Journal of Cellular Biochemistry 96:810–820 (2005)

Amplification Loop Cascade for Increasing Caspase Activity Induced by Docetaxel

Dongxu Cao, Bin Qiao, Zhiqiang Ge, and Yingjin Yuan*

Department of Pharmaceutical Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, Peoples' Republic of China

Abstract The hierarchy of events accompanying induction of apoptosis by the microtubule inhibitor docetaxel was investigated in HL-60 human leukemia cells. Treatment of HL-60 cells with docetaxel resulted in the production of reactive oxygen species (ROS), activation of caspase-3 (-like) protease, c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) activation, bcl-2 phosphorylation and apoptosis. Docetaxel elicited ROS production from NADPH oxidase as demonstrated by specific oxidase inhibitor diphenylene iodonium (DPI). ROS mediated the caspase-3 activation and apoptosis in HL-60 cells. The caspase inhibitor acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) effectively inhibited JNK/SAPK activation, bcl-2 phosphorylation and partially attenuated the ROS production induced by docetaxel. Docetaxel-induced bcl-2 phosphorylation was completely blocked by expression of dominant negative JNK or the JNK/SAPK inhibitor SP600125. Overexpression of bcl-2 partially prevented docetaxel-mediated ROS production and subsequent caspase-3 activation, thereby inhibiting apoptotic cell death. It is thus conferred that such sequent events as ROS production, caspase activation, JNK/SAPK activation, bcl-2 phosphorylation and the further generation of ROS should be parts of an amplification loop to increase caspase activity, thereby facilitating the apoptotic cell death process. J. Cell. Biochem. 96: 810–820, 2005. © 2005 Wiley-Liss, Inc.

Key words: apoptosis; docetaxel; ROS; caspase-3; JNK/SAPK; Bcl-2

c-Jun N-terminal kinase (JNK), a stress-activated protein kinase (SAPK), is a member of mammalian mitogen-activated protein kinase (MAPK) family that mediates intracellular signals originated from diverse extracellular stimuli, including growth factors, cytokines, UV light, heat shock, and a variety of anti-cancer drugs [Osborn and Chambers, 1996; Willaime-Morawek et al., 2003; Sethi and Sodhi, 2004]. The activation of JNK/SAPK involves a protein kinase cascade where mitogen-activated protein kinase kinase-1 (MEKK1) [Davis, 2000] activates SAPK kinase-1 (SEK1) and ultimately activates JNK/SAPK. The JNK/SAPK signaling pathways have been mechanistically implicated in the apoptotic response of cells exposed to stresses [Ip and Davis, 1998]. JNK/SAPK is required for stress-induced neuronal apoptosis [Dickens et al., 1997; Willaime-Morawek et al., 2003]. However, the role of JNK/SAPK in apoptosis of other cell types is controversial and several studies indicated that JNK/SAPK might not mediate apoptotic signaling [Watanabe et al., 2002; Du et al., 2004; Nishitai et al., 2004]. On the other hand, apoptosis itself can be considered as a form of stress, thus, JNK/SAPK activation may be regarded as a stress response secondary to apoptosis, rather than a primary mediator in apoptotic pathways [Ip and Davis, 1998]. Therefore, the basis for the crosstalk between JNK/SAPK signal pathway and apoptotic cell death machinery remains largely unclear.

Abbreviations used: ROS, reactive oxygen species; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; DPI, diphenylene iodonium; dn, dominant negative; MEKK1, mitogen-activated protein kinase kinase-1; SEK1, SAPK kinase-1; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; NAC, *N*-acetyl-L-cysteine; DCFH-DA, 2′,7′-dichlorofluorescein-diacetate.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 20176038, 20236040.

*Correspondence to: Yingjin Yuan, Department of Pharmaceutical Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, P.O. Box 6888, Peoples' Republic of China.
E-mail: yjyuan@public.tpt.tj.cn; yjyuan@tju.edu.cn

Received 3 January 2005; Accepted 31 May 2005
DOI 10.1002/jcb.20563

© 2005 Wiley-Liss, Inc.

Proteases of the ICE/Ced-3 family (caspases) [Hengartner, 2000] are activated during the apoptotic response, including being activated by chemotherapeutic drugs and cleaving specific protein substrates. It is believed that the activation of caspases is a final commitment step for apoptosis. JNK/SAPK has been shown to be involved in the activation of caspases [Wang et al., 1999; Stone and Chambers, 2000; Tournier et al., 2000; Enomoto et al., 2003; Yu et al., 2004]. However, the role of JNK/SAPK in activation of caspases is not straightforward. JNK/SAPK could be either upstream [Wang et al., 1999; Stone and Chambers, 2000; Tournier et al., 2000; Yu et al., 2004] or downstream [Enomoto et al., 2003] of caspase activation or both are activated independently [Toyoshima et al., 1997; Nishitai et al., 2004], depending on the cell types and apoptosis-initiating agents. The JNK kinase kinase (MEKK1) could be cleaved by DEVDase in the early response of genotoxin-induced apoptosis [Widmann et al., 1998]. It has been found that the initial cleavage of MEKK1 generated a pro-apoptotic kinase fragment that was able to activate JNK and subsequent caspase to facilitate the onset of apoptosis. This finding [Widmann et al., 1998] challenges the notion that the interaction between JNK/SAPK pathway and caspase family pathway in the apoptosis induced by various stimuli is simple cell type-specific and agents-specific upstream or downstream relationship.

Docetaxel is an effective antineoplastic agent specifically targeting microtubules in the treatment of lung, breast, malignant melanoma cancers and leukemia [Rowinsky, 1997]. Extensive studies indicated that docetaxel arrested cells at the G_2/M phase of the cell cycle [Choy, 2001] and initiate apoptosis [Goncalves et al., 2001; Bhalla, 2003], but the biochemical events downstream of kinetic stabilization of microtubule dynamics that lead to apoptosis remain largely unclear. Recently, several studies have shown that JNK/SAPK activation induced by microtubules-interfering agents is required for apoptosis in a variety of human cells [Wang et al., 1998, 1999; Stone and Chambers, 2000]. Whether JNK/SAPK activation is required for docetaxel-induced apoptosis or not is still unclear. It has been known that docetaxel induces caspase activation and phosphorylation of Bcl-2 [Haldar et al., 1995, 1996; Kolfschoten et al., 2002], which could be mediated by activated JNK/SAPK [Yamamoto et al., 1999]. Other studies, however, have argued against these findings [Wang et al., 1999; Tournier et al., 2000]. Cellular signaling pathways are also regulated by the intracellular redox state of the cells. Links between oxidative stress and perturbations in MAPK pathways have been extensively documented concerning noxious stimuli [Martindale and Holbrook, 2002]. Reactive oxygen species (ROS) may act as a mediator of apoptosis and play a crucial role in caspase-3 activation [Simizu et al., 1998; Chung et al., 2003]. Furthermore, ROS generation plays a central role in triggering the cell death response through a process involving reciprocal JNK/SAPK activation, mitochondria injury and caspase activation [Yu et al., 2004]. Bcl-2 family of proteins are involved in regulating the redox state of cells [Hengartner, 2000; Chung et al., 2003]. However, little information is known about the relationship between ROS and above-mentioned biochemical events during apoptotic cell death induced by docetaxel.

In this study, our focus is on the roles of ROS and JNK/SAPK in docetaxel-induced caspase activation, bcl-2 phosphorylation and apoptosis. The hierarchy of events accompanying docetaxel-induced apoptosis was investigated in HL-60 human leukemia cells.

MATERIALS AND METHODS

Reagents

Docetaxel was synthesized and purified in our lab, stored as a 100 mM solution in absolute ethanol at -20°C and diluted with the medium prior to use. SP600125 was purchased from Calbiochem (San Diego, CA). 2′,7′-dichlorofluorescein-diacetate (DCFH-DA), Ac-DEVD-CHO, DPI, and *N*-acetyl-L-cysteine (NAC) were obtained from Sigma (St. Louis, MO). All drugs were formulated in sterilized DMSO before. Caspase-3 colorimetric assay kit was purchased from R&D Systems (Minneapdis, MN). Polyclonal antibodies to bcl-2, phospho-JNK, JNK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary anti-rabbit horseradish peroxidase antibodies were from Zsbio (Beijing, China).

Cell Culture, Transfection, and Experimental Procedures

HL-60 cells were obtained from the Chinese Academy of Medical Sciences. The cells were grown in RPMI-1640 (Gibco, Grand Island, NY)

supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were transfected using Cytofectene (Bio-Rad, Richmond, CA) with expression vector for human Bcl-2 cNDA (a gift from Dr. Y. Choi) or dominant negative expression vector for JNK/SAPK (pSR-APF, a gift from Dr. G.L. Johnson). Transfected cells were selected with G418 (800 μg/ml) and then expanded for further studies.

Logarithmically growing cells were seeded at 1.0×10^5/ml and treated with docetaxel for predetermined intervals. When used together with inhibitors, cells were pretreated with the inhibitors for 0.5 h before adding docetaxel. All data were the average of triplicate experiments and the errors were within ±10%.

Assessment of Apoptosis by Morphology Observation

Apoptosis was detected by combined assessment of nuclear morphology and membrane permeability using double staining with Hoechst33342 and propidium-iodide dyes [Yuan et al., 2002]. Briefly, about 10^6 cells were collected, co-stained with 10 μg/ml Hoechst 33342 and 50 μg/ml propidium-iodide in dark at room temperature for 15 min, then observed with a fluorescence microscope (E800, Nikon) and documented by photography. Five different fields were randomly selected for counting at least 500 cells and the percentage of apoptotic cells to that of total cells was calculated.

Measurement of Caspase-3 Activity

The activity of caspase-3 was assayed by the CPP32 ApoAlert colorimetric-assay kit following the manufacturer's instructions. Cells (2×10^6 per sample) were processed and the protease activity was detected by reading samples at 405 nm in a Microplate Reader (Wellscan MK3, Labsystems Dragon). The enzyme activity was expressed as the fold over control samples.

Measurement of ROS Generation

The generation of ROS was detected by using DCFH-DA as described previously [Cao et al., 2004]. Briefly, cells were washed with PBS twice and then incubated with 10 μM DCFH-DA at 37°C for 30 min. The fluorescence intensity of 10,000 cells was monitored with a fluorescent spectrophotometer (RF-5301PC) from an excitation wavelength of 485 nm (bandwidth 5 nm) and an emission wavelength of 524 nm (bandwidth 5 nm).

Western Blot Analysis

Cells were pelleted by centrifugation and lysed immediately with lysis buffer (50 mM Tris-Cl pH 7.5 containing 100 mM NaCl, 2 mM EDTA, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM NaF, 1% NP-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μ/ml leupeptin, and 20 μ/ml aprotinin) at 4°C for 30 min. Aliquots of cell lysate containing equal protein (20 μg) were separated on 12% SDS–polyacrylamide gel electrophoresis (Bio-Rad, Mini-PROTEAN 3 cell System) and transferred to nitrocellulose membrane (Bio-Rad, Mini Trans-Blot Electrophoretic Transfer cell). After being blocked with 5% (w/v) nonfat milk Triton-PBS (0.05%, v/v) solution for 1 h, the blots were probed with the respective antibodies for 2 h at room temperature and then with peroxidase-conjugated secondary IgG antibodies. Detection of specific proteins was carried out with an enhanced chemiluminescence (ECL) Western blotting kit (Santa Cruz Biotechnology) according to the manufacturer's instructions.

Statistical Analysis

Values are expressed as mean ± SEM. For comparisons between groups, the SPSS One Way ANOVA analysis of variance was used. The P values less than 0.05 were considered to be statistically significant.

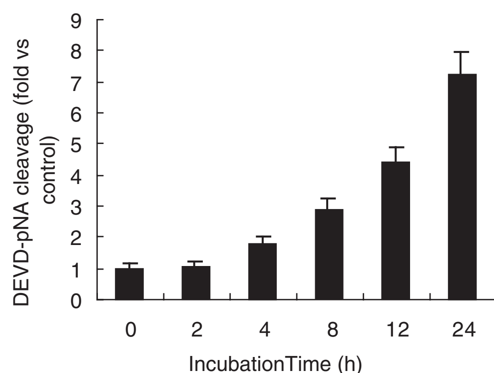
RESULTS

ROS Production and Caspase-3 Activation Mediated by Docetaxel in HL-60 Cells

The caspase-3 activity in HL-60 cells was assayed at 2, 4, 8, 12, and 24 h after addition of 10^{-8}M docetaxel based on the spectrophotometric detection of the chromophore *p*-nitroanilide (pNA) after cleavage from the labeled substrate DEVD-pNA. The comparison of the absorbance of pNA from apoptotic and control cells allows the quantification of the increase in protease activity. Marked increases in DEVD-pNA cleavage activities were observed in the cytosol fractions of HL-60 cells after treatment with docetaxel, indicating the activation of caspase-3, which became visible after 4 h of drug incubation and increased significantly

with time thereafter (Fig. 1A). Figure 1B shows that the DCFH-DA fluorescence in HL-60 cells increased markedly with time after incubation with 10^{-8}M docetaxel, indicating an increased amount of intracellular ROS. An obvious increase of ROS was observed at 2 h (Fig. 1B, 151 ± 13 vs. 100 ± 11, $P < 0.05$) when the activity of caspase-3 had not been initiated (Fig. 1A, 1.07 ± 0.13 vs. 1 ± 0.15, $P > 0.05$). Further analysis of the time-dependent increase in ROS levels showed that DCFH-DA fluorescence increased with a peak occurred at around 24 h (Fig. 1B). These observations revealed that ROS generation initiated by docetaxel came earlier than the activation of caspase-3 in HL-60 cells.

A



B

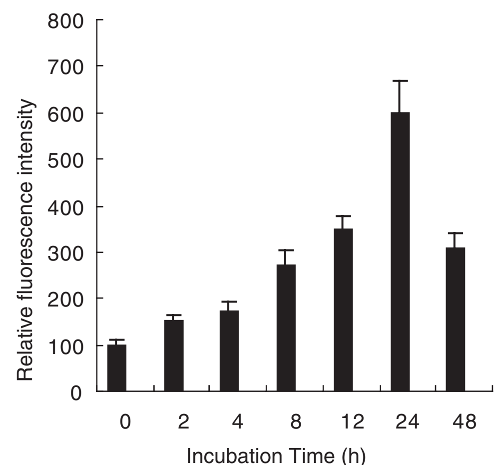


Fig. 1. Effects of docetaxel on caspase-3 activation and reactive oxygen species (ROS) production in HL-60 cells. **A**: Caspase-3 activity. Cytosol was prepared from HL-60 cells after incubation with 10^{-8}M docetaxel for predetermined times. The peptidase activity of caspase-3 was measured as cleavage of the peptide substrate DEVD-pNA. **B**: ROS production. Cells were treated with 10^{-8}M docetaxel for the indicated times and then incubated with DCFH-DA fluorescent probe for 30 min. DCF fluorescence was measured with a fluorescence spectrophotometer. ROS levels are defined as the ratio of the fluorescences of treated and control cells.

Roles of ROS in Caspase-3 Activation and Apoptosis of HL-60 Cells Induced by Docetaxel

To identify whether ROS contribute directly to docetaxel-induced apoptosis or not, HL-60 cells were treated with docetaxel in the presence of antioxidants. The addition of NAC, a potent ROS scavenger, to cells following the docetaxel treatment led to a reduction in ROS levels (Fig. 2A) and caspase-3 activity (Fig. 2C), as well as the attenuation of apoptosis (Fig. 2B). These results suggest that ROS play a role in regulation of the caspase-3 activation and apoptosis of HL-60 cells induced by docetaxel. The effects of DPI, an NADPH oxidase inhibitor, on docetaxel-induced ROS generation, apoptosis and caspase-3 activity in HL-60 cells were also investigated to further test the source(s) of ROS and whether the generation of ROS is a crucial step in docetaxel-induced apoptosis. DCFH-DA fluorescence was completely abolished in docetaxel-treated cells when pre-incubated with DPI compared to cells exposed to docetaxel alone (Fig. 2A), indicating that ROS were generated through NADPH oxidase-dependent pathway. Under these conditions, the activation of caspase-3 was completely blocked and the apoptosis was also obviously attenuated (Fig. 2B,C). On the contrary, docetaxel-induced ROS production was partially eliminated by the presence of Ac-DEVD-CHO (Fig. 2A), an inhibitor of caspase-3 (-like) proteases, which almost completely blocked the caspase-3 activity of HL-60 cells induced by docetaxel (Fig. 2C). Additionally, Ac-DEVD-CHO protected HL-60 cells from docetaxel-induced apoptosis (Fig. 2B), further confirming the requirement of caspase-3 (-like) proteases in this process. It is thus concluded that docetaxel elicited ROS from NADPH oxidase, which involved in the activation of caspase-3 (-like) proteases and subsequent apoptosis in HL-60 cells. ROS acted both upstream and downstream of caspase-3 (-like) proteases activation in docetaxel-induced apoptosis.

Roles of Caspase-3 (-Like) Proteases in Docetaxel-Induced JNK Activation and Apoptosis

Caspases, characterized as protease, could cleave specific protein substrates [Hengartner, 2000]. Previous observations indicated that

MEKK1 could be proteolyzed to specific fragment and activated the JNK/SAPK pathway [Widmann et al., 1998]. So whether caspase (-like) proteases mediate docetaxel-induced JNK/SAPK activation and apoptosis was investigated in HL-60 cells using the caspase tripeptide inhibitors Ac-DEVD-CHO. Figure 3A indicates that the pretreatment of HL-60 cells with 50 μM Ac-DEVD-CHO effectively inhibited docetaxel-induced JNK/SAPK activation, which happened early at 4 h and kept for up to 12 h of drug exposure. These results suggest that caspase play a role in the startup and regulation of JNK/SAPK signal pathway, which may act upstream of the site of JNK/SAPK activation. On the other hand, Figure 3A suggested that where as Ac-DEVD-CHO inhibited JNK, it did not completely inhibit apoptosis. In addition, Ac-DEVD-CHO partially diminished docetaxel-mediated ROS production in HL-60 cells after 4 h of drug treatment (Fig. 3B), while NAC completely blocked the caspase-3 activation up to 12h of docetaxel exposure (Fig. 3C).

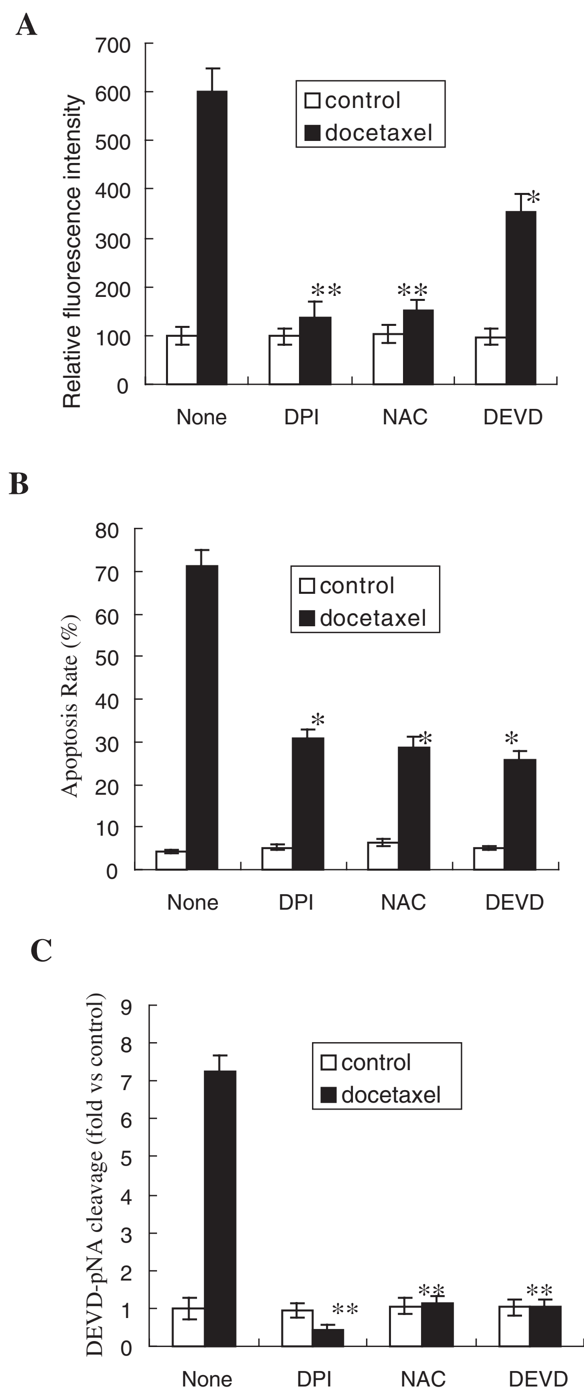


Fig. 2. Effects of ROS on docetaxel-mediated apoptosis and caspase-3 activation in HL-60 cells. Effects of NAC, NADPH oxidase inhibitor (DPI), and caspase-3 inhibitor (Ac-DEVD-CHO) on ROS production (**A**), apoptosis rate (**B**), and caspase-3 activity (**C**) of HL-60 cells induced by docetaxel were measured, respectively. Cells were treated for 24 h with 10^{-8}M docetaxel alone (none) or in the presence of NAC (20 mM) (NAC), DPI (30 μM) (DPI), Ac-DEVD-CHO (50 μM) (DEVD). Apoptosis was measured by double dying with Hoechst33342 and PI. ROS production and caspase-3 activity were assayed as described in "Materials and Methods." $^{**}P < 0.01$, $^{*}P < 0.05$, significantly different from values for cells exposed to docetaxel alone.

Roles of JNK Activation in bcl-2 Phosphorylation, ROS Production, Caspase-3 Activation and Apoptosis

To determine possible downstream effectors of docetaxel-activated JNK/SAPK, we measured phosphorylation of bcl-2, ROS production, caspase-3 activation and apoptosis by transfecting HL-60 cells with dominant negative JNK plasmid [pSR-APF] or by using a specific JNK inhibitor SP600125, followed by treatment with docetaxel for 2, 4, 8, and 12 h. As shown in Figure 4A, docetaxel failed to induce JNK/SAPK activation in the presence of SP600125 (20 μM) as anticipated. Western blot analysis showed that the docetaxel-elicited phosphorylation of bcl-2 was obviously blocked by SP600125, which happened early at 4 h of drug treatment (Fig. 4A). The similar results were also obtained in HL-60 cells that was transfected with dn JNK expression vector (HL-60/dn JNK), confirming that JNK/SAPK was required for the phosphorylation of bcl-2. In consistence with these observations, in the presence of SP600125, HL-60 cells became resistant to apoptosis induced by docetaxel, as quantified by morphology observation with PI and Hoechst33342 co-staining (Fig. 4B). In addition, SP600125 partially diminished docetaxel-mediated ROS production and

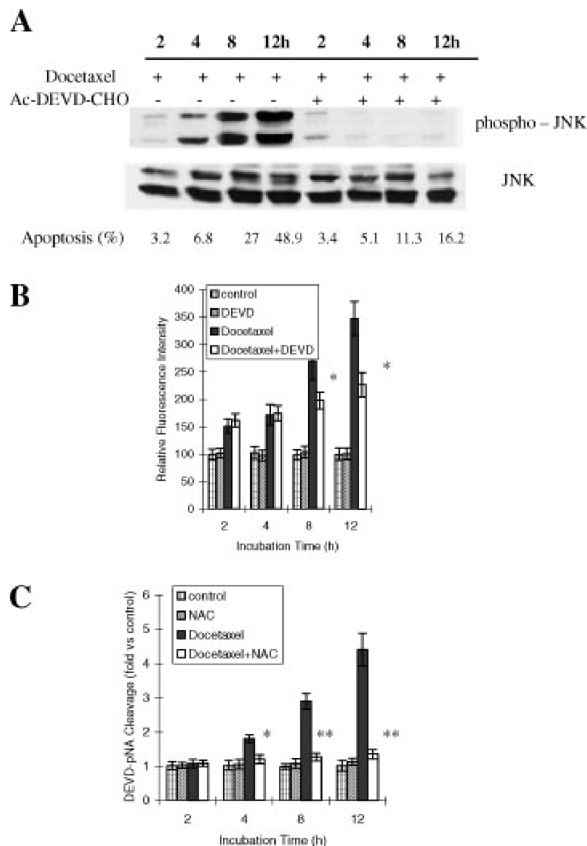


Fig. 3. Effects of caspase inhibition on docetaxel-mediated JNK activation, ROS production, and apoptosis in HL-60 cells. Cells were treated with 10^{-8}M of docetaxel $\pm$ Ac-DEVD-CHO (50 μM) or docetaxel $\pm$ NAC (20 mM) for 2, 4, 8, and 12 h, then the levels of phospho-JNK and apoptosis (**A**), ROS (**B**), and caspase-3 activity (**C**) were monitored as described in ''Materials and Methods.'' $^{**}P < 0.01$, $^{*}P < 0.05$, significantly different from values for cells exposed to docetaxel alone.

caspase-3 activation in HL-60 cells (Fig. 4C,D). These results clearly suggest that the JNK kinase cascade was required for docetaxel-mediated bcl-2 phosphorylation and apoptotic cell death for HL-60 cells, which also involved in ROS production and caspase-3 activation induced by docetaxel. However, all the above-mentioned changes became significant after 4 h of drug treatment. SP600125 almost exerted no effects on the ROS generation, caspase-3 activation and apoptosis of HL-60 cells induced by docetaxel in the former 4 h (Fig. 4B–D).

Roles of bcl-2 in ROS Production of HL-60 Cells Induced by Docetaxel

Evidence in the literature confirms that the members of the bcl-2 family of proteins are involved in regulating the redox state of cells by modulating ROS scavenger activity or by preventing the generation of ROS [Green and Reed, 1998; Kroemer et al., 1998]. So the possible relationship between bcl-2 and ROS generation was investigated in HL-60 cells treated with docetaxel. As illustrated in Figure 5, over-expressed bcl-2 in HL-60 cells effectively diminished the ROS production (Fig. 5A) and the subsequent caspase-3 activation (Fig. 5B) induced by docetaxel. These data suggest that docetaxel-mediated ROS production is blocked by bcl-2 over-expression.

DISCUSSION

Here, the attention was paid to the perturbations in signal transduction pathways such as activation of the JNK/SAPK pathway, bcl-2 phosphorylation, caspase-3 (-like) proteases activation, as well as induction of oxidative stress to elucidate the mechanism by which docetaxel triggers the cell death pathway.

ROS and caspase-3 (-like) protease involved directly in the apoptotic cell death induced by docetaxel (Figs. 1 and 2). Furthermore, the time course of ROS production and caspase-3 activation of HL-60 cells induced by docetaxel (Fig. 1) revealed that ROS production was initiated early at 2 h, while caspase-3 was activated after 4 h of docetaxel treatment, indicating that ROS might play a primary role in triggering the cell death responses, being followed by caspase-3 activation (Fig. 2). However, the relationship between ROS production and caspase-3 activation was not simply linear. Studies with DPI and Ac-DEVD-CHO indicated that docetaxel elicited ROS from NADPH oxidase, which in turn triggered the activation of caspase-3 (-like) proteases as well as subsequent apoptosis and also was partially mediated by caspase-3 in HL-60 cells (Fig. 2). Further time course analysis on the effects of NAC and Ac-DEVD-CHO (Fig. 3B,C) revealed that Ac-DEVD-CHO only partially prevented the ROS production of HL-60 cells after 4 h of docetaxel treatment, while NAC completely blocked the caspase-3 activation which happened early at 4 h and kept for up to 12 h of drug exposure, further indicating that ROS production came first and was followed by caspase-3 activation in the apoptotic cell death process induced by docetaxel. Caspase might also mediate ROS production through some unknown mechanisms. Contrary to our results, recent reports showed that although taxol induced ROS formation in the human lymphoblastic leukemia cells and hepatoma cells,

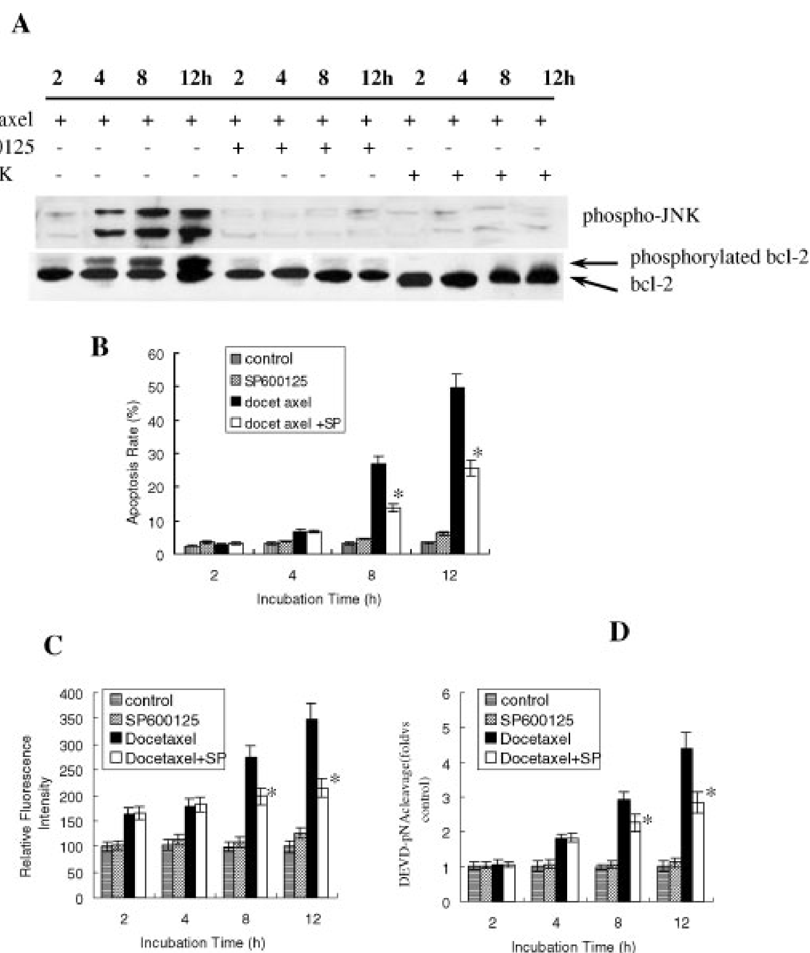


Fig. 4. Effects of JNK inhibition on docetaxel-induced bcl-2 phosphorylation, ROS production, caspase-3 activity and apoptosis in HL-60 cells. Cells pretreated with SP600125 (20 μM) or HL-60/dn JNK cells were treated with 10^{-8}M docetaxel for 2, 4, 8, and 12 h, then the levels of phospho-JNK and bcl-2 phosphorylation (**A**), apoptosis (**B**), ROS (**C**), and caspase-3 activity (**D**) were monitored as described in "Materials and Methods." $^{*}P < 0.05$, significantly different from values for cells exposed to docetaxel alone.

taxanes-triggered apoptosis in these cells is independent of ROS formation [Lin et al., 2000; Park et al., 2004]. Therefore, these results collectively indicate that taxanes use different apoptosis signaling pathways in different cell types. ROS might play cell type-specific roles in the apoptotic cell death process. Our results are consistent with the previous study on the role of ROS in adriamycin-induced apoptosis that acted both upstream and downstream of caspase-3 (-like) protease activation [Simizu et al., 1998]. It has been commonly thought that ROS production through NADPH oxidase acts either upstream [Chung et al., 2003] or downstream [Simizu et al., 1998] of caspase-3 activation. It has been reported that sodium salicylate (NaSal) elicited ROS production from NADPH oxidase, which preceded mitochondrial depolarization leading to the release of cytochrome *c* and activation of caspase cascade [Chung et al., 2003]. On the other hand, it has also been reported that caspase-3 (-like) protease activated NADPH oxidase and mediated the subsequent ROS production through Rac-dependent mechanism or $p47^{phox}$ protein kinase pathway [Na et al., 1996; Simizu et al., 1998]. However, what is the basis of the inter-regulation between ROS and caspase in the apoptosis of HL-60 cells induced by docetaxel is not fully understood at this stage, which promoted us to further investigate the other biochemical events correlated with both ROS and caspase.

JNK/SAPK has been proved to be involved in the regulation of caspase-3 activation [Wang et al., 1999; Stone and Chambers, 2000; Tournier

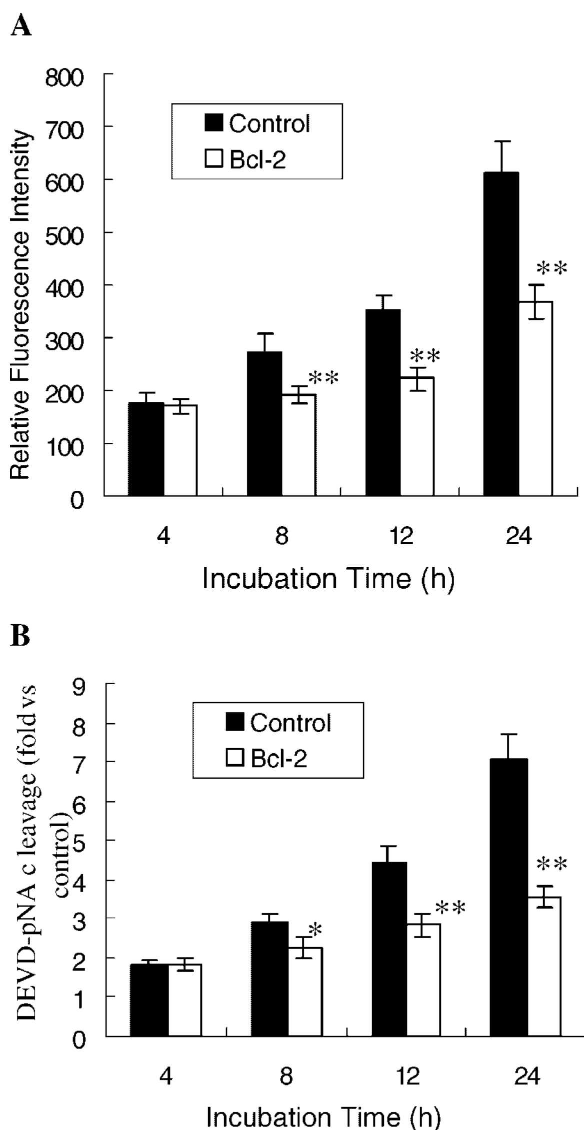


Fig. 5. Effects of bcl-2 on the ROS production and apoptosis of HL-60 cells induced by docetaxel. Control cells and bcl-2 overexpressed HL-60 cells were treated with 10^{-8}M of docetaxel for 4, 8, 12, and 24 h, then the ROS production (**A**) and caspase-3 activity (**B**) were monitored as described in "Materials and Methods." $^{**}P < 0.01$, $^{*}P < 0.05$, significantly different from values to control cells.

et al., 2000; Enomoto et al., 2003; Yu et al., 2004] and the ROS-mediated signal pathway [Martindale and Holbrook, 2002; Michal et al., 2004; Yu et al., 2004]. The apoptosis-promoting role of the JNK/SAPK cascade has been demonstrated in many microtubule-interfering agents-induced apoptotic processes and considered as the common results of microtubule dysfunction [Wang et al., 1998, 1999; Stone and Chambers, 2000]. However, the exact role of JNK/SAPK in taxanes-induced apoptosis still seems to be controversial [Wang et al., 1999; Stone and Chambers, 2000; Du et al., 2004]. Experiments of JNK/SAPK activity modification with specific inhibitor SP600125 (Fig. 4B) demonstrated the involvement of JNK/SAPK in docetaxel-induced apoptosis. The current results are consistent with other studies concerning another taxanes compound paclitaxel [Wang et al., 1998, 1999; Stone and Chambers, 2000], which have demonstrated that the activated JNK/SAPK is essential for paclitaxel-induced apoptosis. Recently, a direct evidence from the study of JNK knockout fibroblasts revealed that the absence of JNK in cells resulted in resistance to apoptosis induced by UV or other chemical drugs [Tournier et al., 2000]. All these results suggest that JNK/SAPK is required for stress-induced apoptosis in a wide variety of cells.

MEKK1 is a 196-kDa kinase and acts upstream of the JNK pathway. It has been demonstrated that MEKK1 is a substrate for caspase-3-like proteases and that the kinase activity of MEKK1 stimulates caspase-3-like activity in cells [Widmann et al., 1998]. Our results support this demonstration. The caspase inhibitor, Ac-DEVD-CHO, almost completely blocked docetaxel-induced JNK/SAPK activation at 4, 8, and 12h (Fig. 3A), suggesting that caspase (DEVDase) may play an initial and critical role in triggering cell-death signals and function upstream of JNK/SAPK activation. JNK/SAPK specific inhibitor SP600125 obviously blocked the bcl-2 phosphorylation of HL-60 cells being treated with docetaxel for 4 h (Fig. 4A), when it did not inhibit the activation of caspase-3 protease (Fig. 4D), further confirming that bcl-2 resides direct downstream of JNK/SAPK. The activation of caspase-3 was followed by JNK/SAPK activation and subsequently phosphorylation of bcl-2, not that of phosphorylation of bcl-2 and subsequently JNK/SAPK activation. The similar results in HL-60/dn JNK cells (Fig. 4A) indicated that the phosphorylation of bcl-2 induced by docetaxel was indeed mediated by JNK/SAPK, excluding the possibility that SP600125 may block bcl-2 phosphorylation through inhibiting cdc2 or other bcl-2 kinases. On the other hand, SP600125 partially blocked docetaxel-mediated ROS production and caspase-3 activation in HL-60 cells after 4 h of docetaxel treatment (Fig. 4C,D), indicating that JNK/SAPK may also act upstream of caspase in these cells. That JNK/SAPK mediated

caspase-3 activation has been found in various types of cells [Wang et al., 1999; Stone and Chambers, 2000; Tournier et al., 2000; Yu et al., 2004]. However, the substrate for JNK/SAPK that results in caspase-3 activation is presently unknown. This process might be mediated through bcl-2 phosphorylation in docetaxel-induced HL-60 cells. As shown in Figure 5, over-expression of bcl-2 blocked the ROS generation and subsequent caspase-3 activation induced by docetaxel, indicating that bcl-2 might function as a cell survival signal through that manners, so it could be speculated that the dysfunction of bcl-2 through phosphorylation led by activation of JNK/SAPK (Fig. 4A) facilitated the ROS production and functioned as an apoptotic stimuli. However, SP600125 decreased apoptosis by 50% (Fig. 4B), suggesting a major role for JNK/SAPK in apoptosis. Figure 4C,D suggested that JNK is not involved in ROS or caspase activation at 2 or 4 h. Taken together this data suggests that ROS can mediate apoptosis partially independently of JNK. JNK increases ROS and caspase-3 after a delay and may be responsible for 50% of apoptosis at 8/12 h. Figure 3A suggested that where as Ac-DEVD-CHO inhibited JNK/SAPK, it did not completely inhibit apoptosis. Therefore, it could be speculated that ROS generation occurs first, being followed by caspase-3 and JNK/SAPK activation. After 4 h of docetaxel treatment, activated JNK/SAPK generated a pro-apoptotic signal and, in turn, triggered a caspase feedback loop that functioned as a branched signal pathway mediating apoptosis. ROS and bcl-2 were also involved in the amplification loop for increasing caspase activity during apoptosis. Caspase feedback loop consisted of JNK, bcl-2 and ROS just facilitated the caspase activation and apoptosis. Without that, the caspase could be activated slowly through ROS-dependent, JNK-independent pathway, leading to the apoptotic cell death.

In conclusion, the present study suggests the following model in which the hierarchy of events accompanying docetaxel-mediated apoptosis in human leukemia HL-60 cells can be well understood (Fig. 6). Docetaxel-mediated ROS production through NADPH oxidase pathway represents the primary trigger for activation of the apoptotic cascade. Antioxidant such as NAC block docetaxel-mediated ROS generation and, as a consequence, all downstream events associated with docetaxel-induced apoptosis.

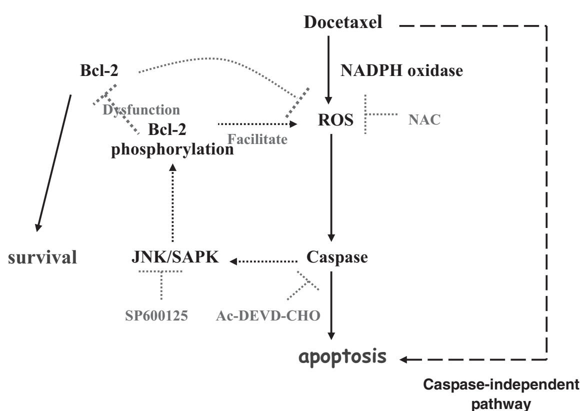


Fig. 6. Proposed model of the hierarchy of events accompanying docetaxel-induced apoptosis in human leukemia HL-60 cells. Docetaxel induces ROS production through NADPH oxidase pathway, which in turn triggers caspase-3 activation and subsequent JNK/SAPK activation as well as bcl-2 phosphorylation. Phosphorylated bcl-2 further promotes the ROS generation and caspase-3 activation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Increased ROS in turn triggers caspase-3 activation, JNK/SAPK activation, and subsequent bcl-2 phosphorylation. Furthermore, docetaxel-mediated ROS production depends, at least in part, upon bcl-2 dysfunction of cell survival through phosphorylation. Phosphorylated bcl-2 further facilitates the ROS generation and caspase-3 activation. The initial stimuli signal is amplified through this loop cascade, resulting in the continuous activation of caspase and ultimate apoptotic cell death. The results that both Ac-DEVD-CHO (Fig. 3B) and SP600125 (Fig. 4C) only partially effected the ROS production after 4 h of docetaxel exposure indicated that the above-mentioned loop cascade just functioned as a branched signal pathway mediating apoptosis.

The results that both ROS scavenger DPI, NAC, and Ac-DEVD-CHO only had partial effect on apoptosis of HL-60 cells induced by docetaxel (Fig. 2B) indicated the ROS-dependent caspase-3 activation may not be the only signal pathway, docetaxel may also mediate HL-60 cell death through caspase-independent manners (Fig. 6).

REFERENCES

Bhalla KN. 2003. Microtubule-targeted anticancer agents and apoptosis. Oncogene 22:9075–9086.

Cao DX, Qiao B, Ge ZQ, Yuan YJ. 2004. Comparison of burst of reactive oxygen species and activation of caspase-3 in apoptosis of K562 and HL-60 cells induced by docetaxel. Cancer Lett 214(1):103–113.

Choy H. 2001. Taxanes in combined modality therapy for solid tumors. Crit Rev Oncol Hematol 37:237–247.

Chung YM, Bae YS, Lee SY. 2003. Molecular ordering of ROS production, mitochondrial changes, and caspase activation during sodium salicylate-induced apoptosis. Free Radic Biol Med 34:434–442.

Davis RJ. 2000. Signal transduction by the JNK group of MAP kinases. Cell 103:239–252.

Dickens M, Rogers JS, Cavanagh J, Raitano A, Xia Z, Halpern JR, Greenberg ME, Sawyers CL, Davis RJ. 1997. A cytoplasmic inhibitor of the JNK signal transduction pathway. Science 277:693–696.

Du L, Lyle CS, Obey TB, Gaarde WA, Muir JA, Bennett BL, Chambers TC. 2004. Inhibition of cell proliferation and cell cycle progression by specific inhibition of basal JNK activity: Evidence that mitotic Bcl-2 phosphorylation is JNK-independent. J Biol Chem 279:11957–11966.

Enomoto A, Suzuki N, Morita A, Ito M, Liu CQ, Matsumoto Y, Yoshioka K, Shiba T, Hosoi Y. 2003. Caspase-mediated cleavage of JNK during stress-induced apoptosis. Biochem Biophys Res Commun 306:837–842.

Goncalves A, Braguer D, Kamath K, Martello L, Briand C, Horwitz S, Wilson L, Jordan MA. 2001. Resistance to Taxol in lung cancer cells associated with increased microtubule dynamics. Proc Natl Acad Sci USA 98: 11737–11742.

Green DR, Reed JC. 1998. Mitochondria and apoptosis. Science 281:1309–1312.

Haldar S, Jena N, Croce CM. 1995. Inactivation of bcl-2 by phosphorylation. Proc Natl Acad Sci USA 92:4504–4511.

Haldar S, Chintapalli J, Croce CM. 1996. Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. Cancer Res 56:1253–1255.

Hengartner MO. 2000. The biochemistry of apoptosis. Nature 407:770–776.

Ip YT, Davis RJ. 1998. Signal transduction by the c-Jun N-terminal kinase (JNK)—From inflammation to development. Curr Opin Cell Biol 10:205–219.

Kolfschoten GM, Hulscher TM, Duyndam MC, Pinedo HM, Boven E. 2002. Variation in the kinetics of caspase-3 activation, Bcl-2 phosphorylation and apoptotic morphology in unselected human ovarian cancer cell lines as a response to docetaxel. Biochem Pharmacol 63:733–743.

Kroemer G, Dallaporta B, Resche-Rigon M. 1998. The mitochondrial death/life regulator in apoptosis and necrosis. Annu Rev Physiol 60:619–642.

Lin HL, Liu TY, Chau GY, Lui WY, Chi CW. 2000. Comparison of 2-methoxyestradiol-induced, docetaxel-induced and paclitaxel-induced apoptosis in hepatoma cells and its correlation with reactive oxygen species. Cancer 89:983–994.

Martindale JL, Holbrook NJ. 2002. Cellular response to oxidative stress: Signaling for suicide and survival. J Cell Physiol 192:1–15.

Michal PY, Drora H, Oded S, Ina F. 2004. The p38 pathway partially mediates caspase-3 activation induced by reactive oxygen species in Fanconi anemia C cells. Biochem Pharmacol 67:539–546.

Na S, Chuang TH, Cunningham A, Turi TG, Hanke JH, Bokoch GM, Danley DE. 1996. D4-GDI, a substrate of CPP32, is proteolyzed during Fas-induced apoptosis. J Biol Chem 271:11209–11213.

Nishitai G, Shimizu N, Negishi T, Kishimoto H, Nakagawa K, Kitagawa D, Watanabe T, Momose H, Ohata S, Tanemura S, Asaka S, Kubota J, Saito R, Yoshida H, Mak TW, Wada T, Penninger JM, Azuma N, Nishina H, Katada T. 2004. Stress induces mitochondria-mediated apoptosis independent of SAPK/JNK activation in embryonic stem cells. J Biol Chem 279:1621–1626.

Osborn MT, Chambers TC. 1996. Role of the stress-activated c-jun NH2-terminal protein kinase pathway in the cellular response to Adriamycin and other chemotherapeuticagents. J Biol Chem 271:30950–90955.

Park SJ, Wu CH, Gordon JD, Zhong XL, Emami A, Safa AR. 2004. Taxol induced caspase-10-dependent apoptosis. J Biol Chem 279:51057–51067.

Rowinsky EK. 1997. The development and clinical utility of taxane class of antimicrotubule chemotherapy agents. Annu Rev Med 48:353–374.

Sethi G, Sodhi A. 2004. Activation of c-Jun N-terminal kinase is required for ultraviolet B-induced apoptosis of murine peritoneal macrophages in vitro. J Photochem Photobiol B 73:133–140.

Simizu S, Takada M, Umezawa K, Imoto M. 1998. Requirement of caspase-3 (-like) protease-mediated hydrogen peroxide production for apoptosis induced by various anticancer drugs. J Biol Chem 273:26900–26907.

Stone AA, Chambers TC. 2000. Microtubule inhibitors elicit differential effects on MAP Kinase (JNK, ERK, and p38) signaling pathways in human KB-3 carcinoma cells. Exp Cell Res 254:110–119.

Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA, Davis RJ. 2000. Requirement of JNK for stress-induced activationof the cytochrome c-mediated death pathway. Science 288: 870–879.

Toyoshima F, Moriguchi T, Nishida E. 1997. Fas induces cytoplasmic apoptotic responses and activation of the MKK7-JNK: SAPK and MKK6-p38 pathways independent of CPP32-like proteases. J Cell Biol 139:1005–1015.

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 TH, Popp DM, Wang HS, Saitoh M, Mural JG, Henley DC, Ichijo H, Wimalasena J. 1999. Microtubule dysfunction induced by paclitaxel initiates apoptosis through both c-Jun N-terminal Kinase (JNK)-dependent and -independent pathways in ovarian cancer cells. J Biol Chem 274:8208–8216.

Watanabe N, Iwamoto T, Dickinson DA, Iles KE, Forman HJ. 2002. Activation of the mitochondrial caspase cascade in the absence of protein synthesis does not require c-Jun N-terminal kinase. Arch Biochem Biophys 405:231–240.

Widmann C, Gerwins P, Johnson NL, Jarpe MB, Johnson GL. 1998. MEK kinase 1, a substrate for DEVD-directed caspases, is involved in genotoxin-induced apoptosis. Mol Cell Biol 18:2416–2429.

Willaime-Morawek S, Brami-Cherrier K, Mariani J, Caboche J, Brugg B. 2003. c-Jun N-terminal kinases/c-Jun and p38 pathways cooperate in ceramide-induced neuronal apoptosis. Neuroscience 119:387–397.

Yamamoto K, Ichijo H, Korsmeyer SJ. 1999. Bcl-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G_2/M. Mol Cell Biol 19:8469–8478.

Yu CR, Rahmani M, Dent P, Grant S. 2004. The hierarchical relationship between MAPK signaling and ROS generation in human leukemia cells undergoing apoptosis in response to the proteasome inhibitor Bortezomib. Exp Cell Res 295:555–566.

Yuan YJ, Ge ZQ, Li JC. 2002. Differentiation of apoptotic and necrototic cells in suspension cultures of *Taxus cuspidata* by the combined use of fluorescent dying and histochemical staining. Biotech Lett 24: 71–76.