

RESEARCH ARTICLE

Inhibitory Effect of Aspirin on Cholangiocarcinoma Cells

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Abstract

Aspirin and other non-steroidal anti-inflammatory drugs reduce the risk of cancer due to their anti-proliferative and apoptotic effects, which are the important mechanisms for their anti-tumor activity. Here, the effect of aspirin on human cholangiocarcinoma cells (KKU-214) and the underlying mechanisms of its action were explored. Cell proliferation was measured by sulforhodamine B (SRB) assay, while cell cycle distribution and apoptosis were determined by flow cytometry. Western blotting was used to explore protein expression underlying molecular mechanisms of anti-cancer treatment of aspirin. Aspirin reduced cell proliferation in a dose- and time-dependent manner, and altered the cell cycle phase distribution of KKU-214 cells by increasing the proportion of cells in the G0/G1 phase and reducing the proportion in the S and G2/M phases. Consistent with its effect on the cell cycle, aspirin also reduced the expression of cyclin D1 and cyclin-dependent kinase 4 (Cdk-4), which are important for G0/G1 cell cycle progression. Treatment with aspirin led to increased induction of apoptosis in a dose-dependent manner. Further analysis of the mechanism underlying the effect of this drug showed that aspirin induced the expression of the tumor-suppressor protein p53 while inhibiting the anti-apoptotic protein B-cell lymphoma-2 (Bcl-2). Correspondingly, the activation of caspase-9 and -3 was also increased. These findings suggest that aspirin causes cell cycle arrest and apoptosis, both of which could contribute to its anti-proliferative effect.

Keywords: Aspirin- cholangiocarcinoma- cell cycle analysis- apoptosis

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Introduction

Cholangiocarcinoma (CCA) is a cancer originating in the biliary epithelial cells and is the second most common primary hepatic tumor, following hepatocellular carcinoma (HCC) (Huether et al., 2007). Over the last decade the mortality rate from CCA, one of the most malignant tumors, has increased worldwide (Patel, 2002; Koh et al., 2005; Yao et al., 2016). Southeast Asian countries, especially Thailand, have the highest incidence of morbidity and mortality from CCA (Sripa and Pairojkul, 2008). This cancer is a major public health problem, particularly in northeastern Thailand (Andrews et al., 2008). CCA has a poor response to currently available chemotherapeutic agents. The most effective treatment to achieve long-term survival is surgical resection, the overall 1-, 2-, and 3-years survival rates were 86%, 63%, and 22%, respectively (Valverde et al., 1999). The five-year survival rates of patients with intrahepatic, distal extrahepatic, and hilar CCA receiving surgical intervention are 22-44%,

27-37%, and 11%-41%, respectively (Hasegawa et al., 2007). It is thus necessary to find effective drugs for treatment of CCA.

Aspirin is a non-steroidal anti-inflammatory drug (NSAID) that inhibits the activity of the cyclooxygenase enzymes (COX-1 and COX-2). The overexpression of COX-2 has been reported to stimulate carcinogenesis by activating cell proliferation, suppressing apoptosis and inducing angiogenesis. COX-2 increases the expression of the regulator protein Bcl-2, which may lead to apoptosis resistance of premalignant cells (Jana, 2008). However, the molecular mechanism of how COX-2 inhibition induces apoptosis is unclear. Aspirin also has an anticancer effect by apoptosis induction (Im and Jang, 2012). In addition, aspirin also has an effect on mitochondria, by inducing mitochondrial permeability transition. Mitochondria play an important role in the apoptotic pathway; many apoptotic signals converge on mitochondria to induce outer mitochondrial membrane permeabilization, leading to cytochrome c release. Cytochrome c directly triggers

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the activation of caspase-3 through the formation of the apoptosome complex consisting of cytochrome c/Apaf-1/caspase-9 (Fulda and Debatin, 2006). The release of cytochrome c is regulated by the Bcl-2 family of proteins. Anti-apoptotic proteins such as Bcl-2 and Bcl-xL prevent the release of cytochrome c, whereas pro-apoptotic proteins such as Bax and Bid induce the release of the cytochrome, leading to the onset of apoptosis (Zimmermann et al., 2000). Recently, Kim et al., (2003) reported that aspirin induced apoptosis in cervical cancer cells through induction of caspase-3 and suppression of Bcl-2 (Kim et al., 2003). Moreover, Shiff et al., (1996) found that aspirin and other NSAIDs (indomethacin, naproxen, and piroxicam) inhibited proliferation and induced cell death by apoptosis in colon adenocarcinoma cells (Shiff et al., 1996). Similarly, Cuesta et al. (2005) reported that aspirin suppressed NF- κ B and oxidative stress and induced apoptosis in tumor cells (Cuesta et al., 2005). Several other studies have found that aspirin and other NSAIDs are capable of apoptosis induction in various cancer cell lines (La Vecchia et al., 1997; Rosenberg et al., 1998; Collet et al., 1999). Moreover, our previous study found that aspirin could delay or inhibit cholangiocarcinoma development in a hamster model (Sudsarn et al., 2016). However, information on the use of aspirin in the treatment of cholangiocarcinoma is limited, and knowledge of its mechanisms of action has continually evolved.

Materials and Methods

Cell Viability Assay

Experiments on the human intrahepatic CCA cell line KKU-214 were performed at the Cholangiocarcinoma Research Institute, Cholangiocarcinoma Screening and Care Program (CASCAP), Khon Kaen University, Thailand. The effect of aspirin on the viability of KKU-214 cells at different concentrations (0, 1, 2, 4, 8, 16, 32 and 64 μ M) and for different time periods (24, 48 and 72 h) was determined by SRB assay, performed as previously described (Pinmai et al., 2008).

Wound Migration Assay

Cells were seeded in 6-well plates at a density of 3×10^5 cells/well and grown to 80–90% confluence. Monolayers of cells were scratched with a 1 mL plastic pipette tip to create a wound, and then rinsed with phosphate-buffered saline (PBS) to remove cell debris. Cells were incubated in medium containing 1% fetal bovine serum (FBS) with or without aspirin (0, 6, 8, 16 and 32 μ M) for 24 h. The width distance of the wound area was monitored by microscopy and digitally photographed.

Cell Cycle Analysis

KKU-214 cells (1×10^5) were plated in 6-well plates for 24 h and then treated with various concentrations of aspirin (0, 6, 8 and 10 μ M). After 24 h, cells were harvested, washed twice with cold PBS, fixed overnight in 70% ethanol at 4 °C, and incubated at room temperature for 30 min in the dark with RNase A (final concentration 2 μ g/mL) and propidium iodide (PI) (final concentration 2.4

μ g/mL). The cell cycle distribution was examined using an FACSCanto™ II flow cytometer (BD Biosciences, San Jose, CA, USA) and the data were analyzed using FACSDiva™ software (BD Biosciences).

Cell Death Detection Assay via Flow Cytometry

The effect of aspirin on apoptosis induction in KKU-214 cells was determined using an Annexin-V-FLUOS staining kit (Roche Diagnostics, Penzberg, Germany). KKU-214 cells (1×10^5 cells/well) were treated with different concentrations of aspirin (0, 6, 8 and 10 μ M) for 24 h. Floating and adherent cells were harvested by trypsinization, washed with cold 1X PBS, and centrifuged at 1,500 rpm for 10 min at 4 °C. Pelleted cells were resuspended in 400 μ L PBS and then stained with 100 μ L incubation buffer containing 2 μ L annexin V-FITC (recombinant annexin V protein conjugated to green fluorescent FITC dye) and 2 μ L PI for 10–15 min at room temperature in the dark. The stained cells were then analyzed by flow cytometry.

Protein Extraction and Western Blot Analysis

Preparation of Total Cell Lysates

The KKU-214 cell line was cultured in 10-cm-diameter dishes (Costar®; Corning, Corning, NY, USA) at a density of 1×10^6 cells per dish and incubated with aspirin (0, 6, 8 and 10 μ M) for 24 h. Harvested cells were washed twice with cold PBS and lysed with ice-cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCL [pH 7.5], 150 mM NaCl, 0.5% Nonidet P-40 detergent, 1 mM EDTA, 1 mM dithiothreitol, 0.5% deoxycholate and 0.1% sodium dodecyl sulfate). Following this, the cell lysate was homogenized and clarified by centrifugation at 13,000 rpm for 30 min at 4 °C. The protein concentration in the total cell lysate was determined by the Bradford method (Bradford, 1976).

Western Blot Analysis

Cell lysates (5 μ g of protein per lane) were fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12%). The proteins were electrically transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in 0.1% Tween 20 in PBS at 37 °C for 1 h, and the protein level was detected using primary antibody. The membrane was incubated with primary antibody at 4 °C overnight against cyclin D1, Cdk-4, p53, Bax, Bcl-2, capase-9, caspase-3 and β -actin, and then incubated with horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The bound secondary antibodies on the nitrocellulose membrane were visualized using an enhanced chemiluminescence reagent (Pierce ECL; Thermo Fisher Scientific, Waltham, MA, USA), quantified by densitometry (ImageQuant LAS 4000; GE Healthcare, Chicago, IL, USA) and analyzed using the Scion Image program (Scion Corp., Frederick, MD, USA). The results were expressed as the relative density of protein normalized to β -actin.

Statistical Analysis

All data were expressed as mean \pm SD of three independent experiments. Significant differences between

groups were analyzed by Student's t-test using SPSS statistical software, version 16.0 (SPSS, Chicago, IL, USA). Statistical significance was indicated by * $p < 0.05$, ** $p < 0.01$ or *** $p < 0.001$.

Results

Cytotoxic Effect of Aspirin on KKU-214 Cells

The effect of aspirin on the viability of KKU-214 cells was assessed by SRB assay. As shown in Figure 1, a dose- and time-dependent decrease in cell viability could be observed after treatment with aspirin. The IC₅₀

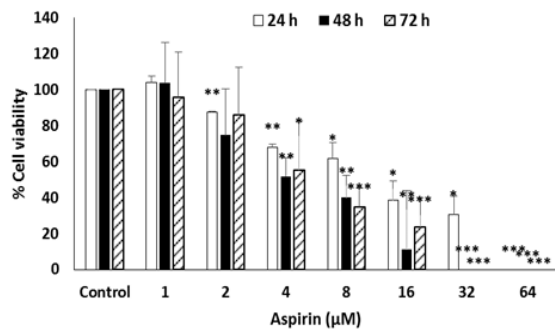


Figure 1. Dose-Dependent Effect of Aspirin on Growth of KKU-214 Cells. KKU-214 cells were plated into a 96-well plate for 24 h, then treated with different concentrations of aspirin (0, 1, 2, 4, 8, 16, 32 and 64 µM) for 24, 48 and 72 h. The percentage of cell viability was determined by SRB assay. Each value represents the mean \pm SD of three independent experiments; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, vs. the control group.

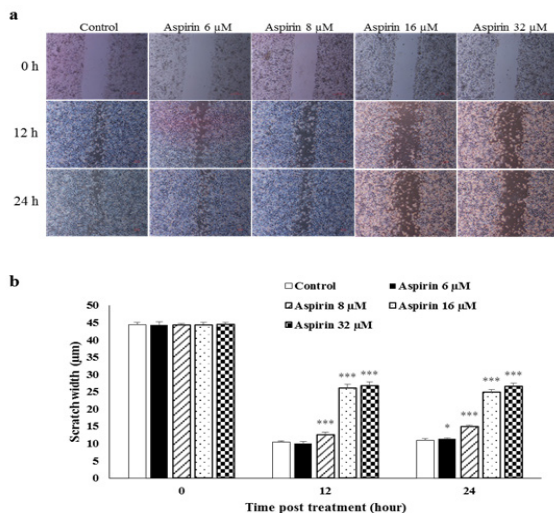


Figure 2. Effect of Aspirin on Cell Migration of KKU-214 Cells. Cells were seeded in 6-well plates in completed medium. After 24 h of incubation, the cells monolayers were wounded by scratched with a 1 mL plastic pipette tip and treated with the indicated concentration of aspirin (0, 6, 8, 16, 32 µM) for 24 h. (a) The wound migration areas were determined by a phase-contrast microscope at 10 \times magnification. (b) The gap distance was measured from photographs of three random fields. Each value represents the mean \pm SD of three independent experiments; * $p < 0.05$ and *** $p < 0.001$, vs. the control group.

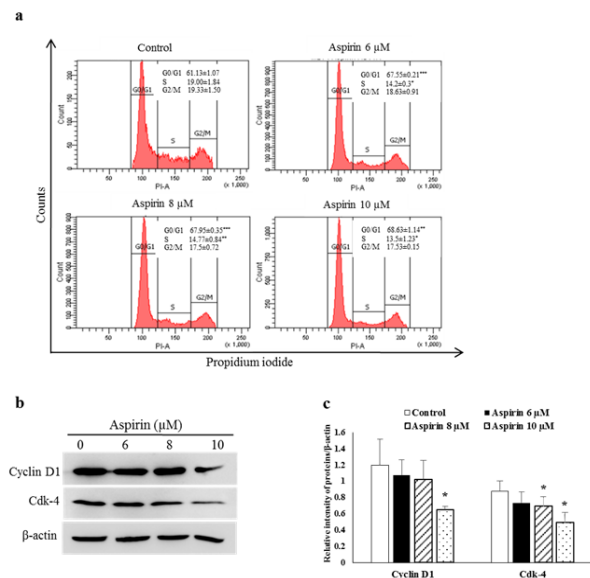


Figure 3. Change in Cell Cycle Distribution and Down-Regulation of G0/G1 Phase-Associated Proteins in KKU-214 Cells. (a) KKU-214 cells were seeded into 6-well plates in complete medium. After incubation, cells were treated with various concentrations of aspirin (0, 6, 8 and 10 µM) and flow cytometry was performed. (b) The total cell lysates were extracted and separated on 12% SDS-PAGE. Blotted proteins on a nitrocellulose membrane were probed with primary antibody specific to cyclin D1 and Cdk-4. (c) The band intensity of cyclin D1 and Cdk-4 was quantitated by densitometry and normalized to β -actin. Statistical analysis of control and treated cells was performed using Student's t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate a significant difference.

value was 6.15, 6.01 and 5.42 µM after 24, 48 and 72 h, respectively.

Anti-migration Effect of Aspirin on KKU-214 Cells

The effect of aspirin on cell migration of KKU-214 cells was determined by wound healing assay. As shown in Figure 2a, aspirin significantly inhibited cell migration in KKU-214 cells in a dose- and time-dependent manner. After treatment for 24 h at concentrations of 6, 8, 16 and 32 µM, gap widths of the wound were 11.30 \pm 0.31, 14.86 \pm 0.39, 24.86 \pm 0.77 and 26.6 \pm 0.85 µm, respectively, vs. 10.85 \pm 0.53 µm for the control (Figure 2b).

Cell Cycle Alteration induced by Aspirin in KKU-214 Cells

Cell cycle distribution in KKU-214 cells after treatment with aspirin was determined using flow cytometry. The results (Figure 3a) showed that KKU-214 cells were arrested in the G0/G1 phase in response to aspirin (6, 8 and 10 µM) treatment in a dose-dependent manner, i.e. 67.55 \pm 0.21%, 67.95 \pm 0.35% and 68.63 \pm 1.14%, respectively, as compared with control cells (61.13 \pm 1.07%). The percentage of the cell population in the S phase was significantly decreased in aspirin-treated (6, 8 and 10 µM) KKU-214 cells (14.2 \pm 0.3%, 14.77 \pm 0.84% and 13.5 \pm 1.23%, respectively) compared with control cells (19 \pm 1.84%). In addition, after treatment with aspirin

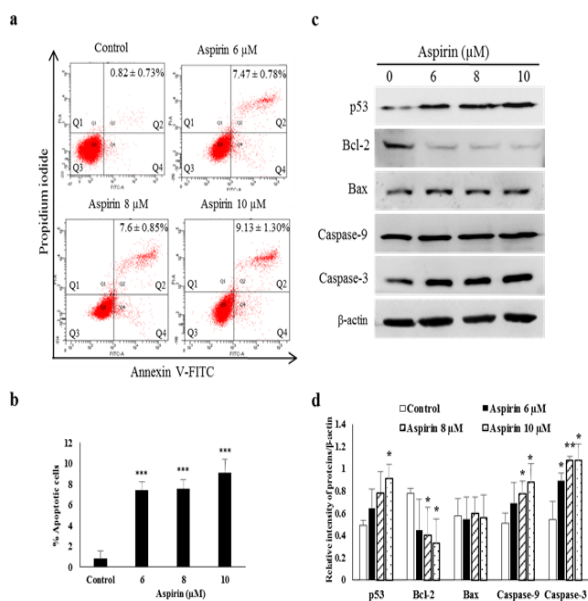


Figure 4. Apoptosis Induction was Increased in KKU-214 Cells Compared with Control Cells. KKU-214 cells were exposed to various concentrations of aspirin for 24 h. (a) Cells were stained with annexin V-FITC/PI and flow cytometry was performed. Flow cytometric analysis was visualized for living cells (Q3), early apoptotic cells (Q4), late apoptotic cells (Q2) and necrotic cells (Q1). (b) The percentage of both early- and late-stage (Q2+Q4) apoptotic KKU-214 cells was analyzed. (c) Results of western blot analysis of apoptosis-regulating protein expression, including p53, Bcl-2, Bax, caspase-9 and caspase-3, and subsequent densitometry. (d) The density of protein expression after normalization to β -actin was measured in three independent experiments. Statistical analysis of cells was performed using Student's t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ indicate a significant difference.

(6, 8 and 10 μ M) the percentage of KKU-214 cells in the G2/M phase ($18.63 \pm 0.91\%$, $17.5 \pm 0.72\%$ and $17.53 \pm 0.15\%$, respectively) was significantly lower compared with control cells ($19.33 \pm 1.50\%$).

Down-regulation of G0/G1 Cell Cycle Phase-regulatory Proteins by Aspirin in KKU-214 Cells

As aspirin induced cell cycle arrest at the G0/G1 phase in the KKU-214 cell line, we assessed the expression of proteins involved in the regulation of G0/G1 cell cycle progression. Expression of the proteins cyclin D1 and Cdk-4 was significantly decreased (Figure 3b, c).

Effect of Aspirin on Apoptosis Induction in KKU-214 cells

After exposure of KKU-214 cells to various concentrations of aspirin for 24 h, apoptosis induction was assessed by flow cytometry with annexin V-FITC/PI staining. The results showed that the percentage of apoptosis was significantly increased in a dose-dependent manner. Interestingly, aspirin (10 μ M) induced apoptosis in 9.13% of KKU-214 cells as compared with 0.82% in the control group (Figure 4a, b).

Aspirin-induced Changes in Apoptotic Regulation in KKU-214 Cells

To determine the possible mechanisms of apoptosis induction in KKU-214 cells, the apoptosis-regulating proteins of the mitochondrial pathway were investigated using western blot analysis. The results demonstrated that p53 was up-regulated while Bcl-2 was down-regulated in KKU-214 cells after treatment with aspirin, but there was no effect on Bax expression (Figure 4c, d). In addition, aspirin increased the expression of caspase-9 and caspase-3 in a dose-dependent manner (Figure 4c, d).

Discussion

In the present study, we found that aspirin could inhibit cell migration and proliferation through G0/G1 phase arrest and induce apoptosis in a cholangiocarcinoma cell line, which corresponded to the results of our previous study in an animal model (Sudsarn et al., 2016). Aspirin has well-documented chemopreventive and anti-proliferative properties against cancer cells. One molecular target of aspirin is COX-2. This is supported by a study by Vane, who reported that aspirin could decrease the production of prostaglandins from COX-2-mediated catalysis of arachidonic acid (Vane, 1971). However, several NSAIDs have anti-proliferative effects but have no effect on COX activity. Treatment with aspirin at high doses has been shown to induce apoptosis by COX-independent mechanisms (Kashfi and Rigas, 2005). Several targets are regulated by aspirin-induced apoptosis via COX-independent mechanisms, such as Par-4, which acts as a pro-apoptotic gene, and Bcl-XL, which acts as an anti-apoptotic gene (Zhang and DuBois, 2000; Zhang et al., 2000). Molecular mechanisms underlying apoptosis induction by aspirin include the mitochondrial pathway by caspase-dependent and -independent mechanisms (Leung et al., 2009), and the protein kinase C (PKC) signaling pathway (Redlak et al., 2007). A study by Mahdi et al. (2006) found that low doses of aspirin in combination with praziquantel could inhibit and/or prevent CCA development and reduce liver fibrosis and tumor size in a hamster CCA model (Mahdi et al., 2006). Apoptosis has been an important mechanism in the development and pharmacology of anticancer agents. Aspirin's role in apoptosis could be attributed to the up-regulation of pro-apoptotic genes, such as Bax, and the down-regulation of anti-apoptotic genes, such as Bcl-2 (Antonsson, 2001). The Bcl-2 family of proteins consists of pro-apoptotic proteins such as Bax and anti-apoptotic proteins such as Bcl-2 (Shi, 2002). Apoptotic stimuli cause the translocation of pro-apoptotic molecules from the cytoplasm to the outer mitochondrial membrane and facilitate the release of cytochrome c and Smac (second mitochondria-derived activator of caspase) into the cytoplasm, resulting in caspase activation. On the other hand, anti-apoptotic molecules interfere with cytochrome c release and caspase activation (Bellosillo et al., 1998). Our findings were consistent with previous reports showing that aspirin induced apoptosis in B-cell chronic lymphocytic leukemia cells and in human colon carcinoma and gastric cancer cells (Wong et al., 1999; Zhou et al., 2001). This process

is regulated by several molecules, such as p53, p21, NF- κ B and c-Myc. Proteins in the Bcl-2 family (Bcl-2, Bax) regulate apoptosis induction by caspase activation, which was found to lead to apoptosis in cancer cells (Stark et al., 2001). In the present study, the mechanisms by which aspirin induced apoptosis in a CCA cell line were mediated by the activation of p53, caspase-9 and caspase-3 and the inhibition of Bcl-2. This result suggested that the mechanism underlying aspirin-induced apoptosis involves p53 activation preceding caspase activation. Aspirin and other NSAIDs can increase the accumulation of p27kip1 or p21waf1/Cip1, resulting in cell cycle arrest at the G1 phase and apoptosis induction (Cui et al., 2005; Luciani et al., 2007). Increased accumulation of p21waf1/Cip1 was found to cause downstream inhibition of cyclin D1 and hypophosphorylation of the retinoblastoma protein Rb, leading to inhibition of E2F gene function and G1 cell cycle arrest (Cui et al., 2005). In the present study, aspirin can induce cell cycle arrest at G1 phase by decrease the cyclin D1 and Cdk-4, which are known to be a critical molecule in regulating the G1 cell cycle progression (Prall et al., 1997).

These results suggest that aspirin induced apoptosis in KKU-214 cells by activation of p53 and caspases, inhibition of Bcl-2 and induced G1 cell cycle arrest by inhibition of cyclin D1 and Cdk-4. Therefore, aspirin shows promising potential as an anticancer agent for treatment of cholangiocarcinoma.

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