Epimedium koreanum Nakai inhibits PMA-induced cancer cell migration and invasion by modulating NF-κB/MMP-9 signaling in monomorphic malignant human glioma cells

WONIL LEE1*, JIN HAN NAM1*, HYUN-JI CHO1*, JU-YOUNG LEE1*, WON-KYUNG CHO3, YEONGYEON KIM1, YOUNG-MAN WE2, JIN YEUL MA3 and HYANG-SOOK HOE1

1Department of Neural Development and Disease, Korea Brain Research Institute (KBRI), Dong-gu, Daegu 41062; 2Oriental Medical Clinic Center, Hyoo Medical Clinic, Gangnam-gu, Seoul 06134; 3Korean Medicine (KM)-Application Center, Korea Institute of Oriental Medicine (KIOM), Dong-gu, Daegu 41068, Republic of Korea

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Correspondence to: Dr Hyang-Sook Hoe, Department of Neural Development and Disease, Korea Brain Research Institute (KBRI), 61 Cheomdan-ro, Dong-gu, Daegu 41062, Republic of Korea
E-mail: sookhoe72@kbri.re.kr
Dr Jin Yeul Ma, Korean Medicine (KM)-Application Center, Korea Institute of Oriental Medicine (KIOM), 70 Cheomdan-ro, Dong-gu, Daegu 41068, Republic of Korea
E-mail: jyma@kiom.re.kr

*Contributed equally

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Abstract. Previously, we showed that the herbal extract EYK (Epimedium koreanum Nakai) can regulate the immune response. Other studies showed that EYK has beneficial effects in human lung cancer, angiogenesis and Alzheimer’s disease (AD). However, it remains unknown whether EYK can affect cancer cell migration and invasion in human brain cancer cell lines. In the present study, we found that pre- or post-treatment with EYK inhibited phorbol 12-myristate 13-acetate (PMA)-induced cancer cell migration and invasion in A172 cells, but not in U373MG or T98G cells. Additionally, pre- or post-treatment with PMA followed by EYK decreased MMP-9 activity in A172 cells. Moreover, treatment with a NF-κB inhibitor significantly decreased cell migration in A172 cells pre- or post-treated with EYK and PMA, suggesting that EYK requires NF-κB to alter cancer cell migration. Either pre- or post-treatment with EYK significantly decreased NF-κB nuclear translocation in comparison with PMA treatment. Taken together, our results suggest that EYK suppresses PMA-induced cancer cell migration in monomorphic malignant human glioma cells by downregulating the NF-κB pathway and decreasing MMP-9 activity.

Introduction

EYK, a herbal plant-based extract derived from Epimedium koreanum Nakai, consists of multiple elements including anhydroicaritin-3-O-α-L-rhamnopyranoside, icariin, icariside II quercetin, epimedin A, epimedin B, ikarisoside, b-sitosterol, daucosterol, campesterol, epimediphine and chlorogenic acid (1). Icariin, acting through the phosphoinositide 3-kinase and protein kinase B (PI3/AKT) and nuclear factor-kappa B (NF-κB) pathways, inhibits lipopolysaccharide (LPS)-induced lung inflammation (2). EYK decreases the abundance of β-amyloid plaque (Aβ) in APP transgenic mice (3) and inhibits adipocyte differentiation by downregulating adipogenic transcription factors in 3T3-L1, a murine preadipocyte cell line (4). Another constituent, icariside II, promotes terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive apoptosis in PC-3 prostate carcinoma cells (5) and exerts antitumor activity in human adenocarcinoma alveolar basal epithelial A549 cells via the mitochondria apoptotic pathway, including bcl2-related X protein/B-cell lymphoma 2 (BAX/Bcl-2) as well as the caspase signaling pathway (6). EYK also has antiviral activity against porcine epidemic diarrhea virus (PEDV), which induces diarrhea in adult pigs and suppresses viral replication in both PEDV-infected cell lines and piglets (7). Thus, EYK extracts confer beneficial effects in various diseases, including human lung cancer (6), prostate carcinoma (5), myeloid acute leukemia (8), angiogenesis (9), Alzheimer’s disease (3) and inflammatory conditions (2). However, it is still not clear whether EYK can affect cancer cell metastasis in human glioblastoma.

Human glioblastoma multiforme (GBM) is a type of brain cancer that arises from astrocytes. Brain cancer is accompanied by symptoms such as seizure, abnormal behavior and memory deficits. Currently, brain tumors are treated by surgery, radiation therapy and chemotherapy with temozolomide (TMZ). However, this regimen can also damage healthy tissues (10) and some cases of GBM are resistant to TMZ therapy (11,12). TMZ is an anti-neoplastic agent that delivers methyl groups to purine bases (guanine at N7 and O6 and adenine at N3) in
DNA of cancer cells, inducing DNA damage and ultimately leading to apoptosis and cytotoxicity. Clinical studies showed that TMZ induces side-effects such as myelosuppression, nausea and vomiting (13). Thus, a new anti-brain cancer drug is necessary for the successful treatment and cure of GBM.

In the present study, we examined the effect of EYK on brain cancer cell migration and invasion. We found that pre- or post-treatment with EYK inhibited cancer cell migration and invasion in monomorphic malignant human glioma cells. We observed that EYK required matrix metalloprotease-9 (MMP-9) activity to inhibit phorbol 12-myristate 13-acetate (PMA)-induced cancer cell migration. In addition, pre- or post-treatment with EYK regulated NF-κB nuclear translocation in PMA-induced A172 cells. Moreover, treatment with NF-κB inhibitor followed by EYK significantly suppressed cell migration in PMA-induced A172 cells in comparison with treatment with PMA and NF-κB inhibitor or PMA and EYK. Taken together, our results suggest that EYK inhibits cell migration in monomorphic malignant human glioma cells by downregulating the NF-κB nuclear translocation and thereby decreasing MMP-9 activity.

Materials and methods

Preparation of Epimedium koreanum Nakai. Epimedium koreanum Nakai (EYK) was as described in our previous studies (7). Briefly, the dried bark of the plant was obtained from a domestic Korean market (Kyung Dong Crude Drugs Market, Seoul, Korea) and boiled in distilled water for 2.5 h at 105˚C; the resultant suspension was filtered and lyophilized. The powder was stored at 4˚C and the extract was dissolved in phosphate-buffered saline (PBS) and diluted with medium before each experiment.

Cell lines and culture conditions. A172, U373MG and T98G cells (human brain cancer cells; Korean Cell Line Bank, Seoul, Korea) were maintained in RPMI-1640 medium (HyClone Laboratories, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories) in a 5% CO2 incubator with 5% fetal bovine serum (FBS; HyClone Laboratories) in a 5% CO2 incubator. A172 cells (1x10⁵/ml) were seeded in ice-cold lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 1% NP-40) containing protease and phosphatase inhibitor (Roche Diagnostics, Indianapolis, IN, USA). Proteins from cell extracts were separated under denatured and reduced conditions using Tris-glycine polyacrylamide gel electrophoresis. Separated proteins were transferred onto polyvinylidene difluoride membrane (PVDF; EMD Millipore, Temecula, CA, USA) at 110 V for 1 h and blocked with 5% skim milk or 5% bovine serum albumin (BSA) in 0.1% TBS-T (Tris-buffered saline with 0.1% Tween-20) for 1 h at room temperature. The blots were incubated overnight at 4˚C with specific primary antibodies: mouse β-actin (1:5,000; Santa Cruz Biotechnology), mouse anti-p-IκBα (Ser32, 1:500; Santa Cruz Biotechnology), rabbit anti-IκBα (1:1,000; Santa Cruz Biotechnology), rabbit anti-NF-κB (P65, 1:1,000; Santa Cruz Biotechnology), rabbit anti-ERK (1:1,000; Cell Signaling Technology), rabbit anti-p-ERK (Thr202/Tyr204, Cell Signaling Technology), rabbit anti-α-actin (Santa Cruz Biotechnology), rabbit anti-IκBα (B-9, Ser32; Santa Cruz Biotechnology), rabbit anti-IκBα (Ser536; Cell Signaling Technology), and PCNA (1:1,000; Abcam). HRP-conjugated secondary antibody was visualized by enhanced chemiluminescence detection reagents (ECL; ATTO Corp., Tokyo, Japan) and image were captured using chemiluminescence imaging system (Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany). Equal amount of protein (10 µg) were used for immunoblotting and all blot images were analyzed using either Fusion software or ImageJ.

Cell migration (wound healing assay). A172, U373MG and T98G cells were seeded in 96-well plates and treated for 24 h with vehicle or EYK at various concentrations (1, 10, 100, 200, or 400 µg/ml) in the absence of serum. After 24 h, cells were treated with 0.5 µg/ml MTT and incubated for 3 h at 37˚C in a 5% CO2 incubator. Plates were read by measuring absorbance at 580 nm.

Transwell invasion assay. Corning Matrigel® was loaded onto the membrane of a culture insert (Corning Transwell System; Corning Inc., Corning, NY, USA) and incubated at 37˚C for 30 min in a 5% CO2 incubator. A172 cells (1x10⁵/ml) were plated onto the Matrigel-coated insert in the upper chamber, which contained serum-free RPMI-1640, and then treated with vehicle, EYK (200 µg/ml) or PMA (75 nM). RPMI-1640 media containing 10% FBS was added to the lower chamber. After 24 h, the cells were fixed in methanol (100%) and stained with hematoxylin-eosin (Sigma-Aldrich). The membrane was cut and mounted on a glass microscope slide. Images were randomly acquired (two or three images from each sample) on a microscope (Carl Zeiss, Oberkochen, Germany).

Gelatin zymography assay. To measure MMP activity, A172 cells were treated with vehicle or PMA (75 nM) for 45 min, and then treated for 24 h with vehicle or EYK (200 µg/ml). After 24 h, the conditioned medium was collected and analyzed on a
10% acrylamide gel containing 0.1% (w/v) gelatin. Following electrophoresis, the gel was washed three times for 10 min each with 2.5% Triton X-100, and then incubated overnight in zymography incubation buffer (2.5 mM CaCl₂, 200 mM NaCl, 50 mM Tris-HCl, pH 7.5). The next day, the gel was stained with 0.2% Coomassie brilliant blue solution R-250 for 1 h, and then washed three times for 10 min each in destaining buffer (10% MeOH, 10% acetic acid in distilled water). Gel images were acquired on a light box (Vilber Lourmat, Seoul, Korea) and analyzed using Image Gauge V4.0 (Fujifilm, Tokyo, Japan).

Cytosolic and nuclear fractionation. A172 cells were lysed on ice for 5 min in buffer A [10 mM HEPES (pH 8.0), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 300 mM sucrose, 0.1% NP-40 and 0.5 mM PMSF]. After 5 min, the cell lysates were centrifuged at 10,000 rpm at 4°C for 1 min, and the supernatant was collected as the cytosolic fraction. Then, the cell pellet was lysed on ice for 15 min in buffer B [20 mM HEPES (pH 8.0), 20% glycerol, 100 mM KCl, 100 mM NaCl, 0.2 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF] and the cell lysates were centrifuged at 15,000 rpm at 4°C for 20 min. The supernatant was collected as the nuclear fraction.

RT-PCR. To examine the effects of EYK on MMP-9 and TIMP-1 mRNA levels, A172 cells were pre-treated with vehicle or PMA (75 nM) for 45 min, and then treated for 24 h with vehicle or EYK (200 µg/ml). After 24 h, RNA was extracted using TRIzol (Ambion, Inc., Austin, TX, USA). RT-PCR was performed using the following primers: MMP-9: 5'-CGG AGC ACG GAG ACG GGT AT and 3'-TGA AGG GGA AGA CGC ACA GC; TIMP-1: 5'-AGC GCC CAG AGA GAC ACC and 3'-CCA CTC CGG GCA GGA TT; GAPDH: 5'-GTT ACC AGG GCT GCC TTC TC and 3'-GTG ATG GCA TGG ACT GTG GT. Image analyses were performed using the Fusion or ImageJ software to measure average band intensities.

Immunocytochemistry. A172 and U373MG cells were fixed for 8 min using ice cold 100% methanol, washed with 1X PBS, and then incubated with the following primary antibodies overnight: rabbit anti-p-NF-κB (Ser536, 1:100; Cell Signaling Technology), mouse anti-β-actin (1:200; Santa Cruz Biotechnology) in GDB buffer (0.1% gelatin, 0.3% Triton X-100, 16 mM sodium phosphate pH 7.4 and 450 mM NaCl) overnight at 4°C. The next day, cells were washed with 1X PBS three times and incubated with the following secondary antibodies in GDB solution for 1 h at room temperature: anti-rabbit-AlexaFluor 488 and anti-mouse-AlexaFluor 555 (1:200; Molecular Probes, Eugene, OR, USA) and cell covered using DAPI containing mount solution (Vector Laboratories, Burlingame, CA, USA). Images were taken on a single plane using a confocal microscope (Nikon) and analyzed using ImageJ software.

Statistical analyses. All data were analyzed by either two-tailed t-test or ANOVA using the GraphPad Prism 4 software. Post hoc analyses were performed using the Tukey’s multiple comparison test with P<0.05 considered to represent significance (P<0.05, P<0.01, P<0.001).

Results

EYK inhibits PMA-induced cell migration in A172 cells. To test the effects of EYK on cell viability in malignant human tumor cells, we treated U373MG, T98G and A172 cells with vehicle or EYK (1, 10, 100, 200 or 400 µg/ml) for 24 h and then MTT assays were performed in U373MG cells, EYK did not influence cell viability at any doses (Fig. 1A). In T98G cells, EYK slightly decreased cell viability at the highest doses, by 13 and 23% at 200 and 400 µg/ml, respectively (Fig. 1B, P<0.01; two-tailed t-test). In A172 cells, EYK caused no toxicity up to a concentration of 200 µg/ml, but had some toxicity at 400 µg/ml (Fig. 1C, P<0.01; two-tailed t-test). Based on these findings, we selected a working concentration of 200 µg/ml for the following experiments.

To determine whether EYK can regulate PMA-induced brain tumor cell migration, we initially optimized the dose and timing for treatment with PMA, a cancer inducer (15), in our culture system. For these experiments, U373MG, T98G and A172 cells were grown in a monolayer, scratched with a pipette tip, and treated with vehicle or PMA (25, 50, 75, 100 or 125 nM) for 24 h. At 0 h (i.e., immediately after

Figure 1. Effect of EYK on cell viability in malignant human glioma cells. (A) U373MG, (B) T98G and (C) A172 cells were treated with vehicle or EYK (1, 10, 100, 200 or 400 µg/ml) for 24 h, and then MTT assays were performed (U373MG: n=16 for each dose; T98G: n=8 for each dose; A172: n=16 for each dose; **P<0.01; two-tailed t-test).
scratching) and 24 h, we acquired images of the wound gap at both time-points and measured the proportion of cells migrated from 0 h to 24 h at different PMA concentrations. Subtracting the surface area at 0-24 h with vehicle treatment...
were listed as 100%, and difference between the areas at 0-24 h at various PMA concentrations were compared with vehicle treatment. PMA dramatically increased cell migration relative to the vehicle at all doses tested in U373MG (Fig. 2A and D, P<0.01; P<0.001; ANOVA with post hoc Tukey’s test), T98G (Fig. 2B and E, P<0.001, ANOVA with post hoc Tukey’s test), and A172 cells (Fig. 2C and F, P<0.01, P<0.001, ANOVA with post hoc Tukey’s test), with an optimal concentration at 75 nM in all three cell lines.

To determine the optimal time for PMA treatment, we treated U373MG, T98G and A172 cells with vehicle or PMA at various times (15, 30, 45 and 60 min) and performed western blotting to detect anti-p-ERK and total ERK, an important signaling for cancer migration (16). PMA significantly increased p-ERK at all time-points in U373MG (Fig. 2G and H, P<0.01, P<0.001, ANOVA with post hoc Tukey’s test), T98G (Fig. 2I and J, P<0.05, ANOVA with post hoc Tukey’s test), and A172 cells (Fig. 2K and L, P<0.05, ANOVA with post hoc Tukey’s test). Based on our findings and literature, we selected a dose of 75 nM and a duration of 45 min for PMA treatment in the following experiments (15).

To determine whether EYK can alter cell migration in human malignant cells, we performed wound healing assays on U373MG, T98G and A172 cells pre-treated with PMA (75 nM) or vehicle for 45 min, and then treated with EYK (200 µg/ml) or vehicle for 24 h. As expected, PMA alone significantly increased cell migration in all three cell lines (Fig. 3A-F). EYK did not alter PMA-induced cell migration in

Figure 3. Pre-treatment with PMA and EYK treatment inhibits PMA-induced cell migration and invasion. (A) U373MG, (C) T98G or (E) A172 cell monolayers were scratched with a pipette tip, pre-treated with vehicle or PMA (75 nM) for 45 min, and then treated with vehicle or EYK (200 µg/ml) for 24 h. Images of the wound gap were acquired at 0 h (i.e., immediately after scratching) and after 24 h. (B, D and F) Quantification of data from (A) (U373MG: con, n=19; PMA, n=27; PMA+EYK, n=15; ***P<0.001; ANOVA with post hoc Tukey’s test), (C) (T98G: con, n=13; PMA, n=18; PMA+EYK, n=10; ***P<0.001; ANOVA with post hoc Tukey’s test), and (E) (A172: con, n=26; PMA, n=26; PMA+EYK, n=28; ***P<0.001; ANOVA with post hoc Tukey’s test). (G) A172 cells were pre-treated with PMA (75 nM) or vehicle for 45 min, treated with vehicle or EYK (200 µg/ml) for 24 h, and then subjected to Transwell invasion assays. (H) Quantification of data from (G) (A172: con, n=7; PMA, n=7; PMA+EYK, n=7; *P<0.05; ANOVA with post hoc Tukey’s test, con vs. PMA, P=0.057; two-tailed t-test). (A, C and E) Scale bar, 200 µm, and (G) 100 µm.
Figure 4. Pre-treatment with PMA followed by EYK treatment decreases MMP-9 activity in A172 cells. (A) A172 cells were pre-treated with vehicle or PMA (75 nM) for 45 min, and then treated with EYK (200 µg/ml) or vehicle for 24 h. After 24 h, gelatin zymography assays were conducted on conditioned medium to measure MMP-9 activity. (B) Quantification of data from A (A172: con, n=6; PMA, n=6; PMA+EYK, n=6; "**P<0.01; ANOVA with post hoc Tukey's test). (C and E) A172 cells were pre-treated with vehicle or PMA (75 nM) for 45 min and treated with EYK (200 µg/ml) or vehicle for 24 h. After 24 h, mRNA levels of MMP-9 (C), TIMP-1 (E) and GAPDH were measured by RT-PCR. (D and F) Quantification of data from (C) (A172: con, n=8; PMA, n=8; PMA+EYK, n=8; "***P<0.001; ANOVA with post hoc Tukey's test) and (E) (A172: con, n=4; PMA, n=4; PMA+EYK, n=4). (G) A172 cell monolayers were scratched with a pipette tip, pre-treated with vehicle or PMA (75 nM) for 45 min, treated with vehicle or CAS 1177749-58-4 (5 µM, MMP-9 inhibitor) for 1 h, and then treated with vehicle or EYK (200 µg/ml) for 24 h. Images of the wound gap were acquired at 0 h (i.e., immediately after scratching) and after 24 h. (H) Quantification of data from (G) (A172: con, n=25; PMA, n=25; PMA+EYK, n=28; PMA+CAS, n=40; PMA+CAS+EYK, n=37; "***P<0.001; ANOVA with post hoc Tukey's test). (I) A172 cells were pre-treated with vehicle or PMA (75 nM) for 45 min, treated with vehicle or CAS 1177749-58-4 (5 µM, MMP-9 inhibitor) for 1 h, and treated with vehicle or EYK (200 µg/ml) for 24 h. After 24 h, gelatin zymography assays were conducted. (J) Quantification of data from I (A172: con, n=28; PMA, n=28; PMA+EYK, n=28; PMA+CAS, n=28; PMA+CAS+EYK, n=28; "**P<0.01; ANOVA with post hoc Tukey's test, PMA+CAS vs. PMA+CAS+EYK; two-tailed t-test). (G) Scale bar, 200 µm.
comparison with PMA in U373MG or T98G cells (Fig. 3A-D, *P*<0.01; ANOVA with post hoc Tukey’s test). By contrast, EYK significantly suppressed PMA-mediated cell migration in A172 cells (Fig. 3E and F, *P*<0.001; ANOVA with post hoc Tukey’s test).

Next, we performed Transwell assays to investigate whether EYK can regulate cell invasion. To this end, A172 cells were pre-treated with PMA or vehicle for 45 min, treated with vehicle or EYK (200 µg/ml) for 24 h, and then subjected to cell invasion assays. PMA increased cell invasion in comparison with vehicle (Fig. 3G and H, *P*<0.05; ANOVA with post hoc Tukey’s test). In addition, EYK inhibited PMA-mediated cell invasion (Fig. 3G and H). Together, these data indicate that EYK can inhibit PMA-mediated cell migration and invasion, at least in monomorphic malignant human glioma cells.

**Pre-treatment with PMA followed by EYK decreased MMP-9 activity in A172 cells**. To determine whether EYK regulates activity and expression of MMPs, which are involved in cell migration and invasion (15,17), we pre-treated A172 cells with PMA (75 nM) or vehicle for 45 min, treated with EYK (200 µg/ml) or vehicle for 24 h, and then conducted gelatin zymography assays. PMA significantly increased MMP-9 activity compared to vehicle (Fig. 4A and B, *P*<0.001; ANOVA with post hoc Tukey’s test). EYK significantly decreased PMA-induced MMP-9 activity (Fig. 4A and B).

In order to examine the effects of EYK on MMP-9 and TIMP-1 mRNA levels, we conducted RT-PCR under the same conditions as described above. We found that PMA significantly increased MMP-9 and TIMP-1 mRNA levels compared to vehicle (Fig. 4C-F). EYK significantly decreased PMA-mediated MMP-9 mRNA levels (Fig. 4C and D, *P*<0.001; ANOVA with post hoc Tukey’s test), but had no effect on TIMP-1 mRNA levels (Fig. 4E and F). These data suggest that EYK can regulate PMA-induced MMP-9 activity as well as its mRNA levels.

We then investigated whether EYK requires MMP-9 activity to alter cell migration. For this purpose, A172 cells were pre-treated with vehicle or PMA (75 nM), treated with CAS1177749-58-4 (an MMP-9 inhibitor, 5 µM) for 1 h, exposed to vehicle or EYK (200 µg/ml) for 24 h, and then subjected to the wound healing assay. Consistent with our findings above, PMA significantly increased cell migration but EYK significantly decreased PMA-induced cell migration (Fig. 4G and H, *P*<0.001; ANOVA with post hoc Tukey’s test). In addition, pretreatment with PMA, MMP-9 inhibitor, and EYK treatment further decreased cell migration compared to treatment with PMA and EYK (Fig. 4G and H). Moreover, we found that treatments with PMA, MMP-9 inhibitor, and EYK significantly reduced MMP-9 activity compared to PMA and EYK treatment (Fig. 4I and J, *P*<0.001; ANOVA with post hoc Tukey’s test, PMA+CAS vs. PMA+CAS+EYK; two-tailed t-test).

**EYK significantly decreased PMA-induced NF-κB translocation to the nucleus**. A transcriptional factor NF-κB plays an important role in cell migration and invasion by regulating MMP activity (18). Hence, we initially investigated whether EYK could alter PMA-induced NF-κB levels in the cytosol vs. the nucleus. For these experiments, A172 cells were pre-treated with PMA (75 nM) or vehicle for 45 min, treated with EYK (200 µg/ml) or vehicle for 45 min, and then subjected to subcellular fractionation. PMA treatment showed a trend toward increased p-IκBα and NF-κB levels, but decreased IκBα levels in the cytosol (Fig. 5A-D, *P*<0.01; ANOVA with post hoc Tukey’s test). EYK did not alter PMA-induced p-IκBα and NF-κB levels, but significantly decreased PMA-mediated NF-κB levels in the cytosol (Fig. 5A-D). In addition, EYK did not alter the PMA-induced NF-κB levels in the nucleus (Fig. 5E and F, *P*<0.01; ANOVA with post hoc Tukey’s test).

We then asked whether EYK could alter NF-κB subcellular localization when administered for longer periods of time. To answer this question, A172 cells were pre-treated with PMA (75 nM) or vehicle for 45 min, treated with EYK (200 µg/ml) or vehicle for 24 h, and then subjected to subcellular fractionation. EYK slightly decreased PMA-induced p-IκBα and NF-κB levels, but did not alter IκBα levels in the cytosol (Fig. 5G-J, *P*<0.001; ANOVA with post hoc Tukey’s test). In addition, EYK significantly decreased PMA-mediated NF-κB levels in the nucleus (Fig. 5K and L, *P*<0.05; *P*<0.001; ANOVA with post hoc Tukey’s test).

To examine whether EYK could alter PMA-induced p-NF-κB levels in the nucleus, A172 cells were pre-treated with PMA (75 nM) or vehicle for 45 min, treated with EYK (200 µg/ml) or vehicle for 24 h, and then immunostaining was performed. PMA significantly increased p-NF-κB levels in the nucleus (Fig. 5M and N, *P*<0.01; ANOVA with post hoc Tukey’s test). EYK significantly decreased PMA-induced p-NF-κB levels in the nucleus (Fig. 5M and N). These data suggest that EYK can alter NF-κB nuclear translocation between subcellular compartments.

**EYK suppresses PMA-mediated cell migration via the NF-κB pathway**. To determine whether EYK requires NF-κB to suppress cancer cell migration, we performed wound healing assays on A172 cells pre-treated with PMA (75 nM) or vehicle for 45 min, treated with BAY 11-7085 (10 µM, a NF-κB inhibitor) or vehicle for 1 h, and then treated with EYK (200 µg/ml) or vehicle for 24 h (Fig. 6A and B, *P*<0.001; ANOVA with post hoc Tukey’s test). EYK significantly decreased PMA-induced p-NF-κB levels in the nucleus (Fig. 5M and N). These data suggest that EYK can alter NF-κB nuclear translocation between subcellular compartments.

**Pre-treatment with EYK and PMA treatment suppresses cell migration**. To determine whether pre-treatment with EYK can modulate cell migration, we performed wound healing assays on A172 cells pre-treated with vehicle or EYK (200 µg/ml) for 45 min, treated with PMA (75 nM) or vehicle for 24 h, and then wound healing assays were conducted. PMA significantly increased cancer cell migration, but this effect was suppressed
Figure 5. Pre-treatment with PMA followed by EYK treatment decreased NF-κB levels in the nucleus. (A) A172 cells were pre-treated with vehicle or PMA (75 nM) for 45 min, treated with EYK (200 μg/ml) or vehicle for 45 min, and then subjected to subcellular fractionation (nucleus vs. cytosol). Western blotting was performed on the cytosolic fraction using antibodies against p-1κBα, 1κBα, NF-κB and β-actin. (B-D) Quantification of data from A (A172: con, n=5; EYK, n=2; PMA, n=5; PMA+EYK, n=5; *P<0.05; **P<0.01; ANOVA with post hoc Tukey's test). (E) Western blotting was performed on the nuclear fraction using antibodies against NF-κB and PCNA. (F) Quantification of data from E (A172: con, n=5; EYK, n=2; PMA, n=5; PMA+EYK, n=5; **P<0.01; ANOVA with post hoc Tukey's test). (G) A172 cells were pre-treated with vehicle or PMA (75 nM) for 45 min, treated with EYK (200 μg/ml) or vehicle for 24 h, and then subjected to subcellular fractionation (nucleus vs. cytosol). Western blotting was performed on the cytosolic fraction using antibodies against p-1κBα, 1κBα, NF-κB and β-actin. (H-J) Quantification of data from (G) (A172: con, n=4; EYK, n=4; PMA, n=4; PMA+EYK, n=4; ***P<0.001; ANOVA with post hoc Tukey's test). (K) Western blotting was performed on the nuclear fraction using antibodies against NF-κB and PCNA. (L) Quantification of data from (K) (A172: con, n=4; EYK, n=4; PMA, n=4; PMA+EYK, n=4; ***P<0.001; ANOVA with post hoc Tukey's test). (M) A172 cells were pre-treated with vehicle or PMA (75 nM) for 45 min, treated with EYK (200 μg/ml) or vehicle for 24 h, and immunostaining were performed using antibodies against p-NF-κBα and β-actin. (N) Quantification of data from M (A172: con, n=63 cells; PMA, n=61 cells; PMA+EYK, n=68 cells; ***P<0.001; ANOVA with post hoc Tukey's test). (M) Scale bar, 20 μm.
by pre-treatment with EYK (Fig. 7A and B, P<0.001; ANOVA with post hoc Tukey’s test).

Next, we performed the Transwell assays to investigate the effects of pre-treatment with EYK on cell invasion and found that PMA significantly increased cell invasion while pre-treatment with EYK significantly inhibited PMA-induced cell invasion (Fig. 7C and D, P<0.05; ANOVA with post hoc Tukey’s test).

We then investigated whether pre-treatment with EYK could alter MMP-9 activity and found that pre-treatment with EYK significantly decreased PMA-induced MMP-9 activity (Fig. 7E and F, P<0.001; ANOVA with post hoc Tukey’s test). Moreover, pre-treatment with EYK significantly decreased MMP-9 mRNA levels, but had no effect on TIMP-1 mRNA levels (Fig. 7G-I, P<0.05; P<0.001; ANOVA with post hoc Tukey’s test).

Pre-treatment with EYK alters cell migration through NF-κB pathway. To determine whether pre-treatment with EYK can regulate PMA-induced NF-κB subcellular translocation, we pre-treated A172 cells with EYK (200 µg/ml) or vehicle for 45 min, treated with PMA (75 nM) or vehicle for 24 h, and then performed subcellular fractionation. Pre-treatment with EYK followed by PMA treatment showed a trend toward decreased p-IκBα levels and did not alter IκBα and NF-κB levels in the cytosol (Fig. 8A-D, P<0.05; ANOVA with post hoc Tukey’s test). In the nuclear fraction, pre-treatment with EYK significantly decreased PMA-induced NF-κB levels (Fig. 8E and F, P<0.01; P<0.001, ANOVA with post hoc Tukey’s test). Moreover, pre-treatment with EYK significantly decreased PMA-induced p-NF-κB levels in the nucleus (Fig. 8G and H, P<0.001; ANOVA with post hoc Tukey’s test).

We then investigated whether pre-treatment with EYK could alter cell migration via NF-κB pathways. To test this, we performed wound healing assays on A172 cells pre-treated with vehicle or BAY11-7085 (10 µM, an NF-κB inhibitor) for 1 h, treated with EYK (200 µg/ml) or vehicle for 45 min, and then treated with PMA (75 nM) or vehicle for 24 h. Pre-treatment with EYK decreased PMA-induced cell migration (Fig. 8I and J, P<0.05; P<0.001, ANOVA with post hoc Tukey’s test; EYK+PMA vs. BAY+EYK+PMA, two-tailed t-test). In addition, pre-treatment with NF-κB inhibitor followed by treatments with EYK and PMA further inhibited cell migration in comparison with treatments with EYK and PMA (Fig. 8I and J). Based on these findings, we conclude that EYK could be used as a drug to prevent cancer cell migration by decreasing the nuclear localization of NF-κB and MMP-9 activity in A172 cells (Fig. 8K).

Discussion

Glioblastoma multiforme (GBM) is characterized by its aggressive cell proliferation and invasive infiltration into the surrounding brain tissue (19), and these features are associated with very poor prognosis (20). Temozolomide (TMZ) in combination with radiation therapy is the typical chemotherapy used to treat GBM (21). However, this approach has several problems, including drug resistance and induction of
proliferation and metastasis (21-23). Therefore, it is necessary to develop a novel antitumor agent in order to successfully treat GBM.

In the present study, we discovered that EYK inhibits monomorphic malignant human glioma cell migration and invasion by downregulating MMP-9 activity and nuclear localization of NF-κB, the transcription factor primarily responsible for expression of MMP-9. Several studies have demonstrated that EYK has antitumor effects including reduction of drug resistance-causing gene mutations in lung cancer cells (24), induction of apoptosis in human prostate cancer cells (5) and non-small cell lung cancer (6), and down-regulation of cell proliferation in osteosarcoma cells (25). However, this is the first study to demonstrate the ability of
EYK to inhibit brain cancer cell migration and invasion in human malignant cells.

Cancer cell migration and invasion are the main biological characteristics associated with tumor malignancy (26). In a previous study of *Epimedium* species, icaritin, a bioactive compound in *Epimedium* extract, inhibited adhesion, migration and invasion of glioblastoma cells via downregulation of extracellular matrix (ECM) and MMPs mediated by the PTEN/AKT/HIF-1α pathway (27). In addition, consistently with those previously published results, our present findings demonstrated that pre-treatment with PMA followed by EYK treatment effectively inhibited migration and invasion (Fig. 3).

Figure 8. Pre-treatment with EYK and PMA decreases translocation of NF-κB to the nucleus. A172 cells were pre-treated with EYK (200 µg/ml) or vehicle for 45 min, treated with vehicle or PMA (75 nM) for 24 h and subjected to subcellular fractionation (nucleus vs. cytosol). (A) Western blotting was performed on the cytosolic fraction using antibodies against p-IκBα, IκBα, NF-κB and β-actin. (B-D) Quantification of data from (A) (A172: con, n=4; EYK, n=4; PMA, n=4; EYK+PMA, n=4; *P<0.05; ANOVA with post hoc Tukey's test). (E) Western blotting was performed on the nuclear fraction using antibodies against NF-κB and PCNA. (F) Quantification of data from (E) (A172: con, n=4; EYK, n=4; PMA, n=4; EYK+PMA, n=4; **P<0.01; ***P<0.001, ANOVA with post hoc Tukey's test). (G) Western blotting was performed on the nuclear fraction using antibodies against NF-κB and β-actin. (H) Quantification of data from (G) (A172: con, n=126 cells; PMA, n=106 cells; PMA+EYK, n=122 cells; **P<0.001; ANOVA with post hoc Tukey's test). (I) A172 cell monolayers were scratched with a scraper, pre-treated with vehicle or BAY 11-7085 (10 µM, NF-κB inhibitor) for 1 h, treated with vehicle or EYK (200 µg/ml) for 45 min, and then treated with PMA (75 nM) or vehicle for 24 h. Images of the wound gap were acquired at 0 h (i.e., immediately after scratching) and after 24 h. (J) Quantification of data from (I) (A172: con, n=68; PMA, n=53; EYK+PMA, n=69; BAY+EYK, n=45; BAY+EYK+PMA, n=58; *P<0.05; **P<0.001, ANOVA with post hoc Tukey's test; EYK+PMA vs. BAY+EYK+PMA, two-tailed t-test). (K) Schematic model of suppression of brain cancer cell migration and invasion by EYK. (G) Scale bar, 20 µm and (I) 200 µm.
cells, but not in two other cell lines, T98G and U373MG. A172 cells are monomorphic and fibroblast-like whereas T98G cells are polymorphic, fibroblast-like and polygonal (28). U373MG cells have pleomorphic features (29). Thus, EYK may selectively alter cell migration and invasion in specific cell types of human brain cancer cells. Indeed, we confirmed that cell migration and invasion were inhibited when EYK was used as a pre-treatment prior to PMA exposure (Fig. 7).

Degradation of ECM is an important process in cancer cell migration and invasion (30). MMPs induce cell migration and invasion by degrading ECM proteins on and around surrounding normal brain tissue (31). One of the MMPs, MMP-9, is overexpressed in experimental glioma models and brain tumor patient tissue samples (32), and plays a major role in invasive infiltration and migration of brain cancer cells (31,33). Accordingly, downregulation of MMP-9 levels prevents tumor growth and invasion in glioblastoma cells lines (34). Quercetin, one of the elements of EYK, inhibits expression of MMP-9 via the AKT/ERK signaling pathway in human glioma cells (35). Our results showed that pre-treatment with PMA followed by EYK treatment dramatically downregulated PMA-induced MMP-9 activity and its mRNA levels in A172 cells (Fig. 4). These results were confirmed in wound healing assays and gelatin zymography assays by treating with CAS 1177749-58-4, MMP-9 inhibitor. Similar to our findings shown in Fig. 4, pre-treatment with EYK and PMA inhibited MMP-9 activity compared to PMA treatment (Fig. 7).

Regulation of MMP-9 is the major anti-oncogenic effect of TIMP-1 (tissue inhibitor of metalloproteinases), an endogenous inhibitor. For instance, B16F10 melanoma-expressing-mice followed by injection of recombinant TIMP-1 decreases pulmonary metastases (36). Hence, we investigated whether EYK regulates MMP-9 activity directly or by modulating levels of TIMP-1. To this end, we asked whether EYK affects TIMP-1 mRNA levels. In this study, neither pre-treatment with PMA followed by EYK nor exposure to the compounds in the opposite order altered TIMP-1 mRNA levels (Figs. 4 and 7). Therefore, we concluded that EYK directly regulates MMP-9 activity to alter cancer cell migration.

Nitrogen-activated protein kinases (MAPK) including ERK, JNK and p38 are involved in cancer cell migration and invasion (37-40) and regulate MMP-9 activity and its expression (41). Notably, we found that PMA in combination with EYK did not alter phosphorylation of ERK, JNK, or p38 (data not shown). We then examined another potential signaling pathways, focal adhesion kinase (FAK) signaling, that are targeted by EYK to regulate cancer cell migration. It is well-established that FAK signaling plays an important role in cancer cell migration as well as invasion (42). For these reasons, we investigated whether EYK can modulate FAK signaling pathway and found that treatment with PMA and EYK in either temporal order did not alter the phosphorylation of FAK (data not shown). Therefore, we concluded that EYK inhibits cell migration independent of PMA-induced MAP kinases and FAK signaling.

To elucidate the molecular mechanism by which EYK inhibits human brain cancer cell migration and invasion, we examined the effect of EYK on NF-κB subcellular localization, which is primarily responsible for regulating MMP-9 expression. In addition, NF-κB activation contributes to PMA-induced MMP-9 activity and glioma cell migration (18). In this study, pre- or post-treatment with EYK decreased PMA-mediated nuclear levels of NF-κB (Figs. 5 and 8). Surprisingly, our results indicated that EYK mainly affects nuclear levels of NF-κB without changing the levels of IkBα, a cellular inhibitor of NF-κB. Thus, EYK may directly regulate subcellular localization of NF-κB or modulate its translocation to the nucleus via an unknown inhibitory factor, thereby altering cancer cell migration. Accordingly, future studies should further investigate the molecular mechanism underlying regulation of NF-κB by EYK in monomorphic malignant human glioma cells.

In summary, this study provides for the first time demonstration that EYK exerts its anticancer effect in monomorphic human glioblastoma by inhibiting MMP-9 activity, mediated by a reduction in nuclear localization of NF-κB. This effect could be achieved by pre-treating with PMA followed by EYK treatment (as a cure condition) or by pre-treating with EYK followed by PMA exposure (as a prevention condition) (Fig. 8K). Taken together, our results suggest that EYK could be used as a drug for the cure and prevention of monomorphic malignant human glioma.

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