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# Inhibitory Effect of Cryptoporic Acid E, a Product from Fungus *Cryptoporus volvatus*, on Colon Carcinogenesis Induced with N-Methyl-N-Nitrosourea in Rats and with 1,2-Dimethylhydrazine in Mice

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**The antitumorigenic effect of cryptoporic acid E (CPA-E), a dimeric drimane sesquiterpenoid isolated from the fungus *Cryptoporus volvatus*, on colon carcinogenesis was investigated. Female F344 rats given an intrarectal instillation of 2 mg of N-methyl-N-nitrosourea 3 times weekly in weeks 1 and 2 were fed diet containing 0.2% CPA-E from week 3. Femal ICR mice given 15 weekly intraperitoneal injections of 10 mg of 1,2-dimethylhydrazine/kg body weight during weeks 1 to 15 were fed diet containing 0.06% CPA-E from week 1. The experiment was terminated at week 35 for rats and at week 25 for mice. The incidence and the number of tumors per animal were reduced in CPA-E-fed animals compared to the controls: 31% vs. 75% ($P<0.05$) and $0.4\pm0.2$ (SEM) vs. $0.9\pm0.2$ ($0.1>P>0.05$) in rats, and 31% vs. 63% ($0.1>P>0.05$) and $0.4\pm0.2$ vs. $2.4\pm0.8$ ($P<0.05$) in mice (16 animals in each group). Intrarectal deoxycholic acid-induced colonic mucosal ornithine decarboxylase activity was significantly lowered in CPA-E-fed animals compared to controls. This shows an antipromoting activity of CPA-E against colon carcinogenesis. Thus, it was concluded that CPA-E inhibits colon cancer development in both rats and mice treated with 2 different colon carcinogens.**

Key words: Colon carcinogenesis — Cancer chemoprevention — Cryptoporic acid E — Ornithine decarboxylase

Cryptoporic acid E (CPA-E, Fig. 1), a dimeric drimane sesquiterpenoid isolated from the fungus *Cryptoporus volvatus* by Asakawa and coworkers,[1] was reported to have a strong inhibitory activity on the release of superoxide anion radicals from guinea pig peritoneal macrophages.[2] Superoxide radicals are known to be associated with tumor promotion. Recently, it was demonstrated by Matsunaga and coworkers that CPA-E has a potent antipromoting effect on mouse skin carcinogenesis.[3] After an initiation dose of 7,12-dimethylbenz-[*a*]anthracene on the skin, topical application of CPA-E significantly inhibited the enhancement of tumor development by the skin tumor promoter, okadaic acid. On the other hand, CPA-E administered orally to mice was found in the colon as well as the serum, and a large amount (90%) of the dose was excreted in the feces (unpublished data). Thus, an antitumorigenic effect of this agent in the colon mucosa, similar to that on mouse skin, was expected. We found that CPA-E in the diet inhibited colon cancer development induced with 2 chemical carcinogens, N-methyl-N-nitrosourea (MNU) and 1,2-dimethylhydrazine (DMH), in rats and mice. Also, the induction by a potent tumor promoter, deoxycholic acid (DCA), of colonic mucosal ornithine decarboxylase (ODC), the activity of which is a useful biological parameter of tumor promotion, was observed to be reduced in both rats and mice fed CPA-E.

## MATERIALS AND METHODS

**Animals** Female F344/NSlc rats and female ICR mice (Shizuoka Laboratory Animal Center, Hamamatsu), 7 weeks of age at the start of the experiment, were used. They were housed in a plastic cage with sterilized wood-chip bedding in a specific-pathogen-free animal room under constant conditions with a 12-h light-dark cycle, a temperature of $22\pm1$°C, a relative humidity of $50\pm10$%, and free access to drinking water and diet.

**Chemicals and diets** MNU, DMH dihydrochloride, and DCA sodium salt were purchased from Nakarai Chemicals Co., Kyoto. CPA-E was extracted and purified by Asakawa, one of the authors, following the reported procedure.[1] Experimental chows containing 0.2% and 0.06% (w/w) CPA-E in CE-2 chow, a standard pelletted laboratory diet, were prepared at CLEA Japan Inc., Tokyo, and stored in a room at 4°C. It was confirmed by chemical analysis that CPA-E is stable during the preparation and storage of the chow.

**Animal treatment** Two groups of 16 rats each received an intrarectal instillation of 0.5 ml of 0.4% MNU aqueous solution 3 times a week in weeks 1 and 2, using a metal feeding tube inserted into the large bowel lumen through the anal orifice as described previously.[4] They were fed the CE-2 chow in the control group or the 0.2% CPA-E chow from week 3 in the CPA-E group. Two

groups of 16 mice each received 15 weekly intraperitoneal injections of 10 mg of DMH/kg body weight dissolved in 0.9% NaCl solution during weeks 1 to 15. They were fed the CE-2 chow in the control group or the 0.06% CPA-E chow from week 1 in the CPA-E group. The experiments with rats and mice were commenced at the same time. The body weights and the amount of diet consumed were measured once a week. The experiment was terminated at week 35 for rats and at week 25 for mice, when all the animals were killed. At autopsy, the large bowel was cut open along its length and carefully inspected grossly. All the tumors and grossly abnormal organs were histologically examined after standard processing, including sectioning and staining with hematoxylin and eosin.

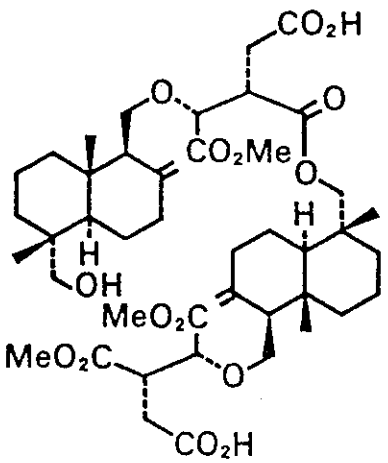


Fig. 1. Structure of cryptoporic acid E.

**Assay of colonic mucosal ODC activity** The rats and mice were fed 0.2% and 0.06% CPA-E chows, respectively, for 2 weeks. Then, they received an intrarectal instillation of 0.5 ml or 0.1 ml of 24 m*M* DCA solution in 0.9% NaCl solution. Control animals had CE-2 chow and received an intrarectal dose of DCA. Four h after the intrarectal dose, 8 animals in each group were killed by cervical dislocation. The large bowel was excised, cut open lengthwise and rinsed with cold 0.9% NaCl solution. The mucosa of the distal large bowel, which had been exposed to the instilled solution, was scraped with a blunt steel plate, then immediately frozen and stored at −80°C. One hundred mg of the mucosa was homoge-

Table I. Body Weight, and Consumed Amounts of Diet and CPA-E in F344 Rats and ICR Mice

| Treatment groups[a)] | W1 | W5 | W10 | W15 | W20 | W25 | W30 | W35 |
|---|---|---|---|---|---|---|---|---|
| | | | | Bodyweight (g/animal) | | | | |
| Rats | | | | | | | | |
| Control | 139±4[b)] | 173±3 | 191±3 | 200±2 | 211±3 | 211±3 | 216±5 | 230±3 |
| CPA-E | 138±1 | 171±3 | 188±2 | 199±2 | 209±2 | 211±3 | 219±2 | 234±4 |
| Mice | | | | | | | | |
| Control | 33±1 | 35±1 | 37±1 | 38±1 | 40±1 | 43±1 | | |
| CPA-E | 33±1 | 35±3 | 39±2 | 40±1 | 42±2 | 45±2 | | |
| | | | | Diet (g/animal/day) | | | | |
| Rats | | | | | | | | |
| Control | 15±1.7 | 11±0.2 | 10±0.7 | 10±0.3 | 10±0.7 | 10±1.1 | 10±1.0 | 11±0.6 |
| CPA-E | 15±1.8 | 12±0.6 | 10±0.9 | 10±0.4 | 10±0.5 | 10±0.9 | 10±1.2 | 10±0.7 |
| Mice | | | | | | | | |
| Control | 4.5±0.1 | 4.7±0.1 | 4.7±0.1 | 4.8±0.1 | 5.2±0.1 | 5.2±0.1 | | |
| CPA-E | 4.8±0.2 | 5.5±0.3 | 5.9±0.3 | 5.7±0.4 | 6.7±0.9 | 6.8±0.3 | | |
| | | | | CPA-E (mg/kg body weight/day) | | | | |
| Rats | | | | | | | | |
| CPA-E | not fed | 145±12 | 105±11 | 102±5 | 95±5 | 94±8 | 95±10 | 99±6 |
| Mice | | | | | | | | |
| CPA-E | 98±6 | 97±5 | 96±8 | 88±5 | 103±15 | 104±15 | | |

*a*) All rats received an intrarectal dose of 2 mg of MNU 3 times a week in weeks 1 and 2, and all mice received an intraperitoneal dose of 10 mg of DMH/kg body weight once a week during weeks 1 to 15. CPA-E groups were fed 0.2% CPA-E diet from week 3 in rats and 0.06% CPA-E diet from week 1 in mice. Each group consisted of 16 animals. The experiment was terminated at week 35 in rats and at week 25 in mice.
*b*) Mean±SEM.

nized in 4 ml of 50 m*M* sodium phosphate buffer (pH 7.2) containing 0.1 m*M* EDTA, 0.1 m*M* pyridoxal phosphate and 1.0 m*M* dithiothreitol. The supernatant fraction obtained after centrifugation at 30,000*g* for 30 min at 2°C was used as the enzyme extract. The enzyme activity was determined in duplicate by measuring the release of $^{14}CO_2$ from DL-[1-$^{14}$C]ornithine hydrochloride (56 mCi/mmol, Amersham Int., Buckinghamshire, England) as a substrate, as described previously.[5] Protein content of the mucosal extract was measured by using a Bio-Rad assay kit (Bio-Rad Lab., Richmond, CA). The average value was expressed as nmol of $CO_2$ liberated in 60 min per mg protein.

**Statistical analysis** Experimental results were tested for statistical significance by using the $\chi^2$ test and Student's *t* test. Differences were considered statistically significant when the *P* value was 0.05 or less.

## RESULTS

**Body weight gain and food consumption** The body weight gain and the amount of consumed diet were the same in the control and CPA-E groups of rats, while in mice, the body weight gains in the control and CPA-E groups were comparable, but the amount of consumed diet was larger in the CPA-E group than in the control group throughout the experiment (Table I). The calculated amounts of CPA-E consumed are shown in Table I: 94–145 mg/kg body weight/day in rats, and 88–104 mg/kg body weight/day in mice.

**Colon cancer development** The incidence and the number of colon tumors at week 35 in rats and at week 25 in mice are summarized in Table II. The incidence in the CPA-E group was significantly lower than that of the control group in rats (31% vs. 75%) and insignificantly lower ($0.05 < P < 0.1$) in mice (31% vs. 63%). The mean number of tumors per animal in the CPA-E group was insignificantly ($0.05 < P < 0.1$) smaller in rats, but significantly smaller in mice than that in the control group.

The tumor-bearing animals had 1 to 3 tumors in rats and 1 to 12 tumors in mice. All the tumors were located diffusely in the distal half of the large bowel in both rats and mice, and they were plaque-shaped or polypoid. Histologically, all the tumors were well-differentiated adenocarcinomas, except for one signet-ring cell carcinoma in a CPA-E-fed rat and one squamous cell carcinoma at the anal region in a control mouse. Most of the tumors were small (1–4 mm in diameter) with minimal extension (within the mucosa or submucosa), without metastasis to lymph nodes or other organs, presumably because the experiment was terminated early at week 35 or at week 25.

The data demonstrate that orally administered CPA-E inhibited the colon cancer development in both rats and mice. Other tumors in the gastrointestinal tract and other organs were not observed, except for one mammary adenocarcinoma each in a control rat and a control mouse.

**Colonic mucosal ODC activity** The level of colonic mucosal ODC activity induced with intrarectal DCA covered a wide range, as shown in Table III. The mean levels of activity in CPA-E-fed groups of rats and mice were significantly lower than those of the respective control groups. The data indicate that orally administered CPA-E suppressed the induction of ODC in the colon mucosa in both rats and mice.

Table II. Inhibitory Effect of CPA-E on Colon Cancer Development Induced with N-Methyl-N-nitrosourea in Rats and with 1,2-Dimethylhydrazine in Mice

| Treatment groups[a] | No. of animals with colon tumors | No. of tumors per animal |
|---|---|---|
| Rats | | |
| Control | 12 (75%) | 0.9±0.2[b] |
| CPA-E | 5 (31%)[c] | 0.4±0.2 |
| Mice | | |
| Control | 10 (63%) | 2.4±0.8 |
| CPA-E | 5 (31%) | 0.4±0.2[c] |

*a*) See Table I or text.
*b*) Mean±SEM.
*c*) Significantly different from the respective controls: $P<0.05$.

Table III. Inhibitory Effect of CPA-E on Deoxycholic Acid-induced Colonic Mucosal Ornithine Decarboxylase Activity in Rats and Mice

| Treatment grops[a] | ODC activity (nmol $CO_2$/60 min/mg protein) | |
|---|---|---|
| | Mean±SEM | Range |
| Rats | | |
| Control | 0.27±0.07 | 0.02–0.56 |
| CPA-E | 0.10±0.02[b] | 0–0.22 |
| Mice | | |
| Control | 0.13±0.01 | 0.09–0.17 |
| CPA-E | 0.07±0.01[b] | 0.01–0.14 |

*a*) Rats and mice in CPA-E groups were fed 0.2% CPA-E chow and 0.06% CPA-E chow, respectively, for 2 weeks, then received an intrarectal dose of 0.5 ml or 0.1 ml of 24 m*M* deoxycholic acid solution. Four hours after the dose, animals were killed and the distal large bowel mucosa was scraped for measuring the enzyme activity. Each group consisted of 8 animals.
*b*) Significantly different from the respective controls: $P<0.01$.

## DISCUSSION

It was clearly demonstrated in the current study that CPA-E inhibits the colon cancer development induced with 2 different chemical carcinogens, MNU and DMH, in both rats and mice, as proposed on the basis of study in which topical application of this agent suppressed the promotion stage of mouse skin carcinogenesis and led to inhibition of tumor development.[3] Oral CPA-E yielded a 50% or more reduction in incidence and number of colon tumors. In rats, CPA-E administration was started at the week after the completion of a 2-week carcinogen treatment. Furthermore, it was confirmed in both rats and mice that short-term feeding for 2 weeks lowered the bile acid-caused ODC activity in the colon mucosa. This enzyme, a first-step and rate-limiting enzyme of polyamine synthesis, has been noted to be involved in tumor promotion by bile acids in experimental colon carcinogenesis,[6,7] as well as by such tumor promoters as 12-O-tetradecanoylphorbol-13-acetate[8,9] and okadaic acid[3] in mouse skin carcinogenesis. Also, high-level ODC induction at an early time after the administration of various kinds of promoters has been demonstrated to be an obligatory event in other organs such as the stomach,[10] liver,[11] urinary bladder[12] and mammary gland.[13] However, it can not be clearly elucidated in mice in the current study, because the mice were given DMH and CPA-E together from week 1. CPA-E has a strong inhibitory effect on the release of superoxide radicals from macrophages,[2] and these radicals are known to have a tumor-initiating activity as well as promoting and cocarcinogenic activities,[14] although the production of superoxide radicals in the colon mucosa was not examined in the current study.

Although the mechanisms of the antipromoting activity of CPA-E have not been elucidated, this agent might inhibit the process of tumor promotion after the binding of the promoter to protein phosphatases in the mouse skin.[3] CPA-E in the diet might directly act on the colon mucosa (it was effective when painted on the skin in a mouse skin carcinogenesis experiment[3]), since a large amount of ingested CPA-E was excreted in the feces and a small amount was found in the blood in mice.

It has been demonstrated in animal models that the promotion phase of colon carcinogenesis is inhibited and the colon cancer development is reduced by various chemical substances, including prostaglandin synthesis inhibitors such as indomethacin,[15] an irreversible ODC inhibitor 2-difluoromethylornithine[16] and a simple monohydroxycembratetraene, sarcophytol A.[17] Importantly, CPA-E used in the current study is less toxic as judged from macroscopic and microscopic observations of animals, and all the animals tolerated well the long-term feeding of this agent at relatively high dosages of 90 to 150 mg/kg body weight/day (approximately the same amount for rats and mice). Thus, CPA-E might be a candidate chemopreventive agent against colon cancer development. It has been indicated that a high fat diet correlates with an increased risk of colon cancer in humans and enhances carcinogen-induced colon cancer development in animal models.[18] In the current study, animals were fed a standard laboratory chow with low fat (5%). Thus, further investigation is needed into the antitumorigenic activity of CPA-E in animal models fed with high-fat diets, as well as its activity in other organs such as the mammary gland, and the mechanisms of its activity.

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## REFERENCES

1) Asakawa, Y., Hashimoto, T., Mizuno, Y., Tori, M. and Fukazawa, Y. Cryptoporic acids A-G, drimane-type sesquiterpenoid esters of isocitric acid from the fungus *Cryptoporus volvatus*. *Phytochemistry*, **31**, 579–592 (1992).
2) Hashimoto, T., Tori, M., Mizuno, Y., Asakawa, Y. and Fukazawa, Y. The superoxide release inhibitors, cryptoporic acids C, D, and E; dimeric drimane sesquiterpenoid esters of isocitric acid from the fungus *Cryptoporus volvatus*. *J. Chem. Soc. Chem. Commun.*, 258–259 (1989).
3) Matsunaga, S., Furuya-Suguri, H., Nishiwaki, S., Yoshizawa, S., Suganuma, M., Hashimoto, T., Asakawa, Y. and Fujiki, H. Differential effects of cryptoporic acids D and E, inhibitors of superoxide anion radical release, on tumor promotion of okadaic acid in mouse skin. *Carcinogenesis*, **12**, 1129–1131 (1991).
4) Narisawa, T., Sato, T., Hayakawa, M., Sakuma, A. and Nakano, H. Carcinoma of the colon and rectum of rats by rectal infusion of N-methyl-N′-nitro-N-nitrosoguanidine. *Gann*, **62**, 231–234 (1971).
5) Chida, K. and Kuroki, T. Inhibition of DNA synthesis and sugar uptake and lack of induction of ornithine decarboxylase in human epidermal cells treated with mouse skin tumor promoters. *Cancer Res.*, **44**, 3226–3230 (1984).
6) Takano, S., Matsushima, M., Ertürk, E. and Bryan, G. T.

Early induction of rat colonic ornithine and S-adenosyl-L-methionine decarboxylase activities by N-methyl-N′-nitro-N-nitrosoguanidine and bile salts. *Cancer Res.*, **41**, 624–628 (1981).
7) Marisawa, T., Takahashi, M., Niwa, M., Fukaura, Y. and Wakizaka, A. Involvement of prostaglandin $E_2$ in bile acid-caused promotion of colon carcinogenesis and anti-promotion by cycooxygenase inhibitor indomethacin. *Jpn. J. Cancer Res.*, **78**, 791–798 (1987).
8) O'Brien, T. G. The induction of ornithine decarboxylase as an early, possibly obligatory, event in mouse skin. *Cancer Res.*, **36**, 2644–2653 (1976).
9) Verma, A. K., Ashendel, C. L. and Boutwell, R. K. Inhibition by prostaglandin synthesis inhibitors of the induction of epidermal ornithine decarboxylase activity, the accumulation of prostaglandins and tumor promotion caused by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Res.*, **40**, 308–315 (1980).
10) Furihata, C., Yamakoshi, A., Takezawa, R. and Matsushima, T. Various sodium salts, potassium salts, a calcium salt and an ammonium salt induced ornithine decarboxylase and stimulated DNA synthesis in rat stomach mucosa. *Jpn. J. Cancer Res.*, **80**, 424–429 (1989).
11) van Rooijen, L. A. A., Uijtewaal, B. and Bisshop, A. Inhibition by indomethacin and 5,8,11,14-eicosatetraynoic acid of the induction of rat hepatic ornithine decarboxylase by the tumor promoters phenobarbital and 12-O-tetradecanoylphorbol-13-acetate *in vivo*. *Carcinogenesis*, **8**, 191–192 (1987).
12) Matsushima, M., Takano, S., Ertürk, E. and Bryan, G. T. Induction of ornithine decarboxylase activity in mouse urinary bladder by L-tryptophan and some of its metabolites. *Cancer Res.*, **42**, 3587–3591 (1982).
13) Abou-El-Ela, S. H., Prasse, K. W., Farrell, R. L., Carroll, R. W., Wade, A. E. and Bunce, O. R. Effects of D,L-2-difluoromethylornithine and indomethacin on mammary tumor promotion in rats fed high n-3 and/or n-6 fat diets. *Cancer Res.*, **49**, 1434–1440 (1989).
14) Totter, J. R. Spontaneous cancer and its relationship to oxygen metabolism. *Proc. Natl. Acad. Sci. USA*, **77**, 1763–1767 (1980).
15) Narisawa, T., Sato, M., Tani, M., Kudo, T., Takahashi, T. and Goto, A. Inhibition of development of methylnitrosourea-induced rat colon tumors by indomethacin treatment. *Cancer Res.*, **41**, 1954–1957 (1981).
16) Kingsnorth, A. N., King, W. W. K., Diekma, A. K., McCann, P. P., Ross, J. S. and Malt, R. A. Inhibition of ornithine decarboxylase with 2-difluoromethylornithine: reduced incidence of dimethylhydrazine-induced colon tumors in mice. *Cancer Res.*, **43**, 2545–2549 (1983).
17) Narisawa, T., Takahashi, M., Niwa, M., Fukaura, Y. and Fujiki, H. Inhibition of methylnitrosourea-induced large bowel cancer development in rats by sarcophytol A, a product from a marine soft coral *Sarcophyton glaucum*. *Cancer Res.*, **49**, 3287–3289 (1989).
18) Weisburger, J. H. Causes, relevant mechanisms, and prevention of large bowel cancer. *Semin. Oncol.*, **18**, 316–336 (1991).