Dietary Feeding of Dibenzoylmethane Inhibits Prostate Cancer in Transgenic Adenocarcinoma of the Mouse Prostate Model

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Abstract

Dibenzoylmethane (DBM), a minor β-diketone constituent of licorice, has been shown to exhibit antineoplastic effects in prostate cancer cell lines by induction of cell cycle arrest and regulation of androgen receptor expression. In the present study, we investigated the in vitro and in vivo efficacy of DBM using TRAMP-C1 cell lines and TRAMP mice. DBM was found to arrest TRAMP-C1 cells at G2-M phase of cell cycle and suppressed phosphorylated retinoblastoma, cyclin D1, and cyclin A. Importantly, DBM was found to be equally effective in suppression of prostate tumor progression in TRAMP mice. At 8 or 12 weeks of age, mice were fed control or 1% DBM-supplemented diets until 24 weeks of age. Our results show that DBM-fed groups had a lower incidence of palpable tumor and high-grade prostatic intraepithelial neoplasia. Subsequent mechanistic studies show that the expression of phosphorylated retinoblastoma, e-myc, cyclin D1, cyclin A, phosphorylated Akt, phosphorylated PDK-1, and phosphorylated S6 was significantly reduced by DBM. Our findings suggest that DBM blocks the growth and progression of prostate cancer in TRAMP mice via modulation of tumor cell cycle regulation and therefore merits its consideration for future clinical intervention of human prostate cancer. [Cancer Res 2009;69(17):7096–102]

Introduction

Prostate cancer is the most common cancer expected to occur in men with an estimated 218,890 new cases leading to 27,050 estimated deaths in 2007 (1). Although localized prostate cancer is curable by traditional clinical interventions, metastatic disease leads to hormone refractory progression and is usually resulting in lethality. Prostate tumorigenesis in humans is an extremely long process involving a cascade of genetic alterations. Progression from preneoplastic lesions known as prostatic intraepithelial neoplasia (PIN) to androgen-independent invasive carcinoma may takes several decades to occur and, therefore, provides a possible opportunity for chemopreventive interventions (2). Epidemiologic studies show that dietary and environmental factors play an important role in prostate carcinogenesis (3). Indeed, high vegetable-and-fruit consumption was inversely associated with prostate cancer, suggesting that the disease may be preventable by a change in life-style or dietary habits (4, 5).

Dibenzoylmethane (DBM), a minor constituent of licorice, has been shown to be a promising anticancer, antimutagenic, and cancer chemopreventive compound. We have recently shown that DBM, when given alone or in combination with sulforaphane, significantly inhibited the development of familial intestinal adenomatous polyposis in ApcMin/+ mouse (6). Likewise, DBM has been reported to inhibit 7,12-dimethylbenz(a)anthracene–induced mammary tumors and lymphomas/leukemias in Sencar mice (7). DBM is also a very strong antimutagenic agent that could effectively inhibit mutagenicity induced by several heterocyclic amines (8). Although DBM has been reported to inhibit the growth of several prostate cancer cell lines, its in vivo antineoplastic activity has yet to be tested.

In the present study, the in vitro and in vivo prostate cancer chemopreventive efficacy of DBM was investigated using TRAMP (transgenic adenocarcinoma mouse prostate)–C1 cells and TRAMP mice, respectively. TRAMP mouse is an autochthonous transgenic animal model of prostate cancer that recapitulates the whole spectrum of human prostate tumorigenesis from the earliest PIN lesions to androgen-independent disease (9). TRAMP mice develop PINs with mild to severe hyperplasia by 8 weeks old. Severe hyperplasia and adenocarcinoma is evidenced in the dorsolateral and ventral lobes of prostate by 18 weeks of age and will eventually develop primary prostate tumors that may metastasize primarily to lymph nodes and lungs by 24 to 26 weeks of age (9). The TRAMP-C1 cells were originated from a heterogeneous 32-week tumor of the TRAMP mouse. Although the IC50 of DBM on TRAMP-C1 cells was found to be >100 μM/L, DBM arrested the cells at G2-M at 50 μM/L with concomitant suppression of some cell cycle–specific biomarkers, such as cyclin D1 and phosphorylated retinoblastoma (pRb). In agreement with the in vitro results, DBM also suppressed the progression of prostate tumorigenesis in TRAMP mice. Mice fed with DBM started at 12 weeks of age developed lower percentage of high-grade PINs (HG PIN) and palpable tumors when compared with their control counterparts. More strikingly, when DBM was given to the TRAMP mice at 8 weeks of age, the time when PINs started to develop, the incidence of palpable tumors and in situ carcinoma was totally blocked.

Materials and Methods

Reagents. DBM was purchased from Sigma Chemicals, Inc. Rabbit polyclonal antibodies against cyclin D1, cyclin A, and glyceraldehyde-3-phosphate dehydrogenase were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against e-Myc, phosphorylated S6 (pS6) ribosomal protein (Ser235/236), phosphorylated Akt (pAkt; Ser473), phosphorylated PDK-1 (p-PDK-1; Ser 241), and pRb were purchased from Cell Signaling.

Note: T.O. Khor and S. Yu contributed equally to this work.

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Cell culture. The TRAMP-C1 cells (10), a generous gift from Dr. Barbara Foster (Roswell Park Cancer Institute), was maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 10 to 8 mol/L 5-androstan-17β-ol-3-one, and antibiotics.

Flow cytometry analysis of cell cycle distribution. The flow cytometry analysis of cell cycle distribution was performed as previously described (11). Briefly, TRAMP-C1 cells were seeded in 60-mm Petri dishes in complete DMEM overnight. The cells were then serum starved for 24 h in serum-free DMEM. Before treatment, the medium was replaced with complete DMEM containing either 0.1% DMSO (as a negative control) or a varying dose of DBM. After DBM treatments, the cells were collected by trypsinization. Cells were collected by centrifugation at 1,000 rpm for 5 min. The cell pellet was then resuspended in 500 μL PBS, and the resulting cell suspension was passed through a 26.5-gauge needle thrice. The cells were then fixed by adding 500 μL ethanol. The fixed cells were then stained by 100 μg/mL RNase A and 10 μg/mL propidium iodide at 4°C for 30 min. Cell cycle distribution was then analyzed by flow cytometry using the fluorescence-activated cell sorting analysis core facility of Rutgers University and University of Medicine and Dentistry of New Jersey.

Animals. Female hemizygous C57BL/Tag TRAMP mice, line PB Tag 8247NG, and male C57BL/6 mice were purchased from The Jackson Laboratory. The animals were bred on same genetic background and maintained in the Animal Care Facility of Rutgers University. Housing and care of the animals were in accordance with the guidelines established by the University’s Animal Research Committee consistent with the NIH Guidelines for the Care and Use of Laboratory Animals. Transgenic males for these studies were routinely obtained as [TRAMP × C57BL/6] F1 or as [TRAMP × C57BL/6] F2 offspring. Identity of transgenic mice was established by PCR-based DNA screening. Throughout the experiment, the animals were housed in cages with wood chip bedding in a temperature-controlled room (68–72°F) with a 12-h light-dark cycle at a relative humidity of 45% to 55%. The animals were fed with irradiated AIN-76A diet or DBM-supplemented AIN-76A diet (DYETS, Inc.).

Study design. After 1 wk of acclimatization, 8 wk old (Group 1; G1) or 12 wk old (Group 2; G2) male TRAMP mice were fed with 1% of DBM (30 mg of DBM per day per mouse) in AIN-76A diet until they are 24 wk old (n = 12). The control animals received AIN-76A diet throughout the experiment. The animals were weighed weekly and monitored on a regular basis for their general health. At each time point, the mice were killed by cervical dislocation, and the genitourinary apparatus consisting of the seminal vesicles, prostate, and bladder were isolated for further analyses.

Histopathology. The dorso-lateral prostate was excised and fixed the proportion of TRAMP-C1 cells at G2-M phase of cell cycle. *, significantly different from the control (the experiment has been repeated at least twice). B, representative histogram showing the dose-dependent increase of G2-M cell population (red circle). Note: The IC50 for DBM on TRAMP-C1 cells is >100 μmol/L as determined by MTS assay (data not shown).

Figure 1. Flow cytometry analysis of cell cycle distribution. A, DBM treatment significantly increased the proportion of TRAMP-C1 cells at G2-M phase of cell cycle. *, significantly different from the control (the experiment has been repeated at least twice). B, representative histogram showing the dose-dependent increase of G2-M cell population (red circle). Note: The IC50 for DBM on TRAMP-C1 cells is >100 μmol/L as determined by MTS assay (data not shown).
The sections were stained with H&E to observe any neoplastic changes. Sections were blinded and evaluated by a histopathologist (X.H.) to classify the PIN lesions. Lesions were classified as PIN I, PIN II, PIN III, and PIN IV, as described by Park and coworkers (12). For the purpose of simplicity, PIN I and PIN II have been grouped as low-grade PIN whereas PIN III and PIN IV have been grouped as high-grade PIN, as we have previously done (13).

**Western blotting.** For in vitro experiment, TRAMP-C1 cells in six-well plates were washed with ice-cold PBS and lysed with 200 μL of cell lysis buffer (Cell Signaling) after DBM treatment. For in vivo study, the dorsal-lateral prostate dissected from mice of each treatment group was weighed, pooled, and treated with cell lysis buffer (Cell Signaling) at a concentration of 10 μg/mL for 40 min on ice, followed by centrifugation at 14,800 × g for 15 min. The protein concentrations of the supernatant were measured using the bicinchoninic acid solution (Pierce). Protein (20 μg) was loaded onto precasted SDS-PAGE gels (Bio-Rad) and, after electrophoresis, transferred onto polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat dry milk in 0.1% Tween 20 in PBS (PBST) for 1 h and incubated with primary antibody in 5% bovine serum albumin–0.1% PBST overnight at 4°C. After three 5-min washes with PBST, the membrane was then incubated with horseradish peroxidase–conjugated secondary antibody in 5% nonfat dry milk–PBST for 1 h at room temperature and then washed with PBST thrice. The transferred proteins were visualized with the Super Signal chemiluminescent substrate (Pierce).

**Quantification of DBM in plasma and prostate tissues using high-performance liquid chromatography.** Plasma and prostate tissue concentrations of DBM in TRAMP mice were measured at the end of 24 wk using a validated high-performance liquid chromatography (HPLC)–UV detection method (14). Briefly, a 50-μL aliquot of the plasma or homogenized prostate tissues samples was added to the internal standard (CHMPP) working solution and extracted twice with 200 μL ethyl acetate/methanol (95:5 v/v) solution. The organic layer was separated by centrifugation and evaporated to dryness under a stream of nitrogen gas at room temperature. The residue was reconstituted in 100 μL of acetonitrile/water (50:50 v/v) solution, and 20 μL were injected onto an HPLC column. Analyses were performed using a Shimadzu HPLC system at 4°C with a reverse-phase column (Gemini C18 column, 150 × 2.0 mm, 5 μm, Phenomenex) protected with a SecurityGuard cartridge system (Phenomenex) and a 0.45-μm in-line filter. The binary gradient mobile phase (mobile phase A (water/methanol, 80:20 v/v, with 0.1% trifluoroacetyl) and mobile phase B (acetonitrile with 0.1% trifluoroacetyl)) were pumped at the flow rate of 0.2 mL/min. The UV detector was set at a single wavelength of 335 nm. The Class-VP software version 7.1.1 (Shimadzu) was used for instrument control and data analysis. The retention time for DBM and internal standard (CHMPP) were 21.4 and 24.0 min, respectively. The lower limit of quantification for DBM was 0.05 μg/mL, and the linear calibration curves were obtained in the concentration ranges of 0.05 to 20 μg/mL.

**Statistical analysis.** Student’s t test or Fisher exact test was used for the statistical analysis. P values of <0.05 were considered significant.

**Results**

**DBM induced G2-M cell cycle arrest in TRAMP-C1 cells.** After 24 hours of treatment of DBM at various doses, flow cytometry analysis was performed. Our results showed that the percentage of TRAMP-C1 cells in G2-M was significantly increased from 19.08% in control to 38.26% and 40.83% at 50 and 100 μmol/L DBM treatment, respectively (Fig. 1).

**DBM-induced G2-M arrest is associated with suppression of cell cycle–related biomarkers.** To investigate the underlying mechanisms by which DBM exerts its cell cycle arrest effect, the protein expression level of several cell cycle–related biomarkers were evaluated. We found that DBM suppressed the expression of pS6, c-myc, pRb, cyclin D1, and cyclin A in a dose-dependent manner (Fig. 2).

**Effects of DBM-supplemented diet on TRAMP mice and general health observations.** The overall health of all the mice was observed on a weekly basis and was found to be good throughout the study period. Gross autopsy of these animals failed to reveal any sign of toxicity or abnormalities. In addition, DBM treatment has no significant effect on the weight of liver, spleen, and kidneys.

**Effect of DBM on prostate tumorigenesis.** The only difference between groups 1 and 2 is that the former were given DBM-supplemented diet earlier when the mice were 8 weeks old, whereas the latter started at the age of 12 weeks old with both sets of mice terminated at the same time at 24 weeks of age (Fig. 3A). The effect of DBM for each experiment on the wet weight of the genitourinary apparatus is shown in Fig. 3B. DBM significantly decreased the genitourinary weight with P values of 0.004 and 0.002, respectively, for groups 1 and 2. In addition, the incidence of palpable tumor was lower in mice from group 2 (not statistically significant) and was totally blocked by DBM treatment in group 1 (Fig. 3C). Histologic examination of prostate tissue by H&E staining revealed that the mice in group 1 have significantly lower proportion of HG PINs compared with the control animals (Fig. 4A). The incidence of poorly differentiated carcinoma was also found to be significantly lower in mice from group 1 (Fig. 4B). Although the incidence of palpable tumor and poorly differentiated carcinoma, as well as proportion of HG PIN, was lower in mice from group 2, they were not statistically significant compared with their control counterpart. Taken together, the results show that DBM treatment suppressed the progression of prostate tumorigenesis in TRAMP mice. More interestingly, the inhibitory effect of...
DBM on progression of prostate cancer is significantly stronger when it was given before the PIN started to develop.

Steady-state levels of DBM in plasma and target tissue. The relationship between the chemopreventive efficacy of DBM in TRAMP mice and the concentration of the compound was investigated in the present study. Steady-state levels were determined in the plasma and prostate, the target tissue, which had received dietary DBM for 16 and 12 weeks for groups 1 and 2, respectively, using HPLC. Mean level of DBM in the plasma was found to be $1.099 \pm 0.306$ and $1.461 \pm 0.237$ mol/L for mice in groups 1 and 2, respectively. In the prostate, DBM concentration was found to be $0.408 \pm 0.050$ and $0.279 \pm 0.104$ nmol/mg protein for mice from groups 1 and 2, respectively.

Effect of DBM on Akt signaling pathway and cell cycle regulation. To investigate the in vivo mechanism of action by which DBM exerts its chemopreventive effect, several biomarkers covering the Akt signal transduction pathway and cell cycle regulation were evaluated. The expression of p-PDK-1, pAkt (Ser473), pS6 ribosomal protein (Ser235/236), c-myc, pRb, cyclin A, and cyclin D1 were all found to be increased gradually concomitant with tumor progression, suggesting that they are appropriate biomarkers for prostate tumorigenesis in TRAMP mice (Fig. 5). Our results show that DBM treatment significantly suppressed the expression of these biomarkers. Stronger suppression on these biomarkers was found in group 1 compared with group 2.

Discussion

Radical prostatectomy and radiation therapies are the most common and also the most effective clinical interventions to treat early or localized prostate cancer (15). However, as the disease progresses, advanced or invasive prostate cancers are far more resistant to most of the therapeutic agents, and as a result, androgen deprivation therapy is needed. Substantial tumor remission can be achieved after the deprivation of androgen, but unfortunately, recurrent tumors arise within a period of 2 to 3 years (16). Given that the progression from the earliest lesions to metastasized prostate cancers may take several decades to establish, chemoprevention by dietary compounds could be an ideal strategy to halt prostate tumorigenesis. In the present study, the efficacy of DBM as a chemopreventive agent for prostate tumorigenesis was tested using TRAMP mice model.

DBM, a $\beta$-diketone analogue of curcumin, has shown antineoplastic effects in several carcinogen-induced preclinical models of cancer. In two independent reports, Singletary and colleagues and Huang and colleagues show that diet supplemented with 1% DBM significantly inhibited both the multiplicity and incidence of 7,12-dimethylbenz($a$)anthracene–induced mammary tumor in rat and Sencar mice, respectively (7, 17). More interestingly, both experiments found that DBM was far more potent than curcumin in inhibiting mammary tumors. In addition to mammary tumors, DBM is also reported to inhibit
7,12-dimethylbenz(a)anthracene–induced lymphomas/leukemias and 7,12-tetradecanoylphorbol-13-acetate–induced skin tumors in mice (7). Carcinogen detoxification through activation of phase II detoxifying machinery has been suggested as the main mechanism of action by which DBM exhibits its antineoplastic effect in the carcinogen-induced preclinical model of cancers. However, we have recently shown that DBM can also prevent adenomatous polyposis in APCmin/+ mice at the promotion stage of carcinogenesis through induction of apoptosis, inhibition of cell growth, and deregulation of cell cycle (6). In addition, DBM has been reported to possess antiinflammatory, antimutagenic, antiangiogenic, and antiandrogenic activity (8, 18–20). DBM has been reported to inhibit the growth of LNCaP, DU145, and PC-3 prostate cancer cells in vitro; however, its in vivo efficacy has not yet been reported. In the present study, we tested the potential of DBM as a chemotherapeutic or/and chemopreventive agent using TRAMP-C1 cell lines and TRAMP mice, respectively. We found that, whereas DBM has an IC50 higher than 100 μmol/L (data not shown), it significantly arrested the cells at G2-M phase of cell cycle. More importantly, our in vivo results showed that DBM possessed strong chemopreventive activity as shown by its ability to suppress the progression of prostate tumor in TRAMP mice. Mice fed with DBM developed significantly lower incidence of HG PIN and poorly differentiated carcinoma when compared with their control counterparts. Strikingly, the chemopreventive efficacy of DBM was stronger when the treatment was started earlier (8 versus 12 weeks of age). Approximately 53% and 27% of HG PIN was inhibited in DBM-treated mice from groups 1 and 2, respectively. Poorly differentiated carcinoma was totally prevented in the mice of group 1 compared with 32% inhibition achieved in group 2. Steady-state levels of DBM in plasma obtained from mice from group 1 were found to be higher when compared with mice from group 2. In contrast, DBM levels in prostate tissues obtained from mice from group 1 were higher than group 2, indicating that steady-state levels of the compound in the target tissue rather than plasma could be a better indicator of predicting the cancer chemopreventive efficacy of compound. It is important to note that the concentration of DBM in the prostate tissues detected in the present study was at least 5-fold lower than the concentration found in the intestinal tissues from our previous study (0.4 nmol/mg protein versus 1.97 nmol/mg protein; ref. 6). In addition, the in vivo concentration of DBM found in the prostate tissues is much lower than the in vitro effective dose (50 μmol/L) based on our cell culture studies. This observation suggests that prostate cancer chemoprevention by p.o. administration of DBM is achievable although at a lower concentration than what could be found in the digestive tissues and in the cell culture setting.

Our current in vitro and in vivo studies indicate that cell cycle regulation could be one of the possible mechanisms by which DBM exhibits its antineoplastic effect (6, 21). In fact, one of the common features of cancers is the deregulation of cell cycle. Overexpression of cyclin D1 has been reported in ~25% of prostate cancer, and its expression has been linked with bone metastasis (22, 23). Likewise, c-myc has been found to be associated with clinical progression in prostate cancer (24). In agreement with these observations, we showed that the cell cycle arrest effect of DBM was associated with suppression of these biomarkers in TRAMP-C1 cells. More importantly, the expression of cyclin A, cyclin D1, pRb, and c-myc was found to be associated with tumor progression in TRAMP mice.
model (Fig. 5). Oral administration of DBM significantly suppressed the expression of these markers, and more interestingly, the suppression effect was stronger when the mice were treated earlier (experiment in G1). Because the overexpression of c-Myc and cyclin D1, as well as pRb, promotes G1-S transition and cell cycling, inhibition of these biomarkers could be one of the possible mechanisms by which DBM retards the progression of prostate cancer in TRAMP mice. It is not clear why suppression of cyclin D1, cyclin A, and pRb in TRAMP-C1 cells did not lead to G0-G1 arrest. It has been reported that cyclin A is required for mitosis until mid prophase (25). Although inhibition of cyclin D1 and pRb are associated with G0-G1 arrest, the G1-S checkpoint could be bypassed by certain molecular events, such as activation of Akt signaling pathway (26).

Ablation of Akt signaling pathway has been reported in human prostate cancer, which is associated with cancer progression (27, 28). We have previously shown that DBM can inhibit the expression of pAkt (Ser473) in APCmin/+ mice (6). To investigate if DBM could affect the Akt signaling pathway, expression of pAkt, p-PDK-1, and pS6 was examined. In agreement with clinical observations, activation of Akt signaling pathway as shown by increased expression of pAkt, p-PDK-1, and pS6 concomitantly with tumor progression was observed in TRAMP mice. DBM treatment significantly suppressed the expression of these biomarkers with stronger effect being observed in mice from group 1. Our data suggest that, in addition to cell cycle regulation, DBM could also inhibit Akt signaling pathway to block the cancer progression in TRAMP mice. It is clear that the effect of DBM was significantly stronger if given before the PIN started to develop at 8 weeks of age.

Taken all these results together, our current study showed that DBM is a potent chemopreventive agent that potentially could be considered for clinical intervention of human prostate cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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