Inhibitory Effects of 1′-Acetoxychavicol Acetate on *N*-Nitrosobis(2-oxopropyl)-amine-induced Initiation of Cholangiocarcinogenesis in Syrian Hamsters

Makoto Miyauchi,[1] Akiyoshi Nishikawa,[1, 4] Fumio Furukawa,[1] Hideaki Nakamura,[1] Hwa-Young Son,[1] Akira Murakami,[2] Koichi Koshimizu,[2] Hajime Ohigashi[3] and Masao Hirose[1]

[1]Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, [2]Department of Biotechnological Science, Faculty of Biology-Oriented Science and Technology, Kinki University, Iwade-Uchita-cho, Naka-gun, Wakayama 649-6493 and [3]Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502

The influence of 1′-acetoxychavicol acetate (ACA) during the initiation stage was investigated in the *N*-nitrosobis(2-oxopropyl)amine (BOP)-initiated hamster tumorigenesis model. Ninety male 5-week-old hamsters were divided into three groups, each consisting of 30 animals, and s.c. injected with 20 mg/kg of BOP twice with a one-week interval. Groups 1 through 3 were fed diet supplemented with ACA at concentrations of 500, 100 and 0 ppm, respectively, for 3 weeks starting one week before the first carcinogen application. At the termination of experimental week 54, the total incidence and multiplicity of cholangiocellular adenomas and carcinomas in group 1 (17.9% and 0.3 ± 0.9) were significantly ($P < 0.05$ and $P < 0.01$) decreased as compared to the group 3 values (50.0% and 0.7 ± 0.8). The ACA treatments also showed a tendency to reduce the development of preneoplastic lesions in the pancreas, a main target organ of BOP, although this was not statistically significant. Our results thus indicate that ACA exerts an inhibitory effect on BOP-induced cholangiocarcinogenesis in hamsters. Taken together with previous findings of inhibited colon, oral and skin carcinogenesis in rats and mice, they suggest that ACA is a candidate chemopreventive agent with a wide spectrum of activity.

Key words: 1′-Acetoxychavicol acetate — *N*-Nitrosobis(2-oxopropyl)amine — Cholangiocarcinogenesis — Hamster

1′-Acetoxychavicol acetate (ACA), extracted from rhizomes of *Languas galanga* (Zingiberaceae), is used as a ginger substitute and as a stomach medicine in Thailand and other countries of south-east Asia.[1] ACA has been extensively investigated for chemopreventive action in rodent carcinogenesis models.[2–6] It was shown to inhibit 12-*O*-tetradecanoylphorbol-13-acetate promotion of skin carcinogenesis in female ICR mice initiated with 7,12-dimethylbenz[*a*]anthracene,[2] 4-nitroquinoline-1-oxide (4-NQO)-induced oral tumor development[3] and azoxymethane (AOM)-induced colon carcinogenesis during both initiation and post-initiation phases,[4, 5] as well as choline-deficient, ʟ-amino acid-defined diet-associated induction of glutathione *S*-transferase-positive liver lesions in male F344 rats.[6] In addition, ACA was demonstrated to inhibit xanthine oxidase, which generates superoxide anions known to be associated with tumor promotion,[7] and also to inhibit tumor promoter-induced Epstein-Barr virus activation *in vitro*.[8] These effects could contribute to chemopreventive activity.

N-Nitrosobis(2-oxopropyl)amine (BOP) is well known to induce lung, pancreatic, liver and kidney tumors in hamsters.[9, 10] It is considered to be particularly advantageous for assessing the modified effects of chemicals on pancreatic and lung carcinogenicity because of the histological and biological similarities of the induced lesions to those observed in man.[11] In the present study, the effects of ACA in the initiation phase were investigated in a BOP carcinogenesis model in hamsters.

MATERIALS AND METHODS

Animals and chemicals A total of 90 male Syrian hamsters (Japan SLC Inc., Shizuoka), 5 weeks old and weighing approximately 80 g at the commencement, were used in this experiment. The animals were housed, five per polycarbonate cage and maintained under standard laboratory conditions: room temperature, $23 \pm 2°C$; relative humidity, $60 \pm 5\%$; a 12h/12h light/dark cycle. The Oriental MF (Oriental Yeast Co., Ltd., Tokyo) basal diet was available *ad libitum*. BOP was obtained from Nakalai Tesque (Kyoto) and ACA (>95% purity) was synthesized according to the method described previously.[12]

[4]To whom correspondence should be addressed.
  E-mail: nishikaw@nihs.go.jp

Experimental procedure Hamsters were divided into three groups, each consisting of 30 males. Animals in groups 1 through 3 were s.c. injected with 20 mg/kg of BOP twice with a one-week interval. They were fed diet supplemented with ACA at concentrations of 500, 100 and 0 ppm, respectively, for 3 weeks starting one week before the initial carcinogen treatment. They were observed daily and weighed weekly during the administration period, and monthly thereafter. At the end of week 54, all surviving animals were killed under ether anesthesia. Moribund animals or those that died during the study were also completely autopsied for histological examination. At autopsy, the main target organs for BOP-induced tumorigenicity, including the pancreas, lung, liver and kidney were carefully examined macroscopically, and then fixed in 10% phosphate-buffered formalin. Four anatomical parts of the pancreas (gastric, splenic and duodenal lobes and head portion) from each animal were sectioned for investigating the multiplicity of dysplasias, as routinely performed in our laboratory.[13] These organs were processed for histological observation by conventional methods, and stained with hematoxylin and eosin (H-E). All proliferative lesions were diagnosed histopathologically and counted in representative sections.

Statistical analysis The tumor incidences were analyzed by the use of Fisher's exact probability test. Variance of data for lesion multiplicities, body weights and organ weights was checked for homogeneity by Bartlett's procedure. If the variance was homogeneous, the data were assessed by one-way analysis of variance (ANOVA). If not, the Kruskal-Wallis test was used. When statistically significant differences were indicated, the unpaired Student's t test or the Mann-Whitney U test was used for comparison of the appropriate pairs of groups. The survival rates were analyzed by the Kaplan-Meier method and evaluated with the log rank test.

RESULTS

Several animals in each group died before the termination at week 54, but there were no significant differences in survival rate among the groups. Except for a few hamsters which were found dead in the early experimental stages, moribund or dead animals were included in the effective numbers. The final body and relative organ weights of hamsters surviving until the termination are summarized in Table I. Final body weights in group 1 were slightly (9.3%) decreased as compared to the group 3 values; this was statistically significant ($P<0.01$). Relative weights of the lungs, liver and kidneys did not significantly vary.

Histopathologically, liver tumors were diagnosed as hepatocellular and cholangiocellular adenomas and carcinomas. As shown in Table II, the total incidence of cholangiocellular adenomas and carcinomas in group 1 (17.9%) was significantly ($P<0.05$) decreased as compared to the group 3 value (50.0%). Similarly, the total multiplicity was significantly ($P<0.01$) decreased (0.3 ± 0.9 as compared to 0.7 ± 0.8). The incidences and multiplicities of hepatocellular adenomas and carcinomas did not significantly differ among the groups.

Cancerous and precancerous ductal lesions observed in the exocrine pancreas were histologically classified as adenocarcinomas and dysplasias as reported previously.[13] Incidence and multiplicity data are summarized in Table III. Although no significant variation was noted, ACA showed a tendency for inhibition of dysplastic lesions. In tumor-bearing animals, the pattern of lesion distribution was comparable among the three BOP-treated groups (data not shown).

Lung tumors were histologically classified into adenomas and adenocarcinomas. The incidences of pulmonary adenomas were 88.5, 77.8 and 87.1%, and those of adenocarcinomas were 7.7, 18.5 and 12.9% in groups 1–3. Therefore, there were no significant intergroup differences. Renal cell carcinomas were observed only in 2 of 28 hamsters in group 1 and 1 of 27 in group 2.

DISCUSSION

The present study indicates that ACA may inhibit initiation of cholangiocarcinogenesis in hamsters by BOP. Final body weights were decreased by dietary administration of ACA at a high dose, but the decrease was less than 10%,

Table I. Body and Relative Organ Weights of Hamsters Surviving until 54 Weeks

Group	No. of animals	BW	Lung	Liver	Kidney
1. BOP+ACA (H)	21	146 ± 17[a],*	0.66 ± 0.11	5.56 ± 1.71	0.79 ± 0.10
2. BOP+ACA (L)	18	162 ± 18	0.59 ± 0.13	6.80 ± 2.19	0.81 ± 0.10
3. BOP	23	161 ± 15	0.60 ± 0.06	6.28 ± 1.50	0.77 ± 0.08

ACA (H): 500 ppm ACA in diet. ACA (L): 100 ppm ACA in diet.
a) Mean±SD.
∗ $P<0.01$ versus BOP alone group.

Table II. Incidences and Multiplicities of Liver Tumors

Tumors	BOP+ACA (H) (28)[a]		BOP+ACA (L) (27)		BOP (30)	
	%	No./animal[b]	%	No./animal	%	No./animal
Cholangiocellular adenoma	14.3	0.3±0.8	22.2	0.3±0.6	33.3	0.4±0.6
Cholangiocellular carcinoma	7.1	0.1±0.3	11.1	0.2±0.6	20.0	0.3±0.6
Total	17.9*	0.3±0.9**	29.6	0.5±0.9	50.0	0.7±0.8
Hepatocellular adenoma	60.7	0.9±1.0	66.7	1.2±1.2	63.3	1.4±1.5
Hepatocellular carcinoma	17.9	0.2±0.4	22.2	0.2±0.4	13.3	0.1±0.4
Total	60.7	1.1±1.2	70.4	1.4±1.3	66.7	1.5±1.6

ACA (H): 500 ppm ACA in diet. ACA (L): 100 ppm ACA in diet.
a) No. of animals.
b) Mean±SD.
∗,∗∗ $P<0.05$, 0.01 versus BOP alone group, respectively.

Table III. Incidences and Multiplicities of Pancreatic Preneoplastic and Neoplastic Lesions

Lesions	BOP+ACA (H) (28)[a]		BOP+ACA (L) (27)		BOP (30)	
	%	No./animal[b]	%	No./animal	%	No./animal
Dysplastic lesion	32.1	0.4±0.6	25.9	0.3±0.5	46.7	0.6±0.9
Adenocarcinoma	39.2	0.5±0.8	55.6	0.6±0.6	40.0	0.7±1.0
Total	60.7	0.9±1.0	74.1	0.9±0.7	60.0	1.3±1.6

ACA (H): 500 ppm ACA in diet. ACA (L): 100 ppm ACA in diet.
a) No. of animals.
b) Mean±SD.

and the mortality and the relative weights of major organs including the liver, kidney, and lung were comparable among the groups, suggesting that the toxicity of ACA was marginal, if present. The mechanisms underlying the chemopreventive effects of ACA against AOM-induced colon carcinogenesis and 4-NQO-induced oral carcinogenesis in rats have been suggested to be inhibition of cell proliferation as well as induction of phase II enzymes.[3, 4] Similar mechanisms could be responsible for inhibitory effects against cholangiocarcinogenesis although this remains to be elucidated in hamsters.

The Syrian hamster is well known to have a greater tendency for bile duct epithelial cells to proliferate than other species.[14] Such proliferation is considered to be a reactive response to severe hepatic cell damage and necrosis.[15, 16] Additional action of carcinogens could promote the development of cholangiofibrosis, cholangiofibroma or cholangiocarcinoma,[17] and it is conceivable that ACA exerts protective effects against carcinogen insult. In hamsters, more than half of the dosed BOP is exhaled as CO_2, and administered BOP is excreted in the urine as *N*-nitroso(2-hydroxypropyl)(2-oxopropyl)amine (HPOP) and *N*-nitroso-(2-hydroxypropyl)amine (BHP).[18–20] Urinary metabolites of HPOP in hamsters are BHP, BHP glucuronide, HPOP, HPOP glucuronide, and HPOP sulfate ester.[21] ACA could reduce the hepatotoxicity of BOP through induction of phase II enzymes, such as UDP-glucuronosyltransferases or sulfotransferases, or other drug-metabolizing enzymes which metabolize BOP. The fact that effects of ACA on pancreatic carcinogenesis were weak or lacking in the present study might be accounted for, at least in part, by differences in tissue distribution of ACA.

It has been demonstrated that a single administration of BOP to hamsters increases 8-hydroxydeoxyguanosine, a biomarker of DNA oxidative damage, in pancreatic nuclear DNA.[22] ACA has been shown to reduce the level of 8-hydroxyguanine in the liver caused by dietary choline deficiency.[6] In addition, it is reported to suppress nitric oxide production in a murine macrophage cell line stimulated with lipopolysaccharide or interferon-γ.[23] Since reactive oxygen or nitrogen species are believed to contribute to carcinogenic processes,[24–27] it is likely that ACA could exert an inhibitory effect partly through inhibition of superoxide generation.[7]

In conclusion, the present study showed that ACA inhibits BOP-induced initiation of cholangiocarcinogenesis. Taken together with previous findings that ACA inhibited colon, oral and skin carcinogenesis in rats and mice, it appears that ACA is a good candidate for a wide-spectrum chemopreventive agent.

REFERENCES

1) Mitsui, S., Kobayashi, S., Nagahori, H. and Ogiso, A. Constituents from seeds of *Alpinia galanga* wild, and their anti-ulcer activities. *Chem. Pharm. Bull.*, **24**, 2377–2382 (1976).

2) Murakami, A., Ohura, S., Nakamura, Y., Koshimizu, K. and Ohigashi, H. 1′-Acetoxychavicol acetate, a superoxide anion generation inhibitor, potently inhibits tumor promotion by 12-*O*-tetradecanoylphorbol-13-acetate in ICR mouse skin. *Oncology*, **53**, 386–391 (1996).

3) Ohnishi, M., Tanaka, T., Makita, H., Kawamori, T., Mori, H., Satoh, K., Hara, A., Murakami, A., Ohigashi, H. and Koshimizu, K. Chemopreventive effect of a xanthine oxidase inhibitor, 1′-acetoxychavicol acetate, on rat oral carcinogenesis. *Jpn. J. Cancer Res.*, **87**, 349–356 (1996).

4) Tanaka, T., Makita, H., Kawamori, T., Kawabata, K., Mori, H., Murakami, A., Satoh, K., Hara, A., Ohigashi, H. and Koshimizu, K. A xanthine oxidase inhibitor 1′-acetoxychavicol acetate inhibits azoxymethane-induced colonic aberrant crypt foci in rats. *Carcinogenesis*, **18**, 1113–1118 (1997).

5) Tanaka, T., Kawabata, K., Kakumoto, M., Makita, H., Matsunaga, K., Mori, H., Satoh, K., Hara, A., Murakami, A., Koshimizu, K. and Ohigashi, H. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by a xanthine oxidase inhibitor, 1′-acetoxychavicol acetate. *Jpn. J. Cancer Res.*, **88**, 821–830 (1997).

6) Kobayashi, Y., Nakae, D., Akai, H., Kishida, H., Okajima, E., Kitayama, W., Denda, A., Tsujiuchi, T., Murakami, A., Koshimizu, K., Ohigashi, H. and Konishi, Y. Prevention by 1′-acetoxychavicol acetate of the induction but not growth of putative preneoplastic, glutathione *S*-transferase placental form-positive, focal lesions in the livers of rats fed a choline-deficient, L-amino acid-defined diet. *Carcinogenesis*, **19**, 1809–1814 (1998).

7) Noro, T., Sekiya, T., Katoh, M., Oda, Y., Miyase, T., Kuroyanagi, M., Ueno, A. and Fukushima, S. Inhibitors of xanthine oxidase from *Alpina galanga*. *Chem. Pharm. Bull.*, **36**, 244–248 (1988).

8) McCord, J. M. and Fridovich, I. The reduction of cytochrome *c* by milk xanthine oxidase, *J. Biol. Chem.*, **243**, 5753–5760 (1968).

9) Nishikawa, A., Furukawa, F., Kasahara, K., Tanakamaru, Z., Miyauchi, M., Nakamura, H., Ikeda, T., Imazawa, T. and Hirose, M. Failure of phenethyl isothiocyanate to inhibit hamster tumorigenesis induced by *N*-nitrosobis(2-oxopropyl)amine when given during the post-initiation phase. *Cancer Lett.*, **141**, 109–115 (1999).

10) Furukawa, F., Nishikawa, A., Imazawa, T., Kasahara, K. and Takahashi, M. Enhancing effects of quinacrine on development of hepatopancreatic lesions in *N*-nitrosobis(2-oxopropyl)amine-initiated hamsters. *Jpn. J. Cancer Res.*, **89**, 131–136 (1998).

11) Furukawa, F., Nishikawa, A., Imaida, K., Mitsui, M., Enami, T., Hayashi, Y. and Takahashi, M. Inhibitory effects of crude soybean trypsin inhibitor on pancreatic ductal carcinogenesis in hamsters after initiation with *N*-nitrosobis(2-oxopropyl)amine. *Carcinogenesis*, **13**, 2133–2135 (1992).

12) Kondo, A., Ohigashi, H., Murakami, A., Suratwadee, J. and Koshimizu, K. 1′-Acetoxychavicol acetate as a potent inhibitor of tumor promotor-induced Epstein-Barr virus activation from *Languas galanga*, a traditional Thai condiment. *Biosci. Biotechnol. Biochem.*, **57**, 1344–1345 (1993).

13) Takahashi, M., Furukawa, F., Toyoda, K., Sato, H., Hasegawa, R., Imaida, K. and Hayashi, Y. Effects of various prostaglandin synthesis inhibitors on pancreatic carcinogenesis in hamsters after initiation with *N*-nitrosobis(2-oxopropyl)amine. *Carcinogenesis*, **11**, 393–395 (1990).

14) Tomatis, L., Magee, P. N. and Shubik, P. Induction of liver tumors in the Syrian golden hamster by feeding dimethylnitrosamine. *J. Natl. Cancer Inst.*, **33**, 341–345 (1964).

15) Farber, E. Ethionine carcinogenesis. *Adv. Cancer Res.*, **7**, 383–474 (1963).

16) Scaffner, F. and Popper, H. Electron microscopic studies of normal and proliferated bile ductules. *Am. J. Pathol.*, **38**, 393–410 (1961).

17) Bannasch, P. Strain and species differences in susceptibility to liver tumor induction. *In* "Modulation of Experimental Carcinogenesis," ed. V. Turusov and R. Montesano, IARC Scientific Publications No. 51, pp. 9–38 (1983). IARC, Lyon.

18) Gingell, R., Wallcave, L., Nagel, D., Kupper, R. and Pour, P. Metabolism of the pancreatic carcinogens *N*-nitroso-bis(2-oxopropyl)amine and *N*-nitroso-bis(2-hydroxypropyl)-amine in the Syrian hamster. *J. Natl. Cancer Inst.*, **57**, 1175–1178 (1976).

19) Gingell, R., Brunk, G., Nagel, D. and Pour, P. Metabolism of three radiolabeled pancreatic carcinogenic nitrosamines in hamsters and rats. *Cancer Res.*, **39**, 4579–4583 (1979).

20) Whalley, C. E., Iqbal, Z. M. and Epstein, S. S. *In vivo* and microsomal metabolism of the pancreatic carcinogen *N*-nitrosobis(2-oxopropyl)amine by the Syrian golden hamster. *Cancer Res.*, **41**, 482–486 (1981).

21) Kokkinakis, D. M., Hollenberg, P. F. and Scarpelli, D. G. Major urinary metabolites in hamsters and rats treated with *N*-nitroso(2-hydroxypropyl)(2-oxopropyl)amine. *Cancer Res.*, **45**, 3586–3592 (1985).

22) Takabayashi, F., Harada, N., Tahara, S., Kaneko, T. and Hara, Y. Effect of green tea catechins on the amount of 8-hydroxydeoxyguanosine (8-OHdG) in pancreatic and hepatic DNA after a single administration of *N*-nitrosobis(2-oxopropyl)amine (BOP). *Pancreas*, **15**, 109–112 (1997).

23) Ohata, T., Fukuda, K., Murakami, A., Ohigashi, H., Sugimura, T. and Wakabayashi, K. Inhibition by 1′-acetoxychavicol acetate of lipopolysaccharide- and interferon-gamma-induced nitric oxide production through suppression of inducible nitric oxide synthase gene expression in RAW264 cells. *Carcinogenesis*, **19**, 1007–1012 (1998).

24) Ames, B. N. Endogenous oxidative DNA damage, aging, and cancer. *Free Radic. Res. Commun.*, **7**, 121–128 (1989).

25) Cerutti, P. Oxy-radicals and cancer. *Lancet*, **344**, 862–863 (1994).

26) Wiseman, H. and Halliwell, B. Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer. *Biochem. J.*, **313**, 17–29 (1996).

27) Kehrer, J. P. Free radicals as mediators of tissue injury and disease. *Crit. Rev. Toxicol.*, **23**, 21–48 (1993).