The anthracyclin doxorubicin (DXR) is a major anti-tumor agent known to cause cellular damage via a number of mechanisms including free radical formation and inhibition of topoisomerase II. It is not clear, however, how the subsequent lesions may lead to the apoptotic death of the cell. We have here examined the effects of DXR on activation of pro-apoptotic members of the Bcl-2 family, all of which are connected to the mitochondrial events of apoptosis. In two human cell lines (lymphoma and myeloma), clinically relevant concentrations of DXR were found to induce apoptosis, first observed after 24 h of treatment. Apoptosis correlated with modulation of Bak and Bax to their active conformations. bax as well as bak-deficient mouse embryonic fibroblasts were resistant to DXR compared with wild-type mouse embryonic fibroblasts further supporting a role for these proteins as main DXR-induced apoptosis regulators. Furthermore, using immunocytochemistry as well as chemical blocking of putative apical pathways we could demonstrate that Bak is activated prior to Bax. In the human cell lines, DXR was furthermore found to induce high protein levels of Bik, another BH3-only protein. DXR-induced apoptosis was completely blocked in Bcl-2-overexpressing U266 cells. Interestingly, in Bcl-2-transfected cells Bak activation was also blocked, while Bax was still partially active in agreement with differential regulation of these two proteins. Furthermore, co-incubation of the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 potentiated the apoptotic response to DXR. This enhanced apoptosis was preceded by enhanced Bak and Bax activation, and both responses as well as apoptosis were blocked in transfectants overexpressing Bcl-2. In summary, several pieces of evidence suggest that DXR induces apoptosis through a sequential and differential activation of Bak and Bax.

The anthracyclin doxorubicin (DXR) is a major anti-tumor agent used for the treatment of a variety of human cancers. Its intracellular effects include free radical formation, inhibition of DNA topoisomerase II, and also nucleotide intercalation, resulting in inhibition of DNA replication. As with many other chemotherapeutic antitumor drugs, the ensuing induction of apoptosis is likely an important reason for its therapeutic effect. DXR-induced apoptosis typically involves cytochrome c (cyt c) release from mitochondria and subsequent caspase activation (1). Accordingly, overexpression of the anti-apoptotic Bcl-2 protein blocks DXR-induced apoptosis (2). However, despite its wide-spread use in the clinic and the many types of cellular damage DXR has been shown to cause, its apoptosis-inducing signaling is far from well characterized (3).

The DXR molecule is amphoteric and binds to cell membranes as well as plasma proteins. Under physiological conditions, redox processing of DXR leads to free radical formation, which in turn may relate to the toxic and apoptotic properties of the drug (3). It has thus been shown that different types of antioxidant treatment, including antisense nitric-oxide synthase, can block DXR-induced toxic and apoptotic effects (3, 4). Other examples of DXR-induced pro-apoptotic signaling include generation of sphingosine and its metabolite ceramide (5). However, it is not clear how these and/or other upstream signaling events lead to the mitochondrial events of DXR-induced apoptosis.

Release of cytochrome c from the mitochondrial intramembrane space to the cytoplasm is commonly mediated by the pro-apoptotic Bcl-2 family proteins Bak and Bax, which in apoptotic cells are suggested to either oligomerize and form pores in the mitochondrial outer membrane (6, 7) or to interact with the proteins of the mitochondrial megapore (VDAC and ANT) (8, 9). Bak and Bax are likely to partially substitute for each other since deficiency for both genes is required to render cells completely resistant to a number of apoptotic agents, while single knockouts for either gene have far less effect on sensitivity (10). The roles of Bak and Bax in DXR-induced apoptosis have, however, not been investigated.

Oligomerization and/or activation of Bak and Bax can be induced by Bid, another pro-apoptotic Bcl-2 family member (7, 11), but also via other mechanisms such as Bid(−/−) MEF cells do not show increased resistance to a number of apoptotic agents (10). During apoptosis, Bid (21 kDa) is proteolytically cleaved to its active truncated form, tBid (15 kDa) by caspase-8 (12) or by calpain (13). Candidate proteins that might have a similar function as Bid are for instance the related Bik and Bim proteins (14). Interestingly, overexpression of Bik has been shown to be sufficient for apoptosis induction in two leukemic cell lines (15).

The activated and oligomerized or otherwise complex-bound Bak and Bax proteins are conformationally modulated, leading to exposure of an occluded N-terminal sequence (16, 17). Using antibodies specific for this epitope, modulation of Bak or Bax to its apoptotic conformations can be quantitated using flow cytometry (16). With this method, cisplatin was found to modulate Bak in all cell lines tested, whereas Bak modulation was...
Bak, Bax, and BOPs in DXR-induced Apoptosis

not seen or occurred only when nuclear fragmentation was already under way (18). Despite the overlapping functions of Bak and Bax as evidenced by knockout experiments, Bak-deficient Jurkat cells proficient for Bax were highly resistant to cisplatin and other agents, and sensitivity was restored by reintroduction of Bak (19). These reports support the idea that Bak and Bax are differently regulated. Moreover, the literature suggests different requirements for Bak or Bax functions; thus, staurosporine-induced apoptosis appears to depend on a Bax-specific function since Bax-/– MEF cells were 10% more resistant compared with Bak-/– and wild-type MEF cells (10).

The PI3K-Akt signaling pathway plays a critical role in mediating survival signals in a wide range of cell types. The recent identification of a number of substrates for the serine/threonine kinase Akt suggests that it blocks cell death both by impinging on the cytoplasmic cell death machinery and by regulating the expression of genes involved in cell death and survival. In more detail, Akt has been shown to phosphorylate the pro-apoptotic Bcl-2 family member, Bad, leading to its sequestration and subsequent inactivation by 14–3–3 proteins. Furthermore, it was recently shown that Akt phosphorylates and inactivates a number of forkhead transcription factors and thereby inhibits transcriptional activation of another proapoptotic Bcl-2 family member, Bim (21). Specific PI3K inhibitors (i.e. LY294002 andwortmannin) were shown recently to significantly increase apoptosis induced by cytotoxic drugs, e.g. DXR in HL60 cells (22). However, the exact mechanism by which PI3-kinase-regulated survival pathways suppress apoptosis is not clear.

We have here studied DXR-induced apoptotic signaling in U266 myeloma and Daudi Burkitt’s lymphoma cells with regard to the kinetics of apoptosis and modulation of Bak and Bax. The involvement of these proteins has been further established by the resistance of bax-/– and bax–/– MEFs to DXR-induced apoptosis. DXR-induced Bak and Bax activation and apoptosis was enhanced in the presence of the PI3K inhibitor LY294002 along with enhanced apoptosis. Bak and Bax activation induced by DXR in the presence or absence of LY294002 was blocked by overexpressed Bcl-2.

EXPERIMENTAL PROCEDURES

Cell Lines, Culture Conditions, and Treatment—A multiple myeloma cell line, U266 (kindly provided by Prof. Kenneth Nilsson, Uppsala University, Uppsala, Sweden), a Burkitt’s lymphoma cell line, Daudi (ATCC, Manassas, VA), and MEF were used. U266 and Daudi cells were cultured in RPMI (GIBCO, Berlin, Germany), supplemented with 10% (v/v) heat-inactivated fetal calf serum (GIBCO), 2 mM l-glutamine, 50 μg/ml streptomycin, 50 μg/ml penicillin, and maintained in a humidified incubator under 5% CO2 at 37 °C. Cells were cultured in similarly supplemented Dulbecco’s minimal essential medium in a humidified incubator under 5% CO2 at 37 °C. Cells were treated with different concentrations of DXR (adriamycin from Amersham Biosciences and Upjohn, Stockholm, Sweden) for 24, 48, and 72 h. The concentrations of DXR were carefully chosen in order to be clinically relevant (3).

Inhibitors and Antibodies—The pan-caspase inhibitor z-VAD-FMK (z-Val-Ala-Asp(Ome)-FMK) (50 μM) and caspase-8 inhibitor z-IETD-FMK (Z-Ile-Glu(OMe)-Thr-Asp(OMe)-FMK) (10 μM) were obtained from Enzyme System Products (Livermore). The p38 MAP kinase inhibitor SB203580 (Calbiochem) was used at 10 μM. The PI3K inhibitor LY294002 (Sigma) was used at 10 μM. The calpain inhibitor calpeptin (Alexis Biochemicals) was used at 10 μM. All inhibitors were added 1 h prior to DXR treatment of the U266 cells and thereafter present in the culture throughout the experiment.

The antibody against Bak is a mouse monoclonal antibody against amino acids 1–52 of Bak (AM05, clone TC100; Oncogene Research Products). The antibody against Bax is a mouse monoclonal antibody against amino acids 12–24 of Bax (clone 6A7; Pharmingen-Becton Dickinson). The antibodies against Bid and Bik are from Cell Signaling Technology and Santa Cruz Biotechnology, respectively. The antibody against cytochrome c was purchased from Pharmingen-Becton Dickinson and was biotinylated by using an EZ-Link Sulfo-NHS biotinylation kit from Pierce.

Assessment of Apoptosis— Redistribution of plasma membrane phosphatidyl serine is a marker of apoptosis and was assessed using annexin V FLUOS (Roche Molecular Biochemicals) according to the manufacturer’s protocol. Briefly, 106 cells per sample were collected, washed in PBS, pelleted, and resuspended in incubation buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl2, 25 mM TMRE) containing 1% annexin V. The samples were kept in the dark and incubated for 15 min prior to addition of another 400 μl of incubation buffer and subsequent analysis on a fluorescence-activated cell sorter Calibur flow cytometer (Becton Dickinson) using Cell Quest software.

Reduction in mitochondrial inner membrane potential, Δψm, is a typical feature of apoptotic cells. The use of the tetramethylrhodamine ethyl ester perchlorate (TMRE; Molecular Probes Inc.) to detect mitochondrial membrane potential change in Δψm, cells were stained with tetramethylrhodamine ethyl ester perchlorate (TMRE; Molecular Probes Inc.). Briefly, 106 cells/sample were collected, whereafter TMRE was added to a final concentration of 25 nM, a concentration that remained throughout the experiment. After 30 min of incubation, cells were pelleted, washed once in PBS and TMRE once, and then incubated for 10 min in the dark in 100 μl of incubation buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl2, 25 mM TMRE) containing 1% annexin V FLUOS. Prior to flow cytometric analysis, another 400 μl of incubation buffer was added. For assessment of caspase-3 activation, see below.

Flow Cytometric Analysis of Bak and Bax Activation—Upon induction of apoptosis, the Bak and Bax protein undergo conformational changes, which expose otherwise inaccessible N-terminal epitopes. In the present study, we have used two antibodies shown to specifically recognize these epitopes (16, 17). Using a fluorescein isothiocyanate (FITC)-conjugated secondary antibody, the increases in accessibility of these epitopes can be monitored by flow cytometry. At specific time points after DXR treatment, caspase-3 was measured by flow cytometry. Results are shown both as frequency histograms and, after quantitation based on median fluorescence intensity values, also as fold induction in bar charts.

Western Blot Analysis—For Western blot analysis 5 × 105 cells were lysed by sonication in LS LD buffer (50 mM HEPES at pH 7.4, 50 mM NaCl, 10% glycerol, 0.1% Tween 20, 0.3 mM sodium-orthovanadate, 50 mM NaF, 80 μM glicero-phosphate, 20 mM sodium-pyrophosphate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μM each of leupeptin and antipain, 5 μg/ml aprotinin, and 100 μg/ml of each of benzamidene hydrochloride and soybean trypsin inhibitor). Forty μg of protein were loaded in each well for resolution on 12% SDS-PAGE and electroblotting to polyvinylidene difluoride-membranes by semi-dry transfer. The membranes were incubated for 1 h each with the appropriate primary and secondary antibodies. Bands were visualized by ECL (Amersham Biosciences).

Transfections—The U266 cells were transfected by electroporation using a conventional protocol giving a full length BCL-2 DNA in a pSFV-neo vector. The pCDNA vector was used to produce a neomycin-resistant pool for control experiments. 5 × 104 cells were resuspended in 300 μl of complete RPMI medium and electroporated in a 0.4-cm cuvette at 250 V, 960 μF using Gene Pulser from Bio-Rad. 10 μl of each plasmid DNA was used. Right after the pulse, the cells were diluted with complete RPMI medium to a concentration of 4 × 106 cells/ml. Two days later, the cells were washed twice in PBS and replaced with RPMI containing 500 μg/ml of G418. The neomycin-resistant pool of living cells was separated on Ficoll gradient 1 week later, and cells were cloned in a 96-well plate by limiting dilution. Bcl-2 expression was confirmed by immunostaining and Western blotting.
**Table of Contents**

1. **Immunostaining**—For Bak or cytochrome c—double staining, cells were cytospun onto glass slides, fixed in 3% paraformaldehyde, washed once with PBS, and permeabilized using digitonin diluted in PBS for 10 min. After one wash in PBS, the staining proceeded as follows: anti-Bax or anti-Bak monoclonal antibodies, three washes in PBS, rabbit anti-mouse FITC-conjugated secondary antibodies, three washes in PBS, normal mouse serum (Chemicon, Int.), biotinylated anti-cytochrome c monoclonal antibody (clone 6E2B4, Pharmingen), three washes in PBS followed by Texas Red streptavidin (Vector Laboratory, Inc.). Slides were mounted using Vectashield with 4',6-diamidino-2-phenylindole (Vector Lab., Inc.).

The images were recorded on DAS Leitz DM RB microscope with a Hamamatsu C4880 dual mode cooled charge-coupled devices camera and further processed using PhotoShop software (Adobe).

**RESULTS**

**Effect of Doxorubicin on Apoptosis**—To determine the dose and time dependence of DXR-induced apoptosis, U266 and Daudi cells were treated with DXR at 10 and 60 ng/ml. Apoptosis, seen as annexin V positivity and mitochondrial depolarization (loss of Δψm), was assessed after 24, 48, and 72 h (Fig. 1, a and b). By 24 h, apoptosis was initiated in both cell lines. At 48 h there was a dose-dependent further increase in apoptosis, and by 72 h both concentrations had induced apoptosis in nearly all remaining cells in both cell lines. Similar data were recorded with annexin V/propidium iodide double stainings to ensure that necrosis was not induced (data not shown).

**Doxorubicin Induces the Modulation of Bak and Bak in Hematopoietic Cell Lines**—Bak and Bak antibodies that recognize their active conformations were used in order to investigate Bak and Bak involvement in the DXR-induced apoptosis. Treatment of U266 cells with 10 ng/ml DXR for 24 h induced no or little activation/modulation of Bak and Bak (Fig. 2, a and b). A higher dose of DXR (60 ng/ml) for 24 h induced an increased activation of both Bak and Bak (Fig. 2, a and b). After 48 h of DXR at both concentrations, Bak (Fig. 2a) and Bak (Fig. 2b) activation was even higher.

Bak and Bak activation was seen also in similarly treated Daudi cells in that no or little Bak and Bak activation was seen at 24 h with both doses, while there is a dose-dependent induction in both Bak (Fig. 2c) and Bak (Fig. 2d) activation after 48 h of DXR treatment.

**Bak Activation Precedes cyt c Release**—The activation of Bak and Bak in relation to cyt c release from mitochondria was investigated by immunocytochemistry. Treatment of U266 cells for 24 h with 60 ng/ml of DXR induced an activation of Bak prior to the release of cyt c from the mitochondria (Fig. 3a). Importantly, active Bak co-localized with cyt c in DXR-treated U266 cells. However, Bak was found to be active only in cells that had already released cyt c and with the fragmented nuclear morphology typical of late apoptosis (Fig. 3b). These data indicate that Bak activation occurs earlier than both Bak activation and cytochrome c release and that DXR differentially regulates these two proteins.

**Involvement of BH3-only Proteins in Doxorubicin-induced Apoptosis**—It has previously been shown that BH3-only proteins (BOPs) have a key regulatory role in activating the pro-apoptotic Bcl-2 family members (14). Involvement of Bid and Bik, two major BH3-only proteins, in DXR-induced apoptosis in U266 cells was examined by assessing drug-induced cleavage of Bid to its active, truncated form tBid, and by analyzing expression levels of Bik. Following treatment with 60 ng/ml, only a little Bid cleavage was seen after 24 h, whereas significant cleavage was seen at 48 h (Fig. 4a). The faint band corresponding to tBid in the control cells (Fig. 4a), probably reflects the low but detectable spontaneous apoptosis observed under standard culture conditions. Bid cleavage induced by DXR after 48 h was blocked by pretreatment with a caspase-8 inhibitor. However, caspase-8 inhibition had no effect on annexin V positivity of DXR-treated U266 cells after 24 or 48 h (data not shown). DXR at 60 ng/ml also induced increased levels of Bik protein already at 24 h, which remained elevated at 48 h (Fig. 4, b and c).

**Roles of Bak, Bak, and Bid in Doxorubicin-induced Apoptosis**—The roles of Bid, Bak, and Bax in DXR-induced apoptosis were further investigated by DXR treatment of wild-type MEF and MEFs deficient for either Bak, Bax, or Bid. Apoptosis induced by increasing concentrations of DXR was assessed as caspase-3 activation after 48 h of treatment (Fig. 5). The results show that bid-deficient cells were at least as sensitive to DXR as WT MEFs (Fig. 5a), indicating that Bid does not have a regulatory role in DXR-induced apoptosis. Similarly treated bax-deficient cells were found to be partially resistant to DXR compared with WT cells (Fig. 5b). In contrast, bak-deficient cells were significantly more resistant to DXR (Fig. 5c). The results are in accordance with involvement of both Bak and Bak (Fig. 2, a and b), whereas they indicate lack of involvement of Bid cleavage (Fig. 4e). This latter observation is also supported by the finding that inhibition of caspase-8 blocks Bid cleavage in this system without affecting Bak and Bak activation (see below).

**Effect of Stress-activated Pathways in Doxorubicin-induced Apoptosis**—A limited number of stress-activating signaling cascades have been implicated as apical activators of the mitochondrial apoptotic pathway. One prominent example are the p38 SAP kinases that have been shown to initiate the apoptotic pathway in response to stress stimuli (e.g. DNA damage, endoplasmic reticulum stress) (23). To investigate the involvement of p38 SAPK in DXR-induced apoptosis, U266 cells were treated with DXR in the presence or absence of SB203580, a p38 SAPK inhibitor. SB203580 was added to U266 cells 1 h prior to DXR (60 ng/ml) and continuously present in the culture throughout the experiment. The resulting Bak and Bak activations were then assessed and related to annexin V positivity. SB203580 was found to have a small blocking effect on annexin V positivity (data not shown), whereas it caused a more pronounced decrease in the activation of both Bak and Bak (Fig. 6, i and ii). These data imply that the p38 SAPK is partially involved in DXR-induced activation of Bak, Bax, and the apoptotic pathway initiated by DXR in U266 cells.

Another example of an enzyme that has been shown also to be an apical activator of the mitochondrial apoptotic pathway is caspase-8. This is a protease that has been demonstrated to induce the activation of Bid and other pro-apoptotic proteins through cleavage (13, 24–25). To examine the role of caspase-8 in DXR-induced apoptosis, calpeptin, a specific inhibitor of calpain, was added to U266 cells 1 h prior to addition of DXR and continuously present in the culture throughout the experiment. Calpeptin had no influence on Bax activation (Fig. 6, iv), whereas it decreased DXR-induced Bak activation, indicating a link between calpain and Bak activation (Fig. 6, iii).

We also examined the involvement of caspases in DXR-induced Bak and Bak activation by pretreating U266 cells with the pan-caspase inhibitor. zVAD induced a small decrease in annexin V positivity as well as Bak and Bak activation suggesting that caspases are marginally involved (Fig. 6, v, vii, viii).

**Protection from Doxorubicin-induced Apoptosis by Bcl-2 Overexpression**—Overexpression of the anti-apoptotic protein Bcl-2 has been shown to protect against a number of apoptotic stimuli in various experimental systems. We have here compared the effects of DXR on U266 cells stably transfected with either vector alone or vector encoding human Bcl-2. The levels of Bcl-2 protein in three overexpressing clones are shown in...
Fig. 7a. The Bcl-2 transfected U266 clones 7, 8, and 13 demonstrated an increased resistance to DXR compared with the neomycin-transfected U266, seen as decreased annexin V positivity following DXR treatment (Fig. 7b). DXR-induced Bak activation was completely abrogated in the Bcl-2-overexpressing U266 clones 7 (Fig. 7c, i) and 13 (Fig. 7c, ii). However, the
activation of Bax was only partially blocked in both clone 7 (Fig. 7c, iii) and clone 13 (Fig. 7c, iv). Although Bax remained partially activated, Bcl-2 overexpression blocked apoptosis as shown in Fig. 7b. The levels of active Bak and Bax in Bel-2 transfected U266 cells were unchanged in relation to neomycin-transfected cells (data not shown). These patterns of inhibition suggest that Bak and Bax activation is differentially regulated by Bel-2.

Inhibition of PI3K Enhances the DXR-induced Apoptosis—Tumor cells show variable sensitivity to chemotherapeutic agents. One potential mechanism is the deregulation of survival pathways such as the PI3K cascade. LY294002, a PI3K inhibitor, was used to examine whether the PI3K pathway counteracts the apoptotic response induced by DXR. Neomycin- and bel-2-stably transfected U266 cells were pretreated with LY294002 1 h prior to addition of 60 ng/ml of DXR and continuously present in the culture throughout the experiment. Treatment of cells with LY294002 alone did not induce apoptosis (Fig. 8a, i). Pretreatment with LY294002 followed by 60 ng/ml of DXR induced an enhanced activation of caspase-3 in neomycin-transfected U266 cells compared with neomycin cells treated with DXR alone (Fig. 8a, ii). Similar data were obtained when the levels of annexin V positivity were analyzed (data not shown). This response was com-

---

**Fig. 2. Effects of doxorubicin on Bak and Bax.** Following treatment with the indicated doses of DXR for 24 and 48 h, U266 (a, b) and Daudi (c, d) cells were probed with antibodies specific for the activated forms of Bak and Bax. Bak or Bax-related immunofluorescence was analyzed by flow cytometry. Gray histogram: control cells; Black line: DXR-treated cells. The results are representative of at least three independent experiments giving similar results.
The enhanced levels of active caspase-3 in the cells treated with LY294002 and DXR correlated well with an increase in the levels of activated Bak (Fig. 8b, ii) and Bax (Fig. 8b, iv). As expected Bcl-2 overexpression was also found to be capable of blocking the Bak activation caused by the combination of LY294002 and DXR as evident by the results for both clone 7 (Fig. 8b, ii) and clone 13 (Fig. 8b, iii). Similar results were found when we examined Bax activation in these two Bcl-2-completely blocked in the Bcl-2-transfected U266 clone 7 (Fig. 8a, iii) and clone 13 (Fig. 8a, iv).

The enhanced levels of active caspase-3 in the cells treated with LY294002 and DXR correlated well with an increase in the levels of activated Bak (Fig. 8b, i) and Bax (Fig. 8b, iv). As expected Bcl-2 overexpression was also found to be capable of blocking the Bak activation caused by the combination of LY294002 and DXR as evident by the results for both clone 7 (Fig. 8b, ii) and clone 13 (Fig. 8b, iii). Similar results were found when we examined Bax activation in these two Bcl-2-completely blocked in the Bcl-2-transfected U266 clone 7 (Fig. 8a, iii) and clone 13 (Fig. 8a, iv).

The enhanced levels of active caspase-3 in the cells treated with LY294002 and DXR correlated well with an increase in the levels of activated Bak (Fig. 8b, i) and Bax (Fig. 8b, iv). As expected Bcl-2 overexpression was also found to be capable of blocking the Bak activation caused by the combination of LY294002 and DXR as evident by the results for both clone 7 (Fig. 8b, ii) and clone 13 (Fig. 8b, iii). Similar results were found when we examined Bax activation in these two Bcl-2-completely blocked in the Bcl-2-transfected U266 clone 7 (Fig. 8a, iii) and clone 13 (Fig. 8a, iv).

The enhanced levels of active caspase-3 in the cells treated with LY294002 and DXR correlated well with an increase in the levels of activated Bak (Fig. 8b, i) and Bax (Fig. 8b, iv). As expected Bcl-2 overexpression was also found to be capable of blocking the Bak activation caused by the combination of LY294002 and DXR as evident by the results for both clone 7 (Fig. 8b, ii) and clone 13 (Fig. 8b, iii). Similar results were found when we examined Bax activation in these two Bcl-2-completely blocked in the Bcl-2-transfected U266 clone 7 (Fig. 8a, iii) and clone 13 (Fig. 8a, iv).

The enhanced levels of active caspase-3 in the cells treated with LY294002 and DXR correlated well with an increase in the levels of activated Bak (Fig. 8b, i) and Bax (Fig. 8b, iv). As expected Bcl-2 overexpression was also found to be capable of blocking the Bak activation caused by the combination of LY294002 and DXR as evident by the results for both clone 7 (Fig. 8b, ii) and clone 13 (Fig. 8b, iii). Similar results were found when we examined Bax activation in these two Bcl-2-completely blocked in the Bcl-2-transfected U266 clone 7 (Fig. 8a, iii) and clone 13 (Fig. 8a, iv).

The enhanced levels of active caspase-3 in the cells treated with LY294002 and DXR correlated well with an increase in the levels of activated Bak (Fig. 8b, i) and Bax (Fig. 8b, iv). As expected Bcl-2 overexpression was also found to be capable of blocking the Bak activation caused by the combination of LY294002 and DXR as evident by the results for both clone 7 (Fig. 8b, ii) and clone 13 (Fig. 8b, iii). Similar results were found when we examined Bax activation in these two Bcl-2-completely blocked in the Bcl-2-transfected U266 clone 7 (Fig. 8a, iii) and clone 13 (Fig. 8a, iv).
transfected clones 7 (Fig. 8b, v) and 13 (Fig. 8b, vi). In conclusion, these data suggest that the PI3K acts upstream to prevent the full activation of Bak and Bax induced by DXR.

**DISCUSSION**

Although apoptosis is a major effect of most chemotherapeutic agents used in the treatment of cancer, the actual mechanisms by which they exert this effect are still highly unclear. This probably limits the efficient use of these drugs in terms of preselection of responsive patients, overcoming resistance, rational combination of therapies, as well as scheduling of treatment. This is certainly also true for the anthracyclin DXR,
which is a backbone agent in the treatment of a large number of common malignant diseases. Several cellular targets have been proposed for this drug, such as DNA damage due to topoisomerase II inhibition, the formation of reactive oxygen species, as well as cytoskeleton damage. However, compelling evidence for specific apoptosis-related pathways involved in DXR induced apoptosis has hitherto been lacking.

In the present study we have therefore begun to investigate

![Fig. 8. Effects of LY on doxorubicin-induced apoptosis.](image_url)
the molecular background to DXR-activated cell death. The studies have focused on the activation of the intrinsic mitochondrial pathway as well as the apical upstream signaling. To our knowledge, this is the first report to show that DXR-induced apoptosis involves the induction of the active conformation of Bak and Bax. Thus, in the two malignant cell lines analyzed, the onset of apoptosis correlated with the appearance of the activated forms of these proteins. Furthermore, through analysis of MEFs nullizygous for either of these proteins, we could also demonstrate that apoptosis caused by DXR is dependent on these proteins.

In an attempt to define the signaling pathways mediating activation of Bak and Bax, the roles of two pathways implicated in apoptosis regulation were analyzed by chemical inhibition of p38 SAPK and calpain respectively. The results suggest involvement of p38 SAPK in Bak and Bax modulation. Calpeptin, a calpain inhibitor, blocked Bak but not Bax activation in further support of differential regulation of these two proteins. Using the pan-caspase inhibitor zVAD, we also found that caspases seem to be marginally involved in Bak/Bax activation.

The protective effect of Bcl-2 against most cytotoxic agents is well established. In this report we show that Bcl-2 overexpression protects against DXR-induced apoptosis through the blockage of the activation of Bak and Bax. The exact mechanism by which this blockade is mediated is not clearly established. However, a known mechanism for Bax and Bak activation is by activation of various BOPs (e.g. Bik, Bid, Bad), and conversely Bcl-2 has been shown to sequester Bak, Bax, and BOPs, thereby preventing Bax- and Bak-mediated apoptosis (7, 20, 26). The fact that Bcl-2 overexpression acts upstream of Bak and Bax activation is compatible with the notion that BOPs are mediating this DXR-induced response, although other mechanisms cannot be excluded.

We report here that both Bid and Bik are affected by DXR treatment. As described with other proapoptotic stimuli, Bid was found to be cleaved to the active form in a caspase-8-dependent manner, whereas the steady state levels of Bik were found to be up-regulated. The relevance for these events in terms of Bax/Bak activation in this system is not clear. Indeed, the contribution of Bid cleavage seems to be minor since inhibition of caspase-8 led to abrogation of Bid cleavage without affecting Bak/Bak activation or the onset of apoptosis. More compelling, Bid−/− cells are still clearly sensitive to DXR-induced apoptosis. In fact, these Bid nullizygous cells were slightly more sensitive to the cytotoxic effect of DXR than the WT control cells. The reason for this is unclear, but this phenomenon has been described also for etoposide (10) and for cis-platinum. As mentioned, the increased Bid expression seen after 24 h of DXR treatment makes this BOP a potential candidate for mediating Bak and Bax activation. Indeed, overexpression of the Bik alone has been shown to be sufficient for induction of apoptosis in leukemic cell lines (15).

The PI3K cascade is an important modulator of cell sensitivity to proapoptotic signals. This study has shown that inhibition of PI3K by LY294002 results in clear sensitization to DXR-induced apoptosis. Furthermore, we also found that the enhanced apoptotic sensitivity in LY294002-treated cells correlates with enhanced Bak and Bax activation. Bcl-2 overexpression blocked DXR- and LY294002-induced apoptosis as well as Bak and Bax activation. One mechanism by which PI3 kinases exert their control on apoptosis involves phosphorylation of Bad, which leads to its sequestration/inactivation by 14–3–3 proteins in the cytoplasm (27). Furthermore, it was recently shown that PI3K phosphorylates and inactivates the forkhead group of transcription factors. Forkhead proteins are able to induce expression of a number of proapoptotic Bcl-2 family members such as Bim, which in turn leads to induction of apoptosis (21, 28). Our data demonstrate that PI3 kinase is acting upstream of Bak and Bax activation and Bcl-2 protection from DXR-induced apoptosis; however, the mechanistic basis for this PI3K-related modulation remains to be determined. The finding that modulation of PI3K signaling can alter the sensitivity to DXR-induced apoptosis also has clear clinical implications. Several tumors have been shown to have genetic aberrations causing hyperactivation of PI3K signaling such as mutations of the ERBB2 and PTEN genes (29, 30). Theoretically this leaves room for further sensitizing tumor cell to anthracyclins.

Importantly, our data support the idea that Bak and Bax are differentially modulated. This is supported by several findings: Bak is activated earlier than Bax, as shown by immunocytochemistry; indeed, Bax activation is seen only in cells with released cyt c and fragmented nuclei; furthermore, bak−/− MEFs were much more resistant to DXR compared with bak−/− supporting the notion that Bak is the main signal transducer in DXR-induced apoptosis; in addition, Bcl-2 overexpression resulted in complete inhibition of apoptosis, which correlated well with complete inhibition of Bak activation, while Bax activation was only partially blocked; and lastly, the calpain inhibitor, calpeptin, blocked Bak but not Bax activation.

The onset of apoptosis, as well as Bik up-regulation, Bid cleavage, and Bax/Bak activation is seen after approximately 24 h of treatment. This time-scale is often seen with several chemotherapeutic agents, e.g. cisplatin or taxol, whereas other cytotoxic agents, e.g. staurosporine, induce apoptosis within hours. The reason for this delay is unclear but might involve the need for induced expression or repression of specific proteins. Alternatively, several parallel signals may be needed to

---

2 M. C. Shosman, unpublished observation.
activate the apoptotic machinery during DXR treatment, and perhaps they do not simultaneously reach their respective critical threshold levels.

In summary, the present investigation, in combination with data from other model systems (20), proposes a hypothetical model for DXR-induced apoptosis where apical signals, such as activation of p38 SAP kinase, are involved in the modulation of the two Bcl-2 family members Bak and Bax into their active proapoptotic conformations (Fig. 9). These proteins are key factors in DXR-induced apoptosis since Bax- or Bak-deficiency causes resistance to DXR. Furthermore, DXR treatment led to increased levels of Bik at a time point that coincides with Bak and Bax activation. This might indicate a requirement for Bik to activate Bak and Bax in response to cytotoxic insult of DXR. The role of Bik in DXR-induced apoptosis remains to be established in future studies. On the other hand, cleavage of the BH3-only protein Bid into its active form tBid occurred at a time point when apoptosis levels were already high. Thus, at 24 h Bid cleavage was still insignificant. The probable lack of involvement of Bid is supported by the bid<sup>−/−</sup> MEFs. Furthermore, the data in this study show that Bcl-2 can confer protection against DXR-induced apoptosis at the step upstream of Bak and Bax activation. Similarly, inhibition of PI3K leads to sensitization to DXR upstream of Bak and Bax. This characterization of the molecular background to DXR-induced apoptosis has yielded important information toward resolving the mechanism of DXR action in malignant disease. Elucidation of the mechanisms of DXR-induced apoptosis may lead to a more efficient use of this drug in the clinic, as well as to greater understanding of the major apoptosis pathways.

Acknowledgments—Dr. Stanley Korsmeyer is thanked for kindly providing various knockout mouse embryo fibroblasts and also for the kind gift of the Bcl-2 vectors. The excellent technical assistance of Ann-Charlotte Björklund is gratefully acknowledged.

REFERENCES
1. Gamen, S., Anel, A., Perez-Galan, P., Lasierra, P., Johnson, D., Pineiro, A., and Naval, J. (2000) Exp. Cell Res. 258, 223–235
2. Decaudin, D., Geley, S., Hirsch, T., Castedo, M., Marchetti, P., Macho, A., Keller, R., and Kroemer, G. (1997) Cancer Res. 57, 82–87
3. Gewirtz, D. A. (1999) Biochem. Pharmacol. 57, 727–741
4. Kalivendi, S. V., Kotamraju, S., Zhao, H., Joseph, J., and Kalyanaraman, B. (2001) J. Biol. Chem. 276, 47266–47276
5. Cuvillier, O., Nava, V. E., Murthy, S. K., Edsall, L. C., Levade, T., Milstein, S., and Spiegel, S. (2001) Cell Death Differ. 8, 571–581
6. Desagher, S., and Martinou, J. C. (2000) Trends Cell Biol. 10, 369–377
7. Wei, M. C., Lindsten, T., Mootha, V. K., Weiler, S., Gross, A., Ashiya, M., Thompson, C. B., and Korsmeyer, S. J. (2000) Genes Dev. 14, 2060–2071
8. Marzo, J., Brenner, C., Zamzami, N., Jurgensmeier, J. M., Susin, S. A., Viera, H. L., Prevost, M. C., Xie, Z., Matsuyama, S., Reed, J. C., and Kroemer, G. (1998) Science 281, 2027–2031
9. Loeffler, M., and Kroemer, G. (2000) Exp. Cell Res. 256, 19–26
10. Wei, M. C., Zong, W. X., Cheng, E. H. Y., Chinnadurai, G., Kanaan, N., Vu, M. D., and Wu, J. (2001) J. Immunol. 166, 3139–3142
11. Desagher, S., Osen-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauger, S., Maundrell, K., Antonsson, B., and Martinou, J. C. (1999) J. Cell Biol. 144, 891–901
12. Luo, X., Budihardjo, I., Zhou, H., Slaughter, C., and Wang, X. (1998) Cell 94, 4184–4190
13. Mandic, A., Viktorsson, K., Strandberg, L., Heiden, T., Hansson, J., Linder, S., and Shoshan, M. C. (2002) Mol. Cell. Biol. 22, 3003–3013
14. Lutz, R. J. (2000) Biochem. Soc. Trans. 28, 51–56
15. Marshansky, V., Wang, X., Bertrand, R., Luo, H., Duguid, W., Chinnadurai, G., Kanaan, N., Vu, M. D., and Wu, J. (2001) J. Immunol. 166, 3139–3142
16. Griffiths, G. J., Dubrez, L., Morgan, C. P., Jones, N. A., Whitehouse, J., Corfe, B. M., Dive, C., and Hickman, J. A. (1999) J. Cell Biol. 144, 903–914
17. Nechushtan, A., Smith, C. L., Hsu, Y., and Youle, R. J. (1999) EMBO J. 18, 2330–2341
18. Mandic, A., Viktorsson, K., Molin, M., Akusjarvi, G., Hansson, J., Linder, S., and Shoshan, M. C. (2001) Mol. Cell. Biol. 21, 3684–3691
19. Wang, Q. Q., Gustman, B. H., Hock, A., Goldstein, L. A., Gambotto, A., Kim, T. H., Fang, B., Rabinovitz, A., Yin, X. M., and Rabinovich, H. (2001) J. Biol. Chem. 276, 34307–34317
20. Cheng, E., Wu, M. C., Weiler, S., Farrow, R. A., Mak, T. W., Lindsten, T., and Korsmeyer, S. J. (2001) Mol. Cell. 8, 705–711
21. Stahl, M., Dijkers, P. F., Kops, G. J., Lens, S. M., Coffey, P. J., Burgering, B. M., and Medema, R. H. (2002) J. Immunol. 168, 5024–5031
22. Cofferman, D. M., McKenzie, S. L., McGahon, A. J., Knox, K. A., and Cotter, T. G. (2000) Leukemia 14, 602–611
23. Pillaire, M. J., Nebreda, A. R., and Darbon, J. M. (2000) Biochem. Biophys. Res. Commun. 278, 724–729
24. Gao, G., and Dou, Q. P. (2000) J. Cell. Biochem. 80, 53–72
25. Kubbata, M. H., and Vousden, K. H. (1997) Cell. 89, 460–468
26. Finnegan, N. M., Curtis, J. F., Prevost, G., Morgan, B., and Cotter, T. G. (2001) Br. J. Cancer 85, 115–121
27. Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. J. (1996) Cell 87, 619–628
28. Dijkers, P. F., Birkenkamp, K. U., Lam, E. W., Thomas, N. S., Lammers, J. W., Koenderman, L., and Coffey, P. J. (2002) J. Cell Biol. 156, 531–542
29. Perez-Tenorio, G., and Stal, O. (2002) Br. J. Cancer 86, 540–545
30. Depewski, P. L., Rosenthal, S. I., and Ross, J. S. (2001) Mod. Pathol. 14, 672–676
Activation of Bak, Bax, and BH3-only Proteins in the Apoptotic Response to Doxorubicin
Theocharis Panaretakis, Katja Pokrovskaia, Maria C. Shoshan and Dan Grandér

J. Biol. Chem. 2002, 277:44317-44326.
doi: 10.1074/jbc.M205273200 originally published online August 21, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M205273200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 29 references, 15 of which can be accessed free at http://www.jbc.org/content/277/46/44317.full.html#ref-list-1