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Putative metabolites derived from dietary combinations of calcium glucarate and *N*-(4-hydroxyphenyl)retinamide act synergistically to inhibit the induction of rat mammary tumors by 7,12-dimethylbenz[*a*]anthracene

HUSSEIN M. ABOU-ISSA*, VALENTINE A. DURUIBE*, JOHN P. MINTON*, SAROJ LARROYA†, CHANDRADHAR DWIVEDI†‡, AND THOMAS E. WEBB†

Comprehensive Cancer Center and the Departments of *Surgery and of †Physiological Chemistry, The Ohio State University, Columbus, OH 43210

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ABSTRACT **Calcium glucarate and *N*-(4-hydroxyphenyl)-retinamide were evaluated individually and in combination in the diet as preventative chemical agents, by using the induction of rat mammary tumors by 7,12-dimethylbenz[*a*]anthracene as the test system. When tested separately over 18 weeks, optimal doses of calcium glucarate (128 mmol/kg of diet) or *N*-(4-hydroxyphenyl)retinamide (1.5 mmol/kg of diet) administered daily inhibited tumor incidence by 50% or 57% and tumor multiplicity by 50% or 65%, respectively. Suboptimal doses of calcium glucarate (32 mmol/kg) and of *N*-(4-hydroxyphenyl)-retinamide (0.75 mmol/kg) inhibited tumor incidence by 15% and 5% but had no inhibitory effect on tumor multiplicity. In contrast, the combination of calcium glucarate (32 mmol/kg) and *N*-(4-hydroxyphenyl)retinamide (0.75 mmol/kg) inhibited tumor incidence and tumor multiplicity by 50%. Similar synergism was observed with the combination of calcium glucarate (64 mmol/kg) and *N*-(4-hydroxyphenyl)retinamide (0.75 mmol/kg), the inhibition being 55–60%. HPLC analysis of the bile of female rats injected intraperitoneally with a single dose of the retinamide [60 mg/kg (body weight)] showed that the excretion of the retinamide and its glucuronide were markedly suppressed by pretreatment with an oral dose of calcium glucarate [4.5 mmol/kg (body weight)].**

The vitamin A analogs retinyl acetate, retinylmethylether and *N*-(4-hydroxyphenyl)retinamide (HPR) have been shown to inhibit the induction of rat mammary cancer by 7,12-dimethylbenz[*a*]anthracene ($Me_2B[a]A$) (1–3), *N*-methyl-*N*-nitrosourea (4, 5), or benzo[*a*]pyrene (6). Retinoids have also been shown to inhibit the neoplastic transformation of mammary tissue by carcinogenic hydrocarbons in whole-organ culture (7). The inhibition of carcinogen-induced tumors by HPR has been confirmed and the mechanisms underlying this inhibition have been investigated (8). The effect of limiting feeding of retinoids to specific time periods of the carcinogenic process has also been reported (7, 9). Although retinyl acetate tends to accumulate in the liver resulting in mild hepatotoxicity, HPR does not have this disadvantage (5). A comprehensive study has been made of HPR metabolism in the rat (10). The overwhelming evidence suggests that retinoids are inhibitory to the initiation and promotion phases of rat mammary carcinogenesis (11).

Studies from this laboratory have shown that dietary calcium glucarate (CGT) is an effective preventative chemical (chemopreventative) agent against cancer induction in several rodent organs, including the mammary gland (12, 13). This activity is believed to derive from the slow conversion of approximately one-third of the CGT to the potent β-glucuronidase inhibitor D-glucaro-1,4-lactone, at the acid pH of the stomach. The increased net glucuronidation could theoretically lead to increased excretion of carcinogens and promoting agents, including steroid hormones, as glucuronide conjugates (12–14). Consequently, CGT has been observed to inhibit the initiation and promotion phases of tumorigenesis.

The present communication describes the efficacy of combinations of low ineffectual or marginally effective levels of dietary HPR and of dietary CGT on $Me_2B[a]A$-induced rat mammary carcinogenesis and the effect of CGT on the clearance of HPR and its glucuronide, both of which have been identified in the bile of HPR-treated rats.

MATERIALS AND METHODS

Carcinogenic Protocols. Fifty-day-old rats of the Sprague Dawley strain (specific pathogen-free; Harlan Sprague Dawley, Indianapolis, IN) were randomly assigned to eight groups (20 rats per group) that were fed various diets beginning 2 weeks before treatment with $Me_2B[a]A$ and continuing throughout the experiment. Each rat received $Me_2B[a]A$ [75 mg/kg (body weight)] (Sigma) in 1.0 ml of sesame oil by gavage with an 18-gauge feeding needle. Beginning 5 weeks after the initiation of $Me_2B[a]A$, the rats were examined (palpated) weekly for mammary tumors. They were weighed every 1 or 2 weeks throughout the experiment.

Chemoprevention Protocols. The eight diets were as follows: (*i*) rat chow (RMH 3200, Pro Lab, Syracuse, NY); (*ii*) rat chow with 1% (wt/wt) CGT·$3.5H_2O$ (32 mmol/kg of diet; Gallard Schlesinger, Carle Place, NY); (*iii*) rat chow with 2% CGT·$3.5H_2O$ (64 mmol/kg); (*iv*) rat chow with 4% CGT·$3.5H_2O$ (128 mmol/kg); (*v*) rat chow with HPR (0.75 mmol/kg); (*vi*) rat chow with HPR (1.5 mmol/kg); (*vii*) rat chow with 2% (wt/wt) CGT·$3.5H_2O$ (64 mmol/kg) and HPR (0.75 mmol/kg); and (*viii*) rat chow with CGT (32 mmol/kg) and HPR (0.75 mmol/kg). The CGT powder was mixed into the meal (powdered chow) with a mechanical mixer and then stored at room temperature. The HPR was first dissolved in 25 ml of a vehicle consisting of ethanol/tricaprylin, (1,2,3-trictanoylglycerol) 1:4 (vol/vol), plus 6% (wt/vol) α-tocopherol, then thoroughly mixed with powdered rat chow or with rat chow fortified with CGT. The same amount of vehicle was also added to the control diet (25 ml/2 kg of chow) and to the diets containing CGT. Diets were prepared weekly and stored at −20°C. From these stocks, the animals

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Abbreviations: HPR, *N*-(4-hydroxyphenyl)retinamide; CGT, calcium glucarate (saccharate); $Me_2B[a]A$, 7,12-dimethylbenz[*a*]anthracene.

‡Present address: College of Pharmacy, South Dakota State University, Brookings, SD.

were fed the respective diets daily. The statistical significance of the results were analyzed with the Student's *t* test.

Effect of Dietary CGT on HPR-*O*-Glucuronide Formation. Because of evidence for two subpopulations of Sprague Dawley rats with different β-glucuronidase levels (15), these studies were performed on the female rats (350–400 g) of the inbred Fischer strain. HPR was obtained from Y. Shealy (Southern Research Institute, Birmingham, AL), through a contract from the National Cancer Institute, Bethesda, MD. HPR-*O*-glucuronide levels were monitored in the bile by HPLC essentially as described by Swanson *et al.* (10). Approximately 4 hr prior to the completion of bile duct cannulation (PE-20 tubing, Clay Adams, Parsippany, NJ), the rats received by gavage (19-gauge feeding needle, Popper & Sons, New Hyde Park, NY) either 1.0 ml of the 10% (wt/vol) aqueous gum acacia vehicle or CGT [4.5 mmol/kg (body weight)] in 1.0 ml of 10% gum acacia. Timing may be important if β-glucuronidase is involved since maximum inhibition is observed in tissues between 3 and 5 hr after administration of CGT (12). The cannula was externalized from the abdominal incision under the skin, exiting to a fraction collector through an incision in the intrascapular region. A portion of the cannula exiting from the intrascapular region was protected with a metal tube as described (16). This procedure allows the rat to be fully mobile during the 24-hr bile collection procedure. After bile duct cannulation the rats received HPR (60 mg/kg) in ethanol/corn oil, 1:15 (vol/vol) by i.p. injection. Aliquots of selected 1.0-hr fractions of the bile were combined and lyophilized before and after incubation with β-glucuronidase (10,000 units/ml) (bovine type B, Sigma) at pH 4.5 for 1 hr at 56°C. The lyophilized aliquots were extracted with methanol and then centrifuged. Aliquots of the methanol extracts before and after treatment with β-glucuronidase were analyzed by HPLC on a C_{18} column. The eluate was monitored at 365 nm. The concentration of HPR and its glucuronide were calculated by using known extinction coefficients (cf. ref. 10). This identification was also confirmed by TLC analysis by using silica gel G plates (Analtech, Newark, DE) and the solvent system chloroform/methanol, 4:1 (vol/vol).

RESULTS

CGT and HPR Dose-Response Curves. The minimum concentration of CGT or HPR in the diet consistent with maximal inhibition of rat mammary tumor induction by $Me_2B[a]A$ can be roughly estimated from the dose–response curve in Fig. 1.

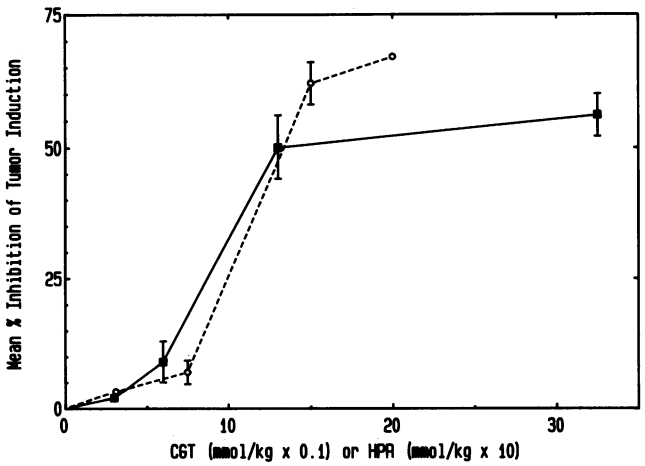

FIG. 1. Dose–response curve for inhibition of $Me_2B[a]A$-induced rat mammary tumorigenesis by dietary CGT (■) or by dietary HPR (○) is shown. Data represent number of tumors 18 weeks after $Me_2B[a]A$ treatment (mean ± SEM) based on three experiments.

These data were obtained by maintaining $Me_2B[a]A$-treated rats on diets containing the indicated concentrations of CGT or HPR from 2 weeks before the treatment to the end of the 18-week experiment. The dose–response curves, based on the number of mammary tumors 18 weeks after $Me_2B[a]A$ treatment, are sigmoidal for both chemopreventative agents. The standard errors are shown for those points based on three separate experiments; the other points are the average of data from two separate experiments. The optimal concentration of CGT is 128 mmol/kg of diet and that for HPR appears to be ≈2.0 mmol/kg of diet, although 92% of maximal inhibition is achieved at 1.50 mmol/kg. Only marginal chemopreventative activity was observed with CGT at 32 mmol/kg or HPR at 0.75 mmol/kg.

HPR–CGT Synergism. The results of selected experiments testing the effect of dietary CGT and HPR, individually and in combination, on the time course of mammary tumor development in $Me_2B[a]A$-treated rats are shown in Figs. 2 and 3, and the data of all experiments are summarized in Table 1. When the number of rats with tumors was measured, CGT at 128 mmol/kg or HPR at 1.5 mmol/kg inhibited incidence of tumors at 18 weeks by 50–60% (Fig. 2 and Table 1). At the lower dosages, of CGT at 32 mmol/kg and HPR at 0.75 mmol/kg, the inhibition of tumor incidence fell to <15% (Fig. 2 and Table 1). However, CGT at 64 mmol/kg plus HPR at 0.75 mmol/kg inhibited tumor incidence 57% or CGT at 32 mmol/kg plus HPR at 0.75 mmol/kg inhibited tumor incidence 50% as compared to the control. Similarly, when the average number of tumors per rat was measured, CGT at 128 mmol/kg or HPR at 1.5 mmol/kg inhibited mammary tumor multiplicity 50% and 65%, respectively (Table 1). Again, when the dosages of CGT and HPR were reduced to 32 and 0.75 mmol/kg, respectively, tumor multiplicity was correspondingly inhibited 0% (Fig. 3 and Table 1). Marginal inhibition was also obtained with CGT at 64 mmol/kg. In contrast, HPR at 0.75 mmol/kg plus CGT at 32 or 64 mmol/kg inhibited tumor multiplicity at least 50% and 55%, respectively ($P < 0.002$).

Uniform Weight Gain on Protocols. Weight loss caused by reduced food intake due to toxicity is known to influence carcinogenesis. However, this was not a factor in these experiments. As shown in Fig. 4, prolonged feeding of diets supplemented with high or low dosages of CGT or HPR, or various combinations, did not significantly affect the weight gain of the rats ($P > 0.15$). The standard error at all points was <5% of the

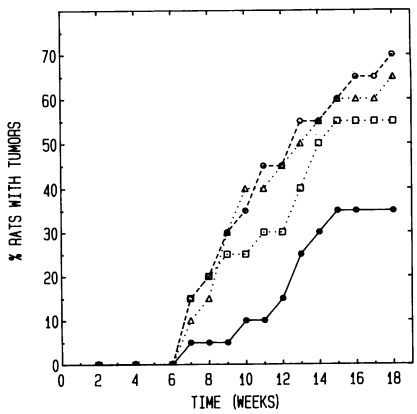

FIG. 2. Effect of dietary CGT and HPR, either alone or in combination, on the time-course change in rat mammary tumor incidence after treatment with $Me_2B[a]A$. The diets were as follows: control diet (○); HPR (0.75 mmol/kg) (△); CGT (32 mmol/kg) (□); CGT (32 mmol/kg) and HPR (0.75 mmol/kg) (●). The difference in degree of the combination as compared to the control HPR- and CGT-treated groups was statistically significant ($P < 0.004$), based on the binomial expansion method.

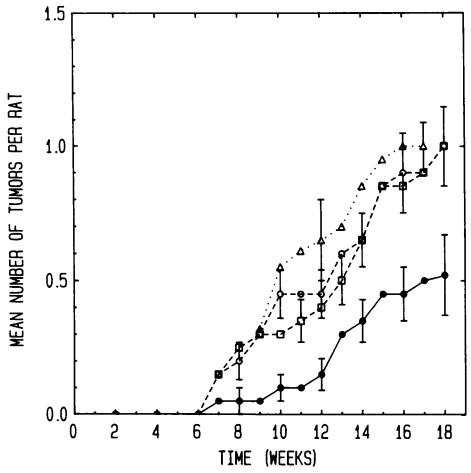

FIG. 3. Effect of dietary CGT and HPR, either alone or in combination with the time-course change in the multiplicity of rat mammary tumors [number of tumors per rat (mean ± SEM)] after treatment with $Me_2B[a]A$. The diets were as follows: control diet (○); HPR (0.75 mmol/kg) (△); CGT (32 mmol/kg) (□); CGT (32 mmol/kg) and HPR (0.75 mmol/kg) (●). The difference in degree of inhibition of the combination as compared to control HPR- and CGT-treated groups was statistically significant ($P < 0.002$).

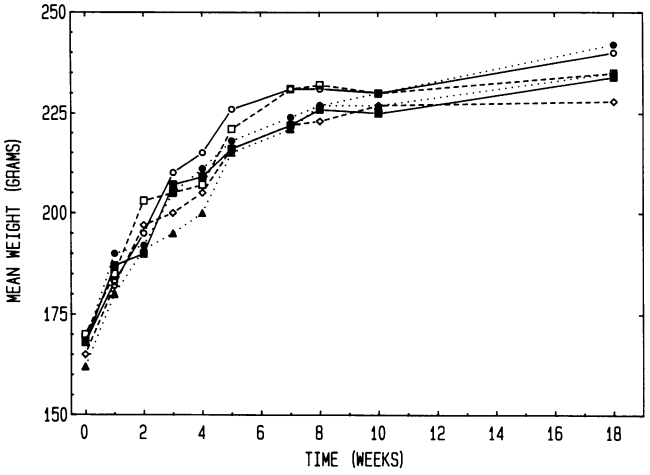

FIG. 4. Mean weight of rats during the experiment. The diets were as follows: control (○); CGT (32 mmol/kg) (□);CGT (32 mmol/kg) and HPR (0.75 mmol/kg) (●);HPR (1.5 mmol/kg) (▲); CGT (64 mmol/kg (■); CGT (64 mmol/kg) and HPR (0.75 mmol/kg) (◇).

mean. Therefore, the observed chemopreventative effects must be due to the specific actions of the agents themselves.

HPR-*O*-Glucuronide in Bile. The retention time of HPR-*O*-glucuronide from the bile on the C_{18} column was identical to that reported by Swanson *et al.* (10). After hydrolysis with β-glucuronidase, the UV-absorbing product had a retention time on the column identical to that of HPR. The effect of dietary CGT on the biliary excretion of HPR-*O*-glucuronide in female rats is summarized in Fig. 5. The control rats excreted HPR and HPR-*O*-glucuronide in the bile in much higher amounts than did rats pretreated with CGT, indicating that CGT pretreatment significantly suppressed the biliary excretion of both components. The differences between the control and treated rats were statistically significant for HPR at 17 hr and for HPR-*O*-glucuronide at 9 hr ($P < 0.05$).

DISCUSSION

The results of the present investigation strongly suggest that low dosages of CGT and of HPR act synergistically to inhibit $Me_2B[a]A$-induced rat mammary carcinogenesis. Although the lowest dosages tested in combination were CGT at 32 mmol/kg and HPR at 0.75 mmol/kg, the similarity of the effects observed with CGT at 32 and 64 mmol/kg suggests that dosages <32 mmol/kg may be equally effective. Whether the dosages of either CGT or HPR can be lowered further without serious loss of efficacy will be dependent upon the nature of their interaction. If, for example, at the lowest concentrations tested, HPR is the actual effector (inhibitor) and CGT has an adjuvant role, then the concentration of HPR will be more critical than that of CGT. Through a further series of long-term carcinogenesis experiments in which the dosages of HPR and CGT in the diet are varied independently over a wide range, it should be possible to elucidate the nature of the synergistic interaction.

That HPR may be the effector with CGT acting in an adjuvant role is suggested by evidence indicating that a large fraction of the HPR excreted in the bile is glucuronidated (10) and that retinoid-glucuronides may be biologically active metabolites (17–20). The biosynthesis of 13-*cis*-retinoic acid and of all-*trans*- and 13-*cis*-retinoyl glucuronides has been identified in the intestinal mucosa of the rat (17). When tested

Table 1. Effect of dietary CGT and HPR on $Me_2B[a]A$-induced rat mammary tumorigenesis

Dietary supplement, mmol/kg of diet		Tumor incidence, % of animals with tumors	Tumor multiplicity, no. of tumors per rat
CGT	HPR		
None	None	70	1.00 ± 0.01
32	None	55	1.00 ± 0.12
64	None	55	1.00 ± 0.05
128	None	35	0.50 ± 0.02
None	0.75	65	1.10 ± 0.10
None	1.50	30	0.35 ± 0.06*
32	0.75	35†	0.50 ± 0.15*
64	0.75	30†	0.45 ± 0.10*

Rats were fed chow diets supplemented with CGT or HPR, as indicated, from 2 weeks before treatment with $Me_2B[a]A$ at 70 mg/kg until the end of the experiment. The data represent the number of tumors 18 weeks after $Me_2B[a]A$ treatment. Tumor multiplicity data are expressed as number of tumors per rat (mean ± SEM).
*Statistically significant difference from control group and from groups treated with CGT or with HPR ($P < 0.002$).
†Statistically significant difference from control group and from groups treated with CGT or with HPR ($P < 0.004$).

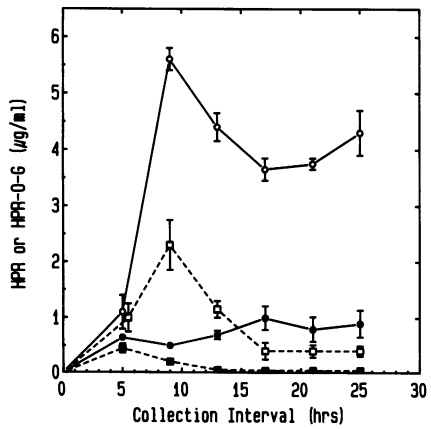

FIG. 5. Time course of excretion of HPR and HPR-*O*-glucuronide (HPR-*O*-G) in the bile of control rats or CGT-treated rats receiving HPR at the time of completion of bile-duct cannulation (zero time). The CGT was administered 4.0 hr before HPR. The data points represent the end of each bile collection interval. Shown is HPR excretion in bile of control (●) and of CGT-treated (■) rats and HPR-*O*-glucuronide excretion in control (○) and in CGT-treated (□) rats. Data are expressed as mean ± SEM, based on three experiments.

in tissue culture against the HL-60 cell line, 1.0 μM retinoyl β-glucuronide inhibited cell proliferation by 55–75% and induced 38–50% of the cells to differentiate (18, 19). The glucuronide of all-*trans*-retinoic acid was 50% less toxic than the parent compound, and the glucuronide is proposed as a more effective alternative to the free retinoid as a cancer chemotherapeutic agent (18). Furthermore, in rats, one of the biologically active forms of vitamin A has been identified as retinoyl β-glucuronide (20).

The results of studies described in the present communication suggest that there is a synergistic interaction between CGT and HPR. Since CGT undergoes a slow partial conversion in the stomach to the potent β-glucuronidase inhibitor D-glucaro-1,4-lactone (12–15), it is possible that CGT enhances the net glucuronidation of HPR, as was shown for bilirubin (15). Therefore, through enhancement of glucuronidation, CGT may cause longer retention of HPR in extrahepatic tissues, in general, and the mammary gland of the female rat, in particular. These results are in accord with the tendency of HPR to concentrate in the mammary gland (5). The concentration of the HPR-*O*-glucuronide in extrahepatic tissues with slow release to circulation of the free HPR as well as reconjugation and excretion of the glucuronide in the bile by the liver would be consistent with the kinetics of appearance of the HPR-*O*-glucuronide in the bile. Other mechanisms of action of CGT that are protective to the organism cannot be ruled out.

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