

# Histone deacetylase inhibitors induced caspase-independent apoptosis in human pancreatic adenocarcinoma cell lines

Pilar García-Morales,<sup>1</sup> Angeles Gómez-Martínez,<sup>1</sup> Alfredo Carrato,<sup>1,2</sup> Isabel Martínez-Lacaci,<sup>1</sup> Víctor M. Barberá,<sup>2</sup> José L. Soto,<sup>2</sup> Estefanía Carrasco-García,<sup>1</sup> María P. Menéndez-Gutiérrez,<sup>1</sup> María D. Castro-Galache,<sup>1</sup> José A. Ferragut,<sup>1</sup> and Miguel Saceda<sup>1,2</sup>

<sup>1</sup>Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, and <sup>2</sup>Hospital General Universitario de Elche, Elche (Alicante), Spain

## Abstract

The antitumor activity of the histone deacetylase inhibitors was tested in three well-characterized pancreatic adenocarcinoma cell lines, IMIM-PC-1, IMIM-PC-2, and RWP-1. These cell lines have been previously characterized in terms of their origin, the status of relevant molecular markers for this kind of tumor, resistance to other antineoplastic drugs, and expression of differentiation markers. In this study, we report that histone deacetylase inhibitors induce apoptosis in pancreatic cancer cell lines, independently of their intrinsic resistance to conventional antineoplastic agents. The histone deacetylase inhibitor-induced apoptosis is due to a serine protease-dependent and caspase-independent mechanism. Initially, histone deacetylase inhibitors increase Bax protein levels without affecting Bcl-2 levels. Consequently, the apoptosis-inducing factor (AIF) and Omi/HtrA2 are released from the mitochondria, with the subsequent induction of the apoptotic program. These phenomena require AIF relocalization into the nuclei to induce DNA fragmentation and a serine protease activity of Omi/HtrA2. These data, together with previous results from other cellular models bearing the multidrug resistance phenotype, suggest a possible role of the histone deacetylase inhibitors as antineoplastic agents for the treatment of human pancreatic adenocarcinoma. [Mol Cancer Ther 2005; 4(8):1222–30]

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**Requests for reprints:** Miguel Saceda, Instituto de Biología Molecular y Celular, Ed. Torregaitan, Universidad Miguel Hernández, 03202 Elche (Alicante), Spain. Phone: 34-96-665-8432; Fax: 34-96-665-8758. E-mail: msaceda@umh.es

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## Introduction

Pancreatic adenocarcinoma is associated with short survival due to several reasons: a delayed diagnosis that makes the tumor nonresectable at detection time, its metastatic potential, and the lack of an effective therapy. Many anticancer agents have been used for the treatment of pancreatic adenocarcinoma, as single chemotherapeutic agents, or in combination regimens (1, 2). Although gemcitabine has been approved as standard therapy for local and metastatic pancreatic adenocarcinoma, based on the clinical benefits observed (3–7), there is not an effective chemotherapy that increases survival in this disease. The use of novel therapeutic approaches at the molecular level, like gene therapy protocols, has been evaluated in the laboratory with discrete results. Due to its frequent mutation in pancreatic cancer, *K-ras* represents an attractive gene target and in fact, antisense constructs (8, 9), dominant-negative mutants (10, 11), and ribozymes (12) targeting *K-ras* have been tested in pancreatic cancer models. Replacement of mutated *p53* with wild-type *p53* by retroviral and adenoviral gene transfer (13, 14), gene transfer of the tumor necrosis factor receptor (15), herpes virus thymidine kinase (16), or other suicidal genes such as cytosine deaminase (17) have been also evaluated. New strategies for pancreatic cancer treatment have to be developed until the treatment at the molecular level becomes a reality.

Recently, it has been shown that regulation of the chromatin structure through modifications of histones by acetylation/deacetylation represents a very important control of gene transcription (18–20). In fact, histone hyperacetylation by specific histone deacetylase inhibitors may critically affect processes such as apoptosis (21–23), cell proliferation (24), and differentiation (25, 26). Particular molecular targets affected by acetylation/deacetylation include the tumor suppressor gene *p53*, which regulates cell growth and apoptosis (27).

We have recently reported that the histone deacetylase inhibitors trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA), dramatically reduce cell viability and promote apoptosis in different drug-resistant cell lines (28). Because pancreatic adenocarcinoma is known to be intrinsically resistant, we decided to investigate whether histone deacetylase inhibitors could show efficacy against this disease. We observed that TSA and SAHA dramatically reduced cell viability and promoted apoptosis in three different pancreatic human adenocarcinoma cell lines, IMIM-PC-1, IMIM-PC-2, and RWP-1 (29). In addition, we have investigated the mechanisms involved in the histone deacetylase inhibitor-induced apoptosis. Our data suggest that histone deacetylase inhibitors might have a therapeutic potential against pancreatic adenocarcinoma.

## Materials and Methods

### Cell Lines and Culture

IMIM-PC-1, IMIM-PC-2, and RWP-1 pancreatic human adenocarcinoma cell lines were kindly donated by Dr. Francisco X. Real (IMIM, Barcelona, Spain). These cell lines have been characterized in terms of their origin; IMIM-PC-1 and IMIM-PC-2 were derived from primary tumors and RWP-1 from a liver metastasis. They have also been studied in terms of several pancreatic adenocarcinoma relevant molecular markers and all of them show mutated *p53* and *K-ras* and no expression of *p16* (29). In addition, we have previously determined their resistance to anthracyclines, showing a different degree of resistance (IMIM-PC-1 > RWP-1 > IMIM-PC-2); as well as their resistance to alkylating agents as determined by the level of expression of *O*<sup>6</sup>-methylguanine methyl transferase, being IMIM-PC-1 the more resistant and RWP-1 the less resistant. The level of expression of resistance-related genes such as *MDR-1* (IMIM-PC-2 > RWP-1 > IMIM-PC-1) and *MRP-1* (IMIM-PC-1 > IMIM-PC-2 > RWP-1) have also been analyzed, as well as the level of expression of differentiation markers such as *MUC-1* (IMIM-PC-2 > IMIM-PC-1 > RWP-1; ref. 29). Cells were grown at 37°C in 5% CO<sub>2</sub>, with DMEM (Life Technologies, Invitrogen, Carlsbad CA) supplemented with 10% FCS (Cambrex Bio Science, Verviers, Belgium), 2 mmol/L L-glutamine (Life Technologies, Invitrogen), 1 mmol/L sodium pyruvate (Life Technologies, Invitrogen), 50 units/mL penicillin, and 50 µg/mL streptomycin (Life Technologies, Invitrogen).

### Flow Cytometric Analysis of Cell Cycle Distribution

IMIM-PC-1, IMIM-PC-2, and RWP-1 cell lines were incubated with TSA or SAHA at selected times up to 24 hours. After harvesting the cells,  $1 \times 10^6$  cells were centrifuged and washed with cold 10 mmol/L phosphate buffer (pH 7.4) supplemented with 2.7 mmol/L KCl and 137 mmol/L NaCl (PBS), and centrifuged again. The pelleted cells were resuspended in 75% cold ethanol, fixed for 1 hour at -20°C, centrifuged, and resuspended in 0.5 mL of PBS supplemented with 0.5% Triton X-100 and 0.05% RNase A. Then, cells were incubated for 30 minutes at room temperature, stained with propidium iodide, and the distribution of cellular DNA content was analyzed by flow cytometry in an Epics XL flow cytometer (Beckman Coulter Co., Miami, FL). Nonviable cells were excluded from the analysis on the basis of their abnormal size.

### Apoptosis Determination

Pancreatic cell lines were treated with histone deacetylase inhibitors in the presence and absence of cell-permeable caspase and serine protease inhibitors at selected times up to 24 hours and then fixed with methanol for 1 hour at -20°C, washed with PBS, incubated with Hoechst 33342 (3 µg/mL) for 30 minutes at room temperature in the dark, and washed again with PBS. Cells were analyzed by fluorescence microscopy. In addition, fluorescent apoptotic cells were visualized by staining with an Annexin V-FITC commercial kit (Calbiochem, Darmstadt, Germany) under a fluorescent light microscope.

### Determination of Caspase Activity

Caspase-8, -9, and -3 activities were determined using a colorimetric kit from Calbiochem (Caspase-8, -9, and -3/Mch6 activity kits).

The inhibition of caspase activity was done by addition of specific inhibitors 1 hour before the apoptosis induction by treatment with TSA. The inhibitors used were inhibitor of caspase-8 II (Z-IETD-FMK, Calbiochem), inhibitor of caspase-9 I (Z-LEHD-FMK, Calbiochem), inhibitor of caspase-3 I (DEVD-CHO, Calbiochem), and a general caspase inhibitor (Z-VAD-FMK, Calbiochem).

### Western Blot Analysis

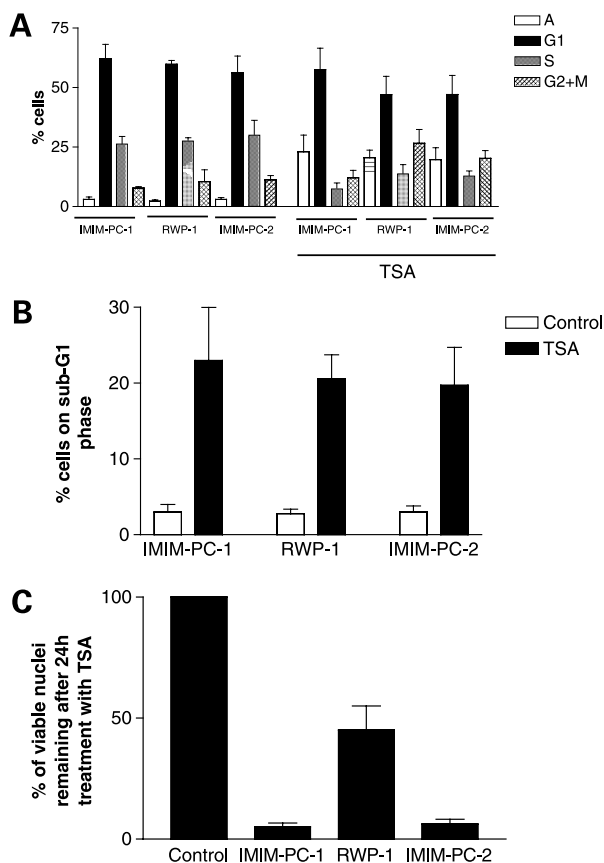
Treated or untreated IMIM-PC-1, IMIM-PC-2, and RWP-1 cells were washed twice with PBS. After scraping the cells with PBS, they were centrifuged at 2,500 rpm for 5 minutes. Cellular pellet was resuspended in radioimmunoprecipitation assay buffer [150 nmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 nmol/L Tris (pH 8)] and proteases and phosphatase inhibitors were added. Cells were kept on ice for 30 minutes and centrifuged at 14,000 rpm for 15 minutes at 4°C. The cellular pellet was discarded, and the protein content of the cell extract (supernatant) was determined using the Bradford method (Bio-Rad, Richmond, CA). Bcl-2, Bax, p21, apoptosis-inducing factor (AIF), poly(ADP-ribose) polymerase (PARP-1), and β-actin expression was determined by Western blot using specific monoclonal antibodies followed by enhanced chemiluminescence (Amersham International, Buckinghamshire, United Kingdom) to develop protein bands.

## Results

### Determination of Apoptosis Induction by Histone Deacetylase Inhibitors in Pancreatic Adenocarcinoma Cell Lines

We have investigated whether histone deacetylase inhibitors were able to induce apoptosis in the three pancreatic cell lines. The cell cycle distribution of these cell lines was studied through determination of the DNA content by flow cytometry, after staining the cells with propidium iodide. Results in Fig. 1A show that treatment with 1 µmol/L TSA induced a decrease of the number of cells in the S phase of the cell cycle in the three cell lines. At the same time, an increase in the sub-G<sub>1</sub> peak was observed, suggesting that the three cell lines underwent apoptosis after TSA treatment (Fig. 1A and B). Similar results were obtained by treatment with SAHA, suggesting that these effects are due to the activity of these compounds as histone deacetylase inhibitors. Apoptosis was also shown by Hoestch 33342 staining, as shown in Fig. 1C, where the viable and not fragmented nuclei present in the three cell lines after 24 hours of treatment with 1 µmol/L TSA were counted, and the number of nuclei was represented as percentage of the viable nuclei in the untreated cell lines.

To confirm that TSA and SAHA were in fact able to induce apoptosis in the three cell lines, time course experiments were done. The three cell lines were treated



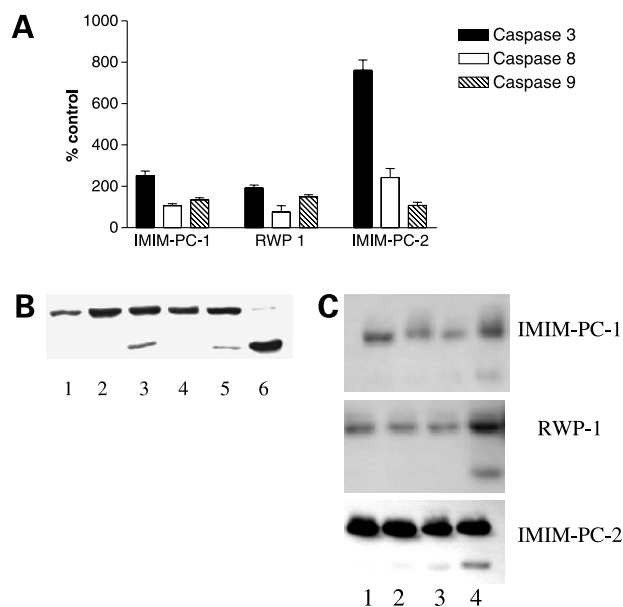
**Figure 1.** **A**, cell cycle distribution determined by flow cytometry in IMIM-PC-1, IMIM-PC-2, and RWP-1 pancreatic cancer cell lines in response to TSA (1  $\mu\text{mol/L}$ ) treatment. Percentage of the different phases of the cell cycle after TSA treatment minus control. **B**, percentage of cells in sub-G<sub>1</sub> phase after 24 h of treatment with 1  $\mu\text{mol/L}$  TSA. **Columns**, mean of four independent experiments; **bars**,  $\pm$ SD. **C**, nuclei from treated or untreated cells with 1  $\mu\text{mol/L}$  TSA for 24 h were stained with Hoechst 33342 and visualized under fluorescence microscopy; viable and not fragmented nuclei were counted in treated and untreated cells. Percentage of the viable nuclei in the untreated cells. **Columns**, mean of three independent experiments with four random selected fields counted per sample and per experiment; **bars**,  $\pm$ SD.

with TSA or SAHA for different times, ranging from 3 to 48 hours, and then stained with FITC-Annexin and propidium iodide or Hoechst 33342 and visualized under a fluorescence microscope. Results are shown in Supplementary Fig. 1<sup>3</sup> and they show that TSA and SAHA induced a massive apoptosis in the three cell lines. After 24 hours of treatment, Annexin-positive cells were predominant. By contrast, after 48 hours of treatment, very few cells remained in the dish and most of them were positive for propidium iodide. In addition, nuclei fragmentation was visible with either propidium iodide or Hoechst 33342. Apoptosis was also evident by superimposing phase contrast and Hoechst 33342 staining.

<sup>3</sup>Supplementary Material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

### Analysis of the Apoptosis Mechanisms Induced by the Histone Deacetylase Inhibitors in Pancreatic Adenocarcinoma Cell Lines

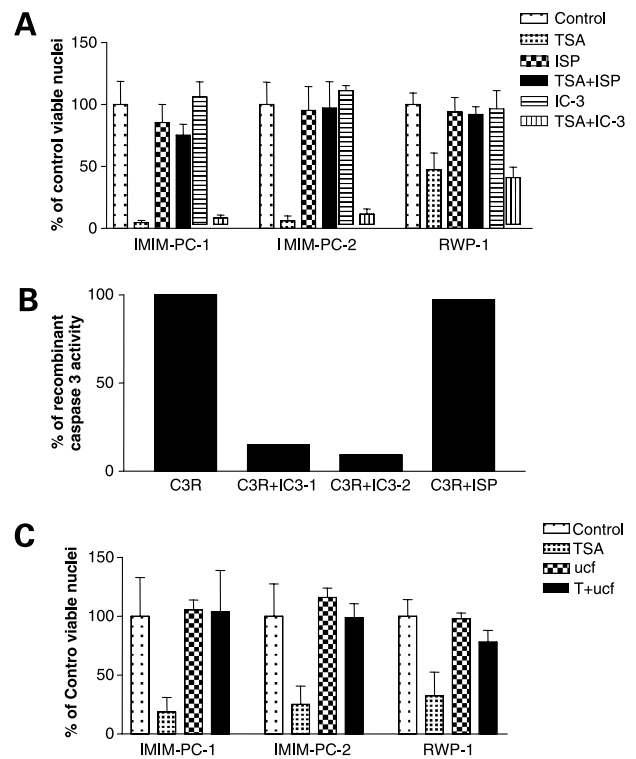
To investigate the apoptosis induction pathways used by histone deacetylase inhibitors in these cell lines, we initially determined the role of different cysteine proteases in the TSA-induced apoptosis. The three cell lines were treated with TSA for 24 hours, and the activity of caspases 3, 8, and 9 were determined by a colorimetric assay and compared with the activity obtained in cell extracts from control nontreated cells. Results in Fig. 2A, show that caspase-3 activity was slightly increased in IMIM-PC-1 and RWP-1 cells but significantly increased in IMIM-PC-2 cells after TSA treatment. Meanwhile, caspase-8 and -9 activities were not significantly increased in any of the cell lines. Because caspase-3 was apparently the only one affected by TSA treatment, we determined the activity of caspase-3 studying PARP-1 processing. As a control of this experiment, we used the murine leukemia cell lines L1210 and L1210R, because L1210R is sensitive to TSA-induced apoptosis and L1210 is resistant to TSA (28). Results in Fig. 2B and C show that PARP-1 is only partially cleaved in the pancreatic cell lines, to the same extent as in the TSA-resistant L1210 cell line but significantly less than in the TSA-sensitive L1210R. The results of three independent experiments were analyzed by optical densitometry and show that only a



**Figure 2.** **A**, TSA (1  $\mu\text{mol/L}$ ) effect on caspase -3, -8, and -9 activities in IMIM-PC-1, IMIM-PC-2, and RWP-1 pancreatic cancer cell lines. Caspase activities were determined by specific colorimetric assays. Normalized to control untreated cells. **B**, TSA (1  $\mu\text{mol/L}$ ) effect on PARP-1 cleavage in L1210 and L1210R murine leukemia cell lines determined by Western blot. **Lanes 1–3**, L1210 control cells and cells treated with TSA for 6 or 24 h, respectively. **Lanes 4–6**, L1210R cells control and treated with TSA for 6 or 24 h, respectively. **C**, TSA (1  $\mu\text{mol/L}$ ) effect on PARP-1 cleavage in IMIM-PC-1, IMIM-PC-2, and RWP-1 pancreatic cancer cell lines determined by Western blot. **Lanes 1–4**, control cells and cells treated with TSA for 3, 6, and 24 h, respectively.

4.6 ± 2%, a 25.7 ± 3.2%, and a 10.6 ± 5.3% of PARP-1 was processed in the IMIM-PC-1, RWP-1, and IMIM-PC-2 cell lines respectively, after 24 hours of treatment with TSA. Meanwhile a 98 ± 3.6% of PARP-1 was processed in the L1210R cell lines under the same treatment with TSA. To further determine the role of the different caspases and their relationship in the three cell lines, cells were treated with TSA in the presence and absence of cell permeable inhibitors of such caspases. Results in Fig. 3A show that the inhibition of the caspase-3 activity by the specific inhibitor IC-3 was unable to block the TSA-induced apoptosis in the pancreatic carcinoma cell lines. Neither inhibition of TSA-induced apoptosis was observed when caspase-8- and -9-specific inhibitors were used (data not shown). Furthermore, a general caspase inhibitor was unable to block TSA-induced apoptosis in these cell lines as shown in Supplementary Fig. 2A,<sup>3</sup> suggesting the TSA-mediated activation of a caspase-independent apoptotic pathway. As a control of the caspase inhibitor activity, the caspase-3 activity that remained after treatment with these inhibitors was determined and compared with the caspase activity in the TSA-treated cell extracts. Our results show that the caspase-3 inhibitor was able to block a 75 ± 11%, a 86.8 ± 8.6%, and a 87.6 ± 3.5% of the caspase-3 activity increase observed in the IMIM- PC-1, RWP-1, and IMIM-PC-2 cell lines treated for 24 hours with TSA, respectively. In addition, we have also shown that the specific caspase-3 inhibitor was able to block daunomycin-induced apoptosis in HL-60 cells (data not shown). Furthermore, we have determined the cytochrome *c* release from the mitochondria in the three cell lines. Our data show that there is not a significant cytochrome *c* release in the IMIM-PC-1 and IMIM-PC-2 cell lines and only a modest increase in RWP-1 cells was observed (Supplementary Fig. 3).<sup>3</sup> In addition, and as a positive control for caspase inhibitor activity, the three cell lines were treated with staurosporine to induce a caspase-dependent apoptosis. Data in Supplementary Fig. 4A<sup>3</sup> show that staurosporine (1–5 μmol/L) was able to induce apoptosis in the RWP-1 and IMIM-PC-2 cell lines, whereas the IMIM-PC-1 cell line remains resistant to the staurosporine-induced apoptosis. The general caspase inhibitor Z-VAD-FMK (25–100 μmol/L) was able to block the staurosporine-induced apoptosis in the IMIM-PC-2 cell line but not in the RWP-1 cell line. The differential effects of the general caspase inhibitor in RWP-1 and IMIM-PC-2 cells was reflected in the caspase-3 induction evoked by staurosporine in the three cell lines, because an increase of the caspase-3 activity after staurosporine treatment was observed only in the IMIM-PC-2 cell line, as shown in the Supplementary Fig. 4B.<sup>3</sup> Interestingly, staurosporine was able to induce apoptosis in the RWP-1 cell line without increasing caspase-3 activity, suggesting that this compound is able to induce apoptosis through a caspase-independent mechanism in this cell line, as it was previously shown in other cellular models (30).

To further show the caspase-independent nature of the histone deacetylase inhibitor-induced apoptosis in this cell model, we tested whether serine protease inhibitors



**Figure 3.** **A**, determination of cell viability in IMIM-PC-1, IMIM-PC-2, and RWP-1 pancreatic cancer cell lines after TSA (1 μmol/L) treatment in the presence and absence of the serine protease inhibitor (ISP) 4-(2-aminoethyl) benzenesulfonylfluoride and in the presence and absence of a caspase-3 inhibitor (IC-3). Hoechst 33342 stained nuclei were counted by fluorescence microscopy. Four different fields were counted for each condition in three independent experiments; mean and SD were then calculated and represented. **B**, caspase-3 activity was determined by a colorimetric assay using caspase-3 – recombinant protein and the specific caspase-3 inhibitors and the serine protease inhibitor 4-(2-aminoethyl) benzenesulfonylfluoride. **C**, determination of cell viability in IMIM-PC-1, IMIM-PC-2, and RWP-1 pancreatic cancer cell lines after TSA (1 μmol/L) treatment in the presence and absence of the omi/HTR2 inhibitor ucf-101. Hoechst 33342 – stained nuclei were counted by fluorescence microscopy. Four different fields were counted for each condition in three independent experiments; mean ± SD were then calculated and represented.

(that have been shown to inhibit apoptosis in systems where caspases are not playing a major role; ref. 31) were able to block the histone deacetylase inhibitor-induced apoptosis in the pancreatic cell lines. Results in Fig. 3A show that the serine protease inhibitor 4-(2-aminoethyl) benzenesulfonylfluoride was able to block the massive cell death induced by TSA in the three cell lines. We further tested the ability of this serine protease inhibitor to inhibit the caspase-3 activity in an *in vitro* assay using recombinant caspase-3. As shown in Fig. 3B, the serine protease inhibitor was unable to inhibit caspase-3 activity showing that this inhibitor is not acting in an unspecific manner. Because it has been reported that Omi/HtrA2, a human serine protease, is released from the mitochondria and participates in apoptosis induction, either through a caspase-dependent or a caspase-independent mechanism (the latter being entirely dependent on its ability to

function as a serine protease; ref. 32), we investigated whether the histone deacetylase inhibitor-induced apoptosis in the three pancreatic adenocarcinoma cell lines was inhibited by a recently developed specific Omi/HtrA2 inhibitor (33). Results in Fig. 3C show that the Omi/HtrA2 inhibitor ucf-101 was able to block the cell death induced by TSA in the three cell lines.

In addition, and using immunocytochemistry, we have been able to show that AIF, a mitochondrial protein associated with caspase-independent apoptosis pathways, is in fact translocated to the nucleus in the three cell lines after TSA treatment (Fig. 4). Moreover, TSA treatment induced a dramatic increase in AIF protein levels as determined by Western blot (Fig. 5A-C).

To determine whether the Bcl-2 family members were playing a role in the TSA-induced apoptosis in these cell lines, we analyzed Bcl-2 expression levels by Western blot because it has been shown that Bcl-2 overexpression is able to block histone deacetylase inhibitor-induced apoptosis (34). We also analyzed Bax expression levels because this proapoptotic member of the Bcl-2 family has been associated with caspase-independent apoptosis (35). Results in (Fig. 5A-C) show that TSA did not modify significantly the levels of Bcl-2 in any of the three cell lines. However, the level of expression of Bax was dramatically increased in the three cell lines after treatment with TSA. Furthermore, the Bcl-2/Bax ratio significantly changed after TSA exposure in all the cell lines. Taken together, our results suggest that the TSA-mediated regulation of the proapoptotic members of the Bcl-2 family may play a role in the TSA-induced apoptosis in these cell lines.

In addition, we have also studied the effect of TSA on the levels of p21, because p21 has been previously related to the histone deacetylase inhibitor-induced apoptosis in different cellular models. Results in Fig. 5A-C clearly show that TSA does not affect p21 protein levels in any of the three cell lines. However, p21 is increased after TSA treatment in PANC-1, another pancreatic adenocarcinoma cell line, as shown in Fig. 5D. Figure 6 shows the changes in Bcl-2, Bax, AIF, and p21 expression as determined by optical densitometry. Results are presented as the ratio of each protein versus  $\beta$ -actin expression in three independent experiments.

## Discussion

Pancreatic cancer has a very poor prognosis as a consequence of a late diagnosis and its aggressive metastatic potential. Despite of increasing efforts to find new agents or to define new chemotherapeutic combinations, many cytotoxic agents have proven to be ineffective in the treatment of this malignancy (reviewed in ref. 1). There is an urgent necessity for the development of new therapeutic agents with significant survival effect on the course of this disease.

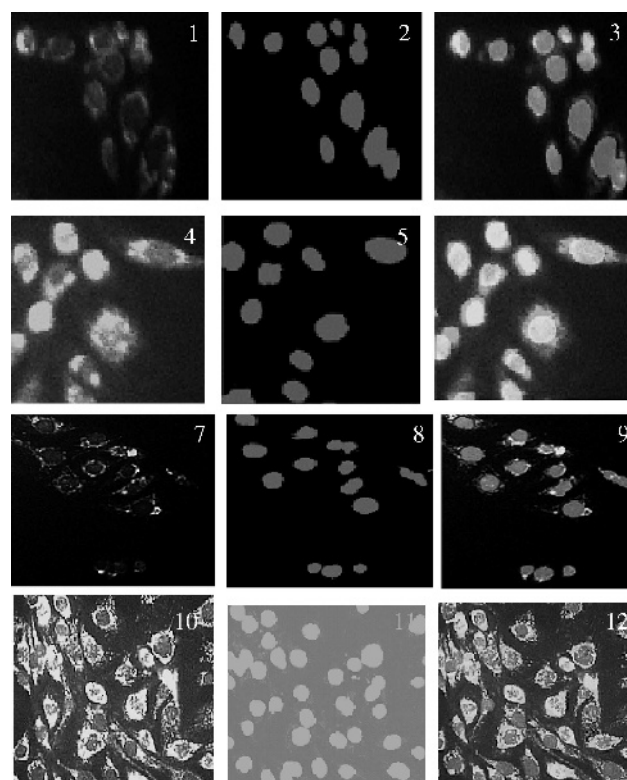
Because histone deacetylase inhibitors are being tested as new antineoplastic agents (36, 37), we have analyzed the effects of two histone deacetylase inhibitors, TSA and SAHA, in three different human pancreatic adenocarci-

noma cell lines, IMIM-PC-1, IMIM-PC-2, and RWP-1. The aim of our study was to determine whether histone deacetylase inhibitors can be considered as effective antitumor drugs against pancreatic cancer.

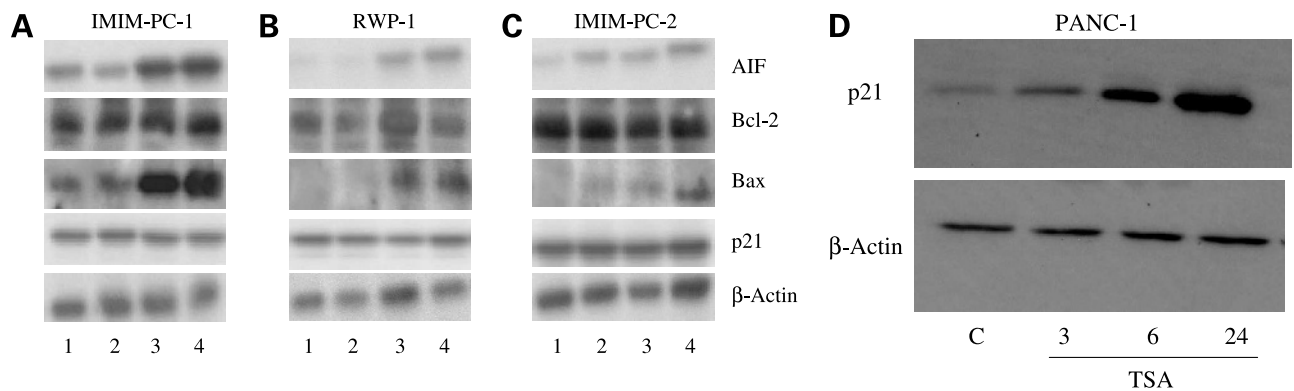
Results from our group and others (28, 38, 39) have shown that histone deacetylase inhibitors were able to induce apoptosis in drug-resistant tumor cells from different tissues. The results presented herein show that histone deacetylase inhibitors were able to induce massive apoptosis in the three pancreatic adenocarcinoma cell lines, independently of their intrinsic resistance to other anticancer drugs.

Histone deacetylase inhibitors induced a dramatic and massive apoptosis in the three cell lines tested. Apoptosis was shown by Annexin-FITC staining and by analysis of cell cycle distribution using flow cytometry. Following treatment with histone deacetylase inhibitors in the three cell lines, we observed a significant increase in the sub-G<sub>1</sub> population.

We have also investigated the apoptotic mechanisms induced by the histone deacetylase inhibitors. To determine the pathways involved in the apoptosis induction, we used an experimental approach that detected simultaneously



**Figure 4.** AIF protein localization in pancreatic adenocarcinoma cell lines treated and untreated with TSA for 6 h. 1–3, IMIM-PC-1 untreated cells, AIF staining (1), nuclei staining with Hoechst 33342 (2), and overlay staining (3). 4–6, IMIM-PC-1 cells treated with 1  $\mu$ mol/L TSA for 6 h, AIF staining (4), nuclei staining with Hoechst 33342 (5), and overlay staining (6). 7–9, IMIM-PC-2 untreated cells, AIF staining (7), nuclei staining with Hoechst 33342 (8), and overlay staining (9). 10–12, IMIM-PC-2 cells treated with 1  $\mu$ mol/L TSA for 6 h, AIF staining (10), nuclei staining with Hoechst (11), and overlay staining (12).



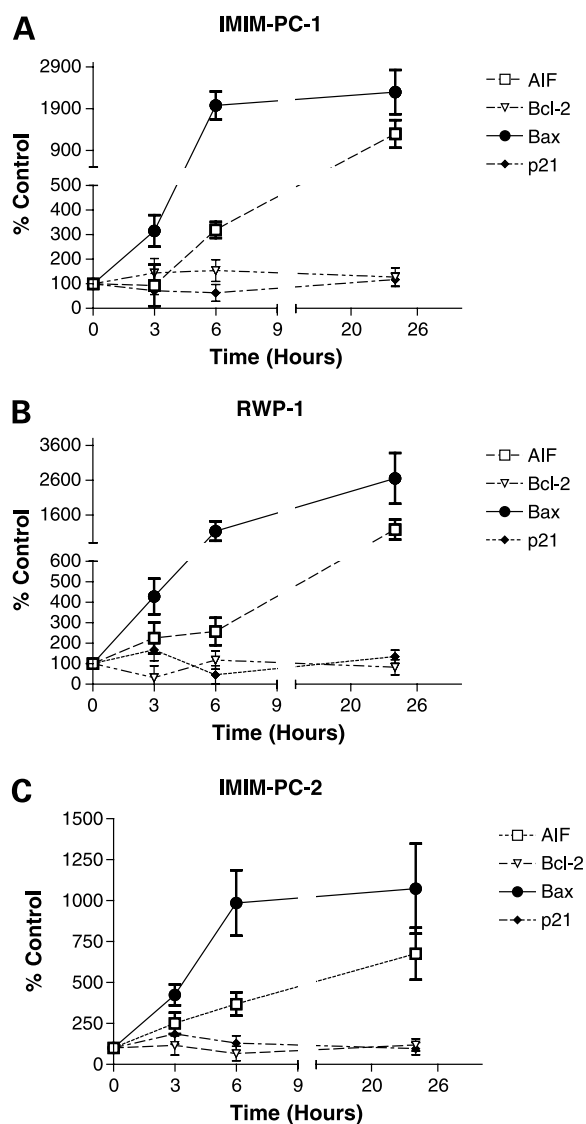
**Figure 5.** AIF, Bcl-2, Bax, p21, and  $\beta$ -actin expression levels determined by Western blot in IMIM-PC-1 (A), RWP-1 (B), and IMIM-PC-2 (C) pancreatic cancer cell lines after TSA (1  $\mu$ mol/L) treatment. Lane 1, control untreated cells. Lanes 2–4, TSA-treated cells for 3, 6, and 24 h, respectively. D, p21 expression levels determined by Western blot in PANC-1 pancreatic adenocarcinoma cell line after TSA (1  $\mu$ mol/L) treatment.

caspase-3, -8, and -9 activities in cellular extracts after TSA and SAHA treatment. This experimental approach is based on the fact that caspase-3 activity has been related to histone deacetylase inhibitor-induced apoptosis in other systems as an executioner caspase (40, 41). It is also known that caspase-3 activation is usually a consequence of the activation of other initiation caspases. For instance, caspase-8, a member of the death receptor apoptosis pathway, is able to activate caspase-3. In fact, it has been reported that histone deacetylase inhibitors are able to increase the expression of FAS and FAS ligand in other systems (21, 23, 42). Additionally, caspase-9 that is activated as a consequence of the mitochondrial pathway of apoptosis induction is able to activate caspase-3. Our results show that there is only a slight induction of caspase-3 activity in two of the three cell lines and that no statistically significant increase in caspases 8 and 9 was found in any of the cell lines. Taken together, our data raised the possibility that the TSA-mediated apoptosis could be due to the activation of a caspase-independent apoptotic pathway, as previously suggested in other cellular models (43, 44).

It is also quite significant that PARP-1 is only partially cleaved after TSA treatment in the three cell lines. To further show the possible induction of a caspase-independent pathway, several experiments with caspase-specific inhibitors were done. We have used specific inhibitors for the three caspases (caspases 3, 8, and 9), although caspase-3 was the only significantly increased in two of the three cell lines after histone deacetylase inhibitor treatment. Our data clearly show that none of the caspase inhibitors alone or in combination were able to block TSA-induced apoptosis in these cell lines. The lack of an effect of the caspase inhibitors was not due to an inappropriate use of the inhibitors, because we have determined the caspase activity remaining in the cells and, as shown in Fig. 3, the caspase-3 inhibitor was able to abrogate the TSA-induced increase in caspase-3 activity in the three cell lines without affecting the TSA-induced

apoptosis. On the other hand, we have also tested all the inhibitors with the appropriate positive controls and we have been able to block apoptosis induced by daunomycin and other agents in HL-60 cells. It is also quite significant that cytochrome *c* is not released from the mitochondria after TSA treatment (Supplementary Fig. 3).<sup>3</sup> In that sense, there is an increasing number of reports about apoptosis induction by different stimuli, showing that AIF is released from the mitochondria before cytochrome *c* release, or even without cytochrome *c* release, suggesting that AIF and cytochrome *c* release are mediated by independent mechanisms (45, 46). Furthermore, we have also shown that a serine protease inhibitor able to block some caspase-independent pathways was able to completely abrogate the histone deacetylase inhibitor-induced apoptosis in the three cell lines. Our data are in agreement with a previous report showing that caspase inhibitors were unable to block histone deacetylase inhibitor-induced apoptosis (43, 44). Even more conclusive is the putative identification of Omi/HtrA2 as the serine protease mediating TSA-induced apoptosis in these cell lines, because Omi/HtrA2 has been reported to participate in caspase-independent apoptosis, being its effects in this case completely dependent of its serine protease activity (31).

A recent report claims that histone deacetylase inhibitor-induced apoptosis is mediated by BID and caspase-3 in a p53-dependent pathway (34). Several considerations in this respect can be made. It is very unlikely that histone deacetylase inhibitor-induced apoptosis could be a p53-dependent phenomenon in all the systems because histone deacetylase inhibitors are able to induce apoptosis in the three different pancreatic carcinoma cell lines that bear mutant p53. Furthermore, we and others have previously reported apoptosis induction by histone deacetylase inhibitors in colon carcinoma cell lines (28), harboring also a mutant p53. Even more conclusive is the case of HL-60, a human promyelocytic leukemia cell line that is sensitive to the histone deacetylase inhibitor-induced apoptosis, which is a p53-null cell line (47).



**Figure 6.** Optical densitometry analysis of AIF, Bcl-2, Bax, and p21 expression levels determined by Western blot in IMIM-PC-1 (A), RWP-1 (B), and IMIM-PC-2 (C) pancreatic cancer cell lines after TSA (1  $\mu\text{mol/L}$ ) treatment. Points, mean of four independent determinations; bars,  $\pm$ SD.

Taken in consideration our data and the controversial data found in the literature, we suggest that histone deacetylase inhibitors induce apoptosis by at least two mechanisms, one of them caspase dependent and the other one caspase independent, being the caspase-independent mechanism dominant in the pancreatic adenocarcinoma cell lines. This model has been observed with other apoptotic stimuli in thymocytes (48). In this case, the caspase-independent mechanism was due to the AIF. We have studied the role of AIF in the histone deacetylase inhibitor-induced apoptosis in pancreatic carcinoma cell lines. Our data clearly show that histone deacetylase inhibitor treatment increases dramatically the

levels of AIF in the three cell lines. Furthermore, we have also found that AIF can be visualized in the cell nucleus in these cell lines after TSA treatment. Taken together, our data clearly support a caspase-independent apoptosis mediated by AIF as the mechanism of histone deacetylase inhibitor-induced apoptosis in pancreatic adenocarcinoma cell lines. This conclusion was further supported after Bcl-2 and Bax expression analysis in response to TSA treatment in these cell lines. We have clearly showed that TSA treatment increased dramatically the protein levels of Bax, without modifying Bcl-2 protein levels. In other systems, Bax has been associated with caspase-independent apoptosis (35). On the other hand, the changes observed in the Bcl-2/Bax ratio as a consequence of the TSA treatment could explain the release of AIF and Omi/HtrA2 from the mitochondria.

Recently, Donadelli et al. have described the effect of TSA on nine pancreatic adenocarcinoma cell lines (49). Interestingly, the nine cell lines used in that study were different from the ones used in this report, which makes our results even stronger because we can conclude that at least 12 different pancreatic adenocarcinoma cell lines undergo apoptosis after TSA and other histone deacetylase inhibitors treatment. However, we have discrepancies according to the mechanism involved in the histone deacetylase inhibitor-induced apoptosis. First, the authors of that report suggest that apoptosis induction by TSA is due to a transcriptional increase in p21. However, as we show in Fig. 5, p21 protein levels remain unchanged in the three cell lines tested after histone deacetylase inhibitor treatment. As a control, we decided to study p21 expression in one of the cell lines used by Donadelli et al. In the PANC-1 cell line, we observed that in fact, p21 was clearly up-regulated after TSA treatment (Fig. 5D). Taken together, our data suggest that an increase in p21 is not an absolute requirement for the histone deacetylase inhibitor-induced apoptosis in these pancreatic adenocarcinoma cell lines. This statement is in agreement with previous reports that show that p21 up-regulation is not necessary for histone deacetylase inhibitor apoptosis induction (50). A second discrepancy is the increase in caspase-3 activity that they associate with the TSA-induced apoptosis in these cell lines. Whereas specific caspase inhibitors are unable to block TSA-induced apoptosis in our cell lines, serine protease inhibitors completely block this apoptosis induction. No data using specific caspase inhibitors were used by Donadelli et al. (49). We used again the PANC-1 cell line to determine the caspase-3 activity evoked by TSA treatment. In agreement with Donadelli et al., we found an increase in caspase-3 activity after TSA treatment (Supplementary Fig. 2B).<sup>3</sup> However, we also determined the TSA-induced apoptosis in the presence and absence of high concentration (100  $\mu\text{mol/L}$ ) of a general caspases inhibitor in our three cell lines as well as in the PANC-1 cell line. Data in the Supplementary Fig. 2A<sup>3</sup> show that independently of the increase in caspase-3 activity, the TSA-induced apoptosis is not blocked by the caspase inhibitor in any of the four

cell lines. Interestingly, in the four cell lines the serine protease inhibitor 4-(2-aminoethyl) benzenesulfonylfluoride is able to block the apoptosis induced by TSA (Supplementary Fig. 2A).<sup>3</sup> Taking together all these data, we believe that the TSA-induced apoptosis in the pancreatic adenocarcinoma cell lines is mostly a caspase-independent phenomenon.

The results presented herein show that histone deacetylase inhibitors inhibit cell growth and induce apoptosis in pancreatic cancer cell lines through a caspase-independent mechanism. These data, together with previous studies in other cellular (28, 38, 39) and animal cancer models (36, 37, 51), suggest that the histone deacetylase inhibitors could be useful as antineoplastic agents for the treatment of human pancreatic adenocarcinoma.

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