Curr Pharmacol Rep (2015) 1:373–381
DOI 10.1007/s40495-015-0033-y

CANCER CHEMOPREVENTION (R AGARWAL, SECTION EDITOR)

Cancer Chemoprevention with Korean Angelica: Active Compounds, Pharmacokinetics, and Human Translational Considerations

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Published online: 19 April 2015

Abstract *Angelica gigas* Nakai (AGN) is a major medicinal herb used in Korea and several other Asian countries. Traditionally, its dried root has been used to treat anemia, pain, infection, and articular rheumatism, most often through boiling in water to prepare the dosage forms. AGN extract or AGN-containing herbal mixtures are sold in the USA and globally as dietary supplements for pain killing, memory enhancement, and postmenopausal symptom relief. Decursin (D) and its isomer decursinol angelate (DA) are the major chemicals in the alcoholic extracts of the root of AGN. The anti-cancer activity of AGN alcoholic extract has been established in a number of animal cancer models, including a transgenic model of prostate carcinogenesis. Cell culture structure-activity studies have uncovered distinct cellular and molecular effects of D and DA vs. their pyranocoumarin core decursinol (DOH) with respect to cancer cells and those associated with their microenvironment. Pharmacokinetic (PK) study by us and others in rodent models indicated that DOH is the major and rapid in vivo first pass liver metabolite of D and DA. Cognizant of metabolic differences among rodents and humans, we carried out a first-in-human PK study of D/DA to inform the translational relevance of efficacy and mechanism studies with rodent models. The combined use of rigorous animal tests and human PK studies can provide stronger scientific rationale to inform design and execution of translational studies to move AGN toward evidence-based herbal medicine.

Keywords *Angelica gigas* Nakai · Decursin · Decursinol · Decursinol angelate · Pharmacokinetics · Translational cancer research

Abbreviations

AGN	*Angelica gigas* Nakai
D	Decursin
DA	Decursinol angelate
DOH	Decursinol

Introduction

We have in a recent article systematically reviewed the anti-cancer and other bio-activities of Korean Angelica and its major pyranocoumarin compounds [1•]. In this paper, we will focus on recent developments concerning the anti-cancer activities and new knowledge concerning the pharmacokinetics (PK) and metabolism of decursin (D) and its isomer decursinol angelate (DA), not only in rodent models but also in humans. Readers are referred to our comprehensive review [1•] and the original references therein for historical information and specific details.

Korean *Angelica gigas* Nakai and Its Major Phytochemicals

Korean *A. gigas* Nakai (AGN) (in Korean Cham Dang-gui) differs from other prominent medicinal Dang-gui species

This article is part of the Topical Collection on *Cancer Chemoprevention*

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including *Angelica sinensis* (Chinese) and *Angelica acutiloba* (Japanese) by its possession of unique pyranocoumarins [1•]. Angelica roots are traditionally used to treat anemia, cold, pain, and other diseases as folk medicines and known to herbalists as "female ginseng." However, these traditional medicinal properties were mostly based on using boiling water to extract the active ingredients. Changing the extraction solvents can lead to the recovery of different chemicals and therefore novel medicinal and pharmacological properties. In fact, many pyranocoumarin compounds have been identified from the alcoholic extracts of AGN roots, which could not be extracted by water.

Chemical composition of plants of the *Angelica* genus has been extensively studied and reviewed [1•, 2]. Pyranocoumarins are the major chemicals identified in the ethanolic or methanolic extracts of AGN. Decursin (D) (Fig. 1a), the most abundant, was first isolated from root of *Angelica decursiva* FR et SAV and later from AGN in the 1960s. Decursinol angelate (DA) (Fig. 1a) is a structural isomer of D on the side chain and about 60 % as abundant as D in the root of AGN. The pyranocoumarin core decursinol (DOH) (Fig. 1a) can be identified with a much lower abundance, if at all, in AGN root. Additional chemicals structurally related to D include 4″-hydroxytigloyldecursinol, 4″-hydroxydecursin, (2″S,3″S)-epoxyangeloyldecursinol, and (2″R,3″R)-epoxyangeloyldecursinol [1•]. The alcohol-extractable D/DA may constitute 3.0–8.2 % of dried AGN root, depending on methods and herbal sources.

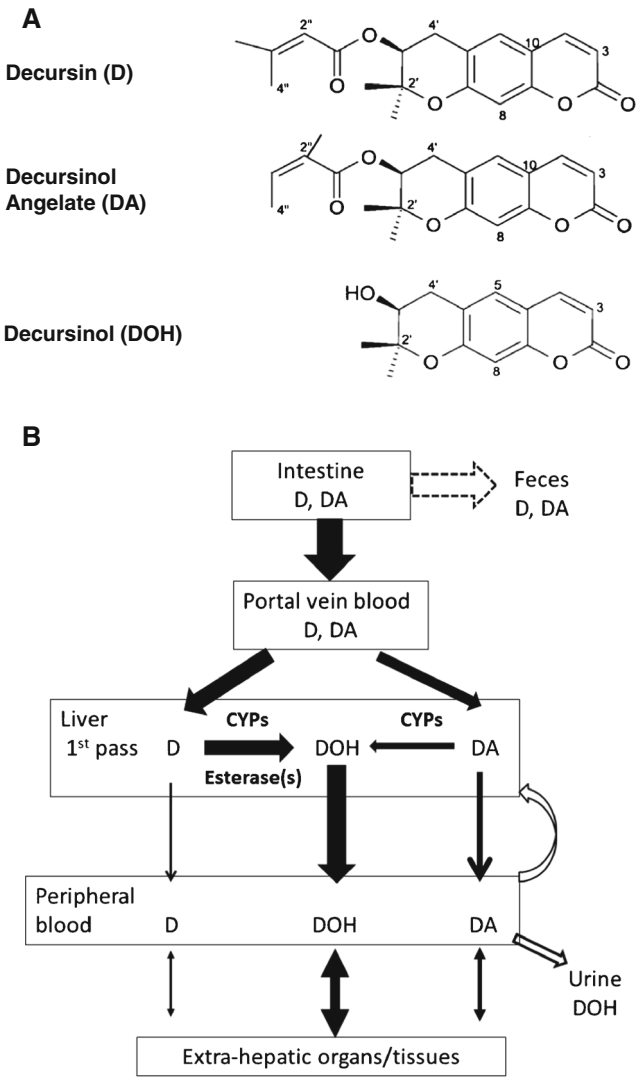

Fig. 1 **a** Chemical structures of decursin (D), decursinol angelate (DA), and decursinol (DOH). **b** Proposed metabolic schema of D and DA in rodents and humans after oral intake. *CYP* cytochrome p450

Classification based on a molecular basis in addition to morphology is very crucial to preventing adulteration of the dried roots of different *Angelica* species available in the herbal markets. Genetic differences among *A. sinensis* (Chinese), *A. acutiloba* (Japanese), and AGN (Korean) have been documented, including 5S-rRNA spacers [3]. They are phylogenetically divided into two clusters: the first group consisting of *A. sinensis* and the second group consisting of *A. acutiloba* and AGN [3]. Chemically, the contents of ferulic acid and Z-ligustilide in the root of *A. sinensis* were much higher than those of *A. acutiloba* and AGN [3]. Others have profiled the volatile components in Angelica species by gas chromatographic/mass spectrometry (GC/MS) [4, 5]. GC/MS profiling was used as a pattern recognition method for the quality control of AGN [6] with a principal component analysis (PCA) subsequently applied to 10 common peaks selected from each chromatogram. Profiling chemical components of AGN from different geographical origins using ^{1}H NMR and UPLC-MS analyses identified D, DOH, nodakenin, marmesin, and 7-hydroxy-6-(2R-hydroxy-3-methylbut-3-ethyl)coumarin as major metabolites that contributed to the discrimination factor [7]. Taken together, D and DA are a robust identifying chemical characteristic of AGN.

In Vivo Anti-cancer Activities of AGN Extracts

We and collaborators reported in vivo inhibitory efficacy of AGN ethanol extracts on androgen-independent DU145 and PC-3 PCA xenografts [8] and in a primary prostate carcinogenesis model [9••]. For the DU145 xenograft study, mice were treated with 100 mg/kg AGN in 1 % Tween 80 by either gavage or intraperitoneal (*i.p.*) injection 7 days before tumor inoculation. By the end of experiment, treatment with AGN by i.p. injection and gavage suppressed the final tumor weight by 74 and 64 %, respectively. None of the treatment negatively affected the body weight of the mice. For the PC-3 xenograft study, treatment with 100 mg/kg AGN by i.p. injection started 4 days after tumor inoculation suppressed final tumor weight by 51 %. Biomarker analyses indicated that anti-proliferation, anti-angiogenesis, and apoptosis were likely

involved in the in vivo suppressing effect of AGN against xenograft tumors.

We have recently shown that AGN inhibits the two lineages of carcinogenesis in the prostate of Transgenic Adenocarcinoma Mouse Prostate (TRAMP) model [9••]. In this study, male C57BL/6 TRAMP mice and wild type littermates were given a daily gavage (5 mg/mouse, ~200 mg/kg, Monday–Friday) of AGN or 1 % Tween 80 vehicle, beginning at 8 weeks of age (WOA). All mice were terminated at 24 WOA, unless earlier euthanasia was necessitated by large tumors. Whereas AGN-treated TRAMP mice decreased dorsolateral prostate growth by 30 % (P=0.009), they developed fewer and smaller neuroendocrine-carcinomas (NE-Ca) (0.12 g/mouse) than vehicle-treated counterparts (0.81 g/mouse, P=0.037). Proteomic and transcriptomic analyses of banked NE-Ca suggest not only multiple cancer cell targeting actions of AGN but also significant impacts on the tumor microenvironments such as angiogenesis, inflammation, and immune surveillance. Prominent among upregulated gene categories by AGN were those involved in immune responses, prostate function/differentiation, ion transport, and tumor and proliferation suppressors [9••]. The immunity category of genes was particularly noteworthy. For example, defensin beta 50 (Defb50) was induced by AGN for 20-folds, and Defb1 for 5.7-folds. Defensin genes code for a family of 3–4 kDa polycationic peptides that act as bactericidal agents through disrupting bacterial membrane integrity. Clinical specimens had high frequencies of loss of Defb1 in malignant prostatic tissue, while high levels of expression were maintained in adjacent benign regions [10]. The finding was in agreement with the mouse model in which the expression of Defb1, Defb2, and Defb4 were decreased in TRAMP tumors [11, 12]. In addition, ectopical expression of Defb1 in DU145 and PC-3 PCA cells resulted in decreased cellular growth accomplished by cytolysis and caspase-mediated apoptosis [10]. Zinc alpha-2-glycoprotein 1 (Azbp1) is a 41-kDa soluble protein synthesized by epithelial cells of many tissues including the prostate gland. Its expression was decreased in TRAMP prostate and tumor at mRNA/protein levels [11, 13]. Recent findings indicated that absent/low Azgp1 expression was an independent predictor of recurrence in localized PCA after radical prostatectomy [14, 15]. Since Azgp1 has a major histocompatibility complex-1 (MHC-1)-like fold in its structure, it might be involved in host immune response to tumor by antigen processing and presentation to exert its tumor suppressing effect. These and many other upregulated changes associated with immune responses suggest a potential promotion of immune surveillance by AGN treatment to inhibit NE-Ca growth.

Other notable changes suggested restoration by AGN of genes inactivated during TRAMP lesion progression. The prostate-specific proteins β-microseminoprotein (Msmb) and serine peptidase inhibitor Kazal type 3 (Spink3) were 27.6 and 7.4-fold higher in AGN-treated NE-Ca, respectively, and we reported earlier their protein levels to be lower in TRAMP prostate than wild-type prostate [13]. GSTM1 and tropomyosin (Tpm2), which are downregulated in TRAMP prostate at protein level [13], and glutathione S-transferase, theta 3 were among tumor suppressor category of genes increased by AGN. The prostate epithelial-specific gene ventral prostate predominant 1 (Vpp1) was significantly induced by AGN treatment in the NE-Ca.

In terms of downregulated genes in the TRAMP NE-Ca, notable categories include neuron signaling and differentiation, oncogenes and proliferation, mast cells and inflammation, Wnt signaling/epithelial-mesenchymal-transition, embryonic morphogenesis, biosynthesis, cell adhesion, motility and invasion, and angiogenesis and hematopoiesis [9••]. The suppressed neuron signaling and differentiation genes could be expected because of the NE-Ca nature of the analyzed tissues and the significantly reduced NE-Ca burden. In particular, synaptotagmin IV (SYT4), known to play an important role in neurotransmitter secretion, was suppressed by AGN treatment as much as sevenfolds. The AGN-suppressed genes in other categories, such as oncogenes/oncofetal antigens and proliferation, Wnt signaling, biosynthesis, cell adhesion, motility and invasion, and angiogenesis and hemopoiesis, were consistent with them as potential contributors to the overall efficacy through not only direct action on cancer cells but also impact on their microenvironments. Several genes related to inflammatory mast cells, including mast cell carboxypeptidase A3 (Cpa3), proteases (Mcpt4; Mcpt6), mast cell chymases (Cma1; Cma2), and mast cell surface marker Fcer1a (immunoglobulin epsilon receptor subunit alpha), were substantially suppressed by AGN treatment. Mast cells (MC) are granulocytic immune cells best known for their role in allergy and anaphylaxis. Pittoni et al. reported that in tumors from TRAM P mice on C57BL/6 background and human patients, MCs were specifically enriched and degranulated in areas of "well-differentiated" adenocarcinoma but not around "poorly differentiated" foci that coexist in the same tumors [16]. They showed that MCs promoted "well-differentiated" adenocarcinoma growth by providing MMP-9 but were dispensable for growth of "poorly differentiated" tumors [16]. Consistent with these findings, Morgenbesser et al. [17] and Kela et al. [11] showed that the expression of another MC surface marker c-kit was increased in TRAMP tumors. These findings suggested the role of MC and inflammation in prostatic carcinogenesis and might be suppressed by AGN to contribute to inhibition of NE-Ca growth.

In work to be published in full length, we compared AGN vs. equimolar intake of D/DA in the TRAMP model [18]. Experimentally, three cohorts of male C57BL/6 TRAMP mice (35–36 mice per group) were gavage-treated with a new vehicle (ethanol: PEG400: Tween 80: 5 % glucose=3:6:1:20), AGN (5 mg/mouse) or equimolar D/DA (3 mg/mouse) from 8 weeks of age (WOA). Mice were euthanized at either 16 WOA or 28 WOA unless large tumors necessitated earlier

sacrifice. The results show that the growth of the TRAMP DLP lobes in AGN- and D/DA-treated mice were inhibited by 66 and 61 %, respectively (ANOVA, $P<0.0001$), from vehicle-control mice by 16 WOA. By 28 WOA, the inhibition of TRAMP DLP growth was 67 and 72 %, respectively (ANOVA, $P<0.001$). Whereas AGN and D/DA decreased the burden of NE-Ca, AGN improved survival of tumor-bearing mice, but D/DA did not. In summary, AGN extract and equimolar intake of D/DA inhibited epithelial lineage lesions to identical extent; the extract was superior to D/DA at suppressing NE-carcinogenesis. Ongoing experiments have focused on (1) testing whether D/DA-hydrolysis product DOH mediates the TRAMP epithelial targeting inhibitory action and (2) identifying non-pyranocoumarin compounds in AGN extract that inhibit NE-carcinogenesis.

Anti-cancer Activities of D, DA, and DOH

Cell culture models have been primarily used as screening tools as well as cellular targets for studying potential anti-cancer activities and "mechanisms" of these compounds. Organ sites included prostate, breast, lung, colon, bladder, sarcoma, and blood cancers and have been extensively reviewed in our recent article [1•]. Here, we will focus on those organ sites with available in vivo efficacy information: prostate cancer, lung cancer, colon cancer, and sarcoma.

Prostate Cancer

Our group first identified D from AGN ethanol extract as a novel anti-androgen receptor (AR) compound with an IC_{50} of 1.3 μM upon 48 h of exposure in the AR-dependent human prostate cancer cell line LNCaP measured by prostate-specific antigen (PSA) secretion [19]. Subsequently, we reported the anti-AR effect of D, DA, and DOH in terms of their structure-activity relationship [20]. Decursin and DA inhibited PSA levels in a dose-dependent manner with IC_{50} to be 1~1.3 μM and suppressed AR levels through proteasomal degradation and blocked androgen-stimulated AR nuclear translocation. In addition, D and DA lacked the agonist activity of AR antagonist drug bicalutamide in the absence of androgen and were more potent than bicalutamide for suppressing androgen-stimulated cell growth [19]. On the other hand, the effect of DOH on PSA expression is biphasic, inhibiting PSA at lower micromolar range yet stimulating at higher concentrations [20]. In a high-throughput imaging-based multiplex screening for AR functional analyses, Szafran et al. also demonstrated a unique response pattern in the presence of synthetic AR ligand methyltrienolone (R1881) [21]. In combination with 10 nM R1881, D caused an antagonistic response (45 % decrease in AR transcriptional reporter gene activity, EC_{50}=13.2 μM). However, it decreased R1881-induced nuclear translocation (95 %, EC_{50}=12.3 μM). The small nuclear pool also exhibited a loss of hyperspeckling (94 % in NVAR, EC_{50}=10.6 μM) [21]. These findings further suggested AR as one target in the in vitro anti-prostate cancer activity of D and DA.

Others reported mechanisms other than AR suppression in the anti-prostate cancer effect of D. Yim et al. reported that D (25–100 μM) inhibited the cell growth in human PCA cells DU145, PC-3 (both lines are AR-negative), and LNCaP [22]. The D-induced growth inhibition was associated with a strong G_1 arrest in DU145 and LNCaP cells and G_1, S as well as G_2-M arrests, depending upon doses and treatment times in PC-3 cells. Interestingly, non-cancerous "normal" human prostate epithelial PWR-1E cells were moderately growth arrested but was not killed. This indicated that D might offer some advantage in drug selectivity [22]. Another study reported that D suppressed the Wnt/β-catenin pathway in PC-3 cells [23]. In this cell model, D promoted β-catenin degradation and suppressed the expression of the related downstream factors cyclin D1 and c-*myc*. Unlike D, DOH had no effect on intracellular level of β-catenin and PC-3 proliferation. More recently, Choi et al. reported that D induced apoptosis in primary malignant tumor-derived human prostate cells, RC-58T/h/SA#4, and investigated the potential mechanisms [24]. They found RC-58T/h/SA#4 cells slightly more sensitive to D than human prostate epithelial RWPE-1 cells. Treatment of the RC-58T/h/SA#4 cells with 80 and 100 μM D for 48 h significantly induced apoptosis, evidenced by nuclear condensation, DNA fragmentation, PARP cleavage, and increased caspase activities.

It is noteworthy that the cellular events mentioned in these papers were observed when cells were treated with D at the concentrations much higher (10–50 fold) than that have been reported for AR suppressing effect [19, 20]. As will be discussed in pharmacokinetics sections later, even single micromolar levels of D or DA will be not likely achievable in rodents or humans by oral route of intake.

Lung Cancer

In a study using mouse Lewis lung cancer (LLC) model, daily administration of D of 4 mg/kg by i.p. injection for 3 weeks reduced tumor growth by 65 % [25]. Tumor microvessel density was dramatically lower in D-treated group as evidenced by CD31 immunohistochemistry staining. The expression levels of phospho-VEGFR2, p-ERK (extracellular signal-regulated kinase), p-JNK (c-Jun N-terminal kinase), and matrix metalloproteinase (MMP)-2 were also significantly reduced compared with control tumors. These observations suggested that an anti-angiogenesis effect of D (or metabolites) might contribute to its tumor suppression efficacy [25]. We also evaluated the effect of D and DOH in the LLC allograft model [8]. In that study, 50 mg/kg D or DOH was administered daily

in 1 % Tween 80 by gavage for 13 consecutive days starting at 5 days after LLC tumor inoculation. Tumor volume and weight were suppressed by about 40 % by either D or DOH. Though the in vitro activities of D and DOH were different as we and others have reported for prostate and breast cancer cells [20, 22, 26], the data suggest that orally administrated D and DOH were equally efficacious against LLC allograft.

Colon Cancer

In murine colon carcinoma CT-26 cells, even though 10 to 20 μM D and DOH marginally reduced the viability and proliferation of CT-26 cells in vitro, both compounds substantially inhibited their invasive activity [27]. DOH exhibited a stronger anti-invasive potential than D. Mechanistic studies indicated that D and DOH suppressed both the expression and activities of MMP-2 and MMP-9, the latter being more important for the invasion of CT-26 cells. Phosphorylation of ERK and JNK, which played a critical role for the invasion of CT26 cells through modulating the expression of MMP-9, was found to be downregulated by D and DOH treatment. In addition, daily oral administration of D and DOH (10 mg/kg for 14 days) starting 30 min prior to the injection of cancer cells through tail vein reduced the formation of tumor nodules in the lungs and decreased lung weight caused by CT-26 metastases. The data suggested that DOH and D (most probably through conversion to DOH, see pharmacokinetic sections) might be anti-metastatic agents targeting MMPs and their upstream signaling molecules [27].

Sarcoma

In a mouse allograft study, D or DA, at doses of 50 and 100 mg/kg by i.p. injection daily for 9 days, suppressed Sarcoma-180 tumor growth in ICR mice [28]. The authors also showed that D or DA each was able to extend the life span of mice inoculated Sarcoma-180 tumor cells by i.p. injection: the median survival times were 32.3 and 34.2 days for 50 and 100 mg/kg DA-treated groups, respectively, and were 29.0 and 39.6 days for 50 and 100 mg/kg D-treated groups, respectively, compared with the median survival time for the control group of just 22.4 days [28].

Anti-angiogenesis Effects

In addition to cancer cell models, it has been reported that D and DOH at non-cytotoxic doses (1–20 μM) reduced VEGF-induced cell proliferation, migration, and capillary tube formation of human umbilical vein endothelial cells (HUVEC) [29]. The anti-angiogenic effect by D and DOH was possibly associated with suppression of ERK and JNK activation. Additionally, D and DOH suppressed microvessel formation on chorioallantoic membranes in fertilized eggs and in mouse Matrigel plugs. Moreover, orally administered D and DOH inhibited VEGF-induced angiogenesis in Matrigel plugs [29]. Others reported similar results about anti-angiogenesis effect with D and DA in association with inhibition of VEGF-induced phosphorylation of VEGFR-2, extracellular signal-regulated kinases, c-Jun N-terminal kinase, and mitogen-activated protein kinases and suppression by D of LLC lung allograft tumor growth in vivo [25].

Anti-cancer Activities of Chemicals in AGN Other Than D/DA

We reviewed other chemicals other than D and DA isolated from AGN that have been evaluated by the NCI Developmental Therapeutics Program for the in vivo ability to increase survival of allograft tumor-bearing mice (http://dtp.nci.nih.gov/docs/dtp_search.html) [1•]. Xanthotoxin might afford a reasonable anti-cancer activity in 25–64 mg/kg dosage range that increased survival time of the treated mice. Its dose-limiting lethality was at 256 mg/kg. Scopoletin was about one tenth as potent for anti-cancer (400 mg/kg), without detectable lethality to the host mice. Marmesin, umbelliferone, and bergapten treatment did not result in a survival advantage in the dosage ranges used. A recent paper indicated that five chemicals isolated form AGN including marmesinin, nodakenin, skimmin, apiosylskimmin, and magnolioside showed no significant cytotoxicity (IC_{50}>100 μM) toward SK-OV-3 ovarian cancer cell line in vitro, though marmesinin and magnolioside exerted notable anti-plasmodial activity against chloroquine-sensitive strains of *Plasmodium falciparum* with IC_{50} of 5.3 and 8.2 μM, respectively [30].

Angelan is a 10-kDa polysaccharide purified from AGN, composed of arabinose, galactose, and galacturonic acid but does not contain glucose [31]. It has been reported to lengthen the survival time of B16F10 tumor-bearing mice when i.p. administered at 30 mg/kg [32]. The primary targets of angelan were assumed to be macrophages and natural killer (NK) cells which were involved in nonspecific immunity [31, 32]. Angelan markedly induced transcription of inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β), and tumor necrosis factor α (TNF-α) in murine macrophage RAW 264.7 cells, which was mediated by specific activation of NF-κB/Rel [33, 34]. Another study claimed the macrophage activation by angelan differed from that induced by bacterial lipopolysaccharide (LPS) without strong data [35]. Further studies indicated that angelan induced NF-κB/Rel activation through CD14 and complement receptor type 3 (CR3) membrane receptor and p38 kinase; the latter is critical for the signal transduction leading to NF-κB/Rel activation in murine macrophages [36]. Later on, the same group investigated the effects of angelan on dendritic cell (DC) maturation and found a crucial role of TLR4 signaling pathways [37]. These results

suggested the possible use of angelan in DC-based immunotherapies. However, the alcoholic extract of AGN sold as dietary supplements should contain little angelan, if any.

Pharmacokinetics of D, DA, and DOH in Rodent Models

Although D and DA are mainly responsible for the cancer cell arrest actions in cell culture models, available rodent studies have shown a rapid conversion of D and DA to DOH, casting grave doubts over the in vivo mechanistic relevance of D and DA. Park et al. detected DOH (~80 μg/ml) in the plasma of rats after administration of 300 mg/kg D or the methanolic or ether extract of AGN while detecting no D [38]. We detected DOH as the major metabolite after i.p. injection and failed to detect the parent compounds in plasma [8]. We then systematically evaluated a method for the simultaneous analysis of D/DA and DOH in biological matrixes by HPLC and applied the method to the detection of plasma or tissue levels of these analytes in several in vivo studies [39]. In male C57BL/6 mice given a single dose of 4.8 mg D/DA (~240 mg/kg) either by gavage or i.p. injection, we detected that plasma concentrations for D/DA and DOH were 11.2 and 79.7 μg/mL, respectively, when D/DA was administered via i.p. injection, and 0.54 and 14.9 μg/mL when given by gavage. D/DA and DOH content in the tumor tissues from nude mouse xenografts correlated very well with that in plasma. Overall, our results affirm the conclusion that majority of D and DA converts to DOH in rodents with a tiny fraction remaining as the parent compounds.

The specific organ site(s) for the conversion of D and DA is likely the liver by first pass metabolism when they are administrated by routes other than *i.v.* infusion, as demonstrated recently [40•]. Plasma D decreased rapidly after i.v. administration with an initial half-life of 0.05 h and was not detectable after 1 h. The concentration of DOH increased rapidly, reaching peak at t_{max}=0.25 h, and the area under the plasma concentration-time curve (AUC_{DOH}) was 4.3-folds of AUC_D. In portal vein-cannulated rats, D was the major pyranocoumarin in blood collected from the portal vein after gavage, whereas DOH was the dominant chemical in blood circulation if D was administered through the portal vein with the fraction of dose escaping the liver extremely low (FH= 0.11).

Absorption of DOH itself in rats was found quite efficient [41]: following i.v. administration, non-linear elimination of DOH was observed; whereas after oral administration, DOH exhibited rapid absorption (t_{max} 0.4–0.9 h), resulting in bioavailability of ~45 % (AUC_{oral}/AUC_{iv}). Following up our mouse study, we compared the plasma profiles of DOH, when equimolar D/DA or DOH was given to rats by gavage [42]. DOH gavage led to a faster attainment of plasma DOH peak (t_{max}~0.7 h) and much higher peak levels than an equimolar amount of D/DA mixture or as AGN ethanol extract, resulting in 2.4-folds higher AUC (65,012 vs. 27,033 h ng/mL for DOH and D/DA groups, respectively). We detected peak levels of D and DA in the plasma of rats administered with D/DA or AGN extract only in the nanomolar range (t_{max}~0.5 h) with a more sensitive UHPLC-MS/MS method.

Contradicting the above, a Korean group reported that both D and DA were detectable at high concentrations in rat plasma up to 72 h after the D/DA mixture was administered by either gavage (200 mg/rat) or i.v. injection (2 mg/rat) [43]. There are many troubling aspects of this study including the near-total absorption of the gavage dose.

In summary, the structural difference between DOH and D/DA is obvious and the natural abundance of DOH in AGN is much lower than D and DA, if at all. We and others documented the different in vitro biological activities of DOH compared to D/DA such as anti-AR and AChE inhibitory effect [20, 44]. On the other hand, others have reported similar in vitro anti-angiogenesis and neuro-protective effects of D, DA, and DOH [25, 29, 45]. It is likely that DOH and/or its further metabolite(s) are the real in vivo "effector" for the many observed effects, i.e., D and DA act as natural prodrugs of DOH.

Pharmacokinetics of D and DA in Humans

Given the first pass liver metabolism in rodents, we compared the metabolic rate of D and DA using liver microsomes from humans and rodents and identified potential metabolites of D and DA in human liver microsomal incubations using LC-MS/MS [46•]. In murine and human liver microsomal incubations in the absence of NADPH, D was efficiently hydrolyzed to DOH presumably by hepatic esterases, but DA was not (see proposed metabolism schema in Fig. 1b). In contrast, formation of DOH from DA was mediated mainly by cytochrome P450(s) requiring NADPH. The human liver S9 fractions metabolized both D and DA more slowly than those of the mouse. We identified nine potential metabolites of D and nine metabolites of DA from human liver microsomal incubations using a hybrid triple quadruple linear ion trap LC-MS/MS system, and many of them were later verified to be also present in plasma samples from rodent PK studies. In contrast, D and DA were not hydrolyzed to DOH with rat intestinal S9 microsomal in vitro metabolism assay with or without NADP H [42].

To address a key question for human translatability of animal model studies of D and DA or AGN extract, we conducted a single oral dose human PK study of D and DA delivered through an AGN-based dietary supplement Cogni.Q (purchased from Quality of Life Labs, Purchase, NY) in 10 healthy men and 10 healthy women, each consuming

119 mg D and 77 mg DA from four vegicaps [47]. Analyses of plasma using UHPLC-MS/MS showed mean time to peak concentration (t_{max}) of 2.1, 2.4, and 3.3 h and mean peak concentration (C_{max}) of 5.3, 48.1, and 2480 nmol/L for D, DA, and DOH, respectively. The terminal elimination half-life ($t_{1/2}$) for D and DA was similar (17.4 and 19.3 h) and each was much longer than that of DOH (7.4 h). The mean AUC_{0-48h} for D, DA, and DOH was estimated as 37, 335, and 27, 579 h nmol/L, respectively. Gender-wise, men absorbed the parent compounds faster and took shorter time to reach DOH peak concentration. The human data supported an extensive conversion (>97 %) of D and DA to DOH, even though they metabolized DA slightly slower than rodents. Therefore, the data generated in rodent models concerning anti-cancer efficacy, safety, tissue distribution, and pharmacodynamic biomarkers will likely be relevant for human translation.

Conclusions and Future Research Directions

PK studies in rodent models and in humans have provided unequivocal evidence that D and DA from dietary supplements or enteral route are extensively converted to DOH by first pass metabolism in the liver, resulting in very low levels of these parent compounds in peripheral circulation (Fig. 1b). Such insights cast grave doubt over the relevance of the "mechanisms" of D and DA in cell culture studies. In a primary carcinogenesis prevention study, we have observed that gavage-administered equimolar D/DA does not fully account for the preventive efficacy of AGN extract on TRAMP neuroendrocrine carcinomas, in spite of equal inhibitory action on the TRAMP prostate epithelial lesions.

Therefore, in the near future, the following should be addressed.

1. Given that DOH has been identified unequivocally as the major and rapid metabolite of D and DA in rodents and humans, experiments are needed to compare its in vivo efficacy with the parent compounds at PK-bioequivalent dosages to determine how much these pyranocoumarins contribute to anti-cancer activities of AGN.
2. Knowledge of DOH further metabolism should be obtained. DOH has been reported to exhibit high stability to oxidative and glucoronic metabolism in human and rat liver microsomes in vitro [41]. The in vivo DOH metabolites should be investigated. Since CYP450 is implicated in DA and, to less extent, D hepatic conversion to DOH through oxygenated intermediates [46•, 48] (Fig. 1b), the extent and contribution of these metabolic intermediates should not be overlooked nor the impact and interactions with CYP450 inhibitor drugs.
3. The pharmacodynamic targets of DOH and its metabolites should be identified, in cancer cells and cells that make up the tumor microenvironment. Our human PK data further motivate such endeavor because the likelihood for D and DA to directly bind proteins such as AR and AChE associated with anti-cancer and neuro-protective effects would be extremely low, if not nil. So far, Kang et al. has identified a few DOH-binding proteins from brain tissue by affinity chromatography and mass spectrometry [49]. They include heat shock protein 90-kDa beta member 1 (GRP94 or HSP90B1), enolase 1 (ENO1), actin gamma 1 (ACTG1), heterogeneous nuclear ribonucleoprotein A2/B1 (HNRNPA2B1), and non-muscle myosin heavy polypeptide 9 (MYH9). The search strategy might be applicable to cancer and other cell types in the cancer microenvironments.
4. Besides pyranocoumarins, other components in AGN extract should be investigated. Whether these low abundant chemicals in AGN mediate or work in synergy with D/DA/DOH to exert anti-cancer activity remain an open question.

Acknowledgments Supported by National Center for Complementary and Integrative Health (NCCIH/NCCAM) grants R01AT007395 (Lü, Xing) and R21AT005383 (Lü).

Compliance with Ethics Guidelines

Conflict of Interest Junxuan Lü, Jinhui Zhang, Li Li, Cheng Jiang, and Chengguo Xing declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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Papers of particular interest, published recently, have been highlighted as:
• Of importance
•• Of major importance

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