Original Article
Dinaciclib prolongs survival in the LSL-Kras$^{G12D/+}$; LSL-Trp53$^{R172H/+}$; Pdx-1-Cre (KPC) transgenic murine models of pancreatic ductal adenocarcinoma

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Abstract: Dinaciclib is a small molecule cyclin-dependent kinase inhibitor with the potential to treat multiple cancers. To better understand its cytotoxic action in pancreatic ductal adenocarcinoma (PDAC), we evaluated dinaciclib therapeutic effects in the transgenic mouse model (LSL-Kras$^{G12D/+}$; LSL-Trp53$^{R172H/+}$; Pdx-1-Cre mice; KPC mice). Tumor growth and microenvironment were dynamically monitored by magnetic resonance imaging (MRI). Dinaciclib therapy significantly delayed tumor progression (P < 0.001) and prolonged survival (P = 0.007) in KPC mice. In vitro assays showed that dinaciclib exerted antiproliferative effects on PDAC cells by increasing surface calreticulin expression and release of ATP. Dinaciclib treatment inhibited proliferation and induced apoptosis in KPC tumor as assessed by Ki67 and cleaved caspase 3, respectively. Particularly, the tumor infiltrating CD8$^{+}$ T cells were increased after dinaciclib treatment in KPC mice. Additionally, the mean apparent diffusion coefficient values of KPC tumor calculated from diffusion weighted MR images were significantly lower after dinaciclib treatment (P = 0.033). These finding suggest that dinaciclib as a single agent can inhibit tumor growth and improve the overall survival in KPC mice.

Keywords: Pancreatic ductal adenocarcinoma, dinaciclib, magnetic resonance imaging, apoptosis

Introduction
Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer, with a median survival of 6 months [1]. PDAC is predicted to become the second leading cause of cancer-related deaths in the United States by 2030 [2]. Hence, there is an urgent need to develop new and effective therapeutic approaches to treat this lethal cancer.

Analyses of human PDAC samples have revealed that TP53 mutation occurs in 50-75% of PDACs following a universal initiating activating mutation in the KRAS gene [3, 4]. Activating mutations in the Kras proto-oncogene is one of the most frequent genetic events driving development of human pancreatic low grade precursor lesions (pancreatic intraepithelial neoplasia-PanIN-) [4, 5]. Mouse models that endogenously express the mutant Kras in pancreatic exocrine lineages can recapitulate human PanIN [6], but progression to PDAC requires additional genetic events. Remarkably, concurrent genetic inactivation of the Trp53 tumor suppressor gene substantially accelerates the PDAC development in the context of Kras mutation [7, 8]. Different pancreatic cancer transgenic mouse models (e.g., Kras$^{G12D}$; Cre model, Kras$^{G12D}$; Trp53$^{flx/flx}$; Cre model, and Kras$^{G12D}$; Trp53$^{mut/mut}$; Cre model) have been created for studying the progression of PDAC and for identifying therapeutic targets [9]. Here, we focused in particular on the KPC mouse model, one of the most commonly studied preclinical transgenic mouse models of
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PDAC, that was established by targeting endogenous expression of oncogenic Kras<sub>G12D</sub> and mutant Trp53<sub>R172H</sub> to the mouse pancreas using Cre-loxP technology. KPC transgenic mice stochastically form pre-invasive PanIN lesions that eventually evolve into invasive PDAC with 100% penetrance [7, 10].

The cyclin-dependent kinases (CDKs), a family of serine/threonine kinases, regulates cell cycle progression. Aberrant CDK activity as well as cell cycle dysregulation is a hallmark of many tumor cells [11]. As such, inhibition of CDK activities represents a potential therapeutic strategy for cancer treatment. CDK inhibitor dinaciclib, also known as SCH 727965 and MK-7965, is a potent CDK2, CDK5, CDK1, and CDK9 inhibitor <i>in vitro</i> with IC50 values of 1, 1, 3, and 4 nmol/L, respectively [12]. It showed promising results in preclinical trials for a range of solid and hematologic malignancies [13-16]. More importantly, a recent study demonstrated that dinaciclib induced a type I IFN gene signature and immunogenic cell death (ICD) in immunocompetent mouse tumor models [17]. ICD response is characterized by apoptotic cell death accompanied by the expression of calreticulin (CRT) on dying tumor cell surfaces, which can re-initiate immune responses suppressed by the tumor microenvironment [18]. Immunologically, the PDAC microenvironment is considered especially suppressive. While several previous studies showed that treatment with dinaciclib effectively inhibited growth and progression of pancreatic cancer xenografts in immunodeficient mice [19-21], the therapeutic role for dinaciclib in PDAC has not yet been fully clarified.

Assessment of response to cancer therapy in pancreatic cancer can often be performed using magnetic resonance imaging (MRI) which allows non-invasive evaluation of tumors over time [22, 23]. Moreover, diffusion-weighted MRI (DW-MRI) can qualitatively and quantitatively assess the molecular function and microarchitecture of solid tumors [24]. Several studies have shown that the apparent diffusion coefficient (ADC) values measured from DW-MRI can serve as an early imaging biomarker for predicting tumor response to chemotherapy for pancreatic cancer [25, 26].

In this study, the KPC mice were used to evaluate the therapeutic effects (including overall survival (OS)) of dinaciclib in PDAC. MRI was used to dynamically quantify and serially assess the therapeutic responses in pancreatic tumor growth and cellularity during dinaciclib treatment. Overall, our results revealed that dinaciclib therapy significantly improved the OS of treated KPC mice and supported clinical practice in this context.

Material and methods

Mice and regents

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Northwestern University and conducted in compliance with the National Institute of Health guidelines for animal research. Kras<sub>G12D</sub><sup>-/+</sup>, Trp53<sub>R172H</sub><sup>-/+</sup>, and pdx1-Cre mice strains, purchased from Jackson Laboratory (Bar Harbor, ME), were interbred to generate KPC mice, as previously described [7]. KPC mice (3-6 months old) with spontaneous pancreatic cancer were used. Tumor growth and development were outlined by serial MRIs as outlined below.

Dinaciclib was purchased from Selleckchem. Inc. (Houston, TX) and formulated in the vehicle 20% hydroxypropyl β cyclodextrin (Sigma-Aldrich, St. Louis, MO). Doxorubicin (DOX) was purchased from Selleckchem. Inc. (Houston, TX).

Cell lines

KPC cells were derived from spontaneous pancreatic tumors originating in KPC mice (6-month-old) as previously described [27, 28]. Single-cell suspensions from tumor fragments were plated on collagen in DMEM/F12 (50:50) medium supplemented 5% Nu-serum IV, 25 μg/mL bovine pituitary extract, 20 ng/mL epidermal growth factor, 0.5% ITS+ Premix, 100 ng/mL cholera toxin, and 1 μM dexamethasone. After 3 passages, the KPC cells were maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. KPC cells did not surpass 12 passages between thawing and use. A murine PDAC cell line Pan02 cell line was obtained from ATCC (Rockville, MD) and was cultured in complete RPMI 1640 medium containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine.
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**Cell viability assay**

Cell Counting Kit-8 (CCK-8) (Dojindo Laboratories, Rockville, MA) was used to conduct the cell viability assay. Cells were seeded in 96-well clear-bottom plates starting from 1 × 10^4 cells per well. After changing the media 24 h later, the cells were treated with different doses of dinaciclib and 1 μM Dox for 24 h. A mixture of 190 μL of OPTI-MEM and 10 μL of CCK-8 was then added into each well. Two hours later, the absorbance at 450 nm was measured.

**CRT expression from the cell lines**

Pan02 cells and KPC cells were seeded on microscope coverslips (Thermo, Rockford, IL) in 24-well plates. Each well contained 2 × 10^4 cells in 1 mL of culture medium. After incubation with 1 μM dinaciclib or 1 μM DOX for 4 h, cells were then fixed and washed 3 times followed by permeabilization with 0.1% TX-100. After incubating for 60 min with blocking buffer, cells were stained with Alexa Fluor® 488-conjugated wheat germ agglutinin (WGA) (Thermo, Waltham, MA) to visualize the cell surface membrane. Then the cells were stained with an anti-CRT antibody (ab2907, 1/500, Abcam, Cambridge, MA) at 4°C overnight followed by Alexa-fluor®555 conjugated secondary antibodies (Life technology, Carlsbad, CA). After washing 3 times with PBS, slides were mounted with Hoechst 33,342 nuclear dye. Finally, all images were obtained with a Nikon confocal microscope. The MFI of immunofluorescent images were quantified by ImageJ 1.52a software (http://imagej.nih.gov/ij).

**Apoptosis assays**

To analyze apoptosis, the cells were seeded into 6-well plates. After growing to 70% to 80% confluence, cells were cultured in increasing doses of dinaciclib for 24 h, as indicated. Dinaciclib-induced PDAC cell death was assessed by flow cytometry using the Annexin V-Propidium Iodide Apoptosis Detection Kit (Life technology, Carlsbad, CA) according to the manufacturer’s instructions.

**ATP release assays**

Supernatants were harvested from Dinaciclib-treated tumor cells. Extracellular ATP levels were measured by ENLITEN ATP Assay System Bioluminescence Detection Kit for ATP (Promega, Madison, WI) using a Multi-Mode Plate Reader (Thermo Fisher Scientific, Waltham, MA).

**Monitoring of tumor growth and therapy**

MRI was performed on 3-6 month-old KPC mice on a 7.0-Tesla scanner (Clinscan, Bruker BioSpin, Ettlingen, Germany) as described previously [29]. KPC transgenic mice underwent treatment or control treatments when the longest tumor diameter reached 0.2-0.5 cm on MRI. Dinaciclib (40 mg/kg) was administered intraperitoneally twice a week for three weeks. OS was defined as the number of days post injection until predefined endpoints requiring euthanasia have been met such as > 20% weight loss, extreme lethargy, decreased mobility, tumor size > 2 cm, or moribund status.

The MRI sequences and parameters were as follows [29]: (a) coronal T2W images: TSE; TR: due to respiratory gating approx. 2100 ms; TE: 40 ms; ST: 0.5 mm; FA: 180º; FOV: 21 × 30 mm²; (b) axial T2-weighted imaging (T2WI): TSE; TR: due to respiratory gating approx. 2100 ms; TE: 40 ms; ST: 0.5 mm; FA: 180º; FOV: 21 × 30 mm²; (c) axial diffusion-weighted imaging (DW-MRI): Echo Planar Imaging (EPI); TR: due to respiratory gating approx. 2700 ms; TE: 40 ms; ST: 1 mm; FA: 90º; FOV: 24 × 30 mm²; b value = 0, and 800 s/mm². DW-MRI was performed in 3 orthogonal directions of the diffusion gradients. ITK-SNAP was used for volume calculations and 3D reconstruction. MATLAB R2018a (Mathworks, Natick, MA) was used for image processing. ImageJ 1.52a was used for ADC calculations.

**Histology analysis and immunohistochemistry**

Histology analysis including H&E, Masson’s Trichrome, anti-mouse cytokeratin 19 (CK19) (Developmental Studies Hybridoma Bank, Iowa City, IA), anti-mouse cleaved caspase 3, rat monoclonal anti-mouse CD8 (Clone 4SM15, Invitrogen), and rabbit monoclonal anti-Ki67 (clone SP6) (Invitrogen, Carlsbad, CA) was performed as described previously [29]. Briefly, tissue was fixed with 10% formalin embedded in paraffin. 4-μm sections were deparaffinized and rehydrated. Heat-induced antigen retrieval was performed 10 mmol/L citrate buffer (pH 6.0). After quenching endogenous peroxidase
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To investigate the antitumor effect of dinaciclib in PDAC, we treated the KPC genetic mice with pancreatic tumors that measured 2-5 mm in diameter on MRI (Figure 2A). Interestingly, dinaciclib treatment significantly delayed tumor growth compared with control group (Figure 2B, 2C). In addition, we evaluated the OS of KPC mice in different groups. Treatment with dinaciclib prolonged the OS of KPC mice from 31 to 57 days (P < 0.01 (Median)) (Figure 2D). Taken together, these results confirmed that dinaciclib can potentially elicit tumor specific antitumor immunity in KPC mice.

Dinaciclib decreases tumor fibrosis in KPC mice

High amounts of fibrosis characterize the tumor microenvironment of human PDAC. We next examined the effect of dinaciclib treatment on fibrosis formation, reflected by collagen deposition as detected by Masson-trichrome staining. We observed that dinaciclib-treated KPC model of PDAC had significantly decreased levels of collagen deposition throughout the pancreas compared with control mice (Figure 3A, 3B).

In order to facilitate clinical translation of dinaciclib, we utilized the ADC values calculated from DW-MRI as it is clinically applicable [25, 26, 31]. Dinaciclib therapy induced a significant increase in tumor mean ADC values, suggesting altered microstructure was induced after the treatment (Figure 3C, 3D). Taken together, these results suggest that dinaciclib treatment reshaped PDAC tumor-induced fibrosis.

Treatment with dinaciclib delays PDAC progression and induces apoptosis

Histological examination of the pancreatic tumor tissues showed the presence of more normal-looking tissue with a desmoplastic reaction in dinaciclib treated KPC mice compared with control group as evaluated by H&E staining (Figure 4A). Importantly, the area of ductal marker CK19 positive structures was larger in the pancreas of dinaciclib treated KPC
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A

PBS

1 μM Dinaciclib

PI

FITC - Annexin V

B

OD450 (CCK8 assay)

**

PBS

0.01 μM

0.1 μM

1.0 μM

DOX

C

Nucleus

CRT

Membrane

Merge

PBS

Dinaciclib

DOX

D

Nucleus

CRT

Membrane

Merge

PBS

Dinaciclib

DOX
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Figure 1. Dinaciclib induced immunogenic cell death in PDAC cells. (A) Representative Annexin V versus PI flow cytometry plots of KPC cells after dinaciclib or PBS treatment for 6 h. (B) KPC cells were treated with increasing concentrations of dinaciclib and 1 μM DOX for 24 h. Cell viability was then measured by the CCK-8 assay. (C) Confocal microscopy showing the induction of the ICD marker, CRT, in Pan02 cells in the presence of PBS, Dinaciclib (1 μM), and DOX (1 μM) for 4 h, and (E) corresponding quantification of the mean immunofluorescent intensity. Scale bar is 20 μm. (D) Confocal microscopy showing the induction of the ICD marker, CRT, in KPC cells in the presence of PBS, Dinaciclib (1 μM), and DOX (1 μM) for 4 h, and (F) corresponding quantification of the mean immunofluorescent intensity. Scale bar is 20 μm. (G) KPC cells were treated with dinaciclib at the indicated concentrations for 24 h. Graphical data show the release of ATP into the culture supernatants. *, P < 0.05, **, P < 0.01, ***, P < 0.001.
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Figure 2. Dinaciclib inhibits tumor growth and prolongs OS in KPC mice. A. Experimental schemes for the MRI scan and treatment of the KPC transgenic mice. B. Representative T2W images at different time points from KPC genetic model of PDAC in control or treated groups. C. Measurement of tumor volumes in KPC transgenic mice (n = 7-8). D. Survival rate of treated KPC transgenic mice. Dinaciclib confers survival advantage to the treated animals (P = 0.007, log-rank test). *, P < 0.001.

mice than in control group (Figure 4A, 4B), again corroborating a less extensive desmoplastic transformation after dinaciclib therapy.

To further evaluate whether the induction of apoptosis after dinaciclib treatment occurs in vivo, KPC tumor samples underwent IHC evaluation. Increased PDAC cell apoptosis was observed in dinaciclib treatment KPC mice compared with control mice (Figure 4A, 4C), as assessed by immunohistochemistry for cleaved caspase 3. Cell proliferation assessed by measuring Ki67 levels also revealed a significant decrease in the dinaciclib group compared to control group (Figure 4A, 4D). These data, demonstrating both induction of apoptosis and reduction of cell proliferation, were consistent with the tumor growth delay results shown earlier. Additionally, the tumor infiltrating CD8+ T cells were significantly increased in treated group than in control group (Figure 4A, 4E), further indicating that dinaciclib treatment induced an immune response in KPC mice.

Discussion

In this study, we demonstrated that dinaciclib can exert potent antitumor effect in the KPC genetic model of PDAC. The results showed that dinaciclib can induce an ICD response in PDAC cells, as reflected by enhanced expression of CRT and release of ATP. Furthermore, the dinaciclib treated KPC mice also showed a higher percentage of apoptotic cells, a lower percentage of proliferating cell, and a higher percentage of tumor infiltrating CD8+ T cells than the control group. Moreover, we also observed an inhibition of tumor growth and a significant reduction of average tumor ADC values after dinaciclib therapy in KPC mice. Importantly, a significant improvement of OS was observed in this study.

Dinaciclib is one of the first drugs in its class that exerts antitumor effects as a single agent in PDAC cells [19, 32]. However, the mechanism underlying its antitumor activity has not been fully elucidated yet. Hu C et al. evaluated
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![Figure 3. Dinaciclib treatment alters tumor fibrosis and mean ADC values in KPC mice. A. Representative images of trichrome staining for collagen connective tissue deposition in PDAC from treated and control KPC genetic model of PDAC. Scale bars represent 100 μm (inset, 25 μm). B. Relative quantification of collagen stained area for each group. C. Representative ADC pseudocolor maps of the tumor overlaid onto a T2W image from KPC genetic model of PDAC in treated and control cohorts are shown. D. ADC values measured from KPC genetic model of PDAC in treated and control groups at baseline and 3 weeks post treatment. n = 6. *, P < 0.05.]

Features of human disease; ii) the KPC mice are immunocompetent and provide a useful platform for studying changes in the tumor environment; and iii) low passage tumor cell lines can be easily derived from tumor-bearing KPC mice for use in in vitro and in vivo studies. Therefore, the KPC mouse is a better model than the subcutaneous and orthotopic models of PDAC.

Although prior studies demonstrated that dinaciclib can inhibit proliferation and induce apoptosis [20]. Accordingly, we demonstrated that administration of dinaciclib alone has considerable inhibitory effects on tumor growth rate in the highly aggressive KC mouse model. KPC mice are excellent tools for assessing therapeutics and understanding mechanisms of drug action and resistance in PDAC. The KPC model has significant advantages over previously described xenograft and implantation models, including that: i) the KPC model recapitulates many of the clinical (e.g. ascites development, bowel and biliary obstruction, metastatic spread, and cachexia), histopathological (e.g. cellular morphology, poor vascularity, and fibrosis), and genetic (e.g. concomitant expression of oncogenic Kras$^{G12D}$ and of Trp53$^{R172H}$) features of human disease; ii) the KPC mice are immunocompetent and provide a useful platform for studying changes in the tumor environment; and iii) low passage tumor cell lines can be easily derived from tumor-bearing KPC mice for use in in vitro and in vivo studies. Therefore, the KPC mouse is a better model than the subcutaneous and orthotopic models of PDAC. Although prior studies demonstrated that dinaciclib can inhibit PDAC tumor growth in different subcutaneous and orthotopic xenograft murine models, OS after dinaciclib treatment has never been assessed [19, 20, 32]. In this study, we showed that treatment with dinaciclib prolonged the OS of KPC mice from 31 to 57 days, which further confirmed the therapeutic effects of dinaciclib in PDAC.

Previous studies have suggested that dinaciclib is a pan-CDK-1/2/5/9 inhibitor that can induce...
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Figure 4. Dinaciclib therapy suppresses PDAC progression and induces apoptosis in KPC mice. A. Representative images of H&E and immunohistochemical analyses in PDAC from KPC mice in treated and control groups are shown. Scale bars represent 100 μm (inset, 20 μm). B. Quantification of CK19 staining from treated and control KPC mice. C. Quantification of cleaved caspase-3 staining from treated and control KPC mice. D. Quantification of stromal and tumor Ki67+ cells from treated and control KPC mice. E. Quantification of stromal and tumor CD8+ T cells from treated and control KPC mice n = 3-4. *, P < 0.05.
apoptosis in different tumor cells, including PDAC cells [19, 32-35]. Dinaciclib has been shown to repress transcription via inhibition of CDK9. One target gene of this repression is the *Myc*, which is amplified in several different cancer types including PDAC [36, 37]. Moreover, a recent study found that tumor cells expressed the hallmarks of ICD including surface CRT expression and release of ATP after dinaciclib treatment [17]. Consistent with this, we showed that *in vitro* treatment with dinaciclib exerts proapoptotic effects in PDAC cells by increasing expression of CRT and ATP, the key damage-associated molecular patterns of ICD that play a beneficial role in antitumor effects [38-41]. Meanwhile, we found that the tumor infiltrating CDB+ T cells and cleaved caspase 3 positive apoptotic cells are increased after dinaciclib treatment in KPC tumor, which further confirmed the ICD response induced by dinaciclib. Dinaciclib also exerts its cytotoxic effects by inhibiting CDKs 1 and 2 [12]; these CDKs have been shown to be potential therapeutic targets for cancer therapy [42] and thus may also contribute to the antitumor efficacy we have shown. Furthermore, one recent study reported that inhibition of CDK by dinaciclib increases casein kinase 1α activity in pancreatic cancer cells [43]. In addition, CDK5 signaling is critical for Ras signaling, which served as a pivotal determinant of the malignant phenotype in PDAC cells [44]. Hu C et al. demonstrated that the combination of dinaciclib and AKT inhibitor MK-2206 was quite effective in patient-derived pancreatic cancer xenograft model by blocking RAS/RAL activation and inhibiting PI3K/AKT pathway [20]. We hypothesize that CDK inhibition induced by dinaciclib synergizes with the ICD response to generate antitumor effects in PDAC. Addressing the antitumor mechanisms of dinaciclib monotherapy could translate into novel rational combinatorial approaches for patients. Combination of dinaciclib with other chemotherapeutics like epirubicin and PARP1/2 inhibitor ABT-888 have been shown to be more effective than monotherapy on malignancies [20, 45]. Further studies are needed to more carefully and closely evaluate the regulatory mechanisms of dinaciclib on PDAC apoptosis. Understanding tumor mechanisms will also enable the development of rational combinations with cancer immunotherapy, including immune-checkpoint blockade, adoptive T-cell strategies, and dendritic cell vaccines, to overcome suppressive tumor microenvironment to induce effective long-term immunity.

Dinaciclib has been approved to enter clinical trials against multiple types of malignant diseases, including PDAC [13-16, 46], but its antitumor efficacy may be limited by the increasing toxicity. Thus, further assessment and optimization of therapeutic strategies are required in preclinical and clinical settings. Herein, we used DW-MRI as an imaging biomarker for early evaluation of the treatment efficacy as it has been found capable of predicting tumor response to chemotherapy for pancreatic cancer [25, 26, 31, 47]. DW-MRI is a highly sensitive imaging technique capable of quantifying and detecting macromolecular and microstructural alterations that impact microscopic water diffusion within tissues [48]. In this study, we showed that DW-MRI is useful for early detection of increases in tumor mean ADC values in KPC mice treated with dinaciclib. The increased mean ADC values indicated that dinaciclib-induced tumor cell apoptosis was achieved in KPC mice. Thus, integration of DW-MRI into a clinical PDAC trial involving evaluation of chemotherapy such as dinaciclib would provide worthwhile information for assessing therapeutic response.

There are some limitations in this study. One limitation is that we only investigated the ICD responses induced by dinaciclib, and we have not yet evaluated the antitumor effects of CDK inhibition on PDAC, which will need to be elucidated in future studies. Second, in this study, no complete tumor elimination was observed in KPC mice bearing established highly invasive tumors, underlining that dinaciclib combination therapies may be important for achieving durable tumor regression in PDAC. Further studies are needed to clarify whether these combination approaches can induce complete and durable tumor regression in PDAC.

In conclusion, our study demonstrated that dinaciclib as a single agent can inhibit tumor growth and improve the OS in KPC mice.

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Disclosure of conflict of interest

None.

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References

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**Figure S1.** Dinaciclib potently induces pancreatic cancer cell apoptosis, related to Figure 1. (A) Representative Annexin V versus PI flow cytometry plots of Pan02 cells after dinaciclib or Dox treatment for 6 h. Pan02 cells were treated with increasing concentrations of dinaciclib and 1 μM DOX for 24 h, (B) the percentage of Pan02 cell apoptosis, (C) cell viability was then measured by the CCK-8 assay, and (D) graphical data show the release of ATP into the culture supernatants. Data are represented as mean ± SEM. **, P < 0.01, ***, P < 0.001 determined by Student's t test.