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

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A R T I C L E   I N F O

A B S T R A C T

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

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-dichloro-dihydrofluorescein 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 MnT-BAP 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 2×10^4 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 non-polar, 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 (1×10^6) 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 phenylmethyl-sulfonyl fluoride). Lysates were mixed and incubated on ice for 10 min, and then cell debris was spun down at a speed of $10,000 \times g$ 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.

3. Results

3.1. Belinostat induces AMPK activation in cultured pancreatic cancer cells

We first examined the effect of belinostat on AMPK activation in PANC-1 pancreatic cancer cells [30]. AMPK activation was reflected by the enhanced phosphorylation of AMPKα (Thr 172) and its downstream target kinase acetyl-CoA carboxylase (ACC, Ser 79) [30]. Western blot results in Fig. 1A demonstrated that belinostat induced AMPK activation in a time-dependent manner (Fig. 1A). Fig. 1B showed that belinostat induced AMPK activation in a dose-dependent manner, and significant AMPK/ACC phosphorylation was observed by 1 or 10 μM of belinostat treatment. Two
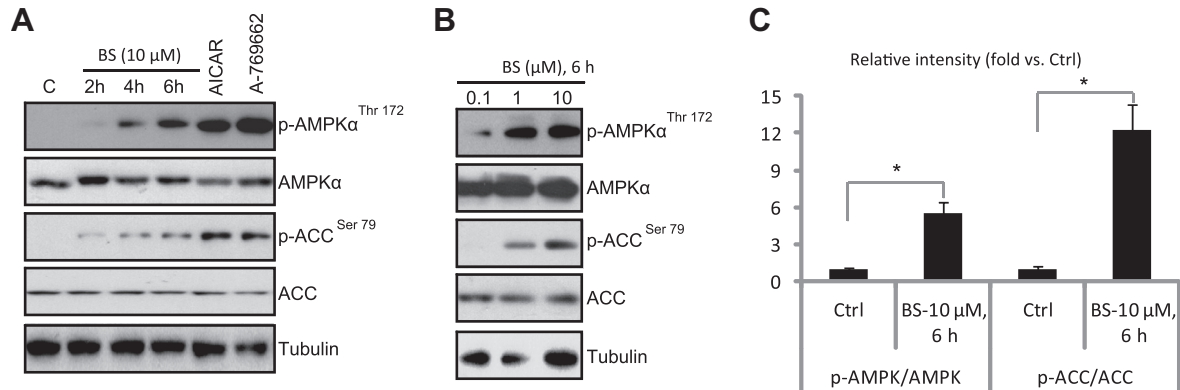
Fig. 1. Activation of AMPK by belinostat in PANC-1 pancreatic cancer cells. Cultured PANC-1 pancreatic cancer cells were treated with vehicle (DMSO, 1%), 10 μM of belinostat (BS, for 2 h, 4 h and 6 h) (A) or AMPK activators AICAR (1 mM, 2 h) (B) or A-769662 (10 μM, 2 h) (A), phospho- and total AMPK and ACC were detected by western blot, tubulin (loading control) was also tested. AMPK and ACC phosphorylation was quantified as described. The number was expressed as fold change vs. vehicle control group (C). Data in this figure was repeated at least three times, and similar results were obtained. $^*p < 0.05$.
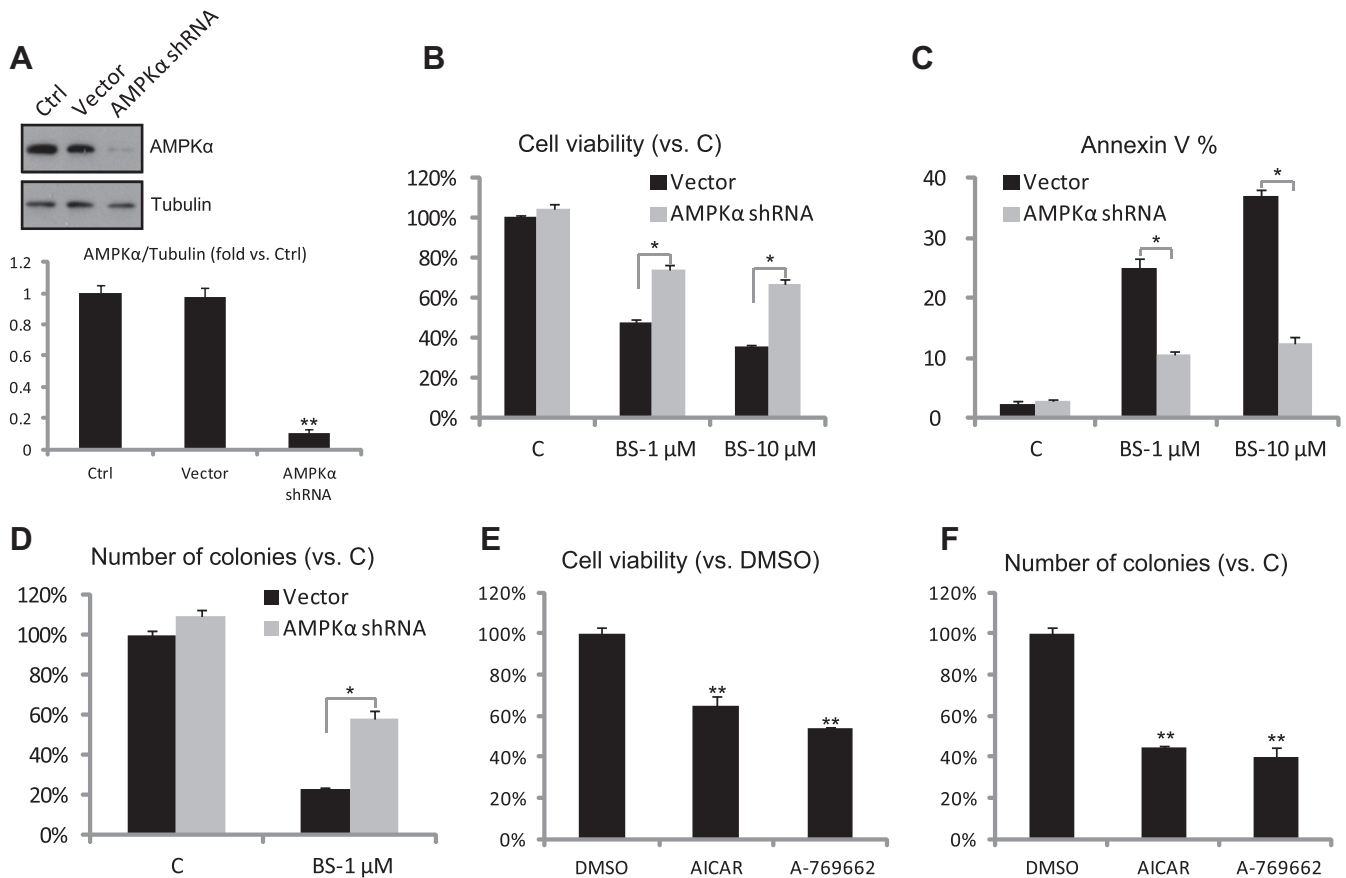
Fig. 2. Activation of AMPK is important for belinostat-induced cytotoxic effects. Reprehensive western blots showing the expression levels of AMPKα and tubulin (loading control) in empty vector- or AMPKα shRNA-transfected stable cells (A). Vector-transfected or AMPKα-shRNA-transfected stable PANC-1 cells were treated with 1 or 10 μM of belinostat for 48 h, cell viability was analyzed by MTT assay (B), Annexin V positive cells were recorded by FACS (C), colonial survival was examined by clonogenicity assay (D). Cultured PANC-1 cells were treated with AMPK activators AICAR (1 mM) or A-769662 (10 μM), cell viability (E) and number of colonies (F) were detected. Data in this figure was repeated at least three times, and similar results were obtained. $^*p < 0.05$ (B–E). $^{**}p < 0.05$ vs. vehicle control group.

well-known AMPK activators AICAR [31] and A-769662 [32] also promoted AMPK activation in PANC-1 cells (Fig. 1A). Belinostat-induced AMPK/ACC phosphorylation was quantified in Fig. 1C.

3.2. Activation of AMPK is important for belinostat-induced cytotoxicity

We established a stable PANC-1 cells expressing AMPKα shRNA. As shown in Fig. 2A, the protein level of AMPKα was significantly reduced in the stable cell line. This cell line was then used as AMPKα knockdown cell line. Results in Fig. 2B–D demonstrated that AMPKα knockdown significantly reduced belinostat-induced cell viability loss (Fig. 2B), apoptosis increase (Annexin V percentage, Fig. 2C) and survival colonies loss (Fig. 2D). Reversely, both AMPK activators AICAR and A-769662 inhibited PANC-1 cell viability (Fig. 2E) and colonies survival (Fig. 2F). These results suggested that activation of AMPK is important for belinostat-induced cytotoxicity in cultured PANC-1 cells.
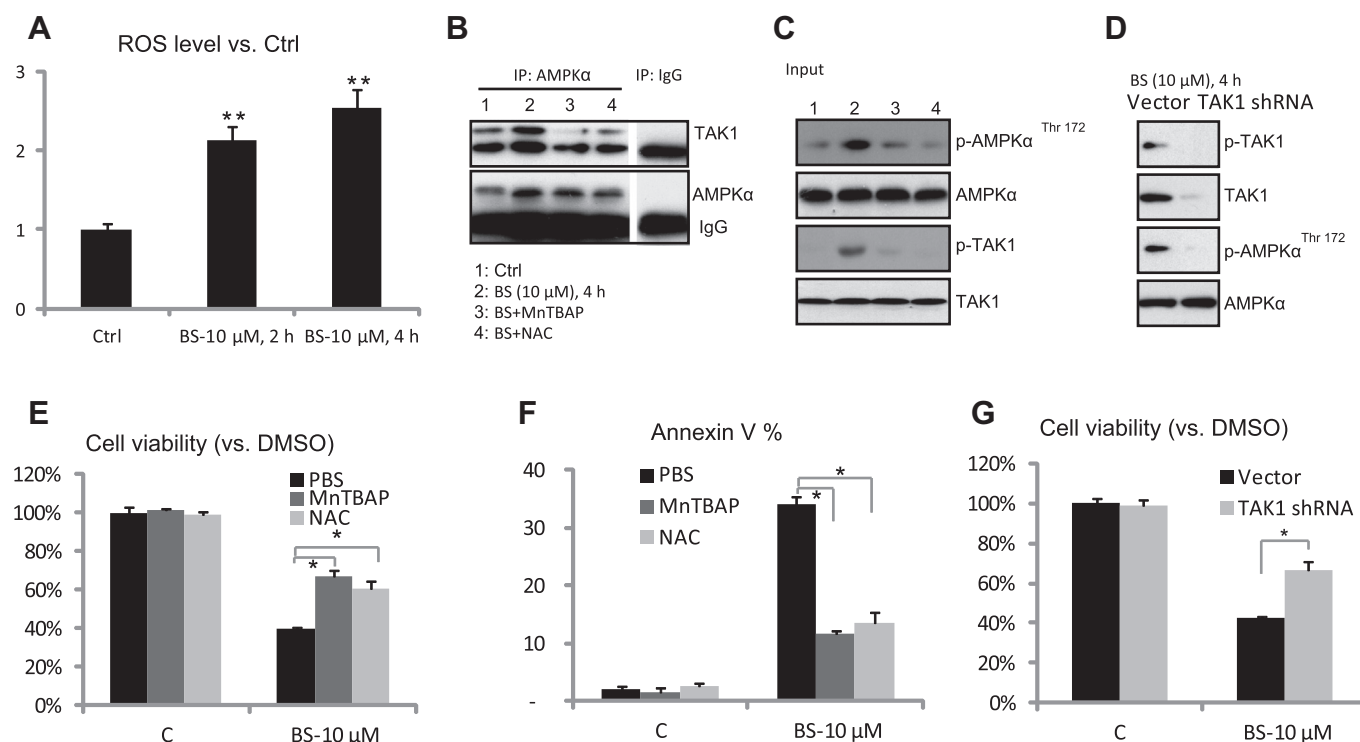
3.3. ROS and TAK1 are involved in belinostat-induced AMPK activation

Next we aimed to understand the signaling pathway required for AMPK activation by belinostat. ROS is a known activator of AMPK [33]. Studies have shown that anti-cancer reagents, including vincristine [14] and temozolomide [18] activate AMPK through ROS production. Therefore, we tested the ROS level in belinostat-treated PANC-1 cells. Results in Fig. 3A showed that intracellular ROS level increased quickly after belinostat treatment in PANC-1 cells. Importantly, two anti-oxidants NAC and MnTBAP inhibited belinostat-induced AMPK activation in PANC-1 cells (Fig. 3C).

Recent studies have confirmed the requirement of TAK1 for ROS-mediated AMPK activation [34,35]. Consistent with these studies, we found that TAK1 was also important for AMPK activation by belinostat. TAK1 shRNA knockdown largely inhibited belinostat-induced AMPK activation in PANC-1 cells (Fig. 3D). Significantly, co-immunoprecipitation (co-IP) results in Fig. 3B showed that TAK1 formed a complex with AMPK after belinostat administration. In PANC-1 cells, antioxidants NAC and MnTBAP almost blocked this interaction by belinostat (Fig. 3B), suggesting that this interaction was ROS dependent. Results in Fig. 3E and F showed that NAC and MnTBAP reduced belinostat-induced cell viability loss (Fig. 3E) and apoptosis induction (Fig. 3F). Meanwhile, TAK1 deficient PANC-1 cells were resistant to belinostat (Fig. 3G). Taken together, these data suggest that belinostat induces ROS production and following TAK1/AMPK complex formation, which is required for AMPK activation.
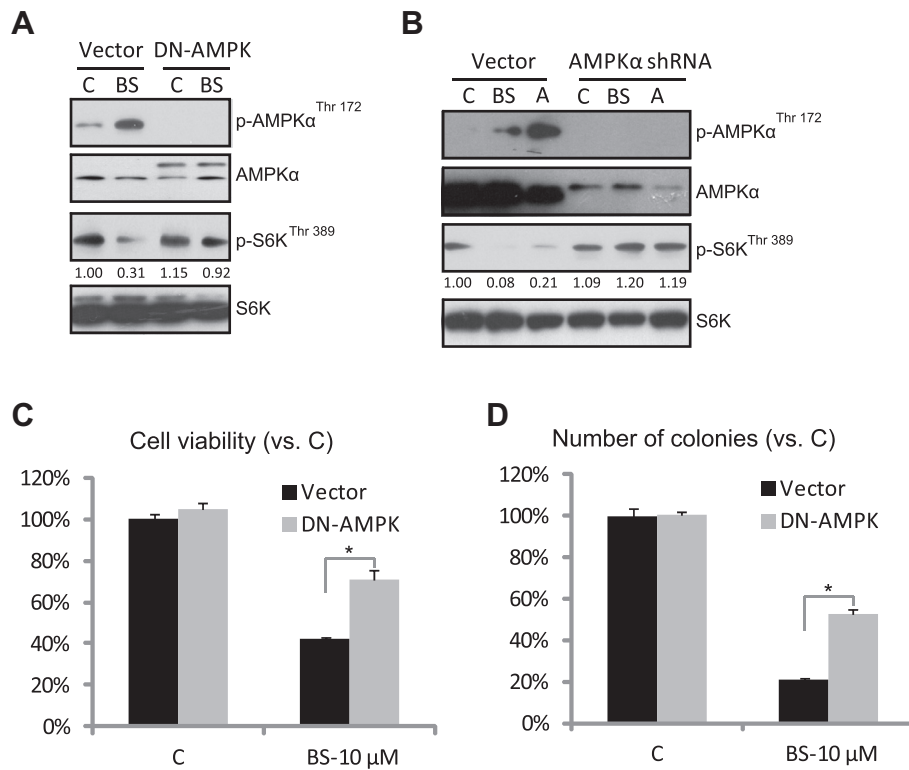
3.4. AMPK activation mediates mTOR inhibition by belinostat

To further confirm that AMPK activation is important for belinostat-induced cytotoxic effect, we introduced a dominant negative (DN) mutation of AMPKα1 (T172A) to PANC-1 cells (see material and methods). Western blot results in Fig. 4A confirmed that stable PANC-1 cells expressing DN-mutant form of AMPKα1 showed no activation of AMPK (AMPKα/ACC phosphorylation) by belinostat. Notably, belinostat-induced S6K inhibition was almost blocked by AMPK mutation (Fig. 4A) in PANC-1 cells. S6K phosphorylation (Thr 389) is major downstream target and indicator of mTOR activation. Similarly, AMPKα shRNA knocking-down also reversed belinostat's and A-769662's effect on S6K phosphorylation

Fig. 3. ROS and TAK1 are involved in belinostat-induced AMPK activation. Cultured PANC-1 cells were treated with belinostat (BS, 10 μM) and cultured for 2 and 4 h, ROS level was detected and quantified (A). PANC-1 cells were pretreated with anti-oxidant NAC (500 μM) or MnTBAP (100 μM) for 1 h, followed by belinostat (BS, 10 μM) treatment, the association between TAK1 and AMPKα was detected by immunoprecipitation (IP) (B), phospho- and total levels of TAK1 and AMPKα were detected by western blots (C). Vector and TAK1 shRNA-transfected stable PANC-1 cells were treated with belinostat (BS, 10 μM), cells were further cultured for 4 h, phospho- and total levels of TAK1 and AMPKα were detected (D). PANC-1 cells were pretreated with anti-oxidant NAC (500 μM) or MnTBAP (100 μM) for 1 h, followed by belinostat (BS, 10 μM) administration for 48 h, cell viability (E) and annexin V positive cells (F) were detected. Empty vector and TAK1 shRNA transfected PANC-1 cells were treated with belinostat (BS, 10 μM) for 48 h, cell viability was analyzed (G). Data in this figure was repeated three times, and similar results were obtained.$^{**}p < 0.05$ vs. vehicle control group (A). $^{*}p < 0.05$ (E–G).

Fig. 4. AMPK activation mediates mTOR inhibition by belinostat. PANC-1 cells expressing empty vector (A), dominant negative (DN) form of AMPK (T172A) (A) or AMPKα shRNA (B) were treated with belinostat (BS, 10 μM) or A-769662 (A, 10 μM) (B) for 12 h, phospho- and total levels of AMPKα (Thr 172) and S6K (Thr 389) were detected by western blots. S6K phosphorylation was quantified. Vector- or dominant negative form of AMPK (T172A)-transfected stable PANC-1 cells were treated with belinostat (BS, 10 μM), cell viability (C) and colonial survival (D) were detected as described. Data in this figure was repeated at least three times, and similar results were obtained. $^*p < 0.05$.

(Fig. 4B). Results in Fig. 4C and D showed that AMPK DN mutation also suppressed belinostat-induced loss of PANC-1 cell viability and survival colonies. These results together suggest that AMPK activation mediates mTOR inhibition by belinostat in PANC-1 cells, which might be responsible for its anti-proliferation effect.

4. Discussion

In cultured pancreatic cancer cells (PANC-1 line), we found that belinostat induced ROS production to activate AMPK signaling cascade, the latter was important for the cell apoptosis and growth inhibition. Although LKB1 is the well-established upstream kinase of AMPK, ROS-induced AMPK activation may not solely depend on LKB1. A recent study by Woods et al., suggested that H_2O_2- and ROS-induced AMPK activation required Ca^{2+}/calmodulin-dependent protein kinase β (CaMKKβ), but not LKB1 [36]. As a matter of fact, it was found that AMPK could still be activated by H_2O_2 in cells without LKB1 [36]. CaMKKβ is an important and alternative kinase for AMPK activation [36,37]. Intriguingly, Alexander et al., showed that ataxia-telangiectasia mutated (ATM) was also important for AMPK activation by H_2O_2 [38]. ATM is the cellular damage sensor that coordinates the cell cycle checkpoints and DNA repair, the authors showed that H_2O_2 potently and rapidly activated cytoplasm ATM, which was responsible for AMPK activation [38].

Our data suggest that ROS-dependent TAK1 activation is responsible for AMPK activation by belinostat. TAK1 shRNA knockdown significantly suppressed belinostat-induced AMPK activation. Further, Co-IP results demonstrated that TAK1 directly associated with AMPK in belinostat-treated PANC-1 cells. This interaction was almost blocked by anti-oxidants. Our results are consistent with a previous publication showing that AMPK activation by TRAIL (tumor necrosis factor-related apoptosis-inducing li-

gand) requires TAK1, but not LKB1 or CaMKK-β [35]. Further, a very recent study shows that TAK1 directly associates and activates AMPK in responsible to H_2O_2 in cultured cardiomyocytes [34]. However, the potential link between TAK1 with other possible AMPK upstream kinases in our system still needs characterization. Also, the detailed mechanism by which TAK1 activates AMPK warrants further investigations.

In the current study, we found that belinostat-induced apoptosis, viability loss and growth inhibition were significantly inhibited in AMPK deficient (shRNA-knockdown) or mutant (DN) cells, indicating that activation of AMPK was required for belinostat-induced cytotoxicity. Meanwhile, inhibition of AMPK upstream molecular ROS and TAK1 also suppressed belinostat's cytotoxicity in our system. These results are consistent with recent publications showing that AMPK activation mediates ROS-induced cytotoxicity in neurons and skin cells [39,40]. Meanwhile, in cardiomyocytes, Chen et al., observed a AMPK-dependent cell death pathway by H_2O_2 [34]. Future studies are needed to test these observations in other pancreatic cancer cell lines, or in primary cells.

In conclusion, we discovered a significant AMPK activation by belinostat in PANC-1 pancreatic cancer cells, which was required for mTOR suppression, PANC-1 cell growth inhibition and apoptosis. ROS and TAK1 were potential upstream molecular for belinostat-induced AMPK activation and the cytotoxic effects.

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