Research Article

Cancer Prevention Research

Pomegranate Ellagitannin–Derived Compounds Exhibit Antiproliferative and Antiaromatase Activity in Breast Cancer Cells *In vitro*

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Abstract

Estrogen stimulates the proliferation of breast cancer cells and the growth of estrogen-responsive tumors. The aromatase enzyme, which converts androgen to estrogen, plays a key role in breast carcinogenesis. The pomegranate fruit, a rich source of ellagitannins (ET), has attracted recent attention due to its anticancer and antiatherosclerotic properties. On consumption, pomegranate ETs hydrolyze, releasing ellagic acid, which is then converted to 3,8-dihydroxy-6H-dibenzo[b,d]pyran-6-one ("urolithin") derivatives by gut microflora. The purpose of this study was to investigate the antiaromatase activity and inhibition of testosterone-induced breast cancer cell proliferation by ET-derived compounds isolated from pomegranates. A panel of 10 ET-derived compounds including ellagic acid, gallagic acid, and urolithins A and B (and their acetylated, methylated, and sulfated analogues prepared in our laboratory) were examined for their ability to inhibit aromatase activity and testosterone-induced breast cancer cell proliferation. Using a microsomal aromatase assay, we screened the panel of ET-derived compounds and identified six with antiaromatase activity. Among these, urolithin B (UB) was shown to most effectively inhibit aromatase activity in a live cell assay. Kinetic analysis of UB showed mixed inhibition, suggesting more than one inhibitory mechanism. Proliferation assays also determined that UB significantly inhibited testosterone-induced MCF-7aro cell proliferation. The remaining test compounds also exhibited antiproliferative activity, but to a lesser degree than UB. These studies suggest that pomegranate ET–derived compounds have potential for the prevention of estrogen-responsive breast cancers. *Cancer Prev Res; 3(1); 108–13. ©2010 AACR.*

Introduction

Treatment of hormone-dependent breast cancers has historically focused on manipulation of the level and/or activity of estrogen. Typically, inhibition of estrogen activity is achievable through antagonism of the estrogen receptor (ER) through selective ER modulators (1–3) or estrogen deprivation through inhibition of estrogen synthesis by the aromatase enzyme (4–6). The aim of chemoprevention research is to identify well-tolerated, highly effective strategies for the prevention of occurrence and reoccurrence of cancer. Currently, interest in a number of fruits high in polyphenolic compounds has been raised due to their reported chemopreventive potential.

Pomegranate juice (*Punica granatum* L) has been shown to be high in antioxidant activity, which is generally attributed to its high polyphenol content. Pomegranate juice is produced by squeezing of the whole fruit, leading to a high content of polyphenols, among which ellagitannins (ET) predominate (7). Pomegranate juice and its purified ETs have been shown to inhibit cancer cell proliferation (8) and inflammatory cell signaling (9) *in vitro*. Previous work has shown that ETs from pomegranate juice are hydrolyzed to ellagic acid (EA), which is then taken up intact and metabolized by human colonic microflora to 3,8-dihydroxy-6H-dibenzo[b,d]pyran-6-one derivatives [urolithin A (UA) and B (UB); refs. 10–13]. These compounds have been shown to inhibit the growth of human prostate and breast cancer cell lines (14). *In vitro* studies have shown that UA and UB are taken up intact in MCF-7 breast cancer cells and converted to sulfate and glucuronate metabolites. In addition, it has been shown that UB is partially converted to UA in MCF-7 cells (15). Finally, *in vivo* studies have shown that pomegranate extract and UA both inhibited the growth of LAPC-4–derived tumors in severe combined immunodeficient mice (14).

A recent study by Larrosa et al. (15) also showed that UA and UB exhibit both estrogenic and antiestrogenic effects in MCF-7 human breast cancer cells and therefore may have selective ER modulator–like qualities. Based on the above information, we investigated the antiaromatase activity of a panel of ET-derived metabolites and synthetic analogues in both a microsome assay and an in-cell

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doi: 10.1158/1940-6207.CAPR-08-0225

aromatase assay. The kinetics of the interaction was determined and we evaluated the ability of the ET metabolites to inhibit testosterone-induced cell proliferation in the MCF-7aro aromatase overexpressing breast cancer cell line. These studies bring us closer to an understanding of how the ET metabolites resulting from pomegranate ingestion can affect the growth of estrogen-responsive breast cancer cells.

Materials and Methods

Reagents and instruments

All solvents were high performance liquid chromatography (HPLC) grade from Fisher Scientific Co. Formic, and phosphoric acids and chemicals used for syntheses of the urolithin derivatives (2-bromobenzoic, 2-bromo-5-methoxybenzoic and acetic acids, resorcinol, potassium dihydrogen phosphate, etc.) were purchased from Sigma-Aldrich. The HPLC with UV detection analyses were carried out on a Waters Alliance 2690 system equipped with a photodiode array detector (Waters Corp.). The HPLC with electrospray ionization mass spectrometry system consisted of an LCQ Classic Finnigan system (ThermoFinnigan), equipped with an Agilent HP 1100 series HPLC system consisting of an autosampler/injector, quaternary pump, column heater, and diode array detector with Xcalibur 1.2 software (Finnigan Corp.).

Test compounds

With the exception of EA (obtained commercially from Sigma-Aldrich) and gallagic acid (GA; isolated in our laboratory from pomegranate fruit extract), UA and UB and their synthetic analogues (either methylated or acetylated) were prepared in our laboratory according to methods previously reported (10, 16). The ET-derived compounds and their analogues included the following: UA, methylated-UA (MUA), dimethylated-UA, acetylated-MUA, UB, methylated-UB (MUB), acetylated-UB, and UB-sulfate (UBS). The structures of the test compounds are shown in Fig. 1.

Cell culture

The ER-positive aromatase-overexpressing MCF-7 cell line, MCF-7aro, was developed previously in our laboratory as previously described (17). The cells were cultured in Eagle's MEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and the selection antibiotic G418 (500 μg/mL; UBS Corporation). Cells were incubated at 37°C with 5% CO_2 and maintained in the linear phase of growth.

Placental microsome assay

This assay was done using human placental microsomes prepared from human placenta as previously described (18). The assay mixture consisted of 20 μg placental microsome, 100 nmol/L (3H)-androstenedione (specific activity 37 MBq/mol), 10 μmol/L progesterone, 1 g/L bovine serum albumin and 67 mmol/L potassium phosphate buffer (pH 7.4), and 12 mmol/L NADPH. Controls consisted of a blank (reaction that is started and stopped immediately), a positive control (water), and a vehicle control (DMSO in equal amount to the highest concentration of test product). Concentrations of test products were 10 times higher in this assay than the in-cell assay, because the chemicals are relatively hydrophobic and are possibly taken up through the cell membrane. Therefore, the actual concentration in cells could be higher than that added to the microsomal mixture. The reaction was run for 20 min at 22°C and the enzymatic activity was terminated with 5% trichloroacetic acid. The supernatant was extracted with dextran-treated charcoal. The suspension was centrifuged and an aliquot was collected and counted for radioactivity. Aromatase activity was calculated as pmol (^{3}H)-H2O

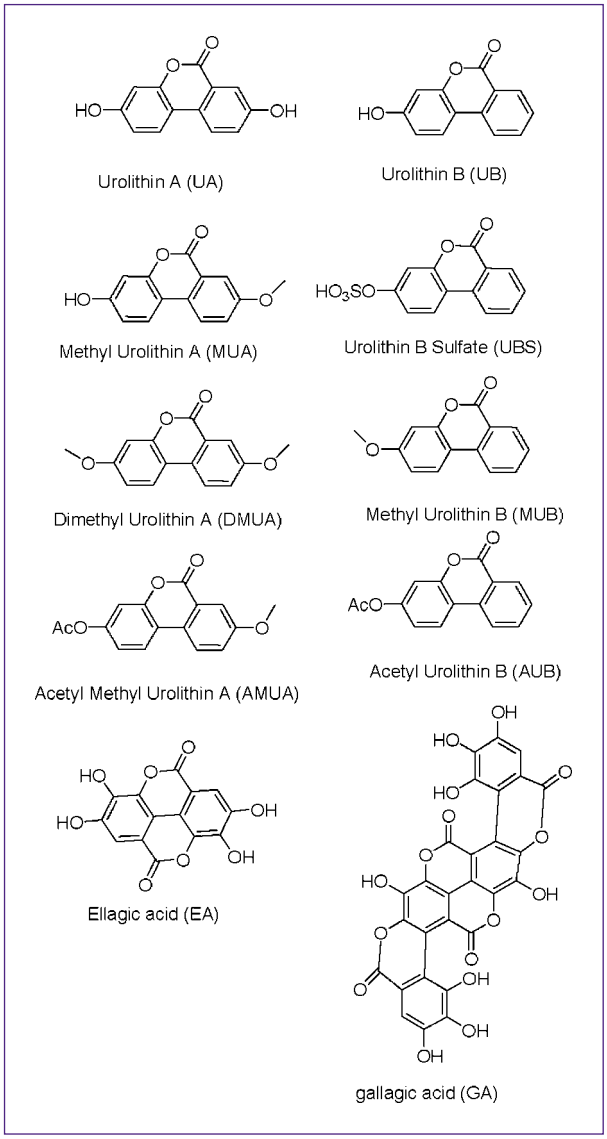

Fig. 1. Chemical structures of test compounds.

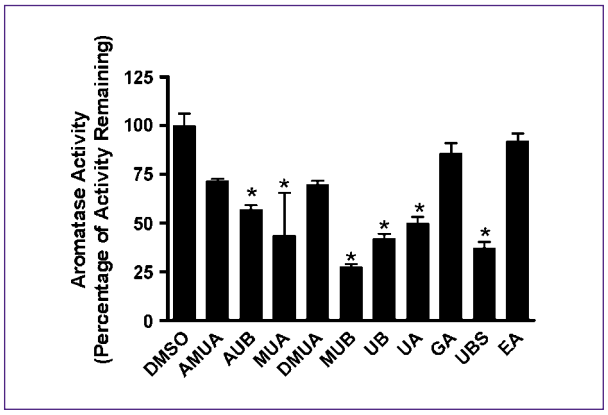

Fig. 2. Inhibition of aromatase by urolithins. *In vitro* human placental aromatase assay was done in the presence of 0.1 μmol/L ^{3}H-androstenedione and urolithin compounds (47 μmol/L). The aromatase activity of vehicle-treated microsomes was set at 100%. The measurements were done in triplicate. Data are expressed as percentage of activity remaining; *columns*, mean; *bars*, SEM; $P \leq 0.01$.

formed/minutes/milligram protein. Analyses were done in triplicate and the data are expressed as mean ± SEM.

Aromatase "in-cell" assay

MCF-7aro cells were cultured in six-well plates in MEM overnight. The following day, the medium was changed to phenol red–free MEM with 10% charcoal dextran–treated FBS (cdFBS) for 24 h. The medium was removed and fresh cdFBS medium alone, with DMSO control or the test compounds (0-4.7 μmol/L), was added plus 0.1 mmol/L (3H)-androstenedione and 500 nmol/L progesterone for 3 h. The supernatant was removed and extracted with dextran-treated charcoal. The suspension was centrifuged (700 × *g* for 7 min) and an aliquot of the supernatant was collected and counted for radioactivity. The cells were solubilized with 1 mL of 0.5 mmol/L NaOH. Protein concentration was determined by the Bradford assay. Aromatase activity was expressed as pmol (3H)-H_2O formed per hour. Analyses were done in triplicate and the data are expressed as mean ± SEM.

Cell proliferation assay

Proliferation was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Technical Bulletin # 288, Promega Corp.). MCF-7aro cells were plated in 96-well plates in MEM overnight. The following day, the medium was removed and changed to phenol red–free MEM with 10% cdFBS for 24 h. Cells were treated with 100 μL cdFBS MEM alone, cdFBS MEM with 1 nmol/L testosterone, or cdFBS with test samples (0-4.7 μmol/L) in the presence of 1 nmol/L testosterone and incubated for 48-h drug exposure duration. After 48 h, the reagent was added and results were read on an Orion Microplate Luminometer (Bertholds Detection Systems). All plates had control wells containing medium without cells to obtain a value for background luminescence, which was subtracted from the test sample readings. This experiment was also carried out using estradiol (1 nmol/L) in the place of testosterone. Data are expressed as percentage of untreated cells, mean ± SEM for three replications.

Kinetic determinations

The inhibition kinetic studies for UB were done using the placental microsome assay. The concentration range of (3H)-androstenedione ranged from 0 to 200 nmol/L. The rate of the reaction was determined to be linear in this range, previously in our laboratory (19–21). Analyses were done in triplicate and the data are expressed as mean ± SEM.

Results

Inhibition of aromatase by ET-derived compounds

To determine the antiaromatase activity of test compounds, we used the placental microsome aromatase assay. Initial studies with the test compounds used a range of concentrations to determine the correct range for further study. The range of 0 to 47 μmol/L was determined to be appropriate. As can be seen from Fig. 2, when they are graphed at an equal concentration (47 μmol/L), the compounds showed varying potency. MUB, UBS, UB, acetylated-UB, UA, and MUA significantly inhibited aromatase activity in this assay ($P \leq 0.01$) where acetylated-MUA, dimethylated-UA, and the polyphenols GA and EA showed no significant antiaromatase activity compared with vehicle-treated controls. These results revealed which metabolites of pomegranate ingestion show antiaromatase activity *in vitro*.

Aromatase inhibition in MCF-7aro cells treated with ET-derived compounds

To determine the inhibitory action of the compounds in a live cell system, active compounds identified in the

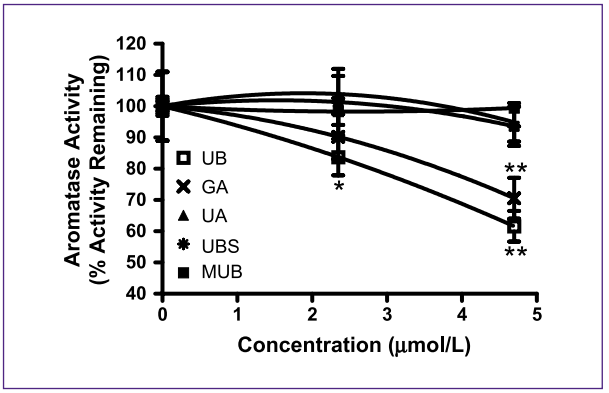

Fig. 3. "In cell" inhibitory effect of urolithin compounds on aromatase in MCF-7aro cells. MCF-7aro cells were maintained in MEM and switched to serum-free medium upon assay. Tritiated androstenedione (0.1 μmol/L) and urolithins (0, 2.35, and 4.7 μmol/L) were administered and incubated for 3 h. *Points*, means ($n = 3$); *bars*, SEM; *, $P \leq 0.05$; **, $P \leq 0.01$.

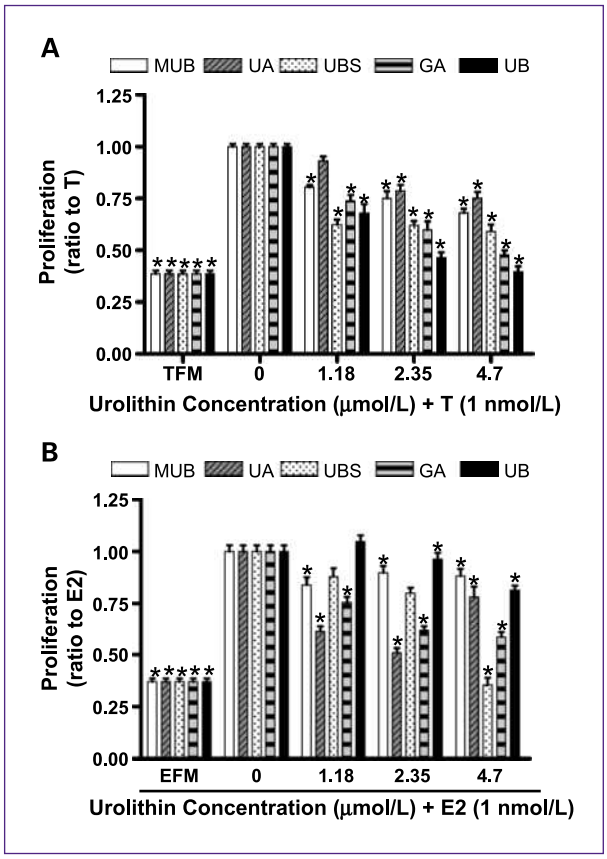

Fig. 4. Effect of urolithin compounds on MCF-7aro cell proliferation. MCF-7aro cells were seeded in 96-well plates and maintained in MEM supplemented with 10% charcoal dextran–treated serum. Proliferation was measured after 48 h under the influence of testosterone (*A*) or estrogen (*B*) + urolithins at the indicated concentrations using the CellTiter-Glo Luminescent Cell Viability Assay. *TFM*, testosterone-free medium; *T*, testosterone; *EFM*, estrogen-free medium; *E2*, estrogen. *Columns*, mean ($n = 9$); *bars*, SEM; *, significant difference from hormone alone (0) group ($P \leq 0.01$).

microsome assay were tested in the MCF-7aro, aromatase-overexpressing cell line. The cells were cultured in medium alone, medium plus vehicle control, or medium plus increasing concentrations of urolithin compounds (Fig. 3). As can be seen from the figure, UB significantly inhibited aromatase activity at 2.35 μmol/L ($P \leq 0.05$) and 4.7 μmol/L ($P \leq 0.01$) in the in-cell assay. In addition, GA also significantly inhibited aromatase activity at 4.7 μmol/L ($P \leq 0.01$). This result shows that although a number of the other test compounds were active in the microsomal assay, UB was the most active in the cell-based assay.

Antiproliferative activity of ET-derived compounds in MCF-7aro cells

In the cell, testosterone is converted to estrogen through a reaction catalyzed by the aromatase enzyme; therefore, we investigated the inhibition of both estrogen- and testosterone-induced proliferation by ET-derived compounds in the MCF-7aro cell line. Results against testosterone-induced proliferation showed that UB had the highest antiproliferative activity at all test concentrations ($P \leq 0.01$), which would be expected as it was the most effective inhibitor of aromatase using the in-cell assay. GA had the second highest activity ($P \leq 0.01$); UBS, UA and MUB showed a mild response ($P \leq 0.01$; Fig. 4A). In the estrogen-induced proliferation assay, UBS was the most potent inhibitor ($P \leq 0.01$), with GA again the second most potent inhibitor. UA, MUB, and UB all showed a mild response, with UB only showing a significant inhibition at the highest dose ($P \leq 0.01$; Fig. 4B).

The results suggest that UB likely inhibits the proliferation of MCF7aro cells primarily through aromatase inhibition where the other test compounds may affect an aromatase-independent mechanism such as direct antagonism of ER signaling or a combination of aromatase-dependent and aromatase-independent mechanisms.

Kinetic analysis of aromatase inhibition with UB

To determine the nature of aromatase inhibition by UB, enzyme kinetic analysis was done using the human

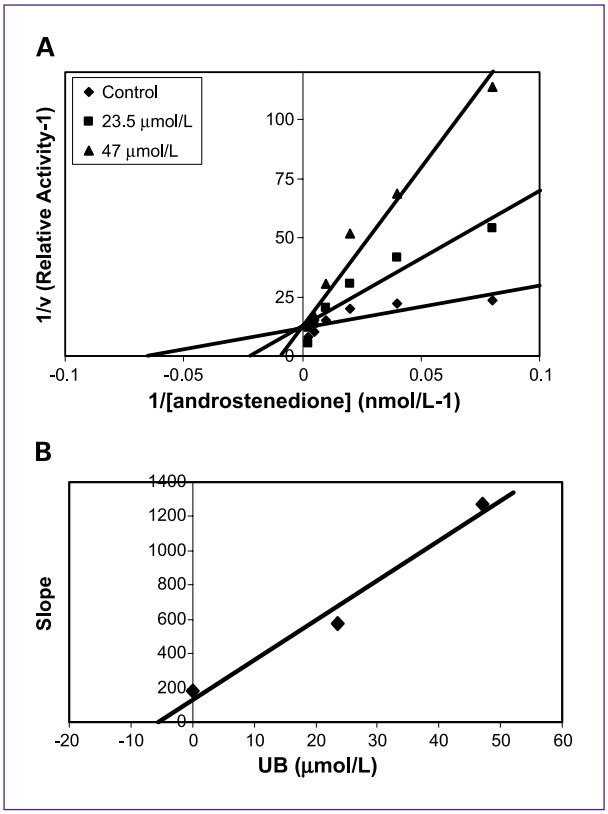

Fig. 5. Aromatase inhibition kinetic profile by UB compound. The enzyme assay was done in the presence of 0, 23.5, and 47 μmol/L of UB with increasing concentrations of [^{3}H]-androstenedione (0-200 nmol/L). The assay was done in triplicate. *A*, Lineweaver-Burk plot (1/v versus 1/[androstenedione]); *B*, secondary plot (slope versus [UB]) used to determine Kı value for UB.

placental microsome assay. Three concentrations (0, 23.5, and 47 μmol/L) of UB were administered to the cells along with increasing concentrations of ^{3}H-androstenedione (0-200 nmol/L). The Lineweaver-Burk plot shows that the presence of UB increased the K_m value and slope with no change in the V_{max}. This indicates that UB is a competitive inhibitor with respect to the substrate, androstenedione (Fig. 5A). An approximate Kι value of 5 μmol/L was determined from the secondary plot (slope versus [I]); Fig. 5B.

Discussion

Six of the ET-derived compounds, MUB, UBS, UB, acetylated-UB, UA, and MUA, exhibited significant antiaromatase activity as measured in the microsome assay. However, this activity did not reach that of known aromatase inhibitor drugs previously tested in our laboratory (22). The test compounds acetylated-MUA, GA, and EA showed no significant antiaromatase activity in this assay. It is possible that a combination of these products may produce a more potent effect, as *in vivo* they would not be found circulating in isolation. For example, a study by Kim et al. (23) showed that polyphenol-rich fractions of pomegranate inhibited aromatase activity in a microsomal assay up to 51%, suggesting that combinations of polyphenols may be more effective than individual compounds. Future studies to examine the synergistic effects of these compounds would answer this question.

Of the urolithin compounds active in the microsomal assay, UB showed the highest antiaromatase activity compared with the other compounds tested in the in-cell assay. This result may be due to a greater absorption of UB into the cells. A recent study by Larrosa (15) et al. showed that both UA and UB were taken up intact in MCF-7 cells and metabolized to their sulfate and glucuronide forms; however, UB uptake was shown to be greater than that of UA. This result could explain the lower activity of UA in the live cell system. In addition to the differences in UB and UA absorption into the cells, UBS and other isomers have been detected in the media but not the cell lysate in significant amounts, suggesting that the other forms of UA and UB were not as active in the in-cell assay perhaps because they must first undergo conversion to UB (15). The proliferation assays were done in a longer time frame (48 hours), and more activity was seen than the shorter term (3 hours) in-cell aromatase assay, suggesting longer term exposure could increase conversion and absorption.

In addition, the flavones chrysin and apigenin are identical in structure with the exception of an additional hydroxyl group on apigenin. In an aromatase assay, this small difference changed the EC_{50} of apigenin to 20 μmol/L where chrysin was 7 μmol/L, illustrating this point (24). Because MCF-7 is the parental line of the MCF-7aro cells used in our study, we suggest that UB absorption is similar in both cell lines and the decreased uptake of UA may account for its lack of aromatase inhibition in the in-cell assay. We further tested UB to determine the nature of its inhibition of aromatase by kinetic analysis using the microsome assay. Results indicated that UB is a competitive inhibitor of aromatase, with an approximate Kι of 5 μmol/L.

Inhibition of testosterone-induced cell proliferation was observed with UB treatment as would be expected from the aromatase assay results. This suggests that the inhibition of aromatase, and therefore estrogen production in the cell, is one mechanism through which UB may inhibit breast cancer cell proliferation. However, because the MCF-7aro cell line also contains a functional ER, the antiproliferative effect may also be due in part to direct antagonism of the ER. To determine the roles of both aromatase and ER signaling in the antiproliferative mechanisms of urolithins in this cell line, proliferation assays using estrogen as an inducer were done. In this assay, UB showed very low activity, further suggesting its role as an inhibitor of aromatase.

Interestingly, the inhibition of testosterone-induced proliferation was also observed in cells treated with UA, GA, MUB, and UBS, suggesting that these ET-derived compounds may inhibit breast cancer cell growth through aromatase-independent mechanisms. In the estrogen-induced proliferation assay, UBS and GA showed the highest antiproliferative activity, suggesting that UBS may antagonize ER signaling. GA on the other hand had activity in both testosterone- and estrogen-induced proliferation assays, suggesting multiple roles for this compound. Although these compounds may not be as readily taken up by the cells as UB, it is possible that their antiproliferative activity could be due conversion to other compounds in the medium. Future studies into the fate of these compounds and their effects on the cell cycle and cell signaling will be carried out to elucidate their mechanisms of action.

The ingestion of pomegranate juice can lead to concentrations of circulating urolithins reaching up to 18 μmol/L in blood. Taken together with the results of current studies and reports of the presence of UA and UB in the blood and urine of human subjects following pomegranate ingestion, the results of these analyses suggest that pomegranate intake may be a viable strategy for the chemoprevention of breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

NIH grants ES08258 and CA44735.

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Received 12/3/08; revised 7/29/09; accepted 7/29/09; published on 1/5/10.

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