



Published in final edited form as:

Pancreas. 2016 January ; 45(1): 101–109. doi:10.1097/MPA.0000000000000411.

Curcumin Induces Pancreatic Adenocarcinoma Cell Death via Reduction of the Inhibitors of Apoptosis

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Abstract

Objectives—The inhibitor of apoptosis (IAP) proteins are critical modulators of chemotherapeutic resistance in various cancers. To address the alarming emergence of chemotherapeutic resistance in pancreatic cancer, we investigated the efficacy of the turmeric derivative curcumin in reducing IAP protein and mRNA expression resulting in pancreatic cancer cell death.

Methods—The pancreatic adenocarcinoma cell line PANC-1 was used to assess curcumin's effects in pancreatic cancer. Curcumin uptake was measured by spectral analysis and fluorescence microscopy. AlamarBlue and Trypan blue exclusion assays were used to determine PANC-1 cell viability following curcumin treatment. Visualization of PANC-1 cell death was performed using Hoffman Modulation Contrast microscopy. Western blot and PCR analyses were used to evaluate curcumin's effects on IAP protein and mRNA expression.

Results—Curcumin enters PANC-1 cells and is ubiquitously present within the cell following treatment. Furthermore, curcumin reduces cell viability and induces morphological changes characteristic of cell death. Additionally, curcumin decreases IAP protein and mRNA expression in PANC-1 cells.

Conclusions—These data demonstrate that PANC-1 cells are sensitive to curcumin treatment. Furthermore, curcumin as a potential therapeutic tool for overcoming chemotherapeutic resistance mediated by IAPs, supports a role for curcumin as part of the therapeutic approach for pancreatic cancer.

Keywords

pancreatic cancer; curcumin; chemotherapy resistance; inhibitor of apoptosis

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Introduction

Pancreatic cancer is an aggressive and devastating disease responsible for the highest mortality rates among cancer types with 94% of patients dying within five years of diagnosis. Pancreatic tumor resection remains the most efficacious treatment with 20 to 25% of the patients surviving 5 years post-surgery. However, early diagnosis, a prerequisite for the surgery, is made difficult by the absence of early signs or symptoms (1-3). As a result, most patients are ineligible for the surgery at the time of diagnosis and are offered chemotherapy (2-4). The gold standard chemotherapeutic for pancreatic cancer is Gemcitabine, which has shown significant clinical benefits, with survival rates ranging from six to fifteen months when resection is not an option and the patients exhibit either non-metastatic or metastatic disease (5-8). Unfortunately, emerging resistance to chemotherapy has hindered the efficacy of chemotherapeutics including Gemcitabine (9), highlighting the need for novel therapeutic approaches that address this rising resistance to therapy.

The inhibitor of apoptosis (IAP) proteins, including Survivin, cellular IAP 1 and 2 (cIAP1 and cIAP2), and X-chromosome linked IAP (XIAP), belong to a family of anti-apoptotic proteins known to confer resistance to treatment modalities such as radiation therapy and chemotherapy (10-13). Additionally, the overexpression of these IAP proteins has been positively correlated with the progression of a variety of cancer types, including pancreatic cancer, resulting in a decline in patient survival after chemotherapeutic treatment (14-21). Pre-clinical and clinical trials aimed at reducing IAP expression via antisense oligonucleotides and/or second mitochondria-derived activator of caspases (Smac) mimetics have yielded promising results in various cancers (14, 22-27); however, such studies have remained inconclusive in pancreatic cancer (28-29). This may be due to a compensatory effect of IAPs to targeted therapies. Further studies involving agents that cause simultaneous reduction in multiple IAPs are needed to investigate this notion.

Curcumin, a turmeric derivative, has been considered as a potential anti-cancer therapy due to its capacity to interrupt signaling pathways that are crucial for the initiation and progression of cancer (30). For instance, studies have demonstrated that curcumin inhibits the progression of various cancers by modulating the expression of anti-apoptotic factors (31-36). Furthermore, curcumin induces apoptosis in pancreatic cancer (37) and regulates IAP expression in a variety of other cancer types (38-39). Preclinical studies involving curcumin in pancreatic cancer have shown that curcumin enhances Gemcitabine sensitivity in vitro and in vivo (40-42). Moreover, Phase I and II clinical trials have yielded promising results on the use of curcumin as part of pancreatic cancer therapeutic strategies (43-48). This recent progress emphasizes the need for a better understanding of the mechanisms by which curcumin counteracts chemotherapeutic resistance. Therefore, the objective of this study was to determine curcumin's impact alone on IAP expression in pancreatic cancer cells.

Materials and Methods

Cells and Culture Conditions

The pancreatic adenocarcinoma cell line PANC-1 was acquired from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's modified Eagle medium (DMEM; ATCC, Manassas, VA) supplemented with Normocin at a final concentration of 100 µg/mL (InvivoGen, San Diego, CA), 100 units of penicillin, 100 µg/mL of streptomycin, 300 µg/mL of L-glutamine and USDA-sourced 10% heat-inactivated fetal bovine serum (Mediatech, Manassas, VA). In all the experiments, cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ to 70-80% confluency prior to use.

Preparation of Curcumin Solutions

Curcumin (Sigma-Aldrich, St. Louis, MO) stock solutions were prepared using DMSO and ethanol as solvents. Subsequent dilutions were made from this stock solution in DMEM. The final concentrations of DMSO and ethanol did not exceed 0.04 % and 0.6%, respectively, and cell viability was not affected at these concentrations (data not shown).

Cell Viability Assays

Cell viability following curcumin treatment was estimated by AlamarBlue and Trypan blue exclusion assays. For AlamarBlue assays, PANC-1 cells were cultured in 96-well plates at 1.0×10^4 cells per well and treated with 10, 50, and 100 µM curcumin for 24, 48 and 72 hours. Subsequently, the AlamarBlue reagent (Life Technologies, Grand Island, NY) was added to each sample (10% final concentration) and incubated at 37°C/5% CO₂ for two hours. Viability was analyzed by detection of absorbance at 570 nm using 600 nm as a reference wavelength in a uQUANT spectrophotometer (Bio-Tek, Winooski, VT). For Trypan blue exclusion assays, cells were cultured in 6-well plates at 3.0×10^5 cells per well and treated with 10, 50, 100 µM curcumin for 24, 48 and 72 hours. Cells were then trypsinized and combined with the Trypan blue reagent (Life Technologies, Grand Island, NY) to calculate viability by counting the cells on a hemocytometer. The results presented are representative of three independent experiments. Data from curcumin-treated samples are normalized to the untreated control.

Hoffmann Modulation Contrast Microscopy

PANC-1 cells were cultured in T25 flasks at 7.0×10^5 cells per flask and treated with 10, 50, and 100 µM curcumin for 24 and 48 hours. Cells were then imaged using an Olympus IX70 microscope with Hoffmann modulation and an Insight Spot 2 Mega Sample camera and software. Three independent experiments were performed and within each experiment three images were captured in different sections of the T25 flasks to obtain a representative image.

Spectral Studies and Fluorescence Imaging

Spectral analysis of PANC-1 cell curcumin content post-treatment was performed as previously described (49-51). Briefly, cells were cultured in T75 flasks at 1.5×10^6 cells per flask and treated with 50 µM curcumin for 24 hours. Cells were then trypsinized, washed

three times with 1X PBS, resuspended in 1 mL of 100% methanol and sonicated to disrupt the cell membrane integrity. The samples were then centrifuged at 10,000 rpm for 5 minutes at 4°C and the supernatant fraction collected for absorbance analysis at 420 nm using a uQUANT spectrophotometer and KC Junior software (Bio-Tek, Winooski, VT). Untreated cells were also lysed in methanol and subjected to spectral analysis to determine baseline auto-fluorescence of PANC-1 cells. Methanol-only, cell-free samples were used as blank controls. Data are representative of three independent experiments. For fluorescence microscopy of PANC-1 cells curcumin content post-treatment, PANC-1 cells were cultured in 6-well plates containing sterile cover slips at 3.0×10^5 cells per well and treated with 50 μ M curcumin for 24 hours. Subsequently, cells were fixed with 4% paraformaldehyde and incubated at -20°C overnight. The following day, the samples were permeabilized using 0.1% Igepal in 1X PBS for 10 minutes at room temperature. The cover slips were then washed three times with 1X PBS and placed onto slides with the nuclear stain DAPI in mounting media for 5 minutes. Stained slides were imaged using a BZ-9000 BIOREVO fluorescence microscope (Keyence, Itasca, IL) with a 40X magnification objective. Results are representative of three independent experiments; within each experiment, three images were acquired from different portions of each slide to obtain a representative image.

Western Blot Analysis

PANC-1 cells were cultured in T25 flasks at 7.0×10^5 cells per flask and treated with 10, 50 and 100 μ M curcumin for 24 and 48 hours. After treatment, lysates were prepared using a lysis buffer composed of 50mM Tris-HCl pH 7.5, 1% Triton-X, 0.25% DOC, 150mM sodium chloride, 1mM sodium orthovanadate, 20mM sodium fluoride, 0.2mM EGTA, 1mM EDTA, 1mM PMSF, 1X protease inhibitor cocktail (Roche Life Science, Indianapolis, IN) and sonicated. To remove cell debris, samples were centrifuged at 13,000 rpm for 20 minutes at 4°C. Protein concentration was determined using the Pierce BCA protein assay (Thermo Scientific, Waltham MA) according to the manufacturer's protocol. Proteins (50 μ g) were heated to 95°C for 5 minutes and fractionated using 10, 12 and 15% Bis-Tris polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes (BioRad, Hercules, CA) and blocked for 1 hour at room temperature in 5% milk (w/v in 1X PBS-0.1% Tween). Subsequently, membranes were incubated overnight at 4°C in the following primary antibody solutions (final dilution 1:1000): rabbit polyclonal anti-Survivin (Novus, Littleton, CO), rabbit monoclonal anti-XIAP, rabbit monoclonal anti-cIAP1, rabbit monoclonal anti-cIAP2, and rabbit monoclonal anti- β -actin (Cell Signaling Technology, Beverly, MA). Membranes were washed with 1X PBST three times for 15 minutes each then probed for 1 hour at room temperature with goat anti-rabbit secondary antibodies labeled with IRDye 680 LT (LI-COR Biosciences, Lincoln, Nebraska), followed by three 15-minute washes in 1X PBST and imaging using the ODYSSEY infrared imaging system (LI-COR, Biosciences, Lincoln, Nebraska). β -actin was utilized as a loading control. Data are representative of 3-4 independent experiments. Densitometry analyses were performed using ImageJ software (<http://imagej.nih.gov/ij/>).

PCR Analysis

PANC-1 cells were cultured in T25 flasks at 7.0×10^5 cells per flask and treated with 10, 50 and 100 μ M curcumin for 24 and 36 hours. Cells were then trypsinized and RNA isolation

and purification was performed using the Tri-Reagent (Sigma-Aldrich, St. Louis, MO) following the manufacturer's protocol. cDNA conversion was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY). These cDNA products were used in combination with forward and reverse primers (IDT Technologies, San Diego, CA) designed for IAP mRNA (Table 1) and Platinum Taq DNA Polymerase (Life Technologies, Grand Island, NY) for PCR in a MasterCycler Gradient Thermo Cycler (Eppendorf, Hamburg, Germany). The PCR products were detected using a 1% agarose gel containing ethidium bromide and visualized using an Alpha Innotech imager (Protein Simple, Santa Clara, CA). Data are representative of three independent experiments. Densitometry analyses were performed using ImageJ software (<http://imagej.nih.gov/ij/>).

Statistical Analysis

All statistical analyses in this study were performed using one-way ANOVA analysis and a probability of less than a 95% confidence limit ($p < 0.05$) was considered to be significant. Data are presented as mean \pm SEM. Statistical analyses were performed using the Prism (Graphpad) software.

Results

Effect of curcumin on PANC-1 cell viability

PANC-1 cells were cultured in increasing concentrations of curcumin for 24-72 hours and analyzed by AlamarBlue (Fig. 1A) and Trypan blue exclusion (Fig. 1B) viability assays. Curcumin demonstrated a significant dose- and time-dependent inhibitory effect on PANC-1 cell viability when compared with untreated controls. Curcumin concentrations (10uM and 50uM) flanking the IC50 were chosen for further experiments.

Effect of curcumin on PANC-1 cell morphology

Apoptosis is a type of cell death with distinctive morphological features. To qualitatively evaluate the effects of curcumin treatment on PANC-1 cellular morphology, images were acquired using Hoffmann modulation contrast microscopy (Fig. 2). These images illustrate an increase in cell death in a dose- and time-dependent manner. The presence of membrane blebs and cell shrinkage, which are main morphological hallmarks of apoptotic cells, were observed following 50 and 100 μ M curcumin treatment for 24 and 48 hours.

Curcumin detection within treated PANC-1 cells

It has been reported previously that curcumin has excitation and emission spectra of 420 and 470 nm, respectively (65-67). These spectral properties of curcumin were used to detect its presence within PANC-1 cells using spectrophotometric studies and fluorescence microscopy. PANC-1 cells were cultured in the presence of 50uM curcumin for 24 hours and subsequently washed, trypsinized, and lysed in 100% methanol. The emission peak of curcumin-treated cell lysates at 420nm (red) was detected compared to cells not treated with curcumin (green) or blank/methanol-only samples (blue) (Fig. 3A). This peak is identical to the emission spectrum of 50uM curcumin in methanol (data not shown). The intrinsic fluorescence of curcumin was also exploited using fluorescence microscopy to visualize

curcumin content in treated versus untreated PANC-1 cells (Fig. 3B). The DNA dye DAPI was used to visualize nuclei (blue). Curcumin demonstrates a pan-cellular staining pattern (green), with cytoplasmic, nuclear, and nucleolar pools visible by fluorescence microscopy. These results indicate that curcumin is capable of entering PANC-1 cells in vitro and is non-specific in its localization, suggesting multiple mechanisms of cytotoxicity.

Curcumin decreases IAP protein and mRNA expression

To determine the effects of curcumin on Survivin, cIAP1, cIAP2 and XIAP expression, Western blot (Fig. 4A) and reverse-transcription PCR (Fig. 5A) were performed following treatment with 10, 50 and 100uM curcumin. Protein expression of cIAP1, cIAP2 and XIAP were significantly decreased at 50 and 100uM curcumin treatment compared to untreated controls at 24 and 48 hours (Fig. 4B). In addition, Survivin protein expression was significantly decreased after 50uM curcumin treatment for 24 hours and after 50uM or 100uM curcumin treatment for 48 hours. Moreover, 10uM curcumin was sufficient to significantly reduce cIAP2 expression after 48 hours of treatment. Similarly, significant reduction in Survivin, cIAP1, cIAP2 and XIAP mRNA expression was observed at 50 and 100uM curcumin compared to untreated controls at 24 and 36 hours (Fig. 5B).

Discussion

Pancreatic cancer is a deadly disease that causes higher mortality rates annually than other cancer types. Currently, surgical resection is one of the most effective therapeutic approaches for pancreatic cancer. However, pancreatic cancer does not exhibit notable signs or symptoms during early stages of development, making an early diagnosis difficult. For this reason, most patients with advanced non-metastatic or metastatic disease are ineligible for surgery and receive chemotherapy. Unfortunately, the efficacy of chemotherapeutic agents is limited by emerging drug resistance (1-4). Therefore, the pancreatic cancer field is moving toward investigating therapeutic approaches that target crucial mediators of chemoresistance. It has been well established that evasion of cell death is a required event in the development of resistance to chemotherapy (52-53) with this resistance linked to the up regulation of anti-apoptotic proteins such as the inhibitor of apoptosis (IAP) family members (54-55).

Apoptosis is a type of cell death highly dependent on the activation of caspases (cysteine-aspartic proteases) that cause endonuclease-mediated fragmentation of DNA and cellular demise. This series of events is a prerequisite for progressive cellular disassembly into apoptotic bodies that are subsequently consumed by phagocytic cells (56). The IAP family, particularly Survivin, cIAP1, cIAP2 and XIAP, are proteins that have substantial roles in modulating the inactivation of apoptosis (23). While the IAPs have been shown to bind caspases, only XIAP directly inhibits caspases (57). In addition to its role in cell cycle regulation, Survivin is thought to bind active caspases through cofactor proteins such as hepatitis B X-interacting protein (HBXIP) to prevent amplification by cleavage of other procaspase isoforms (58-59). cIAP1 and cIAP2, though capable of binding caspases, have been shown to inhibit apoptosis by interrupting caspase activation through their E3 ubiquitin ligase function (60-62).

The nuclear factor-kappa B (NF- κ B) transcription factor has been found to be constitutively activated in pancreatic cancer (63-64) and is known to regulate key mediators of cancer cell survival, proliferation, angiogenesis, and metastasis (65-68). NF- κ B has been shown to regulate the production of certain IAPs, including Survivin, cIAP1, and XIAP (65-69). Furthermore, NF- κ B activity and IAP expression have been implicated in resistance to Gemcitabine (69-70). Gemcitabine induces an increase in IAP expression in pancreatic cancer cells, particularly Survivin and XIAP, as well as cIAP1 in lung cancer cells (71-74).

Indeed, studies targeting NF- κ B (70, 73, 75-76) or IAPs (73, 77-78) have demonstrated increased sensitivity to Gemcitabine. The increased sensitivity to Gemcitabine following IAP reduction is the rationale for the use of second mitochondria-derived activator of caspases (Smac) mimetics. Smac is an endogenous pro-apoptotic protein transcribed by the *DIABLO* gene. This protein promotes apoptosis by direct interaction and inhibition of XIAP and Survivin proteins. Several Smac mimetics are currently under investigation in clinical trials (14). While these Smac mimetics have shown promising results in preclinical trials in vitro and in vivo, both in the reduction of IAP expression and in re-sensitization to Gemcitabine (79), they have no known effects on NF- κ B expression or activity. Recent studies have demonstrated that dual inhibition of NF- κ B activity and IAP expression may have superior benefits than reducing IAP expression alone. Indeed, dual targeting NF- κ B and XIAP was more effective in re-sensitizing pancreatic adenocarcinoma cells to Gemcitabine therapy than XIAP knockdown alone (73). Thus, the optimal “next step” in the development of a therapeutic strategy for pancreatic cancer involves compounds that target upstream mediators of IAP expression, such as NF- κ B, as well as multiple IAPs simultaneously.

Curcumin, a turmeric derivative, is a candidate for such a therapeutic agent. It has been shown to inhibit pancreatic adenocarcinoma cell proliferation, survival, invasion and angiogenesis in vitro and in vivo (41, 80). In addition, studies by Kunukkamara et al. have demonstrated that curcumin attenuates NF- κ B activation, resulting in decreased production of anti-apoptotic factors, including Survivin and cIAP1, as well as pro-angiogenic and metastatic factors, in MiaPaCa-2-derived xenograft tumors (69). Multiple studies have demonstrated synergistic activity between curcumin and Gemcitabine in pancreatic adenocarcinoma cells (40-42). Interestingly, while XIAP is considered to be the most potent regulator of apoptosis in humans, its levels following curcumin treatment remain to be elucidated. Furthermore, the effect of curcumin on mRNA expression of the IAPs remains to be investigated. This information is essential to understanding whether curcumin’s effects on IAP expression are due to transcriptional regulation or post-translational mechanisms. In this study, we explore curcumin’s effects on protein and mRNA expression of a panel of key IAPs, including Survivin, cIAP1, cIAP2 and XIAP in the pancreatic adenocarcinoma cell line PANC-1. Phase I and II clinical trials have been conducted to evaluate the safety and efficacy of curcumin, alone and in combination with standard Gemcitabine-based chemotherapy (45-48). The major challenge to curcumin’s clinical use is poor bioavailability. A recent Phase I clinical trial was conducted using a novel microparticle-based form of curcumin called Theracurmin in combination with standard Gemcitabine-based chemotherapy (48). This study reported promising results, increasing plasma levels

over those reported in previous clinical trials, despite using approximately 5% of the dose of curcumin used in earlier studies (400mg vs. 8g/day) while inducing minimal toxicity in patients.

While some controversy exists as to the Gemcitabine-sensitivity of the pancreatic adenocarcinoma cell line MiaPaCa-2 (73, 75), PANC-1 cells are generally considered to be resistant to Gemcitabine. Therefore, we investigated the sensitivity of these cells to curcumin in vitro using AlamarBlue and Trypan blue exclusion viability assays. Our results are consistent with those published using other viability assays in PANC-1 cells (81-83), demonstrating dose- and time- dependent reduction in cell viability following curcumin treatment (Fig. 1). In addition, Hoffman modulation contrast microscopy illustrates the morphology of PANC-1 cells following curcumin treatment (Fig. 2). Cells treated with curcumin exhibit features characteristic of apoptotic cell death, including cell shrinkage and membrane blebbing.

To further elucidate the possible mechanisms of action of curcumin in PANC-1 cells, the spectral properties of curcumin (Fig. 3A) were used to determine the intracellular localization of the compound as analyzed by fluorescence microscopy (Fig. 3B). Consistent with the notion that curcumin exerts effects on multiple intracellular signaling pathways (84-87); our results demonstrate that curcumin displays a pan-cellular localization.

To determine the effects of curcumin on IAP protein and mRNA expression, we performed Western blot and RT-PCR analyses on curcumin-treated PANC-1 cells. Our data demonstrate that curcumin reduces protein and mRNA levels of Survivin, cIAP1 and cIAP2 and XIAP (Fig. 4 and 5), with the most marked effects on IAP expression demonstrated by cIAPs 1 and 2 and XIAP. Interestingly, while mRNA expression of Survivin diminishes with increasing curcumin concentrations and incubation times, Survivin's protein level appears to be relatively stable at the highest dose (100uM) and time (48h) evaluated, remaining at approximately 50% of the level in untreated PANC-1 cells. These data suggest that mechanisms exist to stabilize Survivin protein under conditions of curcumin treatment, despite a reduction in Survivin mRNA production. This finding carries heavy implications for resistance to therapy, since Survivin itself has been found to bind and stabilize XIAP, enhancing the latter's caspase-9 inhibiting activity (88). Thus, while curcumin exerts potent effects on upstream (NF-kB-mediated) signaling leading to reduced IAP expression, residual Survivin may represent a key mechanism for evasion of cell death in the context of this therapeutic strategy.

In summary, our data demonstrates for the first time that curcumin is effective in reducing expression of multiple IAPs critical to chemoresistance, both at the mRNA and protein level, resulting in increased cell death in vitro. The ability to modulate multiple members of the IAP family may prove to be a key factor in selecting compounds for further study as selection of individual IAP-specific targeting has been less than effective. Furthermore, we demonstrate that, of these IAPs, Survivin shows the least sensitivity to curcumin-mediated downregulation, suggesting a possible role in resistance to curcumin treatment, a phenomena that we are currently investigating in our laboratory.

Acknowledgments

Sources of Financial Support

This work would have been impossible if not for a generous grant from the Hirshberg Foundation for Pancreatic Cancer Research and the friendship, inspiration and mentoring of Agi Hirshberg. This work was also supported by grants from the National Institutes of Health (NIH) National Center on Minority Health and Health Disparities (NIH-NCMHD 5P20MD001632, 1P20MD006988 and 2R25GM060507). Funding was also obtained from a National Merit Test Bed (NMTB) award sponsored by the Department of the Army under Cooperative Agreement Number DAMD17-97-2-7016 (NRW). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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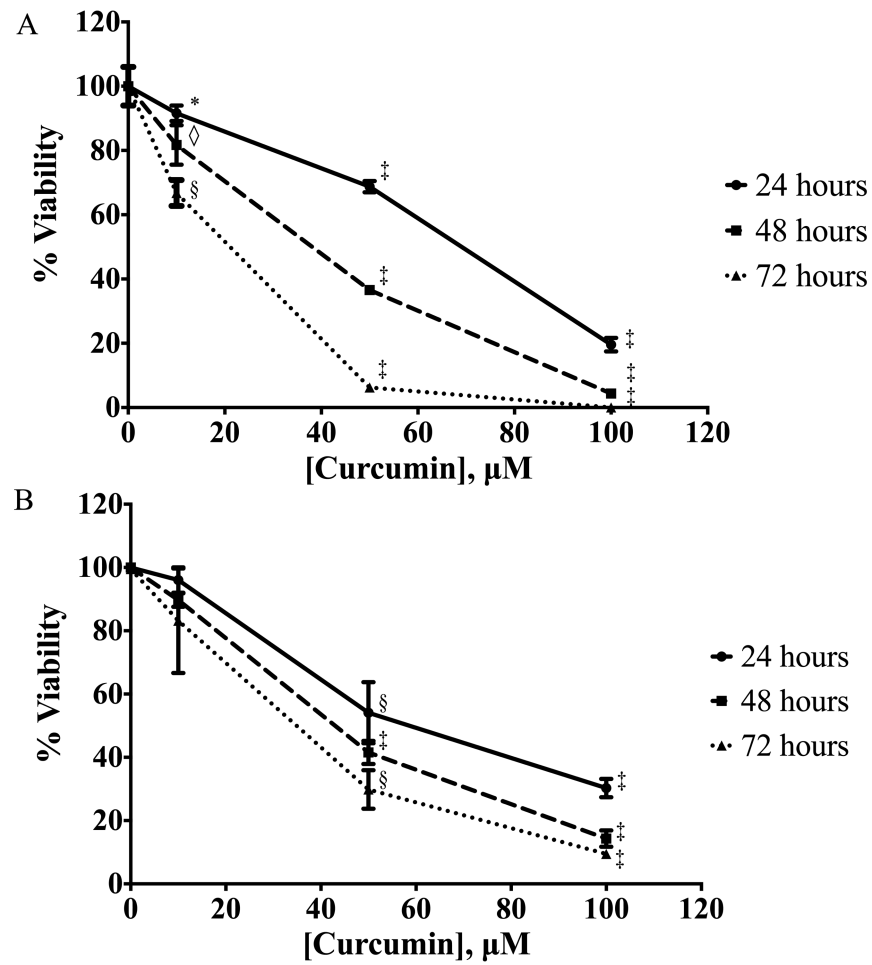


Figure 1. Curcumin induces PANC-1 cell death, analyzed by AlamarBlue and Trypan blue exclusion assays. PANC-1 cells were cultured in medium supplemented with curcumin at increasing doses for 24, 48, and 72 hours followed by (A) AlamarBlue and (B) Trypan blue exclusion viability assays. Data are presented as mean (\pm SEM). * p 0.05, ◇ p 0.01, § p 0.001, † p 0.0001, curcumin treatment versus untreated control.

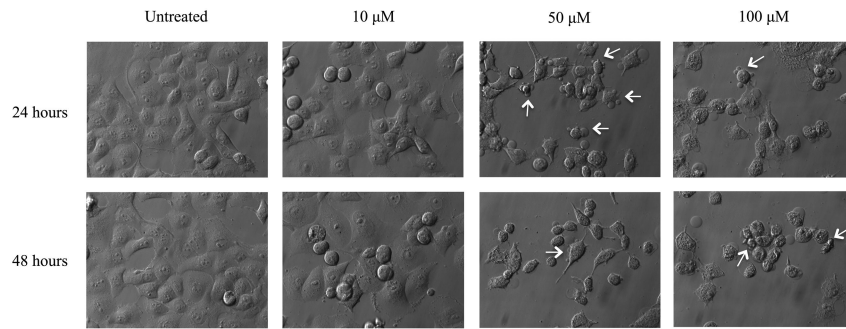


Figure 2. Morphology of curcumin-treated PANC-1 cells, visualized by Hoffman modulation contrast microscopy. PANC-1 cells were cultured in medium supplemented with curcumin at increasing doses for 24 and 48 hours followed by imaging via Hoffman modulation contrast microscopy. White arrows indicate membrane blebs and cell shrinkage, morphological hallmarks of apoptosis. Results depicted in the figure are representative of findings from 3 independent experiments.

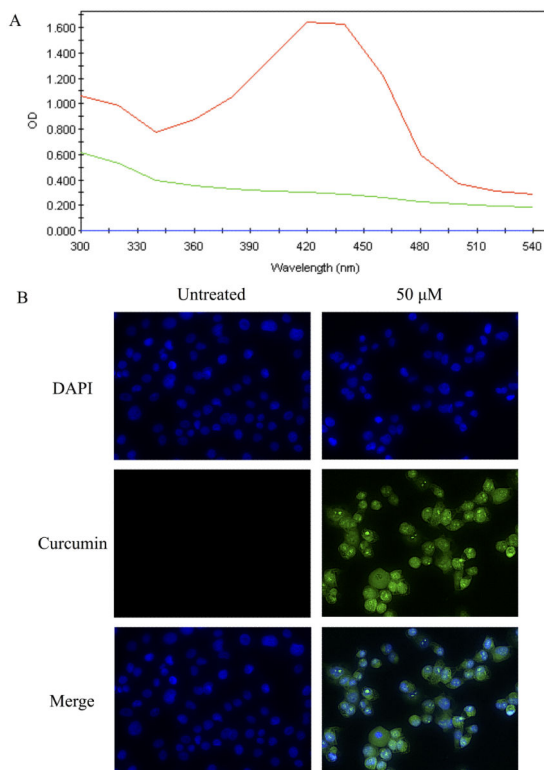


Figure 3. Curcumin in pancreatic cancer cells, analyzed by spectral analysis and fluorescent microscopy. (A) PANC-1 cells were cultured in medium supplemented with 50uM curcumin for 24 hours, trypsinized, lysed in 100% methanol and subjected to spectral analysis on a uQuant spectrophotometer at a 300-540nm wavelength range. Red = curcumin-treated cells; green = untreated cells; blue = methanol as a vehicle control. (B) PANC-1 cells were cultured in medium supplemented with 50uM curcumin (green) for 24 hours and stained with the DNA dye DAPI to show nuclei (blue) followed by imaging via fluorescence microscopy at 40X magnification. Results depicted in the figure are representative of findings from 3 independent experiments.

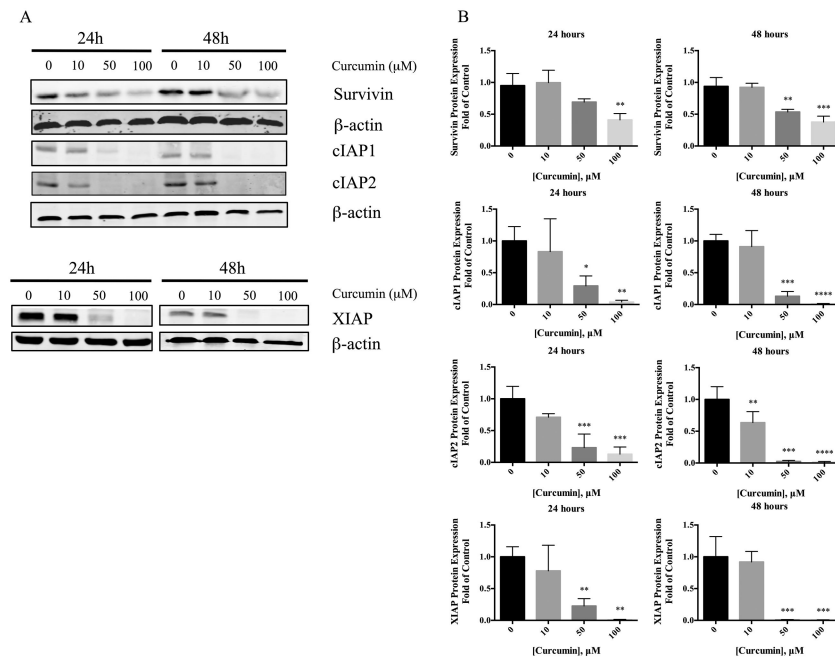


Figure 4. Expression of IAP proteins in PANC-1 cells following curcumin treatment. (A) Cells were cultured in medium supplemented with 10-100uM curcumin for 24 and 48 hours. Whole-cell lysates were prepared and expression levels of Survivin, cIAP1, cIAP2, XIAP and actin proteins were analyzed by Western blot as described in the Materials and Methods. Data are representative of 3-4 independent experiments. (B) Protein levels of IAPs are presented as relative ratios to control cells without curcumin treatment after normalization to B-actin levels. Data are presented as mean (\pm SEM). * $p < 0.05$, $\diamond p < 0.01$, $\S p < 0.001$, $\ddagger p < 0.0001$, curcumin treatment versus untreated control.

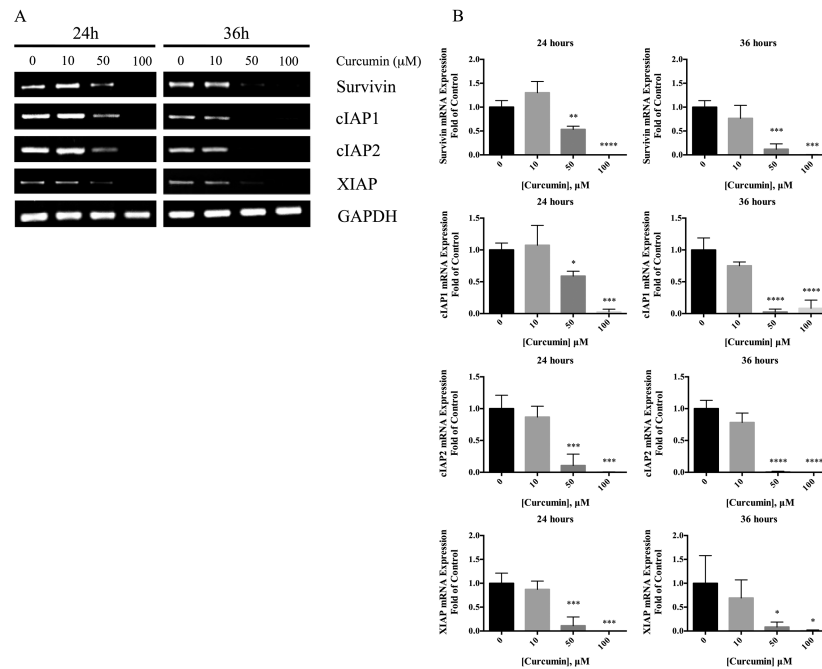


Figure 5.

Expression of IAP mRNA following curcumin treatment in PANC-1 cells. (A) Cells were cultured in medium supplemented with 10-100uM curcumin for 24 and 36 hours. RNA was extracted using the Trizol-chloroform extraction method then converted to cDNA and probed using primers for Survivin, cIAP1, cIAP2, XIAP and GAPDH (Table 1). Data are representative of 3 independent experiments. (B) mRNA levels of IAPs are presented as relative ratios to control cells without curcumin treatment after normalization to GAPDH levels. Data are presented as mean (\pm SEM). * p 0.05, \diamond p 0.01, \S p 0.001, \ddagger p 0.0001, curcumin treatment versus untreated control.

Table 1

Primer sequences targeting IAPs

Name	Primer Sequence
Survivin Fwd	5' – GCA TGG GTG CCC CGA CGT TG – 3'
Survivin Rev	5' – GCT CCG GCC AGA GGC CTC AA – 3'
cIAP1 Fwd	5' – ATT GTG TCA GCA CTT CTT AAT G – 3'
cIAP1 Rev	5' – TTA AGA GAG AAA TGT ACG AAC AGT – 3'
cIAP2 Fwd	5' – TGG AGA AGA CCA TTC AGA AGA T – 3'
cIAP2 Rev	5' – TCA TGA AAG AAA TGT ACG AAC TGT – 3'
XIAP Fwd	5' – ATG ACT TTT AAC AGT TTT GAA GGA – 3'
XIAP Rev	5' – TTA AGA CAT AAA AAT TTT TTG CTT – 3'
GAPDH Fwd	5' – ACG GAT TTG GTC GTA TTG GGC G – 3'
GAPDH Rev	5' – CTC CTG GAA GAT GGT GAT GG – 3'

Primer sequences targeting IAPs. IAP = inhibitor of apoptosis, cIAP1 = cellular inhibitor of apoptosis 1, cIAP2 = cellular inhibitor of apoptosis 2, XIAP = X-linked inhibitor of apoptosis, GAPDH = glyceraldehyde 3-phosphate dehydrogenase, Fwd = forward primer, Rev = reverse primer.