Chemopreventive allylthiopyridazines inhibit invasion, migration and angiogenesis in hepatocarcinoma cells

EUN-JUNG LEE, ILCHUNG SHIN, SOON-KYOUNG KWON, HEA-SOON SHIN and AREE MOON

College of Pharmacy, Duksung Women's University, Seoul 132-714, Korea

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Abstract. Numerous studies have revealed the chemopreventive and hepatoprotective activities of dietary and synthetic organosulfur compounds. We previously showed that synthetic allylthiopyridazine derivatives, designated as K compounds, induced apoptosis in SK-Hep-1 hepatocarcinoma cells. In order to extend our program to pursue the chemopreventive potential of these compounds, we investigated the effects of the K compounds on invasive and migrative properties of the SK-Hep-1 cells in this study. Here, we show that 3-methoxy-6-allylthiopyridazine (K6) and 3-propoxy-6-allylthiopyridazine (K17) efficiently inhibit SK-Hep-1 cell invasion and migration. A prominent downregulation of matrix metalloproteinase (MMP)-2, but not MMP-9, was observed, presenting MMP-2 as a potential target molecule for the anti-invasive and anti-migrative activities of the compounds. Since hepatocarcinoma is characterized as a hypervascular tumor, we examined the effect of the compounds on angiogenesis of human umbilical vein endothelial cells (HUVECs). The K compounds exerted anti-angiogenic activity, supporting that the development of these compounds would be a promising approach for treatment of hepatocarcinoma. Taken in conjunction with the fact that hepatocellular carcinoma is one of the most lethal malignancies, our findings may be critical to the chemopreventive potential of these synthetic organosulfur compounds for hepatocarcinoma.

Introduction

Cancer metastasis represents the most important cause of cancer death and anti-tumor agents that may inhibit this process have been extensively pursued. Invasion and metastasis of malignantly transformed cells is a multi-step process, which involves detachment of cells from the primary tumor, attachment to the extracellular matrix (ECM), degradation of the ECM components and migration of cells through degraded matrix (1). A role for members of matrix metalloproteinase (MMP) family on tumor invasion and metastasis has been suggested, especially, MMP-2 (72 kDa gelatinase A) and MMP-9 (92 kDa gelatinase B), which degrade type IV collagen of ECM (2-4). Angiogenesis, the formation of new blood vessels from pre-existing microvascular, underlies different physiological and pathological processes including reproductive events, development and wound repair, chronic inflammation, and solid tumor growth (5-7). MMPs are thought to be involved in the proteolytic degradation of the basement membrane during the initial stages of the angiogenic process (8).

Numerous studies have revealed the chemopreventive and hepatoprotective effects of garlic (*Allium sativum L.*) and its derivatives, allyl compounds (9). Organosulfur compounds including diallylsulfide have been shown to inhibit proliferation of tumor cells (10-12) and to suppress chemically-induced carcinogenesis (13-15). Synthetic sulfur-containing compounds including oltipraz (16) and sulindac (17,18) exert chemopreventive activities in experimental carcinogenesis as well as hepatoprotective effects (19). Hepatocellular carcinoma is one of the most lethal malignancies and there is no effective preventive measure to date. In an attempt to develop novel hepatoprotective and chemopreventive agents, we synthesized a series of allylthiopyridazine derivatives designated as K compounds and examined the antiproliferative effect of the compounds in SK-Hep-1 hepatocarcinoma cells. We previously revealed that K compounds induced apoptosis in SK-Hep-1 cells through a caspase-3-dependent mechanism (20).

In order to extend our program to pursue the chemopreventive potential of these compounds on hepatocellular carcinoma, we wished to investigate the inhibitory effect of the K compounds on invasive and migrative properties of SK-Hep-1 hepatocarcinoma cells. Here, we report that the K compounds efficiently inhibit invasive phenotype and migration possibly via downregulation of MMP-2 activity, which may contribute to the chemopreventive function of the compounds. Furthermore, we show that the K compounds markedly inhibit the tube formation of human umbilical vein endothelial cells (HUVECs), demonstrating anti-angiogenic potential of the chemopreventive allylthiopyridazine compounds.

Materials and methods

Materials. In Fig. 1 are shown the two allythiopyridazine derivatives used in this study: 3-methoxy-6-allylthiopyridazine

Correspondence to: Dr Aree Moon, College of Pharmacy, Duksung Women's University, 419 Ssangmun-Dong, Tobong-Ku, Seoul 132-714, Korea
E-mail: armoon@duksung.ac.kr

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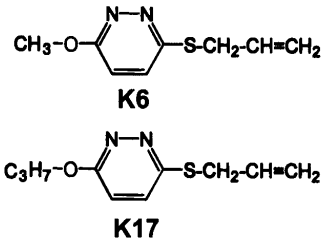

Figure 1. Chemical structures of the allylthiopyridazine derivatives, 3-methoxy-6-allylthiopyridazine (K6) and 3-propoxy-6-allylthiopyridazine (K17).

(K6) and 3-propoxy-6-allylthiopyridazine (K17). These compounds were synthesized as previously described (20). K6 was selected based on the preliminary *in vivo* efficacy data in experimental animals (data not shown). K17 was selected due to its efficient biological activities including induction of apoptosis as previously reported (20). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, penicillin-streptomycin, M199 and heparin were purchased from Gibco BRL (Grand Island, NY). Anti-human MMP-9 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-human MMP-2 antibody was kindly provided by Dr Rafi Fridman (Wayne State University, MI).

Cell lines and culture conditions. SK-Hep-1 cells were purchased from Korean Cell Line Bank (Seoul, Korea). The cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. HUVECs were purchased from Clontics Co. (San Diego, CA) and were grown on a 0.3% gelatin-coated tissue culture plate in M199 medium with 20% fetal bovine serum, 1% penicillin-streptomycin, 5 units/ml heparin and 3 ng/ml basic FGF. The cells were maintained in humidified atmosphere with 95% air and 5% CO_2 at 37˚C.

In vitro invasion assay. *In vitro* invasion assay was performed using 24-well Transwell unit with polycarbonate filters (Corning Costar, Cambridge, MA) as previously described (21). The lower side of the filter was coated with type I collagen, and the upper side was coated with Matrigel (Collaborative Research, Lexington, KY). Lower compartment was filled with serum-free media containing 0.1% BSA. The SK-Hep-1 cells, suspended in 100 µl of serum-free media containing various concentrations of K6 or K17, were placed in the upper part of the Transwell plate, incubated for 17 h, fixed with methanol and stained with hematoxylin for 10 min followed briefly by eosin. The invasive phenotypes were determined by counting the cells that migrated to the lower side of the filter with microscopy at x400. Thirteen fields were counted for each filter and each sample was assayed in triplicate.

In vitro wound migration assay. *In vitro* wound migration assay was performed on the cells preincubated with 25 µg/ml mitomycin C and treated with K6 or K17 for 24 h. Injury line was made on the cells plated in culture dishes at 90% confluency with a tip with 2 mm in width. Following a rinse with PBS, cells were allowed to migrate in complete media and photographs were taken (x40) at indicated time points.

Gelatin zymogram assay. SK-Hep-1 cells cultured in serum-free medium were treated with 1 or 2 mM K compounds for 48 h. Conditioned medium was collected and centrifuged to remove cell debris. Gelatin zymogram assay was performed as previously described (21). Briefly, equal amounts of conditioned media were mixed with 2X Laemmli non-reducing sample buffer, incubated for 15 min at room temperature, and then electrophoresed on 10% SDS-PAGE gels containing 1 mg/ml gelatin. After electrophoresis, the gels were washed and incubated overnight at 37˚C. After staining with 0.1% Coomassie brilliant blue, areas of lysis were observed as white bands against a blue backgound.

Western blot analysis. SK-Hep-1 cells cultured in serum-free medium were treated with 1 or 2 mM K compounds for 48 h. Conditioned media were collected, centrifuged and the total protein content was determined. Equal amounts of protein extracts in supernatnat were subjected to 12% SDS-PAGE and electrophoretically transferred to nitrocellulose membrane. The levels of MMP-2 and MMP-9 were detected using anti-MMP-2 and anti-MMP-9 antibodies. Enhanced chemiluminescence system was used for detection.

Tube formation assay. Growth factor-reduced Matrigel was placed in 48-well plates (150 µl/well) and allowed to gel at 37˚C for 30 min. HUVECs ($5x10^4$ cells/well) were plated onto a layer of Matrigel followed by addition of 1 or 2 mM of K compounds. Matrigel cultures were incubated at 37˚C. Tube formation was observed and photographed (x40) at indicated time points.

MTT assay. HUVECs ($2x10^3$ cells/well) cultured in 0.3% gelatin-coated wells (20 µl/well) were treated with K compounds (1 or 2 mM) for 24 h. MTT (3-(4,5-dimethylthiaxol-2-yl)-2,5-diphenyl tetrazolium bromide) was added to the media and the cells were further incubated for 4 h. After 100 µl of supernatant was replaced with DMSO, absorbance of each well was read at 540 nm with a micro-ELISA reader (Molecular Devices, Sunnylvale, CA). Percent of cell survival was defined as the relative absorbance of treated versus untreated cells.

Results

K compounds inhibit invasive phenotype of SK-Hep-1 cells. In vitro invasion assay was performed on highly invasive SK-Hep-1 cells to investigate the effect of K compounds on invasive phenotype of the hepatocarcinoma cells. As shown in Fig. 2, treatment of K6 or K17 for 17 h inhibited SK-Hep-1 cell invasion in a dose-dependent manner. K6 reduced the number of invaded cells through a reconstituted basement membrane by 35% at a concentration of 1 mM. A stronger anti-invasive activity was shown by K17: the same concentration (1 mM) inhibited invasive phenotype by 70%. The treatment was not cytotoxic (data not shown), demonstrating that the decreased number of invaded cells was due to an inhibitory

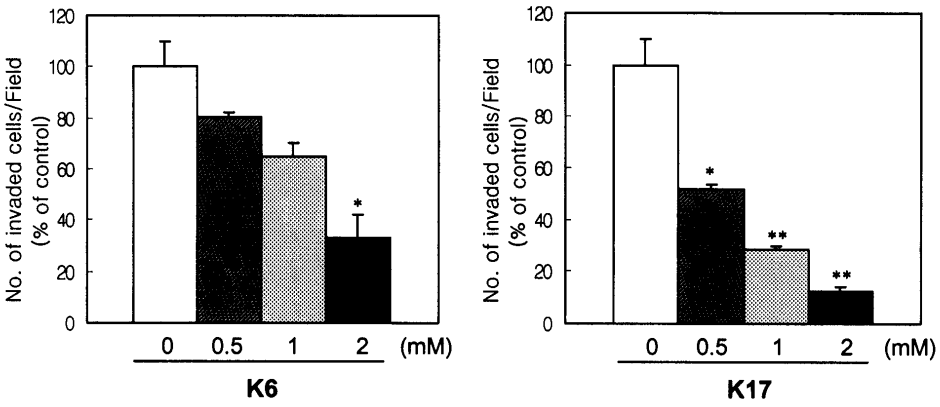

Figure 2. K compounds inihibit invasive phenotype of SK-Hep-1 cells. *In vitro* invasion assay was performed on the cells ($2x10^4$) treated with various concentrations of K6 or K17 for 17 h. Numbers of invaded cells per field were counted under x400 light microscopy. The mean values of triplicates were plotted and the error bars represent means ± SE of triplicates. * and **, statistically different from control at $p<0.05$ and $p<0.01$, respectively, by the independent two sample Student's t-test.

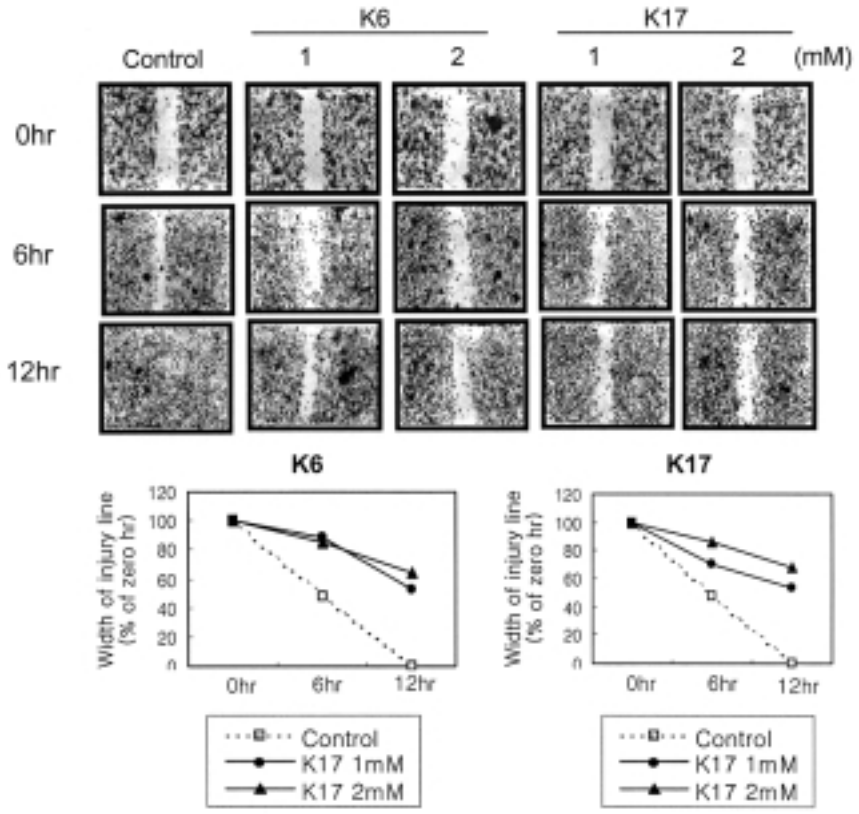

Figure 3. K compounds inhibit SK-Hep-1 cell migration. *In vitro* wound migration assay was performed on the cells preincubated with 25 μg/ml mitomycin C and treated with K6 or K17 for 24 h. Injury line was made on the confluent monolayer of cells and the cells were incubated in complete media containing K compounds. Cell migration was observed with light microscope (x40) at indicated time points. Width of injury line was measured and plotted as % of zero hour for quantification of the inhibitory effect of the K compounds on SK-Hep-1 cell migration.

effect of the K compounds on invasive phenotype and not due to a cytotoxic effect.

K compounds inhibit the SK-Hep-1 cell migration. Since migrative capacity is a prerequisite for cell invasion through the basement membrane, we examined the effect of K compounds on cell migration by *in vitro* wound migration assay. As shown in Fig. 3, migration of SK-Hep-1 cells was inhibited by treatment of K6 and K17. Treatment of K6 exerted a marked decrease of SK-Hep-1 cell migration which was comparable to that of K17. To confirm that the healing of wound is solely due to a migrative property and not due to a proliferative effect, cells were pretreated with mitomycin C (25 μg/ml), a cell cycle blocker at the S phase (22), for 30 min

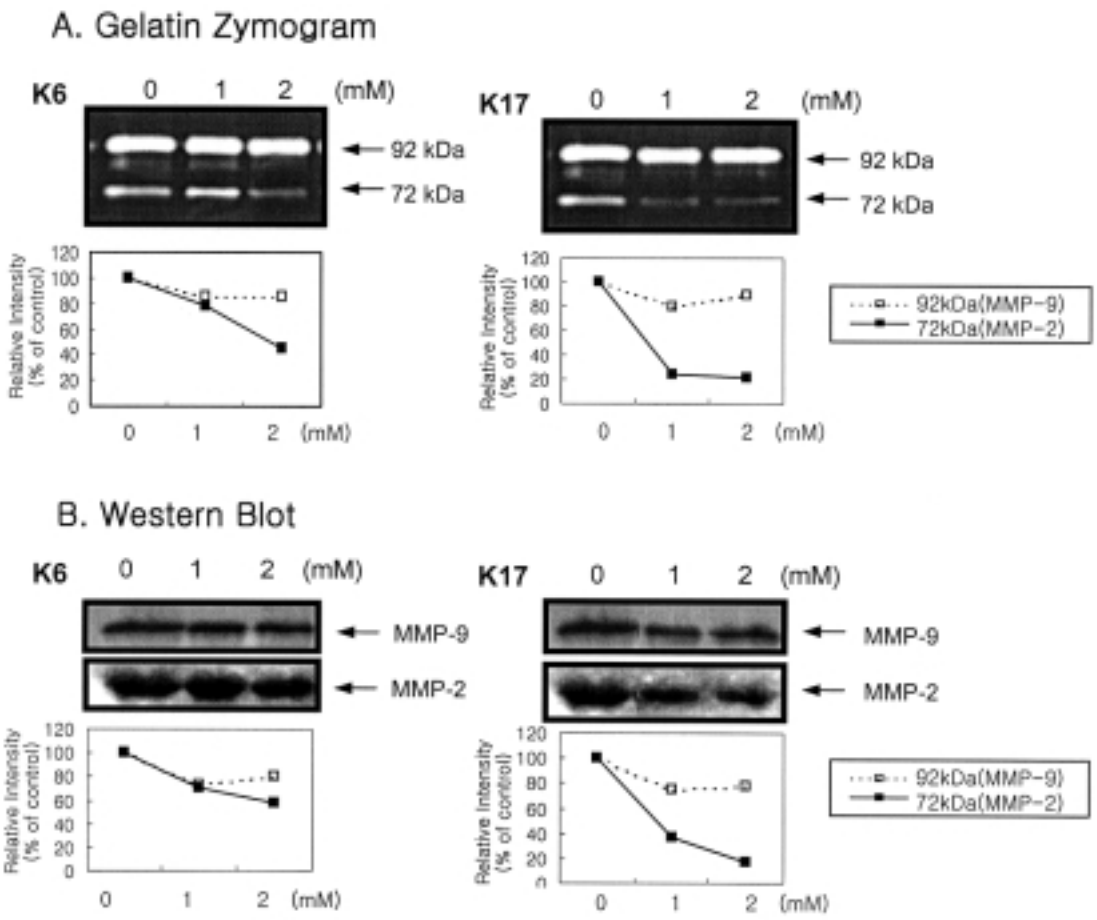

Figure 4. K compounds downregulate MMP-2 in SK-Hep-1 cells. Cells were treated with K6 or K17 for 48 h. (A), Gelatin zymogram assay was performed to analyze gelatinolytic activity. The activities of secreted MMP-2 (72 kDa) and MMP-9 (92 kDa) in the conditioned media were determined with gelatin zymography. Relative intensities of MMP-2 or MMP-9 activities were determined by quantitation of bands by Image analyzer. (B), Conditioned medium of the K-treated cells were concentrated and subjected to Western blot analysis using antibodies against MMP-2 or MMP-9. Relative intensities of MMP-2 bands were quantitated.

before the injury line was made. The results show that both K6 and K17 effectively inhibit cell migration regardless of cell cycle transition, suggesting that the anti-invasive effect of the K compounds in SK-Hep-1 cells may involve inhibition of migrative property of the cells.

K compounds downregulate MMP-2 in SK-Hep-1 cells. Invasive phenotype of cancer cells is often associated with increased expression of MMP-2 and/or MMP-9, which can degrade type IV collagen, the major structural collagen of the basement membrane (23). To determine the effect of K compounds on MMP-2 and/or MMP-9 expression, SK-Hep-1 cells were treated with 1 or 2 mM K compounds for 48 h in serum-free medium. Gelatin-based zymographic assay showed that treatment of K compounds resulted in a marked decrease of a gelatinolytic activity in the harvested conditioned medium, which comigrated with a 72,000 molecular weight marker in a dose-dependent manner (Fig. 4A). As shown in quantitative analysis determined by relative intensities of the bands, K17 downregulated MMP-2 more effectively than K6. When the cells were treated with 1 and 2 mM of K17, MMP-2 activities were decreased by ~75% and ~80%, respectively, while 1 and 2 mM of K6 inhibited MMP-2 activities by ~20% and ~55%, respectively. The gelatinolytic activity of MMP-9 (92,000 molecular weight) was not substantially reduced upon treatment of K compounds: only 10-20% decrease in MMP-9 activity was observed by either K6 or K17. Evidence for the inhibitory effect of K17, and to a lesser degree K6, on MMP-2 expression in SK-Hep-1 cells was also provided by Western blot analysis (Fig. 4B). The data suggest that the inhibitory effect of the K compounds on invasion and migration is associated more closely with downregulation of MMP-2, rather than that of MMP-9.

K compounds inhibit angiogenesis in vitro. Angiogenesis is a complex process, involving motility and degradation of the subendothelial basement membrane (24). Since vascularization is required for expansion of many tumors including hepatocellular carcinoma, we wished to determine the possible inhibitory effect of the K compounds on angiogenesis. We performed *in vitro* tube formation assay on Matrigel of HUVECs. As shown in Fig. 5A, HUVECs formed the elongated and robust tube-like structures after 3 h in culture (control). Treatment of 1 mM K6 or K17 markedly inhibited the tube formation of HUVECs, indicating that the K compounds exerted anti-angiogenic effect in HUVECs.

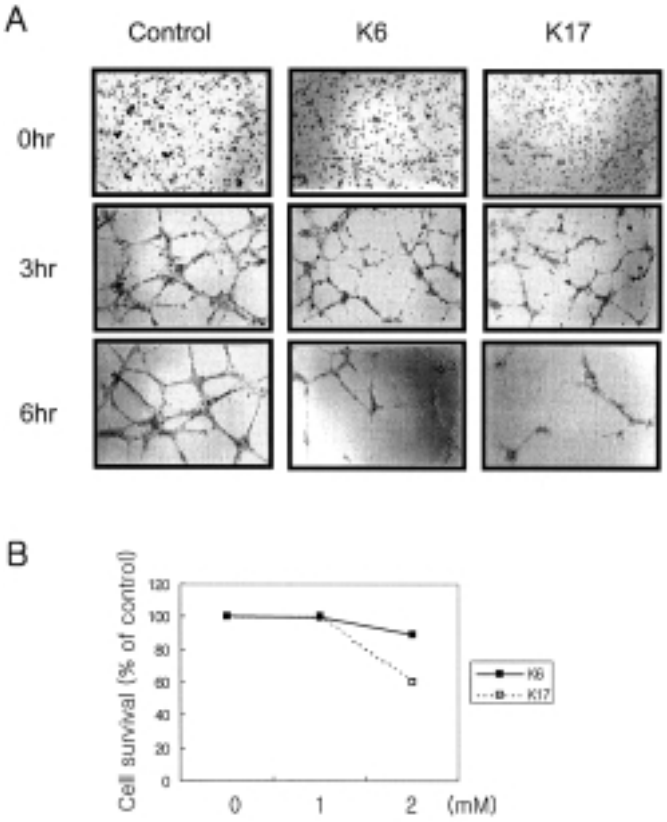

Figure 5. K compounds inhibit angiogenesis of HUVECs. (A), *In vitro* tube formation assay was performed on the HUVECs plated on Matrigel-coated wells. The cells were treated with K6 or K17. Tube formation was observed and photographed (x40). (B), Cytotoxicity of K compounds on HUVECs was tested by MTT assay. HUVECs were treated with the K compounds for 24 h. The results are presented as means ± SE of triplicates.

Because *in vitro* angiogenesis depends on the number of viable cells, we tested the viability of HUVECs treated with K compounds to exclude the possibility that the observed inhibition of angiogenesis was due to a cytotoxic effect. Survival of the HUVECs treated with 1 mM K6 or K17 for 24 h was comparable to that of control cells (Fig. 5B), indicating that the inhibition of tube formation was not due to cytotoxic effect of the K compounds. Treatment of 2 mM K6 did not significantly decrease cell survival whereas that of 2 mM K17 was cytotoxic to HUVECs.

Discussion

Hepatocellular carcinoma is among the most prevalent and deadly cancers worldwide. Recent strategies to prevent hepatocellular carcinoma include treatment of chemopreventive organosulfur compounds such as oltipraz. Identifying and developing pharmaceutical agents that can inhibit processes of invasion, metastasis and angiogenesis may provide an effective tactic for the prevention and treatment of cancer. The major aim of this investigation was to evaluate the inhibitory effects of the K compounds, which was shown to induce apoptosis of SK-Hep-1 hepatocarcinoma cells (20), on invasive and migrative properties of the SK-Hep-1 cells.

An essential part of invasion and metastasis includes degradation of the basement membrane and the stromal extracellular matrix by members of the MMP family (23). Numerous studies show a correlation between the levels of MMP-2 and/or MMP-9 and the invasive phenotype of cancer cells. An imbalance between proteinases and their inhibitors has been demonstrated to be a key factor in many human diseases, including tumorigenesis and angiogenesis (25). We previously suggested the involvement of MMP-2 in H-ras-induced invasive phenotype of MCF10A cells (21). The present study demonstrates that the chemopreventive K compounds exert inhibitory effect on invasiveness of SK-Hep-1 hepatocarcinoma cells in which downregulation of MMP-2, rather than that of MMP-9, is more closely associated. MMP-9 was previously reported to be a key molecule in curcumin-induced invasive phenotype in SK-Hep-1 cells (26), suggesting that the K compounds and curcumin may use different molecular targets to exert the same anti-invasive effects in these cells. Our recent study suggests a critical role for p38 mitogen-activated protein kinase in upregulation of MMP-2, induction of cell motility and invasive phenotype in human breast epithelial cells (27). It would be worthwhile to further investigate the molecular mechanisms underlying the inhibitory effect of the K compounds on invasion, motility and MMP expression in SK-Hep-1 cells.

To move, cells make and break adhesion contacts for cell motility (28) by membrane ruffling (29) and actin re-organization (30). Cell adhesion triggers an intracellular signaling cascade to induce or inhibit MMPs depending on cell systems (31,32). We showed that K compounds can affect SK-Hep-1 cell migration and invasion, suggesting overlapping pathways for regulation of cell migration and ECM degrading activities. It should be mentioned that K17 was more effective in inhibition of invasion and downregulation of MMP-2 than K6 (Fig. 4) while both compounds showed a comparable inhibition of migrative property (Fig. 3). The results suggest that the regulation of migrative and invasive properties may have some discinct molecular effectors in addition to the common pathways. It cannot be ruled out that although the wound migration assay is widely used for the analysis of cell migration, it may have failed to provide a quantitation which is sensitive enough to show the difference in the effects of K6 and K17 on SK-Hep-1 cell migration.

The formation of new blood vessels is permissive for expansion of tumors. Involvement of MMP activities in the initial stages of angiogenesis has been shown (8,25). Hepatocellular carcinoma is generally characterized as a hypervascular tumor of rapid growth. Our results demonstrating the anti-angiogenic activity of K compounds (Fig. 5) further support a notion that the development of K compounds would be a promising approach to prevent and treat hepatocellular carcinoma. Preclinical safety data show that K6 does not exert genotoxicity on bacterial reverse mutation test and it has considerably high maximum tolerable doses for acute toxicity performed on rats and mice (data not shown).

Efforts to elucidate the molecular lesions and processes underlying hepatocellular development identified putative molecular targets for preventive interventions. These include genes and gene products controlling viral replication, carcinogen metabolism, signal transduction, cell cycle arrest,

apoptosis, proliferation and oxidative stress (33). Our previous study revealed caspase-3 as an effector molecule for the K-induced apoptosis in SK-Hep-1 cells. This study presents MMP-2 as a potential target molecule for the anti-invasive and anti-migrative activities of K compounds in SK-Hep-1 cells. Taken together, the present study suggests that K compounds inhibit invasive phenotype and migration of SK-Hep-1 cells through MMP-2 dependent mechanism and angiogenesis which may contribute to the chemopreventive potential of these agents for hepatocarcinoma.

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