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Different contribution of BH3-only proteins and caspases to doxorubicin-induced apoptosis in p53-deficient leukemia cells

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ABSTRACT

Bcl-2 family proteins are key regulators of the intrinsic apoptotic pathway, either facilitating (Bax, Bak, BH3-only) or inhibiting (Bcl-2, Bcl-x_L, Mcl-1, A1) mitochondrial release of apoptogenic factors. The role of caspases in this process is a matter of controversy. We have analyzed the relative contribution of caspases and Bcl-2 family of proteins in the induction phase of apoptosis triggered by doxorubicin in two p53-deficient leukemia cell lines, Jurkat and U937. First, we have found that caspases are dispensable for the induction phase of doxorubicin-induced apoptosis in both cell lines but they are needed to speed up the execution phase in Jurkat cells, not expressing Bax. Thus, down-regulation of Bak expression by siRNA significantly prevented doxorubicin-induced apoptosis in Jurkat but not in U937 cells. Reduction of Mcl-1 protein levels with siRNA increased sensitivity to apoptosis in both cell lines. Moreover, our results indicate that the contribution of BH3-only proteins to apoptosis is cell line specific. In Jurkat cells simultaneous silencing of Bim and PUMA was necessary to reduce doxorubicin-induced apoptosis. In U937 cells silencing of Bim or Noxa reduced sensitivity to doxorubicin. Immunoprecipitation experiments discarded an interaction between Mcl-1 and Bak in both cell lines and underscored the role of Bim and PUMA as mediators of Bax/Bak activation.

1. Introduction

DNA-damaging drugs used in clinical treatment of tumors induce apoptosis, through the so-called "intrinsic pathway", which is characterized by the disruption of mitochondrial membrane potential ($\Delta\Psi_m$) and the subsequent activation of a caspase cascade [1,2]. Although the molecular events controlling the execution phase of this pathway are well characterized, those prior to mitochondrial dysfunction are still imprecisely delineated. In most cases, it seems to be the activity of proapoptotic Bcl-2 family proteins what induces $\Delta\Psi_m$ loss and the release of cytochrome *c*, AIF and other apoptogenic proteins. Cells from Bax and Bak double knockout mice are highly resistant to drug-induced apoptosis [3]. In response to apoptotic signals, Bax and/or Bak undergo a conformational change and oligomerize, forming a pore in the outer mitochondrial membrane. Cell-free experiments indicate that Bax and/or Bak proapoptotic conformational change is caused by the action of one or more BH3-only proteins, whose availability is determined by the multidomain anti-apoptotic proteins Bcl-2, Bcl-x_L and Mcl-1 [4,5]. However, whether BH3-only proteins activate directly Bak and/or Bax, is still matter of controversy. In addition, the indispensability of caspases for apoptosis induced by DNA-damaging drug is still under discussion [6]. Mcl-1 degradation by caspases has been proposed to play a main role in apoptosis induction by chemotherapeutic drugs [7,8]. In this context, some reports have implicated caspases in the induction phase of mitochondrial apoptosis [2,9]. In particular, a number of recent works have pointed out to caspase-2 as the apical caspase responsible for mitochondrial dysfunction and release of cytochrome *c* and Smac/Diablo in apoptosis induced by genotoxic stress [9–11]. However, previous results from our laboratory indicate that, in several cellular models of drug-induced apoptosis, caspase-2 is activated downstream of caspase-3 and its inhibition does not prevent cell death [2,12].

In the present work, we have analyzed the precise role played by proteins of Bcl-2 family and caspases in the induction phase of

Abbreviations: AIF, apoptosis-inducing factor; $\Delta\Psi_m$, mitochondrial transmembrane potential; $DiOC_6(3)$, 3,3′-dihexyloxa-carbocyanine iodide; PS, phosphatidylserine.

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doxorubicin-induced apoptosis using two human p53-deficient leukemia cell lines, Jurkat and U937 [13,14]. We show that while the commitment to death, i.e., the disruption of $\Delta\Psi_m$ and release of cytochrome *c* and AIF, relies entirely on the action of Bcl-2 family proteins, caspase activation may be necessary in some cells to speed up the completion of apoptosis, depending on the efficiency of Bax and/or Bak to cause a sufficient degree of mitochondrial permeabilization. Our results also highlight the redundancy of BH3-only proteins in the induction phase of DNA damage-induced apoptosis. These results could explain the reported apparent dependence of caspases for cell death in certain cell types and may be relevant to predict the responsiveness of leukemia cells to DNA-damaging agents.

2. Materials and methods

2.1. Cell culture and toxicity assays

The human T-cell leukemia Jurkat (clone E6.1) and the human myeloma MM.1S were from the ATCC collection and the promonocytic leukemia U937 was from the culture collection of CIB (Madrid, Spain). Cell lines were routinely cultured at 37 °C in RPMI 1640 medium supplemented with 5% (Jurkat, U937) or 10% (MM.1S) fetal calf serum, 2 mM L-glutamine and penicillin/streptomycin (hereafter, complete medium). Jurkat and U937 cell lines overexpressing Mcl-1 were generated essentially as described previously [15,16]. To evaluate drug toxicity, cells ($3–5 \times 10^5$ cells/ml) were treated in flat-bottom, 24-well or 12-well plates with doxorubicin (Sigma, Spain) in complete medium, as indicated. For caspase inhibition assays, cells were preincubated for 1 h with the following caspase inhibitors: 200 μM Z-VDVAD-fmk, 100 μM Z-VEID-fmk (Calbiochem), 100 μM Z-VAD-fmk, 100 μM Z-DEVD-fmk (Bachem, Switzerland), 100 μM Z-LEHD-fmk (BD Biosciences), alone or in combination, prior to doxorubicin addition.

2.2. DNA electrophoresis

For oligonucleosomal DNA fragmentation analysis, cells (1×10^6) were collected after incubation with doxorubicin, washed and incubated in 500 μl lysis buffer (1% SDS, 50 mM Tris/HCl, 150 mM NaCl, 5 mM EDTA, 200 μg/ml proteinase K, 20 μg/ml RNase A) at 50 °C for 1 h. DNA was extracted with 500 μl of phenol/chloroform/isoamylic alcohol (25:24:1) and precipitated with 2 volumes of cold ethanol and 0.5 volumes of 7.5 M ammonium acetate. Precipitated DNA was washed in 70% ethanol and resuspended in 10 mM Tris/HCl buffer, pH 8.0, 1 mM EDTA containing DNase-free, RNase at a final concentration of 20 μg/ml. DNA was analyzed in 1.5% agarose gels in 40 mM Tris/acetic acid, pH 7.6, 1 mM EDTA. High molecular weight (HMW) fragmentation of DNA was analyzed by FIGE (Field Inversion Gel Electrophoresis). Cells (2×10^6) were resuspended in 50 μl of PBS at 60 °C and mixed with 50 μl of 1% agarose solution (pulse-field agarose, BioRad) and introduced in the moulds (BioRad). After gelification, samples were incubated with 10 mM Tris/HCl, pH 9.5 buffer, containing 0.2 M EDTA, 1% lauryl sarcosine and 600 μg/ml proteinase K at 50 °C for 48 h, renewing the solution every 24 h. Finally, samples were separated in 1% agarose gels using a FIGE-mapper electrophoresis system (BioRad).

2.3. Western blot analysis

Caspase activation and levels of Bcl-2 family proteins were analyzed by Western blot, as described previously [1]. Primary antibodies used were: anti-caspase-8 (clone 5F7) and anti-Bak (#06-536) from Millipore; anti-procaspase-3 (clone 19), anti-active caspase-3 (#557035), and anti-Bax (#554104) from BD Biosciences; anti-caspase-9 (clone 5B4) from MBL; anti-Bcl-2 (sc-783), anti-Mcl-1 (sc-819), anti-Bcl-$x_{L/s}$ (sc-1041) and anti-Bik (sc-1710) from Santa Cruz Biotechnology; anti-Bim from Calbiochem; anti-PUMA (#4976) and anti-Bmf (#4692) from Cell Signalling. Protein loading was verified by reprobing membranes with anti β-actin (clone AC15, Sigma) or anti α-tubulin (clone B-5-1-2, Sigma). Western blots were analyzed for several proteins by a modification of the multiple blotting assay method [17] as described [18]. When necessary, the intensity of protein bands, relative to that of actin, was quantified by densitometry by using Quantity One software (BioRad).

To evaluate cytochrome *c* release from mitochondria to cytosol, cells were lysed by gentle resuspension in 250 mM sucrose, 0.05% digitonin, 1 mM EDTA, 25 mM Tris/HCl pH 6.8, 1 mM DTT, 1 mM leupeptin, 1 mM aprotinin, 1 mM pepstatin, 0.1 mM benzamidine and 0.1 mM PMSF for 30 s on ice [19]. Cell extracts were centrifuged at $12{,}000 \times g$ for 3 min and both supernatants (cytosolic fraction) and pellets (membranous fraction) were recovered. Both fractions were analyzed by SDS-15% PAGE, and analyzed by Western blotting using anti-cytochrome *c* (clone 7H8-2C12, BD Biosciences) and anti-COX-II (clone 12C4, Invitrogen) to assess the purity of cytosolic fractions.

2.4. Inmunofluorescence and flow cytometry analysis

Apoptosis was assessed by flow cytometry by simultaneous quantification of phosphatidylserine (PS) exposure and loss of $\Delta\Psi_m$ as previously described [12]. In some experiments the fluorescent probe TMRE (2 nM; Invitrogen) was used to determine $\Delta\Psi_m$. Conformational changes of Bax and Bak proteins were assessed by intracellular immunostaining with specific antibodies [20–22] as described previously [12]. AIF translocation to nuclei was analyzed by immunofluorescence staining and fluorescence microscopy. Briefly, after the different treatments cells were washed in PBS, fixed with 4% paraformaldehyde for 15 min at room temperature and centrifuged onto poly-L-lysine-coated coverglasses. Cells were permeabilized by immersion in 0.1% saponin and incubated with a 1/200 dilution of a rabbit anti-AIF antibody (A7549, Sigma) at room temperature in a humidified chamber for 30 min. Coverglasses were then washed twice with 0.1% saponin and incubated with either a 1/200 dilution of a FITC-labeled goat anti-rabbit IgG (Caltag) or with a 1/1000 dilution of a Alexa-633-labeled goat anti-rabbit IgG (Invitrogen). Finally, coverglasses were sequentially washed with 0.1% saponin, PBS and distilled water, mounted onto glass slides over a drop of Mowiol (Calbiochem, Madrid, Spain) and analyzed in a Zeiss 310 confocal microscope.

2.5. mRNA quantification by MLPA

Cellular mRNA was analyzed by reverse transcriptase multiplex ligation-dependent probe amplification (RT-MLPA) using SALSA MLPA KIT R011 Apoptosis mRNA from MRC-Holland (Amsterdam, The Netherlands) [23]. Total RNA from 5×10^6 cells was isolated by using the RNeasy Mini Kit and QiaShredder columns (Qiagen, Izasa, Spain). Total RNA (200 ng) was first reverse-transcribed using the gene-specific probe mix and the resulting cDNA was annealed overnight at 60 °C to the MLPA probe mix, and then ligated. Ligation products were amplified by PCR (35 cycles, 30 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C) with one unlabeled and one FAM-labeled primer. PCR amplified fragments were separated by capillary electrophoresis (ABI PRISM 3100 Analyzer, Applied Biosystems). The peak area and height were analyzed with PeakScanner software (Applied Biosystems). Levels of mRNA for each gene were expressed as a normalized ratio of the peak area divided by the peak area of housekeeping control genes (β-glucuronidase and β_2-microglobulin).

2.6. RNA interference assays

Gene silencing of Bim, Bak, Mcl-1, PUMA and caspase-2 in Jurkat and U937 cells was achieved by transfection with 75–150 pmol of the corresponding siRNA duplexes. The sense strands of the siRNAs used were as follows: Bak (Proligo): 5′-CCGACGCUAUGACUCAGAG-3′ (position 457–476 from the mRNA start codon); Casp2 (Dharmacon): 5′-ACAGCUGUUGUUGAGCGAA-3′ (position 94–114); Mcl-1 (Dharmacon): 5′-AAUAACACCAGUACGGACGGG-3′ (position 456–476); Bim (Dharmacon): 5′-GACCGAGAAGGUAGACAAUUG-3′. Lamin A/C (Dharmacon): 5′-GCCAUCCACUAUGCGUUGAAC-3′ (position 334–354) was used as a control. PUMA silencing was performed using a validated oligonucleotide pool from Qiagen. Jurkat and U937 cells were electroporated with Nucleofector Kit-V (Amaxa, Germany), according to manufacturer's instructions. At optimal times for gene silencing (usually 5 h post-transfection for Mcl-1 and 24–48 h for the rest of siRNAs), apoptosis was induced with doxorubicin.

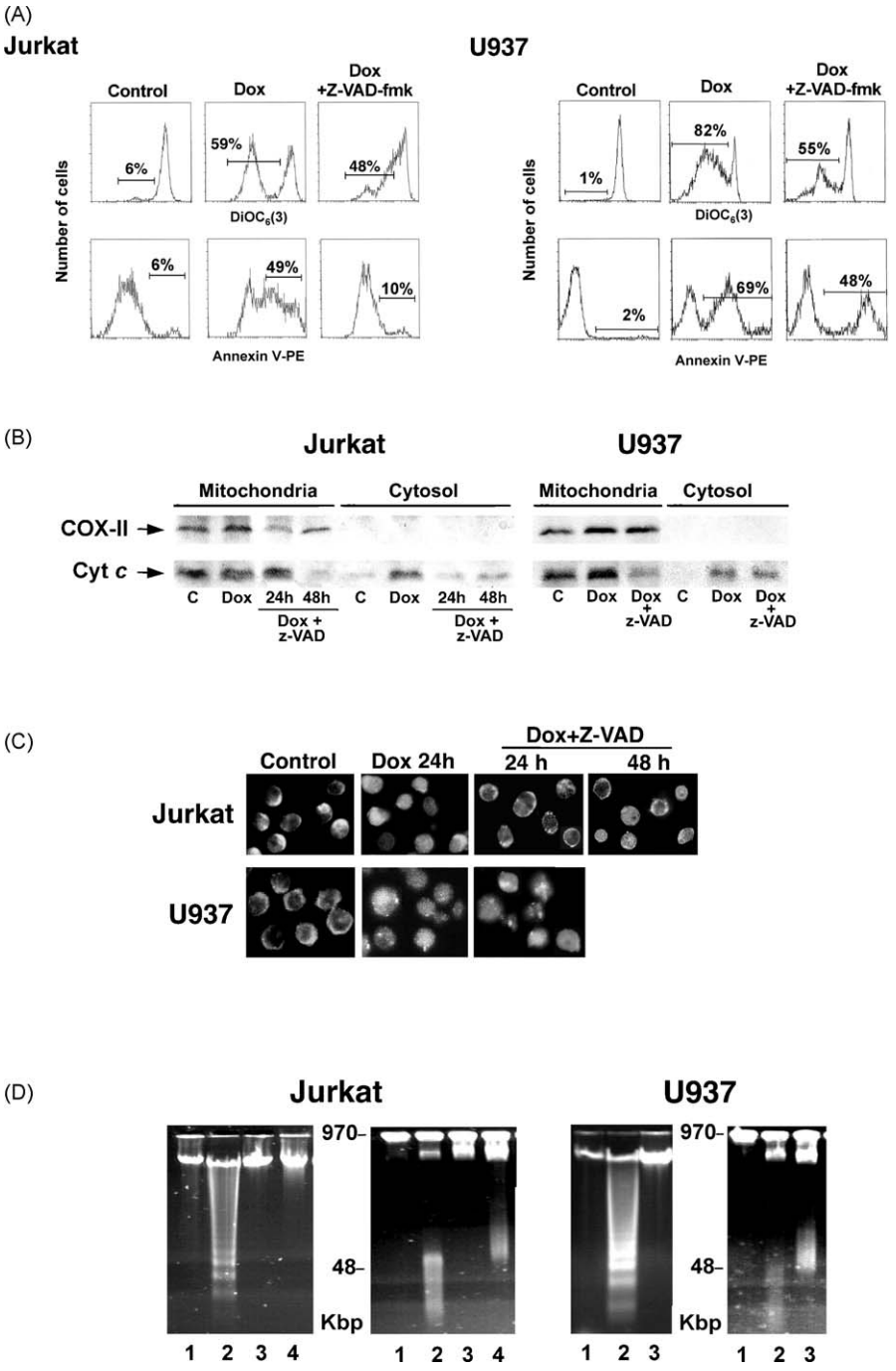

Fig. 1. Caspase inhibition delays loss of $\Delta\Psi_m$ and release of cytochrome *c* and AIF in doxorubicin-induced apoptosis. (A) Jurkat (5×10^5 cells/ml) and U937 (3×10^5 cells/ml) cells were treated for 24 h in complete medium with 1 μM doxorubicin, in the presence or absence of 100 μM Z-VAD-fmk. Then, $\Delta\Psi_m$ and PS exposure were analyzed by flow cytometry with $DiOC_6(3)$ and annexin-V-PE, respectively. (B) The distribution of cytochrome *c* in cytosolic and mitochondrial fractions was analyzed by Western blot in control and doxorubicin-treated cells, in the absence or presence of Z-VAD-fmk. Cytochrome *c* oxidase, subunit II (COX-II), was also analyzed as a control for mitochondrial contamination of cytosolic fractions. (C) Cells were left untreated or incubated for the times indicated, with 1 μM doxorubicin in the absence or in the presence of 100 μM Z-VAD-fmk. After incubation, AIF distribution was assessed by immunostaining with specific anti-AIF antibodies and fluorescence microscopy. Original magnification, 450×. (D) Oligonucleosomal (left panels) and high molecular weight (right panels) DNA fragmentation were analyzed at 0, 24 and 48 h, as described in Section 2. (1) Control; (2) doxorubicin, 24 h; (3) doxorubicin + Z-VAD-fmk, 24 h; (4) doxorubicin + Z-VAD-fmk, 48 h.

In addition, a lentiviral silencing system was used to generate cells stably knocked-down for Bak, Bim or Noxa. Four different sequences were targeted simultaneously to knock-down the proapoptotic protein Bak: 5′-GGTACGAAGATTCTTCAAA-3′, 5′-CCGACGCTATGACTCAGAG-3′, 5′-GCTGCACAGGGACAAGTAAAG-3′ and 5′-AATGCCTATGAGTACTTCACC-3′. Targeted sequences for Bim and Noxa were 5′-GACCGAGAAGGTAGACAATT-3′ and 5′-GCTACT-CAACTCAGGAGATTT-3′, respectively. Oligonucleotides containing the same scrambled sequence (sc) were used to generate a control vector. The pLVTHM, pMD2G and psPAX2, vectors were kindly provided by Dr. Didier Trono (EPFL, Switzerland). Forward and reverse oligonucleotides (Invitrogen) were annealed and cloned into a modified pSUPER.Retro plasmid. The hairpin and promoter fragment from pSUPER.Retro was subcloned in the pLVTHM vector. Packaging 293T cells were transfected by the $Ca_3(PO_4)_2$ method with pLVTHM-Bim, pLVTHM-Bak, pLVTHM-Noxa or pLTHVM-sc plus the packaging and envelope vectors. Supernatants containing viral particles were collected 24 h after transfection. Cells (1×10^6) were resuspended in supernant containing viral particles and centrifuged at $1200 \times g$ and 30 °C for 2 h. Then, cells were resuspended in complete medium and cultured for 24 h before analysis of protein expression by Western blot and selection of individual clones by limiting dilution.

2.7. Immunoprecipitation of Bcl-2 proteins

Total protein extracts from control or doxorubicin-treated cells (5×10^6) were prepared in 500 μl CHAPS buffer (1 mM Hepes pH 7.4, 150 mM NaCl, 1% CHAPS). Cell lysates were subjected to immunoprecipitation with 3 μg of either mouse anti-Mcl-1 mAb (clone 22, BD Biosciences) or rat anti-Bim mAb (clone 14A8, Calbiochem), at 4 °C, overnight, with gentle rotation. Protein–antibody complexes were precipitated by incubating with the corresponding anti-mouse or -rat IgG immobilized on agarose beads for 3 h at 4 °C and brief centrifugation. Beads were washed three times with CHAPS buffer and proteins released by heating for 5 min at 100 °C in electrophoresis sample buffer. Eluted proteins were analyzed by SDS-15% PAGE electrophoresis and Western blotting, using primary rabbit anti-human protein antibodies to avoid detection of rat and mouse immunoglobulins. Membranes were reblotted to confirm Mcl-1 or Bim immunoprecipitation and equal protein loading.

2.8. Statistical analysis

Analysis of significance of results was evaluated by two-tailed Student's *t*-test using GraphPad Prism 4.0 software (GraphPad, USA).

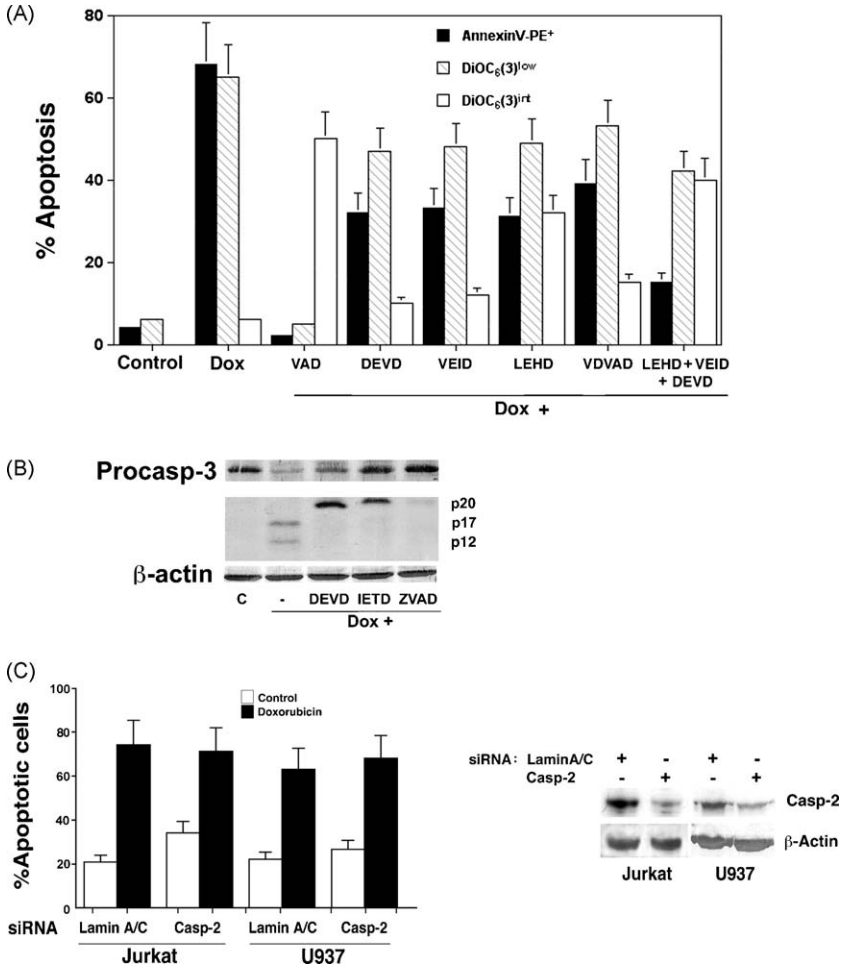

Fig. 2. Role of caspases 3, 6, 9 and 2 in doxorubicin-induced apoptosis in leukemia cells. (A) Jurkat cells (5×10^5 cells/ml) were incubated for 16 h with 1 μM doxorubicin in the absence or presence of either Z-VAD-fmk, Z-DEVD-fmk, Z-VEID-fmk, Z-LEHD-fmk, Z-VDVAD-fmk (all at 100 μM) or the combination of Z-DEVD-fmk + Z-VEID-fmk + Z-LEHD-fmk (100 μM each). PS exposure and $\Delta\Psi_m$ were analyzed as described in Section 2. Data are mean ± SD of three independent experiments. (B) Only Z-VAD-fmk prevents complete processing of procaspase-3. Cells were treated with doxorubicin in the absence or the presence of Z-DEVD-fmk, Z-IETD-fmk or Z-VAD-fmk. Cell lysates were analyzed by Western blot with an anti-caspase-3 antibody as indicated in Section 2. (C) Jurkat or U937 were transfected with siRNAs targeting for lamin A/C or caspase-2. After 24 h, cells were left untreated or incubated with 1 μM doxorubicin for 16 h. Apoptosis was analyzed by $DiOC_6(3)$ and annexin-V-PE staining. Caspase-2 levels were analyzed by Western blot as described in Section 2. Bars are mean ± SD of three independent experiments.

3. Results

3.1. Caspases accelerate doxorubicin-induced apoptosis

We previously reported that doxorubicin-induced apoptosis of Jurkat cells caused the activation of caspases 2, 3, 6, 7, 8 and 9 [1,2]. Co-treatment of Jurkat cells with the pan-caspase inhibitor Z-VAD-fmk prevented PS exposure and delayed $\Delta\Psi_m$ loss induced by doxorubicin (Fig. 1A). Histograms of $\Delta\Psi_m$ analysis by $DiOC_6(3)$ staining showed a high proportion of cell with an intermediate $\Delta\Psi_m$, when compared to untreated cells and cells treated with doxorubicin alone. Cells with intermediate $\Delta\Psi_m$ appear when apoptosis is induced in the absence of caspase activation and are committed to die [6,24]. Cytochrome *c* release caused by doxorubicin was also delayed by Z-VAD-fmk in Jurkat cells (Fig. 1B). In U937 cells, Z-VAD-fmk attenuated doxorubicin-induced PS exposure and $\Delta\Psi_m$ loss (Fig. 1A) but it was unable to inhibit cytochrome *c* release even at 24 h (Fig. 1B). These results were confirmed with the pan-caspase inhibitor Q-VD-OPh (data not shown).

Noteworthy, doxorubicin treatment of Jurkat and U937 cells induced AIF translocation from mitochondria to nucleus, as shown by confocal microscopy (Fig. 1C). Caspase inhibition by Z-VAD-fmk delayed this translocation in Jurkat cells, up to 48 h (Fig. 1C), but it had no effect in U937 cells (Fig. 1C). AIF release nicely correlated with the onset of HMW DNA fragmentation in both cell lines (Fig. 1C and D). To clearly detect HMW DNA fragmentation, experiments were performed in the presence of Z-VAD-fmk to block the action of caspases that induce further oligonucleosomal DNA degradation. As shown in Fig. 1D, doxorubicin-induced oligonucleosomal fragmentation in U937 cells was inhibited by Z-VAD-fmk. However, in these conditions 48 kpb DNA fragments could still be detected. In Jurkat cells Z-VAD-fmk delayed for 48 h the appearance of HMW fragmentation of DNA, typical of AIF action [25].

Peptide inhibitors that preferentially inhibit either caspase-3 (Z-DEVD-fmk), -6 (Z-VEID-fmk), -9 (Z-LEHD-fmk) or -2 (Z-VDVAD-fmk) only attenuated doxorubicin-induced PS exposure in Jurkat cells (Fig. 2A), despite they inhibit nuclear fragmentation (data not shown). The caspase-9 inhibitor reduced the percentage of cells with low $\Delta\Psi_m$ and a significant portion of cells (47%) exhibited an intermediate $\Delta\Psi_m$ (Fig. 2A). Caspase-$9^{-/-}$ MEF also displayed an intermediate $\Delta\Psi_m$ when exposed to various apoptotic insults [6]. According to previous results [12], a mixture of Z-DEVD-fmk, Z-VEID-fmk and Z-LEHD-fmk inhibited $\Delta\Psi_m$ loss more efficiently than the caspase-9 inhibitor alone, but did not achieve the efficiency of Z-VAD-fmk (Fig. 2A). These differences were probably due to the superior performance of Z-VAD-fmk to block full caspase-3 activation into the cells (Fig. 2B). As shown in Fig. 2B, and in agreement with our previous report [2], only Z-VAD-fmk, but not Z-IETD-fmk, which may potentially inhibit caspases 6, 8, 9 and 10 [26,27], was able to prevent the initial processing of caspase-3 in doxorubicin-treated cells.

The pentapeptide Z-VDVAD-fmk has been reported to inhibit caspases 3 and 7 [28], apart from caspase-2. Thus, to better elucidate if caspase 2 was a mediator of $\Delta\Psi_m$ loss in Jurkat cells upon doxorubicin-induced DNA damage, we performed knock-down experiments using RNA interference technology. As shown

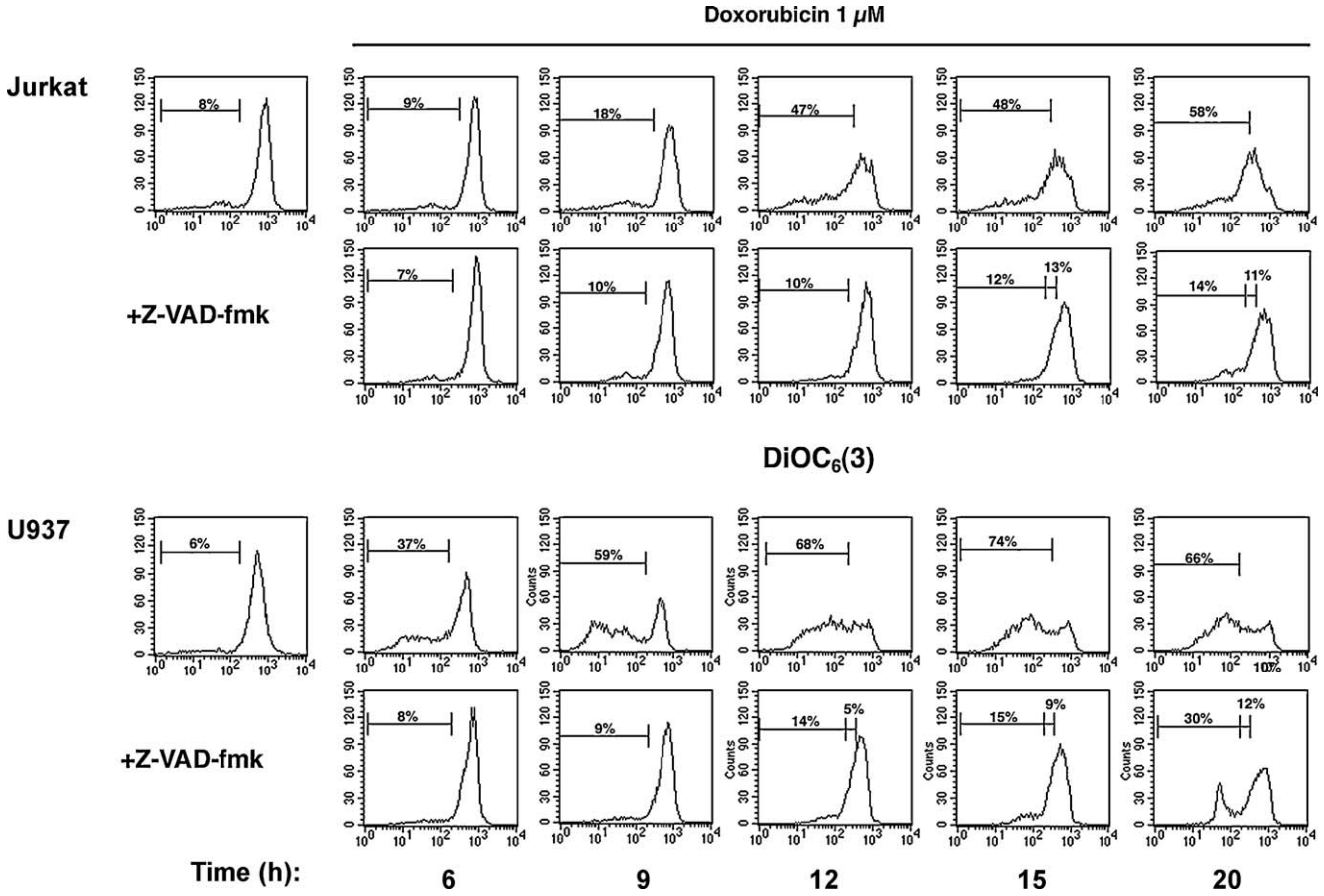

Fig. 3. Caspase-dependence of mitochondrial destabilization in Jurkat and U937 cells. Jurkat (5×10^5 cells/ml) and U937 (3×10^5 cells/ml) cells were treated with 1 μM doxorubicin for the times indicated, in the presence or absence of 100 μM Z-VAD-fmk. At the different time points, $\Delta\Psi_m$ was analyzed by flow cytometry with $DiOC_6(3)$. Results of a representative experiment, out of three, are shown.

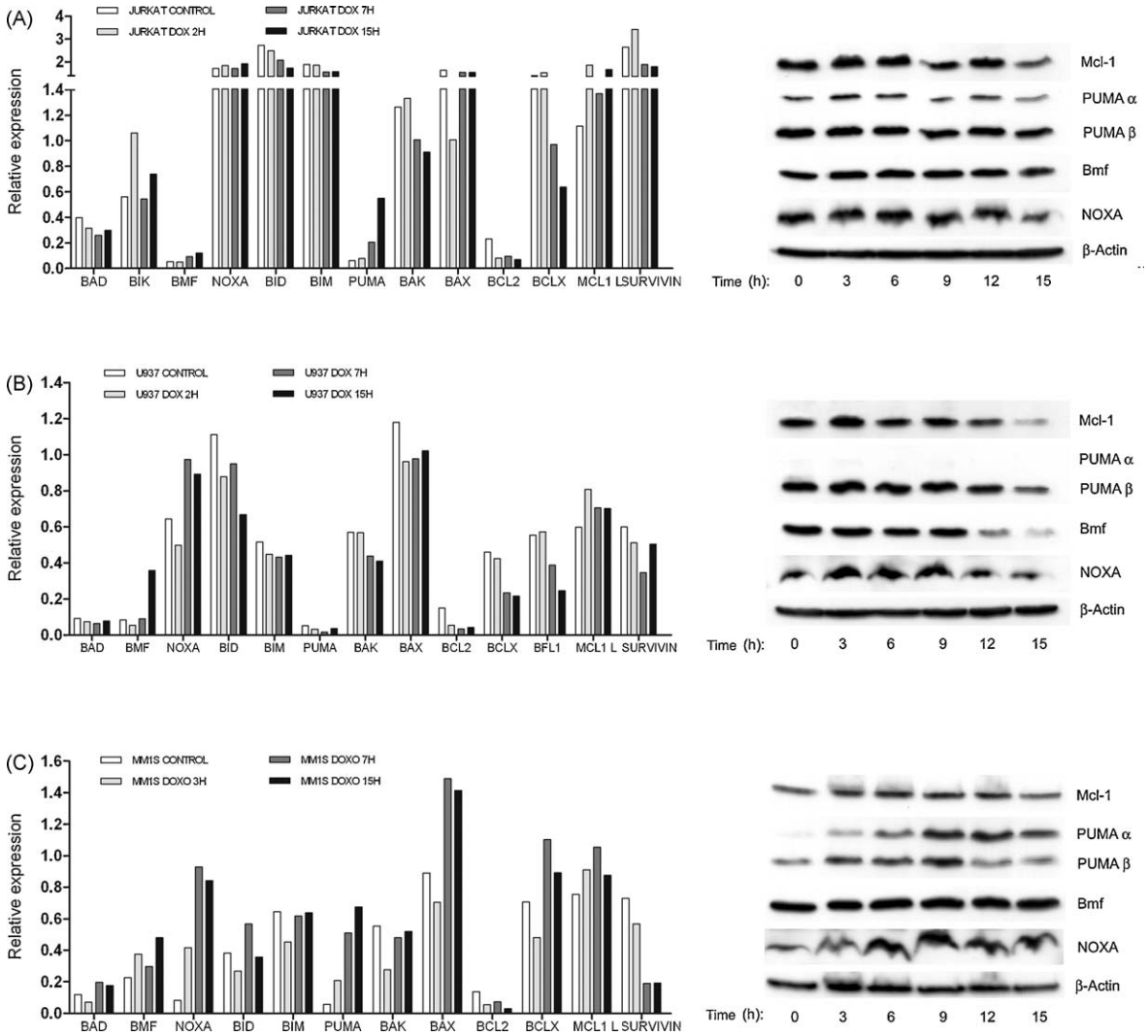

Fig. 4. Doxorubicin induces changes in levels of Bcl-2 family proteins. Jurkat (A), U937 (B) and MM.1S (C) cells (300,000 cell/ml) were either left untreated (white bars) or treated with 1 μM doxorubicin for the times indicated. Left panels: total RNA from of 5×10^6 cells was extracted as described in Section 2 and 200 ng were used for RT-MLPA analysis. The mRNA amount of all genes was normalized with respect to that of β_2-microglobulin. Right panels: Mcl-1, PUMA, Bmf and Noxa protein expression was analyzed by Western blotting during the same time period. Actin was used as a control for equal loading. A representative experiment for each cell line, out of three, is shown.

in Fig. 2C, despite the significant reduction in caspase-2 levels, the degree of apoptosis in response to doxorubicin was comparable to that of untransfected cells (Fig. 2A), or cells knocked-down for a control protein (lamin A/C), as assessed by $DiOC_6(3)$ and annexin-V-PE labeling (Fig. 2C). These results were congruent with the inefficacy of the caspase-2 inhibitor Z-VDVAD-fmk in blocking doxorubicin-induced cell death (Fig. 2A).

Comparative time-course analysis revealed that $\Delta\Psi_m$ loss occurred earlier in U937 than in Jurkat cells and that caspase activity was needed for a complete mitochondrial disruption, especially in Jurkat cells (Fig. 3). Caspase inhibition by co-treatment with Z-VAD-fmk prevented complete $\Delta\Psi_m$ loss induced by doxorubicin in Jurkat but only partially in U937 cells (89% versus 65% in the experiment shown in Fig. 3). In the presence of Z-VAD-fmk, Jurkat cells with an intermediate $\Delta\Psi_m$ started to appear after 16 h of incubation with doxorubicin.

3.2. Doxorubicin induces changes in levels of mRNA and proteins of Bcl-2 family

Changes in the levels of mRNA of several genes implicated in apoptosis, including Bcl-2 family proteins, were simultaneously analyzed by the MLPA technique (Fig. 4). In Jurkat cells, gene expression of the BH3-only protein PUMA was up-regulated at early as 2 h upon doxorubicin treatment and increased progressively (a 10-fold increase at 15 h, Fig. 4A). This increase in mRNA was coincident in time with a moderate increase in the α isoform of PUMA protein (2.2, 1.6 and 1.6 times after 3, 6 and 12 h, respectively, as determined by densitometry) and a decrease at longer times of doxorubicin treatment (Fig. 4A). In U937 cells, mRNA levels of PUMA remained low and no expression of PUMA-α was detected (Fig. 3B). However, Noxa mRNA levels increased from 2 h with a maximum at around 7 h after doxorubicin treatment, with a congruent increase in Noxa protein levels (Fig. 4B). Both in Jurkat and U937 cells, mRNA and protein levels of multidomain proapoptotic Bax and Bak did not significantly change by doxorubicin treatment (Fig. 4A and B and data not shown). Bcl-2 and Bcl-x_L protein levels remained low in Jurkat cells and they did not change in U937 cells (data not shown) despite the reduction of the corresponding mRNA levels (Fig. 3A and B). Mcl-1 gene transcription increased significantly in both cell lines (Fig. 3A and B) and this was confirmed by quantitative RT-PCR (Fig. S1). According to this, Mcl-1 protein levels slightly increased during the first 6 h of treatment with doxorubicin but greatly decreased thereafter (Fig. 4 and Fig. S1). As a control of canonical apoptotic gene response upon DNA damage, MM.1S cells, expressing wild-

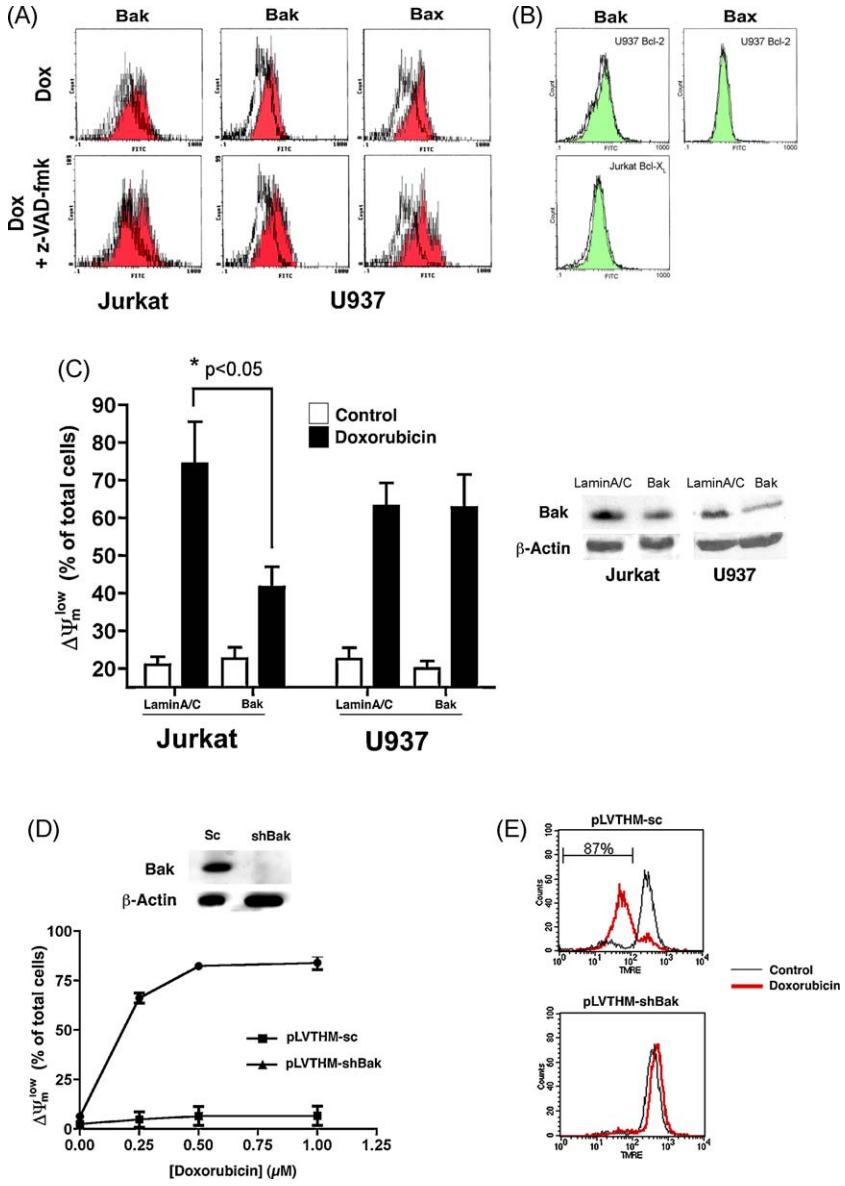

Fig. 5. Activation of Bax and/or Bak is required for doxorubicin-induced apoptosis. (A) Cells were left untreated (empty histograms) or treated with 1 μM doxorubicin (grey histograms) for 16 h in the presence or absence of 100 μM Z-VAD-fmk. Then, cells were fixed and immunostained with conformation-specific monoclonal anti-Bax (6A7) or anti-Bak (Ab-1) antibodies and analyzed by flow cytometry. Histograms shown are representative of four independent experiments. (B) Jurkat-Bcl-X_L and U937-Bcl-2 cells were treated with 1 μM doxorubicin for 16 h and activation of Bax and Bak was analyzed as described in (A). (C) Jurkat or U937 were transfected with siRNAs targeting for lamin A/C or Bak. After 24 h, cells were left untreated or incubated for 16 h with 1 μM doxorubicin and $\Delta\Psi_m$ and PS exposure were analyzed by flow cytometry. Levels of Bak protein were analyzed by Western Blot as described in Section 2. Immunoblots were reprobed with anti-actin antibody as a control for equal loading. Bars are mean ± SD of three independent experiments. Statistically significant differences: $^*p < 0.05$ (D) Jurkat cells were transformed with lentiviral vectors containing shRNA sequences targeting Bak or scrambled sequences (control cells). After infection, stable cell lines were obtained by clonal dilution. pLVTHM-sc or pLVTHM-shBak Jurkat cells were incubated with doxorubicin at the doses indicated for 24 h and $\Delta\Psi_m$ was determined by flow cytometry after TMRE staining. Data are mean ± SD of three independent experiments. (E) pLVTHM-sc or pLVTHM-shBak Jurkat cells were incubated with 1 μM doxorubicin at for 24 h and $\Delta\Psi_m$ was determined by flow cytometry. Histograms shown are representative of six independent experiments.

type p53 [29], were analyzed. In these cells, the p53 targets Noxa and PUMA were strongly up-regulated by doxorubicin both at mRNA and protein level (Fig. 4C). Mcl-1 was also up-regulated at the mRNA level but down-regulated at the protein level (Fig. 4C).

3.3. Bax and Bak activation is an early caspase-independent event in apoptosis induced by DNA damage

Mitochondrial membrane potential loss and release of apoptogenic proteins from mitochondria are mediated by the multidomain proapoptotic proteins Bax and Bak [3]. Jurkat cells do not express Bax due to frameshift mutations resulting in premature termination of translation [30,31]. Doxorubicin treatment induced proapoptotic conformational changes of both proteins in U937 cells and of Bak in Jurkat cells (Fig. 5A). Blockade of caspase activity by Z-VAD-fmk did not prevent the activation of Bax and Bak induced by doxorubicin (Fig. 5A). Overexpression of Bcl-2 in Jurkat and U937 cells completely abrogated doxorubicin-induced Bax and/or Bak proapoptotic conformational changes (Fig. 5B) and fully inhibited all features of apoptosis, including PS exposure and $\Delta\Psi_m$ loss (data not shown). A partial reduction in levels of Bak expression with Bak-siRNA, significantly reduced the percentage of Jurkat cells with low $\Delta\Psi_m$ and positive for annexin-V-PE binding after doxorubicin treatment (Fig. 5C and data not shown).

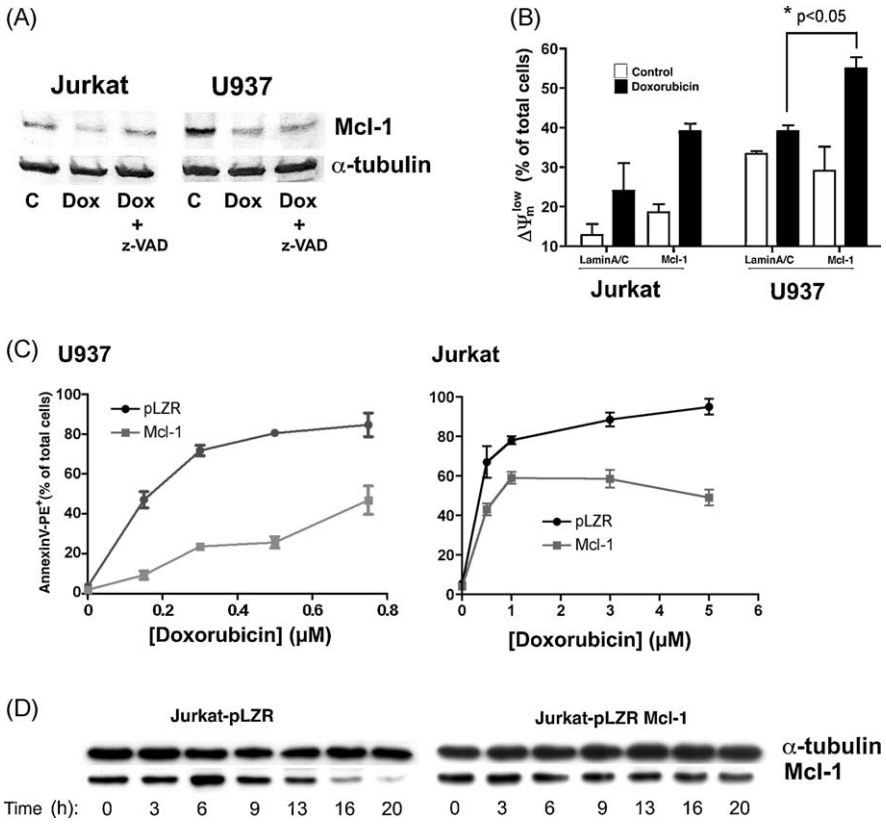

Fig. 6. Role of Mcl-1 in doxorubicin-induced apoptosis. (A) Effect of caspase inhibition on Mcl-1 downregulation during doxorubicin-induced apoptosis. Cells were left untreated or treated for 16 h with 1 μM doxorubicin in the absence or in the presence of Z-VAD-fmk and Mcl-1 protein levels were analyzed by Western blot. (B) Jurkat and U937 were transfected with lamin A/C- or Mcl-1-siRNA. After 6 h, cells were left untreated or incubated for 16 h with 0.25 μM doxorubicin. Apoptosis was quantified by $DiOC_6(3)$ and annexin-V-PE staining. Bars are mean ± SD of three independent experiments. Statistically significant differences: $^*p < 0.05$. (C) U937 (left panel) and Jurkat (right panel) cells overexpressing anti-apoptotic Mcl-1 were treated with doxorubicin at different doses for 24 h. Apoptosis was estimated as by annexin-V-PE staining and flow cytometry. Data are mean ± SD of four independent experiments.

However, reduction of Bak levels with Bak-siRNA in U937 cells did not significantly alter doxorubicin toxicity (Fig. 5C), probably due to these cells still express Bax protein. To further clarify the essentiality of Bak in Jurkat cells, we generated a Jurkat-derived subline completely devoid of Bak by shRNA lentiviral infection (Fig. 5D) and tested its sensitivity to doxorubicin. Jurkat Bax and Bak-null cells (pLVTHM-shBak) exhibited increased resistance to doxorubicin (Fig. 5D). Doxorubicin did not induce the appearance of Jurkat-shBak cells with intermediate $\Delta\Psi_m$ (Fig. 5E) and the release of AIF to cytoplasm and nucleus was also inhibited (Fig. S2).

3.4. Down-regulation of Mcl-1 increases doxorubicin toxicity

The anti-apoptotic member of the Bcl-2 family Mcl-1 is critical for the survival of many hematopoietic cells [32–34] and Mcl-1 degradation by the proteasome [35] or caspases [36–38] is necessary for apoptosis in some models. Doxorubicin induced an early increase followed by a decrease in Mcl-1 levels both in Jurkat and U937 cells (Figs. 4 and 6A and Fig. S1) that was apparent from 12 h of incubation with the drug (Fig. 3). The pan-caspase inhibitor Z-VAD-fmk prevented degradation of Mcl-1 in Jurkat but not in U937 cells (Fig. 6A). Mcl-1 reduction was not due to reduced gene transcription since levels of Mcl-1 mRNA slightly increased in Jurkat and U937 cells during treatment with doxorubicin, as determined by RT-MLPA (Fig. 4) and quantitative PCR (Fig. S1). Jurkat and U937 cells transfected with Mcl-1-siRNA exhibited increased sensitivity to doxorubicin, as compared to cells transfected with LaminA/C siRNA (Fig. 6B), especially in U937 cells. This prominent role of Mcl-1 in U937 cells was further confirmed by overexpression of Mcl-1. Jurkat and specially U937 cells overexpressing Mcl-1 showed reduced sensitivity to doxorubicin compared to cells carrying an empty vector (Fig. 6C). Increased resistance to doxorubicin in Mcl-1-overexpressing Jurkat cells correlated with the delayed degradation of Mcl-1 (Fig. 6D).

3.5. Different BH3-only proteins contribute to doxorubicin-induced apoptosis

The increase in Noxa and PUMA protein levels in U937 and Jurkat cells, respectively, suggested a possible role of these proteins in doxorubicin-induced cell death (Fig. 4). Although Noxa and PUMA are regulated through p53 activation, it has been reported that both proteins can also act in a p53-independent manner [4]. On the other hand, participation of the BH3-only protein Bim in doxorubicin toxicity could explain the increase in cell death caused by Mcl-1 downregulation (Fig. 6B). Silencing of Bim or Noxa by siRNA significantly reduced the apoptotic response to doxorubicin in U937 but not in Jurkat cells (Fig. 7A and B). This partial effect was in accordance with the partial reduction in the corresponding protein levels due to low transfection efficiency (around 40%, data not shown). To overcome this technical limitation, we performed lentiviral depletion of Bim in Jurkat cells. Interestingly, we observed that Bim silencing induced a significant increase in PUMA protein levels (Fig. 7C). Moreover, silencing of PUMA by siRNA also induced a significant increase in Bim levels (Fig. 7D), suggesting that levels of these proteins could be jointly regulated. Silencing of PUMA in Jurkat cells partially

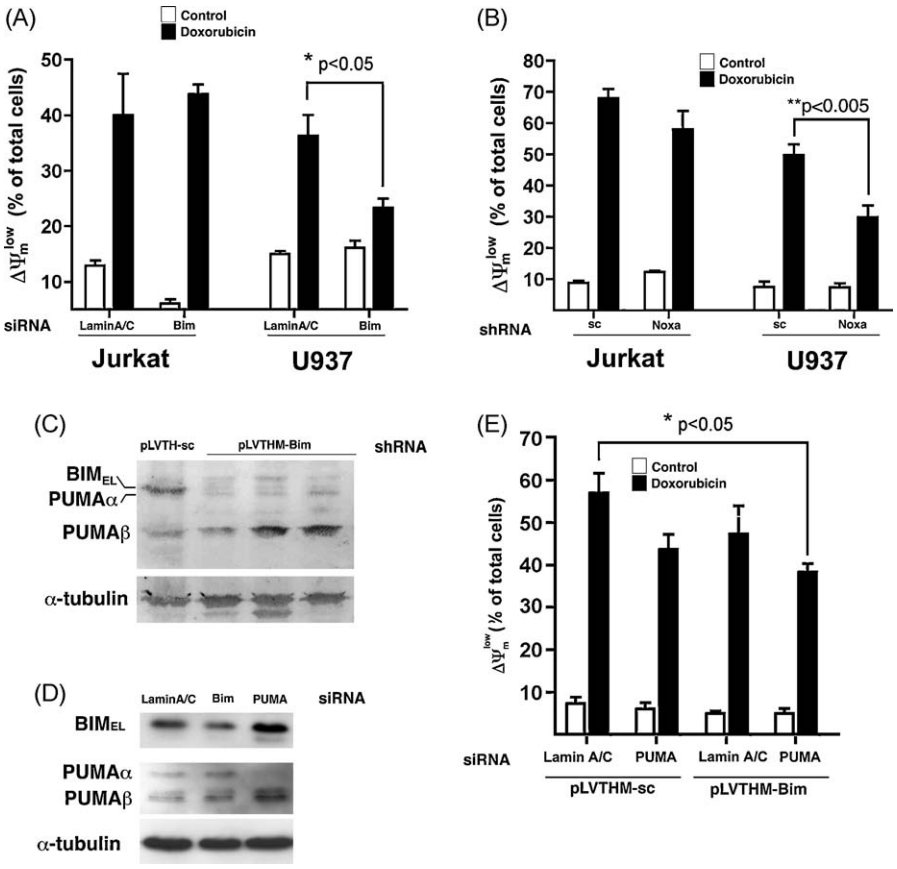

Fig. 7. Contribution of Bim, PUMA and Noxa to doxorubicin-induced apoptosis. (A) Jurkat and U937 were transfected with siRNA targeting lamin A or Bim. (B) Jurkat and U937 cells were transfected with scrambled (sc) or Noxa-siRNA, as indicated. In all cases, 24 h after transfection, cells were left untreated or incubated with 0.25 μM doxorubicin for 16 h and apoptosis was analyzed by simultaneous labeling with $DiOC_6(3)$ and annexin-V-PE. Bars are mean ± SD of three independent experiments. Statistically significant differences: $^*p < 0.05$; $^{**}p < 0.005$. (C) Down-regulation of Bim protein induces overexpression of PUMA in Jurkat cells. Cells were transfected with pLVHTM-sc or pLVTHM-Bim and individual clones were selected by limiting dilution. Expression of Bim and PUMA in three different representative clones was analyzed by Western blot in the same membrane. One representative analysis, out of three is shown. (D) Down-regulation of PUMA-α protein induces overexpression of Bim and PUMA-β in Jurkat cells. Cells were transfected with PUMA-siRNA and levels of PUMA and Bim were analyzed after 24 h. Since PUMA isoforms are generated by alternative splicing form a single gene, silencing of mRNA for α isoform seems to favor expression of β isoform. (E) PUMA-siRNA reduces sensitivity of Jurkat cells knocked-down for Bim. Jurkat-pLVTHM-sc and Jurkat-pLVTHM-Bim were transfected with Lamin A/C- or PUMA-siRNA and treated with doxorubicin for 16 h. Apoptosis was analyzed by annexin-V-PE labeling by flow cytometry. Data are mean ± SD of three independent experiments.

reduced toxicity of doxorubicin with respect to cells transfected with a lamin A/C-targeting siRNA (Fig. 7E). Lentiviral Bim depletion slightly reduced the toxicity of doxorubicin in Jurkat cells, although this reduction was not significant. However, when PUMA was knocked-down in pLVTHM-shBim Jurkat cells, doxorubicin toxicity was significantly reduced (Fig. 7E), indicating the cooperation of both proteins in apoptosis induction.

3.6. Immunoprecipitation of proteins of the Bcl-2 family

The activity of Bcl-2 family proteins is finely tuned by defined interactions among their members. We studied associations among endogenous proteins of this family by co-immunoprecipitation. CHAPS detergent was used to prepare cell extracts in order to avoid non-specific interactions [39]. Immunoprecipitation of Mcl-1 showed that only a very small fraction of Bak interacts with Mcl-1 in untreated cells (Fig. 8A). It must be taken into account that the flow-through fraction is 20-fold diluted with respect to immunoprecipitates. Most Noxa was associated to Mcl-1 both in Jurkat and U937 cells (Fig. 8A). Bim was near completely bound to Mcl-1 in Jurkat but a significant fraction remained unbound in U937 cells (Fig. 8A). No association between Mcl-1 and PUMA in Jurkat cells was detected (Fig. 8A). Incubation with doxorubicin for short times (6 h) significantly increased the amount of Noxa associated to Mcl-1 in U937 (Fig. 8A, left panels), but not in Jurkat cells. Mcl-1/Noxa complexes disappeared at longer times, coinciding with Mcl-1 degradation (Fig. 3). Mcl-1 was associated with Bim in control cells and this association only diminished after 13 h of doxorubicin treatment, both in U937 and Jurkat cells, again coinciding with Mcl-1 degradation. Immunoprecipitation of Bim confirmed the Mcl-1/Bim association. A fraction of Mcl-1 was found in the supernatants, corresponding probably to the Mcl-1 fraction bound to Noxa. Neither Bak protein could be detected in Bim immunoprecipitates in both Jurkat and U937 cells (Fig. 8B) nor Bax in U937 cells (not shown).

4. Discussion

The molecular events of the execution phase of the intrinsic pathway of apoptosis are, for the most part, well known. However the molecular details of the induction phase of apoptosis, leading to mitochondrial destabilization, are more imprecisely identified. Two main questions remain still open: (i) the possible participation of caspases and (ii) the precise role of individual BH3-only proteins and of Mcl-1. In order to shed light on these questions, we have analyzed the molecular events of the induction phase in apoptosis induced by doxorubicin, a topoisomerase II inhibitor and DNA intercalating agent, included in many chemotherapy regimes. We have focused on the relative role of caspases and Bcl-2 family of proteins. Concerning the first question, we have found that Jurkat

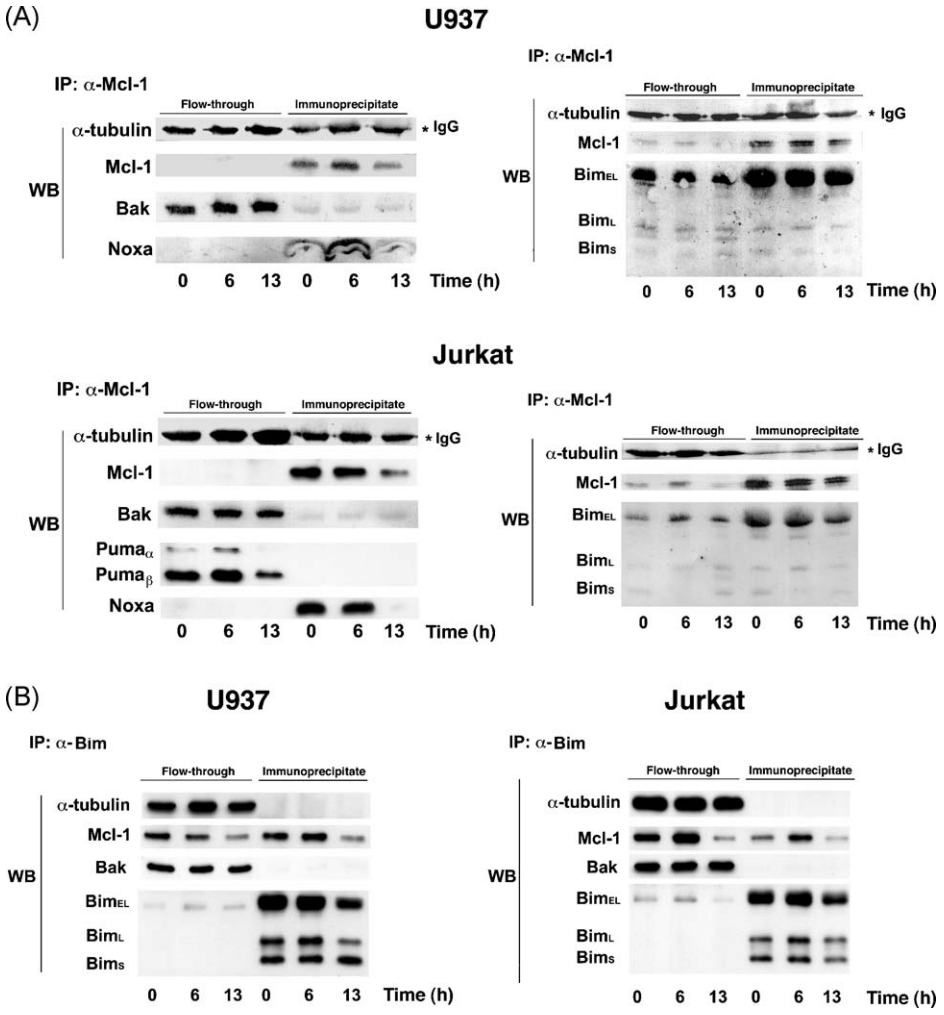

Fig. 8. Mcl-1 interacts with Bim and Noxa, but not with Bak in Jurkat and U937 cells. (A) Protein lysates of control or doxorubicin-treated cells were prepared with CHAPS buffer as described in Section 2. Mcl-1 was immunoprecipitated with a specific mAb (clone 22) and immunoprecipitates were analyzed by Western blot with the antibodies indicated in each panel. Asterisk (*) indicates the position of the heavy chain of immunoprecipitated IgG. (B) Cell lysates were prepared as above, subjected to immunoprecipitation with rat mAb anti-Bim (clone 14A8) and analyzed by Western blotting (WB) with the specified antibodies. Bak was revealed in the same WB membrane prior to Bim. **The position of Bak band in supernatants of Bim_{EL} immunoprecipitation.

and U937 cells differ in the degree of dependence of caspases for apoptosis completion. The pan-caspase inhibitor Z-VAD-fmk delayed late caspase-dependent features of apoptosis, such as DNA laddering and nuclear fragmentation in both cell lines, but had little or no effect in early events, such as proapoptotic conformational changes of Bak and Bax. However, we observed significant differences between Jurkat and U937 cells, namely in the time-course of $\Delta\Psi_m$ loss and release of mitochondrial apoptogenic proteins. General caspase inhibition by Z-VAD-fmk delayed the release of cytochrome *c* and AIF in Jurkat but not in U937 cells. These results fit a two-step model of mitochondrial permeabilization in Jurkat cells in which the second step depends on a caspase amplification loop. The requirement for this amplification loop could be related to the lack of Bax protein in Jurkat cells, resulting in a less efficient mitochondrial permeabilization. Recently, experiments carried out with fibroblasts from double knock-out mice, lacking caspases 3 and 7, demonstrate that these caspases can determine $\Delta\Psi_m$ loss and cytochrome *c* and AIF release during apoptosis [40]. On the contrary, it has been shown that caspases 2 and 9 as well as Apaf-1 are not essential for cell death induced by IL-3 deprivation or drug treatment in mouse myeloid cell lines [41]. These apparently opposing results may reflect differences in the performance of apoptotic machinery among cell models. Cells with a defective mechanism of mitochondrial permeabilization, such as Jurkat cells, would rely on the action of caspases to accelerate the release of cytochrome *c* and caspase-independent apoptogenic factors like AIF to ensure rapid completion of apoptosis (Fig. 9).

Release of apoptogenic proteins from mitochondria is mediated by activation of Bax and Bak through a conformational change that favors their oligomerization [20,22,42–44]. Doxorubicin-induced activation of Bax and Bak in U937 cells and of Bak in Jurkat cells. In both cells lines, Bax/Bak conformational change was caspase-independent, suggesting that caspase activation occurs exclusively downstream mitochondria and that the caspase amplification loop is not involved in Bax/Bak activation. RNAi downregulation of Bak expression greatly reduced cell sensitivity to doxorubicin in Jurkat but not in U937 cells, in agreement with previous reports showing that cells either from $Bax^{-/-}$ or $Bak^{-/-}$ mice are still sensitive to chemical damage and only cells from $Bax^{-/-}Bak^{-/-}$ mice become resistant to drug-induced apoptosis [3]. Knocking down Bak expression in Jurkat cells leads to resistance to doxorubicin-induced apoptosis, since this is the only multidomain proapoptotic protein expressed by cells. Conversely, our preliminary results suggest that the re-expression of Bax protein in Jurkat cells attenuate the degree of caspase-dependence for the efficient

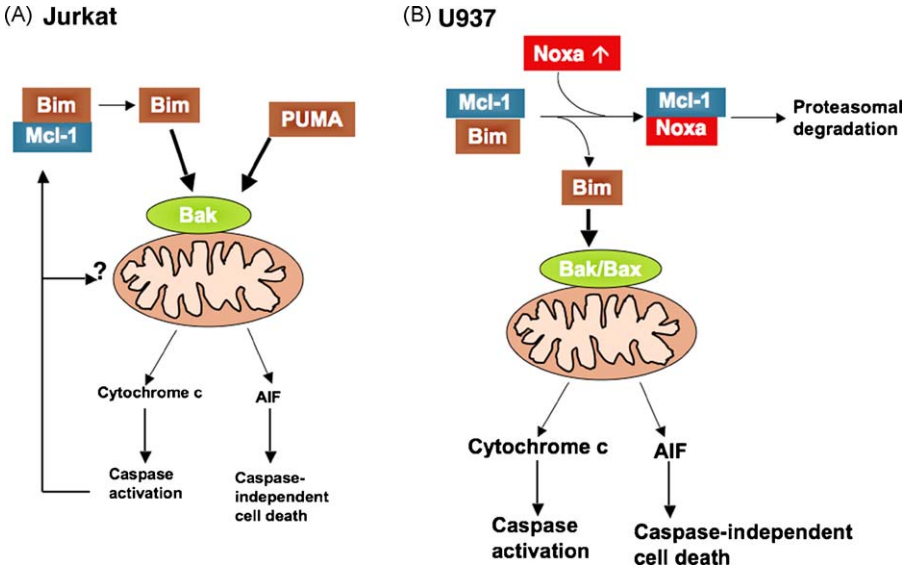

Fig. 9. Proposed models for apoptosis induction and mitochondrial disruption in doxorubicin-induced apoptosis of Jurkat and U937 cells. In Jurkat cells (A), PUMA and, in a lesser extent, Bim contribute to Bak activation. In these cells, the activity of Bak is not enough *per se* to cause a substantial loss of $\Delta\Psi_m$ and the release of AIF from mitochondria in the absence of caspase activation. Under these conditions, the cytochrome *c*-triggered Apaf-1-mediated caspase activation is required for the rapid execution of apoptosis and so apoptosis is delayed in the presence of Z-VAD-fmk. In U937 cells (B), the combined activation of Bax and Bak, induced at least by Bim, causes an important $\Delta\Psi_m$ loss and AIF release and, consequently, caspase inhibition with Z-VAD-fmk does not delay cell death. In U937 cells, Noxa upregulation could mediate the down-regulation of Mcl-1 which allows the release of Bim.

mitochondrial permeabilization (Fig. S3). Furthermore, Jurkat cells are an interesting model to explore since loss-of-function Bax mutations have been found in more than 20% of hematologic neoplasia [45], pointing out the relevance of determining the apoptotic mechanisms activated in these cells. In U937, Bax can still ensure efficient, caspase-independent, mitochondrial permeabilization even after reduction of Bak protein levels by siRNA.

There is now compelling evidence that activation of Bax and Bak is triggered by interaction with a subset of BH3-only proteins [5]. We analyzed the role of BH3-only proteins in doxorubicin-induced apoptosis in Jurkat and U937 leukemia cells. Bim has been proposed to be an activator in the so-called "direct model" of Bak/Bax activation [46]. Downregulation of Bim levels with siRNA or shRNA reduced doxorubicin-induced apoptosis in U937 cells. In Jurkat cells, downregulation of Bim caused an increase of PUMA expression, a protein that could also be an activator of multi-domain proapoptotic proteins [47,48]. This effect could denote a certain functional redundancy of these proteins in cells, as well as a coordinated regulation of their expression to guarantee an effective apoptosis induction after cell damage. Simultaneous silencing of both proteins reduced cell sensitivity to doxorubicin to a greater extent than either Bim or PUMA silencing, indicating that both proteins contribute to the induction of apoptosis in Jurkat cells. Also our results indicate that Bim and Noxa contribute to doxorubicin-induced apoptosis in U937 cells. Although Noxa and PUMA are up-regulated through p53 activation, it has been reported that both proteins can also act in a p53-independent manner [4]. These observations fit with our results indicating that Noxa and PUMA contribute to cell death independently of p53, explaining why p53-deficient are not completely resistant to DNA-damaging agents [49,50]. Indeed, PUMA is induced in response to doxorubicin in p53-deficient cells by the p53-related protein p73 [51], which is expressed in Jurkat cells [52].

How Bcl-2 proteins are regulated through interaction with other family members is at present a matter of controversy. According to the "direct" and "hierarchical" models, some of the BH3-only proteins (Bim, Bid and probably PUMA) [48] can directly activate Bax and Bak, whereas the "displacement model" proposes that the role of BH3-only proteins is to free Bak from Mcl-1 [53]. Most of the results that support both models are based on in vitro experiments with recombinant proteins or in overexpression assays. In this work we have studied the interactions of endogenous Bcl-2 proteins during doxorubicin-induced apoptosis. In Jurkat and U937 leukemia cells we have not observed a significant association between Mcl-1 and Bak, as proposed by the displacement model. Instead, healthy leukemia cells contain complexes of Mcl-1 with Bim or Noxa. In Jurkat cells, in response to doxorubicin, a small increase of PUMA protein occurs which seems to cause a slight mitochondrial destabilization and the release of a small amount of cytochrome *c* and apoptosis is triggered with the engagement of a caspase amplification loop. In U937 cells, which are less dependent on caspases for mitochondrial permeabilization, doxorubicin increases the amount of Mcl-1/Noxa complexes. This association could favor Mcl-1 degradation, reducing its stability through a conformational change [54]. Moreover, a small amount of Bim seems to be released from Mcl-1 after doxorubicin treatment, possibly mediating Bax and Bak conformational activation. We explored the hypothesis of a direct activation of Bak by Bim or PUMA, but our results do not reveal a direct association of these proteins. If Bim or PUMA are implicated in Bax/Bak activation, as supported by previous reports, their action could be either indirect, or mediated by a "hit-and-run" transitory association that remains elusive.

Mcl-1 has a high turnover rate and the shortest half-life among anti-apoptotic Bcl-2 family members [55]. This may facilitate the apoptotic process, since early elimination of Mcl-1 is required for etoposide and UV-mediated apoptosis in HeLa cells [35]. The decrease of Mcl-1 levels by siRNA augmented the sensitivity of Jurkat and U937 cells to doxorubicin-induced apoptosis. In Jurkat cells treated with doxorubicin in presence of Z-VAD-fmk, levels of Mcl-1 are maintained but Bak activation still occurs, suggesting that Mcl-1 destruction is not necessary for Bak activation and could be, in these cells, a secondary event mediated by the activation of caspases. In this sense, we observed that altering Mcl-1 levels by overexpression or RNAi affects the sensitivity of Jurkat cells to doxorubicin to a lesser extent than that of U937. Yet, Mcl-1

caspase-dependent proteolysis and release of Bim could be part of the caspase amplification loop in Jurkat cells. The participation of Bid in this loop can be excluded since tBid is not generated in Jurkat cells overexpressing the serpin CrmA, a strong inhibitor of caspase-8 (Fig. S4), which are sensitive to doxorubicin [2].

These results, taken together, indicate that after the incorporation of a sufficient amount of doxorubicin into DNA, intracellular death signals activate BH3-only proteins causing conformational change and oligomerization of mitochondrial Bax and/or Bak and the release of a limited amount of cytochrome *c* and AIF. Our data indicate that, in Jurkat cells, first PUMA and lately Bim would contribute to Bak activation (Fig. 9A). In these cells, not expressing Bax, the activity of Bak is not enough *per se* to cause a massive loss of $\Delta\Psi_m$ and release of AIF from mitochondria in the absence of caspase activation. Under these conditions, the cytochrome *c*-triggered Apaf-1-mediated caspase activation is required for a rapid execution of apoptosis and so apoptosis is delayed in the presence of Z-VAD-fmk in Jurkat cells. In U937 cells (Fig. 9B), the combined action of Bax and Bak, probably activated by Bim, causes enough $\Delta\Psi_m$ loss and AIF release and consequently caspase inhibition does not delay cell death. In U937 cells, Noxa upregulation could mediate the downregulation of Mcl-1 allowing for the release of Bim (Fig. 9B). Therefore, the commitment to die after DNA damage produced by doxorubicin depends on the action of different subsets of BH3-only proteins to activate Bax and Bak and the need of caspase activation for the rapid completion of apoptosis depends on the ability and efficiency of Bax and/or Bak to cause a sufficient degree of mitochondrial disruption.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.02.010.

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