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Vilas Desai

Satya Murthy Tadinada

Hoora Shaghaghi

Ross Summer

James C.K. Lai

*See next page for additional authors*

Authors
Vilas Desai, Satya Murthy Tadinada, Hoora Shaghaghi, Ross Summer, James C.K. Lai, and Alok Bhushan

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# Combination Treatment of Biochanin A and Atorvastatin Alters Mitochondrial Bioenergetics, Modulating Cell Metabolism and Inducing Cell Cycle Arrest in Pancreatic Cancer Cells

VILAS DESAI[1], SATYA MURTHY TADINADA[2], HOORA SHAGHAGHI[3], ROSS SUMMER[3], JAMES C.K. LAI[4] and ALOK BHUSHAN[1]

*[1]Department of Pharmaceutical Sciences, Jefferson College of Pharmacy, Philadelphia University & Thomas Jefferson University, Philadelphia, PA, U.S.A.;*
*[2]Department of Neuroscience and Pharmacology, Carver College of Medicine, University of Iowa, Iowa City, IA, U.S.A.;*
*[3]Center for Translational Medicine and Jane and Leonard Korman Lung Center, Philadelphia University & Thomas Jefferson University, Philadelphia, PA, U.S.A.;*
*[4]Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, Division of Health Sciences, Idaho State University, Pocatello, ID, U.S.A.*

**Abstract.** *Background/Aim: Pancreatic cancer is an aggressive type of cancer, with a dismally low survival rate of <5%. FDA-approved drugs like gemcitabine have shown little therapeutic success, prolonging survival by a mere six months. Isoflavones, such as biochanin A and daidzein, are known to exhibit anti-cancer activity, whereas statins reportedly have anti-proliferative effects. This study investigated the effects of combination treatment of biochanin A and atorvastatin on pancreatic cancer cells. Materials and Methods: Pancreatic cancer cells AsPC-1, PANC-1, and MIA PaCa-2 were procured from ATCC. The cell viability studies were carried out using MTT & cell count assays. Flow cytometry was used to study cell apoptosis whereas cell metabolism studies were carried out using the Seahorse Mito stress test and XF-PMP assay. The effects of treatment on cell signaling pathways & cell cycle associated proteins were investigated using western blot whereas invasiveness of cancer cells was evaluated using gelatin zymography. Results: The combination treatment decreased the survival and enhanced pro-apoptotic responses compared to single treatments in the pancreatic cancer cells. In PANC-1 cells, the combination treatment decreased invasiveness, reduced expression of activated STAT3 and expression of critical mediators of cell cycle progression. Furthermore, the combination treatment induced a differential inhibition of respiratory complexes in the pancreatic cancer cells. Conclusion: The combination treatment of biochanin A and atorvastatin exerts enhanced anti-cancer effects, inducing apoptosis, down-regulating cell cycle associated proteins and invasiveness in pancreatic cancer cells and merits further investigation for new, improved treatments for pancreatic cancer.*

*Correspondence to:* Dr. Alok Bhushan, Department of Pharmaceutical Sciences, Jefferson College of Pharmacy, Philadelphia University + Thomas Jefferson University, Philadelphia, PA, U.S.A. Tel: +1 2155035039, e-mail: Alok.bhushan@jefferson.edu

*Key Words:* Pancreatic cancer, biochanin A, atorvastatin, combination treatment, cell cycle, cell metabolism.



Pancreatic cancer is the fourth leading cause of cancer-related deaths in the United States and has a very poor prognosis (1). In 2024, an estimated 66,440 new cases and 51,750 deaths are expected to be recorded due to this devastating disease (1). The poor prognosis associated with pancreatic cancer may be attributed to the limited effectiveness of current chemotherapies due to apoptosis resistance of cells in the primary tumor. The highly aggressive nature of the cancer cells and lack of biomarkers for early diagnosis of pancreatic cancer undoubtedly also contribute (2).

Apoptosis resistance in pancreatic cancer has been attributed to several factors including impairment of TRAIL-mediated mechanisms promoting apoptosis and decreased TRAIL receptor expression (3-5). The low survival rate of pancreatic cancer can be mainly attributed to its highly aggressive nature. The tumor cells tend to metastasize to distant organs like the lungs and liver, which explains the primary tumor cells' resistance to anoikis (6-8). Some underlying mechanisms that promote resistance to anoikis and

enhance invasiveness have been elucidated (6, 7, 9-11). For example, several studies have pointed to the importance of Matrix Metalloproteinases (MMPs) and the tumor microenvironment in regulating invasion (12-14). Further, urokinase-type plasminogen activator (uPA) and urokinase-type plasminogen activator receptor (uPAR) in the stroma are known to promote invasion of pancreatic cancer *via* activation of MMPs (15, 16). Although elucidating the mechanisms underlying apoptosis resistance and the aggressive nature of pancreatic cancer may lead to new treatment strategies, pancreatic cancer's poor prognosis and lack of biomarkers for early diagnosis demand new approaches to treat and/or manage this devastating cancer. To address this urgent and unmet need, we investigated our hypothesis that the combination treatment of biochanin A (an isoflavone) and atorvastatin (an antihyperlipidemic drug) induces apoptosis and blocks invasion of pancreatic cancer cells.

Isoflavones such as genistein have been widely studied for their putative anti-cancer properties (17, 18). Genistein enhances the anti-cancer effects of gemcitabine *in vitro* and *in vivo* and of gefitinib *in vitro* (18, 19). However, the clastogenic effects of genistein severely limit its anti-cancer therapeutic potential (20-22). Biochanin A is an analog of genistein but does not possess genistein's clastogenic properties. Thus, we and others have elucidated some of the anti-cancer potential of biochanin A in several types of cancer cells including pancreatic cancer (21-26). These findings suggest biochanin A may be a suitable candidate for combination therapy to treat pancreatic cancer (21-26).

While statins are anti-hyperlipidemic drugs primarily used to manage cardiovascular diseases, some clinical studies noted that individuals who were taking statins showed lower risks of developing cancers, suggesting anti-cancer potential (24, 27-29). The pleiotropic effects of statins, which include down-regulation of the PI3K/AKT pathway and decrease in angiogenesis by inhibiting Inducible nitric oxide synthase (iNOS). This, in addition to their effects on multiple phases of the cell cycle together with their effects on cholesterol biosynthesis, suggest statins can be anti-cancer agents (30-35). Statins have been reported to exert anti-cancer effects *via* modulation of signal transduction pathways and have been demonstrated to exert apoptotic effect as well as decrease invasion *in vitro* (22-29). Increased activities of MAP kinase and PI3K/AKT/mTOR pathways have been observed in pancreatic cancer. Mutated k-RAS up-regulates mitogenic signaling as well as survival signaling mediated by the PI3K/AKT pathway (14, 15, 36-39). k-Ras mutation is frequently observed in pancreatic cancer. Since statins have been shown to inhibit k-Ras, we chose to combine atorvastatin with biochanin A and investigate the effects on pancreatic cancer. Increased expression of growth factors and growth factor receptors can also contribute to activation of these signaling pathways in pancreatic cancer (18, 30, 39-41). Thus, these findings suggest that statins may be suitable candidates for combination treatment of pancreatic cancer.

## Materials and Methods

*Cell culture*. AsPC-1, MIA PaCa-2, and PANC-1 cells were purchased from ATCC (Manassas, VA, USA) and the cells were revived as per the ATCC guidelines. AsPC-1 cells were cultured in RPMI-1640 medium, whereas MIA PaCa-2 and PANC-1 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA). All cell lines were allowed to grow to ~70% confluency for all experiments reported here and were maintained in a humidified atmosphere of 95% (v/v) oxygen and 5% (v/v) carbon dioxide at 37°C.

*Cell viability studies*. MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assay. AsPC-1 and MIA PaCa-2 cells were seeded at a density of 3,000 cells/well (PANC-1 at 4,500 cells/well) in 96-well plates and were allowed to attach to the plates. After approximately 8 h of incubation, cells were treated with specified drug solutions prepared in medium supplemented with serum. Stock solutions of biochanin A (Sigma-Aldrich, St. Louis, MO, USA) and Atorvastatin (LC Laboratories, Woburn, MA, USA) were prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich), and the concentration of DMSO was maintained in the final solution at 0.05% (v/v) for all experiments. Following the desired incubation times, 20 μl of solution of MTT (prepared in 1X PBS at a concentration of 5 mg/ml) was added and incubated for 4 h at 37°C. Following the incubation, the medium with the MTT dye was aspirated, and 100 μl of DMSO was added to each well to solubilize the formazan crystals. The absorbance was measured at 570 nm using a plate reader (16).

*Cell count assay*. Cells ($1\times10^4$ per well) were seeded in a 24-well plate and allowed to attach to the bottom of the plate. Following approximately 8 h of incubation, cells were treated with the drug suspensions prepared in medium and incubated for 72 h. On the day of experiment, the medium with the drug solution was aspirated and cells were washed in 1X phosphate buffered saline (1X PBS). 100 μl of 10X trypsin EDTA (Atlanta Biologicals) was added to each well and incubated for 5 min. Then, 900 μl of isotone (buffered diluent) was added to each well and cells were thoroughly re-suspended. Cells in each well were counted after each treatment using a coulter counter (Beckmann-Coulter counter, Indianapolis, IN, USA) (18).

*Flow cytometry*. A total of $3\times10^5$ AsPC-1 cells were seeded in T-25 flasks and treated with specified drug concentration. After 36 h of incubation, the medium in the flasks was collected and spun at 1,000 × *g* for 5 min to collect the floating cells in the medium. Cells were harvested by trypsinization and suspended in 4 ml of 1X PBS and 1 ml medium. The suspended cells were added to the respective pellets in the centrifuge tubes and spun at 1,000 × *g* for 5 min. The resultant supernatant in each tube was discarded, and the pellet was suspended in 1X binding buffer and left on ice for 15 min. A total of 100 μl of each cell suspension was added to 4 μl of Annexin V and 4 μl of propidium iodide (PI) and left on ice for 15 min. 400 μl of 1X binding buffer was added just before analysis using a Becton Dickinson FACS Calibur Flow Cytometer (San Jose, CA, USA) as per the protocol provided by the vendor for the Aposcreen Annexin V apoptosis kit (Southern Biotech, Birmingham, AL, USA).

Table I. *The buffers and media used for the XF-PMP assay.*

| Media/Buffers for XF-PMP assay | Composition |
|---|---|
| MAS* buffer | Mannitol (220 mM), Sucrose (70 mM), $KH_2PO_4$ (10 mM), $MgCl_2$ (5 mM), HEPES (2 mM), EGTA (1 mM) prepared in di$H_2O$ |
| Washing buffer | MAS + Fatty Acid Free (FAF) BSA: 0.2 (w/v) |
| Assay medium | MAS + ADP (4 mM) + pyruvate (10 mM), malate (1 mM) + XF-PMP (1 nM) |

*The pH of MAS buffer was adjusted to 7.2 using 0.1 M KOH, and the MAS buffer was sterilized by passage through a 0.4 μ filter. MAS: Mitochondrial assay solution.

Table II. *Injection of the XF-PMP assay compounds in different ports of the XFp cartridge.*

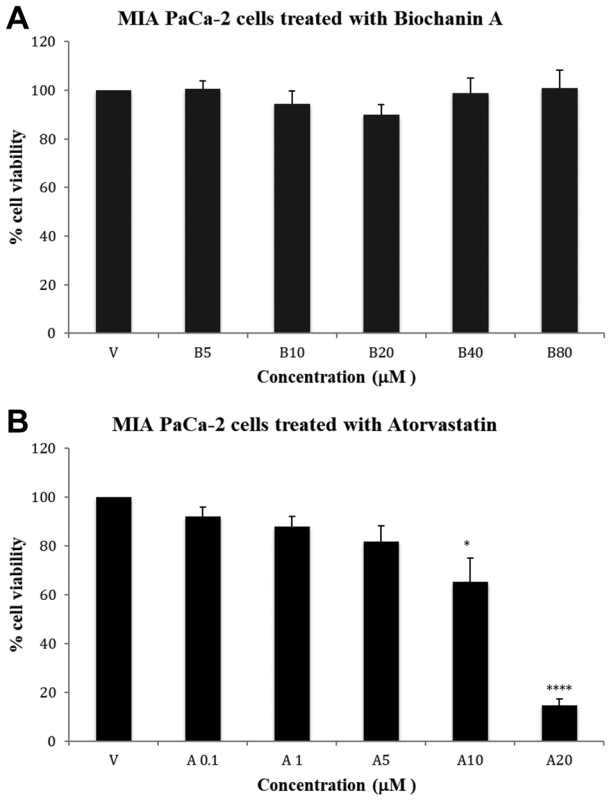
| Port | Injection volume of compounds (μl) | Final concentration |
|---|---|---|
| A | 20 μl of 20 μM Rotenone | 2 μM |
| B | 22 μl of 100 mM Succinate | 10 mM |
| C | 25 μl of 20 μM Antimycin | 2 μM |
| D | 27 μl of 150 mM Ascorbate + 2 mM TMPD | 15 mM/200 μM |

*Cell metabolism studies.* Mito Stress Test. AsPC-1, PANC-1, and MIA PaCa-2 cells were treated with biochanin A (20 μM) and atorvastatin (5 μM) individually and in combination for 72 h. After incubation, $3\times10^4$ cells per well were plated in an 8-well XFp cell culture miniplate (Seahorse Bioscience, Agilent, CA, USA) and incubated overnight at 37˚C in a 5% $CO_2$ (v/v) incubator. After incubation, the growth medium in the miniplate was replaced with the assay medium (XF basal medium containing 1mM sodium pyruvate, 10 mM D-Glucose, and 2 mM L-Glutamine, pH 7.4) and incubated in a non-$CO_2$ incubator at 37˚C for ~1 h. The stock compounds Rotenone/Antimycin A, Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), and Oligomycin were prepared by appropriately diluting the compounds with the assay medium. The stock compounds were loaded into the cartridge (Seahorse Bioscience, Agilent) and hydrated with the XF calibrant to achieve final concentrations of 1.0 μM Oligomycin, 0.5 μM FCCP, and 0.5 μM Rotenone/Antimycin A. The oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) were monitored using Seahorse XFp Extracellular Flux Analyzer (Seahorse Bioscience, Agilent).

*XF-Plasma membrane permeabilizer (PMP) assay.* AsPC-1, PANC-1, and MIA PaCa-2 cells were treated with biochanin A (20 μM) and atorvastatin (5 μM) alone and in combination for 72 h. After incubation, the cells were plated at an optimal density of $3\times10^4$ cells per well in an 8-well XFp cell culture miniplate (Seahorse Bioscience, Agilent) and incubated overnight at 37˚C in a 5% $CO_2$ (v/v) incubator. To evaluate the integrity of Complexes I-IV of the mitochondrial respiratory chain in the cells, OCR was measured as recommended by the manufacturer after cell permeabilization with 1 nM PMP reagent (Seahorse Bioscience, Agilent). Briefly, 10 mM pyruvate and 1 mM malate were used as substrates, in addition to 4 mM Adenosine diphosphate. The buffers/media used for the assay were prepared as shown in Table I.

The final concentrations of the compounds injected into the cartridge were: port A, 2 μM rotenone; port B, 10 mM succinate; port C, 2 μM antimycin A; port D, 150 mM ascorbate plus 2 mM TMPD, as shown in Table II. The protocol and algorithm program for XF-PMP assay were designed using wave 2.4 software. The assay was repeated at least two times for each cohort.

*Western blot analysis.* $6\times10^5$ AsPC-1 cells or $8\times10^5$ PANC-1 cells were seeded in a T-75 flask and allowed to attach to flasks in an incubator. After cells adhered to the flasks, they were treated with specified drug concentrations. Cells were incubated for specific incubation times (AsPC-1 for 36 h and PANC-1 for 72 h), which were based on the differential sensitivity to the drugs as determined by cell viability assay and doubling time of the cell line. On the day of experiment, cells were washed with ice-cold 1X PBS three times and harvested in lysis solution (20 mM Tris; pH 8, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% (v/v) Triton X-100, 1 mM Sodium orthovanadate, 1 mM PMSF (Phenylmethylsulfonyl fluoride), and 40 μl/ml protease inhibitors cocktail). Lysates were placed on ice and were subject to sonication with 6 bursts at position 8 using probe sonicator (Branson Ultrasonics Corp., Danbury, CT, USA) and then centrifuged at 15,000 × *g* for 10 min, and the resultant supernatant was collected. Two parts of lysate were added to one part of 3X Laemmli buffer (pH 6.8), and the resultant solution was heated at 85˚C for 5 min. The protein concentration in the samples was determined using bicinchoninic acid assay (24). Briefly, proteins in the samples were separated using SDS-PAGE. The separated proteins in the samples were transferred onto a PVDF membrane, and the membranes were then blocked in a solution of 5% (w/v) non-fat milk for 2 h and then rinsed three times (10 min



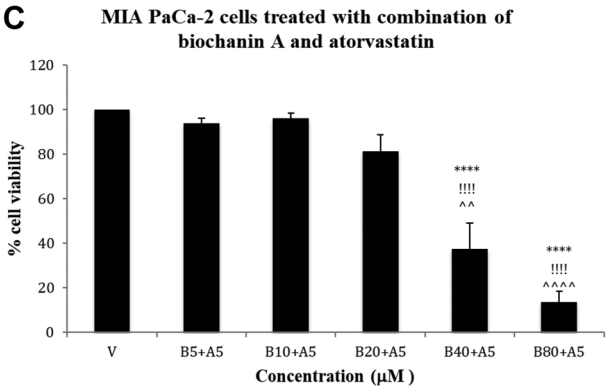




Figure 1. *Effect of Biochanin A on MIA PaCa-2 cells A) Effect of biochanin A on MIA PaCa-2 cells. Cells were seeded at 3000 cells/well in a 96-well plate and after attachment they were treated with biochanin A (B; 5-80 μM) for 72 h. V: vehicle; DMSO). The experiment was replicated, and one-way ANOVA performed with α value set to 0.05 indicated no significant difference between vehicle group and treatment groups. (Statistical analysis: One-way Anova, n=3, *p-value <0.05). B) Effect of atorvastatin on MIA PaCa-2 cells. Cells were seeded at 3,000 cells/well in a 96-well plate and after attachment they were treated with atorvastatin (A; 0.1-20 μM) for 72 h. V: vehicle; DMSO). The experiment was replicated, and one-way ANOVA performed with α value set to 0.05 followed by Tukey's post-hoc analysis. (Statistical analysis: One-way Anova, n=3, *p-value <0.05). C) Effect of combination treatment of biochanin A and atorvastatin on MIA PaCa-2 cells. Cells were seeded at 3,000 cells/well in a 96-well plate. After attachment the cells were treated with biochanin A (B; 5-80 μM) and atorvastatin (A; 5 μM) for 72 h. V: vehicle; DMSO). The experiment was replicated, and one-way ANOVA performed with α value set to 0.05 followed by Tukey's post-hoc analysis. (Statistical analysis: One-way Anova, n=3, *p-value <0.05 as compared to biochanin A single treatment at the same concentration and ^p-value <0.05 as compared to atorvastatin treatment at 5 μM).*

each) in Tris buffered saline and Tween 20 (TBST). Following rinses in TBST, the membranes were incubated overnight with primary antibody solutions prepared in 5% (w/v) bovine serum albumin at suitable dilutions. Following incubation with the primary antibody solutions, membranes were rinsed three times with TBST and incubated for an hour in a solution of 5% (w/v) non-fat milk added with secondary antibody conjugated with HRP at 1:5,000 dilution. Chemiluminescence detection kit was used to visualize the protein bands as recommended by the commercial vendor (Thermo Fisher Scientific, Rockford, IL, USA). β-Actin was used as the loading control (24). Primary antibodies to Akt (SC-8312), p-Akt (SC-16646-R), β-Actin (SC-47778), Erk (SC-094), p-Erk (SC-7976-R), mTOR (cat. #2983), phospho-mTOR (cat. #5536) and phospho-p70S6K (cat. #9234), Cdk1/Cdc2 (SC-954), Cdk2 (SC-163), Cdk4 (SC-260), Cdk6 (SC-177), cyclin A (SC-596), cyclin B1 (SC-245), and STAT3 (SC-7179) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to PARP (cat. #9532) and phospho-STAT3 (cat. #9145) were purchased from Cell Signal Technologies (Danvers, MA, USA). HRP conjugated secondary antibodies to rabbit and mouse were purchased from Thermo Fisher Scientific.

*Gelatin zymography.* A total of $1.5\times10^6$ PANC-1 cells were seeded in a T-75 flask and allowed to attach to the flask. Following incubation, serum containing medium in the flask was switched to serum-free medium and cells were treated with specific drug concentrations. After 72 h of incubation, the medium was collected in centrifuge tubes and spun at 1,000 × *g* for 10 min to pellet any cells and cell debris. The resultant supernatant from each tube was collected, and samples were frozen by snap freezing for 5 min. Samples were then lyophilized and concentrated to 20:1 ratio, and 40 μl of each sample was loaded onto a zymogram gel [10% [w/v] polyacrylamide gel containing 1% [w/v] gelatin (Bio-Rad, Hercules, CA, USA)] and allowed to separate on the gel. After separation, the gel was incubated in renaturation buffer (2.5% [v/v] Triton X-100 in de-ionized water) for 2 h. The gel was then washed and incubated in development buffer (50 mM Tris-base, 200 mM NaCl, 5 mM $CaCl_2$, and 0.02% [w/v] Brij-35, pH 7.6) overnight. The gel was then stained using staining solution (0.5% [w/v] Coomassie blue in 40% [v/v] methanol, 10% [v/v] acetic acid, and 50% [v/v] water) for an hour. De-staining solution containing 40% (v/v) methanol, 10% (v/v) acetic acid, and 50% (v/v) water was used to wash the excess dye and visualize the bands (25).

## Results

*Cell viability assay.* To determine whether the anti-cancer effects of combination treatment of biochanin A and atorvastatin are superior to those of treatment with either agent alone, we compared the effects of the combination treatment with those of either agent alone on the survival of MIA PaCa-

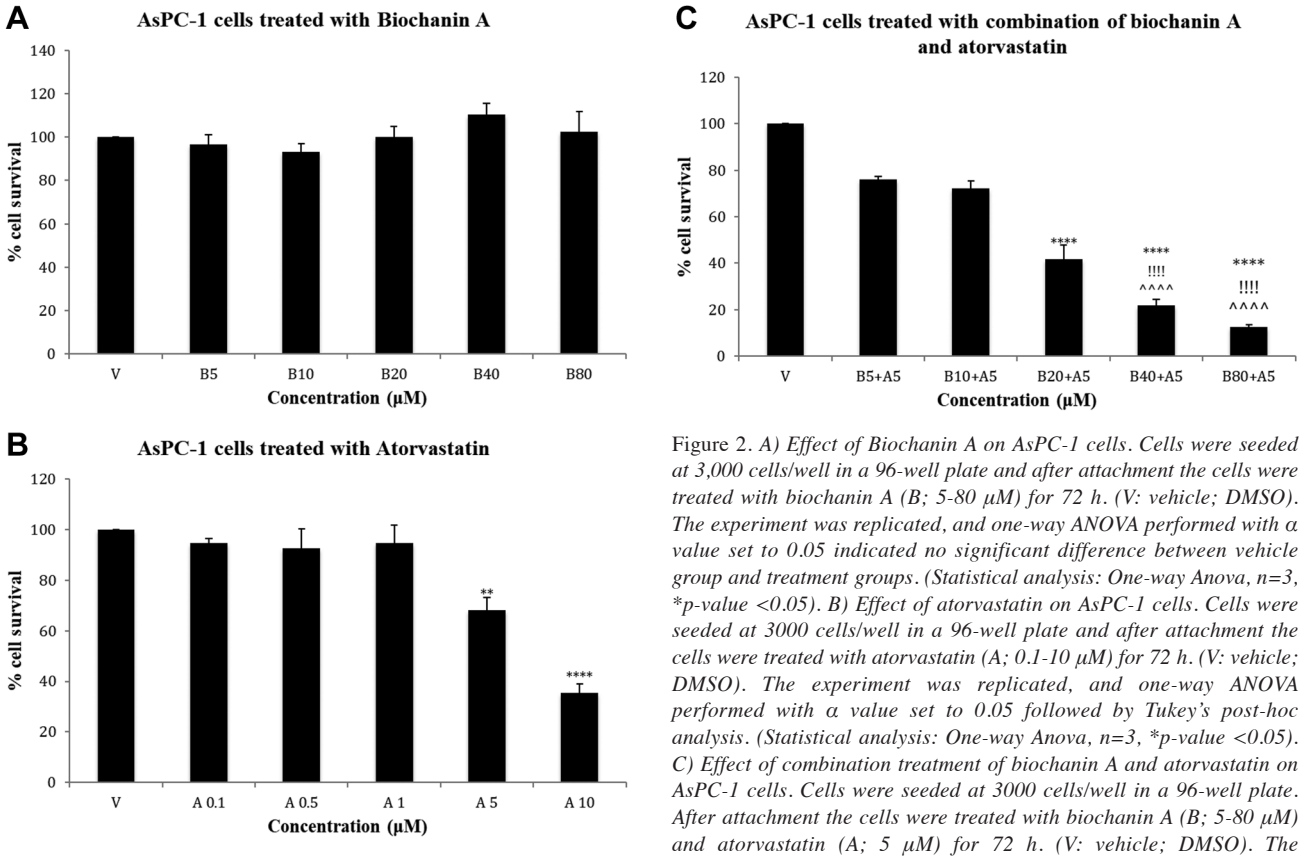



Figure 2. *A) Effect of Biochanin A on AsPC-1 cells. Cells were seeded at 3,000 cells/well in a 96-well plate and after attachment the cells were treated with biochanin A (B; 5-80 μM) for 72 h. (V: vehicle; DMSO). The experiment was replicated, and one-way ANOVA performed with α value set to 0.05 indicated no significant difference between vehicle group and treatment groups. (Statistical analysis: One-way Anova, n=3, *p-value <0.05). B) Effect of atorvastatin on AsPC-1 cells. Cells were seeded at 3000 cells/well in a 96-well plate and after attachment the cells were treated with atorvastatin (A; 0.1-10 μM) for 72 h. (V: vehicle; DMSO). The experiment was replicated, and one-way ANOVA performed with α value set to 0.05 followed by Tukey's post-hoc analysis. (Statistical analysis: One-way Anova, n=3, *p-value <0.05). C) Effect of combination treatment of biochanin A and atorvastatin on AsPC-1 cells. Cells were seeded at 3000 cells/well in a 96-well plate. After attachment the cells were treated with biochanin A (B; 5-80 μM) and atorvastatin (A; 5 μM) for 72 h. (V: vehicle; DMSO). The experiment was replicated, and one-way ANOVA performed with α value set to 0.05 followed by Tukey's post-hoc analysis. (Statistical analysis: One-way Anova, n=3, *p-value <0.05 as compared to biochanin A single treatment at the same concentration and ^p-value <0.05 as compared to atorvastatin treatment at 5 μM).*

2, AsPC-1, and PANC-1 cancer cells. While treatment for 72 h with biochanin A alone at 5-80 μM (Figure 1A) or with atorvastatin alone at 0.1-5 μM (Figure 1B) did not decrease survival of MIA PaCa-2 cells, treatment with atorvastatin alone at a concentration of 10 μM or higher did induce a dose-dependent decrease in survival of these cells (Figure 1B). Combination treatment for 72 h with increasing concentrations (40-80 μM) of biochanin A and a fixed concentration (5 μM) of atorvastatin decreased survival of the MIA PaCa-2 cells more than the treatment at comparable concentrations with either agent alone (Figure 1C).

Treatment for 72 h with biochanin A alone at 5-80 μM (Figure 2A) or with atorvastatin alone at 0.1-1 μM (Figure 2B) did not decrease survival of AsPC-1 cells. Treatment with atorvastatin alone at 5 μM or higher did induce a dose-related decrease in cell survival (Figure 2B). However, a 72-hour combination treatment with increasing concentrations (20-80 μM) of biochanin A and a fixed concentration (5 μM) of atorvastatin significantly decreased the survival of AsPC-1 cells compared to treatment at similar concentrations with either agent alone (Figure 2C). Treatment for 72 h with biochanin A alone at 20-80 μM (Figure 3) or with atorvastatin alone at 2.5-5 μM (Figure 3) did not decrease survival of PANC-1 cells. Combination treatment for 72 h with increasing concentrations (20-80 μM) of biochanin A and increasing concentrations (2.5-5 μM) of atorvastatin substantially decreased the survival of PANC-1 cells compared to treatment with equivalent concentrations of either agent alone (Figure 3).

Consistent with our hypothesis, the overall results of cell survival (Figure 1, Figure 2, Figure 3) strongly suggested that combination treatment of biochanin A and atorvastatin had enhanced effects in lowering the survival of all three pancreatic cancer cells compared to treatment with either agent alone. Further, they indicated that the enhanced effects of the combination treatment were more pronounced in the AsPC-1 cells.

We also examined whether the treatment with biochanin A alone exerted any effect on the viability of pancreatic cancer cells. We examined the effects of treatment with increasing concentrations of biochanin A on the viability of the three pancreatic cancer cells using the cell count assay. Treatment for 72 h with biochanin A alone at increasing

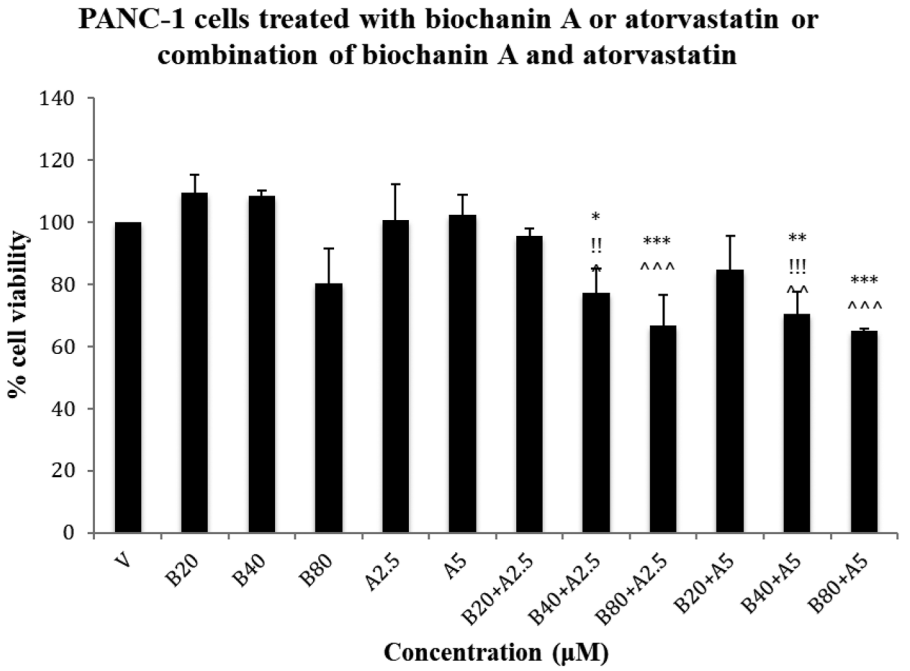



Figure 3. *Effects of biochanin A, atorvastatin, and combination treatment of biochanin A and atorvastatin on PANC-1 cells. Cells were seeded at 4,500 cells/well in a 96-well plate. After attachment the cells were treated with biochanin A (B; 5-80 μM), atorvastatin (A; 5 μM) and combination of biochanin A and atorvastatin for 72 h. (V: vehicle; DMSO). The experiment was replicated, and one-way ANOVA performed with α value set to 0.05 followed by Tukey's post-hoc analysis. (Statistical analysis: One-way Anova, n=3, *p-value <0.05 as compared to biochanin A single treatment at the same concentration and ^p-value <0.05 as compared to atorvastatin treatment at the same concentration).*

concentrations from 20 to 80 μM induced dose-dependent decreases in the viability of MIA PaCa-2 (Figure 4A), AsPC-1 (Figure 4B) and PANC-1 (Figure 4C) cells. Treatment with the maximum concentration of 80 μM induced ~60% decrease in the viability of both AsPC-1 and PANC-1 cells. Of the three pancreatic cancer cells investigated, MIA PaCa-2 cells were the least susceptible to the effect of biochanin A, showing ~40% decrease in cell viability at 80 μM.

*Cell apoptosis assessment using flow cytometry.* Since the combination treatment with biochanin A and atorvastatin lowered the survival of all three pancreatic cancer cells and AsPC-1 cells were more susceptible than the other two cells, we decided to employ AsPC-1 cells to investigate the hypothesis that one cell death mechanism underlying the effect of the combination treatment on the survival of pancreatic cancer cells is apoptosis. We treated AsPC-1 cells for 36 h with biochanin A (20 μM; Figure 5B) or atorvastatin (5 μM; Figure 5C) alone or in combination (Figure 5D); cells treated with vehicle (DMSO; Figure 5A) served as the control. Subsequently, we stained the treated cells with Annexin V (marker for apoptosis) and Propidium Iodide (marker for necrosis) and performed analyses using flow cytometry (Figure 5).

The results indicated that AsPC-1 cells treated with biochanin A alone only showed a slight increase in the numbers of cells in early apoptosis (lower right quadrant, Figure 5B) compared to control cells (lower right quadrant, Figure 5A), but the cells treated with atorvastatin alone showed some increases in numbers of cells in late apoptosis (upper right quadrant, Figure 5C) and in necrosis (upper left quadrant, Figure 5C) compared to control cells (upper right and left quadrant, respectively, Figure 5A). By contrast, AsPC-1 cells treated with both biochanin A and atorvastatin showed substantial increases in the numbers of cells in late apoptosis (upper right quadrant, Figure 5D), some increases in the numbers of cells in early apoptosis (lower right quadrant, Figure 5D), and some increases in the numbers of cells in necrosis (upper left quadrant, Figure 5D) compared to control cells (upper right, lower right, and upper left quadrant, respectively, Figure 5A). Further, compared to cells treated with biochanin A alone (Figure 5B) or atorvastatin alone (Figure 5C), the combination treatment induced some increases in the numbers of cells in early apoptosis (lower right quadrant, Figure 5D) but substantial increases in the numbers of cells in late apoptosis (upper right quadrant, Figure 5D). These results strongly suggested that the combination treatment induced preferential

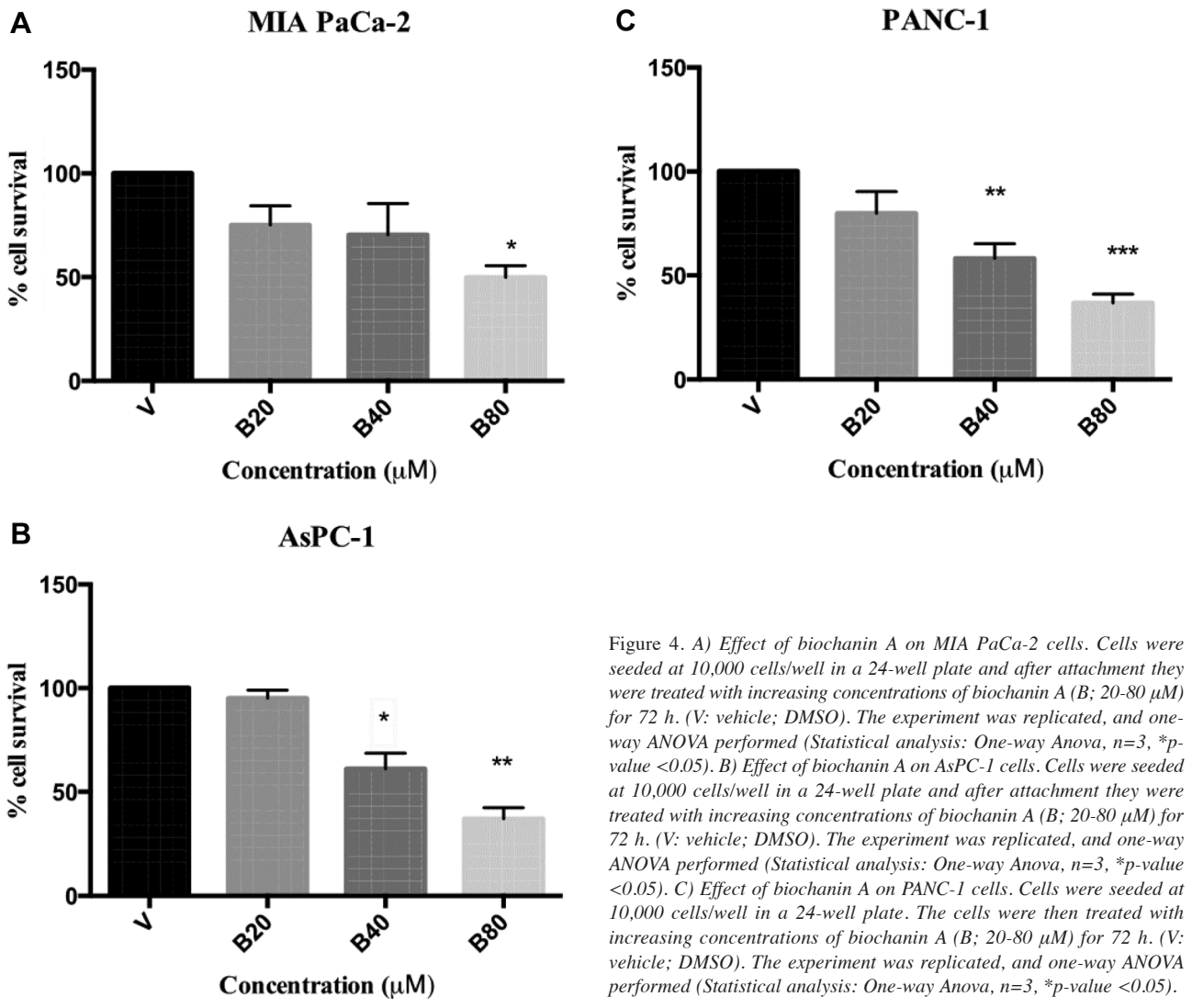



Figure 4. *A) Effect of biochanin A on MIA PaCa-2 cells. Cells were seeded at 10,000 cells/well in a 24-well plate and after attachment they were treated with increasing concentrations of biochanin A (B; 20-80 μM) for 72 h. (V: vehicle; DMSO). The experiment was replicated, and one-way ANOVA performed (Statistical analysis: One-way Anova, n=3, *p-value <0.05). B) Effect of biochanin A on AsPC-1 cells. Cells were seeded at 10,000 cells/well in a 24-well plate and after attachment they were treated with increasing concentrations of biochanin A (B; 20-80 μM) for 72 h. (V: vehicle; DMSO). The experiment was replicated, and one-way ANOVA performed (Statistical analysis: One-way Anova, n=3, *p-value <0.05). C) Effect of biochanin A on PANC-1 cells. Cells were seeded at 10,000 cells/well in a 24-well plate. The cells were then treated with increasing concentrations of biochanin A (B; 20-80 μM) for 72 h. (V: vehicle; DMSO). The experiment was replicated, and one-way ANOVA performed (Statistical analysis: One-way Anova, n=3, *p-value <0.05).*

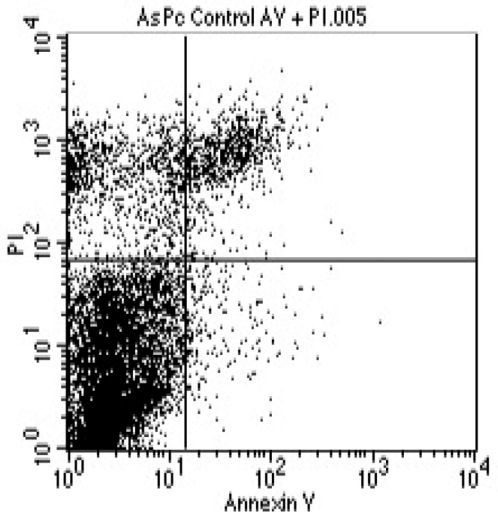
enhancement of late apoptosis and to a much lesser extent early apoptosis in these pancreatic cancer cells.

*Metabolic studies using the Mito stress test and XF-PMP assay.* In accordance with the Warburg hypothesis (also known as Warburg effect) (24, 42-48), we previously demonstrated that altering pancreatic cancer cell metabolism by employing glycolytic enzyme inhibitors can lower their survival (24), as cancer cells are known to metabolically adapt to enhance their survival and proliferation (48). We investigated the possibility that one mechanism underlying the efficacy of the combination treatment employed in our studies is alteration of pancreatic cancer cell metabolism, lowering their survival.

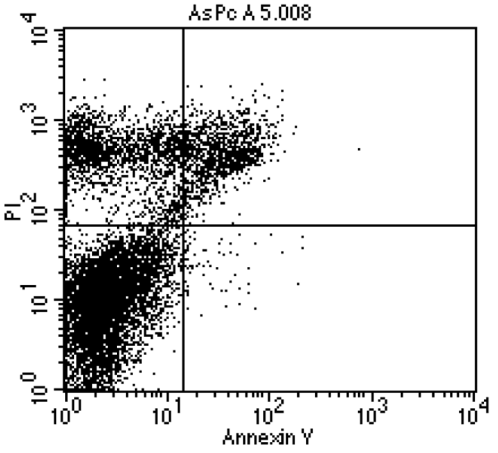
Following the treatment of AsPC-1, MIA PaCa-2, and PANC-1 cells with biochanin A and atorvastatin alone and in combination, we evaluated the mitochondrial respiration (OCR) in permeabilized cells by probing the activity of respiratory complexes using the XF-PMP assay (Figure 6). We observed a significant decrease in OCR in the MIA PaCa-2 cells, affecting complex I, II, and IV of the respiratory chain (Figure 6B), whereas in the PANC-1 cells the effect was observed mainly on complex II (Figure 6C). In AsPC-1 cells, the combination treatment showed higher inhibition of complex I, whereas atorvastatin alone had stronger inhibitory effects on respiratory chain complexes II and IV (Figure 6A).

*Western blot analysis.* Since the Ras/Raf/MEK/MAPK and PI3K/AKT pathways are known to regulate cell survival and/or proliferation and both pathways are frequently deregulated in pancreatic cancer, we examined the effects of

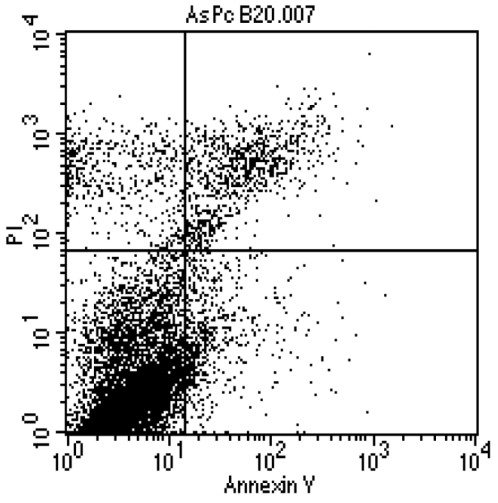
**ANNEXIN V/PI STAINING OF AsPC-1 CELLS**



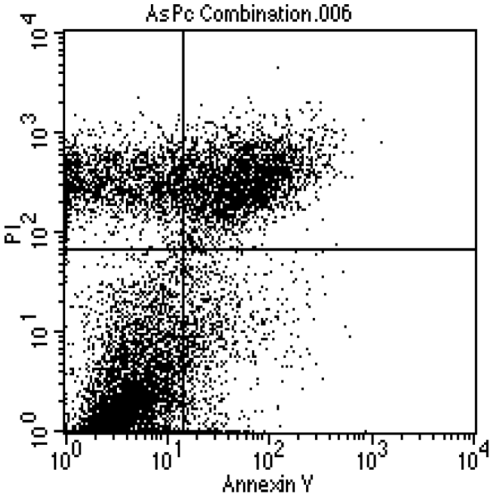

**A** AsPC-1 cells treated with DMSO (Vehicle)




**C** AsPC-1 cells treated with atorvastatin (5 μM)




**B** AsPC-1 cells treated with biochanin A (20 μM)




**D** AsPC-1 cells treated with combination of biochanin A (20 μM) and atorvastatin (5 μM)

Figure 5. *Annexin V/PI stained AsPC-1 cells exposed to different treatments for 36 h were analyzed using flow cytometry indicating increased population of early and late apoptotic cells in cells exposed to combination treatment. $3\times10^5$ AsPC-1 cells per 25-cm$^2$ flask were seeded and incubated for ~4 h. Cells were treated with A) DMSO, B) biochanin A (80 μM), C) atorvastatin (5 μM) or D) the combination of biochanin A and atorvastatin for 36 h. Vehicle (DMSO) was used as a control. The experiments were carried out in duplicate.*

treatment with biochanin A and atorvastatin alone or in combination on the expression of proteins in these two pathways in AsPC-1 and PANC-1 pancreatic cancer cells.

Compared with corresponding values in vehicle-treated (*i.e.*, control) cells, treatment for 36 h with biochanin A (20 μM) or atorvastatin (2.5 and 5 μM) alone or in combination did not induce any changes in the expression of ERK or phospho-ERK in AsPC-1 cells (Figure 7A). Compared with corresponding values in control cells, treatment for 72 h with biochanin A (80 μM) or atorvastatin (5 μM) alone did not

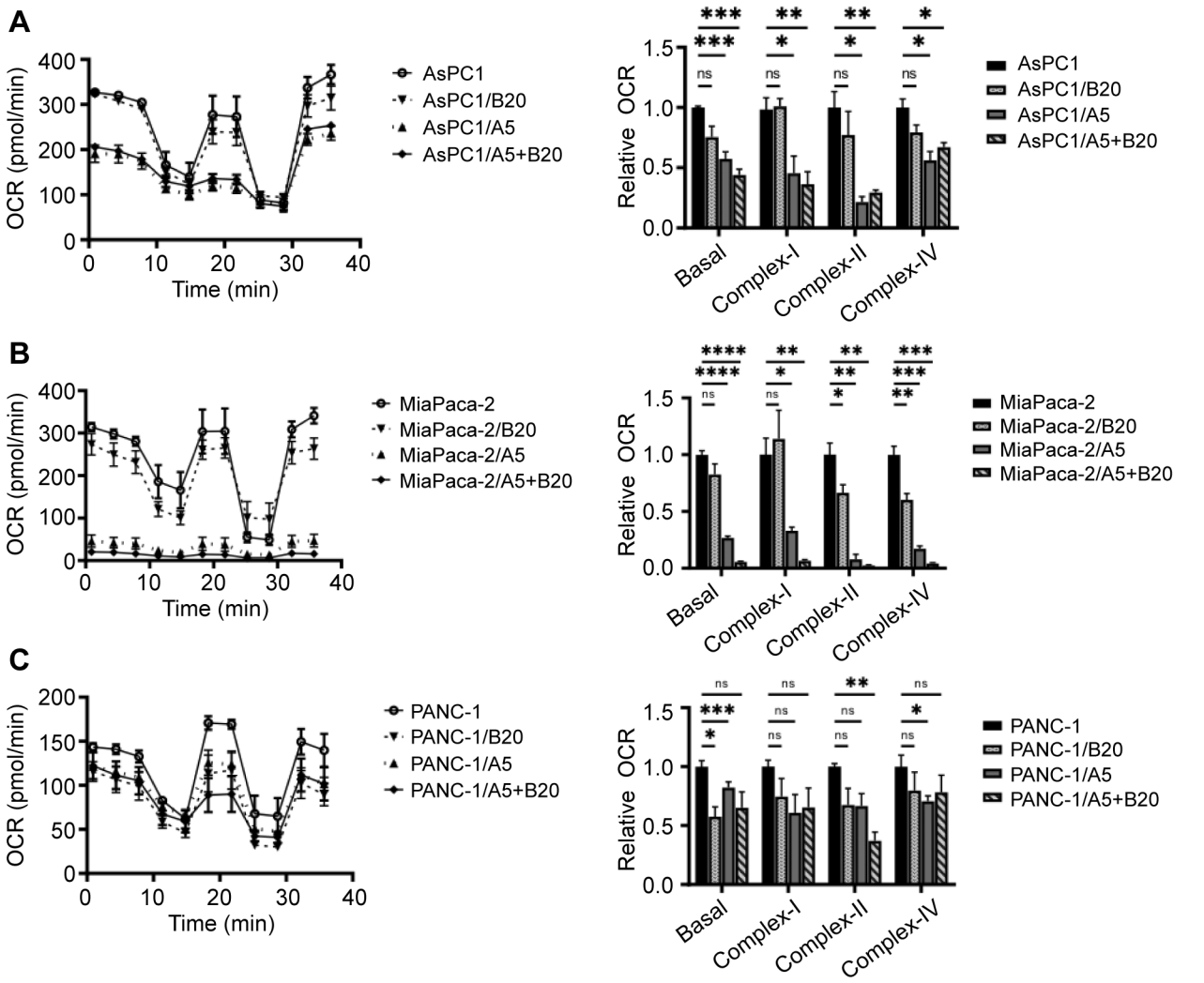



Figure 6. *The XF-PMP assay was performed on (A) AsPC-1, (B) MiaPaca-2 and (C) Panc-1 cells were treated with Biochanin A, atorvastatin, or Biochanin A + atorvastatin. Briefly, the cells were permeabilized with 1 nM XF-PMP reagent and the respiratory chain complexes were probed with specific oxidizable substrates, such as pyruvate + malate (For Complex-I), succinate (Complex-II & III) and ascorbate + TMPD (Complex-IV). Rotenone was used to inhibit the complex-I mediated respiration whereas, antimycin A was used to inhibit complex III mediated respiration. TMPD + ascorbate, is an artificial substrate that by-passes complex-III inhibition and was used to measure the complex-IV mediated respiration. A clear decrease in mitochondrial respiration is observed with the combination drug treatment showing a pronounced deterioration of complex I and II (prominently) in the pancreatic cancer cells. Statistical analysis: AsPC-1: Two-way ANOVA followed by post hoc Dunnett's multiple comparisons test *p<0.05, **p<0.01, ***p<0.001. MiaPaca-2: Two-way ANOVA followed by post hoc Dunnett's multiple comparisons test *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Panc-1: Two-way ANOVA followed by post hoc Dunnett's multiple comparisons test *p<0.05, **p<0.01, ***p<0.001.*

induce any changes in expression of ERK or phospho-ERK in PANC-1 cells, however, combination treatment with the two agents induced a small decrease in expression of phospho-ERK, decreasing the phospho-ERK to ERK ratio (Figure 7B). These results strongly suggested that the MAPK was unlikely to play a significant role in the signaling mechanism underlying the decreases in survival of pancreatic cancer cells induced by combination treatment with biochanin A and atorvastatin.

Critical in regulating cell survival and/or proliferation, the PI3K/AKT pathway mediates the signaling of non-receptor tyrosine kinases, growth factor receptors, and mutated k-RAS. The activation (*via* phosphorylation) of AKT generates an anti-apoptotic response. We therefore investigated whether this pathway is involved in the decrease in survival of pancreatic cancer cells induced by combination treatment with biochanin A and atorvastatin (Figure 1, Figure 2, Figure 3).

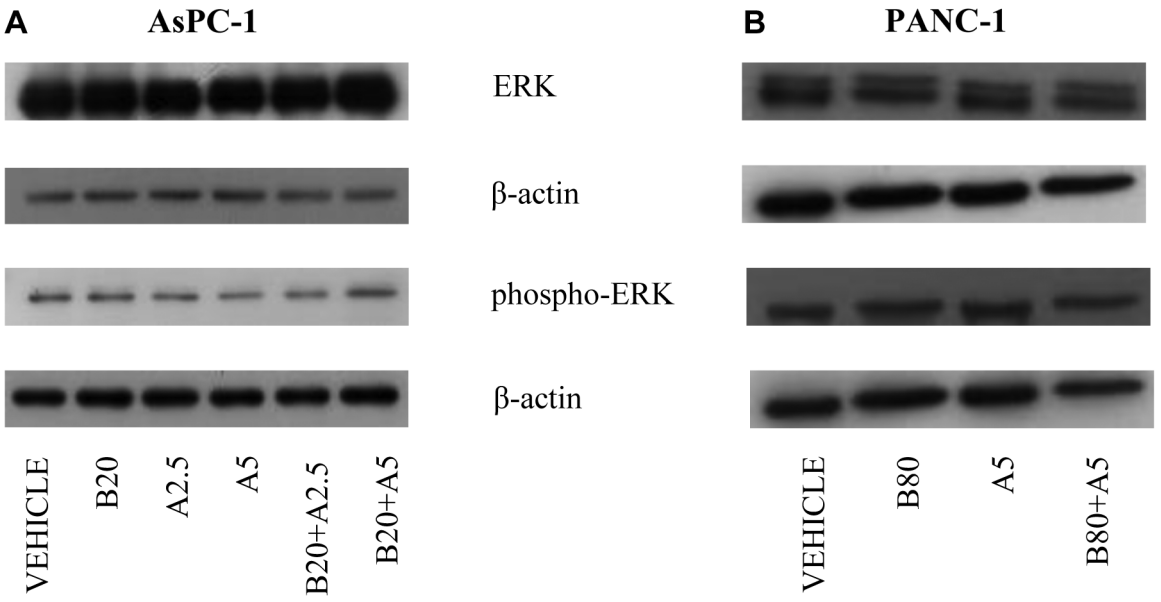



Figure 7. *Effect of biochanin A, atorvastatin, and combination treatments on ERK activation in A) AsPC-1 cells B) PANC-1 cells. Cells were seeded at 6×10$^5$ cells (AsPC-1) and 8×10$^5$ cells (PANC-1) per 75-cm2 flask. The cells were treated with DMSO biochanin A (20 μM- AsPC-1 and 80 μM- PANC-1), atorvastatin (2.5 μM, 5 μM- AsPC-1 and 5 μM- PANC-1) or the combination of biochanin A and atorvastatin for 36 h (AsPC-1) or 72 h (PANC-1). Vehicle (DMSO) was used as control. The experiments were replicated, and blots were digitized using UN-SCAN-IT software (Silk Scientific, Inc. Provo, UT, USA).*

Compared with corresponding values in control cells, treatment for 36 h with atorvastatin (2.5 and 5 μM) alone, but not with biochanin A (20 μM) alone, induced small decreases in the expression of phospho-AKT in AsPC-1 cells (Figure 8A). Further, combination treatment with atorvastatin and biochanin A decreased expression of phospho-AKT in AsPC-1 cells (Figure 8A). However, compared with corresponding values in control cells, expression of AKT in AsPC-1 cells was unaffected by any of the treatments. Consequently, the treatment-induced decreases in phospho-AKT were associated with corresponding decreases in phospho-AKT/AKT ratios (Figure 8A).

Unlike the effects noted in AsPC-1 cells, compared with corresponding values in control cells, treatment for 72 h with biochanin A (80 μM) alone increased expression of phospho-AKT in PANC-1 cells (Figure 8B). Compared with corresponding values in control cells, treatment for 72 h with atorvastatin (5 μM) alone and in combination with biochanin A (80 μM) induced small decreases in the expression of phospho-AKT in PANC-1 cells (Figure 8B), suggesting that the effect induced by atorvastatin was dominant over that of biochanin A. However, none of the treatments affected AKT expression in PANC-1 cells (Figure 8B). The atorvastatin-induced decreased expression of phospho-AKT in AsPC-1 cells but increased expression of phospho-AKT in PANC-1 cells (Figure 8A and B) prompted us to investigate if the mTOR pathway (down-stream of the AKT pathway) was affected by the effect of atorvastatin in these two pancreatic cancer cells.

Compared with corresponding values in control cells, treatment for 36 h with atorvastatin (5 μM) alone, but not with biochanin A (20 μM) alone, induced small decreases in the expression of phospho-mTOR in AsPC-1 cells (Figure 9A). Further, combination treatment with atorvastatin and biochanin A slightly decreased the expression of phospho-mTOR in AsPC-1 cells (Figure 9A). However, compared with corresponding values in control cells, expression of mTOR in AsPC-1 cells was unaffected by any of the treatments. Consequently, the treatment-induced decrease in phospho-mTOR was associated with corresponding decreases in phospho-mTOR/mTOR ratios (Figure 9A). These findings suggested the effects of atorvastatin alone and in combination with biochanin A on the mTOR pathway reflect their effects on the AKT pathway in AsPC-1 cells (Figure 8A, Figure 9A).

Unlike the effects on AsPC-1 cells, compared with corresponding values in control cells, treatment for 72 h with biochanin A (80 μM) alone induced a small increase in the expression of phospho-mTOR and mTOR in PANC-1 cells (Figure 9B). Compared with corresponding values in control cells, treatment for 72 h with atorvastatin (5 μM) in combination with biochanin A (80 μM), but not with

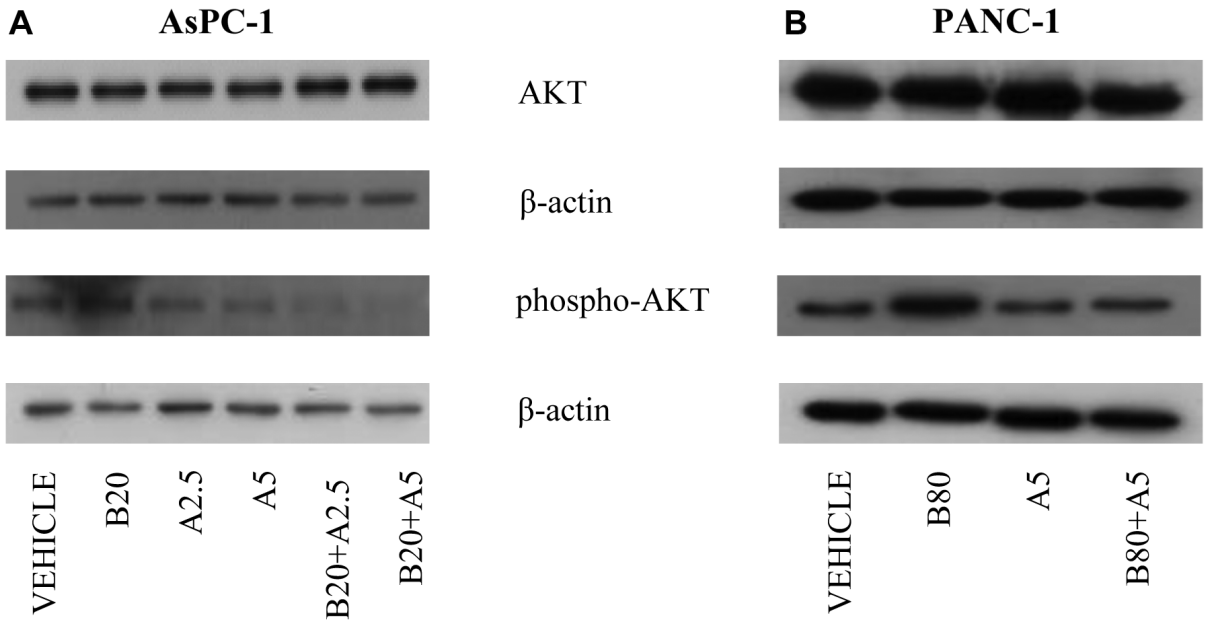



Figure 8. *Effect of biochanin A, atorvastatin, and combination treatments on AKT activation in A) AsPC-1 cells B) PANC-1 cells. Cells were seeded at 6×10$^5$ cells (AsPC-1) and 8×10$^5$ cells (PANC-1) per 75-cm2 flask. The cells were treated with DMSO biochanin A (20 μM- AsPC-1 and 80 μM- PANC-1), atorvastatin (2.5 μM, 5 μM- AsPC-1 and 5 μM- PANC-1) or the combination of biochanin A and atorvastatin for 36 h (AsPC-1) or 72 h (PANC-1). Vehicle (DMSO) was used as control. The experiments were replicated, and blots were digitized using UN-SCAN-IT software, (Silk Scientific, Inc. Provo, UT, USA).*

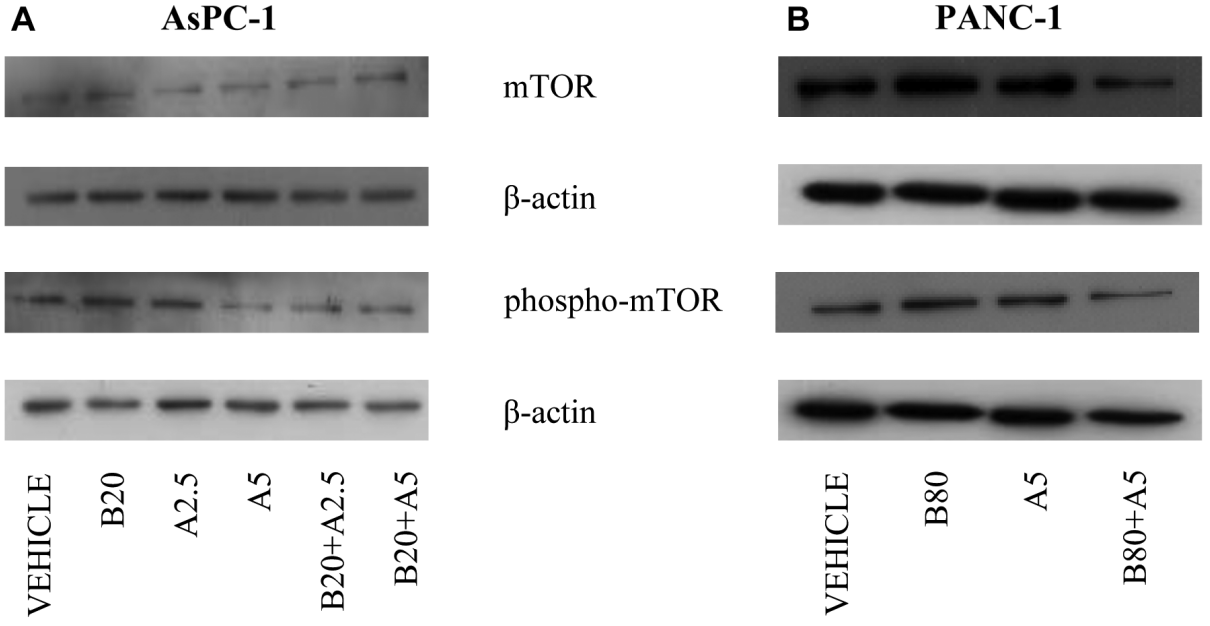



Figure 9. *Effect of biochanin A, atorvastatin, and combination treatments on AKT activation in a) AsPC-1 cells b) PANC-1 cells. Cells were seeded at 6×10$^5$ cells (AsPC-1) and 8×10$^5$ cells (PANC-1) per 75-cm$^2$ flask. The cells were treated with DMSO biochanin A (20 μM- AsPC-1 and 80 μM- PANC-1), atorvastatin (2.5 μM, 5 μM- AsPC-1 and 5 μM- PANC-1) or the combination of biochanin A and atorvastatin for 36 h (AsPC-1) or 72 h (PANC-1). Vehicle (DMSO) was used as control. The experiments were replicated, and blots were digitized using UN-SCAN-IT software (Silk Scientific, Inc. Provo, UT, USA).*

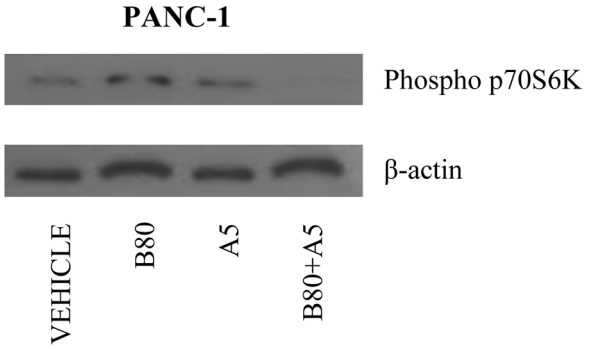



Figure 10. *Effect of biochanin A, atorvastatin, and combination treatment on activation of p70 S6K in PANC-1 cells. Cells were seeded at 8×10$^5$ cells per 75-cm$^2$ flasks. After adhering to the flasks, the cells were treated with DMSO, biochanin A (80 μM), atorvastatin (5 μM) or the combination of biochanin A and atorvastatin for 72 h. Vehicle (DMSO) was used as control. The experiments were replicated, and blots were digitized using UN-SCAN-IT software (Silk Scientific, Inc. Provo, UT, USA).*

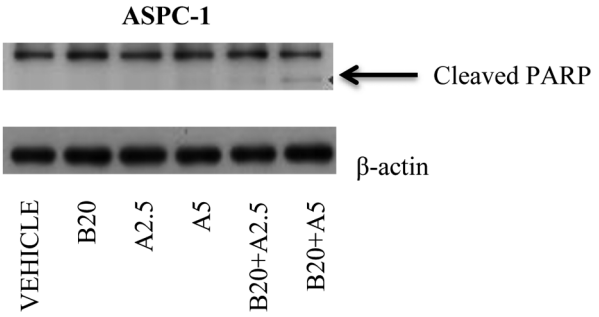



Figure 11. *Effects of biochanin A, atorvastatin and combination treatment on PARP [poly(ADP-ribose) polymerase] cleavage in AsPC-1 cells. Cells were seeded at 6×10$^5$ cells per 75-cm$^2$ flasks. The cells were treated with DMSO (vehicle), biochanin A (B; 80 μM), atorvastatin (A; 5 μM) or the combination of biochanin A and atorvastatin for 72 h. Vehicle (DMSO) was used as the control. The experiments were replicated, and the reproducibility of results observed. Blots were digitized using UN-SCAN-IT software (Silk Scientific, Inc. Provo, UT, USA).*

atorvastatin alone, induced a small decrease in the expression of phospho-mTOR and mTOR in PANC-1 cells (Figure 9B). Further, treatment for 72 h with both biochanin A (80 μM) and atorvastatin (5 μM), but not with either agent alone, substantially decreased the expression of phospho-p70S6K, a protein down-stream of mTOR and modulated by mTOR (Figure 10). Thus, taken together, our results suggested that the effects of treatment with biochanin A and atorvastatin alone or in combination on the AKT and mTOR signaling pathways in AsPC-1 cells differed from the corresponding effects on PANC-1 cells.

*PARP cleavage and apoptosis in AsPC-1 cells*. Because combination treatment with biochanin A and atorvastatin lowered the survival of AsPC-1 pancreatic cancer cells compared to MIA PaCa-2 and PANC-1 pancreatic cancer cells (Figure 1, Figure 2, and Figure 3) and apoptosis was a key mechanism underlying the effects of the combination treatment, we employed AsPC-1 cells to further investigate this mechanism. Treatment for 72 h with both biochanin A (20 μM) and atorvastatin (2.5 and 5 μM), but not with either agent alone, induced cleavage of poly (ADP-ribose) polymerase (PARP), which is a marker of apoptosis (Figure 11). As the atorvastatin concentration was increased from 2.5 to 5 μM, the cleavage of PARP induced by the combination treatment showed a clear and significant increase. Consequently, these results (Figure 11) confirmed the importance of apoptosis as one of the mechanisms underlying the effect of the combination treatment with biochanin A and atorvastatin in lowering the survival of pancreatic cancer cells.

*Expression levels of cell cycle associated proteins*. Preliminary results of Annexin V/Propidium Iodide staining did not indicate apoptosis induction at 24 h and 48 h in PANC-1 cells. However, cell viability assays showed decreased cell viability of PANC-1 cells following exposure to combination treatment. Hence, we investigated the effect of combination treatment compared to single treatments on cell cycle related proteins. Figure 12 shows that the combination treatment effectively decreased the expression levels of Cdk4, Cdk2, and Cdk1 (Cdc2) compared to single treatments. Biochanin A decreased Cdk6 levels compared to the combination treatment, and atorvastatin slightly increased Cdk6 expression.

We further investigated the levels of two cyclins, Cyclin A and Cyclin B1. The cyclins are key to transition between critical phases of the cell cycle. Cyclin A regulates transition from $G_1$ phase to S phase, and Cyclin B1 is critical for the transition from $G_2$ phase to M phase. As seen in Figure 12, the combination treatment decreased expression levels of Cyclin A and Cyclin B1 substantially. We observed a similar but not identical decrease following biochanin A treatment. While the effects on cell cycle progression must be confirmed, our results indicate a possible cell cycle arrest.

*MMPs in invasiveness of pancreatic cancer cells*. Invasion of cancer cells into the extracellular matrix involves digestion of surrounding extracellular matrix. This step is a prerequisite for cancer to metastasize and is known to involve matrix-degrading enzymes such as matrix metalloproteinases. We analyzed the activity of the matrix metalloproteinases using the *in vitro* zymography assay. As shown in Figure 13,

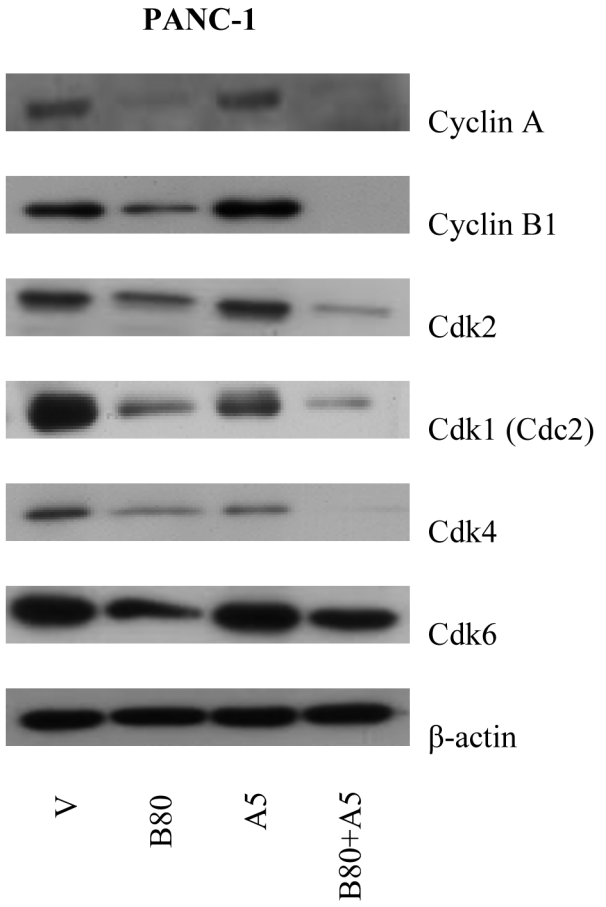



Figure 12. *Effects of biochanin A, atorvastatin, or combination treatment on expression of Cdks and cyclins A and B1 in PANC-1 cells. Cells were seeded at $8\times10^5$ cells per 75-cm$^2$ flask. The cells were treated with DMSO (vehicle), biochanin A (B; 80 μM), atorvastatin (A; 5 μM) or the combination of biochanin A and atorvastatin for 72 h. The vehicle (DMSO) was used as the control. The experiments were replicated, and the reproducibility of results observed. Blots were digitized using UN-SCAN-IT software (Silk Scientific, Inc. Provo, UT, USA).*

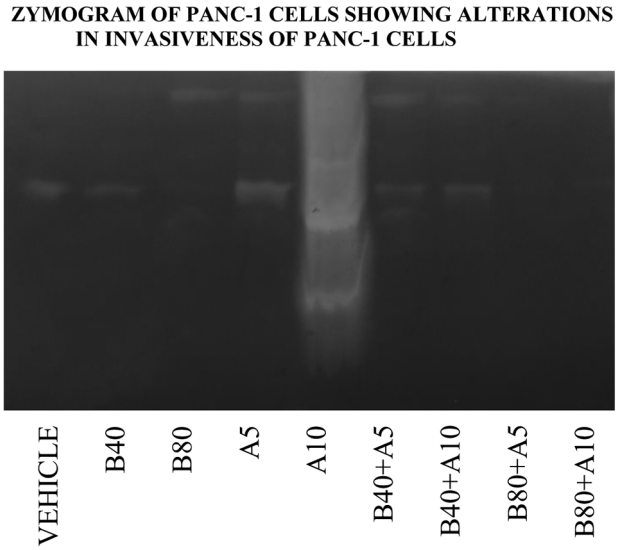



Figure 13. *Effects of biochanin A, atorvastatin, and combination treatments on invasiveness of PANC-1 cells. Cells were seeded at $1.5\times10^6$ cells per 75-cm$^2$ flask. After overnight incubation, the medium in the flasks was replaced with serum free medium and cells were treated with DMSO, biochanin A (40 and 80 μM), atorvastatin (5 and 10 μM) or combination of biochanin A and atorvastatin for 72 h. Vehicle (DMSO) was used as control. The experiments were replicated, and the reproducibility of results observed.*

biochanin A decreased the activity of MMP-2 at 40 μM and 80 μM concentrations but induced the activity of MMP-9 at 80 μM. Atorvastatin increased overall MMP activity, with the intensity and number of bands increasing concurrently with concentrations from 5 μM to 10 μM. However, the combination decreased MMP-2 and MMP-9 activity, as evidenced by decreased band intensities and number of bands.

*STAT3 activation and invasiveness in PANC-1 cells*. The decreased invasiveness of PANC-1 cells treated with combination treatment as assessed by the zymography assay led us to study the effects of combination treatment on STAT3 activation. STAT3 activity has been related to invasiveness of pancreatic cancer and is the transcriptional regulator of many proteins including the MMPs and anti-apoptotic proteins (26). As shown in Figure 14, biochanin A reduced the activation of STAT3 (Y705) in PANC-1 cells. We observed a similar down-regulation in cells treated with the combination. However, atorvastatin did not reduce activation of STAT3, which may explain the intense bands observed in zymography assay.

## Discussion

Pancreatic cancer is a devastating disease with a poor prognosis. Despite the availability of FDA approved drugs, such as 5-Fluorouracil, Gemcitabine, Mitomycin C, Erlotinib, and the combination treatment FOLFIRINOX, the 5-year survival of pancreatic cancer is 11% and at stage 4, the survival is only 3%. Isoflavones can inhibit tumor progression by impeding DNA repair and altering cell signaling pathways like Wnt and NF-κB by inhibiting topoisomerase II and by acting as antioxidants (17-20). To investigate the anti-cancer potential of isoflavones, we used a natural dietary isoflavone, biochanin A, in combination with a statin to elucidate the putative anti-survival, anti-invasive, and pro-apoptotic mechanisms underlying the effects of this combination treatment on pancreatic cancer cells. The effects of combination treatment on the cells

included lowering cell viability (Figure 1, Figure 2, Figure 3) and decreasing activation of AKT (Figure 8) compared to cells exposed to biochanin A or atorvastatin alone.

One mechanism that may account for the effects of the combination treatment-induced decrease in survival of pancreatic cancer cells is apoptosis. Consistent with this hypothesis, flow cytometry analysis of Annexin V/PI staining showed preferentially larger increases in late apoptotic relative to early apoptotic populations of AsPC-1 pancreatic cancer cells after 36 h of incubation with combination treatment compared to apoptotic cells in single treatments and control AsPC-1 cells (Figure 5). Additionally, the combination treatment with both biochanin A and atorvastatin induced some increases in the number of AsPC-1 cells undergoing necrosis (Figure 5), suggesting that necrosis also contributed to lowering pancreatic cancer cell survival induced by the combination treatment.

PARP is a DNA repair protein down-stream of caspase-3, the executioner caspase, and the cleavage of PARP results from excessive DNA breaks and subsequent caspase activation, ultimately leading to PARP inactivation and DNA repair termination (27). To understand this, we examined the pro-apoptotic potential of the combination treatment. Our western blot analysis results clearly indicated that treatment with both biochanin A and atorvastatin, but not with either agent alone, induced cleavage of PARP (Figure 11). Thus, our finding is consistent with earlier reports of PARP cleavage following exposure to biochanin A as well as statins (29-32) and confirmed that the combination treatment enhanced apoptosis as determined by flow cytometric analysis with Annexin V/PI staining. These findings support the hypothesis that apoptosis is one mechanism underlying the lowering of pancreatic cancer cell survival induced by combination treatment with biochanin A and atorvastatin.

The combination treatment with biochanin A and atorvastatin for 24 and 48 h did not result in apoptosis in PANC-1 cells (data not shown), however, the results of cell survival assays showed that the combination treatment decreased PANC-1 cell survival (Figure 3). We therefore investigated the possibility that cell cycle-associated proteins, in particular cyclins and cyclin dependent kinases (Cdks) (33), may be implicated in the combination treatment-induced inhibition of PANC-1 cell cycle based on the following rationale. Activation of Cdk's is not only cyclin dependent but also cyclin specific. Unlike Cdks, expression of cyclins and the duration of their expression is under tight regulation. Hence, the Cdk isoform activation depends on the phase of the cell cycle (33). Our findings indicated that the combination treatment decreased the expression of Cdk1 (Cdc2), Cdk2, and Cdk4 in PANC-1 cells, whereas biochanin A alone decreased the expression of Cdk6 in these cells (Figure 12). Our results are consistent with those reported in earlier studies in other cancer cell lines. For example, biochanin A induced down-regulation of expression of p21 and Cyclin B1 in prostate cancer cells (34), and statins have been shown to decrease the expression of Cdk4 and Cdk6 in prostate cancer cells (35). Our findings in pancreatic cancer cells and those in prostate cancer cells (34, 35) suggest that the effects of combination treatment with biochanin A and statins on the expression of Cdks may vary depending on the cancer cell types.

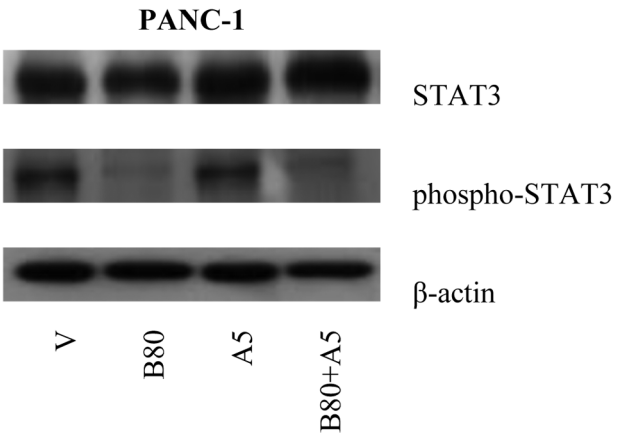



Figure 14. *Effects of biochanin A, atorvastatin, or combination treatment on STAT3 activation in PANC-1 cells. Cells were seeded at $8\times10^5$ cells per 75-cm$^2$ flask. The cells were treated with DMSO, biochanin A (B; 80 μM), atorvastatin (A; 5 μM) or the combination of biochanin A and atorvastatin for 72 h. The vehicle (DMSO) was used as the control. The experiments were replicated, and the reproducibility of results observed. Blots were digitized using UN-SCAN-IT software (Silk Scientific, Inc. Provo, UT, USA).*

Because Cyclin A regulates the transition from $G_1$ phase to S phase and Cyclin B1 is critical for the transition from $G_2$ phase to M phase, we also investigated the combination treatment on the expression of Cyclin A and Cyclin B1 in PANC-1 pancreatic cancer cells. Our results indicate that the combination treatment abolished the expression of both Cyclin A and Cyclin B1 in PANC-1 cells, while treatment with biochanin A alone greatly decreased Cyclin A expression and decreased Cyclin B1 expression to a lesser extent (Figure 12). These results suggest that one mechanism underlying the effects of the combination treatment is the disruption of the cell cycle.

Because cancer cells are known to metabolically adapt to enhance their survival and proliferation (42-48), we investigated the possibility that one mechanism underlying the efficacy of the combination treatment employed in our studies is altering the pancreatic cancer metabolism, lowering their survival. Consistent with this hypothesis, we observed that combination treatment with biochanin A and atorvastatin decreased mitochondrial respiration, affecting complexes I, II, and IV in MIA PaCa-2 pancreatic cancer

cells (Figure 6). The same combination treatment appeared to inhibit complex II in PANC-1 pancreatic cancer cells (Figure 6). The combination treatment was more inhibitory to complex I (compared to that of II and IV) in AsPC-1 pancreatic cancer cells, whereas atorvastatin treatment alone seemed to exert more inhibitory effects on complexes II and IV (Figure 6A). Our results suggest that the combination treatment with biochanin A and atorvastatin induced differential inhibitory effects on the respiratory chain complexes in the three pancreatic cancer cells.

Invasion of cancer cells into their surrounding extracellular matrix through the activation of matrix-degrading enzymes such as MMPs is a prerequisite for cancer growth and metastasis. We previously reported that biochanin A disrupts invasion of breast cancer (25) and glioblastoma *via* inhibition of MMPs. In this study, we investigated the possibility that biochanin A and its combination with atorvastatin disrupts pancreatic cancer cell invasion *via* a similar mechanism. Consistent with our hypothesis, we found that treatment with biochanin A alone decreased MMP-2 activity, and the combination treatment decreased MMP-2 and MMP-9 activities in PANC-1 cells even though treatment with atorvastatin alone appeared to elevate MMP activity (Figure 13). However, the latter observation seems to contradict earlier observations that treatment with statins decreased invasion of human pancreatic (22) and human melanoma (23) cells. Studies have suggested that synergistic statins act as adjuvants with chemotherapy and augment the effects of the chemotherapeutic agents in several cancers (49).

STAT3 is a transcriptional regulator of MMPs and some anti-apoptotic proteins and has been implicated in the invasiveness of pancreatic cancer (26). The STAT3 pathway has been implicated in the anti-apoptotic response and increased invasiveness of pancreatic cancer cells (38-40), but the effect of biochanin A on STAT3 activation in pancreatic cancer has not been reported, which prompted us to study the effects of biochanin A and its combination with atorvastatin on STAT3 in PANC-1 cells. Treatment with biochanin A alone and in combination with atorvastatin decreased phospho-STAT3 formation (Figure 14), which is consistent with our hypothesis and supports the notion that STAT3 activation is implicated in the effects of biochanin A lowering MMPs and decreasing the invasiveness of PANC-1 cells.

## Conclusion

To the best of our knowledge, our study is the first to show that combination treatment with biochanin A and atorvastatin induces apoptosis, down-regulates cell cycle associated proteins, and impedes invasiveness of pancreatic cancer cells. Further, this study demonstrates that one mechanism underlying the combination treatment-induced lowering of pancreatic cancer cell survival is the differential inhibition of their respiratory chain complexes. Our findings strongly suggest that the combination treatment with biochanin A, a natural, nontoxic compound and atorvastatin, a clinically well-tolerated drug, can effectively inhibit the progression of pancreatic cancer with dismally low survival and merits further investigation in animal models of pancreatic cancer.

## Conflicts of Interest

The Authors have no known financial interests in the work reported in this paper.

## Authors' Contributions

AB, ST, VD, and JCKL reviewed related literature and drafted the manuscript. VD, HS, RS contributed to the metabolism experiments. ST, VD, HS, and AB designed and conducted experiments. AB supervised and coordinated the project.

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