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This undertaking relates to the activities of external collaborators and their access to any confidential information in respect of the European Commission Research Collaboration under the Seventh Framework Programme Innovative Medicines Initiative of the European Community. Project title: MARCAR, Grant number 115001 – "Biomarkers and Molecular Tumor Classification for Non-genotoxic Carcinogenesis"

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1. Introduction

For a better understanding of the mode of action of non-genotoxic carcinogens (NGC) in hepatocarcinogenesis, the following parts describe the unique functions and characteristics of the liver and its various cell types.

1.1 The liver: A multitasking organ

1.1.1 Liver structure and its function

The liver is a heterogeneous organ, being composed of five tissue systems: (1) the vascular system, (2) hepatocytes (HC) and hepatic lobule, (3) hepatic sinusoidal cells, (4) biliary system and (5) stroma. The vascular system comprises mainly of the portal vein and the hepatic artery which supply blood to the liver. The general functions of the liver involve the metabolism of amino acids and proteins, carbohydrates, bile acids, cholesterol, lipids and vitamins. Additionally, it is the major organ for biotransformation and defence against foreign macromolecules and xenobiotics. (Malarkey, et al., 2005; Hiromi, et al., 2009)

1.1.2 Liver cell types and "the fantastic four"

Many different cell types are present in the liver. Generally, hepatocytes are denoted as parenchymal cells. Endothelial cells (EC), Kupffer cells (KC), hepatic stellate cells (SC), natural killer (NK) cells, hepatic dendritic cells (DC) and NKT cells are summarized as mesenchymal or non-parenchymal cells (NPC). HC makes about 78% and NPC about 6% of the liver tissue volume. The last 16% of the liver tissue volume are covered by the extracellular space. (Hiromi, et al., 2009) Figure 1 gives a good overview about the whole liver architecture and the localization of the different cell types in the liver. As these thesis concentrates mainly on three cell types (HC, EC, KC), the following paragraphs give a detailed look in their individual functions and characteristics.

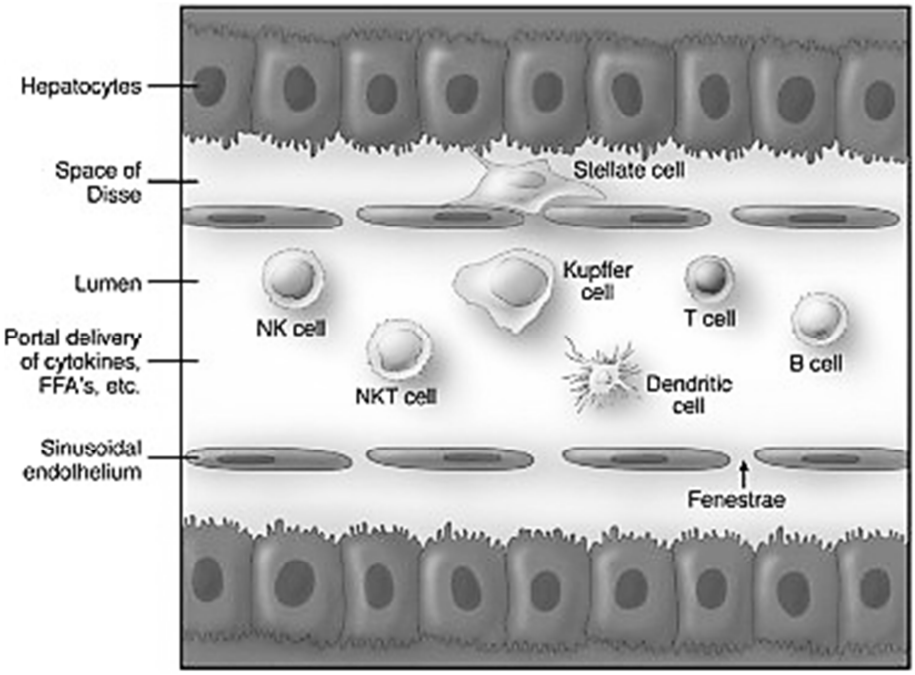

Figure 1: Liver architecture and localization of the different cell types in the liver.
Source: (Shoelson, et al., 2007)

Hepatocytes (HC)

Structurally, HC are large polygonal cells with a 25-30 µm cross section (Grisham, 2009) and six or more surfaces which border on bile canaliculi and the perisinusoidal space. HC are the power stations of the liver as they are equipped with the machinery necessary to carry out all the complex vital functions such as biotransformation. Additionally, HC produce approximately 15 ml bile per kg body weight and day in humans. (Malarkey, et al., 2005)

Endothelial cells (EC)

Specifically, the sinusoidal EC act as a kind of barrier to protect the HC from direct contact with blood. They have fenestrae, a lack of basal lamina and are able to transfer molecules and particles by endocytosis. Further functions include filtration of fluids, solutes and particles between the blood and space of Disse. (Malarkey, et al., 2005)

Kupffer cells (KC)

KC are located within the lumen of the liver sinusoids and as a consequence, they are at first exposed to materials absorbed from the gastrointestinal tract. These cells are defined as resident macrophages responsible for phagocytosis, antigen presentation and production of pro-inflammatory cytokines. Once KC are activated by pathogenic agents, they release inflammatory mediators, growth factors and reactive oxygen species (ROS). Therefore, KC

play an important role in the innate immune response and acute hepatic injury. Furthermore, KC express a variety of toll like receptors (TLRs), such as TLR4, which is involved in uptake and clearance of endotoxins, production of cytokines and ROS. (Hiromi, et al., 2009)

Stellate cells (SC)

The so called fat-storing cells can be found in the space of Disse and are the major source for the production of extracellular matrix. Their functions range from storage of vitamin A to the development of hepatic fibrosis in response to injury. Activated SC transform to myofibroblasts which express desmin and smooth muscle actin filaments. (Malarkey, et al., 2005; Hiromi, et al., 2009)

All cell types of the liver act together. Accordingly, extensive alcohol consumption, unhealthy diet, drugs and xenobiotics mostly affect all cells and lead to profound deregulations of cellular processes. The alterations may lead to the development of liver cancer which is described in detail below.

1.2 Hepatocarcinogenesis

1.2.1 Carcinogenesis

It is well known that tumor development is a multistep process which includes initiation, promotion and progression. Initiation often occurs after a short exposure to a potent initiating agent such as a genotoxic carcinogen. Characteristic for the first stage is that it is an irreversible, heritable process which needs just a short period of time. The next stage (promotion) is a slow, gradual process which includes a longer exposure to the promoting agents. In many rodent tests, NGC have been demonstrated to act in a tumor promoting way. Tumor promotion can be described as the selective amplification of preneoplastic cells by increases in cell proliferation and/or decreases of apoptosis. Finally tumor progression originates from the acquisition of additional mutations and progredient acquisition of a malignant phenotype. (Ruddon, 2007)

The genetic alterations that cause the progressive transformation of normal human cells into highly malignant derivatives have been described by Hanahan and Weinberg in their review of 2011 "*Hallmarks of cancer: The next generation*". Eight essential alterations in cell physiology may collectively lead to malignant growth: (1) on-going proliferative signalling, (2) evading growth suppressors, (3) apoptosis, (4) unlimited replicative potential, (5)

angiogenesis, (6) tissue invasion and metastasis, (7) modifying of cellular metabolism to support neoplastic proliferation and (8) avoiding immune destruction. (Hanahan, et al., 2011)

1.2.2 Hepatocellular carcinoma (HCC)

HCC accounts for about 85% of all primary liver cancers and is one of the most prevalent life-threatening human cancers worldwide. An increase of cases is observable and prognosis is very poor despite recent advances in the understanding of the pathogenesis and some progress in treatment. (Pogribny, et al., 2008; Schattenberg, et al., 2011; Breuhahn, et al., 2006; Frenette, et al., 2012)

The most remarkable risk factors associated with HCC are chronic virus infection with hepatitis B and C virus, exposure to environmental chemicals, alcoholism, obesity and cirrhosis of the liver. (Pogribny, et al., 2008) In most cases, HCC evolves due to a complex interaction between multiple risk factors and not due to a single cause. (Leong, et al., 2005)

1.2.3 Cirrhosis

In 80-90% of patients, cirrhosis is the underlying disease of HCC. (Leong, et al., 2005) Cirrhosis is characterized by replacement of the parenchyma with dense fibrous scars by progredient fibrosis and angiogenesis. This results in profound alterations of the normal hepatic architecture. Growth regulating factors such as cytokines, hepatocyte growth factor (HGF), epithelial growth factor (EGF), transforming growth factor-α (TGFα) and tumor necrosis factor (TNF) play important roles in the formation of regenerative nodules within scarred liver tissue. (Hiromi, et al., 2009)

The knowledge about the biology of cancer grows every day and can be seen as a large renewing handbook used to find further treatment possibilities and therapies. Rather new thematic priorities in cancer research are inflammation and signalling pathways which are also of special interest for this thesis and discussed in the next two chapters.

1.3 Inflammation and cancer

1.3.1 The link between inflammation and liver cancer

In recent years, cancer research highly focuses on the influence of chronic inflammation in tumor promotion and progression. It is already known that chronic inflammation contributes to a tumor supporting microenvironment which plays a huge role in the neoplastic process. In the tumor, there is a shift from cytokines with antitumor activity/immunity towards cytokines that favour tumor progression. (Lin, et al., 2007) The specific functions and pathways of four pro-inflammatory factors, which are also related to the development of HCC, are described below.

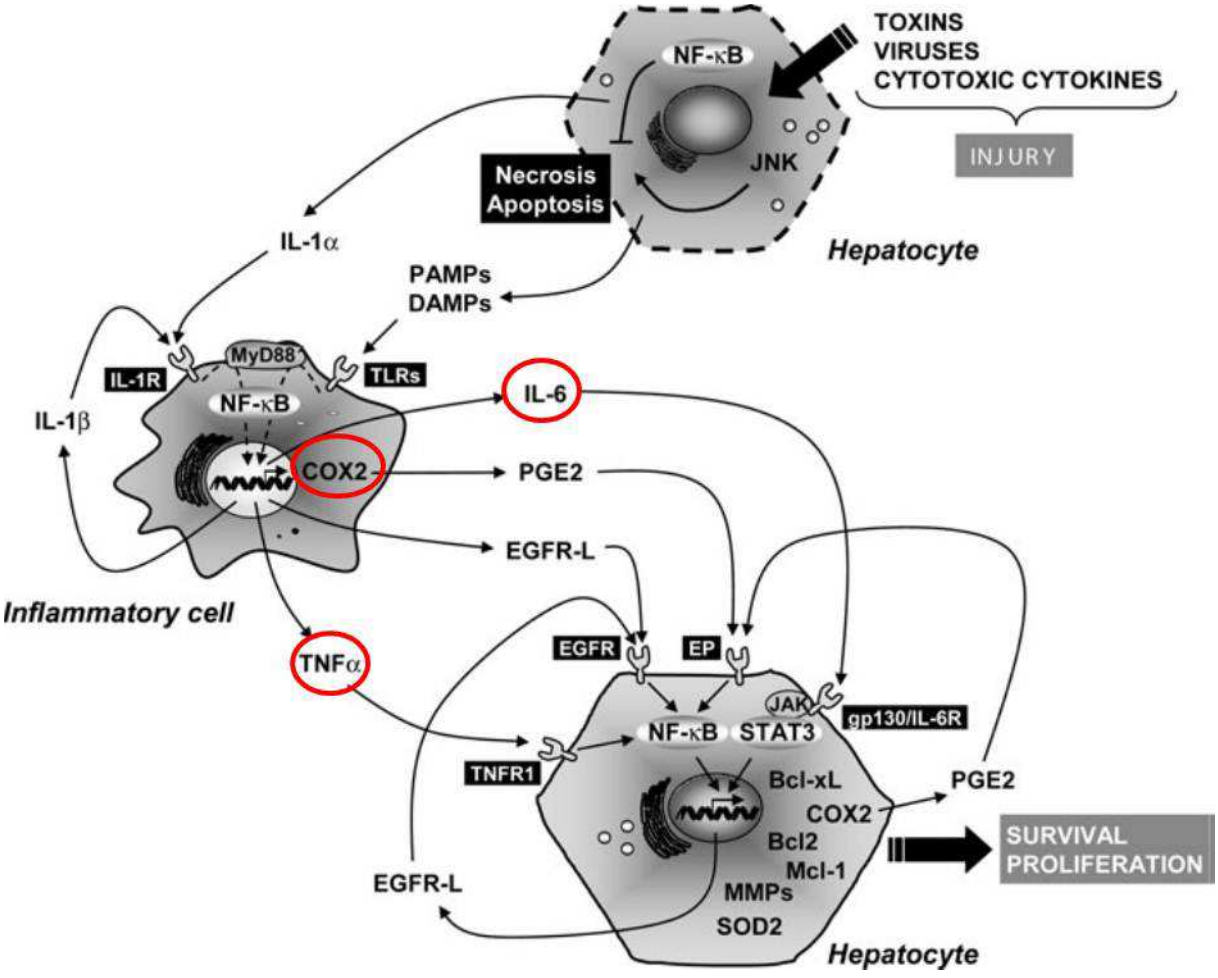

Figure 2: Molecular pathways connecting inflammation and HCC development.

IL-6, COX-2 and TNFα (surrounded) are important key factors for linking inflammation and liver cancer over the NF-κB and JAK/STAT pathways. Source: (Berasain, et al., 2009)

1.3.2 Tumor necrosis factor-alpha (TNFα)

Characteristics

TNFα is a pro-inflammatory cytokine and regulates a cascade of cytokines, chemokines, adhesions, MMPs and angiogenesis. (Coussens, et al., 2002) It is also suggested that TNFα contribute to tumor initiation by enhancing the production of reactive oxygen species. Further functions of TNFα are promotion of metastasis and reduction of immune surveillance by suppressing T cell responses and the cytotoxic activity of activated macrophages. (Lin, et al., 2007)

Pathway

The production of TNFα by tumor cells or inflammatory cells in the tumor microenvironment promotes tumor cell survival through the NF-*κ*B pathway (Figure 2). TNFα activates through its receptor TNFR1 the key inflammatory transcriptional regulator NF-*κ*B which in turn plays an important role during neoplastic transformation in the liver. (Berasain, et al., 2009)

1.3.3 Interleukin 6 (IL-6)

Characteristics

IL-6 is described as a strong pleiotropic inflammatory cytokine that acts as an angiogenic, growth-promoting and anti-apoptotic factor. (Lin, et al., 2007) Additionally, IL-6 has been shown to play an important role in liver cancer development caused by carcinogens. (Zamarron, et al., 2011)

Pathway

The IL-6 signalling pathway (Figure 2) starts at the heterodimeric receptor complex consisting of IL-6Rα and glycoprotein 130 (gp130). The active gp130 induces tyrosine phosphorylation of the `signal transducers and activators of transcription´ (STAT) proteins STAT1 and STAT3. Phosphorylation of the STAT proteins by the janus kinase 1 (JAK1) enables dimerization and translocation to the nucleus where IL-6 target genes get activated. The genes activated are involved in cell cycle progression and suppression of apoptosis which explains the key role of IL-6 in tumorigenesis. (Lin, et al., 2007) There is also the fact of a complex relationship between the NF-*κ*B and STAT3 pathway. An active NF-*κ*B pathway leads to the expression of IL-6 and COX-2 and this again are important activators of STAT3 signalling. (Berasain, et al., 2009)

1.3.4 Cyclooxygenase 2 (COX-2)

Characteristics

COX-2 is an inducible cyclooxygenase and gives response to numerous intracellular and extracellular stimuli in a pro-inflammatory way. (Williams, et al., 1999) Chronic liver inflammation and cirrhosis often show increased COX-2 expression levels. Furthermore, enhanced prostaglandin 2 (PGE2) levels are existent in liver cancer cells which supports tumor cell proliferation, invasion, metastasis, survival and angiogenesis. (Berasain, et al., 2009)

Pathway

COX-2 converts arachidonic acid to prostaglandins such as PGE2 (Figure 2) which in turn contributes to inflammatory response in damaged tissues. (Coussens, et al., 2002) Various researchers have found an interaction between COX-2 and epidermal growth factor receptor (EGFR) signalling pathways. PGE2 is able to transactivate the EGFR receptor which includes binding of PGE2 to its EP1 receptor. Carcinogenic liver cells often show a persistent activation of the EGFR and its pathway. (Berasain, et al., 2009)

1.3.5 Inducible nitric-oxide synthase (iNOS)

Characteristics

Nitric oxide (NO) is a free radical with a short half-life and functions as a mediator of chronic inflammation and as a modulator in tumorigenesis by regulating cell proliferation, survival, angiogenesis and DNA repair. The product NO originates from the conversion of L-arginine to L-citrulline catalysed by the calcium independent enzyme iNOS. Interestingly, patients with cirrhosis and HCC show increased NO plasma levels. However, the detailed mechanism how iNOS interacts with signalling pathways in hepatocarcinogenesis is not completely understood and needs further investigations (Ikeguchi, et al., 2002; Calvisi, et al., 2008)

Pathways

The influence of NO in various pathways correlates with its concentration and depends on the interaction with other molecules such as free radicals, metal ions, proteins and target cells. NO is able to activate the tumor protein 53 (p53) pathway and additionally induces oncogenic mutations in the p53 gene. In general, p53 is involved in cell cycle regulation and acts as a tumor suppressor gene. Besides, iNOS causes unlimited cell growth by inactivating the retinoblastoma protein (pRb) pathway. Normally, pRb is a tumor suppressor and should prevent cells from unrestrained growth. (Calvisi, et al., 2008)

1.4 The role of mitogen-activated protein kinases (MAPKs) in HCC

1.4.1 Function of MAPKs

The MAPKs belong to a family of serine/threonine kinases which consists of four subfamilies: Jun N-terminal kinase (JNK), extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38 MAPK and ERK5. Their main function is the transduction of extracellular stimuli (cytokines, stress signals, growth factors) in a wide range of cellular responses such as cell proliferation, cell survival and death, gene expression, differentiation and migration. MAPKs play key roles in embryonic development, tissue homeostasis and inflammation. Besides regulating these complex cellular functions, the MAPK signalling pathways are often deregulated in many types of human cancers such as HCC. (Min, et al., 2011; Chang, et al., 2001)

1.4.2 Activation of the MAPK signalling pathway

The MAPK signalling pathway proceeds over a multi-stage signal cascade which consists of phosphorylation events of three consecutive components: MAPK kinase kinase (MAP3K), MAPK kinase (MAP2K) and MAPK. The enzymes catalyse the phosphorylation events on the threonine and tyrosine motif. Different external stimuli trigger the activation of MAP3K which activates MAP2K which in turn finally activates MAPK. The outcome is that MAPK activate several transcription factors such as c-Myc or c-Fos. (Min, et al., 2011) In the following paragraph the characteristics and features of the ERK pathway are described.

1.4.3 The ERK pathway

Although ERK1 and ERK2 are serine and threonine kinases with almost different functions, they share more than 80% of its similarity in protein sequences. Ligands bind to extracellular N-terminal domain of tyrosine kinases receptors such as EGFR. After that event, EGFR is able to form dimers and induces phosphorylation of the intracellular C-terminal region that binding sites for adaptor proteins are accessible. In further consequence, Ras GTPases get activated which leads to the activation of Raf kinases. These kinases phosphorylate the dual-specificity kinases MEK1 and MEK2 which in turn phosphorylate ERK1 and ERK2 on threonine and tyrosine residues. Finally active ERK1 and ERK2 regulate a wide variety of cellular processes especially cell proliferation and prevention of cell death. (Min, et al., 2011)

1.4.4 The role of ERK pathway in HCC

Phosphorylated ERK levels are increased in many human HCC samples. Furthermore, constitutively activated ERK1 and ERK2 play an essential role for the proliferation and invasion of human HCC cells. In rodents, the genes Ras and Raf are significantly upregulated in foci of altered hepatocytes, precancerous nodules and HCCs. Recent studies with a siRNA-mediated knockdown of ERK2 show elimination of liver cell proliferation and DNA replication in vitro. (Min, et al., 2011) Moreover, the ERK pathway is involved in the transcriptional regulation of PB induced CYP2B gene expression in rats. (Lu, et al., 2009)

The way how NGC may act over the ERK pathway was one important question to be partially answered in that thesis. The following paragraphs should give a short summary about NGC examined in this thesis.

1.5 Carcinogens

1.5.1 Genotoxic carcinogens (GC) versus non-genotoxic carcinogens (NGC)

NGC can be distinguished from GC by their mode of action. Most GC bind covalently to DNA and damage is caused by the formation of DNA adducts. The cell usually reacts to damage with repair mechanisms, cell cycle arrest or induction of apoptosis. If these lesions are not correctly repaired, mutations lead in further consequence to the formation of tumors. The mode of action of NGC is more complex, excludes DNA damaging but includes a wide variety of cellular processes such as proliferation of peroxisomes or endoplasmatic reticulum, induction of replicative DNA synthesis, suppression of apoptosis and/or oxidative stress. (van Delft, et al., 2004; Mathijs, et al., 2009)

1.5.2 Characteristics of NGC

The carcinogenic potential of NGC is not easy to study due to their tissue and species specificity. This is aggravated by the fact that they act among others as tumor promoters, receptor mediators, immune suppressors or inducers of inflammatory responses. The usual way to detect and study NGC is by performing 2-year cancer bioassays with rodents. (Hernàndez, et al., 2009) The disadvantages of chronic rodent bioassays consist of a high false positive rate, great costs, huge number of animals and a long study period. That is the reason why the development of efficient in vitro assays and the search for reliable biomarkers to predict the carcinogenicity of NGC awake more and more interest. (Mathijs, et

al., 2009) The subsequent paragraphs supplies details about the NGC phenobarbital, cyproterone acetate and wyeth-14,643 as they are the NGC examined in this thesis.

1.5.3 Phenobarbital (PB)

PB belongs to the family of barbiturates (Figure 3) and was established for medical purpose in 1912. It is a common drug for treatment of epilepsy in developed countries and a well-studied liver tumor promoter in rodents. PB causes liver hyperplasia and hypertrophy. Additionally, a promoting dose of PB leads to increased DNA synthesis, decreased apoptosis in murine HC and initially stimulates HC proliferation. On a genetic level, PB induces expression of various cytochrome P450 enzymes (CYP) and causes altered transcriptional regulation. This is mediated through pregnane X receptor (PXR) and constitutive androstane receptors (CAR). Chronic CAR activation in response to PB results in hepatocarcinogenesis. (Kwan, et al., 2004; Waterman, et al., 2010; Phillips, et al., 2009)

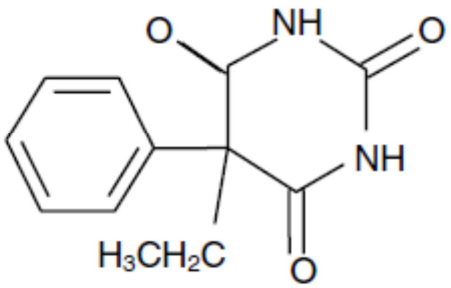

Figure 3: Chemical structure of PB.
Name: 5-ethyl-5-phenylbarbituric acid.
Source: (Kwan, et al., 2004)

1.5.4 Cyproterone acetate (CPA)

CPA is per definition an antiandrogen which acts via competitive inhibition of androgen receptors. It is a synthetic derivative from hydroxyprogesterone (Figure 4) which has also progestational and antigonadotropic activity. Medically CPA is very often used to treat hormonal diseases in women such as hirsutism, androgenic alopecia, acne and seborrhoea. In men, it is applied in high doses to treat prostate carcinoma, sexual drive disorders or is used for gender transformation from male to female. (Kasper, 2001)

The application of CPA in high dosages is necessary for the treatment of malignant prostate diseases. However, high doses of CPA correlate with severe hepatotoxicity. This leads to liver cell proliferation and enlargement of the liver via hyperplasia. Furthermore, the antiandrogen predisposes hepatocytes to undergo apoptosis. DNA damage in the liver is also a consequence of CPA administration. Transcriptionally, CPA is able to activate the nuclear receptor PXR and induce hepatic CYPA3A in rat and human hepatocytes. (Savidou, et al., 2006; Schuetz, et al., 1998)

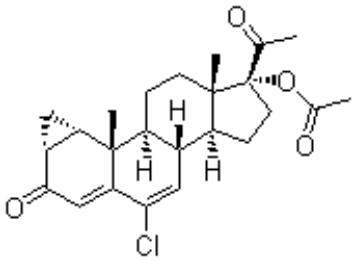

Figure 4: Chemical structure of CPA

Name: 6-chloro-17-acetoxy-1,-2-methylenepregna-4,6-diene-3,20-dione.

Source: http://www.lookchem.com/cyproterone-acetate/

1.5.5 Wyeth-14,643 (WY)

WY is a hypolipidemic drug (Figure 5) and belongs to the group of peroxisome proliferators (PP). Generally, PP are NGC which induce numerical increases of peroxisomes in HC, induction of peroxisomal enzymes and CYP, liver cell proliferation and inhibition of apoptosis in rodents. Long-term treatment of rodents with WY leads to liver hypertrophy, hyperplasia and tumor formation. (Trapp, et al., 2007) WY acts via the nuclear receptor PPARα (see section 1.7.2). Interestingly, cell proliferation, peroxisome proliferation or enzyme induction, and liver tumor formation were not observable in PPARα-knockout mice which were treated with WY. This results lead to the conclusion that PPARα plays an important role in WY-induced hepatocarcinogenesis. (Suga, 2004; Gonzalez, et al., 2008)

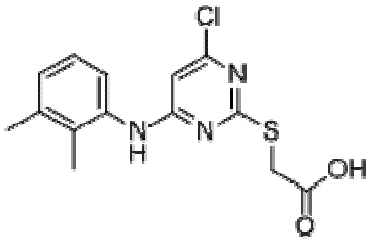

Figure 5: Chemical structure of WY-14,643

Name: 4-chloro-6-(2,3-xylidino)-pyrimidinylthioacetic acid.

Source: http://www.chemicalbook.com/ChemicalProductProperty_DE_CB9345027.htm

The aims of this thesis are to provide a better understanding of the mode of action of NGC and to search for markers indicating their carcinogenic action. As binding to different NR and induction of CYP isoenzymes are key features of many NGC, further information on these aspects are provided in the next two chapters.

1.6 The cytochrome P450 superfamily (CYP)

The CYP enzymes can be defined as heme proteins which catalyse phase I metabolism of xenobiotics such as carcinogens, drugs and environmental pollutants but also of fatty acids, steroids, prostaglandins and bile acids. CYP are located in the endoplasmic reticulum of HC and in the epithelium of the jejunum, lungs, kidney and other organs as well as in the brain. (Carver, 2007) An important characteristic is their broad and overlapping substrate specificity. On the one hand, CYP mediate through detoxification a protective mechanism but on the other hand they are also able to activate substrates to carcinogenic, mutagenic and/or cytotoxic products. (Denison, et al., 1995) In the following the focus lays on CYP enzymes which are induced by PB, CPA and/or WY.

1.6.1 NGC-induced CYP enzymes

It is well known that treatment of rodent HC with PB highly increases the transcription rate of CYP2B1/2 and to lower extent of CYP3A and CYP2C genes. Gene expression of CYP2B1/2 often serves as positive control for the PB effect. The human and rat CYP3A forms are induced by different steroidal hormones. Different studies revealed that anti-hormone representatives such as the anti-mineralocorticoid spironolactone and the antiandrogen CPA were able to induce hepatic CYPA3A in rat and human hepatocytes. The mRNA levels of

CYP4A1 are induced by PPs such as WY. In this context, Cyp4a1 catalyse peroxisomal ß-oxidation of fatty acids and induction of microsomal lauric acid ω-hydroxylase activity. (Denison, et al., 1995; Schuetz, et al., 1998)

1.7 Nuclear receptors (NR)

NR are a superfamily of transcription factors which can be activated by various ligands including hormones, lipids and xenobiotics. Their main function is to regulate many cellular processes such as development, growth and homeostasis. It is anticipated that permanent activation of NR, as occurring during continuous application of NGC, may lead to permanent deregulation of intracellular pathways which may trigger hepatocarcinogenesis. (Shah I., 2011)

The NR superfamily consists of four classes which are based on their dimerization properties and features of binding to DNA. Class I receptors represent the steroid hormone receptors. When induced by ligands, they form homodimers and bind to the response elements in the promoter region of target genes. Class II receptors form heterodimers with the retinoid X receptor (RXR) before interacting with target genes. Noteworthy is that Class II receptors such as PXR and CAR were identified as xenobiotic sensors which induce enzymes involved in phase I detoxification, like CYP enzymes. RXR belong to Class III receptors which are characterized by having no ligands and binding as homodimers. NR belonging to Class IV, such as the liver receptor LRH1, act as monomers. (Lu, et al., 2006; Pirola, 2008; Mangelsdorf, et al., 1995)

1.7.1 Class I receptors

Steroid hormone receptors occur in the inactivated state as monomers bound to heat shock proteins in the cytosol. Ligand binding leads to dissociation of the heat shock proteins, homo-dimerization and translocation to the nucleus to bind to hormone response elements (HRE). In general, they play a crucial role in stress response, metabolism, immune function, growth, development and reproduction. (Lu, et al., 2006; Mohler, et al., 2008)

Mineralocorticoid receptor (MR), progesterone receptor (PGR) and androgen receptor (AR)

MR is highly expressed in epithelial tissues and its principal ligand is aldosterone. PGR can be primarily found in the reproductive tract and progesterone serves as ligand. AR is mainly expressed in prostate, skeletal muscle, liver and central nervous system. Free testosterone and dihydrotestosterone bind to intracellular AR and lead to its activation. (Mohler, et al., 2008)

Estrogen receptor (ER)

In the last two decades it has been recognized that ERα is not the only ER but that additionally ER exist, such as ERβ. ERα and ERβ are not isoforms of each other but are different proteins. (Couse, et al., 1999) Both receptors can be found to be mainly expressed in the reproductive tract and further in liver, pituitary, hypothalamus, bone and cardiovascular system. After binding of the hormone estrogen to the ER, a conformational shift leads to the transcriptionally active form. (Weis, et al., 1996)

1.7.2 Class II receptors

Pregnane X receptor (PXR)

PXR, also often designated as NR1I2, is highly expressed in liver and intestine and less expressed in lungs and kidneys. PXR can be found in the cytoplasm were it is associated with a protein complex. As PXR is equipped with very large binding pockets it can bind many different ligands including the pharmaceutical drugs RU486 and rifampicin but also specific bile acids and hormones. After activation through ligand binding, PXR dimerizes with RXRα and translocates to the nucleus to trigger activation of its target genes. CYP3A is among other phase I metabolism genes known to be transcriptionally regulated by PXR. (Pirola, 2008; Tien, et al., 2006) Additionally, studies of PXR-null mice show that PXR causes downregulation of several hepatic proteins during inflammation. (Teng, et al., 2004)

Constitutive androstane receptor (CAR)

CAR (NR1I3) is detected mainly in the liver but also to a smaller extent in extrahepatic tissues such as the intestine. Well known agonists for CAR are the anti-seizure drug PB and xenobiotics such as 1,4-Bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP). The hormones androstanol and androstenol block the constitutive activity of CAR by acting as inverse agonists. (Pirola, 2008)

This receptor shows unique activation mechanisms compared with other orphan receptors. On the one hand, after direct ligand binding, CAR translocates to the nucleus, heterodimerizes with RXRα and finally binds to the phenobarbital responsive enhancer modules (PBREM). On the other hand, there is strong evidence that CAR gets activated by PB via indirect ligand-independent pathway. Results from different studies lead to the assumption that PB may activate CAR through indirect mechanisms such as co-activation and phosphorylation. (Moore, et al., 2000; Tolson, et al., 2010)

CAR primarily regulates transcription of CYP2B and other drug metabolism genes. Relevant to mention is the role of CAR in the promotion of tumor formation. A study including treatment of mice with the tumor initiator diethyl nitrosamine (DEN) followed by treatment with PB for 36 weeks showed that, in contrast to the wild type mice, the CAR knockout mice did not develop any HCC. (Pirola, 2008; Tien, et al., 2006)

Peroxisome proliferator-activated receptor alpha (PPARα)

PPARα is one of the three members of the peroxisome proliferator-activated receptor family and is highly expressed in rodent liver and kidney. Human HC express PPARα at approximately 5-10% of the levels found in rodent HC. Typical PPs such as WY activate PPARα that act after ligand binding like other type II NR by heterodimerization with RXRα. The heterodimer binds to the peroxisome proliferator response element (PPRE) were it regulates transcription of a large number of genes involved in peroxisome proliferation and the ß-oxidation of fatty acids. Furthermore, PPARα is known as a mediator in cancer development. Studies showed that the chronic treatment of wild type mice with WY cause an increased cell proliferation, upregulation of pro-inflammatory cytokines, decreased apoptosis and as a consequence a high incidence of liver tumors. In contrast, no liver tumor formation could be observed in PPARα-null mice. (Holden, et al., 1999; Gonzalez, et al., 2008)

2. Aims

So far hepatocarcinogenesis is considered to be a mere epithelial disease. However, it is increasingly recognized that mesenchymal cells contribute to the development of HCC as well. It is unknown whether NGC act on the hepatic mesenchyme. The main purpose for that project was the investigation of NGC effects on different liver cell types. The studies included the characterization of the expression levels of NR, growth factors and chemo-/cytokines in parenchymal (HC) and mesenchymal (EC, KC, SC) cells in order to improve our understanding of the mode of action of NGC.

The following points were chosen as important aims for this project:

(1) The induction of some CYP will proof that a given NGC exerts effects on a cell. In order to get to know on which liver cell type NGC may act, the expression levels of various CYP were determined in untreated and NGC treated primary liver cells (HC and NPC).

(2) The activation of NR may play a key role in NGC-driven hepatocarcinogenesis. Therefore, the basal mRNA levels of various NR (CAR, PXR and PPARα) were determined in untreated primary liver cell types (HC, EC and KC). It was also studied whether several NGC, such as CPA, PB and WY, have effects on the expression level of their targeted NR in HC and NPC. Furthermore, chemical inhibitors of CAR and PXR were used to gain more information about the NR involved in the action of NGC.

(3) As the carcinogenic action of NGC may also include interactions between the hepatic parenchyme and mesenchyme, the effects of the secretome of NPC on HC and vice versa were checked. The cells and their supernatant derived from livers of rats treated with PB or solvent. The mRNA levels of the inflammatory mediators TNFα, IL-6, COX-2 and iNOS were determined. Furthermore, the release of TNFα from HC and NPC was measured via ELISA.

(4) The effects of in vivo application of NGC on transcription patterns of HC and NPC were analysed by using the Gene Chip technology.

(5) The ERK pathway may act as a potent signalling pathway involved in the mode of action of NGC. Therefore, expression levels of components of this pathway were determined on transcriptome and/or protein levels in liver cells treated with PB or CPA.

3. Materials and Methods

3.1 Reagents

Table 1: Reagents and Supplier.

Reagent	Supplier
10 mM dNTP Mix	Fermentas (Burlington, Ontario)
10x Complete Mini (protease inhibitor, PMSF)	Roche (Indianapolis, Indiana)
2x Go Taq Green Master Mix (2x MM)	Promega (Madison, WI)
^{3}H-Thymidine	ARC (St. Louis, MO)
5x Biorad protein assay	Biorad (Hercules, CA)
6x Loading Dye	Fermentas (Burlington, Ontario)
Acrylamid	Biorad (Hercules, CA)
Agarose	Biozym (Hessisch Oldendorf, Germany)
Ammonium-persulfate (APS)	Sigma (St. Louis, MO)
Ascorbat	Merck (Darmstadt, Germany)
Bovine serum albumin (BSA)	Sigma (St. Louis, MO)
Calciumchloride	Merck (Darmstadt, Germany)
Chloroform	Merck (Darmstadt, Germany)
Collagenase	Worthington (Lakewood, NJ)
Cyproterone acetate (CPA)	Bayer Schering Pharma (Berlin, Germany)
Detection reagent for western blotting	GE Healthcare (Uppsala, Sweden)
Dexamethasone	Serva (Heidelberg, Germany)
Diethylpyrocarbonate (DEPC)	Sigma (St. Louis, MO)
Dimethylsulfoxide (DMS)	Sigma (St. Louis, MO)
Ethanol	Merck (Darmstadt, Germany)
Ethidiumbromide	Sigma (St. Louis, MO)
Ethylenediaminetetraacetic acid (EDTA)	Sigma (St. Louis, MO)
Fetal Calf Serum (FCS)	PAA (Pasching, Austria)
Formalin	Merck (Darmstadt, Germany)
GeneRuler 50 bp DNA marker	Fermentas (Burlington, Ontario)
Gentamycin	Serva (Heidelberg, Germany)
Glucagon	Sigma (St. Louis, MO)
Glucose	Merck (Darmstadt, Germany)
Glutamax	Invitrogen (Carlsbad, California)
Glycerin	Sigma (St. Louis, MO)
Glycin	Sigma (St. Louis, MO)
Heparin sodium salt	Serva (Heidelberg Germany)
HEPES	Sigma (St. Louis, MO)
Hexamer Primers	Fermentas (Burlington, Ontario)
Igepal CA 630	Sigma (St. Louis, MO)
Insulin	Sigma (St. Louis, MO)
Isopropanol	Merck (Darmstadt, Germany)
KCl	Merck (Darmstadt, Germany)
Mercaptoethanol	Sigma (St. Louis, MO)
Methanol	Merck (Darmstadt, Germany)
MMLV reverse transcriptase	Fermentas (Burlington, Ontario)
N,N,N´,N´-Tetramethylendiamine (TEMED)	Sigma (St. Louis, MO)
Na_2HPO_4	Merck (Darmstadt, Germany)
Na_3VO_4	Sigma (St. Louis, MO)
NaCl	Merck (Darmstadt, Germany)
Na-Deoxycholat	Sigma (St. Louis, MO)
$NaH_2HPO_4*2H_2O$	Merck (Darmstadt, Germany)
NaOH	Merck (Darmstadt, Germany)

N-nitrosomorpholine (NNM)	Sigma (St. Louis, MO)
PageRuler prestained protein ladder	Fermentas (Burlington, Ontario)
Penicillin G sodium salt	Sigma (St. Louis, MO)
Penicillin-Streptormycin	PAA (Pasching, Austria)
Percoll	GE Healthcare (Uppsala, Sweden)
Phenobarbital (PB)	Sigma (St. Louis, MO)
Picrotoxin	Sigma (St. Louis, MO)
PK-11195	Sigma (St. Louis, MO)
Ponceau S	Sigma (St. Louis, MO)
Pyruvate	Sigma (St. Louis, MO)
Recombinant tumor necrosis factor alpha (TNFα)	R&D Systems (Minneapolis, Minnesota)
Skim milk	Sigma (St. Louis, MO)
Sodium dodecyl sulphate	Sigma (St. Louis, MO)
Streptomycin sulphate salt	Sigma (St. Louis, MO)
Sulforaphane (SFN)	Sigma (St. Louis, MO)
TaqMan® gene expression assays	Applied Biosystems (Carlsbad, California)
TaqMan® universal master mix	Applied Biosystems (Carlsbad, California)
TriFast	Peqlab (Erlangen, Germany)
Triiodthyronin	Serva (Heidelberg, German)
Trizma base	Sigma (St. Louis, MO)
Trypan blue	Invitrogen (Carlsbad, California)
Trypsin	Sigma (St. Louis, MO)
Tween 20	Biorad (Hercules, CA)
Wy-14,643 (WY)	Wyeth/Pfizer (New York, NY)

3.2 Buffers and Solutions

BSA buffer pH 7.4 (per litre):

8.3 g NaCl, 0.5 g KCl, 2.4 g HEPES, 6 ml 1M NaOH, 1 g BSA, 10 mg Gentamycin

Collagenase buffer pH 7.5 (per litre):

6.8 g NaCl, 0.4 g KCl, 1 g glucose, 1 g HEPES, 60 mg penicillin G sodium salt, 100 mg streptomycin sulphate salt, 550 mg pyruvate, 294 mg $CaCl_2$

Formalin according to Lillie pH 7.0 (per litre):

4% formalin, 4 g $NaH_2HPO_4*H_2O$ and 6.5 g Na_2HPO_4

10x HBSS (per litre):

80 g NaCl, 4 g KCl, 2g $MgSO_4*7H_2O$, 0.6 g KH_2PO_4, 10 g Glucose, 0.6 g $Na_2HPO_4*2H_2O$

Laemmli electrophoresis buffer (per litre):

3 g Trizma base, 14.4 g Glycin, 1 g SDS

5x Laemmli loading buffer:

300 mM Tris/HCl pH 6.8, 60% Glycerol, 10% SDS, 0.025 % bromophenolblue, 7% ß-mercaptoethanol

Phosphate buffered saline (PBS) pH 7.4 (per litre):

1.44 g Na_2HPO_4, 2.62 g $NaH_2HPO_4*2H_2O$, 5.68 g NaCl

PBST:

PBS supplemented with 0.5% Tween 20

Stock Isoosmotic Percoll (SIP):

90% (=21.6 ml Percoll), 10% (=2.4 ml) 10x HBSS

Stock percoll solution (SPS):

90% Percoll and 10% 10 fold PBS

50% Percoll-solution: 50% SPS + 50% PBS

49% Percoll solution: 49% SPS + 51% PBS

25% Percoll-solution: 25% SPS + 75% PBS

Perfusion buffer pH 7.4 (per litre):

6.8 g NaCl, 0.4 g KCl, 1 g glucose, 1 g HEPES, 60 mg penicillin G sodium salt, 100 mg streptomycin sulphate salt, 12.4 mg heparin sodium salt, 550 mg pyruvate

RIPA buffer:

500 mM NaCl, 50 mM Trizma base pH 7.4, 0.1% SDS, 1% NP-40, 0.5% Na-Deoxycholate, 0.5 mM Na_3VO_4, 1 mM PMSF

Tris-borat-EDTA buffer (TBE) (per litre):

10.8 g Trizma base, 5.5 g boric acid, 4 ml 0.5 M EDTA pH 8.0

Tris buffered saline (TBS) pH 7.6:

0.05 M Trizma base, 0.3 M NaCl

TBST:

TBS supplemented with 0.5% Tween 20

Transfer buffer (per litre):

3 g Trizma base; 14.4 g Glycin, 5% Methanol

3.3 Media

3.3.1 Media for hepatocytes

Wash medium:

Minimum essential medium (Sigma, St. Louis, MO) supplemented with 20 mM HEPES, 10 µg / ml Gentamycin, 10 µl / ml Glutamax

Culture medium (WEII):

Williams-Medium E (Invitrogen, San Diego, CA) supplemented with 20 mM HEPES, 10 µg / ml Gentamycin, 10 µl / ml Glutamax, 0.151 mM Ascorbat, 6.7 nM Insulin, 0.7 nM Glucagon, 10 nM Triiodthyronin, 100 nM Dexamethason

Plating medium:

WEII supplemented with 10% FCS

3.3.2 Media for non-parenchymal cells

Plating medium:

RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10% FCS and 10 µg / ml Gentamycin

Culture medium:

RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10 µg / ml Gentamycin

Medium for endothelial cells:

EBM2 (Lonza, Basel, Switzerland) was applied with EGM-2MV (Lonza)

3.3.3 Media for HepG2 cell line

MNP supplemented with 10% FCS

3.4 Animals and treatment

SPF Wistar rats were kept at the 'Decentralized Biomedical Facilities of the Medical University of Vienna' under standardized "specific pathogen free" (SPF) conditions. The protocol was approved by the Austrian Ethics committee.

Short-term PB treatment:

Male SPF Wistar rats, 8-12 weeks old, were treated with a single dose of PB (50 mg/kg body weight dissolved in autoclaved tap water) by gavage. Perfusion of the livers occurred 24 hours after treatment. The control group was treated with tap water by gavage.

Long-term PB treatment:

Male SPF Wistar rats, 8-12 weeks old, were treated 14 days with PB (50 mg/kg body weight) via drinking water. The control group received tap water only.

Short-term CPA treatment:

Female Wistar rats, 8-12 weeks old, were treated with a single dose of CPA (100 mg/kg body weight dissolved in corn oil) by gavage. The control group was treated with corn oil by gavage. Perfusion of the livers occurred 24 hours after treatment.

Long-term CPA treatment:

Female Wistar rats, 8-12 weeks old, were treated 6 days with a single dose of CPA per day (100 mg/kg body weight dissolved in corn oil) by gavage. The control group was treated 6 days with a single dose of corn oil per day by gavage.

3.5 Liver perfusion

The livers of treated and untreated rats were perfused with collagenase by the colleagues Sandra Sagmeister or Marzieh Nejabat. After the perfusion the softened liver was put into 20 ml of ice cold wash medium. To get the cells out of the liver capsule, the liver was cut more times with a sterile scissors and the cells were shaken out into the wash medium. The resulted cell suspension was filtered through a mesh with 105 µm pores (Polyester precision mesh). This was repeated 3-4 times with fresh and cooled wash medium using a total volume of 100 ml wash medium until no more cells could be collected. Finally the filtered cell suspension was mixed and divided in two Falcons for a consistent cell separation (see section 3.6).

3.6 Cell separation and cell culture

As a first step to separate primary HC from NPC, the obtained cell suspension from the perfused liver was centrifuged at 500 rpm for 5 minutes at 4℃. The supernatant was taken to prepare NPC (see section 3.6.2) whereas the pellet was taken for purification of primary HC (see section 3.6.1).

3.6.1 Isolation and culture of primary hepatocytes

Isolation of primary HC:

After the first centrifugation step, the obtained cell pellet was resuspended with 20 ml wash medium and centrifuged at 300 rpm for 5 minutes at 4℃. In the meantime the SIP was prepared by mixing 21.6 ml Percoll with 2.4 ml 10x HBSS. The cell pellet was resuspended in 25 ml wash medium and mixed well with 24 ml SIP. To separate vital from dead primary HC, the suspension was centrifuged at 500 rpm for 10 minutes at 4℃. Afterwards the supernatant was discarded carefully and the pellet was purified from percoll by resupension with 40 ml wash medium and centrifugation at 500 rpm for 5 minutes at 4℃. If the pellet was split in two Falcons, they were combined by resupension in a final volume of 25 ml wash medium and the last centrifugation step at 300 rpm for 5 minutes at 4℃. Finally the cell pellet was resuspended in 20 ml plating medium and the cell number and vitality was determined (see section 3.6.4).

Culture of primary HC:

If not stated otherwise, HC were seeded on rat-tail collagen coated petri dishes in WEII + 10% FCS. Related to 6-well plates (Falcon) a cell number of 4-5 x 10^5 per well was used whereas for petri dishes with a size of 35 x 10 mm (Falcon) a cell number of 2.5 x 10^5 was used. Change to serum free medium (WEII) occurred 1.5 hours after seeding.

3.6.2 Isolation and culture of non-parenchymal cells

Isolation of NPC:

After the first centrifugation step (see section 3.6) the supernatant was centrifuged again at 2500 rpm for 10 minutes at 4℃. The obtained pellet was resuspended in 10 ml BSA buffer and slowly layered on the top of the percoll gradient (20ml of a 50% Percoll under 19ml of a 25% Percoll). The Percoll cell suspension was centrifuged for 30 minutes at 2500 rpm at 4℃ without any acceleration or deceleration to avoid any mixing of the two Percoll solutions. After the centrifugation two rings can be seen. One on the top of the gradient which contains dead cells and SC; and the second one at the interface of the 25% and 50% Percoll solutions which contains KC and EC. The second ring was collected with a pipette, diluted with BSA buffer to a volume of 50 ml and centrifuged at 2500 rpm for 10 minutes at 4℃. Finally the cell pellet was resuspended in 10-20 ml plating medium (RPMI + 10 % FCS) and the cell number and vitality was determined (see section 3.6.4).

Culture of non-parenchymal cells:

NPC were seeded on collagen-coated petri dishes in RPMI + 10% FCS. If not stated otherwise, a cell number of 3 x 10^6 for 6-well plates was used. After an attachment period of 1.5 hours, the plating medium was removed and the cells were washed twice with sterile and 37℃ warmed PBS. Afterwards a change to serum free medium was done.

3.6.3 Culture of the HepG2 cell line

HepG2, a cell line which is epithelial in morphology, was received from ACC (Manassas, USA) and cultivated in MNP medium with 10% FCS. They were cultured in T25 flasks (Corning) in a cell incubator (New Brunswick Scientific, Innova CO-170) at 37℃ and 5% CO_2. The cells were released from the flask by washing them once with 1 ml trypsin and incubating with another 1ml trypsin at 37℃ for abo ut 5 minutes. Enzymatic digestion was stopped by adding 9 ml medium (MNP + 10% FCS). The cell suspension was pipetted into a Falcon and centrifuged for 5 minutes at 800 rpm. After discarding of the supernatant, the

pellet was resuspended in medium (MNP + 10 % FCS) and the cell number with a Neubauer counting chamber was determined. HepG2 cells were seeded in 6-well plates with a cell number of 2 x 10^5 cells per well. Change of medium was done as required.

3.6.4 Determination of cell number and vitality

The cell number and vitality of primary HC and NPC was determined by using the trypan blue dye exclusion assay. Therefore 50 µl of the cell suspension was mixed with 50 µl of trypan blue and a small volume was put into a Neubauer counting chamber. The vitality can be calculated by counting the amount of living (not coloured) and dead (blue coloured) cells. The living cell number divided by total cell number multiplied with 100 shows the vitality in percent.

3.7 In vitro treatment of cells

3.7.1 Treatment of cells with specific reagents

Treatment of cells was done in medium without FCS approximately 1.5 – 2.0 hours after seeding when cells adhered to the petri dishes. The stock solutions used for treating the cells were diluted in their solvent to their final concentration (Table 2) and sterile filtered with a Millipore filter (Millex®GP Filterunit 0.22 µm, Express PES membrane).

Table 2: In vitro treatment solutions and applied concentrations.

Reagent	Solvent	Stock concentration	Final concentration in medium
Cyproterone acetate	DMSO	5 mM	5 µM or 10 µM
DMSO	Medium	100 %	0.2%
Phenobarbital	Medium	100 mM	0.5 mM or 1 mM
Picrotoxin	DMSO	10 mM	10 µM
PK 11195	DMSO	10 mM	10 µM
Sulforaphane	DMSO	25 mM	10 µM or 25 µM
TNFα	Medium	200 ng	1 ng or 10 ng
WY-14643	DMSO	10 mM	20 µM

3.7.2 Treatment of cells with supernatants

For this experiment, in vivo treated and untreated SPF Wistar rats were used. The treatment procedure is described in chapter 3.4 Animals and treatment. HC and NPC were separated, seeded and kept in culture for 24 hours with a change to fresh RPMI media two hours after seeding. The NPC from untreated rats (CO-NPC) were treated with the supernatant,

obtained from the HC of the PB treated rats (HC-PB). The HC from untreated rats (CO-HC) were treated with the supernatant, obtained from the NPC of the PB treated rats (NPC-PB). Untreated cells cultured in RPMI media served as controls. CO-HC, which were treated with the supernatant of CO-NPC and vice versa, served as an additional control for the experiment. All supernatants were sterile filtered with a Millipore filter (Millex®GP Filterunit 0.22 µm, Express PES membrane) before treatment. The cells were harvested for RNA isolation and further analysis approximately 24 hours after treatment (see sections 3.8.1 and 3.8.6).

3.8 Methods on RNA level

3.8.1 RNA isolation from cells

The supernatant was discarded and cells were washed once with 1x PBS to remove the rest of the medium and non-adherent cells. The appropriate amount (for HC 600 µl/well; for NPC 300 µl/well) of Trifast reagent was pipetted on the cells. The lysed cells were collected with a cell scraper and transferred in an Eppendorf tube. Afterwards they were stored at -80℃ until further usage or RNA was immediately isolated. Therefore a fifth volume chloroform of the used Trifast amount was added followed by 15 seconds inverting and 10 minutes incubation at room temperature. A centrifugation step was done for 10 minutes at 12,000 x g in a 4℃ cooled Eppendorf centrifuge. The upper aqueous phase was transferred to a new tube and put on ice. The half volume isopropanol of the used Trifast reagent was pipetted on the solution and RNA was precipitated overnight at -20°C.

On the next day the RNA-Isopropanol mixture was centrifuged for 10 minutes at 12,000 x g and 4℃. The supernatant was discarded gently and the pellet was washed with the same volume ethanol as the used Trifast reagent. This step was followed by 10 minutes centrifugation at 14,000 x g and 4℃. The supernatant was discarded gently and the pellet was dried before the RNA was solved in the appropriate amount (for HC 50 µl, for NPC 30 µl) of DEPC H_2O. Finally the RNA concentration in µg/µl was determined using a Nanodrop spectrometer.

3.8.2 RNA isolation from tissue

The appropriate amount of Precellys 1.4 mm ceramic beads (Peqlab, Erlangen, Germany) was transferred in RNA isolation tubes (Peqlab, Erlangen, Germany) and 500 µl Trifast was added and put on ice. The liquid nitrogen frozen tissue was cut with a scalpel in 50-150 mg pieces, the weight was noted and the tissue was put with a forceps into the isolation tubes. The tissue was homogenized in with a Precellys® 24 (Peqlab, Erlangen, Germany) for 2x 15 seconds at 6,000 x g. The following RNA isolation steps with chloroform and isopropanol were done as described in section 3.8.1.

3.8.3 Reverse Transcription-PCR (RT-PCR)

If the RNA concentration was high enough, 1-2 µg RNA was transcribed into cDNA. First the RNA was diluted with DEPC H_2O in sterile PCR tubes to a total volume of 15 µl. The MM was prepared as described in table 3. A volume of 0.625 µl random hexamer primers was added to the diluted RNA followed by heating at 70℃ for 2 minutes on a thermo block. Back on ice 9.375 µl of the MM was added, mixed well and placed into a thermocycler (C1000 Thermal Cycler, Biorad) with a program of 1 hour at 42℃ and 5 minutes at 94℃ to transcribe cDNA. The resulted cDNA samples were diluted with DEPC H_2O to an end volume of 100 µl and stored at -20℃ until usage as templates for PCR or qrt-PCR (see sections 0 and 3.8.6).

Table 3: Master mix for RT-PCR.

Reagent	Volume per sample
5x buffer	5.000 µl
dNTPs (10 mM)	1.560 µl
RNase Inhibitor	0.625 µl
MMLV	1.000 µl
DEPC H_2O	1.190 µl
Total volume	9.375 µl

3.8.4 Polymerase Chain Reaction (PCR)

The 1x MM (Table 4) was prepared without adding the template and mixed well. For the reaction 24 µl of the 1x MM was pipetted to 1 µl of the appropriate cDNA in a PCR tube.

Table 4: Master mix for PCR.

Reagent	Volume per sample
Primer forward	1.0 µl
Primer reverse	1.0 µl
2x MM	12.5 µl
DEPC H_2O	9.5 µl
cDNA template	1.0 µl
Total volume of 1x MM	25.0 µl

Finally the tubes were placed into a thermocycler (C1000 Thermal Cycler, Biorad) and the standard programme (Table 5) was used with varying cycles. The used oligonucleotides for PCR are summarized in table 6.

Table 5: Standard program of PCR.

Step	Repeats	Temperature	Time
Cycle 1	1	95℃	5 minutes
Cycle 2	30 – 42		
▪ Denaturation		94℃	30 seconds
▪ Annealing		60℃	30 seconds
▪ Elongation		72℃	30 seconds
Cycle 3	1	72℃	7 minutes
End programme	-	4℃	∞

Table 6: Oligonucleotides (rat) for PCR.

Primer name	Sequence	Product length	Gene-ID (NCBI)
ERα	5´-ATG-ATG-AAA-GGC-GGG-ATA-CG-3´ 5´-TGC-CAG-GTT-GGT-CAA-TAA-GC-3´	303 bp	NM_012689
ERβ	5´-GAG-CAA-AGC-CAA-GAG-AAA-CG-3´ 5´-ACA-TCA-GTC-CCA-CCA-TTA-GC-3´	308 bp	NM_012754
PPARα	5´-TCT-TTC-GGC-GAA-CTA-TTC-GG-3´ 5´-AAG-GCG-GAT-TGT-TGC-TAG-TC-3´	342 bp	NM_013196
PXR (Nr1i2)	5´-CAA-GGA-TTT-CCG-GCT-ACC-TG-3´ 5´-TCT-CTT-TCC-CGT-CGC-TCT-TG-3´	200 bp	NM_052980
CAR (Nr3i2)	5'-TGG-TCC-CAT-CTG-TCC-GTT-TG-3' 5'-GCT-CTT-TCT-GCT-GCT-GAC-TC-3'	209 bp	NM_022941.3
PGR (Nr3c3)	5´-CGA-TGG-AAG-GGC-AGC-ATA-AC-3´ 5´-TTG-ATG-AGT-GGC-GGA-ACC-AG-3´	286 bp	NM_022847
MR (Nr3c2)	5´-GGC-TTC-TGG-GTG-TCA-CTA-TG-3´ 5´-CAG-GCA-GGA-CAG-TTC-TTT-CG-3´	147 bp	NM_013131.1
AR (Nr3c4)	5'-CTG-CCT-GAT-CTG-TGG-AGA-TG-3' 5'-TTT-CCG-GAG-ACG-ACA-CGA-TG-3'	181 bp	NM 012502.1
Cyp1a1	5'-ATT-TGA-GAA-GGG-CCA-CAT-CC-3' 5'-CAT-GAG-GCT-CCA-AGA-GAT-AG-3'	178 bp	NM_012540.2
Cyp2b1	5'-TTC-TGC-GCA-TGG-AGA-AGG-AG-3' 5'-TGG-GAT-ACA-CCT-CAG-TGT-TC-3'	223 bp	NM_001134844.1
Cyp2c12	5'-GGA-GAG-CCA-CAA-GAC-ATT-TC-3' 5'-TTC-CAT-CAC-TAG-CCA-CTC-TG-3'	107 bp	NM_031572.1
Cyp4a1	5'-CTT-TGG-GCA-CAA-GCA-GTT-TC-3' 5'-TTG-GCC-TTT-GGA-TCT-GAT-CG-3'	171 bp	NM_153307.1
Beta Actin	5´-ATG-TTG-CCC-TAG-ACT-TCG-AG-3´ 5´-TCA-TGG-ATG-CCA-CAG-GAT-TC-3´	175 bp	NM_031144.2

3.8.5 Agarose gel electrophoresis

The amplified DNA fragments resulted from PCR (see section 0) were separated by using 1.2% agarose gels and visualized under UV-light by EtBr staining. For that purpose 0.96 g agarose was dissolved in 80 ml 1x TBE. The mixture was heated in a microwave until the agarose was completely melted. After a short cool down of the mixture, 6 µl ethidium bromide were added and the gel was poured in the gel sleigh containing a comb. Following the polymerization step of approximately 40 minutes, the comb was removed and 10 µl of the PCR product and 5 µl of the 50 bp marker were loaded on the gel. 1x TBE was used as electrophoresis buffer and the gel ran about 35 minutes at 125 V. Finally the bands were visualized through a UV-light GelDoc 2000.

3.8.6 Quantitative real time-PCR (qrt-PCR)

As a first step the 1x MM (Table 7) was prepared without adding the template and mixed well. For the reaction 21 µl MM were added to 4 µl of the appropriate cDNA in a 0.5 ml reaction tube.

Table 7: Master mix for qrt-PCR.

Reagent	Volume per sample (x2 for duplicates)
TaqMan® gene expression assays	0.625 µl
2x TaqMan® Gene Expression MM	6.250 µl
DEPC H_2O	3.625 µl
cDNA template	2.000 µl
Total volume	12.500 µl

For duplicates (2 wells per sample), 12 µl per well were transferred to the MicroAmp Fast Optical 96-well reaction plate (Applied Biosystems). The plate was closed with a MicroAmp Optical Adhesive Film (Applied Biosystems) and put into an ABI Prism 7500 Real-Time PCR apparatus (Applied Biosystems). The qrt-PCR cycling program shown in table 8 was used.

Table 8: qrt-PCR cycling program.

Step	Temperature	Time
1x	50℃	2 min
1x	95℃	10 min
40-50x	95℃	15 sec
	60℃	1 min
dissociation control (1x)	95℃	15 sec
	60℃	1 min
	95℃	15 sec

The C_T values for each sample were averaged and this value was used in subsequent calculations. $ß_2$-Microglobulin was chosen as the housekeeping gene Relative gene expression was determined following the ΔΔCt-method. Table 9 summarizes the used TaqMan® gene expression assays (Applied Biosystems).

Table 9: qrt-PCR probes (TaqMan® Gene Expression Assays).

TaqMan® Gene Expression Assays	Assay ID
iNOS	Rn00561646_m1
COX-2	Rn00483828_m1
TNFα	Rn99999017_m1
IL-6	Rn99999011_m1
CAR (Nr1i3)	Rn00576085_m1
PXR (Nr1i2)	Rn00583887_m1
PPARα	Rn00566193_m1
$β_2$-Microglobulin	Rn00560865_m1

3.8.7 Microarray analysis

All Gene Chip experiments were done with in vivo treated rats as indicated in chapter 3.4. Liver perfusion and cell separation were done as described in chapters 3.5 and 3.6 with the only exception that before liver perfusion one small liver lobe was ligated and frozen in liquid nitrogen until further usage.

Total RNA isolation:

HC cell pellets with a cell number of 2 x 10^6 cells and NPC cell pellets with a cell number of 15 x 10^6 were produced. Afterwards the cell pellets were disrupted by adding 700 µl QIAzol Lysis Reagent and homogenized through a QIAshredder (centrifugation at full speed for 2 minutes). RNA isolation was done by using the Qiagen miRNeasy Mini Kit according to manufactures instructions. For the long-term PB in vivo study Qiagen RNeasy Lysis Buffer (RLT buffer) was used and RNA isolation was done by using the Qiagen RNeasy Plus Mini Kit according to manufactures instructions.

RNA quality testing:

As a pre-quality test RNA was separated on an agarose gel to check the degradation level of the RNA. Therefore a 1.5% agarose gel was prepared as described in section 3.8.5. To 1µl RNA sample, 1 µl 6x loading dye and 4 µl DEPC H_2O was added. The total volume of 6 µl was loaded on the gel and run 25 minutes at 80 V. The quality of the 28S and 18S rRNA was checked by visualization of the characteristic bands under UV-light.

The not degraded RNA was used for measuring the RNA Integrity number (RIN) with an Agilent 2100 Bioanalyzer according to manufactures instructions.

Processing of Gene Chip® experiments:

An appropriate amount of each RNA sample from the in vivo studies (treated vs. controls) was used for Gene Chip analysis with the Affymetrix Rat Genome 230 2.0 Array (Affymetrix). All Gene Chip® experiments were kindly done in cooperation with Priv. Doz. Mag. Dr. Martin Bilban of the institution “Core Faciltiy Genomics” of the MUW.

3.9 Methods on DNA-level

3.9.1 Autoradiography

Primary hepatocytes were cultured in (35 x 10 mm) petri dishes. DNA-replication was determined by labelling new synthesized DNA with ^{3}H-thymidine and subsequent autoradiography. Therefore ^{3}H-thymidine was added to medium with a concentration of 1.0 µCi/ml 24 hours before fixing of the cells. For harvesting, the cells were washed twice with 0.9% NaCl solution. Afterwards the cells were fixed with 1 ml per well of 4% buffered formalin according to Lillie for 90 minutes at 4℃. Finally the fixed cells were washed three times with 0.9% NaCl and stored at 4℃ in sterile distilled H_2O. Autoradiography served to determine the percentage of nuclei with incorporated ^{3}H-thymidine. Autoradiography was kindly performed by the technical assistants Birgit Mir-Karner, Krystyna Bukowska and Helga Koudelka.

3.10 Methods on protein level

3.10.1 Isolation of proteins from cells

Protein isolation from primary liver cells was performed at different time points and always on ice for a stable detection of protein phosphorylation. As a first step, the medium was removed and the cells were washed once with ice cold 1x PBS. Afterwards cells of 2-3 wells were lysed with 75 µl RIPA buffer and pooled together for one protein sample. Finally protein samples were homogenized by ultrasonic (Bandelin Sonopuls HD2070) and centrifuged at 14,000 rpm for 5 minutes at 4 ℃. The samples were stored at -20℃ until further usage.

3.10.2 Isolation of proteins from tissue

The appropriate amount of Precellys 1.4 mm ceramic beads (Peqlab, Erlangen, Germany) was transferred in isolation tubes (Peqlab, Erlangen, Germany) and 300 µl RIPA buffer was added on ice. The liquid nitrogen frozen tissue was cut with a scalpel in approximately 50 mg pieces, the weight was noted and the tissue was put with a forceps into the isolation tubes. The tissue was homogenized in with a Precellys® 24 (Peqlab, Erlangen, Germany) for 2 x 15 seconds at 6,000 x g. Afterwards the samples were incubated for 30 minutes on ice and centrifuged at 10,000 x g for 10 minutes at 4℃. The supernatant was transferred into a new tube and centrifuged again at 10,000 x g for 10 minutes at 4℃. Finally the gained

supernatant was used as a total cell lysate for determination of protein concentration and further protein analyses. The protein samples were stored at -80℃.

3.10.3 Determination of protein concentrations

Protein concentration was measured with the Bradford method in a 96-well plate. A standard row with increasing BSA concentrations (ranging from 0-6 µg/µl) was pipetted to get a calibration curve. 1 µl sample was diluted with 9 µl distilled H_2O and pipetted in duplicates into the 96-well plate. The Bradford protein assay kit (Biorad) was diluted 1:5 and 100 µl of the dilution were added to each well. The absorbance was measured with a microplate reader (Tecan Infinite® 200 PRO) at a primary wavelength of 595 nm.

3.10.4 Western blot

SDS-Page:

Firstly, the proteins were separated according to their size and charge on a 12% SDS-Page. The SDS-Polyacrylamide gel consists of two different gels which were prepared from the components listed in table 10.

Table 10: Composition of the SDS-PAGE.

12% Resolving Gel		**4% Stacking Gel**	
Component	Volume in ml (for 2 gels)	Component	Volume in ml (for 2 gels)
40% Acrylamide	3.600	40% Acrylamide	0.500
1,5 M Tris pH 8.8	3.000	1 M Tris pH 6.8	0.625
10% SDS	0.120	10% SDS	0.050
Distilled H_2O	5.200	Distilled H_2O	3.800
10% APS	0.060	10% APS	0.025
TEMED	0.006	TEMED	0.005

As a next step, a specific volume of the protein lysates was filled up with RIPA buffer to reach the same concentration and sample volume. Generally between 5 µg and 20 µg protein were loaded on the gel. Than one fifth of the sample volume 5x Laemmli loading buffer was added and the samples were heated for 5 minutes at 95℃. Finally 5 µl protein ladder and the whole sample volume were loaded on the gel. Laemmli buffer was used as electrophoresis buffer. Electrophoresis of the proteins was done at 60V until the proteins reached the end of the stacking gel (after approximately 30 minutes) and was going on at 125 V for about 1 hour in the resolving gel.

Blotting:

To transfer the proteins from the gel on a PVDF membrane (GE-Healthcare) the tank-blot system with 1x transfer buffer was used. The transfer was done on ice at 30V overnight. To assure that the transfer worked stable, the proteins on the membranes were stained with Ponceau S. The staining was washed out with distilled H_2O and the blots were dried and stored at 4℃ until further usage.

Blocking:

The dried blots were reactivated with methanol and shortly washed with distilled H_2O. Blocking was done with TBST and 3% BSA for approximately 1 hour at room temperature.

Immunodetection:

After blocking, the blots were incubated with the primary antibody (Table 11) over night at 4℃. On the next day, the blots were washed three times in TBST and incubated with horseradish peroxidase conjugated secondary antibodies (Table 11) for 1-2 hours at room temperature.

Table 11: Antibodies used for immunodetection.

Primary Antibodies		**Secondary Antibodies**	
Antibody	Dilution	Antibody	Dilution
pERK-anti-mouse (Sigma)	1:10000 in TBST and 3% BSA	Goat-anti-mouse (Dako)	1:10000 in TBST and 3% BSA
ERK-anti-rabbit (Sigma)	1:1000 in TBST and 2% BSA	Goat-anti-rabbit (Biorad)	1:10000 in TBST and 2% BSA

The blots were washed again three times in TBST and afterwards they were developed with the Amersham ECL plus kit (GE-Healthcare). Chemiluminiscence was detected by using a Hyperfilm ECL (Thermo Scientific, Waltham, MA) on an x-ray machine. Signal intensities were quantified with ImageQuant™ (GE-Healthcare). After the development of the x-ray films, the blots were shortly washed in TBST and dried for storage or incubated with the next primary antibody.

3.10.5 Enzyme-linked immunosorbent assay (ELISA)

TNFα was detected in supernatants of HC and NPC at different time points. The rat TNFα Module Set (Bender MedSystems) was applied according to manufacturers' instructions.

4. Results

4.1 Gene expression pattern of CYP in liver cell types

4.1.1 Basal mRNA level of CYP in untreated primary liver cells

First the basal mRNA levels of specific CYP in different types of untreated primary liver cells were determined. Due to prior results from literature research (Carver, 2007; Denison, et al., 1995), the expression patterns of Cyp1a1, Cyp2b1, Cyp2b2, Cyp2c12 and Cyp4a1 were studied. Therefore specific primers were designed and used to amplify cDNA from untreated HC, EC and KC (Figure 6).

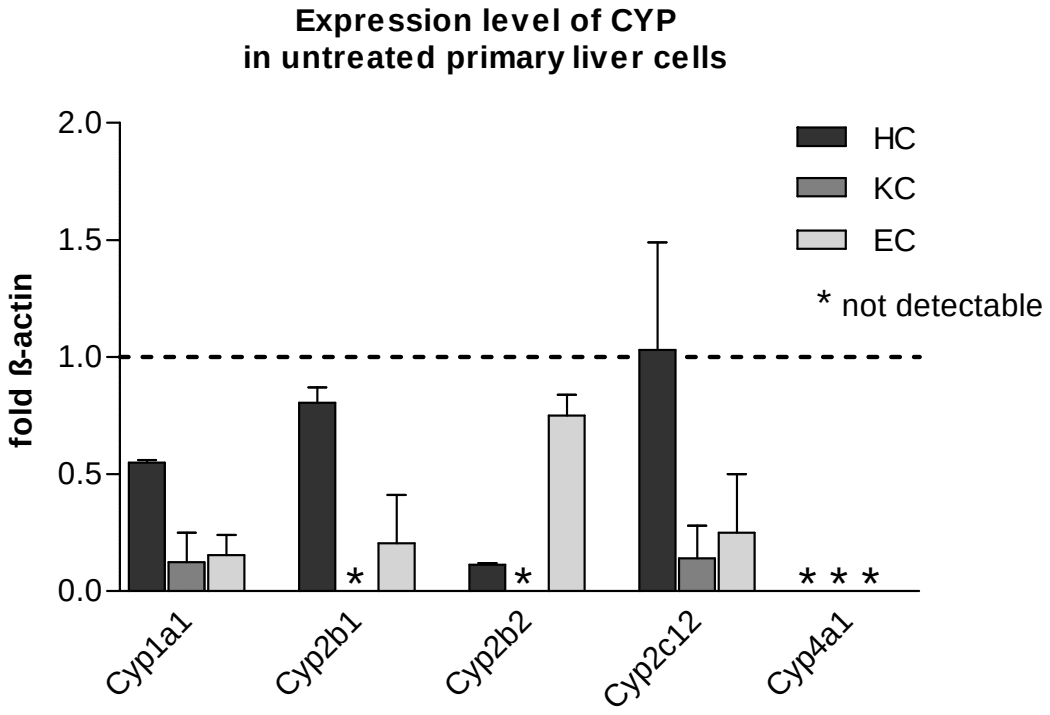

Figure 6: Basal mRNA levels of specific CYP in untreated liver cell types.

HC, KC and EC were isolated from untreated rats and were kept in culture for 24 hours. The cells were harvested, RNA was isolated and cDNA was generated. The cDNA was used as template for PCR amplification. After 40 amplification cycles, products were loaded on a 1.2% agarose gel, stained with EtBr and visualized with UV-light. ß-actin was used as a positive control and reference gene. Volumes of the PCR products were determined by densitometry (Quantity One 4.2.1; Bio-Rad Laboratories, USA). The amount of data obtained for the gene of interest was normalized by β-actin (=fold ß-actin). The data are means +/- SEM of two independent experiments.

At all, HC showed the highest mRNA levels of Cyp1a1, Cyp2b1 and Cyp2b12. In EC, Cyp2b2 showed the maximal mRNA level. Cyp2b1 and Cyp2b2 expressions were not found in KC and transcripts of Cyp4a1 were not detectable in any cell type examined. This indicates that the basal mRNA levels of the selected CYP feature a cell type specific expression pattern and are generally low in the mesenchymal liver cell types.

4.1.2 mRNA level of CYP in treated primary liver cells

In order to determine whether the various liver cell types are able to respond to NGC, they were treated with CPA or PB and the mRNA levels of different CYP were examined.

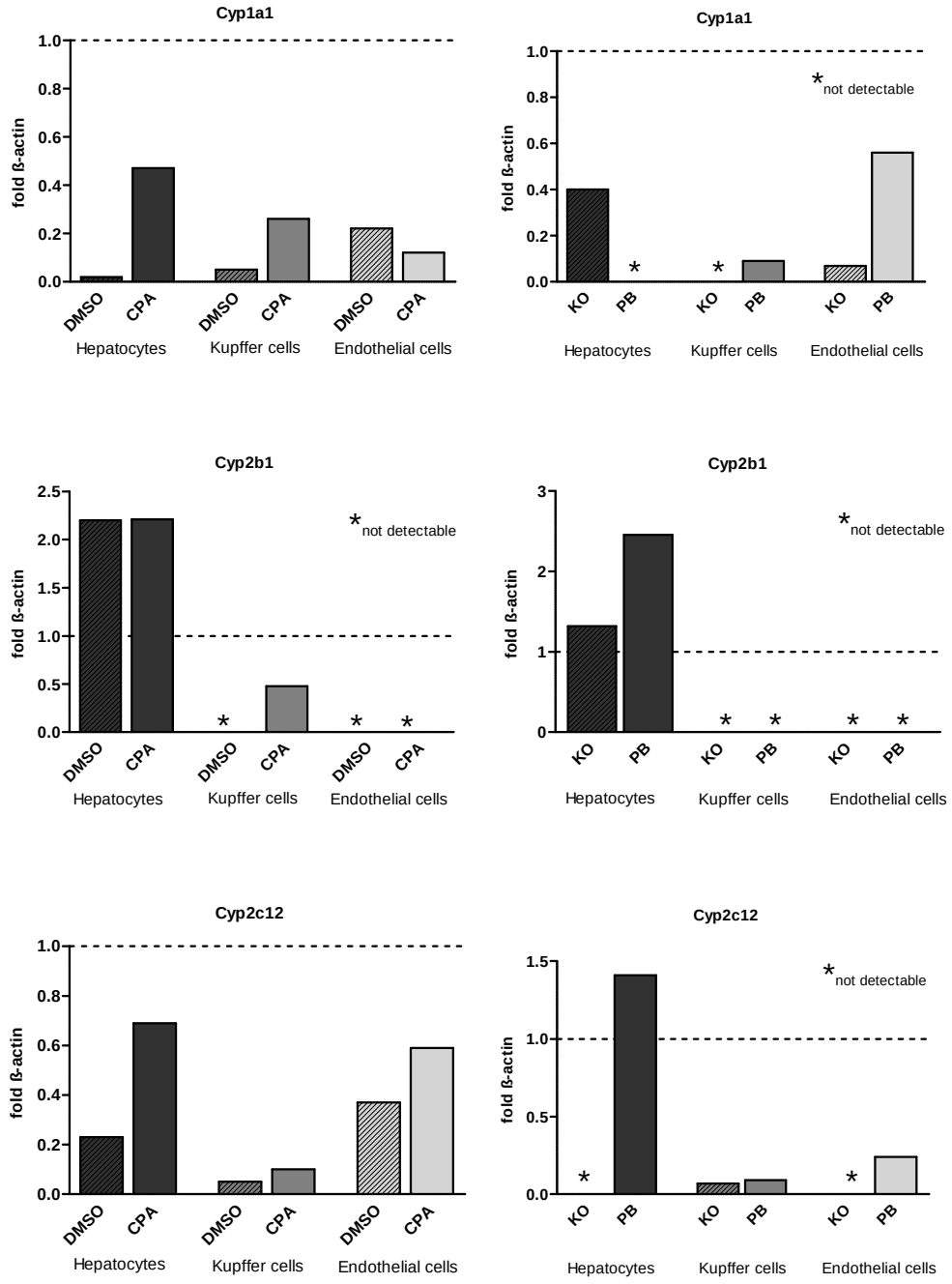

Figure 7: CYP expression induced in liver cell types by treatment with CPA or PB.

HC, KC and EC were isolated from untreated rats and were kept in culture. After two hours in culture, the cells were treated with CPA or PB as follows: CPA was dissolved in DMSO and added to medium at a final concentration of 10 µM. The cells treated with 0.2% DMSO served as control for CPA. PB was dissolved in medium and applied at a final concentration of 1 mM. Untreated cells served as control for PB. 24 hours later, cells were harvested, RNA was isolated and cDNA was generated. The cDNA was used as template for PCR amplification. After 39 amplification cycles, products were loaded on a 1.2% agarose gel, stained with EtBr and visualized with UV-light. ß-actin was used as a positive control and reference gene. Volumes of the PCR products were determined by densitometry (Quantity One 4.2.1; Bio-Rad Laboratories, USA). The amount of data obtained for the gene of interest was normalized by β-actin (=fold ß-actin). The data derive from one experiment.

Figure 7 shows that CPA treatment increased the expression of Cyp1a1 and Cyp2c12 in HC when compared to DMSO. PB treatment of HC increased the mRNA level of Cyp2b1 and significantly induced transcription of Cyp2c12 when compared to the untreated control.

The expression level of Cyp1a1, Cyp2b1 and Cyp2c12 were slightly elevated or induced in CPA treated KC. PB treatment induced transcription of Cyp1a1 to a small extent in KC. Apart from that treatment with PB showed no great effect on the specific CYP transcripts in KC.

In CPA treated EC, Cyp2c12 showed a slight increase in gene expression. There was an increase of Cyp1a1 and induction of Cyp2c12 mRNA when EC were treated with PB. Expression of Cyp2b1 could not be measured in treated and untreated EC after 39 cycles of PCR.

In summary, NGC treatment of primary liver cells lead to a response in mRNA levels of specific CYP, most of all in HC and EC. Cyp1a1 and Cyp2c12 could serve as a transcriptional positive control for the CPA effect in HC. The PB effect could be confirmed by measuring an elevated Cyp2b1 and Cyp2c12 expression in HC.

4.2 Gene expression pattern of NR

4.2.1 Basal mRNA level of NR in untreated primary liver cell types

At first, it was checked if several NR are expressed in untreated HC, KC and EC. For this purpose, the mRNA levels of the receptors PXR, CAR, PGR, ERα, ERβ and PPARα were determined. The specific primers for the appropriate genes were designed and used to amplify cDNA from untreated HC, EC and KC. Figure 8 shows whether a basal expression of the appropriate NR could be determined with PCR or not. Additionally, it was investigated whether the culture time influences the mRNA levels of NR in primary liver cells.

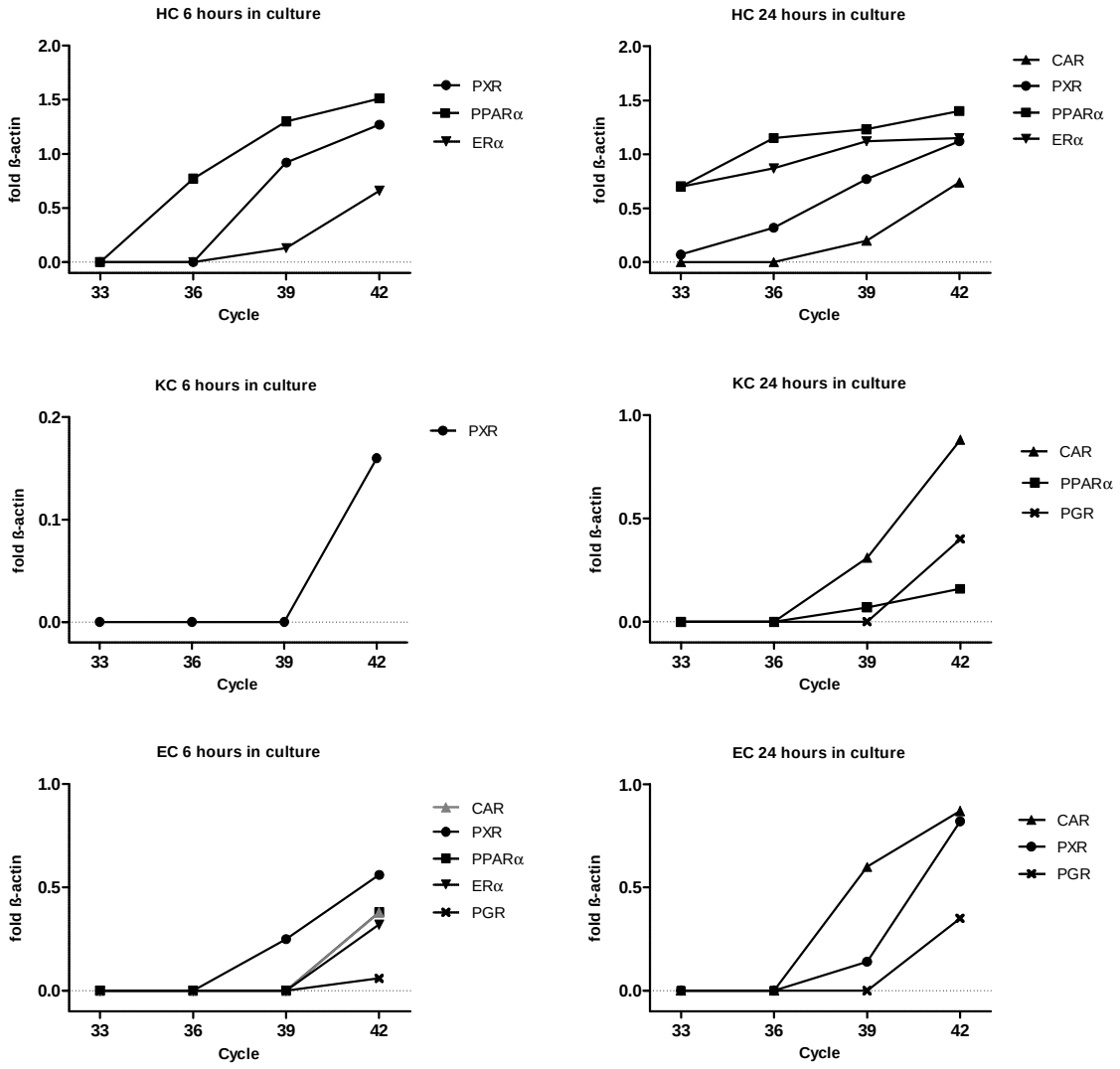

Figure 8: Basal mRNA level of NR in untreated liver cell types in dependence of PCR cycle numbers.
HC, KC and EC were isolated from untreated rats and were kept in culture for 6 hours or 24 hours. The cells were harvested, RNA was isolated and cDNA was generated. The cDNA was used as template for PCR amplification. After 33, 36, 39 and 42 amplification cycles, products were loaded on a 1.2% agarose gel, stained with EtBr and visualized with UV-light. ß-actin was used as a positive control and reference gene. Volumes of the PCR products were determined by densitometry (Quantity One 4.2.1; Bio-Rad Laboratories, USA). The amount of data obtained for the gene of interest was normalized by β-actin (=fold ß-actin). The data derive from one experiment.

In general, HC are best equipped with the tested NR, only the hormone-specific NR ERβ and PGR were not detectable. Especially, PPARα shows the highest expression in HC which were kept in culture for 6 hours or 24 hours. In untreated KC a slight expression of CAR and PPARα was detectable. Expression of PXR and PGR could just be measured upon cycle 42 in KC. Among NR tested in EC, the highest mRNA levels were found for CAR followed by PXR whereas PPARα, PGR and ERα were only detectable upon cycle 42. Additionally, a

small influence of the culture time could be observed as the primary liver cells which were in culture for 24 hours show a higher basal expression level compared to the primary liver cells which were in culture for 6 hours.

As the hormone-specific NR PGR and ERα show very low mRNA levels and ERβ could not be detected in any cell type of the rat liver, it was important to check if the primers designed work without failure. For this purpose, cDNA isolated from highly hormone dependent rat organs, i.e. uterus, ovary and kidney was amplified with these primers. As estimated PGR, ERα and ERβ are strongly expressed in uterus, ovary and kidney of rats (Figure 9). This indicates that the designed primer work specifically and that the results obtained from primary liver cells appear to be reliable.

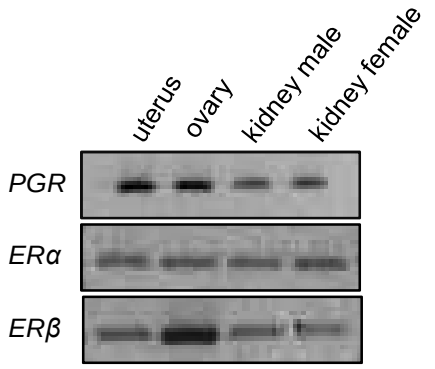

Figure 9: mRNA level of hormone-specific NR in rat uterus, ovary and kidney.

The cDNA obtained from tissue or cells of rat uterus, ovary and kidney were used as template for PCR amplification. After 40 amplification cycles products were loaded on a 1.2% agarose gel, stained with EtBr and visualized with UV-light. For details see section 3.8.4 and 3.8.5 of materials and methods.

4.2.2 mRNA level of specific NR in treated primary liver cell types

Previous studies have shown that NGC can influence gene expression of specific NR. (Zhang, et al., 1999; Moreau, et al., 2007; Gonzalez, et al., 2008; Couse, et al., 1999) For this thesis, it was checked whether NGC induce NR in the different liver cell types (Figure 10).

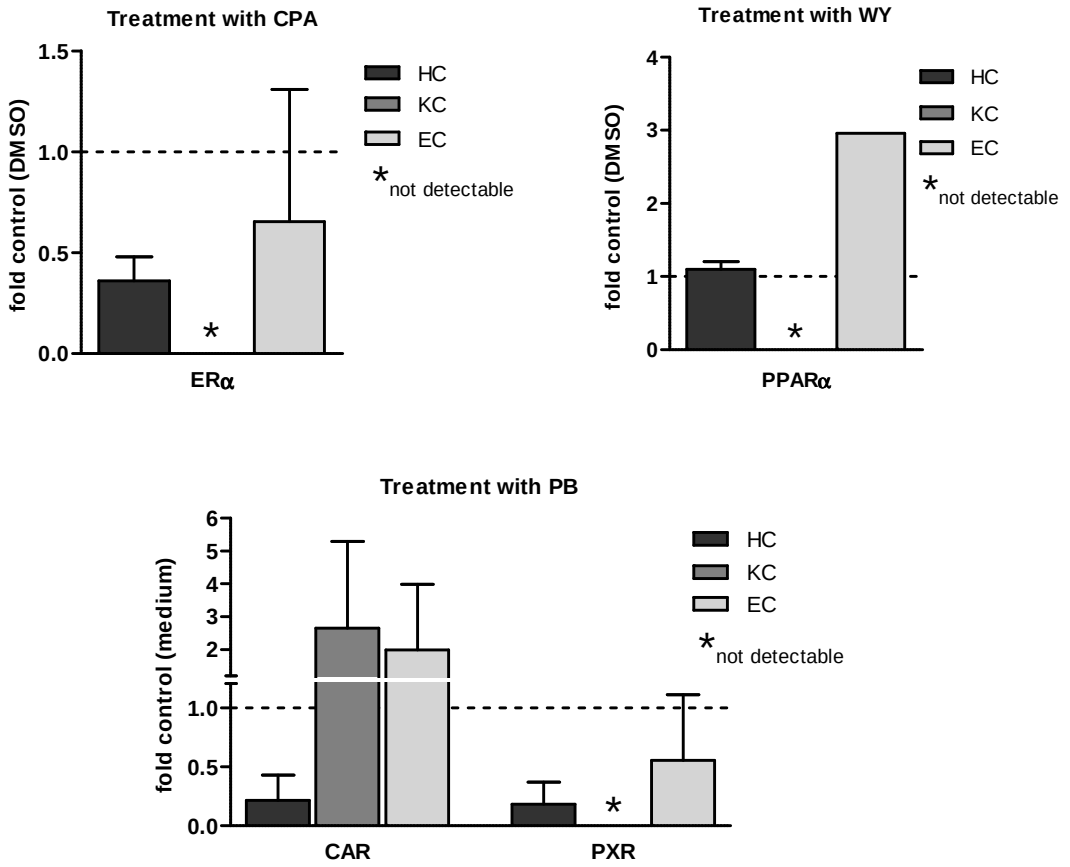

Figure 10: NGC induce mRNA level of specific NR in different liver cell types.

HC, KC and EC were isolated from untreated rats and were kept in culture. After two hours in culture, the cells were treated with CPA, WY or PB as follows: CPA was dissolved in DMSO and added to medium at a final concentration of 10 µM. The cells treated with 0.2% DMSO served as control for CPA. WY was dissolved in DMSO and added to medium at a final concentration of 20 µM. The cells treated with 0.2% DMSO served as control. PB was dissolved in medium and applied at a final concentration of 1 mM. The untreated cells served as control for PB. 24 hours later, the cells were harvested, RNA was isolated and cDNA was generated. The cDNA was used as template for PCR amplification. After 35 amplification cycles (for HC) and 40 amplification cycles (for KC and EC) products were loaded on a 1.2% agarose gel, stained with EtBr and visualized with UV-light. ß-actin was used as a positive control and reference gene. Volumes of the PCR products were determined by densitometry (Quantity One 4.2.1; Bio-Rad Laboratories, USA). The amount of data obtained for the gene of interest was normalized by their specific controls (DMSO or medium). The data are means +/- SEM of two independent experiments.

When HC were treated with CPA for 24 hours, the expression level of ERα decreased by half compared to the control. Treatment of CPA had no significant effect on ERα in EC and failed to induce expression of the hormone receptor in KC. When HC were treated with WY, no change in mRNA level of PPARα could be observed. Treatment of EC with WY resulted in a threefold increase in the expression of PPARα. PPARα remained undetectable in KC. The

mRNA level of CAR showed on the one side an increase in KC and EC but on the other side a decrease in HC when the cells were treated with PB. Treatment of HC and EC with PB reduced the expression level PXR.

Overall, the results obtained with standard PCR show large deviations in mRNA levels. In HC NR transcripts tend to decrease after treatment with NGC while the opposite occurs in EC. Almost no effect was observable in KC. The next step was to verify the data by applying qrt-PCR.

4.2.3 qrt-PCR analyses of NR in treated and untreated primary liver cell types

Next, the expression levels of CAR, PXR and PPARα was quantified by qrt-PCR in HC, KC and EC. Furthermore, it was checked whether treatment with CPA, PB or WY changes mRNA levels of these NR in primary liver cell types (Figure 11).

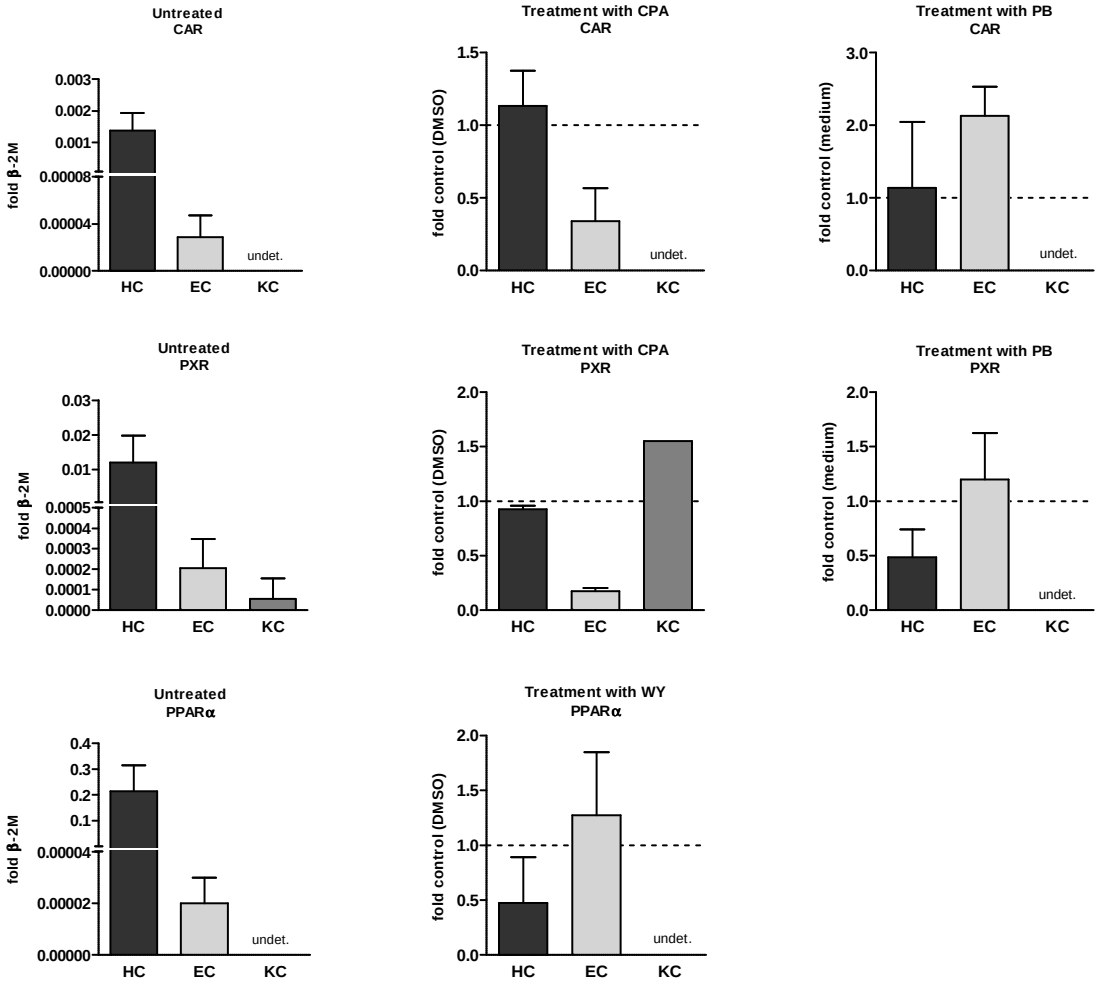

Figure 11: Variable expression of NR in treated and untreated liver cell types.

HC, KC and EC were isolated from untreated rats and were kept in culture. After two hours in culture, the cells were treated with CPA, WY or PB as follows: CPA was dissolved in DMSO and added to medium at a final concentration of 10 µM. The cells treated with 0.2% DMSO served as control for CPA. WY was dissolved in DMSO and added to medium at a final concentration of 20 µM. The cells treated with 0.2% DMSO served as control. PB was dissolved in medium and applied at a final concentration of 1 mM. The untreated cells served as control for PB. 24 hours later, the cells were harvested, RNA was isolated and cDNA was generated. The cDNA was used as template for qrt-PCR. The ABI-Prism PCR standard protocol was used on an ABI-Prism/7500 Sequence Detection System with TaqMan-based assays. The mRNA levels were quantified with an ABI-Prism/7500 SDS-software and normalized to ß2-M. The expression level of treated cells was related to the expression level of their controls. The data are means +/- SEM of three independent experiments.

Expression patterns of CAR

General expression of CAR was detectable to a low extent in untreated HC and EC. Treatment with CPA did not change mRNA level in HC and reduced mRNA level in EC when compared to the control. When EC were treated with PB, a 2-fold increase in gene

expression of CAR was detectable. In contrast to the standard PCR method (Figure 10), CAR was not detectable with qrt-PCR in KC which were treated with PB.

Expression patterns of PXR

Expression of PXR was detectable in all three liver cell types with HC showing the highest mRNA level, followed by EC and KC. Treatment with CPA did not change mRNA level in HC and strongly reduced mRNA level in EC when compared to the control. In KC, treatment with CPA slightly increased gene expression of PXR. Treatment of liver cells with PB showed almost the same effect on PXR expression as already demonstrated with the previous standard PCR experiment (Figure 10).

Expression patterns of PPARα

PPARα is detectable in untreated HC and EC and undetectable in KC. Similar to the preceding standard PCR experiment, the expression of PPARα was slightly elevated when EC were treated with WY.

Overall, the results obtained with qrt-PCR confirm more or less the data generated with conventional PCR, i.e. both approaches showed rather similar expression patterns in primary liver cell types with the exception of KC. Here, CAR could not be detected via qrt-PCR when KC were treated with PB. It is difficult to find reasons for this mismatch but an explanation could be the different primers used in conventional PCR and qrt-PCR or reduction in cDNA quality over time.

4.2.4 Specific inhibitors for PXR and CAR

Specific inhibitors for PXR and CAR were applied in order to find out whether CPA exerts its effect over these NR. Previous studies demonstrated that sulforaphane (SFN), found abundantly in broccoli, blocks xenobiotic-mediated activation of human PXR (Zhou, et al., 2007). Another research group identified 1-(2-chlorophenylmethylpropyl)-3-isoquinoline-carboxamide (PK-11195) as a selective and potent inhibitor of human CAR (Li, et al., 2008). Here, HC were treated simultaneously with the NGC CPA and the inhibitors of PXR and/or CAR. On the one hand, it was important to check the effect of the antagonists on mRNA levels of CAR and PXR (Figure 12). On the other hand, it was investigated whether the specific inhibitors block the CPA-induced DNA synthesis in HC (Figure 13).

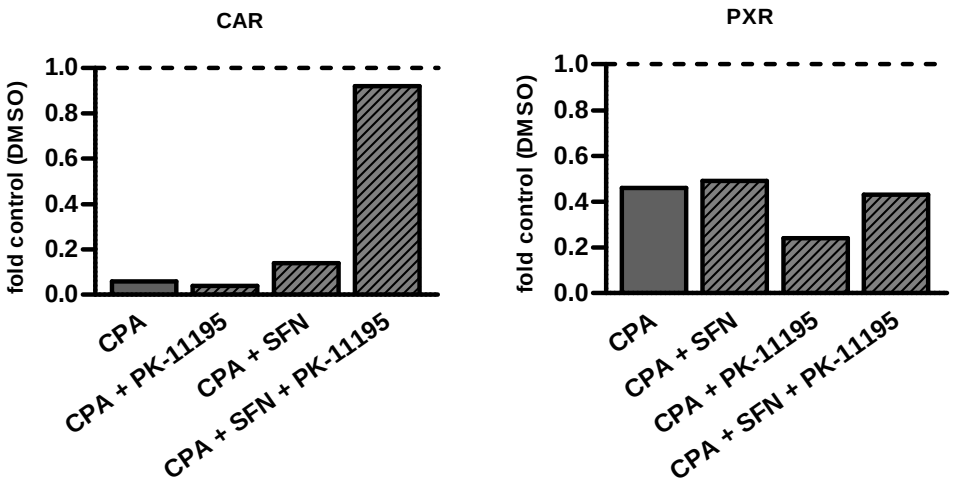

Figure 12: Effect of antagonists of CAR and PXR on mRNA level in HC.

HC were isolated from untreated rats and were kept in culture. After two hours in culture, the cells were treated with CPA and the respective inhibitor as follows: CPA was dissolved in DMSO and added to medium at a final concentration of 10 µM. The PXR inhibitor SFN and the CAR inhibitor PK-11195 were added at a final concentration of 10 µM respectively. The cells treated with 0.2% DMSO served as control. 24 hours later, the cells were harvested, RNA was isolated and cDNA was generated. The cDNA was used as template for qRT-PCR. The ABI-Prism PCR standard protocol was used on an ABI-Prism/7500 Sequence Detection System with TaqMan-based assays. The mRNA levels were quantified with an ABI-Prism/7500 SDS-software and normalized to ß2-M. The expression level of treated cells was related to the expression level of DMSO treated cells (=fold control DMSO). The data derive from one experiment.

The results of this experiment demonstrate that CPA reduced the expression levels of CAR and PXR and that the specific inhibitors SFN and PK-11195 failed to show inhibitory effects on mRNA level of CAR and PXR in rat HC. However, CPA is known to stimulate DNA replication in HC (Löw-Baselli, et al., 2000). Thus it was the next step to find out whether the inhibitors are capable of interfering with the DNA synthesis induction by CPA.

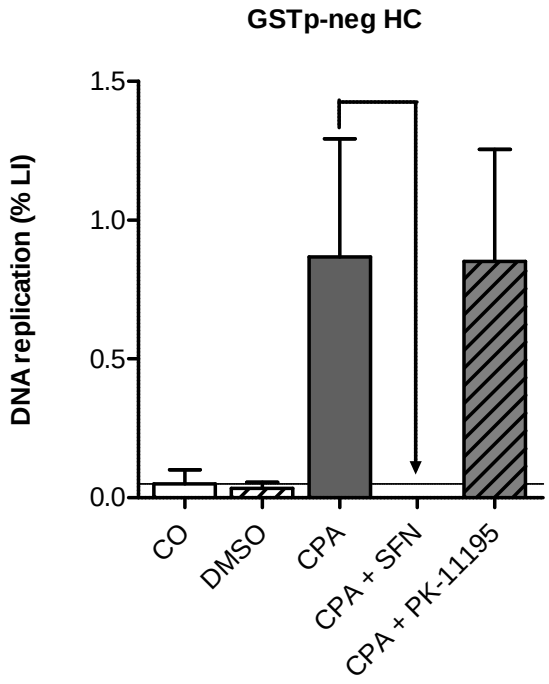

Figure 13:Effect of CAR/PXR-antagonists on DNA replication of HC treated with or without CPA.
HC were isolated from untreated rats and were kept in culture. The inhibitors were added 2 hours after seeding. Treatment was renewed with a medium change 24 hours after seeding. Applied concentrations: PXR inhibitor SFN 25 µM; CAR inhibitor PK-11195 10 µM; CPA 10 µM. Untreated cells and cells treated with 0.2% DMSO served as controls. ^{3}H-thymidine was added 24 hours before harvesting of cells. 72 hours after seeding cultures were fixed and subjected to autoradiography. The nuclei with incorporated ^{3}H-thymidin were counted and the percentages were calculated. In each experiment 1000 nuclei of HC were scored. The data are means +/- SEM from independent experiments on 2 rats.

The application of CPA strongly increased DNA replication in GSTp-neg cells when compared to the untreated or DMSO treated cells. The PXR inhibitor SFN was able block the induction of DNA replication in HC whereas the CAR inhibitor PK-11195 had no effect. Taking these results together, CPA seems to exert its effect on DNA replication of HC via PXR.

4.3 Gene expression pattern of specific pro-inflammatory factors

The connection between hepatocarcinogenesis and inflammation is confirmed. Many previous studies with specific mouse models found important key factors such as TNFα or IL-6 which link these two processes. (Berasain, et al., 2009) Furthermore, microarray analysis (Data not shown) revealed that HC, isolated from PB-exposed livers, tended to counter-react

in order to cope with the action of the pro-inflammatory chemo-/cytokines and to survive in a stressed mesenchyme. The increased production of TNFα by NPC leaves a clear signature in HC consisting of deregulations and counter-regulations within the TNFα-, interferon-, and NFκB-driven pathways. This raises the question whether NPC secrete soluble factors which changes the mRNA level of pro-inflammatory genes in hepatocytes or vice versa. Thus the experiment includes the in vivo part where male Wistar rats were treated for 24 hours or for two weeks with PB (see section 3.4). Subsequently, one cell type was treated with the supernatant of the other cell type (see section 3.7.2). For a better understanding the experimental design is shown in figure 14.

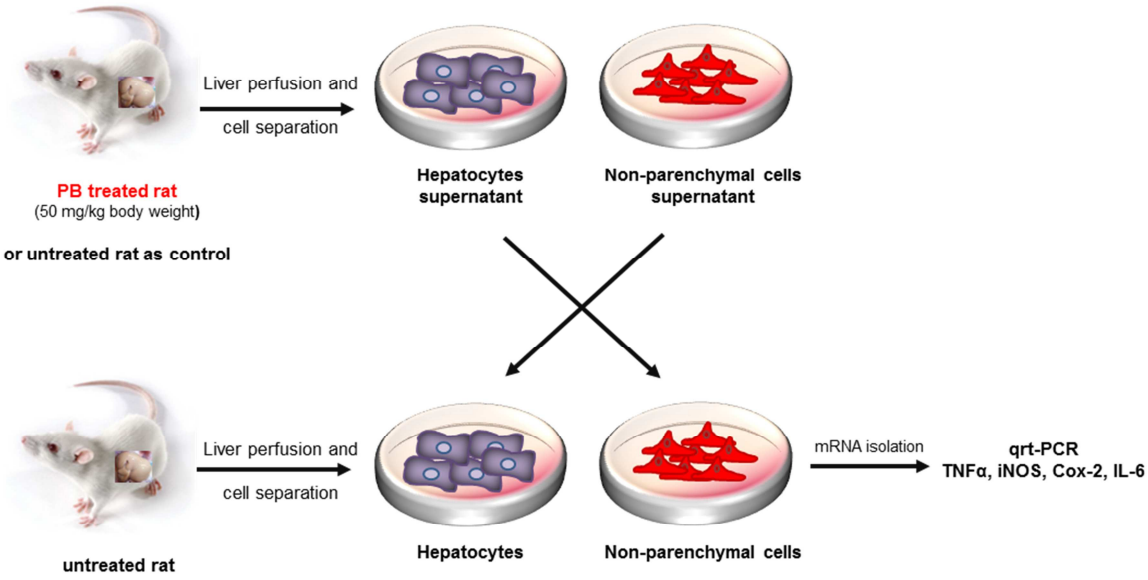

Figure 14: Experimental design of the supernatant experiment.

Male Wistar rats were treated either for 24 hours with a single dose of PB by gavage or for 14 days with PB through their drinking water. Untreated rats served as control. The livers were perfused and cells were separated in HC and NPC and were kept in culture. The HC, obtained from the untreated rats were treated with the supernatant of the NPC, obtained from the PB treated or untreated rats. The NPC, obtained from the untreated rats were treated with the supernatant of the HC, obtained from the PB treated or untreated rats. After 24 hours treatment, the cells were harvested for mRNA isolation. The mRNA levels of the pro-inflammatory genes of interest were determined via qrt-PCR.

4.3.1 Supernatant effects of isolated liver cell types

The following two graphs outline the influence of PB treatment in vivo on the secretome of isolated liver cell types.

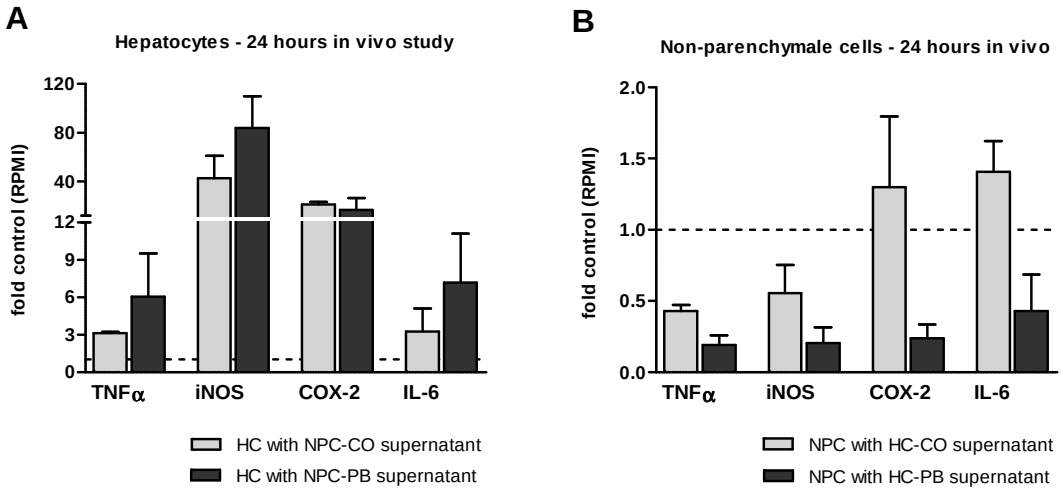

Figure 15: Effect of one day PB treatment in vivo on the secretome of isolated liver cell types.
*Male Wistar rats were treated with a single dose of PB administered by gavage. The livers were perfused; cells were separated in HC and NPC and kept in culture. After 24 hours HC and NPC of untreated rats were treated with the various supernatants as follows: In **(A)** HC were treated with two different supernatants of NPC (NPC-PB supernatant, NPC-CO supernatant) or just with RPMI as control. In **(B)** NPC were treated with two different supernatants of HC (HC-PB supernatant, HC-CO supernatant) or just with RPMI as control. 24 hours later, the cells were harvested, mRNA was isolated and cDNA was generated. The cDNA was used as template for qrt-PCR. The ABI-Prism PCR standard protocol was used on an ABI-Prism/7500 Sequence Detection System with TaqMan-based assays. The mRNA levels were quantified with an ABI-Prism/7500 SDS-software and normalized to ß2-M. The expression level of treated cells was related to the expression level of RPMI treated cells (=fold control). The data are means +/- SEM of ≥ two independent experiments (Figure 15:A) or ≥ three experiments (Figure 15:B).*

Treatment of HC with NPC-CO supernatant induced the mRNA level of pro-inflammatory genes, i.e. COX-2 and iNOS expression were 10- and 40-fold elevated, respectively (Figure 15A). This effect became enhanced when the secretome of NPC, which had been isolated from a PB-treated rat, was used. An 80-fold upregulated iNOS mRNA level was observable when compared to HC kept in pure medium only (Figure 15A).

In contrast, the supernatant of PB-treated HC reduced the expression of pro-inflammatory genes in NPC (Figure 15B). This indicates that HC counteract the pro-inflammatory action of

the PB-activated mesenchyme. Thus PB induces a pro-inflammatory state in the mesenchyme.

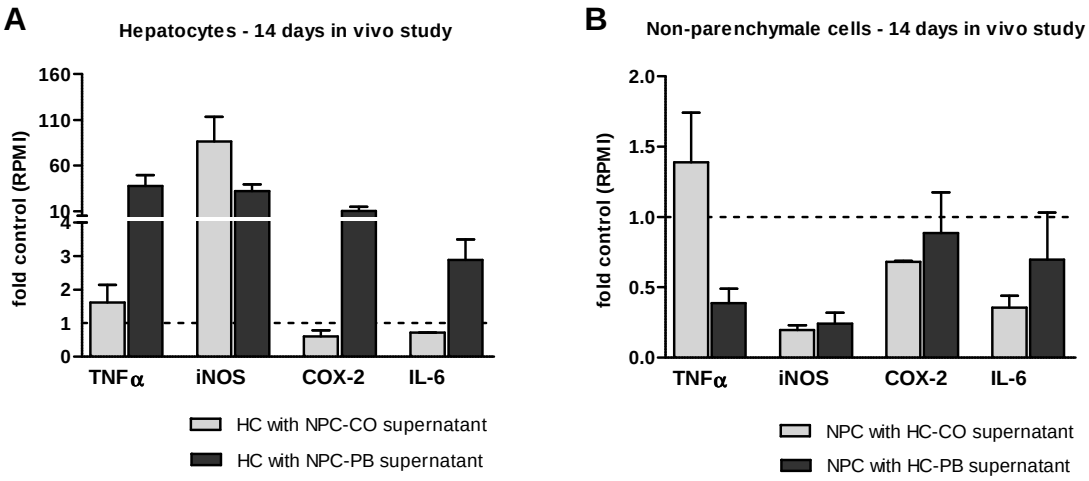

Figure 16: Effect of 14 days PB treatment in vivo on the secretome of isolated liver cell types.
*Male Wistar rats were treated with PB for 14 days via drinking water. The livers were perfused; cells were separated in HC and NPC and kept in culture. After 24 hours, HC and NPC of untreated rats were treated with the various supernatants as follows: In **(A)** HC were treated with two different supernatants of NPC (NPC-PB supernatant, NPC-CO supernatant) or just with medium RPMI as control. In **(B)** NPC were treated with two different supernatants of HC (HC-PB supernatant, HC-CO supernatant) or just with medium RPMI as control. 24 hours later, the cells were harvested, mRNA was isolated and cDNA was generated. The cDNA was used as template for qrt-PCR. The ABI-Prism PCR standard protocol was used on an ABI-Prism/7500 Sequence Detection System with TaqMan-based assays. The mRNA levels were quantified with an ABI-Prism/7500 SDS-software and normalized to ß2-M. The expression level of treated cells was related to the expression level of RPMI treated cells (=fold control). The data are means +/-SEM of ≥ two independent experiments.*

Also after 14 days treatment in vivo, there was a strong effect of the NPC-PB supernatant on HC (from untreated rats) compared to the effect of the NPC-CO supernatant (Figure 16A). All target genes were strongly elevated such as TNFα showing a 37-fold upregulation in HC. Also slight effects of the HC-PB supernatant on NPC could be observed (Figure 16B) when compared to HC-CO supernatant, e.g. 0.5 downregulation of TNFα.

4.4 Analysis of TNFα as pro-inflammatory factor

4.4.1 Treatment of primary liver cells with recombinant TNFα

Considering the findings of the supernatant experiments (chapter 4.3), it was essential to find the factors which lead to the pro-inflammatory effect of the PB-NPC supernatant on HC. The question was whether TNFα may cause this effect. Primary liver cells were treated with the recombinant form of TNFα and mRNA levels of TNFα, COX-2, iNOS and IL-6 were determined (Figure 17).

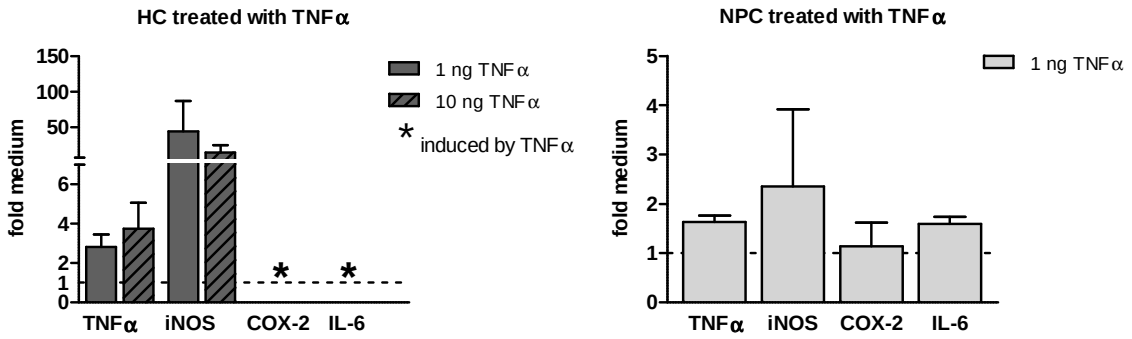

Figure 17: Effect of treatment with recombinant TNFα on primary liver cells.

The livers of untreated rats were perfused and cells were separated into HC and NPC and were kept in culture. After two hours, the cells were treated with 1 ng and/or 10 ng recombinant TNFα. 24 hours later, the cells were harvested, mRNA was isolated and cDNA was generated. The cDNA was used as template for qrt-PCR. The ABI-Prism PCR standard protocol was used on an ABI-Prism/7500 Sequence Detection System with TaqMan-based assays. The mRNA levels were quantified with an ABI-Prism/7500 SDS-software and normalized to ß2-M. The expression level of treated cells was related to the expression level of untreated cells (=fold medium). The data are means +/- SEM of two independent experiments.

As expected, a strong increase in expression of TNFα and iNOS was detectable in HC which were treated with recombinant TNFα. Furthermore, mRNA levels of COX-2 and IL-6 were inducible by the recombinant protein. In comparison to the medium controls, no significant changes of the mRNA level in NPC were detectable.

Taking these results together, the cytokine TNFα has a strong effect on HC and may belong to one of the factors which led to the effects of the supernatant experiment (see section 4.3.1).

4.4.2 TNFα release

Moreover, it was tried to determine the amount of released TNFα in the secretome of primary liver cells. Therefore the supernatants of HC and NPC, from rats treated with PB for 14 days as well as from control rats, were taken at different time-points and the concentration of released TNFα was measured via ELISA (Figure 18).

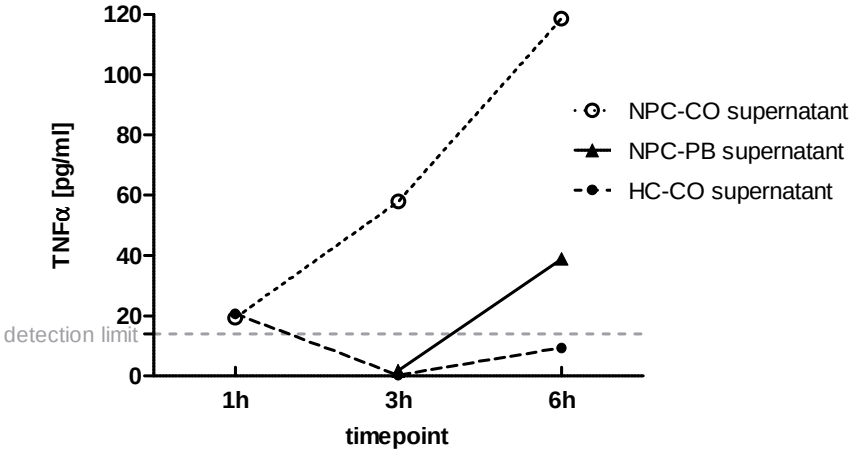

Figure 18: Effect of 14 days PB in vivo treatment on TNFα release of liver cell types.
Male Wistar rats were treated with PB for 14 days via drinking water. The livers were perfused; cells were separated in HC and NPC and kept in culture. The supernatants were collected 1h, 3h and 6h after seeding and concentration of TNFα was determined with ELISA. The data are means of two independent experiments. Detection limit of rat TNFα ELISA is 14 pg/ml according to manufacturer (indicated as dotted grey line).

In general, the concentration of TNFα in the supernatant of primary liver cells was very low or below the detection limit of the used ELISA (detection limit = 14 pg/ml). The highest concentration of secreted TNFα was found in supernatants of NPC controls after 6 hours in culture (Figure 18). TNFα was not detectable in HC-PB supernatants at all. In general, these results revealed that TNFα could not serve as the solitary factor which is responsible for the upregulated pro-inflammatory genes in HC from the supernatant experiment (see section 4.3.1).

4.5 DNA Chip analysis of the 24 hours CPA in vivo treatment

4.5.1 Overview of regulated genes

In vivo treatment of female rats with a single dose of CPA (100 mg/kg body weight) for 24 hours resulted in a wide range of differently expressed genes and altered pathways such as the ERK pathway. In HC more than 500 genes were downregulated and more than 740 genes were upregulated. In NPC, the treatment with CPA caused a downregulation of more than 90 genes and an upregulation of more than 450 genes. The following paragraphs show just a small part of the results from the DNA Chip analysis.

4.5.2 Influence of CPA in vivo treatment on CYP

Downregulated CYP (Figure 19A)

Cyp1a2 expression was reduced by half in HC due to CPA treatment. Cyp2c23 and Cyp4a1, which are generally involved in arachidonic acid metabolism, showed a 50% decrease in HC. Additionally, the steroid hydroxylase Cyp17a1 was downregulated in HC derived from rats which were treated with CPA. In NPC, the CYP26 enzymes with retinoic acid 4-hydroxylase activity showed a 50-80% diminution of their expression levels.

Upregulated CYP (Figure 19B)

It is well known that PB strongly increases Cyp2b1 expression level in primary rat liver cells (Denison, et al., 1995) but in that case CPA also increased Cyp2b1 expression up to 10-fold in NPC and HC after in vivo treatment with CPA. Cyp2c22 is a retinoic acid-metabolizing enzyme in rat liver and was approximately 2.5-fold in HC and more than 14-fold upregulated in NPC. Many steroids and xenobiotics are able to increase Cyp3a1 mRNA level in rat liver and here CPA increased gene expression level of Cyp3a1 in HC more than 2-fold and in NPC almost 20-fold.

At all, it is striking that Cyp2b1, Cyp2c22 and Cyp3a1 are upregulated in parenchymal and mesenchymal cells as a cause of in vivo treatment with CPA. Thus, these three enzymes may play important roles in CPA metabolism of both cell types. Additionally, mRNA analysis of in vitro treated cells shows different results (see section 4.1.2). As opposed to this (Figure 7), here CYP1a1 and Cyp2c12 mRNA level were not found to be changed.

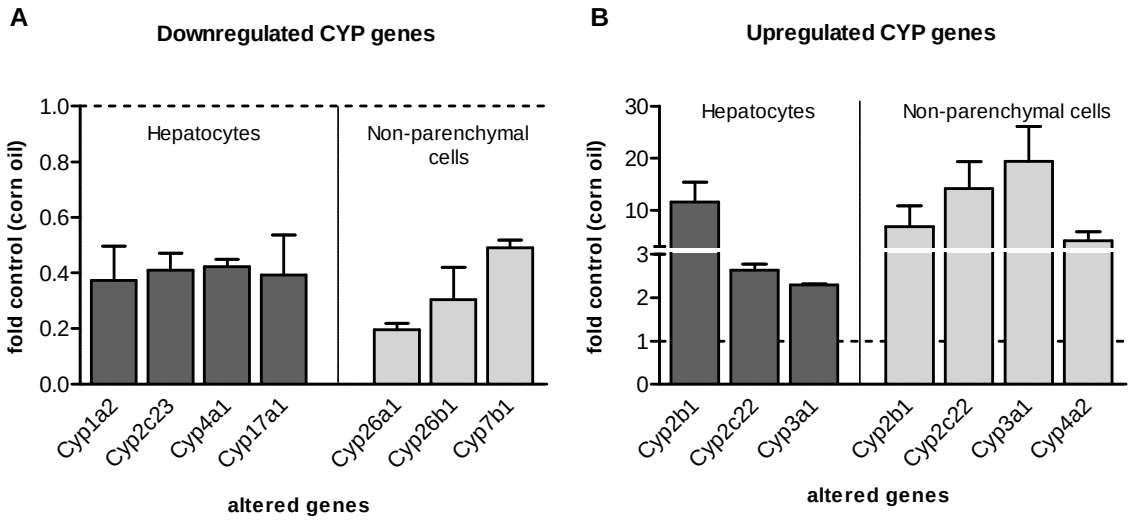

Figure 19: Effect of in vivo treatment with CPA on mRNA level of CYP genes in liver cell types.

Female Wistar rats, 8-12 weeks old, were treated with a single dose of CPA (100 mg/kg body weight dissolved in corn oil) by gavage. The control group was treated with corn oil by gavage. Perfusion of the livers occurred 24 hours after treatment. Total RNA was isolated from cell pellets of HC and NPC. Finally DNA Chip analysis was performed with the Affymetrix Rat Genome 230 2.0 Array (Affymetrix).The data are means +/- SEM of three animals per treatment group.

4.5.3 Influence of in vivo treatment with CPA on the expression of pro-inflammatory genes

For a better analysis, pro-inflammatory genes were roughly separated in chemokines and cytokines, complement activators, activators of prostaglandin synthesis and genes involved in acute phase response (Figure 20). Table 12 lists the meanings of the abbreviated gene names.

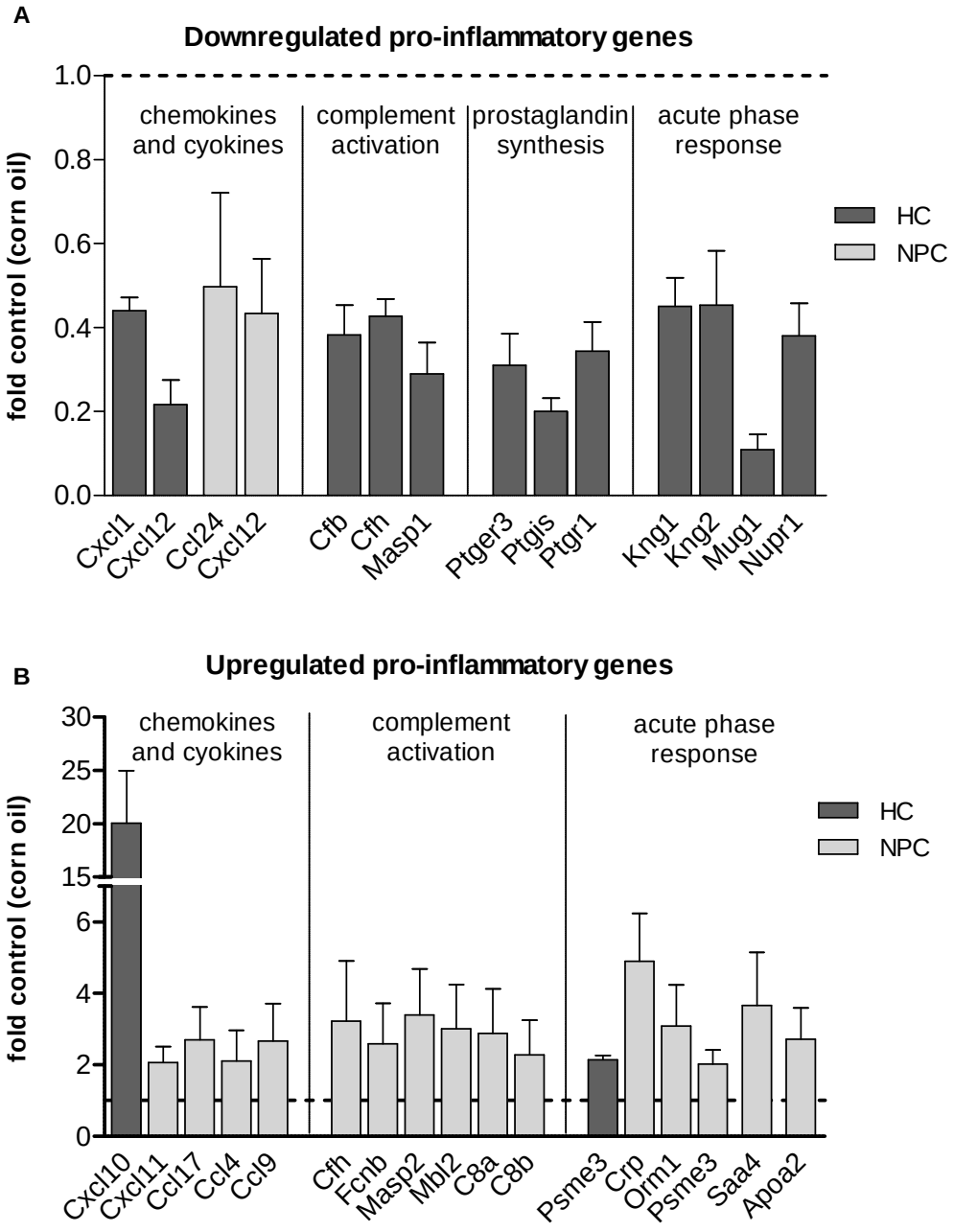

Figure 20: Altered inflammatory response genes in HC and NPC due to CPA treatment in vivo.

Female Wistar rats, 8-12 weeks old, were treated with a single dose of CPA (100 mg/kg body weight dissolved in corn oil) by gavage. The control group was treated with corn oil by gavage. Perfusion of the livers occurred 24 hours after treatment. Total RNA was isolated from cell pellets of HC and NPC. Finally DNA Chip analysis was performed with the Affymetrix Rat Genome 230 2.0 Array (Affymetrix). The data are means +/- SEM of three animals per treatment group.

Figure 20 demonstrates that there is a balance between upregulated and downregulated inflammatory factors in HC and NPC. In vivo treatment with CPA caused a downregulation of the chemokine Cxcl12 in both HC and NPC. The mRNA level of the pro-angiogenic gene

Cxcl1 was decreased by more than 50% in HC. Genes, which activate the complement system, such as Cfb, Cfh and Masp1 showed a significantly diminished expression level in HC. On the other side, the gene expression levels of six complement activating genes (Cfh, Fcnb, Masp2, Mbl2, C8a, C8b) were increased up to 3-fold in NPC. Three genes, which are involved in prostaglandin synthesis, were 2.5-fold downregulated in HC. Many acute phase response genes such as the C-reactive protein (CRP) were significantly elevated in NPC.

Table 12: List of full gene titles of pro-inflammatory genes which show altered gene expression by treatment with CPA in vivo.

Gene Symbol	Gene Title
Apoa2	apolipoprotein A-II
C8a	complement component 8, alpha polypeptide
C8b	complement component 8, beta polypeptide
Ccl17	chemokine (C-C motif) ligand 17
Ccl24	chemokine (C-C motif) ligand 24
Ccl4	chemokine (C-C motif) ligand 4
Ccl9	chemokine (C-C motif) ligand 9
Cfb	complement factor B
Cfh	complement factor H
Crp	C-reactive protein, pentraxin-related
Cxcl1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
Cxcl10	chemokine (C-X-C motif) ligand 10
Cxcl11	chemokine (C-X-C motif) ligand 11
Cxcl12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
Fcnb	ficolin B
Kng1	kininogen 1 /// kininogen 1-like 1
Kng2	kininogen 2
Masp1	mannan-binding lectin serine peptidase 1
Masp2	mannan-binding lectin serine peptidase 2
Mbl2	mannose-binding lectin (protein C) 2
Mug1	murinoglobulin 1
Nupr1	nuclear protein, transcriptional regulator, 1
Orm1	orosomucoid 1
Psme3	proteasome (prosome, macropain) activator subunit 3
Ptger3	Prostaglandin E receptor 3
Ptgis	prostaglandin I2 (prostacyclin) synthase
Ptgr1	prostaglandin reductase 1
Saa4	serum amyloid A4, constitutive

4.5.4 Influence of treatment with CPA on the ERK pathway

Many studies established that altered gene transcription can be observed in the ERK pathway during the development of different types of cancers including HCC (see section 1.4.4). The transcriptional analysis of the CPA short-term in vivo study came to a comparable result (Figure 21).

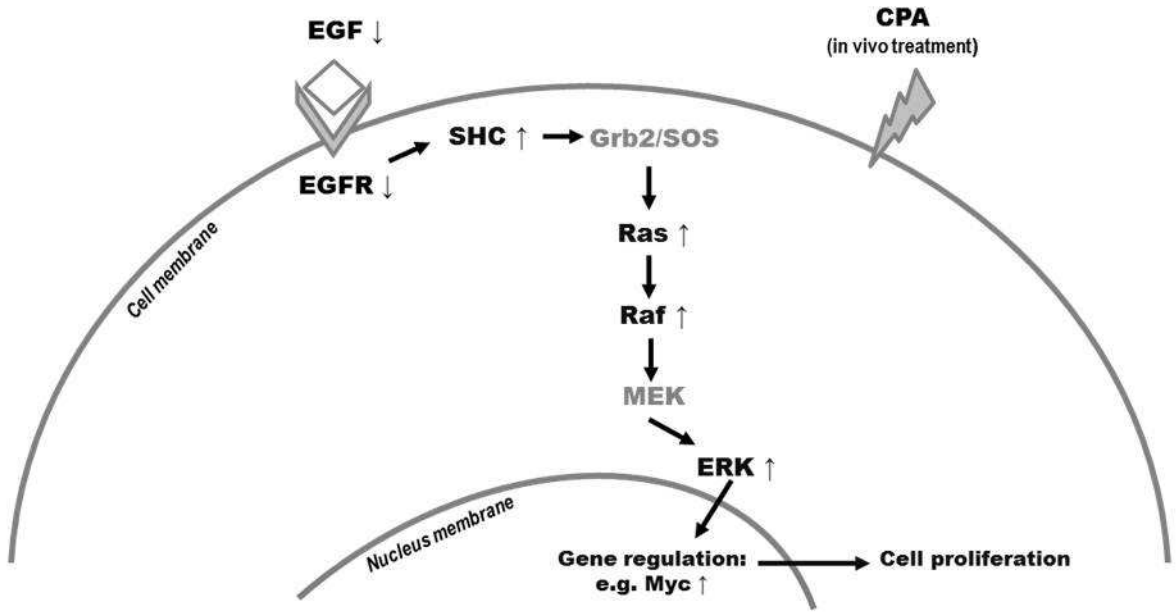

Figure 21: Scheme on ERK pathway components, deregulated in HC due to CPA treatment in vivo.
Symbols: ↓ indicates ≥ 2-fold downregulated genes; ↑ indicates ≥ 2-fold upregulated genes; grey font indicates not regulated genes.

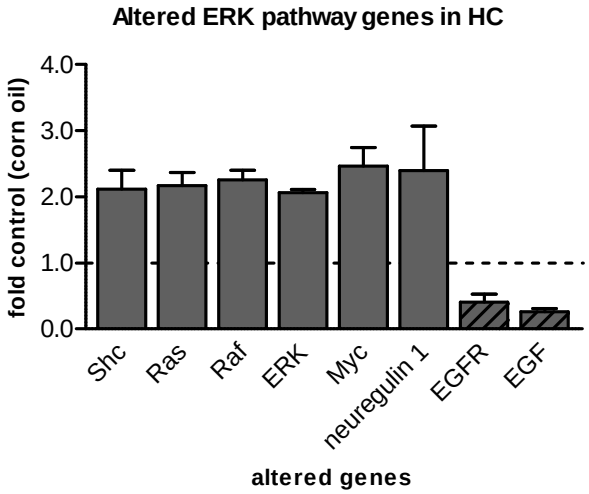

Figure 22: Effect of in vivo CPA treatment on the ERK pathway in HC.
Female Wistar rats, 8-12 weeks old, were treated with a single dose of CPA (100 mg/kg body weight dissolved in corn oil) by gavage. The control group was treated with corn oil by gavage. Perfusion of the livers occurred 24 hours after treatment. Total RNA was isolated from cell pellets of HC and NPC. Finally DNA Chip analysis was performed with the Affymetrix Rat Genome 230 2.0 Array (Affymetrix). The data are means +/- SEM of three animals per treatment group.

As illustrated by figure 21, most of the genes involved in the ERK pathway were altered in HC of rats which were treated in vivo with a single dosage of CPA. By contrast, in NPC no meaningful alterations in the ERK pathway could be found.

The data (Figure 22) revealed a more than 2-fold upregulation of oncogenes, such as Ras and Raf or the apoptosis mediating gene Shc1. One of the possible start points of the pathway, namely EGFR and two of its ligands were deregulated. EGF resulted in a more than 3-fold downregulation and neuregulin 1 was 2-fold upregulated in HC. The key player ERK and its regulated gene Myc showed increased expression levels in HC.

4.6 The MAPK/ERK pathway on protein level

As short-term CPA in vivo study affecting many genes of the ERK pathway (see section 4.5), the protein level of phosphorylated (activated) ERK was examined in cultured HC and NPC which were treated with PB or CPA. The phosphorylation status of the ERK1 and ERK2 proteins was checked from total protein lysates of untreated and treated primary liver cells by Western Blot analysis (see section 3.10.4). The antibodies recognize two distinct protein bands at 44 und 46 kDa representing the two ERK proteins.

4.6.1 Effect of PB treatment on ERK phosphorylation

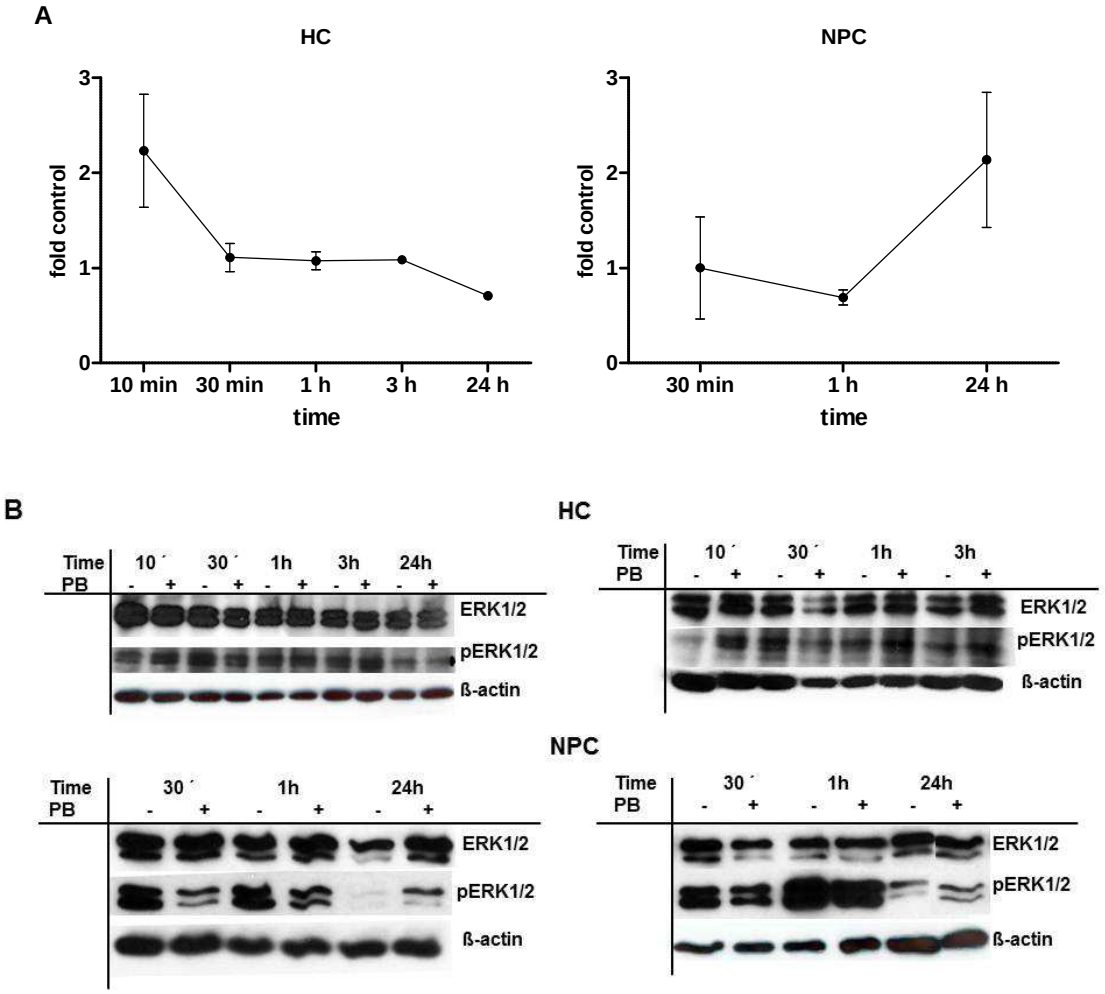

Figure 23: Phosphorylation of ERK1/2 in HC and NPC treated with PB.

The livers of male Wistar rats were perfused; cells were separated in HC and NPC and kept in culture. The cells were treated with PB (1 mM) 2 hours after seeding or stayed untreated as controls. The proteins were harvested 10 min, 30 min, 1h, 3h and 24h after treatment. The proteins were separated via SDS-Page and transferred on a PVDF membrane. The target proteins ERK1/2 and pERK1/2 were detected with specific antibodies and visualized on X-ray films. A ß-actin antibody was used for protein integrity and equivalent loading. The signal intensities were evaluated by densitometry (Quantity One 4.2.1; Bio-Rad Laboratories, USA). At first, the signal intensities of pERK1/2 were normalized to the signal intensities of ERK1/2 (= fold ERK1/2). Finally the values of the treated cells were referred to the values of the untreated cells (= fold control). The data are means +/- SEM of two independent experiments. ***(A)*** *CPA effect on ratio of pERK1/2 to total ERK1/2;* ***(B)*** *Representative Western Blots of ERK1/2, pERK1/2 and ß-actin.*

The degree of ERK phosphorylation in HC was highest when cells were treated 10 min with PB and slowly decreased over time whereas NPC showed a contrary phosphorylation pattern with the highest activity after 24 hours treatment (Figure 23A).

4.6.2 Effect of CPA treatment on ERK phosphorylation

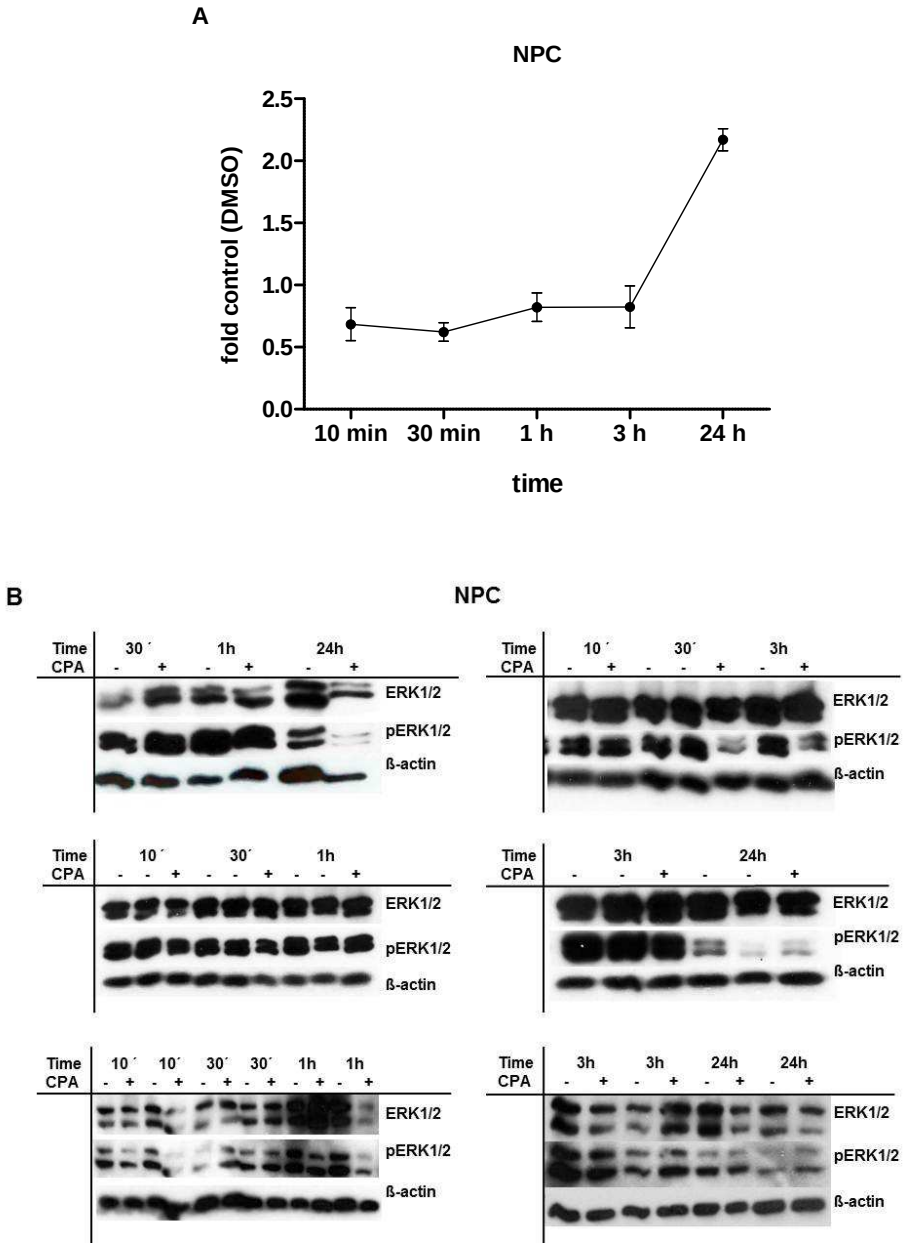

Figure 24: Phosphorylation of ERK1/2 in NPC treated with CPA.

The livers of female Wistar rats were perfused; cells were separated in NPC and kept in culture. The cells were treated with CPA (10 µM) or DMSO (0.2%) as control 2 hours after seeding. The proteins were harvested 10 min, 30 min, 1h, 3h and 24h after treatment. The proteins were separated via SDS-Page and transferred on a PVDF membrane. The target proteins ERK1/2 and pERK1/2 were detected with specific antibodies and visualized on X-ray films. A ß-actin antibody was used for protein integrity and equivalent loading. The signal intensities were evaluated by densitometry (Quantity One 4.2.1; Bio-Rad Laboratories, USA). At first, the signal intensities of pERK1/2 were normalized to the signal intensities of ERK1/2 (= fold ERK1/2). Finally the values of the treated cells were referred to the values of the DMSO treated cells (= fold control DMSO). The data are means +/- SEM of four independent experiments. ***(A)*** *Effects of CPA on ratio of pERK1/2 on total ERK1/2;* ***(B)*** *Representative Western Blots of ERK1/2, pERK1/2 and ß-actin.*

When NPC were treated with CPA, the degree of ERK phosphorylation showed similarity to treatment with PB as the peak with a 2-fold increase in phosphorylation activity was observed after 24 hours (Figure 24A). The other time points showed no significant change compared to the control levels.

At all, these results indicate that PB may influence the activity of the ERK proteins in HC. The impact of the ERK pathway in tumor promotion requires further investigations.

5. Discussion

NGC are a large group of chemicals that induce tumor formation by mechanisms which are different to direct DNA damage. (Mally, et al., 2002) As most of the studies concentrate on hepatocytes, the role of mesenchymal cells in carcinogenesis induced by NGC has found little attention so far. However, NGC have been shown to activate both HC and NPC, resulting in secretion of growth factors, pro-inflammatory cytokines and ROS. (Parzefall, et al., 2001) Besides, some of these growth factors have been found to be selective for preneoplastic liver lesions (PNL), serving as endogenous tumour promoters. Thus, HB-EGF secreted by NPC seems to be part of the growth stimulating circuit of rodent liver PNL. (Sagmeister, et al., 2008) Cooperation between NGC and the altered geno-/phenotype of preneoplasias may result in formation of stable autocrine loops in which growth factors, e.g. TGFα, stimulate their own synthesis and thereby induce steadily excessive growth. (Drucker, et al., 2006) This thesis aims to identify growth factors and chemo-/cytokines from primary liver cells especially mesenchymal cells, driving excessive responses of PNL towards NGC. This knowledge is essential to better understand the carcinogenic mode of action of the model NGC PB, CPA and WY.

5.1.1 Are NPC direct targets of NGC effects?

It is well known that many CYP candidates such as Cyp2b1 serve as markers indicating a response of primary liver cells to NGC. In this project, the in vitro and in vivo effect of NGC on CYP expression was tested separately for HC and NPC.

<u>In vitro effects of NGC on CYP expression</u>

We found very low basal expression levels of the examined CYP enzymes (Cyp1a1, Cyp2b1, Cyp2b2, Cyp2c12 and Cyp4a1) in untreated HC, EC and KC kept for 24 hours in culture. Treatment of HC with PB doubled gene expression of Cyp2b1 when compared to untreated controls. This result confirmed previous studies describing Cyp2b1 to be greatly induced by PB. Moreover, both PB and CPA were found as strong inducers of Cyp2c12 expression in HC and EC. In vitro treatment with CPA sharply increased Cyp1a1 expression in HC and KC whereas treatment with PB increased Cyp1a1 expression in EC. Thus, there is evidence that the mesenchyme show responses to NGC treatment.

In vivo effects of NGC on CYP expression

The data obtained from the in vivo study differ from the in vitro results. Treatment of rats with a single dose of CPA for approximately 24 hours changed gene expression of a broad range of CYP members. The steroid hydroxylase Cyp17a1 plays an important role in androgen biosynthesis and was found to be downegulated by half in HC. This may also confirm the anti-androgenic effect of CPA. Additionally, Cyp2b1 expression may not just serve as a positive control for the PB effect but also for the CPA in vivo effect. We saw a 10-fold upregulation of Cyp2b1 in NPC and HC after in vivo treatment with CPA. To conclude, NPC may react towards NGC as shown by upregulated CYP in this cell compartment.

5.1.2 Do NPC express NR?

NR, like CAR and PXR, are able to regulate drug metabolism which includes the transcriptional regulation of CYP genes. Because of that, the basal NR expression and a possible induction of NR transcripts were investigated separately for HC and NPC in this study.

Basal expression of NR in liver cell types

We found, that untreated HC express many NR such as CAR, PXR and PPARα in a higher level than NPC do. However, KC and EC show also a cell specific expression pattern of the tested NR.

In vitro effects of NGC on NR expression

Treatment of primary liver cells with CPA reduced gene expression of the hormone receptor ERα by half in HC compared to the DMSO control. Additionally, CPA strongly reduced gene expression of PXR in EC. As expected, treatment of primary liver cells with the peroxisome proliferator WY slightly increased expression of PPARα in EC but surprisingly not in HC and KC. The exposure of primary liver cells to PB enhanced CAR expression in EC. At all, the obtained data allow the assumption that EC may contribute to the NR-mediated effect of NGC.

Which NR is the primary target receptor for CPA?

Additionally, we searched for the NR which carries out the effects of CPA. Therefore, specific inhibitors for PXR and CAR, in combination with CPA treatment, were applied. We tested the effects of the PXR inhibitor SFN and the CAR inhibitor PK-11195 on mRNA level and whether the inhibitors are able to block CPA induced DNA synthesis in HC.

On mRNA level, the inhibitor used for PXR (SFN) and CAR (PK-11195) failed to achieve any effect, as has been anticipated. However, the PXR inhibitor SFN was able to block the induction of DNA replication in HC. The CAR inhibitor PK-11195 had no effect. This might indicates that CPA seems to exert its effect on DNA replication of HC via PXR. Future experiments with CAR and PXR knock-out mice might be a good possibility for studying in detail the role of CAR and PXR in NGC-mediated carcinogenesis.

In vivo effect of NGC on NR expression

The short-term CPA in vivo study could not confirm the in vitro results. The expression of NR such as CAR and PXR stayed unchanged with CPA treatment compared to the oil control. It seems that a single application of CPA is not able to induce an effect on NR expression patterns of primary liver cells.

5.1.3 What are the effects of in vivo treatment with CPA on the whole transcriptome?

A solid way to get an overview about the mode of action of NGC on mRNA level is an in vivo experiment in combination with a Gene Chip analysis. We wanted to know whether in vivo application of a single dose of CPA (100 mg/kg body weight) changes the transcriptome in liver cells. The resulting expression levels were related to the expression levels of the control group (oil treated rats).

To summarize, the larger part of altered genes was found in HC, i.e. more than 500 genes were found to be downregulated and more than 740 genes were in the upregulated state. In NPC, the in vivo exposure to CPA caused a downregulation of more than 90 genes and an upregulation of more than 450 genes. Many of these genes play important roles in inflammation, cell cycle, apoptosis, cell proliferation and detoxification. However, this data reflect again the problem that the in vivo and in vitro results differ in many aspects as shown by gene expression levels of different CYP and NR in the present study (see sections 4.1 and 4.2). These discrepancies might be explained by many different factors such as the dedifferentiation of HC in culture caused by the loss of the 3-dimensional configuration of the cell, lack of cell-cell and cell-matrix contacts and the absence of epithelial-mesenchymal interactions. It is well known that P450 expression and activity rapidly decline in conventional HC monolayer cultures, resulting in the loss of responsiveness to some metabolic enzyme inducers, especially PB. (Su, et al., 2004) Co-culture systems or the use of liverbeads would be worth considering as they are closer to the in vivo situation.

Altered inflammatory factors

It is well accepted that chronic inflammation plays a huge role in tumor promotion and that an inflammatory microenvironment is an essential feature of all tumors. (Grivennikov, et al., 2010) Interestingly, only a single application of CPA caused deregulation of inflammatory genes involved in (1) chemo-/cytokine mediated response, (2) prostaglandin synthesis, (3) complement activation and (4) acute phase response. Many pro-inflammatory factors were downregulated in HC (12 downregulated genes vs. 2 upregulated genes) while the opposite was true for NPC (15 upregulated genes vs. 2 downregulated genes). This confirms again the strong reaction of the mesenchyme in response to in vivo treatment with CPA.

It is mentionable that the chemokine Ccl9 was more than 2.5-fold upregulated in NPC due to CPA in vivo treatment. The chemokine Ccl9 is produced by cancer cells in order to recruit specific immune cells (CCR1+ myeloid cells) which in turn promote invasion of cancer cells through secretion of the matrix metalloproteinases MMP2 and MMP9. (Grivennikov, et al., 2010) This alteration might contribute to the potent tumor promoting effects of CPA.

5.1.4 Are their direct or indirect effects of NGC on NPC?

The various cell types of the liver carry out their functions not in a solitary way but rather act as an integrated community in order to fulfil all the complex tasks of this organ. Therefore, we asked whether NGC act in a direct way on NPC or in an indirect way via factors released by HC. This might explain the strong response of NPC towards NGC. Although these cells barely express several important NR. Furthermore, HC may receive the NGC-mediated signal directly or indirectly via NPC.

Interaction between HC and NPC

In detail, we treated rats with PB for 24 hours or for two weeks. Afterwards, the cells were separated in HC and NPC and cultured. 24 hours later the supernatant of NPC, isolated from PB-treated rats, was collected in order to treat HC which were isolated from untreated rats and vice versa. Supernatant derived from HC and NPC, which had been isolated from untreated rats, served as controls. The gene expression levels of the pro-inflammatory mediators TNFα, IL-6, COX-2 and iNOS were studied in order to determine whether the supernatant acts in a pro- or in an anti-inflammatory way.

The supernatants of NPC, obtained from untreated rats, led to a strong overexpression of TNFα, COX-2, iNOS and IL-6 in HC. This was even more pronounced when the supernatant

derived from NPC isolated from PB-treated animals. Surprisingly, when NPC were treated with the secretome of HC, obtained from untreated rats, the expression of the genes studied was significantly lowered which indicates an anti-inflammatory effect of HC on NPC. Interestingly, PB-treated HC had an even stronger anti-inflammatory impact on the mesenchymal cells than control HC.

In conclusion, NPC might react on the PB treatment by secretion of pro-inflammatory factors which lead to an inflammatory signature in HC. It appears possible that a prolonged exposure to NGC would then lead to a chronic low-level inflammation which in return could be the starting point for the development of liver cancer.

Direct and indirect effects of NGC

As already discussed, the weak response of NPC to treatment with CPA, PB or WY in vitro may be caused by dedifferentiation of the cells in culture or by cell separation which disrupts HC and NPC as interacting partners. Moreover, the supernatant studies showed a strong interaction between the parenchymal and mesenchymal cells. This may be mediated by different pro-inflammatory factors such as cytokines. The analysis of the secretome of HC and NPC, derived from PB treated rats, via TNFα ELISA failed to identify TNFα as possible factor, as the measured concentrations were very low. As a consequence, the search for the soluble factors which lead to the strong response in HC and NPC has to be extended in the future.

5.1.5 Which pathway is affected by NGC?

It is common knowledge that the ERK pathway regulates the cell cycle progression via different transcription factors. (Schmitz, et al., 2008) A marked change within this signalling cascade has fatal consequences such as enhanced cell proliferation, which might lead to cancer. (Goodsell, 1999)

In vivo effects of CPA on ERK pathway

We found that the application of a single dose of CPA leads to an altered expression of many genes involved in the ERK pathway of HC. The proto-oncogenes Ras and Raf are related to control cell growth and differentiation and were found to be more than 2-fold upregulated. The EGF is mitogenic for HC through the binding to its receptor EGFR. A relation between the overexpression of the EGFR pathway and the development of HCC has often been described. (Schiffer, et al., 2005) Thus, it is intriguing that we found a more than 50%

reduction in gene expression of EGFR and EGF due to CPA treatment in vivo. This may indicate a counter-regulation following a stimulation of EGFR-mediated signalling. Furthermore, a chronic administration of NGC might have had a completely different effect on the various EGF-receptors and its ligands.

In vitro effects of NGC on ERK activity

Phosphorylation of ERK proteins leads to cellular and/or nuclear responses dependent on the stimulus, the ERK isoform activated (ERK1 or ERK2) and the cell type studied. (McKillop, et al., 2003) We wanted to know whether treatment with different NGC changes the activity of ERK proteins. Therefore, we determined the degree of phosphorylation of ERK1/2 in response to in vitro treatment with PB and CPA via specific antibodies and Western Blot analysis. For a better monitoring of the ERK activity, different treatment time points were used.

HC which were treated for 10 min with PB showed the highest activity of ERK. Additionally, the phosphorylation grade decreased with increasing treatment time. In NPC, activity of ERK increased up to 2-fold after 24 hours treatment with PB. Short treatment (10 min – 3 hours) of NPC with CPA slightly decreased ERK phosphorylation but after 24 hours phosphorylation increased up to 2-fold. However, late phosphorylation probably indicates a result with less significance.

The results confirmed that the activity of the ERK proteins is cell type specific and plays a role in the mode of action of NGC. A higher ERK activity over a long time period might lead to altered cell growth in primary liver cells and in further consequence might contribute to the development of HCC. However, the involvement of the ERK pathway in tumor promotion needs further investigation.

5.1.6 Conclusion and perspectives

Research in NGC-related hepatocarcinogenesis has been reaching big milestones in the last few years. However, there are still many open questions such as the identification of the key factors which promote tumor development and progression. The relationship between inflammation and HCC will be a good basis for further experiments. The overall aim for future studies will be the identification of possible biomarkers in order to prevent the development of liver cancer driven by NGC.

6. Abstract

As particularly aggressive cancer entity, hepatocellular carcinoma (HCC) causes about half a million deaths per year. Therefore, it is important to eliminate putative cancer risk factors and to understand the mechanisms underlying the pathogenesis of this tumor. Many drugs prescribed to millions of people worldwide belong to the class of non-genotoxic hepatocarcinogens (NGC), i.e. they do not feature genotoxicity but produce tumors in long-term rodent bioassays. The significance of these findings for human liver cancer risk is not clear. This thesis aimed to study the mode of action of prototypical NGC (phenobarbital, PB; cyproterone acetate, CPA; and Wyeth, WY) in order to better estimate whether NGC may pose a risk to human health. Hitherto, research has been focusing on hepatocytes (HC) as direct target cells of NGC. Since there is evidence that NGC are able to act also on non-parenchymal liver cells (NPC), the present study was designed to investigate the effects of NGC, separately for HC and NPC.

As main experimental approaches, rats were treated with PB or CPA either once or for a prolonged period of time. The cells were isolated from rat liver by collagenase perfusion, were separated into HC and NPC by percoll gradient centrifugation, were seeded to culture plates and were treated with PB, CPA, or WY for up to 24 hours. Analysis of altered gene expression patterns was performed via conventional polymerase chain reaction (PCR), quantitative Real-Time PCR (qrt-PCR) as well as by Gene Chip analysis of the whole transcriptome. Furthermore, the phosphorylation of extracellular signal-regulated kinase (ERK1/2) proteins was analysed in HC and NPC exposed to PB and CPA in vitro. The impact of the secretome of untreated or PB treated NPC on the pro-inflammatory state of HC was investigated. The same was done with NPC exposed to supernatants derived from untreated or PB treated HC. Changes in mRNA level of pro-inflammatory genes, such as the tumor necrosis factor-alpha (TNFα) or interleukin-6 (IL-6), served as end point.

HC were found to express considerably the nuclear receptors (NR) PXR and PPARα and to lower extent CAR and ERα. Compared to HC, the expression levels of NR in EC and KC were lower or even negligible. In primary culture, HC are able to respond to NGC as indicated by elevated mRNAs of cytochrome P450 enzymes (Cyp1a1, Cyp2b1 and Cyp2c12) while the effect on mesenchymal cells was partially weaker. This was similar with the phosphorylation of ERK1/2 proteins in PB-treated cultured HC. Furthermore, Gene Chip analysis revealed the upregulation of components of the ERK pathway in HC exposed to CPA in vivo. As a consequence, NGC action may involve this signalling pathway.

With regard to epithelial-mesenchymal interactions, the secretome from untreated NPC triggered a pro-inflammatory response in HC which was aggravated when NPC had been exposed to PB in vivo. In contrast, supernatants of untreated HC suppressed transcription levels of pro-inflammatory genes in NPC which became more pronounced when supernatant of PB-exposed HC was applied. The results indicate a profound epithelial-mesenchymal dialogue for the fine tuning of expression levels in liver cell types and that NGC may interfere with these interactions.

In summary, the present work could gain evidence that NGC may promote hepatocarcinogenesis by affecting signalling pathways and altering the pro-inflammatory state in both, mesenchymal and parenchymal liver cells.

7. Zusammenfassung

Das hepatozelluläre Karzinom (HCC), als besonders aggressiver Tumor, führt zu über einer halben Million Todesfälle pro Jahr. Es ist daher von Bedeutung, die potentiellen Risikofaktoren auszuschalten und die Entstehung dieser Krankheit besser zu verstehen. Viele, an Millionen von Menschen weltweit, verschriebene Medikamente zählen zu der Gruppe von nicht gentoxischen Kanzerogenen (NGC), das heißt sie sind nicht gentoxisch, erzeugen aber Tumore in Ratten in Langzeit „Bioassays“. Die Bedeutung dieser Ergebnisse für den humanen Leberkrebs ist nicht eindeutig. Das Ziel dieser Diplomarbeit war die Erforschung der Wirkungsweise von prototypischen NGC (Phenobarbital, PB; Cyproterone Acetat, CPA und Wyeth, WY), um das Risiko für die Gesundheit des Menschen besser einschätzen zu können. Bislang lag der Schwerpunkt der Forschung auf Hepatozyten (HC) als direkte Zielzellen von NGC. Da es Hinweise gibt, dass NGC auch auf nicht parenchymale Zellen der Leber (NPC) wirken, wurden die Experimente in der Weise aufgebaut, dass die Effekte von NGC gesondert für HC und NPC untersucht werden konnten.

Die Versuche basierten darauf, dass die Ratten entweder einmalig oder für einen längeren Zeitraum mit PB oder CPA behandelt wurden. Die Zellen wurden mittels Kollagenase Perfusion aus der Rattenleber isoliert, mittels Percoll Gradienten Zentrifugation in HC und NPC aufgetrennt, auf Kulturplatten ausgesät und mit PB, CPA, oder WY für bis zu 24 Stunden behandelt. Die Analyse von veränderten Genexpressionsmustern erfolgte mit Hilfe der Polymerase-Ketten-Reaktion (PCR), quantitativer Real-Time PCR (qrt-PCR), sowie DNA Chip Analyse des gesamten Transkriptoms. Des Weiteren wurde die Phosphorylierung der „extracellular-signal regulated kinase“ (ERK1/2) Proteine untersucht, die in vitro mit PB oder CPA behandelt worden waren. Die Auswirkung der Zellüberstände von unbehandelten oder PB behandelten NPC auf den pro-entzündlichen Status von HC wurde erforscht. Das Gleiche wurde mit NPC, welche den Zellüberständen von unbehandelten oder PB behandelten HC ausgesetzt wurden, durchgeführt. Die Veränderung der mRNA Level von pro-entzündlichen Genen, wie zum Beispiel des Tumornekrosefaktors (TNFα) oder Interleukin-6 (IL-6), diente als Endpunkt.

Es stellte sich heraus, dass HC hauptsächlich die Nuklearen Rezeptoren (NR) PXR und PPARα und im geringeren Ausmaß CAR und ERα exprimieren. Im Vergleich zu HC waren die Expressionslevel der NR in Endothelzellen und Kupfferzellen geringer oder sogar vernachlässigbar. Es zeigte sich, dass HC in Primärkulturen in der Lage sind auf NGC in Form von erhöhter mRNA der Zytochrom-P450 Isoenzyme (Cyp1a1, Cyp2b1 und Cyp2c12)

zu reagieren, wohingegen der Effekt von NGC auf mesenchymale Zellen teilweise geringer ausfiel. Das Gleiche galt für die Phosphorylierung der ERK1/2 Proteine in PB behandelten HC. Zusätzlich ergab die Gene Chip Analyse von HC, welche aus CPA behandelten Tieren isoliert worden waren, eine Hochregulierung von Genen des ERK Signalweges. Infolgedessen könnte dieser Signalweg bei der Wirkungsweise von NGC eine Rolle spielen.

In Bezug auf die epithelialen-mesenchymalen Wechselwirkungen löste das Sekretom aus unbehandelten NPC eine pro-entzündliche Reaktion in HC aus. Diese wurde verschärft, wenn NPC eingesetzt wurden, die aus PB behandelten Tieren stammten. Im Gegensatz dazu führten die Überstände aus unbehandelten HC zu einer Unterdrückung der Transkriptionslevel von pro-entzündlichen Genen in NPC. Dieser Effekt wurde durch den Einsatz von Überständen aus PB behandelten HC noch verstärkt. Die Ergebnisse deuten auf einen fundierten epithelialen-mesenchymalen Dialog hin, welcher zur Feinabstimmung der Expressionslevel in Leberzelltypen dienen könnte. Es wäre denkbar, dass NGC in diese Interaktionen eingreifen.

Zusammenfassend erlangte diese Arbeit Hinweise darauf, dass NGC möglicherweise die Leberkrebsentstehung durch Beeinflussung von Signalwegen und Veränderungen des pro-entzündlichen Status in mesenchymalen und parenchymalen Leberzellen fördern könnte.

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„Ich habe mich bemüht, sämtliche Inhaber der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.“

9. Appendix

9.1 List of Tables

9.2 List of Figures

9.3 List of abbreviations

APS	Ammonium-persulfate
AR	Androstane receptor
BSA	Bovine serum albumin
CAR	Constitutive androstane receptor
cDNA	Complementary DNA
COX-2	Cyclooxygenase 2
CPA	Cyproterone acetate
CYP	Cytochrome P450 superfamily
DC	Dendritic cells
DEN	Diethylnitrosamine
DEPC	Diethylpyrocarbonate
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EC	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinases
FCS	Fetal calf serum
GC	Genotoxic carcinogen
Gp130	Glycoprotein 130
GTP	Guanine triphosphate
HC	Hepatocytes
HCC	Hepatocellular carcinoma
HGF	hepatocyte growth factor
HRE	Hormone response element
HRP	Horseradish peroxidase
IFN-γ	Interferon γ
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase 2

JAK	Janus kinase
JNK	Jun N-terminal kinase
KC	Kupffer cells
MAPK	Mitogen activated protein kinase
MMLV	Mouse Moloney murine leukemia virus
MR	Mineralcorticoid receptor
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NGC	Non-genotoxic carcinogens
NK	Natural killer
NNM	N-nitrosomorpholine
NPC	Non-parenchymal cells
NR	Nuclear receptor
PAA	Polyacrylamid
PB	Phenobarbital
PBREM	Phenobarbital responsive enhancer modules
PBS	Phosphate Buffer Saline
PCR	Polymerase chain reaction
PGE	Prostaglandin
PGR	Progesterone receptor
PNL	preneoplastic liver lesions
PP	Peroxisome proliferator
PPARα	Peroxisome proliferator activated receptor-alpha
PPRE	Peroxisome proliferator response element
pRb	Retinoblastoma protein
PVC	Polyvinyl chloride
PVDF	Polyvinylidenfluorid
PXR	Pregnane X receptor
RIPA	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse trancriptase polymerase chain reaction
RXR	Retinoid X receptor
SC	Stellate cells
SDS	Dodecylsulfate-Na-Salt
SEM	Standard error of the mean
SFN	Sulforaphane

SPF	Specific Pathogen Free
SPS	Stock Percoll Solution
STAT	Signal transducers and activators of transcription
TBS	Tris buffered saline
TCPOBOP	1,4-Bis[2-(3,5-dichloropyridyloxy)] benzene
TEMED	N,N,N’,N’-Tetramethylendiamine
TGFα	Tumor growth factor-alpha
TLR	Toll like receptor
TNFα	Tumor necrosis factor-alpha
WY	Wyeth-14,643

9.4 Curriculum vitae

M E L A N I E P I C H L B A U E R

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03/2009 – 08/2012	**Masterstudium / Universität Wien** Molekulare Mikrobiologie und Immunbiologie **Schwerpunkt:** Immunbiologie Masterarbeit am Institut für Krebsforschung der Medizinischen Universität Wien mit dem Thema „Mode of action of non-genotoxic carcinogens“ bei Prof. Bettina Grasl-Kraupp.
10/2004 – 02/2009	**Bachelorstudium / Universität für Bodenkultur Wien** Lebensmittel- und Biotechnologie **Schwerpunkt:** Biotechnologie Bachelorarbeit am Institut für Pflanzenbiotechnologie mit dem Thema „Approaches to fight avian influenza – Development of an edible plant-based vaccine“ bei Prof. Margit Laimer da Câmara Machado.
09/1999 – 06/2004	**Handelsakademie / Waidhofen/Ybbs** Abschluss mit Matura **Schwerpunkt**: Wirtschaftsinformatik

B E R U F S E R F A H R U N G

03/2012 – aktuelle Position	**Quintiles Austria** Clinical Trial Assistant (CTA)
11/2010 – 11/2011	**Institut für Krebsforschung / Medizinische Universität Wien** Masterarbeit
09/2008 – 03/2009	**SHT Haustechnik AG, Wien** Kaufmännische Angestellte in der Rechnungskontrolle
07/2006 – 09/2006	**BIUTEC Forschungs- und Entwicklungsgesellschaft m.b.H.** Praktikum in der Umweltanalytik
07/2002 – 06/2005	**Café/Gasthaus Lohnecker, Ertl** Kellnerin

M E T H O D E N

Immunbiologie/Zellkultur	**Institut für Krebsforschung** RT-PCR, qRT-PCR, Agarosegelelektrophorese, RNA-Isolierung, Bioanalyzer Western Blot, ELISA, Floureszenzmikroskopie Virustransfektion mit Adenoviren Isolierung und Präparation von primären Leberzellen aus der Ratte Kultur und Behandlung von Zelllinien und primären Zellen der Leber
Molekularbiologie	**Molekularbiologie der Cyanobakterien** DNA Präparation, Elektroporation, Radioaktivitätsmessung **Molekularbiologische Übungen** Yeast-two-Hybrid, Klonieren, DNA Mini/Midi-Präparation
Angewandte Mikrobiologie	**Fermentation:** Umgang und Kontrolle einer Fermentationsanlage (Batch, Fed-Batch) **Gentechnik:** Klonieren, Elektroporation, Plasmid Präparation **Monitoring:** Luftkeimanalyse, Oberflächen Monitoring, Wasseranalyse

S P R A C H E N , E D V - U N D S O N S T I G E K E N N T N I S S E

Deutsch	**Muttersprache**
Englisch	**Verhandlungssicher** Weiterbildungsseminar „Scientific English“
Französisch	**Grundkenntnisse**
EDV	**MS Office, GraphPad Prism, Image Quant**
Führerschein	**Klasse B**

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