

ORIGINAL ARTICLE

Baicalein flavone targets cisplatin resistant human pancreatic cancer cells via inducing S-phase cell cycle arrest, inhibition of cell migration and invasion, caspase activation and mitochondrial-dependent apoptosis

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Summary

Purpose: Pancreatic cancer (PC) is a lethal disease of the alimentary system and is ranked 4th in cancer-related deaths in United States. PC has a poor prognosis and limited therapeutic options. The main purpose of the current study was to demonstrate the anticancer effects of the naturally occurring Baicalein flavone in human cisplatin-resistant pancreatic carcinoma cell line CAPAN-2.

Methods: Cell viability was examined via MTT cell proliferative assay. Mitochondrial-mediated apoptosis was examined through DAPI and annexin V/propidium iodide (PI) staining using fluorescence microscopy along with estimation of apoptosis-related protein expressions like caspase-3, Bax, Bcl-2 for which western blot was used. Next, wound-healing and transwell assays were performed to find out the effects of Baicalein on cell migration and invasion, respectively.

Results: The results showed that Baicalein induced dose-dependent and selective anticancer effects in CAPAN-2 PC

cancer cells with much less cytotoxicity to normal HTRET-HPNE cells. The antiproliferative effects of Baicalein were due to apoptosis induction as the number of apoptotic cells increased on increasing doses of the test molecule. Western blotting analysis revealed that the expressions of caspase-3 and Bcl-2 were decreased and Bax was increased. The test molecule also induced S-phase cell cycle arrest in PC cells with decreasing the cyclin-B1 expressions. Cell migration and invasion analysis revealed that Baicalein induced dose-dependent suppression in migration and invasion of CAPAN-2 PC cell line

Conclusion: Baicalein is a potential anticancer agent against PC cells and can be considered for PC systemic therapy provided more toxicological and in vivo studies are carried out.

Key words: baicalein, pancreatic cancer, caspase, cell migration, anticancer

Introduction

Pancreatic carcinoma (PC) is a lethal malignancy of the alimentary system, arising due to the development of malignant cells in pancreatic tissues [1,2]. Among all cancer types PC ranks 4th in the number of cancer-related deaths in United States [3]. In United States there is an alarming

frequency of PC patients yearly and alone in 2010 nearly 43,000 new cases of PC were registered [4]. The prevalence of PC is enhancing in China day by day, making it a common mortality factor in cancer-related deaths [5]. It is an extremely lethal distortion of pancreatic tissues that has a very poor

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prognosis due to minimum advancements and improvements made in its early detection [6,7]. Despite being a deadly disease with high morbidity in China, only 15% of the patients receive treatment like surgical removal and postoperative chemotherapy with 5-year overall survival rate under 20%. Metastasis is the main cause of low survival rate of PC [8]. Thus, to increase overall survival rate, prevent mortality and increase chemotherapeutic efficiency there is a pressing need to move to novel strategies and chemotherapeutic agents to control this fatal disease. Natural products, in particular plants and microbes, have assisted as an imperative source of potential chemotherapeutic agents in the past [9-11]. Plants have massive diversity with large number of species and it is supposed that in-depth investigation of plants may provide more potential drugs that can curb deadly diseases like PC [12,13]. Flavones are naturally occurring polyphenolic compounds found in vascular plants (tracheophytes), fruits and vegetables, and have revealed different pharmacological activities, among them anticancer activity as well [14-16]. Baicalein, a flavone, obtained from the roots of *Scutellaria radix* or *Scutellaria baicalensis* has shown potential anticancer activities through inhibition of cell proliferation and cell cycle arrest [17,18]. In a number of human cancer cell lines Baicalein resulted in apoptosis induction, although well-defined mechanisms of apoptosis are still unknown [19,20]. Herein, the current study was performed in order to unveil the anticancer effect of Baicalein on cisplatin-resistant human PC cells via inducing S-phase cell cycle arrest, inhibition of cell migration and invasion, caspase activation and mitochondrial-dependent apoptosis. For that an array of assays was performed

Methods

Cell viability estimation

The human pancreatic carcinoma cell line CAPAN-2 as well as the normal pancreatic cell line HTRET-HPNE were obtained from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China, and plated at a density of 2×10^4 cells per well in 96-well plates. Seeded cells were then treated with changing concentrations of Baicalein (0, 2.5, 5, 10, 20, 40, 80, 160 and 320 μM) for 12 h. After exposure to the test molecule, cells were washed using phosphate buffered saline (PBS) and cultured for 48 h, followed by decanting of DME medium. Both the cell lines were treated with 0.5 mg/mL of MTT assay (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma, St. Louis, USA) and incubated for 5 h. Finally, the cultured treated cells were dissolved in dimethyl sulfoxide (DMSO) and a plate reader (OPTImax Molecular Dynamics) was used to establish the intensity of formazan formed at 545 nm.

Apoptosis determination using DAPI and Annexin V/PI staining assay

For apoptosis determination seeding of the human PC cell line CAPAN-2 was performed at a density of 2×10^4 cells per well in 96-well plates. Seeded cells were then treated with changing concentrations of Baicalein molecule (control, 10, 20 and 40 μM), followed by incubation at 37°C for 12 h. Treated cells were then stained with 4',6-diamidino-2-phenylindole (DAPI) staining. Staining was followed by washing with phosphate buffered saline (PBS) and fixation with 10% formaldehyde. Finally, these cells were investigated under fluorescence microscope (Leica, Wetzlar, Germany). A similar procedure was carried out for annexin V/PI assay but only DAPI staining was replaced by annexin V/PI staining and investigations were performed through flow cytometry (BD, FACSCalibur San Jose, CA, USA).

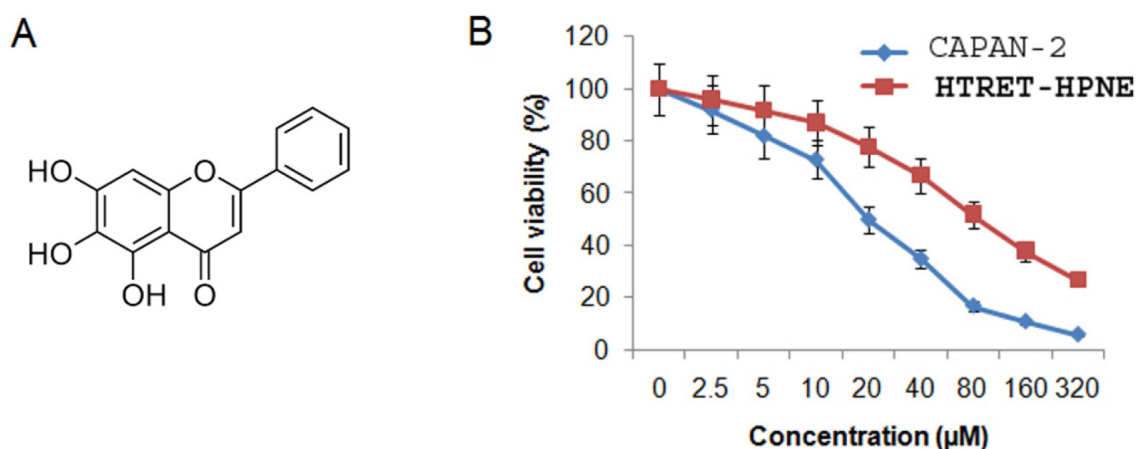


Figure 1. A: Chemical structure of Baicalein flavone. **B:** Baicalein suppressed cell viability of CAPAN-2 pancreatic carcinoma cells as revealed by MTT assay. The cells were subjected to varying doses of the test molecule for 12h which revealed a dose-dependent and selective inhibition in cell viability of these cancer cells showing lower toxicity towards normal pancreatic cells (HTRET-HPNE). The data is the mean \pm SD of three individual experiments ($p < 0.01$).

Cell cycle phase analysis

For cell cycle phase distribution CAPAN-2 PC cell line was harvested and washed twice using PBS. These washed harvested cells were treated with varying doses of Baicalein molecule (control, 10, 20 and 40 μ M), followed by fixation and further washing using 70% ethanol and PBS, respectively. Afterwards, the treated cells were suspended in 50 μ l/ml of PI (Gibco, Gaithersburg, MD, USA), and 250 μ g/ml of RNase1 solution (Gibco, Gaithersburg, MD, USA), followed by incubation for 30 min at 25°C. Finally, the cells were arranged in groups with 10,000 cells in each group and investigations were carried out through fluorescence-activated cell sorting using BD FACSCalibur flow Cytometer.

Wound healing assay for cell migration

Human PC cell line CAPAN-2 was plated at a cell density of 4×10^5 using 6-well plates and RPMI-1640 medium (Gibco, Gaithersburg, MD, USA). A wound was scratched in each well with a 200- μ L pipette tip. Cells were left until they grew up to 90% confluence which was followed by washing with PBS and total decantation of the medium. Wounded well plate cells were then treated with changing concentrations (control, 10, 20 and 40 μ M) of Baicalein molecule. Microscopic pictures were captured at 0 h and 24 h at 6 random fields.

Cell invasion assay

Cell invasion ability of CAPAN-2 PC cells after exposure to different concentrations of Baicalein (control, 10, 20 and 40 μ M) was determined via transwell chambers with Matrigel. Around 200 μ l of cell culture was placed in the upper chambers and medium alone was placed in the lower chambers. Afterwards, cells were incubated for one day, which was followed by fixation with methyl

alcohol. Later, cells were stained using crystal violet and invaded cells were counted under 200x magnification of an inverted microscope.

Western blotting analysis for determination of the effect of Baicalein on expressions of apoptosis and cell cycle related protein expressions

Using a BCA protein assay kit (Beyotime, China) cell lysates were quantified. Equivalent quantities were separated through SDS-PAGE followed by transference to nitrocellulose membranes. These membranes were then subjected to primary antibodies treatment (anti-caspase-3, anti-Bax, anti-Bcl-2 and anti-cyclin B1), followed by secondary antibody treatment at room temperature and incubated for 1 h. Immunocomplexes were then visualized by horseradish peroxidase-conjugated antibody involving chemoluminescence reagent (Millipore, USA) and spotted on a photographic film.

Statistics

All data are conveyed as mean \pm SEM. Statistical analysis was accomplished using GraphPad Prism Vision 5.0. The significance of deviations among controls and experimental groups was evaluated through Student's t-test and 1-way analysis of variance (ANOVA). Statistically significant differences were set at $p < 0.05$.

Results

Effect of Baicalein molecule on cell viability of human cisplatin-resistant pancreatic carcinoma cell line as well as normal pancreatic cell line

Cisplatin-resistant PC cell line CAPAN-2 and normal pancreatic cell line HTRET-HPNE were

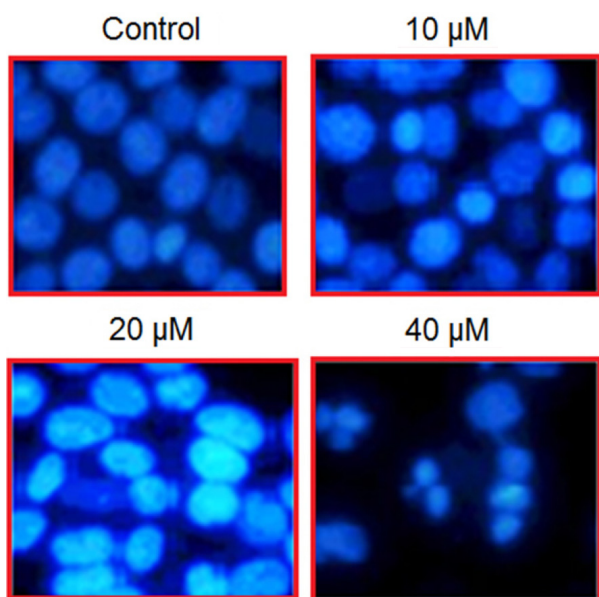


Figure 2. DAPI staining of treated CAPAN-2 pancreatic cancer cells showing membrane blebbing and formation of apoptotic crops with the mentioned doses. The experiments were performed in triplicate.

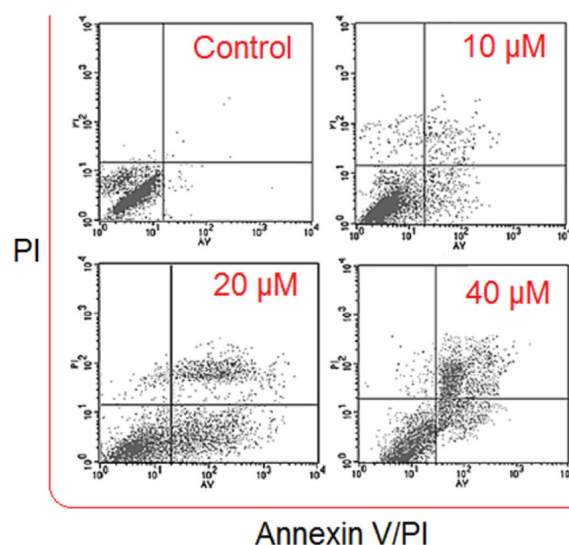


Figure 3. Assessment of apoptotic cell population through annexin V/PI staining at given concentrations of Baicalein. The impact of the test molecule with increasing doses indicates that both early and late apoptotic cells percentage increased significantly. The experiments were performed in triplicate.

treated with different concentrations of Baicalein (0, 2.5, 5, 10, 20, 40, 80, 160 and 320 μM) (Figure 1A) and the results on cell viability were obtained through MTT assay. The results depicted that the viability of PC cancer cells decreased and the decrease was quite significant as compared to that of normal cell line HTRET-HPNE. The proliferation of cancer PC cells was almost limited to zero at 320 μM of drug concentration (Figure 1B), clearly indicating the potential of the test molecule on inhibiting cell viability against cisplatin-resistant PC cell line CAPAN-2 dose-dependently.

Effect of caspase and mitochondrial mediated-apoptosis induction by Baicalein molecule in pancreatic carcinoma cell line CAPAN-2

Apoptosis analysis was performed using DAPI and Annexin V/PI staining to check whether the cytotoxic effects of the test molecule were due to apoptosis induction. After treatment of carcinoma cell line CAPAN-2 with the tested molecule, the DAPI staining revealed that it induced apoptosis as predicted via membrane blebbing and formation of apoptotic bodies (Figure 2). The effect on apoptosis induction was found to depend on the drug dose. Furthermore, on quantifying the rate of apoptosis through annexin V/PI staining it was observed that the percentage of apoptotic cells increased significantly at the dose of 40 μM (Figure 3). Western blotting analysis was carried out to check the expressions of apoptosis-related proteins found in mitochondria, like Bcl-2, Bax and caspase after the test molecule treatment. What was revealed was a significant decrease in caspase-3 and Bcl-2 and

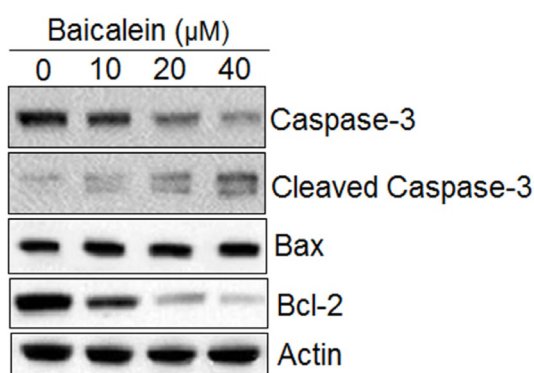


Figure 4. Baicalein induced activation of caspase and mitochondrial mediated apoptosis-related proteins in CAPAN-2 pancreatic cancer cells. After treatment with Baicalein (10, 20 and 40 μM) for 24h, cell lysates were prepared and western blot analysis was performed against caspase-3, Bcl-2 and Bax, Actin was used as a loading control. The results revealed that the expression of cleaved caspase-3 and Bax proteins increased and that of Bcl-2 and caspase-3 proteins decreased on Baicalein exposure. The results shown are illustrative of at least three independent experiments.

increase in cleaved caspase-3 and Bax expressions (Figure 4). Thus it is quite evident that Baicalein induced caspase and mitochondrial-mediated apoptotic cell death in PC CAPAN-2 cells.

Baicalein induced S-phase cell cycle arrest in CAPAN-2 pancreatic carcinoma cell line

Cell cycle is considered as one of the important mechanisms on which antiproliferative drugs exert their effects. Herein, we treated CAPAN-2 PC cell line with different concentrations of the test molecule (control, 10, 20 and 40 μM), and the results were evaluated through fluorescence-activated cell sorting using FACSCalibur flow Cytometer. The results suggested that there is a significant increase in the number of S-phase cells from 30% to nearly 70% and simultaneously the number of G0/G1 phase cells decreased significantly from 50% to near about 10%. It was also observed that the impact of the test molecule on G2/M phase cells was negligible (Figure 5). This was further validated

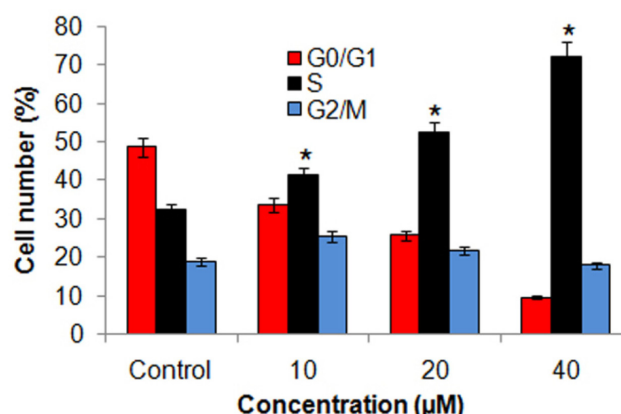


Figure 5. Baicalein resulted in S-phase cell cycle arrest in a dose dependent manner. After treatment of CAPAN-2 pancreatic cancer cells with different doses of Baicalein molecule the number of S-phase cells increased significantly with increased doses. The data is the mean \pm SD of three individual experiments (* $p < 0.01$).

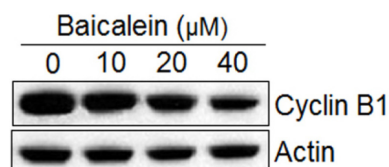


Figure 6. Baicalein resulted in the suppression of cyclin-B1 expressions in CAPAN-2 pancreatic cancer cells. After treatment with Baicalein (10, 20 and 40 μM) for 24h, cell lysates were prepared and western blot analysis was performed against cyclin-B1. Actin was used as a loading control. After Baicalein exposure, the levels of cyclin-B1 in CAPAN-2 cells reduced remarkably in comparison with controls. The results shown are illustrative of at least three independent experiments.

by checking the expression of S-phase-related protein expressions through western blotting analysis, revealing decline in the expression of cyclin-B1 (Figure 6). Thus, it may be concluded that the test molecule induced S-phase cell cycle arrest in CAPAN-2 PC cells in a dose-dependent manner.

Inhibition of cell migration and cell invasion by Baicalein in CAPAN-2 pancreatic carcinoma cell line

The cell migration capability of CAPAN-2 PC cells was checked by wound healing assay. After

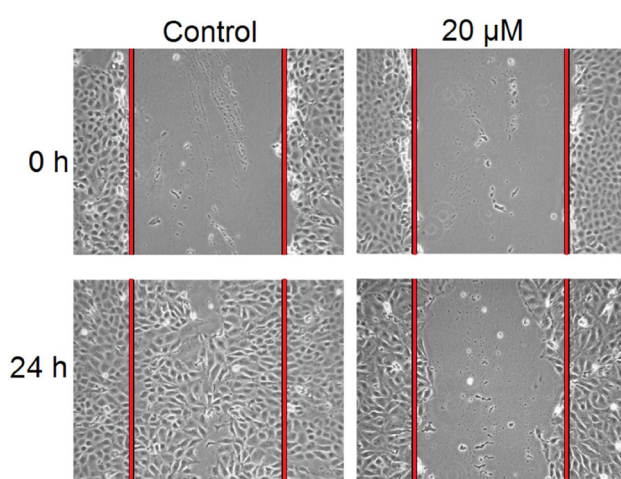


Figure 7. Treatment of CAPAN-2 pancreatic cancer cells resulted in the inhibition of cell migration as shown by wound healing assay. The inhibition was dose-dependent. The experiments were performed in triplicate.

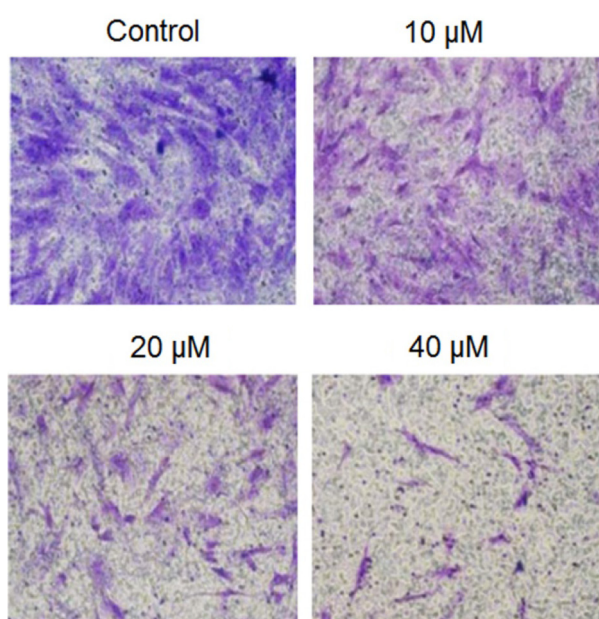


Figure 8. Effect of Baicalein on invasive potential of CAPAN-2 pancreatic cancer cells. Prior to the cell invasion assay, the cells were treated with control, 10, 20 and 40 μ M of Baicalein for 24 h. There was a dose-dependent decrease in the number of invaded cells after 24-h of treatment.

scratching a wound in the cultured cells, treatment with different concentrations of the test molecule was performed. The results in control and 20 μ M were photographed and indicated that the migration capability was significantly decreased at 20 μ M in comparison to control after one complete day of exposure, thus clearly indicating that the test molecule inhibited cell migration of PC carcinoma cells (Figure 7). Transwell assay with Matrigel was used to assess the effect of the test molecule on target cell invasion, on exposure to different molecule doses (control, 10, 20 and 40 μ M) observing a significant decrease in the number of invaded cells as compared to the positive control cells (Figure 8). Thus cell migration and invasion assays unveiled the anti-migration and anti-invasion effects of the test molecule in a dose-dependent manner.

Discussion

PC is one among the ten major fatal malignancies prevailing across the globe with high mortality rates. Due to poor early diagnosis its lethality gets increased. There are limited options towards PC detection as it is asymptomatic or showing only few symptoms; its treatment options are limited to conventional chemotherapy, or surgery. As a result of development of drug resistance in cancer cells the efficacy of chemotherapy gets decreased. Thus, the overall five year survival for PC is only 20% in China, were even only 15% of total PC patients receive medical care. Hence, to curb this malignancy, novel promising and effective drug discoveries and strategies are urgently required. Baicalein (5,6,7-trihydroxyflavone) - a naturally occurring plant flavone obtained from *Scutellaria radix* or *Scutellaria baicalensis* roots - is traditionally used in China. Previous studies regarding the pharmacological activities of Baicalein have reported its anti-inflammatory, antioxidant, free-radical scavenger, cytoprotective, anti-genotoxins and anticancer properties [21-25]. Anticancer activities were reported for the test molecule in various human cancer cell lines. In the current study Baicalein molecule was tested for anticancer potential in the human PC cell line CAPAN-2 via inducing S-phase cell cycle arrest, inhibition of cell migration and invasion, caspase activation and mitochondrial-dependent apoptosis through an array of assays and analyses. MTT cell viability assay was performed to reveal the effects of Baicalein treatment in both PC as well as in normal pancreatic cells, which showed substantial and dose-dependent decrease in cancer cell proliferation. Furthermore, DAPI and annexin V/PI staining were performed to check whether the antiproliferative effects of

the current test molecule was due to its potential of apoptosis induction. The results revealed that the number of apoptotic cells with blebbed membranes and apoptotic crops increased significantly with increased doses of the test molecule. Western blotting analysis also confirmed that the expressions of mitochondrial-associated apoptosis proteins (caspase-3 and Bcl-2) decreased and cleaved caspase-3 and Bax expressions increased. Furthermore, cell cycle analysis was performed to check the test molecule effect on cell cycle of CAPAN-2 PC cells which showed dose-dependent increase in the number of S-phase cells and decrease in G1/G0 phase cells. Western blotting analysis also revealed inhibition effects of Baicalein on cyclin-B1 expression. Next, the effect of the test molecule on cell migration and invasion of CAPAN-2 PC cells was evaluated through wound healing and transwell assays, respectively, indicating that it significantly suppressed both cell migration and migration in a dose-dependent manner. Through all the above assay results and discussions it is clear that Baicalein

is a potential therapeutic agent and can be used in PC treatment.

Conclusions

In conclusion, the outcome of this study indicate that Baicalein shows potent and selective anticancer effects in human pancreatic cancer cells by targeting cell cycle at S-phase, inhibiting cell migration and invasion, caspase activation and inducing mitochondrial-mediated apoptosis.

Conflict of interest: The authors declare that there is no conflict of interest to reveal.

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Conflict of interests

The authors declare no conflict of interests.

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