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Research Paper

Biochanin A Inhibits Breast Cancer Tumor Growth in A Murine Xenograft Model

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Purpose. Our objective was to determine the effect of the flavonoid biochanin A (BCA), administered alone or in combination with the flavonoids quercetin and epigallocatechin-3-gallate (EGCG), on the growth of human breast cancer MCF-7 cells in a murine xenograft animal model.
Materials and Methods. MCF-7 tumors were implanted into mice and groups of mice were treated with vehicle, BCA at 2 doses (5 or 15 mg/kg), quercetin and EGCG (5 mg/kg each), or BCA combined with quercetin and EGCG (5 mg/kg each). The flavonoids were injected once daily intraperitoneally, with treatment starting 4 weeks prior to cell inoculation.
Results. Treatment with 15 mg/kg of BCA or the mixture of the 3 flavonoids resulted in a reduction in tumor incidence. Tumor size in xenograft mice treated with 15 mg/kg BCA was significantly smaller than in the control group. Although quercetin/EGCG administration did not affect tumor size, treatment with the mixture of the 3 flavonoids at doses of 5 mg/kg produced similar effects as seen with 15 mg/kg BCA.
Conclusions. Our findings indicate that BCA inhibits tumor growth in a xenograft animal model; BCA may represent a breast cancer preventive agent, either administered alone or in combination with other flavonoids.

KEY WORDS: biochanin A; breast cancer xenograft; EGCG; flavonoid combination; quercetin.

INTRODUCTION

In the United States, breast cancer is the second leading cause of cancer death (1). An estimated 178,480 new cases of invasive breast cancer and 40,460 breast cancer deaths are expected to occur in women in the US during 2007 (1). Breast cancer is diagnosed more frequently in postmenopausal women and the hormone sensitive tumor incidence is higher in postmenopausal women than in premenopausal women (2). Asian women have a relatively lower incidence of breast cancer. One distinct aspect of Asian diets is the high consumption of soy products. Regular consumption of soy foods by Asian women has been correlated with a lower incidence of breast cancer (3). It has been suggested that the isoflavones provide at least part of the protective effect of soy (4).

Although biochanin A (BCA; Fig. 1) is not present in soy, it is also an isoflavone, representing the major constituent in red clover (*Trifolium pratense*). Popularly sold herbal supplements for alleviation of menopausal symptoms contain high amounts of BCA. BCA has been reported to have health beneficial effects including antioxidant (5), anti-inflammatory (6), and anticarcinogenic effects (7–10). In our previous study, the effect of physiologically-relevant concentrations of BCA on gene expression in normal human mammary epithelial cells (HMEC), immortalized but non-tumorigenic cells (MCF-12A) and tumorigenic (MCF-7) mammary cells was evaluated using gene arrays (11). BCA treatment produced beneficial chemopreventive alterations in gene expression, involving induction of tumor suppressor genes such as MHS2 (mutS homolog 2 (colon cancer, nonpolyposis type1), NF2 (Neurofibromin 2), CD5 (T-cell surface glycoprotein CD5), ATF-2 (Activating transcription factor 2), and WT1 (Wilms' tumor 1), at concentrations that can be obtained following dietary intake (11). BCA also up-regulated the cell cycle arrest gene p18 (cyclin-dependent kinase inhibitor 2C (inhibits CDK4) (11).

Compared to other isoflavones, such as genistein and daidzein, only a few studies (12,13) have been performed examining BCA activity in a human cancer xenograft model. BCA has been reported to have tumor-suppressive effects in cancers of the prostate (12) and gastrointestinal tract (13). Although several *in vitro* studies have examined the growth inhibitory effect of BCA in human breast cancer cell lines (14–16), further *in vivo* studies are required to investigate the breast cancer preventive effects of BCA after long-term exposure, to mimic daily exposure to an isoflavone-rich diet.

Quercetin and EGCG (Fig. 1), two of the most abundant flavonoids in the diet, have been shown to have synergistic effects with other dietary polyphenolic compounds *in vitro* and *in vivo* (17–20). When EGCG and genistein were

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ABBREVIATIONS: BCA, biochanin A; EGCG, epigallocatechin-3-gallate.

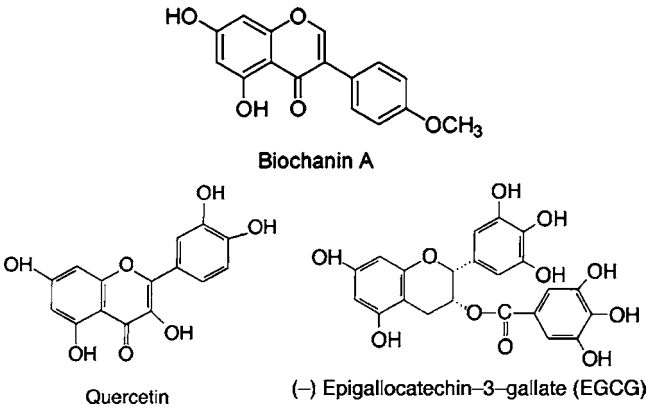

Fig. 1. Chemical structures of biochanin A, quercetin and (−)-epigallocatechin-3-gallate.

combined, synergistic suppression of nitric oxide generation was reported in mouse macrophage RAW 264.7 cells (18). The effect of a combination of quercetin and trans-resveratrol on mitochondrial cytochrome c release and caspase-3 activity was greater than the expected additive response (19). Soy phytochemical concentrate (SPC, containing genistein) and tea components (containing EGCG), when administered alone, had no significant effect on tumor angiogenesis, whereas SPC and tea combinations significantly inhibited MCF-7 tumor angiogenesis in mice (17).

In this study the hypotheses tested are: (1) BCA has inhibitory effects on the growth of estrogen receptor positive human breast cancer MCF-7 cells in a murine xenograft model, and (2) the co-administration of the flavonoids quercetin and EGCG will result in additive or synergistic effects with BCA on MCF-7 cell growth in the xenograft model.

MATERIALS AND METHODS

Materials

BCA, quercetin, EGCG, and β-glucuronidase type H-5 (aryl-sulfate sulfohydrolase from *Helix pomatia*; reported sulfatase activity of 15–40 units/mg and β-glucuronidase activity of 400–600 units/mg), were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640 medium and fetal bovine serum were from Gibco BRL (Buffalo, NY). The MCF-7 human breast cancer cell line was obtained from the National Cancer Institute.

Cell Culture

MCF-7 cells were grown in 75 cm^2 cell culture flasks in RPMI 1640 culture media supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml of streptomycin in a humidified atmosphere of 5% CO_2/95% air at 37°C.

Mouse Xenograft Model

Six-week-old female athymic nude mice were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Mice were housed in a filtered laminar air flow room in standard vinyl cages with air filter tops. Cages and bedding food were autoclaved before use. Water and food were autoclaved and provided *ad libitum*. The experimental protocol is shown in Fig. 2. After 1 week of acclimatization, an estradiol pellet (0.72 mg, 60 day release, Innovative Research of America, Sarasota, FL) was implanted subcutaneously in the interscapular region of mice on the starting day of the treatment (to support the growth of the estrogen-dependent MCF-7 tumor). This mimics premenopausal breast cancer with high estradiol concentrations. Animals were treated with compounds once daily by i.p. injection as follows. Mice were divided into five groups: control (vehicle, DMSO/PEG200/water=40:260:100, n=15), 5 mg/kg of BCA (n=15), 15 mg/kg of BCA (n=15), 5 mg/kg of quercetin combined with 5 mg/kg EGCG (n=8), and the mixture of BCA+EGCG+quercetin, 5 mg/kg each (Mix; n=16). The cell culture media was switched to phenol red-free RPMI 1640 containing 10% charcoal-dextran stripped FBS (Hyclone Laboratories, Inc., Logan, UT, USA) 1 week before the inoculation of MCF-7 cells into the mice. Four weeks later, the mice were anesthetized and injected subcutaneously with 100 μl cell suspension (2×10^6 cells in 100 μl of PBS). Mice exhibiting tumors were counted, and the tumor sizes were measured initially after 2 weeks, with the final measurement taken 4 weeks after tumor cell inoculation.

Tumor length (l) and width (w) were measured by a single observer every three days using a caliper and the tumor volume was determined using the formula for an ellipsoid sphere: $l\times w^2/2$ (17). Body weight was measured weekly but more frequently measured during the first 3 weeks to monitor potential drug-related toxicity. At the end of the study (4 weeks after cell inoculation), animals were sacrificed. Tumors were removed and immersed in 10% neutral buffered formalin. Formalin-fixed tumors were processed for paraffin embedding, sliced, and were stained with hematoxylin and eosin (H&E). Slides were observed using a Zeiss Axiovert 35 microscope (Carl Zeiss, Inc., Oberkochen, West Germany).

Analysis of BCA in Plasma and Liver

On the 56th day of treatment with flavonoids, animals were anesthetized and blood samples were taken 30 min after flavonoid administration. The livers were excised, rinsed with cold 0.9% NaCl solution to minimize blood remaining in the tissue, blotted dry with paper tissue, and homogenized with four volumes of HPLC grade water using a tissue homogenizer.

To measure 'total' BCA (free aglycone plus aglycone following the hydrolysis of the glucuronide and sulfate conjugates), an aliquot of plasma and liver samples were

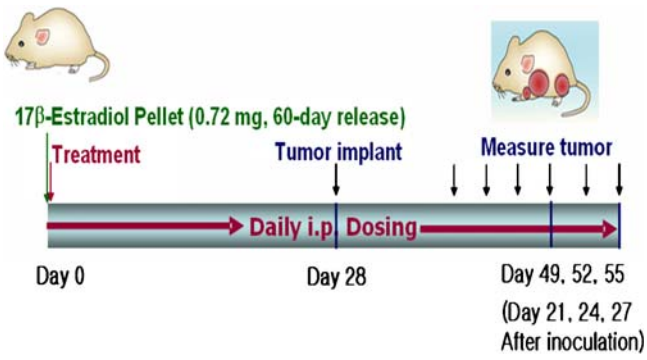

Fig. 2. Experimental protocol.

enzymatically hydrolyzed *in vitro* to determine the total amount of BCA plus conjugates. Sodium acetate buffer (0.2 M, pH 5) with 1,000 units/ml of β-glucuronidase/sulfatase type H-5 (from *Helix pomatia*, Sigma Chemical, St. Louis, MO, USA) were added to samples and incubated for 90 min at 37°C. The reaction was stopped by adding 1 ml of ether, and then centrifuging. The supernatant was removed, dried under nitrogen, and then resuspended in 50:50 methanol/acetonitrile. Free BCA concentrations were measured in the same fashion as described for the total BCA except that 50 µl of 0.2% sodium chloride without enzymes was added to the samples.

The analysis was carried out on a Waters HPLC system (Milford, Massachusetts, USA) equipped with an Alltech Alltima™ column (C18, 250×4.6 mm i.d., 5 µm, W. R. Grace and Co., Deerfield, IL, USA). The mobile phase consisted of 45% acetonitrile in 1% of acetic acid. The UV detector was set at a single wavelength of 260 nm. The assay was linear in the range of 10–1,500 and 100–1,000 ng/ml with r^2>0.99 in both cases, for mouse plasma and liver, respectively. The assay recovery at low, medium and high concentrations was between 93.5% and 104% for both liver and plasma, with a standard deviation of <10%. The lower limit of quantification was 10 ng/ml for plasma and 100 ng/ml for liver.

Data Analysis

One-way ANOVA followed by the Newman–Keuls test and the Student's unequal variance t-test with $p<0.05$ set as the significance level were used for statistical analysis. Survival analysis was constructed using the Kaplan–Meier method. Comparisons of survival curves were carried out using the log-rank test. GraphPad Prism software (GraphPad Software, San Diego, CA, USA) was used to analyze all data.

RESULTS

Effects of BCA and Mix on Tumor Incidence and Tumor Volume in MCF-7 Xenograft Mice

Groups of mice were treated with 5 mg/kg BCA, 15 mg/kg BCA, 5 mg/kg BCA with 5 mg/kg quercetin and 5 mg/kg EGCG (Mix), or 5 mg/kg quercetin combined with 5 mg/kg EGCG. The treatments did not alter body weight significantly (Fig. 3).

Tumor onset was delayed by 5 mg/kg BCA (Day 18), 15 mg/kg BCA (Day 21) or Mix (Day 21) compared to the control group (Day 12; Fig. 4). Comparisons between the survival curves between the various treatment groups showed that 5 mg/kg of BCA (60.0%, p=0.716) and quercetin combined with EGCG (37.5%, p=0.195) did not reduce the tumor incidence compared with control mice (66.7%). However, treatment with 15 mg/kg of BCA (33.3%, p=0.0719) or Mix (31.2%, p=0.0506) led to a marginally significant reduction in tumor incidence compared with that of control (Fig. 4).

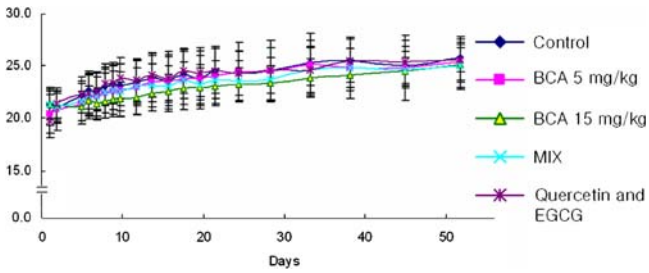

Fig. 3. Body weight measured over time in the treatment groups (control, BCA 5 mg/kg, BCA 15 mg/kg, the mixture of 5 mg/kg of BCA, quercetin and EGCG [mean±SD]). The treatments did not alter the body weight of mice significantly (one way ANOVA followed by Newman–Keuls test).

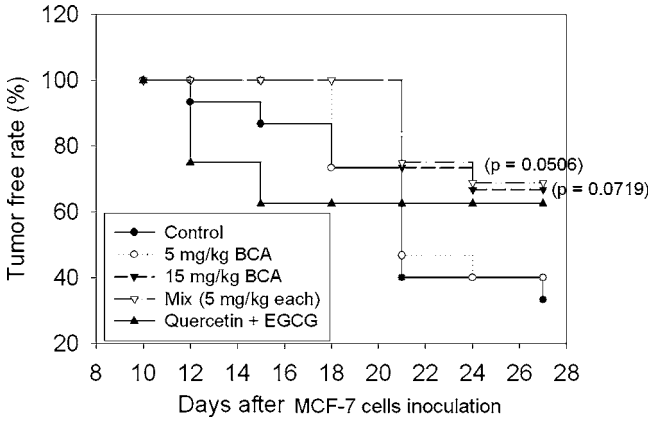

Fig. 4. Kaplan–Meier survival analysis. Tumor onset was delayed by BCA or the flavonoid mixture compared to the control group. Comparisons between the survival curves between the various treatment groups showed that 5 mg/kg of BCA (p=0.716) and quercetin combined with EGCG (5 mg/kg each; p=0.195) did not reduce the tumor incidence compared with control mice. Treatment with 15 mg/kg of BCA (p=0.0719) or the flavonoid mixture (MIX, BCA+quercetin+EGCG, 5 mg/kg of each; p=0.0506) led to a marginally significant reduction in tumor incidence compared with that of the control.

Figure 5 shows the effects of BCA, quercetin plus EGCG and Mix on tumor volume in mice. Tumors in the treatment groups were significantly smaller than that in the control group. The final tumor size from mice treated with 5 mg/kg BCA, 15 mg/kg BCA, and Mix were smaller than the tumor size of control mice by 0.581-fold (p<0.05), 0.417-fold (p<0.01), and 0.363-fold (p<0.01) on Day 27 after cell inoculation (treated for 55 days) with flavonoids. However, the tumor volume in mice treated with quercetin and EGCG was not significantly different from that in the control group. All tumors appeared only at the inoculation sites.

Excised tumors were further examined to determine histological characteristics. Figure 6 shows H&E staining of tumor tissues resected from mice treated with control, BCA (5 mg/kg), BCA (15 mg/kg) and Mix (BCA, quercetin, and EGCG, 5 mg/kg each), quercetin and EGCG (5 mg/kg each). Decreased cellularity and increased necrosis were observed in tumor tissues treated with BCA (5 mg/kg or 15 mg/kg) or Mix compared to the control (Fig. 6). The cell growth inhibitory effects of the treatments were: Mix>BCA (15 mg/kg)>BCA (5 mg/kg)>quercetin/EGCG (5 mg/kg each).

BCA Levels in Plasma and Liver Tissue

Plasma and liver levels of BCA were determined when mice were sacrificed on Day 56 (30 min after the treatment). Because we did not have sufficient quantities of tumor tissues for analysis, we could not measure BCA in tumor tissues. The dose-normalized concentrations of BCA in plasma and liver are shown in Figs 7(A) and (B), respectively. Figure 8 shows

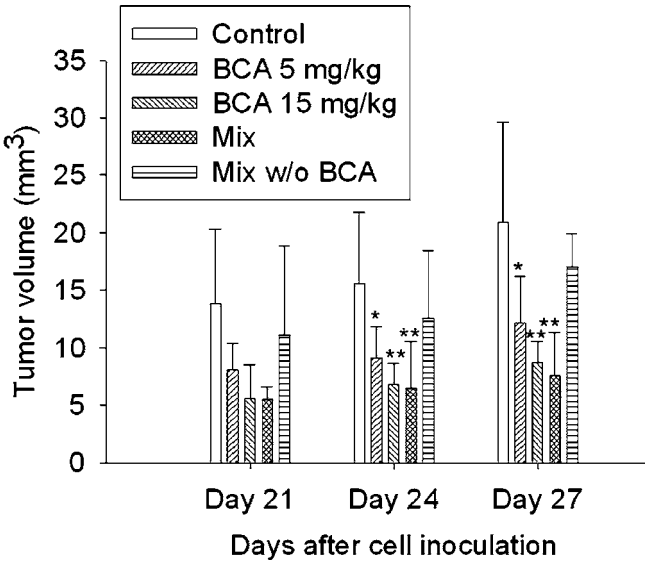

Fig. 5. Tumor growth in xenograft mice. Tumor volume (cubic millimeter) in the treatment groups was significantly less than in the control group (mean±SD, one way ANOVA followed by Newman–Keuls test; *$p<0.05$, **$p<0.01$). The final tumor volume in mice treated with 5 mg/kg BCA, 15 mg/kg BCA, and Mix (BCA, quercetin and EGCG, 5 mg/kg each) were smaller than the tumor volume in the control mice by 0.581-fold ($p<0.01$), 0.417-fold ($p<0.01$), and 0.363-fold ($p<0.01$) on Day 27 after cell inoculation. The tumor volume in mice treated with quercetin and EGCG (Mix without BCA, 5 mg/kg of each) was not significantly different from that in the control group.

the dose-normalized concentrations of total BCA. Plasma concentrations of BCA aglycone in mice receiving the mixture of BCA, quercetin and EGCG (5 mg/kg each) were higher than those in mice receiving 5 mg/kg BCA alone and higher than those in mice receiving 15 mg/kg BCA alone.

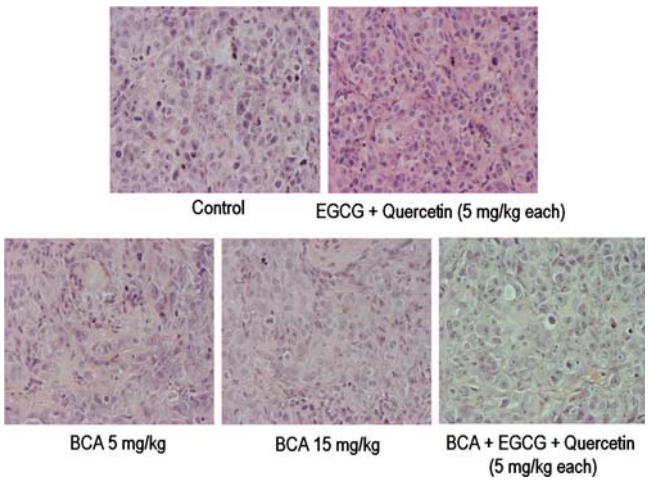

Fig. 6. Histological analysis of resected tumors. H&E staining of tumor tissue from control and from BCA (5 mg/kg), BCA (15 mg/kg), Mix (BCA, quercetin, and EGCG, 5 mg/kg each), quercetin and EGCG (5 mg/kg of each)-treated mice. Nuclei stain blue and cytoplasm stains pink. Decreased cellularity and increased necrosis can be seen in the BCA or Mix-treated tissue compared to the control. The cell growth inhibitory effects of the treatments were: Mix>BCA (15 mg/kg)>BCA (5 mg/kg)>quercetin+EGCG. Three tumors per group were used for staining and the images presented here were randomly chosen from three images. Magnification: ×20

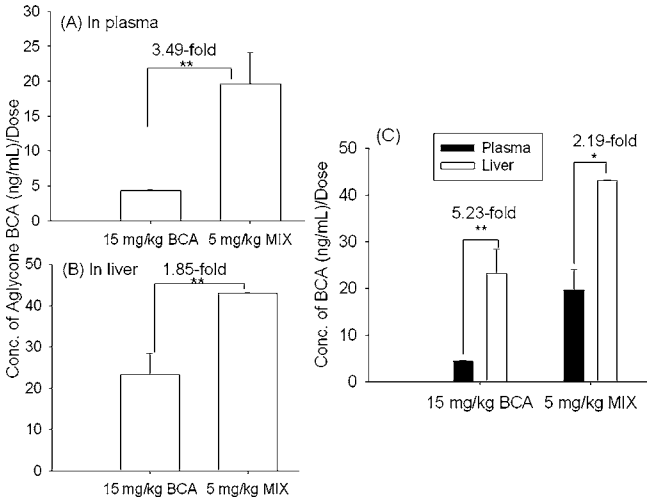

Fig. 7. Dose-normalized concentration of BCA in plasma (**A**) and liver (**B**), and comparison of the concentrations of BCA in plasma and liver (**C**) (mean±SD, *$p<0.05$; **$p<0.01$). Blood and liver samples were taken 30 min after the last flavonoid dose.

In the 15 mg/kg BCA group, the BCA level in liver was significantly higher than that in plasma [Fig. 7(C), 5.23-fold, $p<0.01$]. In the Mix group, the BCA level was also higher in liver than plasma [Fig. 7(C), 2.19-fold, $p<0.05$]. In contrast, the total BCA concentrations [unchanged plus glucuronide and sulfate conjugates, Fig. 8(A)] and conjugated BCA concentrations [Fig. 8(B)] were higher in plasma than liver. This suggests tissue accumulation of BCA aglycone, but not of BCA conjugates, which are more hydrophilic than the aglycone. Liver concentrations of BCA were below the assay detection limit in animals receiving only BCA 5 mg/kg.

DISCUSSION

In the present study, we provide the first *in vivo* evidence for the efficacy of BCA alone or BCA in combination with other flavonoids on estrogen-dependent human breast cancer MCF-7 tumors in mice. MCF-7 cells have been used for many years as a model cell line to investigate hormonally responsive breast cancer (21). The tissue histology confirmed the cell growth inhibitory effect of BCA.

There are several interesting findings from our *in vivo* study. First, BCA, at doses as low as 5 mg/kg (resulting in 73.0 ng/ml [270 nM] of unchanged BCA in plasma), could

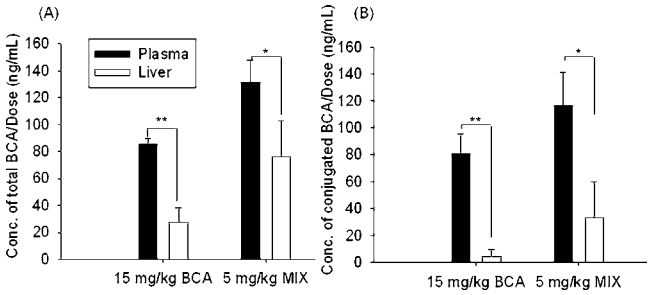

Fig. 8. Dose-normalized concentration of total BCA (unchanged plus glucuronide and sulfate conjugates) in plasma and liver (mean±SD, *$p<0.05$; **$p<0.01$). Blood and liver samples were taken 30 min after the last flavonoid dose.

inhibit tumor growth (but not tumor incidence) in a xenograft mice model. These findings are different from *in vitro* studies where a similar concentration of BCA (330 or 500 nM) had no effect on the cellular proliferation of MCF-7 cells (15). The observed accumulation of BCA in tumor tissue may explain this discrepancy, because BCA can inhibit the growth of estrogen receptor (ER) positive cells at concentrations >20 μM through ER-independent mechanisms, while stimulating the growth at lower concentrations (22,23). Unfortunately we did not have sufficient tumor samples to measure BCA concentrations in tumor tissues. However, we found that BCA concentrations in the liver were five times higher than that in plasma after long-term exposure, suggesting possible tissue accumulation. In a study by Farhan *et al*. (24), the authors reported that after only one dose of genistein given orally, genistein accumulated in the prostate and was present at 10-fold higher levels in the prostatic tissue compared to plasma concentrations. Another study reported the concentrations of the isoflavone equol in samples of plasma and prostatic fluid from normal human subjects (25). In one prostatic fluid sample from a subject in Hong Kong, the concentration of total equol was over 3,200 ng/ml (13.5 μM) when the plasma concentration of equol in this man was 120 ng/ml, suggesting an ability of the prostate to concentrate equol (25). A second reason for the discrepancy between the *in vivo* and *in vitro* results could be related to the presence of different metabolites formed *in vivo* than *in vitro*: BCA and/or its metabolites may have an inhibitory effect of cell growth.

The concomitant administration of BCA with quercetin and EGCG resulted in increased efficacy, having effects similar to that of BCA 15 mg/kg, whereas the administration of quercetin and EGCG had no effect on tumor size. This may reflect pharmacokinetic and/or pharmacodynamic interactions. Plasma concentrations of BCA aglycone in mice receiving the mixture of BCA, quercetin and EGCG 5 mg/kg each were higher than that in mice receiving 5 mg/kg BCA alone, and even higher than that in mice receiving 15 mg/kg BCA alone. This suggests that co-administration of flavonoids may affect the bioavailability of BCA. In fact, we have shown that the administration of quercetin and EGCG with BCA results in an increased oral bioavailability of BCA in rats (26). The co-administration of quercetin and EGCG significantly increases the BCA area under the plasma concentration *vs*. time curve (AUC) in rats, after both i.v. and oral administration of BCA, increasing the oral bioavailability by about three-fold (26). The mechanisms include, at least in part, inhibition of conjugative metabolism and P-glycoprotein-mediated efflux in intestinal cells (26). Therefore, pharmacokinetic interactions are possible, although it is not known whether the increased efficacy of BCA is entirely due to pharmacokinetic interactions.

The mechanism by which BCA inhibits proliferation and induces apoptosis in cancer cells has not been fully elucidated. The chemical structures of isoflavones are similar to 17β-estradiol, and it is known that isoflavones can weakly bind to the estrogen receptor (27). Estrogen plays a key role in estrogen-dependent breast cancer development and growth, exerting effects on estrogen-dependent tumors by binding to the ER and inducing ER-dependent transcriptional expression of estrogen-responsive genes that promote cancer cell proliferation. Although BCA has very little affinity for the ER, BCA can be present at 100 to 1,000 times the concentration of endogenous estrogens. Therefore, BCA may be able to compete with estrogen at the ER and inhibit cellular proliferation. BCA has been also reported to modulate metabolism by both phase I and II pathways and in this way may alter estrogen metabolism (28–30).

The current studies utilized intraperitoneal administration of all flavonoids in order to increase the bioavailability of the flavonoids, compared to that expected after oral administration. Our previous studies with BCA reported a bioavailability of 1.2% following an oral dose of 5 mg/kg and about 40% after an intraperitoneal dose of 5 mg/kg in rats (31). Due to the fact that BCA was administered intraperitoneally in the current study and that higher concentrations would be expected compared with oral administration, the results of this study indicate the plasma concentrations of BCA (about 270 nM), but not the oral dose, at which significant effects on tumor growth are observed. In humans, BCA has poor bioavailability mainly due to extensive first-pass metabolism and biliary elimination, although the absorption of BCA is rapid because of its high permeability (32). The concentrations of BCA achieved in human plasma following dietary administration tend to be in the low nM range (10–20 nM; 33,34), whereas the concentrations following the oral ingestion of dietary supplements (herbal preparations) are above 100 nM (35). These concentrations in human plasma obtained after dietary supplement ingestion, based on our study findings, may be high enough to observe the chemopreventive effects of BCA.

Previous studies in the literature that have examined the breast cancer preventive effects of BCA in animal models support the findings of the present study. BCA is effective in the prevention of *N*-nitroso-*N*-methylurea-induced rat mammary carcinogenesis at doses as low as 10 mg/kg added to the diet (8). As Messina and Loprinzi (36) stated in their review, this is the most pronounced inhibitory effect in response to the administration of an isolated isoflavone that has been reported in the literature. Additionally, in a study in a breast cancer MMTV transgenic mouse model, animals treated with BCA demonstrated a significantly lower incidence of breast cancer than control or daidzein-treated animals (37).

In conclusion, BCA administered once daily i.p. at doses of 5 or 15 mg/kg, or when administered once daily at a 5 mg/kg dose with concomitant quercetin (5 mg/kg) and EGCG (5 mg/kg), were effective in reducing the incidence and the growth of estrogen-dependent MCF-7 tumors in a breast cancer xenograft mouse model. This represents the first study examining the effect of BCA as a breast cancer preventive agent in a breast cancer murine xenograft model. BCA may represent a promising agent for breast cancer prevention either alone or in combination with other flavonoids. The administration of several flavonoids in combination may potentiate the chemopreventive activities of a single flavonoid through pharmacokinetic and/or pharmacodynamic mechanisms.

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REFERENCES

1. www.cancer.org. Cancer Statistics 2007, American Cancer Society, Inc. Available at: http://www.cancer.org/. (2007).
2. A. Brodie, Q. Lu, and J. Nakamura. Aromatase in the normal breast and breast cancer. *J Steroid Biochem Mol Biol* **61**:281–286 (1997).
3. C. A. Lamartiniere. Protection against breast cancer with genistein: a component of soy. *Am J Clin Nutr* **71**:1705S–1707S (2000).
4. S. Barnes, G. Peterson, C. Grubbs, and K. Setchell. Potential role of dietary isoflavones in the prevention of cancer. *Adv Exp Med Biol* **354**:135–147 (1994).
5. C. E. Rufer and S. E. Kulling. Antioxidant activity of isoflavones and their major metabolites using different *in vitro* assays. *J Agric Food Chem* **54**:2926–2931 (2006).
6. A. Kalayciyan, H. Orawa, S. Fimmel, F. H. Perschel, J. B. Gonzalez, R. G. Fitzner, C. E. Orfanos, and C. C. Zouboulis. Nicotine and biochanin A, but not cigarette smoke, induce anti-inflammatory effects on keratinocytes and endothelial cells in patients with Behcet's disease. *J Invest Dermatol* **127**:81–89 (2007).
7. S. Puli, J. C. Lai, and A. Bhushan. Inhibition of matrix degrading enzymes and invasion in human glioblastoma (U87MG) cells by isoflavones. *J Neurooncol* **79**:135–142 (2006).
8. T. Gotoh, K. Yamada, H. Yin, A. Ito, T. Kataoka, and K. Dohi. Chemoprevention of N-nitroso-N-methylurea-induced rat mammary carcinogenesis by soy foods or biochanin A. *Jpn J Cancer Res* **89**:137–142 (1998).
9. Y. S. Lee, J. S. Seo, H. T. Chung, and J. J. Jang. Inhibitory effects of biochanin A on mouse lung tumor induced by benzo(a) pyrene. *J Korean Med Sci* **6**:325–328 (1991).
10. J. M. Cassady, T. M. Zennie, Y. H. Chae, M. A. Ferin, N. E. Portuondo, and W. M. Baird. Use of a mammalian cell culture benzo(a)pyrene metabolism assay for the detection of potential anticarcinogens from natural products: inhibition of metabolism by biochanin A, an isoflavone from Trifolium pratense L. *Cancer Res* **48**:6257–6261 (1988).
11. Y. J. Moon, D. A. Brazeau, and M. E. Morris. Effects of flavonoids genistein and biochanin A on gene expression and their metabolism in human mammary cells. *Nutr Cancer* **57**:48–58 (2007).
12. L. Rice, V. G. Samedi, T. A. Medrano, C. A. Sweeney, H. V. Baker, A. Stenstrom, J. Furman, and K. T. Shiverick. Mechanisms of the growth inhibitory effects of the isoflavonoid biochanin A on LNCaP cells and xenografts. *Prostate* **52**:201–212 (2002).
13. K. Yanagihara, A. Ito, T. Toge, and M. Numoto. Antiproliferative effects of isoflavones on human cancer cell lines established from the gastrointestinal tract. *Cancer Res* **53**:5815–5821 (1993).
14. C. Ying, J. T. Hsu, and S. C. Shieh. Growth inhibition of human endothelial cells by the phyto-oestrogen biochanin A, a metabolite of genistein. *Br J Nutr* **85**:615–620 (2001).
15. J. T. Hsu, H. C. Hung, C. J. Chen, W. L. Hsu, and C. Ying. Effects of the dietary phytoestrogen biochanin A on cell growth in the mammary carcinoma cell line MCF-7. *J Nutr Biochem* **10**:510–517 (1999).
16. T. G. Peterson, L. Coward, M. Kirk, C. N. Falany, and S. Barnes. The role of metabolism in mammary epithelial cell growth inhibition by the isoflavones genistein and biochanin A. *Carcinogenesis* **17**:1861–1869 (1996).
17. J. R. Zhou, L. Yu, Z. Mai, and G. L. Blackburn. Combined inhibition of estrogen-dependent human breast carcinoma by soy and tea bioactive components in mice. *Int J Cancer* **108**:8–14 (2004).
18. A. Murakami, D. Takahashi, K. Koshimizu, and H. Ohigashi. Synergistic suppression of superoxide and nitric oxide generation from inflammatory cells by combined food factors. *Mutat Res* **523–524**:151–161 (2003).
19. M. Mouria, A. S. Gukovskaya, Y. Jung, P. Buechler, O. J. Hines, H. A. Reber, and S. J. Pandol. Food–derived polyphenols inhibit pancreatic cancer growth through mitochondrial cytochrome C release and apoptosis. *Int J Cancer* **98**:761–769 (2002).
20. F. V. So, N. Guthrie, A. F. Chambers, M. Moussa, and K. K. Carroll. Inhibition of human breast cancer cell proliferation and delay of mammary tumorigenesis by flavonoids and citrus juices. *Nutr Cancer* **26**:167–181 (1996).
21. K. B. Horwitz, M. E. Costlow, and W. L. McGuire. MCF-7; a human breast cancer cell line with estrogen, androgen, progesterone, and glucocorticoid receptors. *Steroids* **26**:785–795 (1975).
22. C. Ying, J. T. Hsu, H. C. Hung, D. H. Lin, L. F. Chen, and L. K. Wang. Growth and cell cycle regulation by isoflavones in human breast carcinoma cells. *Reprod Nutr Dev* **42**:55–64 (2002).
23. S. T. Willard and L. S. Frawley. Phytoestrogens have agonistic and combinatorial effects on estrogen-responsive gene expression in MCF-7 human breast cancer cells. *Endocrine* **8**:117–121 (1998).
24. H. Farhan, K. Wahala, H. Adlercreutz, and H. S. Cross. Isoflavonoids inhibit catabolism of vitamin D in prostate cancer cells. *J Chromatogr B Analyt Technol Biomed Life Sci* **777**:261–268 (2002).
25. M. S. Morton, P. S. Chan, C. Cheng, N. Blacklock, A. Matos-Ferreira, L. Abranches-Monteiro, R. Correia, S. Lloyd, and K. Griffiths. Lignans and isoflavonoids in plasma and prostatic fluid in men: samples from Portugal, Hong Kong, and the United Kingdom. *Prostate* **32**:122–128 (1997).
26. Y. J. Moon and M. E. Morris. Pharmacokinetics and bioavailability of the bioflavonoid biochanin A: Effects of quercetin and EGCG on biochanin A disposition in rats. Mol Pharm **4**:865–872 (2007).
27. A. C. Pike, A. M. Brzozowski, R. E. Hubbard, T. Bonn, A. G. Thorsell, O. Engstrom, J. Ljunggren, J. A. Gustafsson, and M. Carlquist. Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *Embo J* **18**:4608–4618 (1999).
28. M. Lacey, J. Bohday, S. M. Fonseka, A. I. Ullah, and S. A. Whitehead. Dose-response effects of phytoestrogens on the activity and expression of 3beta-hydroxysteroid dehydrogenase and aromatase in human granulosa-luteal cells. *J Steroid Biochem Mol Biol* **96**:279–286 (2005).
29. N. R. Bianco, L. J. Chaplin, and M. M. Montano. Differential induction of quinone reductase by phytoestrogens and protection against oestrogen-induced DNA damage. *Biochem J* **385**:279–287 (2005).
30. X. Y. Sun, C. A. Plouzek, J. P. Henry, T. T. Wang, and J. M. Phang. Increased UDP-glucuronosyltransferase activity and decreased prostate specific antigen production by biochanin A in prostate cancer cells. *Cancer Res* **58**:2379–2384 (1998).
31. Y. J. Moon, K. Sagawa, K. Frederick, S. Zhang, and M. E. Morris. Pharmacokinetics and bioavailability of the isoflavone biochanin A in rats. *AAPS J* **8**:E433–442 (2006).
32. X. Jia, J. Chen, H. Lin, and M. Hu. Disposition of flavonoids via enteric recycling: enzyme-transporter coupling affects metabolism of biochanin A and formononetin and excretion of their phase II conjugates. *J Pharmacol Exp Ther* **310**:1103–1113 (2004).
33. M. H. Choi, K. R. Kim, J. K. Hong, S. J. Park, and B. C. Chung. Determination of non-steroidal estrogens in breast milk, plasma, urine and hair by gas chromatography/mass spectrometry. *Rapid Commun Mass Spectrom* **16**:2221–2228 (2002).
34. K. D. Setchell, N. M. Brown, P. Desai, L. Zimmer-Nechemias, B. E. Wolfe, W. T. Brashear, A. S. Kirschner, A. Cassidy, and J. E. Heubi. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J Nutr* **131**:1362S–1375S (2001).
35. J. Howes, M. Waring, L. Huang, and L. G. Howes. Long-term pharmacokinetics of an extract of isoflavones from red clover (Trifolium pratense). *J Altern Complement Med* **8**:135–142 (2002).
36. M. J. Messina and C. L. Loprinzi. Soy for breast cancer survivors: a critical review of the literature. *J Nutr* **131**:3095S–3108S (2001).
37. H. Mizunuma, K. Kanazawa, S. Ogura, S. Otsuka, and H. Nagai. Anticarcinogenic effects of isoflavones may be mediated by genistein in mouse mammary tumor virus-induced breast cancer. *Oncology* **62**:78–84 (2002).