Chemopreventive Effects of Korean Angelica versus Its Major Pyranocoumarins on Two Lineages of Transgenic Adenocarcinoma of Mouse Prostate Carcinogenesis

Su-Ni Tang, Jinhui Zhang, Wei Wu, Peixin Jiang, Manohar Puppala, Yong Zhang, Chengguo Xing, Sung-Hoon Kim, Cheng Jiang, and Junxuan Lu

Abstract

We showed previously that daily gavage of Angelica gigas Nakai (AGN) root ethanolic extract starting 8 weeks of age inhibited growth of prostate epithelium and neuroendocrine carcinomas (NE-Ca) in the transgenic adenocarcinoma of mouse prostate (TRAMP) model. Because decursin (D) and its isomer decursinol angelate (DA) are major pyranocoumarins in AGN extract, we tested the hypothesis that D/DA represented active/prodrug compounds against TRAMP carcinogenesis. Three groups of male C57BL/6 TRAMP mice were gavage treated daily with excipient vehicle, AGN (5 mg per mouse), or equimolar D/DA (3 mg per mouse) from 8 weeks to 16 or 28 weeks of age. Measurement of plasma and NE-Ca (5 mg per mouse) from 8 weeks to 16 or 28 weeks of age. Measurement of plasma and NE-Ca burden. Immunohistochemical and mRNA analyses of DLP showed that AGN and D/DA exerted similar inhibition of TRAMP epithelial lesion progression and key cell-cycle genes. Profiling of NE-Ca mRNA showed a greater scope of modulating angiogenesis, epithelial–mesenchymal transition, invasion–metastasis, and inflammation genes by AGN than D/DA. The data therefore support D/DA as probable active/prodrug compounds against TRAMP epithelial lesions, and they cooperate with non-pyranocoumarin compounds to fully express AGN efficacy against NE-Ca. Cancer Prevention Res; 8(9); 835–44. ©2015 AACR.

Introduction

Prostate cancer is a leading cause of cancer death in the United States and Western countries. Because of the long latency period of prostate cancer formation, chemoprevention using naturally occurring or synthetic chemicals is considered as a plausible approach to block, delay, or even reverse carcinogenesis and progression of prostate cancer. Herbal natural products represent a source of chemopreventive agents with desirable safety attributes and distinct targeting actions for prostate cancer chemoprevention (1). Korean Dang-gui (Angelica gigas Nakai, AGN) is a traditional medicinal herb used in Korea and other Asian countries (2). Its dried root extract is marketed as a dietary supplement for pain relief and memory enhancement in the United States and globally. AGN-containing multiple herbal supplements have been tested and found efficacious for relieving postmenopausal symptoms in U.S. women (3).

Decursin (D) and its structural isomer on the side chain decursinol angelate (DA) are the major pyranocoumarin compounds of the alcoholic extract of the root of AGN (refs. 1, 2, 4; Fig. 1A). Decursin, DA, and their pyranocoumarin core decursinol (DOH) have been reported to exert neuroprotective and pain-killing activities (2). We have earlier demonstrated that in the androgen-dependent LNCaP prostate cancer cell model, ethanol extract of AGN suppressed prostate-specific antigen (PSA) expression at both mRNA and protein levels, inhibited androgen-induced cell proliferation, and blocked the ability of androgen to suppress neuroendocrine differentiation at exposure concentrations that caused G1 arrest but far below those that induced apoptosis (5). In cell culture models, structure–activity comparison by us and others identified D and DA to have identical anti-androgen signaling and antiproliferative activities (5, 6), whereas DOH was much less inhibitory on androgen receptor (AR) signaling, cellular growth, and survival parameters in the same concentration range (5–7). We and others have shown in rodents that D and DA are rapidly and extensively converted to DOH (refs. 8–11; Fig. 1A), and we have confirmed such extensive conversion in healthy human subjects (12).
We reported *in vivo* inhibitory effect of AGN ethanol extract on the growth of androgen-independent DU145 and PC3 prostate cancer xenografts (11) and extended efficacy evaluation into the transgenic adenocarcinoma of mouse prostate (TRAMP) primary carcinogenesis model (13). In TRAMP mice, at least 2 distinct lineages of carcinogenesis exist (14–17), that is, the AR/probasin promoter–driven SV40 antigen–mediated prostate epithelial atypical hyperplasia formation especially in the dorsolateral prostate (DLP) and the SV40 antigen–driven AR-independent neuroendocrine carcinomas (NE-Ca) predominantly originating in the ventral prostate. In the C57BL/6 genetic background, lifetime NE-Ca incidence rate was estimated to be 1 of 5 to 1 of 3 (14, 16, 17). Our data have shown that daily gavage administration of AGN root ethanol extract starting from 8 weeks of age to 24 weeks inhibited the growth of TRAMP DLP and NE-Ca in the TRAMP C57BL/6 mice (13). However, whether D and DA represent active/prodrug compounds against prostate primary carcinogenesis in TRAMP or any other organ sites has not been tested.

In this study, we compared the efficacy of gavage administration of AGN extract and equimolar D/DA to inhibit the 2 lineages of TRAMP carcinogenesis. We investigated associated cellular and molecular changes to provide mechanistic insights into their potential differential targets.

**Table 1.**

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<th>Gavage cohort</th>
<th>Age, weeks</th>
<th>Analyzed tissue</th>
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<th>DOH ng/mL or ng/g</th>
<th>D ng/g</th>
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<tr>
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<td>AGN 28</td>
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<tr>
<td>D/DA 28</td>
<td>NE-Ca</td>
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<td></td>
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Mean ± SD. *Below detection limit of HPLC-UV.*
Materials and Methods

Chemicals and reagents
Alcoholic extract of dried AGN root was prepared by Kim’s group with modification at the Oriental Medical Hospital of Kyunghee University (Seoul, Korea). Instead of manual extraction (5, 13), bulk extraction of powdered AGN root was carried out in a hospital herbal pharmacy extractor with 95% hot alcohol. Chemical fingerprinting of AGN by HPLC-UV showed that content of D plus DA in AGN extract was 60%. Because D and DA possess identical anti-AR, antiproliferative, and apoptotic cellular activities (5, 6, 18) and their majority is converted to DOH (8–11), they were isolated together as a mixture by Xing’s group at University of Minnesota (Minneapolis, MN). The purity of D/DA was more than 99% based on monitoring with NMR and HPLC.

Mayer hematoxylin was obtained from Sigma-Aldrich and eosin Y was purchased from Thermo Fisher Scientific. SV40-T antigen (T-Ag), AR, and synaptophysin antibodies were purchased from BD Pharmingen, EMD Millipore, and BD Transduction Laboratories, respectively. PCR primers were purchased from Integrated DNA Technologies, Inc.

Animal experiment
The animal study was approved by the IACUC of Texas Tech University Health Sciences Center and carried out on the Amarillo campus. TRAMP mice were bred in-house and identified per genotyping protocol as reported before (16, 17). Male C57BL/6 TRAMP mice and their male wild-type (WT) littermates were used. Cohorts of male TRAMP mice (35–36 mice per group) were fed AIN93M-purified diet and gavage-treated with 0.15-mL excipient vehicle (ethanol:PEG-400:Tween-80:50% glucose = 3:6:1:20). AGN (5 mg per mouse), or D/DA (3 mg per mouse, equimolar to that in AGN) 5/d/wk from 8 weeks of age. Fifteen WT littermates were enrolled and treated with vehicle only to serve as the baseline reference for TRAMP prostate lobe expansion. Animals were weighed weekly. Starting 16 weeks, TRAMP mice were palpated for abdominal mass indicative of prostate/gonitotary (GU) tumors. Mice were euthanized at either 16 weeks (n = 11–13 TRAMP mice per group and n = 6 for WT littermates) or 28 weeks unless large tumors necessitated earlier sacrifice.

Blood was taken by cardiac puncture after anesthesia sedation at 3 hours after the last dose. At necropsy, the GU tract was removed en bloc and weighed. Tumors were dissected and weighed. The pelvic lymph nodes were inspected for enlargement (metastasis) and, if visible, were dissected, weighed, and fixed in 10% neutral-buffered formalin for hematoxylin and eosin (H&E) and immunohistochemical (IHC) confirmation of metastasis. The prostate from mice without visible tumors were carefully dissected into anterior prostate, DLP, and ventral prostate and weighed. Left lobes from each mouse were fixed in 10% neutral-buffered formalin for H&E and IHC pathology analyses. Right lobes were snap-frozen on dry ice and stored at −80°C for biochemical and molecular biomarkers analyses. Liver and kidney were inspected for health problems, dissected, weighed, and preserved for later analyses.

Analysis of D, DA, and DOH in mouse plasma and NE-Ca
The D, DA, and DOH levels in plasma were analyzed on an Agilent Infinity 1260 HPLC system by our validated procedure as described before (8). D, DA, and DOH levels in tumor tissues were determined by a Shimadzu Nexera UHPLC system coupled to an AB Scieq 5500 QTRAP MS/MS detector per our validated procedure (9, 19). To extract D, DA, and DOH from tumor tissue, a piece of frozen tumor was homogenized in 4 volumes of water by using a tissue homogenizer. The tumor homogenate (200 μL, equivalent to 50 mg tumor) was then spiked with prednisolone (5 ng, IS) and extracted with ethyl acetate as for plasma samples.

Histology and IHC analyses
Formalin-fixed prostate lobes were processed and embedded in paraffin and routinely stained by H&E as we previously reported (16, 17). For IHC, 5-μm sections were mounted onto adhesive microscope slides. Tissue sections were deparaffinized in xylenes and rehydrated in a series of decreasing concentrations of ethanol. Tissue sections were then heated in 10 mmol/L citrate buffer solution for antigen retrieval. After sections were rinsed in distilled water, 3% hydrogen peroxide was used for quenching. After incubating with normal serum for 30 minutes, sections were applied with primary and secondary antibodies according to the manufacturer’s instructions. The slides were developed in diamino-nobenzidine and counterstained with a weak solution of hematoxylin stain. The stained slides were dehydrated and mounted in Permount. Synaptophysin (NE-Ca), AR (epithelial) expression in all tumors were detected to confirm NE-Ca diagnoses as reported before (16, 17). See Supplementary Fig. S1 for illustrative examples of H&E and IHC characterization.

Scoring of prostate lesions
Mouse prostatic tumors or lobes from TRAMP mice were characterized by H&E and T-Ag staining. Prostatic lesions were scored according to our composite scoring scheme adapted and modified on the basis of that of Suttie and colleagues (ref. 20; see Supplementary Fig. S2 for description). In brief, severity of epithelial lesions was divided into 5 grades: mild lesions as grade 1, moderate lesions as grade 2, severe lesions as grades 3 and 4, and adenoma as grade 5. The lesion grade was modified by their distribution patterns as focal, multifocal, or diffuse. Therefore, scores for grade 1 lesions ranged from 1 to 3 and likewise for grade 5 ranged from 13 to 15. For each lobe, the most severe lesion and its distribution pattern were used to derive the lesion score.

DLP and NE-Ca tissue sampling, RNA extraction, and real-time RT-PCR for expression of selected genes
Because of limited amount of DLP tissue available from each mouse, we pooled them by group into a sample for RNA extraction and qRT-PCR analyses of genes known to be involved in TRAMP epithelial cell-cycle regulation. For NE-Ca, we analyzed 4 IHC-confirmed NE-Ca per group of TRAMP mice treated with vehicle, AGN versus D/DA, respectively. Total RNA was prepared from 30 mg of NE-Ca tissues using the AllPrep DNA/RNA/Protein Mini kit (Qiagen Inc.; refs. 21, 22). RNA (1 μg) was used for cDNA synthesis by using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.) according to the manufacturer’s instructions. qRT-PCR was performed by using the Fast Start Universal SYBR Master with ROX (Hoffmann-La Roche Ltd.) on the CFX96 Touch Real-Time PCR Detection Systems (Bio-Rad Laboratories, Inc.). All reactions were performed in duplicate, and the relative expression of target mRNA in each sample was normalized with that of mean β-actin. The sequences of these primers were as listed in Supplementary Table S1.
Results

Gavage treatment with AGN and equimolar D/DA led to similar DOH levels in mouse plasma or tumor

It is now well established that D and DA are extensively converted to DOH in rodents (refs. 8–11, 19; Fig. 1A). We therefore tested whether daily gavage administration of AGN extract and equimolar D/DA to TRAMP mice led to similar uptake and retention by measuring D, DA, and DOH levels in 8 plasma samples at 16 and 28 weeks of age and 4 tumors collected from each treatment group at 28 weeks. Figure 1B shows representative chromatograms of separating DOH from D and DA of a NE-Ca sample by LC-MS/MS detection. Data in Fig. 1C showed no statistical difference between the plasma DOH concentration of AGN- and D/DA-treated mice at 16 weeks (P = 0.199) and at 28 weeks (P = 0.858), whereas the D and DA levels were below the level of detection of the UV diode detector. The trend for higher plasma DOH at 28 weeks (2,921, 3,076 ng/mL) versus 16 weeks (1,592, 1,927 ng/mL) likely reflected increasing trough level after repeated dosing. Similarly, the NE-Ca DOH content was not statistically different between AGN- and D/DA-treated groups (2,078 vs. 2,573 ng/g, P > 0.05; Fig. 1C). As expected, the D and DA content in the NE-Ca was much lower than DOH (Fig. 1B, note the different mass intensity scales for DOH vs. D and DA); and they were not statistically different between the AGN- and D/DA-treated groups (Fig. 1C). Therefore, our data indicated that other phytochemicals in AGN did not affect the metabolic fate of D and DA and the consumption of AGN extract and equimolar purified D/DA resulted in similar, if not identical, bioavailability of D/DA and their conversion to DOH.

AGN and D/DA treatment did not affect body weight or organ weight of TRAMP mice

At the daily gavage dose of 5 mg AGN and 3 mg D/DA, the body weight of the TRAMP mice was the same as the vehicle-treated TRAMP mice in the 16-week cohort (Fig. 2A). For the 28-week cohort, the body weight of the 3 groups of TRAMP mice remained same for up to 18 weeks and then displayed some variation from large NE-Ca in some TRAMP mice (Fig. 2B). The vehicle-treated WT mice weighed 2 to 3 grams more than the TRAMP mice in the 16-week cohort due, in part, to weight advantage at assignment at 16 weeks (Fig. 2A). In the 28-week cohorts (Fig. 2B), the WT mice had some growth advantage over the TRAMP mice, especially at 24 weeks or older when the large NE-Ca negatively affected health of some TRAMP mice. Normalized to body weight, major organs involved in D/DA metabolism to DOH (liver; refs. 10, 19) and DOH clearance (kidney) were not different among the AGN, D/DA, and vehicle groups (Fig. 2C and D). Overall, the data were consistent with our earlier studies in xenograft models (11) and TRAMP model (13) of the well-tolerated nature of AGN and D/DA.

Gavage with AGN and D/DA exerted the same extent of inhibition of TRAMP dorsolateral prostate epithelial lesions

Because DLP is the most expanded lobe in TRAMP mouse prostate (23) and the anatomic site where atypical hyperplastic epithelial lesions mostly arise (14, 17, 23), we compared DLP weight of the TRAMP mice treated with vehicle, AGN, or D/DA with that of the WT mice. As shown in Fig. 3A and B, the TRAMP DLP expansion was 2- and 3-fold, respectively, over the WT baseline by 16 and 28 weeks of age. The AGN- and D/DA-treated TRAMP mice showed 66% and 61% respective normalization of the DLP weight at 16 weeks (Fig. 3A). At 28 weeks, the inhibition of TRAMP DLP growth by AGN and D/DA was 67% and 72%, respectively (Fig. 3B). The sum weight of the anterior prostate, DLP, and ventral prostate lobes (total prostate) showed some overall suppression by AGN and D/DA at 16 and 28 weeks (Fig. 3C and D), albeit less as dramatic as in DLP, in part, due to the anterior prostate growth was not significantly decreased by AGN or D/DA (Supplementary Table S2). The ventral prostate, smallest of the 3 lobes, expanded to a much less extent than the DLP, and AGN and D/DA suppressed the TRAMP ventral prostate growth to similar percentage as in DLP (Supplementary Table S2).

We next examined the effects of AGN and D/DA on the histopathology of the epithelial lesions in TRAMP DLP. The DLP lobes (from WT and TRAMP) were stained with T-Ag antibody to locate the areas of epithelial lesions (Fig. 4A and see Supplementary Fig. S1A for H&E and IHC verification). WT mouse prostates, as usual, showed negative signal of T-Ag in epithelial cells. The epithelial T-Ag and AR IHC staining (data not shown) was not different among the TRAMP groups. We scored the most severe lesions using a composite scoring scheme (Supplementary Fig. S2) taking into consideration the lesion grade (mild hyperplasia to adenoma) and their distribution patterns (focal, multifocal, or diffuse). The score range was: 1 (focal mild hyperplasia) to 15 (diffuse adenoma). From 16 to 28 weeks, the mean lesion score of DLP increased from 7.9 to 11.1 in TRAMP vehicle mice (Fig. 4B). AGN extract significantly decreased the mean lesion score of DLP from 7.9 to 6.2 (P < 0.05) by 16 weeks and from 11.1 to 7.9 (P < 0.001) by 28 weeks (Fig. 4B). Similarly, D/DA also reduced the mean lesion score of DLP to 7.2 (P < 0.001) by 28 weeks (Fig. 4B). The progression of DLP epithelial lesions was retarded sufficiently by both AGN extract and D/DA in TRAMP DLP so that their lesions at 28 weeks resembled those in the vehicle-treated TRAMP mice at 16 weeks (Fig. 4B).

AGN and D/DA treatment decreased TRAMP DLP proliferation and key cell-cycle genes

Because TRAMP epithelial lesions are principally driven by increased cellular proliferation, we examined the effect of AGN and D/DA treatments on Ki67 proliferation biomarker. As shown is Fig. 4A, Ki67 was highly expressed in some but not all T-Ag+ cells in TRAMP mouse DLP. AGN extract or D/DA decreased Ki67 staining (Fig. 4A). Because of the small amount of DLP tissues, we analyzed pooled samples from each group for the mRNA expression level of key genes known to be involved in the proliferation of TRAMP epithelial lesions such as P27, p21Cip1, p16Ink4a, cyclin A2 (Ccn2; refs. 24, 25, Fig. 4C). Whereas the vehicle-treated TRAMP mice showed elevated expression of all these genes compared with WT DLP (set as unity), AGN and D/DA treatment resulted in the same extent of attenuation of their expressions (Fig. 4C). Taken together, these results demonstrated AGN extract and equimolar D/DA exerted same and profound inhibitory effect.
on DLP epithelial lesions as reflected by decreased lesion score/severity, attenuated expression of cell cycle and proliferative genes, and lower Ki67 staining, cumulating to decreased TRAMP DLP prostate lobe weight.

AGN exerted greater inhibition of growth of NE-Ca than equimolar D/DA

The aggressive growth nature of TRAMP NE-Ca (all verified by IHC staining for positive T-Ag, positive synaptophysin, and negative AR; Supplementary Fig. S1A) necessitated euthanasia as early as 19 weeks of age for vehicle-treated TRAMP mice and 5 mice were sacrificed with NE-Ca weighing 7 to 10 grams before the planned experiment terminal endpoint of 28 weeks (Fig. 5A and B). By 28 weeks, the incidence of dissectible prostate NE-Ca was as follows: vehicle group (7 of 20; 35%), AGN group (6 of 21; 29%), and D/DA group (8 of 22; 36%; Fig. 5A; Supplementary Fig. S1C). The observed NE-Ca incidence rate was in agreement with our and others’ previous findings that the lifetime incidence of NE-Ca in the C57B/6 background is about 1 of 3 (14, 16, 17). Whereas neither AGN nor D/DA decreased the cumulative incidence of NE-Ca by 28-week endpoint (Fig. 5A), only 2 AGN-treated TRAMP mice needed euthanasia before this time, with tumors weighing 4 to 5 grams (Fig. 5B, squares). However, the euthanasia rate (6 of 22 enrolled mice) for D/DA-treated TRAMP mice before 28 weeks was comparable with the vehicle group (5 of 20 enrolled
mice; Fig. 5B, triangle vs. circles). Thus, AGN prolonged survival of mice bearing NE-Ca (Fig. 5C) with the smallest cancer burden overall (Fig. 5D). In comparison, D/DA did not have a survival advantage for NE-Ca–bearing mice (Fig. 5C) but decreased the cancer burden when compared with the vehicle-treated counterparts (Fig. 5D).

**AGN decreased incidence of metastatic lymph nodes**

AGN decreased metastasis of NE-Ca to pelvic lymph nodes as verified by IHC (i.e., 3 of 6 for AGN-treated, NE-Ca–bearing mice vs. 7 of 7 for vehicle-treated NE-Ca–bearing mice; Fig. 5A; Supplementary Fig. S1B and S1C). The size of the metastatic lymph nodes was smaller for AGN-treated (average weight, 53 mg) TRAMP mice than in the vehicle-treated TRAMP mice (average weight, 107 mg; Supplementary Fig. S1C). Whereas the incidence of metastatic lymph node for D/DA-treated TRAMP mice (3 of 8, Fig. 5A) and average lymph node weight (34 mg, Supplementary Fig. S1C) were lower than the vehicle-treated TRAMP mice, data interpretation was confounded by the smaller NE-Ca size when the D/DA-treated, NE-Ca–bearing TRAMP mice had to be euthanized (Fig. 5B).

**Targeted gene expression profiling by qRT-PCR analyses of mRNA from NE-Ca**

To seek molecular insights into the differential NE-Ca inhibition efficacy of AGN versus D/DA, we did targeted profiling of the steady-state mRNA for gene expression patterns in 4 NE-Ca from each group. The choice of genes was based on our earlier report of integrated “omic” profiling of NE-Ca treated with AGN (13), focusing on cell proliferation, angiogenesis (Fgf axis and Vegf axis), epithelial–mesenchymal transition (EMT)/metastasis, and inflammation (Table 1). As a well-recognized cellular proliferation biomarker, NE-Ca Pcn2 was lower in AGN- and D/DA-treated mice, with respective suppression of 61% and 49%. Flap endonuclease 1 (Fen1), an endonuclease that complexes with PCNA in DNA repair (26, 27), was suppressed by AGN and D/DA by 53% and 47%. Stat3, another known proliferation signaling molecule for TRAMP NE-Ca growth (28), was suppressed 73% and 52% by AGN and D/DA, respectively. The differential attenuation of the expression levels of these proliferation molecules roughly correlated with the NE-Ca growth-inhibiting efficacy parameters by AGN and D/DA.

Synaptotagmin IV (Syt4), a gene related to neurosynaptic signaling (29), was suppressed by 94% and 84% in AGN and D/DA-treated NE-Ca, respectively. Saa5, a gene involved in neurogenesis and patterning (30, 31), was 56% lower in AGN- and D/DA-treated NE-Ca than in the vehicle-treated counterpart. These genes reflected the neuroendocrine lineage of the analyzed NE-Ca samples. For angiogenesis-related genes, both AGN and D/DA treatment decreased Fgf10, Mmp2, and Fgfbp3 (Fgf axis), yet AGN, but not D/DA, suppressed Vegf1 and one of its receptors, Flt1 and Nos2, which is known to be stimulated by VEGF in vascular endothelium (Vegf axis). For key EMT/metastasis/invasion genes, AGN suppressed Sna12 (32), Twist1 (33, 34), Nos1 (35), Tgfbr2 (36), and macrophage Mmp12 whereas D/DA only suppressed Sna12. In favor of broader targeting of EMT regulators by AGN over D/DA, AGN increased E-cadherin expression by 2.3-fold whereas D/DA failed. Another prostate epithelium–specific protein, ventral prostate predominant 1 (Vpp1), which is known to be extremely suppressed...
in TRAMP NE-Ca (37), was more dramatically induced by AGN (38-fold) than D/DA (19-fold). These changes indicated a greater reversal of EMT/metastasis in the AGN-treated NE-Ca than in D/DA counterparts. In terms of inflammation-related genes, AGN and D/DA suppressed to the same extent mast cell carboxypeptidases A3 (Cpa3) but also significantly extended the mechanism with aspect to the "active chemicals" for each lineage. Despite different batches of AGN (D/DA varied ~2-fold) and excipients (Tween-80 vs. complex formulation) and different laboratories of the experimentation (MN vs. TX) and different localities of the experimentation (MN vs. TX) and different laboratory personnel delivering treatments, AGN exerted unequivocal inhibition of the DLP epithelial lesion growth in both experiments. An additional experiment has also confirmed the TRAMP epithelial lesion suppression efficacy and dose dependency of D/DA (Wu and colleagues, unpublished data). The present work identified D/DA as active/prodrug compounds for inhibiting the epithelial lineage of TRAMP carcinogenesis. Our data from TRAMP mice (majority without NE-Ca) strongly supported that D/DA recapitulated the inhibitory efficacy of AGN on the epithelial lesion growth (DLP lobe weight, total prostate weight; Fig. 3) as well as by morphologic assessment of the lesion severity and molecular marker profiling (Fig. 4). As D/DA are rapidly and extensively converted to DOH in rodents (8-11) and in humans (12), future studies will need to address whether and how DOH (or its further metabolites, if any) mediate the epithelial lesion inhibitory effect. Because human prostate carcinogenesis is believed to originate from prostatic intraepithelial neoplasia (PIN) and progress to aggressive adenocarcinoma over decades, we believe the demonstrated ability of D/DA as prodrugs in the TRAMP model to inhibit the epithelial lineage lesion growth and progression is significant for extending the research into additional clinically more relevant prostate carcinogenesis models to assess the translatability into human prostate cancer risk reduction in the future.

Regarding NE-Ca lineage, the impact of AGN was consistent across 2 experiments, resulting in inhibition of their growth and decreased the NE-Ca burden of TRAMP mice carrying them

![Figure 4.](https://example.com/figure4.png)

**Figure 4.** Effects of AGN and D/DA on TRAMP dorsolateral prostate lesion progression and associated cell-cycle regulatory genes. A, representative IHC analysis of SV40-T antigen (T-Ag) and Ki67 expression in DLP. Magnification, 200×. Dark brown nuclear staining. B, mean score of DLP lesions. 16 weeks: Vehicle (TRAMP), n = 13; AGN (TRAMP), n = 11; D/DA (TRAMP), n = 10; 28 weeks: Vehicle (TRAMP), n = 13; AGN (TRAMP), n = 15; D/DA (TRAMP), n = 14. C, real-time qRT-PCR analysis of mRNA levels in 28-week pooled DLP. One-way ANOVA followed by the Dunnett post-test: *P < 0.05; **P < 0.01; ***P < 0.001.
and improved their survival to the respective specified termination endpoints (ref. 13 and Fig. 5). Our data on NE-Ca with respect to D/DA indicated measurable inhibition on their growth that was not sufficient enough to improve the survival of mice bearing NE-Ca (Fig. 5). The data suggest that non-pyranocoumarin components in AGN might not only provide cancer-suppressing activity but also sustain host survival benefit to NE-Ca–bearing mice alone or by cooperating with D/DA or DOH. Analyses of plasma DOH in AGN- versus D/DA-treated mice revealed same level of DOH with nondetectable D/DA (Fig. 1C) and therefore indicated that the non-pyranocoumarin components (yet to be identified) would not alter D/DA bioavailability or conversion to DOH as a mechanism to account for the greater anticancer efficacy of the AGN extract.
over D/DA. This was consistent with our early report of no observable pharmacokinetic (PK) difference in rats when purified D/DA was dosed at an equinimal dose with that in AGN (9). Our preliminary profiling of mRNA levels of select genes involved in TRAMP NE-Ca angiogenesis, EMT, invasion–metastasis, and inflammation further support the greater scope of ‘targeting’ by AGN than by D/DA (Table 1). Whereas AGN decreased both Fgf and Vegf axes of angiogenesis (e.g., Fgf10, Fgf17, Vegf, and Fli1/Vegfr), D/DA appeared to only affect the Vegf axis without affecting the Vegfr axis (Table 1). For EMT–invasion–metastasis genes, AGN and D/DA affected Snai2 in common but AGN affected additional and more critical regulators of EMT such as Twist, a known master EMT regulator (33, 34), Notch1 (35), Tgfb2, and E-cadherin. Because chronic inflammation is recognized to be an important contributor to cancer progression and metastasis, our profiling data indicated again the superiority of AGN over D/DA in that 3 of 5 profiled genes were suppressed to a greater extent by AGN (Table 1). In the future, we will attempt to identify the non-pyranocoumarin components and validate their efficacy and mechanism of action.

Given the impressive suppression efficacy of AGN on TRAMP carcinogenesis lineages, it is prudent here to speculate on human dose equivalent of AGN or D/DA by allometric extrapolation. The effective AGN dose of 5 mg per mouse (~200 mg/kg) would translate to 17 mg/kg for humans based on body surface area (38). Assume average adult male body weight of 70 kg, the daily human intake will be 1,167 mg. AGN dietary supplements are currently marketed with recommended dosage of 800 mg daily (e.g., CognI-Q from Quality of Life Laboratories; ref. 12). Therefore, the dosage for effective anti-epithelial and anticancer efficacies should be achievable in humans. Furthermore, our recently completed PK study in 20 healthy human subjects recapitulated D/DA to DOH conversion in pattern and extent as the rodent models (12), providing strong justification of the relevance of the rodent models for efficacy and mechanism studies for translation to humans.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: S.-N. Tang, J. Zhang, Y. Zhang, C. Xing, S.-H. Kim, C. Jiang, J. Liu
Development of methodology: S.-N. Tang, J. Zhang, P. Jiang, Y. Zhang, C. Xing, C. Jiang, J. Liu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.-N. Tang, J. Zhang, W. Wu, P. Jiang, M. Puppala, Y. Zhang, J. Liu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.-N. Tang, J. Zhang, W. Wu, P. Jiang, Y. Zhang, C. Jiang, J. Liu
Writing, review, and/or revision of the manuscript: S.-N. Tang, W. Wu, P. Jiang, Y. Zhang, C. Xing, S.-H. Kim, J. Liu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.-N. Tang, P. Jiang, Y. Zhang
Study supervision: S.-N. Tang, Y. Zhang, C. Xing, C. Jiang, J. Liu

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Table 1. Real-time qRT-PCR analysis of mRNA levels in NE-Ca

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<th>Gene symbol</th>
<th>Gene name</th>
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NOTE: TRAMP vehicle, n = 4; TRAMP AGN, n = 4; and TRAMP D/DA, n = 4, respectively. Gene names in bold denote those commonly affected by AGN and D/DA. Bold numbers in AGN column denote those affected only by AGN.
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References


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