

Saikosaponin D Induced Apoptosis and Anti-Tumor Effect Enhancing Chemotherapy Sensitivity of Gemcitabine in Patient-Derived Cholangiocarcinoma Organoids

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Background: Cholangiocarcinoma (CCA), an aggressive malignancy with limited therapeutic options and poor survival rates, poses an urgent clinical challenge necessitating innovative therapeutic strategies. Saikosaponin D (SSd), a bioactive compound derived from *Bupleurum*, has demonstrated anticancer potential in various malignancies. However, its role in CCA remains unexplored. This study investigated the antitumor effects and potential mechanisms of SSd, both alone and in combination with gemcitabine, utilizing patient-derived cholangiocarcinoma organoids (PDCOs).

Methods: Four PDCO models were established from surgical tumor tissues and metastatic ascites of CCA patients. Histological, immunohistochemical (Cytokeratin 7, Cytokeratin 19 and Ki-67), and immunofluorescence analyses validated the fidelity of organoids to the primary tumors. Drug sensitivity testing evaluated SSd (0.001–50 μM) and gemcitabine alone or in combination (ratios 1:1, 1:2, 1:4) using dose-response curves and combination index (CI) analysis. Apoptosis mechanisms were assessed via TUNEL staining, caspase-3 activity assays, and the JC-1 assay for the mitochondrial membrane potential.

Results: SSd exhibited dose-dependent growth inhibition across PDCOs, with half-maximal inhibitory concentration (IC₅₀) values ranging from 0.9 μM to 13 μM. Gemcitabine showed IC₅₀ values spanning 0.03 μM to 11.1 μM. Notably, the SSd-gemcitabine combination at 1:4 ratio demonstrated synergistic effects (CI=0.0005), significantly reducing organoid viability. Ascites-derived PDCOs (eg, CCA182) displayed lower sensitivity to SSd, correlating with prior chemotherapy exposure. Apoptosis induction by SSd was confirmed through increased TUNEL-positive cells, elevated caspase-3 activity, and mitochondrial depolarization. Histological and molecular profiles of PDCOs closely mirrored those of the primary tumors, preserving spatial heterogeneity (eg, glandular structures in tissue-derived PDCOs vs compact morphology in ascites-derived models).

Conclusion: The combination of SSd and gemcitabine exerts a synergistic antitumor effect in patient-derived cholangiocarcinoma organoids through activating caspase-3 and triggering mitochondrial dysfunction-mediated apoptosis.

Keywords: patient-derived cholangiocarcinoma organoids, PDCOs, gemcitabine, Saikosaponin D, SSd, synergistic effect, apoptosis

Introduction

Cholangiocarcinoma is an aggressive malignancy originating from the epithelial cells of the bile duct system, and it is generally classified into intrahepatic and extrahepatic types.¹ The tumor's distinct anatomical location often complicates early detection, as initial symptoms are typically subtle or nonspecific. Consequently, many patients receive a diagnosis at an advanced stage, which leads to a poor clinical prognosis. Despite notable progress in surgical resection, radiotherapy, chemotherapy, and targeted therapies,^{2,3} the overall survival rate of patients with cholangiocarcinoma remains

low. The five-year survival rate is reported to be between 5% and 15%⁴ with significant risk of recurrence and metastasis post-treatment. Therefore, there is a critical need for the development of novel and more effective therapeutic strategies. Continued research into innovative treatment approaches and early diagnostic methods is essential to improving outcomes for patients with this challenging disease.

Bupleurum, as a traditional Chinese medicinal herb, has been widely used in clinical practice for its therapeutic effects, including heat-clearing, relieving the exterior, liver-Qi regulation, and fortifying Yang energy, according to ancient Chinese philosophy.⁵ Saikosaponins, a class of triterpene saponins extracted from *Bupleurum*, have garnered significant attention due to their diverse biological activities. Among these, Saikosaponin D (SSd) is particularly abundant and has been shown in modern pharmacological studies to possess anti-inflammatory, antiviral, antifibrotic, neuroregulatory, and immune-modulatory properties, with significant potential in cancer therapy.^{6–8} Several studies have demonstrated that SSd can inhibit the growth and proliferation of various cancer cells, such as breast, lung, and liver cancer cells.^{7,9–12} However, its specific antitumor efficacy and underlying mechanisms in CCA remain inadequately explored, highlighting the need for further investigation to assess its potential clinical applications.

In recent years, patient-derived organoids (PDOs) have emerged as a promising three-dimensional in vitro model, offering significant advantages for the study of tumor biology and drug screening.^{13,14} Compared with traditional two-dimensional cell cultures and animal models, organoids more accurately recapitulate the genetic and phenotypic characteristics of patient tumors as cell-cell interactions, the tumor microenvironment,^{15,16} and key genetic mutations.^{17,18} Moreover, organoid technology has revealed remarkable utility in pharmacological evaluation, and can offer personalized predictions regarding clinical treatment responses.^{19–21} This unique capability significantly bolsters the development of customized therapeutic strategies tailored to the specific needs of individual patients. Looking ahead, organoids exhibit the potential to serve as a supplement or even partially substitute for traditional preclinical models, by providing human-relevant platforms for mechanistic drug studies and safety evaluations. This not only helps to address the ethical issues associated with animal experimentation, but also enhances translational research opportunities.

Given this potential, we established a patient-derived cholangiocarcinoma organoid platform to investigate the apoptosis-inducing effects of SSd and evaluate its antitumor efficacy. These findings aim to elucidate SSd's anti-tumor mechanisms, thereby contributing new insights and therapeutic targets for cholangiocarcinoma treatment.

Materials and Methods

Establishment of Patient-Derived Cholangiocarcinoma Organoids

The cholangiocarcinoma tissue samples were all derived from fresh, surgically resected tumor tissues, ensuring collection within 30 minutes post-resection. Tumor samples were finely minced and subjected to enzymatic digestion with a digestion solution (Accurate International, B504) to dissociate tumor cell clusters, followed by filtration. Red blood cells were removed using a red blood cell lysis buffer (Solarbio, R1010), and cell viability was subsequently evaluated via trypan blue staining (Solarbio, C0040). The viable cells were then resuspended in Matrigel (Corning, 356231) and seeded into a 24-well plate. After a 15-minute incubation at 37°C to allow the Matrigel to solidify, 500μL of culture medium (bioGenous, K2104-LB) was added to each well. The culture medium was refreshed every 3–4 days.

The passaging of organoids involves several key steps. Initially, organoids are harvested from the Matrigel using an Organoid Recovery Solution (Corning, 354253) followed by dissociation into small cell clusters or single cells via Organoid Dissociation Solution (bioGenous, E238001) or mechanical trituration. Post-dissociation, cells are washed with Hank's Balanced Salt Solution (HBSS) and centrifuged under specific conditions (speed and duration optimized based on cell type). The cell pellet is then resuspended in fresh Matrigel and reseeded at a ratio conducive to optimal growth and development in subsequent culture. It is crucial to perform all procedures under sterile conditions to prevent contamination and maintain cell viability.

HE, Immunohistochemistry and Immunofluorescence Staining

Hematoxylin and Eosin (HE) staining was performed to compare the histological features of the organoids with those of the primary tumor tissues. HE staining was performed by fixing the organoids with paraformaldehyde and pre-embedding

them in 2% agarose solution. The samples were then subjected to routine dehydration, clearing, and embedding processes, followed by preparation of 4µm-thick paraffin sections. The sections were deparaffinized, rehydrated, and stained with hematoxylin for nuclei and eosin for cytoplasm. Finally, the sections were dehydrated, cleared, and mounted with neutral resin, and then observed under an optical microscope (Olympus, BX53).

Immunohistochemistry was performed by deparaffinizing and rehydrating the sections, followed by blocking of endogenous peroxidase activity using 3% hydrogen peroxide. Antigen retrieval was carried out with citrate buffer under high-pressure conditions, and non-specific binding sites were blocked using 5% normal goat serum. Primary antibodies such as CK7, CEA, and CA19-9 were applied, and the sections were incubated overnight at 4°C. After incubation with HRP-labeled secondary antibodies for 30 minutes, 3,3'-Diaminobenzidine (DAB) was used for visualization, and nuclei were counterstained with hematoxylin. Finally, the sections were dehydrated, mounted, and examined under an optical microscope (Olympus, IX73P1F). Immunofluorescence was performed by fixing organoids or tissue sections with 4% paraformaldehyde and washing with PBS. Antigen retrieval was conducted, followed by blocking of non-specific binding with 5% BSA. Fluorescently labeled primary antibodies, or primary antibodies followed by fluorescently labeled secondary antibodies, were applied and incubated in a humidified chamber. The nuclei were counterstained with DAPI, and the sections were mounted and observed under a fluorescence microscope (Zeiss, LSM800).

Immunohistochemical (IHC) and immunofluorescence (IF) staining were conducted using antibodies such as CK7 (Invitrogen, MA1-06316), CEA (Abcam, ab133633), and CA19-9 (Invitrogen, MA5-12421) and Ki67 (ZSGB-BIO, ZM-0166).

Drug Screening Assays

Organoids of cholangiocarcinoma were recovered from Matrigel using recovery solution, followed by enzymatic digestion and cell counting. Approximately 2000–5000 cells were seeded into each well of a 96-well plate, and after two days of culture, the medium was removed and replaced with fresh medium containing different concentrations of drugs. For drug screening, cholangiocarcinoma organoids were treated with varying concentrations (0.001, 0.01, 0.1, 1, 10, and 50µM) of SSd (MCE, HY-N0250) and gemcitabine (MCE, HY-17026) as single agents, as well as with combinations of SSd and gemcitabine in different ratios (1:1, 1:2, 1:4). alamarBlue® Cell Viability Reagent (Invitrogen, DAL1025) was added directly to each well, and the plates were incubated at 37°C for appropriate time. The absorbance signal was measured by microplate reader (Molecular Devices, VersaMax). Dose-response curves were generated based on the different drug concentrations, and the half-maximal inhibitory concentration (IC₅₀) of each drug was calculated.

Apoptosis Detection

Tunel assay: the organoid pellet was fixed with 4% paraformaldehyde at room temperature for 30 minutes and washed three times with PBS. TUNEL staining was performed using the DAB Detection Kit (Beyotime, C1091). Permeabilization was achieved with 0.5% Triton X-100 in PBS for 5 minutes. The samples were incubated with the TUNEL reaction mixture at 37°C for 30 minutes, followed by the application of the DAB substrate solution for color development. The nuclei were counterstained with hematoxylin for observation. Brown-stained nuclei observed under an optical microscope are indicative of apoptotic cells.

Caspase-3 activity detection: caspase-3 activity in the organoid samples was detected using the Caspase-3 Fluorescence Detection Kit (Beyotime, C1073S). Following organoid recovery, the samples were incubated with GreenNuc™ Caspase-3 Substrate and Mito-Tracker Deep Red 633. The nuclei were counterstained with DAPI, and fluorescence was observed under a fluorescence microscope to evaluate caspase-3 activation as a marker of apoptosis.

JC-1 assay: the JC-1 assay was performed using the JC-1 Detection Kit (Beyotime, C2006) to measure mitochondrial membrane potential. After recovery, the organoids were incubated with JC-1 probe in the dark at 37°C for 20 minutes and then washed with the provided buffer. Fluorescence intensity was analyzed under a fluorescence microscope (Olympus, BX53), with red fluorescence indicating intact mitochondria and green fluorescence indicating depolarized mitochondria, a key indicator of apoptosis.

Statistical Analysis

The quantitative data for TUNEL-positive cells, presented as the percentage of apoptotic cells, were analyzed using a two-way analysis of variance (ANOVA). Post-hoc multiple comparisons were performed using Tukey's honest significant difference test to compare specific groups when a significant main effect or interaction was found. A p-value of less than 0.05 was considered statistically significant. All analyses were conducted using GraphPad Prism software (version 9.0).

Results

The Establishment of Cholangiocarcinoma Organoid (CCAOs)

In this study, four cholangiocarcinoma samples were collected for organoid culture, including one ascites-derived metastatic samples and three surgical tissue samples. Patient information is detailed in Table 1, with the cohort consisting of two males and two females. The predominant tumor histopathological type was identified as cholangiocarcinoma. Using optimized tissue digestion and sample processing methods (Figure 1A), four cholangiocarcinoma organoids were successfully established.

All four established cholangiocarcinoma organoids demonstrated rapid proliferation capacities, with population doubling times ranging from 7.0 ± 1.2 to 25.0 ± 2.1 days (mean $\pm$ SD, $n=6$ passages), and were selected for further analysis. These organoids exhibited diverse growth characteristics and morphological phenotypes (Figure 1), indicative of their distinct molecular and genetic profiles. Specifically, tissue-derived organoid 008 displayed a vacuolated growth pattern, while organoid 086, also tissue-sourced, manifested a spherical and dense structure with cystic cavities. Ascites-derived organoid 182 showed a compact and non-luminal morphology. Notably, tissue-derived organoid 218 presented two distinct morphologies: a dense form with cystic cavities and vacuolated structures (Figure 1B). All organoids maintained stable in vitro passaging for over six generations without significant changes in growth behavior or morphology (Figure 1C). Furthermore, cryopreservation and thawing procedures had no discernible effect on their growth characteristics (Figure 1D).

Histological and Molecular Characterization of Cholangiocarcinoma Organoids

To validate the fidelity of cholangiocarcinoma organoids to their corresponding primary tissues, histological and IHC analyses were performed to verify their molecular characterization. HE staining demonstrated a high similarity between the organoids and the original tumor tissues. The primary tumor cells were enlarged, hyperchromatic, with loss of cellular polarity and moderately to well-differentiated arranged in irregular glandular or tubular structures. The tumor was surrounded by abundant fibrous stroma, lacked a distinct capsule, and demonstrated infiltrative growth pattern. Relatively, the organoids exhibited comparable nuclear atypia, and glandular or luminal growth patterns.

IHC analysis was further performed using key cholangiocarcinoma markers, including cytokeratins (CK7, CK17, CK19 and CK18/8). Results showed strong expression of CK7, CK17, CK18/8 and CK19 in both the organoids and primary tissues, reflecting the epithelial origin of cholangiocarcinoma (Figure 2A). Additionally, IF analysis demonstrated that the organoids expressed CK7 and CK19. Both IHC and IF analyses revealed a higher Ki-67 positivity

Table 1 The Clinical Information of Cholangiocarcinoma Patients

CCA	Gender	Age (years)	Diagnosis	Location	Differentiation	TNM	Medical History and Treatment
CCA008	M	78	CCA	Distal common bile duct	Moderately differentiated adenocarcinoma	T4N0M0	Postoperative radiotherapy and chemotherapy for kidney cancer and colorectal cancer
CCA086	F	68	CCA	Distal common bile duct	Moderately to poorly differentiated Adenocarcinoma	T2N0M0	None
CCA182	F	68	CCA	Widespread metastatic involvement	Poorly differentiated	T4N0M1	Postoperative treatment of cervical squamous cell carcinoma.
CCA218	M	68	CCA	Proximal common bile duct	Moderately to poorly differentiated adenocarcinoma	T2N1M0	None

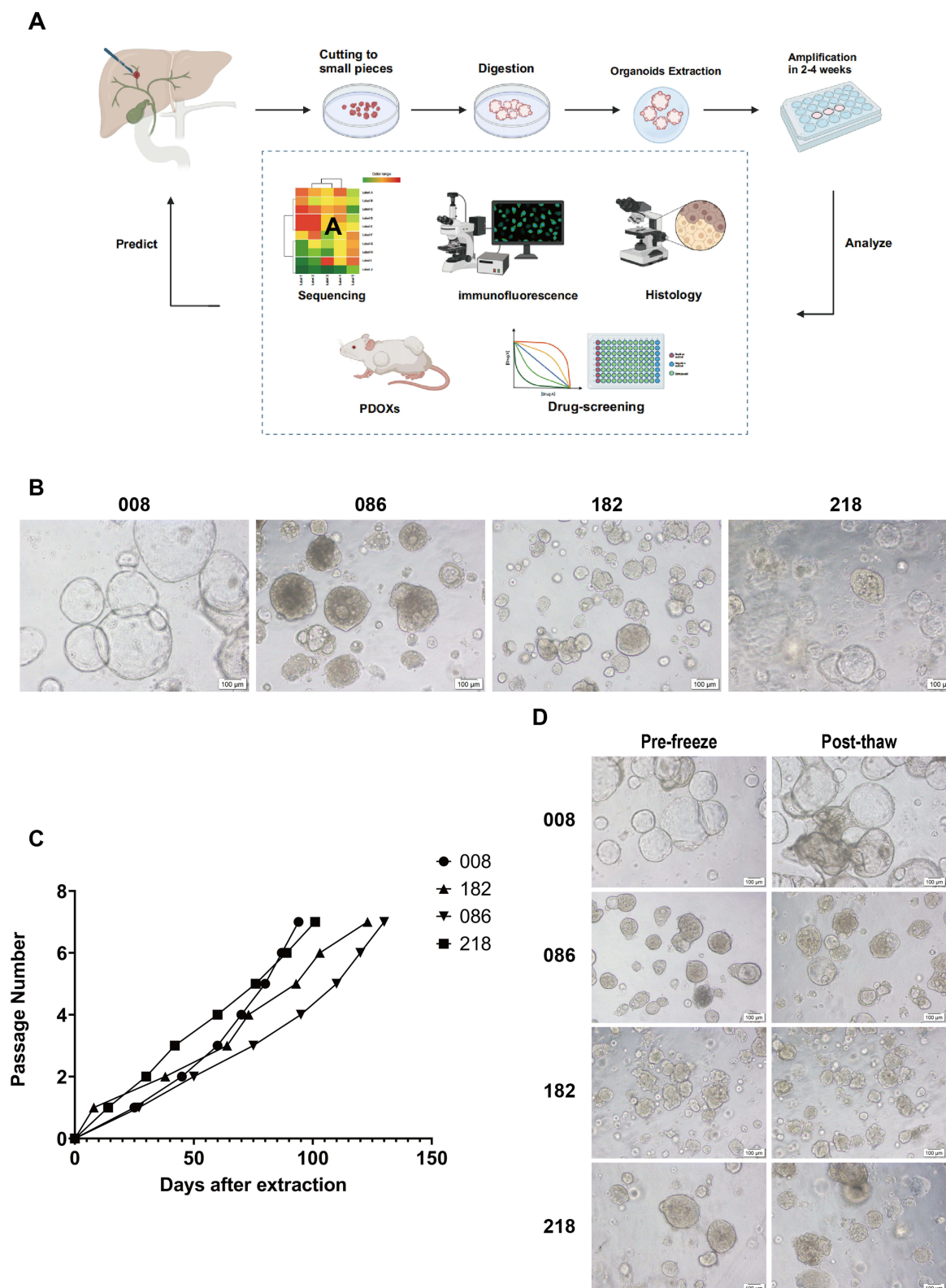


Figure 1 Establishment and characterization of cholangiocarcinoma organoids. **(A)** Workflow for establishing patient-derived organoids via optimized tissue digestion and processing methods. Four organoids were successfully established from one ascites-derived and three surgical tissue samples. **(B)** The organoids displayed distinct growth characteristics and morphological phenotypes, suggesting diverse molecular and genetic profiles. **(C)** All four organoids were stably passaged for over six generations without discernible alterations in growth or morphology. **(D)** Cryopreservation and thawing procedures had no evident effect on the growth characteristics of these organoids. Representative bright-field images of pre-freeze and post-thaw organoids are presented. Scale bar: 100μm.

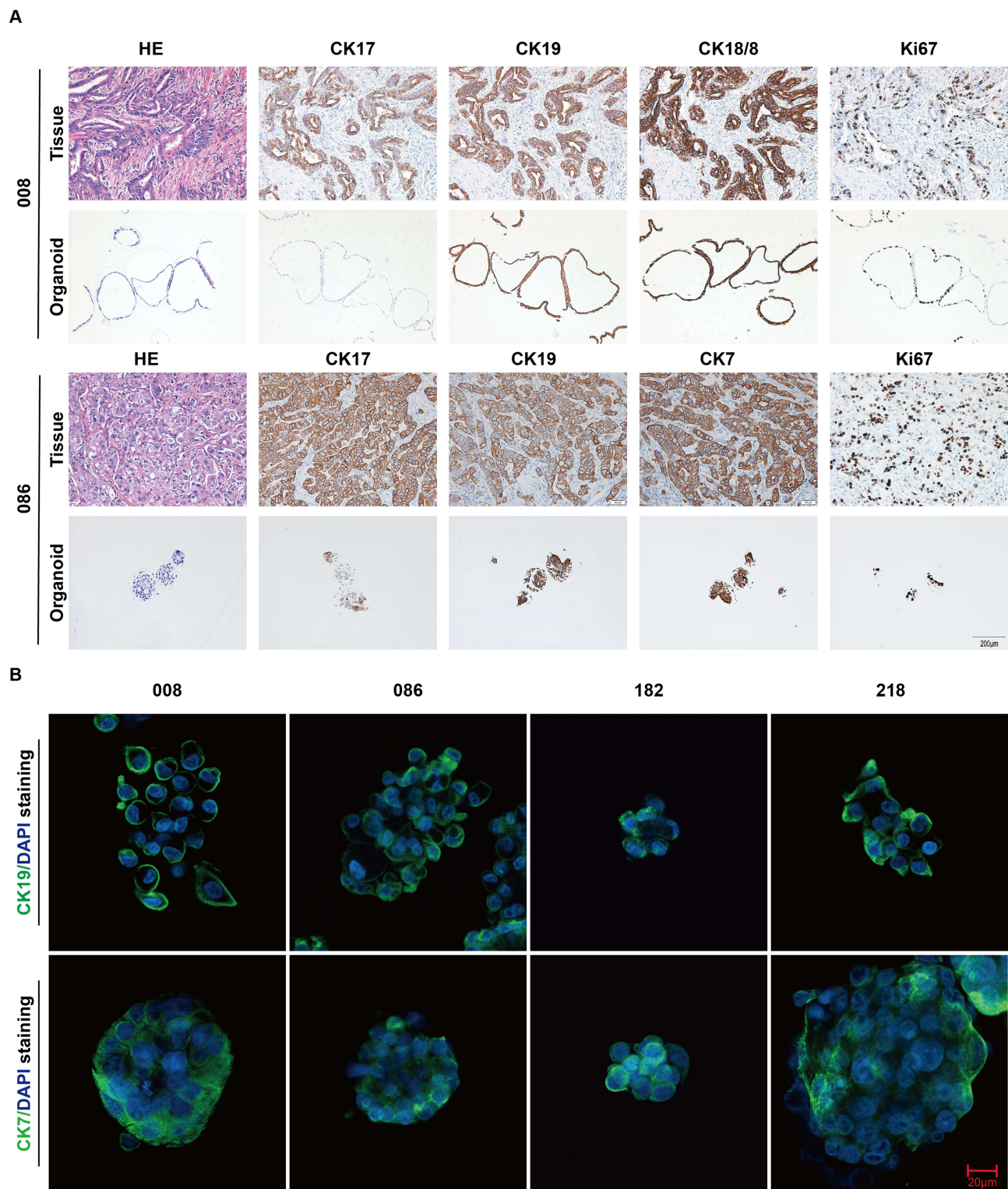


Figure 2 Histological and molecular characterization of cholangiocarcinoma organoids. **(A)** HE staining comparison between primary tumor tissues and their matched organoids. Organoids recapitulate the glandular architecture and vacuolated features of original tumors. Immunohistochemical analysis of CK7, CK19, CK18/8, and Ki-67 expression in organoids and primary tissues. Scale bar: 200µm. **(B)** Immunofluorescence co-staining of CK7 and CK19 in cholangiocarcinoma organoids, with nuclear counterstaining by DAPI (blue). Scale bar: 20µm.

proportion in the organoids compared to the parental tissues, which was consistent with their rapid proliferative capacity (Figure 2B).

These findings indicate that the established cholangiocarcinoma organoids faithfully recapitulate the significant histological and molecular features of cholangiocarcinoma, providing a reliable model for studying tumor biology and therapeutic screening.

Synergistic Enhancement of Chemosensitivity in Cholangiocarcinoma Through Combined Gemcitabine and SSd Treatment

To evaluate the potential therapeutic effects of SSd alone and in combination with gemcitabine, drug sensitivity tests were performed on cholangiocarcinoma organoids. The organoids, recovered from Matrigel, were dissociated into single cells or small clusters, and then seeded in 96-well plates. Subsequently, various concentrations of SSd, gemcitabine, and four different combination ratios were added.

Single-agent sensitivity dose-response curves indicated that SSd had dose-dependent inhibitory effects on cholangiocarcinoma organoids. The IC₅₀ values of SSd ranged from 0.9 to 13 μ M across different organoid samples, demonstrating varying sensitivities among the organoid lines. Organoid 008 (tissue-derived) was the most sensitivity to SSd with an IC₅₀ of approximately 0.98 μ M, while organoid 218 (tissue-derived) exhibited moderate sensitivity with an IC₅₀ of 12.7 μ M. Gemcitabine, a first-line chemotherapeutic agent for cholangiocarcinoma, also demonstrated dose-dependent effects, with IC₅₀ values ranging from 0.03 to 11.1 μ M (Figure 3A–C).

Combination therapy results showed that the SSd and gemcitabine combination produced stronger inhibitory effects compared to either drug alone. Notably, all four cholangiocarcinoma organoid models exhibited enhanced responsiveness when treated with the SSd and gemcitabine combination. Combination index (CI) analysis revealed that certain ratios exhibited synergistic effects. Specifically, in organoids 182 and 218, all tested ratios (1:1, 1:2, 1:4, 4:1) demonstrated significant synergy, especially the 1:4 ratio, which of the CI value was as low as 0.0005, indicating strong synergy between SSd and gemcitabine. Additionally, organoid 008 showed synergy in the 1:1 and 4:1 ratios, and organoid 086 demonstrated synergy in the 1:4 ratio (Figure 3D).

The combination treatment significantly reduced cell viability in cholangiocarcinoma organoids. When SSd was administered in combination, the IC₅₀ of gemcitabine was markedly decreased. This suggests that SSd enhanced the sensitivity of cholangiocarcinoma organoids to gemcitabine. Across all four organoid models, the combination regimen consistently showed improved therapeutic outcomes, highlighting the potential of this dual-drug strategy in cholangiocarcinoma treatment. These findings indicate that the combination of SSd and gemcitabine, particularly in specific ratios, exerts significant synergistic effects in different organoid models, providing robust scientific evidence for further research and clinical application.

SSd Enhancing Chemotherapy Sensitivity of Gemcitabine in Patient-Derived Cholangiocarcinoma Organoids via Apoptosis Induction

To delineate the cell death mechanisms underlying SSd/gemcitabine combinatorial therapy, we conducted a series of experiments to elucidate the precise mode of cell death and the molecular mechanisms involved in cholangiocarcinoma organoids.

First, TUNEL staining was used to detect DNA fragmentation, a hallmark of apoptosis. Results showed a significant rise in TUNEL-positive cells of the SSd-treated group compared to those of the untreated control group, demonstrating the pro-apoptotic effect of SSd in cholangiocarcinoma organoids. Furthermore, the combination treatment group exhibited an even greater increase in TUNEL-positive cells, confirming a synergistic interaction between SSd and gemcitabine in triggering apoptosis. These findings initially suggested that SSd, either alone or in combination with gemcitabine, might exert its cytotoxic effects through the activation of apoptotic cell death pathway (Figure 4A).

To quantitatively confirm these observations and account for potential variability across different patient-derived models, we performed statistical analysis of TUNEL-positive cells. Two-way ANOVA revealed a highly significant main effect of treatment, with post-hoc analysis confirming that the combination therapy elicited a significantly stronger

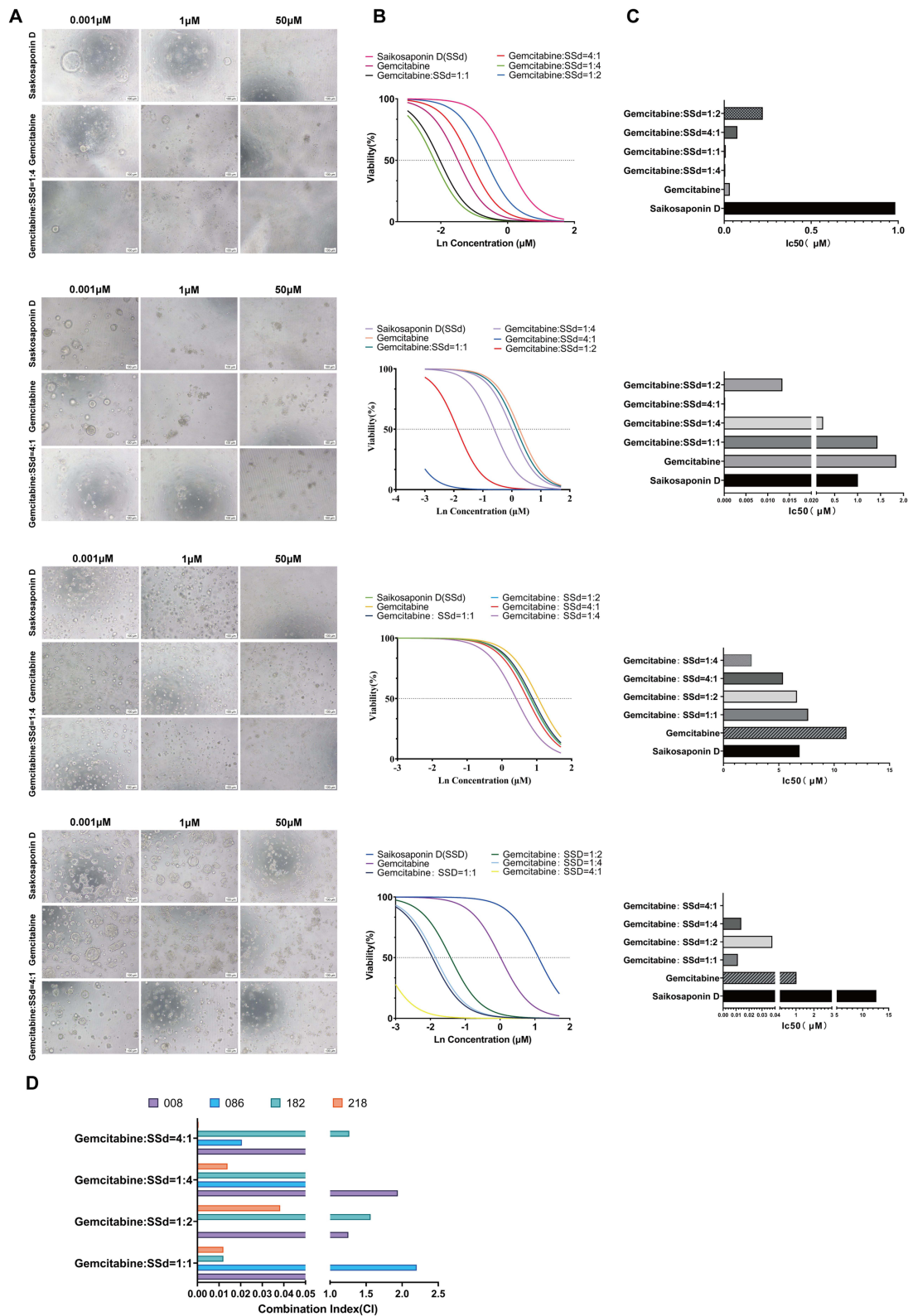


Figure 3 Drug sensitivity profiling of cholangiocarcinoma organoids. **(A)** Representative bright-field images showing cell death in cholangiocarcinoma organoids exposed to different concentrations of monotherapy and combination treatments. Scale bar: 100 μ m. **(B)** Dose-response curves of SSd or gemcitabine monotherapy and their combination treatments across four organoid models. **(C)** Comparison of IC50 values for SSd or gemcitabine monotherapy and their combination treatments in four organoid models. **(D)** CI analysis of SSd and gemcitabine co-treatment in four cholangiocarcinoma organoid models.

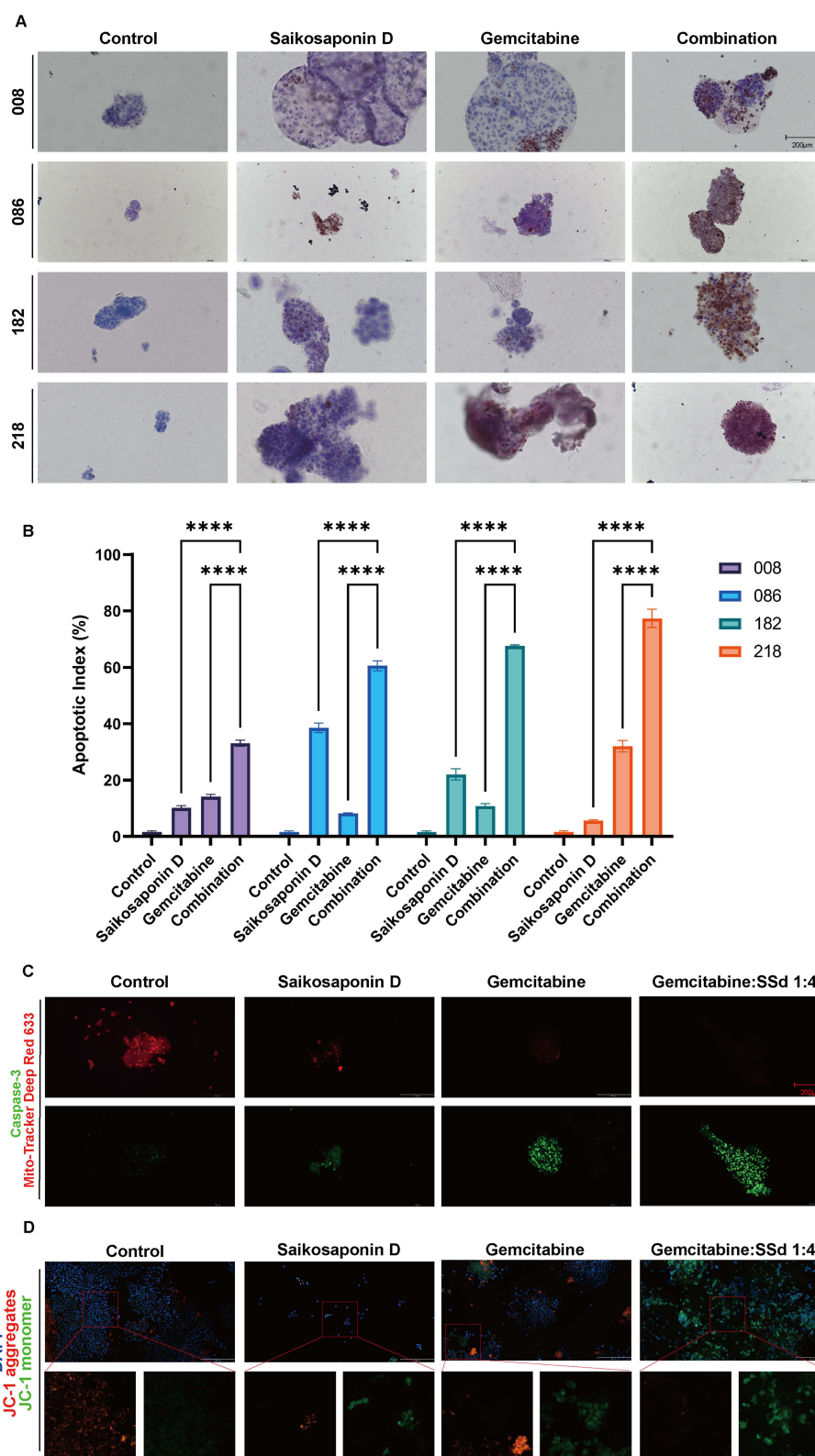


Figure 4 SSd enhancing chemotherapy sensitivity of gemcitabine in patient-derived cholangiocarcinoma organoids via apoptosis induction. **(A)** TUNEL staining was employed to visualize DNA fragmentation (indicated by brown-stained nuclei) in SSd-treated organoids. Scale bar: 200µm. **(B)** Quantitative analysis of TUNEL-positive cells. Data are presented as mean ± SEM (n=3). Statistical significance was determined by two-way ANOVA (factors: treatment and organoid line) with Tukey's multiple comparisons test. The analysis revealed a significant main effect of treatment ($p < 0.001$). For clarity, only select key comparisons are denoted: **** $p < 0.0001$. **(C)** Caspase-3 activation was assessed by GreenNuc™ fluorescence (green) in treatment groups. An intensified signal in the combination group demonstrated synergistic apoptosis induction. Scale bar: 200µm. **(D)** JC-1 assay was utilized to evaluate mitochondrial depolarization, which was manifested as a shift from red to green fluorescence. SSd treatment reduced red fluorescence (intact mitochondria), with further reduction in the combination group. Scale bar: 200µm.

apoptotic response than either monotherapy alone across all organoid lines, providing robust quantitative validation of the synergistic apoptosis induction (Figure 4B).

Next, caspase-3 activity was assessed using the GreenNuc™-DNA complex, which emitted green fluorescence (Ex/Em: 500/530 nm). A marked increase in green fluorescence was seen in the SSd-treated group compared to the control group, indicating higher caspase-3 activity. This effect was more intense in the combination treatment group, further confirming the synergistic impact of SSd and gemcitabine on apoptosis in cholangiocarcinoma organoids. Given the critical role of caspase-3 in apoptosis pathway, these results provide evidence for the apoptotic mode of cell death induced by the combination treatment (Figure 4C).

To further investigate the underlying mechanisms of apoptosis, we assessed mitochondrial function by measuring the mitochondrial membrane potential using Mito-Tracker Deep Red 633, which emitted deep red fluorescence (Ex/Em: 622/648 nm). SSd-treated groups showed a significant reduction or loss of deep red fluorescence, implying decreased mitochondrial membrane potential and mitochondrial dysfunction, an early apoptosis event. The combination treatment group exhibited more severe mitochondrial dysfunction, reinforcing that the SSd and Gemcitabine combination led to mitochondrial impairment (Figure 4D).

Discussion

This study offered patient-derived cholangiocarcinoma organoids (PDCOs) models for evaluating traditional Chinese medicine-derived agents and first-line chemotherapy, revealing novel therapeutic synergies between SSd and gemcitabine, and providing new experimental evidence for precision therapy in cholangiocarcinoma.

The established cholangiocarcinoma organoid biobank (n=4) includes diverse sample sources (primary tumors and metastatic ascites) and clinical features (including advanced metastatic cases). The morphological heterogeneity (eg, vacuolated, dense spheroid structures) closely reflects the histological features of primary tumors. Notably, ascites-derived organoids (eg, CCA182) exhibited compact, non-luminal structures, possibly indicating dedifferentiation of metastatic tumor cells. In contrast, tissue-derived organoids (eg, CCA086) retained the glandular structures typical of primary lesions, demonstrating atypia of histological architecture retention in organoid models. Immunohistochemical analysis further validated identical molecular features between organoids and their corresponding primary tumors. The high CK19 expression confirmed their cholangiocytic origin, and high Ki-67 positivity matched their rapid proliferative ability.

SSd monotherapy exhibited dose-dependent inhibitory effects on the organoids, with IC₅₀ values ranging from 0.9 μM to 13 μM, highlighting significant inter-patient variability. The ascites-derived CCA182 showed lower sensitivity to SSd, with an IC₅₀ value of 12.3 μM. The patient's prior treatment history, which included postoperative therapy for cervical squamous cell carcinoma and lung cancer chemotherapy, might enhance chemoresistance through epigenetic reprogramming or drug-induced selection pressure. This heterogeneity in drug sensitivity, observed for both SSd and gemcitabine (IC₅₀ values ranging from 0.03 μM to 11.1 μM), is not a limitation but rather a key strength of the patient-derived organoid (PDO) model, as it effectively recapitulates the vast inter-patient diversity seen in the clinic. The variability in IC₅₀ values and the resulting synergistic effects (Combination Index < 1) across all models suggest that our PDO platform holds utility for predicting personalized therapy responses. Strikingly, the SSd-gemcitabine combination demonstrated consistent synergistic effects across all organoid models, particularly at a 1:4 ratio, as evidenced by a combination index (CI) of 0.0005. This broad synergy might result from dual-pathway regulation. SSd was known to induce mitochondrial membrane potential collapse, thereby activating the intrinsic apoptosis pathway. On the other hand, gemcitabine disrupted DNA synthesis, creating a synthetic lethality effect when combined with SSd. The convergence of these distinct mechanisms—nuclear DNA damage and mitochondrial stress—likely overwhelms cellular repair capacity, leading to robust synergy even in models with varying baseline sensitivities to either agent. However, the exact molecular targets underlying this synergy remained unclear and warrant further investigation using transcriptomic or proteomic profiling.

Additionally, we further explored the potential mechanism model of the synergistic effect based on literature. Gemcitabine, as an antimetabolite agent, primarily functions by incorporating into DNA strands to terminate synthesis and inhibiting ribonucleotide reductase, leading to S-phase cell cycle arrest and DNA damage-mediated apoptosis.⁵ In

contrast, as a natural compound, SSd has been shown to activate the intrinsic apoptotic pathway by inducing mitochondrial membrane potential collapse, promoting reactive oxygen species (ROS) production, and regulating Bcl-2 family proteins (such as increasing the Bax/Bcl-2 ratio).^{7,9} This mechanistic difference forms the basis of the observed synergy: gemcitabine induces “nuclear catastrophe” (DNA damage), while SSd simultaneously triggers “cytoplasmic crisis” (mitochondrial dysfunction). The combined action of these two stressors may overwhelm cancer cells, significantly lowering their survival threshold. Our experimental data support this multi-target model for instance, DNA fragmentation revealed by TUNEL staining (Figure 4A), elevated caspase-3 activity (Figure 4C), and mitochondrial depolarization shown in the JC-1 assay (Figure 4D) all indicate an enhanced apoptotic pathway. Similar synergistic models have also been reported in other cancers,^{6,10} suggesting this may be a general mechanism by which SSd enhances chemosensitivity.

Although our study utilized TUNEL staining, caspase-3 activity assays, and mitochondrial membrane potential measurements to confirm apoptosis as the primary mechanism of cell death induced by those treatments, several critical questions remained unanswered. First, it is unclear whether SSd modulates the Bcl-2 family of proteins or the death receptor pathways. Second, the impact of combination therapy on tumor stem cell subpopulations has not been fully elucidated. Third, the pharmacokinetic interactions within the organoid microenvironment require further investigation to optimize the therapeutic efficacy of the combination therapy. Additionally, the relatively small cohort size (n=4) in this study limits our exploration of molecular subtypes, such as IDH1/2 mutations and FGFR2 fusions, and their drug responses. Future studies should expand the cohort size and integrate multi-omics data to establish biomarker-guided therapeutic strategies.

This study represents the first to reveal the antitumor activity of SSd and its synergy with gemcitabine in cholangiocarcinoma organoids. Our findings provide a foundation for the development of traditional Chinese medicine-derived agents as potential therapeutic options for cholangiocarcinoma. Successfully modeling of ascites-derived organoids offers a feasible platform for individualized drug testing in advanced metastatic patients. However, clinical translation of these findings requires further validation, including: (1) using patient-derived organoid xenograft (PDOX) models to assess in vivo efficacy and toxicity; (2) dynamically monitoring treatment responses through circulating tumor DNA (ctDNA) analysis; (3) optimizing drug delivery systems to improve SSd bioavailability.

Conclusion

In summary, our experimental results elucidated the mechanisms by which SSd and its combination with gemcitabine induce apoptosis in cholangiocarcinoma organoids. Initially, TUNEL staining confirmed the occurrence of DNA fragmentation, a hallmark feature of apoptotic cell death. Subsequently, caspase-3 activity assays demonstrated the activation of apoptosis-related enzymes. Finally, assessments of mitochondrial membrane potential revealed mitochondrial dysfunction as a key mechanism in SSd-treated cholangiocarcinoma organoids. Collectively, these findings indicate that SSd, when used alone or in combination with gemcitabine, triggers apoptosis via the activation of caspase-3 and the induction of mitochondrial dysfunction. This stepwise experimental approach clearly demonstrated the apoptotic mechanisms underlying SSd-induced cell death in cholangiocarcinoma organoids.

This study provides a translational foundation for cholangiocarcinoma therapy through organoid models, drug sensitivity testing, and exploring initial mechanisms. While the “clinical-organoid-mechanism-clinical” personalized closed-loop has not yet been accomplished, these findings pave the way for identifying the molecular targets of SSd and its combination therapy mechanisms. Future research will focus on mechanistic validation and preclinical studies to advance the clinical application of natural product-chemotherapy combinations for cholangiocarcinoma.

Abbreviations

CCA, Cholangiocarcinoma; PDOs, Patient-Derived Organoids; HE, Hematoxylin and Eosin; IHC, Immunohistochemical; IF, Immunofluorescence; CK7, Cytokeratin 7; CK19, Cytokeratin 19; CEA, Carcinoembryonic Antigen; HRP, Horseradish Peroxidase; DAB, 3,3'-Diaminobenzidine; BSA, Bovine Serum Albumin; DAPI,

4',6-Diamidino-2-Phenylindole; IC50, Half-Maximal Inhibitory Concentration; CI, Combination Index; TNM, Tumor, Node, Metastasis; HBSS, Hank's Balanced Salt Solution.

Data Sharing Statement

The datasets generated and analyzed during this study are available from the corresponding author (Prof. Zhijiang Shao) upon reasonable request. Organoid models and protocols are deposited in the Biobank of Tianjin Fifth Central Hospital.

Ethical Approval and Consent to Participate

Ethical approval for this study was obtained from the Ethics Committee of Tianjin Fifth Central Hospital under the following approved protocols: Approval No. WZX-EC-KY2024038 (Science and Technology Project of Tianjin Binhai New Area Health Commission, 2022BWKQ003). Approval No. WZX-EC-KY2022029 (Tianjin Health Technology Project, TJWJ2022XK041).

Written informed consent was acquired from all participating patients. All procedures strictly adhere to the ethical standards of the Declaration of Helsinki and its subsequent amendments. This declaration has been updated per technical check requirements.

Consent for Publication

All authors have reviewed and approved the final version of this manuscript for submission. The participants provided consent for anonymized data derived from their tissues to be published in scientific journals. No identifiable personal information is disclosed in any figures or text.

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Disclosure

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