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ORIGINAL RESEARCH ARTICLE

Inhibition of Survival Pathways MAPK and NF-kB Triggers Apoptosis in Pancreatic Ductal Adenocarcinoma Cells via Suppression of Autophagy

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Abstract
Background Pancreatic ductal adenocarcinoma (PDAC) is an aggressive disease with a survival rate of 4–6 months from diagnosis. PDAC is the fourth leading cause of cancer-related death in the Western world, with a mortality rate of 10 cases per 100,000 population. Chemotherapy constitutes only a palliative strategy, with limited effects on life expectancy.
Aims To investigate the biological response of PDAC to mitogen-activated protein kinase (MAPK) and NF-kappaB (NF-kB) inhibitors and the role of autophagy in the modulation of these signaling pathways in order to address the challenge of developing improved medical protocols for patients with PDAC.
Methods Two ATCC cell lines, MIAPaCa-2 and PANC-1, were used as PDAC models. Cells were exposed to inhibitors of MAPK or NF-kB survival pathways alone or after autophagy inhibition. Several aspects were analyzed, as follows: cell proliferation, by [^{3}H]TdR incorporation; cell death, by TUNEL assay, regulation of autophagy by LC3-II expression level and modulation of pro-and anti-apoptotic proteins by Western blot.
Results We demonstrated that the inhibition of the MAPK and NF-kB survival pathways with U0126 and caffeic acid phenethyl ester (CAPE), respectively, produced strong inhibition of pancreatic tumor cell growth without inducing apoptotic death. Interestingly, U0126 and CAPE induced apoptosis after autophagy inhibition in a caspase-dependent manner in MIA PaCa-2 cells and in a caspase-independent manner in PANC-1 cells.
Conclusions Here we present evidence that allows us to consider a combined therapy regimen comprising an autophagy inhibitor and a MAPK or NF-kB pathway inhibitor as a possible treatment strategy for pancreatic cancer.

Key Points

Modulation of autophagy is resposible for failure of pancreatic tumor treatment with expiremental drugs.

The inhibition of survival pathways such as MAPK and NF-kB is not enough to kill pancreatic tumor cells; it must allow the pathway inhibitors to lead tumor cells to apoptotic death.

The implementation of a chemotherapy regimen comprising a MAPK or NF-kB pathway inhibitor in combination with an autophagy inhibitor may improve quality of life for oncologic patients.

1 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer-related death in the Western world. Five percent of patients suffering this aggressive malignant disease of the exocrine pancreas have a survival rate of up to 5 years, but patients generally survive 4–6 month after diagnosis of the pathology. PDAC is associated with a mortality rate of 10 cases per 100,000 population, highlighting the poor prognosis of this condition [1–3] which is due to late diagnosis, the absence of specific biomarkers for early screening, premature

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metastatic dissemination, and most importantly, resistance to systemic therapies.

Response to treatment with agents against target molecules of signaling pathways has been shown to result in feedback activation of anti-apoptotic proteins and other cellular pathways [4, 5]. For example, tumor cell survival may be enhanced by induced or constitutively activated mitogen-activated protein–extracellular signal (MAPK-ERK), phosphatidylinositol 3-kinase (IP-3 k) or NF-kB pathways. Pancreatic tumors show a high level of Ras mutation, which in turn is responsible for the constitutive activation of MAPKs [6]. NF-kB factor has also been found constitutively activated in the nucleus of pancreatic adenocarcinoma cells [7], leading to an incremental increase in anti-apoptotic signals. Taking this into consideration, a large number of preclinical assays support the strategy of combining inhibitors of MAPK–ERK and PI3K [8–10] or NF-kB in order to enhance apoptotic death [11].

The inhibitors of apoptosis proteins (IAPs) are a group of anti-apoptotic factors that render cancer cells insensitive to apoptotic stimuli [12–14]. To date, eight members the IAP family have been identified in mammals, including cellular IAP1 (cIAP1), cIAP2, X-linked inhibitor of apoptosis (XIAP), IAP-like protein 2 (ILP2), neuronal apoptosis inhibitory protein (NAIP), livin, survivin and BIR-repeat-containing ubiquitin-conjugating enzyme (BRUCE) [15–17]. As cytoprotective factors, IAPs function as regulators of cellular homeostasis and are involved in cell division, metabolism, and the activation of multiple intracellular signaling pathways, including TGF-β, NF-κB and c-Jun *N*-terminal kinase (JNK) [15]. The overexpression of IAPs is highly correlated with cancer progression and resistance to chemotherapy, and is associated with a poor prognosis [18].

Over the last few years, several authors have demonstrated that autophagy plays an important role in the modulation of sensitivity to anti-cancer drugs [19]. Autophagy is an evolutionarily conserved process whereby cytoplasm components and organelles are degraded in lysosomes [19]. This ubiquitous process involves the recycling of cellular components to sustain metabolism and avoid the accumulation of damaged proteins and organelles during stress conditions [20]. Autophagy offers protection against various pathological conditions, including infection [21, 22], neurodegeneration [23, 24] and tumor development [25, 26]. In contrast, autophagy can also protect cells against several pro-apoptotic stimuli [27]. While it is not clear how autophagy prevents cells from undergoing apoptosis, one possible mechanism involves the sequestration of damaged mitochondria [27], preventing released cytochrome c from forming a functional apoptosome in the cytoplasm. We recently demonstrated that autophagy induction by gemcitabine protected PDAC form the pro-apoptotic effects of this chemotherapeutic agent [28]. The inhibition of this process sensitizes PDAC cells to the effects of gemcitabine, leading to apoptotic death [28].

In this study, we investigated the biological response of PDAC to MAPK and NF-kB inhibitors and the role of autophagy in the modulation of these signaling pathways. We demonstrated that the inhibition of autophagy allowed PDAC cells to enter into the apoptotic process induced by U0126 and caffeic acid phenethyl ester (CAPE), inhibitors of the MAPK and NF-kB pathways. We also provided evidence of a relationship between survivin and autophagy in PDAC, although this interaction requires future intensive study to be completely elucidated.

2 Materials and Methods

2.1 Materials

U0126, Z-VAD-FMK, 3-methyladenine (3-MA), and concanamycin A1 were purchased from Sigma-Aldrich (St. Louis, MO, USA); CAPE was purchased from Calbiochem (San Diego, CA, USA). 3-MA solutions were prepared in Dulbecco's modified Eagle's medium (DMEM) at the time of use. Stock solutions of concanamycin A1, U0126, zVAD-FMK, and CAPE were prepared in dimethyl sulfoxide (DMSO). DMEM, penicillin, and streptomycin were purchased from Invitrogen (Invitrogen Argentina S.A., Buenos Aires, Argentina). Fetal bovine serum (FBS) was purchased from Natocor Laboratories (Córdoba, Argentina).

2.2 Cell Culture and Viability

MIAPaCa-2 (ATCC) and PANC-1 (ATCC) cells were cultured in DMEM containing 10 % heat-inactivated FBS, 2 mM L-glutamine, 20 mM HEPES buffer, 100 mg/ml penicillin, and 150 mg/ml streptomycin. Cell viability was determined by trypan blue exclusion.

2.3 Cell Growth Inhibition Assay

Sensitivity of the cell line to increased doses of U0126 or CAPE was determined by culturing 5×10^4 cells/ml at 37 °C in a 5 % CO2 atmosphere for 24 and 48 h, pulsed with 1 μCi [^{3}H]TdR (DuPont/NEN Products, Boston, MA, USA) for the final 18 h. The cultures were performed in 96-well round-bottom microtitre plates in the presence of different concentrations of U0126 (1.25–20.0 μM) or CAPE (6.25–50.0 μg/ml). After incubation, cells were harvested using a semiautomatic method. Incorporated [^{3}H]TdR was measured in a liquid scintillation beta counter, (Beckman/PerkinElmer, Waltham, MA, USA). Results were calculated from the mean counts per minute (cpm) of [^{3}H]TdR incorporated in triplicate cultures. The percentage of growth inhibition was calculated as:

$$\%growth\,inhibition = \frac{100 - cpm\,treated\,cells}{cpm\,basal\,control}$$

Untreated cells used as basal control represent 0 % inhibition. Cell viability at the beginning of the experiment was higher than 95 %, as assessed by trypan blue exclusion. Each experiment was carried out at least three times, with similar results.

2.4 Apoptotic Assessment

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was carried out as follows. Cells were incubated alone in DMEM 10 % FBS with 3-MA and/or U0126 or CAPE for 72 h. Cells were then resuspended and washed once with ice-cold phosphate-buffered saline (PBS) and fixed in 4 % buffered paraformaldehyde. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling was carried out using the DeadEnd Fluorometric TUNEL System kit (Promega Corporation, Madison, WI, USA) following the manufacturer's recommendations. Images from triplicate samples were recorded at 200× magnification. The images were processed using the Image-Pro Plus 6.2 (Media Cybernetics, Bethesda, MD, USA) software program. A minimum of 500 cells were counted for each condition. Cells with pyknotic nucleus and dark green fluorescent staining were scored as positive. The percentage of positive cells was calculated as follows:

$$\%positive\,cells = \frac{number\,of\,green-fluorescent\,cells}{number\,of\,DAPI-stained\,cells} \times 100$$

2.5 Total Protein Extracts

Cells ($1x10^7$) were lysed with a hypotonic buffer (20 mM Tris pH 8.0, 150 mM NaCl, 100 mM NaF, 10 % glycerol, 2 % Nonidet P-40) and the protease inhibitor cocktail P8340 from Sigma-Aldrich for 30 min at 4 °C followed by centrifugation at 13,000×g for 30 min. The extracts were then stored at −70 °C for further use. Protein concentration was determined by Bradford assay. For Western blotting, equal amounts of protein were loaded into each well and separated by SDS-PAGE gel, then transferred onto PVDF membranes (GE Healthcare, Little Chalfon, UK), and the membranes were blocked by the use of 3 % nonfat dry milk in PBS overnight at 4 °C. The membranes were then incubated with antibodies to LC3 (Cell Signaling Technologies, Danvers, MA, USA), PARP, Bcl-XL, survivin, Bax, Bad, pro-caspase 3 and β-actin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4 °C. Donkey anti-goat IgG secondary antibody and goat anti-rabbit IgG secondary antibody (1:8,000, Santa Cruz Biotechnology, Inc.) were incubated for 1.5 h at 37 °C. Immunoblots were analyzed using a chemiluminescent detection system (Western blotting luminol reagent, Santa Cruz Biotechnology, Inc.). Autoradiography images were obtained using a digital camera (Olympus D-510 Zoom; Olympus Corporation, Tokyo, Japan) and were subjected to densitometry analysis using Image Scion software.

2.6 Statistical Analysis

All data is presented as the mean±SD from at least three independent experiments. Statistical analysis was performed with GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA), using one-way analysis of variance followed by Dunnett or Bonferroni tests. Statistically significant differences were as follows: *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

3 Results

3.1 U0126 and CAPE Strongly Inhibit Pancreatic Tumor Cell Growth

Pancreatic tumors are known to exhibit overexpression in several survival pathways. Two of these are the MAPK and NF-kB pathways, whose role in tumor maintenance is not completely understood. In the present study, we first explored whether treatment with U0126, an inhibitor of MEK1/2, or CAPE, an inhibitor of the binding of NF-kB to its gene promoters, resulted in a reduction in MIAPaCa-2 and PANC-1 cell proliferation as assessed by [3H]-thymidine incorporation. Figure 1 shows that both agents had an inhibitory effect on cell growth after 24 and 48 h of treatment. The maximum inhibitory effect by treatment with U0126 was 64.7±3.1 % and 39.3±9.0 % for MIAPaCa-2 and PANC-1, respectively ($p<0.001$), after 24 h. The highest inhibition of cell proliferation was observed after 48 h of treatment, with inhibition of 92.0±8.7 % for MIAPaCa-2 and 54.7±10.1 % for PANC-1 cell lines ($p<0.001$). In addition, CAPE inhibited cell growth at a rate of 53.0±18.3 % and 64.3±5.0 % for MIAPaCa-2 and PANC-1, respectively ($p<0.001$) after 24 h. The highest inhibition of cell proliferation was observed after 48 h of treatment, with growth inhibition of 65.7±6.4 % and 93.0±2.0 % for the MIAPaCa-2 and PANC-1 cell lines, respectively ($p<0.001$).

The results for exposure of both cell lines to different doses of U0126 and CAPE for 24 and 48 h were time- and dose-dependent. Maximum cell growth inhibition was reached with the highest doses tested.

These results revealed that both agents, U0126 and CAPE, were able to reduce the rates of MIAPACa-2 and PANC-1 proliferation through significant inhibition of this process.

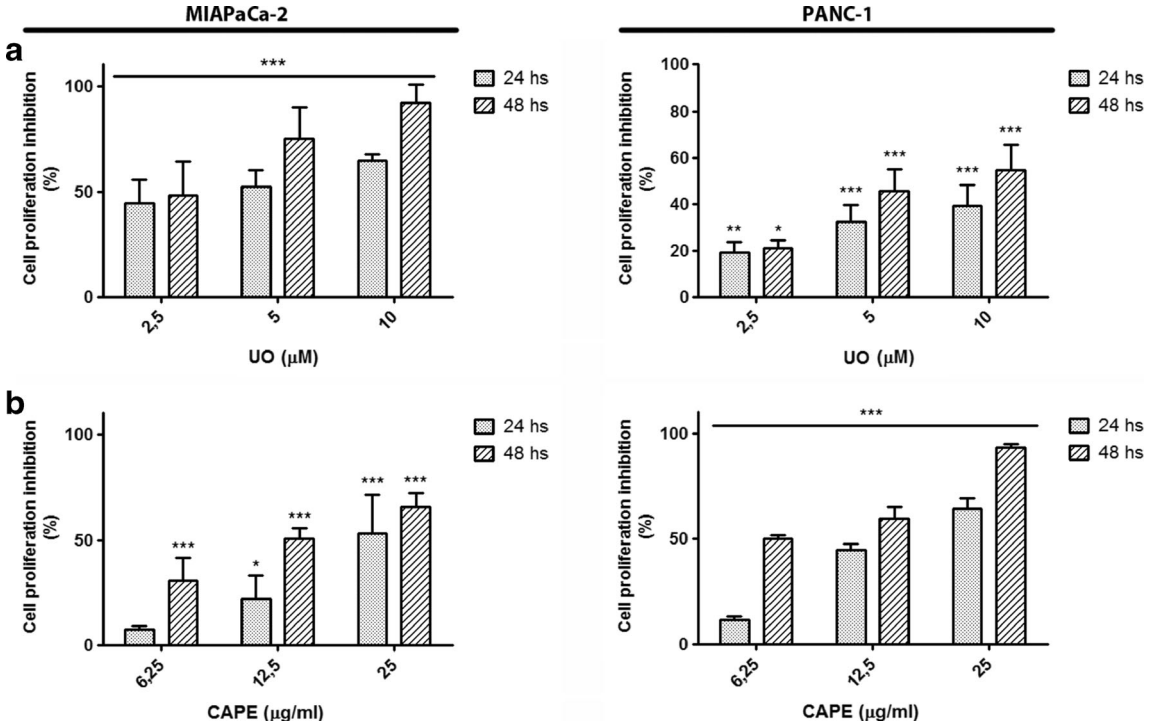

Fig. 1 U0126 and CAPE induce inhibition of cell proliferation. MIAPaCa-2 and PANC-1 cells were exposed in vitro to different concentrations of U0126 (**a**) and CAPE (**b**) for 24 and 48 h. The inhibition of cell proliferation was calculated by measuring [^{3}H]TdR incorporation. *Bars* represent the mean±SD of at least three independent experiments (*p<0.05; **p<0.01 and ***p<0.001)

3.2 Experimental Drugs U0126 and CAPE Are Poor Inducers of Apoptosis

In order to evaluate whether U0126 and CAPE were able to induce apoptosis tumor cell death, we analyzed DNA fragmentation by TUNEL assays in MIAPaCa-2 and PANC-1 cells.

U0126 produced TUNEL-positive cells in 15.0±1.9 % of MIAPaCa-2 cells and 33.0±5.1 % of PANC-1 cells after 72 h of treatment (Fig. 2a). CAPE produced TUNEL-positive cells in 31.4±2.6 % and 16.7±3.2 % of MIAPaCa-2 and PANC-1 cells, respectively, over the same time frame (Fig. 2a).

We then evaluated the expression levels of the pro-apoptotic proteins Bax and Bad and the anti-apoptotic proteins Bcl-XL and survivin by Western blot. We observed that the treatment of MIAPaCa-2 cells with 10 μM of U0126 produced a decrease in intracellular levels of Bcl-XL and an increase in the Bax/Bcl-XL ratio. Despite the diminished levels of Bad, however, the Bad/Bcl-XL ratio was not significantly affected. At the same time, survivin was increased more than 1.5-fold by treatment with U0126.

CAPE at a dose of 25 μg/ml induced a pronounced reduction in Bcl-XL levels and a consequent incremental increase in the Bax/Bcl-XL ratio, even though Bax levels were not modified. A similar situation was observed for the pro-apoptotic protein Bad. CAPE had no affect on Bad levels, but because of the marked decrease in Bcl-XL, the Bad/Bcl-XL ratio increased. The treatment also increased the levels of survivin (Fig. 2b panel I).

The treatment of PANC-1 cells with U0126 induced a reduction in the levels of Bax and Bcl-XL. There was no change, however, in the Bax/Bcl-XL ratio, while the Bad/Bcl-XL ratio increased incrementally, along with survivin levels.

CAPE produced a decrease in Bcl-XL levels, with no change in Bax levels, although the Bax/Bcl-XL ratio increased. The treatment of PANC-1 cells with this agent also resulted in increased levels of Bad and survivin and an increase in the Bad/Bcl-XL ratio (Fig. 2b panel II).

3.3 U0126 and CAPE Inhibit the Autophagy Process in Pancreatic Tumor Cells

The role of autophagy in cancer is controversial. In some cases, this process acts as a mechanism that cooperates with cell death, but in most cases it constitutes a mechanism of resistance to death. We previously described autophagy as the process responsible for the lack of response of pancreatic tumor cells to gemcitabine, a common chemotherapeutic agent used for the treatment of pancreatic cancer [28]. In the present work, we investigated whether autophagy was involved in the survival of pancreatic cancer cells exposed to U0126 or CAPE. We began by analyzing the capability of both drugs to modulate the process of autophagy.

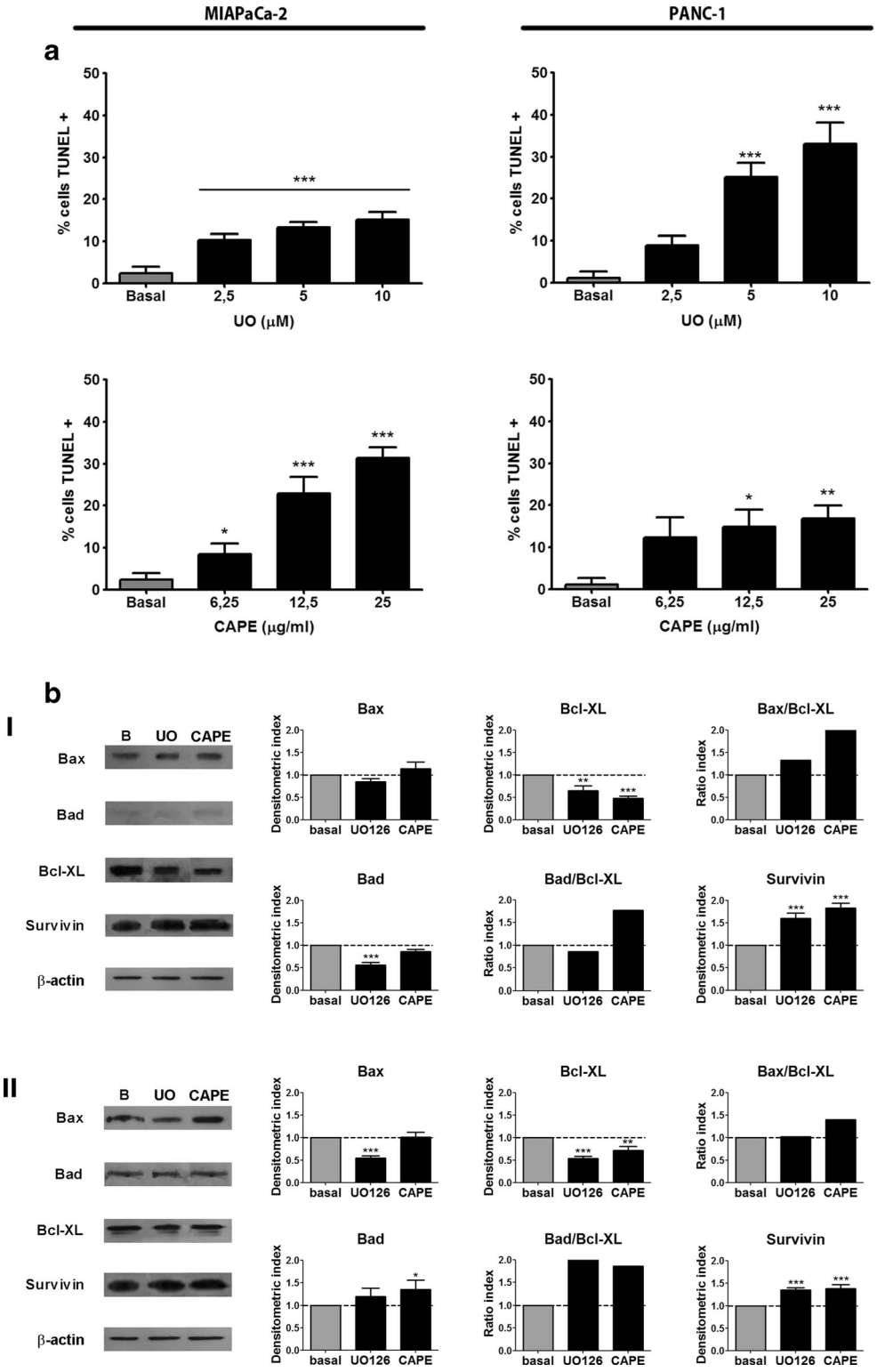

Fig. 2 U0126 and CAPE are poor inducers of apoptotic cell death. Apoptosis was assessed by evaluation of TUNEL assay. **a** Cells were exposed to different concentrations of U0126 and CAPE for 72 h. *Bars* represent the mean±SD of at least three independent experiments. **b** Modulation of pro- and anti-apoptotic proteins. Analysis of intracellular levels of Bax, Bad, Bcl-XL and survivin by Western blot of MIAPaCa-2 cells (I) and PANC-1 cells (II) under basal conditions or after treatment with 10 μM of U0126 or 25 μg/ml of CAPE, after 48 h of treatment. β-actin was used as a loading control (*$p<0.05$; **$p<0.01$ and ***$p<0.001$)

First, we explored the presence of autophagosomes in our two pancreatic tumor cell lines by monitoring the formation of the autophagosome-specific protein LC3. LC3 was present in two forms: LC3-I, the cytosolic form, and LC3-II, the membrane-bound form. When autophagy was induced, LC3-I was covalently conjugated from phosphatidylethanolamine to form LC3-II. LC3-II is specifically targeted to phagophore and remains associated with the expanding limiting membrane, sealed autophagosomes, and mature autophagosomes/

autolysosomes [29]. In addition, it is important to determine whether U0126 and CAPE modulate autophagosome synthesis and/or degradation, by comparing the cleavage of LC3-I to LC3-II by the agents alone or in combination with concanamycin A1, as we previously described [28], following the recommendations of Dr. Rubinsztein and colleagues [30]. To study the effect of U0126 and CAPE on the process of autophagy, we measured the LC3-II levels in the presence of each of these drugs, with and without concanamycin A1, a proton pump inhibitor. For this step, pancreatic cancer cells were exposed to 10 μM of U0126 or 25 μg/ml of CAPE for 24 h and/or 50 nM of concanamycin A1 for 6 h. The levels of LC3-II obtained by treatment of MIAPaCa-2 and PANC-1 cells with U0126+concanamycin A1 or CAPE+concanamycin A1 were lower than those with treatment by concanamycin A1, indicating that both drugs induced inhibition of autophagosome synthesis. In addition, U0126 produced a decrease in LC3-II levels relative to basal conditions of culture in PANC-1 cells, indicating induction of autophagosome degradation (Fig. 3a). We also determined modulation of Beclin-1 and Bcl-XL levels, and evaluated whether U0126 and CAPE were able to modulate the autophagy process. First, we analyzed the modulation of Beclin-1 and Bcl-XL. U0126 produced a decrease of both Beclin-1 and Bcl-XL in MIAPaCa-2 and PANC-1 cells. However, the Beclin-1/Bcl-XL ratio was reduced only in the MIAPaCa-2 cell line (Fig. 3b). CAPE had no effect on Beclin-1 levels in MIAPaCa-2, while Bcl-XL was profoundly decreased, resulting in an elevated Beclin-1/Bcl-XL ratio. CAPE produced a decrease in Beclin-1, Bcl-XL, and the Beclin-1/Bcl-XL ratio (Fig. 3b).

Taken together, these results demonstrate inhibition of autophagy-sensitized tumor cells by the pro-apoptotic effect of both U0126 and CAPE treatment.

3.4 U0126 and CAPE Induce Apoptosis After Autophagy Inhibition

At this time, we evaluated whether this process conferred a protective role to pancreatic tumor cells against the effects of U0126 and CAPE. To do so, we analyzed the percentages of apoptotic cells by treatment with U0126 or CAPE 1 h after treatment with 10 mM 3-MA, an autophagy inhibitor. The experiments were carried out by analyzing the oligonucleosomal fragmentation by TUNEL assay. The percentage of TUNEL-positive cells obtained after incubation with U0126 at a dose of 10 μM was 15.0±1.9 % for MIAPaCa-2 and 33.0±5.1 % for PANC-1 cells. After pre-treatment with 3-MA, the proportion of TUNEL-positive cells increased to 29.6±3.5 % and 45.9±3.9 % for MIAPaCa-2 and PANC-1, respectively (Fig. 4).

CAPE, at a dose of 25 μg/ml, produced TUNEL-positive levels in 31.4±2.6 % of MIAPaCa-2 cells. When cells were pre-treated with 3-MA, percentages increased to 42.9±2.6 %. A similar experiment with PANC-1 cells showed that CAPE induced TUNEL-positive levels in 16.7±3.2 % of cells, while after pre-incubation with 3-MA, the proportion rose to 37.2±4.1 % (Fig. 4).

Lastly, we analyzed the modulation of the pro-apoptotic proteins Bax, Bad, and pro-caspase 3 and the cleavage of PARP, and the anti-apoptotic proteins Bcl-XL and survivin expression, using Western blotting. U0126 induced a small increase in the Bax/Bcl-XL ratio. No changes were observed in the Bad/Bcl-XL ratio despite reduced Bad expression. U0126 also induced an incremental increase in survivin and cleavage of PARP and a reduction in pro-caspase 3 in MIAPaCa-2 cells. When these cells were pre-treated with 3-MA, the Bax/Bcl-XL and Bad/Bcl-XL ratios were notably increased, and the cleavage of PARP and survivin levels decreased (Fig. 5a). CAPE produced a marked reduction in Bcl-XL levels and an increase in the Bax/Bcl-XL and Bad/Bcl-XL ratios, and reduced the level of pro-caspase. However, CAPE induced high expression of survivin. When cells were pre-treated with 3-MA, the Bax/Bcl-XL and Bad/Bcl-XL ratios rose even higher, but in this case, survivin levels were markedly decreased, along with an incremental increase in PARP cleavage (Fig. 5a).

Similar experiments were performed on PANC-1 cells. In this case, U0126 increased the Bad/Bcl-XL ratio, while no variation was observed in the Bax/Bcl-XL ratio. Bcl-XL levels decreased and survivin levels were incrementally increased with U0126 treatment (Fig. 5b). Pre-treatment with 3-MA stimulated the pro-apoptotic effect of U0126, increasing the Bax/Bcl-XL ratio and reducing survivin levels.

Although CAPE inducing an incremental increase in the Bax/Bcl-XL ratio in PANC-1 cells, this ratio increased markedly when cells were pre-treated with 3-MA. The Bad/Bcl-XL ratio increased with treatment by both CAPE and 3-MA+CAPE. The increase in survivin levels produced by CAPE were reversed by pre-treatment with 3-MA. In this cell line, however, no changes were observed in pro-caspase 3 levels or PARP cleavage (Fig. 5b).

3.5 U0126 and CAPE Induce Caspase-Dependent Apoptosis in MIAPaCa-2 Cells and Caspase-Independent Cell Death in PANC-1 Cells

We further investigated the mechanism by which U0126 and CAPE induce apoptotic cell death when autophagy is inhibited. TUNEL assays were used to assess whether Z-VAD-FMK, an inhibitor of pan-caspases, was able to prevent the low pro-apoptotic effect of both drugs and the large effect caused by 3-MA+U0126 or 3-MA+CAPE. Figure 6a shows

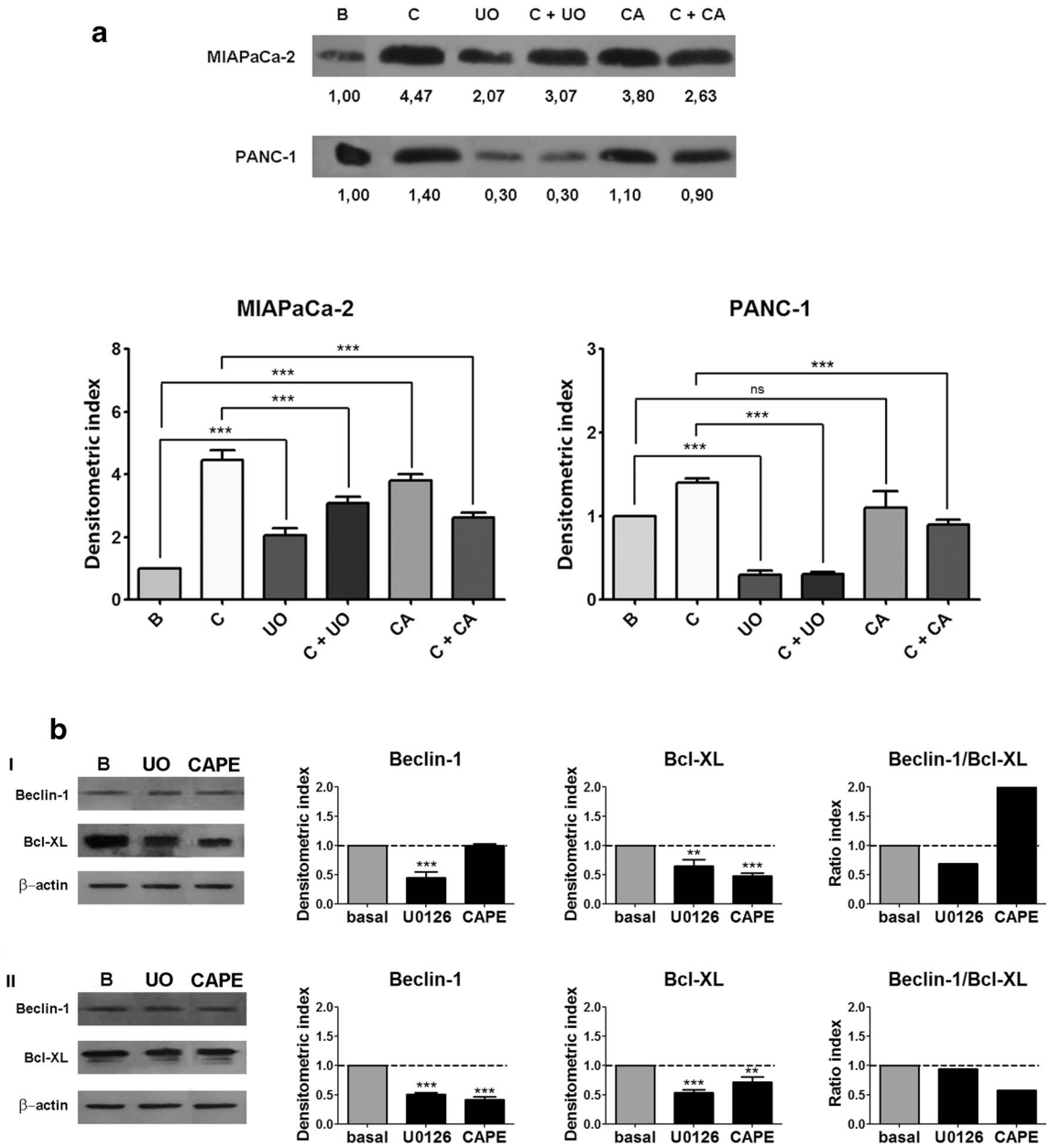

Fig. 3 U0126 and CAPE modulate autophagy. **a** Western blot analysis of endogenous LC3-II levels under basal conditions ("B"), after treatment with 50 nM of concanamycin for 6 h ("C"), after treatment with 10 μM of U0126 for 24 h ("U0"), after treatment with concanamycin+U0126 ("C+U0"), by treatment with 25 μg/ml of CAPE for 24 h ("CA"), and after treatment with concanamycin+CAPE ("C+CA"). **b** Analysis of intracellular levels of Beclin-1 and Bcl-XL by Western blot. MIAPaCa-2 (I) and PANC-1 (II) cells were incubated under basal conditions with 10 mM of 3-MA (3-MA), or treated with 10 μM of U0126 or 25 μg/ml of CAPE. Blots show one representative experiment from among a total of at least three. *Bars* represent the mean±SD of at least three independent experiments. β-actin was used as a loading control (*ns*: not significant; $^{**}p<0.01$ and $^{***}p<0.001$)

that, for all treatments, the pro-apoptotic effect was prevented by pre-incubation with 100 μM Z-VAD-FMK in MIAPaCa-2 cells. In contrast, Z-VAD-FMK was unable to prevent this effect in PANC-1 cells (Fig. 6b). These results suggest that U0126 and CAPE can induce caspase-dependent apoptosis in MIAPaCa-2 cells regardless of whether autophagy is inhibited, but only in a caspase-independent manner in PANC-1 cells.

4 Discussion

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive disease with a survival rate of 4–6 month from the initial diagnosis of the pathology, and it is the fourth leading cause of cancer-related death in the Western world [1–3]. For the majority of patients with PDAC, only palliative therapeutic strategies are available, with limited effects on life expectancy

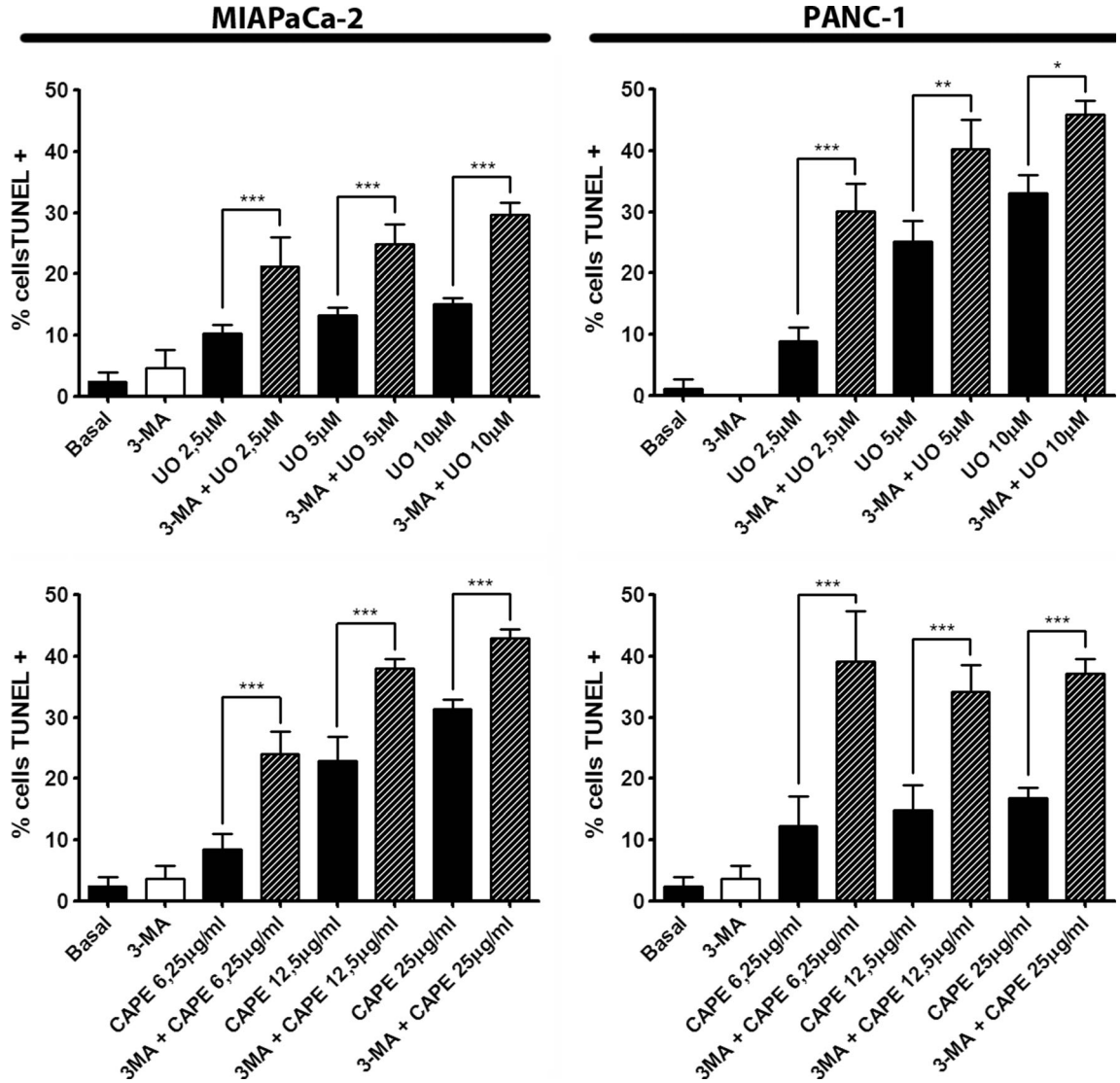

Fig. 4 U0126 and CAPE induce apoptotic cell death more efficiently when autophagy is inhibited. Cells were incubated with 10 mM of 3-MA and/or U0126 ("U0") or CAPE and analyzed for TUNEL-positive cells after 72 h of treatment. *Bars* represent the mean±SD of at least three independent experiments (*$p<0.05$; **$p<0.01$; ***$p<0.001$)

[31, 32]. As such, determining the molecular basis of therapy resistance and the weak points in the biological mechanisms of cell survival represents an important challenge in improving medical protocols among patients with PDAC. In this context, several authors have identified mutations in the KRAS oncogene in a majority if not all PDAC tumors [33, 34], implicating the downstream mitogen-activated protein kinase (MAPK) pathway as a key target for therapeutic intervention in this disease, along with KRAS-mutant subsets of non-small cell lung cancer and colorectal cancer patients [35]. The inhibition of the MAPK pathway alone or in combination with chemotherapy showed promising efficacy in a mutant KRAS-driven genetically engineered mouse model of non-small cell lung carcinoma, which translated into a positive clinical outcome [36, 37]. However, clinical and preclinical studies recently demonstrated the existence of feedback loops that lead to intrinsic and acquired resistance to MAPK pathway inhibitors, frequently involving activation of the phosphoinositol-3 kinase (PI3K) pathway [38]. NF-kB is another downstream effector of oncogenic Ras [39–41]. This transcription factor is constitutively activated in primary pancreatic adenocarcinomas and pancreatic cancer cell lines [39]. Down-regulation of NF-kB with curcumin, a nontoxic phytochemical, constitutes an effective management strategy in patients with pancreatic carcinoma [42]. Several authors have demonstrated that NF-kB regulates the expression of a number of genes whose products are involved in tumorigenesis [43–47], including anti-apoptotic genes (e.g., survivin, XIAP, TRAF, cFLIP, Bfl-1, Bcl-2, and Bcl-xl), inflammatory genes (VEGF, COX-2 and MMP-9), and genes encoding chemokines, adhesion molecules, and cell cycle regulatory genes (e.g., cyclin D1 and c-Myc). Digitoflavone, a common

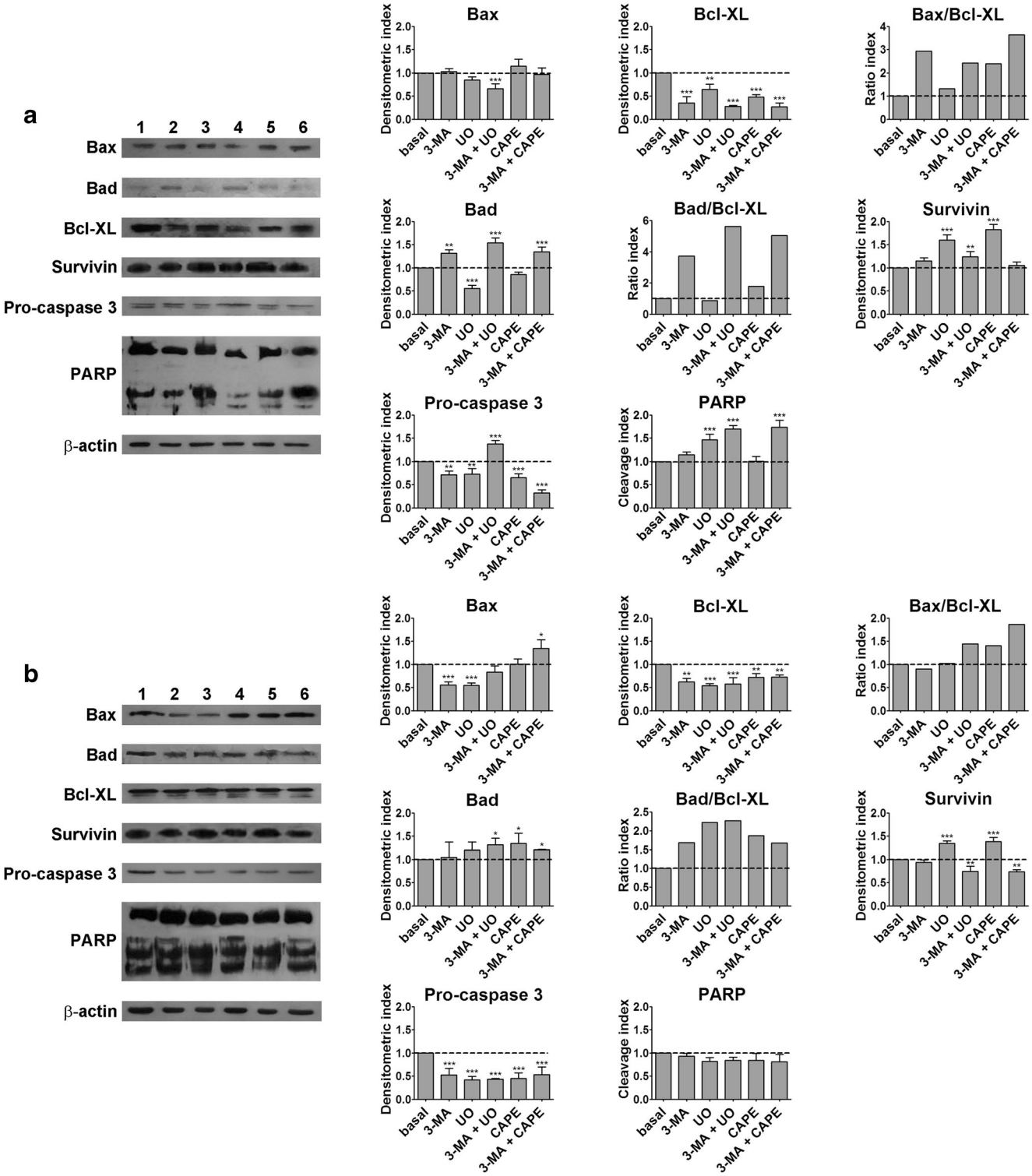

Fig. 5 Modulation of pro- and anti-apoptotic proteins. Analysis of intracellular levels of the pro-apoptotic proteins Bax, Bad, and pro-caspase, and the cleavage of PARP, and the anti-apoptotic proteins Bcl-XL and survivin, by Western blot of MIAPaCa-2 cells (**a**) and PANC-1 cells (**b**) under basal conditions after treatment with 10 mM of 3-MA, 10 μM of U0126 ("U0"), 25 μg/ml of CAPE, or combinations of 3-MA with U0126 or CAPE for 48 h. Blots show one representative experiment from among at least three. *Bars* represent the mean±SD of at least three independent experiments. β-actin was used as a loading control (*p<0,05; **p<0.01 and ***p<0.001)

flavonoid present in many types of plants including fruits, vegetables, and medicinal herbs, was recently described as exhibiting anticancer properties associated with the induction of apoptosis and inhibition of cell proliferation, metastasis,

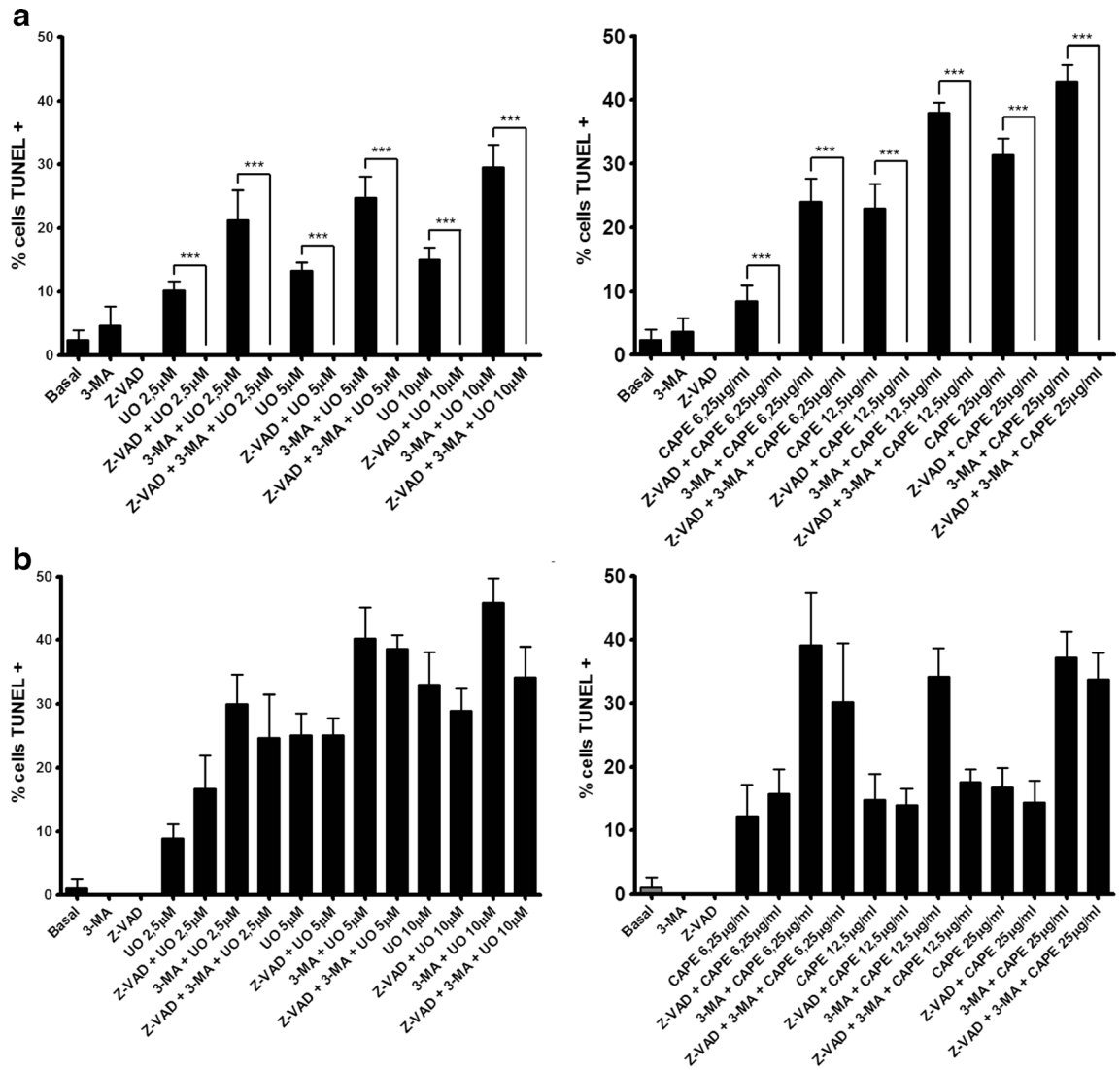

Fig. 6 Apoptotic death characterization. MIAPaCa-2 (**a**) and PANC-1 (**b**) tumor cells were incubated with 10 mM of 3-MA, 100 μM of the pan-caspase inhibitor Z-VAD-FMK, 2.5–10 μM of U0126 ("U0"), 6.25–25 μg/ml of CAPE, or the various combinations thereof as shown in the figure, for 72 h. *Bars* represent the mean±SD of at least three independent experiments (***$p<0.001$)

and angiogenesis [48]. Digitoflavone significantly sensitized cells to TNFα-induced apoptosis in a number of human pancreatic cancer cell lines. Such sensitization is closely associated with the inhibition of NF-kB activation [49].

In this study, we first investigated the biological response of PDAC to MAPK and NF-kB inhibitors. We demonstrated strong inhibition of cell growth with the inhibition of both the MAPK and the NF-kB pathways. To accomplish this, we selected U0126 as an inhibitor of MEK1/2 and CAPE, an active polyphenolic compound of propolis from honeybee hives that inhibits the NF-kB pathway [50]. These compounds, however, were not able to induce apoptotic cell death. Even at high doses, apoptotic cells accounted for just 15.0±1.9 % and 33.0±5.1 % of MIAPaCa-2 and PANC-1 cell lines, respectively, after 72 h of treatment with 10 μM of U0126, and 31.4±2.6 % and 16.7±3.2 % for MIAPaCa-2 and PANC-1 cells, respectively, after 72 h of treatment with 25 μg/ml of CAPE (Fig. 2a). Surprisingly, both compounds increased the levels of the anti-apoptotic protein survivin, as shown in Fig. 2b. Survivin is a member of the IAP family (inhibitor of apoptosis proteins), and is highly expressed in cancer cells while virtually absent in most differentiated normal tissue [51, 52]. Expression levels of survivin have been seen to correlate with poor prognosis in several neoplasia, including multiple myeloma and colorectal, non-small cell lung, prostate, and breast cancer [53–57]. In this work, we present evidence of a link between the process of autophagy and the modulation of survivin in PDAC. We observed that U0126 and CAPE increased the levels of survivin, and the inhibition of autophagy with 3-MA restored basal levels of this anti-apoptotic protein in MIAPaCa-2 cells. Even more significantly, survivin expression decreased below basal levels in PANC-1 cells.

Little evidence exists at this time regarding the possible modulation of autophagy by survivin. Cheng and collaborators recently showed that the inhibition of survivin by YM155 modulated autophagy in a model of tamoxifen-resistant breast cancer. They demonstrated that YM155 induced autophagy by increasing LC3-II expression and the number of autophagosomes [58]. Nothing is known about this effect on PDAC. Moreover, no data have been published regarding the possible modulation of survivin by autophagy. As such, extensive research is needed in order to understand this phenomenon and its relationship with the survival/death signaling balance in tumor cells.

In this work, we demonstrated that both U0126 and CAPE inhibited the autophagy process in PDAC cells. Several authors have published similar results in other tumor models. Wang and collaborators showed that induction of autophagy was dependent on MEK/ERK activation in H4IIE rat hepatoma and human erythroleukemia K562 cells [59]. In other examples, the constitutively activated MEK/ERK pathway is associated with high levels of autophagy, and thus inhibition of this signaling pathway correlates with down-regulation of autophagy in the HT29 human colorectal cancer cell line [60] and the HCT-15 human colon adenocarcinoma cell line [61]. Similarly, cross-modulation between the NF-kB pathway and the autophagy process has been reported. *Sqstm1* has been suggested as a target gene of NF-kB [62]. Interestingly, p65/RelA binds to and activates the beclin-1 promoter in human Jurkat T cells [63].

In this work, we observed that U0126, the inhibitor of MAPK, produced cell death more efficiently in MIAPaCa-2 cells, and the mechanism involved was caspase-dependent apoptosis. At the same time, CAPE, the inhibitor of NF-kB, produced a higher degree of apoptosis in PANC-1 cells via caspase independence. At first glance, we might be tempted to suggest that MAPK is more highly involved in cell death in a caspase-dependent than independent manner, and that NF-kB induces cell death more efficiently when the mechanism involved is caspase-independent apoptosis. However, based on our experience in studying the mechanisms that play a role in the resistance of tumor cells to chemotherapy, let us say that, in general, MIAPaCa-2 cells, independently of the drug under study, die by caspase-dependent apoptosis, while PANC-1 cells, also independently of the agent tested, die by caspase-independent apoptosis [28].

The interaction between the survival pathways MAPK and NF-kB and autophagy, another controversial and intriguing point, requires further study. The different roles of autophagy in cancer models are currently distinguished almost exclusively by determining the impact of autophagy inhibition on drug or radiation sensitivity. Thus it is impossible to ascertain whether the autophagy induced by radiation or chemotherapy is associated with resistance, or to predict circumstances under which autophagy inhibition might be a useful strategy for enhancing sensitivity to therapy. The situation is further complicated by the fact that autophagy can switch its function even within the context of a specific external stress and/or biological cancer model [64]. In PDAC, autophagy has been shown to be highly activated, and it is required for continued malignant growth in vitro and in vivo [65]. We previously reported that gemcitabine enhanced autophagy, transforming this process as a mechanism of chemotherapy. After autophagy inhibition, gemcitabine is able to induce apoptotic cell death [28]. In this context, autophagy inhibition might be a useful strategy for enhancing sensitivity to therapy. To this end, we described the inhibition of autophagy by both U0126 and CAPE, although they were unable to induce cell death. Interestingly, the inhibition of autophagy by 3-MA sensitized cells to the pro-apoptotic stimuli of U0126 and CAPE, showing high levels of apoptotic cell death, as demonstrated in Fig. 4. Understanding the key role played by survivin may represent one step along the path toward understanding tumor resistance and the role of autophagy in this complex process. As we noted previously, extensive work is needed to elucidate this aspect of tumor maintenance.

Finally, different drugs can have different effects on the autophagy process, but the net effect on tumor survival or death is unique to the situation. Here we have presented evidence that allows us to consider a combined therapy comprising an autophagy inhibitor and an inhibitor of MAPK or NF-kB pathways as a possible treatment regimen for pancreatic cancer.

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Compliance with Ethical Standards

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