

## Effect of dietary calcium glucarate on 7,12-dimethylbenz(a)anthracene-induced skin tumorigenesis in CD-1 mice

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■ Dietary calcium glucarate was evaluated as an anti-initiating and anti-promoting agent against skin tumorigenesis in CD-1 mice. A diet supplemented with 4% calcium glucarate inhibited papilloma formation by approximately 50% when fed during either the 7,12-dimethylbenz(a)anthracene-mediated initiation or 12-O-tetradecanoylphorbol-13-acetate-mediated promotion phase of skin tumorigenesis. Papilloma development was decreased in mice receiving calcium glucarate during either the initiation or promotion phases. A calcium glucarate-supplemented diet also inhibited hepatic microsomal  $\beta$ -glucuronidase activity in the mice by about 40%. At the acidic pH of the stomach, calcium glucarate acts as a sustained-release source of D-glucaro-1,4-lactone, a potent  $\beta$ -glucuronidase inhibitor. Both the initiating and promoting agents undergo glucuronidation, therefore, the inhibition of skin carcinogenesis by calcium glucarate is probably due to increased clearance of the initiating and promoting agent by this phase II reaction. However, calcium glucarate may also act by other mechanisms not yet identified.

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**D**IETARY CALCIUM GLUCARATE appears to act as both an anti-initiating and anti-promoting agent in the rodent system. For example, 4% dietary calcium glucarate reduced the frequency of benzo(a)pyrene-induced pulmonary adenomas by 45% when fed only during the initiation phase, by 62% when fed only during the promotion phase, and by 88% when fed during both phases.<sup>1</sup>

Similarly, 10% dietary calcium glucarate was shown to inhibit 7,12-dimethylbenz(a)anthracene(DMBA)-induced rat mammary tumors by 70% when fed during either the initiation or promotion phases.<sup>2</sup> Since calcium glucarate is known to undergo conversion at the

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acid pH of the stomach to the potent  $\beta$ -glucuronidase inhibitor D-glucaro-1,4-lactone, the inhibition is attributed, at least in part, to increased excretion of carcinogens and promoting agents such as glucuronide conjugates. This possibility was supported by the observation that dietary calcium glucarate, one third of which is converted to D-glucaro-1,4-lactone in the stomach, reduced carcinogen binding to target-organ

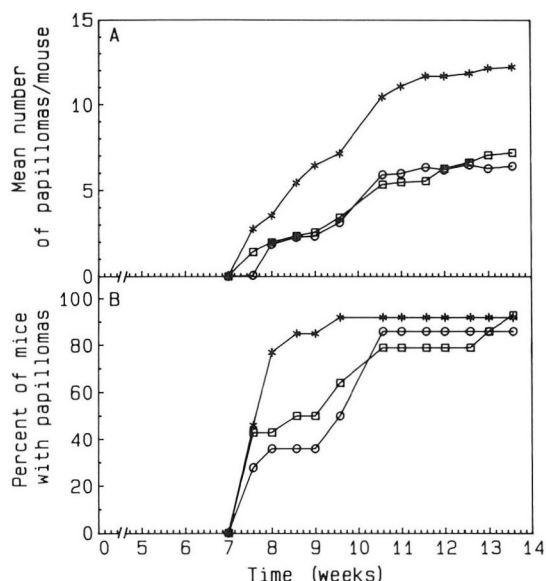


FIGURE 1. The mean number of papillomas per mouse (A) and percent of mice with papillomas (B) were determined over the 13-week period in control mice maintained only on chow diet (asterisks), or in experimental mice maintained on chow plus 4% calcium glucarate only during the initiation phase (circles), or throughout the 12-week promotion phase (boxes). When the experimental groups were compared to the controls using the Student *t*-test, the *P* values were  $<0.05$  at all time points after eight weeks.

DNA and increased the excretion of potential promoting agents, including the steroid sex hormones.<sup>1,2</sup> In contrast to D-glucaro-1,4-lactone, which is rapidly cleared and gives only transitory inhibition of  $\beta$ -glucuronidase in vivo, dietary calcium glucarate maintains  $\beta$ -glucuronidase levels at or below 60% of controls for four to five hours.<sup>1</sup> The sustained-release nature of that calcium salt of glucarate is due to its insolubility.

The aim of this study was to evaluate the effect of dietary calcium glucarate on the DMBA-initiated and 12-O-tetradecanoylphorbol-13-acetate (TPA)-promoted skin tumorigenesis in CD-1 mice. This system has several advantages for such an evaluation. First, it is known to consist of a finite irreversible initiation phase, followed by a prolonged promotion phase under conditions where the initiating agent is applied at a sub-threshold dose.<sup>3</sup> Second, both the initiating agent (DMBA) and the promoting agent (TPA) are known to undergo glucuronidation.<sup>4,5</sup> Therefore, in contrast to earlier systems tested where the nature of the endogenous promoting agent was not rigorously defined, as in lung or mammary tumorigenesis, in this system the development of skin papillomas is strictly dependent on

promotion by TPA.

#### MATERIALS AND METHODS

Female CD-1 mice approximately 30 days old were purchased from Charles River Labs in Wilmington, MA. The mice were randomly assigned to three groups of 15 mice each and were maintained on chow or powdered chow fortified with 4% (w/w) calcium glucarate. A control group C was maintained on mouse chow throughout the experiment. To study the effect of dietary calcium glucarate (calcium saccharate) on the DMBA-initiation phase, the mice in group I were fed the 4% calcium glucarate-containing diet from two weeks before to one week after DMBA treatment. Similarly, to study its effect on TPA promotion, the mice of group P were switched from the chow to the 4% calcium glucarate-supplemented diet one week after treatment with the carcinogen and were maintained on the supplemented diet until termination of the experiment 13 weeks later.

Except for diet, all mice received the same treatment. The protocol selected for these studies, while using a subthreshold dose of DMBA as initiator in combination with TPA promotion, induced papillomas at sufficiently high frequency to permit the detection of low levels of inhibition. A single dose of 200 nmol of DMBA in 100  $\mu$ L of acetone was applied to the shaved back of each mouse under a yellow light. The DMBA-treated mice were housed in containment hoods for one week, whereupon 10 nmol of the promoting agent in 100  $\mu$ L of acetone was applied twice weekly (Tuesday and Friday) to the DMBA-treated area for the duration of the experiment. The backs were shaved carefully, avoiding any cuts, once a week. Papilloma counts were made twice weekly.

The  $\beta$ -glucuronidase activity was assayed in the liver homogenate or in the microsome fraction prepared from liver homogenate using a commercial kit. This assay is based on the hydrolysis of phenolphthalein glucuronide and the quantitation of the phenolphthalein in alkaline medium. The activity is expressed in modified Sigma units (MSUs) where 1.0 MSU is the activity that liberates 1.0  $\mu$ g of phenolphthalein from its glucuronide conjugate in one hour at 56°C. When the whole homogenate was assayed directly, the mouse liver was homogenized in 50 mM Tris-HCl (pH, 7.4). Alternatively, the microsomes were prepared by homogenizing the liver in 0.25 M sucrose, centrifuging for 10 minutes at 12,000 g, then centrifuging the supernatant for 60 minutes at 100,000 g, recovering the pellet, and resuspending the microsomes in 0.15 M KCl.  $\beta$ -glu-

**TABLE 1**  
EFFECT OF DIETARY CALCIUM GLUCARATE ON LIVER MICROSOMAL  $\beta$ -GLUCURONIDASE IN CD-1 MICE\*

Phase on diet	Dietary calcium glucarate		$\beta$ -glucuronidase activity	
	% currently in diet	Hours after feeding	Mean $\pm$ S.E.	Inhibition
None (C)	0	6	18.8 $\pm$ 2.1	—
Initiation (I)	0	6	19.6 $\pm$ 3.4	0
Promotion (P)	4	5	14.8 $\pm$ 0.5	14
	4	6	9.9 $\pm$ 1.4	43 <sup>†</sup>

\* At the termination of the 14-week skin carcinogenesis experiment, mice in groups C, I, and P fasted for 18 hrs, then returned to their diets as indicated.  $\beta$ -glucuronidase activities were identical when determined with or without triton X-100.

<sup>†</sup> Statistically significant ( $P < 0.05$ ).

curonidase was assayed in the homogenates or microsome fraction with or without the addition of triton X-100 (0.2  $\mu$ L of 15% [v/v]/mg protein).

At the termination of the 13-week experiment,  $\beta$ -glucuronidase activity was assayed in each group of mice to demonstrate directly the effect of dietary calcium glucarate. All mice fasted for 18 hours before they received the diets outlined above, then were killed after either five or six hours for evaluation of liver  $\beta$ -glucuronidase activity. Food intake of each group was essentially equivalent.

## RESULTS

Shown in *Figure 1A* is the time course of induction of skin papillomas in the CD-1 mice after a single application of DMBA, followed by twice weekly applications of TPA. Papillomas began appearing at seven weeks and accumulated to an average of 12 per mouse at approximately 12 weeks after carcinogen treatment in the control group (C). The mean number of papillomas per mouse was approximately 50% of group C in both group I, which was fed the calcium glucarate-supplemented diet only during the initiation phase, and in group P, which was fed the supplemented diet throughout the promotion phase. The decrease in the number of papillomas in both groups I and P was statistically significant ( $P < 0.05$ ) after eight weeks. As indicated in *Figure 1B*, the number of papillomas in control group C was a maximum of approximately 90% within nine to 10 weeks. In contrast, papilloma development was delayed in those mice receiving calcium glucarate during either the initiation (group I) or the promotion (group P) phases, maxima of 85% and 80%, respectively, that were attained one to two weeks later than in the control group. In one experiment, animals from group P were returned to normal chow diet. This resulted in a rapid increase in the number of papillomas, reaching close to the number

for the control group within two weeks.

Calcium glucarate is bland-tasting and non-toxic. Both components of this compound are normally present in the body. Incorporation in amounts up to 10% (w/w) does not affect food intake by rodents.<sup>2</sup> At the termination of the experiments described above, the average weight of the mice in groups C, I, and P was 27.0, 26.9, and 27.2 g, respectively. This suggests food intake by the CD-1 mice was not affected by supplementing the chow diet with 4% calcium glucarate, a conclusion reached earlier with A/HeJ mice by Walaszek et al.<sup>1</sup> Because of the low activity per milligrams protein in mouse skin and difficulty in isolating a sufficient microsome fraction from this organ, the effect of dietary calcium glucarate on microsomal  $\beta$ -glucuronidase was assayed in microsomes isolated from the liver at the termination of the experiment at 13 weeks. Initial measurements in the total homogenate indicated little difference among the C, I, and P groups. However, as shown in *Table 1*, when only the microsomal enzyme was measured, significant inhibition (43%,  $P < 0.05$ ) was observed at six hours in group P, receiving calcium glucarate. Groups C and I, receiving chow only, were similar. Nearly the same results were observed whether or not triton X-100 was included in the assay. These results suggest that the D-glucuro-1,4-lactone formed from calcium glucarate is accessible to the microsomal, but not to the lysosomal,  $\beta$ -glucuronidase. A similar pattern was also observed earlier in a time-course study in rats treated with D-glucaro-1,4-lactone and calcium glucarate.<sup>6</sup>

## DISCUSSION

The results of this study show that dietary calcium glucarate can reduce skin carcinogenesis by inhibiting either the initiation or promotion phases. The specific protocol selected used a DMBA dosage to induce a sufficiently high number of papillomas (i.e., a maximum of



12 per mouse in the controls) to permit detection of even low levels of inhibition. However, this DMBA dose (200 nmol per mouse) does not exhibit promoting activity so that the subsequent appearance of papillomas is dependent on promotion with TPA.<sup>3</sup> Since both DMBA and TPA are known to undergo glucuronidation, it seemed possible that both the initiation and promotion phases would be affected in this system. Both phases were, in fact, inhibited by approximately 50% in CD-1 mice when 4% calcium glucarate was included in the diet.

In contrast to the CD-1 mouse system, preliminary experiments with the SENCAR mouse model have not given consistent results. Apparently, the SENCAR mouse skin is more sensitive to the calcium (Dwivedi C, Webb TE, unpublished observation).

At an acidity equivalent to that in the stomach, glucarate is known to undergo conversion to form at equilibrium equimolar mixtures of glucarate, D-glucaro-2,6-lactone, and D-glucaro-1,4-lactone, only the latter being a  $\beta$ -glucuronidase inhibitor.<sup>7</sup> The sustained release of D-glucaro-1,4-lactone is attributed to the water insolubility of calcium glucarate, which dissolves slowly in the stomach. D-glucaro-1,4-lactone and glucaric acid are normally present in most tissues of the body,<sup>7-9</sup> and the metabolism of glucuronolactone to glucarate has been studied in skin.<sup>9</sup> The ability of D-glucaro-1,4-lactone itself to inhibit carcinogenesis has been demonstrated by administering the synthetic precursor 2,5-di-O-acetyl-D-glucaro-1,4:6,3-dilactone, which is completely hydrolyzed in the stomach to the  $\beta$ -glu-

curonidase inhibitor.<sup>10</sup>

The finding that only the microsomal  $\beta$ -glucuronidase is inhibited in response to dietary calcium glucarate is of interest in view of the report by Lloyd and Forster<sup>11</sup> that entry of such compounds into the lysosomes is limited. Thus, the observed inhibition of microsomal  $\beta$ -glucuronidase in liver may be relevant to the inhibition of chemical carcinogenesis observed in these and other systems, assuming the enzyme is inhibited to the same extent in mouse skin. It should be emphasized that although the amount of  $\beta$ -glucuronidase in skin is small (approximately 10% of that in liver), the total activity in this organ is significant because of its large surface area.<sup>12</sup> Both the microsomal  $\beta$ -glucuronidase, bound to the cytoplasmic membrane system by the protein egasyn, and the lysosomal enzyme are coded by the same gene.<sup>13</sup> Despite this similarity, carcinogens, promoters,  $\beta$ -glucuronidase inhibitors, and the like may only be accessible to the microsomal enzyme. D-glucaro-1,4-lactone is bound with high affinity to  $\beta$ -glucuronidase and can only be released by extensive dialysis.<sup>7</sup>

Other chemopreventative agents, such as ellagic acid, inhibit 3-methylcholanthrene-induced skin tumorigenesis in mice by inhibiting the metabolic activation of this polycyclic aromatic hydrocarbon to a carcinogenic metabolite.<sup>14</sup> Since the generation of a reactive metabolite is essential for carcinogenicity, the risk of chemically induced cancers can be reduced by modulating the activity of enzymes that are crucial for both the metabolic activation and inactivation pathways.

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