

Inhibitory effect of isomorellin on cholangiocarcinoma cells via suppression of NF- κ B translocation, the phosphorylated p38 MAPK pathway and MMP-2 and uPA expression

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Abstract. Evidence indicates that most cancer deaths are caused by tumor invasion and metastasis. Cholangiocarcinoma (CCA) is a tumor of the bile duct epithelium characterized by slow growth, rapid metastasis and poor prognosis. Caged xanthenes are extracted from gamboge, a dry resin exuded by *Garcinia hanburyi*. These compounds have been reported to be cytotoxic to several types of cancer cells, without affecting normal cells. The aim of the present study was to determine the effect of isomorellin on the inhibition of CCA cell (KKU-100) viability, migration, invasion and the expression of invasion-regulated proteins. Cytotoxicity of isomorellin was evaluated using a sulforhodamine B assay. The anti-migratory and anti-invasive effects of isomorellin on KKU-100 cells were assessed using wound healing and chamber invasion assays, respectively. Furthermore, the activities of matrix metalloproteinases (MMPs)-2 and -9, and urokinase-type plasminogen activator (uPA) were also investigated. The expression levels of proteins regulating invasion were determined via western blot analysis. The cell viability of KKU-100 cells was decreased following treatment with isomorellin in a dose-dependent manner, with IC₅₀ values at 24, 48 and 72 h of 3.46±0.19, 3.78±0.02 and 4.01±0.01 μ M, respectively. Wound healing and chamber invasion assays indicated that isomorellin significantly inhibited KKU-100 cell migration and invasion in a dose-dependent

manner. In addition, isomorellin significantly inhibited cancer cell migration and invasion abilities via focal adhesion kinase (FAK), protein kinase C (PKC), the phosphorylated (p)-p38 mitogen-activated protein kinase (MAPK) pathway, and nuclear factor (NF)- κ B expression and translocation to the nucleus, thus resulting in downregulation of MMP-2, uPA and cyclooxygenase-2 (COX-2) expression. Therefore, inhibition of MMP-2, uPA and COX-2 expression may result in decreased CCA cell invasion ability. These data demonstrated for the first time that the suppression of KKU-100 cell viability, invasion and migration, and downregulation of NF- κ B, MMP-2, uPA and the p-p38 MAPK pathway, may result in isomorellin-mediated anti-invasiveness.

Introduction

Cholangiocarcinoma (CCA) is a malignant tumor of the bile duct epithelium. This disease has become a major public health concern in Northeastern Thailand (1). Although the growth of CCA is quite slow, the metastasis rate is very high (1). CCA is a rare primary liver tumor worldwide, with an estimated 2,000-3,000 new cases occurring annually in the United States (rate, 1-2 cases/100,000 population between 1999-2014) (2). CCA occurs with a high prevalence in Asia, especially in Northeastern Thailand, where liver fluke *Opisthorchis viverrini* infection is endemic (3). The 5-year survival rates of patients with hilar, intrahepatic and distal extrahepatic CCA receiving surgical intervention are 11-41, 22-44 and 27-37%, respectively (4). The survival rate of patients with CCA depends on the anatomical location and the extent of metastasis (5). The prognosis of patients with CCA is poor due to failure of early diagnosis and the lack of an effective treatment for patients with inoperable CCA (6). Therefore, novel and effective chemotherapeutic agents for the treatment of advanced and metastatic CCA are required.

Previous studies have suggested that cell invasion and metastasis are associated with poor prognosis and increased

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mortality rate in CCA (7,8). Mitogen-activated protein kinases (MAPKs) influence different cellular processes, including gene expression, mitosis, movement, metabolism and apoptosis (9). There are three major subfamilies of MAPKs, including extracellular signal-regulated kinases 1/2 (ERK1 and ERK2), c-Jun NH2-terminal kinases (JNK1, JNK2 and JNK3) and four p38 enzymes, namely p38 α , p38 β , p38 δ and p38 γ (9). The MAPK pathway serves an important role in tumor development and progression via regulating several cellular activities, such as cell apoptosis, proliferation and differentiation (10). ERK 1/2 are proline-directed kinases that are activated via coordinated phosphorylation of tyrosine and threonine residues, resulting in cell proliferation and malignant transformation (10). JNKs are activated in response to environmental stressors, such as inflammatory cytokines, UV irradiation and ischemia or membrane-bound receptor signaling (11,12). Furthermore, JNKs regulate cell viability and proliferation, and induction of apoptosis via modulating the expression of AP-1, p53, c-Myc, nuclear factor (NF)- κ B, Sap-1 and Bcl-2 family members (11,12). The p38 subfamily is a member of the MAPK family and is associated with the development of CCA (13). The activation of the p38 MAPK signaling pathway influences CCA growth via maintaining the transformed cell phenotype in malignant human biliary tract epithelial cells (14).

Natural products extracted from plants, have been used for the prevention and/or treatment of cancer (15). *Garcinia hanburyi* is distributed widely throughout Southeast Asia, including Cambodia and Thailand (15). The latex of *G. hanburyi* is used in Thai traditional medicine for the treatment of potent purgative and infected wounds (15). Several caged xanthenes, including gambogic acid, isomorellin, isomorellinol, forbesione, morellic acid, desoxygambogenin, hanburin, desoxymorellin and dihydroisomorellin are extracted from the latex, fruits and whole *G. hanburyi* plant (16-18). Hahnvajawanong *et al* (19) reported that four caged xanthenes, namely isomorellin, isomorellinol, gambogic acid and forbesione induced apoptosis in CCA cell lines via the mitochondrial-dependent pathway. It has been also reported that forbesione, alone or combined with 5-FU, may strongly induce apoptosis in hamster CCA (Ham-1) cells *in vitro* and *in vivo* (20). Gambogic acid inhibits the invasion of highly invasive human breast carcinoma (MDA-MB-435) cells via protein kinase C (PKC), phosphorylation of ERK1/2 and JNK-mediated MMP-2 and -9 expression (21). Furthermore, gambogic acid has been demonstrated to inhibit human umbilical vascular endothelial cell (HUVEC) proliferation, migration, invasion, tube formation and microvessel growth, via inhibiting vascular endothelial growth factor receptor (VEGF)-2, c-Src, focal adhesion kinase (FAK) and AKT (22). Our previous study revealed that four caged xanthenes, namely isomorellin, gambogic acid, forbesione and isomorellinol, exerted no cytotoxic effect on human peripheral blood mononuclear cells (19). Therefore, the present study aimed to reveal the anti-invasive effect of isomorellin and its underlying mechanism in the CCA cell line KKU-100.

Materials and methods

Materials. Isomorellin (Fig. 1A) was extracted from *G. hanburyi* Hook. f. (family *Guttiferae*) using bioassay-directed fraction-

ation and provided by Professor Vichai Reutrakul, Department of Chemistry, Faculty of Science, Mahidol University (Bangkok, Thailand) as previously described (23). The stock solution of isomorellin was prepared by dissolving in DMSO to a concentration 1.8 mM and stored at -20°C.

Antibodies against PKC (cat. no. sc-17804; 1:500), FAK (cat. no. sc-558; 1:400), phosphorylated (p)-FAK (cat. no. sc-374668; 1:200), ERK1/2 (cat. no. sc-514302; 1:400), p-ERK1/2 (cat. no. sc-7383; 1:400), JNK1/2 (cat. no. sc-7345; 1:400), p-JNK1/2 (cat. no. sc-6254; 1:400), p38 (cat. no. sc-7149; 1:400), p-p38 (cat. no. sc-7973; 1:400), NF- κ B/p65 (cat. no. sc-109; 1:500), MMP-2 (cat. no. sc-53630; 1:400), COX-2 (cat. no. sc-376861; 1:500) and HRP-conjugated secondary antibodies (anti-mouse (cat. no. sc-2005; 1:10,000) or anti-rabbit (cat. no. sc-2004; 1:10,000) and were sourced from Santa Cruz Biotechnology, Inc. Primary antibody against β -actin (cat. no. A1978; 1:3,000) was obtained from Sigma-Aldrich (Merck KGaA) and antibodies against histone-H1 (cat. no. ab11079; 1:10,000) were purchased from Abcam. The BD Biocoat Matrigel invasion chamber was purchased from Becton Dickinson and Company, with 6.5 mm diameter polycarbonate membranes (8- μ m pore size) and coated with Matrigel (Becton Dickinson and Company). The proteolytic enzyme standard of human gelatinases A (MMP-2; cat. no. CC071), B (MMP-9; cat. no. CC079) and uPA (cat. no. CC4000) was purchased from Chemicon International (Thermo Fisher Scientific, Inc.).

Cell culture. The human CCA cell line KKU-100 was established at the Department of Pathology, Faculty of Medicine, Khon Kaen University, Thailand. KKU-100 cells were maintained in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin and cultured at 37°C in a humidified incubator containing 5% CO₂.

Cell viability assay. The sulforhodamine B (SRB) assay (cat. no. 3520-42-1; Sigma-Aldrich; Merck KGaA) was performed as previously described (24). Briefly, KKU-100 cell suspension was seeded into 96-well plates at a density of 1×10^5 cells per well. Following 24-h incubation at 37°C, cells were treated with isomorellin at final concentrations of 0, 0.15, 0.3, 0.6, 1.2, 2.4 and 4.8 μ M for 24, 48 and 72 h, and subsequently the effect of isomorellin on KKU-100 cells was determined using the SRB assay. The IC₅₀ values were obtained using concentration-effect curves following linear regression analysis.

Wound healing assay. Wound healing was performed according to a procedure described by Lu *et al* (25) with certain modifications. Briefly, KKU-100 cells (5×10^5 cells/ml) were seeded into 6-well plates and cultured to 100% confluency. The denuded zone (gap) was generated using a 1 ml plastic pipette tip. Subsequently, cells were starved in 1% FBS medium with or without isomorellin (0, 0.3, 0.6 and 1.2 μ M) for 12 h. The wound area was digitally imaged using a microscope at x10 magnification (Eclipse Ni-U microscope; Nikon Corporation). Finally, the area of the wound closure was calculated using the following formula: (Area of original wound - Area of wound during healing)/Area of original wound.

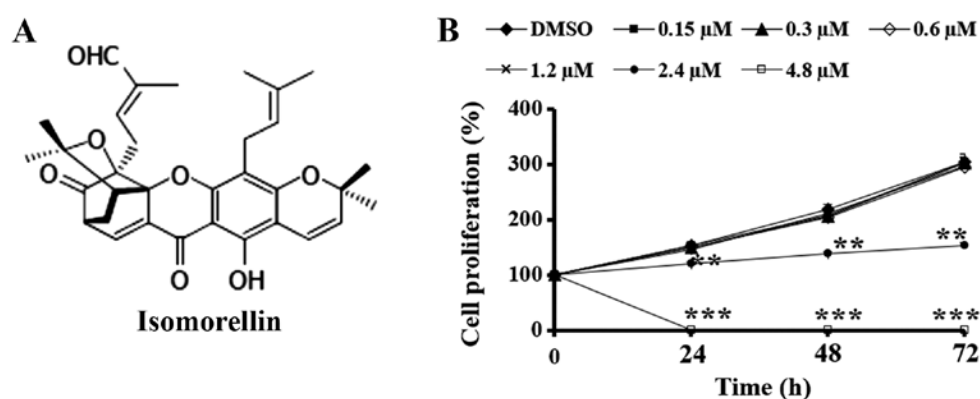


Figure 1. Chemical structure of isomorellin and its inhibitory effect on CCA (KKU-100) cell growth. (A) Isomorellin isolated from *G. hanburyi*. (B) Changes in cell proliferation rate of CCA (KKU-100) cells in response to various concentrations (0–4.8 μ M) of isomorellin at 24, 48 and 72 h. Data are expressed as the mean \pm SD. n=3. **P<0.01, ***P<0.001 vs. DMSO-treated control cells. CCA, cholangiocarcinoma; SD, standard deviation; DMSO, dimethyl sulfoxide.

Chamber invasion assay. The Transwell invasion chamber assay was performed as previously described by Albini *et al* (26). A total of 400 μ l (KKU-100 cells) cell suspension in 10% FBS (density, 1.25×10^5 cells/ml) and 100 μ l of isomorellin (0, 0.3, 0.6 and 1.2 μ M) were added into the upper chamber of a Matrigel invasion chamber. The medium containing 10% FBS was added into the lower chamber as a chemoattractant. Invasion chambers were incubated for 24 h at 37°C, fixed with 4% paraformaldehyde for 30 min at room temperature and stained with 0.4% SRB for 30 min at room temperature. Cells on each membrane were counted under a light microscope and digital images were captured at low-power fields (magnification, x20).

Gelatin zymography and uPA assay. The activity of MMP-2, MMP-9 and uPA was determined using gelatinase zymography assay as previously described, with certain modifications (27). The concentration of the conditioned medium in the homogenate was determined using Bradford reagent (28). Briefly, cells (density, 1.5×10^5 cells/well) were seeded into 6-well plates and incubated at 37°C for 24 h. Cell monolayers were washed with PBS and serum-free medium, and cultured in serum-free medium containing various concentrations of isomorellin (0, 0.3, 0.6 and 1.2 μ M) for 72 h at 37°C. For the gelatin zymography assay, a total of 50 μ g protein lysate was separated in a 10% polyacrylamide gel supplemented with 1 mg/ml gelatin, while a 10% polyacrylamide gel supplemented with 1 mg/ml gelatin (cat. no. 9000-70-8; Sigma-Aldrich; Merck KGaA) and 20 μ g/ml plasminogen (cat. no. 528213; Sigma-Aldrich; Merck KGaA) was used for uPA assay. Gels were washed with 2.5% Triton X-100 and then incubated at 37°C in a 50 mM Tris-HCl buffer containing 5 mM CaCl_2 and 0.02% NaN_3 for 12 h. The gel was stained overnight and discolored using 0.5% Coomassie blue and 10% acetic acid, respectively. The protease activity bands of MMP2, MMP-9 and uPA were imaged using a gel documentation system (Bio-Rad Laboratories, Inc.) and analyzed using Scion Image software (version 4.0.2; Scion Corporation).

Total cell, cytosol, nuclear and membrane protein extraction. Protein extraction was carried out according to the procedure of Wattanawongdon *et al* (29). To prepare total cell lysates,

cells (density, 1×10^6 cells/dish) were seeded and then treated with isomorellin (0, 0.3, 0.6 and 1.2 μ M) at 37°C for 24 h. Subsequently, cells were rinsed and lysed with cold RIPA buffer (50 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.1% sodium dodecyl sulfate and 0.5% deoxycholate) supplemented with protease and phosphatase inhibitor cocktails (cat. no. 78446; Pierce Biotechnology Inc.). The supernatants were collected following centrifugation at $13,000 \times g$ at 4°C for 30 min. For the cytosolic and nuclear proteins, cells were extracted using 500 μ l buffer A (10 mM HEPES, 0.1 mM EDTA, 10 mM KCl, 0.2% NP40, 1.5 mM MgCl_2 , 1 mM DTT and 0.5 mM phenylmethylsulfonyl fluoride) at 4°C for 30 min, followed by vortexing to shear the cytoplasmic membranes. The cell nuclei were collected by centrifugation at $1,000 \times g$ for 30 min at 4°C and nuclear proteins were extracted with 200 μ l of high-salt buffer B (20 mM HEPES, 25% glycerol, 1.5 mM MgCl_2 , 0.1 mM EDTA, 420 mM NaCl, 1 mM DTT and 0.5 mM phenylmethylsulfonyl fluoride) at 4°C for 30 min. Finally, membrane proteins were extracted using translocation buffer containing 0.1% Triton X-100, agitated at 4°C overnight and then centrifuged at $13,000 \times g$ for 30 min. The protein concentration was quantified using the Bradford method (28).

Western blot analysis. Proteins (5 μ g protein/lane) were fractionated in a 12% SDS-PAGE and electrotransferred onto a nitrocellulose membrane (EMD Millipore). Following blocking with 5% skimmed milk dissolved in Tris-buffered saline containing 0.1% Tween-20 at 37°C for 1 h, the membranes were probed with primary antibodies against PKC, FAK, p-FAK, ERK1/2, p-ERK1/2, JNK1/2, p-JNK1/2, p38, p-p38, NF- κ B-p65, COX-2, MMP-2, β -actin or histone H1 at 4°C overnight and then incubated with HRP conjugated secondary antibodies (anti-mouse or anti-rabbit) at room temperature for 1 h. The blots were visualized using an enhanced chemiluminescence kit (Pierce Biotechnology Inc.), quantified using densitometry (Image Quant LAS 4000; GE Healthcare Bio-Sciences) and assessed using the Scion Image software (version 4.0.2; Scion Corporation). The relative intensities of total cell, cytosolic and membrane protein expression were normalized to β -actin, while the protein expression of the nuclear lysate was normalized to histone H1.

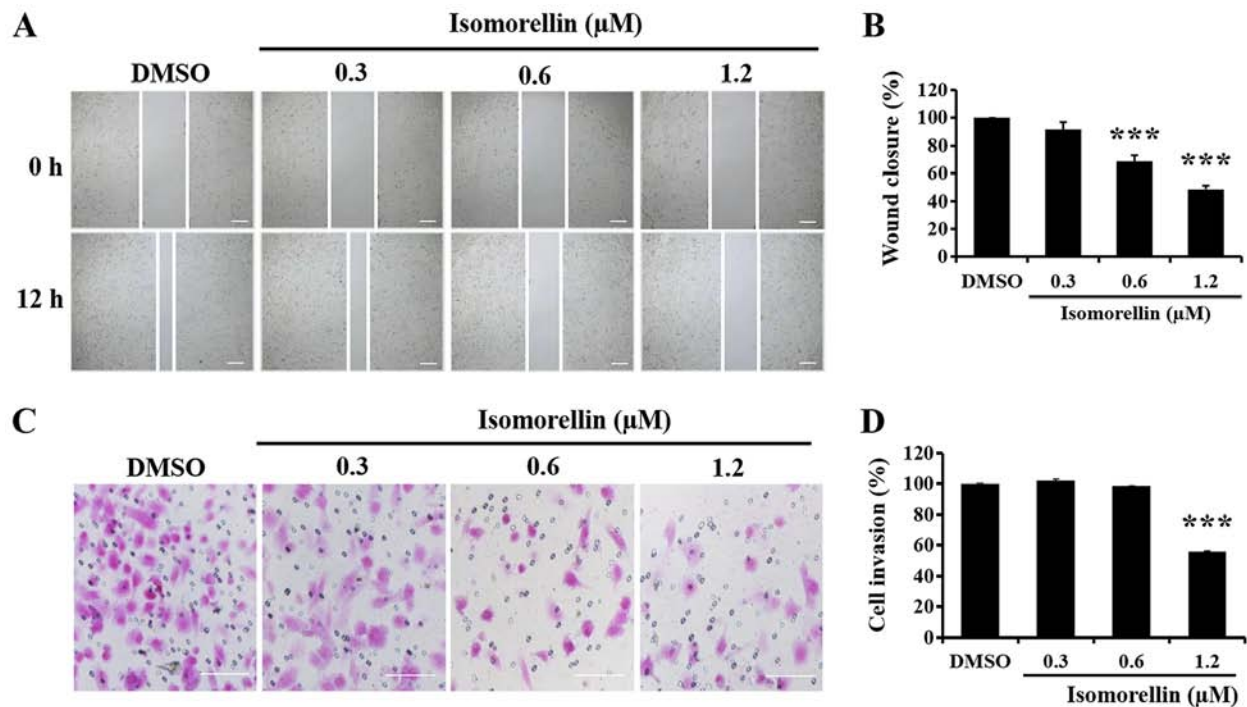


Figure 2. Effect of isomorellin on of KKKU-100 cell migration and invasion. Cells were treated with isomorellin (0, 0.3, 0.6 and 1.2 μM) and cell migration was analyzed using a wound healing assay. (A) Closure of the gap distance was determined using images from three random fields and (B) calculated as the percentage of the closed wound area. (C) Cell invasion was assessed using an invasion chamber and staining with SRB. (D) Cell invasion rate (%) is expressed as the mean \pm SD. Scale bar, 100 μm . *** $P < 0.001$ vs. DMSO-treated group. SRB, sulforhodamine B; SD, standard deviation; DMSO, dimethyl sulfoxide.

Statistical analysis. Data were expressed as the mean \pm standard deviation (SD) from three independent experiments. Comparisons between untreated control cells and treated cells were made using Tukey's post hoc test in the SPSS statistical software, version 16.0 (SPSS, Inc.). Differences were considered significant at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Results

Isomorellin reduces the viability of the CCA cell line KKKU-100. The ability of isomorellin to inhibit KKKU-100 cell viability was assessed using an SRB assay. Following treatment with 2.4 and 4.8 μM of isomorellin for 24, 48 and 72 h, KKKU-100 cell viability was decreased in a dose-dependent manner (Fig. 1B) and the IC_{50} values at 24, 48 and 72 h were 3.46 ± 0.19 , 3.78 ± 0.02 and 4.01 ± 0.01 μM , respectively.

Isomorellin reduces KKKU-100 cell migration and invasion ability. The effect of isomorellin on KKKU-100 cell migration ability was evaluated using a wound healing assay. Compared with the control group, isomorellin (0.6 and 1.2 μM) significantly inhibited the migration of KKKU-100 cells into the wound area in a dose-dependent manner ($P < 0.001$; Fig. 2A and B). Furthermore, an invasion chamber assay was performed to determine the effect of isomorellin on the invasion ability of KKKU-100 cells. Isomorellin (1.2 μM) significantly inhibited the penetration of the Matrigel-coated filter by KKKU-100 cells in a dose-dependent manner ($P < 0.001$; Fig. 2C and D).

Effect of isomorellin on the activity of FAK, PKC and downstream MAPK pathway, and translocation of NF- κB and

I κB - α transcription factors. As revealed in Fig. 3, isomorellin significantly downregulated p-FAK ($P < 0.05$), COX-2 ($P < 0.05$), whole ($P < 0.01$) and membrane PKC ($P < 0.01$) compared with DMSO-treated control cells (Fig. 3A-D). Furthermore, FAK ($P < 0.001$) and cytosolic PKC ($P < 0.01$) expression was significantly increased in isomorellin-treated KKKU-100 cells compared with the control (Fig. 3A and B). Additionally, treatment with isomorellin (1.2 μM) significantly decreased and increased the protein levels of p-p38 ($P < 0.001$) and p38 ($P < 0.001$), respectively, in a dose-dependent manner (Fig. 3E and F). However, isomorellin did not affect the expression of ERK1/2, p-ERK1/2, JNK1/2 and p-JNK1/2 (Fig. 3E and F). Finally, NF- κB expression was significantly downregulated in total cell ($P < 0.001$; 0.6 and 1.2 μM), cytosolic ($P < 0.01$; 1.2 μM) and nuclear fractions ($P < 0.001$; 1.2 μM), and I κB - α was upregulated in total cell ($P < 0.001$; 0.3, 0.6 and 1.2 μM) and cytosolic lysates ($P < 0.001$; 1.2 μM) of isomorellin-treated KKKU-100 cells at 1.2 μM (Fig. 4A-C). These findings indicated that isomorellin inhibited the translocation of NF- κB to the nucleus in a dose-dependent manner.

Isomorellin reduces the activity and expression of MMP-2 and uPA in KKKU-100 cells. It has been reported that the activity of the proteolytic enzymes MMP-2 and uPA serve a critical role in cancer cell invasion and metastasis (30). The results indicated that isomorellin significantly reduced KKKU-100 cell migration and invasion abilities, while DMSO had no effect on the activity of MMP-2 and uPA (Fig. 5). Therefore, the underlying mechanism of the impaired secretion of MMP and uPA was further investigated. Treatment with isomorellin (1.2 μM) significantly reduced the activity of MMP-2 ($P < 0.001$) and

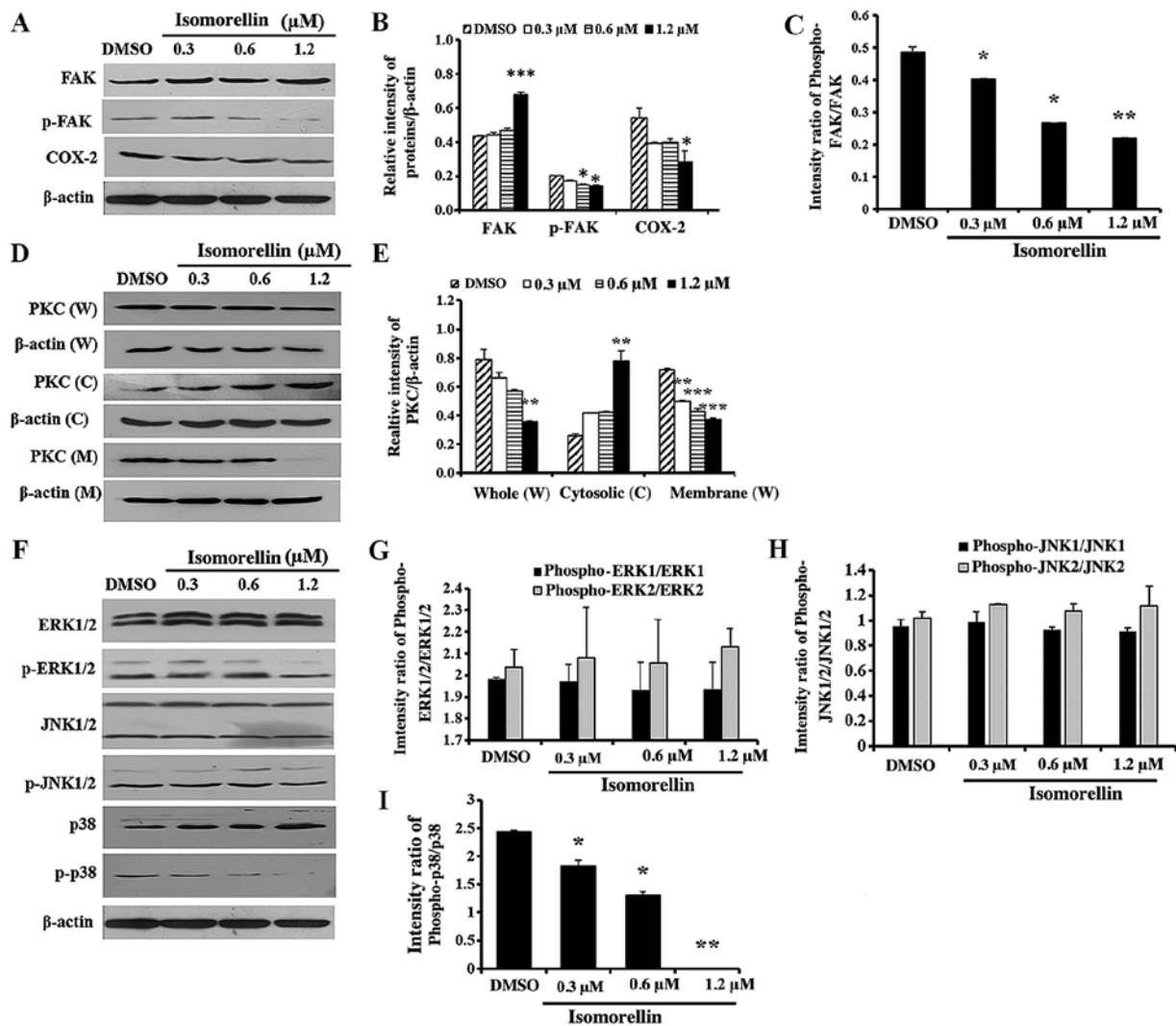


Figure 3. Effect of isomorellin on the expression of FAK, COX-2, PKC and downstream MAPK pathway in K KU-100 cells. (A) Western blot analysis, (B) quantification of the relative intensity of FAK, COX-2 expression and (C) quantification of the intensity ratio of phospho-FAK/FAK. (D) Western blot analysis and (E) quantification of PKC in W, C and M protein extracts. (F) Western blot analysis and (G-I) quantification of the intensity ratio of phospho-ERK1/2-ERK1/2, the intensity ratio of Phospho-JNK1/2/JNK1/2 and the intensity ratio of phospho-p38/p38. The protein expression levels were normalized to β -actin and expressed as relative intensity. Data are expressed as the mean \pm SD of three independent experiments. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. DMSO-treated group. FAK, focal adhesion kinase; COX-2, cyclooxygenase-2; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; ERK1/2, signal-regulated kinases 1/2; p-ERK1/2, phosphorylated ERK1/2; JNK1/2, c-Jun NH2-terminal kinases 1/2; SD, standard deviation; DMSO, dimethyl sulfoxide; W, whole cell; C, cytosolic; M, membrane; phosphor, phosphorylated.

uPA ($P<0.01$) (Fig. 5A-D). Furthermore, isomorellin significantly downregulated MMP-2 protein expression in K KU-100 cells ($P<0.01$) (Fig. 5E and F).

Discussion

The present study provided evidence that isomorellin reduced K KU-100 cell migration and invasion via downregulating FAK, PKC and p-p38 MAPK pathway expression. In addition, isomorellin inhibited the translocation of NF- κ B to the nucleus, thus resulting in decreased activity and expression of MMP-2 and uPA. The present study suggested that the mechanism underlying the inhibitory effects of isomorellin was associated with the inhibition of the transcription factor NF- κ B, the downstream MAPK signaling transduction pathway, MMPs and uPA, thus leading to decreased migration and invasion abilities of CCA cancer cells (Fig. 6).

In recent years, there has been growing use of natural products for medicinal purposes, including anticancer treatment (20,31,32). This may be due to the fact that many plants contain a variety of active phytochemicals, including flavonoids, terpenoids, lignans, sulfides, polyphenolics, carotenoids, coumarins, saporins, plant sterol, curcumins and phthalides, which have been reported to exhibit chemoprotective properties against cancer (33). These active phytochemicals serve an important role in several processes, including antioxidation, electrophile scavenging, immune response activation, induction of detoxification enzymes and inhibition of hormonal function and metabolic pathways in carcinogenesis (33). Recently, a novel approach in cancer therapy targeting the metastatic process has been developed. For example, the anti-metastatic properties of resveratrol (3,4',5-trihydroxystilbene) isolated from *Polygonum* species (*Polygonaceae* family) have been reported (34). Therefore, a previous study demonstrated

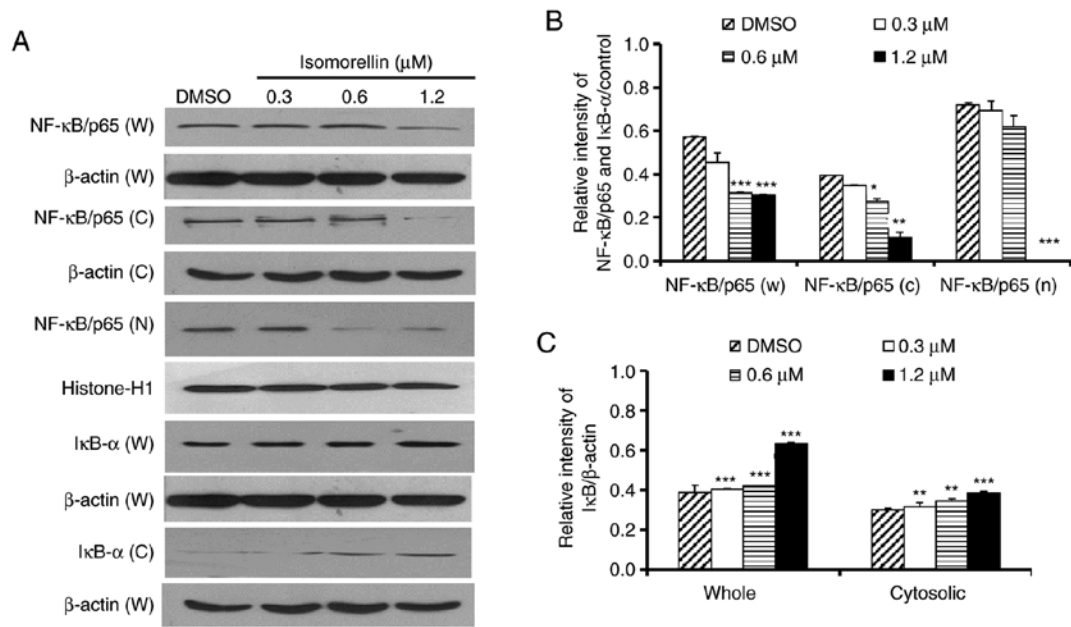


Figure 4. Isomorellin inhibits the expression of the transcriptional factors NF-κB/p65 and IκB-α in KKKU-100 cells. Cells were treated with different concentrations of isomorellin (0, 0.3, 0.6 and 1.2 μM) for 24 h. (A) The protein expression levels of the transcription factor NF-κB/p65 in W, C and N and IκB-α W and C were detected via western blot analysis and normalized to β-actin for total cell and cytosol lysates, and histone-H1 for nuclear lysates. Quantification of (B) NF-κB/p65 blots and (C) IκB-α blots. Data are presented as the relative intensity and expressed as the mean ± SD of three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. DMSO-treated group. NF-κB, nuclear factor κB; IκB-α, NF-κB inhibitor α; SD, standard deviation; DMSO, dimethyl sulfoxide; W, whole cell; C, cytosolic; N, nuclear.

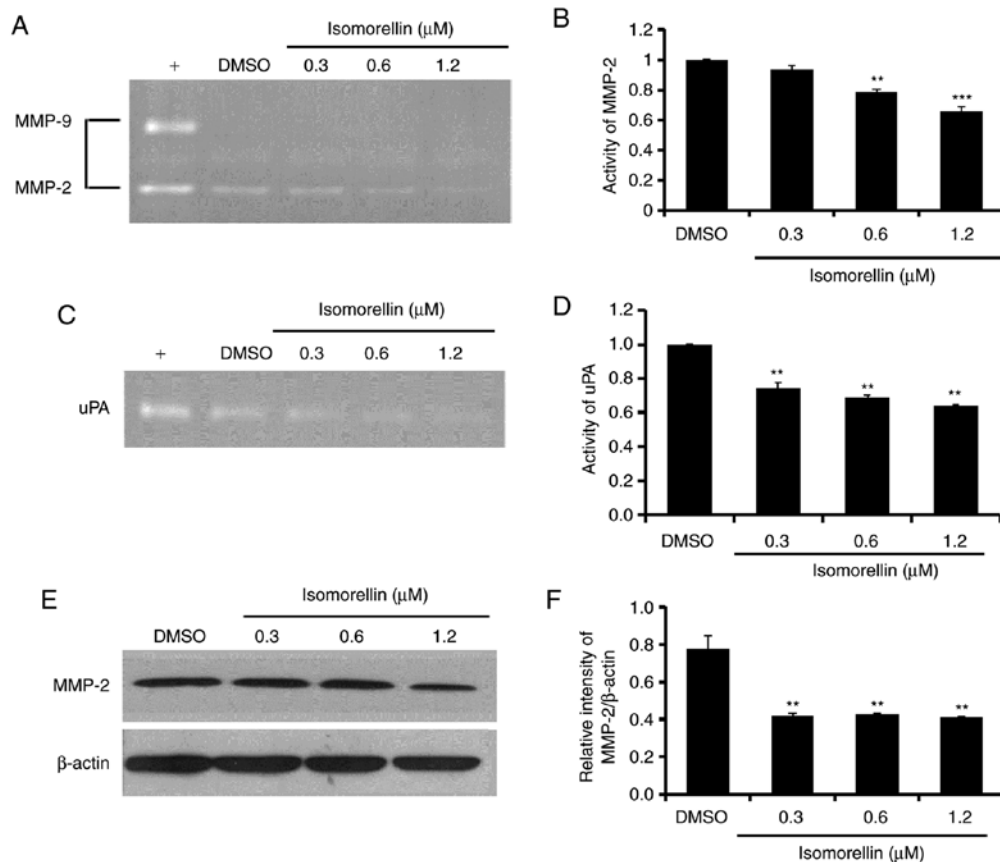


Figure 5. Isomorellin inhibits the activity and expression of MMP-2 and uPA in KKKU-100 cells. Cells were treated with various concentrations of isomorellin (0, 0.3, 0.6 and 1.2 μM) for 72 h and the activity of the condition medium was determined using gelatin and uPA zymography. The activity of MMP-2 was (A) measured and (B) quantified. (C and D) uPA was quantified using densitometry. The protein expression levels of MMP-2 were detected using (E) western blot analysis and (F) quantified. Data are expressed as the mean ± SD of three independent experiments. '+' represents proteolytic enzyme standard of human MMP-2, MMP-9 and uPA as positive controls. **P<0.01, ***P<0.001 vs. DMSO-treated group. MMP-2, metalloproteinase-2; uPA, urokinase-type plasminogen activator; SD, standard deviation; DMSO, dimethyl sulfoxide.

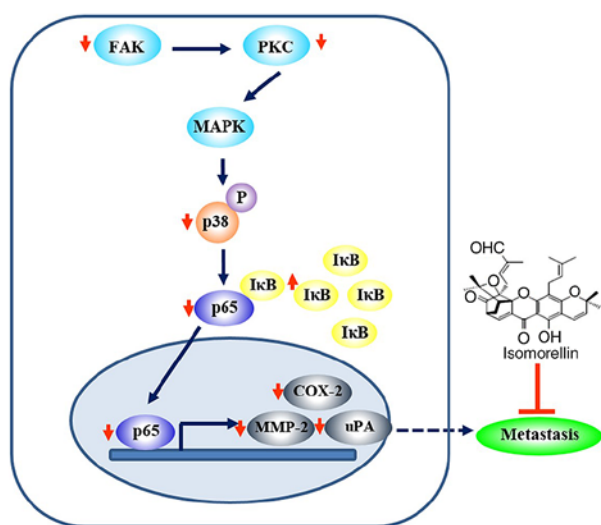


Figure 6. Possible signaling pathway involved in the isomorellin-mediated inhibition of CCA cell migration and invasion. Isomorellin inhibits the FAK, PKC and p38 MAPK pathway, which in turn induces I κ B α expression and inhibits the nuclear translocation of NF- κ B/p65, thus resulting in downregulation of MMP-2, uPA and COX-2 expression in CCA. CCA, cholangiocarcinoma; FAK, focal adhesion kinase; PKC, protein kinase C; MAPK, mitogen-activated protein kinase; I κ B α , NF- κ B inhibitor α ; NF- κ B, nuclear factor κ B; MMP-2, metalloproteinase-2; uPA, urokinase-type plasminogen activator; COX-2, cyclooxygenase-2.

that resveratrol inhibited HUVEC growth via reducing the gelatinolytic activity of MMP-2, tube formation, and endothelial cell attachment to fibronectin and laminin (34). In addition, it has been demonstrated that nobiletin, isolated from citrus fruits, exerts inhibitory effects on highly metastatic human AGS gastric adenocarcinoma cell adhesion, invasion and migration at non-cytotoxic concentrations (35). Furthermore, curcumin, isolated from *Curcuma longa*, decreases cell proliferation, invasion, angiogenesis and metastasis in different types of cancer via interacting with several cell signaling proteins, such as NF- κ B, AP-1 and the MAPK signaling pathway (36). Boueroy *et al* (37) demonstrated that rhinacanthin-C, extracted from *Rhinacanthus nasutus* (L.), inhibited CCA cell growth and metastasis via suppressing the expression of MMP-2, uPA, FAK and the downstream MAPK pathways. Our previous study reported that isomorellin inhibited cholangiocarcinoma cell lines by apoptotic induction in both cell lines (KKU-100 and KKU-M156), indicating a broad spectrum of anticancer activities (19). One limitation of current study is that it only reported the effect of isomorellin against one CCA cell line (KKU-100). However, the inhibition of migration and invasion effects were first demonstrated in the present study. For the evaluation of wound closure potential of compounds, cells were starved for minimizing proliferation and the degree of serum starving has to be worked out for each cell type examined (38). Many studies reported the starvation of cells in media containing 1% FBS for depletes growth factors that could influence the cell migration (39,40).

FAK is a signal transduction molecule associated with the invasive and metastatic potential of different types of cancer (41,42). FAK initiates a cascade of intracellular signals in response to adhesion, including activation of the MAPK pathway (43). PKC is a family of enzymes that serves an

important role in signal transduction pathways associated with the regulation of hormone release, mitogenesis and tumor promotion (44,45). Furthermore, the MAPK signaling pathway is involved in the enhanced invasive and metastatic ability of cancer (46). Additionally, it has been reported that the expression of MMP-2, MMP-9 and uPA inhibits the p-p38 MAPK pathway (46). MMPs serve a key role in the degradation of the extracellular matrix (ECM) and are involved in cell proliferation, migration, invasion and metastasis (30). Two classes of MMPs, namely MMP-2 and MMP-9, serve a key role in cancer cell invasion and metastasis via degrading type IV collagen, which is a major component of the basement membrane (30). The expression and activity of NF- κ B, a transcription factor, mediates cancer cell proliferation, invasion, angiogenesis and metastasis (47,48). Furthermore, the PI3K-AKT and RAS-MAPK pathways regulate the overexpression of NF- κ B (49). Therefore, the aforementioned findings indicate that translocation and downregulation of NF- κ B, which are regulated by the inhibition of the p-p38/MAPK pathway, may mediate the decreased expression and activity of MMP-2 and uPA.

The expression of matrix metalloproteinases (MMP-2 and MMP-9) and COX-2 is also promoted by NF- κ B (50). COX-2 serves a crucial role in cancer metastasis and is associated with the destruction of the ECM, initiation of epithelial-mesenchymal transition (EMT) and angiogenesis (51). Overexpression of COX-2 has been reported in different types of human cancer, including breast cancer, lung cancer, colon cancer and CCA (52,53). Herein, the inhibitory effect of isomorellin on cancer cell migration and invasion was mediated by inhibition of NF- κ B expression and translocation, resulting in decreased expression of MMP-2, uPA and COX-2. The present study also demonstrated that isomorellin significantly decreased the expression of FAK, PKC and the p-p38 MAPK pathway, which are involved in the upstream signal transduction of NF- κ B, resulting in reduced activity and protein expression of MMP-2, uPA and COX-2 in KKU-100 cells.

In conclusion, isomorellin significantly inhibited cancer cell migration and invasion abilities via FAK, PKC, and the p-p38 MAPK and NF- κ B pathways, thus leading to reduced expression levels of MMP-2, uPA and COX-2. Inhibition of MMP-2, uPA and COX-2 may result in decreased CCA cell invasion ability. Therefore, isomorellin may represent a promising therapeutic drug for the treatment of advanced and metastatic CCA. However, further studies should be conducted using *in vivo* models and clinical trials.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CH designed the experiments. TS performed the experiments. VR performed isomorellin separation. TB and AK performed data analysis. PB analyzed the data and wrote and reviewed the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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