). There is also evidence that fenvalerate (with an alpha cyano group) may act trough the classical oestrogen pathway via the oestrogen receptor (Go et al. 1999).

The synergistic effect of oestradiol with pyrethroid compounds in the MCF-7 cell line was detected at concentrations corresponding to the normal human blood concentrations of oestradiol (0.08- 1.6 nM in women and 0.03-0.2 nM in men). This means that pyrethroids can interact with the oestradiol normally present in human subjects. Hormones act at extremely low concentrations and thus exposures to even low levels of hormonally active agents may be significant in inducing breast cancer (Boccinfuso and Korach 1997, Solomon and Schettler 2000). The pyrethroid insecticides imitating oestrogen may also induce expression of proto-oncogene WNT10B and thereby produce abnormal cell proliferation leading to mammary tumours. In human subjects, the expression of this gene was found in a subset of breast adenocarcinomas (Bui et al. 1997a, Bui et al. 1997b).

3. *In vitro* cell culture methods in the evaluation of the toxic effects of pyrethroids

The need to develop acceptable alternatives to conventional animal toxicity testing has been widely recognised as a result of the increasing number of chemicals, the increasing costs and the time required for routine toxicity assessment (Veronesi 1992). One of the aims of *in vitro* techniques is to create suitable cell cultures for mimicking *in vivo* systems as far as possible. *In vitro* systems offer certain unique advantages in toxicity testing compared to *in vivo* experiments. Test chemicals, dissolved in the feeding media, come into direct tissue contact. Because the chemicals are dissolved in the nutrient fluids, toxic exposure is continuous and easily quantitated. The dose of a test chemical can be controlled in terms of the amount of parent compound or of the active metabolite, added either to the cell population or to an individual target cell. *In vitro* systems offer a uniform chemical and physical environment for toxic exposure. The mechanisms of the toxic effect at cellular level can be elucidated, and different factors determining the cell toxicity can be clarified. In addition, cells of human origin can be used in these studies.

However, *in vitro* systems also have their limitations. The simulation of *in vivo* metabolism and systemic distribution remains still very difficult (Freshney 2001). *In vitro* systems lack e.g. toxicokinetics and homeostatic regulations. In

addition, cell cultures of one single cell type are absent from a tissue microenvironment, where interactions of many cell types occur (Freshney 2001). Organotypic cultures reflect the *in vivo* system better than cultures of one cell type only (Hoff et al. 1999). Cocultures of selected cell types and the usage of culture media containing natural growth factors are promising alternative models.

In vitro methods are widely used to clarify the toxic mechanisms (Tähti et al. 1995, Tähti et al. 2003). For the evaluation of the safety of chemicals animal models are still needed. However, in the safety evaluation, *in vitro* studies are often used as screening tests before animal experiments, to get preliminary information about the toxicity of a new compound.

Summary and conclusions

The present *in vitro* study showed that the commercial pyrethroid compounds pyrethrin, permethrin and cypermethrin were already neurotoxic at low (micromolar) concentrations. The toxicity was dose-dependent and varied according to the chemical structure of each pyrethroid compound. Hormone-like effects of all pyrethroid compounds were detected in the studies with breast carcinoma cell line.

1. Pyrethrin decreased clearly the synaptosomal integral protein ATPase activity. PBO potentiated significantly this effect.

2. The synthetic pyrethroids permethrin and cypermethrin decreased the activity of the synaptosomal integral protein ATPase, but there were no clear structure-dependent differences in the ATPase inhibition. The alpha cyano group had no important effect on the function of ATPase.

3. The pyrethroid compounds showed dose-dependent effects in the biochemical cytotoxicity tests (WST-1 and ATP test, which was more sensitive) in SH-SY5Y cell line. The acute cell toxicity of pyrethroids without an alpha cyano group (pyrethrin and permethrin) was lower than that of the pyrethroid with an alpha cyano group (cypermethrin). Microscopic photographs confirmed the results of biochemical cytotoxicity tests. Morphological changes in the exposed cultures were already seen at lower concentrations than in WST-1 and ATP tests. These results suggest the advantages of a wider use of microscopic photographing beside conventional biochemical tests.

4. Pyrethroids showed interaction with the effects of oestradiol in the human breast carcinoma cell line (MCF-7). Pyrethrin potentiated the oestradiol-induced increase of MCF-7 cell proliferation. This result shows a clear synergistic hormone-like effect of pyrethroid compounds.

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