

RESEARCH ARTICLE

Anti-inflammatory Agents Suppress the Prostaglandin E₂ Production and Migration Ability of Cholangiocarcinoma Cell Lines

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Abstract

Prostaglandin E₂ (PGE₂), one of the products catalyzed by cyclooxygenases (COXs), could actuate several pathways implicated in chronic inflammation-related cancer, including apoptosis evasion, cell proliferation, migration and angiogenesis. We hypothesized that blocking of PGE₂ production might be an effective strategy to attenuate the progression of cholangiocarcinoma (CCA). Thus, the aim of this study was to examine the effects of two anti-inflammatory agents, meloxicam, a selective COX-2 inhibitor, and xanthohumol, a natural plant extract, on PGE₂ production and migration ability of human CCA cell lines. The results showed that 100 μM of meloxicam and 10 μM of xanthohumol suppressed the PGE₂ level in the cultured media and wound-induced migration of human CCA cell lines, M139 and M214. The present results revealed that meloxicam and xanthohumol have potential to suppress PGE₂ production and cell migration. These findings may offer alternative approaches for chemoprevention and therapy of CCA.

Keywords: PGE₂ - cholangiocarcinoma - meloxicam - xanthohumol

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Introduction

Recent epidemiological studies and clinical trials indicate that long-term use of aspirin and non-steroidal anti-inflammatory drugs (NSAIDs) can decrease the incidence and recurrence of various cancers. The best known targets of NSAIDs are cyclooxygenase (COX) enzymes, which convert the arachidonic acid to prostaglandins (PGs). Several proofs indicate that the biosynthesis of PGE₂, one of the products of COX activity, can be stimulated by various inflammatory cytokines and mitogenic stimuli including the epidermal growth factor (Han and Wu, 2005), TGF-β (Han et al., 2004), lipopolysaccharide (Miyaura et al., 2003) and bile acids (Yoon et al., 2002). PGE₂ can promote the tumor progression by binding its receptors (EP1-EP4) and activating signaling pathways that control proliferation, migration and apoptosis. NSAIDs, that are the most widely used of the anti-inflammatory drugs by suppressing COX-1 and COX-2 activity in both selective and non-selective forms have lately been addressed as to their potency on the suppression of metastasis and growth in various cancer cell lines such as cholangiocarcinoma (Namwat et al., 2011) and lung cancer cell lines (Liu et al., 2002). The NS-398 and celecoxib, the selective COX-2 inhibitors, have been reported to decrease the PGE₂ level in SK-CHA-1 and CCLP1 human CCA cell lines (Lai et al., 2003; Zhang et al., 2004). Evidence in recent years

has indicated that the regular use of adult-strength aspirin is associated with modest reductions in global cancer incidence in populations in which colorectal, prostate, and breast cancers are prominent (Jacobs et al., 2007). Nonetheless, the concentration of this drug may need to be precisely evaluated as high doses of aspirin may have adverse side effects.

Meloxicam, an oxamic acid derivative belonging to a family of NSAIDs, selectively inhibits the activity of COX-2 over COX-1 at a therapeutic dose (Noble and Balfour, 1996). It has fewer gastrointestinal side effects than other NSAIDs (Engelhardt et al., 1995; Ogino et al., 1997). Previous studies reported that meloxicam inhibited the cancer cell growth including colorectal cancer (Goldman et al., 1998), non-small cell lung cancer (Tsubouchi et al., 2000), ovarian cancer (Xin et al., 2007) and osteosarcoma (Naruse et al., 2006). In addition, meloxicam has been demonstrated its inhibitory effect on cell growth suppression in *N*-nitrosobis (2-oxopropyl) amine induced biliary carcinogenesis in Syrian hamsters suggesting its potential effect on chemoprevention (Tsuchida et al., 2005).

There is increasing evidence that certain natural compounds found in plants may be useful as cancer chemopreventive or chemotherapeutic agents including xanthohumol, the principal flavonoid originally found in the hop plant, *Humulus lupulus* L. (Cannabaceae).

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Xanthohumol has been shown to bear anti-cancer properties (Gerhäuser et al., 2002; Stevens and Page, 2004; Goto et al., 2005). Xanthohumol is characterized as a broad spectrum chemopreventive agent due to its ability to inhibit the initiation, promotion, and progression stages of carcinogenesis. The ability of xanthohumol to induce apoptosis in cancer cells and limit tumor cell invasiveness suggests suppression of post-initiation in cancer development as well (Zhao et al., 2003; Gerhäuser, 2005; Vanhoecke et al., 2005). In addition, xanthohumol has been shown to inhibit activities of COX-1 extracted from ram seminal vesicles and human recombinant COX-2 (Gerhäuser et al., 2002). Xanthohumol also suppresses COX-2 gene transcription in MC3T3-E1 murine osteoblastic cells (Stevens and Page, 2004).

Little is known about the inhibitory effect of meloxicam and xanthohumol on cancer metastasis and the production of PGE₂ in cholangiocarcinoma. Therefore, the aim of this study was to determine effects of meloxicam and xanthohumol on the production of PGE₂ and *in vitro* cell migration in human CCA cell lines.

Materials and Methods

Cell culture, chemicals, and reagents

Cell lines employed in this study were the human intrahepatic CCA cell lines named M139 and M214, which were established at the Liver Fluke and Cholangiocarcinoma Research Center, Khon Kaen University, Thailand. Cells were cultured in Ham's F-12 medium (Gibco/BRL, Grand Island, NY) supplemented with 44 mM NaHCO₃ and 10% fetal bovine serum in a humidified atmosphere containing 5% CO₂. The medium was supplemented with penicillin (100 units/ml) and streptomycin (100 mg/ml) (Life Technologies, Inc.). Meloxicam, PGE₂ and sulforhodamine B (SRB) dye were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PGE₂ Express EIA Kit was purchased from Cayman Chemical Co. (Ann Arbor, Michigan, USA). All other chemicals used were of analytical grade.

Determination of PGE₂ production in culture medium of CCA cell lines

To determine the inhibitory effect of meloxicam and xanthohumol on PGE₂ production of M214 and M139 CCA cells, the PGE₂ concentration in the culture medium was measured by enzyme immunoassay (EIA). Cells were plated in 6-well plates at a concentration of 1×10⁵ cells per well and allowed to attach before adding 100 μM of meloxicam or 20 μM of xanthohumol. The conditioned media was collected after 24 h incubation for EIA assay using a PGE₂ Express EIA Kit (Cayman Chemical, MI) according to the manufacturer's instructions.

Wound-healing assay

To investigate wound-induced CCA cell migration, a wound healing assay was performed as previously described (Lampugnani, 1999) with some modifications. The M139 and M214 CCA cell lines (2×10⁵ cells per well) were seeded in the fibronectin-coated plates (6-well plate format) and cultured until almost confluent (>90%). Cell

monolayers were scratched in a single stripe using a 200 μl pipette tip, and then rinsed several times with phosphate buffered saline (PBS) to remove cell debris. The wound was allowed to heal for 24 h at 37°C. Cells were incubated in the medium containing concentrations of PGE₂ (5 μM) and meloxicam (100 μM) or xanthohumol (10 μM). The average extent of wound closure was evaluated by measuring the width of the wound and photographed using a phase-contrast microscope with a digital camera (Axiovert 40, Germany) at 0-h and 24-h incubation. The distance of the wound area was measured on the images and the migration area was calculated using the following formula: migration area=(area of original wound - area of wound during healing)/area of original wound.

Anti-proliferative assay

To test the effect of meloxicam on cell proliferation, M139 and M214 cells (5×10³ cells per well) were seeded in 96-well plates and allowed to adhere to the bottom of the wells for 24 h before the beginning of treatment. The sub-confluent cells were then exposed to a medium containing various concentrations of meloxicam (10-600 μM) for 24, 48 and 72h. Meloxicam treated and controls with DMSO were tested under the same experimental conditions. All assays were performed in triplicate. At the end of the incubation periods, surviving cells were quantified using the sulforhodamine B (SRB) assay. Its principle is based on the ability of the SRB to bind electrostatically and is pH dependent on basic amino acid residues of trichloroacetic acid-fixed protein in cells. The protocol was according to the original method (Skehan et al., 1990) with some modifications (Namwat et al., 2008).

Statistical analysis

Results were presented as the mean±SD and evaluated for the statistical significance using the Student's t-test. Significant differences were considered at P≤0.05.

Results

Inhibitory effect of meloxicam and xanthohumol on the PGE₂ production in CCA cell lines

PGE₂ is the predominant prostaglandin product in various cancer cell lines including CCA cell lines (Wu, 2005). It has been previously reported that COX-2 inhibitors, such as NS-398 and celecoxib are effective at decreasing PGE₂ levels in SK-CHA-1 and CCLP1 human CCA cell lines (Lai et al., 2003; Zhang et al., 2004). As shown in Figure 1, certain levels of PGE₂ were detected in the cultured media collected from human CCA cell lines, M139 (2.20±0.03 nM) and M214 (2.19±0.13 nM). The PGE₂ level was significantly decreased in the conditioned media of CCA cells treated with the 100 μM of meloxicam (0.35±0.12 nM in M139, 0.30±0.09 nM in M214) or 10 μM of xanthohumol (0.43±0.13 nM in M139, 0.39±0.17 nM in M214).

Meloxicam and xanthohumol suppressed CCA cell migration

The effect of meloxicam and xanthohumol on the migration of M139 and M214 CCA cells was tested

using an *in vitro* wound healing model, in which scrape wounds were produced in confluent cell cultures. As shown in Figure 2A and 2B, cells treated with the 5 μ M of PGE₂ were more migratory than untreated cells, as indicated by a higher number of cells in the stripped areas at 24 h after scratching. The significant induction of migration was 49.05 % (p=0.002) in M139 and 39.0 % (p=0.023) in M214 when compared with their corresponding untreated cells. The M139 and M214 cells with a 100 μ M of meloxicam and 10 μ M of xanthohumol treatment were less motile compared with untreated cells, as shown by fewer cells in the denuded area at 24 h after stripping. The marked reduction of migration by meloxicam was 37.73 % (p=0.008) in M139 and 51.85 % (p=0.012) in M214 when compared with their respective untreated cells. The significant reduction of migration by xanthohumol was 39.62 % (p=0.019) in M139 and 61.11 % (p=0.008) in M214 cells when compared with their corresponding untreated cells. The reduced migratory effect by meloxicam was inhibited in both M139 (24.52 %, p=0.05) and M214 (29.62 %, p=0.043) cells with

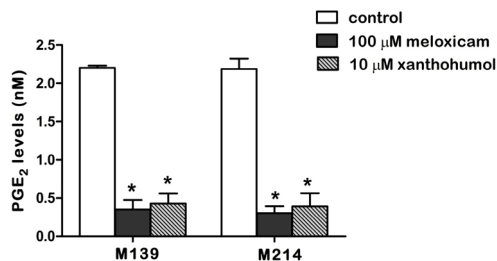


Figure 1. Effects of Meloxicam and Xanthohumol on PGE₂ Production in M139 and M214 Cell Lines. Cells (1×10^5) were plated in 6-well plates cells per well and allowed to attach before adding meloxicam (100 μ M) or xanthohumol (10 μ M). The conditioned media was collected after 24 H incubation for EIA assay using a PGE₂ express EIA kit. Each data point represents the average of three independent experiments

pretreatment of PGE₂ (5 μ M) when compared with untreated cells. The migratory effect was also markedly inhibited in xanthohumol in both M139 (26.78 %, p=0.05) and M214 (62.96 %, p=0.015) cells with pretreatment of PGE₂ (5 μ M) when compared with untreated cells.

Meloxicam inhibited CCA cell proliferation

As shown in Figure 3, the anti-proliferative results were obtained after exposing M139 (Figure 3A) and M214 (Figure 3B) CCA cell lines to different concentrations of meloxicam (10-600 μ M) for 24, 48 and 72 h. The significant inhibition of proliferation in both cell lines was observed after exposure to the 400 and 600 μ M of meloxicam for 24,48 and 72 h (p < 0.0001) when compared with untreated control cells. The concentrations of meloxicam that were equal to or less than 100 μ M had no effect on CCA cell growth inhibition.

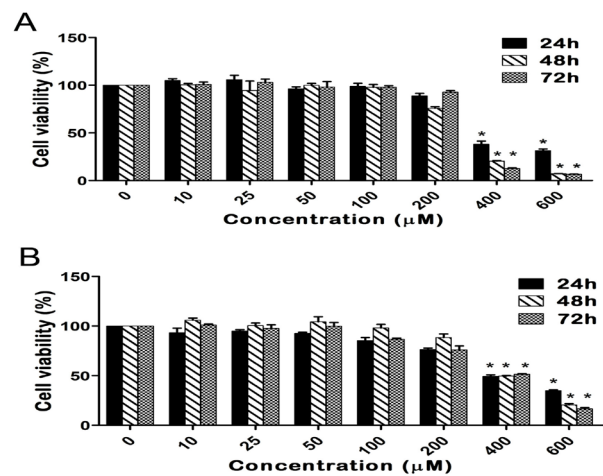


Figure 3. Cell Viability of M139 (A) and M214 (B) CCA Cells. After incubation with different concentrations of meloxicam (10-600 μ M) for 24, 48 and 72h was analyzed by SRB assay. Bars represent mean \pm SD. Each data point represents the average of three independent experiments.

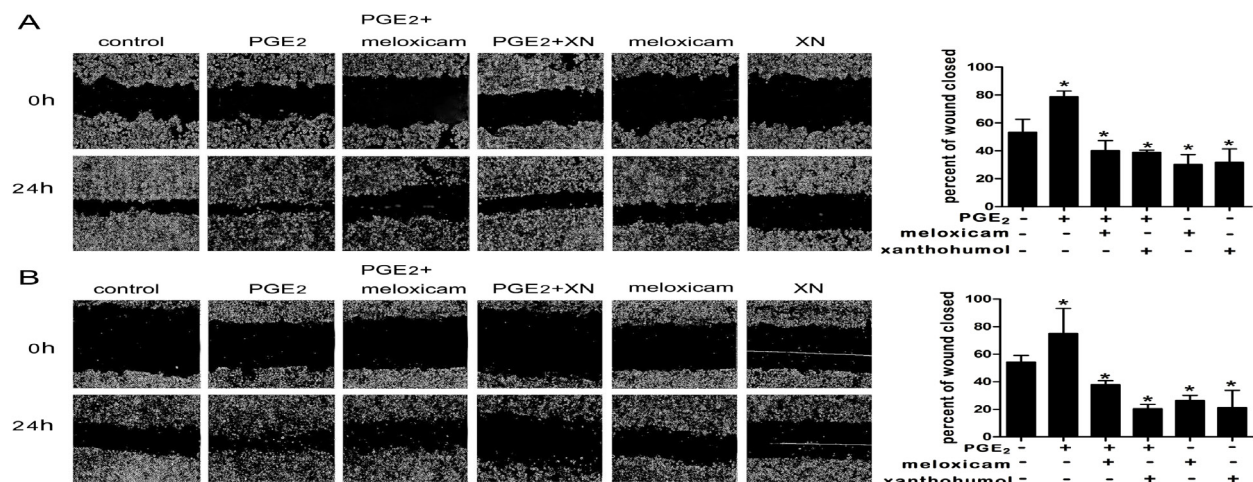


Figure 2. Effects of PGE₂, Meloxicam and Xanthohumol on Migration of M139 (A) and M214 (B) Cells. Cells (2×10^5) were seeded in the fibronectin-coated plates and cultured until almost confluent (>90%). Cell monolayers were scratched in a single stripe using a 200 μ l pipette tip, and then rinsed several times with phosphate buffered saline (PBS) to remove cell debris. The wound was allowed to heal for 24 h at 37°C. Cells were incubated in the medium containing concentrations of PGE₂ (5 μ M), meloxicam (100 μ M) or xanthohumol (10 μ M). The average extent of wound closure was evaluated by measuring the width of the wound and photographed using a phase-contrast microscope with a digital camera at 0-h and 24-h incubation. The migration ability was tested in an *in vitro* wound healing model, in which scrape wounds were produced in confluent cell cultures. The migration percent of wound closed (mean \pm SD). Each data point represents the average of three independent experiments

Discussion

Several lines of evidence indicate that the PGE₂, a major product of COX activity, plays important roles in promoting a metastatic phenotype in various tumor cells including colorectal cancer (Qualtrough et al., 2007), breast cancer (Gupta et al., 2007), and ovarian cancer (Heinonen and Metsa-Ketela, 1984). CCA is recognized as a highly metastatic tumor (Techasen et al., 2010; Boonjaraspinyo et al., 2011; Loilome et al., 2011; Namwat et al., 2011) that expresses high levels of COX-2 (Chariyalertsak et al., 2001). Previous studies demonstrated that PGE₂ (10 μM) promoted cancer cell growth, wound-induced migration and invasion in CCA cell lines including CCLP1, SG231, and HuCCT1 (Han and Wu, 2005; Itatsu et al., 2009). Our study revealed that CCA cell lines, M139 and M214, produced extracellular PGE₂. The 5 μM of exogenous PGE₂ that did not have an effect on CCA cell growth promoted the wound-induced migration ability in CCA cell lines similar to previous studies. Therefore, PGE₂ produced by CCA cell lines possibly activates the migration ability *per se*.

Meloxicam has been proven to inhibit proliferation and migration of different cancer cell cultures but this effect had not yet been investigated in CCA cell lines. Our results demonstrate that meloxicam inhibits CCA cell growth at high concentrations (400 and 600 μM), similar to the growth inhibitory effect on the prostate cancer PC3 cell line in a dose dependent manner (Montejo et al., 2010). Our results are slightly different from the effect of meloxicam on the human HepG2 hepatocellular carcinoma cells that employed lower concentration of meloxicam (50 μM) to inhibit cell growth (Li et al., 2006). Nevertheless, the previous study indicated that meloxicam up to 400 μM did not affect cell viability of the non-tumor cell NIH3T3 (Montejo et al., 2010) suggesting that it is not toxic at these levels. Interestingly, our results showed that a 100 μM of meloxicam markedly inhibits the production of PGE₂ and wound-induced migration in both M139 and M214 cell lines.

In addition to NSAIDs, a large number of natural compounds have been found to have anti-tumor properties such as xanthohumol (Gerhäuser et al., 2002). Xanthohumol modulates pro-carcinogen activating and detoxifying enzymes, besides exhibiting anti-oxidant and free radical-scavenging activity (Gerhäuser, 2005; Lust et al., 2005; Miranda et al., 2000; Stevens and Page, 2004). In addition, xanthohumol suppresses COX-1 and COX-2 activities (Gerhäuser et al., 2002) and COX-2 gene expression (Stevens and Page, 2004). It has been further identified that a 20 μM of xanthohumol inhibits M139 and M214 CCA cell growth (data not shown). The concentration of xanthohumol used in this study was of 10 μM, the dose that did not inhibit CCA cell growth. Our results reveal that treatment of M139 and M214 CCA cells with 10 μM of xanthohumol for 24 h significantly decreased the PGE₂ level and wound-induced cell migration. The possible mechanism by which these agents inhibit CCA cell growth and metastasis *via* COX-2/PGE₂ signaling pathway in both *in vitro* and *in vivo* systems is under investigation.

In conclusion, our results indicate that the meloxicam and xanthohumol decreased PGE₂ production plus growth and wound-induced migration in CCA cells at certain concentrations. Therefore, meloxicam and xanthohumol could be potential anti-cancer or chemopreventive agents. Further studies of the efficacy and safety of this approach will need to be conducted before recommending its clinical use for treatment of CCA patients.

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