

Nuclear Factor- κ B Mediates Up-Regulation of Cathepsin B by Doxorubicin in Tumor Cells

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ABSTRACT

Anthracyclines such as doxorubicin remain among the most effective agents for the treatment of solid tumors and hematological malignancies. To overcome dose-limiting side effects like cardiotoxicity, an intensive effort has been undertaken to develop promising doxorubicin prodrugs that are specifically activated at the tumor site. One approach is the application of peptide prodrugs of doxorubicin. The enzyme cathepsin B catalyzes the activation of these prodrugs, and hence, the regulation of cathepsin B by antitumor agents could influence the efficacy of peptide prodrugs using this protease. In the present investigation, the effects of doxorubicin on cathepsin B expression in the human cervix carcinoma cell line HeLa were examined. Exposure to doxorubicin induced a time- and dose-dependent up-regulation of cathepsin B expression on mRNA, protein, and activity levels. In the cathepsin B gene promoter

region, a potential nuclear factor κ B (NF- κ B) binding site could be identified. Pretreatment of HeLa cells with specific NF- κ B inhibitors abrogated the induction of cathepsin B expression. Doxorubicin-induced degradation of the inhibitory protein I κ B could be prevented by pretreatment with a specific proteasome inhibitor, resulting in a significant reduction of the doxorubicin-induced cathepsin B expression. Finally, binding of NF- κ B subunits p50 and p65 to the NF- κ B binding site in the cathepsin B gene promoter region could be demonstrated by electrophoretic mobility shift assay. In summary, our data clearly indicate that doxorubicin induces cathepsin B expression and activity via NF- κ B. These findings contribute to a better understanding of tumor targeting with peptide prodrugs and help to define a possible mechanism of doxorubicin toxicity in tumor cells.

Pharmacotherapy is the major systemic treatment of most cancer diseases. Anthracyclines such as doxorubicin are widely used in the treatment of many human solid tumors and hematological malignancies, including acute leukemias, lymphomas, Kaposi's sarcoma, bone tumors, and stomach, breast, and ovarian cancers (Danesi et al., 2002). These cytostatic antibiotics intercalate into DNA resulting in genetic damage and cell death. Their therapeutic index is compromised by severe side effects such as irreversible cardiotoxicity, leading to heart failure (Zucchi and Danesi, 2003). One approach to prevent the dose-limiting cardiotoxicity and

other adverse effects is to develop prodrugs of anticancer agents with increased selectivity for tumors by enzymatic activation in the vicinity of the tumor. Realization of such a therapeutic concept depends on the availability of an antitumoral active prodrug and a suitable enzyme that is expressed at high levels in the surrounding tumor. One example of this technique is taken from the activation of prodrugs by tumor-associated enzymes such as peptidases. For the peptide prodrug *N*-L-leucyl-doxorubicin (Leu-Dox), activation and release of doxorubicin by peptidases secreted from either lysosomes or cancer cells was shown, particularly by cathepsin B (Sinhababu and Thakker, 1996). Because of the instability of Leu-Dox in blood, a further candidate prodrug, *N*- β -alanyl-L-leucyl-L-alanyl-L-leucyl-doxorubicin, was developed, releasing Leu-Dox by a two-step activation mediated by pep-

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ABBREVIATIONS: Leu-Dox, *N*-L-leucyl-doxorubicin; NF- κ B, nuclear factor κ B; AMC, 7-amino-4-methylcoumarin; CA-074Me, *N*-[L-*trans*-propylcarbamoyloxirane-2-carbonyl]-L-isoleucyl-L-proline; CA-074, L-3-*trans*-(propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline; DMSO, dimethyl sulfoxide; CAPE, caffeic acid phenylethyl ester; E-64, *trans*-epoxysuccinyl-L-leucylamido-4-(4-guanidino)-butan; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; HNE, 4-hydroxy-2-nonenal; Cat B, cathepsin B; dox, doxorubicin; PBS, phosphate-buffered saline; Z-AMC, 7-N-benzoyloxycarbonyl-L-arginyl-L-arginylamide-4-methyl-coumarin; DTT, dithiothreitol; TBST, Tris-buffered saline/Tween 20; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; MG132, *N*-benzyloxycarbonyl (Z)-Leu-Leu-leucinal.

tidases. Both prodrugs showed improved antitumor efficacy and a decreased toxicity in vivo and in vitro (Boyer and Tannock, 1993). Loadman and coworkers (1999) showed that response to the prodrug PK1, a copolymer-doxorubicin conjugate containing a peptidyl spacer, differed in tumors and was directly related to an increased lysosomal activity of cathepsin B, pointing to a pivotal role for this enzyme in prodrug-based therapy.

The bioactive enzyme cathepsin B is a lysosomal endopeptidase that belongs to the cysteine protease class of the papain superfamily (Turk et al., 2000), which is ubiquitously expressed in mammalian cells. The enzyme was mainly found in the lysosomes of normal tissues and participates in intralysosomal turnover of cellular proteins and molecules assimilated from the extracellular environment as well as in the degradation of extracellular matrix components, prohormone processing (Shinagawa et al., 1990), and turnover of β -amyloids in Alzheimer's disease (Bernstein et al., 1996). Further functions of cathepsin B have been found with the development of cathepsin B-deficient mice. In this model, cathepsin B is significantly involved in the onset of pancreatitis (Halangk et al., 2000) as well as in tumor necrosis factor- α -mediated apoptosis of hepatocytes, hepatic inflammation, and fibrogenesis (Guicciardi et al., 2001; Canbay et al., 2003). In different cancer types, cathepsin B has been shown to be elevated, and its cellular trafficking is frequently altered in malignant cells, resulting in an increased secretion of precursor and active forms of the enzyme (Berquin et al., 1995). In addition, studies using cathepsin B antisense techniques revealed a pivotal role of cathepsin B for invasion and motility of glioblastoma (Mohanam et al., 2001) and osteosarcoma cells (Krueger et al., 1999).

Variation of cathepsin B expression and activity level in different tumors and regulation of this protease could influence the therapeutic efficacy of peptide prodrugs. In HL60 cells, expression of cathepsin B was increased after treatment with differentiating agents such as phorbol esters, calcitriol, and sodium butyrate for monocytic or retinoic acids for granulocytic differentiation (Berquin et al., 1999). From these data and the lack of studies with antineoplastic agents, we investigated the effects of the anthracycline doxorubicin on cathepsin B expression using the cervix carcinoma cell line HeLa. The present study shows that doxorubicin and other anthracycline antibiotics can cause an induction of cathepsin B on mRNA, protein, and activity levels in HeLa cells by an NF- κ B-mediated pathway.

Materials and Methods

Materials. The human cervix adenocarcinoma cell line HeLa was obtained from the American Type Culture Collection (Manassas, VA). Cell-culture media and serum were from Biochrom (Berlin, Germany). Purified human liver cathepsin B, the anthracyclines doxorubicin hydrochloride, daunomycin hydrochloride, and idarubicin hydrochloride, the cysteine protease inhibitor E-64, and 7-amino-4-methylcoumarin (AMC) were from Sigma Chemical (Steinheim, Germany). The cathepsin B inhibitors CA-074 and CA-074Me and the cathepsin B substrate 7-*N*-benzoyloxycarbonyl-L-arginyl-L-arginylamid-4-methyl-coumarin were purchased from Bachem AG (Heidelberg, Germany). The NF- κ B inhibitors caffeic acid phenylethyl ester (CAPE) and 4-hydroxy-2-nonenal (HNE) were from Alexis Biochemicals (Grünberg, Germany). The lysosomotropic fluorochrome LysoTracker Green DND-26 was from Molecular Probes (Eugene,

OR). T4 polynucleotide kinase was from Roche Diagnostics (Mannheim, Germany), and [γ - 32 P]ATP (~ 6000 Ci/mmol) was from Amersham Biosciences (Freiburg, Germany). All materials were obtained in the highest available grade. Stock solutions of doxorubicin, daunorubicin, idarubicin, E-64, CA-074, CA-074Me, CAPE, and HNE were prepared in dimethyl sulfoxide (DMSO) (Roth, Karlsruhe, Germany), AMC was dissolved in acetone, and 7-*N*-benzoyloxycarbonyl-L-arginyl-L-arginylamid-4-methyl-coumarin was dissolved in 50% methanol. Stock solutions were aliquoted and stored at -20°C .

Antibodies. The following antibodies were used for immunodetection, immunofluorescence, and gel shift assays: monoclonal anti-cathepsin B (Ab-2) (Calbiochem, Schwalbach, Germany); polyclonal anti-p50 and anti-p65 (Santa Cruz Biotechnology Inc., Heidelberg, Germany); polyclonal anti-p52 (Upstate Biotechnology, Lake Placid, NY); polyclonal anti-c-Rel (Serotec, Düsseldorf, Germany); monoclonal anti-I κ B α (Alexis Biochemicals); monoclonal anti-glyceraldehyde-3-phosphate-dehydrogenase (Biosdesign International, Kennebunk, ME), and the secondary alkaline phosphatase-conjugated swine anti-rabbit IgG and goat anti-mouse IgG (DakoCytomation, Hamburg, Germany).

Cell Culture and Drug Treatment. HeLa cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and incubated in a humidified atmosphere containing 5% CO₂ at 37°C. The adherent cells were detached from the culture flasks using trypsin/EDTA. Cells were counted in a Casy 1 TT cell counter (Schärfe System GmbH, Reutlingen, Germany). For experiments, cells were plated in six-well plates at a density of 0.4×10^6 cells/well. After 3 days of culture, the medium was changed, and cells were incubated with various concentrations of the compounds for selected time points. When required, cells were preincubated with inhibitors for 1.5 h (CA-074Me, CAPE, HNE, and MG132) or 1 h (dexamethasone) before the addition of the drugs. At the end of the incubation periods, the medium was removed and cells were washed once with phosphate-buffered saline (PBS). Cells were then scraped and used for protein extraction, RNA preparation, or nuclear extract preparation.

Measurement of Protein Content. Cells were scraped, resuspended in lysis buffer (20 mM Tris-HCl, pH 7.4, 100 mM sodium chloride, 0.2% Triton X-100, 5 mM EDTA, and 1 mM Pefabloc) and incubated on ice for 45 min with several intermediate mixing steps. After centrifugation (5 min at 13,000 rpm in a tabletop centrifuge), protein concentration of the supernatant was determined using the bicinchoninic acid method with bovine serum albumin as standard.

Assay for Cathepsin B Activity. Enzyme activity of cathepsin B was determined using the specific fluorogenic substrate 7-*N*-benzoyloxycarbonyl-L-arginyl-L-arginylamid-4-methyl-coumarin (Z-AMC) and calculated by determining the amount of released AMC in micromoles per milligram of protein per hour measuring the fluorescence at 390 nm (excitation) and 460 nm (emission) with the fluorescence microplate reader Wallac 1420 VICTOR² (PerkinElmer Wallac, Gaithersburg, MD). Briefly, HeLa lysates were centrifuged at 13,000 rpm for 1 min to pellet the cell fragments, and the supernatant was used for enzymatic assay. Protein (30 μ g) was incubated at 37°C for 15 min in a volume of 160 μ l of activity buffer (pH 6.0, containing 325 mM KH₂PO₄, 72 mM Na₂HPO₄, 1 mM DTT, and 3.7 mM EDTA). The enzyme reaction was started by the addition of Z-AMC to a final concentration of 200 μ M. After an incubation period of 150 min, the reaction was terminated by the addition of 300 μ l of 1 mM iodoacetate.

Specificity of cathepsin B activity for Z-AMC was demonstrated by incubating the activity assay with E-64, a broad-spectrum inhibitor of cysteine proteases, and the specific cathepsin B inhibitors CA-074 and CA-074Me.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting. Cellular protein from each sample (60 μ g) was mixed with 4 \times Laemmli sample buffer (0.25 M Tris, pH 6.8, 8% SDS, 40% glycerol, 2.5% bromphenol blue, and 2% β -mercaptoethanol), heated at 95°C for 3 min, applied to a 15% acrylamide gel containing 10%

SDS, separated by electrophoresis (SDS-polyacrylamide gel electrophoresis), and subsequently transferred to nitrocellulose membranes (0.2 μ m; Schleicher & Schüll, Dassel, Germany). Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h and incubated for at least 1 h with monoclonal anti-human cathepsin B antibody (Ab-2, mouse) at a dilution of 1:500. After washing six times for 5 min with TBST, blots were incubated with the alkaline phosphatase-conjugated anti-mouse secondary antibody (1:1000) for 1 h. Membranes were washed six times with TBST, and immunoreactive bands were visualized by chemiluminescence (LumiPhos WB; Pierce Chemical, Rockford, IL) and exposure to X-ray films. Equal protein loading was controlled by detection of GAPDH (MAb, 1:1000) with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate staining.

RNA Isolation and Real-Time PCR. Total RNA was prepared from HeLa cells using PeqGold RNAPure (Peqlab, Erlangen, Germany) according to the manufacturer's instructions. RNA (300 ng) was reverse-transcribed using reverse transcription reagents with random hexamer primer (Applied Biosystems, Weiterstadt, Germany) in a 50- μ l reaction volume containing the following components: 1 \times TaqMan RT buffer, 5.5 mM MgCl₂, 500 μ M dNTP mix, 2.5 μ M random hexamer primer, 0.4 U/ μ l RNase inhibitor, 1.25 U/ μ l reverse transcriptase, and 50 μ l of distilled water.

TaqMan assays were performed on the ABI 7700 sequence detection system (Applied Biosystems). The 95-base pair cathepsin B cDNA fragment was amplified using the synthetic primers CatB-For 5'-TGGACAAGAAAGGCCTGGTT-3' and CatB-Rev 5'-CCGTTGACGTGGTGCTCA-3'. The sequence of the 5-carboxyfluorescein-labeled probe was 5'-CCCATGTAGGGTGCAGACCGTACTCC-3'. 18S rRNA primers and probe labeled at the 5' end with the reporter dye VIC and at the 3' end with the quencher dye 6-carboxytetramethyl-rhodamine were from Applied Biosystems. Specificity of the cathepsin B cDNA fragment was proven by sequence analysis.

Reaction mixtures contained 1 \times TaqMan Universal PCR Master Mix (Applied Biosystems), primer/probe mix (300/150 nM for cathepsin B), and 6 ng of cDNA/well for cathepsin B (0.06 ng for 18S rRNA) in a total volume of 20 μ l. Thermal cycling conditions were 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. For quantification, standard curves for cathepsin B and 18S rRNA control were prepared by cloning PCR cDNA fragments into the expression vectors pDrive (for cathepsin B, QIAGEN GmbH, Hilden, Germany) and pGEM-T-Easy (for 18S, Promega, Mannheim, Germany). Serial dilutions of the plasmids containing the cDNA fragments were used as internal standards. Quantification was achieved by comparing the start copy number of the input cDNA with the values of a standard template cDNA that was amplified in the same run. Cathepsin B mRNA expression was calculated in relation to 18S rRNA expression. Experiments were performed in triplicate, and results are given as mean \pm S.D.

Immunofluorescence Staining of Cathepsin B. For immunofluorescence staining, cells were grown on glass slides nearly to confluence, treated with the compounds, and frozen at -80°C until they were used for immunohistochemistry. Cells were fixed with 4% paraformaldehyde in PBS for 30 min on ice and washed with PBS. Monoclonal antibody to cathepsin B was added (1:50 dilution) overnight at 4°C. After washing five times with PBS, cells were incubated with the secondary Alexa Fluor 568-labeled anti-mouse IgG₁ (Molecular Probes), for at least 1 h at room temperature, additionally washed five times with PBS, and embedded with fluorescent mounting medium (DakoCytomation). For staining of lysosomes, we used the acidotropic dye Lysotracker Green DND-26 (Molecular Probes) diluted in PBS. Cells were cultured at 37°C in prewarmed medium containing 10 nM Lysotracker Green for 30 min and then washed three times in PBS.

Nuclear Extract Preparation. For preparation of nuclear extracts, cells were washed once with ice-cold PBS, scraped, and centrifuged for 5 min at 2000g. Cell pellet was resuspended in 400 μ l of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM

EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) by gentle pipetting and allowed to swell for 15 min on ice. After the addition of 25 μ l of 10% Igepal, cell lysates were incubated on ice for 10 min, inverting the tube every minute. The homogenate was centrifuged at 13,000 rpm for 1 min at room temperature, and the supernatant (cytoplasmic extracts) was removed. The nuclear pellet was resuspended in buffer C (20 mM HEPES, pH 7.9, 0.4 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride), incubated for 20 min with vigorous shaking, and centrifuged at 13,000 rpm for 5 min at 4°C. The supernatant containing the nuclear proteins was collected and frozen at -80°C until electrophoretic mobility shift assay (EMSA) was done. Protein was quantified by the bicinchoninic acid method (as described under Measurement of Protein Content).

Electrophoretic Mobility Shift Assay. Nuclear NF- κ B was assessed by EMSA using a 20-base pair oligonucleotide 5'-GCGGGGG-GACTTTTCTAGGC-3' (TIB-Mol Biol, Berlin, Germany) containing the potential NF- κ B binding site of the cathepsin B promoter (boldface letters, position 1460-1471 of cathepsin B promoter region, AF086639). The oligonucleotide was end-labeled with [γ -³²P]ATP using T₄ polynucleotide kinase in 10 \times kinase buffer. The labeled double-stranded oligonucleotide was separated using a NucTrap Probe Purification Column (Stratagene, Heidelberg, Germany). Typically, 5 μ g of nuclear extracts from cells were incubated with radiolabeled oligonucleotide (40,000 cpm), 2 μ g of poly(dI-dC), and DNA binding buffer in a total volume of 20 μ l at room temperature for 30 min. The nuclear protein ³²P-labeled oligonucleotide complexes were then separated on a 6% polyacrylamide gel under nondenaturing conditions in a running buffer of 0.5 \times TBE (50 mM Tris, pH 8.0, 45 mM borate, and 0.5 mM EDTA). Gels were dried on 3MM CHR blotting and chromatography paper in a gel dryer (Whatman Biometra GmbH, Niedersachsen, Germany), and DNA-protein complexes were visualized by autoradiography. For competition studies, an excess of unlabeled oligonucleotide was used to block the binding of activated complexes to labeled NF- κ B probe. For further competition experiments, the following oligonucleotides were used: scrambled oligonucleotide 5'-GATCGAAGTACCGCCCGCGC-CCCGT-3', NF- κ B mutant 5'-GCGGGGAAACGTTTCTAGGC-3', NF- κ B consensus 5'-TTGAGGGGACTTTCCAGGC-3'. To identify NF- κ B subunits of the dimeric complex, nuclear extracts from doxorubicin-treated HeLa cells were preincubated with antibodies against p50, p65, p52, or c-Rel subunits at room temperature for 30 min before the addition of labeled probe.

Statistical Analysis. Results were expressed as mean \pm S.D. The Mann-Whitney *U* test and unpaired *t* test were used to determine statistical significance for all comparisons (Prism; GraphPad Software Inc., San Diego, CA). Values of *p* < 0.05 was considered to be statistically significant.

Results

Induction of Cathepsin B mRNA, Protein, and Activity by Doxorubicin-Time Course. In the first set of experiments, HeLa cells were treated with DMSO (0.1%, control) or 1 μ M doxorubicin for various time points, and cellular extracts were assessed for expression of cathepsin B mRNA, protein, and activity. mRNA content was determined by real-time RT-PCR (TaqMan principle) with specific primers and probe for cathepsin B. The expression of cathepsin B mRNA was normalized to 18S rRNA. Protein content was determined by Western blotting, and cathepsin B activity was measured using a Z-AMC activity assay.

Figure 1a illustrates the time course of cathepsin B mRNA induction by 1 μ M doxorubicin. No increase in cathepsin B mRNA was observed within the first 6 h of doxorubicin treatment. After 8 h, cathepsin B transcripts started to increase (1.5-fold for 8 h and 1.9-fold for 16 h) with a maximum effect

of 2.7-fold mRNA expression at 24 h. Incubation for longer times resulted in a slight decrease of cathepsin B mRNA content (1.9-fold for 32 and 48 h).

Incubation of HeLa cells with 1 μ M doxorubicin for selected time points resulted in a time-dependent increase in cathepsin B protein. As shown in Fig. 1b, Western blot analysis of cathepsin B protein expression resulted in a significant increase of cathepsin B protein content after treatment with 1 μ M doxorubicin for 48 and 72 h. We could detect a prominent band at a molecular size of approximately 30 kDa

representing the single-chain form of cathepsin B and the double-chain variant at 25/26 kDa.

Z-AMC activity assay also showed a time-dependent induction of cathepsin B activity (Fig. 1c). The cathepsin B activity began to increase after 16 h of doxorubicin treatment and increased to a maximum activity with 215% at 48 h compared with the control cells. Incubation of cells for longer time periods did not lead to any further increase in cathepsin B activity; it instead resulted in a slight decrease in cathepsin B activity (208% for 72 h, 192% for 96 h, and 184% for 120 h).

Dose Response. As shown in Fig. 2a, doxorubicin increased the cathepsin B mRNA in a dose-dependent way. A maximum effect of cathepsin B mRNA induction was obtained with 1 μ M doxorubicin in HeLa cells. At this concentration, cathepsin B mRNA was induced approximately 2.5-fold. At higher concentrations of doxorubicin, the induction of cathepsin B did not increase further but strongly decreased at a concentration of 3.3 μ M doxorubicin.

At protein and activity levels, a dose-dependent increase of cathepsin B induced by doxorubicin could also be observed (Fig. 2, b and c). In accordance with the mRNA level, the maximum increase of cathepsin B protein and activity was obtained with 1 μ M doxorubicin, a concentration which can be reached for bolus administration in patients (Gewirtz, 1999). Western blot analysis revealed a band at a molecular size of approximately 30 kDa, representing the single-chain cathepsin B form. In addition, the double-chain form of cathepsin B was detected as bands at 25 to 26 kDa (Fig. 2b). Measurement of cathepsin B activity with the fluorogenic substrate Z-AMC also resulted in a concentration-dependent increase in cathepsin B activity with a maximum effect of 206.6 μ mol/mg \cdot h at 1 μ M doxorubicin compared with the control value of 77.1 μ mol/mg \cdot h (Fig. 2c). Incubation of cells with higher concentration such as 3.3 μ M led to no further increase in cathepsin B activity (203.3 μ mol/mg \cdot h).

Immunofluorescence Detection of Cathepsin B in HeLa Cells. To localize cathepsin B protein, HeLa cells were grown on glass cover slides to approximately 80% confluence and were incubated for 32 h with 1 μ M doxorubicin or DMSO (as control). After fixing with 4% paraformaldehyde/PBS, cells were incubated with an antibody against cathepsin B followed by an anti-mouse-IgG-Alexa Fluor 568 (Fig. 3). In control cells, a faint staining of cathepsin B in the entire cell body could be detected (Fig. 3A). Doxorubicin-treated cells showed an increased cathepsin B staining without change in the localization site (Fig. 3B). In these immunofluorescence studies, a nuclear staining seemed to be present at 488 nm, representing the DNA-intercalated doxorubicin (Fig. 3C). This nuclear staining of doxorubicin could not be seen at 568 nm at which cathepsin B detection was performed. Overlaying of the images from both wavelengths (488 and 568 nm) is demonstrated in Fig. 3D, showing cathepsin B as red fluorescence and doxorubicin-stained nuclei as green fluorescence. Incubation of cells with PBS or the secondary anti-mouse-IgG-Alexa Fluor 568 antibody resulted not in an immunofluorescence staining showing specificity of anti-cathepsin B antibody (data not shown).

Treatment of HeLa Cells with NF- κ B Inhibitors Abrogates the Induction of Cathepsin B Expression. Anthracyclines are known to be able to mediate NF- κ B activation (Das and White, 1997). Therefore, further experiments were carried out to investigate whether NF- κ B is involved in

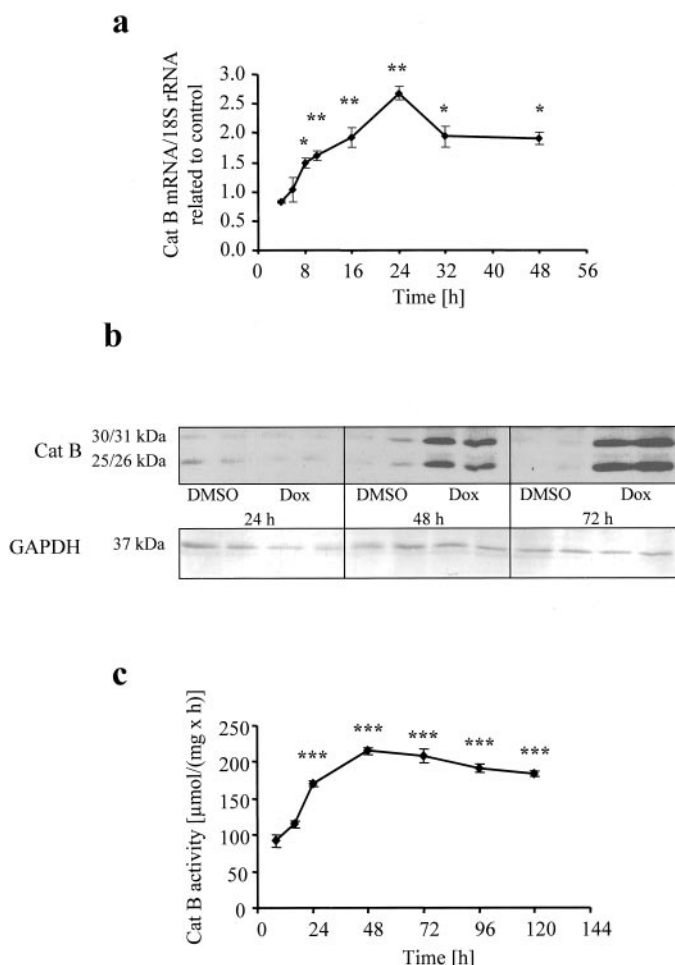


Fig. 1. Time-dependent up-regulation of cathepsin B by doxorubicin. HeLa cells were cultured for 3 days in RPMI 1640 medium containing 10% FCS. Cells were then incubated with 0.1% DMSO (control) or 1 μ M doxorubicin for the indicated times, and then expression of cathepsin B mRNA (a), protein (b), and activity (c) was assessed. For measurement of cathepsin B mRNA, total RNA was prepared and reverse-transcribed, and TaqMan assays were performed as described under *Materials and Methods*. Cathepsin B mRNA was normalized against 18S rRNA. Data are displayed as relative expression to DMSO-treated control cells ($n = 6$; *, $p < 0.05$; **, $p < 0.01$). For detection of cathepsin B protein expression, cellular protein extracts were made, and Western blotting was performed from separate samples as described under *Materials and Methods* using the monoclonal anti-cathepsin B antibody and alkaline phosphatase-conjugated anti-mouse secondary antibody. Bands were visualized by chemiluminescence and exposure to X-ray films. Equal protein loading was controlled by detection of GAPDH. For measurement of cathepsin B activity, cellular protein extracts were prepared as described under *Materials and Methods*. By incubation with the specific cathepsin B substrate Z-AMC for 2.5 h, the cathepsin B activity was determined on a microplate reader at 390 (excitation) and 460 nm (emission). Calculation of significance was performed between the DMSO-treated control and the doxorubicin-treated samples ($n = 6$; ***, $p < 0.001$).

the induction of cathepsin B by doxorubicin. To assess the role of NF- κ B in doxorubicin-mediated induction of cathepsin

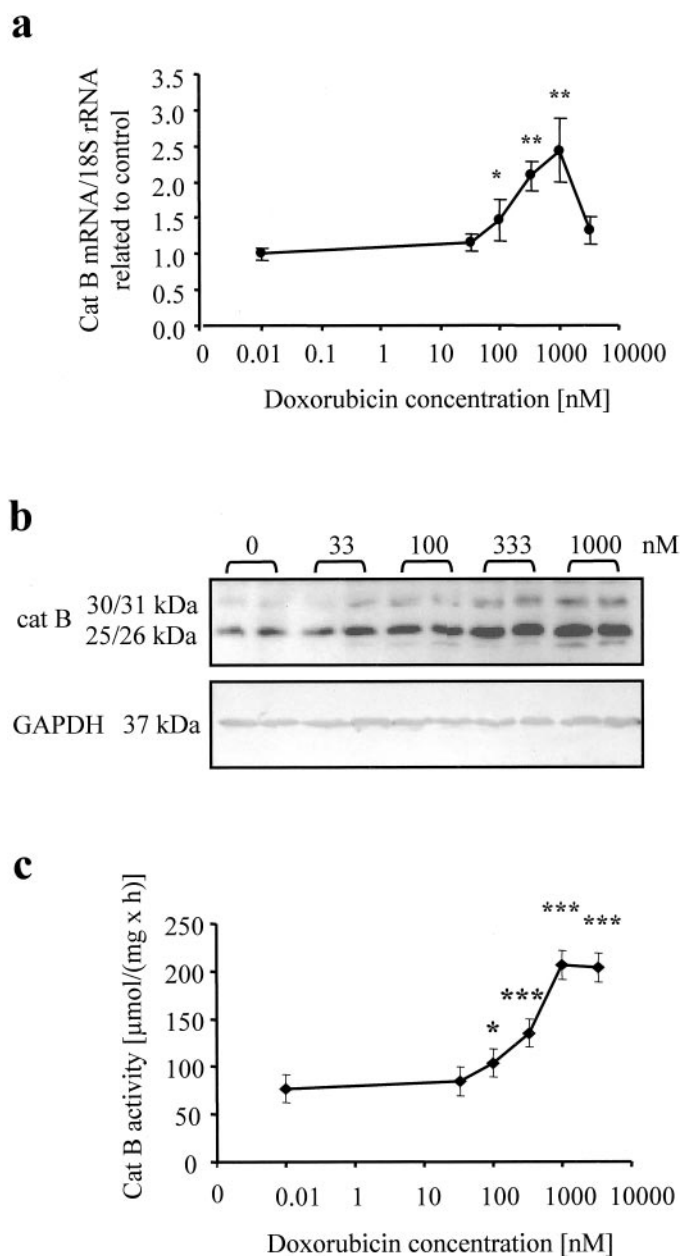


Fig. 2. Concentration-dependent up-regulation of cathepsin B by doxorubicin. HeLa cells were cultured for 3 days in RPMI 1640 medium containing 10% FCS. Cells were then incubated with various concentrations of doxorubicin (0–3.3 μ M) for 24 h (cathepsin B mRNA, a) or 48 h (cathepsin B protein, b; cathepsin B activity, c). For measurement of cathepsin B mRNA, total RNA was prepared and reverse-transcribed, and TaqMan assays were performed as described under *Materials and Methods*. Cathepsin B mRNA was normalized against 18S rRNA. Data are displayed as relative expression to DMSO-treated control cells ($n = 6$; *, $p < 0.05$; **, $p < 0.01$). For detection of cathepsin B protein expression, cellular protein extracts were made, and Western blotting was performed as described under *Materials and Methods* using the monoclonal anti-cathepsin B antibody and alkaline phosphatase-conjugated anti-mouse secondary antibody. Bands were visualized by chemiluminescence and exposure to X-ray films. Equal protein loading was controlled by detection of GAPDH. For measurement of cathepsin B activity, cellular protein extracts were prepared as described under *Materials and Methods*. By incubation with the specific cathepsin B substrate Z-AMC for 2.5 h, the cathepsin B activity was determined on a microplate reader at 390 (excitation) and 460 nm (emission) ($n = 6$; *, $p < 0.05$; ***, $p < 0.001$).

B expression, we used the commercially available inhibitors of NF- κ B HNE and CAPE. HNE inhibits I κ B kinase activity by direct interaction with I κ B kinase (Ji et al., 2001) and thereby prevents I κ B degradation and NF- κ B activation. In contrast, CAPE inhibits translocation of NF- κ B into the nucleus (Natarajan et al., 1996).

To assess whether activation of NF- κ B is involved in doxorubicin-induced increase in cathepsin B expression, we analyzed the effect of pretreatment of HeLa cells with 100 nM HNE and 10 μ M CAPE for 1.5 h followed by exposure to 1 μ M doxorubicin for 18 h (RNA) or 48 h (protein and activity). As disclosed by quantitative RT-PCR, pretreatment with HNE and CAPE effectively blocked the doxorubicin-mediated induction of cathepsin B mRNA expression from approximately 1.65- to 1.23-fold (HNE) and 1.28-fold (CAPE) induction (Fig. 4a). Western blot analysis and Z-AMC activity assay were performed to confirm the results of the quantitative RT-PCR. HNE and CAPE potently reduced the doxorubicin-induced increase of both cathepsin B protein expression and activity (Fig. 4, b and c). As shown in Fig. 4c, after treatment of cells with DMSO, cathepsin B activity amounted to 220 μ mol/mg \cdot h, whereas doxorubicin led to a cathepsin B activity of 574 μ mol/mg \cdot h. After pretreatment with HNE (100 nM) and CAPE (10 μ M) for 1.5 h and incubation with doxorubicin (1 μ M) for 48 h, a loss of doxorubicin-mediated increase of cathepsin B activity from 574 (doxorubicin alone) to 306 (doxorubicin + HNE) and 350 μ mol/mg \cdot h (doxorubicin + CAPE) was observed. This could be asserted by densitometric analysis of the Western blots illustrated in Fig. 4b. Taken together, these findings support the hypothesis that cathepsin B induction in response to doxorubicin treatment results from activation of NF- κ B.

Doxorubicin Mediates I κ B α Degradation in HeLa Cells. NF- κ B activation is dependent on the phosphorylation-induced, proteasome-mediated proteolysis of I κ B. To further examine whether degradation of I κ B α plays a role in the doxorubicin effect, I κ B α protein expression was detected by

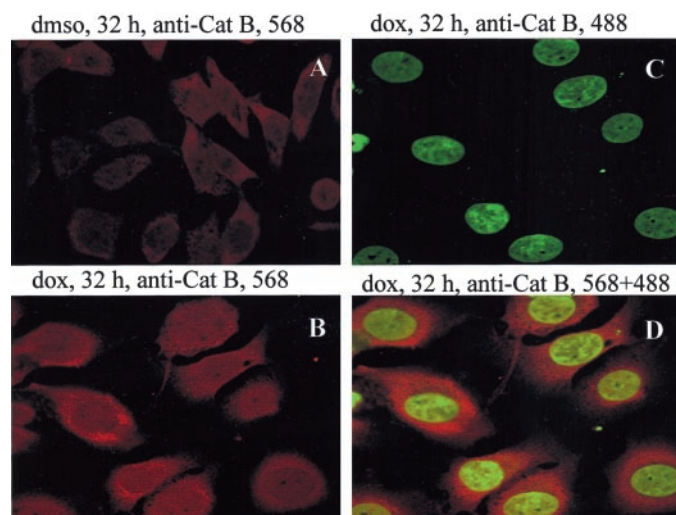


Fig. 3. Immunofluorescence staining of cathepsin B in HeLa cells. HeLa cells were grown in RPMI 1640 medium containing 10% FCS on cover slides for 72 h nearly to confluence. Cells were then incubated with 0.1% DMSO (control) or 1 μ M doxorubicin for 32 h. After fixation of cells with 4% paraformaldehyde/PBS, immunofluorescence detection of cathepsin B was performed as described under *Materials and Methods* using a monoclonal anti-cathepsin B antibody and a secondary Alexa Fluor 568 goat anti-mouse IgG (original magnification, 40 \times).

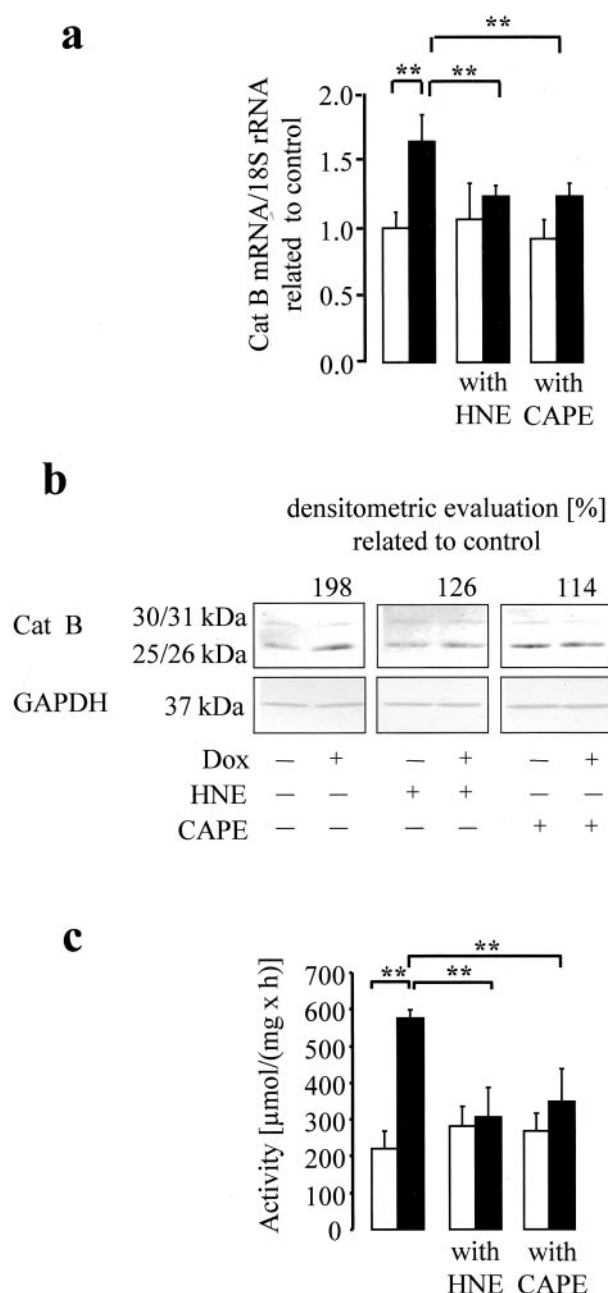


Fig. 4. HNE and CAPE abrogate the doxorubicin-mediated induction of cathepsin B (Cat B). HeLa cells were cultured in RPMI 1640 medium supplemented with 10% FCS for 72 h. Cells were then preincubated with 100 nM HNE or 10 μ M CAPE for 1.5 h followed by incubation with DMSO (control, □) or 1 μ M doxorubicin (■) for an additional 22 h (for mRNA quantification, a) or 32 h (for cathepsin B protein, b; and cathepsin B activity, c). For quantification of cathepsin B mRNA, total RNA was prepared and reverse-transcribed with random hexamer primers followed by semiquantitative RT-PCR analysis. 18S rRNA amplification was used for normalization. Data are displayed as relative expression to DMSO-treated control cells ($n = 6$; **, $p < 0.01$). For detection of cathepsin B protein expression, cellular extracts were prepared, and Western blotting was performed as described under *Materials and Methods* using the monoclonal anti-cathepsin B antibody and alkaline phosphatase-conjugated anti-mouse secondary antibody. Bands were visualized by chemiluminescence and exposure to X-ray films. Equal protein loading was controlled by detection of GAPDH. For measurement of cathepsin B activity, cellular protein extracts were prepared as described under *Materials and Methods*. By incubation with the specific cathepsin B substrate Z-AMC for 2.5 h, the cathepsin B activity was determined on a microplate reader at 390 (excitation) and 460 nm (emission) ($n = 6$; **, $p < 0.01$).

Western blotting using an anti-I κ B α antibody. In addition, the influence of the proteasome inhibitor MG132, which prevents degradation of I κ B α and therefore NF- κ B activation, on cathepsin B expression was investigated. For immunodetection of I κ B α , HeLa cells were incubated with DMSO (0.1%, control) or 1 μ M doxorubicin for 4, 6, and 8 h and cytoplasmic extracts were prepared. As shown in Fig. 5a, after 4 h of treatment with doxorubicin, a marked decrease of immunoreactive I κ B α protein (~ 36 kDa) could be observed, and the maximal reduction occurred after 6 h of incubation with doxorubicin. After 8 h of doxorubicin treatment, the I κ B α protein content slightly increased (Fig. 5a).

For examination of the influence of the proteasome inhibitor MG132 on doxorubicin-induced cathepsin B increase, HeLa cells were pretreated for 1.5 h with 2 μ M MG132. In the presence of MG132 the content of doxorubicin-induced cathepsin B mRNA was reduced from 254% (doxorubicin alone) to 131% (doxorubicin + MG132) (Fig. 5b). This effect could be confirmed at the activity level as seen in Fig. 5c. Cathepsin B activity decreased after pretreatment with MG132 from 327% (doxorubicin alone) to 137% (doxorubicin + MG132) after 32 h of incubation. These data indicate that I κ B α degradation is involved in the induction of cathepsin B by doxorubicin.

p50 and p65 Subunits of NF- κ B Are Involved in Binding to the Response Element. To test the hypothesis that doxorubicin activates the binding of NF- κ B to the NF- κ B response element in the cathepsin B promoter, nuclear proteins were obtained from HeLa cells that were DMSO-treated (control) or stimulated with 1 μ M doxorubicin. EMSA revealed an increase in NF- κ B binding to the radiolabeled oligonucleotide after 6 h of doxorubicin treatment (Fig. 6a). To further proof specificity for the binding, competition experiments with an unlabeled scramble oligonucleotide, an unlabeled oligonucleotide containing the NF- κ B binding site of the cathepsin B promoter, an unlabeled oligonucleotide containing NF- κ B consensus sites, and an unlabeled mutated NF- κ B oligonucleotide were performed. Whereas competition with the unlabeled scramble or the unlabeled mutated NF- κ B oligonucleotide did not change band intensities in the EMSA experiment, competition with the unlabeled oligonucleotides containing NF- κ B consensus sites or the NF- κ B binding region of the cathepsin B promoter resulted in a significant reduction of the band intensities. The radiolabeled scramble oligonucleotide did not show any binding activity specific for NF- κ B (Fig. 6b). To clearly distinguish between the NF- κ B subunits that might participate in the binding to the response element, nuclear extracts were incubated with antibodies against the subunits p50, p52, p65, and c-Rel followed by incubation with radiolabeled oligonucleotide. Incubation with antibodies against p50 and p65 resulted in a supershift compared with doxorubicin-treated probes without antibody incubation that was not seen with anti-c-Rel and anti-p52 antibodies. The specificity of the obtained band shift was assessed by competition experiments with unlabeled NF- κ B oligonucleotides, leading to the disappearance of the NF- κ B shift (Fig. 6c).

Dexamethasone Decreases Doxorubicin-Induced Cathepsin B Expression. Because dexamethasone can inhibit NF- κ B (De Bosscher et al., 1997) and treatment with anthracyclines for acute lymphatic leukemia and non-Hodgkin lymphoma is often combined with dexamethasone, we analyzed

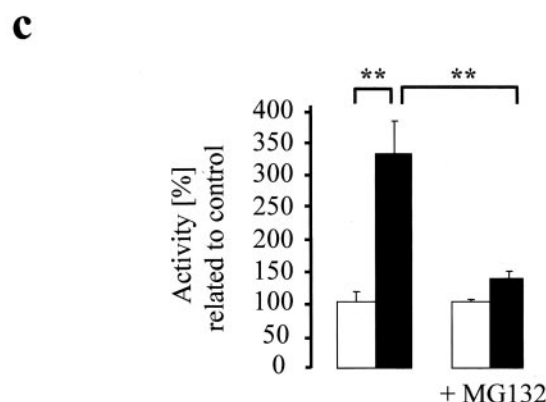
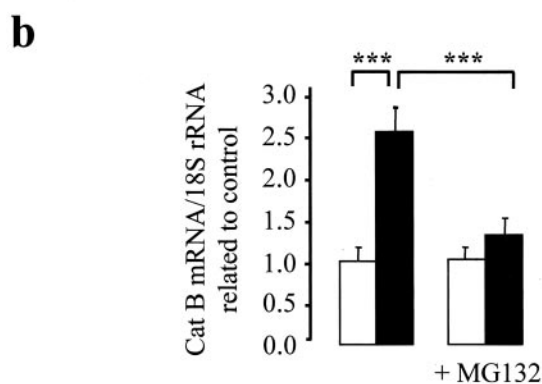
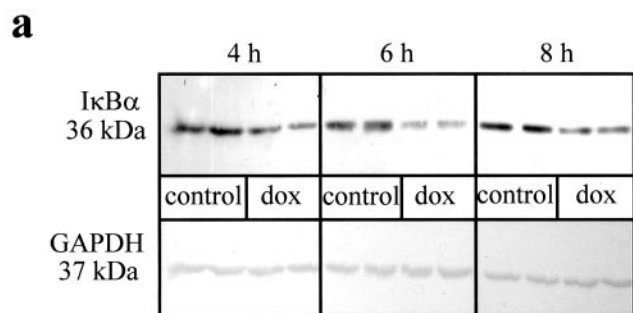


Fig. 5. Doxorubicin mediates $I\kappa B\alpha$ degradation in HeLa cells. HeLa cells were cultured in RPMI 1640 medium containing 10% FCS for 72 h. a, cells were then incubated with DMSO (control) or 1 μ M doxorubicin for 4, 6, and 8 h. For immunodetection of $I\kappa B\alpha$, cellular protein was extracted, and Western blotting was performed as described under *Materials and Methods* using a monoclonal anti- $I\kappa B\alpha$ antibody. Bands were visualized by chemiluminescence and exposure to X-ray films. Equal protein loading was controlled by the detection of GAPDH. (b and c). For assessment of the influence of the proteasome inhibitor MG132, cells were preincubated with 2 μ M MG132 for 1.5 h. Cells were then incubated with DMSO (control, \square) or 1 μ M doxorubicin (\blacksquare) for 24 h (cathepsin B mRNA, b) or 32 h (cathepsin B activity, c). For quantification of cathepsin B mRNA, total RNA was isolated, reverse-transcribed, and analyzed by semiquantitative RT-PCR as described under *Materials and Methods*. Cathepsin B mRNA was normalized to 18S rRNA. Data are displayed as relative expression to DMSO-treated control cells ($n = 6$; $***, p < 0.001$). For measurement of cathepsin B activity, cellular protein extracts were prepared as described under *Materials and Methods*. By incubation with the specific cathepsin B substrate Z-AMC for 2.5 h, the cathepsin B activity was determined on a microplate reader at 390 (excitation) and 460 nm (emission) ($n = 6$; $**$, $p < 0.01$).

the effect of this glucocorticoid on cathepsin B expression. Therefore, HeLa cells were pretreated with 100 nM, 1 μ M, or 10 μ M dexamethasone for 1 h followed by incubation with 1 μ M doxorubicin for 24 h (RNA) or 48 h (protein). Total RNA or cellular protein extracts were prepared, and cathepsin B mRNA, protein, and activity were determined. Real-time RT-PCR revealed a reduction in cathepsin B mRNA expression from approximately 3-fold (doxorubicin) to 2.2-fold for pretreatment with 1 μ M dexamethasone and to 1.9-fold for pretreatment with 10 μ M dexamethasone, whereas 0.1 μ M dexamethasone had no influence on doxorubicin-mediated cathepsin B mRNA induction (Fig. 7a). Western blot analysis showed that the addition of dexamethasone from 0 to 10 μ M

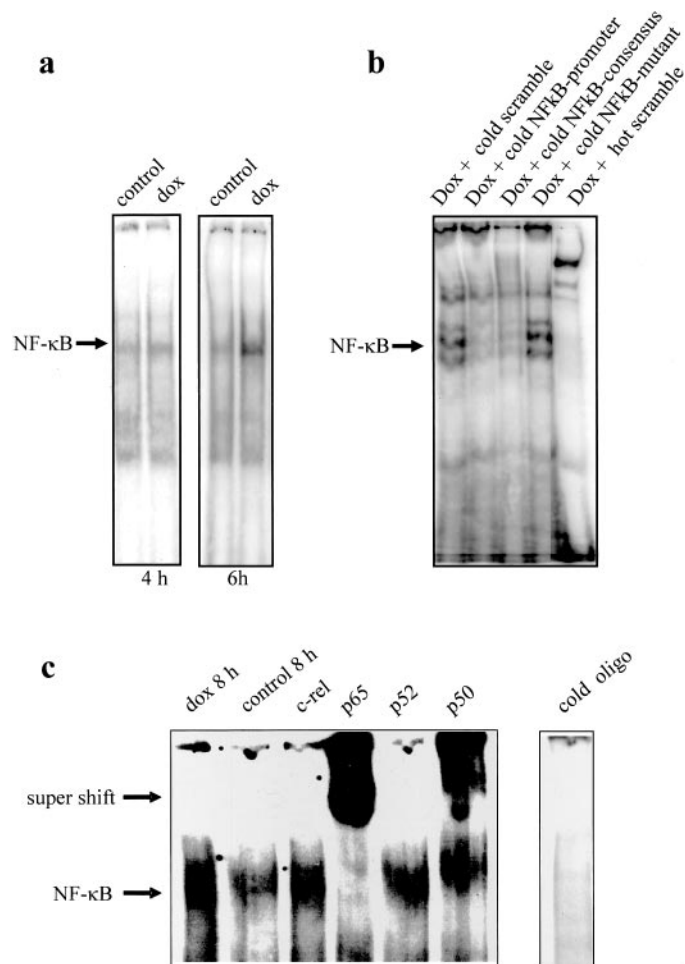


Fig. 6. Doxorubicin induces NF- κ B binding activity in HeLa cells. HeLa cells were cultured in RPMI 1640 medium containing 10% FCS for 72 h. Cells were then incubated with 0.1% DMSO (control) or 1 μ M doxorubicin for 4 and 6 h (a) or 8 h (b and c). Nuclear extract was prepared, and EMSA was performed as described under *Materials and Methods* using a [γ - 32 P]ATP-labeled oligonucleotide containing the potential NF- κ B binding site of the cathepsin B promoter region. b, competition experiments with an unlabeled scramble oligonucleotide, an unlabeled oligonucleotide containing NF- κ B consensus sites, an unlabeled oligonucleotide containing the NF- κ B binding site of the cathepsin B promoter, and an unlabeled mutated NF- κ B oligonucleotide as well as incubation with the radiolabeled scramble oligonucleotide. c, determination of NF- κ B subunit composition. Nuclear extracts of doxorubicin-treated HeLa cells were preincubated with anti-p50, -p52, -p65, or -cRel antibodies before incubation with the [γ - 32 P]ATP-labeled oligonucleotide. Shifts and supershifts were visualized by autoradiography.

resulted in a dose-dependent inhibition of cathepsin B protein expression, with maximal inhibition being achieved at 10 μ M dexamethasone (Fig. 7b). Analysis of cathepsin B activity resulted in approximately 50% reduction with all dexamethasone concentrations used compared with doxorubicin alone (Fig. 7c).

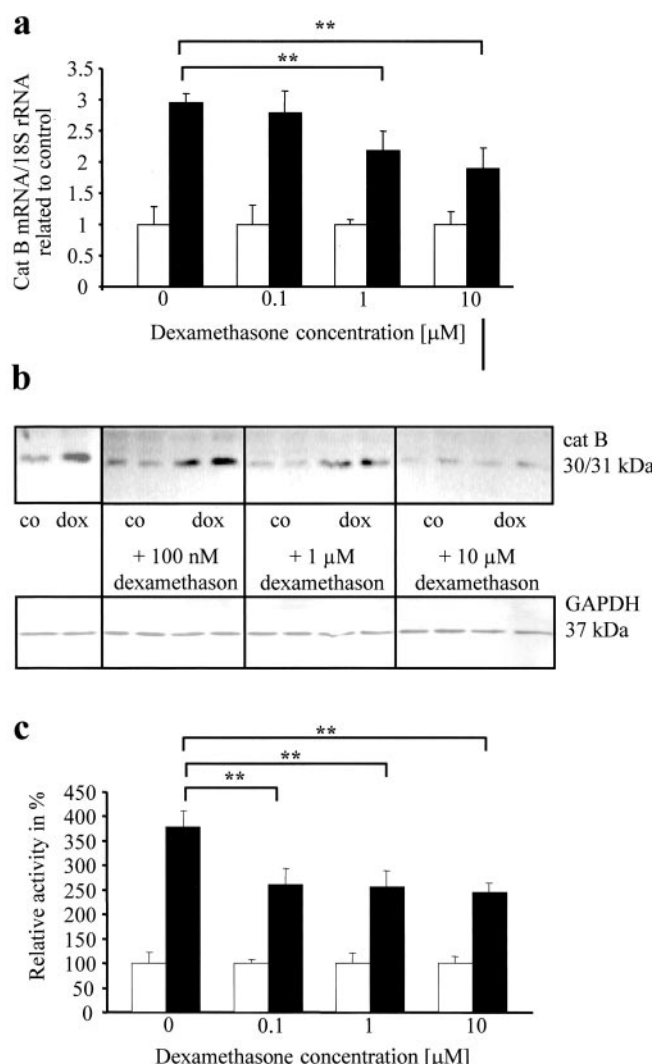


Fig. 7. Dexamethasone decreases doxorubicin-induced cathepsin B expression. HeLa cells were cultured in RPMI 1640 medium supplemented with 10% FCS for 72 h. Cells were then preincubated with dexamethasone (100 nM, 1 μ M, or 10 μ M) for 1 h followed by incubation with DMSO (co, □) or 1 μ M doxorubicin (■) for an additional 24 h (for mRNA quantification, a) or 48 h (cathepsin B protein, b; and cathepsin B activity, c). For quantification of cathepsin B mRNA, total RNA was prepared and reverse-transcribed with random hexamer primers followed by semi-quantitative RT-PCR analysis. 18S rRNA amplification was used for normalization. Data are displayed as relative expression to DMSO-treated control cells ($n = 6$; **, $p < 0.01$). For detection of cathepsin B protein expression, cellular extracts were prepared, and Western blotting was performed as described under *Materials and Methods* using the monoclonal anti-cathepsin B antibody and alkaline phosphatase-conjugated anti-mouse secondary antibody. Bands were visualized by chemiluminescence and exposure to X-ray films. Equal protein loading was controlled by the detection of GAPDH. For the measurement of cathepsin B activity, cellular protein extracts were prepared as described under *Materials and Methods*. By incubation with the specific cathepsin B substrate Z-AMC for 2.5 h, the cathepsin B activity was determined on a microplate reader at 390 (excitation) and 460 nm (emission) ($n = 6$; **, $p < 0.01$).

Induction of Cathepsin B Expression by Daunorubicin and Idarubicin. Effects of the anthracycline antibiotics daunorubicin and idarubicin on cathepsin B expression were investigated to elucidate whether the induction of cathepsin B is limited to doxorubicin. Like doxorubicin, daunorubicin and idarubicin treatment of HeLa cells resulted in a similar induction of cathepsin B expression on mRNA, protein, and activity levels in a dose-dependent manner (Fig. 8). Assessment of cathepsin B mRNA by real-time RT-PCR revealed that daunorubicin and idarubicin led to a doubling of cathepsin B mRNA content after 20 h of treatment (Fig. 8a). Figure 8b shows cathepsin B protein expression detected by Western blot analysis and demonstrates a concentration-dependent increase of cathepsin B protein after treatment of HeLa cells with various concentrations of daunorubicin and idarubicin for 48 h. In accordance with the induction of cathepsin B mRNA and protein, activity also increased from 126 to 289 and 435 μ mol/mg \cdot h after treatment of HeLa cells for 48 h with 333 nM and 1 μ M daunorubicin, respectively, and to 445 and 485 μ mol/mg \cdot h with 333 nM and 1 μ M idarubicin, respectively (Fig. 8c). Treatment with 100 nM of both anthracyclines increased cathepsin B expression to a lesser extent: 186 (daunorubicin) and 224 μ mol/mg \cdot h (idarubicin). Higher concentrations of daunorubicin and idarubicin led to fast cell death and were therefore excluded from further investigations.

Camptothecin, Cisplatin, and Paclitaxel (Taxol) Do Not Induce Cathepsin B Expression in HeLa Cells. To investigate whether the induction of cathepsin B is limited to anthracyclines or whether it is related to other classes of cytostatics, the effects of antitumor compounds with different mechanisms of action were examined. HeLa cells were incubated with various concentrations of the topoisomerase inhibitor camptothecin (1–333 ng/ml), the alkylating agent cisplatin (1–333 μ M), or the mitosis inhibitor paclitaxel (3.3–333 nM) for 48 h. After incubation, cells were harvested and assayed for cathepsin B activity. Under these experimental conditions, no significant increase of cathepsin B activity was seen as a function of concentration and time (data not shown). Similar results were obtained for camptothecin treatment. Furthermore, the cytostatic paclitaxel was also not able to induce cathepsin B expression at the tested concentration range as analyzed by Z-AMC activity assay but resulted in approximately 20% loss of cathepsin B activity (data not shown).

The Effects of Anthracycline on Cathepsin B Expression Are Cell Type-Specific. To determine cell specificity of the anthracycline effect on cathepsin B expression we investigated other cell lines for the inducing effect. Although a variety of cell lines express both NF- κ B and cathepsin B, the screened cells differed in their response to the tested anthracycline concentrations. In contrast to HeLa cells, A549, CCRF/CEM, T47D, human embryonic kidney 293, and ECV cells showed no induction of cathepsin B by either of the tested anthracycline concentrations. In the human colon carcinoma cell line Caco2, a significant 2.5-fold induction of cathepsin B activity and an approximately 3-fold induction of cathepsin B mRNA by doxorubicin treatment was detectable. This could be verified by immunoblot analysis. The human hepatoma cell line HepG2 also responded to anthracyclines in pronounced induction of cathepsin B mRNA, protein, and activity by approximately 3-fold (data not shown).

Discussion

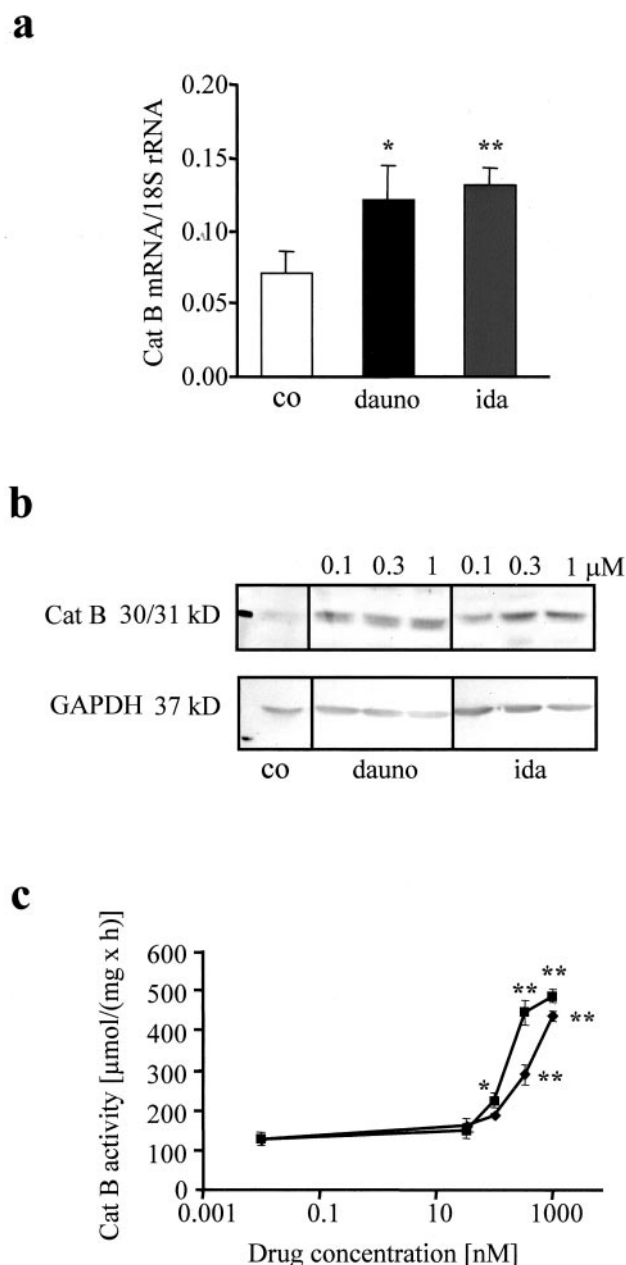


Fig. 8. Induction of cathepsin B expression by daunorubicin (dauno) and idarubicin (ida). HeLa cells were cultured in RPMI 1640 medium containing 10% FCS for 72 h. Cells were then incubated with DMSO (\square), daunorubicin (\blacklozenge), and idarubicin (\blacksquare) at the indicated concentrations for 20 h (cathepsin B mRNA, a) or 48 h (cathepsin B protein, b; and cathepsin B activity, c). For quantification of cathepsin B mRNA, total RNA was prepared and reverse-transcribed with random hexamer primers followed by semiquantitative RT-PCR analysis. 18S rRNA amplification was used for normalization. Data are displayed as relative expression to DMSO-treated control cells ($n = 6$; *, $p < 0.05$; **, $p < 0.01$). For detection of cathepsin B protein expression, cellular extracts were prepared, and Western blotting was performed as described under *Materials and Methods* using the monoclonal anti-cathepsin B antibody and alkaline phosphatase-conjugated anti-mouse secondary antibody. Bands were visualized by chemiluminescence and exposure to X-ray films. Equal protein loading was controlled by detection of GAPDH. For measurement of cathepsin B activity, cellular protein extracts were prepared as described under *Materials and Methods*. By incubation with the specific cathepsin B substrate Z-AMC for 2.5 h, the cathepsin B activity was determined on a microplate reader at 390 (excitation) and 460 nm (emission) ($n = 6$; *, $p < 0.05$; **, $p < 0.01$).

Anthracycline drugs such as doxorubicin remain among the most effective agents for the treatment of solid tumors and hematological malignancies. To overcome their dose-limiting side effects such as cardiac toxicity, there is an intensive effort to develop promising doxorubicin prodrugs that are specifically activated in tumor tissue. One approach is the application of peptide doxorubicin prodrugs like Leu-Dox and *N*- β -alanyl-L-leucyl-L-alanyl-L-leucyl-doxorubicin (Sinhababu and Thakker, 1996; Trouet et al., 2001). Both prodrugs showed improved antitumor efficacy and a decreased toxicity in vivo and in vitro (de Jong et al., 1992; Boyer and Tannock, 1993; Trouet et al., 2001). Activation of these prodrugs is catalyzed by the enzyme cathepsin B. Therefore, regulation of cathepsin B by drugs, especially antitumor agents, could influence the efficacy of peptide prodrugs using this protease.

In the present investigation, effects of the anthracycline antibiotics doxorubicin, daunorubicin, and idarubicin on cathepsin B expression in the human cervix carcinoma cell line HeLa were examined. Exposure to doxorubicin induced a time- and dose-dependent up-regulation of cathepsin B expression on mRNA, protein, and activity levels in vitro at pharmacologically relevant concentrations. Similar effects were obtained for daunorubicin and idarubicin. Proliferation rates of HeLa cells are decreased, and cell-cycle distribution is affected when treated with 1 μ M doxorubicin. However, as determined by cathepsin B inhibition experiments, these cytotoxic effects seem to occur independently of the cathepsin B-inducing effects of doxorubicin, indicating that the regulation of cathepsin B by doxorubicin results directly from transcriptional activation rather than indirectly from cell-cycle regulation.

The 5'-untranslated region of the human cathepsin B gene does not contain a TATA-box and is GC-rich. Therefore, it was initially classified as a housekeeping gene (Berquin et al., 1995). However, further investigations revealed that this protease is regulated at multiple levels, including transcription, post-transcriptional processing, translation, and trafficking by endogenous compounds such as transforming growth factor- β (Gerber et al., 2000), interferon- γ (Li et al., 1998), and granulocyte/macrophage colony-stimulating factor (Ward et al., 1990). Induction of differentiation by differentiating agents such as phorbol esters, calcitriol, sodium butyrate, and retinoic acids resulted in an increased expression of cathepsin B in HL60 cells (Berquin et al., 1999). Transcriptional activation of the cathepsin B promoter by several transcription factors such as USF1, USF2, Sp1, Sp3, and Ets has been reported to play an important role in the regulation of cathepsin B (Yan and Sloane, 2003). The present results indicate for the first time that cathepsin B can be regulated by antineoplastic agents, which opens a new field for enzyme-used drug-targeted therapies.

An induction of cathepsin B by chemotherapeutic substances could have consequences for the success of chemotherapies with peptide prodrugs. As a result of cathepsin B induction, enhanced cleavage of peptide prodrugs may occur, followed by an increased exposure to the cytostatic agent and therefore an increased antitumor efficacy. The particular scenario described in this report is even more favorable because the released active drug up-regulates the expression of

its own activating enzyme. Hence, because of this autoinduction of doxorubicin activation, no additional substance has to be introduced that may increase the risk of interactions and adverse side effects. However, because cathepsin B has been implicated in various diseases including rheumatoid arthritis (Hashimoto et al., 2001), cholestatic liver injury (Guicciardi et al., 2000), and pancreatitis (Halangk et al., 2000), induction of cathepsin B by anthracyclines in nontumoral tissues could increase the risk for adverse side effects at sites other than the tumor. The risk of nontumoral activation of peptide prodrugs, however, is limited by the fact that peptide prodrugs usually are not able to enter the cells and cathepsin B in normal tissue is restricted to the lysosomal compartment.

Anthracyclines are known to be able to mediate NF- κ B activation (Das and White, 1997). Subsequently, a potential NF- κ B binding site could be identified at positions 1456 to 1475 of the cathepsin B gene promoter region (AF086639) by using the programs Genomatix MatInspector (Genomatix Software GmbH, Munich, Germany) and TFSEARCH (Heinemeyer et al., 1998). Therefore, further experiments were carried out to investigate whether NF- κ B is involved in the induction of cathepsin B by doxorubicin. NF- κ B is a dimeric transcription factor that regulates genes associated with stress response such as inflammation, oxidative stress, and apoptosis. In unstimulated cells, NF- κ B is retained in the cytoplasm by interaction with the inhibitory protein I κ B α . Cellular stimuli inactivate I κ B α by phosphorylation, ubiquitination, and proteolytic degradation, which allows NF- κ B to translocate to the nucleus and modulate gene expression. p50, p52, p65 (RelA), and c-Rel are the major components of NF- κ B complexes (Baeuerle and Henkel, 1994). To evaluate the possible role of NF- κ B in the doxorubicin-mediated increase in cathepsin B expression, we pretreated cells with the NF- κ B inhibitors CAPE and HNE. Pretreatment of cells with each of these compounds significantly inhibited the induction of cathepsin B by doxorubicin. Thus, it is most likely that doxorubicin stimulates the activation of NF- κ B and its binding to the NF- κ B response element localized in the cathepsin B promoter region. To further ascertain the role of NF- κ B in doxorubicin-induced cathepsin B expression, we examined the binding of NF- κ B to the potential response element by performing EMSA. These experiments revealed an increase in NF- κ B binding to the potential response element after treatment of HeLa cells with doxorubicin. Analysis of subunits present in the activated complexes of doxorubicin-treated HeLa cells indicated the presence of both p50 and p65 components, which constitute the transcriptionally active NF- κ B heterodimer complex.

Activation of NF- κ B can be the result of many different signaling pathways such as the activation of protein kinase C, which leads to the degradation of the inhibitory protein I κ B and release of active NF- κ B. A role for protein kinase C in the activation of NF- κ B by daunorubicin and doxorubicin was demonstrated by Das and White (1997). Furthermore, phosphatidylinositol-3 kinase/Akt signaling has been implicated in NF- κ B activation (Sizemore et al., 1999). Both protein kinase C and phosphatidylinositol-3 kinase mediate the activation of I κ B kinase α/β and are mainly induced by mitogenic and growth signals. A pathway that is induced by stress and death signals leading to the activation of NF- κ B is mediated by the NF- κ B-inducing kinase (Awane et al., 1999).

NF- κ B-inducing kinase can complex with and activate I κ B kinase resulting in I κ B degradation. The signaling pathways involved in the NF- κ B-mediated induction of cathepsin B by doxorubicin are currently under investigation.

The role of lysosomal proteases in the activation of apoptotic pathways is not clear. However, multiple studies have documented proapoptotic activities of cathepsin B (Guicciardi et al., 2000; Foghsgaard et al., 2001). Vancompernelle and coworkers (1998) suggest that lysosomal proteases such as cathepsin B can directly activate different caspases. Whether cathepsin B plays a role in doxorubicin-mediated apoptosis is currently unknown. In preliminary investigations of the apoptotic events in HeLa cells, we determined the activity of the downstream effector caspase 3 (CPP32). Caspase 3 cleaves a variety of cellular substrates, resulting in apoptotic death. In HeLa cells, we observed that anthracycline treatment caused an increase in caspase 3 activation. Inhibition of cathepsin B by the specific inhibitor CA-074Me diminished caspase 3 activity. These results suggest that NF- κ B-mediated cathepsin B induction in doxorubicin-treated HeLa cells has a proapoptotic stimulus. The specific cathepsin B inhibitor CA-074Me has been described as a proinhibitor that penetrates through cell membranes and is activated by cellular esterases to CA-074 (Buttle et al., 1992). However, effectiveness and selectivity of CA-074Me are discussed controversially in the literature (Jane et al., 2002; Montaser et al., 2002). Therefore, it cannot be ruled out that other cathepsins such as cathepsin L are involved in the proapoptotic effects of doxorubicin as well.

Because cathepsin B has been implicated to play important roles in the complex processes of penetration and degradation of extracellular matrix components, leading to invasion and metastasis of cancer cells (Sloane et al., 1990), it is possible that an induction of cathepsin B could also stimulate the invasiveness and metastatic potential of tumor cells. Results of both in vitro and in vivo models suggest a correlation between cathepsin B expression levels and cancer aggressiveness (Kobayashi et al., 1993; Campo et al., 1994). Down-regulation experiments using antisense and RNA interference technologies have been carried out recently (Zwicky et al., 2002). Inhibition of cathepsin B by antisense oligonucleotides resulted in a decreased cellular motility and invasion of osteosarcoma cells (Krueger et al., 1999) and glioblastoma cells (Mohanam et al., 2001), demonstrating that cathepsin B is involved in the proteolytic processes of invasion. The potential role of NF- κ B in the regulation of prometastatic enzymes was demonstrated by Andela and coworkers (2000), who showed that a blockade of NF- κ B resulted in the down-regulation of prometastatic metalloproteinase-9 and heparinase. Consequently, intravasation of tumor cells was prevented, suggesting that NF- κ B plays a central role in the regulation of tumor metastasis.

Because dexamethasone can inhibit NF- κ B (De Bosscher et al., 1997) and treatment with anthracyclines for acute lymphatic leukemia and non-Hodgkin lymphoma is often combined with dexamethasone, the effect of this glucocorticoid on cathepsin B expression has been analyzed. Because of dexamethasone pretreatment, cathepsin B induction by doxorubicin was reduced. This finding would have clinical consequences for a peptide prodrug-based therapy because the level of cathepsin B activity and subsequent cleavage of peptide prodrugs by this protease would decrease, leading to

diminished therapeutic efficacy. Therefore, in such cases of peptide prodrug therapy, omission of dexamethasone treatment should be considered. Inhibition of the proteasome and hence stabilization of I κ B resulted in the minimization of cathepsin B activation by doxorubicin, confirming the involvement of NF- κ B. Very recently, the proteasome inhibitor bortezomib has been approved for treatment of patients with advanced multiple myeloma. Concomitant administration of doxorubicin and bortezomib may reduce the effect of the anthracycline, particularly during prodrug-based therapies.

Finally, under the experimental conditions applied, induction of cathepsin B by anthracyclines depends on the particular cell type. It might be necessary to determine for each individual cancer type whether cathepsin B is inducible by anthracyclines and whether this induction will be relevant in the apoptotic process and in an increased cytotoxicity of anthracycline derivatives against malignant cells.

In summary, our data clearly indicate that anthracyclines induce cathepsin B via NF- κ B. The data contribute to a better understanding of tumor targeting with peptide prodrugs and help to define a possible mechanism of doxorubicin cytotoxicity in tumor cells.

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