ANALYSIS OF THE ANTI-FIBROTIC EFFECTS OF HOP BITTER ACIDS ON HEPATIC STELLATE CELLS AND THEIR ANTI-TUMORIGENIC EFFECTS ON HEPATOCELLULAR CARCINOMA CELLS

DISSERTATION

ZUR ERLANGUNG DES DOKTORGRADES DER NATURWISSENSCHAFTEN (DR. RER. NAT.) DER FAKULTÄT CHEMIE UND PHARMAZIE DER UNIVERSITÄT REGENSBURG

vorgelegt von

Michael Sebastian Saugspier

aus Deggendorf

im Jahr 2012

Promotionsgesuch eingereicht: Juli 2012

Die Arbeit wurde angeleitet von: Herrn Prof. Dr. Claus Hellerbrand

Prüfungsausschuss:

Vorsitzender: Herr Prof. Dr. Gerhard Franz
1. Gutachter (1. Prüfer):   Herr Prof. Dr. Jörg Heilmann
2. Gutachter (2. Prüfer):   Herr Prof. Dr. Claus Hellerbrand
3. Prüfer: Frau Prof. Dr. Charlotte Wagner

Für meine Eltern

Table of Contents

1 Summary

Bitter acids (BA) from the hop plant *Humulus lupulus* L. exhibit multiple beneficial biological properties. However, no studies are available regarding the effects of hop bitter acids on liver cells or chronic liver diseases, respectively.

Chronic liver diseases are accompanied by chronic hepatic inflammation which can lead to liver fibrosis. If left untreated, liver fibrosis can progress to cirrhosis, which can cause liver failure. Importantly, cirrhosis is also the main risk factor for the development of hepatocellular carcinoma (HCC). Both cirrhosis and HCC are associated with high morbidity and mortality, and up until now, no effective treatment options are available.

The aim of this thesis was to assess the effects of BA on hepatic stellate cells, the central mediators of liver fibrosis, and on hepatocellular carcinoma cells.

First, the effects of hop bitter acids on hepatic stellate cells (HSC) were analyzed. HSC were isolated from murine and human liver tissues and were incubated with a standardized hop bitter acid extract. At a concentration of 25 µg/ml hop bitter acids (BA) started to induce lactate dehydrogenase leakage. Already at lower concentrations BA led to a dose dependent inhibition of HSC proliferation and inhibited IκB-α-phosphorylation and nuclear p65 translocation in a dose dependent way. Accordingly, the same doses of BA inhibited the expression of pro-inflammatory and NFκB-regulated genes, but did not affect expression of genes not related to NFκB signaling. In addition to these effects on activated HSC, BA inhibited the *in vitro* activation of non-activated HSC as evidenced by delayed expression of collagen I and α-sma mRNA and protein. Together, these findings indicate that BA inhibit NFκB activation, and herewith, the activation and development of pro-fibrogenic phenotype of HSC *in vitro*.

Next, we analyzed the effects of BA on hepatocellular carcinoma (HCC) cells. Here, we used two different standardized BA extracts enriched for either α-acids

or β-acids to get a first insight whether biological activity vary between these two groups of bitter acids. At a concentration of 25 µg/ml, only the β-acid rich extract started to induce aspartate aminotransferase (AST) release as marker for cell injury, whereas at a dose of 50 µg/ml both extracts led to a significant increase of AST liberation. Already at lower concentrations both extracts dose-dependently inhibited proliferation and migration of HCC cells. Analysis of different signaling pathways revealed an inhibition of ERK1/2 phosphorylation, down-regulation of AP-1 activity and an alleviation of NFκB activity in HCC cells *in vitro*. Hereby, the β-acid rich extract showed more pronounced effects.

Furthermore, the stability of BA in a rodent chow supplemented with an α-acid rich extract was assessed applying high pressure liquid chromatography diode array detection technology. Mimicking feasible storage conditions we observed a very poor recovery, indicative of a strong degradation of BA. This prompted us not to proceed with application of this particular BA supplemented chow in rodent models, since in addition to imprecise BA-concentration also potential ill-defined degradation products would not have allowed a sound interpretation of such studies.

In conclusion, hop bitter acids alleviate the pro-fibrogenic phenotype of hepatic stellate cells and inhibit central signaling pathways, which are known to play important roles in tumor development and progression, in hepatocellular carcinoma cells. These data suggest the potential use of hop bitter acids as functional nutrient or therapeutical agent for both prevention and treatment of chronic liver diseases. Further studies are needed to verify the beneficial effects in experimental models of liver injury *in vivo*. However, analysis of a newly designed BA-supplemented experimental chow for rodents revealed strong degradation processes even under optimal storage conditions. Therefore, special care has to be taken when planning long term BA-application experiments.

2 Introduction

2.1 Botany

The hop plant *Humulus lupulus* L. is a member of the small family Cannabaceae which consists only of two genera *Humulus* and *Cannabis*. The genus *Humulus* comprises three species, *Humulus lupulus* Lineus, *Humulus japonicus* Siebold & Zucc. and *Humulus yunnanensis* Hu. Originally, this genus belonged to the order Urticales but in 2003 it was incorporated to Rosales (Van Cleemput et al., 2009a; Zanoli and Zavatti, 2008).

Humulus lupulus L. is a perennial herb that typically grows in the moderate climatic zones of the Northern and Southern hemispheres. During spring the plant regrows from the primary root with her succulent rhizome generating twining stems which can reach a height of about 10 meters. This climbing property was determinative involved in its naming as the Roman naturalist Plinius described the plant as "the wolf of the willow" ("lupus salictarius") (Van Cleemput et al., 2009a). For him, wild hop grow behaviour was comparable with a wolf who raptures a sheep. The hop plant is dioecious and almost all seen cultivated hop are female individuals of the species *Humulus lupulus* L. because the female inflorescences (strobiles, cones) contain the lupulin powder which has been traditionally used as a preservative and flavouring agent in beer (Milligan et al., 2002). The selection of plants with specific organoleptic properties for a long time caused the existence of hundreds of named cultivars and recognized chemotypes (Zanoli and Zavatti, 2008). Harvesting time is from August till September when the amount of lupulin has reached its maximum level. Cultivation technique and a flowering female hop plant with inflorescences are shown in Figure 2.1.

Figure 2.1   (A) Cultivation of hop in long rows with supporting aids to allow vertical growth. (B) Female plant rich in inflorescences.

The fine yellow secreted resinous powder (lupulin) is synthesized in the lipophilic lupulin glands, a peltate-type of glandular trichomes, which are located on the abaxial surfaces of the cone bracts and leaves with the highest affinity to the basal part of the bracteols as seen in Figure 2.2 A. The lupulin glands consist of four basal cells, four stalk cells and one large glandular head cell with a typical diameter of 100 – 200 µm (Oliveira et al., 2008). They originate from single epidermis cells after multiple cell divisions and lupulin is secreted in the cuticle-enclosed cavity as seen in Figure 2.2 B (Wärtgen, 1990).
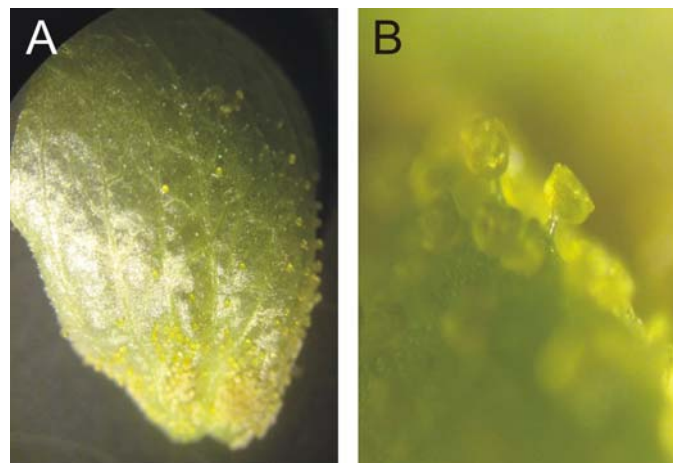
Figure 2.2   (A) A single bract with many trichomes, mainly lupulin glands. (B) Close-up view on lupulin glands.

2.2 Hop Chemistry

2.2.1 Lupulin ingredients

Lupulin is rich in secondary metabolites which can be mainly classified as bitter acids, volatile oil, and polyphenols as listed in table 2.1.

Table 2.1: Average Composition of Air-Dried hop cones

Compound	Percentage (m/m)
α-acids	2 - 17
β-acids	2 - 10
amino acids	0.1
ash/salts	10
cellulose-lignin	40 - 50
monosaccharides	2
oils and fatty acids	1 - 5
pectins	2
polyphenols and tannins	3 - 6
proteins	15
volatile oil	0.5 - 3 (v/m)
water	8 - 12

From Cleemput *et al.* (Van Cleemput et al., 2009a).

The volatile oil is the principal aroma component in hop (Wang et al., 2008). It is composed of a complex mixture of components, mainly terpenoids such as β-myrcene (monoterpene), β-caryophyllene, farnesene and humulene (sesquiterpenes). The terpenoids account for more than 90 % of the total hop oil (Van Cleemput et al., 2009a; Zanoli and Zavatti, 2008). Lupulin-associated polyphenols are rich in prenylated chalcones with xanthohumol (XN) as main constituent (0.1 – 1 % of dry weight) (Zanoli and Zavatti, 2008). Under thermal isomerization XN converts to the prenylflavanone isoxanthohumol which is the main flavonoid in beer. Another encountered prenylchalcone in beer is desmethylxanthohumol, which is the precursor of 8-prenylnaringenin, the most potent phytoestrogen known to date (Milligan et al., 2002). Hop bitter acids are the

quantitatively dominating secondary metabolite in the lupulin glands (about 30 %) and as object of this work will be now explained in more detail.

2.2.2 Hop bitter acids

The use of hop cones in beer brewing has a long history since it is known that hop has preservative and flavouring properties. Most of these properties are attributed to the hop bitter acids (BA).

BA consist of two related series, the α-acids or *humulones* and β-acids or *lupulones*, which are both characterized as prenylated acylphloroglucinols (Van Cleemput et al., 2009a). The two series comprise five analogues each, depending on the nature of the acyl side chain at position C6 as shown in Figure 2.3. The side chains are derived from the hydrophobic amino acids, leucine, valine and isoleucine (Keukeleire D.D., 2000). The analogues are termed humulone / lupulone, cohumulone / colupulone, adhumulone / adlupulone, prehumulone / prelupulone, posthumulone / postlupulone, respectively (Van Cleemput et al., 2009a). The chemical structure of humulone was uncovered by Heinrich Wieland 1925, whereas lupulone was uncovered by Wöllmer (1916 and 1925) and Wieland (1925) (Wöllmer, 1916, 1925; Wieland, 1925).

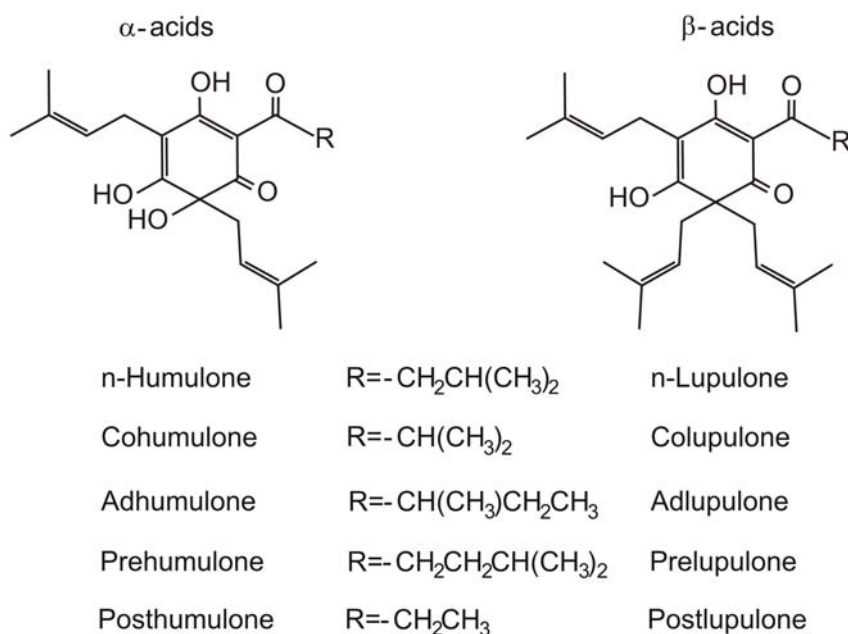|		
n-Humulone	R=-CH₂CH(CH₃)₂	n-Lupulone
Cohumulone	R=-CH(CH₃)₂	Colupulone
Adhumulone	R=-CH(CH₃)CH₂CH₃	Adlupulone
Prehumulone	R=-CH₂CH₂CH(CH₃)₂	Prelupulone
Posthumulone	R=-CH₂CH₃	Postlupulone

Figure 2.3   Basic structures of hop bitter acids and main side chains.

The amount of BA in hop cones strongly depend on strain, cultivation condition and harvesting time, but in general the analogues n-humulone, cohumulone, and adhumulone are the main constituents of hop α-acids, representing 35 – 70 %, 20 – 65 %, and 10 – 15 %, of total levels, respectively (Van Cleemput et al., 2009a). The amount of lupulone and colupulone are similar representing 20 – 55 % and adlupulone with 10 – 15 % of total levels, respectively (Van Cleemput et al., 2009a). Prehumulone / prelupulone and posthumulone / postlupulone are only present in trace quantities.

In beer brewing, BA are added to wort (filtrated sugar solution obtained after enzymatic reaction of the so called mash) and boiled at least for one hour. After removal of BA, the so called "hoppy wort" is fermented using different yeast strains. Importantly, genuine bitter acids can be found only in trace amounts in beer, that are 4mg/L for α-acids and only minor amounts of β-acids (Van Cleemput et al., 2009a). This is based on the fact that BA are extremely sensitive to oxidation as well as their bad solubility in water (wort; pH 5.0 – 5.5). α-acids are the most important compounds responsible for the bitter taste. During wort boiling, α-acids transform to iso-α-acids or isohumulones via an acyloin-type ring contraction (Keukeleire D.D., 2000). Each of the three main α-acids transform into two epimeric iso-α-acid analogues. These iso-α-acids are more water soluble yielding concentrations ranging from 10 up to 100 mg/L in beers without significant differences in their bitterness (Van Cleemput et al., 2009a; Pollock, 1979). Because of their solubility, iso-α-acids account for 80% of total hop derived components in beer. They are responsible for the bitter flavour and due to their tensioactive properties, are also important in foam stability. Therefore, transformation of α-acids to iso-α-acids is the most important reaction in hop chemistry (Pollock, 1979). Otherwise, under exposure of light iso-α-acids decompose, generating three major derivatives called dihydro-, tetrahydro- and hexahydro-iso-α-acids, respectively. This decomposition generates skunky thiols as byproducts which are responsible for the "lightstruck" flavour of beer. Therefore, beer is generally stored in lightproofed bottles (Van Cleemput et al., 2009a). β-acids are more sensitive to oxidation, transforming in the very stable hulupones which are very bitter. However due to their low quantity they play only a minor role in beer quality (Van Cleemput et al., 2009a).

Because of the importance of iso-α-acids for the bitterness of beer and because of the high susceptibility to oxidative degradation of BA in general, different isolation techniques were developed over the last decades. One effective isolation technique of BA is their separation from hop cones by extraction with supercritical carbon dioxide under high pressure. The obtained extracts contain high levels of α-acids and β-acids without the more polar tannins, hard resins, and salts (Van Cleemput et al., 2009a). Further characterization of the extract using high pressure liquid chromatography (HPLC) allows a specific determination of the composition of BA, making them an attractive instrument for controlling organoleptic features in beer brewing.

2.3 Biological effects of hop bitter acids

2.3.1 Anti-bacterial effects

Beer is a beverage with high microbiological stability and only a limited number of species of microorganisms have been reported to spoil beer (Suzuki et al., 2006). This bacteriostatic activity is mainly attributed to BA. First evidences for the anti-bacterial properties of BA were provided in the middle of the 20th century. BA are able to inhibit growth of gram positive bacteria including species of *Bacillus*, *Lactobacillus*, *Micrococcus*, *Staphylococcus* and *Streptococcus*, whereas gram-negative bacteria are either resistant or only affected at very high concentrations (Van Cleemput et al., 2009a; Shimwell, 1937; Teuber and Schmalreck, 1973). In general, β-acids have higher bacteriostatic activity then α-acids (Lewis et al., 1949). A work by Teuber further demonstrated that the two epimeric iso-humulone analogues exhibit less potency against gram-positive bacteria than lupulone and humulone, respectively (Teuber, 1970). Another study by Teuber and Shamwell highlighted that the plasma membrane of susceptible bacteria is the primary target of BA and that the incorporation potency of BA in the cell membrane correlates with their hydrophobicity (Teuber and Schmalreck, 1973). Importantly, Simpson and Smith realized that the pH value is an important determinant of the anti-bacterial activity of BA and that previous studies did not consider this factor. They identified isohumulone to be the most potent inhibitor of bacterial growth at a pH value between 3.8 and 4.4 which is typical for beer (Simpson and Smith, 1992).

BA act as mobile carrier ionophores for undissociated molecules to enter the cell. As BA are weak acids they are only able to bind these molecules in their un-ionized form which in turn is pH depended (Van Cleemput et al., 2009a). Further studies by Chin and colleagues demonstrated the inhibitory effect of lupulone on *Mycobacterium tuberculosis in vitro* and *in vivo* (Chin et al., 1949b; a). Interestingly, a study by Farber and colleagues confirmed the therapeutical effect of lupulone in ten patients suffering from tuberculosis (Farber et al., 1950). However, the number of cases was very small.

It has to be noted that BA also have anti-fungal properties but yeast is not affected by BA which highlight the utility of BA as preserving agent in beer (Mizobuchi and Sato; Michener et al., 1948; Srinivasan et al., 2004).

2.3.2 Anti-inflammatory effects

One of the first experiments identifying the anti-inflammatory potency of hop was done by Yasukawa and co-workers in 1993 (Yasukawa et al., 2006). They screened about 100 edible plant extracts for their anti-inflammatory capability in 12-O-tetradecanoylphorbol-13-acetate (TPA) induced ear oedema in mice. Interestingly, hop showed to be the most potent inhibitor of TPA induced inflammation. A repetition of the experiment with purified humulone revealed an ID_{50} value of 0.2 mg/ear which was similar to the inhibitory potency of indomethacin (ID_{50} 0.3 mg/ear).

A key component in inflammation is the increase of prostanoids, including prostaglandin E2 (PGE2) which results from the transformation of arachidonic acid. In general, two isozymes of prostaglandin forming cyclooxygenases are known, the constitutively expressed cyclooxygenase 1 (COX-1) and the inducible cyclooxygenase 2 (COX-2) (Yamamoto et al., 2000). The induction of COX-2 is triggered by pro-inflammatory mediators and mitogenic stimuli including cytokines, endotoxins, growth factors, oncogenes and phorbol esters (Surh et al., 2001; Hall et al., 2008). Therefore, COX-2 is considered to function primarily as mediator of inflammation and pain in inflamed tissues whereas the housekeeping enzyme COX-1 is thought to be involved in homeostatic function such as cytoprotection of the gastric mucosa or regulation of renal blood flow and platelet activation (Hall et al., 2008).

Several studies with individual hop acids demonstrate their anti-inflammatory potency, by selectively inhibiting COX-2 up-regulation upon pro-inflammatory stimuli (Van Cleemput et al., 2009a). Humulone dose-dependently suppressed tumor necrosis factor α (TNFα) induced PGE2 release in murine osteoblastic MC3T3-E1 cells (IC$_{50}$ 30 nM) (Yamamoto et al., 2000). The authors further demonstrated that humulone dose-dependently suppressed TNFα induced COX-2 mRNA expression and protein activity albeit the IC$_{50}$ level for the catalytic activity was about two orders magnitude higher than the IC$_{50}$ for transcription. Thus, humulone seems to modulate COX-2 at the transcriptional level, and further analysis revealed that nuclear factor kappa B (NFκB) and NF-IL6 are involved. However, the observed effects were independently of glucocorticoid receptors. Importantly, humulone did not affect COX-1 activity which indicates its low gastrointestinal toxicity, a problem of most nonsteroidal anti-inflammatory drugs (NSAIDs) (Van Cleemput et al., 2009a). Hougee and co-workers showed that a standardized hop extract (18.1 % α-acids containing 71.8 % humulone; 2.3 % β-acids) was able to inhibit PGE2 production in lipopolysaccharide (LPS) stimulated human peripheral blood mononuclear cells without affecting the metabolic activity as marker for cytotoxicity (IC$_{50}$ 3.6 µg/ml) (Hougee et al., 2006). They further demonstrated that the hop extract selectively inhibited COX-2 but not COX-1 activity using specific COX-1 (SC-560) and COX-2 (celecoxib) inhibitors in whole blood assays (WBA). Interestingly, orally administered hop extract failed to diminish joint swelling in a zymosan induced acute arthritis model in mice. However, *ex vivo* blood cells from hop treated mice stimulated with LPS showed 24 % lower PGE2 levels compared to control blood cells. In another study, an isomerized α-acid extract (IHE) and pure isohumulone were applied to murine macrophage RAW 264.7 cells, which were stimulated with LPS and interferon γ (INFγ). Both hop products dose-dependently inhibited the LPS and INFγ induced PGE2 production (Nozawa et al., 2005). Further, feeding rats with IHE (0.01 % and 0.04 %) for one week after treatment with azoxymethane (AOM four injections within four weeks) significantly reduced PGE2 level in colonic mucosa. Similar observations with *rho*-iso-α−acids (RIAA), a manmade defined mixture of reduced isohumulones using sodium borohydride, where obtained by Hall and colleagues. They demonstrated a dose-dependent suppression of LPS induced PGE2 release and COX-2 expression in RAW 264.7 macrophages without toxicity effects on a

gastric mucosal cell line (Hall et al., 2008). Another study analyzed if tetrahydro-iso-α-acids (called META060) targets pre-built COX-2 protein in LPS triggered RAW 264.7 cells. META060 failed to abrogate pre-built COX-2 induced PGE2 levels (Desai et al., 2009), which confirmed the assumption that BA inhibit COX-2 at the site of transcription.

Several studies identified the underlying molecular mechanism of the anti-inflammatory potency of individual hop extracts. Using the phorbol ester mouse skin model, Lee and colleagues identified NFκB and activator protein-1 (AP-1) pathways as direct targets of humulone mediated COX-2 suppression in TPA treated mouse skin (Lee et al., 2007).

NFκB functions as a major transcription factor in inflammatory responses and its activation is often detected in cancer (Vallabhapurapu and Karin, 2009). In its inactive form, NFκB consists of a heterotrimer composed of two members of the RelA family (mainly p50 and p65) which are bound to members of the IκB family (mainly IκBα) which function as inhibitors. This heterotrimer is located in the cytoplasm. Pro-inflammatory stimuli lead to phosphorylation of IκBα by protein kinase complex IKK and in turn to its ubiquitination and rapid degradation. The active heterodimer translocate into the nucleus and activate NFκB related genes (Vallabhapurapu and Karin, 2009). Lee and co-workers identified IKK as direct target of humulone with greater extend on IKKβ than IKKα acitivity. Furthermore, humulone exhibited modulatory effects on several kinases. The mitogen-activated protein kinases (MAPK) ERK, p38 and JNK were blunted by humulone (Lee et al., 2007). Desai and colleagues demonstrated a direct functional effect of META060 on LPS induced NFκB binding properties in reporter gene assays. Furthermore META060 inhibited members of the family of Tec tyrosine kinases, particularly BTK (IC_{50} 41 µg/ml), SyK (IC_{50} 60 µg/ml) and BMX (IC_{50} 87 µg/ml) as evidenced in enzyme free cell assays. Members of this family are important for effective LPS triggering of macrophages (Desai et al., 2009). Van Cleemput and colleagues compared α-acids, β-acids and iso-α-acids for their anti-inflammatory potency on L929sA fibroblasts (Van Cleemput et al., 2009b). BA were able to alleviate TNF induced cytokine interleukin-6 (IL-6) mRNA and protein as well as pro-inflammatory chemokine RANTES transcription. The suppression efficiency of α- and β-acids (1-5 µM) was more potent compared to iso-α-acids (50-100 µM).

Further, BA dose-dependently repressed TNF-induced promoter activities of the NFκB-dependent genes IL8 and E-Selectin compared to a synthetic triple κB repeat (α-acids, IC$_{50}$ 0.78 µM; β-acids, IC$_{50}$ 0.70 µM; iso-α-acids IC$_{50}$ 37.04 µM). Many cytokines including IL-6 have binding sites in their promoter region for the pro-inflammatory transcription factors AP-1 and CREB (Van Cleemput et al., 2009b). Reporter gene assays revealed a dose-dependent inhibition of both transcription factors by BA. Intraperitoneal injection of α-acids and iso-α-acids (250 µg each) in zymosan induced inflammation of the footpad markedly alleviated the swelling which persists up to 24 h. In another approach lupulone was tested in a model of colon carcinogenesis. The formation of preneoplastic lesions was initiated by AOM. Orally administered lupulone was able to reduce the amount of lesions. Further analysis of inflammatory cytokines IL-1β and TNFα revealed a reduced expression after lupulone administration (Bousserouel et al., 2011).

2.3.3 Anti-cancer effects

Several natural compounds including hop bitter acids have gained considerable attention as chemopreventive agents. Chemoprevention is recognized as pharmacological intervention to prevent, inhibit or reverse carcinogenesis or to prevent the development of invasive cancer using synthetic or natural agents (She et al., 2003). The first hint for chemopreventive properties of BA was found by Yasukawa and co-workers in the year 1993 since inhibitors of TPA-induced inflammation such as humulone, seem to be roughly in parallel with their inhibitory activities on tumor promotion (Yasukawa et al., 2006).

One strategy in cancer therapy is the specific induction of apoptosis in cancer cells (Thompson, 1995). Apoptosis or programmed cell death is a mechanism that is highly conserved from lower eukaryotes to mammals. It involves the activation of caspases (cystein proteases) which cleave a variety of cellular substrates that contribute to biochemical changes and eventual cell destruction (Chen and Lin, 2004). At least two pathways of caspase activation are known. The extrinsic pathway involves death receptors (Fas, TNFR, DR3, DR4 and DR5) and the intrinsic pathway involves disruption of the mitochondrial membranes driven by Bcl-2 family proteins. Tobe and colleagues demonstrated that humulone (1-100 µg/ml) time- and dose-dependently induced DNA fragmentation in the human

leukemia cell line HL-60. The authors therefore suggested that humulone has apoptosis-inducing activity in this cancer cell line. Further studies tried to unravel underlying molecular mechanisms. Chen and Lin used a defined hop extract containing 49.39 % α-acids and 24.94 % β-acids (Chen and Lin, 2004). BA dose-dependently inhibited cell viability of HL-60 cells (IC_{50} 8.67 µg/ml) and to a lesser extent of human histolytic lymphoma U937 cells (IC_{50} 58.87 µg/ml). Further analysis showed that BA induced DNA fragmentation in both cell lines which was in part mediated by disruption of the mitochondrial membrane as demonstrated by mitochondrial membrane potential measurement, mitochondrial cytochrome c release, active caspase-9 protein level and alteration of Bcl-2 family proteins. Furthermore, BA increased the death receptor FAS and its ligand FASL and downstream activation of caspase-8 and Bid. Similar observations were obtained by Lamy and colleagues (Lamy et al., 2007). Lupulone increased mitochondrial membrane permeability by alteration of Bcl-2 family expression. Furthermore, they identified modulation of FAS and FASL as well as TNF-related apoptosis inducing ligand (TRAIL)-R1 (DR4) and -R2 (DR5) receptor proteins in human colon cancer-derived metastatic SW620 cells stimulated with lupulone. Interestingly, lupulone was able to up-regulate DR4 and DR5 in both TRAIL-sensitive (SW480) and TRAIL-resistant (SW620) cells suggesting that p53 plays only a marginal role in the lupulone triggered apoptosis (Lamy et al., 2010). Analysis of different MAPK, namely JNK, ERK and p38 using specific inhibitors revealed that only p38 plays a major role in lupulone triggered apoptosis (Lamy et al., 2011). Oral administration of lupulone (0.001 % and 0.005 %) led to a 30 and 50 % reduction in the formation of preneoplastic lesions induced by AOM in wistar rats respectively, which highlights the potential therapeutic use of lupulone in colon carcinogenesis (Lamy et al., 2007).

Shimamura and co-workers analyzed the effect of humulone on angiogenesis which describes the formation of new capillary blood vessels (Shimamura et al., 2001). Angiogenesis plays a key role in tumor formation. An important angiogenic factor is vascular endothelial growth factor (VEGF) which is often highly expressed in tumor cells. Humulone dose-dependently inhibited angiogenesis in chick chorioallantoic membranes (CAMs) *in vivo* (ED_{50} 1.5 µg/CAM). Furthermore, humulone suppressed the proliferation of Kop2.16 endothelial cells and reduced the production of VEGF in Kop2.16 and Co26 tumor cells *in vitro*, respectively

(Shimamura et al., 2001). Similar observations with lupulone where obtained by Siegel and colleagues (Siegel et al., 2008). They demonstrated that lupulone is able to inhibit basal as well as VEGF and basic fibroblast growth factor (bFGF) induced proliferation of human umbilical vein endothelial cells (HUVEC). Further, a boyden chamber assay revealed that lupulone dose-dependently inhibited the migratory capacity of HUVEC. Moreover, lupulone reduced capillary-like tube formation of HUVEC *in vitro* and in Matrigel plugs implemented in mice *in vivo*, respectively. Honma and co-workers identified humulone as potent inhibitor of human leukemia U937 cell proliferation (IC_{50} 3.4 µM) (Honma et al., 1998). Moreover, they demonstrated that humulone enhanced the differentiation of U937 cells induced by vitamin D3, TPA, all trans retinoic acid and TNFα. These effects were similar in K562, HEL, Ku182, HL-60, THP-1 and ML-1 cells. The inhibition of proliferation and enhancement of differentiation of premalignant cells are expected to reduce cancer development (Van Cleemput et al., 2009a).

2.3.4 Effects on lipid and carbohydrate metabolism

The application of phytochemicals in foods and in isolated form to provide health benefits has gained more and more attention since metabolic diseases accelerate in the last decades. The so-called "metabolic syndrome" contains a cluster of abnormalities, including insulin resistance, glucose intolerance, dyslipidemia, hypertension, hyperinsulinemia, and microalbuminuria which when occur in combination can lead to heart disease and diabetes (Bruce and Hanson, 2010). Peroxisome proliferator-activated receptor family are dietary lipid sensors that regulate fatty acid and carbohydrate metabolism (Yajima et al., 2004). Modulation of PPARs using synthetic ligands such as fibrates for PPAR α and thiazolidinediones for PPAR γ are useful in the treatment of metabolic disorders (Kersten et al., 2000). Yajima and colleagues analyzed the effect of isohumulones on PPAR α and PPAR γ activation *in vitro* and *in vivo* (Yajima et al., 2004). All three main isohumulone analogues activated PPARs as evidenced in co-transfection studies. Further, feeding isohumulone (0.18 % m/m) and isocohumulone (0.18 % m/m) over a period of two weeks prevented the development of hyperglycemia and hyperlipidemia in a mouse model of non-insulin-dependent diabetes (KK-A$^\gamma$). BA were able to lower the plasma triglyceride

and free fatty acid levels compared to control fed mice, respectively. Moreover the nonfasting plasma glucose levels were reduced compared to control mice, respectively. Interestingly administration of BA did not alter body weight gain compared to a 10 % increase using the insulin sensitizer pioglitazone. Quantitative RT-PCR analysis revealed an up-regulation of acyl-CoA oxidase and fatty acid translocase genes in the liver after BA administration. In contrast, the expression of PPAR γ regulated genes coding for adipose differentiation related protein and for lipoprotein lipase were only slightly increased after isohumulone administration. However, isohumulones reduced adipocyte hypertrophy and induced apoptosis of hypertrophic adipocytes in white adipose tissue. In another approach C57BL/6N were fed a high fat diet to induce hyperglycemia and obesity. Short term co-administration of isohumulone and isocohumulone alleviated insulin resistance and glucose intolerance. Miura and colleagues demonstrated that isohumulones alone and in addition with humulone and lupulone raised total HDL-cholesterol plasma levels and reduced plasma triglyceride content after administration of a high fat and cholesterol diet in mice (Miura et al., 2005). Liver contents of cholesterol and triglycerides were markedly reduced after BA administration. Quantitative RT-PCR analysis revealed an up-regulation of several hepatic genes involved in fatty acid oxidation, but no alteration of genes responsible for cholesterol synthesis and excretion. Similar results were obtained by Shimura and co-workers (Shimura et al., 2005). The administration of a standardized isohumulone extract in water in combination with a high fat diet significantly raised total HDL-cholesterol plasma levels and reduced the plasma triglyceride content. Microarray analysis identified Cyp4a14 as the most regulated gene after BA administration. The strong up-regulation of Cyp4a14 together with other members of the Cyp4a family suggests an increase in microsomal ω-oxidation. The fact, that these effects were abolished using PPAR α deficient mice indicates direct modulation of PPAR α through isohumulones. Recently, the influence of tetrahydro-iso-α-acids (called META060) was also examined in high fat diet induced obese and diabetic mice (Everard et al., 2012). META060 reduced high fat diet induced body weight gain and fat mass development (subcutaneous, visceral and epididymal). Further, META060 improved glucose tolerance and protected against insulin resistance and fasting hyperinsulinemia.

2.3.5 Other effects

Tagashira and co-workers analyzed the anti-oxidative activity of humulone, lupulone and two modified bitter acids (Tagashira et al., 1995). Humulone and lupulone showed radical scavenging activity with IC_{50} values of 32 and 25 µM, respectively. Furthermore both compounds inhibited lipid peroxidation activity (humulone IC_{50} 7.9 µM; lupulone IC_{50} 39 µM). Interestingly, BA were more potent than the antioxidant substances α tocopherol and ascorbic acid. Similar observations were achieved in another report. Here, the following order of radical scavenging activity was observed (IC_{50} [mg/ml]): α-acids 0.21; β-acids 0.96; dihydro-iso-α-acids 1.36; tetrahydro-iso-α-acids 1.40 and hexahydro-iso-α-acids 1.78 (Liu et al., 2007). Namikoshi and co-workers demonstrated that a standardized isohumulone extract (isohumulone, isocohumulone, and isoadhumulone at a ratio of 37:48:15) is able to prevent renal tissue damage in rats after high salt diet. Isohumulone decreased renal reactive oxygen species (ROS) leading to the restoration of bioavailable nitric oxygen which in turn is important for salt and water homeostasis in the kidney (Namikoshi et al., 2007).

Tobe and colleagues identified humulone to be a strong inhibitor of bone resorption (IC_{50} 5.9 nM) in a pit formation assay (Tobe et al., 1997). Adhumulone showed a similar inhibitory effect whereas cohumulone showed almost no inhibitory activity. Thus, humulone might be a therapeutic agent for the treatment of osteoporosis. The authors suggested a role of the PI3K pathway. However, the underlying mechanism remains unclear.

2.4 Toxicology

In general, hop compounds are recognized as safe due to their long-time application in brewing and as herbal medicine. Some *in vivo* studies exhibited that only huge amounts of hop are toxic, which was demonstrated in frogs (Stavén-Grönberg, 1927; Steidle, 1931).

Soderberg and Wachtmeister analyzed the effect of repeated intravenous injections of humulone in cats and rabbits (Soderberg and Wachtmeister, 1955). Humulone (1-10 mg/kg) evoked hyperventilation and an increase in body temperature and when body temperature reached a level of 42 °C polypnoea appeared in cats. These observed effects were much weaker in rabbits.

Interestingly, after death through humulone cats rapidly developed an abnormally severe rigor mortis which suggests that humulone affect muscular metabolism (Van Cleemput et al., 2009a).

Nozawa and colleagues observed no signs of toxicity after oral administration of iso-α-acids (0.01-0.05 %) for seven weeks in rats (Nozawa et al., 2005). Further analysis by Yajima and co-workers revealed a significant decrease in serum GPT and GOT levels after iso-α-acids administration in mice (Yajima et al., 2004). Conversely, Miura *et al.* demonstrated no changes of the transaminase levels, albeit the relative liver weight was higher after BA treatment (Miura et al., 2005). The latter effect was also observed by Shimura and colleagues (Shimura et al., 2005).

Rho-iso-α-acids (RIAA) from hops are generally regarded as safe (Konda et al., 2009). The only statistically significant change after oral administration of RIAA (250 mg/kg day) for 21 days to mice was the increase in absolute and relative liver weights. Histological examinations noted only minor changes in liver and spleen (Hall et al., 2008).

Subchronic oral administration of tetrahydro-iso-α-acids and hexahydro-iso-α-acids in dogs revealed that they are generally well tolerated with no-observed-adverse-effect levels (NOAEL) using 50 up to 100 mg/kg body weight (Chappel et al., 1998). No histopathological changes were found but statistically significant increase in absolute and relative liver weight was observed. Further, serum alkaline phosphatase was increased after 13 weeks of BA administration and the authors suggested that it is from hepatic origin. Moreover, negative results were reported in a number of mutagenicitiy and genotoxicity tests with di-, tetra- and hexa-iso-α-acids, respectively.

Only a small number of reports are available at present addressing the safety of hop bitter acids in humans. No toxicity of liver, kidney, bone marrow and myocardium was detectable in humans that ate 5 g lupulone daily for three months (Farber et al., 1950). However, every patient experienced some degree of gastro-intestinal irritation after taking the first dose of the drug (10 times 0.5 g). These irritations included epigastric burning, abdominal cramping and sometimes water diarrhea that occurred from five minutes to six hours after intake. Another report with NG440, a defined mixture of phytochemical substances including RIAA, revealed no changes in blood pressure, amount of whole blood cells and fecal

calprotectin, a marker for gastrointestinal injury (Minich et al., 2007). A pilot trial with META050, a formula containing phytochemical substances and RIAA, also revealed no serious side effects after administration for eight weeks (Lukaczer et al., 2005).

2.5 Effects of hop bitter acids on the liver

The liver is the largest gland of the human body with several important functions. 70 % of the blood supply comes from the gut via the portal vein. This blood is enriched with nutrients and other substances that were orally administered. Therefore, the liver plays a major role in regulation of carbohydrate (glucose degradation and glycogen storage) and lipid homeostasis (enterohepatic bile acid system, lipogenesis and lipid trafficking) in the body. It is further important in storage and detoxification, in synthesis of hormones and coagulation factors, production and decomposition of red blood cells and plasma protein synthesis, just to mention the most important functions.

Parenchymal cells called hepatocytes are the most abundant cell type in the liver. They account for approximately 80 % of total liver cell mass (Kmieć, 2001). Hepatocytes can be polyploid with numerous mitochondria, and perform the majority of liver functions. The remaining 20 % consist of non-parenchymal cells which can be mainly classified in hepatic stellate cells (formerly called Ito cells), Kupffer cells (macrophages), liver sinusoidal endothelial cells (LSEC), liver-resident lymphocytes and cholangiocytes. Hepatocytes build strong cell junctions between themselves and exhibit cell polarity. Their basolateral surface is directed to the sinusoidal blood vessels. These capillaries consist of LSEC which are fenestrated to enable direct contact between hepatocytes and sinusoidal blood. Hepatic stellate cells (HSC) are located in the perisinusoidal space (or space of Disse), the area between hepatocytes and endothelium.

As mentioned in chapter 2.4 different reports noticed changes in relative liver weight and/or serum transaminases after feeding BA. However, there are only a few reports available which examined the influence of BA on the liver in more detail.

Miura and colleagues analyzed the expression of several hepatic genes after administration of hop extracts in combination with high fat in mice (Miura et al.,

2005). Quantitative RT-PCR analysis of genes involved in lipid metabolism revealed an up-regulation of HMG-CoA synthetase, LDL receptor, acyl-CoA oxidase, acyl-CoA synthetase, fatty acid transport protein, and lipoprotein lipase whereas apolipoprotein B and apolipoprotein CIII were decreased. In addition, no alteration of hydroxymethylglutaryl-CoA reductase and Cyp7A1 gene expression was observed. Shimura and co-workers confirmed these findings using microarray analysis. They further identified several hepatic genes, e.g. cytochrome P450 genes like Cyp4A14 and Cyp4A10, to be regulated by BA (Shimura et al., 2005). Hall and co-workers identified the regulation of several cytochrome P450 genes, which are involved in the metabolism of phase I drugs and xenobiotics (Hall et al., 2008). RIAA demonstrated a strong inhibition of Cyp2B9 (IC_{50} 0.30 µg/ml), a moderate inhibition of Cyp2C19 (IC_{50} 6.3 µg/ml) and a weak inhibitory effect on Cyp3A4 (IC_{50} 12-14 µg/ml) while no inhibitory effect was noted at the highest concentration for Cyp1A2 (>100 µg/ml), Cyp2D6 (>100 µg/ml), and Cyp2E1 (>50 µg/ml). Foster and colleagues analyzed the effect of 35 different sorts of beer on cytochrome P450 metabolism using rat microsomes (Foster et al., 2009). In this study most products showed little or moderate inhibitory effects on Cyp2C19, Cyp3A4, Cyp3A5, Cyp3A7 and Cyp3A19. Furthermore, a homogenous moderate-to-strong inhibition of Cyp2C9 was observed by all sorts of beer. Moreover, correlations between BA content and Cyp inhibition demonstrated that β-acids can modulate Cyp3A4, Cyp3A5, Cyp3A7 and Cyp3A19 isozymes. Teotico *et al.* reported that colupulone and a hop extract were able to induce Cyp3A4, Cyp2B6 and MDR-1 gene expression in human hepatocytes *in vitro* (Teotico et al., 2008). Cyp3A4 and Cyp2B6 are important in drug metabolism and MDR-1 plays a role in drug efflux. Interestingly, BA were able to activate active pregnane X receptor (PXR), a direct regulator of Cyp3A4 using reporter gene assays. Moreover, crystal structure x-ray analysis revealed a possible colupulone PXR interaction through which activation may occur.

Up until now, a detailed analysis of hop bitter acids on hepatic stellate cells as key players in liver fibrosis, and data concerning the chemopreventive effects of BA on hepatocellular carcinoma are missing.

2.6 Liver diseases

2.6.1 Definition and development

Liver diseases describe any type of liver impairment that leads to incomplete liver function. They can be divided in acute liver diseases as a consequence of intoxication or acute infections and chronic liver diseases. The latter can be caused by chronic alcohol or drug abuse, genetic alterations, viral infections and by metabolic disorders or by any combinations thereof. Chronic hepatic injury leads to chronic hepatic inflammation which in turn can manifest in liver fibrosis. Liver fibrosis and its progressive stage called cirrhosis, outline the final common pathway of virtually all chronic liver diseases (Iredale, 2007). Importantly, the long-standing damage of hepatocytes and chronic inflammation within the cirrhotic liver give rise to the development of most cases of hepatocellular carcinoma (80 %) (Alison and Lovell, 2005).

2.6.2 Liver fibrosis

Hepatic fibrosis describes an exuberant wound-healing response to chronic liver injury associated by hepatocellular damage, inflammation and continuous tissue remodelling (Bataller and Brenner, 2001). As a consequence excessive deposition and altered composition of extracellular matrix (ECM) proteins (mainly collagen types I, III and IV) in the liver parenchyma takes place, which in turn distorts the normal hepatic architecture leading to fibrous scars (Iredale, 2007). Therefore, advanced hepatic fibrosis is accompanied by portal hypertension within the liver. Hepatic stellate cells (HSC), first described by Kupffer, account for 5 -8 % of total cells in normal liver. HSC are regarded as central mediators of liver fibrosis because they are the major source of ECM proteins. They are located in the perisinusoidal space between the anti-luminal side of endothelial cells and the basolateral surface of hepatocytes with higher frequency in the periportal area than centrilobularly (Mann and Mann, 2009; Atzori et al., 2009). In normal liver, HSC are mainly in a quiescent state containing droplets of vitamin A in their cytoplasm. Quiescent HSC are involved in ECM homeostasis as they express metalloproteinases (MMPs) for ECM degradation and inhibitors of metalloproteinases (TIMPs) for ECM maintenance, respectively (Atzori et al.,

2009). As response to various stimuli during liver injury, quiescent HSC undergo morphological changes associated with a loss of their vitamin A reservoir. This activation process is a hallmark of fibrogenesis (Friedman and Arthur, 1989). HSC transform into highly proliferative myofibroblast like cells (activated HSC) which express α smooth muscle actin (α sma), a histological marker for activated HSC in injured liver (Mann and Mann, 2009). Activated HSC migrate to and accumulate at the side of tissue repair. They overproduce ECM proteins (mainly collagen type I) and TIMPs (mainly TIMP-1) which in turn block matrix degradation by MMPs. Further, activated HSC up-regulate an array of cytokines (e.g. IL-6), chemokines (e.g. MCP-1), and mitogens (e.g. TGF-β and PDGF) which further activate HSC in an autocrine manner. Therefore, treatment approaches aim to reduce or inhibit either the proliferative or fibrogenic responses of HSC which would reduce the deleterious effects of HSC in the progression of hepatic fibrosis (Gäbele et al., 2003).

2.6.3 Liver cancer

Liver cancer comprises all types of histologically distinct cancers within the liver. Most liver cancers are secondarily that means that these metastases arise from primary tumors in other organs. Primary liver cancer (its origin is within the liver) include hepatocellular carcinoma (HCC), intrahepatic bile duct carcinoma (cholangiocarcinoma), hepatoblastoma, bile duct cystadenocarcinoma, haemangiosarcoma and epitheliod haemangioendothelioma (Farazi and DePinho, 2006).

HCC is the most common type of primary liver cancer, representing about 80% of all cases (Farazi and DePinho, 2006). Furthermore, it is the fifth most frequent cancer worldwide and because of the lack of efficient therapies, the cancer with the third highest mortality after lung and colon cancer (Kubicka et al., 2000; Villanueva et al., 2007). HCC affects all segments of the world population, although geographical variations in the incidence occur due to the large heterogeneity of several risk factors within the population (Farazi and DePinho, 2006; Bruix et al., 2004). The highest incidence of HCC is in most of the Asia-Pacific regions as well as in Africa. However and importantly, the incidence is steadily increasing even in Western countries (Bruix et al., 2004). The most

prominent risk factors are hepatotropic viruses with chronic hepatitis B and C viral infections, being the most relevant ones, respectively. Other main risk factors include chronic alcohol abuse, metabolic liver disease, aflatoxins, and virtually all cirrhosis-induced conditions (Farazi and DePinho, 2006). The molecular mechanisms which are responsible for the development and progression of HCC depend on responsible risk factors and are not well known. In general, hepatocarcinogenesis is a complex process which includes genetic and epigenetic changes that occur during initiation, promotion, and progression (Aravalli et al., 2008). On the cellular level, increased expression of several factors which are responsible for cancerous cell survival were elucidated. Aberrant activation of the transcription factor NFκB as well as the MAP kinase/ERK pathway have been linked to initiation and progression of HCC since these pathways are known to protect cells from apoptosis (Arsura and Cavin, 2005; Ito et al., 1998).

2.7 Aim of the thesis

The aim of this thesis was to analyze the effects of hop bitter acids on liver diseases with the focus on fibrosis and hepatocellular carcinoma. First, the effect of BA on hepatic stellate cell activation and their potential to inhibit molecular processes involved in the pathogenesis of hepatic fibrosis caused by activated HSC was examined. Second, the effect of BA on hepatocellular carcinoma (HCC) cells was assessed to unravel possible anti-tumorigenic effects. Finally, we prepared a BA-supplemented chow for oral application in rodents and performed recovery experiments to test the stability of BA in this chow.

3 Materials and Methods

3.1 Chemicals and Reagents

Anisaldehyde	Sigma, Deisenhofen, Germany
β-Mercaptoethanol	Sigma, Deisenhofen, Germany
Ciprobay	Bayer, Leverkusen, Germany
Collagenase type IV	Sigma, Hamburg, Germany
Cyclohexane	Sigma, Hamburg, Germany
Diflucan	Pfizer, Karlsruhe, Germany
DMEM medium high glucose	PAA Laboratories, Pasching, Austria
DMSO	Sigma, Deisenhofen, Germany
DNAse	Qiagen, Hilden, Germany
Ethyl acetate	Sigma, Deisenhofen, Germany
Fetal calf serum (FCS)	PAN-Biotech, Aidenbach, Germany
GIBCO medium	Invitrogen, Carlsbad, USA
Glacial acetic acid	Sigma, Deisenhofen, Germany
Hexane	Sigma, Deisenhofen, Germany
Hohentanner	Hohentanner Brauerei, Hohentann, Germany
Hop bitter acid extracts	NATECO$_2$, Wolnzach, Germany
Hydrochloric acid	Sigma, Deisenhofen, Germany
Milk powder	Carl Roth, Karlsruhe, Germany
Nonidet® P40	Roche Diagnostics, Mannheim, Germany
Paraformaldehyde	Sigma, Deisenhofen, Germany
PBS buffer	PAA, Pasching, Austria
Penicillin	Invitrogen, Karlsruhe, Germany
Petroleum ether	Sigma, Deisenhofen, Germany
Sodium dodecyl sulfate	Roth, Karlsruhe, Germany
Sodium hydroxide	Roth, Karlsruhe, Germany
Streptomycin	Invitrogen, Karlsruhe, Germany
Sulfuric acid	Merck, Darmstadt, Germany
TNF α	R&D, Wiesbaden-Nordenstadt, Germany
Triton X-100	Sigma, Deisenhofen, Germany
Trypan blue solution	Sigma, Deisenhofen, Germany
Trypsin/EDTA	PAA Laboratories, Cölbe, Germany

3.2 Laboratory expendables

Cell culture flasks (various sizes)	Corning, New York, USA
CryoTube vials	Nunc, Roskilde, Denmark
Eppendorf tubes (1.5 and 2.0 ml)	Eppendorf, Hamburg, Germany
Falcon tubes (15- and 50 ml)	Corning, New York, USA
Glassware (various types)	Schott, Mainz, Germany
Multiwell plates (various sizes)	Corning, New York, USA
Neubauer hemocytometer	Marienfeld GmbH, Lauda-Königshofen, Germany
Pipet tips (various sizes)	Eppendorf, Hamburg, Germany
Pipettes (stripettes® various sizes)	Corning, New York, USA
Silica gel plates GF$_{254}$	Merck, Darmstadt, Germany
Strip tubes (0.2 ml)	Peqlab, Erlangen , Germany

3.3 Laboratory instruments

Heating block:

Thermomixer comfort	Eppendorf, Hamburg, Germany

PCR-cycler:

GeneAmp® PCR System 9700	Applied Biosystems, Foster City, USA

Q-PCR-cycler:

TaqMan® Abi Prism 7900 HT	Applied Biosystems, Foster City, USA

Laminar flow:

Biosafety cabinet	Hera Safe, Heraeus, Osterode, Germany

Pipettes:

Gilson (P2, P20, P200, P1000)	Gilson, Bad Camberg, Germany

Pipette controllers:

Accu-jet®	Brand, Wertheim, Germany

Cell incubator:

Binder series CB	Binder, Tuttlingen, Germany

Shaking devices:

KS 260 Basic Orbital Shaker	IKA® Werke, Staufen, Germany

Power Supplies:

Consort E145	Peqlab, Erlangen, Germany
Power Supply-EPS 301	Amersham Biosiences, Munich, Germany

Spectrophotometer:

EMax® Microplate Reader	MWG Biotech, Ebersberg, Germany

SPECTRAFluor Plus	Tecan, Männedorf, Switzerland

Scale:

MC1 Laboratory LC 620 D	Sartorius, Göttingen, Germany

Water bath:

Haake W13/C10	Thermo Fisher Scientific, Karlsruhe, Germany

Centrifuge:

Biofuge fresco	Heraeus, Hanau , Germany
Megafuge 1.0 R	Heraeus, Hanau, Germany

Microscope:

Olympus CKX41	Olympus, Hamburg, Germany
Zeiss Axioskop2 mot plus microscope	Zeiss, Göttingen, Germany

UV/VIS spectrophotometer:

NanoDrop® ND-1000	Peqlab, Erlangen, Germany

Cell freezing machine:

Nicool LM 10 freezing machine	Air Liquide, Düsseldorf, Germany

Rotary evaporator:

Laborota 4004-control	Heidolph, Schwabach, Germany

3.4 Cell culture

3.4.1 Cell culture medium

General cell culture medium   DMEM (high glucose/300 µg/ml L-glutamine)
supplemented with:

10% (v/v)	FCS
400 U/ml	Penicillin
50 µg/ml	Streptomycin

HSC medium   DMEM
supplemented with:

10 % (v/v)	FCS
10 µg/ml	Diflucan
4 µg/ml	Ciprobay

Freezing medium

5 Vol	DMEM
3 Vol	FCS
2 Vol	DMSO

3.4.2 Cultivation of cells

In general, all cell culture work was perfomed within a laminar flow biosafety cabinet (Hera Safe, Osterode, Germany) to avoid contaminations. Cells were cultivated at 37 °C in a humidified atmosphere of 10 % CO_2 in air. The different cell types were cultured with the appropriate cell culture medium as listed in chapter 3.4.1. For cell transfer adherent cells were washed with PBS and detached with trypsin (0.05 %)/EDTA (0.02 %) at 37 °C. The same volume of DMEM containing 10% FCS was used to stop trypsin activity. Subsequently, cell suspension was transferred to a falcon tube and centrifuged at 500 G for 5 min. The obtained cell pellet was re-suspended in fresh culture medium and cell number was determined (see chapter 3.4.3). A cell density thinning factor of 5 to 10 was used when cells were reseeded to new cell culture flasks. Medium change took place every second day and cell transfer was repeated when cells reached about 80 % of confluence. Cell growth and morphology were microscopically monitored using an Olympus CKX41 microscope and ALTRA20 Soft Imaging System (Olympus, Hamburg, Germany). Accrued cell culture waste was autoclaved before disposal.

3.4.3 Determination of cell number and viability

To determine cell number and cell viability, cells were diluted 1:2 with trypan blue solution and transferred to a Neubauer hemocytometer. A blue staining of cells indicated their impaired cell membrane integrity. Intact cells on the other hand appear white and therefore can be distinguished. To determine total cell number, all four quadrants (each contains sixteen smaller squares) of the hemocytometer were counted and cell number was calculated with the following equation:

$$\textbf{\textit{Cell number/ml = Z x DF x 10}}^{4} \div 4$$

    Z = counted cell number in all four quadrants

  DF = dilution factor (factor = 2)

For determination of cell viability the ratio of living cells to total cell number was calculated.

3.4.4 Cryopreservation of cells

For cryopreservation, 1×10^6 cells were transferred into cryotube vials and centrifuged at 500 G for 5 min. The supernatant was discarded, and the obtained cell pellet was re-suspended in 1 ml of freezing medium. Thereafter, cells were gently cooled down by a stepwise reduction of the temperature using a Nicool LM 10 freezing machine and following program:

Level 4:	30 min
Level 8:	30 min
Level 10:	30 min

The frozen cell suspensions were then transferred to a liquid nitrogen storage tank for long-time cryopreservation.

For cell thawing of cryopreserved cells, stocks were quickly defrosted in a water bath adjusted to 37 °C. Afterwards, defrosted cell suspension was mixed with 8 ml of warm DMEM and centrifuged at 300 G for 5 minutes. The obtained cell pellet was re-suspended in 10 ml of warm DMEM and transferred into a T25 cell culture flask. On the next day medium was exchanged.

3.4.5 Human hepatocellular carcinoma cell lines

The hepatocellular carcinoma cell lines HepG2 (ATCC HB-8065), PLC (ATCC CRL-8024), and Hep3B (ATCC HB-8064) were obtained from the American Type Culture Collection (ATCC).

3.4.6 Isolation of primary human hepatic stellate cells

Isolation of primary human hepatic stellate cells was done in cooperation with the Center for Liver Cell Research (Department of Surgery, University of Regensburg, Germany) using a two-step EGTA/collagenase perfusion procedure with little modifications (Amann et al., 2009; Hellerbrand et al., 1996; Thasler et al., 2003). Remnant liver samples were obtained from patients with informed consent after partial hepatectomy. Human tissue samples used for cell isolation were non-

pathological, which was confirmed by macroscopical as well as microscopical analysis. Moreover, all liver resections have been negatively tested for HBV, HCV and HIV infection. In the first step, cell suspension was centrifuged at 80 G (8 min, 4 °C) to remove parenchymal hepatocytes. The supernatant containing the non-parenchymal cells was centrifuged again at 700 G for 7 min (4 °C). The cell pellet was re-suspended in HSC medium and seeded in T25 or T75 flasks. After 45 min flasks were tapped and medium was changed. Liver sinusoidal endothelial cells are not able to live under these conditions and die within the first 24 h. Medium change was done daily in the first week of cultivation. Thereafter, medium was changed every 2-3 days. *In vitro* activation of HSC can be observed within the first two weeks of cultivation on uncoated plastic surfaces. Activated HSC are highly proliferative and were split 1:3 after two weeks in culture. Hereby, HSC but not Kupffer cells detach, which was confirmed in previously done analyses (Mühlbauer et al., 2006)

3.4.7 Isolation of primary murine hepatic stellate cells

Isolation of primary hepatic stellate cells (HSC) was performed with 8 week old female BALB/c mice (Charles River Laboratories, Sulzfeld, Germany) using the two step collagenase method of Seglen with minor modifications (Seglen, 1976). Following buffers were used and always freshly prepared prior to use.

Perfusion buffer: DMEM

0.25 g/l EGTA (pH 7.3)

Digestion buffer: DMEM

0.02 % Collagenase type IV (440 U/mg solid collagenase activity)

2.0 mM $CaCl_2$

First mice were set under deep ketamine/xylazine anesthetization according to the guidelines of the Central Animal Facility (ZTL) of the University of Regensburg (Germany). In general, 0.2 ml Xylazin 2 % (Serumwerk Bernburg AG, Bernburg, Germany) and 0.6 ml Ketamin 10 % (WDT, Garbsen, Germany) were mixed.

Thereafter, 25 g weighty mice were intraperitoneal injected 30 – 40 µl of this mixture. Both anaesthetics were obtained from the ZTL of the University of Regensburg. After correct anesthetization, an abdominal incision was performed beginning 1 – 2 cm away from the hind legs up to the *sternum* as shown in Figure 3.1. Thereafter, two horizontal cuts were set to expose the liver. Special care must be taken not to induce diaphragmatic hernia.

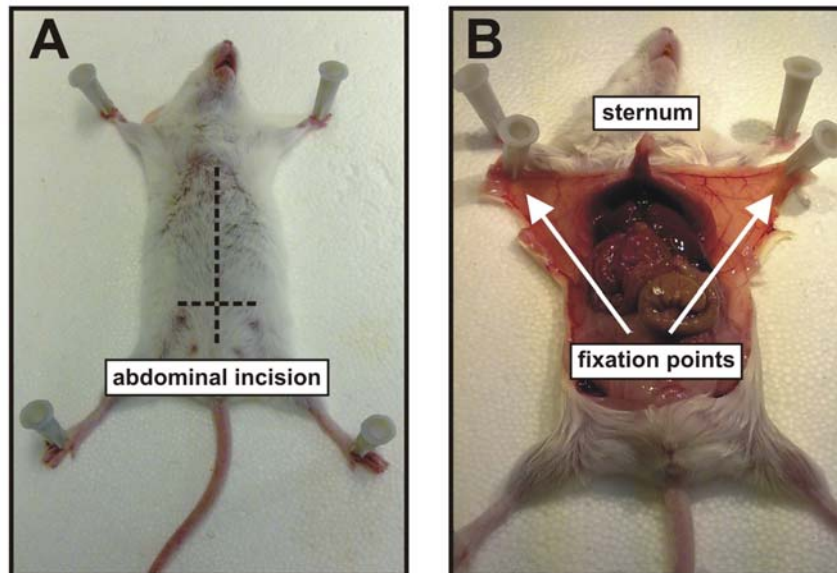**Figure 3.1**   Anesthetized mouse with indicated cutting lines (A) and an open abdomen (B).

Afterwards, the *vena cava inferior* (IVC) and portal vein were carefully ligated loosely with sterile threads as shown in Figure 3.2.
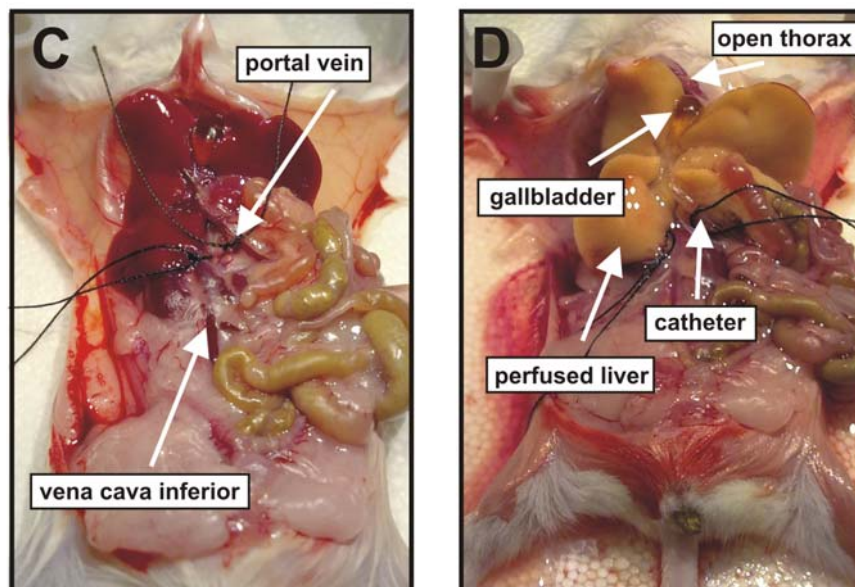

Figure 3.2   (A) open abdomen with prepared ligations of the portal vein and *vena cava inferior*. (B) perfused liver with the catheder inserted in the portal vein.

In the next step, a 22 GA catheter (Optiva 2, Medex Medical, Klein-Winterheim, Germany) was implemented into the portal vein and fixed using the hitherto placed ligature. The catheter was connected to the perfusion system composed of an ISMATEC pump system (IDEX Health & Science GmbH, Wertheim-Mondfeld, Germany), flexible tubings (Novodirect, Kehl/Rhein, Germany) and a water bath adjusted to 37 °C used for buffer as well as exposed tubing warming. The flowrate was set to 4 ml/min. First, the liver was flushed for 5 min with perfusion buffer. Immediately after perfusion start, the IVC was cut behind the ligature to prevent liver from high pressures. To ensure an anterograde perfusion flow, the thorax was opened one minute after perfusion start, heart segmentation was performed and the IVC ligation was tightened. Thereafter, perfusion was implemented with 100 ml of digestion buffer (Figure 3.2 B). Afterwards, liver was separated and the gallbladder was removed. Perfused liver was washed in ice cold PBS, minced in ice cold DMEM and centrifuged at 80 G (8 min, 4 °C) to separate parenchymal cells (hepatocytes). Thereafter, the supernatant containing non-parenchymal cells was centrifuged at 500 G (5 min, 4°C). The pellet was suspended in HSC medium and seeded in T25 flasks. The procedure of further cultivation was the same as described for human HSC (see chapter 3.4.6).

3.5 Hop bitter acid extracts

Hop extracts were provided by NATECO$_2$ GmbH. Two hop bitter acid extracts were used in the experiments. The first one called Herkules, contained 57.2 % (m/m) α-acids (humulone, cohumulone, adhumulone) and 18.3 % (m/m) β-acids (lupulone, colupulone, adhumulone) as determined by HPLC analysis. Therefore, this hop extract can be denoted as α-acid rich. The second extract called Strisselspalter is a β-acid rich extract. HPLC analysis revealed 13.0 % (m/m) α-acids (humulone, cohumulone, adhumulone) and 51.9 % (m/m) β-acids (lupulone, colupulone, adhumulone). For experiments, a stock solution (100 mg/ml) was prepared by dissolving the extracts in DMSO. After a centrifugation step, unsolved compounds were removed and the stock solution was aliquoted and stored at −20 °C. Samples indicated as controls in the particular experiments received DMSO at the same concentration as BA treated samples.

3.6 Enrichment of hop bitter acids

For further enrichment of bitter acids, 10 g hop extract was solved in 100 ml hexane. The obtained solution was transferred to a separating funnel and a quantitative liquid-liquid extraction was done with 100 ml sodium hydroxide (pH 12). The sodium hydroxide solution (containing the deprotonated BA) was acidified with hydrochloric acid (37 %) followed by a quantitative liquid-liquid extraction with petroleum ether as second phase. Subsequently, purified BA as well as the fraction containing remnant lipophilic compounds (not soluble at pH 12) were dried using a rotary evaporator.

Separation success of BA and remnant lipophilic compounds was checked by thin layer chromatography. Anisaldehyde spray reagent was used for visualization of remnant lipophilic compounds (Kritchevsky et al., 1963).

3.7 Preparation of a hop bitter acid-supplemented chow for oral application

The standard chow for mice and rats (Ssniff® R/M-H Cat # V1534-0) contains a balanced mixture of cereals, cereal byproducts, oilseed compounds, minerals, vegetable oils, vitamins and trace elements. In general, the chow is pressed to pellets.

We already used this chow in combination with hop compounds as previously done with the hop chalcone xanthohumol (Dorn et al., 2010, 2012a; b). We provided the chow producing company Ssniff (Soest, Germany) with the BA-extract "Herkules" (see chapter 3.5) to generate a chow with a final concentration of 1.75 % (m/m) of the BA-extract. In general, a typical mouse at 15 weeks of age weighs approximately 25 g and eats approximately 4 g a day. Therefore, the daily BA-intake is approximately 1.6 g/kg/bodyweight α-acids and 0.6 g/kg/bodyweight β-acids, respectively. This concentration was in the range used in previous studies in which BA had been administered to mice or men (Mannering et al., 1992; Farber et al., 1950).

Additionally, we added 5 % (m/m) coconut oil and 1 % (m/m) lecithin to achieve complete homogeneity. Importantly, all transport and producing steps were

performed under cool (4 °C) and dark conditions. Moreover, the generated chow was set under vacuum to avoid oxidation reactions.

3.8 Hop bitter acid-recovery from the hop bitter acid-supplemented chow

BA-recovery experiments (after storage of the chow at 4 °C or -20 °C for two month) were done by NATECO$_2$ GmbH (Wolnzach, Germany) according to the EBC-Proceedings (Maastricht 1997, 223-230). The chow was ground and hop compounds were extracted with a mixture of methanol, ether and hydrochloric acid (12.5:62.5:25). After phase separation, the ether-phase containing the hop compounds was filtered through a 0.2 µm filter and analyzed via high pressure liquid chromatography (250 x 3.0 mm, ProntoSIL 120-C18-AQ, Bischoff, Leonberg, Germany). The elution was carried out with water/H$_3$PO$_4$ (100:0.5) (Phase A) and acetonitrile/H$_3$PO$_4$ (100:0.5) (Phase B) solutions. The following elution concept was used: from 55 % (A) and 45 % (B) to 0 % (A) and 100 % (B), respectively, in a timeframe of 34 min using a flowrate of 0.6ml/min. The whole elution process was recorded using a diode array detection system applying the following wavelengths: 335 nm to detect all α- and β-acids, 270 nm to detect iso-α-acids and reduced iso-α-acids. For absolute quantification different standards (Labor Veritas, Zürich, Swiss/Phytolab, Hamburg, Germany) were used.

3.9 Thin layer chromatography

Thin layer chromatography was used to monitor the enrichment success of hop bitter acids from the extract. Therefore, 1 % (w/v) dichlormethane solutions were made for samples of interest. These standardized solutions were afterwards spotted onto 0.3 mm thick GF254 silica gel plates. A solvent mixture of cyclohexane, ethyl acetate and glacial acetic acid (70:29:1, v/v) was used for separation under saturated conditions. Wavelengths of 256 nm, 330 nm, and daylight were used for visualization, respectively. The retention factor (Rf) of each sample was compared to known Rf values in the literature (Bauer et al., 2009).

3.10 RNA Analysis

3.10.1   RNA Isolation

RNA was used to analyze the state of transcription for genes of interest in cells stimulated with BA. For isolation of total RNA from cells, the RNeasy® mini Kit was used according to manufacturer's instructions (Qiagen, Hilden, Germany). In general, all steps including RNA handling were prepared on ice using sterile plugged pipette tips. To avoid DNA contamination, a genomic DNA digestion step was performed using the RNase-free DNase set (Qiagen, Hilden, Germany). Afterwards, concentration of obtained RNA was determined with the NanoDrop® ND-1000 UV/VIS spectrophotometer (Peqlab, Erlangen, Germany). Ratios of 260/280 and 260/230 were considered to verify purity and isolation success. Isolated RNA was subsequently used for reverse transcription PCR or stored at -80 °C.

3.10.2   Reverse transcription PCR

The reverse transcription PCR (RT-PCR) was used to synthesize stable complementary DNA (cDNA) from RNA. Total RNA was reverse transcribed into cDNA using the reverse transcription system (Promega, Mannheim, Germany). In general, the avian myeloblastosis virus reverse transcriptase enzyme (AMV) and random primers were used. A 25 µl reaction was prepared according to the following setup:

4.9 µl nuclease free water

4.0 µl $MgCl_2$ (25mM)

2.0 µl reverse transcription buffer (10x)

2.0 µl dNTP mixture (10mM)

1.0 µl random primers

0.5 µl recombinant RNasin ® ribonuclease inhibitor

0.6 µl AMV

Afterwards 10 µl of 500 ng RNA/water mixture was added and the reaction was centrifuged briefly. Reverse transcription was performed at 42 °C for 30 min using a PCR cycler. Enzyme inactivation was achieved by a heat step at 95 °C for 5

minutes followed by a chill down to 4 °C. After addition of 75 µl nuclease free water, the generated cDNA was subsequently used for quantitative real time PCR or stored at -20 °C.

3.10.3 Quantitative real time PCR

Quantitative real time PCR (qRT-PCR) was used to quantify specific mRNA expression levels in the cells. It consists of a conventional polymerase chain reaction (PCR) with the addition of a fluorescence dye, e.g. SYBR® Green. Since SYBR® Green intercalates with double-strand DNA, this system offers the possibility to measure the PCR products after each cycle in real time. To quantify the expression level of a gene of interest, the expression of the housekeeper gene 18S was also determined and used for normalization. In general, the ABI Prism® 7900HT (Applied Biosystems, Foster City, USA) was used in combination with the QuantiFast SYBR Green PCR Kit (Qiagen, Hilden, Germany). For expression analysis, oligonucleotides from Sigma-Aldrich (Sigma, Deisenhofen, Germany) were used as listed in table 3.1. Expression of α-smooth muscle actin (α-sma), CCL-5 (RANTES) and keratinocyte growth factor (KGF) was analyzed applying the QuantiTect Primer assays (Qiagen, Hilden, Germany). A 10 µl reaction was prepared according to following setup:

2.5 µl	$H_2O_{dest.}$
0.25 µl	forward primer (20 µM)
0.25 µl	reverse primer (20 µM)
5 µl	QuantiFast® SYBR® Green PCR Master Mix
2 µl	cDNA

Following standard scheme has been used and adapted to the specific melting point temperature of each primer:

Initial denaturation: 95 °C, 900 s

Three step PCR (40 cycles):   95 °C, 15 s

                                    55-65 °C, 20 s

                                    72 °C, 20 s

Analysis of melting curve: 95 °C, 0 s

                                    65 °C, 15 s

                                    95 °C, 0 s

                                    40 °C, 30 s

Amplification products were validated using gel electrophoresis.

Table 3.1   Set of primers used for quantitative RT-PCR analysis.

gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
18S	AAACGGCTACCACATCCAAG	CCTCCAATGGATCCTCGTTA
Col I	CGGGCAGGACTTGGGTA	CGGAATCTGAATGGTCTGACT
MCP-1	TGGGCCTGCTGTTCACA	TCCGATCCAGGTTTTTAATGTA

3.11 Protein Analysis

3.11.1   Isolation of whole cell proteins

For whole cell protein extraction, cell culture medium was removed and cells were washed once with PBS. Thereafter, cells were lysed using an ice-cold cell lysis buffer (Cell signalling technology, Beverly, USA) according to manufacturer's instructions. Afterwards, cells were scraped off with a cell scraper (Corning, NewYork, USA), transferred into 1.5 ml tubes and treated with an ultrasonoscope (Sonoplus hp 70, Bandelin electronics, Berlin, Germany) ten times for three seconds each round. After a centrifugation step at 20,000 G (10 min, 4 °C) the clear supernatant containing the proteins was subsequently used to determine the protein concentration as described in the next chapter. Thereafter, protein concentrations were adjusted, the solutions aliquoted and stored at -80 °C.

3.11.2 Determination of protein concentration

The BCA protein assay kit (Pierce, Rockford, USA) was used for the determination of protein concentrations. In this assay Cu^{2+} is reduced to Cu^{1+} by present proteins under alkaline conditions leading to a blue-colored complex. Thereafter, the cation Cu^{1+} is detected by bicinchoninic acid (BCA) building a water soluble purple-colored chelation-product. In general, 200 µl of alkaline BCA/copper(II) solution (50 parts of solution A mixed with 1 part of solution B) was added to 2 µl of protein solution using a 96-well plate and were incubated for 30 minutes up to one hour at room temperature. A wavelength of 562 nm and a spectrophotometer (EMax® Microplate Reader, MWG Biotech, Ebersberg, Germany) was used to measure the developed purple color. Protein concentration could be determined by doing a parallel quantification of a BSA standard.

3.11.3 Western Blot Analysis

3.11.3.1 SDS PAGE gel electrophoresis

Following buffers and composition of gels were used:

Laemmli buffer	62.5 mM	Tris/HCl; pH 6.8
	2% (w/v)	SDS
	10% (v/v)	Glycerine
	5% (v/v)	β-Mercaptoethanol
Running buffer	25 mM	Tris/HCl; pH 8.5
	200 mM	Glycine
	0.1% (w/v)	SDS

10% Resolving gel	7.9 ml	$H_2O_{dest.}$
	5.0 ml	1.5 M Tris/HCl; pH 8.8
	0.2 ml	10% (w/v) SDS
	6.7 ml	Acrylamide/Bisacrylamide 30%/0.8% (w/v)
	0.2 ml	Ammonium persulfate 10% (w/v)
	0.008 ml	TEMED

5% Stacking gel	2.7 ml	$H_2O_{dest.}$
	0.5 ml	1.0 M Tris/HCl; pH 6.8
	0.04 ml	10% (w/v) SDS
	0.67 ml	Acrylamide/Bisacrylamide (30%/0.8% w/v)
	0.04 ml	Ammonium persulfate 10% (w/v)
	0.004 ml	TEMED

6.7 µl Laemmli buffer was added to 33.3 µl protein solution (standardized amount) and then heated at 95 °C for 5 min. After a short centrifugation step, the denatured proteins were applied on a SDS polyacrylamid gel which consists of a stacking gel and a running gel (detailed composition is listed above). Thereafter, the protein fractionation was separated by size in an electric field at 35 mA/150 V using the XCell SureLock™ Mini-Cell system (Invitrogen, Karlsruhe, Germany). The Full Range Rainbow Molecular Weight Marker (GE Healthcare, Freiburg, Germany) was used as size marker.

3.11.3.2   Protein blot and detection

Following buffer was used for the transfer:

Transfer buffer	10% (v/v)	Methanol
	25 mM	Tris
	190 mM	Glycine

The successfully separated proteins were transferred electrophoretically (220 mA/300 V for 1.5 h) from the gel to a nitrocellulose membrane (Invitrogen, Karlsruhe, Germany) using the XCell II Blot Module (Invitrogen, Karlsruhe, Germany). Afterwards, the membrane was tossed in PBS containing 5% milk powder for at least one hour at room temperature to block unspecific binding sites. Afterwards, the membrane was incubated with a specific primary antibody (listed in table 3.2) over night at 4 °C. The next day, the membrane was washed and then incubated with a secondary horseradish peroxidase (HRP) conjugated antibody (listed in table 3.2) for one hour at room temperature. Unbound antibody was removed by a washing step with PBS and the membrane was incubated with the ECL Plus Western Blotting Detection System solution (GE Healthcare, Freiburg, Germany) for one minute. In this system, HRP catalyzes the conversion of the acridan substrate Lumigen PS-3 to an acridinium ester intermediate which in turn reacts with peroxide under alkaline conditions. In the latter reaction light emits which was detected by autoradiography using a Biomax film (Kodak, Stuttgart, Germany) and a Curix 60 automatic film developer (Agfa, Cologne, Germany). The KS 260 Basic Orbital Shaker (IKA®; Staufen, Germany) was used for the incubation steps.

Table 3.2   Used primary and secondary antibodies for western blot analysis.

Primary antibody	Manufacturer
α−sma	Santa Cruz, Heidelberg, Germany
IκB−α	Cell signalling, Beverly, USA
phospho IκB−α	Cell signalling, Beverly, USA
JNK	Cell signalling, Beverly, USA
phospho-JNK	Cell signalling, Beverly, USA
β-actin	Sigma, Deisenhofen, Germany
Secondary antibody	Manufacturer
anti-mouse HRP	Santa Cruz, Heidelberg, Germany

3.11.4   Immunocytochemistry

The immunofluorescence staining allows the detection of tissue or cell specific proteins, respectively. The primary antibody is specific to the protein of interest. The second antibody which recognizes the first antibody is linked to a fluorophore. For immunofluorescence analysis, cells (5×10^4 cells per well) were seeded in permanox coated 4-well chamber slides (Thermo Fisher Scientific, Karlsruhe, Germany) and cultured overnight. The following day, cells were stimulated with BA for 14 h. For induction of NFκB activity 10 ng/ml TNF (R&D, Wiesbaden-Nordenstadt, Germany) was added for additional 30 min. Subsequently, cells were washed with PBS and fixed using 4 % paraformaldehyd for five minutes. For permeabilization of cell membranes, 0.1 % Triton X-100 (Sigma-Aldrich, Munich, Germany) was added for 15 minutes. After two washing steps with PBS, a blocking solution (PBS and 5 % BSA, w/v) was added for 30 minutes. Thereafter, cells were incubated with a p65 antibody (Santa Cruz Biotechnology, Santa Cruz, USA) overnight at 4 °C. Next day, cells were washed three times with PBS, followed by incubation with the secondary antibody (Invitrogen, Carlsbad, USA) for 1 h. After extensively washing steps, hard set mounting medium including DAPI (Vector Laboratories, Burlingame, USA) was added and images were collected by fluorescence microscopy using a Zeiss Axioskop2 mot plus microscope (Zeiss, Göttingen, Germany).

3.11.5   Quantification of ERK 1/2 and NFκB

Enzyme-linked immunosorbent assay (ELISA) was used to quantifiy phospho-p44/42 and phospho-p65 levels, respectively. Here, we applied the PathScan phospho- sandwich ELISAs (Cell Signalling technology, Beverly, USA) according to manufacturer's instructions. Here, a 96 well plate was coated with the specific capture antibody overnight. Thereafter, cell lysate was added to attach the endogenous p44 and p42 MAP Kinase (Erk1 and Erk2) or phospho-p65 protein to the capture antibodies. After that, captured proteins were linked to a specific detection antibody. Subsequently, this detection antibody was recognized by a horseradish peroxidase (HRP) conjugated antibody. For relative quantification a serial dilution of a strongly positive sample was used. For induction of NFκB

activity, cells were stimulated with conditioned medium from activated hepatic stellate cells.

3.11.6   AP-1 reporter gene assay

In the AP-1 reporter gene assay a plasmid was used which contains a luciferase reporter gene that is controlled by a transcription promoter which consists of seven repeats of the AP-1 element (TGACTAA). For transfection, HCC cells (2×10^5 cells per well) were seeded in six well plates (Corning, New York, USA) and transfected with 0.5 µg pAP-1 luc plasmid (Stratagene, La Jolla, USA) using Lipofectamin Plus reagent (Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions. For normalization of transfection efficiency, cells were co-transfected with 0.2 µg of pRL-TK plasmid resulting in a renilla luciferase acitivity (Promega, Mannheim, Germany). After 24 h, transfection-medium was removed and cells were stimulated with BA for 20 h. Subsequently, cells were lysed and the luciferase activities were measured using a luminometric assay (Promega, Mannheim, Germany).

3.12 Analysis of cell culture supernatants

All analysis were performed at the Department of Clinical Chemistry and Laboratory Medicine (University of Regensburg, Germany) using the Advia 1800 analyzer (Siemens Healthcare Diagnostics, Eschborn, Germany). For determination of cytotoxic effects of BA, cells (4×10^5 cells per well) were seeded in 6 well plates (Corning, New York, USA) and grown overnight followed by stimulation with BA for another 24 h. Thereafter, supernatants were collected and centrifuged at 20,000 G for 5 min to remove detached cells and debris. The amount of lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) in the supernatants were used as marker for cell viability, respectively.

3.13 Functional assays

3.13.1   Proliferation assay (XTT)

To quantify cell proliferation, the colorimetric XTT kit (Roche Diagnostics, Mannheim, Germany) was used. This assay uses the tetrazolium salt XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[phenylamino)carbonyl]-2H-tetrazolium hydroxide) which is reduced to the orange colored formazan by metabolic active cells. Formazan is released into the supernatant. Therefore, dye intensity can be directly read in the supernatant using a spectrophotometer at a wavelength of 450 nm and a reference absorbance wavelength of 650 nm. The intensity directly correlates to the number of metabolically active cells. To analyze the effects of BA on cell proliferation, cells (4 x 10^3 cells per well) were seeded in 96 well plates (Corning, New York, USA) overnight. Thereafter, serum starvation was done for 12 hto achieve cell cycle synchronization. Afterwards, cells were set back to 10 % FCS and stimulated with different concentrations of BA for 24 h and the colorimetric XTT assay was used (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Wavelengths of 450 nm and 650 nm were analyzed using the E max ELISA reader (Molecular Devices, Biberach an der Riss, Germany).

3.13.2   Migration assay

The Cultrex 96 Well Cell Migration Assay (Trevigen, Gaithersburg, USA) was used to quantify the migratory capacity. In general, this assay provides two compartments which are separated by a microporous membrane. Cells are seeded in the upper compartment and are allowed to migrate through the pores forced by chemotactic stimuli located in the lower compartment. After an appropriate cell migration time, migrated cells are carefully detached from the basal side of the membrane and quantified using calcein acetoxymethylester (calcein AM). This Calcein AM is absorbed by the cells, and cleaved to generate free calcein, which in turn can be detected fluorometrically.

For quantification of migratory capacity, cells (0.4 x 10^5 cells/well) were seeded into the upper compartment of the provided 96-well plate. Importantly, only the lower compartment contained 10 % FCS as chemoattractant. After incubation at

37 °C for 8 h cell migration was quantified by fluorimetry using a SPECTRAFluor Plus microplate reader (Tecan, Männedorf, Switzerland).

3.14 Statistical analysis

Values are presented as mean ± SEM. Comparison between groups was made using the Student's unpaired t-test. A p value < 0.05 was considered statistically significant. All calculations were performed using the statistical computer package GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, USA).

4 Results

As described in the introduction hop bitter acids (BA) exhibit multiple beneficial biological properties (see chapter 2.3), however, no data are available at present which describe effects on liver cells or liver diseases, respectively. The aim of this thesis was to address this issue.

In particular, the focus was placed on three aspects:

4.1 Effects of hop bitter acids on hepatic stellate cells as central mediators of liver fibrosis

4.2 Effects of hop bitter acids on the tumorigenicity of hepatocellular carcinoma cells

4.3 BA-recovery experiments from rodent chow supplemented BA-extract

4.1 Effects of hop bitter acids on hepatic stellate cells

4.1.1 Rationale

Liver fibrosis can be regarded as a chronic wound healing process, which in most cases occurs on the ground of chronic liver inflammation; if left untreated liver cirrhosis develops, finally leading to liver failure. Therefore, up until now liver fibrosis represents a major medical problem with significant morbidity and mortality worldwide (Gäbele et al., 2003).

Importantly, current evidence indicates that liver fibrosis is dynamic and can be bidirectional involving phases of progression and regression (Arthur, 2000). Moreover, only advanced fibrosis and cirrhosis are considered to be irreversible (Iredale et al., 1998). Thus, the development of anti-fibrotic drugs is a very attractive strategy to stem against chronic liver diseases.

Here, we analyzed the anti-fibrotic capability of BA (β-rich extract) on hepatic stellate cells as central mediators of liver fibrosis.

4.1.2 *In vitro* activation of HSC

The activation of HSC is accompanied by central pathophysiological changes in the liver and therefore can be regarded as hallmark of liver fibrogenesis (Bataller and Brenner, 2001; Friedman and Arthur, 1989). Our first goal therefore was to analyze the effect of BA on the early onset of activation of murine HSC. *In vitro* activation was achieved by culturing isolated HSC on uncoated plastic surfaces. Two days after isolation, quiescent HSC were exposed to BA at two different doses (2.5 and 5 µg/ml) for three days. Here, and in subsequent experiments, samples indicated as controls received DMSO at the same concentration as used as solvent for BA. No signs of cell toxicity were microscopically observed after incubation with DMSO and BA, respectively (Figure 4.1).
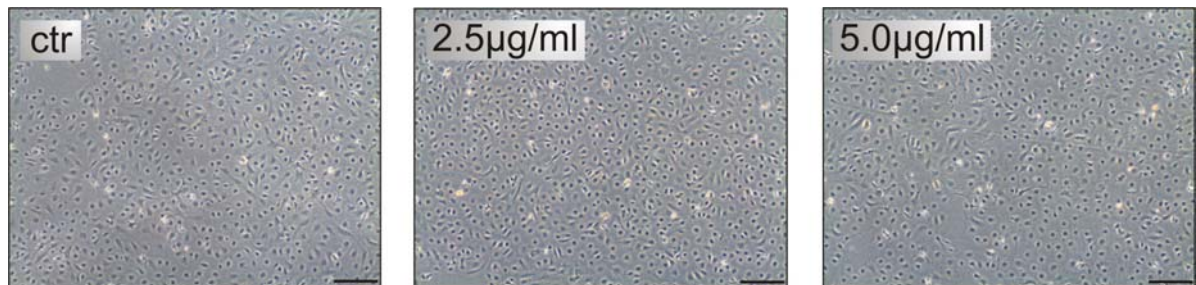

Figure 4.1   Representative phase-contrast images of cultured HSC after 72 h incubation with the indicated concentrations of BA. Bar represents 200 µm in size.

To identify the influence of BA on HSC activation, mRNA expression of two established markers of HSC activation, namely collagen type I (col I) and α-smooth muscle actin (α-sma), was determined by quantitative RT-PCR analysis. Treatment with BA reduced the expression of collagen type I and α-smooth muscle actin compared to control HSC (Figure 4.2).
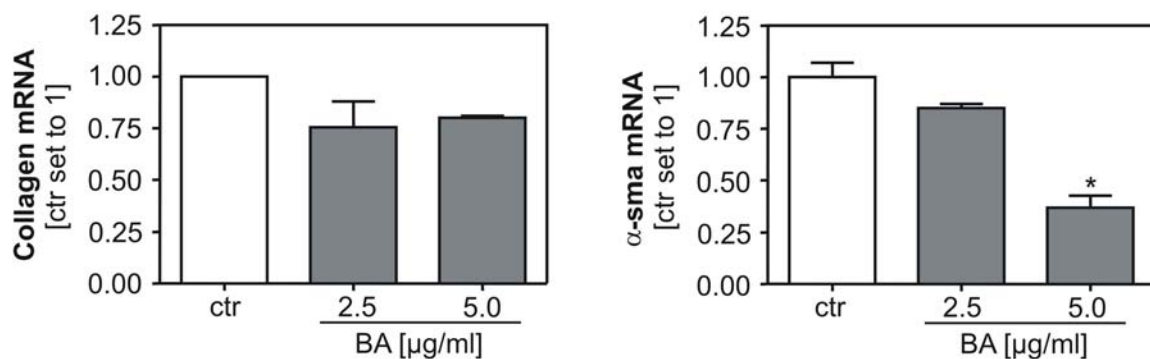

Figure 4.2   Collagen type I and α-sma mRNA expression in HSC after 72 h incubation with indicated concentrations of BA was analyzed by qRT-PCR analysis. *$p < 0.05$ compared to control.

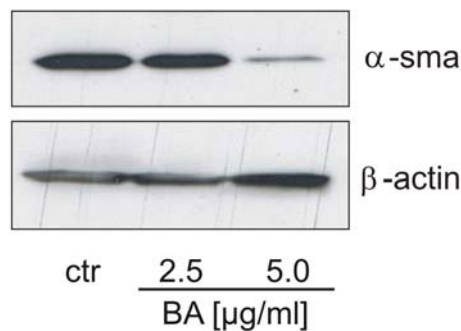Reduction of α-sma expression was also confirmed on the protein level by Western blot analysis (Figure 4.3).

Figure 4.3   Western blot analysis of α-sma expression in HSC cells after 72 h incubation with indicated concentrations of BA.

4.1.3 Proliferation and cell viability

Once they are activated HSC exhibit a high proliferation rate, a mechanism that plays a key role in the progression of liver fibrosis since activated HSC are the main source of ECM deposition (Bataller and Brenner, 2001; Friedman and Arthur, 1989). Thus, we assessed the effect of different BA concentrations on the proliferation of activated human HSC *in vitro*. Incubation with 5, 10 and 25 µg/ml of BA, respectively, led to a dose dependent inhibition of cell growth whereas proliferative activity was completely inhibited at a concentration of 25 µg/ml of BA (Figure 4.4).
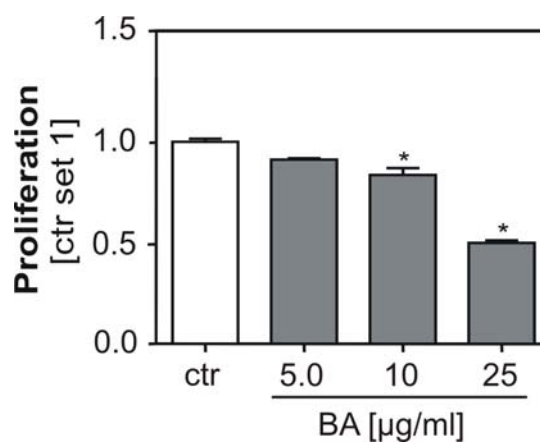

Figure 4.4   Effect of BA on the proliferation of activated HSC after 24 h stimulation. $*p < 0.05$ compared to control.

Microscopical analysis revealed first signs of cell bubbling of activated HSC stimulated with 25 µg/ml of BA, and incubation with 50 µg/ml of BA led to a complete detachment of HSC after 24 h (Figure 4.5).
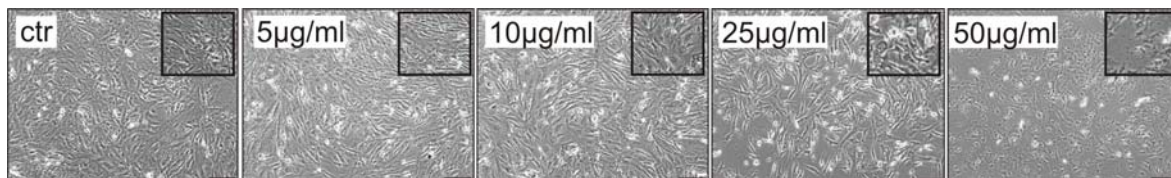

Figure 4.5   Phase-contrast images of activated HSC after 24 h incubation with the indicated concentrations of BA. Bar represents 200 µm in size.

In line with these data, LDH concentration in the supernatant of HSC treated with BA up to a concentration of 10 µg/ml differed only slightly from control cells, while LDH levels were markedly increased upon incubation with BA at a concentration of 25 µg/ml for 24 h (Figure 4.6).
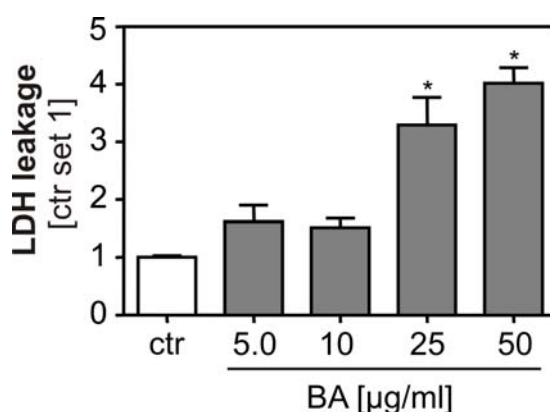

Figure 4.6   Twenty-four hours after stimulation with BA, the amount of lactate dehydrogenase (LDH) in the supernatants of activated HSC was measured as marker for cell membrane integrity. $*p < 0.05$ compared to control.

These analyses have been performed with a supercritical carbon dioxide hop bitter acid extract. To confirm that BA and not remnant hop compounds in the extract account for the observed effects, we separated BA from the extract by liquid-liquid extraction. Figure 4.7 depicts a thin layer chromatogram of the purified BA and the residual lipophilic compounds.
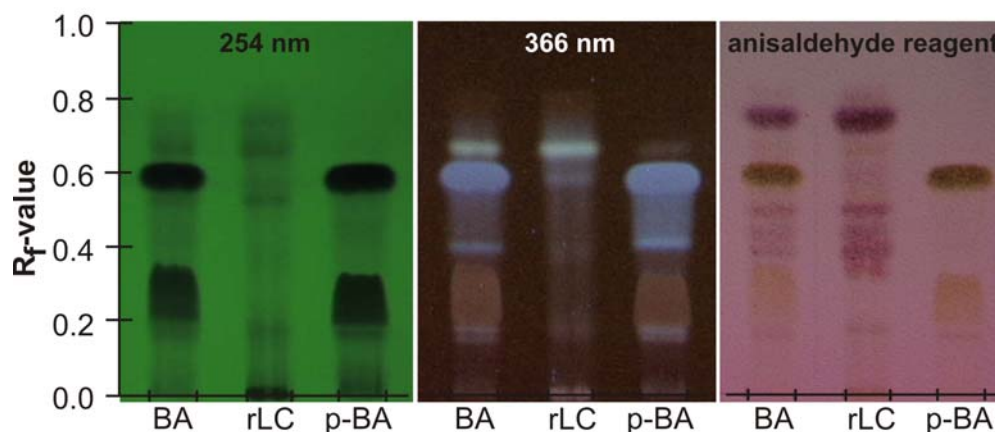
Figure 4.7 Thin layer chromatogram for hop bitter acid extract (BA), remnant lipophilic compounds (rLC) and purified bitter acids (p-BA). At a wavelength of 254 nm separation of main compounds was visualized. A wavelength of 366 nm was chosen for detection of brown fluorescent humulones with a retardation factor (Rf) of 0.3 and the blue fluorescent lupulones with Rf =0.58, respectively. Remnant lipophilic compounds were visualized by detection with anisaldehyde spray reagent (purple).

A subsequent proliferation assay revealed that purified BA have the same anti-proliferative potential as the hop extract, while the residual lipophilic compounds did not affect HSC proliferation (Figure 4.8).
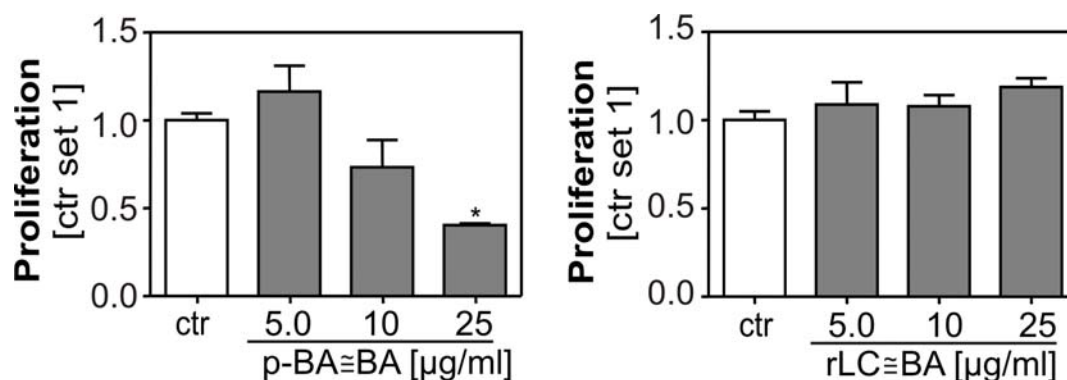

Figure 4.8 Effect of purified BA (p-BA) and remnant lipophilic compound (rLC) on the proliferation of activated HSC after 24 h stimulation. p-BA and rLC were applied in corresponding concentrations as present in stimulation experiments with the crude hop extract. *$p < 0.05$ compared to control.

4.1.4 NFκB activity and pro-inflammatory gene expression

Recent studies identified NFκB as one target of the anti-inflammatory effects of BA (Lee et al., 2007; Van Cleemput et al., 2009b). It has been shown that activation of the transcription factor NFκB plays a critical role in HSC activation (Hellerbrand et al., 1998b; a; Elsharkawy et al., 2005). Moreover, increased hepatic NFκB activity and responsiveness during activation of HSC is known to promote and perpetuate hepatic inflammation (Lee et al., 1995; Hellerbrand et al., 1998a; Lang et al., 2000).

Here, we found that 10 µg/ml BA repressed basal NFκB activity in activated HSC. In contrast, activation of c-Jun N-terminal kinase (JNK) was not impaired, indicating the specificity of the BA-mediated effects on the NFκB pathway (Figure 4.9).

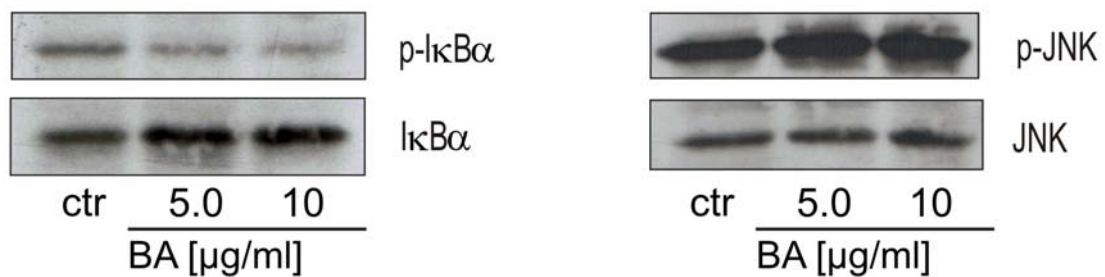**Figure 4.9**   Analysis of phospho-IκB-α and phospho-JNK protein levels in BA treated HSC compared to control cells by Western blotting.

In accordance, immunofluorescence staining revealed that BA inhibit nuclear translocation of p65 in TNF stimulated activated HSC (Figure 4.10).
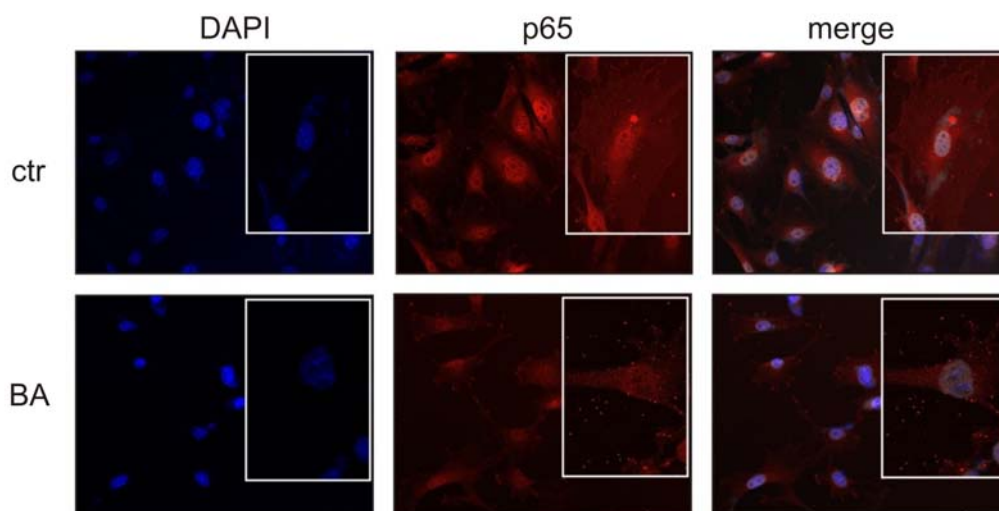

Figure 4.10   Immunofluorescence analysis of TNF induced p65 localization in HSC pre-treated with BA.

In line with these findings, incubation with 10 µg/ml BA reduced gene expression of monocyte chemotactic protein-1 (MCP-1) and CCL-5 (RANTES) in activated HSC. Both pro-inflammatory chemokines are *de novo* expressed during HSC activation and are highly regulated via activation of the transcription factor NFκB in activated HSC (Hellerbrand et al., 1998b). In contrast, the keratinocyte growth factor (KGF) expression was not altered by BA. Also KGF is *de novo* expressed during HSC activation (Steiling et al., 2004) but its expression is not regulated by NFκB (Figure 4.11).
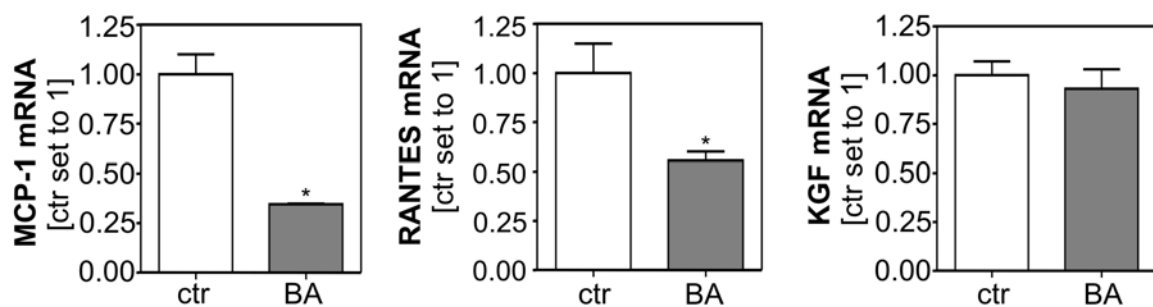

Figure 4.11 Analysis of mRNA levels of MCP-1, CCL-5 and KGF expression in BA treated and control HSC by quantitative RT-PCR. *p<0.05 compared to control.

4.1.5 Summary

BA inhibit the *in vitro* activation process of freshly isolated HSC as evidenced by delayed expression of collagen I and α-sma mRNA and protein. Further, BA led to a dose dependent inhibition of cell proliferation and induced cell death in activated HSC. Already at lower concentrations BA inhibited IκB-α-phosphorylation, nuclear p65 translocation and binding activity in a dose dependent way (up to 10 µg/ml) with no effects on the JNK pathway. Accordingly, the same BA-doses inhibited the expression of pro-inflammatory and NFκB regulated genes as MCP-1 and RANTES, but did not affect expression of genes not related to NFκB signaling. Together, these findings indicate that BA inhibit NFκB activation, and herewith, the activation and development of the pro-fibrogenic phenotype of HSC. Thus, hop bitter acids appear as potential functional nutrient for the prevention or treatment of hepatic fibrosis.

4.2   Effects of hop bitter acids on hepatocellular carcinoma cells

4.2.1   Rationale

As already mentioned in Chapter 2.6.3 HCC has the third highest mortality due to inconspicuous symptoms in early stages as well as insufficient disease-specific diagnosis. Until now, surgical resection or transplantation is still the most promising treatment option of HCC. However, there is a high rate of disease recurrence because most patients are diagnosed at an advanced stage. In addition it has to be considered that the tumor develops in diseased liver with a variable degree of function derangement (Bruix et al., 2004). Thus, new therapeutic treatment options are highly needed. Hop bitter acids have gained considerable attention as chemopreventive agents but no data concerning the anti-tumorigenic effects of BA on HCC are available. Here, we analyzed the effect of BA on the tumorigenicity of HCC.

4.2.2   Cell viability

Our first aim was to define the effective dose range of BA on different human HCC cell lines. Cells were incubated with indicated concentrations of an α-acid rich (αE) and a β-acid rich extract (βE) for 24 h. Microscopic analysis revealed no alterations in BA treated HepG2, Hep3B and PLC cells up to a concentration of 10 µg/ml. Higher concentrations led to first morphological changes, and a concentration of 50 µg/ml caused cell bubbling in all three HCC cell lines (Figure 4.12).
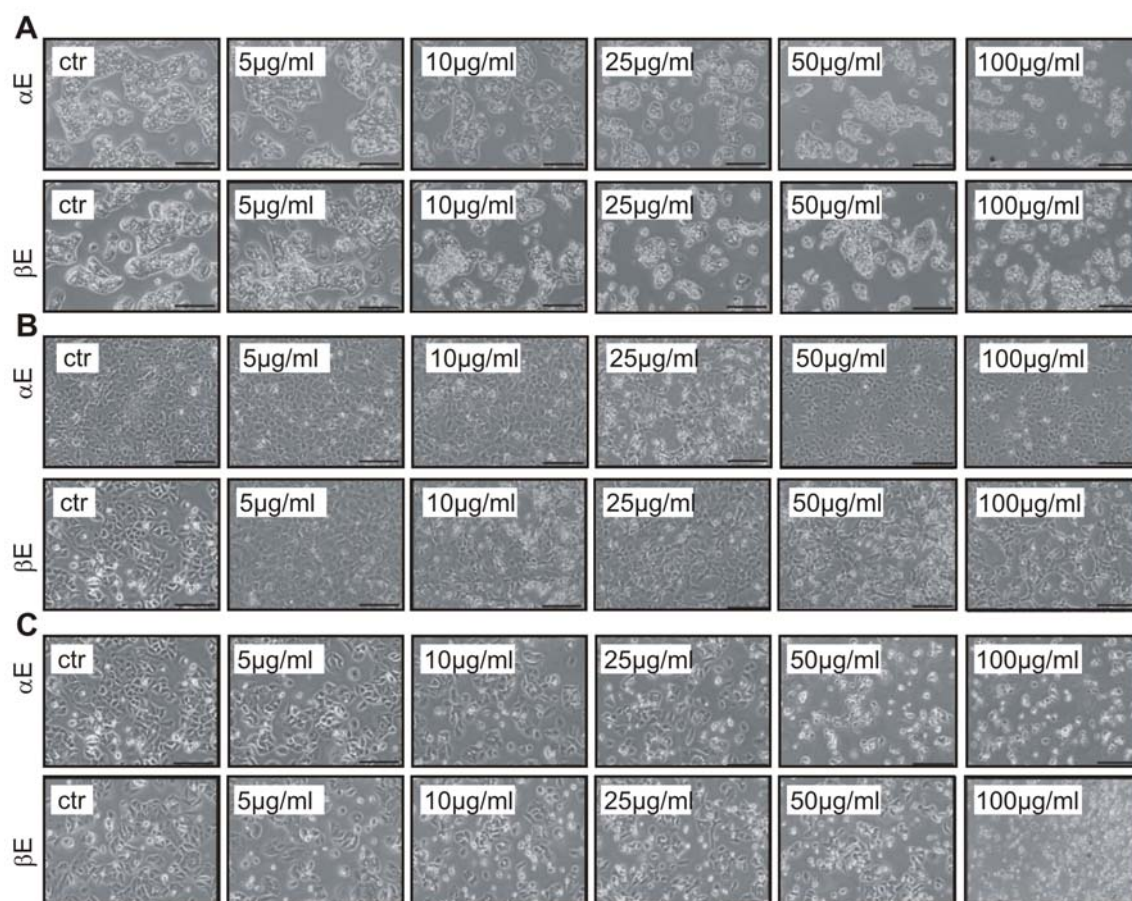
Figure 4.12 Representative phase-contrast images of (A) HepG2, (B) Hep3B and (C) PLC cells after 24 h incubation with the indicated concentrations of an α-acid rich (αE) and a β-acid rich extract (βE), respectively . Bar represent 200 µm in size.

In line with these data, aspartate aminotransferase (AST) concentration in the supernatant of HepG2 cells treated with the two hop extracts did only slightly differ from control cells up to a concentration of 25 µg/ml, while AST levels were markedly increased upon incubation with concentrations of 50 µg/ml indicating cell injury (Figure 4.13). Interestingly, β-extract showed more pronounced effects than α-extract regarding morphological changes and induction of AST release.
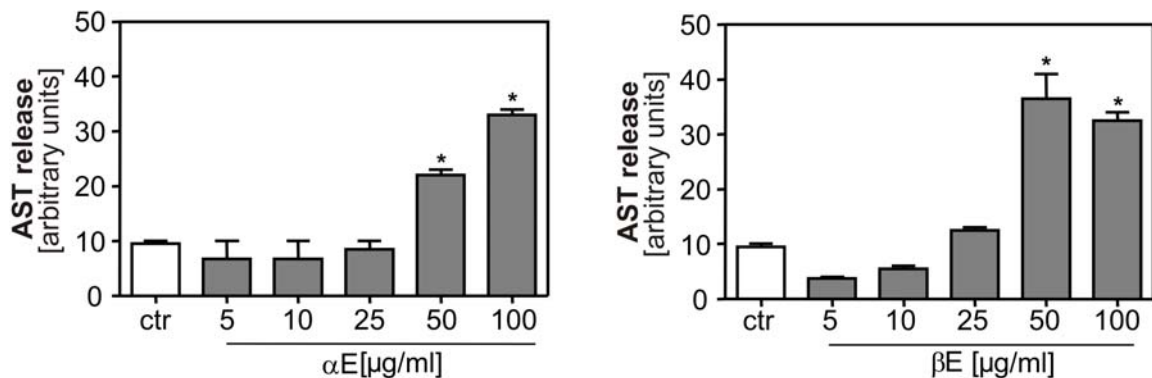
Figure 4.13   After 24 h stimulation, the amount of aspartate aminotransferase (AST) in the supernatants of HepG2 cells treated with (A) an α-acid rich extract (αE) and (B) a β-acid rich extract (βE) was measured as biomarker for cell injury. *p<0.05 compared to control.

4.2.3 Proliferation and migratory potential

Acquisition of a highly proliferative and invasive phenotype is a main characteristic of tumor cells. Therefore, we investigated the effect of the two hop extracts on cell cycle synchronized HepG2 cells. Incubation with 15 µg/ml β-extract for 24 h led to a significant inhibition of the proliferation rate whereas a concentration of 25 µg/ml α-extract was required to achieve an anti-proliferative effect on HCC cells (Figure 4.14).
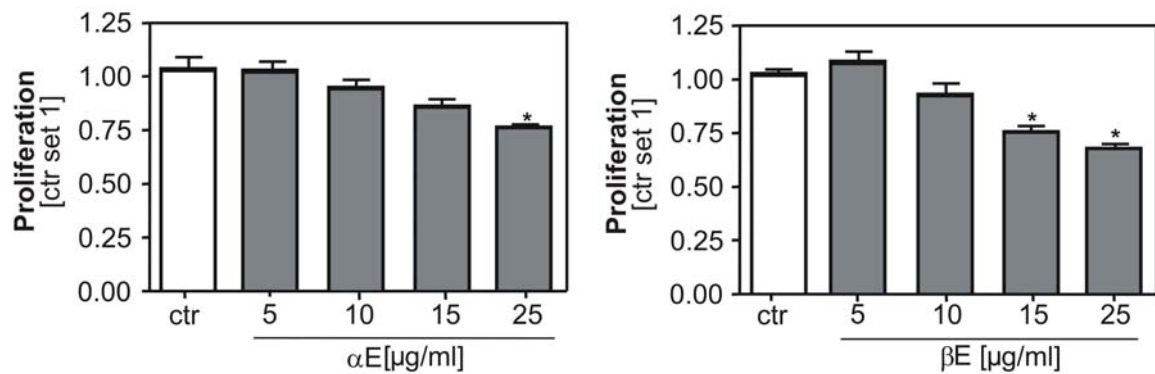
Figure 4.14   Effect of an α-rich (αE) and a β–rich (βE) hop extract on the proliferation of HepG2 cells after 24 h stimulation. *p<0.05 compared to control.

Next, we analyzed the effect of the hop extracts on the migratory capacity of HCC cells. Here, we applied a low concentration of BA-extracts (5 μg/ml) to exclude interfering effects on cell viability and proliferation, respectively. At this concentration both α- and β-extract exhibited a similar inhibitory effect on HepG2 cell migration (Figure 4.15).
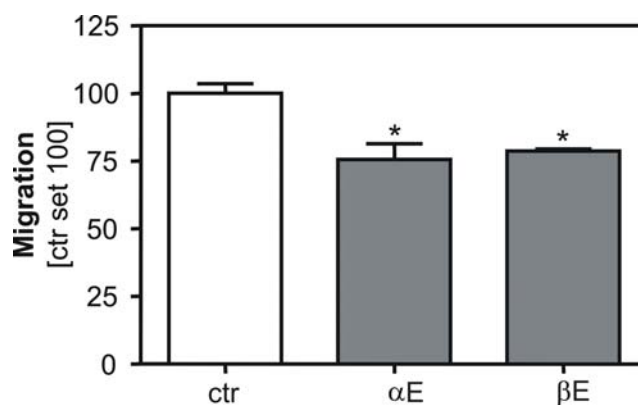
Figure 4.15   Quantification of the migratory capacity of HepG2 cells incubated with 5 μg/ml of an α-rich (αE) and a β-rich (βE) hop extract compared to untreated control cells. *p<0.05 compared to control.

4.2.4 ERK1/2, AP-1 and NFκB activity

To get an insight into the molecular mechanism by which BA affect proliferation and migration of HCC cells, we analyzed different pathways which are known to affect HCC progression. Extracellular signal-regulated kinase (ERK1/2) is often deregulated in HCC, and its induction correlates with poor prognosis of HCC patients (Ito et al., 1998; Schmitz et al., 2008; Tsuboi et al., 2004). Stimulation with 5 μg/ml of both hop extracts significantly reduced the amount of phosphorylated

ERK1/2 in HepG2 cells (Figure 4.16). Interestingly, the inhibitory effect of β-extract was more potent compared to α-extract.
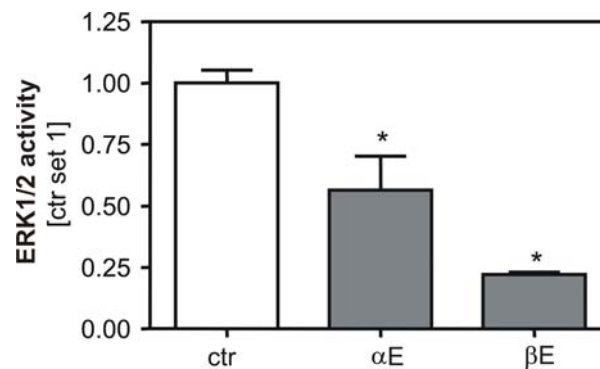
Figure 4.16   Quantification of phospho-ERK1/2 after 24 h stimulation with 5 µg/ml of an α-rich (αE) or a β-rich (βE) hop extract compared to untreated control cells applying the ELISA technique. *p<0.05 compared to control.

Once activated, ERK1/2 pathway induces different transcription factors including the activator protein-1 (AP-1) (Ito et al., 1998). Therefore, we next applied a reporter gene assay to assess AP-1 activity in HepG2 cells stimulated with the two hop extracts. Both of them markedly inhibited AP-1 activity in HepG2 cells (Figure 4.17).
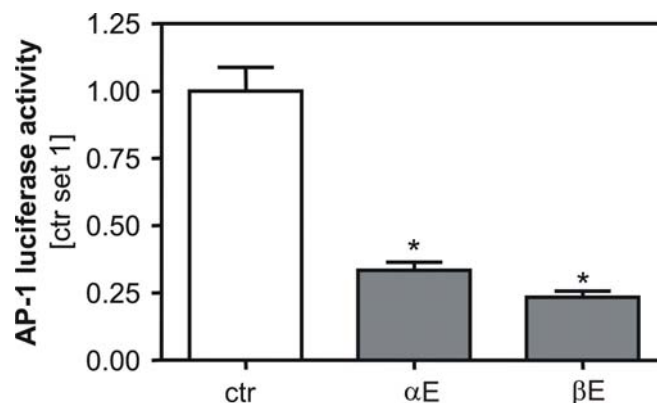
Figure 4.17   AP-1 activity after treatment with 5 µg/ml of an α-rich (αE) and a β-rich (βE) hop extract for 20 h using a reporter gene assay. *p<0.05 compared to control.

Moreover, ERK-activation can also directly affect the transcription factor NFκB (Nakano et al., 1998; Zhao and Lee, 1999), and it has been shown that NFκB plays an important role in HCC progression (Amann et al., 2009; Arsura and Cavin, 2005; Pikarsky et al., 2004). Therefore the influence of BA on NFκB activity

was investigated. Both hop extracts significantly attenuated basal NFκB activity at a concentration as low as 5 µg/ml (Figure 4.18).
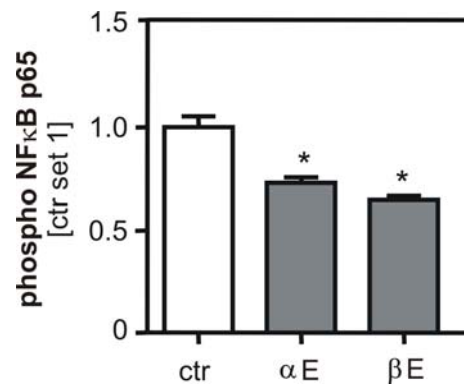
Figure 4.18   Quantification of the basal NFκB activity in HepG2 cells which were incubated with 5 µg/ml of an α-rich (αE) and a β-rich (βE) hop extract for 16 h. *$p<0.05$ compared to control.

Further, the effect of both hop extracts on induced NFκB activity was assessed. To induce NFκB activation, HepG2 cells were stimulated with conditioned medium from activated hepatic stellate cells (HSC). Activated HSC secrete a variety of pro-inflammatory cytokines, and previously, the influence of HSC on tumor aggressiveness has been demonstrated (Amann et al., 2009). Importantly, both hop extracts significantly alleviated HSC induced NFκB activity at concentrations as low as 5 µg/ml (Figure 4.19).
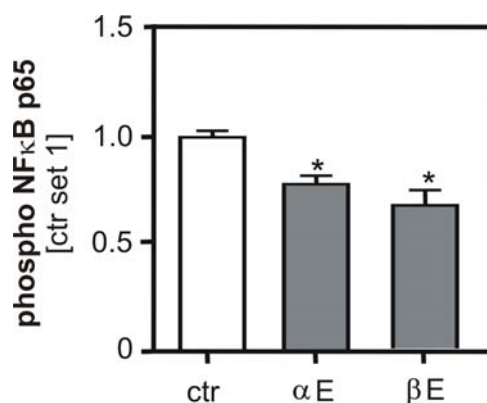
Figure 4.19   Quantification of induced NFκB activity caused by conditioned medium from activated HSC after pretreatment with an α-rich (αE) and a β-rich (βE) hop extract for 16 h. *$p<0.05$ compared to control.

4.2.5 Comparison of bitter acids and remnant lipophilic compounds

Finally, we wanted to analyze whether concomitant compounds present in the α- and β-extracts contribute to the observed anti-tumorigenic effects on HCC cells. We separated BA from the β-extract by liquid-liquid extraction. Separation success was checked by thin layer chromatography (see Figure 4.7). Afterwards, HepG2 cells were stimulated with the obtained purified bitter acids (pBA) and the remnant lipophilic compounds (rLC) at equimolar concentrations. Importantly, treatment with p-BA revealed the same anti-proliferative effect as the original β-extract whereas treatment with the remnant lipophilic compounds alone had no influence on proliferation (Figure 4.20). These findings confirmed that BA and not remnant hop compounds in the extracts account for the observed effects on HCC cells.
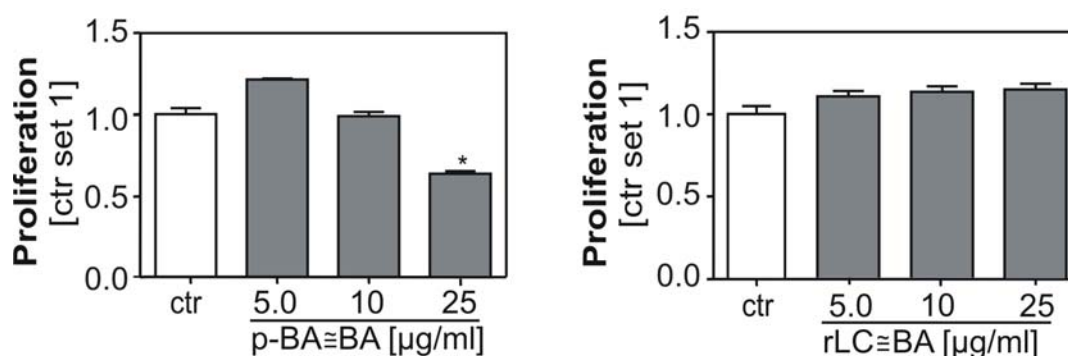
Figure 4.20   Effect of purified bitter acids (p-BA) (A) and remnant lipophilic compounds (rLC) (B) on the proliferation of HepG2 applying the colorimetric XTT assay. p-BA and rLC were applied in corresponding concentrations as present in stimulation experiments with the original β-bitter acid extract. *$p<0.05$ compared to control.

4.2.6 Summary

Hop bitter acid extracts induced cell death in HCC cells at a concentration of 50 µg/ml. Already at lower concentrations (5 – 25 µg/ml) both extracts led to a dose dependent inhibition of proliferation, and migration was suppressed at a concentration as low as 5 µg/ml. Analysis of different signaling pathways revealed an inhibition of ERK1/2 phosphorylation, down-regulation of AP-1 activity and an alleviation of NFκB activity in HCC cells by incubation with 5 µg/ml of either of both extracts. The β-acid rich extract showed more pronounced effects in all experiments. In summary, ERK1/2, AP-1 and NFκB, which are important factors in

tumor development and progression, were identified as targets of hop BA. These data suggest the potential use of BA as functional nutrients for both prevention and treatment of HCC.

4.3 BA-recovery experiments from rodent chow supplemented BA-extract

In vivo studies are desirable to verify the anti-fibrotic and anti-tumorigenic effects of BA seen *in vitro.* For this reason, we generated a chow for mice and rats supplemented with 1.75 % (m/m) of the standardized hop extract "Herkules". This concentration is equivalent to 1 % (m/m) of (total) α-acids. A typical mouse at 15 weeks of age weighs approximately 25 g and eats approximately 4 g a day. Thus, the concentration of BA can be translated to approximately 1.6 g/kg/bodyweight α-acids and 0.6 g/kg/bodyweight β-acids, respectively.

Since it is known that BA are very sensitive to oxidation and degradation, we tried to minimize these reactions during the whole process of chow production and storage. After production, the BA-chow was either stored at 4 °C or at -20 °C over a period of two month to mimic feasible storage conditions for *in vivo* application. Afterwards, the composition of BA in the chow was analyzed using the HPLC-Diode Array Detection system in collaboration with NATECO$_2$ GmbH (Wolnzach, Germany).

After 2 month of storage at -20 °C, analysis of the chow revealed 0.39, 0.27 and 0.091 % (m/m) for the three main analogues of α-acids (n-,co-,ad-) and 0.062, 0.105 and 0.018 % (m/m) for the three main analogues of β-acids (n-,co-,ad-), respectively (table 4.1). Further, 0.01 % (m/m) of pre-humulone derivatives was detected. The recovery was approximately 75 % for genuine α-acids and approximately 58 % for genuine β-acids. The amount of degradation products was 0.037 % (m/m) for humulinic acid derivatives, the main α-acids degradation product, and 0.048 % (m/m) for hulupones, the main β-acids degradation product, respectively.

Next, we assessed the chow stored at 4 °C for 2 month. Here, we detected a much more pronounced decrease of the three main analogues of α-acids (n-,co-, ad-) to 0.044, 0.033, and 0.01 % (m/m), respectively (table 4.1). Similarly, the three main analogues of β-acids (n-,co-,ad-) dropped significantly to 0.004, 0.007

and 0.001 % (m/m), respectively. Pre-humulone derivatives were not detectable any more, whereas the humulinic acid derivatives and hulupones as main α- and β-acid degradation products, respectively, increased to 0.047 % (m/m) and 0.085 % (m/m). In summary, the recovery of genuine α-acids dropped to 9 % and approximately 4 % for genuine β-acids, respectively, after a 2 month storage interval at 4 °C.

These findings indicate a stronger decay of BA during storage at 4 °C compared to -20°C. Recovery of genuine α- and β-acids significantly dropped from 75 % and 58%, when stored at -20 °C, to 9 % and 4 % when stored at 4 °C, respectively (table 4.2). The detailed composition of BA in the chow stored at 4 °C or at -20 °C is listed in table 4.1. Concentration and recovery of total BA is shown in table 4.2

Table 4.1   Comparison of BA and BA-degradation products in chow supplemented with the BA-extract Herkules stored at 4 °C or -20 °C.

Compound [% m/m]	storage at 4 °C	storage at -20 °C
n-humulone	0.044	0.39
co-humulone	0.033	0.27
ad-humulone	0.01	0.091
pre-humulone-derivatives	n.d.	0.01
n-lupulone	0.004	0.062
co-lupulone	0.007	0.105
ad-lupulone	0.001	0.018
humulinic acid-derivatives	0.047	0.037
hulupones	0.085	0.048
co-isohumulone	n.d.	n.d.
n-isohumulone	n.d.	n.d.
ad-isohumulone	n.d.	n.d.

(n.d. = not detectable)

Table 4.2   Concentration and recovery of total BA in chow supplemented with BA-extract Herkules stored at 4 °C or -20 °C.

Compounds	4 °C [% m/m]	recovery at 4 °C [%]	-20 °C [% m/m]	recovery at -20 °C [%]
Σ α-acids (co-, n-, -ad)	0.09	9	0.75	75
Σ β-acids (co-, n-, -ad)	0.012	4	0.185	58
Σ iso-α-acids	n.d.	-	n.d.	-

(n.d. = not detectable)

In vivo application of BA is a desirable step to unravel the mechanisms of action of BA. However, since BA are very sensitive to oxidation and degradation it is essential that the individual composition of BA during the application is well controlled. Furthermore, optimal storage conditions are required to diminish potential ill-defined oxidation and degradation products. Together, our data clearly show that degradation and oxidation processes occur even under cooled storage conditions, at least in the present chow and the used BA-extract, respectively. Based on these data we decided not to proceed to analyse the effect of this BA-supplemented chow in rodent models of fibrosis or hepatocellular cancer.

5 Discussion

Hop bitter acids (BA) are multipotent bioactive compounds (see chapter 2.3), however, no data concerning the effects on liver cells or liver diseases, respectively, are available at present. Therefore, the aim of this thesis was to address this issue. In particular, we analyzed the effects of BA on hepatic stellate cells as central mediators in hepatic fibrosis (see chapter 4.1) as well as BA effects on hepatocellular carcinoma cells (see chapter 4.2).

In the following, the discussion is divided into three main chapters in accordance to the arrangement of the results in chapter 4:

5.1 Hop bitter acids and hepatic stellate cells

5.2 Hop bitter acids and hepatocellular carcinoma

5.3. Poor recovery of hop bitter acids in animal chow supplemented with a bitter acid-extract

5.1 Hop bitter acids and hepatic inflammation and fibrosis

In the present study we aimed to analyze the effects of hop bitter acids (BA) on hepatic stellate cells (HSC). The activation of these cells in response to liver injury is considered as the key event of hepatic fibrosis (Bataller and Brenner, 2001). Activated HSC are the cellular source of the excessive deposition of extracellular matrix during chronic liver injury (Mann and Mann, 2009). Further, the activation process of HSC is paralleled by *de novo* expression of several cytokines and chemokines, and by this, activated HSC also significantly contribute to hepatic inflammation and perpetuation of fibrosis.

Noteworthy, BA exhibited a profound effect on several pathophysiological characteristics of HSC *in vitro*. The activation of HSC was significantly inhibited at BA concentrations of 2.5 µg/ml and 5 µg/ml. In a similar dose range, BA also reduced proliferation of activated HSC and their expression of the pro-inflammatory chemokines MCP-1 and RANTES. Both chemokines are regulated by NFκB and increased levels are associated with fibrosis progression in chronic liver disease (Jarrar et al., 2008; Wouters et al., 2008). Further, NFκB activation is

a central pathophysiological mechanism during HSC activation (Hellerbrand et al., 1998a; b; Elsharkawy et al., 2005). Importantly, our study revealed that BA inhibit NFκB activity in activated HSC *in vitro*. In contrast, neither activation of JNK nor the expression of genes not related to NFκB activity were affected, which confirms that the observed effects are specific for the NFκB pathway and are not related to (unspecific) cytotoxic effects.

In this study we applied a hop extract enriched for bitter acids, which harbored the possibility that remnant hop compounds contribute to the anti-fibrogenic effects in addition to BA. However, we separated BA from the extract by liquid-liquid extraction and observed a striking similarity between the anti-fibrogenic effects of these purified BA and the original extract. In contrast, residual lipophilic compounds did not exhibit any effects on HSC. Together, these findings strongly suggest that indeed BA exclusively account for the observed effects on HSC.

Little is known about the metabolism and bioavailability of BA. In one study by Desai *et al.* after a single oral uptake of 940 mg of isomerized α-acids (called META060) serum concentrations of 4-15 µg/ml were detected in men with the highest concentrations between 2 and 4 hours after intake (Desai et al., 2009). Although according analysis are missing, it can be expected based on the anatomical situation that after oral intake BA concentration is significantly higher in the portal vein than in systemic circulation. Further, it has to be considered that HSC are located in the liver in the space of Disse (or perisinusoidal space) *i.e.* between the sinusoid and the hepatocytes. Herewith, HSC are directly exposed to the BA concentration reaching the liver via the portal vein irrespective of (subsequent) metabolisms in hepatocytes. Thus, it can be speculated that the dose range of 5-10 µg/ml shown to reveal anti-fibrogenic effects on HSC *in vitro*, can be achieved with significantly lower oral doses of BA than used in the study of Desai and colleagues (Desai et al., 2009).

Still it has to be noted, that there are substantial variations of used BA, extraction methods as well as analysis of the composition in the literature (Van Cleemput et al., 2009a). Therefore, it is hard to compare our observations with other experiments. However, most of the observed effects of BA on liver cells (5-10 µg/ml) were in a dose range that was also observed as effective in other studies using similar hop extracts and other cell lines.

Beer is the major dietary source of BA, but the average content of BA in beer is probably not high enough to produce a (hepato)protective effect in humans. Thus, Foster *et al.* revealed that the amount and composition of BA strongly varies in 35 different sorts of beers (Foster et al., 2009). Furthermore, there is certainly unanimous hesitancy among researchers to recommend drinking alcohol to avoid any kind of disease because of the fine line between moderate and binge drinking. Certainly, this is even truer in the case of chronic liver disease. However, different methods (i.e. using liquid supercritical carbon dioxide) were developed to isolate BA from hop cones in large quantities and thus, independent of beer intake BA may be used as hepatoprotective dietary supplement.

Still, caution has to be taken and more research is needed before it is known whether the findings of the present study can be applied to humans. Importantly, we found that BA exhibit their anti-fibrogenic effects on both primary murine and human HSC, and a safety study by Farber *et al.* did not reveal toxic effects on liver, kidney, bone marrow or myocardium in man after oral application of BA at a concentration of 5.0 g per day for three months (Farber et al., 1950). Furthermore, recent reports indicate that BA have a positive effect on lipid metabolism, glucose tolerance and insulin resistance in animals (Yajima et al., 2004; Miura et al., 2005; Yajima et al., 2005). Still, further safety studies as well as efficacy studies in experimental *in vivo* models of hepatic fibrosis are required. However, the present study indicates the potential of BA as functional nutrient to ameliorate inflammation and fibrosis in chronic liver disease.

5.2 Hop bitter acids and hepatocellular carcinoma

In the second part this thesis aimed to analyze the effects of bitter acids (BA) on tumorigenicity of HCC cells. To get a first insight in differential effects of α-BA and β-BA we used hop extracts enriched with either one of these two related series and found that both extracts exhibited a profound effect on ERK1/2 as well as on AP-1 and NFκB activation. These signaling pathways are known to be pathophysiologically relevant in HCC cells (Amann et al., 2009; Ito et al., 1998; Schmitz et al., 2008; Nakano et al., 1998; Arsura and Cavin, 2005; Pikarsky et al., 2004), and in line with this, both extracts also revealed inhibitory effects on proliferation and migration of HCC cells *in vitro*. Importantly, we could exclude that

the observed effects derived from remnant lipophilic compounds in hop extracts. However and interestingly, individual anti-tumorigenic effects were stronger upon stimulation with β-extract compared to α-extract-mediated effects. One might speculate that the additional isoprenyl side chain of β-BA, which contributes to a higher lipophilicity and increased steric hindrance, may positively influence their effects on HCC cells. However, Cattor *et al.* demonstrated a fast and effective uptake of α-BA into colon cancer cells *in vitro* (Cattoor et al., 2010). Furthermore and notwithstanding the slight differences between α- and β-BA regarding anti-tumorigenic effects on HCC, it has to be noted that α-BA are the most abundant type of BA in beer whereas β-BA are only present in trace amounts (Foster et al., 2009). Still, different methods were developed to isolate BA from hop cones in large quantities (*i.e.* using liquid supercritical carbon dioxide), and thus, independent of beer intake, extracts enriched for either α- or β-BA may be potentially used as hepatoprotective dietary supplement.

In the previous chapter it has been described that available studies indicate a high safety profile of BA. Still, it has to be considered that HCC mainly develops in cirrhotic livers (Kirchner et al., 2010), which are characterized by an impaired metabolic and detoxifying capacity. Therefore, special caution has to be taken in patients with chronic liver disease, and further studies in experimental fibrosis and HCC models are required. Still, the present study indicates the potential of BA as functional nutrient for both prevention and treatment of HCC.

5.3 Poor recovery of hop bitter acids in animal chow supplemented with a bitter acid-extract

For a more advanced insight into the bioavailability and safety profile of BA more *in vivo* studies would be wanted. Further, it would have been desirable to verify the beneficial effects of BA seen *in vitro* in the *in vivo* situation.

Mice and rats are the most frequently used species to experimentally mimic hepatic fibrosis and hepatocancerogenesis. Established models are for example the bile duct ligation model or chronic toxic liver injury applying carbon tetrachloride or thioacetamide (Brenner, 2009). Further, models leading to non-alcoholic steatohepatitis (NASH) are increasingly getting attention (Nagarajan et al., 2012). All these models last at least several weeks. Rodent models leading to

hepatocellular carcinoma (HCC) last even longer. In the diethylnitrosamine (DEN) model, the most frequently used model of HCC, tumors develop usually about 8-12 month after intraperitoneal injection of DEN into young mice. Due to these long time spans, it is not practicable to apply or test experimental substances (e.g. hop extracts) by repeated intravenous or intraperitoneal injections, respectively. In any case, oral application is most desirable also with regards to clinical application since the course of liver disease in men typically last several years. Moreover, as described above, based on the anatomical situation oral application of BA is reasonable for liver diseases. Still, also gavage is not possible due to the long experimental duration of the models. Moreover, the administration of BA in water is also not practical, because the hydrophobic BA are poorly soluble in water (Simpson and Smith, 1992). Thus, we intended to generate a chow supplemented with BA extracts to test the efficacy of BA in a defined concentration in established rodent models. Here, we used a concentration of 1.75 % (m/m) of BA-extract. This can be translated into a daily dose of approximately 1.6 g/day/bodyweight for α-acids and 0.6 g/day/bodyweight for β-acids, respectively. This concentration is in the range of concentrations used in previous studies in men and mice (Mannering et al., 1992; Farber et al., 1950).

Mice are active during night and eat more or less continuously over the 12 hours of darkness if housed under controlled conditions with day/night cycles, resulting in a mainly steady calorie intake over these 12 hours. Mice are usually housed at a temperature of about 20 °C. For logistical reasons food has to be provided in one-day-portions (or 2- to 3-days-portions on weekends), and therefore, cannot be stored continuously at -20 °C.

Our analysis revealed that even under optimal practical storage conditions, i.e. storage at -20 °C, only 75 % of genuine α-acids (n-,co-,ad-) and about 58 % of genuine β-acids (n-,co-,ad-) could be recovered after 2 months of storage. Currently, we can only speculate whether this loss occurred already during the transport and production process. However and remarkably, the recovery even further dropped below 10 % for genuine α-acids (n-,co-,ad-) and 4 % for genuine β-acids (n-,co-,ad-) if the chow was stored at 4 °C. Although not systematically tested, one can speculate that the concentrations of BA would be even lower if stored at room temperature, *e.g.* during application.

In addition to the uncertain amount of actually applied BA, the formation of ill-defined degradation products, which may have unknown biological effects, depicts another potential problem. A first evidence for the formation of degradation products was the observed increase of humulinic-acid derivates and hulupones as the main degradation products of α- and β-acids, respectively, when stored at 4 °C. Stability or degradation problems, respectively, may also be the reason that different studies with the same hop compounds are often difficult to compare.

These data show that at least with the formulation and the hop extract used here, respectively, this chow is not suitable for analyzing the effects of a defined concentration of BA *in vivo*. Together, these data prompted us not to proceed with *in vivo* experiments in which we could have tested the (beneficial) effect of BA-application in rodent models of (chronic) liver injury. For a sound interpretation and comparison of studies it appears mandatory that further approaches have to be performed to achieve this goal.

5.4 Conclusion

Hop bitter acids are multipotent bioactive compounds, however, no data concerning the effects on liver cells or liver diseases, respectively, are available at present. We could demonstrate that hop bitter acids exhibit beneficial effects on hepatic stellate cells as central mediators of liver fibrogenesis as well as on hepatocellular carcinoma cells *in vitro*. Importantly, there is a direct link between the pro-fibrogenic phenotype of hepatic stellate cells and the development and aggressiveness of hepatocellular carcinoma progression. Furthermore, recent reports indicate that bitter acids have a positive effect on lipid metabolism, glucose tolerance and insulin resistance in animals. Since metabolic disorders steadily increase all over the world leading to chronic liver inflammation and consequently to liver damage, hop bitter acids might be an attractive natural drug in the battle against chronic liver diseases. Still, further studies are needed to verify the beneficial effects in experimental models of liver injury *in vivo*. However, our studies with a BA supplemented chow for rodents revealed a bad stability of BA. Therefore, special care has to be taken and the stability of BA has to be monitored during *in vivo* application. Nevertheless, based on the very promising data revealed in this thesis with regards to the anti-fibrotic and anti-tumorigenic effects

of BA *in vitro*, it appears worth to emphasize *in vivo* approaches to study the potential of BA as functional nutrient in chronic liver disease.

6 References

Alison, M.R., and M.J. Lovell. 2005. Liver cancer: the role of stem cells. *Cell Prolif.* 38:407–421.

Amann, T., F. Bataille, T. Spruss, M. Mühlbauer, E. Gäbele, J. Schölmerich, P. Kiefer, A.-K. Bosserhoff, and C. Hellerbrand. 2009. Activated hepatic stellate cells promote tumorigenicity of hepatocellular carcinoma. *Cancer Sci.* 100:646–653.

Aravalli, R.N., C.J. Steer, and E.N.K. Cressman. 2008. Molecular mechanisms of hepatocellular carcinoma. *Hepatology.* 48:2047–2063.

Arsura, M., and L.G. Cavin. 2005. Nuclear factor-kappaB and liver carcinogenesis. *Cancer Lett.* 229:157–169.

Arthur, M.J. 2000. Fibrogenesis II. Metalloproteinases and their inhibitors in liver fibrosis. *Am. J. Physiol. Gastrointest. Liver Physiol.* 279:G245–249.

Atzori, L., G. Poli, and A. Perra. 2009. Hepatic stellate cell: a star cell in the liver. *Int. J. Biochem. Cell Biol.* 41:1639–1642.

Bataller, R., and D.A. Brenner. 2001. Hepatic stellate cells as a target for the treatment of liver fibrosis. *Semin. Liver Dis.* 21:437–451.

Bauer, R., W. Blaschek, W. Buff, K. Hiller, J.J. Lichius, D. Loew, and M. Wichtl. 2009. Teedrogen und Phytopharmaka: Ein Handbuch für die Praxis auf wissenschaftlicher Grundlage. 5., vollst. überarb. u. erw. Aufl. Wissenschaftliche Verlagsgesellschaft. 783 pp.

Bousserouel, S., V. Lamy, F. Gossé, A. Lobstein, J. Marescaux, and F. Raul. 2011. Early modulation of gene expression used as a biomarker for chemoprevention in a preclinical model of colon carcinogenesis. *Pathol. Int.* 61:80–87.

Brenner, D.A. 2009. Molecular pathogenesis of liver fibrosis. *Trans. Am. Clin. Climatol. Assoc.* 120:361–368.

Bruce, K.D., and M.A. Hanson. 2010. The developmental origins, mechanisms, and implications of metabolic syndrome. *J. Nutr.* 140:648–652.

Bruix, J., L. Boix, M. Sala, and J.M. Llovet. 2004. Focus on hepatocellular carcinoma. *Cancer Cell.* 5:215–219.

Cattoor, K., M. Bracke, D. Deforce, D. De Keukeleire, and A. Heyerick. 2010. Transport of Hop Bitter Acids across Intestinal Caco-2 Cell Monolayers. *J. Agric. Food Chem.* 58:4132–4140.

Chappel, C.I., S.Y. Smith, and M. Chagnon. 1998. Subchronic toxicity study of tetrahydroisohumulone and hexahydroisohumulone in the beagle dog. *Food Chem Toxicol.* 36:915.

Chen, W.-J., and J.-K. Lin. 2004. Mechanisms of cancer chemoprevention by hop bitter acids (beer aroma) through induction of apoptosis mediated by Fas and caspase cascades. *J. Agric. Food Chem.* 52:55–64.

Chin, Y.-C., H.H. Anderson, G. Alderton, and J.C. Lewis. 1949a. Antituberculous Activity and Toxicity of Lupulon for the Mouse. *Proc Soc Exp Biol Med.* 70:158–162.

Chin, Y.-C., N.-C. Chang, and H.H. Anderson. 1949b. Factors influencing the antibiotic activity of lupulon. *J Clin Invest.* 28:909–915.

Van Cleemput, M., K. Cattoor, K. De Bosscher, G. Haegeman, D. De Keukeleire, and A. Heyerick. 2009a. Hop (Humulus lupulus)-derived bitter acids as multipotent bioactive compounds. *J. Nat. Prod.* 72:1220–1230.

Van Cleemput, M., A. Heyerick, C. Libert, K. Swerts, J. Philippé, D. De Keukeleire, G. Haegeman, and K. De Bosscher. 2009b. Hop bitter acids efficiently block inflammation independent of GRα, PPARα, or PPARγ. *Molecular Nutrition & Food Research.* 53:1143–1155.

Desai, A., V.R. Konda, G. Darland, M. Austin, K.S. Prabhu, J.S. Bland, B.J. Carroll, and M.L. Tripp. 2009. META060 inhibits multiple kinases in the NF-kappaB pathway and suppresses LPS--mediated inflammation in vitro and ex vivo. *Inflamm. Res.* 58:229–234.

Dorn, C., F. Bataille, E. Gaebele, J. Heilmann, and C. Hellerbrand. 2010. Xanthohumol feeding does not impair organ function and homoeostasis in mice. *Food Chem. Toxicol.* 48:1890–1897.

Dorn, C., J. Heilmann, and C. Hellerbrand. 2012a. Protective effect of xanthohumol on toxin-induced liver inflammation and fibrosis. *Int J Clin Exp Pathol.* 5:29–36.

Dorn, C., S. Massinger, A. Wuzik, J. Heilmann, and C. Hellerbrand. 2012b. Xanthohumol suppresses inflammatory response to warm ischemia-reperfusion induced liver injury. *Experimental and molecular pathology.*

Elsharkawy, A.M., F. Oakley, and D.A. Mann. 2005. The role and regulation of hepatic stellate cell apoptosis in reversal of liver fibrosis. *Apoptosis.* 10:927–939.

Everard, A., L. Geurts, M. Van Roye, N.M. Delzenne, and P.D. Cani. 2012. Tetrahydro iso-Alpha Acids from Hops Improve Glucose Homeostasis and Reduce Body Weight Gain and Metabolic Endotoxemia in High-Fat Diet-Fed Mice. *PLoS ONE.* 7:e33858.

Farazi, P.A., and R.A. DePinho. 2006. Hepatocellular carcinoma pathogenesis: from genes to environment. *Nat. Rev. Cancer.* 6:674–687.

Farber, S.M., J.M. Masten, H.H. Anderson, R.W. Gentry, and Y.C. Chin. 1950. Tolerance and Effects of Lupulon in Man. *Chest.* 18:10–15.

Foster, B.C., N. Kearns, J.T. Arnason, A. Saleem, C. Ogrodowczyk, and S. Desjardins. 2009. Comparative study of hop-containing products on human cytochrome p450-mediated metabolism. *J. Agric. Food Chem.* 57:5100–5105.

Friedman, S.L., and M.J. Arthur. 1989. Activation of cultured rat hepatic lipocytes by Kupffer cell conditioned medium. Direct enhancement of matrix synthesis and stimulation of cell proliferation via induction of platelet-derived growth factor receptors. *J Clin Invest.* 84:1780–1785.

Gäbele, E., D.A. Brenner, and R.A. Rippe. 2003. Liver fibrosis: signals leading to the amplification of the fibrogenic hepatic stellate cell. *Front. Biosci.* 8:d69–77.

Hall, A.J., J.G. Babish, G.K. Darland, B.J. Carroll, V.R. Konda, R.H. Lerman, J.S. Bland, and M.L. Tripp. 2008. Safety, efficacy and anti-inflammatory activity of rho iso-alpha-acids from hops. *Phytochemistry.* 69:1534–1547.

Hellerbrand, C., C. Jobin, Y. Iimuro, L. Licato, R.B. Sartor, and D.A. Brenner. 1998a. Inhibition of NFκB in activated rat hepatic stellate cells by proteasome inhibitors and an IκB super - repressor. *Hepatology.* 27:1285–1295.

Hellerbrand, C., C. Jobin, L.L. Licato, R.B. Sartor, and D.A. Brenner. 1998b. Cytokines Induce NF-κB in Activated but Not in Quiescent Rat Hepatic Stellate Cells. *Am J Physiol Gastrointest Liver Physiol.* 275:G269–G278.

Hellerbrand, C., S.C. Wang, H. Tsukamoto, D.A. Brenner, and R.A. Rippe. 1996. Expression of intracellular adhesion molecule 1 by activated hepatic stellate cells. *Hepatology.* 24:670–676.

Honma, Y., H. Tobe, M. Makishima, A. Yokoyama, and J. Okabe-Kado. 1998. Induction of differentiation of myelogenous leukemia cells by humulone, a bitter in the hop. *Leuk. Res.* 22:605–610.

Hougee, S., J. Faber, A. Sanders, W.B. Berg, J. Garssen, H.F. Smit, and M.A. Hoijer. 2006. Selective inhibition of COX-2 by a standardized CO_2 extract of Humulus lupulus in vitro and its activity in a mouse model of zymosan-induced arthritis. *Planta Med.* 72:228–233.

Iredale, J.P. 2007. Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *J. Clin. Invest.* 117:539–548.

Iredale, J.P., R.C. Benyon, J. Pickering, M. McCullen, M. Northrop, S. Pawley, C. Hovell, and M.J. Arthur. 1998. Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. *J. Clin. Invest.* 102:538–549.

Ito, Y., Y. Sasaki, M. Horimoto, S. Wada, Y. Tanaka, A. Kasahara, T. Ueki, T. Hirano, H. Yamamoto, J. Fujimoto, E. Okamoto, N. Hayashi, and M. Hori. 1998. Activation of mitogen - activated protein kinases/extracellular signal - regulated kinases in human hepatocellular carcinoma. *Hepatology.* 27:951–958.

Jarrar, M.H., A. Baranova, R. Collantes, B. Ranard, M. Stepanova, C. Bennett, Y. Fang, H. Elariny, Z. Goodman, V. Chandhoke, and Z.M. Younossi. 2008.

Adipokines and cytokines in non-alcoholic fatty liver disease. *Aliment. Pharmacol. Ther.* 27:412–421.

Kersten, S., B. Desvergne, and W. Wahli. 2000. Roles of PPARs in health and disease. *Nature.* 405:421–424.

Keukeleire D.D. 2000. Fundamentals of beer and hop chemistry. *Quimica Nova.* 23:108 –112.

Kirchner, G., G. Kirovski, A. Hebestreit, J. Schölmerich, H.J. Schlitt, O. Stoeltzing, and C. Hellerbrand. 2010. Epidemiology and survival of patients with hepatocellular carcinoma in Southern Germany. *Int J Clin Exp Med.* 3:169–179.

Kmieć, Z. 2001. Cooperation of liver cells in health and disease. *Adv Anat Embryol Cell Biol.* 161:III–XIII, 1–151.

Konda, V.R., A. Desai, G. Darland, J.S. Bland, and M.L. Tripp. 2009. Rho iso-alpha acids from hops inhibit the GSK-3/NF-κB pathway and reduce inflammatory markers associated with bone and cartilage degradation. *Journal of Inflammation.* 6:26.

Kritchevsky, D., D.S. Martak, and G.H. Rothblat. 1963. Detection of bile acids in thin-layer chromatography. *Analytical Biochemistry.* 5:388–392.

Kubicka, S., K.L. Rudolph, M. Hanke, M.K. Tietze, H.L. Tillmann, C. Trautwein, and M. Manns. 2000. Hepatocellular carcinoma in Germany: a retrospective epidemiological study from a low-endemic area. *Liver.* 20:312–318.

Lamy, V., S. Bousserouel, F. Gossé, C. Minker, A. Lobstein, and F. Raul. 2010. p53 Activates Either Survival or Apoptotic Signaling Responses in Lupulone-Treated Human Colon Adenocarcinoma Cells and Derived Metastatic Cells. *Transl Oncol.* 3:286–292.

Lamy, V., S. Bousserouel, F. Gossé, C. Minker, A. Lobstein, and F. Raul. 2011. Lupulone triggers p38 MAPK-controlled activation of p53 and of the TRAIL receptor apoptotic pathway in human colon cancer-derived metastatic cells. *Oncol. Rep.* 26:109–114.

Lamy, V., S. Roussi, M. Chaabi, F. Gossé, N. Schall, A. Lobstein, and F. Raul. 2007. Chemopreventive effects of lupulone, a hop ß-acid, on human colon cancer-derived metastatic SW620 cells and in a rat model of colon carcinogenesis. *Carcinogenesis.* 28:1575–1581.

Lang, A., R. Schoonhoven, S. Tuvia, D.A. Brenner, and R.A. Rippe. 2000. Nuclear factor kappaB in proliferation, activation, and apoptosis in rat hepatic stellate cells. *J. Hepatol.* 33:49–58.

Lee, J.-C., J.K. Kundu, D.-M. Hwang, H.-K. Na, and Y.-J. Surh. 2007. Humulone Inhibits Phorbol Ester-Induced COX-2 Expression in Mouse Skin by Blocking Activation of NF-κB and AP-1: IκB Kinase and C-Jun-N-Terminal Kinase as Respective Potential Upstream Targets. *Carcinogenesis.* 28:1491–1498.

Lee, K.S., M. Buck, K. Houglum, and M. Chojkier. 1995. Activation of hepatic stellate cells by TGF alpha and collagen type I is mediated by oxidative stress through c-myb expression. *Journal of Clinical Investigation*. 96:2461–2468.

Lewis, J.C., G. Alderton, J.F. Carson, D.M. Reynolds, and W.D. Maclay. 1949. Lupulon and Humulon-Antibiotic constituents of hops. *Journal of Clinical Investigation*. 28:916–919.

Liu, Y., X. Gu, J. Tang, and K. Liu. 2007. Antioxidant Activities of Hops (Humulus lupulus) and Their Products. *Journal of the American Society of Brewing Chemists*.

Lukaczer, D., G. Darland, M. Tripp, D. Liska, R.H. Lerman, B. Schiltz, and J.S. Bland. 2005. A pilot trial evaluating Meta050, a proprietary combination of reduced iso-alpha acids, rosemary extract and oleanolic acid in patients with arthritis and fibromyalgia. *Phytother Res*. 19:864–869.

Mann, J., and D.A. Mann. 2009. Transcriptional regulation of hepatic stellate cells. *Adv. Drug Deliv. Rev.* 61:497–512.

Mannering, G.J., J.A. Shoeman, and L.B. Deloria. 1992. Identification of the antibiotic hops component, colupulone, as an inducer of hepatic cytochrome P-4503A in the mouse. *Drug Metab. Dispos.* 20:142–147.

Michener, H.D., N. Snell, and E.F. Jansen. 1948. Antifungal activity of hop resin constituents and a new method for isolation of lupulon. *Arch Biochem*. 19:199–208.

Milligan, S., J. Kalita, V. Pocock, A. Heyerick, L. De Cooman, H. Rong, and D. De Keukeleire. 2002. Oestrogenic activity of the hop phyto-oestrogen, 8-prenylnaringenin. *Reproduction*. 123:235–242.

Minich, D.M., J.S. Bland, J. Katke, G. Darland, A. Hall, R.H. Lerman, J. Lamb, B. Carroll, and M. Tripp. 2007. Clinical safety and efficacy of NG440: a novel combination of rho iso-alpha acids from hops, rosemary, and oleanolic acid for inflammatory conditions. *Can. J. Physiol. Pharmacol.* 85:872–883.

Miura, Y., M. Hosono, C. Oyamada, H. Odai, S. Oikawa, and K. Kondo. 2005. Dietary isohumulones, the bitter components of beer, raise plasma HDL-cholesterol levels and reduce liver cholesterol and triacylglycerol contents similar to PPARalpha activations in C57BL/6 mice. *Br. J. Nutr.* 93:559–567.

Mizobuchi, S., and Y. Sato. Antifungal activities of hop bitter resins and related compounds. *Report of the Research Laboratories of Kirin Brewery Co., Ltd.* 39–44.

Mühlbauer, M., M. Fleck, C. Schütz, T. Weiss, M. Froh, C. Blank, J. Schölmerich, and C. Hellerbrand. 2006. PD-L1 is induced in hepatocytes by viral infection and by interferon-alpha and -gamma and mediates T cell apoptosis. *J. Hepatol.* 45:520–528.

Nagarajan, P., M.J. Mahesh Kumar, R. Venkatesan, S.S. Majundar, and R.C. Juyal. 2012. Genetically modified mouse models for the study of nonalcoholic fatty liver disease. *World J. Gastroenterol.* 18:1141–1153.

Nakano, H., M. Shindo, S. Sakon, S. Nishinaka, M. Mihara, H. Yagita, and K. Okumura. 1998. Differential regulation of IkappaB kinase alpha and beta by two upstream kinases, NF-kappaB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. *Proc. Natl. Acad. Sci. U.S.A.* 95:3537–3542.

Namikoshi, T., N. Tomita, S. Fujimoto, Y. Haruna, M. Ohzeki, N. Komai, T. Sasaki, A. Yoshida, and N. Kashihara. 2007. Isohumulones derived from hops ameliorate renal injury via an anti-oxidative effect in Dahl salt-sensitive rats. *Hypertens. Res.* 30:175–184.

Nozawa, H., W. Nakao, F. Zhao, and K. Kondo. 2005. Dietary supplement of isohumulones inhibits the formation of aberrant crypt foci with a concomitant decrease in prostaglandin E2 level in rat colon. *Mol Nutr Food Res.* 49:772–778.

Oliveira, M.M., M. Salomk, and S. Pais. 2008. Glandular trichomes of Humulus lupulus var. Brewer's Gold: Ontogeny and histochemical characterization of the secretion. *Nordic Journal of Botany.* 8:349–359.

Pikarsky, E., R.M. Porat, I. Stein, R. Abramovitch, S. Amit, S. Kasem, E. Gutkovich-Pyest, S. Urieli-Shoval, E. Galun, and Y. Ben-Neriah. 2004. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature.* 431:461–466.

Pollock, J.R.A. 1979. Brewing science. Academic Press. 628 pp.

Schmitz, K.J., J. Wohlschlaeger, H. Lang, G.C. Sotiropoulos, M. Malago, K. Steveling, H. Reis, V.R. Cicinnati, K.W. Schmid, and H.A. Baba. 2008. Activation of the ERK and AKT signalling pathway predicts poor prognosis in hepatocellular carcinoma and ERK activation in cancer tissue is associated with hepatitis C virus infection. *J. Hepatol.* 48:83–90.

Seglen, P.O. 1976. Preparation of isolated rat liver cells. *Methods Cell Biol.* 13:29–83.

She, Q.-B., W.-Y. Ma, M. Wang, A. Kaji, C.-T. Ho, and Z. Dong. 2003. Inhibition of cell transformation by resveratrol and its derivatives: differential effects and mechanisms involved. *Oncogene.* 22:2143–2150.

Shimamura, M., T. Hazato, H. Ashino, Y. Yamamoto, E. Iwasaki, H. Tobe, K. Yamamoto, and S. Yamamoto. 2001. Inhibition of angiogenesis by humulone, a bitter acid from beer hop. *Biochem. Biophys. Res. Commun.* 289:220–224.

Shimura, M., A. Hasumi, T. Minato, M. Hosono, Y. Miura, S. Mizutani, K. Kondo, S. Oikawa, and A. Yoshida. 2005. Isohumulones modulate blood lipid status through the activation of PPAR alpha. *Biochim. Biophys. Acta.* 1736:51–60.

Shimwell, J.L. 1937. On the relation between the staining properties of bacteria and their reaction towards hops antiseptic. *J. Inst. Brewing,.* 43:111.

Siegel, L., A. Miternique-Grosse, C. Griffon, C. Klein-Soyer, A. Lobstein, F. Raul, and D. Stephan. 2008. Antiangiogenic properties of lupulone, a bitter acid of hop cones. *Anticancer Res.* 28:289–294.

Simpson, W.J., and A.R.W. Smith. 1992. Factors affecting antibacterial activity of hop compounds and their derivatives. *Journal of Applied Microbiology.* 72:327–334.

Soderberg, U., and C.A. Wachtmeister. 1955. Pharmacological effects of humulone on cats and rabbits. *Acta Physiol. Scand.* 34:90–98.

Srinivasan, V., D. Goldberg, and G.J. Haas. 2004. Contributions to the Antimicrobial Spectrum of Hop Constituents. *Economic Botany.* 58:S230–S238.

Stavén-Grönberg, A. 1927. Über die Wirkungen der Hopfensubstanzen bei Fröschen. *Naunyn-Schmiedeberg's Archives of Pharmacology.* 123:272–281.

Steidle, H. 1931. Zur Pharmakologie des Hopfens. *Naunyn-Schmiedeberg's Archives of Pharmacology.* 161:154–162.

Steiling, H., M. Mühlbauer, F. Bataille, J. Schölmerich, S. Werner, and C. Hellerbrand. 2004. Activated hepatic stellate cells express keratinocyte growth factor in chronic liver disease. *Am. J. Pathol.* 165:1233–1241.

Surh, Y.J., K.S. Chun, H.H. Cha, S.S. Han, Y.S. Keum, K.K. Park, and S.S. Lee. 2001. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. *Mutat. Res.* 480-481:243–268.

Suzuki, K., K. Iijima, K. Sakamoto, M. Sami, and H. Yamashita. 2006. A Review of Hop Resistance in Beer Spoilage Lactic Acid Bacteria. *J. Inst. Brew.* 112:173–191.

Tagashira, M., M. Watanabe, and N. Uemitsu. 1995. Antioxidative activity of hop bitter acids and their analogues. *Biosci. Biotechnol. Biochem.* 59:740–742.

Teotico, D.G., J.J. Bischof, L. Peng, S.A. Kliewer, and M.R. Redinbo. 2008. Structural basis of human pregnane X receptor activation by the hops constituent colupulone. *Mol. Pharmacol.* 74:1512–1520.

Teuber, M. 1970. Low Antibiotic Potency of Isohumulone. *Appl. Microbiol.* 19:871–871.

Teuber, M., and A.F. Schmalreck. 1973. Membrane leakage in Bacillus subtilis 168 induced by the hop constituents lupulone, humulone, isohumulone and humulinic acid. *Archives of Microbiology.* 94:159–171.

Thasler, W.E., T.S. Weiss, K. Schillhorn, P.-T. Stoll, B. Irrgang, and K.-W. Jauch. 2003. Charitable State-Controlled Foundation Human Tissue and Cell Research: Ethic and Legal Aspects in the Supply of Surgically Removed Human Tissue For Research in the Academic and Commercial Sector in Germany. *Cell Tissue Bank.* 4:49–56.

Thompson, C.B. 1995. Apoptosis in the Pathogenesis and Treatment of Disease. *Science.* 267:1456–1462.

Tobe, H., Y. Muraki, K. Kitamura, O. Komiyama, Y. Sato, T. Sugioka, H.B. Maruyama, E. Matsuda, and M. Nagai. 1997. Bone resorption inhibitors from hop extract. *Biosci. Biotechnol. Biochem.* 61:158–159.

Tsuboi, Y., T. Ichida, S. Sugitani, T. Genda, J. Inayoshi, M. Takamura, Y. Matsuda, M. Nomoto, and Y. Aoyagi. 2004. Overexpression of extracellular signal-regulated protein kinase and its correlation with proliferation in human hepatocellular carcinoma. *Liver Int.* 24:432–436.

Vallabhapurapu, S., and M. Karin. 2009. Regulation and Function of NF-κB Transcription Factors in the Immune System. *Annual Review of Immunology.* 27:693–733.

Villanueva, A., P. Newell, D.Y. Chiang, S.L. Friedman, and J.M. Llovet. 2007. Genomics and signaling pathways in hepatocellular carcinoma. *Semin. Liver Dis.* 27:55–76.

Wang, G., L. Tian, N. Aziz, P. Broun, X. Dai, J. He, A. King, P.X. Zhao, and R.A. Dixon. 2008. Terpene biosynthesis in glandular trichomes of hop. *Plant Physiol.* 148:1254–1266.

Wärtgen, K. 1990. Über den Einfluss äusserer Faktoren auf die Stabilität der Hopfenbitterstoffe. 195 pp.

Wieland, H. 1925. Über die chemische Natur der Hopfenharz - Säuren (I.). (In Gemeinschaft mit Walter Schneider und Erwin Martz). *Berichte der deutschen chemischen Gesellschaft (A and B Series).* 58:102–112.

Wöllmer, W. 1916. Über die Bitterstoffe des Hopfens. *Berichte der deutschen chemischen Gesellschaft.* 49:780–794.

Wöllmer, W. 1925. Über die Bitterstoffe des Hopfens. *Berichte der deutschen chemischen Gesellschaft (A and B Series).* 58:672–678.

Wouters, K., P.J. van Gorp, V. Bieghs, M.J. Gijbels, H. Duimel, D. Lütjohann, A. Kerksiek, R. van Kruchten, N. Maeda, B. Staels, M. van Bilsen, R. Shiri-Sverdlov, and M.H. Hofker. 2008. Dietary cholesterol, rather than liver steatosis, leads to hepatic inflammation in hyperlipidemic mouse models of nonalcoholic steatohepatitis. *Hepatology.* 48:474–486.

Yajima, H., E. Ikeshima, M. Shiraki, T. Kanaya, D. Fujiwara, H. Odai, N. Tsuboyama-Kasaoka, O. Ezaki, S. Oikawa, and K. Kondo. 2004. Isohumulones, bitter acids derived from hops, activate both peroxisome proliferator-activated receptor alpha and gamma and reduce insulin resistance. *J. Biol. Chem.* 279:33456–33462.

Yajima, H., T. Noguchi, E. Ikeshima, M. Shiraki, T. Kanaya, N. Tsuboyama-Kasaoka, O. Ezaki, S. Oikawa, and K. Kondo. 2005. Prevention of diet-induced obesity by dietary isomerized hop extract containing isohumulones, in rodents. *Int J Obes (Lond).* 29:991–997.

Yamamoto, K., J. Wang, S. Yamamoto, and H. Tobe. 2000. Suppression of cyclooxygenase-2 gene transcription by humulon of beer hop extract studied with reference to glucocorticoid. *FEBS Letters*. 465:103–106.

Yasukawa, K., A. Yamaguchi, J. Arita, S. Sakurai, A. Ikeda, and M. Takido. 2006. Inhibitory effect of edible plant extracts on 12 - O - tetradecanoylphorbol–13 - acetate - induced ear oedema in mice. *Phytotherapy Research*. 7:185–189.

Zanoli, P., and M. Zavatti. 2008. Pharmacognostic and pharmacological profile of Humulus lupulus L. *J Ethnopharmacol*. 116:383–396.

Zhao, Q., and F.S. Lee. 1999. Mitogen-activated protein kinase/ERK kinase kinases 2 and 3 activate nuclear factor-kappaB through IkappaB kinase-alpha and IkappaB kinase-beta. *J. Biol. Chem.* 274:8355–8358.

7 Abbreviations

AOM	azoxymethane
α-sma	α-smooth muscle actin
AST	aspartate aminotransferase
AP-1	activator protein-1
BA	bitter acids
BW	body weight
°C	degree Celsius
cDNA	complementary DNA
col I	collagen type I
COX1	cyclooxygenase 1
COX2	cyclooxygenase 2
CREB	cAMP response element-binding protein
Ctr	control
d	day
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
ED_{50}	effective dose (half-maximal inhibitory dose)
e.g.	*exempli gratia*
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMSA	electromobility shift assay
ERK	extracellular-signal-regulated kinases
et al.	*et alii*
FCS	fetal calf serum
g	gram
G	gravitational acceleration
GRα	glucocorticoid receptor α

GOT	glutamic oxaloacetic transaminase
GPT	glutamic pyruvic transaminase
h	hour
HCC	hepatocellular carcinoma
HDL	high density lipoprotein
HL-60	premyocytic leukemia cell line
HPLC	high pressure liquid chromatography
HSC	hepatic stellate cells
HRP	horse radish peroxidise
IC$_{50}$	half-maximal inhibitory concentrations
ID$_{50}$	half-maximal inhibitory dose
IHE	isomerized α-acid extract
IκBα	inhibitory *kappa* B *alpha*
IKK	IκB kinase
IL-6	interleukin-6
INF γ	interferon γ
IVC	vena cava inferior
JNK	c-Jun N-terminal kinases
KGF	keratinocyte growth factor
l	liter
LPS	lipopolysaccharide
LSEC	liver sinusoidal endothelial cells
M	molar; mol/l
mA	miliampere
MAPK	mitogen-activated protein kinases
MC3T3-E1	murine osteoblastic cell line
MCP-1	monocyte chemotactic protein; *CCL-2*
META060	tetrahydro-iso-α-acids
mg	milligram
min	minute
mM	milimolar
m/m	mass per mass
MMPs	matrix metalloproteinases
mRNA	messenger ribonucleic acid

nm	nanometer
µg	microgram
µl	microliter
µm	micrometer
NAFLD	non-alcoholic fatty liver disease
NASH	non-alcoholic steatohepatitis
NOAEL	no-observed-adverse-effect levels
NSAIDs	nonsteroidal anti-inflammatory drugs
NFκB	nuclear factor *kappa* B
PBS	phosphate buffered saline
PDGF	plated derived growth factor
PGE2	prostaglandin E2
PI3K	phosphatidylinositol 3-kinase
PPAR	peroxisome proliferator-activated receptor
PXR	pregnane X receptor
q-RT-PCR	quantitative real time PCR
Rf	retention factor
RIAA	*rho*-iso-α-acids
RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
s	second
TIMPs	tissue inhibitors of metalloproteinases
TNF α	tumor necrosis factor α
TPA	12-O-tetradecanoylphorbol-13-acetate
TRAIL	TNF-related apoptosis inducing ligand
U	unit
V	volt
VEGF	vascular endothelial growth factor
v/m	volume per mass
v/v	volume/volume
WBA	whole blood assays
w/v	weight per volume
XN	xanthohumol

8 Appendix

8.1 Curriculum Vitae

Michael Sebastian Saugspier

Geburtsdatum: 10.12.1981

Geburtsort: Deggendorf

Staatsangehörigkeit: deutsch

09/88 bis 08/92	Volksschule St. Martin II, Deggendorf
09/92 bis 07/01	Robert-Koch Gymnasium, Deggendorf
09/01 bis 06/02	Zivildienst bei Erholungshilfe Sozialtherapeutische Erlebnisreisen e.V. Hannover
10/02	Beginn des Diplomstudienganges Biologie im WS 02/03 an der Universität Regensburg
09/05	Vordiplom
11/05 bis 01/06	Pharmazeutische Biologie III Praktikum (Prof. Dr. J. Heilmann)
01/06 bis 02/06	Botanisches Großpraktikum I
02/06 bis 04/06	Studentische Hilfskraft am BIOTA II Projekt (Prof. Dr. P. Poschlod)
05/06 bis 07/06	Molekularbiologisches Großpraktikum (Block A, B und C)
10/06 bis 11/06	Botanisches Forschungspraktikum (Prof. Dr. C. Oberprieler)
01/07	Studentische Hilfskraft (Prof. Dr. C. Oberprieler)
02/07 bis 03/07	Schwerpunktpraktikum am Lehrstuhl für Zellbiologie (Prof. Dr. T. Dresselhaus)
04/07 bis 05/07	Forschungspraktikum am Lehrstuhl für Pharmazeutische Biologie (Prof. Dr. J. Heilmann)
06/07 bis 07/07	Forschungspraktikum am Lehrstuhl für Humangenetik (Prof. Dr. T. Langmann)
12/07	Mündliche Diplomprüfungen
01/08 bis 10/08	Diplomarbeit am Lehrstuhl für Humangenetik (Prof. Dr. T. Langmann)
11/08	Dipl. –Biol. Univ. (1,3)
02/09	Beginn des Promotionsvorhabens am Lehrstuhl für Pharmazeutische Biologie an der Universität Regensburg

8.2 Advanced training courses

30.-31. März 2009	Fortbildungsveranstaltung für Projektleiter und Beauftragte für Biologische Sicherheit (BBS) in Regensburg
2010	Weiterbildungsveranstaltung "Versuchstierkunde und Tierschutz" in Regensburg

8.3 Publications

- Morczeck C., Schmalz G., Reichert T.E., Völlner F., Saugspier M., Viale-Bouroncle S., Driemel O.
  Gene expression profiles of dental follicle cells before and after osteogenic differentiation *in vitro. Clin Oral Investig.* **13(4)**, 383-391 (2009).

- Ernst W., Saugspier M., Felthaus O., Driemel O., Morczeck C.
  Comparison of murine dental follicle precursor and retinal progenitor cells after neural differentiation *in vitro. Cell Biol Int.* **33(7)**, 758-764 (2009).

- Morczeck C., Völlner F., Saugspier M., Brandl C., Reichert T.E., Driemel O., Schmalz G.
  Comparison of human dental follicle cells (DFCs) and stem cells from human exfoliated deciduous teeth (SHED) after neural differentiation *in vitro. Clin Oral Investig.* **14(4)**, 433-440 (2010).

- Saugspier M., Felthaus O., Viale-Bouroncle S., Driemel O., Reichert T.E., Schmalz G., Morczeck C.
  The differentiation and gene expression profile of human dental follicle cells. *Stem Cells Dev.* **19(5)**, 707-717 (2010).

- Dorn C., Riener M.O., Kirovski G., Saugspier M., Steib K., Weiss T.S., Gäbele E., Kristiansen G., Hartmann A., Hellerbrand C.
  Expression of fatty acid synthase in nonalcoholic fatty liver disease. *Int J Clin Exp Pathol.* **3(5)**, 505-514 (2010).

- Klein M.S., Dorn C., Saugspier M., Hellerbrand C., Oefner P.J., Gronwald W.

  Discrimination of steatosis and NASH in mice using nuclear magnetic resonance spectroscopy. *Metabolomics* **7**, 237-246 (2011)

- Saugspier M., Dorn C., Thasler W.E., Gehrig M., Heilmann J., Hellerbrand C.

  Hop bitter acids exhibit anti-fibrogenic effects on hepatic stellate cells *in vitro. Exp Mol Pathol.* **92(2)**, 222-228 (2012).

- Saugspier M., Dorn C., Czech B., Gehrig M., Heilmann J., Hellerbrand C.

  Hop bitter acids inhibit tumorigenicity of hepatocellular carcinoma cells *in vitro. Oncology report (in press).*

8.4 Poster presentations

27. Jahrestagung der Deutschen Arbeitsgemeinschaft zum Studium der Leber
(28. - 29. Jan. 2011, Regensburg, Germany):

- Saugspier M., Dorn C., Gehrig M., Heilmann J., Hellerbrand C.

  Hop bitter acids exhibit anti-fibrogenic effects an hepatic stellate cells *in vitro. Z Gastroenterol.* **49(1)**, 79 (2011)

- Saugspier M., Heilmann J., Hellerbrand C.

  Hop bitter acids inhibit tumorigenicity of hepatocellular carcinoma cells. *Z Gastroenterol.* **49(1)**, 105 (2011)

8.5 Danksagung

Die vielen fachlichen Diskussionen und Ratschläge, aber auch der Rückhalt und die aufmunternden Worte vieler Menschen, haben mich während meiner Doktorarbeit begleitet. Daher möchte ich nun meinen ganz besonderen Dank aussprechen:

Ich danke **Herrn Prof. Dr. Claus Hellerbrand**, der mir mit seinem Fachwissen stets zur Seite stand und mir die wissenschaftliche Herangehensweise aufzeigte. Vielen Dank für die offene und freundschaftliche Art und den Rückhalt und das Vertrauen in mich !

Ich danke **Herrn Prof. Dr. Jörg Heilmann,** der immer an mich geglaubt hat. Ohne ihn wäre diese Doktorarbeit nicht möglich gewesen. Vielen Dank für die fachlichen Diskussionen und Ratschläge und das familiäre Arbeitsklima !

Ich danke **Frau Prof. Dr. Charlotte Wagner** für Ihre Bereitschaft als Drittprüfer zu fungieren.

Ich danke **Herrn Prof. Dr. Gerhard Franz** für die Übernahme des Vorsitzes der Prüfungskommission.

Auch bedanken möchte ich mich bei **Herrn Dr. Manfred Gehrig, Herrn Roland Schmidt und Frau Sabine Massinger von NATECO₂** für die Bereitstellung der Extrakte, die Analysen und Ihr eingebrachtes Fachwissen auf dem Gebiet der Hopfenbitterstoffe.

Ein großer Dank gilt der „Lebergruppe": **Abdo Mahli, Andreas Koch, Barbara Czech, Birgitta Ott-Rötzer, Dr. Christoph Dorn, Daniela Valletta, Diane Lochbaum, Georgi Kirovski, Heidi Gschwendtner, Karin Dostert, Karin Grünberger, Kornelia Elser, Leandra Koletzko, Margit Nützel, Marina Fink, Monika Artinger, Ruth Schewior, Sabine Kiessling und Dr. Thomas Amann** für die gemeinsame Zeit und die vielen schönen Momente. Ein besseres Arbeitsklima kann man sich nicht vorstellen.

Ebenso möchte ich mich bei allen **Kollegen des Forschungsbaus H1** für die stets große Hilfsbereitschaft und die vielen schönen Momente bedanken!!

Ein großer Dank gilt auch der „PharmBio" Arbeitsgruppe: **Dr. Anne Freischmidt, Anne Grashuber, Dr. Birgit Kraus, Daniel Bücherl, Gabi Brunner, Dr. Guido Jürgenliemk, Hedwig Ohli, Katharina Zenger, Magdalena Motyl, Marcel Flemming, Dr. Matej Barbic, Dr. Regina Schlegl, Rosmarie Scherübl, Dr. Sarah Sutor, Sebastian Schmidt, Dr. Susann Haase, Susanne Knuth, Dr. Susanne Vogel**. Bei Euch habe ich mich immer wohl gefühlt!!

Natürlich möchte ich mich bei meinen Freunden bedanken, die mir es nicht krumm genommen haben, mal keine Zeit für sie zu haben. Vielen Dank **Malte, Johannes, Schos, Christina und Manuel**.

Ein ganz großes Dankeschön an **meine Familie,** allen voran **meinen Eltern, meinem Bruder Daniel**, **meiner Oma**, **meinem Onkel Robert** und **meinen Schwiegereltern** für alles, was sie mir ermöglicht haben. Danke für Euer Verständnis und Euren Rückhalt!!

Am meisten möchte ich mich bei Dir bedanken liebe Steffi!! Es ist schön zu wissen, dass Du immer für mich da bist und an mich glaubst.

8.6 Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

Weitere Personen waren an der inhaltlich-materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

....
         (Ort, Datum)   (Unterschrift)