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Sodium butyrate sensitises human pancreatic cancer cells to both the intrinsic and the extrinsic apoptotic pathways

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Abstract

Pancreatic cancer is characterised by a highly malignant phenotype with a marked resistance to conventional therapies and to apoptotic activators. Here, we demonstrate that sodium butyrate (NaBt), an inhibitor of histone deacetylases, sensitises human pancreatic cancer cell lines to both mitochondria- and Fas-mediated apoptosis. The analysis of anti-apoptotic and pro-apoptotic members of the Bcl-2 family in untreated pancreatic cancer cell lines shows a generalised low expression of Bcl-2 and a strong expression of Bcl-x_L. NaBt treatment results in a marked down-regulation of Bcl-x_L expression, mitochondrial membrane depolarization, cytochrome *c* release from mitochondria, activation of caspase-9 and -3 and apoptosis induction. Furthermore, NaBt sensitises pancreatic cancer cells to Fas-mediated apoptosis as well. In fact, the combined treatment with NaBt and the agonistic antibody anti-Fas (CH11) is able to induce apoptosis at an early time, in which neither NaBt nor CH11 alone induce apoptosis. Down-regulation of FLIP and activation of caspase-8 allow apoptosis to occur. These findings suggest that sodium butyrate could represent a good candidate for the development of new therapeutic strategies aimed at improving chemotherapy and immunotherapy in pancreatic cancer.

Keywords: Apoptosis; Pancreatic cancer; Sodium butyrate; Fas; FLIP; Bcl-2 family

1. Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death in industrialised countries and its incidence almost equals its mortality [1]. The high death rate is due to the difficulty of achieving early diagnosis, rapid tumour progression and resistance to chemotherapy, radiotherapy, immunotherapy and anti-hormonal treatment [2–4]. Tumour development and progression as well as resistance to most cancer therapies mainly depend on the lack of response to apoptosis induction [5].

Apoptosis is a cell death process that plays a critical role both in animal development and tissue homeostasis and in many diseases [6]. Two major pathways for induction of apoptosis have been identified, namely the extrinsic and the intrinsic apoptotic pathways. The extrinsic pathway is induced via death receptors and utilises protein interaction modules known as death domains (DDs) and death effector domains (DEDs) to assemble signaling complexes that induce apoptosis [7]. Fas (APO-1/CD95) is a member of the death receptor family, whose binding with its natural ligand, Fas-L or with agonistic antibodies activates the extrinsic pathway of apoptosis in Fas-expressing cells [8]. Fas triggering results in the recruitment of the adaptor molecule Fas-associated death domain (FADD) and procaspase-8 in the so-called Death Inducing Signaling Complex, DISC. This aggregation allows the activation of caspase-8, an essential step for the propagation of Fas-mediated apoptotic signal [9].

The intrinsic pathway of apoptosis, that involves mitochondria and the release of caspase-activating proteins, is regulated mainly by the Bcl-2 gene family. This family includes both anti-apoptotic (Bcl-2, Bcl-x_L) and pro-

Abbreviations: NaBt, Sodium butyrate; PI, Propidium iodide; FLIP, FLICE-Inhibitory Protein

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apoptotic (Bax, Bak, Bcl-x_S) members which share structural homologies and can physically interact with each other to form homo- or hetero-dimers [10]. The balance of anti-apoptotic and pro-apoptotic proteins determines, in part, how cells will react to apoptotic signals. Cancer cells are usually resistant to both apoptotic pathways.

Recently, numerous differentiation-inducing agents have been shown to have potent activities against various types of cancer cells. Butyric acid is the major short-chain fatty acid produced by fermentation of dietary fibers in the colon [11]. Butyrate has been shown to cause inhibition of proliferation and induction of differentiation and/or apoptosis in a variety of cancer cells [12]. Although the molecular mechanisms by which butyrate exerts these effects are not well understood, it is known that butyrate is an inhibitor of histone deacetylase (HDAC) [13]. Acetylation and deacetylation of histones play an important role in the regulation of gene expression [14], and butyrate has been shown to modify expression of genes involved in both cell cycle and apoptosis [15,16]. Among the genes that are differentially regulated by butyrate and that are involved in apoptosis, there is the *bcl-2* gene family. Numerous studies have highlighted that NaBt-treated cancer cells down-regulate the anti-apoptotic molecules Bcl-2 or Bcl-x_L [16,17] or up-regulate pro-apoptotic molecules such as Bax, Bak and Bcl-x_S [18,19]. In this way, NaBt plays a key role in favouring the intrinsic pathway of apoptosis. Moreover, it has been reported that NaBt may also influence the extrinsic pathway of apoptosis, although the molecular mechanisms are still unclear [20–22].

In the present study, we investigate the effects of NaBt treatment on cell growth inhibition and apoptosis in pancreatic cancer cell lines. Our results demonstrate that NaBt is able to influence both the intrinsic and the extrinsic pathway of apoptosis. Moreover, the analysis of genes and proteins involved in both pathways allow us to suggest that the decrease of Bcl-x_L and FLIP expression represent an important molecular mechanism of NaBt-mediated cell death in pancreatic cancer cells. These results assume particular relevance in this form of cancer, resistant to most known therapies and suggest the use of butyrate to develop new strategies in treating pancreatic cancer.

2. Materials and methods

2.1. Reagents

NaBt was purchased from Sigma Chemical Co. (St. Louis, MO). A stock solution (5 M) of NaBt was prepared with water and diluted with RPMI 1640 to the required concentrations before each experiment.

RPMI 1640, fetal bovine serum (FBS), Penicillin/Streptomycin solution 100× and L-glutamine 200 mM were purchased from EuroClone (EuroCLone Ltd. U.K.). The Trypsin-EDTA solution C was purchased from Biological Industries.

The mouse-monoclonal anti-Fas (human activating), clone CH11 was purchased from Upstate Biotechnology.

2.2. Cell culture

The human pancreatic cancer cell lines ASPC-1 and PANC-1 were obtained from the American Type Culture Collection (Rockville, MD, USA); PACA44 from Dr. M. v Bulow (University of Mainz, Germany) and PT45 from Dr. H. Kalthoff (University of Kiel, Germany). All the pancreatic cell lines were grown in 5% CO_2 saturated humidity, at 37 °C and cultured as monolayers in RPMI 1640 supplemented with 1× penicillin/streptomycin, 2 mM L-glutamine and 10% FBS. Cells were always detached using Trypsin-EDTA.

2.3. MTT assay

The in vitro growth-inhibitory effects of NaBt on pancreatic cancer cell lines were assessed by the Mono-tetrazolium (MTT) assay. Briefly, 1×10^4 cells were treated with various concentrations of NaBt (1 mM, 5 mM, 10 mM) for 24 h, 48 h, 72 h and 96 h in 96-well microtiter plates. Following each incubation, 20 μl of 5 mg/ml MTT (Sigma Diagnostic, St. Louis, MO) in PBS 1× was added to each well for 4 h at 37 °C. The formazan crystals were dissolved in 100 μl anhydrous isopropanol with 0.1 N HCl (Sigma Diagnostic, St. Louis, MO). The optical density was determined with a microculture plate reader (BIO-RAD MICROPLATE READER) at 590 nm. Each assay was performed in triplicate. Absorbance values were normalised to the values for the vehicle-treated cells to determine the percent of survival. Results of the MTT assay have been shown to correlate well with ^{3}H-thymidine incorporation.

2.4. DNA labeling technique and flow cytometric analysis of apoptosis

Hypodiploid DNA was analysed using the method of propidium iodide (PI) staining and flow cytometry. Briefly, after detachment with trypsin-EDTA, NaBt-treated or untreated cells (3×10^5) were washed in PBS 1× and resuspended in 300 μl hypotonic fluorochrome solution (50 μg/ml of PI, 0.1% sodium citrate plus 0.1% Triton-X-100). The samples were incubated 15–20 min at 37 °C and then transferred to 4 °C in the dark for 1 h. The DNA content was measured by a FACScan flow cytometer (Becton Dickinson, U.S.A.). Cell debris was excluded from analysis by increasing the forward scatter threshold. Cells containing a DNA content lower and a Side Scatter higher than that of G0/G1 cells were considered to be apoptotic cells. Data are representative of at least five independent experiments.

2.5. Western blot analysis

After detachment with trypsin and EDTA, 10^6 cultured cells were washed in PBS 1×, lysed in NP40 lysis buffer

and centrifuged for 20 min at 14000 rpm. The protein concentration was measured by using the Bio-Rad Protein Assay (BIO-RAD laboratories GmBH). Equal amounts of proteins (20 μg) were separated by SDS-PAGE using a 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane. Primary antibodies used in this study are: mouse monoclonal antibodies to Bcl-x_L and to Bax (Santa Cruz Biotechnology, CA.); to Bcl-2 (Ancell Immunology Research Products); to caspase-8 clone 5F7 (Upstate Biotechnology); to β-actin (Sigma); rabbit polyclonal antibody to Fas (C-20) or to caspase-3 (H-277) or to FLIP (H-202) (Santa Cruz Biotechnology, CA). Following four washes in PBST, the blots were incubated with peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham, Les Ullis, France). The Amersham ECL Western Blotting Analysis System was subsequently used for protein detection.

2.6. Isolation of RNA and reverse transcription

Total RNA was isolated from cell pellets by the method of Chomczynski and Sacchi [23]. RNA quality was checked by agarose gel. cDNA was synthetised from oligo dT-primed RNA in 20 μl of reverse transcriptase buffer and 200 U of Moloney murine leukemia virus reverse transcriptase (GIBCO, Bethesda Research Laboratories) incubated at 37 °C for 1 h.

2.7. PCR amplification

CDNA was amplified by PCR consisting of 30 cycles of denaturation at 94 °C for 30 s, annealing at the right temperature for each pair of primers and extension at 72 °C for 30 s Primer sequences used were: β-actin 5′ GTGGGGCGCCCCAGGCACCA, β-actin 3′ CTCCTTAATGTCACGCACGATTTC (548 bp fragment); Fas 5′ GTACAGAAAACATGCAGAAAGCAC, Fas 3′ CTCTGCAAGAGTACAAAGATTGGC (342 bp fragment); Bcl-2 5′ GCGTCAACCGGGAGATGTCGCCC, Bcl-2 3′ TTTCTTAAACAGCCTGCAGCTTTG (348 bp fragment); Bcl-x_L 5′ ATTGGTGAGTCGGATCGCAGC, Bcl-x_L 3′ AGAGAAGGGGGTGGGAGGGTA (262 bp fragment) and Bax 5′ AGCTCTGAGCAGATCATGAAG, 3′ CTCCCGGAGGAAGTCCAATG (396 bp fragment). PCR products were size fractionated by agarose gel electrophoresis and normalised according to the amount of β-actin detected in the same sample. Photographs were taken with positive/negative 665 film (Polaroid Corp.) of DNA bands visualised by ethidium bromide staining and UV transillumination.

2.8. Analysis of cytochrome c release

ASPC-1 and PACA44 cells (3×10^6) were treated with NaBt 5 mM for 48 h. Following treatment, cells were detached using Trypsin-EDTA, washed twice with PBS and resuspended in 200 μl digitonin lysis buffer (75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 250 mM sucrose, 190 μg/ml digitonin). Digitonin is a weak nonionic detergent that, at low concentration, selectively renders the plasma membrane permeable, releasing cytosolic components from cells but leaving other organelles intact [24].

After 5 min on ice, cells were spun for 10 min at 14.000 rpm at 4 °C in an Eppendorf microcentrifuge. Supernatants were transferred to fresh tubes, and the pellets were resuspended in Triton lysis buffer (25 mM Tris–HCl pH 8; 0.1% Triton X-100). Aliquots (70 μl) of both pellet and supernatant for each sample were added to 30 μl of SDS-loading buffer (0.5 M Tris–HCl pH 6.8; 1 M 2-ME, 10% (w/v) SDS, 10% (v/v) glycerol, 0.05% (w/v) bromophenol blue) and boiled for 10 min. Boiled samples (25 μl for pellets, 50 μl for supernatants) were loaded onto 12.5% polyacrylamide gels, followed by electrophoresis and transfer to nitrocellulose membranes. Membranes were blocked overnight at 4 °C in PBS containing 0.1% (v/v) Tween20 (PBS-T) and 10% no fat dried milk. Blocked membranes were incubated with a monoclonal anti-human cytochrome *c* Ab (clone 7H8.2C12) (1:500 dilution in PBS-T and 5% milk) for 1 h at room temperature. Membranes were washed three times (5 min each) with PBS-T, followed by incubation with HRP-conjugated anti-mouse IgG secondary Ab. Detection of cytochrome *c* on blots was performed using enzyme-linked chemiluminescence (ECL, Amersham).

2.9. Immunofluorescence staining and flow cytometry

After detachment with trypsin-EDTA, 5×10^5 cells were washed in 1× PBS and resuspended in 50 μl of 4% paraformaldheyde for 15 min at 4 °C in order to fix them. Cells were then rinsed twice in ice-cold 1× PBS and incubated with 10 μg/ml mAb against human Fas/APO1 (clone ZB4 Upstate Biotech) for 60 min at 4 °C. Cells were washed in ice-cold 1× PBS and then incubated with an anti-mouse IgG/IgM-FITC conjugated (SIGMA) for an additional 60 min at 4 °C in the dark. Subsequently, cells were washed twice in ice-cold 1× PBS and resuspended in 300 μl of PBS 1× and analysed on FACScan (Becton Dickinson, San Jose, CA). For intracellular staining cells were treated with 0.1% saponin in 1× PBS for 15 min at 4 °C. Then, cells were washed twice in 0.1% saponin in PBS and incubated with 5 μg/ml mAb against human Bcl-2 (clone 100, Ancell) diluted in 0.1% saponin in 1× PBS for 60 min. The secondary antibody was the same as for Fas, and was used as reported above, but with the addition of 0.1% saponin in PBS.

2.10. Determination of mitochondrial membrane potential ($\Delta\Psi$)

To measure mitochondrial membrane potential the lipophilic cationic dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-

benzimidazolylcarbocyanine iodide (JC-1, Molecular Probes) was used; a JC-1 stock solution was prepared at 5 mg/ml in dimethylsulfoxide (DMSO).

After treatment, cells were detached from culture plates by means of trypsin-EDTA, washed twice and stained with JC-1 at a final concentration of 10 μg/ml in 500 μl of complete medium for 15 min at 37 °C in air shielded from light.

After incubation, cells were washed twice, resuspended in 400 μl of PBS 1× and analysed on a FACScan using CellQuest Software (Becton Dickinson) in FL-1 and FL-2. Mitochondrial membrane is accompanied by a change of JC-1 colour from greenish orange (analysed in FL-2) to green (analysed in FL-1).

2.11. Caspases activation assay

To evaluate caspases activity, cell lysates were incubated with the appropriate 7-Amino-4-Methyl-Coumarin (AMC)-conjugated substrates. The substrates were Ac-DEVD-AMC for caspase-3/7-like activity, Ac-IETD-AMC for caspase-8-like activity, and Ac-LEHD-AMC for caspase-9-like activity (Alexis Biochemicals).

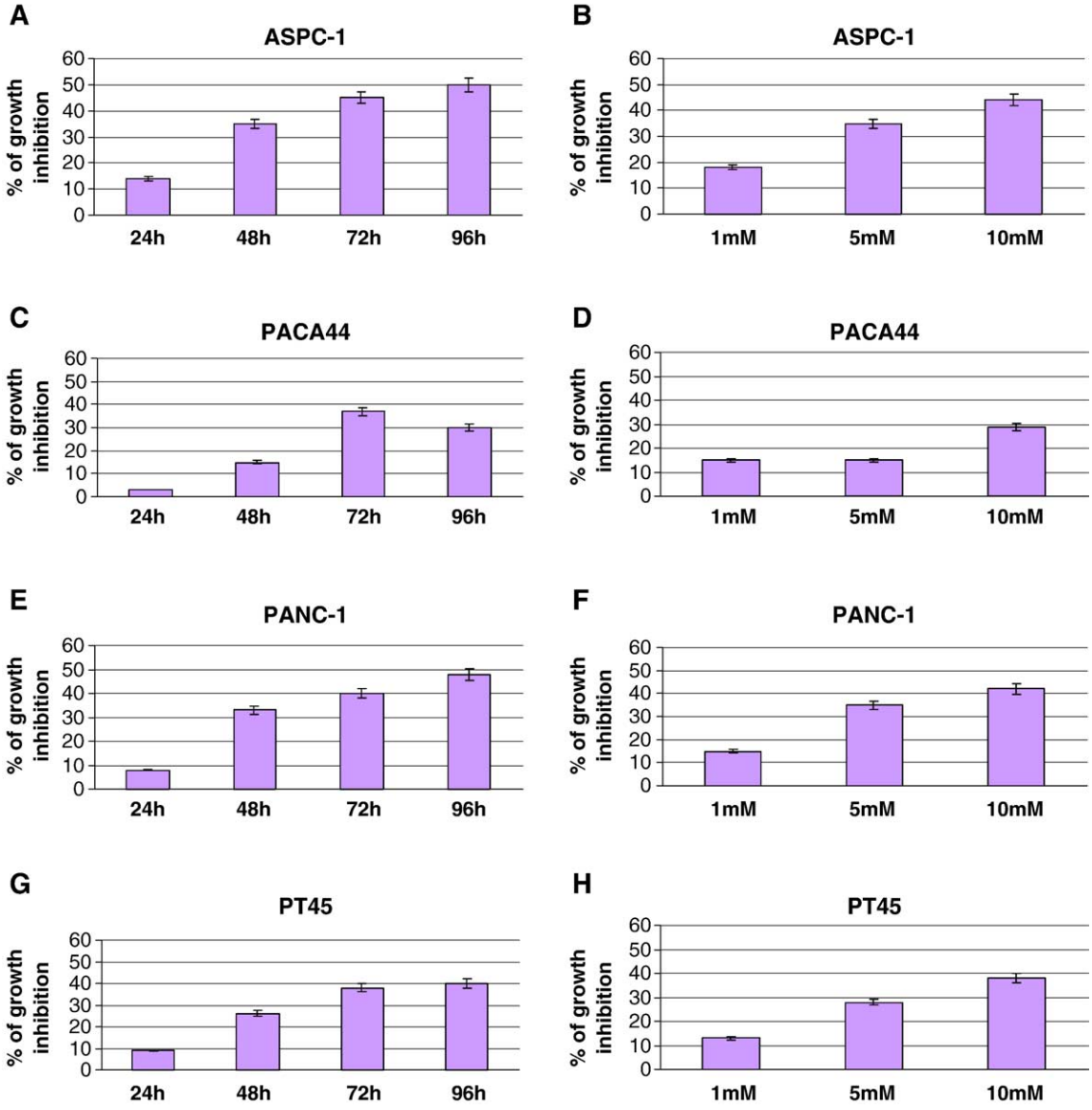

Fig. 1. NaBt inhibits cell proliferation The four pancreatic cancer cell lines ASPC-1, PACA44, PANC-1 and PT45 were treated with NaBt and proliferation was evaluated by MTT assay. In panels A, C, E and G, time-course experiments were performed. Cells were treated for different periods of time (24, 48, 72 and 96 h) with 5 mM NaBt. In panels B, D, F and H dose-response was studied with 1 mM, 5 mM and 10 mM NaBt for 48 h. Viability was determined and reported as % of growth inhibition. Data represent the mean ± standard deviations (bar) determined from the results obtained from three replicative wells. Statistical significance was performed by Student's *t*-test, as compared to the value in untreated cells, $P \leq 0.05$. A representative of three independent experiments is shown.

Lysates (10 μg of protein) were incubated in a 100-μl reaction buffer with the specific caspase substrates (40 μM caspase-8 and -9 substrates, and 20 μM caspase-3 substrate). The caspase activity was measured reading the plate every 10 min for 1 h in a microtiter plate fluorometer (excitation wavelength of 360 nm and emission wavelenght of 480 nm). Activity is expressed as fold induction over the activity of the control after correction for baseline activity.

3. Results

3.1. NaBt-induced cell growth inhibition in pancreatic cancer cells

To determine the effect of NaBt in pancreatic cancer cell lines, the rate of proliferation was evaluated by the MTT assay. Four pancreatic cancer cell lines, ASPC-1, PANC-1, PACA44 and PT45, were treated with different amounts of butyrate for 24, 48, 72 and 96 h. As shown in Fig. 1, NaBt reduces the proliferation of all the cell lines studied in a dose-response and time-dependent manner. Data were confirmed by ^{3}H-thymidine incorporation and by cell count (data not shown).

3.2. NaBt-mediated apoptosis in pancreatic cancer cells

To determine whether NaBt is able to induce cell death in pancreatic cancer cell lines, apoptosis analysis was performed. Cells were treated or not with NaBt (1 mM, 5 mM and 10 mM) and cultured for different periods of time. At the end of incubation, cells were stained with propidium iodide (PI) and analysed by flow cytometry to evaluate the DNA content. Fig. 2 shows that all the four cell lines undergo apoptosis induction albeit with a different susceptibility.

3.3. Effect of NaBt on Bcl-2 family member expression

It has been reported that NaBt influences cell sensitivity to apoptosis either by down-regulating anti-apoptotic or up-regulating pro-apoptotic members of the Bcl-2 protein family in different cancer cell lines (17–19). To verify whether NaBt treatment could affect the Bcl-2 gene family in pancreatic cancer cells, we examined the expression of both anti-apoptotic (*bcl-2* and *bcl-x$_L$*) and pro-apoptotic (*bax* and *bcl-x$_S$*) members by RT-PCR and Western blot analysis in the four cell lines, ASPC-1, PACA44, PANC-1 and PT45. As shown in Fig. 3A, a different amount of Bcl-2 protein, which is not

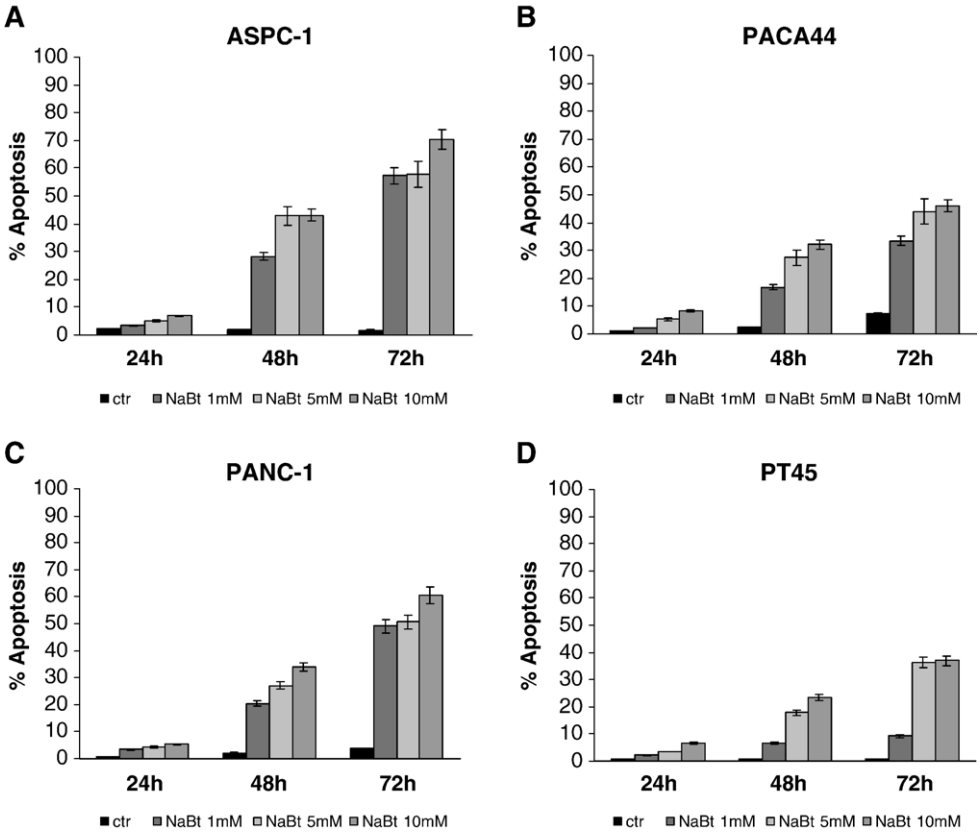

Fig. 2. NaBt induces apoptosis The four pancreatic cancer cell lines ASPC-1, PACA44, PANC-1 and PT45 were treated with different concentrations of NaBt, 1 mM, 5 mM and 10 mM for 24, 48 and 72 h. After incubation, cells were stained with propidium iodide and analysed by flow cytometry. Results are expressed as percent apoptosis. The mean and standard deviations (bar) were determined from the results obtained from five independent experiments. Statistical significance was performed by Student's *t*-test, as compared to the value in untreated cells, $P \leq 0.05$.

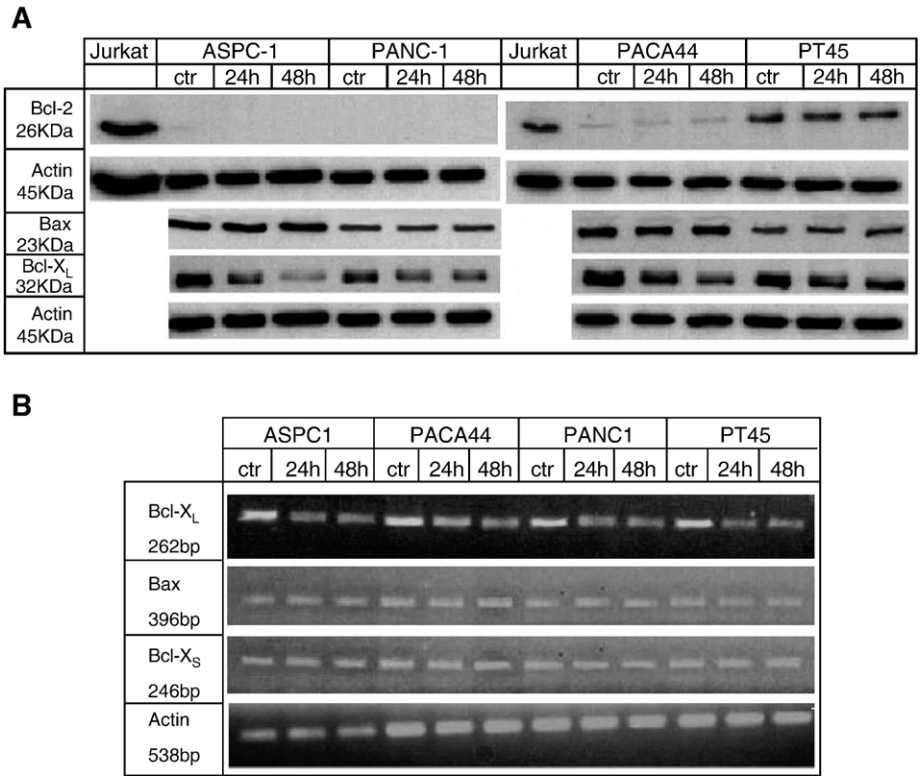

Fig. 3. NaBt down-regulates Bcl-x_L The four pancreatic cancer cell lines ASPC-1, PACA44, PANC-1 and PT45 treated with 5 mM NaBt for 24 and 48 h were analysed by Western blot (A) and RT-PCR (B). Equal amounts of protein (25 μg), from cell extracts, were analysed by antibodies directed against Bcl-2, Bcl-x_L, Bax and β-Actin and detected by ECL assay (A). cDNA was amplified using different conditions according to specific primers for *bax*, *bcl-x_L* and *bcl-x_S*. *Actin* was used as control (B).

modified after NaBt treatment, is expressed by the four cell lines. Moreover, the further analysis by immunofluorescence staining (data not shown) confirmed that PT45 is the cell line that expresses the greatest amount of Bcl-2 protein, PACA44 and PANC-1 are weakly positive and ASPC-1 is negative. Bax and Bcl-x_L were expressed at relatively high levels and in similar amounts among the cell lines, but were differently regulated by NaBt. In fact, Bax expression was not altered by NaBt treatment, whereas Bcl-x_L expression was strongly down-regulated by NaBt in a time-dependent manner. Moreover, NaBt had no effect on *bcl-x_S* expression as demonstrated by RT-PCR (Fig. 3B). RT-PCR analysis confirmed the results of protein expression identifying in *bcl-x_L* the target for NaBt treatment in pancreatic cancer cell lines and demonstrated that Bcl-x_L is down-regulated at the transcriptional level.

3.4. NaBt triggers the intrinsic pathway of apoptosis

To evaluate whether down-regulation of bcl-x_L is able to influence the intrinsic pathway of apoptosis we analysed the effect of NaBt treatment on mitochondrial membrane potential ($\Delta\psi$) with the fluorescent probe JC-1. The data reported in Fig. 4A indicates that NaBt induces membrane depolarization, which begins to be detectable at 48 h of NaBt treatment and reaches high levels at 72 h.

Since mitochondrial membrane depolarization causes pro-apoptotic proteins to be released from the mitochondrial membrane interspace to the cytosol, thus allowing activation of caspase-9 and consequently activation of the caspase cascade, we investigated the effect of NaBt treatment on the activity of caspase-3, as the principal member of the caspase cascade. Accordingly, NaBt treatment provoked an increase of caspase-3 activation that became evident in all the cell lines as attested both by enzymatic activity assay and by Western blot (Fig. 4, panel B and C). Since caspase-3 is an effector caspase, which actually may be activated both by caspase-9 and caspase-8, analysis of caspase-9 and -8 activation was performed by activity assay for caspases. As shown in Fig. 4 (panel E and F), NaBt treatment induces caspase-9 enzymatic activity, but has no effect on caspase-8. Moreover, to evaluate that activation of caspase-9 was due to the pro-apoptotic proteins released by mitochondria membrane depolarization we evaluated the release of cytochrome *c* from mitochondria. The Western blot analysis of cytochrome *c* on the membrane fraction (including mitochondria) and the cytosol fraction confirmed that, following treatment with NaBt for 48 h, cytochrome *c* is translocated from the membrane fraction to the cytosol (Fig. 4D). Altogether these results demonstrate that in pancreatic cancer cell lines the intrinsic pathway of apoptosis is functional and can be activated by NaBt.

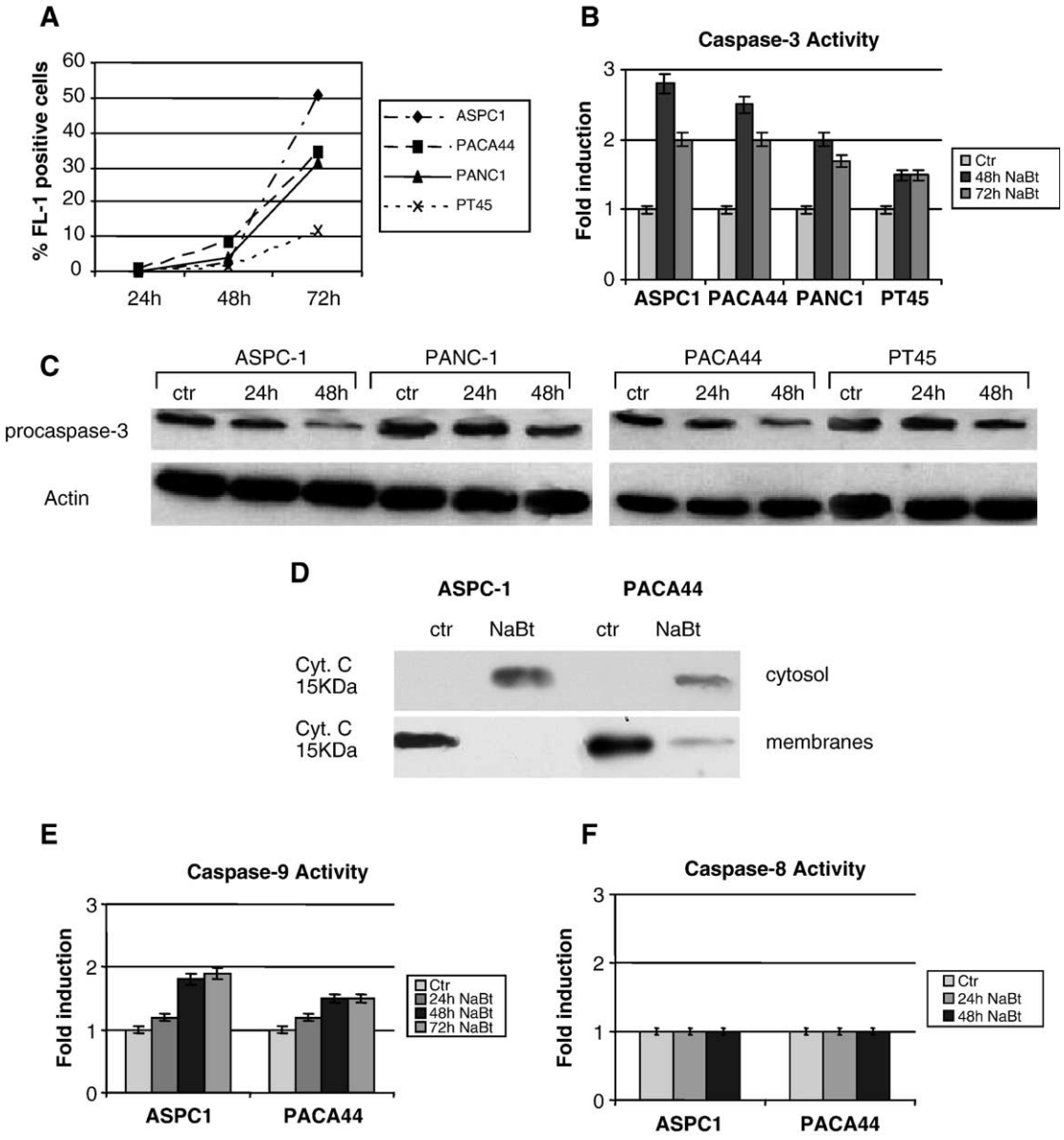

Fig. 4. NaBt triggers the mitochondrial apoptotic pathway. To evaluate the effect of NaBt treatment on mitochondrial membrane potential ($\Delta\Psi$) cells were stained with the fluorescent probe JC-1 at different period of time and analysed by cytofluorimeter (A). Activation of the mitochondrial caspase cascade was established by studying activity of caspase-3 (B), -9 (E) and -8 (F) by enzymatic assays. Statistical significance was performed by Student's *t*-test, as compared to the value in untreated cells, $P \leq 0.05$. Procaspase-3 cleavage has also been investigated by Western blot in cell lysates treated or not with NaBt for different period of time (C). Cytochrome *c* release has been studied by Western blot analysis of membrane and cytosolic fractions in ASPC1 and PACA44 treated and untreated cell lysates (D).

3.5. NaBt favours the extrinsic pathway of apoptosis sensitising pancreatic cancer cell lines to Fas-mediated signals

It is known that often, in cancer cells, stimulation of death receptors does not succeed in triggering the extrinsic pathway of apoptosis. Since in some models resistance to Fas-induced apoptosis has been shown to be the consequence of a decreased expression of Fas, we examined the expression of Fas receptors on the cell surface of the four cell lines ASPC-1, PACA44, PANC-1 e PT45. FACS analysis revealed that all the cell lines express high levels of Fas (Fig. 5A). However, when cells were treated with the agonistic antibody to Fas, CH11, cells did not undergo apoptosis induction even at high doses (500 ng/ml) and for long periods of time (72 h) (Fig. 6A). Usually, when Fas is triggered DISC is recruited and procaspase 8 (FLICE) is activated. In the aim of determining whether the apoptosis signal is blocked upstream or downstream of caspase-8 activation, we analysed levels of procaspase 8 by Western blot. Fig. 5B shows that Fas-triggering failed to activate procaspase 8 in all the pancreatic cancer cell lines.

In order to evaluate if NaBt could play a role in favouring Fas-mediated apoptosis, we co-treated pancreatic

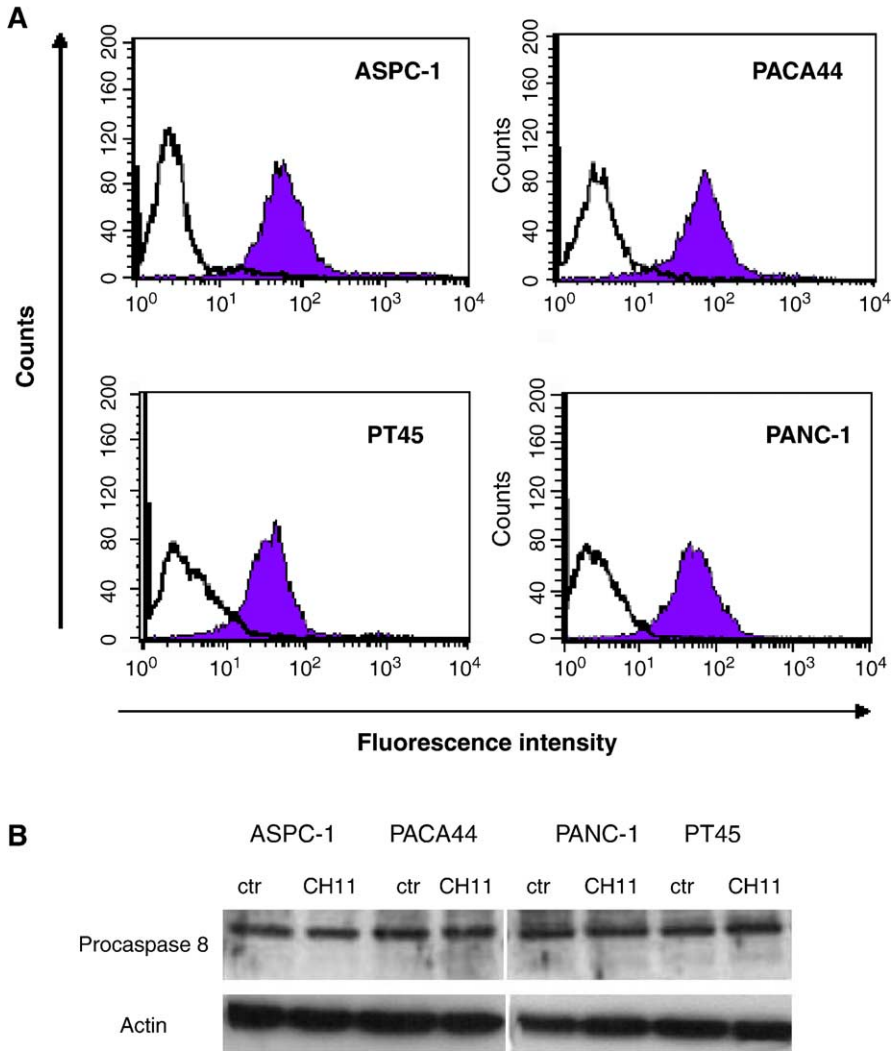

Fig. 5. Fas is expressed but fails to induce apoptosis Flow cytometric analysis of Fas expression on ASPC-1, PACA44, PANC-1 and PT45 using ZB4 mAb (gray-filled curves) and control staining (black lines)(A). Pancreatic cancer cell lines were treated or not with 500 ng/ml CH11 for 24 h. Protein expression was assessed by immunoblotting with antibodies directed against caspase-8 (B).

cancer cells with both NaBt and CH11. As Fig. 6A shows, at 24 h, time point in which neither Fas nor NaBt, alone in culture, was able to induce apoptosis, all the four cell lines did undergo apoptosis when the two stimuli were combined. The synergistic effect is maintained at 48 h and 72 h. Moreover, in order to examine whether apoptosis induction was effectively the result of the extrinsic pathway activation, we evaluated caspase-8 and caspase-3 activation. As shown with the two representative cell lines ASPC-1 and PT45, caspase-8 (Fig. 6B) and caspase-3 (Fig. 6C) are strongly activated only by co-treatment with NaBt and CH11 for 24 h. These results suggest that the intrinsic and the extrinsic pathways of apoptosis are favoured by the combination of the two signals.

3.6. NaBt inhibits FLIP expression

Finally, we wanted to evaluate the role of FLIP, a known inhibitor of caspase-8 activation, in resistance to Fas-mediated apoptosis in pancreatic cancer cells. To this aim, we studied the expression of FLIP in the four cell lines ASPC-1, PACA44, PANC-1 e PT45. As expected, all the cell lines expressed FLIP evaluated by both RT-PCR and Western blot analyses (Figs. 7A and B). To investigate whether NaBt could exert its effect on the extrinsic pathway of apoptosis modulating FLIP, we studied FLIP expression in cells treated with NaBt alone or in combination with CH11 mAb. As shown in Fig. 7, FLIP was strongly reduced by NaBt treatment both as mRNA (Fig. 7A) and as protein (Fig. 7B). These results point out for the first time that NaBt favours Fas-mediated apoptosis by down-regulating the expression of FLIP.

4. Discussion

The aim of the present study was: (i) to examine the efficacy of NaBt in inducing apoptosis in pancreatic carcinoma cells by influencing both intrinsic and extrinsic

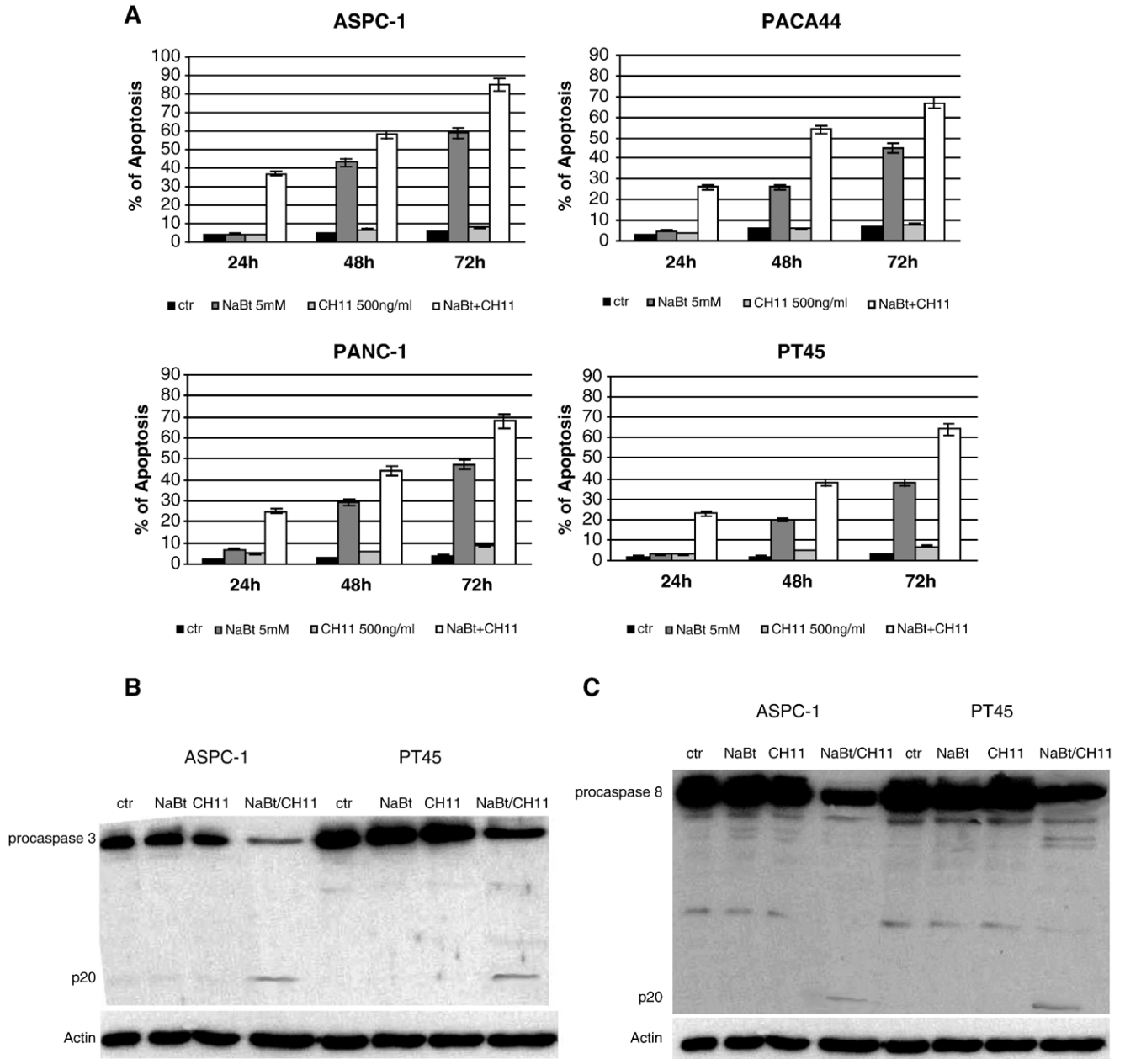

Fig. 6. NaBt and Fas co-treatment induces apoptosis and activates caspase-3 and caspase-8. The four pancreatic cancer cell lines ASPC-1, PACA44, PANC-1 and PT45 were treated with 5 mM NaBt and 500 ng/ml CH11 alone or in combination for 24, 48 and 72 h to evaluate apoptosis (A). After incubation, cells were stained with propidium iodide and analysed by flow cytometry. Results are expressed as percent of apoptosis. The means and standard deviations (bar) were determined from the results obtained from five independent experiments. Statistical significance was performed by Student's *t*-test, as compared to the value in untreated cells, $P \leq 0.05$ (A). Equal amounts of protein (25 μg) from cell extracts were analysed by antibodies directed against caspase-8, caspase-3 and actin and detected by ECL assay (B and C).

pathways and (ii) to identify the molecular mechanisms responsible of such phenomena.

In the last few years, many authors have claimed that inhibitors of histone deacetylase are potentially effective anticancer agents [25]. Among these, the short-chain fatty acid NaBt induces apoptosis in a variety of cancer cells and different tissue-specific targets of its action have been identified [12,26]. Since pancreatic cancer is resistant to all the classical cancer therapies, mostly because it is resistant to apoptosis induction, we asked whether treatment with NaBt might represent a good adjuvant in chemo-, radio- and immuno-therapies in pancreatic cancer. Our results demonstrate that this is the case. In fact, NaBt treatment renders pancreatic cancer cells prone to mitochondria-mediated and to Fas-mediated apoptosis by down-regulating Bcl-x_L and FLIP proteins and by inducing caspases activation.

In order to study the ability of NaBt to induce apoptosis in pancreatic cancer cell lines, we performed experiments in which the effect of NaBt was evaluated for different periods of time and using different doses. Our results,

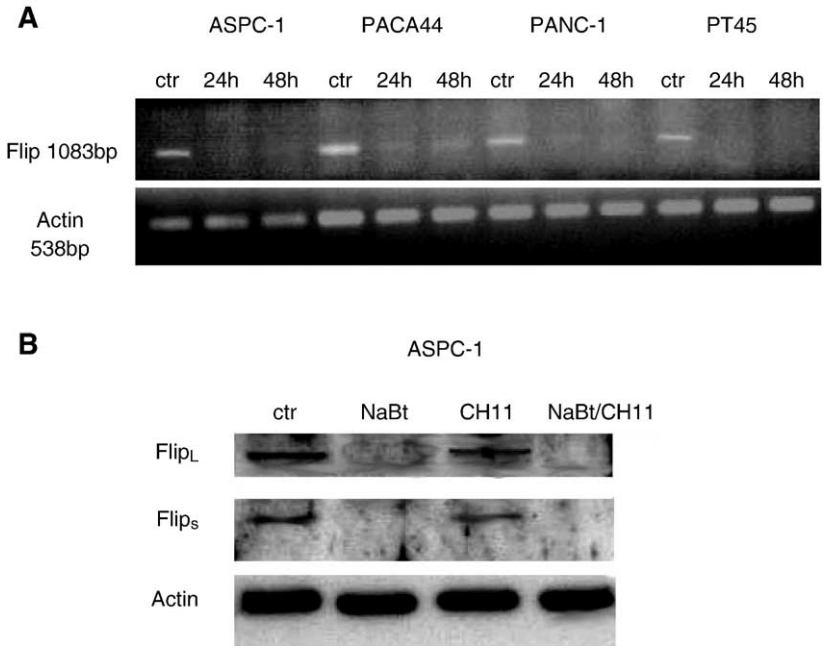

Fig. 7. NaBt down-regulates FLIP. The four pancreatic cancer cell lines ASPC-1, PACA44, PANC-1 and PT45 treated or not with 5 mM NaBt for 24 and 48 h were analysed by RT-PCR using specific primers for *flip* expression. Actin was used as control (A). Cell extracts from ASPC-1 treated with 5 mM NaBt and 500 ng/ml CH11 alone or in combination for 24 h were analysed for FLIP expression with a polyclonal anti-FLIP antibody by ECL assay. (B) Actin was used as control loading.

showing that NaBt inhibits cell growth and induces apoptosis, not only confirm but add new information to previous observations on the effects of NaBt [27–29]. Moreover, to date, the molecular mechanisms mediated by NaBt in pancreatic cancer cells have not been investigated. To approach this issue, we studied the amount of expression of Bcl-2, Bcl-x_L, Bax and Bcl-x_S and the effect of NaBt treatment on the expression of these genes in four pancreatic cancer cell lines. Previous papers reported that the level of Bcl-2 expression is normal or even decreased in pancreatic cancer cells [30,31], while Bcl-x_L is markedly over-expressed [32,33]. Our data show that all the cell lines express high levels of Bcl-x_L, which is strongly down-regulated by NaBt treatment. The effect of NaBt treatment is exerted at the transcriptional level, as evidenced by RT-PCR analysis. The two pro-apoptotic members Bax and Bcl-x_S are expressed in the four cell lines and NaBt does not modify their expression either at the transcriptional level or at the protein level (Bax). Bcl-2 analysis shows heterogeneous expression of Bcl-2 protein in the four cell lines. In particular, Western blot and immunofluorescence analyses evidence that Bcl-2 is undetectable in ASPC-1, barely detectable in PACA44 and PANC-1 (immunofluorescence, not shown) and strongly detectable in PT45. The comparison of Bcl-2 protein expression among the cell lines shows that Bcl-2 expression is inversely correlated with apoptosis sensitivity. In fact it can be noted that ASPC-1 is the most sensitive line to apoptosis (40–50% cell death in 48 h), followed by PACA44 and PANC-1 (30% cell death in 48 h) and as last PT45 (20% cell death in 48 h). However, Bcl-2 expression does not restrain apoptosis induction. Altogether, these data indicate that Bcl-2 may influence the amount of apoptosis induction, but Bcl-x_L renders pancreatic cancer cells resistant to cell death. NaBt, down-regulating Bcl-x_L levels, modifies the ratio between pro- and anti-apoptotic proteins favouring the induction of apoptosis in pancreatic cells. These data are in agreement with previous papers reporting that increased expression of Bcl-x_L correlated with a worse prognosis in pancreatic cancer patients and the down-regulation of Bcl-x_L levels by antisense oligonucleotides resulted in a decreased viability of pancreatic cancer cells and enhanced apoptosis [33–35]. A further evidence, supporting these conclusions, is represented by the paper of Scarpa et al. which describes that, following treatment with a different histone deacetylase inhibitor, the trichostatin A (TSA) the ratio between the levels of expression of pro-apoptotic and anti-apoptotic genes was significantly increased in pancreatic cancer cells [36]. Moreover, the data, reported here, highlight that NaBt induces apoptosis activating the mitochondrial pathway, as demonstrated by membrane depolarization, release of cytochrome *c* in the cytosol and caspase-9 activation. In this context, treatment with NaBt may be of great usefulness in restoring chemotherapy sensitivity in pancreatic cancer.

Furthermore, it has been recently demonstrated that the effects of NaBt are additionally or synergistically enhanced by combining different types of agents, such as phorbol ester and Fas [21,37]. Resistance to Fas-mediated apoptosis in pancreatic cancer cells has been previously reported [38–40],

but the mechanism responsible for this phenomenon has remained unclear. Moreover, the effect of NaBt in Fas-mediated apoptosis in pancreatic cancer has never been investigated. Our results point out that pancreatic cancer cells express high levels of FLIP, an inhibitory protein of death receptor-mediated apoptosis. Moreover, for the first time, our data highlight that FLIP down-regulation by NaBt sensitises to Fas-mediated apoptosis allowing caspase-8 activation by Fas triggering. Bernhard et al. have already claimed that NaBt could have a role in sensitising cancer cells to Fas-mediated apoptosis and, using the acute lymphoblastic leukemia cell line CCRF-CEM, showed that butyrate enhanced Fas-mediated apoptosis downstream of Fas but upstream of caspase-8 activation. Moreover, these authors found that NaBt did not modify FLIP expression and suggest that a new 37 kDa protein, which was up-regulated by butyrate and co-precipitated with caspase-8, could play a role in inducing Fas-mediated apoptosis [41]. Moreover, the ability of NaBt to decrease FLIP expression was previously identified by Hernandez et al. in colon cancer cell lines [22]. In fact, in their paper, they have shown that the reduction of FLIP protein levels by NaBt renders TRAIL-resistant human colon cancer cells sensitive to TRAIL-mediated apoptosis. This scenario confirms that NaBt may have a synergistic effect with Fas, and may induce apoptosis in resistant cells, even if it seems that different mechanisms are used in different cell types. Furthermore, it has recently been shown that NaBt down-regulates NF-κB expression [42]. Since NF-κB has been implicated in the regulation of both FLIP and Bcl-x_L expression, and seems to be constitutively activated in human pancreatic cancer cells, we can imagine that NaBt influences the two pathways of apoptosis by acting on this common regulator [43].

In conclusion, our evidence that NaBt sensitises pancreatic cancer cells to mitochondria-mediated and Fas-mediated pathways of apoptosis strongly suggests that NaBt treatment may be useful to improve chemotherapy and immunotherapy in cancer and therefore may provide a novel regimen for clinical treatment of pancreatic cancer.

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