Characterization of *in vitro* and *in vivo* models for the investigation of hepatotoxicity

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2 Abbreviations

AHR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
ATP	Adenosine-5'-triphosphate
BBD	γ-butyrobetaine dioxygenase
BSA	Bovine serum albumin
BSEP	Bile salt export pump
CACT	Carnitine-acylcarnitine translocase
CAT	Carnitine acetyltransferase
cDNA	Complementary Deoxyribonucleic acid
CoA-SH	Acetyl conenzyme A
CPT I	Carnitine palmitoyltransferase I
CPT II	Carnitine palmitoyltransferase II
CYP	Cytochrome P450
DILI	Drug-induced liver injury
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EMEA	European Medicines Agency
FACS	Fluorescence activating cell sorting
FBS	Foetal bovine serum
GABA	γ-aminobutyric acid
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GHB	γ-hydroxbutyrate
GSH	Glutathione
GSSG	Oxidized glutathione
HEPES	N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HRT	Hormone replacement therapy
HTML	3-hydroxy-6-*N*-trimethyl-lysine
JC-1	5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-

	carbocyanide iodide
JVS	Juvenile visceral steatosis
LCA Cn	Long-chain-acylcarnitine
LDH	Lactate dehydrogenase
LS180	Human colon carcinoma cell line type LS180
MDR	Multi-drug resistance
mRNA	Messenger ribonucleic acid
MRP	Multi-drug resistance associated protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCE	New chemical entity
NEAA	Non essential amino acids
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
OCTN	Carnitine/organic cation transporter
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PXR	Pregnane X receptor
RPMI	Roswell Park Memorial Institute 1640 Medium
RXR	Retinoic X receptor
SCA Cn	Short-chain-acylcarnitine
SCD	Systemic Carnitine Deficiency
SRB	Sulforhodamine B
SV40Tag	Simian virus 40 large T antigen
TAS Cn	Total acid soluble carnitine
TMBA	4-trimethylaminobutyraldehyde
TMBA-DH	4-trimethylaminobutyraldehyde dehydrogenase
TML	6-*N*-trimethyl-lysine
TMLD	6-*N*-trimethyl-lysine dioxygenase
VPA	Valproic acid
WEM	Williams E Medium
WT	Wild type
zFA-fmk	Z-Phe-Ala-fluoromethylketone
zVAD-fmk	Z-Val-Ala-Asp-fluoromethylketone

3 Summary

The liver is the primary site of drug metabolism and plays a major role in metabolism, digestion, detoxification, and elimination of drugs and toxins from the body. Consequently, drugs affect the liver more frequently than any other organ and place the liver at increased risk for toxic damage. Drug-induced liver injury (DILI) is a common cause of acute liver failure and the most frequent reason for the withdrawal of approved drugs, representing a serious challenge for the pharmaceutical industry. The risk of developing hepatotoxicity is not only due to the chemical properties of the drug but also to environmental factors, pre-existing diseases and genetic factors, leading to the classification into either predictable (high incidence) or unpredictable (low incidence) hepatotoxicity. Drugs that produce predictable liver injury are generally a result of direct liver toxicity of the parent drug or its metabolites. However, the majority of adverse drug-induced hepatic events are unpredictable and the underlying mechanisms are mostly unknown, but assumed to be either immune-mediated hypersensitivity reactions or idiosyncratic and are able to alter the susceptibility to adverse events. In recent years mitochondrial dysfunction has been recognized as β-oxidation of fatty acids, inhibition or uncoupling of the respiratory chain, or through a primary effect on the mitochondrial genome.

One aim of this thesis was to investigate the juvenile visceral steatosis (jvs) mouse, which is characterized by microvesicular steatosis of the liver and to impaired renal reabsorption leading to systemic carnitine deficiency. The main focus was put on the assessment of the hepatic toxicity of valproate, an antiepileptic drug known to induce liver injury, and to investigate whether the underlying carnitine deficiency is a risk factor for valproate-associated hepatotoxicity. Furthermore, *in vitro* studies using several hepatic cell lines were performed to estimate the suitability as screening systems for hepatic metabolism and CYP induction, and one study was conducted to evaluate the hepatotoxic effect of the plant cimicifuga racemosa.

Initially we assessed the carnitine homeostasis and energy metabolism in carnitine-deficient (jvs-/-) mice after cessation of carnitine substitution (Chapter 6). It is well established that sufficient carnitine plasma and tissue levels in jvs mice can be obtained by carnitine substitution, correcting carnitine deficiency. We studied the

kinetics of carnitine loss from plasma and tissue carnitine stores and markers of energy metabolism after carnitine deprivation for a maximum of ten days. The total carnitine concentrations in plasma, liver and skeletal muscle were significantly decreased, whereas carnitine concentration decreased rapidly in plasma but much slower in tissue. Deprivation of carnitine was also associated with a further drop in the plasma β-hydroxybutyrate levels and hepatic fat accumulation.

In a second *in vivo* experiment (Chapter7) we investigated whether carnitine deficiency is a risk factor for valproate-associated hepatotoxicity in jvs mice, and we assessed the effects of valproate on carnitine plasma and tissue stores in these mice. Therefore, we treated heterozygous jvs+/- and the corresponding wild type mice with subtoxic oral doses of valproate for two weeks. Our study shows that jvs+/- mice treated with VPA have impaired hepatic mitochondrial β-oxidation and increased hepatic fat accumulation, findings associated with increased activities of serum transaminases and alkaline phosphatase, and hepatocellular damage. Furthermore, the effect of VPA treatment on the carnitine plasma and tissue stores was much more dramatic in JVS+/- than in wild type mice, leading to additional and substantial losses in the plasma and tissue carnitine pools. In conclusion, hepatic toxicity of VPA was more pronounced in JVS+/- mice than in corresponding wild type mice, and systemic carnitine deficiency can therefore be considered to be a risk factor for hepatotoxicity associated with VPA.

In an *in vitro* study using hepatic cell lines (Chapter 8), drug-induced changes in the activity of cytochrome P450 isoforms were assessed. Since the activity of most CYPs can be regulated by induction and/or inhibition by specific drugs, and possibly affecting the metabolism of other drugs or even their own metabolism, we investigated the expression and induction of several CYP isozymes and the human pregnane X receptor in immortalized human hepatocytes for their suitability as screening systems for hepatic drug metabolism. Our investigations demonstrated that hHepLT5 cells contain the main human CYP isozymes CYP1A2 and CYP3A4 which are important for drug metabolism. Summarized, hHepLT5 cells appear therefore to be a valuable alternative for primary human hepatocytes for studying pharmacological and toxicological features of new drug entities.

The last described study (Chapter 9) was conducted to assess the hepatotoxicity of cimicifuga racemosa in experimental animals *in vivo*, in hepatocyte cultures and in isolated liver mitochondria. Ethanolic cimicifuga racemosa extract was administered orally to rats and liver sections were analyzed for microvesicular steatosis by electron microscopy. Tests for cytotoxicity, mitochondrial toxicity and apoptosis/necrosis were performed using HepG2 cells, and mitochondrial toxicity was studied using isolated rat liver mitochondria. The main findings *in vivo* and *in vitro* were hepatic mitochondrial toxicity, as evidenced by microvesicular steatosis and inhibition of β-oxidation, eventually resulting in apoptotic cell death. These findings suggest that inhibition of β-oxidation is the initial hepatotoxic event of cimicifuga extract, which eventually may result in apoptosis of the hepatocytes.

4 Aim of the thesis

The major purpose was to characterize *in vitro* and *in vivo* systems for the evaluation of drug-induced hepatotoxicity. For *in vivo* experiments we used a mouse model with systemic carnitine to estimate whether a pre-existing mitochondrial dysfunction due to inhibition of the β-oxidation of fatty acids represents a risk factor for susceptibility to drug-induced hepatotoxicity. *In vitro* studies were performed with hepatic cell lines, namely hepatocellular carcinoma cells and immortalized human hepatocytes. These cells were characterized by studying the expression and induction of drug metabolizing enzymes as a useful tool to study the hepatic metabolism of different drugs and for toxicological investigations.

Summarized, the following issues were studied:

I) Carnitine homeostasis and energy metabolism in carnitine-deficient (jvs-/-) mice after cessation of carnitine substitution

II) Toxicity of valproic acid in jvs mice with impaired β-oxidation associated with carnitine deficiency

III) Expression and inducibility of cytochrome P450 isozymes in immortalized human hepatocytes

IV) Hepatotoxic effects of cimicifuga racemosa (black cohosh).

5 Introduction

5.1 In vivo investigations

5.1.1 Carnitine – Functions

Carnitine (β-hydroxy-4-*N*-trimethylaminobutyric acid) has several important intracellular functions.
Primarily, it represents an essential cofactor for the transport of activated long-chain fatty acids across the inner mitochondrial membrane to the mitochondrial matrix (Figure 2), where β-oxidation takes place (Bremer, 1983; Rebouche and Paulson, 1986). Cytosolic long-chain fatty acids, which are present as CoA esters, are activated by a specific acyl-CoA synthase at the outer mitochondrial membrane. The long-chain acyl-CoAs (e.g. palmitoyl-CoA) are further conjugated to carnitine by carnitine palmitoyltransferase I (CPT I). The resulting long-chain acylcarnitine esters are transported over the inner mitochondrial membrane via the specific carrier carnitine-acylcarnitine translocase (CACT) and reconverted to long-chain acyl-CoAs in the mitochondrial matrix by carnitine palmitoyltransferase II (CPTII). In the mitochondria, the long-chain acyl-CoAs undergo β-oxidation, resulting in the production of acetyl-CoA. Short and medium-chain acyl-CoAs can be reconverted into acylcarnitines by the enzyme carnitine acetyltransferase (CAT) and can then leave the mitochondria via CACT for another round of transport.

Carnitine plays also an important role in the transfer of products of the peroxisomal β-oxidation, e.g. acetyl-CoA, to the mitochondria for the oxidation to CO_2 and H_20 in the Krebs cycle (Wanders et al., 1995). Other functions of carnitine include the modulation of the free CoA/acyl-CoA ratio, the storage of energy as acetylcarnitine and the detoxification of potentially toxic, poorly metabolized acyl groups by excreting them as carnitine esters (Bremer, 1983; Bieber, 1988; Steiber et al., 2004).

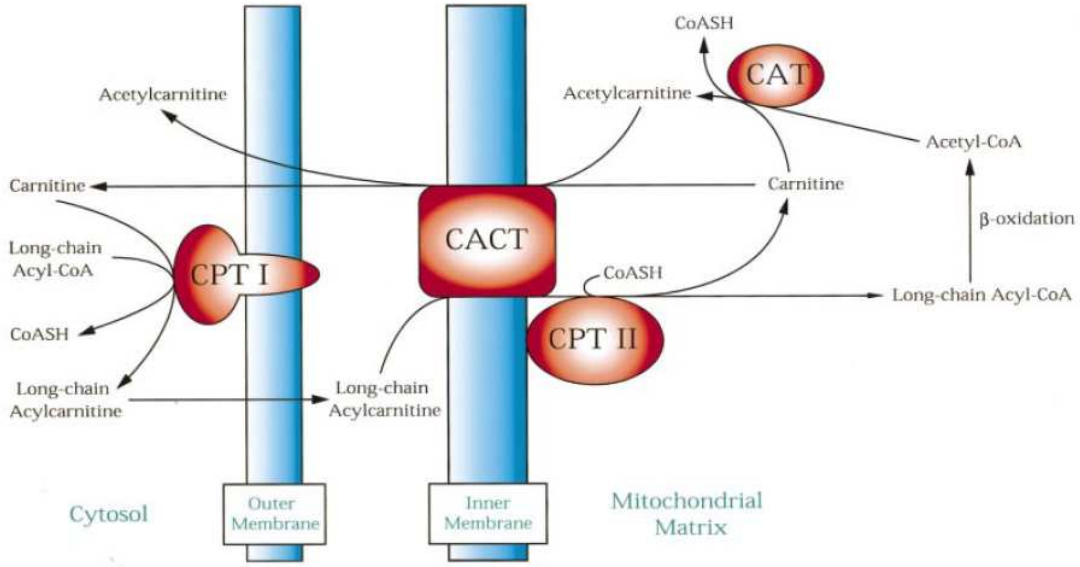


Figure1: Function of carnitine in the transport of mitochondrial long-chain fatty acid oxidation and regulation of the intramitochondrial free CoA/acyl CoA ratio

5.1.2 Carnitine – Biosynthesis

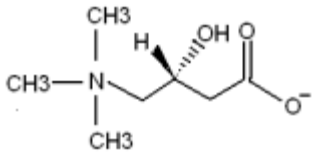


Figure 2: L-Carnitine

Carnitine, a water-soluble zwitterion, is a chemically simple substance, physiologically presented as L-enantiomer and containing a negatively charged carboxylate at C1 and a positively charged quaternary nitrogen at C4 at physiological pH (Figure 2). Most of the carnitine needed is obtained from the diet, in particular by meat and dairy products. The rest is biosynthesized, starting from the amino acids lysine and methionine, whereas lysine provides the carbon backbone and the 4-*N*-methyl groups originate from methionine (Tanphaichitr et al., 1971; Horne and Broquist, 1973).

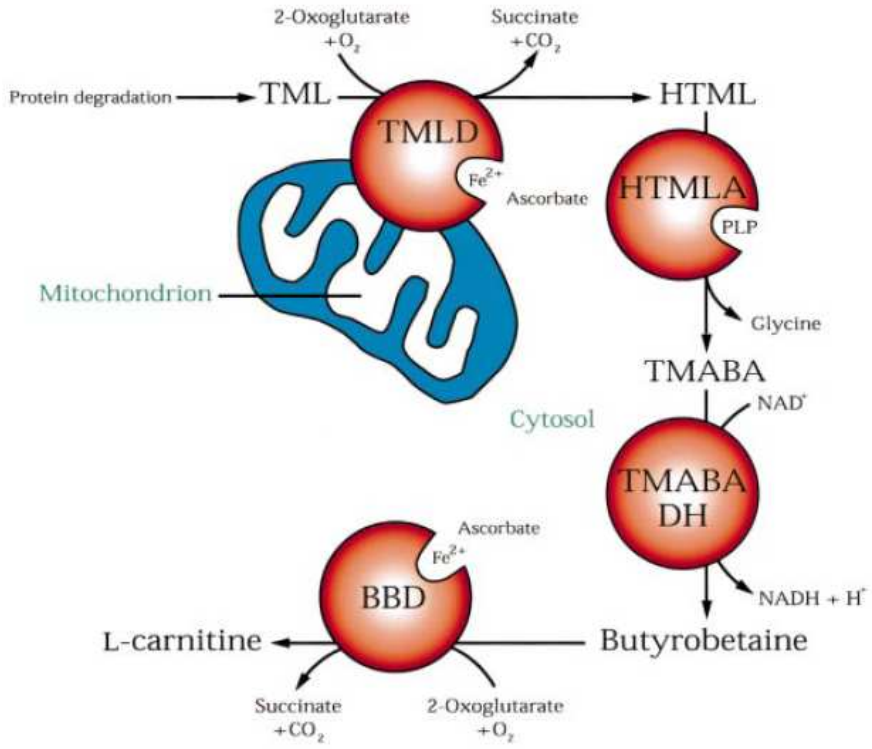
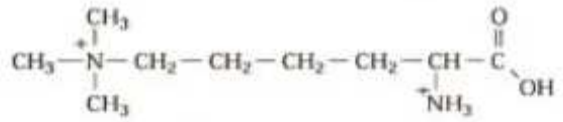


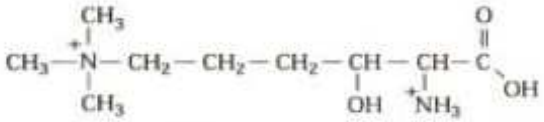
Figure 3: Pathway of carnitine biosynthesis

In mammals, the carnitine biosynthesis is initiated by the N-methylation of the protein linked L-lysine. This reaction is catalyzed by specific methyltransferases, which use S-adenosyl-L-methionine as a methyl donor (Paik and Kim, 1971; Cox and Hoppel, 1973). Lysosomal hydrolysis of these proteins results in the release of 6-*N*-trimethyl-lysine (TML), the first metabolite of carnitine biosynthesis (LaBadie et al., 1976; Dunn and Englard, 1981). The following hydroxylation on the 3-position by TML dioxygenase (TMLD) yields 3-hydroxy-TML (HTML), which is cleaved to 4-trimethylaminobutyraldehyde (TMABA) and glycine, a reaction catalysed by the HTML aldolase (HTMLA). Dehydrogenation of TMABA by the TMABA dehydrogenase (TMABA-DH) results in the formation of 4-*N*-trimethylaminobutyrate (butyrobetaine). In the last step, butyrobetaine is hydroxylated on the 3-position by γ-butyrobetaine dioxygenase (BBD). Human skeletal muscle, heart, liver, kidney and brain are capable to the biosynthesis of carnitine from methionine and lysine to its immediate precursor γ-butyrobetaine (Rebouche and Engel, 1980). Final conversion of γ-butyrobetaine to L-carnitine by γ-butyrobetaine hydroxylase can only be done in liver, kidney and brain in humans (Englard, 1979). The chemical structure of the

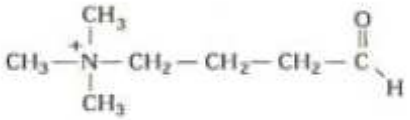
intermediates and the enzymes of the carnitine biosynthesis are shown in Figure 3 and 4.



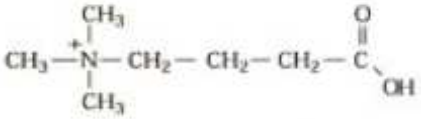
N^6-trimethyllysine (TML)



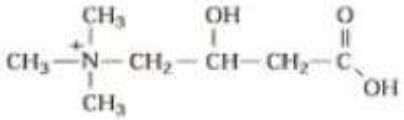
3-hydroxy-N^6-trimethyllysine (HTML)



4-*N*-trimethylaminobutyraldehyde (TMABA)



4-*N*-trimethylaminobutyrate (butyrobetaine)



L-carnitine

Figure 4: Metabolites of the carnitine biosynthesis

5.1.3 Carnitine – Absorption, Metabolism and Elimination

In omnivores, approximately 75% of carnitine sources are from the diet and about 25% from endogenous synthesis, whereas in strict vegetarians, endogenous carnitine synthesis provides >90% of the total available carnitine (Rebouche, 1992). Carnitine homeostasis in mammals is maintained by a combination of absorption of carnitine from dietary sources, a modest rate of endogenous synthesis, efficient reabsorption from the glomerular, and mechanisms present in most tissues that establish and maintain substantial concentration gradients between intracellular and extracellular carnitine pools. The rate of carnitine biosynthesis in humans is estimated to be about 1.2 µmol per kg body weight per day, which was evaluated from the steady-state rate of excretion of carnitine by strict vegetarian adults and children (Lombard et al., 1989). The major dietary sources of carnitine are meat, poultry, fish and dairy products (Rebouche and Engel, 1984). An average omnivorous diet provide 2 to 12 µmol of carnitine per kilogram of body weight per day, in contrast to strict vegetarians consuming less than 0.1 µmol of carnitine per kilogram of body weight per day. Skeletal muscle contains over 90% of total body carnitine (Rebouche, 1992), and the plasma carnitine concentration is regulated largely by the renal threshold, which is approximately 40 µmol/l (Engel et al., 1981).

Since carnitine is found in very high concentrations in skeletal muscle, heart and epididymal fluid – tissues that lack the ability to synthesize carnitine - it is obvious that an active transport takes place, which has been reported to be sodium dependent (Rebouche and Mack, 1984). Absorption of carnitine results from a two-component system, namely a linear absorption, probably representing a passive diffusion, and a saturable system suggesting the presence of an active transport system (Hamilton et al., 1986). Additionally, carnitine absorption was shown to be dependent on the intake amount, whereas humans do not absorb all of the consumed carnitine, proving the theory that a specific active transporter, which can be saturated even with a normal dietary intake, might be involved (Harper et al., 1988).

It has been shown that carnitine is extensively metabolized in microorganisms, whereas bacteria are able to metabolize the trimethylammonium compound of carnitine in three different ways. Depending on the species and the cultivation

conditions used (e.g. aerobiosis, anaerobiosis) L-carnitine is catabolized by various pathways. Some, especially *Pseudomonas* species, assimilate carnitine as a unique source of carbon and nitrogen. The first catabolic step is the oxidation of the β-hydroxy group of carnitine with formation of 3-dehydrocarnitine, which is catalyzed by the L-carnitine dehydrogenase. 3-dehydrocarnitine is degraded to glycine betaine and further metabolized by step demethylation to glycine (Lindstedt et al., 1970; Kleber, 1997). Others, for instance, *Acinetobacter* species are able to degrade only the carbon backbone of L-carnitine with formation of trimethylamine (Kleber et al., 1977). A third group of carnitine metabolizing microorganisms comprises different *Enterobacteriaccae.* These bacteria have the ability to metabolize L-carnitine, via crotonbetaine, to γ-butyrobetaine in the presence of carbon and nitrogen sources during anaerobic growth (Seim et al., 1980).

In contrast to microorganisms, mammals lack the enzymes which are responsible for the degradation of carnitine (Rebouche et al., 1984; Seim et al., 1985). It was shown, after oral administration of radioactive-labeled carnitine in rats, that urine and feces contained two radiolabeled metabolites which were identified as trimethylamine N-oxide and γ-butyrobetaine. For rats that received intravenous labelled carnitine or germ-free rats receiving the isotope orally or intravenously, the radioactivity recovered was in the form of carnitine and the mentioned metabolites were not found (Rebouche et al., 1984; Seim et al., 1985). It was concluded that the indigenous flora, but not the tissues of mammals, is responsible for carnitine degradation in the gastrointestinal tract. Same results were found in human studies, in which a tracer dose of radioactive-labeled carnitine was administered orally. The major metabolites found were trimethylamine N-oxide (primarily in urine) and γ-butyrobetaine (primarily in feces), whereas the formation of these metabolites was attributed to the bacterial flora in the gastrointestinal tract of humans (Rebouche and Chenard, 1991).

Under normal homeostasis conditions, carnitine is mainly eliminated by excretion in urine. In rats, 1 to 2 µmol of carnitine is excreted per 100 g body weight per day, whereas this amount represents 5 to 7% of the total body pool (Cederblad and Lindstedt, 1976; Brass and Hoppel, 1978). In these animals, the glomerular filtration rate is about 5 liters per day, contrary to the carnitine clearance with only 5 ml per day, which implies that 99.9% of the filtered carnitine is reabsorbed in the kidney (Brass and Hoppel, 1978). In healthy humans, the serum clearance of carnitine is

about 1 ml per day, the daily excretion in urine is 100 to 300 µmol and the tubular reabsorption in the kidney is 90 to 98% (Maebashi et al., 1976). In strict vegetarians, dietary carnitine supplementation did not significantly increase plasma carnitine concentration and did not alter the glomerular filtration rate. At normal physiological plasma carnitine concentrations, the rate of carnitine excretion was increased and the rate of carnitine reabsorption was decreased by carnitine supplementation. It was concluded that the kidney adapts to carnitine intake by reducing the efficiency of carnitine reabsorption (Rebouche et al., 1993). Excretion of carnitine also takes place into milk, whereas the carnitine concentration was shown to increase in the first week post-partum from 39 to 63 µmol/l and was stabilized at 45 µmol/l after one month (Borum, 1981).

5.1.4 Carnitine – Transport

Since the cloning of the first organic cation transporter OCT1 from rat kidney in 1994 many of other transport members belonging to the OCT family have been described. A subfamily of the organic cation transporter family, namely the carnitine/organic cation OCTN transporters have been isolated and characterized in mice (Tamai et al., 2000). The members include the low affinity transporter OCTN1, the high affinity transporter OCTN2 and the intermediate affinity transporter OCTN3, which have the ability to transport carnitine, but with variable characteristics. The primary function of these transporters is the elimination of cationic drugs and other xenobiotics. Carnitine transport through cation transporters has a pharmacological importance since the OCTN2 transports drugs such as valproate, verapamil and quinidine (Wu et al., 1999).

OCTN1, originally cloned from a human fetal kidney library, is widely expressed in various tissues (Tamai et al., 1997). Rat OCTN1, cloned from placenta, is expressed particularly in liver, intestine, kidney, brain and placenta. There is a very low affinity interaction between carnitine and rat OCTN1, and this transporter does not mediate Na^+-coupled carnitine transport to a significant extent (Wu et al., 2000). However, mouse OCTN1 can mediate carnitine transport in a Na^+-dependent manner, illustrating an apparent species difference in the specifity for the same transporter type (Tamai et al., 2000).

OCTN2 was first isolated from a human placental trophoblast cell line (Wu et al., 1998) and from a human kidney cDNA library (Tamai et al., 1998). This transporter is widely expressed in human tissues such as heart, skeletal muscle, kidney, placenta, small intestine and some brain areas (Tamai et al., 1998; Wu et al., 1998; Wu et al., 1999). OCTN2 functions as a Na^+-dependent carnitine transporter as well as Na^+-independent transporter for other organic cations. The Na^+-dependent L-carnitine transport by OCTN2 is done with high affinity with the apparent K_m value of 4.3 µM (Tamai et al., 1998). Several anionic drugs such as valproate, as well as cationic drugs (e.g. verapamil, emetine) and short-chain acyl esters of carnitine (e.g. acetyl-L-carntine), used as therapeutic agents in the treatment of a wide range of disorders, are also transported by OCTN2 (Wu et al., 1999) and consequently inhibit the OCTN2-mediated carnitine uptake (Figure 5).

Name of the compound	Formula	Therapeutic use
Carnitine	$C_7H_{15}NO_3$	Antihyperlipoproteinemic
Acetylcarnitine	$C_9H_{17}NO_4$	Neurotropic
Betaine	$C_5H_{11}NO_2$	Hepatoprotectant
Cephaloridine	$C_{19}H_{17}N_3O_4S_2$	Antibacterial
Choline	$[C_5H_{14}NO]^+$	Lipotropic
Emetine	$C_3H_7NO_2S$	Antiamebic and used in lung worm infection
Pyrilamine	$C_{17}H_{23}N_3O$	Antihistaminic
Quinidine	$C_{20}H_{24}N_2O_2$	Antiarrhythmic Cardiac depressor
Tetraethylammonium (TEA)	$C_8H_{20}N$	Nicotinic cholinergic antagonist
Valproate	$C_8H_{16}O_2$	Anticonvulsant Antiepileptic
Verapamil	$C_{27}H_{38}N_2O_4$	Antiangina Antiarrhythmic

Figure 5: Therapeutic uses of carnitine and certain organic cation drugs transported by OCTN2

The last member of the OCTN family is termed OCTN3 and was isolated from mice. The mouse OCTN3 was expressed predominantly in testis and weakly in kidney. Functionally, mouse OCTN3 mediates carnitine transport in a Na^+-independent manner, contrary to mouse OCTN1 and OCTN2 which transport carnitine in a Na^+-dependent mode, and additionally, OCTN3 has a higher specificity for carnitine transport than OCTN1 and OCTN2 (Tamai et al., 2000).

5.1.5 Mutations in OCTN2

The physiological significance of the transporters in the body is confirmed by the identification of hereditary diseases caused by mutations of genes encoding various transporters (Sesaki, 2000). In the case of OCTN2, the ultimate proof of its importance derives from mutations in the gene encoding the protein, which cause an autosomal recessive disease named primary systemic carnitine deficiency (SCD) (Nezu et al., 1999). There are numerous studies describing patients with nonsense or missense mutations in OCTN2 and different clinical manifestations. In SCD homozygous patients, who manifest symptoms like cardiomyopathy, progressive skeletal weakness, non-ketotic hypoglycaemia and hyperammonemia, many mutations in the OCTN2 protein have been identified (Figure 6).

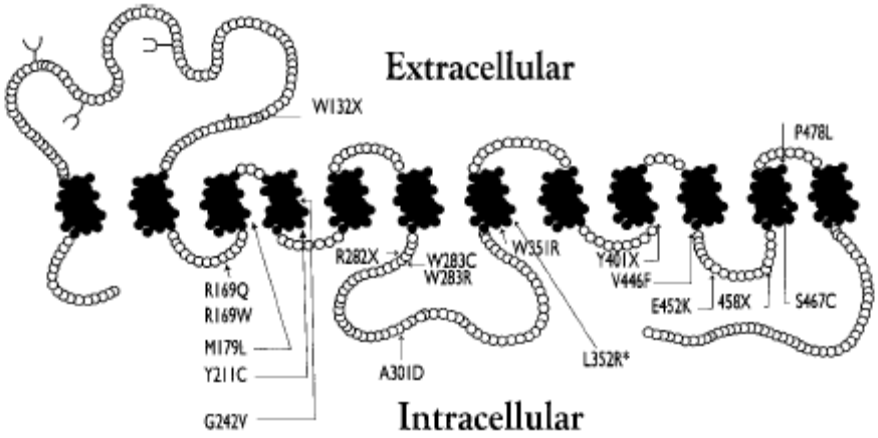


Figure 6: Mutations in the carnitine/organic cation transporter OCTN2 in humans

5.1.6 Systemic Carnitine Deficiency

Carnitine deficiency can be characterized by low plasma and tissue carnitine concentrations and can be defined as a decrease of intracellular carnitine, leading to an accumulation of acyl-CoA esters and an inhibition of acyl-transport via the mitochondrial inner membrane. Due to the two main functions of carnitine, namely the transport of long-chain fatty acids into the mitochondrial matrix for beta-oxidation to provide cellular energy and the modulation of the rise in intramitochondrial acyl-

CoA/CoA ratio, which relieves the inhibition of many intramitochondrial enzymes, the main consequence of carnitine deficiency is impaired energy metabolism and pathological changes in different tissues such as liver, muscle, heart and brain (Engel and Angelini, 1973; Karpati et al., 1975; Pons and De Vivo, 1995). Since the first description of human myopathic carnitine deficiency (Engel and Angelini, 1973), different forms of carnitine deficiency have been reported. According to their different ethiologies, human carnitine deficiency can be either hereditary or acquired. Hereditary carnitine deficiency can be grouped into three clinical entities: myopathic carnitine deficiency, systemic carnitine deficiency, and organic acidurias. Acquired carnitine deficiency is due to inadequate intake, increased requirement, and increased loss of carnitine (Angelini et al., 1992; Kerner and Hoppel, 1998).

5.1.6.1 Primary Carnitine Deficiency

Primary carnitine deficiency is defined as a decrease in intracellular carnitine content, which is associated with impaired fatty acid oxidation and with no other identifiable systemic disease that might deplete tissue carnitine stores (Millington and Roe, 1989). There are two forms of primary carnitine deficiency, depending on the tissue distribution of the low carnitine level: The systemic carnitine deficiency with low carnitine levels in plasma and the affected tissues, and the muscle carnitine deficiency, with low carnitine concentration restricted to muscle (Engel and Angelini, 1973; Karpati et al., 1975).

I. Systemic Carnitine Deficiency (SCD)

Primary systemic carnitine deficiency (SCD; OMIM 212140) is an autosomal recessive disorder characterized by progressive cardiomyopathy, skeletal myopathy hypoglycaemia and hyperammonemia (Karpati et al., 1975; Treem et al., 1988). It was first described in 1975 (Karpati et al., 1975), and is differentiated from myopathic carnitine deficiency (OMIM 212160). The defects in this disorder result from an impaired carnitine uptake into cells and are associated with a deficient renal carnitine transporter (Treem et al., 1988). There have been identified numerous point mutations in the gene encoding for the high affinity carnitine transporter OCTN2 in SCD patients (Nezu et al., 1999; Tang et al., 1999; Vaz et al., 1999; Wang et al., 1999; Wang et al., 2000). Treatment of SCD consists of daily high doses of orally

administered carnitine (100 to 200 mg/kg body weight), to ensure its absorption and to reverse or attenuate the clinical symptoms, whereas carnitine concentrations increase slightly in skeletal muscle and reach nearly normal levels in the liver, but without restoring totally the tissues carnitine stores (Angelini et al., 1992).

II. Myopathic Carnitine Deficiency (MCD)

In muscle carnitine deficiency, lipid storage myopathy occurs with low muscle carnitine but normal liver and serum carnitine and affected patients suffer from progressive muscle weakness and some of them from lipid storage myopathy (Engel and Angelini, 1973; Markesbery et al., 1974; VanDyke et al., 1975). Due to normal plasma levels, it has been assumed that MCD is associated with a defect in the low affinity muscle-specific carnitine transporter (Martinuzzi et al., 1991), and that this form of carnitine deficiency can be inherited as an autosomal recessive disorder, since parents also had low muscle carnitine levels (VanDyke et al., 1975). Carnitine treatment has been beneficial on muscle strength in some patients only, whereas the muscle carnitine content was increased with variable success, but carnitine stores were only rarely replenished (Hosking et al., 1977; Shapira et al., 1993). However, in order to achieve full recovery the duration of therapy should probably continue for longer periods, with a dose of not less than 100 mg/kg body weight/day (Shapira et al., 1993).

5.1.6.2 Secondary carnitine deficiency

Secondary carnitine deficiency, manifested by decreased plasma or tissue carnitine levels, is associated primarily with a wide range of genetic diseases, caused by metabolic disorders (Pons and De Vivo, 1995). These disorders are associated with impaired oxidation and accumulation of atypical acyl-CoA intermediates, and include fatty acid oxidation disorders and amino acid oxidation defects, and are characterized by plasma and tissue carnitine levels of 25 to 50% of normal (Stanley, 1987). Fatty acid oxidation defects, inherited in an autosomal recessive manner, can be subdivided into defects of the carnitine cycle for the transport of the long-chain fatty acids into mitochondria and defects of the β-oxidation cycle, that occur within the

mitochondria (Pons and De Vivo, 1995). Defects of the enzymes involved in the carnitine cycle implicates carnitine-acylcarnitine translocase deficiency (Stanley et al., 1992) and carnitine palmitoyltransferase I and II deficiencies (Angelini et al., 1981). Defeciences due to the enzymes involved in the β-oxidation cycle comprise the short-chain (Turnbull et al., 1984), the medium-chain (Roe et al., 1986), the long-chain (Hale et al., 1985) and the very long-chain acyl-CoA dehydrogenases (Bertrand et al., 1993). The postulated mechanism of carnitine deficiency in these disorders is an imbalance between the urinary excretion of the accumulated acylcarnitines and the sum of the dietary intake and biosynthesis of carnitine, resulting in the accumulation of the corresponding acyl-CoA esters in mitochondrial matrix and a characteristic increase of the acylcarnitine to carnitine ratio (Chalmers et al., 1984; Rebouche and Paulson, 1986).

Several drugs such as the branched fatty acid valproic acid (VPA), pivalic acid containing pro-drugs, cisplatin or carnitine derivates are involved in secondary carnitine deficiency (Opala et al., 1991; Holme et al., 1992; Heuberger et al., 1998; Brass et al., 2003). It has been shown that these drugs have inhibitory effect on OCTN2-mediated carnitine transport, whereas the most potent blockers were the antibiotic emetine and the ion channel blockers quinidine and verapamil (Ohashi et al., 1999; Wu et al., 1999; Wagner et al., 2000; Wu et al., 2000). Since no significant inhibition of carnitine transport by VPA was found, it was suggested that the deficiency induced by valproate therapy is due to a different mode of action.

5.1.7 Juvenile Visceral Steatosis (jvs) Mouse

In 1988, Koizumi et al. (Koizumi et al., 1988) described a C3H-H-2° strain of mouse, autosomal recessively associated with microvesicular fatty infiltration of viscera. The mice, later renamed juvenile visceral steatosis (jvs) mice (Hayakawa, 1990), show, beside severe lipid accumulation in the liver, other features of carnitine deficiency such as hyperammonemia, hypoglycemia, cardiac hypertrophy, mitochondrial abnormalities in skeletal muscle and progressive growth retardation (Horiuchi et al., 1993; Kaido et al., 1997). The hyperammonemia in jvs mice has been described as a consequence of a decrease of all the urea cycle enzyme activities resulting from

suppressed transcription during development (Imamura et al., 1990; Tomomura et al., 1992; Tomomura et al., 1994). They also have cardiac hypertrophy that can be significantly suppressed after carnitine administration (Horiuchi et al., 1993), and they were shown to have a marked decrease of carnitine levels in serum, liver and muscle, in comparison with controls (Kuwajima et al., 1991). After carnitine treatment, all the symptoms disappear, and carnitine substitution corrects carnitine deficiency and also reduces cardiac hypertrophy and hepatic accumulation of fat (Horiuchi et al., 1992). The jvs mice are therefore established as a model for SCD since they also show symptoms similar to those observed in SCD patients (Koizumi et al., 1988).

The metabolic defect in jvs mice was suspected to be primarily due to impairment of the renal carnitine transport system, and studies on the renal reabsorptional capacity of carnitine in the jvs mice revealed that the affected homozygous mice showed a higher rate of carnitine excretion ten days after birth (Horiuchi et al., 1994; Horiuchi et al., 1997). Biochemical studies of carnitine transport, using cultured fibroblast from normal and mutant jvs mice, indicated that the mutant jvs had significantly lower rates of Na^+-dependent carnitine uptake than controls (Kuwajima et al., 1996). The jvs phenotype is inherited in an autosomal recessive manner (Hayakawa, 1990) and the jvs locus has been identified within a 1.6 cM region on mouse chromosome 11 (Nikaido et al., 1995; Okita et al., 1996). The missense mutation in the jvs mouse was identified as L352R and is characterized on the molecular level by a point mutation from CTG to CGG that substituted from leucine to arginine at amino acid position 352 in the mouse homologue of OCTN2 (Lu et al., 1998). All these findings indicate that jvs mice represent a valid animal model for human primary carnitine deficiency.

5.1.8 Valproic Acid (VPA)

Valproic acid (N-dipropylacetic acid) or valproate (VPA) is a branched, medium-chain fatty acid composed of eight carbons (Figure 7), which is structurally unrelated to other antiepileptic drugs. VPA is a broad-spectrum antiepileptic drug which was introduced into the anticonvulsant market in 1968 in Europe and in 1978 in the United States (Zafrani and Berthelot, 1982). It is routinely used for both partial and generalized seizures, and it is effective against abscences (typical petit mal)

seizures, atypical absence seizures, myoclonic and tonic-clonic (grand mal) seizures and can be used as second choice medication in status epilepticus (Koch-Weser and Browne, 1980). The most common side effects are gastrointestinal disturbances (anorexia, nausea, vomiting), sedation, coagulation disorders (thrombocytopenia, decreased serum fibrinogen, prolonged prothrombin time), alopecia and hepatic toxicity (Pinder et al., 1977; Bruni and Wilder, 1979; Koch-Weser and Browne, 1980). Shortly after introduction, cases of fulminant liver failure in patients treated with VPA have been reported (Zafrani and Berthelot, 1982; Zimmerman and Ishak, 1982; Dreifuss et al., 1987; Konig et al., 1994; Krahenbuhl et al., 1995), but the underlying mechanism of VPA induced hepatotoxicity is still not fully known.

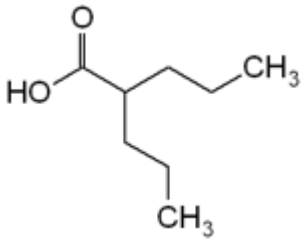


Figure 7: Valproic Acid

5.1.8.1 Pharmacology of VPA

VPA potentiates γ-aminobutyric acid (GABA) ergic inhibitory effects in some specific brain regions that are involved in the control of seizure generation and propagation by increasing both GABA synthesis and release (Bolanos and Medina, 1997; Loscher, 2002). Additionally, VPA also interacts with the metabolism of γ-hydroxbutyrate (GHB), a metabolite of GABA, reducing the GHB release and attenuating the neuronal excitation induced by *N*-methyl-D-aspartate type glutamate receptors (Loscher, 2002).

Therapeutic serum concentrations range from 50 to 125 µg/ml. At such therapeutic concentrations VPA is 80 to 90% bound to serum proteins. The binding is concentration-dependent, whereas the percentage decreases at higher VPA levels (Gugler and von Unruh, 1980; Chadwick, 1985). The protein bound fraction is less in

patients with renal disease, chronic hepatic disease, in the elderly, during pregnancy and in the presence of other protein bound drugs (Klotz and Antonin, 1977; Davis et al., 1994).

The metabolism of VPA follows at least five main metabolic pathways in the liver including glucuronidation, mitochondrial β-oxidation and cytosolic ω-oxidation (catalyzed by microsomal cytochrome P450) to produce multiple metabolites (Figure 8). However, because of their low plasma and brain concentrations, it is unlikely that they contribute significantly to the anticonvulsant effects of VPA (Davis et al., 1994; Loscher, 2002). Nevertheless, some of them may be involved in toxic effects of VPA, whereas the exact mechanism is not fully elucidated.

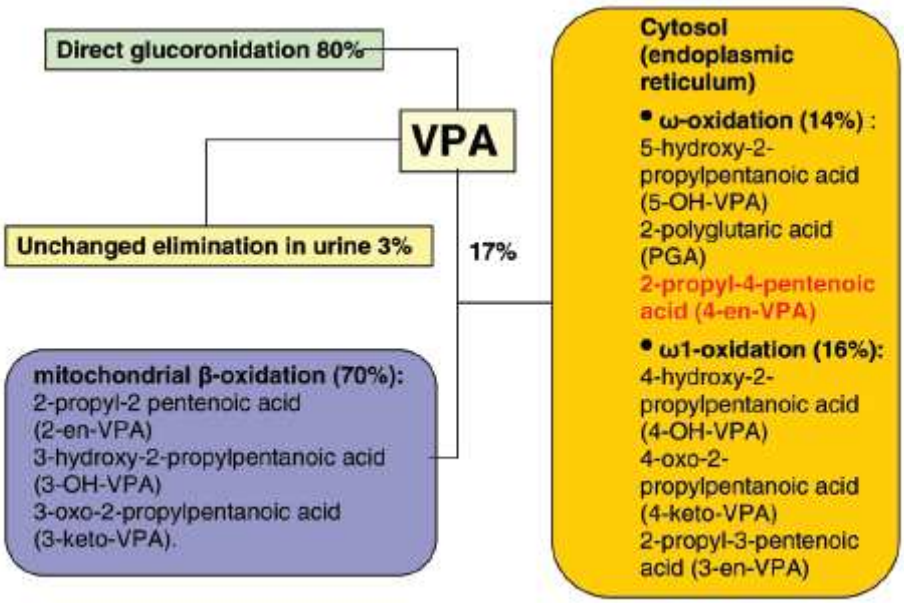


Figure 8: Liver metabolism and metabolites of VPA

Mitochondrial β-oxidation of VPA involves its transport within the mitochondrial matrix using the same pathway as long-chain fatty acids. This pathway consists of several steps and is called the “carnitine shuttle” (Figure 9). First, VPA is activated in the cytosol and links with coenzyme A (CoA-SH) to form valproyl-CoA. Valproyl-CoA then crosses the outer mitochondrial membrane. Under the effect of carnitine palmitoyltransferase I, valproylcarnitine is formed. Valproylcarnitine is then exchanged for free carnitine by carnitine-acylcarnitine translocase. In the mitochondrial matrix, carnitine palmitoyltransferase II transforms valproylcarnitine into

valproyl-CoA, which is able to enter a β-oxidation process (Millington et al., 1985; Li et al., 1991; Ketter et al., 1999).

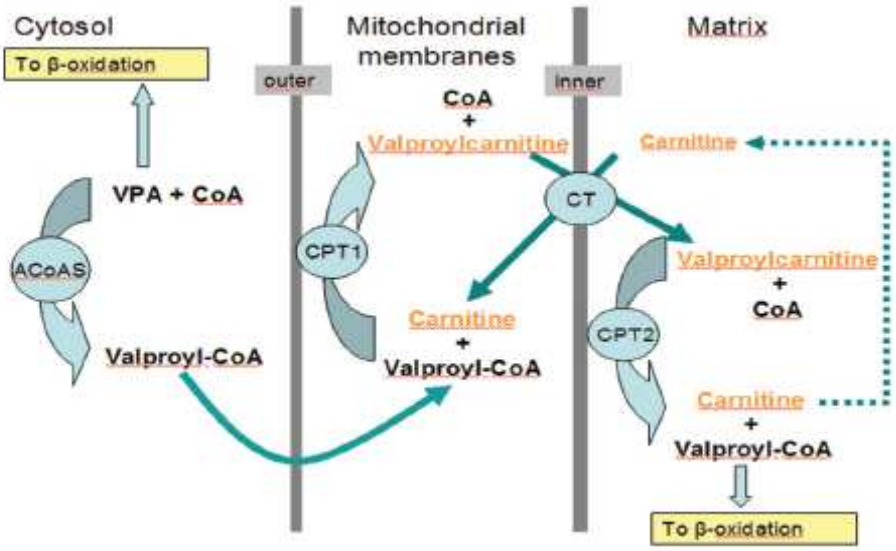


Figure 9: The 'carnitine shuttle'. ACoAs, acyl-CoA synthetase; CoA, coenzyme A; CPT, carnitine palymitoyltransferase; CT, carnitine translocase

5.1.8.2 VPA-associated hepatotoxicity

The hepatotoxicity associated with VPA has been well documented (Sussman and McLain, 1979; Dickinson et al., 1985; Eadie et al., 1988). The type I VPA-mediated hepatotoxicity is associated with dose-dependent changes in serum aminotransferase activity and low plasma fibrinogen levels that are normalized with either dose reduction or drug discontinuation. This dose-related toxicity occurs during the first three months of therapy in up to 44% of recipients (Sussman and McLain, 1979; Coulter et al., 1980). The type II VPA-mediated hepatotoxicity is considered to be rare, but often fatal and irreversible idiosyncratic reactions characterized by centri- and midzonal microvesicular steatosis that is sometimes accompanied by centrizonal necrosis (Zafrani and Berthelot, 1982; Zimmerman and Ishak, 1982; Dreifuss et al., 1987). This severe form of hepatotoxicity is not clearly dose-dependent, as it can arise with either low doses (or low VPA plasma concentrations) or high doses (or

high VPA plasma concentrations) (Zimmerman and Ishak, 1982; Dreifuss et al., 1987).

Although the mechanism of the type II VPA-mediated hepatotoxicity is not fully elucidated, mitochondrial dysfunction was considered as principal cause of VPA-induced liver failure (Fromenty and Pessayre, 1995). In agreement with this concept, microvesicular steatosis, the principal histological finding in valproate induced hepatotoxicity, is also detected in other types of liver disease with decreased mitochondrial β-oxidation such as Reye's syndrome, Jamaican vomiting sickness, mitochondrial cytopathies and acute fatty liver of pregnancy (Bioulac-Sage et al., 1993; Ponchaut and Veitch, 1993).
Since VPA is activated to both CoA and carnitine derivates, depletion of hepatic free CoA and free carnitine represents a potential mechanism, whereas this sequestration of CoA and carnitine is thought to be the major cause for the inhibition of mitochondrial β-oxidation by VPA (Ponchaut et al., 1992b). Another mechanism may be direct inhibition of mitochondrial β-oxidation by VPA metabolites, namely 4-ene VPA (Δ^4-VPA) and its subsequent metabolite 2,4-diene VPA, resulting in an inactivation of mitochondrial β-oxidation enzymes (Thurston et al., 1983; Turnbull et al., 1983; Granneman et al., 1984; Rettenmeier et al., 1985; Ponchaut et al., 1992b). A third possible mechanism is a decreased activity of complex IV (cytochrome c oxidase) of the respiratory chain, associated with a significant loss in cytochrome aa3 in liver mitochondria (Ponchaut et al., 1991a; Ponchaut et al., 1991b; Ponchaut and Veitch, 1993). Furthermore, it has been proposed that pre-existing mitochondrial diseases, e.g. impaired β-oxidation and/or impaired function of the respiratory chain, may increase susceptibility for VPA-induced mitochondrial dysfunction, in particular for liver failure (Chabrol et al., 1994; Lam et al., 1997; Krahenbuhl et al., 2000a).

5.2 In vitro investigations

5.2.1 Immortalized human hepatocytes

Primary human hepatocytes are widely used for xenobiotic metabolism, toxicity studies and the design for bioartificial liver devices. Nevertheless, there are several disadvantages occurring with this screening system. Primary hepatocytes have limited and unpredictable availability, restricted growth activity and lifespan, and show significant inter-individual differences in the expression of drug metabolizing enzymes and responses to toxicants. Huge variations in functional activities, especially P450 levels, as well as in the magnitude of P450 induction after treatment with prototypical inducers, have been reported from one human hepatocyte population to another (Guillouzo et al., 1993; Madan et al., 2003).

In contrast to primary human hepatocytes, immortalized hepatocytes could be taken into consideration for investigations on hepatic metabolism or drug toxicity. These cells are readily available, can be passaged and used over a longer time period, retaining the activity of major drug-metabolizing enzymes. On a cellular basis, mortality is defined as the death of a lineage of cells, immortality would be defined as infinite survival, a life span without time limits, unlimited proliferative potential and maintenance of critical liver functions (Cascio, 2001). The most widely used immortalizing agent that allow normal cells to overcome senescence signals and continue proliferating, is the simian virus 40 large T antigen (SV40TAg). The common mode of action of this viral oncogene is the inactivation of the cell cycle regulatory proteins pRB and p53 by various mechanisms (Bryan and Reddel, 1994; Mathon and Lloyd, 2001).

The development of an immortalized hepatocyte cell line would be beneficial for the pharmaceutical industry and an enormous need exists for an *in vitro* human hepatocyte assay system for high throughput testing of the pharmacological properties and toxicology of new chemical entities (NCE). A differentiated human hepatocyte cell line, especially one which exhibits P450 function, would find immediate and widespread application in pharmacology and toxicology. The used immortalized human hepatocyte cell line was generated in our laboratory by

transducing the SV40TAg gene into primary human hepatocytes, using a HIV-derived lentiviral vector as described by Salmon et al. (Salmon et al., 2000).

5.2.2 Hepatic cell lines

A frequently used alternative for the screening of hepatic metabolism and toxicity of several drugs is the utilization of hepatic cell lines, deriving from hepatoblastoma or hepatocellular carcinoma.

5.2.2.1 HepG2

The hepatocellular carcinoma HepG2 cell line is a perpetual adherent cell line which has been isolated primarily from a liver tissue of a 15 year old Caucasian male with a well differentiated hepatocellular carcinoma. These cells are epithelial in morphology and have a model chromosome number of 55. The cells secrete a variety of major plasma proteins, e.g. albumin, α-2 macroglobulin, α-1 antitrypsin, transferrin and plasminogen, and have provided a tool for extensive studies of biochemical functions of liver cells and used to test a wide variety of compounds over the last years (Bouma et al., 1989; Javitt, 1990). In our studies this cell line was used as a comparator to investigate the expression and induction of several CYP isozymes and the human pregnane X receptor (hPXR).

5.2.3 Cytochrome P450 enzymes

Cytochome P450s (CYPs) are a large group of heme-containing monooxygenase enzymes responsible for the oxidative metabolism of drugs and other xenobiotics, as well as many endogenous compounds, whereas NADPH is required as a coenzyme and O_2 is used as a substrate. They are classified in the same family (symbolized by an Arabic number) when their amino acid sequence similarity is greater than 40% and to the same subfamily (symbolized by an upper case letter) when their amino acid sequence similarity is above 55% (Nebert et al., 1987; Nebert and Gonzalez, 1987). CYPs are located on the membrane of the endoplasmic reticulum and are

highly concentrated in the liver and in the small intestine, and they are also found in the mitochondrial membrane (Modi et al., 1995). Presently, there are more than 270 different CYP gene families, with 18 recommended in mammals (Nebert and Russell, 2002) (Figure 10). Up to now, three main P450 families (1, 2 and 3) have been identified as mainly involved in xenobiotic metabolism (Gonzalez, 1988; Nelson et al., 1993), and drug-drug interacations (DDI) are of increasing interest due to the occurrence of adverse drug reactions and/or loss of therapeutic effect (Li et al., 1997b; Michalets, 1998; Madan et al., 2003). Induction of CYP3A4 gene expression is caused by a variety of marketed drugs, including rifampin, phenobarbital, clotrimazole and dexamethasone (Meunier et al., 2000; Sahi et al., 2000; Luo et al., 2002; Madan et al., 2003) and represents the basis for a number of common drug-drug interactions. CYP1A2 is inducible by 3-methylcholanthrene, β-naphtofavone and tetrachlorodibenzodoxin (Li et al., 1998; Breinholt et al., 1999; Meunier et al., 2000; Madan et al., 2003). CYP2C9 can be induced by rifampin and phenobarbital, whereas the magnitude of induction is less than that for CYP3A4 (Li et al., 1997b; Madan et al., 2003). Knowledge of possible CYP-induction or -inhibition potential of drug candidates in drug discovery or the early preclinical phase of development would be therefore helpful for the prediction of drug-drug interactions. Beside involvement in drug metabolism, CYPs also play a major part in cholesterol biosynthesis and metabolism, bile-acid biosynthesis, steroid synthesis and metabolism, vitamin D3 synthesis and metabolism, and retinoic acid hydroxylation (Nebert and Russell, 2002).

Family	Number of subfamilies	Number of genes	Substrates and functions
CYP1	2	3	Foreign chemicals, arachidonic acid, eicosanoids
CYP2	13	16	Foreign chemicals, arachidonic acid, eicosanoids
CYP3	1	4	Foreign chemicals, arachidonic acid, eicosanoids
CYP4	5	12	Fatty acids, arachidonic acid, eicosanoids
CYP5	1	1	Thromboxane A_2 synthase
CYP7	2	2	Cholesterol, bile acid synthesis
CYP8	2	2	Prostacyclin synthase, bile-acid synthesis
CYP11	2	3	Steroidogenesis
CYP17	1	1	Steroid 17α-hydroxylase, 17/20-lyase
CYP19	1	1	Aromatase to form oestrogen
CYP20	1	1	Unknown
CYP21	1	1	Steroid 21-hydroxylase
CYP24	1	1	Vitamin D_3 24-hydroxylase
CYP26	3	3	Retinoic acid hydroxylation
CYP27	3	3	Bile-acid biosynthesis, vitamin D_3 hydroxylations
CYP39	1	1	24-hydroxycholesterol 7α-hydroxylase
CYP46	1	1	Cholesterol 24-hydroxylase
CYP51	1	1	Lanosterol 14α-desmethylase

Figure 10: Substrates and functions of human CYP gene families.

5.2.3.1 CYP1A enzymes

Members of the CYP1A (CYP1A1 and CYP1A2) subfamily have been identified in a wide range of vertebrates, including fish, amphibians, birds, and mammals, and are involved in the oxidation of a wide range of endogenous compounds and xenobiotics. The expression of members of the CYP1A family is inducible by polycyclic aromatic hydrocarbons, such as those found in charbroiled foods and cigarette smoke acting through the aryl hydrocarbon receptor (AHR), a transcription factor (Hahn and Stegeman, 1994). CYP1A2 is responsible for about 10 to 15% of the total CYP content of human liver and is the major CYP isozyme involved in the metabolism of important drugs, e.g. imipramine, propranolol, clozapine, olanzapine, theophylline

and caffeine (Brosen, 1995). Figure 11 shows the substrates, inhibitors and inducers of CYP1A2.

Substrates, Inhibitors and Inducers of CYP1A2

Substrates
Amitriptyline (Elavil)
Clomipramine (Anafranil)
Clozapine (Clozaril)
Imipramine (Tofranil)
Propranolol (Inderal)
R-warfarin
Theophylline
Tacrine (Cognex)

Inhibitors
Fluvoxamine (Luvox)
Grapefruit juice
Quinolones
Ciprofloxacin (Cipro)
Enoxacin (Penetrex) > norfloxacin (Noroxin) > ofloxacin (Floxin) > lomefloxacin (Maxaquin)

Inducers
Omeprazole (Prilosec)
Phenobarbital
Phenytoin (Dilantin)
Rifampin (Rifadin, Rimactane)
Smoking
Charcoal-broiled meat

Figure 11: Substrates, inhibitors and inducers of CYP1A2.

5.2.3.2 CYP2C enzymes

The CYP2C subfamily is also important for drug metabolism, accounting for approximately 18% of the CYP protein content in human liver and for approximately 20% of the CYP-mediated metabolism of drugs (Rendic and Di Carlo, 1997). CYP2C9 is a member of the CYP2C subfamily, which includes in humans at least three other members, e.g. CYP2C8, CYP2C18 and CYP2C19. The CYP2C9 isozyme is, among others, responsible for the metabolism of several substrates including warfarin, phenytoin and various non-steroidal anti-inflammatory agents (Rettie et al., 1992; Bajpai et al., 1996; Hamman et al., 1997; Miners and Birkett, 1998). CYP2C19 has been shown to exhibit genetic polymorphism, and is completely absent in 3

percent of Caucasians and 20 percent of Japanese (Wedlund et al., 1984; Nakamura et al., 1985). It plays a role in the metabolism of phenytoin (Levy, 1995), and is involved in the metabolism of omeprazole and diazepam (Andersson et al., 1993; Jung et al., 1997) (Figure 12).

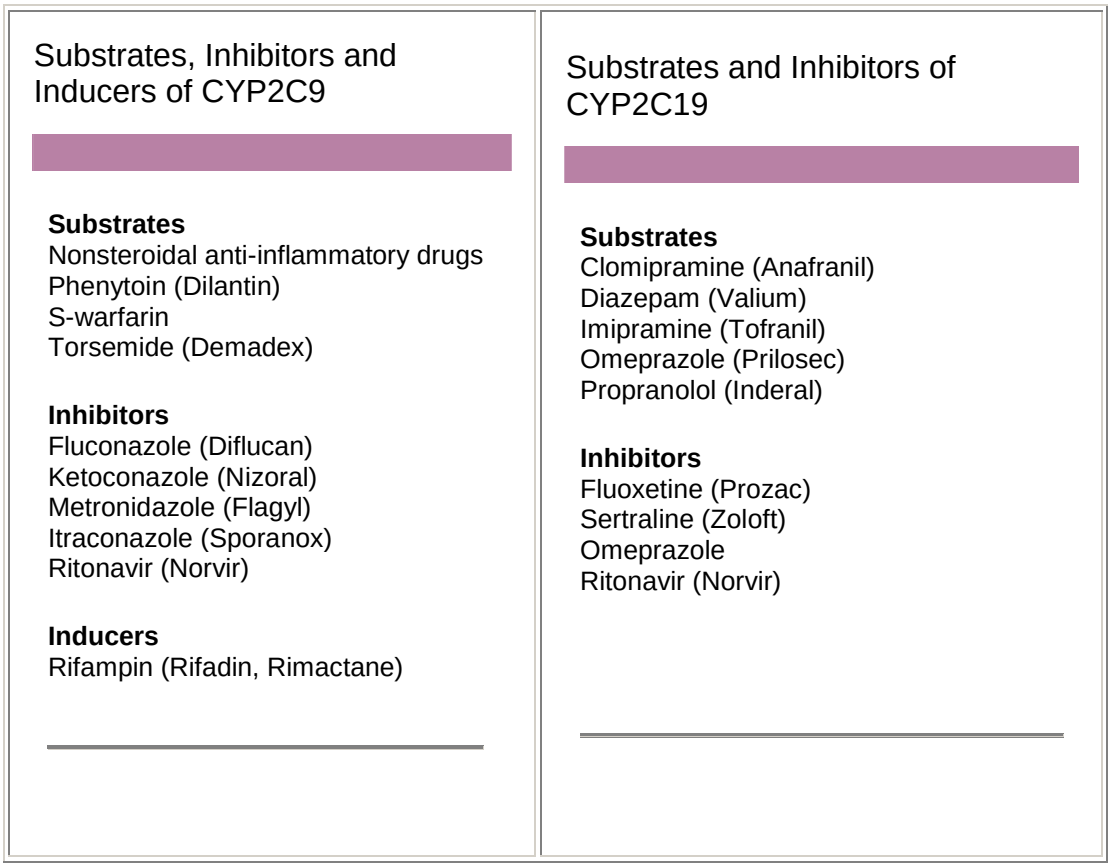


Figure 12: Substrates, inhibitors and inducers of CYP2C9, and substrates and inhibitors of CYP2C19.

5.2.3.3 CYP2D6 enzyme

CYP2D6, the only known functional member of the CYP2D subfamily in humans, metabolizes a wide variety of substances including many psychotherapeutic agents (e.g. amitriptyline, haloperidol, risperidone) and also beta-blockers (e.g. metoprolol). This enzyme is genetically polymorphic, leading to impaired metabolism in 5 to 10% of Caucasians of many centrally acting drugs and toxins (Steiner et al., 1988; Meyer et al., 1990). Individuals with normal CYP2D6 activity are termed extensive metabolizers. These ultra-rapid metabolizers show increased metabolism and decreased drug effects of CYP2D6 substrates, such as tricyclic antidepressants (Dalen et al., 1998). Ethnic differences are indicated in this genetic polymorphism,

since Asians and blacks are less likely than Caucasians to be poor metabolizers (Relling et al., 1991; Bertilsson et al., 1992). Poor metabolizers are at risk for drug accumulation and toxicity from drugs metabolized by this isoform. Conversely, when formation of an active metabolite is essential for drug action, poor metabolizers of CYP2D6 can exhibit less response to drug therapy compared with extensive metabolizers. The substrates and inhibitors of CYP2D6 are shown in Figure 13.

Substrates and inhibitors of CYP2D6

Substrates

Antidepressants*
- Amitriptyline (Elavil)
- Clomipramine (Anafranil)
- Desipramine (Norpramin)
- Doxepin (Adapin, Sinequan)
- Fluoxetine (Prozac)
- Imipramine (Tofranil)
- Nortriptyline (Pamelor)
- Paroxetine (Paxil)
- Venlafaxine (Effexor)

Antipsychotics
- Haloperidol (Haldol)
- Perphenazine (Etrafon, Trilafon)
- Risperidone (Risperdal)
- Thioridazine (Mellaril)

Beta blockers
- Metoprolol (Lopressor)
- Penbutolol (Levatol)
- Propranolol (Inderal)
- Timolol (Blocadren)

Narcotics
- Codeine, tramadol (Ultram)

Inhibitors

Antidepressants
- Paroxetine > fluoxetine > sertraline (Zoloft) > fluvoxamine (Luvox), Nefazodone (Serzone), Venlafaxine > clomipramine (Anafranil) > amitriptyline

Cimetidine (Tagamet)

Fluphenazine (Prolixin)

Antipsychotics
- Haloperidol
- Perphenazine
- Thioridazine

Figure 13: Substrates and inhibitors of CYP2D6.

5.2.3.4 CYP3A enzymes

CYP3A enzymes are the most abundantly expressed cytochrome P450 enzymes in the liver and is considered to be the major drug metabolizing subfamily. Its members are localized in the organs most associated with drug disposition, including the liver, gastrointestinal tract, and kidney. CYP3A4 is the predominant cytochrome P450 enzyme, accounting for up to 30% of total hepatic CYP protein content (Shimada et al., 1994), and is known to metabolize a large variety of xenobiotics (among them amiodarone, lipophilic HMG-CoA reductase inhibitors, cyclosporine, tacrolimus and sirolimus, and various anticancer drugs) and endogenous substances, such as steroids (Brian et al., 1990; Araya and Wikvall, 1999). Other isoforms are CYP3A43,

CYP3A5 and CYP3A7. Among them, it has been estimated that about 60% of all clinically used drugs are metabolized by CYP3A4 (Bertz and Granneman, 1997). Members of this subfamily are involved in many clinically important drug interactions (Slaughter and Edwards, 1995). Several potent inducers of CYP3A are known, including rifampicin, dexamethasone and phenorbarbital (Meunier et al., 2000; Sahi et al., 2000; Luo et al., 2002; Madan et al., 2003), leading to clinically important drug-drug interactions when these substances are administered concurrently with drugs, which are metabolized by these CYPs. Substrates, inhibitors and inducers of CYP3A are listed in Figure 14.

CYP3A43 gene expression was found in liver, kidney, pancreas, and prostate as well as fetal liver and fetal skeletal muscle (Domanski et al., 2001). The highest expression level of CYP3A43 mRNA was found in prostate, and in liver it could be induced by rifampicin (Gellner et al., 2001). CYP3A43 is expressed at 0.1% and 2% of the levels of CYP3A4 and CYP3A5 (Westlind et al., 2001).

CYP3A5 was isolated from a liver cDNA library and was first termed PCN3, sharing 85% sequence similarity with CYP3A4 (Aoyama et al., 1989). It is present at only 10 to 30% of CYP3A4 levels (Kuehl et al., 2001). It is well established that only approximately 20% of livers express CYP3A5. The most common reason for the absence of expression is a splice site mutation (Kuehl et al., 2001; Lin et al., 2002). In individuals who express CYP3A5, the percentage contributed to total hepatic CYP3A by this isoform is still unclear, with estimates ranging from 17% to 50%, wheras CYP3A5 was more frequently expressed in livers of African Americans (60%) than in those of Caucasians (33%) (Kuehl et al., 2001). CYP3A5 is also expressed in a range of extrahepatic tissues such as small intestine, colon esophagus and lung (Ding and Kaminsky, 2003).

CYP3A7 is expressed specifically in fetal livers and accounts for up to 50% of the total fetal hepatic CYP content (Wrighton and Vandenbranden, 1989). It has been shown that CYP3A7 is expressed in placental and endometrial microsomes that increases dramatically from the first to the second trimester of pregnancy. An increased expression of a CYP3A7 transcript was found in endometria of pregnant compared with nonpregnant women as well as an increase from the first to the second trimester of pregnancy (Schuetz et al., 1993). The level of expression of

CYP3A7 varies with gestational age and is higher in the 20-week fetus than at 40 weeks of age, and decreases dramatically after birth (Kitada et al., 1987). Initially, CYP3A7 was thought to be unique to the fetal liver (Wrighton and Vandenbranden, 1989), however, its presence has now been noted in placental, endometrial (Schuetz et al., 1993), and adult hepatic tissue (Schuetz et al., 1994).

Substrates, Inhibitors and Inducers of CYP3A

Substrates	**Inhibitors**
Amitriptyline (Elavil)	Antidepressants
Benzodiazepines	Nefazodone > fluvoxamine (Luvox) > fluoxetine (Prozac) > sertraline
Alprazolam (Xanax)	Paroxetine (Paxil)
Triazolam (Halcion)	Venlafaxine
Midazolam (Versed)	Azole antifungals
Calcium blockers	Ketoconazole (Nizoral) > itraconazole (Sporanox) > fluconazole (Diflucan)
Carbamazepine (Tegretol)	Cimetidine (Tagamet)
Cisapride (Propulsid)	Clarithromycin (Biaxin)
Dexamethasone (Decadron)	Diltiazem
Erythromycin	Erythromycin
Ethinyl estradiol (Estraderm, Estrace)	Protease inhibitors
Glyburide (Glynase, Micronase)	**Inducers**
Imipramine* (Tofranil)	Carbamazepine
Ketoconazole (Nizoral)	Dexamethasone
Lovastatin (Mevacor)	Phenobarbital
Nefazodone (Serzone)	Phenytoin (Dilantin)
Terfenadine (Seldane)	Rifampin (Rifadin, Rimactane)
Astemizole (Hismanal)	
Verapamil (Calan, Isoptin)	
Sertraline (Zoloft)	
Testosterone	
Theophylline	
Venlafaxine (Effexor)	
Protease inhibitors	
Ritonavir (Norvir)	
Saquinavir (Invirase)	
Indinavir (Crixivan)	
Nelfinavir (Viracept)	

Figure 14: Substrates, inhibitors and inducers of CYP3A4.

5.2.4 Human pregnane X receptor (PXR)

The pregnane X receptor (PXR), an orphan nuclear receptor, is one of the key transcriptional regulators of cytochrome P450 CYP3A monooxygenases and other drug metabolizing enzymes and transporters, such as CYP2B6, CYP2C8/9 and CYP3A7, as well as the drug transporters MDR1, OATPC, bile salt export pump (BSEP) and MRP2 (Schuetz et al., 2001; Kast et al., 2002; Pascussi et al., 2003; Tirona et al., 2003). PXR is a member of the nuclear receptor family of ligand-activated transcription factors that includes the steroid, retinoid, and thyroid hormone receptors as well as many orphan receptors for which physiological ligands have yet to be identified (Mangelsdorf et al., 1995; Giguere, 1999), and is highly expressed in

the liver and intestine. The human PXR, that binds to the rifampicin/dexamethason response element in the CYP3A4 promoter (Lehmann et al., 1998), is activated by a variety of endogenous (e.g. steroids and bile acids), and exogenous compounds (e.g. rifampicin, phenytoin and hyperforin) through direct interaction with these ligands. After activation by the ligand, PXR forms heterodimers with the 9-cis retinoic X receptor (RXR), another nuclear receptor. The emerging heterodimer binds to specific DNA sequences and regulates the expression of its targets. The elucidation of the three-dimensional structure of the PXR ligand binding domain has provided important insights into the structural basis for the promiscuous ligand binding properties of this nuclear receptor (Watkins et al., 2001). PXR activation assays can be used to predict the induction of CYP3A gene expression by drug candidates and therefore to predict drug-drug interactions (Lehmann et al., 1998; Goodwin et al., 2002; Kliewer et al., 2002).

5.2.5 Cimicifuga racemosa (Black cohosh)


Cimicifuga racemosa—Plant and rhizome. Note the pinnately decompound leaf and the wand-like racemes which bear white flowers.

Figure 15: Cimicifuga racemosa – plant and rhizome.

Cimicifuga racemosa (Actaea racemosa) (Figure 15), commonly known as black cohosh, is an herb native to Eastern North America. Traditionally, the root and rhizome was used by North American Indians for joint aches, myalgias, neuralgias and rheumatic disorders, but also for menopausal complaints and pain during labour.

Nowadays, ethanolic or isopropanolic extracts of cimicifuga racemosa are most commonly used in the treatment of menopausal symptoms, menstrual dysfunction and other gynaecological disorders, although not all studies have shown a better effect than placebo (McKenna et al., 2001; Frei-Kleiner et al., 2005; Nappi et al., 2005; Pockaj et al., 2006; Uebelhack et al., 2006).

The therapeutic activity of black cohosh was originally suggested to derive from an activation of estrogen receptors (Jarry et al., 1985; Duker et al., 1991; Kruse et al., 1999; Liu et al., 2001b), however, in other studies, estrogenic or estrogen receptor-binding effects were not found (Einer-Jensen et al., 1996; Liu et al., 2001a). Since it is unclear if black cohosh has an estrogenic effect or not and due to its potential ability to stimulate uterine contraction, it is contraindicated during pregnancy (Mahady et al., 2002).

Data from clinical studies and spontaneous reporting programs suggest that adverse events associated with cimicifuga racemosa are rare, generally mild and reversible. Gastrointestinal upset and rashes were the most common adverse events reported (Dog et al., 2003; Huntley and Ernst, 2003). In mostly uncontrolled clinical trials and post-marketing studies including more than 2,800 patients, adverse events had an incidence of 5.4%. Of the reported adverse events, 97% were minor or mild, none of them resulting in discontinuation of the therapy. When higher doses than those recommended are used, however, cimicifuga racemosa can cause dizziness, headaches, nausea, and vomiting (Dog et al., 2003). In their review, which includes all post-marketing programs of cimicifuga extracts, Huntley et al. also described patients with hepatic adverse events (Huntley and Ernst, 2003). They reported one case with hepatic failure, three cases with hepatitis and three cases with increased liver enzymes. In addition, several case reports have been published about patients developing acute hepatitis (Whiting et al., 2002; Cohen et al., 2004) or fulminant liver failure (Lontos et al., 2003; Levitsky et al., 2005; Lynch et al., 2006) while being treated with cimicifuga extracts. Since an association of hepatotoxicity with cimicifuga appears to be possible and there are only limited data available, it is of great interest to further investigate the toxicological profile and the factors contributing to the potential toxicity of black cohosh.

6 Effect of carnitine deprivation on carnitine tissue stores and energy metabolism in mice with systemic carnitine deficiency

Running title: Carnitine deprivation in jvs-/- mice

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6.1 Abstract

Aims: To study carnitine homeostasis and energy metabolism in carnitine-deficient (jvs-/-) mice after cessation of carnitine substitution.
Methods: Homozygous jvs-/- mice starved overnight were studied 3, 6 and 10 days after cessation of carnitine substitution and compared to wild-type and heterozygous jvs+/- mice.
Results: In comparison to wild type mice, jvs-/- mice treated with oral carnitine had a higher liver weight and hepatic fat accumulation, and decreased plasma β-hydroxybutyrate levels. The total carnitine concentrations in plasma, liver and skeletal muscle were decreased by 58%, 16% and 17%, respectively. After cessation of carnitine administration, the plasma carnitine levels fell rapidly, reaching 2.3 μmol/L after 10 days. After 10 days of carnitine deprivation, the hepatic and skeletal muscle carnitine content had dropped to 51% and 66%, respectively, of carnitine-treated jvs-/- mice. Carnitine deprivation was associated with a further drop in the plasma β-hydroxybutyrate levels and hepatic fat accumulation. In skeletal muscle, the glycogen content decreased and the lactate levels increased with carnitine deprivation, whereas tissue ATP levels were maintained.
Conclusions: Although the tissue stores of carnitine are quite resistant to carnitine deprivation in mice with systemic carnitine deficiency, the margin between adequate function and failure of energy metabolism is narrow.

Key words: jvs-/- mice, systemic carnitine deficiency, β-oxidation, glycogen, ATP
Abbreviations: JVS, juvenile visceral steatosis; OCTN2, organic cation / carnitine transporter; SCD, systemic carnitine deficiency

6.2 Introduction

Carnitine (β-hydroxy-4-*N*-trimethylaminobutyric acid) represents an essential cofactor for the transport of activated long-chain fatty acids across the inner mitochondrial membrane to the mitochondrial matrix, the place of β-oxidation (Bremer, 1983; Rebouche and Paulson, 1986). Since fatty acids are an important energy source for many organs, carnitine deficiency is associated with cardiomyopathy, muscle weakness, encephalopathy and/or hepatopathy including Reye's syndrome (Karpati

et al., 1975; Chapoy et al., 1980; Treem et al., 1988). Carnitine is a polar molecule with a high intracellular concentration, necessitating an active transport from the plasma into cells. OCTN2 is a carnitine transporter, which has been characterized intensively on both the molecular and on the functional level (Tamai et al., 1998). The functional importance of OCTN2 is evidenced in patients with mutations in the corresponding gene, leading to primary systemic carnitine deficiency due to a lack of renal carnitine reabsorption (Nezu et al., 1999; Seth et al., 1999; Vaz et al., 1999; Wang et al., 1999). Primary systemic carnitine deficiency (SCD; OMIM 212140) is an autosomal recessive disorder of fatty acid oxidation, clinically characterized by progressive cardiomyopathy, skeletal myophathy, hypoglycemia and hyperammonemia (Karpati et al., 1975; Treem et al., 1988). C3H.OH (formerly C3H-H-2ᵒ) mice, which are characterized by microvesicul ar steatosis of the liver and triglyceride accumulation in other visceral organs, were first described 1988 by Koizumi et al. (Koizumi et al., 1988) and were later renamed juvenile visceral steatosis (jvs) mice (Hayakawa, 1990). The jvs phenotype is inherited in an autosomal manner (Hayakawa, 1990) and is characterized on the molecular level by a point mutation in the mouse homologue of OCTN2, leading to an exchange of an amino acid (L352R) (Lu et al., 1998). Beside liver steatosis, jvs mice show other features of carnitine deficiency such as hyperammonemia, hypoglycemia, cardiac hypertrophy, mitochondrial abnormalities in skeletal muscle and progressive growth retardation (Horiuchi et al., 1993; Kaido et al., 1997). The accumulation of lipids occurs within 5 days of birth whereas hypoglycemia, hyperammonemia and growth retardation appear approximately 2 weeks later (Horiuchi et al., 1994). Hyperammonemia has been described as a consequence of a decrease in urea cycle enzyme activities due to reduced transcription of the corresponding genes (Imamura et al., 1990; Tomomura et al., 1992; Tomomura et al., 1994). As expected, as a consequence of impaired renal reabsorption of carnitine, plasma and tissue carnitine levels are substantially decreased in jvs as compared to wild type mice (Kuwajima et al., 1991). Carnitine substitution corrects carnitine deficiency and transcription of the urea cycle enzymes at least partially and also reduces cardiac hypertrophy and hepatic accumulation of fat (Horiuchi et al., 1992). These findings demonstrate that jvs mice represent an excellent animal model for primary systemic carnitine deficiency in humans.

While it is well established that sufficient carnitine plasma and tissue levels can be obtained in jvs mice by carnitine substitution, the kinetics of carnitine loss from plasma and tissues after carnitine deprivation and the consequences on energy metabolism have not been described so far. We therefore studied plasma and tissue carnitine stores and markers of energy metabolism after cessation of carnitine substitution in jvs mice.

6.3 Materials and Methods

Animals

The juvenile visceral steatosis (jvs) mice were obtained from Prof. Masahisa Horiuchi from the University of Kagoshima, Japan. The breeding pairs (wild type and heterozygous jvs+/-) and the offsprings were supplemented with carnitine (1g/250ml drinking water) before weaning to maintain the survival rate. After weaning, the supplementation with carnitine was continued for the homozygous jvs-/- mice. For genotyping the littermates (wild type, heterozygous jvs+/- and homozygous jvs-/- mice), DNA was extracted and purified from a piece of tail using a DNA extraction kit (kit No. 740952.250, Macherey-Nagel, Oensingen, Switzerland). A TaqMan allelic discrimination method was established which combines PCR and mutation detection in a single step. Two allele-specific TaqMan probes were used, one for each allele (Applied Biosystems, UK). Each probe consisted of an oligonucleotide with a 5' reporter dye (FAM for the detection of the wild type L352 allele and VIC for the detection of the mutant L352R allele) and a 3' quencher dye (TAMRA for both probes). The probes were as follows: FAM, 5'-atatggtc**a**gcctgca-3' and VIC, 5'-tatggtc**c**gcctgca-3'. The primers used (Microsynth, Switzerland) were identically for both alleles and were designed as follows: forward primer, 5'-tccccatgcaagttaggagtgt-3', reversed primer, 5'-tgctgctccagctctcttctg-3'. TaqMan analysis was carried out on a 7900HT Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland) and identification of the mutation in Octn2 was achieved using an allelic discrimination plot (Todesco et al., 2003). Cycling conditions were 10 min at 95℃ for initial denaturation and activation of the DNA polymerase, followed by 40 cycles of 15 s at 95℃ for denaturation and 1 min at 60℃ for co mbined annealing and primer extension. Fluorescence from the FAM reporter only reflects the presence of wild type alleles, whereas fluorescence from the VIC reporter only indicates mutant

alleles. Accordingly, fluorescence from both reporters reflects the heterozygous population.

Study Design

All experiments had been reviewed and accepted by the Animal Ethics Committee of the State of Basel Stadt. Experiments were performed with animals of 6 to 8 weeks old.

Six groups of jvs mice were studied. Wild type (WT), heterozygous jvs+/- and homozygous jvs-/- with carnitine supplementation (n=5 per group) were starved overnight before entering the final part of the study. After having obtained a blood sample from the tail vein, the mice were killed by decapitation. Tissue samples were obtained from the liver and skeletal muscle (quadriceps femoris), frozen rapidly by immersion in liquid nitrogen and stored at -70℃ un til analysis. These tissue samples were analyzed for carnitine and markers of energy metabolism. Frozen liver tissue was also used for staining with Sudan Black B (see below). Additional liver and skeletal muscle samples were treated with 4% formaldehyde for histological analysis. The other 3 groups of animals were homozygous jvs-/- mice deprived from carnitine (drinking water without containing carnitine) for 3, 6 or 10 days (n=5 per group). After the respective carnitine deprivation period, mice were starved overnight and killed by decapitation the next morning. Blood, liver and muscle samples were collected and stored as described above.

Characterization of the animals

The animals were characterized by their body and liver weights, levels of glycogen, lactate and ATP in liver and muscle and their plasma concentration of β-hydroxybutyrate. Lactate, ATP and glycogen were measured spectrophotometrically as described by Harris et al. (Harris et al., 1974), after adaptation of the method for a SpectraMax 250 absorbance microplate reader (Molecular Devices Corp.). β-Hydroxybutyrate was determined fluorimetrically according to Olsen (Olsen, 1971), after adaptation of the method for a SpectraMax Gemini XS (Molecular Devices Corp.) plate reader.

Determination of plasma and tissue carnitine

The carnitine concentrations in plasma, liver and muscle were determined radioenzymatically as described by Brass and Hoppel (Brass and Hoppel, 1978). Plasma and tissue samples were treated with perchloric acid (final concentration 3%) resulting in a supernatant and a pellet. Analysis of the supernatant yields free carnitine and, after alkaline hydrolysis, total acid soluble carnitine. The pellet yields the long-chain acylcarnitines after alkaline hydrolysis (acyl group chain length ≥10 carbons). The short-chain acylcarnitine fraction (acyl group chain length <10 carbons) can be calculated from the difference between total acid soluble and free carnitine. The sum of total acid soluble and long-chain acylcarnitines represents the total carnitine content.

Histological analysis of liver tissue

Pieces of liver and skeletal muscle fixed in 4% formaldehyde were used for staining with hematoxilin-eosine for histological analysis. The frozen tissue was cut into sections and stained with Sudan Black B for the visualization of fat (Lison, 1934). The estimation of fat accumulation in the liver was carried out by light microscopy of the stained sections.

Statistical analysis

All analyses were performed in duplicate. For each treatment group (n=5 per group) the results are presented as mean ± SD. Significant differences between groups were determined by ANOVA/Bonferroni multiple comparison post hoc test. P values <0.05 were considered to be statistically significant.

6.4 Results

The aim of the current study was to characterize carnitine homeostasis and energy metabolism in OCTN2-deficient (homozygous jvs-/-) mice during carnitine deprivation and to compare the findings with heterozygous jvs+/- and wild type mice.

Body and liver weight

As shown in Table 1, there was no difference in the body weight at the end of the study between wild type, heterozygous jvs+/- and homozygous jvs-/- mice, and

carnitine deprivation did not affect body weight. Liver weight was increased by 14% in homozygous jvs-/- mice as compared to heterozygous jvs+/- mice, but not significantly different from wild type mice. Homozygous jvs-/- mice showed a further significant increase in their liver weights during carnitine deprivation, reaching approximately 15% after 6 or 10 days of carnitine withdrawal compared to jvs-/- mice supplemented with carnitine.

Glycogen, lactate and ATP in liver and muscle

As shown in Table 1, homozygous jvs-/- revealed a 215% increase in the hepatic glycogen content as compared to wild type mice and a 108% increase compared to heterozygous jvs+/- mice. After carnitine deprivation, the hepatic glycogen content did not change significantly. On the other hand, the skeletal muscle glycogen content was not significantly different between wild type mice, heterozygous jvs+/- and homozygous jvs-/- mice, but was decreased by 53% after 3 and 6 days of carnitine deprivation and by 55% after 10 days of carnitine deprivation. The liver lactate content was decreased in homozygous jvs-/- mice compared to heterozygous jvs+/- (67% decrease) or wild type mice (72% decrease). After carnitine deprivation, the liver lactate content showed no further decrease in homozygous jvs-/- mice. In contrast, the skeletal muscle lactate content was not different between homozygous jvs-/-, heterozygous jvs+/- and wild type mice, and significantly increased during carnitine deprivation in homozygous jvs-/- mice. This increase reached 154% after 6 days and 338% after 10 days of carnitine withdrawal. Hepatic ATP levels were approximately doubled in homozygous jvs-/- mice compared to heterozygous jvs+/- or wild type mice, and remained constant 6 and 10 days after carnitine withdrawal. In contrast, the ATP content in skeletal muscle was slightly decreased in homozygous jvs-/- mice compared to wild type mice (decrease by 37%), but not significantly different from heterozygous jvs+/- mice. Similar to liver, carnitine deprivation did not significantly affect the skeletal muscle ATP content in homozygous jvs-/- mice.

β-Hydroxybutyrate in plasma

The plasma β-hydroxybutyrate concentration was decreased by 53% in homozygous jvs-/- mice compared to wild type or heterozygous jvs+/- mice. As expected, the plasma β-hydroxybutyrate concentrations showed a further drop with carnitine

deprivation in homozygous jvs-/- mice, reaching almost undetectable levels 10 days after carnitine withdrawal (Table 1).

	Wild type	Heterozygous	Homozygous (jvs-/-)			
	(jvs+/+)	(jvs+/-)	basal	day3	day6	day10
Body weight (g)	18.1±3.3	18.2±0.7	18.1±2.9	18.4±0.6	18.3±0.9	18.5±2.0
Liver weight (% body weight)	5.8±0.4	5.4±0.4	6.1±0.4§	6.1±0.7	7.0±0.3†	7.0±0.4†
Glycogen in liver (µmoles/g)	35.6±17.2	53.9±14.3	112.1±17.9*§	107.8±23.9	94.3±26.2	83.4±13.5
Glycogen in muscle (µmoles/g)	31.1±2.7	34.8±3.5	32.0±1.9	15.1±1.8†	14.9±4.9†	14.5±3.4†
Lactate in liver (µmoles/g)	12.1±2.4	10.4±4.2	3.4±1.8*§	5.9±2.8	4.0±2.0	6.3±2.4
Lactate in muscle (µmoles/g)	3.8±1.4	3.4±1.1	4.8±3.1	10.0±3.0	12.2±1.7†	21.0±3.3†
ATP in liver (µmoles/g)	1.4±0.2	1.3±0.2	2.5±0.6*§	1.0±0.2†	3.2±0.6	2.4±0.6
ATP in muscle (µmoles/g)	3.8±0.9	2.7±0.4	2.4±0.8*	2.0±0.8	2.4±1.1	3.1±1.0
ß-Hydroxybutyrate in plasma (µmol/l)	220±20	230±20	100±20*§	66±14†	45±7†	27±4†

* p<0.05 vs. jvs+/+, † p<0.05 vs. jvs-/-, § p<0.05 vs. jvs+/-

Table 1: *Characterization of the animals*. The mice were starved overnight before the final experiments, n = 5 for each group. Tissue concentrations are expressed per g tissue wet weight. Homozygous jvs-/- mice were studied while treated with carnitine (basal), and 3, 6 and 10 days after carnitine deprivation. Results are presented as mean±SD.

Plasma carnitine pool

As shown in table 2, the free carnitine levels in plasma were significantly lower in heterozygous jvs+/- mice (decrease by 42%) and homozygous jvs-/- (decrease by 62%) compared to wild type mice. After carnitine deprivation, the plasma free carnitine concentration showed a further decrease in homozygous jvs-/- mice, reaching a concentration of 1 µmol/L after carnitine withdrawal for 10 days. Similar results were obtained for the other carnitine fractions, namely short- and long-chain acylcarnitines and total carnitine. Interestingly, the ratio short-chain acylcarnitines to total acid soluble carnitine increased significantly in homozygous jvs-/- mice after carnitine withdrawal.

	Wild type	Heterozygous	Homozygous (jvs-/-)			
	(jvs+/+)	(jvs+/-)	basal	day3	day6	day10
Free Carnitine	28.9±1.7	16.8±1.5*	11.1±1.0*§	1.2±0.7†	1.5±0.2†	1.0±0.2†
SCA Carnitine	4.7±2.1	2.7±1.7	0.4±0.2*	2.0±0.9	1.0±0.4	0.8±0.2
TAS Carnitine	33.6±0.9	19.5±1.2*	11.5±0.9*§	3.2±0.6†	2.5±0.3†	1.8±0.2†
LCA Carnitine	7.6±0.5	7.1±0.5	5.8±0.7*§	2.7±0.6	2.6±0.2	0.5±0.3
SCA/TAS Carnitine	0.14±0.06	0.13±0.08	0.03±0.02	0.54±0.20†	0.39±0.15†	0.46±0.09†
Total Carnitine	41.2±0.9	26.6±1.5*	17.3±1.2*§	5.9±0.4†	5.1±0.4†	2.3±0.5†

*p<0.05 vs. jvs+/+, † p<0.05 vs. jvs-/-, § p<0.05 vs. jvs+/-

Table 2: *Plasma concentration of carnitine*. The mice were starved overnight before the final experiments, n = 5 for each group. The carnitine concentrations are expressed as µmol/L. Homozygous jvs-/- mice were studied while treated with carnitine (basal), and 3, 6 and 10 days after carnitine deprivation. Results are presented as mean±SD. LCA, long-chain-acylcarnitines; SCA, short-chain-acylcarnitines; TAS, total acid soluble carnitine.

Liver carnitine pool

As shown in Table 3, the free carnitine content in the liver was not different between wild type, heterozygous jvs+/- and homozygous jvs-/- mice. After carnitine

withdrawal, there was a rapid and significant drop in the hepatic free carnitine content in homozygous jvs-/- mice, resulting in free carnitine levels in the range of 40 to 50% percent of those in homozygous jvs-/- mice. Similar results were obtained for the other carnitine fractions, i.e. short- and long-chain acylcarnitines and total carnitine. The ratio between free carnitine and short-chain acylcarnitines did not show significant differences between wild type, heterozygous jvs+/- and homozygous jvs-/- mice, but had a tendency to increase after carnitine withdrawal in homozygous jvs-/- mice.

	Wild type	Heterozygous	Homozygous (jvs-/-)			
	(jvs+/+)	(jvs+/-)	basal	day3	day6	day10
Free Carnitine	143±22	124±14	133±18	44±19†	37±11†	51±7†
SCA Carnitine	198±38	194±36	176±23	141±18	129±27†	97±26†
TAS Carnitine	341±20	318±26	309±13	185±10†	166±16†	148±28†
LCA Carnitine	95±11	79±16	55±12	46±7	41±8	38±7†
SCA/TAS Carnitine	0.58±0.08	0.61±0.07	0.57±0.06	0.76±0.10†	0.77±0.08†	0.67±0.05
Total Carnitine	436±11	397±39	363±22*	230±10†	207±16†	186±32†

*p<0.05 vs. jvs+/+, † p<0.05 vs. jvs-/-, § p<0.05 vs. jvs+/-

Table 3: *Liver carnitine content*. The mice were starved overnight before the final experiments, n = 5 for each group. The carnitine content is expressed as nmol/g wet tissue. Homozygous jvs-/- mice were studied while treated with carnitine (basal), and 3, 6 and 10 days after carnitine deprivation. Results are presented as mean±SD. LCA, long-chain-acylcarnitines; SCA, short-chain-acylcarnitines; TAS, total acid soluble carnitine

Skeletal muscle carnitine pool

In contrast to liver, the free carnitine content in skeletal muscle was decreased in heterozygous jvs+/- (decrease by 24%) and in homozygous jvs-/- mice (decrease by 33%) as compared to wild type mice. Similar to liver, carnitine deprivation was associated with a significant decrease in the skeletal muscle carnitine content in homozygous jvs-/- mice. After 10 days of carnitine withdrawal, the skeletal muscle carnitine content had dropped to 35% of the level in homozygous jvs-/- mice supplemented with carnitine. Similar results were obtained for the other carnitine

fractions, i.e. short- and long-chain acylcarnitines and total carnitine. Similar to liver, the ratio between free carnitine and total acid soluble carnitine increased with carnitine deprivation, reaching significance 10 days after carnitine withdrawal.

Kinetics of total carnitine loss

As shown in figure 1, the velocity of the drop in the total carnitine levels is different between plasma, liver and skeletal muscle. As expected when renal reabsorption of carnitine is lacking, the carnitine concentration drops most rapidly in plasma. Interestingly, the initial drop in the tissue carnitine content was faster in liver than in skeletal muscle, suggesting that export of carnitine and acylcarnitines from hepatocytes is easier than from myocytes. Ten days after carnitine withdrawal, the tissue content was still in the range of 60-70% percent of the initial values, demonstrating that the plasma membranes represent a strong barrier for cellular carnitine excretion.

Fig. 1

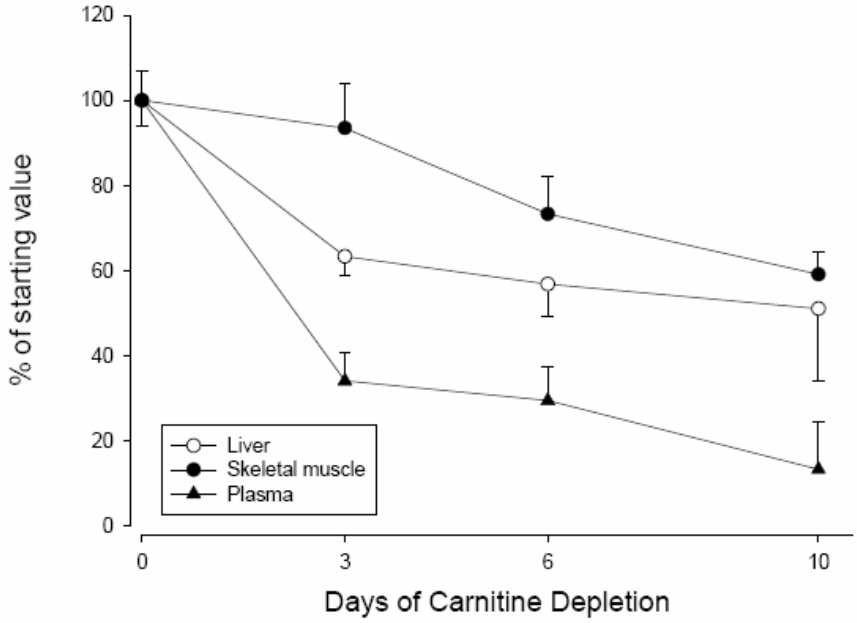

Figure 1: Carnitine plasma and tissue stores after cessation of carnitine treatment in homozygous jvs-/- mice. After carnitine deprivation, there is a rapid fall in the plasma total carnitine content, whereas the tissue carnitine content falls less rapidly. The fractional elimination rate of carnitine from skeletal muscle is in the order of 4% per day or 0.2% per hour.

Microvesicular liver steatosis

Microvesicular liver steatosis, a consequence of impaired hepatic β-oxidation (Fromenty and Pessayre, 1995; Spaniol et al., 2001b), is a hallmark of homozygous jvs-/- mice (Koizumi et al., 1988; Kaido et al., 1997). As shown in Figure 2, microvesicular steatosis was much more accentuated in homozygous jvs-/- mice as compared to wild type or heterozygous jvs+/- mice. After carnitine withdrawal, microvesicular liver steatosis increased proportionally with the duration of carnitine deprivation. Accordingly, the highest fat content was observed in livers from homozygous jvs-/- mice 10 days after carnitine withdrawal.

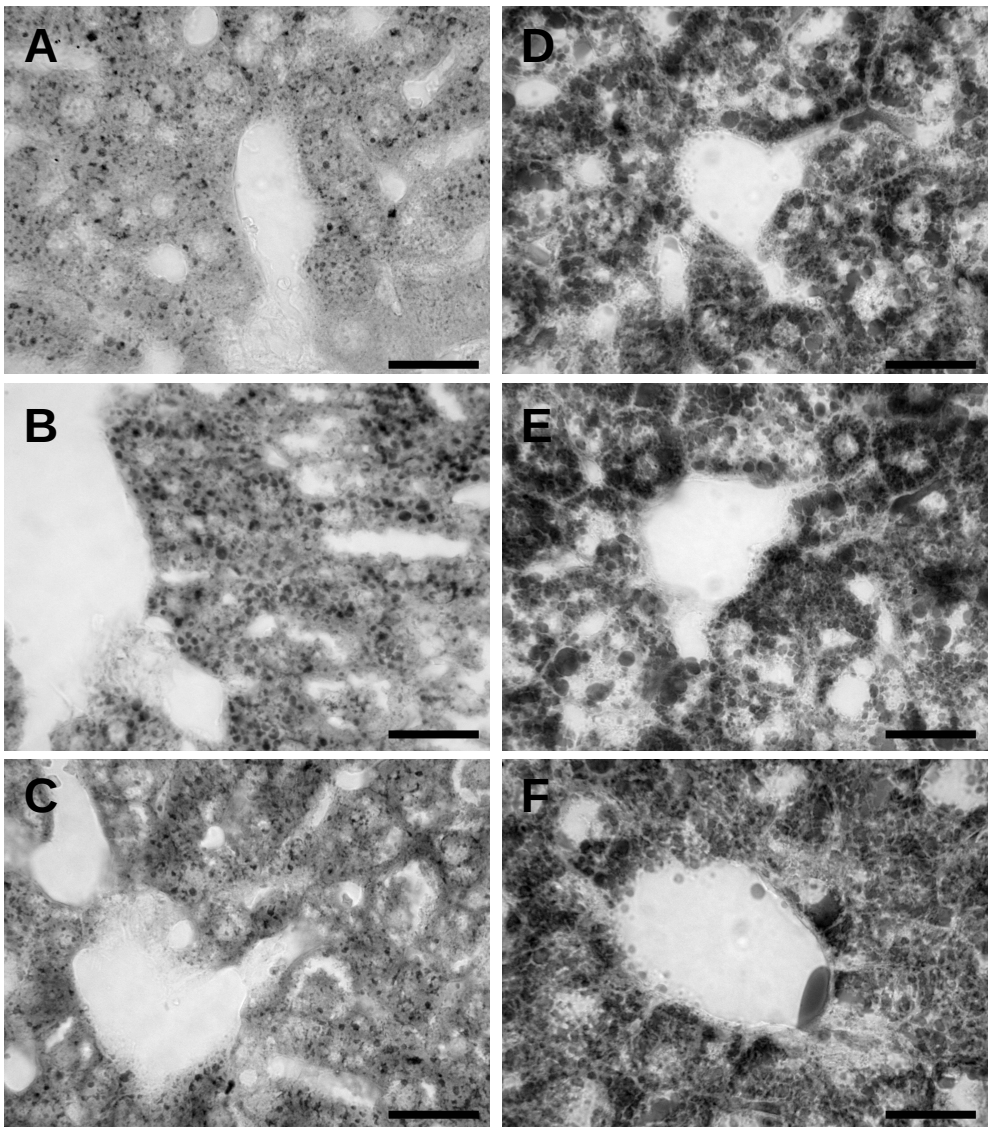

Figure 2: Hepatic accumulation of fat in wild type (A), heterozygous jvs+/- (B) and homozygous-/- mice before (C) and after carnitine deprivation for 3 (D), 6 (E) and 10 days (F). Homozygous jvs-/- mice reveal a slight increase in the hepatic fat content compared to wild type or heterozygous jvs+/- mice. Carnitine deprivation is associated with a rapid and massive increase in the hepatic fat content in homozygous jvs-/- mice. Sudan Black B stain, magnification 63x, the micron bars represent 25 µm.

6.5 Discussion

While the free and total carnitine plasma concentrations were clearly decreased in heterozygous jvs+/- and carnitine-treated homozygous jvs-/- mice compared to wild type mice, the tissue carnitine pool was not decreased in heterozygous jvs+/- mice and only slightly decreased in carnitine-treated homozygous jvs-/- mice. These findings suggest that the plasma membranes of hepatocytes and myocytes are quite resistant for the transport of carnitine, protecting the tissues from carnitine losses. This statement is underscored by the findings during carnitine deprivation in homozygous jvs-/- mice. While the plasma carnitine concentration decreased rapidly in jvs-/- mice after cessation of carnitine administration, the carnitine tissue levels showed a much slower decrease (see Figure 1).

The rapid fall in the plasma carnitine concentration after carnitine deprivation could be expected, since the renal carnitine excretion fraction of carnitine increases from <0.05 to approximately 1 in patients or mice with systemic carnitine deficiency (Treem et al., 1988; Kuwajima et al., 1991), demonstrating that OCTN2 is the most important or even the only carrier for renal reabsorption of carnitine. Since carnitine is a polar molecule, transporters are needed for its efficient transition across biological membranes. Taking into account the large concentration difference between the intracellular and the plasma carnitine levels (between 1 and 2 orders of magnitude) and in vitro findings with isolated rat skeletal muscle (Brass et al., 1993), diffusion may also play a role. While the transport into cells is mediated by OCTN2 and possibly other carriers, which use the sodium gradient between plasma or interstitial fluid and the intracellular milieu as a driving force (Stieger et al., 1995; Tamai et al., 1998; Berardi et al., 2000), the export of carnitine from cells has so far not been characterized on the molecular level. In perfused rat livers, an active transport mechanism has been described, which can be blocked by mersalyl but not oubain and which has a K_m in the range of 300 μmol/L and a maximal transport capacity of approximately 2.5 nmol/g liver per minute (Sandor et al., 1985). Using these values, the livers of the jvs-/- mice (weighing 1 to 1.5 g) should have been completely carnitine-depleted in less than 3 hours after carnitine deprivation. In contrast to this prediction, the livers of jvs -/- mice had lost only approximately 50% of their carnitine stores after carnitine deprivation for 10 days. Assuming that the observations reported in the literature are correct and that the data obtained in rats are also valid for mice, two explanations can be offered to resolve this apparent discrepancy. The

first one is uptake of a sufficient amount of carnitine by the food. Mice eat per day approximately 10% of their body weight (approximately 2g per day) and the food for rodents used by us contains approximately 15 nmoles carnitine per g (Spaniol et al., 2003), resulting in a daily carnitine ingestion of approximately 30 nmoles. This amount is lower than the daily excretion in the urine, which is in the range of 100 nmoles/day for wild type or heterozygous jvs -/+ mice (Knapp AC and Krähenbühl S, unpublished results) or even higher in jvs -/- mice (Horiuchi et al., 1994). Carnitine intake by the food is therefore not sufficient to explain our findings. A second possible explanation is hepatic carnitine biosynthesis. In rodents, the liver is the most important organ for the final step in carnitine biosynthesis, the conversion of butyrobetaine to carnitine (Krahenbuhl et al., 2000b). Butyrobetaine is formed in most tissues by trimethylation of protein-bound lysine, which is subsequently transformed over several steps (including proteolysis and decarboxylation) to butyrobetaine (Hoppel and Davis, 1986; Krahenbuhl et al., 2000b; Vaz and Wanders, 2002). Since the highest amount of butyrobetaine is formed in skeletal muscle (Davis and Hoppel, 1986; Krahenbuhl et al., 2000b), butyrobetaine would have to be transported from skeletal muscle into the liver, where it is hydroxylated to carnitine. Similar to carnitine, the transport into the liver is active and sodium-dependent, and has a K_m in the range of 5 µmol/L (Berardi et al., 1998), suggesting that butyrobetaine is transported by a carrier similar to or identical with OCTN2. Since jvs -/- mice are able to form carnitine from butyrobetaine in vivo (Higashi et al., 2001), it can be assumed, however, that other transporters than OCTN2 exist that can transport butyrobetaine at a sufficient amount into the liver. Hepatic synthesis of carnitine is therefore a possible explanation for the slow decrease of the hepatic carnitine stores in jvs -/- mice.

On the other hand, the slow decay of the carnitine content in skeletal muscle could be expected. Rebouche et al. have investigated the export of carnitine from tissues such as skeletal muscle in humans with or without carnitine deficiency and have described a fractional elimination rate in the range of 0.5% per hour (Rebouche and Engel, 1984). In our studies, the fractional elimination rate of carnitine from skeletal muscle is in the range of 4% per day or approximately 0.2% per hour, which is close to the values reported by Rebouche et al. (Rebouche and Engel, 1984). These observations show directly that the tissue carnitine stores are tried to be kept at a high level as long as possible by a high resistance of the plasma membranes for

carnitine transport and possibly also by synthesis of carnitine in specific tissues such as the liver.

Nevertheless, these measures are not sufficient to keep the carnitine tissue stores at a high enough level to avoid negative consequences on energy metabolism in jvs -/- mice. While carnitine-treated jvs-/- mice had lower β-hydroxybutyrate plasma levels after starvation for 12 h than wild type or heterozygous jvs-/+ mice (suggesting impaired hepatic β-oxidation (Brass and Hoppel, 1978), accumulation of hepatic fat was not dramatic and skeletal muscle energy metabolism was not disturbed. After cessation of carnitine treatment, however, plasma β-hydroxybutyrate levels fell rapidly and hepatic accumulation of fat increased dramatically, suggesting that hepatic β-oxidation had almost vanished. Nevertheless, jvs-/- mice were still able to maintain the tissue ATP levels up to 10 days of carnitine deprivation. In skeletal muscle, glycogenolysis and glycolysis were increased as suggested by the decreasing tissue glycogen content and increasing lactate concentrations after cessation of carnitine administration. Lactate produced in skeletal muscle may have been transported to the liver for gluconeogenesis, possibly explaining the increased hepatic glycogen stores in jvs -/- mice, which were quite resistant to carnitine deprivation.

In conclusion, the current studies show directly that the carnitine tissue stores are maintained over a long time after cessation of carnitine administration in jvs -/- mice due to the tightness of the plasma membranes towards transition of carnitine and most probably also due to hepatic carnitine biosynthesis. Nevertheless, the consequences of carnitine depletion on fatty acid and carbohydrate metabolism appear rapidly after cessation of carnitine administration in jvs -/- mice, showing that the margin between apparent well functioning and demise of energy metabolism is very narrow in mice, and possibly also humans, with systemic carnitine deficiency.

7 Toxicity of valproic acid in in mice with decreased plasma and tissue carnitine stores

Running title: Carnitine deficiency and valproate toxicity

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7.1 Abstract

The aims of this study were to investigate whether carnitine deficiency is a risk factor for valproate (VPA)-associated hepatotoxicity and to explore the effects of VPA on carnitine plasma and tissue stores in mice with carnitine deficiency. Therefore, we treated heterozygous jvs+/- mice, an animal model for systemic carnitine deficiency due to impaired renal reabsorption of carnitine, and the corresponding wild type mice with subtoxic oral doses of VPA (100 mg/kg bw) for 2 weeks. In jvs+/- mice, but not in wild type mice, treatment with VPA was associated with increased serum activities of aspartate aminotransferase and alkaline phosphatase, reduced palmitate metabolism assessed *in vivo* and microvesicular steatosis of the liver. Creatine kinase activities were not affected by treatment with VPA. In isolated liver mitochondria, VPA was associated with decreased oxidative metabolism of L-glutamate, succinate and palmitate as well as impaired β-oxidation of palmitate, both in wild type and jvs+/- mice. In comparison to vehicle-treated wild type mice, vehicle-treated jvs+/- mice had decreased carnitine plasma, liver and skeletal muscle levels. Treatment with VPA was associated with further substantial decreases in carnitine plasma and tissue levels and with a shift of the carnitine pools towards short-chain acylcarnitines. We conclude that jvs+/- mice reveal a more accentuated hepatic toxicity by VPA than the corresponding wild type mice. Systemic carnitine deficiency can therefore be regarded as a risk factor for hepatotoxicity associated with VPA.

7.2 Introduction

Valproic acid (N-dipropylacetic acid) or valproate (VPA) is a branched, medium-chain fatty acid composed of eight carbons, which is structurally unrelated to other antiepileptic drugs. VPA is a broad-spectrum antiepileptic drug which was introduced into the anticonvulsant market in 1968 in Europe and 10 years later in the United States (Zafrani and Berthelot, 1982). Shortly after introduction, cases of fulminant liver failure in patients treated with VPA have been reported (Zafrani and Berthelot, 1982; Zimmerman and Ishak, 1982; Dreifuss et al., 1987; Konig et al., 1994; Krahenbuhl et al., 1995), but the underlying mechanism of VPA induced hepatotoxicity is still not fully understood. One principal cause of liver failure associated with VPA therapy is most probably the inhibition of hepatic mitochondrial β-oxidation. Microvesicular steatosis of the liver, one of the most important

histological findings in VPA-induced liver failure (Zafrani and Berthelot, 1982; Zimmerman and Ishak, 1982; Dreifuss et al., 1987; Krahenbuhl et al., 1995), is considered to result from impaired hepatic β-oxidation (Fromenty and Pessayre, 1995; Spaniol et al., 2001b). Different mechanisms have been proposed explaining inhibition of mitochondrial β-oxidation by VPA, among them microsomal production of toxic metabolites, e.g., 4-ene-VPA and 2,4-diene-VPA (Gram and Bentsen, 1985; Tennison et al., 1988; Ponchaut et al., 1992b; Ishikura et al., 1996), decreased activity of complex IV of the respiratory chain and/or depletion of the hepatic pools of coenzyme A and/or carnitine (Ponchaut and Veitch, 1993; Krahenbuhl et al., 1995). Pre-existing mitochondrial diseases, e.g. impaired β-oxidation or impaired function of the respiratory chain, have been proposed to represent risk factors for VPA-associated mitochondrial dysfunction and therefore for liver failure (Chabrol et al., 1994; Lam et al., 1997; Krahenbuhl et al., 2000a). Since carnitine is an essential cofactor for hepatic β-oxidation (Fromenty and Pessayre, 1995), systemic carnitine deficiency (SCD; OMIM 212140) is associated, among others, with microvesicular steatosis of the liver (Spaniol et al., 2001b). We therefore hypothesized that mice with SCD are more susceptible to hepatic and possibly also skeletal muscle adverse effects associated with VPA treatment compared to control mice. To test our hypothesis, we used juvenile visceral steatosis (jvs) mice (formerly named C3H-H-2ᵒ), which were first described 1988 by Koizumi et al. (Koizumi et al., 1988). Jvs mice have a mutation in the gene coding for OCTN2 (Lu et al., 1998), leading to impaired renal absorption of carnitine and systemic carnitine deficiency. These mice are phenotypically characterized by liver steatosis and other features of carnitine deficiency such as hyperammonemia, hypoglycemia, cardiac hypertrophy, mitochondrial abnormalities in skeletal muscle and progressive growth retardation (Horiuchi et al., 1993; Kaido et al., 1997). For our studies, we used heterozygous (jvs+/-) and not homozygous (jvs-/-) mice, since the carnitine tissue levels of jvs+/- mice are approximately half of wild type mice and, in contrast to homozygous jvs-/- mice, jvs+/- mice can survive without carnitine replacement. An additional question that we wanted to address with our studies was the effect of VPA on carnitine homeostasis in an animal model with decreased carnitine stores such as jvs-/+ mice.

7.3 Materials and Methods

Reagents

[1-^{14}C] palmitic acid was purchased from Amersham Pharmacia Biotech (Dübendorf, Switzerland). Sodium valproate (VPA) and all other chemicals used in this study were obtained from Sigma Aldrich (Buchs, Switzerland) and were of the highest purity available.

Animals

The juvenile visceral steatosis (jvs) mice were obtained from Prof. Masahisa Horiuchi from the University of Kagoshima, Japan. The breeding pairs (wild type and jvs+/- mice) and the offsprings were supplemented with carnitine (1g/250ml drinking water) before weaning to maintain an optimal survival rate. After weaning, the supplementation with carnitine was continued for the homozygous jvs-/- mice. For genotyping the littermates (wild type, jvs+/- and jvs-/- mice), DNA was extracted and purified from the mouse tails with a DNA extraction kit (kit No. 740952.250, Macherey-Nagel, Oensingen, Switzerland) and analyzed using a TaqMan allelic discrimination method, which combines PCR and mutation detection in a single step. Two allele-specific TaqMan probes were used, one for each allele (Applied Biosystems, UK). Each probe consisted of an oligonucleotide with a 5' reporter dye (FAM for the detection of the wild type L352 allele and VIC for the detection of the mutant L352R allele) and a 3' quencher dye (TAMRA for both probes). The probes were as follows: FAM, 5'-atatggtc**a**gcctgca-3' and VIC, 5'-tatggtc**c**gcctgca-3'. The primers used (Microsynth, Switzerland) were identically for both alleles and were designed as follows: forward primer, 5'-tccccatgcaagttaggagtgt-3', reversed primer, 5'-tgctgctccagctctcttctg-3'. TaqMan analysis was carried out on a 7900HT Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland) and identification of the mutation in Octn2 was achieved using an allelic discrimination plot (Todesco et al., 2003). Cycling conditions were 10 min at 95°C for initial denaturation and activation of the DNA polymerase, followed by 40 cycles of 15 s at 95°C for denaturation and 1 min at 60°C for combined annealing and primer extension. Fluorescence from the FAM reporter only reflects the presence of wild type alleles, whereas fluorescence from the VIC reporter only indicates mutant alleles. Accordingly, fluorescence from both reporters reflects the heterozygous population.

All experiments had been reviewed and accepted by the Animal Ethics Committee of the canton Basel Stadt. Experiments were performed with animals 9 to 12 weeks old.

Study design and VPA administration

For this study, 4 groups of jvs mice were investigated. Wild type mice treated with VPA or 0.9% NaCl (vehicle), and heterozygous jvs+/- mice treated with VPA or 0.9 % NaCl (n=5 per group). VPA (0.1 mg/g body weight/day) or vehicle were administered p.o. in a volume of 10 µl/g body weight once a day for 2 weeks. The used VPA dose was subtoxic, as established in earlier studies (Letteron et al., 1996; Schnackenberg et al., 2006). The mice were starved overnight before being used for the experiments. Urine of the mice was collected individually for 24 hours and a blood sample was obtained from the tail vein before the mice were killed by decapitation. Tissue samples were obtained from the liver and skeletal muscle (quadriceps femoris) for carnitine analysis. These samples were quickly frozen in liquid nitrogen and stored at -80℃ until analysis. Additional liver samples were treated with 4% formaldehyde for histological analysis after staining with hematoxylin-eosin or with Sudan Black B. The remainder of the liver was quickly removed, put on ice and used for the isolation of mitochondria.

Characterization of the animals

The animals were characterized by their body and liver weights, activities of aspartate aminotransferase, alkaline phosphatase and creatine kinase. These parameters were analyzed with commercially available kits on a MODULAR analyzer (Hoffmann-La Roche Diagnostics, Basel, Switzerland).

In vivo oxidation of palmitate

To collect breath of the mice, they were placed in a cylindrical vessel attached to a vacuum pump. [1-^{14}C] palmitic acid (3 µCi/kg, 57.0 mCi/mmol) was diluted in thistle oil and administered i.p. at 0 min. To collect the $^{14}CO_2$ resulting from the oxidation of [1-^{14}C] palmitate, the exhaled air was pulled through successive solutions of ethanol (to dry the exhaled breath) and ethanolamine (4M in ethanol) to trap exhaled $^{14}CO_2$. The exhaled $^{14}CO_2$ was quantified over 120 min by scintillation spectroscopy.

Isolation of liver mitochondria

The mitochondrial fraction of mouse livers was obtained by differential centrifugation according to the method of Hoppel (Hoppel et al., 1979). The mitochondrial protein content was determined using the biuret method with BSA as a standard (Gornall et al., 1949).

Oxygen consumption and in vitro β-oxidation of intact mitochondria

Oxygen consumption by freshly isolated liver mitochondria was measured in a chamber equipped with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH) at 30℃ as described previously (Hoppel et al., 1979). The concentrations of the substrates were 20 mmol/l for L-glutamate and succinate, and 40 µmol/l for palmitoyl-CoA. The incubation with palmitoyl-CoA contained in addition 2 mmol/l L-carnitine and 5 mmol/l L-malate.

In vitro β-oxidation of intact mitochondria

The β-oxidation of [1-^{14}C] palmitic acid by liver mitochondria, which measures the formation of acid-soluble products from mitochondrial palmitate metabolism, was determined with freshly isolated liver mitochondria according to the method of Freneaux et al. (Freneaux et al., 1988) with some modifications as described by Spaniol et al. (Spaniol et al., 2001a).

Determination of carnitine in plasma, tissue and urine

The carnitine concentrations in plasma, liver, muscle and urine were determined radioenzymatically as described by Brass and Hoppel (Brass and Hoppel, 1978). Plasma and tissue samples were treated with perchloric acid (final concentration 3%), resulting in a supernatant and a pellet. Analysis of the supernatant yields free carnitine and, after alkaline hydrolysis, total acid soluble carnitine. The pellet yields the long-chain acylcarnitines (acyl group chain length ≥10 carbons) after alkaline hydrolysis. The short-chain acylcarnitine fraction (acyl group chain length <10 carbons) can be calculated from the difference between total acid soluble and free carnitine, and the sum of total acid soluble and long-chain acylcarnitine represents total carnitine.

Histological analysis of liver tissue

Pieces of the liver were fixed in 4% formaldehyde for histological analysis after staining with hematoxylin-eosin or immunohistochemistry for caspase 3.

For caspase 3 staining, paraffin sections were rehydrated and heated in EDTA buffer (pH 8.0; 100℃/ 5min). Slides were then incubated in a quench solution (1.0 M sodium azide in a solution of 4:1 methanol and 30% hydrogen peroxide, v:v) and after that incubated with blocking solution (normal goat serum) for 30min. Next, sections were incubated with caspase-3 antibody (cleaved caspase-3 antibody from Cell Signalling Technology, Beverly, MA, USA) diluted 1:100 in a phosphate-buffered saline pH 7.1–7.3 (Antibody Diluting Buffer, ChemMate, Ventana Medical Systems, Illkirch, France) for 1h at room temperature. Negative controls were performed by omitting the primary antibody. Following primary antibody incubation, slides were washed three times with TBS containing 0.05% Tween 20, and then incubated for 30min at room temperature with a cocktail of biotinylated secondary antibodies in Antibody Dilution Buffer. The slides were again washed and then incubated for 30 minutes at room temperature with avidin-biotin complex (Vectastain Elite ABC kit, Vector, Burlingame, CA, USA). Staining was visualized by incubating for 10min in DAB solution (K3466, Dako, Baar, Switzerland), after which the slides were rinsed in water, counterstained with hematoxylin, dehydrated and coverslipped.

The frozen liver tissue was cut into sections and stained with Sudan Black B (Lison, 1934) for the determination of fat accumulation. The estimation of fat accumulation and the investigation of pathological changes in the liver were carried out by light microscopy of the stained sections.

Statistical analysis

All analyses were performed in duplicate. For each treatment group (n=5 per group) the results are presented as mean ± SD. Significant differences between groups were determined by ANOVA/Bonferroni multiple comparison post hoc test. P values <0.05 were considered to be significant.

7.4 Results

The aims of this study were to investigate whether moderate carnitine deficiency is a risk factor for VPA-associated hepatotoxicity and possibly myotoxicity, and to explore the effect of VPA on carnitine homeostasis in jvs-/+ mice.

Body and liver weight, biochemical parameters

As shown in Table 1, no difference was found in the body weight before and at the end of the study between wild type and jvs+/- mice. Treatment with VPA had no significant effect on body weight. Liver weight adjusted to body weight was increased by 25% in jvs+/- mice treated with VPA compared to jvs+/- mice treated with vehicle. In contrast, treatment with VPA did not affect liver weight in wild type mice. In comparison to wild type mice treated with VPA, jvs+/- mice treated with VPA showed an increase of 49% in aspartate aminotransferase activity and of 50% in alkaline phosphatase activity. In comparison to vehicle-treated jvs+/- mice, this increase was 110% for aspartate aminotransferase and 68% for alkaline phosphatase. Activities of creatine kinase were not significantly different between the groups, suggesting that carnitine status and/or treatment with VPA had no significant effect on skeletal muscle.

	Wild type (jvs+/+)		Heterozygous (jvs+/-)	
	vehicle	VPA	vehicle	VPA
Body weight (start of study) (g)	20.6 ± 1.8	19.3 ± 1.5	22.7 ± 4.0	17.8 ± 2.5
Body weight (end of study) (g)	21.0 ± 2.2	19.7 ± 1.4	22.9 ± 3.7	18.0 ± 2.6
Liver weight (mg per g body weight)	41.6 ± 3.2	45.4 ± 3.5	41.0 ± 4.7	51.2 ± 2.3#
Aspartate aminotransferase (U/l)	49 ± 14	69 ± 18	49 ± 11	103 ± 8†,#
Alkaline phosphatase (U/l)	58 ± 10	82 ± 12	73 ± 19	123 ± 17†,#
Creatine kinase (U/l)	101 ± 50	115 ± 60	95 ± 40	123 ± 47

† p<0.05 VPA-treated jvs+/- vs. VPA-treated wild type; # p<0.05 VPA-treated jvs+/- vs. vehicle-treated jvs+/-

Table 1: *Characterization of the animals*. The mice were starved overnight before the final experiments, n = 5 for each group. Wild type and jvs+/- mice were treated orally with normal saline or VPA for 14 days. Results are presented as mean±SD.

In vivo oxidation of palmitate

As shown in figure 1, VPA-treated jvs+/- mice showed a lower peak exhalation of $^{14}CO_2$ (30 min after injection) compared to vehicle-treated jvs+/- mice (21% decrease) and to VPA-treated wild type mice (20 min after injection, 23% decrease).

Additionally, the exhalation of $^{14}CO_2$ over 2 hours was significantly lower in VPA-treated jvs+/- mice. The decrease reached 23% compared to vehicle-treated jvs+/- mice and 20% compared to VPA-treated wild type mice. On the other hand, there was no difference in these parameters between vehicle-treated wild type mice and vehicle-treated jvs+/- mice, as well as between vehicle-treated and VPA-treated wild type mice. These findings indicate that both carnitine deficiency and treatment with VPA are necessary to impair hepatic β-oxidation *in vivo*.

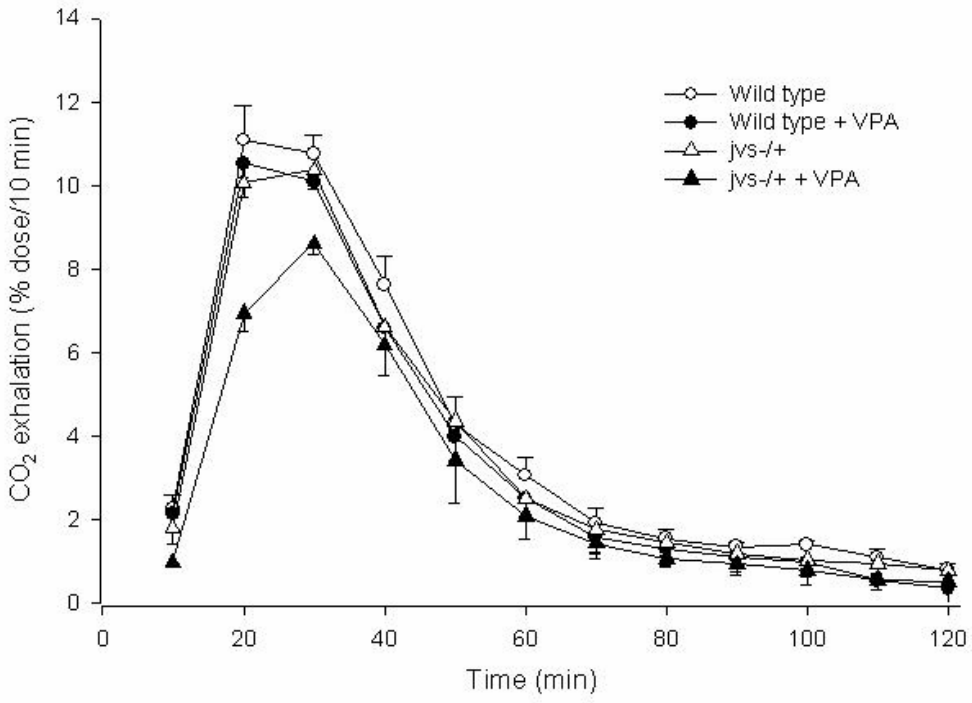

Figure 1: *In vivo* palmitate metabolism by wild type or jvs+/- mice treated with oral saline or VPA (100 mg/kg body weight) for 14 days. A trace amount of [1-^{14}C] palmitic acid was injected intraperitoneally and exhalation of $^{14}CO_2$ was determined over 2 hours. VPA-treated jvs+/- mice show a lower peak exhalation of $^{14}CO_2$ (after 30 min) compared to vehicle-treated jvs+/- mice, vehicle-treated wild type mice or VPA-treated wild type mice (8.6 ± 0.3 vs. 10.6 ± 0.4, 11.3 ± 0.8 or 10.7 ± 0.3% of dose per 10 min, respectively, $p<0.05$ against all other groups). In addition, the total amount of $^{14}CO_2$ exhaled over 2 hours was also less in VPA-treated jvs+/- mice compared to vehicle-treated jvs+/- mice, vehicle-treated wild type mice or VPA-treated wild type mice (33.5 ± 4.2 vs. 41.8 ± 3.9, 46.5 ± 3.9 or 41.7 ± 4.8% of dose, respectively, $p<0.05$ against all other groups).

Histological findings in the liver

In agreement with *in vivo* β-oxidation, fat accumulation in liver was lowest in vehicle-treated wild type mice (Figure 2A) and slightly higher in wild type mice treated with VPA or in vehicle-treated jvs-/+ mice (Figures 2B and C). The combination of carnitine deficiency (jvs-/+ mice) and treatment with VPA was associated with the highest extent of fat accumulation (Figure 2D). The accumulated fat was from the microvesicular type, compatible with impaired β-oxidation (Fromenty and Pessayre, 1995; Spaniol et al., 2003).

Stains with hematoxylin-eosin confirmed the presence of microvesicular steatosis predominantly in VPA-treated jvs+/- mice but showed only a small number of cells undergoing apoptosis (eosinophilic hepatocytes) (Figure 3A). Accordingly, only few cells were caspase-3 positive (Figure 3B), suggesting that apoptosis was rare, even in livers from VPA-treated jvs-/+ mice. Similar histological findings have been reported in another study (Jezequel et al., 1984).

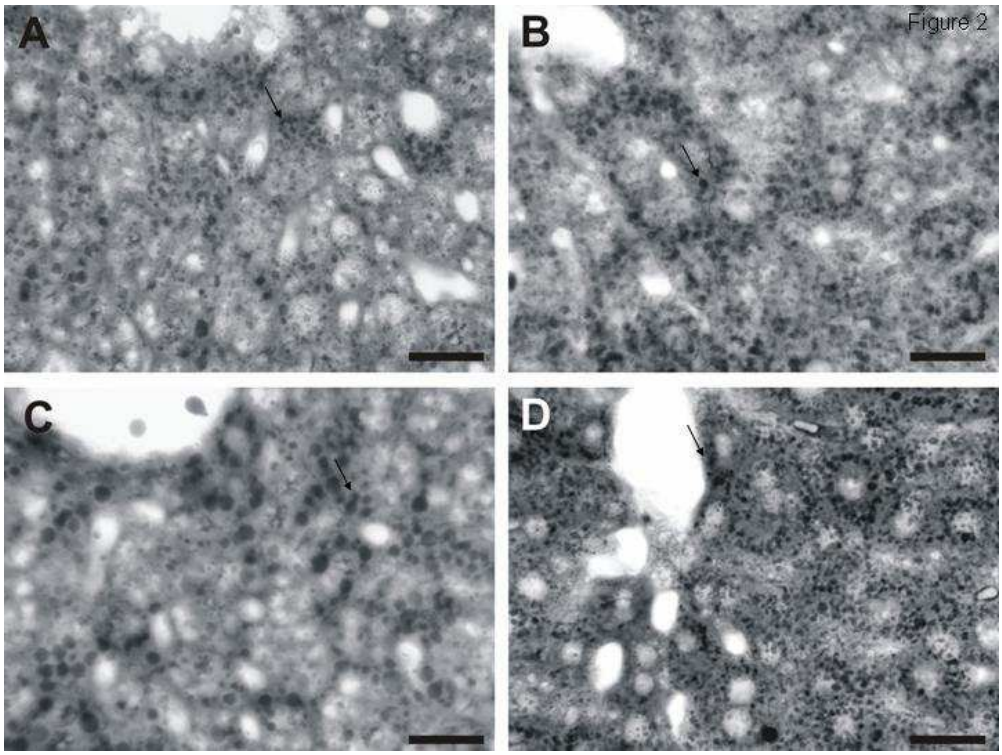

Figure 2: Hepatic accumulation of fat in vehicle-treated wild type (A), VPA-treated wild type (B), vehicle-treated jvs+/- mice (C) and VPA-treated jvs+/- mice (D). Vehicle-treated wild type livers contain only few hepatocytes with Sudan B stainable material (small intracellular dark droplets, see arrow) (A). VPA treatment of wild type mice for two weeks (B) or heterozygousity for OCTN2 (vehicle-treated jvs+/- mice) (C) is associated with a slight increase in microvesicular fat. VPA-treated jvs+/- mice show the highest accumulation of microvesicular fat, mainly in the pericentral region of liver lobules (D). Sudan black B staining, the micron bars represent 20 µm.

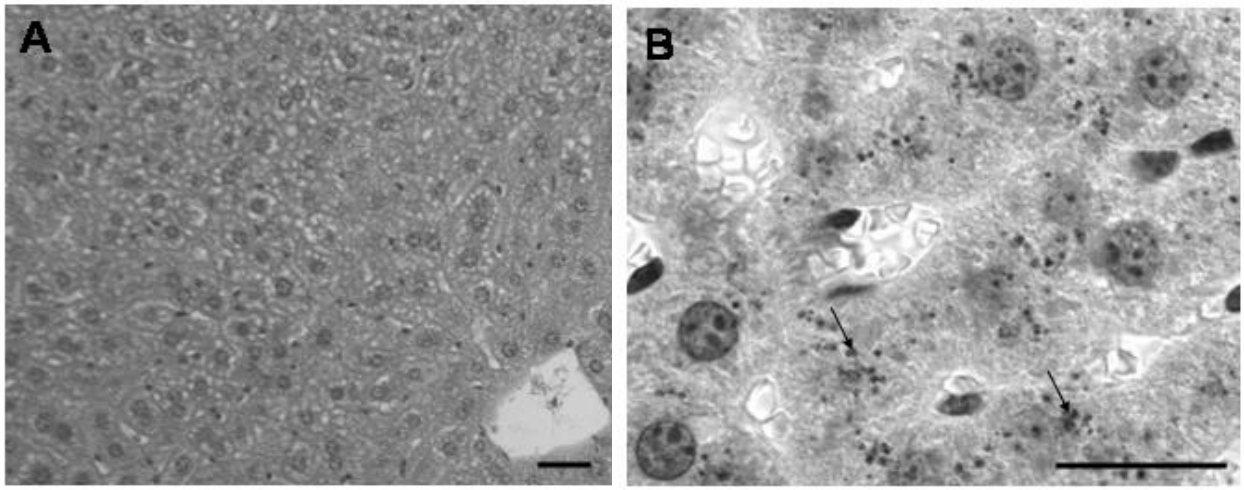

Figure 3: Histological changes in liver sections stained with hematoxylin-eosin and for caspase-3 in liver sections of VPA-treated jvs+/- mice. Staining with hematoxylin-eosin of livers from VPA-treated jvs+/- mice shows cytoplasmic vesicles compatible with microvesicular steatosis, mononuclear portal infiltrates (not shown) and occasional hypereosinophilic hepatocytes (3A). Staining with caspase-3 is slightly positive in such hepatocytes (3B), compatible with hepatocyte apoptosis. In sections of vehicle-treated wild type livers no such changes could be observed. HE-staining, the micron bars represent 20 µm.

Oxygen consumption by isolated liver mitochondria

Since impairment of mitochondrial function associated with VPA has been shown before (Ponchaut and Veitch, 1993), we examined the function of the respiratory chain in the presence of different substrates. As shown in table 2, the state 3 oxidation rate of L-glutamate was decreased by 32% in VPA-treated compared to vehicle-treated wild type mice. Similarly, a decrease of 58% was found in VPA-treated heterozygous jvs+/- versus vehicle-treated jvs+/- mice. The state 3 oxidation rate of succinate was also significantly lower in VPA-treated jvs+/- mice (decrease by 52%) compared to vehicle-treated heterozygous jvs+/- mice, whereas no significant decrease was found in VPA-treated wild type compared to vehicle-treated wild type mice. Palmitoyl-CoA state 3 oxidation rates were decreased by 43% in VPA-treated wild type mice and by 73% in VPA-treated jvs+/- mice compared to their vehicle-treated controls.

In vitro β-oxidation by intact liver mitochondria

Due to the fact that VPA has been shown to impair mitochondrial β-oxidation (Levy et al., 1990; Ponchaut et al., 1992b; Fromenty and Pessayre, 1995), we also investigated the effect of VPA on the metabolism of palmitate by isolated liver

mitochondria. As shown in table 2, VPA treatment significantly decreased palmitate oxidation by 44% in wild type and by 35% in jvs+/- mice compared to their vehicle-treated controls. For the interpretation of these results, it is important to take into account that the experiments were performed under saturating conditions regarding palmitate and in the presence of exogenous L-carnitine, which is different from the *in vivo* palmitate oxidation experiments.

	Wild type (jvs+/+)		Heterozygous (jvs+/-)	
	vehicle	VPA	vehicle	VPA
State 3 oxidation rates				
L-Glutamate (20 mM)	56 ± 12	38 ± 8*	48 ± 8	28 ± 2#
Succinate (20 mM)	120 ± 20	88 ± 28	122 ± 28	64 ± 10#
Palmitoyl-CoA (40 µM)	42 ± 12	24 ± 8*	30 ± 8	8 ± 2†,#
Mitochondrial ß-Oxidation				
ß-Oxidation of ^{14}C-1-palmitate	0.27 ± 0.04	0.15 ± 0.02*	0.26 ± 0.04	0.17 ± 0.04#

* p<0.05 Vehicle-treated jvs+/- or VPA-treated wild type vs. vehicle-treated wild type; † p<0.05 VPA-treated jvs+/- vs. VPA-treated wild type; # p<0.05 VPA-treated jvs+/- vs. vehicle-treated jvs+/-

Table2: *Function of mouse liver mitochondria.* The mice were starved overnight before the final experiments, n = 5 for each group. Wild type and jvs+/- mice were treated orally with normal saline or VPA for 14 days. Mitochondria were isolated by differential centrifugation. State 3 oxidation rates were determined using different substrates and the in vitro ß-oxidation was measured with ^{14}C-1-palmitate. Units are natoms oxygen/min/mg mitochondrial protein for the oxidation rates and nmol/min/mg mitochondrial protein for ß-oxidation. Results are presented as mean±SD.

Plasma carnitine concentration

As shown in table 3, the free and total carnitine levels in plasma were significantly lower in wild type mice treated with VPA compared to vehicle-treated wild type mice (decrease by 58% and 24%, respectively). Similar results were obtained for VPA-treated jvs+/- mice, where the decrease was 65% for free carnitine and 39% for total carnitine, respectively, versus vehicle-treated jvs+/- mice. A comparison of VPA-treated jvs+/- versus VPA-treated wild type mice revealed a decrease of 40% in free carnitine and a decrease of 37% in total carnitine in jvs+/- mice. Treatment with VPA was associated with a decrease in the total carnitine concentration in both groups, namely by 24% in wild type and by 39% in jvs+/- mice. Remarkably, the short-chain acylcarnitine/total acid soluble carnitine ratio showed a 120% increase in VPA-treated wild type and a 76% increase in VPA-treated heterozygous jvs+/- mice compared to their vehicle-treated controls.

	Wild type (jvs+/+)		Heterozygous (jvs+/-)	
	vehicle	VPA	vehicle	VPA
Free Carnitine	31.3 ± 2.7	13.1 ± 1.7*	22.4 ± 2.0*	7.9 ± 1.7†,#
SCA Carnitine	10.1 ± 3.4	14.6 ± 2.1*	11.0 ± 1.5	11.1 ± 1.4
TAS Carnitine	41.4 ± 1.2	27.6 ± 1.4*	33.6 ± 1.2*	19.0 ± 0.9†,#
LCA Carnitine	6.6 ± 1.3	9.0 ± 0.9*	4.2 ± 1.2*	4.1 ± 0.5†
SCA/TAS Carnitine	0.24 ± 0.08	0.53 ± 0.06*	0.33 ± 0.04	0.58 ± 0.08#
Total Carnitine	48.0 ± 1.2	36.6 ± 2.3*	37.8 ± 2.2*	23.1 ± 1.2†,#

* p<0.05 Vehicle-treated jvs+/- or VPA-treated wild type vs. vehicle-treated wild type; † p<0.05 VPA-treated jvs+/- vs. VPA-treated wild type; # p<0.05 VPA-treated jvs+/- vs. vehicle-treated jvs+/-

Table 3: *Plasma carnitine content*. The mice were starved overnight before the final experiments, n = 5 for each group. Wild type and jvs+/- mice were treated orally with normal saline or VPA for 14 days. Carnitine was determined using a radioenzymatic method, units are µmol/L. Results are presented as mean ± SD. LCA, long-chain-acylcarnitine; SCA, short-chain-acylcarnitine; TAS, total acid soluble carnitine.

Urinary excretion of carnitine

As shown in table 4, treatment with VPA over two weeks was associated with an increased excretion of free carnitine in wild type and of free and total carnitine in jvs+/- mice. In jvs+/- mice treated with VPA, the excretion of free carnitine was increased by 114% compared to vehicle-treated jvs+/- mice, whereas the increase in total carnitine excretion was 108%. Compared to vehicle-treated control mice, the renal clearance of free carnitine was approximately 6-fold higher in VPA-treated jvs+/- mice and approximately 3-fold higher in VPA-treated wild type mice.

	Wild type (jvs+/+)		Heterozygous (jvs+/-)	
	vehicle	VPA	vehicle	VPA
Free Carnitine	85.1 ± 11.0	102.8 ± 17.5	91.3 ± 25.5	195.8 ± 15.3†,#
Total Carnitine	144.0 ± 12.1	137.5 ± 14.7	133.2 ± 34.7	277.4 ± 24.7†,#
SCA Carnitine	58.9 ± 13.1	34.7 ± 7.4	41.9 ± 15.7	81.6 ± 19.1†,#
Renal clearance, free carnitine	2.8 ± 0.5	8.0 ± 1.4*	4.10 ± 1.2	25.5 ± 5.10†,#

* p<0.05 Vehicle-treated jvs+/- or VPA-treated wild type vs. vehicle-treated wild type; † p<0.05 VPA-treated jvs+/- vs. VPA-treated wild type; # p<0.05 VPA-treated jvs+/- vs. vehicle-treated jvs+/-

Table 4: *Urinary excretion of carnitine*. The mice were starved overnight before the final experiments, n = 5 for each group. Wild type and jvs+/- mice were treated orally with normal saline or VPA for 14 days. Carnitine was determined using a radioenzymatic method. Units are nmol/day for carnitine and ml/day for renal clearance of free carnitine. Results are presented as mean ± SD.

Liver carnitine content

As shown in Table 5, the hepatic free carnitine content was 33% lower in vehicle-treated jvs+/- compared to vehicle-treated wilt type mice. While treatment with VPA did not affect significantly the free carnitine content in wild type mice, VPA decreased the free carnitine content in jvs+/- mice by 53%. The short chain-acylcarnitine content was increased by VPA treatment by a factor of 2 to 3 in both wild type and jvs+/- mice compared to the respective vehicle-treated groups. Accordingly, the short-chain acylcarnitine/total acid soluble carnitine ratio was increased in both VPA-groups versus the vehicle-treated controls, reaching 157% in the wild type and 195% in jvs+/- mice, respectively. The total carnitine content was decreased by 26% in vehicle-treated jvs+/- compared to wild type mice. In both groups, treatment with VPA was not associated in significant changes in the total carnitine content.

	Wild type (jvs+/+)		Heterozygous (jvs+/-)	
	vehicle	VPA	vehicle	VPA
Free Carnitine	200.6 ± 43.1	166.6 ± 40.6	134.3 ± 24.7*	63.7 ± 12.2†,#
SCA Carnitine	31.8 ± 17.6	93.0 ± 26.9*	31.2 ± 15.3	82.6 ± 16.4#
TAS Carnitine	232.5 ± 27.0	259.7 ± 32.6	165.8 ± 11.4*	146.3 ± 14.0†
LCA Carnitine	71.1 ± 4.7	59.6 ± 2.4*	57.8 ± 3.4*	49.7 ± 5.4†,#
SCA/TAS Carnitine	0.14 ± 0.09	0.36 ± 0.11*	0.19 ± 0.08	0.56 ± 0.07†,#
Total Carnitine	303.6 ± 33.3	319.3 ± 37.4	223.6 ± 11.7*	196.1 ± 18.9†

* p<0.05 Vehicle-treated jvs+/- or VPA-treated wild type vs. vehicle-treated wild type; † p<0.05 VPA-treated jvs+/- vs. VPA-treated wild type; # p<0.05 VPA-treated jvs+/- vs. vehicle-treated jvs+/-

Table 5: *Liver carnitine content*. The mice were starved overnight before the final experiments, n = 5 for each group. Wild type and jvs+/- mice were treated orally with normal saline or VPA for 14 days. Carnitine was determined using a radioenzymatic method. Units are nmol/g wet tissue. Results are presented as mean ± SD. LCA, long-chain-acylcarnitine; SCA, short-chain-acylcarnitine; TAS, total acid soluble carnitine.

Skeletal muscle carnitine content

Free carnitine levels were not different between vehicle-treated wild type and jvs+/- mice. Treatment with VPA decreased the free carnitine content by 31% in jvs+/- mice, but had no significant effect in wild type mice. The short-chain acylcarnitine content and the short-chain acylcarnitine/total acid soluble carnitine ratio were not different between vehicle-treated wild type and jvs+/- mice and were not affected by

VPA treatment. The total carnitine content was 16% lower in vehicle-treated jvs+/- mice compared to the respective wild type mice. Treatment with VPA was associated with a 15% drop in the total carnitine content in jvs+/- mice, but did not affect significantly the total carnitine content in wild type mice (Table 6).

	Wild type (jvs+/+)		Heterozygous (jvs+/-)	
	vehicle	VPA	vehicle	VPA
Free Carnitine	276.5 ± 38.0	283.4 ± 21.9	262.7 ± 54.0	194.7 ± 18.7†
SCA Carnitine	204.0 ± 24.9	204.2 ± 55.0	147.4 ± 18.3	143.9 ± 22.9
TAS Carnitine	480.4 ± 14.3	487.6 ± 42.0	410.7 ± 35.6*	338.6 ± 12.4†,#
LCA Carnitine	140.3 ± 4.4	106.9 ± 17.8*	109.5 ± 20.3*	117.7 ± 7.7
SCA/TAS Carnitine	0.43 ± 0.06	0.41 ± 0.08	0.36 ± 0.06	0.42 ± 0.06
Total Carnitine	620.7 ± 12.8	594.5 ± 44.2	520.2 ± 31.6*	443.9 ± 26.1†,#

* p<0.05 Vehicle-treated jvs+/- or VPA-treated wild type vs. vehicle-treated wild type; † p<0.05 VPA-treated jvs+/- vs. VPA-treated wild type; # p<0.05 VPA-treated jvs+/- vs. vehicle-treated jvs+/-

Table 6: *Muscle carnitine content.* The mice were starved overnight before the final experiments, n = 5 for each group. Wild type and jvs+/- mice were treated orally with normal saline or VPA for 14 days. Carnitine was determined using a radioenzymatic method. Units are nmol/g wet tissue. Results are presented as mean ± SD. LCA, long-chain-acylcarnitine; SCA, short-chain-acylcarnitine; TAS, total acid soluble carnitine.

7.5 Discussion

Our study shows that jvs+/- mice treated with VPA have impaired hepatic mitochondrial β-oxidation and increased hepatic fat accumulation, findings associated with increased activities of serum transaminases and alkaline phosphatase, and hepatocellular damage.

In vivo determination of hepatic β-oxidation revealed a decrease in palmitate metabolism in VPA-treated jvs+/- mice, which was not the case for VPA-treated wild type or vehicle-treated jvs+/- mice. In combination with the liver enzyme elevations, these findings suggest that VPA is more toxic in jvs+/- mice than in wild type mice, supporting our initial hypothesis that carnitine deficiency is a risk factor for hepatotoxicity associated with VPA. VPA is metabolized primarily by conjugation with glucuronic acid or carnitine, and to a lesser extent by mitochondrial β-oxidation, microsomal ω-oxidation and ω-1-oxidation (Zaccara et al., 1988). Microsomal VPA metabolism has been shown to be catalyzed by various cytochrome P450 (CYP) isozymes, among them CYP2C9, 2A6 and 2B6 (Kiang et al., 2006). These oxidative

pathways can yield potentially hepatotoxic products, e.g. pentanoate and propionate, as well as 4-en-VPA and others. It is conceivable that a reduction in the hepatic availability of carnitine in jvs+/- mice can be associated with reduced conjugation of VPA, shifting more VPA into the oxidative pathways and possibly leading to hepatic toxicity. The observed decrease in the hepatic free carnitine content in vehicle-treated jvs+/- versus wild type mice and the even more pronounced decrease in the hepatic carnitine content of jvs+/- mice treated with VPA support such a mechanism. In addition, a reduction in the free carnitine pool is associated with similar changes in the CoA pool (Ponchaut et al., 1992b; Krahenbuhl et al., 1995), because these pools are connected with each other by the carnitine acyltransferases. A drop in cellular CoASH should impair enzymes and/or metabolic pathways using CoASH, for instance pyruvate dehydrogenase and β-oxidation of fatty acids.

If only production and presence of toxic metabolites were responsible for hepatic toxicity of VPA, this toxicity could be expected to decrease or even to disappear in isolated mitochondria, due to loss of toxic metabolites during the isolation procedure (Spaniol et al., 2003). As shown in Table 2, this was clearly not the case in the current investigations, suggesting that mitochondrial changes on the gene expression and/or structural level are associated with VPA treatment. Earlier studies by Hayasaka et al. (Hayasaka et al., 1986) and by Ponchaut et al (Ponchaut et al., 1992a) have indeed demonstrated that long-term treatment with VPA is associated with changes in the composition of cytochrome c oxidase (complex IV), namely a loss cytochrome *aa3*. A reduced activity of complex IV associated with VPA treatment explains not only impaired oxidation of succinate and L-glutamate, but also of palmitate, as observed in our studies (table 2). On the other hand, the reduced activity of mitochondrial β-oxidation, which has been described in other studies assessing hepatic toxicity of VPA (Turnbull et al., 1983; Baldwin et al., 1996), can be explained most probably by interactions of toxic metabolites with enzymes involved in β-oxidation (Ito et al., 1990; Baldwin et al., 1996).

A comparison of palmitate oxidation *in vivo* and *in vitro* reveals that *in vivo*, β-oxidation was impaired only in VPA-treated jvs+/- mice, whereas *in vitro*, palmitate oxidation was reduced also in liver mitochondria from VPA-treated wild-type mice. It has to be taken into account that *in vivo* only a tracer dose was administered whereas saturating concentrations of palmitate were used in the *in vitro* studies. Furthermore, palmitate could have been metabolized *in vivo* to a minor part also by

extrahepatic tissues, rendering small differences in hepatic activity of β-oxidation more difficult to detect. Finally, it can be argued that reduced *in vivo* β-oxidation of palmitate is primarily due to hepatic carnitine deficiency, which was most accentuated in livers from VPA-treated jvs+/- mice (Table 5).

The fact that the skeletal muscle carnitine pools were much less affected by OCTN2 activity and by VPA administration than the hepatic carnitine pools may serve as one possible explanation for reduced toxicity of VPA in skeletal muscle. In addition, the CYPs involved in the metabolism of VPA have the highest expression in liver (Gonzalez, 1992), and not in skeletal muscle. Nevertheless, VPA fat accumulation and morphological mitochondrial abnormalities have been described both in children and in rats with long-term VPA treatment (Melegh and Trombitas, 1997).

Body carnitine homeostasis was affected by both activity of OCTN2 (jvs+/- vs. wild type mice) and treatment with VPA. As expected, jvs+/- mice had clearly reduced plasma and liver carnitine pools compared to wild type mice, demonstrating the importance of renal carnitine reabsorption associated with OCTN2. Interestingly, the effect of partial loss of OCTN2 activity was less accentuated for skeletal muscle than for liver, a finding which can at least partially be explained by the resistance of the plasmalemmal membrane for transport of carnitine (Rebouche and Engel, 1984). The effect of VPA treatment on the carnitine plasma and tissue stores was much more dramatic in JVS+/- than in wild type mice, leading to additional and substantial losses in the plasma and tissue carnitine pools. As shown in Table 4, this is a consequence of a massive increase in the renal excretion of carnitine and of acylcarnitines, in this case most probably valproylcarnitine (Muro et al., 1995). Although precise data are lacking, the increase in renal carnitine excretion can most probably be explained by the competition between valproylcarnitine (and possible other acylcarnitines) with carnitine for proximal tubular reabsorption by OCTN2 (Okamura et al., 2006).

In conclusion, hepatic toxicity of VPA is more pronounced in JVS+/- mice than in corresponding wild type mice. Carnitine deficiency can therefore be considered to be a risk factor for VPA-associated hepatotoxicity, showing the importance of a sufficient hepatic carnitine pool in patients treated with this drug.

8 Expression and inducibility of cytochrome P450 isozymes in immortalized human hepatocytes compared to HepG2 cells

Running title: Expression and induction of CYP450 in hepatic cell lines

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8.1 Abstract

Drug-induced changes in the activity of drug-metabolizing enzymes, e.g. cytochrome P450 (CYP), are an important cause of drug-drug interactions, possibly resulting in loss of efficacy. Prediction of such changes is therefore a prerequisite of early drug development. Since availability and quality of fresh human hepatocytes is problematic, hepatocyte cell lines may serve as an alternative. In this study, we have investigated expression and induction of several CYP isozymes and the human pregnane X receptor (hPXR) in the immortalized human hepatocyte cell line hHepLT5 and in HepG2 cells used as a comparator. hPXR, CYP1A2 and CYP3A4 expression was observed in HepG2 as well as in hHepLT5. CYP2D6 and CYP2C9 were detectable only in HepG2 but not in hHepLT5, and CYP2C19 expression could not be found in both cell lines. Treatment with 50 µM omeprazole did not affect CYP1A2 mRNA induction in both cells. Treatment with 25 µM rifampicin was associated with a 3.4-fold increase in CYP3A4 mRNA in HepG2 but no increase in hHepLT5 cells. In conclusion, hHepLT5 cells contain the hPXR and the CYP isozymes 1A2 and 3A4 but could not be induced by prototypical incucers compared to HepG2 cells.

8.2 Introduction

Drug-drug and food-drug interactions caused by changes in the expression of cytochrome P450 (CYP) genes play an important role in the occurrence adverse drug reactions and/or loss of therapeutic effect of drugs (Li et al., 1997b; Michalets, 1998). Therefore, it is of great interest to investigate the inductive, but also the inhibitory potential of new chemical entities to predict possible changes in drug metabolism. CYPs, representing monooxygenases containing a heme group, are the most important group of enzymes involved in phase I metabolism of xenobiotics, primarily in the liver (Guengerich, 1990). The activity of most CYPs can be regulated by induction and/or inhibition by specific drugs, possibly affecting the metabolism of other drugs (Tanaka and Misawa, 1998; Jones et al., 2000; Lin and Lu, 2001) or even their own metabolism, e.g. by autoinduction (Strolin Benedetti et al., 1990).

The pregnane X receptor (PXR), an orphan nuclear receptor, is one of the key transcriptional regulators of cytochrome P450 CYP3A monooxygenases and other

drug metabolizing enzymes and transporters. PXR is activated by a variety of endogenous (e.g. steroids and bile acids), and exogenous compounds (e.g. rifampicin, phenytoin and hyperforin) through direct interaction with these compounds. PXR activation assays can be used to predict the induction of CYP3A gene expression by drug candidates and therefore to predict drug-drug interactions (Lehmann et al., 1998; Goodwin et al., 2002; Kliewer et al., 2002).

Primary human hepatocytes are considered as the gold standard and as a common model for the *in vitro* assessment of cytochrome P450 manipulation by new drug candidates (LeCluyse, 2001), and there are several studies using primary human hepatocytes for pharmacological and toxicological investigations (Donato et al., 1995; Guillouzo et al., 1997; Kern et al., 1997; Li et al., 1997a). Nevertheless, there are several disadvantages occurring with this screening system. For instance, primary cultures of human hepatocytes lose the expression of CYPs during a short culture period, they show interindividual variability and different quality, and the availability is limited. A possible alternative is the use of HepG2 or LS180 cells, which are used widely as in vitro screening systems. However, it is known that the expression of most CYPs in HepG2 cells is very low or even lacking and can change during culture (Rodriguez-Antona et al., 2002; Wilkening and Bader, 2003; Wilkening et al., 2003; Westerink and Schoonen, 2007).

In order to circumvent some of these drawbacks, we investigated immortalized human hepatocytes, a cell line produced in our laboratory. The cells were characterized by studying the mRNA expression and activity of their CYPs and by studying CYP induction.

8.3 Materials and Methods

Reagents

Dulbecco's Modified Eagle Medium (DMEM), phosphate buffered saline (PBS), fetal bovine serum (FBS), penicillin/streptomycin, GlutaMAX, HEPES and non essential amino acids (NEAA) for the culture of the cell lines were purchased from Invitrogen (Basel, Switzerland). Williams E Medium (WEM) was from Cambrex (Verviers, Belgium). Rifampicin (RIF), omeprazole (OME), insulin, dimethyl sulfoxide (DMSO)

and dexamethason were obtained from Sigma Aldrich (Buchs, Switzerland). All chemicals used in this study were of the highest purity available.

Hepatocyte cell lines and cell culture conditions

HepG2 cells (hepatocellular carcinoma cell line) were kindly provided by Professor Dietrich von Schweinitz (University Hospital Basel, Switzerland) and were used between passage 4 – 8.

The hHepLT5 cell line was generated in our laboratory by transducing the simian virus 40 large T antigen (SV40TAg) gene into primary human hepatocytes (Becton Dickinson, Woburn, US) using a HIV-derived lentiviral vector as described by Salmon et al. (Salmon et al., 2000). Clones (named hHepLT) were generated by single cell cloning using serial dilution and selected according to their morphological features and culture properties.

hHepLT5 cells were cultured in Biocoat collagen I-coated plates (BD biosciences, Allschwil, Switzerland) in Williams E Medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 10 mmol/l HEPES buffer, pH 7.4, 2 mmol/l GlutaMAX, penicillin/streptomycin 100 U/ml, insulin 20 mU/ml and dexamethason 100 nmol/l.

For the investigation of CYP1A2 enzyme activity, both cell lines were plated on 24 well plates at density of $2x10^5$ cells per well. For mRNA expression studies, the cells were used after they reached confluence.

Total RNA extraction and cDNA synthesis

At the end of the culture period, medium was removed and total RNA was isolated from cultured cells using the RNeasy Mini Kit from Qiagen (Hombrechtikon, Switzerland) and quantified with a Nano Drop ND-1000 spectrophotometer (Witec AG, Littau, Switzerland). The absorption ratio at 260 nm/280 nm (Nano Drop ND-1000 spectrophotometer) was in the range of 1.8 – 2.0. After DNase I digestion (Invitrogen, Basel, Switzerland), synthesis of cDNA was carried out using 1 – 5 µg total RNA which was reversed transcribed by Superscript II Reverse Transcriptase (Invitrogen, Basel, Switzerland) according to the manufacturer's protocol using random hexamer primers.

Real time polymerase chain reaction (TaqMan assay)

TaqMan analysis was performed using 10 ng of cDNA in a total volume of 10 µl per TaqMan reaction as a template for quantitative real time PCR analysis on a 7900HT Sequence Detection System (Applied Biosystems, Rotkreuz, Switzerland). Primers and probes were designed according to the guidelines of Applied Biosystems with Primer Express 2.0 software (Applied Biosystems) and were synthesized by Eurogentec (Seraing, Belgium). The probes consisted of an oligonucleotide with a 5' reporter dye (FAM) and a 3' quencher dye (TAMRA). Primers and probes for all isoforms were used at concentrations of 900 nm and 200 nm, respectively. Sequences of primers and probes are listed in Table 1. TaqMan Universal PCR Mastermix was purchased from Eurogentec (Seraing, Belgium). Cycling conditions were 10 min at 95°C for initial denaturation and activation of the DNA polymerase, followed by 40 cycles of 15 s at 95°C for denaturation and 1 min at 60°C for combined annealing and primer extension. All samples were run in triplicates and were quantified using the ΔΔCt method. The threshold cycles (Ct) of the genes of interest in all samples were normalized to these of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ($Ct_{gene\ of\ interest} - Ct_{GAPDH} = \Delta Ct$). For induction experiments, the effect of the compounds tested on the genes of interest were normalized to the dimethyl sulfoxide (DMSO) control sample ($\Delta Ct_{compound} - \Delta Ct_{DMSO} = \Delta\Delta Ct$). Fold changes in the expression of the genes of interest were calculated by taking 2 to the power of the ΔΔCt value ($2^{-\Delta\Delta Ct}$) as described in the user bulletin of Applied Biosystems.

Gene	Primer sense	Sequence (5'? 3')	GenBank accession number
GAPDH	Forward	GGTGAAGGTCGGAGTCAACG	X01677
	Probe	CGCCTGGTCACCAGGGCTGC	
	Reverse	ACCATGTAGTTGAGGTCAATGAAGG	
hPXR	Forward	GGCCACTGGCTATCACTTCAA	AF061056
	Probe	AGCCCTTGCATCCTTCACATGTCATGA	
	Reverse	GTTTCATGGCCCTCCTGAAA	
CYP1A2	Forward	TGTTCAAGCACAGCAAGAAGG	AF182274
	Probe	CTAGAGCCAGCGGCAACCTCATCCCA	
	Reverse	TGCTCCAAAGACGTCATTGAC	
CYP2C19	Forward	GAACACCAAGAATCGATGGACA	NM_000769
	Probe	TAATCACTGCAGCTGACTTACTTGGAGCTGGG	
	Reverse	TCAGCAGGAGAAGGAGAGCATA	
CYP2C9	Forward	GACATGAACAACCCTCAGGACTTT	NM_000771
	Probe	AAAACACTGCAGTTGACTTGTTTGGAGC	
	Reverse	TGCTTGTCGTCTCTGTCCCA	
CYP2D6	Forward	CCTACGCTTCCAAAAGGCTTT	NM_000106
	Probe	CAGCTGGATGAGCTGCTAACTGAGCACA	
	Reverse	AGAGAACAGGTCAGCCACCACT	
CYP3A4	Forward	GATTGACTCTCAGAATTCAAAAGAAACTGA	AF182273
	Probe	AGGAGAGAACACTGCTCGTGGTTTCACAG	
	Reverse	GGTGAGTGGCCAGTTCATACATAATG	

Table 1: Sequences of gene specific primers and probes used for real time polymerase chain reaction.

Statistical analysis

All analyses were performed in triplicate. The results are presented as mean ± SD from four separate experiments. Significant differences between groups were determined by ANOVA/Bonferroni multiple comparison post hoc test. P values <0.05 were considered to be significant.

8.4 Results

The aims of this study were to prepare a cell line of immortalized human hepatocytes and to characterize these cells regarding the expression pattern and inducibility of different CYP isozymes and of the human pregnane X receptor (hPXR). The results from these cell lines were compared to those obtained in HepG2 cells.

Immortalization of human hepatocytes

Transduction of human hepatocytes with the simian virus 40 large T antigen (SV40TAg) gene (Salmon et al., 2000) yielded several clones, which were selected according to morphology and culture properties. The clone hHepLT5 showed comparable morphological characteristics as primary cultured hepatocytes (see Figure 1) and was easy to culture. This clone was therefore used for the current studies.

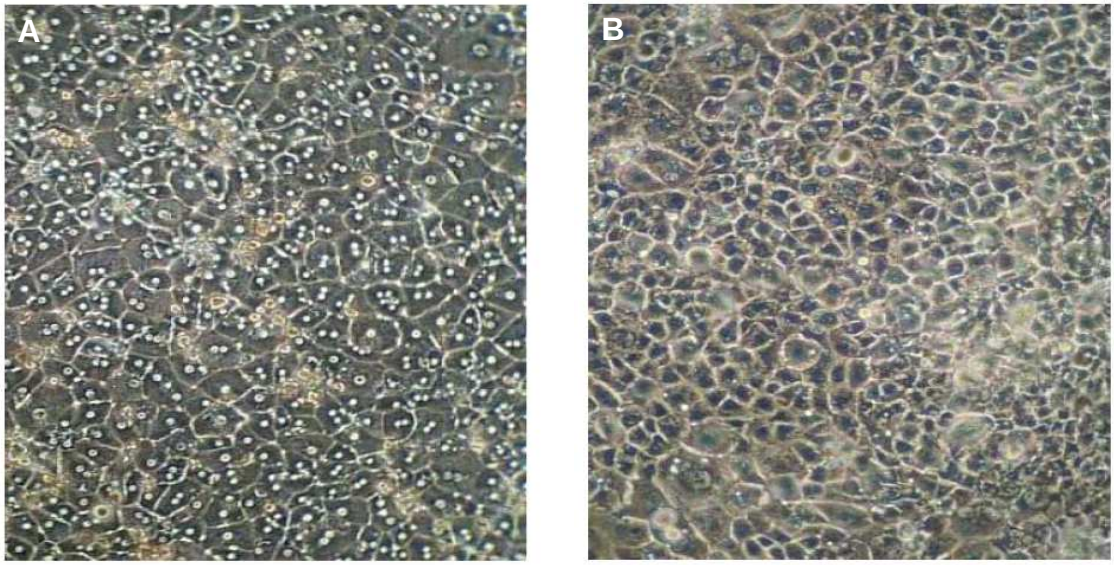

Figure 1: *Morphology of hHepLT5 cells*. The hHepLT cell lines were generated by transducing the simian virus 40 large T antigen (SV40TAg) gene into primary human hepatocytes using a HIV-derived lentiviral vector. Clones were obtained by single cell cloning using serial dilution as described in Methods. The hHepLT5 cell line (Figure 1B) shows morphological features close to human hepatocytes (Figure 1A) and was therefore used for CYP characterization.

mRNA expression of hPXR

As shown in Figure 2, hPXR mRNA was expressed in HepG2 and in hHepLT5. The hPXR mRNA levels in hHepLT5 cells contained approximately 5 times less hPXR mRNA than HepG2 cells.

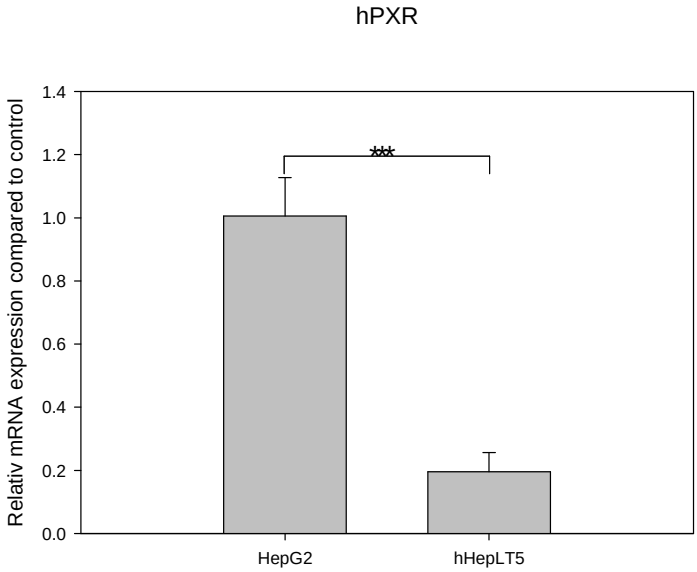

Figure2: *mRNA expression of hPXR*. Expression of hPXR mRNA could be detected in HepG2 and hHepLT5 cells. The hPXR mRNA levels were significantly lower in hHepLT5 cells vs. HepG2 cells. ***P < 0.001 vs. HepG2 cells.

Gene expression of CYP1A2, CYP2D6, CYP3A4, CYP2C9 and CYP2C19

To compare the CYP isozyme mRNA expression levels in the immortalized human hepatocytes cell line, the mRNA expression was normalized to that of untreated HepG2 cells. As shown in Figure 3, CYP1A2 was expressed in both cell lines. In comparison to HepG2 cells, the mRNA expression levels were elevated 2.7-fold in hHepLT5 cells. CYP3A4 mRNA expression could also be detected in HepG2 and in hHepLT5 cells, whereas the mRNA levels were approximately 2-fold lower in hHepLT5, compared to HepG2 cells. The CYP2D6 and CYP2C9 mRNA expression was observed in HepG2, but not in hHepLT5 cells. CYP2C19 mRNA expression was not detectable in HepG2 and in hHepLT5 cells (data not shown).

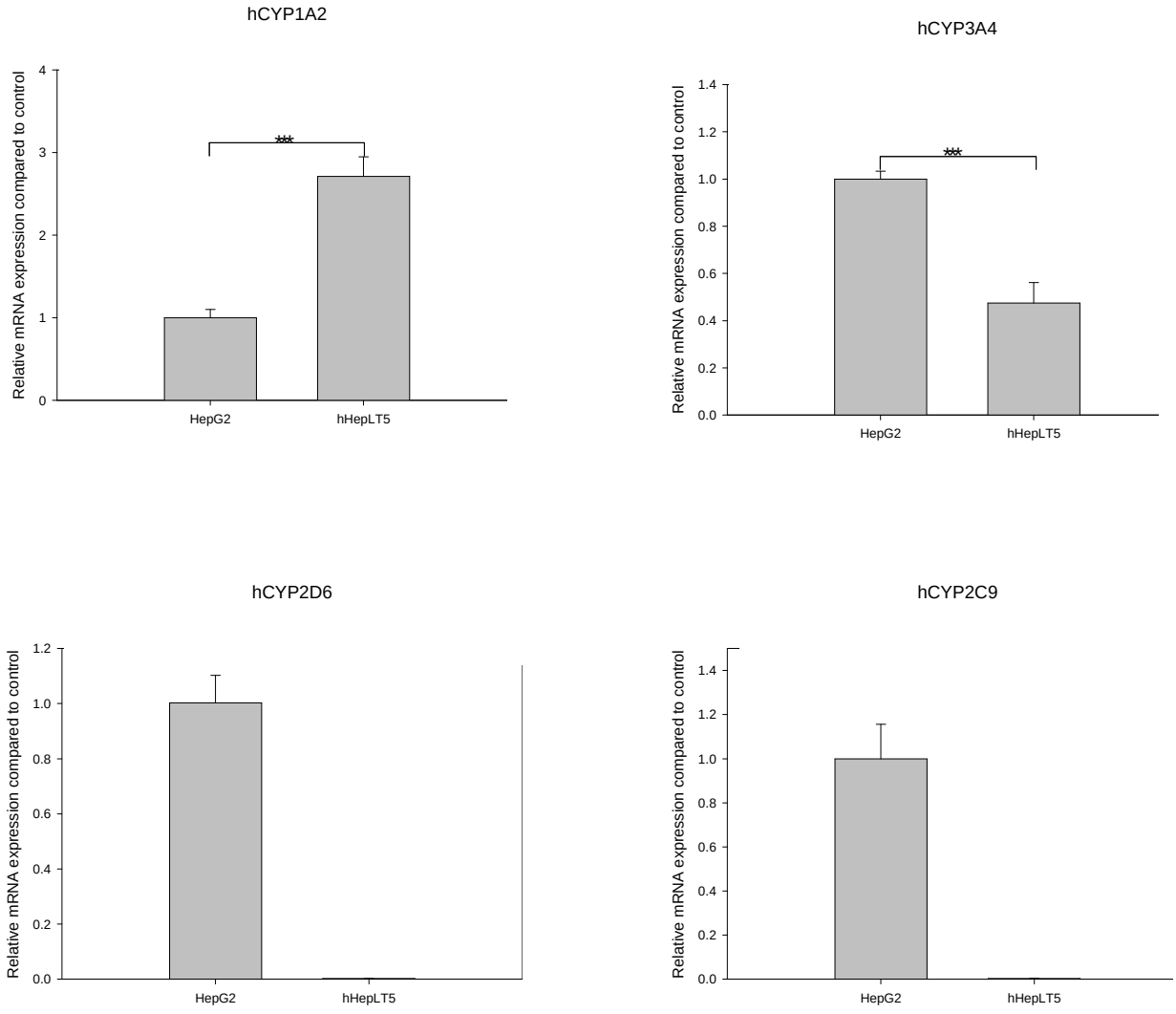

Figure 3: *mRNA expression of several CYP isozymes.* The immortalized human hepatocyte cell line hHepLT5 revealed mRNA expression of CYP1A2 and CYP3A4, but not of CYP2D6 and CYP2C9. *** $P < 0.001$ vs. HepG2 cells.

Inducibility of CYP isozymes

Cells were incubated with medium containing 0.1% DMSO as vehicle control, with rifampicin (10 and 25 µM) for the induction of CYP3A4, CYP2C19 and CYP2C9, or with omperazole (25 and 50 µM) for the induction of CYP1A2 for 72 hours. After treatment with rifampicin, CYP2C9, CYP2C19 and CYP2D6 were not induced in both cell lines (results not shown). As shown in Figure 4, HepG2 cells showed a concentration-dependent significant increase in CYP3A4 mRNA levels in the cells treated with rifampicin as compared to the untreated group. CYP3A4 mRNA levels in HepG2 were increased 1.6-fold and 3.4-fold after treatment with 10 µM and 25 µM rifampicin, respectively. In hHepLT5 cells no CYP3A4 mRNA induction could be observed after treatment with 10 µM and 25 µM rifampicin. Furthermore, the CYP1A2 mRNA levels were not affected by treatment with omeprazole in HepG2 and in hHepLT5 cells.

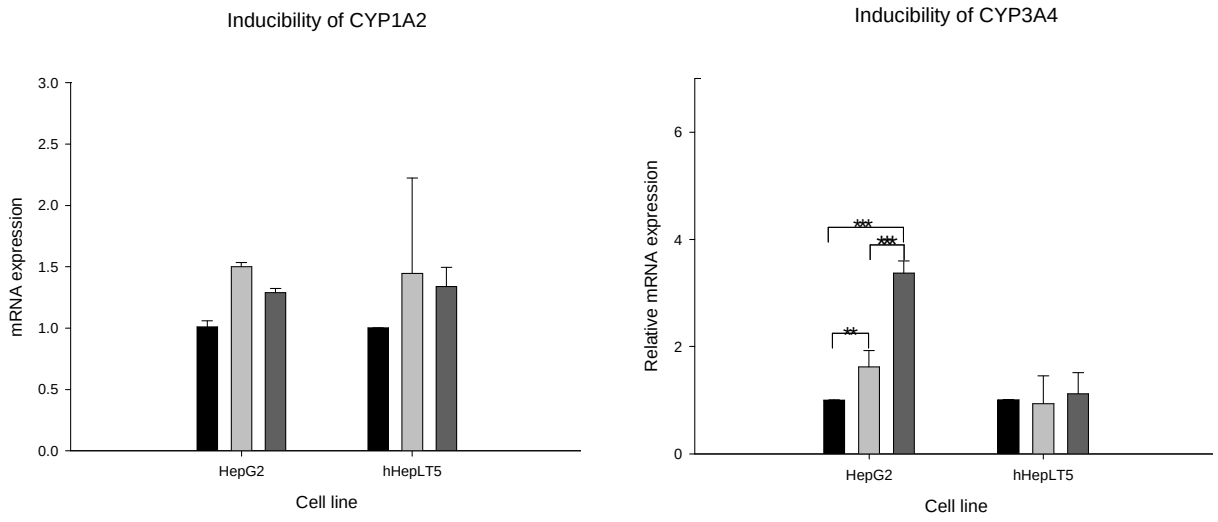

Figure 4: *mRNA expression of CYP3A4 after induction with rifampicin.* Both cell lines were treated for 72 hours with medium only, or with rifampicin or omeprazole in different concentrations. The mRNA expression is shown relative to the respective control incubations (DMSO 0.1%). Results are presented as fold change compared to DMSO 0.1%. OME= omeprazole, RIF = rifampicin. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. DMSO 0.1%.

8.5 Discussion

In the current study, we have investigated the expression and the inducibility of several CYP isozymes in a hepatocellular carcinoma cell line and immortalized human hepatocytes. Since CYP3A4, 2D6, 1A2 and 2C are the main human CYP isozymes associated with drug metabolism, information about inhibitory and/or inducing characteristics of chemical substances may be helpful for the prediction of drug-drug interactions (Michalets, 1998).

CYP1A2 is responsible for about 10 to 15% of the total CYP content of human liver and is the major CYP isozyme involved in the metabolism of important drugs, e.g. imipramine, propranolol, clozapine, olanzapine, theophylline and caffeine (Brosen, 1995). mRNA expression of CYP1A2 could be detected in both cell lines investigated (Figure 2). In comparison to HepG2 cells, the mRNA expression levels were significantly higher in hHepLT5 cells. These results are in accordance with previous reports, where mRNA levels of CYP1A2 in HepG2 cells were very low (Rodriguez-Antona et al., 2002; Wilkening et al., 2003; Westerink and Schoonen, 2007). After induction with omeprazole, which has been shown to be an efficient CYP1A2 inducer (Diaz et al., 1990; Daujat et al., 1992; Curi-Pedrosa et al., 1994), CYP1A2 induction could not be detected in both cell lines investigated.

CYP3A4 is the predominant cytochrome P450 enzyme found in human liver, accounting for up to 30% of total hepatic CYP protein content (Shimada et al., 1994). CYP3A4 is known to metabolize a large variety of xenobiotics (among them amiodarone, lipophilic HMG-CoA reductase inhibitors, cyclosporine, tacrolimus and sirolimus, and various anticancer drugs) and endogenous substances, such as steroids (Brian et al., 1990; Araya and Wikvall, 1999). The results of our studies indicate that CYP3A4 mRNA expression is lower in hHepLT5 cells compared to HepG2. Rifampicin, a known inducer of CYP3A4 and other CYP isozymes (Kern et al., 1997; Rae et al., 2001; Raucy et al., 2002), was able to induce CYP3A4 mRNA levels in a concentration-depending manner in HepG2 cells, but not in hHepLT5 cells. CYP3A4 mRNA induction correlates with mRNA expression of hPXR, which showed a high expression in HepG2 cells, but a low or even lacking expression in hHepLT5 cells. In agreement with CYP3A4 mRNA induction, also CYP3A4 activity was induced by rifampicin in HepG2.

The CYP2C subfamily is also important for drug metabolism, accounting for approximately 18% of the CYP protein content in human liver and for approximately

20% of the CYP-mediated metabolism of drugs (Rendic and Di Carlo, 1997). The CYP2C9 isozyme is, among others, responsible for the metabolism of warfarin, phenytoin and various non-steroidal anti-inflammatory agents (Rettie et al., 1992; Bajpai et al., 1996; Hamman et al., 1997; Miners and Birkett, 1998). CYP2C19 plays a role in the metabolism of phenytoin (Levy, 1995), and is involved in the metabolism of omeprazole and diazepam (Andersson et al., 1993; Jung et al., 1997). In our study, CYP2C9 mRNA was detectable in HepG2, but not in hHepLT5 cells. In comparison, CYP2C19 mRNA expression was neither detectable in HepG2 nor in hHepLT5 cells. As shown in other studies, the expression of CYP2C9 and 2C19 is generally low or even inexistent in HepG2 cells and can change during culture (Ogg et al., 1999; Pourreyron et al., 2003; Plant, 2004; Westerink and Schoonen, 2007). As discussed above, our results show that the hPXR activator rifampicin is able to induce CYP3A4 in HepG2 cells. On the other hand, rifampicin was not able to induce CYP2C9 despite the presence of hPXR. This finding differs from other studies with cultured hepatocytes (Gerbal-Chaloin et al., 2001; Chen et al., 2004). At least in HepG2 cells, hPXR expression is therefore not sufficient for CYP2C9 mRNA expression by rifampicin. Regarding CYP2C9 and 2C19, HepG2 cells can be used to test drug metabolism, but are not suitable to investigate enzyme induction.

CYP2D6 is responsible for the metabolism of many psychotherapeutic agents (e.g. amitriptyline, haloperidol, risperidone) and also of beta-blockers (e.g. metoprolol). Expression of CYP2D6 mRNA could only be detected in HepG2, but not in hHepLT5 cells. CYP2D6 appears not to be inducible by the hPXR inducer rifampicin, as described in other studies (Li et al., 1997b; Edwards et al., 2003; Glaeser et al., 2005). Similar to our statement regarding CYP2C9 and 2C19, only HepG2 cells could possibly be used to test the metabolism of drugs CYP2D6.

In conclusion, hHepLT5 cells contain the CYP isozymes CYP1A2 and 3A4 which are involved in the metabolism of a variety of drugs. The immortalized human hepatocytes cell line hHepLT5 appear therefore to be a suitable alternative for primary human hepatocytes for studying at least certain pharmacological and toxicological features of new drug entities.

9 Hepatic effects of cimicifuga racemosa extract *in vivo* and *in vitro*

Running title: Hepatic effects of cimicifuga racemosa

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9.1 Abstract

Extracts of cimicifuga racemosa are used frequently for menopausal complaints. Cimicifuga is well tolerated, but can occasionally cause liver injury. To assess hepatotoxicity of cimicifuga in more detail, ethanolic cimicifuga racemosa extract was administered orally to rats and liver sections were analyzed by electron microscopy. Tests for cytotoxicity, mitochondrial toxicity and apoptosis/necrosis were performed using HepG2 cells. Mitochondrial toxicity was studied using isolated rat liver mitochondria. Microvesicular steatosis was found in rats treated with >500µg/kg body weight cimicifuga extract. *In vitro*, cytotoxicity was apparent at concentrations ≥75µg/mL and mitochondrial β-oxidation was impaired at concentrations ≥10µg/mL. The mitochondrial membrane potential was decreased at concentrations ≥100µg/mL and oxidative phosphorylation was impaired at concentrations ≥300µg/mL. The mechanism of cell death was predominantly apoptosis. Cimicifuga racemosa exerts toxicity *in vivo* and *in vitro*, eventually resulting in apoptotic cell death. The results are compatible with idiosyncratic hepatotoxicity as observed in patients treated with cimicifuga extracts.

Key words: Cimicifuga racemosa, hepatotoxicity, mitochondria, apoptosis, HepG2

9.2 Introduction

Hormone replacement therapy (HRT) has been considered to be the standard treatment of menopausal disturbances. The association of HRT with breast and uterus cancer (Rossouw et al., 2002) and the desire of many women for a “natural treatment” were the main reasons why alternative therapies became increasingly popular. Especially extracts of the plant cimicifuga racemosa are currently used for this indication. Cimicifuga racemosa, also called actaea racemosa or black cohosh, is a member of the buttercup family (ranunculaceae), and originates from the Eastern part of the United States and Canada. Traditionally, the rhizome was used by North American Indians for joint aches, myalgias, neuralgias and rheumatic disorders, but also for menopausal complaints and pain during labour. Nowadays, ethanolic or isopropanolic extracts of cimicifuga racemosa are most commonly used for the symptomatic treatment of menopausal disorders and of the premenstrual syndrome

(McKenna et al., 2001; Frei-Kleiner et al., 2005; Nappi et al., 2005; Uebelhack et al., 2006), although not all studies have shown a better effect than placebo (Pockaj et al., 2006).

Data from clinical studies and spontaneous reporting programs suggest that adverse events associated with cimicifuga racemosa are rare, generally mild and reversible. Gastrointestinal upset and rashes were the most common adverse events reported (Dog et al., 2003; Huntley and Ernst, 2003). In mostly uncontrolled clinical trials and post-marketing studies including more than 2,800 patients, adverse events had an incidence of 5.4%. Of the reported adverse events, 97% were minor or mild, none of them resulting in discontinuation of the therapy. When higher doses than those recommended are used, however, cimicifuga racemosa can cause dizziness, headaches, nausea, and vomiting (Dog et al., 2003). In their review, which includes all post-marketing programs of cimicifuga extracts, Huntley et al. also described patients with hepatic adverse events (Huntley and Ernst, 2003). They reported one case with hepatic failure, three cases with hepatitis and three cases with increased liver enzymes. In addition, several case reports have been published about patients developing acute hepatitis (Whiting et al., 2002; Cohen et al., 2004) or fulminant liver failure (Lontos et al., 2003; Levitsky et al., 2005; Lynch et al., 2006) while being treated with cimicifuga extracts. The European Medicines Agency (EMEA) recently assessed the case reports of hepatotoxicity associated with ingestion of cimicifuga racemosa root extracts (EMEA/HMPC/88766/2006). The conclusion was that all the cases reported in the literature and all pharmacovigilance reports are poorly documented and that these adverse events should be interpreted as a signal. Systematic investigations and careful assessment of the present and possibly future cases by the marketing authorization holders is appreciated by the EMEA.

Since an association of hepatotoxicity with cimicifuga appears to be possible, we decided to investigate the potential for hepatotoxicity of cimicifuga extracts in experimental animals *in vivo*, in hepatocyte cultures and in isolated liver mitochondria.

9.3 Material and Methods

Chemicals

The cimicifuga extract was obtained from Max Zeller Söhne AG (Romanshorn, Switzerland, batch number V2009). The extraction solvent was 60% ethanol (v:v) with a ratio of native herbal drug to drug preparation of 4.5-8.5:1 (w:w), depending on the content of triterpene glycosides (≥6%). Solutions of the extract were made by dissolving the extract in DMSO. Caffeic acid and ferulic acid were purchased from Fluka (Buchs, Switzerland) and cimiracemoside A was from ChromaDex (Santa Ana, CA, USA). JC-1 and propidium iodide were from Molecular Probes (Eugene, OR, USA); Z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) and Z-Phe-Ala-fluoromethyl-ketone (zFA-fmk) were from Enzyme Systems Products (Livermore, CA, USA). Alexa Fluor 633 labelled annexin V was a generous gift of Dr. Felix Bachmann, Aponetics Ltd. (Witterswil, Switzerland). [1-^{14}C] palmitic acid was obtained from Amersham Pharmacia Biotech (Dübendorf, Switzerland). The scintillation cocktail was from Perkin Elmer (Boston, MA, USA). The Cy3^{TM} conjugated anti-sheep IgG was purchased from Jackson Laboratories (West Grove, PA, USA). All other chemicals were from Sigma (Buchs, Switzerland) and of highest quality available when not otherwise stated.

In vivo hepatotoxicity of cimicifuga extract

Groups of 5 female Wistar rats were fed each with daily doses of 1, 10, 100, 300 or 1000mg/kg body weight of cimicifuga racemosa extract. The extract was administered as a suspension in a solution of Arabic gum in water by means of esophageal gavage over a period of 21 days. After anesthesia and decapitation, the livers of the animals were perfused for fixation, tissue blocks were excised and prepared for electron microscopy as described previously in detail (Spornitz et al., 1999). The study protocol had been accepted by the Animal Ethics Committee of the Canton of Basel.

Cell culture

The human hepatocarcinoma cell line HepG2 was kindly provided by Dr. Dietrich von Schweinitz (Department of Pediatric Surgery, Children's Hospital, University of Basel). The cell line was grown in RPMI 1640 medium supplemented with

GlutaMAX^{TM}-I, 25mM HEPES, 10% (v/v) heat-inactivated fetal bovine serum and 100U/mL penicillin/streptomycin (all from Gibco, Paisley, UK). Culture conditions were 5%CO_2 and 95% air atmosphere at 37°C. Experiments were performed when the cells had reached a confluence of about 80%.

Cytotoxicity tests

To examine cell viability and reductive activity, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed in HepG2 as described originally by Mosmann (Mosmann, 1983), but including an additional washing step (Bruggisser et al., 2002).

In addition, the sulforhodamine B (SRB) test was performed according to the protocol of Skehan (Skehan et al., 1990) and the lactate dehydrogenase (LDH) assay according to Vassault (Vassault, 1983).

Isolation of rat liver mitochondria

Male Sprague Dawley rats (Charles River, Les Onins, France) were anesthetized with carbon dioxide and killed by decapitation. Liver mitochondria were isolated by differential centrifugation according to the method of Hoppel et al (Hoppel et al., 1979). The mitochondrial protein content was determined using the biuret method with bovine serum albumin as a standard (Gornall, 1949).

In vitro mitochondrial β-oxidation

Beta-oxidation with freshly isolated liver mitochondria was assessed as the formation of ^{14}C-acid-soluble β-oxidation products from [1-^{14}C] palmitic acid in the presence of the cimicifuga extracts. Experiments were performed as described initially by Freneaux et al. (Freneaux et al., 1988) with the modifications described by Spaniol et al (Spaniol et al., 2001a).

Oxygen consumption

Polarographic monitoring of oxygen consumption in rat liver mitochondria was carried out in a 1mL chamber equipped with a Clark-type oxygen electrode (Yellow Springs

Instruments, Yellow Springs, OH, USA) at 30℃ as de scribed previously (Krahenbuhl et al., 1991). Subsequent experiments with the F_1F_0-ATPase inhibitor oligomycin (5µg/mL) were performed to check for uncoupling of oxidative phosphorylation (Kaufmann et al., 2005).

Mitochondrial membrane potential

To determine the mitochondrial membrane potential, the dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide) was used according to the protocol of Molecular Probes. Cells were incubated with different concentrations of cimicifuga extract for 24 hours. After the addition of JC-1 and 10 minutes of incubation, cell fluorescence was determined by flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA) (Kaufmann et al., 2006).

Determination of intracellular GSH and GSSG content

In order to assess the redox status of the treated HepG2 cells and possible formation of reactive metabolites, determination of GSH (glutathione) and GSSG (oxidized glutathione) was performed using the enzymatic recycling assay of Tietze (Tietze, 1969), with the modifications described by Griffith et al. (Griffith, 1980).

Apoptosis / Necrosis

Discrimination between apoptosis and necrosis was done with the AnnexinV/ propidium iodide stain. HepG2 cells were incubated for 24 hours with the extract. After trypsinization and centrifugation, cells were resuspended in RPMI medium (adjusted to 2.5mM calcium), stained with Alexa Fluor 633 labelled annexin V and propidium iodide (final concentration 1µg/mL) and analyzed by FACS (FACSCalibur, Becton Dickinson) (Kaufmann et al., 2005).

ATP determination

The ATP content of HepG2 cells treated with cimicifuga extract was determined with the luciferin/luciferase method using the ATP bioluminescence assay kit from Sigma as described previously (Kaufmann et al., 2005).

Hoechst staining

HepG2 cells were incubated with cimicifuga extract for 24 hours, stained with 0.1mM Hoechst 33342 dye and visualized with an inverted fluorescent microscope (Olympus IX50, Hamburg, Germany).

Cytochrome c staining

HepG2 cells (10^5 cells) were seeded into a 8-well chamber slide (Nunc Labtek, Naperville, IL, USA) and cultured for two days. Subsequently, cells were incubated for 24 hours with cimicifuga extracts as described in the result section. The cells were fixed in 4% paraformaldehyde and analyzed for cytochrome c as described previously (Kaufmann et al., 2005).

Statistical methods

Data represent mean ± SEM of at least 4 replicates. Statistical analysis of differences between control incubations and incubations with cimicifuga extract was performed using analysis of variance (ANOVA) and Dunnett's multiple comparison test as posthoc test to localize differences obtained by ANOVA. A p-value <0.05 was considered to be statistically significant.

9.4 Results

Our aims were first to find out whether cimicifuga is hepatotoxic *in vivo* in rats and second, if *in vivo* hepatotoxicity could be demonstrated, to find out its mechanisms by *in vitro* investigations. There were no differences in food consumption and body weight increase between the rats treated with cimifuga extract and the respective control rats (data not shown). Rats treated with 10mg per kg body weight showed a slight increase in the volume of hepatocellular mitochondria (mitochondrial swelling) and an enlargement of bile canaliculi (data not shown). Rats fed with 100 or 300mg/kg body weight showed more marked mitochondrial swelling and alterations in mitochondrial morphology such as vacuoles in the matrix (data not shown). Rats treated with 1000mg/kg body weight developed microvesicular steatosis of the

hepatocytes (see Figure 1), glycogen depletion and fragmentation of the rough endoplasmic reticulum.

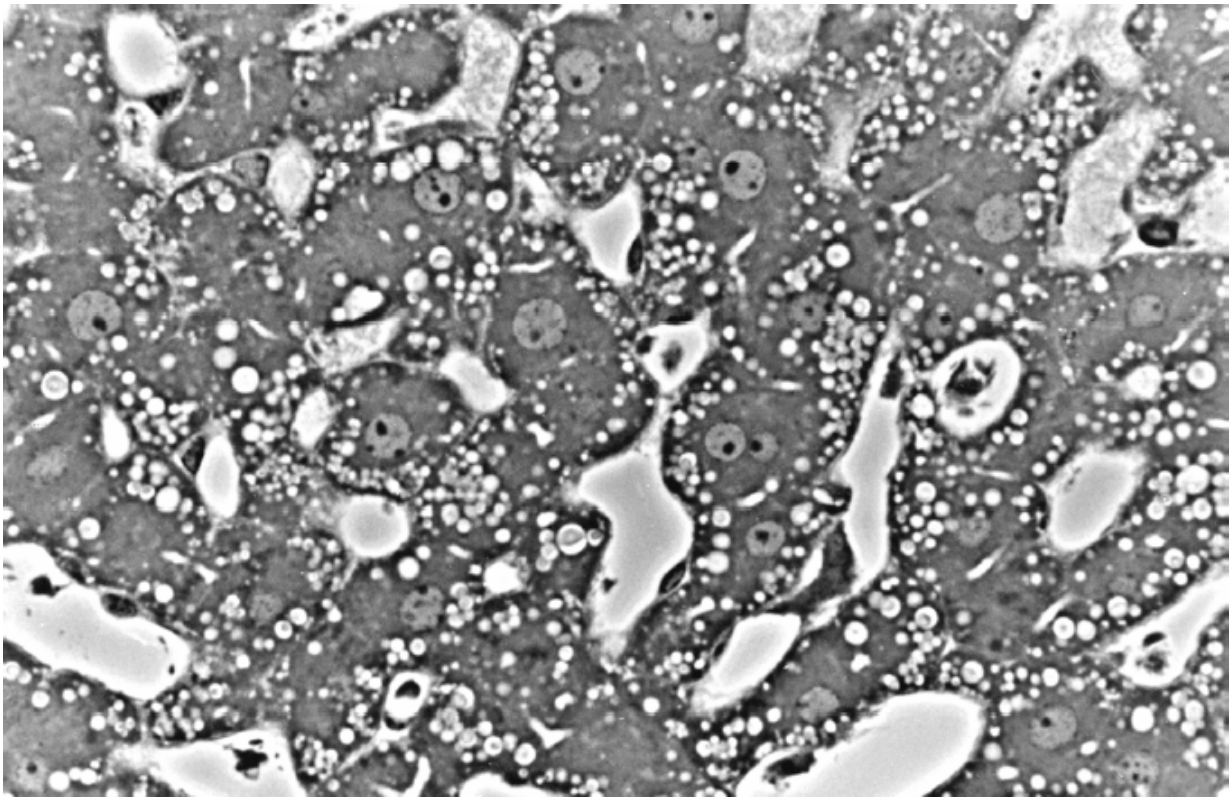

Figure 1: Semi-thin section of a liver form a rat treated with 1000 mg/kg body weight of cimicifuga extract for 21 days. The majority of the intracellular vesicles are small lipid droplets, which are located in the cytoplasm of the hepatocytes and do not displace the nuclei, signs typical for microvesicular steatosis. The sections have been stained with paraphenylene diamine (PPD) as described in methods.

Since microvesicular steatosis usually reflects a mitochondrial damage and can be associated with cytotoxicity (Fromenty and Pessayre, 1995; Mahler et al., 1997), the MTT test was carried out on HepG2 cells. As shown in Figure 2, cimicifuga extract displayed a concentration-dependent toxicity starting at 75µg/mL. Cytotoxicity of cimicifuga could be confirmed using the LDH and the sulforhodamine B tests (data not shown). In contrast, specific components of the cimicifuga extract, namely caffeic acid, ferulic acid or cimiracemoside A, which were investigated at concentrations calculated to be equivalent to those in the extract, were not cytotoxic (data not shown).

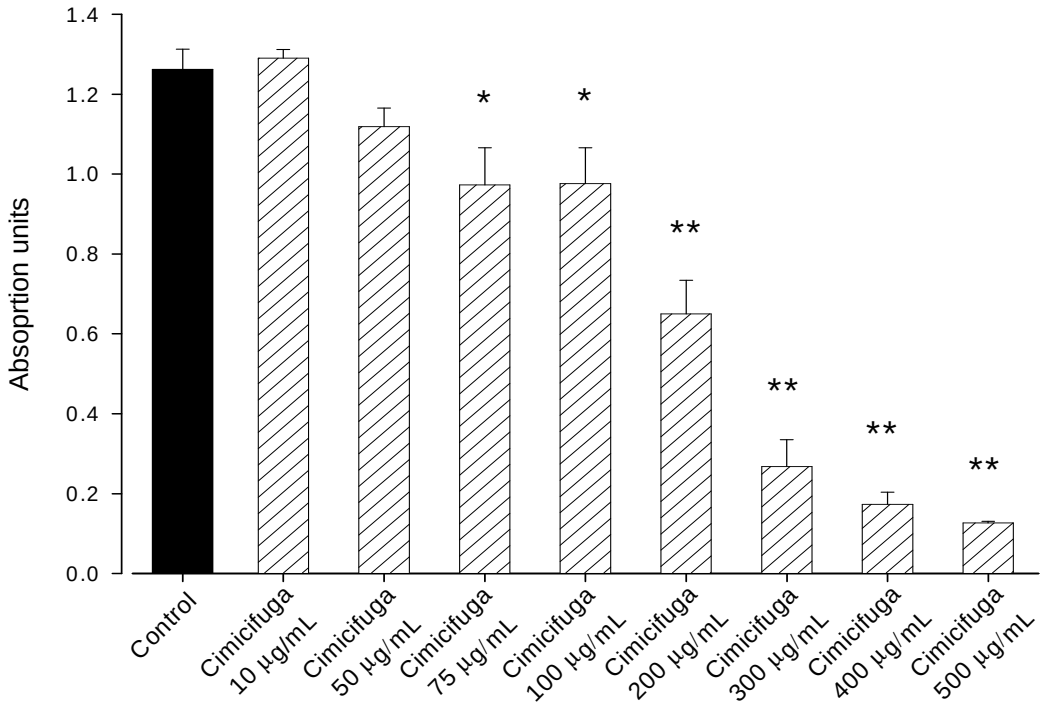

Figure 2: Reductive capacity and integrity of HepG2 cells investigated using the MTT test after incubation with cimicifuga extracts for 24 hours. In intact cells, MTT is metabolically converted to its blue formazan form, whose absorption can be measured at 550 nm, which is presented here. Cytotoxicity is detectable beginning at a concentration of 75 µg/mL of cimicifuga extract. Results are expressed as mean ± SEM of 10 determinations. *P < 0.05, **p < 0.01 vs. control values.

Microvesicular steatosis and a signal in the MTT test were compatible with mitochondrial toxicity associated with cimicifuga extract. The mitochondrial membrane potential was therefore determined in HepG2 cells treated with cimicifuga extract using JC-1 as a marker (Kaufmann et al., 2006). These experiments revealed a dose-dependent decrease in the membrane potential, starting at a concentration of 100µg/mL (see Figure 3).

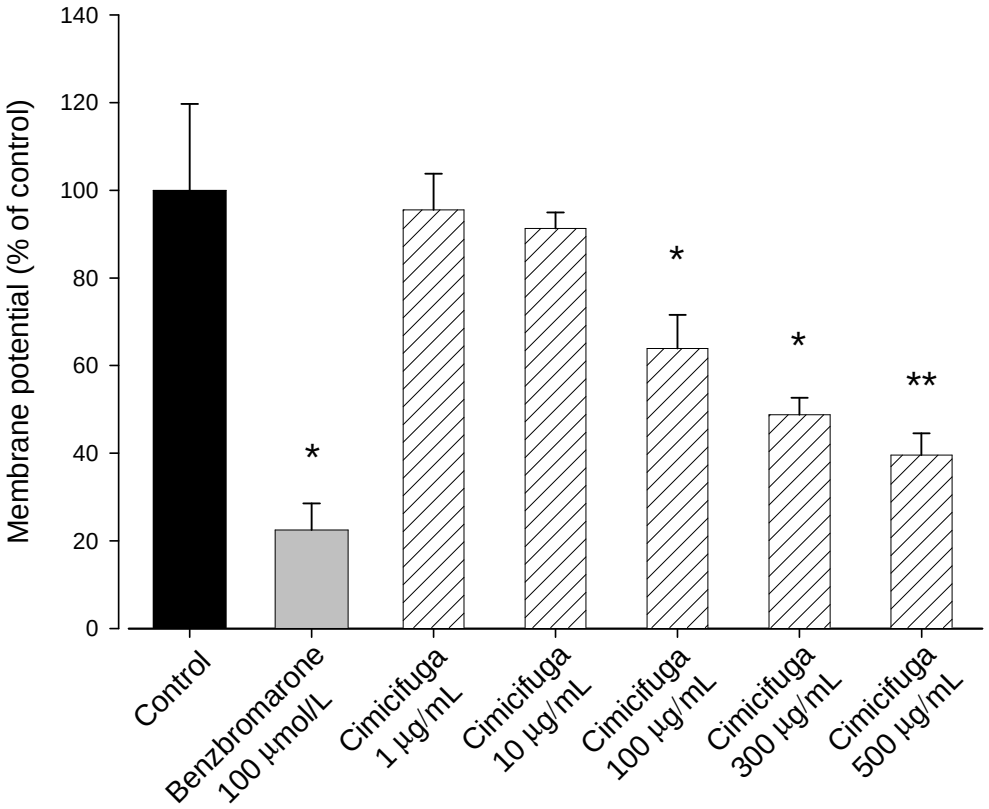

Figure 3: Mitochondrial membrane potential in HepG2 cells incubated with cimicifuga extract. Cells were incubated with cimicifuga extract for 24 hours. After this incubation, JC-1 was added (final concentration 7.5µM), and cell fluorescence was analyzed using a cell sorter after an incubation of 10 minutes (Kaufmann et al., 2006). The mitochondrial membrane potential starts to drop at a concentration of 100 µg/mL cimicifuga extract, corresponding well with the MTT test shown in Figure 2. Results are expressed as the percentage of the membrane potential compared to control values, which was set at 100%. Results are presented as mean ± SEM of 3 determinations. *P < 0.05 vs. control values.

In order to find out the reasons for the observed decrease in the mitochondrial membrane potential, specific mitochondrial functions were studied using freshly isolated rat liver mitochondria. The investigation of palmitate metabolism revealed that cimicifuga racemosa inhibited mitochondrial β-oxidation in a dose-dependent fashion, starting at a concentration of 10µg/mL (Figure 4). At a concentration of 500µg/mL, the residual activity was only 8.5%.

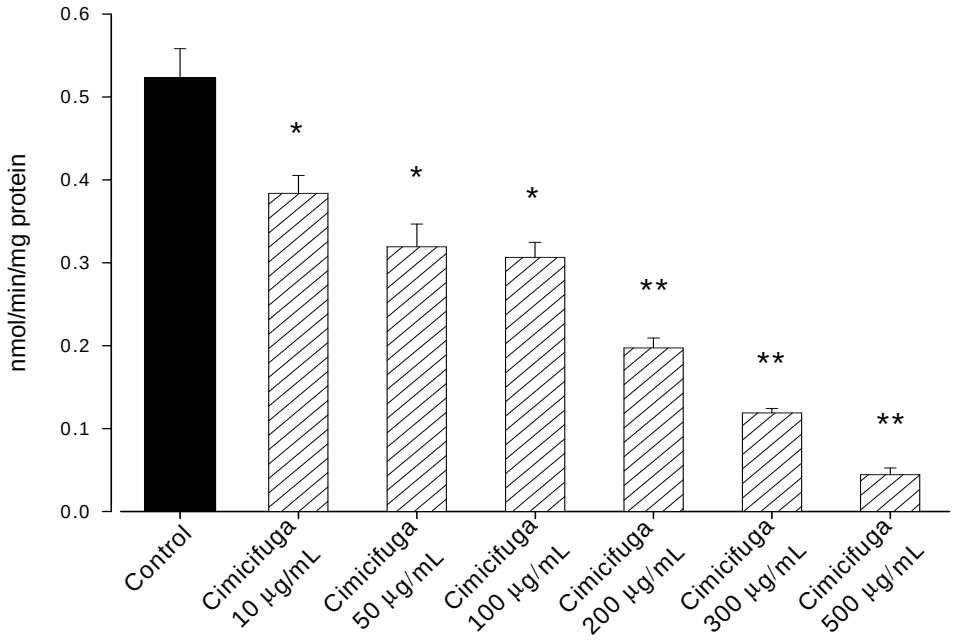

Figure 4: Activity of the β-oxidation pathway of isolated rat liver mitochondria in the presence of cimicifuga extract. Beta-oxidation was determined as the formation of ketone bodies using [1-^{14}C]palmitate as a substrate. Cimicifuga extract shows a dose-dependent toxicity starting at a concentration of 10µg/mL. Results are expressed as mean ± SEM of 3 determinations. *P < 0.05, **p < 0.01 vs. control values.

Oxidative phosphorylation is another important metabolic process in mitochondria, which is sensitive to toxicants (Waldhauser et al., 2006). As shown in Table 1, state 3 oxidation rates in the presence of L-glutamate were decreased by 20% starting at a concentration of 300 µg/mL of the extract, whereas state 4 oxidation rates were increased by 53% or 132% at 300 or 500µg/mL, respectively. In contrast, cimiracemoside A did affect neither state 3, nor state 4 oxidation rates (Table 1). In order to proof uncoupling of oxidative phosphorylation (as suggested by increased state 4 oxidation rates), state 4u was induced by the addition of oligomycin, an inhibitor of F_1F_0-ATPase. As shown in Figure 5, 500µg/mL cimicifuga extract led to a significant increase in state 4u oxygen consumption, similar to the known uncoupler dinitrophenol. In contrast, such an increase could not be shown in the presence of cimiracemoside A.

Incubation	State 3	State 4
Control (1% DMSO)	70±4	9.3±0.4
Cimicifuga 10 µg/mL	75±7	9.6±0.5
Cimicifuga 50 µg/mL	70±7	10.3±0.9
Cimicifuga 100 µg/mL	61±6	8.6±0.7
Cimicifuga 200 µg/mL	61±3	11.4±0.5
Cimicifuga 300 µg/mL	56±4*	14.2±1.1*
Cimicifuga 500 µg/mL	56±3*	21.6±0.9*
Cimiracemoside A 0.05 µg/mL	73±7	9.3±1.3
Cimiracemoside A 0.5 µg/mL	86±20	9.6±1.6
Cimiracemoside A 5.0 µg/mL	73±15	8.2±1.3

Table 1: *Effect of cimicifuga racemosa on mitochondrial oxidative metabolism*. L-Glutamate (20 mmol/L) was used as a substrate. Oxygen consumption by freshly isolated rat liver mitochondria was determined using an oxygen electrode as described in methods. Oxygen consumption is expressed as natoms/min/mg mitochondrial protein. Data are presented as mean±SEM, n ≥4 individual observations. * P < 0.05 vs. control incubations.

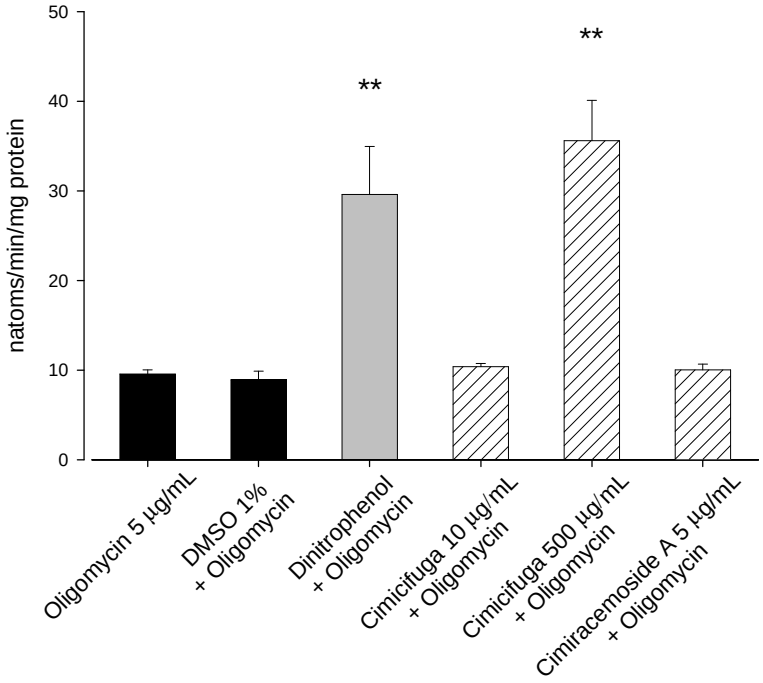

Figure 5: Oxygen consumption in the presence of L-glutamate (20mmol/L) as a substrate and of the F_1F_0-ATPase inhibitor oligomycin (5 µg/mL). In the presence of oligomycin, any increase in oxygen consumption has to be due to uncoupling of the respiratory chain, since ADP cannot be phosphorylated to ATP. Cimicifuga extract starts to uncouple oxidative phosphorylation at a concentration of 300µg/mL, which is in accordance with state 4 oxidation rates shown in Table 1. In contrast, cimiracemoside A does not act as an uncoupler. Dinitrophenol was used as a positive control. Results are expressed as mean ± SE of 4 determinations. **P < 0.01 vs. control incubations.

Since impairment in mitochondrial function can be associated with increased production of ROS (Kaufmann et al., 2005; Kaufmann et al., 2006), the redox status of HepG2 cells was assessed by determining their glutathione content. However, neither raised GSSG levels, nor an increased GSSG/GSH ratio were detectable in the presence of cimicifuga extract up to 500µg/mL, suggesting that ROS do not play a significant role in cimicifuga hepatotoxicity (data not shown).

As it is well established that mitochondrial damage can be associated with apoptosis and/or necrosis (Wolvetang et al., 1994; Kaufmann et al., 2005; Kaufmann et al., 2006), we assessed these possibilities using annexin V and propidium iodide staining of HepG2 cells. As shown in Figure 6, cimicifuga extract induced a concentration-dependent increase in early apoptotic and, to a smaller extent, also late apoptotic/necrotic cells, starting at a concentration of 300µg/mL. The specificity of this mechanism was shown by adding the pancaspase inhibitor zVAD-fmk to the incubations, which was able to prevent early (but not late) apoptosis significantly. In contrast, the cysteine protease inhibitor zFA-fmk, which does not affect caspases, had no effect on apoptosis associated with cimicifuga extract. To further confirm these results, Hoechst 33342 stains were performed, which confirmed that treatment with cimicifuga extract is associated with apoptosis of HepG2 (data not shown).

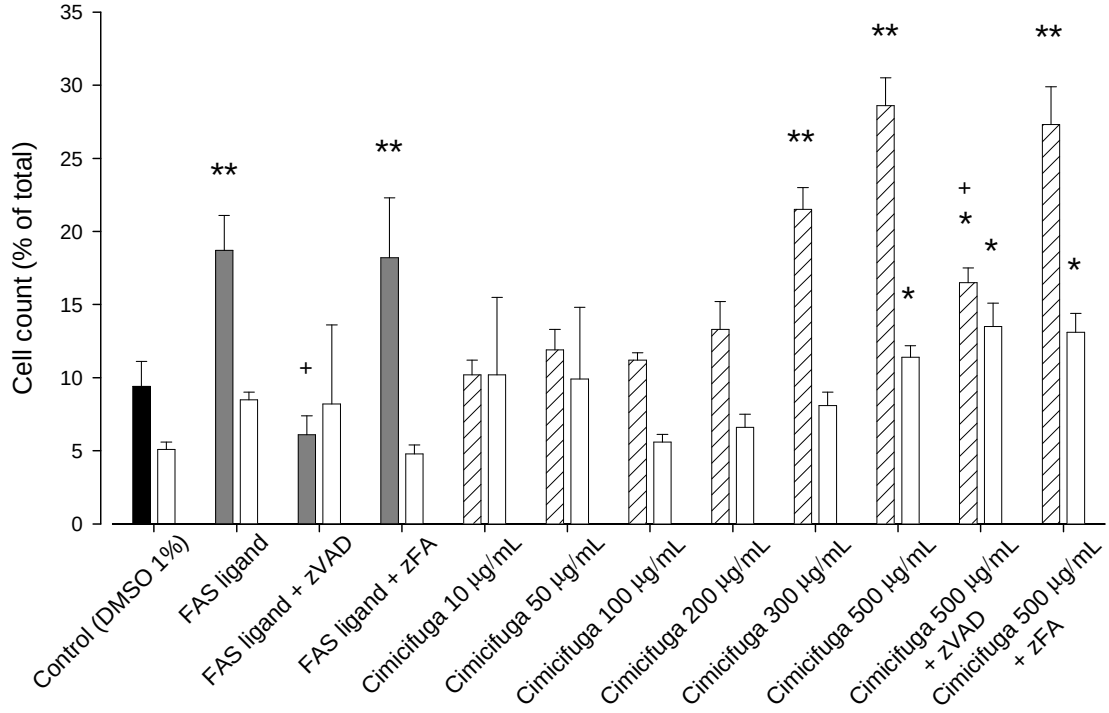

Figure 6: Early apoptosis (black, grey or shaded columns) and late apoptotis/necrosis (white columns) of HepG2 cells incubated with different concentrations of cimicifuga extract and other test compounds. After incubation with the test compounds for 24 hours, cells were stained with annexin V and propidium iodide and cell fluorescence was analyzed using a cell sorter as described in methods. This method allows allocation of cells into the categories living cells, early apoptotic cells and late apoptotic/necrotic cells (Kaufmann et al., 2005). While control incubations contain only a small percentage of apoptotic or necrotic cells, incubation with FAS ligand (positive control) is associated with a significant increase in early apoptotic cells. This increase can be inhibited by co-incubation with zVAD, a pancaspase inhibitor. In contrast, the increase is not inhibited in the presence of zFA, a cysteine protease inhibitor without activity against caspases. Cimicifuga extract is associated with early apoptosis (and to smaller extent also late apoptosis/necrosis) starting at a concentration of 300µg/mL. Early apoptosis, but not late apoptosis/necrosis, can at least partially be prevented by the addition of zVAD, but not zFA. Results are expressed as mean ± SEM of 3 determinations. *P < 0.05, **p < 0.01 vs. the respective control incubations. $^{+}$ P< 0.05 vs. the respective incubation containing no zVAD.

In order to discriminate better between late apoptosis and necrosis in the annexin V/propidium iodide stain, the ATP content of HepG2 cells treated with cimicifuga extract was determined. For the occurrence of apoptosis, normal levels of ATP are necessary, whereas low levels of ATP are indicative for necrosis (Leist et al., 1997). The ATP levels of the cells treated with cimicifuga extract were not decreased compared to untreated control cells, indicating the occurrence of apoptosis and not necrosis (data not shown).

To investigate the possible contribution of mitochondria in the development of apoptosis associated with cimicifuga extract, mitochondrial leakage of cytochrome c

was investigated using an immunohistological method (Kaufmann et al., 2005; Kaufmann et al., 2006). As shown in Figure 7, cimicifuga extract was associated with mitochondrial leakage of cytochrome c into the cytoplasm of HepG2 cells, starting at a concentration of 200µg/mL.

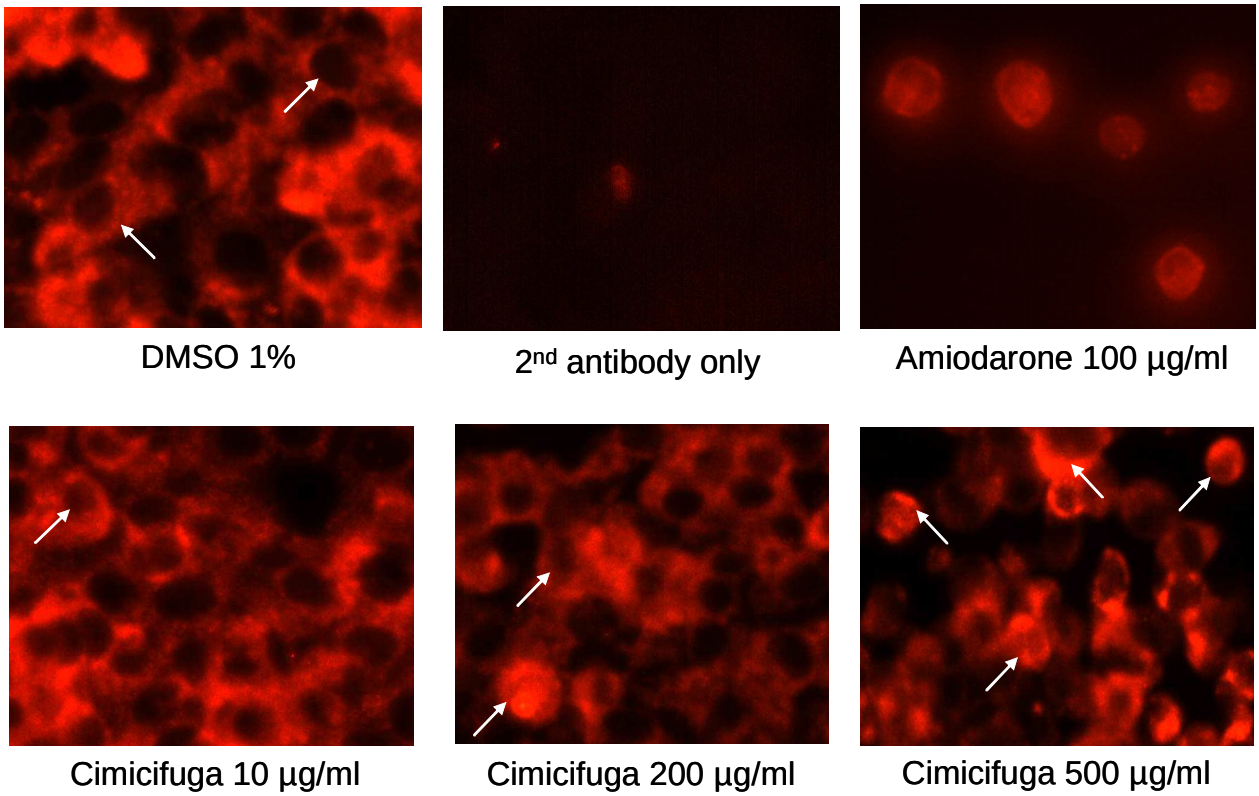

Figure 7: Immuno-cytological staining of cytochrome c in HepG2 cells. Cells (n = 10^5) were incubated for 24 hours with cimicifuga extracts as described in methods. After treatment, they were fixed with 4% paraformaldehyde, washed, incubated with anti-cytochrome c antibody, and, after washing, with Cy3 conjugated anti-sheep IgG (Kaufmann et al., 2005). In control incubations (1% DMSO), cytochrome c has a granular structure and does not cover the nucleus (arrows). In the presence of 10 µg/mL cimicifuga extract, the pattern of cytochrome c does not change. In the presence of 200µg/mL cimicifuga extract, the granular appearance of cytochrome c is changing to more diffuse appearance and cytochrome c starts to cover the nucleus (arrows). Some cells are completely covered by cytochrome c (arrows). In the presence of 500µg/mL cimicifuga extract, these changes are even more accentuated (arrows). In comparison, treatment with 100 µmol/L amiodarone (positive control) is associated with complete staining of the cells by cytochrome c, but cells detach from the plate, indicating loss of cell integrity. Incubation with the second antibody only was used as a negative control.

9.5 Discussion

The main findings *in vivo* and *in vitro* were hepatic mitochondrial toxicity, as evidenced by microvesicular steatosis and inhibition of β-oxidation, eventually leading to apoptotic cell death. Severe inhibition of hepatic mitochondrial β-oxidation is associated with cellular accumulation of long-chain fatty acids, e.g. palmitate, which, depending on the localization of the defect, may still be activated to the respective acyl-CoAs and form triglycerides (Spaniol et al., 2003). Triglycerides can be stored in hepatocytes, possibly leading to liver steatosis, or can be exported from hepatocytes as VLDL particles (Fromenty and Pessayre, 1995; Fromenty and Pessayre, 1997; Spaniol et al., 2003). The microvesicular type of steatosis is thought to result from accumulation of triglycerides, acyl-CoAs and long-chain fatty acids due to inhibited β-oxidation (Fromenty and Pessayre, 1995; Fromenty and Pessayre, 1997), which is different from the macrovesicular type of liver steatosis, where accumulation of triglycerides is predominant.

Our studies suggest that inhibition of β-oxidation is the initial hepatotoxic event of cimicifuga extract, which eventually may result in apoptosis of the hepatocytes. Since long-chain acyl-CoAs accumulate in the cytoplasm of hepatocytes when β-oxidation is impaired (Spaniol et al., 2003), these fatty acid metabolites may be associated with hepatocellular toxicity. Saturated long chain fatty acids such as palmitate have indeed been shown to be associated with apoptosis both *in vivo* (Feldstein et al., 2003) and in cell cultures including hepatocytes (Sparagna et al., 2000; Feldstein et al., 2003; Wei et al., 2006). Palmitoyl-CoA can induce mitochondrial membrane permeability transition, release of cytochrome c into the cytoplasm and apoptosis via caspase-dependent pathways (Furuno et al., 2001). Many of our findings, e.g. prevention of apoptosis by the pancaspase inhibitor zVAD and release of cytochrome c into the cytoplasm HepG2 cells, are in agreement with this mechanism.

Palmitate and/or palmitoyl-CoA can induce apoptosis by different mechanisms. One possibility is increased formation of ceramide, which can induce mitochondrial membrane permeability transition (Arora et al., 1997) and is important for apoptosis associated with TNF-α (Osawa et al., 2005). However, while the addition of palmitate was associated with increased ceramide formation by cultured hepatocytes, inhibition of ceramide synthesis did not prevent apoptosis (Wei et al., 2006). In addition, in rat neonatal cardiomyocytes, decreased cardiolipin synthesis was found in the presence

of palmitate, which was associated with increased release of cytochrome c into the cytoplasm and initiation of apoptosis (Ostrander et al., 2001).

The main constituents of cimicifuga racemosa are triterpene glycosides (e.g. actein, deoxyactein, cimifugoside, cimiracemosides), aromatic acids and their derivatives (e.g. ferulic acid, isoferulic acid, caffeic acid, fukinolic acid, cinnamic acid esters and cimicifugic acid A and B), flavonoids, volatile oils and tannins (He et al., 2006; Jiang et al., 2006). The components responsible for the pharmacological activity have so far not been identified. Regarding toxicity, Hostanska et al. investigated triterpene glycosides and cinnamic acid esters for their ability to induce apoptosis (Hostanska et al., 2004b; Hostanska et al., 2004a; Hostanska et al., 2005). Both types of substances were found to be associated with caspase-dependent apoptosis in breast cancer (Hostanska et al., 2004b; Hostanska et al., 2004a) and prostate cancer cell lines (Hostanska et al., 2005). In another investigation, the formation of quinine metabolites (possibly generated from phenolic acids and derivatives) has been proposed to be the cause of cimicifuga toxicity (Johnson and van Breemen, 2003). In our investigations, however, we had no evidence for the development of reactive metabolites. Furthermore, in the *in vitro* study performed by Hostanska et al. (Hostanska et al., 2004b), addition of microsomes to the incubations did not increase the toxicity of cimicifuga constituents, arguing against the formation of toxic metabolites. In order to find out precisely which component of cimicifuga extract is hepatotoxic, the most important components of cimicifuga extract would have to be studied individually, but most of them are not commercially available.

To assess whether the toxic concentrations are in the range of the blood concentrations reached after ingestion of cimicifuga tablets, a rough estimate can be made. Assuming that a preparation containing 5-10 mg ethanolic extract is ingested and that the entire extract is absorbed rapidly, a maximal plasma concentration in the range of 1.5 -3 µg/mL could be reached (assuming rapid intravascular distribution, a plasma volume of 3.5 L and absence of significant metabolism). This value almost approaches the concentrations found to inhibit β-oxidation in our *in vitro* experiments (lowest inhibitory concentration 10µg/mL), but is 30 or 150 times lower than the lowest concentration associated with cytotoxicity (figure 2) or apoptosis (figure 6), respectively. On the other hand, in rats *in vivo*, microvesicular steatosis of the liver was observed at 1000mg extract per kg body weight. Assuming a rapid and complete absorption of the extract and a plasma volume of 15mL, the maximal plasma

concentration would be in the range of 20mg/mL, a concentration too high to be tested *in vitro* due to solubility problems. These considerations suggest that toxic concentrations can most probably not be reached in humans treated with the recommended doses. This is in agreement with the toxicity profile of the drug, showing hepatic adverse events only in a small fraction of patients, possibly with so far unknown risk factors (Dog et al., 2003; Huntley and Ernst, 2003).

In conclusion, ethanolic cimicifuga extract is associated with hepatic mitochondrial toxicity, both *in vivo* in rats and *in vitro* using cell cultures and isolated rat liver mitochondria. This toxicity is in most patients not clinically relevant but may become important in patients with underlying risk factors.

10 Conclusion and outlook

Drug-induced liver injury (DILI) is a major health problem that challenges not only health care professionals but also the pharmaceutical industry. As a consequence, it is also the most frequent reason for the withdrawal of approved drugs from the market. Although the exact mechanism of DILI remains widely unknown, it appears to involve two pathways – direct hepatoxicity and idiosyncratic hepatic injury. In most cases, DILI is initiated by the direct action of a drug, or more often a reactive metabolite of a drug (e.g. acetaminophen), and is dose-related. Other hepatotoxic drugs induce steatoheptatitis (e.g. ethanol, tamoxifen) or cholestasis (cyclosporine A, chlorpromazine). Whereas the above forms of hepatotoxicity are to a large extent predictable as a result of pre-clinical toxicology, a second category of drugs can cause so-called idiosyncratic hepatic injury where hepatotoxicity develops in only 1 in 500–50,000 of exposed patients (e.g. phenytoin, isoniazid, valproic acid). Little is known about the cause of delayed, non-allergic forms of idiosyncratic hepatotoxicity. However, in idiosyncratic allergic drug hepatotoxicity, both the innate and adaptive immune system play a determinant role in the pathogenesis of liver injury (Larrey, 2000; Kass, 2006).
Another major concern in clinical practice and for the pharmaceutical industry in multidrug therapy is the Cytochrome P450 induction-mediated interaction. There are two crucial issues associated with CYP induction. First, induction may cause a reduction in therapeutic efficacy of co-medications. For drugs whose effect is produced primarily by the parent drug, induction would increase the drug's elimination, resulting in lower drug concentrations, and decrease the drug's pharmacological effect. Second, induction may create an undesirable imbalance between detoxification and activation as a result of increased formation of reactive metabolites, leading to an increase in the risk of metabolite-induced toxicity (Lin and Lu, 1998; Lin, 2006).

In this thesis, we present an *in vivo* model for the investigation of new or approved drugs on their hepatotoxic potential, especially drugs initiating idiosyncratic hepatotoxicity. Our mouse model (juvenile visceral steatosis mouse) is characterized by a mitochondrial dysfunction due to impaired hepatic β-oxidation, whereas this pre-existing mitochondrial disease can be considered to be a risk factor for drug-induced hepatotoxicity and may increase susceptibility for the occurrence of severe

hepatotoxic reactions. Therefore, this *in vivo* model can be used to screen drugs for their capability to initiate idiosyncratic hepatotoxicity and to evaluate the mitochondrial involvement in drug-induced hepatic injury.

Several *in vitro* models have been established to assess the potential of CYP induction, including liver slices, immortalized cell lines, and primary human hepatocytes (Silva et al., 1998; Kostrubsky et al., 1999; LeCluyse, 2001). Among these models, we present an immortalized human hepatocyte cell line, namely hHepLT5, which is a suitable screening system for pharmacological and toxicological properties of drugs. hHepLT5 cells contain CYP1A2 and 3A4 isozymes involved in drug metabolism. Furthermore, hepatic cell lines show several advantages compared to primary hepatocytes, including maintenance of the activity of major drug-metabolizing enzymes, permanent availability, constant quality, and cultivability. Consequently, hHepLT5 cells are a useful tool to investigate certain pharmacological and toxicological aspects, facilitating the prediction of several effects of new drug entities.

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12 Curriculum Vitae

Personal data	Andrea-Caroline Knapp Genossenschaftsstrasse 4 4132 Muttenz Phone: 0041 61 461 88 20 Mobile: 0041 79 772 60 37 Date of Birth: 02.06.1976 in Basel, Switzerland Citizenship: Austria Marital status: Single

Education

01/2003 – 10/2007	**PhD thesis**, Division of Clinical Pharmacology and Toxicology, University Hospital Basel, Switzerland
11/2002	**Master of Science (Pharmacy)**, University of Innsbruck, Austria
05/2002 – 09/2002	**Diploma Thesis**, Institute of Pharmaceutical Chemistry, University of Innsbruck, Austria • Thesis topic: "Synthesis of thioureas as potential NNRT inhibitors"
1995 – 2002	**Study of Pharmacy**, University of Innsbruck, Austria
1990-1995	**BHS Commercial School**, Feldkirch, Austria

Professional Experiences

11/2007 – current **Clinical Research Coordinator**, CCRC (Clinical Cancer Research Center), Department of Oncology, University Hospital Basel, Switzerland

- Tasks:
  - Participation in planning of local study conduct
  - General conduction of trials
  - Clinical data management
  - In site monitoring
  - Drug accountability, ordering and storage
  - Monitoring of medication administration and dose modification
  - Report SAEs and resolve queries
  - Contribute to the preparation of clinical study budget
  - Preparing key documents for submission to Ethics Committee
  - Safeguarding adherence to ICH-GCP, protocol, national regulations and local SOPs

01/2003 – 10/2007 **PhD student**, Division of Clinical Pharmacology and Toxicology, University Hospital Basel, Switzerland

- Thesis topic: "Characterization of *in vitro* and *in vivo* models for the investigation of hepatotoxicity"

- Other tasks:
  - Clinical Pharmacological Service of the Univerisity Hospital Basel (KLIPS)
    - Answering inquiries concerning pharmacological, toxicological or drug safety questions
    - Presentation of current pharmacological topics
  - Therapeutic Drug Monitoring (TDM)
    - Monitoring and Evaluation of drug concentrations and dose adjustments
  - Assisting in clinical trials

2003 – 2006 **Assistant at the practical course "Pharmacology and Toxicology"**, University Hospital Basel, Switerzland

05/2005 – 10/2005 **Supervisor of a diploma student**, Division of Clinical Pharmacology and Toxicology, University Hospital Basel, Switzerland

06/2003 – 10/2003 **Internship at the Division of Clinical Pharmacology and Toxicology**, University Hospital Basel, Switzerland

06/1999 – 11/1999 **Research associate**, Institute of Pharmaceutical Chemistry, University of Innsbruck, Austria
- Synthesis of antiviral agents

1997 & 1998 **Part time employee at Hilcona AG,** Schaan, Liechtenstein
- Quality assurance
- Secreterial work

1995 & 1996 **Internship at the Hospital Pharmacy of the Liechtensteinisches Landesspital**, Vaduz, Liechtenstein

Qualifications & Additional Courses

2008 **SAKK Annual Training** for Clinical Research Coordinators

Pharmathemen – New Therapies in Oncology

2006 **"Good Clinical Practice"**, EKBB and University Hospital Basel, Switzerland

2003 – 2005 Graduate Study Program and passed exam in **"Key Issues in Drug Discovery and Development"**

2004	Advanced training course **"Strategies and trends in pharmaceutical development and production"**, Pharmacenter Basel-Zürich, University Basel, Switzerland

Computer skills

MS-Office (Word, Excel, Powerpoint), Internet Explorer, Literature retrieval, Corel Draw and Paint, Adobe Photoshop, Primer Express, Endnote, GraphPrism, SigmaPlot etc

Languages

German	mother tongue
English	good speech and writing abilities
Spanish	intermediate speech and writing abilities
French	basic speech and writing abilities
Italian	basic speech and writing abilities

Memberships

Since 2003	Member of the SAV (Swiss society of pharmacy)
Since 2007	Member of the Swiss Group for Clinical Cancer Research (SAKK)

Publication Record

Andrea Caroline Knapp, Liliane Todesco, Michael Török, Konstantin Beier, Stephan Krähenbühl. Effect of carnitine deprivation on carnitine homeostasis and energy metabolism in mice with systemic carnitine deficiency. Ann Nutr Metab 2008 Apr; 52:136.

Andrea Caroline Knapp, Liliane Todesco, Konstantin Beier, Luigi Terracciano, Hans Sägesser, Jürg Reichen, Stephan Krähenbühl. Toxicity of valproic acid in an animal model with impaired β-oxidation associated with carnitine deficiency. J Pharmacol Exp Ther. 2008 Feb;324:568.

Saskia Lüde, Michael Török, Sandy Dieterle, Andrea Caroline Knapp, Robert Kaeufeler, René Jäggi, Udo Spornitz, Stephan Krähenbühl. Hepatic effects of cimicifuga racemosa extract *in vivo* and *in vitro*. Cell Mol Life Sci. 2007;64:2848.