Biochemical Pharmacology, Vol. 38, No. 14, pp. 2279–2283, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

INDUCTION OF DNA DAMAGE IN EHRLICH ASCITES TUMOUR CELLS BY EXPOSURE TO EUPATORIOPICRIN

HERMAN J. WOERDENBAG,*† JOOST C. C. VAN DER LINDE,† HARRIE H. KAMPINGA,‡ THEO M. MALINGRÉ† and ANTONIUS W. T. KONINGS‡

†Department of Pharmacognosy, University Centre for Pharmacy, University of Groningen, Ant. Deusinglaan 2, 9713 AW Groningen, The Netherlands, and ‡Department of Radiobiology, University of Groningen, Bloemsingel 1, 9713 BZ Groningen, The Netherlands

(*Received* 28 *October* 1988; *accepted* 18 *January* 1989)

Abstract—The sesquiterpene lactone eupatoriopicrin (EUP) from *Eupatorium cannabinum* L. has been shown to be cytotoxic in a glutathione (GSH)-dependent way. In order to assess possible DNA damage as a cause for cell death, the study reported was initiated. After 2 hr incubation of Ehrlich ascites tumour cells with EUP, the DNA damage, determined by the use of an alkaline DNA unwinding method, followed by hydroxylapatite column chromatography of degradated DNA, was observed at concentrations only slightly higher than those causing cell death in a clonogenic assay. The amount of EUP, requested to demonstrate DNA damage after a 24-hr post-incubation period lay within the concentration range that was effective in the clonogenic assay (1–10 μg/ml). Glutathione (GSH) depletion of the cells to about 99%, by use of buthionine sulphoximine (BSO), enhanced the extent of DNA damage. It is concluded that EUP-induced DNA damage may play a role in the observed cytotoxicity.

The cytostatic activity of eupatoriopicrin (EUP§; Fig. 1), a sesquiterpene lactone from *Eupatorium cannabinum* L., has been described in several *in vitro* and *in vivo* studies, performed in our laboratory [1–4]. The mechanism of action of this compound is unclear. Interference of cytotoxicity with the cellular antioxidant glutathione (GSH) has been observed [5, 6].

The purpose of this study was to determine whether treatment of cells *in vitro* with EUP can cause DNA damage. Lesions in DNA may possibly relate to the cytotoxic action of EUP. The presence of DNA damage (strand breaks and alkali-labile sites) in Ehrlich ascites tumour (EAT) cells, exposed to EUP, was assayed by use of an alkaline unwinding technique, followed by hydroxylapatite column chromatography [7, 8].

It is known that EUP affects the level of GSH and that GSH depletion enhances the cell killing effect of EUP [5, 6]. To investigate the role of GSH with respect to EUP-induced DNA lesions, part of the EAT cells were depleted from GSH before exposure to EUP.

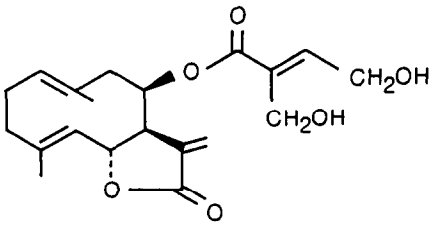

Fig. 1. Structural formula of eupatoriopicrin (EUP).

METHODS

Cell culturing. EAT cells were grown in suspension culture in RPMI 1640 medium (Flow Laboratories, Irvine, Scotland), supplemented with 10% fetal calf serum (Gibco, Paisley, Scotland), 50 μg/ml streptomycin (Mycopharm, Delft, The Netherlands) and 50 IU/ml penicillin G (Mycopharm), in a shaking incubator at 37°, as described in [8]. The doubling time was 12–14 hr. Exponentially-growing cells were used in all experiments. The viability of the cells used in the experiments exceeded 95%, as determined with Trypan Blue.

DNA labelling. DNA labelling was obtained by growing the cells (about 1×10^5/ml) in culture medium containing 3 μM [^{3}H]thymidine (New England Nuclear, Dreieich, F.R.G.) and 5 μM unlabelled thymidine, for a period of 36–48 hr (3–4 doubling times) [8]. The cells were centrifuged (5 min, 200 *g*) and the medium was replaced by fresh medium containing 5 μM unlabelled thymidine. The cells were chased for 60–90 min, centrifuged and resuspended in normal growth medium at a density of about 1×10^6 cells/ml.

Conditions for incubation with EUP. EUP (M_r = 362), isolated from *Eupatorium cannabinum* L. (Asteraceae) as described in [9], was dissolved in 96% (v/v) ethanol. To study the effect of EUP on DNA of EAT cells, 25 μl of EUP solution were added to 2.5 ml cell suspension (1×10^6 cells/ml), yielding final EUP concentrations between 0 and 20 μg/ml. The ethanol present in all samples (1% (v/v)) was shown not to affect the experiments performed. The suspensions were incubated for 2 hr in a shaking water bath at 37°. After the incubation

* All correspondence to: Dr H. J. Woerdenbag, Department of Pharmacognosy, University Centre for Pharmacy, University of Groningen, Ant. Deusinglaan 2, 9713 AW Groningen, The Netherlands.

§ Abbreviations used: EUP, eupatoriopicrin; GSH, glutathione; EAT, Ehrlich ascites tumour.

period the samples were either washed free of external EUP and incubated again or placed on ice. Initial DNA damage was assessed in the samples directly placed on ice. The possibility of repair of the initial DNA damage was monitored by post-incubations up to 24 hr at 37° after exposure to EUP.

All cells incubated with EUP were washed twice by centrifugation (5 min, 200 *g*, 4°) in drug-free medium before further treatment.

To deplete cells of GSH, they were cultured overnight with 500 μM buthionine sulphoximine (BSO: Chemalog, South Plainfield, U.S.A.), as described in [10], prior to the treatment with EUP (GSH no longer detectable; depletion > 99%). GSH values for untreated cells were 20–25 nmol/mg protein.

Determination of DNA damage. DNA damage (strand breaks and alkali labile sites) was determined by the method of Ahnström and Erixon [7], as modified by Jorritsma and Konings [8]. Shortly, for the alkaline unwinding ice-cooled samples of 100 μl (about 1×10^5 cells) were rapidly mixed with an alkaline buffer (pH = 12.6) and kept in the dark at 20° for 30 min. After rapid neutralization sodium dodecylsulphate (SDS) solution was added and the samples were stored at −20°.

After thawing, the samples were sonicated (20 sec, 50 W) and submitted to hydroxylapatite column chromatography [8]. Single-strand DNA fractions (SS) were eluted with 0.150 M Na–phosphate buffer (pH = 6.8) and double-strand DNA fractions (DS) with 0.4 M Na–phosphate buffer (pH = 6.8). To obtain counting gels, Hydroluma (Lumac, Schaesberg, The Netherlands) was added. The gels were allowed to cool and counted in a liquid scintillation counter.

Damage to DNA was calculated as %DS, using the formula:

$$\%DS = \frac{DS \times 100\%}{SS + DS}.$$

EUP-induced DNA damage was related to X-ray-induced strand breaks and expressed as Gy-equivalents [8]. X-irradiation was performed using a Philips-Müller MG 300 machine operating at 200 kV and 15 mA at a dose rate of 6 Gy/min. The X-rays were filtered with 0.5 mm Cu and 0.5 mm Al. EUP-induced DNA damage of the BSO treated and non-treated cells was calculated on the basis of X-ray dose response curves from parallel experiments.

Determination of cell survival. Cell survival was determined as colony forming ability of the cells on soft agar. A total of 1×10^6 cells/ml were incubated with various concentrations of EUP at 37° for 2 hr. Further experimental conditions were similar to those described earlier [6, 8]. Colonies were counted after about 7 days incubation in a humidified incubator at 37°, gassed with 5% CO_2. Plating efficiency was always higher than 85%.

Statistics. For the statistical evaluation of the data the unpaired Student's *t*-test was used. A P-value < 0.05 was considered significant.

RESULTS

To determine EUP cytotoxicity in terms of cell death, cell survival after EUP exposure was determined using a clonogenic assay. Progressive killing was observed after 2-hr incubation with 1–10 μg/ml EUP (Fig. 2). When cellular GSH was depleted (>99%), the cells were found to be more sensitive for the cell killing effect of EUP. The BSO treatment itself had no cytotoxic effects. With 5 and 10 μg/ml EUP the surviving fraction of BSO-treated cells was significantly smaller than of normal cells. The level of 10% survival was reached with 5.3 μg/ml EUP for normal cells and 3.2 μg/ml for BSO treated cells yielding a dose enhancement factor 1.7.

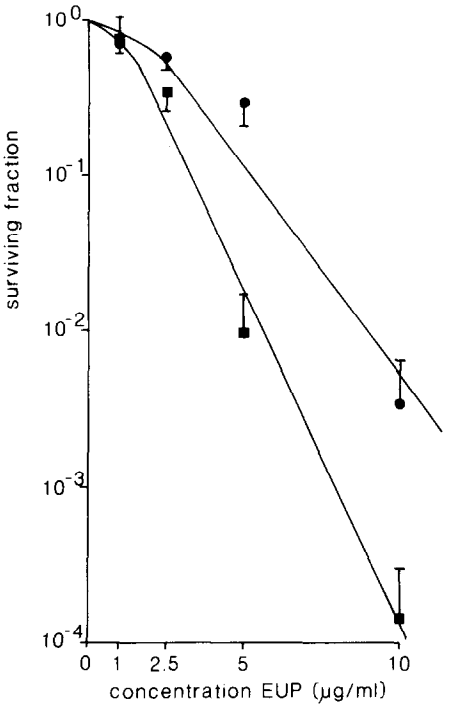

Fig. 2. The effect of EUP on cell survival of EAT cells as measured by the clonogenic assay. Normal cells (●) and GSH depleted cells (■) were incubated for 2 hr with different EUP concentrations. For each point the mean value (N = 3) ± SEM (vertical bar) is given.

Exposure of EAT cells to the EUP concentrations used resulted in DNA damage as illustrated in Fig. 3. Immediately after 2-hr exposure to EUP concentrations from 15 μg/ml, significant formation of DNA damage was observed. GSH depletion caused potentiation of EUP-induced DNA damage induction. Significant damage induction in GSH depleted cells was observed after treatment with 10 μg/ml EUP or more.

To investigate the possibility of repair in normal as well as in GSH depleted cells, the concentrations of EUP for both conditions causing iso-damage were compared, as well as an equal concentration of EUP (20 μg/ml), with and without GSH depletion. The initial damage in normal cells, caused by 20 μg/ml EUP corresponded with the initial damage in BSO treated cells caused by 10 μg/ml EUP (see Fig. 3), being *ca.* 5 Gy-equivalents. DNA damage was measured between 0 and 90 min of incubation at 37°, after washing the EUP-treated cells. From the

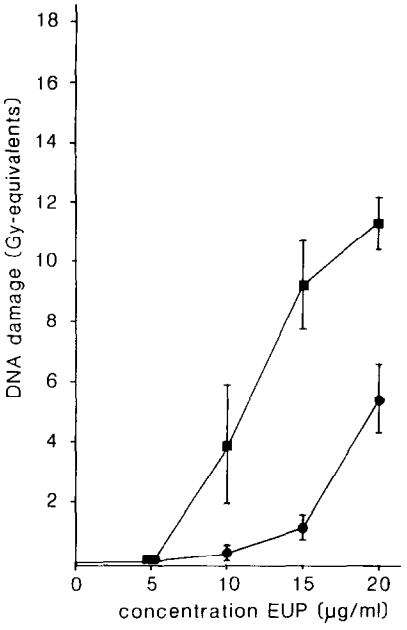

Fig. 3. Initial DNA damage induction by EUP in EAT cells. Normal cells (●) and GSH-depleted cells (■) were incubated for 2 hr with different EUP concentrations and immediately assayed for DNA damage. For each point the mean value (N = 6–11) ± SEM (vertical bar) is given.

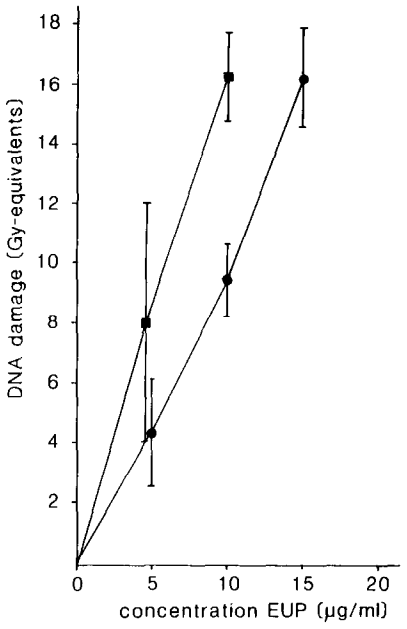

Fig. 4. Late DNA damage induction by EUP in EAT cells. Normal cells (●) and GSH-depleted cells (■) were incubated for 2 hr with different EUP concentrations, washed, post-incubated 24 hr at 37° and assayed for DNA damage. For each point the mean value (N = 3–5) ± SEM (vertical bar) is given.

Table 1. DNA damage in normal EAT cells, exposed to 20 μg/ml EUP for 2 hr (A) and GSH-depleted EAT cells, exposed to 10 μg/ml EUP (B) or 20 μg/ml EUP (C) for 2 hr.

Post-incubation time (min)	A	B	C
0	100	100	226
3	135 ± 12	107 ± 7	192 ± 15
7	168 ± 34	103 ± 5	181 ± 2
15	190 ± 50	115 ± 10	262 ± 16
45	180 ± 36	137 ± 12	269 ± 16
90	180 ± 35	145 ± 24	276 ± 12

After a 2-hr incubation period with EUP the cells were washed, post-incubated 0–90 min at 37° and assayed for DNA damage. The initial DNA damage (100%) was about equal (5 Gy-equivalents) for A and B (Fig. 3). The initial damage of C is expressed as percentage of the initial damage of A and B (5 Gy-equivalents, 100%). The mean values (N = 3) ± SD are given.

results, listed in Table 1, it can be seen that no decrease in the amount of DNA damage occurred during the post-incubation time, but an increase for all conditions was found. After 90 min more DNA damage was observed in normal cells treated with 20 μg/ml EUP than in the GSH-depleted cells treated with 10 μg/ml EUP. Furthermore, the initial DNA damage in GSH depleted cells, incubated with 20 μg/ml EUP, was much higher than in untreated cells incubated with the same EUP concentration.

From Fig. 4 it can be seen that the extent of DNA damage in EAT cells, incubated for 2 hr with a range of EUP concentrations, followed by a 24-hr period of post-incubation at 37°, is still enlarged, as compared with the initial damage (Fig. 3). Already at concentrations as low as 5 μg/ml EUP significant DNA damage could be detected. Again, the BSO-treated cells appeared to be more sensitive for EUP. The extensive GSH depletion (>99%) itself did not induce DNA damage (not shown).

DISCUSSION

DNA is an important target for cell killing caused by a number of cytostatic acting agents. Several types of DNA lesions may occur. They include single- and double-strand breaks, base damage, inter- and intrastrand DNA cross-links, DNA-protein and DNA-drug cross-links [11].

The initial DNA damage, i.e. the DNA damage present immediately after 2-hr exposure of the EAT cells to EUP (Fig. 3), was measured at supralethal EUP concentrations, when compared with those used for the survival experiments (Fig. 2). After washing the cells, followed by a post-incubation period at 37°, the extent of DNA damage increased (Table 1, Fig. 4). Post-incubations up to 24 hr led to detectable DNA damage, at lower EUP concentrations, approaching the concentrations used for determination of cell survival (e.g. 5–10 μg/ml). On the other hand, Trypan Blue exclusion performed on unlabelled EAT cells, incubated with these EUP concentrations and post-incubated for 24 hr, did not

reveal cell killing. However, the possibility that the DNA damage measured here may be a *post-mortem* effect, cannot be excluded.

The cellular GSH level, at the time of EUP exposure, has been shown to have effects on EUP-induced cytotoxicity on FIO 26 cells *in vitro*, as well as on the antitumour effect of EUP *in vivo* [6]. EAT cells, depleted >99% of GSH by BSO, showed also significantly enhanced sensitivity for EUP cytotoxicity (Fig. 2). So, this observation is consistent with the enhanced cytotoxicity of EUP, at comparable concentrations, against FIO 26 tumour cells, depleted of GSH [6]. Chemosensitization for EUP-induced DNA damage, initial (Fig. 3) as well as on longer terms (Fig. 4), occurred after BSO mediated GSH depletion of the cells. This suggests that DNA damage is involved in EUP-induced cytotoxicity.

It is not known at the moment how EUP may elicit initial DNA damage. It may occur directly, e.g. via intercalation, or indirectly, via metabolites of EUP, EUP-induced free radicals, reactive oxygen or the action of peroxides. DNA damage may also occur through the activity of endogenous enzymes of the dead cells. The EUP-induced DNA damage may be well-related to the occurrence of lipid peroxidation. It is known that reactive oxygen intermediates, as generated in chain reactions during the process of lipid peroxidation, can cause oxidative DNA damage [12–15]. Degradation products, generated during this process, such as malondialdehyde and arachidonic acid peroxides, are potent inducers of DNA breaks [16–18]. The occurrence of lipid peroxidation in murine liver and tumour tissue, incubated *in vitro* with EUP, was shown recently [19].

Several different mechanisms of action of clinically applied chemotherapeutics, acting on DNA, can be distinguished. Alkylating agents (nitrogen mustards, cisplatin) damage DNA by alkylation of a single-strand of the DNA or by causing cross-links. Antimetabolites disturb the DNA synthesis by interference with purine or pyrimidine. Natural products, such as vinca-alkaloids and colchicin are mitotic inhibitors. Anthracyclin antibiotics (doxorubicin, daunorubicin, dactinomycin) act via intercalation and frequently the generation of free radical species is found in this group [11, 20].

In general, at physiological temperatures, the majority of radiation-induced DNA damage, as well as DNA damage induced by several drugs, is repaired within 2 hr [11, 21]. From our results (Table 1, Fig. 4) it seems that no repair of EUP-induced DNA damage occurs in EAT cells. The data show an increase of DNA damage upon post-incubation time. DNA damage that was not or hardly detectable directly after certain EUP treatments, became evident 24 hr later (compare Figs 3 and 4, Table 1). Apart from the *post-mortem* effect mentioned earlier, the increase also suggests the possibility that, even after the washing procedures, EUP, its metabolites or reaction products (lipid peroxidation) are still present in the cell and as such cause DNA damage during incubation after EUP had been washed out. However, the data do not rule out the possibility of repair of EUP-induced damage to the DNA. The increase might be a net result of the balance between DNA repair and further DNA damage, the latter being prevalent. The observed difference between the extent of DNA damage after 90 min post-incubation, caused by 20 μg/ml EUP in normal and 10 μg/ml EUP in BSO-treated cells, may possibly be explained in terms of elevated intracellular levels of EUP or its metabolites, under the first-mentioned condition. The role of GSH in the extent of EUP-induced DNA damage is obvious when the damage, caused by 20 μg/ml EUP is compared for normal and GSH-depleted cells (Table 1).

In conclusion, interaction of EUP with EAT cells leads to damaged DNA. Initial unrepaired damage or faulty DNA repair may play a role in EUP cytotoxicity.

REFERENCES

1. Woerdenbag HJ, Meijer C, Mulder NH, De Vries EGE, Hendriks H and Malingré ThM, Evaluation of the *in vitro* cytotoxicity of some sesquiterpene lactones on a human lung carcinoma cell line using the Fast Green dye exclusion Assay. *Planta Med* **52**: 112–114, 1986.
2. Woerdenbag HJ, Hendriks H, Malingré ThM, Van Stralen R, Van den Berg KJ and Konings AWT, *In vitro* cytotoxicity of sesquiterpene lactones from *Eupatorium cannabinum* L. and semi-synthetic derivatives from eupatoriopicrin. *Phytotherapy Res* **2**: 109–114, 1988.
3. Woerdenbag HJ, Lemstra W, Hendriks H, Malingré ThM and Konings AWT, Investigation of the antitumour action of eupatoriopicrin against the Lewis Lung tumour. *Planta Med* **53**: 518–522, 1987.
4. Woerdenbag HJ. Malingré ThM, Lemstra W and Konings AWT, Cytostatic activity of eupatoriopicrin in fibrosarcoma bearing mice. *Phytotherapy Res* **1**: 76–79, 1987.
5. Woerdenbag HJ, Malingré ThM, Lemstra W and Konings AWT, Reduced levels of glutathione in liver and tumour tissue of the mouse after administration of eupatoriopicrin. *Phytotherapy Res* **2**: 80–84, 1988.
6. Woerdenbag HJ, Lemstra W, Malingré ThM and Konings AWT, Enhanced cytostatic activity of the sesquiterpene lactone eupatoriopicrin by glutathione depletion. *Br J Cancer* **59**: 68–75, 1989.
7. Ahnström G and Erixon K, Radiation induced strand breakage in DNA from mammalian cells. Strand separation in alkaline solution. *Int J Radiat Biol* **23**: 285–289, 1973.
8. Jorritsma JBM and Konings AWT, Inhibition of repair of radiation-induced strand breaks by hyperthermia, and its relationship to cell survival after hyperthermia alone. *Int J Radiat Biol* **43**: 506–516, 1983.
9. Woerdenbag HJ, *Eupatorium cannabinum* L. A review emphasizing the sesquiterpene lactones and their biological activity. *Pharm Weekbl [Sci]* **8**: 245–251, 1986.
10. Konings AWT and Penninga P, On the importance of the level of glutathione and the activity of the pentose phosphate pathway in heat sensitivity and thermotolerance. *Int J Radiat Biol* **48**: 409–422, 1985.
11. Lown JW, Molecular mechanisms of action of anticancer agents involving free radical intermediates. *Adv Free Radical Biol Med* **1**: 225–264, 1985.
12. Brawn K and Fridovich J, DNA strand scission by enzymatically generated oxygen radicals. *Arch Biochem Biophys* **206**: 414–419, 1981.
13. Lesko SA, Lorentzen RJ and Ts'o POP, Role of superoxide in deoxyribonucleic acid strand scission. *Biochemistry* **19**: 2023–2028, 1980.
14. Fridovich I, Superoxide radical: an endogenous toxicant. *Annu Rev Pharmacol Toxicol* **23**: 239–247, 1983.
15. Moody CS and Hassan HM, Mutagenicity of oxygen free radicals. *Proc Natl Acad Sci USA* **79**: 2855–2859,

1982.
16. Ames BN, Hollstein MC and Cathcart R, Lipid peroxidation and oxidative damage to DNA. In: *Lipid Peroxides in Biology and Medicine* (Ed. Yagi K), pp. 339–351. Academic Press, New York, 1982.
17. Kozumbo WJ, Muehlematter D, Jörg A, Emerit I and Cerutti P, Phorbol ester-induced formation of clastogenic factor from human monocytes. *Carcinogenesis* **8**: 521–526, 1987.
18. Muehlematter D, Larsson R and Cerutti P. Active oxygen induced DNA strand breakage and poly ADP-ribosylation in promotable and non-promotable JB6 mouse epidermal cells. *Carcinogenesis* **9**: 239–245, 1988.
19. Woerdenbag HJ, Edwards ACM, Budé EJM, Malingré ThM and Konings AWT, Eupatoriopicrin-induced lipid peroxidation in liver and tumour tissue of the mouse. *Biochem Pharmacol*, in press.
20. Calabresi P and Parko RE Jr, Chemotherapy of neoplastic diseases. In: *The Pharmacological Basis of Therapeutics* (Eds. Goodman Gilman A, Goodman LS, Rall TW and Murad F), pp. 1240–1306. Macmillan, New York, 1985.
21. Pommier Y, Schwartz RE, Kohn KW and Zwelling LA, Formation and rejoining of deoxyribonucleic acid double-strand breaks induced in isolated cell nuclei by antineoplastic intercalating agents. *Biochemistry* **23**: 3194–3201, 1984.