Effects of Pomegranate Chemical Constituents/Intestinal Microbial Metabolites on CYP1B1 in 22Rv1 Prostate Cancer Cells

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The cytochrome P450 enzyme, CYP1B1, is an established target in prostate cancer chemoprevention. Compounds inhibiting CYP1B1 activity are contemplated to exert beneficial effects at three stages of prostate cancer development, that is, initiation, progression, and development of drug resistance. Pomegranate ellagitannins/microbial metabolites were examined for their CYP1B1 inhibitory activity in a recombinant CYP1B1-mediated ethoxyresorufin-*O*- deethylase (EROD) assay. Urolithin A, a microbial metabolite, was the most potent uncompetitive inhibitor of CYP1B1-mediated EROD activity, exhibiting 2-fold selectivity over CYP1A1, while urolithin B was a noncompetitive inhibitor with 3-fold selectivity. The punicalins and punicalagins exhibited potent CYP1A1 inhibition with 5−10-fold selectivity over CYP1B1. Urolithins, punicalins, and punicalagins were tested for their 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced CYP1 inhibitory activity in the 22Rv1 prostate cancer cell line. Urolithins A and B showed a decrease in their CYP1-mediated EROD inhibitory IC_{50} values upon increasing their treatment times from 30 min to 24 h. Urolithin C, 8-*O*-methylurolithin A, and 8,9-di-*O*-methylurolithin C caused a potent CYP1-mediated EROD inhibition in 22Rv1 cells upon 24 h of incubation. Neutral red uptake assay results indicated that urolithin C, 8-*O*-methylurolithin A, and 8,9-di-*O*-methylurolithin C induced profound cytotoxicity in the proximity of their CYP1 inhibitory IC_{50} values. Urolithins A and B were studied for their cellular uptake and inhibition of TCDD-induced CYP1B1 expression. Cellular uptake experiments demonstrated a 5-fold increase in urolithin uptake by 22Rv1 cells. Western blots of the CYP1B1 protein indicated that the urolithins interfered with the expression of CYP1B1 protein. Thus, urolithins were found to display a dual mode mechanism by decreasing CYP1B1 activity and expression.

KEYWORDS: CYP1B1; ellagitannins; urolithins; pomegranate juice; prostate cancer; chemoprevention

INTRODUCTION

Dietary intervention to prevent carcinogenesis has been well-established in epidemiological studies. The consumption of fruits and vegetables is considered to be a safeguard against various forms of cancers. Polyphenols are the major constituents of fruit and vegetable diets and are believed to elicit a number of biological properties due to their antioxidant and anticarcinogenic activities (*1*). A number of flavonoids such as quercetin, chrysin, apigenin, and luteolin have been investigated for their cytochrome P450 1 (CYP1) enzyme inhibition activities, indicating that flavonoid-related anticarcinogenesis is mediated in part by CYP1 inhibition (*2*). Ellagic acid, the hydrolysis product of ellagitannins, exhibits anticarcinogenic effects by inhibition of CYP1A1-dependent activation of procarcinogens. A number of ellagic acid analogues showed similar inhibitory activities against

CYP1-mediated benzo[*a*]pyrene activation (*3*). Ellagic acid is a major polyphenol in pomegranate juice. Pomegranate juice polyphenols showed a strong inhibitory activity against estrogen-dependent MCF-7 cell lines. In in vivo studies, pomegranate juice exhibited 47% inhibition of cancerous lesion formation induced by the carcinogen, 7,12-dimethylbenz[*a*]anthracene (*4*), indicating its potential use as an adjuvant therapeutic in human breast cancer treatment. Pomegranate juice components are also believed to exert cancer chemopreventive activity against skin and colon cancer. Importantly, the consumption of pomegranate juice decreased the clinical reemergence of prostate cancer-specific antigen in prostate cancer patients after primary therapy (*5*, *6*). Pomegranate ellagitannins are transformed by human colonic flora into bioavailable organic molecules called urolithins (*7*). The pomegranate microbial metabolites preferentially accumulate in prostate, colon, and intestinal tissues relative to other organs in a mouse model and exert their beneficial effects to a greater extent in those tissues (*5*). Pomegranate constituents

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inhibited prostate cancer cell growth by affecting their proliferation, gene expression, invasion, and apoptosis but had a less profound effect on normal prostate epithelial cells. However, the concentrations at which they exhibited antiproliferation activity against human prostate cancer cell lines were higher than the physiologically available concentrations (18.6 μM when 1 L of juice is consumed for 5 days) (*5*, *8*). These results suggest that there might be some other pathways through which pomegranate constituents exert cancer chemoprevention. To explore the other possible prostate cancer chemopreventive pathways, we studied the effects of pomegranate juice ellagitannins and their microbial metabolites on CYP1B1-induced carcinogenesis. Previously, it was shown that pomegranate juice consumption resulted in lowered total hepatic CYP content and also decreased CYP1A2 and CYP3A. Therefore, the anticarcinogenic effects of pomegranate juice could be partly attributed to their ability to inhibit CYP activity/expression (*9*). Our work represents the first report concerning the effects of pomegranate ellagitannins on CYP1B1 inhibition as a means for cancer chemoprevention.

CYPs are responsible for the bioactivation of endogenous compounds, drugs, dietary chemicals, and xenobiotics. The CYP1 isoforms, CYP1A1, CYP1A2, and CYP1B1, are of major importance because they activate a number of polycyclic aromatic hydrocarbons (PAHs) to genotoxic compounds leading to tumorogenesis (*10*). CYP1B1 is abundantly expressed extrahepatically in steroidogenic (ovaries, testes, and adrenal glands) and steroid-responsive (breast, uterus, and prostate) tissues. The CYP1B1 enzyme plays an important role not only in the initiation and promotion of cancer but also in the development of drug resistance. The CYP1B1 enzyme alone accounts for activation of 15 PAHs, six heterocyclic amines, and two nitropolycyclic hydrocarbons into mutagenic and carcinogenic compounds, which cause DNA damage and initiate cancer formation. CYP1B1 is also involved in the metabolism of endogenous compounds such as 17β-estradiol to an active metabolite, 4-hydroxyestradiol (4-OH-E$_2$), which has been implicated in breast cancer initiation. In comparison, CYP1A1 converts 17β-estradiol into 2-hydroxyestradiol (2-OH-E$_2$), which is relatively noncarcinogenic as compared to 4-OH-E$_2$ and plays no role in cancer (*11*). CYP1B1 levels are overexpressed in prostate, lung, esophageal, oral, and colon cancers but not in the corresponding normal tissues. The increased expression of CYP1B1 could generate an excessive number of genotoxic metabolites, which may attack the DNA of normal cells, thus allowing for cancer promotion. Although the augmented CYP1B1 expression does not cause tumor invasion or metastasis, it leads to deactivation of anticancer drugs such as flutamide in prostate cancer treatment and docetaxel in breast cancer treatment (*12*). Considering the crucial role played by CYP1B1 in cancer initiation, promotion, and resistance development, it is an attractive molecular target for cancer chemoprevention. The expression of the CYP1 family is regulated by the aryl hydrocarbon receptor (AhR). The ligands of AhR range from environmental contaminants to plant- or diet-derived constituents such as curcumin and carotenoids (*13*). Because CYP1B1 is a therapeutic target in prostate cancer, we hypothesized that pomegranate constituents/metabolites might exert prostate cancer chemoprevention through CYP1B1 inhibition as one of the plausible mechanisms. Our results indicate a previously unexplored pathway through which pomegranate juice constituents may contribute to prostate cancer chemoprevention.

MATERIALS AND METHODS

Isolation and Identification of Pomegranate Juice Ellagitannins. The extraction of ellagitannins was performed by a procedure described previously, by use of a step gradient consisting of an increasing amount of methanol in water. The commercial POMx (100 mL) was diluted to 500 mL with Millipore purified water and successively partitioned with EtOAc (3 × 200 mL) and *n*-BuOH (3 × 200 mL).

The *n*-BuOH extract (2.0 g) was concentrated and subjected to Amberlite XAD-16 column chromatography (500 g, 6 cm × 35 cm) and eluted with H$_2$O (2.0 L) and MeOH (2.0 L) successively. The MeOH fraction on removal of solvent under reduced pressure afforded a tannin fraction (XAD-*n*-BuOH) (1.3 g). This was further purified on Sephadex LH-20 CC (6 cm × 55 cm) and eluted with H$_2$O:MeOH (2:8, 350 mL), H$_2$O:MeOH (1:9, 500 mL), MeOH (450 mL), and MeOH:Me$_2$CO (1:1, 600 mL) to give nine fractions. A follow-up of fractionation and further purification of all of the fractions on Sephadex LH-20 column chromatography using H$_2$O:MeOH gradient, MeOH, and MeOH:Me$_2$CO gradient system afforded the compounds gallic acid, hexahydroxydiphenic acid (HHDP), gallagic acid, punicalins, and punicalagins. The latter compounds (punicalins and punicalagins) exist in solution as the α- and β-anomers as well as acyclic hydroxyaldehyde analogues (*14*) (**Figure 1**). The compounds were identified using LC-MS retention time, UV absorption pattern, molecular mass, and ^{1}H NMR spectra. The LC-MS system consisted of Waters Micromass ZM^{TQ} mass spectrophotometer, Waters 2695 Separation Module, and Waters 996 Photodiode Array Detector. Mass spectra were recorded in negative mode, using a capillary voltage of 4000/3500 V and a gas temperature of 300 °C. The column used was a 150 mm × 3.0 mm i.d., 5 μm, Luna C18 100 Å (Phenomenex, Torrance, CA). The analysis was performed using a 2.5% acetic acid in water (solvent A) and 2.5% acetic acid in methanol (solvent B), starting from 100% A for 5 min, 0−60% B for 15 min, and 60−100% B for the next 15 min. The flow rate was 0.3 mL/min with the pressure set at 900−1500 mmHg.

Synthesis of Urolithins. *Chemicals.* Resorcinol, ReagentPlus (99%), 2-bromobenzoic acid (97%), 2-bromo-4,5-dimethoxybenzoic acid (98%), and chlorobenzene were purchased from Sigma Aldrich (St. Louis, MO). 2-Bromo-5-methoxybenzoic acid (98%) was purchased from Alfa Aesar (Ward Hill, MA). Pyrogallol (ACS grade) was purchased from Acros Organics. CuSO$_4$, NaOH, and AlCl$_3$ were purchased from Fisher Scientific (Pittsburgh, PA).

Purification of Compounds. The high-performance liquid chromatography (HPLC) system consisted of a Waters Delta 600, a Waters 600 controller, a Waters 996 Photodiode Array Detector, and a 3.0 mm × 150 mm column (Phenomenex, ODS 5 μm C18 100 Å). Analyses were performed in the gradient system A, 2.5% aqueous acetic acid, and B, 2.5% acetic acid in methanol, starting from 100% A for 5 min, 0−60% B for 15 min, and 60−100% B for 15 min. The flow rate was 1 mL/min, and the pressure was 600−800 mmHg. The elution of metabolites was monitored at 254 nm.

Urolithins (urolithin B, 8-*O*-methylurolithin A, urolithin A, 8,9-di-*O*-methylurolithin C, urolithin C, 8,9-di-*O*-methylurolithin D, and urolithin D) were synthesized by the condensation of resorcinol or pyrogallol with an appropriately substituted benzoic acid by the modified protocols described by Ito et al. (*15*). The structures of urolithins were confirmed by their molecular mass and comparison of observed and reported ^{1}H NMR data with reported data (**Figure 1**).

Recombinant CYP1 Ethoxyresorufin-O-deethylase (EROD) Assay and Inhibition Kinetics. To study the effects of pomegranate chemical constituents and their microbial metabolites on recombinant CYP1A1 and CYP1B1, a 96-well plate EROD assay was used (*16*). Concentrations of the test compounds ranged from 0.5 to 30 μM. Inhibition kinetics of CYP1B1-mediated EROD activity was determined similarly. Concentrations of 0.5 and 1 μM were used for urolithins A and B in triplicate.

22Rv1 Prostate Cell EROD Assay. To evaluate the effects of pomegranate constituents and microbial metabolites in a cell-based CYP1 activity, an EROD assay was conducted using 22Rv1 cells in a 48-well plate format (*32*). The test compounds were studied for their effects on cell-based 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced CYP1-mediated EROD activity. The cells were treated with TCDD for 24 h to induce CYP1 expression. The cells were also cotreated with compounds, for either 30 min or 24 h, at concentrations ranging from 6.75 to 50 μM. The cells were treated with DMSO, ellagitannins, and urolithins alone to evaluate their ability to induce CYP1 expression in the absence of TCDD.

Microsome Preparation. Cells were seeded in 150 cm^2 culture plates and exposed to various treatments for 24 h. The cells were harvested,

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GALLIC ACID

ELLAGIC ACID

HEXAHYDROXYDIPHENIC ACID

GALLAGYLDILACTONE

PUNICALAGINS

PUNICALINS
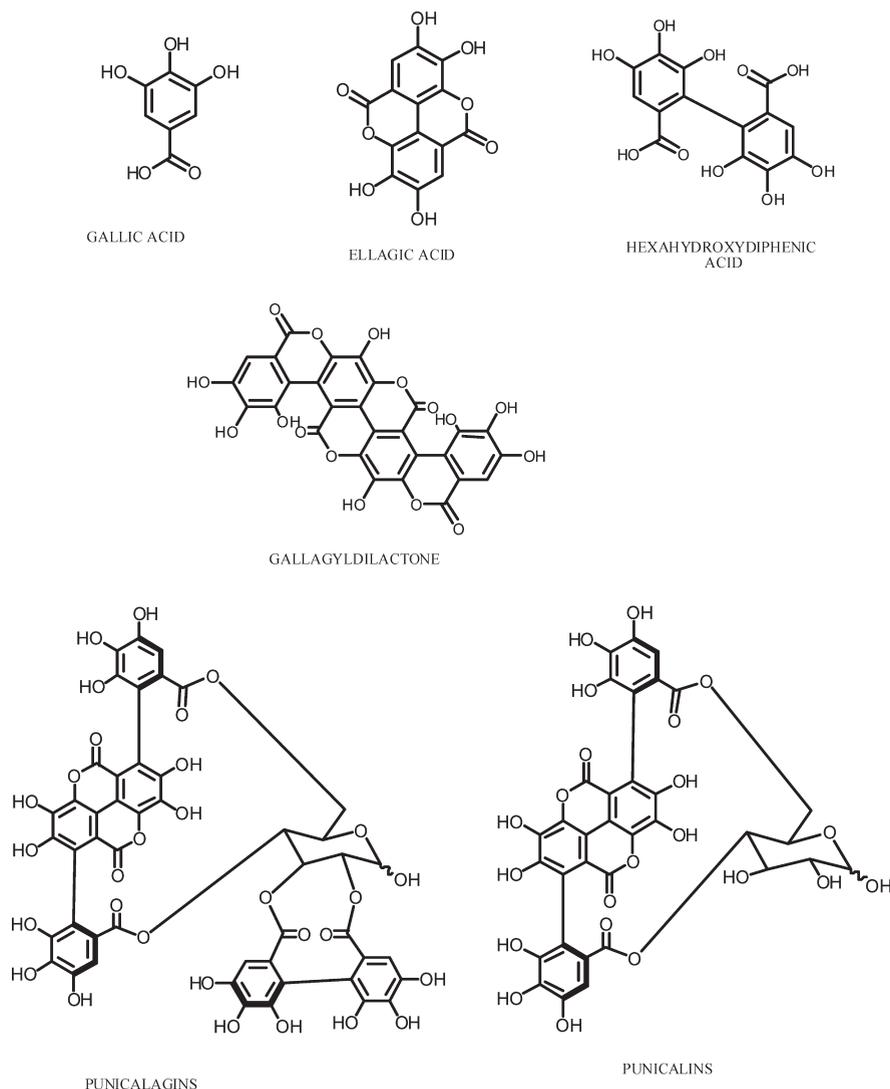
Figure 1. Structures of ellagitannins and urolithins.

washed, and spun down (250g/5 min/22 °C). An appropriate amount of the lysis buffer [10 mM Tris, pH 7.5, 10 mM KCl, and 0.5 mM ethylenediaminetetraacetic acid (EDTA)] was added, and the cells were transferred to the glass tube of a Teflon homogenizer and kept on ice for 10 min. This was followed by the addition of an appropriate amount of homogenization buffer [0.25 M KH_2PO_4, 0.15 M KCl, 10 mM EDTA, and 0.25 mM phenylmethanesulfonyl fluoride (PMSF)]. The cells were then broken by 12 manual strokes on a tight-fitting Teflon homogenizer. After centrifugation at 15000g/20 min/4 °C, the supernatant was again centrifuged at 105000g/90 min/4 °C. Microsomal pellets were resuspended in microsomal dilution buffer [0.1 M KH_2PO_4, 20% glycerol, 10 mM EDTA, 0.25 mM PMSF, and 0.1 mM dithiothreitol (DTT)] and stored in aliquots at −80 °C.

Western Blotting. Microsomal protein (5−10 μg) samples were prepared by heating at 95 °C for 5 min in the sample buffer comprising 0.5 μM Tris HCl (pH 6.8), 10% glycerol, 2% sodium dodecyl sulfate (SDS), 5% mercaptoethanol, and 0.001% bromophenol blue. The samples were resolved by precast criterion SDS-polyacrylamide gel electrophoresis (PAGE) gel (10%) at 200 V for 45−50 min (Bio-Rad Laboratories, Hercules, CA). The proteins were then transferred to a PVDF membrane (Bio-Rad Laboratories) at 100 V for 90 min. Following transfer, the membranes were blocked in blocking buffer for 1 h followed by incubation in CYP1B1 primary antibody (1:1000, antirat CYP1B1 polyclonal antibody, kindly donated by Dr. Thomas R. Sutter). After they were washed, the membranes were incubated in buffer containing the horseradish peroxidase-conjugated secondary antibody (1:30000, antigoat IgG peroxidase conjugate, Sigma Chemicals) for 2 h. The membrane was washed and

developed using LumiGLO Reserve chemiluminescent substrate (KPL, Inc., Gaithersburg, MD). The signals were detected using a CCD camera (VersaDoc Imaging System, Bio-Rad Laboratories). Human CYP1B1 supersomes were used to make standard curves of known protein concentrations (0.25−2 pmol). Standard curves were used to quantitate the CYP1 protein amounts in the samples using the Quantity One quantitation software (Bio-Rad Laboratories). Statistical differences between the control and the treated samples were determined using one-way analysis of variance (ANOVA) followed by Newman−Keuls posthoc ($p < 0.05$) using GraphPad Prism software.

Neutral Red Cytotoxicity Assay. The assay was performed in 96-well microplates. Cells were seeded at a density of 10000 cells/well and allowed to settle for 30 min at 37 °C. The compounds, diluted appropriately in RPMI-1640 medium, were added to the cells and again incubated for 48 h. The number of viable cells was determined using the neutral red assay procedure (17).

Induction of Phase II Conjugating Enzymes Assay. The assays were performed according to standard procedures described by Kirlin et al. (18)

Cellular Uptake of Urolithins A and B. 22Rv1 cells were incubated with 20 μM urolithins A and B in the RPMI-1640 media for 0.5, 6, 12, and 24 h time periods. After incubation, the cells were washed and harvested by trypsinization. The cells were extracted with acidified MeOH. The samples were analyzed by reverse phase HPLC at 288 nm detection. The experiment was done in triplicate. A standard curve of urolithins A and B was prepared from which the amount of urolithin uptake was determined. The amount of urolithin uptake was adjusted to the amount of protein.

Table 1. Results of Recombinant CYP1-Mediated EROD Assay and Kinetic Parameters[a]

	Section A				
	CYP1B1		CYP1A1		
	IC$_{50}$ ± SEM (μM)	K_i ± SEM (μM)	IC$_{50}$ ± SEM (μM)	K_i ± SEM (μM)	K_i(CYP1A1:CYP1B1)
UA	1.15 ± 0.65	0.25 ± 0.14	12.4 ± 4.7	0.51 ± 0.18	2.05
UB	1.55 ± 0.49	0.34 ± 0.17	26.8 ± 12.9	1.11 ± 0.53	3.28
UC	39.9 ± 29	8.74 ± 0.65	790 ± 75	32.7 ± 3.11	3.74
UD	137 ± 11.08	30.10 ± 25	2907 ± 168	120.3 ± 69.6	3.99
MUA	1.49 ± 0.39	0.327 ± 0.08	59.8 ± 8.7	2.47 ± 0.36	7.57
DMUC	89.6 ± 9.7	19.6 ± 2.13	657 ± 74.3	27.2 ± 3.00	1.39
PL	2.82 ± 0.33	0.618 ± 0.07	1.5 ± 0.32	0.062 ± 0.00	0.10
PG	2.6 ± 0.79	0.58 ± 0.02	2.67 ± 0.48	0.109 ± 0.20	0.19

	Section B				
	urolithin A			urolithin B	
	DMSO	0.5 μM	1 μM	DMSO	0.5 μM	1 μM
V_{max}	339 ± 148	97.3 ± 14.6	66 ± 6.02	355 ± 103	150.9 ± 14.9	99.5 ± 8.53
K_m	3.414 ± 2	2 ± 0.47	1.6 ± 0.25	4.78 ± 1.81	2.10 ± 0.33	1.9 ± 0.26

[a] IC$_{50}$ and K_i values (±SEM) and the ratio of CYP1A1 to CYP1B1 K_i for urolithins A (UA), B (UB), C (UC), D (UD), 8-*O*-methylurolithin A (MUA), 8,9-di-*O*-methylurolithin C (DMUC), punicalins (PL), and punicalagins (PG) mediated inhibition of EROD acitivity using recombinant human CYP1B1 and CYP1A1 enzymes. For section B, kinetic parameters, V_{max} (pmol/mg/min), and K_m (μM) ± standard errors (n = 3) for the inhibition of recombinant human CYP1B1 by urolithin A and B (0.5 and 1 μM), determined by nonlinear regression curve fit using the Michaelis−Menten equation ([S] vs V) plot using GraphPad Prism.

RESULTS AND DISCUSSION

Pomegranate fruit consists of two major classes of polyphenols, flavonoids and ellagitannins. The flavonoids include quercetin, kaempferol, and myricetin (*19*). These flavonoids have been reported to exhibit CYP1 inhibitory activities (*16*). Pomegranate juice consumption has been found to decrease the expression/activity of total hepatic CYP content (*9*). Pomegranate juice obtained by hydrostatic pressing of whole fruit predominantly contains ellagitannins. Ellagitannins, with the exception of ellagic acid, have not been previously studied for their CYP1 inhibitory activities. Ellagic acid has been shown to inhibit CYP2A2, 3A1, 2C11, 2B1, 2B2, and 2C6 in rat liver microsomes (*20*) and also inhibited the CYP1A1-dependent activation of benzo-[*a*]pyrene (BaP) (*3*). Inhibition of CYP1 protein expression and activity by some flavonoids, such as diosmin, diosmetin, quercetin, kaempferol, and myricetin, was believed to be through AhR antagonism or their effects on the downstream products of AhR signal transduction pathways (*21*). However, ellagic acid decreased CYP1A1-dependent BaP activity independent of the AhR-responsive element (*3*). The proposed mechanism of inhibition of CYP1A1-dependent BaP activation involved scavenging of the carcinogen by ellagic acid through chemical binding (*22*). Therefore, our objective was to study the effects of a selection of pomegranate ellagitannins and urolithins on the inhibition of CYP1-dependent carcinogen activation.

The ability of ellagitannins and urolithins to inhibit CYP1 activity was tested in a recombinant CYP1A1- and CYP1B1-dependent EROD assay. The IC$_{50}$ values for CYP1B1 inhibition ranged from 1.15 ± 0.65 μM for urolithin A to 137 ± 11.08 μM for urolithin D. CYP1A1 IC$_{50}$ values ranged from 1.5 ± 0.32 μM for punicalins to 2907 ± 168 μM for urolithin D (**Table 1**, section A). Urolithins exhibited higher selectivity toward CYP1B1 EROD inhibition as compared to CYP1A1, although the selectivity was not significant. The K_i values of CYP1B1 and CYP1A1 depicted in **Table 1**, section A, indicate that 8-*O*-methylurolithin A exhibited a 7.5-fold selectivity toward CYP1B1 inhibition. Punicalins and punicalgins were 10- and 5-fold more selective toward CYP1A1 inhibition.

Urolithins A and B are the major microbial metabolites of pomegranate chemical constituents detected in human systemic circulation. These metabolites exhibited lower IC$_{50}$ values in recombinant CYP1 inhibition as compared to all other tested compounds. Therefore, a study was conducted to investigate the mechanism of action of CYP1B1 inhibition by urolithins A and B. The concentrations of inhibitors used were in the vicinity of their calculated IC$_{50}$ values, that is, 0.5 and 1 μM. EROD activities were determined with substrate concentrations ranging from 0.1 to 2.0 μM. Kinetic parameters, V_{max} and K_m, were calculated using the Michaelis−Menten equation ([S] vs V curve). Double reciprocal plots were plotted using 1/[S] and 1/V (**Figure 2**) from which K_i values were calculated (**Table 1**, section B). The calculated K_i values for urolihins A and B (1.51 ± 0.91 and 1.33 ± 0.08 μM) were not statistically different from those calculated by using Cheng−Prusoff equations (*16*). The calculated V_{max} and K_m for urolithin A changed significantly with an increasing concentration of inhibitor, suggesting an uncompetitive type of inhibition. However, the V_{max} and K_m of urolithin B did not differ significantly upon increase of inhibitor concentration, suggesting a noncompetitive type of inhibition.

In our study, urolithins A and B, structural analogues of ellagic acid, exhibited a significant inhibition of CYP1-dependent EROD activity. The results suggested that a hydroxy group at C-8 and C-3 (urolithins A and B), corresponding to the C-4 and C-4′ position of ellagic acid, were required for full CYP1-dependent EROD activity inhibition in accordance with a previous study by Barch et al. (*7*). However, in our study, additional hydroxy groups at C-4 and C-9 of the urolithin pharmacophore (urolithins C and D) resulted in decreased CYP1-dependent EROD inhibitory activity. Methylation of the hydroxy groups to give 8-*O*-methylurolithin A and 8,9-di-*O*-methylurolithin C decreased the activity, suggesting that the phenolic hydroxy groups are important for CYP1 inhibitory activity. To probe the importance of the lactone group for CYP1-dependent EROD activity inhibition, HHDP and gallic acid were tested for their CYP1-dependent EROD activity.
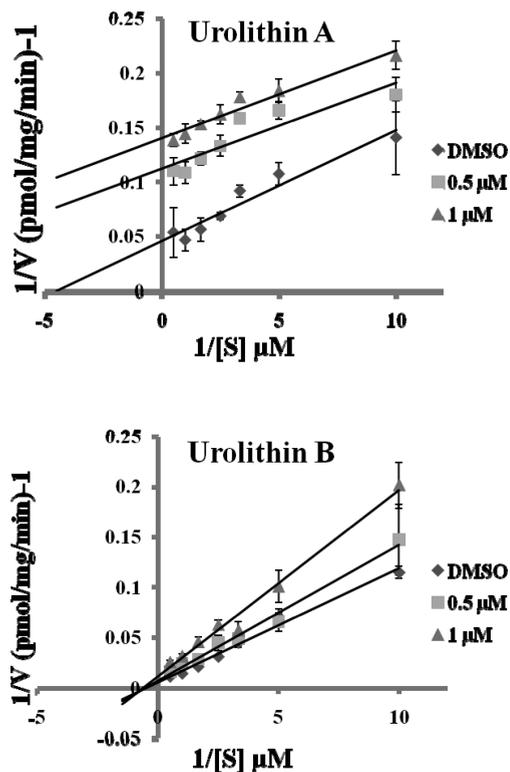
Figure 2. Double reciprocal plots for the inhibition of in vitro EROD activity of CYP1B1 by urolithins A and B at 0.5 and 1 μM. EROD substrate concentrations used were 0.1, 0.2, 0.3, 0.4, 0.6, 1.0, and 2.0 μM. Recombinant CYP1B1 was preincubated with the inhibitor or DMSO prior to initiation of reaction. Each experiment was done three times in duplicate.

The results showed that hydrolysis of the lactone functionality did not result in CYP1 inhibition (data not shown).
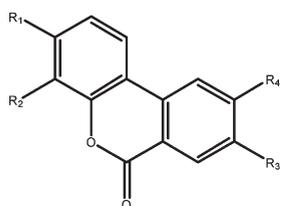
Urolithin pharmacophore

	R$_1$	R$_2$	R$_3$	R$_4$
Urolithin B (UB)	-OH	-H	-H	-H
Urolithin A (UA)	-OH	-H	-OH	-H
Urolithin C (UC)	-OH	-H	-OH	-OH
Urolithin D (UD)	-OH	-OH	-OH	-OH
8-*O*-methylurolithin A (MUA)	-OH	-H	-OCH$_3$	-H
8,9-Di-*O*-methylurolithin C (DMUC)	-OH	-H	-OCH$_3$	-OCH$_3$
8,9-Di-*O*-methylurolithin D (DMUD)	-OH	-OH	-OCH$_3$	-OCH$_3$

The punicalins and punicalagins were potent inhibitors of CYP1-dependent EROD activity with K_i values (**Table 1**, section A) comparable to the dietary flavonoids. The dietary flavonoids, inhibiting CYP1-dependent metabolic activation of procarcinogens, include quercetin, kaempferol, apigenin, myricetin, and rutin. Quercetin and kaempferol, which are the predominant flavonoids in the human diet, inhibited CYP1-mediated EROD activities. The apparent K_i values of inhibition of human recombinant CYP1B1 and CYP1A1 were 14 $\pm$ 3 and 52 $\pm$ 2 nM for kaempferol, whereas they were 23 $\pm$ 2 and 77 $\pm$ 5 nM for quercetin (*23*). In a different study, quercetin inhibited epoxidation

of 7,8-dihydro-7,8-benzo[*a*]pyrene-7,8-diol by CYP1A1 allelic variants with K_i values ranging from 2.0 to 9.3 μM with mixed type inhibition (*24*). In another study, quercetin inhibited recombinant CYP1A1 and CYP1B1 activities with K_i values of 0.25 $\pm$ 0.04 and 0.12 $\pm$ 0.02 μM with a mixed type inhibition (*16*). The bioavailability of the flavonoids depends upon the source of food; for example, quercetin absorption from tomato puree, apples, and onions was 0.082, 0.34, and 0.74 μM, respectively (*25, 26*). Kaempferol plasma concentrations ranged from 0.01, 0.05, and 0.1 μM upon consumption of onion, tea, and endive, respectively (*27, 28*). Bioavailable concentrations of quercetin and kaempferol from some food sources were less than their reported K_i values of CYP1 inhibition. Apigenin, a flavone present in parsley, exhibits CYP1B1 and CYP1A1 inhibition with K_i values ranging from 60 nM to 0.2 μM. These concentrations are bioavailable (127 $\pm$ 81 nM) upon consumption of 2 g of blanched parsley (*14*). Bioavailability of rutin from tomato puree as detected in plasma was calculated to be 0.1 μM (*25*), which was around 60-fold lower than the reported K_i of CYP1B1 inhibition. The bioavailability of many other flavonoids is still unclear. However, dietary flavonoids could inhibit CYP1-mediated bioactivation of environmental and dietary carcinogens into genotoxic compounds and prevent cancer initiation in alimentary canal-related cancers because they come into direct contact with the digestive epithelium of the digestive system (*29*). However, the prostate cancer chemopreventive effects of flavonoids (*30*) depending on the bioavailability are still debated. It is therefore important to choose an appropriate dietary supplement that can release adequate amounts of cancer chemopreventive compounds into plasma, whenever it is consumed, with an intended pharmacological activity.

Pomegranate juice ellagitannins have been extensively studied for their bioavailability and their biological effects. In one study, it was established that the punicalagins hydrolyze into ellagic acid and other smaller polyphenols that are responsible for the bioactivity of ellagitannins. In a study performed on human subjects, consumption of 180 mL of pomegranate juice (equivalent to 25 mg of ellagic acid and 319 mg of punicalagins) resulted in detection of ellagic acid in plasma with a maximum concentration of 31.9 ng/mL (0.1 μM) (*31*). Previous studies also indicated that the bioavailability of ellagic acid from pomegranate juice, pomegranate liquid concentrate extract, and pomegranate powder extract was not statistically different (*32*). In our study, punicalins and punicalagins inhibited CYP1A1 with K_i values of 0.062−0.109 μM and CYP1B1 with K_i values 0.618 and 0.58 μM, respectively. Punicalins and punicalagins exhibited approximately a 10- and 5-fold selectivity for CYP1A1 over CYP1B1. Thus, consumption of pomegranate juice could be beneficial in decreasing CYP1-mediated oral, esophageal, and colon cancers. However, these ellagitannins cannot exhibit a systemic CYP1 inhibition activity because they are metabolized in the colon by microflora into smaller organic molecules called urolithins.

Bioavailability studies indicate that maximum plasma concentrations of urolithins A and B reach concentrations of 4−18 μM in human subjects (*8, 32*). Urolithins A and B inhibited human recombinant CYP1A1 and CYP1B1 with K_i values in their bioavailable concentration range. Our particular interest was to explore the beneficial effects of urolithins in prostate cancer. Studies indicate that 15% of prostate cancer patients, who have undergone a radical prostectomy, had a biochemical recurrence of prostate-specific antigen (PSA). Among them, 34% of patients developed distant metastases within 15 years (*33*). It was evident that consumption of pomegranate juice delayed the doubling time of the PSA by 39 months after primary therapy (*7*). The effects were ascribed to the antiproliferative, apoptotic, and antioxidant

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A.

B.
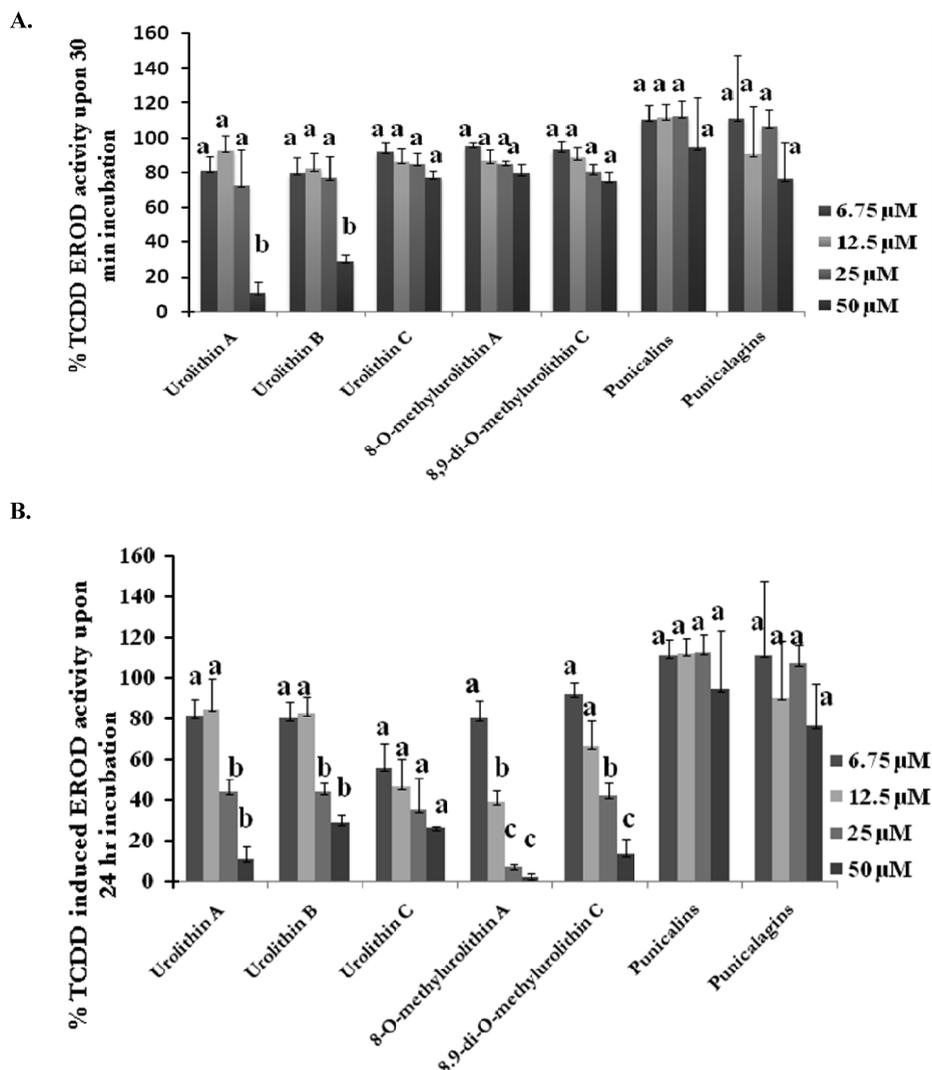
Figure 3. Effects of increasing concentration (6.75, 12.5, 25, and 50 μM) of urolithin A (UA), urolithin B (UB), urolithin C (UC), 8-*O*-methylurolithin A (MUA), 8,9-di-*O*-methylurolithin C (DMUC), punicalins (PL), and punicalagins (PG) on 50 nM TCDD-induced EROD activity in intact 22Rv1 human prostate cancer cells. The cells were exposed to the compounds for (**A**) 30 min or (**B**) 24 h prior to the EROD measurement. Each experiment was done three times in triplicate. Global one-way (ANOVA) with a Student−Newman−Keuls posthoc test was used to determine treatment effects. In the same treatment groups, bars with different letters are statistically different.

effects of pomegranate constituents, observed in LNCaP, PC-3, 22Rv1, and DU 145 prostate cancer cell lines (*8*, *34*). A study about gene polymorphisms and risk of prostate cancer showed that polymorphisms in CYP1B1 and PSA genes increased the risk and aggressiveness of prostate cancer (*35*). Any dietary constituent with CYP1B1 inhibitory activity could potentially lead to prostate cancer chemoprevention. There were no previous reports about the ability of pomegranate constituents/metabolites to inhibit CYP1B1-dependent carcinogenesis.

Therefore, we studied the capability of pomegranate constituents to inhibit CYP1B1-induced metabolic activation in a prostate cancer cell line, 22Rv1. The cells were treated with TCDD for 24 h to induce CYP1B1 protein expression. Then, the cells were treated with punicalins, punicalagins, or urolithins for 30 min. Following 30 min of incubation, urolithins A and B significantly decreased TCDD-induced EROD activity at the highest concentration used (**Figure 3A**). The IC$_{50}$ values calculated for cell-based CYP1 inhibition by urolithins A and B were 32 ± 8.9 and 38.2 ± 3.94 μM, respectively (**Table 2**). The results indicate a 28- and 26-fold increase in IC$_{50}$ values for cell-based CYP1-mediated EROD activity of urolithins A and B, respectively, as compared

Table 2. IC$_{50}$ Values for Pomegranate Chemical Constituents/Microbial Metabolite Mediated Inhibition of EROD Activity in TCDD-Induced 22Rv1 Prostate Cancer Cells

	IC$_{50}$ ± SEM (μM)	
chemical constituent	30 min	24 h
urolithin A	32 ± 8.9	13.3 ± 1.3
urolithin B	38 ± 3.9	17.9 ± 1.8
urolithin C	NA	26.89 ± 2.5
8-*O*-methylurolithin A	NA	14.8 ± 2.2
8,9-di-*O*-methylurolithin C	NA	11.5 ± 2.8
punicalins	NA	NA
punicalagins	NA	NA

to their in vitro recombinant CYP1-mediated EROD inhibitory IC$_{50}$. Punicalins and punicalagins did not inhibit cell-based CYP1-mediated EROD activity at the highest concentration used (50 μM). To ascertain whether a decrease in IC$_{50}$ occurred upon longer incubation, the cells were allowed to grow in the presence of compounds and TCDD for 24 h (**Figure 3B**). The EROD activity results indicated that the compounds more effectively inhibited

CYP1-mediated EROD activity and had IC_{50} values lower than those following 30 min of incubation. After 24 h of cotreatment, urolithins A and B inhibited TCDD-induced EROD activity in prostate cells with IC_{50} values of 13.3 ± 1.32 and $17.9 \pm 1.8 \mu M$, respectively, which were in the vicinity of their bioavailability (8, 32). Punicalins and punicalagins did not exhibit EROD inhibition even upon 24 h of incubation. Urolithin C, 8-O-methylurolithin A, and 8,9-di-O-methyl urolithin C demonstrated IC_{50} values of 26.8 ± 2.5, 14.8 ± 2.24, and $11.5 \pm 2.8 \mu M$, respectively, which were lower than those of urolithins A and B (**Table 2**). The compounds alone did not induce EROD activity after 24 h of incubation. Because the prostate cells were treated with the test compounds for 24 h, it was imperative to investigate if the compounds exhibited any cytotoxicity. A neutral

Table 3. IC_{50} Values for Urolithin-Mediated Cytotoxicity of 22Rv1 Prostate Cells

chemical constituents	$IC_{50} \pm SEM$ (μM)
urolithin A	98.7 ± 4.2 (a)
urolithin B	108 ± 3.9 (a)
urolithin C	36 ± 3.5 (b)
8-O-methylurolithin A	23.3 ± 3.9 (c)
8,9-di-O-methylurolithin C	20.6 ± 4.6 (c)

Table 4. Uptake of Urolithins A and B by 22Rv1 Cells over a Period of 0.5−24 h

time (h)	uptake in $\mu mol/mg$ protein	
	urolithin A	urolithin B
0.5	5.2 ± 0.4	5.7 ± 0.8
6	9.6 ± 0.4	8.0 ± 1.1
12	20.1 ± 0.5	21.9 ± 1.1
24	23.9 ± 0.6	25.2 ± 1.6

red dye uptake assay was used to measure the cytotoxicity of the test compounds. The data were probit transformed followed by linear regression and IC_{50} calculation. The cytotoxic IC_{50} values ranged from $20.6 \pm 4.58 \mu M$ for 8,9-di-O-methylurolithin C to 108 ± 3.994 μM for urolithin B (**Table 3**). The results indicate that the cytotoxicities of urolithin A and B had no contribution toward decreased CYP1-mediated EROD activity. The results also indicate that urolithin C, 8,9-di-O-methylurolithin C, and 8-O-methylurolithin A were false positives in the prostate cell EROD assay. The activity was not because of CYP1 inhibition but due to cytotoxicity. This conclusion was verified based on four facts: (1) These compounds inhibited recombinant CYP1-mediated EROD activity at higher concentrations as compared to urolithins A and B; (2) these compounds did not inhibit prostate cell EROD activity upon 30 min of incubation; (3) they inhibited prostate cell EROD activity upon 24 h of treatment, with IC_{50} values lower than those exhibited in the recombinant CYP1-mediated EROD assay; and (4) they exhibited cytotoxicity in the vicinity of their prostate cell EROD inhibition IC_{50} values.

Following cell-based EROD assays, cellular uptake experiments were performed for urolithins A and B. These experiments were performed to determine if the decrease in IC_{50} values for CYP1-mediated EROD inhibition in 22Rv1 cells was related to increased uptake over a 24 h time period. The experiments indicated that there was a 4.5-fold increase in urolithin uptake upon 24 h of incubation as compared to 30 min of incubation. The results also indicated that there was a dramatic increase in urolithin uptake between 6 and 12 h, beyond which the uptake was constant. The results suggested that increased availability of urolithins (from 30 min to 24 h) could have contributed to the decrease in IC_{50} of CYP1 inhibition (**Table 4**). The results also indicated that urolithins were metabolically stable in 22Rv1 cells for up to 24 h.
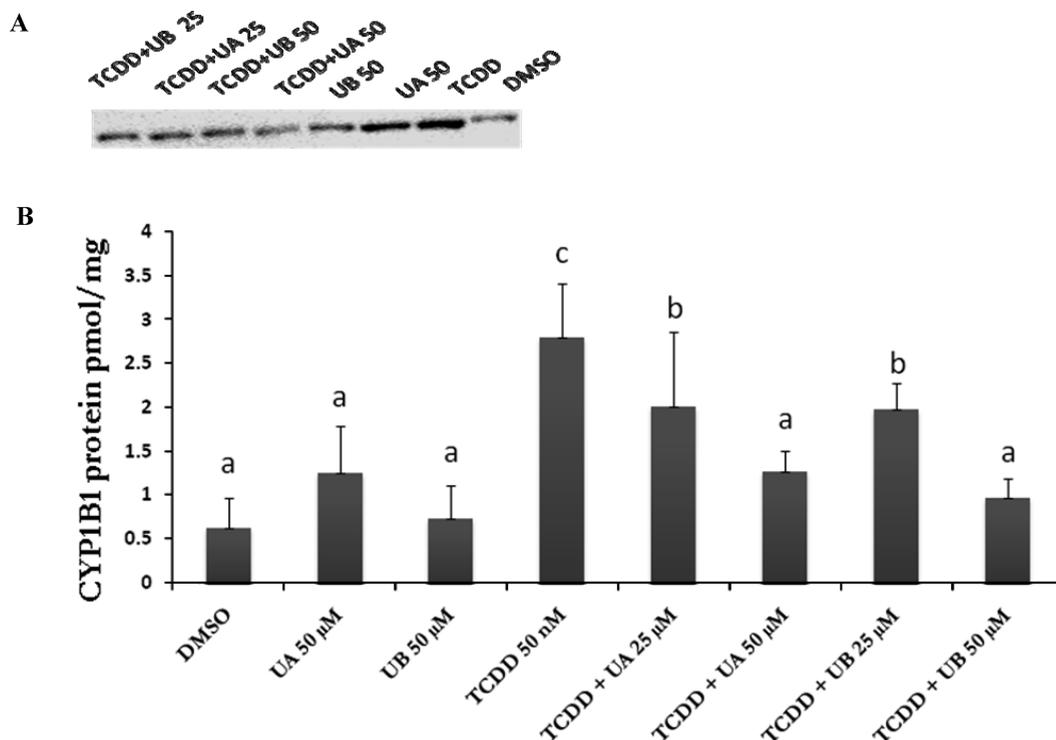
Figure 4. (**A**) Western blot showing the effect of urolithins A and B and their cotreatment with TCDD on CYP1B1 protein expression. Microsomes were prepared after treating cells for 24 h with different treatments (DMSO, 50 nM TCDD, UA 50 μM, UB 50 μM, TCDD + UA 50 μM, TCDD + UA 25 μM, TCDD + UB 50 μM, and TCDD + UB 25 μM), along with standards, were loaded on 10% SDS-PAGE. Protein levels were expressed in pmol/mg. (**B**) Effect of urolithin A and B on the TCDD-induced CYP1B1 protein expression in 22Rv1 prostate cancer cells. Each experiment was repeated three times. Global one-way ANOVA with a Student−Newman−Keuls posthoc test was used to determine treatment effects. Bars with different letters are statistically different.

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The decrease in IC_{50} values could also be attributed to the decrease in the TCDD-induced CYP1B1 protein expression. To examine if the treatment affected CYP1 protein expression levels, Western blots were performed (**Figure 4**). Cell treatments were DMSO, TCDD (50 nM), UA (50 μM), UB (50 μM), TCDD + UA (50 μM), TCDD + UB (50 μM), TCDD + UA (25 μM), and TCDD + UB (25 μM). CYP1B1 protein levels were significantly increased ($\sim$4.5-fold) by TCDD (2.8 $\pm$ 0.6 pmol/mg) as compared to DMSO (0.62 $\pm$ 0.28 pmol/mg). Cotreatment of TCDD with urolithin A (50 μM) significantly decreased CYP1B1 protein (1.26 $\pm$ 0.38 pmol/mg) production by 54%, while urolithin A (25 μM) decreased CYP1B1 protein (2.00 $\pm$ 0.5 pmol/mg) production by 28% as compared to TCDD-induced cells. Cotreatment of TCDD with urolithin B (50 μM) decreased CYP1B1 protein (0.96 $\pm$ 0.4 pmol/mg) production by 65%, and urolithin B (25 μM) treatment decreased protein (1.9 $\pm$ 0.5 pmol/mg) production by 29% as compared to TCDD-induced levels. Western blot analyses suggest that none of the urolithins induce CYP1B1 basal levels significantly as compared to DMSO. However, cotreatments with urolithins A and B 50 μM and TCDD decreased CYP1B1 protein expression levels as compared to TCDD alone (**Figure 4**).

Urolithins A and B inhibited CYP1 EROD activity by inhibiting both the protein expression and the activity of CYP1B1. While the influence of urolithin C, 8,9-di-*O*-methylurolithin C, and 8-*O*-methylurolithin A on CYP1B1 activity/expression was not clear, they are believed to exert an antiproliferative activity on prostate cancer cells, in accordance with previous studies (*32, 34*). An ideal anticarcinogenic agent would inhibit phase I enzymes, involved in carcinogen activation while inducing the phase II enzymes, responsible for the deactivation of carcinogens by assisting their excretion via increased water solubility. The pomegranate ellagitannins and urolithins were tested for their capacity to induce glutathione *S*-transferase and quinone *O*-reductase enzymes. The basal levels of quinone *O*-reductase and glutathione *S*-transferase enzymes in 22Rv1 cells were determined to be 1.2 $\pm$ 0.32 and 0.51 $\pm$ 0.17 μmol min^{-1} mg protein^{-1}, respectively. However, none of the compounds exhibited (data not shown) induction of quinone *O*-reductase or glutathione *S*-transferase as compared to normal proliferating cells.

In conclusion, our study has asserted a previously unknown mechanism of action of pomegranate juice constituents, which could potentially contribute to prostate cancer chemoprevention. In a study to evaluate the safety of consuming large quantities of pomegranate ellagitannins, it was shown that the consumption of pomegranate ellagitannin-enriched extracts (providing 435 and 870 mg of gallic acid equivalents) was safe in humans (*36*). The study also demonstrated the ability of pomegranate ellagitannins to release antioxidant principles into plasma. In our study, we proved that systemically available metabolites of pomegranate juice are effective inhibitors of CYP1B1 enzyme activity/expression and could lower the incidence of prostate cancer initiation and sustenance. However, some of the cytotoxic metabolites may exhibit their prostate cancer inhibition activity by exerting an antiproliferative effect. These metabolites may also decrease the incidence of drug resistance mediated by CYP1B1-related drug inactivation, if used as an adjuvant during chemotherapy. It is also well-known that prostate cancer typically possesses long latency periods and develops in older men; therefore, cancer chemoprevention by dietary supplement-based intervention is a desirable form of chemotherapy. Pomegranate juice consumption, thus, may be of considerable advantage in prostate cancer chemoprevention, not only in patients with a genetic predisposition toward prostate cancer but also in patients undergoing cancer therapy.

ABBREVIATIONS USED

CYP, cytochrome P450; PAHs, polycyclic aromatic hydrocarbons; 4-OH-E$_2$, 4-hydroxyestradiol; AhR, aryl hydrocarbon receptor; HHDP, hexahydroxydiphenic acid; EROD, ethoxyresorufin-*O*-deethylase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol BaP, benzo[*a*]pyrene.

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