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# Belinostat-induced apoptosis and growth inhibition in pancreatic cancer cells involve activation of TAK1-AMPK signaling axis

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ABSTRACT

Pancreatic cancer accounts for more than 250,000 deaths worldwide each year. Recent studies have shown that belinostat, a novel pan histone deacetylases inhibitor (HDACi) induces apoptosis and growth inhibition in pancreatic cancer cells. However, the underlying mechanisms are not fully understood. In the current study, we found that AMP-activated protein kinase (AMPK) activation was required for belinostat-induced apoptosis and anti-proliferation in PANC-1 pancreatic cancer cells. A significant AMPK activation was induced by belinostat in PANC-1 cells. Inhibition of AMPK by RNAi knockdown or dominant negative (DN) mutation significantly inhibited belinostat-induced apoptosis in PANC-1 cells. Reversely, AMPK activator AICAR and A-769662 exerted strong cytotoxicity in PANC-1 cells. Belinostat promoted reactive oxygen species (ROS) production in PANC-1 cells, increased ROS induced transforming growth factor-β-activating kinase 1 (TAK1)/AMPK association to activate AMPK. Meanwhile, anti-oxidants N-Acetyl-Cysteine (NAC) and MnTBAP as well as TAK1 shRNA knockdown suppressed belinostat-induced AMPK activation and PANC-1 cell apoptosis. In conclusion, we propose that belinostat-induced apoptosis and growth inhibition require the activation of ROS-TAK1-AMPK signaling axis in cultured pancreatic cancer cells.



## 1. Introduction

Over 43,000 new pancreatic malignancy cases and 36,800 related deaths were reported in the United States in 2010 [1]. Worldwide, about 250,000 pancreatic cancer individuals were diagnosed annually [2]. Pancreatic cancer has a high mortality rate, as it is typically diagnosed at an advanced stage, and surgery can no longer remove the entire tumor [2]. Radiation plus gemcitabine is the "golden" standard therapy for advanced pancreatic cancers [3]. A number of small molecule kinase inhibitors have been developed recently. However, the efficiency of these inhibitors are also limited [4]. One key hurdle is the molecular heterogeneity of pancreatic cancers, which impedes uniform application of specific molecularly targeted agents [5,6]. Over 90% of pancreatic cancers have a K-RAS mutation and constitutive activation (CA) mutation in multiple downstream pathways, including phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) cascade [1,2,7].

The histone deacetylases inhibitors (HDACis) have demonstrated a promising anti-cancer abilities [8–10]. A number of HDACis have shown significant treatment potential for pancreatic cancer with encouraging results at well-tolerated doses [11]. Recent studies demonstrated the anti-proliferation activities of belinostat, a novel pan-HDACi in pancreatic cancer cells [9,12]. Belinostat induced significant cell cycle arrest, growth inhibition and apoptosis in a total of 14 human pancreatic cancer cell lines [12]. Further, belinostat inhibited pancreatic cancer cells in vivo growth in a mice xenograft model [9,12]. Studies showed that belinostat inhibited Akt-mTOR activation, which might count for its anti-proliferation effects in pancreatic cancer cells [12]. However, the underlying molecular mechanisms were not fully understood [12].

AMPK-activating kinase (AMPK) is the master regulator of cell metabolism [13]. Recent studies have shown that AMPK activation promotes cell apoptosis and growth inhibition under stress conditions. As a matter of fact, multiple anti-cancer drugs, including vincristine [14,15], taxol [16,17], temozolomide [18] and doxorubicin [19,20] are able to activate AMPK-dependent cell apoptosis pathway. It is now known that AMPK promotes cell apoptosis through regulating its downstream signaling targets. For example, AMPK activation inhibits mTOR-dependent cell growth [21]. AMPK also activates pro-apoptotic p53 [22] and JNK signaling [23]. In the

*Abbreviations:* AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; HDACi, histone deacetylases inhibitor; NAC, N-acetyl-cysteine; (mTOR), mammalian target of rapamycin; ROS, reactive oxygen species; TAK1, transforming growth factor-β-activating kinase 1; $H_2O_2$, hydrogen peroxide.

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current study, we observed a significant AMPK activation by belinostat in PANC-1 pancreatic cancer cells, which was required for apoptosis and anti-proliferation effect.

## 2. Material and methods

### 2.1. Chemical and reagents

Lipofectamine™ 2000, 5-(and-6)-carboxy-20 and 70-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA) were purchased from Invitrogen (Shanghai, China). Anti-S6K, AMPK, Acetyl-CoA carboxylase (ACC), transforming growth factor-β-activating kinase 1 (TAK1) and tubulin antibodies were purchased from Santa Cruz biotechnology (Santa Cruz, CA). All other antibodies used in this study were purchased form Cell Signaling Tech (Denver MA). The enhanced chemiluminescence (ECL) western blot reagent kit was purchased from Pierce (Rockford, IL). AICAR(5-Aminoimidazole-4-carboxamide ribonucleotide), N-Acetyl-Cysteine (NAC) and MnTBAP were purchased form Sigma (Shanghai, China). A-769662 was obtained from selleck.cn (Shanghai, China).

### 2.2. Cell culture

PANC-1 pancreatic cancer cells were maintained in RPMI 1640 (Gibco Life Technologies, Carlsbad, CA) supplemented with 10% FBS, penicillin/streptomycin (1:100, Sigma), in a humidified incubator at 37 °C and 5% $CO_2$.

### 2.3. Cell viability detection

As previously reported [24], cell viability was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay. Briefly, PANC-1 cells were seeded in 96-well plates at a density of $2 \times 10^4$ cells/well. Forty-eight hours after the indicated treatment, MTT tetrazolium salt (Sigma, 0.20 mg/ml) was added to each well, cells were further incubated in $CO_2$ incubator for 2–3 h. Afterwards, DMSO (150 μl/well) was added to dissolve formazan crystals, the absorbance of each well was observed by a plate reader at a wavelength of 490 nm.

### 2.4. Clonogenic survival

As previously reported [25], a total of 1000 cells were seeded onto 100-mm dish and allowed to attach overnight. Three dishes were used for each treatment condition. Cells were treated with indicated drugs. Seven days after exposure, colonies were stained with Giemsa solution and counted. Clonogenic experiments were repeated at least three times.

### 2.5. Annexin V detection

Cell apoptosis was detected by the Annexin V Apoptosis Detection Kit (Promega, San Jose, CA) according to the manufacturer's protocol. Briefly, 36 h after indicated treatments, PANC-1 cells were stained with PE-Annexin V and Propidium iodide (PI) fluorescence dye. The apoptosis percentage was reflected by Annexin V/PI percentage, detected by a flow cytometry machine (BD Bioscience).

### 2.6. ROS assay

The ROS level in the PANC-1 cells was determined by carboxy-H2DCFDA staining assay, which was based on the fact that the nonpolar, nonionic H2-DCFDA crosses cell membranes and was hydrolyzed into non-fluorescent H2-DCF by intracellular esterase. In the presence of ROS, H2-DCF was rapidly oxidized to become highly fluorescent DCF. After indicated treatment, PANC-1 cells were incubated with 1 μM of carboxy-H2-DCFDA at 37 °C for 30 min with. PANC-1 cells ($1 \times 10^6$) were then resuspended in phosphate-buffered saline (PBS, pH7.4) and sent to flow cytometry analysis (BD Bioscience). The percent of fluorescence-positive cells was recorded on a spectofluotometer using excitation and emission filters of 488 and 530 nm. ROS level in drug treated group was normalized to vehicle control group.

### 2.7. Western blotting and data quantification

Cells were collected by scraping, washed with cold PBS, and resuspended in whole-cell lysis buffer (50 mM Tris [pH 8.0], 250 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate, 5 mM EDTA, 2 mM $Na_3VO_4$, 10 mM $Na_2P_2O_7$, 10 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride). Lysates were mixed and incubated on ice for 10 min, and then cell debris was spun down at a speed of 10,000×*g* for 10 min. Proteins were separated by SDS–PAGE gel and electro-transferred to a PVDF membrane (Bio-Rad). Primary antibodies used were mentioned above. Protein bands were visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz) and an enhanced chemiluminescence (ECL) kit. The intensity of each phosphorylated kinase was normalized to the intensity of corresponding non-phosphorylated kinase. The number was expressed as fold change vs. vehicle control group, vehicle group was labeled as "1.00".

### 2.8. Stable siRNA knockdown of TAK1 and AMPKα1/2

The lentiviral particles containing AMPKα1/2 [26] or TAK1 shRNA were purchased from Santa Cruz Biotech (Santa Cruz, CA), lentiviral shRNAs were added to the PANC-1 cells for 36 h, and stable clones expressing scramble-, AMPKα1/2- or TAK1-shRNA were selected by puromycin (2.0 μg/ml). Cell culture medium was replaced with fresh puromycin-containing medium every 24 h, until resistant colonies can be identified. The expression level of AMPKα1/2 or TAK1 in stable cells was always tested by western blot.

### 2.9. AMPK dominant negative mutation and stable cell selection

Human AMPK-α1 cDNA was amplified from PANC-1 cell cDNA and sub-cloned into the BamHI site of pcDNA3.1 (Invitrogen). The dominant-negative (DN) mutant of AMPK-α1 (AMPK-α1-T172A) was created by mutating the Thr172 residue into Ala as previously reported [27,28]. DN-AMPK-α1 cDNA was transfected to the PANC-1 cells through lipofectamine 2000 protocol [27], stable cells were selected through neomycin (2 μg/ml). Empty vector (pcDNA3.1) transfected stable cells were used as control.

### 2.10. Co-Immunoprecipitation (Co-IP)

Similar to previously reported [29], PANC-1 cell lysate (600–800 μg) in 1 mL Co-IP lysis buffer (350 μL 1% Triton and 0.3% CHAPS buffer) was rotated overnight at 4 °C with 1 μg of anti-AMPKα1/2 antibody (Santa Cruz). Protein A/G-agarose (25 μl) was then added to the supernatants for 2–3 h at 4 °C. Pellets were washed a few times with PBS, resuspended in lysis buffer, and then assayed in western-blots to detect TAK1.

### 2.11. Statistical analysis

The data was expressed as means ± SE. Statistical difference was analyzed by one-way ANOVA followed by multiple comparisons performed with post hoc Bonferroni test (SPSS version 15). Value of $p < 0.05$ was considered statistically different.