An oriental herbal cocktail, ka-mi-kae-kyuk-tang, exerts anti-cancer activities by targeting angiogenesis, apoptosis and metastasis

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Introduction

Cancers of the lung, prostate and colorectum account for the majority of cancer mortality in the USA and in the Western countries (1). Chemotherapy using available cytotoxic anti-cancer drugs for advanced-stage malignancies in the lung and prostate offers little survival benefit (2,3). All current chemotherapy modalities have significant side effects and dose-limiting toxicities (2,3). It is now being increasingly recognized that intervening critical processes of cancer growth and development including angiogenesis and metastasis with naturally occurring herbal and phytochemical agents to achieve chemoprevention is crucial to decreasing the morbidity and mortality of these and other cancers.

Chinese/Oriental herbal medicine has long been used for treating malignancies. Whereas single herbs are seldom used alone, herbal cocktails take advantage of synergy and interactions among a myriad of phytochemicals present in the different herbs to achieve therapeutic efficacy targeting multiple biological and pathological processes while minimizing side effects. However, herbal remedies are yet to be integrated into mainstream medicine due to a number of challenges, including herbal standardization and quality control issues, safety and toxicity concerns, interactions with existing therapeutic modalities, a lack of proven efficacy by standard clinical trials and a lack of mechanistic details, to name a few (4). Rigorous in vitro and pre-clinical animal studies will be essential and necessary to evaluate their efficacy and safety before clinical trials can be contemplated for the chemoprevention and treatment of these major cancers in humans and to transform traditional herbal practices into ‘evidence-based medicine’.

Here we report the evaluation of an anti-cancer formula of ten Oriental herbs, Ka-mi-kae-kyuk-tang (KMKKT) (5), with a battery of in vitro and in vivo pre-clinical tests. In addition to tumor cell proliferation and apoptosis, we focused on angiogenesis assays because of the central importance of angiogenesis in supporting tumor growth and metastasis (6–9). Angiogenesis in embryonic development, reproduction and wound healing is tightly regulated by a balance between the angiogenic inhibitors and activators and in neoplasia, the delicate balance is tilted to favor increased angiogenesis (6–9). In terms of the angiogenic activators, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) have been well documented (10–12). Even though VEGF is a primary mediator of angiogenic responses, bFGF is more potent than VEGF for stimulating the vascular endothelial mitogenesis. Hence bFGF is the most often used angiogenic polypeptide for experimental studies (13) and we used it for our angiogenesis tests in the current work. Tumor

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Abbreviations: bFGF, basic fibroblast growth factor; CAM assay, Chick chorioallantoic membrane assay; CM, conditioned medium; ERK, extracellular signal-regulated kinase; HIF, hypoxia-inducible factor; IHC, immunohistochemistry; KMKKT, Ka-mi-kae-kyuk-tang; LLC, Lewis lung carcinoma; PCNA, proliferating cell nuclear antigen; PARP, poly(ADP ribose) polymerase; VEGF, vascular endothelial growth factor.

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growth and progression require angiogenesis because in its absence tumor growth is restricted to 1–2 mm in diameter due to the physical constraint set by simple diffusion of nutrients and oxygen (6–12). In addition, angiogenesis and vascularization provide an important avenue for the extravasation of metastatic tumor cells to the circulation and for them to re-establish in the other organ sites. Consequently, the inhibition of neo-angiogenesis has been considered an attractive and rationale approach to cancer chemoprevention and therapy (14,15). Thus, in the present study, we focused on the anti-angiogenic effects of KMKKT for screening purposes. We tested the in vivo efficacy of this formula against three models of mouse and human solid tumor growth or metastasis. The results support the merit of this cocktail for the chemoprevention and therapy of cancers of multiple organ sites.

Materials and methods

Ka-mi-kae-kyuk-tang
This formula consists of ten Oriental medicinal herbs (Table 1). It was formulated for use by lung cancer patients. The ethanol extraction procedure was as described previously (5). The ethanol extract was dried in vacuo and reconstituted in either dimethylsulfoxide (DMSO) for the in vitro studies or in 1% Tween-80 for the animal tumor studies as appropriate.

Cell culture
HUVECs were isolated from fresh human umbilical cord veins by collagenase treatment (16,17). The cells were cultured in M199 medium (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (FBS), 3 ng/ml bFGF (R&D systems, Minneapolis, MN), 5 U/ml heparin and 100 U/ml antibiotic-antimycotics in 0.1% gelatin-coated flasks. Mouse Lewis lung carcinoma (LLC) cells were kindly provided by Dr Ikuko Saiki (Toyama Medical and Pharmaceutical Univ., Toyama, Japan) and cultured in EMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 100 U/ml antibiotic-antimycotics and 2.2 g/l sodium bicarbonate (17). A metastatic mouse colon cancer cell line, colon 26-L5, was also obtained from Dr Ikuko Saiki (18) and cultured with RPMI 1640 medium supplemented with 10% FBS, 100 U/ml antibiotic-antimycotics and 2 g/l sodium bicarbonate. The human androgen-independent PC-3 prostate cancer cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were grown in F-12K medium with 10% FBS without antibiotics. All cells were grown at 37°C in a humidified atmosphere containing 5% CO2 except specially noted for hypoxia treatment.

Cell viability and proliferation assays
The effect of KMKKT on the viable cell number was assessed using a mitochondrial reduct substrate XTT as described previously (17). Cell proliferation was determined using a 5-bromo-2'-deoxyuridine (BrdU) assay kit (Roche, Sanhofer, Mannheim) according to the manufacturer’s protocols as described previously (17). Briefly, HUVECs (5 x 104 cells/well) were seeded onto 0.1% gelatin-coated 96 well plates and incubated in a humidified incubator for 24 h. After an incubation for 6 h in M199 medium containing 5% heat-inactivated FBS to decrease mitogen signaling, the cells were exposed to various concentrations of KMKKT in the presence or absence of bFGF (5 ng/ml) for 48 h at 37°C. To label proliferative cells actively engaged in DNA synthesis, BrdU was added for an additional 6 h. The cells were fixed and incubated with anti-BrdU and detected by the substrate reaction. The absorbance was measured using a microplate reader (Molecular Devices, USA) at 450 with 690 nm correction.

Migration/invasion assays
HUVEC migration assay was performed using a modified 48-well microchemotaxis chamber (Nuero Probe, Cabin John, MD). Polyester membranes (12 μm pores) were coated with 0.1% gelatin for 2 h and dried. The bottom chamber was loaded with M199 medium containing 0.1% bovine serum albumin (BSA) and bFGF (5 ng/ml). The coated membrane and upper chamber were laid over the bottom chamber. The upper chamber wells were then loaded HUVECs (4 x 104) in M199 medium containing 0.1% BSA with variable levels of KMKKT. After 3 h incubation, the membrane was fixed with Diff-Quick fixative and stained with Diff-Quick Sol. I and II and randomly chosen fields were photographed at x100 magnification and migrated cells were counted. For the invasion assay of colon 26-L5 cells through Matrigel-coated filter pores, cells were loaded and simultaneously treated for 24 h with KMKKT, processed and counted as above for HUVECs.

Tube formation assay
HUVEC tube formation on Matrigel was performed as described previously (17,19). Matrigel (200 µl, Becton Dickinson Labware, Bedford, MA) was added to 24-well plates and allowed to solidify for 30 min at 37°C. HUVECs (3 x 104 cells/well) were treated with various concentrations of KMKKT in the absence or presence of bFGF (5 ng/ml). After 7 h, cells were fixed with 4% paraformaldehyde and randomly chosen fields were photographed at x100 magnification.

Chick chorioallantoic membrane (CAM) assay
The anti-angiogenic activity was assessed using the CAM assay as described previously (17). Briefly, KMKKT and bFGF (100 ng) were spotted onto 1/4 piece of thermomonox disk (Nunc, Naperville, IL). The dried thermomonox disk was applied to the CAM of a 10-day-old embryo. After 72 h incubation, a fat emulsion was injected under the CAM for better visualization of the blood vessels. The number of newly formed blood vessels was counted. The experiment was repeated twice with 15 eggs for each group.

Mouse Matrigel plug assay
This in vivo angiogenesis assay was performed as described previously (17,20,21). Briefly, 6-week-old C57BL/6 mice (Daehan Biolink, Chungbuk, Korea) were each given a subcutaneous injection of 0.5 ml of the growth factor-reduced Matrigel containing KMKKT, bFGF (300 ng) and heparin (10 U). After 7 days, mice were killed, and the Matrigel plugs were removed. To quantify the formation of functional blood vessels, the amount of hemoglobin (Hb) was measured as reported in Passaniti et al. (20) (kit 525, Sigma Chemical, St Louis, MO).

Mouse LLC-tumor model
We chose this model because of its rapid growth in the conventional syngenic mice and its strong dependence on angiogenesis. We have used this model to study the in vivo effects of a number of novel agents (17,22,23). LLC cells (3 x 105 in 100 µl PBS) were subcutaneously injected into the right flank of C57BL/6 mice. Three days after tumor inoculation, mice were each given an intraperitoneal (i.p.) injection of either solvent vehicle (1% Tween-80) or KMKKT every other day. Tumor dimensions were measured with a caliper, and the volume was calculated according to the formula [(L x W²)/2], where L and W stand for length and width, respectively. All mice were killed 10 days after tumor inoculation and the tumors were excised, weighed and fixed in 10% neutral buffered formalin overnight for histology and immunohistochemistry (IHC).

Mouse colon cancer hepatic metastasis model
Five-week-old female Balb/c mice were purchased from Daehan Biolink, (Chungbuk, Korea) and given food and water ad libitum. The mice were each given a daily i.p. injection of either solvent vehicle or KMKKT throughout the study duration. Briefly, on Day 9 of treatment, mice were inoculated with colon 26-L5 cells through an intraperitoneal injection as reported (18). Mice were killed 15 days after tumor inoculation, and the liver weight was measured. Livers were fixed in 10% neutral buffered formalin overnight. Thin sections of 4 μm were stained with hematoxylin and eosin. Representative fields (x100) for each group were photographed.

### Table I. The composition of KMKKT

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<td>China</td>
<td>15</td>
<td>8.62</td>
</tr>
<tr>
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<td>15</td>
<td>8.62</td>
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<td>Panax ginseng (root)</td>
<td>Korea</td>
<td>15</td>
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</tr>
<tr>
<td>Phaseolus angularis (seed)</td>
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<td>17.24</td>
</tr>
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<td>12</td>
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</tr>
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<td>8.62</td>
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<tr>
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**Human PC-3 prostate cancer xenograft model**

This animal use protocol was approved by IACUC of the University of Minnesota. Six-week-old male athymic Balb/C nude mice were purchased from NxGen, San Diego, CA. The mice were maintained in the HEPA-filtered caged room and quarantined for 2 weeks in a limited-access specific pathogen free room at the Hormel Institute Animal Facility (Austin, MN). Mice were given free access to a commercial rodent chow and water. PC-3 cells (1 x 10^6, in 0.1 ml volume containing 20% Matrigel) were injected subcutaneously into the left flank of each mouse. Tumor size was measured twice per week as above. Starting 3 days after the tumor cell inoculation, KMKKT or the solvent vehicle (1% Tween-80) was administered by i.p. injection once a day for 7 days, then three times per week during the experiment. The tumors were excised and weighed. A portion of each large tumor (>0.3 g) was snap-frozen in liquid nitrogen for biochemical analyses by western blot, and the rest of the tumor as well as all small tumors were fixed in 10% neutral buffered formalin for histology and IHC.

**IHC and western analyses**

Analyses by IHC of the proliferating cell nuclear antigen (PCNA) as an IHC and western analyses by western blot, and the rest of the tumor as well as all small tumors were fixed in 10% neutral buffered formalin for histology and IHC.

**Results**

**KMKKT inhibits multiple in vitro angiogenic attributes of HUVEC**

The angiogenic response by the vascular endothelial cells involves a number of concerted events such as the angiogenic factor-stimulated membrane receptor-tyrosine kinase signaling to ERK/MAPKs (extracellular signal-regulated kinase/mitogen-activated protein kinases) and other protein kinases, an increased motility, and matrix remodeling and eventually the endothelial cell division to produce needed cells for elongating the growing capillary 

(KMKKT inhibited not only HUVEC migration toward bFGF when evaluated in a Borden Chamber assay (Figure 1C), but also the formation of capillary-like structures sustained by the bFGF stimulation when the HUVECs were seeded on Matrigel (Figure 1D). These responses were more striking than the anti-proliferative action, each being very profound at the treatment concentration of 25-100 µg/ml without changing the total ERK levels when examined at 30 min after the bFGF treatment was initiated (total KMKKT exposure duration is 1 h) (Figure 1A).)

To assess the anti-angiogenic potential of KMKKT, we carried out a battery of biochemical and cell-based assays using HUVECs as the endothelial cell target. Pre-incubation with KMKKT for 30 min blocked the bFGF-stimulated ERK1/2 phosphorylation within the treatment concentration range of 25-100 µg/ml without changing the total ERK levels when examined at 30 min after the bFGF treatment was initiated (total KMKKT exposure duration is 1 h) (Figure 1A).

(KMKKT inhibited the bFGF-stimulated AKT phosphorylation within this time frame (Figure 1A). KMKKT inhibited the bFGF-stimulated BrdU incorporation as a measure of cell proliferation to the same level as the unstimulated cells by a treatment concentration of 125 µg/ml, but not at 62 µg/ml (Figure 1B).

KMKKT inhibited not only HUVEC migration toward bFGF when evaluated in a Borden Chamber assay (Figure 1C), but also the formation of capillary-like structures sustained by the bFGF stimulation when the HUVECs were seeded on Matrigel (Figure 1D). These responses were more striking than the anti-proliferative action, each being very profound at the treatment concentration of 25 µg/ml and above. This battery of tests showed that KMKKT potently modulated a number of the endothelial cellular processes important for angiogenesis.

Regarding the tumor epithelial cells, hypoxia is common in the rapidly growing tumor mass and is a potent inducer of the expression of the primary angiogenic factor VEGF in the tumor epithelial cells. The induction of VEGF is principally mediated transcriptionally by the hypoxia-inducible factor (HIF)-1α (24,25). Indeed, when the mouse LLC cells were subjected to hypoxia, both the HIF-1α and VEGF mRNA (Figure 1E, lane 1, hypoxia versus lane 3, normoxia) and protein expression levels (Figure 1E, lane 5, hypoxia versus lane 4, normoxia) were greatly increased compared with normal oxygen tension. Inclusion of KMKKT significantly decreased the induction of either factor by hypoxia at the mRNA level (Figure 1E, lane 2 versus lane 1) and the protein level (Figure 1E, lane 6 versus lane 5). As a result, the VEGF secretion by the hypoxic LLC cells into the conditioned medium (CM) was decreased by ~50% (Figure 1E, bar graph, KMKKT versus control). The potency of CM from the KMKKT-treated LLC cells to induce HUVEC tube formation was decreased accordingly (Figure 1F, panel b versus a).

Taken together, these biochemical and cell-based assays suggest a strong anti-angiogenic potential of the KMKKT formula through not only a suppression of the bFGF-stimulated endothelial membrane receptor-tyrosine kinase signaling to ERK1/2, cell motility and capillary differentiation, and to a less extent their mitogenesis, but also an inhibition of the hypoxia-induced tumor epithelial expression of HIF1α and VEGF, dampening the angiogenic stimuli.

**KMKKT inhibits the bFGF-induced angiogenesis in CAM and Matrigel plug models**

Next, we used the chick CAM and mouse Matrigel plug assays to further establish the anti-angiogenic activities of KMKKT. KMKKT applied to the bFGF-impregnated filter significantly decreased the capillary formation in the CAM (Figure 2A) at both doses tested. In the Matrigel plug assay, the bFGF-loaded plugs from the control mice exhibited bright red color indicating abundant red blood cells in the newly formed vascular structure, whereas the KMKKT/bFGF-loaded plugs showed light yellowish color (Figure 2B, representative photographs). KMKKT significantly decreased the functional angiogenesis as shown by the hemoglobin content at the high dose and the slope of the response curve suggested a trend for a dose-dependent inhibition (Figure 2B, bar graph). These experiments provided strong evidence of the anti-angiogenic activities of the KMKKT cocktail in vivo.

**KMKKT suppresses tumor growth in LLC-bearing mice**

The strong anti-angiogenic properties of KMKKT prompted us to evaluate its safety and efficacy to inhibit the mouse LLC-tumor growth in syngenic C57BL/6 mice. Tumor growth in this model is very rapid and has been shown to be efficiently inhibited by several natural or synthetic agents with anti-angiogenesis and anti-tumor activities (17,22,23). To ensure efficient uptake bypassing gastrointestinal processing, KMKKT was administered by i.p. injection every other day starting from Day 3 after the subcutaneous inoculation of LLC cells. KMKKT did not cause any change in body weight during the experiment (Figure 3A). KMKKT inhibited the tumor growth kinetics (Figure 3B) and decreased the final tumor size (Figure 3C, photograph) and weight at necropsy (Figure 3C, bar graph) in a dose-dependent manner.

IHC analyses of PCNA (Figure 3D, picture and graph) revealed that KMKKT treatment decreased the number of proliferative cells. TUNEL assay showed that KMKKT treatment increased the tumor cell apoptosis by ~3-fold (Figure 3E). In addition, KMKKT-treated tumors contained fewer vWF-positive stained microvessels (Figure 3F),
consistent with a suppressed tumor angiogenesis in this mouse lung cancer model.

**KMKKT inhibits the invasiveness of mouse colon 26-L5 cells and decreases its metastatic growth in mouse liver**

Motility and invasion through the extracellular matrix are important for the extravasation of cancer cells to disseminate to the other organ sites to form distant metastasis (26,27). Because angiogenesis provides an important avenue for this step, we therefore analyzed the invasion property of the mouse colon 26-L5 carcinoma cells, which are highly metastatic toward the liver (18), in the Matrigel-coated 48-well microchemotaxis chambers. After 24 h treatment, randomly chosen fields were photographed and the cells that had migrated through the filter were counted. As shown in Figure 4A, KMKKT significantly decreased the cell invasion with an IC_{50} of \( \sim 25 \) μg/ml. Within the treatment period of 24 h, the cancer cell viability was not significantly affected by KMKKT in the concentration range tested (Figure 4B).

These in vitro observations prompted us to test the ability of KMKKT to inhibit the experimental liver metastasis produced by an intraportal injection of colon 26-L5 cells to the syngenic Balb/c mice (18). The mice were each given a daily i.p. injection of either solvent vehicle (1% Tween-80) or KMKKT throughout the experiment duration. On Day 9 of treatment, they were inoculated with the colon 26-L5 cells. Fifteen days after tumor inoculation, the liver of vehicle-treated mice contained numerous visible pale tumor nodules (Figure 4C; compare control with normal liver). KMKKT treatment significantly improved the gross appearance of the
liver toward the size and dark red color of the normal mice as its dosage was increased (Figure 4C, pictures). The liver weight was increased by the metastatic tumor load in the vehicle-treated control mice by $>5$-fold in comparison with normal mice not inoculated with the tumor cells, and KMKKT treatment resulted in a dose-dependent reduction of liver weight, attaining a complete normalization with the 100 mg/kg dose (Figure 4C, graph). Histological examination of the liver sections confirmed the displacement of hepatocytes by the metastatic colon 26-L5 cells in the vehicle-treated control mice, and increasing normalization of the liver morphology as the KMKKT treatment dosage was increased (Figure 4D). These results provided strong support for a potential for KMKKT to prevent and inhibit cancer metastasis and could be useful for the secondary prevention of cancer recurrence in cancer patients.

**KMKKT inhibits the growth of PC-3 human prostate cancer xenograft in nude mice**

Because the LLC lung cancer model and colon 26-L5 liver metastasis model were of mouse origin in the immunocompetent syngenic mice strains, we next evaluated the efficacy of KMKKT on the growth of PC-3 human prostate cancer cells as xenografts in the immunocompromised athymic Balb/C nude mice. PC-3 cells are androgen-independent, representing a very aggressive stage of prostate cancer. Treatment was started 4 days after cell inoculation with daily injection of KMKKT for the first 7 days, then...
three times (M, W, F) per week until 36 days when the mice were sacrificed. The KMKKT treatment did not result in any adverse effect on the body weight of the mice (Figure 5A). Instead, the vehicle-treated control mice experienced severe body weight loss in the last week of the experiment, which was prevented by the KMKKT treatments (Figure 5A). The KMKKT treatment resulted in a dose-dependent reduction of tumor weight at necropsy (Figure 5B). The 100 mg/kg dose led to 68% reduction ($P = 0.007$, two tailed $t$-test). These results have been replicated in a second experiment, with a 45% reduction of tumor weight at the 100 mg/kg dose (0.64 ± 0.1 to 0.35 ± 0.1 g, $n = 15$ mice, $P = 0.015$, two tailed $t$-test). These data indicate that KMKKT is also effective against the growth of aggressive human prostate cancer xenograft in the immunodeficient mouse hosts and does not require immunocompetence to exert the anti-cancer activities.

By IHC analysis, KMKKT-treated tumors contained fewer vWF-positive stained microvessels (Figure 5C), consistent with a suppressed tumor angiogenesis in the xenograft model as well. The TUNEL assay showed a >2-fold increase of the apoptosis index in the PC-3 tumors from the KMKKT-treated mice (Figure 5D). Western blot analyses of selected tumors obtained from the control and the KMKKT-treated mice showed an increased cleavage of PARP in most
KMKKT-tumors (Figure 5E), supporting the caspase involvement in apoptosis in vivo. Furthermore, the abundance of the VEGF 42 and 21 kDa isoforms was lower in the KMKKT-treated group than in the vehicle-control group (Figure 5E). These results were consistent with the involvement of an inhibition of VEGF expression and angiogenesis and an induction of caspase-mediated apoptosis in the human cancer xenografts by the KMKKT treatment.

Discussion

Oriental medicinal herbs are rich sources of potential cancer chemopreventive and therapeutic agents, but require rigorous and systematic in vitro and in vivo pre-clinical evaluations as exemplified in the current work to transform traditional herbal practices into mainstream evidence-based medicine. To this end, we focused our work on a battery of angiogenesis functional assays for screening purposes to provide the rationale for supporting the in vivo tests of the anti-tumor efficacy and safety. The results presented in Figures 1 and 2 strongly support anti-angiogenesis properties of the KMKKT formula and reveal potential cellular and molecular pathways that are affected. These include the bFGF-stimulated endothelial ERK phosphorylation, cell motility, capillary differentiation, on one hand, and the hypoxia signaling to VEGF in the tumor cells, on the other. Further work will be necessary to identify the active herbs and chemical compounds responsible for these activities.

As far as the in vivo anti-tumor/metastasis efficacy and safety are concerned, we demonstrated a dose-dependent suppression by KMKKT of the mouse LLC-tumor growth (Figure 3) as well as the establishment of liver metastasis by...

Fig. 4. (A) Effect of KMKKT on the invasion of the mouse colon 26-L5 cells through Matrigel-coated 48-well microchemotaxis chamber. The cell loading was simultaneous with KMKKT treatment. Randomly chosen fields were photographed at ×100 magnification 24 h later and the migrated cells were counted. Values represent means ± SD, n = 6. ***P < 0.001 versus unstimulated control; **P < 0.01, and ***P < 0.001 versus +BFGF control. (B) Effect of KMKKT on the viability of the attached monolayer colon 26-L5 cells after 24 h treatment and detected by XTT assay. Values represent means ± SD, n = 6. (C) Effect of KMKKT on the experimental liver metastasis produced by an intraportal injection of the mouse colon 26-L5 carcinoma cells in Balb/c mice. Mice were each given a daily i.p. injection of vehicle or KMKKT throughout the study period and were inoculated with the colon 26-L5 cells on Day 9. Fifteen days after the tumor inoculation, mice were sacrificed and the liver weight was measured. The values in the graph represent means ± SD, n = 10. **P < 0.01 and ***P < 0.001 versus control. (D) Histological appearance of representative livers from the mice in C. The livers were fixed in 10% neutral buffered formalin and stained with hematoxylin and eosin. ×100 magnification.
the mouse colon cancer cells (Figure 4). Furthermore, we showed that KMKKT was effective against human PC-3 PCa xenograft growth (Figure 5). In the mouse LLC model (Figure 3D–F) and the PC-3 human cancer xenograft model (Figure 5C–E), our data showed changes of the in vivo biomarkers that were consistent with the involvement of an inhibition of angiogenesis (decreased vWF staining and VEGF expression), an induction of caspase-mediated apoptosis (cleaved PARP, TUNEL-positive staining), and an inhibition of cancer proliferation (decreased PCNA staining) to mediate the anti-cancer growth activity. The PC-3 human xenograft studies are important for two reasons. The first is the demonstration of the in vivo efficacy of KMKKT against human cancer cell growth. Especially considering that PC-3 cells represent advanced androgen-independent prostate cancer, it is reasonable to suggest that KMKKT could be more efficacious against early lesions during carcinogenesis in a chemoprevention context. The second is the immunodeficient nature of the host mice. Because Chinese and Oriental herbal medicine often emphasizes on the enhanced immuno-surveillance and immuno-protection to account for anti-cancer activities, the results of the xenograft studies therefore suggest that KMKKT can exert anti-tumor effects under immuno-compromised conditions, which can be quite common in cancer patients during or after intense chemotherapy and radiation therapies. Taken together, our data strongly suggest that the KMKKT cocktail can be a promising chemopreventive and therapeutic modality by targeting multiple biological and pathological processes critical for cancer growth and metastasis. It should be recognized that in the current work aiming more for the proof of concept than practicality, we used i.p. injection of the KMKKT extract to bypass gastrointestinal processing to establish the anti-cancer efficacies. It will be very important for us to evaluate its efficacies and bioavailability with oral delivery in future work for the practical application of KMKKT in cancer chemoprevention and treatment. Concerning the potential therapeutic use of KMKKT, we want to point out that the manners in which we have evaluated the anti-cancer activities in the LLC model and the PC-3 xenograft model by delivering KMKKT several days after tumor inoculation, and the anti-metastatic activity by administering the formula after 9 days of colon tumor cell inoculation support such a treatment hypothesis for established tumors. This should be tested in future studies.

In addition to the demonstration of the anti-tumor and anti-metastasis efficacies, a salient feature of the animal studies is the lack of any observable adverse effect of KMKKT on the
body weight of the treated mice. As shown in both the immuno-competent C57B mice (Figure 3A) and the immunodeficient Balb/C nude mice (Figure 5A), treatment with the highest tested dose of 100 mg of KMKKT per kg body weight through i.p. injection did not decrease the body weight when compared to the control mice. Especially in the nude mice studies, KMKKT treatment reversed the body weight loss in the PC-3 tumor bearing mice (Figure 5A). This effect could be important for invigorating the cancer patients during and after chemotherapeutic and radiation treatments and for averting the cachexia caused by advanced-stage cancers.

The current work identified multiple biological processes including angiogenesis, apoptosis and metastasis as potential targets of the anti-cancer activities of the KMKKT formula. Guided by the reductionistic paradigm of Western medicine, we are carrying out additional studies to identify the active herb(s) and particularly their active chemical compounds for the various activities observed here. As a test of the feasibility of the activity-guided approach, we have recently discovered a potent anti-androgen signaling activity of KMKKT and identified the pyranocoumarin compound decursin from the Angelica gigas root in KMKKT as a novel anti-androgen compound (5). Conceptually, it will be interesting and important to determine whether the anti-tumor efficacies of KMKKT can be reconstituted by a few key herbs or the active chemicals identified from each.

In summary, the KMKKT cocktail at an i.p. dosage of 100 mg/kg is safe to the mice and is efficacious against angiogenesis, solid tumor growth and metastasis in four in vivo models. These desirable activities merit a serious consideration for the evaluation of KMKKT in primary carcinogenesis models for cancer chemoprevention and in additional pre-clinical models for therapeutic applications to lay a solid foundation for translational work in humans.

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Conflict of Interest Statement: None declared.

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