CDK7 inhibition augments the response against multidrug chemotherapy in pancreatic cancer

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Abstract

Background: Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer with a dismal prognosis. Although combined treatment with gemcitabine and albumin-bound paclitaxel has improved the prognosis of PDAC, both intrinsic and acquired chemoresistance cloud the outcomes. Hence, there is an urgent need to explore new therapeutic targets and formulate innovative strategies for the more effective treatment of PDAC.

Methods: We transduced pancreatic cancer cells with a protein kinase library, and transfected cells were selected by puromycin treatment, followed by continuous exposure to vehicle control or gemcitabine/paclitaxel chemotherapy for 21 days. Next-generation sequencing as well as MAGeCK-VISPR analysis were performed to identify candidate genes. Finally, we used the CRISPR-Cas9 to knockout the target gene, confirming that it is a viable target for overcoming chemoresistance *in vivo* and *in vitro*.

Results: We identified several cell cycle checkpoint kinases and DNA damage-related kinases as targets for overcoming chemoresistance. Among them, cyclin-dependent kinase 7 (CDK7) ranked highly in both screenings. We demonstrated that both gene knockout and pharmacological inhibition of CDK7 by THZ1 result in cell cycle arrest, apoptosis induction. Furthermore, THZ1 synergized with gemcitabine and paclitaxel *in vitro* and *in vivo*, resulting in enhanced antitumor effects.

Conclusions: Our findings support the application of CRISPR-Cas9 screening in identifying novel therapeutic targets and provide new strategies for overcoming chemoresistance in pancreatic cancer.

Background

PDAC is the fourth leading cause of cancer-related mortality globally and is expected to overtake lung cancer as the second leading cause of cancer-related death by 2030 [1]. According to the Global Cancer Statistics 2020, a total of 495,773 new cases of pancreatic cancer (PC) and 466,003 related deaths were recorded worldwide in 2020, and the mortality rate is nearly as high as the incidence rate [2]. Despite the advances in the therapy of pancreatic cancer, the 5-year survival rate remains at 9% [3, 4]. The poor prognosis associated with PC is primarily due to the low rate of early detection, rapid progression and limited therapy options [5–7]. Currently, the first-line chemotherapy regimens for patients with advanced or metastatic pancreatic cancer are combinations of 5-fluorouracil/leucovorin with irinotecan and oxaliplatin (FOLFIRINOX), or gemcitabine plus nanoparticle albumin-bound paclitaxel (nab- never been compared head-to-head in a clinical trial, real-world retrospective analyses reveal that younger, fitter patients are more likely to receive FOLFIRINOX and have a better overall survival rate when compared to gemcitabine plus nab-paclitaxel [8, 9]. Unfortunately, the effectiveness of both schemes is frequently plagued by a lack of response against these agents [10, 11]. Hence, there is an urgent need to unravel the molecular mechanisms that limit the clinical application of chemotherapy and formulate innovative strategies for the more effective treatment of PDAC.

CRISPR-Cas9-based screening of sgRNA libraries has evolved into a potent tool for elucidating the molecular processes underlying drug response and discovering novel targets for chemotherapy [12–14]. As major regulators at nearly every developmental stage, protein kinases modulate most of the signal transmission in eukaryotic cells [15–17]. The dysregulation of protein kinases is associated with a number of carcinogenic processes including chemoresistance [18, 19]. Therefore, they represent interesting targets for CRISPR-Cas9 screens.

We applied CRISPR-Cas9 screening of protein kinases and PDAC-related genes to analyze their role in the drug sensitivity of PC cells against gemcitabine and paclitaxel. We were able to identify several candidates and we observed that deletion of CDK7 in human and murine cell lines produced the greatest degree of phenotypic change, suggesting that CDK7 expression is required for drug resistance.

CDK7, along with MAT1 and cyclin H, forms the CDK-activating complex (CAK), which directs the cell cycle advancement via T-loop phosphorylation of the cell cycle CDKs [20, 21]. Meanwhile, as a component of the multi-protein basal transcription factor TFIIH, CDK7 plays a vital role in transcription initiation and elongation by phosphorylating the C-terminal domain (CTD) of RNA polymerase II (RNAPII) [20]. Preclinical investigations have demonstrated that selective CDK7 inhibitors (CDK7i) have the potential to overcome chemoresistance by arresting the cell cycle, increasing apoptosis, and inhibiting transcription [21]. Currently, several CDK7i, CT7001 (NCT03363893), SY-1365 (NCT03134638), SY-5609 (NCT04247126), XL102 (NCT04726332) and LY3405105 (NCT03770494) have progressed to Phase I/II clinical trials (www.clinicaltrials.gov). Numerous covalent CDK7 inhibitors have also been discovered, the first of which is THZ1, that specifically targets a cysteine residue (Cys312) on the C-terminal extension of CDK7 just beyond the ATP-binding site and exhibits potent action against a variety of cancer types [20, 21].

Materials And Methods

Cell culture and reagents

Human pancreatic cancer cell lines PANC-1 (ATCC, Cat. #CRL-1469, RRID: CVCL_0480), AsPC-1 (ATCC, Cat. #CRL-1682, RRID: CVCL_0152), BxPC-3 (ATCC, Cat. #CRL-1687, RRID: CVCL_0186), MIA PaCa-2 (ATCC, Cat. #CRM-CRL-1420, RRID: CVCL_0428), SUIT-2 (JCRB, Cat. #JCRB1094, RRID: CVCL_3172), TKCC-10, Mayo4636 and murine pancreatic cancer cell line TB32047 were used and listed in Supplementary Table S1. PANC-1, AsPC-1, BxPC-3 and MIA PaCa-2 were purchased from American Type Culture Collection (ATCC). PANC-1, AsPC-1 and BxPC-3 were cultured in RPMI-1640 medium (Cat. #21875-034, Gibco) supplemented with 10% FBS (Cat. #A3160401, Gibco). MIA PaCa-2 cells were cultured in DMEM (Cat. #30966-021, Gibco) medium with 10% FBS and 2% horse serum (Cat. #16050-130, Gibco). SUIT-2 cells were purchased from Japanese Collection of Research Bioresources Cell Bank (JCRB) and cultured in MEM (Cat. #31095-029, Gibco) supplemented with 10% FBS. Patient-derived cell lines (PDCLs) TKCC-10 were cultured in conditions specifically formulated and were previously described [22]. Mayo4636 were cultured in DMEM/F12 (Cat. #11320-074, Gibco) supplemented with 10% FBS. Primary

murine pancreatic cancer cell line TB32047 was kindly provided by David Tuveson, Cold Spring Harbor Laboratory (CSHL) and cultured in DMEM medium supplemented with 10% FBS. HEK293TN (BioCat, Cat# LV900A-1-GVO-SBI, RRID: CVCL_UL49) cells were obtained from BioCat and maintained in DMEM supplemented with 10% FBS. All cell lines were grown in monolayers at 37°C in a humidified environment containing 5% CO₂ and tested for Mycoplasma contamination. Human cell line identity was confirmed by STR analysis. All cells were harvested by 0.25% Trypsin-EDTA (Cat. #25200-072, Gibco). DNA fingerprinting by highly polymorphic short tandem repeat (STR) analysis was used to authenticate cells, and mycoplasma testing was performed on a regular basis to ensure cells were free from mycoplasma contamination.

The chemotherapy drugs gemcitabine (GEM, 40 mg/mL) and paclitaxel (PTX, 6 mg/mL) were purchased from the pharmacy of the Universitätsklinikum Erlangen in ready-made solutions and nab-Paclitaxel (Pazenir, 5 mg/ml) was purchased from Ratiopharm GmbH, Germany. The small-molecule inhibitor targeting CDK7 (THZ1, Cat. #HY-80013) was purchased from MedChemExpress.

sgRNA library and lentivirus production

The pooled-sgRNA library (Mouse Brie kinome pooled library) targeting murine kinome was a gift from John Doench and David Root (Addgene, RRID: Addgene_75316) with additional sgRNAs for genes involved in pancreatic cancer [23] (library files, please see Supplementary table). The lentivirus was produced as described previously [20] and the plasmids are listed in Supplementary Table S2. Briefly, three T175 flasks of HEK293TN cells were plated at 30% confluence in antibiotic-free DMEM supplemented with 10% FBS. Transfection was performed with Lipofectamine™ 3000 Transfection Reagent (Cat. # L3000001, Invitrogen). For each flask, 13.8 µg of pooled-sgRNA library, 9.2 µg of pMDLg/pRRE (Cat. #12251, Addgene), 4.6 µg of pRSV-REV (Cat. #12253, Addgene), 4.6 µg of pMD2.G (Cat. #12259, Addgene), 64.4 µL P3000 Enhancer Reagent and 129 µL of Lipofectamine™ 3000 diluted in OptiMEM (Cat. #31985070, Gibco) were mixed and added to the HEK293TN cells. pMDLg/pRRE (Addgene; RRID: Addgene_12251), pRSV-REV (Addgene; RRID: Addgene_12253) and pMD2.G (Addgene, RRID: Addgene_12259) were gifts from Didier Trono [24]. Medium was changed 6 hours post transfection, and virus-containing medium was collected 24 hours later and filtered through a 0.45 µm strainer.

CRISPR-Cas9 knockout screening with protein kinases library

The CRISPR screen was performed essentially as described previously [12]. Briefly, 1.8 million TB32047 cells were transduced triplicates with kinome-wide sgRNA lentivirus (MOI~0.3). Cells were then selected with puromycin (10 µg/mL) for 3 days. Ten million transfected cells were harvested as baseline (D0 treatment). The rest of survival cells were divided into three groups (T75 flask/group) following treatment with vehicle (PBS), gemcitabine (IC₉₀= 50 nM) or paclitaxel (IC₇₀ = 10 nM) for 21 days. At least 10 million cells for each group were collected for DNA sequencing at the end of the treatment.

Genomic DNA sequencing and data analysis

The isolation of genomic DNA (gDNA) was performed with a NucleoSpin Blood L kit (Cat. #740954.20, Macherey-Nagel) and followed by the PCR procedure to amplify sgRNAs. For the PCR reaction, 10 µg DNA was used in order to achieve 300X coverage. For each sample, Q5 master mix was used to perform 2 separate 100 µl reactions with 5 µg of genomic DNA in each reaction and then combined the resulting amplicons. All PCR primers are list in Supplementary Table S2 and were synthesized by Eurofins. The resulting PCR amplicons from the PCR reactions were purified and sequenced by NovaSeq 6000 (Illumina). The number of reads for each sgRNA was quantified and normalized to total reads of all sgRNAs using MAGECK-VISPR software as described previously [25]. The result of the sequencing can be found at EBI database accession: PRJEB48728.

Cell viability assay

Cell viability was performed as described previously [26]. Briefly, cells were plated as 10,000 cells per well in grown medium on a 96-well black plate (Cat. #3603, Corning) for 24 h. After that, the chemotherapy drug was added to the well to final concentration. Cells were cultured for another 72 h and stained with DAPI by Hoechst 33342 (Cat. #H3570, Life Technologies) and imaged in 20 fields for each well by an Evos FL Auto 2 imaging system (Cat. #AMAFD2000, Invitrogen). Images were counted by HCS studio cell analysis software V2.0 (Thermo, SX000041A, Waltham, MA, USA). Each data point was normalized to untreated control and generated in triplicate and each experiment was done three times (n=3). IC50 curves were generated in GraphPad Prism [(log (inhibitor) vs response (-variable slope four parameters)]. Combination Index (CI) was calculated by ComboSyn software (ComboSyn Inc., <http://www.combosyn.com/>).

Clonogenic assay

In brief, clonogenic assay was done using exponentially grown cells seeded in 6-well plates (1,000–2,000 cells/well). Cells were treated with drugs for 3 days and cultured without drugs for 7 to 10 days depending on the growth rate, colonies were stained with 0.5% crystal violet (Cat. #61135, Sigma Aldrich). Colonies of at least 50 cells were counted and the surviving fraction was normalized to the corresponding untreated control group.

Luminescent caspase 3/7 activity assay

Luminescent caspase 3/7 activity assay was conducted with Caspase-Glo 3/7 Assay Systems (Cat. #G8091, Promega) as described by the manufacturer. The appropriate amounts of cells was seeded in white 96-well plates (Cat. #10158721, Thermo Fisher Scientific) and incubated for 24 h. Subsequently, drugs and DMSO were added to the cells and incubated for a further 24h. An equal amount of Caspase-3/7-Glo reagent was added to the wells, gently mix contents of wells and incubated for one hour at room temperature in dark. Luminescence value was detected by a luminescent plate reader (Infinite 200 PRO, Tecan). Background signal (medium and Caspase 3/7 Glo reagent only) was subtracted and data were normalized to vehicle-treated cells.

Western blot

Western blot analysis was performed as described [26]. In brief, cells were lysed in RIPA buffer (Cat. #89900, Thermo Fisher Scientific) containing protease and phosphatase Inhibitor (Cat. #78442, Thermo Fisher Scientific), and the protein concentration was determined photometrically using BCA Protein Assay Kit (Cat. #23250, Thermo Fisher Scientific). Equal amounts of total protein were separated on 4-12% NUPAGE Bis-Tris gels (Cat. #NP0322BOX; Thermo Fisher Scientific) using the Mini Gel Tank chamber system (Cat. #A25977, Invitrogen), and proteins were transferred to a nitrocellulose membrane (Cat. #GE10600003, Sigma Aldrich). Membranes were then blocked in blocking buffer (Cat. # A0830.1000, AppliChem GmbH) for 1 hour at room temperature and incubated with appropriate primary antibodies (Supplementary Table S3) overnight at 4°C. HRP-linked anti-rabbit IgG or anti-mouse IgG were used as the secondary antibody. Signal detection was performed by an Amersham Imager 600 (Pittsburgh, PA, USA) with SignalFire™ ECL Reagent (Cat. #6883S, Cell Signaling Technology).

Cell-cycle analysis and apoptosis assays

Treated cells were harvested and fixed with 75% ethanol at -20°C overnight, washed with ice-cold PBS and incubated with RNase (200 µg/ml) for 60min at 37°C and stained with propidium iodide (PI; 50 µg/ml, Cat. #421301, BioLegend), quantitative analysis was performed by flow cytometry on a BD Biosciences LSRII flow cytometer.

For apoptosis assay, cells treated with vehicle or drugs in the supernatant were detached with Trypsin-EDTA, collected, washed with PBS and stained with FITC Annexin V Apoptosis Detection Kit I (Cat. #556547, BD Pharmingen) according to the manufacturer's instructions. Subsequently measured on a BD Biosciences LSRII flow cytometer. The flow cytometry results were analyzed by using FlowJo™ v10.8 Software (BD Life Sciences).

In vivo mouse experiments

Experiments were performed on female 8–10-week-old C57BL/6JRj mice from Janvier-Labs weighing 18–20 g. Experiments were conducted following the German legislation on the protection of animals and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council; NIH Guide, vol. 25, no. 28, 1996). Murine pancreatic ductal organoid culture was described previously [27]. In brief, TB32047 cells were harvested and evenly mixed with Matrigel (Cat. #354230, VWR). Then 50 µL domes of the cell/Matrigel suspension were plated into wells of a pre-warmed 24-well plate. After Matrigel was solidified, 500 µL of pre-warmed Feeding Medium was added. The components of Feeding Medium were listed in Supplementary Table S1. During culturing, medium was refreshed after 2 to 3 days. 7 days after seeding, organoids were harvested and for each mouse, 50,000 organoids suspended in 20 µl of fresh Matrigel and orthotopically injected to the tail of the pancreas via laparotomy. The tumor-bearing mice, when tumor sizes reached 70-120 mm³, were randomized into 4 groups (5-8 mice/group) namely Vehicle (V, 10 µL/g 0.85% NaCl + DMSO, ip), THZ1 (T, 5 mg/kg, ip, 5 days/week), gemcitabine and nab-paclitaxel (GP, 50 mg/kg GEM ip, 5 mg/kg nab-PTX iv, Q4Dx4), gemcitabine and nab-paclitaxel combined with THZ1 (GPT, 50 mg/kg GEM ip, 5 mg/kg nab-PTX iv, Q4Dx4, 5 mg/kg, THZ1 ip, 5 days/week). Tumor growth and progression were monitored daily in mice,

and tumor weight was estimated using the formula: tumor weight (mg) = (length in mm) × (width in mm)²/2. Tumor-bearing mice (treatment, vehicle) were sacrificed on day 16 or when they became moribund before tumor volume reached 2000 mm³. At the end of each experiment, tumors were removed from the mice of all groups for further analysis.

Statistical analysis

Statistical analyses were performed by using GraphPad Prism (GraphPad Prism, RRID:SCR_002798; <http://www.graphpad.com>) version 8.0 unless otherwise indicated. The data in all experiments represent biological replicates (n) and are shown as mean standard deviation (mean ± SD) as described in the figure legends. The unpaired, two-tailed Student *t* test, one-way ANOVA, or two-way ANOVA with Tukey multiple comparisons test were used to compare mean values, as shown in the figure legends. In all analyses, *P* < 0.05 were considered statistically significant.

Results

CRISPR-Cas9 screening identifies genes whose deficiency sensitizes pancreatic cancer cells to chemotherapy.

Currently, weak treatment response represents a crucial factor in restricting the survival rate of pancreatic cancer. To explore new mediators of chemotherapy sensitivity, we performed CRISPR-Cas9 knockout screening in pancreatic cancer cell line TB32047 derived from the KPC mouse model. We transduced TB32047-cas9 cells with a protein kinase library with additional sgRNAs enriched for PDAC associated genes containing 3438 small-guide RNAs (sgRNA) that target 920 murine genes and 110 non-targeting control sgRNAs. Transfected cells were selected by puromycin treatment, followed by continuous exposure to vehicle control or gemcitabine/paclitaxel chemotherapy for 21 days (Fig. 1A). We experimentally determined the optimal concentration of the drugs in TB32047 cells (Supplementary Fig. S1A and S1B). Continuously treatment of transfected TB32047 cells with gemcitabine at IC90 and paclitaxel at IC70 resulted in a strong cell proliferation inhibition and cell death. Subsequently, genomic DNA was isolated from transfected and puromycin-selected cells treated with vehicle control, gemcitabine or paclitaxel, and referred to as TB-C3W and TB-G3W/TB-P3W, respectively. sgRNAs were quantified by next-generation sequencing (Supplementary Fig. S1C, Supplementary Table S2).

We then analyzed the sgRNA counts of each group and scored genes targeted by sgRNA in TB-C3W versus TB-G3W/TB-P3W. We prioritized targets according to commonalities among sgRNAs, meaning that genes were selected which had multiple sgRNAs significantly reduced in TB-C3W compared with TB-G3W/TB-P3W. We ranked individual sgRNAs based on log₂ fold-change (FC) < -1 and adjusted *P* < 0.05, and finally identified 33 genes as candidates in gemcitabine drug screening (Fig. 1B; Supplementary Table S4), while 47 genes were determined in paclitaxel drug screening (Fig. 1C; Supplementary Table S4). Gene ontology (GO) terms biological process analysis (DAVID Bioinformatics Resources 6.8) indicated that these genes were involved in cell division, protein phosphorylation, cell cycle, mitotic

nuclear division, DNA repair, apoptotic process, and cellular response to DNA damage stimulus (Supplementary Fig. S1D and S1E).

We took the intersection of the top 20 genes in the two drug screenings, with a total of four identical gene hits, namely Cdk7, Wee1, Fgfr1 and Havcr2 (Fig. 1D), whose loss of function by specific sgRNAs led to lethality in presence of gemcitabine and paclitaxel. Furthermore, Cdk7 was took second and first place in screening for gemcitabine and paclitaxel, respectively (Supplementary Table S4). Interestingly, CDK7 is involved in a wide spectrum of biological processes, including cell-cycle regulation and DNA damage response [28]. The majority of CDK7-targeting sgRNA standardized read counts were considerably decreased following treatment with gemcitabine and paclitaxel (Fig. 1E). Data from TCGA-PDAC indicated that increased CDK7 expression is statistically associated with poor overall survival in PDAC, and CDK7 is upregulated in pancreatic cancer as compared to normal tissues (Fig. 1F and G). Furthermore, CDK7 is expressed in PC cell lines of different subtypes (Fig. 1H). Therefore, we focused our subsequent analyses on the role of CDK7 in the cellular response to chemotherapy.

Knockout of CDK7 combined with gemcitabine and paclitaxel chemotherapy enhances the antiproliferative effects and apoptosis in pancreatic cancer cells.

To confirm CDK7 as a potential therapeutic target for enhancing the impact of chemotherapy, we produced CDK7 stable knockout (KO) subclones by transfecting TB32047 and MIA PaCa-2 cells with four sgRNAs (Fig. 2A; Supplementary Table S4). Western blotting confirmed that sgRNA1, sgRNA2, sgRNA4 sequences in TB32047 and sgRNA2, sgRNA3, sgRNA4 sequences in MIA PaCa-2 were able to effectively knockout CDK7. Therefore, we selected these three subclones for follow-up experiments (Fig. 2A). Dose-dependent viability assays and clonogenic assays indicated that CDK7-deficient cell lines were significantly more sensitive to both GEM and PTX than cells transduced with scrambled control sgRNA (NC) (Fig. 2B; Supplementary Fig. S2A). Consistently, CDK7 knockout also dramatically induced apoptosis in the presence of GEM and PTX (Fig. 2C-F). Flow cytometry-based apoptotic analysis revealed that in the presence of chemotherapy, the early and late apoptosis of CDK7 knockout cells were significantly enhanced compared with non-targeting control (NC). Additionally, CDK7 knockout cell lines demonstrated higher caspase 3/7 activity (Fig. 2G and H), suggesting that CDK7 knockout induced more considerable cell damage and led to more apoptosis in GEM and PTX treated cells. Interestingly our results showed that CDK7 knockout also slightly increased cell apoptosis without chemotherapy (Fig. 2D and F), which might be related to the role of CDK7 in transcriptional control of DNA repair networks [28, 29].

Following that, we investigated the mechanism through which CDK7 deficiency results in chemosensitivity. CDK7 is required for RNAPII-mediated transcription and also functions as an integral component of the transcription factor TFIIH, which is involved in transcription initiation and DNA repair [28, 30]. Therefore, we performed western blot analysis of apoptosis and DNA repair associated proteins in TB32047 and MIA PaCa-2 KO cell lines treated with vehicle or GEM and PTX. Interestingly, we observed a substantial down-regulation of phosphorylated STAT3 protein expression in the absence of CDK7,

which was more pronounced in the presence of GEM and PTX (Fig. 2I). The phosphorylation level of STAT3 in CDK7 knockout cells decreased more drastically after chemotherapy. Meanwhile, we also detected a decrease in the protein level of MCL1 after CDK7 deletion. Similarly, we determined the amount of expression of CHK1, an MCL1 regulatory target involved in cell cycle control and DNA damage repair. CHK1 consistently decreased in the presence of CDK7 knockout, and CHK1 was significantly decreased following 24 hours therapy. According to the foregoing findings, inactivating CDK7 sensitizes PDAC cells to GEM and PTX chemotherapy via the STAT3-MCL1-CHK1 axis (Fig. 6C).

Targeted inhibition of CDK7 enhanced gemcitabine and paclitaxel chemotherapy response in pancreatic cancer in vitro.

Next, we sought to confirm this effect by using the CDK7-specific inhibitor THZ1. Dose-response curves performed on a panel of human PDAC cell lines (MIA PaCa-2, AsPC-1, SUIT-2, BxPC-3, Mayo4636, TKCC-10) and TB32047 indicated that THZ1 inhibited PDAC cell viability in a dose-dependent manner, with the IC50 ranging from 26.08nM to 423.7nM (Fig. 3A; Supplementary Fig. S2B).

To analyze whether CDK7 inhibitor could work synergistically with GEM and PTX in suppressing PDAC growth, we used THZ1 at a dose inhibiting proliferation by less than 20% (IC20) in the aforesaid PDAC cells in conjunction with a concentration gradient of GEM and PTX for 72 hours. As expected, GEM and PTX reduced PDAC cell viability in a dose-dependent manner, while the combination of THZ1 with GEM/PTX generated a substantial synergistic effect ($CI < 1$), resulting in a more severe reduction of cell viability than the GEM/PTX regimen (Fig. 3B and C; Supplementary Fig. S2C-S2G). Clonogenic assays revealed that the combination treatment group had considerably fewer colonies than groups treated with THZ1 or GEM/PTX alone (Fig. 3D and F). According to the caspase 3/7 activity assay, the combined treatment group exhibited significantly higher caspase 3/7 activity than either chemotherapy or THZ1 alone, suggesting that the cytotoxicity generated by combination treatment was more effective (Fig. 3E and G). Further, the flow cytometry analysis confirmed that the combination treatment was superior to single agents, manifested by a more pronounced activation of apoptotic cell death induced by combined THZ1 and GEM/PTX (Fig. 3H and I; Supplementary Fig. S2H). In conclusion, these results confirmed that the targeted inhibition of CDK7 amplifies the toxicity of chemotherapy, rendering PDAC cells more vulnerable to GEM/PTX chemotherapy.

Combined CDK7 inhibition with standard chemotherapy suppresses PDAC tumor growth in vivo.

Next, we evaluated the *in vivo* efficacy of CDK7 inhibitor combined with GEM and nab-PTX chemotherapy. To establish an orthotopic mouse model of pancreatic cancer, we used TB32047 organoids. Tumor-bearing mice were treated with GEM (50mg/kg) and nab-PTX (5mg/kg) once every 4 days and THZ1 (5mg/kg) 5 consecutive days a week. Notably, the dose of chemotherapy and THZ1 used in our study were below clinically achievable concentrations. As a consequence, none of the combinations caused substantial weight loss in mice, and no additional toxicity was observed during the therapy (Supplementary Fig. S2I).

Treatment with GEM and nab-PTX slightly reduced the tumor volume, which confirmed the effectiveness of the chemotherapy regimen. Although THZ1 alone had no effect on tumor volume, the combination treatment considerably increased chemotherapy effectiveness in an orthotopic pancreatic cancer mice model by significantly decreasing tumor size (Fig. 4A), volume and weight (Fig. 4B and C) when compared to the vehicle group. Hence, the combination of CDK7 inhibitor together with the standard care GEM + nab-PTX exhibited a superior anti-tumorigenic activity compared to chemotherapy alone. The high synergistic impact of THZ1 was consistent with our *in vitro* results (Fig. 2 and Fig. 3). We performed western blots to evaluate the expression levels of cell cycle and apoptosis-related proteins in tumor samples from different treatment groups to confirm the better efficacy of the combination therapy. Combination treatment for PDAC tumors resulted in lower WEE1 and CHK1 expression than monotherapy. The expression of MCL1 in PDAC tumors was reduced following the combination of treatments, suggesting an increase in apoptosis (Fig. 4D). Additionally, hematoxylin and eosin (H&E) staining demonstrated that the combination of THZ1 with GEM and nab-PTX dramatically decreased the tumor cell content compared to the single components and control group (Fig. 4E). Briefly summarized, our *in vivo* results validate that combined CDK7 inhibition is an effective strategy to promote standardized treatment and overcome chemoresistance of PDAC.

CDK7 inhibition induces cell-cycle arrest, apoptotic cell death and DNA damage through the STAT3-MCL1-CHK1 axis.

To investigate the potential molecular mechanism of chemotherapy and CDK7 inhibition in combination, we first interrogated the Gene Expression Omnibus (GEO) datasets and performed gene set enrichment analysis (GSEA) on the RNA-sequencing data of BXPc-3, MIA PaCa-2, and PANC-1 cells treated with THZ1[30]. Mechanistic investigations demonstrated that CDK7 inhibition significantly decreased gene transcription, with a preference for mitotic cell cycle and NF- κ B signaling-related transcripts preferentially repressed [30]. Simultaneously, GSEA analysis revealed that THZ1 substantially decreased the gene features of DNA damage repair (DDR), the G2-M checkpoint, and regulate apoptosis (Fig. 5A). Notably, CDK7 is also strongly positively correlated with the expression of key genes involved in cell cycle and DNA repair through the analysis of TCGA PDAC patient transcriptomic data, in particular CDC25C, CCNB1, CDC25B, CDK1, NHEJ1, CHK1 and RAD51, with Pearson's correlation coefficients being 0.42, 0.51, 0.31, 0.41, 0.48, 0.24 and 0.24, respectively (Supplementary Fig. S3A).

To demonstrate that THZ1 therapy resulted in significant alterations in cell cycle-related proteins, TB32047 and MIA PaCa-2 cells were treated with THZ1 in concentration gradient and time gradient to ascertain the effects of CDK7 inhibition on cell cycle regulation. Importantly, p-CTD^{Ser7} progressively decreased in a time- and dose-dependent manner, which was related to the role of CDK7 in RNAPII-mediated transcription and confirmed the effectiveness of THZ1. Meanwhile, WEE1, phosphorylated WEE1, phosphorylated CDK1, and phosphorylated CDK2 all reduced dose- and time-dependently. Simultaneously, when the dose or time was increased, the cleaved PARP was up-regulated, indicating that THZ1 also regulated apoptosis in a time and dose-dependent manner (Supplementary Fig. S3C and S3D).

Through cell cycle analyses, we revealed increased G2-M arrest in a dose-dependent way in the aforementioned two cells (Supplementary Fig. S3E and S3F).

We have established that deletion of CDK7 results in cell cycle arrest and increased apoptosis through the STAT3-MCL1-CHK1 pathway, rendering PDAC susceptible to GEM/PTX therapy. As a result, we were interested in determining if inhibiting CDK7 may improve chemotherapeutic effectiveness through the same mechanism. In order to determine the interaction between CDK7 and the above pathway proteins, we conducted endogenous co-immunoprecipitations and found that CDK7 was immunoprecipitated with STAT3, MCL1 and CHK1 in the reciprocal experiment in TB32047 cells, respectively (Supplementary Fig. S3B). Next, we treated TB32047 and MIA PaCa-2 cells for 24 hours with THZ1 at a dosage that has no impact on cell proliferation in conjunction with chemotherapy. Consistent with CDK7 deletion, targeted inhibition of CDK7 with GEM/PTX chemotherapy also decreased the amount of transcription factor STAT3 and its phosphorylation, despite the fact that a single medication had no effect on their expression. Following combination therapy, the cell cycle checkpoint protein CHK1 and its Ser296 phosphorylation site were also considerably suppressed, showing that the severity of cell cycle arrest and DNA damage increased. The level of the anti-apoptotic protein MCL1 was also considerably decreased, demonstrating that apoptosis was induced, which was compatible with the apoptosis experiment results (Fig. 5B). Immunofluorescence experiments confirmed that the combined treatment significantly increased the number of γ H2AX foci compared to the THZ1 or GEM and PTX treatment group (Fig. 5C), revealing that the combined treatment resulted in the highest percentage of DNA double-strand breaks (DSB) that caused increased DNA damage and cell death.

In conclusion, our findings suggest that inhibiting CDK7 enhances the efficacy of standard chemotherapy in generating cell cycle arrest, DNA damage, and ultimately apoptosis in PDAC.

Targeted inhibition of CDK7 reversed chemoresistance in pancreatic cancer cells.

In the following step, we investigated whether CDK7 contributes to resistance to GEM and PTX. To simulate acquired resistance in patients, we produced gemcitabine- and paclitaxel-resistant cells (TBGPR and MIAGPR) by chronically treating TB32047 and MIA PaCa-2 cells with stepwise incremental doses of GEM and PTX. The dose response curve displayed a 5-10-fold increase in IC₅₀ of chemoresistant cells compared with parental cells (Fig. 3B and C; Fig. 6B). It is sufficient to provide a model of drug resistance in refractory pancreatic cancer for subsequent studies.

Interestingly, CDK7, STAT3, P-STAT3^{Tyr705}, CHK1, and MCL1 signal activation were clearly increased in chemoresistant cells compared to parental cells (Fig. 6A). Notably, consistent with the results of chemotherapy on parental cells, the addition of THZ1 restored GEM/PTX sensitivity in TBGPR and MIAGPR cells. The cell viability assay revealed a high synergistic effect between THZ1 and GEM/PTX in the two resistant cell lines (Fig. 6B). Surprisingly, chemoresistant cell lines demonstrated increased susceptibility to THZ1 (Supplementary Fig. S4A and S4B), mainly due to the persistently high CDK7 expression in TBGPR and MIAGPR. These results indicated that CDK7 contributes to chemoresistance

and THZ1 abolished this process, rendering refractory PDAC cells susceptible to GEM/PTX chemotherapy (Fig. 6C).

Discussion

Despite the fact that cancer treatment has improved dramatically due to targeted therapy, PDAC remains one of the deadliest malignant cancers where only classical chemotherapy is applied [2, 7, 31]. The options for therapeutic intervention in pancreatic cancer are limited, partly due to the ineffectiveness of pharmacological therapy and the consequent chemoresistance [7]. As a result, the development of novel targeted therapies or chemosensitizers has become the ultimate focus of investigation. Here, we used a sgRNA library focused on kinases as well as PDAC-associated genes and identified CDK7 as a candidate for chemotherapy sensitization of pancreatic cancer by adopting a systematic approach of negative selection of CRISPR-Cas9. We demonstrate that the efficient inhibition of CDK7 improved cell apoptosis and DNA damage after chemotherapy by decreasing the signal transduction of STAT3-MCL1-Chk1 pathway, so as to promote the chemotherapy impact of GEM and PTX. THZ1, a covalent inhibitor of CDK7, has shown promising therapeutic effects in preclinical models for a range of malignancies, including high-grade glioma [32, 33], hepatocellular carcinoma [34, 35], and non-small cell lung cancer [20]. We further examined the therapeutic impact of CDK7 inhibition in PDAC preclinical tumor model and discovered that targeting CDK7 would provide a new strategy for enhancing the chemotherapy of pancreatic cancer. Moreover, we revealed evidence that acquired chemoresistance was linked with the up-regulation of STAT signal-dependent transcription genes required for sustaining a malignant phenotype, thus, enabling chemoresistant pancreatic cancer cells to be particularly sensitive to THZ1. Combined therapy successfully induced chemo sensitization, thereby providing a novel approach to overcome chemoresistance in pancreatic cancer [36]. In summary, our results provided mechanistic basis for the combination of CDK7 inhibitors and standard chemotherapy in the treatment of pancreatic cancer as well as the support for the application of CRISPR-Cas9 functional genomics to identify novel therapeutic targets and develop new targeted drugs to enhance more effective chemotherapies.

Apart from its normal involvement in the cell cycle, CDK7 is essential for RNA transcription mediated by RNA polymerase II [20]. Inhibiting CDK7 specifically reduced the activity of STAT3 and other cell-death related proteins. STAT3 mediates the expression of multiple genes in response to cellular stimuli and therefore plays a key role in many cellular processes such as cell growth and apoptosis [37, 38]. Weakening the STAT pathway enhanced the susceptibility of surviving cells to GEM and PTX treatment. Even in cells with a STAT3 mutation, the STAT signaling system is particularly vulnerable to THZ1, according to the literature. STAT3 chromatin binding and highly transcribed target genes such as MYC, PIM1, MCL1, CD30 and IL2RA are reduced in mutant cells where CDK7 is inhibited [37–39]. MCL1 and BCL-XL expression in tumor cells is decreased by THZ1, making tumor cells more responsive to BH3 mimics [37]. Therefore, the combination of THZ1 and BH3 mimics is also a potential targeted medication therapy option.

Cancer cells escape apoptosis by a variety of mechanisms, including increased production of pro-survival proteins (BCL2, BCLXL, or MCL1) [40]. The BCL2 protein family is critical for tumor cell survival [40, 41], and PDAC, in particular, is strongly dependent on the anti-apoptotic protein MCL1 [41, 42]. Our data demonstrated that CDK7 deletion dramatically reduced the amount of MCL1 protein, however, this impact was enhanced when coupled with GEM/PTX therapy. Thus, when chemotherapy was combined with THZ1 at a dosage that did not affect proliferation, MCL1 was also considerably reduced, indicating that THZ1 may effectively combine chemotherapy to induce tumor cell death synergistically. In addition, our experimental data confirm the function of CDK7 in cell cycle control. Inhibition of CDK7 decreased the phosphorylation of G2-M cell cycle-related proteins WEE1, CDK1, and CDK2, resulting in G2-M phase gradient-dependently arrested by THZ1 and reducing cell proliferation. Interestingly, it has been shown that the inhibition of CDK7 may reduce CDK1 and CDK2 activity, which would lead to the degradation of MCL1 [43].

In addition to its typical role in the cell cycle, CDK7 is also involved in DNA repair [28]. As a transcriptional activator, the tumor suppressor protein p53 regulates the cell response to DNA damage by inducing apoptosis and cell cycle arrest [28]. p53 and the CAK complex share a high degree of commonality in terms of cell cycle and transcriptional control, and CAK phosphorylates p53 at Ser33 site, the regulation of DNA damage repair [28, 33]. As a result, we concentrated on the relationship between CDK7-mediated transcriptional regulation and DNA damage. We observed that combining THZ1 with chemotherapy enhanced γ H2AX foci, a marker of an early DNA double-strand break [44], and reduced CHK1 phosphorylation, therefore increasing radiation-induced apoptotic signaling. As a comprehensive result, this dramatically increased G2-M cell cycle arrest, DNA damage, and apoptosis. These findings provide evidence for a causal connection between DNA damage and checkpoint mechanisms generated by the combined use of THZ1 and GEM/PTX.

Conclusions

In conclusion, our work demonstrated that CDK7 is a novel therapeutic target for pancreatic cancer, and the molecular basis for the synergistic effect appears to involve increased DNA damage cell cycle arrest and apoptosis, leading to an improved therapeutic effect than GEM/PTX or CDK7 inhibition alone. Notably, the combined medication had no adverse effects in preclinical mice models as compared to monotherapy, exceeding the requirements for synergistic combination therapy. Therefore, we report a possible novel treatment regimen for overcoming PDAC chemoresistance. This regimen is noteworthy clinically relevant due to its potential outstanding effectiveness, minimal toxicity, and excellent selectivity. There have been several CDK7i clinical studies in a variety of malignancies, including breast cancer, ovarian cancer, and prostate cancer, but no relevant CDK7i clinical trials in pancreatic cancer have been initiated. The majority of published research focuses only on the impact of a single inhibitor and still remains in preclinical stages. Our results underscore the need of conducting more clinical trials to evaluate this newly discovered synergistic drug combination in the treatment of patients with PDAC and other malignancies.

Abbreviations

PDAC
Pancreatic ductal adenocarcinoma
PC
Pancreatic cancer
CDK7
Cyclin-dependent kinase 7
CAK
CDK-activating complex
CTD
C-terminal domain
KO
Knockout
ATCC
American Type Culture Collection
PDCLS
Patient-derived cell lines
CSHL
Cold Spring Harbor Laboratory
GEO
Gene Expression Omnibus
GSEA
Gene set enrichment analysis
DDR
DNA damage repair
DSB
Double-strand break.

Declarations

Ethics approval and consent to participate

In vivo experiments were performed by skilled experimenters trained according to FELASA guidelines. Animal protocols were approved by the Preclinical Animal Facility of Universitätsklinikum Erlangen.

Consent for publication

All authors agree to submit the article for publication.

Availability of data and materials

Raw data of the CRISPR/Cas9 screening data are available at EBI database accession: PRJEB48728.

Competing interests

The authors declare that they have no other competing interests.

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Authors' Contributions

Conceptualization: SYZ, BL, RG and CP; methodology: SYZ, BL, XFR, SMZ, ME and NBL; software: SYZ, BL and DS; validation: SYZ, BL, XFR, SMZ and IK; formal analysis: SYZ, BL, DS and XFR; resources: IK, SP, RG, PB, DC, DM, NBL, AD, HY, ME and CP; data curation: SYZ, SMZ and XFR; Original draft preparation: SYZ; writing, review and editing: SYZ, BL, NBL, PB, DM, DC, AB, RG and CP; visualization: AB, AD, DM, IK, SP and RG; supervision: RG and CP; project administration: CP; funding acquisition: CP. All authors read and approved the final manuscript.

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Figures

Figure 1

CRISPR-Cas9 knockout screens to identify chemosensitivity targets in pancreatic cancer cells treated with gemcitabine and paclitaxel. **A.** Schematic diagram of the timeline and experimental procedure for CRISPR-Cas9 screening using protein kinases library. **B, C.** Volcano plots showing $\log_2$ -transformed sgRNA normalized fold change and $-\log_{10}$ -transformed p-value of gemcitabine (B) and paclitaxel (C) versus vehicle-treated TB32047 cells, with significant genes in positive and negative selection highlighted in red and blue, respectively. **D.** The Venn diagram showing the common genes of the top 20 candidate genes for gemcitabine and paclitaxel negative screening are *Cdk7*, *Wee1*, *Fgfr1* and *Havcr2*. **E.** Scatterplots showing $\log_2$ -transformed sgRNA normalized read counts of gemcitabine(**left**) and paclitaxel (**right**) versus vehicle-treated TB32047 cells, with sgRNAs targeting *Cdk7* highlighted. **F.** Data from TCGA-PDAC was applied for survival analysis. Kaplan–Meier survival analysis shows that higher CDK7 mRNA expression is associated with a tendency of poor overall survival. **G.** The analysis of CDK7 expression in PDAC tissues and normal pancreas tissues was conducted with TCGA (PDAC) and GTEx (Pancreas) database. The results jointly indicated that CDK7 expression level was significantly higher in pancreatic cancer than in the corresponding normal pancreas tissue. **H.** Western blot analysis revealed a variation in CDK7 expression between pancreatic cancer subtypes. *, $P < 0.05$; ***, $P < 0.001$ and ****, $P < 0.0001$ was calculated by the unpaired Student *t* test.

Figure 2

CDK7 knockout in combination with gemcitabine and paclitaxel treatment inhibits proliferation and increases apoptosis in pancreatic cancer cells. **A.** Western blot of TB32047 and MIA PaCa-2 cells transfected with CDK7 sgRNAs or non-targeting control. **B.** The dose response curves for GEM and PTX in TB32047 and MIA PaCa-2 CDK7 KO or NC cells. 72 hours following treatment, cell survival was measured and normalized. Data are presented as mean of three independent experiments ($n = 3$). **C-F.** CDK7-WT (NC sgRNA) and CDK7-knockout TB32047 (C, D) cells, as well as MIA PaCa-2 (E, F) cells, were treated with vehicle or chemotherapy for 24 hours and then analyzed for apoptosis by flow cytometry. Data are presented as mean of three independent experiments ($n = 3$). **, $P < 0.01$; ***, $P < 0.001$ and ****, $P < 0.0001$ by two-way ANOVA with Tukey multiple comparisons test. **G** and **H.** TB32047(G) and MIA PaCa-2(H) cells expressing CDK7 or control sgRNAs were treated with GEM and PTX, caspases 3/7 activity was measured 24 hours later. Data are presented as mean $\pm$ SD ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ and ****, $P < 0.0001$ by two-way ANOVA with Tukey multiple comparisons test. **I.** Western blot analysis of TB32047(left) and MIA PaCa-2(right) cells transfected with non-targeting control (NC sgRNA) or CDK7-targeting sgRNAs (CDK7-sg1, CDK7-sg2, CDK7-sg3, CDK7-sg4) and treated for 24 hours with vehicle or GEM plus PTX.

Figure 3

Targeted inhibition of CDK7 enhanced gemcitabine and paclitaxel chemotherapy response in pancreatic cancer *in vitro*. **A.** Dose–response curves of TB32047 and MIA PaCa-2 wild type cells to THZ1. Cell proliferation was evaluated 72 hours after drug treatment. Data are presented as mean of three independent experiments (n=3). **B** and **C.** The CDK7 inhibitor THZ1 enhances the antiproliferative impact of GEM and PTX in pancreatic cancer cells synergistically. Proliferation analysis was performed on TB32047(**B**) and MIA PaCa-2(**C**) cells treated for 72 hours with THZ1 and GEM/PTX alone or in combination. The plot of fraction affected versus combination index is shown underneath. $CI < 1.0$, synergism. **D** and **F.** Clonogenic assay of TB32047(**D**) and MIA PaCa-2(**F**) cells treated with vehicle or the indicated drugs for 72 hours and cultured without drugs for additional 7–10 days. The remaining cells after the treatment were fixed and stained with crystal violet. Representative images(**left**) and quantification (**right**) of three independent experiments (n = 3) are shown. *, $P < 0.05$ and **, $P < 0.01$ by one-way ANOVA with Tukey multiple comparisons test. ns, not significant. **E** and **G.** Caspase 3/7 activity was evaluated in TB32047(**E**) and MIA PaCa-2(**G**) cells treated for 72 hours with vehicle or the indicated drugs. Data are presented as mean $\pm$ SD (n = 3). *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$ by two-way ANOVA with Tukey multiple comparisons test. **H** and **I.** Flow cytometry–based apoptotic assay of TB32047 and MIA PaCa-2 cells treated for 72 hours with the indicated drugs or drug combination for 72 hours. Representative images(**H**) and quantification (**I**) of three independent experiments (n = 3) are shown. **, $P < 0.005$ by two-way ANOVA with Tukey multiple comparisons test.

Figure 4

Combined CDK7 inhibition with standard chemotherapy suppresses PDAC tumor growth *in vivo*. **A.** Images of TB32047 orthotopic mouse model of pancreatic cancer after treated for 16 days. **B** and **C.** Relative tumor weights (**B**) and volume (**C**) of TB32047 orthotopic pancreatic cancer mouse model after the treatment. ***, $P < 0.001$ and ****, $P < 0.0001$ by one-way ANOVA with Tukey multiple comparisons test. ns, not significant. **D.** Western blot analysis of tissue samples from mice with pancreatic cancer that had been treated with vehicle, GEM/nab-PTX or in combination. **E.** Hematoxylin and eosin (H&E) staining demonstrated that the combination of THZ1 and GEM with nab-PTX dramatically decreased tumor sections compared to the single components and control group. Scale bar (black), 50 μ m.

Figure 5

CDK7 inhibition induces cell-cycle arrest, apoptotic cell death and DNA damage through the STAT3-MCL1-CHK1 axis. **A.** Gene set enrichment analysis (GSEA) on the RNA-sequencing data of BXPc-3, Mia PaCa-2, and PANC-1 cells treated with THZ1, a selective CDK7 inhibitor. Transcriptomic gene expression data are based on the GEO dataset GSE121273. **B.** Western blot of TB32047 and MIA PaCa-2 cells treated with vehicle (DMSO), THZ1, and GEM plus PTX, alone or in combination for 24 hours. **C.** Immunofluorescence of TB32047 cells treated with vehicle (DMSO), THZ1, and GEM/PTX, alone or in combination for 24 hours. Cells were subsequently stained with antibodies against γ H2AX (green) and DAPI (blue; nuclei). Scale bar (white), 20 μ m.

Figure 6

Targeted inhibition of CDK7 reversed chemoresistance in pancreatic cancer. **A.** Western blot of TB32047 and MIA PaCa-2 gemcitabine and paclitaxel resistant cells (TBGPR, MIAR) and parental cells (TBWT, MIAWT). **B.** The CDK7 inhibitor THZ1 synergistically enhances the antiproliferative effects of GEM and PTX in TBGPR and MIAGPR cells. The proliferation was determined after 72 hours of treatment with THZ1 and GEM/PTX alone or in combination. Below is a plot of the fraction impacted vs the combo index. $CI < 1.0$, synergism. **C.** Diagrammatic representation of the research outcome. Gemcitabine and paclitaxel combined CDK7 inhibition induces DNA damage, G2-M arrest, and subsequent apoptosis.

Supplementary Files

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