The anti-tumor effect of a novel agent MCL/ACT001 in pancreatic ductal adenocarcinoma

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Research Article

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Abstract

Purpose

Pancreatic ductal adenocarcinoma (PDAC) is a major challenge in cancer therapy, there are more than four hundred thousand deaths a year and the 5-year survival rate less than 10%. The incidence continues to rise. Treatment with classic drugs offers limited therapeutic benefits. The aim of this study was to investigate a new agent ACT001, the active metabolite is Micheliolide (MCL), study the mechanism and effect in vitro and in vivo against PDAC.

Methods

MTT assay, wound healing assay and flow cytometry were used to assess the effects of MCL/ACT001 in vitro. DCFH-DA assay was to assess ROS accumulation. Western blotting, Immunohistochemical staining and TUNEL assay were also conducted to determine the mechanisms. PANC-1-Luc cells and bioluminescent reporter imaging were used to assess anti-tumor effect of ACT001 using a GSC orthotopic xenograft model in vivo.

Results

MCL/ACT001 inhibited cell growth in PDAC in a dose-dependent manner significantly, induced cell apoptosis, cell migration and reactive oxygen species (ROS) accumulation in vitro. In vivo, ACT001 (400 mg/kg/day) inhibited PDAC tumor growth in orthotopic xenograft mice. We verified that EGFR, Akt were overexpressed in PDAC cells and patient tumors markedly. Mechanism investigations revealed that MCL exerted its anti-tumor activity via regulation of EGFR-Akt-Bim signaling pathway thus inducing Bim expression both in vitro and in vivo.

Conclusion

MCL/ACT001 is a highly promising agent in the treatment of PDAC patients.

1. Introduction

Pancreatic ductal adenocarcinomas (PDAC) make up the majority (~95%) of all pancreatic malignancies and are associated with poor overall survival (Connor and Gallinger, 2022; Conway et al., 2019). It is the major death cause of cancer patients. In the past 25 years, the number of cases has more than doubled around the world (Klein, 2021). Most patients with PDAC present with symptomatic, surgically unresectable disease (Singhi et al., 2019). Treatment with the classic drugs gemcitabine, FOLFIRINOX or nab-paclitaxel offers limited therapeutic benefits and the overall survival did not improved significantly because of drug resistance (Zhu et al., 2018). Even though many molecular targeted agents are being
tested in clinical trials, there are still very few new drugs treated PDAC application for marketing (Neoptolemos et al., 2018). Hence, it is urgent to find new agent that are safe and effective for the treatment of PDAC.

Epidermal growth factor receptor type1 (HER1/EGFR) is overexpressed in PDAC and is associated with tumorigenesis and poor prognosis. Ardito, C. M. and Navas, C. showed that the development of PDAC, which was driven by K-Ras oncogenes, is totally dependent on EGFR signaling (Ardito et al., 2012; Navas et al., 2012). Bruns, C. J. showed that EGFR-Akt signaling inhibitors could inhibit the pancreatic tumor growth and metastasis in animal model. Moore, M. J. revealed that EGFR inhibitor Erlotinib combined gemcitabine could improve the anticancer effects and prolonged the progression-free survival compared to gemcitabine alone (Bruns et al., 2000; Moore et al., 2007). Furthermore, Lihua Huang suggested EGFR-Akt signaling pathway played an important role in the progression of cell apoptosis (Huang and Fu, 2015). Chen F revealed EGFR-Akt signaling has close relationship with Bim, a critical mediator of apoptosis. His group found EGFR activation suppressed Bim expression (Chen et al., 2018). Chen, H. found low expression of Bim has relationship with EGFR inhibitor resistance (Chen et al., 2017). Coloff JL revealed Bim's key role in Akt-mediated cell survival in cancer (Coloff et al., 2011). Reynolds C and Yue D found inhibition of Akt promoted Bim induced apoptosis in hepatocellular carcinoma and leukemia (Reynolds et al., 2014; Yue and Sun, 2018). Reactive oxygen species (ROS) also play an essential role in the process of tumor development (Gorrini et al., 2013; Moloney and Cotter, 2018). ROS overload could accelerate cell death.

Micheliolide (MCL) is a kind of guaianolide sesquiterpene lactone. It extracted from michelia compressa or synthesized from parthenolide (PTL), a natural product with many anti-tumor effects (Ghantous et al., 2013; Zhai et al., 2012). The half-life of MCL is 2.64 h in mouse plasma, while PTL is 0.36 h, which makes MCL to be a promising candidate for cancer therapy (Jin et al., 2007; Zhang et al., 2012). ACT001, the pro-drug of MCL, is synthesized and developed by Professor Yue Chen and could sustained release of MCL over 8 h in humans (Xi et al., 2019). Studies found that MCL or ACT001 has anti-tumor effect in many cancers, such as colitis-associated cancer (Viennois et al., 2014), rhabdomyosarcoma (Xu et al., 2019), breast cancer (Jia et al., 2015a; Jin et al., 2018), leukemia (Ji et al., 2016; Li et al., 2018) and glioma (An et al., 2015; Guo et al., 2019). A phase clinical trial for ACT001 to treat glioma patients is in progress. However, the study with MCL/ACT001 on PDAC have not been reported.

In this study, we investigated the anti-tumor effect of MCL/ACT001 in PDAC. MCL, the active metabolite of ACT001, could not only induce ROS generation, mitochondrial dysfunction, but also inhibits EGFR-Akt signaling and promotes Bim induced apoptosis in PDAC cells. These results suggest that MCL/ACT001 could be a novel potential agent for the treatment of PDAC.

2. Materials And Methods

Cell lines and Cell culture
The human PDAC cell lines PANC-1, Mia-PaCa-2 and human pancreatic ductal epithelial cell HPDE6-C7 were cultured in DMEM medium containing 10% fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin); HEK293T, BxPC-3 were cultured in RPMI1640 medium containing 10% FBS and antibiotics (penicillin and streptomycin); CFPAC-1 was cultured in IMDM medium containing 10% FBS and antibiotics (penicillin and streptomycin). They were all cultured in 5% CO$_2$ incubator at a constant temperature 37 $^\circ$C.

**Antibodies and Reagents**

The antibodies were used as follows: anti-EGFR (Santa# sc03), anti-P-EGFR (Santa# sc-57545), anti-Akt (CST# 4685X), anti-P-Akt (Ser473) (AF0016), anti-cl-Caspase3(CST# 9661L), anti-Bim (Santa# sc-11425), anti-β-actin (easybio), anti-Ki67 (CST# 9449), MCL and ACT001 were synthesized by Yue Chen group.

**Generation of luciferase-expressing stable cell lines**

PLenti-CMV-Puro-Luc plasmid was transfected into HEK293T cells together with auxiliary plasmids (pLP1, pLP2 and VSV-G) to package the lentivirus. The viral particles were collected at 24 h and 48 h. PANC-1 cells transfected with the viral particles in plate with 50% confluence. After infection, PANC-1-Luc stable transfected cells were selected using 2 µg/mL of puromycin (Solarbio, #P8230).

**Cell viability assay**

In exponential growth period, the cells were counted and mixed well, then plated 100 µL/well cell suspension into 96-well plates. After incubated for 12 h, the cells were added 100 µL/well MCL or ACT001. When the time was up, 20 µL MTT (5 mg/mL) reagent added to each well and cells were incubated for 2 h in the incubator. Then discarded the supernatant and added 100 µL DMSO into the cells. After 10 min incubation, the absorbance was measured using an Tecan InfiniteF50 microplate reader.

**Wound healing assay**

In exponential growth period, the cells were plated into 6-well plates and then incubated for 24 h in the CO2 incubator. When the cells overgrown, a 200 µL pipette tip was used to put a scratch on the cell monolayer. Then discarded the medium, washed with PBS twice and added fresh medium with or without MCL into each well. Micrographs were obtained at 0 h and 24 h post-scratch.

**JC-1 analysis for mitochondrial membrane potential (MMP)**

5×10$^5$ cells/well were plated into 6-well plates, incubated for 12 h and then treated with MCL for 6 h. Subsequently, the cells were collected, centrifuged and incubated with JC-1 (2 µM) for 30 min at 37°C, then washed, resuspended in PBS and analyzed with Fluorescence Activated Cell Sorter (FACS). MMP was quantitated by the ratio of red fluorescence to green fluorescence.
**Measurement of ROS levels**

For the ROS measurement, the cells were plated into black 96-well plates (Thermo # 165303). After 12 h incubation, 30 µM MCL was added and then treated the cells for indicated times, then discarded the supernatant and added 10 µM oxidant-sensing fluorescent probe DCFH-DA (Beyotime), after 20 min incubation at 37°C in the dark, the fluorescence was examined using an Tecan InfiniteF50 microplate reader with the green fluorescence channels (488 nm excitation/525 nm emission).

**Immunoblotting**

Cells or tumor tissues were lysed using cold RIPA buffer on the ice for 20 min. The lysate was centrifuged for 25 min at 12000rpm at 4°C. Then the supernatant was collected and the protein concentration was measured using Pierce BCA protein assay kit (Thermo # SH251403), then added loading buffer. The protein sample was added into SDS-PAGE gels at 20 µg/well and electrophoresed, then transfer onto PVDF membrane at low temperature, after that, the membranes were blocked with 5% BSA diluted in TBST for 1 h and incubated with primary antibody overnight and second antibody for 1 h.

**Cell apoptotic assay**

5×10^5 cells/well were plated into 6-well plates and incubated for 12 h followed by incubating with various concentrations of MCL for 24 h. Cells were collected, centrifuged, washed twice with PBS and suspended in 500 µL binding buffer, stained with Annexin V-APC/7-AAD apoptosis kit (MultiSciences Biotech, China), then determined using FACS.

**Mouse xenograft studies**

Female Balb/c nude mice (Vitalriver Beijing, China) at the age of 6 to 8 weeks were used. Surgical implantation was operated to perform orthotopic model. 100 µL 5×10^6 luciferase-expressing PANC-1 cell suspended with PBS/Matrigel (1:1) (BD Biosciences) was injected into the pancreas of the anesthetized mice (isoflurane, RWD Life Science Co.,Ltd). After the operation, the mice were injected with ketoprofen (Yuanye Biotechnology Co. Ltd.) (Kowolik et al., 2019). When bioluminescent intensity was up to 1×10^8, the mice were randomized into two groups: vehicle control (n = 10) and ACT001 (n = 10; 400 mg/kg/day, p.o.). Bioluminescent imaging was photographed weekly. Animal health was monitored daily during the experiments. After 6 weeks, mice were euthanized and the tumors were removed for the following studies.

**Bioluminescent reporter imaging**

Before imaging, intraperitoneal injection with D-luciferin (120 mg/kg, i.p.; PerkinElmer Inc.; #122799) was performed. Then the mice were turned sideways on the black board of the instrument and the bioluminescent signals of pancreas region were recorded using the IVIS Spectrum System.

**Immunohistochemistry (IHC)**
The paraffin-embedded tissue sections were sliced to 5 µm and dewaxed with xylene, rehydrated with gradient ethanol, citrate buffer was used to repair antigen. Then, the slices incubated with fresh 3% H₂O₂ (diluted with methanol) for 10 min and blocked with 10% goat serum (diluted with PBS) at room temperature for 30 min, incubated with primary antibodies at 4°C overnight and secondary antibodies at room temperature for 30 min. After that, the sections were stained with DAB solution (Solarbio, DA1010) and hematoxylin for appropriate time.

**TUNEL assay**

The apoptosis of tumor tissues was determined by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay according to the kit (absin, abs50021). Staining was assessed using fluorescing microscopy.

**Patients and tissue samples**

The pancreatic tumor and adjacent normal tissues from three PDAC patients were provided by Peking Union Medical College Hospital. All patients obtained the written consent.

**The siRNA oligos**

<table>
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<th>Antisense(5'-3')</th>
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<td>UUCAGGAUUACCUUGGGCTT</td>
</tr>
<tr>
<td>BIM-2</td>
<td>CCCAUGAGUUGUGACAAUUTT</td>
<td>AUUUGUCACAACUCAUGGTT</td>
</tr>
<tr>
<td>BIM-3</td>
<td>GCGUAUUGGAGACGAGUUUTT</td>
<td>AAACUCGUCUCCAAUAAGCCTT</td>
</tr>
</tbody>
</table>

Data was represented as mean ± SEM. We used Graphpad prism 8.0 to analyze statistics. All the experiments were repeated at least three times unless otherwise stated. We used Student’s t test to compare two groups. p value less than 0.05 indicated statistically significant.

### 3. Results

**3.1 MCL inhibited proliferation and migration in PDAC cells.** To investigate the effect of MCL/ACT001 on PDAC cell survival in vitro, PDAC cells BxPC-3, Mia-Paca-2, CFPAC-1, PANC-1 and the control pancreatic ductal cell HPDE6-C7 were treated with different concentrations of MCL/ACT001 for 48 h, and MTT assay was used to monitor the cell survival. The growth of five cell lines was suppressed by MCL/ACT001 in a dose-dependent manner (Fig. 1A-1C). The IC50s of MCL against BxPC-3, Mia-Paca-2, CFPAC-1 and PANC-1 cells were 14.43±0.22, 7.21±0.33, 11.15±0.42 and 13.43±1.14 µM respectively, while the IC50 of HPDE6-C7 cell was 41.3±9.1 µM. The IC50s of ACT001 against BxPC-3, Mia-Paca-2,
CFPAC-1 and PANC-1 cells were 27.11±0.18, 10.02±0.33, 23.8±0.5 and 23.98±2.82 μM respectively, the IC50 of HPDE6-C7 cell was 30.1±4.31 μM, indicating that normal cells were resistant to MCL/ACT001 treatment.

We further evaluated the effect of MCL on the migratory ability of PDAC cells. The data showed that the migratory ability of PANC-1 cells treated with 20 μM (43.5%±6%) and 30 μM (22.9%±8.77%) MCL was much weaker than control (70.69%±7.88%) (Fig. 1D). The migratory ability of CFPAC-1 cells treated with 10 μM (29.2%±6.55%) and 20 μM (11.41%±3.06%) MCL was weaker than control (42.92%±6.7%) (Fig. 1E). These results suggested that MCL treatment suppressed cell proliferation and migration in a dose-dependent manner in PDAC cells.

3.2 MCL induced MMP decrease, ROS generation and apoptosis in PDAC cells. To investigate whether MCL induces PDAC cell death via apoptosis, we treated PDAC cells with MCL for 24 h to perform FACS analysis (Annexin V and 7-AAD) using a flow cytometer. Annexin V was used to indicate early apoptosis, and cells with positive for 7-AAD and Annexin V indicated late apoptosis. As shown in Fig. 2, MCL significantly induced PANC-1, CFPAC-1, BxPC-3 and Mia-PaCa-2 cells apoptosis in a dose-dependent manner. The decline of MMP is considered as one of the symbolic events of early cellular apoptosis. Changes of MMP could be assessed by monitoring JC-1, which exhibiting red fluorescent at high membrane potential accumulates in mitochondria and exhibit green fluorescent monomer exits in cytosol. MMP was measured in PDAC cells. PANC-1 and CFPAC-1 cells treated with MCL showed reduction of red fluorescent and increase of green fluorescent (Fig.3A). In our analysis, Previous research found that MCL exerted cytotoxic effects by generating ROS (Ji et al., 2016, Jia et al., 2015b, Wang et al., 2019, Xu et al., 2019), thus the level of ROS in PDAC cells was also detected after the cells treated with MCL for indicated times. Our results showed that ROS accumulated in MCL-treated PDAC cells (Fig. 3B). These data indicated that MMP and ROS might contribute to the effects of MCL on PDAC cells.

3.3 MCL inhibited EGFR/Akt/Bim signaling in PDAC cells. EGFR-Akt hyperactivation and overexpression were found in many cancers including PDAC (Ardito et al., 2012, Li et al., 2021, Nedaeinia et al., 2014). EGFR-Akt signaling was chosen to explore the potential mechanism of MCL induced cell apoptosis. We searched the TCGA database (http://gepia.cancer-pku.cn/index.html) and found that EGFR and Akt were significantly overexpressed in pancreatic tumors compared to normal tissues (Fig. 4A). We then explored whether EGFR and Akt protein expression associated with overall survival, Kaplan–Meier survival analysis was performed in 89 high expressed PDAC patients and 89 lower expressed PDAC patients, and found that higher EGFR, Akt expression positively correlated with reduced overall survival (Fig. 4B). To investigate the roles of EGFR and Akt in PDAC, we performed immunoblot with PDAC cells and human tissues. We found EGFR-Akt signaling was hyperactivated in PDAC cells and tumors compared to HPDE6-C7 cells and the paired noncancerous tissue regions (Fig. 4C, 4D). Treatment with EGFR-Akt inhibitors induced Bim upregulation and apoptosis in leukemia (Reynolds et al., 2014). Researchers revealed a key role of Bim in Akt-mediated cell survival through Akt-dependent stimulation in cancer (Coloff et al., 2011). Whether MCL has an effect on EGFR-Akt-Bim signaling pathway? Fig. 4E-4H showed that MCL treatment for 24 h has little effect on ERK or STAT3 but inhibited EGFR, Akt phosphorylation, up-regulated
apoptosis-associated proteins Bim and cl-Caspase3 expression in PANC-1 and CFPAC-1 cells. To evaluate the role of Bim in the process of MCL-induced cell death, cells transfected with Bim-siRNA or control-siRNA, and then treated with MCL. We found that Bim-siRNA transfected cells had statistically higher survival rate than the control-siRNA group after MCL treatment (Fig. 4I, 4J). These data indicated that the efficacy of MCL induced apoptosis via inhibiting EGFR-Akt-Bim signaling pathway.

3.4 MCL inhibited tumor growth in xenograft tumor model. To study the efficacy of MCL/ACT001 against PDAC in vivo, tumor xenografts were established by injecting luciferase-tagged PANC-1 cells orthotopically into nude mice. The mice were randomized to ACT001 treatment and vehicle control until bioluminescent intensity was up to $1 \times 10^8$. The tumor growth was observed and photographed weekly by bioluminescence imaging (Fig. 5A). Bioluminescent imaging revealed the tumor was statistically significant (2.5-folds difference) between the treatment and the control groups (Fig. 5B, 5C). The mice were euthanized after 6 weeks of treatment. These results indicated that MCL/ACT001 inhibited tumor growth. Furthermore, the tissue sections were stained with hematoxylin-eosin and the vehicle-treated groups exhibited high grade adenocarcinoma. IHC analysis revealed that vehicle-treated tumors contained more Ki-67-positive cells and fewer Bim-positive cells compared to ACT001-treated group, and P-EGFR or P-Akt positive cells were reduced in the tumors treated with ACT001 (Fig. 5D). TUNEL assay revealed that ACT001-treated tumors displayed increased apoptotic cells (Fig. 5E). Immunoblot assay also showed that EGFR-Akt signaling was inhibited, and apoptosis-related proteins Bim, cl-Caspase3 upregulated in ACT001 treated tumors (Fig. 5F). Collectively, our findings emphasized MCL/ACT001, as a new agent, has anti-tumor effect on PDAC in vivo and in vitro.

4. Discussion
PDAC is one of the most lethal epithelial malignancies, the incidence and overall mortality rate was rising every year. Advanced PDAC patients respond worse to the conventional chemotherapy and target therapy because of individual differences and drug resistance. It is very urgent to find new and effective drugs. ACT001 is synthesized and developed by professor Yue Chen in our university and has been designated an orphan drug by FDA. It’s safety and tolerability in advanced glioma patients has been evaluated. In a phase I dose-escalation study, ACT001 dose levels were ranged from 100 mg BID to 1200 mg BID. Patients were well tolerated with no dose-limiting toxicity. It currently in a phase II clinical trials for the treatment of recurrent glioma. The clinical trials of ACT001 are not limited to glioma. A b/ a clinical trial for the treatment of neuromyelitis carried out in PLA General Hospital in China and a phase II clinical trial for the treatment of pulmonary fibrosis carried out in Australia. In these clinical trials, the mechanisms underlying the anticancer and anti-inflammatory of ACT001 are its regulatory effects on inflammatory processes and the cancer immune microenvironment, such as NF-κB, STAT3 signaling pathways inhibition and T cells regulation. MCL, the active metabolite of ACT001, has specific cytotoxicity in many kinds of cancers. MCL inhibits intestinal inflammation and colitis-associated cancer cell viability (Viennois et al., 2014), induces the breast cancer cells death in vitro (Jia et al., 2015b), inhibits glioma (An et al., 2015) and rhabdomyosarcoma (Xu et al., 2019) cell growth, induces acute myelogenous leukemia
stem cells apoptosis in vitro (Ji et al., 2016). It also exerts antitumor effects on hepatocellular carcinoma (Yao et al., 2020). But to date, there have no reports about the effect of MCL/ACT001 on PDAC. In this study, we revealed that the novel agent MCL/ACT001 had anti-tumor growth effect on PDAC in vitro and in vivo.

In our research, we compared the sensitivity to MCL between PDAC cells and human pancreatic ductal cell. The IC50s of MCL in PDAC cells ranged from 7.21 to 14.43 µM, while the IC50 of human pancreatic ductal cell was 41.30 µM, which was 2.86 to 5.73 folds of those in PDAC cells. These data suggested that MCL is more sensitive to PDAC cells than normal cells and it might be a potentially valuable and safe drug for PDAC therapy. What really matters for a good drug is its low toxicity and high efficacy. In the following study, we found that MCL inhibited PDAC cells migration and induced PDAC cell apoptosis. What is the molecular mechanism regulated by MCL?

EGFR frequently overexpressed and EGFR-Akt signaling pathway hyperactivated in different types of human cancers. It is a classic target for anti-tumor drug design (Nedaeinia et al., 2014; Sigismund et al., 2018). Akt also plays an important role in cell apoptosis (Tang et al., 2016). We interrogated the TCGA database and performed immunoblot assay, and found that EGFR and Akt were overexpressed in PDAC tissues and cells. Kaplan–Meier survival analysis revealed high EGFR or Akt expression positively correlated with reduced overall survival in PDAC patients. Researcher shows EGFR inhibition is effective against KRAS-wild-type PDAC patients, altered tumor immune microenvironment and sensitized other chemotherapy drugs in PDAC (Li et al., 2021) (Sidaway, 2017). ACT001 could inhibit PD-L1 transcription and modulates antitumor immune response as well (Tong et al., 2020). PDAC patients who treated with gemcitabine combined with erlotinib have a survival benefit (NCT01303029). Combined inhibition of EGFR and C-RAF completely regressed PDAC (Blasco et al., 2019). We can see that EGFR inhibition combined with other chemical drugs will be more effective on PDAC therapy.

The underlying mechanisms associated with the EGFR-Akt signaling pathway of MCL have been investigated. We found MCL inhibited EGFR, P-EGFR, Akt and P-Akt proteins expression and increased pro-apoptosis protein Bim in PDAC cells. Immunoblot assay showed a remarkable increase of Bim and cl-Caspase3 protein following MCL treatment. Bim, a tumor suppressor, is markedly reduced in human cancer (Greenhough et al., 2010; Tan et al., 2005) and negatively regulated by Akt. Akt inactivation promotes Bim accumulation (Yue and Sun, 2018). Knockdown of Bim partially blocked MCL-induced cell death, indicating that Bim might partially be participated in the process of the MCL-induced apoptosis. Previous studies found MCL induces cancer cell death by decreasing MMP and increasing ROS generation (Jia et al., 2015b). Consistently, MCL treatment induced ROS production and decreased MMP in PDAC cells. Most importantly, the results of animal experiment (Kowolik et al., 2019) indicated that MCL/ACT001 could efficiently inhibit the pancreatic tumor growth with a maximal inhibitory rate of 60.0%.

Taken together, MCL/ACT001 effectively inhibited PDAC cell growth and induced cell apoptosis in vitro and in vivo, the effect of MCL/ACT001 was partially mediated by EGFR-Akt-Bim pathway, and oxidative
stress might also be involved in MCL-induced apoptosis. We hope provide a new choice for PDAC combination therapy.

**Declarations**

**Author contributions** C L and J Y designed this study, completed the data analysis, drafted all figures, and wrote of the manuscript, J Y, X H, Y L and Z L performed the experiment, data collection and revised the manuscript. X P participated in the animal experiment. All authors read and agree with the final manuscript. C L designed, supervised, and guided this study and revised the final manuscript.

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**Data availability** All data generated that are relevant to the results presented in this article are included in this article. Other data that were not relevant for the results presented here are available from the corresponding author (C L) upon reasonable request.

**Conflicts of interest** All authors declare no potential conflicts of interest.

**Ethics statement** All animal studies were approved by the Institutional Animal Care and Use Committee at Nankai University. Human experiments were approved by the human experimentation committee and informed consent was obtained from all subjects.

**Consent to participate** Informed consent was obtained from all individual participants included in the study.

**References**


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**Figures**

![Figure A](image1)

![Figure B](image2)

**Table 1: IC₅₀ values for MCL and ACT001**

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<td>HPDE6-C7</td>
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![Figure C](image3)

**Figure C**

![Figure D](image4)

![Figure E](image5)
Figure 1

**MCL inhibited proliferation and migration in PDAC cells.** (A) The chemical structure of MCL and ACT001. ACT001 is a prodrug, slowly release MCL in HEPES buffer, pH = 7.4 or mouse plasma. (B and C) Four human PDAC cells BxPC-3, Mia-Paca-2, CFPAC-1, PANC-1, one pancreatic ductal cell HPDE6-C7 were treated with indicating concentrations of MCL (B) and ACT001 (C) for 48 h, growth curves were measured by MTT assay and IC50 was calculated. (D and E) MCL inhibited PANC-1 (20 μM and 30 μM) and CFPAC-1 (10 μM and 20 μM) cells migration significantly at 24 h, representative images (left panel) and quantitative analysis (right panel) of the wound healing rates are shown. Scale bars, 100 μm. **P < 0.01, ***P < 0.001, ****P < 0.0001.
**Figure 2**

**MCL induced apoptosis in PDAC cells.** PANC-1, CFPAC-1, BxPC-3 and Mia-PaCa-2 cells treated with 20 μM or 40 μM MCL for 24 h and the rate of apoptosis was quantified using annexin V-APC and 7-AAD staining. Cells were stained with Annexin V-APC/7-AAD Apoptosis Detection Kit. Cell apoptosis was determined by flow cytometry. **P < 0.01, ***P < 0.001.
**Figure 3**

**MCL induced MMP decrease and ROS generation in PDAC cells.** (A) Flow cytometry analysis of mitochondrial membrane potential (MMP) with JC-1 in PANC-1 and CFPAC-1 cells treated with 0, 20, 40 μM MCL for 6 h. Reduction of MMP was detected by the change in JC-1-derived fluorescence from red to green. The quantitative MMP from each group was marked. (B) PANC-1 and CFPAC-1 cells were treated with 30 μM MCL for 0, 2, 4, 6 h, BxPC-3 cells were treated with 30 μM MCL for 0, 3, 6 h, Mia-Paca-2 cells were treated with 30 μM MCL for 0, 1, 2 h and the activity of ROS was detected with DCFH-DA probes. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 4

**MCL inhibited EGFR/Akt/Bim signaling in PDAC cells.** (A) Boxplot of EGFR and Akt gene expression levels in PDAC tissues (n=179) and normal tissues (n=171) from the GEPIA database. (B) Kaplan–Meier analysis with optimal cutoff value of EGFR and Akt expression in human PDAC samples (n (high) = 89, n(low) = 89). (C) Immunoblot analysis of EGFR, P-EGFR, Akt, P-Akt in PDAC patients compared to adjacent normal tissues (N: Normal, T: Tumor). (D) Immunoblot analysis of EGFR, P-EGFR, Akt, P-Akt in
PDAC cells compared to HPDE6-C7. (E and F) Immunoblots of lysates from PANC-1 cells treated with 10 μM, 20 μM MCL for 24 h, detect EGFR, P-EGFR, Akt, P-Akt, p-ERK, p-STAT3, STAT3, apoptosis-related Bim and cl-Caspase3 protein expression. (G and H) Western blotting assay was performed to examine the expression of EGFR, P-EGFR, Akt, P-Akt, p-ERK, p-STAT3, STAT3, apoptosis-related Bim and cl-Caspase3 in CFPAC-1 cells treated with 20 μM MCL. (I) Bim protein level was detected by immunoblot assay. (J) PANC-1 cells were transfected with Bim-siRNA or control-siRNA then treated with 1 μM, 5 μM MCL, cell survival was detected by MTT assay. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
**Figure 5**

**MCL inhibited tumor growth in xenograft tumor model.** (A) Bioluminescence imaging of orthotopically implanted 5×10^6 PANC-1-Luc cells in live mice. The mice were treated with the prodrug ACT001 (400 mg/kg/day, p.o.) or vehicle for 6 weeks (n=10). (B) The data are the relative mean bioluminescence intensity in tumors from ten mice per group. (C) Tumors of PANC-1-Luc cells implanted orthotopically from mice treated with either vehicle or ACT001. (D) H&E staining and IHC analysis of Bim, Ki-67, P-EGFR, P-Akt in xenograft tumors from mice treated with ACT001 or vehicle. Scale bars for HE, 50 μm. Scale bars for IHC, 25 μm. (E) The apoptosis of tumors treated with ACT001 was measured by the TUNEL method and examined using confocal microscopy. Scale bars, 25 μm. (F) Immunoblot analysis of EGFR, P-EGFR, Akt, P-Akt, Bim and cl-Caspase3 in xenograft tumors from mice treated with ACT001 or vehicle. (G) Action model of MCL on PDAC cells. ***P< 0.001.