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Strong Inhibition of 2-Amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole-induced Mutagenesis and Hepatocarcinogenesis by 1-*O*-Hexyl-2,3,5-trimethylhydroquinone

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The effects of 3-*O*-dodecylcarbomethylascorbic acid (3-*O*-DAsA), 3-*O*-ethylascorbic acid (3-*O*-EAsA) and 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ) on 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]-imidazole (Glu-P-1)-induced mutagenesis and hepatocarcinogenesis were examined. In a *Salmonella* assay, addition of 2.5 to 20.0 mg of HTHQ to *Salmonella* TA 98 in the presence of S-9 mixture dose-dependently inhibited Glu-P-1-induced mutagenesis. The highest dose showed a 99% reduction in revertants. 3-*O*-DAsA and 3-*O*-EAsA were without effect. In an animal study using the medium-term bioassay system for the detection of hepatocarcinogens or hepatopromoters in F344 male rats, treatment with Glu-P-1 alone was associated with a significant increase in the number and area of GST-P-positive foci (47.5±8.9 and 11.1±4.7, respectively). Combined treatment with 1.0% HTHQ significantly reduced the number and area of GST-P-positive foci (to 8.1±2.1 and 0.6±0.2) while 3-*O*-DAsA exerted marginal inhibition and 3-*O*-EAsA had no effect. On the other hand, all three of these compounds slightly enhanced the numbers and areas of foci when given alone. The results indicate that HTHQ is a potent chemopreventer of Glu-P-1-induced hepatocarcinogenesis.

Key words: Chemoprevention — Hepatocarcinogenesis — Heterocyclic amine — Antioxidant — Anti-mutagenesis

During the last fifteen years several mutagenic and carcinogenic heterocyclic amines have been isolated as pyrolysis products from amino acids and proteins.[1] 2-Amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1), found in glutamic acid pyrolysates, was shown to induce malignant tumors in the liver, large intestine, small intestine and Zymbal's glands of male and female F344 rats, and in the liver and blood vessels of CDF_1 mice.[2,3] Although the carcinogenic dose is thousands of times higher than the estimated human ingestion level, these heterocyclic amines could conceivably still play a part in human environmental carcinogenesis, since small amounts of a variety of heterocyclic amines were detected in human urine and feces after ingestion of fried ground beef,[4] and DNA adducts could be formed in mouse liver treated with only one-thousandth of the carcinogenic dose for 12 weeks.[5] Therefore it is important to determine whether the potential hazard from heterocyclic amines could be eliminated by chemopreventive measures. Antioxidants are an interesting class of chemicals in this regard. Many phenolic compounds, vitamins, flavonoids, sulfur compounds and seleno compounds have been shown to inhibit carcinogen-induced mutagenesis and to block chemical carcinogenesis when applied at the initiation stage.[6,7] The novel lipophilic and water-soluble ascorbic acid derivatives 3-*O*-dodecylcarbomethylascorbic acid (3-*O*-DAsA) and 3-*O*-ethylascorbic acid (3-*O*-EAsA), respectively, and the lipophilic phenolic compound 1-*O*-hexyl-2,3,5-trimethylhydroquinone (HTHQ) all possess strong antioxidative activity.[8–11] In the present experiment, the effects of 3-*O*-DAsA, 3-*O*-EAsA and HTHQ on Glu-P-1-induced mutagenesis were studied in the Ames assay, and their influence on Glu-P-1-induced hepatocarcinogenesis was determined by using the medium-term bioassay system for the detection of hepatocarcinogens or hepatopromoters in F344 male rats.

For the anti-mutagenesis assay, the methods of Ames *et al.*[12] were basically followed, with the use of the preincubation modification of Yahagi *et al.*[13] Test components were added as follows: (i) S9 mixture (Oriental Yeast Co., Tokyo), 0.5 ml; (ii) overnight bacterial culture of strain TA 98, 0.1 ml and (iii) one of four doses of test drugs along with 0.02 or 0.005 μg of Glu-P-1, each in 0.1 ml of dimethyl sulfoxide (DMSO) (3-*O*-DAsA, HTHQ and Glu-P-1) or 0.1 *M* phosphate buffer (3-*O*-EAsA). In the case of lipophilic 3-*O*-DAsA and HTHQ, the dose of Glu-P-1 was increased to 0.02 μg because

the higher amount of DMSO (0.2 ml) decreased the number of His^+ revertants. Tubes containing reaction mixtures were incubated for 20 min in a small shaker at 37°C and 2 ml of soft agar was added to each tube. The tubes were vortexed and their contents were poured onto minimal glucose agar plates, which were allowed to stand for 30 min and then incubated for 48 h at 37°C. At the end of the incubation, the number of growing colonies of histidine revertants on each plate was counted. Each assay included a set of negative controls (only DMSO or phosphate buffer added) and positive controls (only mutagen added). The mean number of colonies from duplicate or triplicate plates was used to determine the percentage inhibition of mutagenicity by test drugs. 3-*O*-DAsA, 3-*O*-EAsA and HTHQ were obtained from Dainippon Ink and Chemicals, Inc., Tokyo. Their purity were over 99.9% by HPLC analysis. Glu-P-1 (Glu-P-1·HCl) was from the Nard Institute, Osaka. Bacterial strain TA 98 was kindly supplied by Dr. T. Nohmi of the National Institute of Hygienic Sciences, Tokyo.

For the animal study, a total of 120 6-week-old F344 male rats (Charles River Japan Inc., Atsugi) were given an intraperitoneal injection of 200 mg/kg body weight diethylnitrosamine (DEN, Tokyo Kasei Kogyo Co., Ltd., Tokyo). Two weeks after the DEN treatment, 15 animals each were maintained on powdered diet (Oriental MF, Oriental Yeast Co., Tokyo) supplemented with 0.03% Glu-P-1 alone, 0.03% Glu-P-1 plus 1.0% 3-*O*-DAsA or 1.0% 3-*O*-EAsA or 1.0% HTHQ, one of the antioxidants alone, or basal diet alone for 6 weeks. All rats were subjected to two-thirds partial hepatectomy at the end of week 3 and killed at the end of week 8. Immediately upon killing, the livers were excised and cut into 2–3 mm thick slices, three of which, one from the expanded caudate lobe and two from the right anterior lobe, were fixed in ice-cold acetone for subsequent immunohistochemical examination of glutathione *S*-transferase placental form (GST-P) expression. GST-P immunohistochemistry was performed as previously described.[14] The numbers and areas of GST-P-positive foci of more than 0.2 mm in diameter and the total areas of the liver sections examined were measured with the aid of a video image processor (VIP-21C, Olympus Co., Tokyo). Fisher's exact test and Student's *t* test were used for the statistical analysis of the data.

In the mutagenesis assay, Glu-P-1 at a dose of 0.02 μg induced 517±58 His^+ revertants/plate. Values are given after subtraction of the background levels of about 70 His^+ revertants. Addition of HTHQ dose-dependently reduced the numbers of His^+ revertants, and 99% inhibition was found at a dose of 20 μg. 3-*O*-DAsA and 3-*O*-EAsA did not exert any influence on Glu-P-1-induction of His^+ revertants at any of the doses applied in the present experiment (Table I). None of the chemicals showed any bacteriocidal effect against TA 98 at the dose applied.

In the animal study, average final body weights did not significantly differ among the Glu-P-1-treated groups. In contrast, average liver weights were significantly increased in animals treated with Glu-P-1 plus HTHQ compared with Glu-P-1 alone. Average body weights in the groups treated with antioxidant alone without Glu-P-1 were all slightly lower than the basal diet-alone group value, although the average liver weights were increased in the 3-*O*-DAsA and HTHQ-treated groups (Table II).

Table I. Anti-mutagenic Effects of Antioxidants on Glu-P-1-induced Mutagenesis

Glu-P-1 /plate (μg)	Test chemical	Test chemical /plate (μg)	His^+ rev. /plate[a]	% inhibition[b]
0.02	3-*O*-DAsA	0	517±58	
		12.5	603±39	–
		25.0	588±46	–
		50.0	537±62	–
		100.0	506±62	–
0.005	3-*O*-EAsA	0	301±15	
		12.5	314±19	–
		25.0	291±32	–
		50.0	291±18	–
		100.0	302±18	–
0.02	HTHQ	0	517±58	
		2.5	163±29[c]	68
		5.0	96±10[c]	81
		10.0	40±9[c]	92
		20.0	1±9[c]	99

a) Mean±SD of values for three plates.

b) $100 - \frac{\text{revertants/plate with Glu-P-1 plus test chemical}}{\text{revertants/plate with Glu-P-1}}$

c) Significantly different from control value at $P<0.001$.

Table II. Final Body and Liver Weights

Treatment	No. of rats	Body weight	Liver weight (g/100 g body weight)
Glu-P-1	16	262±9	3.60±0.25
Glu-P-1 +3-*O*-DAsA	12	255±11	3.66±0.22
+3-*O*-EAsA	14	255±15	3.42±0.14[b]
+HTHQ	13	264±12	3.88±0.14[a]
3-*O*-DAsA	15	265±10[d]	3.40±0.21[c]
3-*O*-EAsA	14	268±19	2.76±0.36[d]
HTHQ	13	262±15[c]	3.66±0.21[c]
Basal diet	15	278±16	2.99±0.11

Significantly different at *a*, $P<0.01$; *c*, $P<0.001$; *b*, *d*, $P<0.05$ compared with Glu-P-1 alone (*a*, *b*) or basal diet alone (*c*, *d*) group values.

Table III. Quantitative Data for GST-P-positive Liver Foci

Treatment	No. of rats	GST-P-positive foci (mean±SD)	
		No. (/cm²)	Area (mm²/cm²)
Glu-P-1	16	47.5±8.9	11.1±4.7
Glu-P-1 +3-*O*-DAsA	12	38.2±7.6[b]	7.9±3.5[c]
+3-*O*-EAsA	14	39.1±14.5	11.2±5.4
+HTHQ	13	8.1±2.1[a]	0.6±0.2[a]
3-*O*-DAsA	15	8.3±3.2[d]	1.0±0.6[d]
3-*O*-EAsA	14	6.1±2.4[e]	0.5±0.3[f]
HTHQ	13	9.5±3.0[d]	0.9±0.5[e]
Basal diet	15	3.6±1.6	0.3±0.1

Significantly different at *a, d,* $P<0.001$; *b, e,* $P<0.01$; *c, f,* $P<0.05$ compared with Glu-P-1 alone (*a, b, c*) or basal diet alone (*d, e, f*) group values.

Glu-P-1 strongly enhanced the induction of GST-P-positive foci in terms of number (47.5±8.9) and area (11.1±4.7) as compared to the basal diet group (3.6±1.6 and 0.3±0.1, respectively). Additional treatment with HTHQ markedly reduced the number (8.1±3.2, $P<0.001$) and area (0.6±0.2, $P<0.001$) of foci. 3-*O*-DAsA also reduced the Glu-P-1-associated foci development but the effect was much weaker than with HTHQ. 3-*O*-EAsA was without apparent influence. Treatment with the antioxidants alone, without Glu-P-1, in all cases slightly but significantly enhanced the development of GST-P-positive foci. The number and area values were: 3-*O*-DAsA, 8.3±3.2 and 1.0±0.6; 3-*O*-EAsA, 6.1±2.4 and 0.5±0.3; HTHQ, 9.5±3.0 and 0.9±0.5; basal diet alone group, 3.6±1.6 and 0.3±0.1 (Table III).

The present experiments thus clearly showed that combination treatment with HTHQ potently inhibits Glu-P-1-induced mutagenesis *in vitro* and Glu-P-1-induced hepatocarcinogenesis *in vivo*. The lipophilic phenolic compound HTHQ has stronger antioxidative activity than butylated hydroxytoluene, α-tocopherol or nordihydroguaiarrhetic acid as assayed in a liver microsome Fe^{3+}/ADP system.[10] Although its biological activities have not been fully established, it is likely to be an inducer of drug-metabolizing or detoxifying enzymes, like other phenolic antioxidants,[15] since it potently increases liver weight without causing apparent toxic injury. Heterocyclic amines including Glu-P-1 are metabolically converted by cytochrome P450s in the liver to hydroxyamines, and further activated by acetyltransferase or sulfotransferase, forming esters with acetic acid and sulfuric acid, respectively. These esters may be the ultimate forms producing DNA adducts.[16-19] On the other hand, heterocyclic amines or their metabolites can be detoxified through glucuronyl or sulfate conjugation and then excreted into the feces via bile.[17] The observed inhibition of Glu-P-1-induced hepatocarcinogenesis and mutagenesis by HTHQ may be due to alteration of metabolic pathways. It could inhibit hydroxylation, acetylation or sulfation of Glu-P-1, or might accelerate conjugation enzymatically, or it may directly bind to the ultimate active form of Glu-P-1. Further studies are necessary to elucidate the mechanisms underlying the observed strong inhibition. The effects of HTHQ are complex, because when it is given to rats alone without Glu-P-1 in combination, it significantly enhanced the development of GST-P-positive foci as compared to the basal diet alone group. Similarly 3-*O*-DAsA and 3-*O*-EAsA, without Glu-P-1, caused increases in both number and area of foci, suggesting that these compounds all share weak hepatopromoter character. It should be stressed, however, that in our observations, continuous dietary treatment with 1.0% HTHQ, 3-*O*-DAsA or 3-*O*-EAsA for one year did not induce any neoplastic lesions in the livers of F344 male rats (unpublished observation). 3-*O*-DAsA and 3-*O*-EAsA are novel ascorbic acid derivatives which act as scavengers of active oxygen species and free radicals, and inhibit lipid peroxidation of rat liver microsomes.[8-11] It is of interest that lipophilic 3-*O*-DAsA but not water-soluble 3-*O*-EAsA weakly inhibited Glu-P-1-induced hepatocarcinogenesis in spite of the lack of anti-mutagenic activity against *Salmonella* strain TA-98. Thus, *in vitro* anti-mutagenic activity does not necessarily correlate with *in vivo* anti-carcinogenesis, and differences in lipophilicity may partly account for the observed differences in anti-carcinogenesis of ascorbic acid derivatives. Although they may act as weak hepatopromoters, it might be expected that these strong antioxidative agents, like other lipophilic ascorbic acid derivatives,[20] could also inhibit carcinogenesis in experimental systems involving active oxygen species. Long-term experiments with these compounds using other experimental models are in progress.

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REFERENCES

1) Wakabayashi, K., Nagao, M., Esumi, H. and Sugimura, T. Food-derived mutagens and carcinogens. *Cancer Res.* (Suppl.), **52,** 2092s–2098s (1992).
2) Takayama, S., Masuda, M., Mogami, M., Ohgaki, H., Sato, S. and Sugimura, T. Induction of cancers in the intestine, liver and various other organs of rats by feeding mutagens from glutamic acid pyrolysate. *Gann,* **75,** 207–213 (1984).
3) Ohgaki, H., Matsukura, N., Morino, K., Kawachi, T., Sugimura, T. and Takayama, S. Carcinogenicity in mice of mutagenic compounds from glutamic acid and soybean globulin pyrolysates. *Carcinogenesis,* **5,** 815–819 (1984).
4) Hayatsu, H., Hayatsu, T. and Ohara, Y. Mutagenicity of human urine caused by ingestion of fried ground beef. *Jpn. J. Cancer Res,* **76,** 445–448 (1985).
5) Yamashita, K., Adachi, M., Nakagawa, H., Sato, S., Nagao, M. and Sugimura, T. Exposure of MeIQx to rats: DNA modification and expression of multidrug-resistant gene. *Proc. Jpn. Cancer Assoc., 47th Annu. Meet.,* 97 (1988).
6) Wattenberg, L. W. Inhibition of carcinogenesis by minor dietary constituents. *Cancer Res.* (Suppl.), **52,** 2085s–2091s (1992).
7) Ito, N. and Hirose, M. Antioxidants — carcinogenic and chemopreventive properties. *Adv. Cancer Res.,* **53,** 247–302 (1989).
8) Nihro, Y., Miyataka, H., Sudo, T., Matsumoto, H. and Satoh, T. 3-*O*-Alkylascorbic acids as free-radical quenchers: synthesis and inhibitory effect on lipid peroxidation. *J. Med. Chem.,* **34,** 2152–2157 (1991).
9) Nihro, Y., Sogawa, S., Sudo, T., Miki, T., Matsumoto, H. and Satoh, T. 3-*O*-Alkylascorbic acids as free radical quenchers. II. Inhibitory effects on some lipid peroxidation models. *Chem. Pharm. Bull.,* **39,** 1731–1735 (1991).
10) Nihro, Y., Sogawa, S., Furukawa, H., Matsumoto, H. and Satoh, T. Medical chemical studies on cytoprotective agents (5): synthesis and antioxidative activity of a novel series of monoalkylhydroquinones. *Proc. 112th Annu. Meet. Jpn. Pharma. Assoc.,* 267 (1992).
11) Nihro, Y., Sogawa, S., Izumi, A. Sasamori, A., Sudo, T., Miki, T., Matsumoto, H. and Satoh, T. 3-*O*-Alkylascorbic acid as free radical quenchers. 3. Protective effect on coronary occlusion-reperfusion induced arrhythmias in anesthetized rats. *J. Med. Chem.,* **35,** 1618–1623 (1992).
12) Ames, B. N., Durston, W. E., Yamasaki, E. and Lee, F. D. Carcinogens are mutagens: a simple test system combining liver homogenates for activation and bacteria for detection. *Proc. Natl. Acad. Sci. USA,* **70,** 2281–2285 (1973).
13) Yahagi, T., Degawa, Y., Seino, T., Matsushima, M., Nagao, M., Sugimura, T. and Hasimoto, Y. Mutagenicity of carcinogenic azo dyes and their derivatives. *Cancer Lett.,* **1,** 91–96 (1975).
14) Tatematsu, M., Mera, Y., Ito, N., Satoh, K. and Sato, K. Relative merits immunohistochemical demonstrations of placental A, B, and C forms of glutathione S-transferase and histochemical demonstration of γ-glutamyltransferase as markers of altered foci during liver carcinogenesis in rats. *Carcinogenesis,* **6,** 1621–1626 (1985).
15) Kahl, R. Synthetic antioxidants: biochemical actions and interference with radiation, toxic compounds, chemical mutagens and chemical carcinogenesis. *Toxicology,* **33,** 185–228 (1984).
16) Hashimoto, Y., Shudo, K. and Okamoto, T. Modification of DNA with potent mutacarcinogenic 2-amino-6-methyldipyrido[1,2-a:3′,2′,-d]imidazole isolated from a glutamic acid pyrolysate; structure of the modified nucleic acid base and initial chemical event caused by the mutagen. *J. Am. Chem. Soc.,* **104,** 7636–7640 (1982).
17) Kato, R. and Yamazoe, Y. Metabolic activation and covalent binding to nucleic acids of carcinogenic heterocyclic amines from cooked foods and amino acid pyrolysates. *Jpn. J. Cancer Res.,* **78,** 297–311 (1987).
18) Alexander, J. and Wallin, H. Metabolic fate of heterocyclic amines from cooked food. *In* "Mutagens in Food: Detection and Prevention," ed. H. Hayatsu, pp. 143–156 (1989). CRC Press, Boca Raton, FL.
19) Snyderwine, E. G., Schut, H. A. J., Adamson, R. H., Thorgeirsson, U. P. and Thorgeirsson, S. S. Metabolic activation and genotoxicity of heterocyclic arylamines. *Cancer Res.* (Suppl.), **52,** 2099s–2102s (1992).
20) Kushida, H., Wakabayashi, K., Suzuki, M., Takahashi, S., Imaida, K., Sugimura, T. and Nagao, M. Suppression of spontaneous hepatocellular carcinoma development in C3H/HeNCrj mice by the lipophilic ascorbic acid, 2-*O*-octadecylascorbic acid (CV-3611). *Carcinogenesis,* **13,** 913–915 (1992).