In vivo Anti-Cancer Activity of Korean Angelica Gigas and its Major Pyranocoumarin Decursin

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Abstract: We have reported that a 10-herbal traditional formula containing Korean Angelica gigas Nakai (AGN) exerts potent anti-cancer efficacy and identified decursin and decursinol angelate (DA) from AGN as novel anti-androgens. Here, we determined whether AGN would exert in vivo anti-cancer activity and whether decursin or DA could account for its efficacy. The AGN ethanol extract was tested against the growth of mouse Lewis lung cancer (LLC) allograft in syngenic mice or human PC-3 and DU145 prostate cancer xenograft in immunodeficient mice. The pharmacokinetics of decursin and DA were determined. The AGN extract significantly inhibited LLC allograft growth (30 mg/kg) and PC-3 and DU145 xenograft growth (100 mg/kg) without affecting the body weight of the host mice. Biomarker analyses revealed decreased cell proliferation (Ki67, PCNA), decreased angiogenesis (VEGF, microvessel density) and increased apoptosis (TUNEL, cPARP) in treated tumors. Decursin and DA injected intraperitoneally were rapidly hydrolyzed to decursinol. Decursinol and decursin at 50 mg/kg inhibited LLC allograft growth to the same extent, comparable to 30 mg AGN/kg. Therefore the AGN extract possessed significant in vivo anti-cancer activity, but decursin and DA only contributed moderately to that activity, most likely through decursinol.

Keywords: Oriental Herbal Extracts; Pyranocoumarin; Pharmacokinetics; Prostate; Lung Cancer.

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Introduction

Cancers of the lung and prostate account for the majority of cancer mortality in men in the USA (Jemal et al., 2008) and globally (Parkin, 2001; Parkin, 2004). Chemotherapy using available cytotoxic anticancer drugs for advanced-stage malignancies in the lung and prostate offers limited survival benefits (Raez and Lilenbaum, 2004; Evans, 2005; Petrylak, 2005). It is now being increasingly recognized that intervening critical processes of cancer growth and development with naturally occurring herbal and phytochemical agents to achieve chemoprevention is crucial to decreasing the morbidity and mortality of these and other cancers.

Oriental herbal medicine has long been used for treating malignancies. Medicinal herbs are rich sources of novel anti-cancer agents (Ozaslan et al., 2007; Norikura et al., 2008). Recently, we have reported KMKKT (ka-mi-kae-kyuk-tang) cocktail, an ethanol extract of a mixture of dried Korean Angelica gigas Nakai (Cham Dang Gui, AGN) root and 9 other Korean or Chinese herbs, exerts anti-angiogenic, tumor growth inhibitory and anti-metastatic effects using a number of cancer models including mouse Lewis lung cancer (LLC) allograft and human PC-3 prostate cancer xenograft (Lee et al., 2006b). From this mixture, we performed activity-guided fractionation using cell culture assays combined with mechanistic studies and identified pyranocoumarin compound decursin and its isomer decursinol angelate (DA) from AGN as members of a novel class of anti-androgen receptor signaling agent (Jiang et al., 2006; Guo et al., 2007). Decursin and DA have been reported to induce cytotoxic activity of leukemia cell lines and various human cancer cell lines (Kim et al., 2005; Ahn et al., 1996; Ahn et al., 1997). In prostate and breast adenocarcinoma cells, decursin and DA induce G1 cell cycle arrest and caspase-mediated apoptosis (Yim et al., 2005; Jiang et al., 2006; Guo et al., 2007; Jiang et al., 2007). Only one article has been published showing decursin and DA to be active in vivo against Sarcoma-180 growth (Lee et al., 2003). These two compounds, when administered by i.p injection for 9 consecutive days at 50 and 100 mg/kg, caused a significant increase in the life span of acetes-bearing mice and a significant decrease in the tumor volume and final tumor weight of mice with subcutaneously-inoculated tumor cells (Lee et al., 2003). Decursin and DA are the major chemical components of the root of AGN, constituting 3.3–5.9% and 2.4–2.9% of its dry matter, respectively (Ryu, 1967; Ahn et al., 2008). These studies prompt the speculation that AGN with its pyranocoumarin compounds may be one of the active herbs in the KMKKT cocktail for the anti-cancer efficacy.

The present work was designed to answer the following questions: Is AGN ethanol extract active against in vivo cancer growth? Does oral delivery of AGN extract lead to comparable in vivo efficacy as i.p injection? What are the pharmacokinetic characteristics of decursin and DA in vivo? Can pyranocoumarins account for in vivo anti-cancer activity? We used bioassays with LLC allograft model and human prostate cancer xenograft models to evaluate the in vivo efficacy of the AGN ethanol extract. When our pharmacokinetics study showed a rapid conversion of decursin and DA to the pyranocoumarin core decursinol in vivo, we compared the growth inhibitory efficacy of decursinol with decursin side-by-side in the LLC model. Collectively, these studies established an impressive anti-cancer efficacy
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of the AGN ethanol extract and demonstrated its oral bioavailability and safety to the hosts. Our results suggest the presence of additional active compounds besides decursin and DA to mediate the anti-cancer activity of AGN through a number of cellular processes involving proliferation, apoptosis and angiogenesis.

Materials and Methods

Extraction of KMKKT and Constituent Herbs and Preparation of Decursin and Decursinol

Herbal extraction and compound preparations were as described previously. (Lee et al., 2006b; Jiang et al., 2006; Guo et al., 2007) The ethanol extracts of individual herbs or the mixture were dried in vacuo. The herbal extracts or the pure compounds were reconstituted in Tween-80 as solvent vehicle (1% aqueous solution) for animal studies.

Cell Culture

Human DU145 and PC-3 prostate cancer cells were obtained from the American Type Culture Collection (ATCC), Manassas, VA. They were grown in Minimum Essential Eagle’s Medium and F-12K supplemented with 10% fetal bovine serum without antibiotics, respectively. LLC cells were grown in Minimum Essential Eagle’s Medium with 10% fetal bovine serum without antibiotics. Cells were expanded and used for inoculation within 3–4 passages after thawing from liquid nitrogen storage.

Mouse LLC Allograft Tumor Model

We chose to use this model because of its rapid growth in conventional syngenic mice. We have used this model to study the in vivo effects of KMKKT and a number of novel herbal or synthetic agents (Huh et al., 2005; Lee et al., 2006a; Lee et al., 2006b; Lee et al., 2007). LLC cells (3 \times 10^5 in 100 \mu l PBS) were subcutaneously injected into the right flank of C57BL/6 mice. Five days after tumor inoculation, mice were given i.p injection of Tween-80 (final 1%)-reconstituted ethanol extract of each of the constituent herbs of KMKKT once a day (group size range from n = 5 to 6 mice). Tumors were measured with a caliper, and their volumes were calculated according to the formula [(L \times W^2)/2], where L and W stand for length and width, respectively. All mice were sacrificed 13 days after tumor inoculation and the tumors were excised, weighed and photographed.

For comparison of decursin and decursinol, cell inoculation was same as above. Five days after tumor inoculation, mice were given orally decursin, decursinol or vehicle (1% Tween-80) by a feeding needle once daily for 13 consecutive days. Each group contained 7 mice. Tumor growth was measured as above. All mice were sacrificed and the tumors were excised, photographed and weighed.
Human PC-3 and DU145 Xenograft Models

Animal use protocol was approved by Institutional Animal Care and Use Committee of University of Minnesota. In the experiment with PC-3 xenograft, 6-week-old male athymic Balb/C nude mice were purchased from NxGen, San Diego, CA. The mice were maintained in clean HEPA-filter top covered cages and quarantined for 2 weeks in a limited-access specific pathogen free room at the Hormel Institute Animal Facility. Mice were given free access to rodent chow and water. PC-3 cells (1 × 10^6, in 0.1 ml volume containing 20% matrigel) were inoculated per site to the left flank of each mouse. For each group of mice (n = 16), KMKKT, AGN or vehicle was administrated by i.p. injection once a day starting 4 days after inoculation for 7 days, then 3 times a week (M, W and F) until termination at 50 days of inoculation. Tumor size was measured twice a week as above for LLC model. The tumors were excised and weighed. For large tumors, a piece was frozen on dry ice and stored at −70°C for analyses later. A piece of each large tumor and all small tumors were fixed in 10% neutral buffered formalin and processed for histology staining and immunohistochemistry (IHC).

For the experiment with DU145 xenograft, 4–5 weeks-old male athymic Balb/C nude mice were purchased and maintained as above. After necessary quarantine, groups of mice (n = 16) received vehicle (1% Tween-80) or AGN extract by i.p. administration whereas another group of mice (n = 10) received oral gavage by using a feeding needle, once a day for 7 consecutive days. On the 7th day, DU-145 cells (1 × 10^6, in 0.1 ml volume containing 20% matrigel) were inoculated per site to the left flank of each mouse. Tumor size was measured twice per week as above. AGN extract administrations were continued 3 times per week (M, W and F) until termination at 44 days of inoculation. The tumors were excised, weighed and processed as above for PC-3 study.

Pharmacokinetic Study of Decursin and DA

The maximally tolerated dose of decursin and decursinol angelate (DA) by daily i.p. injection for 4 weeks was determined to be 300 mg/kg in mice, without any adverse effect on their body weight gain (data not shown). A single dose of decursin or DA was administered by i.p. injection to male mice (~6–8 weeks of age). For each agent, 3 mice were used per time point. Blood was collected at 0.08, 0.25, 0.5, 1, 2, 4, 8, 16, 24, 48 and 72 hour (11 time points). A blood sample of 0.5~1 ml was obtained by cardiac puncture. The blood samples were centrifuged at 3,000 rpm for 15 min immediately and a 100 µl aliquot of each plasma sample was stored in a −70°C freezer until HPLC analysis.

HPLC separation and analysis of decursin, DA and Decursinol: 100 µl acetonitrile was added to 100 µl-aliquot of each plasma sample. After vortex-mixing and centrifugation at 13,000 rpm for 15 min, 100 µl of the supernatant was recovered for decursin, DA and decursinol measurement by HPLC as follows: A reverse-phase C18 column (Waters, Atlantis®, 5 µm, 4.6 × 250 mm) was used with the mobile phase consisting of 45/55% acetonitrile/water (vol/vol), with the addition of 0.05% trifluoroacetic acid for HPLC, at a flow rate of 0.8 ml/min. Diode array detection was set at 330 nm and the injection volume was 50 µl. Standard curves show linear detection of all 3 analytes.
Immunohistochemistry

Cell proliferative index was evaluated by IHC staining for Ki67 antigen with the MIB-1 antibody (Lab Vision Corporation, CA, USA). Apoptosis was detected by Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) kit according to manufacturer’s instruction (Calbiochem, Darmstadt, Germany). Immunostaining for vascular endothelial growth factor (VEGF) was performed with antibody from Santa Cruz Biotechnology (Santa Cruz, CA). CD34 antibody (Abcam, MA, USA) was used to identify microvessels in tumor. Immunohistochemistry color development used indirect avidin-biotin-enhanced horseradish-peroxidase method. Antigen retrieval was performed after deparaffinization and rehydration of the tissue sections (4 µm) by microwave for 10 min in 10 mM citrate buffer. Sections were cooled to room temperature, treated with 3% hydrogen peroxide in methanol for 10 min, and blocked with 6% horse serum for 40 min at room temperature. Sections were then incubated with the primary antibody to Ki67 (diluted 1:200, Labvision), VEGF (diluted 1:200, Santa Cruz Biotechnology) and CD34 (diluted 1:50, Abcam) at 4°C overnight. Sections were washed in PBS and incubated with secondary antibody (biotinylated goat anti-rabbit (1:150, Vector Laboratories) or biotinylated rabbit anti-rat IgG (1:150, Abcam) for 30 min. After further washes, the color development was carried out with the Dako Cytomation ABC/HRP kit (Dako Cytomation) and with 3,3’-diaminobenzidine tetrahydrochloride as substrate.

For semi-quantitation, ten 200× power photomicrographs were taken for each slide with a digital camera, avoiding gross necrotic areas. The positively-stained cancer epithelial cells within each photomicrograph were counted. The counting of total cancer cells was aided with the ImagePro+ image processing program.

Western Blot Analyses

For frozen tumor samples, vascular endothelial growth factor (VEGF), proliferative cell nuclear antigen (PCNA), cleaved poly(ADP-ribose)polymerase (PARP) and β-actin were analyzed by Western blotting as previously described (Lee et al., 2006b).

Statistical Analyses

Statistical analyses were carried out with Prism and Sigma plot softwares, and p < 0.05 was considered statistically significant. The data were analyzed by one-way ANOVA followed by Bonferroni t test for pairwise multiple comparisons or other appropriate tests.

Results

AGN was an Active Herb in KMKKT Against LLC Allograft Growth

To identify which herb(s) contributed to the in vivo anti-cancer activity for KMKKT, we used LLC model as a rapid screening bioassay to compare the tumor growth inhibitory activity of the individual herbal extracts. LLC cells were injected subcutaneously into the right flank of
each mouse. Five days after tumor inoculation, mice were each given once daily i.p injection of the solvent vehicle or the vehicle-reconstituted ethanol extract of either *Angelica gigas Nakai* (AGN), *Benincasa hispida* (BH), *Panax ginseng* (PG), *Phaseolus angularis* (PA), *Zanthoxylum piperitum* (ZP), *Tulipa edulis* (TE), *Astragalus membranaceus* (AM), *Patrinia villosa* (PV) or *Bletilla striata* (BS). Due to the extreme low extraction yield from *Asini Gelatinum* (Jiang et al., 2006), it was not tested. We chose a single dosage of 30 mg/kg body weight for each extract. The rationale was such a dose would provide 2 to 4 times of each herb’s contribution to the KMKKT cocktail when it was given at the effective dose of 100 mg/kg. The results show that none of the herbs at the tested dose decreased body weight of the mice (Fig. 1a). AGN extract inhibited tumor growth significantly (*p* < 0.05, by 42%), so did BH and PG (Fig. 1b). Whereas the effect of PA approached statistical significance (*p* = 0.064), the rest of the herbs did not produce statistically significant effect on LLC growth at the dose tested (Fig. 1b).

![Figure 1](image)

**Figure 1.** Comparison of growth inhibitory efficacy of ethanol extract of individual herbs of KMKKT cocktail against LLC tumors in C57BL/6 mice. LLC cells (5 × 10⁵ in 200 µl PBS) were injected subcutaneously into the right flank of C57BL/6 mice. Five days after tumor inoculation, mice were given i.p injection of Tween-80 (1%) as solvent vehicle or Tween-80-reconstituted extract of each of the constituent herbs of KMKKT once a day. *Angelica gigas Nakai* (AGN), *Benincasa hispida* (BH), *Panax ginseng* (PG), *Phaseolus angularis* (PA), *Zanthoxylum piperitum* (ZP), *Tulipa edulis* (TE), *Astragalus membranaceus* (AM), *Patrinia villosa* (PV) and *Bletilla striata* (BS). Dosage = 30 mg/kg body weight. (a) Body weight of mice. Mean ± SE, n = 5–6. (b) Final tumor weight at necropsy 13 days after inoculation. Mean ± SE, n = 5–6 mice per group.
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AGN Extract Inhibits Human PC-3 Prostate Cancer Xenograft Growth in vivo

Since the LLC allograft model indicated AGN as the most active, we evaluated its efficacy against human PC-3 prostate cancer cells as cellular targets in comparison with the efficacy of KMKKT formula in immuno-compromised athymic Balb/C nude mice. Treatment was started 4 days after cell inoculation with the daily i.p. injection of KMKKT (100 mg/kg) or AGN extract (100 mg/kg) for the first 7 days, then 3 times per week until 50 days of inoculation when the mice were sacrificed. The results show that both the KMKKT treatment and AGN treatment inhibited the tumor growth kinetics with similar growth curves (Fig. 2a). By the end of experiment, the tumor weight in the AGN-treated mice was decreased by 51.2% and in the KMKKT treated mice by 43.9% (Fig. 2b). Neither KMKKT nor AGN treatment caused any adverse effect on the body weight of the mice (Fig. 2c). In fact, the treatment by AGN or KMKKT ameliorated body weight loss that occurred in the control mice in the last 2 weeks of the experiment (Fig. 2c).

To determine whether apoptotic cell death was involved in mediating the in vivo efficacy of AGN extract, we did TUNEL assay on the PC-3 tumors. AGN-treated tumors displayed an apoptosis rate of 7.4%, whereas the KMKKT-treated tumors were around 5%, compared to 2% in the control tumors (Fig. 3a). These results were corroborated by Western blot analyses of the tumor tissue extracts using cleaved PARP as a biomarker of caspase-mediated apoptosis (Fig. 3b), in that AGN-tumors showed slightly stronger cPARP than KMKKT-tumors. In terms of a cell proliferation biomarker, Western blot revealed a decreased PCNA level in AGN-treated tumors (25–40% according to densitometric analyses) and KMKKT-treated tumors (20–30%). Such analyses also indicated that KMKKT was more active than AGN to decrease the expression of VEGF (Fig. 3b). VEGF staining was localized in the cytosol in the control tumor (Fig. 3c). The immunoreactivity was decreased more by KMKKT than by AGN treatment, corroborating the Western blot data. These results indicate that in spite of similar anti-cancer activity with KMKKT in the PC-3 model, AGN extract may preferentially target apoptosis and cell proliferation rather than KMKKT whereas KMKKT contains active herbs that affect the angiogenesis processes more, as we have shown earlier (Lee et al., 2006b).

AGN Extract Inhibits DU-145 Prostate Cancer Xenograft Growth by Both Oral Administration and i.p Injection

The results from the PC-3 prostate cancer xenograft model prompted us to test whether the inhibitory ability of AGN extract could be translatable to another human prostate cancer cell target, the DU-145 xenograft model. In addition, we tested whether AGN could be given orally to achieve in vivo efficacy. The second issue is important because oral administration is one of the most practical and non-invasive means to deliver anti-cancer agents in a chemoprevention context for non-skin cancers. To increase our chance of detecting an inhibitory effect by either route, we pretreated the mice for 7 days with daily oral administration or i.p injection of AGN extract at 100 mg per kg dose before cancer cell inoculation, then continued the treatments 3 times per week (M, W and F) until termination at 44 days of inoculation.
Figure 2. Comparison of effects of ethanol extract of Angelica gigas Nakai with KMKKT on human PC-3 prostate cancer xenograft growth in Balb/C athymic nude mice subcutaneously inoculated with cancer cells. Treatment was started 3 days after cancer cell inoculation with once daily i.p injection for the first 7 days and then continued 3 times (M, W, F) per week until 50 days of inoculation. (a) Tumor growth kinetics. (b) Final tumor weight at necropsy. (c) Body weight. Mean ± SE, n = 16 mice. *p < 0.05, **p < 0.01, ***p < 0.001 vs. the control.
Figure 3. Effects of ethanol extract of Angelica gigas Nakai and KMKKT on selected biomarkers related to apoptosis, cell proliferation and angiogenesis in human PC-3 prostate cancer xenograft. (a) Apoptosis index estimated by TUNEL-positive cells, n = 10. Values in the bar graph represent mean ± SE. ANOVA, p < 0.001. Means bearing different letters are significantly different. (b) Immunoblot analyses of proliferative cell nuclear antigen (PCNA, proliferation), the cleaved PARP (caspase-mediated apoptosis) and vascular endothelial growth factor (VEGF, angiogenesis) proteins in selected tumors from the control mice and the KMKKT- and AGN-treated mice. (c) Representative immunostaining for VEGF from different groups. Photograph (200 × magnification).

Treatment with AGN by i.p injection (AGNI) and by oral gavage (AGNO) resulted in a significant inhibition of the xenograft tumor growth over time (Fig. 4a), suppressing the final tumor weight by 73.6% and 63.6%, respectively (Fig. 4b). AGN treatment did not negatively affect the body weight of the mice (Fig. 4c), indicating good tolerance of mice at this dosage without adverse side-effects.
Figure 4. Effects of intraperitoneal versus oral administration of Angelica gigas Nakai extract on the growth of DU-145 human prostate cancer xenografts in Balb/C athymic nude mice. Tween-80-reconstituted AGN extract or vehicle (Tween-80, 1%) was given by i.p. injection (AGNI) or feeding needle (AGNO) once a day starting for 7 days before cancer cell inoculation by subcutaneous injection. Then the treatments were continued 3 times per week (M, W and F) until termination at 44 days of inoculation. (a) Tumor growth kinetics. *p < 0.05 vs. control. (b) Final tumor weight at necropsy. Means bearing different letters are significantly different at p < 0.05. (c) Body weight. Mean ± SE, n = 16 for vehicle and AGNI groups and n = 10 for AGNO group.

IHC staining of Ki-67 positive cells (Fig. 5a) indicated that both AGNI (AGN by i.p. injection) and AGNO (AGN by oral administration) decreased the number of proliferative cells (Fig. 5d). The TUNEL assay for apoptotic cells showed a low level of cell death in the non-necrotic epithelial compartments in the DU-145 tumors and was not significantly increased by AGNI or AGNO (Fig. 5e). As a biomarker for angiogenesis, we did IHC staining for CD34 positive vascular endothelial cells to detect new microvessels (Fig. 5c). AGNI- and AGNO-treated tumors displayed decreased microvessel density in the DU-145 tumors (Fig. 5f). These data indicate that AGN extract inhibited the growth of DU145 human prostate cancer xenograft in nude mice and, oral administration had comparable efficacy as i.p. injection. The efficacy of AGN against DU145 xenograft model was principally associated with anti-proliferative and anti-angiogenesis activities.
Figure 5. Effects of intraperitoneal versus oral administration of Angelica gigas Nakai extract on Ki67 proliferation index, CD34 positive microvessel density and TUNEL apoptosis index of DU145 human prostate cancer xenografts in Balb/C athymic nude mice. Photomicrographs show representative immunostaining patterns for Ki67 as a biomarker for cell proliferation (a), for TUNEL-positive cells for apoptosis (b) and for CD34 as a vascular endothelial marker for tumor microvessel density (c). Ki-67 and CD34 staining used DAB substrate staining (brown) and counterstained with Mayer’s hematoxylin solution (blue). TUNEL staining was counterstained with methyl green. 200 × magnification. Graphs show quantification of Ki67 index (d), Apoptosis (e) and CD34 + microvessel density (f) in control and AGN-treated xenograft tumors. Values in the bar graphs represent means ± SE, n = 10. Means bearing different letters are significantly different at p < 0.05.
Figure 6. Pharmacokinetics of intraperitoneally-injected decursin and decursinol angelate (a) and (b) and comparison of the inhibitory efficacy of decursin and decursinol against LLC tumors in C57BL/6 mice (c) and (d). (a) Chemical structure of decursin, decursinol angelate and decursinol. (b) The concentration of plasma decursinol as a function of time after i.p. injection of decursin (solid circles) and DA (empty circles) at 300 mg/kg body weight to mice. Mean ± SE, n = 3 mice per time point. (c) Tumor volume as a function of time and treatment by decursin and decursinol (50 mg/kg). Decursin or decursinol was administered orally once daily for 13 consecutive days, starting at 5 days after LLC tumor inoculation as in Fig. 1. (d) Final weight of dissected LLC tumors. Mean ± SE, n = 7. ANOVA, p < 0.01. Means bearing different letters are significantly different.

**The Major Pyranocoumarin Compounds Decursin and DA Converted to Decursinol upon i.p Injection**

Since we have identified decursin and DA from AGN as a novel class of anti-androgen/AR agent (see Fig. 6a for structure) (Jiang et al., 2006; Guo et al., 2007), together with their reported anti-proliferative and apoptotic activities in prostate cancer cells (Yim et al., 2005; Jiang et al., 2006; Guo et al., 2007), breast cancer cells (Jiang et al., 2007) and leukemia cells (Ahn et al., 1996; Ahn et al., 1997) *in vitro* as well as their *in vivo* inhibitory activity against sarcoma (Lee et al., 2003), these compounds could be the putative active agents for...
the *in vivo* anti-cancer activity of AGN. To shed light on this issue, we determined next their *in vivo* pharmacokinetic characteristics.

After an i.p. injection of 300 mg/kg (the maximally tolerated dose) to mice, neither decursin nor DA was detectable in the plasma at any time point studied (5 min to 72 hours). However, decursinol was detected in the plasma as early as 5 min and increased sharply, peaking at 30 min and subsided thereafter with $t_{1/2} \sim 2–3$ hours to below the limit of detection after 8 hours (Fig. 6b). These data support a rapid conversion of decursin and DA to decursinol during their transit from the intraperitoneal space to the blood stream.

Both decursinol and decursin exerted a modest *in vivo* anti-cancer activity

The pharmacokinetic data suggest the *in vivo* hydrolysis product decursinol, rather than the parent compounds, will likely mediate the *in vivo* anti-cancer activity of decursin and DA as reported previously in the sarcoma model (Lee et al., 2003). To test this hypothesis, we compared the anti-tumor activity of decursin with decursinol in the LLC tumor growth model by oral gavage starting 5 days after tumor inoculation. The solvent vehicle (1% Tween-80), 50 mg/kg of decursin or decursinol were given once daily for 13 consecutive days. The decursinol treatment led to virtually the same extent of inhibition of the tumor growth kinetics as decursin (Fig. 6c). At necropsy time, the tumor weight for the decursin-treated mice was indistinguishable from the decursinol-treated mice, being 33% less than the tumors from vehicle-treated mice (Fig. 6d).

**Discussion**

An ever increasing number of cancer patients turn to herbal medicine for alternative therapy or to complement their standard therapies in hope of preventing cancer recurrence or metastasis (Ernst et al., 2006; Guo et al., 2008). In spite of the growing demand on herbal remedies, their efficacies are in most cases not proven. Rigorous studies using pre-clinical cancer models to establish their *in vivo* activity and to identify mechanisms of action are necessary first steps to make progress in this arena.

In the present study, we used the mouse LLC allograft model as an efficient bioassay to evaluate the anti-cancer efficacy of ethanol extracts of the individual herbs that made up the KMKKT anti-cancer herbal cocktail and identified AGN as the most active (Fig. 1). We confirmed and extended the *in vivo* anti-tumor efficacy of AGN against 2 human PCa xenograft models: PC-3 (Fig. 2) and DU145 (Fig. 4), which represent aggressive androgen independent human prostate cancers. Because the *in vivo* anti-cancer efficacy of AGN was found in both the immune-competent host mice for the LLC allograft model and the immuno-compromised host nude mice for the human cancer cells in the xenograft models, the data strongly suggest that AGN contains active chemicals that can suppress tumor growth irrespective of possible immunomodulatory actions of the extract.

In addition, we established that AGN given by oral administration was as effective as by i.p. injection in the DU145 xenograft model (Fig. 4). This finding was important for establishing the gastrointestinal bioavailability of the active compounds present in AGN.
to enter the blood circulation and reach the target cancer cells. Oral intake is the most practical and non-invasive means to deliver anti-cancer agents to internal organs in a cancer chemoprevention context.

As for the active anti-cancer compounds in AGN, impressive data from cell culture studies have strongly implicated decursin and DA, as described in the Introduction. However, our pharmacokinetic study for these 2 compounds strongly disputed this hypothesis. As shown in Fig. 6, decursin and DA were not stable \textit{in vivo}, rapidly converting to decursinol during their transit from the intraperitoneal space to the blood stream. Similar to our data, a research group published in the Korean literature a pharmacokinetic study of decursin in the rats after oral administration (Park \textit{et al.}, 2001). Decursin was given at 300 mg/kg to each rat by gavage, and then, the concentration of decursinol in the urine and plasma was determined by HPLC. According to their results, decursin was not detected in the blood, whereas decursinol rose in the blood peaking at 2 hours and then declined. Urinary decursinol excretion within the first 24 hours was approximately 95% of the decursin dose administered (Park \textit{et al.}, 2001), supporting the absorption of decursinol into and clearance of it from the blood. The authors speculated that decursin was hydrolyzed by esterases in the intestine or liver. Taken together, the results of these 2 independent pharmacokinetic studies in rats and mice suggest that decursinol, rather than decursin and DA, may exert \textit{in vivo} anti-cancer activity that was reported for these 2 parent compounds (Lee \textit{et al.}, 2003).

This hypothesis was supported by our direct comparison of decursin with decursinol (Figs. 6c and 6d). They resulted in an equal \textit{in vivo} growth suppression of LLC when each was given at a daily dosage of 50 mg/kg. Pyranocoumarins (decursin, DA plus decursinol) are major constituents of the ethanol extract of dried AGN root, accounting for approximately 20% (Ryu \textit{et al.}, 1967; Ahn \textit{et al.}, 2008). In the LLC model, 30 mg/kg of AGN (∼6 mg decursinol equivalent of pyranocoumarins) was shown to be as effective as (Fig. 1b) the pure compounds at 50 mg/kg (Figs. 6c and 6d). We therefore speculate that additional active compounds must exist in AGN extract to contribute to its \textit{in vivo} anti-cancer efficacy demonstrated here in multiple models. Identification of these compounds is currently being planned.

Mechanistically, the analyses of the biomarker changes in PC-3 xenograft samples comparing AGN with KMKKT showed that when provided on an equal weight basis, KMKKT cocktail preferentially targeted angiogenesis (e.g., suppression of VEGF expression) whereas AGN induced more apoptosis (e.g., higher TUNEL index and more cleaved PARP) (Fig. 3). These analyses also indicated that AGN extract exerted a modest anti-proliferative activity (e.g., decreased PCNA). These results are not surprising because the other herbs in KMKKT may contain more active chemicals for anti-angiogenesis activities or anti-metastasis activities, which were demonstrated in our earlier study (Lee \textit{et al.}, 2006b). Interestingly, in the DU145 xenograft model, we did not detect a significant increase in apoptosis in the non-necrotic epithelial compartments (Fig. 5), although significant effects on Ki67 proliferative index and microvessel density were detected. The reasons for the 2 human PCa models to differ in the cellular processes involved for AGN efficacy were not immediately apparent and will be dealt with in future studies.
In summary, our study using multiple prostate and lung cancer growth models established an impressive in vivo anti-cancer efficacy of the AGN ethanol extract and demonstrated its oral bioavailability and safety to the hosts. These data support further development of AGN ethanol extract as an effective dietary herbal supplement or therapeutic product for cancers of multiple organ sites. The pharmacokinetic study of the main pyranocoumarin compounds combined with direct comparison of the anti-cancer efficacy of decursinol with decursin ruled out decursin or DA as in vivo active compounds, rather through their conversion to decursinol or further metabolite(s), exerted a modest in vivo cancer inhibitory activity. Based on these results, we speculate the presence of other active compounds in AGN in addition to the pyranocoumarins to contribute to the observed anti-cancer activity through a number of cellular processes involving proliferation, apoptosis and angiogenesis.

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