The Effects of Ibuprofen, Naproxen and Diclofenac on cell Apoptosis, Cell Proliferation and Histology Changes in Human Cholangiocarcinoma Cell Lines

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Abstract

Objective: To examine the effects of ibuprofen, naproxen and diclofenac, non-steroidal anti-inflammatory drugs (NSAIDs) on cell proliferation activity of the human CCA cell lines. Methods: KKU-M139 and KKU-213B cell lines were used in this study. The cell viability was assessed by the MTT assay. Lipid synthesis determined by Oil red O staining and colorimetric assay. An inverted phase-contrast light microscope was used to investigate the histological change of the cells. Caspases 3/7 activity and Annexin V/PI were used to assess apoptosis by multiple microplate reader. Results: The results showed that ibuprofen, naproxen and diclofenac suppressed the viability of the KKU-M139 and KKU-213B cells in a dose-dependent manner, as measured especially diclofenac. However, these three NSAIDs slightly decreased lipid synthesis determined by Oil red O staining and colorimetric assay. The histological change observations showed the shrinking cell and become star-shaped in high dose treated groups. Interestingly, these NSAIDs exhibited in both of KKU-M139 and KKU-213B cell lines, the diclofenac-treated cells had the most injury cells. The cells exhibited cell injury features. In addition, the detection of caspase 3/7 and Annexin V/PI in this investigation revealed early cell apoptotic characteristics. Conclusion: These finding suggest that ibuprofen, naproxen and diclofenac suppress cell viability. The results reveal that ibuprofen, naproxen and diclofenac, which induce the histological change and apoptosis. This study indicates that these NSAIDs may be used as an anti-proliferation agent for the treatment of CCA in the future.

Keywords: Cholangiocarcinoma- apoptosis- ibuprofen- naproxen- diclofenac
Materials and Methods

Cell Culture
The current study was based on two human intrahepatic CCA cell lines; namely KKU-M139 and KKU-213B, all of which were established from the CCA patients residing in opisthorchiasis endemic areas in Northeastern Thailand at the Department of Pathology, Faculty of Medicine, Khon Kaen University. KKU-M139 was derived from a patient with squamous carcinoma and KKU-213B from patients with moderately differentiated adenocarcinomas. All cell lines were cultured in Ham’s F12 containing 100 U/ml penicillin and 100 µg/ml streptomycin and supplemented with 10% fetal bovine serum at 37°C under 5% CO₂ incubator (Namwat et al., 2011). Hydrogen peroxide (H₂O₂) was used to induce CCA cells apoptosis, and the death cell control was heated to a 55°C for 90 minutes.

Chemicals
CCA cells were treated with ibuprofen (sigma-aldrich, product no. I4883), naproxen (sigma-aldrich, product no. 46482), and diclofenac sodium salt (sigma-aldrich, product no. D6899) in low doses of 500 µM and high doses of 2 mM.

Cell viability assay
The MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to determine the cytotoxicity of drugs on CCA cells. Cells were seeded on 96-well plates (2×10⁴ cells/well) and treated with various concentrations of ibuprofen, naproxen, and diclofenac (0-2 mM) for 8 hours. The culture medium was subsequently removed, and 100 µl incomplete-medium containing MTT dye (0.25 mg/ml at final volume) was added. The treated cells were further incubated for 4 hours. After removed medium solution, formazan crystals were dissolved with 100 µl DMSO. The solution was measured at 540 nm by using the microplate reader (EZ read 2000 microplate reader, biochrom, Holliston, Germany). The percentage of cell viability versus the drug concentrations was plotted as previously reported estimating the drug concentrations necessary to inhibit cell proliferation by 50% (IC₅₀).

Oil red O staining
Oil red O staining was performed according to the protocol. To illustrate, cells were seeded in 24-well plates (2 × 10⁴ cells/well), incubated overnight and treated with 2mM of ibuprofen, naproxen, and diclofenac for day 1 and day 3. Cells were washed with phosphate buffer saline (PBS) and fixed with 10% formalin. The stained cells were visualized by the light microscope and photographed using an inverted microscope (Olympus).

Observations on the histological changes
Cell suspensions (2×10⁴ cells/well) were seeded in 24-well plates and incubated for 24 hours at 37°C in a humidified 5% CO₂ atmosphere. Then the cells were treated with various concentrations of ibuprofen, naproxen and diclofenac (500 µM and 2 mM) for 48 hours and incubated at 37°C in a humidified 5% CO₂. Cells were visualized by bright field inverted microscope and photographed using a ZEISS microscope.

Fluorescence microscopy assays
The cells were treated with high doses of ibuprofen, naproxen, and diclofenac (2mM) before being incubated for 24 hours. Untreated cells, apoptosis cells, death cells, and drug-treated cells were all incubated with Annexin V-FITC/PI (5 µl each) before being analyzed under a fluorescent microscope (magnification, x200; Olympus IX73).

Lipid measurement
The cells (1×10⁴ cells/dish) were treated with 2mM ibuprofen, naproxen, and diclofenac for 72 hours. The untreated control cells and the drugs-treated cells were harvest with trypsin/EDTA and placed into a tube. The cells suspension was analyzed using Beckman Coulter AU680 analyser for measured cholesterol and triglyceride levels.

Caspase activity assay
The Caspase-Glo 3/7 Assay (Promega, Madison, USA) was used for detection of active caspases 3 and 7. The assay was performed as recommended by the manufacturer. The CCA cells (2×10⁴ cells/well) were seeded in 96 well plate and treated with 2 mM ibuprofen, naproxen and diclofenac for 24 hours. Apoptotic cells control was induced by 750 µM H₂O₂ and primary necrosis cell control was heated to 55°C 90 minutes, for 24 hours. The untreated control cells and the drugs-treated cells were added 100 µl of Caspase-Glo 3/7 Reagent to each well and then analyzed under a luminescence multiple microplate reader (BMG, CLARIO Star).

Annexin V-FITC/PI detection
Treated CCA cells were stained using the FITC Annexin V Apoptosis Kit (cat 556547, BD Pharmingen) according to the manufacturer’s, cells were cultured in 96-well plates (2×10⁴ cells/well) and treated with 2 mM ibuprofen, naproxen and diclofenac. Apoptotic cells control was induced by 750 µM H₂O₂ and primary necrosis cell control was heated to 55°C for 90 minutes. The cells were then subjected to incubation for 24 hours and visualized under a fluorescent microscope (LED fluorescence Olympus IX73) and analyzed under a fluorescence multiple microplate reader (BMG, CLARIO Star).

Statistical analysis
All data were expressed as mean ± SEM and mean ± SD of three independent experiments. We analyzed for significant differences between groups by two-way ANOVA using GraphPad prism statistical software, version 9. Statistical significance was indicated by * p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001
Results

Cytotoxic effect of ibuprofen, naproxen and diclofenac on cell viability KKU-M139 and KKU-213B cells

The cytotoxic effect of ibuprofen, naproxen and diclofenac on the viability of CCA cells was assessed using MTT assay. A dose-dependent decrease of cell viability was observed after treatment with ibuprofen, naproxen and diclofenac for 48 hours. The 50 percent inhibitory concentration (IC$_{50}$) of ibuprofen, naproxen and diclofenac were 1.87 mM, 2.49 mM and 1.24 mM, respectively on KKU-M139 cells (Figure 1), and the IC$_{50}$ of ibuprofen, naproxen and diclofenac were 1.63 mM, 6.95 mM and 1.12 mM for, respectively on KKU-213B cells (Figure 2).

Lipid droplets staining and histology on KKU-M139 and KKU-213B cell lines

The effects of ibuprofen, naproxen and diclofenac on lipogenesis were observed by staining of lipid droplet with Oil red O in cytoplasm of CCA cells. After treatment of KKU-M139 and KKU-213B cells with 2 mM ibuprofen, naproxen and diclofenac and observed under microscope at day 1 and day 3. There was no difference in Oil red O staining patterns between ibuprofen, naproxen and diclofenac-treated KKU-M139 and KKU-213B cells when compared with their control group at day 1 and day 3 after treatment (Figure 3 and Figure 4).

In this investigation, we discovered that the treated group had histological abnormalities, particularly in the diclofenac-treated group, where shrinking cells were discovered. They turn to be star-shape, as opposed to the control group, which has the polyclonal shape of cell lines. The results of KKU-M139 and KKU-213B cells exposed to ibuprofen-, naproxen-, and diclofenac-treated group cells displayed retraction cells, star shape cells, and separation from the flask bottom after treatment. We discovered membrane blebbing, which is a sign of cell injury or damage. The number of cells in the treated group es was reduced after a high-dose treatment shown

![Image 1](https://example.com/image1.png)

Figure 1. The Effect of Ibuprofen, Naproxen and Diclofenac on Viability of KKU-M139 Cells for 48 Hours. This data represents the mean ±SEM of three times independent experiment. Statistically significant differences is indicated: *p < 0.05 when compared with the control group.

![Image 2](https://example.com/image2.png)

Figure 2. The Effect of Ibuprofen, Naproxen and Diclofenac on Viability of KKU-213B Cells for 48 Hours. This data represents the mean ±SEM of three times independent experiment. Statistically significant differences is indicated: *p < 0.05, **p <0.01 when compared with the control group.
in Figure 5.

**Fluorescence Microscope**

In both cell lines, KKU-M139 and KKU-213B, treated group and \( \text{H}_2\text{O}_2 \) exposed early apoptotic cell death. The PI intensity of diclofenac-treated cells was higher than ibuprofen and naproxen treatments. Diclofenac caused early apoptotic CCA cell death as shown in figure 6. Meanwhile, 55°C-heated both cells exhibited late apoptosis.

**Caspase activity assay**

Diclofenac and \( \text{H}_2\text{O}_2 \) (early apoptosis marker) increased caspase 3/7 activity higher than ibuprofen and naproxen treated of KKU-M139 cells compared with control cells. However, the caspase 3/7 activity of H2O2 treatment on KKU-213B cells has higher than the others NSAIIDs. These findings suggested that diclofenac, ibuprofen, and naproxen induced early apoptosis in both KKU-M139 and KKU-213B cells via increasing caspase 3/7 activation. However, CCA cells were heating to 55°C, late apoptosis marker, showed the highest caspase 3/7 activity in both cells (Figure 7).

**Annexin V-FITC/PI detection**

The ratio of Annexin V-FITC/PI on untreated cells, ibuprofen-treated, naproxen-treated, diclofenac-treated, \( \text{H}_2\text{O}_2 \)-treated and 55°C-heated KKU-M139 cells were 85.37/14.63, 87.64/12.36, 85.98/14.02, 82.32/17.68, 88.30/11.70, and 50.81/49.19 respectively. Similarly, the ratio of Annexin V-FITC/PI on untreated cells, ibuprofen-treated, naproxen-treated, diclofenac-treated, \( \text{H}_2\text{O}_2 \)-treated and 55°C-heated KKU-213B cells were 83.28/16.72, 83.26/16.74, 85.72/14.28, 75.77/24.23, 89.75/10.25, and 47.88/52.12 respectively.

Ibuprofen, naproxen, diclofenac and H2O2 showed early apoptotic cell death in KKU-M139 and KKU-213B cell lines, according to observations. However, 55°C-heated both cells showed late apoptosis/necrotic cell death (Figure 8).

**Lipid measurement**

The cholesterol and triglyceride levels on CCA-treated cells were measured by using colorimetric assay. In

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Figure 3. Overview of Oil Red O Staining of KKU-M139 Cell Line with Ibuprofen, Naproxen and Diclofenac with Concentrations of 2 mM. (A1, A2): untreated control cell KKU-M139 day1 and day3 (B1, B2): treated with ibuprofen day1 and day3 (C1, C2): treated with naproxen day1 and day3 (D1, D2): treated with diclofenac day1 and day3, respectively (x40 magnifications)

Figure 4. Overview of Oil Red O Staining of KKU-213B Cell Line with Ibuprofen, Naproxen and Diclofenac with Concentrations of 2 mM. (A1, A2): untreated control cell KKU-213B day1 and day3 (B1, B2): treated with ibuprofen day1 and day3 (C1, C2): treated with naproxen day1 and day3 (D1, D2): treated with diclofenac day1 and day3, respectively (x40 magnifications)
KKU-M139 cells, triglyceride level in untreated control group was 11.09 mg/dl, whereas ibuprofen-treated, naproxen-treated and diclofenac were, 9.59, 10.71 and 9.93 mg/dl, respectively. While the cholesterol level in the untreated control, ibuprofen-treated, naproxen-treated and diclofenac-treated groups were 2.0, 2.12, 2.40, and 2.16 mg/dl, respectively. This result suggested that the triglyceride levels in untreated control group was higher than treated-group in KKU-M139 cells. Similarly, in KKU-213B cells, the triglyceride level in the untreated group was 13.47 mg/dl, while the ibuprofen-treated, naproxen-treated, and diclofenac-treated group showed 11.36, 13.15, and 11.44 mg/dl, respectively. While the cholesterol level in the untreated control, ibuprofen-treated, naproxen-treated and diclofenac-treated groups were 2.22, 2.10, 2.18, and 2.31 mg/dl, respectively (Figure 9).

The results showed that no difference in lipid droplets patterns and the cholesterol levels between treated and untreated group. However, in the treated group, triglyceride levels were non-significantly decreased.

**Discussion**

Ibuprofen and naproxen are the propionic acid derivative while diclofenac is a benzene acetic acid
derivative, they inhibit cyclooxygenase (COX1-2) activity and prostaglandin E2 synthesis. Diclofenac has well-documented as chemopreventive and anti-proliferative properties against cancer cells such as colorectal cancer, neuroblastoma, ovarian cancer and other cell types of cancer (Pantziarka et al., 2016). In this study, we found that the potential of ibuprofen, naproxen and diclofenac suppressed the proliferation of KKU-M139 and

![Figure 6. Annexin V and PI Staining in CCA Cells Treated with 2 mM Diclofenac.](image)

![Figure 7. Caspase 3/7 Activity of CCA Cells Treated with 2 mM Ibuprofen, Naproxen and Diclofenac. This data represents the mean ±SD of three times independent experiment. Statistically significant are indicated: ***p <0.001, ****p < 0.0001 when compared with the control group.](image)

![Figure 8. Fluorescence Intensity of Annexin V and PI Staining in CCA Cell Treated with 2 mM Ibuprofen, Naproxen and Diclofenac. (A): KKU-M139 (B): KKU-213B. This data represents the mean ±SD of three times independent experiment: **p < 0.01, ***p <0.001, when compared with the control group.](image)
Effects of NSAIDs in CCA Cell Lines

Figure 9. Cholesterol and Triglyceride Levels of CCA Cells Treated with 2 mM Ibuprofen, Naproxen and Diclofenac. Treatment for 3 days (A): KKU-M139 cells (B): KKU-213B cells, treatment for 1 and 3 days. This data represents the mean ±SD of three times independent experiment.

KKU-213B cells. Moreover, aspirin can inhibit the growth of hepatocellular carcinoma cells (Yang et al., 2017) and human intrahepatic CCA cells (KKU-213B). Therefore, aspirin inhibits the migration and induces apoptosis via G0/G1 arrest on KKU-213B cells (Boueroy et al., 2017).

In the previous study, ibuprofen inhibited survival of bladder cancer cells (Khwaja et al., 2004). Naproxen suppressed chondrocyte cells proliferation and prechondrocytic precursor cell differentiation (Karaarslan et al., 2018).

High rate of lipid uptake and lipogenesis are frequently found in cancer cells (Menendez & Lupu, 2007). The abnormal of lipogenesis enhances development of hepatocellular carcinoma (Patterson et al., 2011). Our experiments were investigated lipogenesis and lipid deposition by colorimetric assay and Oil red O staining, respectively in CCA cells. The results showed that ibuprofen, naproxen and diclofenac may not be able to significantly suppress lipogenesis and lipid droplets deposition in KKU-M139 and KKU-213B cell lines. The numbers of lipid droplet in the cytoplasm were not significantly different as well. It has been reported that CCA might receive lipid for membrane synthesis through exogenous fatty acid uptake (Li et al., 2016). Our data are inconsistent with the previous study showing aspirin reduced lipogenesis in hepatocellular carcinoma (HCC) (Yang et al., 2017), suggested that it might be having different mechanisms of ibuprofen, naproxen and diclofenac on cell viability and different affecting in deferent cancer cell types.

In addition, this technique can determine the proliferation reducing of these drugs that may cause cell injury or cell damage using histological study. When observed KKU-M139 and KKU-213B cell lines under the bright field inverted microscope and fluorescence microscope, the result found that it was significantly different histological changes when compared with untreated group of KKU-M139 and KKU-213B cell lines. Our data which recorded images under inverted light microscopy after ibuprofen, naproxen and diclofenac treatment showed star shape cells that caused by cell retraction and detachment on the bottle surface. The treated cell occurred membrane blebbing like a typical of cell injury or cell damage especially in diclofenac-treated cells. Furthermore, Caspases 3/7 activity and AnnexinV/PI staining were used to confirmed cell apoptosis. The results demonstrated that these drugs had slightly effects on both cell lines. Nevertheless, according to several examinations, there was no dose-dependent effects on CCA cells. The ratio of apoptotic cell death was measured using Annexin V and PI to validate the findings. In addition, the result of caspase 3/7 is comprised of the findings of Annexin V and PI detection, according to the caspase test activity assay. Ibuprofen and naproxen exhibited a lower caspase 3/7 activity than diclofenac on KKU-M139 and KKU-213B cells. Moreover, aspirin induced cell cycle arrested in the G0/G1 phase in cholangiocarcinoma (HuCCT1) (Shi et al., 2021).

Although they have the anti-inflammatory properties, more studies are still needed. The differences in outcomes could be shown by a different active component in these medications. CCA is a sort of chronic inflammation caused by a liver fluke, according to this study’s findings. An optimum dose of these NSAIDs in the early stages of infection may be able to reduce CCA inflammation.

However, there are some limitations to this study, including the lack of instructional tools, a verification check, and timing. Because a global coronavirus pandemic is one of the reasons for the need to adapt procedures that are appropriate for time and equipment. As a result, the method of this research is suitable for the period. Moreover, further experimental studies require to investigation.

In conclusion, the present results suggest that ibuprofen, naproxen and diclofenac can inhibit proliferation of KKU-M139 and KKU-213B cells lines that could contribute to the anti-proliferation of CCA. There is no significant inhibition in lipogenesis determined by Oil red O staining and colorimetric assay. The results of histology changes, both of them showed a typical of cell
injury or cell damage in high dose treatment especially in diclofenac. However, the Annexin V/PI showed apoptosis characteristic and the difference by caspase assay was still not statistically significant. Consequently, these drugs could be alternative drugs for anti-proliferation agent of CCA cells. Further study on the knowledge of the mechanisms of CCA cell lines proliferation suppression by these drugs should be continually evolved.

Author Contribution Statement

The study was created and designed by RTN, KJN and LTP. Under the supervision of RTN, KJN and LTP, KMR conducted research, provided research materials and collected and organized data. With RTN, Prism evaluated and interpreted data. All authors contributed to the first and final drafts of the paper, as well as providing appropriately resolved information. The manuscript’s content and similarity index are the responsibility of all authors, who have critically examined and approved the final draft.

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Study Approval

This manuscript is a part of an approved student thesis by College of Medicine and Public Health, Ubon Ratchathani University.

Ethical approval

Because it was an in-vitro experiment. In this research, the cell lines KKU-M139 and KKU-213B were used, which have been examined and published for a long period of time. This is an experiment that was accepted by a committee at Ubon Ratchathani University and does not require ethical approval.

Availability of data

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Conflicting Interest

The authors have no conflict of interest to declare.

References

Boueroy P, Aukkanimart R, Muangnoi P, et al (2017). Inhibitory Effect of Aspirin on Cholangiocarcinoma Cells. Asian Pac J Cancer Prev, 18, 3091-6.

Charonpongsuntorn C, Piyasatit P, Muntham D, Chommairtee P, Muangnoi P (2019). Clinical Prognostic Factors and Treatment Outcomes for the Survival of Patients with Cholangiocarcinoma in the Eastern Region of Thailand. Asian Pac J Cancer Care, 4, 101-5.

Huether A, Hopfner M, Baradari V, Schuppan D, Scherubl H (2007). Sorafenib alone or as combination therapy for growth control of cholangiocarcinoma. Biochem Pharmacol, 73, 1308-17.

Karaarslan N, Batmaz AG, Yilmaz I, et al (2018). Effect of naproxen on proliferation and differentiation of primary cell cultures isolated from human cartilage tissue. Exp Ther Med, 16, 1647-54.

Khwaia F, Allen J, Lynch J, Andrews P, Djakiew D (2004). Ibuprofen inhibits survival of bladder cancer cells by induced expression of the p75NTR tumor suppressor protein. Cancer Res, 64, 6207-13.

Li L, Che L, Tharp KM, et al (2016). Differential requirement for de novo lipogenesis in cholangiocarcinoma and hepatocellular carcinoma of mice and humans. Hepatology, 63, 1900-13.

Menendez JA, Lupu R (2007). Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat Rev Cancer, 7, 763-77.

Namwat N, Puetsakichonpashuta 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.

Pantziarka P, Bouche G, Sukhatme V, et al (2016). Repurposing Drugs in Oncology (ReDO)-Propranolol as an anti-cancer agent. Ecancermedicalscience, 10, 680.

Patterson AD, Maurhofer O, Beyoglu D, et al (2011). Aberrant lipid metabolism in hepatocellular carcinoma revealed by plasma metabolomics and lipid profiling. Cancer Res, 71, 6590-6600.

Shi T, Gong J, Fujita K, et al (2021). Aspirin inhibits cholangiocarcinoma cell proliferation via cell cycle arrest in vitro and in vivo. Int J Oncol, 58, 199-210.

Tawarungruang C, Khuntikeo N, Chamadol N, et al (2021). Survival after surgery among patients with cholangiocarcinoma in Northeast Thailand according to anatomical and morphological classification. BMC Cancer, 21, 497.

Warner TD, Nylander S, Whatling C (2011). Anti-platelet therapy: cyclo-oxygenase inhibition and the use of aspirin with particular regard to dual anti-platelet therapy. Br J Clin Pharmacol, 72, 619-33.

Yang G, Wang Y, Feng J, et al (2017). Aspirin suppresses the abnormal lipid metabolism in liver cancer cells via disrupting an NFKappaB-ACSL1 signaling. Biochem Biophys Res Commun, 486, 827-32.