THE DEVELOPMENT OF
2,3,7,8-TETRACHLOROPHENOTHIAZINE
AS A DRUG LEAD

By

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requirements for the degree of Doctor of Philosophy

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that this is the approved version of the following dissertation:

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ABSTRACT

The dioxin analogue 2,3,7,8-tetrachlorophenothiazine (TCPT) was synthesized and developed as a drug lead with potential applications in chemoprevention, body weight control, type II diabetes, contraception, and longevity. These well-documented effects of dioxins are believed to be mediated through two key mechanisms of action: AhR binding with downsteam induction of enzyme activity, and reduced IGF-1 signaling. The effects of TCPT on these pathways were investigated quantitatively.

TCPT showed an affinity to AhR similar to that of TCDD, and it induced EROD activity with high efficacy *in vitro*. Its potency regarding enzyme induction, however, was lower by two orders of magnitude compared to that of TCDD. Metabolism was demonstrated to yield low-potency derivatives.

Dose-response relationships were developed for TCPT and TCDD regarding the reduction of serum IGF-1 levels in the rat. TCPT displayed in this regard also high efficacy, but very low potency compared to TCDD.

Unlike TCDD, TCPT did not affect thyroid homeostasis at doses that lowered IGF-1 signaling. These findings suggest that TCPT could become a suitable drug lead.

To my Family

How can I describe to you the beauty, the grandeur, the freedom of these Kansas prairies? Undulating to the right and left on either side, a sea of verdure – unfenced, unbroken – unadorned, yet beautiful in their sunny repose of light and shade in their hills and hollows.

R. A. Parks, June 26, 1868

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LIST OF ABBREVIATIONS

AhR   aryl hydrocarbon receptor

ARNT   aryl hydrocarbon receptor nuclear translocator

CPZ   chlorpromazine

DRE   dioxin response element

EROD   ethoxyresorufin-O-deethylase

HIF   hypoxia-inducible factor

HpCB   heptachloro biphenyl

HpCDD   heptachlorodibenzo-*p*-dioxin

HpCDF   heptachlorodibenzofuran

HxCB   hexachlorobiphenyl

HxCDD   hexachlorodibenzo-*p*-dioxin

HxCDF   hexachlorodibenzofuran

IGF-1   insulin-like growth factor-1

IGFBP   insulin-like growth factor binding protein

LDR	loading dose rate (x_0^*)
Me-TCPT	N-methyl-2,3,7,8-tetrachlorophenothiazine
Me-TCPT-O	N-methyl-2,3,7,8-tetrachlorophenothiazine-5-oxide
Me-TCPT-O$_2$	N-methyl-2,3,7,8-tetrachlorophenothiazine-5,5-dioxide
MDR	maintenance dose rate (x_0)
NIOSH	National Institute for Occupational Safety and Health
OCB	octachlorobiphenyl
OCDD	octachlorodibenzo-p-dioxin
OCDF	octachlorodibenzofuran
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-p-dioxin
PCDF	polychlorinated dibenzofuran
PeCB	pentachlorobiphenyl
PeCDD	pentachlorodibenzo-p-dioxin
PeCDF	pentachlorodibenzofuran

PEPCK	phosphoenolpyruvate carboxykinase
PHAH	polyhalogenated aromatic hydrocarbon
T_3	free triiodo-thyronine
T_4	free thyroxine
TBF	total body fat
TCB	tetrachlorobiphenyl
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TCDF	tetrachlorodibenzofuran
TCPT	2,3,7,8-tetrachlorophenothiazine
TCPT-O	2,3,7,8-tetrachlorophenothiazine-5-oxide
TCPT-O_2	2,3,7,8-tetrachlorophenothiazine-5,5-dioxide
TEF	toxic equivalency factor
TEQ	toxicity equivalent
TT_3	total triiodo-thyronine
TT_4	total thyroxine

NOTE

All *in vivo* studies were conducted under kinetic steady-state conditions for the minimization of experimental variabilities. To achieve a rapid onset of equilibrium, doses were administered as loading dose rate (LDR) followed by maintenance dose rates (MDRs).

$$dose = LDR + \sum_{n=1}^{n} MDR$$

The LDR it is referred to as dose rate despite its single application per dosing regimen, because it represents a part of the total, or cumulative, dose.

CHAPTER 1

INTRODUCTION

1.1 Dioxins and Persistent Dioxin-Like Compounds (Fried KW and Rozman KK, 2007)

Polyhalogenated aromatic hydrocarbons (PHAHs) are mostly anthropogenic organic compounds, which share general dispositional and toxicological properties due to their structural similarities. The most prominent members of this class of toxicants are polychlorinated dibenzo-*p*-dioxins (PCDDs), two classes of dioxin-like compounds (polychlorinated dibenzofurans [PCDFs] and polychlorinated biphenyls [PCBs]), and some pesticides, such as *p,p'*-dichlorodiphenyl-trichloroethane (DDT) and hexachlorobenzene (HCB). Brominated and mixed halogen congeners have similar physical and biological properties, resulting in comparable disposition and effects (U.S. Department of Health & Human Services, 1995). PHAHs are ubiquitous environmental pollutants. Many are persistent and detrimental to the environment, as well as to humans and have been listed with other persistent organic pollutants (POPs) as the "Dirty Dozen" (Table 1-1). All of these compounds have been banned or are being phased out in accordance with the Stockholm Convention of 2001, which was signed by 122 countries including the U.S. It became effective on May 17th, 2004, when it was ratified by 50 countries (UNEP, 2003).

Table 1-1: The Dirty Dozen, as identified by the Stockholm Convention. Almost half of the compounds are PHAHs (*).

Compound	Use	Structure
Aldrin	Insecticide	Chlorinated 1,4:5,8-dimethanonaphthalene derivative
Chlordane	Insecticide	Chlorinated 4,7-methanoindane derivative
DDT*	Insecticide	Chlorinated diphenylethane derivative
Dieldrin	Insecticide	Chlorinated 1,4:5,8-dimethanonaphthalene derivative
Endrin	Insecticide, rodenticide	Chlorinated 1,4:5,8-dimethanonaphthalene derivative
HCB*	Fungicide, industrial compound and byproduct	Perchlorinated benzene
Heptachlor	Insecticide	Chlorinated 4,7-methanoindene derivative
Mirex	Insecticide	Perchlorinated cyclobuta[cd]pentalene
PCBs*	Industrial compounds	Chlorinated biphenyls
PCDDs*	Combustion/industrial byproducts	Chlorinated dibenzo-*p*-dioxins
PCDFs*	Combustion/industrial byproducts	Chlorinated dibenzofurans
Toxaphen	Insecticide	Chlorinated norbornane derivatives

1.1.1 PCDDs and PCDFs
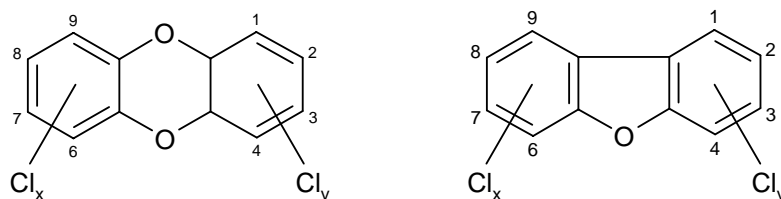
Figure 1-1: Chemical structures of PCDDs (left) and PCDFs (right).

PCDD/Fs are undesired byproducts arising mainly from anthropogenic activity. Different chlorination patterns combined with the consideration of symmetry result in 75 possible dioxin and 205 furan congeners. The most toxic ones, being halogenated in the 2,3,7,8 positions, are strictly planar molecules (Boer *et al.*, 1972).

In 1872, Merz & Weith reported the first documented synthesis of PCDDs (Merz and Weith, 1872). The high biological potency of this class of compounds, however, was not noted until 1957, when Sandermann conducted research on the fungicide pentachlorophenol (PCP) (Sandermann *et al.*, 1957). He discovered PCDDs as byproducts of a process for manufacturing plywood using PCP as a wood preservative. The perchlorinated congener (octachlorodibenzo-*p*-dioxin, OCDD) showed no activity towards termites or mold, whereas tetrachloro-diphenylene dioxide (later referred to as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD) was highly effective against both. Researchers involved in this discovery soon developed symptoms of exposure, most prominently chloracne, which led to the end of Sandermann's research on the pyrolysis of PCP (Sandermann, 1984). Kimmig &

Schulz linked these symptoms to identical clinical signs in workers at plants manufacturing trichlorophenoxyacetic acid (2,4,5-T) (Kimmig and Schulz, 1957a; Kimmig and Schulz, 1957b; Schulz, 1957). It was found that dioxins were generated as byproducts in the industrial synthesis of this chlorinated pesticide as well as other chloroorganic syntheses, such as the production of trichlorobenzene and polyhalogenated biphenyls. PCDDs were found as contaminants of 2,4,5-T in the defoliant Agent Orange, which was used in Operation Ranch Hand during the Vietnam War. The concentration of TCDD ranged from 0.1-47 μg TCDD per g Agent Orange (Young *et al.*, 1976). It was public awareness of the health problems in veterans that started research on dioxins around 1970. Reports of industrial accidents with the release of PCDDs date back as early as 1949 (Ashe and Suskind, 1949), but a major accident in a 2,4,5-T producing plant in Seveso, Italy, in 1976 became the most infamous episode. Estimates of the amount of TCDD, the most potent dioxin congener, released range from 300 g - 34 kg (Bisanti *et al.*, 1978; DiDomenico *et al.*, 1990). The most widely accepted estimate is ca. 1.3 kg, which contaminated an area of approximately one square mile affecting about 37,000 people (Cikryt, 1991).

The main sources of dioxins and furans in the environment are thermal processes (production of metals and mineral products), combustion (waste incineration, heat and power generation, open burning processes) and the chloroorganic industry. Each production condition generates a characteristic cluster of congeners. Therefore, environmental samples can be linked to emission sources. PCDD/Fs are not commercially synthesized except in small amounts for scientific

research. The thermal formation of PCDDs is a combination of de novo synthesis

(Vogg and Stieglitz, 1986) and generation from precursors (Dickson *et al.*, 1989) in a

temperature window of 250-350 ºC, as found in smelters and outdated waste

combustion plants (Lenoir and Fiedler, 1992). PCDD/Fs are also generated during

natural processes including forest fires, biodegradation and biosynthesis (Klimm,

1996; Klimm *et al.*, 1998), and geothermal activities. Furthermore, congener clusters

have been found in historical and pre-historical sediments world-wide (Australia

(Müller *et al.*, 1999; Gaus *et al.*, 2001; Gaus *et al.*, 2001), Germany (Jobst and Aldag,

2000), China (Hashimoto *et al.*, 1995), Japan (Hashimoto *et al.*, 1990), USA (Ferrario

et al., 2000)). The composition of those mixtures is not related to any pattern

generated by anthropogenic processes (Gaus *et al.*, 2001), indicating a truly natural

formation. The United Nations Environmental Programme (UNEP) published a

comprehensive inventory of contributions to dioxin release, which allow countries to

estimate total emissions according to their domestic economy (UNEP, 2005).

The environmental fate of TCDD is determined by its persistence. Studies

after the accidental release in Seveso showed a half-life in soil of about 9-12 months

(Homberger *et al.*, 1979). Studies at test sites in Florida and Utah revealed a climate-

dependency of the half-life and reported 6.3 and 11 months, respectively (WHO,

1977). The number of bacterial strains capable of anaerobic, reductive dehalogenation

in soil/sediment is limited and still under investigation for purposes of developing

large scale decontamination methods (Bunge *et al.*, 2003). The degradation of TCDD

in the gas phase is rapid due to its susceptibility to photolysis by UV-light ($\lambda = 290$).

Its theoretical half-life under photolytic conditions is 1 h, under conditions of radical

reactions with ·OH, its half-life in air is about 8.3 days (Podoll *et al.*, 1986). The

persistence of TCDD in surface water depends on the extinction-coefficient of the

respective body of water and on seasonal changes in UV-radiation. It varies between

21 h (summer) and 118 h (winter) (Mabey *et al.*, 1982).

1.1.2   PCBs

co-planar   non-planar
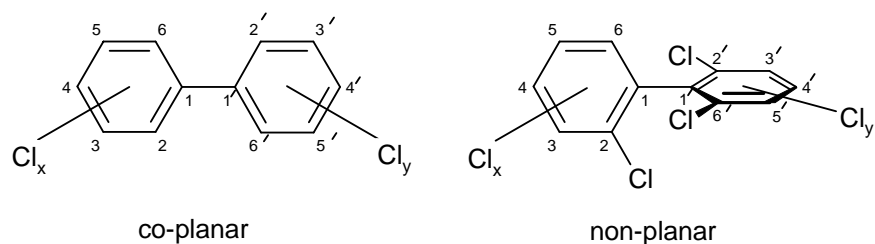
Figure 1-2: Stereochemical structures of PCBs.

This class of industrial chemicals consists of 209 congeners, the toxicological

potencies of which differ by the degree as well as by the pattern of chlorination.

There are co-planar and non-planar PCBs, which have differential toxicity profiles.

PCBs are no longer used in developed countries.

PCBs possess favorable technical properties, which was the basis for their

wide-spread application in industry. These properties include a very high dielectric

coefficient and high boiling point. PCBs are virtually non-combustible, heat resistant,

chemically stable, and show very low acute toxicity. Their viscosity varies with the

degree of chlorination. Therefore, PCBs have found use in electric transformers and

capacitors, and in heat exchangers, as lubricants, flame retardants, plasticizers, and additives in print inks and lacquers (Erickson, 2001). An estimated cumulative total of 1.2 million metric tons of PCBs were produced worldwide (Holoubek, 2001), half of which was manufactured by the USA. Production in America and new use was prohibited in 1978 under the Toxic Substances Control Act (TSCA).

PCBs are cost-effectively produced by chlorination of the biphenyl parent compound. Technical mixtures were marketed according to chlorine content (30-60 wt. %), which was often indicated by their commercial trade name (e.g. Aroclor 1242 with 42 wt. % chlorine). IUPAC Nomenclature of individual congeners is based on ascending numeric order of chlorination: PCB congeners were assigned numbers from 1-209 (Ballschmiter and Zell, 1980; Ballschmiter *et al*., 1992). According to the Stockholm Convention, the use of equipment containing PCBs must be phased out by 2028.

The biological effects of PCBs greatly depend on their pattern of chlorination and, thus, on their conformation. Meta, para and mono-ortho substituted congeners are co-planar and show a toxicological profile similar to PCDDs, but at much lower potency. The rings rotate freely around the central C-C bond. Bi-ortho chlorinated PCBs show a rotation barrier of about 80 kJ/mole, which still allows racemization at room temperature. Higher chlorination in ortho positions, however, greatly increases the rotation barrier, because only cisoid transition states are possible. The energy required for C-C rotation is ca. 180 kJ/mole for tri- and 246 kJ/mole for tetra-ortho

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chlorinated congeners, preventing these compounds from racemization (Lehmler and Robertson, 2001), even at higher temperatures. Therefore, a planar transition state cannot be attained by these compounds under physiological or environmental conditions. It has been reported that some (+) and (-) enantiomers differ in toxic potency. Kinetic studies have shown slight differences in disposition (Püttmann *et al*., 1986), providing a possible explanation for their different potencies. Tri- and tetra-ortho congeners elicit a toxicity profile different from those of planar PHAHs, by being also neurotoxic (Schantz and Widholm, 2001; Seegal, 2001), which may result from altering calcium signaling (Pessah and Wong, 2001).

The first reported mass-poisoning with PCBs occurred 1968 on Kyushu Island, Japan. PCBs were used in the heat-exchanging system of a rice oil manufacturing plant. Due to leaky pipework, PCBs entered the final product and were consumed by approximately 1800 people (Urabe and Asahi, 1985). It was later shown that repeated heating of PCBs used in the heat exchanger caused the formation of traces of PCDFs in the product. Therefore, the symptoms observed in the affected population such as chloracne and skin pigmentation, are considered to result from a combination of PCB and PCDF toxicity (Guo and Hsu, 2001).

In the U.S. most of the PHAHs covered by the Stockholm Convention are categorized as hazardous air pollutants under the Clean Air Act (CAA) and/or as priority toxic pollutants under the Clean Water Act (CWA) (EPA, 2005).

1.1.3 Properties

PHAHs share common characteristics, such as high lipophilicity, low vapor pressure, a high melting point, and slow biodegradation, leading to biomagnification and environmental persistence.

The hydrophobicity of a compound increases with the degree of halogenation. This effect is amplified in PHAHs that lack further functional groups. Besides creating steric hindrance for enzymatic reactions, a high degree of halogenation also causes increased molecular weight, with elevated melting points and very low vapor pressure (Table 1-2). These properties are the predominant reasons for the persistence of PHAHs in the environment.

Due to (at least partial) planarity as a result of attached aromatic rings, PHAHs easily intercalate in microlayered minerals, such as clay, leading to geoaccumulation. Their Henry constants indicate low volatility from aqueous solution into the gas phase, which is quite independent of environmental conditions. Therefore, their vertical mobility in soil and sediment is very limited. Horizontal mobility occurs by erosion only. Consequently, PHAHs show high compartmental persistence. Ecotoxicological evaluations need to consider the resulting low bioavailability for these compounds from adsorbed states. The high $pK_{O/W}$ values of PHAHs indicate their high lipophilicity, causing bioaccumulation (i.e. uptake from medium and food) particularly in aquatic animals, and consequently, biomagnification (i.e. uptake from food only) throughout the food chain.

Table 1-2: Physical properties of selected PHAHs at 20-25 ºC.

	TCDD (Podoll *et al.*, 1986; Lenoir and Sandermann, 1993)	Aroclor 1242 (U.S. Department of Health & Human Services, 1997)
Molecular weight [g/mol]	321.97	266.5
Melting point [°C]	305	N/A
Boiling point [°C]	N/A	325-366
Water solubility	7.9 ng/l	0.10-0.34 mg/l
$pK_{O/W}$	6.79	5.6
Vapor pressure [mmHg]	1.5×10^{-9}	4.06×10^{-4}
Henry constant [atm m^3/mol]	1.62×10^{-5}	5.2×10^{-4}

These compounds are slowly degraded by chemical and biochemical processes. However, they are subject to photolysis. Dehalogenation of higher chlorinated PCDD/Fs and PCBs can lead to the formation of more toxic congeners, increasing the total toxicity of a mixture. In aqueous media, OCDD is preferentially dechlorinated in the peri-positions (C1,4,6,9), yielding the more potent heptachloro congener (Vollmuth *et al.*, 1994). It has also been shown that UV-radiation of PHAHs can result in the formation of other PHAH classes that were previously not present. Irradiation of water samples containing PCP resulted in the formation of PCDDs and PCDFs in similar ratios (Vollmuth *et al.*, 1994). Therefore, degradation of PHAHs can lead to an increase or decrease in total toxicity depending on substrates and conditions.

1.1.4 Toxicity

The toxicity profiles of PCDDs, PCDFs and co-planar PCBs are qualitatively very similar (henceforth referred to as dioxin toxicity). However, amongst congeners, potency can differ by orders of magnitude. This relative potency can be expressed in terms of Toxic Equivalency Factors (TEFs), normalized to TCDD, which is the most potent member of the family.

The most toxic congeners of PCDDs, PCDFs and PCBs are chlorinated in the 2,3,7,8- or 3,3′,4,4′-positions, respectively. These chlorination patterns are required for the compound to interact with the target site, and they render these compounds largely resistant to metabolism by sterically blocking ring hydroxylation. Therefore, they persist long enough to reach the target site at concentrations high enough to elicit an effect. The potency of these PHAHs is ranked in terms of TEFs (Table 1-3). The respective numbers were determined based on a combination of acute effects *in vitro* and *in vivo*. Evaluations of data by different groups of individuals led to slightly different TEF values between agencies.

Table 1-3: TEF values of PCDDs, PCDFs, and PCBs according to NATO (NATO, 1988) and WHO (Leeuwen et al., 2000) determinations. (Pe = penta, Hx = hexa, Hp = hepta, O = octa). Instances of difference between NATO and WHO values are bolded.

Congener	NATO	WHO
PCDDs		
2,3,7,8-TCDD	1	1
1,2,3,7,8-PeCDD	0.5	1
1,2,3,4,7,8-HxCDD	0.1	0.1
1,2,3,7,8,9-HxCDD	0.1	0.1
1,2,3,6,7,8-HxCDD	0.1	0.1
1,2,3,4,6,7,8-HpCDD	0.01	0.01
1,2,3,4,6,7,8,9-OCDD	0.001	0.0001
PCDFs		
2,3,7,8-TCDF	0.1	0.1
1,2,3,7,8-PeCDF	0.05	0.05
2,3,4,7,8-PeCDF	0.5	0.5
1,2,3,4,7,8-HxCDF	0.1	0.1
1,2,3,7,8,9-HxCDF	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1
1,2,3,4,6,7,8-HpCDF	0.01	0.01
1,2,3,4,7,8,9-HpCDF	0.01	0.01
1,2,3,4,6,7,8,9-OCDF	0.001	0.0001

IUPAC #	Structure	NATO	WHO
PCBs			
77	3,3',4,4'-TCB		0.0001
81	3,4,4',5-TCB		0.0001
105	2,3,3',4,4'-PeCB		0.0001
114	2,3,4,4',5-PeCB		0.0005
118	2,3',4,4',5-PeCB		0.0001
123	2',3,4,4',5-PeCB		0.0001
126	3,3',4,4',5-PeCB		0.1
156	2,3,3',4,4',5-HxCB		0.0005
157	2,3,3',4,4',5'-HxCB		0.0005
167	2,3',4,4',5,5'-HxCB		0.00001
169	3,3',4,4',5,5'-HxCB		0.01
189	2,3,3',4,4',5,5'-HpCB		0.0001

It has been unequivocally demonstrated that high-dose acute toxicity (Safe, 1986; Safe, 1990; Stahl *et al*., 1992; Weber *et al*., 1992a; Weber *et al*., 1992b) as well as carcinogenicity (Walker *et al*., 2005) of PCDDs, PCDFs, and co-planar PCBs are additive. More recently, additivity of medium-dose effects has also been demonstrated for reproductive endpoints (Hamm *et al*., 2003). A mixture of 2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HxCDD, 2,3,4,7,8-PeCDF, and 3,3',4,4',5-PeCB (PCB 126) inhibited ovulation in an entirely additive manner (Gao *et al*., 2000). Additivity allows the calculation of the total toxicity of mixtures based on

the concentration of its individual components and the respective TEFs (Equation

1-1). The sum of these products is expressed as Toxicity Equivalent (TEQ):

$$TEQ = \sum_{k=1}^{n}(c_k \cdot TEF_k)$$

TEQ = toxicity equivalent

c = concentration

TEF = toxic equivalency factor

k, n = unique for specific congeners

Equation 1-1: Formula to calculate the TEQ of a mixture applying the TEF concept.

The TEF of the most potent congener (TCDD) is defined as follows: TEQ

represents the theoretical amount of TCDD eliciting a response identical to that of a

given mixture. Therefore, TEQ is also referred to as TCDD-equivalent.

The effects of PCDDs and PCDFs as well as PCBs are thought to occur by the

same or similar mechanisms. Therefore, most studies have focused on the most potent

member of these substance classes, namely on TCDD. It serves as the standard model

compound for toxicological studies. The LD_{50} of TCDD varies greatly among species

(Table 1-4). The most sensitive mammal is the guinea pig (Schwetz *et al*., 1973) with

an LD_{50} between 0.7-2.0 μg/kg. It is well over 1000-times more sensitive than the

hamster (LD_{50} = 5051 μg/kg), the most resistant species (Eisler, 1986). LD_{50} values

also vary greatly among strains, as is most prominently revealed by two rat models:

The LD_{50} in the sensitive Long Evans rat is 10 μg/kg (Pohjanvirta *et al*., 1993),

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differing by a factor of 1000 from the resistant Han/Wistar rat ($LD_{50} > 9600$ µg/kg) (Unkila *et al*., 1995). To a lesser degree, this difference has also been observed in mice. C57BL mice were found to be about 30-times more sensitive than DBA mice (Weber *et al*., 1995).

Table 1-4: LD_{50} values for TCDD in selected mammals (Geyer et al., 1990).

Species	LD_{50} of TCDD [µg/kg]	Total Body Fat [% of Body Weight]
Guinea pig (Dunkin-Hartley)	0.7-2.0	4.5
Rat (Sprague Dawley)	45	8.9
Rhesus monkey	50	10.3
Mouse (C57BL/6J)	100	7.9
Rabbit (New Zealand White)	115	10.1
Dog (Beagle)	1,000	13.8
Hamster (golden Syrian)	~ 5000	17.3

Although the explanation for some intra-species/inter-strain differences has turned out to be quite complex, a striking general correlation ($R^2 = 0.834$, P<0.0001) has been identified between total body fat content in % of body weight (TBF) of a species and the acute toxicity (LD_{50}) of TCDD (Geyer *et al*., 1990) (Equation 1-2).

$$LD_{50} = 6.03 \cdot 10^{-4} (TBF)^{5.30}$$

TBF = total body fat [% of body weight]

Equation 1-2: Formula to calculate the approximate LD_{50} of TCDD in mammals (Geyer et al. 1990).

A correlation between the toxicity of highly lipophilic compounds and TBF appears intuitively correct considering the disposition of such compounds. The larger the compartment that serves as a sink or pool (peripheral compartment) for the compound is, the lower is its concentration in the circulation, and, thus, at the target site (assuming the target site is not part of the peripheral compartment).

The above mentioned empirical formula can be used to calculate a hypothetical acutely lethal dose for humans. The calculated LD_{50} of 6230 μg/kg TCDD for the "Reference Western Man" (70 kg body weight, 21 % TBF) supports the common notion that adult humans are not as sensitive to acute dioxin toxicity as some animal models. Furthermore, newborns (13.6 % TBF) would be 10-times more sensitive to the acute toxicity of TCDD than adult humans (Geyer *et al*., 1990).

The effects of PCDD/Fs and PCBs are quite diverse and well-characterized in experimental animals. They include a wasting syndrome (Harris *et al*., 1973; Seefeld *et al*., 1984) and carcinogenicity (lungs, liver) (Kociba *et al*., 1978) at high doses, liver injury, immuno-suppression (Sharma *et al*., 1978), reproductive effects (Peterson *et al*., 1993) and lowered serum IGF-1 levels at medium doses, and effects on thyroid hormones (Potter *et al*., 1983; Rozman *et al*., 1984; Croutch *et al*., 2005), thymic atrophy (Gupta *et al*., 1973), and enzyme inductions (Poland and Glover, 1978) at low doses. In the rabbit, the initial symptom of chloracne is dermatitis. This occurs two to four weeks after administration of TCDD, followed by swollen follicles and cysts several days later.

In humans, chloracne is the most sensitive symptom of elevated TCDD body burdens. It occurs approximately two weeks after exposure, manifested by swollen hair follicles. Three to five weeks post exposure, these turn into comedones (Homberger *et al*., 1979). In susceptible humans (young girls), symptoms may occur at TCDD concentrations of 800 ppt (= 800 pg/g) based on serum lipid content. In adolescents, differential diagnosis of acne-like skin conditions is notoriously difficult. Most individuals do not show signs below 11,000 ppt (= 11,000 pg/g) (Williams & Wilkins, 1992). The highest recorded level of TCDD in humans was 144,000 ppt (= 144,000 pg/g) blood fat in a 30-year-old woman, corresponding to a dose of 25 μg/kg TCDD (Geusau *et al*., 2001). Symptoms included severe chloracne, nausea, vomiting and gastrointestinal pain as well as cessation of menstruation. The hypothalamic-pituitary axis appeared to be unaffected (Geusau *et al*., 2001).

Epidemiological studies could not clearly link TCDD-exposure to increased cancer mortality (Zober *et al*., 1990; Fingerhut *et al*., 1991). However, exposure to dioxins significantly above background levels correlated with increased cancer incidences (Huff *et al*., 1994; McGregor *et al*., 1998). Most recently, the National Research Council summarized the available epidemiological data:

> *"The committee concludes that the weight of epidemiological*
> *evidence that TCDD is a human carcinogen is not strong, but the*
> *human data available from occupational cohorts are consistent*

with a modest positive association between relatively high body

burdens of TCDD and increased mortality from all cancers."

(National Research Council of the National Academies, 2006)

Some studies associated occupational PCB exposure with hepatic, biliary, and intestinal cancers as well as skin melanomas (Prince *et al.*, 2006; Ruder *et al.*, 2006). Except for liver and biliary tract cancer, confirmatory evidence in animal models is lacking. Investigations after accidental exposure suggest increased risks of digestive tract and respiratory tract cancer in TCDD-exposed smokers (Ott and Zober, 1996). This association supports the notion that TCDD is a carcinogen even though it is not a mutagen. Workers exposed to high concentrations of TCDD over prolonged periods of time showed significantly elevated cancer incidence ≥ 20 years after initial exposure (Zober *et al.*, 1990; Fingerhut *et al.*, 1991). However, when considering the total cohort, no increased mortality rate was found in comparison to the general population. Another cohort of 5100 workers, however, showed a decreased mortality rate from strokes and gastrointestinal diseases (Fingerhut *et al.*, 1991). In summary, epidemiological data suggests that TCDD might have a weak tumorigenic effect at higher doses, particularly because in rats, high doses of dioxins cause tumors of the lungs and liver (Kociba *et al.*, 1978). A lack of evidence for genotoxicity *in vitro* and *in vivo* suggests that TCDD, like PCBs and several other chlorinated hydrocarbons, exert their carcinogenic effect through an epigenetic mechanism (e.g. promotion).

In many if not most species, TCDD has immunosuppressive effects at doses much lower than those causing acute toxicity.

Doses of 1 μg/kg/wk TCDD caused an increased susceptibility of mice to *Salmonella* infections (Thigpen *et al*., 1975). Immunosuppression has been observed in various lymphoid organs such as the thymus, spleen and lymph nodes in different species exposed to a wide range of doses. Rats showed a decrease in cell-mediated immunity after a dose of 40 μg/kg TCDD, whereas 10 μg/kg TCDD caused an increase. The most prominent effect is thymic atrophy with impaired differentiation of T-lymphocytes. Antibody-mediated humoral immunity is also reduced (Fan *et al*., 1996). There are contradictory reports regarding effects on the immune system in humans. Epidemiological studies in Seveso cohorts could not establish an association (Homberger *et al*., 1979).

In laboratory animals, dioxins have been shown to affect development and reproduction, and to be feto- and embryotoxic as well as teratogenic (Neubert and Meister, 1987). In humans, cessation of menstruation has been reported after TCDD exposure (Geusau *et al*., 2001).

Studies in animal models demonstrated effects of TCDD on fertility (Murray *et al*., 1979), pre- and post-parturitional growth and development. At higher doses, these effects included miscarriages and stillbirths (Sparschu, 1971; Peterson *et al*., 1993). Morphological aberrations in the ovaries and uterus of the rat were seen after

1 μg/kg/d over a period of 13 weeks (Kociba *et al*., 1976). The ED_{50} for the inhibition

of ovulation in the immature rat model was found to be 3-10 μg/kg TCDD (Li *et al*.,

1995). A NOAEL of 0.03 μg/kg/d TCDD was reported for rats when administered

from days 6-15 of gestation (Schwetz *et al*., 1973). In mice, teratogenic effects,

including cleft palate, hydronephrosis and thymic hyperplasia, were observed at doses

below maternally toxic doses. The same effects have been reported in rats and

hamsters, however, at doses that caused significant toxicity in the dam (Courtney and

Moore, 1971; Thomas and Thomas, 2001). A delayed onset of puberty was found in

the offspring of rats when pregnant dams were dosed with 0.8 μg TEQ/kg on

gestation day 15 (Hamm *et al*., 2003). Studies in primates also showed reduced

fertility, lowered birth weights and increased prenatal mortality, often accompanied

by considerable toxicity in the pregnant female (Allen *et al*., 1977; Allen *et al*., 1979;

Barsotti *et al*., 1979; Schantz *et al*., 1979). Epidemiological studies on the Seveso

cohorts showed no increase in miscarriages or teratogenic effects (Homberger *et al*.,

1979) but did show an increase in the number of female offspring (Mocarelli *et al*.,

1996). This was assumed to be linked to the father (Mocarelli *et al*., 2000), but could

not be confirmed in cohorts of Operation Ranch Hand (Michalek *et al*., 1998), which

leaves a considerable uncertainty regarding reliability of the Seveso finding.

Other alleged effects associated with human exposure include hyperkeratosis,

hyperpigmentation, hirsutism, liver damage, elevated blood fat content and

cholesterol levels, intestinal effects such as diarrhea, cardiovascular effects, headache,

peripheral neuropathy, reduced sensory performance, loss of libido, and psychiatric

changes (Abel, 1987). Some of these effects are also age-related and, therefore, it is difficult to assess if dioxins are indeed contributory or not. This demonstrates once more that the lack of information on dose or dose rates and exposure time in epidemiological studies often leads to equivocal conclusions.

TCDD has been shown to reduce hormone levels, such as thyroid hormones, insulin and insulin-like growth factor-1 (IGF-1), causing endocrine disruption and also dysregulation of intermediary metabolism.

It has been determined that TCDD causes a decrease in serum levels of total thyroxine (TT_4) in rats within four days after a dose of 1 μg/kg (Gorski and Rozman, 1987). The concentration of total triiodo-thyronine (TT_3) was, however, unaffected. Both hormones were lowered in a dose-dependent manner in C57BL (LOEL 0.1 μg/kg) and DBA mice (LOEL 100 μg/kg) (Weber *et al.*, 1995). Sprague Dawley rats undergo a transient hypoinsulinemea at doses of 25 μg/kg together with insulin-hypersensitivity (Gorski and Rozman, 1987). It has been reported recently that rats maintained at steady-state after a 3.2 μg/kg TCDD loading dose rate show a decrease in IGF-1 signaling within 8 days (Croutch *et al.*, 2005). Similar doses were shown to decrease ovulation, but also to prolong the life of experimental animals as well as to reduce cancer rates below controls. Scheme 1-1 illustrates that these effects could be mediated by decreased IGF-1 signaling (Arking, 2003).

```
┌─────────────────┐             ┌─────────────────┐
│  IGF-1 Signal   │             │  IGF-1 Signal   │
│      ON         │             │      OFF        │
└─────────────────┘             └─────────────────┘
    ↙        ↘                      ↙        ↘
┌──────────┐ ┌──────────┐      ┌──────────┐ ┌──────────┐
│  stress- │ │  growth  │      │  stress- │ │  growth  │
│ resistant│ │   genes  │      │ resistant│ │   genes  │
│   genes  │ │ activated│      │   genes  │ │ repressed│
│ repressed│ │          │      │ activated│ │          │
└──────────┘ └──────────┘      └──────────┘ └──────────┘
     ⇓            ⇓                  ⇓            ⇓
┌───────────────────────────┐  ┌───────────────────────────┐
│ increased reproduction    │  │   increased longevity     │
│ and growth but aging      │  │ but decreased reproduction│
│                           │  │        and growth         │
└───────────────────────────┘  └───────────────────────────┘
```
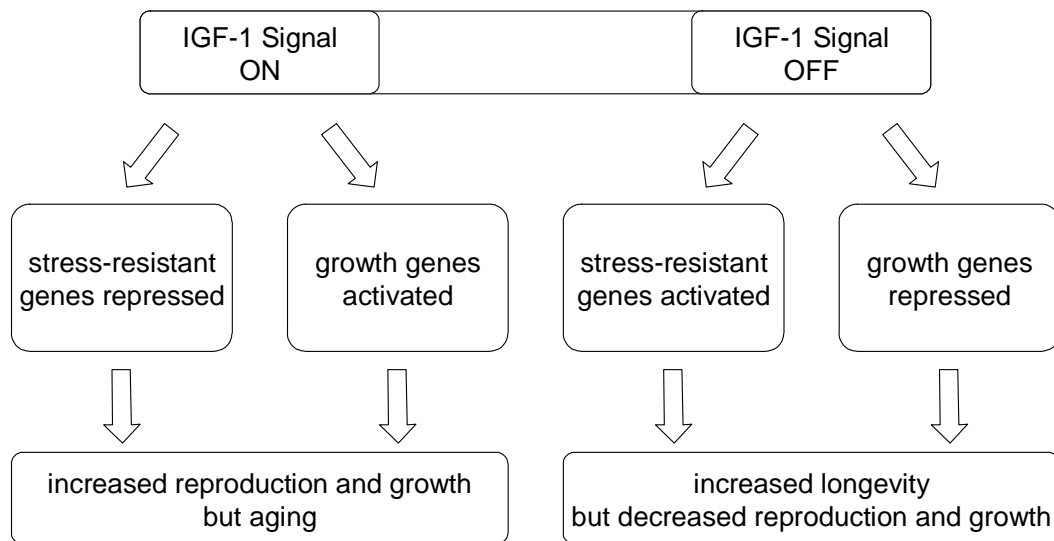
Scheme 1-1: Arking's theory of IGF-1 signaling and associated effects.

1.1.5   Mechanisms of Action

1.1.5.1   The Aryl Hydrocarbon Receptor (AhR)

Much research has been conducted to elucidate the mechanism of dioxin

toxicity, most of it related to the AhR (Poiger *et al.*, 1982; Thunberg, 1984;

McKinney *et al.*, 1985). However, it has also been argued that a single mechanism is

unlikely to explain a complex toxicity profile such as that displayed by dioxins

(Rozman, 1989; Rozman, 1992). Furthermore, TCDD altered the expression of 32 out

of 456 probe sets investigated in AhR knock-out mice, demonstrating the existence of

AhR-independent pathways (Tijet *et al.*, 2006).

Cytochrome P450s play a major role in phase I metabolism. Compounds can

influence the transcription of these proteins via promoters. As first described by

Poland in 1976, TCDD induces CYP1A1 activity by interacting with its gene through

a receptor-mediated mechanism (Poland *et al.*, 1976). The cytoplasmic AhR and its

nuclear partner ARNT (AhR nuclear translocator) play key roles in this signal

transduction (Figure 1-3). Several variants of the AhR have been reported, and two

point mutations have been detected in the AhR of the rat strain that is most resistant

to TCDD toxicity (Pohjanvirta *et al*., 1998). However, downstream induction of

EROD activity was only minimally affected (Pohjanvirta *et al*., 1988). Splice variants

of ARNT and its isoform ARNT2 were confirmed not to contribute to TCDD

resistance amongst rat strains (Korkalainen *et al*., 2003). Although many AhR ligands

are known, TCDD is the most potent AhR agonist. Therefore, TCDD is used as a

model compound for AhR binding kinetics and the study of downstream effects. The

best known AhR signaling pathway is initiated by an agonist binding to the receptor.

In this process, proteins associated with the AhR are released. The AhR/agonist

complex translocates into the nucleus and binds to the ARNT. This heterotrimer

attaches to the dioxin response element (DRE), a core heptanucleotide sequence in

the DNA, and acts as transcription factor. Mediated through translation, the AhR

signaling cascade elicits a biological response, such as CYP1A1 induction *in vitro*

and *in vivo*.

The associated proteins mentioned above serve as chaperones, guiding the

signaling process. Unligated AhR exists as a tetrameric structure, composed of the

ligand binding subunit, a dimer of heat shock protein 90 (hsp90), and the AhR-

interacting protein (AIP), also referred to as immunophilin-like X-associated protein

2 (XAP2), or AhR associated protein 9 (ARA9) (Petrulis and Perdew, 2002). AIP

binds to both, hsp90 and Ahr, and therefore stabilizes the tetramer (Bell and Poland,

2000). AIP prevents nucleocytoplasmic shuttling and, therefore, regulates nuclear translocation of the unbound tetramer (Petrulis and Perdew, 2002). The two hsp90 molecules bind to distinctly different sites of the ligand binding subunit, supporting proper folding of the subunit and interaction with ligands.

Several other factors influence AhR signaling, such as coactivators (e.g. ERAP140, SRC-1, NCoA-2, CoCoA) or corepressors (e.g. SMRT, SHP) (Nguyen *et al.*, 1999; Ke *et al.*, 2001; Kim and Stallcup, 2004; Harper *et al.*, 2006). Furthermore, physiological alterations caused by aging or cancer can also down- or upregulate AhR levels (Harper *et al.*, 2006).
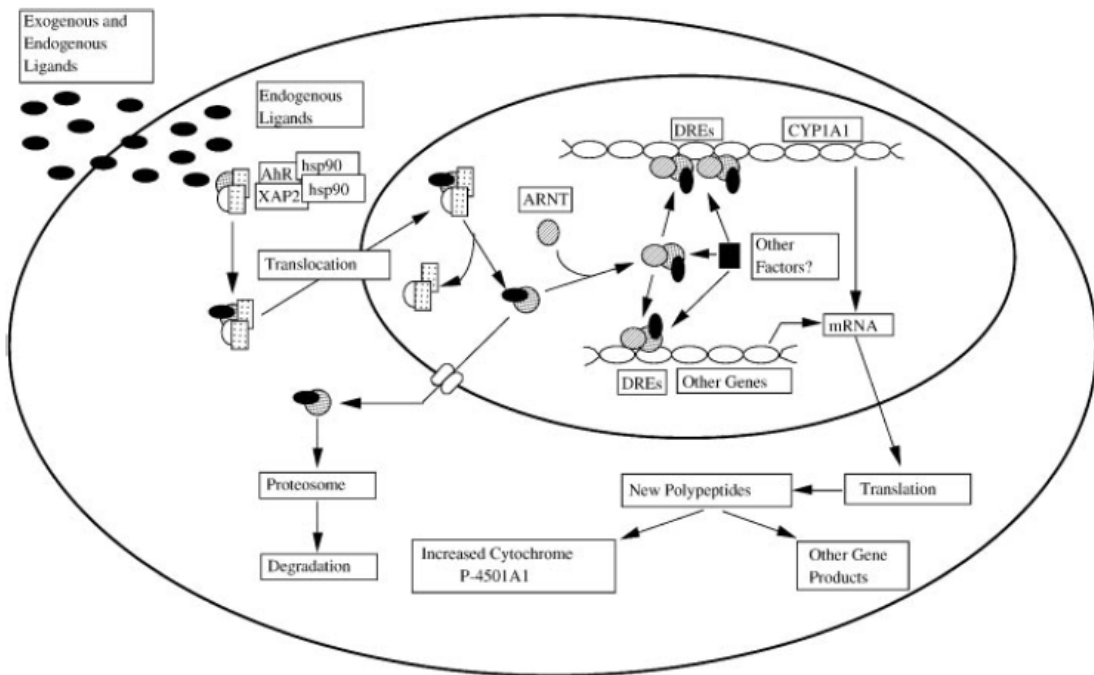
Figure 1-3: AhR signaling pathway (Denison and Nagy, 2003).

Although CYP1A1 metabolizes many exogenous compounds, an endogenous ligand for the AhR has yet to be identified. In the rat, the highest tissue-concentrations of AhR were found in the thymus, lung , liver and kidney (Carlstedt-Duke, 1979). Some researchers attribute most or all toxic effects of PCDDs to AhR interaction (Gallo *et al*., 1991; DeVito *et al*., 1995). However, it has also been argued that the AhR might not be the universal key to acute toxicity (Rozman, 1989) as the ED_{50} for CYP1A1 induction in rats is about two orders of magnitude lower (Sprague-Dawley 0.2-0.3 µg/kg TCDD) than the LD_{50} (43 µg/kg TCDD) (Rozman *et al*., 1993). Furthermore, calculations of protein content in the liver yield a saturation of the AhR at 1.27 ng TCDD/g tissue (Rozman *et al*., 1993), whereas sublethal doses (2.5 µg/rat) cause 100-times higher liver concentrations (117 ng/g). It has been argued that a saturated receptor cannot cause a variable such as a dose-response to occur at much higher doses (Rozman *et al*., 1993). The study of sensitive and resistant rat strains also yields controversial results. The specific binding affinity of the AhR to TCDD does not differ between sensitive (Long Evans, 20 fmoles/mg cytosolic protein) and resistant (Han/Wistar, 23 fmoles/mg cytosolic protein) rat strains (Pohjanvirta *et al*., 1988). In addition, different effects are produced at different doses of TCDD in sensitive and resistant mice (Weber *et al*., 1995) (Table 1-5). Whereas C57BL mice show a decrease in thyroid hormones at 0.1 µg/kg TCDD, the same effect is only elicited in DBA mice by doses almost 1,000 times higher. The ED_{50}s for CYP1A1 induction, however, only differ by a factor of 15.

Table 1-5: Differences in doses and effects between mice strains sensitive (C57BL)
and resistant (DBA) to TCDD toxicity.

Mouse Strain	ED$_{50}$ CYP1A1 induction (liver)	LD$_{50}$	ED$_{50}$ reduced serum glucose, activity of PEPCK and glucose-6-phosphatase	Effects reduced T$_3$, T$_4$ serum levels
C57BL (μg/kg)	1.1	100	100	0.1
DBA (μg/kg)	16	> 3000	1000	97.5
Difference between strains	15-fold	> 30-fold	10-fold	975-fold

Studies in AhR knock-out mice showed at least 10 times higher resistance to
TCDD as compared to the wild type (Fernandez-Salguero *et al*., 1996). However,
TCDD-treated knock-out mice displayed scattered necrosis of the liver and
lymphocytic infiltration of the lungs, which were effects also seen in TCDD-treated
wild type mice (Fernandez-Salguero *et al*., 1996). Although less severe, the very
presence of these effects suggests an AhR-independent component in the
manifestation of toxicity.

Further studies with AhR knock-outs as well as wild type animals must be
conducted to address contradictions and to fully reveal if and to what extent this
receptor/mechanism modulates or mediates dioxin toxicity.

1.1.5.2 Phosphoenolpyruvate Carboxykinase (PEPCK)

The hallmark of dioxin-toxicity is the wasting syndrome in animal models: reduced feed intake, combined with decreased body weight, and derailment of intermediary metabolism. This eventually leads to a lethal hypoglycemia, the apparent cause of acute death by TCDD.

Overall energy metabolism is directly related to the respiratory quotient (the ratio of CO_2 output to oxygen usage). In TCDD-treated animals, this quotient is reduced as compared to pair-fed controls when the wasting syndrome has progressed beyond the stage of glycogen depletion (Muzi *et al.*, 1989). This has led to the suggestion that intermediary metabolism is impaired in animals treated with TCDD. In further studies, gluconeogenesis was confirmed as the target of that alteration (Gorski *et al.*, 1990). It has been shown in rats that a key enzyme of hepatic gluconeogenesis, PEPCK, is inhibited by TCDD in a dose-dependent manner (Weber *et al.*, 1991), leading to a decreased activity to the extent of 44 % of pair-fed controls (Weber *et al.*, 1991). This reduction of liver PEPCK activity was found in the same dose-range as acute toxicity occurs (Rozman, 1992). In addition, hepatic tryptophan 2,3-dioxigenase was also dose-dependently reduced in rats and, consequently, plasma tryptophan was dose-dependently increased (Fan and Rozman, 1994), which is compatible with the feed intake reduction observed. Subsequent studies in mouse strains with different sensitivity to TCDD (C57BL/6J and DBA/2J) revealed a plateau in the decreasing activity of PEPCK in liver, coinciding with the onset of acute

toxicity in the same dose range (Weber *et al*., 1995). At lethal doses, PEPCK activity was reduced by 80 % in mice livers. However, hepatic tryptophan 2,3-dioxygenase activity and plasma tryptophan were unchanged in mice, which is compatible with a lack of reduced feed intake and a lack of reduced body weight in mice. Still, lethality ensuing from the PEPCK inhibition was apparently more pronounced in mice than in rats.

Studies on glucose homeostasis in the most TCDD-resistant and the most TCDD-susceptible species, the hamster and the guinea pig, respectively, did not support the notion of hepatic PEPCK being the sole mediator of lethal TCDD toxicity in all species. A decreased PEPCK activity but unaffected liver glycogen levels were observed in hamsters at doses that did not induce body weight loss. Additionally, in hamsters a dose-dependent decrease in serum free fatty acids was also detected. Whereas guinea pigs showed a dose-dependent decrease in liver glycogen levels, they revealed a dose-dependent increase in serum free fatty acids. No inhibition of liver PEPCK activity was seen in liver cytosol of guinea pigs (Unkila *et al*., 1995). It is noteworthy that in guinea pigs most of the hepatic PEPCK is located in mitochondria, whereas in both rats and mice PEPCK is mainly found in cytosol.

This leads to the conclusion that lethality in rats, mice, hamsters and guinea pigs after the administration of TCDD is due to severe impairment of intermediary metabolism. However, a uniform mechanism of action for all species is unlikely to be

discovered because of very different regulation of intermediary metabolism between

hibernators (hamsters), herbivores (guinea pigs), and omnivores (rats, mice, humans).

1.1.6 Metabolism

Biotransformation is the slowest and hence rate-limiting step in PCDD-

elimination (Neal *et al*., 1984). Therefore, non-biliary intestinal elimination by

desquamating enterocytes and by re-distribution of PHAHs into fecal fat becomes the

main route of excretion.

PHAHs can be ring-hydroxylated, which requires vicinal unsubstituted aryl

positions (Poiger *et al*., 1982). Lacking these, biotransformation occurs on a very

slow time-scale, leading to long half-lives of the different congeners. Therefore,

PCDD/Fs chlorinated in the 2,3,7,8 positions (Figure 1-1) are poor substrates for both

oxidation and reductive dehalogenation. The same holds true for PCBs with meta and

para substituents (Figure 1-2). These enzymatic reactions can also involve a chlorine-

switch from a lateral position towards the central ring, as shown by identification of

the minor TCDD metabolite 2-OH-1,3,7,8-TCDD. Other metabolic pathways are

oxygen bridge cleavage in PCDD/Fs, leading to dihydroxy-tetrachlorodiphenyl ether

or hydroxylated PCBs, respectively (Poiger and Buser, 1984). Perchlorinated PHAHs

have to undergo reductive dehalogenation in order for further metabolism to occur.

Epoxide intermediates as well as hydroxylated metabolites, once formed in low yield,

are readily biotransformed by phase II metabolism. The derivatized PCB glutathione

adduct 4,4-bis (methylsulfonyl)-2,2',5,5'-tetrachlorobiphenyl was reported to

accumulate in lungs after i.p. administration to rats (Brandt *et al.*, 1985; Lund *et al.*, 1985). Further methylsulfone derivatives of PCBs were identified in liver and adipose tissue after chronic exposure, suggested to be due to protein binding. However, metabolites are generally less toxic than the parent compounds and much more rapidly excreted, due to increased hydrophilicity and additional phase II metabolism. Overall, biotransformation plays a minor role in the disposition of PHAHs.

1.1.7 Enzyme Induction

PHAHs are potent enzyme inducers *in vitro* and *in vivo*. Enzyme induction is one of the most sensitive effects of TCDD and related compounds.

It has been shown *in vitro* that prior exposition of hepatocytes to TCDD increases the rate of its metabolism 3.2-fold for subsequent exposures (Wroblewski and Olson, 1985). However, an auto-induction of TCDD-metabolism *in vivo* could not be confirmed (Van den Berg *et al.*, 1994). The previously described AhR signaling pathway is the best known route of enzyme induction by co-planar PHAHs. It leads predominantly to the induction of CYP1A1 and 1A2 by a factor of 50-100 compared to controls (Abraham *et al.*, 1988). The activity of NAD (P)H-quinone oxidoreductase was also induced by TCDD, however, to a far lesser degree. It has been also reported that PCBs induce activity of the phenobarbital group (CYP2B, 2C, 3A) as well as CYP2A1, epoxide hydrolase, and aldehyde dehydrogenase activity. PCB atropisomers display differential induction patterns of enzymes by (+) and (-)

enantiomers, including patterns observed by the phenobarbital-type inducers (Lehmler and Robertson, 2001).

PHAHs also induce phase II enzymes, but fewer studies have been conducted in this field. It was demonstrated that TCDD induces UDP-GT about 25-fold (Rozman *et al.*, 1987), whereas activities of glutathione-S-transferase (GST), sulfotransferase and N-acetyltransferase remained essentially unaffected (Thompson *et al.*, 1982).

1.1.8   Hormesis/Hormoligosis

Hormesis is the overcompensation response of an organism to an inhibitory signal, whereas hormoligosis is overcompensation to a stimulatory signal (Rozman and Doull, 2003). Both are manifestations of non-mutational evolutionary principles: Homeostasis and optimization. Hormesis and hormoligosis should be strictly separated from homeopathy, the basis of which lacks scientific evidence.

Reports on differences between low-dose and high-dose effects of chemicals date back to the early 19th century (Schulz, 1887). The first documented medical study was the self-administration of a high dose of camphor by Jörg in 1825. This physician observed the opposite of the known low-dose effects; instead of CNS stimulation, he experienced CNS depression. The occurrence of low- vs. high dose effects is not limited to humans and animals. Yeast has been used as a toxicological test system for more than a century. A study on chromate, a fermentation poison,

showed an early increase in fermentation rate followed by inhibition at later time points (Schulz, 1888). The early stimulatory effect can be explained by the slow uptake of chromate by yeast. When stimulation was observed, a minimal amount of chromate had entered the cells. The overcompensation by yeast for the inhibitory effects of chromate resulted in increased fermentation. Once intracellular concentrations exceeded hormetic levels, however, the organisms could no longer counteract the effects and inhibition of fermentation was observed.

Low doses trigger a system to be removed from equilibrium (homeostasis), which can be counteracted by responses on the same or a different pathway, leading to homeostatic overcompensation. Effects can be reversed based on the kinetics of a compound or the dynamics of its effects. Kinetic reversibility includes distribution, biotransformation, and/or excretion. An organism adapts by increased elimination, e.g. by enzyme induction. Dynamic reversibility can be due to repair or reversibility of receptor interactions, with adaptation occurring by protein up- or down-regulation.

High doses, however, force the system beyond kinetic or dynamic recovery. An effect is either driven by kinetics or dynamics, depending on which is the rate-determining step:

$$\frac{dA}{dE} = \frac{dA}{dD} \cdot \frac{dD}{dK} \cdot \frac{dK}{dE} \qquad \text{(a)}$$

$$\frac{dA}{dE} = \frac{dA}{dD} \cdot \frac{dD}{dE} \qquad \text{(b)}$$

A = action (effect)

E = exposure (duration/frequency)

D = rate - determining dynamic process

K = rate - determining kinetic process

Equation 1-3: Differential changes in effect as a result of kinetics driven by exposure that in turn drives dynamics (a), or as a result of dynamics driven by exposure (b).

The action of compounds with short half-lives but long-lasting effects, such as the antiplatelet effect of aspirin, is driven by dynamics (Equation 1-3 (a)). However, the biological effects of PHAHs are kinetics-driven (Equation 1-3 (b)) because of the persistence of these compounds. To avoid large variability in effects at later time points, studies on such compounds should be conducted at steady-state levels, i.e. administration of loading and maintenance dose rates (Equation 1-4).

$$x_0 = x_0^* \cdot (1 - e^{-k\tau})$$

x_0 = maintanence dose rate

x_0^* = loading dose rate

k = elimination rate constant

τ = dosing interval

Equation 1-4: Formula for the calculation of maintenance dose rates

The principle of an overcompensation to effects at low doses that is overcome by higher doses suggests that low-dose effects can be the opposite of high-dose effects, although not necessarily by the same mechanism of action. Table 1-6 depicts respective observations on TCDD, supporting this notion. Dioxins are known carcinogens in the rat model at high doses. However, cancer rates below control levels have been observed in animals treated with low doses of dioxins, demonstrating their chemoprotective potential. Whereas high doses of TCDD suppressed the immune system in rats, low doses stimulated it. Other medium to low dose effects of dioxins are not necessarily the opposite of high dose effects, however, they seem to be related. The wasting syndrome is a hallmark effect of high-dose dioxin toxicity in rats, causing reduced feed intake and a decrease in body weight. At medium doses, however, only the set-point for body weight is reduced. This effect could find application in weight-control. Some of the symptoms of dioxins at medium to low doses are believed to be modulated by the effects of reduced IGF-1 signaling. As depicted in Scheme 1-1, this can also potentially be used for weight control, for contraception, and longevity.

The hormetic effects of TCDD were first documented by Kociba in 1978 in the rat (Kociba *et al*., 1978). At a life-long dose rate of 0.1 μg/kg/day TCDD, they observed a reduction in the occurrence of age-related tumors in the pituitary, uterus, pancreas, adrenal, and especially mammary tissue. Only 49 % of the female rats treated with TCDD showed benign mammary tumors, and none showed malignant tumors, compared to 85 % and 9 %, respectively, in controls.

Table 1-6: High and medium to low dose effects of dioxins and the potential use of drugs acting through similar mechanisms. [a] Single dose. [b] LDR + MDRs

High dose effects	Medium to low dose effects	Potential as
Carcinogenicity (Rozman *et al.*, 2005) (SD rat: 2.8 mg/kg HpCDD[a] $\approx$ 22 μg/kg TEQ)	↓ lung, liver tumors (Rozman *et al.*, 2005) (SD rat: 1 mg/kg HpCDD[a] $\approx$ 8 μg/kg TEQ)	Cancer prophylactic
Immunosuppression (Fan *et al.*, 1996) (SD rat: 40 μg/kg TCDD[a])	Immunostimulation (Fan *et al.*, 1996) (SD rat: 1-20 μg/kg TCDD[a])	Immunostimulation
Wasting syndrome (Seefeld *et al.*, 1984) (SD rat: 25 μg/kg TCDD[a])	↓ BW set-point (Seefeld *et al.*, 1984) (SD rat: 15 μg/kg TCDD[a])	Anti-obesity drug
	Inhibition of ovulation (Li *et al.*, 1995) (SD rat: 3-10 μg/kg TCDD[a])	Contraceptive
	↓ IGF-1 signaling (Croutch *et al.*, 2005) (SD rat: 3 μg/kg TCDD[b])	Anti-obesity drug, longevity, contraceptive

Recently, chronic toxicity including carcinogenicity of 1,2,3,4,6,7,8-HpCDD has been investigated in female Sprague Dawley rats (Rozman *et al.*, 2005). Lung cancer was the major effect and overall chronic toxicity obeyed Haber's Rule of Inhalation Toxicology (Haber, 1924), in that increasing doses reduced the time to death in an entirely predictive manner (Equation 1-5).

$$c \cdot t = k$$

c = concentration

t = time

k = constant

$k_{\text{(Lethality HpCDD)}} = 1212 \pm 53.4 \, mg / kg \cdot day$

$k_{\text{(Life Prolongation HpCDD)}} = 598 \pm 28.7 mg / kg \cdot day$

Equation 1-5: Haber's Rule of Inhalation Toxicology with constants for different effects of HpCDD.

A subthreshold dose (1,000 µg/kg) of HpCDD prolonged the life of rats by about two months over controls, with no lung or liver cancer occurring in these rats. According to *in vivo* studies, the TEF for HpCDD is 0.007 TCDD-equivalents (Viluksela *et al*., 1997). Thus, the corresponding c·t product for TCDD is 4.19 mg/kg·day. These rats lived to be an average 777 days old (controls 721 days) corresponding to 5.4 µg/kg daily intake of TCDD. The half-life of TCDD in rats is about 21 days, in humans about 7.8 years. Therefore, over 40 years, this daily rat dose rate would accumulate in humans to a steady state concentration 136 times higher than in rats, requiring a correction of the rat daily dose intake for kinetics, yielding a human daily dose rate of 40 ng/kg/day of TCDD that could be expected to have life-prolonging effects in humans as well.

1.1.9 Kinetics

After an initial distribution phase, PHAHs are deposited mainly in adipose tissue and also in liver. Due to their persistence they have very limited elimination, and thus long elimination half-lives. Because of these very long half-lives, the rate-limiting steps in the toxicity of this class of compounds are driven by their kinetics.

The bioavailability of TCDD after a single *per os* administration ranges from about 50 % in the guinea pig (Nolan *et al.*, 1979) to 70-85 % in the rat (Piper *et al.*, 1973; Allen *et al.*, 1975; Rose *et al.*, 1976) and is also dependent on the choice of vehicle. Human data are available only on one self-administered dose (Poiger and Schlatter, 1986). It was determined as > 86 % derived from fecal excretion of [1,6-^{3}H] TCDD. Feces are the main elimination pathway also in the rat. Urinary excretion plays a minor role at only 5-13 % of dose (Piper *et al.*, 1973; Allen *et al.*, 1975). Following absorption, the initial distribution of TCDD depends on physiological parameters such as perfusion rate and relative size of tissues. Final distribution of TCDD follows the affinity of the compound to liver (5 % of dose/g tissue) and white adipose tissue (1 % of dose/g tissue) within the first 24 h (Weber *et al.*, 1993). The half-life of TCDD in rats is 11 days for the parent compound in serum and 21 days in the liver. In guinea pigs, TCDD has a half-life of approximately 30 days (Gasiewicz and Neal, 1979), whereas the mean half-life of TCDD in humans is 7.8 years (Geyer *et al.*, 2002). The higher the degree of chlorination, the higher is the biological half-life of a PHAH as compared to its congeners.

A critical review of the kinetics of persistent xenobiotics in rats and humans led to an empirical formula to estimate the elimination half-life of these compounds based on animal data (Geyer *et al.*, 2002) (Equation 1-6).

$$t_{\frac{1}{2}(human)} = 17.78 \cdot \left(t_{\frac{1}{2}(rat)}\right)^{1.34}$$

$t_{\frac{1}{2}}$ = elimination half-life

Equation 1-6: Formula for the extrapolation of elimination half-lives of persistent xenobiotics from rats to humans (Geyer et al. 2002).

This equation also allows the calculation of elimination half-lives of very persistent compounds, such as OCDD. Exact experimental studies on kinetics would require measuring the elimination process during several half-lives. With an estimated half-life of 112 years for OCDD in humans, this is not feasible. The difference in half-lives between rat and human not only determines species-dependent persistence of these chemicals in the body, but also steady state levels during chronic exposure (Equation 1-7).

$$c_{ss} = \frac{1.44 \cdot t_{\frac{1}{2}} \cdot f \cdot dose}{V_d \cdot \tau}$$

c_{ss} = steady-state concentration
f = fraction absorbed
V_d = volume of distribution
τ = dosing interval

Equation 1-7: Formula for the calculation of steady-state concentrations during chronic exposure.

Small species to species variations in the volume of distribution and in the fraction absorbed only have a minor impact on this equation. Differences in half-lives, however, are orders of magnitude larger and directly proportional to the steady state levels achieved. The half-life in rats is approximately 140-times shorter than in humans. It takes 6.64 elimination half-lives to reach 99 % of steady state during chronic exposure. Therefore, rats reach it considerably faster than humans (Equation 1-8). Furthermore, at identical daily dose rates, humans will eventually reach a steady-state about 140-times higher than rats, which represents the factor needed when extrapolating data obtained in rats to humans.

$$C_p = C_0 \cdot e^{-kt}$$

$$C_p = 0.5 \cdot C_0 \longrightarrow t_{\frac{1}{2}} = \frac{\ln 2}{k}$$

$$C_p = 0.01 \cdot C_0 \longrightarrow t_{\frac{1}{100}} = \frac{\ln 100}{k} = 6.64 \cdot t_{\frac{1}{2}}$$

C_p = concentration in plasma t = time

C_0 = concentration in plasma at $t = 0$ $t_{\frac{1}{2}}$ = elimination half - life

k = elimination rate constant $t_{\frac{1}{100}}$ = time to reach 99 % elimination

Equation 1-8: Calculation of eliminated fractions.

The long period of time to steady state also affects bioconcentration factors (BCFs). This is the reason why exposure studies with PHAHs in aquatic species must be extended for at least 6.64 half-lives. In the 1970s, a lack of appropriate study designs led to the assumption that highly chlorinated PCDDs would not

bioconcentrate. This was clearly refuted both theoretically and experimentally (Geyer *et al*., 1994). Lipophilicity of PHAHs leads to high concentrations in breast milk. With the onset of lactation, lipophilic compounds are re-distributed into this newly formed lipophilic compartment. This results in high concentrations, especially for the first pregnancy, when the body burden of decades of PHAHs accumulation is available for redistribution into milk fat. In later pregnancies, levels in breast milk are significantly lower, because the body burden has been reduced by the previous lactational elimination. Because of more frequent lactations, PHAH levels in cow's milk are considerably lower than in breast milk.

1.2   The Dioxin-Analogue 2,3,7,8-Tetrachlorophenothiazine (Fried *et al*., 2007)

The medium to low dose effects of dioxins inspired the development of a structural analogue as a drug lead because of its potential therapeutic benefit (Chapter 1.1.8, Table 1-6). As previously described, TCDD has been reported to exert a broad range of effects *in vitro* and *in vivo*. The most biologically effective dioxins are halogenated in the 2, 3, 7 and 8 positions, with TCDD being the most potent congener. Different dioxin congeners vary in potency (NATO, 1988; Leeuwen *et al*., 2000). Their effects are additive and diverse but well-characterized. They include a wasting syndrome (Harris *et al*., 1973; Seefeld *et al*., 1984) and carcinogenicity (lungs, liver) (Kociba *et al*., 1978) at high doses, liver injury, immuno-suppression, (Sharma *et al*., 1978) reproductive effects (Peterson *et al*., 1993) and reduced serum IGF-1 (insulin-like growth factor-1) levels (Croutch *et al*., 2005) at medium doses,

and effects on thyroid hormones, (Potter *et al*., 1983; Rozman *et al*., 1984) thymic

atrophy, (Gupta *et al*., 1973) and enzyme induction (Poland and Glover, 1978) at low

doses. TCDD is a teratogen in the rat, with a NOEL (no observable effect level) at a

cumulative dose of 0.3 µg/kg TCDD administered orally during gestation (Sparschu,

1971).

During the search for a new dioxin-like compound, the striking structural

similarity between dioxins and the medicinally important class of phenothiazines

became apparent (Figure 1-4). Both classes consist of a heterocyclic central ring in a

tricyclic twelve-carbon structure.
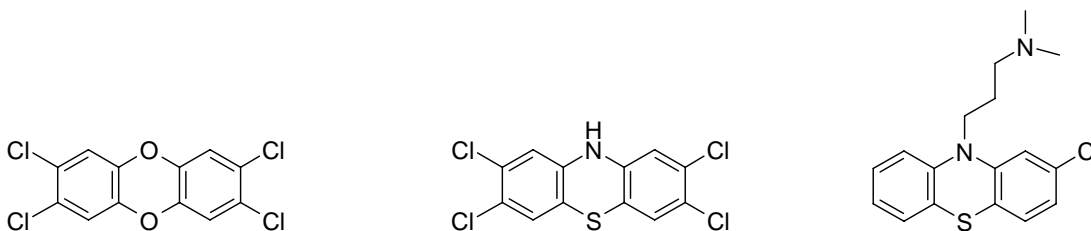
***Figure 1-4. Structural comparison of TCDD (left), TCPT (middle) and a
representative classical phenothiazine (chlorpromazine, right).***

Although the chloro-substituent in the first big-market neuroleptic

phenothiazine drug, chlorpromazine (CPZ), was documented to be a key component

for its efficacy (Jovanovic and Biehl, 1987), no attempt had yet been made to

investigate the effects of higher chlorinated, N-unsubstituted phenothiazine

derivatives. Historically, the pharmacological activity of phenothiazine therapeutics

was mostly attributed to their N-substituent. In contrast, a lateral chlorination pattern

of polychlorinated dibenzo-*p*-dioxins (PCDDs) is known to be the determinant for

their biological activity. Therefore, to develop a new dioxin-analogue, the N-unsubstituted laterally chlorinated phenothiazine was of interest: 2,3,7,8-Tetrachlorophenothiazine (TCPT).

Phenothiazines have long been known for their anti-psychotic as well as their sedative, antihistaminic, anti-emetic, antidiabetic and anthelmintic effects (Hardman *et al.*, 2001). These drugs show a wide therapeutic index and a broad dose-response curve, rendering them ideal for therapeutic use (Hardman *et al.*, 2001).

Adverse reactions include cardiovascular effects; however, these have been mostly observed after suicidal self-administration (Mehtonen *et al.*, 1991). Most phenothiazine drugs showed no fetotoxic, embryotoxic or teratogenic effects in rodent models at doses that did not affect the pregnant female; only doses that were toxic to the dam were also toxic to the fetus and embryo (Walker and Patterson, 1974). Epidemiological data in humans could not associate phenothiazine drugs administered during pregnancy to increased mortality or morbidity of mother nor baby (Sobel, 1960; Milkovich and Van Den Berg, 1976; Slone *et al.*, 1977).

1.2.1   Synthesis

Previously, the synthesis of 2,3,7,8-TCPT had been reported (Li *et al.*, 1988; Huang *et al.*, 1997) in which the unsubstituted phenothiazine was exposed to chlorine gas in acidic solution in the presence of a Friedel-Crafts catalyst. However, attempts to reproduce this synthesis led only to the isolation of 1,3,7,9-TCPT and its sulfoxide

as analyzed by GC/MS and finally confirmed by crystal structure analysis (Fried,

2000). Variation of the conditions for catalyzed chlorination also did not produce any

2,3,7,8-TCPT (Fried, 2004).

Phenothiazine derivatives have been used for their therapeutic effects since

the 1950s. Numerous synthetic approaches to the phenothiazine backbone have been

developed (Bodea and Silberg, 1968; Sayeed *et al.*, 1987) and were applied in the

attempted synthesis of TCPT. However, only one of these routes successfully

produced the target compound in isolable quantities.
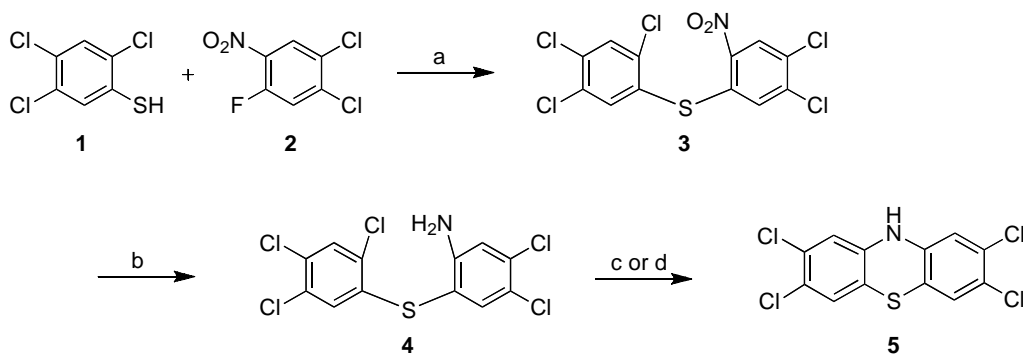
a Reagents and conditions: (a) K_2CO_3, $CaCO_3$, 3Å molecular sieves, CH_2Cl_2, reflux, 21 h, 97%; (b) Fe filings, glacial HOAc, acetone, DI H_2O, reflux, 2.5 h, 86%; (c) Cu, CuI, Na_2CO_3, DMF, reflux, 24 h, 5%; (d) $Pd(OAc)_2$, 2-(dicyclohexylphosphano)-biphenyl, Na^tBuO, DMF, 200 °C microwave, 2 min, 37%.

Scheme 1-2. Synthesis of TCPT in three steps, based on commercially available starting material: Formation of the diarylsulfide, reduction to the amine, then alternatively intramolecular Ullmann-type condensation or Maes-modified Buchwald-Hartwig coupling.

As the compound was to be used in biological testing, a synthetic approach was needed which could be easily reproduced and readily scalable. The final synthetic route (Scheme 1-2) was chosen based upon the commercial availability of the starting materials, the ease of preparation of the cyclization substrate, and the possibility for new methods to be developed in the ultimate ring-closing step.

The three-step reaction sequence commenced with the coupling of the aromatic thiol **1** and the aryl fluoride **2** in the presence of potassium carbonate under anhydrous conditions, producing diarylsulfide **3** (Scheme 1-2). The yield of the coupling reaction was found to be solvent dependent, with polar solvents such as DMF and acetone giving low yields (32% and 35%, respectively). However, a less polar solvent (dichloromethane) improved the coupling step, resulting in a 97% yield. In the second step, the nitro group was reduced with Fe in acetic acid to the corresponding amine **4** via the method of Bechamp (Béchamp, 1854; Béchamp, 1854) in 86% yield after purification. These reduction conditions proved to be superior to other methods in which $SnCl_2$ (10% yield) or Pd/H_2 (18% yield) were used.

Compound **4** was then set up for an intramolecular cyclization to yield the target compound **5**. Ullmann-type coupling conditions modified from historical phenothiazine syntheses (Grotta *et al.*, 1967) resulted in a 5% yield of the desired tricyclic compound. Critical for inducing cyclization was the use of purified cuprous iodide and freshly prepared copper. Further investigations into the Ullmann-type coupling reaction kinetics identified an increase in production of TCPT up to 24 h,

followed by decay of the cyclized compound presumably due to the harsh conditions, despite the continuous presence of starting material. Additional supplementation of the reaction with catalysts after 24 h did not increase the yield nor influence the kinetics of formation or degradation.

In an attempt to shorten the reaction time and improve the yield, a microwave-mediated Ullmann-type coupling was attempted. Ullmann-type couplings under microwave conditions have been reported to reduce reaction time and to improve yields (Wu *et al.*, 2003). However, these reaction conditions allowed no conversion to product, and only starting material was recovered.

Key to our synthesis of multi-gram quantities of the compound was the discovery that a Buchwald-Hartwig amination could be used to induce the cyclization.

After minimal success with the Ullmann-type coupling, a catalytic system employing palladium was used to perform the cyclization and to increase the yield of the ultimate step. The Buchwald-Hartwig amination is known to proceed better for substrates having electron-deficient ring systems, as the carbon-halogen bond becomes more activated for oxidative insertion (Maes *et al.*, 2004). However, Buchwald has demonstrated that functional groups located ortho to the coupling position of the substrate reduces reactivity due to steric hindrance (Wolfe *et al.*,

2000). The reactivity of the catalytic system has also been shown to be sensitive to the electronics and size of the palladium ligand used (Hartwig, 1998).

Bearing these facts in mind, a catalytic system was chosen because of its ability to couple many types of activated and unactivated substrates. This catalytic system, based upon the work of Maes (Maes *et al*., 2003), was shown to successfully couple aryl chlorides with amines under microwave heating. The ligand used in the reaction, 2-(dicyclohexylphosphino)-biphenyl (DCPB), has been demonstrated by Buchwald to be effective in Suzuki couplings of sterically hindered substrates (Wolfe *et al*., 1999) as well as in coupling of both activated and unactivated reactants (Maes *et al*., 2003). Using Pd (OAc)$_2$ and DCPB as the ligand, the reaction was completed after two minutes of microwave irradiation at 200 ˚C using 10 mol % catalyst, resulting in a 37 % yield after recrystallization from chloroform (Table 1-7, entry 3). Attempts to reduce the amount of catalyst resulted in decreased yields and incomplete conversion, even after prolonged reaction times. Yields decreased when the reaction time was extended for longer than two minutes using 10 % catalyst (entries 4 and 5), presumably due to product decomposition as was observed in the Ullmann-type coupling. The only solvent that produced the desired compound was N,N-dimethyl-formamide; toluene and dioxane both proved unsuitable for cyclization.

Table 1-7. Variation of the reaction conditions in the palladium-catalyzed ring closing to form TCPT (Scheme 1 (d)). [a] After recrystallization. [b] The reaction was run under normal reflux conditions

#	Time (min)	Temp (ºC)	% Catalyst	Yield[a]
1	5	150	1	No Reaction
2	10	150	1	No Reaction
3	2	200	10	37 %
4	4	200	10	27 %
5	5	200	10	26 %
6	60	150	10	35 %[b]

A control experiment was run using conventional heating methods in order to determine whether microwave heating displayed any distinct advantages over conventional heating. The Buchwald-Hartwig amination was heated in an oil bath at 150 ºC for 1 h – conditions corresponding to 200 ºC and 2 min in the microwave reactor. Although some starting material remained after 1 h, the reaction produced the desired product, which was subsequently purified and recrystallized to yield 35% of TCPT (Table 1-7, entry 6). This control experiment proved that a reduction in reaction time was the only inherent advantage in utilizing microwave heating, thus providing more suitable access for large-scale synthesis. Maes-modified Buchwald-Hartwig animation improved the total yield from 4.2 % (Ullmann-type condensation) to 30.9 %.

Determining the crystal structure of TCPT revealed the expected butterfly structure of the compound (Figure 1-5). The two central heteroatoms form an axis

along which the molecule is folded, causing a deviation from planarity by 18.5 º. This

supports the validity of *semi-empirical* calculations, which predicted

19.8 º ± 0.7 º(Fried, 2000).

Solid TCPT is not completely stable under ambient conditions, resulting in

oxidation of trace amounts. When dissolved in acetone or THF and exposed to light,

TCPT is rapidly photolyzed, because these solvents can act as photosensitizers.

TCPT is insoluble in most other common solvents. However, TCPT is highly soluble

(280 mg/mL) and stable when dissolved in dimethyl sulfoxide (DMSO) at room

temperature even when exposed to light.
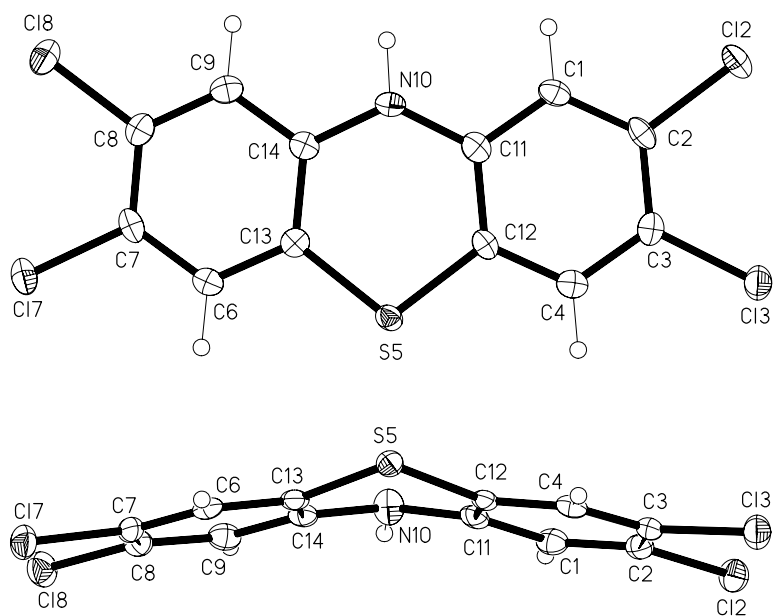
Figure 1-5. Crystal Structure of TCPT, displaying its deviation from planarity by 18.5 º.

1.2.2 *In vitro* **Enzyme Induction**

Following synthesis and chemical characterization, TCPT was assayed in a

pilot study for its effects *in vitro* to investigate the analogy of this compound to

TCDD. As described above (1.1.7), enzyme induction is a sensitive endpoint of

exposure to TCDD. Therefore, H4IIEC/T3 rat hepatoma cells were exposed to

different concentrations of TCPT and for different periods of time. The induced

CYP1A1 activity was quantified as ethoxyresorufin-O-deethylase (EROD) activity.

The cleaving of ethoxyresorufin was quantified photometrically, allowing the direct

comparison of TCPT to TCDD when assayed in parallel.

EROD activity in rat hepatoma cells increased over time after exposure to

TCDD. The induction of EROD activity by TCPT, however, declined after 24 h and

was virtually completely reversed for the concentrations tested after 72 h (Figure

1-6). An ED_{50} of 85.1 pg/well (= 851 pg/ml) was calculated for the induction of

EROD by TCPT after 24 h, as compared to 0.23 pg/well (= 2.3 pg/ml) by TCDD,

demonstrating a 370-fold difference in terms of induction potency.

This illustrated clearly the influence of the underlying tricyclic backbone on

potency: The high potency as displayed by dioxins was diminished by the

introduction of different heteroatoms without substantially compromising efficacy of

TCPT regarding the induction of CYP1A1-specific enzyme activity.

The average ED_{50} across all time points tested was ED_{50} = 90.5 ± 6.78 pg/well (905 pg/ml) for TCPT, and ED_{50} = 0.20 ± 0.03 pg/well (2.0 pg/ml) for TCDD. The decline of induction by TCPT at the concentrations tested after 24 h is indicative of its metabolism by hepatocytes and the formation of inactive metabolites. This conclusion is further supported by constant ED_{50}s throughout the time points and concentrations studied. Potency is a substance-specific property and, thus, independent of dose or concentration. Therefore, 24 h data correlated well with the 48 h and 72 h measurements for both compounds across the studied dose range. The maximal induction of EROD activity after 24 h incubation with TCDD was 636.7 pmol/min mg protein, whereas TCPT elicited a calculated induction of 494.1 pmol/min mg protein, representing 77.6 % efficacy of the maximal induction by TCDD. The concentrations used in this assay were non-cytotoxic.
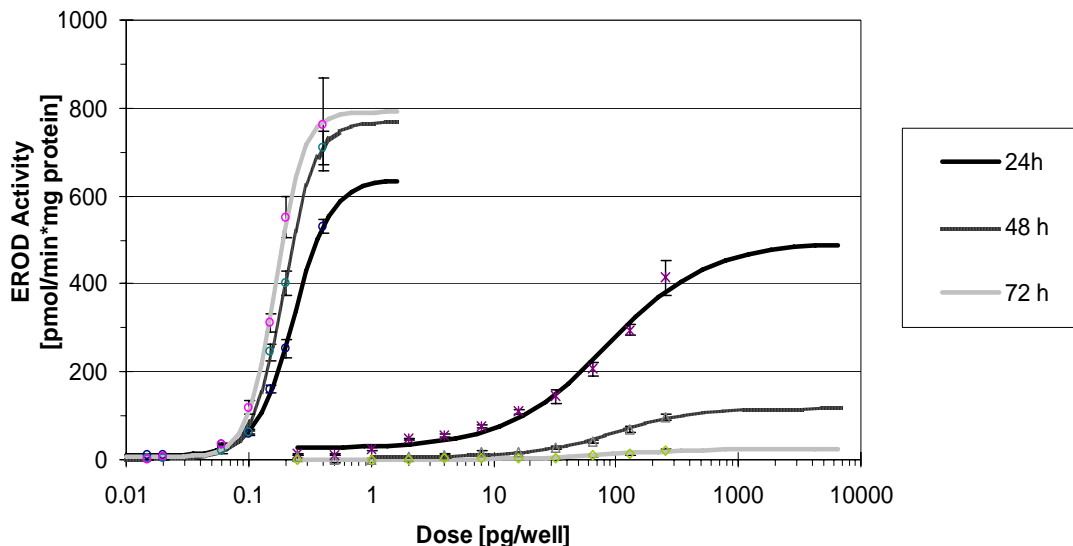
Figure 1-6. Time-dependent dose-responses of TCDD (left (o), increasing with time) and TCPT (right (x), decreasing with time) in an in vitro EROD-assay.

The binding of potential drug candidates to the AhR with subsequent

induction of CYP1A1 activity is considered detrimental because of the alleged

association of CYP1A1 with the metabolic activation of potential carcinogens

(Nakatsuru *et al*., 2004; Shimada and Fujii-Kuriyama, 2004). However, CYP1A1

knock-out mice displayed increased lethality to benzo[*a*]pyrene (BaP) compared to

wild type mice (Uno *et al*., 2004). The concentrations of BaP-DNA adducts in most

tissues were higher in CYP1A1 knock-out mice than in wild type animals,

demonstrating that CYP1A1 is not necessarily required for bioactivation.

Pretreatment with TCDD lowered the levels of DNA adducts in all genotypes,

indicating a protective mechanism unrelated to CYP1A1 (Uno *et al*., 2001). A

possible cause is the induction of other enzymes, e.g. CYP1A2, which is still present

in CYP1A1 knock-out mice (Dalton *et al*., 2000). In contrast to observations with

BaP toxicity, CYP1A1 knock-out mice were more resistant to lethality by TCDD than

respective wild type mice (Uno *et al*., 2004).

It has been stated that "intact animal data contradict pharmaceutical company

policies" that eliminate drug leads that induce CYP1 activity "for fear of possible

toxic or carcinogenic effects" (Nebert *et al*., 2004). Induction of CYP1A1 activity is,

however, almost certainly needed for some beneficial low-dose effects of dioxins.

Furthermore, the induction of CYP1A1 activity is not only a hallmark effect of many

polychlorinated organic compounds, also many naturally occurring compounds like

indoles from cruciferous vegetables, chrysin derivatives and carotinoids are AhR

ligands (Denison *et al*., 2002; Denison and Nagy, 2003). Furthermore, caffeine

(Goasduff *et al*., 1996) and drugs such as omeprazole (Daujat *et al*., 1992) and chlorpromazine (Kerecsen *et al*., 1994) are known CYP1A1 inducers. Although the elimination half-lives of naturally occurring compounds and drugs are comparatively short, daily intake still causes chronic exposure. As evidenced by these compounds, induction of CYP1A1 can occur without a documented increase in cancer prevalence. In fact, induction of CYP1A1 by HpCDD during the entire life span of rats resulted in decreased cancer prevalence (Kociba *et al*., 1978; Rozman *et al*., 2005). Therefore, the induction of EROD activity should not compromise TCPT as a potential drug.

1.2.3   Serum Kinetics in Rats and Guinea Pigs after Intravenous Injection

Rapid metabolism is expected to occur at the thioether moiety of TCPT, which can be readily oxidized to the sulfoxide or sulfone, and the secondary amine, which can be oxidized to the hydroxylamine, conjugated by phase II enzymes and readily excreted. In contrast, biotransformation is the slowest and hence rate-limiting step in PCDD-elimination (Neal *et al*., 1984), making non-biliary intestinal elimination by desquamating enterocytes and re-distribution of PCDDs into fecal fat to become the main route of very slow excretion.

Female rats were administered 5 mg/kg TCPT *i.v.* in freshly prepared acetone solution (1 mL acetone/kg), female guinea pigs received 10 mg/kg TCPT *i.v.* in acetone (0.5 mL/kg acetone). Blood samples were drawn from cannulas in the jugular vein in 500 μl volumes from both species at different time intervals after dosing and analyzed by HPLC.

The kinetics of TCPT were identified by classical curve feathering as well as computational regression to follow a two-compartment model in both species investigated:

Rat: $\qquad C_p = 3.700e^{-0.843t} + 1.862e^{-0.128t} \qquad R^2 = 0.99 \ (n = 8)$

Guinea Pig: $\quad C_p = 6.132\,e^{-1.658t} + 4.518e^{-0.256t} \qquad R^2 = 0.99 \ (n = 9)$

The calculated half-lives in rats were $t_{1/2} = 0.8$ h for distribution and $t_{1/2} = 5.4$ h for elimination (Figure 1-7). In guinea pigs, half-lives were $t_{1/2} = 0.4$ h and $t_{1/2} = 2.7$ h, respectively (Figure 1-8). The concentration of TCPT in the last two blood samples of the guinea pigs was near the limit of quantification, resulting in a larger standard deviation than the earlier time points. Because of the limited reliability of these data points, they were omitted from curve feathering. Computational regression assigned less weight to standard deviations at lower concentrations than at more reliable high concentration data points. Both methods yielded comparable results, leading to the curve fit depicted in Figure 1-8.
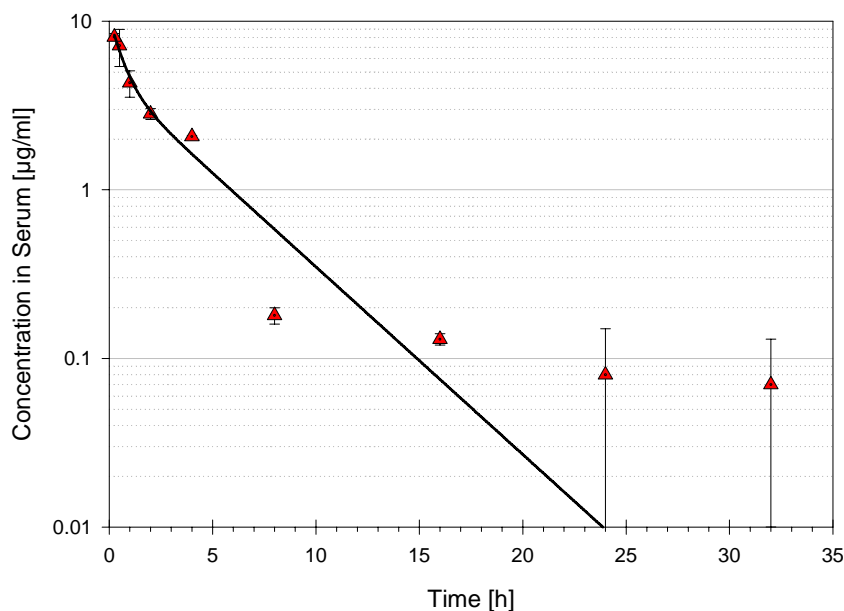
Figure 1-7. Serum profile of TCPT in rats after administration of **i.v.** *5 mg/kg TCPT with two-compartment curve-fit (*$C_p = C_1 e^{-k_1 t} + C_2 e^{-k_2 t}$*).* **C$_1$ = 3.700 (SD 0.744), k$_1$ = 0.843 (SD 0.284), C$_2$ = 1.862 (SD 0.832), k$_2$ = 0.128 (SD 0.057), R^2 = 0.99 (n = 8), t$_{½ (distribution)}$ = 0.82 h, t$_{½ (elimination)}$ = 5.42 h.**

The volumes of distribution were $V_{d \, (central)}$ = 0.9 l/kg, $V_{d \, (apparent)}$ = 1.8 l/kg, $V_{d \, (peripheral)}$ = 2.3 l/kg in rats and $V_{d \, (central)}$ = 0.9 l/kg, $V_{d \, (apparent)}$ = 2.6 l/kg, $V_{d \, (peripheral)}$ = 4.1 l/kg in guinea pigs. The apparent volumes of distribution indicate a distribution slightly larger than total body water. One possible explanation is that TCPT, like TCDD, exerts binding affinity to proteins such as the AhR or CYP1A2, resulting in tissue sequestration, but to a much lesser extent than TCDD.
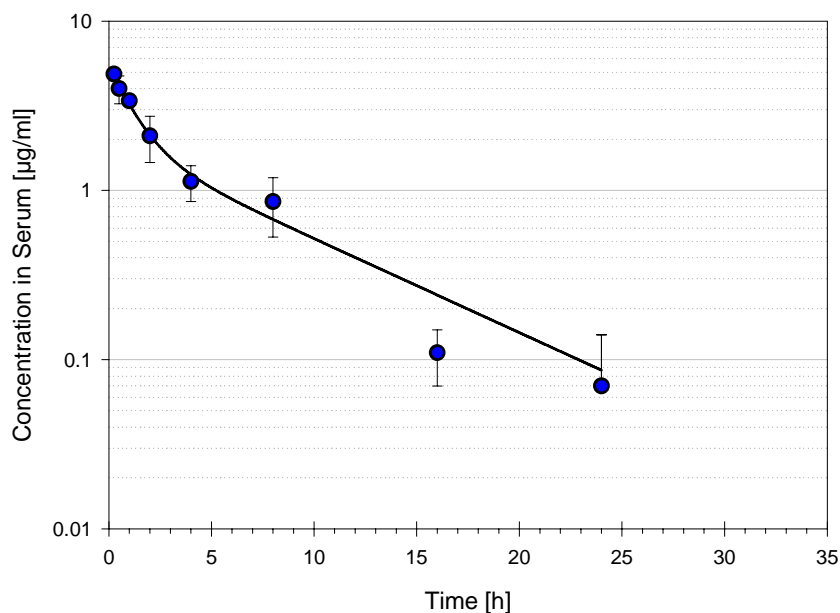
Figure 1-8. Serum profile of TCPT in guinea pigs after administration of i.v. 10 mg/kg TCPT with two-compartment curve-fit ($C_p = C_1 e^{-k_1 t} + C_2 e^{-k_2 t}$). C_1 = 6.132 (SD 1.840), k_1 = 1.658 (SD 1.022), C_2 = 4.518 (SD 2.254), k_2 = 0.256 (SD 0.125), R^2 = 0.99 (n = 9), $t_{1/2\ (distribution)}$ = 0.42 h, $t_{1/2\ (elimination)}$ = 2.71 h.

1.3   Tetrachlorophenothiazine as an Analogue of Tetrachlorodibenzo-*p*-dioxin

The potency of TCPT to induce CYP1A1 activity over 24 h was $^1/_{370}$th that of TCDD *in vitro*. The elimination half-life of the parent compound in serum was 5.4 h in the rat and 2.7 h in the guinea pig, compared to 11 and 30 days, respectively, for TCDD. Compared to TCDD, TCPT is more readily eliminated and has a reduced potency regarding enzyme induction, while maintaining high efficacy regarding the induction of CYP1A1 enzyme activity as demonstrated in this study. These initial findings clearly differentiated TCPT from TCDD and provided the basis for further studies of this compound (Fried *et al.*, 2007).

CHAPTER 2

STATEMENT OF PURPOSE

2.1 General Background

The mechanism of action for the potentially beneficial lower-dose effects of dioxins has been explored in a rudimentary fashion in the past. However, it has been reported that TCDD reduced serum IGF-1 levels (Croutch *et al*., 2005), which is assumed to be a key mediator for several medium- to low-dose effects (Arking, 2003), such as longevity (Kurosu *et al*., 2005; Rozman *et al*., 2005; Van Heemst *et al*., 2005), inhibition of ovulation, (Li *et al*., 1995; Li *et al*., 1995) and sustained body weight reduction. Another presumed mechanism of action for low-dose effects, such as reduced cancer rates (Kociba *et al*., 1978; Rozman *et al*., 2005), seems to be mediated by the AhR. Agonists of this receptor-mediated pathway induce cytochrome P450 CYP1A1 activity. This induction of phase I metabolism of ubiquitous toxic exogenous and endogenous compounds could account for reducing concentrations at target sites from reaching threshold levels for effects, such as cancer (Rozman *et al*., 1996), to occur.

Given these beneficial effects of dioxins, it was reasonable to contemplate possible medicinal uses of this class of compounds (Kayajanian, 2003). Effects such as reduced cancer rates, lowered body weight, increased insulin sensitivity (Gorski and Rozman, 1987) and inhibition of ovulation are all under certain circumstances desirable effects and suitable analogues could find application in the prevention of cancer, treatment of obesity and type II diabetes, or as contraceptives. It is the unfavorable kinetics and, to a limited extent, also the high toxic potency of dioxins

that preclude their use as therapeutic agents. With an average elimination half-life of

7.8 years (Geyer *et al.*, 2002) and a calculated LD_{50} value of 6 mg/kg (Geyer *et al.*,

1990) in humans, TCDD poses risks that far outweigh potential benefits. The use of

congeners with lower potency (i.e. higher degree of chlorination) is prohibitive as

well, since the elimination half-life increases with increasing chlorination to the point

of exceeding human life-expectancy (Geyer *et al.*, 2002). These two exclusionary

properties (kinetics and potency) had to be modified for the development of dioxin

analogues with potential therapeutic applications.

The new TCDD analogue 2,3,7,8-tetrachlorophenothiazine (TCPT) was

developed to take advantage of the low-dose effects of dioxins that have potential

application in therapeutics. Its development marked a deviation from the traditional

scope of phenothiazine drug design by modifying aryl substituents rather than the

traditional alteration of the N-substituent. The pharmacokinetic profile of

phenothiazines differs dramatically from that of PCDDs, addressing the first

exclusionary property of dioxins, *viz.* unfavorable kinetics. The most prominent

member of the class, chlorpromazine (CPZ), has an elimination half-life of 9.1 h in

the rat (Mahju and Maickel, 1969) and 30 h in humans (Hardman *et al.*, 2001). CPZ,

like all conventional phenothiazine drugs, forms sulfoxo-, ring hydroxyl- and side

chain metabolites. However, TCPT does not contain side chains, and its aryl positions

are sterically hindered by chloro-substituents, preventing ring hydroxylation from

occurring. Its elimination half-life in rats is 5.4 h. Based on a comparison with CPZ,

an extrapolation of the elimination half-life from rats to humans suggests an approximate elimination half-life of 18 h for TCPT in humans.

Concerns regarding potency, which was the second exclusionary property for a potential drug lead, were addressed by modifying the stereochemistry of phenothiazines. Whereas dioxins are essentially planar molecules (Boer *et al*., 1972; Senma *et al*., 1973), phenothiazines are angled along the N-S axis. Because AhR-mediated CYP1A1 induction has typically been described for planar ligands (Denison *et al*., 2002; Denison and Nagy, 2003), this slight steric difference between phenothiazines and dioxins was expected to affect interactions with endogenous targets and to result in reduced potency of TCPT as compared to TCDD. Most importantly, TCPT could become a drug lead only if it retained some of the medium to low dose effects of dioxins.

2.2   Specific Research Goals

Because the two major exclusionary properties for the medicinal exploitation of low-dose effects of dioxins were eliminated by the synthesis of TCPT, detailed studies were needed to characterize the analogy in terms of effects between these two (classes of) compounds, or a lack thereof:

1) The AhR signaling pathway plays a central role in the mechanism of action for low-dose effects of dioxins. Therefore, the interaction of TCPT and selected derivatives with this receptor as well as down-stream effects were expected to play a critical role in the investigation of the analogy. Standard *in vitro* assays were to be employed in the investigation of the binding affinity of TCPT and derivatives to the AhR and compared to the induction of CYP1A1 enzyme activity with EROD as a surrogate substrate. The null hypothesis for these *in vitro* studies was that the binding affinity of phenothiazines to the AhR and the induction of EROD activity would be mechanistically the same or similar to but in their extent much lower than those reported for TCDD.

2) Based on the proposed mechanisms of action of dioxins in the medium-dose range, the effects of TCPT on IGF-1 serum levels were of interest to furthering the potential of this compound as a drug lead. However, effects of TCDD on decreasing serum IGF-1 levels in rats were described in the past for only one dose. In order to compare the effects of TCPT with those of TCDD quantitatively, dose-response curves of both compounds had to be developed. The hypothesis for this study was that TCPT reduces serum IGF-1 levels dose-dependently, albeit with much reduced potency as compared to TCDD.

CHAPTER 3

RELATIONSHIP BETWEEN ARYL HYDROCARBON RECEPTOR-AFFINITY AND THE INDUCTION OF EROD ACTIVITY BY 2,3,7,8-TETRACHLORINATED PHENOTHIAZINE AND DERIVATIVES

3.1   Abstract

The development of TCPT was based on its structural analogy to dioxins, specifically to TCDD. TCDD binds readily to the aryl hydrocarbon receptor (AhR), and thus exerts many of its biological effects *in vitro* and *in vivo* through AhR activation.

Reported herein are semi-empirical calculations of the molecular geometry of TCDD, TCPT, TCPT-sulfoxide (TCPT-O), TCPT-sulfone (TCPT-O_2), N-methyl-TCPT (Me-TCPT), N-methyl-TCPT-sulfoxide (Me-TCPT-O), and N-methyl-TCPT-sulfone (Me-TCPT-O_2), the characterization of their AhR binding affinity in rat hepatic cytosol, and their ability to induce EROD activity in a rat hepatoma cell line *in vitro*.

Semi-empirical calculations yielded detailed information about the stereochemistry and the preferred conformation of each of these compounds. These results in combination with observations reported in this paper were used to determine structure-activity relationships of these compounds.

[3]H-TCDD binds specifically to the AhR in rat hepatic cytosol, and *in vitro* displacement was measured by increasing concentrations of the respective ligands. This assay revealed a strong binding affinity of TCPT to the AhR with a K_i value of 1.08 nM (= 0.36 ng/ml). TCDD has a K_i value of 0.54 nM (= 0.17 ng/ml) , i.e. the K_i of TCPT is only twice as high as that of TCDD. The affinity of TCPT derivatives

for the AhR decreased with increasing degree of oxidation. Moreover, N-methylation

further lowered the affinity, so that the N-methyl sulfone derivative of TCPT

displayed the highest K_i at ~ 1200 nM (= 460.4 ng/ml).

A corresponding trend was observed regarding the potency of TCPT and

derivatives to induce EROD activity *in vitro*. However, the potencies were

considerably lower than that of TCDD. Enzyme induction was measured in a rat

hepatoma cell line by quantification of ethoxyresorufin-O-deethylase (EROD)

activity. Induction was measured at 12, 24, 48 and 72 h to determine time-

dependence. Sulfoxidated and N-methylated phenothiazines displayed a lower

potency than their respective parent compounds. TCPT and all derivatives induced

enzyme activity at an efficacy similar to TCDD at all time points measured.

3.2 Introduction

Polychlorinated dibenzo-*p*-dioxins (PCDDs) are among the most toxic and

persistent xenobiotics in the environment. Their effects are diverse and range from

hormetic effects (Kociba *et al*., 1978) and enzyme induction (Poland *et al*., 1976) at

low doses to endocrine effects at medium doses (Peterson *et al*., 1993) and

carcinogenic (Van Miller *et al*., 1977) and acutely toxic effects at still higher doses

(Harris *et al*., 1973; Schwetz *et al*., 1973). Much research has been conducted to

elucidate the mechanism of these effects, most of it related to the AhR (Poland and

Knutson, 1982; Thunberg, 1984; McKinney *et al*., 1985). However, it has also been

argued that a single mechanism is unlikely to explain a complex toxicity profile such as that displayed by dioxins (Rozman, 1989; Rozman, 1992).

TCDD is one of the most potent AhR agonist known. Therefore, it is used as a model compound for AhR binding kinetics as well as to study downstream effects, such as CYP1A1 induction. The best known AhR signaling pathway is initiated by a ligand binding to the receptor. In this process, proteins associated with the AhR are released. The AhR/agonist complex translocates into the nucleus and binds to the AhR nuclear translocator (Arnt). This heterotrimer attaches to the dioxin response element (DRE) on DNA and acts as a transcription factor. Mediated through translation, the AhR signaling cascade elicits a biological response, such as CYP1A1 induction.

Steric requirements for AhR ligands were initially postulated by Poland (Poland and Knutson, 1982). Planarity and a molecular size of 10.0 x 3.0 Å were proposed to be required for rectangular, halogen-atom centered molecules to fit the AhR binding pocket. Molecular modeling later led to the refinement of the requirements, yielding a box-shaped binding site of maximally 14.0 x 12.0 x 5.0 Å (Waller and McKinney, 1995).

Competitive displacement assays using radiolabeled high affinity ligands were used in the past to quantitatively study interactions of compounds with the AhR *in vitro*. Initially, ^{125}I-2-iodo-7,8-dibromodibenzo-*p*-dioxin was used because of the high

specific activity of this isotope (Bradfield and Poland, 1988). However, displacement of ^{3}H-TCDD (Bradfield *et al*., 1988) allows a more direct comparison of affinities to that of TCDD without the need of further validation of the standard.

TCDD induces CYP1A1 and CYP1A2 in rats up to 50-100 fold (Abraham *et al*., 1988). CYP1A1 activity is most frequently measured by its ethoxyresorufin-O-deethylase (EROD) activity. The ED_{50} for EROD-induction in rats by TCDD is 0.2-0.3 μg/kg (Roth *et al*., 1988), whereas the LD_{50} value is 45 μg/kg (Schwetz *et al*., 1973). No lethality occurs at doses of complete AhR saturation, in fact, saturation is reached at doses 100-times lower than what is known to be a sublethal dose of TCDD (Rozman *et al*., 1993). In the most TCDD-susceptible (Long Evans) and the most TCDD-resistant (Han/Wistar) rat strains, *i.p.* LD_{50}s for TCDD have been reported as 10 and > 3000 μg/kg, respectively (Pohjanvirta *et al*., 1988). However, neither AhR binding affinities (Pohjanvirta *et al*., 1999) nor related EROD activities (Pohjanvirta *et al*., 1990) were different between these two strains of rat supporting the view that binding to the AhR may be a first step in the interaction of TCDD with an organism, but not the ultimate cause of toxicity.

In contrast to the predominantly negative perception of dioxins, they have been also shown to exert beneficial effects in animal models, such as immunostimulation (Fan *et al*., 1996), reduced cancer rates (Kociba *et al*., 1978; Rozman *et al*., 2005) and prolongation of life (Rozman *et al*., 2005) at (hormetic) doses in the range of AhR binding. The binding of potential drug candidates to the

AhR, however, is generally considered undesirable and usually stops the development of a drug candidate. This, in spite of the presence of naturally occurring AhR agonists in food. The expected induction of CYP1A1 activity is still associated with the metabolic activation of potential carcinogens. Therefore, a separation of AhR interaction and CYP1A1 induction could save drug leads that otherwise would have been discarded.

The study of TCPT was a result of these considerations. This compound was developed as a potential drug lead for aforementioned indications. A pilot study confirmed EROD-inducing activity *in vitro* (Fried *et al*., 2007), a hallmark effect and proposed mechanism of action for many low-dose effects of dioxins. The study of putative metabolites as part of the biological characterization of TCPT was undertaken to predict the influence of biotransformation on overall compound activity.

Phenothiazines structurally differ from dioxins by deviating from planarity. Whereas dioxins are essentially planar (Boer *et al*., 1972; Senma *et al*., 1973; Cordes and Fair, 1974), the phenothiazine structure is bent along the N-S axis (McDowell, 1969; Jovanovic and Biehl, 1987), suggesting altered interactions with molecular targets. Therefore, TCPT and putative metabolites were scrutinized regarding their stereochemistry, AhR binding affinity, and the hallmark downstream effect of AhR signaling, CYP1A1 activity (measured as EROD activity).

3.3 Materials & Methods

3.3.1 Semi-Empirical Calculations

The molecular geometries of all the compounds were calculated using PM3 methodology (Stewart; Dewar *et al*., 1985) employed by Hypercube Inc., Gainesville, FL, in their software HyperChem 6.0. The following parameters were used in the calculations: Convergence limit 10^{-6}, iteration limit 150, spin pairing RHF, lowest state, no configuration interaction. Algorithms were calculated according to Polack-Ribiere under the termination condition of an RMS gradient of 10^{-4}. π-electrons in aromatic systems were considered delocalized for all calculations. Length was calculated from $C\ell 2(3)$-$C\ell 8(7)$. Width was the longest distance between two atoms perpendicular to the length axis but not necessarily on the plane created by the four chloro substituents. Height was calculated as the elevation of the N-S axis from the $C\ell 2,3,7,8$ plane.

3.3.2 Competitive AhR Ligand Displacement Assay

Male Sprague Dawley rats weighing 225-250 g were obtained from Harlan, Indianapolis, IN. Animals were housed in wire-bottom cages at a 12/12 h light/dark cycle, 22 °C ambient temperature and a relative humidity of 40-60 %. All rats had *ad libitum* access to water and 8604 Rodent Diet supplied by Harlan Teklad, Madison, WI. The research presented was conducted in compliance with NIH Guidance for the Care and Use of Animals and was approved by the KUMC Institutional Animal Care and Use Committee. Preparation of cytosolic protein and the binding assay were performed as described elsewhere (Bradfield *et al*., 1988; Bradfield and Poland,

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1988). In brief, rats received a lethal injection of pentobarbital and livers were perfused with cold 150 mM $KCl_{(aq)}$, adjusted to pH 7.4. The tissue was homogenized in MDENG buffer containing 25 mM 3-(N-morpholino)-propane sulfonic acid, 0.02 % sodium azide, 1 mM ethylenediaminotetraacetate, 10 % (w/v) glycerol, 1 mM dithiothreitol and adjusted to pH 7.5. A 12,000 g supernatant was prepared by centrifugation at 4 ºC for 20 min. Ultracentrifugation (440,000 g for 25 min) yielded hepatic cytosol, containing the AhR in the supernatant. Aliquots were stored at -80 °C until use. Protein content was determined using the Better Bradford assay by Pierce Biotechnology, Inc., Rockford, IL, and cytosol was diluted with buffer to 5 mg/ml protein for assaying. The solvent for all compounds was dimethylsulfoxide. Four experiments were run in triplicate, each using 200 μl cytosol, in order to measure:

a) *Total binding of TCDD:* Cytosol + 1 μl of 200 nM ^{3}H-TCDD,

b) *non-specific binding of TCDD:* a) + 1 μl of 20 μM 3,3',4,4'-tetrachloroazoxybenzene

c) *displacement of TCDD from AhR (specific binding) by test compound:*
a) + 1 μl increasing concentration of test compound, and

d) *non-specific binding of test compound:* b) + 1 μl increasing concentration of test compound.

After incubation at 4 °C for 16-18 h, 2 mg dextran-coated charcoal was added to remove unbound compounds during 10 min on ice. Following centrifugation at 20,000 g at 4 ºC for 10 min, the 150 μl supernatants were used in 5 ml Emulsifier Safe scintillation cocktail purchased from PerkinElmer Life and Analytical Sciences,

Boston, MA and radioactivity was counted. Curve fits, respective parameters and standard errors were calculated using Sigma Plot 8.0 from SPSS Inc., Chicago, IL.

3.3.3 *In vitro* Induction of EROD Activity

An *in vitro* EROD assay with the H4IIEC/T3 rat hepatoma cell line (Reuber, 1961; Pitot *et al*., 1964) was conducted according to SOP (Doods, 2003). In brief, cells were plated at a density of ~ 10,000 cells/well in 96 well plates and cultured for 72 h prior to exposure. Culture media was then replaced with 100 µl media containing TCDD or test compounds. All compounds were dissolved in dimethylsulfoxide /isopropanol 4/1 (v/v). The concentration of organic solvents in media was 0.5 % in the final assay mixture. Various concentrations of compounds were added and the plates were incubated for 12, 24, 48, and 72 h. The highest concentration of TCPT and derivatives represented the respective limit of solubility. After incubation, the media was discarded and hepatocytes were exposed to 8 µM 7-ethoxyresorufin in 100 µl media for 30 min. Subsequently, the resorufin thus generated was quantified by detecting fluorescence at 590 nm after excitation at 535 nm. Protein content was measured employing the Pierce assay. Cytotoxicity was determined by the resazurine assay, based on the potential of living cells to reduce this agent to resorufin, detectable as described above. Each dose was represented in triplicate, error bars depict the standard error of the mean. Curve fits, respective parameters, confidence intervals and standard errors were calculated using Sigma Plot 8.0 from SPSS Inc., Chicago, IL.

3.4 Results

3.4.1 Semi-Empirical Calculations

The overall size of the compounds based on the plane created by the four chlorine atoms ranges from 10.04 Å (TCDD) to 10.47 Å (Me-TCPT) in length and 3.06 Å (TCDD) to 3.07 Å (phenothiazines) in width. Therefore, it can be considered equivalent for all molecules. Maximum width and three-dimensional structure, however, differed (Table 3-1). Deviations from planarity by derivatization caused a rise of the tricyclic backbone structure of up to 1.9 Å, adding to the perimeter extension by the respective substituent (Figure 3-1 and Figure 3-2).

Table 3-1: Semi-empirically calculated structural dimensions of TCDD, TCPT, and its sulfoxo- and N-methyl derivatives. Maximum width reported as distance between H1 and H4 (*) or between N and S substituents (‡).

Compound	Length Cℓ2-8 [Å]	Width Cℓ2-3 [Å]	Max. Width [Å]	Elevation of Backbone [Å]	Bond Length of N or S Substituent(s) [Å]	Angle of Deviation from Planarity
TCDD	10.04	3.06	5.02*	planar	N/A	0º ± 0
TCPT	10.46	3.07	4.99*	0.91	N/A	19.8º ± 0.07
TCPT-O	10.27	3.07	5.32‡	1.41	S-O = 1.54	30.8º ± 0.31
TCPT-O_2	10.39	3.07	5.29‡	1.19	S-O = 1.46	25.7º ± 0.21
Me-TCPT	10.47	3.07	4.99*	0.87	N-Me = 1.49	18.9º ± 0.13
Me-TCPT-O	10.31	3.07	5.71‡	1.87	N-Me = 1.48 S-O = 1.55	39.8º ± 0.24
Me-TCPT-O_2	10.35	3.07	5.72‡	1.74	N-Me = 1.48 S-O = 1.46	37.1º ± 0.21
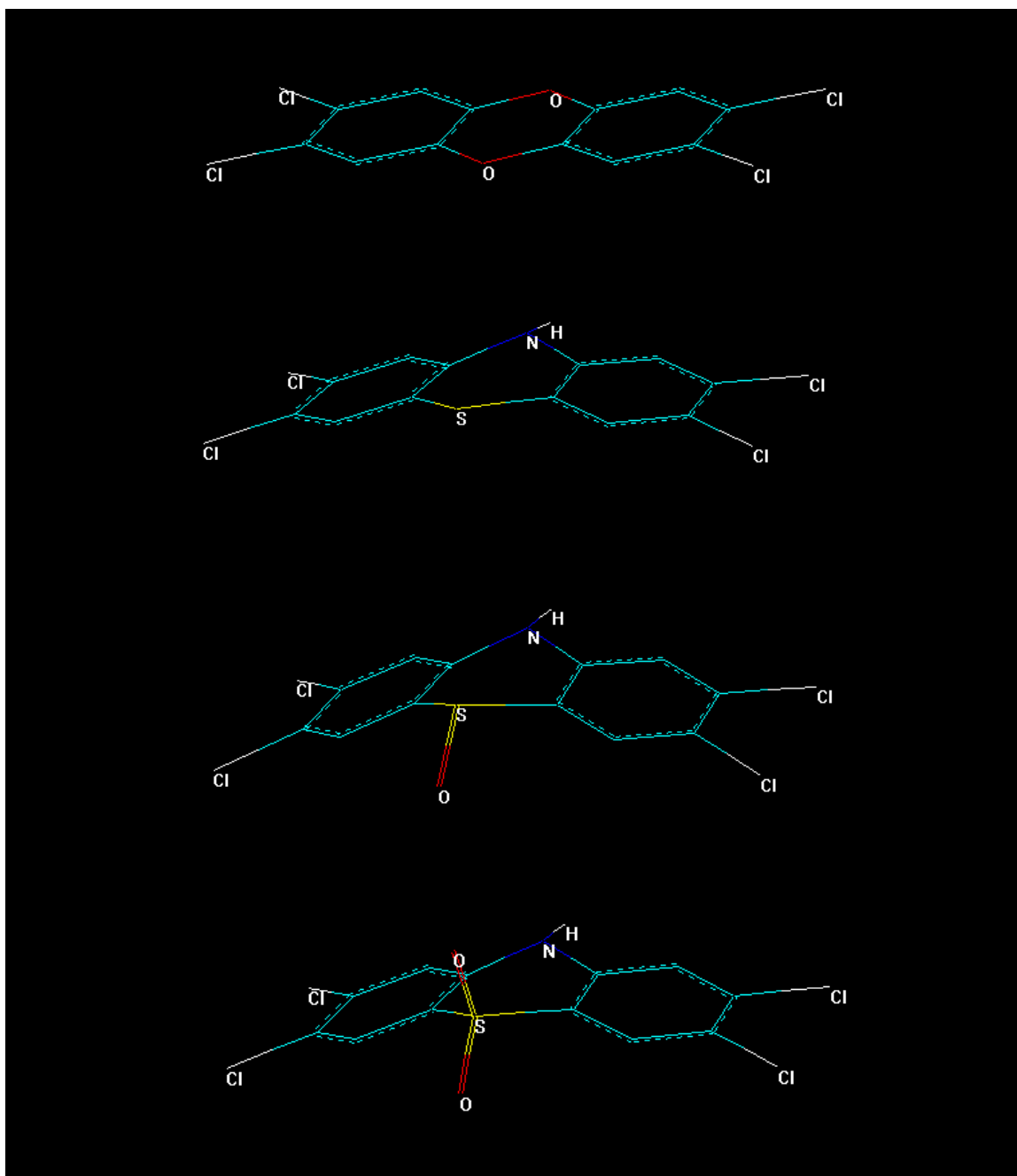
Figure 3-1: Semi-empirically calculated stereochemical structures of TCDD, TCPT, TCPT-O, and TCPT-O₂.
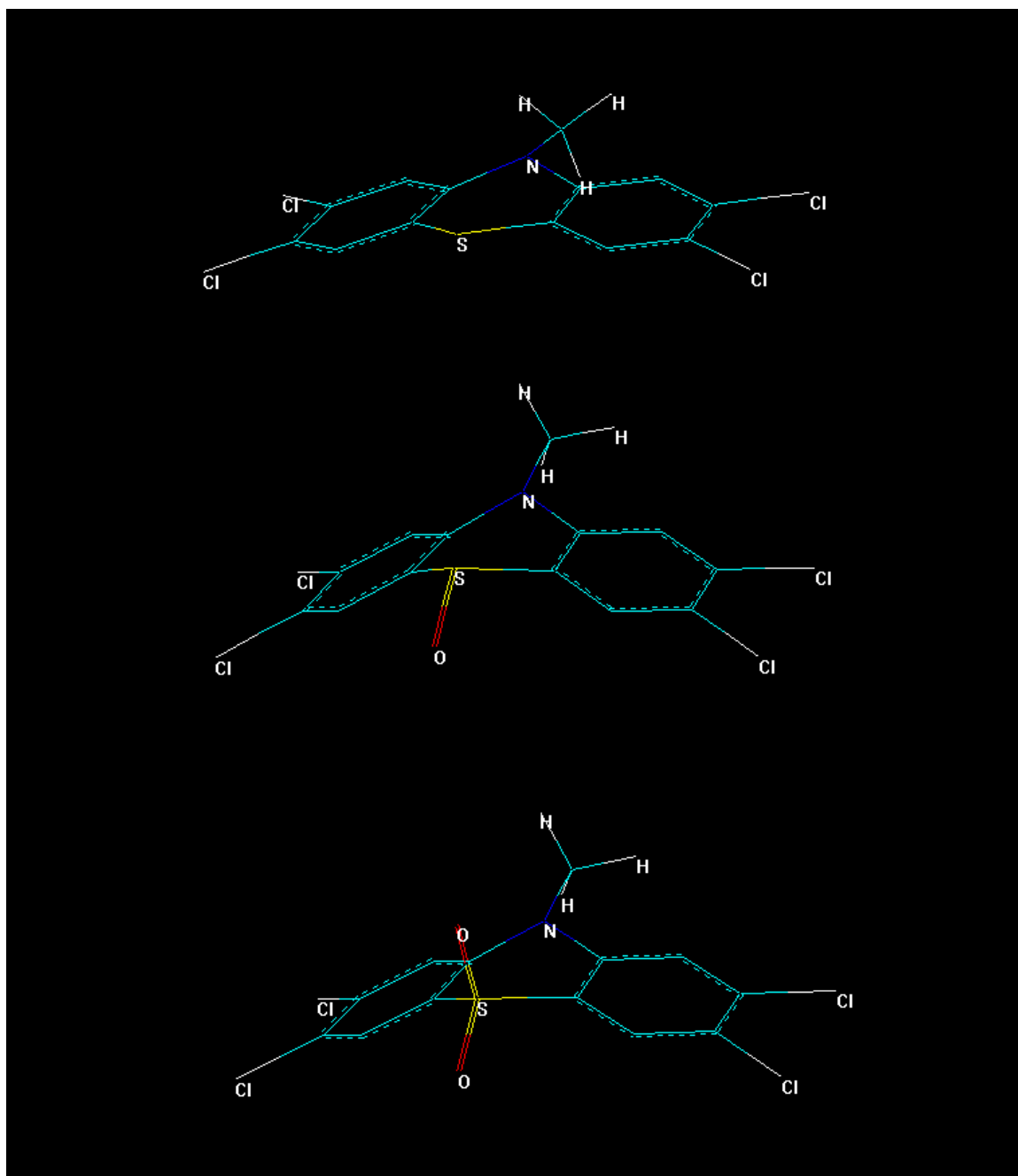
Figure 3-2: Semi-empirically calculated stereochemical structures of Me-TCPT, Me-TCPT-O, and Me-TCPT-O₂.

3.4.2 Competitive AhR Ligand Displacement Assay

^{3}H-TCDD bound to the AhR was quantitatively displaced by increasing concentrations of TCPT or derivatives (Figure 3-3 and Figure 3-4). TCPT exerted, after TCDD, the second highest affinity among the compounds tested. The K_i of TCPT of 1.08 nM (= 0.36 ng/ml) was only two times higher than that of TCDD (K_i = 0.54 nM, or 0.17 ng/ml).
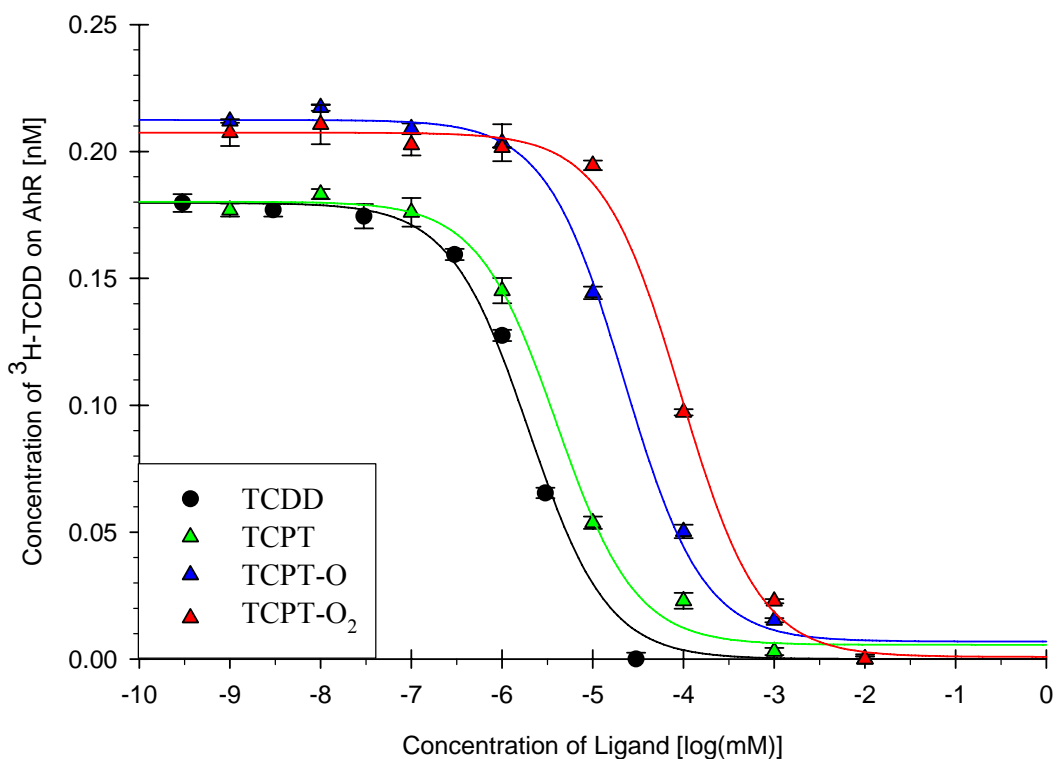
Figure 3-3: Competitive displacement of ^{3}H-TCDD from its specific binding site on the AhR by increasing concentrations of TCDD, TCPT and its sulfoxides.
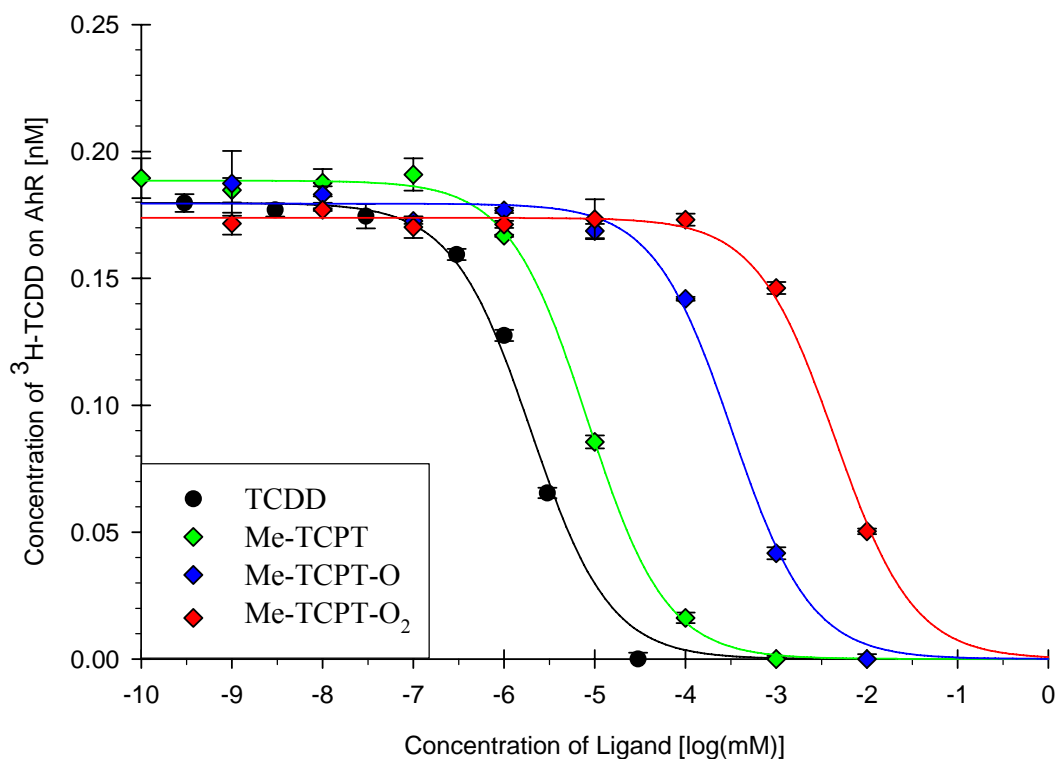
Figure 3-4: Competitive displacement of ^{3}H-TCDD from its specific binding site on the AhR by increasing concentrations of TCDD, Me-TCPT and its sulfoxides.

The N-methylated derivative also exhibited high affinity to the AhR with a K_i of 2.25 nM (0.79 ng/ml), i.e. its affinity was within a five-fold range of that of TCDD. Non-specific binding of all compounds was independent of concentration (data not shown).

Table 3-2: Inhibition constants and EC₅₀ levels for TCDD, TCPT, and its sulfoxo-and N-methyl derivatives regarding competitive displacement from AhR.

Compound	EC_{50} [nM] (95% confidence interval)	EC_{50} [ng/ml]	K_i [nM] (95% confidence interval)	K_i [ng/ml]	K_i Normalized to TCDD
TCDD	1.98 (1.15 – 3.42)	0.64	0.54 (0.31 – 0.92)	0.17	1.0
TCPT	4.00 (2.64 – 6.05)	1.35	1.08 (0.72 – 1.63)	0.36	2.0
TCPT-O	22.25 (16.05 – 30.85)	7.86	6.01 (4.34 – 8.33)	2.12	~11
TCPT-O$_2$	93.65 (69.83 – 125.6)	34.56	25.29 (18.86 – 33.92)	9.33	~47
Me-TCPT	8.33 (6.91 – 10.04)	2.94	2.25 (1.87 – 2.71)	0.79	4.2
Me-TCPT-O	327.0 (197.7 – 541.1)	120.0	88.31 (53.39 – 146.1)	32.42	~165
Me-TCPT-O$_2$	4452 (2299 – 8621)	1705	1202 (620.9 – 2328)	460.4	~2200

3.4.3 *In vitro* Induction of EROD Activity

Induction of EROD activity was measured after 12, 24, 48, and 72 h (Figure
3-5 to Figure 3-10). No concentration of any test compound was cytotoxic, except for
1 and 10 µg/well (= 10 and 100 ug/ml) of TCPT, which also resulted in decreased
induction of enzyme activity. These data points were eliminated from curve
regression but included in the graph (Figure 3-5).
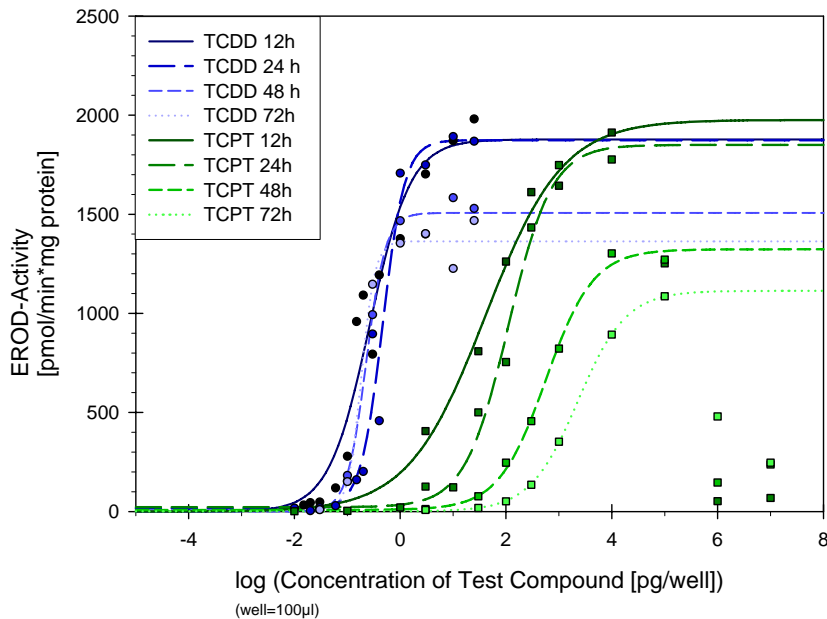
*Figure 3-5: Induction of EROD activity in vitro by TCDD and TCPT after 12, 24,
48, and 72 h of incubation. Concentrations of 1 and 10 µg/well (10 and 100 mg/µl)
TCPT were cytotoxic and not included in data evaluation.*
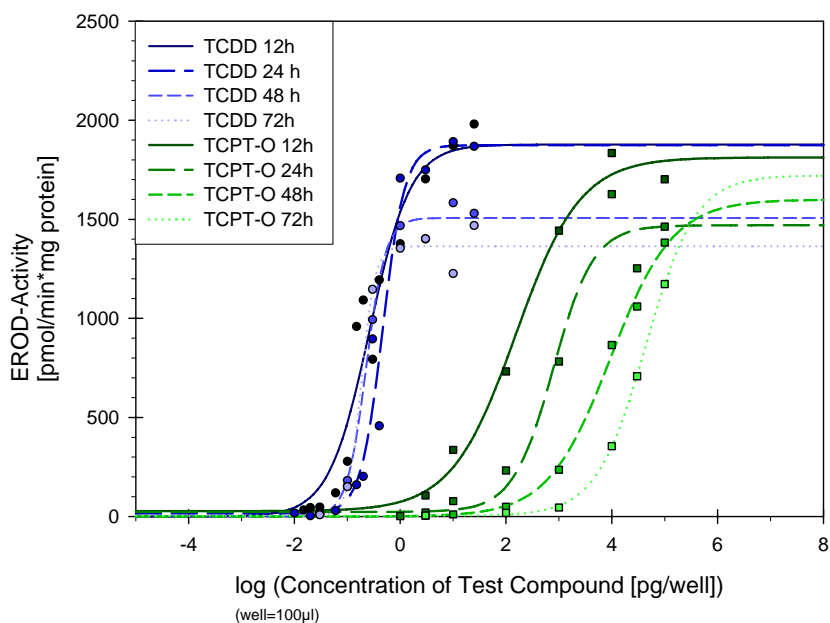
Figure 3-6: Induction of EROD activity in vitro by TCDD and TCPT-O after 12, 24, 48, and 72 h of incubation.
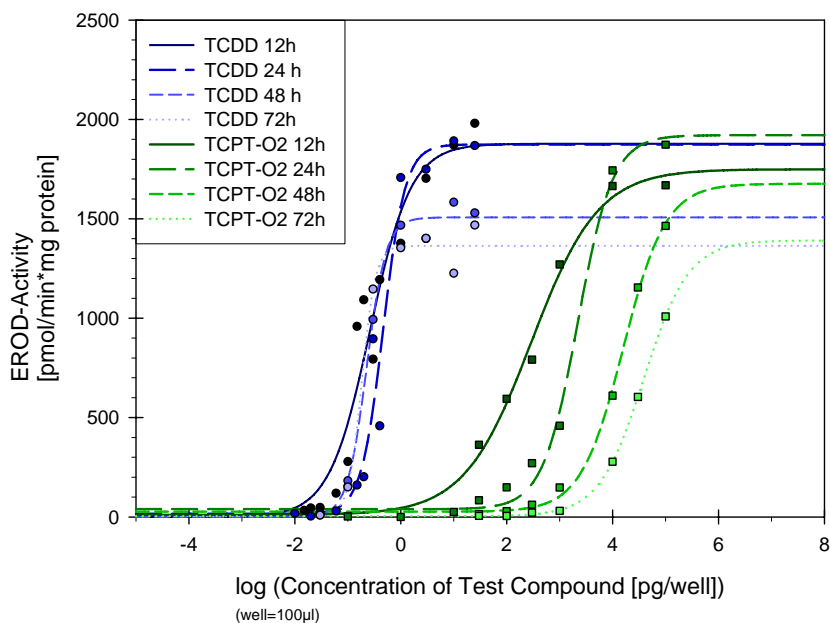
Figure 3-7: Induction of EROD activity in vitro by TCDD and TCPT-O$_2$ after 12, 24, 48, and 72 h of incubation.
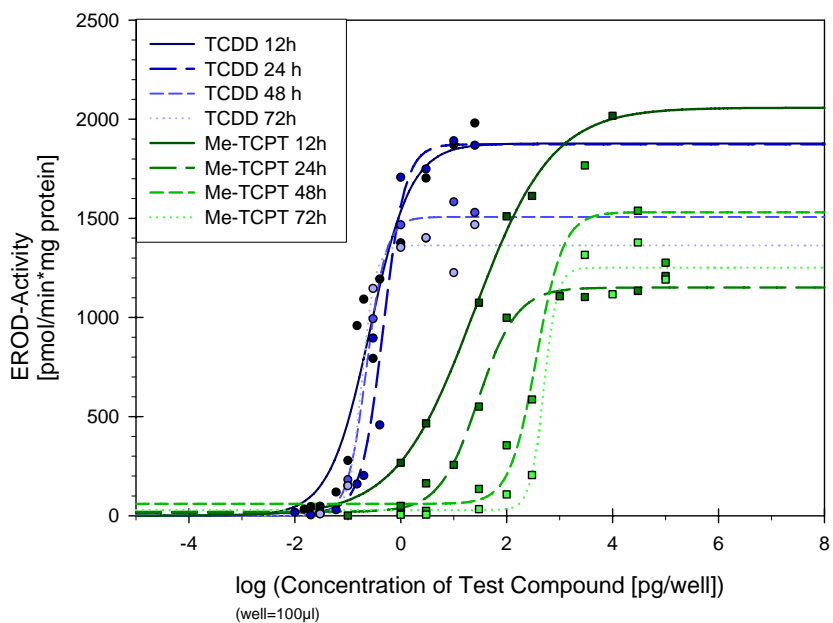
Figure 3-8: Induction of EROD activity in vitro by TCDD and Me-TCPT after 12, 24, 48, and 72 h of incubation.
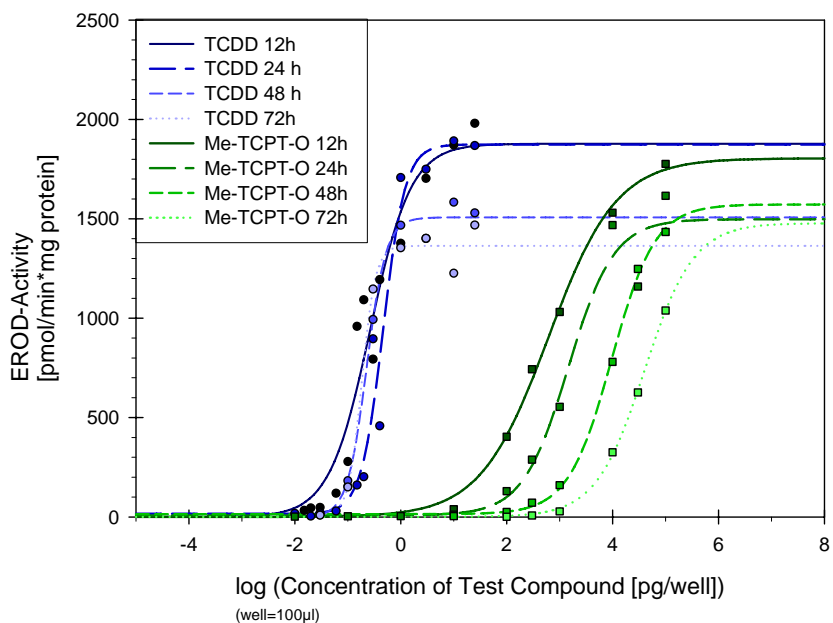
Figure 3-9: Induction of EROD activity in vitro by TCDD and Me-TCPT-O after 12, 24, 48, and 72 h of incubation.
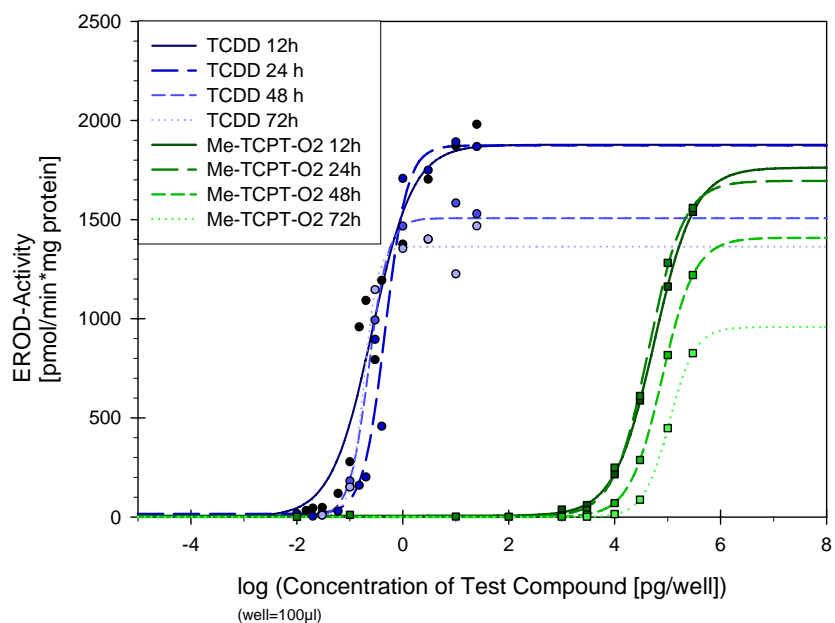
***Figure 3-10: Induction of EROD activity in vitro by TCDD and Me-TCPT-O₂ after
12, 24, 48, and 72 h of incubation.***

The efficacy of all compounds regarding the induction of EROD activity was

in the same order of magnitude as that of TCDD at all time points (Figure 3-11).

Normalized to the efficacy of TCDD, the lowest efficacy observed was 70 % after

72 h. In contrast, efficacy differed 7 % or less after 12 h incubation (Table 3-3). The

ED_{50} levels of all compounds except TCDD and Me-TCPT-O₂ decreased with

increasing induction time (Figure 3-12). TCDD and Me-TCPT-O₂, however,

displayed very little variability in ED_{50} values among time points (Figure 3-10). The

standard error for Me-TCPT-O₂ at the 72 h time point was high (Figure 3-12 and

Figure 3-13) because the limit of solubility was reached, preventing the measurement

of a full dose response curve (Figure 3-10). The exponential regression of ED_{50}

values over time (Figure 3-12) yielded an average half-life for the decline in apparent

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potency of 9.7 ± 0.79 h for all phenothiazines except Me-TCPT-O_2 (52.1 h) (Table 3-4). Comparison of all derivatives revealed an inverse relationship between the degree of oxidation and potency (Table 3-4, Figure 3-13).
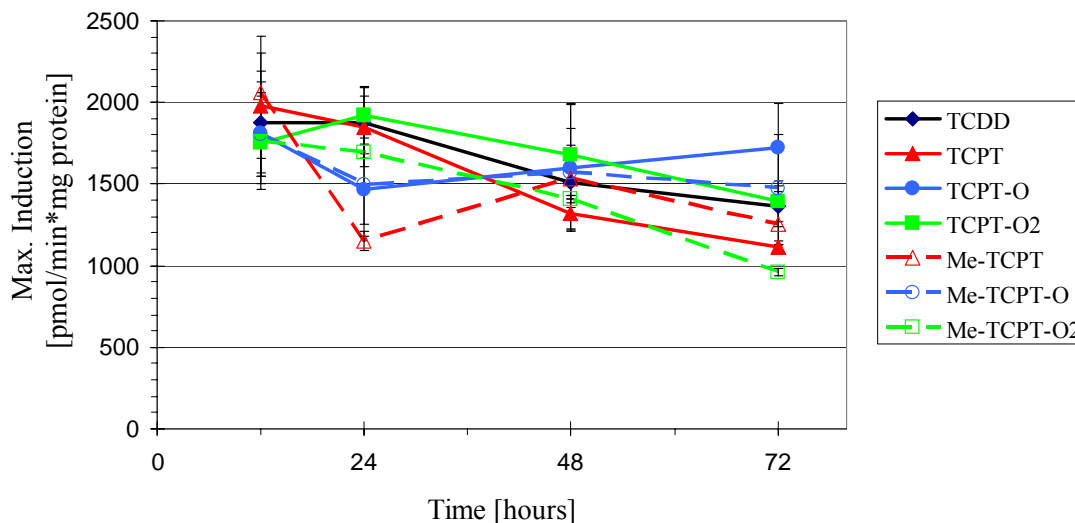
Figure 3-11: Efficacy of TCDD, TCPT, and its sulfoxo- and N-methyl derivatives regarding EROD induction after incubation for 12 to 72 h.

Table 3-3: Normalized efficacy of TCDD, TCPT, and its sulfoxo- and N-methyl derivatives regarding EROD induction after incubation for 12-72 h.

	12 h	24 h	48 h	72 h
TCDD	1.00	1.00	1.00	1.00
TCPT	1.05	0.99	0.88	0.82
TCPT-O	0.97	0.78	1.06	1.26
TCPT-O_2	0.93	1.03	1.11	1.02
Me-TCPT	1.10	0.61	1.02	0.92
Me-TCPT-O	0.96	0.80	1.04	1.08
Me-TCPT-O_2	0.94	0.90	0.93	0.70
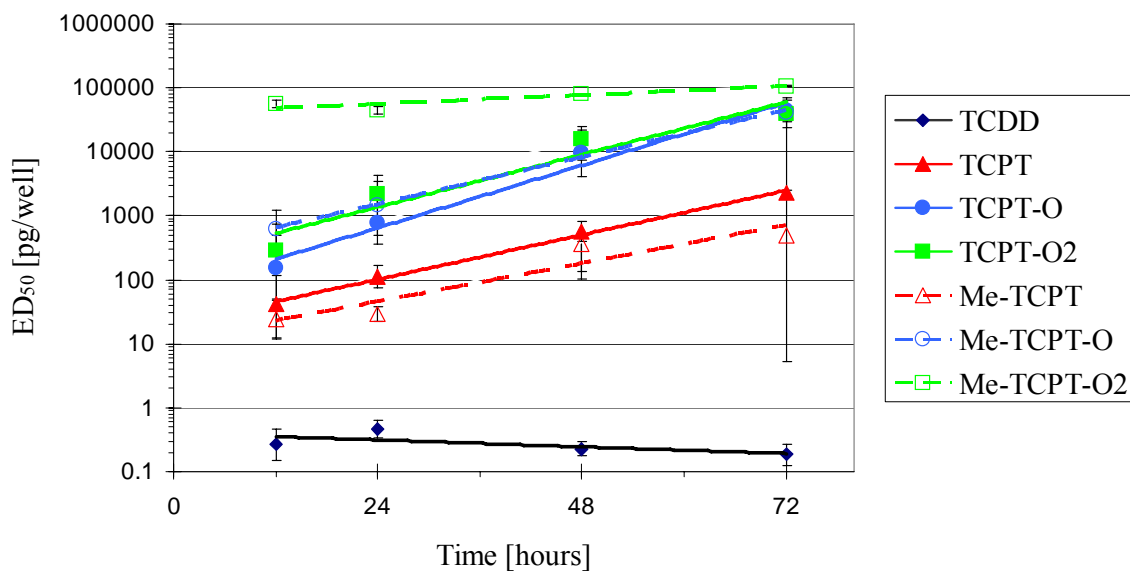
Figure 3-12: Exponential regression of ED_{50} values of TCDD, TCPT, and its sulfoxo- and N-methyl derivatives regarding EROD induction after incubation for 12 to 72 h.
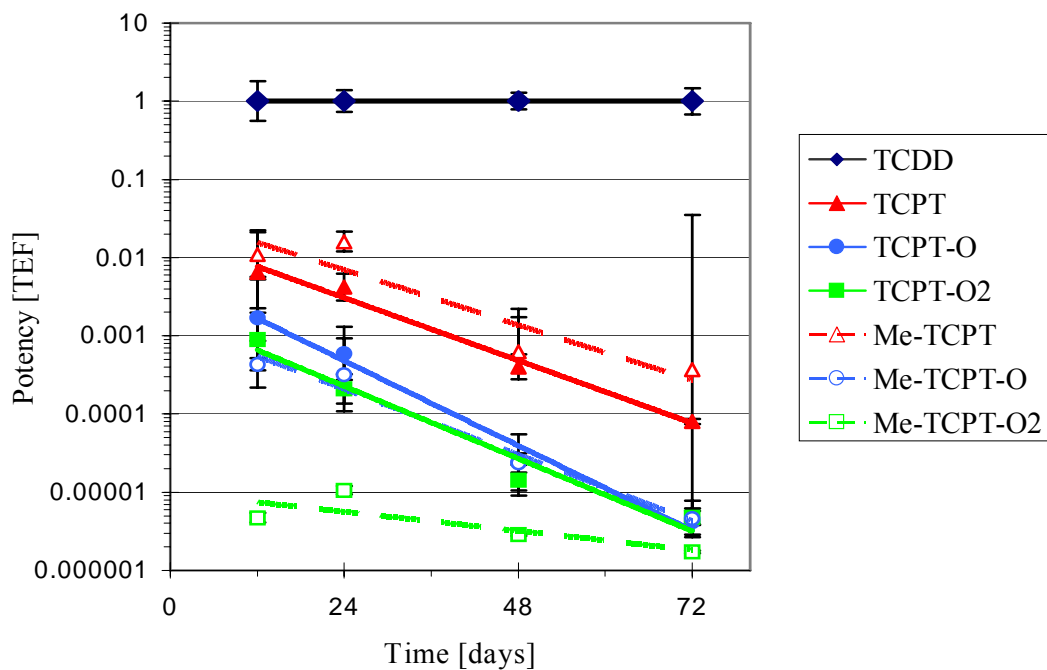
Figure 3-13: Exponential regression of normalized apparent potency of TCDD, TCPT, and its sulfoxo- and N-methyl derivatives regarding EROD induction after incubation for 12 to 72 h.

Table 3-4: Normalized apparent potency of TCDD, TCPT, and its sulfoxo- and N-methyl derivatives regarding EROD induction after incubation for 12 to 72 h. Half-lives of shifting apparent potencies were calculated from original data (ED$_{50}$s) and were not normalized.

	12 h	24 h	48 h	72 h	$t_{1/2}$ (95% confidence interval)
TCDD	1	1	1	1	68.0 h (54.2 - 92.4)
TCPT	1/155	1/238	1/2,495	1/12,390	- 10.4 h [(- 8.5) – (- 13.5)]
TCPT-O	1/583	1/1,679	1/41,569	1/238,615	- 7.4 h [(- 6.6) – (- 8.4)]
TCPT-O$_2$	1/1,109	1/4,740	1/70,232	1/211,357	- 8.8 h [(- 7.8) – (- 10.0)]
Me-TCPT	1/92	1/62	1/1,599	1/2,718	- 12.1 h [(- 5.9) – 45.3]
Me-TCPT-O	1/2,325	1/3,139	1/42,306	1/218,821	-9.9 h [(- 9.0) – (- 10.9)]
Me-TCPT-O$_2$	1/212,647	1/94,484	1/347,961	1/580,855	-52.1 h [(- 45.3) – (- 61.3)]

3.4.4 Comparison of AhR Affinity and Potency Regarding Induction of EROD Activity

The affinity of ligands to the AhR was compared to the potency of these ligands to induce EROD activity at the earliest time point examined (12 h). As shown in Figure 3-14, an increased degree of oxidation lowered affinity of the respective compounds to the AhR and also decreased apparent potency in the *in vitro* EROD assay. Both effects change in an almost parallel fashion, though separated by 1 to 2 orders of magnitude (Table 3-5, Figure 3-14).
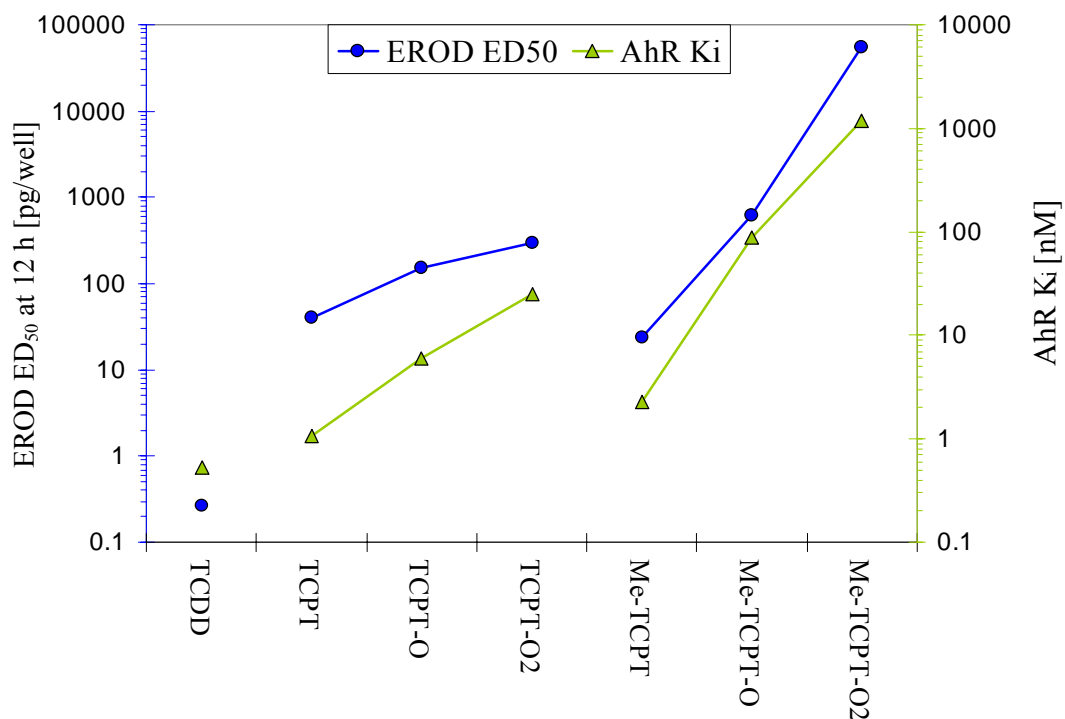
Figure 3-14: Comparison of the potency of TCDD, TCPT, and its sulfoxo- and N-methyl derivatives regarding EROD induction after 12 h of incubation (left axis, blue graph) and AhR affinity (right axis, green graph).

Table 3-5: Comparison of potencies regarding the induction of EROD activity after 12 h of incubation and affinities to the AhR, normalized to TCDD.

Compound	Normalized Potency (EROD)/ Normalized Affinity (AhR)
TCDD	1.0
TCPT	77.0
TCPT-O	52.0
TCPT-O$_2$	23.5
Me-TCPT	21.9
Me-TCPT-O	14.1
Me-TCPT-O$_2$	94.8

3.5   Discussion

Six phenothiazine derivatives were investigated for their stereochemistry, their interaction with the AhR, and a resulting downstream effect compared to TCDD. Previously published studies on CPZ demonstrated a low potency in inducing EROD activity compared to TCDD, suggesting that phenothiazine derivatives are probably weak AhR ligands (Rozman *et al.*, 1993).

The size of the AhR recognition site initially postulated by Poland (Poland and Knutson, 1982) was only specified for the two dimensions of the plane created by four chlorine substituents (10 x 3 Å). Semi-empirical calculations demonstrated that all of the derivatives fit not only these steric requirements (Table 3-1) but also the three-dimensional requirements later identified by molecular field analysis through the evaluation of a larger selection of ligands *in silico* (14 x 12 x 5 Å). Even in the case of full periplanar extension of a methyl group and/or oxygen(s), none of the TCPT derivatives exceeded the latter specifications. This analysis was based on the energetically preferred conformation of each compound.

The phenothiazine backbone, however, has been reported to be flexible in solution, bending along the N-S axis (Leonard and Sutton, 1948). Furthermore, the introduction of functional groups capable of forming hydrogen bridges, such as sulfoxides and sulfones, can influence greatly the stereochemistry and binding affinity of these molecules.

Therefore, although the energetically optimized conformation would fit into the recognition site of the AhR, not every possible transitional conformation, much less with hydration, necessarily meets these requirements. This applies particularly to compounds with two or more substituents that can extend in different directions and can influence electronic interactions with solvent and target. For this reason alone, the residence time on the receptor decreases with increasing degree of oxidation, even in the absence of significant metabolism (see Table 3-2 to Table 3-4 for comparison).

As a result of this, the binding affinity of oxidized phenothiazine derivatives was lower than that of the parent compounds (Table 3-2). Contrary to expectations, TCPT and Me-TCPT both had very high affinity to the AhR (K_i = 1.08 and 2.25 nM, or 0.36 and 0.79 ng/ml, respectively) compared to TCDD (K_i = 0.54 nM, or 0.17 ng/ml). Their respective sulfoxides and sulfones exhibited progressively reduced binding affinity with K_i values of 6.01 (= 2.12 ng/ml) and 25.29 nM (= 9.33 ng/ml) (TCPT-O and TCPT-O_2), and 88.31 nM (= 32.42 ng/ml) and 1202 nM (= 460.4 ng/ml) (Me-TCPT-O and Me-TCPT-O_2). The largest molecule, Me-TCPT-O_2, demonstrated the lowest affinity of all compounds examined, suggesting flexibility beyond the calculated stereochemistry with or without hydration, thereby limiting the fit of the compound and thus the residence time at the AhR binding site.

All compounds induced EROD activity with high efficacy at all time points. However, a shift of dose response curves to higher doses at later time points was observed for all compounds except TCDD and Me-TCPT-O_2. Enzyme induction seen

12 h after application of low doses ($< ED_{50}$) of test compounds was almost completely reversed by 72 h. The activity induced by high doses was also decreased after 72 h; even though higher concentrations caused sustained induction, leading to the observed shift of each curve. As a consequence, these compounds displayed an apparent decrease in potency over time (Figure 3-5 to Figure 3-9). However, potency is a constant and does not vary with time. Instead, the shift of ED_{50}s to higher values with increasing time indicated elimination of the compound by metabolism. The assumption of rapid metabolism is supported by a short TCPT half-life of 5.4 h in the rat *in vivo* (Fried *et al.*, 2007).

TCPT and derivatives contain two functional groups that can be metabolized: An amine and a thioether (Figure 3-15). Their aryl positions are sterically hindered by chloro-substituents and, thus, not readily hydroxylated. Flavin monooxygenases, as well as CYP3A4 enzymes, have been reported to catalyze the oxidation of secondary amines to hydroxylamines and the oxidation of thioethers to sulfoxides and sulfones in phenothiazine drugs (Goodwin, 1976; Parkinson, 2001). The predominant conjugation (and elimination) step for hydroxylamines is glucuronidation because of the high capacity of this phase II pathway.
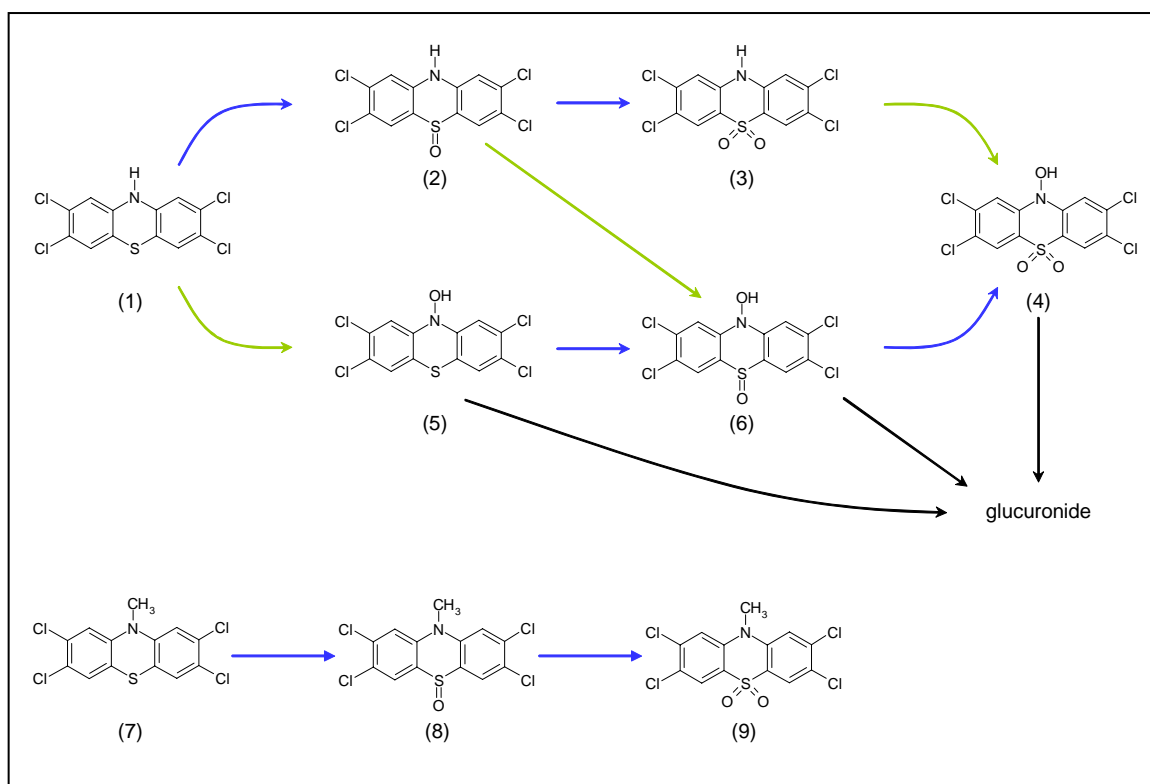
Figure 3-15: Putative biotransformation of TCPT and its derivatives. The parent compound (1) is expected to undergo combined sulfoxidation (blue arrows) and N-hydroxylation (green arrows), ultimately leading to the glucuronide. In contrast, TCPT-O₂ (3) and Me-TCPT (7) are only subject to either pathway, with Me-TCPT-O₂ being the ultimate metabolite of Me-TCPT.

Each of the two metabolizable centers was selectively blocked in individual derivatives to distinguish the influence of each site on overall metabolism of TCPT. Sulfoxidation completed metabolism on the sulfur (TCPT-O_2), allowing only hydroxylation of the amine. The formation of the hydroxylamine, in turn, was blocked by methylation in Me-TCPT. Both sites are theoretically targets of a wide spectrum of metabolic reactions, such as sulfoxide reduction by thioredoxin-dependent enzymes, reductive dehalogenation by P450 enzymes, or oxidation of

tertiary amines by flavin monooxygenases (leading to N-oxides) and P450 enzymes (resulting in N-dealkylation). These reactions apparently do not occur as readily as oxidation of a thioether or hydroxylation of a secondary amine. This is evident because there was not much change in EROD induction and presumably in AhR binding affinity over a 72 h incubation period with Me-TCPT-O_2.

Me-TCPT is a suitable compound to study the role of sulfoxidation on changing induction potency. The apparent potency of Me-TCPT decreased over time and was 2,718-times less than that of TCDD after 72 h (Table 3-4). The expected metabolite, Me-TCPT-O, displayed almost the same potency (2,325-times less) after 12 h, suggesting that Me-TCPT was probably completely transformed to Me-TCPT-O within 72 h. The apparent potency of Me-TCPT-O, in turn, decreased to ~219,000-times less than that of TCDD within 72 h, which corresponded to the potency of its expected metabolite, Me-TCPT-O_2 (~213,000-times less after 12 h), suggesting that Me-TCPT-O was converted to Me-TCPT-O_2 during 72 h. This metabolite displayed almost constant potency (variability about a factor of 2) across all time points, supporting the assumption that not much further biotransformation occurred on the N-methyl group. This series of compounds provides indirect evidence for the progression of sulfoxidation of tetrachlorinated phenothiazines, and a lack of reactivity of the tertiary amine in a rat hepatoma cell line.

The sulfoxidized derivative of TCPT (TCPT-O_2) provides evidence for the effects of metabolism on the second metabolizable center, the secondary amine. The

induction of EROD activity by TCPT-O$_2$ decreased from $\sim^1/_{1,100}$-times that of TCDD

after 12 h, to $\sim^1/_{211,000}$-times after 72 h, which is almost the same as for Me-TCPT-O$_2$,

indicating that the hydroxylamine of TCPT-O$_2$ had about the same potency as the

N-methylated sulfone. This inference is supported by the metabolism of TCPT-O,

which at the end of the incubation period (72 h) had an apparent potency of

$\sim^1/_{239,000}$-times that of TCDD.

All derivatives except Me-TCPT-O$_2$ displayed comparable half-lives

(9.7 ± 0.79 h) for their shift in apparent potency (Table 3-4), which leads to three

conclusions: First, rates of sulfoxidation and formation of the hydroxylamine do not

differ sufficiently to result in differential declines in EROD induction over time.

Second, TCPT with both centers available for metabolism was not biotransformed

much faster than when only one center was available, suggesting that the velocity of

the two reactions (sulfoxidation and N-hydroxylation) is similar. Third, the half-lives

for the dose-response-shifts of all inductions (9.7 ± 0.79 h except for Me-TCPT-O$_2$)

were lower than those of inducible proteins. The half-lives of P450 enzymes have

been reported to be 12-16 h in rats (Shiraki and Guengerich, 1984). Kinetic

calculations on the half-lives of proteins show that, upon removal of enzyme inducers

by biotransformation, over 90% of enzyme activity is abolished within 2-3 days, as

was observed here. The kinetic half-lives of the tested compounds (except

Me-TCPT-O$_2$) were considerably shorter than the reversibility half-life of the

induction. Therefore, dynamics of the effect, and not kinetics of the compounds

provide the rate-determining step in the observed shifts of EROD activities.

The sustained induction of EROD activities at high doses indicated a requirement for the continuous presence of the respective inducer, suggesting saturation of the metabolic capacity of this *in vitro* system. This resulted in high efficacy of the induction for all compounds. In essence, two processes seemed to occur simultaneously: Compounds induced enzyme activity, at the same time, they also were metabolized.

Considering that induction after 12 h exposure was at or near maximum, yet metabolism of the compounds affected the original concentration (dose) to the least extent, this time point was used for comparison of all compounds with regard to AhR binding affinity. There was an almost parallel trend in the effects of derivatization on AhR binding and enzyme induction. All compounds were much less potent in terms of enzyme induction than in terms of AhR binding (Table 3-5). The potency of TCPT to induce *in vitro* EROD activity was about 155-times less than that of TCDD after 12 h (Table 3-4), whereas the affinity of TCPT to the AhR was only about a factor of two lower (Table 3-2). The only other compound known to have such a discrepancy between EROD induction and AhR activity is methylcholanthrene (Riddick *et al.*, 1994). The reason for this discrepancy between K_i and induction of P450 has not been satisfactorily explained. However, it suggests an intricate regulatory system beyond simple receptor-binding.

One variable that must be considered in this comparison is the use of hepatic cytosol from Sprague Dawley rats for the binding assay but the use of a hepatoma cell

line from an AXC rat strain (Reuber, 1961) for the study of enzyme induction. However, the affinity of the AhR to TCDD is similar across species (Poland and Knutson, 1982). Furthermore, rat strains, even those displaying differential lethality (Pohjanvirta *et al*., 1999), showed no difference regarding the induction of enzyme activity by TCDD (Pohjanvirta *et al*., 1990). Therefore, other steps within the AhR signaling cascade are assumed to become rate-limiting, e.g. the effects of ligands on binding to the AhR/ARNT or binding of the heterotrimer to DNA. Thus, binding to the AhR might be the first and most sensitive step in the interaction of AhR ligands with an organism. But there are certainly other steps as well that contribute to their differential effect profile.

3.6   Conclusions

TCPT is a potent AhR ligand in the liver. Its activity regarding downstream enzyme induction, however, is over two orders of magnitude lower than that of TCDD. Evidently, EROD induction is more complex than simple binding to the AhR and downstream effects. Putative metabolites of TCPT are less avid ligands for the AhR and also less potent inducers of EROD activity. A decline of enzyme activity occurred over time, indicating rapid metabolism of all but one derivative (Me-TCPT-O$_2$) to progressively less active metabolites. Based on these findings, TCPT might be viewed as a partial AhR agonist, suggesting possible applications as a competitive TCDD antagonist.

CHAPTER 4

2,3,7,8-TETRACHLOROPHENOTHIAZINE LOWERS IGF-1 SERUM LEVELS IN RATS DOSE-DEPENDENTLY WITHOUT AFFECTING TOTAL THYROXINE LEVELS

4.1   Abstract

2,3,7,8-Tetrachlorophenothiazine was studied for its effects on serum IGF-1 levels in rats as part of its *in vivo* characterization as a potential drug lead. Twice daily treatment with *i.v.* TCPT significantly reduced IGF-1 levels within two days, and IGF-1 levels were maximally reduced within 4 days at a dose rate of 5 mg/kg/12 h TCPT, whereas lower doses exhibited a delayed time-response. An ED_{50} of 0.82 mg/kg/12 h TCPT ($R^2 = 0.98$) was calculated for day 8 when serum IGF-1 levels decreased to 54% of controls.

Steady-state exposure to sub-lethal doses of dioxins has previously been reported to significantly reduce serum IGF-1 levels in rats within 8 days, with a maximum reduction between 16-32 days. This effect was further analyzed in a dose-response study. Treatment with TCDD reduced IGF-1 levels to a minimum of 35% of controls by day 21. An ED_{50} (loading dose rate) of 1.1 µg/kg ($R^2 = 0.97$) was calculated.

Both dose-response studies were designed to yield rapid steady state concentrations, allowing a direct comparison of doses and effects. Calculations of average blood concentrations of each compound at the ED_{50} yielded 213 µg/L for TCPT and 17 ng/L for TCDD, representing an approximately 12,500-fold difference in potency regarding the reduction of serum IGF-1 levels in the rat. Repeated dose rates of TCPT and TCDD maximally reduced serum IGF-1 levels by 46 and 65%

compared to controls, respectively, indicating that the efficacy of TCPT was 71 % of that of TCDD.

While TCPT altered IGF-1 levels in serum, total thyroxine (TT_4) levels remained at control levels throughout the studied time-period even at the highest examined dose of 5 mg/kg/12h TCPT *i.v.*

The potency of TCPT regarding the IGF-1 effect is orders of magnitude lower than that of TCDD, whereas the difference in efficacy is less than two-fold. These results indicate that TCPT exerts some dioxin-like effects, albeit at much higher doses. The reduction of serum IGF-1 levels without affecting TT_4 suggests that TCPT could be used as a drug lead for applications in obesity treatment, contraception, and longevity.

4.2   Introduction

The phenothiazine structure is considered a traditional and versatile pharmacophore in medicinal chemistry. The most prominent member of the family, CPZ, entered the market in 1951 as the first widely-used neuroleptic drug (Shen, 1999). In addition to the use of different derivatives as anti-psychotics, numerous phenothiazines have since been applied as sedatives, antihistamines, anthelmintics, and anti-emetics (Hardman *et al.*, 2001). In addition, more recent studies focused on possible applications as anti-viral drugs (Bishop, 1998; Kristiansen and Hansen, 2000), anti-oxidants (Dalla Libera *et al.*, 1998), inhibitors of osteoclastic bone

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resorption (Hall *et al.*, 1996), in cancer treatment (Jones, 1996), and as prophylactic

antibacterials (Komatsu *et al.*, 1997). The effects of phenothiazine drugs have almost

exclusively been attributed to their different amine-substituents, whereas only one

publication has indicated the importance of the chloro aryl-substituent for the efficacy

of CPZ (Jovanovic and Biehl, 1987).

Dibenzo-*p*-dioxins are strikingly similar to the phenothiazine pharmacophore

regarding their chemical structure (Fried *et al.*, 2007). Both classes of compounds are

tricyclic with a heterocyclic central ring. Although oxygen atoms are the central

heteroatoms in the dioxin structure, a secondary amine and a thioether are substituted

in phenothiazines (Figure 4-1). The most biologically active dioxin congeners are

chlorinated at least in the 2,3,7,8-positions, of these the tetrachlorodibenzo-*p*-dioxin

possesses the highest potency.
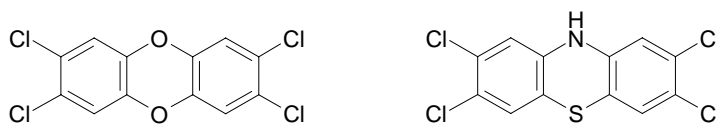
Figure 4-1: Structures of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (left) and its analogue 2,3,7,8-tetrachlorophenothiazine (TCPT) (right).

Dioxins are persistent environmental pollutants that have been well

characterized during the past 30 years. They exert potentially beneficial effects at

medium-to-low doses in addition to their well-described deleterious high-dose effects.

Their effects at high doses (>15 μg/kg TCDD) include a wasting syndrome (Harris *et*

al., 1973; Seefeld *et al*., 1984), suppression of the immune system (Sharma *et al*., 1978; Fan *et al*., 1996), and carcinogenicity (lungs, liver) (Kociba *et al*., 1978). Medium dose effects (1-15 µg/kg TCDD) include increased liver weight, stimulation of the immune system (Fan *et al*., 1996), reproductive effects (Peterson *et al*., 1993; Li *et al*., 1995), and a reduction of the set-point for body weight (Seefeld *et al*., 1984). Amongst the low dose effects (<1 µg/kg TCDD) are alterations in thyroid hormone homeostasis (Potter *et al*., 1983; Rozman *et al*., 1984), thymic atrophy (Gupta *et al*., 1973), and enzyme induction (Poland and Glover, 1978). It has been reported that dioxins reduce serum IGF-1 levels (Croutch *et al*., 2005) at medium doses. The IGF-1 signaling pathway is known to influence aging, reproduction, and body mass (Arking, 2003). Because TCDD has been reported to reduce serum IGF-1 levels at a dose range where longevity (Kociba *et al*., 1978; Rozman *et al*., 2005), inhibition of ovulation (Li *et al*., 1995; Li *et al*., 1995), and sustained body weight reduction (Peterson *et al*., 1984; Seefeld *et al*., 1984) were also observed, IGF-1 is assumed to be a key mediator of these effects exerted by dioxins.

The pharmacophore phenothiazine structurally resembles the dioxin backbone. As compared to dioxins, its derivatives possess short half lives combined with reduced potency. TCPT was developed as a crossover between these two structures to combine medicinally favorable kinetics with potentially beneficial lower-dose effects of dioxins. Therefore, its effects on the key biomarker IGF-1 were investigated to further develop this drug lead.

4.3   Materials & Methods

Animals: Female Sprague Dawley rats weighing 225-250 g were obtained from Harlan, Indianapolis, IN, for the study of TCPT. Surgical services by the supplier were employed to obtain animals cannulated in the *vena jugularis*. Animals were housed in wire-bottom cages at a 12/12 h light/dark cycle, 22 °C ambient temperature and a relative humidity of 40-60 %. All rats had *ad libitum* access to water and 8604 Rodent Diet supplied by Harlan Teklad, Madison, WI. The research presented was performed in compliance with NIH Guidance for the Care and Use of Animals and was approved by the KUMC Institutional Animal Care and Use Committee. **TCDD study:** Groups of 5 rats each were dosed *per os* with loading dose rates (LDRs) of 1.6 and 6.4 µg/kg TCDD followed by maintenance dose rates (MDRs) (one tenth of the respective loading dose rate) in 3-day intervals. Controls received vehicle only (4 ml/kg corn oil). Trunk blood was collected after 21 days. Data for 0.0125, 0.05, 0.2, 0.8, and 3.2 µg/kg LDR groups were obtained from a published study of identical design (Croutch *et al*., 2005). **TCPT study:** Groups of rats with cannulated jugular vein received a solution of 0.1 (one rat), 1 (4 rats), 2.5 (4 rats) and 5 mg/kg/12h (7 rats) TCPT *i.v.* every 12 hours for 8 days. Controls received vehicle only (0.5 ml/kg dimethylsulfoxide [DMSO]). Blood samples of 0.5 ml were drawn daily from controls and from lower-dose groups, and on days 4 and 8 from the high-dose group. The volume withdrawn was substituted with saline. Animals of the lower-dose groups were euthanized on day 8. For animals of the high-dose group, dosing was continued in 24 h intervals for another three days. Due to gradually

developing occlusion of the catheters, scheduled blood withdrawals were limited as

shown in Table 4-1.

Table 4-1. Number of animals from which blood was drawn in each dose group per day. Shaded fields: recovery period with increased dosing interval.

Day	Dose Rates [mg/kg/12 h (24 h)]					
	Controls	0.1	1	2.5	5	Controls
0	5	1	4	4	7	7
1	5	1	4	4	NA	NA
2	5	1	4	4	NA	NA
3	5	1	3	4	NA	NA
4	4	1	2	3	3	7
5	4	1	2	3	NA	NA
6	4	1	1	2	NA	NA
7	4	1	1	1	NA	NA
8	4	1	3	4	3	7
9	NA	NA	NA	NA	1	1
10	NA	NA	NA	NA	1	1
11	NA	NA	NA	NA	2	3

Analytics: Trunk blood and blood withdrawn from the jugular vein were

stored on ice until centrifuged at 4 ºC for 15 min. After sedimentation at 9,000 g,

serum aliquots were stored at -80 ºC. Serum IGF-1 and TT_4 levels were measured

using radio immuno assays (RIA) obtained from Diagnostic Systems Laboratories,

Webster, TX and Diagnostic Products Corp., Los Angeles, CA, respectively. Sample

preparation and analyses were conducted according to manufacturers' instructions.

Data Processing: Individual dose-response curves and respective parameters

were calculated using the program Sigma Plot 8.0 from SPSS Inc., Chicago, IL.

Steady-state concentrations were calculated using Equation 4-1 (Gibaldi and Perrier,

1982). Half-lives of 5.4 h for TCPT (Fried *et al*., 2007) and 20 days for rats (Geyer *et*

al., 2002) were used in the calculations.

$$c_{ss} = \frac{1.44 \cdot t_{1/2} \cdot f \cdot dose\ rate}{V_d \cdot \tau}$$

c_{ss} = steady-state concentration
$t_{1/2}$ = elimination half-life
f = fraction absorbed
V_d = volume of distribution
τ = dosing interval

Equation 4-1: Equation for the calculation of normalized steady-state
concentrations, allowing the comparison of different study designs for TCDD and
TCPT.

Statistical Analysis: Data are presented as average; error bars represent the

standard error of the mean. All data were analyzed using GraphPad InStat 3.06, San

Diego, CA. Statistics were conducted by an unpaired t-test, assuming Gaussian

distribution of samples in populations with equal SD. A p-value of ≤ 0.05 was set to

indicate a statistically significant difference in data, depicted by (*) in graphs.

4.4   Results

Published reports on homeostatic serum IGF-1 levels in rats vary from 431 ±
32 ng/ml (Perrien *et al*., 2007) to 2181 ± 118 ng/ml (Han *et al*., 2006). Croutch *et al.*
(2005) reported control levels of 1025 ± 75 ng/ml IGF-1 in serum. In contrast, control
levels for the studies presented herein were measured at 1410 ± 47 ng/ml (TCDD
study), 1277 ± 62 ng/ml (controls for low-dose TCPT study), and 1376 ± 108 ng/ml
(controls for high-dose TCPT study). Therefore, serum IGF-1 levels had to be
normalized to controls to allow the integration of data for quantitative dose-response
analysis.

4.4.1   Effects of TCPT on Body Weight and Feed Intake

Body weight and feed intake were normalized to allow the statistical
comparison of dose groups. Absolute body weights varied because studies were
staggered.

A decreased gain in body weight was observed in rats of the two highest
treatment groups, beginning on day 4 (Figure 4-2). This stagnation of body weight
was later reversed in animals receiving 2.5 mg/kg/12 h TCPT. Body weight of these
rats and of those in the highest dosage group approached control levels by day 7.
Decreased gain in body weight was preceded by decreased feed intake (Figure 4-3).
Feed intake fluctuated but was consistently reduced at the mid-point of the study.
Feed intake returned to control levels by day 7 in all dosage groups.

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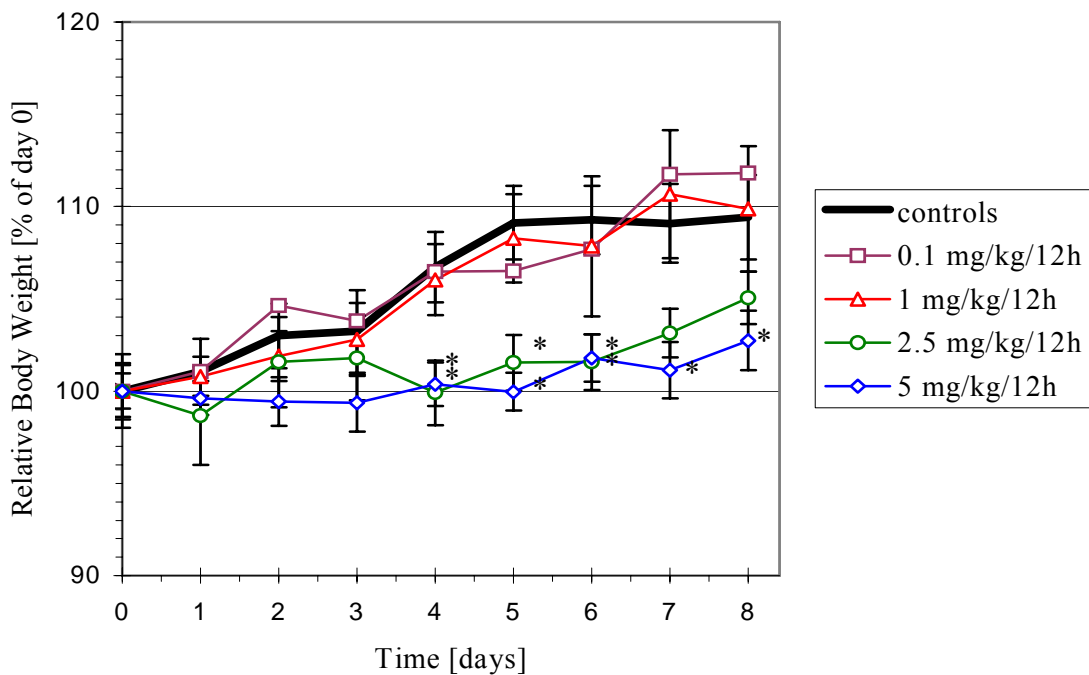
Figure 4-2: Relative body weight of rats treated with TCPT, normalized to their initial body weight on day 0. (*) = different from controls.
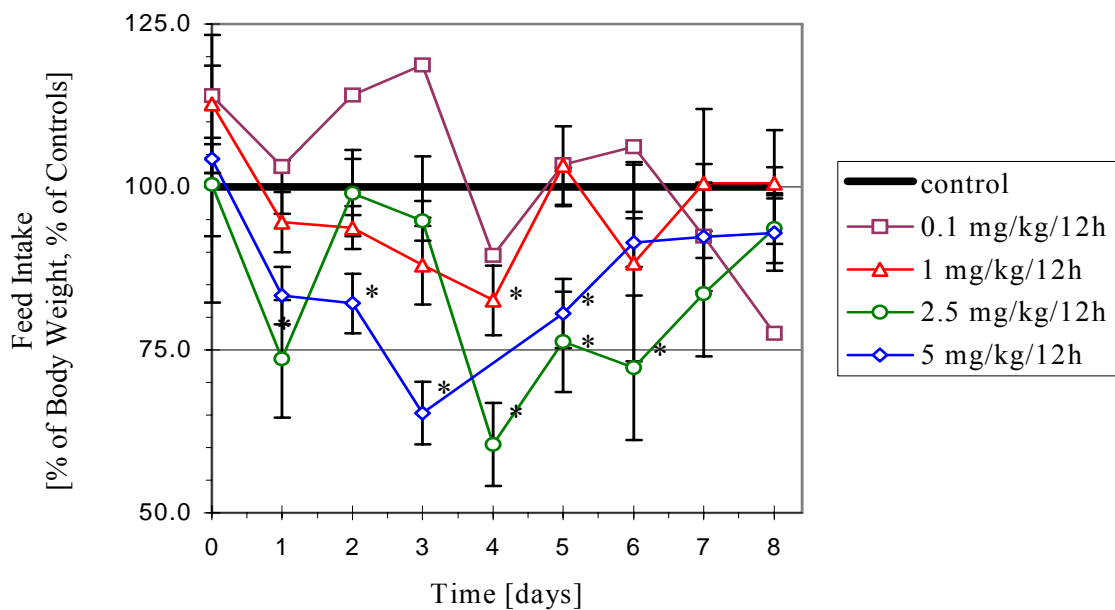
Figure 4-3: Relative feed intake of rats treated with TCPT. Averages were calculated in % of body weight and then normalized to controls. (*) = different from controls.

4.4.2 Effects of TCPT and TCDD on TT_4

Hypothyroidism is an often observed side-effect of phenothiazine drugs (Sauvage *et al.*, 1998). However, none of the dose rates of TCPT affected serum TT_4 levels (Figure 4-4) at any time point. Even at the highest dose rate of 5 mg/kg/12 h no deviation from control levels was seen. In contrast, TCDD was reported to lower serum TT_4 levels within four days by 42 % at an LDR of 3.2 µg/kg (Croutch *et al.*, 2005).
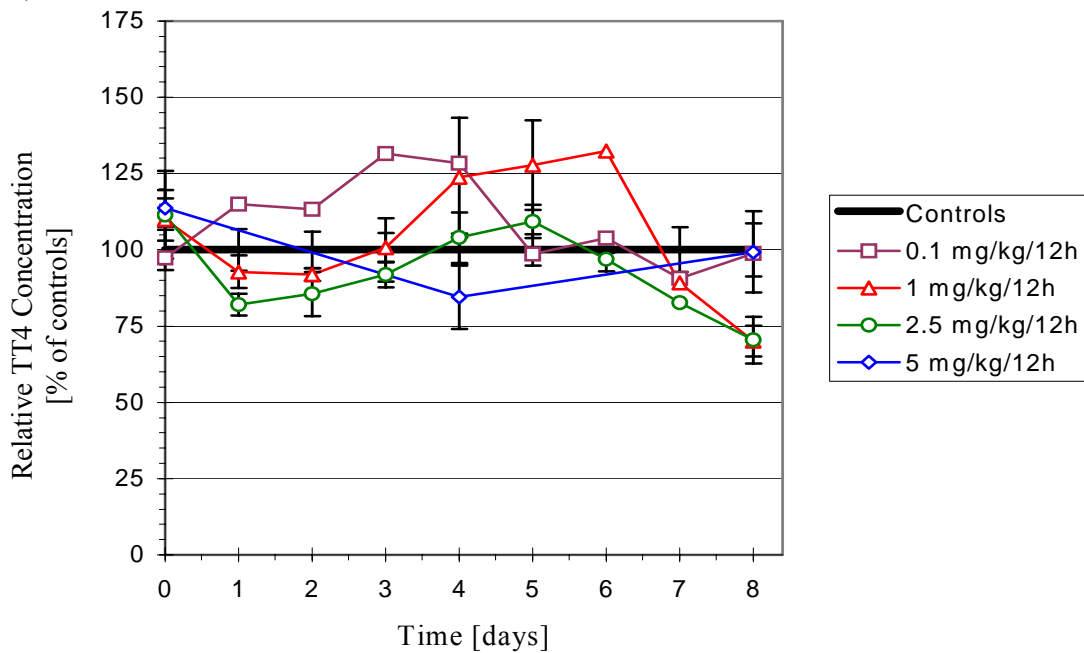
Figure 4-4: Relative total thyroxine concentrations in serum during treatment with TCPT. No treatment group differed from control levels.

4.4.3 Quantitative Dose-Response of Serum IGF-1 to TCDD

The design of this study was identical to previously published research (Croutch *et al.*, 2005) in order to combine data for the development of a dose-response curve. It has been reported that TCDD lowered serum IGF-1 levels with a lowest observable effect level (LOEL) of 3.2 µg/kg LDR of TCDD. The no observable effect level (NOEL) was reported at an LDR of 0.8 µg/kg TCDD.
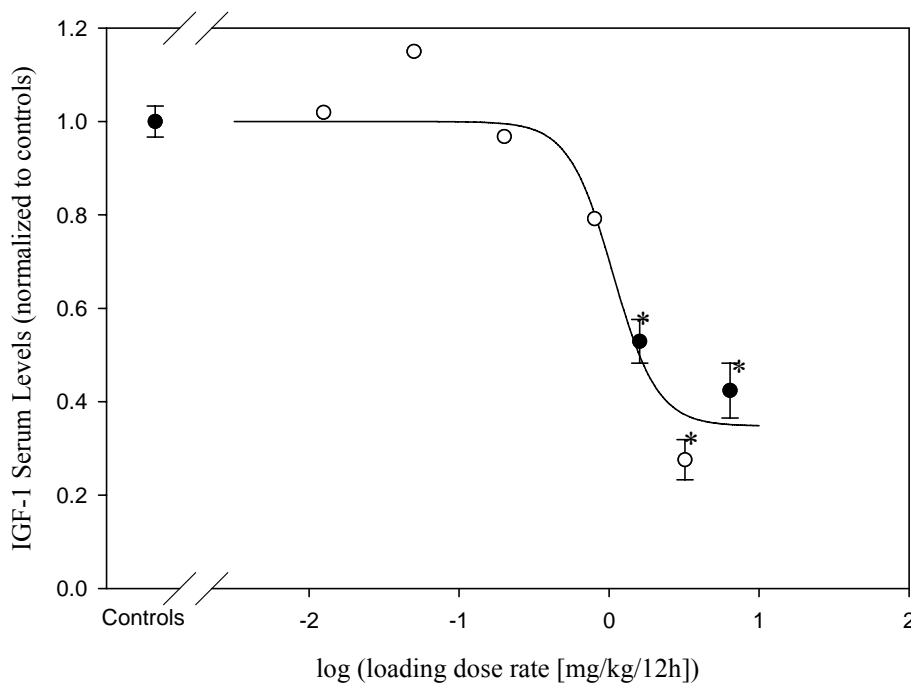
Figure 4-5: Dose-response of serum IGF-1 levels to TCDD after 21 days at steady-state concentrations. Maximum effect was 65 % lower than control levels. An ED_{50} was calculated at an LDR of 1.1 µg/kg TCDD. Graph shows data combined from a published study (○)(Croutch et al., 2005) and new data (●). R^2= 0.97. (*)= different from controls.

Plotting and analyzing all IGF-1 data revealed a calculated ED_{50} of 1.1 µg/kg LDR of TCDD for this effect (Figure 4-5). An LDR of 1.6 µg/kg TCDD resulted in significantly reduced IGF-1 levels as compared to controls. Therefore, this value is the new, experimentally determined LOEL. Maximum reduction of serum IGF-1 level was 65% lower than controls by day 21.

4.4.4 Quantitative Dose- and Time-Response of Serum IGF-1 to TCPT

TCPT lowered serum IGF-1 levels dose- and time-dependently (Figure 4-6). At a high dose rate of 5 mg/kg/12 h, serum IGF-1 levels were maximally reduced by 4 days into dosing. Dose rates of 2.5 mg/kg/12 h resulted in reduced IGF-1 serum levels by day 2 and in maximum and sustained reduction after 5 days into dosing.
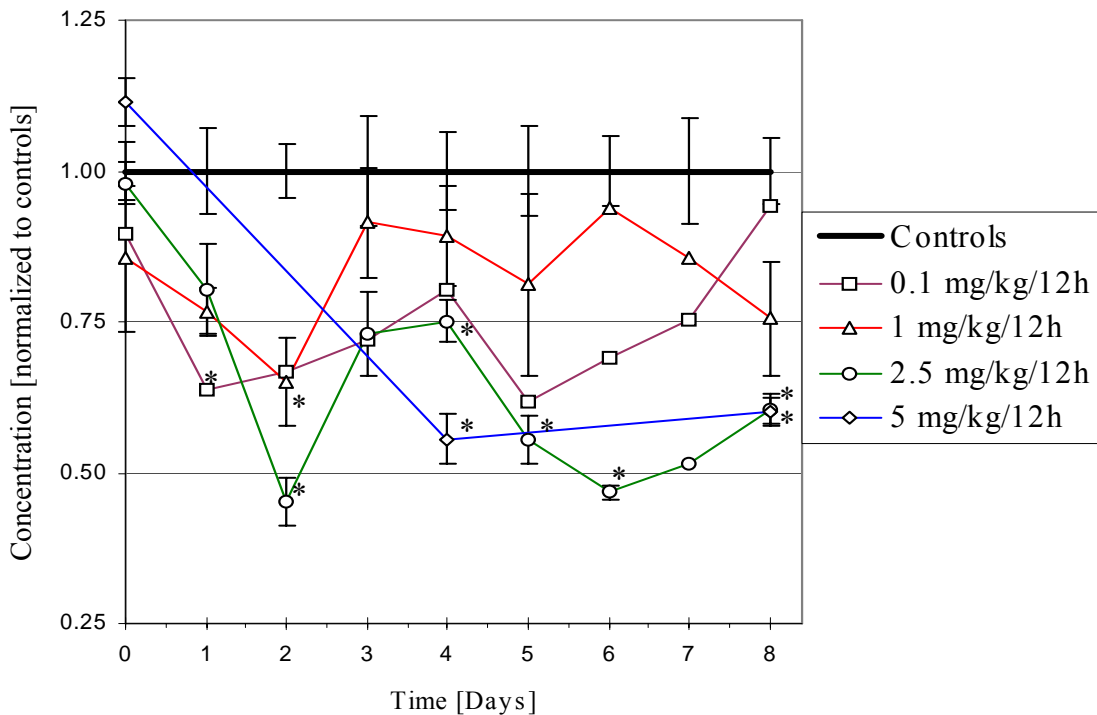
Figure 4-6: Time-course of serum IGF-1 levels in rats treated with different dose rates of TCPT. (*) = different from controls.

Lower dose rates also seemed to cause an initial reduction of serum IGF-1, but most values were not significantly different from controls. Data of day 8 analyzed by sigmoidal curve regression indicated a dose-response relationship with an ED_{50} of 0.82 mg/kg/12 h TCPT. This effect plateaued at 2.5 mg/kg/12 h with 46 % reduction compared to controls (Figure 4-7).
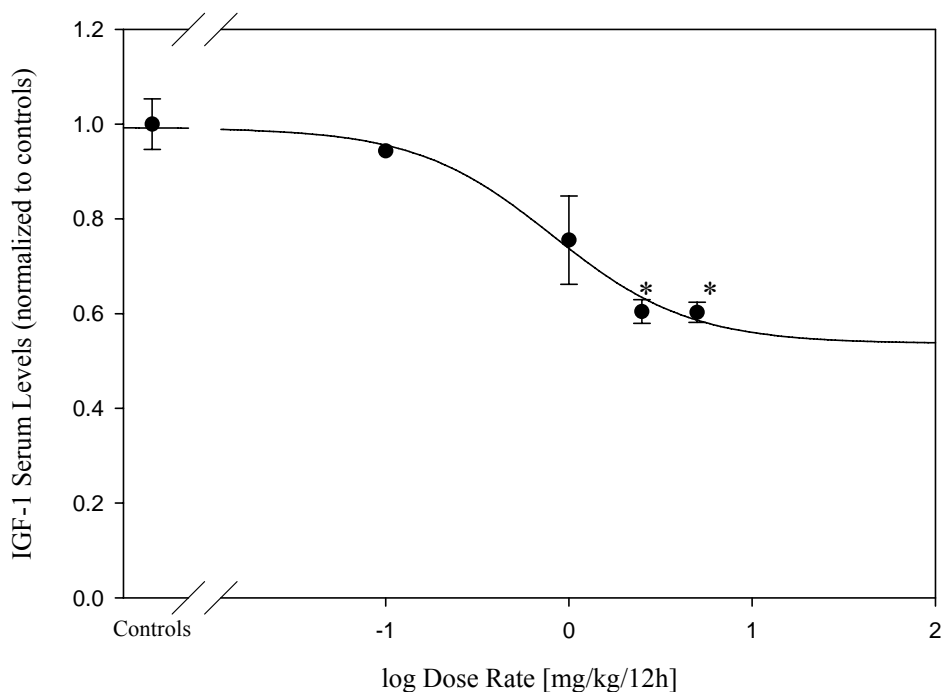
Figure 4-7: Dose-response curve of serum IGF-1 levels in rats dosed with different dose rates of TCPT every 12 h for 8 days. Maximum reduction was 46% lower than controls. An ED_{50} was calculated at 0.82 mg/kg/12 h TCPT. $R^2 = 0.98$. (*) = different from controls.

4.4.5   Comparison of the Effect of TCPT and TCDD on Lowering Serum IGF-1

In order to compare the potencies of TCPT and TCDD, average blood levels of each compound during each study and for each dose group were calculated using

Equation 4-1. Sigmoidal curve regression yielded an EC$_{50}$ of 213 µg/L in blood for TCPT and 17 ng/L for TCDD (Figure 4-8), revealing an approximately 12,500-fold difference in potency regarding the reduction of serum IGF-1 levels.

The efficacy of TCPT to reduce serum IGF-1 levels was 71% of the efficacy of TCDD. However, maximum reduction by TCPT was reached almost two weeks earlier than the effect started plateauing out for TCDD (Croutch *et al.*, 2005).
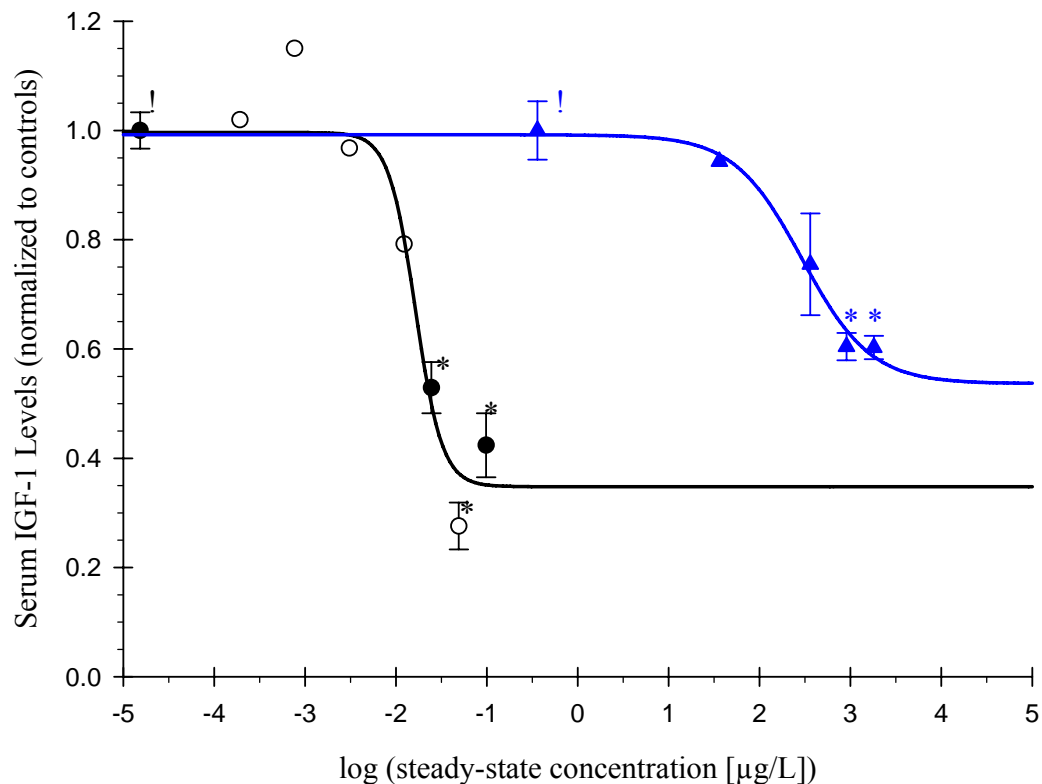
Figure 4-8: Comparison of steady-state concentrations of TCPT (blue) and TCDD (black) regarding reduction of serum IGF-1 levels. Potencies differed by a factor of 12,500, while the efficacy of TCPT was 71 % of that of TCDD. (!) = respective controls. Graph shows data combined from published studies (○)(Croutch et al., 2005) combined with new data (●, ▲). (*) = different from controls.

4.4.6 Reversibility of the Effects of TCPT After Increasing the Dosing Interval

To investigate the reversibility of the effects of TCPT, the dosing interval of the high-dose group was increased from 12 to 24 h beginning on day 8 (indicated by shaded areas in Figure 4-9 to Figure 4-11). Considering the elimination half-life of TCPT of 5.4 h, only 4.6 % of the original dose remained in the rats after 24 h as compared to 21.5 % after 12 h.

Body weight of treated rats returned to control levels by day 9, the first day of prolonged dosing interval (Figure 4-9). This development was preceded by the reversal of initially reduced feed intake (see 4.4.1).
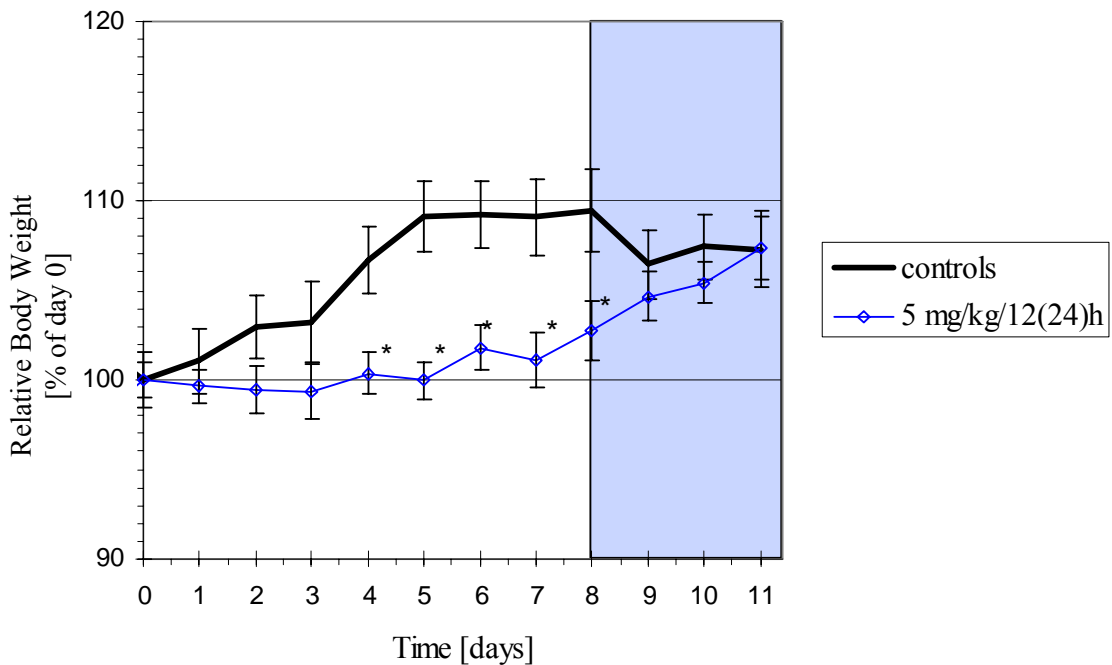
Figure 4-9: Body weight of rats treated with TCPT at steady-state (days 0-8) and during increased dosing interval leading to recovery (days 8-11, shaded area). (*) = different from controls.

Decreased body weight of the controls by day 9 was an artifact due to reduction in animal numbers and, therefore, a change in the number of rats of the control group. When the catheter of a treated rat was blocked that rat was euthanized and a control rat was sacrificed for comparison. For animal numbers, see 4.3 Materials & Methods.

Feed intake of treated animals returned to control levels by day 6 and was already indistinguishable from controls when the recovery phase of the study began (Figure 4-10). Although the change was not statistically significant, feed intake seemed to be increased the day after initiation of a prolonged dosing interval (day 9).
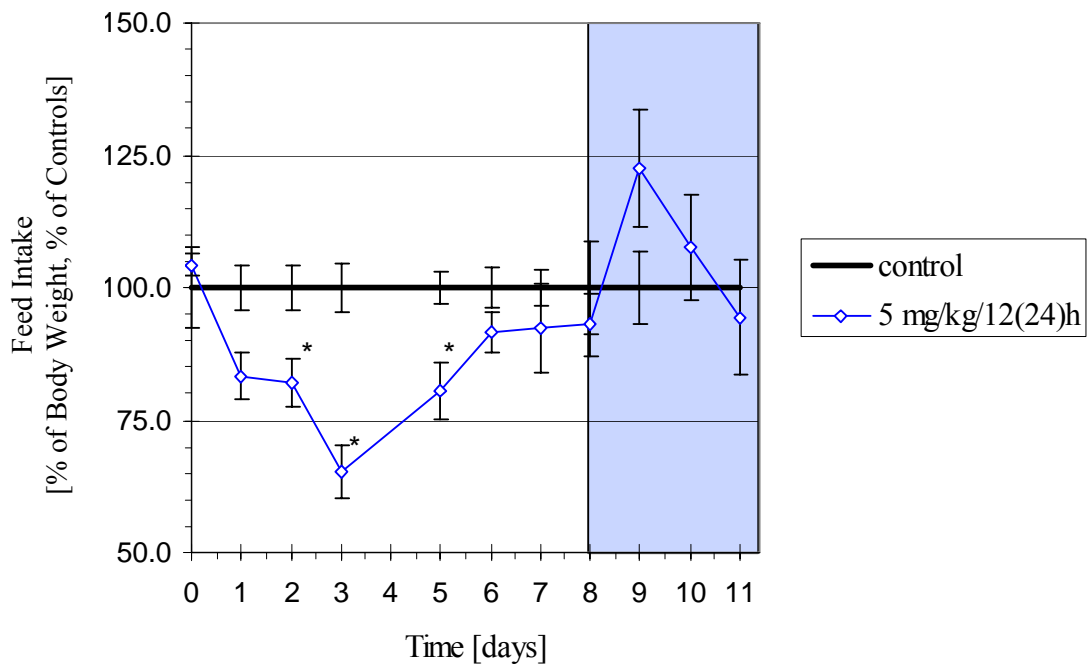
Figure 4-10: Feed Intake of rats treated with TCPT at steady-state (days 0-8) and during increased dosing interval leading to recovery (days 8-11, shaded area). (*) = different from controls.

The effect of TCPT on IGF-1 was reversed within three days of increased dosing interval (Figure 4-11).
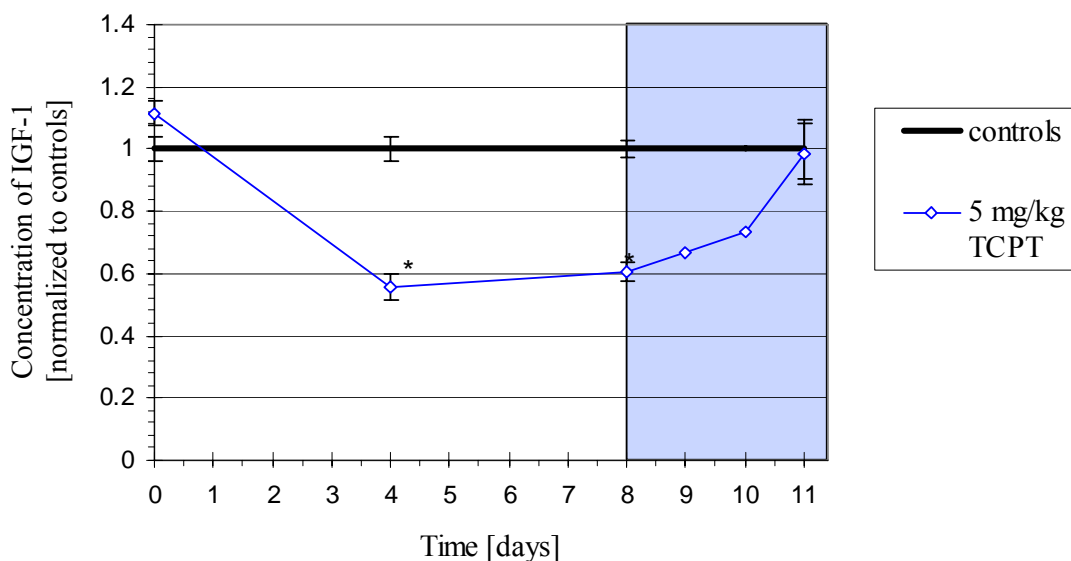
Figure 4-11: Reversibility of serum IGF-1 levels after increasing the dosing interval of TCPT from 12 h (days 0-8) to 24 h (days 8-10, shaded area). (*) = different from controls.

4.4.7   Observations

Doses of 1 mg/kg/12 h of TCPT and higher administered *i.v.* caused hematuria in several rats as early as 24 h within the first dose rate. Some controls displayed the same toxicity at later time points, an apparent vehicle effect. Other observations in this and higher dose groups included convulsion of the animals upon injection and subsequent uncoordinated movements such as stumbling. Duration of these effects extended from seconds (1 mg/kg/12 h TCPT) to minutes in the higher dose groups. Dose rates of 2.5 mg/kg/12 h and higher after two days of dosing caused aggression in some animals while being handled. The highest dose rate was lethal for two out of

seven rats. Death occurred on days 5 and 8 after profound disorientation, labored breathing, hematuria and oral discharge of blood.

4.5   Discussion

The presented subacute studies on the effects of TCPT *in vivo* represent the first reports on this compound other than acute toxicity. TCPT caused a reduction in body weight in the two higher dose groups during the second half of the study (Figure 4-2), which initially appeared to be due to reduced feed intake. However, feed intake returned to control levels within 7 days of administration of TCPT (Figure 4-3), indicating that the animals adapted to this effect of the compound. In general, adaptation is either a dynamic or kinetic process. Previously published studies identified TCPT as an inducer of CYP 1A1 enzyme activity *in vitro* (Fried *et al*., 2007). Therefore, these findings are compatible with kinetic adaptation, suggesting that TCPT induced enzyme activity also *in vivo*, leading to an increase of its own biotransformation. The reduction of body weight by TCDD is a well-characterized effect. In contrast to the reports with TCDD (Peterson *et al*., 1984; Seefeld *et al*., 1984; Seefeld *et al*., 1984), TCPT does not alter the set-point for body weight permanently.

Treatment with TCPT did not affect TT_4 levels at any dose, even at dose rates approaching lethality (Figure 4-4). In contrast, the reduction of serum T_4 levels is a hallmark of dioxin toxicity (Potter *et al*., 1986; Gorski *et al*., 1988). Within 2 - 4 days, and therefore clearly preceding effects on IGF-1 levels, serum TT_4 was dose-

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dependently reduced in rats treated with doses of 1 μg/kg of TCDD or higher (Gorski

and Rozman, 1987). TSH and TT_3 concentrations remained unaltered in rats for up to

32 days after single doses of TCDD. Dioxins have been shown to affect thyroid

hormone status in part by by tissue-specific up- and downregulation of deiodinase

activities (Raasmaja *et al.*, 1996). The effects of dioxins on serum TT_4 are of

importance regarding the presented data on IGF-1 levels because T_4 influences IGF-1

homeostasis indirectly by affecting the growth hormone (GH) axis (Miki *et al.*, 1992;

Näntö-Salonen *et al.*, 1993). It has been documented that dioxins and dioxin-like

compounds such as PCBs bind to thyroxine-specific sites in rat liver (McKinney *et

al.*, 1987). The liver is the main production site of IGF-1, although paracrine

production has also been reported for white adipose tissue, and to a lesser degree for

spleen, heart, and skeletal muscle (Gosteli-Peter *et al.*, 1994). The synthesis of IGF-1,

a 70 amino acid peptide (Rinderknecht and Humbel, 1978), is primarily stimulated by

growth hormone (GH) (Mathews *et al.*, 1986; Doglio *et al.*, 1987; Tollet *et al.*, 1990),

but it is also insulin- (Pao *et al.*, 1992) and estradiol-dependent (Ernst and Rodan,

1991). A negative feedback of IGF-1 in the pituitary regulates GH release. It has been

reported also that thyroid hormones play an important role in IGF-1 synthesis and

secretion. Not only is thyroid homeostasis essential for GH synthesis and secretion

(Peake *et al.*, 1973; Wilkins *et al.*, 1974), but thyroid hormones have also been

reported to act in combination with GH in controlling IGF-1 production and release

(Wolf *et al.*, 1989).

Despite a strong correlation between thyroid hormones and serum IGF-1 levels in dioxin toxicity, no such correlation was seen regarding the effects of TCPT. Serum IGF-1 levels decreased within days of commencing dosing with TCPT, and, thus, more rapidly (Figure 4-6) than in animals treated with TCDD, where this effect became manifested within weeks (Croutch et al., 2005). But thyroxine homeostasis remained unaffected by TCPT. Because of their structural similarity, it was reasonable to assume a related mechanism of action on IGF-1 regulation for TCPT and TCDD. In that case, however, the reduction of TT_4 and the lowering of IGF-1 after TCDD exposure must be unrelated.

The onset of maximum IGF-1 reduction by TCPT, is 2-3 times faster than by TCDD (8 days compared to 16-32 days). Possible explanations for this difference are differential disposition and compartmentalization. TCDD is a highly non-polar compound, rapidly sequestered into the lipophilic compartment or bound to high-affinity proteins in the liver such as CYP 1A2. Studies in CYP1A2 knock-out mice demonstrated the lack of sequestration of dioxins in the liver in the absence of this protein (Diliberto et al., 1997; Diliberto et al., 1999). Because of sequestration, the volume of distribution for TCDD is about 50 l/kg (Weber et al., 1993). In contrast, much less is known about the exact disposition of TCPT (Fried et al., 2007) due to the chemical instability of the parent compound under extraction conditions, and the lack of availability of radio-labeled substance. However, judging from its volume of distribution ($V_{d(apparent)}$ = 2.6 l/kg), it is not sequestered in tissues nearly as

extensively as dioxins, and therefore is more readily available for interaction at sites of IGF-1 regulation.

The potency of TCPT is $^1/_{12,500}$th that of TCDD (Figure 4-8). This difference in potency could be a result of differences in stereochemistry because dioxins are nearly planar but TCPT deviates from planarity by 18.5°. This non-planarity was expected to affect target organ interactions and to cause reduced potency as indeed observed in these experiments.

Effects of TCPT on body weight (Figure 4-9), feed intake (Figure 4-10) and serum IGF-1 levels (Figure 4-11) were readily reversible when increasing the dosing interval for TCPT. Prolongation of the dosing interval led to declining body burdens (steady state concentrations) because of TCPT's short half life (5.4 h). After one day, less than 5% of the TCPT dose remains as body burden. However, recovery of IGF-1 did not occur until 3 days later (Figure 4-11), implying that the dynamics of the effect rather than the kinetics of TCPT provide the rate-determining step for recovery. Many inducible proteins have *in vivo* half-lives of 12-16 h (Shiraki and Guengerich, 1984), requiring about 4 half lives (2-3 days) for steady state to be reestablished. This was also observed in this study, when TCPT administration continued at 24 h time intervals and IGF-1 levels returned to normal after 3 days.

4.6 Conclusions

This study of TCDD and its structural analog TCPT revealed some similarities but also an important difference between the two compounds. TCPT affected body weight and feed intake, two of the hallmark effects of TCDD toxicity. Furthermore, both compounds lowered serum IGF-1 levels in a dose-dependent manner, however, TCPT displayed a much lower potency regarding this effect. A decrease in TT_4 levels in serum that is typical for exposure to low doses of dioxins, however, was not observed with TCPT at the doses examined. Thus, although the effects of TCDD on IGF-1 and serum TT_4 clearly coincide, they do not coincide for TCPT, indicating a lack of cause/effect relationship between these two responses.

The analogy between TCPT and TCDD regarding serum IGF-1 levels suggests that IGF-1-mediated effects such as inhibition of ovulation and longevity that are observed with TCDD may also be expected for TCPT. This and the lack of correlation regarding serum TT_4 homeostasis support the notion that TCPT could become a suitable drug lead. Interference with thyroid hormone homeostasis would have caused many undesired side-effects that would have made TCPT unsuitable as a drug lead for above mentioned indications.

CHAPTER 5

SUMMARY AND CONCLUSIONS

5.1 TCPT is an AhR Ligand and Inducer of *in vitro* EROD Activity

Semi-empirical calculations of the structures of TCPT and five sulfoxo- and N-methyl derivatives did not reveal striking conformational differences (Table 3-1). All phenothiazines fit a three-dimensional AhR binding site predicted by *in silico* modeling (Waller and McKinney, 1995). Therefore, stereochemistry alone could not predict differential binding to or activation of the AhR by TCPT and its derivatives. However, the calculated structures qualitatively supported the notion of tetrachlorinated phenothiazines and derivatives being AhR ligands.

TCPT and derivatives were then studied *in vitro* for their affinity to the AhR and their ability to induce CYP 1A1, measured as EROD activity. Previous studies with CPZ documented low potency of this compound to induce EROD activity (Rozman *et al*., 1993), demonstrating decreased AhR signaling by some phenothiazines compared to the strictly planar dioxins. Therefore, similar properties were also expected for TCPT and derivatives. More recently, TCPT was shown in a pilot study to induce EROD activity at low potency (370-fold lower than TCDD, see Chapter 1) (Fried *et al*., 2007). However, that study did not cover the whole dose-response range, and it did not include exposure times shorter than 24 h, nor did it address the influence of metabolic pathways on apparent relative potency.

5.1.1 Affinity to the AhR

All compounds studied quantitatively displaced TCDD from specific binding sites (AhR) at high concentrations (Figures 3-3 and 3-4). This demonstrated that binding of tetrachlorinated phenothiazines and derivatives occurred at the same binding site as for TCDD.

Contrary to expectations, both TCPT and Me-TCPT turned out to be high affinity ligands for the AhR. Sulfoxidation is a known metabolic pathway for phenothiazine drugs (Goodwin, 1976). Therefore, sulfoxides and sulfones were expected to be metabolites of both TCPT and Me-TCPT (Figure 3-15). The respective sulfoxides showed reduced affinity to the AhR as expected, with the binding affinity of the sulfones being even lower (Table 3-2), indicating that sulfoxo-metabolism will cause decreased downstream effects, such as enzyme induction. Reduced AhR binding of the sulfoxides and sulfones is in agreement with the known molecular flexibility of phenothiazines in solution (Leonard and Sutton, 1948), which might be further enhanced in aqueous media by hydrogen bridges with available electron pairs on nitrogen and sulfoxide/sulfone. Although semi-empirical calculations have yielded dimensions within that of the AhR binding site for all compounds, binding and conformational flexibility are apparently more complex than accounted for by the calculations.

5.1.2 Induction of EROD activity

All compounds induced EROD activity with an efficacy similar to that of TCDD (Figure 3-11). Enzyme induction was time-dependent for all compounds except Me-TCPT-O_2 and TCDD, in that dose-response curves shifted to higher doses with increasing duration of exposure (Figure 3-12).

A similar shift relative to TCDD between AhR binding and downstream effects has previously only been reported for 3-methylcholanthrene (Riddick *et al.*, 1994), however, without a satisfactory explanation. In general, this shift in the dose response indicates the existence of one or more additional rate-limiting steps between binding of a ligand to the AhR and the induction of enzyme activity. Potential sites for this interaction could be the effects of ligands on binding to ARNT or the DNA binding site, because these interactions of the AhR/ligand complex are known to be key steps in the signaling cascade (Figure 1-1).

Potency, however, is a constant at steady-state concentration of a ligand and thus should not be influenced by time. The reason for the observed time-dependence of induction-potency must be due to metabolism of ligands by the hepatoma cells.

Tetrachlorinated phenothiazines contain two metabolizable centers: An amine and a thioether (Figure 3-15). In some derivatives, either one (Me-TCPT, TCPT-O_2) or both (Me-TCPT-O_2) metabolizable centers were blocked, allowing for separation of these two metabolic pathways.

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N-methylated derivatives showed a decline in induction potency clearly correlated to metabolism from the parent compound to the sulfoxide to the sulfone (Figure 3-13). Enzyme activity induced by TCPT-O_2 decreased at the same rate as that induced by Me-TCPT, indicating that rates of metabolism of the amine and sulfoxidation cannot be distinguished in this hepatoma cell line. Induction by TCPT, with both metabolizable centers available, did also not differ much from either of the above compounds (Table 3-4), indicating that for compounds with half-lives shorter than those of induced proteins, the dynamics of the effect were rate-determining. Blockage of both metabolizable centers, however, increased the persistence of the compound and thus the persistence of induction potency greatly. Thus, in the case of Me-TCPT-O_2, the kinetics of the compound became rate-determining.

Because induction and metabolism occurred simultaneously, the earliest time point (12 h) was used for a comparison of TCPT and derivatives with TCDD. At this time point, induction was at or near maximum because compounds had been available for metabolism for the shortest time period. TCPT and Me-TCPT displayed the highest potencies regarding EROD induction (Figure 3-13). These potencies were, however, two orders of magnitude lower than that of TCDD. Sulfoxides showed still lower potencies than either parent compound, and potencies were even further decreased for their respective sulfones. These compounds are expected metabolites of TCPT and/or Me-TCPT, indicating that the parent compounds are mostly responsible for effects.

5.1.3   Conclusions with Regard to the Hypothesis

The null hypothesis for these in vitro studies was that the binding affinity of phenothiazines to the AhR and the induction of EROD activity would be mechanistically the same or similar to but in their extent much lower than those reported for TCDD.

All compounds were demonstrated to bind to the AhR at the TCDD binding site. The affinities of TCPT and Me-TCPT to the AhR were similar to that of TCDD (Table 3-2), which is in contrast to the expected much lower potency. However, they were two orders of magnitude less potent than TCDD in their induction of EROD activity (Table 3-4). Binding affinity and induction though changed in parallel (Figure 3-14) in that TCPT and Me-TCPT showed the highest AhR affinity and also the most pronounced EROD induction, and an increasing degree of sulfoxidation caused decreasing binding affinity as well as diminished enzyme induction.

5.2   TCPT Lowered Serum IGF-1 Levels in Rats

Previously published data indicated that steady-state concentrations of TCDD reduced serum IGF-1 levels during subchronic exposure (Croutch *et al*., 2005). Additional doses were used to allow a quantitative assessment of this effect (Figure 4-5). An analogous study was then conducted for TCPT, accounting for the short elimination half life of this compound which required intravenous dosing. TCPT lowered serum IGF-1 levels with an ED_{50} of 0.82 mg/kg/12 h (Figure 4-7).

5.2.1 Comparison of TCDD and TCPT

The lowering of serum IGF-1 levels by TCPT occurred with a much earlier onset of the effect than after administration of TCDD. Serum levels of IGF-1 were reduced within days rather than weeks. The efficacy of TCPT was only slightly lower than that of TCDD. However, its potency was four orders of magnitude lower than that of TCDD when the comparison was made at steady-state blood concentrations (Figure 4-8).

5.2.2 Biological Significance

Doses of TCPT that maximally lowered serum IGF-1 levels also caused a transient decrease in body weight gain (Figure 4-2). However, significant toxicity, including lethality, was observed at these higher doses of TCPT. Reduced feed intake and body weight are well-known indicators of toxicity, so that the changes caused by TCPT could be attributed to acute toxicity which, in turn, requires increased cell maintenance for accelerated repair of injured cells. Therefore, this could trigger a reduction in IGF-1 levels indirectly, and also provide an explanation for the early onset of the effect. In contrast, TCDD reduced serum IGF-1 at doses one order of magnitude below acute toxicity as well as at much later time points.

5.2.3 Reversibility of Effects

TCPT has a short elimination half-life in the rat (Fried *et al*., 2007). Only frequent dosing maintained steady state levels in treated animals. Thus, a prolongation of the dosing interval allowed more elimination to occur, thereby enabling the organism to recover, which indeed occurred after extending the dosing interval from 12 h to 24 h.

Feed intake was initially reduced during treatment with TCPT, but it returned to control levels before the extension of the dosing interval (Figure 4-10). Body weight followed with a lag period, and it rapidly returned to control levels with an increased dosing interval (Figure 4-9). Serum IGF-1 levels also returned to control levels during the recovery phase (Figure 4-11). The time-response of the reversal of the reduction in serum IGF-1 levels suggests that the half-life of TCPT was not the rate-determining step. Instead, it indicated that protein half-lives were more likely to be responsible for the time course of reversibility.

5.2.4   Conclusions with Regard to the Hypothesis

The hypothesis for this study was that TCPT reduces serum IGF-1 levels dose-dependently, albeit with much reduced potency as compared to TCDD.

TCPT lowered serum IGF-1 levels in a dose-dependent manner like TCDD. However, much higher concentrations of TCPT were required to elicit this effect. It was not possible to determine if TCPT reduced serum IGF-1 levels directly or indirectly via toxicity.

Nevertheless, the findings clearly confirmed the hypothesis.

5.3   Further Discussion of Results

5.3.1   AhR Binding/Enzyme Induction and IGF-1 Signaling Share a Protein

Binding to the AhR/enzyme induction and the reduction of IGF-1 signaling occur at such different doses/concentrations, that a direct cause-effect relationship between these two events can be excluded.

However, IGF-1 has been reported to stimulate hypoxia-inducible factor (HIF) signaling by inducing the expression and nuclear translocation of HIF1α (Treins *et al.*, 2006). HIF1α and AhR share the same heterodimeric protein, referred to as ARNT in AhR signaling, or hypoxia-inducible factor 1β (HIF1β) in HIF signaling. In both signaling cascades, a primary binding partner relocates into the nucleus and forms a hetero-complex with this protein. The resulting complex then binds to

respective response elements on the DNA and induces transcription of regulated genes. Since both pathways utilize the same protein, it was conceivable that they could compete for ARNT/HIF1β in the nucleus. Microarray analysis, however, revealed no consistent AhR/HIF cross-talk (Lee *et al*., 2006).

In conclusion, theoretical consideration of differences in dose-responses and experimental studies of both pathways indicate that AhR and IGF-1 signaling are independent of each other although they both utilize ARNT/HIF1β.

5.3.2   Importance of Findings for Developing TCPT as a Drug Lead

As hypothesized, TCPT was confirmed to share two key mechanistic pathways with dioxins: AhR-mediated enzyme induction and the reduction of serum IGF-1 levels in rats.

5.3.2.1   AhR Ligand and Inducer of *In vitro* EROD Activity

Drug leads that induce CYP 1A1 (EROD) activity have been avoided by the pharmaceutical industry without much scientific basis (Nebert *et al*., 2004). Some procarcinogens, e.g. benzo[*a*]pyrene (BaP), are inducers of, as well as substrates for, CYP 1A1. They are metabolically activated by the enzymes they or other compounds induce. This induction of enzyme activity, however, is primarily a protective response and not necessarily a cause of toxicity. Common dietary CYP 1A1 inducers such as indole-3-carbinol possess chemopreventive properties without causing acute (Pohjanvirta *et al*., 2002) or chronic toxicity (Leibelt *et al*., 2003). Animals with life-

long induction of EROD activity had reduced cancer rates and lived longer than controls (Rozman *et al*., 2005). Furthermore, CYP 1A1 knockout mice responded with increased toxicity when treated with doses of BaP that were non-toxic to wild type animals (Uno *et al*., 2004). Although cancer- and enzyme-induction have been clearly separated for dioxins (Rozman *et al*., 1996), this information has not had an impact on the pharmaceutical industry's attitude towards AhR agonists. However, a recent study revealed that 239 out of 596 investigated compounds induced CYP1A1 mRNA expression in one or more tissues in the rat. Of these inducers,158 (84 %) were FDA-approved drugs (Hu *et al*., 2007).

Closely related to CYP1A1 induction is the induction of CYP1A2 activity. TCDD induces proteins of CYP1A1 and CYP1A2 in female Sprague Dawley rats at an ED_{50} of 0.22 μg/kg and 0.40 μg/kg, respectively (Santostefano *et al*., 1997). Both enzymes are exclusively induced *via* the AhR signaling pathway, as demonstrated by a lack of inducibility of either enzyme in two different AhR knock-out mouse strains (Lahvis and Bradfield, 1998). These animals, however, also displayed phenotypical deviations from wild type mice, such as reduced growth, decreased fertility (Lahvis and Bradfield, 1998), and partial portal shunting of hepatic blood flow to the *vena cava* (Bunger *et al*., 2003), indicating important physiological roles for the AhR. TCDD induces predominantly CYP1A1 activity in rat liver, but induces mainly CYP1A2 activity in human hepatocytes (Xu *et al*., 2000). Although mRNA levels of CYP1A1 and CYP1A2 were equally induced in human hepatocytes, activation kinetics were markedly increased for CYP1A2 (Zhang *et al*., 2006). Therefore, the

latter could be expected to be a more sensitive endpoint for human exposure to dioxins. This has recently been confirmed *in vivo* by the caffeine test (Guzelian *et al.*, 2006). An epidemiological evaluation demonstrated that CYP1A2 activity is only elevated in highly exposed individuals (> 1,000 ppt TCDD based on serum lipid [= >1,000 pg/g]) (Guzelian *et al.*, 2006). Therefore, it is reasonable to conclude that exposure at or near background levels of TCDD does not cause any observable effects.

It is of interest in regard to AhR signaling and downstream effects that the National Research Council recently published the following statement:

> *"Carcinogenicity of DLCs is not solely and quantitatively related to receptor binding. […] Because multiple additional steps are necessary, each probably with homeostatic mechanisms functional at low doses but perhaps overwhelmed at high doses, sublinearity with a response approaching zero at low doses would be expected.*

> *(National Research Council of the National Academies, 2006)*

In conclusion, this statement is consistent with the idea of developing TCPT and other AhR ligands as drug leads. The high affinity of TCPT to the AhR and its ability to induce enzyme activity appear to be a beneficial rather than a detrimental effect.

5.3.2.2 Serum IGF-1 Levels

IGF-1 signaling is believed to play a central role in aging. Epidemiological studies linked reduced serum IGF-1 levels to extended life spans in seniors (Paolisso *et al*., 1997), and genetically modified rodent models exhibited the same correlation (Holzenberger *et al*., 2003; Shimokawa *et al*., 2003). This pathway, however, has not yet been successfully targeted for anti-aging drug development.

Mechanistically, reduced IGF-1 signaling causes longevity by induction of cell maintenance genes. This induction is mediated via reduced deactivation of transcription factors (Corton and Brown-Borg, 2005). The effects of IGF-1 on growth and body mass are likely to be associated with inhibition of gluconeogenesis. A reduction in IGF-1 signaling has been reported to downregulate PEPCK by upregulation of a gene-silencing protein (Corton and Brown-Borg, 2005). The inhibition of PEPCK is a well-documented medium-dose effect of TCDD. However, the dose-response studies presented here on the effect of TCDD on serum IGF-1 levels revealed that they occur at doses slightly lower than those reported for PEPCK inhibition (Weber *et al*., 1991).

Reduced IGF-1 signaling has also been associated with decreased reproduction, which is another documented effect of TCDD (Li *et al*., 1995). Both effects occur at similar doses, suggesting a probable mechanistic link.

Although the reduction of serum IGF-1 levels by TCPT was an important finding for drug development purposes, the therapeutic window was very narrow. This might be different for a bioavailable oral formulation, since in that case TCPT would reach the target site for IGF-1 production, the liver, before it would enter the systemic circulation. This might allow for reduced serum concentrations and thereby widening the therapeutic window.

In conclusion, TCPT reversibly reduced serum IGF-1 levels, a key target for many applications such as weight loss, contraception, and longevity. Further studies are needed to evaluate the safety and efficacy of this compound for therapeutic purposes.

5.4   Outlook

Several new lines of investigations have already been initiated, focused on the bioavailability of TCPT and alternate routes of administration. Membrane permeability studies with intestinal cells and metabolism studies with liver homogenate are being conducted to resolve the question of bioavailability. Prodrug-design will possibly lead to the synthesis of an orally available formulation of TCPT, which at the present time is the rate-determining factor in the development of this compound.

APPENDIX

Chemicals & Solvents (if not stated otherwise)

Sigma Aldrich Chemical Company, St. Louis, MO

highest purity available

TCAOB

AccuStandard, Inc., New Haven, CT

purity 98.6 %

TCDD

Cambridge Isotope Laboratories, Inc., Woburn, MA

purity > 99%

[1,6]^{3}H-TCDD

EaglePicher Pharmaceuticals Services, LLC, Lenexa, KS

Specific activity: 27.7 mCi/mmol

TCPT and derivatives

Synthesized by Samson Speciality Fine Chemicals PVT LTD, Bangalore, India,

following a patented synthetic route (Rozman *et al.*, 2005)

purity > 98%

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