Involvement of p53 and Nuclear Factor-kappaB Signaling Pathway for the Induction of G1-Phase Cell Cycle Arrest of Cholangiocarcinoma Cell Lines by Isomorellin

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Cell cycle arrest is closely linked to apoptosis. Isomorellin—a caged xanthone isolated from Garcinia hanburyi—induced apoptosis in cholangiocarcinoma (CCA) cell lines. To elucidate potential anticancer mechanisms, we investigated the effects of isomorellin on the growth, cell cycle progression, cell cycle regulated protein expression and nuclear factor-kappa B (NF-κB) activation of KKU-100 and KKU-M156 CCA cell lines; using sulforhodamine B assay, flow cytometry and Western blot analysis. The growth of both CCA cell lines was significantly inhibited by isomorellin treatment in a time- and dose-dependent manner. The respective IC50 value of isomorellin for KKU-100 cells was 6.2±0.13, 5.1±0.11 and 3.5±0.25 µM at 24, 48 and 72 h. By comparison, the respective IC50 value for KKU-M156 cells was 1.9±0.22, 1.7±0.14 and 1.5±0.14 µM at 24, 48 and 72 h. The growth inhibition of CCA cells by isomorellin was through the G0/G1 phase arrest mediated by inhibition of NF-κB activation, up-regulation of p53, p21 and p27 and down-regulation of cyclin D1, cyclin E, Cdk4 and Cdk2 protein levels. Our research suggests that isomorellin induces cell cycle arrest and apoptosis in CCA cell lines through p53 and the NF-κB-signaling pathway. The growth inhibitory potential of isomorellin was comparable to that of gambogic acid. Isomorellin shows potential as a therapeutic agent against human cholangiocarcinoma.

Key words isomorellin; cell cycle arrest; cholangiocarcinoma; Garcinia hanburyi; nuclear factor-kappa B; p53

Cholangiocarcinoma (CCA) is a tumor of the bile duct epithelium. The highest worldwide incidence is in the northeast region of Thailand and the bordering areas of Lao PDR and Cambodia.1 Recent epidemiologic studies have documented an increase in the worldwide incidence and mortality of CCA.2,3 Although a curative therapy can be achieved by surgical treatments and chemotherapy—if found early—the prognosis for late-stage CCA patients is poor, with a 5-year survival of <5%.4,5

The specific molecular events that initiate and drive the progression of CCA are debated. The hallmark of cancer in general is deregulation of the mechanisms (on/off-switching proteins) controlling cellular growth, arrest, apoptosis and/or survival.5 In several human cancers, there is an association between aggressive tumor behavior and the expression of cell cycle regulatory proteins (e.g., pRb, p21, p27, p53 and cyclin D1).6

In CCA, an overexpression of cyclin D1 protein has frequently been found; which in turn has been associated with poor histological differentiation, high cellular proliferation, lymph node metastasis and a poor prognosis.7 The low expression of the cyclin-dependent kinase inhibitor p27 correlated with vascular invasion and lymph node metastasis8 and low or absent expression with poor survival among CCA patients.9 The involvement of p53 tumor suppressor gene inactivation has also been reported in the development, carcinogenesis and progression of several cancers including CCA.10 The association between the transcription nuclear factor-kappa B (NF-κB) activation and CCA development has also been reported in an animal model11 and a high expression of the NF-κB has been found in CCA patient tissues (Seubwai et al., manuscript in preparation). Ongoing research is therefore needed to test new therapeutic agents that inhibit tumor cell growth or activate cell death.

In recent years, there has been growing interest in using natural products, especially those derived from plants, for medicinal purposes including anticancer agents. Isomorellin—one of the caged xanthones isolated from Garcinia hanburyi Hook. f. (family Guttiferae)—is a Thai traditional medicine used as a potent purgative and for treating infected wounds.12 Several caged xanthones—isolated from G. hanburyi, e.g., isomorellin, isomorellinol, gambogic acid and forbesione—exhibit cytotoxic effects in several mammalian cancer cell lines13–15 and anti-cancer and anti-tumor activities.14 Among the caged xanthones, gambogic acid causes an anticancer activity through down-regulation of telomerase,16 reduction of CDK7 kinase activity17 and inhibition of the catalytic activity of topoisomerase II alpha.18 Recently, both an

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anti-angiogenesis9) and an anti-invasive effect20) by gambogic acid have been reported. In addition, gambogic acid has been shown to induce cell cycle arrest at the G2/M phase in human gastric carcinoma BGC-823 cells.17) Recent studies have demonstrated that gambogic acid inhibits the NF-κB signaling pathway and induces apoptosis through its interaction with the transferrin receptor.21) Owing to these significant anti-tumor activities, the Chinese Food and Drug Administration recently approved a phase II clinical trial of gambogic acid injection as an antitumor treatment.20) It is therefore possible that other caged xanthones inhibit cancer cell growth and/or induce apoptosis through modulation of NF-κB expression and induction of cell cycle arrest.

Our group recently reported that four caged xanthones—including isomorellinol, isomorellin, gambogic acid and forbesione—have selective anti-proliferative activities against the cholangiocarcinoma cell lines KKU-100 and KKU-M156; by inducing apoptosis through both caspase-dependent and -independent mitochondrial pathways.22) The objective of the current study was to determine how and when isomorellin affects cell cycle progression and relatedly the expression of NF-κB and p53 in the CCA KKU-100 and KKU-M156 cell lines.

MATERIALS AND METHODS

Materials RPMI 1640 media, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL Products (Grand Island, NY, U.S.A.). Dimethylsulfoxide (DMSO), pifithrin-α (PFT-α), sulforhodamine B (SRB), Nonidet P-40, ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), sodium dodecyl sulfate (SDS), leupeptin, E-64, deoxycholate, phenylmethylsulfonylfluoride (PMSF), ethylene glycol tetraacetic acid (EGTA), Triton X-100 and antibody against β-actin (AC-15) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). GUAVA® cell cycle reagent was from GUAVA Technologies Inc. (U.S.A.). Antibodies against cyclin D1 (C-20), cyclin E (M-20), Cdk2 (M2), Cdk4 (C-22), p21 (C-19), p27 (N-20), NF-κBp65 (C-20), inhibitor of NF-κB (IxB-α) (C-21), Histone H1 (AE-4), horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) and goat anti-rabbit IgG antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The p53 antibody (BP53-12) was from Upstate (U.S.A.). The Coomassie protein assay kit reagent, Super signalaminol substrate, CL-XPosure film and Western blot stripping buffer were from Pierce Biotechnology, Inc. (Rockford, U.S.A.). Hybond ECL nitrocellulose membrane and an enhanced chemiluminescence kit were from Amersham Pharmacia Biotech (U.K.). Other reagents were of the highest grade available.

Preparation of Isomorellin, Gambogic Acid and Doxorubicin The caged xanthones isomorellin and gambogic acid (Fig. 1) were isolated from G. hanburyi Hook. f. (family Guttiferae) using bioassay-directed fractionation15) and dissolved in DMSO at 8 mM concentration and stored at −20°C. Doxorubicin purchased from Boryung Pharmaceutical Co., Ltd. (Korea) was aliquoted and stored at −20°C.

Cell Culture The cholangiocarcinoma cell lines KKU-100 (poorly-differentiated adenocarcinoma) and KKU-M156 (moderately-differentiated adenocarcinoma) were isolated from Thai CCA patients.20) Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL of penicillin and 100 µg/mL of streptomycin at 37°C in a humidified incubator containing 5% CO2.

Cell Proliferation Assay Effect of isomorellin on the cell proliferation was determined by sulforhodamine B (SRB) assay as described.24) Briefly, 200 µL of cell suspension (1.9×104 cells/well) were seeded into 96-well plates (Costar, Cambridge, MA, U.S.A.) and incubated at 37°C. After 24 h incubation, cells were treated with 0–8 µM of isomorellin in triplicate for 0, 24, 48 or 72 h. Since gambogic acid coexists with isomorellin in the same plant and is a well-known caged xanthone having potent anticancer effects,16–22) 0–8 µM of gambogic acid was used as a reference compound for treating cells.

Doxorubicin and 0.1% DMSO were used as the positive and negative controls, respectively. At the end of each exposure time, the medium was removed. The cells were then fixed with 20% (w/v) cold trichloroacetic acid (TCA) at 4°C for 30 min and washed 5 times with distilled water. The TCA-fixed cells were stained with 0.4% (w/v) SRB in 1% acetic acid for 30 min at room temperature. The bound dye was solubilized with 200 µL of 10 mM Tris buffer (pH 10). The absorbance of each well was measured using ELISA plate reader (Sunrise-TECAN, U.S.A.) at 510 nm. Three independent experiments were performed. Percent cell viability and the 50% inhibitory concentration (IC50) were determined as previously described.24)

Effects of p53 Inhibitor (Pifithrin-α, PFT-α) on Isomorellin Treatment KKU-100 cells were seeded at an appropriate density for 24 h. Cells were pre-treated with PFT-α (40 µM) for 5 h then treated with isomorellin (8 µM) for 24 h. Cells cultured in DMSO or PFT-α alone were used as controls. All assays were done in triplicate. The viability of the cells was analyzed using SRB assay, apoptotic cell death determined by FACSCalibur flow cytometer, and p53, p21 and Bax protein expression examined using Western blot analysis.

Cell Cycle Analysis Cells (1×106) were seeded 24 h before treatment in 10-cm dishes (Corning Incorporation, MA, U.S.A.). Cells treated with isomorellin (0–8 µM) or gambogic

Fig. 1. Chemical Structures of Isomorellin and Gambogic Acid Isolated from G. hanburyi
All analyses were performed using SPSS version 10.0 (SPSS Inc., U.S.A.).

RESULTS

Inhibition of CCA Cells Growth by Isomorellin After treating CCA KKU-100 and KKU-M156 cells with various concentrations of isomorellin (0–8 µM), gambogic acid (0–8 µM) or doxorubicin (0–0.125 µM) and SRB assaying at 24, 48 and 72 h, the growth inhibitory effects were determined. Growth in both cell lines was significantly decreased when the isomorellin concentration and time of incubation were increased (Figs. 2A, B). The respective IC_{50} value of isomorellin for KKU-100 cells at 24, 48 and 72 h was 6.2±0.13, 5.1±0.11 and 3.5±0.25 µM and for KKU-M156 cells 1.9±0.22, 1.7±0.14 and 1.5±0.14 µM. Growth inhibition of isomorellin on both CCA cell lines was in a dose- and time-dependent manner. Gambogic acid also significantly inhibited the growth of both CCA cell lines in a dose- and time-dependent manner (Figs. 2C, D) with the respective IC_{50} value at 24, 48 and 72 h for KKU-100 cells being 3.22±0.17, 2.83±0.18 and 2.47±0.03 µM and for KKU-M156 cells 1.96±0.01, 1.30±0.15 and 0.76±0.14 µM. Doxorubicin significantly inhibited the growth of both CCA cell lines at 72 h in a dose-dependent manner with an IC_{50} value of 0.66±0.07 µM and 0.01±0.01 µM for the KKU-100 and KKU-M156 cells, respectively.
and KKU-M156 cells, respectively. The growth inhibition effects of isomorellin, gambogic acid and doxorubicin were stronger on the KKU-M156 than on the KKU-100 cell line. Since isomorellin and gambogic acid showed different IC$_{50}$ values for growth inhibition on each CCA cell line, further study of each compound was done depending on the IC$_{50}$ values: (a) the isomorellin treatment of both CCA cell lines and the gambogic acid treatment of KKU-M156 cells for 24 h; and, (b) the gambogic acid treatment of KKU-100 cells for 36 h.

Induction of G0/G1 Phase Cell Cycle Arrest in CCA Cell Lines by Isomorellin In our previous study,22) isomorellin inhibited cell growth of KKU-100 and KKU-M156 cells through induction of apoptosis. Induction of apoptosis is due, at least in part, to cell cycle arrest.25,26) To determine whether isomorellin-induced growth inhibition of the CCA cell lines is mediated via alterations in cell cycle regulation, the effect of isomorellin on cell cycle distribution was evaluated by DNA cell cycle analysis. When KKU-100 and KKU-M156 cell lines were treated with isomorellin (0–8 μM for KKU-100 and 0–4 μM for KKU-M156 cells) for 24 h, a dose-dependent accumulation of cells in the G0/G1 phase (from 53 to 75% and from 47 to 72%, respectively) was observed with a decrease of cells in both the S phase (from 10 to 4% and from 16 to 7%, respectively) and the G2/M phase (from 36 to 21% and from 37 to 21%, respectively) (Fig. 3). After isomorellin-treatment at 4 μM, the proportion of G0/G1 phase cells in the KKU-M156 cells was slightly higher (1.5-fold of the control) than that of the KKU-100 cells (1.3-fold of the control).

When both CCA cell lines were treated with various doses (0.25–2 μM) of gambogic acid (36 h for KKU-100 and 24 h for KKU-M156), a cell cycle arrest at the G0/G1 phase was also observed (Table 1). After gambogic acid (2 μM)-treatment, the proportion of G0/G1 phase cells in the KKU-M156 cells was slightly higher (1.5-fold of the control) than that of the KKU-100 cells (1.3-fold of the control).

After 24 h pre-incubation, cells untreated or treated with 0.1% DMSO or indicated doses of isomorellin for 24 h. After fixation with 70% ethanol, cells stained with GUAVA® cell cycle reagent and analyzed by flow cytometry using CellQuest software. The respective percentage of cells in G0/G1, S and G2/M phase of cell cycle expressed as mean±S.E.M. of three independent experiments. *p<0.05 and **p<0.01 versus the 0.1% DMSO-treated cells.

Fig. 3. Effect of Isomorellin on Cell Cycle Progression in CCA KKU-100 (A) and KKU-M156 Cells (B)

After 24 h pre-incubation, cells untreated or treated with 0.1% DMSO or indicated doses of isomorellin for 24 h. After fixation with 70% ethanol, cells stained with GUAVA® cell cycle reagent and analyzed by flow cytometry using CellQuest software. The respective percentage of cells in G0/G1, S and G2/M phase of cell cycle expressed as mean±S.E.M. of three independent experiments. *p<0.05 and **p<0.01 versus the 0.1% DMSO-treated cells.
The reference compound can induce cell cycle arrest at the G0/G1 phase. The degree of cell cycle arrest by both compounds was slightly higher in KKU-M156 than in KKU-100 cells.

Effect of Isomorellin on the Expression of G0/G1 Phase Cell Cycle Regulatory Proteins

To determine the molecular mechanisms of isomorellin-induced G0/G1 phase arrest in CCA cell lines, expression of G0/G1 phase cell cycle regulatory molecules were examined. Among the cyclin and cyclin-dependent kinases (Cdks), the changes in the protein expression levels of cyclin D1, cyclin E, Cdk2, and Cdk4—which operate in the G0/G1 phase of the cell cycle—were

![Fig. 4. Effect of Isomorellin on Expression of G1 Phase Cell Cycle-Regulating Proteins in CCA KKU-100 (A) and KKU-M156 Cells (B)](image)

Data are means±S.E.M. of three independent experiments. *p<0.05, **p<0.01 and ***p<0.001 versus the DMSO-treated cells.

Table 1. Effect of Gambogic Acid on Cell Cycle Distribution in Cholangiocarcinoma KKU-100 and KKU-M156 Cell Lines

<table>
<thead>
<tr>
<th>Gambogic acid (µM)</th>
<th>% cells (mean±S.E.M.)</th>
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<tbody>
<tr>
<td></td>
<td>KKU-100</td>
</tr>
<tr>
<td></td>
<td>G0/G1</td>
</tr>
<tr>
<td>Media</td>
<td>57.09±0.94</td>
</tr>
<tr>
<td>DMSO</td>
<td>59.73±0.76</td>
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<tr>
<td>0.25</td>
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<tr>
<td>0.5</td>
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</tr>
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<td>1</td>
<td>68.97±0.90*</td>
</tr>
<tr>
<td>2</td>
<td>73.75±0.18**</td>
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</tbody>
</table>

Comparison between two CCA cell lines analyzed by Mann–Whitney test. ##p<0.01 and ###p<0.001.

Fig. 4. Effect of Isomorellin on Expression of G1 Phase Cell Cycle-Regulating Proteins in CCA KKU-100 (A) and KKU-M156 Cells (B)

After 24 h pre-incubation, cells treated with 0.1% DMSO or indicated doses of isomorellin for 24h. The whole cell lysate from untreated and treated cells separated on SDS-PAGE. Blotted proteins on nitrocellulose membrane separately probed with an antibody specific to cyclin D1, cyclin E, Cdk2, Cdk4, p21, p27, p53 and β-actin. Protein bands visualized using chemiluminescence system. Protein bands of isomorellin-treated KKU-100 cells (A). Protein bands of isomorellin-treated KKU-M156 cells (B). The data are mean ratio of density reading of target protein normalized to β-actin of isomorellin-treated to DMSO-treated KKU-100 and KKU-M156 cells (n=3). *p<0.05, **p<0.01 and ***p<0.001 versus the DMSO-treated cells.

Comparison between two CCA cell lines analyzed by Mann–Whitney test. ##p<0.01 and ###p<0.001.
examined by Western blotting analysis. Isomorellin-treatment (0–8 \( \mu M \) for KKU-100 and 0–4 \( \mu M \) for KKU-M156) for 24 h resulted in a significant decrease of the protein expression of cyclin D1, cyclin E, Cdk2 and Cdk4 in both cell lines in a dose-dependent manner (Fig. 4).

Since cell cycle progression was negatively regulated by the tumor suppressor protein p53 and Cdk inhibitors (CDKIs), including p21 and p27, the protein expression levels of p53, p21 and p27 were also examined. Isomorellin treatment significantly increased the protein expression of p53, p21 and p27 in a dose-dependent manner (Fig. 4). These results suggest that the G0/G1 isomorellin-induced phase arrest in both CCA cell lines was associated with the altered expression of the cell cycle regulatory molecules operative in the G0/G1 phase.

After isomorellin treatment at 1, 2 and 4 \( \mu M \), the relative amounts of cyclin D1, cyclin E, Cdk2 and Cdk4 protein levels in CCA cell lines were decreased several-fold, whereas that of p53, p21 and p27 protein were increased several-fold over against those in the DMSO-treated cells (Fig. 4C). The suppressive effects of isomorellin (4 \( \mu M \)) on cyclin D1, cyclin E, Cdk2 and Cdk4 were significantly stronger on the KKU-M156 cells than the KKU-100 cells. Conversely, the degree of up-regulation of the protein levels of p53, p21 and p27 by isomorellin (at 4 \( \mu M \)) was significantly greater in the KKU-100 cells than the KKU-M156 cells (Fig. 4C).

**Involvement of p53 in Isomorellin Induced CCA Cell Death** To determine whether isomorellin-induced apoptosis is regulated by the tumor suppressor p53, the effects of p53 inhibitor, PFT-\( \alpha \), on isomorellin-induced cell growth inhibition, induction of apoptosis and p53, p21 and Bax protein

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**Fig. 5. Involvement of p53 in Isomorellin-Induced Apoptosis in CCA KKU-100 Cells (A, B, C)**

After 24 h pre-incubation, cells were cultured in presence or absence of the pifithrin-\( \alpha \) (PFT-\( \alpha \)) (40 \( \mu M \)) for 5 h then treated with isomorellin (8 \( \mu M \)) for 24 h. Cells cultured in 0.1% DMSO or PFT-\( \alpha \) alone were used as control cells. Cell viability of untreated or treated cells was determined by SRB assay. Isomorellin-induced growth inhibition inhibited by PFT-\( \alpha \) (A). Apoptosis induction of untreated or treated cells was determined by FACS Calibur flow cytometer. Isomorellin-induced apoptosis decreased by PFT-\( \alpha \) (B). Cell lysate from untreated or treated cells was used for Western blot analysis. Expressions of p21 and Bax proteins induced by isomorellin decreased by PFT-\( \alpha \) (C).

**Fig. 6. Involvement of p53 in Isomorellin-Induced Apoptosis in CCA KKU-M156 Cells (D)**

After 24 h pre-incubation, cells were cultured in presence or absence of the pifithrin-\( \alpha \) (PFT-\( \alpha \)) (40 \( \mu M \)) for 5 h then treated with isomorellin (8 \( \mu M \)) for 24 h. Cells cultured in 0.1% DMSO or PFT-\( \alpha \) alone were used as control cells. Cell viability of untreated or treated cells was determined by SRB assay. Isomorellin-induced growth inhibition inhibited by PFT-\( \alpha \) (A). Apoptosis induction of untreated or treated cells was determined by FACS Calibur flow cytometer. Isomorellin-induced apoptosis decreased by PFT-\( \alpha \) (B). Cell lysate from untreated or treated cells was used for Western blot analysis. Expressions of p21 and Bax proteins induced by isomorellin decreased by PFT-\( \alpha \) (C).

**Mean±S.E.M. of density reading of target protein normalized to \( \beta \)-actin of isomorellin (Iso)- or Iso+PFT-\( \alpha \)-treated KKU-100 cells (D) \((n=3)\). *\( p<0.05 \) and **\( p<0.01 \) versus the DMSO- or DMSO+PFT-\( \alpha \)-treated cells. Mean±S.E.M. of density reading of target protein normalized to \( \beta \)-actin of Iso-treated KKU-100 cells \((n=3)\). †\( p<0.05 \) and ††\( p<0.01 \) versus the Iso+PFT-\( \alpha \)-treated KKU-100 cells.
expression were evaluated. When KKU-100 cells were treated with isomorellin (8 µM) plus PFT-α (40 µM) for 24 h, the percentage of cell viability was significantly increased as compared to isomorellin treatment alone (Fig. 5A). This result suggests that the p53 function is required for isomorellin-induced growth inhibition of KKU-100 cells.

Flow cytometric analysis revealed that PFT-α did not affect the proportion of sub-G1 population (corresponding to the apoptotic cells) in KKU-100 cells compared to DMSO-treated cells (Fig. 5B). When KKU-100 cells were treated with isomorellin (8 µM) plus PFT-α (40 µM) for 24 h, the sub-G1 population was significantly decreased compared to the isomorellin treatment alone (Fig. 5B). These results suggest isomorellin-induced apoptosis in KKU-100 cells is mediated via the p53 pathway.

Western blot analysis revealed that p53, p21 and Bax protein expression were significantly increased by isomorellin treatment in KKU-100 cells (Figs. 5C, D). The increase in p21 and Bax protein expression induced by isomorellin was significantly inhibited by PFT-α (Figs. 5C, D). These results suggest that p53 function is important in isomorellin-induced activation of p21 and Bax protein expression.

Inhibition of NF-κB Activation in CCA Cell Lines by Isomorellin NF-κB is a nuclear factor known to activate the expression of genes involved in cell proliferation (cyclin D1) and cell survival (anti-apoptotic proteins: Bcl-2, survivin). Since the down-modulation of NF-κB activity in the cytosol and nucleus is associated with an apoptotic response among eukaryotic cells, the effect of isomorellin on the protein expression of NF-κB/p65 in both CCA cell lines was examined. Using Western blotting analysis, isomorellin treatment of KKU-100 (0–8 µM) and KKU-M156 (0–4 µM) cell lines for 24 h was found to significantly reduce the protein expression of NF-κB/p65 in whole cell, cytosol and nuclear lysates in a dose-dependent manner in both CCA cell lines over against the control cells (Fig. 6). These results indicate that isomorellin suppresses NF-κB/p65 protein expression and translocation into the nucleus.

Since NF-κB activation is regulated by an IκB-α, we examined whether IκB-α was involved in the inhibition of NF-κB activation by isomorellin. The level of IκB-α in cytosol lysates was significantly increased in both the isomorellin-treated KKU-100 (0–8 µM) and KKU-M156 (0–4 µM) cell lines in a dose-dependent manner (Fig. 6).
When the relative intensities of NF-κB and IκB-α proteins to β-actin of isomorellin-treated cells (at 1, 2, 4 μM) were compared to those of the control (DMSO-treated) cells, the NF-κB protein level decreased several-fold and the IκB-α protein level increased several-fold in both the CCA cell lines (Fig. 6C). The degree of decrease in the NF-κB protein level (total, cytosol and nuclear fractions) and the increase of IκB-α protein level in the KKU-M156 cells was more prominent than in the KKU-100 cells at the same concentration of isomorellin (1, 2, 4 μM) tested. The decrease in the total NF-κB protein level in the KKU-100 cells was marginal (1, 0.99, 0.90-fold) at the low to medium doses of isomorellin (1, 2, 4 μM), but significant (0.62-fold) at the 8 μM dose. By contrast, the isomorellin treatment on the KKU-M156 cells caused a significant decrease of NF-κB in a dose-dependent manner at the 2 and 4 μM doses (Fig. 6C). These results suggest that isomorellin has a stronger effect on the KKU-M156 cells than the KKU-100 cells.

**DISCUSSION**

Induction of cell cycle arrest and/or apoptosis by manipulation of cancer cell growth has been used for screening potential chemotherapeutic agents. Many chemotherapeutic agents can cause DNA damage, leading to cell cycle arrest and/or apoptosis. Recently, caged xanthones— isomorellin, isomorellinol, gambogic acid and forbesione— isolated from G. hanburyi have been reported to inhibit the growth of various mammalian cancer cell lines (including the cholangiocarcinoma cell lines KKU-100 and KKU-M156). These four caged xanthones selectively killed cancer cells; without any negative influence on normal human peripheral blood mononuclear cells.

In a study of healthy rats, four intravenous injections of gambogic acid (6, 3, 1.5 mg/kg) on alternate days did not influence body weight, number of white blood cells or karyotypes in the marrow.

At the molecular level, these four caged xanthones inhibited KKU-100 and KKU-M156 cell growth by inducing apoptosis through both caspase-dependent and -independent mitochondrial signaling pathways. In addition, gambogic acid was found to induce G2/M phase cell cycle arrest in human gastric cancer BGC-823 cells, induce apoptosis in human gastric cancer BGC-823 and MGC-803 cells and inhibit the telomerase enzyme in human hepatoma SMMC-7721 cells. Although isomorellin—another caged xanthone—can also suppress tumor cell growth, the mechanism of its anticancer properties has yet to be defined.

In the present study, we first confirmed that isomorellin and gambogic acid strongly inhibited the growth of KKU-100 and KKU-M156 CCA cell lines in a dose- and time-dependent manner (Fig. 2). The growth inhibitory potency of gambogic acid was slightly higher than isomorellin (Fig. 2); however, according to the IC_{50} values, the growth inhibitory potential of isomorellin was comparable to gambogic acid. The small difference may be due to the prenyl methyl groups; functionalized as an aldehyde functional group in isomorellin and as a carboxylic acid functional group in gambogic acid (Fig. 1).

The growth inhibitory effect of isomorellin was greater on the KKU-M156 than on the KKU-100 cell line, perhaps due to their respective histologic types and drug sensitivities. Since the IC_{50} values of isomorellin, which exert growth inhibition to each CCA cell line, were different, isomorellin concentrations ranging from 1 to 8 μM for the KKU-100 cells and 0.5 to 4 μM for KKU-M156 cells for 24 h treatment were used for the further study. Similarly, based on the growth inhibitory results, treatments with 0.25–2 μM gambogic acid for 36 h for KKU-100 and 24 h for KKU-M156 were used for further study.

Deregulation of cell cycle progression is a hallmark of tumor growth. In the time-course study of tumor growth inhibition with isomorellin on both CCA cell lines, the IC_{50} values for isomorellin gradually decreased with exposure time, suggesting that the anti-proliferative effect is due to the blocking of some steps of cell proliferation. We, therefore, sought to determine whether the growth inhibitory effect of isomorellin was due to cell cycle arrest; hence a cell cycle analysis was done in the current study, which revealed that isomorellin treatment caused significant growth arrest at the G0/G1 phase of both CCA cell lines (Fig. 3). The degree of isomorellin-induced cell cycle arrest at the G0/G1 phase was higher in the KKU-M156 cells than the KKU-100 cells (Fig. 3). Similar results were obtained when both CCA cell lines were treated with gambogic acid (Table 1), which induced cell cycle arrest at the G0/G1 phase in human chronic myeloid leukemia cell line K562 through down-regulation of both mRNA and protein levels of the steroid receptor coactivator-3.

In a study of rat aortic smooth muscle cells, gambogic acid-induced cell cycle arrest at the G0/G1 phase was mediated through down-regulation of Cdk2, cyclin E, Cdk4 and cyclin D1 protein expression. By contrast, gambogic acid-induced cell cycle arrest at the G2/M phase in human gastric carcinoma BGC-823 cells was via disrupting Cdk7-mediated phosphorylation of Cdc2/p34 with no effect on cyclin D, E and A, and Chk1/2 and Cdc25. The cell cycle arrest by gambogic acid at the G2/M phase in human breast carcinoma MCF-7 cells has been attributed to a depolymerization of microtubules.

Taking the previous results and our own together, cell cycle arrest by gambogic acid appears to be caused by several different pathways. The exact mechanisms of the cell cycle arrest induced by isomorellin and gambogic acid are therefore only partially understood and require further focused study. The different susceptibility of two CCA cell lines for isomorellin/gambogic acid-induced cell cycle arrest should provide an interesting model system for the detailed analysis of the pathways of cell cycle arrest.

The cyclin-dependent kinases (Cdks) play important roles in the various phases of eukaryotic cell cycle progression: the cyclins and Cdk inhibitors (CDKIs) positively and negatively regulate the Cdk activities, respectively. The cyclin D1/Cdk4 complex can activate cell cycle progression early in the G1 phase by phosphorylation of pRb, while the Cdk2/cyclin E complex plays a role in the transition from the G1 to S phase. The cell cycle progression was negatively regulated by the tumor suppressor protein p53 and CDKIs, including p21 and p27. The p53 protein enhances the transcription of several genes including p21 and Bax while suppressing Bcl-2 expression. Binding of p21 and p27 to the cyclin-Cdk complex therefore results in an inhibition of a kinase activity; thereby interfering with phosphorylation of pRb and inducing arrest of cell-growth.

Modulation of the expression of key regulatory molecules in cell cycle progression can lead to cell cycle arrest. Consistent with previous reports, we found that the respective expression of p53, p21 and p27 in both CCA cell
lines was markedly increased by isomorellin treatment; by contrast, expression of the cyclin D1, cyclin E, Cdk2 and Cdk4 proteins was decreased (Fig. 4). The increase of protein expression levels of p53, p21 and p27 was significantly higher in the KKU-100 cell line than the KKU-M156 cell line, whereas the respective decrease of protein expression of cyclin D1, cyclin E, Cdk2 and Cdk4 was significantly stronger in the KKU-M156 cell line than the KKU-100 cell line (Fig. 4). Our results indicate that isomorellin-induced cell cycle arrest at the G0/G1 phase is via modulation of cell cycle regulatory molecules. The degree of isomorellin-induced cell cycle arrest at the G0/G1 phase was higher in the KKU-M156 cells than the KKU-100 cells: this may depend on the sum of incoming signals and the cellular context of these two different CCA cell lines.

The underlying mechanism of cell growth inhibition involves the modulation of cell cycle regulation leading to apoptotic cell death. In the present study, treatment with isomorellin resulted in cell cycle arrest at the G0/G1 phase at 24 h. In our previous study,22 apoptotic cells were observed in the KKU-M156 and KKU-100 cell lines at 24 and 36 h after treatment, respectively, and apoptosis was even more pronounced after longer incubation. The present results confirm our previous report22 that isomorellin treatment (at 36 h) induced a higher degree of apoptotic cell death in KKU-M156 cells than KKU-100 cells. Thus, growth inhibition by isomorellin seems to be initiated by cell cycle arrest at the G0/G1 phase before apoptosis occurred.

At the molecular level, a significant increase in p53 and p21 protein levels at 24 h was observed in both CCA cell lines treated with isomorellin. In our previous study, a gradual increase in Bax and a decrease in Bcl-2 protein levels were observed from 12 to 48 h in the isomorellin-treated KKU-100 and KKU-M156 cell lines.22 Treatment of both CCA cell lines with isomorellin resulted in concomitant up-regulation of p53, p21 and Bax and down-regulation of Bcl-2. It is probable that the increase in p21 and Bax and the decrease in Bcl-2 proteins in the isomorellin-treated CCA cell lines is due to up-regulation of p53.41,45 These results suggest that isomorellin induces apoptosis of both CCA cell lines through cell cycle arrest at the G0/G1 phase via up-regulation of the p53 protein.

In the current study, to determine the role of p53 in isomorellin-induced apoptosis, the p53 activity was attenuated by a p53 specific inhibitor, PFT-α. In KKU-100 cells, PFT-α treatment resulted in a significant decrease of isomorellin-induced growth inhibition by 23.2% (Fig. 5A), apoptosis induction by 43.8% (Fig. 5B), and a decrease in the expression of the p53-regulated proteins, p21 and Bax (Fig. 5C). These results suggest the involvement of p53 in isomorellin-induced apoptosis in KKU-100 cells. It is interesting to note that isomorellin-induced growth inhibition was not completely inhibited by PFT-α treatment in KKU-100 cells, suggesting that isomorellin may induce apoptosis through other pathways. Recently, steroid receptor coactivator-3 (SRC-3)—also called amplified in breast cancer 1 (AIB1), a member of the nuclear receptor coactivators—was reported to play an important role in tumor genesis and progression.46 Overexpression of SRC-3 was found to inhibit apoptosis through enhanced Akt and NF-κB activities and increased Bcl-2 levels.47 Overexpression of AIB1 protein has been reported in human CCA specimens and CCA cell lines.48 AIB1 was found to regulate the Bcl-2 expression through activation of the Akt pathway in CCA cells.49 Down-regulation of AIB1 caused G2/M arrest and decreased the expression of cyclin A, cyclin B, Cdk1 and Bcl-2 protein through the suppression of the Akt pathway, leading to inhibition of cell growth and induction of apoptosis in CCA cells.50 Gambogic acid is known to induce apoptosis in human chronic myelogenous leukemia K562 cell line through down-regulation of the SRC-3 expression, then inhibition of Akt kinase activity and its downstream targets p70 S6 kinase (S6K1) and glycogen synthase kinase 3b (GSK3b) and down-regulation of Bcl-2 expression.51 From the previous results and our own, isomorellin-induced apoptosis of KKU-100 and KKU-M156 cells may have been caused by both p53-dependent and -independent pathways, which require further detailed investigations.

The p53 has itself a direct apoptotic effect.50 In response to apoptotic signals, p53 will form complexes with the anti-apoptotic Bcl-XL and Bcl-2 proteins leading to (a) permeabilization of the outer mitochondrial membrane (b) release of cytochrome c and (c) activation of the caspase cascades.50 In our previous study, the activation of caspase-9 and -3 were also observed in isomorellin- and gambogic acid-treated CCA cell lines.22 Our results are supported by previous studies in which gambogic acid caused apoptosis of rat C6 glioma cells by triggering an intrinsic mitochondrial pathway for apoptosis.50 Dulxanthone A—an active cytotoxic xanthone isolated from Garcinia cowa Roxb (family Guttiferae)—induced cell cycle arrest and apoptosis via up-regulation of p53 through mitochondrial pathway in HepG2 cells.52

An increased accumulation of nuclear NF-κB in the bile duct epithelial cells of Opisthochis viverini-infected hamsters is hypothesized to play an important role in inflammation-associated CCA development.21 Relatively, a high expression of NF-κB (p50, p52 and p65) was found in 100% (58 cases) of CCA patients’ tissues compared with normal cholangiocytes from cadaveric donors (Seubwai et al., manuscript in preparation). NF-κB is a nuclear factor known to activate the expression of genes involved in cell proliferation (cyclin D1) and cell survival (anti-apoptotic proteins; Bcl-2, survivin).28,29

NF-κB exists as a heterodimer of p50 and p65 subunits bound to an endogenous inhibitor IκB to form an inactive complex in the cytoplasm. Following cellular stimulation, phosphorylation of IκB proteins by IκB-α kinase (IKK) occurs leading to IκB proteolysis degradation.29 The NF-κB/p65 is then phosphorylated by IKK-α, leading to nuclear translocation, bind to DNA to initiate transcription of target genes.29,53 An association between the down-modulation of NF-κB activity in the cytosol and the nucleus with an apoptotic response of the eukaryotic cells has been reported.30 Recent research shows that gambogic acid suppresses the NF-κB activation by various inflammatory agents and carcinogens.21 Gambogic acid inhibited tumor necrosis factor (TNF)-induced NF-κB activation through inhibition of IKK activation leading to suppression of NF-κB/p65 phosphorylation and translocation of NF-κB/p65 into the nucleus resulting the increase of cytosol IκB-α protein level.21 In accord with this report, we found the level of nuclear NF-κB/p65 protein was decreased by isomorellin treatment. We also found that the total and cytosol NF-κB/p65 protein level were decreased by isomorellin treatment. Moreover, the level of cytosol IκB-α was increased by the isomorellin treatment. Taken together, the inhibition of
NF-κB activation by isomorellin treatment is possibly mediated through decreased NF-κB protein expression, inhibition of IKK activation leading to suppressed phosphorylation and degradation of IκB-α, suppression of phosphorylation and nuclear translocation of NF-κB/p65.

According to the multi-fold change of NF-κB and IκB-α protein expression (Fig. 6C), the effects of isomorellin (1, 2, 4 μM) treatment on IκB-α and NF-κB levels of KKU-100 cells were just marginal and not dependent on the doses. By comparison, in the KKU-M156 cells, isomorellin treatment caused significant increase of IκB-α level in a dose-dependent manner and caused the concomitant decrease of nuclear NF-κB level. These results suggest that the translocation of NF-κB into nucleus may be related to the IκB-α level in the cytosol. In KKU-M156 cells, isomorellin showed a stronger inhibitory effect on NF-κB activity than the KKU-100 cells.

Different efficacy of isomorellin on different cell types may be due to the difference in endogenous gene expression profiles of the cell cycle regulatory molecules such as cyclin D1, NF-κB and IκB-α. These results suggest that the growth inhibitory effect of isomorellin on CCA cell lines is possibly associated with the inhibition of NF-κB function. The molecular mechanisms of an increase in cytosol IκB-α protein level need further investigation.

According to our previous work and the present study, the inhibition of NF-κB activation in the isomorellin-treated CCA cell lines was confirmed by a decrease in protein expression of Bel-2, survivin and cyclin D1, which are NF-κB-regulated gene products. The tumor suppressor p53 and its family members play important roles in therapy-induced cancer cell death and proliferation inhibition. Distinct suppression mechanisms of p53 functions by NF-κB have been reported. The response of p53 to DNA damage was inhibited by NF-κB, through inducing the expression of E3 ubiquitin ligase Hdm2 (Mdm2 in mice) that destabilizes p53. Importantly, the following corroborative associations have been reported of (a) cyclin D1 over-expression (or low expression of CDKp27) with poor survival among CCA patients and (b) p53 inactivation or NF-κB activation with the development of CCA.

In CCA cell lines, isomorellin mediated activation of p53 and the inhibition of NF-κB activation led to cell cycle arrest at the G0/G1 phase as well as induction of apoptosis (Fig. 7): these results strongly infer that the activation of p53 and inactivation of NF-κB may be an important dual anticancer mechanism of isomorellin in CCA.

In conclusion, the current research has demonstrated for the first time that isomorellin inhibits cell growth of both KKU-100 and KKU-M156 cell lines through cell cycle arrest at the G0/G1 phase; most likely by inhibition of NF-κB activation, up-regulation of p53, p21 and p27 and down-regulation of cyclin D1, cyclin E, Cdk2 and Cdk4. The KKU-M156 cells were much more susceptible than the KKU-100 cells to cell growth inhibition by isomorellin. The growth inhibitory potential of isomorellin was comparable to gambogic acid, which indicates a potential role for isomorellin in cancer therapy. Further work on isomorellin should include (a) studies on molecular mechanisms (b) testing the effects in in vivo models and (c) clinical trials on CCA patients.

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