Original Research

Turk J Immunol 2024;12(3):114-24
DOI: 10.4274/tji.galenos.2025.29290

Evaluation of the Anti-Cancer Efficacy of Cyclooxygenase Inhibition in Combination with Nutrient Starvation on Pancreatic Ductal Adenocarcinoma *In Vitro*

Ayşe Nur Çaldıran, Gizem Gürel, Başak Aru

Yeditepe University Faculty of Medicine, Department of Immunology, İstanbul, Turkey

Cite as: Çaldıran AN, Gürel G, Aru B. Evaluation of the Anti-Cancer Efficacy of Cyclooxygenase Inhibition in Combination with Nutrient Starvation on Pancreatic Ductal Adenocarcinoma *In Vitro*. Turk J Immunol. 2024;12(3):114-24

Received: 25.10.2024 **Accepted:** 07.01.2025

Corresponding Author: Başak Aru, Yeditepe University Faculty of Medicine, Department of Immunology, İstanbul, Turkey
E-mail: basak.aru@yeditepe.edu.tr **ORCID:** orcid.org/0000-0002-2987-0523

Abstract

Objective: Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer related deaths. Despite the advancements in cancer management, novel targets to improve treatment outcomes for PDAC are still needed. Herein, we aimed to evaluate the anti-cancer efficacy of nonselective non-steroidal anti-inflammatory drug (NSAID) diclofenac on PDAC *in vitro*, either alone or in combination with starvation.

Materials and Methods: Two different PDAC cell lines, PANC-1 and MIA PaCa-2, were treated with diclofenac either alone or after starvation with culture medium or Hank's balanced salt solution. Apoptosis, autophagy and cyclooxygenase (COX) levels were evaluated by flow cytometry.

Results: Diclofenac decreased both COX isoforms compared to untreated cells. However, the differences in COX-2 levels between starvation modalities were not significant. Furthermore, starvation followed by diclofenac treatment did not decrease COX-2 levels in the PDAC cell lines tested compared to diclofenac treatment alone.

Conclusion: This study demonstrated that diclofenac treatment can induce apoptosis in PDAC by suppressing both COX-1 and COX-2 levels, although starvation does not have a major impact on its anticancer efficacy. Further studies should focus on determining the optimal duration of starvation prior to NSAID treatment. In addition, the combinatorial effects of starvation and NSAID treatment with conventional treatment options for PDAC should be evaluated.

Keywords: Pancreatic ductal adenocarcinoma, cyclooxygenases, non-steroidal anti-inflammatory drugs, apoptosis, autophagy

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths and accounts for more than 90% of all pancreatic cancers (1-3). Currently, chemotherapy, radiotherapy and surgical resection are widely used to treat the disease. For patients with early-stage PDAC, surgical resection in combination with chemotherapy has improved survival, although this treatment regimen may not be sufficient for patients with late-stage disease (2). Due to the poor prognosis of PDAC, identification of novel targets to enhance treatment efficacy is required.

The role of chronic stress in cancer initiation and progression has been recognized for more than a decade, and the role of cyclooxygenases (COXs) in cancer-related inflammation is well documented (4,5). COX catalyzes the first step in the synthesis of prostanoids; prostaglandins (PG) and thromboxane A2 from arachidonic acid to induce inflammation. Two human COX isoforms have been identified, COX-1 and COX-2, which differ in their biological roles: COX-1 is constitutively expressed in tissues, whereas COX-2 is generally expressed upon induction and is associated with inflammation and carcinogenesis (5,6). Involvement of COX-2 in PDAC has been previously studied and is proposed as an important target for its treatment (7,8).

By inhibiting COX activity, non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to promote

ORCID: A.N. Çaldıran: 0009-0002-8025-3253, G.Gürel: 0000-0002-4073-3343, B.Aru: 0000-0002-2987-0523

Copyright© 2024 The Author. Published by Galenos Publishing House on behalf of the Turkish Society of Immunology.
This is an open access article under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 (CC BY-NC-ND) International License.

apoptosis and sensitize PDAC cells to conventional cancer treatment strategies by inducing tumor-suppressive autophagy (9,10). On the other hand, autophagy has also been reported to support PDAC initiation and progression (11,12). Recently, starvation has been suggested to enhance the impact of anti-cancer treatments in PDAC while it is also stated that nutrient deprivation can promote autophagy in PDAC to inhibit apoptosis and in turn, lead to chemotherapy resistance (11,13-15). Altogether, these data indicate the controversial roles of autophagy and starvation in PDAC, underlining the requirement of further studies.

In this study, we aimed to evaluate the anti-cancer efficacy of COX inhibition on PDAC *in vitro* by treating two different PDAC cell lines, PANC-1, and MIA PaCa-2 with non-selective NSAID diclofenac and investigated two programmed cell death pathways, apoptosis, and autophagy. Then, we coupled nutrient deprivation with COX inhibition to compare two different starvation modalities' effect on diclofenac-induced anti-cancer effect.

Materials and Methods

Cell Culture Conditions

Involvement of COX-1 and COX-2 in PDAC and the correlation of these enzyme levels' correlation with overall survival (OS) and disease-free survival (DFS) rates are investigated through Gene Expression Profiling Interactive Analysis (16). Even though both COX isoforms do not have a major impact in terms of OS or DFS in PDAC (Supplementary Figure 1), cancer and matched normal tissue (The Cancer Genome Atlas normal + Genotype-Tissue Expression normal) analysis revealed that both COX-1 (Supplementary Figure 2a) and COX-2 (Supplementary Figure 2b) expressions were significantly enhanced in cancer tissue, suggesting targeting COX enzymes may be a promising strategy for PDAC treatment. In our study, two different PDAC cell lines, PANC-1 (ATCC #CRL-1469) and MIA PaCa-2 (ATCC #CRL-1420) were included. Characteristic features and oncogenic mutations detected for both cell lines are presented in Supplementary Table 1 while representative micrographs are provided in Supplementary Figure 3.

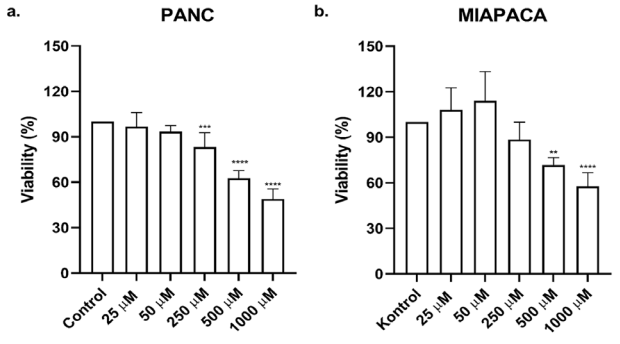


Figure 1. Bar graphics indicating viability ratios of (a) PANC-1 cell line and (b) MIA PaCa-2 cell line upon treatment with diclofenac at the doses between 25 and 1000 µM for 24 hours.
***: p<0.01, ***: p<0.001, ****: p<0.0001*

Cell lines were maintained in high glucose Dulbecco's Modified Eagle Medium (DMEM) basal medium (Thermo Fisher Scientific, #41966029) supplemented with antibiotic mixture (100 U/mL of penicillin and 100 µg/mL of streptomycin, Thermo Fisher Scientific, #15140122) and 10% fetal bovine serum (FBS) or horse serum for PANC-1 and MIA PaCa-2 cells, respectively. Trypsin (Thermo Fisher Scientific, #25200056) was used for detaching cells. Cells were seeded into 96-well cell culture plates at 5×10^3 cells per well for viability screening, while for flow cytometric analyses, 5×10^5 cells were seeded into 60 mm culture dishes. For all evaluations, cells were incubated overnight for attachment before the treatment. To investigate the effects of nutrient starvation, culture media was discarded, and PANC-1 and MIA PaCa-2 cells were incubated for six hours either with Hank's balanced salt solution (HBSS) (HBSS starvation group; Wisent Bioproducts, #311-510-CL) or DMEM w/o serum supplementation (DMEM starvation group). To examine the effect of nutrient starvation before diclofenac treatment, cells underwent starvation for six hours by incubating with HBSS or DMEM w/o serum supplementation followed by treatment with diclofenac for 24 hours. This study is an *in vitro* study that does not involve any primary human or animal tissue or cell samples. All cell lines used in this study are commercially available, therefore ethics committee approval and patient informed consent were not required.

Colorimetric Determination of Viability

For determining the optimal dose of diclofenac on PANC-1 and MIA PaCa-2 cell lines, cells were treated with diclofenac (Sigma Aldrich, #D6899) at the concentrations of 25, 50, 100, 250 and 500 µM for 24 hours. At the end of incubation period, 10% (v/v) MTS reagent (Abcam #ab197010) were added to wells, and plates were further incubated at 37°C under humidified atmosphere for one hour (New Brunswick, USA). Untreated cells were included as the control group and wells containing equal volume of complete culture media were used as blank. Absorbance at 490 nm was measured with a spectrophotometer (Epoch, BioTek, USA), and relative viability was calculated according to the formula given below. The lowest dose that significantly decreased viability compared to the control was used for mechanistic studies.

$$\text{Relative viability (\%)} = \frac{\text{Sample OD (490 nm)-Blank OD (490 nm)}}{\text{Control OD (490 nm)-Blank OD (490 nm)}} \times 100$$

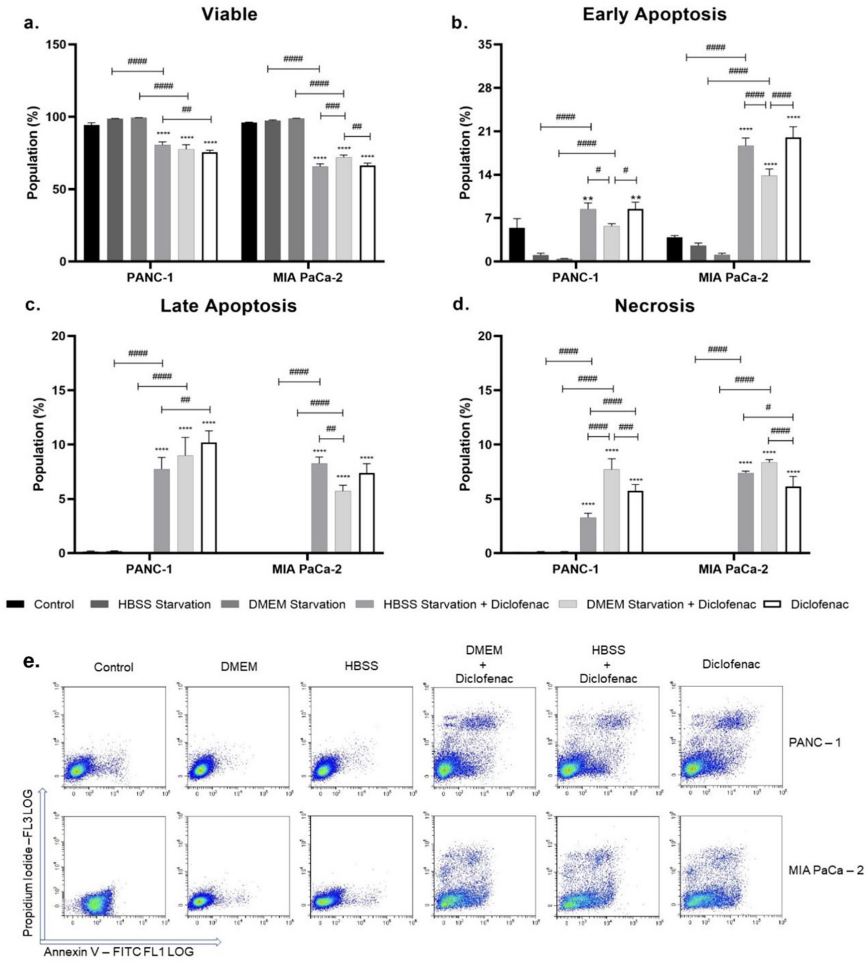


Figure 2. Bar graphics indicating (a) viability, (b) early apoptosis, (c) late apoptosis and (d) necrosis levels in PANC-1 and MIA PaCa-2 cell lines upon treatment with diclofenac for 24 hours, either in the presence of HBSS or DMEM starvation for 12 hours or not. (e) Representative density plots. HBSS starvation before diclofenac treatment protected PANC-1 cells from apoptosis while increasing necrosis. In MIA PaCa-2 cells, diclofenac treatment after HBSS starvation significantly enhanced late apoptosis along with increasing necrosis compared to diclofenac group.
**: Denotes differences among control and treatment groups while, #: denotes differences between treatment groups, **: p<0.01, ****: p<0.0001; #: p<0.05, ##: p<0.01, ###: p<0.001, ####: p<0.0001, DMEM: Dulbecco's Modified Eagle Medium, HBSS: Hank's balanced salt solution*

Annexin V/Propidium Iodide Staining

One of the early events in apoptosis is the loss of membrane asymmetry that leads to translocation of phosphatidylserine (PS) residues to the outer leaflet of the plasma membrane. Annexin V can bind to PS in the presence of calcium and labels apoptotic cells (17). When used in conjunction with Annexin V, propidium iodide (PI) can penetrate cells that have lost membrane integrity, allowing differentiation between early apoptotic (Annexin V+ / PI-), viable (Annexin V- / PI-), late apoptotic (Annexin V+ / PI+) and necrotic (Annexin V- / PI+) cell populations. After treatment, cells were collected and the pellet was suspended in 1 mL of Annexin V Binding Buffer (Abcam, #ab14084). Cells were labelled with Annexin V-FITC (1 µL, BioVision #1001) and PI (1 µL of 250 µg/mL in DPBS, Thermo Fisher Scientific, #P3566) by incubating at 4°C under dark conditions for 15 minutes and immediately acquired by DxFLEX flow cytometry system (Beckman Coulter, USA). 2.5×10^4 events per tube were read. Analyses were conducted with CytExpert software.

Protein Expression Analysis

The expression levels of the COXs, COX-1 and COX-2; Beclin-1 and active LC3B, which are involved in autophagy; and the apoptosis indicator active caspase-3 were evaluated by flow cytometry. Briefly, cells were collected by trypsinization and fixed with 1% paraformaldehyde solution in DPBS, followed by permeabilization with DPBS containing 0.1% Triton X-100. Permeabilization solution was discarded by centrifugation at 350×G for 5 minutes, and cells were either labelled with COX-1 (Clone #345305, Alexa Fluor 488 conjugated, R&D Systems #FAB3740G-100 µg) and COX-2 (polyclonal, Abcam, #ab15191), or Beclin-1 (polyclonal, Alexa Fluor 750 conjugated, Novus Biologicals, # NB500-

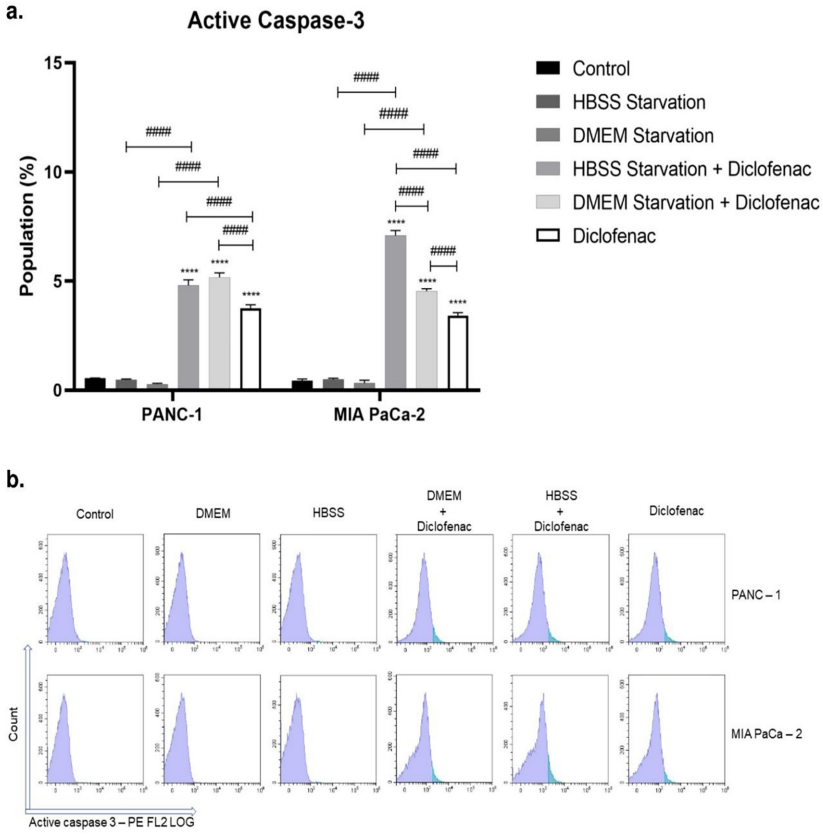


Figure 3. (a) Bar graphic indicating active caspase-3 protein levels in PANC-1 and MIA PaCa-2 cell lines upon treatment with diclofenac for 24 hours, either in the presence of HBSS or DMEM starvation for 12 hours or not. (b) Representative histogram plots. Debris was omitted when gating cells. Active caspase-3 positive cells are shown in turquoise. Diclofenac treatment after both starvation modalities significantly increased active caspase-3 levels compared to the control as well as respective starvation groups.
**: Denotes differences among control and treatment groups while, #: denotes differences between treatment groups, ****: p<0.0001; ####: p<0.0001, DMEM: Dulbecco's Modified Eagle Medium, HBSS: Hank's balanced salt solution*

249AF750) and active LC3 (polyclonal, Aviva Systems Biology, #OAAB13518), or active caspase-3 (Clone #C92-605, PE conjugated, BD Biosciences, # 561011) antibodies by incubating at 4°C under dark for 30 minutes. Primary antibody solutions were discarded by centrifuging cells at 350×G for 5 minutes. PE conjugated anti-rabbit antibody (Abcam, #ab7007) or DyLight 488 conjugated anti-rabbit antibody (Abcam, #ab96923) were used as secondary antibodies by incubating the samples at room temperature for 15 minutes under dark. Cells were acquired by Beckman Coulter DxFLEX flow cytometry system (Beckman Coulter, USA) and 2.5×10^4 events per tube were read. Analyses were conducted with CytExpert software.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism software (version 8). The Shapiro-Wilk test was used to determine whether the data were normally distributed. One-way ANOVA followed by Dunnett's multiple comparison test was used to determine the dose of diclofenac on cell lines. Annexin V/PI staining and protein expression results were analyzed by two-way ANOVA followed by Sidak's multiple comparison test. P-values less than 0.05 were considered statistically significant.

Results

Diclofenac Decreases Viability in Pancreatic Cancer Cell Lines in a Dose Dependent Manner

Diclofenac decreased viability of PANC-1 and MIA PaCa cells over 250 µM and 500 µM, respectively in 24 hours treatment duration (Figure 1). In terms of MIA PaCa cell line, a slight but non-significant increase in viability upon treatment with low doses (25 µM and 50 µM) of the drug was observed. Further analyses were conducted with the lowest doses that significantly decrease viability of PANC-1 (250 µM) and MIA PaCa- 2 (500 µM) cells.

Nutrient Starvation Has Contradictory Effects on Pancreatic Cancer Cell Lines in Terms of Apoptosis

As confirmed with both Annexin V/PI staining (Figure 2) and active caspase-3 protein levels (Figure 3), diclofenac treatment increased apoptosis and necrosis in the cell lines

tested compared to the control as well as starvation groups (p<0.0001). In PANC-1 cells, HBSS starvation protected cells from diclofenac-induced apoptosis as viability levels of HBSS + diclofenac group were significantly higher than their diclofenac treated counterparts without starvation (p=0.0069) (Figure 2a). Yet, DMEM + diclofenac group had significantly decreased early apoptosis rates compared to both diclofenac and HBSS + diclofenac groups (p=0.015) (Figure 2b). In HBSS + diclofenac group, late apoptosis (Figure 2c) and necrosis (Figure 2d) levels were significantly lower than those treated with diclofenac (p=0.0054 and p<0.0001, respectively). On the other hand, DMEM starvation prior to diclofenac treatment led to a significant increase in necrosis compared to the diclofenac treatment (p=0.0003) as well as HBSS starvation followed by diclofenac treatment (p<0.0001) in this cell line (Figure 2d), although no differences among diclofenac and DMEM + diclofenac groups in terms of viability (Figure 2a) and late apoptosis (Figure 2c) were observed (p>0.05). When active caspase-3 levels are evaluated, both HBSS + diclofenac and DMEM + diclofenac groups had significantly higher active caspase-3 levels compared to the diclofenac group (Figure 3a) though no difference among groups underwent starvation was observed (p>0.05).

In terms of MIA PaCa-2 cell line, DMEM + diclofenac group had significantly higher viability rates compared to HBSS + diclofenac (p=0.0003) and diclofenac (p=0.0015) groups (Figure 2a) and significantly lower early apoptosis rates (p<0.0001) (Figure 2b). Similarly, DMEM + diclofenac group had significantly lower late apoptosis rates compared to the HBSS + diclofenac group (p=0.003) (Figure 2d). On the other hand, like PANC-1 cells, MIA PaCa-2 cells also had significantly higher necrosis rates in DMEM + diclofenac group in comparison with the diclofenac group (Figure 2d). In this cell line, active caspase-3 levels of the diclofenac group were found to be significantly lower than DMEM + diclofenac and HBSS + diclofenac groups (p<0.0001) while HBSS + diclofenac group had significantly higher active caspase-3 levels compared to both DMEM + diclofenac and diclofenac groups (p<0.0001) (Figure 3).

Autophagy is Promoted by COX Inhibition and Further Enhanced by Nutrient Starvation

Both starvation modalities led to a significant increase in active LC3B levels in PANC-1 (p<0.0001 and p=0.0002 for DMEM and HBSS starvation, respectively) and MIA PaCa-2 (p<0.0001) cells (Figure 4a). In PANC-1, starvation before diclofenac treatment significantly enhanced active LC3B levels compared to the diclofenac group (p<0.0001 and p=0.0003 for DMEM and HBSS starvation, respectively). When the impact of the starvation modality prior to diclofenac treatment was compared, DMEM + diclofenac had significantly higher active LC3B levels than HBSS + diclofenac group (p<0.0001). In MIA PaCa- 2, starvation before diclofenac treatment significantly increased active LC3B levels in comparison with the respective starvation modality and the control group (p<0.0001). Comparison between starvation modalities revealed that active LC3B levels of HBSS + diclofenac group were higher than DMEM + diclofenac group (p=0.0012).

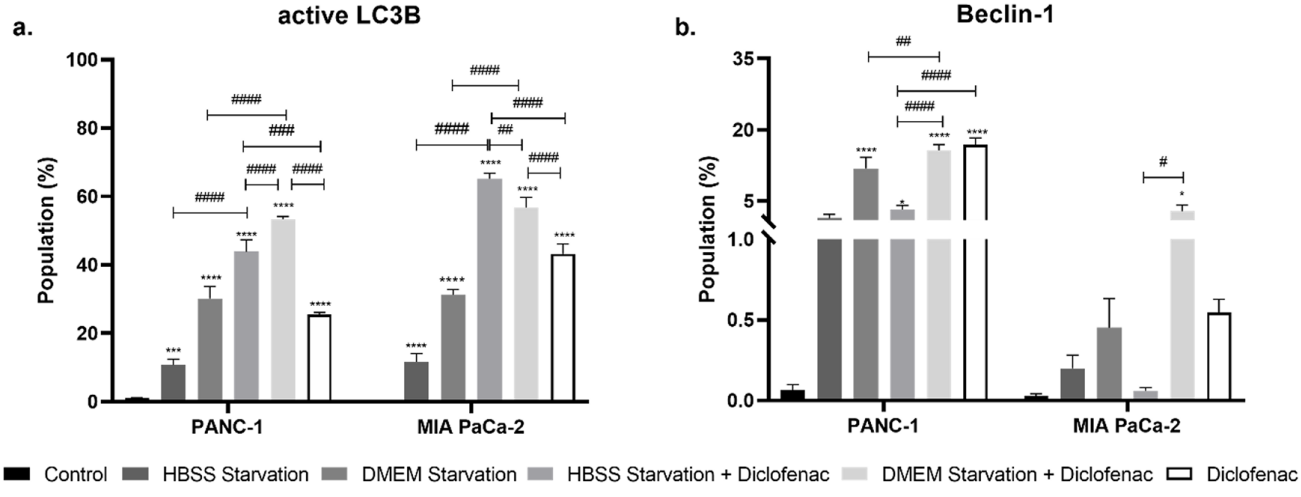


Figure 4. Bar graphics indicating (a) active LC3B and (b) Beclin-1 protein levels upon treatment with diclofenac for 24 hours, either in the presence of HBSS or DMEM starvation for 12 hours or not. Both starvation modalities promoted active LC3B levels in PANC-1 and MIA PaCa-2 cells while diclofenac treatment after starvation further increased the protein's levels, both in comparison with the respective starvation group as well as the diclofenac group. In MIA PaCa-2 cells, Beclin-1 was increased in DMEM + diclofenac group compared to the control.
**: Denotes differences between control and treatment groups while, #: denotes differences between treatment groups, *: p<0.05, ***: p<0.001, ****: p<0.0001; #: p<0.05, ##: p<0.01, ###: p<0.001, ####: p<0.0001, DMEM: Dulbecco's Modified Eagle Medium, HBSS: Hank's balanced salt solution*

Both HBSS + diclofenac and DMEM + diclofenac groups had significantly higher Beclin-1 levels in PANC-1 cells in comparison with the control group (p=0.011 and p<0.0001, respectively) (Figure 4b). In MIA PaCa-2 cell line, Beclin-1 levels were significantly increased in DMEM + diclofenac group compared to the control group (p=0.02); no difference between the diclofenac group and the groups underwent starvation before diclofenac treatment was observed (p>0.05). When different starvation modalities followed by diclofenac treatment were compared, DMEM + diclofenac groups had significantly higher Beclin-1 levels in comparison with HBSS + diclofenac groups (p<0.0001 and p=0.022 for PANC-1 and MIA PaCa-2 cell lines, respectively). Representative flow cytometry histograms are given in Supplementary Figure 4.

Starvation Followed by COX Inhibition Has Different Consequences in Terms of COX Protein Levels

Diclofenac treatment successfully decreased COX-1 and COX-2 levels in both cell lines tested, either under starvation conditions or not (Figure 5). Representative flow cytometry histograms are given in Supplementary Figure 5. In addition, DMEM starvation alone also led to a subtle but significant decrease in COX-1 levels in both cell lines (p=0.0034 and p=0.0019 for PANC-1 and MIA PaCa-2, respectively). In PANC-1, no differences between HBSS + diclofenac and DMEM + diclofenac groups were observed (p>0.05), though both had significantly higher COX-1 levels in comparison with the diclofenac group (p<0.0001 and p=0.0002, respectively). On the contrary, in MIA PaCa-2, HBSS + diclofenac group had lower COX-1 levels in comparison with diclofenac group (p=0.0023), yet the difference between DMEM + diclofenac and diclofenac groups was not significant (p>0.05) (Figure 5a). In PANC-1 cells, DMEM + diclofenac group had lower COX-2 levels in comparison with HBSS + diclofenac group (p=0.009). For MIA PaCa-2 cells, the opposite was observed as HBSS + diclofenac group had significantly lower COX-2 levels compared to DMEM + diclofenac group (p=0.03) (Figure 5b). However, no difference between starvation and diclofenac groups were determined in both cell lines (p>0.05).

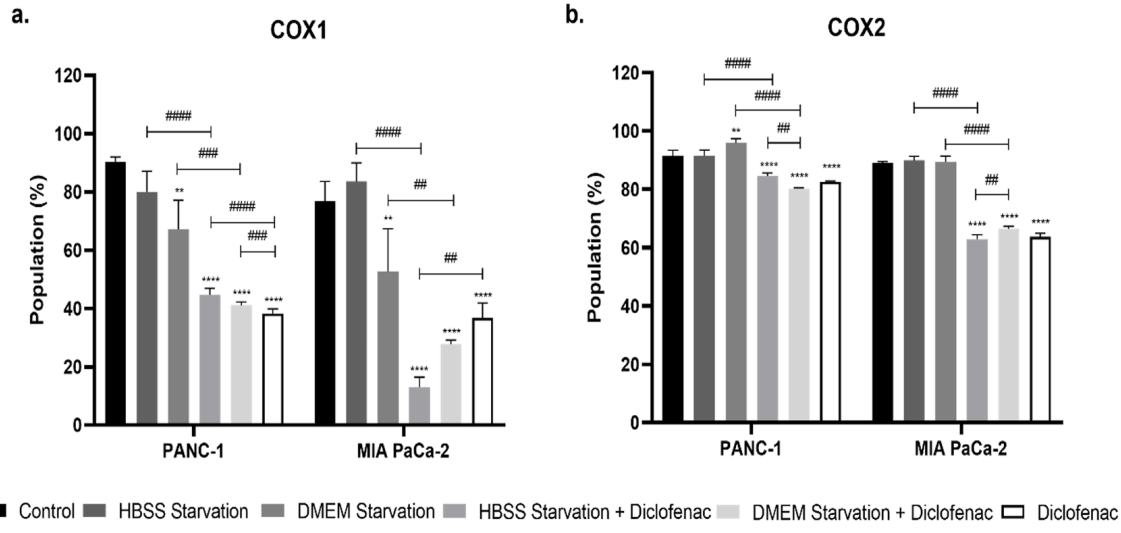


Figure 5. Bar graphics indicating (a) COX-1 and (b) COX-2 protein levels upon treatment with diclofenac for 24 hours, either in the presence of HBSS or DMEM starvation for 12 hours or not. Diclofenac significantly decreased both COX-1 and COX-2 levels in both cell lines, and starvation prior drug treatment further decreased COX-1 levels in MIA PaCa-2 cells.

**:Denotes differences between control and treatment groups while, #: denotes differences between treatment groups, **: p<0.01, ***: p<0.001, ****: p<0.0001; ##: p<0.01, ###: p<0.001, ####: p<0.0001, COX: Cyclooxygenase*

Discussion

Despite the advancements in cancer treatment, PDAC remains one of the most challenging cancers to treat (3,18,19). Low survival rate of the disease is associated with the fact that PDAC at early-stage is usually asymptomatic, and 80% patients are generally diagnosed at advanced stages with nonresectable disease (3). Currently, gemcitabine and fluoropyrimidine-based combination therapies are regarded as standard chemotherapeutic regimens for metastatic PDAC patients, though median OS rates remain around 1 year (3).

Recently, a growing body of evidence has suggested the critical role of altered cellular metabolism in PDAC initiation, progression, and treatment to meet the enhanced needs of these cells for unlimited tumor progression (20). Metabolic reprogramming in PDAC contributes to chemotherapy, radiotherapy and immunotherapy resistance and is associated with poor prognosis, which may be improved by therapies targeting metabolic regulation (20). *In vitro* studies of chemotherapy-resistant PDAC cells have reported increased rates of aerobic glycolysis and decreased accumulation of reactive oxygen species compared to their parental cell lines, facilitating epithelial-mesenchymal transition and chemoresistance (21).

Impact of the amino acid metabolism on chemoresistance in PDAC remains relatively unclear (20). Glutamine is the most investigated amino acid in chemotherapeutic drug resistance (22). In PDAC, increased glutamine metabolism promotes hexosamine biosynthetic pathway and glycosylation (20). Glutamine contributes to nicotinamide adenine dinucleotide phosphate production that plays role in redox homeostasis and enhances the mammalian target of rapamycin pathway that both leads to gemcitabine resistance (20,23). Herein, we evaluated the impact of amino acid starvation on PDAC cell lines PANC-1 and MIA PaCa-2 in combination with a COX inhibitor, diclofenac.

In our study, nutrient deprivation either did not decrease viability or did not promote apoptosis in the PDAC cell lines tested, while diclofenac treatment promoted cell death under both conditions. However, in PANC-1 cells, the highest levels of late apoptosis were detected in the diclofenac group; and in MIA PaCa-2 cells, both starvation modalities did not alter late apoptosis levels compared to the diclofenac group, suggesting that DMEM or HBSS starvation prior to diclofenac exposure does not have a major influence in enhancing apoptosis. On the other hand, in accordance with previous studies, starvation led to a significant increase in autophagy, as indicated by the increase in active LC3B protein levels (24,25). Increased autophagy along with apoptosis levels observed in our study may highlight the double-faced role of autophagy in cancer (26,27). On the contrary, Beclin-1, the protein involved in the formation of functional autophagosomes did not elevate upon starvation in MIA PaCa- 2 cells; and in PANC-1 cell line, only DMEM starvation increased its protein levels which is further enhanced in the case of DMEM starvation followed by diclofenac treatment though it should be noted that Beclin-1 does not take essential part in LC3B lipidation (28). Taken together, our findings regarding apoptosis and autophagy demonstrate that starvation increases active LC3B levels, hence autophagy in PDAC cell lines, which can be further increased by subsequent diclofenac treatment without increasing late apoptotic cell populations.

The impact of starvation on COX expression *in vitro* is rather limited. It has been stated that serum starvation led to a decrease in COX-2 levels in BXPC-3 and Capan-1 cell lines, which was induced upon FBS supplementation (7). These results highlight the modulatory effects of growth signals on COX-2 expression in PDAC. In addition to PDAC, Li et al. (29) showed that amino acid starvation increases COX-2 expression, which promotes vascular endothelial growth factor expression in glioma cells. However, there are several reports underlining the involvement of COX, especially COX-2 in PDAC and is associated with worse prognosis (30,31). In PDAC, COX-2 activates PI3K/AKT pathway to contribute tumorigenesis (8). Enhanced COX-2 expression also promotes PGE2 production that favors not only tumor progression, but also evasion of immunosurveillance (32). Given the involvement of COX in PDAC, we hypothesized that its suppression by serum starvation may favor the anticancer efficacy of diclofenac. We revealed that diclofenac successfully decreases both COX-1 and COX-2 protein levels compared to untreated cells, in addition, in MIA PaCa-2 cells, HBSS starvation followed by diclofenac treatment further decreased COX-1 levels in comparison with diclofenac treated cells. However, the differences in terms of COX-2 levels between starvation modalities were not notable; similarly, starvation followed by diclofenac treatment failed to decrease COX-2 levels in both PDAC cell lines tested when compared to sole diclofenac treatment.

As stated before, COX-2 is the main isoform involved in PDAC (30,31), and further studies investigating longer starvation intervals followed by administration of COX-2 specific drugs such as celecoxib may be considered for an enhanced anti-cancer efficacy (33). Moreover, targeting COX-2 has been shown to exert synergistic effect with immune checkpoint blockade by promoting intratumoral accumulation of effector T-cells, and administration of NSAIDs may be a novel strategy for switching the tumor inflammatory profile from cold to hot (34).

Conclusion

In summary, our findings reveal that diclofenac treatment can induce apoptosis in PDAC by suppressing both COX-1 and COX-2 levels *in vitro*, but starvation does not have a major effect on the drug's efficacy on COX-2. It should be noted that the optimal duration of starvation before NSAID treatment is yet to be elucidated, and future studies could explore the effects of different periods of starvation on PDAC. In addition, NSAID treatments, either coupled with starvation or alone, may be more effective when used in combination with other anti-cancer agents. Future studies could investigate the combinatorial effects of starvation and NSAID treatment with chemotherapy, radiation therapy, or immunotherapy. Lastly, as stated previously, COX-2 inhibition has been shown to activate AKT pathway and induce therapeutic resistance. Thus, PI3K-AKT pathway should also be investigated while evaluating the anti-cancer mechanisms of NSAIDs' anti-cancer activities.

Ethics

Ethics Committee Approval: This study is an *in vitro* study that does not involve any primary human or animal tissue or cell samples. All cell lines used in this study are commercially available, therefore ethics committee approval was not required.

Informed Consent: This study is an *in vitro* study that does not involve any primary human or animal tissue or cell samples. All cell lines used in this study are commercially available, therefore patient informed consent was not required.

Acknowledgements

This study was performed in Immunology Laboratories of Yeditepe University. Prof. Dr. Gülderen Yanıkkaya Demirel, MD, PhD, head of the Yeditepe University Faculty of Medicine, Department of Immunology, is gratefully acknowledged. We thank Prof. Dr. Devrim Atilla from Gebze Technical University, Department of Chemistry, for sharing diclofenac; and Prof. Dr. Ömer Faruk Bayrak from Yeditepe University Faculty of Medicine, Department of Medical Genetics, for providing PANC-1 and MIA PaCa-2 cell lines.

Footnotes

Authorship Contributions

Concept: B.A., Design: G.G., B.A., Data Collection or Processing: A.N.Ç., G.G., Analysis or Interpretation: A.N.Ç., G.G., B.A., Literature Search: A.N.Ç., B.A., Writing: A.N.Ç., G.G.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declare that they have no relevant financial interest.

References

1. Brand RE, Persson J, Bratlie SO, Chung DC, Katona BW, Carrato A, et al. Detection of early-stage pancreatic ductal adenocarcinoma from blood samples: results of a multiplex biomarker signature validation study. Clin Transl Gastroenterol. 2022;13:e00468.
2. Sarantis P, Koustas E, Papadimitropoulou A, Papavassiliou AG, Karamouzis MV. Pancreatic ductal adenocarcinoma: Treatment hurdles, tumor microenvironment and immunotherapy. World J Gastrointest Oncol. 2020;12:173-81.
3. Hosein AN, Dougan SK, Aguirre AJ, Maitra A. Translational advances in pancreatic ductal adenocarcinoma therapy. Nat Cancer. 2022;3:272-86.
4. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646-74.
5. Pannunzio A, Coluccia M. Cyclooxygenase-1 (COX-1) and COX-1 inhibitors in cancer: a review of oncology and medicinal chemistry literature. Pharmaceuticals (Basel). 2018;11:101.
6. Liu B, Qu L, Yan S. Cyclooxygenase-2 promotes tumor growth and suppresses tumor immunity. Cancer Cell Int. 2015;15:106.
7. Yip-Schneider MT, Barnard DS, Billings SD, Cheng L, Heilman DK, Lin A, et al. Cyclooxygenase-2 expression in human pancreatic adenocarcinomas. Carcinogenesis. 2000;21:139-46.
8. Hill R, Li Y, Tran LM, Dry S, Calvopina JH, Garcia A, et al. Cell intrinsic role of COX-2 in pancreatic cancer development. Mol Cancer Ther. 2012;11:2127-37.
9. Fu X, Tan T, Liu P. Regulation of autophagy by non-steroidal anti-inflammatory drugs in cancer. Cancer Manag Res. 2020;12:4595-604.
10. Li J, Luo M, Wang Y, Shang B, Dong L. Celecoxib suppresses fibroblast growth factor-2 expression in pancreatic ductal adenocarcinoma PANC-1 cells. Oncol Rep. 2016;36:1345-52.
11. Hashimoto D, Bläuer M, Hirota M, Ikonen NH, Sand J, Laukkarinen J. Autophagy is needed for the growth of pancreatic adenocarcinoma and has a cytoprotective effect against anticancer drugs. Eur J Cancer. 2014;50:1382-90.
12. Gillson J, Abd El-Aziz YS, Leck LYW, Jansson PJ, Pavlakis N, Samra JS, et al. Autophagy: a key player in pancreatic cancer progression and a potential drug target. Cancers (Basel). 2022;14:3528.
13. Sun S, Kim MJ, Omar AM, Duy Phan N, Aoike M, Awale S. GDP Induces PANC-1 human pancreatic cancer cell death preferentially under nutrient starvation by inhibiting PI3K/Akt/mTOR/autophagy signaling pathway. Chem Biodivers. 2021;18:e2100389.
14. Zalyte E, Cicenas J. Starvation mediates pancreatic cancer cell sensitivity to ferroptosis via ERK1/2, JNK and changes in the cell mesenchymal state. Int J Mol Med. 2022;49:84.
15. Kim SE, Park HJ, Jeong HK, Kim MJ, Kim M, Bae ON, et al. Autophagy sustains the survival of human pancreatic cancer PANC-1 cells under extreme nutrient deprivation conditions. Biochem Biophys Res Commun. 2015;463:205-10.

16. Tang Z, Kang B, Li C, Chen T, Zhang Z. GEPIA2: an enhanced web server for large-scale expression profiling and interactive analysis. Nucleic Acids Res. 2019;47:556-60.

17. van Genderen HO, Kenis H, Hofstra L, Narula J, Reutelingsperger CP. Extracellular annexin A5: functions of phosphatidylserine-binding and two-dimensional crystallization. Biochim Biophys Acta. 2008;1783:953-63.

18. Anderson EM, Thomassian S, Gong J, Hendifar A, Osipov A. Advances in pancreatic ductal adenocarcinoma treatment. Cancers (Basel). 2021;13:5510.

19. Bockorny B, Grossman JE, Hidalgo M. Facts and hopes in immunotherapy of pancreatic cancer. Clin Cancer Res. 2022;28:4606-17.

20. Qin C, Yang G, Yang J, Ren B, Wang H, Chen G, et al. Metabolism of pancreatic cancer: paving the way to better anticancer strategies. Mol Cancer. 2020;19:50.

21. Zhao H, Duan Q, Zhang Z, Li H, Wu H, Shen Q, et al. Up-regulation of glycolysis promotes the stemness and EMT phenotypes in gemcitabine-resistant pancreatic cancer cells. J Cell Mol Med. 2017;21:2055-67.

22. Yoo HC, Han JM. Amino acid metabolism in cancer drug resistance. Cells. 2022;11:140.

23. Feng M, Xiong G, Cao Z, Yang G, Zheng S, Qiu J, et al. LAT2 regulates glutamine-dependent mTOR activation to promote glycolysis and chemoresistance in pancreatic cancer. J Exp Clin Cancer Res. 2018 Nov;37:274.

24. New M, Tooze S. The role of autophagy in pancreatic cancer-recent advances. Biology (Basel). 2019;9:7.

25. Raufi AG, Liguori NR, Carlsen L, Parker C, Hernandez Borrero L, Zhang S, et al. Therapeutic targeting of autophagy in pancreatic ductal adenocarcinoma. Front Pharmacol. 2021;12:751568.

26. Kroemer G, Levine B. Autophagic cell death: the story of a misnomer. Nat Rev Mol Cell Biol. 2008;9:1004-10.

27. Yun CW, Lee SH. The roles of autophagy in cancer. Int J Mol Sci. 2018;19:3466.

28. He R, Peng J, Yuan P, Xu F, Wei W. Divergent roles of BECN1 in LC3 lipidation and autophagosomal function. Autophagy. 2015;11:740-7.

29. Li Z, Chang CM, Wang L, Zhang P, Shu HG. Cyclooxygenase-2 induction by amino acid deprivation requires p38 mitogen-activated protein kinase in human glioma cells. Cancer Invest. 2017;35:237-47.

30. Hu H, Han T, Zhuo M, Wu LL, Yuan C, Wu L, et al. Elevated COX-2 expression promotes angiogenesis through EGFR/p38-MAPK/Sp1-dependent signalling in pancreatic cancer. Sci Rep. 2017;7:470.

31. Gonnermann D, Oberg HH, Kellner C, Peipp M, Sebens S, Kabelitz D, et al. Resistance of cyclooxygenase-2 expressing pancreatic ductal adenocarcinoma cells against γδ T cell cytotoxicity. Oncoimmunology. 2015;4:e988460.

32. Sreeramkumar V, Fresno M, Cuesta N. Prostaglandin E2 and T cells: friends or foes? Immunol Cell Biol. 2012;90:579-86.

33. Koki AT, Masferrer JL. Celecoxib: a specific COX-2 inhibitor with anticancer properties. Cancer Control. 2002;9:28-35.

34. Pelly VS, Moeini A, Roelofsen LM, Bonavita E, Bell CR, Hutton C, et al. Anti-inflammatory drugs remodel the tumor immune environment to enhance immune checkpoint blockade efficacy. Cancer Discov. 2021;11:2602-19.

35. Lieber M, Mazzetta J, Nelson-Rees W, Kaplan M, Todaro G. Establishment of a continuous tumor-cell line (panc-1) from a human carcinoma of the exocrine pancreas. Int J Cancer. 1975;15:741-7.

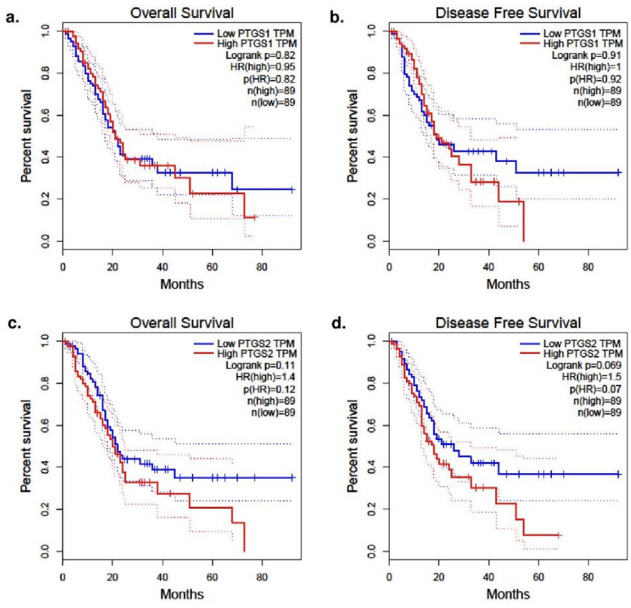
36. Gradiz R, Silva HC, Carvalho L, Botelho MF, Mota-Pinto A. MIA PaCa-2 and PANC-1-pancreas ductal adenocarcinoma cell lines with neuroendocrine differentiation and somatostatin receptors. Sci Rep. 2016 ;6:21648.

37. Yunis AA, Arimura GK, Russin DJ. Human pancreatic carcinoma (MIA PaCa-2) in continuous culture: sensitivity to asparaginase. Int J Cancer. 1977;19:128-35.

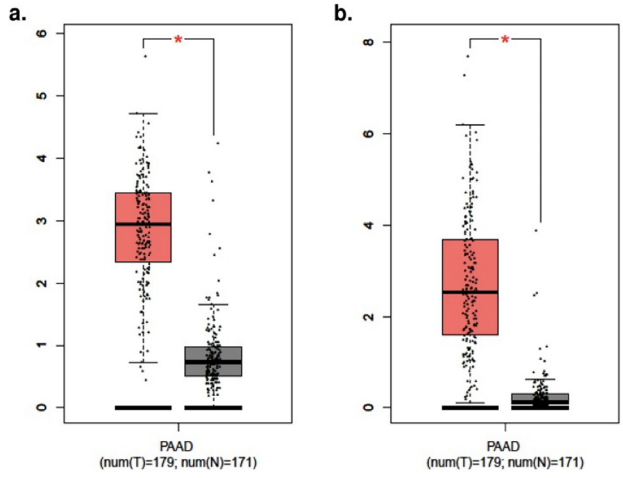
Supplementary Table 1. Characteristic features of PANC- and MIA-PaCa-2 cell lines

Cell Line	Donor	Morphology	Mutations
PANC-1	56-years old female patient (35)	Epithelial (35)	KRAS: homozygous p.G12D; GGT > GAT (36) TP53: p.P72R; CCC > CGC, p.R273H; CGT > CAT CDKN2A/p16: homozygous deletion of exons 1, 2 and 3
MIA-PaCa-2	65-years old male patient (37)	Epithelial (37)	KRAS: homozygous p.G12C; GGT > TGT (35) TP53: homozygous p.R248W; CGG > TGG CDKN2A/p16: homozygous deletion in exons 1, 2 and 3

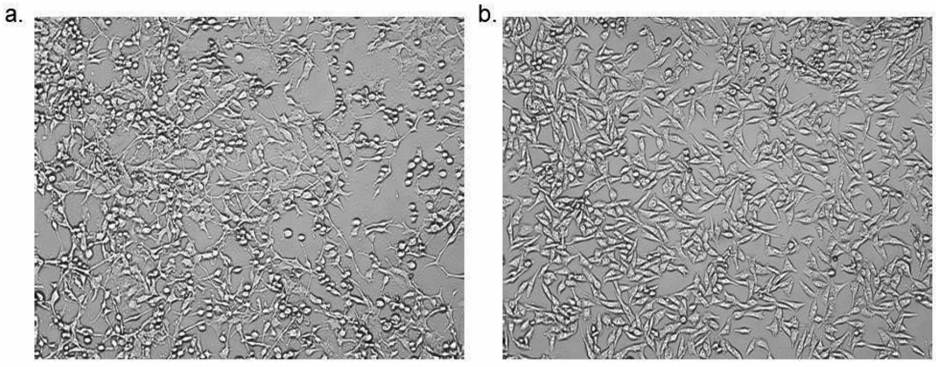
KRAS: Kirsten rat sarcoma virus, TP53: Tumor suppressor protein 53, CDKN2A: Cyclin-dependent kinase inhibitor 2A



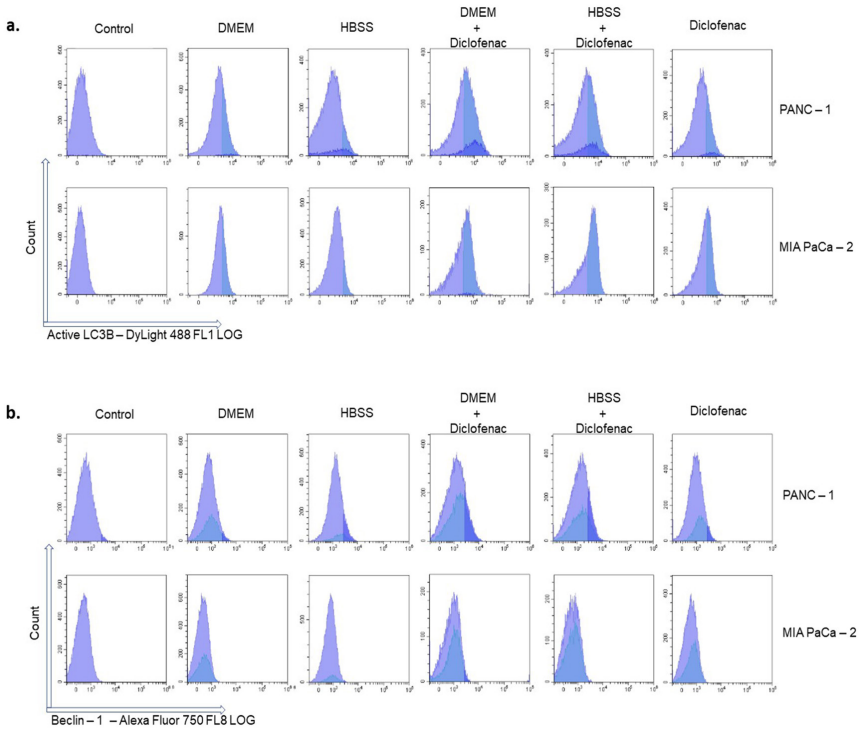
Supplementary Figure 1. Kaplan-Meier plots indicating the correlation between COX-1 (a and b) and COX-2 (c and d) mRNA levels and the overall survival (OS) or disease-free survival (DFS) in PDAC patients. Analysis was performed on GEPIA with log-rank test, and COX proportional hazard ratio as well as 95% confidence intervals calculated. P-values lower than 0.05 were stated statistically significant. These results suggest that both COX isoforms do not have a major impact in terms of OS or DFS in PDAC.
PDAC: Pancreatic ductal adenocarcinoma, COX: Cyclooxygenase, GEPIA: Gene Expression Profiling Interactive Analysis



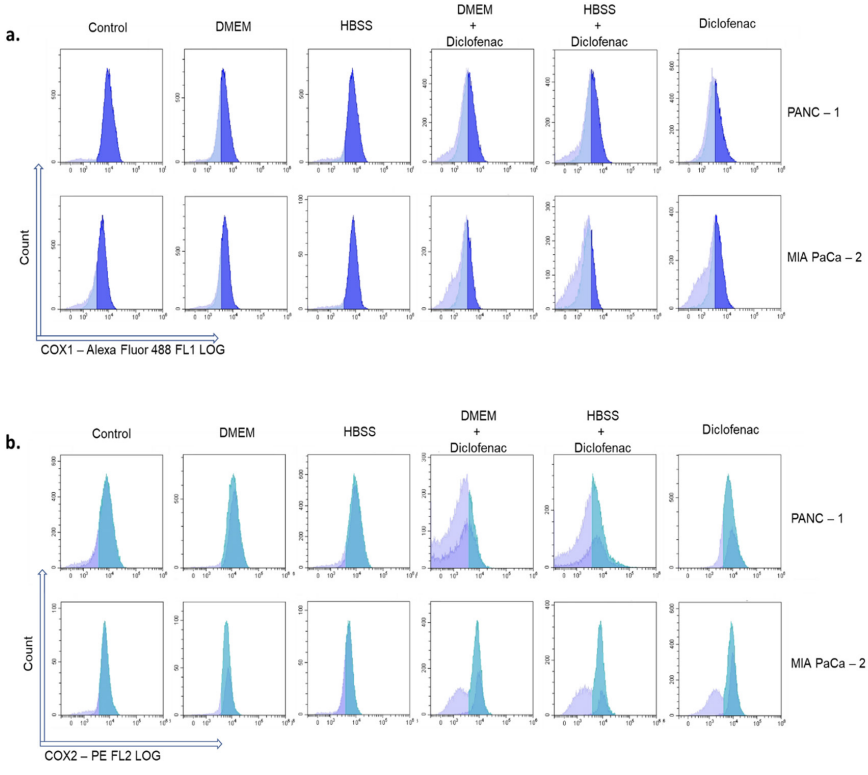
Supplementary Figure 2. Boxplots presenting either (a) COX-1 or (b) COX-2 mRNA expression profiles in human cancer and matched normal tissue (TCGA normal + GTEx normal) datasets. Analysis was performed with Gene Expression Profiling Interactive Analysis. Red and grey colors represent cancer and healthy tissues, respectively. A p-value lower than 0.05 was stated statistically significant (*p<0.05).
COX: Cyclooxygenase, TCGA: The Cancer Genome Atlas, GTEx: Genotype-Tissue Expression



Supplementary Figure 3. Representative micrographs of (a) PANC-1 and (b) MIA-PaCa-2 cell lines. The images were taken with JuLI Br&FL cell analyzer (NanoEntek, South Korea) at 4X magnification.



Supplementary Figure 4. Representative flow cytometry histograms regarding (a) active LC3B and (b) Beclin-1 evaluations. Debris was omitted when gating cells. Positive populations are shown in dark blue and turquoise for active Beclin-1 and active LC3B, respectively.
DMEM: Dulbecco's Modified Eagle Medium, HBSS: Hank's balanced salt solution



Supplementary Figure 5. Representative flow cytometry histograms regarding (a) COX-1 and (b) COX-2 evaluations. Debris was omitted when gating cells. Positive populations are shown in dark blue and turquoise for COX- 1 and COX-2, respectively.
COX: Cyclooxygenase, DMEM: Dulbecco's Modified Eagle Medium, HBSS: Hank's balanced salt solution