[CANCER RESEARCH 63, 8516–8522, December 1, 2003]

Suppression of Estrogen Biosynthesis by Procyanidin Dimers in Red Wine and Grape Seeds

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ABSTRACT

In breast cancer, *in situ* estrogen production has been demonstrated to play a major role in promoting tumor growth. Aromatase is the enzyme responsible for the conversion of androgen substrates into estrogens. This enzyme is highly expressed in breast cancer tissue compared with normal breast tissue. A wine extract fraction was recently isolated from red wine that exhibited a potent inhibitory action on aromatase activity. Using UV absorbance analysis, high-performance liquid chromatography profiling, accurate mass-mass spectrometry, and nanospray tandem mass spectrometry, most of the compounds in our red wine fraction were identified as procyanidin B dimers that were shown to be aromatase inhibitors. These chemicals have been found in high levels in grape seeds. Inhibition kinetic analysis on the most potent procyanidin B dimer has revealed that it competes with the binding of the androgen substrate with a K_i value of 6 μM. Because mutations at Asp-309, Ser-378, and His-480 of aromatase significantly affected the binding of the procyanidin B dimer, these active site residues are thought to be important residues that interact with this phytochemical. The *in vivo* efficacy of procyanidin B dimers was evaluated in an aromatase-transfected MCF-7 breast cancer xenograft model. The procyanidin B dimers were able to reduce androgen-dependent tumor growth, indicating that these chemicals suppress *in situ* estrogen formation. These *in vitro* and *in vivo* studies demonstrated that procyanidin B dimers in red wine and grape seeds could be used as chemopreventive agents against breast cancer by suppressing *in situ* estrogen biosynthesis.

INTRODUCTION

Estrogens play an important role in breast cancer development. Approximately 60% of premenopausal and 75% of postmenopausal patients have estrogen-dependent carcinomas. Aromatase, a cytochrome P450, is the enzyme that synthesizes estrogens by converting C19 androgens into aromatic C18 estrogenic steroids. Our study and the studies of others have shown that there is a high expression of aromatase in breast cancer tissue and in surrounding stroma when compared with normal breast tissue (1–4). When compared to circulating estrogen, *in situ*-produced estrogen has also been shown to play a significant role in breast cancer growth (5, 6). Aromatase inhibitors have been found to be valuable in treating these estrogen-dependent and aromatase-mediated diseases including breast cancer (7). Two new aromatase inhibitors, anastrozole and letrozole, were recently approved by the United States Food and Drug Administration for use as first-line agents against estrogen-responsive cancer in postmenopausal women. In the recent Arimidex, Tamoxifen, Alone or Combination (ATAC) trial, anastrozole was found to be more effective than tamoxifen in the treatment of ER-positive breast cancer in postmenopausal women, and anastrozole treatment was shown to significantly prevent contralateral cancers (8). In addition, letrozole was found to be very effective in treating Her-2-overexpressing and ER-positive breast cancer (9). Therefore, suppression of *in situ* estrogen formation in the breast of postmenopausal women by aromatase inhibitors is considered to be a useful way to prevent and treat breast cancer in these women.

Many epidemiological studies have shown that a diet high in fruits and vegetables can reduce cancer incidence, including that of breast cancer (10–12). Previous studies from our laboratory have found that grape juice and red wine contain chemicals that suppress estrogen biosynthesis *in vitro* as well as *in vivo* (13, 14). Grapes and red wine are a rich source of flavonoids known for their strong antioxidant properties. Over the past several years, multiple studies have looked at the nutritional and pharmacological benefits of wine and grape products. Moderate red wine consumption has also been linked to a decreased risk in cardiovascular disease (15, 16) and to a reduction in cancer risk (17). In the latter report, after adjusting for other sources of alcohol, wine intake among postmenopausal women was found to significantly affect the percentage of breast density, where white wine showed a positive association, and red wine showed an inverse association. Women with very dense breast tissue as determined by mammography have a risk of breast cancer that is 1.8–6 times that of women of the same age with normal density (18). Additional studies have demonstrated that specific components isolated from wine are responsible for its chemopreventive activities (19, 20).

The association of alcohol consumption as a breast cancer risk factor has been established in numerous studies (21). Our results on white wine suggest a weak inductive effect of alcohol on aromatase activity (22). However, the inductive effect of alcohol was not seen when aromatase activity was suppressed by chemicals in red wine. The opposite action of alcohol and phytochemicals in red wine on aromatase activity may complicate the evaluation of the effect of wine drinking on breast cancer. Therefore, we have set our goals to separate the effect of alcohol from the action of phytochemicals in red wine and to identify the chemicals in red wine that have the ability to suppress estrogen biosynthesis. The antiaromatase action of alcohol-free red wine extract has been examined using a transgenic mouse model in which aromatase is overexpressed in the mammary tissues (14). It was found that, similar to the treatment with the selective and potent aromatase inhibitor letrozole, the intake of the extract by gavage completely abrogated aromatase-induced hyperplasia as well as other changes in the mammary tissue. This was a direct demonstration of the chemopreventive effect of red wine chemicals against breast cancer by suppressing *in situ* estrogen formation.

MATERIALS AND METHODS

Preparation of Wine Fractions. One hundred ml of complete red wine (1999 Pinot Noir; Hacienda, Sonoma, CA) were applied to each 5 g/60 ml capacity polyamide column (Discovery DPA-6S SPE; Supelco, Bellefonte, PA). Fractions were eluted by a step gradient (50 ml of each step) of increasing methanol to water. The 70% methanol-water fraction (70M) (50 ml) was rotor

Received 7/16/03; revised 9/8/03; accepted 9/22/03.

Grant support: This work was supported by NIH-National Center for Complementary and Alternative Medicine predoctoral fellowship F31 AT00059 (to E. T. E.), NIH Grants ES08258 and CA44735 (to S. C.), and the California Breast Cancer Research Program 4PB-0115 (to S. C.).

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evaporated to dryness and then redissolved in 2 ml of deionized water to produce the 1× extract or redissolved in 0.67 ml of water to produce the 3× concentrate.

The HPLC[4] system used was a Beckman Gold System Programmable Solvent Module 126 with a Shimadzsu SPD-6A UV spectrophotometric detector set at 214 nm. The active wine extract was separated on a Discovery C18 column (5 μM, 25 cm × 4.6 mm; Supelco) using a shallow gradient at a 1 ml/min flow rate with solvent A (0.1% trifluoroacetic acid; Pierce) and solvent B [90% acetonitrile (Burdick and Jackson), 0.1% trifluoroacetic acid]: 5–10% B (5 min), 10–30% B (60 min), and 30–98% B (15 min).

MS. The accurate mass-mass spectral analysis was performed using a Mariner time-of-flight mass spectrometer (PerSeptive Biosystems, Framingham, MA). Tandem MS of isolated HPLC peaks was performed on a LCQ quadropole ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA) through ESI-MS.

***In Vitro* Aromatase Assay.** Aromatase activity was determined using the tritiated water release method developed by Thompson and Siiteri (23). The assay was performed using placental microsomes or microsomal fractions prepared from aromatase-expressing CHO cells. A detailed procedure was described previously (14). Briefly, the assay reaction mixture (500 μl) contained the substrate [1β-^{3}H(*N*)]androst-4-ene-3,17-dione (specific activity, 24.7 Ci/mmol; 100 nM), microsomal preparations (20 μg), progesterone (10 μM), BSA (0.1%), and potassium phosphate [67 mM (pH 7.4)]. Progesterone was required to suppress endogenous 5α-reductase in the cell homogenates that also consume the androgen substrate. After incubation for 10 min at room temperature, 50 μl of NADPH (12 mM) were added to the mixture and incubated in a 37°C water bath for 10 min. At the end of 10 min, the reaction was stopped by the addition of 500 μl of 5% trichloroacetic acid. After a 10-min centrifugation at 1000 × *g*, supernatants were removed to new glass tubes and mixed with an equal volume of chloroform to remove unreacted substrate. For the second extraction, the upper aqueous phase was transferred to microcentrifuge tubes containing a dextran-charcoal pellet. Charcoal mixtures were vortexed and subsequently pelleted by centrifugation at 15,000 × *g* for 5 min. For each sample, a 300-μl aliquot of the supernatant containing the tritiated water product was mixed with 3 ml of Scintisafe (Fisher's Scientific, Tustin, CA) 30% liquid scintillation mixture and counted in a Tri-Carb Liquid Scintillation Analyzer 1600CA (Packard, Downers Grove, IL).

To determine the aromatase suppression activity of wine chemicals, the assay was performed in the presence of wine fractions at the indicated amounts. Inhibition kinetic analysis on wine chemicals was performed with various concentrations of the substrate androstenedione at 20, 40, 60, 100, and 200 nM.

***In Vivo* Experiments.** In the experiments using intact animals, 5–6-week-old female BALB/c-*nu-nu*, athymic, nonovariectomized mice were purchased (Charles River Laboratories). At about 8 weeks of age, mice received s.c. implant with 5 mg/60 day time-release androstenedione pellets (Innovative Research of America, Sarosota, FL). A week later, mice were individually gavage fed (p.o. force-fed) with 100 μl of water control or 25, 50, or 100 μl of a 1× concentrated wine polyamide extract (in water). Each animal received daily gavage treatment for 42 consecutive days. At 10 weeks old, mice were given two s.c. injections of MCF-7aro cells. These cells were grown in MEM with nonessential amino acids, sodium pyruvate, and Earle's salts in 10% FCS. The MCF-7aro cells were harvested and resuspended in an equal volume of Matrigel (BD Biosciences) to a final concentration of 1×10^7 cells/ml. Body weights were monitored weekly as an indicator of the overall health of the animals. At the end of 6 weeks of gavage treatment, mice were euthanized, blood samples were collected, and tumors and ovaries-uteri were removed, weighed, and sent for H&E histological staining through the City of Hope Pathology Department Core Facility.

Estrogen concentrations were determined from mouse sera. Mouse blood was obtained through cardiac puncture and immediately combined with heparin. Whole blood was separated to serum and plasma in a tabletop centrifuge. Sera were frozen until later evaluation by radioimmunoassay for estrogen and estrone levels.

For the animal experiments using ovariectomized animals, athymic nude ovariectomized female mice were used (Charles River Laboratories). Mice also received the same s.c. androstenedione 60 day time-release pellet implants as described in the previous experiment. Control animals were given androstenedione pellet and gavage fed 100 μl of sterile water. Treatment groups were given androstenedione pellets and either 1× or 3× concentrate of the Pinot Noir polyamide 70% methanol fraction in a 100-μl volume. All other parameters (*i.e.*, number of cells injected, duration of experiment, and experimental analyses) were identical to the animal experiment using intact mice described previously. For the animal experiments using MCF-7 cells (the cells without aromatase), MCF-7 cells and estrogen pellets, instead of androgen pellets, were used. Other conditions were identical to those described for the experiments using MCF-7aro cells.

RESULTS AND DISCUSSION

Isolation of Antiaromatase Chemicals from Red Wine. To better understand the molecular nature of red wine's antiaromatase activity, we decided to isolate and characterize the chemicals that are capable of inhibiting aromatase. Whole red wine was applied to the polyamide column (Discovery DPA-6S SPE; Supelco). The elution solvent consisted of a step gradient of methanol and water. This particular SPE column produced fractions exhibiting a range of inhibitory activity with the best inhibitory fractions seen with 60–80% methanol-water gradients (data not shown). When the active polyamide fractions were injected onto a reverse-phase HPLC system, we obtained 12 peaks. The UV abs_{max} of HPLC peaks 1–6 and 8 were between 279 and 280 nm. Both HPLC peaks 9 and 12 had UV abs_{max} values of 264 nm. Compounds found in HPLC peaks 1–6 and 8 had similar UV absorbance characteristics to procyanidins B1 and B2 that had UV abs_{max} of 279–279.5 nm and to catechin and epicatechin standards that had UV abs_{max} of 277.5–278.5 nm. Peaks 9 and 12 were similar to gallic acid that also had a UV abs_{max} of 264 nm. Once concentrations were determined for the 12 unknown compounds (described below), they were evaluated for their ability to inhibit aromatase in human placental microsomes. Increasing concentrations of each HPLC peak compound were assayed in the presence of 100 nM androstenedione substrate. The aromatase activity results show that all HPLC fractionated compounds inhibited aromatase in a dose-dependent manner (Fig. 1). The most effective inhibition curve was observed with HPLC peak 1, the IC_{50} of which was between 15 and 20 μM (calculated using M_r 579; discussed further below). The reconstituted mixture was significantly more effective than any single chemical (see Fig. 1).

Accurate Mass-MS. Because HPLC peak 1 chemical had the best inhibitory activity on aromatase, a Mariner time-of-flight mass spectrometer was used to determine the precise mass or *m/z* for better estimating the elemental composition. To calibrate every run, an internal standard was added to each sample. Because the UV spectral analysis suggests that the compound might be a procyanidin, the internal standard used was polyethylene glycol, M_r 600 (polyethylene glycol 600), which has a well-established mass spectra profile and contains product ions that border the $m/z = 579$ (the molecular weight of a procyanidin dimer) without overlapping at the same position. The Mariner Data Explorer software performs a mass calibration based on the internal standard. Once an exact mass had been determined, the Data Explorer software also calculated the possible elemental compositions and scored them based on statistical probability. We analyzed our unknown compound HPLC peak 1 combined with polyethylene glycol 600 as the internal standard in the Mariner mass spectrometer. The observed positive parent ion mass (M+) for HPLC peak

[4] The abbreviations used are: HPLC, high-performance liquid chromatography; CHO, Chinese hamster ovary; abs_{max}, maximal absorbance; ppm, parts/million; ESI, electrospray; MS, mass spectrometry; NMR, nuclear magnetic resonance; ER, estrogen receptor; 70M, 70% methanol-water fraction; CK18, cytokeratin 18; GSP, grape seed polyphenolic fraction; CDK, cyclin-dependent kinase.

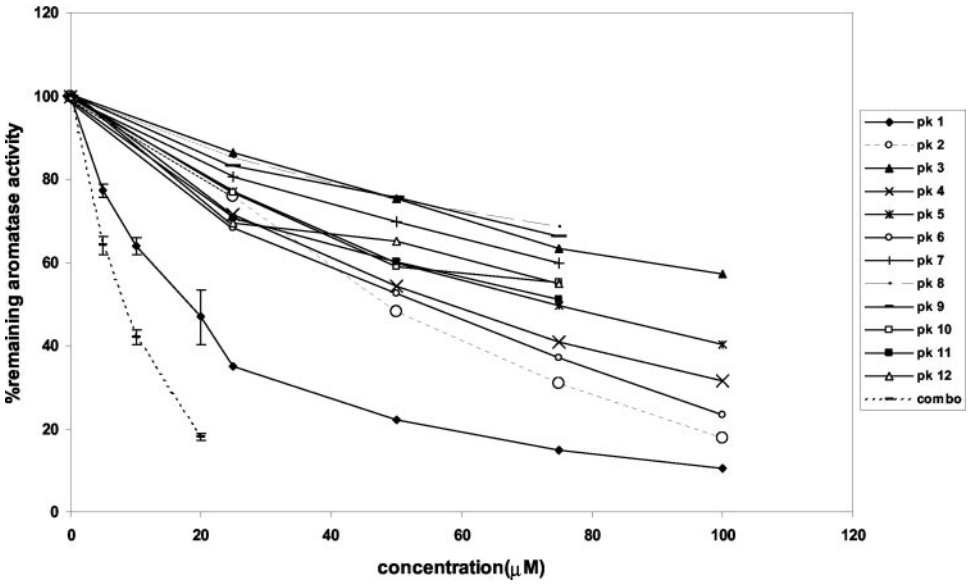

Fig. 1. Dose-dependent inhibition of human placental aromatase by the chemicals in the 12 HPLC peaks isolated from the polyamide 70M. *combo* represents the 12 isolated peaks pooled together and evaluated in the aromatase inhibition assay. Data are represented as a percentage of the water control.

1 was 579.1484, which represented an accurate mass with an error of just 3 ppm when compared with the theoretical mass for a procyanidin B dimer (Fig. 2). For HPLC peak 1 chemical, the elemental analysis gave the most likely chemical formula as $C_{30}H_{27}O_{12}$ with an 89.1% isotope match score. In practice, the mass of a compound measured with an error of <10 ppm from the theoretical mass is considered an exact mass. Therefore, HPLC peak 1 exhibited a mass (M+) of 579 and an elemental formula of $C_{30}H_{27}O_{12}$, which confirmed that HPLC peak 1 is a procyanidin B dimer.

The procyanidin B dimers consist of eight stereoisomers composed of catechin and epicatechin monomers that can be divided into two subgroups based on their 4-8 or 4-6 linkages. Only three procyanidin B dimers are commercially available; these are procyanidin B1, B2, and B3. Using analytical reverse-phase HPLC, procyanidins B1, B2, and B3 were found to coelute with HPLC peaks 2, 6, and 3, respectively. Therefore, peak 1 is a procyanidin B dimer that is different from procyanidin B1–B3.

The HPLC fractions were also analyzed by ESI-MS with collision-induced dissociation. Tandem fragmentation and ionization of the parent ion can help elucidate chemical and structural features of the molecule. As expected, HPLC peaks 2, 3, and 6 had identical tandem MS profiles up to MS (3) as did procyanidin B1, B3, and B2. HPLC peaks 1 and 5 had a different fragmentation pattern from HPLC peaks 2, 3, and 6, as seen by their MS (2) fragment ion of 301 compared with MS (2) fragment ion of 427. Perhaps there is no noticeable difference among dimers B1, B2, or B3 because they all belong to the same subgroup of procyanidin dimers with the 4-8 linkage. Thus, HPLC peaks 1 and 5 could be procyanidin B dimers with the 4-6 linkage. It would be ideal to analyze the subgroup of authentic procyanidin B dimers with the 4-6 linkage, which could confirm the nature of HPLC peak 1. However, no compounds from the 4-6 linkage subgroup are commercially available. Thus, using mass spectral analyses, we have determined that the major chemicals in the 60–80% methanol-eluted polyamide fraction are isomers of procyanidin B dimer, and HPLC peak 1 is probably a procyanidin B dimer with 4-6 linkage (see Fig. 3). Finally, NMR analysis was carried out and confirmed that

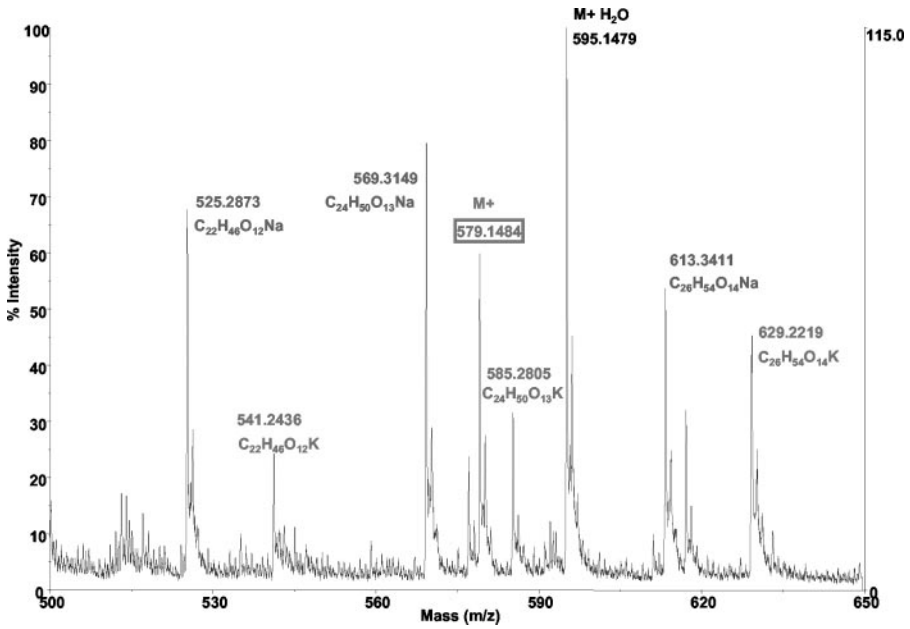

Fig. 2. Accurate mass determination of the chemical in HPLC peak 1. The accurate masses for the positive parent ion, M+, and the hydrated positive parent ion, M+ H_2O, are shown. These masses were determined based on the masses of sodiated polyethylene glycol standards and potassiated polyethylene glycol standards. The chemical formulas of the standards are indicated. Exact mass determination of HPLC peak 1 was performed on an ESI time-of-flight spectrometer Mariner Biospectrometry Workstation with Data Explorer software version 3.2 (PerSeptive Biosystems). The spray tip potential was 806, and quad and nozzle potential was 140. Samples were diluted with 90% acetonitrile containing 2% acetic acid. The internal standard used was polyethylene glycol M_r 600 (Sigma) that became sodiated or potassiated after nanospray ESI.

peak 1 is a procyanidin B dimer, but it was not able to determine which isomer.

Enzyme Kinetic Analysis for HPLC Peak 1 Procyanidin Dimer. To understand the molecular basis of peak 1 procyanidin B dimer inhibition of aromatase, enzyme kinetic analysis was performed using the human placenta microsome aromatase assay. The results show that the dimer from HPLC peak 1 inhibited aromatase in a competitive manner with respect to the substrate (Fig. 4). A K_i value of 6 μM for HPLC peak 1 was determined from the secondary plot (1/v *versus* [I]). Therefore, this procyanidin B dimer inhibits aromatase/estrogen biosynthesis in a specific manner with a K_i value similar to the known aromatase inhibitor, aminoglutethimide (24).

Aromatase Site-Directed Mutagenesis Studies. The specificity of the interaction of peak 1 procyanidin B dimer with aromatase was further evaluated using aromatase active site mutants. The wild type and six human aromatase mutants (I133Y, E302D, D309A, T310S, S478T, and H480Q) were used. Previous studies performed in our laboratory have determined that these mutated amino acids are present in the active site of aromatase (24). With increasing concentrations of HPLC peak 1 procyanidin B dimer, the analysis revealed that mutations I133Y and E302D did not alter the inhibition profile obtained from HPLC peak 1 when compared with the wild-type aromatase (Fig. 5). Mutant D309A produced the most dramatic effect by completely eliminating the ability of HPLC peak 1 dimer to inhibit aromatase. Two other mutants, S478T and H480Q, also proved to be significant in reversing the inhibitory action of HPLC peak 1. Interestingly, the T310S mutant produced a more effective dose response after treatment with HPLC peak 1 procyanidin B dimer when compared with the wild-type aromatase. These results support the finding from enzyme kinetic analysis that this compound binds to the active site of aromatase, and they indicate that Asp-309, Thr-310, Ser-478, and His-480 are involved in the interaction with the peak 1 procyanidin B dimer.

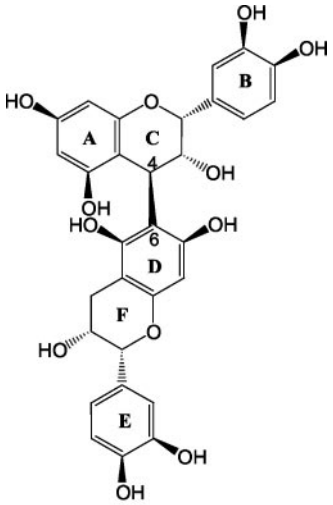

Fig. 3. The chemical structure of procyanidin B5. Procyanidins B5–B8 are dimers with the 4-6 linkage and stereoisomers at position C-3. The *capital letters* in the procyanidin structure represent the standard ring assignments.

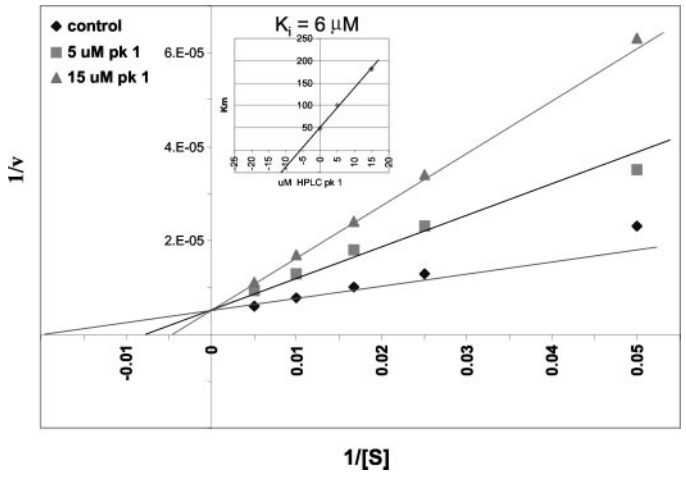

Fig. 4. Kinetic analysis of HPLC peak 1 procyanidin B dimer in the human placenta microsome aromatase assay. The substrate used was [1β-^{3}H]androstenedione (NEN-DuPont) at 20, 40, 60, 100, and 200 nM. Water control, ◆; 5 μM HPLC peak 1, □; 15 μM HPLC peak 1, △. The *inset graph* is the secondary plot (1/v *versus* [I]) used to determine the K_i value for HPLC peak 1 procyandin dimer.

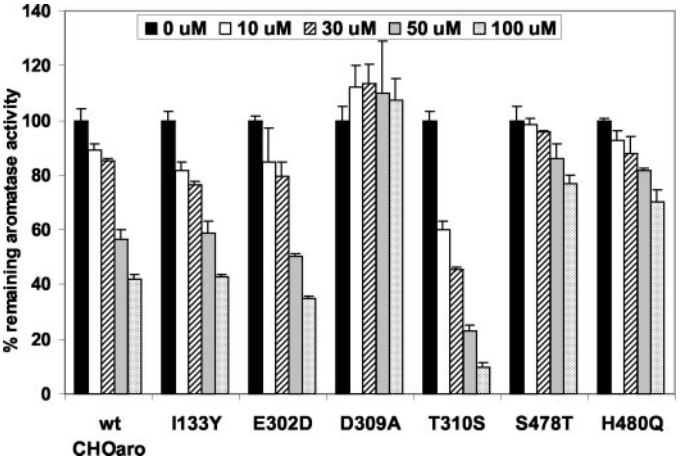

Fig. 5. Effect of the mutations at the active site region of aromatase on the inhibitory activity of HPLC peak 1 procyanidin B dimer. In the presence of various concentrations of HPLC peak 1 procyanidin B dimer, *in vitro* microsome assay was performed on the cell homogenates prepared from the wild type and mutant human aromatase-transfected CHO cells. The activities of the untreated samples were taken as 100%. CHO cells transfected with human wild type and mutant aromatase were grown in Ham's medium, 1 mM sodium pyruvate, 2 mM L-glutamine, 1× penicillin/streptomycin, 15 mM HEPES, and 10% fetal bovine serum optimized for CHO cells (Hyclone).

***In Vivo* Experiments.** Our *in vitro* studies have found that most of the chemicals in the 60–80% methanol-eluted polyamide fraction are procyanidin B dimers. These compounds have been shown to be inhibitors of aromatase. We also found that the mixture was more active than individual chemicals. In addition, because there was not a quick high-yield method to purify enough isolated HPLC peaks to perform animal experiments, we decided to perform animal experiments using the entire polyamide 60–80% methanol-water fraction that contains mainly procyanidin B dimers. The aromatase inhibitory action of the polyamide Pinot Noir wine extract was first analyzed in a mouse xenograft model using BALB/c-*nu-nu* athymic intact (with ovaries) mice. Mice were given two s.c. injections (0.2 ml/site) of MCF-7aro cells diluted in an equal volume of Matrigel to a final concentration of 1×10^7 cells/ml. MCF-7aro cells are ER positive and overexpress aromatase (25). These cells proliferate in an androgen-dependent manner. It was found that mice fed daily with the polyamide 70M at 50- and 100-μl doses had a significant reduction in tumor growth when compared with the control ($P < 0.03$). The average tumor weights for each group were as follows: 58.5 ± 23.2 (control), 44.8 ± 30.5 (70M wine extract, 25 μl), 36.4 ± 25.9 (70M wine extract, 50 μl), and 33.3 ± 26.8 mg (70M wine extract, 100 μl). The ovaries and uteri were also removed to examine any effects from the wine extract on other endocrine glands that either produce estrogen

(ovaries) or require estrogen for their cell maintenance (uterus). The average ovary-uterine weights for each group were as follows: 172.8 ± 16.2 (control), 170.1 ± 31 (70M wine extract, 25 μl), 131.4 ± 44 (70M wine extract, 50 μl), and 134.1 ± 41 mg (70M wine extract, 100 μl). Again, the ovary-uterine weights of mice fed daily with the polyamide 70M at 50 and 100 μl were significantly smaller than the weights of the control mice.

Serum from each mouse was also collected and analyzed for estradiol and estrone concentrations. Mice treated with increasing concentrations of wine extract showed a decreasing trend in the levels of estradiol and estrone compared with the control. The average estrogen concentration values for wine extract-treated mice did show a clear difference when compared with mice treated with water. Unfortunately, the SD values were too large to determine any statistical significance. The large SD values may be because of the intact ovaries in the mice that contribute to the variation in circulating estrogen levels. Over the course of the 2-month experiment, the weights of animals in each group were recorded where all groups had similar body weight.

To eliminate the effect of estrogens generated endogenously by the ovary, in the second type of animal experiments, athymic nude-ovariectomized female mice were used. Additionally, a more concentrated polyamide wine extract was used in these experiments; *i.e.*, at a 100-μl dose each, a 1× or 3× concentrated extract was used as compared with just a 1× concentrate used at different volumes (25, 50, and 100 μl). All mice received a 5 mg/60-day release androstenedione pellet that was implanted s.c. For 6 weeks, mice were gavage fed daily with either 100 μl of 1× or 3× concentrated 70M wine extract or water. After the first week of gavage treatment, each mouse was given a 0.2-ml s.c. injection into each hind flank containing MCF-7aro cells suspended in equal volume Matrigel to a final concentration of 1×10^7 cells/ml. At the completion of 6 weeks of gavage treatment, mice were sacrificed, and blood, tumor, and uteri

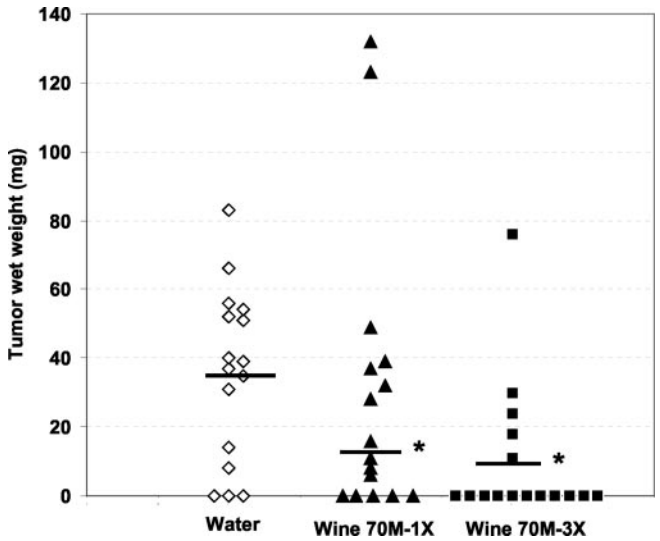

Fig. 6. Tumor and uterine weight comparison of athymic mice after 6 weeks of gavage feeding with procyanidin B dimers. Seven- to eight-week-old athymic nude ovariectomized female mice (Charles River Laboratories) received s.c. implant with 5 mg/60 day time-release androstenedione pellets (Innovative Research of America). The feeding started 1 week after the implantation of androgen pellets. The mice were gavage fed daily for 6 weeks with 100 μl of 1× concentrated (Wine 70M-1×) or 3× concentrated (Wine 70M-3×) Pinot Noir polyamide 70% methanol fraction (in water) or water. Two weeks after the implantation of androgen pellets, mice were given a 0.2-ml s.c. injection in each hind flank; the injection contained MCF-7aro cells mixed with an equal volume of Matrigel (BD Biosciences) to a final concentration of 1×10^7 cells/ml. * indicates significantly different groups compared with water control group ($P < 0.01$). The *bars* represent the average weight in each group. Statistics were performed using the two-tailed Student's *t* test.

Table 1 *Summary of tumor numbers in individual athymic nude mice gavage fed with Pinot Noir polyamide 70% methanol fraction*

Treatment group	Tumors on 2 sides	Tumors on 1 side only	No tumors on both sides	Percentage of injected sites with tumors
And+[a]/H_2O ($n = 8$)	5[b] (62.5%[c])	3 (37.5%)	0	81.3%
And+/wine 1× ($n = 8$)	4 (50%)	3 (37.5%)	1 (12.5%)	68.8%
And+/wine 3× ($n = 8$)	2 (25%)	1 (12.5%)	5 (62.5%)	31.3%

[a] Androstenedione implanted pellet.
[b] Number of animals per group.
[c] Percentage of mice in group.

were removed, weighed, and sent out for histological evaluation. Mice treated with 1× and 3× concentrated 70M wine extract showed a significant reduction ($P < 0.01$) in tumor size compared with the androstenedione control mice fed with water (15.7 ± 16.2 and 9.9 ± 20.2 *versus* 35.4 ± 25.2 mg, respectively; Fig. 6). A more detailed analysis of individual mice per treatment group is summarized in Table 1. The positive control group And+/H_2O was the group that had the highest percentage (62.5%) of mice with tumor growth on each flank. Fifty percent of the 1× concentrate-treated group had growth of two tumors. Whereas the group with the lowest number of mice having two tumors was the mice treated with the 3× concentrate, the 3× concentrate-treated mice had the most dramatic suppression of tumor growth, as indicated by the high percentage (62.5%) of animals that had no MCF-7aro growth at either injection site. Interestingly, only 12.5% of the 1× concentrate-treated mice had complete tumor suppression, whereas 37.5% of the mice had just one tumor with an average tumor weight of 22.7 mg. To evaluate whether these treatments caused any deleterious effects on other endocrine glands, the uteri were removed and weighed. The wet uterine weights of all wine extract-treated mice were not found to be statistically different from the uteri of androstenedione control mice. Blood samples were taken, and serum was analyzed for the circulating estrogen. The concentrations of circulating estrogen in ovariectomized mice were found to be significantly lower than those found in intact animals (with ovary). Furthermore, the results suggested only a slight trend in reduced blood estrogen concentrations after treatment with increasing concentrations of wine extract. The results from uterine weight measurements and circulating estrogen levels support the hypothesis that *in situ*-produced estrogen plays a larger role in aromatase expressing and ER-positive breast tumor growth than circulating estrogen.

Because procyanidin B dimers are competitive inhibitors, not irreversible inhibitors, the specific aromatase activity (per tumor weight) should be the same when measured on tumors that have been removed from mice. We did not detect any difference in specific enzyme activity by measuring aromatase activity in homogenized tumor tissue from nontreated or treated mice.

These mice tumors were further evaluated using immunohistochemistry with M30 CytoDEATH mouse monoclonal antibodies that bind to an early apoptotic marker, the caspase-cleaved product of human CK18 cytoskeletal protein. The M30 CytoDEATH antibody recognizes a specific caspase-cleavage site in CK18 that is not detected in native CK18 of normal cells. The tumors of mice treated with wine extract showed a slight increase in the number of apoptotic cells when compared with tumors of control mice; however, the difference did not appear to be statistically significant. These results suggest that the observed reduction in tumor growth in the wine extract-treated mice was attributable to the inhibition of aromatase and not because of a nonspecific cytotoxic effect.

We have also examined the effect of polyamide wine extract in a MCF-7 breast cancer xenograft model. In these *in vivo* studies, athymic nude-ovariectomized female mice were given two s.c. injections of MCF-7 instead of MCF-7aro cells, and the tumor formation was

induced using 5 mg/60-day release 17β-estradiol pellet (implanted s.c.). Other experimental conditions were identical to those described for the experiments using MCF-7aro cells. MCF-7 cells have minimal aromatase activity. We found that 3× concentrated 70M wine extract fed daily at 100 μl was not able to suppress the growth of MCF-7 tumors ($P = 0.286$). The average tumor weights for the estrogen control and 3× concentrated extract-treated mice were 44.2 ± 60.3 *versus* 52.4 ± 45.9 mg, respectively. The weights of the tumors from mice without estrogen pellet were 21.0 ± 15.4 mg. These results are very crucial for confirming the antiaromatase activity of procyanidin B dimers in red wine.

Although our studies have demonstrated a highly specific inhibitory activity of procyanidin B dimers against aromatase/estrogen biosynthesis, these chemicals are thought to exhibit additional activities in cells. For example, it has been shown by Agarwal *et al.* (26) that a GSP largely composed of procyanidins could irreversibly inhibit breast carcinoma MDA-MB468 cells by reducing constitutive extracellular signal-regulated kinase 1/2 and p38 activation. Additionally, GSP arrested MDA-MB468 cells at G_1 as seen by the increase of Cip1/p21 levels and the decrease of CDK4 and cyclin D1 G_1-phase regulators. Similarly, this same group evaluated the GSP on human prostate cancer DU145 cells. This fraction caused a decrease in extracellular signal-regulated kinase 1, an increase in Cip1/p21, and a decrease in CDK2, CDK4, and cyclin E that also led to G_1 cell cycle arrest (27). Previously, this group examined the anti-tumor-promoting activity of the same GSP in a 12-*O*-tetradecanoylphorbol-13-acetate-promoted SENCAR mouse skin two-stage initiation-promotion model. The grape seed fraction reduced tumor occurrence and tumor volume (28). Others have also studied grape seed proanthocyanidin extract and shown that it inhibited growth of MCF-7 human breast cancer, A-427 human lung cancer, and CRL 1730 human gastric adenocarcinoma cells in a concentration- and dose-dependent manner (29). The GSP contains procyanidin B dimers (28). These published results suggest that grape seed extract may modulate cell proliferation, but it has not yet been determined whether procyanidin B dimers are directly responsible for the reported cell growth suppression activity of grape seed extract. Because our procyanidin B preparation did not suppress the growth of MCF-7 tumors in nude mice, the cytotoxic effect of the grape seed extract may result from different phytochemicals in the extract.

In summary, previous studies from this laboratory have shown that red wine contains phytochemicals that inhibit aromatase activity *in vitro* and suppress aromatase-mediated breast tumor formation *in vivo*. In this study, we have isolated a bioactive fraction from red wine. The chemicals in this fraction were identified to be procyanidin B dimers that were shown to be aromatase inhibitors. The most active procyanidin B dimer was found to inhibit aromatase with a K_i value of 6 μM, and the preparation of procyanidin B dimer mixture was found to be much more potent than any one of the individual procyanidin B dimers. Using a MCF-7aro tumor induction model, oral intake of the procyanidin B dimer mixture was found to be effective in suppressing aromatase-mediated tumor formation *in vivo*. On the other hand, the procyanidin B dimer mixture was not able to suppress the formation of MCF-7 tumors. These *in vitro* and *in vivo* studies demonstrated that procyanidin B dimers in red wine could be used as chemopreventive agents against breast cancer by suppressing *in situ* estrogen biosynthesis. Five ml of red wine can produce 100 μl of a 1× fraction. A typical conversion factor for determining mouse to human dosage of chemotherapeutic agents is 25, calculated based on body surface area (30). Therefore, consumption of 125 ml of red wine/day would provide adequate amounts of procyanidin B dimers to suppress *in situ* aromatase in an average postmenopausal woman.

ACKNOWLEDGMENTS

We thank Wei-dong Hu, the City of Hope NMR Core Facility Manager, for running the NMR and for assistance in the interpretation, and Dr. Sei-Ryang Oh for helpful discussions on the MS and NMR analysis. We also thank Sophia Loera, the City of Hope Pathology Core Facility Manager, for help with immunohistochemistry staining of the mouse tissues. In addition, we thank Dr. Mark Dizik for proofreading this manuscript.

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Suppression of Estrogen Biosynthesis by Procyanidin Dimers in Red Wine and Grape Seeds

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Cancer Res 2003;63:8516-8522.