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Research Article

Hepatic effects of *Cimicifuga racemosa* extract *in vivo* and *in vitro*

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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 *C. 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 >500 µg/kg body weight cimicifuga extract. *In vitro*, cytotoxicity was apparent at concentrations ≥ 75 µg/mL, and mitochondrial β-oxidation was impaired at concentrations ≥ 10 µg/mL. The mitochondrial membrane potential was decreased at concentrations ≥ 100 µg/mL, and oxidative phosphorylation was impaired at concentrations ≥ 300 µg/mL. The mechanism of cell death was predominantly apoptosis. *C. 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.

Keywords. *Cimicifuga racemosa*, hepatotoxicity, mitochondria, apoptosis, HepG2.

Introduction

Hormone replacement therapy (HRT) has been considered to be the standard treatment for menopausal disturbances. The association of HRT with breast and uterine cancer [1] and the desire of many women for a "natural treatment" are the main reasons why alternative therapies have become increasingly popular; especially extracts of the plant *Cimicifuga racemosa* are currently used for this indication. *C. 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 to treat joint aches, myalgias, neuralgias and rheumatic disorders but also for menopausal complaints and pain during labour. Nowadays, ethanolic or isopropanolic extracts of *C. racemosa* are most commonly used for the symptomatic treatment of menopausal disorders and premenstrual syndrome [2–5], although not all studies have shown a better effect than placebo [6].

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Data from clinical studies and spontaneous reporting programs suggest that adverse events associated with *C. racemosa* are rare, generally mild and reversible. Gastrointestinal upset and rashes were the most common adverse events reported [7, 8]. In mostly uncontrolled clinical trials and post-marketing studies including more than 2800 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, *C. racemosa* can cause dizziness, headaches, nausea and vomiting [8]. In their review, which includes all post-marketing programs of cimicifuga extracts, Huntley et al. also described patients with adverse hepatic events [7]: 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 [9, 10] or fulminant liver failure [11–13] while being treated with cimicifuga extracts. The European Medicines Agency (EMEA) recently assessed the case reports of hepatotoxicity associated with ingestion of *C. racemosa* root extracts (EMEA/HMPC/88766/2006) and concluded that the cases reported in the literature as well as the pharmacovigilance reports are mostly poorly documented and that these adverse events should be interpreted with caution. Systematic investigation of the present and possible future cases by the marketing authorization holders and pharmacovigilance units 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*, hepatocyte cultures and isolated liver mitochondria.

Materials 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 ($\geq$ 6 %). Solutions of the extract were made by dissolving it 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), and Z-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) and Z-Phe-Ala-fluoromethylketone (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 the highest quality available when not otherwise stated.

***In vivo* hepatotoxicity of cimicifuga extract.** Groups of five female Wistar rats were fed daily with doses of 1, 10, 100, 300 or 1000 mg/kg body weight *C. 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 anaesthesia and decapitation, the livers of the animals were perfused for fixation, and tissue blocks were excised and prepared for electron microscopy as described previously in detail [14]. 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, 25 mM Hepes, 10 % (v : v) heat-inactivated fetal bovine serum and 100 U/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 [15] but including an additional washing step [16]. In addition, the sulforhodamine B (SRB) test was performed according to the protocol of Skehan [17] and the lactate dehydrogenase (LDH) assay according to Vassault [18].

Isolation of rat liver mitochondria. Male Sprague Dawley rats (Charles River, Les Onins, France) were anaesthetized with carbon dioxide and killed by decapitation. Liver mitochondria were isolated by differential centrifugation according to the method of Hoppel et al. [19]. The mitochondrial protein content was determined using the biuret method with bovine serum albumin as a standard [20].

***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. [21] with the modifications described by Spaniol et al. [22]. Cimicifuga extract was added to the incubation mixture just before starting the experiment.

Oxygen consumption. Polarographic monitoring of oxygen consumption in rat liver mitochondria was carried out in a 1-mL chamber equipped with a Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH, USA) at 30°C as described previously [23]. Subsequent experiments with the F_1 F_0-ATPase inhibitor oligomycin (5 μg/mL) were performed to check for uncoupling of oxidative phosphorylation [24]. Cimicifuga extract was added to the incubation mixture just before starting the experiment.

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 h. After the addition of JC-1 and 10 min of incubation, cell fluorescence was determined by flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, USA) [25].

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 [26], with the modifications described by Griffith et al. [27].

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

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 [24].

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

Cytochrome c staining. HepG2 cells (105 cells) were seeded into an 8-well chamber slide (Nunc Labtek, Naperville, IL, USA) and cultured for 2 days. Subsequently, cells were incubated for 24 h with cimicifuga extracts as described in the Results. The cells were fixed in 4 % paraformaldehyde and analyzed for cytochrome c as described previously [24].

Statistical methods. Data represent the mean ± SEM of at least three 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 a posthoc test to localize differences obtained by ANOVA. A p value <0.05 was considered to be statistically significant.

Results

Our aims were to find out whether cimicifuga is hepatotoxic *in vivo* in rats and, 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 vehicle only showed no signs of hepatic toxicity. Rats treated with 10 mg 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 300 mg/kg body weight showed more marked mitochondrial swelling and alterations in mitochondrial morphology such as vacuoles in the matrix (Fig. 1a). Rats treated with 1000 mg/kg body weight developed microvesicular steatosis of the hepatocytes (see Fig. 1b), glycogen depletion and fragmentation of the rough endoplasmic reticulum.

Since microvesicular steatosis usually reflects mitochondrial damage and can be associated with cytotoxicity [28, 29], the MTT test was carried out on HepG2 cells. As shown in Fig. 2, cimicifuga extract displayed a concentration-dependent toxicity starting at 75 μg/mL. Cytotoxicity of cimicifuga could be confirmed using the LDH and 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

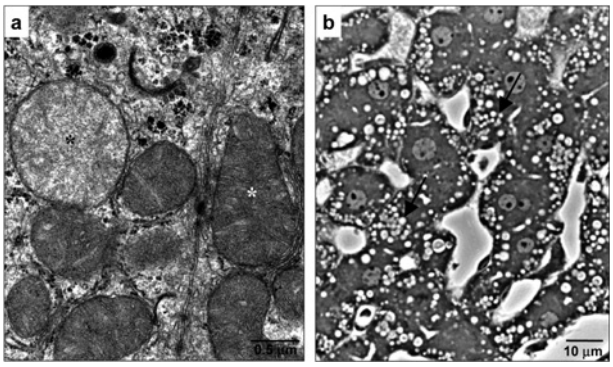

Figure 1. Electron micrograph of a liver from a rat treated with 300 mg/kg body weight (*a*) shows swollen mitochondria (marked with stars) beside mitochondria with a normal size. The bright, swollen mitochondrion (black star) has a loose matrix and is probably non-functioning. A semi-thin section of a liver from a rat treated with 1000 mg cimicifuga extract/kg body weight for 21 days (*b*) shows hepatocytes with intracellular accumulation of small lipid droplets, which are located in the cytoplasm and do not displace the nuclei, typical for microvesicular steatosis.

specific mitochondrial functions were studied using freshly isolated rat liver mitochondria. The investigation of palmitate metabolism revealed that *C. racemosa* inhibited mitochondrial β-oxidation in a dose-dependent fashion, starting at a concentration of 10 µg/mL (Fig. 4). At a concentration of 500 µg/mL, the residual activity was only 8.5 %.

Oxidative phosphorylation is another important metabolic process in mitochondria that is sensitive to toxicants [30]. 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 500 µg/mL, respectively. In contrast, cimiracemoside A affected neither state 3 nor state 4 oxidation rates (Table 1). In order to prove uncoupling of oxidative phosphorylation (as suggested by the increased state 4 oxidation rates), state 4u

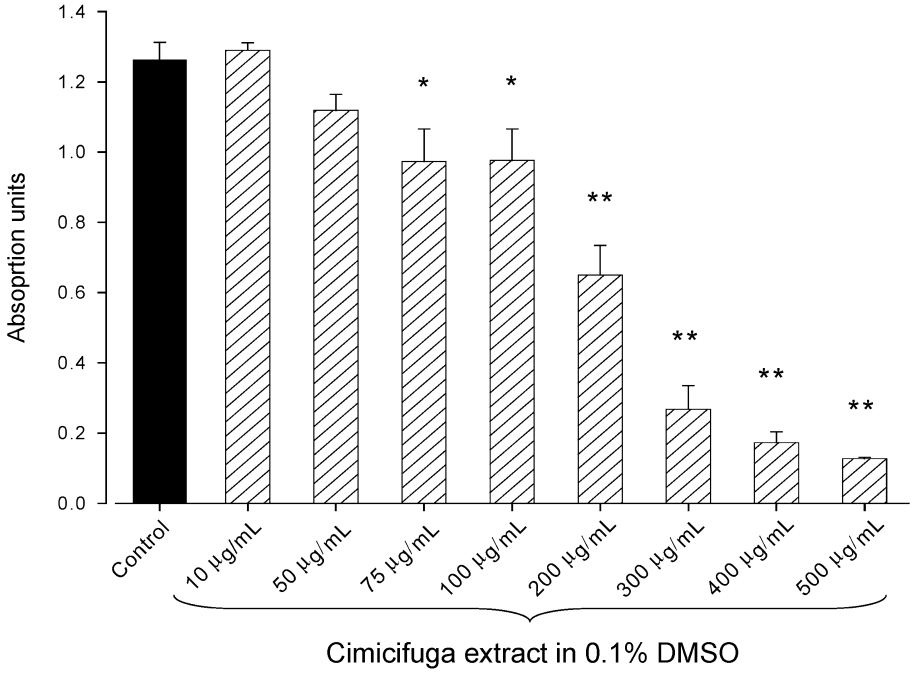

Figure 2. Reductive capacity and integrity of HepG2 cells investigated using the MTT test after incubation with cimicifuga extracts for 24 h. The concentration of DMSO was 0.1 % in all incubations. In intact cells, MTT is metabolically converted to its blue formazan form, whose absorption can be measured at 550 nm (presented here). Cytotoxicity is detectable beginning at a concentration of 75 µg/mL cimicifuga extract. Results are expressed as mean ± SEM of ten determinations (*$p < 0.05$, **$p < 0.01$ vs. control values).

concentrations calculated to be equivalent to those in the extract, were not cytotoxic (data not shown).

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 [25]. These experiments revealed a dose-dependent decrease in the membrane potential, starting at a concentration of 100 µg/mL (see Fig. 3). In order to determine the reasons for the observed decrease in the mitochondrial membrane potential, was induced by the addition of oligomycin, an inhibitor of F_1F_0-ATPase. As shown in Fig. 5, 500 µg/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.

Since impairment of mitochondrial function (*e.g.* impaired activity of the respiratory chain) can be associated with increased production of ROS [24, 25], the redox status of HepG2 cells was assessed by determining their glutathione content; neither raised GSSG levels nor an increased GSSG/GSH ratio was

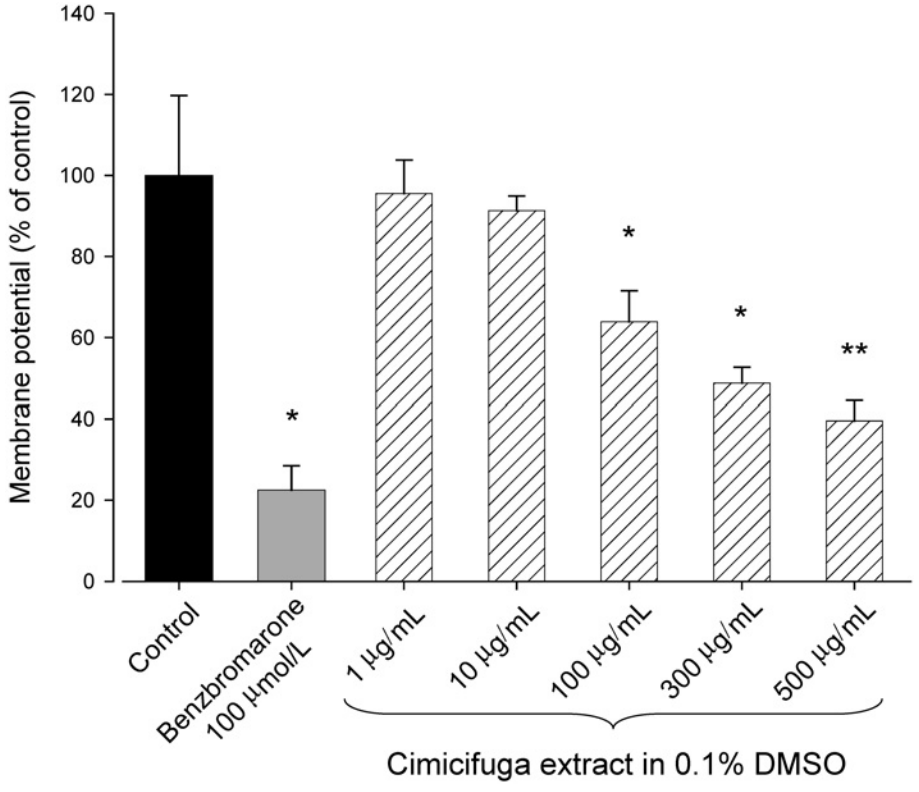

Figure 3. Mitochondrial membrane potential in HepG2 cells incubated with cimicifuga extract. Cells were incubated with cimicifuga extract for 24 h. After this incubation, JC-1 was added (final concentration 7.5 µM), and cell fluorescence was analyzed using a cell sorter after an incubation of 10 min [25]. The concentration of DMSO was 0.1 % in all incubations. The mitochondrial membrane potential started to drop at a concentration of 100 µg/mL cimicifuga extract, corresponding well with the MTT test shown in Fig. 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 three determinations (*$p < 0.05$ vs. control values).

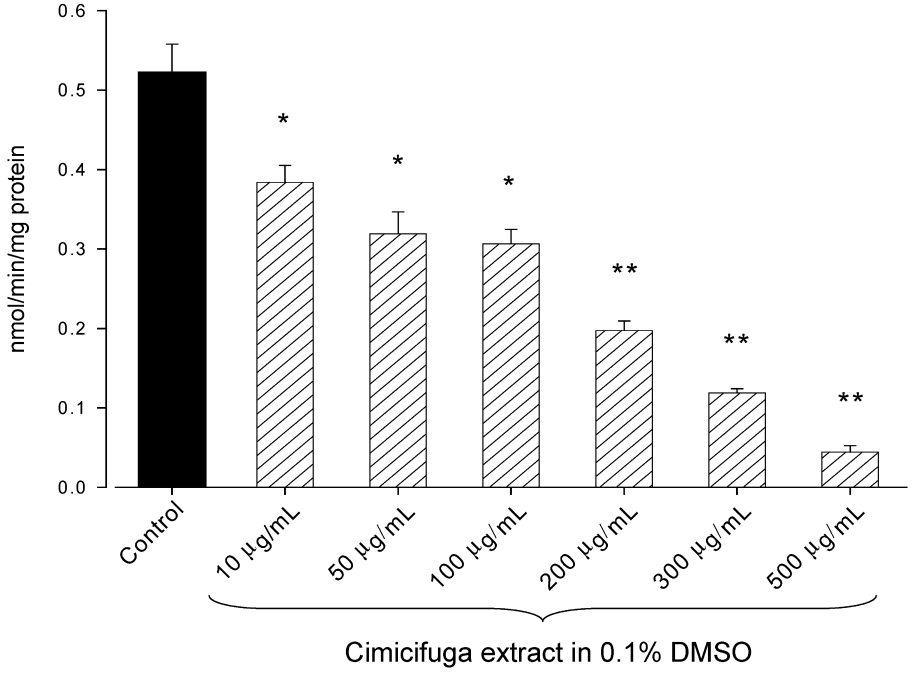

Figure 4. Activity of the β-oxidation pathway of isolated rat liver mitochondria in the presence of cimicifuga extract. β-oxidation, measured as the formation of ketone bodies using [1–^{14}C] palmitic acid as a substrate, was determined using freshly isolated mitochondria. Cimicifuga extract was added to the incubation mixture just before starting the experiment. The concentration of DMSO was 0.1 % in all incubations. Cimicifuga extract showed dose-dependent toxicity starting at a concentration of 10 µg/mL. Results are expressed as mean ± SEM of three determinations (*$p < 0.05$, **$p < 0.01$ vs. control values).

detectable in the presence of cimicifuga extract (up to 500 µg/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 [24, 25, 31], we assessed these possibilities using annexin V and propidium iodide staining of HepG2 cells. As shown in Fig. 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 300 µg/mL. The specificity of

Table 1. Effect of *Cimicifuga racemosa* on mitochondrial oxidative metabolism.

Incubation	State 3	State 4
Control (0.1 % 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

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 the Materials and methods. Cimicifuga extract was added to the incubation mixture just before starting the experiment. The concentration of DMSO was 0.1 % in all incubations. Oxygen consumption is expressed as natoms/min/mg mitochondrial protein. Data are presented as mean ± SEM ($n \geq 4$ individual observations; $^*p < 0.05$ vs. control incubations).

this mechanism was shown by adding the pan-caspase 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 staining was performed, which confirmed that treatment with cimicifuga extract is associated with apoptosis of HepG2 cells (data not shown).

In order to better discriminate between late apoptosis and necrosis in the annexin V/propidium iodide staining, 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 of necrosis [32]. 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 [24, 25]. As shown in Fig. 7, cimicifuga extract was associated with mitochondrial leakage of cytochrome c into the cytoplasm of HepG2 cells starting at a concentration of 200 μg/mL.

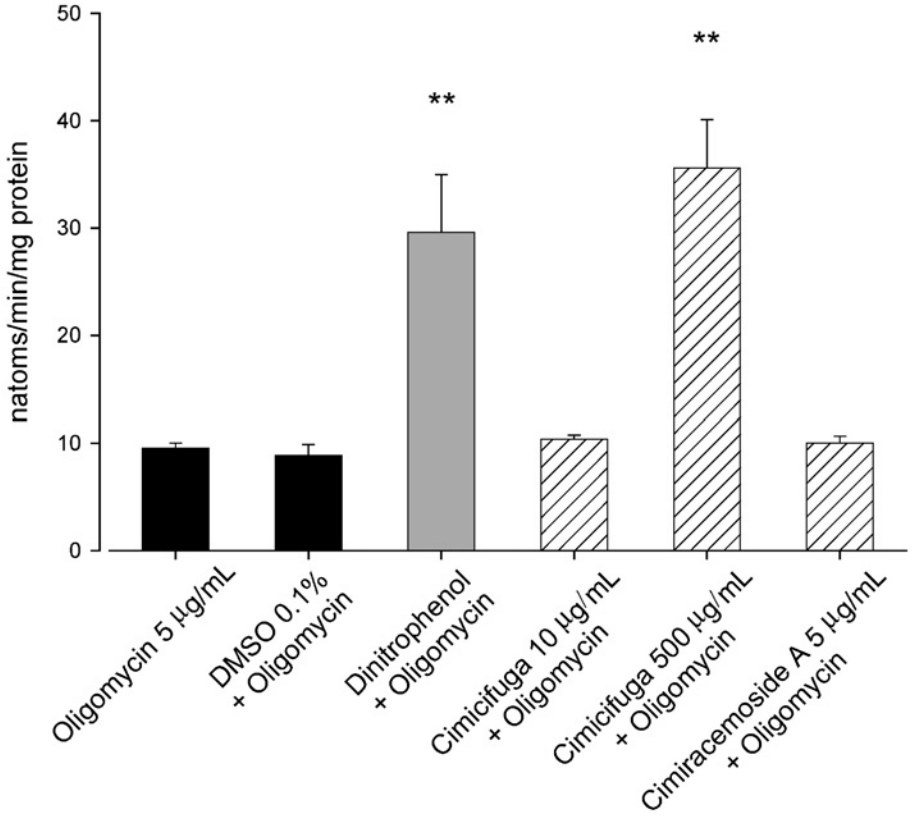

Figure 5. Oxygen consumption in the presence of L-glutamate (20 mmol/L, as a substrate) and the F_1F_0-ATPase inhibitor oligomycin (5 μg/mL). In the presence of oligomycin, any increase in oxygen consumption has to be due to uncoupling of the respiratory chain, since ADP cannot be phosphorylated to ATP. Cimicifuga extract was added to the incubation mixture just before starting the experiment. The concentration of DMSO was 0.1 % in all incubations. Cimicifuga extract started to uncouple oxidative phosphorylation at a concentration of 300 μg/mL, which is in accordance with the state 4 oxidation rates shown in Table 1. In contrast, cimiracemoside A did not act as an uncoupler. Dinitrophenol was used as a positive control. Results are expressed as mean ± SEM of four determinations ($^{**}p < 0.01$ vs. control incubations).

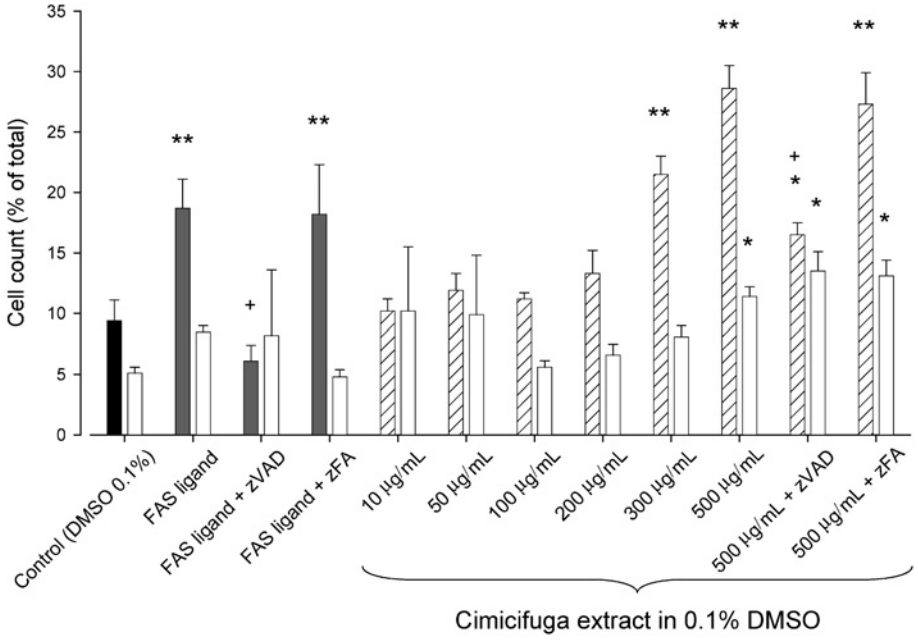

Figure 6. Early apoptosis (black, grey or shaded columns) and late apoptotis/necrosis (white columns) of HepG2 cells incubated with different concentrations of cimicifuga extract and other test compounds. After incubation with the test compounds for 24 h, cells were stained with annexin V and propidium iodide, and cell fluorescence was analyzed using a cell sorter. The concentration of DMSO was 0.1 % in all incubations. This method allows allocation of cells into the categories living cells, early apoptotic cells and late apoptotic/necrotic cells [24]. 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 pan-caspase 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 300 µg/mL. Early apoptosis but not late apoptosis/necrosis could at least partially be prevented by the addition of zVAD but not zFA. Results are expressed as mean ± SEM of three determinations (*$p < 0.05$, **$p < 0.01$ vs. the respective control incubations, $^{+}p < 0.05$ vs. the respective incubation containing no zVAD).

Discussion

The main finding *in vivo* and *in vitro* was hepatic mitochondrial toxicity, as evidenced by microvesicular steatosis and inhibition of β-oxidation and the respiratory chain, eventually leading to apoptotic cell death. While inhibition of the respiratory chain and uncoupling of oxidative phosphorylation could be demonstrated starting at concentrations of 300 µg/mL, inhibition of β-oxidation appeared at much lower concentrations (starting at 10 µg/mL) and was quite strong, suggesting that it represents the most relevant mechanism of mitochondrial toxicity associated with cimicifuga. 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-coenzymes A (CoAs) and form triglycerides [33]. Triglycerides can be stored in hepatocytes, possibly leading to liver steatosis, or can be exported from hepatocytes as very low-density lipoprotein (VLDL) particles [28, 33, 34]. 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 [28, 34], 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 caused by 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 [33], 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* [35] and in cell cultures including hepatocytes [35–37]. Palmitoyl-CoA can induce mitochondrial membrane permeability transition, release of cytochrome c into the cytoplasm and apoptosis via caspase-dependent pathways [38]. Many of our findings, *e.g.* prevention of apoptosis by the pan-caspase 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

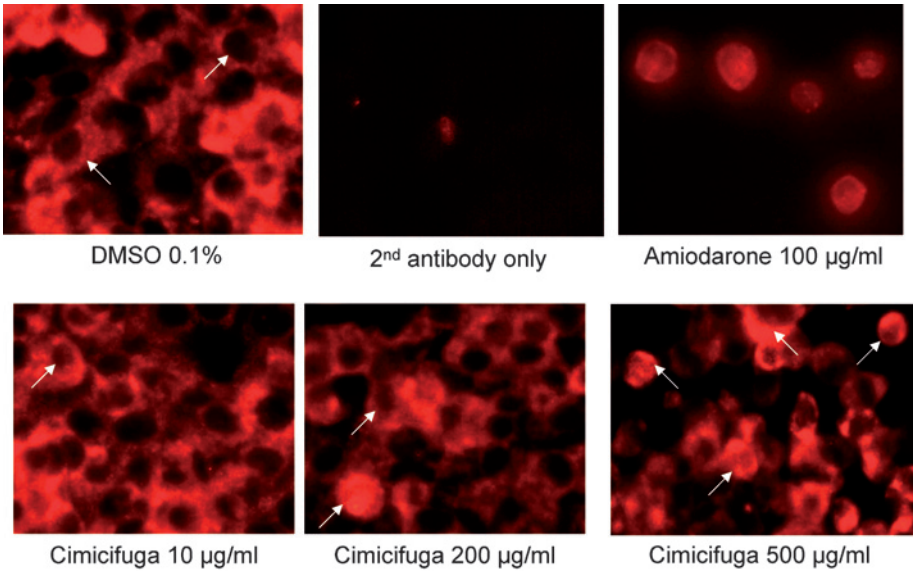

Figure 7. Immunohistological staining of cytochrome c in HepG2 cells. Cells ($n = 10^5$) were incubated for 24 h with cimicifuga extracts. The concentration of DMSO was 0.1 % in all incubations. After treatment, the cells were fixed with 4 % paraformaldehyde, washed and incubated with anti-cytochrome c antibody and, after washing, with Cy3-conjugated anti-sheep IgG [24]. 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 200 µg/mL cimicifuga extract, the granular appearance of cytochrome c changes to a 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 500 µg/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.

formation of ceramide, which can induce mitochondrial membrane permeability transition [39] and is important for apoptosis associated with TNF-α [40]. However, while the addition of palmitate was associated with increased ceramide formation by cultured hepatocytes, inhibition of ceramide synthesis did not prevent apoptosis [37]. 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 [41].

The main constituents of *C. 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 [42, 43]. 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 [44–46]. Both types of substances were found to be associated with caspase-dependent apoptosis in breast cancer [44, 45] and prostate cancer cell lines [46]. 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 [47]. On the other hand, in the *in vitro* study performed by Hostanska et al. [44], addition of microsomes to the incubations did not increase the toxicity of cimicifuga constituents, arguing against the formation of toxic metabolites. In our investigations, we did not focus on the formation of metabolites, although it is possible that some of the constituents may have been metabolized by HepG2 cells during the 24 h incubation period. 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 10 µg/mL) but is 30 or 150 times lower than the lowest concentration associated with cytotoxicity (Fig. 2) or apoptosis (Fig. 6), respectively. On the other hand, in

rats *in vivo*, microvesicular steatosis of the liver was observed at 1000 mg extract per kg body weight. Assuming a rapid and complete absorption of the extract and a plasma volume of 15 mL, the maximal plasma concentration would be in the range of 20 mg/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 as-yet-unknown risk factors [7, 8].

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 not clinically relevant for most patients but may become important in patients with underlying risk factors.

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