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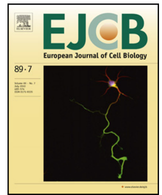

Doxorubicin induces cell death in breast cancer cells regardless of Survivin and XIAP expression levels

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ABSTRACT

Breast cancer is the leading cause of deaths in women around the world. Resistance to therapy is the main cause of treatment failure and still little is known about predictive biomarkers for response to systemic therapy. Increasing evidence show that Survivin and XIAP overexpression is closely associated with chemoresistance and poor prognosis in breast cancer. However, their impact on resistance to doxorubicin (dox), a chemotherapeutic agent widely used to treat breast cancer, is poorly understood. Here, we demonstrated that dox inhibited cell viability and induced DNA fragmentation and activation of caspases-3, -7 and -9 in the breast cancer-derived cell lines MCF7 and MDA-MB-231, regardless of different p53 status. Dox exposure resulted in reduction of Survivin and XIAP mRNA and protein levels. However, when we transfected cells with a Survivin-encoding plasmid, we did not observe a cell death-resistant phenotype. XIAP and Survivin silencing, either alone or in combination, had no effect on breast cancer cells sensitivity towards dox. Altogether, we demonstrated that breast cancer cells are sensitive to the chemotherapeutic agent dox irrespective of Survivin and XIAP expression levels. Also, our findings suggest that dox-mediated modulation of Survivin and XIAP might sensitize cells to taxanes when used in a sequential regimen.

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Introduction

Breast cancer is the leading cause of deaths in women around the world, despite recent advances regarding novel therapeutic options. Resistance to chemotherapy remains the major hurdle for the success of antineoplastic treatment. Several mechanisms have been implicated in chemoresistance in breast cancer such as TP53 mutations (Knappskog and Lonning, 2012), changes in the DNA repair machinery (Dhillon et al., 2011), overexpression of ATP-binding cassette drug transporters (Wind and Holen, 2011), modifications of drug targets such as topoisomerase II (Konecny et al., 2010) and disruption in apoptotic signaling pathways such as the inhibitor of apoptosis proteins (IAPs) overexpression (Foster et al., 2009). Survivin is the smallest member of the IAP family (Ambrosini et al., 1997), whose function also extends to cell division regulation and control of mitotic spindle assembly (Li et al., 1998). Survivin is poorly expressed in normal breast despite its overexpression in neoplastic breast tissues (Nassar et al., 2008), where it was found to be an independent prognostic factor of poor outcome (Hinnis et al., 2007; Ryan et al., 2006). Survivin has been shown to inhibit apoptosis through an IAP-IAP complex, which stabilizes XIAP expression, preventing its proteasome degradation and enabling its caspase-inhibitory activity (Dohi et al., 2004). XIAP is another IAP protein, known for its ability to bind and effectively inhibit caspases-3, -7 and -9 (Eckelman et al., 2006). Although XIAP expression is highly prevalent in breast carcinomas, its role in prognosis still remains controversial. While some studies have demonstrated that XIAP expression was closely related to a more aggressive clinical behavior (Jaffer et al., 2007) and shortened survival (Zhang et al., 2011), Hinnis and colleagues (2007) observed no correlation between its expression and clinical-biological parameters. In preclinical and clinical models, different approaches directed to inhibit IAPs sensitize breast cancer cells to apoptosis and reduce tumor growth (Aird et al., 2008; Flygare et al., 2012; Gonzalez-Lopez et al., 2011; Li et al., 2006), even in chemoresistant tumors (Dean et al., 2009; Yamanaka et al., 2011), supporting their potential use as therapeutic targets. Consistent with this, other groups have demonstrated that Survivin can prevent apoptosis mediated by a variety of stimuli such as tamoxifen (Moriai et al., 2009), paclitaxel (Wang et al., 2010), transtuzumab (Zhu et al.,

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2010) and radiotherapy (Papanikolaou et al., 2011). Nevertheless, little is known about Survivin and XIAP role in resistance to doxorubicin (dox), an anthracyclic chemotherapeutic agent widely used to treat breast cancer (Burnell et al., 2010).

In this study, we investigated the potential role of Survivin and XIAP as dox resistance factors in breast-derived cancer cell lines. Our data show that Survivin-induced overexpression does not prevent dox-mediated cell death in both invasive and non-invasive breast cancer cells. Similarly, knockdown of Survivin by siRNA, alone or in combination with XIAP silencing, cannot sensitize cells to cytotoxic stimuli. Taken together, these findings suggest that Survivin and XIAP expression have no influence on dox resistance in breast cancer cells.

Materials and methods

Materials

Dox (Rubidox®) was provided by Bergamo (São Paulo, Brazil). The drug was diluted in NaCl 0.9% and serial dilutions in culture media were performed prior to use. Tris (trishydroxyimethylaminomethane) was purchased from Merck and MTT (3-(4,5-dimethylthiazlol-2-yl)-2,5-diphenyltetrazolium bromide), from USB. PBS (phosphate buffered saline), RNAse (ribonuclease A), DMSO (dimethyl sulfoxide) and β-actin antibody (1:5000 dilution) were obtained from Sigma Aldrich (St. Louis, MO, USA). Antibodies for Survivin, XIAP, procaspase-7 and procaspase-9 were from R&D Systems (1:1000 dilution; Minneapolis, MN, USA). Procaspase-3 and p53 antibodies were from BD Biosciencies (1:1000 dilution; San Jose, CA, USA) and Dako (1:500 dilution; Glostrup, DK), respectively. Mouse and rabbit secondary antibodies were purchased from GE Healthcare (Buckinghamshire, UK).

Cell lines

The human breast cancer cell lines MCF7, a non-invasive, estrogen receptor (ER) positive, and MDA-MB-231 (invasive, basal-like ER-negative) were cultured in RPMI 1640 and Dulbecco's modified Eagle's medium (Gibco; BRL, UK), respectively, supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and maintained in a humidified atmosphere at 37 °C and 5% CO_2. Cells in exponential growth were exposed to clinically relevant dox doses (1 and 2 μM) and to a concentration used mainly for mechanistic purposes (5 μM) (Gewirtz, 1999). The cell lines were genotyped for confirmation of authenticity by the Sonda Laboratory at Federal University of Rio de Janeiro and monitored for mycoplasm contamination. The cell lines express similar levels of both Survivin and XIAP (Supplementary material A).

Measurement of cell metabolic viability

Cell viability was evaluated by the MTT assay. Breast cancer-derived cells (10^5 ml^{-1}) were left to adhere onto 96-well plates for 24 h and treated with dox for 24, 48 and 72 h. After incubation with MTT (5 mg/ml in PBS) for 4 h at 37 °C, the supernatant was removed and 150 μl of DMSO were added to dissolve the formazan salt. Absorbance at 492 nm was measured with an ELISA reader (DTX 800 Multimode Detector from Beckman Coulter, Fullerton, CA, USA) and the percentage of cell viability was assessed as: (absorbance of dox treated cells/absorbance of untreated cells) × 100. The concentrations and experiments were conducted in triplicate.

Assessment of DNA fragmentation

After dox treatment, cells were harvested by trypsinization (trypsin 0.125%), washed twice in PBS and incubated with 300 μl of propidium iodide (PI) staining solution (PI 50 μg/ml diluted in citrate buffer 4 mM and Triton X-100 0.3%) and 300 μl RNAse (100 μg/ml diluted in citrate buffer 40 mM) for 15 min at room temperature. DNA content was determined on a flow cytometer (Cyan ADP; Dako) and a total of 10,000 events were acquired per sample. Analysis was performed using the Summit v4.3 software and DNA fragmentation was quantified by the percentage of cells in sub-G0/G1 phase.

Detection of apoptosis

For detection of caspase-mediated apoptosis, Western blotting was performed as previously described (Nestal de Moraes et al., 2011). Caspases activation was analyzed by reduction of procaspase levels or detection of cleaved caspase bands.

Western blot analysis

For Survivin, XIAP and p53 detection, pellets of 2×10^6 trypsinized cells were washed three times in PBS and lysed in resuspension buffer (40 mM Tris–HCl; 40 mM EDTA; pH 7.5) and 10% SDS (dodecyl sodium sulfate) for 15 min at 100 °C. Total protein was determined using Bio-Rad protein assay solution and 80 μg of lysates were subjected to SDS-PAGE onto 15% polyacrylamide gels. After electrophoresis and transfer to Hybond-P membranes, blots were stained with Ponceau red to assure equal protein loading and blocked for 2 h at room temperature with 5% nonfat dry milk TBS containing 0.2% Tween-20. Subsequently, membranes were incubated with primary antibodies overnight at 4 °C, prior to incubation with secondary horseradish peroxidase-labeled anti-rabbit, anti-mouse (1:1000 dilution; GE Healthcare, Buckinghamshire, UK) or anti-goat antibodies (1:2000 dilution; Novus Biologicals, Littleton, CO, USA). Antibody complexes were visualized by the ECL detection system (GE Healthcare) and band intensities were quantified using the VisionWorks software. Protein expression was normalized with respect to β-actin.

Detection of p53 expression by flow cytometer

After exposure to dox for 24 h, MCF7 cells were incubated for 10 min at room temperature with 2 ml of a mixture of paraformaldehyde (final concentration: 2%; diluted in PBS) and Becton Dickinson's FACS lysing solution (1:10 dilution in distilled water) for fixation and permeabilization. Afterwards, cells were centrifuged and supernatant was discarded and then washed with 2 ml of PBS/0.5% Tween 20. The fixed cells were incubated with 10 μl of anti-p53 directly labeled with FITC (Dako; 1:500) in a single step for 30 min, followed by two washes with PBS/0.5% Tween 20 and resuspended in 500 μl of 1% formaldehyde/PBS, prior to flow cytometer analysis. For each test, isotype-matched monoclonal antibody was used as a negative control (IgG 1-FITC; Dako; 1:500). The p53 antibody reacts with both wild-type and mutant p53 proteins.

Real time PCR quantification (TaqMan) of Survivin and XIAP mRNA

For Survivin and XIAP mRNA analysis, total RNA was isolated from dox-treated and untreated cells using the Trizol reagent (Trizol®, Invitrogen, Carlsbad, CA, USA) and quantified (Nanodrop® ND-1000). Subsequently, cDNA was synthesized through the Superscript method (Superscript® II Reverse Transcriptase; Invitrogen). Survivin (Hs000153353_m1) and XIAP (Hs01597783_m1) mRNA was amplified by using TaqMan probes obtained from Applied Biosystems (Foster City, CA, USA). Real-time monitoring

of PCR amplification of cDNAs was carried out using TaqMan Universal master mix (Applied Biosystems). Relative quantification of target gene expression was performed by employing a comparative Ct method (ΔΔCt), using β-actin (Hs99999903_m1) as a reference gene. All experiments were carried out in the StepOne™ Real-Time PCR System (Applied Biosystems).

Transfection of Survivin gene into breast cancer cells

The plasmid-encoding human survivin pcDNA3-myc-survivin and the empty vector pcDNA3-myc were kindly provided by Dr. John C. Reed (The Burnham Institute, La Jolla, CA) (Tamm et al., 1998). Transfections were performed using the Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions. After 24 h of transfection, cells were trypsinized and left to adhere, prior to dox exposure for 72 h.

Transfection of siRNA targeting Survivin and XIAP into MCF7 cells

Small interfering RNAs (siRNAs) targeting Survivin (4392420; ID:s1455) and XIAP (4392420; ID:s1459) and the non-silencing control – scramble – (4390843) were purchased from Life Technologies (Foster City, CA, USA). Transfections were performed using the Lipofectamine RNAimax reagent, according to the protocol provided by the manufacturer (Invitrogen). After 24 h of simultaneous or individual transfection with the siRNA sequences, cells were exposed to dox for 24 h and viability was assessed.

Statistical analysis

Statistical validation of data was performed using the Graph Pad Prism 4.0 software. The Student's *t*-test was used to compare differences between dox-treated and untreated cells. A value of $p < 0.05$ was considered statistically significant.

Results

Dox induces apoptosis in breast cancer cells

First, we evaluated the effects of dox on non-invasive MCF7 and invasive MDA-MB-231 cells. We could observe that cell viability was inhibited in a time-dependent manner when cells were exposed to increasing concentrations of dox (Fig. 1A). To investigate cell death mechanisms, we assessed DNA fragmentation and procaspases levels after cells exposure to dox. Our results showed that dox induced DNA fragmentation in both cell lines, as observed by the hypodiploidy cells in sub-G0/G1 phase (Fig. 1B). The increase in dox concentrations had an impact on MDA-MB-231 toxicity, but not on MCF7 cells, where DNA fragmentation occurred mostly time-dependent and was more expressive after a 72-h incubation. In addition, we demonstrated that procaspases-9, -7 and -3 levels decreased after a 72-h dox treatment (Fig. 1C). Altogether, these data suggest that dox triggers apoptosis in breast cancer cells.

Dox-induced apoptosis occurs in cells lines with different p53 status

As an anthracycline, dox acts by intercalating DNA and inhibiting topoisomerase II activity (Gewirtz, 1999; Lothstein et al., 2001), which leads to DNA damage and p53 activation (Gewirtz et al., 2000). To investigate if dox-induced apoptosis involves p53 induction in our model, we examined p53 levels in mutant p53 MDA-MB-231 cells, which display high levels of undegraded nonfunctional p53 protein, and in wild-type p53 MCF7 (Fig. 2A). As shown in Fig. 2B, approximately 27% and 44% of MCF7 cells had an increase in p53 levels after incubation with 1 μM and 5 μM dox, respectively. To further confirm this result, we performed Western blot experiments and observed that dox was able to induce p53 expression in all concentrations tested in MCF7 cells, but not in MDA-MB-231 whose expression levels remained unaltered (Fig. 2C). These results indicate that dox is able of inducing apoptosis in breast cancer cells displaying different p53 status.

Survivin and XIAP expression is down-regulated at both mRNA and protein levels after dox treatment

It is well known that IAPs such as Survivin can be regulated at both transcriptional and post-transcriptional levels (Zhang et al., 2006). Next, we investigated if dox could modulate the expression of antiapoptotic proteins Survivin and XIAP. Survivin and XIAP mRNA levels were down-regulated on dox-treated when compared to untreated cells (Fig. 3A and B). These results were mirrored in protein expression analysis, where we observed the decrease in XIAP expression as early as 24 h of dox treatment whereas Survivin had its expression diminished when cells were exposed to 2 and 5 μM dox (Fig. 3C and D) Interestingly, there is no significant difference in Survivin levels in MDA-MB-231 cells other than the one observed after a 72-h exposure to 5 μM dox (Fig. 3C). Actually, Survivin expression was increased after MDA-MB-231 cells were exposed to 1 μM dox, whose cytotoxic effects are less pronounced (Fig. 1). These results indicate that Survivin and XIAP are down-regulated at the transcriptional level by dox and that their modulation profile correlates to dox-induced apoptosis.

Survivin overexpression does not confer resistance to dox-mediated cell death

As dox treatment decreased Survivin expression, we sought to investigate if its decreased expression was facilitating cell death induction by dox. Transiently transfected cells with either a Survivin-encoding plasmid and an empty vector were compared in terms of dox cytotoxic effects. Upon an efficient transfection (87% and 95% efficiency for MDA-MB-231 and MCF7, respectively – data not shown), we did not observe changes in the cell cycle profile of both cell lines (Fig. 4A). Although Survivin overexpression attenuated processing of caspase 9 (Fig. 4B), it did not block cell death induced by dox, as evaluated by DNA fragmentation (Fig. 4C) and changes in cell viability (Fig. 4D). Moreover, Survivin overexpression did not stabilize XIAP expression, whose levels were still reduced upon dox treatment (Fig. 4B). It is important to emphasize that a 5-day kinetics was performed after cell transfection with the vectors (supplementary material B). Even though Survivin expression reached its peak about 2 days after transfection, its overexpression was sustained until the day in which cells were harvested for cytotoxicity assays. Altogether, these findings show that the increase in Survivin expression does not counteract dox-induced cell death in breast cancer cells.

Survivin silencing, alone or in combination with XIAP silencing, does not sensitize breast cancer cells to dox apoptotic stimuli

Next, we tested the hypothesis that a further decrease in Survivin expression would enhance cell death induced by dox in breast cancer cell lines. As a model, we chose MCF7 cells that were shown to be more sensitive to dox effects. By using Survivin and XIAP siRNAs, we found that silencing either isolated or simultaneous Survivin or XIAP expression clearly stimulated processing of procaspases-7 and -9 (Fig. 5A). However, it did not have an impact on dox-induced cell death (Fig. 5B). Nonetheless, when XIAP was silenced, the decrease in Survivin expression is enhanced

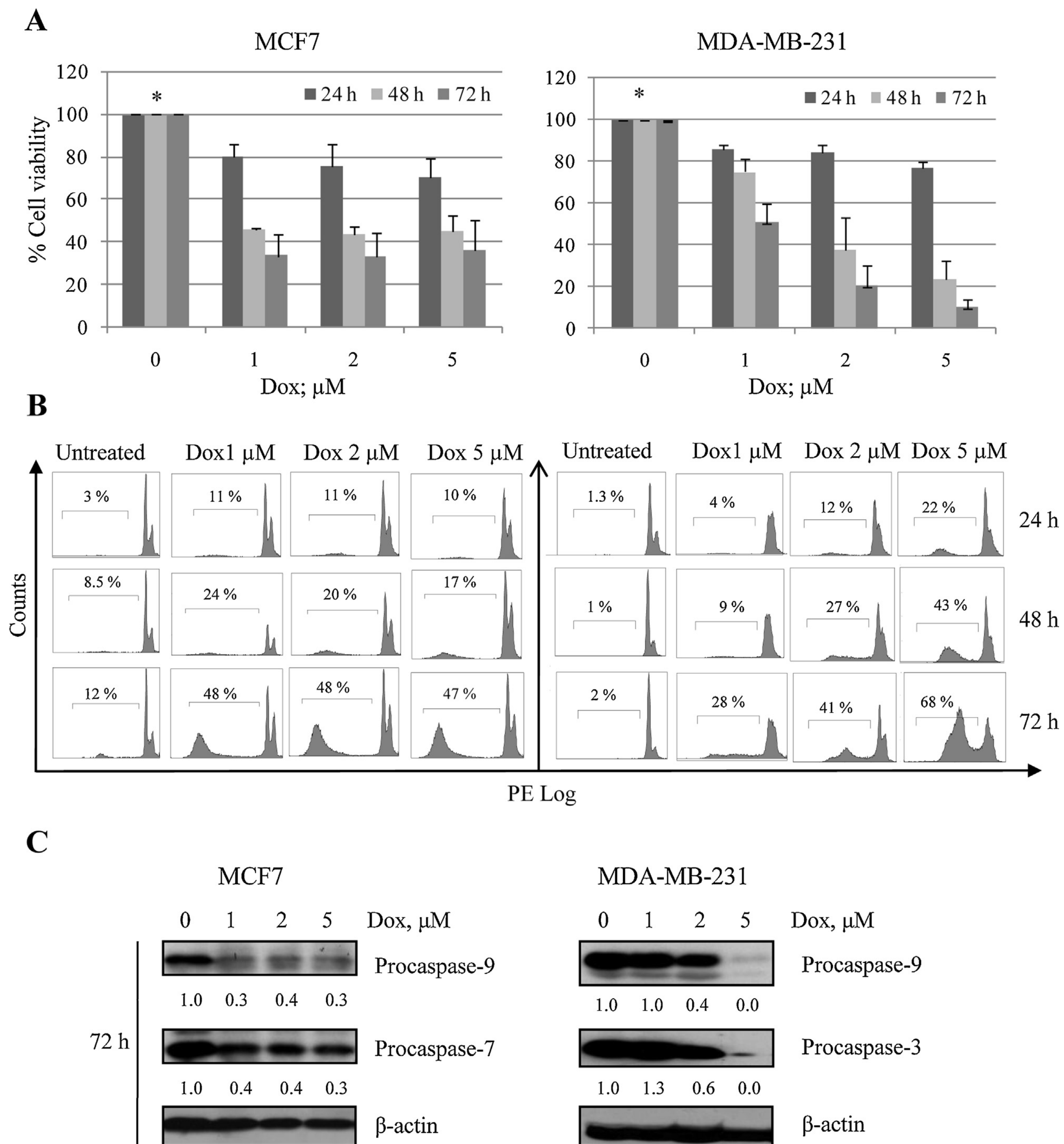

Fig. 1. Doxorubicin (dox) induces apoptosis in breast cancer cells. MCF7 and MDA-MB-231 cell lines were left to adhere for 24 h and were exposed to dox for the indicated times. (A) MTT assay. Data are expressed as the mean ± standard deviation from three independent experiments; *$p < 0.05$; Student's *t*-test. (B) Flow cytometry analysis of dox effect on DNA fragmentation, evaluated by the appearance of a sub-G0/G1 peak. (C) Cells were lysed for Western blotting. The values below the blots indicate the densitometric ratio between the protein and β-actin. The images are representative of three independent experiments.

in the presence of dox (compare data on Survivin expression in Figs. 3C and 5A), suggesting that lack of XIAP expression may render Survivin more unstable. We should highlight that Survivin and XIAP expression was analyzed exactly at the same day that cytotoxicity assays were performed. Altogether, our findings suggest that dox-induced cell death is not enhanced by a further decrease in Survivin and/or XIAP expression levels in breast cancer cells. However, Survivin expression levels appear to be stabilized by XIAP, corroborating data in the literature regarding their functional interaction (Dohi et al., 2004).

Long-term Survivin inhibition results in cytotoxicity

Survivin function is not only limited to apoptosis inhibition but also to control of mitotic spindle assembly and cell cycle progression (Li et al., 1998). Although Survivin silencing did not alter sensitivity to dox, we observed that, after 72 h of inhibition of Survivin expression, there was an increase in DNA fragmentation when compared to cells transfected with the scramble siRNA (Fig. 6). We also observed this effect in cells transfected with both Survivin and XIAP siRNAs, but not in XIAP-silenced cells, in which

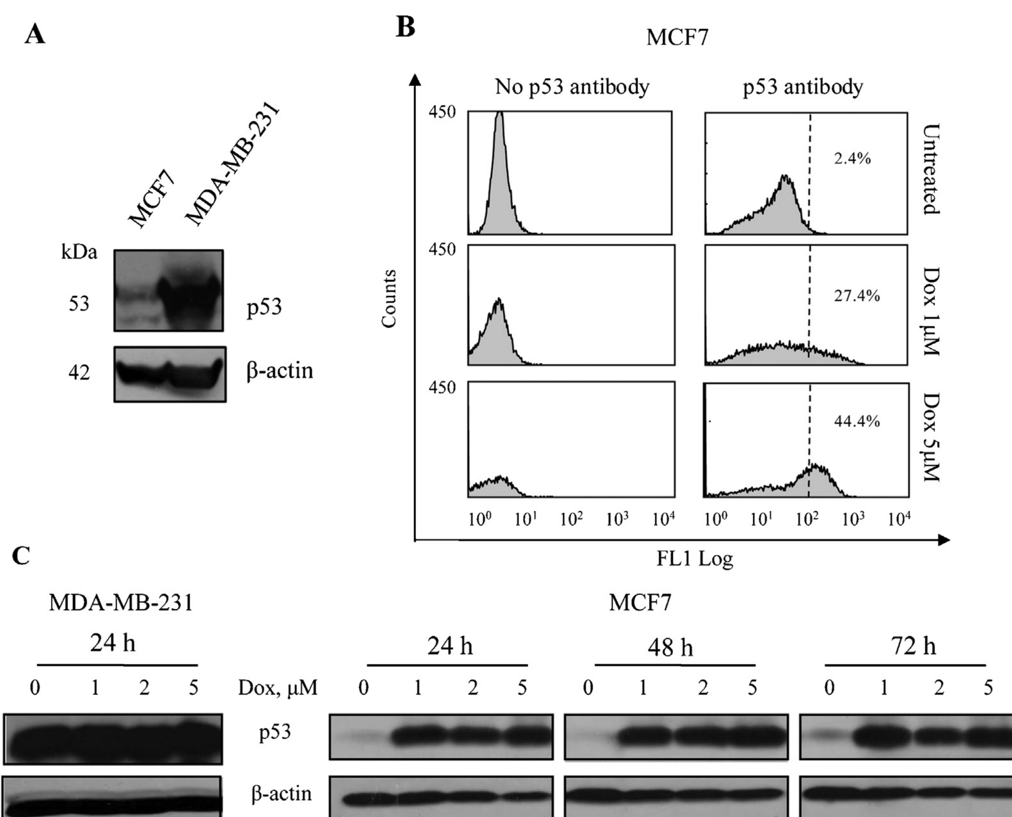

Fig. 2. Dox-induced apoptosis occurs in cell lines with different p53 status. Wild-type MCF7 and mutant MDA-MB-231 cells (A) were left to adhere for 24 h and were exposed to increasing concentrations of dox. (B) Cells were permeabilized for the flow cytometry experiment after 24 h of dox treatment. (C) Cells were lysed for Western blotting at the indicated time points. The figures are representative of three independent experiments.

the cytotoxicity was less pronounced. This result shows that long-term Survivin inhibition leads to cytotoxicity and confirms Survivin cytoprotective role.

Discussion

Despite many advances in the treatment of breast cancer, with the development of novel therapeutic modalities and identification of prognostic and predictive factors, resistance to chemotherapy remains the major obstacle for successful treatment. To assign numbers, resistance usually accounts for 90% of patients receiving chemotherapy in metastatic disease (Coley, 2008). Therefore, the search for biomarkers known to confer resistance to chemotherapy has the potential to avoid overtreatment and unnecessary exposure to toxic and ineffective therapy. Although the microarray gene expression profiling technology has arisen as a strategy to classify patients according to their molecular signature and possibly identify those who could benefit from specific therapies, still there are limitations that derail its incorporation into clinical practice and treatment decisions (Lavasani and Moinfar, 2012). Thus, the better understanding of mechanisms responsible for causing tumor resistance can provide tools to developing new strategies for overcoming chemoresistance.

Dox is considered to be one of the most effective agents in the treatment of breast cancer and, despite first used decades ago (Fisher et al., 1990), it is still included in the standard chemotherapy protocol for this disease. In vitro, dox has been shown to counteract viability of breast cancer cells by triggering apoptosis (Di et al., 2009). Accordingly, in the present study, we analyzed apoptotic features and demonstrated that dox can induce DNA fragmentation and cleavage of procaspases-3, -7 and -9 in both invasive MDA-MB-231 and non-invasive MCF7 cells. In addition, we also observed reduced viability upon dox treatment, as measured by the MTT metabolic assay. The time-dependent dox cytotoxic effects were not dependent upon of p53 status, as it occurred in wild-type MCF7 and mutant MDA-MB-231 p53 bearing cells. Also, our data shows that dox induced p53 expression in wild-type MCF7 cells, confirming previous data reporting dox-induced p53 phosphorylation (Yeh et al., 2004) and differential p53 modulation between dox and paclitaxel, a taxane also used in breast cancer treatment (Srivastava et al., 1998). On the other hand, the high levels of inactive mutant p53 remained unaltered in MDA-MB-231 cells, whose signaling pathways associated to chemosensitivity have been shown to be mediated by p73 up-regulation (Wong et al., 2011). Consistent with this, Vayssade and colleagues (2005) demonstrated that the p73 transcription factor may assume some of the p53 functions in dox-treated p53-deficient cells. Our finding that p53 status does not influence the response of these cells to dox might provide clinically relevant information, as approximately 30% of all breast tumors display *TP53* mutations (Borresen-Dale, 2003).

Our next step was to investigate whether Survivin would confer dox resistance and protect breast cancer cells from apoptosis. First, we observed that dox could reduce Survivin and XIAP levels, concomitantly with apoptosis induction, pointing to a possible correlation between IAPs and dox resistance. Dox-induced Survivin downregulation is an effect which has been previously observed in lung cells (Mirza et al., 2002), but was never reported in breast cancer cells. In the aforementioned study, overexpression of Survivin rescued A549 cells from dox-induced cell death, reducing the levels of apoptosis in a dose-dependent manner. When we transfected cells with a Survivin-encoding plasmid, we found that caspase-9 cleavage was attenuated, suggesting that, at least in part,

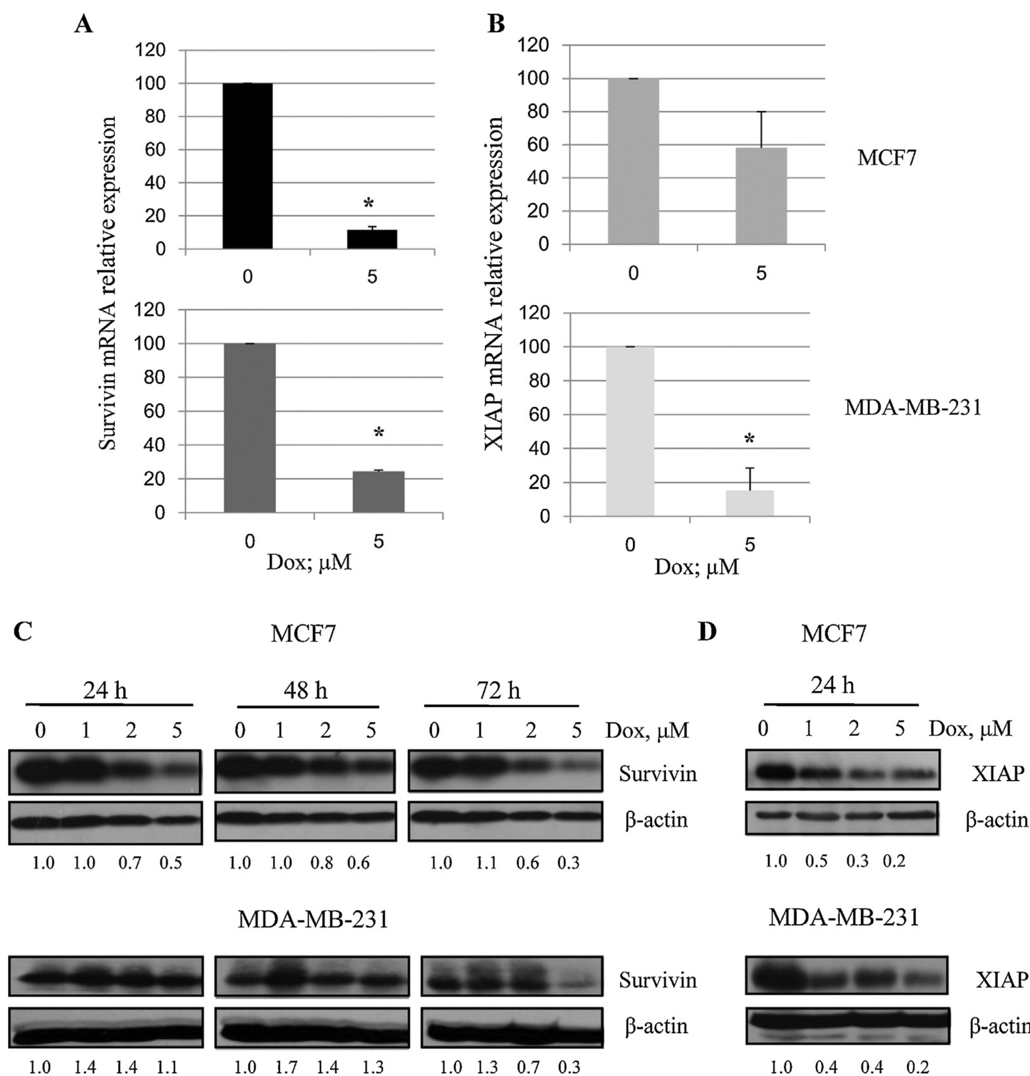

Fig. 3. Survivin and XIAP expression is down-regulated at both mRNA and protein levels upon dox treatment. MCF7 and MDA-MB-231 cell lines were left to adhere for 24 h and were exposed to dox for the indicated times. (A,B) After a 24-h incubation with the drug, RNA was extracted from dox-treated and untreated cells and c-DNA was synthesized by the Superscript Method. β-actin was used as an internal control to normalize Survivin and XIAP mRNA levels. $p < 0.05$; Student's *t*-test. (C,D) Cells were lysed for Western blot. The values below the blots indicate the densitometric ratio between the protein and β-actin. The figures are representative of three independent experiments.

Survivin overexpression can counteract dox-mediated apoptosis. However, we observed no changes in dox sensitivity between Survivin-overexpressing cells and cells transfected with the vector control regarding changes in cell viability and DNA fragmentation. This finding is in contrast with the current knowledge regarding Survivin role as a powerful resistant factor in breast cancer. By conducting similar experiments, Moriai and colleagues (2009) found a decrease in the number of apoptotic cells induced by tamoxifen after overexpression of Survivin. A recent study demonstrated that Survivin acts downstream the human epidermal growth factor receptor-2 (Her2) and 3 (Her3)/PI3K/Akt pathway and is required for mediating paclitaxel resistance in Her2-overexpressing breast cancer cells (Wang et al., 2010). Another group has shown that Survivin-overexpressing cells had little changes in viability upon transtuzumab treatment, indicating that Survivin rescues transtuzumab-induced cell growth inhibition (Zhu et al., 2010). Finally, Survivin knockdown by siRNA has been shown to enhance sensitivity to irradiation effects (Papanikolaou et al., 2011). Altogether, these results show that Survivin role in resistance is most likely therapy-specific. However, it is noteworthy that our results might offer an explanation for the relative efficiency of therapeutic regimens combining dox and taxanes for breast cancer patients (e.g. AC-P: sequence of dox and cyclophosphamide – 4x – and paclitaxel – 4x) (Henderson et al., 2003). As the anthracycline is given first in the treatment sequence, it is possible that the degradation of Survivin induced by dox might act as a sensitizing mechanism to the posterior action of paclitaxel, which resistance by tumors is clearly mediated by Survivin (Wang et al., 2010). Supporting this hypothesis, a recent study evaluated pathological complete response to GAT (dox on day 1, paclitaxel and gemcitabine on day 2, every 14 days for six cycles) and its correlation with tumors biomarkers and found that tumors exhibited decreased numbers of Survivin-positive cells after treatment, suggesting that Survivin degradation might be involved in the response to GAT (Sanchez-Rovira et al., 2012).

We have also questioned if a further decrease in Survivin and XIAP expression would enhance cell death induced by dox in breast cancer cells, as it was previously described that Survivin and XIAP may act in complex to counteract apoptosis (Dohi et al., 2004). For this purpose, we inhibited their expression and found that Survivin- and XIAP-silenced cells have the processing of procaspases-7 and -9 stimulated, but did not display a more sensitive phenotype towards

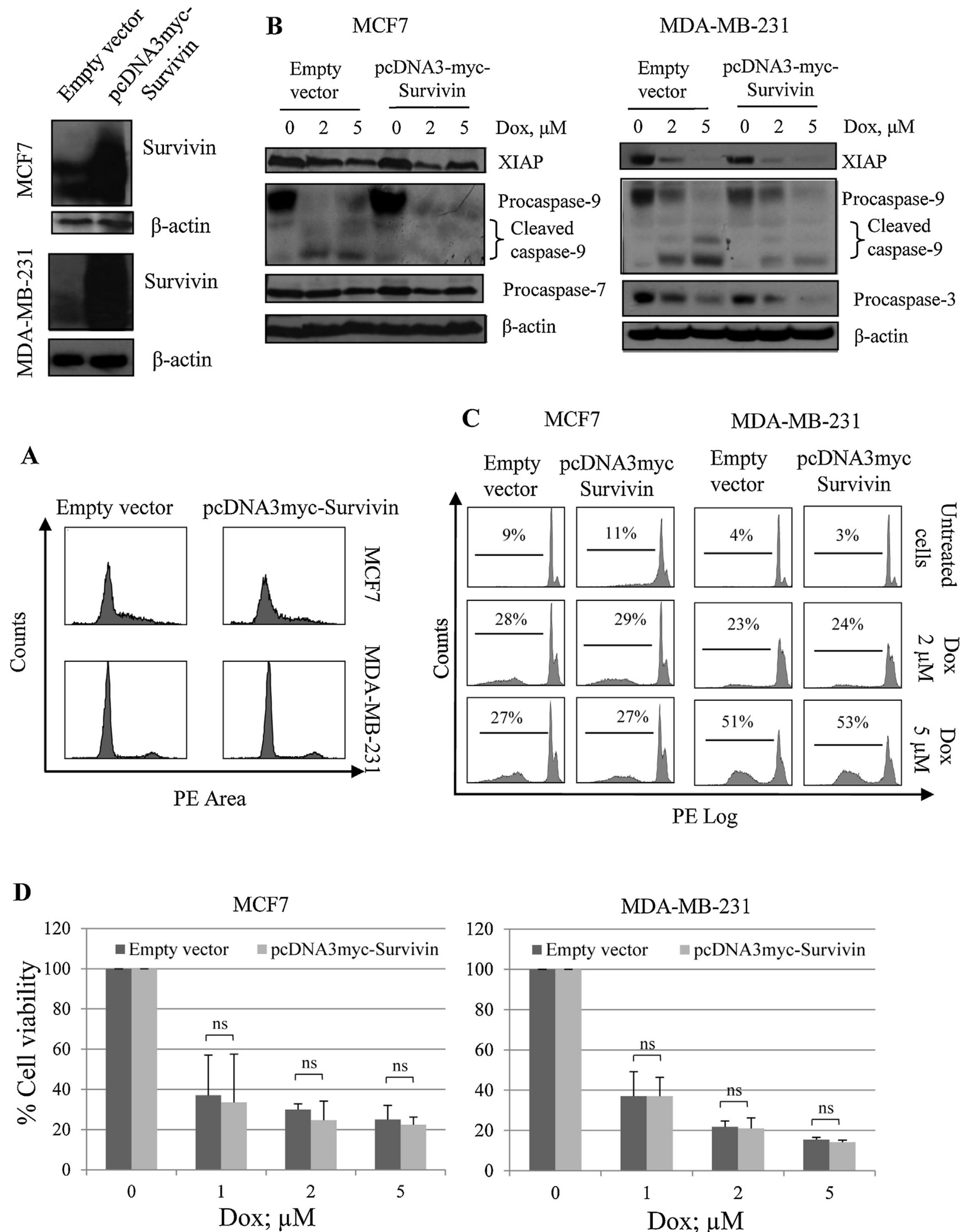

Fig. 4. Survivin overexpression does not confer resistance to dox-mediated cell death. MCF7 and MDA-MB-231 cells were left to adhere for 24 h prior to transfection with the pcDNA3myc (empty vector) or pcDNA3myc-Survivin vector using the Lipofectamine 2000 reagent. Following 24 h of transfection, cells had cell cycle profile (A) assessed by flow cytometry. Cells were also exposed to dox for 72 h and harvested for analysis of caspase levels (B), DNA fragmentation (C) and cell viability (D). Data are expressed as the mean ± standard deviation from three independent experiments; ns: non-significant; Student's *t*-test. The figures are representative of three independent experiments.

dox, as DNA fragmentation remained unchanged in this experimental paradigm, indicating that depleting IAPs expression cannot render breast cancer cells less resistant. A recent study showed that XIAP overexpression alone could not confer resistance to Hela cells against several drugs, such as etoposide, staurosporine, vincristine and also dox (Seeger et al., 2010). A resistant-phenotype was only observed when XIAP overexpression was combined with ablation of its negative regulator and proapoptotic protein Smac.

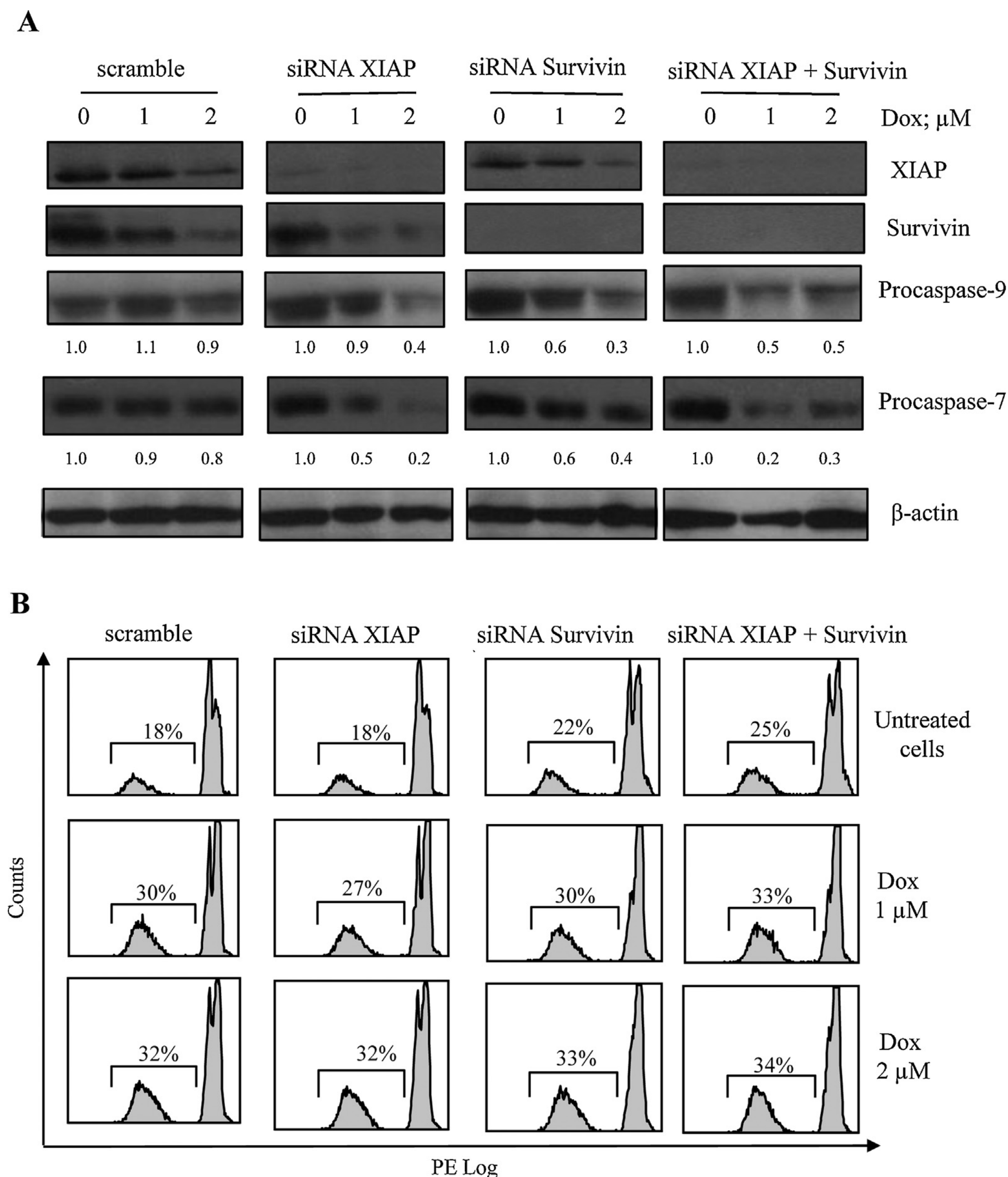

Fig. 5. Survivin and XIAP silencing, alone or in combination, does not sensitize MCF7 cells to dox. MCF7 cells were transfected with the Survivin and/or XIAP-targeted siRNA or with the scramble-siRNA sequences using the Lipofectamine RNAimax reagent. On the following day, dox was added to the culture medium. After 24 h of dox exposure, cells were harvested for Western blotting (A) and flow cytometry DNA content analysis (B). The figures represent three independent experiments.

Like Survivin, XIAP silencing alone has been shown to reduce resistance to transtuzumab in breast cancer cells (Foster et al., 2009). However, the data presented here support the idea that Survivin and XIAP expression does not modify the response to dox-based therapy in breast cancer cells. Together with the Survivin overexpression data, these results strongly support the idea that manipulation of Survivin and XIAP levels can affect the threshold for dox-induction of apoptosis but cannot influence significantly DNA fragmentation, the final event of cell death. It suggests that, by partly preventing apoptosis, IAPs overexpression can favor the induction of other types of cell death by dox. Reinforcing our hypothesis, dox has been shown to counteract viability of breast cancer cells in vitro by triggering different types of cell death: apoptosis, senescence and autophagy (Di et al., 2009).

In addition to these findings, we could observe that Survivin silencing had an impact on spontaneous cell viability after 72 h of transfection rather than on protection from dox-induced cytotoxic effects. This result is in accordance with several studies which demonstrated that Survivin plays a crucial role in cell cycle regulation and cytoprotection. Therefore, Survivin depletion results in aberrant progression into mitosis and pleiotropic division defects (Li et al., 1998). We also observed that XIAP silencing leads to a destabilization of Survivin expression, suggesting an interplay between these two molecules that regulates Survivin stability. These findings are relevant in the context of breast cancer because Survivin and XIAP expression is often observed in breast cancer samples (Ryan et al., 2006; Zhang et al., 2011). In the future, it might be interesting to establish the ratio of XIAP and Survivin and test its prognostic power in a cohort of breast cancer patients, instead of interrogating both proteins in an independent manner.

According to our data, Survivin and XIAP are down-regulated upon apoptosis induction by dox, but do not influence dox resistance in breast cancer cells. These conclusions prompted us to speculate about the mechanisms underlying their negative regulation. Indeed, Survivin and XIAP are downstream factors for multiple

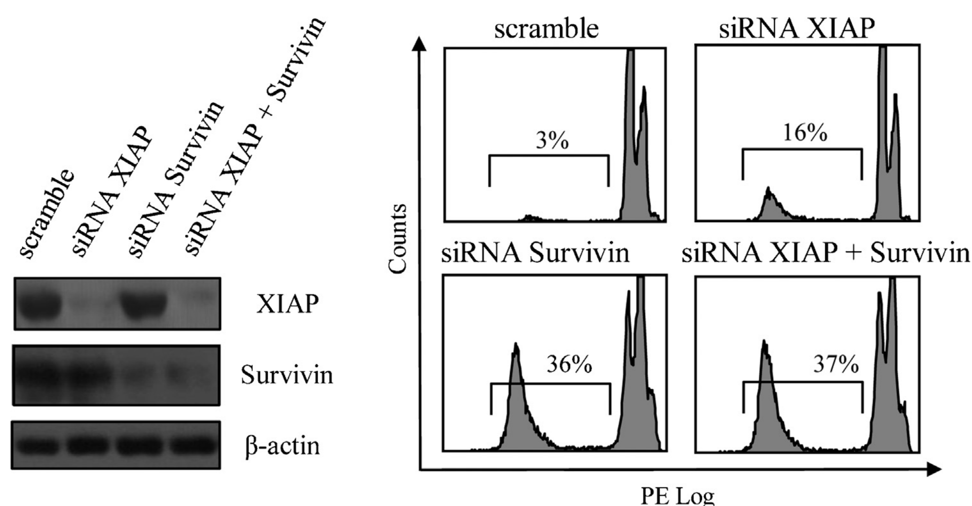

Fig. 6. Long-term Survivin inhibition results in cytotoxicity. MCF7 cells were transfected with the Survivin and/or XIAP-targeted siRNA sequences using the Lipofectamine RNAimax reagent. After 72 h of transfection, DNA content was evaluated using flow cytometry. The histograms are representative of three independent experiments.

oncogenic signaling pathways (for review: Galban and Duckett (2010); Guha and Altieri, 2009) and most likely, their regulation occurs at the level of transcription as a consequence of deregulation of upstream regulators and transcription factors. In this study, we also showed that both Survivin and XIAP are down-regulated at mRNA levels, which supports our hypothesis that they might be transcriptionally repressed following dox exposure. Thus, it would be interesting to explore what transcription factors, whether activated or inhibited, can account for IAPs negative regulation in response to cytotoxic chemotherapy-induced stress. Interestingly, dox was shown to impair the transcriptional response of the hypoxia-inducible factor (HIF) by inhibiting the binding of the HIF-heterodimer to the consensus enhancer element (RCGTG), and this effect was accompanied by a co-ordinated down-regulation of HIF targets (Tanaka et al., 2012). Survivin was already shown to be regulated by HIF1 alpha on some models (Chen et al., 2009; Peng et al., 2006), rendering HIF-1 alpha impaired function a putative culprit for the decreased expression of Survivin upon dox treatment.

Another point to highlight is that, despite having based our experiments in totally different breast-derived cell lines, the results we obtained were mostly similar. Unlike MCF7 which is a non-invasive, luminal-like cell type, MDA-MB-231 has a highly invasive, basal-like phenotype and is usually considered a resistant cell line model. Moreover, there are data in the literature reporting distinct molecular lesions harbored by these cell lines. MCF7 is wild-type for KRAS, EGFR (epithelial growth factor receptor) and BRAF, but it displays a missense PI3K (phosphatidylinositol 3-kinase) mutation in exon 9 (E545K), rendering this kinase constitutively active. Of note, MCF7 cells do not express caspase-3. As for MCF7, MDA-MB-231 is wild-type for EGFR, but it harbors activating mutations in KRAS (G13D) and BRAF (G464V) and is wild-type for PI3K (Sanchez et al., 2011; Simi et al., 2008). In spite of the differences in these clinically relevant molecular lesions and different expression pattern of key molecules such as ER and Her2, we observed that both cell lines were similarly sensitive to dox-mediated apoptosis. As breast cancer is a heterogeneous disease (Curtis et al., 2012), results obtained from in vitro experiments based on a single cell line do not resemble breast cancer biology.

In summary, we demonstrated that breast cancer cells are sensitive to the chemotherapeutic agent dox irrespective of Survivin and XIAP expression levels and that molecular manipulation of their levels does not modify the response to dox. Nonetheless, our findings suggest that dox might act as a sensitizer agent to taxanes when used in a sequential regimen and that the ratio of Survivin and XIAP should be explored in the future as a prognostic/predictive factor to taxane use. Currently, we are conducting a study in a cohort of breast cancer patients in order to explore these findings in vivo. Our experimental data can contribute to a better understanding of molecular mechanisms underlying chemoresistance in breast cancer.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejcb.2013.08.001.

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