Valproic acid induces apoptosis in prostate carcinoma cell lines by activation of multiple death pathways

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Valproic acid is a well-known antiepileptic drug that was recently discovered to have a wide-spectrum antitumoral action in several tumors. In our work, we tested the proapoptotic activity of valproic acid in prostate cancer. Valproic acid-induced apoptosis was described by several in-vitro assays in three prostate cancer cell lines: two representing the prototype of advanced, clinically untreatable stages of prostate progression, PC3 and DU145, and one resembling a more differentiated androgen-sensitive tumor, LNCaP. We observed that valproic acid was a potent and early apoptotic inducer, mainly in less-differentiated prostate cancer cell lines. The molecular analysis of the apoptotic machinery involved in valproic acid action revealed a central role in Bcl-2 downmodulation. When prostate cancer cells were treated for a longer time with valproic acid, we detected an enhancement of Fas-dependent apoptosis associated with an overexpression in Fas and Fas ligand. Our data indicate that the use of valproic acid may be a suitable therapeutic agent in the control of prostate cancer progression and its action appears particularly relevant in the control of refractory stages of prostate cancer. *Anti-Cancer Drugs* 17:1141–1150 © 2006 Lippincott Williams & Wilkins.

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Introduction

Transformation of prostate epithelial cells from a preneoplastic lesion into an intraepithelial neoplasm requires a great number of sequential genetic alterations [1]. Some of these aberrations are able to alter the dependence on survival and proliferative stimuli, whereas others determine the resistance in prostate cancer (PCa) cells to apoptosis. To date, several studies have indicated that resistance to apoptosis is an essential feature in the progression of many tumors. Although the bases of this phenomenon are largely unknown, the actual knowledge indicates that tumor cells maintain their ability to undergo apoptotic death in response to many drugs.

Androgen ablation therapy remains the most widely used treatment for advanced PCa. Unfortunately, this therapeutic approach frequently fails to eradicate tumor cells completely and after a variable period the emerging tumor is characterized by androgen-refractory growth [2]. Moreover, current chemotherapeutic regimens do not increase long-term survival in patients with PCa [3] and this has been linked to an increased resistance to the activation of specific apoptotic programs [4].

Several anticancer drugs are dependent on Fas receptor activation in order to induce apoptosis [5]. The Fas membrane receptor is a potent inducer of apoptosis. Fas is expressed mainly in hematological cells, where it regulates the immunity homeostasis. Many PCa cell lines and primary tumors, independently from their grade, express Fas and its ligand (FasL), but their action results in a modest or null induction of the apoptotic response [6,7]. The resistance to Fas-induced apoptotic death has been linked to the presence of specific inhibitors of caspase-8 as well as to an increased expression in Bcl-2 [4]. Treatments able to sensitize Fas-mediated apoptosis have been described both *in vitro* and *in vivo*, suggesting that Fas may represent a suitable therapeutic target in PCa [8,9].

Prostate basal epithelial cells and androgen-independent PCa express a high level of Bcl-2 conferring resistance to the PCa cells exposed to androgen ablation: a condition that normally induces apoptosis [10,11]. For this reason, increased expression of the Bcl-2 proto-oncogene could be involved in the development of androgen-independent PCa. By the use of Bcl-2 antisense oligonucleotides in

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preclinical and clinical PCa models, it has been demonstrated that inhibition in Bcl-2 expression delayed development of androgen independence and enhanced the effects of chemotherapy [12,13].

In recent years, new studies have suggested that the short-chain fatty valproic acid (VPA) and VPA analogs potently modulate the biology of several tumor cell types by inducing differentiation, inhibiting proliferation, increasing apoptosis and immunogenicity, and by decreasing metastatic and angiogenic potential [14]. The antitumor effect of VPA has been associated to its role as an effective histone deacetylase (HDAC) inhibitor [15]. Increasing evidence has indicated HDAC inhibitors as promising anticancer agents, and some of them are currently in phase I and phase II clinical trials [16,17]. In addition or alternatively to HDAC inhibition, other functions have been described for VPA action, including apoptosis induction [18]. Current data suggest that VPA exerts its functions through a largely obscure pleiotropic mechanism able to induce specific response in dependence of different timing and dosages.

To date, only few studies have been conducted in order to elucidate the possible role of VPA in the control of PCa growth. Some authors indicated that VPA is able to induce apoptosis in an androgen-sensitive tumor cell line, LNCaP [19]. The wide spectrum of anticancer effects in combination with its good tolerability, however, renders VPA a promising candidate also in the treatment of tumors, which normally are refractory to conventional therapy, like advanced grade PCa.

Here, we report the effect of VPA on PCa cells, as a substantial inductor of growth arrest and programmed cell death. Indeed, we observed that VPA strongly induced the apoptosis process through Bcl-2 downregulation as an early event, and through following upregulation of Fas and Fas-L. Moreover, less-differentiated and hormone-independent PCa cell lines demonstrated a higher susceptibility to VPA-induced apoptosis.

Material and methods

Reagents and cell cultures

The human prostatic cell lines PC3, derived from a bone metastasis, DU145, from a cerebral metastasis, and LNCaP, from a lymph-node metastasis were obtained from the American Type Culture Collection (Rockville, Maryland, USA), and were maintained in 10% fetal calf serum (FCS) supplemented with Dulbecco's modified Eagle's medium (DMEM; Sigma, St Louis, Missouri, USA) (PC3) or in 10% FCS supplemented RPMI1640 (Sigma) (DU145 and LNCaP). Antibodies anti-phospho-Tyr (PY99), anti-Fas, anti-FasL, anti-Bcl-2, anti-Akt, anti-phospho-Akt and anti-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA), whereas antibodies anti-caspase-3 and anti-caspase-8 were from Becton Dickinson (Franklin Lakes, New Jersey, USA). Agonist anti-human Fas IgG and isotype control IgG were purchased from Becton Dickinson.

Growth and inhibitory curves

A classical growth curve analysis was used to determine the effect of VPA. Cells were cultured in DMEM (PC3) or RPMI 1640 (DU145 and LNCaP) plus 10% FCS, then trypsinized, counted using a hemocytometer and adjusted to 1×10^4 cells per 50-mm diameter Petri dish in serum-free DMEM. After 24 h, three dishes were harvested for cell counting (time 0) to measure the baseline cell number. The remaining dishes received fresh medium containing 1 or 5 mmol/l VPA. For cell counting, cells were trypsinized, resuspended in 20 ml saline and counted in a Coulter Counter (LabRecyclers, Gaithersburg, Maryland, USA). Five independent counts were made from each dish. IC_{50}s were calculated by Kaleidagraph 3.6 (Synergy Software, Reading, Pennsylvania, Massachusetts, USA) utilizing data recorded at 72 h in the presence of increasing concentrations of VPA (from 0.01 to 10 mmol/l).

Western blotting

Total cell lysates were obtained by resuspending the cells in a buffer containing 1% Triton, 0.1% sodium dodecylsulfate, 2 mmol/l $CaCl_2$, 100 μg/ml phenylmethyl sulfonyl fluoride. Protein content was determined using the Protein Assay Kit 2 (Bio-Rad, Hercules, California, USA). Eighty micrograms of proteins were electrophoresed in 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis and then electrotransferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), which was then blocked overnight with 10 mmol/l Tris–HCl, pH 8.0, 150 mmol/l NaCl, 0.05% Tween-20 (TBS-T) containing 10% nonfat dry milk. The membrane was then incubated with 1 μg/ml of primary antibody in TBS-T and with specific horseradish peroxidase-conjugated secondary antibodies in TBS-T. Protein bands were visualized using a chemiluminescent detection system (Amersham Biosciences, Piscataway, New Jersey, USA) and their volume was analyzed by a public domain software (ImageJ v1.3 by Wayne Rasband; National Institutes of Health, Bethesda, Maryland, USA)

Fluorescence analysis by fluorescence-activated cell sorting

Quantification of membrane-associated proteins was performed by flow cytometric analysis (FACScan; Becton Dickinson, Mountain View, California, USA). Cells were trypsinized, centrifuged and left at 37°C for 1 h in DMEM/10% FCS in polypropylene tubes in order to reconstitute cellular external membrane. Cells were washed in saline buffer, fixed for 10 min at 4°C with a 4% buffered formalin solution, washed and resuspended at 1×10^6 cells/ml. Cells were incubated with 1 μg/ml primary antibody at 4°C for 1 h, and then washed twice

and incubated with fluorescein isothiocyanate-conjugated anti-mouse secondary antibody at 4°C for 1 h. Finally, after two more washes, cells were resuspended in 1 ml of phosphate-buffered saline (PBS) and were analyzed by flow cytometery. All the flow cytometry measurements were made under the same instrument settings and at least 5000 cells were measured for each sample. Expression levels were evaluated as fluorescence intensity in the presence of the relevant antibody and reported as the ratio between this value and background value obtained by staining cells incubated with fluoreiscinated secondary antibody only (Fluorescence Index)

Cell cycle and apoptosis analysis

The adherent cells were trypsinized, pooled with the culture supernatant containing the apoptotic cells already detached from the dish and centrifuged. Cells (1×10^6) were washed in PBS and fixed for 30 min by the addition of 1 ml 70% ethanol. After 30 min, the cells were pelleted by centrifugation (720 *g*; 5 min), and rinsed in 1 ml of DNA staining solution (PBS containing 200 mg/ml RNase A, 20 mg/ml propidium iodide and 0.1% Triton X-100) and stained by incubation at room temperature for 60 min. All cells were then measured on a FACScan flow cytometer with an argon laser at 488 nm for excitation and analyzed using Cell Quest software (Becton Dickinson, Sunnyvale, California, USA). All flow cytometry measurements were made using the same instrument settings and at least 10 000 cells were measured in each sample. Apoptotic cells were detected by a quantifiable peak in sub-G_1 phase corresponding to the red fluuorescent light emitted by subdiploid nuclei of cells and the results were expressed as the percentage of death by apoptosis induced by a particular treatment.

Mitochondrial membrane potential

Alterations in the mitochondrial membrane potential ($\Delta\psi_m$) were analyzed by flow cytometry using the $\Delta\psi_m$-sensitive dye JC1 (5,5′6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; Molecular Probes Eugene, Oregon, USA). Briefly, following treatment, cells were harvested, washed once and then resuspended in PBS 1 ×, incubated with 1 μmol/l JC1 at 37°C for 10 min. Stained cells were then washed once in PBS 1 × and analyzed by flow cytometry. A Becton Dickinson FACScalibur (Becton Dickinson) was used to analyze a minimum of 10 000 cells per sample. Data were acquired in list mode and evaluated using a CellQuest software package (Becton Dickinson). Forward and side scatter were used to gate viable populations of cells. JC1 monomers emit at 527 nm (FL-1 channel) and 'J-aggregates' emit at 590 nm (FL-2 channel). Protonophore carbonylcyanide *m*-chlorophenylhydrazone-treated cells were used for compensation (FL-1/FL-2).

Total histone deacetylase enzymatic activity

HDAC activity was measured by using HDAC Activity Assay Kit (Upstate, colorimetric detection, Charlottesville, VA, USA). Experimental procedures were designed and performed according to the protocol provided within the kit. In brief, cell lysates (prepared according to the same procedure described in the Western blot analysis section) from untreated or treated cells (72 h of exposure to different doses of VPA) were used as sources for HDAC activity. The absorbance (405 nm) was measured using Sirio-S (SEAC; Radim Group, Calanzano, Florence, Italy). HDAC assay buffer plus 4 μmol/l trichostatin A has been used as control.

Assay for cytochrome *c* release

The test was performed according to a previously described procedure [20]. In brief, cells were harvested and permeabilized for 5 min on ice with 50 μg/ml digitonin solution. Then cells were fixed in 4% w/v paraformaldehyde, washed in PBS and incubated for 1 h in blocking solution (3% w/v albumin, 0.05% v/v saponin in PBS). The cells were incubated overnight at 4°C with 1 μg/ml anti-cytochrome *c* antibody and for 1 h with fluorescein isothiocyanate-labeled secondary antibody. Expression level of released cytochrome *c* was evaluated by flow cytometry as fluorescence intensity in the presence of the cytochrome *c* antibody, and reported as the ratio between this value and the background staining of cells incubated with fluorescent secondary antibody (FI). The loss of cytochrome *c* from mitochondrion of cells treated with 5 mmol/l in respect of control cells was evaluated as ΔIF (IF control–IF treated cells).

Statistical analysis

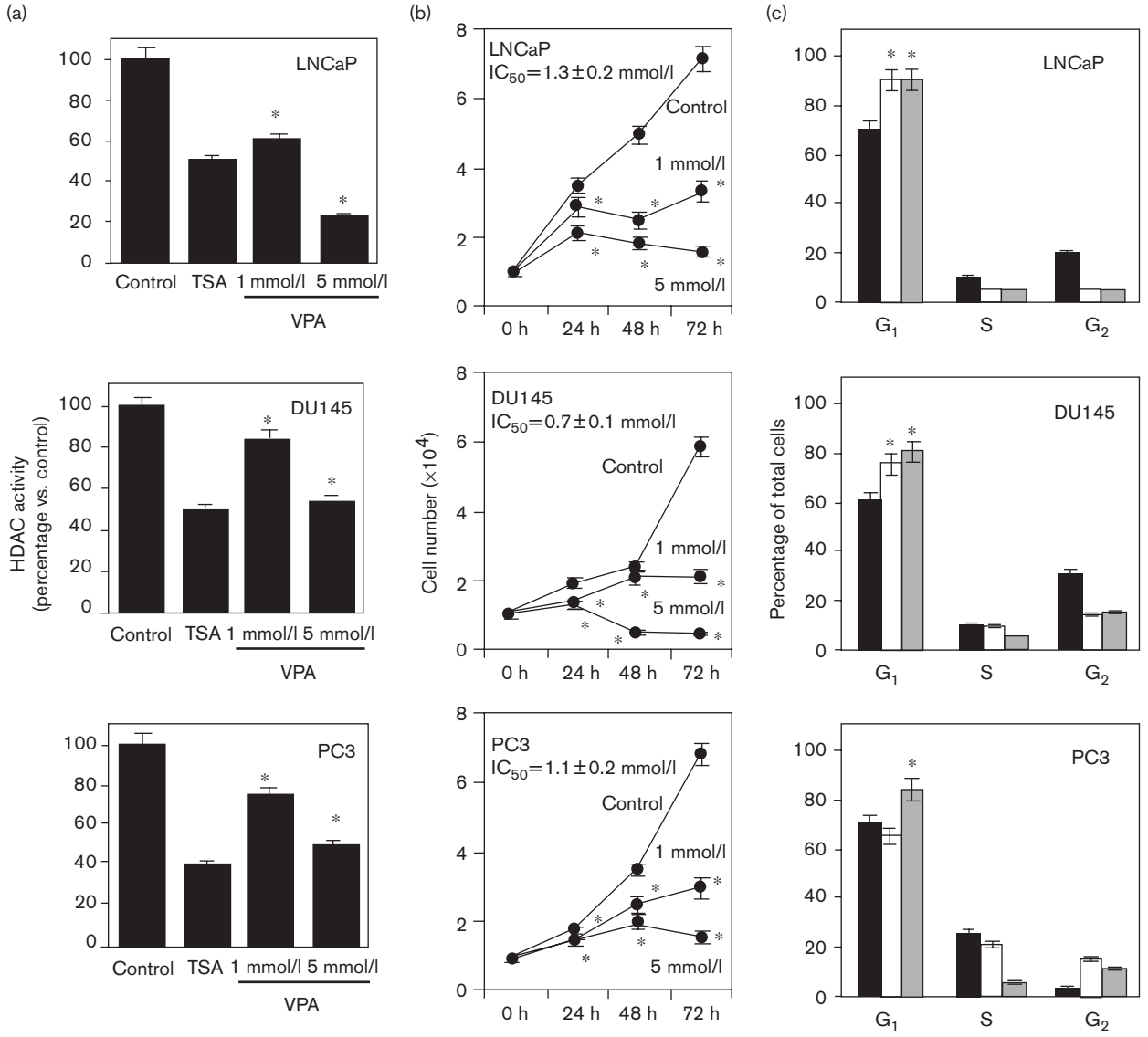
Results are expressed as means ± SD for at least three distinct experiments. Demonstration of significant differences among means was performed by Student's *t* analysis considering 0.05 the threshold value of *P*. All statistical analyses were performed using Kaleidagraph 3.6 (Synergy Software).

Results

Valproic acid inhibits prostate cancer cell proliferation and induces apoptosis

VPA and other butyrate analogs are effective HDAC inhibitors. The HDAC activity inhibition by VPA was examined on three different PCa cell lines, the androgen-sensitive LNCaP, and the androgen-independent DU145 and PC3 cells. We observed that VPA was able to inhibit *in vitro* the total HDAC activity in all the three PCa cell lines. This effect was evident starting from 24 h (data not shown) and reached a maximum after 72 h of incubation with 5 mmol/l VPA (Fig. 1a). In parallel, dose-dependent inhibition of cell growth was observed in all the cell lines with IC_{50} at 72 h between 0.7 and 1.3 mmol/l. The addition of 1 and 5 mmol/l VPA was

Fig. 1

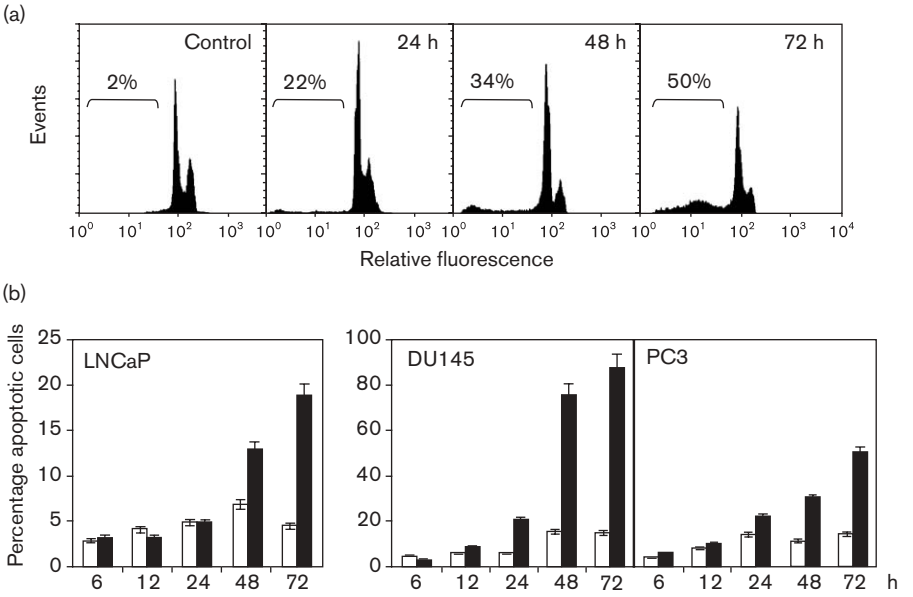


VPA inhibits HDAC activity and has an anti-proliferative effect in PCa cell lines. (a) The HDAC activity was evaluated in total extracts of PCa cells 72 h after treatment with 4 μmol/l Trichostatin A (positive control) and VPA (1 and 5 mmol/l). Results are expressed as percentage (±SD) with respect to control considering 100% the HDAC activity in the control cells. Experiments were performed in triplicate and means are considered. *$P<0.01$. (b) Growth curves in the presence of two different concentrations of VPA (1 and 5 mmol/l). IC_{50}s (mmol/l) at 72 h are shown (insets). PCa cells were cultured in standard conditions and viable cells were counted daily for 3 days. Means of the data obtained from three independent experiments were plotted. * $P<0.05$ (b) Cell cycle analysis by cytofluorimetry. The three cell lines were treated with 1 mmol/l VPA and cells were stained with propidium iodide, and cell cycle analyzed at 0 (black bar), 24 (white bar) and 72 h (gray bar). For each cycle phase, G_1, S and G_2, the mean percentage of cells with respect to total cell population was reported. Black bar represents the basal cell distribution. The experiment was repeated 3 times and means (±SD) were calculated. *$P<0.01$. VPA, valproic acid; HDAC, histone deacetylase; PCa, prostate cancer.

sufficient to significantly reduce cell proliferation with respect to control 24 h after the treatment (Fig. 1a). When cultured in the presence of 5 mmol/l, the number of viable cells clearly decreased with respect to initial seeded cells suggesting a prevalence of cell mortality. The cytofluorimetric analysis showed that the cells treated with 1 mmol/l VPA were progressively blocked in the G_0/G_1 phase of the cell cycle (Fig. 1b).

Furthermore, the analysis of DNA content by cytofluorimetry demonstrated the presence of a hypodiploid peak, indicative of apoptotic DNA fragmentation (Fig. 2a, DU145 cells). Apoptotic cell rate was detected in all the PCa cell lines in a dose- and time-dependent trend. The addition of 1 mmol/l VPA stimulated the apoptotic death in a percentage of cells inferior to 20% with respect to control, reaching a plateau after 24 h of treatment. Indeed, the percentage of apoptotic cells remained

Fig. 2



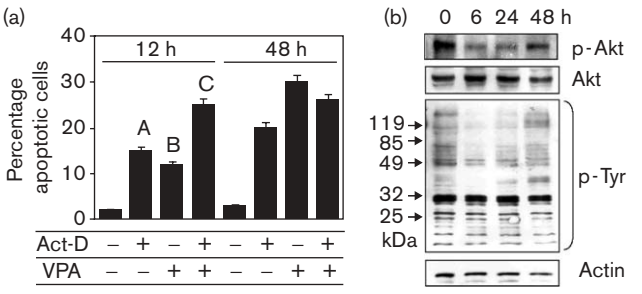
VPA induces apoptotic cell death. (a) Example histograms showing DNA content analysis by cytofluorimetry of PC3 cells in the presence of 5 mmol/l VPA. Cells were recovered at 24, 48 and 72 h, and for each experimental condition, percentage of hypodiploid events was indicated. (b) Cytofluorimetry analysis of the DNA content was performed in all three cell lines in the presence of 1 mmol/l (white bar) and 5 mmol/l (black bar) VPA. Percentage of apoptotic cells with respect to the total cell population was recorded at 12, 24, 48 and 72 h. Values are expressed as means (± SD) of three different experiments. VPA, valproic acid.

substantially unvaried until 72 h after the treatment (Fig. 2b). Subsequent observations adding 1 mmol/l of VPA every 3 days demonstrated the permanence of a steady state in cellular number for a period of time exceeding 3 weeks (data not shown). When we treated PCa cells with 5 mmol/l of VPA there was a progressive increase in apoptotic cells with a maximum percentage reached at 72 h with more than 50% cell mortality. Dissimilar behavior was observed in LNCaP cells that in all the experimental conditions showed no more than 20% of apoptotic cells.

Valproic acid stimulates transcription-independent early events

In order to investigate the role of the de-novo mRNA transcription in VPA activity, PC3 cells were pretreated with the transcription inhibitor actinomycin D for 6 h before the addition of 5 mmol/l VPA (Fig. 3a [21]). The results were expressed as percent of apoptotic cells determined by cytofluorimetry. The blockade of transcription in PC3 cells determined an increment in the number of apoptotic cells that reached after 48 h about 20% of the total cell population. The incubation with 5 mmol/l VPA and actinomycin D determined a synergic effect (Drewinko index >1 [21]) in inducing apoptosis 12 h after VPA treatment, suggesting the existence of a transcription-independent mechanism of VPA activity. On the contrary, when we analyzed the apoptotic effect of the combination 48 h after VPA treatment, we observed a competitive action of the two compounds demonstrating the importance of the transcription in determining the late VPA effects.

Fig. 3



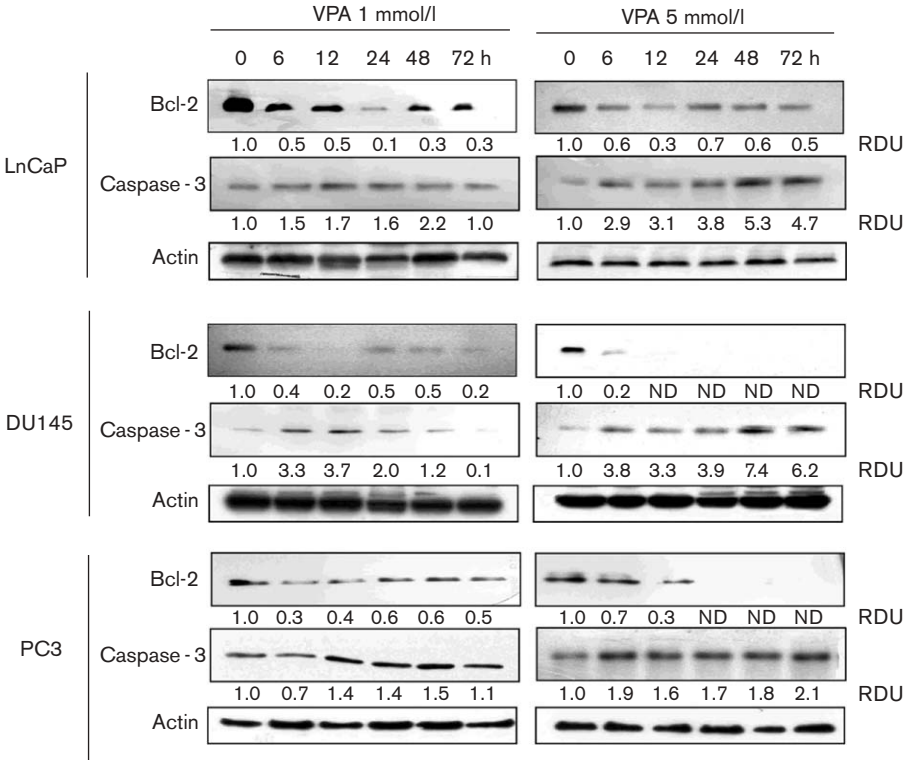
(a) Effects of transcriptional inhibitor actinomycin D (Act-D) on VPA-induced apoptosis in PC3 cells and detected by cytofluorimetry. Cells were pretreated with 0.05 μmol/l actinomycin D prior to the addition of 5 mmol/l VPA. The presence of apoptosis was expressed as mean percentage of hypodyploid events at 12 and 24 h. The statistical significance was evaluated by Student's *t*-test and $P<0.01$ when confronting A vs. C and B vs. C. Drug interaction was examined according to the method of Drewinko *et al.* [21]. (b) Detection of p-Akt, Akt and p-Tyr in PC3 total cell lysates by Western blot. PC3 cells were treated with 5 mmol/l VPA, and the content of proteins was analyzed 6, 24 and 48 h after VPA addition. For p-Tyr blot, approximate molecular weights are indicated on the left. As loading control actin content is shown. VPA, valproic acid.

Evidence obtained by actynomycin D prompted us to hypothesize that VPA was able to induce an early effect by the modulation of signal transduction pathways. As several of these intracellular ways are regulated by tyrosine phosphorylation, we thought to evaluate the time-dependent pattern of p-Tyr in total cell lysates from PC3 cells treated with 5 mmol/l VPA (Fig. 3b). The treatment with VPA determined at 6 h high reduction in the presence of total p-Tyr, mainly in a molecular weight range more than 32 kDa. The initial p-Tyr expression was slowly recovered by cells and only after 48 h did we observe a comparable expression pattern between VPA-treated and control cells. A similar trend was also observed in the expression of the active form of Akt, while the expression levels of total Akt, were unchanged (Fig. 3b).

Valproic acid stimulates an early mitochondrion-dependent apoptosis

The induction of apoptotic death was confirmed by evaluating the level of the apoptosis executive caspase-3 activated form (Fig. 4). Whereas an increment in activated caspase-3 was observed after 12 h with both 1 and 5 mmol/l VPA, its level remained high until 72 h only in the presence of the higher concentration of VPA. The expression profile of activated caspase 3 in the presence of 1 mmol/l VPA was characterized by a transient upregulation that reached the maximum level between 12 h (LNCaP and DU145) and 48 h (PC3) followed by an evident decrease as indicated by densitometric analysis (Fig. 4). Then we evaluated the implication of the mitochondrial pathway in the apoptosis induction by VPA. All three PCa cell lines expressed detectable levels of Bcl-2 in the basal condition (Fig. 4). The expression profiles of Bcl-2 at the two different concentrations were similar to those observed for active caspase-3. VPA at the concentration of 1 mmol/l induced a decrease in Bcl-2 level that reached the highest downregulation between 6 h (PC3) and 24 h (LNCaP). A partial stabilization in Bcl-2 expression level was observed in a longer time, but without reaching the basal value as indicated by densitometric analysis (Fig. 4). PC3 and DU145, but not LNCaP cells responded to 5 mmol/l VPA with a drastic reduction in Bcl-2 level that became undetectable within 24 h. The consequence of Bcl-2 downregulation was evaluated by determining the mitochondrial membrane potential ($\Delta\Psi_m$) and the release of cytochrome *c*. Figure 5(a) shows representative JC1 fluorescence dot plots of DU145 cells treated or not with 5 mmol/l VPA. As positive control of JC1 fluorescence was used the

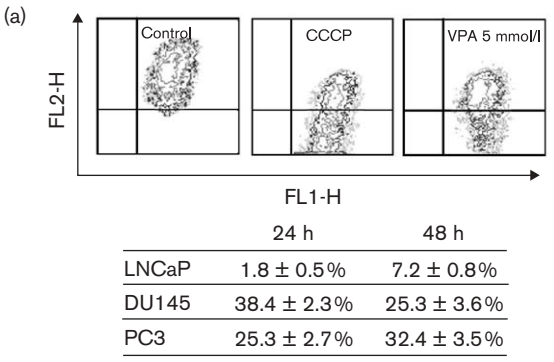
Fig. 4



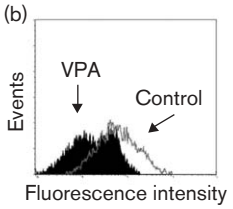
Western blot analysis of Bcl-2 and of the active form of caspase-3. The three cell lines were treated with 1 mmol/l (left panels) and 5 mmol/l (right panels), and total cell lysates were recovered at the indicated times. For each band, the densitometric value with respect to control (set to 1.0) is indicated (RDU = relative densitometric unit). The same blot was probed with both Bcl-2 and caspase-3, and actin was used as loading control.

protonophore carbonylcyanide *m*-chlorophenylhydrazone. In the bottom table, we have summarized the percentage of cells showing depolarization of mitochondrial $\Delta\Psi_m$ after 24 and 48 h of treatment with 5 mmol/l VPA. The data obtained from the analysis of $\Delta\Psi_m$ are consistent with those obtained with the investigation of Bcl-2 protein expression. In fact, we observed 38.4% of DU145 cells with loss of $\Delta\Psi_m$ after 24 h of treatment with VPA corresponding to the drastic downregulation in Bcl-2 protein. On the other hand, LNCaP revealed only 7.2% of $\Delta\Psi_m$ loss, after 48 h of treatment with 5 mmol/l VPA, at the same time in which we observed an increment in Bcl-2 protein expression. Then, we quantified in the same experimental conditions the amount of cytochrome *c* released from mitochondria. In the presence of cytoplasmatic cytochrome *c*, a brief treatment with digitonin determined the loss of cytochrome *c* released from mitochondria and this reduction can be visualized using fluorescent antibodies by flow cytometry (Fig. 5b, left figure). In the presence of 5 mmol/l VPA for 24 and 48 h, we observed the release of cytochrome *c* from mitochondria in all the PCa cell lines, with the highest values in PC3 and DU145 cells.

Fig. 5



	24 h	48 h
LNCaP	1.8 ± 0.5%	7.2 ± 0.8%
DU145	38.4 ± 2.3%	25.3 ± 3.6%
PC3	25.3 ± 2.7%	32.4 ± 3.5%



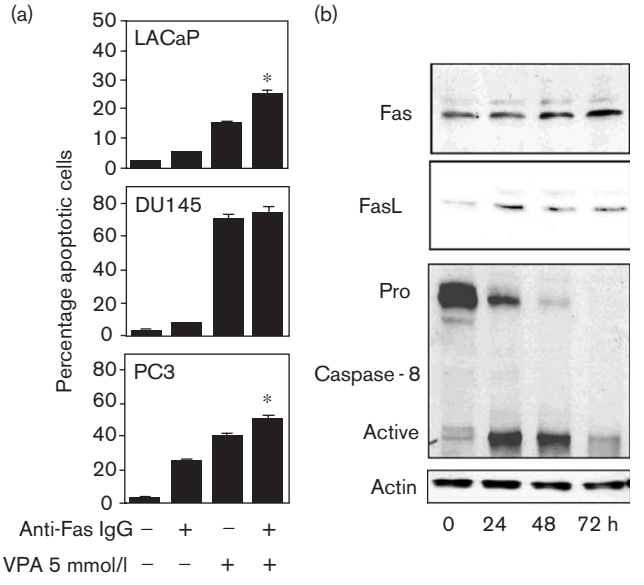
	24 h	48 h
LNCaP	2.03 ± 0.09	5.34 ± 0.14
DU145	13.12 ± 1.05	9.01 ± 0.68
PC3	8.93 ± 1.01	11.24 ± 0.91

(a) Detection of the mitochondrial membrane potential by fluorescence analysis of the dye JC1. Dot plots show example results obtained in DU145 cells in basal conditions (control), in the presence of depolarization inducer (protonophore CCCP) and of 5 mmol/l VPA. Dots in the bottom right square represent cells with depolarized mitochondria. Percentage of cells with depolarized mitochondria with respect to total events in all the three cell lines 24 and 48 h after 5 mmol/l VPA treatment was recorded (bottom table). Values are expressed as means (± SD) of three different experiments. (b) Quantitative levels of cytochrome *c* in PCa cells treated with 5 mmol/l at 24 and 48 h were evaluated by flow cytometry and reported in the right table. Data represent means (± SD) of three different experiments. Representative histograms of PC3 cells treated with 5 mmol/l VPA at 48 h are shown: black profile represents the fluorescence shift due to the loss of cytochrome *c* from mitochondria. CCCP, carbonylcyanide *m*-chlorophenylhydrazone; VPA, valproic acid.

Valproic acid stimulates the late expression of Fas and FasL

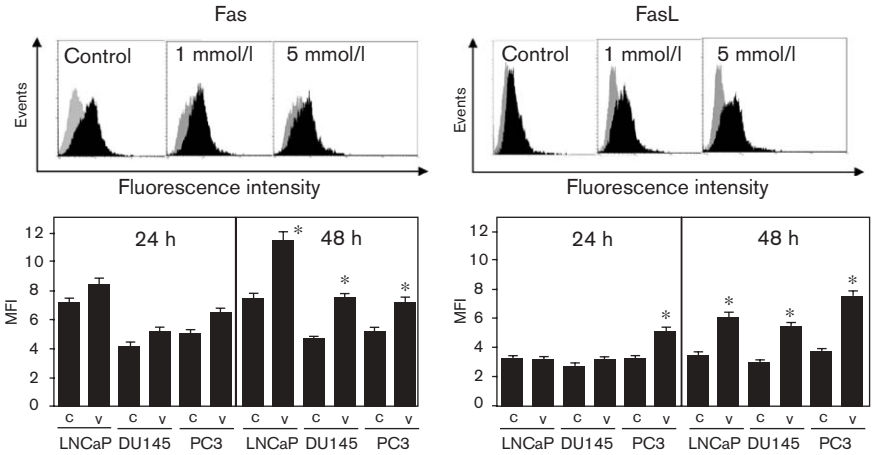
PCa cell lines were relatively resistant to Fas-mediated killing. As shown in Fig. 6(a), the use of agonist anti-Fas IgG determined after 48 h the apoptotic death in a percentage of cells from 5% (LNCaP) to 25% (PC3). When we added anti-Fas IgG together with 5 mmol/l VPA, the fraction of dead cells increased significantly also with respect to VPA treatment alone (Fig. 6a, asterisks). Then we verified by Western blot the status of Fas expression following VPA treatment. Protein analysis revealed that untreated PC3 cells express the death receptor Fas and very low levels of its ligand FasL. We observed that VPA determined an increment in Fas that was noticeable after 48 h of drug addition, while FasL became detectable as early as 24 h after treatment. Then we evaluated the time course of caspase-8 activation in PC3 cells (Fig. 6). At time 0, only the proform was evident, while after 48 and 72 h the entire procaspase-8 was converted in the active form, supporting a continued Fas activation even in the late stages of VPA action. Further, we evaluated, by cytofluorimetry, the expression on cellular membrane of

Fig. 6



(a) Fas transactivation of PCa cell lines. Cells were cultured in the presence of isotype IgG (control, 10 μg/well), agonist anti-Fas (10 μg/well) and 5 mmol/l VPA, and after 48 h the percentage of apoptotic cells was evaluated by cytofluorimetry. *$P < 0.05$ (Student's *t*-test). (b) FasL and caspase-8 expression by Western blot analysis. PC3 cells were incubated with 5 mmol/l VPA for 24, 48 and 72 h. Total cell lysates were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis and the same blot was used for the detection of all the antigens analyzed. Antibody against caspase 8 binds both the proenzymatic form (high molecular weight) and the active form (low molecular weight) of the enzyme. PCa, prostate cancer.

Fig. 7



Detection of Fas and FasL expression on the cellular membranes in LNCaP, DU145 and PC3 cells. Fas and FasL were detected by the use of fluorescent monoclonal antibodies and cytofluorimetric analysis. Data are shown as MFI values from three different experiments. All cell lines expressed at basal conditions appreciable levels of Fas and FasL proteins (c bars); VPA treatment (v bars) determined in all the three cell lines at 48 h a significant increment (*$P<0.01$) of both proteins and at 24 h a weak increment only in Fas expression. An increment in FasL expression was visible at 24 h in PC3 cells. Representative histograms of PC3 cells treated with 1 and 5 mmol/l VPA at 48 h are shown: gray profiles represent autofluorescence in control panels and basal level of proteins in 1 and 5 mmol/l treatment panels; black profiles represent basal level of proteins in control panels and expression levels in 1 and 5 mmol/l treatment panels. MFI, mean fluorescence index; VPA, valproic acid.

the death receptor Fas and its ligand. All three cell lines expressed on their cell surface elevated levels of Fas antigen, but very low levels of FasL (Fig. 7). Figure 7 (upper panels) shows representative results from cytofluorimetric analysis of Fas and FasL in PC3 cells in the presence of 1 and 5 mmol/l VPA at 72 h of treatment. An increment in Fas and FasL mainly in the presence of 5 mmol/l VPA is evident. Bottom histograms report the mean values in PCa cells 24 and 48 h after 5 mmol/l VPA treatment. While the expression of FasL was not significantly different from basal values in LNCaP and DU145 cells treated with VPA for 24 h, Fas and FasL expression was significantly upregulated in all three cell lines 48 h after the treatment with VPA.

Discussion

VPA reduced PCa cell proliferation, inducing apoptosis through the modulation of different death pathways. The apoptotic effect of VPA has been recently connected to the ability of short-chain fatty acids, such as phenyl butyrate, phenyl acetate and sodium butyrate, to act as potent HDAC inhibitors. HDACs are associated with a number of well-characterized cellular oncogenes and tumor-suppressor genes, leading to aberrant recruitment of HDAC activity, altered gene expression and the development of hematological tumors [22,23]. The mechanisms through which HDAC inhibitors affect tumor behavior in so severe a manner are largely unclear. As histone acetylation is associated with activation of gene transcription, it is plausible that a leading role in tumor suppression is played by the transcription of repressed tumor-suppressor genes [24]. Our data confirm that VPA is able to affect gene transcription of PCa cells leading to the upregulation of Fas and FasL. The PCa cell lines are generally resistant to FasL-mediated cytotoxicity [25] and the block of the apoptotic pathway appears to occur upstream of caspase-8 [6]. In our cellular system, we observed that caspase-8 activation followed the upregulation of FasL. Moreover, the addition of agonist anti-Fas antibody resulted in intense cell death only in conjunction with VPA treatment. This aspect together with the 'de-novo' expression of FasL in the tumor cells suggests the existence of a mechanism based on Fas/FasL-mediated autologous killing. We cannot, however, exclude that the leaning to Fas killing is an indirect event linked to the presence of low levels of Bcl-2.

It should be noted that Fas and FasL protein reached maximal levels after the detection of caspase-3 activation. Moreover, we described for the first time that the early VPA-induced apoptosis was gene transcription-independent. Interestingly, VPA administration determined a rapid reduction in several tyrosine-phosphorylated proteins, according to the earliest signs of apoptosis. These might include key factors whose activation allows the maintenance of survival signals in PCa. Few data are actually available about a role of VPA that is not correlated to HDAC inhibition. In fact, a study conducted on its role as mood stabilizer indicated that VPA exerts in neuronal cells a protective role activating the extracellular signal-regulated kinase pathway and inducing extracellular signal-regulated kinase pathway-mediated neurotrophic action [26]. On the other hand, our observations support the induction of an early and transient block in survival

signal pathways, and this in turn may determine, as indicated in the literature, an apoptotic death controlled by mitochondria. According to our data, this early event may be controlled, at least in part, through the inhibition of Akt phosphorylation. In fact, Akt is a key molecule in the activation of the survival pathway targeting Bcl-2 and its involvement is in agreement with the mitochondrial depolarization observed within 24 h of VPA exposure. Moreover, a transient imbalance in ATP concentration caused by VPA has been previously described in rat hepatoma cells, and could be the key element responsible for the mitochondrial dysfunction and consequent cell death [27]. VPA-induced Bcl-2 downmodulation was demonstrated also by Takai *et al.* [28] in endometrial cancer cells, but only at 72 h. Interestingly, leukemia cells resistant to VPA-induced apoptosis overexpressed Bcl-2 and the addition of a Bcl-2 antagonist rapidly restored apoptosis in this cell line [29].

Bcl-2 overexpression is a useful marker in progression of PCa and in predicting hormone resistance [30]. By deregulating programmed cell death, alteration in this gene may prevent patients from responding to androgen ablation or allow them to escape hormonal control of the disease. Our data confirm that the Bcl-2 downmodulation plays an important role especially in hormone-refractory cell lines, DU145 and PC3, in which the levels of Bcl-2 became undetectable as early as 12 h after VPA exposure and the subsequent mitochondrial depolarization was higher than in hormone-responsive LNCaP cells. Although LNCaP cells showed a significant increment in Fas and FasL expression, the percentage of apoptotic cells at 72 h was lower than in DU145 and PC3 cells. At the same time, VPA induced in LNCaP a mitochondrial depolarization to a lower extent with respect to other PCa cell lines. This phenomenon may be due to the maintenance in LNCaP of a protective mechanism against Fas-mediated death. Although we ignore the molecular feature determining this partial resistance, the higher level of Bcl-2 expression observed in LNCaP cells in the presence of VPA than in DU145 and PC3 cells suggests the existence of a protective mechanism based on Bcl-2. Hence, it is evident that although VPA-induced apoptosis occurs probably through activation of different and complementary routes (complementary mechanisms), Bcl-2 and the intrinsic pathway (mitochondrial depolarization) are the main modulators of VPA-induced apoptosis.

In accordance with previously reported data obtained in several tumors including leukemia [18], hepatoma [27], endometrial tumors [28], ovarian carcinoma [31], thyroid cancer [32] and PCa [19], we confirm that VPA could be a suitable therapeutic tool in cancer therapy. As advanced stages of PCa have demonstrated to be resistant to conventional chemotherapy regimens and other available therapies, VPA, whose inhibitory action seems to be effective on undifferentiated tumor cells, appears particularly useful for the treatment of not curable hormone-refractory PCa.

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