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Prostate Cancer Xenograft Inhibitory Activity and Pharmacokinetics of Decursinol, a Metabolite of *Angelica Gigas* Pyranocoumarins, in Mouse Models

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Abstract

We have previously shown that the ethanol extract of dried *Angelica gigas* Nakai (AGN) root exerts anti-cancer activity against androgen receptor (AR)-negative human DU145 and PC-3 prostate cancer xenografts and primary carcinogenesis in the transgenic adenocarcinoma of mouse prostate (TRAMP) model. The major pyranocoumarin isomers decursin (D) and decursinol angelate (DA), when provided at equi-molar intake to that provided by AGN extract, accounted for the inhibitory efficacy against precancerous epithelial lesions in TRAMP mice. Since we and others have shown in rodents and humans that D and DA rapidly and extensively convert to decursinol, here we tested whether decursinol might be an *in vivo* active compound for suppressing xenograft growth of human prostate cancer cells expressing AR. In SCID-NSG mice carrying subcutaneously inoculated human LNCaP/AR-Luc cells overexpressing the wild type AR, we compared the efficacy of 4.5 mg decursinol per mouse with equi-molar dose of 6 mg D/DA per mouse. The result showed that decursinol decreased xenograft tumor growth by 75% and the lung metastasis, whereas D/DA exerted a much less effect. Measurement of plasma decursinol concentration, at 3 hours after the last dose of respective dosing regimen, showed higher circulating level in the decursinol-treated NSG mice than in the D/DA-treated mice. In a

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No potential conflicts of interest were disclosed.

subsequent single-dose pharmacokinetic experiment, decursinol dosing led to 3.7-fold area under curve (AUC) of plasma decursinol over that achieved by equi-molar D/DA dosing. PK advantage notwithstanding, decursinol represents an active compound to exert *in vivo* prostate cancer growth and metastasis inhibitory activity in the preclinical model.

Keywords

Angelica gigas Nakai; decursinol; decursin; decursinol angelate; *in vivo* anti-cancer efficacy; pharmacokinetics

Introduction

Prostate cancer (PCa) is the most-frequently diagnosed non-cutaneous malignancy in US men with the second highest mortality rate next to lung cancer, leading to 26,000 adult male deaths each year in the United States alone (Siegel *et al.*, 2016). Because of its long latency and slow growth, natural herbal extracts or compounds have been considered as safe and practical chemopreventive agents to inhibit the carcinogenesis or as intercepting drugs to delay the progression of PCa (Ting *et al.*, 2014). In spite of more than 3 decades of intensive research, there is currently no FDA-approved small molecule drug for PCa chemoprevention or early intervention (Thompson *et al.*, 2014).

Our long-term goal is to develop herbal modalities or compounds from *Angelica gigas* Nakai (AGN) for the chemoprevention of prostate carcinogenesis and the precision therapy of recurrent prostate cancer after localized treatments. Known as Korean Angelica or Cham Dong Quai, the dried roots of AGN are used in traditional medicine in Korea for treatment of anemia, infection and articular rheumatism, mostly through its water-soluble herbal prescriptions (Zhang *et al.*, 2012). Herbal products containing AGN are marketed as dietary supplements in the United States for relief of minor aches and pain, memory enhancement and post-menopausal women's hot flashes (Zhang *et al.*, 2012). Ethanolic extract of dried AGN root has been shown by us to exert anti-cancer activity in androgen receptor (AR)-negative human PCa xenograft models in immunocompromised athymic nude mice (Lee *et al.*, 2009) and against primary carcinogenesis in the transgenic adenocarcinoma of mouse prostate (TRAMP) model (Tang *et al.*, 2015; Zhang *et al.*, 2015c). The pyranocoumarin decursin (D) and its isomer decursinol angelate (DA) (Fig. 1A) are the major chemicals in the alcoholic extract of the root of AGN (Sarker and Nahar, 2004; Zhang *et al.*, 2012). In a sarcoma allograft model, D and DA by i.p. injection were shown to inhibit *in vivo* murine tumor growth in syngeneic mice (Lee *et al.*, 2003). In our recent study with TRAMP mice (Tang *et al.*, 2015), we have demonstrated that AGN ethanol extract can significantly suppress the growth of AR-negative neuroendocrine (NE)-lineage cancer and inhibit prostate epithelial precancerous growth. The mixture of D and DA, at equi-molar intake to that provided by AGN, afforded the same epithelial lesion growth suppression efficacy as judged by weight of prostate lobes and lesion severity (Tang *et al.*, 2015). Since pharmacokinetic (PK) studies by us and others have shown that D/DA rapidly and extensively convert to decursinol, in rodents (Lee *et al.*, 2009; Li *et al.*, 2012; Li *et al.*, 2013b; Park *et al.*, 2012) and humans alike (Lu *et al.*, 2015; Zhang *et al.*, 2015a), involving 2 major cytochrome p450

(CYP) isoforms and carboxylesterase-2 (CES-2) (Zhang *et al.*, 2015b) (Fig. 1B), here we tested the hypothesis that decursinol might be an *in vivo* “active” compound for PCa growth suppression.

In the present study with SCID-NSG mice carrying AR-overexpressing human LNCaP/AR-Luc cells inoculated subcutaneously, we compared the efficacy of D/DA (6 mg per mouse) with equi-molar dose of 4.5 mg decursinol per mouse. The LNCaP/AR-Luc cells and xenografts in immunodeficient mice have been workhorse models for the development of next-generation anti-AR drug enzalutamide/Xtandi (formerly MDV3100) by Charles Sawyers and coworkers (Tran *et al.*, 2009). Once we observed that decursinol treatment decreased tumor growth more potently than equi-molar dosing of D/DA in association with higher plasma decursinol level in the decursinol-treated mice, we embarked on a PK study to investigate whether the greater anti-cancer efficacy of decursinol administration was linked to its PK advantage over the administration of the parent compounds.

Materials and methods

Chemicals and reagents

Decursin (D) and decursinol angelate (DA) were purified together from the ethanol extract of the dried root of AGN by silica column chromatography by MP and CX as described previously (Tang *et al.*, 2015) (Li *et al.*, 2013b). Decursinol was prepared from the hydrolysis of D/DA (Li *et al.*, 2013b). The purities of D/DA and decursinol were verified to be higher than 99% by HPLC and thin layer chromatography (TLC). PEG 400, Tween 80, and ethyl acetate were purchased from Sigma-Aldrich Company (St. Louis, MO). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA). D-Luciferin was obtained from Caliper Life Sciences (Hopkinton, MA).

Dosage preparations

For efficacy experiments, an excipient solution made up of ethanol: PEG 400: Tween 80: 5% glucose (EPTG) with the ratio of 3:6:1:20 was used to prepare AGN, D/DA and decursinol, as described previously (Li *et al.*, 2013b; Tang *et al.*, 2015). Briefly, AGN extract, D/DA or decursinol was first dissolved in stock preparations in 30% ethanol, 60% PEG 400, and 10% Tween 80 and kept frozen at -20°C . Before each day's dosing, two volumes of 5% glucose solution were added to dilute the stock to the working formula.

Due to suboptimal dissolution of decursinol in the above working formula, we further adjusted the ratio of EPT to G to 3:6:1:5 as an improved excipient vehicle which proved to be better suited for administering decursinol and D/DA. The improved vehicle was used for subchronic safety evaluation and PK experiment.

Cell line choice

LNCaP/AR-Luc cells were generously provided by Charles Sawyers, MD of Memorial Sloan Kettering Cancer Center, New York (Tran *et al.*, 2009). They were originally generated by the Sawyers group based on the findings that in isogenic hormone-sensitive (passaging the parental lines in intact mice) and hormone-refractory (passaging the parental

lines in castrated mice with similar passage numbers) human PCa xenograft pairs, that the hormone-refractory tumors had more AR protein than their hormone-sensitive counterparts (Chen *et al.*, 2004). Wide-type AR cDNA was introduced into hormone-sensitive LNCaP cells by retroviral infection, and the threefold increase in AR abundance in the LNCaP/AR-Luc cells mimicked the expression difference observed in the hormone-refractory/hormone-sensitive pairs (Tran *et al.*, 2009). In this cell model, co-transfection of constitutively expressed firefly luciferase made possible for detection of metastatic lesions by *in vivo* or *ex vivo* bioluminescence. The LNCaP/AR-Luc cells were cultured in RPMI 1640 Medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA) without any antibiotics. The cells were expanded and used for inoculation within 3–4 passages after thawing from liquid nitrogen storage.

Authentication of the LNCaP/AR-luc and LNCaP cell lines was accomplished by next-gen DNA whole genome sequencing (Macrogen Clinical Laboratory, Rockville, MD) and RNA sequencing (Penn State Hershey Genome Sciences Facility, Hershey, PA). At the genotype level, the cells display XY gender trait and the characteristic T877A mutation in the AR gene of the parental LNCaP cells. At the mRNA level, these cells express *KLK3* (PSA) at 1/4 of parental LNCaP cells as expected from the Sawyers' original report (Tran *et al.*, 2009). Western Blot analysis confirmed the increased total AR protein and decreased PSA as compared to the LNCaP cells (data not shown).

LNCaP/AR-Luc xenograft Model

The animal use protocol was approved by the Institution Animal Care and Use Committee (IACUC) of Texas Tech University Health Sciences Center. Male SCID-NSG mice at 7 weeks of age were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were maintained in clean HEPA-filter top covered cages and were housed 3 per cage with purified AIN-93M pellet diet (Harlan Teklad, Madison, WI). After acclimation for one week, the mice were assigned into groups stratified by body weight (n = 20 per group). In the first experiment, they received 0.3 mL volume of excipient vehicle (ethanol: PEG400: Tween80: 5% glucose = 3:6:1:20), 5 or 10 mg AGN, respectively, via gavage once daily (6 days a week). In the second experiment, they were treated with D/DA (6 mg/mouse, matching that in 10 mg AGN) or decursinol (4.5 mg/mouse, equi-molar dose of 6 mg D/DA) via gavage once daily (7 days a week) until the termination of the study. After one week of pre-treatments with AGN, D/DA or decursinol, 20,000 LNCaP/AR-Luc cells (in 100 μ L of serum-free medium and Matrigel mix 1:1 v/v) were subcutaneously inoculated into the lower right flank of the mice. The xenograft tumor size was measured twice a week using a caliper and the volume was calculated according to the formula $[(L \times W^2)/2]$, in which L and W stand for length and width, respectively. At necropsy, tumors were dissected and weighed. For large tumors, one portion was fixed in neutral-buffered formalin and the remaining piece was frozen and stored at -80°C . Small tumors were fixed in formalin only. Lungs from tumor bearing mice were imaged *ex vivo* by bio-luminescence for detection of metastasis.

Safety evaluation of decursinol in improved excipient vehicle in CD-1 mice

A total of 24 male CD-1 mice (genetic background closer to SCID) at 7 weeks of age were purchased from Charles River Laboratory (Wilmington, MA) and maintained on purified

AIN-93M diet. After two weeks of acclimation, the mice were assigned into four groups stratified by body weight and received 2, 3, and 4 mg of decursinol per 25 g body weight (80, 120, and 160 mg/kg body weight) in the improved excipient vehicle (EPTG 3:6:1:5) or 200 μ L vehicle daily (7 days per week). Body weight was measured once a week and used for dose adjustment for the following week. After four weeks of treatment, all the mice were euthanized. Heparinized blood was collected for plasma by cardiac puncture and organs were dissected and weighed. Plasma biochemistry panel, including ALT (alanine aminotransferase), AST (aspartate transaminase), ALP (alkaline phosphatase), ALB (albumin), amylase (to test exocrine pancreatic integrity), BUN (blood urea nitrogen), creatinine, glucose, cholesterol and total bilirubin, was measured at University of Minnesota College of Veterinary Medicine Diagnostic Laboratory (St Paul, MN).

Single dose pharmacokinetic study in CD-1 mice

A total of 112 male CD-1 mice at 7 weeks of age were purchased from Charles River Laboratory and maintained on purified AIN-93M diet at least 1 week before use. Mice were assigned to one of 9 time points randomized by body weight (5–7 mice per group). Half of them (56 mice) were given a single dose of D/DA (4 mg per 25 g body weight or 160 mg/kg body weight), and the other half of mice were each given a single dose of decursinol (3 mg per 25 g body weight or 120 mg/kg body weight), at equi-molar dose of D/DA, by gavage in 0.2 mL of improved excipient vehicle (EPTG 3:6:1:5). The mice were euthanized at 0.1, 0.25, 0.5, 1, 2, 3, 6, 12 and 24 hours post-dose by isoflurane sedation and their blood was taken by cardiac puncture. Sodium heparin was used as anti-coagulant. The blood was held on ice and then centrifuged at 5000 g for 5 minutes to collect plasma. Liver and other organs were also collected at each time point. All the samples were stored in -80°C freezer until analysis following extraction method as described above. The pharmacokinetic analysis was performed by a non-compartmental approach using Phoenix WinNonlin software Version 6.1 (Pharsight Corporation) to calculate area under the curve (AUC).

Analysis of decursinol by HPLC

An Agilent Infinity 1260 HPLC system (Wilmington, DE) was equipped with a binary pump, a degasser, a diode array detector, an auto sampler, and a thermostatted column compartment. The sample preparation method was the same as previously reported by our group (Li *et al.*, 2012; Li *et al.*, 2013a; Li *et al.*, 2013b). In brief, 100 μ L of mouse plasma was spiked with 10 μ L of chrysin solution as internal standard (100 $\mu\text{g}/\text{mL}$) and 10 μ L of methanol by vortexing for 30 seconds and extracted with 0.8 mL of ethyl acetate by vortex-mixing for 3 minutes. After centrifugation at 3500 g for 3 minutes, the organic phase was quantitatively transferred to a clean Eppendorf tube. A second extraction was performed by adding 0.7 mL of ethyl acetate to the same plasma-containing tube and vortexed for another 3 minutes. The sample was centrifuged again at 5000 g for 5 minutes. The ethyl acetate supernatant was collected and combined with the first fraction, and then dried in a Speedvac at room temperature. The residue was reconstituted in 100 μ L of methanol, and vortex-mixed for 2 minutes, followed by centrifugation at 16000 g for 10 min. Finally, 50 μ L of the solution was injected into the HPLC system for analysis.

Liver tissue samples were rinsed with physiological saline to remove the blood, and blotted on filter paper. Approximately 0.2 g of liver was weighed and homogenized in 2X volume of saline, with a tissue homogenizer. The liver homogenate was spiked with 10 μ L of chrysin (Internal Standard (IS), 500 μ g/mL) as well as 10 μ L of methanol and subjected to liquid/liquid extraction with ethyl acetate by using the same method described above for plasma samples.

The chromatographic separation of analytes was carried out on a 5 μ m Cliepus C18 column (250 mm $\times$ 4.6 mm, Higgins Analytical, Mountain View, CA), protected by a C18 guard column (5 μ m, 4.0 mm $\times$ 2.0 mm), at room temperature. The mobile phase was composed of water (A) and acetonitrile (B). The 23-min HPLC gradient elution program was as follows: 20% B-90% B at 0–15 min and 90% B in another 8 minutes. The flow rate was 1 mL/min and the detection wavelength was 330 nm.

Statistical analysis

Statistical analyses were carried out with GraphPad Prism 6 software (La Jolla, CA), and $p < 0.05$ was considered statistically significant. Differences among groups were analyzed by one-way ANOVA. For comparison involving only two groups, the Student t test was used.

Results

AGN extract inhibited LNCaP/AR-Luc xenograft growth and lung metastasis

Given that our earlier studies demonstrated that daily gavage dosing or *i.p.* injection with AGN extract suppressed xenograft growth of AR (–) PCa cells such as DU145 and PC-3 (Lee *et al.*, 2009), we tested whether AR(+) LNCaP/AR-Luc xenograft responded to AGN in a dose-dependent manner with gavage dosages of 5 and 10 mg per mouse. Neither of AGN dosages affected the body weight of the SCID-NSG mice throughout the duration of treatment (Fig. 2A), consistent with its well tolerated nature. As common with many xenograft models, we observed significant variance of the tumor growth rate in the SCID-NSG mice, especially when only 20,000 cancer cells were inoculated per site. By 9 weeks of cancer cell inoculation, approximately 50% of the mice bore a measureable tumor. Since mice without tumors were not informative, we analyzed tumor data from the top 10 mice (out of 20 mice per group) for each group. Fig. 2B presented mean tumor volume measurements as a function of time after inoculation. Fig. 2C showed the mean tumor weight after necropsy dissection. The results showed growth inhibition of tumor xenograft in SCID-NSG mice in a dose-dependent manner. Fig. 2D illustrated the distribution of individual tumors of each group by weight ranking.

Through *ex vivo* bioluminescence imaging as shown in Fig 2E, metastasis in the lung was detected in mice bearing large primary *s.c.* xenograft. After 10 weeks of AGN treatments, lung metastasis rates of *s.c.* xenograft tumors were decreased from 60% in control mice (6 out of 10) to 40% (AGN5, 4 out of 10) and 20% (AGN10, 2 out of 10).

Decursinol exerted greater inhibitory effect on LNCaP/AR-Luc xenograft growth than equi-molar dosing of D/DA

In our recent publication in the TRAMP model (Tang *et al.*, 2015), we have shown that D/DA, when provided by daily gavage at equi-molar dosage to that provided by AGN, accounted for the suppression efficacy against TRAMP epithelial precancerous growth, but was less efficacious to inhibit the growth of the very aggressive and fast growing AR(–) neuroendocrine carcinomas. We thereby compared daily gavage administration of decursinol with equi-molar D/DA to suppress LNCaP/AR-Luc xenograft growth. Whereas D/DA treatment for 12 weeks did not affect body weight of tumor-bearing SCID-NSG mice, those receiving decursinol showed a progressive body weight disadvantage, noticeable at 7–8 weeks of initiating treatment (Fig. 3A). Because we did not adjust dosage according to body weight in this experiment, the body weight loss was more pronounced in mice that weighed less from the start. Gavage treatment with decursinol decreased LNCaP/AR-Luc xenograft tumor growth by 75% and D/DA treatment effect was marginal (23%) for the top 10 tumors in each group (Fig. 3B). Fig. 3C documented *ex vivo* bioluminescence imaging detectable (as shown in Fig 2D) lung metastasis rate from 100% in control group (10 out of 10) to 60% in the decursinol group (6 out of 10), whereas D/DA was less as efficacious (9 out of 10).

At study termination, the weight of major organs of drug metabolism such as the liver and kidney was not statistically different from vehicle- or D/DA-treated mice (Fig. 3D). Nor were the male reproductive organs such as testes and prostate of decursinol-treated mice (Fig. 3D).

Histopathological findings of the xenograft tumors

A board-certified veterinarian pathologist (TKC) examined H&E stained slides without knowledge of treatment groups, noting inflammation, necrosis (Fig. 4A, C), acute hemorrhage (Fig. 4B, D), tumor surface superficial angiogenesis and tumor emboli in vessels (precursor to metastasis) (Fig. 4B). Irrespective of treatment groups, inflammatory cells were not observed, consistent with the severe immune deficiency nature of the SCID-NSG mice. Necrosis was observed in all tumors ranging from 10% to over 40% of each sampled tumor section. Necrosis extent in decursinol or AGN treatment groups did not noticeably differ from the vehicle groups within each experiment.

Acute hemorrhage was a common histopathological feature noted in all tumors from the vehicle group, whereas there was a trend for less acute hemorrhage in tumors from AGN-treated mice, but not in tumors from decursinol-treated mice in spite of their greatly reduced tumor size (Fig. 4B, D). The hemorrhagic feature was consistent with the gross appearance of the freshly harvested vehicle-treated control tumors being in various shades of dark (blood filled) color. Presence of intravascular tumor emboli was noted in about 70% of the control tumors and less frequently (20%, 2 out of 10) in AGN-treated tumors, consistent with the decreased lung metastasis incidence through *ex vivo* bioluminescence detection (Fig. 2E). Overall, in spite of trends of changes in the AGN- or decursinol-treated tumors, the vast extent of intra-tumoral and inter-tumor histomorphological variabilities prohibited objective quantitative assessment of these metrics. Immunohistochemical staining of AR and Ki67 proliferative cell antigen yielded expected nuclear-specific signals for each protein in

non-necrotic tumor areas within tumor sections, however, quantitative assessment was not amenable to discern treatment-related effect (Data not shown).

Gavage treatment with decursinol led to higher plasma level than dosing with equi-molar D/DA

Since D and DA are rapidly and extensively converted to decursinol in rodents (Li *et al.*, 2012; Li *et al.*, 2013b; Park *et al.*, 2012) and humans alike (Lu *et al.*, 2015; Zhang *et al.*, 2015a) (see Fig 1B), we measured decursinol in plasma (see examples of HPLC detection in Fig. 5 B–D) from D/DA- vs. decursinol-treated SCID-NSG mice inoculated with LNCaP/AR-Luc cells, at 3 hours after the last dose. Repeated daily dosing with decursinol led to 1.7 folds higher decursinol level in plasma than equi-molar D/DA-treated mice (Fig. 5E), with mean decursinol concentration of 4.1 and 2.4 µg/mL, respectively. Notably, the decursinol dosing preparation made fresh each day in EPTG (3:6:1:20) formula, so as to be consistent with D/DA dosing used in our earlier efficacy studies (Li *et al.*, 2012; Li *et al.*, 2013b; Tang *et al.*, 2015), did not stay in a uniformly dispersed state for more than 20 minutes of dilution from the aliquoted stock concentrate (Li *et al.*, 2013b). The difference in systemic decursinol level from the two dosing sources might have been underestimated due to the suboptimal solubility of decursinol in this working excipient formula.

In addition, we noted the presence of a UV absorbing peak (peak X) that eluted faster than decursinol from the reverse phase HPLC column (Fig. 5, C and D), suggesting a more polar metabolite(s) than decursinol in the plasma of decursinol- as well as in D/DA-treated mice, perhaps as a consequence of phase II hydrophilic modification of decursinol. Comparison with synthetic decursinol-3'-sulfate ruled out this particular sulfate form as the identity for peak X (to be reported elsewhere in future).

Safety assessment of decursinol in improved excipient formulation in CD-1 mice

The suboptimal solubility of decursinol in the original working excipient formulation motivated us to improve the EPTG formula by step-wisely decreasing the amount of 5% glucose to the new EPTG (3:6:1:5) in which decursinol remained as clear solution for more than 1 hour. We assessed the safety of daily gavage dosing with a dose-titration design: 2, 3 and 4 mg per 25 g mouse with weekly adjustment according to body weights (80, 120, and 160 mg decursinol/kg body weight). Daily dosage as high as 4 mg/25 g or 160 mg per kg did not cause body weight change (Fig. 6A). At the end of 4 weeks of decursinol treatment, the plasma decursinol level (3 h post last dose) was found to increase in a dose-dependent manner (Fig. 6B) and higher than previously achieved with the original working excipient formula of an even higher dosage of decursinol (4.5 mg per mouse) (Fig. 3E).

The subchronic dosing treatment for 4 weeks did not affect the weights of liver, kidney, prostate, spleen, lung, heart, testes and brain (Table 1). Moreover, plasma biochemistry assays showed that ALP (alkaline phosphatase), AST (aspartate aminotransferase), ALT (alanine aminotransferase), Amy (amylase), ALB (albumin), BUN (blood urea nitrogen), Gluc (glucose), Chol (cholesterol), and T. Bili (total bilirubin) were not statistically different among the 4 groups (Table 2), consistent with functional integrity of liver, pancreas and kidney.

Single dose PK revealed a greater bioavailability advantage for decursinol over D/DA

With the improved working excipient formulation, we completed a detailed PK study with a well-tolerated dose of decursinol at 3 mg/25 g or 120 mg/kg body weight in comparison with equi-molar dose of D/DA (4 mg/25 g or 160 mg/kg body weight). The AUC_{0–24h} of plasma decursinol was 193.4 mg/L*h for mice dosed with decursinol, which was approximately 3.7 folds higher than that of plasma decursinol from D/DA dosing, 52.8 mg/L*h (Fig. 7A). The liver decursinol mirrored the plasma patterns from both dosing sources (Fig. 7B). The data established an unequivocal PK advantage of delivering decursinol directly vs. through D/DA as the prodrugs.

Discussion

To our knowledge, this is the first study in which LNCaP/AR-Luc xenograft mouse model was used to evaluate the anti-cancer efficacy of AGN (Fig. 2), D/DA vs. decursinol (Fig. 3). Co-transfection of constitutively expressing firefly luciferase in these cells enabled detection of metastatic lesions with bioluminescence as illustrated in Fig. 2E. In pilot studies, we found that by inoculating the same number of cells into SCID-NSG mice, LNCaP/AR-Luc cells formed more and bigger tumors than LNCaP cells during the same time frame (unpublished data). Lung metastases were detected through *ex vivo* imaging in the LNCaP/AR-Luc-bearing mice in positive association with tumor size grown at the s.c. inoculation site. Notably, daily gavage of AGN extract resulted in dose-dependent suppression of growth of tumors at the s.c. inoculation site (Fig. 2C) and lung metastatic growth (Fig. 2E). This model therefore should facilitate future studies of the anti-metastasis mechanism of AGN and pyranocoumarins, so far not amenable with other s.c. PCA xenograft models.

Since our initial discovery of D and DA as novel AR suppressing compounds in cell culture model (Guo *et al.*, 2007; Jiang *et al.*, 2006), we and others have provided conclusive evidence for their rapid and extensive conversion to decursinol in rodent models (Lee *et al.*, 2009; Li *et al.*, 2012; Li *et al.*, 2013b; Park *et al.*, 2012) and humans (Lu *et al.*, 2015; Zhang *et al.*, 2015a) (Fig. 1B). Decursinol exhibited much inferior direct cancer cell cytotoxic and anti-AR actions in cell culture models as compared to D/DA (Guo *et al.*, 2007; Jiang *et al.*, 2006; Yim *et al.*, 2005). Nevertheless, we confirmed that equi-molar D/DA exerted similar inhibition of TRAMP prostate precancerous epithelial growth as the AGN extract (Tang *et al.*, 2015). These findings led to our hypothesis for the present study that decursinol might be an *in vivo* “active” chemical for suppressing the AR (+) PCa. Our efficacy data (Fig. 3) are consistent with this overall hypothesis to suggest decursinol might be a promising standalone anticancer agent, and D and DA work as natural pro-drugs. Pertinent to the potential decursinol metabolite(s), we observed a more polar peak (Peak X) on reverse phase HPLC spectrum (Fig. 5C, D). We did not detect the speculated decursinol-3'-glucuronate in mouse plasma (insensitive to glucuronidase digestion; no signal from LC/MS/MS daughter ion search, data not shown), consistent with a report that decursinol was resistant to Phase II glucuronidation at least in *in vitro* rat liver microsomal systems (Song *et al.*, 2011). Through chemical synthesis, we ruled out decursinol-3'-sulfate as Peak X (to be reported elsewhere

in future). Further effort is underway to positively identify and verify the compound or compounds in Peak X.

Our detailed PK comparison of D/DA and decursinol delivered in an improved working excipient formula in mice highlights the necessity to use PK-adjusted bio-equivalent dosing to carry out a fair comparison of parent-product pairs. The murine PK data of 3.7-fold AUC advantage for dosing decursinol directly over equi-molar intake of D/DA (Fig. 7A, B) agreed well with our previously reported PK comparison in the rat model (Li *et al.*, 2013b). Multi-steps are involved in D/DA absorption in the gastrointestinal tract, portal transport and hepatic conversion to decursinol (Zhang *et al.*, 2016) vs. the direct absorption of decursinol itself to raise its systemic level (Fig. 1B). The observed PK/AUC advantage for dosing decursinol over equi-molar intake of D/DA (Fig. 7) most likely accounted for the greater anti-cancer efficacy of decursinol over the parental compounds (Fig. 3B). Future work will focus on comparing D/DA and decursinol at PK-bioequivalent doses to determine how much these pyranocoumarins contribute to the tumor- and metastasis-suppressing efficacy of AGN.

In summary, our data support decursinol to exert *in vivo* inhibitory activity against LNCaP/AR-Luc xenograft growth and lung metastasis. Given its lackluster cell arrest and apoptotic actions on this and other cancer cell types in direct exposure cell culture models (Guo *et al.*, 2007; Yim *et al.*, 2005), decursinol further metabolite(s) as well as cancer-extrinsic mechanisms such as the tumor microenvironment should be factored in for future mechanistic investigations.

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Fig.1. Chemical structures (Panel A) of decursin (D), decursinol angelate (DA) and decursinol (DOH), and likely metabolic pathway (Panel B) of D and DA based on rodent data and human studies *in vitro* and *in vivo*. CYP, cytochrome p450. CES-2, carboxyesterase-2.

Fig. 2.

Effects of daily gavage treatment with AGN extract on LNCaP/AR-luc xenograft growth and lung metastasis. Male SCID-NSG mice were treated with 300 μ L of vehicle (ethanol: PEG400: Tween80: 5% dextrose 3:6:1:20) or AGN (5 or 10 mg/mouse/day, 6 times per week, excluding Sundays) once daily. Treatment was started 7 days before cancer cell s.c. inoculation (100 μ L of 2×10^4 cells with a 1:1 mixture of Matrigel and serum-free medium). (A) Body weight, (B) Growth kinetics of top 10 tumors from each group. (C) Weight of top 10 tumors per group after necropsy. Mean $\pm$ SD, n=10 mice in each group. *, p < 0.05 vs. the control. (D) Individual tumor weight at necropsy in ranking order. (E) Illustrative example of *ex vivo* imaging detection of lung metastasis and the incidence of detectable lung metastasis in each group.

Fig. 3. Effect of gavage administration of D/DA vs. equi-molar decursinol on LNCaP/AR-luc xenograft growth. Male SCID-NSG mice were treated with 300 μ L of vehicle (ethanol: PEG400: Tween80: 5% dextrose 3:6:1:20) or D/DA (6 mg/mouse/day) or decursinol (4.5 mg/mouse/day, equi-molar dose of D/DA) once daily, 7 days per week. Treatment was started 7 days before cancer cell s.c. inoculations (100 μ L of 2×10^4 cells in a 1:1 mixture of Matrigel and serum-free medium). **(A)** Body weight, Mean $\pm$ SD, n=20 mice in each group; **(B)** Weight of top 10 tumors in each group after necropsy, n=10; **(C)** Lung metastasis detection by *ex vivo* bioluminescence imaging. **(D)** Relative weight of key metabolism and detoxification organs (liver, kidney) and male related organs (prostate and testes) normalized to 25 g BW. Mean $\pm$ SD, n=20 mice in each group. *, $p < 0.05$ vs. the control.

Fig. 4. Photomicrographs of H&E-stained LNCaP/AR-luc xenograft tumor sections featuring necrosis, hemorrhage and intravascular cancer cell embolus. (**A, B**) Sections of tumors from vehicle-treated mice. (**C, D**) Sections of tumors from decursinol-treated mice. N denotes necrotic area. H denotes hemorrhage area. Arrow points to an intravascular cancer cell embolus in (**B**).

Fig. 5. HPLC analyses of plasma samples obtained at 3 h after the last gavage dose from male SCID-NSG mice. Typical chromatograms of blank mouse plasma (**A**), blank mouse plasma spiked with decursinol (DOH) and IS (**B**), mouse plasma sample after oral administration of D/DA (**C**), and mouse plasma sample after oral administration of decursinol (**D**) with a HPLC gradient elution (0–15min: 20–90% acetonitrile, 15–23min: 90% acetonitrile), and diode array detection at 330 nm wavelength. DA marks expected elution time of DA peak. Peak X marks a more polar potential metabolite(s) of decursinol. (**E**) Plasma decursinol concentration of mice at 3 h after the last dose of respective treatment.

Fig. 6. Safety assessment of sub-chronic (4 weeks) decursinol daily gavage in improved vehicle (ethanol: PEG400: Tween80: 5% dextrose 3:6:1:5) in male CD-1 mice beginning at 9 weeks of age. DOH2, DOH3, DOH4 indicate body weight-adjusted dosages of 2, 3 or 4 mg per 25 g body weight. **(A)** Body weight assessed in weekly intervals, Mean $\pm$ SEM, N=6. **(B)** Plasma decursinol concentration at 3 h after the last dose as a function of decursinol dosage. Mean $\pm$ SEM, N=6.

Fig. 7. Pharmacokinetic characterization of (A) plasma decursinol (DOH) concentration- and (B) liver decursinol content-time profiles in male CD-1 mice after a single oral dose of decursinol (120 mg/kg) or D/DA mixture (160 mg/kg, equi-molar dose) delivered in improved vehicle of ethanol: PEG400: Tween80: 5% dextrose (3:6:1:5). Mean $\pm$ SEM, N=5–7 mice at each time point per drug.

Table 1.

Effect of 4-week subchronic gavage intake of decursinol on body weight and select organs of male CD-1 mice.
N = 6 mice per group

Group	Body Weight (g)	Organ weight (Mean $\pm$ SD)							
		Liver (g)	Kidney (g)	Prostate (g)	Spleen (g)	Lung (g)	Heart (g)	Testes (g)	Brain (g)
Control	36.83 $\pm$ 2.01	1.86 $\pm$ 0.22	0.60 $\pm$ 0.07	0.12 $\pm$ 0.02	0.09 $\pm$ 0.02	0.22 $\pm$ 0.02	0.18 $\pm$ 0.01	0.25 $\pm$ 0.04	0.54 $\pm$ 0.02
DOH-2	36.52 $\pm$ 1.71	1.62 $\pm$ 0.14	0.67 $\pm$ 0.10	0.12 $\pm$ 0.01	0.09 $\pm$ 0.01	0.23 $\pm$ 0.02	0.18 $\pm$ 0.01	0.27 $\pm$ 0.03	0.53 $\pm$ 0.03
DOH-3	35.97 $\pm$ 2.10	1.58 $\pm$ 0.14	0.59 $\pm$ 0.08	0.10 $\pm$ 0.02	0.09 $\pm$ 0.02	0.20 $\pm$ 0.02	0.19 $\pm$ 0.01	0.25 $\pm$ 0.03	0.53 $\pm$ 0.03
DOH-4	35.38 $\pm$ 1.71	1.54 $\pm$ 0.17	0.60 $\pm$ 0.08	0.12 $\pm$ 0.02	0.09 $\pm$ 0.02	0.22 $\pm$ 0.01	0.20 $\pm$ 0.01	0.24 $\pm$ 0.05	0.53 $\pm$ 0.02
ANOVA significance	N	N	N	N	N	N	N	N	N

Table 2.

Effect of 4-week subchronic gavage intake of decursinol on plasma biochemical safety indicators of liver and kidney functions. N = 6 mice per group

Group	Plasma biochemistry (Mean $\pm$ SD)								
	ALP (U/L)	AST (U/L)	ALT (U/L)	Amy (U/L)	ALB (g/dL)	BUN (mg/dL)	Gluc (mg/dL)	Chol (mg/dL)	T.Bili (mg/dL)
Control	49.8 $\pm$ 9.2	58.7 $\pm$ 4.5	22.7 $\pm$ 8.3	938.3 $\pm$ 138.3	2.9 $\pm$ 0.1	13.3 $\pm$ 2.6	299.2 $\pm$ 36.4	184.7 $\pm$ 31.7	0.18 $\pm$ 0.04
DOH-2	44.2 $\pm$ 6.1	79.5 $\pm$ 19.7	33.3 $\pm$ 21.2	772.7 $\pm$ 38.3	2.7 $\pm$ 0.2	9.7 $\pm$ 1.8	336.3 $\pm$ 37.2	167.3 $\pm$ 28.3	0.23 $\pm$ 0.05
DOH-3	47.2 $\pm$ 14.6	82.5 $\pm$ 29.6	34.3 $\pm$ 21.7	780.2 $\pm$ 70.1	2.7 $\pm$ 0.2	12.3 $\pm$ 2.5	367.3 $\pm$ 69.0	182.0 $\pm$ 38.8	0.27 $\pm$ 0.05
DOH-4	45.5 $\pm$ 9.1	72.3 $\pm$ 29.9	21.0 $\pm$ 7.6	668.0 $\pm$ 117.1	2.3 $\pm$ 0.3	14.0 $\pm$ 2.2	408.3 $\pm$ 130.0	161.0 $\pm$ 20.5	0.32 $\pm$ 0.10
ANOVA significance	N	N	N	N	N	N	N	N	N