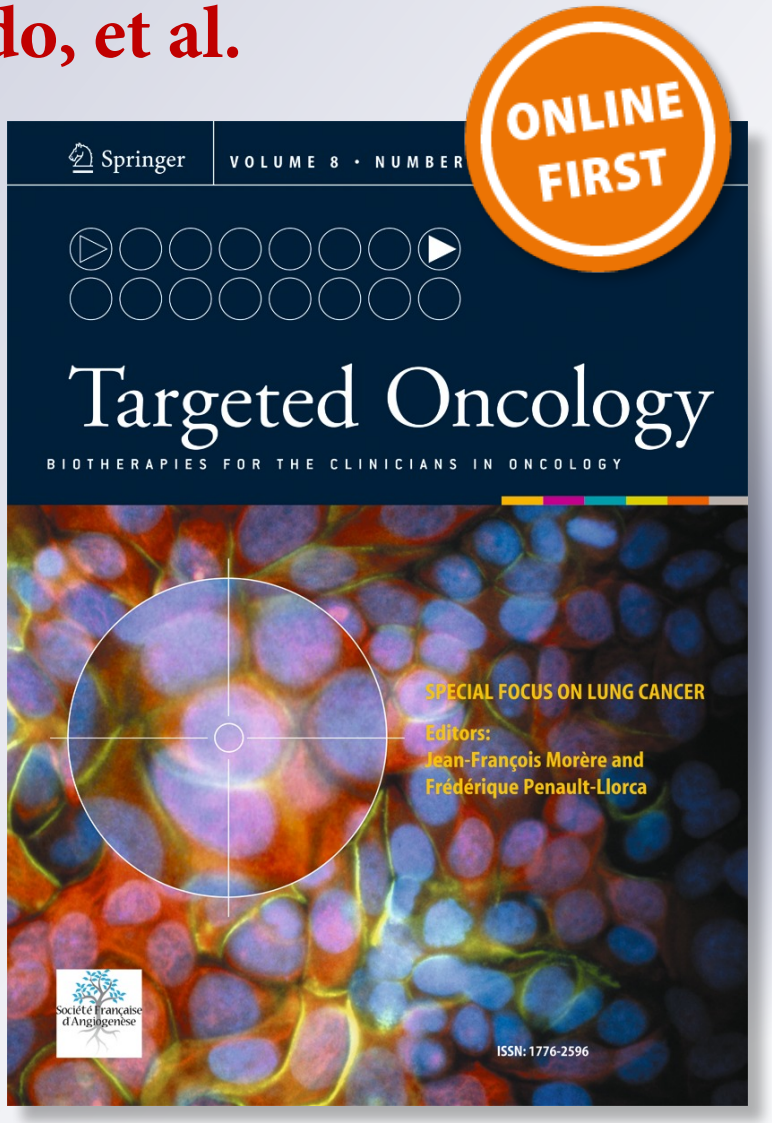
Interplay between autophagy and apoptosis in pancreatic tumors in response to gemcitabine

Daniela Laura Papademetrio, Victoria Cavaliere, Tania Simunovich, Susana Costantino, María Dolores Campos, Tomás Lombardo, et al.

Targeted Oncology

ISSN 1776-2596

Targ Oncol
DOI 10.1007/s11523-013-0278-5



Springer

Targ Oncol
DOI 10.1007/s11523-013-0278-5

ORIGINAL RESEARCH

Interplay between autophagy and apoptosis in pancreatic tumors in response to gemcitabine

Daniela Laura Papademetrio · Victoria Cavaliere · Tania Simunovich · Susana Costantino · María Dolores Campos · Tomás Lombardo · Claudio Marcelo Fader Kaiser · Élida Álvarez

Received: 16 January 2013 / Accepted: 3 April 2013

Abstract Pancreatic cancer is an aggressive disease. Its incidence has increased over the last two decades. It is currently the fourth cause of death among cancers in the western world. Unfortunately, systemic chemotherapy still relies on just a few drugs which until now have produced unsatisfactory results. Gemcitabine (2′-2′-difluorodeoxycytidine) is currently the standard chemotherapy treatment at all stages of pancreatic adenocarcinoma. Survival benefit and clinical impact however remain moderate due to a high degree of intrinsic and acquired resistance. Autophagy plays an important role in cell death decision but can also protect cells from various apoptotic stimuli. We investigated the function of autophagy in pancreatic carcinoma cells, which are frequently insensitive to standard chemotherapeutic agents. Here, we demonstrate that autophagy is one of the mechanisms responsible for the refractory response of pancreatic tumors to gemcitabine. We present evidence in vitro and in vivo that proves autophagy plays a protective role in pancreatic ductal carcinoma cells, preventing them from entering the apoptotic pathway after stimulus with gemcitabine, thus contributing to treatment resistance. A better understanding of the role in the process may help in the discovery of new strategies to overcome tumor drug resistance in this aggressive disease.

D. L. Papademetrio (✉) · V. Cavaliere · T. Simunovich · S. Costantino · M. D. Campos · T. Lombardo · É. Álvarez
IDEHU-CONICET, Cátedra de Inmunología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junin 956, 4° piso,
Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina
e-mail: dpapademetrio@ffyb.uba.ar

C. M. F. Kaiser
Laboratorio de Biología Celular y Molecular, IHEM-CONICET, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza, Argentina

Published online: 16 April 2013

Keywords Apoptosis · Autophagy · Drug resistance · Pancreatic tumor

Introduction

Pancreatic cancer is at present the fourth leading cause of death among cancers in the western world. Nearly 90 % of pancreatic tumors are ductal adenocarcinomas. Pancreatic cancer incidence has a mortality rate of 10 out of every 100,000 cases, highlighting the poor prognosis of this condition [1]. There has been little improvement in prognosis over the past 20 years, with a survival of just 4–6 months for the metastatic disease and an overall 5-year survival of <4 % [1, 2]. Radical surgery increases the median to survival time of between 13 and 15 months with a 5-year survival rate of approximately 10 % [3]. Several single chemotherapeutic agents have been tried in the treatment of pancreatic cancer. Currently, gemcitabine (2′-2′-difluorodeoxycytidine) represents the standard chemotherapy for the treatment of all stages of pancreatic adenocarcinoma [4]. Its survival benefit and clinical impact however remain moderated due to the high degree of intrinsic and acquired resistance. Although many randomized gemcitabine combination trials have been carried out in the last years, they have failed to demonstrate a statistically significant survival advantage over gemcitabine alone, with the exception of erlotinib combinations, which have provided a minor benefit [5].

Autophagy is an evolutionarily conserved process by which cytoplasm and cellular organelle are degraded in lysosomes [6].This ubiquitous process plays dual roles in cell death and survival. On the one hand, it induces type II programmed cell death, often termed as autophagic cell death, which is different from apoptosis. On the other hand, it recycles cellular components to sustain metabolism and avoid the accumulation of damaged, toxic proteins, and

organelles during stress [7]. Autophagy is important in development and diverse pathophysiological conditions in eukaryotic cells [8]. It offers protection against neurodegeneration [9, 10], infections [11, 12], and tumor development [13, 14]. Autophagy has an important role in cell death decisions and can protect cells against various apoptotic stimuli [15]. It is not clear how autophagy prevents cells from undergoing apoptosis; one suggested mechanism is the sequestration of damaged mitochondria [15], which prevents released cytochrome c from forming a functional apoptosome in the cytoplasm. Recent work has suggested that autophagy can also protect cells from caspase-independent death, which occurs after mitochondrial outer membrane permeabilization (MOMP) is in the presence of caspase inhibitors. Interestingly, this study shows that while energy can still be generated, cells can use autophagy to survive MOMP, the release of cytochrome c and other apoptogenic proteins, thus being able to recover and continue growing [16]. An increment in autophagy levels can protect cells from apoptosis as well as from this kind of caspase-independent death [17].

In this case, autophagy is considered as an important survival mechanism not only in normal cells, but also in tumor cells. In this study, we investigated in-depth the function of autophagy in pancreatic carcinoma cells, which are frequently insensitive to standard chemotherapeutic agents. We clearly demonstrated that autophagy is indeed one of the mechanisms responsible for the refractory response of pancreatic tumors to gemcitabine. A better understanding of the role in this process may lead us to new methods to overcome tumor drug resistance in this aggressive disease. Here, we present evidence in vitro and in vivo that proves autophagy plays a protective role in pancreatic ductal carcinoma cells, which prevents them from entering the apoptotic pathway after stimulus with gemcitabine, thus contributing to the resistance of treatment.

Materials and methods

Materials 3-Methyladenine (3-MA) was purchased from SIGMA (St Louis, MO, USA) and gemcitabine was kindly provided by Richmond (Buenos Aires, Argentina). Both were resuspended in DMEM containing 10 % fetal bovine serum (FBS). z-VAD-fmk was obtained from SIGMA. DMEM, penicillin, and streptomycin were purchased from Invitrogen (Buenos Aires, Argentina). FBS was purchased from Natocor (Córdoba, Argentina). Plasmid pRFP-LC3 was generously supplied by Dr. Noboru Mizushima (Department of Physiology and Cell Biology, Tokyo Medical and Dental University, Tokyo, Japan) and Dr. Tamotsu Yoshimori (Department of Cellular Regulation, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan).

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.

Cell growth inhibition assay Sensitivity of the cell line to increased doses of gemcitbine was determined by culturing 5×10^4 cells/ml at 37 °C in a 5 % CO2 atmosphere for 48 and 72 h, pulsed with 1 μCi [3H]TdR (DuPont, Nen Products, Boston, MA, USA) for the last 18 h. The cultures were performed in 96-well round-bottomed microtitre plates in the presence of different concentrations of gemcitabine (0.01–1000 μg/ml). After incubation, cells were harvested with a semiautomatic method. Incorporated [3H]TdR was measured in a Liquid Scintillation beta Counter, (Beckman, Maryland, USA). Results were calculated from the mean cycles per minute (cpm) of [3H]TdR incorporated in triplicate cultures. Each experiment was repeated at least three times with similar results. Inhibition of growth percentage was calculated as

$$\%\,\text{Growth inhibition} = \frac{100–[\text{cpm}-\text{treated cells}\times 100]}{\text{cpm basal control}}$$

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.

Cell transfection Seventy-five percent confluent MiaPaCa2 cells were transfected with pRFP-LC3 using DMRIE-C transfection reagent (Invitrogen, Buenos Aires, Argentina) as indicated by the manufacturer. The transfected cells were incubated for 48 h in DMEM supplemented with 10 % FBS in 24-well plates and then treated with DMEM supplemented with 10 % FBS, 3-MA 10 mM, gemcitabine 100 μg/ml or 3-MA 10 mM+gemcitabine 100 μg/ml. Cells expressing RFP-LC3 were directly observed using a fluorescent microscope equipped with a CCD camera.

Flow cytometric analysis of cell stress

Annexin V-FITC Percentage of annexin+cells was determined by staining the annexin V-fluorescein isothiocyanate (annexing V-FITC Apoptosis Detection Kit., Invitrogen, CA, USA) assay. Cells were incubated alone in DMEM 10 % FBS, with 3-MA (10 mM), gemcitabine (10–1,000 μg/ml), 3-MA+gemcitabine or z-VAD-fmk+3-MA+gemcitabine for 72 h. Single-cell suspensions were analyzed by Flow Cytometry (Partec II, Germany) and data was analyzed using the WinMDI 2.9 software (Scripps Institute, La Jolla, CA, USA). Annexin positive cells were scored as Annexin V+, PI−, and Annexin V+, PI+.

Apoptotic assessment

Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling: Cells were incubated alone in DMEM 10 % FBS, with 3-MA (10 mM), gemcitabine (10–1,000 μg/ml), 3-MA+gemcitabine or z-VAD-fmk+3-MA+gemcitabine for 72 h. The cells were then resuspended and washed once with ice-cold phosphate-buffered saline (PBS) and fixed in 4 % buffered paraformaldehyde. Terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) was carried out using the DeadEnd Fluorometric TUNEL System kit (Promega, 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) and Adobe Photoshop (Adobe, Inc., Mountain View, CA, USA) programs.

Total protein extracts Cells (1×10^7) 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, for 30 min at 4 °C followed by centrifugation at 13,000×*g* for 30 min. The extracts were then stored at −70 °C until further use. Protein concentration was determined by the Bradford assay.

Western blot Equal amounts of protein were loaded into each well and separated by SDS-PAGE gel, then transfered onto PVDF-membranes (GE Healthcare, Argentina). 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), Beclin-1, PARP, Bcl-XL, Survivin, Bax, Bad, Pro-caspase 3, AIF, and β-Actin (Santa Cruz Biotechnology, CA, USA) overnight at 4 °C. Donkey antigoat IgG secondary antibody and goat antirabbit IgG secondary antibody (1:8,000, Santa Cruz Biotechnology) 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, Camedia, D-510 ZOOM) were subjected to densitometry analysis using Image Scion software.

Analysis of caspase activity Treated with gemcitabine (1,000 μg/ml) either with or without pretreatment with 3-MA (10 mM), during 24 h was 1.5×10^4 cells/well. The activity of caspase 3/7 and 9 was determined following the manufacturer's instructions provided in Caspase-Glow® 3/7 and Caspase–Glow® 9 detection kit (Promega, Argentina). Luminescence production was measured on a Victor plate-reading luminometer (Perkin Elmer). The signal obtained was proportional to caspase 3/7 activity in cell culture. Caspase activity in samples was scored as Activity Index which was calculated as follows:

$$\text{Activity index} = \text{Luminescence units of treated cells}/\text{luminescence units of control cells}$$

Tumor xenograft studies Tumors were induced by s.c. injection of 5×10^6 MiaPaCa2 cells in 10 % FBS DMEM in immunodeficient nude mice. When tumors reached an average size of 25–30 mm^3, the animals were randomly assigned to four groups and injected peritumorally for 21 days with the different drugs. Group 1 was injected with 200 μl of sterile physiological solution; group 2 with 200 μl of 100 μg/g 3-MA; group 3 with 100 μl of 50 μg/g gemcitabine, group 4 with 200 μl of 100 μg/g 3-MA, and 1.5 h later with 100 μl of 50 μg/g gemcitabine. Tumors were measured with an external caliper and their volumes were calculated as $(4\pi/3)\times(\text{width}/2)^2\times(\text{length}/2)$.

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

Results

Gemcitabine inhibits pancreatic cancer cell proliferation

As gemcitabine is the antineoplastic agent commonly included in most pancreatic induction protocols, we explored whether the treatment of MIAPaCa-2 and PANC-1, two pancreatic cell lines with gemcitabine resulted in a reduction of cell proliferation as assessed by [^{3}H]-thymidine incorporation. All doses tested exhibited an antiproliferative effect after 48 h with maximal values of 40.4±7.3 and 48.1±2.5 % of growth inhibition for MIAPaCa-2 and PANC-1 cells, respectively ($p<0.001$; Fig. 1a). The highest inhibitions however were reached after 72 h of treatment with growth inhibition values of 69.6±1.0 % for MIAPaCa-2 and of 65.8 ±5.3 % for PANC-1 ($p<0.001$). We then analyzed the tumor growth inhibition caused by gemcitabine in a xenograft model. As shown in Fig. 1b, peritumoral treatment notably reduced the growth of the established pancreatic tumors at the two concentrations evaluated. These results revealed that

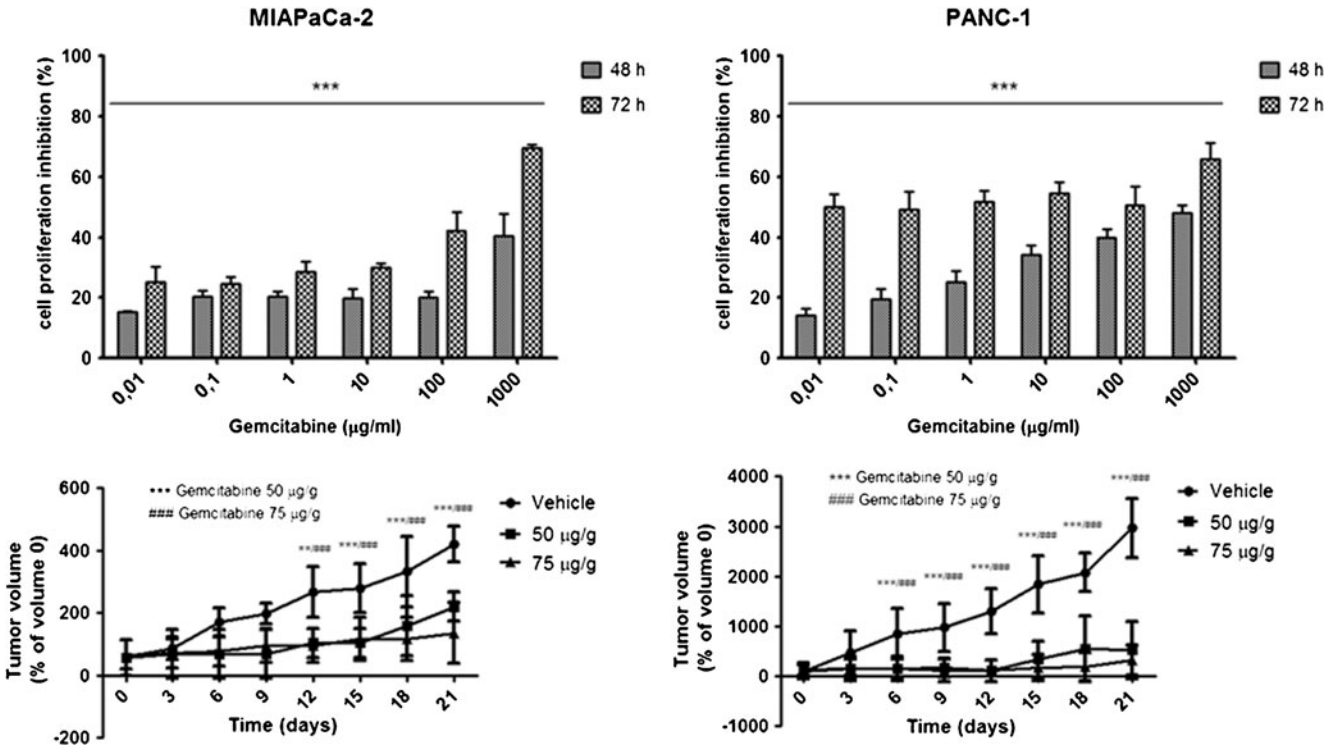


Fig. 1 Gemcitabine induces inhibition of cell proliferation. **a** MIAPaCa-2 and PANC-1 cells were exposed in vitro to different concentrations of gemcitabine for 48 and 72 h. The inhibition of cell proliferation was calculated by measuring [^{3}H]TdR incorporation. *Bars* mean±SD of at least three independent experiments (***$p<0.001$). **b** Gemcitabine treatment reduces the growth of pancreatic tumor cells in vivo. S.c. tumors were generated as described in the "Materials and methods" section. When tumors reached the desired size (day 0), animals were treated with either vehicle or gemcitabine for 21 days ($n=10$ for each experimental group). Tumor volume was measured at the indicated times. Results are expressed as the percentage of tumor volume growth relative to day 0 (***/###$p<0.001$)

gemcitabine was able to interfere with both the MIAPaCa-2 and PANC-1 proliferation rate through significant inhibition of this process, as demonstrated both in vitro and in vivo.

Gemcitabine modulates autophagy in pancreatic cancer cells

As the role of autophagy in cancer is still controversial, we investigated whether autophagy was involved in the survival of pancreatic cancer cells. We first explored the presence of autophagosomes in our two pancreatic tumor cell lines. As shown in Fig. 2a, both MIAPaCa-2 and PANC-1 presented autophagy in basal culture conditions, a process that was inhibited by the treatment of 10 mM of 3-MA. We then determined the induction of autophagy 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 [18]. This proves that the LC3-II levels correlate to the number of autophagosomes. According to western blot, LC3-II is seen as a faster migrating band and can also be assessed by scoring GFP-LC3-positive vesicles (scoring fluorescent LC3 positive vesicles) [19]. In addition, it is important to determine whether gemcitabine modulates autophagosome synthesis and/or degradation [20]. To study the effect of gemcitabine on the autophagic process, we measured the LC3-II levels in the presence of gemcitabine and/or concanamycin, a proton pump inhibitor. For this, pancreatic cancer cells were exposed to 100 µg/ml of gemcitabine for 48 h and/or 50 nM of concanamycin for 6 h. Both Treatments of MIAPaCa-2 cells with gemcitabine and gemcitabine+concanamycin in basal conditions showed an increase in LC3-II levels when compared to treatment with concanamycin alone. In contrast, treatment in basal conditions of PANC-1 cells with gemcitabine and gemcitabine+concanamycin caused a decrease in LC3-II levels when compared to the levels obtained by treatment with concanamycin alone or basal conditions, respectively (Fig. 2b). In support of this data, cells were transfected with RFP-LC3 and then cultured under the conditions mentioned above. The treatment of MIAPaCa-2 cells with gemcitabine showed an increase in the size but not in the number of LC3-II-positive vesicles when compared to the basal conditions. Gemcitabine+concanamycin caused an increase in both the number and size of LC3-II-positive vesicles with the concanamycin treatment.

As expected, the treatment of PANC-1 cells with gemcitabine decreased the number of LC3-II-positive vesicles in basal conditions. Gemcitabine+concanamycin however, reduced the size of the LC3-II-positive vesicles with no alterations to the number of autophagosomes (Fig. 2c). In addition, beclin-1 levels as well as the beclin-1/Bcl-XL ratio increased 1.7, compared to the controls in MIAPaCa-2 cells treated with 100 μg/ml of gemcitabine. In the case of PANC-1, we did not observe any changes to the levels of beclin-1. The beclin-1/Bcl-XL ratio to controls did however increase by 1.34-fold (Fig. 2d). Our results demonstrate that gemcitabine enhances autophagy in MIAPaCa-2 cells but delays the synthesis of autophagosomes in PANC-1 cells.

Gemcitabine induces apoptosis after autophagy inhibition in vitro and in vivo

As several authors have suggested that autophagy constitutes a resistance mechanism developed by tumor cells to survive [21–23], we decided to test whether pharmacological inhibition of this process by treatment with gemcitabine sensitizes pancreatic tumor cells to death. We first tested the effect of gemcitabine in the presence of 3-MA. For this, we incubated pancreatic tumor cells with 10 mM of 3-MA for 1 h and then added gemcitabine at a final concentration of 10–1,000 μg/ml. We then collected the cells to monitor the different aspects of death. Treatment of both tumor cell lines with gemcitabine alone and after pretreatment with 3-MA showed a significant increase in the number of annexin V-FITC-positive cells, a marker of cell stress in all the doses tested (Fig. 3a). We then analyzed treated cells using TUNEL to determine the percentage of apoptotic death induced by the different drugs. In all cases, the number of TUNEL-positive cells obtained by the treatment with gemcitabine after pre-incubation with 3-MA was significantly higher than those obtained by the treatment of gemcitabine alone (Fig. 3b). These results suggest that gemcitabine can induce apoptotic cell death more efficiently in MIAPaCa-2 and PANC-1 cells when autophagy is inhibited.

We then analyzed the modulation of pro- and anti-apoptotic protein expression by western blot. Figure 4a shows that gemcitabine induced an anti-apoptotic balance in MIAPaCa-2 cells, with a decrease in the Bax/Bcl-XL ratio and an increase in the pro-caspase 3 levels. The pretreatment with 3-MA led gemcitabine to have a pro-apoptotic effect, as shown by the increase in Bax/Bcl-XL levels, the decrease in survivin and pro-caspase 3 expression and the increase in PARP cleavage. Figure 4b shows that the bad levels were modulated by the treatment with gemcitabine in PANC-1 cells and that after treatment with 3-MA, gemcitabine induced a decrease in 3 levels of procaspase without variations in the levels of PARP cleavage.

In order to evaluate the effect of gemcitabine in combination with 3-MA in vivo, we generated tumor xenograft by s.c. injection of the MIAPaCa-2 cells in immunodeficient mice. Peritumoral treatment with 3-MA+gemcitabine as well as treatment with gemcitabine alone notably reduced the growth of the established pancreatic tumors (Fig. 5a and b). The analysis of tumor histology showed a more extensive necrotic area with 3-MA plus gemcitabine treatment than the area observed under basal conditions (Fig. 5c). Finally, we determined the death mechanism involved by analyzing the tumor cell morphology by electron microscopy. As shown in Fig. 5d, gemcitabine enhanced the number of autophagosomes. In contrast, treatment with 3-MA+gemcitabine induced apoptotic cell death. This data supports the in vitro conclusions, demonstrating that gemcitabine enhances autophagy. Once the process is inhibited however, it can induce apoptotic death.

Gemcitabine induces caspase-dependent cell death in MIAPaCa-2 but not in PANC-1 cells

We further investigated the mechanism of which gemcitabine induces apoptotic cell death when autophagy is inhibited. For this, we first analyzed using TUNEL assay whether z-VAD-fmk, an inhibitor of pan-caspases, was able to prevent the low pro-apoptotic effect of gemcitabine and the high effect caused by 3-MA+gemcitabine. Figure 6a shows that the pro-apoptotic effect caused by both treatments is prevented by pre-incubation with z-VAD-fmk 100 μM in MIAPaCa-2 cells. In contrast, z-VAD-fmk did not prevent the effect caused by gemcitabine or 3-MA+gemcitabine in PANC-1 cells. In addition, we analyzed the modulation of caspase-3 by luminescence. As expected, the treatment of MIAPaCa-2 cells with gemcitabine after 3-MA resulted in a 2.7-fold increase in the activity of caspase-3. In contrast, this treatment caused no changes to the caspase-3 levels of PANC-1 cells (Fig. 6b). We also analyzed the levels of AIF in the nucleus and cytoplasm. As shown in Fig. 6c, the AIF nuclear/cytoplasm ratio in PANC-1 cells treated with 3-MA+gemcitabine increased fourfold. No changes were observed in MIAPaCa-2 cells. These results suggest that gemcitabine can induce caspase-dependent apoptosis when autophagy is inhibited in MIAPaCa-2 cells, but only in a caspase-independent way in PANC-1 cells.

Discussion

Pancreatic cancer, a complex disease, is caused by the mutations of oncogenes and/or tumor suppressor genes that cause alterations in survival signaling pathways. The great challenge underlying anticancer therapy is the uncertain and intricate molecular mechanism involved in this pathology.

It is currently acknowledged that apoptosis and autophagy, two types of programmed cell death can be used for tumor

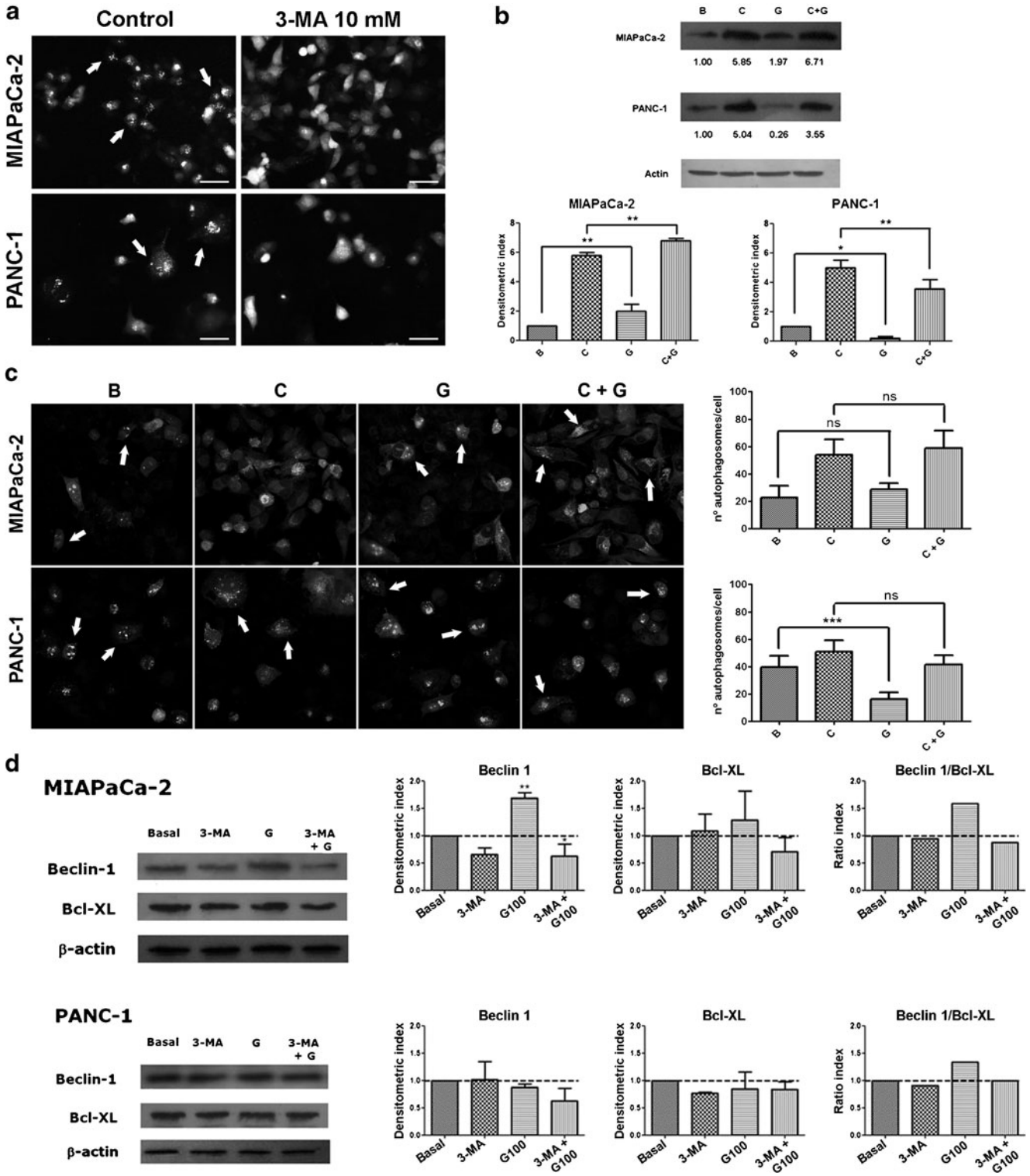


Fig. 2 Gemcitabine modulates autophagy. **a** Tumor cells were transfected with RFP-LC3 plasmid, incubated under basal conditions or with 10 mM of 3-MA for 1 h, and then observed by fluorescence microscopy. *Bars* 60 μm. *Arrows* cells with punctate LC3-II, indicating the presence of autophagosomes. **b** 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 100 μg/ml of gemcitabine for 24 h (*G*), and after treatment with concanamycin+gemcitabine (*C+G*). **c** Transfected cells were incubated with the different drugs and observed by confocal microscopy. The number of LC3-II-positive autophagosomes per cell was counted. Magnifications ×600. *Arrows* cells with punctate LC3-II, indicating the presence of autophagosomes. **d** Analysis of intracellular levels of beclin-1 and Bcl-XL by western blot. Cells were incubated under basal conditions (*B*) with 10 mM of 3-MA (*3-MA*), treated with 100 μg/ml of gemcitabine (*G*), and finally with concanamycin+gemcitabine (*C+G*)

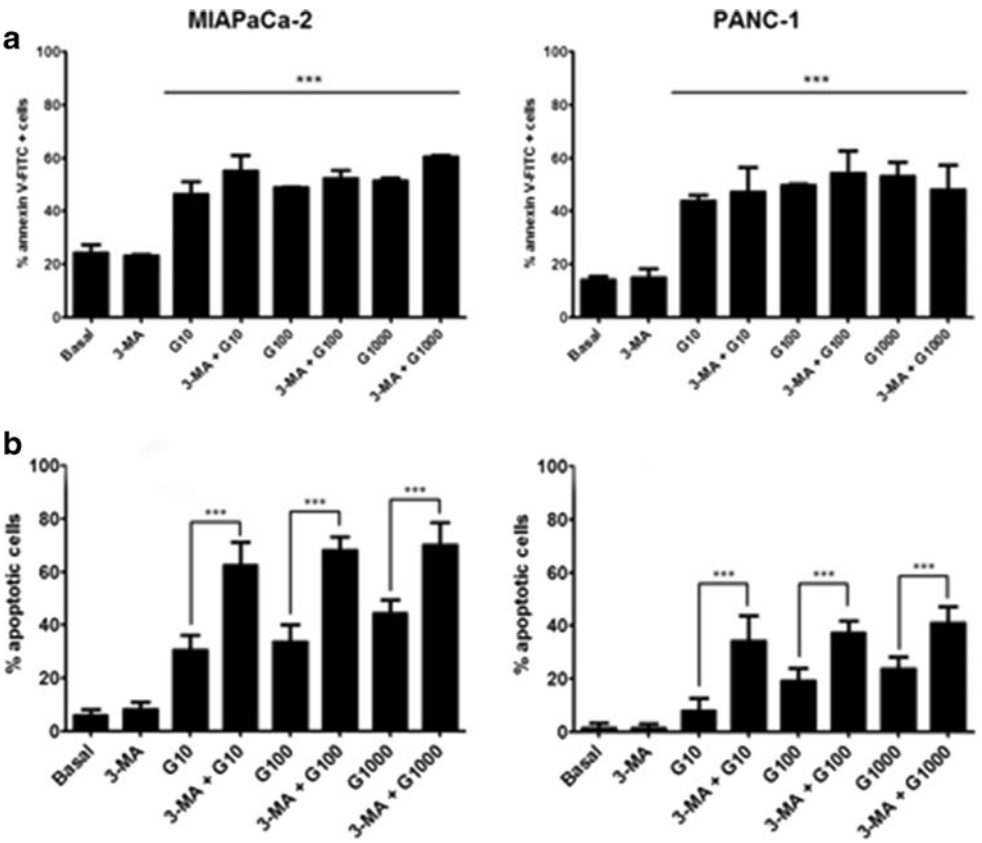


Fig. 3 **a** Gemcitabine induces stress in MIAPaCa-2 and PANC-1 cells. Tumor cells were treated with 10–1,000 μg/ml of gemcitabine alone or after 1 h pretreatment with 10 mM of 3-MA for 72 h. The number of stressed cells was calculated by annexin V-FITC/IP dye, following the manufacturer's recommendations, as described in the "Materials and methods" section. The number of stressed cells corresponds to the sum of annexin V-FITC+/IP- and annexin V-FITC+/IP+. **b** Gemcitabine induces apoptotic cell death more efficiently when autophagy in inhibited. As described before, cells were incubated with 3-MA+ gemcitabine or gemcitabine alone and analyzed by TUNEL assay after 72 h. The percentage of apoptotic cells was calculated as number of TUNEL+cells/ number of total cells ($p<0.001$)

treatment thanks to their regulating of cancer cell death capabilities. Apoptosis invariably contributes to the death of cancer cells. Autophagy on the other hand, plays dual roles in cancer cell survival or death. This makes autophagy the most intricate part in tumor treatment [24, 25]. Additionally, a positive or negative relationship between apoptotic and autophagic cell death may exist.

The responsibility of autophagy in cancer progression has remained ambiguous; its role in cell fate decision remains a matter of controversy. Colorectal cancer cells express functional autophagy and this mechanism allows the prolongation of the cell's survival during shortages of nutrients [26]. Several studies have indicated that activated autophagy is associated with pancreatic cancer cells and that LC3 expression of pancreatic cancer cells is significantly correlated with a poor outcome [27].

It is known that gemcitabine can inhibit tumor cell proliferation. The reasons why this drug is unable to induce cell death remain completely misunderstood. Previously, we showed evidence that early induction of autophagy by suboptimal doses of gemcitabine were able to induce apoptosis of pancreatic cancer cells [28]. In culture, MIAPaCa-2 cells treated with 50 μg/ml of gemcitabine for 48 h induced a 30 % apoptotic cell death. That was reversed by pretreatment with 10 mM of 3-MA. In this study, we investigated the role of autophagy on gemcitabine-treated cells under conditions comparable to that of the doses used in patient protocols. For this, we used 1,000 μg/ml of gemcitabine and evaluated cell death after 72 h. In this setting, we demonstrated that autophagy was one of the mechanisms responsible for the refractory response of pancreatic tumors to therapuetic doses of gemcitabine. Data presented here demonstrates that pharmacological inhibition of autophagy leads to pancreatic cancer death by treatment of gemcitabine. The death mechanism implicated under these conditions is apoptosis. Kimmelman et al. [23] have shown that autophagy is highly activated in pancreatic ductal adenocarcinoma (PDAC) and that it is required for continued malignant growth in vitro and in vivo. In most cells, autophagy is induced by nutrient deprivation, chemotherapeutic agents, and hypoxia [29, 30], meanwhile pancreatic tumor cells exhibit constitutive autophagy under basal conditions. Autophagy may help in promoting tumorigenesis in other types of cancer. It may not however be as pronounced as PDAC, in which the majority of tumors are dependent on this process [23]. The positive role of autophagy in the maintenance of pancreatic tumors contrasts a number of other malignancies, in which inactivation of autophagy can promote tumorigenesis [13, 31].

Previous studies have suggested that in certain contexts, autophagy can contribute to chemotherapeutic resistance rather than to tumor maintenance. For example, Thompson et al. [21] demonstrated in a lymphoma model that the treatment of mice with chloroquine results in the growth impairment of tumors but with no tumor regression unless combined with restoration of p53 expression or alkylating

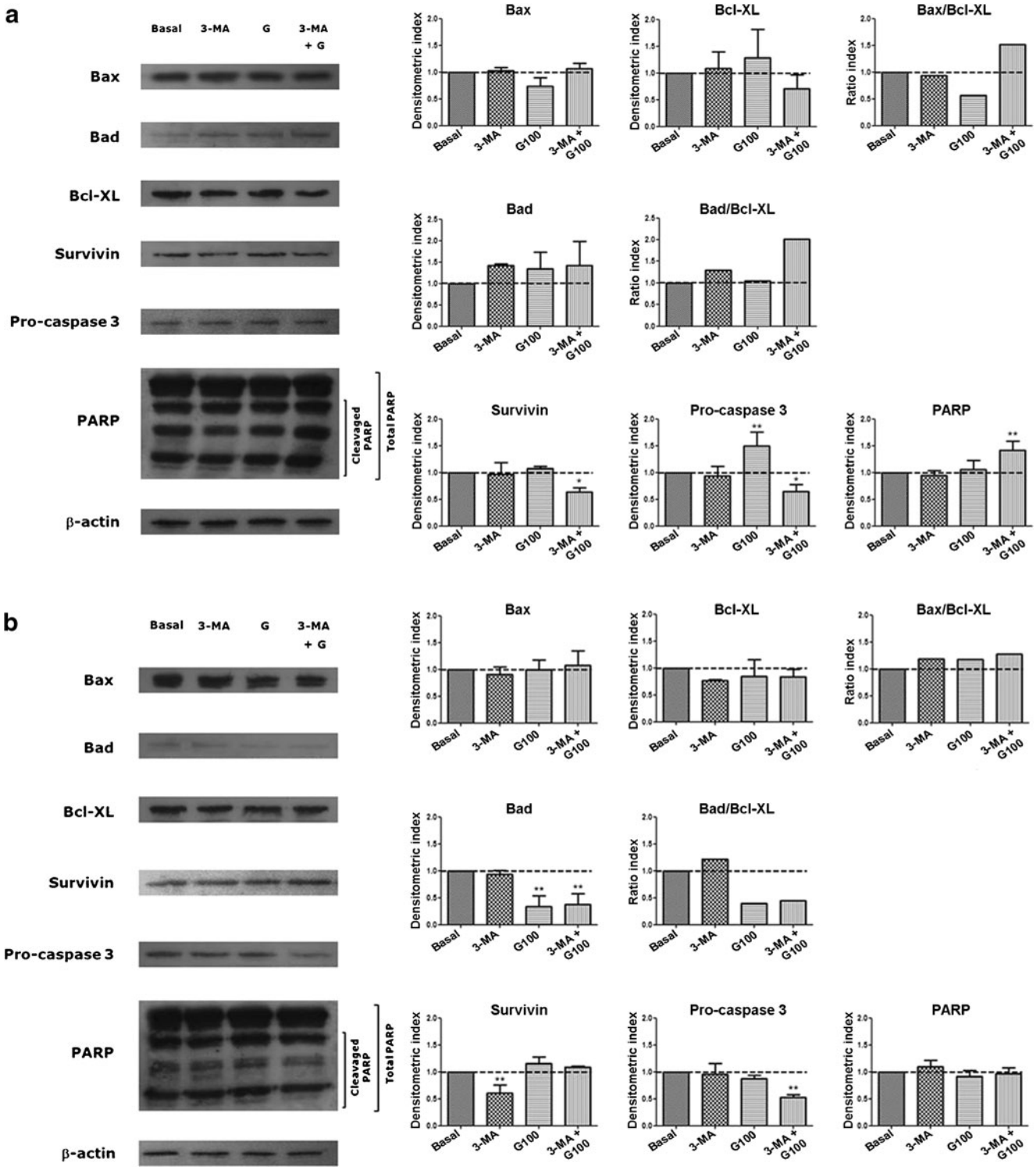


Fig. 4 Modulation of pro- and anti-apoptotic proteins. Analysis of intracellular levels of the pro-apoptotic proteins Bax, Bad, pro-caspase, and cleavage of PARP, and the anti-apoptotic proteins Bcl-XL and survivin by western blot of MIAPaCa-2 cells (**a**) and PANC-1 cells (**b**) under basal conditions (*B*), after treatment with 10 mM of 3-MA (*3-MA*), after 48 h of treatment with gemcitabine, (*G*), or after incubation with 3-MA+gemcitabine (*3-MA+G*; $*p<0.05$, $**p<0.01$)

chemotherapy. Cooperation of autophagy inhibition with chemotherapy has also been observed in leukemia [32] and may reflect the upregulation of autophagy as a possible survival mechanism in response to chemotherapeutic agents [33]. Other authors however have labeled autophagy a death mechanism [34, 35].

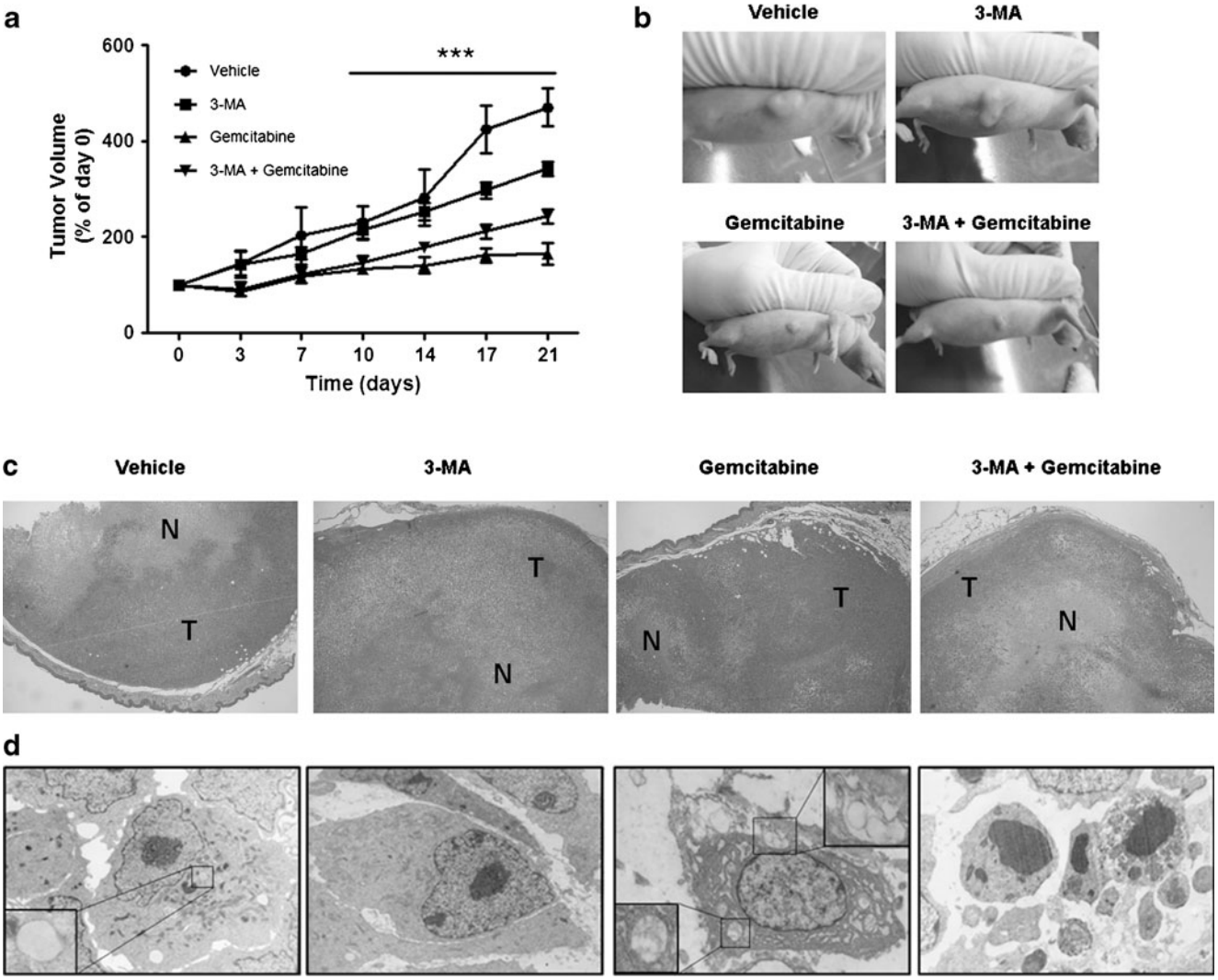


Fig. 5 Tumor growth inhibition. S.c. tumors were generated as described in Materials and Methods. When tumors reached the desired size (day 0), animals were distributed randomly into four groups. Group 1 was injected with 200 μl of sterile solution, group 2 with 200 μl of 3-MA in a concentration of 100 μg/g, group 3 with 50 μg/g of gemcitabine, and group 4 with 200 μl of 3-MA (100 μg/g) and 1.5 h after with gemcitabine (50 μg/g). **a** and **b** Gemcitabine and 3-MA+gemcitabine inhibit tumor growth. Results are expressed as the percentage of tumor volume growth relative to day 0 (***$p<0.001$). **c** Histology of xenograft tumors. Hemaoxylin and eosin dye of tumors showing tumor (*T*) and necrotic (*N*) areas. **d** Electron microscopy of tumors under the different treatments after 21 days. Magnification, ×3,000

In contrast, PDAC is characterized by its critical role of autophagy in tumorigenicity under basal conditions. Here, we demonstrate that gemcitabine enhances autophagy in MIAPaCa-2 cells and delays it in PANC-1 cells. Figure 2 shows the modulator effect of this drug on autophagy. We also demonstrate that gemcitabine can induce high levels of apoptosis, in vitro and in vivo, when autophagy is inhibited (Figs. 3 and 5) contrary to data published by those authors who claimed that autophagy constitutes a death mechanism when pancreatic cancer cells are treated with gemcitabine and other chemotherapeutic agent such as ionizing radiation [36] or cannabinoids [37]. Furthermore, recent studies have shown that induction of autophagy by drug-resistant esophageal cancer cells promotes their survival. Cells that do not respond to chemotherapeutic agents with apoptosis undergo autophagy and cell populations can recover when cytotoxic drugs are withdrawn. Their ability to recover may explain recurrent disease and may be a decisive factor in current treatments. Both selective inhibition of proteins involved in the formation of autophagosomes and indirect inhibition of autophagy can reduce the recovery of cancer cells following cytotoxic drug treatment. This indicates the importance of autophagy in this recovery [38]. Data published by ourselves and several other authors force us to think about the bivalent participation of autophagy in pancreatic cancer cell growth. Although autophagy may be a prosurvival process for tumor cells in the first steps of tumor formation, the induction of autophagy could in fact lead to apoptotic cell death. This model represents the autophagy paradox where both

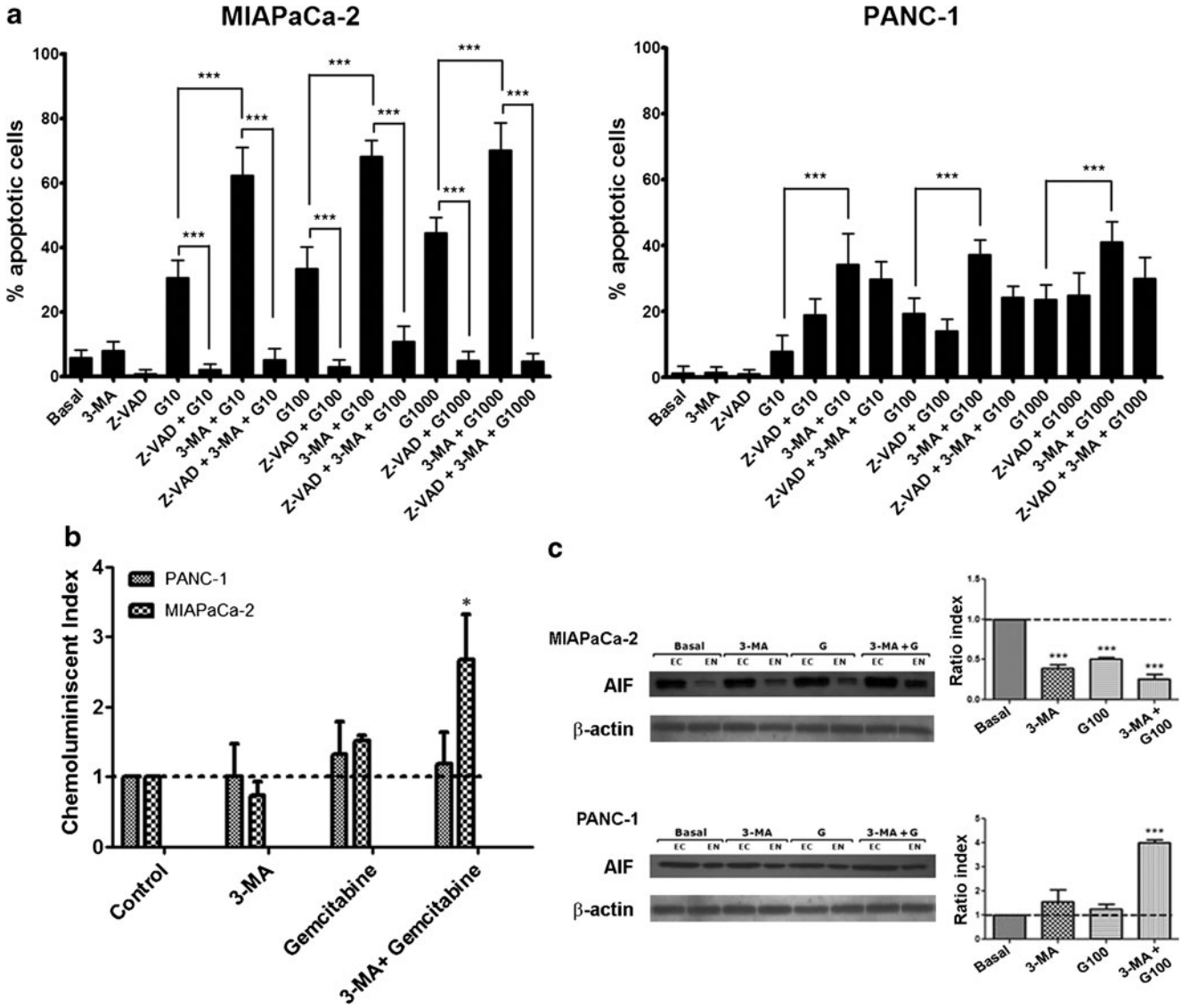


Fig. 6 Apoptotic death characterization. **a** Tumor cells were incubated with 10 mM of 3-MA, 100 μM of the pan-caspase inhibitor Z-VAD-fmk, 10–1,000 μg/ml of gemcitabine or the different combinations between them as shown in the figure for 72 h. **b** Analysis of caspase-3 activity by luminescence as described in the "Materials and methods" section. **c** Study of subcellular localization of AIF after 24 h of treatment with 3-MA, gemcitabine, or 3-MA+gemcitabine

autophagy inhibition and stimulation have the same net effect, to inhibit tumor growth but with differences in the efficacy of cell death induction.

Finally, we describe the death mechanism involved when gemcitabine acts after autophagy inhibition. Several studies have been performed in an attempt to understand the death mechanisms induced by natural compounds in tumor cells. It has been shown that Plumbagin may induce apoptosis in human pancreatic cancer cells, PANC-1, and BxPC-3 by caspase-dependent and independent cascades [39]. Indolequinone ES936 induces caspase-dependent apoptosis in MIAPaCa-2 and BxPC-3 cells but not in PANC-1 cells [40]. Other substances such as Isoegomaketone on DLD1 human colon cancer cells [41] and Decursin on malignant tumor (RC-58 T/h/SA#4)-derived human prostate cells are able to induce both types of apoptotic cell death [42]. However, there is no report that shows which mechanism is activated when gemcitabine acts on MIAPaCa-2 and PANC-1 cells after autophagy inhibition. Interestingly, our results show that MIAPaCa-2 cells die by a caspase-dependent apoptotic pathway, contrary to PANC-1 cells, which die from a caspase-independent apoptotic mechanism (Figs. 4 and 6).

In summary, we identified one of the mechanisms involved in the resistance of pancreatic tumors to the treatment of gemcitabine. The identification of this mechanism will help in the development of more efficient therapies against this aggressive pathology thus leading to an improvement in the patient's life.

Conflict of interest The authors declare no conflict of interest

Grant support This study was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) PIP no. 0199 and Secretaría de Ciencia y Tecnología Universidad de Buenos Aires B011 and 20020100100185.

References

1. Verslype C, Van Cutsem E, Dicato M, Cascinu S, Cunningham D, Diaz-Rubio E, Glimelius B, Haller D, Haustermans K, Heinemann V, Hoff P, Johnston PG, Kerr D, Labianca R, Louvet C, Minsky B, Moore M, Nordlinger B, Pedrazzoli S, Roth A, Rothenberg M, Rougier P, Schmoll HJ, Tabernero J, Tempero M, van de Velde C, Van Laethem JL, Zalcberg J (2007) The management of pancreatic cancer. Current expert opinion and recommendations derived from the 8th World Congress on Gastrointestinal Cancer, Barcelona, 2006. Ann Oncol 18(7):vii1–vii10. doi:10.1093/annonc/mdm210
2. Sakorafas GH, Tsiotou AG, Tsiotos GG (2000) Molecular biology of pancreatic cancer; oncogenes, tumour suppressor genes, growth factors, and their receptors from a clinical perspective. Cancer Treatment Reviews 26(1):29–52. doi:10.1053/ctrv.1999.0144
3. Neoptolemos JP, Dunn JA, Stocken DD, Almond J, Link K, Beger H, Bassi C, Falconi M, Pederzoli P, Dervenis C, Fernandez-Cruz L, Lacaine F, Pap A, Spooner D, Kerr DJ, Friess H, Buchler MW (2001) Adjuvant chemoradiotherapy and chemotherapy in resectable pancreatic cancer: a randomised controlled trial. Lancet 358(9293):1576–1585
4. Burris HA 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD (1997) Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol 15(6):2403–2413
5. Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA, Campos D, Lim R, Ding K, Clark G, Voskoglou-Nomikos T, Ptasynski M, Parulekar W (2007) Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol 25(15):1960–1966. doi:10.1200/JCO.2006.07.9525
6. Reggiori F, Klionsky DJ (2002) Autophagy in the eukaryotic cell. Eukaryot Cell 1(1):11–21
7. Shintani T, Klionsky DJ (2004) Autophagy in health and disease: a double-edged sword. Science 306(5698):990–995. doi:10.1126/science.1099993
8. Cuervo AM (2004) Autophagy: in sickness and in health. Trends Cell Biol 14(2):70–77. doi:10.1016/j.tcb.2003.12.002
9. Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, Yokoyama M, Mishima K, Saito I, Okano H, Mizushima N (2006) Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 441(7095):885–889. doi:10.1038/nature04724
10. Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, Ueno T, Koike M, Uchiyama Y, Kominami E, Tanaka K (2006) Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature 441(7095):880–884. doi:10.1038/nature04723
11. Kirkegaard K, Taylor MP, Jackson WT (2004) Cellular autophagy: surrender, avoidance and subversion by microorganisms. Nat Rev Microbiol 2(4):301–314. doi:10.1038/nrmicro865
12. Ogawa M, Yoshimori T, Suzuki T, Sagara H, Mizushima N, Sasakawa C (2005) Escape of intracellular *Shigella* from autophagy. Science 307(5710):727–731. doi:10.1126/science.1106036
13. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B (1999) Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 402(6762):672–676. doi:10.1038/45257
14. Liang XH, Yu J, Brown K, Levine B (2001) Beclin 1 contains a leucine-rich nuclear export signal that is required for its autophagy and tumor suppressor function. Cancer Res 61(8):3443–3449
15. Ravikumar B, Berger Z, Vacher C, O'Kane CJ, Rubinsztein DC (2006) Rapamycin pre-treatment protects against apoptosis. Hum Mol Genet 15(7):1209–1216. doi:10.1093/hmg/ddl036
16. Colell A, Ricci JE, Tait S, Milasta S, Maurer U, Bouchier-Hayes L, Fitzgerald P, Guio-Carrion A, Waterhouse NJ, Li CW, Mari B, Barbry P, Newmeyer DD, Beere HM, Green DR (2007) GAPDH and autophagy preserve survival after apoptotic cytochrome C release in the absence of caspase activation. Cell 129(5):983–997. doi:10.1016/j.cell.2007.03.045
17. Shen S, Kepp O, Kroemer G (2012) The end of autophagic cell death? Autophagy 8(1):1–3. doi:10.4161/auto.8.1.16618
18. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 19(21):5720–5728. doi:10.1093/emboj/19.21.5720
19. Klionsky DJ, Abeliovich H, Agostinis P, Agrawal DK, Aliev G, Askew DS, Baba M, Baehrecke EH, Bahr BA, Ballabio A, Bamber BA, Bassham DC, Bergamini E, Bi X, Biard-Piechaczyk M, Blum JS, Bredesen DE, Brodsky JL, Brumell JH, Brunk UT, Bursch W, Camougrand N, Cebollero E, Cecconi F, Chen Y, Chin LS, Choi A, Chu CT, Chung J, Clarke PG, Clark RS, Clarke SG, Clave C, Cleveland JL, Codogno P, Colombo MI, Coto-Montes A, Cregg JM, Cuervo AM, Debnath J, Demarchi F, Dennis PB, Dennis PA, Deretic V, Devenish RJ, Di Sano F, Dice JF, Difiglia M, Dinesh-Kumar S, Distelhorst CW, Djavaheri-Mergny M, Dorsey FC, Droge W, Dron M, Dunn WA Jr, Duszenko M, Eissa NT, Elazar Z, Esclatine A, Eskelinen EL, Fesus L, Finley KD, Fuentes JM, Fueyo J, Fujisaki K, Galliot B, Gao FB, Gewirtz DA, Gibson SB, Gohla A, Goldberg AL, Gonzalez R, Gonzalez-Estevez C, Gorski S, Gottlieb RA, Haussinger D, He YW, Heidenreich K, Hill JA, Hoyer-Hansen M, Hu X, Huang WP, Iwasaki A, Jaattela M, Jackson WT, Jiang X, Jin S, Johansen T, Jung JU, Kadowaki M, Kang C, Kelekar A, Kessel DH, Kiel JA, Kim HP, Kimchi A, Kinsella TJ, Kiselyov K, Kitamoto K, Knecht E, Komatsu M, Kominami E, Kondo S, Kovacs AL, Kroemer G, Kuan CY, Kumar R, Kundu M, Landry J, Laporte M, Le W, Lei HY, Lenardo MJ, Levine B, Lieberman A, Lim KL, Lin FC, Liou W, Liu LF, Lopez-Berestein G, Lopez-Otin C, Lu B, Macleod KF, Malorni W, Martinet W, Matsuoka K, Mautner J, Meijer AJ, Melendez A, Michels P, Miotto G, Mistiaen WP, Mizushima N, Mograbi B, Monastyrska I, Moore MN, Moreira PI, Moriyasu Y, Motyl T, Munz C, Murphy LO, Naqvi NI, Neufeld TP, Nishino I, Nixon RA, Noda T, Nurnberg B, Ogawa M, Oleinick NL, Olsen LJ, Ozpolat B, Paglin S, Palmer GE, Papassideri I, Parkes M, Perlmutter DH, Perry G, Piacentini M, Pinkas-Kramarski R, Prescott M, Proikas-Cezanne T, Raben N, Rami A, Reggiori F, Rohrer B, Rubinsztein DC, Ryan KM, Sadoshima J, Sakagami H, Sakai Y, Sandri M, Sasakawa C, Sass M, Schneider C, Seglen PO, Seleverstov O, Settleman J, Shacka JJ, Shapiro IM, Sibirny A, Silva-Zacarin EC, Simon HU, Simone C, Simonsen A, Smith MA, Spanel-Borowski K, Srinivas V, Steeves M, Stenmark H, Stromhaug PE, Subauste CS, Sugimoto S, Sulzer D, Suzuki T, Swanson MS, Tabas I, Takeshita F, Talbot NJ, Talloczy Z, Tanaka K, Tanida I, Taylor GS, Taylor JP, Terman A, Tettamanti G, Thompson CB, Thumm M, Tolkovsky AM, Tooze SA, Truant R, Tumanovska LV, Uchiyama Y, Ueno T, Uzcategui NL, van der Klei I, Vaquero EC, Vellai T, Vogel MW, Wang HG, Webster P, Wiley JW, Xi Z, Xiao G, Yahalom J, Yang JM, Yap G, Yin XM, Yoshimori T, Yu L, Yue Z, Yuzaki M, Zabirnyk O, Zheng X, Zhu X, Deter RL (2008) Guidelines

for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy 4(2):151–175
20. Rubinsztein DC, Cuervo AM, Ravikumar B, Sarkar S, Korolchuk V, Kaushik S, Klionsky DJ (2009) In search of an "autophagomometer". Autophagy 5(5):585–589
21. Amaravadi RK, Yu D, Lum JJ, Bui T, Christophorou MA, Evan GI, Thomas-Tikhonenko A, Thompson CB (2007) Autophagy inhibition enhances therapy-induced apoptosis in a Myc-induced model of lymphoma. J Clin Invest 117(2):326–336. doi:10.1172/JCI28833
22. White E, DiPaola RS (2009) The double-edged sword of autophagy modulation in cancer. Clin Cancer Res 15(17):5308–5316. doi:10.1158/1078-0432.CCR-07-5023
23. Yang S, Wang X, Contino G, Liesa M, Sahin E, Ying H, Bause A, Li Y, Stommel JM, Dell'antonio G, Mautner J, Tonon G, Haigis M, Shirihai OS, Doglioni C, Bardeesy N, Kimmelman AC (2011) Pancreatic cancers require autophagy for tumor growth. Genes and Developments 25(7):717–729. doi:10.1101/gad.2016111
24. Levine B, Kroemer G (2008) Autophagy in the pathogenesis of disease. Cell 132(1):27–42. doi:10.1016/j.cell.2007.12.018
25. Mathew R, Karantza-Wadsworth V, White E (2007) Role of autophagy in cancer. Nat Rev Cancer 7(12):961–967. doi:10.1038/nrc2254
26. Sato K, Tsuchihara K, Fujii S, Sugiyama M, Goya T, Atomi Y, Ueno T, Ochiai A, Esumi H (2007) Autophagy is activated in colorectal cancer cells and contributes to the tolerance to nutrient deprivation. Cancer Res 67(20):9677–9684. doi:10.1158/0008-5472.CAN-07-1462
27. Fujii S, Mitsunaga S, Yamazaki M, Hasebe T, Ishii G, Kojima M, Kinoshita T, Ueno T, Esumi H, Ochiai A (2008) Autophagy is activated in pancreatic cancer cells and correlates with poor patient outcome. Cancer Science 99(9):1813–1819. doi:10.1111/j.1349-7006.2008.00893.x
28. Pardo R, Lo Re A, Archange C, Ropolo A, Papademetrio DL, Gonzalez CD, Alvarez EM, Iovanna JL, Vaccaro MI (2010) Gemcitabine induces the VMP1-mediated autophagy pathway to promote apoptotic death in human pancreatic cancer cells. Pancreatology 10(1):19–26. doi:10.1159/000264680
29. Kundu M, Thompson CB (2008) Autophagy: basic principles and relevance to disease. Annual Review of Pathology 3:427–455. doi:10.1146/annurev.pathmechdis.2.010506.091842
30. Mizushima N, Levine B, Cuervo AM, Klionsky DJ (2008) Autophagy fights disease through cellular self-digestion. Nature 451(7182):1069–1075. doi:10.1038/nature06639
31. Mathew R, Karp CM, Beaudoin B, Vuong N, Chen G, Chen HY, Bray K, Reddy A, Bhanot G, Gelinas C, Dipaola RS, Karantza-Wadsworth V, White E (2009) Autophagy suppresses tumorigenesis through elimination of p62. Cell 137(6):1062–1075. doi:10.1016/j.cell.2009.03.048
32. Bellodi C, Lidonnici MR, Hamilton A, Helgason GV, Soliera AR, Ronchetti M, Galavotti S, Young KW, Selmi T, Yacobi R, Van Etten RA, Donato N, Hunter A, Dinsdale D, Tirro E, Vigneri P, Nicotera P, Dyer MJ, Holyoake T, Salomoni P, Calabretta B (2009) Targeting autophagy potentiates tyrosine kinase inhibitor-induced cell death in Philadelphia chromosome-positive cells, including primary CML stem cells. J Clin Invest 119(5):1109–1123. doi:10.1172/JCI35660
33. Hippert MM, O'Toole PS, Thorburn A (2006) Autophagy in cancer: good, bad, or both? Cancer Research 66(19):9349–9351. doi:10.1158/0008-5472.CAN-06-1597
34. Du J, Martin SM, Levine M, Wagner BA, Buettner GR, Wang SH, Taghiyev AF, Du C, Knudson CM, Cullen JJ (2010) Mechanisms of ascorbate-induced cytotoxicity in pancreatic cancer. Clin Cancer Res 16(2):509–520. doi:10.1158/1078-0432.CCR-09-1713
35. Newman RA, Kondo Y, Yokoyama T, Dixon S, Cartwright C, Chan D, Johansen M, Yang P (2007) Autophagic cell death of human pancreatic tumor cells mediated by oleandrin, a lipid-soluble cardiac glycoside. Integrated cancer imaging and therapy 6(4):354–364. doi:10.1177/1534735407309623
36. Mukubou H, Tsujimura T, Sasaki R, Ku Y (2010) The role of autophagy in the treatment of pancreatic cancer with gemcitabine and ionizing radiation. Int J Oncol 37(4):821–828
37. Donadelli M, Dando I, Zaniboni T, Costanzo C, Dalla Pozza E, Scupoli MT, Scarpa A, Zappavigna S, Marra M, Abbruzzese A, Bifulco M, Caraglia M, Palmieri M (2011) Gemcitabine/cannabinoid combination triggers autophagy in pancreatic cancer cells through a ROS-mediated mechanism. Cell Death and Disease 2: e152. doi:10.1038/cddis.2011.36
38. O'Donovan TR, O'Sullivan GC, McKenna SL (2011) Induction of autophagy by drug-resistant esophageal cancer cells promotes their survival and recovery following treatment with chemotherapeutics. Autophagy 7(5):509–524
39. Chen CA, Chang HH, Kao CY, Tsai TH, Chen YJ (2009) Plumbagin, isolated from *Plumbago zeylanica*, induces cell death through apoptosis in human pancreatic cancer cells. Pancreatology 9(6):797–809. doi:10.1159/000210028
40. Yan C, Shieh B, Reigan P, Zhang Z, Colucci MA, Chilloux A, Newsome JJ, Siegel D, Chan D, Moody CJ, Ross D (2009) Potent activity of indolequinones against human pancreatic cancer: identification of thioredoxin reductase as a potential target. Mol Pharmacol 76(1):163–172. doi:10.1124/mol.109.055855
41. Cho BO, Jin CH, Park YD, Ryu HW, Byun MW, Seo KI, Jeong IY (2011) Isoegomaketone induces apoptosis through caspase-dependent and caspase-independent pathways in human DLD1 cells. Biosci Biotechnol Biochem 75(7):1306–1311
42. Choi SR, Lee JH, Kim JY, Park KW, Jeong IY, Shim KH, Lee MK, Seo KI (2011) Decursin from *Angelica gigas* Nakai induces apoptosis in RC-58T/h/SA#4 primary human prostate cancer cells via a mitochondria-related caspase pathway. Food and Chemical Toxicology 49(10):2517–2523. doi:10.1016/j.fct.2011.06.016