

Effect of arenobufagin on human pancreatic carcinoma cells

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Abstract. Pancreatic carcinoma (PC) is a deadly form of cancer with poor overall survival. Currently, chemotherapy such as gemcitabine and 5-fluorouracil (5-FU) are the most popular medications that can improve survival, but rapid drug-resistance makes the search for more effective drugs urgent. Upon looking for natural components to treat PC, it was found that arenobufagin, a cardiac glycosides-like compound, showed significant effects on the gemcitabine-resistant pancreatic carcinoma cell line Panc-1 and the gemcitabine-sensitive cell line ASPC-1 at nanomolar concentrations. The present study used MTT and clonogenic survival assays to examine survival and proliferation, and western blotting to assess changes in the associated mitogen activated protein kinase and phosphoinositide 3-kinase pathways and expression of apoptosis-related proteins. The current study also detected the cell cycle by flow cytometry. Arenobufagin inhibited cell survival and proliferation, decreased the phosphorylation of key downstream proteins of K-Ras, including protein kinase B and extracellular signal related kinase, induced cell cycle G2/M phase arrest and apoptosis, and downregulated the level of phosphorylated epidermal growth factor receptor. Notably, the present data also showed that arenobufagin can enhance the sensitivity of PC cells to gemcitabine and 5-FU. In conclusion, arenobufagin could enhance the effect of gemcitabine and 5-FU on PC cells by targeting multiple key proteins. Therefore, arenobufagin has potential as an adjuvant therapy for the treatment of PC.

Introduction

Pancreatic cancer (PC) ranks as the fourth leading cause of cancer-associated mortality in the USA, with the worst prognosis of all solid tumors. It is estimated that 40,560/48,960 patients diagnosed with PC in the USA will succumb to this disease in 2015 (1). Despite advancements in the understanding of the genetics of PC and application of combined chemotherapy and targeted biological agents, the management of this lethal malignancy remains one of the greatest oncological challenges (2). Currently, the only successful treatment for a local pancreatic tumor is surgery, and adjuvant chemotherapy after surgery is indicated to delay relapse, but the effect is limited (3). For those who have advanced and metastatic PC, chemotherapy is a primary treatment and gemcitabine has become the most popular first-line therapy for the treatment of pancreatic ductal adenocarcinoma (PDAC) (4,5). However, the response ratio of PDAC to gemcitabine in clinical research is <25%, and those patients showing initial response generally develop drug resistance during therapy (6,7). The development of rapid resistance to gemcitabine may be due to either stem-like subpopulations of tumor cells, which have innate resistance to chemotherapy, or be caused by molecular changes in cancer cells, such as alternations of transport and metabolism of gemcitabine or the upregulation of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) or the DNA repair pathway (8-12). In addition to monotherapy, there are clinical treatments with gemcitabine in combination with other biological or chemotherapeutic targeted agents, such as the epidermal growth factor receptor (EGFR) inhibitors erlotinib, tipifarnib and gefitinib (13,14), but the combinations are limited, with unsatisfactory efficacy. Only patients developing a rapid response upon erlotinib could benefit from EGFR-targeted therapy (13,15). Overall, there is an urgent requirement to identify novel chemotherapeutic agents or an effective combination scheme for this malignancy.

Arenobufagin is a cardiac glycoside agent and one of the main active ingredients of toad secretions. Secretions of the postauricular and skin glands of *Bufo gargarizans* and *Duttaphrynus melanostictus* have been used as a traditional Chinese medicine (TCM) in numerous diseases, including cancers, heart failure and sore throat (16). A previous study suggested cardiac glycosides as potent inhibitors of cancer cell growth (17). Previous studies have demonstrated that arenobufagin is a potent Na⁺-K⁺ pump inhibitor that depresses the delayed rectifier K⁺ current in myocytes (18,19). It has been

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Abbreviations: 5-FU, 5-fluorouracil; EGFR, epidermal growth factor receptor; PC, pancreatic carcinoma; PDAC, pancreatic ductal adenocarcinoma; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; TCM, traditional Chinese medicine

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reported that arenobufagin can suppress cell adhesion, migration and invasion and induce apoptosis and autophagy via inhibition of the PI3K/Akt/mammalian target of rapamycin pathway in a human hepatoma cell line (20,21) as well as block vascular endothelial growth factor (VEGF)-mediated angiogenesis to prevent carcinogenesis (22). However, to the best of our knowledge, the effects and the mechanism of arenobufagin in PC cells have not been studied.

To uncover the effect of arenobufagin on PC cells and the assistance to first-line medicine, the gemcitabine-resistant pancreatic carcinoma cell line Panc-1 and the gemcitabine-sensitive cell line ASPC-1 were used in the present study.

In the current study, it was found that arenobufagin effectively suppressed the proliferation of PC cells by blocking the phosphorylation of both Akt and extracellular signal-regulated kinases (Erk), as well as inducing G2/M phase cell cycle arrest and apoptosis in PC cells. In order to find a new strategy for combination therapy, the present study determined the effect of arenobufagin in combination with gemcitabine or 5-fluorouracil (5-FU), revealing a significant impact on cell proliferation. These results indicate that arenobufagin may be used as a potential adjuvant to overcome the resistance to chemotherapy in PC.

Materials and methods

Materials. Arenobufagin was isolated from toad secretions, as previously described (23). Gemcitabine and 5-FU were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). All 3 agents were dissolved in dimethyl sulfoxide (DMSO) as a stock solution (10 mM) and stored at -20°C. The culture media containing different concentrations of these agents were freshly prepared for each experiment. The final concentration of DMSO was <0.1%. Propidium iodide (PI) for cell cycle analysis was purchased from Sigma-Aldrich (Merck KGaA). Rabbit monoclonal antibodies against Akt (#4691), phosphorylated Akt^{Ser473} (p-Akt) (#4060), Erk1/2 (#4695), phosphorylated Erk1/2 (p-Erk; #4370), EGFR (#4267), phosphorylated epidermal growth factor receptor^{Tyr1068} (p-EGFR; #3777), caspase-3 (#9665), poly (ADP-ribose) polymerase (PARP; #9542), cleaved PARP (Asp214; #5625), caspase-9 (#9508) and cleaved caspase-9 (Asp330; #7237), mouse monoclonal GAPDH (#97166) and β -actin (#3700), and anti-mouse (#7076) and anti-rabbit (#7074) IgG-horseradish peroxidase (HRP)-linked antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell lines and cell culture. The human PC Panc-1 and ASPC-1 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). These two cell lines were incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% (v/v) penicillin-streptomycin at 37°C under 5% CO₂.

MTT assay. The viability of the Panc-1 and ASPC-1 cells was detected using MTT assay. Cells were planted on 96-well plates with 3 replicates at a density of 5x10³ cells per well. After culturing in DMEM medium containing 10% FBS for

12 h to obtain a confluent monolayer, the medium was replaced with DMEM containing 5% FBS and arenobufagin at different concentrations (0, 1, 10 and 100 nM) for 24, 48 and 96 h. Subsequently, 10 μ l MTT (5 mg ml⁻¹ in PBS) was added to each well at the indicated time points. The culture medium was removed and MTT formazan was dissolved in 150 μ l DMSO per well 4 h later. The plates were agitated for 15 min, and OD_{490nm} was measured using an absorbance reader.

Clonogenic survival assay. Cell proliferation ability was measured using a colony formation assay. Approximately 500 cells were seeded in each well on a 12-well plate as a single cell suspension. After 24 h, different concentrations of arenobufagin (0, 5, 10 and 50 nM) were added and the cells continued to be maintained at 37°C with a humidified atmosphere of 5% CO₂ for 15-20 days. Visible colonies were then stained with crystal violet and manually counted.

Western blot analysis. Cells were treated with arenobufagin (0, 5 and 10 nM) at the indicated time (0, 0.5 and 1 h). Whole-cell extracts were prepared using radioimmunoprecipitation assay buffer supplemented with proteinase inhibitors (Sigma-Aldrich, Merck KGaA) at 4°C. Following centrifugation at 13,000 x g for 20 min, the supernatant was collected and quantified using the bicinchoninic acid protein assay. Total protein (50 μ g per well) was separated using 8-12% SDS-PAGE and transferred to nitrocellulose transfer membranes. The membranes were blocked in TBST (10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 0.1% Tween-20) with 5% non-fat milk for 2 h. The membranes were then incubated with specific primary antibodies (all antibodies were diluted at a ratio of 1:1,000) overnight at 4°C, followed by treatment with HRP-linked secondary antibodies (24). The protein bands were visualized using ECL agent (Thermo Fisher Scientific, Inc.) and detected using a Gel imaging system (Bio-Rad Laboratories, Inc., Hercules, California, USA).

Detection of cell cycle. Cells were seeded on a 6-well plate and treated with 100 nM arenobufagin for 24 h. The cells were then harvested by trypsin (up to 5x10⁶ cells), washed twice with PBS, and fixed with cold 70% ethanol at 4°C overnight. The cells were stained with 50 μ g/ml PI [PBS 480 μ l; 5 μ l PI (5 mg/ml); 5 μ l RNase (10 mg/ml); 10 μ l Triton-100 (10%)] at 37°C for 30 min and were kept in the dark. The cells were then suspended in PBS and cell cycle distribution was detected. Samples were analyzed using a FACSC flow cytometer. Additional analyses were processed by Flow Jo software (Tree Star, Inc., Ashland, OR, USA) (24).

Statistical analysis. The data were analyzed by Student's t-test or one-way analysis of variance (Dunnett's test and Least Significant Difference test). P<0.05 was considered to indicate a statistically significant difference. All analysis was performed using SPSS13.0 (SPSS, Inc., Chicago, IL, USA).

Results

Arenobufagin inhibited survival and proliferation of PC cells. The chemical structure of arenobufagin is shown in Fig. 1A. The cytotoxic efficacy of arenobufagin was first tested on the

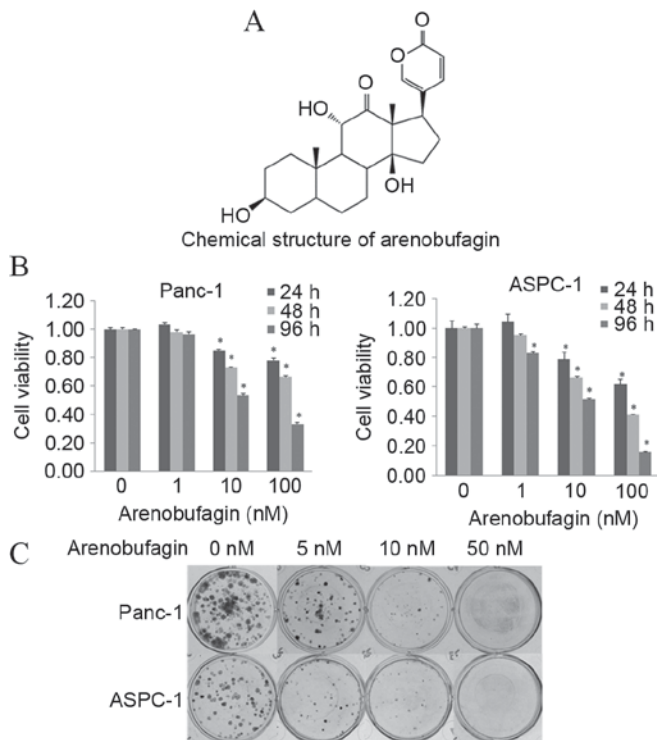


Figure 1. Arenobufagin inhibited cell survival and proliferation in human PC cells. (A) The chemical structure of arenobufagin. (B) Cell viability was measured by MTT. Panc-1 and ASPC-1 cell lines were treated with different concentrations of arenobufagin (0-100 nM) for 24, 48 and 96 h, and cell viability was evaluated by optical density at 490 nm. The results showed that arenobufagin inhibits the survival of PC cells significantly at concentrations as low as 10 nM. (C) Cell proliferation was tested using a clonogenic survival assay. Each cell line was treated with arenobufagin at 0, 5, 10 and 50 nM. As shown, the proliferation of PC cells was evidently inhibited by arenobufagin, even at 5 nM. The data shown are representative of at least three experiments. * $P < 0.05$ compared with the control; cells treated with 0 nM were used as the control. PC, pancreatic cancer.

PC Panc-1 and ASPC-1 cell lines by MTT analysis. Subsequent to treatment with different concentrations (0-100 nM) of arenobufagin for 24, 48 and 96 h, cell proliferation was significantly suppressed in a dose- and time-dependent manner, as shown in Fig. 1B. To confirm the inhibition of cellular proliferation, the present study processed the colony formation assay (Fig. 1C). The MTT assay and the colony formation assay showed that cellular viability and proliferation could be inhibited by arenobufagin even at a concentration as low as 1 nM.

Arenobufagin suppressed the phosphorylation of Akt, Erk and EGFR. The present study investigated whether arenobufagin could inhibit key downstream pathways of cell survival and proliferation, and whether they play a role in arenobufagin-mediated cell death. As shown in Fig. 2A, western blot analysis showed that the phosphorylation of Akt and Erk decreased in ASPC-1 and Panc-1 cell lines within 1 h when treated with arenobufagin at 10 nM. To uncover the mechanism of arenobufagin on PC cells, the present study examined the state of EGFR. Subsequent to treatment with arenobufagin at 10 nM for 0 and 0.5 h, the levels of p-EGFR decreased in the Panc-1 cell line. There was no evident change in the gemcitabine-sensitive ASPC-1 cells, but it should be noted that the initial level of p-EGFR was low in the ASPC-1 cell line, as shown in Fig. 2B.

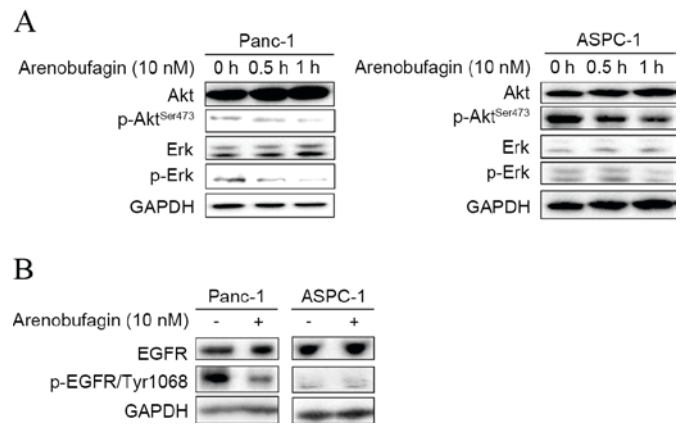


Figure 2. Arenobufagin decreased the phosphorylation of Akt, Erk and EGFR. (A) Levels of p-Erk and p-Akt^{Ser473} in total cell lysates from Panc-1 and ASPC-1 cells treated with arenobufagin at 10 nM for 0.5 and 1 h were decreased compared with the control, which was untreated. (B) Cells were treated as aforementioned. The levels of total EGFR and phosphorylated EGFR were detected and the phosphorylated proteins were decreased. GAPDH was used as a loading control, and the ratios were compared to the GAPDH at 0 h. The western blots shown here are representative of at least three independent experiments. p-, phosphorylated; Erk, extracellular signal-regulated kinases; EGFR, epidermal growth factor receptor.

Arenobufagin induced cell cycle G2/M phase arrest and G0/S phase decline. The inhibition of cellular proliferation is usually caused by cell cycle arrest and cell death. Given the significant decrease in cell viability, a dose of 10 nM of arenobufagin was used to explore the underlying mechanism of arenobufagin-induced inhibition on cellular proliferation. Subsequent to treatment with the indicated concentration for 24 h, the percentage of cells in the G2/M phase increased from 10.3-22.74% in Panc-1 cells and 16.46-30.65% in ASPC-1 cells, along with a decrease in the S phase, as shown in Fig. 3A and B. The statistical analysis also showed that the increase in the G2/M phase percentage was significant ($P < 0.05$).

Arenobufagin affected the apoptosis-related pathway. In addition to cell cycle arrest, the effect of arenobufagin on the apoptosis pathway in PC cells was examined. Western blot analysis revealed not only specific cleavage of PARP but also a decrease in PARP, pro-caspase-3 and pro-caspase-9 was induced by arenobufagin treatment (Fig. 4A). The decrease in caspase-9 (Fig. 4B) indicated that the mechanism of apoptosis may be associated with a mitochondrial-dependent pathway.

Arenobufagin significantly enhanced the effects of both gemcitabine and 5-FU. The present study assessed the effect of arenobufagin on the inhibition of proliferation induced by gemcitabine in the gemcitabine-resistant Panc-1 and gemcitabine-sensitive ASPC-1 cell lines. The two cell lines were treated with arenobufagin and gemcitabine at the indicated concentrations, either alone or in combination for 48 h. The combination of arenobufagin and gemcitabine inhibited the growth of Panc-1 and ASPC-1 cells more than either of the agents alone, with the cell viability of Panc-1 cells declining from 89% in the gemcitabine group and 71% in the arenobufagin group to 50% in the combination treatment group. In the ASPC-1 cell line, the viability of cells declined from 71% in the gemcitabine group and 51% in the

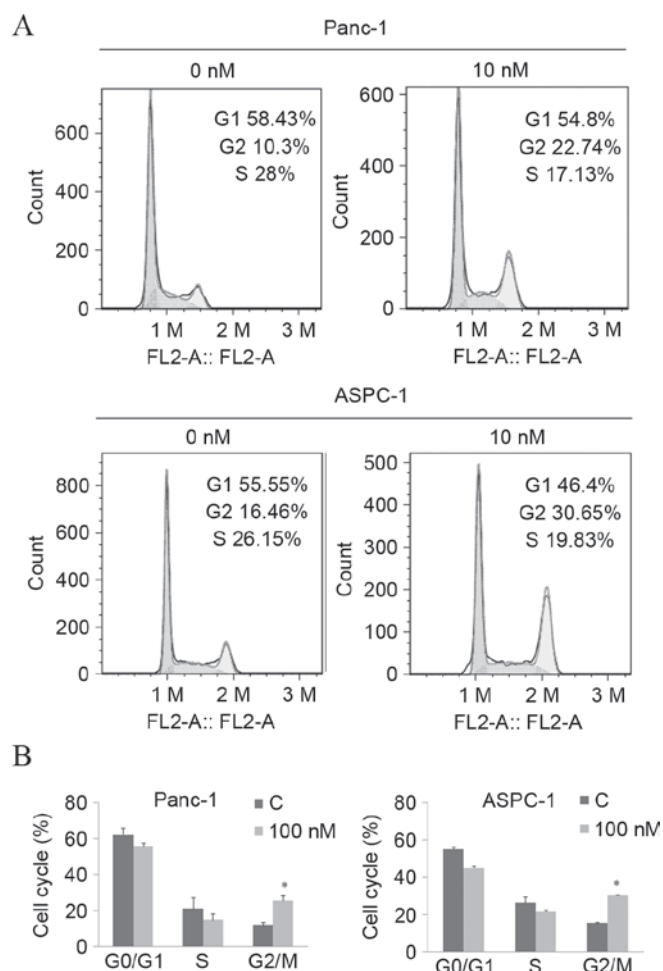


Figure 3. Arenobufagin leads to cell cycle G2/M phase arrest. (A) Cells were exposed to arenobufagin at 10 nM for 24 h and were collected by trypsinization and analyzed by flow cytometry subsequent to staining with propidium iodide. Arenobufagin induced G2/M phase cell cycle arrest in Panc-1 and ASPC-1 cells. (B) Three independent experiments were performed, the histogram is presented according to statistical analysis. * $P < 0.05$ compared with control; groups treated for 0 h were used as the control. C, control.

arenobufagin group to 41% in the combination treatment group. The decline between the single treatment groups and the combination groups was statistically significant ($P < 0.05$; Fig. 5A). In addition, the present study identified that the combination treatment of arenobufagin at 10 nM and 5-FU at 50 μM for 48 h significantly enhanced ($P < 0.05$) the effect of 5-FU, with the cell viability of Panc-1 cells decreasing between 82% in the 5-FU group and 50% in the combination group. The viability of ASPC-1 cells decreased between 68% in the 5-FU group to 42% in the combination treatment group (Fig. 5B).

Discussion

Arenobufagin, an effective cardiac glycoside, is one of the most active compounds found in toad secretions, and is listed in the Chinese Pharmacopoeia (20). Arenobufagin has been used to treat hepatic carcinoma in TCM. Certain cardiac glycosides have been reported as potent inhibitors of cancer cell growth (17). To the best of our knowledge, the present study demonstrated for the first time that arenobufagin is an

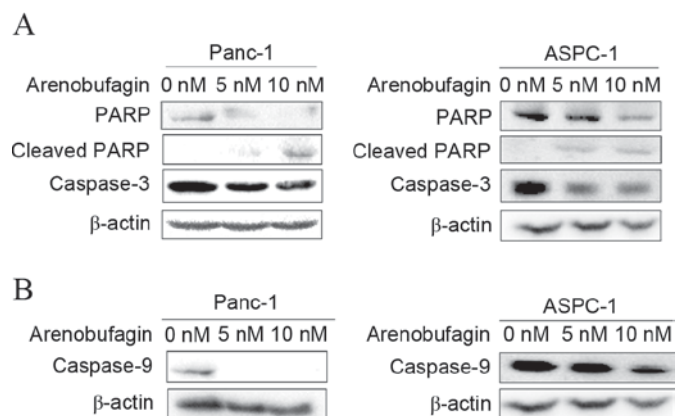


Figure 4. Effects of arenobufagin on apoptosis in prostate cancer cells. (A) Panc-1 and Asp-1 cells were treated with different concentrations of arenobufagin (0, 5 and 10 nM) for 48 h. Western blot analysis demonstrated that the expression of the apoptosis-related proteins PARP and caspase-3 decreased and the expression of cleaved PARP increased. (B) Cells were treated as in (A) and the level of caspase-9 was detected. The groups treated with arenobufagin also showed decrease in caspase-9. β -actin was used as the internal control. The western blots shown are representative of at least 3 independent experiments with similar results. PARP, poly (ADP-ribose) polymerase.

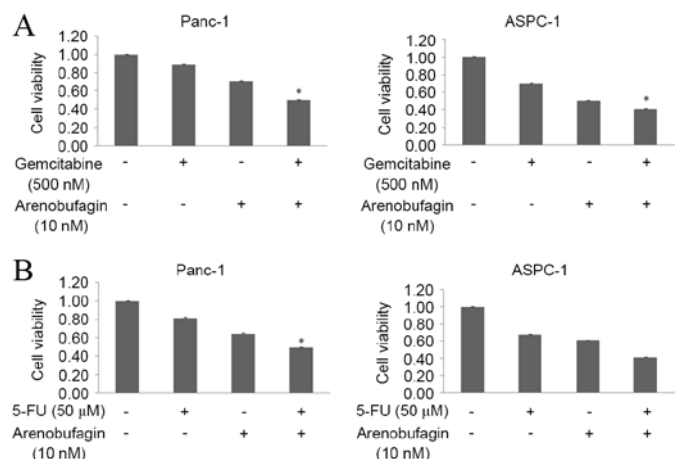


Figure 5. Arenobufagin enhanced the effects of gemcitabine and 5-FU. (A) Panc-1 and ASPC-1 cells were treated with arenobufagin (10 nM) and/or gemcitabine (500 nM) for 48 h. The combination of the two drugs significantly inhibited the proliferation of Panc-1 more than either arenobufagin or gemcitabine alone. (B) Panc-1 and ASPC-1 cells were treated with arenobufagin (10 nM) and/or 5-FU (50 μM) for 48 h. The combination of the two drugs inhibited the proliferation of Panc-1 more than either arenobufagin or 5-FU alone. The data shown are representative of at least three independent experiments. * $P < 0.05$ compared with the monotherapy group. 5-FU, poly (ADP-ribose) polymerase.

effective agent in PC cell lines, including the drug-resistant Panc-1 cell line.

K-Ras has previously been identified as a small GTPase that is mutated in 90% of human pancreatic carcinomas (25). Certain genetic studies have shown that K-Ras activation and mutation is necessary for the initiation of PC (26-28), and an inducible pancreas-specific expression system was used to show that K-Ras expression is also required for tumor maintenance (29). These mutations lock K-Ras and its downstream proteins, such as Akt and Erk, in a constitutively activated form. In turn, this leads to enhanced cell proliferation and a

growth advantage to the cancer cell, as well as playing a key role in tumorigenesis and resistance to standard therapies, such as chemotherapy and radiation (30,31). Thus, the associated signaling pathways are critical targets for which specific inhibitors are expected to exert antitumor efficacy. It is notable that arenobufagin could evidently downregulate the phosphorylation of Erk and Akt in the K-Ras mutant Panc-1 cell line, suggesting that arenobufagin may be an efficient inhibitor in Ras mutation cancer cell lines. In addition, the present study showed that this downregulation was more evident on the level of p-Erk compared with p-Akt when treated with arenobufagin, which is consistent with previous studies (32).

A new study recently showed that EGFR signaling is essential for K-Ras oncogene-driven PDAC (33). EGFR belongs to the tyrosine kinase receptor erbB family, and is important in tumor growth, metastasis and disease recurrence (34,35). Overexpression of EGFR commonly occurs in PC, and this overexpression is associated with a poor outcome (36-38). Although EGFR inhibitors have exerted significant clinical benefits, clinical research has shown that only patients who developed a rapid response upon erlotinib treatment benefit from EGFR inhibitors (13), which suggests a limited efficacy of EGFR inhibition and the requirement for agents targeting multiple signaling pathways. Considering the inhibitory effects of arenobufagin on the phosphorylation of Erk and Akt in K-Ras mutant Panc-1 cell lines and the cross-talk between EGFR and K-Ras, it was hypothesized that a possible target by which arenobufagin may affect cell growth and death was EGFR. The present study detected significant decreases of phosphorylated EGFR subsequent to treatment with arenobufagin in PC cells, thus confirming that arenobufagin is an efficient agent for the treatment of Ras mutated PC.

Previously, it has been reported that arenobufagin induces apoptosis in hepatocellular carcinoma cells. Similar to the present findings for arenobufagin, certain studies have reported that bufalin, another representative cardiac glycoside compound from secreted toad toxins, inhibited cell proliferation in various cancer cells and induced apoptosis and cell cycle arrest in PC cells (21,39-41). The exact mechanism by which arenobufagin induces apoptosis in pancreatic cells is unclear. The present finding on the reduction of caspase-9 indicated that arenobufagin may induce apoptosis via a mitochondrial pathway in PC cells. In addition, to the best of our knowledge, the present data showed for the first time that arenobufagin induced cell cycle G2/M phase arrest and a decrease in the population of cells in the S phase, inhibiting the proliferation of PC cells.

Several studies have indicated that chemotherapeutic and biological targeted agents could be used in combination with clinical chemotherapeutic drugs, such as gemcitabine and 5-FU to overcome drug resistance and improve the efficiency of treatments (42,43). The classic model for PDAC treatment is treatment with gemcitabine as a single agent or in combination with EGFR inhibitors. Since it has been reported that the levels of EGFR and Erk phosphorylation in PDAC were increased in Panc-1 and BXPC-1 cell lines when treated with gemcitabine, this may be a mechanism of drug resistance (15). The phosphorylation of Erk is associated with cell proliferation, differentiation, migration and transcription. Notably, the present western blot analysis showed that arenobufagin could

significantly downregulate the phosphorylation of EGFR, Erk and Akt. Cell growth analysis testified to the effect of arenobufagin in combination with gemcitabine or 5-FU, and the results demonstrated that the involvement of arenobufagin could significantly enhance the inhibiting effect of either gemcitabine or 5-FU against PC cells. Thus, the present results indicated that arenobufagin could be used as a new drug to enhance the effect of gemcitabine through inhibiting EGFR, Erk, Akt and NF- κ B associated pathways.

In summary, the present study determined that arenobufagin could cause cell cycle G2/M phase arrest and apoptosis in PC cells, and downregulate the levels of p-Erk, p-Akt and p-EGFR even in drug-resistant Panc-1 cell line, which could in turn offset the adverse effects of gemcitabine. Thus, the present study identified arenobufagin as a potential effective agent with a marked effect on pancreatic cancer cells, and therefore causing us to consider arenobufagin as a promising candidate for combination therapy in PC.

Acknowledgements

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