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Pomegranate Ellagitannin-Derived Metabolites Inhibit Prostate Cancer Growth and Localize to the Mouse Prostate Gland

NAVINDRA P. SEERAM,*,† WILLIAM J. ARONSON,‡ YANJUN ZHANG,† SUSANNE M. HENNING,† AUNE MORO,† RU-PO LEE,† MARYAM SARTIPPOUR,† DIANE M. HARRIS,† MATTHEW RETTIG,§ MARC A. SUCHARD,|| ALLAN J. PANTUCK,‡ ARIE BELLDEGRUN,‡ AND DAVID HEBER†

Center for Human Nutrition, Department of Urology, Department of Medicine at the Greater Los Angeles VA Medical Center, and Biomathematics and Human Genetics, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, California 90095

Our group has shown in a phase II clinical trial that pomegranate juice (PJ) increases prostate specific antigen (PSA) doubling time in prostate cancer (CaP) patients with a rising PSA. Ellagitannins (ETs) are the most abundant polyphenols present in PJ and contribute greatly towards its reported biological properties. On consumption, ETs hydrolyze to release ellagic acid (EA), which is then converted by gut microflora to 3,8-dihydroxy-6H-dibenzo[*b*,*d*]pyran-6-one (urolithin A, UA) derivatives. Despite the accumulating knowledge of ET metabolism in animals and humans, there is no available data on the pharmacokinetics and tissue disposition of urolithins. Using a standardized ET-enriched pomegranate extract (PE), we sought to further define the metabolism and tissue distribution of ET metabolites. PE and UA (synthesized in our laboratory) were administered to C57BL/6 wild-type male mice, and metabolite levels in plasma and tissues were determined over 24 h. ET metabolites were concentrated at higher levels in mouse prostate, colon, and intestinal tissues as compared to other tissues after administration of PE or UA. We also evaluated the effects of PE on CaP growth in severe combined immunodeficient (SCID) mice injected subcutaneously with human CaP cells (LAPC-4). PE significantly inhibited LAPC-4 xenograft growth in SCID mice as compared to vehicle control. Finally, EA and several synthesized urolithins were shown to inhibit the growth of human CaP cells in vitro. The chemopreventive potential of pomegranate ETs and localization of their bioactive metabolites in mouse prostate tissue suggest that pomegranate may play a role in CaP treatment and chemoprevention. This warrants future human tissue bioavailability studies and further clinical studies in men with CaP.

KEYWORDS: Pomegranate; ellagitannins; urolithins; metabolite; tissue disposition; prostate cancer

INTRODUCTION

Recently, our group reported that pomegranate juice (PJ) consumption by prostate cancer (CaP) patients with rising prostate specific antigen (PSA), following primary therapy, significantly increased the mean PSA doubling time from 15 to 54 months ($p < 0.001$) (*1*). Ellagitannins (ETs) are the most abundant polyphenols found in PJ, obtained from squeezing of the whole fruit, and are also found in other berry fruits such as strawberries, red raspberries, black raspberries, and muscadine grapes, some nuts, and oak-aged beverages. Published studies have shown that ETs and their hydrolysis product, ellagic acid (EA), inhibit CaP cell growth through cell-cycle arrest and stimulation of apoptosis (*2–4*). PJ and pomegranate extracts (PEs) have also been shown to inhibit CaP growth in vitro (*5, 6*). Likewise, PEs administered in animal diets have been shown to inhibit CaP xenograft growth in severe-combined immunodeficient (SCID) mice (*7, 8*).

Our group and others have shown that in human volunteers, ETs from PJ are hydrolyzed to EA and then further converted by gut bacteria to yield bioavailable 3,8-dihydroxy-6H-dibenzo[*b*,*d*]pyran-6-one [urolithin A (UA)] derivatives (*9–11*) (see **Figure 1** for structures). In fact, urolithins appear in blood, urine, and feces of human volunteers 12–24 h after consumption of a single dose of several ET-rich foods and persist in vivo for up to 48–56 h after ingestion (*9–12*). Despite these advances in understanding ET metabolism in rodents (*13, 14*) and humans (*9–12*), there have been no studies determining the disposition

* To whom correspondence should be addressed. Tel: 310-825-6150. Fax: 310-206-5264. E-mail: nseeram@mednet.ucla.edu.
† Center for Human Nutrition.
‡ Department of Urology.
§ Department of Medicine at the Greater Los Angeles VA Medical Center.
|| Biomathematics and Human Genetics.

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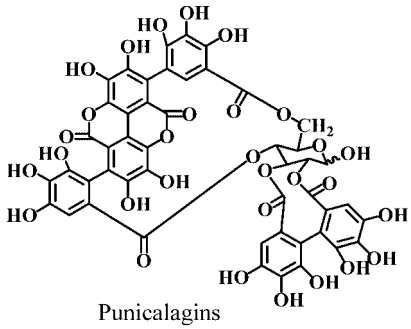

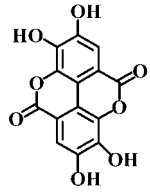

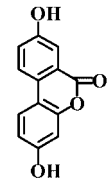

Figure 1. Chemical structures of the major pomegranate ET, punicalagin (occurs as a pair of anomers hence referred to as punicalagins), and its metabolites, EA and UA.

of ET metabolites to tissues of interest including the prostate. Therefore, we planned the current studies to explore (i) the bioavailability and tissue distribution of PE and UA (synthesized in our laboratory) metabolites administered orally and intraperitoneally to C57BL/6 wild-type mice, (ii) the in vivo effects of PE on CaP xenograft growth administered orally to SCID mice, and (iii) the in vitro effects of EA and synthesized UA derivatives on the growth of human androgen-dependent (LNCaP) and androgen-independent (LNCaP-AR, DU145, and 22RV1) CaP cell lines.

MATERIALS AND METHODS

General Reagents and Instruments. All solvents were high-performance liquid chromatography (HPLC) grade from Fisher Scientific Co. (Tustin, CA). Ellagic, 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 (St. Louis, MO). The HPLC with ultraviolet (UV) detection analyses were carried out on a Waters Alliance 2690 system equipped with a photodiode array detector (Waters Corp., Milford, MA), and data handling was with Waters Millenium version 3.02 software. The HPLC with electrospray ionization mass spectrometry (ESI/MS) system consisted of an LCQ Classic Finnigan system (ThermoFinnigan, San Jose, CA), equipped with an Agilent HP 1100 series HPLC (Santa Clara, CA) system consisting of an autosampler/injector, quaternary pump, column heater, and diode array detector (DAD) with Xcalibur 1.2 software (Finnigan Corp., San Jose, CA).

PE. An ET-enriched PE derived from the skin of pomegranate fruit (*Punica granatum* L., Wonderful Variety, Paramount Farms, Lost Hills, CA), was standardized to 37% ETs (as punicalagin isomers) and 3.5% free EA as reported (*15*). PE was administered to animals in doses based on the amount of ETs (as punicalagins) in a single serving of PJ (240 mL of single strength juice, ~80 mg of ETs) (*9*). The equivalent dose that would be administered to a 70 kg human was calculated for a mouse of 25 g body weight to be 0.03 mg of ETs. Ten times (10×) this dose was used in the animal studies (0.8 mg PE per dose per animal) to enhance our ability to detect phytochemical distribution. For studies administering UA, doses were calculated assuming complete conversion of ETs to UA. This results in a dose of 0.3 mg of synthesized UA per animal. Both PE and UA were suspended in 50 μL of phosphate-buffered saline or 10% aqueous glucose solution for intraperitoneal or oral administration, respectively.

Tissue Disposition Studies. Male C57BL/6 mice ($n = 156$ mice, retired breeders, 7 months old, 25–30 g) from Charles River laboratories (Wilmington, MA) were fed AIN 93G diets and water ad libitum and fasted 6 h prior to dosing. Mice were dosed orally or intraperitoneally with either PE (0.8 mg/mouse/dose; $n = 72$), UA (0.3 mg/mouse/dose; $n = 72$), or vehicle control (50 μL; $n = 12$ mice). Blood samples were collected at 0.5, 1, 2, 4, 6, and 24 h after dosing, and prostate, liver, kidney, lung, colon, intestine, and brain tissues were collected and stored at −80 °C. Harvested tissue (200 mg) was homogenized in methanol with 0.1% acetic acid (1 mL) and centrifuged at 3000*g* for 10 min. The supernatant was dried on a SpeedVac and then reconstituted with 200 μL of the HPLC mobile phase for a final injection volume of 20 μL.

Plasma and Tissue Extraction and Analysis. For tissue analyses, a weighed sample of tissue (~200 mg) was homogenized (IKA Ultra-Turrax T8, IKA Works, Inc., Wilmington, NC) in methanol with 0.1% acetic acid (1 mL) as previously reported (*13*). The homogenate was centrifuged at 3000*g* for 10 min, and the supernatant was dried on the SpeedVac and then reconstituted with 200 μL of the HPLC mobile phase for a final injection volume of 20 μL. Using these extraction methods (*13*), metabolites were not initially detected in xenograft tumor tissues collected from the SCID mice. Therefore, tumor tissues were subjected to acid hydrolysis as previously described for animal tissue extraction studies with soy isoflavones (*16*). Briefly, tissue homogenates were mixed with hydrochloric acid for a final concentration of 6 M and heated to boiling for 1 h prior to chromatography. Mouse diet (AIN 93G) was also subjected to acid hydrolysis and analyzed by chromatography.

To estimate concentrations of the ET metabolites in experimental samples, linear calibration curves were constructed using a spiked internal standard method with EA or UA using plasma or liver tissues collected from control mice as described previously (*9*). Calibration curves were prepared between the concentration ranges of 7.8125–1000 ng/mL. At the time of the current study, we did not have an authentic standard for UA-glucuronide; therefore, concentrations for this metabolite were estimated by using UA as an internal standard. We have since obtained UA-glucuronide by chromatographic isolation from urine collected from healthy volunteers who consumed PJ (unpublished results). ET metabolite concentrations were determined from the peak area as previously described (*9, 17*). The extraction efficiency was determined by comparison of a calibration curve prepared in mobile phase with a calibration curve for the same concentrations extracted from the plasma or liver tissues. The percentage recovery for EA has been previously reported (*17*). The percentage recovery of UA ranged from 104 to 113% (for plasma) and from 59 to 72% (for tissues). The lower limits of quantitation for EA and UA were 3 ng/mL (for plasma) and 5 ng/g (for tissue), respectively. The extraction methods were validated for the analyses of the liver tissues where the precision (expressed as coefficient of variation) was in the range of 5–10%. Accuracy values ranged from 91 to 108% for the quality control samples containing the EA or UA standard.

Syntheses of ET Metabolites. UA, methylated urolithin A (mUA), dimethylated urolithin A (dmUA), and UA-sulfate were synthesized in our laboratory according to methods previously reported (*9, 18*). We are currently synthesizing a number of EA and other UA derivatives for future studies.

Liquid Chromatography Mass Spectrometry (LC-MS). Peak identities of ET metabolites were obtained by matching their molecular ions ($M - H^+$) obtained by LC-MS and LC-MS/MS with the expected theoretical molecular weights from literature data as follows: EA = M – H *m/z* 301; UA = M – H *m/z* 227; mUA = M – H *m/z* 241; UA-sulfate = M – H *m/z* 307; and UA-glucuronide = M – H *m/z* 403 (*9*).

Xenograft Study. All mice received a standard research diet (AIN 93G, Dyets, Bethlehem, PA) ad lib throughout the experiment. Androgen-dependent LAPC-4 prostate cancer cells (200000 cells per animal; gift from Charles Sawyers) were implanted subcutaneously into the shoulders of 24 5 week old SCID mice (Taconic Farm, Germantown, NY). When tumors became palpable, mice were administered either

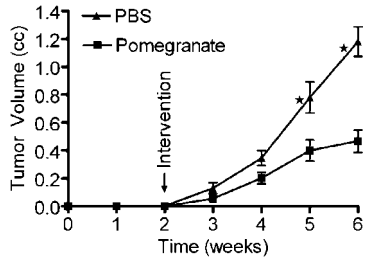

Figure 2. Inhibition of tumor xenograft (LAPC-4) growth in immunocompromised (SCID) mice treated with PE as compared to vehicle control. Inhibition of growth was significant beginning at 2 weeks after initiation of PE administration (0.8 mg/mouse/dose) orally ($p < 0.05$) with greater than 50% inhibition of tumor volume by 6 weeks after tumor injection. PE or vehicle control was administered when tumors became palpable 2 weeks after injection of 200000 prostate tumor cells (LAPC-4).

PE ($n = 12$ mice) or vehicle control ($n = 12$ mice) orally 5 days per week (Monday–Friday). Oral dosing was done using an animal feeding needle (Biomedical Needles, Popper and Sons Inc., New Hyde Park, NY). Tumors were measured twice weekly, and mice were weighed weekly. Tumor volumes were calculated using the formula: length × width × height × 0.5236 (*19*). At sacrifice, primary tumors were excised and blood was collected. All animal protocols were approved by University of California, Los Angeles Chancellor's Animal Care Committee.

In Vitro Proliferation Studies. DU145 and LNCaP cells (ATCC, Rockville, MD), 22Rv1 cells (gift from Pinchas Cohen), and LNCAP-AR cells (gift from Charles Sawyers) were maintained below passage 20 and used in experiments during the linear phase of growth as previously described (*5*). Compounds were dissolved in dimethylsulfoxide (DMSO) and then added to media to achieve the final desired test concentration with a final DMSO concentration not exceeding 0.1%. Proliferation was determined by tritiated thymidine uptake as previously described (*20*). All assays were performed in triplicate.

Statistical Analysis. For the cell proliferation assays, data were expressed as a percentage of untreated cells (i.e., treatment value blank/vehicle value blank), mean ± SE for at least three separate experiments. Data were analyzed by either Student's *t* test or one-way analysis of variance followed by Dunnett's multiple range test ($\alpha = 0.05$) with Graph Pad Prism 3.0 (Graph Pad Software Inc.) as appropriate. The statistical package R.2.5.1 was used to compute descriptive statistics and generate the plots displayed in **Figure 2**.

RESULTS

Inhibition of Xenograft Growth in SCID Mice. Oral PE administration led to a significant inhibition of LAPC-4 xenograft growth relative to the vehicle control group (**Figure 2**). Tumor volumes were significantly reduced in the PE-treated group relative to the control group at 4 (0.35 ± 0.19 vs 0.20 ± 0.14 cm^3; $p = 0.0280$), 5 (0.78 ± 0.39 vs 0.39 ± 0.26 cm^3; $p = 0.005$), and 6 weeks ($1.18 + 0.37$ vs $0.47 + 0.28$ cm^3; $p = 0.0002$) after tumor cell injection.

Tumor tissues collected from SCID mice in the xenograft study were extracted using published methods (*13*) and analyzed by LC-MS/MS methods for metabolites. However, no metabolites were initially detected. Therefore, tumor tissues were subjected to acid hydrolysis as previously described for animal tissue extraction studies conducted with soy isoflavones (*16*). Unfortunately, because we expended tumor tissues in our method development efforts and are also utilizing these limited tumor tissues for other planned biomarker evaluation assays (reported elsewhere), we only had sufficient tissue mass from three PE-treated mice for the metabolite quantification study reported here. EA was found at statistically higher concentrations in tumor tissues collected from the three PE-treated mice (1030 ± 124.9 ng/g) as compared to mice receiving vehicle control (316.3 ± 54.1 ng/g; $p = 0.0008$). UA and its metabolites were not detected in xenograft tissues.

Tissue Distribution of Pomegranate ET Metabolites in C57BL/6 Wild-Type Mice. After oral administration of PE, EA was detected in plasma at 0.5 h and was cleared after 2 h. Intraperitoneally administered PE gave higher plasma EA levels at 0.5 h as compared to oral dosing (134.5 ± 12 vs 11.5 ± 1.4 ng/mL) and persisted in plasma for 6 h. EA was not detected in prostate tissue after oral PE administration, whereas it was detected at high concentrations in prostate tissue (676 ± 172 ng/g) and to a lesser extent in intestine, colon, and liver after intraperitoneal administration. Neither UA nor its conjugates were detected in plasma or tissues of mice following PE administration.

Orally administered UA was rapidly absorbed and taken up in highest concentrations in the prostate gland followed by the small intestine and colon with a peak at 4 h (**Figure 3**A). Intraperitoneally administered UA concentrated in the colon, prostate, and intestine with a peak at 2 h (**Figure 3**B). Among the UA metabolites, UA-sulfate and mUA were detected primarily in the prostate gland whereas UA-glucuronide was found in greatest concentration in liver and kidney tissues.

Bioactivity of ET Metabolites In Vitro. EA (commercial standard purchased from Sigma) and UA and UA derivatives (synthesized in our laboratory) were incubated at 10–100 μmol/L concentrations with androgen-dependent (LNCaP) and -independent CaP cell lines (LNCaP-AR, DU145, and 22RV1). All ET metabolites demonstrated dose-dependent antiproliferative effects on all cell lines tested. The IC_{50} of UA was lower than that for EA in all cell lines (**Table 1**). Among the UA metabolites, mUA had the greatest bioactivity in inhibiting LNCaP and 22RV1 cell growth. The IC_{50} values for each ET metabolite are summarized in **Table 1**.

DISCUSSION

In the current study, we sought to determine the tissue disposition of pomegranate ET metabolites, EA, and urolithins and to determine if the metabolites localize in the prostate gland and prostate tumor xenografts. Therefore, UA was studied in detail, since prior studies demonstrated that UA conjugates were the most prevalent metabolites of EA in human subjects and that they persist for up to 56 h after ingestion of ETs (*9*, *10*, *12*).

Oral administration of PE to wild-type mice led to increased plasma levels of EA, but EA was not detected in the prostate gland. On the other hand, intraperitoneal administration of PE led to 10-fold higher EA levels in the plasma and detectable and higher EA levels in the prostate, intestine, and colon relative to other organ systems. The detectable EA levels in prostate tissue following intraperitoneal but not oral administration were likely due to higher plasma levels attained after intraperitoneal administration.

Intraperitoneal and oral administration of synthesized UA led to uptake of UA and it conjugates in prostate tissue, and UA levels were higher in prostate, colon, and intestinal tissues relative to other organs. It is unclear why pomegranate ET metabolites localize at higher levels in prostate, colon, and intestinal tissues relative to the other organs studied. Importantly, the predilection of bioactive pomegranate ET metabolites to localize in prostate tissue, combined with clinical data demonstrating the anticancer effects of PJ, suggest the potential for pomegranate products to play a role in CaP chemoprevention. Whether urolithins in human prostate tissue can be used as a

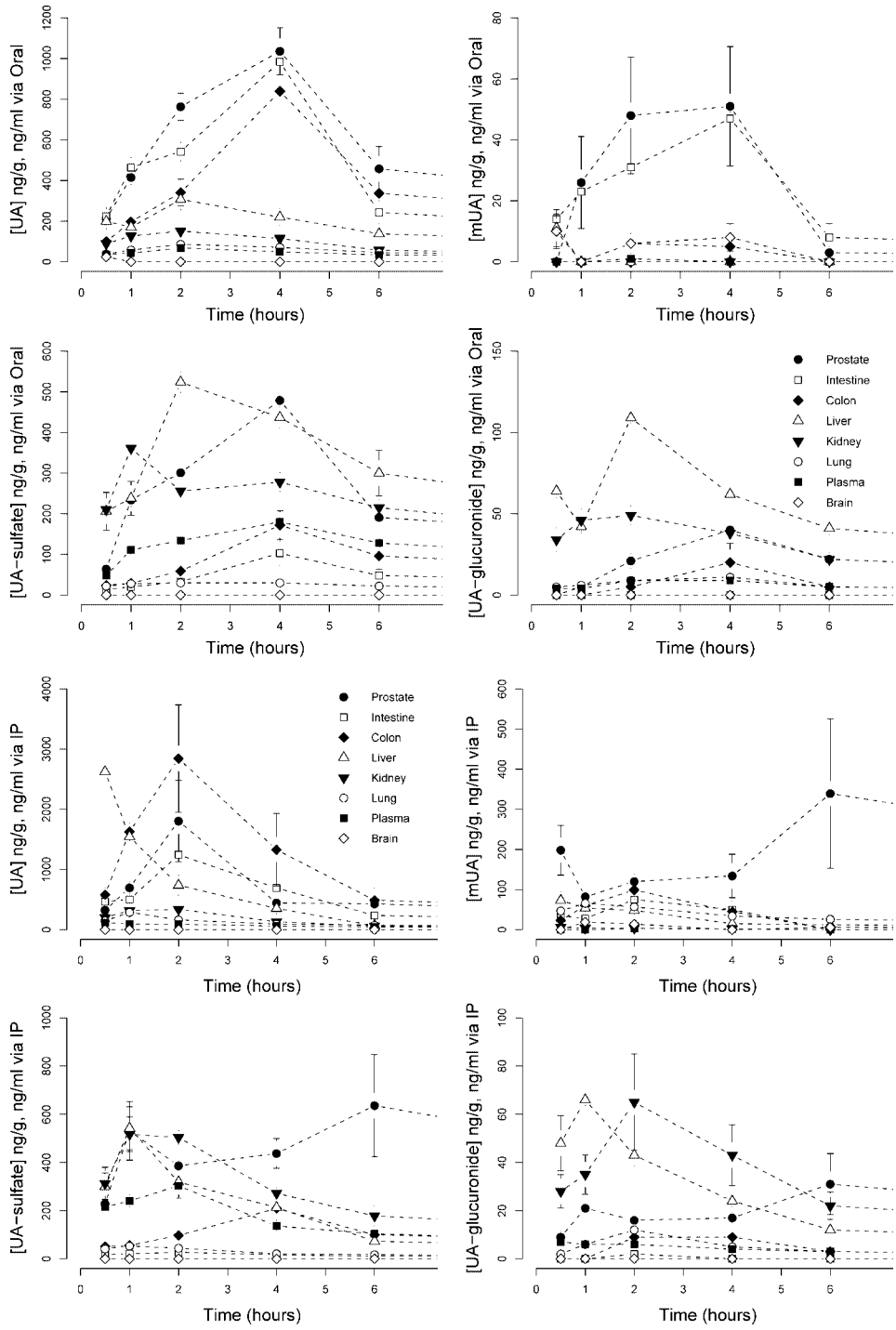

Figure 3. (**A**) Concentrations of UA and UA conjugates: methylated-UA (mUA), UA-sulfate, and UA-glucuronide in plasma (ng/mL) and tissues (ng/g) of male C57BL/6 wild-type mice that received UA (0.3 mg/mouse/dose) by the oral route. Metabolite levels at the 24 h time point were below the detectable limit (3 ng/mL for blood and 5 ng/g for tissues) and are therefore not shown. Data show the means ± SEs for $n = 6$ mice per time point. (**B**) Concentrations of UA and UA conjugates: mUA, UA-sulfate, and UA-glucuronide in plasma (ng/mL) and tissues (ng/g) of male C57BL/6 wild-type mice that received UA (0.3 mg/mouse/dose) by the intraperitoneal route. Metabolite levels at the 24 h time point were below the detectable limit (3 ng/mL for blood and 5 ng/g for tissues) and are therefore not shown. Data show the means ± SEs for $n = 6$ mice per time point.

biomarker following long-term administration of PJ or PE remains to be determined.

Following administration of UA, a number of UA conjugates were detectable in the prostate gland. UA and other polyphenols are metabolized by hepatic phase II enzymes including glucuronosyl transferases to form glucuronides, sulfotransferases to form sulfates, and catechol-*o*-methyl transferases to form methylated derivatives (*9*). These phase II metabolic processes facilitate the excretion of xenobiotics. UA-sulfate was the major metabolic product of UA in this study, suggesting that among these phase II enzymes, the sulfotransferases apparently play a major role in UA transformation.

In the wild-type mice bioavailability experiment, we were surprised that urolithins were not detected in the mouse prostate following PE dosing, given that high urolithin levels were found in human plasma and urine following oral PJ administration (*9*, *10*). Likewise, although EA was detectable in the SCID mice xenograft tissues, again, urolithins were not present. Metabolism

Table 1. IC_{50} (μmol/L) Values of Pomegranate ET Metabolites on Growth of Human Prostate Cancer Cells[a]

cells	EA	UA	mUA	dmUA
LNCaP	62.4 ± 4.2	31.3 ± 0.5	15.9 ± 4.7	71.4 ± 13.5
LNCaP-AR	78.7 ± 3.2	43.3 ± 2.8	126.7 ± 7.3	ND
DU145	74.3 ± 5.2	49.8 ± 3.9	43.3 ± 3.5	70.7 ± 13.0
22RV1	108.7 ± 16.6	47.8 ± 3.2	6.2 ± 0.8	85.0 ± 8.6

[a] Data are expressed as a percentage of untreated cells (i.e., treatment value blank/vehicle value blank); means ± SEs for at least three separate experiments.

of EA to urolithins by colonic microflora has only been reported in humans and rats (*9*, *10*, *15*), and it is possible that mice are not able to metabolize EA. We conducted a separate experiment with wild-type C57BL/6 mice receiving oral PE once daily for 5 days per week for a total of 5 weeks and still did not detect urolithins in plasma or tissue samples (data not shown). This implied that the wild-type mice used in our study probably lack the colonic microflora required to produce urolithins. Also, because SCID mice are immunocompromised, they may harbor even fewer colonic bacteria than wild-type mice, further contributing to their inability to produce urolithins.

We observed that the urolithins inhibited the growth of both androgen-dependent and androgen-independent CaP cell lines, with IC_{50} values lower than EA. Future studies to evaluate the mechanistic basis for the antiproliferative effects of urolithins are required. It should be noted that the involvement of the cyclin kinase inhibitor-cyclin-cyclin-dependent kinase network has been implicated in the antiproliferative effects of a pomegranate fruit extract against CaP cells (*8*). On the basis of current knowledge of polyphenol bioavailability (*21*), the IC_{50} values that we observed in the antiproliferative assays far exceed physiologically achievable levels. It is noteworthy, however, that total circulating urolithins have been estimated to reach levels of 18.6 μM concentrations when 1 L per day of PJ (containing 4.3 g ETs) is consumed by humans for 5 days (*10*). Future studies of prostatectomy tissue in humans will be needed to determine whether these metabolites and conjugates accumulate in human prostate tissue. Of interest, in our preliminary phase II clinical trial with CaP patients, serum collected from men had antiproliferative and proapoptotic effects on human CaP cells (LNCaP) in an ex vivo assay (*1*). Future studies should be designed to determine if EA, urolithins, and related metabolites in patient sera are responsible for these antiproliferative and proapoptotic effects.

In conclusion, we have shown that pomegranate ET metabolites are concentrated to a high degree in mouse prostate tissues. Given our recent observations of the effects of PJ in CaP patients (*1*), the current study contributes to the increasing body of evidence demonstrating the CaP chemopreventive potential of pomegranate ETs. Future animal studies using preclinical chemoprevention models and human studies evaluating metabolite uptake into human prostate tissue, together with studies of the effects of these metabolites on relevant tissue biomarkers, are warranted.

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