JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Inhibition of Rat Mammary Tumorigenesis by Concord Grape Juice Constituents

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The effects of Concord grape juice constituents on the promotion of chemically induced rat mammary tumor development and on the proliferation of a rat mammary adenocarcinoma cell line were studied. Isocaloric grape juice formulations provided in the drinking fluid of rats at concentrations of 489 and 651 mg of phenolics/dL of fluid significantly inhibited mammary adenocarcinoma multiplicity compared to controls. Final tumor mass also was significantly decreased for animals provided these two grape juice concentrations compared to controls. In addition, DNA synthesis of the rat mammary adenocarcinoma RBA cell line was significantly inhibited in a dose-dependent manner for cells treated with a grape extract, with an IC_{50} dose of ~14 μg of phenolics/mL. This inhibition of DNA synthesis was not accompanied by changes in 8-oxodeoxyguanosine formation or by substantial cell cycle arrest. These studies thus indicate that Concord grape juice constituents can inhibit the promotion stage of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced rat mammary tumorigenesis, in part by suppressing cell proliferation.

KEYWORDS: Grape; breast cancer; 7,12-dimethylbenz[*a*]anthracene

INTRODUCTION

Breast cancer is a leading contributor to cancer-related deaths in the United States. Approximately 180,000 new cases and 40,000 deaths due to breast cancer were reported in the United States in 2000. It has been estimated that a considerable portion of cancer incidence and mortality may be associated with dietary behavior (*1*). In particular, an increased intake of fruits and vegetables continues to be a factor that is associated with decreased cancer incidence and mortality (*2*, *3*). In light of this epidemiologic data, the field of cancer chemoprevention has emerged, in which these natural as well as synthetic chemicals are being identified for use in inhibiting or reversing carcinogenesis (*4*, *5*). Cancer preventive phytochemicals have been shown to suppress or block carcinogenesis by a variety of mechanisms including acting as antioxidants or antiproliferative agents (*6*, *7*). Among the many classes of plant chemicals studied in this regard, phenolic compounds have been identified as anticancer agents and are consumed by humans in a variety of plant foods and beverages, such as tea (*8*). Other dietary sources of naturally derived bioactive phenolics that are widely consumed are grapes and grape-derived beverages. Interest in the health benefits of grape products has increased markedly in large part due to recent reports that several constituents of grapes or grape extracts may be potential cancer chemopreventive agents (*9*, *10*). For example, resveratrol, a stilbene phytoalexin and antioxidant found in grapes, has been reported to inhibit neoplastic cell proliferation by altering receptor- and non-receptor-mediated cell signaling pathways (*11*). Grape proanthocyanidins present in the skin and seeds also have been identified as inhibitors of neoplastic cell growth (*12*−*15*). Piceatannol, a structural analogue of resveratrol, has cancer-suppressing actions as well (*16*, *17*). Although less well studied as bioactive components of grapes, the anthocyanin pigments nonetheless are receiving increased scrutiny for their cancer preventive actions (*18*).

Although many of the collective benefits are attributed to the phytochemicals present in grapes, little is known about the capacity of the components of the richly pigmented Concord grape to inhibit breast carcinogenesis. Therefore, the present studies were conducted to determine the capacity of dietary Concord grape juice to inhibit breast carcinogenesis in an animal

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10.1021/jf030278l CCC: $25.00
Published on Web 11/01/2003

model. The in vivo influence of Concord grape juice consumption on the promotion stage of 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced rat mammary tumorigenesis was examined, in particular, and was followed by an in vitro experiment to evaluate the antiproliferative action of a Concord grape extract toward a rat mammary adenocarcinoma cell line.

MATERIALS AND METHODS

Reagents. AIN-93G diet ingredients were obtained from Harlan Teklad (Madison, WI). Grape juice concentrate and grape color extract were obtained from Welch's Foods Inc. (Billerica, MA). The grape color extract was freeze-dried for use in cell culture experiments. This dried extract was chosen for the in vitro studies because of its lower content of carbohydrates and organic acids compared to the grape juice concentrate. Glucose, fructose, malic acid, tartaric acid, DMBA, RNase A, phosphate-buffered saline (PBS), propidium iodine, NP-40, trichloroacetic acid, and standards and reagents for phenolic acid analyses (gallic acid, Folin−Ciocalteu reagent, and sodium carbonate) were obtained from Sigma-Aldrich (St. Louis, MO). All HPLC reagents (phosphoric acid, glacial acetic acid, and acetonitrile) were of HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ).

Preparation of Dried Grape Color Extract. The procedure for the freeze-drying of the grape color extract was performed with the assistance of the Department of Food Science and Human Nutrition at the University of Illinois. Trays with grape color extract were placed into a Virtis Unitop 800L (with Virtis Freezemobile 12) chamber precooled to a temperature of −45 °C (with the Freezemobile condenser set to −80 °C) and the pressure reduced to 30 mTorr. Samples were allowed to dry at −45 °C and a pressure of 30 mTorr for a period of 2−3 h. After this period, the temperature of the chamber was increased stepwise to −10 °C over 48 h. Following completion of the drying, the resultant powder was packaged, sealed, and frozen at −20 °C. This procedure concentrates phenolics ∼4.3-fold (w/w).

Grape Juice Analyses. Monomeric anthocyanin content was determined according to the pH-differential method described by Giusti and Wrolstad (*19*). Polymeric color and color density were determined by using the bisulfite bleaching method (*19*). Pigment content was calculated as cyanidin 3-glucoside, using an extinction coefficient of 26900 L cm^{-1} mol^{-1} and a molecular weight of 449.2 g mol^{-1} (*20*). Anthocyanins were separated by a Symmetry C_{18}, 5 μm, 4.6 × 150 mm column (Waters Corp., Milford, MA), fitted with a 22 × 4.6 mm Symmetry 2 micro guard column (Waters Corp.) using a Waters Delta 600 high-pressure liquid chromatograph (HPLC), equipped with a Waters 996 photodiode array detector, a Waters 717 plus autosampler, and Millenium^{32} software (Waters Corp.). Samples were eluted with a linear gradient from 0 to 35% B in 35 min with A = 1% phosphoric acid, 10% acetic acid, 5% acetonitrile, 84% water and B = 100% acetonitrile. Spectra and retention times of each peak were compared to those of a commercial sample of concord grape juice concentrate of known composition (*21*).

Total phenolics were measured using a modification of the Folin−Ciacalteu method for total phenol analysis (*22*, *23*) and were calculated as gallic acid equivalents based on the gallic acid standard curve.

For resveratrol and piceatannol measurements a sample of grape juice concentrate (2.0090 g) was added to17 mL of methanol. To a portion of the lyophilized grape color extract was added 1 mL of deionized water and vortex-mixed, and then 17 mL of methanol was added. The mixtures were stir mixed using a stir bar for 16 h and then centrifuged at 3000*g* for 5 min. The supernatant was collected. The residue was rinsed once with 10 mL of methanol and centrifuged for 5 min at 3000*g*. The supernatant was collected and combined with the supernatant from the first extraction. The combined supernatant was dried under vacuum using a Savant Speedvac. An aliquot of the dried extract was used directly for analysis of resveratrol and analogues by gas chromatography−mass spectrometry (GC-MS).

To determine the occurrence of glycosylated resveratrol, to another portion of dried extract was added 10 mL of deionized water, the pH was adjusted to 6 using 0.1 M NaOH, and then the mixture was subjected to enzymatic hydrolysis by incubation with 18 mg of β-D-glucosidase (4.0 units/mg; Sigma-Aldrich) at 37 °C for 18 h (*24*). Thereafter, the solution was extracted with 10 mL of ethyl acetate (four times), and the combined extracts were evaporated to dryness in a Rotavap. The dried ethyl acetate extract was used for GC-MS analysis of resveratrol and analogues.

For GC-MS analysis of resveratrol and analogues, 1 mg of methanol or ethyl acetate extract in a GC vial was added to 100 μL of derivatizing reagent [bis(trimethylsilyl)trifluoroacetamide/dimethylformamide/methanol, 3.5:1:0.5]. The vial was capped and heated at 70 °C in a heating block for 1 h. After cooling to room temperature, the sample was analyzed by GC-MS on a JEOL (JEOL USA, Inc., Peabody, MA) GCMate II system. The GC temperature program was as follows: initial, 150 °C; increased to 260 °C at a rate of 25 °C/min, increased to 270 °C at a rate of 1 °C/min, increased to 320 °C at a rate of 60 °C/min; and held at this temperature for 2 min. The GC capillary column used was a ZB-50 (0.25 mm i.d., 0.25 mm film thickness, 30 m length; Phenomenex, Torrance, CA). The carrier gas was ultrahigh-purity helium (nexAir, Batesville, MS) at a 1 mL/min flow rate. The inlet (splitless), GC interface, and ion chamber temperatures were 250, 250, and 200 °C, respectively. The volume of sample injected was 2 μL.

Resveratrol, piceatannol, and pterostilbene (retention times of 8 min, 41 s; 9 min, 29 s; and 10 min, 8 s, respectively) were analyzed in a selected ion monitoring mode. Resveratrol was monitored for *m*/*z* 444 (and 429, 371, and 355 as qualifier ions). Piceatannol was monitored for *m*/*z* 532 (and 517, 444, and 429 as qualifier ions). Pterostilbene was monitored for *m*/*z* 328 (and 313, 297, and 281 as qualifier ions). Quantitation was done using external standards of commercial samples of resveratrol (Sigma-Aldrich) and piceatannol (Calbiochem-Novabiochem Corp., San Diego, CA) and a synthetic sample of pterostilbene.

Tumor Study. Female Sprague−Dawley rats were obtained from Harlan Sprague−Dawley, Inc. (Indianapolis, IN) at 40 days of age, housed individually in wire-bottom cages in rooms with controlled temperature, humidity, and lighting, and provided an AIN-93G semipurified diet ad libitum. Each animal was administered a single dose of DMBA (37 mg/kg of body weight, ig, in corn oil) at 50 days of age. Beginning 1 week post-DMBA, animals were randomized into four groups and fed for the remainder of the study a Concord grape juice-supplemented drinking fluid containing total phenolics at 0 (n = 32 rats), 326 (n = 29 rats), 489 (n = 29 rats), and 651 (n = 29 rats) mg/dL concentrations. The latter three fluids were obtained by preparing dilutions of grape juice concentrate/water as 1:3, 1:2, and 2:1, respectively. All drinking fluids were isocaloric, with concentrations of glucose and fructose calculated as 194.6 mg/mL and of malic and tartaric acids as 16 mg/mL. Animals were weighed weekly and palpated weekly beginning at week 8. At the termination of the study tumors were removed and classified histopathologically (*25*).

Cell Proliferation Measurement. The RBA cell line was obtained from the American Type Culture Collection (Manassas, VA) and is an adenocarcinoma cell line isolated from a DMBA-induced rat mammary tumor. Cells were grown in Corning T-75 tissue culture flasks containing MEM medium plus 10% fetal bovine serum. Samples of freeze-dried grape color extract were added to cell cultures at phenolics concentrations of 0, 14, 28, 41, and 55 μg/mL and were made such that concentrations of glucose, fructose, malic acid, and tartaric acid were equivalent among all groups. Cell proliferation was determined by [^{3}H]thymidine incorporation according to the assay of Denton (*26*). Briefly, 4 × 10^3 RBA cells/mL were added to Corning 48 well plates, allowed to attach overnight, and then treated for 48 h with control and grape color extract-supplemented media. After 48 h, 1 μCi of [^{3}H]-thymidine was added to each well for 24 h, followed by 5% trichloroacetic acid. The precipitate was washed with absolute methanol and treated with 23.6 M formic acid, and radioactivity was quantitated by liquid scintillation.

Cell Cycle Analyses. For cell cycle analyses, RBA cells were treated with either 0 or 55 μg/mL phenolics from the grape color extract for 24, 48, or 72 h. Cells in PBS were fixed in 70% cold ethanol, stained by the addition of 0.1 mg/mL RNase A, 0.25 mg/mL propidium iodine, and 0.1% NP-40 in PBS, and analyzed by flow cytometry using an EPICS XL-MCL four-color flow cytometry analyzer (Beckman-Coulter International, Miami, FL) with EPICS XL System II version 3.0 software.

Table 1. Anthocyanin Profile of Grape Extracts[a]

peak	name	RT	% area: color extract	% area: concentrate
1	Dpd-3-glu	3.59	31.2	26.4
2	Cyd-3-glu	5.29	15.5	12.4
3	Ptd-3-glu	6.68	8.1	10.2
4	Pnd-3-glu	10.0	2.8	2.2
5	Mvd-3-glu	11.51	3.7	4.7
6	Dpd-3,5-diglu-p-coum[b]	14.05	1.5	4.5
7	Cyd-3,5-diglu-p-coum	16.79	5.5	9.0
8?, 9?	Ptd-3,5-diglu-p-coum[b]	18.48	2.8	4.7
10	Dpd-3-glu-p-coum	19.75	15.1	10.6
11, 12	Pnd-3,5-diglu-p-coum and Mvd-3,5-diglu-p-coum	21.03	1.3	5.2
13	Cyd-3-glu-p-coum	21.68	5.1	3.2
14	Ptd-3-glu-p-coum[b]	22.19	2.7	2.4
15	Mvd-3-glu-p-coum[b]	24.41	1.1	1.1
	other acylated acns		2.8	4.4
	total nonacylated anthocyanins		60.6	55.3
	total acylated monoglucosides		23.7	17.2
	total acylated diglucosides		12.8	23.1

[a] RT, retention time; Dpd, delphinidin; Cyd, cyanidin; Ptd, petunidin; Pnd, peonidin; Mvd, malvidin; glu, glucoside; diglu, diglucoside; p-coum, acylation with *p*-coumaric acid. [b] Tentative peak assignments.

8-Oxodeoxyguanosine (8-Oxo-dG) Determination. RBA cell pellets were collected following treatment of cells with 0, 28, and 41 μg/mL grape color extract. DNA was subsequently isolated, digested, and 8-oxo-dG quantitated by LC-MS/MS as described as by Hua et al. (*27*).

Statistical Analyses. Statistical significance among treatment means was determined by ANOVA with Fisher's least significant difference test for post-hoc comparisons. Significant differences between tumor incidence values was determined using the chi-squared test. Differences were considered to be statistically different at $p < 0.05$.

RESULTS

Composition of Grape Extracts. Anthocyanin profiles and content of the grape juice concentrate and grape color extract are presented in **Table 1**. The grape juice concentrate and color extract both exhibited similar proportions of nonacylated and acylated anthocyanins. Monomeric anthocyanin pigments were 12 and 33% of the total phenolics for the grape juice concentrate and grape color extract, respectively. Delphinidin, cyanidin, and petunidin 3-glucosides were the major anthocyanins present, constituting ~50% of the total anthocyanin content. As expected, the grape color extract had high monomeric anthocyanin content and color density (**Table 2**), containing ~3 g of pigment per 100 g of the freeze-dried powder. The resveratrol concentrations of grape juice concentrates were 253.7 and 21.7 μg/mL for samples with and without glucosidase treatment, respectively. The resveratrol contents of the freeze-dried grape color extracts were 36.0 and 2.8 μg/g for samples with and without glucosidase treatment, respectively. Piceatannol concentrations of the grape concentrates were 25.8 and 18.3 μg/mL for samples with and without glucosidase treatment, respectively, and in the grape color extracts were 6.4 and 9.2 μg/g for samples with and without glucosidase treatment, respectively. Because the piceatannol values did not differ significantly between those with and without enzyme hydrolysis, it can be inferred that the piceatannol did not occur as a glycoside.

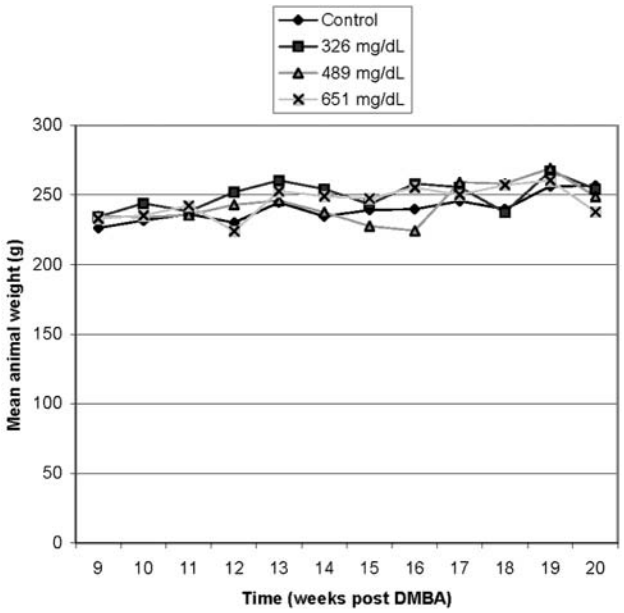

Figure 1. Effect of grape juice consumption on animal growth.

Tumor Study. There was no significant difference in body weights among groups fed the grape juice-supplemented drinking fluid throughout the study (**Figure 1**). On the basis of a daily consumption of ~10 mL of fluid/animal, rats fed the 326, 489, and 651 mg/dL fluids obtained about 33, 49, and 65 mg of grape phenolics/day, respectively. Cumulative palpable mammary tumor incidence was not significantly affected by consumption of the grape juice containing fluids, except for weeks 9 and 10 (**Figure 2**). For week 9, rats fed the 489 and 651 mg/dL diets exhibited significant 84 and 69% reductions in tumor incidence, respectively, compared to controls. At week 10, only rats fed the 489 mg/dL diet exhibited a significant 55% decrease in tumor incidence compared to controls. Histopathologic examination indicated that adenocarcinomas constituted 96% of all tumors. The remaining tumors were identified as adenomas, fibroadenomas, and hyperplasic lesions. The trends for adenocarcinoma multiplicity measured for weeks 9−20 were significantly lower (by two-way ANOVA) for rats fed the 489 and 651 mg/dL drinking fluids compared to controls (**Figure 3**). Tumor mass measured at the end of the experiment (**Figure 4**) decreased significantly by 28 and 36% for animals fed the 489 and 651 mg/dL fluids, respectively, compared to controls.

Cell Proliferation Studies. The proliferation of RBA cells as measured by [^{3}H]thymidine incorporation was significantly

Table 2. Phenolic Concentration and Color Density of Grape Extracts

sample	monomeric anthocyanins (mg/100 mL)	color density	polymeric color (%)	total phenolics (mg/100 mL)	anthocyanins[a] (%)
grape juice concentrate	117	60	16.2	977	12
grape color extract	2880[b]	1300	13.5	8770[b]	33

[a] Anthocyanin % is the percent of total phenolics represented by anthocyanins. [b] mg/100 g.

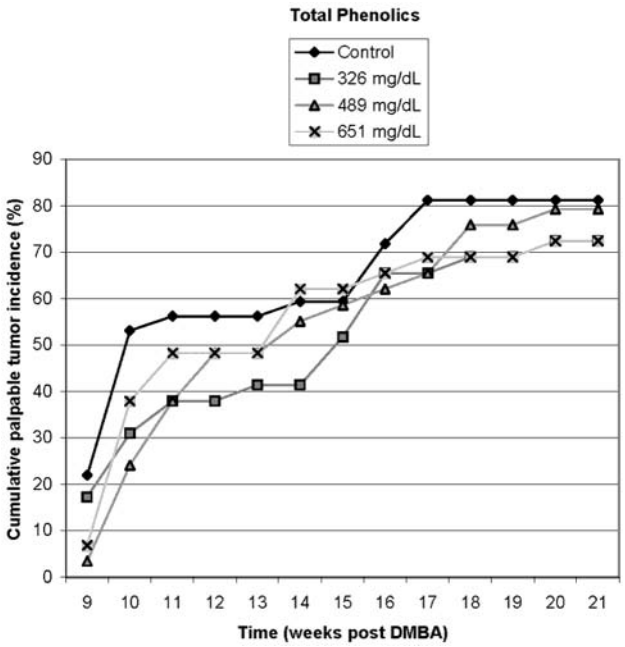

Figure 2. Effect of grape juice consumption on cumulative palpable mammary tumor incidence. After week 17, incidence curves for the animals fed the 326 and 651 mg/dL diets are identical.

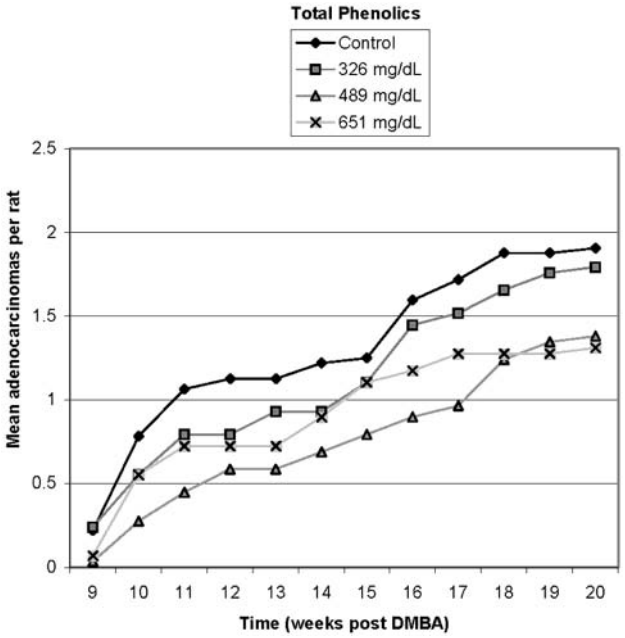

Figure 3. Effect of grape juice consumption on adenocarcinoma multiplicity. Multiplicity trends during weeks 9–20 for rats fed the 489 and 651 mg/dL fluids were significantly different from controls. The numbers of rats per group were 32, 29, 29, and 29 for animals fed the 0, 326, 489, and 651 mg/dL fluids, respectively.

reduced by 48, 64, 83, and 91% for cells treated with the color extract at 14, 28, 41, and 55 μg/mL concentrations, respectively, compared to controls (**Figure 5**). There was no decrease in oxidative DNA damage as measured by 8-oxo-dG formation for cells treated with grape color extract, compared to controls. Values (means ± SD) for cells treated with 0, 28, and 41 μg/mL phenolics were 0.8 ± 0.1, 0.6 ± 0.1, and 0.7 ± 0.2 8-oxo-dG/10^6 dG, respectively. On the basis of flow cytometric analyses, there was a small (16%) but significant accumulation of cells at the G1 phase (**Figure 6**) for cells treated with grape color extract at 55 μg/mL compared to controls.

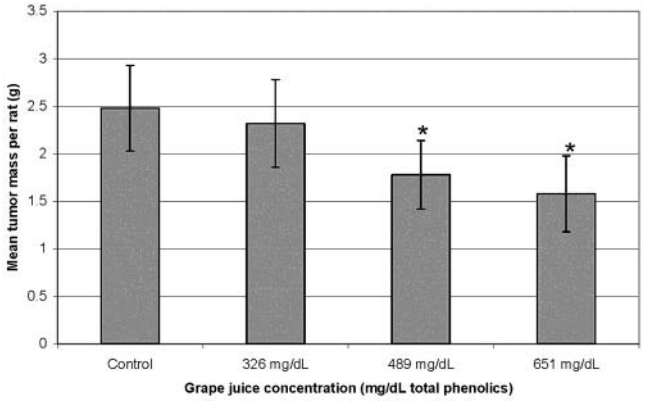

Figure 4. Effect of grape juice consumption on final mammary tumor mass per rat. Values represent means ± SD. *, $p < 0.05$ versus controls.

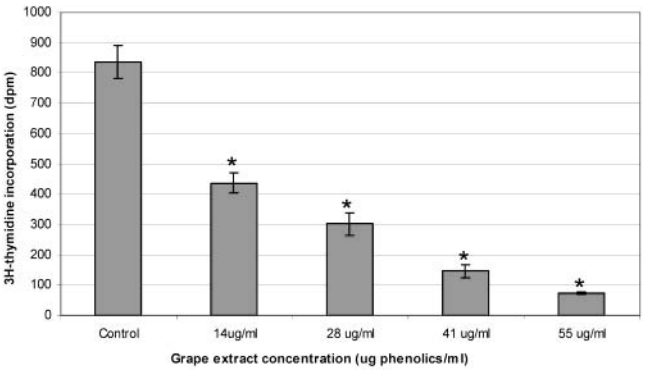

Figure 5. Effect of freeze-dried grape color extract on RBA cell [^{3}H]-thymidine incorporation. Values represent means ± SD for four separate determinations. *, $p < 0.05$ versus controls.

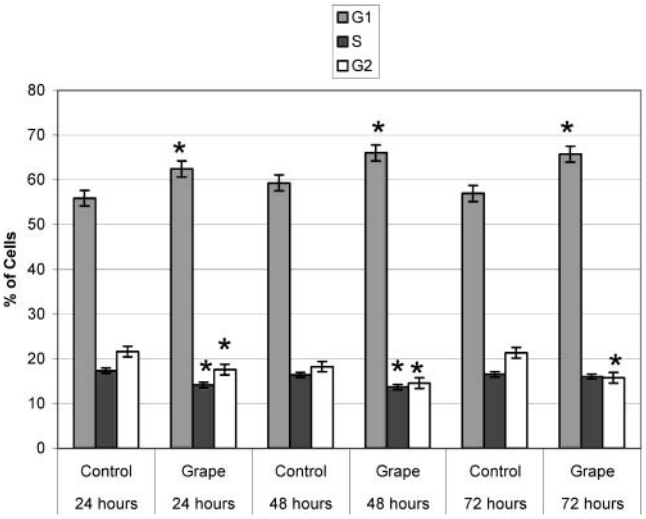

Figure 6. Effect of freeze-dried grape color extract on RBA cell cycle progression. Values are means ± SD for four determinations. *, $p < 0.05$ for treatment versus controls at each time point.

DISCUSSION

The present studies provide evidence that Concord grape juice constituents can inhibit the promotion of chemically induced rat mammary tumorigenesis. Because the studies were designed such that calories, simple sugars, and organic acids were equivalent in all groups, it is likely that phenolic constituents of the grape juice are the major contributors to this anticancer effect. The significant inhibition of tumor multiplicity and mass at the 489 and 651 mg/dL concentrations indicates that juice

constituents in part are suppressing adenocarcinoma proliferation. This is supported by our observation that constituents of the grape color extract significantly inhibited DNA synthesis of RBA cells even at the lowest doses examined. The cancer suppressive action of Concord grape juice in our studies is similar to that observed by Chen et al. (*28*). They reported that nude mice implanted with MCF-7 cells and fed grape juice exhibited a reduction in growth of the MCF-7 xenografts. Our results are also similar to those of others who reported that colored plant extracts inhibited rodent tumorigenesis (*29*−*35*). There are several individual components or combinations of grape constituents that might contribute to the cancer inhibitory action of the grape juice that we observed. Although the composition of the non-anthocyanin phenolics in this juice was not determined, it has been reported that cinnamic acids, flavanols, and polyflavan-3-ols are present (*36*, *37*), all of which have been reported to possess anticancer activity (*38*−*40*). The stilbene resveratrol and its analogue piceatannol were detected in our grape samples. Resveratrol has received considerable attention as a cancer inhibitory agent (*41*−*46*) and in part may be responsible for the effect we observed. Piceatannol is a naturally occurring analogue of resveratrol that can inhibit tyrosine kinase signaling cascades and was identified as a potential cancer chemopreventive agent (*47*, *48*). Ellagic acid has been determined to be a major contributor to the anticancer effects of berry extracts in one study (*35*); however, the amount of this polyphenolic compound in our juice is not known. On the basis of our analysis of the juice and color extract, ∼12−33% of the phenolics were characterized as anthocyanins, with delphinidin being most prominent. These anthocyanins flavonoids are present in other plant products and have been reported to inhibit proliferation and stimulate apoptosis in neoplastic cells (*49*−*53*). Proanthocyanidins are present in grapes and also have been reported to suppress tumorigenesis, although bioavailability and bioefficacy may be limited (*13*, *15*, *54*−*56*). On the basis of our findings and evidence from the literature, the relative magnitude of different phenolic constituents of the Concord grape juice as contributors to the suppression of mammary adenocarcinoma formation warrants further characterization.

Our findings of an inhibition of DNA synthesis and modest block in cell cycle progression for RBA cells treated with grape color extract indicate that one or more phenolic constituents of the extract are acting as antiproliferative agents. The identity of the components is not known. Our findings are similar to those reported by others who observed that polyphenolic compounds from grape products inhibited several breast cancer cell lines (*12*, *57*−*59*). There have been numerous mechanisms by which plant phenolics, including those from grapes, are hypothesized to be acting, including suppression of cellular oxidation, modulation of mitogenic signaling, suppression of aromatase activity, inhibition of inflammatory processes and angiogenesis, and induction of apoptosis (*60*−*76*). On the basis of our analytical data, the extract contains delphinidin and cyanidin glycosides as major anthocyanins, suggesting that anthocyanins warrant further evaluation for potential breast cancer inhibitory actions (*49*, *77*). It should be noted that our data indicate that oxidative DNA damage in RBA cells was not decreased and that only a small accumulation of cells at the G1 stage was associated with treatment of cells with grape color extract. This suggests that this extract likely inhibited RBA cell growth through mechanisms other than antioxidant action and in addition to cell cycle arrest. The impact of this grape extract and its constituents on apoptosis thus warrants further study.

In summary, the present studies indicate that phenolic constituents of grape juice can inhibit the promotion stage of DMBA-induced rat mammary tumorigenesis. Also, rat mammary adenocarcinoma cell proliferation was inhibited by an anthocyanin-rich extract of grape juice, without a substantial change in 8-oxo-dG formation. Grape extract treatment was associated with a modest stage-specific cell cycle arrest. Thus, grape juice constituents, most likely the anthocyanins and other phenolic constituents, have potential for breast cancer prevention. Future research should focus on the characterization of the constituents of the Concord grape phenolic mix in order to identify individual chemopreventive compounds that may be most active and to clarify their mechanisms of action, especially toward induction of apoptosis.

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Received for review April 11, 2003. Revised manuscript received September 11, 2003. Accepted September 12, 2003. This research was funded in part by the Illinois Council on Food and Agricultural Research and the Illinois Agricultural Experiment Station.

JF030278L