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# Eupatoriopicrin-induced lipid peroxidation in liver and tumour tissue of the mouse

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Eupatoriopicrin (EUP; Fig. 1)*, the principal sesquiterpene lactone from *Eupatorium cannabinum* L., is known to exhibit cytostatic activity *in vitro* as well as *in vivo* [1–4]. Interaction with biological sulphydryl containing molecules such as glutathione (GSH) has been proposed to be one of the possible mechanisms of cytostatic action of sequiterpene lactones [5].

Recently, we reported that EUP is able to reduce cellular GSH levels both *in vitro* and *in vivo* [6, 7]. We also demonstrated that the cytostatic action of EUP depends on the cellular GSH concentration at the moment of exposure to the drug. After lowering the GSH content in the cells by use of buthionine sulphoximine (BSO), a specific inhibitor of GSH synthesis [8], the cytostatic action of EUP was significantly enhanced. BSO extensively reduced cellular GSH levels, without exhibiting cytostatic action [7], showing that reducing the cellular GSH content as such is not the cause of cytostatic action of EUP. Moreover, EUP was cytotoxic *in vitro* at concentrations below those producing a significant GSH reduction [7]. In another study, we showed that EUP could induce DNA damage in Ehrlich Ascites Tumour cells, but the EUP concentrations that caused detectable DNA damage were somewhat higher than those inducing cell death [9].

To further examine the mechanism of action, a possible role of free radical reactions was investigated. Polyunsaturated fatty acids in cellular membranes are targets for free radical mediated oxidative degradation, a process called lipid peroxidation. As an antioxidant and a free radical scavenger, GSH may provide protection against lipid peroxidation. A possible role of GSH in EUP-induced lipid peroxidation was investigated by means of GSH depleted tissues as well as by addition of GSH.

## *Methods and materials*

*Chemicals.* EUP ($M_r$ = 362) was isolated from ground dried aereal parts of *Eupatorium cannabinum* L. [10]. The identity and purity were confirmed using spectroscopical and chromatographic techniques. D,L-buthionine-*S*,*R*-sulphoximine (BSO) was from Chemalog (South Plainfield, U.S.A.). Reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), Ellman's reagent and diethyl maleate (DEM) were from Sigma Chemical Co. (St Louis, MO). Tetraethoxypropane (TEP) was from Schuchardt (Munich, F.R.G.). Folin-Ciocalteus reagent and 2-thiobarbituric acid (TBA) were from Merck (Darmstadt, F.R.G.).

*Mice.* Three-month-old syngeneic C57Bl mice (Department of Radiobiology, Groningen, The Netherlands), with free access to food and water, were used in all experiments.

The experiments with liver tissue were performed with female mice (body wt 20–25 g). The livers weighed 1.0–1.3 g. The FIO 26 fibrosarcoma, a solidly growing tumour, was maintained at the Department of Radiobiology by serial passage in male C57Bl mice, by subcutaneous transplantantation of about $4 \times 10^6$ viable tumour cells [3, 4]. In the experiments described, passages 6–10 were used. About three weeks after transplantation, the tumours, weighing about 2 g, were used for the experiments.

Reduction of hepatic and tumour GSH was achieved by intraperitoneal (i.p.) injection of 4 mmol/kg BSO, 4 hr before being killed, followed by 400 mg/kg DEM, 1 hr before the mice were killed. BSO was dissolved in 0.9% NaCl solution and injected at 0.3 ml/20 g body weight. DEM was administered in sesame oil, at 0.1 ml/20 g body weight.

In vitro *lipid peroxidation.* Mice were killed by cervical dislocation. All steps to prepare tissue homogenates were carried out at 4°. Livers of tumours were extirpated and necrotic parts of the tumours (if any) were removed. After washing with 0.15 M KCl solution, the tissue was minced with scissors and a 10% homogenate in 0.15 M KCl solution was prepared with the aid of a Potter–Elvehjem homogenizer. The homogenates were diluted with 0.15 M KCl solution, to obtain a protein content of about 3 mg/ml and used for the lipid peroxidation experiments.

Erlenmeyer flasks of 25 ml were used to contain 10 ml of the homogenate. The level of the suspension was always about 1 cm from the bottom of the flask. EUP was dissolved in 96% (v/v) ethanol and 100 μl/10 ml homogenate of the EUP solutions were added, yielding a final concentration of ethanol of 1% (v/v) and EUP between 0 and 1.5 mM. Ethanol, at this concentration, did not cause lipid peroxidation in the tissue homogenates.

In the experiments in which external GSH was added to the incubation mixture, at concentrations between 0 and 1.5 mM, EUP was added after a 15 min pre-incubation period of the homogenates with the GSH at 37°.

The Erlenmeyer flasks were stoppered with a cotton plug, to allow sufficient air supply, and incubated at 37° in a vigorously shaking water bath. Samples of 800 μl were

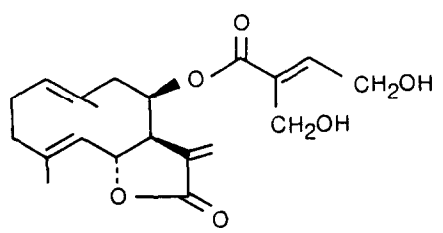


Fig. 1. Structural formula of eupatoriopicrin (EUP).

* Abbreviations: BSO, D,L-buthionine-*S*,*R*-sulphoximine; DEM, diethyl maleate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); EUP, eupatoriopicrin; GSH, glutathione (reduced); GSSG, glutathione (oxidized); i.p., intraperitoneal; MDA, malondialdehyde; TBA, 2-thiobarbituric acid; TEP, tetraethoxypropane.

taken before incubation and at different time points after incubation and immediately stored at −20° until analysed.

*Biochemical assays.* Lipid peroxidation was assayed by spectrophotometrical measurement at 532 nm of the chromogen, which is formed upon reaction of TBA with the lipid peroxidation product malondialdehyde (MDA), as described by Wilbur *et al.* [11] and modified by Konings and Drijver [12]. TEP was used as the standard. Total glutathione, referred to as GSH, was assayed according to Griffith [13]. GSSH was used as the standard. MDA and GSH concentrations were expressed per mg protein. Protein was assayed by the method of Lowry *et al.* [14], with bovine serum albumin as the standard. Before analysing for protein content, the thawed samples were sonicated (50 W, 30 sec).

*Results and discussion*

Exposure of murine liver and tumour homogenates to EUP resulted in lipid peroxidation, as shown in Figs 2 and 3 respectively. During this process preferential polyunsaturated fatty acids are peroxidized by free radical mechanisms. The amount of MDA, formed as a major degradation product of polyunsaturated fatty acids with three or more double bonds, can be used as a measure for lipid peroxidation [15].

For liver tissue, the lowest EUP concentration causing measurable lipid peroxidation was about 0.25 mM. After 1 hr incubation, MDA production was observed for 1.5 mM EUP only, whereas the MDA production was significant for all EUP concentrations used after 2 hr incubation. Increasing the EUP concentration from 0.5–1.5 mM enhanced the amount of MDA formed, although not in a linear way. The maximal amount of MDA formed during the time of the assay was 15 nmol/mg protein. After 1 hr incubation of the homogenates with 0.5–1.5 mM EUP, the GSH content dropped rapidly to about 50%. During the subsequent period of the assay, the GSH concentration decreased steadily by an additional 10–20%. Under control conditions (0 mM EUP), the GSH concentration reduced to about 80% after 4 hr incubation.

In tumour tissue, a marked increase in MDA production was found already after 1 hr incubation. There was not much difference between the effects of 0.5, 1.0 and 1.5 mM EUP. The maximal amount of MDA formed in the presence of EUP was 8 nmol/mg protein.

Comparing liver tissue with tumour tissue, it is seen that the peroxidizing capacity in the tumour is lower. This may possibly be ascribed to the lower availability of polyunsaturated fatty acids in tumour cells [16]. By means of gas chromatographic analysis of the methylated fatty acids, we indeed found that in liver tissue more polyunsaturated fatty acids were available as substrates for lipid peroxidation, as compared with FIO 26 tumour tissue (unpublished observation).

Reduction of the GSH level in liver and tumour was achieved by treating the mice with 4 mmol/kg BSO i.p. and 400 mg/kg DEM i.p., respectively, 4 and 1 hr before they were killed. Hepatic GSH was over 90% depleted. The GSH values were 73 nmol/mg protein in normal tissue and 1.5 nmol/mg protein after treatment. In the tumour the GSH content dropped from 77 nmol/mg protein to 20 nmol/mg protein (about 75% reduction). After GSH depletion, 0.1 mM EUP already caused lipid peroxidation in liver tissue. The GSH depleted control (without EUP)

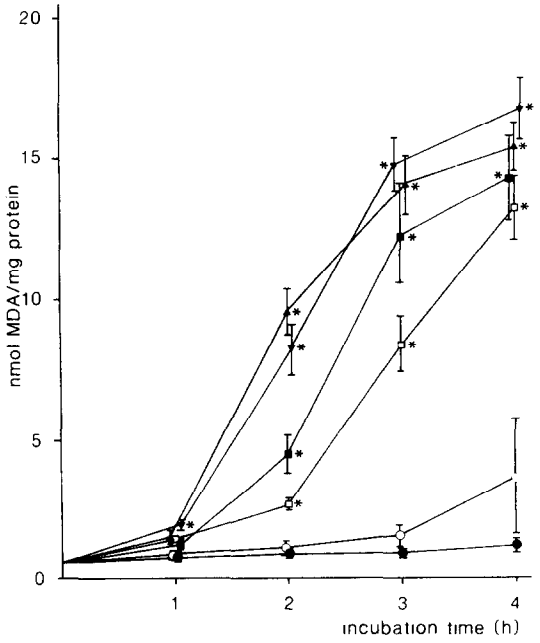


Fig. 2. EUP-induced lipid peroxidation in liver homogenate during 4 hr incubation at 37°. Each point represents the mean value ± SE (vertical bar). Control (●), N = 8; 0.1 mM EUP (○), N = 3; 0.25 mM EUP (□), N = 3; 0.5 mM EUP (■), N = 6; 1 mM EUP (▲), N = 6; 1.5 mM EUP (▼), N = 15. * $P < 0.05$, compared with the control (unpaired Student's *t*-test).

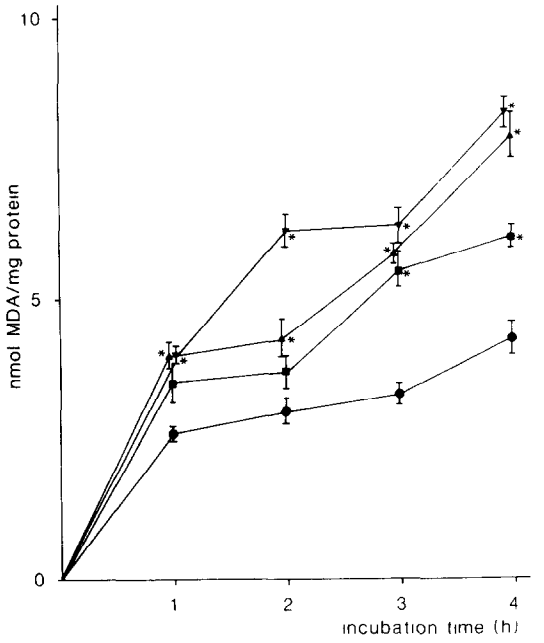


Fig. 3. EUP-induced lipid peroxidation in FIO 26 tumour homogenate during 4 hr incubation at 37°. Each point represents the mean value ± SE (vertical bar). Control (●), N = 3; 0.5 mM EUP (■), N = 3; 1 mM EUP (▲), N = 3; 1.5 mM EUP (▼), N = 3. * $P < 0.05$, compared with the control (unpaired Student's *t*-test).

showed an increase to about 5 nmol MDA/mg protein in both liver and tumour. This control MDA production together with the EUP-induced MDA production equalled the final amount of MDA formed.

GSH is the predominant cellular nonprotein thiol. As an antioxidant and nucleophile, GSH plays an important role in the cellular defence against toxic compounds and thus in protecting the cells against injury, e.g. caused by lipid peroxidation [17, 18]. Lipid peroxidation in GSH depleted liver tissue, achieved by BSO and DEM, without added EUP was significantly stimulated upon incubation at 37°. It has been described before that spontaneous lipid peroxidation may occur in liver tissue depleted of GSH [19] and that DEM can elicit lipid peroxidation in mouse liver homogenates [20].

No enhancement of EUP-induced lipid peroxidation was found after incubation with EUP in liver tissue of mice injected with 4 mmol/kg BSO i.p. only, resulting in about 50% GSH reduction [6]. The critical threshold of GSH for lipid peroxidation is suggested [21] to be reached by depleting both the pools in the cytoplasm and in the mitochondria. This situation could only be achieved by combining BSO with DEM.

To investigate the GSH-dependent protection against EUP-induced lipid peroxidation further, external GSH was added to liver tissue homogenates before incubation with EUP. The results of these experiments are depicted in Fig. 4. The influence of externally added GSH, in a concentration range of 0–1.5 mM, on the EUP-induced lipid peroxidation is shown after 2, 3 and 4 hr incubation. GSH, at a concentration of 0.5 mM, tended to reduce the process of lipid peroxidation. A concentration of 1 mM GSH significantly inhibited the formation of MDA, whereas 1.5 mM GSH completely halted the process at all EUP concentrations used.

The amount of GSH required to halt EUP-induced lipid peroxidation in liver tissue *in vitro*, was more or less stoichiometric, although a tendency exists toward a slight surplus of GSH. The homogenates, as incubated with EUP, contained 0.2–0.3 mM GSH and at least 0.25 mM EUP was necessary to elicit significant lipid peroxidation (Fig. 2). It was noticed by us that during incubation of GSH with culture medium (no cells, no EUP) the concentration dropped steadily (unpublished observation). This may explain the observation that more GSH had to be added to completely counteract EUP-induced lipid peroxidation. Because we found reduced GSH levels in liver and tumour tissue of the mouse after injection of EUP [6] and in tumour cells cultured *in vitro* and exposed to EUP [7], it is assumed that in the presence of EUP the amount of GSH may become reduced by thioether conjugation, resulting in a deactivation of EUP. High conjugating activity of a number of sesquiterpene lactones, possessing an $\alpha$-methylene $\gamma$-lactone moiety, with GSH in aqueous buffer has been described by Arrick *et al.* [22]. Apart from removal of GSH by conjugation, GSH may also scavenge free radicals or reactive oxygen, possibly induced by EUP.

The role of GSH as an important protective compound against lipid peroxidation in tissue homogenates has been demonstrated earlier by Konings and Oosterloo [23]. They showed that *in vitro* lipid peroxidation in liver tissue, induced by exposure of mice to X-irradiation or ozone, could be counteracted by injecting the animals with reduced GSH just before exposure. So a relatively high level of antioxidant was maintained.

Although EUP-induced lipid peroxidation may, in principle, cause cell death, no clear evidence for this has been established yet. The EUP concentrations used in the experiments performed in this study exceeded those required to cause cell death as measured in cell survival experiments [7, 9]. Furthermore, lipid peroxidation in tissue homogenates may not directly be compared with lipid peroxidation in intact cells. More research on this subject has

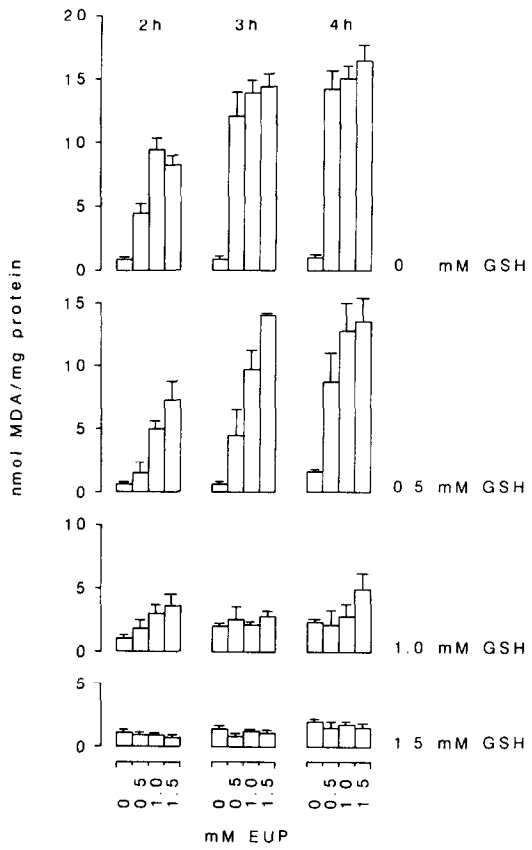


Fig. 4. The effect of GSH (0, 0.5, 1.0 and 1.5 mM) added to liver homogenates, on EUP-induced (0, 0.5, 1 and 1.5 mM) lipid peroxidation, after 2, 3 and 4 hr incubation at 37°. Given is the mean value ± SE of at least triplicate experiments.

to be done with intact cells and by measuring MDA levels *in vivo*. In addition, more sensitive and specific methods are required to demonstrate the occurrence of lipid peroxidation at the lower EUP concentrations. As an indicator of free radical production, the method used by us has its limitations for determination of lipid peroxidation because its nonspecific nature. It has also to be kept in mind that degradation products, from other than unsaturated lipids, may be formed that are not detected. Additive lipid peroxidation reactions may be analysed by other techniques, such as HPLC, fluorescence spectrophotometry or by gas chromatographic analysis of polyunsaturated fatty acids before and after incubation. The occurrence of DNA damage in Ehrlich Ascites Tumour cells after exposure to EUP, as reported recently by us [9], may also be related to attack by reactive oxygen species or by lipid peroxides, generated during chain reactions [24]. As is the case for lipid peroxidation, the extent of DNA damage induction depended on the GSH content of the cells [9].

The observation that liver tissue is more sensitive for EUP-induced lipid peroxidation than tumour tissue, may be of relevance if the general toxicity of this sesquiterpene lactone *in vivo* is considered.

In summary, addition of EUP to homogenates of murine liver and FIO 26 tumour tissue increased the rate of lipid peroxidation, as determined by measuring TBA-reactive

substances. *In vivo* lowering the GSH concentrations to 10% in the liver and 25% in the tumour, increased the rate of lipid peroxidation in the tissue homogenate, but did not provide more than an additive effect when combined with EUP.

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