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Evidence that activation of nuclear factor-κB is essential for the cytotoxic effects of doxorubicin and its analogues

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Abstract

Several reports within the last 5 years have suggested that nuclear factor (NF)-κB activation suppresses apoptosis through expression of anti-apoptotic genes. In the present report, we provide evidence from four independent lines that NF-κB activation is required for the cytotoxic effects of doxorubicin. We used doxorubicin and its structural analogues WP631 and WP744, to demonstrate that anthracyclines activate NF-κB, and this activation is essential for apoptosis in myeloid (KBM-5) and lymphoid (Jurkat) cells. All three anthracyclines had cytotoxic effects against KBM-5 cells; analogue WP744, was most potent, with an IC_{50} of 0.5 μM, and doxorubicin was least active, with an IC_{50} of 2 μM. We observed maximum NF-κB activation at 1 μM with WP744 and at 50 μM with doxorubicin and WP631, and this activation correlated with the IκBα degradation. Because the anthracycline analogue (WP744), most active as a cytotoxic agent, was also most active in inducing NF-κB activation and the latter preceded the cytotoxic effects, suggests that NF-κB activation may mediate cytotoxicity. Second, receptor-interacting protein-deficient cells, which did not respond to doxorubicin-induced NF-κB activation, were also protected from the cytotoxic effects of all the three anthracyclines. Third, suppression of NF-κB activation by pyrrolidine dithiocarbamate, also suppressed the cytotoxic effects of anthracyclines. Fourth, suppression of NF-κB activation by NEMO-binding domain peptide, also suppressed the cytotoxic effects of the drug. Overall our results clearly demonstrate that NF-κB activation and IκBα degradation are early events activated by doxorubicin and its analogues and that they play a critical pro-apoptotic role.

Keywords: NF-κB; Cytotoxicity; Doxorubicin; Transcription factor; RIP; NBD peptide

1. Introduction

Doxorubicin, an anthracycline antibiotic, is widely used for the treatment of a wide variety of different cancers [1]. Anthracyclines mediate their anticancer effect in part by targeting topoisomerase II, which leads to DNA damage. In comparison, antimetabolites such as 5-fluorouracil or methotrexate prevent DNA synthesis, taxanes (e.g. paclitaxel) stabilize the microtubules, and Vinca alkaloids (such as vinblastine and vincristine) induce depolymerization of microtubules [2,3]. Doxorubicin can induce apoptosis through a wide variety of mechanisms including production of reactive oxygen species, alkylation of cellular macromolecules, DNA intercalation and cross-linking, lipid peroxidation, cell membrane damage, ceramide production [4], and p53 induction [5].

Besides inducing apoptosis [6], one of the characteristic features of all the above-described chemotherapeutic agents, including doxorubicin, is that they all activate NF-κB [7–9]. NF-κB is a ubiquitous nuclear transcription factor that plays a major regulatory role in apoptosis and inflammation [10,11]. It resides in inactive state in cytoplasm as a heterotrimer consisting of p50, p65, and inhibitory subunit of NF-κB (IκBα) subunits [12]. The p50–p65 heterodimer is retained in the cytoplasm by the inhibitory subunit IκBα. On activation of the complex, IκBα sequentially undergoes phosphorylation, ubiquitination, and degradation, thus releasing the p50–p65 hetero-

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Abbreviations: TNFR1, TNF receptor 1; PIS, preimmune serum; IκBα, inhibitory subunit of NF-κB; RIP, receptor-interacting protein; MIS, mullerian-inhibiting substance; PDTC, pyrrolidine dithiocarbamate; NEMO, NF-κB essential modulator; NBD, NEMO-binding domain peptide.

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dimer for translocation to the nucleus [13]. Treatment of cells with various inflammatory and oxidative stress stimuli activates the phosphorylation, thus leading to the degradation of IκBα and activation of the transcription factor [14].

Work within last 5 years has shown that activation of NF-κB can suppress apoptosis induction by cytokines and chemotherapeutic agents [15–17]. A large number of NF-κB-regulated genes that can abrogate apoptosis including TRAF1, TRAF2, cIAP1, cIAP2, XIAP, COX2, and survivin have been identified [18,19]. Paradoxically, however, most agents that activate NF-κB, also induce apoptosis. Thus, the activation of NF-κB has been shown to mediate chemoresistance [20–23]. There are some other reports that suggest that NF-κB activation is either unrelated to apoptosis [24,25] or mediates apoptosis [26–29]. For instance mullerian-inhibiting substance (MIS) has been shown to activate NF-κB, and this activation was shown to be essential for MIS-induced anti-proliferative effects against breast cancer cells [27,28]. Similarily, taxol has been shown to induce apoptosis in human breast, ovarian, and epidermoid cancer cells, and this is mediated through the upregulation of IκBα kinase-mediated NF-κB activation [29]. Doxorubicin has been shown to activate both NF-κB [7,8,30] and apoptosis [6,31]. Whether NF-κB activation is required for doxorubicin-induced apoptosis is not fully understood [32–34]. Therefore, in the present report we used several approaches to investigate the role of NF-κB in doxorubicin-induced apoptosis. Our results demonstrate that the structural analogues of doxorubicin that are more active in inducing NF-κB activation are also more potent in inducing apoptosis. Additionally, cells in which doxorubicin and its analogues failed to activate NF-κB also did not undergo apoptosis. Overall our results suggest that NF-κB activation plays a proapoptotic role in doxorubicin-mediated cellular responses.

2. Materials and methods

2.1. Materials

Doxorubicin and its structural analogues WP631 and WP744 (see Fig. 1) were synthesized in our laboratory as described [35,36]. They were prepared in distilled sterile water as 1 mM solutions and then further diluted in the cell culture medium. Iscove's Modified Dulbecco's medium (GIBCO), RPMI 1640 medium, FBS, 0.4% trypan blue stain, penicillin, and streptomycin were obtained from Life Technologies. BSA was obtained from Sigma. Bacteria-derived human rTNF, purified to homogeneity with a specific activity of 5×10^7 unit/mg, was kindly provided by Genentech. Antibodies against IκBα, p50, p65, cyclin D1 and β-actin were purchased from Santa Cruz Biotechnology. Antibodies against phospho-IκBα was purchased from New England Bio Labs, Inc. Pyrrolidine dithiocarbamate (PDTC) was obtained from Sigma. [^{32}P]ATP was purchased from ICN Pharmaceuticals, and polynucleotide kinase kit from New England Bio Labs, Inc. Cell-permeable NF-κB essential modulator (NEMO; also called IKKγ)-binding domain peptide (NBD), NH_2-DRQIKIWFQNRRMKWKK-*TALDWSWLQTE*-$CONH_2$, and control

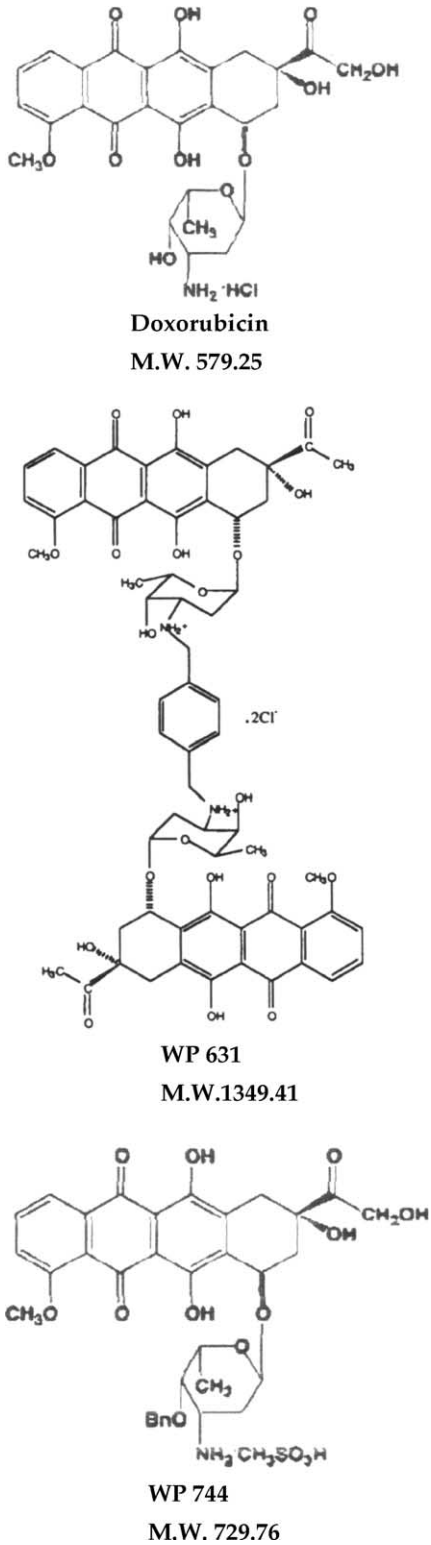

Fig. 1. Structure of doxorubicin, WP631, and WP744.

peptide NEMO-C, NH_2-DRQIKIWFQNRRMKWKK-$CONH_2$ [37] were kind gifts from Imgenex.

2.2. Cell lines

Human myeloid KBM-5 cells (kindly supplied by Dr. Nicholas Donato from our department), human T cell leukemia Jurkat cells (kindly provided by Dr. Ken Fujise of the University of Texas Health Science Center) and human non-small cell lung carcinoma cells (H1299) were originally obtained from the American Type Culture Collection. The receptor-interacting protein (RIP)-deficient clone of Jurkat cells was kindly provided by Dr. Brian Seed (Harvard Medical School). It has been shown that RIP-deficient Jurkat cells are unable to activate TNF-mediated NF-κB-dependent reporter gene expression [38,39]. KBM-5 cells were cultured in Iscove's Modified Dulbecco's medium with 15% FBS. Jurkat and H1299 cells were maintained in RPMI 1640 with 10% FBS. RIP-deleted Jurkat cells were maintained in Iscove's Modified Dulbecco's medium with 10% FBS. Both of the cell lines were cultured in the presence of penicillin (100 unit/mL) and streptomycin (100 μg/mL) and were mycoplasma free as tested by Hoechst staining and custom PCR for mycoplasma DNA.

2.3. NF-κB activation assay

To determine NF-κB activation, we carried out EMSA essentially as described [40]. Briefly, nuclear extracts prepared from drug-treated cells (2×10^6 mL^{-1}) were incubated with ^{32}P end-labeled 45-mer double-stranded NF-κB oligonucleotide (4 μg protein with 16 fmol DNA) from the human immunodeficiency virus long terminal repeat, 5′-TTGTTACAA**GGGACTTTC**CGCTG **GGGACTTTC** CAGGGA GGCGT GG-3′ (boldface indicates NF-κB binding sites) for 15 min at 37°, and the DNA–protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5′-TTGTTACAA**CTCACTTTC** CGCTG**GTCACTTTC**CAGGGAGG CGTGG-3′, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from drug-treated cells were incubated with antibodies against either p50 or p65 of NF-κB for 30 min at room temperature before the complex was analyzed by EMSA. Antibodies against cyclin D1 and PIS were included as negative controls. The dried gels were visualized, and radioactive bands quantitated by a PhosphorImager (Molecular Dynamics) using Imagequant software.

2.4. Degradation of IκBα

To determine the levels of IκBα, postnuclear (cytoplasmic) extracts were prepared [9] from TNF-treated cells and resolved on 10% SDS–polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to nitrocellulose filters, probed with rabbit polyclonal antibodies against IκBα, and detected by chemiluminescence (ECL). The bands obtained were quantitated using a Personal Densitometer Scan v. 1.30 with Imagequant software version 3.3 (Molecular Dynamics).

2.5. Cytotoxicity assays

The cytotoxic effect were measured by three different methods. For the trypan blue dye-exclusion method 5×10^3 cells were plated in 0.09 mL of medium in 96-well flat-bottom plates. Then 0.01 mL of different concentrations of doxorubicin, WP631, or WP744 was added to make the final concentration. After 24 hr of incubation at 37°, trypan blue was added and viable cells counted by hemocytomter.

In second method, the thymidine incorporation method [41], cells were plated at 5000 per well in 0.09 mL medium in 96-well flat-bottom Falcon plates. Different concentrations of doxorubicin, WP631, or WP744 were added in an additional 0.01 mL of medium, and the plates were incubated at 37° for 24 hr. During the last 6 hr before harvesting, [^{3}H]thymidine (5 mCi/mmol) was added to each well (0.5 μCi per well), and then cells were harvested with the aid of a Filter 96 harvester (Packard Instruments). Radioactivity bound to the filter was measured with automated direct beta counter (Matrix 9600, Packard Instruments Company).

The third method involved determination of cytotoxicity by the modified tetrazolium salt 3-(4-5-dimethylthiozol-2-yl)2-5-diphenyl-tetrazolium bromide (MTT) assay [42]. Briefly, cells (5000 cells per well) were incubated in the presence or absence of the indicated test sample in a final volume of 0.1 mL for 72 hr at 37°. Thereafter, 0.025 mL of MTT solution (5 mg/mL in PBS) was added to each well. After a 2-hr incubation at 37°, 0.1 mL of the extraction buffer (20% SDS, 50% dimethyl formamide) was added. After an overnight incubation at 37°, the optical densities at 570 nm were measured using a 96-well multiscanner autoreader (Dynatech MR5000), with the extraction buffer as blank. The percent cytotoxicity was determined as follows: $(1 - (A_{570}\text{ of test sample})/(A_{570}\text{ of control sample}) \times 100)$.

3. Results

In the present report, we investigated the role of NF-κB in doxorubicin-induced apoptosis by using four independent approaches. The first approach involved the use of doxorubicin and novel structural analogues WP774 and WP631 (see Fig. 1). The second approach involved the use of PDTC, which is known to suppress NF-κB activation. The third approach involved the use of a

RIP-deficient cell line that is known not to activate NF-κB in response to TNF. The fourth approach involved the use of NF-κB-specific inhibitory peptides [37]. The dose and time at which doxorubicin-induced NF-κB activation had no effect on the morphology of the cells (data not shown).

3.1. WP744 is more active than WP631 or doxorubicin in suppressing growth of myeloid cells

The effect of doxorubicin and its structural analogues on the cytotoxic effects of KBM-5 cells was examined. As shown in Fig. 2A, doxorubicin and its analogues inhibited

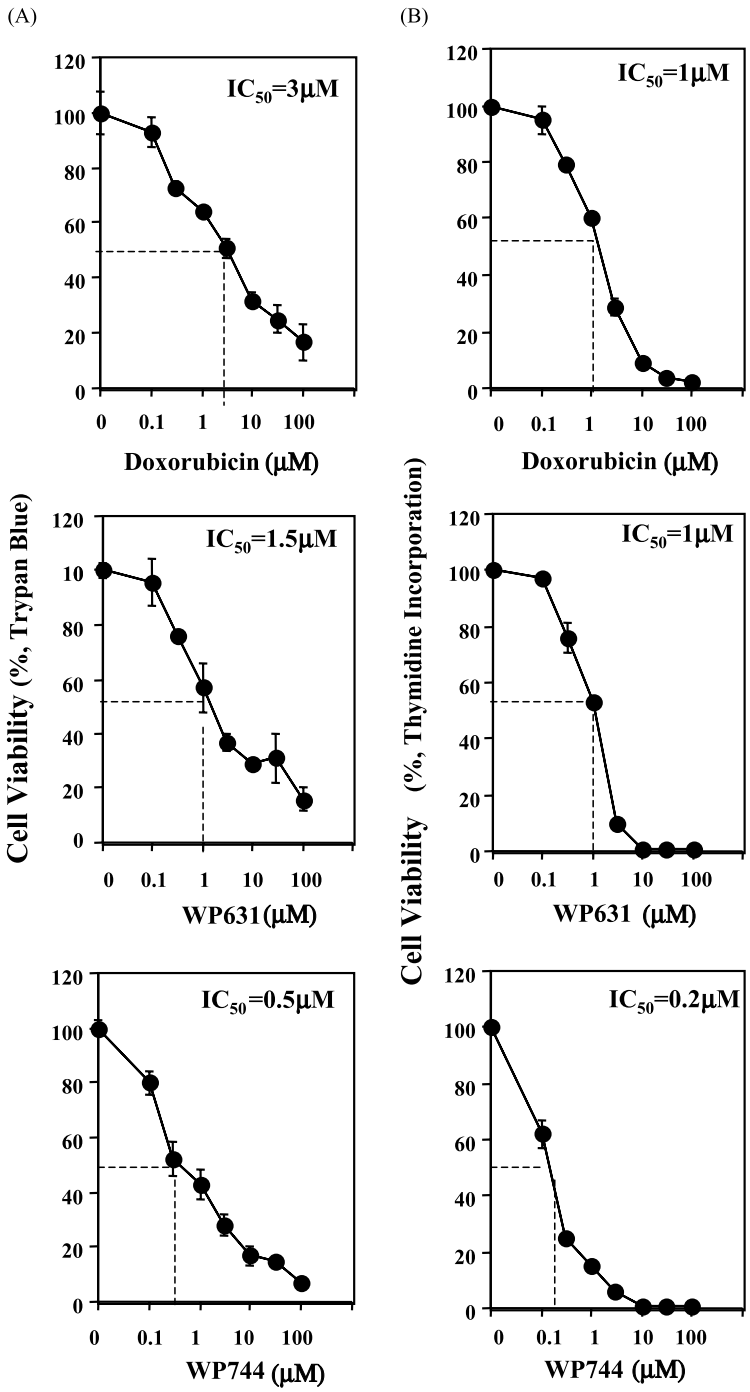

Fig. 2. (A) Dose-dependent effect of doxorubicin, WP631, and WP744 on the viability of KBM-5 cells, 5×10^3 cells (0.1 mL) in 96-well plates were incubated with doxorubicin, WP631, or WP744 at 37° for 24 hr, and the relative cell viability was determined by staining with trypan blue. The values are mean ± SD and are representative of three independent experiments with similar results. (B) Dose-dependent effect of doxorubicin, WP631, and WP744 on thymidine incorporation by KBM-5 cells 5×10^3 cells (0.1 mL) in 96-well plates were incubated with doxorubicin, WP631, and WP744 at 37° for 24 hr, and thymidine incorporation was determined as described in Section 2. The values are mean ± SD and are representative of three independent experiments with similar results.

cell growth in a dose-dependent manner. The analogue WP744 was most active, with an IC_{50} of 0.5 μM and doxorubicin was least active, with an IC_{50} of around 3 μM.

3.2. WP744 is more active than WP631 or doxorubicin in inhibiting DNA synthesis of myeloid cells

The effect of doxorubicin and its structural analogues on the proliferation of KBM-5 cells was determined by thymidine incorporation for 8 hr. As shown in Fig. 2B, doxorubicin and its analogues inhibited the cells' thymidine incorporation in a dose-dependent manner. The analogue WP744 was most active, with an IC_{50} of around 0.2 μM, and doxorubicin was least active, with IC_{50} of around 1 μM.

3.3. WP744 is more active than WP631 or doxorubicin in activating NF-κB in myeloid cells

Doxorubicin and its analogues activated NF-κB in a dose-dependent manner. WP744 was most active, reaching maximum activation at a 1 μM concentration, and doxorubicin least active, reaching maximum at 50 μM (Fig. 3A).

The effect of doxorubicin and its structural analogues on the time-course of NF-κB activation was also determined.

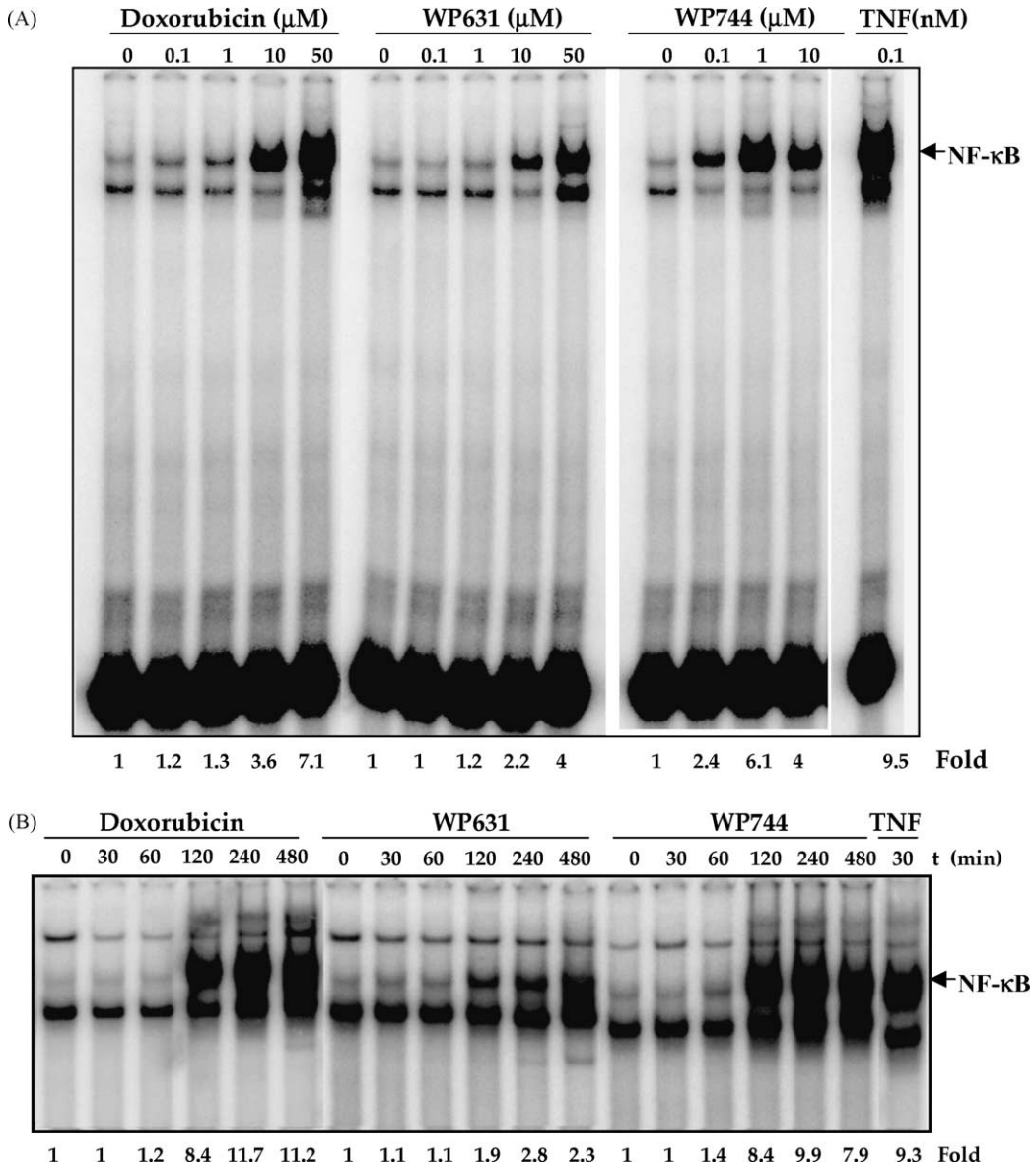

Fig. 3. (A) Dose-dependent effect of doxorubicin, WP631, and WP744 on the activation of NF-κB. KBM-5 cells (2×10^6 mL^{-1}) were preincubated at 37° for 4 hr with different concentrations of doxorubicin, WP631, and WP744. After treatment, nuclear extracts were prepared and then assayed for NF-κB, as described in Section 2. (B) Time-dependent effect of doxorubicin, WP631, and WP744 on the activation of NF-κB. KBM-5 cells (2×10^6 mL^{-1}) were preincubated at 37° for 4 hr, and then incubated for different times with 50 μM doxorubicin or WP631 or 1 μM WP744. After treatment, nuclear extracts were prepared and then assayed for NF-κB, as described in Section 2.

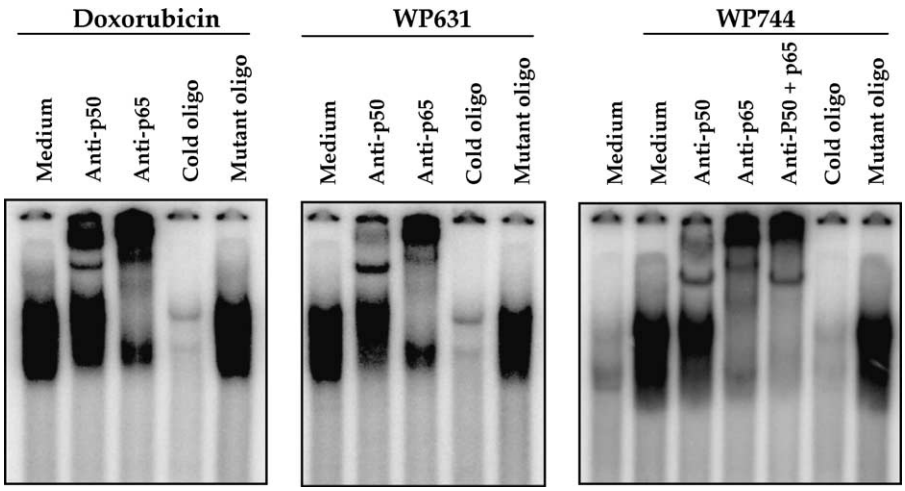

Fig. 4. Supershift and specificity of NF-κB activation. Nuclear extracts were prepared from untreated and doxorubicin (50 μM), WP631 (50 μM), and WP744 (1 μM) treated KBM-5 cells (2×10^6 mL^{-1}), incubated for 15 min with the indicated antibodies and unlabeled NF-κB, and then assayed for NF-κB, as described in Section 2.

As shown in Fig. 3B, NF-κB was activated in a time-dependent manner. The analogue WP744 at 1 μM and doxorubicin at 50 μM activated NF-κB at 120 min, whereas WP 631 at 50 μM activated NF-κB at 240–480 min.

Since NF-κB is a family of proteins, various combinations of Rel/NF-κB protein can constitute an active NF-κB heterodimer that binds to a specific sequence in DNA [43]. To show that the retarded band visualized by EMSA in doxorubicin-, WP744-, and WP631-treated cells was indeed NF-κB, we incubated nuclear extracts from treated cells with antibody to either the p50 (NF-κB1) or the p65 (RelA) subunit of NF-κB. Both shifted the band to a higher molecular mass (Fig. 4), thus suggesting that the doxorubicin-, WP744-, and WP631-activated complex consisted of p50 and p65 subunits. Neither PIS nor the irrelevant antibody as anti-cyclin D1 had any effect. Excess unlabeled NF-κB (100-fold) caused complete disappearance of the band.

3.4. WP744 is more active than WP631 or doxorubicin in degradation of IκBα

The translocation of NF-κB to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκBα [13]. To determine whether doxorubcin-induced NF-κB activation was due to IκBα degradation, we treated the cells with doxorubicin and its analogues for different times; we then examined the cells for IκBα by Western blot. TNF was used as a control. As shown in Fig. 5, TNF-induced IκBα degradation could be seen as early as 5 min and reached maximum at 10 min, and resynthesis of IκBα could be noted at 60 min. In contrast, doxorubicin and its structural analogues induced IκBα degradation that first appeared at 120 min. The analogue WP744 was most active, with degradation occurring at 1 μM, and doxorubicin was least active with degradation occurring at 50 μM. No resynthesis of IκBα could be seen up to 480 min with either doxorubicin or its structural analogues.

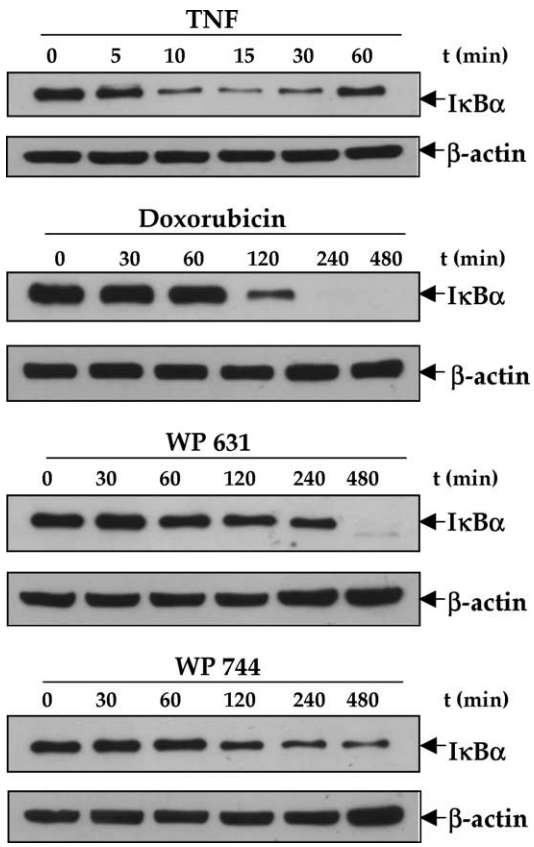

Fig. 5. Effect of TNF, doxorubicin, WP631, and WP744, on degradation of IκBα. KBM-5 cells (2×10^6 mL^{-1}) were pretreated for the indicated times with 0.1 nM TNF, 50 μM doxorubicin or WP631, or 1 μM WP744 at 37°, and then assayed for IκBα in cytosolic fraction by Western blot analysis as described in Section 2.

3.5. Cells defective in NF-κB activation are protected from cytotoxic effects of doxorubicin and its analogue

To further confirm the role of NF-κB in the cytotoxic effects of doxorubicin, we used cells defective in NF-κB activation in response to the anthracyclines. That distinct signal transduction pathways could mediate NF-κB induction in different cell types has been demonstrated [44]. Therefore, we examined the relationship of NF-κB to apoptosis in a lymphoid cell line. The deletion of RIP protein in Jurkat cells has been shown to suppress TNF-induced NF-κB reporter activity [39]. The effect of RIP on doxorubicin-induced NF-κB activation, however, is not known. We found that the doxorubicin analogues activated NF-κB in the control Jurkat cells but failed to activate NF-κB in the RIP-deleted cells. This paralleled the TNF-induced NF-κB activation (Fig. 6A). These results for the first time demonstrate that RIP is also required for doxorubicin-induced NF-κB activation.

We also determined whether the abrogation of NF-κB affects doxorubicin-induced cytotoxicity in control and RIP-deleted Jurkat cells. As shown in Fig. 6B, doxorubicin and its analogues inhibited cell growth in a dose-dependent manner in control cells but had minimal effect on RIP-deleted Jurkat cells. These results thus suggest that NF-κB activated through RIP protein is essential for the cytotoxic effects of doxorubicin and its analogue.

3.6. Suppression of NF-κB by PDTC inhibits cytotoxicity induced by doxorubicin and its analogue

Our results till now indicate that the anthracycline analogue (WP744), most active as a cytotoxic agent, was also most active in inducing NF-κB activation and the latter preceded the cytotoxic effects. This may suggest that NF-κB activation mediates the doxorubicin-cytotoxicity. To further establish the relationship of NF-κB activation to doxorubicin-induced cytotoxicity, we examined the

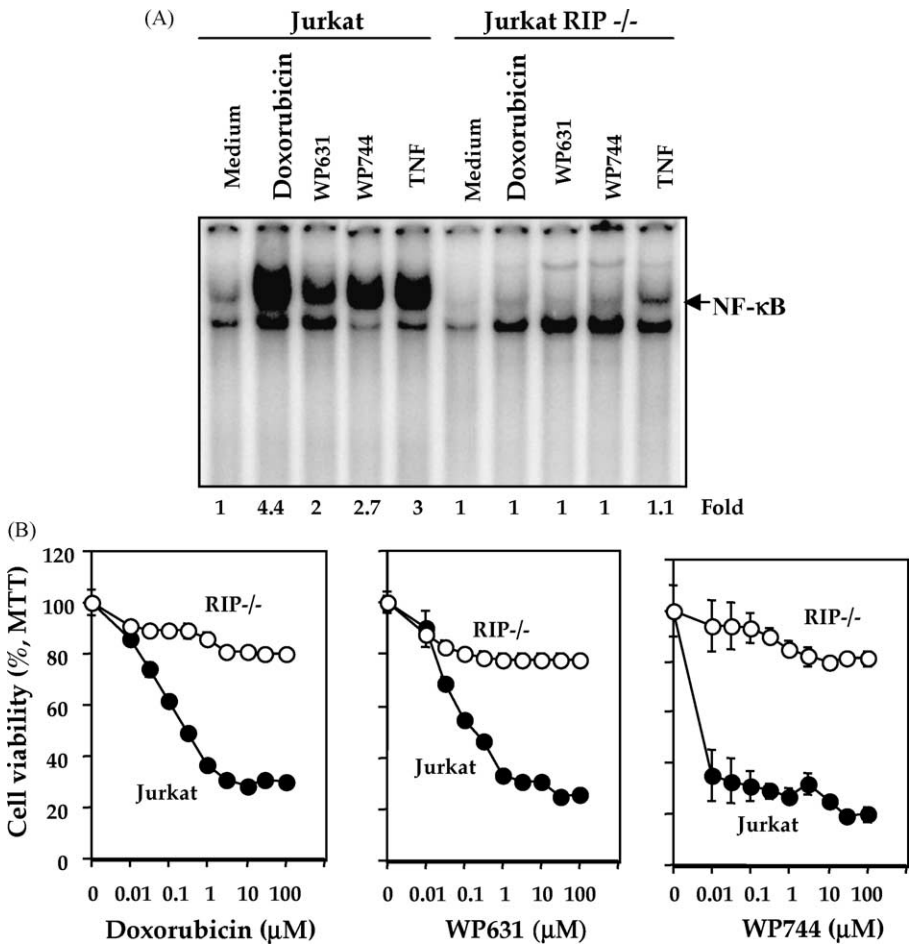

Fig. 6. (A) Effect of doxorubicin, WP631, WP744, and TNF on Jurkat and RIP-deleted Jurkat cells. Jurkat and RIP-deleted Jurkat cells (2×10^6 mL^{-1}) were preincubated at 37° for 4 hr with doxorubicin (50 μM), WP631 (50 μM) or WP744 (1 μM), or with 0.1 nM TNF for 30 min. After treatment, nuclear extracts were prepared and then assayed for NF-κB, as described in Section 2. (B) Dose-dependent anti-proliferative effects of doxorubicin, WP631, and WP744 against control and RIP-deleted Jurkat cells. 5×10^3 cells (0.1 mL) were incubated in 96-well plates with indicated concentrations of doxorubicin, WP631, or WP744 at 37° for 72 hr, and the relative cell viability was determined by the MTT method as described in Section 2. The values are mean ± SD and are representative of three independent experiments with similar results.

effect of PDTC, an antioxidant known to suppress NF-κB activation induced by a wide variety agents. We first examined the ability of PDTC to suppress doxorubicin-induced NF-κB activation. For this, KBM-5 cells were preincubated for 1 hr with or without PDTC, treated with doxorubicin and assayed for NF-κB. The results in Fig. 7A indicate that doxorubicin-induced NF-κB activation was significantly suppressed by PDTC. We next examined the effect of PDTC on the cytotoxic effects of doxorubicin, WP631, and WP744. For this cells were pretreated with or without PDTC for 1 hr, then exposed to the indicated concentrations of doxorubicin, WP631, or WP744 for 72 hr and then cell viability measured by the MTT method. The results in Fig. 7B indicate a complete suppression of cytotoxic effects of all the three anthracyclines.

3.7. Suppression of NF-κB by NBD peptide inhibits cytotoxicity induced by doxorubicin

IKK is composed of IKKα, IKKβ and IKKγ (also called NEMO). The amino-terminal α-helical region of NEMO has been shown to interact with the C-terminal segment of

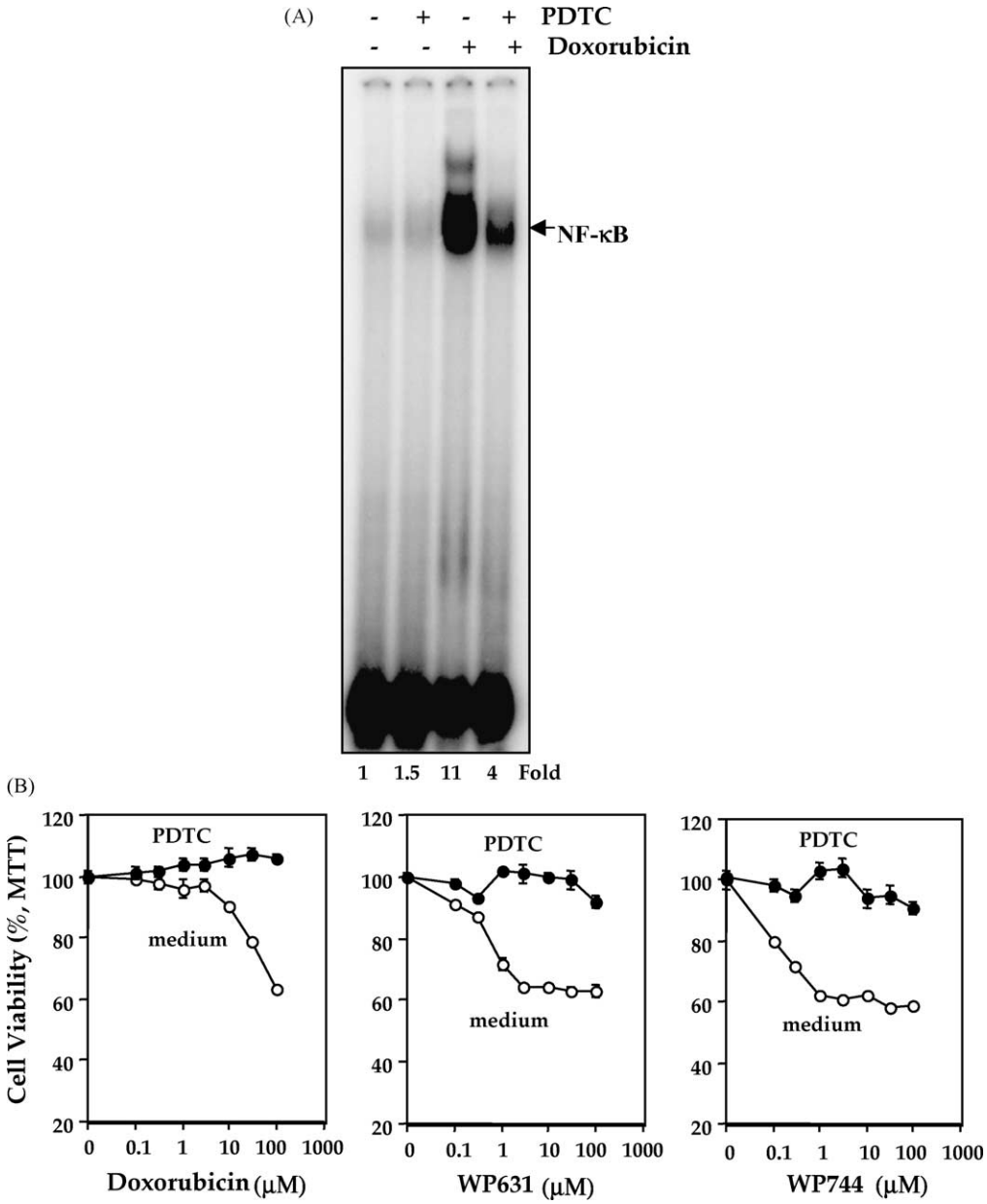

Fig. 7. (A) Effect of PDTC on doxorubicin-induced NF-κB activation on KBM-5 cells. KBM-5 cells (2×10^6 mL^{-1}) were preincubated at 37° for 1 hr with or without PDTC, and then treated with or without doxorubicin (50 μM) for 4 hr. After treatment, nuclear extracts were prepared and then assayed for NF-κB, as described in Section 2. (B) Effect of PDTC on cytotoxic effects of doxorubicin, WP631, and WP744 in KBM-5 cells. 5×10^3 cells (0.1 mL) were preincubated in 96-well plates with or without PDTC for 1 hr. And then added indicated concentrations of doxorubicin, WP631, or WP744 at 37° for 72 hr, and the relative cell viability was determined by the MTT method as described in Section 2. The values are mean ± SD and are representative of three independent experiments with similar results.

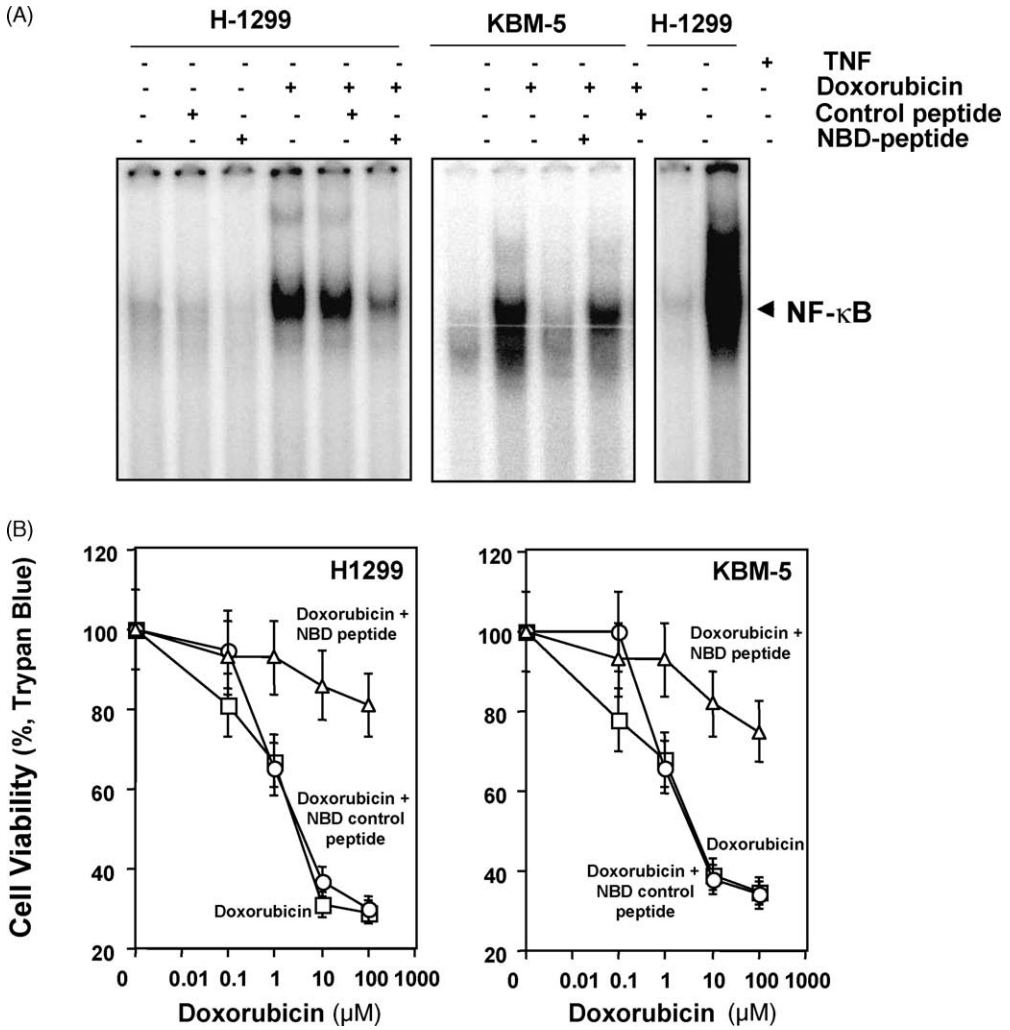

Fig. 8. (A) Effect of NBD peptide on doxorubicin-induced NF-κB activation in KBM-5 and H1299 cells. KBM-5 and H1299 cells (2×10^6 mL^{-1}) were preincubated at 37° for 4 hr with or without NBD peptide or control peptide, and then left untreated or treated with doxorubicin (50 μM) for 4 hr. After treatment, nuclear extracts were prepared and then assayed for NF-κB, as described in Section 2. TNF was used as a positive control. (B) Effect of NBD peptide on cytotoxic effects of doxorubicin in KBM-5 and H1299 cells. 5×10^3 cells (0.1 mL) were preincubated in 96-well plates with or without NBD peptide or control peptide for 4 hr. And then added indicated concentrations of doxorubicin for 24 hr, and the relative cell viability was determined by the trypan blue method as described in Section 2. The values are mean ± SD and are representative of three independent experiments with similar results.

IKKα and IKKβ. A small peptide from the C-terminus of IKKα and IKKβ, NEMO has been shown to block this interaction. To make it cell permeable, the NBD peptide was conjugated to a small sequence from the antennapedia homeodomain. This peptide has been shown to specifically suppress NF-κB activation. The peptide without the antennapedia homeodomain protein sequence, was used as a control.

To further confirm the proapoptotic role of NF-κB, we examined the ability of NF-κB essential modulator (NEMO) binding domain (NBD) peptide to abrogate doxorubicin-induced NF-κB activation in KBM-5 and H1299 cells. The results in Fig. 8A indicate that doxorubicin-induced NF-κB activation was significantly suppressed by NBD peptide in both myeloid and epithelial cell lines, however, the NBD control peptide had no effect on doxorubicin-induced NF-κB activation. Similarly, NBD peptide also abrogated the cytotoxic effects of doxorubicin in both KBM-5 and H1299 cells (Fig. 8B). Control peptide again has no effect on doxorubicin-induced cytotoxicity.

4. Discussion

Several mechanisms explaining how doxorubicin exhibits its cytotoxic effects have been described over the years. In the present report, we demonstrate by using four different approaches that NF-κB activation is required for doxorubicin-induced cytotoxicity. The first approach involved the use of doxorubicin and its structural analogues WP631 and WP744. Our results indicate that all three anthracyclines activated NF-κB, induced IκBα degradation, and were cytotoxic against KBM-5 cells, in a dose- and time-dependent manner. The anthracycline analogue,

WP744, most active in inducing NF-κB activation and IκBα degradation was also maximally cytotoxic, suggesting that NF-κB activation may mediate cytotoxicity. Furthermore, NF-κB activation and IκBα degradation preceded the cytotoxic effects of doxorubicin. The second approach involved the use of cells deficient in RIP, which is required for NF-κB activation. We found that all three anthracyclines activated NF-κB in control Jurkat cells but not in RIP-deleted Jurkat cells. Similarily, all three anthracyclines were cytotoxic to the control cells, but RIP-deleted Jurkat cells were completely resistant. The third approach involved the use of a pharmacological inhibitor, PDTC, known to suppress NF-κB activation. Suppression of NF-κB by PDTC suppressed the cytotoxic effects of doxorubicin. The fourth approach involved the use of a specific NBD peptide that is known to inhibit NF-κB through inhibition of IKK [37]. Suppression of NF-κB by NBD peptide suppressed the cytotoxic effects of doxorubicin. These results again suggest that NF-κB activation is essential for apoptosis induced by doxorubicin and its analogues.

Our results indicate that doxorubicin and its structural analogues can activate NF-κB and that the kinetics of activation is slower than that by TNF. That doxorubicin can activate NF-κB is consistent with previous reports [7,8,30]. How doxorubicin activates NF-κB, however, is poorly understood. We found that doxorubicin-induced NF-κB activation requires the presence of RIP, as cells with deleted RIP failed to activate NF-κB. This is the first report to our knowledge to indicate that RIP is needed for NF-κB activation by any chemotherapeutic agent. Previously, the requirement for RIP had been implicated in NF-κB reporter activation induced by TNF and phorbol ester [39]. We showed that doxorubicin-induced NF-κB activation correlates with IκBα degradation in KBM-5 myeloid cells, in agreement with previously reported correlations for HL-60, another myeloid cell line [30,34]. Like TNF, doxorubicin has been shown to activate IκBα kinase [33], which in turn phosphorylates IκBα, thus leading to its degradation through the ubiquitin-dependent pathway.

Our results also indicate that compound WP744 was at least 50 times more potent than WP631 or native doxorubicin for NF-κB activation. Why WP744 is more potent is not clear. These results, however, are consistent with an enhanced cytotoxicity of this compound reported in the present studies against KBM-5 and Jurkat cells, and against leukemic cells reported previously by our laboratory [36].

Whether NF-κB activation is needed for cytotoxic effects of doxorubicin and its structural analogues was also investigated. Four independent approaches suggested NF-κB is essential for the cytotoxicity. The first approach was based on the relative potency of doxorubicin and its analogues, i.e. the compound maximally active for NF-κB was also most cytotoxic. The second approach involved the RIP-deleted cells, which failed to activate NF-κB, and also did not undergo apoptosis in response to doxorubicin or its analogues. The third approach involved the suppression of NF-κB by PDTC. Since suppression of NF-κB by PDTC or RIP-deleted cells may not be specific, we undertook a fourth approach involving the use of NF-κB-specific NBD peptide. All approaches uniformly suggested that suppression of NF-κB abrogates doxorubicin-induced cytotoxicity.

Our results disagree with reports that indicate NF-κB activation inhibits doxorubicin-induced apoptosis [32,33,45,46]. For instance Arlt *et al*. [32] showed that inhibition of NF-κB sensitizes human pancreatic carcinoma cells to apoptosis induced by doxorubicin. In their studies, the sensitization of cells to NF-κB inhibitors was observed at concentrations of doxorubicin that did not activate NF-κB, suggesting an NF-κB-independent mechanism.

Our results on the proapoptotic effects of NF-κB, however, are consistent with a recent study by Bian *et al.*, who showed that in neuroblasotma cells PDTC inhibits doxorubicin-induced NF-κB-mediated reporter activity, thus protecting cells from doxorubicin-induced killing. Similar to our RIP-deletion experiments, Bian *et al*. [34] showed that cells expressing a super repressor for IκBα were fully resistant to doxorubicin-induced cell death. That NF-κB could mediate proapoptotic effects is valid not only for doxorubicin but also for such other chemotherapeutic agents as taxol [29] and dopamine [47], in neurons undergoing focal ischemia [48], Jurkat cells exposed to either VP-16 or UV, and hepatocellular carcinoma cell lines treated with doxorubicin. Similarly, p53-mediated apoptosis also requires NF-κB activation [49]. Because p53 expression is regulated by NF-κB [50], it is possible that doxorubicin-induced NF-κB activation leads to p53 expression, which in turn causes apoptosis. While Tergaonkar *et al.* showed that doxorubicin-induced NF-κB activation decreases p53 stability [45]; Lavon *et al.* showed that the genotoxic effects of doxorubicin are p53 independent [46]. Alternatively, doxorubicin-induced NF-κB activation could mediate apoptosis through the transcriptional activation of Fas (CD95) or TRAIL [51]. Thus, our results suggest that signals upstream and downstream of NF-κB may play a major role in doxorubicin-induced cell death. For instance, tumor cells that express low or no RIP protein may be resistant to doxorubicin-induced killing. These possibilities should be explored in the future.

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