RESEARCH ARTICLE

Anti-inflammatory Agents Suppress the Prostaglandin E_2 Production and Migration Ability of Cholangiocarcinoma Cell Lines

Jurairat Jongthawin[1,2], Anchalee Techasen[1,2], Watcharin Loilome[1,2], Puangrat Yongvanit[1,2], Nisana Namwat[1, 2]*

Abstract

Prostaglandin E_2 (PGE_2), 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 PGE_2 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 PGE_2 production and migration ability of human CCA cell lines. The results showed that 100 μM of meloxicam and 10 μM of xanthohumol suppressed the PGE_2 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 PGE_2 production and cell migration. These findings may offer alternative approaches for chemoprevention and therapy of CCA.

Keywords: PGE_2 - cholangiocarcinoma - meloxicam - xanthohumol

Asian Pacific J Cancer Prev, **13**, 47-51

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 PGE_2, 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). PGE_2 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 PGE_2 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 oxicam 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).

*[1]Departments of Biochemistry, [2]Liver Fluke and Cholangiocarcinoma Research Center, Faculty of Medicine, Khon Kaen University, Khon Kaen 40002, Thailand *For correspondence: nisana@kku.ac.th*

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 PGE_2 in cholangiocarcinoma. Therefore, the aim of this study was to determine effects of meloxicam and xanthohumol on the production of PGE_2 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 $NaHCO_3$ and 10% fetal bovine serum in a humidified atmosphere containing 5% CO_2. The medium was supplemented with penicillin (100 units/ml) and streptomycin (100 mg/ml) (Life Technologies, Inc.). Meloxicam, PGE_2 and sulforhodamine B (SRB) dye were purchased from Sigma Chemical Co. (St. Louis, MO, USA). PGE_2 Express EIA Kit was purchased from Cayman Chemical Co. (Ann Arbor, Michigan, USA). All other chemicals used were of analytical grade.

Determination of PGE_2 production in culture medium of CCA cell lines

To determine the inhibitory effect of meloxicam and xanthohumol on PGE_2 production of M214 and M139 CCA cells, the PGE_2 concentration in the culture medium was measured by enzyme immunoassay (EIA). Cells were plated in 6-well plates at a concentration of 1×10^5 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 PGE_2 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 (2×10^5 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 PGE_2 (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 (5×10^3 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 $P \leq 0.05$.

Results

Inhibitory effect of meloxicam and xanthohumol on the PGE_2 production in CCA cell lines

PGE_2 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 PGE_2 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 PGE_2 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 PGE_2 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 μM of PGE_2 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 μM of meloxicam and 10 μM 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 pretreatment of PGE_2 (5 μM) 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 PGE_2 (5 μM) when compared with untreated cells.

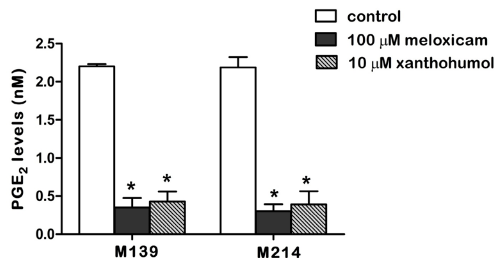

Figure 1. Effects of Meloxicam and Xanthohumol on PGE_2 Production in M139 and M214 Cell Lines. Cells (1×10^5) were plated in 6-well plates cells per well and allowed to attach before adding meloxicam (100 μM) or xanthohumol (10 μM). The conditioned media was collected after 24 H incubation for EIA assay using a PGE_2 express EIA kit. Each data point represents the average of three independent experiments

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 μM) for 24, 48 and 72 h. The significant inhibition of proliferation in both cell lines was observed after exposure to the 400 and 600 μM 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 μM had no effect on CCA cell growth inhibition.

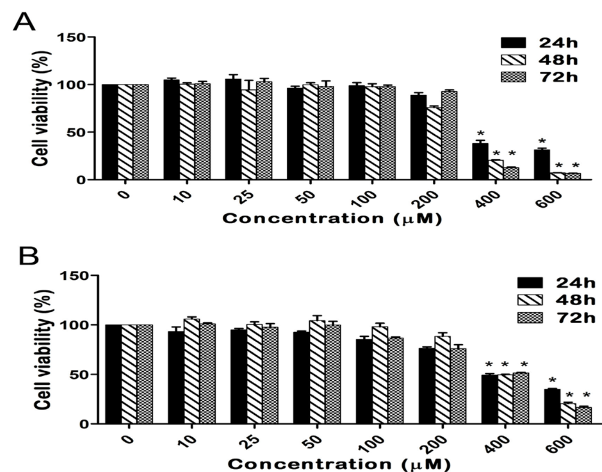

Figure 3. Cell Viability of M139 (A) and M214 (B) CCA Cells. After incubation with different concentrations of meloxicam (10-600 μM) for 24, 48 and 72h was analyzed by SRB assay. Bars represent mean±SD. Each data point represents the average of three independent experiments.

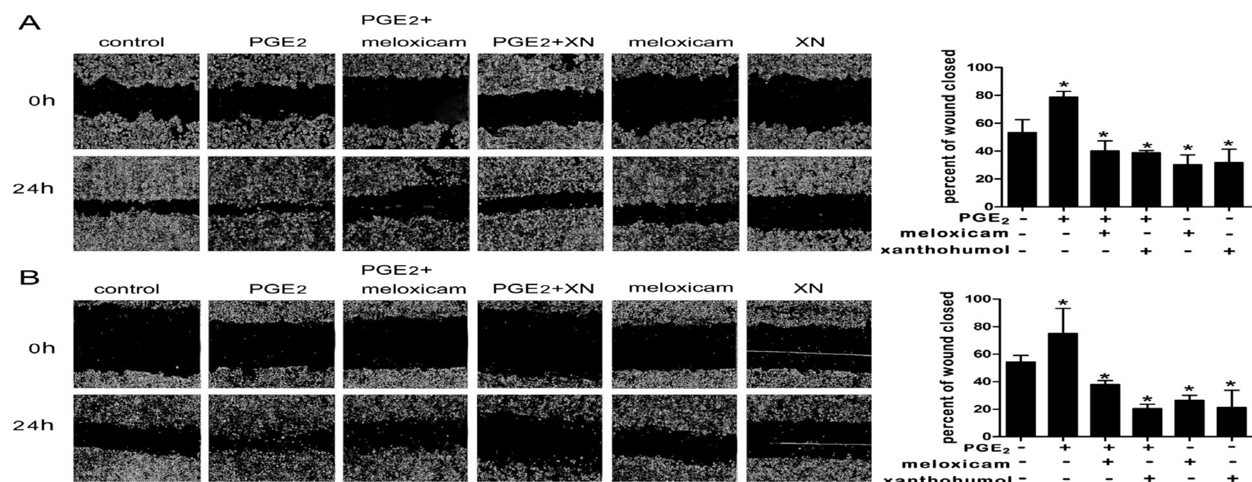

Figure 2. Effects of PGE_2, Meloxicam and Xanthohumol on Migration of M139 (A) and M214 (B) Cells. Cells (2×10^5) were seeded in the fibronectin-coated plates and cultured until almost confluent (>90%). Cell monolayers were scratched in a single stripe using a 200 μl pipette tip, and then rinsed several 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 PGE_2 (5 μM), 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 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. Bars represent percent of wound closed (mean±SD). Each data point represents the average of three independent experiments

Discussion

Several lines of evidence indicate that the PGE_2, 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 PGE_2 (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 PGE_2. The 5 μM of exogenous PGE_2 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, PGE_2 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 PGE_2 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 PGE_2 level and wound-induced cell migration. The possible mechanism by which these agents inhibit CCA cell growth and metastasis *via* COX-2/PGE_2 signaling pathway in both *in vitro* and *in vivo* systems is under investigation.

In conclusion, our results indicate that the meloxicam and xanthohumol decreased PGE_2 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.

Acknowledgements

This work was supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, through the Health Cluster (SHeP-GMS) Khon Kaen University to N.N., a grant from a Mid-Career Grant (RSA5280007), Thailand Research Fund, to N.N., the Invitation Research Grant (I55219) to J.Jand a grant fund for the Ph.D. program, Faculty of Medicine, Khon Kaen University,to J.J.

References

Boonjaraspinyo S, Wu Z, Boonmars T, et al (2011). Overexpression of PDGFA and its receptor during carcinogenesis of Opisthorchis viverrini-associated cholangiocarcinoma. *Parasitol Int*, **61**, 145-50.

Chariyalertsak S, Sirikulchayanonta V, Mayer D, et al (2001). Aberrant cyclooxygenase isozyme expression in human intrahepatic cholangiocarcinoma. *Gut*, **48**, 80-6.

Engelhardt G, Homma D, Schlegel K, et al (1995). Anti-inflammatory, analgesic, antipyretic and related properties of meloxicam, a new non-steroidal anti-inflammatory agent with favourable gastrointestinal tolerance. *Inflamm Res*, **44**, 423-33.

Gerhauser C (2005). Beer constituents as potential cancer chemopreventive agents. *Eur J Cancer*, **41**, 1941-54.

Gerhauser C, Alt A, Heiss E, et al (2002). Cancer chemopreventive activity of xanthohumol, a natural product derived from hop. *Mol Cancer Ther*, **1**, 959-69.

Goldman A P, Williams C S, Sheng H, et al (1998). Meloxicam inhibits the growth of colorectal cancer cells. *Carcinogenesis*, **19**, 2195-9.

Goto K, Asai T, Hara S, et al (2005). Enhanced antitumor activity of xanthohumol, a diacylglycerol acyltransferase inhibitor, under hypoxia. *Cancer Lett*, **219**, 215-22.

Gupta G P, Nguyen D X, Chiang A C, et al (2007). Mediators of vascular remodelling co-opted for sequential steps in lung metastasis. *Nature*, **446**, 765-70.

Han C, Demetris A J, Liu Y, et al (2004). Transforming growth factor-beta (TGF-beta) activates cytosolic phospholipase A2alpha (cPLA2alpha)-mediated prostaglandin E_2 (PGE)2/EP1 and peroxisome proliferator-activated receptor-gamma (PPAR-gamma)/Smad signaling pathways in human liver cancer cells. A novel mechanism for subversion of TGF-beta-induced mitoinhibition. *J Biol Chem*, **279**, 44344-54.

Han C, Wu T (2005). Cyclooxygenase-2-derived prostaglandin E_2 promotes human cholangiocarcinoma cell growth and invasion through EP1 receptor-mediated activation of the epidermal growth factor receptor and Akt. *J Biol Chem*, **280**, 24053-63.

Heinonen P K and Metsa-Ketela T (1984). Prostaglandin and thromboxane production in ovarian cancer tissue. *Gynecol Obstet Invest*, **18**, 225-9.

Itatsu K, Sasaki M, Yamaguchi J, et al (2009). Cyclooxygenase-2 is involved in the up-regulation of matrix metalloproteinase-9 in cholangiocarcinoma induced by tumor necrosis factor-alpha. *Am J Pathol*, **174**, 829-41.

Jacobs E J, Thun M J, Bain E B, et al (2007). A large cohort study of long-term daily use of adult-strength aspirin and cancer incidence. *J Natl Cancer Inst*, **99**, 608-15.

Lai G H, Zhang Z, Sirica A E (2003). Celecoxib acts in a cyclooxygenase-2-independent manner and in synergy with emodin to suppress rat cholangiocarcinoma growth *in vitro* through a mechanism involving enhanced Akt inactivation and increased activation of caspases-9 and -3. *Mol Cancer Ther*, **2**, 265-71.

Lampugnani M G (1999). Cell migration into a wounded area *in vitro*. *Methods Mol Biol*, **96**, 177-82.

Li J, Chen X, Dong X, et al (2006). Specific COX-2 inhibitor, meloxicam, suppresses proliferation and induces apoptosis in human HepG2 hepatocellular carcinoma cells. *J Gastroenterol Hepatol*, **21**, 1814-20.

Liu X H, Kirschenbaum A, Lu M, et al (2002). Prostaglandin E(2) stimulates prostatic intraepithelial neoplasia cell growth through activation of the interleukin-6/GP130/STAT-3 signaling pathway. *Biochem Biophys Res Commun*, **290**, 249-55.

Loilome W, Wechagama P, Namwat N, et al (2011). Expression of oxysterol binding protein isoforms in opisthorchiasis-associated cholangiocarcinoma: a potential molecular marker for tumor metastasis. *Parasitol Int*, **61**, 136-9.

Lust S, Vanhoecke B, Janssens A, et al (2005). Xanthohumol kills B-chronic lymphocytic leukemia cells by an apoptotic mechanism. *Mol Nutr Food Res*, **49**, 844-50.

Miranda C L, Yang Y H, Henderson M C, et al (2000). Prenylflavonoids from hops inhibit the metabolic activation of the carcinogenic heterocyclic amine 2-amino-3-methylimidazo[4, 5-f]quinoline, mediated by cDNA-expressed human CYP1A2. *Drug Metab Dispos*, **28**, 1297-302.

Miyaura C, Inada M, Matsumoto C, et al (2003). An essential role of cytosolic phospholipase A2alpha in prostaglandin E_2-mediated bone resorption associated with inflammation. *J Exp Med*, **197**, 1303-10.

Montejo C, Barcia E, Negro S & Fernandez-Carballido A (2010). Effective antiproliferative effect of meloxicam on prostate cancer cells: development of a new controlled release system. *Int J Pharm*, **387**, 223-9.

Namwat N, Amimanan P, Loilome W, et al (2008). Characterization of 5-fluorouracil-resistant cholangiocarcinoma cell lines. *Chemotherapy*, **54**, 343-51.

Namwat N, Puetkasichonpasutha J, Loilome W, et al (2011). Downregulation of reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) is associated with enhanced expression of matrix metalloproteinases and cholangiocarcinoma metastases. *J Gastroenterol*, **46**, 664-75.

Naruse T, Nishida Y, Hosono K & Ishiguro N (2006). Meloxicam inhibits osteosarcoma growth, invasiveness and metastasis by COX-2-dependent and independent routes. *Carcinogenesis*, **27**, 584-92.

Noble S and Balfour J A (1996). *Meloxicam*. *Drugs*, **51**, 424-30.

Ogino K, Hatanaka K, Kawamura M, et al (1997). Evaluation of pharmacological profile of meloxicam as an anti-inflammatory agent, with particular reference to its relative selectivity for cyclooxygenase-2 over cyclooxygenase-1. *Pharmacology*, **55**, 44-53.

Qualtrough D, Kaidi A, Chell S, et al (2007). Prostaglandin F(2alpha) stimulates motility and invasion in colorectal tumor cells. *Int J Cancer*, **121**, 734-40.

Skehan P, Storeng R, Scudiero D, et al (1990). New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst*, **82**, 1107-12.

Stevens J F and Page J E (2004). Xanthohumol and related prenylflavonoids from hops and beer: to your good health! *Phytochemistry*, **65**, 1317-30.

Techasen A, Loilome W, Namwat N, et al (2010). Myristoylated alanine-rich C kinase substrate phosphorylation promotes cholangiocarcinoma cell migration and metastasis via the protein kinase C-dependent pathway. *Cancer Sci*, **101**, 658-65.

Tsubouchi Y, Mukai S, Kawahito Y, et al (2000). Meloxicam inhibits the growth of non-small cell lung cancer. *Anticancer Res*, **20**, 2867-72.

Tsuchida A, Itoi T, Kasuya K, et al (2005). Inhibitory effect of meloxicam, a cyclooxygenase-2 inhibitor, on *N*-nitrosobis (2-oxopropyl) amine induced biliary carcinogenesis in Syrian hamsters. *Carcinogenesis*, **26**, 1922-8.

Vanhoecke B, Derycke L, Van Marck V, et al (2005). Antiinvasive effect of xanthohumol, a prenylated chalcone present in hops (*Humulus lupulus* L.) and beer. *Int J Cancer*, **117**, 889-95.

Wu T (2005). Cyclooxygenase-2 and prostaglandin signaling in cholangiocarcinoma. *Biochim Biophys Acta*, **1755**, 135-50.

Xin B, Yokoyama Y, Shigeto T, et al (2007). Inhibitory effect of meloxicam, a selective cyclooxygenase-2 inhibitor, and ciglitazone, a peroxisome proliferator-activated receptor gamma ligand, on the growth of human ovarian cancers. *Cancer*, **110**, 791-800.

Yoon J H, Higuchi H, Werneburg N W, et al (2002). Bile acids induce cyclooxygenase-2 expression via the epidermal growth factor receptor in a human cholangiocarcinoma cell line. *Gastroenterology*, **122**, 985-93.

Zhang Z, Lai G H & Sirica A E (2004). Celecoxib-induced apoptosis in rat cholangiocarcinoma cells mediated by Akt inactivation and Bax translocation. *Hepatology*, **39**, 1028-37.

Zhao F, Nozawa H, Daikonnya A, et al (2003). Inhibitors of nitric oxide production from hops (*Humulus lupulus* L.). *Biol Pharm Bull*, **26**, 61-5.