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Pronounced Inhibitory Effect of Chlorcyclizine (CCZ) in Experimental Hepatocarcinoma

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Abstract. *The piperazine chlorcyclizine HCl (CCZ), possessing significant antimetabolic as well as virucidal and virustatic activities against the human immunodeficiency virus (HIV) and other retroviruses, was selected to determine its anticarcinogenic potential. The anticancer activity of CCZ was evaluated against procarcinogen n-diethylnitrosamine (NDA)-initiated hepatocarcinogenesis, which was subsequently promoted by phenobarbital (PB) in male Sprague-Dawley rats. The anticancer efficacy of CCZ was monitored by estimating some potential markers of neoplastic and preneoplastic hepatic conditions, e.g., glutathione (GSH), glutathione-S-transferase (GST) and γ-glutamyl transpeptidase (γGTP). CCZ exhibited antineoplastic activity on a long-term therapeutic basis. Furthermore, this drug restricted the exponential increase of the antioxidant markers in the hyperplastic nodule and the surrounding liver tissues in comparison with the carcinogen-controlled rats during the entire period of treatment. A decrease in the number of nodules was observed in the CCZ-treated group.*

Rat liver carcinogenesis can be induced by some procarcinogens such as 2-acetylamino fluorine, *n*-nitrosodiethylamine (NDA), 3-methyl-4-dimethylamino-azobenzene and aflatoxin B1. An appropriate rodent model may elucidate the sequential steps leading to carcinogenesis, and may also serve as a screening tool to study the effect of drugs interfering with this process (1). Alteration of the enzymatic patterns in these induced models, as well as in tumors that have been transplanted, allow studies on the process of carcinogenesis and cancer chemotherapeutic control (2-5).

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A piperazine derivative of chlorcyclizine (CCZ) had been found to possess high antimetabolic and antiviral activity (6). Our recent studies have revealed CCZ to be virustatic, as well as, in an appropriate tissue culture system, virucidal against HIV (7). The present study was designed to determine the anticarcinogenetic potential of CCZ to serve against a two-stage rat liver carcinogenesis initiated with NDA and promoted by phenobarbital (PB).

Materials and Methods

Animals and diet. Male Sprague-Dawley rats, obtained from the Indian Institute of Chemical Biology (CSIR), Calcutta, weighing on average 100-120 g, were used for the entire study. The rats were acclimatized to the standard laboratory conditions (temperature 24±1˚C, relative humidity 55±5% and 12-hour light/dark cycle) in suspended wire-meshed galvanized cages (4-5 rats per cage) for one week before commencement of the experiment. During the entire period of the study, the rats were maintained on a semi-purified basal diet (Lipton^{R}, India) with water supplied *ad libitum*.

The basic experimental regimen. Twenty Sprague-Dawley rats were divided into four batches of five rats each. Batch 1 received only the vehicles, *i.e.*, normal saline instead of NDA and distilled water in place of CCZ or PB in equal volumes, respectively, and served as the normal vehicle controls outlined in Table I. To the second group (Batch 2) of rats, a neurogenic dose of NDA was given (a single *i.p.* injection of 200 mg/kg body weight followed by PB as a promoter, 0.05% in basal diet, 5 days/week) and they served as the NDA + PB control. The PB supplementation started 4 weeks after challenge with NDA and continued thereafter till 12 weeks of experimentation (8). The third group (Batch 3) of rats received CCZ at 6 mg/kg basal diet, which started 4 weeks before NDA challenge or 8 weeks before PB treatment and continued thereafter till 12 weeks. Similarly, Batch 4 of rats received CCZ only for an identical time-span as per Batch 3 and served as the CCZ control. Batch 4 was not given any challenge. As shown in Table I, both Batches 3 and 4 received CCZ for 16 weeks, being started 4 weeks before the application of NDA and continued thereafter until the end of the regimen. All rats were maintained on the basal diet without any treatment, of either the control or experimental

Table I. *Pattern of administration of hepatocarcinogen (NDA) plus phenobarbital (PB) and chlorcyclizine (CCZ) in different batches.*

Batch	Challenge (NDA/NS)	Promoter (PB)	Treatment (CCZ/DW)	Period of study (week)
1	NS	-	DW	12
2	NDA	PB	DW	12
3	NDA	PB	CCZ	4*+12
4	NS	-	CCZ	4*+12

Abbreviations: NDA: *n*-nitrosodimethylamine; NS: normal saline; PB: phenobarbital; CCZ, chlorcyclizine; DW: distilled water.
*All the batches received treatment for 12 weeks from the first day of administration of NDA (or NS). Batches 3 and 4 were given CCZ for 4 weeks prior to the challenge by NDA.

groups, for 1 week before being killed by proper anaesthesia on an empty stomach.

Morphology and histology. Soon after the sacrifice, the livers were promptly excised, weighed and examined on the surface for visible macroscopic lesions (nodular hyperplasias). The nodules with approximate spheres were measured in two perpendicular directions to the nearest mm into three categories, *viz.*, > = 3 < 3 > = 1 and < = 1 mm, according to published criteria (9). Representative liver slices, mostly from the right and central lobes, were fixed in 10% neutral buffered formalin for routine haematoxylin and eosin (H&E) stain-based histopathology (5-μm-thick sections were examined using an Adcon-5591 light microscope). The histopathological slides were coded to avoid observer bias.

Preparation of liver cytosolic fraction. The rats were sacrificed with an empty stomach by proper anaesthesia. The liver of either lobes were excised, minced and homogenized with ice-cold 1.15% (w/v) KCl solution in a Teflon-coated glass homogenizer to make a 10% (w/v) homogenate (9). The cytosolic fraction was prepared by differential centrifugation. Firstly, the homogenate was centrifuged at 9,000 xg for 30 minutes; the resultant supernatant fraction was centrifuged at 105,000 xg for 90 minutes in an OTD-50B Sorvall-ultracentrifuge. The supernatant of the 105,000 xg centrifugation represented the cytosolic fraction and was stored at –20˚C until further use, while the pellet of the same process was resuspended in 1/10th volume of the homogenizing buffer and served as the microsomal fraction. All operations were carried out at 0–4˚C.

Enzyme assays and estimation. The total hepatic cytosolic glutathione (GSH) content was determined by the method of Ellman (10) with 4% (w/v) sulfosalicylic acid and 0.1 mM 5,5'-dithio-*bis*-2-nitrobenzoic acid, while cytosolic glutathione-*S*-transferase (GST) activity was determined against 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate, according to the method proposed by Habig *et al.* (11). Cytosolic protein concentrations were estimated by the method of Lowry *et al.* (12) using bovine serum albumin as a standard. All spectrophotometric readings for the enzyme assays and estimations were obtained by using an UV/VIS DW-2a spectrophotometer (Pye Unicam, Cambridge, UK).

Statistical analysis. Each enzyme assay and biochemical estimation was performed about 4-6 times from every treated and control group(s) under the experimental conditions. All the data were pooled from each treated and control regimen and the statistical analysis was carried out using the volunteer's-test.

Results

Liver cell pathology. Histopathological examination of liver sections of the normal untreated group or CCZ controls (Batches 1 and 4, respectively) revealed normal parenchymal cells with granular cytoplasm and small uniform nuclei radially arranged around the central vein (Figure 1). In contrast, rats of Batch 2, having received NDA + PB, showed phenotypically altered hepatocyte populations in the form of altered liver cell foci throughout the liver parenchyma (Figure 2). These foci were considerably enlarged, largely vesiculated and mostly binucleated. The nuclei were enlarged and the chromatin showed erratic condensation. Treatment with CCZ in Batch 3 elicited a detectable protection against the NDA + PB regimen. The configuration of the sinusoid appeared normal with normal Kupffer cells. The size of the nuclei mostly resembled that of normal cells. Chromatin condensation was extremely poor with fewer binucleated cells, particularly in the perifocal regions. In contrast with the NDA – PB challenge (Batch 2), treatment with CCZ in rats offered a detectable and clear protection, which showed lesser hypereosinophilic and basophilic focal proliferation with abundance of clear cell foci coupled with larger sinusoids (Figure 3). In contrast, feeding rats on a similar regimen with CCZ alone did not reveal any toxic manifestation, at least at the 6 mg/kg dosage studied herein (Figure 4).

Effect of CCZ on NDA-induced nodule formation in liver. Table II depicts the effect of CCZ on hepatic nodular hyperplasias induced by NDA and promoted by PB. A variable focus of hyperplasia could be observed after a single *i.p.* injection of NDA followed by promotion with PB with/without CCZ treatment. The appearance of hepatic nodules, as assessed by their incidence (%), total number and multiplicity, was more pronounced in the NDA + PB-treated rats (Batch 2) compared with Batch 3, which received NDA-PB along with CCZ treatment.

In NDA + PB-treated rats, the incidence of hepatic nodular lesions reached higher statistical significance ($p<0.001$) compared with the vehicle controls. However, continuous supplementation of the CCZ for a total period of 16 weeks significantly ($p<0.05$) reduced the number of nodular hyperplasia compared with that of the NDA-PB

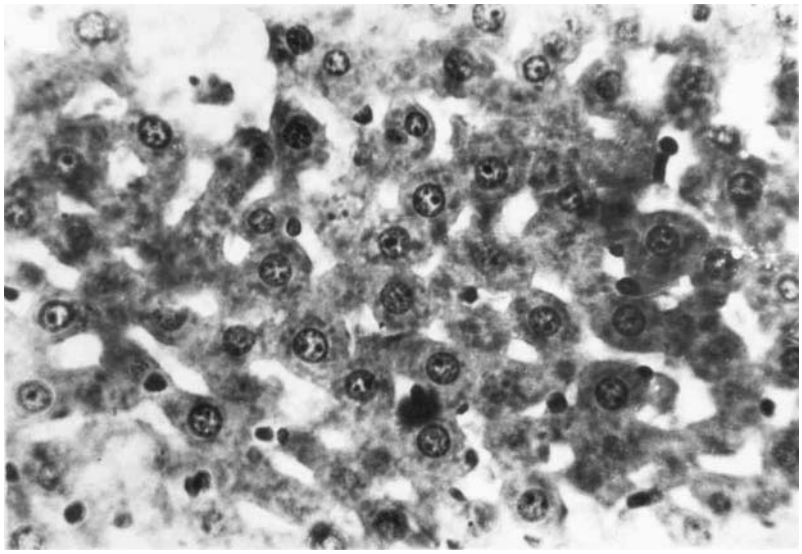

Figure 1. *Thin section [x100] of the right liver lobe showing normal hepatic architecture with a large central vein and sinusoids without any detectable staining intensity difference in the hepatic parenchyma. The chromatin bodies are stained with eosin (E) and the cytoplasm with haematoxylin (H).*

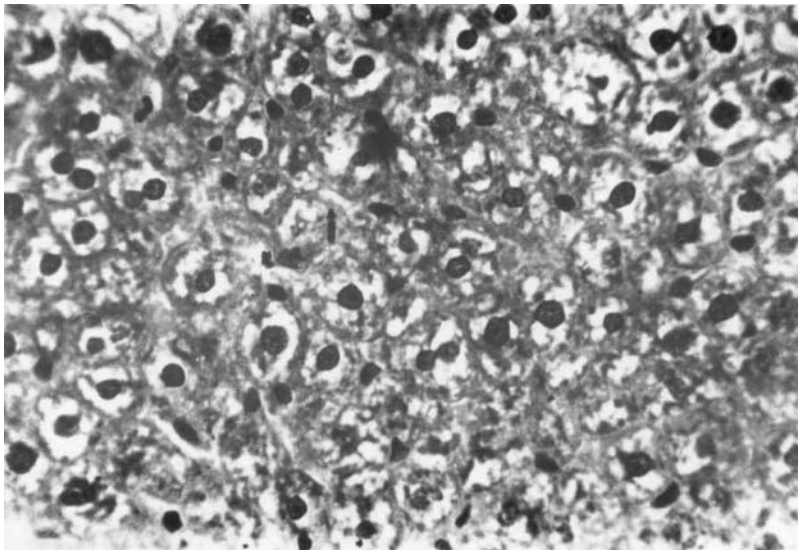

Figure 2. *Thin section of the right liver lobes of rat treated with NDA + PB for 16 weeks, representing a detectable abnormal pathology stained with H & E, (x 100), showing scattered eosinophilic focal development with variable (low to high) intensity in cytoplasm staining. A number of hepatocytes are seen with higher intensity in the basophilic stain, probably due to a rise in nuclear DNA content. These are clearly seen in the plate, called mixed cell focus. Sinusoids are comparatively less or extremely few in number, showing greatly increased mitotic index (higher cell proliferation). Also showing is a typical hyperbasophilic cluster with extreme pycnosis in the nucleus coupled with strong hyper-acidophilic staining in the cytoplasm. Compare the plate with the normal one of Figure 1.*

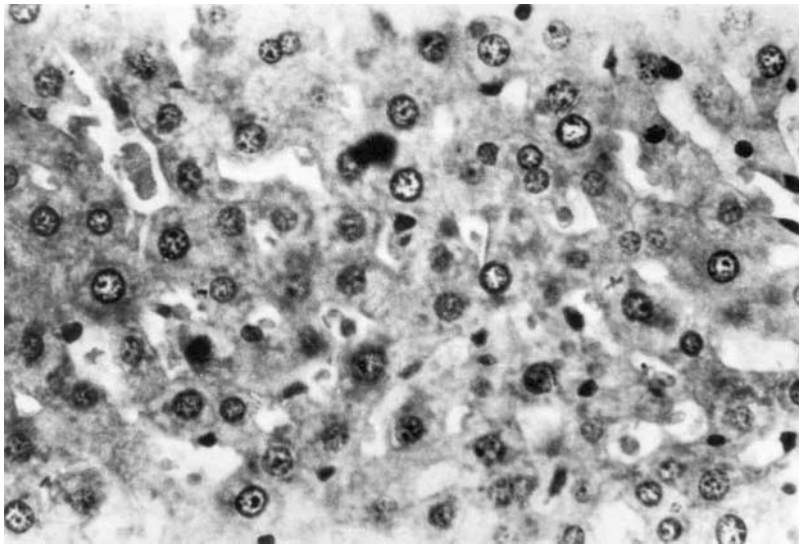

Figure 3. *Thin section of the right liver lobes of rats fed with CCZ previously treated with NDA + PB for 16 weeks, representing a gross recovery compared with the NDA + PB regimen (x100), showing a majority of clear cell foci coupled with eosinophilic and still with strong hyper-acidiophilic staining in the cytoplasm. Sinusoids are comparatively more in number compared with the NDA + PB controls.*

Table II. *Effect of CCZ on the incidence and spread of nodular hyperplasias in the liver challenged with the NDA-PB regimen.*

Group	No. of rats with nodules/total rats (Total no. of nodules)	Nodule incidence (%)	Nodule multiplicity	Relative size of nodule (% of total no.)		
				<1 mm	1 mm-3 mm	3 mm<
Control (Batch 2)						
NDA + PB	6/6	100.00	1.51±0.22	4	5	3
	(12)					
Experimental (Batch 3)						
CCZ plus	6/9	66.66	1.32±0.20	4	4	1
NDA-PB	(8)					

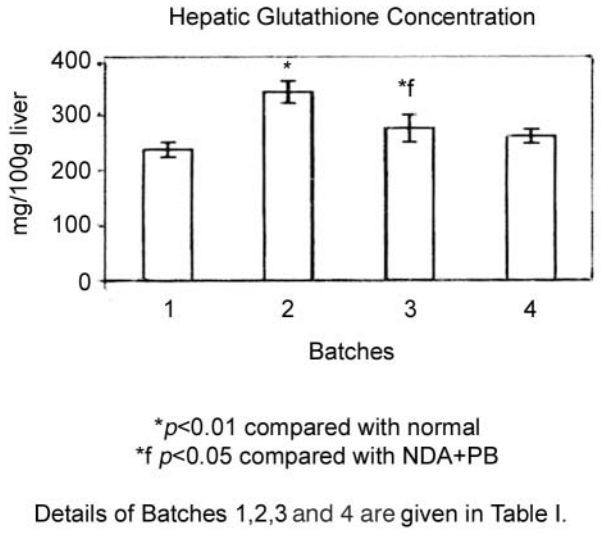

Figure 4. *Hepatic glutathione concentration.*

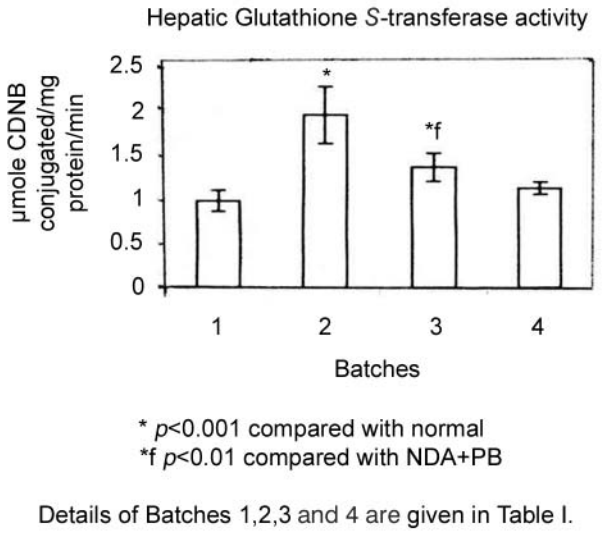

Figure 5. *Hepatic glutathione S-transferase activity.*

control (Batch 3). A major difference could be observed when the occurrence of larger (<3 mm in diameter) nodules was considered between these two groups. No nodular lesion could be seen in the CCZ control (Batch 4) compared to that of the vehicle counterparts.

Effect of CCZ administration on hepatic cytosolic glutathione concentration and glutathione-S-transferase (GST) activity. Among the major antioxidants tested, both the hepatic cytosolic glutathione (GSH) concentration and CDNB conjugated glutathione-*S*-transferase (GST) were increased significantly ($p<0.01$-0.001) in the rats (Batch 2) challenged with the NDA-PB regimen compared with those of the normal vehicle controls (Batches 1, 4). These are presented in Figures 4 and 5. More than a 2-fold increase in hepatic GSH activity and less than a 1.5-fold increase in total hepatic GST concentration could be observed upon treatment with NDA-PB compared with those of the vehicle control. In contrast, supplementation with CCZ significantly reduced this high increase in both total hepatic GSH concentration ($p<0.05$) and GST activity ($p<0.01$) in Batch 3 compared with Batch 2. An insignificant increase in the total hepatic GSH concentration was detected, but there was no apparent effect on the cytosolic GST activity in the CCZ group (Batch 4).

Discussion

It has been postulated that the evolution of thiol-dependent detoxification pathways was initially the result of the availability of molecular oxygen (13). It is probable that the cellular defense against oxygen free radicals and peroxide is of considerable importance to cell survival. In the present study, there was an elevation in endogenous GSH content and enhancement of GST activity after administration of NDA (Batch 2). The increased level of GSH in NDA control rats may be due to the fact that GST catalyses the degradation of extra-cellular GSH and is perhaps involved in the production of a precursor that can be used in the intracellular synthesis of GSH (14). This elevation of the GSH level in Batch 2 rats following NDA-PB treatment was an attempt by the host cells to combat the existing oxidative stress imposed by the hepatocarcinogen.

Moreover, preneoplastc hepatocytes, characterized by an induction of cytochrome P-450, may also be due to the ATP/ADP ratio by the inhibition of oxidative phosphorylation, thereby increasing the NADPH content rapidly for the mixed function oxidase system to act (15). Again, it has been shown that cytochrome oxidase I and II were up-regulated by certain anticancer drugs and antioxidants (13). Therefore, for preneoplastic cells in hyperplastic nodules, an increase in cytochrome P-450 content might be favourable for their escape from contact with cytotoxic metabolites derived from a carcinogen and for their growth and progression to cancer cells (13).

In contrast, Batch 3 rats, which received CCZ, showed a significant decrease in the level of cytosolic GSH without any significant effect in the CCZ control counterparts. This may be a reflection of the interaction with some active oxygen compounds and the formation of additional pyridine nucleotides which, thereby, abate the growth and spread of neoplastic nodules, as observed therein.

From the cancer chemotherapy point of view, GST-mediated conjugation of antineoplastic agents with GSH results in their inactivation (16). In most cases, a strong correlation could be observed between an increase in GST activity, metabolic inactivation and resistance to several anticancer agents (17). In view of this, intensive efforts are underway to design and develop new selective inhibitors of GST to overcome the problem of drug resistance in tumour cells (18). In the present investigation, the increased activity of the GST enzyme in NDA control rats was evident and the subsequent depletion of the enzyme in the treatment group (Batch 3), which had received CCZ for the entire period of the experiment (16 weeks). In tumours where increased GST activity has been implicated in drug resistance, the use of combination chemotherapy with GST inhibitors has been a successful approach to additive apoptosis (18). Induction of high γ-glutamyl transpeptidase (γGTP) levels in preneoplastic foci is frequently an early event in hepatocarcinogenesis. The increase in the metabolism of extracellular GSH by γGTP may lead to a radical-rich microenvironment in close proximity to the focal development. Moreover, suppression of the intracellular levels of GSH and GST by CCZ clearly suggests that a progressive loss of growth capacity by putative preneoplastic cells and their differentiation into normal-appearing hepatocytes takes place.

A large body of evidence on a close correlation between γGTP activation and the process of carcinogenesis has been reported (19). A marked increase of the number of γGTP-positive foci could well be correlated with a higher nodule increase; its total number coupled with a larger hepatic parenchyma occupied by these nodules and foci in hepatic tissue, as can be judged from routine histopathology. Although it is evident that not all the hepatocyte nodules become cancerous during the lifespan of the animals, numerous observations support the concept that the nodules are the precursors of hepatic cancer (1, 20). A clear reflection of the action of CCZ *in vivo* could be judged by NDA-induced hepatic nodulogenesis and routine pathology. Although no significant CCZ inhibition could be observed on nodule incidence and multiplicity, it was found to be effective in abating the growth and spread of this nodule in the hepatic parenchyma, compared with that of the NDA-PB control regimen.

Regardless of the biochemical mechanism of action, the present observation strongly supports that the progression of NDA-induced hepatic tumorigenesis is abated by CCZ at the phase of initiation, without any significant toxicity as manifested by enzymology or in routine pathology.

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References

1 Farber E: The sequential analysis of liver cancer induction. Biochem Biophys Acta *605*: 149-166, 1980.
2 Sato K: Glutathione transferases as markers of preneoplasia and neoplasia. Adv Cancer Res *52*: 205-255, 1990.
3 Scherer E: Neoplastic progression in experimental hepatocarcinogenesis. Biochem Biophys Acta *738*: 219-236, 1984.
4 Guengerich EP: Roles of cytochrome P_{450} enzymes in chemical carcinogenesis and cancer chemotherapy. Cancer Res *48*: 2946-2954, 1998.
5 Farber E: The biochemistry of preneoplastic liver: a common metabolic pattern in hepatocyte nodules. Cancer J Biochem Cell Biol *62*: 486-494, 1994.
6 Hossain M, Dastidar SG, Das S and Chakrabarty AN: Anti-metabolic activity of some commonly used drugs. Indian J Exp Biol *725*: 866-868, 1988.

7 Chakrabarty AN, Mookerjee M and Dastidar SG: Screening anti HIV-drugs that can combine virucidal and virustatic activities synergistically. Int J Antimicrob Agents *14*: 249-251, 2000.
8 Sarkar A, Bishayee A and Chatterjee M: Beta-carotene prevents lipid peroxidation and red blood cell membrane protein damage in experimental hepatocarcinogenesis. Cancer Biochem Biophys *15*: 111-125, 1995
9 Sarkar A, Mukherjee B and Chatterjee M: Inhibitory effect of β-carotene on chronic 2-acetylaminofluorine induced hepatocarcinogenesis in rat: reflection in hepatic drug metabolism. Carcinogenesis *15*: 1055-1060, 1994.
10 Ellman GL: Tissue sulfhydryl groups. Arch Biochem Biophys *82*: 70-77, 1959.
11 Habig WH, Pabst MJ and Jacoby WB: Glutathione-*S*-transferase, the first enzymatic step in mercapturic acid formation. J Biol Chem *249*: 7130-7139, 1974.
12 Lowry OH, Rosenbrough NJ, Farr AL and Randall RJ: Protein measurement with the folin phenol reagent. J Biol Chem *193*: 265-275, 1951.
13 Tsuda H, Moore MA and Asamoto M: Effect of modifying agent on the phenotypic expression of cytochrome P-450, glutathione-*s*-transferase molecular forms, microsomal epoxide hydrolase, glucose 6-phosphate dehydrogenase and γ-glutamyl transpeptidase in the rat liver preneoplastic lesions. Carcinogenesis *9*: 547-557, 1988.
14 Stark AA, Zieger E and Pagano DA: Glutathione mutagenesis in *Salmonella typhimurium* is a γ-glutamyl transpeptidase enhanced process involving active oxygen species. Carcinogenesis *9*: 771-778, 1988.
15 Degawa M, Miura S and Hashimoto Y: Expression and induction of cytochrome P-450 isozymes in hyperplastic nodules of rat liver. Carcinogenesis *12*: 2151-2156, 1991.
16 Tsuchida H and Sato K. Glutathione transferases and cancer. CRC Crit Rev Biochem Mol Biol *27*: 337-384, 1992.
17 Gallagher BC, Rudolph DB and Hinton BT: Differential induction of γ-glutamyl transpeptidase in primary cultures of rat and mouse hepatocytes parallels induction during hepatocarcinogenesis. Carcinogenesis *19*: 1251-1255, 1998.
18 Mannervik B and Danielson UH: Glutathione transferases – structure and catalytic activity. CRC Crit Rev Biochem *23*: 283-287, 1988.
19 Fiala ES: Activation of chemical carcinogens of γ-glutamyl transpeptidase in rat and mouse liver. J Natl Cancer Inst *51*: 151-158, 1973.
20 Moreno FS, Rizzi MBS and Dagli MLZ: Inhibitory effects of β-carotene on preneoplastic lesions induced in Wistar rats by the resistance hepatocyte model. Carcinogenesis *12*: 1817-1822, 1991.

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