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Inhibition of Constitutive Activity of Nuclear Transcription Factor kappaB Sensitizes Doxorubicin-Resistant Cells to Apoptosis

Charitha Gangadharan, Maikho Thoh, and Sunil K. Manna*

Laboratory of Immunology, Centre for DNA Fingerprinting & Diagnostics, Nacharam, Hyderabad 500076, India

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ABSTRACT

Doxorubicin is one of the most effective agents used in the treatment of various tumors. Its use is restricted by the development of resistance to apoptosis, the mechanism of which is not fully understood. Nuclear transcription factor kappaB (NF-κB) has been shown both to block apoptosis and to promote cell proliferation, and hence has been considered as an important target for anticancer drug development. We found that in wild type and Dox-revertant MCF-7 cells, Doxorubicin induced NF-κB was transient and Dox-resistant cells showed high basal activity of NF-κB and expression of genes dependent on it. Moreover, in resistant cells Doxorubicin was unable to induce apoptosis as detected by assays for reactive oxygen intermediates generation, lipid peroxidation, cytotoxicity, PARP degradation and Bcl-2 expression. High basal expressions of multi-drug resistant protein and transglutaminase were found in Dox-resistant cells and inhibition of NF-κB decreased those amounts and also sensitized these cells by Doxorubicin. These observations collectively suggest that high NF-κB activity confers resistance to Doxorubicin and its inhibition potentiates apoptosis. This study indicates that NF-κB plays an important role in chemoresistance and establishes the fact that inhibition of NF-κB will be a novel approach in chemotherapy. J. Cell. Biochem. 107: 203–213, 2009. © 2009 Wiley-Liss, Inc.

KEY WORDS: NF-κB; DOXORUBICIN; APOPTOSIS; DRUG RESISTANCE; P_3-25

Development of resistance to chemotherapeutic agents is a major problem in treatment of cancer. Doxorubicin, an anthracycline is one of the most effective chemotherapeutic agents available to treat breast cancer patients. The drug is able to induce regression of metastatic breast cancer alone [Blum and Carter, 1974; Harris et al., 1993] and constitutes the core of most combination chemotherapeutic regimes currently used to treat breast cancer. Aberrantly active NF-κB complexes can contribute in tumorigenesis by regulating genes that promote the growth and survival of cancer cells [Giri and Aggarwal, 1998; Manna and Aggarwal, 1999; Barre and Perkins, 2007]. Although many tumor cells display some level of constitutive activity of nuclear NF-κB, the transcription potential of it can be further enhanced in response to certain types of chemotherapy [Chuang et al., 2002]. Constitutive expression of NF-κB has been implicated as one of the causes for drug resistance in tumors [Cusack et al., 1999; Wang et al., 1999a,b]. This study indicates that NF-κB plays an important role in chemoresistance and establishes NF-κB inhibition may have implication for refining systemic chemotherapy in future.

NF-κB is a heterodimer of two subunits p50 (NF-κB 1) and p65 (RelA). It is normally present in the cytoplasm in an inactive state in complex with an inhibitory subunit of kappaB (IκBα). Upon phosphorylation and subsequent degradation of IκBα, a nuclear localization signal on the p50-p65 heterodimer is exposed, leading to nuclear translocation of NF-κB. The p50–p65 heterodimer binds with a specific sequence in DNA, which in turn results in gene transcription. Phosphorylation of p65 of NF-κB (RelA) is required for effective NF-κB-dependent gene transcription [Jobin and Sartor, 2000; Bonizzi and Karin, 2004; Hayden and Ghosh, 2004]. Treatment of BAY 11-7082 has shown to inhibit IκBα phosphorylation [Mori et al., 2002] thereby blocks proteasomal degradation of IκBα, and hence sequestering NF-κB in the cytoplasm in an inactivated state. P_3-25 is shown to block p65 phosphorylation and to inhibit NF-κB-dependent reporter gene transcription [Manna et al., 2007].

Doxorubicin, an anthracycline is a major anti-tumor agent used in the treatment of a variety of cancers. Evidences demonstrate that cells respond to Doxorubicin is highly regulated by multiple

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*Correspondence to: Dr. Sunil K. Manna, Laboratory of Immunology, Centre for DNA Fingerprinting & Diagnostics, ECIL Road, Nacharam, Hyderabad 500076, India. E-mail: manna@cdfd.org.in

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signaling events, including generation of sphingosine [Reynoldsa et al., 2004; Jurisicova et al., 2006] and activation of different caspases, c-Jun N-terminal kinase (JNK) [Mikami et al., 2006], transcription factors [Chuang et al., 2002], and Fas/Fas ligand system [Kalivendi et al., 2005]. The development of cellular resistance mechanism to chemotherapeutic drugs includes altered expression of different proteins like multi-drug resistant (TGase), Bcl-2, etc., deficient intracellular calcium pools, and loss of p53 function [Chen et al., 2002; Jin et al., 2007; Verma and Mehta, 2007]. The mechanisms of Doxorubicin induced apoptosis and resistance against it, have not yet been fully understood. Attaining resistance to chemotherapy by tumors is a common clinical problem in cancer.

In the laboratory conditions, development of Doxorubicin resistance in a human breast cancer cell line (MCF-7) is a result of propagation of an inherently resistant sub clone. The drug resistant subline was established from the parental MCF-7 cells by culturing in continuous presence of 2 μM Doxorubicin. Only few viable cells were observed after 7 days of culture. Single clone started dividing after 2 weeks of culture and became confluent in 4 weeks time. We then used these cells to characterize the drug-resistance phenomenon. A revertant MCF-7 cells subline was also established by culturing the drug-resistant subline in the absence of Doxorubicin for 6 months [Devarajan et al., 2002]. While culturing, these cells might change some genetic make-up that leads to decrease in several molecules involved in resistance against Doxorubicin behaves like wild type cells.

As NF-κB is currently being used as a target for cancer therapy, it is of interest to see the possible interplay of NF-κB and Doxorubicin resistance. In this study, we demonstrate that Doxorubicin induces NF-κB in wild type and Dox-revertant, but not in Dox-resistant MCF-7 cells. Dox-resistant cells show high basal NF-κB activity and expression of the genes dependent on NF-κB. We also demonstrate that high NF-κB activity confers resistance to Dox-resistant cells and down regulation of NF-κB in these cells (by transfecting with *IκBα-DN* construct or treating with BAY 11-7082 (known as BAY), an IκBα kinase (IKK) inhibitor and P_3-25 [5-(4-methoxyarylimino)-2-*N*-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine], a synthetic derivative of thiadiazolidine sensitizes the cells to apoptosis. High basal NF-κB activity in Doxorubicin-resistant cells confers basal expression of Bcl-2, superoxide dismutase (SOD), multi-drug resistance gene (Mdr), and transglutaminase. Basal amounts of reactive oxygen intermediates (ROI) might maintain high basal activity of NF-κB in Dox-resistant cells that leads to expression of its dependent genes like Bcl-2, Mdr, TGase, or superoxide dismutase (SOD). The SOD1 (Cu/Zn SOD), the most abundant and ubiquitous isoform, has great physiological significance and therapeutical potential in several neurodegenerative disorders and it is a NF-κB-dependent gene product [Rojo et al., 2004]. This SOD did not allow further increase in ROI generation by Doxorubicin treatments in Dox-resistant cells. Hence, understanding the molecular mechanisms of Doxorubicin-mediated apoptosis and a comparative study on Doxorubicin sensitive and resistant tumors will help to improve the potency of cytotoxicity and decrease the resistance of tumor and adopt combination therapy for effective treatment of tumor.

MATERIALS AND METHODS

MATERIALS

Doxorubicin, MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide)], glycine, 4-methyl umbelliferyl phosphate (4-MUP) and anti-tubulin antibody were obtained from Sigma (St Louis, MO). DMEM and fetal bovine serum (FBS) were obtained from Life Technologies (Grand Island, NY). Dihydrorhodamine, DAPI, and goat anti-rabbit IgG-Alexa Flour were purchased from Molecular Probe, The Netherlands. Oct1 double-stranded oligonucleotide and antibodies against p65, IκBα, cyclooxygenase (Cox)2, Transglutaminase (TGase) 2, multi-drug resistance protein (Mdr), intercellular adhesion molecule (ICAM) 1, superoxide dismutase (SOD) 1, CRM1, and Bcl-2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). BAY 11-7082 (designated as BAY) and poly ADP-ribose polymerase (PARP) and phospho-IκBα antibodies were purchased from Calbiochem (San Diego, CA). The 5-(4-methoxyarylimino)-2-*N*-(3,4-dichlorophenyl)-3-oxo-1,2,4-thiadiazolidine (P_3-25) was synthesized and provided by Prof. K.K. Narang, Department of Applied Chemistry, Banaras Hindu University, Varanasi, India. The plasmid construct for *NF-κB-SEAP* and *Cox-2 luciferase* were kind gift from Prof. B.B. Aggarwal of the University of Texas, M. D. Anderson Cancer Center (Houston, TX).

CELL LINES

Wild type MCF-7 cells obtained from American Type Culture Collection (Manassas, VA). Doxorubicin resistant and revertant MCF-7 cells were obtained from Prof. Kapil Mehta, MD Anderson Cancer Center, Houston, USA. Doxorubicin resistant cells were cultured in presence of 2 μM Doxorubicin as reported earlier [Devarajan et al., 2002]. Dox-resistant colony was picked up and grown in presence of doxorubicin (2 μM). Cells were cultured in MEM medium containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml).

NF-κB ACTIVATION ASSAY

Activation of NF-κB was determined by gel shift assay [Manna et al., 1998]. Briefly, cells, after different treatments were used to prepare cytoplasmic and nuclear extracts. Nuclear extract proteins (8 μg) were incubated with ^{32}P end-labeled double-stranded NF-κB oligonucleotide of HIV-LTR, 5′-TTGTTACAAGGGACTTTCCGCT-GGGGACTTTCCAGGGAGGCGTGG-3′ for 30 min at 37°C, and the DNA–protein complex was separated from free oligonucleotide on 6.6% native PAGE. Similarly, Oct1 was assayed using specific double stranded labeled oligonucleotides. Visualization of radioactive bands was done in a PhosphorImager (Fuji, Japan).

NF-κB-DEPENDENT SEAP REPORTER GENE ASSAY

Cells were transiently transfected with Qiagen SuperFect transfection reagent (Hilden, Germany) with reporter plasmid containing NF-κB binding site cloned upstream of heat-stable secretory alkaline phosphatase (SEAP) designated as *NF-κB-SEAP* and *GFP* (0.5 μg each) constructs for 3 h and cultured for 12 h. GFP positive cells were counted and indicated for transfection efficiency. After different treatments, cell culture-conditioned medium (25 μl) was analyzed for SEAP activity essentially as per the Clontech protocol

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(Palo Alto, CA) and reported as fold activation with respect to vector-transfected cells [Manna et al., 2007].

COX-2-DEPENDENT LUCIFERASE GENE TRANSCRIPTION ASSAY

Cells were transiently transfected with SuperFect transfection reagent containing 0.5 μg of each reporter plasmid containing Cox-2 binding site cloned upstream of luciferase (designated as *Cox-2-luciferase*) and *GFP* constructs [Manna et al., 2007]. After 3 h of transfection, cells were cultured for 12 h followed by treatment with Doxorubicin for different times. The cell pellets were extracted with lysis buffer and luciferase activity was measured using firefly luciferin (substrate, Promega).

CYTOTOXICITY ASSAY

The cytotoxicity was measured by MTT assay [Manna et al., 2007]. Briefly, cells (1×10^4 cells/well of 96-well plate) after different treatments were incubated with 25 μl of MTT solution (5 mg/ml in PBS). After 2 h incubation, 100 μl extraction buffer (20% SDS in 50% dimethylformamide) was added and absorbance was read at 570 nm after 12 h incubation with the extraction buffer as blank.

IMMUNOCYTOCHEMISTRY

The amounts of p65 and multi-drug resistant protein (Mdr) were examined by the immunocytochemical method. Briefly, cells were plated chamber slides, washed after different treatments, air-dried, fixed with 3.5% formaldehyde, permeabilized with 0.5% of Triton X-100, blocked by 5% goat serum, and incubated with anti-p65 or -Mdr Ab for 8 h followed by incubation with goat anti-rabbit IgG-Alexa Fluor for 1 h. Slides were mounted with mounting medium with DAPI and analyzed under a fluorescence microscope.

DETERMINATION OF LIPID PEROXIDATION AND MEASUREMENT OF REACTIVE OXYGEN INTERMEDIATES (ROI)

Lipid peroxidation was determined by detection of thiobarbituric acid-reactive MDA, an end product of the peroxidation of polyunsaturated fatty acids as described [Manna et al., 2000]. The production of ROI was determined by flow cytometry as described [Sreenivasan et al., 2003].

RESULTS

In this study, we examined the effect of Doxorubicin in MCF-7 (designed as Wild), Doxorubicin-resistant (Dox-resistant) and revertant (Dox-revertant) MCF-7 cells. Dox-resistant cells were cultured in presence of 2 μM Doxorubicin. The Doxorubicin, BAY, and P_3-25 were used as a solution in DMSO at 10 mM concentration. Further dilution was carried out in cell culture medium. The concentrations and duration of exposure of these chemicals employed in these studies had no effect on cytolysis as detected by lactate dehydrogenase (LDH) assay from culture supernatant of treated cells (data not shown).

DOX-RESISTANT CELLS SHOW CONSTITUTIVE ACTIVATION OF NF-κB

MCF-7 cells (Wild, Dox-resistant, and Dox-revertant) were treated with 1 μM Doxorubicin for different times; nuclear extracts were prepared, and assayed for NF-κB by gel shift assay. As shown in Figure 1A, Dox-resistant cells showed high basal NF-κB activity, whereas the Wild type or Dox-revertant cells showed transient induction of NF-κB. Oct1 DNA binding activity did not alter in those samples as shown by gel shift assay (Fig. 1B). Nuclear extracts from Doxorubicin-treated MCF-7 cells were incubated with antibodies (Abs) to p50 and p65 alone or in combination, and then conducted gel shift assay to study the specificity and composition of the retarded band. Abs to either subunit of NF-κB shifted the band to a higher molecular weight (Fig. 1C), thus suggesting that the retarded complex consisted of both p50 and p65 subunits. Neither preimmune serum nor irrelevant Ab such as anti-c-Rel had any effect on the mobility of NF-κB. This complex completely disappeared in the presence of 50-fold molar excess of cold NF-κB and was unable to bind with mutant oligonucleotide, indicating its specificity.

DOX-RESISTANT CELLS SHOW CONSTITUTIVE ACTIVATION OF NF-κB-DEPENDENT GENES

To determine the effect of Doxorubicin on NF-κB dependent reporter gene expression, we transiently transfected MCF-7, Dox-resistant, and Dox-revertant cells with the NF-κB SEAP reporter construct then treated with different concentrations of Doxorubicin for 6 h. Dox-resistant cells had high basal activity of SEAP. This SEAP activity was not altered significantly by treatment with increasing concentrations of Doxorubicin. In wild type and Dox-revertant cells, the SEAP activity increased with the increased concentrations of Doxorubicin (Fig. 1D).

DOX-RESISTANT CELLS SHOW HIGH BASAL EXPRESSION OF COX-2 AND ICAM1 AND COX-2-DEPENDENT LUCIFERASE

In MCF-7 cells (Wild type), Doxorubicin increased the amounts of Cox-2 (2A) and ICAM1 (2C) at 3 h and 6 h and reduced partially at 12 h as shown by Western blot. In Dox-resistant cells, the high basal amount of Cox-2 was observed and this amount did not alter with the increasing time of Doxorubicin treatments (Fig. 2A). MCF-7 cells (Wild type), transiently transfected with the Cox-2-luciferase and GFP constructs for 3 h were cultured for 12 h. Cells were then treated with 1 μM Doxorubicin for different times. The induction of Cox-2 dependent luciferase activity was transient in wild type (Fig. 2B). Dox-resistant cells showed constitutive activation of luciferase activity (Fig. 2B). These results demonstrate Dox-resistant cells show constitutive expression of Cox-2 and ICAM1; whereas the expression in wild type is transient.

DOX-RESISTANT CELLS SHOW HIGH BASAL LEVELS OF IκBα, PHOSPHO-IκBα, AND P65

Doxorubicin treated cells showed degradation of IκBα after 1 h in wild-type and Dox-revertant cells. Phospho-IκBα was observed at 0.5 and 1 h of Doxorubicin treatment (Fig. 3A). High basal expression of IκBα and phospho-IκBα were observed in Dox-resistant cells and these were not altered on Doxorubicin treatment. The amount of p65 was decreased with increasing time of

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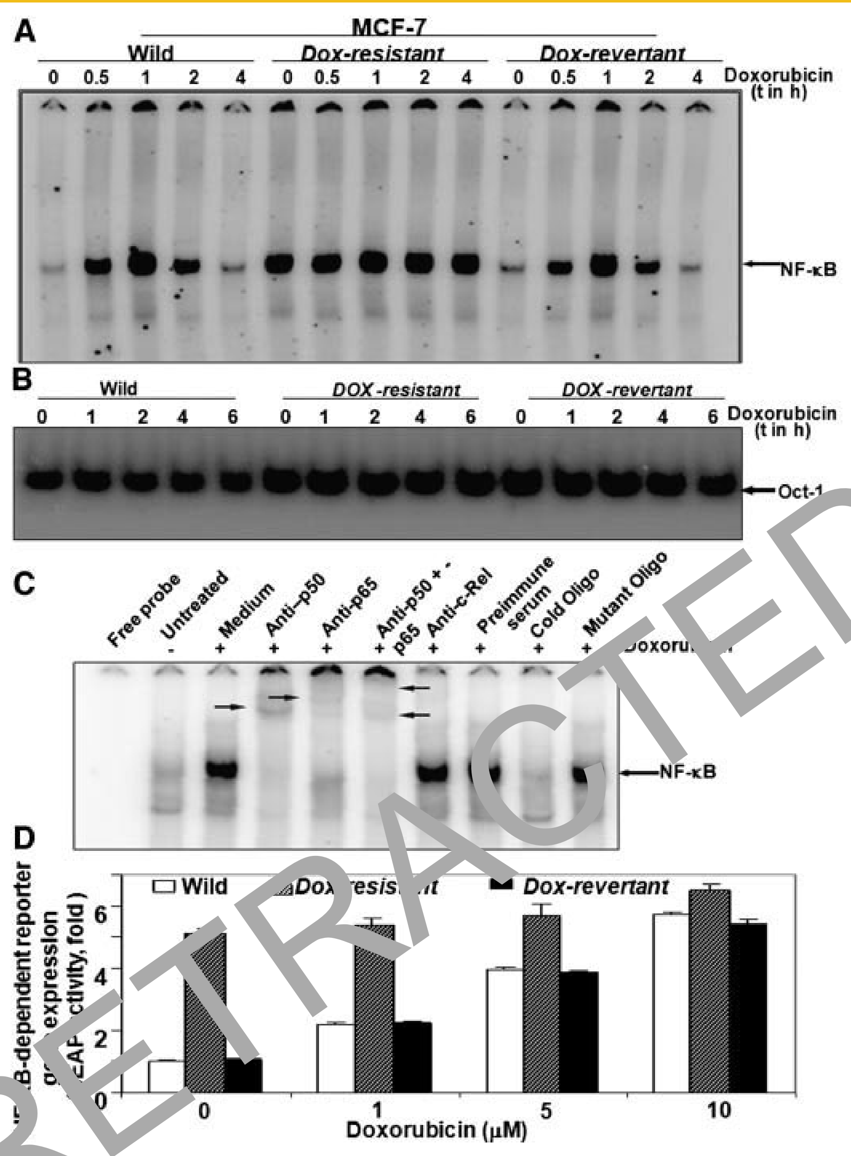

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Fig. 1. Effect of Doxorubicin on NF-κB activation in wild type, Dox-resistant and Dox-revertant cells. MCF-7 (Wild type, Dox-resistant and Dox-revertant) cells were treated with 1 μM Doxorubicin for different times. After these treatments, cytoplasmic (CE) and nuclear extracts (NE) were prepared and NE was assayed for NF-κB (A) and Oct1 (B) by gel shift assay. NE was prepared from untreated or Dox-treated wild type cells, incubated for 15 min with different Abs and unlabeled and mutated NF-κB oligonucleotides (50-fold), and then assayed for NF-κB (C). Wild type, Dox-resistant and Dox-revertant cells were transiently transfected with *NF-κB-SEAP* construct for 3 h, cultured for 12 h, then treated with different concentrations of Doxorubicin for 6 h. Cells culture supernatant was assayed for secreted alkaline phosphatase (SEAP) activity as described in Materials and Methods Section and mean SEAP activity was indicated as fold of activation above vector-transfected cells (D).

Doxorubicin treatment in wild-type and Dox-revertant cells in cytoplasmic extracts. The amount of p65 in nuclear extracts was increased till 1 h of Doxorubicin treatment and decreased thereafter. In Dox-resistant cells high basal level of p65 was observed both in cytoplasmic and nuclear extracts and this level did not alter due to Doxorubicin treatment (Fig. 3B). The p65 concentration was increased at 3 h in nucleus and decreased at 6 h both in cytoplasm and nucleus in Doxorubicin-treated wild type cells. The p65 level did not alter upon Doxorubicin treatment in Dox-resistant cells (Fig. 3C).

DOX-RESISTANT CELLS DO NOT SHOW APOPTOSIS

Cytotoxicity as measured by MTT assay was increased in wild type and Dox-revertant, but not Dox-resistant cells with increasing concentrations of Doxorubicin (Fig. 4A). Apoptosis is reflected by caspase-mediated cleavage of PARP (poly-ADP-ribose polymerase). As shown in Figure 4B, Doxorubicin induced cleavage of PARP with increasing time of incubation in wild type and Dox-revertant cells, but not in Dox-resistant cells. Bcl-2, an antiapoptotic protein, prevents release of cytochrome *c* from mitochondria. Therefore, the effect of Doxorubicin on the concentration of Bcl-2 was

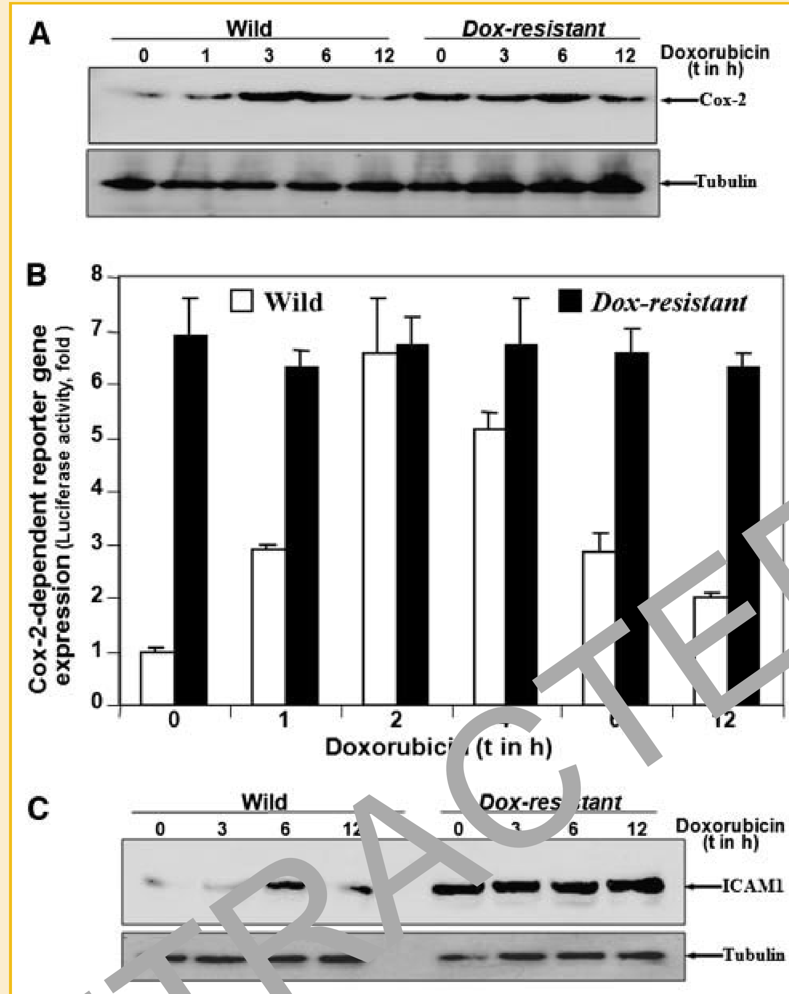

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Fig. 2. Effect of Doxorubicin on C[illegible]-2, ICAM1, and Cox-[illegible]dependent luciferase expression. Wild type and Dox-resistant cells were treated with 1 μM Doxorubicin for varying time periods. The Cox-2 (A) and I[illegible]M1 (C) [illegible]re detected fr[illegible]m whole cell extract by Western blot. The blot was reprobed with anti-tubulin antibody as loading control. Wild type and Dox-resistant [illegible]re tr[illegible]cted with *Cox-2-luciferase* expression vector and *β-galactosidase* gene for 3 h, culture for 12 h, then treated with 1 μM Doxorubicin for different times [illegible] lucife[illegible]se and [illegible]-galac[illegible]dase enzymes activity was measured from whole cell extracts as described in Materials and Methods Section (B).

assessed. As shown in Figure 4C, treatment of cells with Doxorubicin increased the concentration of Bcl-2 at 6 h and then decreased at 12 and 24 h. A high basal level of Bcl-2 was detected in Dox-resistant cells and this was not altered at any time of Doxorubicin treatment.

DOX-RESISTANT CELLS SHOW HIGH BASAL LEVEL OF ROI, BUT NOT LIPID PEROXIDATION

Doxorubicin induced ROI in a dose-dependent manner in wild type and Dox-revertant, but not in Dox-resistant cells (Fig. 4D). Dox-resistant cells showed high basal level of ROI and this level did not alter at any concentrations of Doxorubicin. Doxorubicin at 10 μM concentration induced ROI generation in wild type and Dox-revertant cells beyond the basal of Dox-resistant cells. Doxorubicin induced lipid peroxidation in a dose-dependent manner in wild type and Dox-revertant, but not in Dox-resistant cells (Fig. 4E). These results further suggest that Dox-resistant cells are also resistant to Doxorubicin-induced apoptosis.

DOX-RESISTANT CELLS SHOW BASAL EXPRESSION OF SUPEROXIDE DISMUTASE (SOD) 1

Doxorubicin did not increase ROI generation of Dox-resistant cells. We have checked the amount of ROI detoxifying factors like SOD1. The amount of SOD1 was increased in Dox-resistant cells than wild type or Dox-revertant MCF-7 cells (Fig. 4F). Doxorubicin treatment did not alter the basal amount of SOD1 in these cells. These results suggest that Doxorubicin-induced ROI generation was not observed in Dox-resistant cells may be due to high basal expression of SOD1.

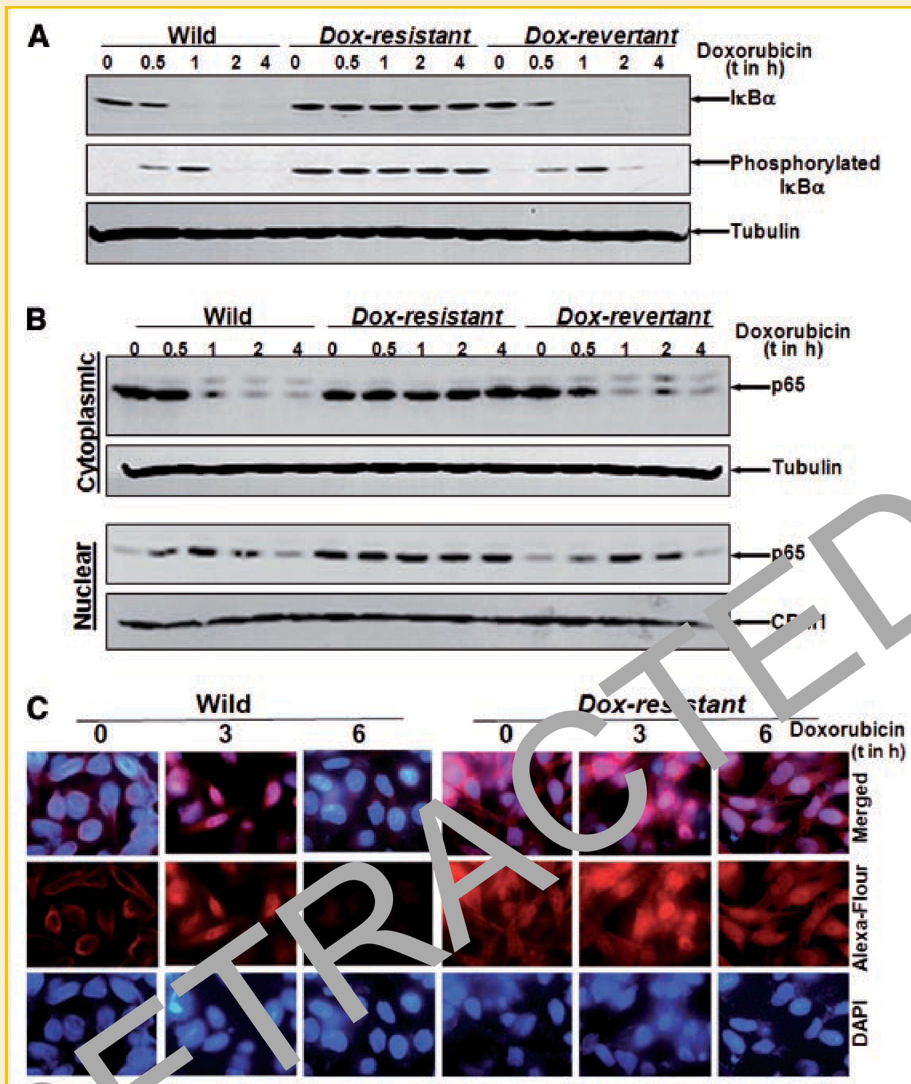

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Fig. 3. Effect of Doxorubicin on the amounts of IκBα, phospho-IκBα, and p65 in wild type, Dox-resistant, and Dox-revertant cells. Wild type, Dox-resistant and Dox-revertant cells were treated with 1 μM Doxorubicin for varying time periods. CE and NE were prepared. CE was assayed for IκBα and phospho-IκBα by Western blot (A). The blot was reprobed for anti-tubulin antibody. The p65 was detected from CE and NE by Western blot and blots were reprobed with anti-tubulin and anti CRM1 antibodies respectively (B). The level of p65 was examined by the immunofluorescence using anti-p65 antibody followed by goat-anti-rabbit IgG-Alexa-Flour antibody. Cells were then mounted on a slide and visualized under fluorescence microscope (C). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

INHIBITION OF NF-κB SENSITIZES THE DOX-RESISTANT CELLS TO APOPTOSIS

NF-κB was downregulated in Dox-resistant cells by multiple ways: (i) transfecting with *IκBα-DN* plasmid; (ii) treating with 5 μM BAY for 6 h; and (iii) treating with 100 nM P_3-25 for 6 h. Dox-resistant cells showed high basal activity of NF-κB. *IκBα-DN* transfected or BAY-treated, but not P_3-25-treated cells showed decrease in NF-κB DNA binding (Fig. 5A). Expression of NF-κB-dependent reporter gene, SEAP, was decreased in *IκBα-DN* transfected, BAY-treated, or P_3-25-treated Dox-resistant cells (Fig. 5B). Doxorubicin-treated cells did not alter NF-κB DNA binding or NF-κB-dependent SEAP activities. Doxorubicin induced cytotoxicity in these NF-κB down-regulated Dox-resistant cells in a concentration dependent manner (Fig. 5C). They showed 20–25% cell death due to NF-κB down-regulation. These results suggest that inhibition of NF-κB activity can induce cell death in Dox-resistant cells.

Low level of ROI was observed in NF-κB downregulated cells than untreated Dox-resistant cells. Doxorubicin induced ROI generation in NF-κB-inhibited cells were more than the high basal level of ROI in Dox-resistant cells (Fig. 5D). Doxorubicin alone did not induce lipid peroxidation in these cells. When NF-κB was downregulated, cells showed increase in lipid peroxidation by Doxorubicin treatment in a concentration dependent manner (Fig. 5F). The high basal expression of SOD1 was decreased in NF-κB-downregulated Dox-resistant cells (Fig. 5E1). These results support that the decrease in the amount of SOD1 by inhibiting NF-κB may induce ROI

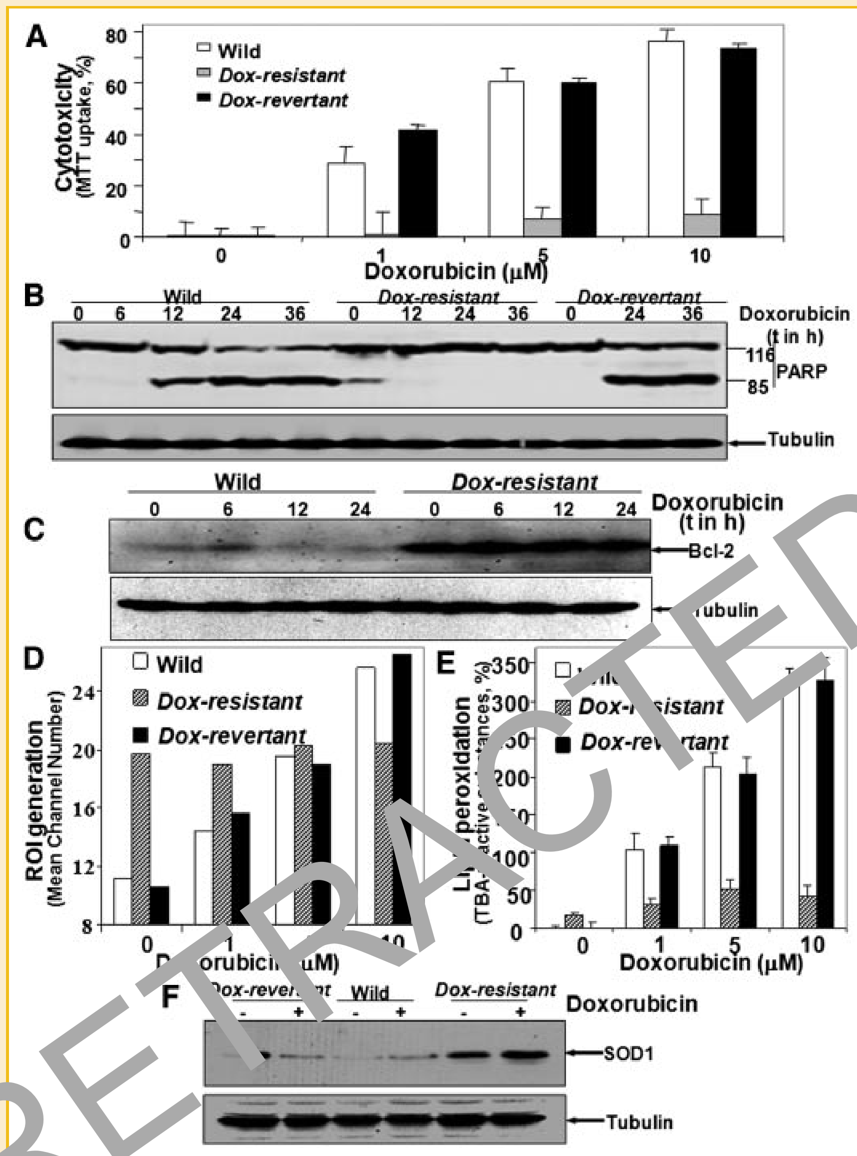

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Fig. 4. Effect of Doxorubicin on apoptosis in wild type, Dox-resistant and Dox-revertant MCF-7 cells. Cells were incubated with varying concentration of Doxorubicin for 72 h. Then cytotoxicity was assayed by MTT assay (A). Results are represented as inhibition of cell viability in percentage. Wild type, Dox-resistant, and Dox-revertant MCF-7 cells were treated with 1 μM Doxorubicin for different time periods. Then whole cell extracts were prepared and 100 μg proteins were analyzed by 7.5% SDS–PAGE to detect PARP by Western blot using anti-PARP Ab (B). 100 μg protein was analyzed by 10% SDS–PAGE and Bcl-2 was detected by Western blot using anti-Bcl-2 Ab (C). Both the blots were then reprobed for tubulin. Wild type, Dox-resistant, and Dox-revertant cells were treated with varying concentration of Doxorubicin for 6 h. ROI was then measured in flow cytometer (D). The results shown are representative of two independent experiments. Wild type, Dox-resistant and Dox-revertant cells were treated with varying concentration of Doxorubicin for 6 h. Cell pellets were extracted by three times freeze–thaw method with addition of 200 μl water and 500 μg protein sample was used to measure malondialdehyde by TBA-SDS buffer as described in Materials and Methods (E). The results shown are representative of two independent experiments. Untreated cells showed 0.524 ± 0.076 nmol of MDA equivalents/mg protein. The amount of SOD1 was detected from wild type, Dox-resistant, and Dox-reveretant MCF-7 cells after treatment with Doxorubicin (5 μM) for 6 h by Western blot (F). The same blot was reprobed with anti-tubulin antibody.

generation by Doxorubicin in Dox-resistant cells as shown in Figure 5D. As shown in Figure 4C, Dox-resistant cells showed high basal level of Bcl-2, an antiapoptotic protein. NF-κB down-regulation decreased the level of Bcl-2 and this level did not alter due to Doxorubicin treatment in these cells (Fig. 5E2). These data suggest that Dox-resistant cells become sensitive to Doxorubicin when NF-κB is downregulated.

INHIBITION OF NF-κB DECREASES BASAL EXPRESSION OF MDR AND TGASE IN DOX-RESISTANT CELLS

In wild type and Dox-revertant cells, the amount of Mdr was not detected but high basal expression of Mdr was detected in Dox-resistant cells. The amount of Mdr decreased in Dox-resistant cells when NF-κB was downregulated (by incubating cells with BAY, P_3-25, or transfecting with *IκBα-DN* construct) (Fig. 6A). The

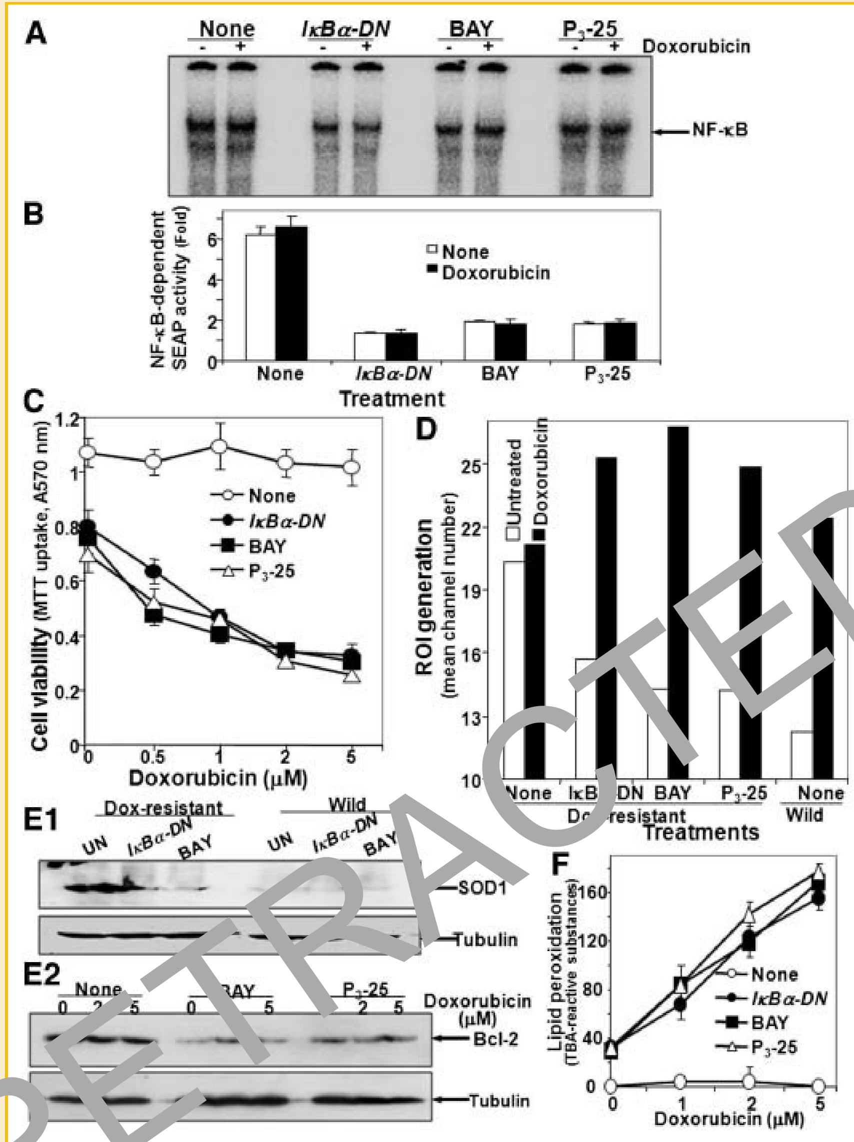

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Fig. 5. Effect of Doxorubicin on NF-κB downregulated Dox-resistant cells. Dox-resistant cells were pretreated with BAY (5 μM for 6 h); P_3-25 (100 nM for 6 h); or transiently co-transfected with *IκBα-DN* and *GFP* constructs. Cells, in these conditions were treated with 1 μM Doxorubicin for 12 h. NF-κB DNA-binding activity was assayed from nuclear extracts (A). Dox-resistant cells were transfected with *NF-κB-SEAP* alone or together with *NF-κB-SEAP* and *IκBα-DN* constructs for 3 h and cultured for 12 h. *NF-κB-SEAP* transfected cells were treated with BAY or P_3-25 last 6 h while culturing. All these cells were treated with 1 μM Doxorubicin for 12 h. SEAP activity was assayed from culture supernatant and indicated as fold activation (B). Dox-resistant cells were pretreated with BAY or P_3-25, or transiently transfected with *IκBα-DN*. Cells, in these conditions were treated with different concentrations of Doxorubicin for 72 h. Cell viability was assayed by MTT dye uptake and indicated in mean absorbance from triplicate samples (C). ROI generation was measured in Dox-resistant cells (pretreated with BAY or P_3-25, or transiently transfected with *IκBα-DN*) and wild type cells, treated with 1 μM Doxorubicin for 6 h using Dihydrorhodamine fluorescent dye in FACS (D). Dox-resistant and wild type cells (pretreated with BAY or transiently transfected with *IκBα-DN*) were cultured for 12 h and the SOD1 was measured by Western blot (E1). Dox-resistant cells (pretreated with BAY or P_3-25 for 6 h), treated with different concentrations of Doxorubicin for 12 h. Cell extracts were prepared and 100 μg proteins were used to detect Bcl-2 by Western blot (E2). The same blots were reprobed for tubulin. Dox-resistant cells (pretreated with BAY or P_3-25, or transiently transfected with *IκBα-DN*), treated with different concentrations of Doxorubicin for 12 h. Cell extracts were prepared by the freeze–thaw method and 500 μg proteins were used for TBA-SDS reactive MDA assay as described in Materials and Methods (F). The results are indicated as MDA production in percentage above control.

high basal expression of Mdr was decreased kinetically by BAY-treated Dox-resistant cells as shown by Western blot (Fig. 6B) and immunofluorescence (Fig. 6C). In wild type and Dox-revertant cells, the amount of TGase was not observed but high basal expression was detected in Dox-resistant cells. The amount of transglutaminase (TGase) decreased in Dox-resistant cells when NF-κB was downregulated (Fig. 6D). The high basal amount of TGase was decreased in Dox-resistant cells kinetically by BAY-treatment (Fig. 6E). These results suggest that Dox-resistant cells show high basal expression of Mdr and TGase and by inhibiting NF-κB these expressions are reduced.

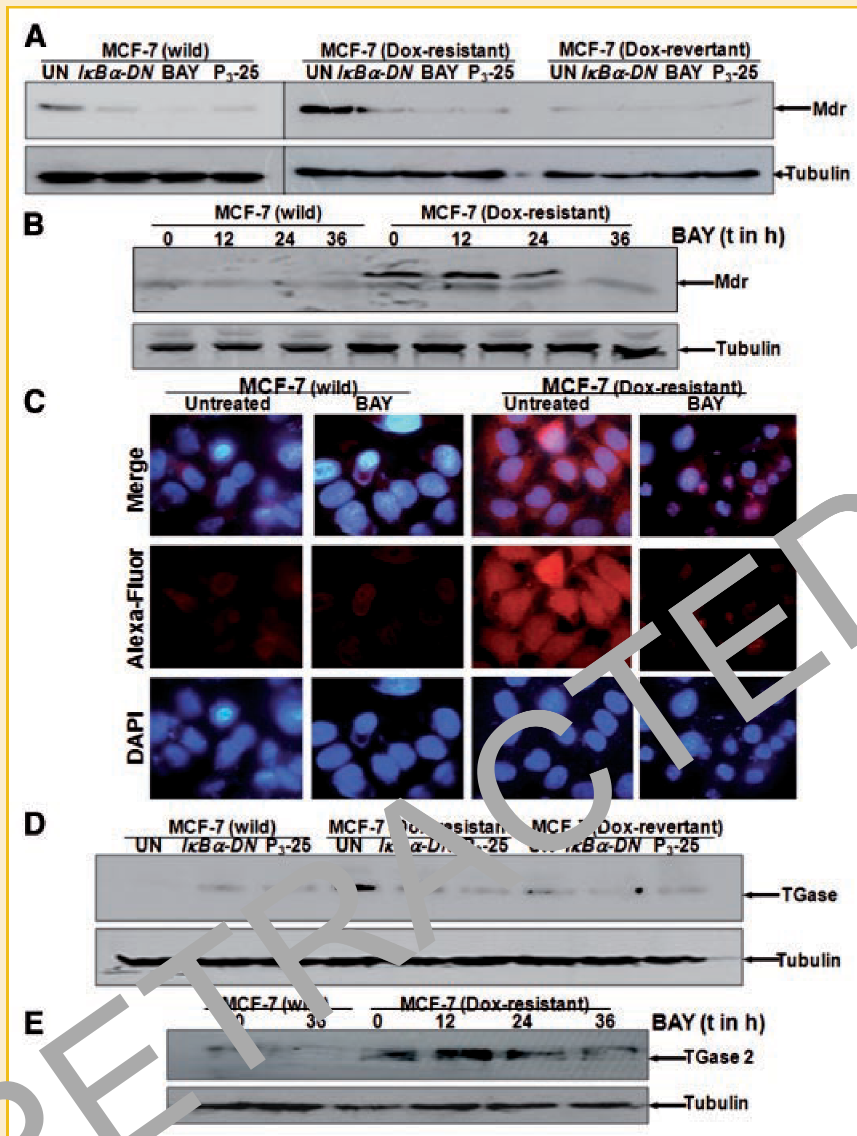

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Fig. 6. Effect of BAY and P_3-25 on expression of Mdr and TGase in Dox-resistant cells. MCF-7 (wild type, Dox-resistant, and Dox-revertant) cells were either treated with BAY (5 μM), P_3-25 (100 nM), or transfected with *IκBα-DN* construct for 36 h. The amount of Mdr was measured by Western blot (A). MCF-7 (wild type and Dox-resistant) cells were treated with BAY (5 μM) for different times and whole cell extracts (150 μg proteins) were used to detect Mdr by Western blot (B). The same blot was reprobed against tubulin. Cells were treated with BAY for 36 h and Mdr was detected using anti-Mdr antibody followed by goat-anti-rabbit IgG-Alexa-Flour antibody. Cells were then mounted on a slide and visualized through a fluorescence microscope (C). MCF-7 (wild type, Dox-resistant, and Dox-revertant) cells were either treated with P_3-25 (100 nM) or transfected with *IκBα-DN* construct for 36 h. The amount of TGase was measured by Western blot (D). Cells were treated with BAY for different times and amount of TGase 2 was detected by Western blot. The same blot was reprobed for tubulin (E). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

This study provides evidence in support of the concept that high levels of NF-κB and expression of genes dependent on it confer resistance to Doxorubicin in breast tumor cell lines. Doxorubicin induced NF-κB transiently may in some way be responsible for some of the cells becoming resistant to cell death. In the laboratory conditions, a few populations of MCF-7 breast tumor cell lines attain resistance, which later on grow happily in presence of Doxorubicin even at higher concentration (10 μM) [Devarajan et al., 2002]. How these cells attain resistance still remains elusive. While culturing these resistant cells in absence of Doxorubicin for 6 months, a few populations showed cell death against Doxorubicin. These populations were isolated and considered as Dox-revertant cells. These cells showed low expression of NF-κB, SOD1, Mdr, and TGase. Considering Doxorubicin's usefulness in treating different tumors alone or in combination generating resistance against it caused much distress to these patients. Understanding the mechanism of this resistance against Doxorubicin is of value. How Doxorubicin induces cell death has not yet been fully established. To address

these issues, we used Doxorubicin resistant and revertant MCF-7 cells to compare the signaling mechanism in these cells targeting for better and effective treatment of tumor.

We found that NF-κB DNA binding activity was initially induced and later on decreased in Doxorubicin treated wild type and Dox-revertant cells. Dox-resistant cells, however, showed high basal NF-κB activity. Doxorubicin induced NF-κB DNA binding activity till 2 h of treatment on wild type or Dox-revertant cells and then this level decreased significantly with increasing time. Though we did not notice any cytolysis till 72 h of Doxorubicin treatment as detected by LDH assay (data not shown) it induced cell death 24 h onwards. So, decrease in NF-κB DNA binding is not due to reduction in the cell number. It is also observed that the Oct1 DNA binding activity did not alter at any time of Doxorubicin treatment, though decrease in NF-κB binding. These data support the genuine downregulation of NF-κB activity in wild type or Dox-revertant cells. High basal activity of NF-κB DNA binding was detected in nuclear extracts of Dox-resistant cells. Though 4 h onwards NF-κB DNA binding activity was decreased, but 6 h of incubation with different concentrations of Doxorubicin showed gradual increase in reporter gene expression. As SEAP is secreted from cells into the culture supernatant, the decrease in NF-κB DNA binding at later time points with Doxorubicin was not reflected in the results. The concentrations of Cox-2 and ICAM1 were reflected with the NF-κB activity in all these three types of cells as both are NF-κB-dependent genes. Doxorubicin-mediated NF-κB activation is correlated with phospho-IκBα and IκBα levels in wild type and Dox-revertant cells. In Dox-resistant cells, high basal levels of phospho-IκBα and IκBα were observed. IκBα is an NF-κB-dependent gene product. Constitutive activity of NF-κB might lead to expression of high IκBα and rapid turnover of NF-κB in the nucleus may be required for constitutive level of phospho-IκBα in Dox-resistant cells. The p65 subunit of NF-κB is known to be vital for its function by heterodimerizing with p50 subunit. Though p65 level is decreasing from cytoplasm with increasing time of Doxorubicin treatment, it is translocating to nucleus at early time of Doxorubicin treatment. Surprisingly, at later time points, nuclear p65 levels decreased without shuttling back to cytoplasm. How the amount of p65 decreases from cells due to Doxorubicin treatment in wild type and Dox-revertant cells needs to be studied. Involvement of ubiquitination for degradation of p65 needs to be addressed.

Doxorubicin induced cell death in wild type and Dox-revertant, but not Dox-resistant cells. Doxorubicin-mediated cell death was observed at 12 h onwards with Doxorubicin treatment which correlates with downregulation of NF-κB. Dox-resistant cells with constitutive active NF-κB are fully resistant to cell death even at 10 μM concentration of Doxorubicin, indicating its possible role in proliferating tumor cells and also aiding them to abate apoptosis. Bcl-2, an NF-κB-dependent gene product [Christman et al., 1998; Sarkar et al., 2004], also transiently increased by Doxorubicin treatment in wild type cells and constitutive expression was shown in Dox-resistant cells. In Dox-resistant cells, Doxorubicin was unable to induce lipid peroxidation and ROI generation, further indicating that the cells are not following the apoptotic pathway. Dox-resistant cells showed a constitutive level of ROI, which did not alter due to Doxorubicin treatment. NF-κB activation is redox-sensitive [Anddrassy et al., 2002; Shimokawa, 2004; Fischer et al., 2006]. Certain amounts of ROI are required for NF-κB activation. The basal amounts of ROI, detected in Dox-resistant cells, may be threshold to attain resistance against Doxorubicin, which is also important to keep its high basal NF-κB activity. Induced generation of ROI above this level might have deleterious effect such as lipid peroxidation and other oxidative stress that leads to apoptosis. We did not see any lipid peroxidation in Dox-resistant cells. Our data suggest that the basal expression of SOD1 in Dox-resistant cells might have role in inhibiting Doxorubicin-induced ROI generation in these cells. High basal expression of NF-κB induced the expression of SOD1 as downregulation of NF-κB decreased the expression of SOD1. As SOD1 expression is dependent of NF-κB, the basal SOD1 expression was shown in high basal NF-κB containing cells like Dox-resistant cells. Though several reports suggest that high SOD can inhibit redox-sensitive NF-κB activation, how Dox-resistant cells have both high NF-κB and SOD1 needs to further study. The high level of ROI in Dox-resistant cells might induce other antioxidant enzymes such as peroxidase, gamma glutamyl synthetase, etc. [Manna et al., 1999; Sarkar et al., 2004] which neutralize the toxic effect of high ROI level.

As NF-κB is an ideal target for anticancer drug development, switching off aberrant NF-κB activity could have a major therapeutic benefit. Hence we investigated whether inhibition of NF-κB can sensitize the Dox-resistant cells to apoptosis. In these NF-κB downregulated cells Doxorubicin induced apoptosis, increased ROI generation over its basal level, induced lipid peroxidation, and decreased Bcl-2 and SOD1 expressions, further indicating that the cells are following the apoptotic pathway by Doxorubicin treatment. Inhibition of NF-κB decreases high basal expression of Mdr and TGase. Mdr is a NF-κB-dependent gene product [Deng et al., 2001; Kuo et al., 2002]. Possibly, multi-drug resistant proteins are inhibiting Doxorubicin's effect in the drug-resistant cells and decreasing these levels, cells are becoming sensitive again to Doxorubicin. Downregulation of p65, an important subunit of NF-κB in wild type or Dox-revertant cells, potentiates cell death, which is an important observation. Dox-resistant cells showed constitutive activation of NF-κB whose inhibition resulted in sensitization to cell death by the causal agent, Doxorubicin.

As NF-κB is currently being used as a target for cancer therapy, it is important to understand the possible interplay of NF-κB and drug resistance. Overall, our results suggest that Doxorubicin-resistant cells shows high basal NF-κB activity and bypasses apoptosis. The high NF-κB activity confers resistance to Doxorubicin in Dox-resistant cells and down regulation of NF-κB in these cells potentiates apoptosis. This study will help to understand Doxorubicin mediated chemoresistance and further designing for combination chemotherapy in intervene several tumors involving Doxorubicin.

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