universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

Impact of stereochemistry on biological effects of permethrin: induction of apoptosis in human hepatoma cells (HCC-1.2) and primary rat hepatocyte cultures.

angestrebter akademischer Grad

Magister/Magistra der Naturwissenschaften (Mag. rer.nat.)

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Wien, am 01. August 2008

This diploma thesis was performed on the
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1 INTRODUCTION

Pesticides constitute a considerable fraction of large-scale products with world-wide distribution and according to the US Environmental Protection Agency (EPA), almost a quarter of them contain a chiral center [GARRISON, 2006]. Many of these compounds such as pyrethroid-type insectides (α-cypermethrin, λ-cyhalothrin, β-cyfluthrin) or herbicides (glufosinate) are approved in Austria (RL 91/414 Appendix I).

Stereochemistry plays a crucial role in determining the toxicological profile of many chiral xenobiotics as exemplified by the insecticide permethrin. The insecticidal (neurotoxic) action of mixtures containing the four stereoisomers of permethrin is essentially brought about by the (1*R*,*cis*)- and (1*R*,*trans*)-forms. As to the mode of action (MOA), permethrin interferes with the closure of voltage-sensitive sodium channels in both, insects and mammals. In addition to its neurotoxic action, permethrin may exert biological effects in other target cells/organs. For instance, permethrin has been reported to induce drug metabolizing enzymes and growth of rodent liver, it may also inhibit mitochondrial respiratory chain in hepatocytes [GASSNER *et al.*, 1997; KOSTKA *et al.*, 2000; HEDER *et al.*, 2001], all of which are relevant endpoints in elucidating a chemical's mode of action and toxicological risk assessment. However, no data on potential stereoselectivity of these permethrin hazards are available; the published studies on the induction of cytochrome P450 enzymes and inhibition of mitochondrial function were performed with four-isomer mixtures of permethrin only.

Gathering information on stereochemistry-dependent biological effects of chiral pesticides is important for both regulatory as well as industrial decisions [KURIHARA *et al*., 1997]. The basic reasons for the separate testing of isomers and isomer-mixtures can be summarized as follows:

(1) Isomers may possess different persistence in environment / exposed organism.

(2) Isomers may have different effect profiles, both qualitatively and quantitatively.

(3) One isomer may modulate the toxicokinetic and -dynamic profile of the other(s).

For example, if only one pesticide isomer exerts the desired effect ("eutomer"), while the other is essentially inactive ("distomer"), application of the isomer-mixture leads to an increased environmental and in consequence, human burden due to the presence of "isomeric ballast" in the formulation. For example, chiral organophosphates and pyrethroids are two prominent classes of insecticides with high stereoselectivity in biodegradation and aquatic toxicity [LIU *et al.*, 2005]. Hence, envisaging *green chemistry* it is rational to develop and apply appropriate isomer-containing formulations of chiral pesticides rather than isomer mixtures to maximize the ratio desired *vs*. undesired effects and to keep dosage and, thus, environmental and consumer load minimal.

Taken together, it is obvious that the consideration of stereoselectivity in risk assessment of permethrin and related chiral pyrethroid burden is highly demanding [ALI *et al*., 2005]. However, regulatory guidelines for chiral pesticides are not yet as stringent as for chiral drugs and not at least due to this reason, the overwhelming number of chiral pesticides are still marketed and applied as isomer mixtures despite the (partial) knowledge about stereoselective biological fate and effects [MULLER *et al.*, 2004; GARRISON, 2006]. This issue is even more problematic as isomer-mixtures of permethrin are for example used in medical applications for humans and pets all over the world including Austria (Infectopedicul®, Lyclear®, etc.).

An important prerequisite and common bottleneck in single stereoisomer testing is the availability of the respective compounds in highly purified form and stereochemical stability during the incubations. Both is fulfilled for permethrin as single stereoisomers are available in stereochemical purity exceeding 99 % [BICKER, 2006] and chiral centers are not considered susceptible to racemization. Need for individual stereoisomers of chiral pesticides by international health agencies which highlights the attention paid to chirality issues by competent authorities was recognized by the fact that the group of Prof. Dr. W. Lindner, University Vienna, recently served as a supplier of individual permethrin stereoisomers for environmental monitoring studies of the

US EPA. Owing to the broad relevance of permethrin and due to the in-house availability of permethrin stereoisomers, a joint project of the Institute of Organic Chemistry/Christian Doppler Laboratory (Bicker, Lämmerhofer, Lindner, University of Vienna) and the Research Unit Toxicology and Prevention, Clinics of Medicine I, Institute of Cancer Research (Bursch, Dornetshuber, Medical University of Vienna) was initiated to shed light on the stereoselectivity of non-neurotoxic profile of permethrin and its stereoisomers. Constituting a part of this project, the present diploma thesis addresses two topics:

1. the validity of newly established human hepatoma cell line HCC-1-2 for toxicological studies.
2. the action of commercial [***cis*-racemate / *trans*-racemate** ~ 25/75] and stereosisomers [(trans(+)-(1S,3R)- permethrin (**11-permethrin**); trans(-)-(1R,3S) permethrin (**12-permethrin**); cis(-)-(1S,3S) permethrin (**21-permethrin**); Cis(+)-(1R,3R) permethrin (**22-permethrin**)] on hepatocellular death (apoptosis).

2 STATE OF RESEARCH

2.1 Evaluation of the validity of the newly established human hepatoma cell line HCC-1-2 for toxicity studies.

Since a long line of years, the toxicological research at the Institute of Cancer Research (ICR), Medical University of Vienna, is focused on liver growth regulation (cell proliferation, apoptosis) and its disturbance in chemical hepatocarcinogenesis. In the context of the present diploma thesis, two of the prominent outcomes of toxicological research on rodents *in vivo* are briefly addressed: (1) Non-genotoxic hepatocarcinogens (liver tumor promoter such as sedatives, hypolipidemic drugs, hormones and environmental pollutants) favor cell multiplication and inhibit apoptosis, thereby accelerating hepatocarcinogenesis. (2) Transforming growth factor beta-1 (TGF-β1) constitutes a major death signal in rat liver, acting in concert with liver tumor promoter and nutritional factors to maintain liver cell number homeostasis [BURSCH *et al.*, 2004].

Briefly, **TGF-β1** is a member of a family of dimeric polypeptide growth factors that includes bone morphogenic proteins and activins [RODGARKIA-DARA *et al.*, 2006]. TGF-β1 is a multi-functional cytokine involved in cell proliferation, differentiation, angiogenesis, motility, adhesion, and cell death [FLANDERS *et al.*, 2001; M. O. LI *et al.*, 2006; PRUD'HOMME, 2007; HOWE, 2003]. In the liver, TGF-β1 plays a key role in the regulation of homeostasis liver cell by inhibiting hepatocelluar proliferation and concomitantly, by inducing apoptosis; liver tumor promoting agents such as phenobarbital interfere with mechanisms maintaining the balance between birth and death of cells (fig.2.1) [OBERHAMMER *et al.*, 1991; BURSCH *et al.*, 1993; MICHALOPOULOS, 2007].

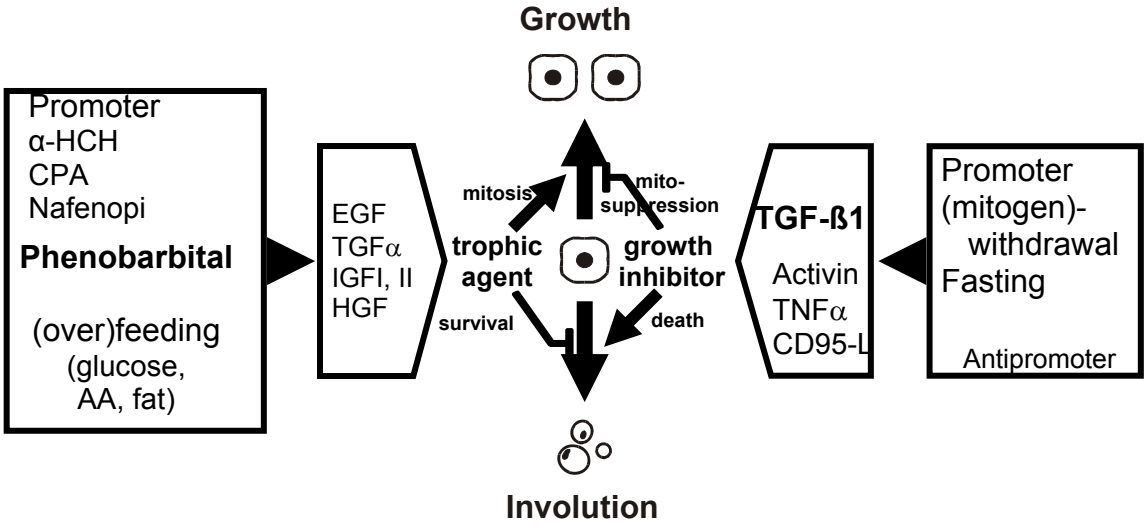

Fig. 2.1 Regulation of birth and death of cells in the liver.
The balance between replication and death of cells is tightly controlled according to the physiologcal needs of the organism. Some mitogenic factors also act as survival factors. Examples are transforming growth factor α (TGF-α), epidermal growth factor (EGF), or insulin-like growth factors I and II (IGF I, II). Mitosis-inhibiting factors may induce cell death, for example, transforming growth factor β1, (TGF-β1), activin A and tumor necrosis factor (TNFα,β). A further important death signal is the ligand to the CD95 (= fas, Apo I) receptor. Tumor promoters interfere with mechanisms maintaining the balance between birth and death of cells [RODGARKIA-DARA *et al.*, 2006].

In the context of the present study it should be noted that TGF-β1 expression in the liver may be also enhanced during certain stages of chemical liver injury, for instance upon intoxication with carbon tetrachloride, possibly providing an autocrine loop tuning the regenerative response of the liver to cell loss. TGF-β1 regulates cellular processes by binding to three high-affinity cell-surface receptors known as types I, II, and III, signal-transduction involve Smad-molecules, finally resulting in apoptosis execution via “intrinsic pathway” , see below; for review: [RODGARKIA-DARA *et al.*, 2006].

Concepts of cell death. The occurrence of **cell death** under a variety of physiological and pathological conditions in multicellular organisms has been documented many times during the past 160 years [CLARKE, 1990]. In pathology and toxicology, based upon the pioneering work of Rudolf Virchow in 1850ies cell death (usually called “necrosis”) in living organisms traditionally was considered as a passive, degenerative phenomenon resulting from external

insults by numerous agents. This view of cell death was revolutionized in the early 1970ies by a group of British-Australian pathologists (John Kerr, Andrew Wyllie, Alistair Currie) proposing two broad cell death categories: 1. necrosis, resulting from massive cell injury and breakdown of cellular homeostasis [KERR *et al.*, 1972; WYLLIE *et al.*, 1980]. Morphologically necrosis is characterised by swelling of the cell and bursting its content in the area, which usually causes an inflammatory response. 2. The new term „apoptosis or programmed cell death was coined to describe an orchestrated collapase of a cell, staging membrane blebbing, cell shrinkage, chromatin condensation, DNA and protein degradation, accomplished by phagocytosis of corpses by neighbouring cells [KERR *et al.*, 1972; WYLLIE *et al.*, 1980].

Apoptosis gained considerable credit when it became clear that it constitutes an essential part of life for any multicellular organism. Apoptotic cell death can be triggered by various death stimuli, including endogenous (physiological) death ligands as well as cytotoxic chemicals. Endogenous death ligands, for instance, comprise TGF-β1 (see above), tumor necrosis factor α (TNF-α), FasL/CD95/Apo1, and TNF-related apoptosis-inducing ligand (TRAIL). Chemical or chemotherapeutic agents inducing apoptosis, for instance, comprise organotin compounds, arsen, cisplatin, doxorubicin, 5-Fluoruracil, immunotoxins, antibodies [MEYERS *et al.*, 2004; BILLINGSLEY *et al.*, 2006; BREMER *et al.*, 2006; VALKO *et al.*, 2006]. Modern techniques provided insights into the molecular pathways triggered by such death stimuli; these revealed to be conserved from worm to mammals [DEGTEREV *et al.*, 2008]. Thus, in a number of biological settings apoptosis involves the action of caspases (cysteine aspartic acid-specific proteases) as major players. For instance, most of the stereotypic morphological features of apoptotic cells are brought about by caspases [LOCKSHIN *et al.*, 2004a; LOCKSHIN *et al.*, 2004b; I. LAVRIK *et al.*, 2005a]. Caspases belong to a large family of highly conserved proteins that have been found in hydra, insects, nematodes and mammals; a number of them constitute a set of sequentially acting ‘‘initiator’’ and ‘‘executioner’’ caspases, mediating a wide range of physiological and non-physiological pro-apoptotic signals down to a final coordinated self-destruction

of the cell. To date, over 400 substrates for the effector caspases have been identified [CHANDRA *et al.*, 2000; HAIL *et al.*, 2006; LUTHI *et al.*, 2007].

Mitochondria constitute a major site for integration of diverse pro-apoptotic signals ["intrinsic pathway" via caspase-9 activation (apoptosome) as opposed to "extrinsic pathway", triggered by activation of caspase-8 via death receptors of the TNF/NGF-family; both pathways join at the level of caspase-3 (fig. 2.2.)

[LAVRIK *et al.*, 2005a; LAVRIK *et al.*, 2005b]. The pro-apoptotic action of numerous stress signals incl. chemical agents has been shown to mediated to final cell death execution by the intrinsic pathway [BREMER & HELFRICH, 2006; HETZ, 2007; LEMASTERS, 2007; FADEEL *et al.*, 2008]. Apart from mitochondria the endoplasmic reticulum [PIZZO *et al.*, 2007], lysosomes [STOKA *et al.*, 2005] and the trans-Golgi-Network [NAKAGOMI *et al.*, 2008] play important roles as well. Thus, each organelle possesses sensors that detect specific alterations, locally activates signal transduction pathways and emits signals that ensure inter-organellar cross-talk.

Along with this gain in knowledge, however, morphological, biochemical and molecular observations revealed that active self-destruction of cells is not confined to apoptosis but cells may use different pathways to commit suicide, thereby severly challenging the initial necrosis-apoptosis dichotomy [CLARKE, 1990; ZAKERI *et al.*, 1995; KITANAKA *et al.*, 1999; BURSCH, 2001; WYLLIE *et al.*, 2001; YUAN *et al.*, 2003; BURSCH, 2004; EDINGER *et al.*, 2004; GOZUACIK *et al.*, 2004; LOCKSHIN & ZAKERI, 2004a; LOCKSHIN & ZAKERI, 2004b; SHINTANI *et al.*, 2004; LEVINE *et al.*, 2005; STEFANIS, 2005].

For instance, cell death induced by apoptotic stimuli such as CD95-L or TNF exhibit hallmarks of necrosis under conditions of caspase-inhibition ("programmed necrosis") [YUAN *et al.*, 2003; EDINGER & THOMPSON, 2004]. Moreover, caspase-independent cell death may also ensue with the morphology of apoptosis [LOCKSHIN & ZAKERI, 2004a; LOCKSHIN & ZAKERI, 2004b]. Notably, early morphological and histochemical studies revealed no evidence for lysosomal or autophagic events in apoptotic cells in vivo [KERR *et al.*, 1972; BURSCH *et al.*, 1985; WYLLIE & GOLSTEIN, 2001]. To date, the autophagic-lysosomal compartment has been implicated in the

initiation of programmed cell death, either upstream or independent of caspase cascades, often denoted “type II programmed cell death” or “autophagic cell death” [SCHWEICHEL *et al.*, 1973; CLARKE, 1990; ZAKERI *et al.*, 1995; BURSCH, 2001; BURSCH, 2004; GOZUACIK & KIMCHI, 2004; LOCKSHIN & ZAKERI, 2004a; LOCKSHIN & ZAKERI, 2004b; ARTAL-SANZ *et al.*, 2005].

In summary, it appears that diverse cell death programs emerged during evolution, the conservation of which apparently equips cells with a high degree of flexibility in assembling such elements to a cell death pathway according to the (patho)physiological conditions and needs.

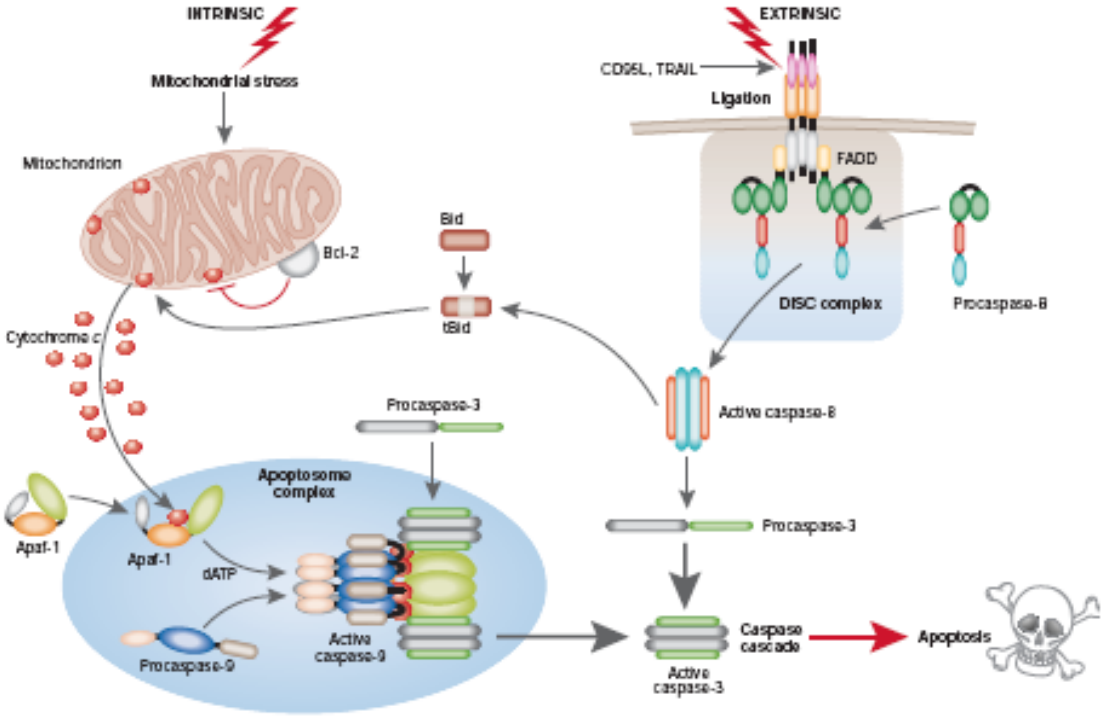

Fig. 2.2 Apoptosis: “Extrinsic” and “intrinsic” pathway to caspase activation [MACFARLANE *et al.*, 2004].

The **extrinsic** or caspase 8/10 dependent pathway is initiated by death receptors of the tumor necrosis factor–family (TNF-family). Membrane-proximal caspases (caspase-8 and -10) are activated by ligation of transmembrane death receptors (like Fas/CD95, TNF receptor, and TNF-alpha related apoptosis-inducing ligand (TRAIL) receptor) with their respective ligands (FasL, TNF, and TRAIL). Ligand binding of the receptor causes the assembly of a series of proteins called the DISC (death-inducing signalling complex) which then activates procaspase-8 which in turn cleave and activate effector caspases such as caspase-3 and –7. [I. LAVRIK *et al.*, 2005a; I. N. LAVRIK *et al.*, 2005b]. The **intrinsic** (mitochondrial) pathway of apoptosis functions in response to various types of intracellular stress and requires permeabilitation of the mitochondrial membrane and the release of mitochondrial proteins in the cytosol, such as cytochrome c which is normally confined in the mitochondrial intramembrane space. Once cytochrome c is released it binds with apoptotic protease-activating factor 1 (Apaf-1) and the presence of dATP, which then bind to pro-caspase-9 to create a protein complex termed apoptosome. The apoptosome cleaves the pro-

caspase-9 to its active form, which in turn activates the effector caspase-3, to orchestrate the biochemical execution of programmed cell death [MACFARLANE & WILLIAMS, 2004; GOGVADZE *et al.*, 2008; OBERST *et al.*, 2008]. In addition, the release of cytochrome c from the intramembrane space, the intramembrane content released also contains for example apoptosis inducing factor (AIF) to facilitate DNA fragmentation, and Smac/Diablo proteins to inhibit the inhibitor of apoptosis (IAP) [HAGUE *et al.*, 2004; GOGVADZE *et al.*, 2008; OBERST *et al.*, 2008].
The regulation of the intrinsic pathway is subjected to the control of proteins of the Bcl-2 family . Furthermore, agents that promote the mitochondrial permeability transition (MPT) such as ceramide, reactive oxygen species (ROS) or Ca2+ are key players in apoptosis execution [HAIL *et al.*, 2006].

Available liver cell culture models for studying the mode of action (MOA) of chemicals. To elucidate interactions (mode of action, MOA) of non-genotoxic carcinogens with the complex growth regulatory network of the liver incl. cell death (apoptosis), along with the general need for alternative test models in toxicology, the research efforts at ICR aim to establish appropriate hepatocellular cell culture models that can be integrated into hazard and risk assessment for humans; for this purpose, an approach as schematically illustrated in figure 2.3 has been chosen.

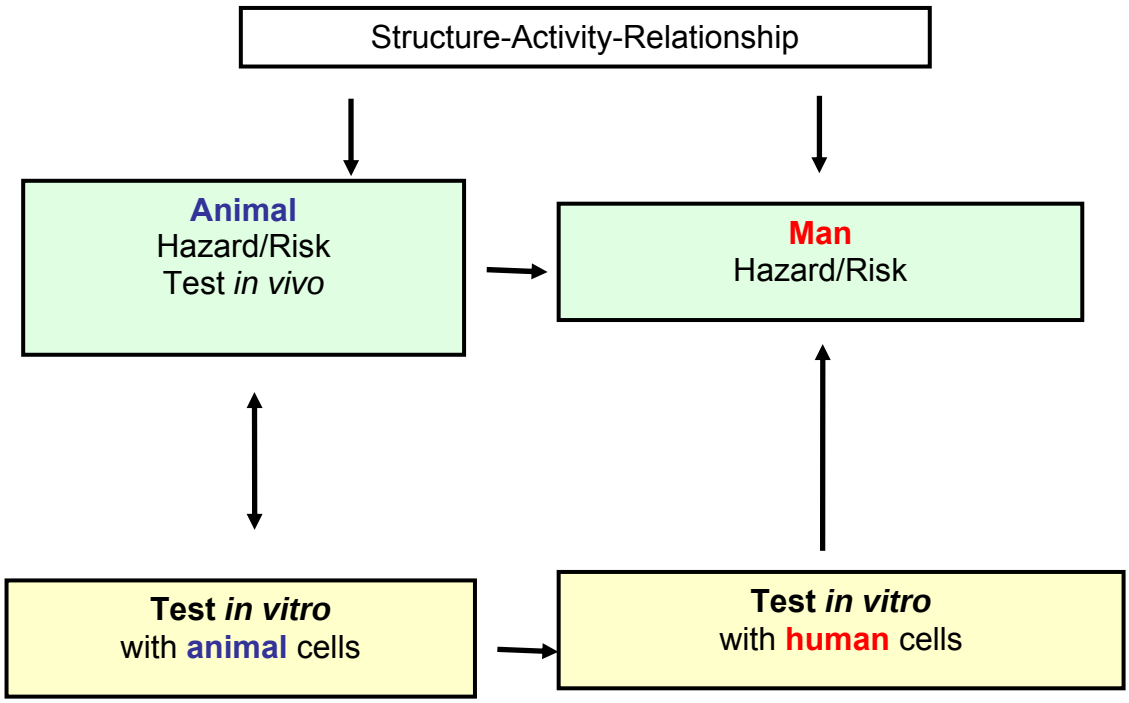

Fig. 2.3 Approach using knowledge on chemical structure along with animal experiments and cultured human cells for human hazard and risk assessment.

Briefly, weighing validity of cell culture findings is guided by their concordance with *in vivo* observations. Thus, in a first step, based upon **rat *in vivo*** data, a

primary rodent hepatocyte culture system (incl. preneoplastic hepatocytes) was developed, followed by **primary human hepatocyte culture** [PARZEFALL *et al.*, 1991; SCHULTE-HERMANN *et al.*, 1999; LOW-BASELLI *et al.*, 2000; PARZEFALL *et al.*, 2002; SCHAUSBERGER *et al.*, 2003; DRUCKER *et al.*, 2006]. These studies were complemented by investigations with **established human hepatoma cell lines** such as HepG2 and Hep3B [GÄRTNER, 2000; GRUSCH *et al*., 2006; BURSCH *et al*., unpublished results].

With regards to growth-regulation related endpoints and the need for standardized test conditions, the data revealed limitations of the investigated test systems. For instance, in primary hepatocyte cultures of rat or human origin, liver-specific functions are usually decreased and instable exhibiting variable and different changes with time in culture. Furthermore, use of primary human hepatocytes is limited by scarce and unpredictable availability, new donors are needed repeatedly, and considerable inter-individual variations do exist (for rats additional inter-species variations have to be considered). As to available human hepatoma cell lines, these are barely authenticated (no informed consent, few data on etiology and pathology) as well as ill characterized (may be even contaminated by other cells) and lack important functions of viable liver cells such as metabolic competence and the response to growth regulating cytokines. For instance, HepG2 and Hep3B have been found unsuitable for mimicking *in vivo* normal parenchymal cells as exemplified by their sensitivity to TGF-β1 and metabolic competence: HepG2 cells were resistant to TGF-β1-induced growth arrest and apoptosis [BUENEMANN *et al.*, 2001], but at least have retained little cytochrome P450 enzyme activity; on the other hand, Hep3B cells are sensitive to the pro-apoptoc action of TGF-β1 [ROLIN *et al.*, 1989; GRESSNER, 1998; HUANG *et al.*, 1998; OGINO *et al.*, 2002; WILKENING *et al.*, 2003].

Taken together, both primary human cells and current cell lines are not considered suitable for stringent standardized testing and, thus, deficiency of well-defined human hepatocytes culture systems / cell lines still constitutes a bottleneck for standardized compound testing according to REACH (**R**egistration, **E**valuation and **A**pproval of **Ch**emicals; European Community

Regulation on chemicals and their safe use (EC 1907/2006), entered into force on 1 June 2007). Most recently, Grasl-Kraupp and colleagues [SAGMEISTER *et al.*, in press] succeeded in establishing a human hepatoma cell line (HCC-1.2) and the first part of the present diploma thesis addresses the validity of HCC-1.2 cells as an alternative test system for hepatotoxicity. Based on the approach schematically shown in fig. 2.3, two questions were raised:

1. Does TGF-β1 induce apoptosis of HCC-1.2 cells?
2. Does the liver tumor promoter phenobarbital inhibit TGF-β1 induced apoptosis?

According to the validation procedure schematically shown in fig. 2.3., a positive answer to these questions would render this cell culture model suitable to tackle questions concerning the hepatocellular toxicity of permethrin and its stereoisomers.

2.2 Permethrin and cell death (apoptosis) in the liver

2.2.1 Permethrin: chemical structure

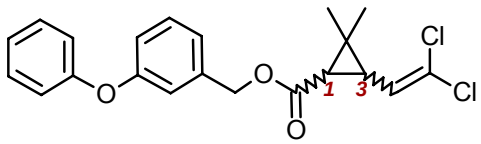

Fig. 2.4 Structure of Permethrin

Permethrin [3-phenoxybenzyl (±) *cis/trans*-3(2,2-dichlorovinyl)-2,2-dimethylcyclo-propane-1- carboxylate] is an insecticide and belongs to the group of pyrethroids. Pyrethroid insecticides are synthetic chemicals and similar to the natural pyrethrins contained in flowers of the genus Chrysanthemum. All pyrethroids are ester-containing compounds consisting of various acid and alcohol moieties. Permethrin and other pyrethroids with a basic cyclopropane

carboxylic ester structure are classified as type I pyrethroids. Type II pyrethroids have additionally an alpha-cyano group [BRADBERRY *et al.*, 2005].

Owing two asymmetric substituted carbon atoms (stereogenic centres) in the cyclopropane ring system of permethrin four stereoisomers, two pairs of enantiomers (non-superimposable mirror images of each other) and two pairs of diastereoisomers (e.g. *cis*/*trans* isomerism) are possible. The two stereogenic centres are located in the 1 and 3 position of the cyclopropane ring. The spatial orientation of the C-3 subsitutients relative to the C-1 carboxylic acid moiety at the cyclopropane ring is either in *cis* or *trans* configuration. (fig. 2.4 is shows the *cis* configuration)

Many of today's permethrin formulations are marketed as four-isomer mixtures. Typical *cis* to *trans* ratios are 25:75 and 40:60 [RAY *et al.*, 2006]. Permethrin is widely used for agricultural and domestic pest control and possesses relatively low acute mammalian toxicity and low environmental persistence. Although for agricultural applications the use of permethrin is now forbidden in Austria and other European countries, it is still widely employed for indoor pest control like in textiles such as carpets, wallpapers, furniture, and clothes as well as in human and veterinary medicine for the treatment of lice and scabies [MACAN *et al.*, 2006].

2.2.2 Toxicokinetics

Uptake of permethrin by humans predominantly ensues through ingestion, dermal absorption, or inhalation of dust or aerosol droplets; because of the very low volatility of pyrethroids, inhalation of vapour is of minor importance [RAY & FRY, 2006]. The initial biotransformation of permethrin (Phase I) involves attack by either esterases at the central ester bond or cytochrome P 450-dependent monooxygenases at the acid or alcohol moieties (fig. 2.5). In rats, permethrin was hydrolyzed by carboxylesterase, the resulting alcohol moiety (3-phenoxybenzyl alcohol (PBOH) was oxidized to 3-phenoxybenzaldehyde (not shown) and 3-phenoxybenzoic acid (PBA) by the cytochrome P450 system [NAKAMURA *et al.*, 2007]. Notably, *trans*-isomers of permethrin are much more susceptible to esterase attack than *cis*-isomers [NAKAMURA *et al.*, 2007].

Further metabolism of these intermediate products involves conjugation reactions with amino acids, sugars, sugar acids or sulfate prior to excretion [IARC 53, 1991].

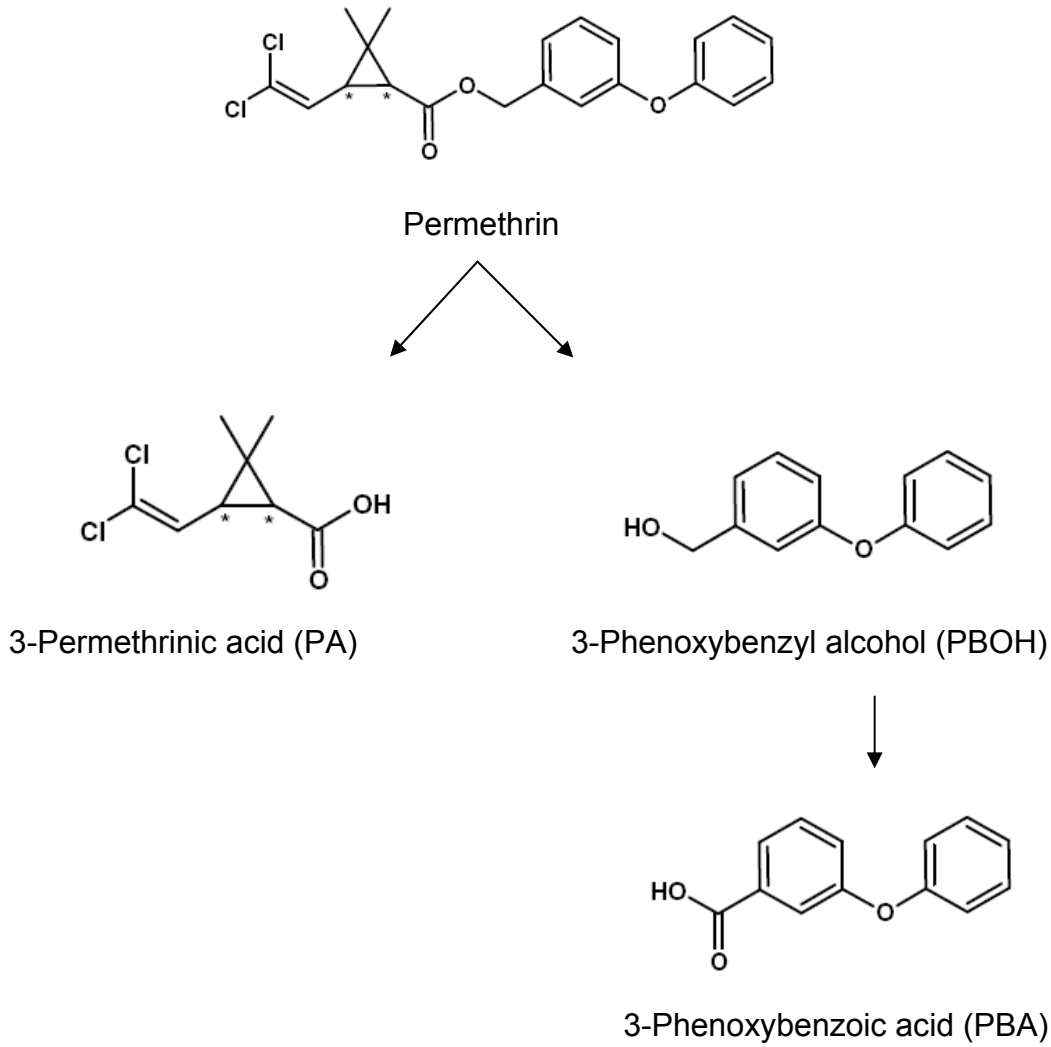

Fig. 2.5 Metabolites of permethrin in rat hepatocytes cultured identified by GC/MC analysis [HEDER *et al.*, 2001].

Serum concentrations of permethrin peaked 3-4 hours after ingestion and then declined. Levels of the *trans* isomer were below the limits of detectability within 25 hours after exposure, whereas cis-permethrin was still present at detectable levels 10 days after exposure. [SODERLUND *et al.*, 2002].

2.2.3 Toxicodynamics

Permethrin, like all synthetic pyrethroids, kills insects by strongly exciting their nervous systems. The insecticidal action as affected by stereochemistry is shown in the following figure.

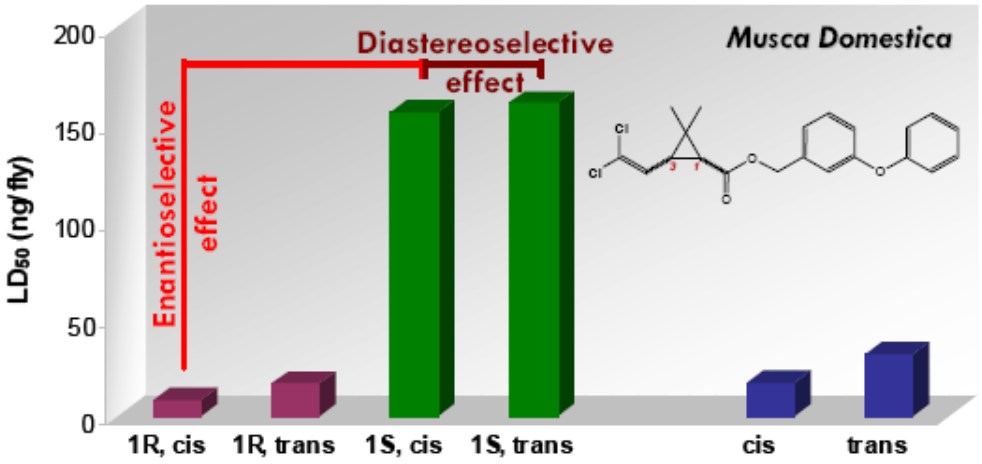

Fig. 2.6 Acute Toxicity (LD50) of individual permethrin stereoisomers and racemic mixtures of *cis* and *trans*-permetrhin, respectively, towards the house fly (musca domestica). The "cis" and "trans" nomenclature refers to the spatial orientation of the substituent at C-3 in relation to the one at C-1 of the cyclopropane moiety [BICKER 2006].

As illustrated by fig. 2.6, LD_{50} values of permethrin towards Musca domestica (the amount of permethrin that kills 50 percent of a population within a given time frame) varies by almost a factor of 10 between the individual stereoisomers. Thus, 1R-isomers exert a much stronger insecticidal effect compared to the 1S-isomers; the insecticidal potency of racemic mixtures of the *cis*- and the *trans*- enantiomers is dominanted by the R-configuration (blue bars on the right). Overall, permethrin is toxic to honey bees and other beneficial insects, fish, aquatic insects, crayfish, and shrimp. For many species, concentrations of less than one part per billion are lethal. Permethrin causes deformities and other developmental problems in tadpoles, and reduces the number of oxygen-carrying cells in the blood of birds.

In mammals, permethrin has complex effects on the nervous system as well. As in insects, it causes repetitive nerve impulses. It also inhibits a number of key molecules essential for homoeostasis of the nervous system: (a) the enzyme

ATPase, resulting in an increased release of the neurotransmitter acetylcholine; (b) monoamine oxidase-A, both enzymes are involved in neurotransmitter turnover; (c), GABA receptor, resulting in excitability and convulsions; (d) mitochondrial respiratory chain (for review: US-EPA 1997). Humans exposed to high doses exhibit symptoms including tremors, incoordination, elevated body temperature, increased aggressive behaviour, and disruption of learning. Laboratory tests suggest that permethrin is more acutely toxic to children than to adults.

Mode of neurotoxic action. Pyrethroids act on the axons of the peripheral and central nervous system of insects and mammals, with the voltage-sensitive ion channels (especially sodium) as primary targets. Voltage-sensitive sodium channels are responsible for the generation of the inward sodium current that maintains the action potential in most cells and are closed at normal resting potentials. Pyrethroids modify the gating characteristics of these channels and thereby delay their closure. Since sodium channels are held open longer, more sodium ions are allowed to cross and depolarize the neuronal membrane. Such a protected sodium influx can lower the action potential threshold and can cause repetitive firing of action potentials. This may be the mechanism of type I pyrethroids leading to paraesthesia and other syndromes like tremor, prostration, paralysis and eventual death [BRADBERRY *et al.*, 2005; RAY & FRY, 2006]. Pyrethroids are much more neurotoxic to insects than to mammals because insects have a higher sodium channel sensitivity, a smaller body size as well as a lower body temperature. Moreover, mammals are specifically protected towards pyrethroid toxicity owing to a lower dermal absorption and a relative rapid and esterase-mediated biotransformation to non neurotoxic metabolites [BRADBERRY *et al.*, 2005].

Permethrin and cell death. Only a few studies were found addressing the effect of permethrin on cell death: screening the pubmed-database (05.06.2008) using the search term “permethrin” retrieved 1783 references, but only 11 in combination with “cell death”. These studies cover neurotoxicity, immuno-toxicity and endocrine organ toxicity [ABDEL-RAHMAN *et al.*, 2001¸ ABOU-DONIA *et al.*, 2003; PRATER *et al.*, 2002; ABU-QARE *et al.*, 2003; ABDEL-

RAHMAN *et al.*, 2004; GREENLEE *et al.*, 2004; KAKKO *et al.*, 2004; OLGUN *et al.*, 2004; ELWAN *et al.*, 2006]. For instance, as to the role of cell death for immunotoxic risks associated with multiple pesticide exposure, both apoptosis and necrosis of thymocytes were obsreved upon lindane, malathion, and permethrin, either separately or in mixtures of two pesticides [OLGUN *et al.*, 2004]. In rat brain, increased apoptosis was observed upon combined exposure to permethrin and the insect repellent DEET (N,N-diethyl-m-toluamide), but not upon permethrin alone; apoptosis was activated via release of mitochondrial cytochrome c (intrinsic pathway). None of the studies on permethrin and cell death addressed a potential selective action of its stereoisomers.

Therefore, in the present study the effect of the commercial [***cis*-racemate / *trans*-racemate** ~ 25/75] and the stereosisomers [(trans(+)-(1S,3R)- permethrin (**11-permethrin**); trans(-)-(1R,3S) permethrin (**12-permethrin**); cis(-)-(1S,3S) permethrin (**21-permethrin**); Cis(+)-(1R,3R) permethrin (**22-permethrin**)] on hepatocellular death (apoptosis) on cell death was investigated.

3 MATERIAL AND METHODS

3.1 Chemicals and suppliers

RPMI 1640	Sigma-Aldrich, Vienna, Austria
TGF-β1	Eubio, Vienna, Austria
BSA (Bovine Serum Albumin) *Albumine Bovine Fraction Powder*	Sigma-Aldrich, Vienna, Austria
DMSO	Fluka, Buchs, Switzerland
Phenobarbital	Fluka, Buchs, Switzerland
Rotenone	Sigma-Aldrich, Vienna, Austria
Permethrin (four isomer mixture) Permethrin-metabolites: 3-permethrinic acid (PA) 3-phenoxybenzyl alcohol (PBOH) 3-phenoxybenzoic acid (PBA) Permethrin-stereoisomers, permethrin pure	Agrochemia, Budapest, Hungary Agrochemia, Budapest, Hungary Fluka, Buchs, Switzerland Fluka, Buchs, Switzerland Dr. Wolfgang Bicker, Department of Analytical Chemistry and Food Chemistry, University Vienna, Austria

Tab. 3.1 Chemicals and suppliers

3.2 Cell culture

3.2.1 Stock culture

Human Hepatoma cell lines: HCC-1.2 and Hep3B

When working with cells sterile and antiseptic conditions were maintained. Cell cultures were grown in sterile T75 bottles in the cell incubator at 37°C and 5 % CO_2. As growth medium the commercial available RPMI 1640 containing 10 % FCS (fetal calf serum) and P/S (Penicillin/Streptomycin) was used. The growth medium (10-20 ml) was usually changed every second day. When confluence of the cells was reached, the cell culture was splitted, normally once a week.

Splitting of the cell culture: The RPMI (+ 10% FCS + P/S) medium was discarded and the T75 bottle was washed with 4 ml trypsin. Then the cells were incubated with 2.5 ml trypsin for 3 minutes in the incubator until they have detached from the bottom of the bottle. Immediately 10 ml RPMI + 10% FCS + P/S were added to neutralise trypsin and to stop the reaction. Afterwards the cell suspension was removed into 50 ml tubes and centrifuged for 5 min at 1500 rpm. Subsequently, the pellet was resuspended with 5 ml RPMI + 10% FCS + P/S. Depending on the cell charge, the cells are reseeded 1:10 – 1:40 in T75 bottles.

3.3 Experimental Protocol

3.3.1 Hepatoma cell line HCC-1.2

Preparation (Fig. 3.1): For the apoptosis / mitosis assay, $1x10^5$ cells were seeded in 3.5 Ø dishes for 24 hours in RPMI 1640-medium containing 10 % FCS to allow for recovery/attachment; for controls and in each experiment 2 dishes were used. Subsequently, FCS was removed for 48 hours before commencing treatment with cytokines and/or permethrin.

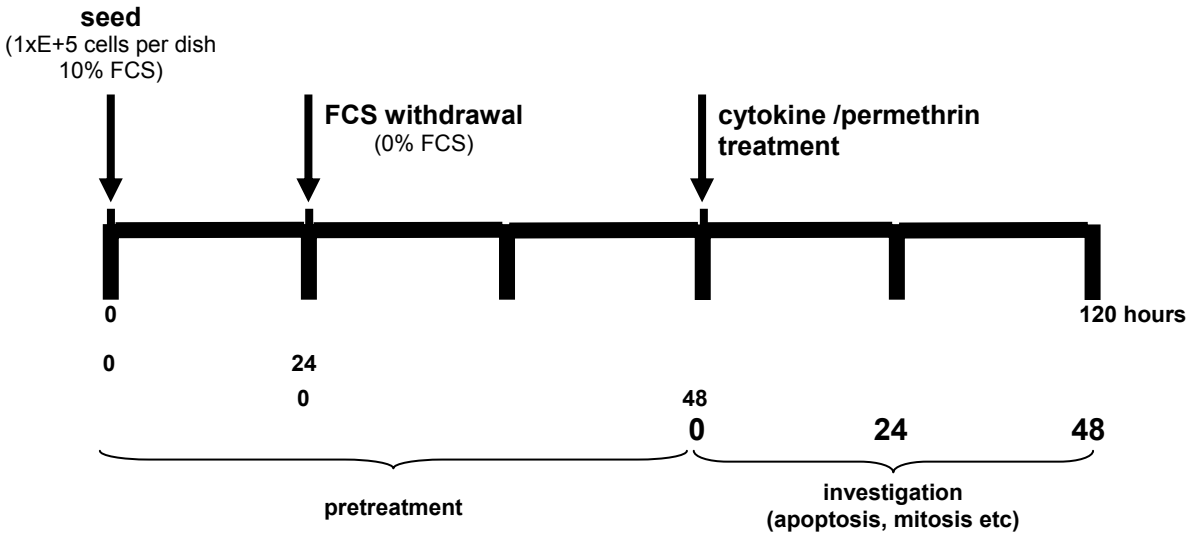

Fig. 3.1 Experimental protocol

3.3.2 Hepatoma cell line Hep3B

Hep3B cells were in general treated as described in “Hepatoma cell line HCC-1.2”. However, cell density varies. For the apoptosis / mitosis assay $2x10^5$ cells were seeded in 3.5Ø dishes.

3.3.3 Primary hepatocyte cultures

Reagents:

Washing medium and William’s E medium was always prepared freshly before application.

Washing medium (MEME)

100 ml MEME
1 ml Glutamax
1 ml HEPES
20 µl Tresylol
100 µl Gentamycin

William’s Medium E (WMEII)

100 ml William’s Medium
1,04 ml Glutamax
1,04 ml Hepes
1,04 ml Ascorbat
1,04 ml H_2
104 µl Gentamycin

Rat tail collagen

The collagen was diluted 1:10 with H_2O_{dd}.
Each dish was covered with 1,25 ml and dried over night.

SIP (Stock Isoosmotic Percoll)

21,6 ml HBSS

2,4 ml Percoll

Male SPF Wistar rats obtained from the animal facilities of the Medical University of Vienna at the age of 3–4 weeks were used as donors. Hepatocytes were isolated by collagenase perfusion as described previously by Parzefall et al. 1989. Hepatocytes were collected from the initial cell suspension by centrifugation at 600 rpm for 5 minutes at 4°C. After the supernatant was discarded the pellet was resuspended with 20 ml MEME and centrifuged at 300 rpm for 5 minutes (4°C). 25 ml of the resuspended cell suspension was mixed with 24 ml SIP (density: 1,06 g/ml) and centrifuged at 550 rpm for 10 minutes (4°C). The purified hepatocytes were washed twice with MEME: The supernatant was removed and the pellet was resuspended in 40 ml MEME and centrifuged at 550 rpm for 2 minutes (4°C). Then the pellet was resuspended in 25 ml MEME and centrifuged at 300 rpm for 5 minutes. Finally 20 ml MEME were added after the supernatant was discarded. Cell viability and total cell count were assessed by trypan blue index.

Isolated hepatocytes were seeded in WMEII + 10 % FCS on plastic dishes covered with rat tail collagen at a density of $3x10^4$ cells/cm^2. The hepatocytes were allowed to attach to the collagen gel for 4 h. Treatment commenced after cells were twice washed with 1 ml MEME. The time of addition was defined “time point 0”. After 30 and 44 hours cells were fixed and stained as described in histological determination of apoptosis and mitosis.

3.3.4 Treatment of cell cultures

Reagents

Stock solutions:

TGF-β1 (Transforming Growth Factor-β1): 2.5 ng/µl (BSA)

(dissolved in BSA)

After a quick spin 100 µl H_2O_{dd} were added to 5 µg recombinant human TGF-β1. Afterwards the solution was diluted with 1,9 ml BSA (2 mg/ml) and transferred à 100 µl into tubes and stored at -20°C.

BSA: 2 mg/ml
(dissolved in $H_2O_{dd;}$ stored at 4-7°C)
Phenobarbital: 100 mM
(dissolved in DMSO; stored at -20°C)
Permethrin (four isomer mixture, stereoisomers and metabolites): 10 mM
(dissolved in DMSO; stored at -20°C)
Rotenone: 10 mM
(dissolved in DMSO; stored at -20°C)

For each cell culture dish 2 ml medium (RPMI + P/S) were used. TGF-β1 was applied in a concentration of 10 ng/ml. Phenobarbital was tested in the concentrations of 1 µM, 10 µM, 25 µM and 50 µM. Permethrin was applied in the concentrations of 25 µM and 50 µM, whereas experiments with the permethrin-stereoisomers were performed with 2 µM, 10 µM and 50 µM. For all permethrin metabolites a concentration of 50 µM was used. Rotenone was tested with the concentrations of 0,1 µM, 1 µM and 10 µM. Immediately before application, permethrin, permethrin-stereoisomers, permethrin metabolites, phenobarbital and rotenone were diluted 1:200 in RPMI + P/S, thus the various final concentrations were dissolved in 0,5 % DMSO.

3.4 Histological determination of apoptosis and mitosis

Reagents

10 x PBS

80g NaCl, 2g KCl, 18g $Na_2HPO_4 \cdot 1H_2O$ and 2,4g KH_2PO_4 were added to 1000 ml H_2O_{dd} and adjusted with NaOH to pH 7,4. Subsequently, 10 x PBS was autoclaved.

4% Paraformaldehyde

8% Paraformaldehyde was diluted 1:1 with 2 x PBS

HOECHST

10 x stock of bisbenzimide H33258 (80µg/ml H_2O_{dd})

Before the application the bisbenzimide H33258 stock was dissolved 1:10 in 1x PBS (8µg/ml).

Geltol

6g glycerol and 2,4g mowiol were added to 6ml H_2O_{dd} and soaked for ≥ 2 hours at room temperature. Then 12 ml 0,2M tris-Cl (pH 8,5) were supplemented and stirred for 10 minutes at 50°C. Finally, the solution was centrifuged at 3000 rpm for 15 minutes.

After the RPMI 1640 + P/S medium was removed and the cells were fixed with 1 ml 4% paraformaldehyde per dish for 10 min at RT. The dishes were washed 2 times with 1 ml 1x PBS. Subsequently, the cells were stained with HOECHST (1:10 dilution in 1x PBS) for 10 min, followed by 2 further washing steps with 1x PBS. The dishes were dried in the dark, the dish plates were cut out and covered with 12 µl geltol. The plates were left in the dark over night until analysis by microscopy.

3.4.1 Morphological Evaluations

Chromatin was stained with the DNA-specific stain H33258. Chromatin condensation and fragmentation indicative of apoptosis as illustrated in fig. 3.2 were counted for quantitative analysis of apoptosis scoring 500-1000 cells per dish.

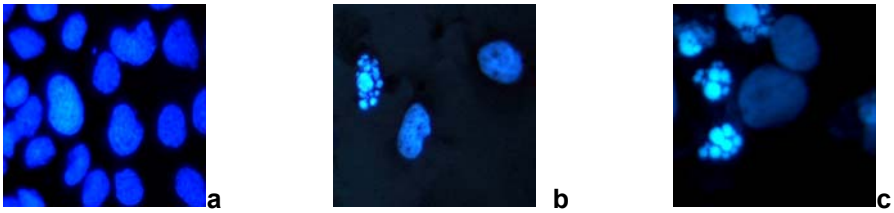

Fig. 3.2 Morphology of HCC-1.2 treated with TGF-β1. Fluorescence microscopy (40x) of H33258 staining. (a) Control: intact chromatin, (b and c) condensation and fragmentation of the chromatin.

3.5 Statistical analysis

Statistical analysis was performed with the software InStat (Version 3.06, created 2003). All data were examined for normality by Kolmogorov Smirnov-Test. Statistical differences for data which passed the normality test were evaluated using the One-way Analysis of Variance (ANOVA) and Tukey-Kramer Multiple Comparisons Test whereas data which deviated from normality were compared with Kruskal-Wallis Test (Nonparametric ANOVA) and Dunn's Multiple Comparisons Test. The P value < 0.05 was considered significant.
(*** = P < 0.001; ** = P< 0.001; * = P<0.05)

4 RESULTS

4.1 Functional characterisation of human hepatoma cell lines (HCC.1-2, Hep3B) and primary rat hepatocytes based upon sensitivity to TGF-β1 and phenobarbital

4.1.1 Human hepatoma cell line HCC-1.2

A first series of experiments aimed at elucidating whether the newly established human hepatoma cell line HCC-1.2 [SAGMEISTER *et al*., in press], would exhibit a sufficient concordance with *in vivo* observations on the role of Transforming Growth Factor beta 1 (TGF-β1) and liver tumor promoter in regulation of liver cell proliferation and apoptosis, namely

(1) a high sensitivity towards the anti-proliferative and pro-apoptotic action of TGF-β1;

(2) the inhibition of TGF-β1- induced apoptosis by liver tumor promoter, as exemplified by phenobarbital.

4.1.1.1 Effect of TGF-β1 on apoptosis, mitosis and cell density

The **apoptotic response** of HCC-1.2 cells to TGF-β1 (10 ng/ml) was determined morphologically at 24 hours and 48 hours after treatment. As shown in fig. 4.1 a, the basal (control) apoptotic activity was about 1.5 – 2 % at 24 and 48 hours. TGF-β1 (10 ng/ml) caused an increase in apoptoses to about 7 % at 24 hours, followed by further enhancement to approximately 11 % at 48 hours after treatment. To account for variances of the basal apoptotic activity among the individual experiments, apoptoses of 24 hour controls were set as 1 and those of 48 hour controls as well TGF-β1 treated cell cultures were expressed as x-fold change. As shown in fig. 4.1 b, essentially the same results were obtained.

The **mitotic activity** of control cultures declined significantly with time (fig. 4.2. a; P< 0.001 48 vs. 24 hours). At both time points of investigation, TGF-β1 caused an approximately 30 % inhibition of mitosis. Again, essentially the same results were obtained when mitosis were expressed as "x-fold of 24 hour control" (fig. 4.2 b).

As a result of the pro-apoptotic and the anti-mitotic action of TGF-β1 on HCC-1.2 cells, **cell density** progressively declined by approximately 30 % at 24 hours and by about more than 50 % at 48 hours after TGF-β1 (fig. 4.1 e).

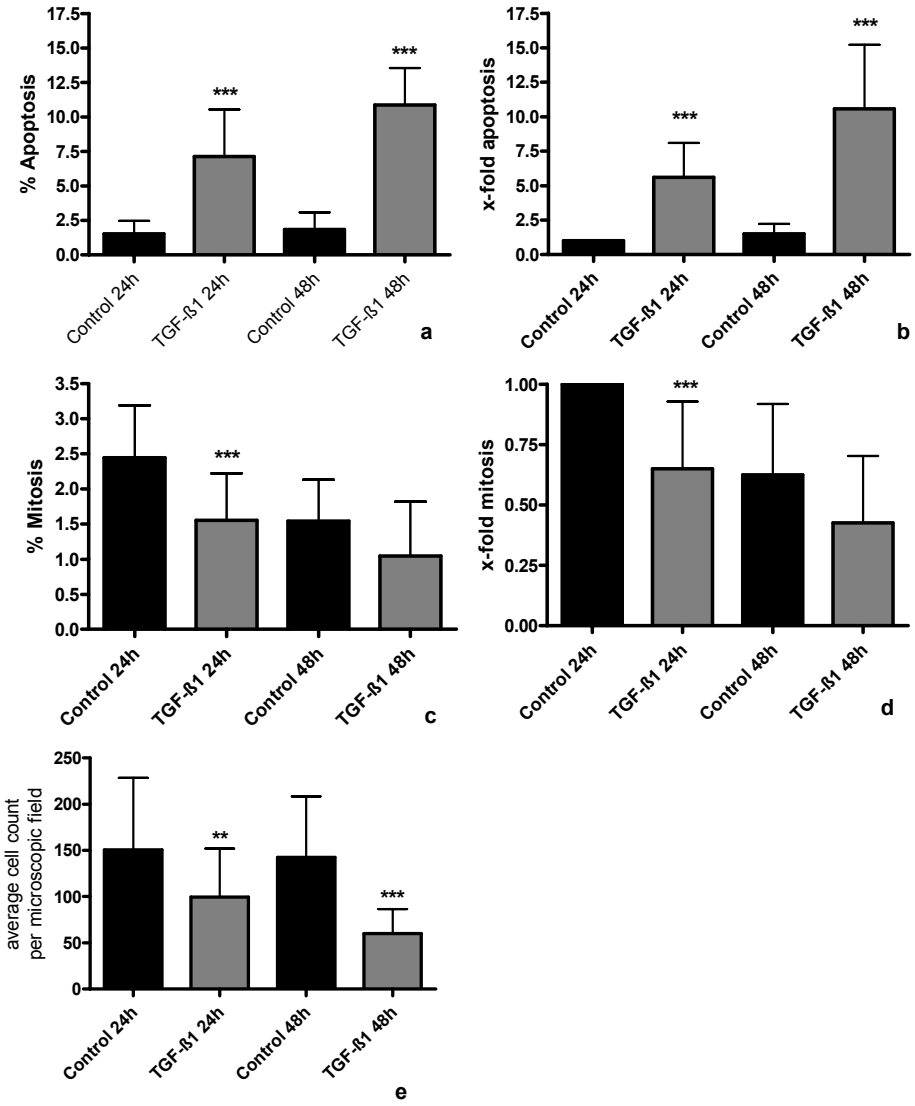

Fig. 4.1 Effect of TGF-β1 on apoptosis, mitosis and cell density of HCC-1.2 cell cultures

10 ng/ml TGF-β1. Time points of investigation: 24 h and 48 h. (a, b) apoptosis; (c, d) mitosis; (e) cell density (number of cells per microscopic field). Means (±SD) of 12-15

experiments are shown; numbers are listed in appendix, table 1. Statistical comparisons: (a, c, d, e) ***P<0.001, **P<0.01 using Dunn's Multiple Comparisons, Co vs. TGF-β1; (b) ***P<0.001 using Tukey-Kramer Multiple Comparisons Test, Co vs. TGF-β1.

4.1.1.2 Effect of phenobarbital on TGF-β1 induced responses: apoptosis, mitosis and cell density

A prominent feature of liver tumor promoter such as phenobartital is to inhibit apoptosis of hepatocytes and thereby, to accelarate development of frank neoplasia in rat liver [BURSCH *et al.*, 1984; SCHULTE-HERMANN *et al.*, 1990], most likely in a concerted action with cytokines of the transforming growth factor-beta family [RODGARKIA-DARA *et al.*, 2006]. Consequently, HCC-1.2 cells were probed for their sensitivity to phenobarbital as inhibitor of TGF-β1 induced apoptosis.

In fact, a time- and dose-dependent inhibitory effect was found (fig. 4.2 a): a 40-50 % inhibition of TGF-β1 induced **apoptosis**, though not significant, occurred at 24 hours after treatment with 25 µM to 50 µM phenobarbital (fig. 4.2 b). A more pronounced inhibitory effect (P<0.001) became obvious at 48 hours after phenobarbital-treatment (fig. 4.2 a, b). Again, essentially the same results were obtained when apoptosis where expressed as “x-fold of 24 hour control”.

As to **mitosis** and cell death density, phenobarbital did not produce a significant effect but revealed a consistent trend to increase both, mitoses and the average cell count per microscopic field in TGF-β1 treated cultures (fig. 4.2 d, e).

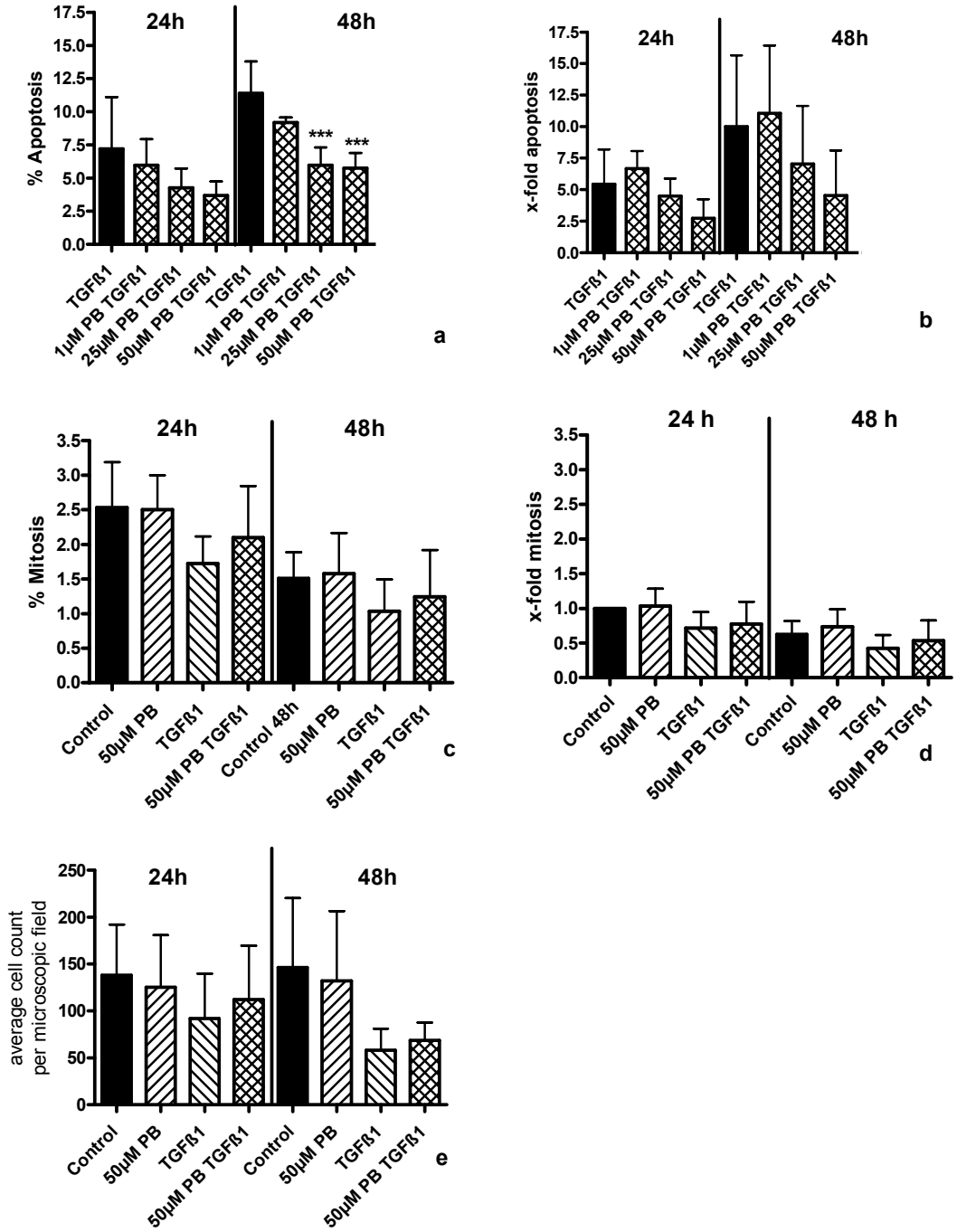

Fig. 4.2 Effect of phenobarbital onTGF-β1 induced response: apoptosis mitosis and cell density of HCC1.2 cell cultures

10 ng/ml TGF-β1; 1 µM, 25 µM and 50 µM PB (phenobarbital). Time points of investigation: 24 h and 48 h. (a, b) apoptosis; (c, d) mitosis; (e) cell density (number of cells per microscopic field). Means (±SD) of 2-6 experiments are shown; numbers are listed in appendix, table 8.2. Statistical comparisons: (a) ***P<0.001 using Tukey-Kramer Multiple Comparisons Test, TGF-β1 vs. PB+TGF-β1, (b, c, d, e) using Dunn's Multiple Comparisons, TGF-β1 vs. PB+TGF-β1.

4.1.2 Hep3B cells: Effect of TGF-β1 and phenobarbital on apoptosis, mitosis and cell density

In a second step, the experiments with the newly established HCC-1.2 cells were supplemented by studies with a human hepatoma cell line widely used by the scientific community since many years, namely Hep3B.
Treatment of Hep3B-cells with TGF-β1 caused a significant increase in **apoptotic activity** at 24 and 48 hours (Fig 4.3 a), i.e. 3.1 and 2.1-fold, respectively (fig 4.3 b). Furthermore, 50 µM phenobarbital alone tended to increase apoptosis of Hep3B cells but produced a significant inhibition of TGF-β1 induced apoptosis (approximately 55 % at 24 and 48 hours, 4.3.a).
Mitotic activity of Hep3B tended to be reduced after TGF-β1 treatment and somewhat more pronounced upon combined treatment with TGF-β1 plus Phenobarbital, but none of the differences was significant (fig. 4.3 c, d). As a result, **cell density** appears to grossly meet with apoptotic activity (fig.4.3 e).

In summary, TGF-β1 exhibited a pro-apoptotic action on Hep3B cells which, however, was less pronounced as compared to HCC-1.2 (cf. fig. 4.3 b, 4.1 b).

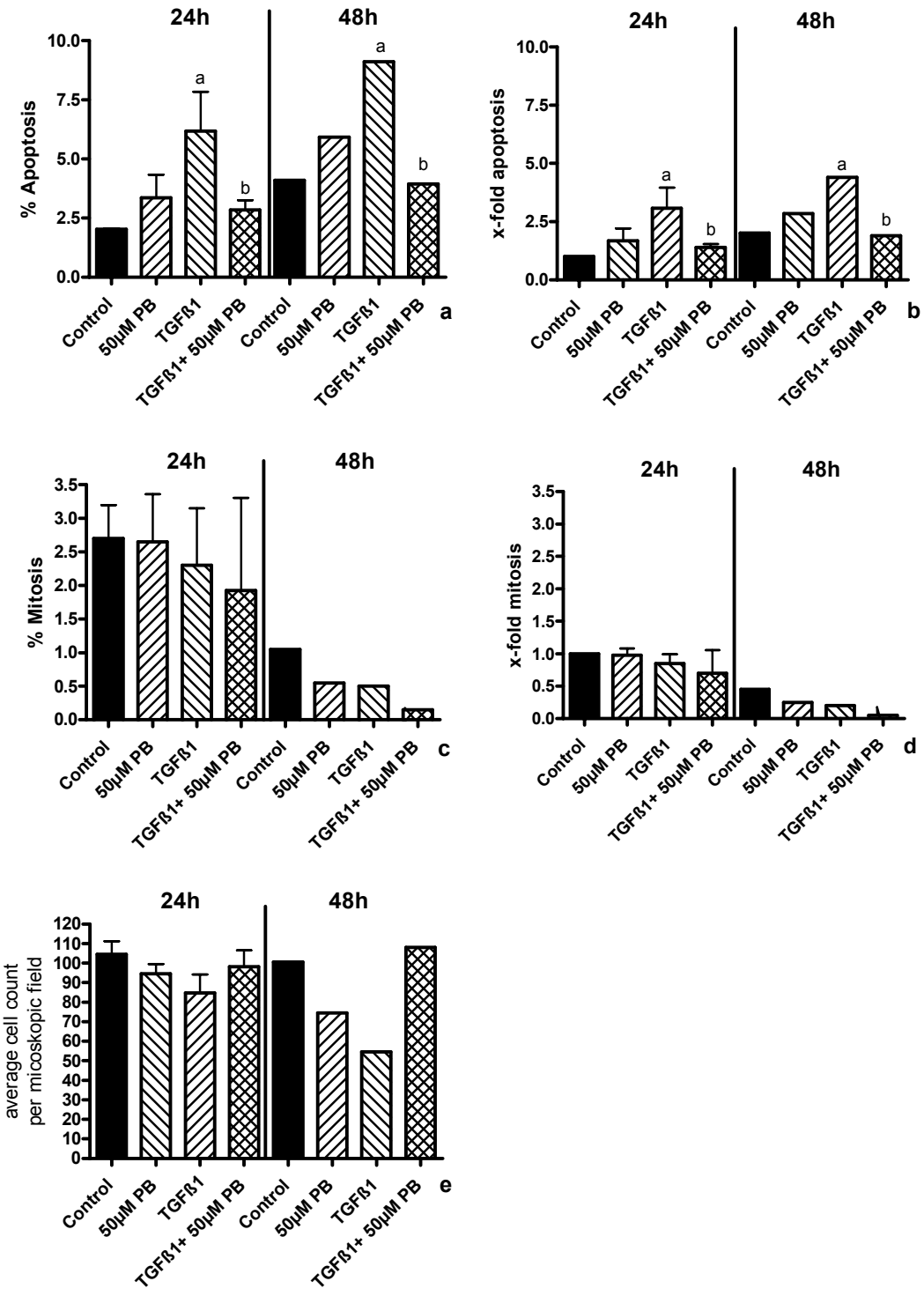

Fig. 4.3 Hep3B cells: Effect of TGF-β1 and phenobarbital on apoptosis, mitosis and cell density.

10 ng/ml TGF-β and 50 µM PB (phenobarbital). Time points of investigation: 24 h and 48 h. (a, b) apoptosis; (c, d) mitosis; (e) cell density (number of cells per microscopic field). Means (±SD) of 1-2 experiments are shown; numbers are listed in appendix, table 8.3. Statistical comparisons: [a)] P<0.05, Co vs. TGF-β1; [b)]TGF-β1vs. PB+TGF-β1 using Tukey-Kramer Multiple Comparisons Test.

4.1.3 Primary rat hepatocytes: Effect of TGF-β1 and phenobarbital on apoptosis, mitosis and cell density

In a third step, the experiments with human hepatoma cells were supplemented by studies with primary hepatocytes as a model for healthy cells. Primary rat hepatocytes are known to be highly sensitive to TGF-β1 and based on previous findings from [OBERHAMMER *et al.*, 1991] cultured primary rat hepatocytes were treated with 3 ng/ml TGF-β1.

The **apoptotic activity** in primary rat hepatocyte culture remained at a low level of about 0.5 % at 30 and 44 hours, respectively (fig. 4.4 a). TGF-β1 (3 ng/ml) led to a significant increase in apoptotic nuclei to about 7.5 % and 5 % at 30 and 44 hours, respectively (fig. 4.1 a). Furthermore, addition of 50 µM phenobarbital significantly ($P < 0.001$) antagonized the pro-apoptotic action of TGF-β1 (inhibition by approximately 40-45 %, fig. 4.4 a). Essentially the same response pattern was obtained when the data were expressed as “x-fold of 24 hour control”.

As to **mitosis**, control hepatoycte cultures displayed 0.15 % mitotic cells, which was considerably less compared to HCC-1.2 cell cultures with mitosis levels of approximately 2.5%. Treatment of primary rat hepatocyte cultures with phenobarbital and TGF-β1, either alone or in combination, did not significantly affect mitotic activity (fig. 4.4 e). The low mitotic activity is typical of primary hepatocyte cultures, even upon stimulation with liver mitogenes such as epidermal growth factor [PARZEFALL *et al.*, 1989]. Consequently, **cell density** as measured at 48 hours after TGF-β1 or phenobarbital-treatment appeared to be determined predominantly by apoptotic activity (fig. 4.4 e).

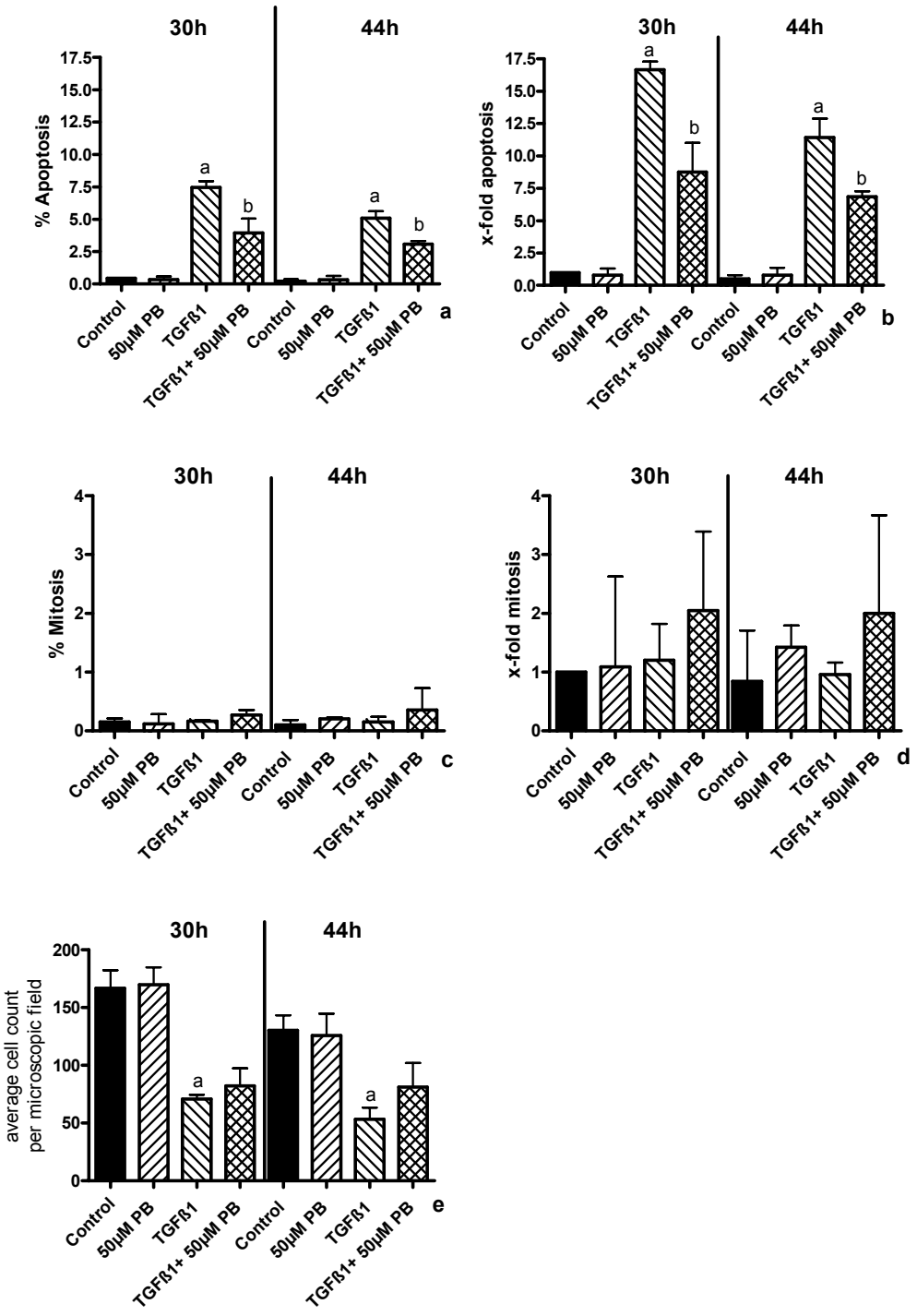

Fig. 4.4 Primary rat hepatocytes: Effect of TGF-β1 and phenobarbital on apoptosis, mitosis and cell density

10 ng/ml TGF-β and 50 µM PB (phenobarbital). Time points of investigation: 30 h and 44 h. (a, b) apoptosis; (c, d) mitosis; (e) cell density (number of cells per microscopic field). Means (±SD) of 2 experiments are shown; numbers are listed in appendix, table 8.4. Statistical comparisons: [a)] P<0.01, Co vs. TGF-β1; [b)]TGF-β1vs. PB+TGF-β1 using Tukey-Kramer Multiple Comparisons Test.

4.2 EFFECT ON PERMETHRIN AND ITS STEREOISOMERS ON HUMAN HEPATOMA CELLS (HCC.1-2, Hep3B) AND PRIMARY RAT HEPATOCYTES

4.2.1 Effect of stereoisomer mixture on apoptosis, mitosis and cell density of HCC1.2 cells.

First, a commercial mixture consisting of about 25 % *cis*-permethrin and 75 % of *trans*-permethrin racemate (chemical purity ~96% as determined by HPLC) was tested. As shown in fig. 4.5 a, 25 µM and 50 µM permethrin alone led to a 2-3 fold, but statistically insignificant, increase in apoptosis at both time points of investigation. TGF-β1 exerted a pro-**apoptotic** activity as in the previous experiments (approximately 5-fold above control), which was further enhanced by combined treatment with 25 or 50 µM permethrin (about 10 fold above control; Fig. 4.5 b).

As to **mitosis**, permethrin, with and without TGF-β1, tended to inhibit mitosis. As a result of its pro-apoptotic, along with the less pronounced anti-mitotic action, permethrin caused a significant loss in the average cell count per microscopic field (fig. 4.5 d).

In conclusion, commercial permethrin possesses a pro-apoptotic action on HCC-1.2 cells, along with a weak anti-mitotic activity. Notably, as illustrated in fig 4.6, the pro-apoptotic action of permethrin was found to be additive to that of TGF-β1.

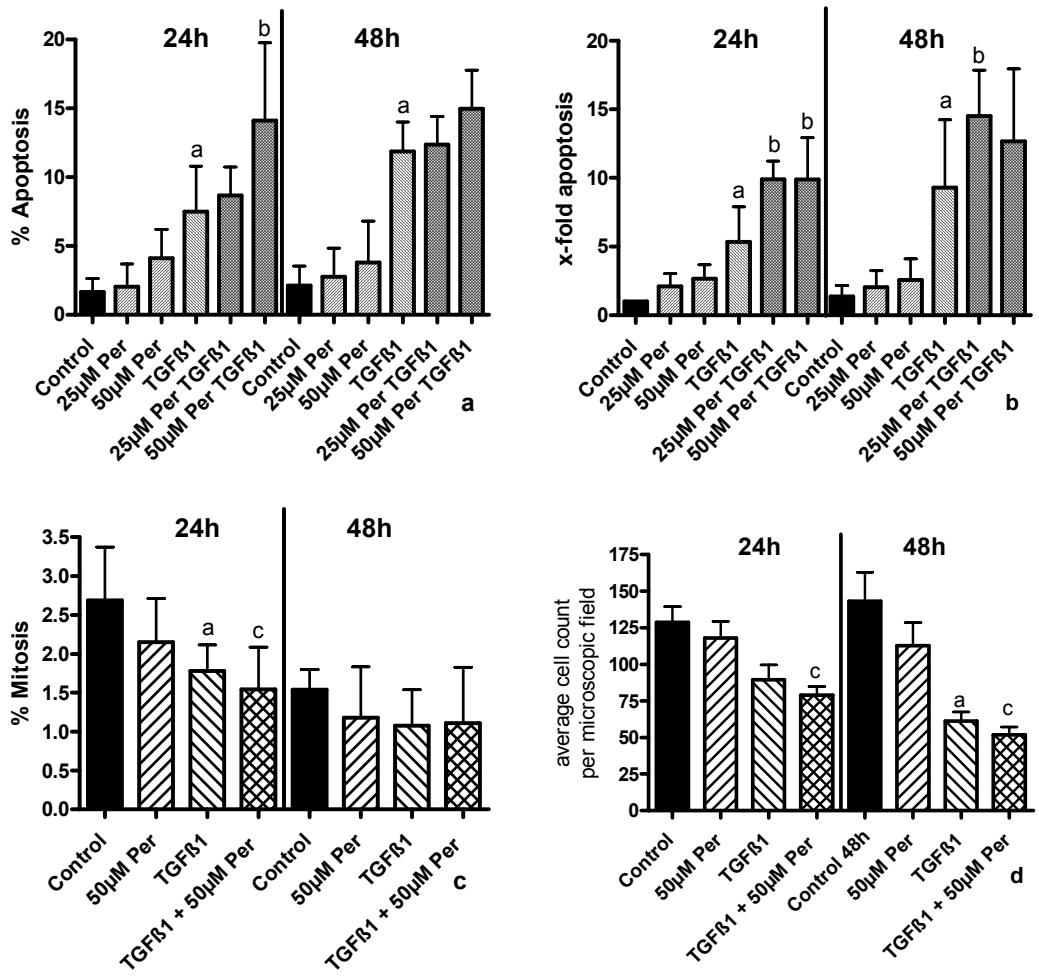

Fig. 4.5 Effect of commercial permethrin on apoptosis, mitosis and cell density in HCC-1.2 cultures.
10 ng/ml TGF-β1; 25 µM and 50 µM permethrin (Per). Time points of investigation: 24 h and 48 h. (a, b) apoptosis; (c) mitosis; (d) cell density (number of cells per microscopic field). Means (±SD) of 4-10 experiments are shown; numbers are listed in appendix, table 8.5. Statistical comparisons**:** [a)] P<0.05, Co vs. TGF-β1; [b)] P<0.05 TGF-β1vs. Per+TGF-β1 [c)] P<0.01, Co vs. Per+TGF-β1, (a, b, d) using Dunn's Multiple Comparisons Test and (c) Tukey-Kramer Multiple Comparisons Test.

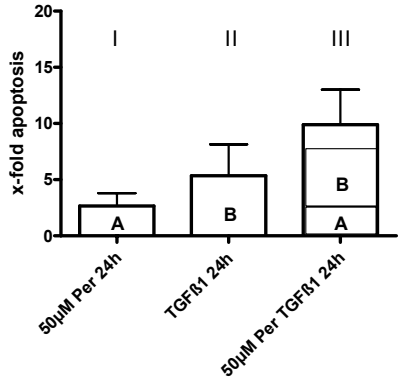

Fig. 4.6 Additive effect of 50µM permethrin and 10 ng/ml TGF-β1 after 24 hours.
(I: Per alone = A; II: TGF-β1 alone = B; III: Per + TGF-β1 ≈ A+B)

4.2.2 Potential effect of chemical impurities of commercial permethrin on apoptosis of HCC-1.2 cells

To check for the possibility that chemical impurities of the commercial permethrin might contribute to the pro-apoptotic effect, the commercial lot was purified by HPLC (chemical purity > 99 %) and subjected to the same test protocol as the non-purified material. As shown in Fig. 4.7 no significant differences between commercial and purified permethrin were found at 24 and 48 hours after treatment, with or without TGF-β1. Thus, commercial permethrin proved to be a suitable positive control for subsequent experiments with purified stereoisomers of permethrin.

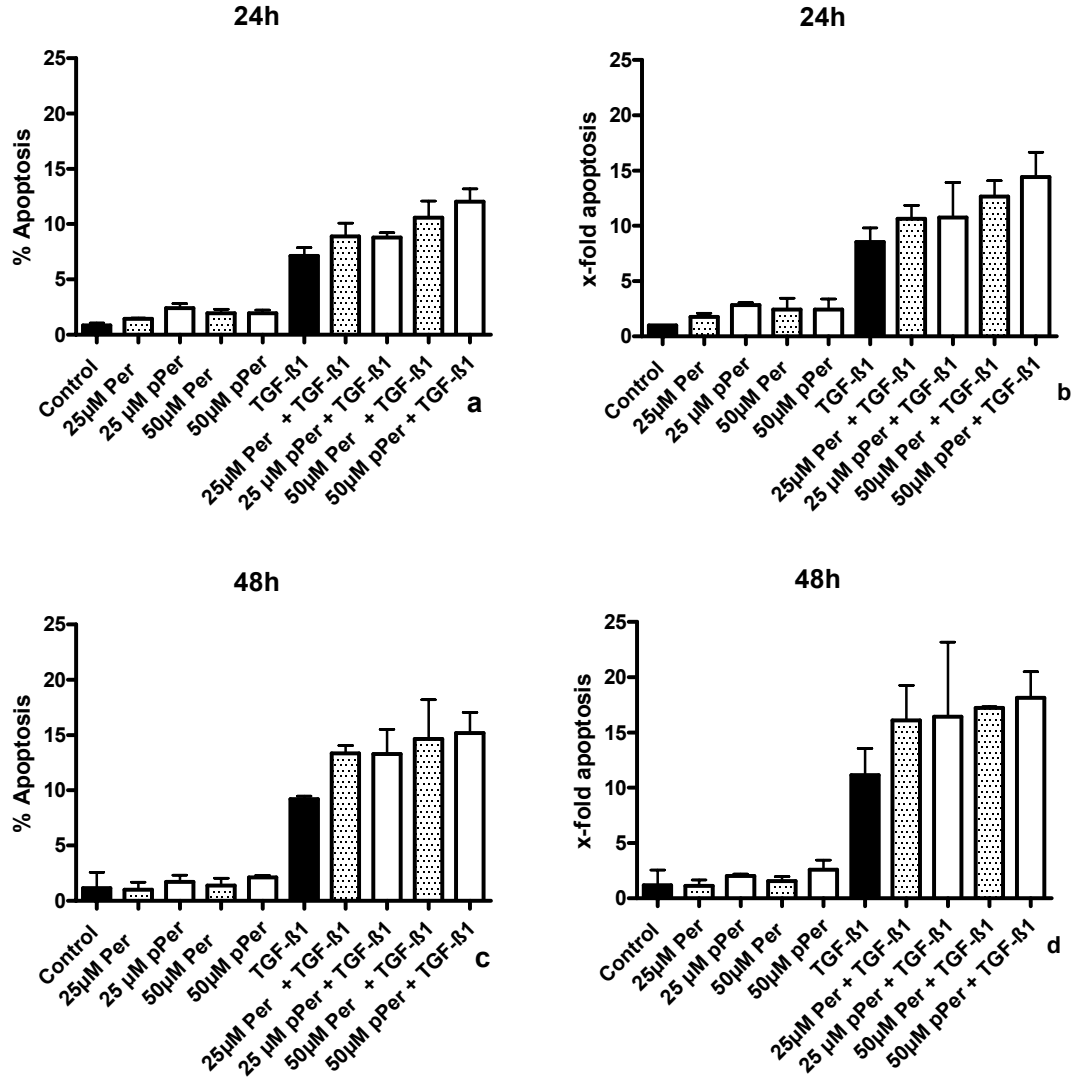

Fig. 4.7 Potential effect of chemical impurities of commercial permethrin on apoptosis of HCC-1.2 cells

10 ng/ml TGF-β1, 25 µM and 50 µM commercial permethrin (Per; dotted columns), pure permethrin (pPer; white columns), respectively. Time points of investigation: 24 h (a, b) and 48 h (c, d). Means (±SD) of 2 experiments are shown; numbers are listed in appendix, table 8.6. No significant differences using Tukey-Kramer Multiple Comparisons Test Per vs. pPer.

4.2.3 Effect of permethrin on TGF-β1-induced apoptosis of human hepatoma cells Hep3B and primary rat hepatocytes

In a further series of experiments, the pro-apoptotioc effect of permethrin on HCC-1.2 was compared Hep3B cells and to primary rat hepatocytes.

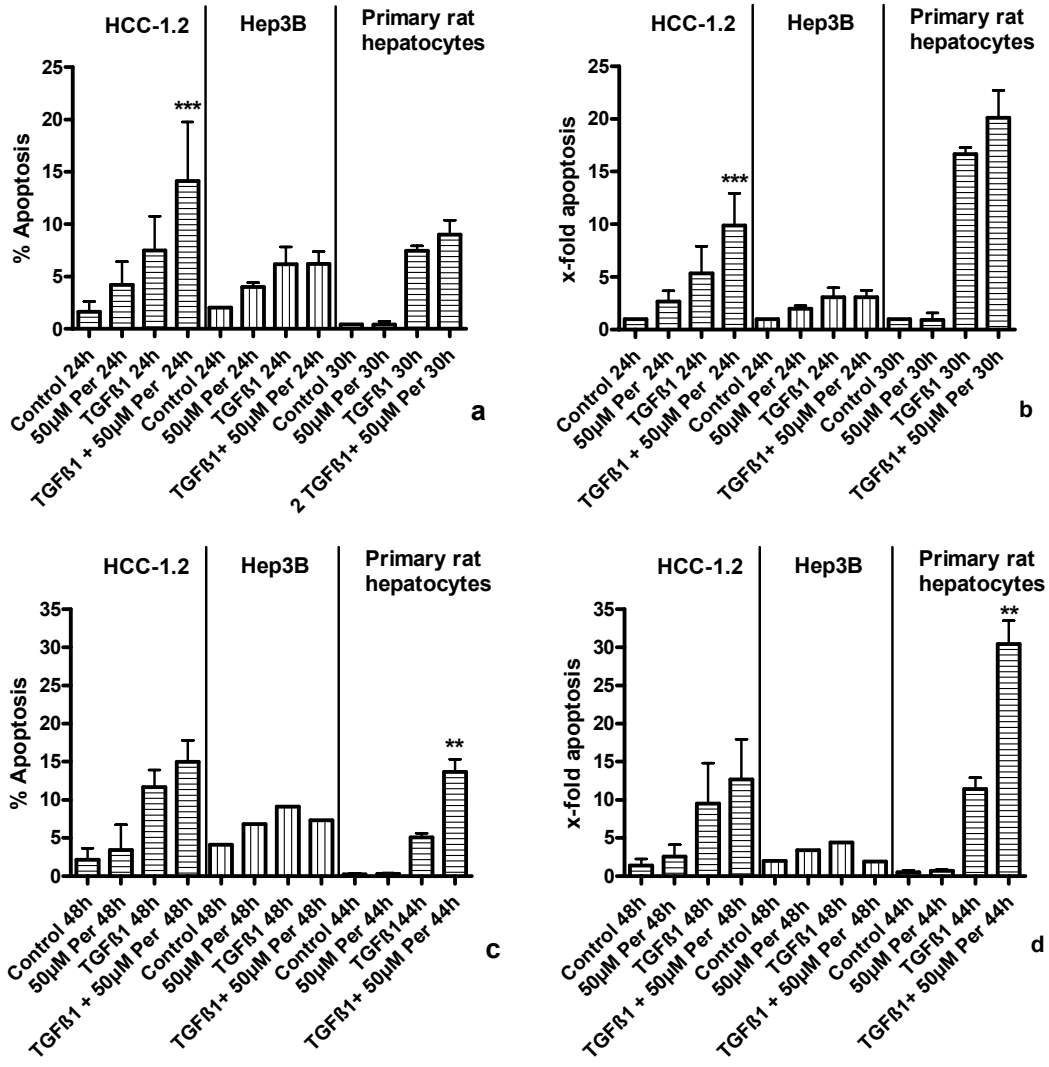

Fig. 4.8 Effect of permethrin on apoptosis of human hepatoma cells Hep3B and primary rat hepatocytes

Effect of 50 µM permethrin (Per) and 10ng/ml TGF-β1 in HCC-1.2 and Hep3B cells after 24 hours (a, b) and 48 hours (c, d). Effect of 50 µM permethrin (Per) and 3ng/ml TGF-β1 in primary rat hepatocytes after 30 hours (a, b) and 44 hours (c, d). Means (±SD) of 1-10 experiments are shown; numbers are listed in appendix, table 8.7. Statistical comparisons: HCC-1.2: ***P<0.001 using Dunn's Multiple Comparisons Test, TGF-β1 vs. Per+TGF-β1. Hep3B and primary rat hepatocytes: **P<0.01 using Tukey-Kramer Multiple Comparisons Test, TGF-β1 vs. Per+TGF-β1.

HCC-1.2 cells responded to TGF-β1 and permethrin as in previous experiments. As to Hep3B cells, permethrin alone (50 µM) tended to enhance apoptotic activity and TGF-β1 caused a moderate increase in apoptosis; combined treatment of TGF-β1 and permethrin did not alter the moderate pro-apoptotic action of TGF-β1. Furthermore, in cultured primary rat hepatocytes permethrin alone (50 µM) did not affect apoptotic activity as compared to controls. In combination with TGF-β 1, permethrin caused at most a slight (20%) enhancement of apoptoses after 24 hours (fig. 4.8b), a seemingly more pronounced effect of combined treatment resulted at 48 hours. However, the data available at present do not allow a final conclusion, further studies incl. more than two time points are required.

4.2.4 Stereoselective apoptotic effects of permethrin

Stereoisomers of permethrin [(trans(+)-(1S,3R)- permethrin (**11-permethrin**); trans(-)-(1R,3S) permethrin (**12-permethrin**); cis(-)-(1S,3S) permethrin (**21-permethrin**); Cis(+)-(1R,3R) permethrin (**22-permethrin**); enantiomeric excess > 99 % each, chemical purity > 99 % each] were tested with HCC-1.2 cells at concentrations of 2 µM, 10 µM and 50 µM for 24 hours, with or without TGF-β1. For comparison, cells were treated with commercial permethrin (*cis*-racemate / *trans*-racemate ~ 25/75).

As shown in fig. 4.9, HCC-1.2 cells responded to TGF-β1 and 50 µM commercial permethrin as in the previous experiments (cf. fig. 4.5 a, b). Treatment with 2 µM, 10 µM and 50 µM of the individual permethrin stereoisomers for 24 hours revealed a dose dependent pro-apoptotic effect (fig. 4.9 a-d). 50 µM of each isomer exhibited the same pro-apoptotic potency as the the equimolar concentration of the commercial formulation.

In the presence of TGF-β1, the stereoiosomers exhibited a dose-dependent additive pro-apoptotic action, becoming manifest at 10 µM and statistically significant, at 50 µM of either isomer (fig. 4.9. a-d).

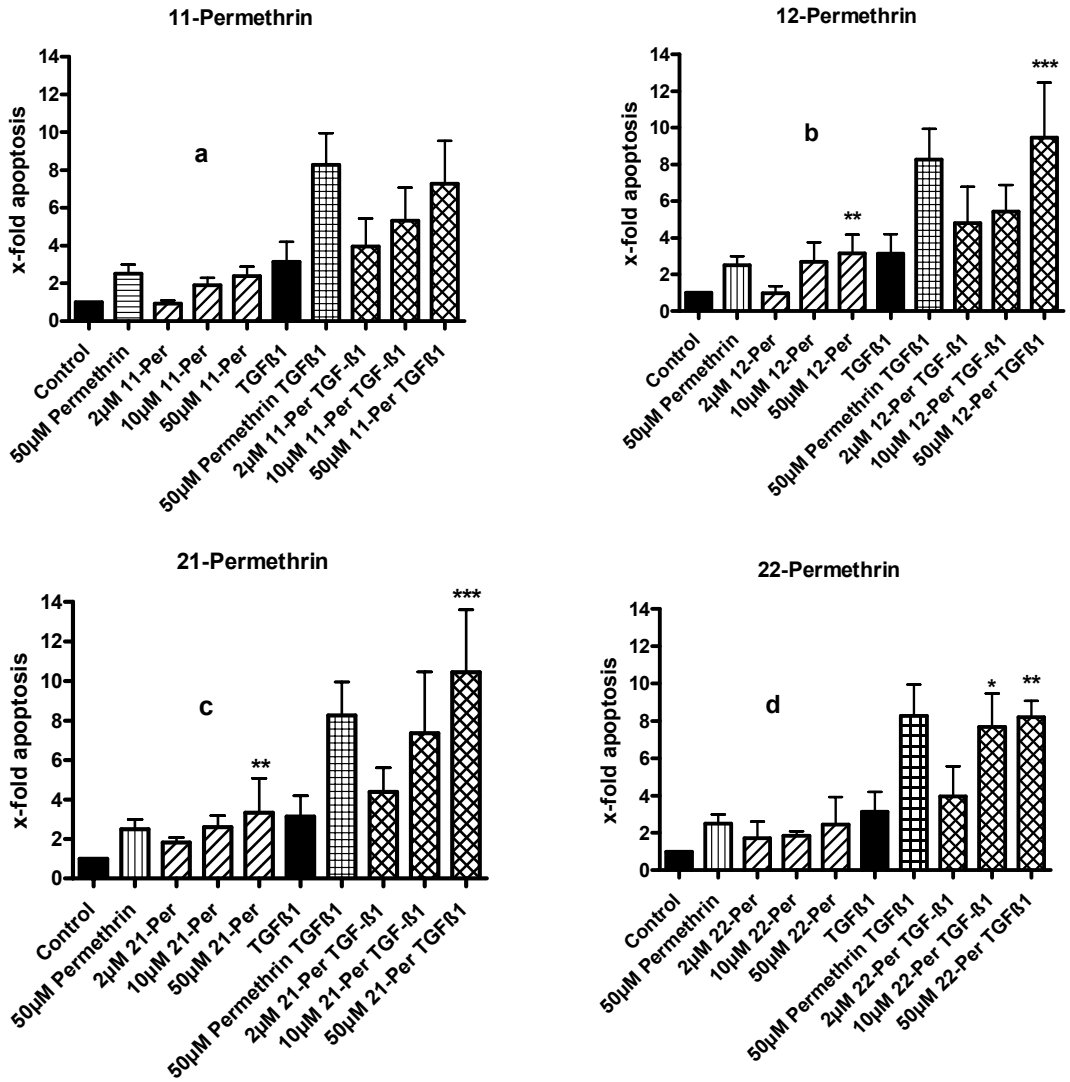

Fig. 4.9 Stereoselective apoptotic effects of permethrin
HCC-1.2 treated with 10 ng/ml TGF-β1, 50 µM commercial permethrin, 2µM, 10µM and 50 µM permethrin-stereoisomers. Time point of investigation: 24h. Means (±SD) of 2-3 experiments are shown; numbers are listed in appendix, table 8.8. Statistical comparisons:
(b) **P<0.01 Co vs. 50µM 12-Per; ***P<0.001 TGF-β1 vs. 50µM 12-Per+TGF-β1.
(c) **P<0.01 Co vs. 50µM 21-Per; ***P<0.001 TGF-β1 vs. 50µM 21-Per+TGF-β1.
(d) *P<0.05 TGF-β1 vs. 10 µM 22-Per+TGF-β1; **P<0.01 TGF-β1 vs. 50µM 12-Per+TGF-β1.
using Tukey-Kramer Multiple Comparisons Test.

As to potential differences in the pro-apoptotic potency among the stereoisomers, the data as plotted in fig. 4.10 revealed neither consistent nor significant differences between 11, 12, 21 and 22 permethrin. These investigations demonstrated that, in contrast to the pronounced differences in the insecticidal (neurotoxic) action of permethrin stereoisomers (isomers with 1*R*-configuration being most effective, cf. Figure 2.2), the pro-apoptotic action of permethrin does not depend on steroisomeric properties of the chemical.

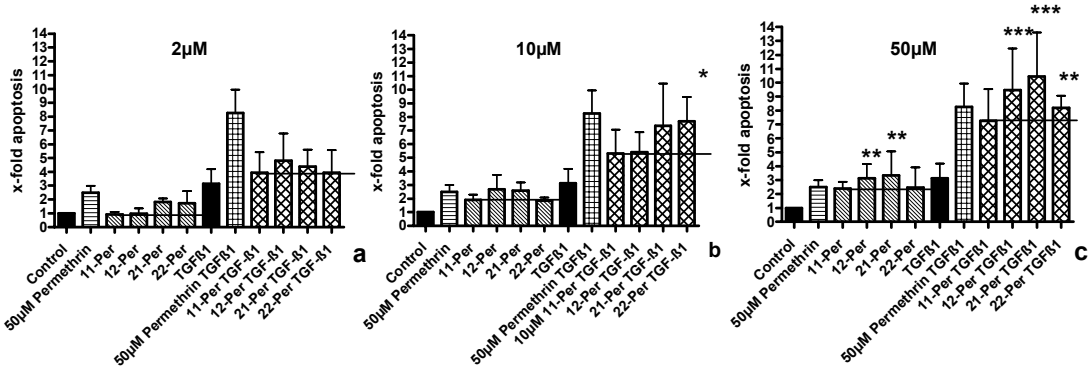

Fig. 4.10 Same data as shown in fig. 4.9. For easier comparison, columns of for each stereoisomer are juxtaposed. Statistical comparisons: see fig. 4.9.

4.2.5 Effects of permethrin metabolites

Finally, to check whether phase I metabolism of permethrin to 3-permethrinic acid (PA), 3-phenoxybenzyl alcohol (PBOH) and 3-phenoxybenzoic acid (PBA), respectively, might modulate the pro-apoptotic action of permethrin, complementary investigations were carried out with these three compound. As reference substances 50µM phenobarbital, 50µM permethrin and 1µM rotenone were applied. Phenobarbital served as reference to take into account potential inhibitory effects of the metabolites, rotenone potential pro-apoptotic effects of metabolites. Retonone was choosen as it known to induce apoptosis of HL-60 cells, involving mitochondrial production of ROS as anticipated for permethrin (see discussion, 5.2.) [N. LI *et al.*, 2003]. In a preliminary, dose-finding experiment HCC-1.2 cells were tested for rotenone sensitivity. As shown in fig. 4.11 rotenone showed a dose and time dependent induction of apoptosis; the concentration of 1µM was chosen for the subsequent experiments with permethrin metabolites.

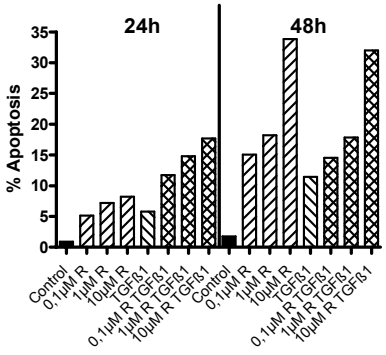

Fig. 4.11 Response of HCC-1.2 to 0,1 µM, 1 µM and 10 µM rotenone (R) and 10 ng/ml TGF-β1.
Time point of investigation: 24h and 48h. Data of individual cell cultures of one experiment are shown; numbers are listed in appendix, table 8.9.

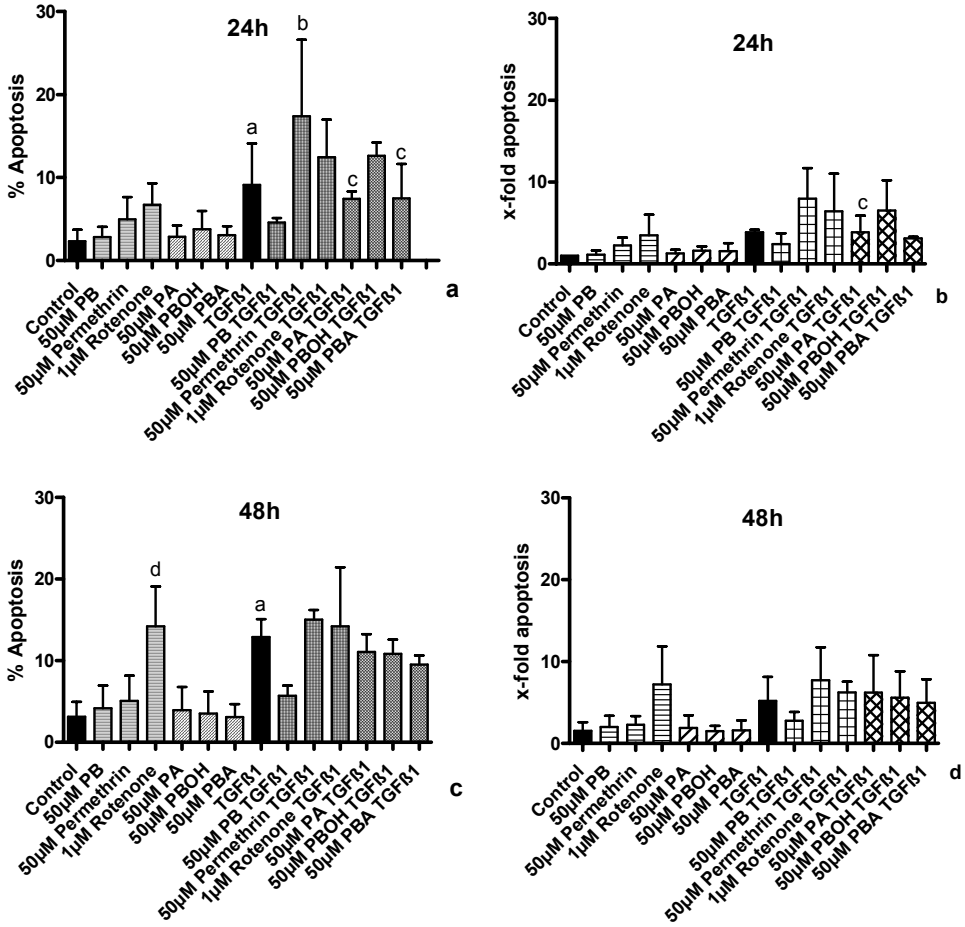

Fig. 4.12 Effects of permethrin metabolites
HCC-1.2 treated with 10 ng/ml TGF-β1, 50 µM permethrin, 50 µM phenobarbital, 1 µM rotenone, 50 µM 3-permethrinic acid (PA), 50 µM 3-phenoxybenzyl alcohol (PBOH) and 50 µM 3-phenoxybenzoic acid (PBA). Time point of investigation: 24h and 48h. Means (±SD) of 3 experiments are shown; numbers are listed in appendix, table 8.10. Statistical comparisons: [a)]P<0.05 Co vs. TGF-β1; [b)]P<0.01 TGF-β1 vs. TGF-β1 + Per; [c)]P<0.05 TGF-β1+Per vs. TGF-β1 + metabolites; [d)]P<0.01 Co vs. Rotenone, no significant differences of "TGF-β1 alone" vs. TGF-β1 + metabolite" as well as among the metabolites were found using (a, b, c) Tukey-Kramer Multiple Comparisons and (d) Dunn's Multiple Comparisons Test.

All permethrin metabolites, along with the parental compound, were tested at the concentration of 50 μM, with and without TGF-β1, at 24 and 48 hours after treatment (fig. 4.12 a-d). In this experimental series permethrin alone caused an approximately 2-fold, TGF-β1 about 4-fold induction of apoptoses (fig. 4.11). Again, the combination of both resulted in an additive effect (fig. 4.11), i.e. an about 8-fold enhancement of apoptotic activity. The permethrin metabolites PA, PBOH and PBA, alone or in combination with TGF-β1, exhibited a less, if any, pro-apoptotic activity as compared to parental permethrin. Essentially the same response pattern was observed at 24 and 48 hours after treatment (4.12 a, b vs. 4.12 c, d). In conclusion, hydrolysis of the ester-bond appears sufficient for greatly reducing the pro-apoptotic potency of permethrin.

5 DISCUSSION

5.1 Evaluation of HCC-1-2 cells as test system for toxicological studies

Previous *in vivo* studies revealed TGF-β1 as a major player in liver growth regulation in rats, namely acting as inhibitor of cell birth and inducer of cell death [OBERHAMMER *et al.*, 1991; BURSCH *et al.*, 1993; SCHULTE-HERMANN *et al.*, 1995; RODGARKIA-DARA *et al.*, 2006]. Furthermore, TGF-β1 induced apoptosis was found to ensue via the intrinsic pathway as demonstrated by caspase analysis *in vivo* [ECKLE *et al.*, 2004]. These *in vivo* findings were supported by studies on primary hepatocyte cultures [OBERHAMMER *et al.*, 1991; BURSCH *et al.*, 1993; SCHULTE-HERMANN *et al.*, 1995; RODGARKIA-DARA *et al.*, 2006]. The first series of experiments the newly established human hepatoma cell line HCC-1.2 confirmed and extended these observations (table 5.1).

Tab. 5.1 Response of liver cells in vivo and in cell culture to TGF-β1 and Phenobarbital

Parameter	In vivo (rat)	Primary rat Hepatocytes	HCC-1.2	Hep3B
TGF-β1: inhibition cell proliferation	+	n.d.	+	n.d.
TGF-β1: induction of apoptosis	+	+	+	+
Apoptosis via the intrinsic pathway	+	n.d.	+	n.d.
Inhibition of TGF-β1-induced apoptosis by Phenobarbital	+	+	+	+

Briefly, HCC-1.2 cells revealed a high sensitivity towards the pro-apoptotic action of TGF-β1. In addition, TGF-β1 exhibited an anti-mitotic effect on HCC-1.2 cells. As to the cellular mechanism of apoptosis execution, Western blot analysis revealed cleavage of caspase 3 and 9, but not of caspase 8 in HCC-1.2 cells [KARWAN *et al*., 2007; BURSCH *et al.,* 2008]. Thus, TGF-β1 induced apoptosis of HCC-1.2 cells ensues via the intrinsic pathway as *in vivo* [ECKLE *et al.*, 2004].

A prominent feature of liver tumor promoter such as phenobartital is to inhibit apoptosis of hepatocytes and thereby, to accelarate development of frank neoplasia in rat liver [BURSCH *et al.*, 1984; SCHULTE-HERMANN *et al.*, 1990], most likely in a concerted action with cytokines of the transforming growth factor-beta family [RODGARKIA-DARA *et al.*, 2006]. Likewise, phenobarbital as well as another liver tumor promoter, 2,3,7,8-Tetrachlorodibenzodioxine, inhibited UV-induced apoptosis in primary rat hepatocytes cultures [BOHNENBERGER *et al.*, 2001; SCHRENK *et al.*, 2004]. The present study confirmed and extended these observations showing that phenobarbital inhibited TGF-β1 induced apoptosis of hepatoma cells (HCC-1.2, Hep3B) in a dose-dependent manner.

Furthermore, the pro-apoptotic effect of TGF-β1 on HCC-1.2 cells were compared with that on primary rat hepatocytes and the human hepatoma cell line Hep3B. In all cell types tested, TGF-β1 exerted a pro-apoptotic action; the pro-apoptotic activity was more pronounced in cultured primary rat hepatocytes than in HCC-1.2 and in Hep3B cells at both after 24 hours and 48 hours.

Taken together, the responses of HCC-1.2 cells to TGF-β1 and phenobarbital are highly concurrent with previous *in vivo*-observations. Furthermore, TGF-β1-induced apoptosis of the cultured hepatoma cells ensue via the intrinsic pathway, the identical pathway activated by normal (healthy) and preneoplastic hepatocytes *in vivo* [ECKLE *et al.*, 2004]. In view of validating toxicological test systems, the higher sensitivity of primary rat hepatocytes to the pro-apoptotic action of TGF-β1 as compared to HCC-1.2 cells is considered to be counterbalanced by the advantage of using a cell line of human origin and (b) by reducing the use animals (hepatocyte donors). In conclusion, HCC-1.2 cells

provide a valuable test system for cell cultures tackling to questions such as on toxicity of permethrin, permethrin-stereoisomers and permethrin-metabolites.

5.2 Studies on permethrin

The present study revealed that the four-isomer mixture of commercial permethrin [cis-racemate / trans-racemate ~ 25/75; 25 and 50 µM] exerted pro-apoptotic action at 24 and 48 hours after treatment of HCC-1.2 cells and somewhat less pronounced, Hep3B cells. Cultured primary rat hepatocytes cells, however, revealed no apoptotic response to permethrin under the experimental conditions used (50µM, 24 and 48 hours).

Likewise, the four isomer mixture of permethrin induced apoptosis in murine SK-DAT cells [ELWAN *et al.*, 2006] and in murine thymocytes [PRATER *et al.*, 2002]. Combined exposure of permethrin along with pyridostigmine-bromide (anti-nerve gas drug) and N;N-diethyl m-toluamide (DEET; insect repellent) caused apoptosis in testicular germ-cell in stressed rats; this kind of study was performed because military personnel was exposed to such a combination during the Persian Gulf War [ABOU-DONIA *et al.*, 2003]. The pesticide mixture lindane, malathion and permethrin (reflecting agricultural exposure scenarios) induced apoptotic and necrotic cell-populations in murine thymocytes [OLGUN *et al.*, 2004].

In the present study, permethrin was tested in combination with TGF-β1. As outlined above, TGF-β1 possibly provides an autocrine loop tuning the response of the liver to chemical injury. Thus, the approach of combining permethrin with TGF-β1 as used in the present study represents the combination of a potentially cytotoxic agent with an endogenous growth regulating cytokine. The series of experiments on combined treatment of permethrin (50 µM) with TGF-β1 (10 ng/ml) revealed an additive effect on apoptosis in HCC-1.2 cells, but only a weak in Hep3B or even none in primary rat hepatocyte cultures. The causes underlying this differential sensitivity are not yet elucidated.

Notably, as commercial permethrin (cis-racemate / trans-racemate ~ 25/75) was used in the first series of experiments, the potential effect of impurities on

apoptosis has to be taken into account. Consequently, HPLC-purified fractions (purity > 99 %) of the commercial permethrin were tested for pro-apoptotic activity on HCC-1.2 cells. No difference in the pro-apoptotic activity of either test material was found; therefore, the commercial permethrin lot was used for all further experiments

As to the mechanism(s) underlying the pro-apoptotic action of permethrin on hepatocytes, Gassner et al. (1997) suggested that permethrin blocks mitochondrial complex I and consequently, cytotoxicity might involve ROS formation and disturbance of energy supply. Meanwhile, the specific targeting of complex I by permethrin has been challenged. Thus, recent studies on submitochondrial particles in mitochondrial bovine heart by Staniek, Bicker and Bursch [personal communication] strongly suggest a permethrin action downstream of complex I, involving inhibition of the NADH-oxidase– activity. Notably, the permethrin phase I metabolites permethrinic acid, 3-phenoxybenzyl alcohol and 3 phenoxybenzoic acid did not inhibit NADH-oxidase-activity in submitochondrial particles [STANIEK, BICKER, BURSCH, unpublished observations, personal communication]. This is in line the results of the present study, revealing a reduced or even lacking pro-apoptotic potency of permethrin metabolites as compared to the parent compound.

In summary, permethrin seem to cause apoptosis via inhibition of mitochondrial respiration, which in turn is known to result in permeabilisation of the outer mitochondrial membrane (OMM), finally leading to apoptosis execution via the intrinsic apoptotic pathway. As the pro-apoptotic action of TGF-β1 is mediated via the same pathway down to final apoptosis execution, the initial events triggered by permethrin and TGF-β1 may well join at the mitochondrial level, thereby providing a plausible explanation for their additive effect on apoptosis (fig. 5.1.).

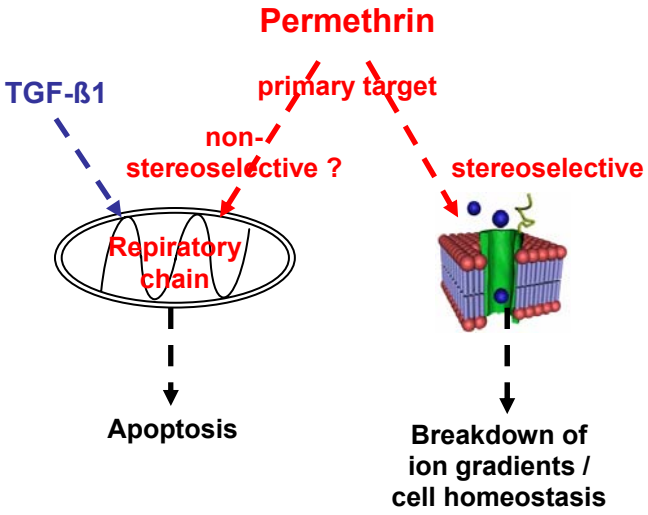

Fig. 5.1 Stereoselectivity of effects caused by permethrin

Finally, the pro-apoptotic potency of the four-isomer mixture of permethrin as well as that of its stereoisomers [(trans(+)-(1S,3R)- permethrin **(11-permethrin)**; trans(-)-(1R,3S) permethrin **(12-permethrin)**; cis(-)-(1S,3S) permethrin **(21-permethrin)**; Cis(+)-(1R,3R) permethrin **(22-permethrin)**] essentially revealed to be equal, as opposed to the insecticidal action of permethrin based upon its interaction with ion-channels. The present study along with the currently ongoing studies by Staniek, Bicker and Bursch suggest that permethrin primarily targets the mitochondrial respiratory chain independent of its stereochemistry and thereby, triggers apoptosis (fig. 5.1). At the current state of knowledge, however, potential stereoselective interactions of permethrin with other initial target molecules cannot be ruled out, but if occurring, the final outcome seems to be dominated by mitochondrial events leading to apoptosis execution.

6 CONCLUSION

The present study revealed the following results:

(a) The newly established hepatoma cell line HCC-1.2 exhibits (1) a high sensitivity towards the anti-proliferative and pro-apoptotic action of TGF-β1; (2) inhibition of TGF-β1-induced apoptosis by liver tumor promoter, as exemplified by Phenobarbital.

(b) The commercial four-isomer mixture (cis-racemate / trans-racemate ~ 25/75) exhibited a dose-dependent pro-apoptotic effect.

(c) Joint application of permethrin with TGF-β1 led to an additive effect on apoptosis in HCC-1.2.

(d) The apoptosis-inducing action of the commercial permethrin mixture and that of highly purified permethrin did not differ, rendering impurities of the commercial batch most unlikely to account for its pro-apoptotic potency.

(e) As compared to HCC-1.2 cells, Hep3B cells exhibited only a weak response to permethrin under the experimental conditions used (50µM, 24 and 48 hours). In cultured primary rat hepatocytes, permethrin alone did not exhibit a pro-apoptotic effect; the data on combined treatment with TGF-β1 do not allow a final conclusion yet.

(f) The pro-apoptotic potency of permethrin was largely reduced upon metabolism to main phase I metabolites.

(g) The permethrin stereoisomers (1R,*cis*, 1S,*cis*, 1R,*trans* and 1S, *trans*) did not exhibit pronounced differences in their pro-apoptotic action on HCC1-2 cells.

In conclusion, HCC-1.2 cell are considered to provide a usefull human liver cell culture system, based upon its ability to detect known *in vivo* activities of TGF-β1 and phenobarbital. Furthermore, permethrin was found to posses a pro-apoptotic activity on human hepatoma cells (HCC-1.2), additive to TGF-β1. Most probably, the pro-apoptotic signalling cascades triggered by TGF-β1 and permethrin join at the level of mitochondria. In contrast to the neurotoxic (insecticidal) action of permethrin, the pro-apoptotic action on liver cells was found not to dependent on the stereoisomeric properties of the chemical.

7 SUMMARY

Stereochemistry plays a crucial role in determining the toxicological profile of many chiral xenobiotics. For instance, the insecticidal (neurotoxic) action of permethrin based upon interaction with sodium-channels is highly stereoselective, with 1R-isomers being much more toxic than 1S-isomers. However, studies on the toxicity of permethrin in other target organs such the liver were carried out with the isomer mixture of permethrin only. Therefore, the present project was initiated to shed light on stereoselectivity of non-neurotoxic profile of permethrin and its stereoisomers in mammalian cells. The present diploma thesis addresses two topics:

1. the validity of newly established human hepatoma cell line HCC-1-2 for toxicity studies

2. the action of commercial four-isomer mixture and stereosisomers on hepatocellular apoptosis.

1. Experimental models:

(a) two established cell culture models, namely primary rat hepatocytes and the human hepatoma cell line Hep3B

(b) a newly established hepatoma cell line HCC-1.2 [SAGMEISTER *et al*., in press; KARWAN *et al*., 2007; BURSCH *et al*., 2008].

2. Substances tested:

(a) commercial four-isomer mixture (cis-racemate / trans-racemate ~ 25/75)

(b) permethrin isomers: (trans(+)-(1S,3R)- permethrin (**11-permethrin**); trans(-)-(1R,3S) permethrin (**12-permethrin**); cis(-)-(1S,3S) permethrin (**21-permethrin**); Cis(+)-(1R,3R) permethrin (**22-permethrin**)

(c) phase I metabolites of permethrin: permethrinic acid, 3-phenoxybenzyl alcohol, and 3-phenoxybenzoic acid.

3. Morphological detection of apoptosis:

chromatin condensation/fragmentation as visualized by the DNA specific stain H33258.

4. Results

(a) The newly established hepatoma cell line HCC-1.2 exhibits (1) a high sensitivity towards the anti-proliferative and pro-apoptotic action of TGF-β1; (2) inhibition of TGF-β1-induced apoptosis by liver tumor promoter, as exemplified by Phenobarbital. Furthermore, Karwan et al. (2007) have shown that TGF-β1 induced apoptosis of HCC-1.2 cells is mediated via the intrinsic pathway.

(b) The commercial four-isomer mixture (cis-racemate / trans-racemate ~ 25/75) exhibited a dose-dependent pro-apoptotic effect.

(c) Joint application of permethrin with TGF-β1 led to an additive effect on apoptosis in HCC-1.2.

(d) The apoptosis-inducing action of the commercial permethrin mixture and that of highly purified permethrin did not differ, rendering impurities of the commercial batch most unlikely to account for its pro-apoptotic potency.

(e) As compared to HCC-1.2 cells, Hep3B cells exhibited only a weak response to permethrin under the experimental conditions used (50μM, 24 and 48 hours). In cultured primary rat hepatocytes, permethrin alone did not exhibit a pro-apoptotic effect; the data on combined treatment with TGF-β1 do not allow a final conclusion yet.

(f) The pro-apoptotic potency of permethrin was largely reduced upon metabolism to main phase I metabolites.

(g) The permethrin stereoisomers (1R,*cis*, 1S,*cis*, 1R,*trans* and 1S,*trans*) did not exhibit pronounced differences in their pro-apoptotic action on HCC1-2 cells.

Zusammenfassung

Stereochemie spielt bei der Ermittlung des toxikologischen Profils eine äußerst wichtige Rolle. Die neurotoxische Wirkung von Permethrin, basierend auf Interaktionen mit Natrium-Kanälen, ist stark von der Stereochemie beeinflusst. 1R-Isomere zeigen eine deutlich höhere toxische Wirkung verglichen zu den 1S-Isomeren. Studien, die jedoch andere Zielorgane untersuchten, wurden nur mit der Vier-Isomer Mischung von Permethrin durchgeführt. Ziel dieses Projektes ist die selektive Wirkung von Permethrin-Stereoisomern in Säugetierzellen zu untersuchen.

Diese Diplomarbeit behandelt zwei Themen:

1. Die Validierung einer neu etablierten humanen Hepatom-Zelllinie (HCC-1.2) für toxikologische Studien und

2. die Wirkung einer kommerziell erhältlichen Vier-Isomer Mischung von Permethrin bzw. Permethrin-Stereoismeren auf hepatozelluläre Apoptosis.

1. Experimentelle Ausführung:

(a) Zwei etablierte Zellkultur Modelle: primäre Rattenhepatozyten und die menschliche Hepatom-Zelllinie Hep3B und
(b) die neu etablierte Hepatom-Zelllinie HCC-1.2.

2. Getestete Substanzen:

(a) die Vier-Isomer Mischung von Permethrin [*cis*-Racemat / *trans*-Racemat ~ 25/75]
(b) Permethrin Stereoisomere [(trans(+)-(1S,3R)- Permethrin; trans(-)-(1R,3S) Permethrin; cis(-)-(1S,3S) Permethrin; Cis(+)-(1R,3R) Permethrin]
(c) Phase I Metaboliten: Permethrinsäure, 3-Phenoxabenzylalkohol and 3-Phenoxybenzoesäure

3. Morpholgische Auswertung der Apoptose:

Chromatin Kondensation/Fragmention visualisiert mittels des DNA spezifischen Farbstoff H33258.

4. Resultate:

(a) Die neu etablierte Hepatom-Zelllinie HCC-1.2 zeigt eine hohe Sensitivität gegenüber der anti-proliferativen und pro-apoptotischen Wirkung von TGF-β1 sowie der Hemmung von TGF-β1 induzierter Apoptose durch Tumorpromotoren am Beispiel von Phenobarbital.

(b) Die Vier-Isomer Mischung von Permethrin weist eine dosisabhängige pro-apoptotische Wirkung auf.

(c) Die gemeinsame Behandlung von Permethrin und TGF-β1 führt zu einem additiven Effekt in HCC-1.2.

(d) Zwischen der kommerziell erhältlichen Permethrin Mischung und dem hoch gereinigten Permethrin bestehen keine Unterschiede in der pro-apoptotischen Wirkung. Verunreinigungen sollten daher keinen Einfluss auf die pro-apoptotische Wirkung von Permethrin haben.

(e) Verglichen zu HCC-1.2 Zellen weisen Hep3B und primäre Rattenhepatozyten nur eine schwache Reaktion gegenüber der Behandlung mit Permethrin auf.

(f) Die pro-apoptotische Wirkung von Permethrin wurde größtenteils durch den Phase I Metabolismus reduziert.

(g) Die Permethrin-Stereoismere weisen keine signifikanten Unterschiede in der pro-apoptotischen Wirkung auf.

8 APPENDIX

Tables

Tab. 8.1 Apoptosis rate of HCC-1.2 cells after 24 h and 48 h, respectively, cultivation with 10 ng/ml TGF-β1 (Fig. 4.1 a-e).

	Apoptosis Mean (%)	Apoptosis x-fold
Control 24h (n=15)	1.5 ± 1.0	1
TGF-β1 24h (n=15)	7.1 ± 3.4	5.6 ± 2.5
Control 48h (n=12)	1.8 ± 1.2	1.5 ± 0.7
TGF-β1 48h (n=12)	10.8 ± 2.7	10.6 ± 4.6

	Mitosis Mean (%)	Mitosis x-fold	Cell density Mean
Control 24h (n=15)	2.5 ± 0.7	1	151 ± 78
TGF-β1 24h (n=15)	1.6 ± 0.7	0.7 ± 0.3	100 ± 52
Control 48h (n=12)	1.5 ± 0.6	0.6 ± 0.3	142 ± 66
TGF-β1 48h (n=12)	1.1 ± 0.8	0.4 ± 0.3	60 ± 27

Tab. 8.2 Response of HCC-1.2 to 1µM, 25µM, 50µM and 100µM phenobarbital (PB) and 10 ng/ml TGF-β1 after 24h and 48h (Fig. 4.2 a-e).

	Apoptosis Mean (%)		Apoptosis Mean (%)
TGF-β1 24h (n=8)	7.2 ± 3.9	100µM PB TGF-β1 24h (n=2)	3.9 ± 0.7
TGF-β1 48h (n=8)	11.4 ± 2.4	1µM PB TGF-β1 48h (n=2)	9.2 ± 0.4
1µM PB TGF-β1 24h (n=2)	6.0 ± 1.9	25µM PB TGF-β1 48h (n=3)	6.0 ± 1.3
25µM PB TGF-β1 24h (n=3)	4.3 ± 1.5	50µM PB TGF-β1 48h (n=6)	5.7 ± 1.2
50µM PB TGF-β1 24h (n=6)	3.7 ± 1.1	100µM PB TGF-β1 48h (n=2)	6.0 ± 1.8
	Apoptosis (x-fold)		Apoptosis (x-fold)
TGF-β1 24h (n=8)	5.4 ± 2.7	100µM PB TGF-β1 24h (n=2)	6.5 ± 1.1
TGF-β1 48h (n=8)	10.0 ± 5.6	1µM PB TGF-β1 48h (n=2)	11.1 ± 5.4
1µM PB TGF-β1 24h (n=2)	6.7 ± 1.4	25µM PB TGF-β1 48h (n=3)	7.1 ± 4.6
25µM PB TGF-β1 24h (n=3)	4.3 ± 1.4	50µM PB TGF-β1 48h (n=6)	4.5 ± 3.6
50µM PB TGF-β1 24h (n=6)	2.7 ± 1.5	100µM PB TGF-β1 48h (n=2)	10.0 ± 3.0

	Mitosis Mean (%)	Mitosis x-fold	Cell density Mean (%)
Control 24h (n=6)	2.5 ± 0.7	1	138 ± 4
50µM PB 24h (n=6)	2.5 ± 0.5	1.0 ± 0.3	125 ± 55
TGF-β1 24h (n=6)	1.7 ± 0.4	0.7 ± 0.2	92 ± 48
50µM PB TGF-β1 24h (n=6)	2.1 ± 0.7	0.8 ± 0.4	112 ± 57
Control 48h (n=6)	1.5 ± 0.4	0.6 ± 0.2	146 ± 74
50µM PB 48h (n=6)	1.6 ± 0.6	0.7 ± 0.3	132 ± 74
TGF-β1 48h (n=6)	1.0 ± 0.5	0.4 ± 0.2	58 ± 23
50µM PB TGF-β1 48h (n=6)	1.2 ± 0.7	0.5 ± 0.3	69 ± 19

Tab. 8.3 Response of Hep3B cells to 50µM phenobarbital (PB) and 10 ng/ml TGF-β1 after 24h and 48h (Fig. 4.3 a-e).

	Apoptosis Mean (%)	Apoptosis x-fold
Control 24h (n=2)	2.0 ± 0.3	1
50µM PB 24h (n=2)	3.4 ± 0.9	1.7 ± 0.5
TGF-β1 24h (n=2)	6.2 ± 1.6	3.1 ± 0.9
TGF-β1+ 50µM PB 24h (n=2)	2.8 ± 0.4	1.4 ± 0.1
Control 48h (n=1)	3.7; 4.5	1.8; 2.2
50µM PB 48h (n=1)	5.2; 6.6	2.5; 3.2
TGF-β1 48h (n=1)	9.1; 9.1	4.4; 4.4
TGF-β1+ 50µM PB 48h (n=1)	4.2; 3.7	2.0;1.8

	Mitosis Mean (%)	Mitosis x-fold	Cell density Mean
Control 24h (n=2)	2.7 ± 0.5	1	105 ± 7
50µM PB 24h (n=2)	2.7 ± 0.7	1.0 ± 0.1	95 ± 5
TGF-β1 24h (n=2)	2.3 ± 0.8	0.9 ± 0.1	85 ± 10
TGF-β1+ 50µM PB 24h (n=2)	1.9 ± 1.4	0.7 ± 0.4	98 ± 8
Control 48h (n=1)	1.2; 0.9	0.5; 0.4	101; 100
50µM PB 48h (n=1)	0.7; 0.4	0.3; 0.2	77; 72
TGF-β1 48h (n=1)	0.7; 0.3	0.3; 0.1	54; 55
TGF-β1+ 50µM PB 48h (n=1)	0.1; 0.2	0.0; 0.1	140; 76

Tab. 8.4 Response of primary rat hepatocytes to 3ng/ml TGF-β1, 50µM phenobarbital (PB) after 30h and 44h (n=2) (Fig. 4.4 a-e).

	Apoptosis Mean (%)	Apoptosis x-fold
Control 30h	0.45 ± 0.15	1
50µM PB 30h	0.35 ± 0.24	0.78 ± 0.53
TGF-β1 30h	7.46 ± 0.47	16.65 ± 0.63
TGF-β1+ 50µM PB 30h	3.94 ± 1.11	8.75 ± 2.26
Control 44h	0.22 ±0.14	0.50 ±0.28
50µM PB 44h	0.33 ± 0.29	0.80 ± 0.57
TGF-β1 44h	5.09 ± 0.53	11.43 ± 1.45
TGF-β1+ 50µM PB 44h	3.07 ± 1.25	6.85 ± 0.42

	Mitosis Mean (%)	Mitosis x-fold	Cell density Mean
Control 30h	0.15 ± 0.06	1	167 ± 16
50µM PB 30h	0.12 ± 0.17	1.09 ± 1.54	170 ± 15
TGF-β1 30h	0.17 ± 0.02	1.21 ± 0.63	71 ± 4
TGF-β1+ 50µM PB 30h	0.27 ± 0.08	2.05 ± 1.34	82 ± 16
Control 44h	0.10 ± 0.08	0.84 ± 0.87	130 ± 13
50µM PB 44h	0.21 ± 0.03	1.42 ± 0.38	126 ± 19
TGF-β1 44h	0.15 ± 0.09	0.96 ± 0.20	53 ± 10
TGF-β1+ 50µM PB 44h	0.36 ± 0.37	2.00 ± 1.67	81 ± 21

Tab. 8.5 Response of HCC-1.2 to 25µM, 50µM commercial permethrin (Per) and 10 ng/ml TGF-β1 after 24h and 48h (Fig. 4.5 a-d).

	Apoptosis Mean (%)	Apoptosis x-fold
Control 24h (n=10)	1.7 ± 1.0	1
25µM Per 24h (n=4)	2.1 ± 1.6	2.1 ± 0.9
50µM Per 24h (n=10)	4.1 ± 2.1	2.7 ± 1.0
TGF-β1 24h (n=10)	7.5 ± 3.3	5.3 ± 2.6
25µM Per TGF-β1 24h (n=4)	8.7 ± 2.1	9.9 ± 1.3
50µM Per TGF-β1 24h (n=10)	14.1 ± 5.7	9.9 ± 3.0
Control 48h (n=7)	2.1 ± 1.4	1.4 ± 0.8
25µM Per 48h (n=4)	2.8 ± 2.1	2.0 ± 1.2
50µM Per 48h (n=7)	3.8 ± 3.0	2.6 ± 1.5
TGF-β1 48h (n=7)	11.9 ± 2.2	9.3 ± 4.9
25µM Per TGF-β1 48h (n=4)	12.4 ± 2.1	14.5 ± 3.4
50µM Per TGF-β1 48h (n=7)	15.0 ± 2.8	12.7 ± 5.3

	Mitosis Mean (%)	Cell density Mean
Control 24h (n=10)	2.7 ± 0.7	129 ± 43
50µM Per 24h (n=10)	2.2 ± 0.6	118 ± 46
TGF-β1 24h (n=10)	1.8 ± 0.3	90 ± 41
TGF-β1 + 50µM Per 24h (n=10)	1.5 ± 0.5	80 ± 24
Control 48h (n=7)	1.5 ± 0.3	143 ± 70
50µM Per 48h (n=7)	1.2 ± 0.7	113 ± 50
TGF-β1 48h (n=7)	1.1 ± 0.5	61 ± 22
TGF-β1 + 50µM Per 48h (n=7)	1.1 ± 0.7	52 ± 17

Tab. 8.6 Response of HCC-1.2 to commercial permethrin (P commercial), permethrin pure (P pure) and TGF-β1 after 24 and 48 hours (n=2) (Fig. 4.7 a-d).

	24 h Apoptosis Mean (%)	48 h Apoptosis Mean (%)	24 h Apoptosis x-fold	48 h Apoptosis x-fold
Control	0.9 ± 0.2	1.2 ± 1.4	1	1,2 ± 1,4
25µM P commercial	1.5 ± 0.1	1.0 ± 0.7	1.8 ± 0.4	1.1 ± 0.5
25 µM P pure	2.4 ± 0.4	1.7 ± 0.6	2.9 ± 0.2	2.0 ± 0.2
50µM P commercial	2.0 ± 0.4	1.4 ± 0.7	2.4 ± 1.0	1.6 ± 0.4
50µM P pure	2.0 ± 0.3	2.1 ± 0.2	2.4 ± 1.3	2.6 ± 0.8
TGF-β1	7.1 ± 0.7	9.2 ± 0.3	8.6 ± 1.3	11.1 ± 2.4
25µM P commercial + TGF-β1	8.9 ± 1.2	13.4 ± 0.7	10.6 ± 1.2	16.1 ± 3.2
25 µM P pure + TGF-β1	8.8 ± 0.4	13.3 ± 2.2	10.8 ± 3.2	16.5 ± 6.7
50µM P commercial + TGF-β1	10.6 ± 1.5	14.6 ± 3.6	12.7 ± 1.4	17.2 ± 0.1
50µM P pure + TGF-β1	12.0 ± 1.2	15.2 ± 1.9	14.4 ± 2.2	18.2 ± 2.3

Tab. 8.7 Effect of 50 µM commercial permethrin (P) and 10ng/ml TGF-β1 in HCC-1.2 and Hep3B cells after 24 hours and the effect of 50 µM permethrin (P) and 3ng/ml TGF-β1 in primary rat hepatocytes after 30 hours (Fig. 4.8 a-d).

	Apoptosis Mean (%)	Apoptosis x-fold
Control 24h (HCC-1.2) (n=10)	1.7 ± 1.0	1.0
50µM P 24h(HCC-1.2) (n=10)	4,2 ± 2,2	2,7 ± 1,0
TGF-β1 24h (HCC-1.2) (n=10)	7.5 ± 3.3	5.3 ± 2.6
TGF-β1+ 50µM P 24h(HCC-1.2) (n=10)	14.1 ± 5.7	9.9 ± 3.0
Control 24h (Hep3B) (n=2)	2.0 ± 0.0	1.0
50µM P 24h (Hep3B) (n=2)	4,0 ± 0,4	2,0 ± 0,3
TGF-β1 24h (Hep3B) (n=2)	6.2 ± 1.7	3.1 ± 0.9
TGF-β1+ 50µM P 24h (Hep3B) (n=2)	6.2 ± 1.2	3.1 ± 0.7
Control 30h (primary rat hepatocytes) (n=2)	0.45 ± 1.01	1.0
50µM P 30h (primary rat hepatocytes) (n=2)	0,41 ± 0,30	0,93 ± 0,67
TGF-β1 30h (primary rat hepatocytes) (n=2)	7.46 ± 0.47	16.70 ± 0.64
TGF-β1+ 50µM P 30h (primary rat hepatocytes) (n=2)	9.01 ± 1.38	20.10 ± 2.62

	Apoptosis Mean (%)	Apoptosis x-fold
Control 48h (HCC-1.2) (n=7)	2.1 ± 1.5	1.4 ± 0.9
50µM P 48h(HCC-1.2) (n=7)	3,4 ± 3,3	2,6 ± 1,5
TGF-β1 48h (HCC-1.2) (n=7)	11.7 ± 2.2	9.5 ± 5.3
TGF-β1 + 50µM P 48h(HCC-1.2) (n=7)	15.0 ± 2.8	12.7 ± 5.3
Control 48h (Hep3B) (n=1)	3.7; 4.5	1.8; 2.2
50µM P 48h (Hep3B) (n=1)	5.0¸8.6	2.5; 4.3
TGF-β1 48h (Hep3B) (n=1)	9.1; 9.1	4.4; 4.4
TGF-β1+ 50µM P 48h (Hep3B) (n=1)	8.4; 6.2	2.0; 1.8
Control 44h (primary rat hepatocytes) (n=2)	0.22 ± 1.14	0.50 0.28
50µM P 44h (primary rat hepatocytes) (n=2)	0,30 ± 0,09	0,67 ± 0,18
TGF-β1 44h (primary rat hepatocytes) (n=2)	5.09 ± 0.53	11.43 ± 1.45
TGF-β1+ 50µM P 44h (primary rat hepatocytes) (n=2)	13.60 ±1.78	30.40 ± 3.04

Tab. 8.8 HCC-1.2 treated with 10ng/ml TGF-β1, 50µM permethrin and permethrin-stereoisomers (2 µM, 10 µM and 50 µM) for 24 hours (Fig. 4.9 a-d).

	11-Per Apoptosis Mean (%)	11-Per Apoptosis x-fold	12-Per Apoptosis Mean (%)	12-Per Apoptosis x-fold
Control (n=3)	2.0 ± 0.5	1	2.0 ± 0.5	1
50µM Permethrin (commercial) (n=3)	4.8 ± 0.3	2.5 ± 0.5	4.8 ± 0.3	2.5 ± 0.5
2µM Per-stereoisomer (n=2)	1.9 ± 0.2	0.9 ± 0.2	2.1 ± 1.3	1.0 ± 0.4
10µM Per-stereoisomer (n=3)	3.7 ± 0.9	1.9 ± 0.4	5.0 ± 2.3	2.7 ± 1.1
50µM Per-stereoisomer (n=3)	4.6 ± 0.8	2.4 ± 0.5	6.0 ± 1.1	3.2 ±1.0
TGF-β1 (n=3)	6.0 ± 1.4	3.1 ± 1.1	6.0 ± 1.4	3.1 ± 1.1
50µM Permethrin (commercial) TGF-β1 (n=3)	15.8 ± 1.3	8.3 ± 1.7	15.8 ± 1.3	8.3 ± 1.7
2µM Per-stereoisomer TGF-β1 (n=2)	7.9 ± 09	4.0 ± 1.5	9.5 ± 1.5	4.8 ± 2.0
10µM Per-stereoisomer TGF-β1 (n=3)	10.1 ± 2.0	5.3 ± 1.8	10.2 ± 0.8	5.4 ± 1.5
50µM Per-stereoisomer TGF-β1(n=3)	13.9 ± 3.0	7.3 ± 2.3	17.8 ± 2.7	9.5 ± 3.0
	21-Per Apoptosis Mean (%)	21-Per Apoptosis x-fold	22-Per Apoptosis Mean (%)	22-Per Apoptosis x-fold
Control (n=3)	2.0 ± 0.5	1	2.0 ± 0.5	1
50µM Permethrin (commercial) (n=3)	4.8 ± 0.3	2.5 ± 0.5	4.8 ± 0.3	2.5 ± 0.5
2µM Per-stereoisomer (n=2)	3.8 ± 0.5	1,8 ± 0,2	3.8 ± 2.8	1.7 ± 0.9
10µM Per-stereoisomer (n=3)	5.0 ± 1.5	2.6 ± 0.6	3.7 ± 1.4	1.9 ± 0.2
50µM Per-stereoisomer (n=3)	6.2 ± 2.4	3.3 ± 1.7	5.3 ± 4.5	2.5 ± 1.5
TGF-β1 (n=3)	6.0 ± 1.4	3.1 ± 1.1	6.0 ± 1.4	3.1 ± 1.1
50µM Permethrin (commercial) TGF-β1 (n=3)	15.8 ± 1.3	8.3 ± 1.7	15.8 ± 1.3	8.3 ± 1.7
2µM Per-stereoisomer TGF-β1 (n=2)	8.8 ± 0.1	4.4 ± 1.5	7.8 ± 1.3	4.0 ± 1.6
10µM Per-stereoisomer TGF-β1 (n=3)	13.7 ± 4.7	7.4 ± 3.1	14.7± 1.8	7.7 ± 1.8
50µM Per-stereoisomer TGF-β1(n=3)	19.5 ± 2.2	10.5 ± 3.2	15.6 ± 2.2	8.2 ± 0.9

Tab. 8.9 Response of HCC-1.2 to 0.1 µM, 1 µM and 10 µM rotenone (R) and 10 ng/ml TGF-β1 after 24h and 48h (n=1) (Fig. 4.11).

24 h	Mean (%)	**48h**	Mean (%)
Control	0.8; 1.0	Control	1.6; 1.9
0,1µM R	6.7; 3.6	0,1µM R	18.0; 12.1
1µM R	7.1; 7.3	1µM R	17.2; 19.2
10µM R	10.2; 6.2	10µM R	40.0; 27.7
TGF-β1	5.8; 5.8	TGF-β1	10.4; 12.5
0,1µM R TGF-β1	11.4; 12.0	0,1µM R TGF-β1	16.9; 12.2
1µM R TGF-β1	13.8; 15.8	1µM R TGF-β1	16.9; 18.8
10µM R TGF-β1	15.0; 20.4	10µM R TGF-β1	28.4; 35.6

Tab. 8.10 HCC-1.2 treated with 10 ng/ml TGF-β1 50 µM permethrin, 50 µM phenobarbital, 1 µM rotenone, 50 µM 3-permethrinic acid (PA), 50 µM 3-phenoxybenzyl alcohol (PBOH) and 50 µM 3-phenoxybenzoic acid (PBA) for 24 hours and 48 hours (n=3) (Fig. 12 a-d).

24h	Apoptosis Mean (%)	Apoptosis x-fold		Apoptosis Mean (%)	Apoptosis x-fold
Control	2.4 ± 1.4	1	TGF-β1	9.1 ± 5.0	3.9 ± 0.3
50µM PB	2.8 ± 1.2	1.2 ± 0.5	50µM PB TGF-β1	4.6 ± 0.6	2.4 ± 1.3
50µM Permethrin	5.0 ± 2.7	2.3 ± 0.9	50µM Permethrin TGF-β1	17.4 ± 9.2	8.0 ± 3.7
1µM Rotenone	6.7 ± 2.6	3.5 ± 2.5	1µM Rotenone TGF-β1	12.5 ± 4.5	6.4 ± 4.6
50µM PA	2.9 ± 1.4	1.3 ± 0.4	50µM PA TGF-β1	7.4 ± 0.9	3.9 ± 2.0
50µM PBOH	3.8 ± 2.2	1.6 ± 0.5	50µM PBOH TGF-β1	12.6 ± 1.6	6.5 ± 3.2
50µM PBA	3.1 ± 1.1	1.6 ± 1.1	50µM PBA TGF-β1	7.5 ± 4.1	3.1 ± 1.1

48h	Apoptosis Mean (%)	Apoptosis x-fold		Apoptosis Mean (%)	Apoptosis x-fold
Control	3.1 ± 1.8	1.5 ± 1.1	TGF-β1	12.9 ± 2.2	5.2 ± 3.0
50µM PB	4.2 ± 2.8	2.0 ± 1.4	50µM PB TGF-β1	5.7 ± 1.3	2.8 ± 1.1
50µM Permethrin	5.0 ± 3.1	2.3 ± 1.1	50µM Permethrin TGF-β1	15.0 ± 1.1	7.7 ± 4.0
1µM Rotenone	14.2 ± 4.9	7.2 ± 4.7	1µM Rotenone TGF-β1	15.0 ± 1.1	6.2 ± 1.3
50µM PA	3.9 ± 2.8	1.9 ± 1.5	50µM PA TGF-β1	11.1 ± 2.2	6.2 ± 4.6
50µM PBOH	3.5 ± 2.7	1.5 ± 0.6	50µM PBOH TGF-β1	10.8 ± 1.8	5.6 ± 3.3
50µM PBA	3.1 ± 1.6	1.6 ± 1.2	50µM PBA TGF-β1	9.4 ± 1.3	4.9 ± 2.9

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Danksagung

An erster Stelle bedanke ich mich bei Herrn Prof. Dr. Ibrahim Elmadfa, der es mir ermöglichte diese Diplomarbeit durchzuführen.

Ein herzliches Dankeschön an Herrn Prof. Dr. Wilfried Bursch und an Hern Dr. Wolfgang Bicker für die ausgezeichnete wissenschaftliche Betreuung der Diplomarbeit.

Außerdem bedanke ich mich bei Herrn Dr. Bicker, bei Herrn Prof. Dr. Lämmerhofer und bei Herrn Prof. Dr. Lindner am Institut für Analytische Chemie für die gute Zusammenarbeit und finanzielle Unterstützung.

Weiters bedanke ich mich herzlich bei Anneliese Karwan für die ausgezeichnete Einschulung und Unterstützung.

Mein Dank gilt auch meiner Zimmerkollegin Mag. Miriam Mayer für ihre tatkräftige Unterstützung.

Ein besonderer Dank gebührt meiner Familie die mir dieses Studium ermöglicht haben und immer ein wichtiger Rückhalt für mich waren.

Abschließend danke ich meinen Freunden und Studienkollegen, die mir während meines Studiums stets zur Seite standen.

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Wien, August 2008