SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

"THE ANTICARCINOGENIC EFFECTS OF SECONDARY BILE ACIDS IN PANCREATIC ADENOCARCINOMA"

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Introduction

Pancreatic Cancers

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive cancer types. It is the 12th most common cancer and ranks 6th in cancer-related mortality worldwide. In 2022, 510,566 new cases and 467,005 deaths were reported. Patients are typically diagnosed at an advanced stage due to the lack of early diagnostic markers and the asymptomatic nature of the disease in its early stages. The 5-year survival rate of pancreatic cancer patients is approximately 10%. The high metastatic potential and resistance to chemotherapy contribute to the poor prognosis, highlighting the critical need for a better understanding of PDAC pathogenesis.

Pancreatic Adenocarcinoma

Pancreatic adenocarcinoma develops from non-invasive precancerous neoplasms, which are classified as low- or high-grade based on the degree of epithelial dysplasia. These lesions are curable if detected and treated early. The most common precursor of invasive PDAC is pancreatic intraepithelial neoplasia (PanIN), a microscopic lesion found in small pancreatic ducts. Risk factors for developing the disease include smoking, obesity, poor dietary habits, diabetes, and chronic pancreatitis.

PDAC often arises in the background of chronic pancreatitis and is associated with an inflammatory microenvironment. There is substantial evidence supporting that inflammation in pancreatic tissue expressing oncogenic KRAS (Kirsten rat sarcoma viral oncogene homolog) accelerates tumor progression and promotes the emergence of neoplastic precursor lesions such as acinar-to-ductal metaplasia (ADM) and PanIN. Mutations resulting in the active form of KRAS occur in more than 95% of PDACs and are widely believed to contribute to tumor development.

The combination of surgical resection and systemic chemotherapy remains the only hope for long-term survival or cure in non-metastatic pancreatic cancer patients. In 1997, the nucleoside analogue gemcitabine was introduced for treating PDAC. Later, several

chemotherapeutic agents, including topoisomerase inhibitors (irinotecan), nucleoside analogues (capecitabine), platinum compounds (oxaliplatin), and antimetabolites (5-fluorouracil), were approved for PDAC treatment. However, these options did not provide a significant survival advantage over gemcitabine monotherapy. In 2011, the combination therapy FOLFIRINOX (leucovorin, 5-fluorouracil, irinotecan, and oxaliplatin) showed substantial improvement in patient survival compared to gemcitabine alone. This treatment, however, causes notable side effects, including diarrhea, nausea, fatigue, myelosuppression, and neuropathy, which can only be partially controlled with medications.

The improved survival benefit observed with combination therapy over single-agent gemcitabine suggests that combined chemotherapeutic regimens offer a viable treatment option for patients with pancreatic cancer. Nevertheless, due to their toxicity, these treatments are suitable only for patients in generally good health.

Dysbiosis

The human microbiota is the community of commensal, symbiotic, and pathogenic microorganisms—such as viruses, fungi, and bacteria—that inhabit the surface and interior of the human body. The human microbiome refers to these microorganisms and their collective genome. The study of the microbiome has significantly advanced our understanding of the microbiota's impact on health at every stage of life. Of the approximately ~10^{12} microbial species on Earth, the International Association for Cancer Registries (IACR) has classified 11 as “human carcinogens” or “oncogenic microbes.” Some of these microbes, such as *Fusobacterium nucleatum* and various *Salmonella* strains, possess virulence factors that promote tumor formation through the E-cadherin–Wnt–β-catenin signaling pathway and are responsible for an estimated 2.2 million cancer cases annually.

The gut microbiota consists of over 1,500 species, classified into more than 50 different phyla. The dominant phyla in the gut are Bacteroidetes, Firmicutes, Proteobacteria, Fusobacteria, Tenericutes, Actinobacteria, and Verrucomicrobia, collectively accounting for 90% of the human microbial population.

The microbiota exists in symbiosis with the host and influences physiological development from early life. It plays a crucial role in digestion, absorption of micronutrients, and synthesis of vitamins and bile acids. The microbiota affects numerous physiological processes, including immune homeostasis, maintenance of intestinal barrier function, adipose tissue regulation, metabolism, blood pressure, glucose homeostasis, and coagulation risks.

Several factors influence the composition of the gut microbiome, such as age, hygiene, diet, use of pre- and probiotics or antibiotics, alcohol consumption, and smoking, as well as invasion by certain pathogens. Alterations in microbiome composition are referred to as dysbiosis. Gastrointestinal dysbiosis can trigger pathological processes, including chronic inflammation and DNA damage caused by oxidative stress, leading to genomic instability. Dysbiosis specifically associated with cancer is called "oncobiosis," and the altered microbiome is referred to as the "oncobiome."

Microbial dysbiosis—i.e., the imbalance of the gut flora—is most frequently associated with cancers of organs colonized by bacteria, such as the stomach, intestines, or oral cavity. However, the impact of the microbiome is not limited to these organs; bacterial metabolites can reach distant organs through the bloodstream, where they may promote or inhibit tumor formation. Some bacterial metabolites are by-products of normal bacterial metabolism—e.g., phenols, indoles, hydrogen sulfide, or other aromatic amines—which can cause chronic inflammation, DNA damage, or epigenetic modifications in host cells over time. Additionally, some metabolites become biologically active only after further modification by the host's enzymatic systems, often exerting even stronger biological effects.

Secondary Bile Acids

The gut microbiome has significant biosynthetic capacity, producing various metabolites that act locally or reach distant tumor cells via the bloodstream and influence their behavior. Among these metabolites are secondary bile acids, such as lithocholic acid (LCA), deoxycholic acid (DCA), and ursodeoxycholic acid (UDCA), formed from primary bile acids by gut bacteria. The amount of synthesized bile acid is regulated at the transcriptional level, where bile acids inhibit further synthesis by binding to the farnesoid X receptor (FXR).

Bile acid production consumes about 500 mg of cholesterol per day, making it a key mechanism for regulating cholesterol levels in the human body. Following a meal, bile acids are released into the small intestine through the bile ducts under the influence of the hormone cholecystokinin (CCK), where they aid in fat digestion, micelle formation, and the

emulsification and solubilization of fat-soluble nutrients and vitamins (A, D, E, K1), as well as maintaining normal cholesterol levels. The bile acids are then absorbed in the ileum by enterocytes and transported back to the liver via the portal circulation, a process known as enterohepatic circulation.

Approximately 500 mg of bile acids exit the enterohepatic cycle daily and are converted into secondary bile acids through various reactions. Bile acids not only participate in digestion but also function as signaling molecules. They can activate membrane receptors such as the G-protein-coupled bile acid receptor (GPBAR1, also known as TGR5), sphingosine-1-phosphate receptor 2 (S1PR2), and muscarinic receptors (CHRM2 and CHRM3). They also bind to nuclear receptors including farnesoid X receptor (FXR, NR1H4), pregnane X receptor (PXR, NR1H2), vitamin D receptor (VDR, NR1H1), constitutive androstane receptor (CAR, NR1H3), and liver X receptors (LXR, NR1H2-3). Through these receptors, bile acids influence immune responses, gastrointestinal mucosal barrier function, pregnancy, carcinogenesis, and metabolic diseases. Activation of bile acid receptors triggers various signaling pathways that regulate glucose, lipid, and energy homeostasis and play critical roles in cancer development.

Cell Proliferation in PDAC

The expression of proteins and signaling pathways that regulate the growth of healthy, normally functioning cells is tightly controlled to maintain tissue structure and function. Malignant cells evade these regulatory mechanisms and maintain constitutive growth signals. Sustained proliferative signaling is made possible by the emergence of somatic mutations, commonly resulting in constant activation of signaling pathways such as MAPK, PI3K, and AKT. In PDAC, somatic mutations in the KRAS gene occur in approximately 90% of cases. Numerous studies have demonstrated that mutations in genes involved in the MAPK signaling pathway influence cellular processes such as growth, differentiation, migration, and apoptosis, and play key roles in the initiation and progression of cancers, including pancreatic adenocarcinoma.

In a significant proportion of tumors, including PDAC, tumor suppressor genes are mutated. Mutations in the p53 protein increase the resistance of PDAC cells to chemotherapeutic agents such as gemcitabine. Recent research also indicates that elevated PTEN expression and inhibition of the PI3K/Akt signaling pathway are associated with poorer clinical outcomes and worse prognosis in PDAC.

Epithelial-Mesenchymal Transition (EMT) in PDAC

Most adult tissues and organs are formed through repeated cycles of epithelial–mesenchymal transition (EMT) and its reverse process, mesenchymal–epithelial transition (MET). These cycles are classified as primary, secondary, and tertiary EMT. Primary EMT occurs during early embryonic development, such as implantation. Secondary EMT is involved in wound healing, tissue regeneration, and fibrosis, while tertiary EMT is characteristic of tumors, contributing to stemness, drug resistance, immune evasion, and metastasis.

Epithelial cells form tight junctions with neighboring cells and establish an apico-basal polarity through the sequential organization of adherens junctions, desmosomes, and tight junctions. In contrast, mesenchymal cells are loosely arranged in a three-dimensional extracellular matrix and form connective tissues. Key steps in the EMT process include: 1) loss of cell–cell and cell–basement membrane contacts; 2) loss of apico-basal polarity; and 3) activation of genes that define the mesenchymal phenotype.

Genes crucial in EMT induction include members of the SNAIL transcriptional repressor family (SNAIL1 and SNAIL2, also known as Snail and Slug), the zinc-finger E-box-binding ZEB family, and the TWIST protein family. Members of the WNT protein family inhibit the expression of epithelial marker genes (such as E-cadherin, claudins 1-7 (CLDN1-7), and zonula occludens 1 (ZO-1 or TJP1)), leading to the breakdown of tight junctions. Receptor tyrosine kinases involved in EMT induction include transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), epidermal growth factor (EGF), and hepatocyte growth factor (HGF) families.

The tertiary EMT process, characteristic of tumors, has also been observed in PDAC. EMT activation is a critical step in PDAC progression, enabling cancer cells to acquire increased motility and invasive capabilities. The mesenchymal phenotypic traits acquired during EMT, such as elongated morphology and directed cell migration, contribute to early tumor dissemination, including in PDAC. During the neoplastic EMT process, primary tumor cells can enter the bloodstream via intravasation and form metastases.

Oxidative/Nitrosative Stress in PDAC

Over the past decade, our understanding of oxidative stress has significantly expanded. It is now seen not just as an imbalance between pro- and antioxidant molecules but as a complex process rooted in genetic mechanisms and gene expression regulation. Central to this new

understanding is the transcription factor NRF2 (nuclear factor erythroid 2-related factor 2), a key regulator of the human antioxidant response.

In cancer cells, redox homeostasis is disrupted, closely tied to NRF2 function. Under normal conditions, NRF2 forms a complex with Kelch-like ECH-associated protein 1 (KEAP1) in the cytoplasm. This NRF2/KEAP1 complex prevents NRF2 from translocating to the nucleus and initiating transcription of its target genes, such as NAD(P)H quinone dehydrogenase 1 (NQO1), heme oxygenase 1 (HO-1), glutathione peroxidase (GPX), and catalase (CAT).

Reactive oxygen and nitrogen species are naturally produced in physiological processes and are essential in signal transduction. However, disruption of the pro-/antioxidant balance can trigger neoplastic processes. Increased free radical production and reduced antioxidant response lead to oxidative stress, causing damage to vital molecules, eventually resulting in necrosis or apoptosis.

Excessive ROS production can cause genomic and/or mitochondrial DNA damage, somatic mutations, activation of oncogenes, inhibition of tumor suppressor genes, and alterations in metabolic and signaling pathways—all of which contribute to cellular damage, disease development, and carcinogenesis.

In the pancreas, KRAS expression induces NRF2 activation, which in turn promotes cancer cell proliferation. In PDAC cases with KRAS mutations, abnormal NRF2 pathway activation is frequently observed and is associated with tumor progression. Thus, NRF2 represents a potential therapeutic target in the development of new strategies for treating PDAC.

Stemness in PDAC

Normal stem cells can be found in embryonic, fetal, and adult tissues. They represent a class of undifferentiated cells with high self-renewal and proliferative capacity, capable of differentiating into one or more cell lineages.

Recent studies support the role of cancer stem cells (CSCs), also referred to as tumor-initiating cells, in tumor development, metastasis formation, chemoresistance, and tumor recurrence. In various tumor types, CSC markers have been identified to isolate and characterize cancer stem cell populations. Membrane glycoproteins (such as CD44, CD24, and CD133) are commonly used to identify pancreatic CSCs. Additional research has shown that the enzyme aldehyde dehydrogenase 1 (ALDH1) can also be used to identify pancreatic cancer stem cells.

In pancreatic cancer, CSCs play a central role in the development of drug resistance, tumor aggressiveness, and metastasis formation. In PDAC, the expression of several stem cell marker genes (e.g., CD24, CD44) is elevated in high-grade dysplasias. Currently available drugs are effective in destroying the bulk of tumor cells but often leave CSCs untouched, leading to recurrence and the formation of metastases. Therefore, there is a critical need to develop new approaches for understanding the molecular mechanisms underlying stemness in order to create targeted therapies.

Cell Metabolism in PDAC

To sustain their unlimited proliferation and metastatic potential, malignant cells require a sufficient supply of energy. PDAC is an extremely aggressive tumor type, characterized by hypovascularization and a desmoplastic reaction, resulting in a tumor microenvironment that is nutrient-deprived and highly hypoxic. Tumor cells undergo metabolic reprogramming, where aerobic glycolysis (the Warburg effect) predominates, characterized by increased glucose uptake and elevated lactate production. This metabolic reprogramming is crucial for the survival and growth of tumor cells. Enhanced aerobic glycolysis, reduced oxidative phosphorylation (OXPHOS), and increased lactic acid production are hallmarks of malignant tumors.

In PDAC, glycolysis promotes rapid tumor cell growth, and the enzymes and intermediates of glycolysis also participate in the regulation of metastasis formation. The reduced efficiency of OXPHOS further contributes to the dominance of glycolysis. Mitochondrial dysfunction may result from mitochondrial DNA mutations, errors in the electron transport chain, and increased ROS production, all of which can activate oncogenic pathways and promote the expression of glycolytic enzymes. In PDAC cells, there is a marked increase in mitochondrial DNA mutations, associated with decreased oxygen consumption and increased glycolysis. This metabolic reprogramming contributes to the development of drug resistance in malignant tumors, including pancreatic cancer. Understanding the metabolic features and energy regulation of PDAC cells may help in the development of new treatment strategies to overcome drug resistance.

Objectives

In the context of the microbiome–cancer relationship, the microbiome can influence tumor behavior through the secretion of bacterial metabolites. Once in circulation, these bacterial metabolites may reach distant tumor cells and affect their function, essentially exerting hormone-like effects.

Among such bacterial metabolites are secondary bile acids, such as lithocholic acid (LCA) and ursodeoxycholic acid (UDCA). In my research, I examined how secondary bile acids affect the function of pancreatic cancer cells through the analysis of classical cancer hallmarks.

The main goal of my study was to identify the mechanisms by which bile acids exert antitumor effects on pancreatic ductal adenocarcinoma (PDAC) cells.

The following research questions were addressed:

- How do bile acids affect tumor cell growth and the epithelial–mesenchymal transition (EMT) process?
- Do bile acids influence the redox homeostasis of tumor cells?
- Can correlations be identified between patient survival in pancreatic adenocarcinoma and the expression of antioxidant genes using *in silico* analysis?
- What effects do bile acids have on cancer stemness?
- Do bile acids affect the energy metabolism of tumor cells?
- Which receptors mediate the effects of bile acids in pancreatic adenocarcinoma cells?
- Can bile acids influence the efficacy of chemotherapeutic drugs commonly used in PDAC treatment?

Materials and Methods

Reagents

The bile acids used in our experiments—lithocholic acid (LCA, cat # L6250; Sigma-Aldrich, St. Louis, MI, USA) and ursodeoxycholic acid (UDCA, cat # U5127; Sigma-Aldrich)—were dissolved in dimethyl sulfoxide (DMSO, cat # D8418; Sigma-Aldrich) to prepare 100 mM stock solutions. LCA was used at a concentration of 0.03 μM and UDCA at 0.3 μM, corresponding to the concentrations found in human serum [10], [229], [230]. Control cells were treated with culture medium containing 0.001% DMSO.

Reduced glutathione (GSH; cat # G4251; Sigma-Aldrich) was used at a final concentration of 5 mM. Pegylated catalase (pegCAT; cat # C4963; Sigma-Aldrich) was used at a concentration of 500 U/ml.

Bile acid receptor antagonists—NF449 (cat # 1391), a Gsα-selective antagonist [231]; CINPA1 (cat # 5605), a constitutive androstane receptor (CAR) antagonist [232]; DY268 (cat # 5656), a farnesoid X receptor (FXR) antagonist [233]; and GSK2033 (cat # 5694), a liver X receptor (LXR) antagonist [234]—were purchased from Tocris Bioscience (Bristol, UK) and used at a final concentration of 5 μM. Ketoconazole (cat # K0600000), an inhibitor of pregnane X receptor (PXR) signaling [235], was obtained from Sigma-Aldrich and also used at a final concentration of 5 μM.

siRNAs targeting the TGR5 G-protein-coupled bile acid membrane receptor (GPBAR1-siRNA ID: s195791), vitamin D receptor (VDR/NR1I1-siRNA ID: s14777), FXR receptor (NR1H4-siRNA ID: s19371), and negative control siRNA (cat # 4390843) were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and used at a final concentration of 30 nM.

The chemotherapeutic agents 5-fluorouracil (5FU, cat # F6627) and oxaliplatin (OXA, cat # O9512) were purchased from Sigma-Aldrich and dissolved in DMSO to a stock concentration of 100 mM. The highest concentrations used were 300 μM for 5FU and 19.2 μM for oxaliplatin.

Cell Lines and Cell Culture

The human pancreatic ductal adenocarcinoma cell lines Capan-2, BxPC-3, and PancTu-1 were obtained from the American Type Culture Collection (ATCC).

Capan-2 cells were cultured in MEM (cat # M8042; Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS, cat # F2442; Sigma-Aldrich), 1% penicillin/streptomycin (cat # P4333; Sigma-Aldrich), and 2 mM glutamine (cat # G7513; Sigma-Aldrich) at 37°C in a 5% CO_2 incubator.

BxPC-3 and PancTu-1 cells were cultured in RPMI 1640 medium (cat # R5886; Sigma-Aldrich) containing 10% FBS, 2 mM glutamine, and 1% penicillin/streptomycin, also at 37°C in a 5% CO_2 incubator.

Human primary fibroblast cells were cultured in DMEM (cat # D5546; Sigma-Aldrich) supplemented with 20% FBS, 1% penicillin/streptomycin, and 2 mM L-glutamine at 37°C in a 5% CO_2 incubator.

Cell Viability Assay (MTT)

Capan-2 cells were seeded in 96-well plates (3000 cells/well) in 200 µl of culture medium. On the following day, after cell adhesion, they were treated with LCA (0.03 µM–66 µM), UDCA (0.3 µM), or DMSO (as a control). After 48 hours of treatment, cell viability was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; cat # A2231.000; VWR).

MTT solution (20 µl of 5 mg/ml) was added to the cells and incubated at 37°C for 1.5 hours. After removing the supernatant, the formazan crystals were dissolved in 100 µl DMSO per well, and absorbance was measured at 540 nm using a spectrophotometer (Thermo Labsystems Multiskan MS, Waltham, MA, USA).

Sulforhodamine B Assay

To study cell proliferation, sulforhodamine B (SRB, cat # 230162; Sigma-Aldrich) staining was used. For the assay, Capan-2 cells were cultured in 96-well plates (3000 cells/well) in 200 µl of cell culture medium. On the day following cell adhesion, the cells were treated with LCA (0.03 µM) or UDCA (0.01–1 µM), with DMSO (0.001%) used as the control. After 48 hours of treatment, the cells were fixed with 10% final concentration trichloroacetic acid (TCA, cat # T6399; Sigma-Aldrich) for 1 hour at 4°C. Cells were then washed five times with distilled water and stained with 0.4% SRB solution (prepared in 1% acetic acid) for 10 minutes. Unbound dye

was removed using 1% acetic acid. Bound dye was dissolved in 100 µl of 10 mM Tris base per well, and absorbance was measured at 540 nm.

Detection of Cell Death

To evaluate changes in necrotic and apoptotic cell death, Annexin V/PI dual staining was performed (cat # V13242; Thermo Fisher Scientific). Capan-2 cells were seeded in 6-well plates (150,000 cells/well) and treated with UDCA (0.3 µM) for 48 hours. The cells were then stained with 100 µg/ml PI solution and 5 µl FITC Annexin V for 15 minutes at room temperature. The number of apoptotic and necrotic cells was determined using a FACS Calibur flow cytometer (Beckton Dickinson, Franklin Lakes, NJ, USA).

Cell Invasion Assay

Cell invasion assays were conducted using Corning BioCoat Matrigel invasion chambers (cat # 354480; Corning, NY, USA) with 8.0 µm PET membranes in 24-well plates. Capan-2 cells (20,000 cells/well) were seeded in serum-free medium in the upper chamber and incubated overnight. The following day, the cells were treated with LCA (0.03 µM) or UDCA (0.3 µM) in 0.5 ml of serum-free medium. The lower chamber contained 0.75 ml of medium supplemented with serum, LCA and/or UDCA, and 100 ng/ml SDF1-alpha (cat # SRP4388; Sigma-Aldrich) as chemoattractant.

After 48 hours of treatment, non-migrating cells were removed from the upper side of the membrane using PBS. Cells that had migrated to the underside of the membrane were fixed with 100% methanol and stained with 4',6-diamidino-2-phenylindole (DAPI). Migrated cells were counted using the Opera Phoenix High Content Screening System, and images were analyzed using Harmony 4.6 Software.

The invasion index was calculated based on the ratio of cells that passed through the Matrigel membrane compared to control membranes using the following formulas:
% Invasion = (average number of cells that passed through the Matrigel membrane / average number of cells that passed through the control membrane) × 100 Invasion Index = % invasion of treated cells / % invasion of control (untreated) cells.

Western Blot

For cell lysis, we used RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF, and a protease inhibitor cocktail). Protein concentrations were determined spectrophotometrically using BCA reagent (cat # A65453; Pierce Biotechnologies, Rockford, IL, USA). Proteins (20 μg per lane) were separated by SDS-PAGE on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Subsequently, any remaining free protein-binding sites on the nitrocellulose membrane were blocked with a 5% BSA solution (in 1× TBS-Tween buffer) for 1 hour at room temperature. The membranes were then incubated overnight at 4 °C with primary antibodies under constant shaking. Next, the membranes were washed three times for 10 minutes each with 1× TBS-Tween buffer, followed by a 1-hour incubation at room temperature under constant shaking with IgG HRP-conjugated secondary antibodies. Finally, the membranes were washed three times for 10 minutes each with 1× TBS-Tween buffer. Antibody binding was detected by chemiluminescence using the ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA, USA). Band intensities were quantified by densitometry using Image Lab 6.1 software. β-actin was used for normalization.

Aldefluor Assay

The activity of the aldehyde dehydrogenase (ALDH1) tumor stem cell marker gene in bile acid-treated cells was measured using Aldefluor staining (cat # 01700; StemCell Technologies, Vancouver, Canada) [113]. Capan-2 cells were cultured in 6-well plates (100,000 cells/well), and on the day after seeding, they were treated with LCA (0.03 μM) or UDCA (0.3 μM) for 48 hours. The cells were then incubated in 0.5 ml Aldefluor assay buffer containing the ALDH substrate (5 μl/ml) for 45 minutes at 37°C. As a negative control, cells were treated with 5 μl diethylamino-benzaldehyde (DEAB; 50 mmol/l), a specific ALDH inhibitor. The percentage of ALDH-positive cells was determined by flow cytometry, and the analysis was performed using Flowing Software 2.5.1.

RNA Isolation, Reverse Transcription, and Quantitative RT-PCR

Capan-2 cells were seeded in 6-well plates (100,000 cells/well), and treated with LCA (0.03 μM) the day after attachment. Following a 48-hour incubation, total RNA was isolated using TRIzol reagent (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. RNA purity and concentration were determined using a NanoDrop1000 spectrophotometer (Thermo Labsystems Multiskan MS, Waltham, MA, USA). RNA samples were treated with DNase I enzyme (cat #10104159001; Merck), and cDNA synthesis was performed using the High Capacity Reverse Transcription Kit (cat #4368814; Applied Biosystems, Waltham, MA, USA), following the manufacturer's protocol.

The qPCR reactions were performed in a final volume of 10 μl containing 20 ng of cDNA template, 500 nM of each primer (see Table 4), and qPCRBIO SyGreen Lo-ROX Supermix (PCR Biosystems Ltd., London, UK). Reactions were carried out using a LightCycler 480 detection system (Roche, Basel, Switzerland). For normalization of mRNA expression levels, the geometric mean of 36B4 and cyclophilin (CYCLO) gene expression was used.

Mitochondrial Oxidation and Glycolysis

To monitor oxygen consumption and extracellular acidification in real time, we used a Seahorse XF96 oximeter (Agilent Technologies, Santa Clara, CA, USA). The oxygen consumption rate (OCR), which characterizes mitochondrial respiration, and the extracellular acidification rate (ECAR), an indicator of pH change and glycolysis, were measured in LCA- and UDCA-treated Capan-2 cells using the Seahorse system.

Cells were cultured overnight in a 96-well Seahorse plate (5000 cells/well), then treated with bile acids (LCA [0.03 μM]; UDCA [0.3 μM]) for 48 hours. After treatment, cells were incubated in pre-warmed XF Seahorse medium (cat # 103334-100) at 37°C for 1 hour in a CO_2-free incubator. Baseline OCR was recorded five times at five-minute intervals. The following inhibitors were used during the experiments: Etomoxir (50 μM), a CPT-1 inhibitor blocking fatty acid oxidation; Oligomycin (10 μM), an ATP synthase inhibitor; Antimycin (10 μM), an inhibitor of mitochondrial respiratory complex III.

Each OCR value was measured five times over five-minute intervals. OCR and ECAR values were normalized to protein content (as described in section 5.4, SRB assay), and normalized values were used in calculations.

Basal respiration was defined as baseline OCR minus antimycin-resistant respiration (baseline – antimycin). The etomoxir-resistant OCR (etomoxir – antimycin) was interpreted as oxygen consumption linked to glucose and amino acid oxidation. Fatty acid oxidation was calculated from the etomoxir-sensitive OCR (baseline – etomoxir). Oligomycin-resistant respiration (oligomycin – antimycin) reflected proton leak, while the oligomycin-sensitive fraction (baseline – oligomycin) was defined as ATP-linked respiration.

Gene Silencing

For transient transfection, Capan-2 cells were treated with 30 nM of siRNAs specific to TGR5, VDR, FXR, or a negative control siRNA. Transfections were performed using Lipofectamine RNAiMAX (cat # 13778150; Thermo Fisher Scientific) for 48 hours, with or without LCA treatment.

Measurement of Mitochondrial Membrane Potential

To measure mitochondrial membrane potential, cells were stained with DioC6 (3,3′-Dihexyloxacarbocyanine iodide) (cat # HY-D0084; MedChemExpress). Capan-2 cells were seeded in 6-well plates (150,000 cells/well) and, after 48 hours of treatment with UDCA (0.3 μM), were stained with 40 nM DioC6 for 30 minutes. Cells were then washed with PBS and collected using trypsin for flow cytometric analysis (FACS Calibur, BD Biosciences). Control cells were treated with 10 μM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) to uncouple mitochondrial membrane potential. FCCP-treated values were subtracted from all experimental groups.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism 8.0.1 software. Results are expressed as mean ± SEM. Normality was tested using the D'Agostino–Pearson test. Paired *t*-tests were used to compare bile acid- and vehicle-treated samples. For comparisons involving

multiple groups, one-way or two-way ANOVA was performed with Dunnett's or Tukey's post hoc test. Nonlinear regression and IC_{50} values were determined using GraphPad's "[Inhibitor] vs. response-variable slope (four parameters)" tool.

Results

1. Investigating the Effects of Lithocholic Acid in Pancreatic Adenocarcinoma Cells

LCA Inhibits the Proliferation of Pancreatic Adenocarcinoma Cells

We first examined whether LCA influences the proliferation of Capan-2 cells. The cells were treated with varying concentrations of LCA (0.003 μM–66 μM), and cell viability was assessed using the MTT assay. LCA significantly reduced the viability of Capan-2 cells at concentrations including those found in human serum (0.01–0.03 μM). Similarly, using the sulforhodamine B staining method, we found that LCA at 0.03 μM slowed the proliferation of Capan-2 cells. Importantly, within the tested concentration range (0.01 μM–10 μM), LCA did not affect the proliferation of non-transformed human primary fibroblasts, suggesting that the effects of LCA are specific to PDAC tumor cells.

LCA Reduces the Expression of EMT Marker Genes and Cell Invasion in Pancreatic Adenocarcinoma Cells

Next, we investigated whether LCA can modulate the epithelial–mesenchymal transition (EMT) process. Our results showed that LCA significantly decreased the expression of mesenchymal markers involved in EMT, including the Snail protein in Capan-2 cells and β-catenin protein expression in both Capan-2 and BxPC-3 cells. The epithelial marker ZO1 protein level increased in Capan-2 cells following LCA treatment. Interestingly, LCA reduced the expression of Claudin-1, a tight junction protein, in both examined cell lines. In line with these findings, cell invasion assays demonstrated that LCA-treated cells exhibited significantly reduced invasive capacity compared to DMSO-treated controls. Overall, these results suggest that LCA may inhibit the EMT process by downregulating key EMT-associated proteins and reducing the invasive potential of pancreatic adenocarcinoma cells.

High Antioxidant Expression Levels Are Associated with Poor Prognosis in Pancreatic Adenocarcinoma Patients

We examined the expression levels of several antioxidants (NRF2, GPX2, SOD1, SOD2) and NRF2 target genes (NQO1, HMOX1, TXN) using data from the TCGA/GTEx pancreatic adenocarcinoma (PAAD) database, including 179 tumor and 171 normal tissue samples. Interestingly, the expression of both antioxidants and NRF2 target genes was elevated in pancreatic adenocarcinoma compared to normal tissues. Furthermore, high antioxidant expression was associated with poorer overall survival in patients. These findings suggest that elevated antioxidant expression is a characteristic feature of pancreatic adenocarcinoma and may contribute to unfavorable disease outcomes.

LCA-Induced Oxidative/Nitrosative Stress Contributes to the Downregulation of EMT-Related Genes in Pancreatic Adenocarcinoma Cells

As previously observed, antioxidant genes are overexpressed in pancreatic adenocarcinoma tissues compared to normal tissues, and this overexpression correlates with reduced patient survival. In an earlier study, we demonstrated that LCA induces cytostasis via oxidative stress in breast cancer. Here, we investigated how LCA affects oxidative stress pathways in pancreatic adenocarcinoma cells. LCA treatment reduced NRF2 protein expression in Capan-2 pancreatic adenocarcinoma cells, similar to our findings in breast cancer cells. NRF2 is a key regulator of cellular antioxidant defense mechanisms. Additionally, LCA treatment increased the levels of 4HNE protein adducts, indicating enhanced lipid peroxidation. The expression of inducible nitric oxide synthase (iNOS) was also elevated, suggesting an increase in nitric oxide (NO) production. The reaction between NO and superoxide generates peroxynitrite, a highly reactive and damaging molecule capable of modifying aromatic amino acids. Consistently, LCA treatment led to elevated nitrotyrosine levels in cells, indicative of peroxynitrite formation. Furthermore, our results showed that antioxidants such as reduced glutathione (GSH) and pegylated catalase (pegCAT) prevented the LCA-induced downregulation of β-catenin and Snail expression. Collectively, these findings suggest that LCA-induced oxidative/nitrosative stress plays a key role in the suppression of EMT in pancreatic adenocarcinoma cells.

LCA Reduces the Expression of Cancer Stem Cell Markers in Pancreatic Adenocarcinoma Cells

We further investigated the effect of LCA on stemness by analyzing the expression of selected cancer stem cell markers. LCA treatment significantly reduced the protein level of aldehyde dehydrogenase 1 (ALDH1) and decreased the number of ALDH-positive Capan-2 cells. Consistently, the expression of CD133 protein also declined following LCA treatment.

Together, these findings suggest that LCA may reduce the proportion of cancer stem cells within pancreatic adenocarcinoma cell populations.

LCA Induces Mitochondrial Oxidative Phosphorylation in Pancreatic Adenocarcinoma Cells

To assess metabolic changes in LCA-treated Capan-2 cells, we utilized a Seahorse XF96 analyzer. This instrument simultaneously measures the oxygen consumption rate (OCR), indicative of mitochondrial respiration, and the extracellular acidification rate (ECAR), reflective of glycolysis. Our experiments showed that LCA significantly increased basal respiration, etomoxir-sensitive respiration (fatty acid oxidation), etomoxir-resistant respiration (glucose and amino acid oxidation), and oligomycin-sensitive ATP-linked respiration. LCA did not significantly affect the oligomycin-resistant fraction of mitochondrial respiration, which represents ATP-independent oxygen consumption. Moreover, glycolytic activity (ECAR) remained unaffected by LCA treatment in Capan-2 cells. These findings suggest that LCA plays a critical role in enhancing mitochondrial oxidative metabolism in pancreatic adenocarcinoma cells.

LCA-Induced Effects Are Mediated Through the Nuclear Receptors FXR, CAR, and VDR

Bile acids are known to activate nuclear receptors such as FXR, CAR, PXR, LXR, and VDR, as well as membrane-bound receptors like TGR5. To identify the receptors responsible for mediating the effects of LCA, we first employed receptor antagonists and/or inhibitors, including CINPA1 (CAR receptor antagonist), DY268 (FXR receptor antagonist), NF449 (inhibitor of TGR5 downstream signaling), GSK2033 (LXR receptor antagonist), and ketoconazole (PXR receptor antagonist). The LCA-induced reduction in cell invasion was abolished by treatment with CINPA1 and DY268, whereas other antagonists had no significant effect. Both DY268 and CINPA1 prevented the LCA-induced downregulation of β-catenin and NRF2 expression.

To gain a more comprehensive understanding, we performed siRNA-mediated knockdown of the receptors in Capan-2 cells. In addition, the vitamin D receptor (VDR), another known

target of LCA, was included in these experiments. Knockdown of FXR and VDR prevented the LCA-induced decrease in β-catenin protein levels. In contrast, silencing of TGR5 did not affect the cellular responses to LCA. These findings suggest that the effects of LCA in pancreatic adenocarcinoma cells are mediated predominantly through the nuclear receptors CAR, FXR, and VDR.

2. Investigation of the Effects of Ursodeoxycholic Acid in Pancreatic Adenocarcinoma Cells

Ursodeoxycholic Acid Reduces the Proliferation of Pancreatic Adenocarcinoma Cells

Capan-2 cells were treated with various concentrations of UDCA (0.01 μM, 0.03 μM, 0.1 μM, 0.3 μM, and 1 μM), followed by sulforhodamine B (SRB) staining. UDCA inhibited the proliferation of Capan-2 cells in a concentration- and time-dependent manner. A significant antiproliferative effect was observed at 0.3 μM, which corresponds to physiological concentrations of UDCA in human serum. Based on these results, this concentration was used in subsequent experiments.

To determine whether the observed reduction in proliferation was due to UDCA-induced cytotoxicity, we assessed cell viability and death. UDCA did not increase the proportion of propidium iodide (PI)-positive, Annexin V-FITC/PI double-positive (necrotic), or Annexin V-FITC-positive (apoptotic) cells at any of the tested concentrations, indicating that the antiproliferative effect was not a result of cytotoxicity.

Ursodeoxycholic Acid Inhibits the EMT Process in Capan-2 Cells

After confirming that UDCA exhibits cytostatic effects on pancreatic adenocarcinoma cells, we investigated its influence on other cancer hallmarks. UDCA treatment decreased the protein expression of key mesenchymal markers involved in epithelial–mesenchymal transition (EMT), including β-catenin, Snail, Slug, and Vimentin. Conversely, the expression of epithelial markers ZO1 and E-cadherin was upregulated upon UDCA treatment. Interestingly, similar to LCA, UDCA reduced the expression of Claudin-1, a tight junction protein. Notably, low Claudin-1 expression has been associated with improved survival in pancreatic adenocarcinoma patients. In line with the downregulation of EMT-associated genes, UDCA also reduced the invasive potential of Capan-2 cells.

UDCA Effects Are Reproducible in Other Human PDAC Cell Lines but Absent in Normal Human Fibroblasts

The effects of UDCA were further evaluated in two additional pancreatic adenocarcinoma cell lines, BxPC-3 and PancTu-1. UDCA treatment reduced the expression of the mesenchymal marker Snail in both cell lines and significantly increased E-cadherin expression in PancTu-1 cells, consistent with our findings in Capan-2 cells. Importantly, UDCA had no effect on the expression of the mesenchymal marker Slug in normal human fibroblast cells. These results suggest that UDCA does not affect the EMT process in non-transformed, normal human cells, indicating that its effects are selective for PDAC cells.

Ursodeoxycholic Acid Induces Mitochondrial Activity

Altered cellular metabolism is a hallmark of cancer. To assess whether UDCA influences mitochondrial respiration, we performed Seahorse analysis to evaluate mitochondrial oxidative metabolism. Our results demonstrated that UDCA enhanced all measured components of mitochondrial respiration, including basal respiration, etomoxir-sensitive respiration (fatty acid oxidation), etomoxir-resistant respiration (glucose and amino acid oxidation), oligomycin-sensitive ATP-linked respiration, and oligomycin-resistant (non-ATP-linked) respiration. In addition, UDCA increased DioC6 fluorescence, indicating an elevation in mitochondrial membrane potential. Together with the absence of cell death induction, these observations suggest the presence of a more coupled and efficient mitochondrial system following UDCA treatment.

Ursodeoxycholic Acid Does Not Affect the Kinetic Properties of Chemotherapeutic Agents Used in PDAC Treatment

An increasing number of studies indicate that bacterial metabolites can influence the efficacy of therapeutic agents. In our experiments, we evaluated the combined effects of UDCA with chemotherapeutic agents commonly used in the treatment of pancreatic adenocarcinoma. We tested 5-fluorouracil (5-FU) and oxaliplatin (OXA) at various concentrations, both alone and in combination with UDCA (0.3 μM), and assessed their impact on PDAC cell viability.

Our results showed that UDCA did not alter the kinetic properties of these chemotherapeutic agents. Specifically, there were no significant changes in the IC50 values or Hill coefficients (reflecting cooperative binding or effect) in modulating Capan-2 cell proliferation. These findings suggest that UDCA does not interfere with the pharmacodynamic profile of 5-FU or OXA in this model.

Discussion

The Effects of Lithocholic Acid in Pancreatic Adenocarcinoma Cells

Cancer development is often accompanied by alterations in the microbiome—a phenomenon known as oncobiosis—which may promote tumor progression. The microbiome exerts its influence in part through the secretion of bacterial metabolites that affect tumor behavior. In this study, we identified lithocholic acid (LCA) as a bacterial metabolite with antitumor activity. Interestingly, bile acids were historically regarded as carcinogenic agents. However, recent studies have demonstrated that bile acids can also exert antitumor effects, and their pro- or anticarcinogenic properties depend on the specific bile acid involved—even in pancreatic adenocarcinoma (PDAC). For instance, chenodeoxycholic acid (CDCA) exhibits procarcinogenic features, whereas cholic acid (CA) and ursodeoxycholic acid (UDCA) show tumor-suppressive activity, and deoxycholic acid (DCA) has mixed effects. LCA has demonstrated antineoplastic effects in several malignancies beyond PDAC, including breast cancer, neuroblastoma, prostate cancer, hepatocellular carcinoma, gallbladder cancer, and nephroblastoma. These effects appear to be selective for neoplastic cells, as LCA does not affect non-transformed cells. Notably, in our study, LCA was used at concentrations equivalent to those found in human serum, which are significantly lower than the doses applied in previous studies.

The antineoplastic activity of LCA is multifaceted, encompassing cytostasis, inhibition of epithelial–mesenchymal transition (EMT), suppression of cancer stem cell properties, and induction of mitochondrial oxidative phosphorylation. We showed that LCA inhibits EMT by downregulating mesenchymal markers such as β-catenin and Snail, while increasing the expression of the epithelial marker ZO1. Furthermore, LCA reduced the expression of Claudin-1—a tight junction protein associated with disease progression in PDAC—similarly to mesenchymal markers. In line with EMT inhibition, LCA decreased the invasive capacity of

pancreatic cancer cells and promoted the loss of stem cell-like properties. These stem-like traits are closely linked to several tumor types, including PDAC.

Metabolic reprogramming is a hallmark of cancer, and PDAC is characterized by profound metabolic alterations, including changes in glycolysis, mitochondrial oxidative phosphorylation (OXPHOS), the Krebs cycle, lipid metabolism, and glutaminolysis. Despite this heterogeneity, suppression of mitochondrial OXPHOS is a common feature. Our study demonstrated that LCA treatment enhances mitochondrial oxygen consumption in PDAC cells, including fatty acid oxidation, glucose and amino acid oxidation, and ATP-linked respiration. Elevated mitochondrial respiration may reduce the availability of biosynthetic substrates and contribute to metabolic inflexibility, rendering cancer cells more vulnerable to nutrient fluctuations.

A key mechanism underlying the antitumor effects of LCA is its ability to disrupt redox homeostasis. We showed that LCA induces oxidative and nitrosative stress in PDAC cells by downregulating the expression of the transcription factor NRF2 and upregulating inducible nitric oxide synthase (iNOS). This mechanism plays a central role in EMT inhibition and may also contribute to reduced proliferation in other tumor models. Multiple studies have demonstrated the oncogenic role of NRF2 in PDAC. Consistent with this, ROS overproduction sensitizes PDAC cells to cell death. PDAC progression is frequently associated with mutations in the Keap1 gene, resulting in constitutive activation of NRF2. Elevated nuclear NRF2 expression correlates with reduced survival in PDAC patients. We confirmed that antioxidant genes are overexpressed in PDAC tissues and that high antioxidant expression is associated with poorer clinical outcomes.

Our experiments demonstrated that the antitumor effects of LCA in PDAC models are mediated by the nuclear receptors CAR, FXR, and VDR. VDR is highly expressed in pancreatic tumor cells and in the surrounding tumor stroma. Activation of VDR signaling improves therapeutic response and clinical outcomes in PDAC, and VDR also plays a role in suppressing cancer stem cell-like features. The role of FXR is more complex, as its impact on cell survival is somewhat controversial. The function of the CAR receptor in PDAC remains largely unexplored. Since bile acids can activate numerous receptors, similar signaling pathways may be involved in mediating LCA's effects in other cancer types (e.g., colorectal cancer). Additionally, other receptors may also be responsible for mediating cytostatic effects in distinct malignancies, such as TGR5 and CAR in breast cancer.

While our experimental data clearly show that LCA inhibits multiple cancer hallmarks in PDAC cell models, it remains uncertain how these effects translate to human pancreatic cancer. Bile acids have been associated with PDAC risk factors, including obesity, diabetes,

pancreatitis, and hypertriglyceridemia. For example, elevated secondary bile acid levels (e.g., DCA) have been reported in type 2 diabetes; ob/ob mice exhibit increased plasma bile acid concentrations; and total circulating bile acid levels are elevated in pancreatitis. In PDAC patients, plasma and serum levels of conjugated bile acids (most notably glycocholic acid) are elevated compared to healthy controls. Conversely, levels of unconjugated bile acids are reduced in the plasma of PDAC patients. However, in bile samples collected from the common bile duct (CBD), unconjugated bile acid levels are higher in PDAC patients than in those with benign disease. These increases may be due to the presence of hydroxylase-producing bacteria and CBD stones, which impair bile flow and promote bacterial overgrowth. Serum bile acid profiles may serve as biomarkers to distinguish PDAC patients from those with benign pancreatic conditions or healthy individuals.

To our knowledge, no studies to date have examined a direct link between bacterial LCA production and pancreatic cancer progression. It is worth noting that among secondary bile acids, LCA is present at the lowest concentrations in healthy human serum, making its detection challenging. In other cancer types, such as breast adenocarcinoma, we have previously shown that microbial LCA synthesis is reduced in early stages (stages 0–1), suggesting that low systemic LCA levels may contribute to tumorigenesis.

The Effects of Ursodeoxycholic Acid in Pancreatic Adenocarcinoma

In our experiments, we demonstrated that ursodeoxycholic acid (UDCA), a bacterial metabolite, exerts cytostatic effects in pancreatic ductal adenocarcinoma (PDAC) cell models. The beneficial effects of UDCA have been described in multiple cancer types, including glioblastoma, neuroblastoma, PDAC, prostate cancer, melanoma, hepatocellular carcinoma, oral squamous cell carcinoma, leukemia, gastric cancer, esophageal cancer, and colorectal cancer—consistent with the findings presented in this study. Importantly, in most previous studies, UDCA was applied at supraphysiological concentrations, often 2–4 orders of magnitude higher than the reference concentration found in human serum. However, our group and others have demonstrated that several bile acids, including UDCA, can exert biologically relevant effects even at low, near-physiological concentrations in various tumor models.

Among secondary bile acids, many can influence PDAC cell behavior—including UDCA (as shown here), DCA, and LCA—while in other malignancies, such as ovarian or breast cancer, only one specific secondary bile acid tends to be effective. In our study, UDCA treatment reduced the expression of key mesenchymal markers involved in EMT, including β-catenin,

Snail, Slug, and Vimentin, while increasing the expression of epithelial markers ZO1 and E-cadherin. UDCA also decreased the expression of the tight junction protein Claudin-1, whose lower tumor expression correlates with better survival outcomes in PDAC patients. Furthermore, UDCA significantly reduced the invasive capacity of Capan-2 cells.

We also showed that UDCA treatment induces mitochondrial oxidative phosphorylation. Increased OXPHOS activity indicates a metabolic shift toward mitochondrial energy production. This metabolic reprogramming was observed not only with UDCA but also with other bile acids and has been associated with both cytostasis and the induction of apoptosis. Mitochondrial metabolic changes are also known to play a central role in cancer stem cell reprogramming. These findings suggest that UDCA exerts multifaceted effects contributing to the reprogramming of PDAC cells toward a less aggressive phenotype. Metabolic reprogramming may therefore represent a promising therapeutic avenue in PDAC.

We also examined whether UDCA influences the efficacy of chemotherapeutic agents commonly used in PDAC treatment. Bile acids have been reported to act synergistically with anticancer drugs. In pediatric cancer patients receiving chemotherapy, elevated levels of circulating bile acids have been associated with faster recovery. The bile acid derivative tauroursodeoxycholic acid (TUDCA), used as a dietary supplement, improved recovery after 5-FU treatment in mice by attenuating ER stress responses. Microbial metabolites also play an important role in modulating response to therapy in PDAC.

The complex interaction between the microbiome and chemotherapy may involve:
a, bacterial metabolism of chemotherapeutic agents, which alters drug pharmacokinetics and efficacy; **b,** chemotherapy-induced shifts in microbiome composition, which in turn can impact treatment outcomes; and **c,** bacterial metabolites that modulate the activity of chemotherapeutic agents, in both PDAC and other malignancies.

For example, certain bacterial metabolites, such as indole derivatives, have been shown to enhance chemotherapy efficacy and modulate the activity of drugs like 5-fluorouracil, doxorubicin, and paclitaxel. UDCA has been shown to enhance apoptosis induced by DNA topoisomerase I inhibitors in several cancer cell lines. Furthermore, the combination of UDCA and the COX-2 inhibitor celecoxib reduced the growth of colorectal cancer cells. Interestingly, UDCA also demonstrated synergistic effects with the multikinase inhibitor sorafenib in hepatocellular carcinoma cells.

Overall, we demonstrated that UDCA, at concentrations equivalent to those found in human serum, exerts beneficial effects on PDAC cells without interfering with chemotherapy. This avoids the potential risks associated with high-dose supraphysiological applications, such as

toxicity and adverse side effects. UDCA may be used as a standalone agent or in combination with chemotherapeutics. Even at pharmacologically relevant concentrations, UDCA exhibits low toxicity. These findings highlight the potential utility of UDCA in PDAC treatment. Given its low toxicity and favorable activity profile, UDCA represents a particularly promising candidate, especially considering the limited effectiveness of current therapeutic options and the urgent need for novel treatment strategies in pancreatic cancer.

Summary

A diverse array of bacterial species inhabit the surfaces and internal cavities of the human body in a symbiotic relationship with the host, and they influence physiological processes and pathological conditions through the biosynthesis of bacterial metabolites. Both external and internal factors—such as age, diet, hygiene, genetic background, immune function, and antibiotic use—can affect microbial composition. Dysbiosis of the gastrointestinal microbiome has been linked to various diseases, including pancreatic cancer. The gut microbiome produces small-molecule metabolites that can exert hormone-like effects and inhibit the progression of distant tumors.

In this study, we demonstrated that the secondary bile acid lithocholic acid (LCA), produced by gut bacteria, exhibits antitumor properties in pancreatic ductal adenocarcinoma (PDAC) cells. LCA inhibits tumor cell proliferation, epithelial–mesenchymal transition (EMT), the expression of cancer stem cell markers, and promotes mitochondrial oxidative phosphorylation. LCA induces oxidative and nitrosative stress, which underlies its antineoplastic activity. These effects are mediated through the nuclear receptors CAR, FXR, and VDR in PDAC cells. Our findings indicate that LCA is a non-toxic compound with potent antitumor activity and highlight the therapeutic potential of nuclear receptors activated by LCA.

We also showed that another secondary bile acid, ursodeoxycholic acid (UDCA), likewise exhibits cytostatic properties in PDAC cell models. At concentrations corresponding to physiological levels in human serum, UDCA reduced PDAC cell proliferation, inhibited EMT, and induced mitochondrial oxidation. Importantly, UDCA did not interfere with the efficacy of standard chemotherapeutic agents used in PDAC treatment.

These results support the concept that bacterial metabolites play a significant role in tumor progression. Our findings also demonstrate that different tumors respond to different bile acids, and the effect of a specific bile acid can vary depending on the tumor type—likely due to differences in receptor expression profiles across tumor types. Understanding the specific roles of bacterial metabolites in cancer may lead to novel therapeutic approaches. Identified receptors for these metabolites may serve as potential drug targets, and the bacterial signatures responsible for metabolite production could offer promising biomarkers for early detection and prognosis.

Keywords

Lithocholic acid, Ursodeoxycholic acid, Pancreatic ductal adenocarcinoma, PDAC, Microbiome, Epithelial–mesenchymal transition, Oxidative-nitrosative stress, Stemness, Cell metabolism, Cell proliferation

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List of publications related to the dissertation

1. Kovács, P., **Schwarcz, S.**, Nyerges, P., Bíró, T. I., Ujlaki, G., Bai, P., Mikó, E.: Anticarcinogenic effects of ursodeoxycholic acid in pancreatic adenocarcinoma cell models.
   Front. Cell. Dev. Biol. 12, 1-14, 2024.
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2. **Schwarcz, S.**, Kovács, P., Nyerges, P., Ujlaki, G., Sipos, A., Uray, K., Bai, P., Mikó, E.: The bacterial metabolite, lithocholic acid, has antineoplastic effects in pancreatic adenocarcinoma.
   Cell Death Discov. 10 (1), 1-12, 2024.
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List of other publications

3. Sipos, A., Kerekes, É., Szeőcs, D., Szarvas, F., **Schwarcz, S.**, Tóth, E., Ujlaki, G., Mikó, E., Bai, P.: Ursodeoxycholic acid prompts glycolytic dominance, reductive stress and epithelial-to-mesenchymal transition in ovarian cancer cells through NRF2 activation.
   Cell Death Discov. 11 (1), 1-10, 2025.
   DOI: http://dx.doi.org/10.1038/s41420-025-02398-9
   IF: 6.1 (2023)
4. **Schwarcz, S.**, Nyerges, P., Bíró, T. I., Janka, E. A., Bai, P., Mikó, E.: Cytostatic Bacterial Metabolites Interfere with 5-Fluorouracil, Doxorubicin and Paclitaxel Efficiency in 4T1 Breast Cancer Cells.
   Molecules. 29 (13), 1-16, 2024.
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5. Ujlaki, G., Kovács, T., Vida, A., Kókai, E., Rauch, B., **Schwarcz, S.**, Mikó, E., Janka, E. A., Sipos, A., Hegedűs, C., Uray, K., Nagy, P., Bai, P.: Identification of bacterial metabolites modulating breast cancer cell proliferation and epithelial-mesenchymal transition.
Molecules. 28 (15), 1-18, 2023.
DOI: http://dx.doi.org/10.3390/molecules28155898
IF: 4.2

6. **Schwarcz, S.**, Kovács, P., Kovács, T., Ujlaki, G., Nyerges, P., Uray, K., Bai, P., Mikó, E.: The pro- and antineoplastic effects of deoxycholic acid in pancreatic adenocarcinoma cell models.
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