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ORIGINAL PAPER

Doxorubicin induces atypical NF-κB activation through c-Abl kinase activity in breast cancer cells

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Abstract
Purpose NF-κB transcription factor has been associated with cancer development and chemoresistance. We studied the signaling pathway activated by doxorubicin (DOX) leading to NF-κB activation in breast cancer cells.
Methods NF-κB activity was evaluated by electrophoretic mobility shift in T47D, ZR75.30 and primary culture (MBCDF) from a ductal infiltrating carcinoma. Cell viability was measured by crystal violet. Western blotting was performed to check the expression and phosphorylation of IκBα Ser-32/36. c-Abl was inhibited with Imatinib or by overexpressing a dominant negative form of c-Abl (K290R).
Results We found a correlation between sensitivity to DOX and amplitude of NF-κB activation. In cells least sensitive to DOX, NF-κB remained activated for longer time (T47D and MBCDF). The opposite effect was observed in cells sensitive to DOX (ZR75.30). DOX did not induce IκBα degradation or Ser-32/36 phosphorylation. Instead, there were modifications in the levels of IκBα tyrosine phosphorylation, suggesting an atypical NF-κB activation. In DOX-resistant cells, Imatinib treatment reduced IκBα tyrosine phosphorylation and NF-κB activity. The Imatinib–DOX combination significantly enhanced cell death of T47D and MBCDF breast cancer cells. Overexpression of c-Abl K290R in T47D and MBCDF cells reduced basal and DOX-induced NF-κB activation as well as IκBα tyrosine phosphorylation. In c-Abl K290R cells, DOX treatment did not mimic the combination Imatinib–DOX-induced cell death.
Conclusions Inhibition of c-Abl inactivated IκBα/NF-κB pathway is associated with IκBα tyrosine phosphorylation in breast cancer cells. These results also raise the potential use of a combined therapy with Imatinib and DOX for breast cancer patients.

Keywords NF-κB · Breast cancer · IκBα · Imatinib · Tyrosine kinase inhibitor · EMSA

Abbreviations

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B cell inhibitor, alpha
DOX	Doxorubicin
EMSA	Electrophoretic mobility shift assay

Introduction

The transcription factor NF-κB is activated by a wide variety of stimuli including inflammatory cytokines, growth factors and immune-related stress such as bacterial

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infections and their products (lipopolysaccharides) among others (Garg and Aggarwal 2002; Karin and Lin 2002). All these stimuli participate in the regulation of several cellular processes such as proliferation, migration, survival and cell death (Pahl 1999). Aberrant NF-κB activation has been linked to different pathological conditions such as atherosclerosis, diabetes, AIDS and cancer (Baldwin 2001; Karin 2006). Inactive NF-κB homo/heterodimers are usually sequestered in the cytoplasm bound to their inhibitory proteins (IκBs) that prevent their translocation into the nucleus. Inflammatory cytokines activate NF-κB through the canonical pathway that requires IκBα Ser-32/36 phosphorylation and degradation (Karin et al. 2002; Hayden and Ghosh 2004; Moynagh 2005). Non-classical stimuli, such as hypoxia/reoxygenation, hydrogen peroxide stimulation and growth factors (NGF, EGF and PDGF), show an atypical NF-κB activation that in some cases is independent of IKKs and IκBα degradation (Imbert et al. 1996; Mukhopadhyay et al. 2000; Schoonbroodt et al. 2000). Instead, IκBα is phosphorylated at tyrosine residues; phosphorylation at Tyr 42 has been attributed to different kinases such as Syk and c-Src in response to atypical stimuli (Imbert et al. 1996; Singh et al. 1996; Kawai et al. 2002; Perkins 2006; Yang et al. 2012). Tyr 305 is phosphorylated by c-Abl and is clearly implicated in c-Abl-dependent cell death increasing IκBα stability (Kawai et al. 2002).

NF-κB activity has been implicated in several aspects of oncogenesis and chemoresistance (Basseres and Baldwin 2006; Kim et al. 2006; Cusack et al. 2000; DiDonato et al. 2012; Zhang et al. 2012). These include its ability to induce transcription of genes associated with proliferation and survival, such as cyclin D1, c-IAP-2 and Bcl-xL (Baldwin 2001; Karin and Greten 2005; Karin 2006). NF-κB has also been found constitutively activated in a variety of cancers. There are only few studies trying to define whether this activation follows the canonical or atypical pathways (Bargou et al. 1997; Cogswell et al. 2000; Nakshatri et al. 1997; Wang et al. 1999; Biswas et al. 2004; Wu and Kral 2005). NF-κB activation by chemotherapeutic agents has been associated with chemoresistance. For these reasons, in recent years, there has been a growing pharmacological interest aimed to inhibit NF-κB activity.

Breast cancer represents the most common malignancy in women worldwide. Despite recent improvements in screening programs and therapies, it remains the second cause of cancer death for women in North America (Jemal et al. 2008; DeSantis et al. 2008). Anthracyclines such as DOX and Epirubicin are common agents for cancer treatment. However, they produce several adverse effects, and cancer cells develop drug resistance with time. In order to gain a better understanding on the signaling of NF-κB triggered by DOX and its relation to chemotherapy resistance, we studied molecular markers of NF-κB activation in breast cancer cells. We found two different kinetics of NF-κB activation that correlate with the sensitivity to DOX. IκBα was already phosphorylated at tyrosine residues without stimulation in three breast cancer cells analyzed. This tyrosine phosphorylation was modified negatively by DOX treatment in cells highly sensitive. Furthermore, we demonstrated that treatment with the c-Abl kinase inhibitor, Imatinib, enhances the cell death induced by DOX alone with a parallel decrease in IκBα tyrosine phosphorylation and NF-κB DNA-binding activity. The overexpression of c-Abl K290R, a dominant negative form, induced a considerable reduction in IκBα tyrosine phosphorylation and NF-κB DNA-binding activity. DOX treatment in these cells did not produce an effect as potent as the one induced by the DOX–Imatinib combination. However, cytotoxicity was significantly increased with this combination in cells overexpressing c-Abl K290R. These data suggest that c-Abl is necessary but not sufficient in DOX-induced cell death and that other tyrosine kinases sensitive to Imatinib are involved. Nevertheless, inhibiting c-Abl kinase activity efficiently blocks basal and DOX-induced NF-κB activation in breast cancer cells. Together, these results indicate that in breast cancer cells, DOX induces NF-κB activity through an atypical pathway.

Materials and methods

Reagents

Antibodies against phosphotyrosine (4G10) were obtained from Millipore Corp (Bedford, MA); primary antibodies against IκBα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-tubulin antibodies, Crystal Violet, proteasome inhibitor MG132 and DOX were purchased from Sigma (St Louis, MI). Pharmacological c-Abl inhibitor Imatinib was obtained from Novartis Pharmaceuticals (Basel, Switzerland).

Cell lines

Breast cancer cell lines T47D and ZR75.30 were kindly provided by Dr. Vilma Maldonado Lagunas and Dr. Jorge Meléndez-Zajgla, National Cancer Institute (INCAN). Cells were maintained in RPMI phenol red-free (MicroLabs S.A. Mexico City) supplemented with 10 % fetal bovine serum. MBCDF cells were generated from explants derived from a biopsy obtained from a radical mastectomy in a patient diagnosed with ductal infiltrating carcinoma stage IV with bone metastasis (protocol approved by the

Ethics Committee and Research of the National Institute for Medical Sciences and Nutrition "Salvador Zubirán" (INCMNSZ), Ref. 1549, BQO-008-06/9-1). Tissue was cut into small pieces and seeded as explants in RPMI supplemented with 10 % fetal bovine serum. Cells that grew out of the explants were left to fill the tissue culture plate. These primary cell isolates were trypsinized and grown as a regular cell line. Immortalization was assumed by continued growth in vitro for over 2 months. Experiments have been performed in these cells for over a year with similar phenotype.

Cytotoxicity assay

Cytotoxicity assays were performed by seeding cells in 24-well plates at a density of 1×10^4 cells/cm^2 in RPMI phenol red-free supplemented with 10 % FBS. The next day defined doses of DOX were added. Cell viability was evaluated 48 h after treatment by crystal violet assay. Dye was dissolved in 400 µl of 10 % acetic acid, and the OD was read at 570 nm in a spectrophotometer. Results are expressed as the percentage of the absorbance of treated cells over the absorbance of untreated control cells. Experiments were performed in triplicate in at least three independent experiments.

Western blotting and immunoprecipitation

Cells were treated with 1 µg/ml of DOX for the time points indicated and lysed in a buffer containing 50 mM HEPES (pH 7.4), 1 mM EDTA, 250 mM NaCl, 1 % Nonidet P-40, 10 mM NaF, 1 mM sodium vanadate and protease cocktail inhibitor (Complete, EDTA-free, Roche Diagnostics, Indianapolis, IN). Thirty micrograms of protein was run in SDS–PAGE and transferred to Immobilon-P PVDF membranes (Millipore Corp., Bedford, MA). Western blots were probed with the following primary antibodies against phosphotyrosine (4G10), IκBα, phospho-IκBα (Ser-32/36) or tubulin. HRP-conjugated anti-mouse or anti-rabbit antibodies (Thermo Scientific, Rockford, IL) were used as secondary antibodies. The blots were revealed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL). The chemiluminescence was visualized by exposing to a film (Kodak, Rochester, NY). For IκBα immunoprecipitation, 600 µg of protein from total cell lysates was incubated with anti-IκBα antibody and protein A Sepharose (Invitrogen, San Diego, CA) overnight at 4 °C with gentle rotation. Immunocomplexes were washed five times with lysis buffer, and protein was eluted with 2X SDS–sample buffer. Samples were run in SDS–PAGE and transferred. Western blot analysis was performed as mentioned above.

Electrophoretic mobility shift assays

EMSAs were conducted as previously described in Ibarra-Sánchez et al. (2001) using 10 µg of nuclear protein from serum-starved breast cancer cells stimulated with 1 µg/ml of DOX at indicated time points. Briefly, 10 µg of nuclear extracts was incubated with ^{32}P-labeled double-stranded oligonucleotide corresponding to the NF-κB consensus site (underlined) (5′ AGTTGAGGGGACTTTCCCAGGC 3′, Santa Cruz Biotechnology) in a buffer of HEPES 20 mM, pH 7.9, DTT 1 mM, KCl 50 mM, EDTA 0.2 mM, glycerol 10 % and poly(dI-dC)-poly(dI-dC) 1 µg per reaction for 20 min. Specific binding was determined by competing with 100 times of unlabeled probe. Complexes were resolved in 4 % polyacrylamide gels under native conditions. Gels were dried and exposed on a phosphorimager screen. The screen was read in a Typhoon 9400 (Amersham Biosciences) and analyzed with the software ImageQuant TL v2005.

Transfection of c-Abl K290R

T47D and MBCDF were transfected by lipofectamine with a pcDNA3 vector containing c-Abl K290R mutant. Cells were selected with 400 µg/ml of geneticin (Invitrogene, San Diego, CA). Single clones were obtained by limiting dilution.

Results

Sustained DOX-induced NF-κB DNA-binding activity correlates with reduced cytotoxic effect of DOX on breast cancer cells

Genotoxic cancer drugs such as DOX induce NF-κB activation in several types of cancers. However, the mechanism of activation remains controversial. For this reason, we were interested in clarifying whether the NF-κB activation maintains any correlation with the cytotoxic effect induced by DOX in breast cancer cells. We analyzed DNA-binding activity of NF-κB in breast cancer cells treated with DOX and correlated it with DOX cytotoxicity. We used a primary breast cancer cell culture MBCDF as well as two established breast cancer cell lines (T47D and ZR75.30). Following treatment with 1 µg/ml of DOX between 0 and 2 h, NF-κB activation was evaluated by EMSA on nuclear extracts. We noted that NF-κB DNA-binding activity increased after DOX treatment with different kinetics among the different breast cancer cells. In MBCDF, we observed an upward trend of NF-κB activation after DOX stimulation that reached up to almost twofold increase at 2 h. In T47D,

DOX increased NF-κB activity 1.3-fold after 30 min, reaching 1.6-fold at 2 h (Fig. 1a, upper and middle panels). In contrast, in ZR75.30 cells, DOX induced a transient effect with a peak increase in NF-κB activation at 30 min (1.8-fold increase) that returned to the basal value at 2 h (Fig. 1a, lower panel). These data suggest that NF-κB is activated by DOX with different kinetics in the three breast cancer cells analyzed. It is worth mentioning that all breast cancer cells presented a basal NF-κB DNA-binding activity consistent with the previous reports of constitutive NF-κB activation in breast cancer cells (Biswas et al. 2004; Wu and Kral 2005). Next, we evaluated the cytotoxic effect of increasing doses of DOX on breast cancer cells. We found that all breast cancer cells died in a dose-dependent manner; however, they displayed different sensitivity to DOX. T47D and MBCDF were less susceptible to DOX. Cell viability was reduced with increasing amounts of DOX; at 0.1 μg/ml we observed 73 and 76 % of cell viability for T47D and MBCDF, respectively; at 0.5 μg/ml it fell up to 58 and 48 % and at 1.0 μg/ml reached 42 and 31 %, respectively. In contrast, ZR75.30 viability fell off rapidly; at 0.1 μg/ml viability reached 45 % and remained steady at 0.5 or 1.0 μg/ml of DOX with a viability of 20 % (Fig. 1b).

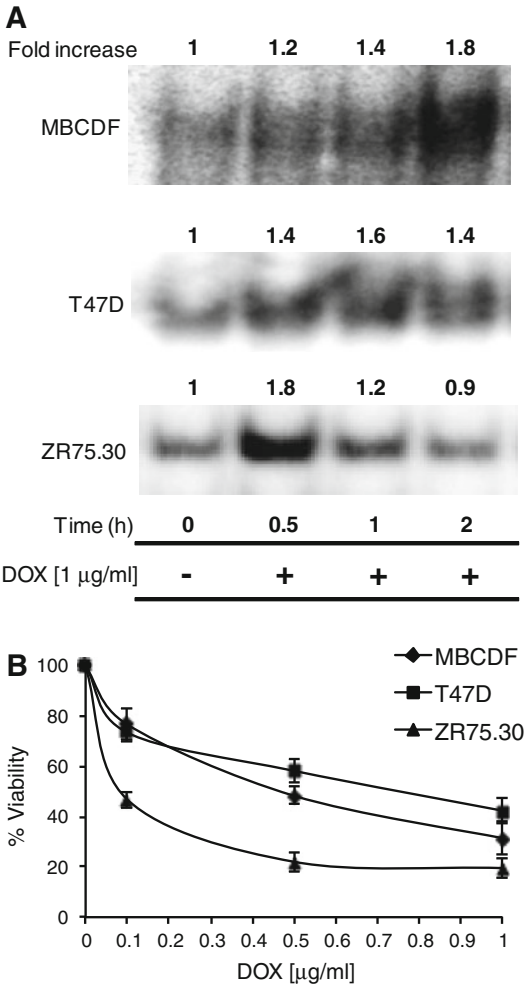

Fig. 1 DOX induced different kinetics of NF-κB activation in various breast cancer cells. **a** MBCDF, T47D and ZR75.30 were treated with 1 μg/ml of DOX at the indicated time points. EMSA for NF-κB DNA-binding activity was performed using a ^{32}P-labeled double-stranded oligonucleotide corresponding to the NF-κB consensus site and 10 μg of nuclear protein. Numbers above the lanes represent fold increase over the control. **b** DOX dose-dependent curve was plotted on MBCDF, T47D and ZR75.30 breast cancer cells. Cells were seeded in 24-well plate at 10^4 cells/cm^2, and DOX was added at 0, 0.1, 0.5 and 1 μg/ml. Cell viability was evaluated by crystal violet 48 h after DOX treatment. Results are expressed as mean ± SD of the percentage of OD of untreated cells. Experiments were seeded in triplicate, and graphs are representative of three independent experiments with similar results

DOX did not induce IκBα Ser-32/36 phosphorylation or degradation in breast cancer cells

Next, we investigated canonical DOX-dependent NF-κB activation. We evaluated IκBα Ser-32/36 phosphorylation or degradation in the three breast cancer cells used. TNF was used as a positive control. Our results showed that DOX did induce slight changes, but not as strong as with TNF. In T47D cells at 0.5 and 1 h, it seems that IκBα Ser-32/36 phosphorylation is reduced, and for ZR75.30 and MBCDF, there were no significant changes. It is worth to mention that a basal phosphorylation is observed in all breast cancer cells used (Fig. 2a). In accordance with this result, we found that IκBα was not degraded with DOX. In contrast, TNF treatment showed proper IκBα degradation as expected (Fig. 2b).

DOX reduces IκBα tyrosine phosphorylation in breast cancer cells sensitive to DOX

Since tyrosine phosphorylation of IκBα has been linked to NF-κB activity through an atypical pathway (Tergaonkar et al. 2003), we investigated whether IκBα phosphorylation on tyrosine residues was modified in response to DOX in the three breast cancer cells. Untreated breast cancer cells showed a basal IκBα tyrosine phosphorylation. In T47D, DOX did not have effect over IκBα tyrosine phosphorylation after half an hour; however, we observed a fold decrease of 0.5 after 1 h treatment. In ZR75.30 cells, DOX induced a fold decrease of 0.6 at 0.5 h and came back to basal levels after 1 h. DOX did not induce significant change on IκBα tyrosine phosphorylation in MBCDF cells at 0.5 h; however, at 1 h there was an increase in IκBα tyrosine phosphorylation up to 1.2-fold (Fig. 2c).

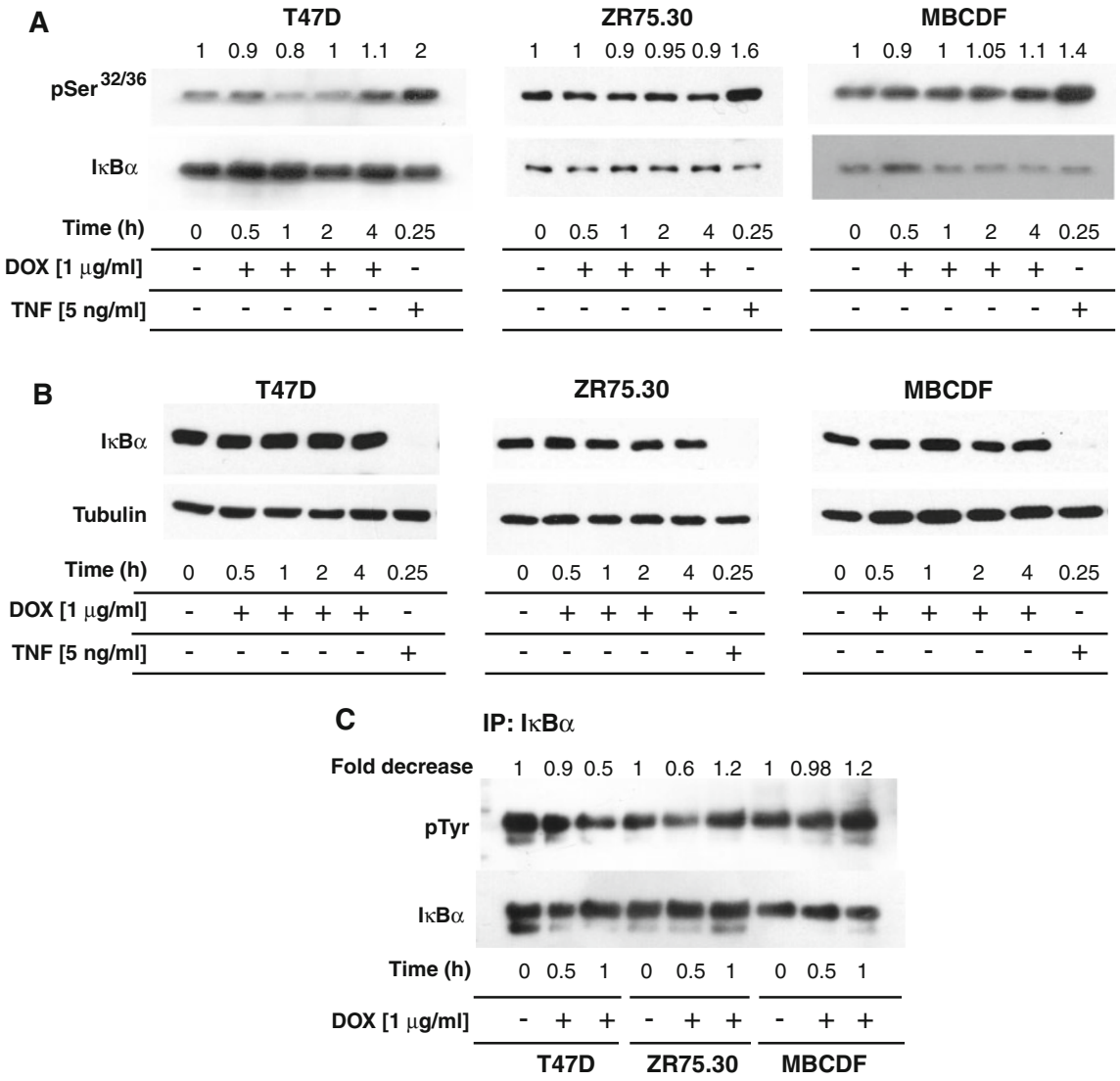

Fig. 2 IκBα is neither Ser-32/36 phosphorylation nor degraded by DOX; instead, DOX affects its phosphorylation at tyrosine residues in breast cancer cells. **a** MBCDF, T47D and ZR-75-30 and breast cancer cells were treated with 20 μM of the proteasome inhibitor MG-132 2 h before the addition of DOX (1 μg/ml) or TNF (5 ng/ml). Whole-cell lysates were collected at the indicated time points. pSer-32/36 IκBα was immunodetected using a phosphor-specific antibody. Membrane was stripped and reblotted for IκBα. **b** MBCDF, T47D and ZR75.30 cells were treated with either 1 μg/ml of DOX or 5 ng/ml of TNF. Whole-cell lysates were obtained at the time points indicated. Degradation of IκBα was detected by Western blot analysis using anti-IκBα antibody. Membranes were stripped and reblotted with anti-tubulin antibody as loading control. **c** MBCDF, T47D and ZR75.30 cells were treated with DOX. Cells were harvested at 0.5 and 1 h after treatment with 1 μg/ml of DOX. Whole protein extracts were immunoprecipitated overnight with anti-IκBα antibody at 4 °C. A phosphotyrosine Western blotting was performed using 4G10 antibody, and then membranes were stripped and reblotted with anti-IκBα antibody as loading control

Imatinib reduces IκBα tyrosine phosphorylation and NF-κB DNA-binding activity

Since we found that DOX affected IκBα tyrosine phosphorylation in ZR75.30, but not in MBCDF or T47D cells, we tested the putative participation of c-Abl in IκBα phosphorylation using its inhibitor, Imatinib. MBCDF and T47D cells were treated with 10 μM of Imatinib for 2 h before the addition of 1 μg/ml of DOX. As observed in Fig. 2c, IκBα tyrosine phosphorylation was not significantly affected by DOX treatment. In MBCDF cells, Imatinib alone induced only a slight decrease in IκBα tyrosine phosphorylation levels compared to MBCDF cells; however, Imatinib–DOX combination provoked significant loss of IκBα tyrosine phosphorylation. In T47D, Imatinib was able to decrease the tyrosine phosphorylation of IκBα; Imatinib–DOX combination did not further decrease the level of tyrosine phosphorylation (Fig. 3a).

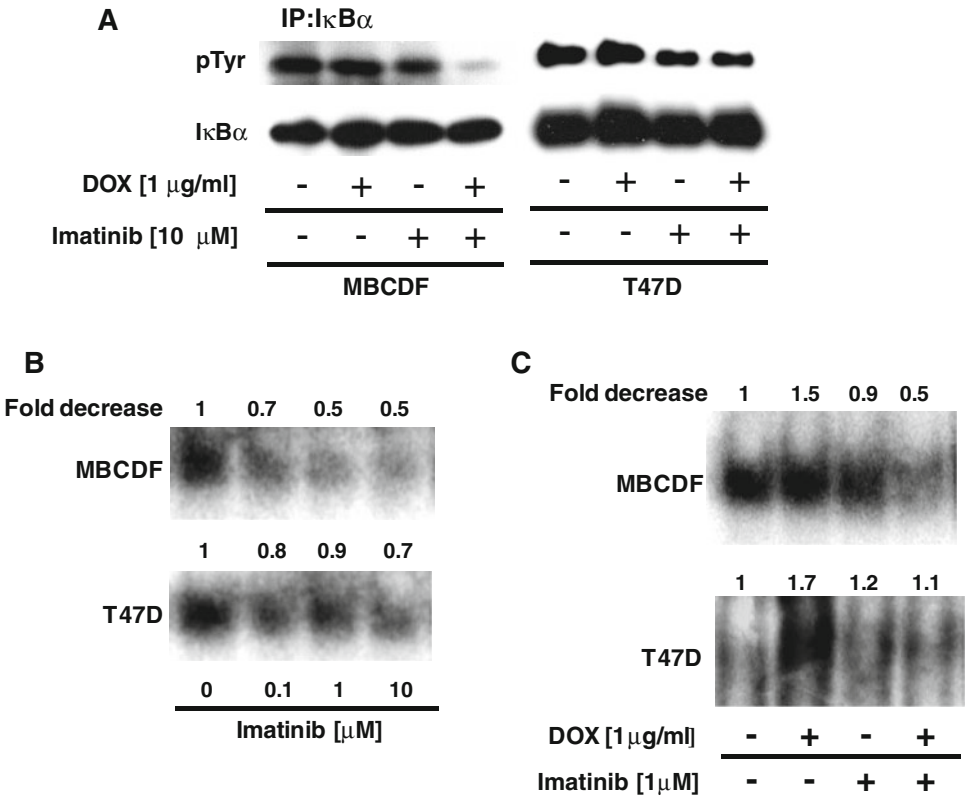

Fig. 3 IκBα tyrosine phosphorylation and NF-κB DNA-binding activity are modified by Imatinib. **a** Immunoprecipitation of IκBα from MBCDF and T47D cells treated with or without 10 μM of Imatinib 2 h before adding 1 μg/ml of DOX for 30 min. Whole-cell lysates were immunoprecipitated as in Fig. 2b. Western blotting of anti-phosphotyrosine was performed, and then the membranes were stripped and reblotted for total IκBα as loading control. **b** MBCDF and T47D breast cancer cells were treated with 0.1, 1 and 10 μM of Imatinib for 30 min. EMSA for NF-κB was performed as in Fig. 1a. **c** MBCDF and T47D breast cancer cells were treated with 1 μM of Imatinib for 2 h before the addition of 1 μg/ml of DOX. EMSA for NF-κB was performed as in Fig. 1a. Numbers above the lanes represent fold increase over the control

Next, we evaluated the effect of Imatinib on NF-κB DNA-binding activity, by performing EMSAs in MBCDF and T47D treated with increasing doses of Imatinib for 30 min. In MBCDF cells, Imatinib induced a slight decrease in NF-κB DNA-binding activity at 0.1 μM and fell down to half at 1 and 10 μM (Fig. 3b, upper panel). In the case of T47D cells, Imatinib produced a small decrease in NF-κB DNA-binding activity, reaching only 0.7-fold decrease at the highest concentration (Fig. 3b, lower panel). To investigate whether Imatinib interferes with NF-κB DNA-binding activity induced by DOX as described in Fig. 1, we pre-treated MBCDF and T47D cells with Imatinib (1 μM) for 2 h before the addition of DOX (1 μg/ml). EMSA was performed 30 min later. The results showed that NF-κB DNA-binding activity decreased by half in MBCDF cells treated with the combination. The 1.5-fold activation mediated by DOX was reverted by the combination Imatinib–DOX (Fig. 3c, upper panel). In T47D cells, the effect of Imatinib induced a similar effect, but NF-κB DNA-binding activity returned to basal levels (Fig. 3c, lower panel).

Imatinib increases DOX-induced cell death in breast cancer cells

We evaluated the effect of Imatinib on cell death induced by DOX-resistant cells (MBCF and T47D). First, we plotted a dose–response curve of Imatinib to investigate its effect on these cells. In MBCDF cells, cell viability decreased in a dose-dependent manner from 0.01 to 10 μM with sharp decrease at 50 μM. In T47D cells, Imatinib treatment decreased 25 % of cell viability at 0.01 μM, remaining steady until 10 μM. Similar to MBCDF, T47D cells had also a sharp reduction of cell viability at 50 μM (Fig. 4a). Next, we investigated whether Imatinib pre-treatment affected the cell death induced by DOX. Imatinib was added at different concentrations to T47D and MBCDF cells 2 h before treatment with DOX (Fig. 4b, c). We found that pre-treatment with Imatinib enhanced cell death induced by DOX in both T47D and MBCDF cells (Fig. 4b, c). These results indicate that Imatinib–DOX combination can increase cell death of breast cancer cells that normally present a poor response to DOX. These data also correlate with a reduction in persistent IκBα tyrosine phosphorylation levels and NF-κB DNA-binding activity observed in these breast cancer cells.

Expression of the c-Abl K290R-inactive kinase abolishes NF-κB DNA-binding activity

In order to examine the relevance of c-Abl kinase in DOX-induced NF-κB signaling, MBCDF and T47D cells were transfected with plasmid containing the cDNA of c-Abl with the mutation K290R. We selected two stable

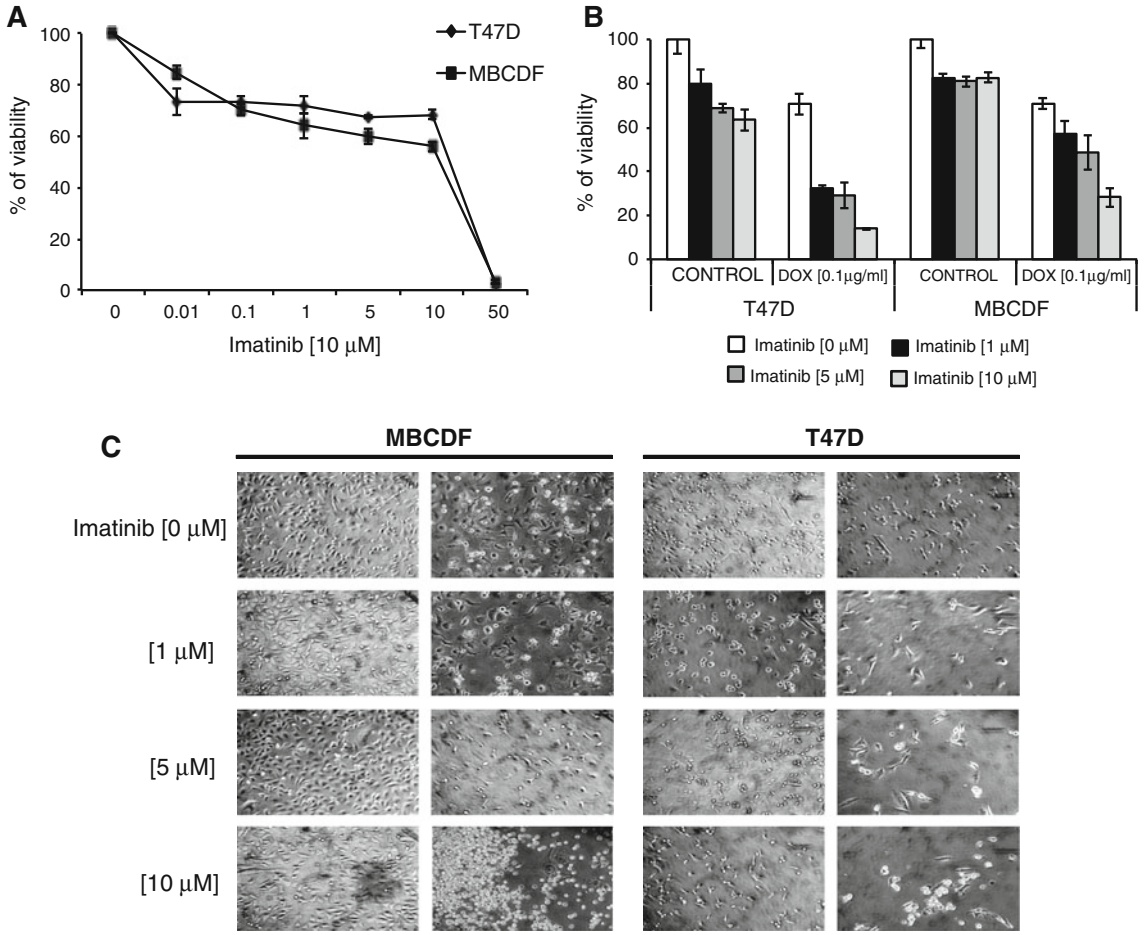

Fig. 4 Imatinib increases the DOX-induced cell death of resistant breast cancer cells. **a** MBCDF and T47D cells were seeded as in Fig. 1b, and then cells were treated with increasing doses of Imatinib. Cell viability was evaluated by crystal violet assay 48 h after Imatinib addition. **b** T47D and MBCDF cells were seeded as in **a**, and then cells were stimulated with 1, 5 and 10 μM of Imatinib before adding 0.1 μg/ml of DOX. Cell viability was evaluated by crystal violet assay 48 h after the addition of drugs. Results in **a** and **b** are mean ± SD of a plate seeded in triplicate, and they are representative of three independent experiments. **c** Pictures from cytotoxicity assays were taken under inverted microscope at the magnification of 400 × 48 h after the addition of drugs

transfectants one from each cell line containing either the empty vector or c-Abl K290R. Western blot analysis against c-Abl showed that T47D-C6 and MBCDF-C5 overexpress c-Abl K290R compared to T47D-EV and MBCDF-EV that were transfected with the empty vector (Fig. 5a, upper panel). The stripped membrane was reblotted against tubulin that was used as a loading control (Fig. 5a, lower panel). Next, we evaluated whether overexpression of c-Abl K290R affected NF-κB DNA-binding activity induced by DOX, using the same protocol as in Fig. 1a. The results showed that DOX induced NF-κB activation after 30 min of stimulation in control cells (MBCDF-EV and T47D-EV) as shown before (Fig. 5b). Overexpression of c-Abl K290R reduced NF-κB basal activity around 40 % for MBCDF-C5 and 60 % for T47D-C6 cells. In MBCDF-C5, DOX treatment induced an increase of 1.7-fold of NF-κB DNA-binding activity and in T47D-C6 did not have any effect. Treatment with TNF induced NF-κB activation in both overexpressing c-Abl K290R cells and the cells transfected with EV (Fig. 5b). The data suggest that c-Abl kinase activity plays a role in both basal and DOX-induced NF-κB activity.

Expression of the c-Abl K290R-inactive kinase reduces total IκBα tyrosine phosphorylation levels in breast cancer cells

Next, we analyzed IκBα tyrosine phosphorylation in T47D-EV, T47D-C6, MBCDF-EV and MBCDF-C5 cells treated as in Fig. 5b. MBCDF-EV and T47D-EV treated with DOX showed no significant changes in IκBα tyrosine phosphorylation as we had previously shown in Fig. 3a. Meanwhile, the overexpression of c-Abl K290R in MBCDF-C5 and T47D-C6 cells showed a severe reduction in the IκBα tyrosine phosphorylation levels that were almost undetectable (Fig. 5c, lanes 3 and 7). DOX did not

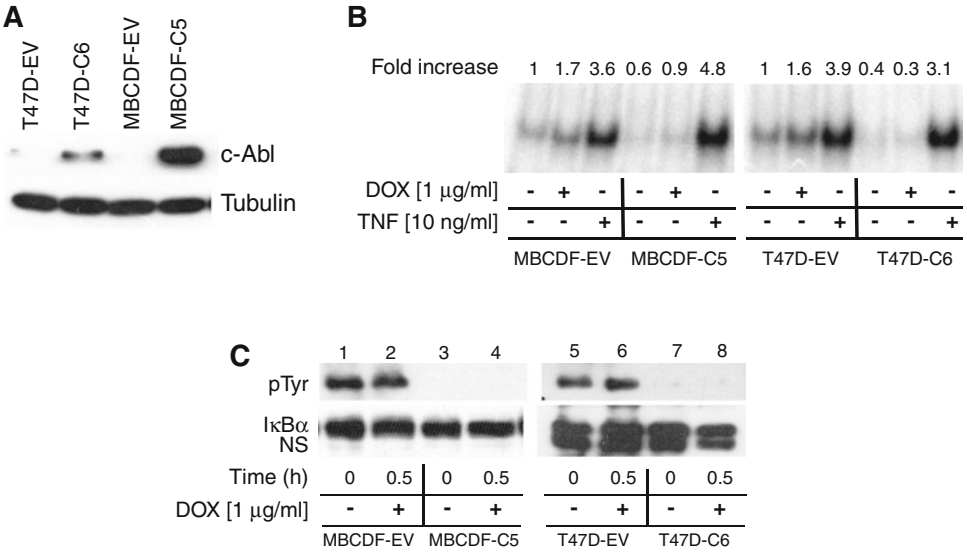

Fig. 5 Overexpression of c-Abl K290R interferes with NF-κB DNA-binding activity and IκBα tyrosine phosphorylation. **a** MBCDF and T47D breast cancer cells were transfected with a plasmid containing the c-Abl K290R cDNA, and clones overexpressing c-Abl K290R were isolated using 100 μg/ml of geneticin. Western blot analysis of T47D-EV (transfected with empty vector), T47D-C6 (transfected with pcDNA3 c-Abl K290R), MBCDF-EV (transfected with empty vector) and MBCDF-C5 (transfected with pcDNA3 c-Abl K290R). Equal amounts of protein whole-cell lysates from each clone were analyzed by Western blotting using a polyclonal antibody against c-Abl. Membranes were stripped and reblotted for tubulin as loading control. **b** T47D-EV, T47D-C6, MBCDF-EV and MBCDF-C6 cells were treated with 1 μg/ml of DOX for 30 min, and then nuclear protein was obtained to perform EMSA for NF-κB. Numbers above the lanes represent fold increase over the control. **c** T47D-EV, T47D-C6, MBCDF-EV and MBCDF-C6 cells were treated as in **b**, and then whole protein lysates were immunoprecipitated with anti-IκBα antibody as in Fig. 3b. Western blotting of anti-phosphotyrosine and anti-IκBα was performed

induce further effect over IκBα tyrosine phosphorylation (Fig. 5c, lanes 4 and 8). These data demonstrate that pharmacological inhibition of c-Abl with Imatinib or overexpressing a dominant negative form without kinase activity triggers a reduction in the IκBα tyrosine phosphorylation levels.

Overexpression effect of c-Abl K290R on DOX-induced cell death

Since we observed that overexpression of c-Abl K290R blocks NF-κB DNA-binding activity and IκBα tyrosine phosphorylation, we investigated whether the c-Abl dominant negative form increases sensitivity to DOX. We performed cytotoxicity assays as in Fig. 1b, using the clones T47D-EV, T47D-C6, MBCDF-EV and MBCDF-C5. Cells were treated with 0.1 and 0.5 μg/ml or without DOX, evaluating cell viability 48 and 96 h after treatment (Fig. 6a). MBCDF-C5 and T47D-C6 were more susceptible to DOX-induced cell death. MBCDF-C5 cells presented 10 % more cell death than MBCF-EV when were treated with 0.1 μg/ml for 48 h. Treatment with a higher dose of DOX (0.5 μg/ml) increased cell death over 37 % more than MBCDF-EV. At 96 h cell death is further increased in cells overexpressing c-Abl K290R compared with cells transfected with the empty vector (Fig. 6a, upper graph). Cell viability in T47D-C6 cells treated with 0.1 μg/ml for 48 h decreased 38 % compared to T47D-EV; this difference was further augmented with 0.5 μg/ml of DOX. Longer treatment (96 h) also induced higher percentage of cell death in T47D-C6 cells (Fig. 6a, lower graph). Overexpression of c-Abl K290R increased susceptibility to the cytotoxic effect of DOX. However, this effect is not as strong as the one observed with the Imatinib–DOX combination. These data suggest that c-Abl kinase activity participates partially in the cell death induced by DOX in breast cancer cells and implies that other tyrosine kinases are involved.

Imatinib enhances DOX-induced cell death in breast cancer cells overexpressing c-Abl K290R

To prove the putative participation of other tyrosine kinases in DOX-induced cell death, we decided to treat cells overexpressing c-Abl K290R with the combination Imatinib–DOX. We performed cytotoxicity assays as in Fig. 4b. Clones MBCDF-EV, MBCDF-C5, T47D-EV and T47D-C6 were pre-treated with 1, 5 and 10 μM of Imatinib for 2 h before the addition of 0.1 μg/ml of DOX, evaluating cell viability 48 h after treatment (Fig. 6b). The results showed that Imatinib decreased cell viability in MBCF-EV and T47D-EV cells similar to parental cells as shown in Fig. 4a. In these clones, Imatinib–DOX combination

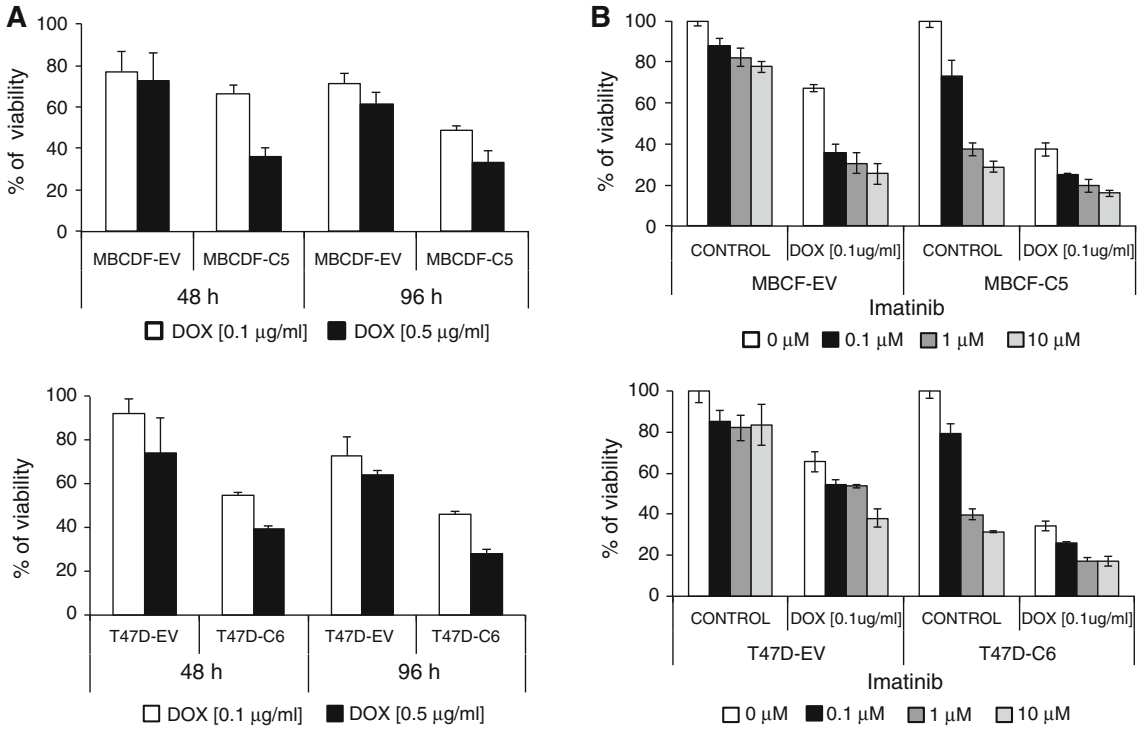

Fig. 6 Overexpression of c-Abl K290R partially increases DOX-induced cell death. **a** T47D-EV, T47D-C6, MBCDF-EV and MBCDF-C6 cells were seeded as in Fig. 1b. DOX was added at 0.1 and 0.5 µg/ml. Cell viability was evaluated by crystal violet assay after 48 h. **b** T47D-EV, T47D-C6, MBCDF-EV and MBCDF-C6 cells were seeded as in **a**, and then cells were stimulated with 1, 5 and 10 µM of Imatinib before adding 0.1 µg/ml of DOX. Cell viability was evaluated 48 h after drug addition by crystal violet assays. Results in **a** and **b** are mean ± SD of a plate seeded in triplicate, and it is representative of three independent experiments

increased cell death compared with DOX, reproducing the results obtained in the parental cells. MBCDF-C5 and T47D-C6 cells died in a dose-dependent manner with Imatinib treatment. In the cells overexpressing c-Abl K290R treated with DOX, cell viability decreased an average of 30 % more than cells transfected with the empty vector. Comparing the effect of the Imatinib–DOX combination between cells overexpressing c-Abl K290R with cells transfected with the empty vector, the cell death increased between 10 and 35 % (Fig. 6b). These results suggest that Imatinib has an effect on other tyrosine kinases that participate in DOX-induced cell death that was not blocked by the expression of c-Abl K290R.

Discussion

The importance of NF-κB in cancer has been emphasized with the finding that inhibition of NF-κB activation can sensitize cancer cells to chemotherapy agents, an effect that has been linked to transcription of anti-apoptotic genes (Cusack et al. 2001; Wang et al. 1999; Basseres and Baldwin 2006; Baldwin 2001; Miura et al. 2009). In recent years, it has become evident that pathways leading to NF-κB activation in response to a particular inducer differ depending on the specific cell type. In particular, IκBα tyrosine phosphorylation has not been well characterized, but represents a potential target for the development of new therapeutic strategies. The role of IKKs upstream of IκBα phosphorylation remains controversial (Tergaonkar et al. 2003; Janssens et al. 2005; Mabb et al. 2006; Bednarski et al. 2008).

In this work, we studied the signaling pathway induced by DOX in two breast cancer cell lines and one primary cell culture. We found that DOX activates NF-κB with different kinetics; the first one has an upward trend of NF-κB activation that correlates with steady levels of IκBα tyrosine phosphorylation and poor response to the cytotoxic effect of DOX (T47D and MBCDF). The second displays transient NF-κB activation that correlates with a reduction in the IκBα tyrosine phosphorylation levels and high sensitivity to DOX (ZR75.30). DOX did not produce IκBα degradation in the same manner as TNF did, suggesting a NF-κB activation through an atypical pathway. We found that T47D and MBCDF have steady levels of IκBα tyrosine phosphorylation after half-an-hour treatment with DOX that are not further modified in MBCDF, but in T47D returned to basal levels correlating with a decrease in NF-κB DNA-binding activity. Interestingly, ZR75.30 showed a decrease in IκBα tyrosine phosphorylation after

half-an-hour treatment with DOX, which correlates with the peak of NF-κB DNA-binding activity and the high sensitivity to DOX. Phosphorylation of IκBα at Tyr 42 and Tyr 305 by certain non-classical stimuli has been reported. Tyr 42 is phosphorylated by non-receptor tyrosine kinases (Schoonbroodt et al. 2000), whereas Tyr 305 is phosphorylated by the tyrosine kinase, c-Abl, postulated to increase IκBα protein stability (Kawai et al. 2002). The fact that c-Abl activity has been related to an invasive phenotype of breast cancer cells (Srinivasan and Plattner 2006; Srinivasan et al. 2008) and our findings showing that cells resistant to DOX displayed steady levels of IκBα tyrosine phosphorylation raise a putative relationship between c-Abl activity and IκBα/NF-κB signaling. In agreement with this model, we found that c-Abl kinase inhibitor (Imatinib) negatively modulates IκBα tyrosine phosphorylation. These changes in phosphorylation correlate to a decrease in the basal and DOX-induced NF-κB DNA-binding activity. Furthermore, these two molecular changes are related to a significant increase in the DOX-induced cell death.

Imatinib inhibits efficiently the c-Abl tyrosine kinase family; however, it can also inhibit other tyrosine kinases such as PDGFR and c-Kit that may contribute in the effect observed. When we analyzed the relevance of c-Abl, we found that overexpression of c-Abl K290R leads to a marked reduction in NF-κB DNA-binding activity and IκBα tyrosine phosphorylation in both basal and DOX-treated cells. These data suggest that c-Abl inhibition either by Imatinib or by overexpression of a dominant negative form blocks NF-κB activity and decreases IκBα tyrosine phosphorylation. It is worth to mention that c-Abl dominant negative form did not affect NF-κB activation induced by TNF, suggesting that inhibition of c-Abl affects an atypical pathway in these breast cancer cells. These results suggest that c-Abl may phosphorylate IκBα maintaining its stability and NF-κB heterodimer active. Indeed, we have found an interaction between c-Abl and IκBα (data not shown), suggesting that c-Abl phosphorylates directly IκBα. After inhibition of c-Abl, IκBα reduces its phosphorylation on tyrosine residues, most likely affecting the interaction with NF-κB and then producing a reduction in its DNA-binding activity; how this interaction is modified remains to be elucidated. As mentioned above, IκBα is phosphorylated at Tyr 42 and Tyr 305, being the last residue the target of c-Abl. The fact that c-Abl K290R induced a complete abrogation of IκBα tyrosine phosphorylation might be due to a direct inhibition of Tyr 305 and indirect blocking of another signaling pathway involving phosphorylation of Tyr 42.

Although inhibition of c-Abl appears to be sufficient to abolish IκBα tyrosine phosphorylation and NF-κB DNA-binding activity, for cell death, overexpression of c-Abl dominant negative form did not have the same effect as the combination Imatinib–DOX. These data suggest that Imatinib is inhibiting other tyrosine kinases that are also mediating cell death independently of NF-κB. This hypothesis was supported by the fact that the combination Imatinib–DOX augmented cell death in cells overexpressing c-Abl dominant negative form. These results are in agreement with a recent report where the combination of Imatinib with radiotherapy or standard chemotherapy leads to a decrease in cell proliferation through inhibition of PDGFR (Weigel et al. 2010). We conclude that DOX-induced cell death of breast cancer cells is a complex process that involves other tyrosine kinases in addition to c-Abl; likely candidates are PDGFR or c-Kit.

Conclusion

Together, our results suggest that NF-κB activation in breast cancer cells with poor response to DOX involves c-Abl-dependent IκBα tyrosine phosphorylation. We also showed that inactivation of c-Abl and other Imatinib-sensitive kinases play an important role in DOX-induced cell death. This model is valid in established breast cancer cell lines (T47D) as well as primary breast cancer cell culture from a patient with advanced breast cancer (MBCDF), suggesting that this is not a particular fact of T47D cells. It is important to test the combination Imatinib–DOX in more primary breast cancer isolates to prove the efficiency of this combination. Furthermore, our results raise the potential use of a combined therapy with Imatinib (c-Abl inhibitor) and DOX in breast cancer patients.

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Conflict of interest None.

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