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 I 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
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 UA derivatives on the growth of human androgen-dependent (LNCaP) and androgen-independent (DU145, and LNCaP-AR) 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-bromo-benzoic, 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 Milenium 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% ETs (occurs as a pair of anomers hence referred to as punicalagins), and its metabolites, EA and UA.

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.

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 into heparinized tubes 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 3000g 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 3000g 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 Sawyer) were implanted subcutaneously into the shoulders of 24–5 week old SCID mice (Tacomic Farm, Germantown, NY). When tumors became palpable, mice were administered either
Data were analyzed by either Student's t expressed as a percentage of untreated cells (i.e., treatment value blank/test concentration with a final DMSO concentration not exceeding 0.1%.

AR cells (gift from Charles Sawyers) were maintained below passage 20 and used in experiments during the linear phase of growth as previously described (20 and used in experiments during the linear phase of growth as previously described (20). Compounds were dissolved in dimethylsulfoxide (DMSO) and then added to media to achieve the final desired concentration.

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).

In 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).
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
of EA to urolithins by colonic microflora has only been reported in humans and rats, 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 IC50 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 are required. It should be noted that the involvement of the cyclin kinase inhibitor-cyclin-cyclin-dependent kinase network is implicated in the antiproliferative effects of a cyclin kinase inhibitor-cyclin-cyclin-dependent kinase network is implicated in the antiproliferative effects of a cyclin kinase inhibitor-cyclin-cyclin-dependent kinase network.

Table 1. IC50 (µmol/L) Values of Pomegranate ET Metabolites on Growth of Human Prostate Cancer Cells

<table>
<thead>
<tr>
<th>cells</th>
<th>EA</th>
<th>UA</th>
<th>mUA</th>
<th>dmUA</th>
</tr>
</thead>
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<td>31.3</td>
<td>0.5</td>
</tr>
<tr>
<td>LNCaP-AR</td>
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<tr>
<td>DU145</td>
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<td>5.2</td>
<td>40.9</td>
<td>3.9</td>
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<tr>
<td>22RV1</td>
<td>108.7</td>
<td>16.6</td>
<td>47.8</td>
<td>3.2</td>
</tr>
</tbody>
</table>

*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.

**LITERATURE CITED**


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