2005

THE EFFECT OF POLYCHLORINATED BIPHENYLS ON LIVER TUMOR PROMOTION: A ROLE FOR KUPFFER CELLS?

Rodica Petruta Bunaciu

University of Kentucky

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THE EFFECT OF POLYCHLORINATED BIPHENYLS ON LIVER TUMOR PROMOTION: A ROLE FOR KUPFFER CELLS?

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School at the University of Kentucky

by
Rodica Petruta Bunaciu
Lexington, Kentucky

Director: Dr. Howard Glauert, Professor, Graduate Center for Nutritional Sciences

Lexington, Kentucky
2005
THE EFFECT OF POLYCHLORINATED BIPHENYLS ON LIVER TUMOR PROMOTION: A ROLE FOR KUPFFER CELLS?

Polychlorinated biphenyls (PCBs) are ubiquitous lipophilic environmental pollutants. At least some of the PCB congeners and mixtures are hepatic tumor promoters. The mechanisms are not fully understood and might be multifactorial. Besides being the most abundant congener in the environment, 2,2',4,4',5,5'-hexachlorobiphenyl (PCB-153), has been previously shown to increase hepatocyte proliferation 48h after exposure in rats. The goal of this study was to determine whether hepatic Kupffer cells are important in the promoting activity of PCBs. The hypothesis of this study was that modulation of Kupffer cell activity by PCBs may contribute to PCB-induced liver tumor promotion. The experimental approach consisted of three in vivo models (tumor promotion model and two short term exposure models) and one in vitro model. In the tumor promotion model, glycine inactivation of Kupffer cells did not significantly influence the promoting activity of PCB-77 (3,3',4,4'-tetrachlorobiphenyl) or PCB-153. For the short term exposure model, we investigated the effect of Kupffer cell inactivation by glycine and the effect of Kupffer cell depletion on PCB-153’s impact on hepatocyte proliferation. The oil used as a vehicle had no significant effect on any of the end points considered. Inhibition of Kupffer cells with glycine or the absence of Kupffer cells did not affect cell proliferation or NF-κB activation after PCB treatment compared to the control. In vitro, PCB-153 increased DNA binding activity of NF-κB in Kupffer cells but did not significantly increase the TNF-α concentration in the medium. In conclusion, PCB-153 increased the number of preneoplastic foci per liver in the casein group but had no significant effect on cell proliferation, and Kupffer cells do not seem to play a role in hepatocyte proliferation.

KEYWORDS: Polychlorinated biphenyls; Tumor promotion; Kupffer cells; Nuclear factor-κB; Hepatocyte proliferation.
THE EFFECT OF POLYCHLORINATED BIPHENYLS ON LIVER TUMOR
PROMOTION: A ROLE FOR KUPFFER CELLS?

By

Rodica Petruta Bunaciu

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DISSECRATION

Rodica Petruta Bunaciu

The Graduate School
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ACKNOWLEDGMENTS

Over the years, many people have contributed to this project. All the experimental designs and the research funding for this project were provided by Dr. Howard P. Glauert. This dissertation would not have been possible without his guidance and encouragement, and I am very grateful for everything he did for this project. I am also grateful to the other members of my doctoral committee: Drs. Geza Bruckner, Brett T. Spear, and Steven I. Shedlofsky for their encouragement and input on this project. I am especially grateful to Drs. Brett T Spear, Donald Cohen, and Sandra H. Burnett for their constant focus toward the putative molecular mechanisms involved in this project and for helping me utilize the transgenic mice model developed in Dr. Cohen’s laboratory. Finally, I thank Dr. Zimmer for taking the time and effort to serve in my committee as the outside member.

I also want to thank my laboratory colleagues--Job, Karen, Jason, Divinia, Jill, Parvaneh, Aysegul, Maria, Casey, Amita and Sam—for all their help. I especially want to thank Drs. Job C. Tharappel and Karen Mason who made critical contributions toward the completion of this project. Dr. Tharappel's assistance with all my experiments and laboratory equipment was immeasurable. I thank Dr. Cohen’s laboratory, Drs. Robertson, Lehmler (for providing the PCBs), Ludwig (for cell culture advice), and Lee (for histology expertise). I am grateful to Dr. Chantal A. Rivera (Baylor College of Medicine, TX) and Dr. Ion Deaciuc (University of Louisville) for providing me the Kupffer cell isolation protocol and useful information regarding the glycine model.

While in the nutrition graduate program, I have been fortunate to receive support from the Graduate Center for Nutritional Sciences. I was also fortunate in receiving funding through a superfund training grant from the National Institute of Environmental Health Sciences, a Commonwealth Research Award, and a Students Travel Award from the Graduate School. The opportunities that I was given to present the research of this project at national and international
meetings were greatly supported by these fellowships along with travel money from grants from Dr. Glauert and the Graduate Center for Nutritional Sciences.

I can not leave this stage of my life without acknowledging and thanking Dr. Linda Chen for her guidance and constant and peaceful encouragement. I would like also to thank Mrs. Carol Cottrill for her friendship and effective help with everything bureaucratic.

I also would like to thank all my former mentors and professors from my prior studies in Bucharest--especially Dr Dana Iordachescu. And finally, I mention my dear sister Monica and Paolo, the doctor who cured her of leukemia and who served as a sterling example of Christian professionalism. I am most indebted to my parents, Dr. and Mrs. Petru Bunaciuc for all their love and sacrifice for me. But above all, I want to give thanks to God for all His assistance.
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Chapter 1: Background and Literature Review on Hepatocarcinogenesis, PCBs, and their tumor promoting activity

1. Liver tumor promotion

Hepatocarcinogenesis

Cancer might be seen as an abdication of a cell from its integrative role in an organism and a selfish behavior of proliferation and survival of its own clone at the expense of the host organism. Carcinogenesis can be seen as a successful rebellion of the transformed cell against the organism and its orderly structure and function; an intracellular and intercellular fight involving multiple genes, signaling pathways, cells as a whole (for example in the phagocytic activity of macrophages against tumor cells), and humoral factors such as cytokines, nitric oxide, and hormones. When the transformed cell and its clones proliferate, secure a nutrient supply by angiogenesis, and progress to metastasis, the death of the host organism is assured. This carcinogenesis process is an intricate interaction between genetic and environmental factors. An illustration of this interaction is the worldwide incidence in hepatocellular carcinoma, with higher rates in Asia, especially when hepatitis B or C is present, but also among Chinese living in San Francisco that are not infected with hepatitis (Clavien, 1999).

There are known genes with high impact on carcinogenesis, as BRCA1 and BRCA2 in breast and ovarian cancer, and there are many cancer types where the combined effect of many genes, with modest effects individually, are responsible. In any case, as Armitage and Doll showed as early as in 1954 that, based on the observation that the incidence of tumors usually increases with the sixth power of age, malignancy manifests itself after six or seven ‘specific,’ ‘discrete,’ ‘stable’ events that “must proceed in a unique order” in the transformed cells (Armitage and Doll, 2004). This observation is the beginning of the multistage carcinogenesis theory. Many other theories preceded and followed it, each highlighting one or another aspect of the complex process of carcinogenesis.
The initiator-promoter model (two-stage theory) of carcinogenesis was proposed by Berenblum and Shubik in 1949. This model is a practical way of production of experimental induced tumors, using initiating, mutagenic agents, followed by nonmutagenic promoters such as the inflammatory phorbol esters. The initiating agents can be chemical, physical or viral, and they induce irreversible genetic alterations. The initiation, however, is successful only when the cell fails the two protective systems: protection of the cell by DNA repair mechanisms and protection of the organism by induction of apoptosis in the transformed cell. Promotion is an epigenetic change and has as its main characteristic the increase in proliferation of the transformed cell and expression of genes that are not expressed in the original, untransformed cells.

In the liver, these clusters of cells form altered foci. Many animal studies have used altered hepatic foci as an endpoint in studying hepatocarcinogenesis because these foci can be detected relatively soon and easily after beginning promotion. These altered hepatic foci can be measured by abnormal existence or deficiency of certain enzymatic activities. Commonly used markers are γ-glutamyl transpeptidase (GGT), placental glutathione-S-transferase (PGST), glucose-6-phosphatase (G6Pase) and adenosine triphosphatase (ATPase). The foci, through progression, may lead to autonomous neoplasms, either benign or malignant.

Human hepatic cancers are associated with poor prognosis (Clavien, 1999). Usually, a case has clinical symptoms only at a late stage. Little is known about the natural history of human liver malignant tumors, and therefore cell culture and animal models are critical and also problematic due to species differences. Table 1.1. presents a classification of human liver tumors.

The liver is a very special organ. Its central role in the metabolism, including the metabolism of xenobiotics, its unique immunological characteristics, its position between the gut and the rest of the body, and the hepatocyte proliferation characteristics, make it both essential for the body and vulnerable to a wide array of insults, including those making it prone to both develop carcinogenesis and to harbor metastasis.
The immunological characteristics of the liver are very interesting. While being very efficient in cleansing immunological dangers, it also induces immunological tolerance for other types of antigens. Liver growth regulation (the balance between cell proliferation and apoptosis) is perturbed during hepatocarcinogenesis. Furthermore, hepatocytes are a type of specialized epithelial cells that, in a healthy liver, proliferate at an extremely low rate. It is striking, however, that after partial hepatectomy or liver transplantation (with less liver mass than optimum for the body weight of the donor), or injury (such as necrosis induced by hepatotoxicants, e.g. CCl$_4$), hepatocytes promptly leave G$_0$, replicate their DNA and divide. TNF-$\alpha$ released locally has been shown to be central to these processes (Diehl and Rai, 1996; Rusyn et al., 1998; Yamada and Fausto, 1998; Rose et al., 1999c). Kupffer cells, the resident macrophages of the liver, are a very important source of TNF-$\alpha$. But, TNF-$\alpha$ is not the only cytokine involved in initiating hepatocyte proliferation, as IL-1$\alpha$ also has been shown to be important in nafenopin-induced liver carcinogenesis, though using a different signal transduction pathway than TNF-$\alpha$ (West et al., 1999).

Reactive oxygen species are hypothesized, and in some cases proved, to be involved in the mechanism by which xenobiotics induce cancer (Rose et al., 1999b). Reactive oxygen species (ROS) can lead to oxidative DNA damage, modulate gene expression (activate immediate early genes or transcription factors (i.e. NF-$\kappa$B)), stimulate protein kinase C, and block gap junctional intercellular communication. All of these can be mechanisms by which tumor promoters function (Klaunig et al., 1995; Rusyn et al., 2000). The antioxidants can have a direct role against ROS, but also can have metabolic roles; both of these are protective against ROS-induced tumor promotion (Kawada et al., 1998).
### A. Primary Tumors of the Liver

#### A.1. Primary Epithelial Tumors of the Liver

<table>
<thead>
<tr>
<th><strong>Hepatocellular Carcinoma (hepatoma)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>- Etiology: multifactorial. Important factors are: cirrhosis and chronic hepatitis, but also aflatoxin exposure and exaggerated alcohol consumption. Genetic hemochromatosis and hereditary tyrosinemia increase risk.</td>
</tr>
<tr>
<td>- Arise from hepatocytes</td>
</tr>
<tr>
<td>- Alpha-fetoprotein levels are usually elevated in HCC, but also in liver diseases that predispose to HCC: viral, alcoholic, and chronic active hepatitis, and primary biliary cirrhosis.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Hepatoblastoma</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>- Alpha-fetoprotein levels are elevated</td>
</tr>
<tr>
<td>- Etiology: unknown. Loss of heterozygosity on chromosome 1 and loss of heterozygosity on chromosome 11 were reported</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Cholangiocarcinoma</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>- Malignant bile duct tumors; arise from the biliary epithelium</td>
</tr>
<tr>
<td>- Etiology: usually unknown. Probably, fibropolycystic liver diseases, chronic inflammatory lesions of the bile ducts due to primary sclerosing cholangitis, parasitic infections with liver flukes, bacterial cholangitis with hepatolithiasi, use of anabolic steroids.</td>
</tr>
<tr>
<td>- Serum levels of alpha-fetoprotein are normal in these patients</td>
</tr>
</tbody>
</table>

#### Mixed Hepatocellular Carcinoma/Cholangiocarcinoma

<table>
<thead>
<tr>
<th><strong>Biliary Cystadenocarcinoma</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A.2. Primary Hepatic Sarcomas</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Angiosarcoma</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>- Etiology: there are associations with exposure to vinyl chloride, Thorotrast, arsenical compounds, oral contraceptives, estrogens, anabolic steroids.</td>
</tr>
</tbody>
</table>
Mutations in the K-ras-2 gene have been detected in some patients.

<table>
<thead>
<tr>
<th>Epithelioid Hemangioendothelioma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Undifferentiated Sarcoma (Embryonal Sarcoma)</strong></td>
</tr>
<tr>
<td>• Negative for alpha-fetoprotein</td>
</tr>
<tr>
<td>• Peak incidence: 6-10 years, but there are also adult cases</td>
</tr>
<tr>
<td>• Multipotential differentiation of the primitive tumor cell</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
</tr>
<tr>
<td>Malignant Fibrous Histiocytoma</td>
</tr>
<tr>
<td>Malignant Schwannoma</td>
</tr>
<tr>
<td>Dedifferentiated Liposarcoma</td>
</tr>
</tbody>
</table>

**A.3. Primary Hepatic Lymphoma**

- Malignant lymphoma frequently involves the liver, however, primary hepatic lymphomas are rare.

**B. Secondary Tumors (metastatic tumors)**

- 95% of all liver malignancies and 50% of malignancies in the cirrhotic liver
- Usually, the primary tumor site is known when the patient is referred for liver biopsy; exceptions consists: carcinomas of the lung, pancreas, stomach and the neuroendocrine tumors, when many times a liver biopsy reveals a metastasis from previously undetected primaries.

Adapted from (Clavien, 1999)
2. Polychlorinated biphenyls (PCBs)

2.1. Chemistry of the PCBs

Polychlorinated biphenyls are a class of chemicals characterized by a biphenyl structure with the general formula C_{12}H_{10-x}Cl_x (where x may take values from 1 to 10). As a class, PCBs comprise 209 possible congeners (isomers differing by number and location of chlorine). These chemicals do not exist naturally, but are synthesized for their thermal stable properties. In addition to intentionally synthesized PCBs, some are an unintended by product of municipal solid-waste incineration (Choi et al., 2002). When still legal, batch synthesis of PCBs consisted of chlorination of biphenyl with Cl_2. Therefore, the end product comprised a mixture of congeners. These mixtures were used as they were, without purification of congeners because the only important parameter was considered to be the degree of chlorination of the batch. In the United States, PCB mixtures were produced and commercialized under the name Aroclor followed by a 4 digit number, the first two digits being 12, representing the 12 C of biphenyl, and the last two digits representing the percentage of chlorine by weight in the mixture. Historic, worldwide production of PCBs has been estimated to be at least 1 million metric tons (Erickson, 2001).

As a result of industrial and commercial use, PCBs are found in dielectric fluids of electrical capacitors (about 50% of the total amount of PCBs produced were used in capacitors (Erickson, 2001)) and electrical transformers, plasticizers in synthetic resins and rubbers, and in hydraulic fluids, vacuum pumps, gas-transmission turbines, heat transfer systems, inks, carbonless copy papers, lubricants, waxes, cutting oils, adhesives, and immersion oils for microscopes. But, the same properties that made them desirable (thermal and chemical stability) also have helped them persist in the environment (they are a class of persistent organic pollutants (POPs) and therefore prone to bioaccumulation and biomagnification). Other useful properties of PCBs are low flammability and low electrical conductance. PCB mixtures are liquid oils or sticky resins, and their viscosity depends on the degree of chlorination. PCBs have low vapor pressures, low water solubility, and
high solubility in most organic solvents and lipids (Erickson, 2001). Most PCB congeners are colorless or are white crystals. In the environment PCBs can be degraded by photolysis and biodegradation (Seeger et al., 1999; Bedard, 2001; Erickson, 2001), but human controlled disposal and degradation usually requires very high temperatures (Erickson, 2001).

2.2. Distribution of PCBs

PCBs are in the environment. Some quantities are still in use (for example in electrical capacitors and transformers), while others are present as ubiquitous pollutants (Erickson, 2001), released either intentionally (disposal) or accidentally into the air, surface water, groundwater, soils, sediment, plants and animals. The main site of PCBs pollutants is soil, which contains about 99% of pollutant PCBs (Erickson, 2001). Urban areas usually have more PCBs than rural areas, and developed countries usually have more PCBs than the rest of the world. Recently, a redistribution of PCBs has been observed, with increased concentrations in the polar regions (Sanders et al., 1994), possibly due to the lower volatility of PCBs at lower temperatures. The main mechanisms of PCB redistribution are human intervention (disposal) and atmospheric transport (Erickson, 2001).

Approximately 900,000 kilograms of PCBs cycle through the atmosphere annually in the United States (Erickson, 2001). The main route of human exposure is through food (mainly seafood and meat of animals fed with recycled fat), but also through exposure to airborne particles and skin penetration (Erickson, 2001; Schepens et al., 2001).

PCBs bioaccumulate as they go up the food chain, with increased accumulation of highly chlorinated congeners at the top of the food chain (Erickson, 2001). Mono- and dihydroxychlorobiphenyls and their conjugates are the main metabolites in higher organisms and are eliminated in the urine, but; the degree of metabolism decreases with increased chlorination (Erickson, 2001).

Human hair contains between 2 and 24 ng of individual PCB per gram of hair for each of about 25 congeners tested, with PCB-153 being one of the most
abundant. Indeed, PCB-153 has a mean value of 21.6 ng/g powdered human hair (Gill et al., 2004). Human organs such as adipose tissue, muscle and liver contain even higher amounts. The mean concentrations of total PCBs were found to be 29.4, 35.3, 10.6, and 11.8 ng/g wet weight, which corresponds to 579, 1139, 386, 2691 ng/g lipid in liver, muscle, kidney, and brain, respectively (Chu et al., 2003). The most abundant PCB was PCB-153 with 7.5 ng/g liver, 9.5 ng/g muscle, 2.6 ng/g kidney, 3.1 ng/g brain. PCB-153 is one of the most abundant congeners in the environment, and both animals and humans (Duarte-Davidson et al., 1994; Falandysz et al., 1994; Lanting et al., 1998; Mariottini et al., 2000; Chu et al., 2003; Gill et al., 2004). The low-chlorinated PCBs are not present in high concentrations in human tissues, except for the lungs (Bachour et al., 1998; Chu et al., 2003). Other studies found the mean concentrations of total PCBs to be up to 1200 ng/g lipid in liver and 380 - 1780 ng/g lipid in adipose tissue (Falandysz et al., 1994; Chu et al., 2003). The differences between studies might be due to different exposure levels of the subjects (Robinson et al., 1990). For example, PCB concentrations in human adipose tissue were higher in Italian samples (from Siena) than in those from Chile (from Concepción) (493 and 53 ng/g wet wt., respectively), when the determination was done by the same laboratory (Mariottini et al., 2000). The most abundant PCB congeners were the same in both groups: PCB-118, PCB-138, PCB-153, PCB-170, PCB-180, PCB-187. PCB-153 represented more than 20% of the total PCB residues in both groups, and the other six congeners represented approximately 70%. The coplanar congeners were below 1 pg/g wet wt. In the adipose tissue samples from Siena, PCB-77, PCB-126, and PCB-169 were present at about the same concentrations, whereas in the adipose tissue samples from Concepción, PCB-77 was at a much higher concentration than PCB-126 and PCB-169 (Mariottini et al., 2000).

It is interesting that there is a species variation regarding the organ distribution. Some animals such as foxes and deer accumulate PCBs in the liver, but humans seem to accumulate PCBs mainly in the adipose tissue and muscle tissue fat but not in the liver or brain (Bachour et al., 1998; Lanting et al., 1998). The brain, despite its high lipid content, is not the richest organ in PCBs (except in fish),
probably due to the blood-brain barrier. Interestingly, the blood serum levels in PCB-exposed people is increased even years after exposure (Guo et al., 1997; Erickson, 2001), demonstrating a continuous redistribution in the body. The serum levels positively correlate with initial exposure and for women negatively correlate with breast feeding (Guo et al., 1997).

There are no differences between the organ distribution of coplanar and noncoplanar PCBs (Bachour et al., 1998). The adipose tissue from older humans usually has more PCBs than in younger ones and generally contains a relatively high proportion of the more persistent and higher chlorinated congeners (Duarte-Davidson et al., 1994). In the Welsh population, there are no significant differences in the PCB concentrations between men and women, between people living in rural and urban areas, or between people with different body weights at the time of their death (Duarte-Davidson et al., 1994). However, in a study of people from the Great Lakes region of Canada, residue levels for PCB-126 were significantly higher in females compared to males (Williams and LeBel, 1991).

In a study on 44 women from Belgium, the cord blood delivery of all newborns had detectable levels of PCBs (120 and 1580 pg/ml) and correlated with the level in the maternal blood, proving that PCBs have an efficient transplacental transfer (Covaci et al., 2002). Those observations were confirmed by a study on tissue distribution of PCBs in fetuses that died in utero. The total concentration of PCBs and the congener distribution in fetal adipose tissue were similar to those in human breast milk (of Dutch women) (Lanting et al., 1998). The trans-membrane crossing and intracellular distribution of each congener as well as their transplacental transfer and dermal absorption, depend on their octanol-water partition coefficient (Jackson et al., 1993).

2.3. Health effects of PCBs

Human exposure to PCBs is widespread, and their impact on the human health might have multiple facets.
The coplanar congeners are the congeners that are chlorine substituted in both para positions and at least one meta position, but are not chlorine substituted in any of the ortho positions or have only one ortho-chlorine substituent. They therefore have greater freedom in adopting a more coplanar structure, even if not truly coplanar, since the angle between rings is at least 48 degrees (Erickson, 2001). Their structure allows them to interact with the Ah receptor and to have similar responses as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or 3-methylcholanthrene (3-MC) (Safe et al., 1985). The coplanar PCBs induce cytochrome 1A1/2 in liver (Safe et al., 1985). The non-coplanar congeners are those with two or more chlorines in the ortho positions and chlorines in the para positions and are sometimes referred as non-TCDD-like PCB congeners and have different toxic profile (Denomme et al., 1983). They activate the constitutive androstane receptor (CAR) which translocates to the nucleus to heterodimerize with the retinoid X receptor (RXR) to activate PBREM and in this way to induce cytochrome 2B1/2 in liver (Safe et al., 1985). CAR activation has deep implications on the energy metabolism and drug/steroid metabolism (Ueda et al., 2002), besides its impact on the Cyp 450 gene expression. The non-coplanar congeners, including PCB-153, are the most potent PCBs in decreasing dopamine content and in inhibiting calcium homeostasis mechanisms in brain cells (Tilson HA, 1997).

**Accidental exposure**

Accidental exposure usually occurs orally. PCBs can contaminate food directly as in the case of Yusho, Yucheng or Belgian PCB/dioxin crises, or the environment first, as in the major environmental contamination sites: Anison, AL; Hudson River, NY; New Bedford Harbor, MA; and the Great Lakes, in central North America.

In 1999, in Belgium, 500 tons of feed were contaminated with approximately 50 kg of PCBs and 1 g of dioxins from transformer oil and distributed to animal farms mainly in Belgium but also in the Netherlands, France, and Germany. Approximately, 10 million Belgians were exposed in this way to about 25 μg PCBs/Kg
body weight (van Larebeke et al., 2001). The health affects of this exposure, however, is not yet clear.

In 1979, in central Taiwan, four counties were affected by a poisoning due to the consumption of PCBs- and dibenzofurans-contaminated rice oil--Yucheng i.e. “oil disease” (Hsu et al., 1985; Ikeda, 1996). The rice oil for cooking was contaminated with the oil of the press machine, oil that contained PCBs as heat exchangers. One year after the accident, 1843 cases (11 to 20 years old) were reported with symptoms (mainly skin symptoms) of mild or moderate severity (Hsu et al., 1985). Chloracne, acne-like eruption, pigmentation of the skin, conjunctiva and nails, and Meibomian gland hypersecretion were the most frequent symptoms (Ikeda, 1996). Beside these, sensory-dominant neuropathy, headaches, eyelid swelling, fever, abnormal liver tests (especially high triglycerides in serum), and pulmonary disorders were reported (Ikeda, 1996). In most of the patients, clinical manifestations became apparent three to four months after the ingestion of the contaminated oil (Hsu et al., 1985). The amount of PCB intake per victim was between 0.7 and 1.84 g (Hsu et al., 1985). The highest blood level was 1156 ppb and the lowest 3 ppb (Hsu et al., 1985), with 27.6% of the 613 patients having PCB blood levels over 100 ppb (Hsu et al., 1985). 3.5 years after the accident, 2061 cases were reported (Hsu et al., 1985). At that time, except for a few severe cases, the skin symptoms were improved but problems in the victim’s descendents became apparent. Also, Yucheng cases presented significantly increased mortality compared to controls from hepatoma, liver cirrhosis, or liver diseases with hepatomegaly (Hsu et al., 1985; Yu et al., 1997). Remarkably, the low-chlorinated PCBs had a shorter half-life than the high-chlorinated congeners (Ikeda, 1996). Yucheng women had blood serum PCB concentrations one to two orders of magnitude higher than controls even 14 years after the exposure (Guo et al., 1997). More of the Yucheng women had children that died during childhood than in unexposed women (Yu et al., 2000). Anemia in women of this cohort, and joint and spine diseases in men of this cohort, were more frequent than in the control subjects (Guo et al., 1999). Also, the children born of Yucheng parents had lung immunology dysfunctions (Yu et al., 1998).
In 1968, in Japan, a similar accident occurred, and it is referred to as Yusho, i.e. “oil disease” in Japanese. The difference in PCB composition was that, in the Yusho case, the PCB mixture was Kanechlor 400 but, in the Yucheng case, it was a Kanechlor 400-500 mixture (Ikeda, 1996). The number of patients was 1866, the average estimated intake PCBs per patient was 2g (higher than for Yucheng), and the symptoms are very similar to those of the Yucheng cases (Ikeda, 1996). Oxidative stress was reported, in the form of increased nitric oxide, 30 years after the accident (Shimizu K et al., 2002). The blood levels were lower in the Japanese patients than in the Taiwanese patients; therefore in the Yusho patients the dermatological manifestations were much more prevalent than any other signs and symptoms and correlated with the PCB blood levels (Tokunaga and Kataoka, 2001). However, even in Yusho patients endocrinologic, neuronal and immune pathologies were described (Masuda et al., 2001).

Irregular menstrual cycles and altered immune responses were reported in both Yusho or Yucheng patients (Aoki, 2001). One year after exposure, Yucheng patients presented decreased concentration of IgM and IgA; decreased percentage of total T-cells, activated T-cells and helper T-cells; normal percentage of B-cells and suppressor T-cells; suppression of delayed type response; increase of lymphocyte spontaneous proliferation; and increase of lymphocyte proliferation after stimulation. These symptoms were decreased three years after exposure (Lu and Wu, 1985).

Hyperpigmentation was a common symptom in the children from PCB-poisoned mothers and many of these children (8 out of 39) died soon after birth (Hsu et al., 1985). The children of the Yucheng cohort mothers had low body weights at birth, with dysmorphic physical characteristics (the most common and persistent being nail abnormalities), and had delayed cognitive development compared with control children (Guo et al., 1995; Hsu et al., 1995). The girls were more affected than the boys of Yucheng mothers and the children of fathers exposed did not differ from the general population (Guo et al., 1994). The degree of delayed cognitive development of offspring of Yucheng mothers did not correlate with the PCB concentration in the children’s blood (Chen et al., 1994).
Occupational exposure

The main routes for occupational exposure are inhalation and skin absorption. One paper reviewed the data on overall mortality and cancer mortality on capacitor workers and concluded that those parameters are not increased comparative to the general population in men or in women (Kimbrough et al., 2003). For similar blood PCBs levels in accidental exposure (e.g. Yusho and Yucheng) and occupational PCB exposure, the health effects are greater in the cases of accidental exposure, possibly due to the presence of other compounds as dioxins, polychlorinated quaterphenyls and polychlorinated dibenzofurans (pyrolysis products of PCBs) (Kashimoto et al., 1981; Fischbein et al., 1985; Takamatsu et al., 1985; Kimbrough, 1995; Ikeda, 1996; van Larebeke et al., 2001).

Low PCB occupational exposure usually is not reported to induce pathological symptoms. However, there are such reports of increased serum liver enzymes with low PCB exposure (Chase et al., 1982; Ikeda, 1996). For example, out of 80 electrical workers exposed to PCB mixtures with 42% mean chlorine content for many years, with PCB concentrations from 41 to 1319 µg/L blood, 15 had skin diseases—chloracne (4), folliculitis (4), oil dermatitis (1), juvenile acne (1), and dermatitis due to irritative or allergic agents (5); 16 presented liver pathology—hepatomegaly with an increase in serum GGT, AST, ALT, and OCT values; and 2 had bleeding cavernous haemangiomas, with one case associated with chronic myelocytic leukaemia. (Maroni et al., 1981). High levels of PCBs in blood (up to 3330 µg/L) from occupationally exposed workers from the paint industry (even higher levels than in Yusho or Yucheng patients) did not produce remarkable symptoms; even the dermatological signs were not always present (Takamatsu et al., 1985). The serum triglyceride levels were elevated in some cases with really high PCB levels, and dizziness, headache and abnormal tactile sense in face and hands were reported (Ikeda, 1996). Regarding PCB occupational exposure and liver enzymes, women seem to be more sensitive than men (Fischbein, 1985).

Serum triglyceride levels are elevated both in accidental or occupational exposure cases, even at levels of PCBs that do not induce other symptoms, which
might indicate a PCB effect on lipid metabolism (Baker et al., 1980; Chase et al., 1982).

The half-life of highly-chlorinated congeners in the serum of capacitor manufacture factory workers, such as those present in Aroclor 1254, is longer than that of the lower-chlorinated congeners, such as those present in Aroclor 1242--4.8 years versus 2.6 years (Phillips et al., 1989). This difference may have implications for long-term effects such as cancer risk.

3. PCBs and cancer

Epidemiological studies

Overall mortality rates are not higher in PCB exposed groups than in non-exposed groups (Kuratsune et al., 1987; Sinks et al., 1992; Bosetti et al., 2003). Some studies find no association between cancer mortality and PCBs whatsoever (Kimbrough et al., 2003; Rusiecki et al., 2004). But, other data suggest correlation between PCB exposure and carcinogenesis. A general problem in epidemiological studies is the elimination of possible confounding factors. Possibly, confounding factors, synergic factors, or different congener composition may account for the different conclusions of the conflicting studies. A study performed in Poland, found that adipose tissue samples of patients who died of liver cancer contained 4.7 µg/g of PCBs (23% of which was PCB-153), while in samples from cancer-free subjects, the level was between 0.75 and 1.9 µg/g of PCBs (Falandysz et al., 1994). Twelve deaths among 2061 victims of the Yucheng 1979, Taiwan rice-oil poisoning, 3.5 years after the accident were due to hepatoma, liver cirrhosis or liver diseases with hepatomegaly (Hsu et al., 1985). Autopsies of 12 Yusho patients revealed five cases of carcinomas: two of the liver, two of the lung, and one of the esophagus (Kikuchi, 1984). Another study on Yusho patients reported that in males, the incidence of liver cancer was significantly higher in those patients than in unexposed males (Kuratsune et al., 1987). Two electrical workers out of 80 presented bleeding cavernous haemangiomas, in one of them associated with chronic myelocytic
leukaemia (Maroni et al., 1981). A meta-analysis of six studies on occupational PCB exposure found no increase in overall cancer mortality, no significant increased mortality due to liver cancer (12 deaths from liver cancer compared with 9.5 expected; standardized mortality ratio 126), and no increase in cancers of the breast or of the lymphatic and haematopoietic system (were lower in workers group) (Bosetti et al., 2003). A retrospective cohort analysis found also no increase in overall mortality, in all- causes mortality, but found an increase due to malignant melanoma and cancer of the brain and nervous system (Sinks et al., 1992). PCB-153 and PCB-180, two PCBs moderate chlorinated phenobarbital-type inducers (constitutively active receptor (CAR) agonists) are positively associated with DHEA hormone levels and prostate cancer cases (Ritchie et al., 2005). Although some studies did not find a correlation between breast cancer and PCB exposure (Rusiecki et al., 2004; Safe, 2004), a recent study established that the genotype of the cytochrome P-450 1A1 gene determines the breast cancer risk following PCB exposure. The study comprised the comparison of 374 women with histologically confirmed breast cancer and 406 noncancerous controls for serum PCB level and CYP1A1 genotype and found that women who had the CYP1A1 m2 variant genotype have a higher incidence of breast cancer if they have high serum PCB levels than the women with other genotypes and comparable PCB levels and that the CYP1A1 m1 and m4 genotypes confer low breast cancer risk even in conditions of PCB exposure (Zhang et al., 2004).

Animal studies examining the hypothesis that PCBs are hepatic tumor promoters

Before 2000, as reviewed by Glauert H.P. et al (2001), 39 studies examined the promotion of altered hepatic foci by PCB industrial mixtures or congeners (tetra-, penta-, or hexa-chlorobiphenyl) in rat liver (using one or more of the following markers: \(\gamma\)-glutamyl transpeptidase (GGT), placental glutathione-S-transferase (PGST), glucose-6-phosphatase (G6Pase), or adenosine triphosphatase (ATPase) (Glauert et al., 2001). Most of these studies used DEN as initiating agent. Seven of
these studies, performed by different laboratories, investigated the promoting activity of PCB-77 and all were positive, providing strong evidence that this congener is a liver tumor promoter. Four studies investigated the promoting activity of PCB-153 and all were positive. One study (Berberian et al., 1995), used exactly the same protocol as the studies presented in the following chapters of this work for initiation and promotion but completely different markers for altered hepatic foci (ATP-negative, GGT-positive, G6Pase-negative), whereas we used PGST-positive.

Aroclor 1254 promotes GGT–positive foci after initiation by 2/3 partial hepatectomy followed 24 h later by DEN. Aroclor 1254 was administered as one, two or three doses (Aroclor 1254 was administered to each rat 7, 28 and 49 days after the DEN) and the rats were euthanized 3 weeks after the last administration they received. This study showed that even one single oral administration (7 days after DEN) promotes altered liver foci (Pereira et al., 1982).

Clophen A 50 administrated alone (with no chemical or surgical initiation) initiated few hepatic altered foci and many disappeared before 12 weeks after Clophen A 50 administration, indicating an existing but weak initiating activity (Oesterle and Deml, 1983). Regarding its tumor promoting activity after initiation with a genotoxic compound there is strong evidence given by the 6 studies reviewed by Glauert et al (2001) that this mixture has promoting activity, and that weanling rats are more sensitive than adults (Oesterle and Deml, 1983). An interesting study was that of Deml and collaborators showing that PCBs can act as initiating agents at high doses (Deml et al., 1983).

Dean et al showed that individual PCB-153 and PCB-126 treatment lead to dose dependent increases in liver and adipose concentrations and that hepatic PCB-153 retention was increased after combined exposure. PGST-positive foci were increased both in number and volume by PCB-153 or PCB-126 in a dose dependent manner. The mixture of these two compounds, however, had an antagonistic effect in the formation of both foci area and number (Dean et al., 2002).

As reviewed by Glauert et al, (2001) there are multiple studies showing the promotion of gross tumors by PCBs. Of particular interest is the study of Preston et al (1981). A general critique of the conclusions of epidemiological studies is that
they do not ascertain whether the observed negative health effects are caused by PCBs or by other contaminants such as furans. One study, showed that in a rat tumor promotion model with DEN as the initiator, the incidence of hepatocellular carcinoma after exposure to Aroclor 1254 with impurities removed was higher than after exposure to non-purified Aroclor 1254 (Preston et al., 1981).

Possible mechanisms by which PCBs might function as hepatic tumor promoters

Regarding the mechanism by which PCBs may act as tumor promoters, one possibility could be the increased reactive oxygen species production in the presence of PCBs. Higher chlorinated classes of PCBs activate at least one class of Cyp450 (Tatematsu et al., 1979; Nakanishi and Shigematsu, 1991). For example, PCB-153 activates Cyp450 2B1/2. Another possible mechanism by which PCBs may alter the hepatocyte cell-cycle is by the activation of transcription factor NF-κB, which has been reported to be antiapoptotic and to increase cell proliferation in some conditions. Several studies in our laboratory have shown NF-κB DNA binding activity to be increased with PCB-treatment in mice and rats (Tharappel et al., 2002; Lu et al., 2003; Lu et al., 2004). Another mechanism of PCB induced tumor promoting activity was proposed to involve effects on vitamin A metabolism (Glauert et al., 2001). Intercellular communication seems to be disrupted by PCBs (Nakanishi and Shigematsu, 1991) and may be a mechanism of their tumor promotion. There are multiple ways this can happen, one being disrupter of gap junctions (Machala et al., 2003). Another mechanism might be the alteration of the normal soluble mediator release rate, for example by Kupffer cells, and their impact on the parenchymal population.

4. Kupffer cells and hepatotoxicity/hepatopathology

Kupffer cells are the resident macrophages of the liver and represent about 10% of total liver cells and 30% of the nonparenchymal cells (Roberts et al., 2000). To study the involvement of Kupffer cell in liver pathology, investigators have used different modalities of Kupffer cell depletion, or inactivation. Some of these methods
were even used to prevent or alleviate liver pathology. However, none of these methods are perfect and to date there is no method to specifically inhibit only Kupffer cells.

Glycine inactivation or GdCl₃ inactivation of Kupffer cells proved useful in liver protection in a number of cases: hepatotoxicity [e.g. alcohol (Yin et al., 1998), endotoxin (Rose et al., 2000), peroxisome proliferators (Rose et al., 1999a), CCl₄ (Rivera et al., 2001), thioacetamide (Andres et al., 2003), cocaine (Labib et al., 2003)], liver transplantation (Schemmer et al., 1998; Schemmer et al., 1999), liver reperfusion injury (Thurman et al., 1998), and hemorrhagic shock (Zhong et al., 1999).

Gadolinium chloride (GdCl₃) leads to the destruction of Kupffer cell populations preferentially: primarily ED2-positive mature Kupffer cells are depleted (Neyrinck et al., 2002), and ED1-positive Kupffer cells are 75% depleted (Rivera et al., 2001). As the result of depletion, after the initial increase in TNF-α concentration due to release from the apoptotic Kupffer cells, the liver loses the phagocytic and secretory activities specific to Kupffer cells. There are controversies regarding the decrease of TNF-α in the GdCl₃ treated animals. Kinoshita et al (2005) confirmed Diehl's group observations from the '90's (that GdCl₃ initially enhances TNF-α concentrations (Rai et al., 1997)) and demonstrated that GdCl₃ depletes only the superoxide-producing Kupffer cells, but at the same time enhances the function of TNF-α –producing Kupffer cells (Kinoshita et al., 2005). Glycine preserves the phagocytic activity (colloidal carbon uptake) of Kupffer cells (Neyrinck et al., 2002), but inhibits the secretory activity of these resident macrophages (Ikejima et al., 1997) without leading to the TNF-α peak characteristic after gadolinium administration. Glycine inactivates the secretory activity of Kupffer cells by activating the Cl⁻ -selective ion channels in the cell membrane of these cells(Ikejima et al., 1997; Rose et al., 2000). Hyperpolarization of the cell membrane is a consequence of Cl⁻ selective ion channels activation. In this condition, the increase in intracellular calcium is not possible. The intracellular calcium surge is a necessary condition for some signaling molecules (including TNF-α) to be produced.
and released by the Kupffer cells (Dieter et al., 1988; Kawada et al., 1992; Ikejima et al., 1996; Lichtman et al., 1996; Watanabe et al., 1996; Ikejima et al., 1997).

Gadolinium chloride is more frequently used as a model to study Kupffer cell function, but in terms of prevention in patients, it is not as useful due to its toxicity. In pretreatment of liver transplants, glycine is clearly better in preventing transplant failure than gadolinium chloride (Rentsch et al., 2002). Glycine is also more effective than gadolinium chloride in reducing the TGF-β induced by CCl₄ (Rivera et al., 2001).

The main mechanism of Kupffer cell influence upon hepatotoxicity seems to be through TNF-α and seems to involve cAMP, PKC and NF-κB upstream of TNF-α. Other important mediators are NO, reactive oxygen species and PGE2 (Ikejima et al., 1996; Bojes et al., 1997; Rose et al., 2000; Ding et al., 2003). Proteolysis, impaired hepatic microcirculation, and a hypermetabolic state are also induced by Kupffer cells and are alleviated by glycine or gadolinium chloride (Schemmer et al., 2000; Schemmer et al., 2001; Ito et al., 2003). Some injuries at remote sites have an effect on liver and the process is mediated by Kupffer cells. For example, in patients with 30% of body surface area burns, the gut permeability is increased and consequently, the gut-derived endotoxins induce Kupffer cells to secrete TNF-α (Enomoto et al., 2004).

Kupffer cells are also the main source of systemic IL-10, a cytokine with both proinflammatory and immunosuppressive activities, and also a source of IL-4 and IL-13, cytokines with a role in Th2 differentiation of hepatic T cells (Emmanuilidis et al., 2001). The induction of the CD14 system in the Kupffer cells seems to be very important in the synthesis of another inflammatory cytokine, IL-1β, which produced in excess leads to hepatic injury (Takeda et al., 2003).

As demonstrated by Roth’s group, however, sometimes inflammation and Kupffer cell involvement is only secondary to the liver injury, as in the case of the plant toxin monocrotaline (Copple et al., 2003). Whatever be the case, with Kupffer cell initiating the liver injury or amplifying it, a general hope prevails that the ability to manipulate interactions between Kupffer cells and hepatocytes may have important therapeutic implications.
5. **Kupffer cells and cancer**

Kupffer cells might have a dual role: promotion of carcinogenesis by releasing soluble factors such as TNF-α, NO, PGE2, and IL-1 (Ikejima et al., 1996; Bojes et al., 1997; Rose et al., 2000; Ding et al., 2003); and an anticarcinogenic effect by their phagocytic activity, especially against tumors that express non-self antigens (Clavien, 1999). Kupffer cell inhibition by glycine was shown to inhibit the development of liver tumors promoted by Wy-14,643 (Rose et al., 1999a). The mechanism was proposed to involve TNF-α. Anti-TNF-α antibody abrogated the hepatocyte proliferation induced by Wy-14,643 in rats (Bojes et al., 1997). But, studies with mice nullizygous for TNF-receptor 1 (TNFR1), TNFR2, or both receptors showed that TNF-α signaling is not involved at all in this process (Anderson et al., 2001). Also, studies with TNF-α knockout mice produced the same results (Lawrence et al., 2001). Contradictory results were also obtained regarding the TNF-α mRNA levels after Wy-14,643 treatment: some studies noticed a 2-2.5-fold increase (Bojes et al., 1997; Rose et al., 1997b; Rusyn et al., 1998), whereas others noticed a 50% decrease (Lawrence et al., 2001). But the lack of consensus could be due to multiple factors; since it is well established and yet hard to explain why some cancer types overexpress TNF-α and also TNF-α is used for the treatment of some other cancer types in humans (Demetri et al., 1989; Senzer et al., 2004). It is evident that TNF-α is a key mediator of inflammation and in the liver is produced primarily by the Kupffer cells (Climuro et al., 1997). The transcriptional factor NF-κB is involved in TNF-α gene transcription (Goto et al., 1996). As described below, inflammation is proposed to play an important role in carcinogenesis.

6. **Inflammation and cancer**

Both acute inflammation and cancer in other parts of the body leads to increased synthesis of acute phase proteins (C-reactive protein (CRP), fibrinogen, serum amyloid protein, and haptoglobin), and decreased albumin synthesis in the
liver and these processes are in part mediated by IL-6 (Kishimoto, 2005). Further IL-6 might act as a growth factor for hepatocytes inducing their proliferation. IL-6 might be released by T cells (mainly), but also by many other cells including macrophages, fibroblasts, synovial cells, endothelial cells, glia cells, and keratinocytes. Proinflammatory cytokines, such as IL-1 and TNF-\(\alpha\), and microbial agents can induce IL-6 synthesis. The involvement of NF-\(\kappa\)B, Ras protein and other many proteins in both cancer and chronic inflammation suggest that many different pathways might be interrelated (Karin, 2005).

6.1. NF-\(\kappa\)B and cancer

Nuclear factor \(\kappa\)B (NF-\(\kappa\)B) is a transcription factor able to transfer various information from the cell membrane to the nucleus (Rothwarf and Karin, 1999), and is implicated in a complex array of pathways and in controlling genes with diverse physiological roles. NF-\(\kappa\)B consists of either homo- or heterodimers of a vast family of proteins \([p50, p52, c-Rel, Rel A (p65), \text{and Rel B}]\) but the predominant form in many cell types is p50:p65. All eukaryotic cell types contain the transcription factor NF-\(\kappa\)B. In mature B cells and plasma cells, NF-\(\kappa\)B is constitutively activated and involved in maintaining the differentiation state of these cells (Ghosh \textit{et al.}, 1998). In the rest of the cell types, NF-\(\kappa\)B dimers are sequestered in the cytosol (and therefore kept in an inactive form) by a member of the inhibitory protein family I\(\kappa\)B (I\(\kappa\)B\(\alpha\), I\(\kappa\)B\(\beta\), I\(\kappa\)B\(\epsilon\)). Each of these proteins can associate with the NF-\(\kappa\)B dimer and mask its nuclear localization signal. NF-\(\kappa\)B can be activated by radiation, and cytokines such as TNF-\(\alpha\) or IL-1. NF-\(\kappa\)B may be activated by reactive oxygen species (Schreck and Baueurele, 1991; Schreck \textit{et al.}, 1991; Schreck \textit{et al.}, 1992; Remacle \textit{et al.}, 1995; Baldwin, 1996; Li and Karin, 1999), yet controversies exist regarding the physiological role of the antioxidants in this process. Some researchers appreciate that the protective effect of antioxidants against NF-\(\kappa\)B activation is a proof of the ROS involvement in the activation of this transcription factor (Meyer \textit{et al.}, 1993; Liu \textit{et al.}, 1995; Hill \textit{et al.}, 1999), whereas others propose that this protective effect is due to other properties of those compounds commonly referred as antioxidants and
that NF-κB is not activated by ROS (Bowie and O'Neill, 2000; Hayakawa et al., 2003). Whatever the extracellular activation signal may be, the multisubunit IκB kinase (IKK) transduces and converges most stimuli that activate NF-κB through phosphorylation of the IκB isoforms (DiDonato et al., 1997; Hoffmann et al., 2002). IKK has two kinase subunits, IKKα and IKKβ, and a non-kinase subunit, IKKγ/NEMO (Zandi et al., 1997; Pomerantz and Baltimore, 2002). The three IκB isoforms provide a temporal control of NF-κB activation. IκBα is involved in rapid NF-κB activation (Ghosh and Baltimore, 1990); it is the first of the three isoforms that is degraded following stimulus (Hoffmann et al., 2002). This isoform also has a rapid and strong negative feedback, i.e. a NF-κB inactivation because the IκBα gene has a highly NF-κB-responsive promoter (Scott et al., 1993). The isoforms IκBβ and IκBε respond more slowly to stimuli; their synthesis is steady-state and they therefore act to minimize the long-term oscillations of the NF-κB system (Hoffmann et al., 2002). Once phosphorylated by IKK, the IκB unit is degraded (DiDonato et al., 1995) by the ubiquitin/26S proteasome pathway. Phosphorylation and subsequent degradation of IκB allows the NF-κB dimers to translocate into the nuclei and recognize in their target genes a DNA sequence motif known as the κB site. This site has variability depending on the gene, but the canonical sequence is considered to be: 5′-G₅G₄G₃R₂N₁N₀Y₊₁Y₊₂C₊₃C₊₄₋₃′ (where R represents a purine, N could be any nucleotide and Y represents a pyrimidine, and 0 is the “ideal center of the structure) (Natoli et al., 2005).

In macrophages, IKKα is reportedly involved in quenching NF-κB pro-inflammatory activity by accelerating both the turnover of the NF-κB subunits RelA and c-Rel, and their removal from pro-inflammatory gene promoters (Lawrence et al., 2005). But Lawrence et al (2005) found that Rel B is not affected. Therefore, the difference between Rel family members might be important for maintaining a balance between pro- and anti-inflammatory responses. IKKα is required for activation of an alternative NF-κB signaling pathway, i.e. processing of the NF-κB2/p100 precursor protein, which associates with RelB in the cytoplasm. This alternative pathway leads to activated RelB:p52 dimers, required for organization of
secondary lymphoid organs (Bonizzi et al., 2004). For mice with mutant IKKα isoforms that can not be activated, macrophage inflammatory protein (MIP)-2, MIP-1α, interleukin (IL)-12p40, inhibitor of apoptosis protein (IAP)-2 and inducible nitric oxide synthase (iNOS) are elevated and all these are pro-inflammatory and / or anti-apoptotic proteins yielding an overall exacerbated pro-inflammatory and anti-apoptotic response (Lawrence et al., 2005). This fact is considered a link between an aberrant NF-κB pathway and cancer. Inflammation is linked more to cancer initiation whereas deregulation in expression of inhibitor of apoptosis proteins prevents the elimination of tumor cells with DNA damage and induces resistance to cancer therapy. In a murine cancer metastasis model in which a colon adenocarcinoma cell line generates lung metastases, LPS-induced metastatic growth response depends on both TNF-α production by host hematopoietic cells and NF-κB activation in tumor cells (Luo et al., 2004). Ras-like proteins have been shown to interact with IκBβ such that the phosphorylation and degradation of this subunit requires additional signals from other signaling pathways besides the classical IKK-induced activation and since Ras is involved in so many cancer types, also suggests a possible link between cancer and NF-κB aberrant activation (Chen et al., 2004).

Through the products of a multitude of genes that have κB response elements, NF-κB controls aspects of immune response and inflammation (COX-2, TNF-α, TNF–β, IL-1, IL-2, IL-2R, IL-6, IL-8, G-CSF, GM-CSF, IFN-β, angiotensinogen, serum amyloid protein, C3 complement, complement factor B), cell proliferation (IL-6, Cyclin D1), apoptosis (p65/- mice die during embryogenesis due to high apoptosis of hepatocytes) and cell migration (VCAM-1, ELAM-1, ICAM-1) (Libermann and Baltimore, 1990; Ghosh et al., 1998; Jobin et al., 1998; Guttridge et al., 1999; Mayo and Baldwin, 2000; Cao et al., 2001; Karin et al., 2002; Nadjar et al., 2005). PGE2, the downstream product of COX-2 is an important molecule in angiogenesis. NF-κB is regarded most of the time as a cell survival molecule. Many types of cancer (such as many hematological malignancies or some types of breast cancer) have this transcription factor constitutively activated (Scott et al., 1993; Karin
et al., 2002). The proto-oncogene Bcl-3 share structural homologies with \( \text{I} \kappa \text{B}_\alpha,\beta,\gamma \) proteins, having ankyrin repeats and similar amino acid sequence, therefore is considered to be a member of \( \text{I} \kappa \text{B} \) family (Ghosh et al., 1998). In contrast with the inhibitory activity of \( \text{I} \kappa \text{B}_\alpha,\beta,\gamma \) proteins, Bcl-3 promotes activation of gene transcription by p50 homodimer (Fujita et al., 1993; Nolan et al., 1993). NF-\( \kappa \text{B} \) p50 homodimer is usually transcriptionally repressive (Ghosh et al., 1998), but when Bcl-3 binds to the DNA-bound p50 homodimers, the transcription is activated (Fujita et al., 1993).

6.2. TNF-\( \alpha \) and cancer

TNF-\( \alpha \) is a cytokine produced and released by monocytes, macrophages, granulocytes, fibroblasts, endothelial cells, and epithelial cells (Spriggs et al., 1992; Diehl et al., 1994). TNF-\( \alpha \) has a dual role in cancer, depending on many factors. Reportedly, in early stages, it facilitates tumor cell adhesion, but at later cancer stages, inhibits the formation of liver metastasis (Sturm et al., 2003). Therefore, the time point of its manipulation can have beneficial or harmful effects.

The human TNF-\( \alpha \) was first cloned in 1984 (Pennica et al., 1984). The TNF-\( \alpha \) gene is highly conserved across species and in humans and mouse is located in close proximity to the HLA-B. The 5' regulatory region of the TNF-\( \alpha \) gene contains consensus sequences for the AP-1, AP-2, and NF-\( \kappa \text{B} \) transcription factors, and also contains the cAMP-responsive element (Spriggs et al., 1992). The human TNF-\( \alpha \) gene has 4 \( \kappa \text{B} \) binding sites in its promoter and transcription of this gene takes place only when NF-\( \kappa \text{B} \) heterodimers p50/p65 bind to all four sites (Goto et al., 1996). As mentioned above, AP and NF-\( \kappa \text{B} \) families of transcription factors are involved in liver growth regulation. The TNF-\( \alpha \) gene is upregulated by the presence in the medium of its own product, TNF-\( \alpha \), but also by other cytokines such as IL-1, also by LPS, calcium flux, and radical oxygen species. Apparently, these diverse stimuli act through different mechanisms to induce the TNF-gene expression.

TNF-\( \alpha \) expression and secretion are highly controlled processes due to the need of maintaining very narrow concentration limits. TNF-\( \alpha \) expression is regulated both at the transcriptional and posttranscriptional levels (Spriggs et al., 1992). In
leukocytes and HL-60 cells, the induction of TNF-α expression is dependent upon phospholipase A2, arachidonic acid and the lipoxygenase pathway (Spriggs et al., 1990; Spriggs et al., 1992). A downstream product of COX-2, PGE2 provides a negative feedback and, by raising the cAMP concentrations in the cell, inhibits TNF-α expression (Spriggs et al., 1992). The secretion of this important cytokine requires G binding proteins (Spriggs et al., 1992) and a surge in intracellular Ca levels (Edwards et al., 1993).

TNF-α acts via 2 distinct cell surface receptors: TNF-R1 and TNF-R2 (Imamura et al., 1987), with TNF-R1 being involved in most of the biological processes. After receptor binding, TNF-α is internalized together with the receptor (Imamura et al., 1987). TNF-α has multiple and diverse functions: cytolysis, differentiation, and mitogenesis, lipid metabolism (Yamada et al., 1998), and modulation of electrophysiological properties of myenteric neurons (Rehn et al., 2004). In vitro, hepatocytes cultivated with TNF-α, insulin and epidermal growth factor (EGF) had significantly higher rate of proliferation than hepatocytes cultivated with insulin and EGF without TNF-α, evidence that TNF-α is able to prime the hepatocytes to divide and make them more susceptible to mitogens (Diehl et al., 1994). This point of view was strengthened by an experiment using transforming growth factor β (TGF-β1), a cytokine that is a known physiological negative regulator of liver growth. This study demonstrated that TNF-α is able to induce hepatocyte proliferation and to suppress apoptosis induced by TGF-β1, both in rats and in mice (Rolfe et al., 1997). TNF-α is important in initiating the regenerative growth after partial hepatectomy (Diehl et al., 1994; Yamada et al., 1997; Yamada et al., 1998). Liver regeneration after partial hepatectomy was inhibited by anti-TNF-α antibody (Akerman et al., 1992) and was greatly delayed in TNF-receptor 1 (TNFR1) knock-out mice (Yamada et al., 1997). In addition, TNFR1 knock-out mice had impaired liver regeneration after CCl₄-induced necrosis (Yamada and Fausto, 1998). For both partial hepatectomy as well as CCl₄ toxicity after the establishment of necrosis, the liver undergoes roughly the same sequence of events, initiated by TNF-α and culminating with hepatocyte proliferation. The steps involve TNF-α release and
binding to the TNFR1 (but not TNFR2) receptor with subsequent NF-κB activation and IL-6 release, STAT 3 activation, and cyclin D1 upregulation leading to DNA synthesis in the hepatocytes (Yamada et al., 1997; Yamada and Fausto, 1998; Yamada et al., 1998; Guttridge et al., 1999). TNF-α also induces c-jun expression and increases AP-1 DNA binding activity (Diehl et al., 1994), but these actions are relayed through both TNFR1 and TNFR2 receptors (Yamada et al., 1998) and seems to be not as critical as NF-κB activation for liver regeneration (Yamada and Fausto, 1998; Yamada et al., 1998). Also, it seems that IL-6 release is also an upstream event of AP-1 activation (Yamada et al., 1997).

Chemically induced carcinogenesis is often associated with a similar but exacerbated mechanism that is present after partial hepatectomy. Nafenopin is one of the peroxisome proliferators that induces DNA synthesis in hepatocytes and cell division after increasing the TNF-α concentration and subsequent NF-κB activation (West et al., 1999). As in the case of partial hepatectomy, TNFR1 is involved more than TNFR2 (West et al., 1999). Moreover, chemical carcinogens such as peroxisome proliferators often block apoptosis (Christensen et al., 1998; James et al., 1998; West et al., 1999; Roberts et al., 2000; Ichikawa et al., 2005).

TNF-α was proposed by Thurman’s group as central in liver carcinogenesis promoted by the peroxisome proliferators Wy-14,643. Anti-TNF-α antibody abrogated the hepatocyte proliferation induced by Wy-14,643 in rats (Bojes et al., 1997). However, studies with mice nullizygous for TNF-receptor 1 (TNFR1), TNFR2, or both receptors showed that TNF-α signaling is not involved at all in this process (Anderson et al., 2001). Also, studies with TNF-α knockout mice reached the same result (Lawrence et al., 2001). Contradictory results were also obtained regarding the TNF-α mRNA levels after Wy-14,643 treatment: some studies noticed a 2-2.5-fold increase (Bojes et al., 1997; Rose et al., 1997b; Rusyn et al., 1998), whereas others noticed a 50% decrease (Lawrence et al., 2001).

Some cancer types overexpress TNF-α and also TNF-α is used for the treatment of some other cancer types in humans (Demetri et al., 1989; Senzer et al., 2004). TNF-α is constitutively expressed in leukemic blasts from patients with acute
myeloblastic leukemia (Wakamiya et al., 1989; Westermann et al., 1996). TNF-α has been shown to induce apoptosis but with different potencies in apoptotically-resistant compared with apoptotically-sensitive cancer cells, and the mechanism of resistance was demonstrated to involve constitutive expression of TNF-α (Spriggs et al., 1987) and NF-κB activation (Weldon et al., 2001) in the resistant cells. This demonstrates once more that the narrow concentration window of TNF-α is essential for normal balance between cell division and apoptosis. A recent review (Watts, 2005) points out the necessity of a transient signaling via TNFR family for an appropriate anticancer response, which underlines the importance not only of an appropriate concentration of TNF-α, but also of an appropriate duration of the signal.

**Hypothesis and specific aims**

The unavoidable human exposure to the lipophilic pollutants PCBs and the reported cases of liver pathology, including hepatomegaly and hepatoma, after accidental rice-oil PCB exposure (Hsu ST, 1985) prompted many research teams around the globe to study their hepatocarcinogenic activity. Animal studies show they are hepatic tumor promoters (Glauert et al., 2001), but despite the efforts to elucidate the mechanism, the molecular details of PCBs' impact on the liver are still unknown.

We focused on PCB-153, the most abundant congener in the environment, in our studies. This is a non-coplanar PCB, able to activate Cytochrome P-450 2B1 in a phenobarbital-like manner.

Our working model is based on the idea that PCB-153 acts upon both the Kupffer cell and hepatocyte populations (see figure 1.1) and that the end result is an amplification of this effect. Our working hypothesis is that in the Kupffer cells, PCB-153 activates the transcriptional factor NF-κB and this leads to Kupffer cell activation. The Kupffer cell activation also usually involves protein kinase C (Rose et al., 2000) and NF-κB and results in the secretion of chemotactic and mitogenic mediators such as tumor necrosis factor-α (TNF-α), hepatocyte growth factor, prostaglandin E2, and superoxide radical (Rose et al., 2000). All these mitogenic mediators could impact the hepatocyte's cell-cycle. TNF-α, for example, subsequent to its binding to the TNF-receptors, can activate NF-κB in hepatocytes. Our working
model also envisions a direct action of PCB-153 upon the hepatocyte: it can activate NF-κB and also bind to the CAR receptor. The result of both these events can be an up regulation of Cytochrome P-450 2B1, since the promoter of the P-450 2B1 gene has κB response elements (Shaw PM, 1996) and PB-responsive enhancer module (PBREM) (Honkakoski et al., 1998; Sueyoshi et al., 1999). The induction of cytochrome P-450 2B1 by PCB-153 is well documented and increased P450 activity was reported to increase the formation of reactive oxygen species (Schlezinger et al., 1999; Schlezinger et al., 2000; Coteur et al., 2001; Schlezinger and Stegeman, 2001; Fadhel et al., 2002; Mariussen et al., 2002; Shertzer et al., 2004). PCB-153 is not a substrate of this enzyme, and it was shown for toluene, ethanol and phenobarbital that activation of cytochrome P-450 in the absence of substrate produces the maximum amount of reactive oxygen species, comparing with the case when the substrate is present (Bondy SC, 1994). Reactive oxygen species may provide a positive feedback on NF-κB activation and all these factors—reactive oxygen species, NF-κB activation and probably a direct PCB-153 effect upon the other target genes—could lead to increased hepatocyte proliferation and decreased apoptosis, with the net result being increased genetic instability, which may lead to pre-neoplastic foci formation and to cancer.

Based on this model, we hypothesized that modulation of Kupffer cell activity by PCBs may contribute to PCB induced liver tumor promotion. We also hypothesized that PCB-153 modulates the Kupffer cell activity by increasing DNA binding activity of the transcription factor NF-κB in these cells, which might lead to increased cytokines (e.g. TNF-α) released by these cells therefore effecting hepatocyte proliferation.

To address these hypotheses, three specific aims were proposed:

- Specific aim 1: To investigate whether Kupffer cell activity modulates the formation of preneoplastic foci induced by PCB-77 and PCB-153.
- Specific aim 2: To investigate whether Kupffer cells modulate the amplitude of hepatocyte proliferation induced by PCB-153.
- Specific aim 3: To investigate if the Kupffer cells are activated in vitro by PCB-153.
Figure 1.1. Working model
Chapter 2: Kupffer Cell Inhibition by Dietary Glycine Does Not Prevent the Tumor Promoting Activity of PCBs

Introduction

Polychlorinated biphenyls (PCBs) are persistent environmental pollutants. Chemically, they are a diverse class of substances. Along with chemical diversity comes a wide range of effects that vary between different subclasses. PCBs were produced as mixtures (industrial mixtures) and are present in the environment as mixtures (environmental mixtures, containing the parent congeners and some thermal or metabolic derivatives). Their removal is very costly and some methods are congener specific, therefore, it is important to know which congeners pose serious health threats. Also, detailed information about toxic congeners and their mechanism of toxicity is important in order to diminish the effects on humans. Human exposure to PCBs is ubiquitous and once the PCBs are in the body, important quantities of the parent congeners or their metabolites are retained for long periods of time (Guo et al., 1997; Chu et al., 2003). Therefore, the real human health concern is the long term effects of PCBs in the body. As reviewed by Glauert H.P. et al (2001), strong evidence shows that PCB mixtures and medium- and highly-chlorinated individual congeners are hepatic tumor promoters in laboratory rats. The rodent model is considered a very good indicative of potential hepatic damage in humans (Hayes et al., 1982). Seven studies in rats revealed that PCBs promote gross liver tumors and 42 studies demonstrated that PCBs promote altered hepatic foci in rats (Glauert et al., 2001; Whysner and Wang, 2001; Dean et al., 2002; Tharappel et al., 2002; Glauert et al., 2005).

PCB-153 is one of the most abundant congeners both in the environment and in organisms (Duarte-Davidson et al., 1994; Falandysz et al., 1994; Mariottini et al., 2000; Gill et al., 2004). PCB-77 is present in some dietary supplements for human consumption such as cod liver oil (Storelli et al., 2004). Also, in human adipose tissue samples from Concepción, Chile, PCB-77 was in much higher concentration
than PCB-126 and PCB-169 (Mariottini et al., 2000). Both PCB-77 and PCB-153 have tumor promoting activity (Glauert et al., 2001). The mechanisms of their promoting activities are still unclear, though one possibility is an imbalance between increased cell replication and suppressed apoptosis in the hepatocyte population. Most of the PCB congeners do not directly interact with DNA, but they may promote or amplify in the hepatocyte the consequences of mutagenic events that would otherwise be repaired. The increased cell proliferation and decreased apoptosis increase the probability that a spontaneous mutation could be propagated and that successive mutations accumulate into the genotype of some cells with a net result of promotion and progression of a tumor. But, what is the incentive for increased hepatocyte turnover to start with? One possibility is that PCBs act upon other types of hepatic cells and these cells in turn induce hepatocytes to proliferate. Kupffer cells, when activated, release cytokines, prostaglandins, and reactive oxygen species, all of which can cause tissue injury in certain conditions (Edwards et al., 1993; Clìmuro et al., 1997). Exacerbated Kupffer cell activation might have multiple causes, one being the presence of xenobiotic toxic compounds. Peroxisome proliferators are another class of nongenotoxic carcinogens. Wy-14,643, a member of this class, activates NF-κB in the Kupffer cells (Rose et al., 2000), and stimulates the Kupffer cells to release mitogenic cytokines such as TNF-α (Rose et al., 1997a) and reactive species such as superoxide (Rose et al., 1999b; Rusyn et al., 2000). These chemical messengers subsequently induce hepatocyte proliferation (Rose et al., 1997a; Rose et al., 1999c).

We have hypothesized that the activation of Kupffer cells by PCB-153 and PCB-77 is necessary for the formation of hepatic preneoplastic foci. Our working model is that PCBs activate Kupffer cells to secrete mitogenic factors such as TNF-α and superoxide, and these factors alter gene expression in hepatocytes, leading to an increased mitogenic rate, conditions favorable to genetic instability and mutation accumulation with the net result being preneoplastic foci formation.

To test our hypothesis, we studied the effects of Kupffer cell inhibition on two-stage hepatocarcinogenesis using PCB-153 (a non-coplanar PCB) or PCB-77 (a coplanar PCB) as promoters. Diethylnitrosamine (DEN, 150 mg/kg) was used as the
initiator. There are several ways to inhibit Kupffer cell secretory activity, including dietary administration of the amino acid glycine (Ikejima et al., 1996; Ikejima et al., 1997; Rose et al., 1997a) and we chose this model. Glycine or casein (as control) was fed in an unrefined diet at 5% starting two weeks after DEN administration. The rats received four biweekly i.p. injections of vehicle (corn oil) or of PCB-77 or PCB-153. We quantified the formation of preneoplastic focal lesions expressing placental glutathione S-transferase and the incidence of DNA synthesis in these foci and in the normal tissue.
Materials and Methods

Materials:
1. PCB-153 (2,2',4,4',5,5'-hexachlorobiphenyl) and PCB-77 (3,3',4,4'-tetrachlorobiphenyl) were generous gifts from Drs. Hans Lehmler and Larry Robertson. The purity of this PCB was greater than 98%, as assayed by gas-chromatography (Lehmler and Robertson, 2001).
2. The diet for the entire study was Harlan Teklad, Global 18% protein Rodent Diet 2918 (Madison, WI).
3. Vitamin E-stripped corn oil was obtained from Acros Organics (Morris Plains, NJ).
4. Casein was from Teklad (Madison, WI).
5. Glycine was obtained from Sigma (G-7403, Sigma, St. Louis, MO).
6. Diethylnitrosamine (DEN) was from Sigma (N0258).

Experimental design and animal treatment:
Fifty female Sprague-Dawley rats weighing 100-125g at arrival (Harlan Sprague Dawley, Indianapolis, IN), were housed three per hanging-wire, stainless-steel cage in a temperature- and light-controlled room and were fed a powdered irradiated unrefined rodent diet (Harlan Teklad, Global 18% protein Rodent Diet 2918) and water ad libitum. The experimental protocols and procedures that involved rats were approved by the Institutional Animal Care and Use committee of the University of Kentucky and were in accordance to all policies for the use and care of laboratory research animals as stipulated by the NIH. Upon arrival, the animals were allowed to adjust for 1 week before starting the experiment. DEN was administrated by gavage, 150mg/Kg body weight. Glycine or casein (casein contains 1.8% glycine) was fed in the unrefined diet at 5% starting on the 14th day after DEN initiation until the end of the experiment. The diet was administered ad libitum in glass feeding cups. On the third day after starting the diets, PCB-77 (300 μmol/kg), PCB-153 (300 μmol/kg), or corn oil were injected i.p. The PCB injections were repeated 3 times every 14 days. Euthanasia was by
overexposure to carbon dioxide, on the 10th day after the last PCB injection. 72 hours before the euthanasia, the rats were surgically implanted with Alzet osmotic pumps containing BrdU (20mg BrdU/ml PBS, 10 µL/h). The livers were immediately removed and weighed; a portion of each was frozen in liquid nitrogen and stored at –80°C until used. For each rat, a piece of liver was randomly taken from 4 different lobes and fixed in 10% buffered formalin. Most of the methods used in these studies are slight modifications of the standard protocols from Dr. Glauert’s laboratory and most of them were first published in detail by Calfee - Mason, (2001).

**Surgical implantation of osmotic pumps:**

**Aim:**
The continuously infused 5-bromo-2’-deoxyuridine, an analog of thymidine, is capable of incorporating into newly replicated DNA during S-phase of the cell cycle and it is a very frequently used marker for in vivo cell proliferation quantification (Wynford-Thomas and Williams, 1986). Anti-5-bromo-2’-deoxyuridine monoclonal antibody, commercially available, can be used to immunohistochemically detect in the liver tissue slices all the cells that have undergone DNA replication during the last 3 days of the experiment, and therefore to estimate the cell proliferation during that time. The administration of the BrdU via osmotic pumps increases the sensitivity compared to the one time s.c. injection.

**Materials:**
- Alzet osmotic pumps (model 2ML1) were obtained from Alza Scientific Products (Palo Alto, CA).
- NaCl (Sigma Chemical Co. S3014)
- KCl (Sigma Chemical Co P- 4504)
- Na₂HPO₄ (Sigma Chemical Co S- 5136)
- KH₂PO₄ (Sigma Chemical Co P- 3786)
- 5-bromo-2’-deoxyuridine (BrdU) (Sigma Chemical Co. B5002)
- Sterilized scissors, forceps, gauze and wound clips (Fisher Scientific 01-804-5 or Becton Dickinson 427631).
**Solutions**

1. Phosphate Buffered Saline (PBS), pH 7.6
   
   (0.8% NaCl, 0.02% KCl, 0.144 % Na$_2$HPO$_4$, 0.024 % KH$_2$PO$_4$) adjusted to pH 7.6 with HCl.

2. 20 mg/ml 5-bromo-2'-deoxyuridine (BrdU)
   
   made in PBS, pH 7.6

Notes:

- BrdU is light sensitive, therefore direct light should be avoided during weighting of the BrdU and BrdU solution should be covered with aluminum foil
- BrdU is not easily soluble in PBS; solution should be heated at 39°C for an hour and shaken to facilitate the BrdU solubilization

**Procedure:**

1. **Pump preparation**
   
   a. We used pumps with the following characteristics: 2ml/pump at 20 mg/ml BrdU with a flow rate of 1 µl/hour for 3 days.
   
   b. The BrdU solution was filtered through a 0.22 µM filter (Costar 8110) into a sterile tube. All procedures from this point were performed in a sterilized manner.
   
   c. 2ml of the sterile BrdU solution were aspirated into a syringe and attached to the needle provided by Alza.
   
   d. BrdU was added carefully to each pump and then the flow restrictor was inserted into the same opening.
   
   e. The pump was kept sterile until the surgery.

2. **Surgical implantation**
   
   a. Three days before the sacrifice date, the pump was inserted subcutaneously into the dorsal side of each rat by an incision on the skin above the hind legs region; the pump was pushed deep into the forelimb region above the spine; inserting of the pump was made with the flow controller (cap) pointing inwards
b. The rats were anesthetized with isofluorane in an Anesthesia System during the procedure.
c. The incision was closed with sterile wound clips.

**PGST and BrdU Immunohistochemical Staining:**

**Aim:**
The preneoplastic foci express a protein normally not expressed in hepatocytes, placental glutathione S transferase (PGST). In order to assay the liver preneoplastic foci, immunostaining of this protein in tissue sections was performed. The rate of replicative DNA synthesis in the foci and in the normal tissue (the rate of hepatocyte proliferation) was determined by immunostaining for 5-bromo-2'-deoxyuridine (BrdU).

**Principle:**
BrdU, a synthetic analog of thymidine can be incorporated into the DNA of replicating cells during the S phase. The PGST is pathologically expressed in transformed cells. Immunohistochemical techniques using a monoclonal antibody against BrdU and a polyclonal antibody against PGST (raised in rabbit) allow for specific detection. Briefly, the primary antibody recognizes and binds the BrdU or the PGST, respectively. The primary antibody is recognized by a biotinylated secondary antibody. This biotin is further recognized by an avidin-biotinylated enzyme macromolecular complex. The enzyme used for the BrdU staining was horseradish peroxidase, the substrate was diaminobenzidine tetrahydrochloride (DAB) and the enzyme for PGST was alkaline phosphatase, the substrate—a phosphate containing substance— and the capture reagent—a substance that binds the soluble product of the alkaline phosphatase catalyzed reaction and forms a colored insoluble precipitate— were supplied by Vector \textsuperscript{®} Red kit.(Hsu et al., 1981b; Hsu et al., 1981a; Gratzner, 1982; Sugihara et al., 1986; Wynford-Thomas and Williams, 1986; Murdoch et al., 1990)

**Reagents:**
1. 10x Automation Buffer (Biomedia Corp - M30)
2. Ammonium hydroxide (J.T. Baker Chemical Co.- 9721)
3. Anti BrdU antibody (Becton Dickinson-347580); (San Jose, CA)
4. 10 X Antigen Retrieval Citra (Bio Genex- HK086-5K)
5. Anti-GST (placenta/acidic) antibody (Novocastra- NCL-GSTpi) (New Castle upon Tyne, England ordered through Vector)
7. Biotinilated anti-rabbit IgG
8. DAB tablet set (Sigma- D4168)
9. 100% ethanol
10. 30% H$_2$O$_2$ (Sigma Chemical Co. H0904)
11. Harris hematoxylin solution, modified (Sigma- HHS-32)
12. 10mM Tris-HCl, pH 7.4-8.0 Sigma T-2663
13. HCl (Sigma- H7020)
15. Hydrogen peroxide (Sigma- H0904)
16. Normal goat serum (Vector- S-1000)
17. Normal horse serum (Vector- S-2000)
18. Permount medium (Fisher Scientific Co.-SP15-500)
19. Sodium chloride (Sigma- S3014)
20. Sodium phosphate dibasic (Sigma Chemical Co S- 5136)
21. Sodium phosphate monobasic (Sigma S- 5011)
22. Vectastain ABC peroxidase kit (mouse IgG) Elite series (Vector- PK-6102) contains: horse normal serum; vectastain A; vectastain B; anti-mouse Ig G biotinylated antibody.
23. Vectastain ABC-AP (alkaline phosphatase) kit standard (Vector- AK-5000) contains reagent A and reagent B
**Solutions:**

1. PBS (phosphate buffered saline), pH 7.4
   - a. 0.026% sodium phosphate (monobasic)
   - b. 0.113% sodium phosphate (dibasic)
   - c. 0.8% sodium chloride

2. 96% Ethanol

3. H$_2$O$_2$ solution (should be made fresh)
   - a. 180 ml methanol
   - b. 20 ml 30% H$_2$O$_2$

4. 1x Antigen Retrieval Citra (diluted fresh)
   - a. 63 ml distilled water
   - b. 7 ml 10x antigen retrieval citra

5. diluted normal horse serum
   - a. 3 drops of normal horse serum (Vector Laboratories Peroxidase Kit PK-6102)
   - b. 10 ml 1x automation buffer

6. diluted primary antibody (BrdU) 1:40

7. diluted biotinylated anti-mouse IgG
   - a. 1 drop of biotinylated IgG (Vector Laboratories Peroxidase Kit PK-6102)
   - b. 3 drops of diluted normal horse serum
   - c. 10 ml 1x automation buffer

8. ABC solution (allow solution to sit 20-30 minutes before use)
   - a. 3 drops of A (Vector Laboratories Peroxidase Kit PK-6102)
   - b. 3 drops of B
   - c. 10 ml 1x automation buffer

9. DAB staining mix (make fresh- supplied by kit)
   - a. Sigma tablet A 1
   - b. Sigma tablet B 1
   - c. 1 ml distilled water

10. Diluted normal goat serum
a. 3 drops of normal serum  
b. 10 ml of 1X automation buffer

11. Biotinylated Anti-rabbit IgG  
a. 1 drop of Biotinylated IgG  
b. 3 drops Normal goat Serum  
c. 10 ml 1X Automation Buffer

12. diluted GSTpi antibody 1:40

13. 1% acid alcohol  
a. 140 ml absolute ethanol  
b. 60 ml distilled water  
c. 2 ml HCl

14. diluted ABC-AP (let stand 20-30 min before use)  
a. 2 drops A  
b. 2 drops B  
c. 10 ml 1X Automation Buffer,

15. Ammonium hydroxide  
a. 200 ml distilled water  
b. 10-15 drops of ammonium hydroxide

16. Vector Red staining mix (made according to Vector protocol)  
a. 5 ml 100 mM tris HCl (pH 8.2-8.5) buffer  
b. 2 drops of reagent 1  
c. mix well  
d. 1 drop of levamisole  
e. mix  
f. 2 drops of reagent 2  
g. mix  
h. 2 drops of reagent 3  
i. mix well

**Procedure:**  
Deparafination and hydratation of samples:
1. The formalin-fixed liver tissues were paraffin-embedded, 5 μm sectioned, and laid on glass slides.

2. The melting of the paraffin was made in a 60°C oven for 30 minutes on a flat surface.

3. The slides were submerged in Hemo De, using a staining jar, for a total of three changes (15 minutes 1st and 5 minutes for 2nd and 3rd change), 100% ethanol for 2 changes (5 minutes each), in 96% ethanol for 5 minutes.

4. The slides were rinsed well with tap water.

**Epitope retrieval and endoperoxidase activity block**

5. The slides were placed in the methanol, hydrogen peroxide solution (made fresh) for 10 minutes.

6. The slides were rinsed well with tap water.

7. While being submerged in 1x antigen retrieval citra, the slides were heated in a microwave for 15 minutes in 5-minute intervals (1st 5 minutes on 20% power and last 10 minutes on 10% power). Caution was taken in making sure the sections were always covered with the citra; the plastic standing reservoir was placed in a beaker and the microwave was stopped from time to time to pour the citra from the glass wear into the reservoir.

8. The slides were cooled in the citra for 30 minutes and then rinsed very gently with tap water 3 times.

**Immunohistochemical detection of BrdU**

9. Diluted normal horse serum was added to the slides for 10 minutes at 37°C (∼3 drops/slide).

10. After the horse serum was drained, the Avidin Block (undiluted) was added to the slides for 15 minutes (2 drops/slide).

11. The slides were dipped briefly in PBS.

12. The Biotin Block was added to the slides for 15 minutes at room temperature (2 drops/slide).

13. The slides were drained (not rinsed).
14. The diluted primary antibody (BrdU) was added to the slides for 30 minutes at 37°C with high humidity (100 µl/slide using a 1:40 dilution of BrdU).
15. The slides were rinsed 2 times with PBS (5 minutes each).
16. Two drops of diluted Biotinylated Anti-mouse IgG were added to each slide for 15 minutes at 37°C.
17. The slides were rinsed twice with PBS (5 minutes each).
18. ABC solution was applied to each slide for 15 minutes at 37°C.
19. The slides were rinsed with PBS twice (5 minutes each).
20. The DAB staining solution was added to each slide for 2-10 minutes at room temperature. This solution was made fresh, and each slide was monitored using a microscope to determine when staining was complete.
21. The slides were gently but thoroughly rinsed in tap water.

**Immunohistochemical detection of PGST**

22. Two drops of the diluted normal goat serum was applied per slide for 10 min at 37°C.
23. After the excess serum was drained, Avidin Block (undiluted) was applied for 15 min, room temperature, 2 drops/slide.
24. The slides were dipped briefly in PBS.
25. Biotin Block (undiluted) was applied for 15 min, room temperature, 2 drops/slide.
26. The slides were drained.
27. The primary antibody (GSTpi) was applied; 100 µl/slide (1:40 diluted in 1X automation buffer), incubate 30 min in a humid 37°C oven.
28. The slides were rinsed in PBS, 2 changes, 5 minutes each.
29. 2 drops/slide of Biotinylated Anti-rabbit IgG, were applied, and let sit 15 minutes in the 37°C humid oven.
30. The slides were rinsed in PBS 2 changes 5 min.
31. 2 drops /each slide of ABC-AP were applied and let sit 15 min at 37°C.
32. The slides were rinsed in PBS 2 changes 5 minutes each.
33. Stain in Vector Red staining mix for 20 to 30 minutes, at room temperature and in darkness.
34. The slides were rinsed in PBS for 5 min
35. The slides were rinsed gently in tap water

**Countrastaining, dehydration and mounting:**
36. The slides were dipped in Hematoxylin (freshly filtered) for 1 minute.
37. The slides were washed in running tap water.
38. The slides were quickly dipped in 1% acid alcohol solution.
39. The slides were dipped several times in tap water.
40. Three dips were made in the ammonium hydroxide solution.
41. The slides were gently rinsed in running tap water for 5 minutes.
42. In order to dehydrate the slides, they were dipped 10 times in each of the following jars: 95% ethanol, 100% ethanol (3 jars) and Hemo De (3 jars).
43. The slides were mounted with Permount medium and covered with cover slips (Clay Adams 3322).
44. The slides were allowed to air dry in darkness before analyzing the imaging.

**Counting of the BrdU-stained nuclei:**
a. Cells that had incorporated BrdU were visualized as having brown nuclei due to diaminobenzidnne (DAB). 3635±549 hepatocellular nuclei per slide were counted randomly, an average of 400 being from PGST-positive foci.
Labeling index (%)=100Xnumber of labeled nuclei / total number of nuclei counted
b. The labeling index was calculated separately for intra- and extra-foci.

**Quantitation of preneoplastic hepatic foci:**
The incidence and growth (number and volume) of PGST-positive foci were measured using a quantitative stereology computer digitizing software system developed at University of Wisconsin in Dr Pitot’s group and graciously offered to us by them. The images were captured using a Nikon microscope. The number of foci/cm$^3$ (Saltykov method), foci/liver (Saltykov method), the mean focal
volume (Saltykov method), and the volume fraction (Delesse method) were analyzed.

**Statistical analysis:**
The results for BrdU labeling index were analyzed by ANOVA test. The number of foci per liver and number of foci per cubic centimeter data was analyzed by negative binomial regression model with logarithm as the link function by Dr Srinivasan, Department of Statistics, UK. The statistical comparison was made between the PCB treatment groups with the respective corn oil groups (control groups), separately for casein and glycine diets. The goodness of fit of the model was assessed by Pearson $X^2$ value adjusted for overdispersion and the parameters of the model were estimated by the method of maximum likelihood. The Wald's asymptotic procedure was used to determine the p values for significance of the differences between the PCB treatment groups and the control groups. The statistical software PROC GENMOD of SAS, version 8, was used to carry out the above mentioned statistical analyses. We used the negative binomial regression model for the analysis of data obtained for the number of foci per liver and number of foci per cubic centimeter because of the discrete nature of these measurements and it was published that for analyzing discrete measurements negative binomial regression model or Poisson model are more appropriate (Espandiar et al., 2003). The data for the mean focal volume and the volume fraction were analyzed by two way analysis of variance. Individual differences between means were determined using the Bonferroni post hoc test. The results were expressed as means ± standard error of the mean (SEM). The results were considered significant at p<0.05.

**Protein assay:**

**Principle:** The protein contained in the sample reduces Cu$^{+2}$ to Cu$^{+1}$ in an alkaline environment (Reagent A). Cu$^{+1}$ newly formed is chelatated by two molecules of bicinchoninic acid-BCA-(Reagent B) forming a purple complex, with absorbance at 562 nm.
**Reagents:**

- Reagent A (Pierce Chemical Company 23223)
- Reagent B (Pierce Chemical Company 23224)
- Standards – BSA (bovine serum albumin, fraction V) (Pierce Chemical Company 23209)

**Procedure:**

- 2mg/ml BSA stock solution was diluted with PBS to the working standard concentrations: 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml.
- 190 µl of mixed reagent (1 part Reagent A and 49 parts Reagent B) was added to each well of a 96-well plate.
- 10 µl of each diluted sample and 10 µl of each standard was added to each well in duplicate.
- The 96-well plate was mixed and then incubated for 30 minutes at 37°C.
- The plate was cooled to room temperature for 5 minutes and read at 562 nm with a Bio-Tek plate reader Model 312 (Bio-Tek Instruments, Inc.). Using the KinetiCalc software (Bio-Tek Instruments, Inc.), the concentration of protein (mg/ml) was calculated for the samples based on the standard curve and considering the average of the values for the duplicates.

**Isolation of nuclear extracts:**

Nuclear extracts were prepared from frozen liver tissue by a modification of the method of Deryckere and Gannon (Deryckere and Gannon, 1994).

**Reagents:**

1. Aprotinin (Sigma Chemical Co. A1153)
2. autoclaved nanopure water
3. Benzamidine (Sigma Chemical Co. B6506)
4. DMSO (Sigma Chemical Co. D8418)
5. DTT (Bio-Rad 161-0611)
6. EDTA (Sigma Chemical Co. E5134)
7. 100% Ethanol (Aaper alcohol and chemical Co., Shelbyville, Kentucky)
8. Glycerol (Sigma Chemical Co. G5516)
9. (N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]) Hepes (Sigma Chemical Co. H4034)
10. IGEPAL CA-630 (Sigma Chemical Co. I3021)
11. KOH pellets (Sigma Chemical Co P-6310)
12. Leupeptin (Sigma Chemical Co. L2884)
13. MgCl₂ (Sigma Chemical Co. M2670)
14. NaCl (Sigma Chemical Co. S3014)
15. Pepstatin A (Sigma Chemical Co. P4265)
16. PMSF (Boehringer Mannheim 837 091)

Stock solutions:
1. 5 M NaCl
2. 1 M Hepes-KOH, pH 7.9
3. 0.5 M EDTA, pH 8.0
4. 1 M MgCl₂
5. 0.1 M Phenylmethylsulfonyl fluoride (PMSF) made in ethanol. NOTE: The half-life in solution with water is less than 2 hours.
6. 0.1 M Dithiothreitol (DTT)
7. 0.1 M Benzamidine
8. 0.33% mg/ml protease inhibitor cocktail
   a. 1 mg aprotinin
   b. 1 mg leupeptin
   c. 1 mg pepstatin A (dissolved first in 100 µl DMSO)
   d. 3 ml H₂O

Buffers:
1. Buffer A: homogenization buffer
<table>
<thead>
<tr>
<th>Stock</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGEPAL CA-630</td>
<td>0.6%</td>
</tr>
<tr>
<td>5M NaCl</td>
<td>150 mM</td>
</tr>
<tr>
<td>1 M Hepes, pH 7.9</td>
<td>10 mM</td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>1 mM</td>
</tr>
<tr>
<td>0.1 M PMSF (added last)</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>Sterilized water</td>
<td></td>
</tr>
</tbody>
</table>

2. Buffer C: extraction buffer

<table>
<thead>
<tr>
<th>Stock</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% Glycerol</td>
<td>25% Glycerol</td>
</tr>
<tr>
<td>1 M HEPES-KOH, pH 7.9</td>
<td>20 mM</td>
</tr>
<tr>
<td>5 M NaCl</td>
<td>420 mM</td>
</tr>
<tr>
<td>1 M MgCl$_2$</td>
<td>1.2 mM</td>
</tr>
<tr>
<td>0.05 M EDTA</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>0.1 M PMSF</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>0.1 M DTT</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>0.1 M Benzamidine</td>
<td>2 mM</td>
</tr>
<tr>
<td>0.33 mg/ml aprotinin</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>sterilized water</td>
<td></td>
</tr>
</tbody>
</table>

Note: Buffer A and Buffer C may be prepared in advance and stored at 4°C, except for protease inhibitors, DTT, benzamidine that should be added shortly prior use because they are not stable in aqueous solutions.

**Procedure:**

**Nuclei isolation**
1. The liver taken from the -80°C freezer was broken into smaller pieces avoiding thawing.

2. Approximately 0.5 g of tissue and 5 ml of cold Buffer A were added to a 15-ml Dounce tissue homogenizer on ice. Eight strokes were made and the homogenate was transferred to a 15 ml tube.

3. The homogenate was centrifuged at 270 x g for 1 min to get rid of unbroken tissue (1000 rpm for Mistral 3000i centrifuge).

4. The supernatant was transferred to a 15 ml tube and kept on ice for at least 5 minutes.

5. The supernatant was centrifuged at 2980 x g for 20 minutes (3600 rpm for Mistral 3000i centrifuge).

6. During the 20 minute spin, protease inhibitors, DTT, benzamidine were added to the incompletely buffer C that was stored at 4°C.

7. The supernatant was discarded. The walls of the tube were cleaned with an autoclaved cotton swab in order to rid the fat that clings to the walls.

8. The pellet was resuspended in 1 ml of buffer A and transferred to a microcentrifuge tube.

9. The resuspended pellet was centrifuged at 7,000 rpm for 3 minutes.

**Nuclear extract isolation**

10. The supernatant was discarded, and the pellet was resuspended with buffer C and kept on ice for 1 hour for high-salt extraction.

11. The tubes were centrifugated at 14000 rpm for 10 minutes in microcentrifuge to get rid of the nuclear membranes. The nuclear extract (the supernatant) was aliquoted and stored at -80°C. One aliquot was diluted 1:3 in PBS to measure the protein concentration using the BCA method (Pierce method).

**Radiolabeling and purification of NF-κB oligonucleotide (probe synthesis)**

*Principle:*

T4-polynucleotide kinase is used to label the 5'-OH group of the NF-κB oligonucleotide with [γ-32P] ATP.
**Reagents**

1. \([\gamma^{32}P] \text{ATP- } 6000\text{Ci/mmol at 10mCi/ml (NEN Life Science BLU/NEG/002Z)}\)
2. 0.5 M EDTA, pH 8.0
3. nuclease free water
4. NF-κB consensus oligonucleotide:  (Promega Corporation E329A) (Madison, WI)
   
   NF-κB (5’-AGT TGA GGG GAC TTT CCC AGG C-3’)
5. T4 Polynucleotide Kinase (New England Biolabs M0201S)
6. T4 Polynucleotide Kinase Buffer (10x) – supplied with T4 Polynucleotide Kinase
7. Tris-HCl, pH 7.5
8. Bio-gel column (Bio-Rad Laboratories, Richmond, CA)

**Solutions:**

1. TE buffer (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA)

**Procedure:**

a. The **phosphorylation reaction** was prepared in 3 sterile microcentrifuge tubes. The following were added to each tube:

   - Nuclease-free water 5 µl
   - NF-κB consensus oligonucleotide (1.75 pmol/µl) 2 µl
   - T4 Polynucleotide Kinase 10x Buffer 1 µl
   - \([\gamma^{32}P] \text{ATP} \) 1 µl
   - T4 Polynucleotide Kinase (10 U/µl) 1 µl

b. The reactions were incubated at 37°C for 15 minutes.

c. Each reaction was stopped with 1 µl of 0.5 M EDTA.

d. The contents of the tubes were combined and 1 µl phenol red was added.
Purification of Labeled Probe

The products of the above mentioned reaction (the labeled oligonucleotide mixed with unincorporated nucleotides) were purified by passing the reactions through a Bio-gel column (Bio-Rad Laboratories, Richmond, CA). The components of the 3 reactions (mixed together) were added to the center of the column and washed with TE buffer. The radioactive peak was collected, and 1 µl was counted using a liquid scintillation counter. The average cpm for 1 µl was 50,000. The radiolabeled probe was stored at -20°C until use.

Electrophoretic mobility shift assay (EMSA):

Aim:

NF-κB DNA binding activities were measured using electrophoretic mobility shift assay.

Principle:

NF-κB transcriptional factor present in the nucleus interacts with the radioactive-labeled sequence specific oligonucleotide and the products of the protein/DNA specific binding are separated by the rest of the components of the reaction mix by nondenaturing polyacrylamide gel electrophoresis. In order to verify DNA specific binding, antibodies to the p50 and p65 subunits of NF-κB were added to the reaction resulting in a shifted band.

Reagents:

1. 30% Acrylamide (29:1) (Bio-Rad Laboratories 161-0156)
2. Ammonium Persulfate (Sigma Chemical Co. A9164)
3. Autoclaved nanopure water
4. IGEPAL CA-630 (Sigma Chemical Co. I3021)
5. KCl (Sigma Chemical Co. P4504)
7. p50 (sc-114x) antibody, Santa Cruz,
8. p65 (sc-372G) antibody, Santa Cruz
9. Tris-HCl 1M pH 7.4 Sigma T 2663
10. 0.5 M EDTA pH8 Fluka 03690
11. DTT (Bio-Rad 161-0611)
12. 100% Glycerol (Sigma Chemical Co. G5516)
13. TEMED (Sigma Chemical Co. T7024)
14. 10x TBE (Invitrogen 15581-044) Carlsbad, CA

**Solutions:**
1. probe obtained previously
2. nuclear extract obtained previously
3. 10% Ammonium Persulfate
4. Poly (dl-dC)-(dl-dC) was dissolved in TE (10 mM Tris- HCl and 1 mM EDTA, pH 7.5) to the concentration of 2mg/ml and stored to -20°C until use
5. 1M KCl
6. 0.1 M Dithiothreitol (DTT)
7. 5x NF-κB DNA binding buffer (according to Promega):

<table>
<thead>
<tr>
<th>Stock</th>
<th>5x binding buffer (stock)</th>
<th>1x binding buffer (final concentration in reaction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Hapes, pH 7.9</td>
<td>50 mM</td>
<td>10 mM</td>
</tr>
<tr>
<td>1 M KCl</td>
<td>250 mM KCl</td>
<td>50 mM KCl</td>
</tr>
<tr>
<td>0.5 M EDTA</td>
<td>1 mM EDTA</td>
<td>0.2 mM EDTA</td>
</tr>
<tr>
<td>0.1 M DTT</td>
<td>12.5 mM DTT</td>
<td>2.5 mM DTT</td>
</tr>
<tr>
<td>100% glycerol</td>
<td>50% glycerol</td>
<td>10% glycerol</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------</td>
<td>--------------</td>
</tr>
<tr>
<td>IGEPAL CA-630</td>
<td>0.25% IGEPAL CA-630</td>
<td>0.05% IGEPAL CA-630</td>
</tr>
<tr>
<td>0.22 µM Filtered H₂O</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Procedure:**

a. 7% polyacrylamide gel was prepared (17.0 x 15 cm, 0.75 thickness)

<table>
<thead>
<tr>
<th>30 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>water</td>
</tr>
<tr>
<td>30% acrylamide/bis(29:1)</td>
</tr>
<tr>
<td>TBE (10x)</td>
</tr>
<tr>
<td>10% APS</td>
</tr>
<tr>
<td>TEMED</td>
</tr>
</tbody>
</table>

The gel was allowed to solidify for at least 45 minutes.

b. The reaction was prepared:

- Nuclear extracts  3 µg
- 5x binding buffer  4 µl
- Poly (dl-dC)  1 µg for the EMSA with individual samples or 0.8 µg for the EMSA with the pooled samples

Note: we performed titration reactions using Poly (dl-dC) 0.6, 0.8, 1 µg per reaction and protein 3 µg, to determine the optimal report for these 2 reactants for each new batch of Poly (dl-dC) or nuclear extract.

- NF-κB probe  2 µl (100,000 cpm)
- Water  brought total volume to 20 µl

c. All contents except for the radiolabeled probe were pre-incubated on ice for 5 minutes. The radiolabeled NF-κB probe was added to the reaction and incubated for 20 minutes at 37°C.
d. **Supershift**: In order to confirm binding specificity of NF-κB, 1 µg of antibody raised against p50, p65, or nonlabeled oligo (cold competition) were added to the reaction before adding the NF-κB probe (during the 10 minute pre-incubation).

e. **Electrophoresis**: The comb was removed and the wells were rinsed with 0.5 X TBE buffer using a needle and syringe. The 20 µl reaction mixture was added to each well of the 7% polyacrylamide gel and electrophoresed for 1.5 hours at 175 volts using 0.5 X TBE as the running buffer.

f. **Results**: After removing the gel from the running apparatus, the gel was attached to filter paper and covered with plastic wrap. The gel was dried under vacuum and exposed overnight at -80°C to Kodak XOMAT-AR film. Gels were then re-exposed to a phosphorimaging screen and analyzed using the STORM Phosphoimager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant 5.0 software (Molecular Dynamics).

g. **Statistical analysis**: sample counts were analyzed using ANOVA followed by Bonferroni for examination of differences in treatment means using the statistical software package Systat V.8 (SPSS, INC).

---

**Western blotting:**

**Reagents:**

1. Tris base (Boehringer Mannheim 1814 273)
2. Sodium dodecyl sulfate (SDS) (Sigma Chemical Co. L4390)
3. Phenylmethylsulfonyl fluoride (PMSF) (Boehringer Mannheim 837 091)
4. Aprotinin (Sigma Chemical Co. A1153)
5. Leupeptin (Sigma Chemical Co. L2884)
6. Pepstatin A (Sigma Chemical Co. P4265)
7. DMSO (Sigma Chemical Co. D8779)
8. Na₂HPO₄ (Sigma Chemical Co. S5136)
9. NaH₂PO₄ (Sigma Chemical Co. S5011)
10. NaCl (Sigma Chemical Co. S3014)
11. NaOH (Sigma Chemical Co. S0899)
12. Glycerol (Sigma Chemical Co. G5516)
13. 14.3 M β-mercaptoethanol (Sigma Chemical Co. M6250)
14. bromophenol blue
15. Glycine (Gibco 15527-013)
16. Tween-20 (Sigma Chemical Co. P7949)
17. Ponceau S (Sigma Chemical Co. P3504)
18. trichloroacetic acid (Sigma Chemical Co. T4885)
19. sulfosalicylic acid (Sigma Chemical Co. S0640)

**Solutions:**
1. 1 M Tris-HCl, pH 6.8; stored at 4°C
2. 1.5 M Tris-HCl, pH 8.8; stored at 4°C
3. 10% Sodium dodecyl sulfate (SDS) (w/v); stored at room temperature
4. 10 mg/ml Phenylmethylsulfonyl fluoride (PMSF)
   Dissolved in 100% ethanol
5. 0.33 µg/µl protease inhibitor cocktail
   1 mg aprotinin
   1 mg leupeptin
   1 mg pepstatin A dissolved in 100 µl DMSO
   bring all mixture to 3 ml with autoclaved nanopure water

**Buffers:**
1. 1 x Phosphate Buffered Saline (PBS), pH 7.4

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>9.1 mM</td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td>1.7 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>150 mM</td>
</tr>
</tbody>
</table>

Adjusted pH to 7.4 with NaOH
2. Lysis/homogenization buffer

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonidet P-40 (Sigma Chemical Co. N6507)</td>
<td>1%</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.1%</td>
</tr>
<tr>
<td>10 mg/ml PMSF</td>
<td>0.1 mg/ml</td>
</tr>
<tr>
<td>Protease inhibitors cocktail</td>
<td>2 µg/ml</td>
</tr>
<tr>
<td>1x PBS</td>
<td></td>
</tr>
</tbody>
</table>

3. Gel loading buffer (2x): according to Santa Cruz

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>17.3%</td>
</tr>
<tr>
<td>14.3 M β-mercaptoethanol</td>
<td>1.25 M</td>
</tr>
<tr>
<td>10% SDS</td>
<td>5.2%</td>
</tr>
<tr>
<td>1 M Tris pH 6.8</td>
<td>0.22 M</td>
</tr>
<tr>
<td>1-2 mg bromophenol blue</td>
<td></td>
</tr>
</tbody>
</table>

Should be made fresh and kept at room temperature for the day or a stock can be made and kept at -20°C, if the mercaptoethanol is not added.

4. 10x Running buffer:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris base</td>
<td>250 mM</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.92 M</td>
</tr>
</tbody>
</table>
SDS | 1%  
---|---
Can be kept at room temperature

5. 1x Transfer Buffer:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>188 mM</td>
</tr>
<tr>
<td>Tris base</td>
<td>24.8 mM</td>
</tr>
<tr>
<td>100% methanol</td>
<td>20%</td>
</tr>
</tbody>
</table>

Made fresh and kept at -20°C until use

6. 10x TBS (pH 7.5):

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-base</td>
<td>10 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>150 mM</td>
</tr>
</tbody>
</table>

pH with conc HCl to 7.5; can be kept at room temperature

7. Wash buffer (TTBS):

1x TBS (1 liter)

500 µl Tween-20 (0.05% final concentration)

Should be stored at 4°C

8. 5% Blocking buffer (w/v):

10 g fat-free dry instant powdered milk

200 ml TTBS buffer

Can be stored at 4°C up to a week

9. 10x Ponceau S stain

2 g Ponceau S

30 g trichloroacetic acid
Procedure:

1. Sample preparation

From each experimental group, two animals were randomly taken. The nuclear extracts from the livers of these animals were made as described for EMSA. The correspondent volume of nuclear extract for 13 µg protein was taken from each sample and the two samples from the same group were pooled. 3 µg of the pooled sample was used for pooled-sample EMSA and the remaining 23 µg were used for Western.

The pooled samples for Western from each group were brought to the same volume with autoclaved nanopure water. An equal volume of the gel loading buffer (2x) was added mixed together and placed in boiling water for 3 to 5 minutes to denature the proteins. The samples were immediately loaded onto a gel.

2. Gel electrophoresis

For the analysis of p50 and p65 proteins from the nuclear extract, a vertical acrylamide gel (17.0 x 15 cm, 0.75 thickness), made of 8% separating gel (bottom) and 4% stacking gel was used to run the samples.

<table>
<thead>
<tr>
<th>Component</th>
<th>8%</th>
</tr>
</thead>
<tbody>
<tr>
<td>DiH₂O</td>
<td>11.68 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH 8.8</td>
<td>6.25 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>250 µl</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>6.67 ml</td>
</tr>
</tbody>
</table>
10% APS | 125 µl
TEMED | 25 µl
Total | 25 ml

Stacking Gel

<table>
<thead>
<tr>
<th>Component</th>
<th>4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>diH₂O</td>
<td>10.94 ml</td>
</tr>
<tr>
<td>1.0 M Tris-HCl, pH 6.8</td>
<td>1.88 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>150 µl</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>1.95 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>75 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>15 µl</td>
</tr>
<tr>
<td>Total</td>
<td>15 ml</td>
</tr>
</tbody>
</table>

The proteins were electrophoresed with 1x running buffer at 175 volts for 2 hours

2. Protein Transfer

a. The gel was removed between the electrophoresis glasses, and the stacking gel was trimmed from the separating gel and discarded. The gel was cut after electrophoresis to fit the Mini Trans-Blot Transfer Unit (Bio-Rad). Using filter paper to adhere to the gel, the gel was moved to a pre-cooled transfer buffer.
b. All components of the transfer unit were pre-soaked in cold transfer buffer for at least 15 minutes before starting the transfer of the proteins from the gel to the nitrocellulose membrane (Gibco 11467-016).

c. The transfer cassette was assembled in a container of cold transfer buffer as indicated in the manual.

d. The cassette was placed in the transfer unit with a stir bar, ice block, and transfer buffer.

e. The entire transfer box was placed in a Pyrex dish filled with ice. Cold buffer and the stirring of the buffer during transfer prevent the buffer from overheating.

f. The parameters of transfer were: 100 volts for 1 hour.

g. The membranes were rinsed in wash buffer and a successful transfer was checked by 1) being able to see the pre-stained biomarker and 2) the membrane was stained with 1x Ponceau S stain. The Ponceau S stain was easily removed with wash buffer.

3. Blocking of the membranes

a. It was used 5% blocking buffer for 1 hour at room temperature on the shaker.

b. After one hour, the membranes were rinsed once for 5 minutes with 1x wash buffer.

4. Primary antibody labeling

a. Each primary antibody was diluted to 0.2 $\mu$g/ml in 5% blocking buffer with a total volume of 20 ml and added for 1 hour to the membranes (on the shaker, room temperature). The following primary antibodies were examined:

- NF-$\kappa$B p65(C-20)-G, Santa Cruz sc-372-G, a goat affinity purified polyclonal antibody raised against a peptide mapping at the carboxy terminus of NFkB p65 of human origin (identical to corresponding mouse sequence)
- NF-$\kappa$B p50(C-19)X, Santa Cruz sc-1190X, a goat affinity purified polyclonal antibody raised against a peptide mapping at the carboxy terminus of NFkB p50 of human origin (identical to corresponding mouse sequence)
b. The membranes were rinsed with the wash buffer 2 times for 5 minutes and once for 15 minutes.

5. Secondary antibody
a. The membranes were incubated with a donkey anti-goat IgG HRP-conjugated secondary antibody (Santa Cruz sc-2020) for one hour at room temperature on the shaker in 20 ml total volume. The concentration was 80 ng/ml. The membranes were rinsed with wash buffer two times for five minutes each and 2 times for 15 minutes each.

6. Chemiluminescence
a. Ten ml of the peroxide solution and luminol/enhancer solution from Pierce SuperSignal West Pico Chemiluminescent Substrate Kit (34080) were mixed together and added to each membrane for 5 min.
b. The membranes were covered in plastic wrap and exposed to Kodak XOMAT-AR film for 1 minute.
c. The membranes were placed back into the wash buffer and later stripped of the antibodies and detection reagents.
d. The procedure for stripping the membranes was as follows: in a 50 ml tube, 42.55 ml of 0.235 M Glycine pH 2.2, 5 ml 5M NaCl, 2.45 ml sH$_2$O were mixed together. The membranes were placed in a pre-heated hybridization oven for 30 minutes at 50°C. The membranes were removed from the glycine buffer and washed with 0.1M NaOH for 10 minutes at room temperature with shaking. The membranes were rinsed in wash buffer and the proteins were checked with 1x Ponceau stain. The membrane was either dried and stored or re-probed with another antibody.

Preparation of whole liver homogenate (WLH):

Materials:
EDTA (Gibco, 15575-038)
KCl (Sigma Chemical Co. P4504)

Procedure:
Frozen liver pieces were homogenized in 4°C cooled homogenization buffer (1.15% KCl with 0.1 mM EDTA, pH 7.4) with an Ultra-Turrax homogenizer (Tekmar, Cincinnati, OH) on ice as following:

1. The frozen liver tissues were removed from the -80°C freezer and ≈ 0.3 g of tissue was weighed and placed in 13 ml tubes (rounded base)
2. 2 ml homogenization buffer were added to the tube.
3. The liver pieces were homogenized for 2 times, 10 seconds each at 60 output using the Ultra-Turrax homogenizer, with 10 seconds break between them.
4. The sample was kept in ice at all times, and the homogenizer was cleaned with deionized water between every sample.

Cytosolic and microsomal isolation:
The microsomal fraction was isolated from the whole-liver homogenate using the method described by Schramm (Schramm et al., 1985)

1. Approximately 1ml of the WLH was diluted with 2 ml 1.15% KCl-0.1 mM EDTA buffer.
2. The diluted WLH was centrifuged for 20 minutes at 10,000 x g.
3. The pellet was discarded, and the supernatant was transferred to a thick-walled tube suitable for ultracentrifugation (Nalgene 3425-1613) and equalized.
4. Using the Beckman 70.1 Ti rotor, the samples were centrifugated for 1 hour at 100,000 x g (33,000 rpm).
5. The supernatant (cytosol) was collected and stored at -80°C.
6. The microsomal fraction, the pellet, was resuspended in 1 ml 1.15% KCl-0.1 mM EDTA, aliquoted in 5 tubes and stored at -80°C.
7. The protein concentration of the microsomes (diluted 1:10 in PBS) was determined (by Pierce method)
Alkoxyresorufin O-dealkylation assay

**Principle:**

7-Benzyloxyresorufin and ethoxyresorufin were used as specific substrate for the Cytochrome P-450 2B1/2 and P-450 1A1 isozymes respectively of the microsomal fraction in the alkoxyresorufin O-dealkylation method (Burke and Mayer, 1974b; Burke et al., 1985). The absorbance of resorufin newly formed can be detected by a fluorometer at an excitation wavelength of 530 nm and an emission wavelength of 590 nm.

**Reagents:**

1. DMSO (Sigma Chemical Co. D8779)
2. Na$_2$HPO$_4$ (Sigma Chemical Co. S5136)
3. NaH$_2$PO$_4$ (Sigma Chemical Co. S5011)
4. Resorufin (Sigma Chemical Co: R3257)
5. 7-Benzyloxyresorufin (Sigma Chemical Co. B1532)
6. Ethoxyresorufin (Sigma Chemical Co. E3763)
7. β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt; (Sigma Chemical Co. N 1630)
8. Acetonitrile (EM Science, UN 1648, Gibbstown, NJ)

**Solutions:**

Note: All reagents should be kept on ice; avoid direct light.

1. Phosphate buffer 50 mM, pH 8.0
2. Resorufin stock solution 1 mM in DMSO
3. Ethoxyresorufin stock solution 1 mM in DMSO
4. Benzyloxyresorufin stock solution 1 mM in DMSO
5. Standard Curve Dilutions
### Resorufin standard curve (100 µl work dilution 100 µM (1:10))

<table>
<thead>
<tr>
<th>Tube</th>
<th>Concentration (pmol/µl)</th>
<th>Resorufin in 10 µl (pmol)</th>
<th>Work Solution (µl)</th>
<th>Methanol (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>30</td>
<td>300</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>11</td>
<td>20</td>
<td>200</td>
<td>20</td>
<td>80</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>100</td>
<td>10 (15)</td>
<td>90 (135)</td>
</tr>
<tr>
<td>9</td>
<td>7.5</td>
<td>75</td>
<td>7.5</td>
<td>92.5</td>
</tr>
<tr>
<td>8</td>
<td>5</td>
<td>50</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>7</td>
<td>2.5</td>
<td>25</td>
<td>2.5</td>
<td>97.5</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>10</td>
<td>10 (15)</td>
<td>90 (135)</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>5</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>4</td>
<td>0.25</td>
<td>2.5</td>
<td>2.5</td>
<td>97.5</td>
</tr>
<tr>
<td>3</td>
<td>0.1</td>
<td>1</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>0.05</td>
<td>0.5</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

#### 6. Master mix:
- 34.34 mg NADPH
- 27.8 ml Phosphate buffer
- 377.8 µl Ethoxyresorufin
- 1.84 ml MeOH

#### Procedure:

Note: each, sample and standard is in duplicate on the plate

1. The phosphate buffer is added to the 96 plate well
   - 76 µl for standards and samples
   - 86 µl for blank
2. 10 µl diluted resorufin is added to the wells for standards
3. 10 µl samples are added to the wells for samples
4. 135 µl master mix is added to all the wells
5. The plates are incubated at 37°C for 30 minutes in dark
6. 50 µl acetonitrile is added in all the wells to stop the reaction
7. The plates are read in the fluorimeter after 15 min to scan for resorufin with a 530 nm excitation filter and a 590 nm emission filter and cut off at 590 nm.
8. The readings for the samples are interpolated on the standard curve by the fluorimeter’s software SOFT max PRO.

**Statistical analysis:**

All statistical analyses were performed using SYSTAT V.8 (SPSS, Chicago, IL) software. Results were first analyzed by two-way analysis of variance (ANOVA). Individual differences between the treatment means were determined with the Bonferroni post-hoc test. The results were reported as means ± standard error of the mean (SEM). Results were defined as statistically significant for P<0.05.
**Results**

The body weights were not significantly modified by any of the treatments (Fig. 2.1). The relative liver weights were significantly increased in the rats treated with PCB-77 but not with PCB-153; glycine treatment had no effect (Fig. 2.2).

PCB-77 significantly increased the number of PGST-positive foci per liver and per cubic centimeter as well as the mean focal volume and the total focal volume as a percentage of liver volume both in the casein and in the glycine groups (Table 2.1.). PCB-153 increased significantly the number of foci per liver compared to the corn oil group in the casein diet only, whereas in the glycine diet, the number of foci was only slightly increased (p=0.2). The number of foci per cubic centimeter was slightly increased in the casein diet by PCB-153 (p=0.08) but not for the glycine diet. PCB-153 did not significantly increase the mean focal volume nor the percentage of liver occupied by foci. There were no significant differences between the casein and glycine diets in rats administered vehicle, PCB-77, or PCB-153.

To measure hepatocyte proliferation in normal and PGST-positive hepatocytes, we quantified the labeling indexes after 3 days of continuous BrdU infusion using Alzet pumps. The BrdU labeling index was similar among all groups in both normal hepatocytes and PGST-positive hepatocytes. The labeling index was not different between different lobes of the same animal, regardless of the treatment. As expected, cell proliferation was higher in the PGST-positive foci than in the normal tissue. Interestingly, there were some clustered BrdU positive hepatocytes (as presented in Fig. 2.5 c) that were not positive for PGST, suggesting that maybe another type of foci were present, for example GGT-positive or ATPase-negative foci. Glycine did not significantly decrease hepatocyte proliferation.

NF-κB DNA-binding activity (assayed by EMSA) was not affected by PCB treatment, nor by glycine. We performed Western blotting for p50 and p65 from the same pool of nuclear extract as we did EMSA to verify the lack of change in
this transcription factor and found no significant differences between treatments, but it was surprising to find an important quantity of p105. The specificity of the NF-κB band on the EMSA was demonstrated using cold competition and supershift analyses.

Dietary glycine augmented the PCB-induced increases in cytochrome P-450 related enzyme activities for both PCB-153 and 77, but the level of significance was reached only by PCB-153. BROD and EROD activities were induced by PCB-153 and PCB-77 respectively.
Figure 2.1 Effect of PCBs and glycine on body weight.

Results are expressed as mean ± SEM (n=6-9). The values were analyzed by ANOVA and the level of significance was p<0.05.
Figure 2.2 Effect of PCBs and glycine on relative liver weight.

Results are expressed as mean ± SEM (n=6-9). The values were analyzed by ANOVA followed by Bonferroni, and the level of significance was p<0.05.
Table 2.1 Effect of PCBs and glycine on the induction of PGST-positive foci

<table>
<thead>
<tr>
<th>Glycine</th>
<th>Treatment (nr of animals)</th>
<th>Foci/cm³</th>
<th>Foci/liver</th>
<th>Mean focal volume (mm³ x 10⁻³)</th>
<th>Focal volume (% of liver volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>Corn oil(9)</td>
<td>2258±285</td>
<td>23658±3371</td>
<td>3.0±0.4</td>
<td>0.6±0.09</td>
</tr>
<tr>
<td></td>
<td>PCB-77(9)</td>
<td>7462±910*</td>
<td>104667±13003*</td>
<td>4.4±0.5*</td>
<td>3.3±0.5*</td>
</tr>
<tr>
<td></td>
<td>PCB-153(8)</td>
<td>3033±319</td>
<td>36093±5301*</td>
<td>4.1±0.4</td>
<td>1.3±0.2</td>
</tr>
<tr>
<td>Present</td>
<td>Corn oil(9)</td>
<td>2692±202</td>
<td>29190±2246</td>
<td>3.3±0.4</td>
<td>0.9±0.1</td>
</tr>
<tr>
<td></td>
<td>PCB-77(9)</td>
<td>7054±384*</td>
<td>92801±6295*</td>
<td>4.4±0.5*</td>
<td>3.1±0.3*</td>
</tr>
<tr>
<td></td>
<td>PCB-153(6)</td>
<td>2976±563</td>
<td>35629±7790</td>
<td>3.1±0.6</td>
<td>1.0±0.3</td>
</tr>
</tbody>
</table>

Results are expressed as mean±SEM.

* Values are significantly different from their respective controls treated with corn oil (p<0.05). The statistical analysis is detailed in the materials and methods part.
Figure 2.3 Quantitation of hepatocyte proliferation in normal tissue.

The labeling index was determined by BrdU immunohistochemical staining. Values represent mean ± SEM with n = 6-9. The statistical level of significance was p<0.05.
Figure 2.4 Quantitation of hepatocyte proliferation in PGST-positive foci.

The labeling index was determined by BrdU immunohistochemical staining.

Values represent mean ± SEM with n = 6-9. The statistical level of significance was p<0.05.
Figure 2.5 Immunohistochemically stained rat liver tissue.

a. PGST-positive foci
b. BrdU positive hepatocytes inside the PGST-expressing preneoplastic foci and into the normal tissue
c. Clustered BrdU positive hepatocytes, negative for PGST.
Figure 2.6. Effect of PCBs and glycine on the rat hepatic DNA binding activity of NF-κB. NF-κB DNA binding activity was determined using the electrophoretic mobility shift assay as described in the Materials and Methods. Each line contains extract from a single animal.
Figure 2.7. Determination of NF-κB specific binding.

Electrophoretic mobility shift assay was performed using the same NF-κB radiolabeled oligonucleotide used for the assay presented in figure 2.6, nuclear extract (7µg protein) from a PCB-153 treated animal on a casein diet, anti-p50 and anti-p65 antibodies. The first lane contains the radiolabeled NF-κB probe with no nuclear extract; the second line contains all the components of the reaction used in figure 2.6; the third line differs of the second line by having an excess of unlabeled NF-κB oligonucleotide (cold competition). The last two lanes were incubated with antibodies to p50, and p65, respectively.
Figure 2.8. Effect of PCBs and glycine on the rat hepatic DNA binding activity of NF-κB. NF-κB DNA binding activity was determined using the electrophoretic mobility shift assay as described in the Materials and Methods. Each line contains pooled nuclear extract from 2 animals belonging to the same group; from the same pool, some nuclear extract was used for this EMSA, and some was used for Western.
Figure 2.9. Protein levels of nuclear p50 and p65; Western analysis.
Figure 2.10 Effect of PCBs and glycine on ethoxyresorufin-O-dealkylase (EROD) activity in rat liver microsomes.
Values represent means ± SEM for 6-9 rats. * Significantly different from controls (p<0.05)
Figure 2.11  Effect of PCBs and glycine on 7-benzoyloxyresorufin-O-dealkylase (BROD) activity in rat liver microsomes.
Values represent means ± SEM for 6-9 rats. Values with different letters are significantly different from one another (p<0.05)
Discussion

In this study, we tested the hypothesis that the secretory activity of Kupffer cell is required for the promoting activity of polychlorinated biphenyls. For this, we examined the ability of dietary glycine to inhibit PCB-induced tumor promotion, as glycine was previously reported to be able to inhibit the secretory activity of Kupffer cells (Rose et al., 1997a).

Cell proliferation was not increased by PCB treatment in this study neither in the normal hepatocytes nor in the PGST-positive hepatocytes. As expected, cell proliferation was higher in the PGST-positive foci than in the normal tissue. Former studies using the same protocol on rats on the same strain, sex and age (Berberian et al., 1995; Tharappel et al., 2002; Glauert et al., 2005), found that cell proliferation in both focal and nonfocal hepatocytes was increased by PCB-77 (unlike our study) but was not affected by PCB-153 (as in our study).

In this study, the PGST-positive foci were strongly promoted by both PCBs. In previous studies using the same protocol, (Tharappel et al., 2002; Glauert et al., 2005), PCB-77 increased the numbers of PGST-positive foci per cubic centimeter and per liver and the total focal volume as a percentage of liver volume but not the mean focal volume and PCB-153 did not increase significantly either the number, or the volume of foci. Berberian et al (1995) used exactly the same protocol for initiation and promotion but completely different markers for AHF (ATP-negative, GGT-positive, G6Pase-negative) (Berberian et al., 1995), compared with our studies and PCB-153 was a promoter as assayed by these markers. In this study, PCB-77 increased all four parameters and PCB-153 significantly increased (P=0.03) the number of foci per liver as compared to the corn oil group in the casein diet.

NF-κB was not increased by either PCB in our tumor-promotion experiment. We performed Western analysis for p50 and p65 from the same pool of nuclear extract as we did EMSA to verify the lack of change in this transcription factor and found no differences between treatments, but it was
surprising to find a significant quantity of p105. The presence of p105 protein might indicate that the peak of NF-κB DNA binding activation was at an early time point and lead to new p105 synthesis. It has been reported that p105 might act as an inhibitor of p50 (Liou et al., 1992). Another possibility would be that the diet prevented NF-κB activation. Two other studies in our laboratory examined NF-κB DNA binding in female Sprague-Dawley rats (the same strain and sex as we used) treated with PCB77 and PCB-153 at the same concentrations as in this study and both found an increase in NF-κB binding activity with PCB-77 treatment but not regarding PCB-153, congener for which one found no activation (Glauert et al., 2005), the other found activation (Tharappel et al., 2002). The different results for NF-κB activation between the studies could be due to the different type of diets; for this study we used an unrefined diet, for the study of Glauert et al, a purified diet freshly made was used, and for the study of Tharappel et al another type of unrefined diet was used. The level of antioxidants or the activity of the antioxidants could be very important factors. One possibility would be that in our study, a component of the diet shifted the events to earlier time points; the NF-κB activation and the surge in hepatocyte proliferation might have been at an earlier time point and therefore our results are negative for these end points but more significant for foci promotion than in the other 2 studies mentioned (Glauert et al., 2001; Tharappel et al., 2002). This factor might be the extra protein/amino acid that we added. Indeed, the dietary protein/amino acid quantity and quality are important factors in liver preneoplastic foci induction and promotion (Schulsinger et al., 1989; Youngman and Campbell, 1991). It has been found that at even a slight increase in dietary casein as the protein source (10-12% versus 6-8% required for maximum body weight gain), the formation of GGT positive foci significantly increased after AFB1 exposure (Dunaif and Campbell, 1987a; Dunaif and Campbell, 1987b). Another similar study obtained similar results, using both GGT positive and PGST positive foci (Blanck et al., 1992). Microsomes isolated from rats exposed to Aroclor (but not phenobarbital) were more effective at activating aflatoxin if the rats were fed
higher casein levels comparing to the optimal casein levels (Woodall et al., 1996).

However, higher casein levels are not always detrimental for the induction of altered hepatic foci. Other dietary factors also seem to be important. In a phenobarbital promotion study with DEN as the initiator, a higher level of casein, but a lower level of corn oil and different carbohydrate composition inhibited foci formation: a diet containing 30% casein, 10% partially hydrogenated corn oil, 40% sucrose, and 15% cornstarch induced fewer foci compared to a diet containing 20% casein, 5% corn oil, 65% cornstarch, and 5% cellulose (Hendrich et al., 1989) Dietary vitamin A deficiency increased (compared to normal vitamin A levels) and a casein–based diet decreased (compared to a cereal-based diet) tetrachlorodibenzo-p-dioxin (TCDD)- and phenobarbital-induced liver tumor promotion in female Sprague-Dawley rats as assayed by GGT-positive altered hepatic foci (Flodstrom et al., 1991). These aspects of casein-PCBs interaction might be very important to study since all humans are now PCB contaminated, and the PCBs are transferred from mother to the fetus transplacentally (Covaci et al., 2002) and then to the child through breast milk - and an important component of the milk is casein.

Interestingly, glycine had just one effect: increased cytochrome P-450 activity for both PCBs treatments, reaching statistical significance for PCB-153. This result may explain in part the mainly negative results for dietary glycine, since active oxygen released from cytochromes may contribute to the tumor promoting activities of PCBs (Glauert et al., 2001). However, our finding that glycine (Kupffer cell inhibition) activates cytochrome P-450 in hepatocytes is not contradictory to our hypothesis that Kupffer cells play a permissive role for tumor promotion by PCBs. It was reported that if Kupffer cells are co-cultured with hepatocytes and treated with LPS (well known Kupffer cell activator) and simultaneously treated with either phenobarbital or 3-methylcholanthrene, the induction by phenobarbital of cytochrome P-450 2B1 mRNA and the activity of PROD are significantly reduced (85%) but 3-methylcholanthrene-induced affects cytochrome P-450 1A1 only marginally (15%) (Milosevic et al., 1999). It is very
exciting that our experiment inhibits cytokine production from Kupffer cells and obtained a similar conclusion with this experiment from the literature that has an opposite approach: activation. In our case, inhibition of Kupffer cells increased activity of Cytochrome P-450 1A1 by about 20%, whereas the activity of cytochrome P-450 2B1/2 was increased by PCB-153 by about 50%. Cytokines, released by macrophages, are likely responsible for the down-regulation of cytochrome P-450 2B1/2B2 activities. Consequently when we inhibited Kupffer cell secretory activity, cytochrome P-450 activity went up. Reports show that TNF-α, IL-1β, IL-2 are able to reduce cytochrome P-450 2B1 and 1A1 levels (Pous et al., 1990; Ferrari et al., 1992; Cantoni et al., 1995; Milosevic et al., 1999).

The direct aim of this study was to see if dietary glycine can inhibit the promotion of putative preneoplastic lesions by PCB-153 and PCB-77. The results of our study demonstrate that the preneoplastic lesions were induced by both PCBs, but glycine did not have any significant effect on the promoting activity at this time point. Also, the cell proliferation and NF-κB binding activities were not induced by PCB treatment at this time point, but cytochrome P-450 activity was increased by PCBs as expected and this increase was exacerbated by glycine. These facts suggest that the two congeners could indeed pose a carcinogenic threat, that Kupffer cells might play a role and that our model should be refined to explain the cell proliferation and NF-κB data.
Chapter 3: Neither Dietary Glycine nor the Type of Oil Used as Vehicle for PCB-153 Influence the Effect of this PCB on Hepatic Cell Proliferation in Rats

Introduction

Exposure of humans to environmental PCBs is unavoidable. Indeed, 2,2’, 4,4’, 5,5’-hexachlorobiphenyl (PCB-153) is ubiquitous in our habitat and has nervous and endocrine systems effects (Tilson HA, 1997; Miyazaki et al., 2004). The huge PCB-153 quantities present into the environment, and the ability of PCB-153 to increase the activity of cytochrome P450 2B1/2 (Fadhel et al., 2002), raise the concern that this compound might pose carcinogenic risk even if it is a low-metabolisable compound in humans and rodents and does not directly bind to DNA. A question of importance is whether PCB-153 increases the cell proliferation and therefore increases the risk of mutation.

In the previous chapter, PCB-153 was shown to promote hepatic preneoplastic lesions, but cell proliferation was not higher, either in normal or preneoplastic hepatocytes. However, a peak in cell proliferation followed by a return to normal levels is characteristic after exposure to many other tumor promoters. Previous experiments carried out in our laboratory have shown that PCB-153 increases hepatocyte proliferation two days after one single administration (Lu et al., 2003). Based on these results, we hypothesized that promotion of the preneoplastic lesions by PCB-153 involves hepatocyte proliferation prompted by the secretory activity of the Kupffer cell and that these processes take place shortly after PCB-153 administration.

There are multiple approaches to assess Kupffer cell involvement in chemically induced liver toxicity. The most frequently used is administration of gadolinium chloride. However, it has major actions on the preapoptotic Kupffer cells, such as increased tumor necrose factor-α (TNF-α) release, and on hepatocytes, such as increased AP-1 activation, increased hepatocyte
proliferation, increased expression of proliferating cell nuclear antigen, TNFR1 and transforming growth factor-β (TGF-β), increased interleukin-6 (IL-6) release and decreased IL-8 release (Rai et al., 1996; Fukuda et al., 2004; He et al., 2005; Kinoshita et al., 2005). Gadolinium chloride (GdCl₃) treatment leads to the destruction of Kupffer cell populations preferentially: primarily ED2-positive mature Kupffer cells are depleted (Neyrinck et al., 2002), and ED1-positive Kupffer cells are 75% depleted (Rivera et al., 2001). As the result of depletion, after the initial increase in TNF-α concentration due to release from the apoptotic Kupffer cells, the liver loses the phagocytic and secretory activities specific to Kupffer cells. There are research groups that do not even agree with the decrease of TNF-α in the GdCl₃ treated animals. Kinoshita et al (2005) confirmed the observation that GdCl₃ initially enhances TNF-α concentrations (Rai et al., 1997) and further demonstrated that GdCl₃ depletes only the superoxide-producing Kupffer cells, but at the same time enhances the function of TNF-α-producing Kupffer cells (Kinoshita et al., 2005). In vitro, Kupffer cells respond differently than in vivo to GdCl₃, their TNF-α production not being affected by GdCl₃ (Lee et al., 2004).

Another approach for the study of Kupffer cell involvement in chemically induced liver pathology is to inhibit the secretory activity of these resident macrophages with the dietary amino acid glycine. Glycine preserves the phagocytic activity (colloidal carbon uptake) of Kupffer cells (Neyrinck et al., 2002), but inhibits the secretory activity of these cells (Ikejima et al., 1997) without leading to the TNF-α peak characteristic after gadolinium administration. This model was successfully used in a number of studies looking at liver toxicity induced by alcohol (Yin et al., 1998), endotoxin (Rose et al., 2000), peroxisome proliferators (Rose et al., 1997a; Rose et al., 1999a), and CCl₄ (Rivera et al., 2001). The mechanism underlying the effect of glycine on Kupffer cells involves chloride (Cl⁻) channels. Many physiological processes depend upon Cl⁻ ion channels function. Molecularly, there are known at least three different classes of Cl⁻-selective ion channels: postsynaptic GABA and glycine receptors, CIC-type Cl⁻ channels, and the cystic fibrosis transmembrane conductance regulator.
(CFTR) (Pusch, 2005). GABA/glycine receptors mediate inhibitory postsynaptic responses in the nervous system (Pusch, 2005). In the Kupffer cells, the physiological role of these channels is not as clear. Glycine inactivates the secretory activity of Kupffer cells by activating the Cl$^{-}$-selective ion channels in the cell membrane of these cells (Ikejima et al., 1997; Rose et al., 2000). A consequence of Cl$^{-}$ selective ion channels activation is the hyperpolarization of the cell membrane. In this condition, the increase in intracellular calcium is not possible. An intracellular calcium surge is a necessary condition for some signaling molecules (including TNF-$\alpha$) to be produced and released by the Kupffer cells (Dieter et al., 1988; Kawada et al., 1992; Ikejima et al., 1996; Lichtman et al., 1996; Watanabe et al., 1996; Ikejima et al., 1997).

In this study, we investigated if inhibiting Kupffer cell activation with dietary glycine would inhibit cell proliferation in PCB-153-treated or control rats 2 days after the PCB administration. Glycine or valine (as control) was fed in an unrefined diet at 5% for the entire study (6 days). PCB-153 was injected i.p. two days before euthanasia; Wy-14,643 was fed for 2 days as a positive control.
Materials and Methods

Materials:

1. PCB-153 (2,2′,4,4′,5,5′-hexachlorobiphenyl) was a generous gift from Drs. Hans Lehmler and Larry Robertson. The purity of this PCB was greater than 98%, as assayed by gas-chromatography (Lehmler and Robertson, 2001).
2. Wy-14,643 ([4- chloro- 6- (2,3- xylindino)- 2- pyrimidinylthio]- acetate) was obtained from Chemsyn Science Laboratories (Lenexa, KS).
3. Vitamin E-stripped corn oil was obtained from Acros Organics (Morris Plains, NJ).
4. Medium chain triglyceride oil (MCT) oil was obtained from Mead Johnson Nutritionals (Evansville, IN).
5. Olive oil was obtained from Sigma (O-1500, Sigma, St. Louis, MO).
6. Alzet osmotic pumps (model 2ML1) were obtained from Alza Scientific Products (Palo Alto, CA).
7. The diet for the entire study was Harlan Teklad, Global 18% protein Rodent Diet 2018 (Madison, WI).
8. Glycine was obtained from Sigma (G-7403, Sigma, St. Louis, MO).
9. Valine was obtained from Sigma (V-0513, Sigma, St. Louis, MO).

Experimental design and animal treatment:

Male Sprague-Dawley rats weighing 175-200 g (Harlan Sprague Dawley, Indianapolis, IN), were housed three per cage in a temperature- and light-controlled room and fed an unrefined rodent diet ad libitum in feeding cups (Harlan, 18% protein powdered for studies 1 and 3 and pelleted for study 2). The experimental protocols were approved by the Institutional Animal Care and Use committee of the University of Kentucky. Upon arrival, the animals were allowed to adjust for 1 week before starting the experiment. Glycine or valine (as control) was fed in the diet at 5% (yielding a protein plus amino-acid percent of 22.1) for the entire study (6 days), for each of the studies 1 and 3.
PCB-153 was injected intraperitoneally (i.p.) three days after starting the diets and 48 hours before euthanasia. We used two doses: 100 µmol/kg body weight and 300 µmol/kg body weight, using medium chain triglyceride (MCT) oil as the vehicle (study 1), and only 300 µmol/kg body weight using MCT, corn oil or olive oil as vehicles (studies 2 and 3). For study 1 we injected 0.2 ml oil containing PCBs/100g rat and for the other two studies we injected 1ml oil containing PCBs /100g rat. The same number of rats, with the same diet as those injected with PCB dissolved in oil, received a vehicle only injection in each of the three studies. As a positive control for hepatocyte proliferation we fed Wy-14,643 (0.05%) for 48 hours.

For studies 2 and 3 the rats were injected s.c. with 5-bromodeoxyuridine (BrdU, 100 mg/kg; 20mg BrdU/ml PBS ) 2 h before euthanasia. For study 1, the rats were implanted with Alzet osmotic pumps containing BrdU (20mg BrdU/ml PBS, 10 µL/h) 48 h before euthanasia. Euthanasia was by overexposure to carbon dioxide. The livers were immediately removed and weighed; a portion of each was frozen in liquid nitrogen and stored at –80°C until used. For each rat, a piece of liver was randomly taken from 4 different lobes and fixed in 10% buffered formalin.

5-bromo-2’-deoxyuridine administration:

Aim:

5-bromo-2’-deoxyuridine, an analog of thymidine, is capable of incorporating into newly replicated DNA during S-phase of the cell cycle. Anti-5-bromo-2’-deoxyuridine antibody, can be used to immunohistochemically detect in the liver tissue slices all the cells that have undergone DNA replication since BrdU administration. For our first study we assessed all the DNA replication since treatment, since we implanted Alzet pumps in the same time we injected with PCB and/or oil and started the Wy-14,643 diet. For the other two studies, we assessed the DNA replication taking place during the 46th -48th hours after treatment since we injected BrdU s.c. two hours before euthanasia

Procedure:
The BrdU solution (20mg BrdU/ml) was prepared in the same way for the pumps and injection and the details are mentioned in chapter two.

The pump implantation was performed as described in chapter two, with the difference that the rats were anesthetized with Nembutal (Abbot Laboratories, NDC 0074-3778-05) instead of isoflurane.

**BrdU Immunohistochemical Staining:**

The rate of replicative DNA synthesis (the rate of hepatocyte proliferation) was determined in the formalin-fixed tissues by immunohistochemically staining for BrdU. The formalin-fixed liver tissues were paraffin-embedded, sectioned, laid on glass slides, stained with an anti-BrdU antibody to identify nuclei that had incorporated BrdU, and counter-stained with hematoxylin. The procedure and materials are described in chapter two 2, the only difference is that in this case we stopped at step of DAB staining and continued directly with hematoxylin staining, since we do not need double staining for these three experiments. Cells that had incorporated BrdU were identified as those with brown nuclei. We counted at least 3000 hepatocellular nuclei randomly per slide (rat) with 1000 nuclei from each of three different lobs. The labeling index was expressed as the percentage of number of labeled hepatocyte nuclei out of total number of hepatocyte nuclei counted.

**Protein assay:**

It was performed as described in chapter two.

**Isolation of nuclear extracts:**

Nuclear extracts were prepared from frozen liver tissue by a modification of the method of Deryckere and Gannon (Deryckere and Gannon, 1994), as described in chapter two.
**Radiolabeling and purification of NF-κB oligonucleotide (probe synthesis)**

It was performed as mentioned in chapter two.

**Electrophoretic mobility shift assay (EMSA):**

NF-κB DNA binding activities from nuclear extracts were measured using electrophoretic mobility shift assay essentially as described in chapter two. 2.5 µg of nuclear extract from each liver was incubated with 0.5 µg poly (dl-dC) in a binding buffer (50 mM KCl, 10 mM HEPES-KOH, pH 7.9, 6.5 mM dithiothreitol, and 10% glycerol) on ice for 5 min, and then for 15 min at room temperature after the addition of radiolabeled NF-κB probe (approximately 100000 cpm). After incubation, samples were resolved by electrophoresis on a 7% polyacrylamide gel at 175 V for 2 h with 0.5× TBE as the running buffer. After the electrophoresis, gels were dried under vacuum and exposed overnight at -80°C to Kodak XOMAT-AR film.

**Preparation of whole liver homogenate (WLH):**

It was performed as described in chapter two.

**Cytosolic and microsomal isolation:**

The microsomal fraction was isolated from the whole-liver homogenate using the method described by Schramm (Schramm et al., 1985) and detailed in chapter two.

**Alkoxyresorufin O-dealkylation assay**

*Principle:*
7-Benzyloxyresorufin was used as specific substrate for the CYP2B1/2B2 isozyme of the microsomal fraction in the benzyloxyresorufin O-dealkylase method (Burke and Mayer, 1974a). The fluorescence of resorufin newly formed was detected with a fluorescence spectrophotometer at an excitation wavelength of 556 nm and an emission wavelength of 589 nm. This is a variation of the method presented in chapter two, but NADPH is not added directly - it is regenerated from NADP and glucose 6-phosphate in the presence of glucose-6-phosphate dehydrogenase.

**Reagents:**
1. Tris base (Boehringer Mannheim 1814 273)
2. K$_2$HPO$_4$ (Sigma Chemical Co. P8281)
3. KH$_2$PO$_4$ (Sigma Chemical Co. P5655)
4. NADP (Sigma Chemical Co. N3886)
5. Glucose 6-Phosphate (Sigma Chemical Co. G7879)
6. MgCl$_2$ (Sigma Chemical Co. M2670)
7. Glucose 6-Phosphate Dehydrogenase (Sigma Chemical Co. G8289)
8. resorufin (Sigma Chemical Co. R3257)
9. dimethylsulfoxide (DMSO) (Sigma Chemical Co. D8779)
10. 7-Benzoyloxyresorufin (Sigma Chemical Co. B1532)
11. HPLC grade methanol (Fisher Scientific A454-4)

**Solutions:**
1. 0.1 M Tris-HCl buffer, pH 7.4
2. 20 mM Potassium phosphate, pH 7.4 (using 5 M NaOH)
3. NADPH regenerating system
   - 0.1 M NADP 20 mM K$_3$PO$_4$
   - 0.5 M Glucose 6-Phosphate
   - 0.1 M MgCl$_2$ in 10 ml 20 mM K$_3$PO$_4$
   - 100 U/ml Glucose 6-Phosphate Dehydrogenase in 20 mM K$_3$PO$_4$ and adjusted pH with 1 M NaOH
4. 0.5 mM Resorufin in dimethylsulfoxide
5. 0.5 mM 7-Benzoyloxyresorufin in DMSO
Procedure:

1. The microsomal fraction was diluted 15 times (and for some samples that first were too concentrated and did not fit into the standard curve 30 times) for protein determination using Pierce method as described in chapter two.

2. The following 13 x 100 mm glass tubes (Fisher Scientific 14-961-27) were labeled per sample:
   a. the reaction
   b. background containing no regenerating system
   c.d. 1 ml of reaction mixture after incubation (duplicate)

3. The reaction included:
   a. a microsomal fraction containing 200 µg of total protein was added (studies 1 and 2). Before reading all the samples, linearity of the protein and incubation times were checked for two samples from each group.
   b. substrate (i.e. 0.5 mM Benzyloxyresorufin) – added 38.8 µl (10 µl/ml is needed and total reaction volume before adding 120 µl of regenerating system is 3880 µl)
   c. Tris buffer – brought total volume to 3880 µl
   d. Regenerating system – this was added to the reaction last (120 µl total) only after removing 1 ml of the above volume to act as a background control

<table>
<thead>
<tr>
<th>Reagent</th>
<th>For 3 ml Reaction</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M NADP</td>
<td>30 µl (10 µl/ml)</td>
<td>0.001 M</td>
</tr>
<tr>
<td>0.5 M G6P</td>
<td>30 µl</td>
<td>0.005 M</td>
</tr>
<tr>
<td>0.3 M MgCl₂</td>
<td>30 µl</td>
<td>0.003 M</td>
</tr>
<tr>
<td>100 U/ml G 6-PD</td>
<td>30 µl</td>
<td>1 U/ml</td>
</tr>
</tbody>
</table>

4. one ml methanol was added already to 3 different glass tubes in order to stop the reaction.
5. The reaction mixture without the regenerating system was pre-incubated at 37°C for 2 minutes in a shaking water bath. A 1 ml aliquot of the reaction mixture was removed and added to 1 ml methanol acting as a blank for the reaction.

6. 120 µl of the regenerating system were added to the first reaction. The sample was incubated with the regenerating system for 10 minutes in the shaking water bath. Following the 10-minute incubation, two 1 ml aliquots were removed and added to the tube containing 1 ml methanol, in order to stop the reaction.

7. The samples were protected from direct light and covered with paraffin until read. The absorbance was read on a Shimadzu Fluorescence Spectrophotometer with an excitation wavelength of 556 nm and an emission wavelength of 589 nm (study 2). For the study 1, the reaction was as in above mentioned protocol, just that the reading was done simultaneously for 11 samples at a time in a microplate reader, with an excitation wavelength of 556 nm, an emission wavelength of 589 nm and a cut off of 590 nm.

8. A resorufin standard curve was prepared from the 0.5 mM Resorufin stock solution with the following final concentrations: 0, 0.05, 0.1, 0.5, 1, and 1.5 nmol/ml. (the stock solution is equivalent to 500 nmol/ml).

9. Calculations: Alkoxyresorufin o- Dealkylase activity(nmol/mg protein/min) = Conc of resorufin formed (nmol/ml) / 10 min x mg protein in 1 ml reaction mixture

**Fatty acyl-CoA oxidase assay**

**Principle:**
Lauroyl-CoA used as substrate was oxidized by the fatty acyl-CoA oxidase from the whole liver homogenate to produce trans-2-dodecenoyl-CoA by reducing FAD to FADH$_2$ at pH 7.4. The FADH$_2$ donates the electrons to molecular oxygen to form H$_2$O$_2$. The added horseradish peroxidase reduces the produced H$_2$O$_2$ to water by oxidizing the substrate 4-hydroxyphenylacetic acid. The oxidized product is 6,6'-dihydroxy-(1,1'-biphenyl)-3,3'-diacetic acid, which is
fluorescent and is formed at a directly proportional rate with the activity of FAO. (Poosch and Yamazaki, 1986). The readings were done with a Gilford Fluoro IV fluorometer at the excitation wavelength of 318 nm and the emission wavelength at 405 nm.

**Reagents:**

1. KH$_2$PO$_4$ (Sigma Chemical Co. P5655)
2. K$_2$HPO$_4$ (Sigma Chemical Co. P3786)
3. KOH (Sigma Chemical Co. P5958)
4. p-hydroxyphenylacetic acid (Sigma Chemical Co. H4377)
5. Triton X-100 (Sigma Chemical Co. T8787)
6. horseradish peroxidase (Sigma Chemical Co. P6140)
7. FAD (Sigma Chemical Co. F6625)
8. lauroyl-CoA (Sigma Chemical Co. L9012)
9. sodium carbonate (Sigma Chemical Co. S2127)
10. KCN (Fisher Scientific Co. P-226)
11. hydrogen peroxide (Sigma Chemical Co. H0904 30% stock)

**Solutions:**

1. 60mM Potassium Phosphate Buffer, pH 7.4 (with KOH)
2. 50 mM p-hydroxyphenylacetic acid in potassium phosphate buffer
3. 10 mg/ml Triton X-100 in potassium phosphate buffer
4. 200 U/ml Horseradish Peroxidase in potassium phosphate buffer
5. 1 mM FAD in potassium phosphate buffer
6. 5 mM Lauroyl-CoA in potassium phosphate buffer
7. 100 mM Sodium Carbonate Buffer with 2 mM Potassium Cyanide, pH 10.4 with KOH
8. Hydrogen Peroxide Stock Standard (diluted 1:50)
9. working fatty acyl-CoA oxidase (WFAO) reagent (on ice):
   
<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mM, pH 7.4 Potassium phosphate buffer</td>
<td>31.5 ml</td>
</tr>
<tr>
<td>50 mM p-hydroxyphenylacetic acid</td>
<td>0.7 ml</td>
</tr>
<tr>
<td>10 mg/ml Triton X-100</td>
<td>0.7 ml</td>
</tr>
</tbody>
</table>
200 U/ml horseradish peroxidase 0.7 ml
1 mM FAD 0.7 ml
5 mM Lauroyl-CoA 0.7 ml
10. control fatty acyl-CoA oxidase (CFAO) cocktail (on ice)
60 mM, pH 7.4 Potassium phosphate buffer 32.2 ml
50 mM p-hydroxyphenylacetic acid 0.7 ml
10 mg/ml Triton X-100 0.7 ml
200 U/ml horseradish peroxidase 0.7 ml
1 mM FAD 0.7 ml

Procedure:
1. label 13 x 100 mm tubes for:
   1) seven standards (0,1.0, 2.0, 4.0, 6.0, 8.0, 10.0 nmol/ml)
   2) two tubes/sample – one labeled “test” and the other “control”
2. samples were diluted using 60 mM potassium phosphate buffer. An initial assay for FAO activity was performed on treated and control samples of different concentration to assure the linearity of FAO activity. Dilutions used in this experiment were 1:400 for Wy-14,643 -treated WLH and 1:30 for all the others WLH.
3. Standard curve:

<table>
<thead>
<tr>
<th>Std conc (nmol/ml)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:50 diluted H$_2$O$_2$ (µl)</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>60 mM Potassium Phosphate Buffer (µl)</td>
<td>100</td>
<td>95</td>
<td>90</td>
<td>80</td>
<td>60</td>
<td>40</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

4. 50 µl of each diluted sample and 50 µl 60 mM potassium phosphate buffer were added to the “test” and “control” tubes.
5. 500 µl of WFAO reagent was added to all standard tubes and “test” tubes
6. 500 µl of CFAO reagent was added to all “control” tubes and mixed.
7. All tubes were incubated in a 37°C water bath for 30 minutes.
   The water bath was covered.
8. After the 30-minute incubation, all tubes were placed in an ice bath.
9. In order to stop the reaction, 2 ml ice-cold sodium carbonate/KCN buffer were added to each tube and mixed. All samples were kept away from direct sunlight or fluorescent light.

10. Samples were read in the Gilford Fluoro IV fluorometer using 1 ml quartz cuvettes at excitation wavelength 318 nm and emission wavelength 405 nm. Auto zero was done with 0 nmol/ml std. Calibrate (100% fluorescence) at 10 nmol/ml std. The fluorometer was recalibrated every 5 samples.

**Calculations:**

1. Performed linear regression analysis between the standard concentrations and their respective fluorometric readings. The correlation coefficient for the standard curve should be >96.

2. Determined the FAO activity (nmol) for each sample:
   
   
   \[
   \text{FAO Activity (nmol)} = (\text{“test”-“control” readings}) \times (X \text{ coefficient}) + \text{(constant)}
   \]

3. FAO Activity (nmol/min/mg protein) = \[
\frac{2.6 \text{ (ml)} \times \text{nmol FAO activity} \times \text{Dilution Factor}}{[30 \text{ (min)} \times 0.5 \text{ (ml)} \text{ protein conc for that sample (mg/ml)}]}
\]

**Statistical analysis:**

All statistical analyses were realized using SYSTAT V.8 (SPSS, Chicago, IL) software. Results were first analyzed by two-way analysis of variance (ANOVA). Individual differences between the treatment means were determined with the Bonferroni post-hoc test. The results were reported as means ± standard error of the mean SEM. Results were defined as statistically significant for \( P<0.05 \).
Results

The main aim of this set of experiments was to test the hypothesis that secretory activity of Kupffer cells is important for the short-term induction of cell proliferation by PCB-153. The second aim was to assess if the vehicle for PCB delivery has any influence on the hepatocyte proliferation. The PCB experiments in animals are traditionally done using corn oil. Before the beginning of our experiments, it was reported that corn oil administrated orally increased hepatocyte proliferation in rats, via increased NF-κB DNA binding activity in Kupffer cells and TNF-α release (Rusyn et al., 1999).

In the first experiment of this series, a group of rats received PCB-153 (100 μmol/Kg or 300 μmol/Kg) in MCT oil, another group received only oil and other two groups received no injection—one of them receiving Wy-14,643 in the diet serving as positive control for hepatocyte proliferation and the other one serving as baseline control. In each of these groups, half of the animals received glycine and the other half received valine in the diet for the entire study (6 days). PCB-153 was injected intraperitoneally (i.p.) 3 days after starting the diets and the animals were euthanized 48h after the PCB injection. The volume of oil injected in this study was 0.2 ml /100g rat. To assess the cell proliferation, the rats were implanted with Alzet osmotic pumps containing BrdU 48 h before euthanasia.

The body weights were not statistically different among the groups (Fig.3.1). The relative liver weights were significantly increased only by Wy-14,643 administration (Fig.3.2). Cytochrome P-450 2B1/2 activity was increased by PCB-153 significantly (Fig.3.3). The fatty acyl-CoA oxidase activity was increased by Wy-14,643 significantly (Fig. 3.4). Glycine presented a trend of decreasing the increases of cytochrome P-450 2B1/2 activity and of fatty acyl-CoA oxidase activity but this effect was not significant (Figs.3.3. and 3.4.).

Only Wy-14,643 significantly increased cell proliferation compared to the controls (animals receiving no injections) and only glycine decreased significantly the Wy-14,643 cell proliferation (Fig.3.5.). MCT oil decreased the cell
proliferation by about 50% comparing with the groups that did not receive any injections (Fig.3.5). The pathology laboratory reported that liver slides stained with hematoxylin and eosin presented no abnormalities.

The DNA binding activity of NF-κB was not changed (did not reach the significance level) by PCB treatment. However glycine decreased NF-κB activation compared to the valine (p=0.043) (Fig. 3.6).

We observed increases in cell proliferation in our positive control groups (Wy-14,643 groups), however, unlike in our other experiments with PCB-153, we did not observed an increase in cell proliferation in rats receiving PCB-153, in either the valine or glycine groups. Based on the inhibition on cell proliferation in rats only receiving MCT oil, we hypothesized that MCT oil was inhibiting the hepatocyte proliferation in PCB-153-treated rats. We therefore did another experiment using three types of oil as a vehicle for PCB-153: MCT oil, corn oil and olive oil. We used the same strain, age and sex of rats as in the first study. No glycine or valine was administered in the diet and the dose of PCB-153 was 300 μmol/Kg. Six rats were not injected at all and served as base line. Each type of oil was injected 1ml/100g body weight. BrdU was administered s.c. 2 h before euthanasia.

The body weights were not significantly different among groups (Fig.3.7.). Relative liver weights had the tendency to be higher in the PCB-treated animals, but not significantly (Fig.3.8.). PCB-153 significantly increased cytochrome P450 2B1/2 activity but the oil had no effect (Fig.3.9).

MCT oil again decreased the hepatocyte proliferation by about 50% compared to the group that did not receive any injections (Fig. 3.10.). Cell proliferation in rats receiving corn oil or olive oil was slightly higher than in rats receiving MCT oil, but this was not statistically significant. In rats receiving PCB-153, the trend was that hepatocyte proliferation was higher than their respective controls for corn oil and olive oil but not for MCT oil.

NF-κB DNA binding activities (Fig.3.11) were not different among the groups.
Since we did not observe the corn oil to be a confounding factor for the
effect of PCB-153 on hepatocyte proliferation, we designed a third experiment,
testing the effect of glycine and PCB-153 on hepatocyte proliferation when the
vehicle was corn oil. As in study 2, we used 300 µmol/Kg PCB-153, 1ml oil/100g
rat and BrdU injected s.c. 2 h before euthanasia. As in study 1, we used Wy-
14,643 as positive control for cell proliferation and administered glycine to inhibit
Kupffer cells. For nitrogen balance, valine was used in the groups with normal
Kupffer cells.

As expected, and confirming what we obtained in study 1, the body
weights were not different among the groups (Fig. 3.12) and the relative liver
weights were significantly increased only by the Wy-14,643 compound (Fig.3.13).
Glycine inhibited cell proliferation, PCB-153 did not increase cell proliferation.
The control for cell proliferation, Wy-14,643, behaved as expected increasing cell
proliferation, this effect being blunted by glycine (Fig.3.14).

PCB-153 again did not induce a significant increase in NF-κB activation,
possibly due to the existence of 2 unusually high values in the glycine control
group and one unusually high value in the valine oil group (Fig. 3.15).
Figure 3.1. Effect of PCB-153 and glycine on body weight. (Study 1)
**Figure 3.2. Effect of PCBs and glycine on relative liver weight.** (Study 1)

Results are expressed as mean ± SEM. The values were analyzed by ANOVA followed by Bonferroni, and the level of significance was p<0.05.
Figure 3.3. Effect of PCB-153 and glycine on the cytochrome P-450 2B1/2 activity. (Study 1)
Figure 3.4. Effect of PCB-153 and glycine on fatty acyl-CoA oxidase activity. (Study 1)
Hepatocyte proliferation

Figure 3.5. Effect of PCB-153 and glycine on the quantitation of hepatocyte proliferation (Study 1)

Results are expressed as mean ± SEM. The values were analyzed by ANOVA followed by Bonferroni, and the level of significance was p<0.05
A

Quantitation of NF-κB bands

Net radioactive counts for NF-κB activity

Valine  Glycine

control PCB-153
100 µmol  300 µmol

B

Quantitation of NF-κB bands

Net radioactive counts for NF-κB activity

Valine  Glycine

control PCB-153
100 µmol/kg  300 µmol/kg
Figure 3.6. Effect of PCBs (in MCT) and glycine on the rat hepatic DNA binding activity of NF-κB. A. NF-κB DNA binding activity was determined using the electrophoretic mobility shift assay as described in the Materials and Methods. Each line contains extract from a single animal. B. Quantitation of the specific bands; net radioactive counts were determined subtracting the background counts from the NF-κB band counts. Results are expressed as mean ± SEM. The values were analyzed by ANOVA followed by Bonferroni, and the level of significance was p<0.05. * Values are significantly different from the valine groups (Study 1).
Figure 3.7. Effect of PCB-153 and vehicle on body weight. (Study 2)
Figure 3.8. Effect of PCB-153 and vehicle on relative liver weight. (Study 2)
Figure 3.9. Effect of PCB-153 and vehicle on the cytochrome P-450 2B1/2 activity. (Study 2)
Figure 3.10. Effect of PCB-153 and vehicle on the quantitation of hepatocyte proliferation (Study 2)

Results are expressed as mean ± SEM. The values were analyzed by ANOVA followed by Bonferroni, and the level of significance was p<0.05
Figure 3.11. Effect of PCBs and vehicles on the rat hepatic DNA binding activity of NF-κB. NF-κB DNA binding activity was determined using the electrophoretic mobility shift assay as described in the Materials and Methods. Each line contains extract from a single animal (Study 2).
Figure 3.12. Effect of PCB-153 on body weight. (Study 3)
Figure 3.13. Effect of PCBs and glycine on relative liver weight. (Study 3)

Results are expressed as mean ± SEM. The values were analyzed by ANOVA followed by Bonferroni, and the level of significance was p<0.05.
Figure 3.14. Effect of PCB-153 and glycine on the quantitation of hepatocyte proliferation (Study 3)

Results are expressed as mean ± SEM. The values were analyzed by ANOVA followed by Bonferroni, and the level of significance was p<0.05
Quantitation of NF-κB bands

Net radioactive counts for NF-κB activity

- Valine
- Glycine

B

- Control
- PCB-153 300 μmol/kg
- Corn oil
Figure 3.15. Effect of PCBs (in corn oil) and glycine on the rat hepatic DNA binding activity of NF-κB. A. NF-κB DNA binding activity was determined using the electrophoretic mobility shift assay as described in the Materials and Methods. Each line contains extract from a single animal B. Quantitation of the specific bands; net radioactive counts were determined subtracting the background counts from the NF-κB band counts. Results are expressed as mean ± SEM. The values were analyzed by ANOVA followed by Bonferroni, and the level of significance was p<0.05. (Study 3)
Discussion

We hypothesized that promotion of the preneoplastic lesions by PCB-153 reported in the previous chapter involves hepatocyte proliferation prompted by the secretory activity of the Kupffer cell and these processes take place shortly after PCB-153 administration. Specifically, we had two goals for these three short term experiments: first, to investigate if inhibiting Kupffer cell activation with dietary glycine would inhibit cell proliferation in PCB-153-treated or control rats, as assayed 2 days after the PCB administration and second to assess if the vehicle for PCB delivery has any influence on the cell proliferation.

To quantify the rate of hepatocyte proliferation, we assayed the labeling index after rats were administered BrdU. Only Wy-14,643 significantly increased the cell proliferation compared to the controls. And, glycine significantly decreased the cell proliferation only in Wy-14,643-treated rats (Figs. 3.5., 3.10. and 3.14). The increase in cell proliferation by Wy-14,643 is well established (Rose et al., 1997a) and our control behaved as expected. The significant decrease of cell proliferation by glycine in the Wy-14,643 treated group has also been reported (Rose et al., 1997a) and used as control in our experiments. The lack of an increase in cell proliferation by PCB-153 treatment was not expected. Previously it was reported that the cell proliferation was increased (Lu et al., 2003), as assayed after 2 days after 300 µmol/kg PCB-153 treatment.

For both studies where we used MCT oil, cell proliferation was decreased by about 50% compared with the groups that did not receive any injections (Figs. 3.5. and 3.10). But, when we used corn oil or olive oil (Fig. 3.10), hepatocyte proliferation was increased 2-3 fold compared to MCT oil. Corn oil was used in previous PCB experiments in our and other laboratories (Jin et al., 2001; Lu et al., 2003; Lu et al., 2004; Lyche et al., 2004; Nyska et al., 2004; Glauert et al., 2005).

The DNA binding activity of NF-κB was not significantly increased in this series of experiments by PCB treatment, or by different types of oil (Figs. 3.6.,
The glycine effect was not consistent (Figs 3.6 and 3.15). The lack of PCB effect on NF-κB activity, as on hepatocyte proliferation, is unlike the previously reported study (Lu et al., 2003). This difference might be due to the different diets that were used: in these studies we used a Harlan Teklad Rodent Diet 2018, Lu et al used a Purina Mill 5001 diet (see Appendix). Purina Mill 5001 diet contains 22 IU/g vitamin A for example whereas our diet (used for all three experiments) contains 30.7 IU/g vitamin A. Retinyl palmitate was reported to inhibit the promotion of foci by PCB-153 (Berberian et al., 1995). Vitamin A is involved in cellular growth and differentiation, being able to activate retinoic acid receptors (RAR) and retinoid X receptors (RXR) (Makita et al., 2005). We can only speculate that vitamin A dietary levels contribute to the different results between Lu’s study and the present one, since the diets also differ for components other than vitamin A; both diets are nonpurified diets and therefore the non-nutrient components might have an impact of the endpoints that we studied. Even if our study does not clarify which of these possibilities is the case, it nevertheless demonstrates that diet can be an important factor for the PCB-153 toxicity and therefore this aspect deserves further investigation.

Cytochrome P-450 2B1/2 activity was increased by PCBs as expected, and fatty acyl-CoA oxidase activity was increased by Wy-14,643 as expected (Figs. 3.3., 3.4., 3.9.). Glycine did not significantly inhibit either the PCB-induced increase of cytochrome P-450 2B1/2 activity, or the Wy-14,643-induced increase of fatty acyl-CoA oxidase activity. The observation that fatty acyl-CoA oxidase activity was not significantly reduced by glycine is well established (Rose et al., 1997a) and again this demonstrates that our experimental system works and it is worthwhile investigating what in our experiment diminished the PCB-153 effect or what increased its effect in the study of Lu et al, (2003). Both diets used are recommended for the rodents and the differences are not considered to impair animal’s health in normal conditions. Nevertheless we noticed this different response. In this case we do not consider the protein/amino acid level to be a factor, for in studies 1 and 3 our diet had 18 percent and we added 5 percent glycine of valine that led to a total protein concentration of 22.1 percent, and the
diet used in Lu’s experiment was 23 percent protein. In our study 2, the percent was 18 percent but the response to PCB-153 in corn oil was not as different in this study comparing to the study 3 which had 22.1 percent. Nevertheless, this does not elucidate if the source of the protein is not the reason for the differences between our 3 experiments and the study of Lu et. al, (2003).

The responses after glycine treatment in Wy-14,643- and PCB-153-treated rats were different. The mechanisms by which peroxisome proliferators increase cell proliferation might be different of those for PCBs. Non-coplanar PCBs, as PCB-153, were reported to be potent inhibitors of Ca homeostasis in the neurons (Tilson HA, 1997). It is well known that calcium is important for the well functioning of these resident macrophages (Bermelin and Decker, 1983). In our model, a calcium surge after treatment was hypothesized as an important factor that leads to cytokine release by Kupffer cell, cytokines that might affect gene expression in hepatocytes and therefore the cell proliferation/apoptosis ratio. One hypothesis for further investigations would be that perhaps PCB-153 inhibits the calcium surge in Kupffer cells and therefore these cells might not be able to release TNF-α and other soluble factors; in other words maybe the toxic effects induced by PCB-153 are not due to an increase in calcium concentrations and therefore an activation of Kupffer cells but rather to an inhibition of the activity of these macrophages by inhibiting the calcium surge. To investigate that possibility, the use of oligofructose, a dietary factor shown to activate Kupffer cells, might be useful.
Chapter 4: Transgenic Macrophage Fas-Induced Apoptosis (Mafia) Mice as Models for the Role of Kupffer Cells in PCB and Wy-14,643 Hepatotoxicity

Introduction

Polychlorinated biphenyls (PCBs) are persistent environmental pollutants. Although they were banned both in USA and Europe about 3 decades ago, they are still among the most ubiquitous and persistent environmental contaminants. The mechanism by which they act as hepatocarcinogens and tumor promoters is largely unknown. Due to the number of different cells in the liver, the mechanism is likely very complex. Kupffer cells are active, hepatic resident macrophages. They are involved in host defense by removing endotoxin, tumor cells, viruses, bacteria, and parasites. A number of approaches are available to study Kupffer cells’s role in pathological processes. Some of them aim to prevent Kupffer cell activation while others aim to destroy Kupffer cells. Among the methods used to prevent Kupffer cell activation are the administration of the following agents: glycine (Ikejima et al., 1996), uridine (Stachlewitz et al., 1999), taurine (Warskulat et al., 1997; Wettstein and Haussinger, 1997; Seabra et al., 1998; Kim and Kim, 2002), betaine (Wettstein and Haussinger, 1997; Kim and Kim, 2002), and methyl palmitate (Rose et al., 2000). The methods used to destroy Kupffer cells are as follows: administration of lanthanides as gadolinium in the form of gadolinium chloride (Mizgerd et al., 1996), use of liposome-encapsulated dichloromethylene diphosphonate (clodronate) (Buiting and Van Rooijen, 1994; Imamura et al., 1995), administration of silica (Schindler et al., 1984), and administration of desulfated iota-carrageenan (Ishizaka et al., 1989).

One problem with chemical inactivation/depletion is low specificity for one cell type. Uridine perturbs calcium homeostasis not only in Kupffer cells but also in the hepatocytes, endothelial cells and stellate cells (vom Dahl et al., 1999). Glycine inhibits T lymphocyte growth (Stachlewitz et al., 2000), and the same
glycine-gated chloride channels are present in the central nervous system; therefore the glycine treatment might have an inhibitory effect on the nerve cells with those channels. Glycine also might directly inhibit the stellate cell’s production of collagen (Rivera et al., 2001). GaCl$_3$ attenuates stellate cell activation (Canbay et al., 2003). Glycine and taurine can lower the toxicity of some compounds, especially lipophilic compounds, by increasing their excretion through the bile, since bile acids are usually secreted conjugated with one of these two amino acids. There are reports showing that glycine influences the lipid metabolism in the liver. In fasted rats, inhibition of Kupffer cells by glycine leads to increased esterification of fatty acids and their accumulation in the liver (Neyrinck, 2004). This might result in a higher lipid-soluble substance (e.g. PCBs) accumulation in the liver of glycine treated rats than in the control ones. Dichloromethylene diphosphonate leaks out of the liposomes and the real distribution might be different than the theoretical one (Buiting et al., 1996). Gadolinium chloride has major actions on the preapoptotic Kupffer cells as induce massive TNF-α release, and on the hepatocyte population as increases DNA binding activity of transcription factor AP-1, increases hepatocyte proliferation, increases expression of proliferating cell nuclear antigen, TNFR1, TGF-β, increases IL-6 release and decreases IL-8 release (Rai et al., 1996; Fukuda et al., 2004; He et al., 2005; Kinoshita et al., 2005). Gadolinium chloride treatment leads to the destruction of Kupffer cell populations preferentially: primarily ED2-positive mature Kupffer cells are depleted (Neyrinck et al., 2002), and ED1-positive Kupffer cells are depleted in proportion of about 75% (Rivera et al., 2001). As the result of depletion, after the initial increase in TNF concentration due to release from the apoptotic Kupffer cells, the liver loses the phagocytic and secretory activities specific to Kupffer cells. Research groups do not even agree with the decrease of TNF-α in the GdCl$_3$ treated animals. Reportedly, GdCl$_3$ depletes only the superoxide-producing Kupffer cells but it is also reported to enhance the function of TNF-α-producing Kupffer cells (Kinoshita et al., 2005). Kupffer cells respond differently in vitro than in vivo to GdCl$_3$ in that TNF-α production is not being affected in vivo (Lee et al., 2004).
Glycine, on the other hand, preserves the phagocytic activity (colloidal carbon uptake) of Kupffer cells (Neyrinck et al., 2002), but inhibits the secretory activity of these resident macrophages (Ikejima et al., 1997) without the TNF-\(\alpha\) peak characteristic after gadolinium administration.

To avoid all the above mentioned problems with chemical depletion and inactivation, we used a transgenic Kupffer cell knockout mouse model for the study of PCB and Wy-14,643 induced toxicity. This mouse model bears an inducible Fas suicide/apoptotic system in the macrophage, dendritic cell lineage (Burnett et al., 2004). The principle of this construct is that the transgene protein is expressed in the membrane of all macrophages and dendritic cells. The mice are normal before dimerizing drug injection. FK-binding protein domains are able to preferentially bind a dimerizing drug (AP20187) and when this is added, the neighboring proteins from the membrane dimerize and the cytoplasmic Fas domains from neighboring proteins become cross-linked and activated, inducing further the activation of the caspase-8 pathway, and apoptosis. The main advantage of this system is that the depletion is inducible (therefore embryonic development is normal) and independent of the cell cycle of the monocytes (therefore the mature Kupffer cells are depleted as well as the immature ones).
Materials and Methods

Materials:

1. PCB-153 (2,2',4,4',5,5'-hexachlorobiphenyl) was synthesized and characterized as described previously (Schramm et al., 1985) by Dr Lehmler. The purity was greater than 99%, as assayed by gas-chromatography (Lehmler and Robertson, 2001)
2. The diet for the entire study was Harlan Teklad, Global 18% protein Rodent Diet 2918 (Madison, WI)
3. Vitamin E-stripped corn oil was obtained from Acros Organics (Morris Plains, NJ).
4. The dimerizer drug AP 20187 was a gift from Ariad Pharmaceuticals
5. Polyethylene glycol, PEG-400, was purchased from Sigma, P 3265
6. Polyoxyethylenesorbitan monolaurate, Tween 20, Sigma P 1379
7. 100% Ethanol (Aaper alcohol and chemical Co., Shelbyville, Kentucky)
8. 5-bromo-2'-deoxyuridine, Sigma, B 5002

Experimental design and animal treatment:

Nineteen male wild type C57Bl/6J mice and 46 male homozygous transgenic macrophage Fas-induced apoptosis (Mafia) (about eight weeks old at the first retroorbital injection) were used for this experiment. The wild type C57Bl/6J mice were purchased from Harlan. The Mafia mice were from Dr Cohen’s colony, University of Kentucky. The mice can be purchased as hemizygotes from the Jackson Laboratory (Bar Harbor, Maine), under the name: C57BL/6J-Tg(Csf1r-EGFP-NGFR/FKBP1A/TNFRSF6)2Bck/J (former name: C57BL/6J-Tg(Csf1r-GFP, NGFR/FKBP12)2Bck/J). We used homozygotes from Dr. Donald A. Cohen’s colony, University of Kentucky, Dr Cohen being the donating investigator for the colony existing in Jackson Laboratory. As their name says, these mice are transgenic on a C57Bl/6J background. The detailed procedure for obtaining these mice and a full description of their characteristics is available in the paper written by their producers (Burnett et al., 2004).
transgene is under the control of the promoter for mouse CSF-1r, colony stimulating factor 1 receptor including intron 2. The presence of intron 2 of the CSF-1r promoter determines a macrophage-specific expression. Enhanced green fluorescence protein gene follows. Green Fluorescent Protein (GFP) is a very frequently used reporter molecule derived from the jellyfish Aequorea victoria. Recently, many laboratories use as reporter a genetically manipulated form that has enhanced fluorescence intensity (EGFP). Tissue expressing sufficient amounts of GFP will appear green when exposed to a 488 nm light source. In order to enhance the visibility of this marker, antibodies anti GFP were made and the slides can be immunohistochemically stained. Immediately downstream of the GFP gene, was inserted the Fas suicide construct. This construct contains, in this order: an internal ribosome entry site (IRES) sequence, human low affinity nerve growth factor receptor (ΔLNGFR) for membrane insertion, 2 copies of a mutant human FK506 binding protein 1A, 12 kD (FKBP12), the intracellular domain region of the Fas gene and ends with the SV40 early mRNA polyadenylation signal.

The mice were housed in plastic microisolator cages. Water and food were administered *ad libitum*. The experimental protocols and procedures that involved these mice were approved by the Institutional Animal Care and Use Committee of the University of Kentucky and were in accordance to all policies for the use and care of laboratory research animals as stipulated by the NIH. The mice received the dimerizer for 5 consecutive days, let to rest for 2 days and treated with PCB (400 µmol/10 ml oil/kg body weight) or only corn oil or 0.05% Wy-14,643 on the eighth day. After 2 days, 2 hours before euthanasia, the mice were injected BrdU. All mice were euthanized by CO₂ inhalation at 48h after the PCB injection, and the livers were immediately removed and weighed. A piece of liver was randomly sliced from three different lobes of the liver and placed in a tissue cassette (Sakura Finetek, Torrance, CA), and then in a 10% buffered formalin (Fisher Scientific SF100-4) solution. The remainder of liver was flash frozen in liquid nitrogen and stored at -80°C until assayed for NF-κB. The formalin tissues were paraffin embedded and sectioned at 5 µm and mounted on
collagen coated glass slides by the Pathology Department at the University of Kentucky. Some of the slides were coming back and we stained them for BrdU, and some were stained by the Pathology Department for GFP using the anti-GFP- biotin (Vector Laboratories) primary antibody at 1:200 dilution.

**Dimerizer administration:**

The lyophilized AP 20187 was dissolved in 100% ethanol to make a stock solution of 62.5 mg/ml. Just before use, an aqueous injecting solution was made: 2.5 mg/ml dimerizer, 4% ethanol, 10% PEG-400, 1.72% Tween. (dimerizer stock solution was mixed with PEG and then 2% Tween in water was added.)

The solution was injected retro-orbitaly to the mice under anesthesia with isoflourane. Each mouse received 10mg AP 20187/ kg body weight each day for 5 consecutive days.

**5-bromo-2′-deoxyuridine administration:**

**Aim:**

5-bromo-2′-deoxyuridine (BrdU), an analog of thymidine, is capable of incorporating into newly replicated DNA during S-phase of the cell cycle. Anti-5-bromo-2′-deoxyuridine antibody, commercially available, can be used to immunohistochemically detect in the liver tissue slices all the cells that have undergone DNA replication during the last two hours of the experiment, and therefore to estimate the cell proliferation happened that time.

**Materials:**

**Solutions**

3. Phosphate Buffered Saline (PBS), pH 7.6
   (0.8% NaCl, 0.02% KCl, 0.144 % Na₂HPO₄, 0.024 % KH₂PO₄) adjusted to pH 7.6 with HCl.

4. 20 mg/ml 5-bromo-2′-deoxyuridine (BrdU) made in PBS, pH 7.6

**Procedure:**

20 mg/ml BrdU solution was injected to the mice 0.005 ml/g mice (that is 100mg BrdU /kg body weight).
**BrdU Immunohistochemical Staining:**

**Aim:**
The rate of replicative DNA synthesis in the hepatocytes (the rate of hepatocyte proliferation) was determined by immunostaining for 5-bromo-2'-deoxyuridine (BrdU).

**Procedure:**
BrdU staining was identical to the one described in chapter two.

**Counting of the BrdU-stained nuclei:**
Cells that had incorporated BrdU were visualized as having brown nuclei due to diaminobenzidine (DAB). An average of 4600 hepatocellular nuclei per slide (a minimum of 3400 / slide) were counted randomly, with at least 1000 cells from each three different lobes.

Labeling index was calculated as percent of cells undergoing proliferation from total number of cells.

**Protein assay:**

**Principle:** The protein contained in the sample reduces Cu\(^{+2}\) to Cu\(^{+1}\) in an alkaline environment (Reagent A). Cu\(^{+1}\) newly formed is chelated by two molecules of bicinchoninic acid-BCA-(Reagent B) forming a purple complex, with absorbance at 562 nm.

**Isolation of nuclear extracts:**

Nuclear extracts were prepared from frozen liver tissue by a modification of the method of Deryckere and Gannon (Deryckere and Gannon, 1994) as described in chapter two.

One difference is that EDTA was purchased as 0.5M EDTA, pH 8.0 from Fluka (03690)
Radiolabeling and purification of NF-κB oligonucleotide (probe synthesis)

T4-polynucleotide kinase was used to label the 5’-OH group of the NF-κB oligonucleotide with [γ-32P] ATP as described in chapter two.

Electrophoretic mobility shift assay (EMSA):

NF-κB DNA binding activities were measured using electrophoretic mobility shift assay as described in chapter two.
0.2μg/well Poly dIdC and 5μg protein/ well were used. A 7% polyacrylamide, 0.75 mm thick gel was used to resolve the bands in one hour and a half at 170V in 0.5 x TBE running buffer. Gels were exposed to a phosphoimaging screen and analyzed using the STORM Phosphoimager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant 5.0 software (Molecular Dynamics).

Kupffer cell isolation:

Reagents:
1. Hanks 10x, Sigma H4641
2. NaHCO3, Sigma, S5761
3. Albumin, Sigma, A9418
4. antibiotic/antimycotic (100x), Atlanta Biologicals, B22110
5. autoclaved nonopure water
6. collagenase type IV, Sigma, C5138
7. heparin, Sigma, H3149
8. Nembutal, Abbot Laboratories, NDC 0074-3778-05
9. 70% EtOH
10. NH4Cl, Sigma, A4514
11. Tris-HCl 1M pH 7.2, Sigma T 2538
12. Percoll, Amersham Biosciences 17-0891-01
13. Hepes Buffer Solution 1M Gibco 15630-080
Solutions:

1. NH4Cl-Tris:
8.3g NH4Cl
800 ml H2O
100 ml Tris-HCl pH 7.2
adjust to pH 7.2, add H2O to 1 l; autoclave in 100 ml bottles.

2. Hanks working solution:
200 ml Hanks 10x
0.7g NaHCO3
5g albumin
20 ml antibiotic/antimycotic
add H2O to 2 l
filter through 0.22µm filter
pH should be 7.4-7.6, if needed adjust with Hepes Buffer

3. Hanks solution for digestion (made just prior use)
Hanks working solution wormed at 37°C
0.2% collagenase type IV
1000 U/ml heparin

Procedure:
1. The mice were anesthetized with nembutal
2. The abdomen was cleaned with 70% EtOH and laparotomy was performed
3. The livers were removed and placed in Hanks with heparin (in stomacher’s bags)
4. The livers were placed in stomacher for 30 sec at medium speed
5. For further digestion, the bags with liver cells were placed at 37°C, with agitation
6. Everything is filtered through BD Falcon Cell strainer 70µm Nylon (cat nr 352350) into 50 ml conical tubes. Filtration will require additional cold Hanks working solution (no collagenase).
7. Centrifuge at 100 x g, 3 min, 5°C to pellet hepatocytes. REPEAT
8. Spin supernatant 300 x g for 7 min to pellet Kupffer cells.
9. Add NH4Cl-Tris; place in dark for 2 min.
10. Centrifuge at 300 x g, 7 min, 5°C
11. Place 15 ml of 25% percoll in a 50 ml conical tube. Very carefully layer 20 ml 50% percoll UNDER the 25% layer. Place cell suspension on top of the 25% layer.
12. Centrifuge at ~600 x g, 15 min (BREAK OFF), 20°C
13. Keep cell layer just below hepatocytes and debris
14. Resuspend in 50 ml Hanks and centrifuge 300 x g 7 min to wash away percoll.

**Staining for flow-cytometry**

1. The cells are resuspended in PBS
2. Two aliquots (100µl) are taken; one is left unstained as negative control.
3. 3 µl of Fc blocker (CD16/CD32) are added to the aliquot to be stained
4. Incubate on ice for 15 min.
5. 3 µl F4/80-RPE Cy5 are added
6. Incubate on ice in dark for 15 min
7. 3 ml PBS are added as rinse, followed by 7 min centrifugation at 2000 rpm
8. The cells are resuspended in 100 µl PBS and read.
Results

After the five dimerizer/mock injections, the drug receiving animals lost weight (24%±1.2) whereas the mock animals did not. At the end of the experiment, the animals that received the drug did not completely recover their body weight deficit (Fig. 4.1). Only the group receiving the drug and the Wy-14,643 has an increase in relative liver weight (Fig. 4.2).

The flow cytometry data demonstrates that Mafia mice express GFP in their macrophages (F4/80 positive), whereas the wild type mice do not (Fig. 4.3.).

By counting the number of cells per 30 photo frames per slide (animal), we estimated the size of hepatocytes (Fig. 4.4). It is interesting that there was no difference due to the corn oil versus PCB-153 treatment. There is a decrease in the size of hepatocytes with the solvent administration (PEG-Tween solution in water) and even more so with the dimerizer. For the Wy-14,643 group, this effect is not present most probably due to the hypertrophy induced by Wy-14,643.

We quantified hepatocyte proliferation by BrdU immunohistochemical staining. In the wild type mice, Wy-14,643 significantly increased cell proliferation, but PCB-153 increased the cell proliferation just about 30% comparing to the corn oil wild type group (Fig. 4.5). It is interesting to notice that, for the PCB and Wy-14,643 groups, the groups receiving just the PEG-Tween solution in water had a lower labeling index compared to their respective control mice that did not receive retroorbital injection. The drug had also a similar effect but not as marked.

NF-κB DNA binding activity was determined using the electrophoretic mobility shift assay. The only increase in DNA binding activity was due to the drug and was not affected by oil, PCB or Wy-14,643 treatment (Fig. 4.6).
Figure 4.1 Body weight at the end of the experiment
Figure 4.2. Relative liver weight.
Figure 4.3. Flow cytometry data

a) for a wild type mice

b) for a Mafia mice

Two markers were used: F4/80 for the entire macrophage population and GFP for the macrophages expressing the transgene.
Figure 4.4. Estimation of hepatocyte size
Hepatocyte proliferation

Figure 4.5 Quantitation of hepatocyte proliferation

The labeling index was determined by BrdU immunohistochemical staining.

Values represent mean ± SEM
Figure 4.6. Mice hepatic DNA binding activity of NF-κB. NF-κB DNA binding activity was determined using the electrophoretic mobility shift assay. Each line contains extract from a single animal.
**Discussions**

The mice receiving the dimerizer lost about 24% of their body weight whereas the mice receiving mock injections neither lost nor gained weight during the five injection days. Previously, this weight lost was associated with depletion (Burnett *et al.*, 2004). In our study, however, despite the fact that all the mice injected with the dimerizer were losing weight, not all of them were depleted of liver macrophages, as indicated by the anti-GFP antibody immunohistochemically stained liver tissues. It has been previously published (Burnett *et al.*, 2004) that GFP and suicide construct expression was detected in 78% of isolated peritoneal cells. In AP20187 treated mice, apoptosis was induced in more than 90% of peritoneal and bone marrow macrophages and dendritic cells, and in more than 70% of macrophages of blood, spleen, lung and thymus (Burnett *et al.*, 2004). But, in the same study, in the brain and lymph nodes, the depletion was not induced (Burnett *et al.*, 2004). Before our study, no flow cytometry data has been obtained regarding the GFP and suicide construct expression in the liver, but the degree of depletion induced by the dimerizer was significant as has been shown by immunohistochemistry with anti-GFP antibody. In our study, the flow cytometry data revealed comparing levels of transgene expression with the other organs; however the extent of depletion, as revealed by immunohistochemistry for GFP was lower than expected. One possibility was that the strain of mice has lost the ability to be depleted along the generations; another possibility would be that our retro orbital administration of the dimerizer was not as effective in depleting the liver resident macrophages as the reported ip administration.

The animals in the Wy-14,643 group treated with the dimerizer developed inflammatory lesions with neutrophil infiltration. This finding agrees with other reports showing that macrophages undergoing Fas-mediated apoptosis cause strong inflammatory response both by releasing proinflammatory cytokines, such as macrophage inflammatory protein-2, TNF-α and IL-1β and by releasing neutrophil chemotactic factors (Mizgerd *et al.*, 1996; Rai *et al.*, 1996; Hohlbaum
et al., 2001; Hohlbaum et al., 2002). All these effects might be exacerbated by Wy-14,643, reason for which at this time point, among the groups receiving the dimerizer, the Wy-14,643 group (not the control or the PCB groups) presented inflammatory lesions. The release of TNF-α could explain also the NF-κB activation data: the groups receiving the drug had higher NF-κB activation. And this end point might be even more sensitive than the neutrophil infiltration to the cytokine release triggered by Fas-induced apoptosis.

In conclusion, we failed to uniformly deplete Kupffer cells in the livers of Mafia mice. However, the fact that in the Wy-14,643 group the vehicle of the drug in the PEG-solution as vehicle decreased cell proliferation so drastically, it is an important observation and might provide a method of decreasing the cell proliferation induced by such potent hepatotoxicants as Wy-14,643 at a remarkable degree without affecting the Kupffer cells. The fact that PEG is a component of many pharmaceutical compounds, might give importance to our results for clinical practice. It would be interesting to study the effect of this solution on hepatocyte proliferation in wild type mice and on a route of administration more relevant for clinic than the retroorbital administration.
Chapter 5: Effects of PCB-153 on Kupffer Cells In Vitro

Introduction

Polychlorinated Biphenyls (PCBs) are a group of chlorinated aromatic hydrocarbons, which were widely used in industry for their physical properties, such as low reactivity and thermal stability, but the same properties allow them to persist in nature causing accumulation in biological systems. PCB-153 is one of the most abundant congeners. In the second chapter we described that this congener was able to increase the preneoplastic foci formation. Our hypothesis was that Kupffer cells are involved in the promoting effect of PCB-153. But, we did not detect any effect of Kupffer cell presence or secretory activity on foci promotion or hepatocyte proliferation in any of our animal experiments. We questioned if this is a result of Kupffer cells not being activated by this congener or being activated but without a net effect on hepatocyte proliferation and carcinogenesis. Kupffer cells are involved in other processes that are traumatic for the liver: hepatotoxicity [e.g. alcohol (Yin et al., 1998), endotoxin (Rose et al., 2000), peroxisome proliferators (Rose et al., 1999a), CCl₄ (Rivera et al., 2001), thioacetamide (Andres et al., 2003), cocaine (Labib et al., 2003)], liver transplantation (Schemmer et al., 1998; Schemmer et al., 1999), liver reperfusion injury (Thurman et al., 1998), and hemorrhagic shock (Zhong et al., 1999). Their wide implication in liver pathology might be due to the fact that they are the resident macrophages of the liver with both phagocytic and secretory activities and having a major role in the liver's microenvironment composition. Regarding carcinogenesis, Kupffer cells might have dual role: promotion of carcinogenesis by releasing soluble factors such as TNF-α, NO, PGE2, and IL-1 (Ikejima et al., 1996; Bojes et al., 1997; Rose et al., 2000; Ding et al., 2003) and an anticarcinogenic effect by their phagocytic activity, especially against tumors that express non-self antigens (Clavien, 1999). Kupffer cell inhibition by glycine was shown to inhibit the development of liver tumors promoted by Wy-14,643 (Rose et al., 1999a). The mechanism was proposed to involve TNF-α. TNF-α is a
cytokine produced and released by monocytes, macrophages, granulocytes, fibroblasts, endothelial cells and epithelial cells (Spriggs et al., 1992; Diehl et al., 1994; Climuro et al., 1997). TNF-α has a dual role in cancer, depending on the many other factors. In early stages, TNF-α facilitates tumor cell adhesion but, at later cancer stages, it inhibits the formation of liver metastasis (Sturm et al., 2003). Thus, the time point of its manipulation can have beneficial or harmful effects. TNF-α has multiple and diverse functions: cytolysis, differentiation, mitogenesis, lipid metabolism and modulation of electrophysiological properties of myenteric neurons (Yamada et al., 1998; Rehn et al., 2004). In vitro, hepatocytes cultivated with TNF-α, insulin and epidermal growth factor (EGF) had a significantly higher rate of proliferation than hepatocytes cultivated with insulin and EGF without TNF-α, evidence that TNF-α is able to prime the hepatocytes to divide and make them more susceptible to mitogens (Diehl et al., 1994). As noted above, TNF-α was proposed as central in liver carcinogenesis promoted by Wy-14,643 (Bojes et al., 1997; Rose et al., 1997a; Rose et al., 1997b; Rusyn et al., 1998). And, Anti-TNF-α antibody abrogated the hepatocyte proliferation induced by Wy-14,643 in rats (Bojes et al., 1997). But, studies with mice nullizygous for TNF-receptor 1 (TNFR1), TNFR2, or both receptors showed that TNF-α signaling is not involved at all in this process (Anderson et al., 2001). Also, studies with TNF-α knockout mice reached the same result (Lawrence et al., 2001). Contradictory results are also obtained regarding the TNF-α mRNA after Wy-14,643 treatment: some studies noticed a 2-2.5-fold increase (Bojes et al., 1997; Rose et al., 1997b; Rusyn et al., 1998), whereas others noticed a 50% decrease (Lawrence et al., 2001).

The transcriptional factor NF-κB is involved in TNF-α gene transcription; the human TNF-α gene has 4 κB binding sites in its promoter and transcription of this gene takes place only when NF-κB heterodimers p50/p65 bind to all four sites (Goto et al., 1996). The products of a multitude of genes under NF-κB control are involved in biological functions such as immunity, including inflammation, cell growth and differentiation, cell proliferation and apoptosis,
angiogenesis and cell migration (Rose et al., 2000; Karin et al., 2002). After Wy-14,643 treatment, NF-κB is activated in Kupffer cells in two hours and in hepatocytes in eight hours, with the early NF-κB activation in Kupffer cells leading to TNF-α activation (Rusyn et al., 1998). Once released by Kupffer cells, TNF-α is a mitogenic cytokine that acts upon hepatocytes and activates NF-κB in these cells (Rusyn et al., 1998; Rose et al., 2000).

To determine whether PCB-153 activates Kupffer cells directly, we isolated and cultured rat Kupffer cells, and NF-κB DNA binding activity and TNF-α production were then assessed.


**Materials and Methods**

**Materials and animals:**

1. PCB-153 (2,2',4,4',5,5'-hexachlorobiphenyl) was synthesized and characterized as described previously (Schramm et al., 1985) by Dr Lehmler. The purity was greater than 99%, as assayed by gas-chromatography (Lehmler and Robertson, 2001)

2. Medium chain trygliceride oil (MCT) oil was obtained from Mead Johnson Nutritionals (Evansville, IN),

**Animals:**

Male Wistar rats, 200g body weight, were used to isolate Kupffer cells

**Kupffer cell isolation:**

**Reagents:**

1. Hanks 10x, Sigma H4641
2. NaHCO3, Sigma, S5761
3. Albumin, Sigma, A9418
4. antibiotic/antimycotic (100x), Atlanta Biologicals, B22110
5. autoclaved nanopure water
6. collagenase type IV, Sigma, C5138
7. heparin, Sigma, H3149
8. Nembutal, Abbot Laboratories, NDC 0074-3778-05
9. 70% EtOH
10. NH4Cl, Sigma, A4514
11. Tris-HCl 1M pH 7.2, Sigma T 2538
12. Dulbecco’s phosphate buffered saline with Mg 10X, Sigma D1283
13. Dulbecco’s phosphate buffered saline without Mg 10X, Sigma D1408
14. Hapes Buffer Solution 1M Gibco 15630-080
15. EGTA Sigma E 3889

**Solutions:**
1. NH4Cl-Tris:
   1. 8.3g NH4Cl
   2. 800 ml H2O
   3. 100 ml Tris-HCl pH 7.2
   4. adjust to pH 7.2, add H2O to 1 l; autoclave in 100 ml bottles.

2. Hanks working solution:
   1. 200 ml Hanks 10x
   2. 0.7g NaHCO3
   3. 5g albumin
   4. 20 ml antibiotic/antimycotic
   5. add H2O to 2 l
   6. filter through 0.22µm filter
   7. pH should be 7.4-7.6, if needed adjust

3. Buffer 1
   1. 50 ml DPBS without Mg
   2. 95 mg EGTA
   3. 5 ml antibiotic/antimycotic
   4. 175 mg NaHCO3
   5. add to 500 with autoclaved water
   6. filter through 0.22µm filter
   7. pH should be 7.4-7.6, if needed adjust

4. Buffer 2
   1. 50 ml DPBS with Mg
   2. 0.5g glucose
   3. 5ml antibiotic/antimycotic
   4. 300 mg NaHCO3
   5. add to 500 with autoclaved water
   6. filter through 0.22µm filter
7. pH should be 7.4-7.6, if needed adjust

5. Buffer 2 for digestion (made just prior use)

1. Buffer 2 wormed at 37°C
2. 0.2% collagenase type IV
3. 1000 U/ml heparin

Procedure:
1. The rat was anesthetized with nembutal
2. The abdomen was cleaned with 70% EtOH and laparotomy was performed
3. The liver was perfused with about 50 ml Buffer 1 at 37°C
4. Buffer 2 for digestion was used next for perfusion at 37°C
5. The liver was excised and placed in leftover buffer 2 for digestion
6. The capsulae was peeled away and the cell suspension was filtered through a mesh using COLD buffer 2 with NO collagenase
7. To pellet hepatocytes, it was performed centrifugation at 100 x g, 3 min, 5°C to. This step was repeated
8. Spin supernatant 300 x g for 7 min to pellet Kupffer cells.
9. Add NH4Cl-Tris; place in dark for 2 min.
10. Centrifuge at 300 x g, 7 min, 5°C
11. Resuspend in 15 ml Hanks and centrifuge 300 x g 7 min to wash away NH4Cl.
12. Pellet was resuspended in 10 ml Hanks and loaded into the elutriaion in two steps. The elutriaion was set for 3200 rpm and 4°C.
13. Collected cells are spin at 1400 rpm 8 minutes
14. Pellet is resuspended in RPMI-1640 with 10% serum and 1% antibiotic/antimycotic
**Cell culture**

The cells obtained as mentioned above, were plated in 6 well plastic-culture plates. Cells were grown in presence of 5% CO$_2$, 37°C, in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin, streptomycin, amphotericin.

One hour after first plating, the medium was changed to improve the purity of the Kupffer cell culture since this type of cells is the first one to adhere to the plate among the liver cells.

In most of our experiments, 24 hours after first plating, the 48 hours treatment was started. We also did a 6,12, 24 h time-course study. We had every time control (wells with Kupffer cells cultured just in regular medium), oil control (wells with Kupffer cells cultured in regular medium and MCT oil 0.5% in final medium mix) and PCB-153 treated cells (wells with Kupffer cells cultured in regular medium and with 10 µM PCB 153 in MCT MCT 0.5% in final medium mix).

At the end of the treatment, the culture medium was removed and stored at -80°C until the ELISA was performed and the cells were used to isolate nuclear extracts as described below.

**Isolation of nuclear extracts:**

Buffer A was prepared in autoclaved water and contained:

1. 10 mM HEPES pH7.9
2. 1.5 mM MgCl$_2$
3. 10 mM KCl
4. 0.5 mM DTT
5. 0.2 mM PMSF

Buffer C was prepared in autoclaved water and contained:

1. 20 mM HEPES pH7.9
2. 1.5 mM MgCl$_2$
3. 0.5 mM DTT
4. 0.2 mM PMSF
5. 25% Glycerol
6. 420 mM NaCl
7. 0.2 mM EDTA
8. 2 mM benzamidine
9. 5 µg/ml aprotinin
10. 5 µg/ml leupeptin
11. 5 µg/ml pepstatin

Nuclear extracts were prepared fresh cells as following:

1. cells were harvested, washed with PBS and transferred to an eppendorf tube
2. the cells were spin down at 3000 rpm for 10 minutes
3. the pellet was resuspended in 1ml buffer A, vortexed and kept on ice for 10 minutes
4. the tubes were spin down at 3000 rpm for 5 minutes
5. 70 µl cold buffer C was added to the pellet
6. the samples were kept on ice for 20 minutes
7. the membranare debries were pelleted down by centrifugation at 14000 rpm for 5 minutes and the sup was aliquoted and stored at -80°C.

**Radiolabeling and purification of NF-κB oligonucleotide (probe synthesis)**

T4-polynucleotide kinase was used to label the 5'-OH group of the NF-κB oligonucleotide with [γ-32P] ATP as described in chapter two.

**Protein assay:**

Protein concentrations were assayed as described in chapter two using the bicinchoninic acid-BCA method.
Electrophoretic mobility shift assay (EMSA):

NF-κB DNA binding activities were measured using electrophoretic mobility shift assay as described in chapter two.

0.2µg/well Poly dIdC and 3µg protein/ well were used. A 7% polyacrylamide, 0.75 mm thick gel was used to resolve the bands in one hour and a half at 170V in 0.5 x TBE running buffer. Gels were exposed to a phosphoimaging screen and analyzed using the STORM Phosphoimager (Molecular Dynamics, Sunnyvale, CA) and ImageQuant 5.0 software (Molecular Dynamics).

Enzyme-linked immunosorbent assay (ELISA):

The ELISA assay was performed to assay the amount of rat tumor necrosis factor contained in rat Kupffer cell culture supernatant, using BD OptEIA Set, cat nr 558870 purchased from BD Biosciences Pharmingen and following the manufacturer’s instructions.

Briefly, the procedure was as following:
1. each well was coated with capture antibody overnight and then washed
2. each well was blocked with assay diluent for 1h at room temperature and then washed
3. standards and samples were added to the respective wells, incubated for 2 hours at room temperature and then washed
4. detection antibody was added for 1 hour at room temperature and then washed
5. avidin-HRP was added for 30 minutes at room temperature and then washed
6. TMB substrate solution was added for 30 minutes, room temperature, dark
7. stop solution was added and the reactions were read at a microplate reader at 450 nm with λ correction 570 nm.
Results

In this study, we examined the ability of PCB-153 to activate the resident macrophages of liver Kupffer cells. Rat Kupffer cells were isolated by the collagenase-elutriation method and cultured in RPMI medium. The exact conditions of elutriation were established by collecting cells at different fluid speeds and assessing them by flow cytometry as described in chapter four to detect the richest population in Kupffer cells. PCB-153 was added to the cells in a MCT oil solution since we noticed that DMSO or DMFA are toxic for the cells. Our model was that PCB-153 induces NF-κB activation in Kupffer cells, fact that leads to TNF-α release.

Our data showed that in Kupffer cells, PCB-153 is able to increase NF-κB at 48 hours after treatment (Fig. 5.1). But, we did not obtain consistent activation at 48 hours every time when we repeated the experiment in the same conditions, and DNA binding activity was not increased to any other time point that we tested (Fig. 5.2.). The release of TNF-α was not increased compared to the control not even for the media collected from the cells that showed increase in NF-κB (table 5.1.).
Figure 5.1. Rat hepatic DNA binding activity of NF-κB after 48h treatment.

NF-κB DNA binding activity was determined using the electrophoretic mobility shift assay.
Figure 5.2. Rat hepatic DNA binding activity of NF-κB – time course. NF-κB DNA binding activity was determined using the electrophoretic mobility shift assay.
Table 5.1. TNF-α concentrations following 48 hour treatment.

<table>
<thead>
<tr>
<th>TREATMENT</th>
<th>TNF-α (pg/ml) Individual samples</th>
<th>TNF-α (pg/ml) Average per treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no treatment)</td>
<td>136, 168</td>
<td>152</td>
</tr>
<tr>
<td>MCT oil treatment</td>
<td>153, 173</td>
<td>163</td>
</tr>
<tr>
<td>PCB-153 in MCT treatment</td>
<td>175, 175</td>
<td>175</td>
</tr>
</tbody>
</table>
Discussion

The aim of the cell culture part was to determine if PCB-153 was able to activate NF-κB and to stimulate TNF-α release in Kupffer cells. We obtained a variable response to the first part of the question and a negative answer for the latter. This result, however, is not very surprising for this congener. There are 6 published studies on TNF-α and PCBs, 4 of them published after we started our project, and none of them observing TNF-α induction with PCBs in non-malignant cells. Ours is the first to look at the Kupffer cell population. TNF-α production by in vitro human peripheral blood leukocytes is marginally decreased by PCB-153 (Devos et al., 2004) and by more than 60% by either PCB-77 or PCB-126 (Ahne and Jarre, 2002) when the TNF-α production by the whole blood was stimulated by lipopolysaccharides/phytohemagglutin in vitro. TNF-α levels were not different in monkeys administered Aroclor 1254 p.o. compared to the control group (Tryphonas et al., 1991). PCB-153 does not enhance endotoxicity or TNF-α or IL-6 production by Kupffer cells (Hoglen, 1993). The secretion of TNF-α requires G binding proteins (Spriggs et al., 1992) and a surge in intracellular Ca concentrations (Edwards et al., 1993). PCB-153 is very potent in inhibiting calcium homeostasis mechanisms in the brain cells. (Tilson HA, 1997). If this also occurred in Kupffer cells, it may explain why TNF-α release was not changed despite the NF-κB activation.

Two more reports, might shed light on our results: In regenerating livers, the bile ducts and veins were found to be the major sources of TNF-α, not Kupffer cells (Loffreda et al., 1997). Reports also indicate that sometimes Kupffer cells behave completely different in vivo and in vitro. Ethanol modifies the function of L-type voltage-dependent Ca2+ channels in Kupffer cells after intragastric administration to rats but has no effect when it is added directly to the Kupffer cell culture (Hijioka et al., 1993).
Summary

Polychlorinated biphenyls (PCBs) are ubiquitous lipophilic environmental pollutants. Due to their lipophilicity, PCBs accumulate in the food chain and tend to concentrate toward the top of this chain i.e. into humans. At least some of the PCB congeners and mixtures of congeners are non-genotoxic hepatic tumor promoters. The mechanism is not fully understood and is likely to be multifactorial. A complex intercellular communication usually accompanies any kind of cancer. The aim of this study was to investigate if the resident liver macrophages, Kupffer cells, are important for the promoting activity of PCBs. The hypothesis of this study was that modulation of Kupffer cell activity by PCBs may account for PCB induced liver tumor promotion. The second hypothesis was that PCB-153 modulates the Kupffer cell activity by increasing DNA binding activity of the transcriptional factor NF-κB in these cells leading to increased cytokine (e.g. TNF α) release, followed by increased hepatocyte proliferation.

In our first study, we investigated if Kupffer cell activity modulates the formation of preneoplastic foci induced by PCB-77 and PCB-153 in a two stage carcinogenesis model. PCB-153 did not significantly increase the focal volume, but increased the number of foci formed, PCB-77 increased both the foci number and their volume, and glycine (an inhibitor of Kupffer cell secretory activity) had no effect for either PCB.

In our subsequent four experiments, we investigated if Kupffer cells modulate the amplitude of hepatocyte proliferation induced by PCB-153. The model used was a short term exposure model, with Kupffer cell secretory activity inhibition, for the first three experiments, and with Kupffer cell depletion for the forth experiment. PCB-153 did not increase the cell proliferation. The oil used as vehicle had no significant effect on any of the end points considered. Inhibition of Kupffer cells with glycine did not affect the cell proliferation and NF-κB activation
compared with the valine control after PCB treatment. The absence of Kupffer cells did not affect hepatocyte proliferation.

Using an *in vitro* approach, we examined if rat Kupffer cells are activated by PCB-153 added to the culture. PCB-153 increased DNA binding activity of NF-κB in cultured Kupffer cells and did not significantly increase the TNF-α concentration in the medium.

**Conclusions and Future Directions**

In conclusion, in our experimental models, PCB-153 as the only challenge increased tremendously the Cyp450 2B1/2, and also increased the number of preneoplastic foci per liver in the casein group but had no significant effect on cell proliferation. Kupffer cells seem not to play a significant role on hepatocyte proliferation in this model. This is in correlation with other findings. PCB-153 alone does not significantly upregulate or downregulate any gene expression, in rat, with the exception of the genes for cytochrome 2B1/2 that are strongly upregulated (Vezina *et al.*, 2004). Another study, looking at the effect of PCB-153 on expression of proteins controlling G0/G1-S-phase transition and S-phase progression, found no effect for this congener (Vondracek *et al.*, 2005).

The fact that in cell culture NF-κB was activated by PCB-153 shows that Kupffer cells are responsive to this congener. The apparent discrepancy between the NF-κB activation in Kupffer cells by PCB-153 and lack of downstream effects (TNF-α release by Kupffer cells, induction of hepatocyte proliferation, etc) needs further investigation. One reason might be that PCB alone is not sufficient to elicit from Kupffer cells a strong enough response to influence the hepatocyte proliferation. Kamendulis *et al.*, (2005) found out that using LPS as promoter after DEN initiation, Kupffer cells were involved in the promoting process. Kupffer cells are involved in PCB-126 enhancement of LPS induced endotoxicity and hepatotoxicity via TNF-α but PCB-153 does not enhance endotoxicity or TNF-α production neither IL-6 production (Hoglen,
It would be interesting to study the importance of hepatic Kupffer cells in the promoting activity of dioxin-like PCBs such as PCB-77 and PCB-126 in conditions of low LPS challenge. This would also be important for the PCB exposed population, since everybody is exposed almost constantly to low LPS doses during life. For some chemicals, the Kupffer cells are the first to respond to the insult, as in the case of Wy; for other compounds, as CCl$_4$, Kupffer cells are activated by free radicals released from hepatocytes after CCl$_4$ metabolism and by endotoxin from the gut (Rivera et al., 2001). It might be that Kupffer cells are not the main player, or not the initial player in the PCB-153 induced hepatotoxicity, but they might have a role in the combination of immunological challenge and hepatotoxicity challenge. An important factor in LPS-induced intrahepatic cholestasis is the modulation of transporter proteins as multi drug resistance proteins (Mrp2, Mrp6, Mdr1a), organic anion-transporting polypeptide (Oatp1, Oatp2, Oatp4), Ntcp, bile salt export pump, organic cation transporters (Oct1, and Oat3), all dramatically decreased (Cherrington et al., 2004). These events were independent of Kupffer cells; and it is also known that bile duct cells are major producers of TNF-α in some conditions (Loffreda et al., 1997). The National Toxicology Program found that cholangioma was the only effect after two years exposure to PCB-153. As of July, 2005, this finding is reported on the internet at http://ntp.niehs.nih.gov/INDEX.CFM?OBJECTID=FB5B77E2-F1F6-975E-3E47DD3ACDDA48D. Thus, it might be interesting to look at the expression of liver protein transporters in PCB exposed rats that are exposed to LPS. Cholangioma might progress to cholangiosarcoma in those conditions.

We did not find NF-κB activation in hepatocytes, and this might mean that our sampling was before or after the NF-κB peak. IL-6 was reported to be released by hepatocytes as a result of NF-κB activation (Yamada et al., 1997). In the same study, it has been shown that IL-6 promotes hepatocyte proliferation only in a narrow concentration range, and once the superior threshold is reached, the hepatocytes can not divide. As we did not find hepatocyte proliferation, either, but we found increased preneoplastic foci formation with the PCB treatment, it would be interesting to measure the levels of IL-6 in serum and
mRNA for IL-6 in the liver samples. It would be of interest to study IL-1α and IL-1β, as they are also proinflammatory cytokines and were reported to be able to induce hepatocyte proliferation after nafenopin treatment (West et al., 1999). It has been recently reported that in the macrophages IKKα is involved in quenching NF-κB pro-inflammatory activity by accelerating both the turnover of the NF-κB subunits RelA and c-Rel, and their removal from pro-inflammatory gene promoters (Lawrence et al., 2005). It would be interesting to assess by Western analyses the levels of IKK subunits, IκB isoforms and p50, p52, c-Rel, Rel A (p65), and Rel B proteins both in cytosol and in the nucleus after PCB treatment.

PCBs are lipid soluble compounds. Some likely effects of their presence in the organism are lipid metabolism perturbation, integration in the membranes, and membrane domain perturbation. PCB-77 was shown to be distributed mainly in the cytosol (Pereg et al., 2001); it would be interesting to see where PCB-153 is localized. My hypothesis is that it would be mainly in the membranes and that interferes with the cell membrane and organelle membrane structure. In the liver, it is likely to be higher in stellate cells, since they have higher lipid content. Inhibition of Kupffer cells, either with gadolinium chloride or with glycine, affects lipid metabolism by increasing the fatty acid release by adipose tissue, triglyceride synthesis and steatosis in the liver (Neyrinck et al., 2002). This fact might result in more PCB accumulation in the liver in our glycine groups, due to lipid redistribution. To test this possibility, we sent samples from our tumor promotion study to an analytical chemistry laboratory to determine the liver concentrations of PCBs in the different groups. The non-coplanar congeners, including PCB-153, are the most potent PCBs in decreasing dopamine content and in inhibiting calcium homeostasis mechanisms in brain cells. (Tilson HA, 1997). PCB-153 is highly retained in the lipid-rich structures of the body, and has a great impact on the nervous and endocrine systems (Tilson HA, 1997; Miyazaki et al., 2004); the neuroendocrine impact is another mechanism this congener can affect the metabolism. If the calcium homeostasis is perturbed by PCB-153 in the macrophages as well, it might explain the lack of responsiveness to glycine.
It is well known that calcium is important for the functioning of these resident macrophages (Bermelin and Decker, 1983; Rose et al., 1997a). In our model, a calcium surge after treatment was hypothesized as an important factor that leads to cytokine release by Kupffer cells, cytokines that might affect gene expression in hepatocytes and therefore the cell proliferation/apoptosis ratio. One direction for further investigations would be to see if PCB-153 blunts the calcium surge in Kupffer cells. In that case these cells might not be able to release TNF and other soluble factors, in other words, maybe the toxic effect induced by PCB-153 are not due to an increase in calcium concentrations and therefore a activation of Kupffer cells but rather by an inhibition of the activity of these macrophages by inhibiting calcium surge. To investigate that theory, it may be useful to compare the response to PCB in rodents receiving a regular diet, glycine as a Kupffer cell inhibitor, or oligofructose (reported to increase the phagocytic activity and production of Kupffer cell –derived mediators (Neyrinck, 2004)). If there are differences, a corollary would be the finding of dietary recommendations for hepatoprotection against PCBs.

Another PCB, PCB-164 is able to induce CAR nuclear translocation (Kawamoto et al., 1999), to trans-activate phenobarbital response element (Sueyoshi et al., 1999) and to induce the human CYP 450 2B6 (Sueyoshi et al., 1999). A transcription factor array would be interesting to see what transcription factors are affected by PCB-153.

An interesting aspect to study could be the interaction between diet and PCB toxicity. PCB-153 is a phenobarbital like compound and for phenobarbital it has been shown that dietary restriction protects against hepatic preneoplastic lesions. (Kolaja et al., 1996) However, for the PCBs (lipophylic moieties) the effect of caloric restriction and weight loss is controversial, because it may lead to PCB release from the adipose tissue. Interesting, caloric restriction would modulate hepatic metabolism and that might have an effect upon foci formation.
Appendix


**Proximate analysis:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude Protein</td>
<td>18.90%</td>
</tr>
<tr>
<td>Crude Oil</td>
<td>6.00%</td>
</tr>
<tr>
<td>Crude Fiber</td>
<td>3.80%</td>
</tr>
<tr>
<td>Ash</td>
<td>5.90%</td>
</tr>
<tr>
<td>NFE</td>
<td>55.40%</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>57.33%</td>
</tr>
<tr>
<td>Starch</td>
<td>41.24%</td>
</tr>
<tr>
<td>Sugar</td>
<td>4.93%</td>
</tr>
<tr>
<td>Digestible Energy</td>
<td>3.4 (14.03)Kcal/g (MJ/kg)</td>
</tr>
<tr>
<td>Metabolizable Energy</td>
<td>3.3 (13.70)Kcal/g (MJ/kg)</td>
</tr>
</tbody>
</table>

**Minerals:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td>1.01%</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.65%</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.23%</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.68%</td>
</tr>
<tr>
<td>Chloride</td>
<td>0.40%</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.20%</td>
</tr>
<tr>
<td>Zinc</td>
<td>77.00mg/kg</td>
</tr>
<tr>
<td>Manganese</td>
<td>118.00mg/kg</td>
</tr>
<tr>
<td>Copper</td>
<td>15.21mg/kg</td>
</tr>
<tr>
<td>Iodine (added)</td>
<td>11.55mg/kg</td>
</tr>
<tr>
<td>Iron</td>
<td>226.00mg/kg</td>
</tr>
</tbody>
</table>
Selenium 0.20mg/kg
Cobalt 0.63mg/kg
Chromium 0.53mg/kg

**Amino Acids:**
Aspartic Acid 1.42%
Glutamic Acid 3.70%
Alanine 1.08%
Glycine 0.79%
Threonine 0.67%
Proline 1.55%
Serine 0.97%
Leucine 1.94%
Isoleucine 0.85%
Valine 0.95%
Phenylalanine 0.99%
Tyrosine 0.61%
Phe + Tyr 1.60%
Methionine 0.35%
Cystine 0.33%
Met + Cyst 0.69%
Lysine 0.92%
Histidine 0.47%
Arginine 1.06%
Tryptophan 0.20%
Available Lysine 0.83%

**Vitamins:**
<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>15.40 iu/g</td>
</tr>
<tr>
<td>Retinol</td>
<td>4.65 mg/kg</td>
</tr>
<tr>
<td>Vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1.54 iu/g</td>
</tr>
<tr>
<td>Cholecalciferol</td>
<td>38.39 g/kg</td>
</tr>
<tr>
<td>Vitamin E (α-tocopherol)</td>
<td>101.00 mg/kg</td>
</tr>
<tr>
<td>Vitamin K&lt;sub&gt;3&lt;/sub&gt; (menadione)</td>
<td>51.00 mg/kg</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;1&lt;/sub&gt; (thiamine)</td>
<td>16.50 mg/kg</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;2&lt;/sub&gt; (riboflavin)</td>
<td>14.90 mg/kg</td>
</tr>
<tr>
<td>Avail. Niacin (nicotinic acid)</td>
<td>41.20 mg/kg</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;6&lt;/sub&gt; (pyridoxine)</td>
<td>18.50 mg/kg</td>
</tr>
<tr>
<td>Pantothenic Acid</td>
<td>33.00 mg/kg</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt; (cyanocobalamin)</td>
<td>0.08 mg/kg</td>
</tr>
<tr>
<td>Avail. Biotin</td>
<td>0.30 mg/kg</td>
</tr>
<tr>
<td>Folate</td>
<td>3.34 mg/kg</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>0.00 mg/kg</td>
</tr>
<tr>
<td>Choline</td>
<td>1120.00 mg/kg</td>
</tr>
<tr>
<td>B Carotene</td>
<td>2.47 mg/kg</td>
</tr>
<tr>
<td>Inositol</td>
<td>1455.00 mg/kg</td>
</tr>
</tbody>
</table>

**Fatty Acids:**

**Saturated:**
- C4:0 Butyric 0.00 g/kg
- C6:0 Caproic 0.00 g/kg
- C8:0 Caprylic 0.00 g/kg
<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>C10:0 Capric</td>
<td>0.00g/kg</td>
</tr>
<tr>
<td>C12:0 Lauric</td>
<td>0.26g/kg</td>
</tr>
<tr>
<td>C14:0 Myristic</td>
<td>0.06g/kg</td>
</tr>
<tr>
<td>C15:0 Pentadecanoic</td>
<td>0.00g/kg</td>
</tr>
<tr>
<td>C16:0 Palmitic</td>
<td>7.64g/kg</td>
</tr>
<tr>
<td>C17:0 Margaric</td>
<td>0.00g/kg</td>
</tr>
<tr>
<td>C18:0 Stearic</td>
<td>1.50g/kg</td>
</tr>
<tr>
<td>C20:0 Arachidic</td>
<td>0.10g/kg</td>
</tr>
<tr>
<td>C22:0 Behenic</td>
<td>0.03g/kg</td>
</tr>
<tr>
<td>C24:0 Lignoceric</td>
<td>0.00g/kg</td>
</tr>
</tbody>
</table>

**Mono-unsaturated:**

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:1ω7 Palmitoleic</td>
<td>0.07g/kg</td>
</tr>
<tr>
<td>C17:1ω8 Heptadecenoic</td>
<td>0.00g/kg</td>
</tr>
<tr>
<td>C18:1ω9 Oleic</td>
<td>12.59g/kg</td>
</tr>
<tr>
<td>C20:1ω9 Gadoleic</td>
<td>0.17g/kg</td>
</tr>
<tr>
<td>C22:1ω9 Erucic</td>
<td>0.01g/kg</td>
</tr>
</tbody>
</table>

**Polyunsaturated:**

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18:2ω6 Linoleic</td>
<td>31.35g/kg</td>
</tr>
<tr>
<td>C18:3ω3 Linolenic</td>
<td>2.76g/kg</td>
</tr>
<tr>
<td>C18:4ω3 Octadecatetraenoic</td>
<td>0.00g/kg</td>
</tr>
<tr>
<td>C20:2ω6 Eicosadienoic</td>
<td>0.00g/kg</td>
</tr>
<tr>
<td>C20:3ω6 Dihomo-gamma-linolenic</td>
<td>0.00g/kg</td>
</tr>
<tr>
<td>C20:4ω6 Arachidonic</td>
<td>0.00g/kg</td>
</tr>
</tbody>
</table>
C20:5\(\omega3\)  
Eicosapentanoic  
0.00g/kg

C22:5\(\omega3\)  
Clupanodonic  
0.00g/kg

C22:6\(\omega3\)  
Docosahexaenoic  
0.00g/kg

Nutrient levels are calculated from raw material data and are adjusted to 10% moisture level in the diet. Reported nutrient values may vary due to the inherent variability in laboratory analysis.

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The above mentioned table is located at :http://www.teklad.com/

Purina Mill 5001 diet (http://www.labdiet.com/indexlabdiethome.htm)

Chemical composition

**Nutrients**

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>23.4%</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.38%</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.32%</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.20%</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.55%</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>1.18%</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.70%</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.42%</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.43%</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.03%</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.68%</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.91%</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.29%</td>
</tr>
<tr>
<td>Valine</td>
<td>1.21%</td>
</tr>
<tr>
<td>Serine</td>
<td>1.21%</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>2.83%</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>4.54%</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.44%</td>
</tr>
<tr>
<td>Prolline</td>
<td>1.55%</td>
</tr>
<tr>
<td>Component</td>
<td>Percentage</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Taurine</td>
<td>0.02%</td>
</tr>
<tr>
<td>Fat (ether extract)</td>
<td>4.5%</td>
</tr>
<tr>
<td>Fat (acid hydrolysis)</td>
<td>5.5%</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>200ppm</td>
</tr>
<tr>
<td>Linoleic Acid</td>
<td>1.16%</td>
</tr>
<tr>
<td>Linolenic Acid</td>
<td>0.07%</td>
</tr>
<tr>
<td>Arachidonic Acid</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>Omega-3 Fatty Acids</td>
<td>0.26%</td>
</tr>
<tr>
<td>Total Saturated Fatty Acids</td>
<td>1.50%</td>
</tr>
<tr>
<td>Total Monounsaturated Fatty Acids</td>
<td>1.58%</td>
</tr>
<tr>
<td>Fiber (Crude)</td>
<td>5.3%</td>
</tr>
<tr>
<td>Neutral Detergent Fiber³</td>
<td>14.35%</td>
</tr>
<tr>
<td>Acid Detergent Fiber⁴</td>
<td>6.8%</td>
</tr>
<tr>
<td>Nitrogen-Free Extract (by difference)</td>
<td>49.9%</td>
</tr>
<tr>
<td>Starch</td>
<td>31.9%</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.23%</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.30%</td>
</tr>
<tr>
<td>Sucrose</td>
<td>3.68%</td>
</tr>
<tr>
<td>Lactose</td>
<td>1.67%</td>
</tr>
<tr>
<td>Total Digestible Nutrients</td>
<td>76.0%</td>
</tr>
<tr>
<td>Gross Energy</td>
<td>4.00 kcal/gm</td>
</tr>
<tr>
<td>Physiological Fuel Value⁵</td>
<td>3.34 kcal/gm</td>
</tr>
<tr>
<td>Metabolizable Energy</td>
<td>3.04 kcal/gm</td>
</tr>
</tbody>
</table>

**Minerals**

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash</td>
<td>6.9%</td>
</tr>
<tr>
<td>Calcium</td>
<td>0.95%</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>0.67%</td>
</tr>
<tr>
<td>Phosphorus (non-phytate)</td>
<td>0.40%</td>
</tr>
<tr>
<td>Potassium</td>
<td>1.10%</td>
</tr>
<tr>
<td>Magnesium</td>
<td>0.21%</td>
</tr>
<tr>
<td>Sulfur</td>
<td>0.28%</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.40%</td>
</tr>
<tr>
<td>Chlorine</td>
<td>0.65%</td>
</tr>
<tr>
<td>Fluorine</td>
<td>18 ppm</td>
</tr>
<tr>
<td>Iron</td>
<td>270 ppm</td>
</tr>
<tr>
<td>Zinc</td>
<td>70 ppm</td>
</tr>
<tr>
<td>Manganese</td>
<td>64 ppm</td>
</tr>
<tr>
<td>Copper</td>
<td>13 ppm</td>
</tr>
<tr>
<td>Cobalt</td>
<td>0.6 ppm</td>
</tr>
<tr>
<td>Iodine</td>
<td>0.8 ppm</td>
</tr>
<tr>
<td>Nutrient</td>
<td>Amount</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Chromium</td>
<td>2.0 ppm</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.27 ppm</td>
</tr>
</tbody>
</table>

**Vitamins**

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carotene</td>
<td>4.5 ppm</td>
</tr>
<tr>
<td>Vitamin K (as menadione)</td>
<td>0.5 ppm</td>
</tr>
<tr>
<td>Thiamin Hydrochloride</td>
<td>17 ppm</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>8.0 ppm</td>
</tr>
<tr>
<td>Niacin</td>
<td>124 ppm</td>
</tr>
<tr>
<td>Pantothenic Acid</td>
<td>24 ppm</td>
</tr>
<tr>
<td>Choline Chloride</td>
<td>2250 ppm</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>5.9 ppm</td>
</tr>
<tr>
<td>Pyridoxine</td>
<td>6.0 ppm</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.2 ppm</td>
</tr>
<tr>
<td>B12</td>
<td>22 mcg/kg</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>22 IU/gm</td>
</tr>
<tr>
<td>Vitamin D₃ (added)</td>
<td>4.5 IU/gm</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>49 IU/kg</td>
</tr>
<tr>
<td>Ascorbic Acid</td>
<td>--</td>
</tr>
</tbody>
</table>

**Calories provided by:**

<table>
<thead>
<tr>
<th>Category</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>28.049 %</td>
</tr>
<tr>
<td>Fat (ether extract)</td>
<td>12.137%</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>59.814%</td>
</tr>
</tbody>
</table>

* Product Code
1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly.
2. Nutrients expressed as percent of ration except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.

The above mentioned table is located at: [http://www.labdiet.com/indexlabdiethome.htm](http://www.labdiet.com/indexlabdiethome.htm)
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Vita

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Biochemist, Molecular Genetics Lab, Institute of Biology and Animal Nutrition-Balotesti Bucharest-Romania (1996-2000)

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- Member of the American Physiological Society
- Member of the American Society for Pharmacology and Experimental Therapeutics

Publications:

Published papers


Oral Presentation


Abstracts

- Bunaciu RP, Spear BT, Glauert HP. The effect of Kupffer cell inhibition on hepatocyte cell proliferation in rats administered PCB-77 or PCB-153.


- **Bunaciu, R.P.,** Vior, C., Iordachescu, D. Stimulating activity of a diphenyltin (IV) derivative on humoral immune response of chicks. Proceedings of the