

## Chemopreventive Effects of Coumaperine from Pepper on the Initiation Stage of Chemical Hepatocarcinogenesis in the Rat

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This study was designed to investigate the chemopreventive action of three natural products, coumaperine, auraptin and an extract from rosemary, against the initiation stage of rat hepatocarcinogenesis. Coumaperine has been isolated from white pepper as a naturally occurring antioxidative agent, but its potential modifying effects on carcinogenesis remain unclear. In experiment 1, a modification of the model developed by Tsuda *et al.* was applied, with assessment of numbers and areas of induced glutathione S-transferase placental form (GST-P)-positive hepatocellular foci in male F344 rats. Coumaperine, auraptin and the extract from rosemary were administered i.g. at 100 mg/kg/day once daily for 5 days with initiation by diethylnitrosamine (DEN) on day 4 (20 mg/kg, i.p.). Numbers and areas of GST-P-positive foci in each group given test chemicals tended to be decreased as compared to the vehicle control group values, significance being achieved for number with coumaperine. Experiment 2 was planned to investigate the mechanism of the inhibitory effects of coumaperine. Livers at 8 h after initiation by DEN were examined with coumaperine administered at 100 mg/kg/day once daily for 3 days. Proliferating cell nuclear antigen (PCNA)-positive cells tended to be decreased as compared to the vehicle control, but no effects on apoptosis or cytochrome P-450 (CYP) 2E1 expression were apparent. Our results suggest that coumaperine provides protection against initiation of hepatocarcinogenesis, and that this is related to inhibition of cell proliferation.

Key words: Coumaperine — Chemoprevention — Rat hepatocarcinogenesis

Recently, much importance has been attached to chemoprevention. Several candidates from plants are known to be efficacious, including some natural products with low toxicity.<sup>1–4</sup> Further examples, coumaperine from pepper, auraptin from citrus fruit and N-hexane extract (acidulous fraction) from rosemary, were tested in the present study.

Coumaperine, N-5-(4-hydroxyphenyl)-2E,4E-pentadienoyl piperidine, isolated from *Piper nigrum* L. (white pepper),<sup>5</sup> is present at a concentration of about 6 ppm in the dry fruits. It has antioxidative activity against linoleic acid oxidation<sup>6</sup> and although this does not necessarily indicate preventive potential,<sup>7</sup> it was chosen for examination. N-Hexane extract (acidulous fraction) from rosemary (*Rosmarinus officinalis* L.) also has antioxidative activity.<sup>8</sup> Auraptin, derived from citrus fruit (*Citrus hassaku*), has antimicrobial and spasmolytic activity, but not antioxidative activity.<sup>9,10</sup> It has also been reported that auraptin

inhibits chemical tumorigenesis in tongue and skin,<sup>11,12</sup> and Tanaka *et al.*<sup>13</sup> found that it reduced the development of aberrant crypt foci in the colon.

In the present study, potential modifying effects of coumaperine, auraptin and the extract from rosemary on the initiation stage of rat hepatocarcinogenesis were examined, using a modification of the model developed by Tsuda *et al.*<sup>14</sup> Takada *et al.*<sup>1</sup> adopted this approach and demonstrated an inhibitory effect of S-methylcysteine. To cast light on possible mechanisms of action, an examination of cell proliferation, apoptosis, phase I enzymes and early response gene expression was included, along with double staining of GST-P and AgNOR.

### MATERIALS AND METHODS

**Animals** A total of 76 male 7-week-old F344 rats were purchased from Charles River Japan Inc., Hino, Shiga, and housed in an animal room maintained with a 12 h (7:00–19:00) light-dark cycle, at a constant temperature of 25±2°C, and a relative humidity of 55±5%. The animals were observed daily, and were used after a 1-week acclimation period for the experiments detailed below.

**Chemicals** In this study, coumaperine was prepared as

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Abbreviations: GST-P, glutathione S-transferase placental form; AgNORs, silver-stained nucleolar organizer regions; DEN, diethylnitrosamine; 2-AAF, 2-acetylaminofluorene; PCNA, proliferating cell nuclear antigen; CYP, cytochrome P-450; ABC, avidin-biotin complex; NEL, nick-end labeling.

described previously.<sup>15</sup> Aurapten was isolated from the precipitated fraction of *C. hassaku* juice oil as described earlier.<sup>9</sup> The chemical structures of these compounds are shown in Fig. 1. The N-hexane extract (acidulous fraction) from rosemary was as reported.<sup>8</sup> DEN was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo) and 2-AAF from Sigma-Aldrich Japan (Tokyo).

**Treatment** Experiment 1 was performed according to the method of Takada *et al.*,<sup>1</sup> itself a modification of the procedure developed by Tsuda *et al.*<sup>14</sup> The experimental design is shown in Fig. 2. Seventy male 8-week-old F344 rats were allocated into 5 groups (groups 1–4: 15 rats/group, group 5: 10 rats/group). All rats were given a single i.p. injection of DEN (20 mg/kg b.w.) dissolved in saline to initiate hepatocarcinogenesis. Coumaperine (group 1), extract from rosemary (group 2) and aurapten (group 3) were administered i.g. at 100 mg/kg/day b.w. dissolved in 60% ethanol, once a day from 3 days prior to DEN injection to one day after (total 5 days). Ethanol (60%) as the vehicle (group 4) and saline (group 5) were given in the control groups. The dose volume was 3 ml/kg b.w. All rats were fed 0.01% 2-AAF in powdered diet from weeks 2 to 4 and subjected to two-thirds partial hepatectomy at week 3. All survivors were killed under ether anesthesia at the end of week 5. The livers were subjected to immunohistochemical staining for GST-P. Double staining of GST-P and AgNORs was carried out for the coumaperine (group 1) and vehicle control (group 4) groups.

Experiment 2 was planned to examine the mechanism of the inhibitory effect of coumaperine on hepatocarcinogenesis. Six rats were divided into two groups, and given a single i.p. injection of DEN (20 mg/kg b.w.) as in experiment 1. Coumaperine (group 1) was administered i.g. at 100 mg/kg/day b.w. dissolved in 60% ethanol (3 ml/kg b.w.), once a day from 2 days prior to DEN injection to the day of DEN injection. Group 2 received the ethanol (60%) vehicle. All rats were killed under ether anesthesia 8 h after administration of DEN and their livers were examined for immunohistochemical staining of PCNA, CYP2E1, c-Jun, c-Myc and *in situ* analysis of apoptosis.

**Tissue processing** At autopsy, livers were quickly dissected out and weighed, then 2–3 mm thick sections from three lobes were fixed in 10% buffered formalin and embedded in paraffin wax. Sections cut at 4  $\mu$ m were used for immunohistochemistry, *in situ* analysis of apoptosis and double staining of GST-P and AgNORs.

**Immunohistochemical staining of GST-P, PCNA, CYP2E1, c-Jun and c-Myc** The avidin-biotin complex (ABC) method was used.<sup>16</sup> After deparaffinization, liver sections were treated sequentially with 3% H<sub>2</sub>O<sub>2</sub>, normal goat serum or horse serum, first antibody, biotin-labeled goat anti-rabbit IgG or horse anti-mouse IgG and avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories,

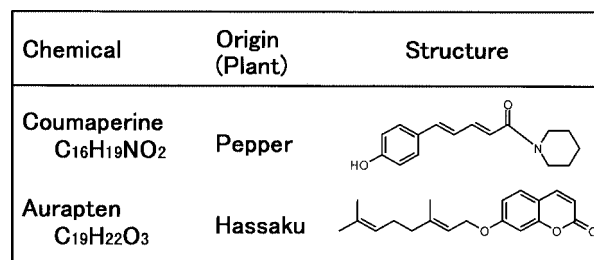


Fig. 1. Chemical structures of coumaperine and aurapten.

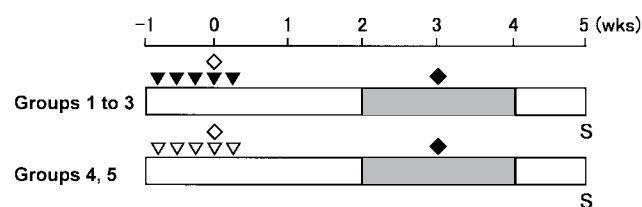


Fig. 2. Protocol for examination of modifying effects on the initiation stage of hepatocarcinogenesis (experiment 1).  $\diamond$ , DEN, 20 mg/kg b.w. (i.p.);  $\blacktriangledown$ , test chemicals, 100 mg/kg/day b.w. (i.g.);  $\nabla$ , ethanol (60%) or saline;  $\blacklozenge$ , partial hepatectomy;  $\blacksquare$ , 0.01% 2-AAF in the diet; S, animals killed.

Burlingame, CA). The sites of peroxidase binding were demonstrated by the diaminobenzidine method. The tissue sections were lightly counterstained with hematoxylin to facilitate orientation. As first antibodies, rabbit anti rat GST-P antibody (1:2000) (MBL, Nagoya), mouse anti PCNA antibody (1:200) (DAKO Japan Co., Ltd., Kyoto), rabbit anti rat CYP2E1 antibody (1:300),<sup>17</sup> rabbit anti c-Jun/AP-1 antibody (1:100) (Oncogene Research Products, Cambridge, MA),<sup>18</sup> and mouse anti c-Myc antibody (1:20) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were employed at 4°C overnight. As negative controls, normal rabbit or mouse sera were applied instead of a first antibody.

**Detection of DNA fragmentation *in situ*** To detect DNA fragmentation *in situ* associated with apoptosis, tissue sections were stained for nick-end labeling (NEL) according to the method of Gavrieli *et al.*,<sup>19</sup> with an *in situ* apoptosis detection kit (peroxidase) (Apop Tag, Oncor, Inc., Gaithersburg, MD). After deparaffinization, liver sections were treated according to the manufacturer's instructions. The sites of DNA fragmentation were demonstrated by the diaminobenzidine method.

**Double staining for AgNORs and GST-P** First, GST-P staining was performed using the ABC method. After deparaffinization, liver sections were treated sequentially with 3% H<sub>2</sub>O<sub>2</sub>, normal goat serum, rabbit anti rat GST-P antibody (1:2000, 4°C, overnight), biotin-labeled goat anti

Table I. Final Body and Relative Liver Weights, and Total 2-AAF Intake (Experiment 1)

Group	Chemical	Effective no. of rats	Final body weight (g)	Relative liver weight (%)	2-AAF consumption (mg/rat)
1	Coumapherine	15	236±11 <sup>a)</sup>	3.0±0.3	19.1±2.5 <sup>b)</sup>
2	Extraction of rosemary	14	253±14	3.1±0.2	19.8±3.1 <sup>b)</sup>
3	Aurapten	10	235±14	3.0±0.2	18.4±2.5 <sup>c)</sup>
4	Ethanol (60%)	12	237±18	3.1±0.3	16.3±1.3
5	Saline	8	234±14	3.1±0.2	16.9±3.1

a) Mean±SD.

b) Significantly different from the vehicle control group (group 4) at  $P<0.01$  (Student's *t* test).

c) Significantly different from the vehicle control group (group 4) at  $P<0.05$  (Student's *t* test).

rabbit IgG and alkaline phosphatase labeled avidin-biotin complex (ABC-AP standard kit, Vector Laboratories). The sites of alkaline phosphatase binding were demonstrated by using an alkaline phosphatase substrate (Vector red, Vector Laboratories), which stains the target antigen red.

Next AgNOR staining was performed following a modification of Chiu's method.<sup>20, 21)</sup> The sections were treated with an acetic acid-ethanol (1:3) mixture for 5 min, followed by absolute ethanol, and 70% ethanol, then placed in deionized water. After this pretreatment, silver colloid solution was prepared by dissolving 2 g/dl gelatin in 1 g/dl aqueous formic acid and mixing this with one volume of 50% (w/w) aqueous silver nitrate solution. Specimens were incubated in silver colloid for 30 min at room temperature and finally washed with deionized water and fixed with 5% sodium thiosulfate for 5 min.

**Quantitative assessment of GST-P-positive foci** In this study, a two-dimensional evaluation of foci was used. The numbers and areas of GST-P-positive foci larger than 0.2 mm diameter and the total areas of the liver sections were measured using a color image processor (VIP-21 C; Olympus-Ikegami Tsushin, Tokyo), and the numbers and areas of foci/cm<sup>2</sup> of liver section were calculated.

**Evaluation of PCNA positivity and apoptosis** The numbers of PCNA-positive cells were counted in 8000 cells per rat (2000 cells×4 sites) in randomly selected areas, and the average was calculated as the labeling index. The numbers of apoptotic cells were measured and numbers of cells/cm<sup>2</sup> of liver section were calculated.

**Evaluation of GST-P and AgNORs double staining** The number of AgNOR dots in both GST-P-positive and -negative cells was assessed in 200 cells for each sample. Previous studies have indicated that an increase in the size of AgNOR dots rather than their number correlates positively with elevated cell proliferation.<sup>22, 23)</sup> Therefore the roundness and areas of AgNORs were measured quantitatively with the aid of a color video image processor (IPAP, image processor for analytical pathology; Sumika Technos, Osaka) in about 50 cells in each site.<sup>24)</sup> The value for roundness was calculated as  $4\pi S/P^2$  (*S*, area of AgNORs;

Table II. Numbers and Areas of GST-P-positive Foci in Rat Livers (Experiment 1)

Group	Chemical	Effective no. of rats	GST-P	
			Numbers	Areas
1	Coumapherine	15	9.3±4.1 <sup>a, b)</sup>	1.3±0.9
2	Extraction of rosemary	14	9.9±5.0	1.4±1.0
3	Aurapten	10	10.6±6.2	1.5±1.3
4	Ethanol (60%)	12	13.7±6.0	2.0±1.4
5	Saline	8	18.2±6.7	3.0±2.7

a) Mean±SD.

b) Significantly different from the vehicle control group (group 4) at  $P<0.05$  (Student's *t* test).

*P*, perimeter of AgNORs). The value for the AgNOR area was expressed per nuclear area (dot-to-nuclei area ratio).

**Statistical evaluation** Statistical analysis of AgNORs data was conducted using the multiple comparison test (Tukey type). Other data were evaluated using Student's *t* test. The analysis was performed using the SAS system (Release 6.12, SAS Institute Inc., Cary, NC).

## RESULTS

**Experiment 1** The cause of death of these animals was partial hepatectomy in all cases, and no animals died due to chemical toxicity. There was no appreciable difference in clinical signs (general observation) between test chemical groups and control groups. Final body and relative liver weights, and 2-AAF intake data for experiment 1 are shown in Table I. Final average body weights and relative liver weights in groups 1 to 3 did not significantly differ from control group values (group 4). Average intake of 2-AAF in the test chemical groups 1 to 3 was significantly increased as compared to the vehicle control group 4 value.

Data for GST-P positive foci (per cm<sup>2</sup> of liver tissue) in experiment 1 are given in Table II. Values for the numbers

and area in groups 1 to 3 treated with test chemicals tended to be decreased, as compared to those in group 4 (vehicle control), the number in the coumaperine group being significantly reduced.

Because coumaperine significantly decreased the number value of GST-P-positive foci in rat liver, AgNORs were compared between the coumaperine (group 1) and vehicle control (group 4) cases. Data for numbers of AgNOR dot/cell in GST-P-positive foci and background parenchyma are summarized in Table III. No statistically significant differences were observed. Quantitative data for roundness and area are shown in Table IV. Relative area of AgNORs against nuclear area (dot-to-nuclei area ratio) in GST-P positive foci was significantly increased and roundness decreased as compared to the value background parenchyma in both groups 1 and 4 (Fig. 3), but no intergroup differences were found.

**Experiment 2** The results of immunohistochemical staining for PCNA and *in situ* analysis of apoptosis are summarized in Fig. 4. PCNA-positive cells tended to be decreased in coumaperine-treated liver tissue, as compared to the control group. In addition, the numbers of apoptotic cells also tended to be reduced.

In the livers of all animals, the centrilobular zone was stained with antibody to CYP2E1 protein (Fig. 5). However, no intergroup differences were apparent.

Table III. Numbers of AgNOR Dots per Cell in GST-P-positive Foci and in Background Parenchyma (Experiment 1)

Group	Chemical	Effective no. of rats	Number of dots	
			In foci	In background parenchyma
1	Coumaperine	15	2.4±0.3 <sup>a)</sup>	2.3±0.3
4	Ethanol (60%)	12	2.4±0.2	2.4±0.2

a) Mean±SD.

No cells were positive for c-Jun or c-Myc immunohistochemical staining in this experiment.

## DISCUSSION

In the coumaperine group, the number of GST-P-positive foci in rat liver was significantly decreased in experiment 1, indicating inhibition of the initiation stage, possibly linked to depressed cell proliferation. Auraptin from *C. hassaku* and extract from rosemary also exhibited weakly inhibitory effects.

No toxicity was evident in data for body or liver weights, in line with data from a preliminary single oral dose toxicity study (500 mg/kg b.w., i.g.) (unpublished data). The doses of test chemicals were selected as relatively high, as compared to conceivable human intake in pepper, because this examination was a screening test for exploring chemopreventive potential. The dose was thus large, but produced no sign of toxicity under the present experimental conditions. In future, a dose-response study is planned, and the relationship between inhibitory effect and administration period should also be examined. Furthermore, the issue of chronic toxicity is very important for chemopreventive agents. In a preliminary study, we examined single exposure toxicity to control for experiments 1 and 2 only. In this study, we administered chemicals 3–5 times to assess modification effects on initiation activity, and we judged that the administration caused no problem with regard to toxicology. In future, however, a chronic toxicity test will be needed. The reason why total intake of 2-AAF in the test chemical group was increased as compared to the vehicle control group values is unclear but despite this increased carcinogen exposure, coumaperine in the initiation phase was associated with decreased development of GST-P-positive foci. In addition, we used 60% ethanol as a vehicle in this study to enhance the absorption of chemicals. There are many

Table IV. Relative Area (Dot-to-nuclei Area) and Roundness of AgNOR Dots per Cell in GST-P-positive Foci and in Background Parenchyma (Experiment 1)

Group	Chemical	Effective no. of rats	Areas ( $\mu\text{m}^2/\mu\text{m}^2$ )		Roundness	
			In foci	In background	In foci	In background
1	Coumaperine	15	0.17±0.04 <sup>a)</sup>	0.10±0.01 <sup>b)</sup>	0.87±0.04	0.92±0.04 <sup>c)</sup>
4	Ethanol (60%)	12	0.18±0.02	0.08±0.02 <sup>d)</sup>	0.86±0.05	0.93±0.03 <sup>d)</sup>

a) Mean±SD.

b) Significantly different from the focus value (group 1) at  $P<0.001$  (Tukey type multiple comparison test).

c) Significantly different from the focus value (group 1) at  $P<0.05$  (Tukey type multiple comparison test).

d) Significantly different from the focus value (group 4) at  $P<0.001$  (Tukey type multiple comparison test).

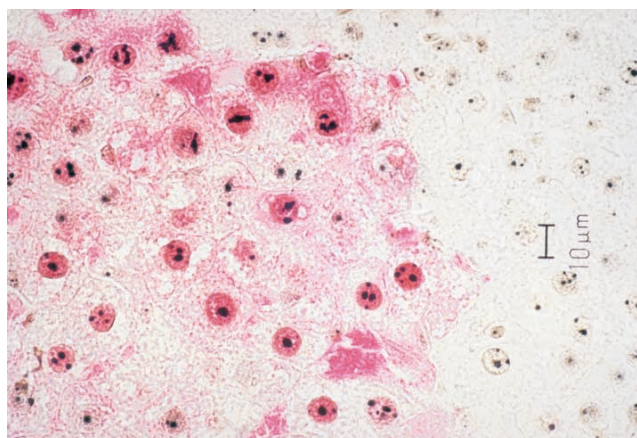


Fig. 3. Double staining of GST-P and AgNORs in rat liver (experiment 1). Large and bizarre dots in the nucleus are evident inside a GST-P-positive focus. Small and rounded dots are apparent outside the GST-P-positive focus.

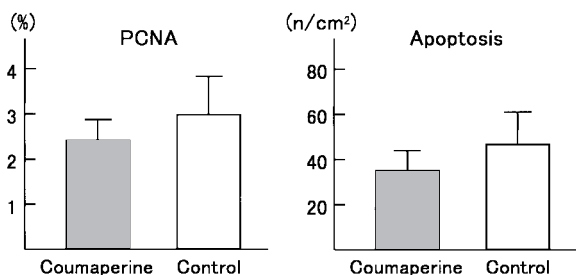


Fig. 4. Data for immunohistochemical staining of PCNA and *in situ* analysis of apoptosis (experiment 2). Both tended to be decreased with coumapherine.

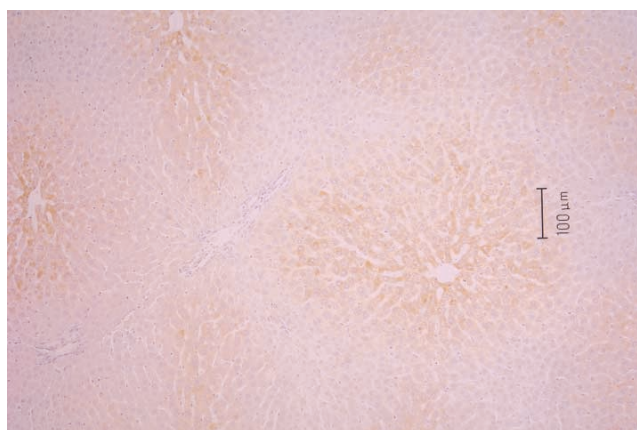


Fig. 5. Immunohistochemical staining for CYP2E1 in the liver of a rat given coumapherine (experiment 2). Hepatocytes in the centrilobular zone were positive. However, no difference in staining intensity was observed between the coumapherine-treated and non-treated groups.

reports about modification of hepatocarcinogenesis by ethanol.<sup>25)</sup> However, no significant difference was observed in the GST-P data between the ethanol vehicle control group and saline control group. We therefore considered the observed effect to be solely due to coumapherine.

The number and area of AgNORs are related not only to cell proliferation, but also to cell cycle phase,<sup>26)</sup> and have been used to estimate the chemopreventive effects of several candidate drugs.<sup>27, 28)</sup> The AgNORs area and shape rather than number reflect the cell proliferation,<sup>22, 23, 29)</sup> but no modification of AgNORs by coumapherine was seen in this experiment. To investigate the effects of coumapherine on cell proliferation, we examined PCNA at an early stage (8 h after DEN treatment) and AgNORs at a late stage (5 weeks after DEN administration). Coumapherine tended to inhibit cell proliferation in the early stage immediately after coumapherine administration but no statistically significant effect was evident. In the late stage, the effects of partial hepatectomy or 2-AAF might have masked any modification of cell proliferation by coumapherine. We believe that coumapherine inhibits cell proliferation at an early stage, right after DNA damage.

In order to cast light on the inhibitory effects on liver cell proliferation by coumapherine in the early stage of chemical hepatocarcinogenesis, expression of CYP2E1 protein in the liver tissue was examined, since it is known that DEN is oxidized to genotoxic products by rat liver microsomes with a major role for this enzyme<sup>30)</sup> CYP2E1 is present in normal rat hepatocytes, also contributing to the metabolism of lipids and ketone bodies.<sup>31, 32)</sup> We thought that coumapherine might modulate the activity of normal CYP2E1, but no clear change was apparent with the present immunohistochemical procedure. We then examined the expression of *c-jun* and *c-myc* right after DEN and coumapherine treatment, because a previous study had suggested that early response genes may be related to chemopreventive effects on hepatocarcinogenesis.<sup>1)</sup> However, no expression of these proteins was observed in the early stage. Previous reports indicated that expression of *c-jun*<sup>18)</sup> and *c-myc*<sup>33)</sup> is elevated in liver tumors and some foci. However, we examined *c-jun* and *c-myc* only in experiment 2 (8 h after DEN administration), when no foci or tumors were present. The lack of any increase in apoptosis might suggest no increase in DEN toxicity, or may result from inhibition of cell proliferation by coumapherine. Thus the mechanism remains to be clarified.

Antioxidative effects are not always related to inhibition of carcinogenesis because they may markedly differ from organ to organ.<sup>7)</sup> Whether oxidative stress is involved might be determined with a marker such as 8-hydroxydeoxyguanosine.<sup>34)</sup> Suzuki *et al.*<sup>35)</sup> have shown that GST-P mRNA is over-expressed on a whole-liver basis as a part of the immediate detoxifying reaction within 5 h after

DEN administration, reaching a peak level at 12 h. Inhibitory action could be related to induction of phase II enzymes in the initiation stage of carcinogenesis.<sup>36, 37)</sup>

In conclusion, coumaperine may protect against liver carcinogenesis in the initiation stage, and this action may be related to inhibition of cell proliferation.

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