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Title: VPA is more effective than TSA in the induction of pancreatic cancer cell death by inhibiting ERK activation and reducing mutant p53 expression.

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Abstract: Histone deacetylase inhibitors (HDACis) are antineoplastic agents that affect cell growth, differentiation and invasion in a variety of different cancer types, although the underlying mechanisms have not been completely clarified yet. In this study, we compared the effects of two HDACis, namely Trichostatin A (TSA) and Valproic Acid (VPA) in the induction of both cell death and autophagy in the pancreatic cancer cell lines PaCa44 and Panc1. These cells are characterized by a high metastatic capacity and display k-RAS/p53 double mutation. We found that both HDACis reduced cell survival and induced pancreatic cancer cells apoptosis by triggering mitochondrial membrane depolarization, cytochrome C release and caspase 3 activation, although VPA was more effective than TSA, especially in Panc1. Among the underlying molecular mechanism/s leading to the HDACis-mediated cytotoxic effect, we found that ERK1/2 was de-phosphorylated and c-Myc and mutant p53 protein levels were reduced by VPA and to a lesser extent by TSA. Up-regulation of p21 and Puma was also observed, suggesting a possible wild-type p53 re-activation, concomitantly with mutant p53 degradation. In addition, in both cell lines VPA increased the pro-apoptotic Bim, reduced the anti-apoptotic Mcl-1, induced ROS production and autophagy while TSA was able to mediate these effects only in PaCA44 cells. These results suggest that VPA, that specifically inhibits class I HDAC, may have a stronger and broader cytotoxic effect in comparison with TSA against pancreatic cancer and could represent a better choice in pancreatic anticancer therapy, alone or in combination with other drugs.

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BBA Molecular Cell research

Prof Lienhard Schmitz, Executive Editor

Dear Prof. Schmitz, please find enclosed our MS entitled “VPA is more effective than TSA in the induction of pancreatic cancer cell death by inhibiting ERK activation and reducing mutant p53 expression” by Gilardini Montani et al.

I hope you will find it suitable for publication in BBA Molecular Cell Research.

The manuscript is original and has not been submitted for publication elsewhere.

Kind Regards

Mara Cirone

1 VPA is more effective than TSA in the induction of pancreatic cancer cell death by inhibiting ERK
2 activation and reducing mutant p53 expression.

3

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19

20 *Running title:* VPA and TSA inhibit ERK activation, down-regulate mutant p53 and induce pancreatic
21 cancer cell death.

22 *Abbreviations:* Histone Deacetylase, HDAC; Histone Deacetylase inhibitors, HDACis; Valproic acid,
23 VPA; Trichostatin A, TSA; Extracellular Receptor Kinase, ERK; Reactive Oxygen Species, ROS.

24 **Abstract**

25 Histone deacetylase inhibitors (HDACis) are antineoplastic agents that affect cell growth,
26 differentiation and invasion in a variety of different cancer types, although the underlying mechanisms
27 have not been completely clarified yet. In this study, we compared the effects of two HDACis, namely
28 Trichostatin A (TSA) and Valproic Acid (VPA) in the induction of both cell death and autophagy in the
29 pancreatic cancer cell lines PaCa44 and Panc1. These cells are characterized by a high metastatic
30 capacity and display k-RAS/p53 double mutation. We found that both HDACis reduced cell survival
31 and induced pancreatic cancer cells apoptosis by triggering mitochondrial membrane depolarization,
32 cytochrome C release and caspase 3 activation, although VPA was more effective than TSA, especially
33 in Panc1. Among the underlying molecular mechanism/s leading to the HDACis-mediated cytotoxic
34 effect, we found that ERK1/2 was de-phosphorylated and c-Myc and mutant p53 protein levels were
35 reduced by VPA and to a lesser extent by TSA. Up-regulation of p21 and Puma was also observed,
36 suggesting a possible wild-type p53 re-activation, concomitantly with mutant p53 degradation. In
37 addition, in both cell lines VPA increased the pro-apoptotic Bim, reduced the anti-apoptotic Mcl-1,
38 induced ROS production and autophagy while TSA was able to mediate these effects only in PaCA44
39 cells. These results suggest that VPA, that specifically inhibits class I HDAC, may have a stronger and
40 broader cytotoxic effect in comparison with TSA against pancreatic cancer and could represent a better
41 choice in pancreatic anticancer therapy, alone or in combination with other drugs.

42

43

44 *Keywords:* HDACis, VPA, TSA, apoptosis, autophagy, ERK, mutant p53, ROS and pancreatic cancer

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49 1. Introduction

50

51 Pancreatic cancer is the fourth cause of death by cancer in the western world, although its incidence
52 accounts only for 3% of all cancers [1]. This is due to the lack of markers for early diagnosis, to its
53 aggressive behaviour and, more importantly, to the high resistance to the radio- and chemotherapy.
54 Thus, is crucial to find new strategies to improve the outcome of pancreatic cancer therapy [2, 3]. It is
55 known that posttranslational modifications of proteins, such as acetylation and methylation, play an
56 important role in the regulation of gene transcription [4]. Histone deacetylases (HDACs), initially
57 identified for their activity on histone proteins, have been later found to deacetylate also non-histone
58 proteins, such as those regulating cell cycle, apoptosis and differentiation [5, 6]. Since HDACs are
59 often overexpressed in tumours [7], in the past few decades a variety of structurally heterogeneous
60 compounds, able to inhibit the different HDACs and so called HDAC inhibitors (HDACis), have been
61 developed. Their ability to reduce cancer cell growth, to induce cell cycle arrest and promote cell death
62 or differentiation has been demonstrated *in vitro* and *in vivo* in several cancer types [8-10]. Although
63 the anticancer effect of HDACis seems to be very promising, the underlying molecular mechanisms
64 have not been completely elucidated yet. Also because they may vary among the several classes of
65 HDACis and they can be cancer specific. HDACis mainly trigger the intrinsic mitochondrial apoptotic
66 pathway in cancer cells [11, 12] which occurs through the modulation of the expression of Bcl-2 family
67 proteins that control the mitochondrial membrane stability. This family comprises anti- and pro-
68 apoptotic proteins that interact with each other to control the oligomerization and activation of the
69 multi-domain proteins Bak and Bax. Once activated, Bak and Bax induce mitochondrial outer
70 membrane permeabilization (MOMP), process essential for the activation of the intrinsic apoptosis [13,
71 14]. HDACis can induce the extrinsic apoptotic pathway too, as it has been demonstrated for butyrate
72 that reduces the expression of anti-apoptotic molecule c-Flip [15]. In this study, we compared the
73 effects of Valproic Acid (VPA) and Trichostatin A (TSA) in the induction of apoptosis and autophagy
74 in two pancreatic cancer cell lines, namely Panc1 and PaCa44. VPA is a short-chain fatty acid that
75 specifically inhibits class I HDACs and induces the proteasomal degradation of HDAC2 [16]. VPA has
76 been safely used for over three decades in the long-term therapy of epilepsy and more recently it has

77 been found to be very effective also in the inhibition of cancer cell growth and in the induction of cell
78 differentiation and death [17, 18]. TSA, a hydroxamic acid derived from fermentation of Streptomyces,
79 is considered to be a HDAC pan-inhibitor [19]. It was initially employed as an antifungal agent and
80 later on its anticancer properties were also discovered. In this study, we found that VPA was more
81 effective than TSA in triggering a dose- and time-dependent pancreatic cell apoptosis, especially
82 against Panc1. Since both PaCa44 and Panc1 cell lines display k-RAS/p53 double mutation and a
83 constitutive ERK activation, that play an important role in cell survival, the cytotoxic effect mediated
84 by these HDACis was correlated with their capacity to reduce the expression and/or the activation of
85 these pro-survival molecules.

99 **2. Materials and Methods**

100

101 *2.1 Cell Culture and Reagents*

102 The human pancreatic cancer cell line Panc1 was obtained from the American Type Culture Collection
103 (ATCC Manassas, VA, USA) and PaCa44 from Dr. M. v Bulow (University of Mainz, Germany). Both
104 the pancreatic cell lines were cultured as monolayers in RPMI 1640 (Sigma Aldrich, R0883), 10%
105 Fetal Bovine Serum (FBS) (Sigma Aldrich, F7524), L-glutamine (Aurogene, AU-X0550) and
106 streptomycin (100 µg/ml) and penicillin (100 U/ml) (Aurogene, AU-L0022) in 5% CO₂ saturated
107 humidity at 37°C. Cells were always detached using Trypsin-EDTA solution (Euroclone). Valproic
108 Acid (VPA) (P4543) and Trichostatin A (TSA) (T8552) were purchased from Sigma Chemical Co. (St.
109 Louis Mo), Bortezomib (BZ) (sc-217785) was from Santa Cruz and Caspase 9 inhibitor Z-LEHD
110 (550381) was from BD Pharmingen.

111

112 *2.2 Cell viability*

113 PaCa44 and Panc1 cells were plated in 6-well plates at a density of 2×10^5 cells/well. Then, the
114 following day, cells were treated with VPA (10 mM) and TSA (500 nM) and Bortezomib (10 nM)
115 alone or in combination. After 48 hrs, a trypan blue (Euroclone, 72571) exclusion assay was performed
116 to test cell viability. Live cells were counted by light microscopy using a Neubauer hemocytometer.

117

118 *2.3 MTT assay*

119 *In vitro* growth-inhibitory effects of HDACis on pancreatic cancer cell lines were assessed by the
120 Monotetrazolium (MTT) assay. Briefly, 1×10^4 cells were treated with 10 mM VPA or 500 nM TSA for
121 24h, 48h and 72h in 96-well plates. Following each incubation, 20 µl of 5 mg/ml MTT (Sigma
122 Diagnostic St. Louis MO.) diluted in 1X PBS was added to each well for 4h at 37°C. The formazan

123 crystals were dissolved in 100 µl anhydrous isopropanol with 0,1N HCl (Sigma Diagnostic St. Louis
124 MO.). The optical density was determined with a microculture plate reader (BIO-RAD MICROPLATE
125 READER) at 590 nm. Each assay was performed in triplicate. Absorbance values were normalized to
126 the values for the vehicle-treated cells to determine the percent of survival.

127

128 *2.4 Apoptosis detection and sub-G1 cell cycle analysis.*

129 Early apoptosis was evaluated in PaCa44 and Panc1 cells treated with VPA (10 mM) and TSA (500
130 nM) for 24 hrs by staining with 0.5 mg/ml Annexin V kit (Molecular Probes Inc., Invitrogen, UK). For
131 cell cycle analysis, the DNA content was analyzed using the method of Propidium Iodide (PI) staining
132 and flow cytometry. VPA- and TSA-treated or untreated 3×10^5 cells were washed with 1X PBS and
133 fixed in 70% ethanol on ice for at least 1 h. Cell pellet was washed three times with 1X PBS and
134 stained with 50 µg/ml PI for 15 min at 37°C. DNA content was measured by a BD Biosciences
135 FACSCalibur. Cell debris was excluded from analysis by increasing the forward scatter threshold.
136 Cells with a DNA content lower and a Side Scatter higher than that of G0/G1 cells, were considered as
137 apoptotic cells, sub-G1. Data are representative of at least five independent experiments.

138

139 *2.5 Antibodies*

140 In this work we used the following primary antibodies: rabbit polyclonal anti-caspase 3 (1:200) (Santa
141 Cruz sc-7148) mouse monoclonal anti-cytochrome C (1:500) (BD Pharmingen 556433) rabbit
142 polyclonal anti-Bim (1:200) (Santa Cruz sc-11425), rabbit polyclonal anti-Bmf (1:1000) (Cell
143 Signaling 4692), mouse monoclonal anti-Bad (1:700) (BD Pharmingen 610391), rabbit polyclonal anti-
144 Mcl-1 (1:500) (Cell Signaling 5453P), mouse monoclonal anti-Bax for immunoprecipitation (6A7
145 Sigma B8429), rabbit polyclonal anti-Bax (Santa Cruz sc-493), rabbit polyclonal anti-PARP (1:500)
146 (Cell Signaling, 9542), mouse monoclonal anti-p-ERK (1:100) (Santa Cruz sc-7383), rabbit polyclonal

147 anti-ERK 1 (1:500) (Santa Cruz sc-93), rabbit polyclonal ERK 2 (1:500) (Santa Cruz sc-154), rabbit
148 polyclonal anti-cMyc (1:500) (Cell Signaling, 5605), mouse monoclonal anti-p53 (1:200) (Santa Cruz
149 sc-126), mouse monoclonal anti-p21 (BD Pharmingen, 556430), rabbit polyclonal anti-Puma (1:1000)
150 (Cell Signaling 4976) , rabbit polyclonal anti-LC3 (1:1000) (Novus Biologicals, NB100-2220), mouse
151 monoclonal anti-p62 (1:500) (BD 610833), rabbit polyclonal anti-HSP70 (1:100) (Santa Cruz sc-
152 66049). Mouse monoclonal anti- β -actin (1:10000) (Santa Cruz sc-137179), mouse monoclonal anti- α -
153 tubulin (1:10000) (Sigma T6199) and anti-GAPDH (Santa Cruz sc-25778) were used as markers of
154 equal loading. Goat polyclonal anti-mouse IgG-HRP (Santa Cruz Biotechnology Inc., sc-2005) and
155 anti-rabbit IgG-HRP (Santa Cruz Biotechnology Inc., sc-2004) were used as secondary antibodies. All
156 the primary and secondary antibodies used in this study were diluted in a PBS- 0.1% Tween 20
157 solution containing 3% BSA.

158

159 2.6 Western blot analysis

160 Following 10 mM VPA or 500 nM TSA treatments, pancreatic cancer cells (1×10^6) were detached with
161 trypsin and EDTA, washed in 1X PBS, lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-
162 HCl (pH 8), 0.5% deoxycholic acid, 0.1% SDS, protease and phosphatase inhibitors) and centrifuged at
163 14000 rpm for 20 min. The protein concentration was measured by using the Bio-Rad Protein Assay
164 (BIO-RAD laboratories GmbH). To evaluate caspase 3, Bim, Bmf, Bad, Bax, Puma, p53 and p21 equal
165 amounts of proteins were separated by SDS-PAGE using a 12,5% polyacrylamide gel. To detect Mcl-1,
166 PARP, pErk, Erk, LC3, p62 and HSP70, protein lysates were subjected to electrophoresis on 4-12%
167 NuPage Bis-Tris gels (Life Technologies, N00322BOX) according to the manufacturer's instruction.
168 Then, the gels were transferred to Nitrocellulose Membranes (Biorad, Hercules, 162-0115) for 2 hrs in
169 Tris-Glycine buffer. The membranes were blocked in 1 X PBS-0.1% Tween20 solution containing 3%
170 of BSA, probed with specific antibodies and developed using ECL Blotting Substrate (Advansta, K-

171 12045-D20).

172

173 2.7 Immunoprecipitation

174 Cells were lysed in 300 µl CHAPS lysis buffer (HEPES 10 mM, NaCl 150 mM, 1% CHAPS and
175 proteases inhibitors) for 30 min on ice. Then lysates were centrifuged, supernatants were discard and
176 the pellets were incubated over night (O.N.) with 2 µg mouse monoclonal anti-Bax (6A7 Sigma) that
177 interacts with active Bax. The cellular extracts were mixed with 50 µl of protein G coupled agarose
178 beads and incubated. at 4 °C. Then, the Bax/anti-Bax complex was recovered by centrifugation,
179 washed three times with lysis buffer and separated by SDS-PAGE for western blot analysis.

180

181 2.8 Determination of mitochondrial membrane potential ($\Delta\Psi_m$)

182 To measure mitochondrial membrane potential the lipophilic cationic dye 5,5',6,6'-tetrachloro-
183 1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1, Molecular Probes) was used; a JC-1 stock
184 solution was prepared at 5 mg/ml in dimethylsulfoxide (DMSO).

185 After treatment, cells were detached from culture plates by means of trypsin-EDTA, washed twice and
186 stained with JC-1 at a final concentration of 10 µg/ml in 500 µl of complete medium for 15 min at
187 37°C in air shielded from light. After incubation, cells were washed twice, resuspended in 400 µl of
188 PBS 1X and $\Delta\Psi_m$ was analyzed by a BD Biosciences FACSCalibur flow cytometer using CellQuest
189 Software (Becton Dickinson) by a biparametric analysis of FL-1 versus FL-2. Depolarization of
190 Mitochondrial membrane is accompanied by a change of JC-1 colour from greenish orange (analysed
191 in FL-2) to green (analysed in FL-1).

192

193

194

195 2.9 Cytochrome c release

196 Pancreatic cancer cells (5×10^6) were treated with 10 mM VPA or 500 nM TSA for 48h. Following
197 treatment, cells were detached using Trypsin-EDTA, washed twice with PBS and resuspended in 200
198 μ l digitonin lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4 , 8 mM Na_2HPO_4 , 250 mM sucrose, 190
199 μ g/ml digitonin) to recover cytosolic protein extract. The extracts were then centrifuged at 14.000 rpm
200 for 10 min at 4°C , supernatants transferred to fresh tubes, and 70 μ l of each sample were added to 30 μ l
201 of 1 X SDS-loading buffer (0,5 M Tris-HCl pH 6,8; 1 M 2-ME, 10% (w/v) SDS, 10% (v/v) glycerol,
202 0,05% (w/v) bromophenol blue) and boiled for 10 min. Samples (50 μ l) were loaded onto 12,5%
203 polyacrylamide gels, followed by electrophoresis and transfer to nitrocellulose membranes (Biorad,
204 Hercules, 162-0115) for 2 hrs in Tris-Glycine Buffer.

205

206 2.10 Immunofluorescence staining and flow cytometry analysis

207 TSA- and VPA-treated and untreated PaCa44 and Panc1 cells (5×10^5) were washed in 1X PBS and
208 fixed with 0,25% paraformaldehyde 5 min at room temperature (RT). Then cells were washed,
209 incubated with anti-Bak mAb (AM03, Calbiochem) for 30 min on ice, washed with ice-cold 1X PBS
210 and incubated with an anti-mouse IgG/IgM-FITC conjugated (Sigma) for an additional 40 min at 4°C .
211 Subsequently, cells were washed twice in ice-cold 1X PBS, resuspended in 300 μ l of 1X PBS and
212 analysed on FACScalibur (Becton Dickinson, San Jose, CA).

213

214 2.11 Measurement of intracellular Reactive Oxygen Species production

215 To measure reactive oxygen species (ROS) production the 2',7'-dichlorofluorescein diacetate (DCFDA)
216 (Molecular Probes, CA) was used. DCFDA is a fluorogenic dye that, after diffusion in to the cell, is
217 oxidized by ROS into 2',7'-dichlorofluorescein (DCF), a highly fluorescent compound which can be
218 detected by fluorescence spectroscopy. To measure ROS production, PaCa44 and Panc1 cells were

219 seeded in a 6 well plate and, the day after, treated with VPA and TSA. After 24 hrs, cells were washed
220 with pre-warmed PBS and then were incubated at 37 °C with 10 µM DCFDA for 15 min in PBS. Then,
221 cells were washed and analyzed in FL-1 by a FACScalibur flow cytometer (BD, USA). For each
222 analysis 10,000 events were recorded.

223

224 *2.12 Densitometric analysis*

225 The quantification of proteins bands was performed by densitometric analysis using the Image J
226 software, which was downloaded from NIH web site (<http://imagej.nih.gov>).

227

228 *2.13 Statistical analysis*

229 Data are represented by the mean $\pm$ standard deviation (SD) of at least three independent experiments.

230 Kern Index was used to calculate drug synergistic, antagonistic or additive effects.

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242 3. Results

243 3.1 VPA and to a lesser extent TSA reduce pancreatic cancer cell survival in dose and time dependent 244 fashion

245

246 We investigated the ability of TSA and VPA to inhibit PaCa44 and Panc1 pancreatic cancer cell
247 survival. We found that VPA, more effectively than TSA, reduced cell survival in a dose- dependent
248 fashion, especially in Panc1 (Fig.1A and 1B). Similar results were obtained in a time-dependent cell
249 proliferation assay (MTT), using TSA at 500 nM and VPA at 10mM, for 24, 48 and 72 h (Fig.1C and
250 1D).

251

252 3.2 TSA and VPA induce an apoptotic cell death in PaCa44 and Panc1 cells

253 We then analyzed the type of cell death induced by these HDACis in PaCa44 and Panc1 cells. To this
254 aim, annexin-V membrane exposure was evaluated in these cells treated with VPA or TSA. The results
255 shown in Fig. 2A indicate that VPA- and TSA-treated cells expressed annexin-V on their surface,
256 suggesting the occurrence of an apoptotic cell death that was further indicated by the dose-dependent
257 increase of sub-G1 events (Fig.2B and 2C). Next, apoptosis was confirmed by the activation of caspase
258 3, in both cell lines (Fig.2D). Of note, TSA was less effective than VPA in the induction of the
259 apoptotic features, especially in Panc1, accordingly to its reduced cytotoxic effect exerted against these
260 cells, as shown in Fig.1A and 1B.

261

262 3.3 VPA and TSA activate the intrinsic apoptotic pathway in PaCa44 and Panc1 cells

263 In order to evaluate whether the intrinsic pathway was involved in apoptosis induced by HDACis in
264 PaCa44 and Panc1 cells, the mitochondrial membrane potential ($\Delta\Psi$) was investigated by using the

265 fluorescent probe JC-1. We found that VPA induced mitochondrial outer membrane depolarization
266 (MOMP) in both cell lines and that TSA was less effective in mediating such effect (Fig. 3A).
267 Accordingly, a higher amount of cytochrome C was released in the cytosol of cells undergoing VPA
268 treatment in comparison with those treated with TSA (Fig. 3B). Next, we investigated the expression of
269 some pro-apoptotic molecule belonging to the Bcl-2 family protein and involved in the regulation of
270 the mitochondrial membrane stability [20]. Bim expression was increased by VPA and to a lesser
271 extent by TSA in PaCa44, while it in Panc1 Bim was up-regulated only by VPA (Fig.3C). Differently
272 from Bim, Bmf and Bad were slightly affected by both HDACis (Fig.3D). Similar results were then
273 obtained at mRNA level, by analysing Bim, Bmf and Bad expression by Real Time PCR (data not
274 shown). Subsequently, the anti-apoptotic Bcl-2 protein Mcl-1, known to interact and inhibit Bim
275 activity [21] was evaluated. Interestingly, we found that VPA treatment down-regulated the expression
276 of this protein in both pancreatic cell lines while, once again, TSA reduced Mcl-1 only in PaCa44
277 (Fig.3E). Finally, the analysis of the activation of the apoptotic effector molecules Bax and Bak showed
278 that they were activated by VPA and to a lesser extent by TSA. The activation of Bak was evidenced
279 by FACS analysis (Fig. 3F) using a monoclonal antibody recognizing an amino terminal portion of Bak
280 whose exposure indicated its activation [22]. While the activation of Bax was investigated by
281 immunoprecipitation and western blot analysis, using a monoclonal antibody able to detect amino
282 terminal portion of Bax (Fig.3G), whose exposure indicated its activation [23] (Fig.3F). Finally, the
283 involvement of the intrinsic apoptotic pathway in HDACis-cytotoxicity was confirmed by using Z-
284 LEHD caspase 9 inhibitor, which partially counteracted cell death (Fig.3H) and reduced HDACis-
285 mediated PARP cleavage (Fig.3I).

286

287

288 *3.4 VPA and TSA inhibit ERK1/2 phosphorylation and reduce c-Myc and mutant p53 protein*
289 *expression in pancreatic cancer cells*

290

291 Searching for the molecular mechanism/s leading to the cytotoxic effect mediated by HDACis in
292 pancreatic cancer cells, we found that, although at different extent, VPA and TSA induced ERK1/2 de-
293 phosphorylation (Fig.4A). ERK activation represents one of the most important pro-survival pathways
294 in these cells as well as in other cancer cells displaying k-RAS mutations [24]. Thus, ERK inhibition is
295 likely involved in the HDACis-mediated cytotoxic effect observed in PaCa44 and Panc1 cells. The
296 importance of ERK activation in the survival of these cells was confirmed by the cytotoxic effect
297 mediated by PD98059 ERK specific inhibitor in both cell lines (data not shown). ERK activation
298 contributes to the stabilization of c-Myc in cells displaying constitutively activated RAS [25] and
299 indeed Myc is frequently overexpressed in pancreatic cancers [26]. It has also been reported that
300 HDACis may down-regulate c-Myc expression by increasing its acetylation [27]. So, we then treated
301 PaCa44 and Panc1 cells with VPA and TSA and found that c-Myc expression was reduced by these
302 HDACis in both cell lines (Fig.4B). Given the pro-survival role of c-Myc over-expression in cancer
303 cells, its down-regulation by HDACis likely contributes to their-mediated cytotoxicity.

304 Besides k-RAS, PaCa44 and Panc1 cells display different p53 mutations [28], which occur in about 50-
305 75% of the pancreatic cancers. P53 mutations mainly rely in the DNA binding domain (DBD) with loss
306 of sequence-specific DNA binding to the canonical wild-type p53-binding site of target genes for its
307 oncosuppressor function [29]. In some cases, though, mutant p53 proteins may acquire pro-oncogenic
308 functions, contributing to tumor progression and chemoresistance [30]. Thus targeting mutant p53 is a
309 promising strategy for cancer chemotherapeutics [31, 32]. Interestingly, we found that VPA and to a
310 lesser extent TSA reduced mutant p53 protein levels in both PaCa44 and Panc1 cell lines (Fig4C).
311 More importantly, these HDACis induced a concomitant up-regulation of p21 and Puma level in these

312 cells (Fig.4C), suggesting a re-activation of wild-type p53 activity, although it has been reported that
313 their expression can also be up-regulated independently of p53 [33, 34].

314

315 *3.5 VPA induces ROS production and promotes autophagy in PaCa44 and Panc1 cells while TSA*
316 *mediates such effects only in PaCa44*

317

318 The increase of ROS production, which is strictly inter-connected with mitochondrial damage [35], has
319 been previously reported in several cancer cells undergoing HDACis treatments [36]. Here, we
320 investigated whether VPA and TSA would induce ROS production in PaCa44 and Panc1 cells. As
321 shown in Fig.5A and 5B, VPA increased ROS production in both cell lines while TSA was able to
322 mediate such effect only in PaCa44. Besides being involved in cell death, ROS have been also reported
323 to promote autophagy [37]. The latter plays indeed an important role in the elimination of proteins and
324 organelles damaged by the oxidative stress [38]. Thus, we next investigated whether these HDACis
325 would induce autophagy in PaCa44 and Panc1 cells. The increase of the lipidated form of LC3 (LC3-
326 II) in both cell lines treated with VPA and in PaCa44 treated with TSA, and observed in the presence of
327 chloroquine (CQ) (Fig.5C), indicate the induction of a complete autophagic flux. CQ indeed, by
328 impairing LC3-II degradation, allows evaluate the formation of LC3-II, which reflects autophagy
329 induction [39]. The finding that autophagy was activated by the same treatments and in the same cells
330 in which ROS production was induced, suggests that oxidative stress could be involved in the
331 HDACis-mediated autophagy induction. To confirm this hypothesis, we pre-treated Panc1 cells with N-
332 Acetyl Cysteine (NAC) ROS scavenger, before TSA or VPA treatment. The results, shown in Fig5D,
333 indicate that the expression level of p62, a protein mainly degraded through autophagy, was reduced by
334 VPA and not by TSA, confirming that only the former induced autophagy in Panc1. They also indicate

335 that NAC pre-treatment counteracted VPA-mediated autophagy induction since it was able to prevent
336 p62 down-regulation mediated by VPA.

337

338 *3.6 VPA and TSA synergize with bortezomib in the induction of pancreatic cancer cell death*

339 It has been previously reported that HDACis can synergize with bortezomib in anticancer therapy, even
340 if the reasons leading to this synergistic effect are not completely clarified [40]. In this study, we asked
341 whether bortezomib could potentiate VPA- and TSA -induced cytotoxic effect in PaCa44 and Panc1
342 cells. We found that bortezomib was able to increase the cytotoxicity mediated by both HDACis
343 (Fig.6A and 6B) and increase PARP cleavage (Fig.6C and 6D) in both cell lines. Searching for the
344 molecular mechanism/s of such synergistic effect, we found that bortezomib up-regulated Heat Shock
345 Protein 70 (HSP70) in PaCa44 and Panc1 cells (Fig.6E and 6F), as we have previously found in
346 primary effusion lymphoma (PEL) [41]. Interestingly, we found that VPA and TSA reduced HSP70
347 expression in both PaCa44 and Panc1 (Fig.6E and 6F), according to a previous study in which similar
348 results were obtained with TSA [42]. Since the inhibition of HSP70 exerted a stronger cytotoxic in
349 cells highly expressing it [43, 44] we hypothesize that HSP70 upregulation by bortezomib could be one
350 of the possible mechanisms leading to the synergistic cytotoxic effect mediated by its combination with
351 HCDAi against these pancreatic cancer cells.

352

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357

358 4 Discussion

359 Despite the efforts in the search for effective therapies, pancreatic cancer has still a very low
360 percentage of 5-year survival [1], which can be partially explained by its high resistance to cell death
361 induction. Thus, further studies on the understanding the molecular mechanisms of pancreatic cancer
362 cell resistance to chemotherapy are needed. HDACis have been widely used to reduce cell survival of
363 haematological and solid cancers, given their ability to target the HDACs that often aberrantly
364 expressed and/or regulated in cancers [45]. HDACis are divided in different classes based on their
365 ability to target the eighteen different classes of HDACs [46]. VPA and TSA belong to this family, the
366 former being a class I HDAC inhibitor and the latter being a pan HDAC inhibitor [47].

367 In this study, we show that VPA exerted a stronger cytotoxic activity in comparison with TSA against
368 two pancreatic cancer cell lines PaCa44 and Panc1 and the lower TSA-mediated cytotoxicity was
369 particularly evident against Panc1. Cell death induction by VPA and TSA correlated with their ability
370 to inhibit ERK phosphorylation and to reduce mutant p53 expression. ERK represents an important pro-
371 survival pathway, frequently hyper-activated in cancer cells such as those that display K-RAS
372 mutations [24]. ERK can contribute to cell proliferation also by increasing the stability and the
373 expression of c-myc, an oncogenic molecule whose expression is frequently up-regulated in cancer
374 [48]. Interestingly, concomitantly to ERK inhibition, c-myc was down-regulated by VPA and TSA in
375 the pancreatic cell lines used in this study. To this effect could also contribute c-myc acetylation
376 increased by HDACis, as previously reported [27]. C-myc reduction is likely involved in VPA- and
377 TSA-mediated cytotoxic effect, since it may be considered, per se, a potential therapeutic target in
378 pancreatic cancers [26].

379 Another key role in VPA and TSA-mediated cell death was played by the reduction of mutant p53
380 protein levels. Mutant p53 proteins indeed, besides being not able to recognize wild-type p53 DNA

381 binding sites in the promoter of p53 target genes, may activate the transcription of several oncogenic
382 proteins [49, 50]. This has been suggested by the finding that patients carrying p53 mutations together
383 with the expression of a mutant p53 protein had a significantly earlier cancer onset in comparison to
384 patients with mutations p53 resulting in the loss of p53 protein expression [51, 52].

385 Moreover, in this study we found that, concomitantly to mutant p53 reduction, a reactivation of wild-
386 type p53 occurred upon VPA and TSA treatment, as indicated by the up-regulation of p21 and puma,
387 targets of wild-type p53. These findings are in agreement with previously reported studies showing that
388 some treatments can induce both mutant p53 reduction and wild-type p53 reactivation [53, 54], in line
389 with the finding that mutant p53 protein exhibits dominant-negative activities over wild-type p53 [55].

390 The lower cytotoxic effect mediated by TSA against Panc1 in comparison to PaCa44 also correlated with
391 the lack of Mcl-1 reduction and of ROS production that was instead induced by VPA in both cell lines.
392 ROS have been previously reported to promote autophagy [38, 56] and accordingly in this study, we
393 showed that autophagy was induced by VPA in both PaCa44 and Panc1 and by TSA in PaCa44, in
394 correlation to their capacity to induce ROS production in the same cells. These findings, together with
395 the inhibition of autophagy mediated by NAC, suggest that ROS were strongly involved in autophagy
396 induction by VPA and TSA in pancreatic cancer. Regarding HDACis, it has been previously reported
397 that they have a different impact on the autophagic process, depending on the HDACis used and on the
398 cancer cell types [57-59]. More frequently they induce autophagy, as for example has been reported
399 that suberoylanide hydrodynamic acid (SAHA) do so by inhibiting mTOR [60] or, in another study, that
400 TSA and SAHA promote a pro-survival autophagy by activating the transcriptional activity of forkhead
401 box protein 1 (Foxo1) [61].

402 The different modulation of the bcl-2 protein expression by HDACis [62] and in particular by VPA and
403 TSA, as observed in this study, could also contribute to autophagy regulation, besides their impact on
404 cell death. Indeed, it has been reported that BH3 only pro-apoptotic bcl-2 proteins, such as Puma,

405 promote autophagy and that the anti-apoptotic bcl-2 proteins, such as Mcl-1, inhibit it [63-65]. Another
406 important result of this study was that both VPA and TSA cytotoxicity against pancreatic cancer could
407 be increased their combination with bortezomib. It has been previously reported that bortezomib
408 synergized with HDACis against other cancers and among the underlying mechanisms involved it has
409 been reported that bortezomib can affect the expression of HDAC or that HDACis inhibit aggresomes
410 formation [40]. HDACis and bortezomib combination has been also previously exploited in particular
411 against pancreatic cancer, since both drugs participate to the reduction of NF-kB activation and Bcl-xL
412 expression [40, 66]. As a possible explanation for the synergistic cytotoxicity of bortezomib in
413 combination with VPA or TSA observed this study, we show that HDACis reduced HSP70 in PaCa44
414 and Panc1, the same cells in which bortezomib increased its expression. Our and other's laboratories
415 [44] have previously shown that HSP70 inhibition plays a stronger cytotoxic effect in cells in which
416 this chaperone is highly expressed. After all, this represents the main rationale of the higher cytotoxic
417 effect exerted by HSP70 inhibition against cancer cells in comparison with normal cells in which
418 HSP70 is expressed at low level in baseline conditions [43].

419 In summary, in this study we show that VPA and TSA effectively induced apoptotic cell death and
420 autophagy in pancreatic cancer cells. We also show that VPA has a stronger and broader effect in
421 comparison with TSA and unveil the molecular mechanisms leading to their different effects. Another
422 important finding of this study is that both VPA- and TSA-mediated cytotoxic effect could be
423 strengthened by their combination with bortezomib, encouraging the use of such combination to
424 improve the outcome of the therapy of this highly malignant cancer whose prognosis remains still very
425 poor.

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428 **Conflict of interest**

429 The authors declare no conflict of interest.

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434 **Legend to figures**

435 **Fig. 1.** TSA and VPA inhibit pancreatic cell viability.

436 The pancreatic cancer cell lines PaCa44 (A) and Panc1 (B) were treated with different concentrations
437 of VPA (2,5 mM, 5 mM, 10 mM and 20 mM) or TSA (80 nM, 200 nM, 500 nM and 1,25 μ M) or mock
438 treated for 48 hours. Cell viability was evaluated by Trypan blue exclusion assay. Histograms represent
439 the mean plus standard deviations (SD) of the percentage of viability of treated cells determined from
440 five independent experiments. To evaluate VPA and TSA effect on cell growth at different times, time
441 course experiments were performed. PaCa44 and Panc1 cancer cell lines were treated with 10 mM
442 VPA and 500 nM TSA or left untreated and cell growth was evaluated by MTT assay and reported as
443 percent of growth inhibition at 24, 48 and 72 hours. Data represent the mean plus SD determined from
444 the results obtained from three replicative wells of five independent experiments.

446 **Fig. 2.** TSA and VPA induce pancreatic cancer cell apoptosis.

447 The pancreatic cancer cell lines PaCa44 and Panc1 were treated with 500 nM TSA or 10 mM VPA or
448 untreated (CT) for 24 hours and surface expression of Annexin V was evaluated and reported as
449 percent of positive cells, by FACS analysis (A). DNA content of PaCa44 and Panc1 treated with TSA
450 (80 nM, 200 nM, 500 nM and 1,25 μ M) and VPA (2,5 mM, 5 mM, 10 mM and 20 mM) for 48 hours
451 was measured with Propidium Iodide (PI) staining and analyzed by flow cytometry. Histograms
452 representing the mean plus SD of the percentage sub-G1 events (panel B), while FACS profile from a
453 representative experiment in which PaCa44 and Panc1 were treated with 500 nM TSA and 10 mM
454 VPA is shown in panel C. Caspase 3 activation by 500 nM TSA and 10 mM VPA in PaCa44 and
455 Panc1, as analysed by western blot. A representative experiment and mean of the ratio caspase
456 3/GAPDH densitometric analysis of three independent experiments is also shown (D).

457 **Fig. 3.** TSA and VPA trigger an intrinsic apoptotic pathway.

458 The pancreatic cancer cell lines PaCa44 and Panc1, treated with 500 nM TSA or 10 mM VPA or
459 untreated for 48 (panel A) were stained with the fluorescent probe JC-1 to analyze the depolarization of
460 the mitochondrial membrane by FACS analysis. Western blot analysis of cytochrome C, present in
461 cellular cytosolic fractions of PaCa44 and Panc1 treated with 500 nM TSA or 10 mM VPA or untreated
462 for 48, is shown in panel B. GAPDH was used as control. Expression of the pro-apoptotic Bim (panel
463 C), Bmf and Bad (panel D) and the anti-apoptotic molecule Mcl-1 (panel E) was evaluated by western
464 blot analysis and the histograms represent the mean of the ratio plus SD of densitometric values of the
465 specific proteins on GAPDH or β -Actin. FACS analysis of the pro-apoptotic activated bak protein in
466 PaCa44 and Panc1 cells treated with TSA or VPA (full black histograms show bak expression on
467 untreated control cells, while empty histograms indicate bak expression on treated cells). The
468 percentage of bak positive cells treated with VPA or TSA is also reported (panel F). Western blot
469 analysis of the immunoprecipitated (IP) bax protein (G). TSA- and VPA-treated Panc1 cells were pre-
470 treated or not pre-treated with Z-LEHD (caspase 9 inhibitor) and cell viability and PARP cleavage
471 were evaluated. In panel H, histograms represent the mean plus SD of the percentage of cell viability of
472 three independent experiments, as measured by trypan blue exclusion. In panel I PARP cleavage was
473 evaluated by western blot analysis in treated or untreated control cells in the presence of Z-LEHD. The
474 histograms represent the ratio of the mean plus SD of densitometric values of clPARP versus tubulin of
475 three independent experiments.

476

477 **Fig. 4.** VPA and TSA inhibit ERK and reduce c-Myc and mutant p53 expression. PaCa44 and Panc1
478 cancer cell lines were treated with 500 nM TSA or 10 mM VPA and the expression of phosphorylated
479 ERK (A) c-Myc (B) and mutant p53 (C) was evaluated by western blot analysis. Histograms represent
480 the ratio of the mean plus SD of densitometric values of pERK versus ERK, ERK and β -Actin (A), c-
481 Myc versus β -Actin (B) and p53 versus GAPDH (C) of three independent experiments. The expression
482 of p21 and Puma in PaCa44 and Panc1 cancer cell lines treated with VPA and TSA was investigated by

western blot analysis. The histograms represent the ratio of the mean plus SD of densitometric values of p21 and puma versus GAPDH (C) of three independent experiments.

485

Fig. 5. VPA induces ROS production and autophagy in both PaCa44 and Panc1 cell lines while TSA mediates these effects in PaCa44 only.

ROS production was investigated by FACS analysis using DCF-DA staining in PaCa44 and Panc1 cancer cell lines treated for 24 hours with VPA and TSA. Black histograms represent the amount of ROS in untreated (CT) cells, the grey histograms represent the amount of ROS in VPA or TSA treated cells while the dotted black line represent the isotype control. The values of Mean Fluorescence Intensity (MFI) is also reported (A). Histograms represent the values of the mean plus SD of MFI of DCF-DA produced by VPA- or TSA-treated PaCa44 and Panc1 cell lines of three independent experiments (B). Autophagy induction, was investigated by evaluating the expression of LC3-I and LC3-II by western blot in cells treated with VPA or TSA in the presence or in the absence of chloroquine (CQ) (C). The histograms represent the ratio of the mean plus SD of densitometric values of LC3-II versus β -Actin of three independent experiments. The role of ROS in autophagy induction in Panc1 cells pretreated or not with N-Acetyl Cysteine before VPA and TSA treatment was investigated by western blot analysis. The histograms represent the ratio the mean plus SD of densitometric values of p62 or clPARP versus β -Actin of three independent experiments (D).

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Fig. 6. VPA and TSA synergize with bortezomib in the induction of pancreatic cancer cell death.

PaCa 44 (A) and Panc1 (B) cancer cell lines were treated with TSA (500 nM), VPA (10 mM) or bortezomib (BZ) (10 μ M), alone or in combination, for 24 hours. Histograms shown in panel A and B represent the mean plus SD of the percentage of cell viability as measured by trypan blue exclusion from three independent experiments. PARP cleavage was evaluated by western blot analysis and the histograms representing the ratio of the mean plus SD of densitometric values of clPARP versus α -Tubulin of three independent experiments are shown (C and D). HSP70 protein expression as evaluated

510 by western blot analysis in PaCa44 and Panc1 cells treated with TSA (500 nM), VPA (10 mM) or BZ
511 (10 μ M). The histograms representing the ratio of the mean plus SD of densitometric values of HSP70
512 on β -Actin of three independent experiments are shown in panel E and F.

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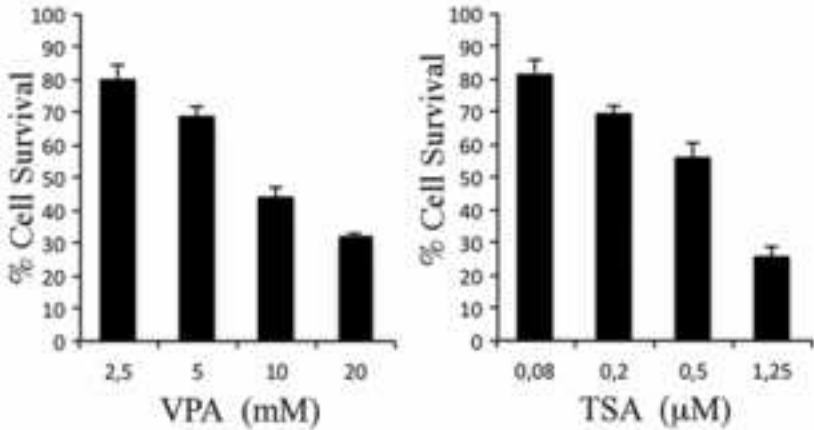
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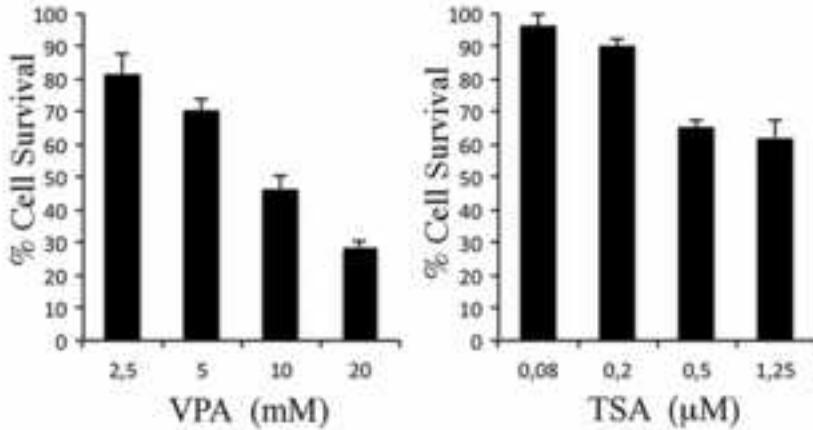
Figure 1
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A



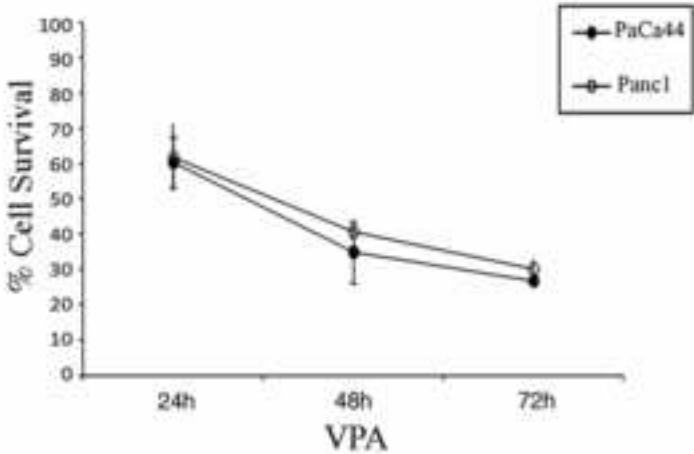
PaCa44

B



Panc1

C



D

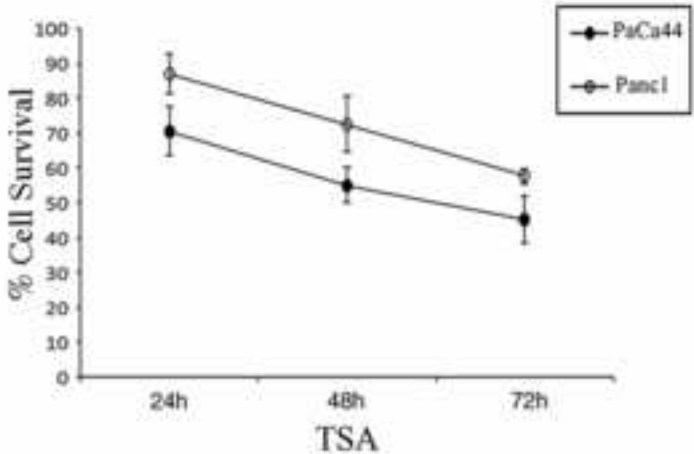


Figure 1

Figure 2
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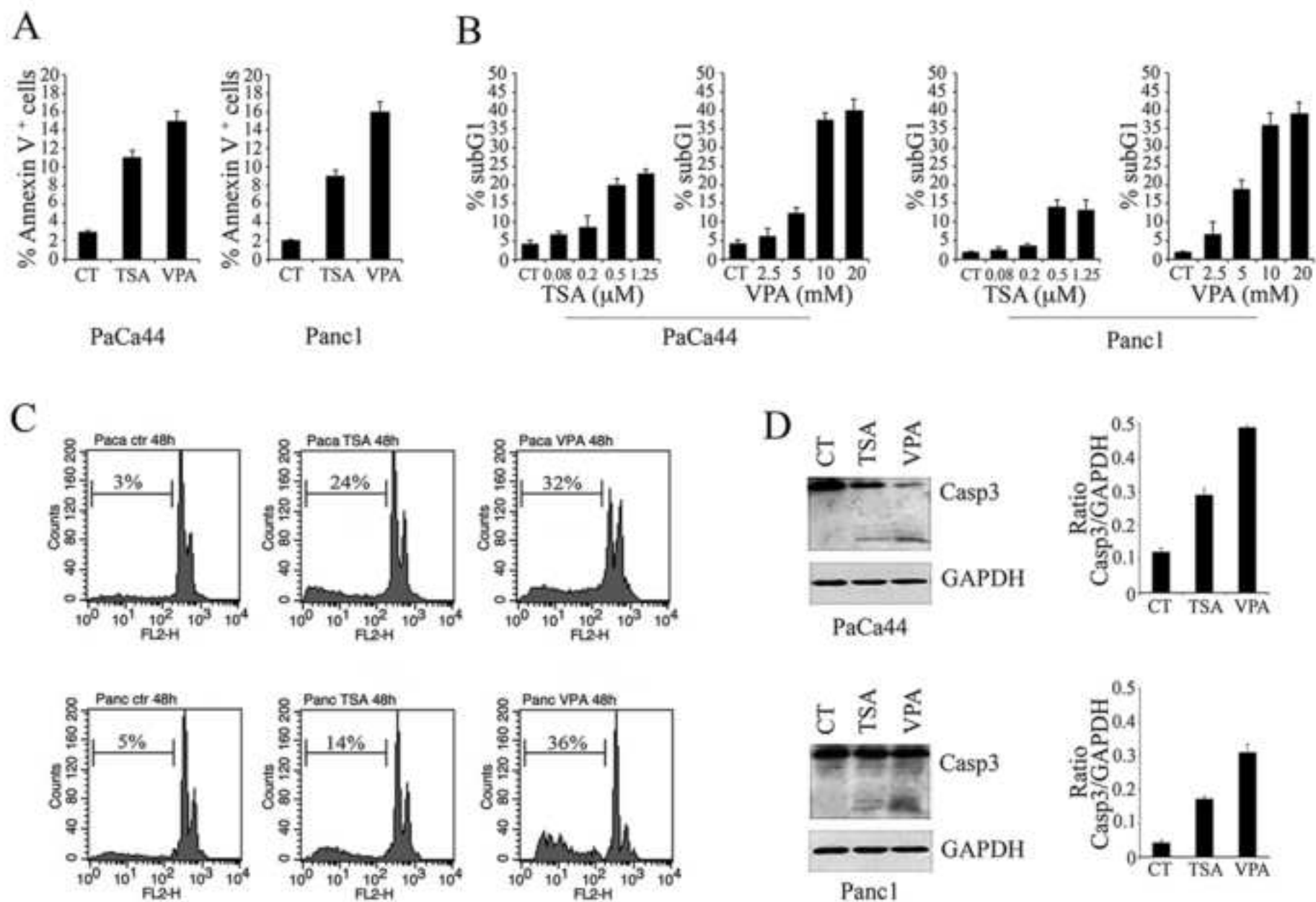


Figure 2

Figure 3
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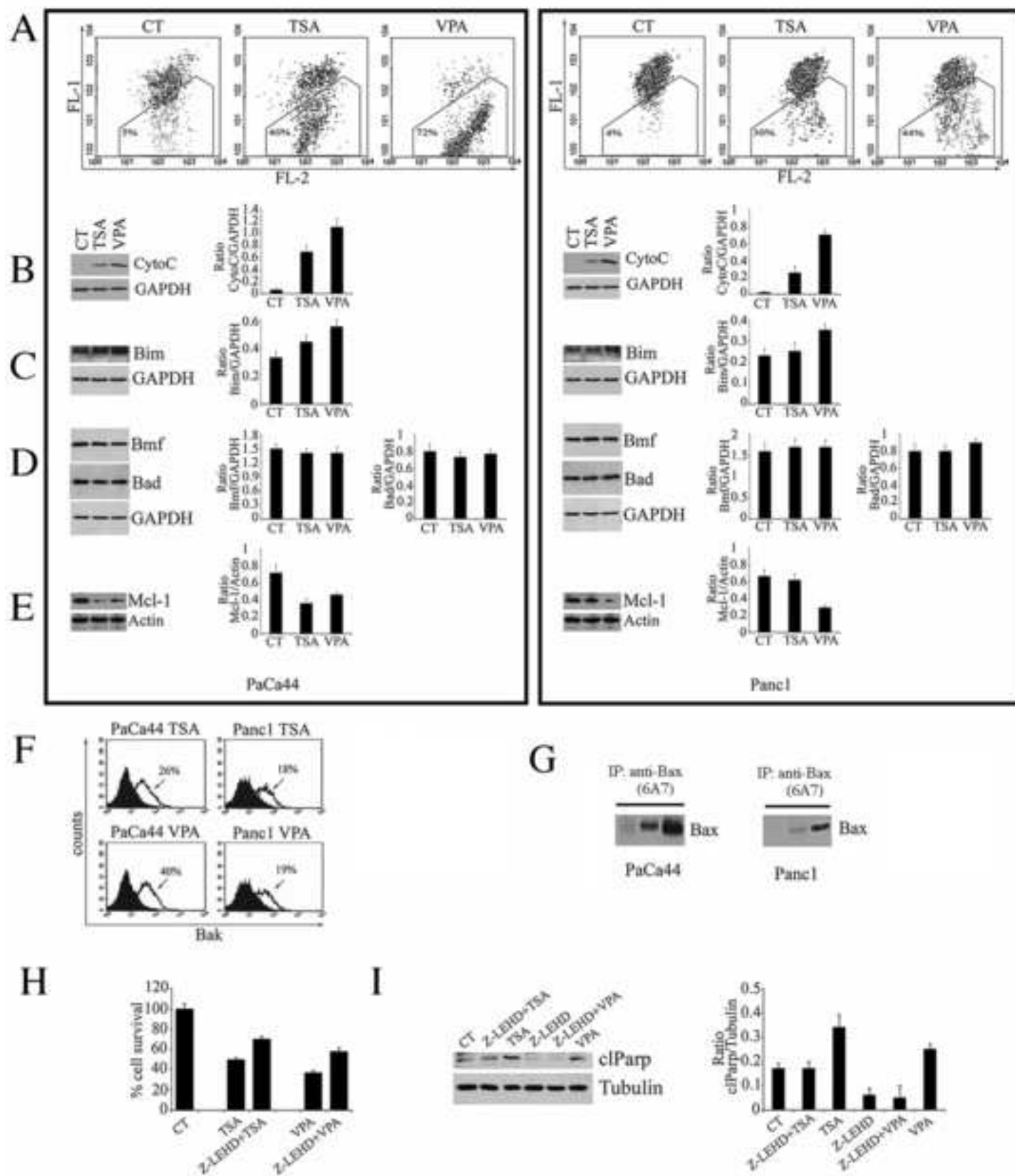


Figure 3

Figure 4
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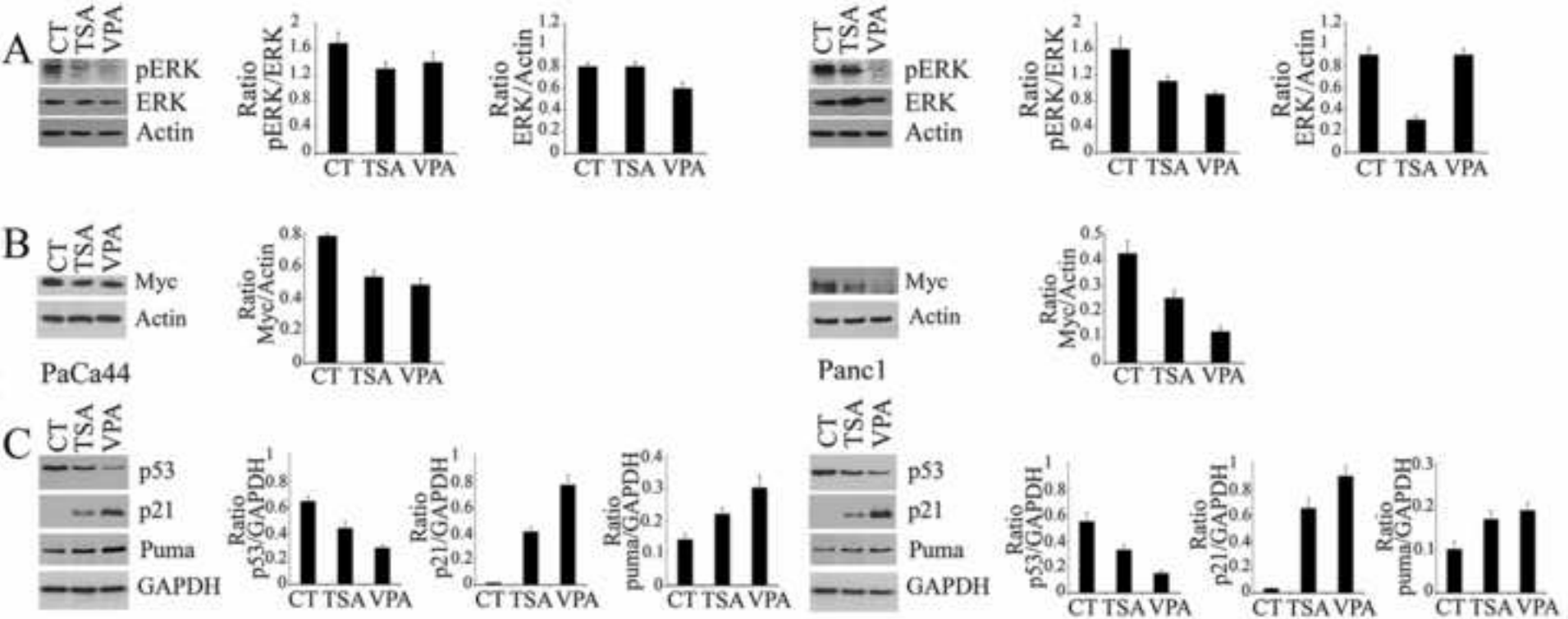


Figure 4

Figure 5
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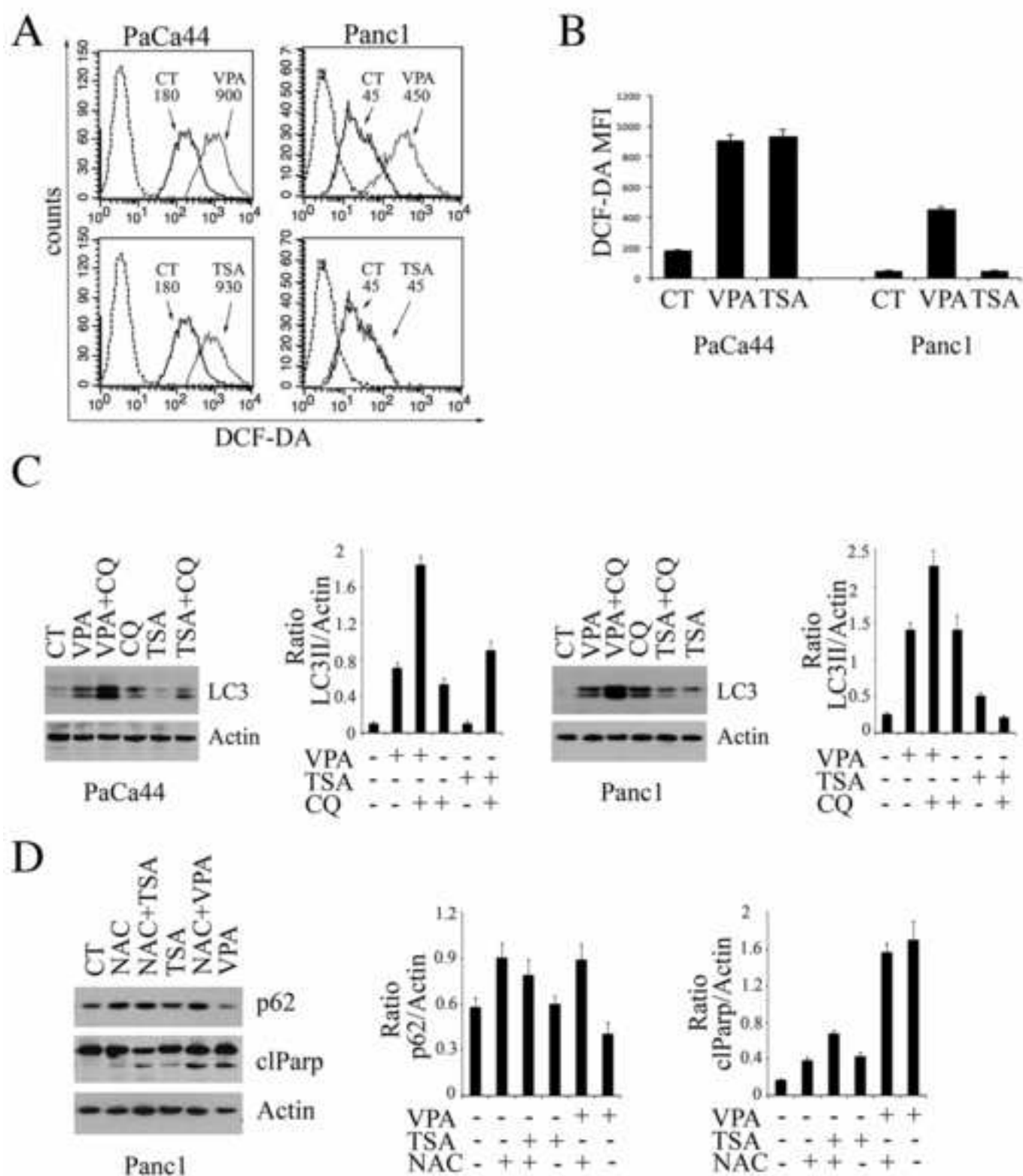


Figure 5

Figure 6
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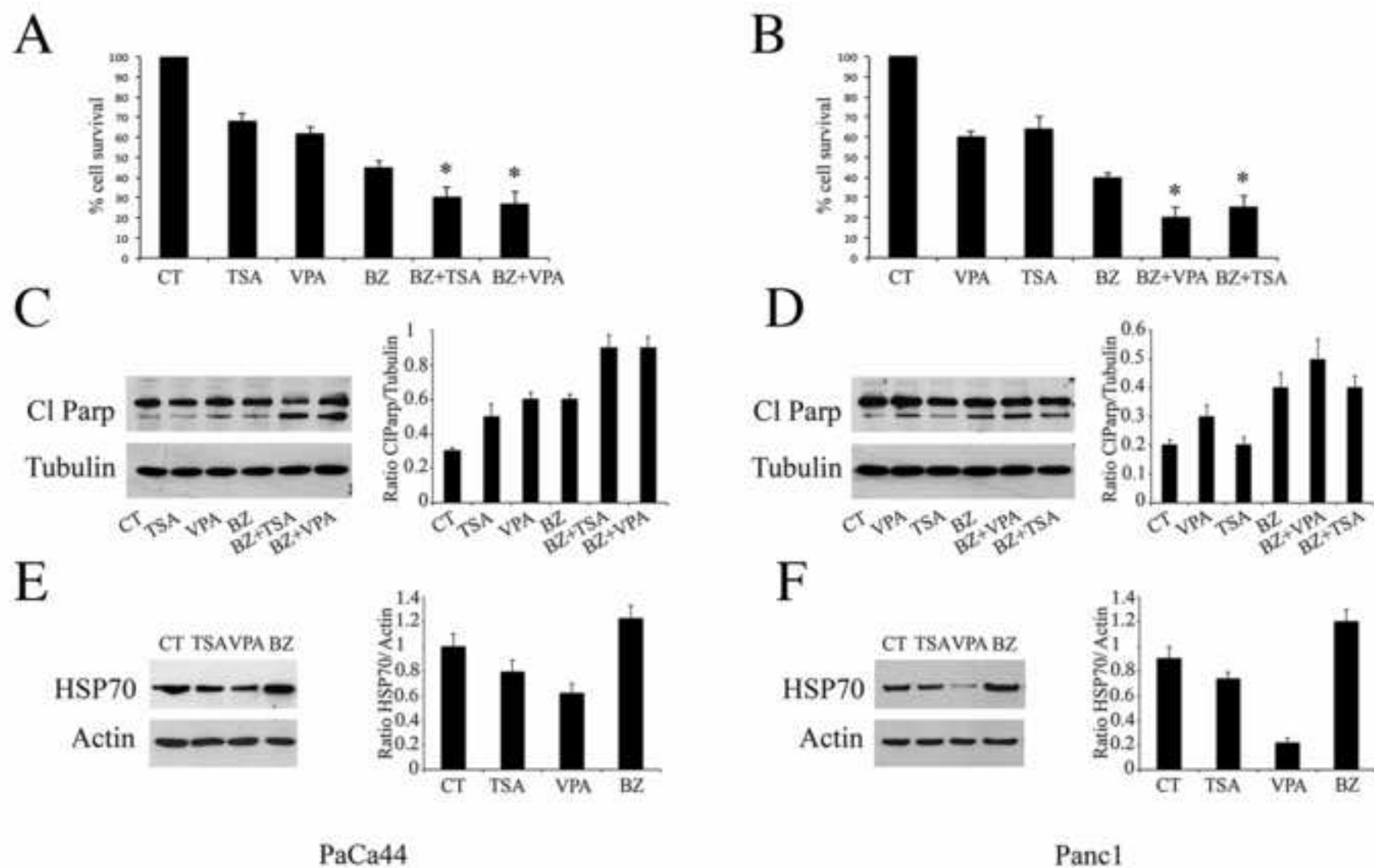


Figure 6

***Conflict of Interest**

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