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Different Roles of 8-Hydroxyguanine Formation and 2-Thiobarbituric Acid-reacting Substance Generation in the Early Phase of Liver Carcinogenesis Induced by a Choline-deficient, L-Amino Acid-defined Diet in Rats

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The present study was performed to assess the roles of hepatocellular oxidative damage to DNA and constituents other than DNA in rat liver carcinogenesis caused by a choline-deficient, L-amino acid-defined (CDAA) diet by examining the effects of the antioxidant *N,N′*-diphenyl-*p*-phenylenediamine (DPPD). The parameters used for cellular oxidative damage were the level of 8-hydroxyguanine (8-OHGua) for DNA and that of 2-thiobarbituric acid-reacting substance (TBARS) for constituents other than DNA. A total of 40 male Fischer 344 rats, 6 weeks old, were fed the CDAA diet for 12 weeks with or without DPPD (0.05, 0.10 or 0.20%) or butylated hydroxytoluene (BHT, 0.25%). In the livers of the rats, the numbers and sizes of glutathione *S*-transferase (EC 2.5.1.18) placental form (GSTP)- and/or γ-glutamyltransferase (GGT, EC 2.3.2.2)-positive lesions and levels of 8-OHGua and TBARS were determined. The GSTP-positive lesions of 0.08 mm² or larger were all stained positively for GGT as well in cross-sectional area, whereas the smaller lesions were generally negative for GGT. DPPD and BHT reduced the size of the GSTP-positive lesions without affecting their total numbers. At the same time, they reduced TBARS generation without affecting 8-OHGua formation in DNA. The present results indicate that oxidative DNA damage (represented by 8-OHGua formation) and damage to constituents other than DNA (represented by TBARS generation) may play different roles in rat liver carcinogenesis caused by the CDAA diet; the former appears to be involved in the induction of phenotypically altered hepatocyte populations while the latter may be related to the growth of such populations.

Key words: Choline-deficient, L-amino acid-defined diet — 8-Hydroxyguanine — 2-Thiobarbituric acid-reacting substance — Enzyme-altered putative preneoplastic liver lesion — *N,N′*-Diphenyl-*p*-phenylenediamine

Oxidative stress is recognized as being involved in various stages of carcinogenesis[1] and has also been proposed to play a significant role in "endogenous" carcinogenesis.[2] Hence, explorations of how oxidative stress is related to carcinogenic mechanisms are of clear importance. Oxidative stress, once it has overwelmed cellular defense systems, injures all cellular constituents, resulting in formation of DNA adducts, peroxidation of membrane lipids, deenergization of mitochondria, degradation of proteins or sugars and so on.[3–5] 8-Oxo-7,8-dihydroguanine (8-OHGua[4]) is a major oxidative DNA adduct[6] and has attracted particular attention since it is relevant to mutagenic and/or carcinogenic processes.[7–10] The significance of 8-OHGua for chemical carcinogenesis has been supported by its exclusive induction in target organ DNA by a variety of oxidative carcinogens.[11–14] 8-OHGua moieties excised from DNA have been detected in human biological fluids.[15,16] TBARS generation has been widely used as a marker of *in vitro*[17] lipid peroxidation, one of the major mechanisms by which oxidative stress lethally injures cells.[3–5] It may be inappropriate, however, to use this parameter as a marker of *in vivo* lipid peroxidation since, as is well known, a variety of materials other than lipid peroxidative products can react with 2-thiobarbituric acid. Nevertheless, TBARS is often detected exclusively *in vivo* in the target organ, in an amount dependent on the degree of oxidative stress, and its generation can be modified by manipulations of such oxidative stress.[18–21] Since TBARS generation can be dissociated from oxidative DNA damage in cultured rat hepatocytes,[22] this parameter should be available as a marker of oxidative damage to cellular constituents other than DNA.

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[4] The abbreviations used are: 8-OHGua, 8-hydroxyguanine; TBARS, 2-thiobarbituric acid-reacting substance; CDAA diet, choline-deficient, L-amino acid-defined diet; DPPD, *N,N′*-diphenyl-*p*-phenylenediamine; BHT, butylated hydroxytoluene; GSTP, glutathione *S*-transferase placental form; GGT, γ-glutamyltransferase; CSAA diet, choline-supplemented, L-amino acid-defined diet; 8-OHdGuo, 8-hydroxydeoxyguanosine; dGuo, deoxyguanosine; MDA, malondialdehyde.

We have developed a model for liver carcinogenesis that involves feeding a CDAA diet to male Fischer 344 rats.[13, 20, 21, 23] In this model, 8-OHGua in liver DNA and TBARS in total liver homogenate can be detected from day 3 with progressive accumulation at least up to week 12,[20] suggesting a possible contribution of oxidative stress. Dietary iron deficiency regimens and the co-administration of a lipophilic or hydrophilic derivative of vitamin C or E reduce the levels of both 8-OHGua and TBARS as well as the numbers and sizes of enzyme-altered putative preneoplastic lesions.[20, 21] It is thus evident that iron-dependent oxidative stress, inducing change in these parameters, somehow contributes to rat liver carcinogenesis associated with consumption of the CDAA diet. What remains to be definitively clarified is the respective contributions of the two parameters.

In this context, we designed the present study to examine the influence of a potent lipophilic antioxidant, DPPD, on the numbers and sizes of enzyme-altered putative preneoplastic lesions while allowing simultaneous comparison with levels of 8-OHGua in DNA and TBARS in the livers of rats fed the CDAA diet for 12 weeks. Another potent lipophilic antioxidant, BHT, was included for positive control purposes as this agent is known to inhibit rat liver carcinogenesis by a semi-purified choline-deficient diet.[24] GSTP (an isozyme of EC 2.5.1.18) was chosen as a marker for detection of preneoplastic liver lesions since its expression can be clearly distinguished even in lesions consisting of only a few altered hepatocytes.[25] Data for these GSTP-positive lesions were compared with those for GGT (EC 2.3.2.2)-positive lesions.

MATERIALS AND METHODS

Animals A total of 40 male Fischer 344 rats, 5 weeks old, were obtained from Japan SLC Inc., Hamamatsu, Shizuoka and housed in stainless-steel wire cages in an air-conditioned atmosphere at 25°C with a 12-h dark/light cycle. Rats were allowed access to food and tap water *ad libitum* throughout the experiment. After a 1-week acclimation period on Oriental MF diet (Oriental Yeast Co., Ltd., Tokyo) in pellet form, the rats were divided into eight experimental groups, each consisting of 5 animals.

Diets The CDAA diet and a CSAA diet were produced by Dyets Inc., Bethlehem, PA, both in powder form. The compositions of these diets were described elsewhere.[13, 23] They were stored at 4°C immediately on their arrival, and the experiment was completed with consumption of a single batch of each diet.

Chemicals DPPD and BHT were purchased from Eastman Fine Chemicals, Eastman Kodak Co., Rochester, NY, and Sigma Chemical Co., St. Louis, MO, respectively, and administered to rats by admixture into the CDAA or CSAA diet at concentrations as specified below. Each antioxidant-admixed diet was freshly prepared every Monday and Friday.

Experimental protocol During the 12-week experiment, the respective diets were continuously administered. Group 1 received the CDAA diet alone. Groups 2, 3 and 4 received the CDAA diet containing DPPD at the concentrations of 0.05, 0.10 and 0.20%, respectively. Group 5 received the CDAA diet containing BHT at the concentration of 0.25%, in line with the literature.[24] Group 6 received the CSAA diet alone. Groups 7 and 8 received the CSAA diet containing DPPD (0.20%) and BHT (0.25%), respectively. All rats were weighed weekly for the first 4 weeks and bi-weekly thereafter. Diets were replaced every Monday and Friday when average food intake was monitored. At week 12, all rats were killed under light ether anesthesia, and their livers were immediately excised, weighed and utilized for the analyses described below.

Histopathological, histochemical and immunohistochemical examinations Liver slices 5-mm thick from three major lobes were obtained, fixed in an ice-cold mixture of dehydrated ethyl alcohol and glacial acetic acid at a ratio of 19:1 for 3 h followed by overnight incubation in 99.5% ethyl alcohol at 4°C, and embedded in paraffin. Three serial 4-μm thick sections were prepared from each fixed liver slice. The first section was processed routinely for hematoxylin and eosin staining for histopathological assessment. The second was histochemically stained for GGT activity by the method of Rutenberg *et al.*[26] The third was immunohistochemically stained for demonstration of anti-GSTP binding by the avidin-biotin-peroxidase complex method as previously described[27] using rabbit anti-rat-GSTP polyclonal antibody (Medical and Biological Laboratories Corp., Nagoya) and a Vectastain Elite ABC kit (rabbit IgG; Vector Laboratories, Inc., Burlingame, CA). Quantitative analyses of the numbers and sizes of GGT- and GSTP-positive lesions were performed using an image analyzing system as described elsewhere,[21] featuring the three-dimension correction procedure of Campbell *et al.*[28] Lesions consisting of more than five enzyme-altered hepatocytes in cross-section were counted. In the case of GGT, even the smallest positively stained lesions had an area of 0.08 mm^2.

Determination of 8-OHGua formation in DNA 8-OHGua formation in DNA was assessed as detailed elsewhere.[20] Briefly, from liver samples of approximately 2 g wet weight, DNA was isolated and digested into deoxynucleosides by combined treatment with nuclease P1 (EC 3.1.30.1, Yamasa Shoyu Co., Ltd., Chiba) plus alkaline phosphatase (EC 3.1.3.1, Sigma) by the method of Takagi *et al.*[12] The level of 8-OHdGuo in each resultant preparation was determined by high-performance

liquid chromatography with electrochemical detection by an adaptation of the methods of Floyd *et al.*[29] and Kasai *et al.*[30] An authentic sample of 8-OHdGuo was generously provided by Dr. Hiroshi Kasai (Department of Environmental Oncology, Institute of Industrial Ecological Sciences, University of Occupational and Environmental Health, Fukuoka), and dGuo was obtained from Sigma for control purposes. Levels of 8-OHGua formation in DNA were expressed as the numbers of 8-OHdGuo formed per 10^5 total dGuo nucleosides.

Determination of TBARS generation TBARS generation was assessed by an adaptation of the method of Yagi[31] as described previously.[19] Briefly, acid-soluble fractions of total liver homogenates were reacted with 2-thiobarbituric acid (Sigma) solution. The reaction mixture was then extracted with 1-butanol. The fluorescence intensities of the resultant samples at 553 nm (emission) and 515 nm (excitation) were collated against those of standard amounts of MDA (Aldrich Chemical Co., Inc., Milwaukee, WI) and standardized for protein values measured using the BCA Protein Assay employing bicinchoninic acid (Pierce Chemical Co., Rockford, IL).

Statistical evaluations The statistical significance of inter-group differences in quantitative data was determined with the paired Student's *t* test.

RESULTS

All rats survived throughout the experimental period without differences among groups in terms of food intake. Final body weights of Groups 1, 4 and 5 were significantly lower than that of Group 6, but there were no significant differences among Groups 1–5 or 6–8. Relative liver weights (per 100 g body weight) in Groups 1, 4 and 5 were significantly greater than that of Group 6, but there were no significant differences among Groups 1–5 or 6–8.

Histopathologically, fatty cirrhosis was evident in the livers of all rats of Groups 1–5. No qualitative differences in terms of the degree of fatty cirrhosis development could be distinguished among those groups. There were no significant histopathological changes in the livers of any rats in Groups 6–8. Putative preneoplastic liver lesions ranging from altered hepatocyte foci to adenomas

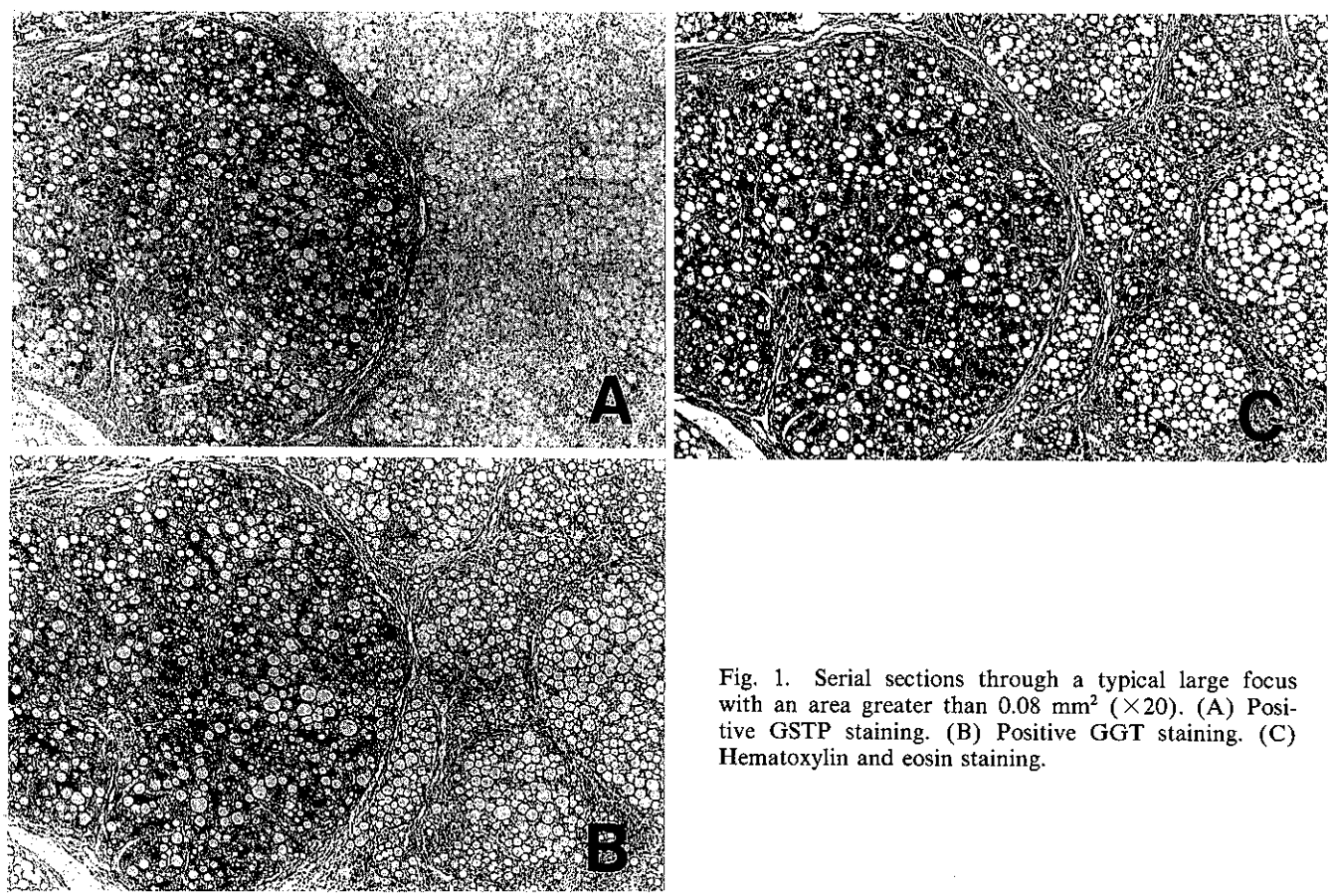

Fig. 1. Serial sections through a typical large focus with an area greater than 0.08 mm^2 (×20). (A) Positive GSTP staining. (B) Positive GGT staining. (C) Hematoxylin and eosin staining.

Table I. Effects of DPPD and BHT on GSTP- and GGT-positive Lesion Induction, 8-OHGua Formation in DNA and TBARS Generation in the Livers of Rats Fed the CDAA Diet for 12 Weeks

Group	Diet	Chemical	GSTP-positive lesions				GGT-positive lesions		8-OHGua in DNA (8-OHdGuo/10^5 dGuo)	TBARS (pmol MDA equivalent/mg protein)
			Number/$cm^3/10^2$			Size of lesions ≥ 0.08 mm^2 (mean volume) (mm^3)	Number/$cm^3/10^2$	Size (mean volume) (mm^3)		
			<0.08 mm^2	≥ 0.08 mm^2	Total					
1	CDAA	—	$121\pm53^{a,b)}$	0.43 ± 0.10	$121\pm50^{b)}$	5.43 ± 1.72	0.39 ± 0.03	6.21 ± 1.17	$10.58\pm1.03^{b)}$	$40\pm8^{b)}$
2	CDAA	DPPD (0.05%)	$122\pm66^{b)}$	0.26 ± 0.13	$122\pm58^{b)}$	$2.57\pm0.26^{c,d)}$	$0.24\pm0.04^{c,d)}$	$2.63\pm1.01^{c)}$	$9.79\pm1.00^{b)}$	$14\pm3^{b,c,d)}$
3	CDAA	DPPD (0.10%)	$124\pm72^{b)}$	$0.15\pm0.04^{c)}$	$124\pm68^{b)}$	$1.56\pm0.15^{c)}$	$0.13\pm0.03^{c)}$	$1.58\pm0.73^{c)}$	$10.18\pm1.59^{b)}$	$7\pm1^{b,c)}$
4	CDAA	DPPD (0.20%)	$120\pm33^{b)}$	$0.10\pm0.11^{c)}$	$120\pm36^{b)}$	$0.65\pm0.24^{c,d,e)}$	$0.09\pm0.02^{c,e)}$	$0.78\pm0.55^{c,e)}$	$10.88\pm1.21^{b)}$	$2\pm1^{c,d,e)}$
5	CDAA	BHT (0.25%)	$122\pm48^{b)}$	$0.08\pm0.03^{c)}$	$122\pm48^{b)}$	$0.72\pm0.28^{c)}$	$0.07\pm0.02^{c)}$	$1.03\pm0.43^{c)}$	$10.11\pm1.22^{b)}$	$3\pm2^{c)}$
6	CSAA	—	12 ± 2	0	12 ± 2	—	0	—	1.30 ± 1.00	3 ± 2
7	CSAA	DPPD (0.20%)	12 ± 3	0	12 ± 3	—	0	—	1.28 ± 0.89	2 ± 1
8	CSAA	BHT (0.25%)	13 ± 4	0	13 ± 4	—	0	—	1.35 ± 0.78	2 ± 2

a) Results are means $\pm$ standard deviations of the determinations for five rats.
b) Significantly higher than Group 6 value ($P<0.02$).
c) Significantly lower than Group 1 value ($P<0.02$).
d) Significantly different from Group 3 value ($P<0.01$).
e) Significantly lower than Group 2 value ($P<0.02$).

were detected in all animals of Groups 1–5. Distinguished by positive GSTP-immunohistochemical staining, those with areal dimensions of 0.08 mm^2 or larger (Fig. 1A) also generally demonstrated a positive GGT reaction (Fig. 1B), but were not easily discernible in hematoxylin and eosin-stained sections (Fig. 1C). In contrast, the GSTP-positive lesions smaller than 0.08 mm^2 were negative for GGT while being practically indistinguishable in hematoxylin and eosin-stained specimens. Quantitative data for the numbers and sizes of the GSTP- and/or GGT-positive lesions are presented in Table I. The total numbers of GSTP-positive liver lesions in Groups 1–5 were all significantly higher than the Group 6 value without any statistically significant differences among themselves. However, DPPD exhibited a clear dose-dependent reducing influence on the number of the GSTP-positive lesions with areas of 0.08 mm^2 or larger, and this was also seen with 0.25% BHT. There were no GSTP-positive lesions of 0.08 mm^2 or larger in Groups 6–8. The numbers of GGT-positive lesions in each group were essentially similar to those of GSTP-positive lesions of 0.08 mm^2 or larger. Since most of the GSTP-positive lesions were smaller than 0.08 mm^2 and because the standard deviations were rather large, values for sizes of the total GSTP-positive lesion population were not statistically analyzed. However, the size of the GSTP-positive lesions was clearly reduced by both DPPD and BHT, without any alteration in their total numbers. The average size of the GGT-positive lesions in each group closely mirrored that of the GSTP-positive lesions of 0.08 mm^2 or larger.

Quantitative data for levels of 8-OHGua in DNA and TBARS generation in total liver homogenate detected in each group are also shown in Table I. The levels of 8-OHGua in liver DNA of Groups 1–5 were significantly greater than the Group 6 value but showed no clear variation when compared with each other. In contrast, DPPD reduced the TBARS generation induced in the livers of rats fed the CDAA diet in a dose-dependent fashion. DPPD at the concentration of 0.20% as well as BHT at the concentration of 0.25% completely prevented the CDAA diet-induced TBARS generation.

DISCUSSION

The results presented above provide strong evidence that oxidative DNA damage, represented by 8-OHGua formation, and oxidative damage to cellular constituents other than DNA, represented by TBARS generation, play different roles in the CDAA diet-induced hepatocarcinogenesis, i.e., the former appears to be involved in the induction of phenotypically altered hepatocyte populations while the latter seems to influence the growth of such cell populations. It is generally accepted that number and size of preneoplastic foci are, respectively, related to the induction and growth of preneoplastic cell populations,[32] and the remarkable correlations between lesion size and TBARS level on the one hand, and lesion number and 8-OHGua level on the other, are of obvious significance in this respect.

8-OHGua has been identified as a physiological form of DNA damage. *Escherichia coli* has a sophisticated enzyme-complex to protect it from 8-OHGua-derived mutagenesis,[9, 33, 34] and a similar system has been suggested to be ubiquitously present in aerobes.[35, 36] 8-OHGua formation induces mutations, either G·C→

T·A or A·T→C·G transversion, in *E. coli*[7,8] and in simian kidney cells.[10] The G·C→T·A transversion is observed at specific sites of the p53 gene in benzo[*a*]-pyrene-induced murine skin tumors[37] and also in human hepatocellular carcinomas[38–40] as well as non-small cell lung carcinomas,[41] suggesting a possible contribution of 8-OHGua formation to the mechanisms underlying such tumorigenesis. In this context, it is conceivable that 8-OHGua formation may contribute to the induction of preneoplastic hepatocyte populations in the livers of rats fed the CDAA diet via its ability to induce genetic alterations. However, further studies to test this hypothesis are needed since no G·C→T·A and only rare A·T→C·G transversions were detected in the p53 gene of hepatocellular carcinomas in rats fed a semipurified choline-deficient diet, though p53 mutation may not be an obligatory step in the genesis of such tumors.[42] It is possible that critical 8-OHGua-derived genetic alterations may occur in genes other than p53 under dietary choline deficiency[42] or that the carcinogenic mechanisms of the CDAA diet may be different from those of a semipurified choline-deficient diet.[43]

Our present data indicate that, even if 8-OHGua formation in DNA indeed results in the induction of phenotypically altered hepatocyte populations in the livers of rats fed the CDAA diet, such cell populations require further stimulation in order to grow into more advanced lesions, and TBARS generation may be involved in this step. As stated in the introduction, TBARS generation *in vivo* does not reflect a single particular phenomenon but does indicate overall oxidative damage to cellular constituents other than DNA. Such damage includes lipid peroxidation, mitochondrial deenergization and protein or sugar degradation, among which at least the former two are major oxidative mechanisms by which hepatocytes are lethally injured.[5] Liver cell death and subsequent regenerative cell proliferation are accepted to play critical roles in liver carcinogenesis in rats fed a semipurified choline-deficient diet.[44,45] The CDAA diet also induces hepatocellular necrosis[20] and, in turn, can induce regenerative cell proliferation. Cell proliferation is capable of facilitating "fixation" of genetic alterations in preneoplastic cell populations, which then grow to form focal preneoplastic cell lesions.[46] Thus, it is conceivable that TBARS generation, induced by iron-dependent oxidative stress,[20,21] lethally injures hepatocytes and, in turn, induces cell proliferation which then gives a growth advantage to phenotypically altered populations induced by the genetic alterations resulting from 8-OHGua formation in DNA by virtue of the same oxidative stress in the livers of rats fed the CDAA diet. As an alternative interpretation, TBARS generation might influence protooncogenes related to hepatocellular growth. Various oxidative stress situations, including the feeding of a semipurified choline-deficient diet, alter protooncogenes such as c-*fos*, c-*myc* and c-Ha-*ras*.[47–50] The liver cell proliferation induced by a semipurified choline-deficient diet may not only be a consequence of liver cell necrosis but also may result from a possible primary mitogenic activity of such a diet.[44] Although the CDAA diet might be different from a semipurified choline-deficient diet in terms of carcinogenic mechanisms,[43] the former could exert effects similar to those of the latter.

DPPD is widely used in the industrial field and inhibits a variety of oxidative stress-induced *in vivo* disorders.[18,51,52] It failed, however, to inhibit rat liver carcinogenesis induced by *N*-2-fluorenylacetamide or by *N*-hydroxy-*N*-2-fluorenylacetamide[53] or to alter spontaneous tumorigenesis in male or female Fischer 344 rats,[54] implying a possible specificity of its inhibitory potential. DPPD might have particular advantages for further investigations of the roles played by oxidative stress in neoplasia.

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