Journal of Cellular Biochemistry 91:621–632 (2004)

Distinctive Regulation and Function of PI 3K/Akt and MAPKs in Doxorubicin-Induced Apoptosis of Human Lung Adenocarcinoma Cells

Yanqiu Zhao,[1,2] Han You,[2] Yu Yang,[1] Lin Wei,[1] Xin Zhang,[1] Libo Yao,[2] Daiming Fan,[2] and Qiang Yu[1,2]*

[1]Pulmonary Center, Department of Medicine, and Department of Biochemistry, Boston University Medical Center, Boston, Massachusetts 02118
[2]The International Molecular Biology Research Center, Fourth Military Medical University, Xian, China 710032

Abstract Regulation and function of PI 3K/Akt and mitogen-activated protein kinases (MAPKs) in doxorubicin-induced cell death were investigated in human lung adenocarcinoma cells. Doxorubicin induced dose-dependent apoptosis of human lung adenocarcinoma NCI-H522 cells. Prior to cell death, both Akt and the MAPK family members (MAPKs: ERK1/2, JNK, and p38) were activated in response to the drug treatment. The kinetics of the inductions for Akt and MAPKs are, however, distinct. The activation of Akt was rapid and transient, activated within 30 min of drug addition, then declined after 3 h, whereas the activations of three MAPKs occurred later, 4 h after addition of the drug and sustained until cell death occurred. Inhibition of PI 3K/Akt activation had no effect on MAPKs' activation, suggesting that the two pathways are independently activated in response to the drug treatment. Inhibition of PI 3K/Akt and p38 accelerated and enhanced doxorubicin-induced cell death. On the contrary, inhibition of ERK1/2 or JNK had no apparent effect on the cell death. Taken together, these results suggest that PI 3K/Akt and MAPKs signaling pathways are all activated, but with distinct mechanisms, in response to doxorubicin treatment. Activation of PI 3K/Akt and p38 modulates apoptotic signal pathways and inhibits doxorubicin-induced cell death. These responses of tumor cells to cancer drug treatment may contribute to their drug resistance. Understanding of the mechanism and function of the responses will be beneficial for the development of novel therapeutic approaches for improvement of drug efficacy and circumvention of drug resistance. J. Cell. Biochem. 91: 621–632, 2004. © 2004 Wiley-Liss, Inc.

Key words: doxorubicin; PI 3K; Akt; MAPK; apoptosis; cancer; lung

Doxorubicin (Adriamycin), an anthracycline antibiotic, is one of the most effective and widely used anti-neoplastic agents in cancer chemotherapy. They have been used in treating a broad range of malignancies including hematopoietic malignancies and various solid tumors [Weiss, 1992]. At physiologically relevant concentrations, doxorubicin induces apoptosis of many types of cancer cells in vitro. Although whether tumor cell apoptosis is the major therapeutic effect of doxorubicin in vivo remains to be proven, understanding the molecular mechanism and regulation of doxorubicin-induced apoptosis is of therapeutic value in designing more effective and less toxic anticancer drugs. The cytotoxic mechanism of doxorubicin is uncertain and is complex. Damage of DNA is generally considered as the primary cause of doxorubicin-induced cell death. The exact mechanism and biochemical events that lead the doxorubicin-induced DNA damage signal to cell death have not been defined. Likewise, factors and biochemical pathways that influence the cytotoxicity of doxorubicin have not been well-characterized. A number of studies have shown that two classes of cell growth and survival/death signaling pathways, the PI 3K/Akt and MAPKs pathways, play important roles in regulating DNA

Yanqiu Zhao and Han You contributed equally to the paper.

Grant sponsor: NIH Grant (from the National Institute of General Medical Sciences); Grant number: R01 GM59678.

*Correspondence to: Qiang Yu, Pulmonary Center, Boston University Medical Center, 715 Albany Street, Boston, MA 02118. E-mail: qyu@lung.bumc.bu.edu

Received 22 July 2003; Accepted 29 September 2003
DOI 10.1002/jcb.10751

© 2004 Wiley-Liss, Inc.

damage agents-induced cell death [Persons et al., 1999; Wang et al., 2000]. But the results of these studies on function and regulation of these enzymes in the anti-cancer drug-induced apoptosis have been controversial. There is very limited information about the regulation and function of the two signaling pathways in doxorubicin-induced apoptosis [Yu et al., 1996; Kang et al., 2000; Guise et al., 2001; Negoro et al., 2001].

PI 3K/Akt signaling pathway is the best-characterized and most prominent pathway in transmission of anti-apoptotic signals in cell survival [Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Kennedy et al., 1997; Kulik et al., 1997]. Overexpression of PI 3K and Akt protect cells from cell death, whereas inhibition of the two enzymes causes increased cell death and decreased cell survival [Plo et al., 1999; Nakashio et al., 2000; Ng et al., 2000, 2001; Yuan et al., 2000; Page et al., 2000b; Brognard et al., 2001; Negoro et al., 2001; Tang et al., 2001]. Overexpression and constitutive activation of Akt have been observed in a number of human cancers including ovarian [Yuan et al., 2000], breast [Nakatani et al., 1999; Page et al., 2000a], prostate [Graff et al., 2000], and lung cancers [Brognard et al., 2001]. Many studies have reported that PI 3K/Akt signaling pathway is involved in regulating chemotherapeutic drug-induced cell death [Plo et al., 1999; Nakashio et al., 2000; Ng et al., 2000, 2001; Yuan et al., 2000; Page et al., 2000b; Brognard et al., 2001; Tang et al., 2001]. Cytotoxic drugs can either inhibit or activate Akt under different conditions. Inhibition of Akt potentiates drug-induced cell death [Plo et al., 1999; Nakashio et al., 2000; Ng et al., 2000, 2001; Yuan et al., 2000; Page et al., 2000b; Brognard et al., 2001; Tang et al., 2001], whereas activation of Akt delays or decreases drug-induced cell death [Plo et al., 1999; Brognard et al., 2001; Del Bufalo et al., 2002; Li et al., 2002]. The function and regulation of PI 3K/Akt signaling pathway in tumor cells in response to doxorubicin treatment have not been studied.

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases comprising extracellular signal-regulated kinase (ERK), p38 MAPK, and c-jun N-terminal kinase (JNK). These enzymes are important components of signaling pathways that transduce extracellular stimuli into intracellular responses. MAPK family members are involved in regulating diverse biological functions including cell proliferation, differentiation, and survival/death.

ERK is activated in response to a variety of mitogenic stimuli. Its major role is to transmit cell growth and differentiation signals. Activation of ERK usually confers a survival advantage to cells. Recent studies have shown that constitutive activation of ERK is associated with neoplastic phenotypes of a large number of human tumor cells [Hoshino et al., 1999; Kim et al., 1999; Morgan et al., 2001]. Many anti-cancer drugs, such as paxlitaxol, cisplatin, and doxorubicin, induce activation of ERK [Wang et al., 2000; Guise et al., 2001; Seidman et al., 2001]. The same drug can also inhibit ERK activation in different cells or under different experimental conditions [Seidman et al., 2001]. The role of ERK in regulating the anti-cancer drug-induced cell death also varies considerably. In most cases, ERK activation protects cells from drug-induced cell death [Seidman et al., 2001], in others, it contributes to cell death [Wang et al., 2000; Guise et al., 2001]. The regulation and function of ERK in lung tumor cells in response to doxorubicin has not been studied.

JNK is preferentially activated by stress signals such as UV irradiation, hyperthermia, inflammatory cytokines, and chemotherapeutic agents. Its activation is most frequently associated with cell death. Many studies have demonstrated that JNK is activated by cytotoxic drugs, and the activation plays a role in drug-induced cell death [Wang et al., 1998; MacKinnon et al., 2000; Mandlekar et al., 2000; Stadheim and Kucera, 2002]. Although there are evidences showing that JNK can be activated by doxorubicin [Yu et al., 1996], its role in regulating doxorubicin-induced cell death is unclear.

p38, like JNK, is activated primarily by cellular stresses. The role of p38 in cell death is somewhat controversial. In some instances, p38 functions as a cytoprotective kinase [Zechner et al., 1998; Communal et al., 2000], in others, it is a cytotoxic kinase [Kang et al., 2000; Sanchez-Prieto et al., 2000; Deschesnes et al., 2001; Hu et al., 2001; Nelson and Fry, 2001]. In cardiomyocytes and NIH 3T3 cells, p38 is activated in response to doxorubicin and is required for doxorubicin-induced apoptosis [Jiang et al., 1996; Kang et al., 2000; Sanchez-Prieto et al., 2000]. The regulation and function

of p38 in doxorubicin-treated human tumor cells have not been studied.

In this study, we sought to examine the regulation and function of PI 3K/Akt and MAPKs signaling pathways in doxorubicin-induced cell death in human lung adenocarcinoma cells. We found that although Akt and the three MAPKs were all activated in response to doxorubicin treatment, the kinetics of the inductions were distinct. Furthermore, we found that only PI 3K/Akt and p38 signaling pathways were functionally involved in regulating doxorubicin-induced apoptosis. The activation of JNK and ERK1/2 had no apparent effect on the induction of cell death. These findings are of therapeutic values in designing new combinational regimens for treating tumors.

MATERIALS AND METHODS

Cell Culture

All human lung adenocarcinoma cell lines were purchased from America Type Culture Collection (Manassas, VA) and were maintained at 37°C with 5% CO_2 in standard culture DMEM medium (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Chemicals and Antibodies

Doxorubicin, the mitogen-activated protein kinase kinase (MEK1/2) inhibitor PD98059, p38 inhibitor SB203580, JNK inhibitor II, and the PI 3K inhibitor LY294002 were purchased from Calbiochem (La Jolla, CA). Stock solutions were prepared in deionized water (doxorubicin) or DMSO (for inhibitors). The highest concentration of DMSO used was 0.2%. Annexin V-FITC Apoptosis Detection Kit was purchased from Oncogene (Boston, MA). Mouse anti-human PARP antibody was purchased from Pharmingen (Temecula, CA), anti-phospho-JNK antibody, horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies were purchased from Sigma (St. Louis, MO); the other antibodies were from Cell Signaling Technology (Beverly, MA). The antibodies were used as recommended by the manufacturers.

Drug Treatment

Exponentially growing cells were seeded 1 day before experiments (2×10^5 cells per ml). Cells were incubated in drug-free medium, and then treated with doxorubicin for various lengths of time. For experiments using combinations of doxorubicin and other inhibitors, cells were pre-incubated with the inhibitors PD98059 (100 μM), SB203580 (20 μM), JNK inhibitor II (40 μM), or Ly294002 (50 μM) for 1 h before addition of doxorubicin, the same volumes of corresponding solvents were added to the controls.

Assays of Cell Survival and Apoptosis

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test was employed to measure cell survival as described [McFadyen et al., 2001]. Briefly, cells were seeded at 5×10^5 per well in 50 μl volume in a 96-well plate (use phenol Red-free media only). After treatment, 5 μl MTT (M5655, Sigma, 5 mg/ml in phenol red-free RPMI) was added to the cultures and incubated at 37°C for 2–4 h (until live cells became blue). Fifty microliters of 0.08 N HCl in isopropanol (1:10 dilution of 0.8 N HCl) was added and the cultures were pipeted up and down vigorously to dissolve all cells. The optical density (OD) was measured in an EL-312 microplate spectrophotometer (Biotek Instruments, Winooski, VT) at 562 and 650 nm. The percentage of cell survival was calculated by dividing the OD of drug-treated cell minus blank by mean OD of control cell minus blank. MTT tests were repeated at least three times from separate cell cultures.

Annexin V assay for apoptosis: after induction of apoptosis, approximately 5×10^5 cells were transferred to a microfuge tube, washed with cold PBS, then resuspended in 0.5 ml cold 1× binding buffer, followed by addition of 1.25 μl Annexin V-FITC. The mixture was incubated for 15 min at room temperature in the dark, then analyzed by Flowcytometry immediately.

DNA laddering assay for apoptosis: cytosolic nucleic acids, which contain both fragmented genomic DNA and RNA, were extracted with a 0.6 ml solution of 0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris (pH7.4), then phenol–chloroform extracted three times, ethanol precipitated and analyzed on a 1.5% agarose gel. The gel was stained with ethidium bromide, and incubated in RNase A containing solution (5 μg/ml) to digest away the RNAs before photographing.

Immunoblotting

Cells were lysed with Laemmli buffer (Sigma). Cell lysates were subjected to 8% SDS–PAGE

and transferred to nitrocellulose membranes. The membranes were incubated for 1 h at room temperature in a blocking solution containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% (v/v) Tween-20, and 3% (w/v) non-fat dry milk. After preincubation, the membranes were incubated with various primary antibodies at 4°C overnight. The antibodies were used as recommended by the manufacturers. The membranes were then washed three times with the blocking solution and incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies, followed by enhanced chemiluminescence (ECL) detection kit from Amersham (Aylesbury, UK).

RESULTS

Doxorubicin Induced Dose-Dependent Cell Death in Human Lung Adenocarcinoma Cells

To understand the regulation and function of PI 3K/Akt and MAPKs in doxorubicin-induced apoptosis in lung cancer cells, we screened six human lung adenocarcinima cell lines for their sensitivity to doxorubicin-induced cell death. After 24 h of 2 μM doxorubicin treatment, the cell death ranged from 19.9 to 99% (Fig. 1). Among the cell lines screened, the NCI-H522 cell line is the most sensitive cell line in response to doxorubicin treatment. We then focused on this cell line for further analysis. Doxorubicin induced dose-dependent cell death of H522 cells (Fig. 2). At the concentrations above 2 μM, most of the cells were dead after 24 h doxorubicin treatment. At low concentration, less than 2 μM, the cells died apparently through apoptosis, as evidenced by the appearance of fragmented DNA in the DNA laddering assay and the cleavage of caspase substrate PARP (Fig. 2B,C). We chose to use 1 μM doxorubicin for subsequent experiments, because it is also the initial plasma concentration in cancer patients undergoing doxorubicin treatment.

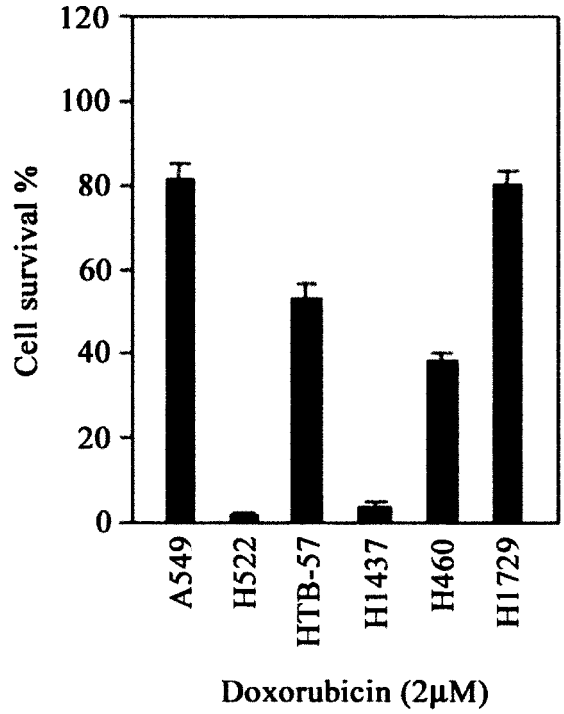

Fig. 1. Induction of cell death by doxorubicin in human lung cancer cells six different human lung cancer cell lines were treated with 2 μM of doxorubicin for 24 h, cell viability was measured by the MTT assay as described in "Materials and Methods." The experiment was repeated three times and the data were the average of triplicates from one experiment.

Doxorubicin Induced Activation of Akt and MAPKs With Distinctive Kinetics

We next analyzed the changes in phosphorylation/activation of Akt, ERK1/2, p38, and JNK during doxorubicin-induced cell death. The phosphorylation of Akt at both Ser-473 and Thr-308 sites were induced at 30 min after addition of 1 μM doxorubicin (Fig. 3). The activation began to decrease after 5–8 h treatment, and then gradually returned to the basal level. The decrease of Akt phosphorylation coincided with the onset of PARP cleavage, which may indicated the onset of apoptosis (Fig. 3). In contrast, the activation of all three MAPKs occurred much later, beginning at 4 h following exposure to doxorubicin (Fig. 3). The increased levels of the MAPKs phosphorylation were maintained throughout the rest of the period of the doxorubicin treatment. The final decrease of the MAPKs phosphorylation at 24 h was likely the consequence of cell death because more than 90% of cells were dead after 24 h drug treatment. These data suggest that the PI 3K/Akt and the MAPKs pathways are all activated by doxorubicin, but the mechanisms of the induction are distinct.

Function of PI 3K and MAPK in Doxorubicin-Induced Apoptosis

To determine whether the activations of Akt and MAPKs are functionally involved in doxorubicin-induced cell death, we inhibited the

activation of Akt and MAPKs by pretreating the cells with their respective inhibitors or inhibitors to their upstream kinases before addition of doxorubicin. Quantitation of cell death with FITC-labeled Annexin V reveled that 24 h after drug treatment, 51% of cells were dead by doxorubicin treatment alone; 92% of cell death with LY294002 co-treatment; and 83% with SB203580 co-treatment. LY294002 alone induced 8% of apoptosis; SB203580, MEK, or JNK inhibitors alone had no apparent effect on cell death, nor did they affect the doxorubicin-induced cell death (Fig. 4A). Cell death induced by doxorubicin alone, as indicated by the cleavage of caspase substrate PARP in Figure 4B, occurred around 12 h. With addition of PI 3K inhibitor Ly294002, which inhibit phosphorylation/activation of its substrate Akt, cell death began to occur as early as at 6 h (Fig. 4B). Addition of p38 inhibitor SB203580 also advanced and increased the induction of cell death by doxorubicin, but to a less extent (Fig. 4B). These results indicate that activations of Akt and p38 function to protect cells from doxorubicin-induced apoptosis. Inhibition of Akt or p38 activation accelerates and increases doxorubicin-induced cell death.

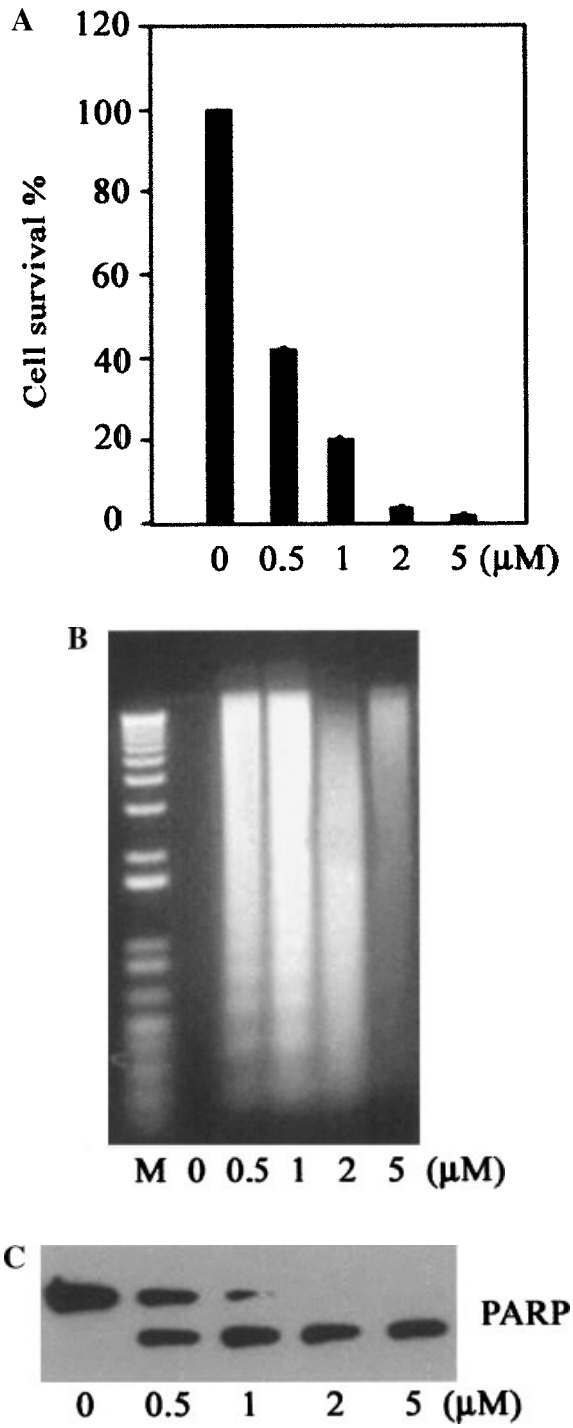

Fig. 2. Induction of cell death by doxorubicin in H522 cells H522 cells were treated with different doses of doxorubicin, as indicated, for 24 h. Cells without drug treatment were used as a control (0). **A**: Cell viability was measured by MTT assay. The experiment was repeated three times and the data were the average of triplicates from one of the experiments. **B**: DNA fragmentation assay for apoptosis. Cytoplasmic DNAs were isolated from the doxorubicin-treated cells and analyzed by agarose gel electrophoresis as described in ''Experimental Procedures.'' Equal amount of DNA was loaded in each lane. **M**: The 1-kb DNA marker. **C**: PARP cleavage was analyzed by Western blotting with an anti-PARP antibody, which detects the intact (116 kDa) and cleaved (85 kDa) products.

PI 3K/Akt and MAPKs Signaling Pathways Were Independently Regulated

Since that Akt activation proceeds the activation of MAPKs and that crosstalk between PI 3K/Akt and MAPKs have been reported previously [Souza et al., 2001; Laprise et al., 2002], we tested whether PI 3K/Akt played a role in regulating the MAPKs activation in response to doxorubicin. We examined the induction of MAPKs phosphorylation by doxorubicin in the presence of the PI 3K inhibitor LY294002. Inhibition of PI 3K had no apparent effects on the doxorubicin-induced phosphorylation of the MAPKs (Fig. 5), suggesting that activation of Akt and the MAPKs were independent events and are through distinct mechanisms.

It has been reported that a late induction of the MAPKs by cisplatin requires de novo protein synthesis [Persons et al., 1999]. We, therefore, tested whether the induction of the MAPKs by doxorubicin also depended on de

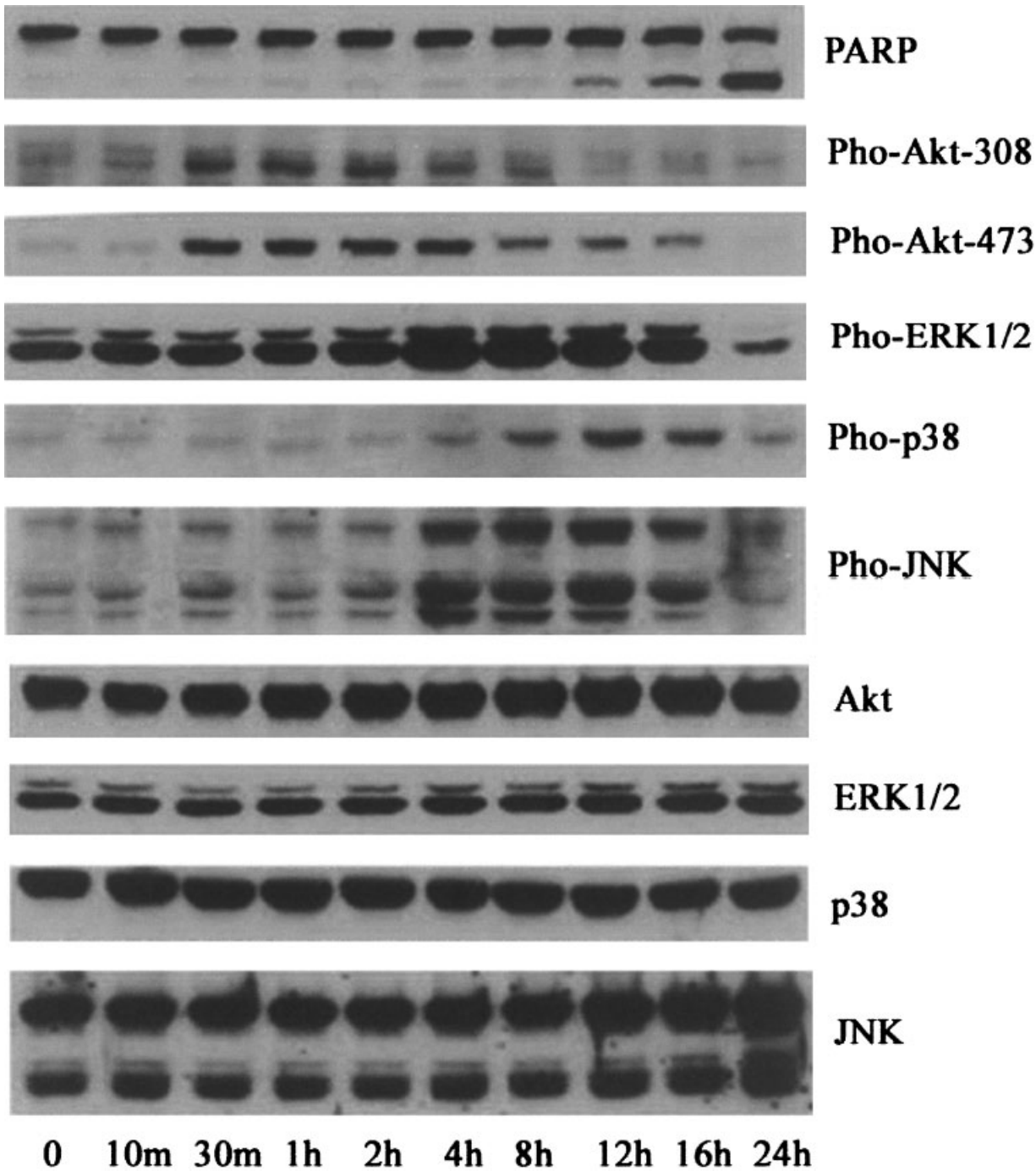

Fig. 3. Induction of Akt and mitogen-activated protein kinases (MAPKs) activation by doxorubicin H522 cells were exposed to 1 μM doxorubicin for indicated time periods. Cells were lysed and the cell lysates were resolved by SDS–PAGE. Akt and MAPKs activation was analyzed by Western blotting with anti-phospho-Akt, anti-phospho-Erk, anti-phospho-p38, or anti-phospho-JNK specific antibodies as indicated. Equal protein loading was confirmed by probing the membranes with antibodies detecting the respective unphosphorylated proteins.

novo protein synthesis. We treated cells with doxorubicin in the presence of protein synthesis inhibitor cycloheximide. As shown in Figure 6, cycloheximide inhibited the phosphorylation of ERK whether or not doxorubicin was present, but it did not affect the induction of phosphorylation of Akt and MAPKs by doxorubicin. These data suggested that induction of Akt and MAPKs phosphorylation by doxorubicin does not require new protein synthesis.

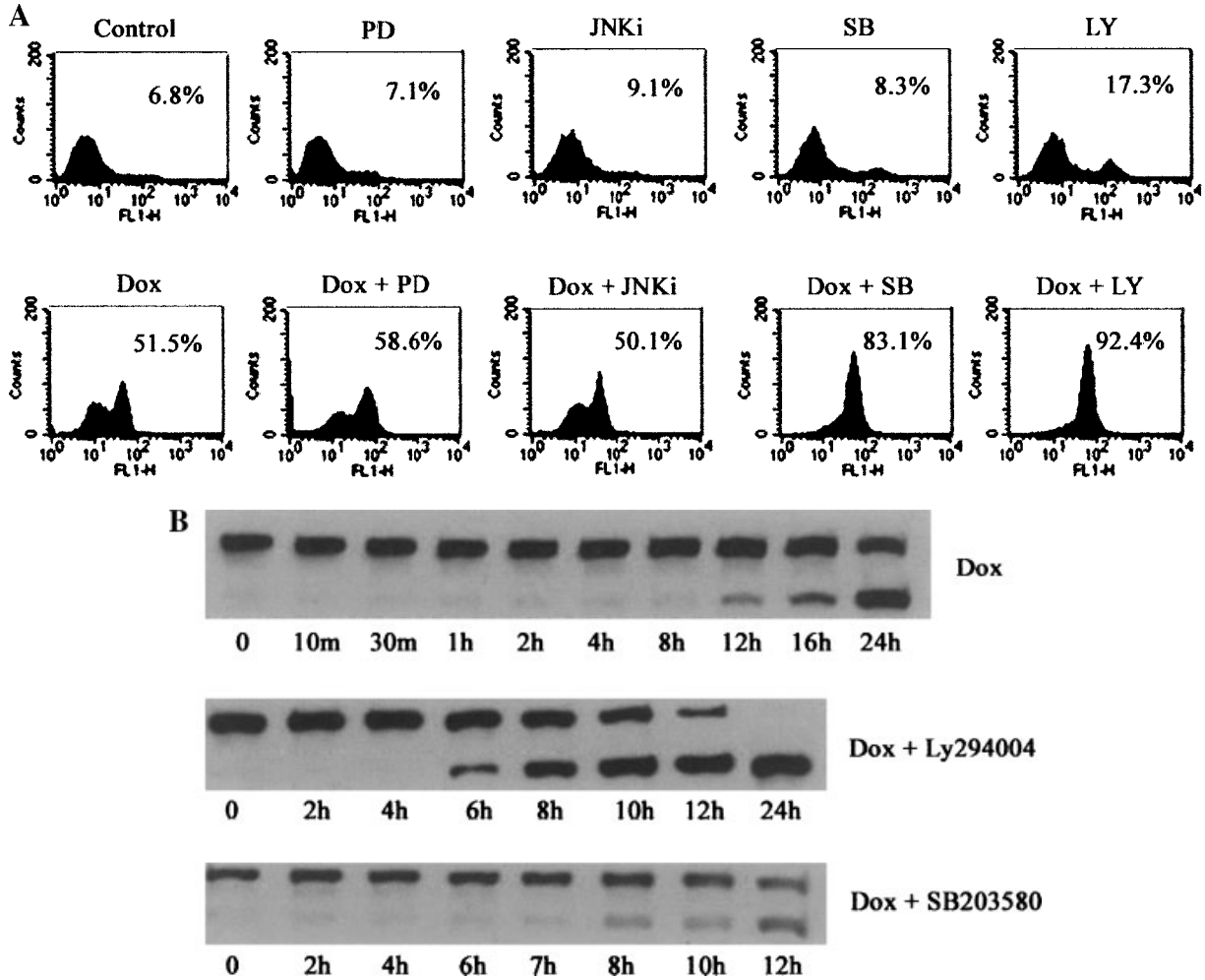

Fig. 4. Effects of PI 3K inhibitor and MAPKs inhibitors on doxorubicin-induced cell apoptosis in H522 cells (**A**). H522 cells were treated with either PD98059 (100 μM), SB203580 (20 μM), JNK inhibitor II (JNKi) (40 μM), or LY294002 (50 μM) alone for 24 h, or pretreated with the signaling molecule inhibitors for 1 h, then treated with doxorubicin (1 μM) for 24 h. Cell death was analyzed by Annexin V binding assay as described in ''Experimental Procedures.'' Percentages of cell death (Annexin V positive cells in the second peak) are indicated. Cells without drug treatment were used as a control. **B**: PI 3K inhibitor and p38 inhibitor accelerated cell death caused by doxorubicin. H522 cells were treated with doxorubicin (1 μM) for the indicated time periods in the absence or presence of pretreatment of SB203580 (20 μM) or LY294002 (50 μM) for 1 h, then analyzed by Western blotting with anti-PARP antibody.

DISCUSSION

Doxorubicin is one of the most effective and widely used anti-neoplastic agents in cancer chemotherapy. It has been used in treating leukemia, lymphomas, and a variety of carcinomas. Its effect on lung cancer cells has not been well studied. One of the notable consequences of doxorubicin treatment is the induction of apoptosis. A number of studies indicate that the range of dose of doxorubicin inducing apoptosis varies depending on the types of cancer cells. In general, doxorubicin induces apoptosis at low concentrations, less than 5 μM, but induces necrosis at higher concentrations [Ling et al., 1993; Skladanowski and Konopa, 1993; Zaleskis et al., 1994]. Our data are consistent with the previous reports. Apoptosis in the doxorubicin-treated lung carcinoma cells is more prominent at low concentrations than that at higher concentrations.

The effects of chemotherapeutic agents on ERK phosphorylation vary considerably depending on the cell types as well as the types of agents. They can increase, decrease, or have no effect on the activation of ERK [Osborn et al., 1996; Yu et al., 1996; Persons et al., 1999; Wang et al., 2000; Guise et al., 2001; Liu et al., 2001].

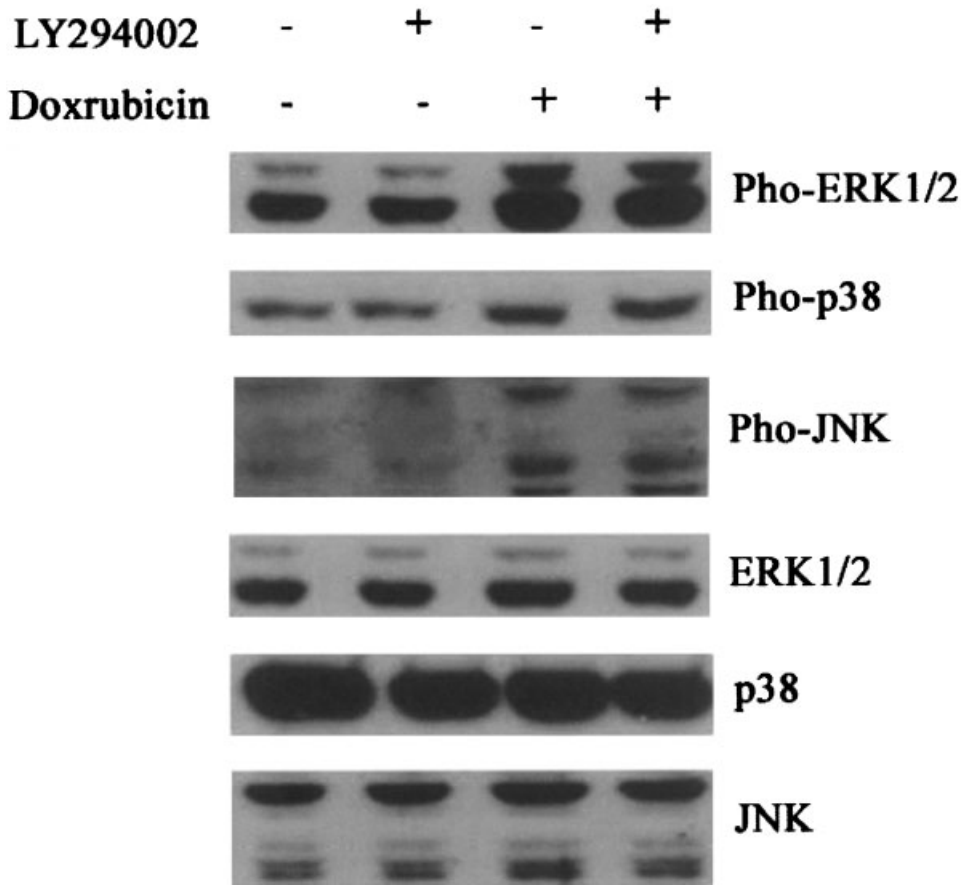

Fig. 5. Effect of inhibition of PI 3K on the phosphorylation of the MAPKs H522 cells were treated with doxorubicin (1 μM) for 10 h with or without LY294002 pretreatment. The cell lysates were then analyzed by Western blotting analysis using antibodies against phosphorylated (pho-) or unphosphorylated MAPKs as indicated. The anti-phospho-JNK and anti-JNK antibodies recognize both JNK1 and JNK2.

Likewise, the functions of ERK activation in the drug-induced cell death also differ from experiments to experiments [Persons et al., 1999; Wang et al., 2000; Liu et al., 2001]. There are very limited studies on the regulation and function of ERK in doxorubicin-induced cell death [Osborn et al., 1996; Yu et al., 1996; Guise et al., 2001]. Two reports showed that doxorubicin had no effect on ERK activation in KB-3 carcinoma cells and H9 leukemia cells, respectively [Osborn et al., 1996; Yu et al., 1996]. In our experiments, doxorubicin induced a delayed activation of ERK in human lung adenocarcinoma H522 cells. Inhibition of ERK had no significant effect on the doxorubicin-induced cell death. Doxorubicin has also been reported to induce ERK activation in neuroblastoma SK-N-SH cells [Guise et al., 2001]. But the activation of EKR in the neuroblastoma cell line appeared to enhance apoptosis since inhibition of ERK reduced the doxorubicin-induced apoptosis [Guise et al., 2001]. We noticed a difference in the timing of induction in the two cell lines. The induction of ERK activation in the neuroblastoma cells occurred as early as 2 h after the addition of doxorubicin (earlier time points were not analyzed in that study). In our study, the activation of ERK did not occur until 4 h after the drug addition. This difference may indicate different induction mechanisms and different factors that are involved in the two cell lines. Therefore, the effects of the ERK activation on the drug-induced cell death in the two cell lines may differ. This is again another example that the activation and function of ERK in response to anti-cancer agents are cell-type and agent dependent.

JNK is activated by many cytotoxic drugs and the activation generally plays a role in mediating the drug-induced cell death [Wang et al., 1998; MacKinnon et al., 2000; Mandlekar et al., 2000; Stadheim and Kucera, 2002]. Although

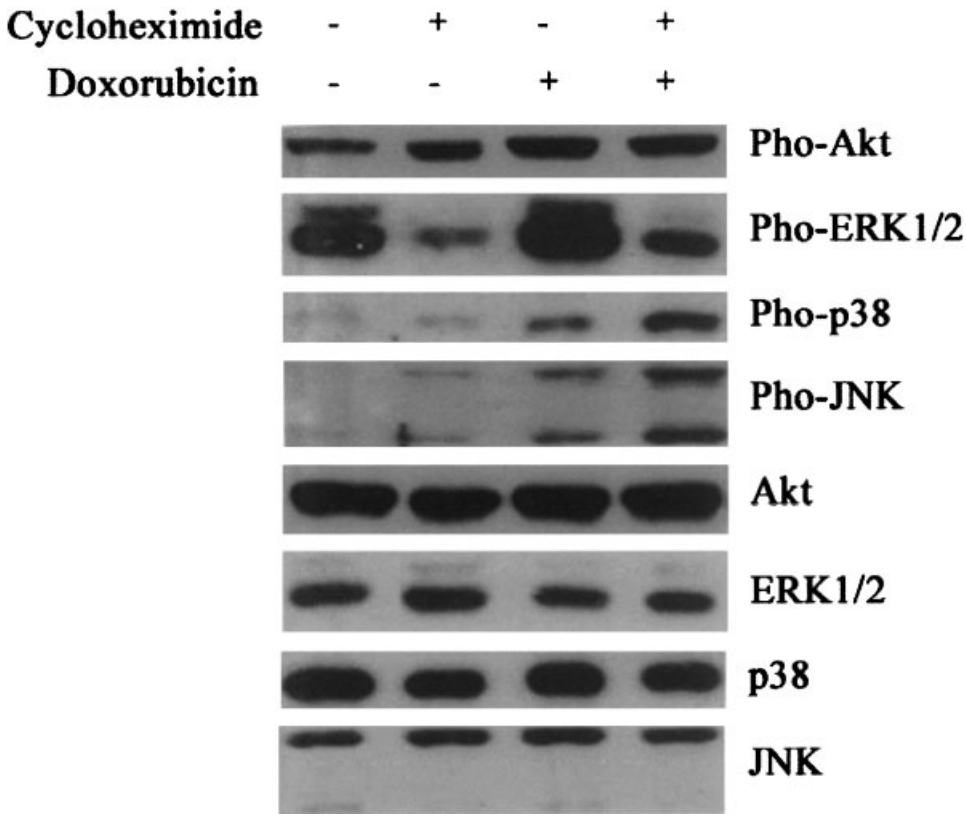

Fig. 6. Effects of inhibition of de novo protein synthesis on activation of Akt and MAPKs induced by doxorubicin in H522 cells H522 cells were treated with 1 μg/ml of cycloheximide for 1 h prior to doxorubicin (1 μM) exposure for 1 h (for Akt analysis) or 10 h (for MAPKs analysis). The cell lysates were then analyzed by Western blotting analysis using antibodies against phosphorylated (pho-) or unphosphorylated Akt or MAPKs as indicated.

there have been reports showing that JNK is activated by doxorubicin [Osborn et al., 1996; Yu et al., 1996], its role in regulating doxorubicin-induced cell death is unclear. Our data showed that both JNK1 and JNK2 were activated by doxorubicin. But inhibition of JNK activation did not affect the doxorubicin-induced apoptosis. Similar to ERK, the activation of the JNKs induced by doxorubicin was delayed. It has been suggested that the activation time course of JNK and the status of other survival factors are important for determining the outcome of JNK activation [Barr and Bogoyevitch, 2001]. The simultaneous induction of ERK, p38 and perhaps other unidentified pro-survival factors by doxorubicin may play a role in affecting the function of JNK in doxorubicin-induced cell death.

p38 was also activated, with the same induction kinetics as the other two MAPKs, by doxorubicin. Inhibition of p38 activation increased doxorubicin-induced apoptosis, suggesting that p38 plays an anti-apoptotic role in the doxorubicin-induced cell death. p38 has been shown to either stimulate or inhibit apoptosis in various cell lines under different stimuli [Nemoto et al., 1998; Zechner et al., 1998; Communal et al., 2000; Kang et al., 2000; Sanchez-Prieto et al., 2000; Deschesnes et al., 2001; Hu et al., 2001]. The reason for the contradictory roles of p38 is not clear. Distinct functions of different isoforms of p38 have been suggested to explain the seemingly contradictory role of p38 [Nemoto et al., 1998]. The differential effects of p38 inhibition on apoptosis in various cell types may be due to the heterogeneity of the expression and/or activation of various p38 kinase isoforms [Wang et al., 1998; Dashti et al., 2001]. Activation of p38 by doxorubicin has been reported in cardiomyocytes and NIH 3T3 cells [Kang et al., 2000; Sanchez-Prieto et al., 2000]. In both cases, the activation of p38 by doxorubicin played pro-apoptotic roles [Kang et al., 2000; Sanchez-Prieto et al., 2000], which are contradictory to our observation. It is possible that different isoforms of p38 were activated in different cell types. On this notion, it is worth mentioning

that both the cardiomyocytes and NIH 3T3 cells are non-transformed cells, whereas the H522 cell line in the present study is a tumor cell line. There could be a difference in the expression of different isoforms of p38 between non-transformed versus transformed cells. Alternatively, the differences in the presence and level of other cell survival factors in tumor versus non-tumor cells may influence the function of p38 in the drug-induced apoptosis.

Akt is a key player in mediating cell survival signals from extracellular stimuli. The function and regulation of Akt in cell death control have been extensively studied. Overexpression and/or constitutive activation of Akt have been observed in a number of cancer cells [Graff et al., 2000; Yuan et al., 2000; Brognard et al., 2001]. Inhibition of Akt activation generally enhances anti-cancer drug-induced apoptosis in the cancer cells [Plo et al., 1999; Nakashio et al., 2000; Ng et al., 2000, 2001; Yuan et al., 2000; Page et al., 2000b; Tang et al., 2001]. There are only limited studies on the regulation of Akt in response to anti-cancer drug treatment [Plo et al., 1999; Nakashio et al., 2000; Negoro et al., 2001; Tang et al., 2001]. Two of them showed an increase of Akt activation in response to daunorubicin [Plo et al., 1999], staurosporine, and etoposide [Tang et al., 2001], whereas the other two showed an inactivation of Akt by doxorubicin [Negoro et al., 2001] and topotecan [Nakashio et al., 2000]. By comparing these studies, we noticed a difference in the time points at which the phosphorylations of Akt were analyzed. In the studies that showed an increase of Akt phosphorylation in response to the drug treatment, the increased phosphorylations of Akt occurred transiently. It was induced within minutes after addition of the drugs, and was then decreased prior to cell death. On the other hand, in the studies that showed an inhibition of Akt phosphorylation by the drugs, the phosphorylation of Akt were only analyzed at late stages of the drug treatment, 12 and 16 h after drug addition, respectively. The status of Akt phosphorylation at earlier time points was not analyzed in these two studies. In the present study, we also observed a transient activation of Akt in response to doxorubicin, which lasted less than 10 h. After 10 h, the phosphorylation of Akt returned to its basal level and cell death began. Inhibition of the Akt activation accelerated and increased the doxorubicin-induced cell death. Therefore, these seemingly contradictory observations may in fact not be different from each other. It is likely that in all of these cases, the activation of Akt is a transient response of the cells to an apoptotic stimulus, which functions as a self-defense mechanism to protect cells from cell death. An eventual decrease in the Akt phosphorylation may be necessary for the induction of apoptosis in these cells.

The mechanisms that regulate the induction of the Akt and the MAPKs in response to doxorubicin treatment are not clear at present. But the mechanisms that regulate the Akt induction are apparently distinct from that of the MAPKs. Induction of Akt was quick, within minutes of drug addition, whereas the induction of the MAPKs was a delayed response and was not regulated by PI 3K/Akt. A better understanding of the signal transduction pathways that modulate the doxorubicin response will be beneficial in the development of novel therapeutic approaches for improvement of doxorubicin efficacy and circumvention of doxorubicin resistance.

REFERENCES

Barr RK, Bogoyevitch MA. 2001. The c-Jun N-terminal protein kinase family of mitogen-activated protein kinases (JNK MAPKs). Int J Biochem Cell Biol 33: 1047–1063.

Brognard J, Clark AS, Ni Y, Dennis PA. 2001. Akt/protein kinase B is constitutively active in non-small cell lung cancer cells and promotes cellular survival and resistance to chemotherapy and radiation. Cancer Res 61: 3986–3997.

Communal C, Colucci WS, Singh K. 2000. p38 mitogen-activated protein kinase pathway protects adult rat ventricular myocytes against beta-adrenergic receptor-stimulated apoptosis. Evidence for Gi-dependent activation. J Biol Chem 275:19395–19400.

Dashti SR, Efimova T, Eckert RL. 2001. MEK6 regulates human involucrin gene expression via a p38alpha- and p38delta-dependent mechanism. J Biol Chem 276:27214–27220.

Del Bufalo D, Di Castro V, Biroccio A, Varmi M, Salani D, Rosano L, Trisciuoglio D, Spinella F, Bagnato A. 2002. Endothelin-1 protects ovarian carcinoma cells against paclitaxel-induced apoptosis: Requirement for Akt activation. Mol Pharmacol 61:524–532.

Deschesnes RG, Huot J, Valerie K, Landry J. 2001. Involvement of p38 in apoptosis-associated membrane blebbing and nuclear condensation. Mol Biol Cell 12: 1569–1582.

Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME. 1997. Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 275:661–665.

Graff JR, Konicek BW, McNulty AM, Wang Z, Houck K, Allen S, Paul JD, Hbaiu A, Goode RG, Sandusky GE,

Vessella RL, Neubauer BL. 2000. Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27Kip1 expression. J Biol Chem 275:24500–24505.

Guise S, Braguer D, Carles G, Delacourte A, Briand C. 2001. Hyperphosphorylation of tau is mediated by ERK activation during anticancer drug-induced apoptosis in neuroblastoma cells. J Neurosci Res 63:257–267.

Hoshino R, Chatani Y, Yamori T, Tsuruo T, Oka H, Yoshida O, Shimada Y, Ari-i S, Wada H, Fujimoto J, Kohno M. 1999. Constitutive activation of the 41-/43-kDa mitogen-activated protein kinase signaling pathway in human tumors. Oncogene 18:813–822.

Hu X, Janssen WE, Moscinski LC, Bryington M, Dangsupa A, Rezai-Zadeh N, Babbin BA, Zuckerman KS. 2001. An IkappaBalpha inhibitor causes leukemia cell death through a p38 MAP kinase-dependent, NF-kappaB-independent mechanism. Cancer Res 61:6290–6296.

Jiang Y, Chen C, Li Z, Guo W, Gegner JA, Lin S, Han J. 1996. Characterization of the structure and function of a new mitogen-activated protein kinase (p38beta). J Biol Chem 271:17920–17926.

Kang YJ, Zhou ZX, Wang GW, Buridi A, Klein JB. 2000. Suppression by metallothionein of doxorubicin-induced cardiomyocyte apoptosis through inhibition of p38 mitogen-activated protein kinases. J Biol Chem 275:13690–13698.

Kauffmann-Zeh A, Rodriguez-Viciana P, Ulrich E, Gilbert C, Coffer P, Downward J, Evan G. 1997. Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. Nature 385:544–548.

Kennedy SG, Wagner AJ, Conzen SD, Jordan J, Bellacosa A, Tsichlis PN, Hay N. 1997. The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. Genes Dev 11:701–713.

Kim SC, Hahn JS, Min YH, Yoo NC, Ko YW, Lee WJ. 1999. Constitutive activation of extracellular signal-regulated kinase in human acute leukemias: Combined role of activation of MEK, hyperexpression of extracellular signal-regulated kinase, and downregulation of a phosphatase, PAC1. Blood 93:3893–3899.

Kulik G, Klippel A, Weber MJ. 1997. Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. Mol Cell Biol 17: 1595–1606.

Laprise P, Chailler P, Houde M, Beaulieu JF, Boucher MJ, Rivard N. 2002. Phosphatidylinositol 3-kinase controls human intestinal epithelial cell differentiation by promoting adherens junction assembly and p38 MAPK activation. J Biol Chem 277:8226–8234.

Li B, Desai SA, MacCorkle-Chosnek RA, Fan L, Spencer DM. 2002. A novel conditional Akt 'survival switch' reversibly protects cells from apoptosis. Gene Ther 9: 233–244.

Ling YH, Priebe W, Perez-Soler R. 1993. Apoptosis induced by anthracycline antibiotics in P388 parent and multidrug-resistant cells. Cancer Res 53:1845–1852.

Liu XM, Wang LG, Kreis W, Budman DR, Adams LM. 2001. Differential effect of vinorelbine versus paclitaxel on ERK2 kinase activity during apoptosis in MCF-7 cells. Br J Cancer 85:1403–1411.

MacKinnon AC, Waters C, Rahman I, Harani N, Rintoul R, Haslett C, Sethi T. 2000. [Arg(6), D-Trp(7,9), *N*(me)Phe(8)]-substance P (6-11) (antagonist G) induces AP-1 transcription and sensitizes cells to chemotherapy. Br J Cancer 83:941–948.

Mandlekar S, Yu R, Tan TH, Kong AN. 2000. Activation of caspase-3 and c-Jun NH2-terminal kinase-1 signaling pathways in tamoxifen-induced apoptosis of human breast cancer cells. Cancer Res 60:5995–6000.

McFadyen MC, McLeod HL, Jackson FC, Melvin WT, Doehmer J, Murray GI. 2001. Cytochrome P450 CYP1B1 protein expression: A novel mechanism of anticancer drug resistance. Biochem Pharmacol 62:207–212.

Morgan MA, Dolp O, Reuter CW. 2001. Cell-cycle-dependent activation of mitogen-activated protein kinase kinase (MEK-1/2) in myeloid leukemia cell lines and induction of growth inhibition and apoptosis by inhibitors of RAS signaling. Blood 97:1823–1834.

Nakashio A, Fujita N, Rokudai S, Sato S, Tsuruo T. 2000. Prevention of phosphatidylinositol 3′-kinase-Akt survival signaling pathway during topotecan-induced apoptosis. Cancer Res 60:5303–5309.

Nakatani K, Thompson DA, Barthel A, Sakaue H, Liu W, Weigel RJ, Roth RA. 1999. Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. J Biol Chem 274: 21528–21532.

Negoro S, Oh H, Tone E, Kunisada K, Fujio Y, Walsh K, Kishimoto T, Yamauchi-Takihara K. 2001. Glycoprotein 130 regulates cardiac myocyte survival in doxorubicin-induced apoptosis through phosphatidylinositol 3-kinase/Akt phosphorylation and Bcl-xL/caspase-3 interaction. Circulation 103:555–561.

Nelson JM, Fry DW. 2001. Akt, MAPK (Erk1/2), and p38 act in concert to promote apoptosis in response to ErbB receptor family inhibition. J Biol Chem 276:14842–14847.

Nemoto S, Xiang J, Huang S, Lin A. 1998. Induction of apoptosis by SB202190 through inhibition of p38beta mitogen-activated protein kinase. J Biol Chem 273: 16415–16420.

Ng SSW, Tsao MS, Chow S, Hedley DW. 2000. Inhibition of phosphatidylinositide 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells. Cancer Res 60:5451–5455.

Ng SS, Tsao MS, Nicklee T, Hedley DW. 2001. Wortmannin inhibits pkb/akt phosphorylation and promotes gemcitabine antitumor activity in orthotopic human pancreatic cancer xenografts in immunodeficient mice. Clin Cancer Res 7:3269–3275.

Osborn MT, Chambers TC, Cloud-Heflin BA, McMasters RA. 1996. Role of the stress-activated/c-Jun NH2-terminal protein kinase pathway in the cellular response to adriamycin and other chemotherapeutic drugs. Expression, subcellular distribution, and response to phorbol esters of protein kinase C (PKC) isozymes in drug-sensitive and multidrug-resistant KB cells evidence for altered regulation of PKC-alpha. J Biol Chem 271: 30950–30955.

Page C, Huang M, Jin X, Cho K, Lilja J, Reynolds RK, Lin J. 2000a. Elevated phosphorylation of AKT and Stat3 in prostate, breast, and cervical cancer cells. Int J Oncol 17: 23–28.

Page C, Lin HJ, Jin Y, Castle VP, Nunez G, Huang M, Lin J. 2000b. Overexpression of Akt/AKT can modulate chemotherapy-induced apoptosis. Anticancer Res 20: 407–416.

Persons DL, Yazlovitskaya EM, Cui W, Pelling JC. 1999. Cisplatin-induced activation of mitogen-activated protein kinases in ovarian carcinoma cells: Inhibition of extracellular signal-regulated kinase activity increases sensitivity to cisplatin. Clin Cancer Res 5: 1007–1014.

Plo I, Bettaieb A, Payrastre B, Mansat-De Mas V, Bordier C, Rousse A, Kowalski-Chauvel A, Laurent G, Lautier D. 1999. The phosphoinositide 3-kinase/Akt pathway is activated by daunorubicin in human acute myeloid leukemia cell lines. FEBS Lett 452:150–154.

Sanchez-Prieto R, Rojas JM, Taya Y, Gutkind JS. 2000. A role for the p38 mitogen-acitvated protein kinase pathway in the transcriptional activation of p53 on genotoxic stress by chemotherapeutic agents. Cancer Res 60:2464–2472.

Seidman R, Gitelman I, Sagi O, Horwitz SB, Wolfson M. 2001. The role of ERK 1/2 and p38 MAP-kinase pathways in taxol-induced apoptosis in human ovarian carcinoma cells. Exp Cell Res 268:84–92.

Skladanowski A, Konopa J. 1993. Adriamycin and daunomycin induce programmed cell death (apoptosis) in tumour cells. Biochem Pharmacol 46:375–382.

Souza K, Maddock DA, Zhang Q, Chen J, Chiu C, Mehta S, Wan Y. 2001. Arsenite activation of P13K/AKT cell survival pathway is mediated by p38 in cultured human keratinocytes. Mol Med 7:767–772.

Stadheim TA, Kucera GL. 2002. c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for mitoxantrone- and anisomycin-induced apoptosis in HL-60 cells. Leuk Res 26:55–65.

Tang D, Okada H, Ruland J, Liu L, Stambolic V, Mak TW, Ingram AJ. 2001. Akt is activated in response to an apoptotic signal. J Biol Chem 276:30461–30466.

Wang TH, Wang HS, Ichijo H, Giannakakou P, Foster JS, Fojo T, Wimalasena J. 1998. Microtubule-interfering agents activate c-Jun N-terminal kinase/stress-activated protein kinase through both Ras and apoptosis signal-regulating kinase pathways. J Biol Chem 273:4928–4936.

Wang X, Martindale JL, Holbrook NJ. 2000. Requirement for ERK activation in cisplatin-induced apoptosis. J Biol Chem 275:39435–39443.

Weiss RB. 1992. The anthracyclines: Will we ever find a better doxorubicin? Semin Oncol 19:670–686.

Yu R, Shtil AA, Tan TH, Roninson IB, Kong AN. 1996. Adriamycin activates c-jun N-terminal kinase in human leukemia cells: A relevance to apoptosis. Cancer Lett 107:73–81.

Yuan ZQ, Sun M, Feldman RI, Wang G, Ma X, Jiang C, Coppola D, Nicosia SV, Cheng JQ. 2000. Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. Oncogene 19:2324–2330.

Zaleskis G, Berleth E, Verstovsek S, Ehrke MJ, Mihich E. 1994. Doxorubicin-induced DNA degradation in murine thymocytes. Mol Pharmacol 46:901–908.

Zechner D, Craig R, Hanford DS, McDonough PM, Sabbadini RA, Glembotski CC. 1998. MKK6 activates myocardial cell NF-kappaB and inhibits apoptosis in a p38 mitogen-activated protein kinase-dependent manner. J Biol Chem 273:8232–8239.