

Inhibition of AMPK/PFKFB3 Mediated Glycolysis Synergizes with Penfluridol to Suppress Gallbladder Cancer Growth

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Research Article

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Abstract

Background

Penfluridol (PF) is an FDA approved antipsychotic drug, which shows anticancer activity recently. However, the anticancer effects and underlying mechanisms of PF on gallbladder cancers (GBCs) is not well-established.

Methods

Herein, cytotoxicity, cell proliferation, cell apoptosis, and cell metastasis assays were used to investigate the anticancer activity of PF on GBCs. Glucose consumption and lactic production assays were used to detect the glycolysis alteration. Western blotting was used to detect the corresponding signaling change after PF treatment. Nude mice were utilized to study the anticancer activity of PF in vivo.

Results

Here, we first observed that PF significantly suppress GBC cells proliferation and metastasis. After PF treatment, the glucose consumption and lactic production of GBCs were significantly increased. In addition, we found that inhibition of glycolysis enhanced the anticancer activity of PF. Further studies demonstrated that glycolysis was mediated by the activation of AMPK/PFKFB3 signaling pathway. Mechanistically, we demonstrated that AMPK/PFKFB3 signaling pathway mediated glycolysis was a resistant mechanism of PF in GBCs.

Conclusions

Inhibition of AMPK enhanced the anticancer effects of PF on GBCs. therefore, our studies provided a novel insight into repurposing PF as anticancer agent for GBCs, and AMPK inhibition in combination with PF could be a potential therapeutic approach for GBCs.

Background

Gallbladder cancers (GBCs) are rare biliary tract malignancies with rising prevalence particularly among American Indians and Southeast Asians [1, 2]. Surgical resection is the first-line treatment for patients with early GBCs, which contributes a near-perfect long-term survival. For patients diagnosed as unresectable or metastatic GBCs, gemcitabine plus cisplatin is recommended as the primary treatment [3]. Unfortunately, adverse events are commonly observed and prognosis remains unsatisfactory after chemotherapy for advanced GBCs [4]. Therefore, it is necessary to explore adequate treatments to improve prognosis with fewer side effects for GBC patients.

In recent years, several non-anticancer drugs have been repurposed for cancer treatment, such as metformin for colorectal cancer, and nitroxoline for pancreatic cancer [5, 6]. The rationale for repurposing antipsychotic medications for cancer therapy stemmed from multiple clinical studies, demonstrating that patients receiving antipsychotic medications had a lower cancer incidence [7]. Penfluridol (PF), a first-generation FDA-approved antipsychotic drug, exhibited anticancer activity [8]. PF affected several hallmark aspects of cancers, including tumor-promoting inflammation, immune evasion, activation invasion and metastasis, cell death resistance, and sustaining proliferative signaling [9]. While multiple mechanisms were reported, such as inhibiting the integrin signaling pathway in breast cancer and inducing autophagy to promote cancer apoptosis, the exact one in GBCs remained unclear [10, 11].

Reprogramming energy metabolism is common in malignancies and causes tumor progression [12, 13]. In the aerobic condition, cancer cells mostly rely on glycolysis, which has low energy production efficiency but provides multiple intermediates, to maintain cancer biosynthesis and redox homeostasis [14, 15]. Notably, PF was shown to modulate tumor-specific glycolysis, suggesting a novel anticancer mechanism. A latest study demonstrated that PF downregulated hexokinase-2 (HK2) to suppress glycolysis and further inhibited colorectal cancer cells proliferation [16]. However, there is no study conducted to explore the relationship between PF treatment and glycolysis in GBCs.

In this study, we repurposed the anticancer activity of PF on GBCs. Additionally, we further investigated that activation of AMPK/PFKFB3 signaling pathway mediated glycolysis after PF treatment attenuated the anticancer effects of PF. Thus, in combination of inhibiting AMPK/PFKFB3 signaling pathway and PF treatment would be a potential therapeutic strategy for GBCs.

Methods

Reagents and antibodies

The following reagents were used in the study: Penfluridol (PF), 2-Deoxy-D-glucose (2-DG), A-769662, dorsomorphin dihydrochloride/Compound C (CC) were purchased from MedChemExpress (MCE, Shanghai, China), Cell Counting Kit-8 (CCK-8) were obtained from Yeason (Shanghai, China), Annexin V/PI Apoptosis staining Kit, and cell cycle staining kit were purchased from MULTI SCIENCES (Hangzhou, China), si-AMPK (5'-GGTTGGCAAACATGAATTG 3') were obtained from Ribio (Guangzhou, China), Glucose colorimetric assay kit, and L-Lactic Acid colorimetric assay kit were purchased from Elabscience (Wuhan, China), Lipofectamine 3000 reagent, and BCA protein assay kit were purchased from Invitrogen (CA, USA), 4% paraformaldehyde, 0.1% crystal violet, and RIPA were purchased from FUDE BIOLOGICAL (Hangzhou, China), ibidi Culture-insert was purchased from ibidi (Martinsried, Germany), transwell plate were purchased from Corning (Corning, NY, USA).

The anti- β -actin (FD0060), secondary antibodies goat-anti-rabbit (FD0128) and goat anti-mouse (FD0142) IgG-HRP antibody was purchased from FUDE BIOLOGICAL Technology (Hangzhou, China), Anti-Caspase-3 (ER30804), anti-activated caspase-3 (ET1602-47), anti-PARP (ET1608-56), anti-cleaved-PARP

(ET1608-10), anti-activate + pro caspase-9 (ET-1610-95) were purchased from HUABIO (Hangzhou China). Anti-Bcl-2 (EPR17509), anti-AMPK (ab207442), and anti-PFKFB3 (ab181861) were purchased from Abcam (Cambridge, UK), anti-Noxa was purchased from Cell Signaling Technology (Beverly, MA, USA), anti-PFKFB3^{S461} (TA3581), and anti-AMPK^{T172} (TP56027) were purchased from Abmart (Shanghai, China), anti-HK2 (sc-374091) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

Human GBC cell lines EH-GB1 and GBC-SD were purchased from a cell bank (Chinese Academy of Sciences, Shanghai, China). The SGC-996 cell line was provided by Dr. Ying-bin Liu's lab at Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, China. These cells were maintained in DMEM (EH-GB1 and GBC-SD) and RPMI-1640 (SGC-996) medium containing 10% fetal calf serum (FBS) from cellMax (Beijing, China), penicillin (100 units/ml) and streptomycin (100 µg/ml) in a 5% CO₂ humidified incubator at 37°C (Thermo Scientific).

Cell viability assay

Cell viability was assessed by CCK-8. Cells (3×10^3) were seeded in 96-well plates and allowed to attach overnight in a 5% CO₂ incubator. The cells were then treated with agents at different concentrations for 24h or 48h. CCK-8 (100 µl/ml) was added to each well and incubated for 1h at 37°C. The absorbance at 450nm was measured with a Multiscan spectrophotometer (Thermo Scientific).

Colony formation assay

1×10^3 cells/well were seeded in triplicate in a six-well plate for 24h. Then, the original medium was replaced with medium-containing agents at each concentration tested. Cells were incubated for another 14 days, photographed after being fixed by 4% paraformaldehyde and stained with 0.1% crystal violet and counted.

Wound-healing assay

GBC cells were pre-treated for 12h as indicated. Then, cells were digested and resuspended with a culture medium. 2×10^4 cells/well were seeded into ibidi Culture-insert on 12-well plates for 12h. Later, the insert was gently removed, and the medium was replaced with FBS-free medium. Photomicrographs were taken by a microscope (Zeiss, Germany) at the indicated time.

Transwell assay

GBC cells were pre-treated for 12h as indicated. Then, cells were digested and resuspended with FBS-free medium. 2×10^4 cells/well were seeded into the upper chamber of transwell 24-well plates with 8 µm pore size. Then, the lower chamber was added with a medium containing 20% FBS. After incubation of 24h or 48h, the upper surface of the chamber was cleaned, and migrated cells of the lower surface were stained with 0.1% crystal violet for 4 minutes. The level of migration was observed by a microscope.

Detection of cell apoptosis

Cell apoptosis was detected by staining using Annexin V/PI Apoptosis staining Kit according to the manufacturer's instructions. Briefly, GBC cells were digested with EDTA-free trypsin after agent treatment and resuspended in 500 μ L of binding buffer. After incubation with 5 μ L of Annexin V-FITC and PI staining for 15min at room temperature in the dark, data acquisition and analysis were carried out using flow cytometry.

Detection of cell cycle

Cell cycle was detected by staining using a cell cycle staining kit according to the manufacturer's instructions. Briefly, GBC cells were digested with EDTA-free trypsin after agent treatment and resuspended in 1ml of DNA staining solution. After incubation with 10 μ L of Permeabilization solution for 30min at room temperature in the dark, data acquisition and analysis were carried out using flow cytometry.

Western blot

Cells were seeded into a six-well plate (80,000 cells/well) overnight and treated with agents. After treatment, cells were collected by being scraped and washed with PBS. Then, the cells were lysed with RIPA buffer. Protein concentrations in the supernatants were determined with a BCA protein kit. Equal amounts of proteins were separated on SDS-polyacrylamide gels and then electroblotted onto polyvinylidene fluoride membranes (Sigma-Aldrich). The membranes were blocked with TBST plus 5% skimmed milk for 2h and incubated with primary antibodies overnight at 4°C. After being washed three times with TBST, the membranes were incubated with secondary antibodies (1:5000) for 1h at room temperature. Before development, the membranes were washed three times again, and the immunoblots were visualized with an ECL system.

Glucose consumption measurement

Cell glucose consumption was detected by Glucose colorimetric assay kit according to the manufacturer's instructions. Briefly, cells were planted in the six-well plates (80,000 cells/well) and incubated for 24h. Then, the culture medium was replaced with a medium with different agents. After 24h, the supernatant was collected and measured for glucose concentration. The cells left were lysed by RIPA for the detection of protein concentration to standardize glucose consumption levels.

Lactic acid production measurement

Cell lactic acid production was detected by L-Lactic Acid colorimetric assay kit according to the manufacturer's instructions. Briefly, cells were planted in the six-well plates (80,000 cells/well) and incubated for 24h. Then, the culture medium was replaced with a medium with different agents. After 24h, the supernatant was collected and measured for lactic acid concentration. The cells left were lysed by RIPA for the detection of protein concentration to standardize glucose consumption levels.

Patient-derived xenograft (PDX) model establishment

Tumor tissues from the patient were evaluated by pathologists and macro-dissected for implantation. 4-week-old female nude mice were purchased from Hangzhou Ziyuan Experimental Animal Technology Co., Ltd (Hangzhou, China) and maintained in a pathogen-free environment. The mice were transplanted with tumor tissues subcutaneously. During the grafting, all mice were intact. Initial tumor growth was detected at 4 months post-implantation. The tumors were serially implanted into new female nude mice *via* the same procedure as the original implants. A line was considered established if there was active growth after at least five passages. Tumor samples were harvested from later passages (>3) for characterization.

In vivo study

Xenograft tumors were established by subcutaneous transplantation. After 10 days, to assess the anticancer effects of agents on tumor growth, tumor-bearing mice were randomly divided into four groups: the control group (10% DMSO, 40% polyethylene glycol, and 5% Tween-80 in saline), CC treatment group (10mg/kg CC, intratumor injection, twice every week), PF treatment group (10mg/kg, ig, qd), and CC plus PF treatment group. Tumor volumes were measured every 3 days and calculated by the followed equation: $\text{Volume} = (\text{Length} \times \text{Width}^2)/2$. On day 15, mice were sacrificed for the tumor tissue collection.

Immunohistochemical staining

Tumor tissues were acquired and fixed overnight in 4% paraformaldehyde and then dehydrated and coated with wax. Paraffin-embedded specimens were cut into 3- μm thick sections. Slices were either dyed with hematoxylin and eosin or immunostained with the primary antibodies.

Statistical analysis

All experiments were performed at least three times in triplicate to ensure reproducibility. Statistical analyses used unpaired tailed student's *t*-tests, Kaplan-Meier survival analysis, and log-rank tests with GraphPad Prism 7 (GraphPad Software, Inc., La Jolla, CA). The results were shown as mean \pm SD. $P < 0.05$ was considered statistically significant.

Results

Penfluridol reduced cell viability and metastasis of GBC cells

We adopted a series concentration (2 μM ~10 μM) of PF to investigate the anticancer effects on human GBC cells, including SGC-996, EH-GB1, and GBC-SD. The cell viabilities were measured at 24h and 48h using a CCK-8 assay, and PF showed anticancer activity in a dose-dependent manner (Fig. 1A). A colony assay for GBC cells was also performed to determine the long-term effects of PF on GBC cells (Fig. 1B). The results indicated that 2 μM of PF significantly reduced the cloning formation rate for GBC cells. Additionally, the metastasis ability of GBC cells was reduced after PF treatment by wound healing assay and transwell migration assay (**Supplementary Fig. S1A-B**). These studies demonstrated that PF monotherapy had the potential for GBC treatment.

Penfluridol induced the apoptosis of GBC cells

Besides inhibiting the proliferation of GBC cells, we also investigated the ability of PF to induce GBC cell death. First, Annexin V/PI staining analysis was performed to detect the increase of PF in early and late apoptosis (Fig. 1C). The Western blot results revealed that PF treatment increased the expression of Noxa, reduced the expression of Bcl-2, and was associated with the elevated cleaved caspase9, caspase3, and PARP, indicating the activation of intrinsic apoptotic pathway (Fig. 1D). To further identify the apoptosis induced by PF, we performed a FACS analysis to detect the cell cycle distribution. A dose-dependent accumulation of G1 population in all SGC-996, EH-GB1, and GBC-SD cells were detected (Fig. 1E). Cyclin D1 associated with cyclin-dependent kinase 4 was crucial for the transition from G1 to S phase [17]. The Western blot results showed a decrease of both cyclin D1 and CDK4 (Fig. 1F) that was consistent with the FACS results. Taken together, PF induced cell apoptosis to suppress GBC.

Inhibiting glycolysis enhanced the anticancer effects of penfluridol

Given that promoted aerobic glycolysis is crucial for cancer cell response to the microenvironment changes and drug treatment [18], we investigated the role of glycolysis during PF treatment. Glycolysis was detected by measuring the glucose consumption and lactic acid production of the GBC cells pre-treated with different concentrations of PF. Increased glycolysis was detected with the increased PF concentrations (Fig. 2A-B).

We hypothesized that the promoted glycolysis was critical for the anticancer effects of PF. Thus, we examined whether glycolysis inhibitor 2-DG would influence the anticancer effects of PF on GBC cells. Using 2-DG could reduce the glycolysis level after PF treatment (Fig. 2C-D). Expectedly, 2-DG combining with PF significantly reduced cell growth and clone formation ability, which indicated a synergetic interaction between 2-DG and PF for GBC cells (Fig. 2E and **Fig. S2A**). To further determine the synergetic interaction, Annexin V/PI staining analysis was performed and showed that the combination of 2-DG and PF significantly induced apoptosis in GBC cells compared to monotherapy and control groups (Fig. 2F). Western bolt also showed that combination therapy significantly elevated cleaved caspase levels, indicating more potent apoptosis (Fig. 2G). Additionally, we also performed wound healing assay and transwell migration assay and found that combining 2-DG with PF suppressed metastasis abilities of GBC cells more significantly compared to PF treatment alone (**Fig. S2B-C**). These findings suggested that promoted glycolysis was a cytoprotective process in GBC cells in response to PF treatment.

Activation of AMPK/PFKFB3 mediated the glycolysis after penfluridol treatment

Activation of AMPK, as well as its downstream factors within glycolysis PFKFB3, were detected after PF treatment for 24h in all three GBC cell lines (Fig. 3A). Additionally, HK2 did not have significant expression changes after different concentrations of PF treatment at 24h. Thus, we hypothesized that activation of the AMPK/PFKFB3 pathway was the major mechanism that mediated glycolysis after PF treatment.

To identify the exact effects that AMPK promoted glycolysis after PF treatment, AMPK knockdown by siRNA and A-769662 (AMPK activator) was used to further evaluate the role of AMPK signaling during PF treatment. After PF treatment, the glucose consumption and lactic acid production of AMPK knockdown groups were less than knockdown control groups, and A-769662 combination treatment augmented the glycolysis level compared to PF monotherapy (Fig. 3B-E). Western blot detected reduced phosphorylation levels of p-AMPK and p-PFKFB3 in the AMPK knockdown group and increased phosphorylation levels of p-AMPK and p-PFKFB3 in the A-769662 combination treatment group, respectively (Fig. 3F-G). These results indicated that activation of AMPK/PFKFB3, especially the phosphorylation levels, was the critical pathway to mediate the glycolysis during PF treatment.

AMPK inhibitor dorsomorphin dihydrochloride enhanced the anticancer effects of penfluridol

Given that the activation of the AMPK/PFKFB3 pathway promoted glycolysis, which impaired the anticancer effects of PF, we used the selective AMPK inhibitor CC to identify the function of the AMPK/PFKFB3 pathway within the anticancer functions of PF in GBC cells. First, we identified that CC treatment could significantly reduce glucose consumption and lactic acid production after PF treatment at 24h (Fig. 4A-B). Then, the CCK8 cell viability assay indicated that co-treated with CC and PF could significantly reduce cell viabilities of GBC cells compared to PF treatment alone (Fig. 4C). As the results showed, the co-treatment group had worse clone formation abilities compared to monotherapy groups and the control group (Fig. 4D). Moreover, CC had a significant synergistic effect with PF to induce apoptosis in GBC cells, accompanied by the accumulation of cleaved caspase and inhibition of AMPK/PFKFB3 phosphorylation (Fig. 4E-F). Additionally, GBC cells treated with CC and PF combination regimen also showed weaker invasion and migration ability compared to PF monotherapy (Fig. S3A-B). In general, the *in vitro* studies revealed that PF had potential anticancer effects in GBC cells, and activation of AMPK/PFKFB3 signaling pathway mediated glycolysis after PF treatment could attenuate its anticancer effects. Co-treated with CC suppressing the phosphorylated AMPK/PFKFB3 signaling level could augment the anticancer effects of PF. The mechanism was shown in Fig. 4G.

Penfluridol in combination with dorsomorphin dihydrochloride effectively suppress the tumor xenografts in vivo

To further explore the feasibility of the combination regimen *in vivo*, we built a mice PDX model. After being transplanted with tumor tissue for 10 days, mice were randomly separated into four groups (n=5, control group, CC group, PF group, and CC+PF group). The tumor-bearing mice were treated with vehicle control, CC (peritumoral injection, 10mg/kg, twice a week), PF (intragastric administration, 10mg/kg, q.d.), or CC+PF combination for 15 days (Fig. 5A). The weights of mice in treatment groups decreased at the beginning of the treatment and reached normal weight compared to the mice in the control group (Fig. 5B). Treatment with CC or PF alone both inhibited tumor growth, while CC+PF combination

treatment decreased tumor weights more significantly (Fig. 5C). The tumor growth curve data also indicated the potent anticancer effects of these combination regimens (Fig. 5D-E).

To examine the effects of CC and PF treatment on apoptosis and the related mechanism, H&E and immunohistochemistry were used to determine the anticancer effects of CC and PF cotreatment. Lower Ki67 was induced by CC or PF treatment, and especially, CC and PF co-treatment (Fig. 5F). Additionally, more significant p-AMPK and cleaved-caspase 3 were induced in tumors obtained from the CC+PF group compared to vehicle control or monotherapy mice. Western blotting demonstrated that *in vivo* anticancer mechanism was consistent with *in vitro* studies (Fig. 5G).

Discussion

In this study, we demonstrated that PF could induce apoptosis to effectively suppress GBC cells proliferation. Moreover, activation of AMPK/PFKFB3-mediated glycolysis attenuated PF response in GBC cells. The AMPK inhibitor CC suppressed the AMPK/PFKFB3 mediated glycolysis, which enhanced the anticancer effects of PF compared to PF monotherapy in GBC cells. The synergetic effects of AMPK inhibition and PF was further validated in a PDX model.

The tumor-specific metabolism, also known as the Warburg effects, supports rapid cancer cells proliferation and tumor growth by converting OXPHOS into glycolysis even in the aerobic environment [12]. In GBCs, glycolysis is a crucial process to support tumor initiation and progression, which could be developed for cancer therapy [19, 20]. For instance, the anti-estrogen drug, tamoxifen, suppressed GBC viability by impairing glycolysis [21]. LncRNA PVT1 could modulate glycolysis by regulating miR-143-HK2 to promote GBC growth [19]. On the contrary, promoted glycolysis is also associated with drug resistance in GBCs [22]. Interestingly, knockdown of UCP2 inhibited GBC cells proliferation and glycolysis, and these cells became sensitive to gemcitabine treatment, indicating the role of glycolysis in the chemoresistance [22]. In our study, suppression of glycolysis significantly enhanced the anticancer effects of PF in GBC cells, demonstrating that glycolysis also contributed to the PF resistance in GBC cells.

AMPK is a highly conserved energy regulator that plays a crucial role in maintaining energy homeostasis [23]. Several studies demonstrated that activation of the AMPK pathway was a crucial process for cancer growth, even drug resistance, under energy stress conditions [24–26]. AMPK activation under energy stress could induce the NADPH generation pathway to maintain cellular redox hemostasis and inhibit cell death [27]. Moreover, for cells during mitosis, promoted glycolysis mediated by the AMPK PFKFB3 signaling pathway was a determinant for survival [28]. In our study, AMPK activation was detected after PF treatment, and the activated AMPK promoted glycolysis by activating its downstream factor PFKFB3. Inhibition of AMPK/PFKFB3 signaling mediated glycolysis enhanced the anticancer effects of PF in GBC cells. Therefore, AMPK/PFKFB3 mediated glycolysis was important for PF response in GBC cells.

The anticancer mechanism of PF has been proposed by previous studies [9, 10, 29–31]. D₂ receptor and T-type channel were major antipsychotic-related targets of PF to suppress cancer proliferation [32, 33].

Other signaling pathways such as integrin signaling pathway and autophagy pathway were also reported in the anticancer mechanism of PF. However, the connection among PF, glycolysis, and AMPK was rarely studied. The suppressive role of PF on glycolysis was reported by downregulating HK2 [16]. For AMPK, PF could activate it to further induce autophagy, while the connection between PF-induced activation of AMPK and glycolysis was not studied [8]. As mentioned above, glycolysis is crucial for the GBC progression and even chemoresistance. Our study demonstrated that glycolysis mediated by activation of the AMPK/PFKFB3 signaling pathway was important in the anticancer mechanism of PF in GBCs.

There were several limitations to be addressed in the study. On the one hand, although a novel anticancer strategy (a combination of PF treatment and AMPK inhibition) was proposed for GBCs, further studies would be conducted to explore the underlying anticancer mechanisms. On the other hand, we found that the regulation of AMPK/PFKFB3 signaling pathway in GBCs after PF treatment may be a potential cause of PF resistance, so the resistance mechanism of PF would be further investigated to improve its anticancer effects.

Conclusion

In summary, our study repurposed the anticancer effects of PF on GBCs, while its anticancer activity was limited due to AMPK/PFKFB3 activation mediated glycolysis. Therefore, the combination of AMPK inhibition and PF treatment would be a potential therapeutic strategy for GBCs.

Abbreviations

PF
pemfluridol
GBC
gallbladder cancer
HK2
hexokinase-2
2-DG
2-Deoxy-D-glucose
CC
dorsomorphin dihydrochloride/Compound C
CCK-8
Cell Counting Kit-8.

Declarations

Author contributions

MYC and XJC conceived and designed the experiments. JHH, JSC, and BZ performed the experiments. WT and SJ wrote the manuscript. SJL, TEC, and ZYL composed the Figures. All authors have read and agreed with the published version of the manuscript.

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Availability of data and materials

The raw images files of western blots used in the manuscript are included within the article and its additional files. Other datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Human-related ethics was approved by the Sir Run-Run Shaw Hospital (SRRSH) of Medicine Institutional Review Board (IRS reference number: 20210716-37) for the collection of human gallbladder cancer tissues from surgeries performed at Sir Run-Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, China.

All animal experimental protocol was approved by the SRRSH of Medicine Institutional Review Board and followed the National Guidelines for animal protection.

Consent for publication

All authors agreed on the manuscript.

Competing Interests

Competing interests: The authors declared no competing interests.

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References

1. Misra S, Chaturvedi A, Misra NC, Sharma ID: **Carcinoma of the gallbladder.** *Lancet Oncol* 2003, **4**(3):167–176.
2. Goetze TO: **Gallbladder carcinoma: Prognostic factors and therapeutic options.** *World J Gastroenterol* 2015, **21**(43):12211–12217.
3. Benson AB, D'Angelica MI, Abbott DE, Anaya DA, Anders R, Are C, Bachini M, Borad M, Brown D, Burgoyne A *et al.*: **Hepatobiliary Cancers, Version 2.2021, NCCN Clinical Practice Guidelines in Oncology.** *J Natl Compr Canc Netw* 2021, **19**(5):541–565.
4. Valle J, Wasan H, Palmer DH, Cunningham D, Anthoney A, Maraveyas A, Madhusudan S, Iveson T, Hughes S, Pereira SP *et al.*: **Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer.** *N Engl J Med* 2010, **362**(14):1273–1281.
5. Xie J, Xia L, Xiang W, Wenzhuo H, Yin H, Wang F, Gao T, Qi W, Yang Z, Yang X *et al.*: **Metformin selectively inhibits metastatic colorectal cancer with the KRAS mutation by intracellular accumulation through silencing MATE1.** *Proc Natl Acad Sci USA* 2020, **117**(23):13012–13022.
6. Veschi S, De Lellis L, Florio R, Lanuti P, Massucci A, Tinari N, De Tursi M, di Sebastiano P, Marchisio M, Natoli C *et al.*: **Effects of repurposed drug candidates nitroxoline and nelfinavir as single agents or in combination with erlotinib in pancreatic cancer cells.** *Journal of Experimental & Clinical Cancer Research* 2018, **37**.
7. Dalton SO, Johansen C, Poulsen AH, Nørgaard M, Sørensen HT, McLaughlin JK, Mortensen PB, Friis S: **Cancer risk among users of neuroleptic medication: a population-based cohort study.** *Br J Cancer* 2006, **95**(7):934–939.
8. Varalda M, Antona A, Bettio V, Roy K, Vachamaram A, Yellenki V, Massarotti A, Baldanzi G, Capello D: **Psychotropic Drugs Show Anticancer Activity by Disrupting Mitochondrial and Lysosomal Function.** *Front Oncol* 2020, **10**:562196.
9. Tuan NM, Lee CH: **Penfluridol as a Candidate of Drug Repurposing for Anticancer Agent.** *Molecules* 2019, **24**(20).
10. Hedrick E, Li X, Safe S: **Penfluridol Represses Integrin Expression in Breast Cancer through Induction of Reactive Oxygen Species and Downregulation of Sp Transcription Factors.** *Mol Cancer Ther* 2017, **16**(1):205–216.
11. Hung W-Y, Chang J-H, Cheng Y, Cheng G-Z, Huang H-C, Hsiao M, Chung C-L, Lee W-J, Chien M-H: **Autophagosome accumulation-mediated ATP energy deprivation induced by penfluridol triggers nonapoptotic cell death of lung cancer via activating unfolded protein response.** *Cell death & disease* 2019, **10**(8):538.
12. Hanahan D, Weinberg RA: **Hallmarks of cancer: the next generation.** *Cell* 2011, **144**(5):646–674.
13. Pavlova NN, Thompson CB: **The Emerging Hallmarks of Cancer Metabolism.** *Cell Metab* 2016, **23**(1):27–47.

14. Ganapathy-Kanniappan S, Geschwind J-FH: **Tumor glycolysis as a target for cancer therapy: progress and prospects.** *Mol Cancer* 2013, **12**:152.
15. Obre E, Rossignol R: **Emerging concepts in bioenergetics and cancer research: metabolic flexibility, coupling, symbiosis, switch, oxidative tumors, metabolic remodeling, signaling and bioenergetic therapy.** *Int J Biochem Cell Biol* 2015, **59**:167–181.
16. Wang N-N, Zhang P-Z, Zhang J, Wang H-N, Li L, Ren F, Dai P-F, Li H, Lv X-F: **Penfluridol triggers mitochondrial-mediated apoptosis and suppresses glycolysis in colorectal cancer cells through down-regulating hexokinase-2.** *Anat Rec (Hoboken)* 2021, **304**(3):520–530.
17. Qie S, Diehl JA: **Cyclin D1, cancer progression, and opportunities in cancer treatment.** *Journal of Molecular Medicine-Jmm* 2016, **94**(12):1313–1326.
18. Cao Y: **Adipocyte and lipid metabolism in cancer drug resistance.** *J Clin Invest* 2019, **129**(8):3006–3017.
19. Chen J, Yu Y, Li H, Hu Q, Chen X, He Y, Xue C, Ren F, Ren Z, Li J *et al*: **Long non-coding RNA PVT1 promotes tumor progression by regulating the miR-143/HK2 axis in gallbladder cancer.** *Mol Cancer* 2019, **18**(1):33.
20. Xue C, He Y, Hu Q, Yu Y, Chen X, Chen J, Ren F, Li J, Ren Z, Cui G *et al*: **Downregulation of PIM1 regulates glycolysis and suppresses tumor progression in gallbladder cancer.** *Cancer Manag Res* 2018, **10**:5101–5112.
21. Huang S, Wang H, Chen W, Zhan M, Xu S, Huang X, Lin R, Shen H, Wang J: **Tamoxifen inhibits cell proliferation by impaired glucose metabolism in gallbladder cancer.** *J Cell Mol Med* 2020, **24**(2):1599–1613.
22. Yu J, Shi L, Lin W, Lu B, Zhao Y: **UCP2 promotes proliferation and chemoresistance through regulating the NF- κ B/ β -catenin axis and mitochondrial ROS in gallbladder cancer.** *Biochem Pharmacol* 2020, **172**:113745.
23. Garcia D, Shaw RJ: **AMPK: Mechanisms of Cellular Energy Sensing and Restoration of Metabolic Balance.** *Mol Cell* 2017, **66**(6):789–800.
24. Wang Z, Liu P, Chen Q, Deng S, Liu X, Situ H, Zhong S, Hann S, Lin Y: **Targeting AMPK Signaling Pathway to Overcome Drug Resistance for Cancer Therapy.** *Curr Drug Targets* 2016, **17**(8):853–864.
25. Lee H, Zandkarimi F, Zhang Y, Meena JK, Kim J, Zhuang L, Tyagi S, Ma L, Westbrook TF, Steinberg GR *et al*: **Energy-stress-mediated AMPK activation inhibits ferroptosis.** *Nat Cell Biol* 2020, **22**(2).
26. An Y, Wang B, Wang X, Dong G, Jia J, Yang Q: **SIRT1 inhibits chemoresistance and cancer stemness of gastric cancer by initiating an AMPK/FOXO3 positive feedback loop.** *Cell death & disease* 2020, **11**(2).
27. Jeon S-M, Chandel NS, Hay N: **AMPK regulates NADPH homeostasis to promote tumour cell survival during energy stress.** *Nature* 2012, **485**(7400):661–665.
28. Doménech E, Maestre C, Esteban-Martínez L, Partida D, Pascual R, Fernández-Miranda G, Seco E, Campos-Olivas R, Pérez M, Megias D *et al*: **AMPK and PFKFB3 mediate glycolysis and survival in response to mitophagy during mitotic arrest.** *Nat Cell Biol* 2015, **17**(10):1304–1316.

29. Xue Q, Liu Z, Feng Z, Xu Y, Zuo W, Wang Q, Gao T, Zeng J, Hu X, Jia F *et al*: **Penfluridol: An antipsychotic agent suppresses lung cancer cell growth and metastasis by inducing G0/G1 arrest and apoptosis.** *Biomed Pharmacother* 2020, **121**:109598.
30. Gupta N, Gupta P, Srivastava SK: **Penfluridol overcomes paclitaxel resistance in metastatic breast cancer.** *Sci Rep* 2019, **9**(1):5066.
31. Wu S-Y, Wen Y-C, Ku C-C, Yang Y-C, Chow J-M, Yang S-F, Lee W-J, Chien M-H: **Penfluridol triggers cytoprotective autophagy and cellular apoptosis through ROS induction and activation of the PP2A-modulated MAPK pathway in acute myeloid leukemia with different FLT3 statuses.** *J Biomed Sci* 2019, **26**(1):63.
32. Weissenrieder JS, Neighbors JD, Mailman RB, Hohl RJ: **Cancer and the Dopamine D Receptor: A Pharmacological Perspective.** *J Pharmacol Exp Ther* 2019, **370**(1):111–126.
33. Dziegielewska B, Gray LS, Dziegielewski J: **T-type calcium channels blockers as new tools in cancer therapies.** *Pflugers Arch* 2014, **466**(4):801–810.

Figures

Figure 1

The anticancer effects of penfluridol on GBC. A SGC-996, EH-GB1, and GBC-SD cells were treated with PF as indicated for 24h/48h. Cell viability was measured by the CCK-8 assay. The IC50 was determined by GraphPad Prism 7.0. B SGC-996, EH-GB1, and GBC-SD cells were seeded in 6-well plates and treated with PF as indicated for 14 days. C SGC-996, EH-GB1, and GBC-SD cells were treated with PF as indicated for 24h. The cell apoptosis was quantified by the Annexin V/PI assay. D SGC-996, EH-GB1, and GBC-SD were treated with PF as indicated for 24h before Western blotting. E SGC-996, EH-GB1, and GBC-SD cells were treated with PF as indicated for 24h. The cell cycle was quantified by the cell cycle assay. F SGC-996, EH-GB1, and GBC-SD were treated with PF as indicated for 24h before Western blotting. *P <0.5, **P <0.01, ***P <0.001, ****P <0.0001.

Figure 2

Inhibition of glycolysis enhanced the anticancer effects of penfluridol. A SGC-996, EH-GB1, and GBC-SD cells were treated with PF as indicated for 24h before glucose consumption measurement. B SGC-996, EH-GB1, and GBC-SD cells were treated with PF as indicated for 24h before lactic acid production measurement. C&D SGC-996, EH-GB1, and GBC-SD cells were treated with control, 2-DG (2mM), PF (5µM), or 2-DG+PF before glucose consumption and lactic acid production measurement. E SGC-996, EH-GB1, and GBC-SD cells were treated with controls, 2-DG (2mM), PF (1µM), or 2-DG+PF for 14 days before colony formation assay. F SGC-996, EH-GB1, and GBC-SD cells were treated with control, 2-DG (2mM), PF

(5 μ M), or 2-DG+PF for 24h before Annexin V/PI assay. G SGC-996, EH-GB1, and GBC-SD were treated with control, 2-DG (2mM), PF (5 μ M), or 2-DG+PF for 24h before Western blotting. *P <0.5, **P <0.01, ***P <0.001, ****P <0.0001.

Figure 3

Activation of AMPK/PFKFB3 signaling pathway mediated the glycolysis after penfluridol treatment. A SGC-996, EH-GB1, and GBC-SD cells were treated with PF as indicated for 24h before Western blotting. B SGC-996, EH-GB1, and GBC-SD cells were treated as indicated with or without PF (5 μ M) before glucose consumption measurement. C SGC-996, EH-GB1, and GBC-SD cells were treated as indicated with or without PF (5 μ M) before lactic acid production measurement. D SGC-996, EH-GB1, and GBC-SD cells were treated with control, A-769662 (100 μ M), PF (5 μ M), and A-769662+PF before glucose consumption measurement. E SGC-996, EH-GB1, and GBC-SD cells were treated with control, A-769662 (100 μ M), PF (5 μ M), and A-769662+PF before lactic acid production measurement. F SGC-996, EH-GB1, and GBC-SD cells were treated as indicated with or without PF (5 μ M) before Western blotting. G SGC-996, EH-GB1, and GBC-SD cells were treated with control, A-769662 (100 μ M), PF (5 μ M), and A-769662+PF for 24h before Western blotting. **P <0.01, ***P <0.001, ****P <0.0001.

Figure 4

AMPK inhibitor CC enhanced the anticancer effects of penfluridol. A SGC-996, EH-GB1, and GBC-SD cells were treated with control, CC (10 μ M), PF (5 μ M), and CC+PF before glucose consumption measurement. B SGC-996, EH-GB1, and GBC-SD cells were treated with control, CC (10 μ M), PF (5 μ M), and CC+PF before lactic acid measurement. C SGC-996, EH-GB1, and GBC-SD cells were treated with PF as indicated with or without CC (10 μ M) for 24h/48h. Cell viability was measured by the CCK-8 assay. D SGC-996, EH-GB1, and GBC-SD cells were treated with control, CC (10 μ M), PF (5 μ M), and CC+PF for 14 days for colony formation assay. E SGC-996, EH-GB1, and GBC-SD cells were treated with control, CC (10 μ M), PF (5 μ M), and CC+PF for 24h. The cell apoptosis was quantified by the Annexin V/PI assay. F SGC-996, EH-GB1, and GBC-SD cells were treated with control, CC (10 μ M), PF (5 μ M), and CC+PF for 24h before Western blotting. G The brief graph shows the molecular mechanism of the anticancer effects of PF in GBC cells. *P<0.05, ***P<0.001, ****P<0.0001.

Figure 5

Penfluridol in combination with CC effectively suppresses the tumor xenografts in vivo. A The working model shows the process of our animal studies. Mice were transplanted with tumor tissues for 10 days and randomly divided into four groups (control, CC (10mg/kg), PF (10mg/kg), CC+PF; n=5/group). Then, the tumor-bearing mice were treated with intragastric administration and intratumor injection with a

dissolvent, intratumor injection with CC, intragastric administration with PF, and CC+PF co-treatment. All mice were sacrificed on day 15, and the tumors were collected for subsequent experiments. B The body weight change of mice after indicated treatment. C Tumor weight of the mice. D Tumor growth curves of tumor tissues after indicated treatment. E Photograph of the transplanted tumors after indicated treatment. F The H&E results and IHC staining assay for Ki-67, Cleaved-caspase3, p-AMPK T172 of the tumors. G Western blotting assay result of the tumors with indicated treatment. **P<0.01, ****P<0.0001.

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