

The JNK, ERK and p53 pathways play distinct roles in apoptosis mediated by the antitumor agents vinblastine, doxorubicin, and etoposide

Cheryl Brantley-Finley^a, Christopher S. Lyle^a, Lihua Du^a, Mary E. Goodwin^a, Toria Hall^a,
Dominika Szewdo^a, G.P. Kaushal^{a,b}, Timothy C. Chambers^{a,*}

^aDepartment of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA

^bDepartment of Medicine, University of Arkansas for Medical Sciences, Little Rock, AR 72205, USA

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Abstract

Assessment of specific apoptosis and survival pathways implicated in anticancer drug action is important for understanding drug mechanisms and modes of resistance in order to improve the benefits of chemotherapy. In order to better examine the role of mitogen-activated protein kinases, including JNK and ERK, as well as the tumor suppressor p53, in the response of tumor cells to chemotherapy, we compared the effects on these pathways of three structurally and functionally distinct antitumor agents. Drug concentrations equal to 50 times the concentration required to reduce cell proliferation by 50% were used. Vinblastine, doxorubicin, or etoposide (VP-16) induced apoptotic cell death in KB-3 carcinoma cells, with similar kinetic profiles of PARP cleavage, caspase 3 activation, and mitochondrial cytochrome *c* release. All three drugs strongly activated JNK, but only vinblastine induced c-Jun phosphorylation and AP-1 activation. Inhibition of JNK by SP600125 protected cells from drug-induced cytotoxicity. Vinblastine caused inactivation of ERK whereas ERK was unaffected in cells exposed to doxorubicin or VP-16. Inhibition of ERK signaling by the MEK inhibitor, U0126, potentiated the cytotoxic effects of vinblastine and doxorubicin, but not that of VP-16. Vinblastine induced p53 downregulation, and chemical inhibition of p53 potentiated vinblastine-induced cell death, suggesting a protective effect of p53. In contrast, doxorubicin and VP-16 induced p53, and inhibition of p53 decreased drug-induced cell death, suggesting a pro-apoptotic role for p53. These results highlight the differential roles played by several key signal transduction pathways in the mechanisms of action of key antitumor agents, and suggest ways to specifically potentiate their effects in a context-dependent manner. In addition, the novel finding that JNK activation can occur without c-Jun phosphorylation or AP-1 activation has important implications for our understanding of JNK function.

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1. Introduction

Anticancer drugs exert their lethality by inducing apoptosis in tumor cells *in vitro* and *in vivo*, and both the mitochondrial and death receptor pathways have been implicated in various studies [1–5]. The triggering of apoptosis

in response to chemotherapy can involve the induction or activation of various mediators such as p53, ceramide, or Fas L, as well as modulation of the expression or function of members of the Bcl-2 family or other apoptotic regulators. Conversely, tumor cells often respond to chemotherapy by engaging protective mechanisms, and survival signaling can antagonize chemotherapy. For example, NFκB activation is a common anti-apoptotic response to chemotherapy [6]. Thus, the balance and integration of multiple survival and death pathways dictates the overall outcome, and it has become evident that a greater understanding of the molecular mechanisms of action of antitumor agents will require elucidation of these complex signaling pathways.

* Corresponding author. Tel.: +1-501-686-5755; fax: +1-501-686-8169.

E-mail address: chamberstimo@c@uams.edu (T.C. Chambers).

Abbreviations: MAPK, mitogen-activated protein kinase; JNK, c-Jun NH₂-terminal kinase; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PARP, poly (ADP-ribose) polymerase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

MAPKs, which include the ERK, JNK, and p38 subgroups, play key roles in survival, proliferation, and apoptosis [7]. A large body of evidence has accumulated to show that antitumor agents alter the activity of different MAPK subgroups in many cancer cell lines [8]. Importantly, pharmacological or molecular modulation of MAPK signaling has been shown in many cases to influence the apoptotic response to antitumor agents [8,9]. Such results suggest that MAPKs may mediate destructive and/or protective responses to these drugs. However, the roles played by MAPKs tend to be strongly context-dependent, influenced by the cell type, drug concentration and duration of exposure, and on the type of assay used to monitor apoptosis or cell survival [8]. For example, while ERK activation is a common response to cisplatin, inhibition of MEK/ERK sensitizes ovarian cancer cell lines to cisplatin, suggesting a protective role for ERK [10], whereas inhibition of MEK/ERK blocks cisplatin-induced apoptosis in HeLa cells, suggesting instead an active role for ERK in cell death [11]. Similarly, the role of JNK signaling in the response of tumor cells to anticancer drugs is complex, and both destructive and protective roles have been proposed in different systems [8]. Another example is represented by the key protein p53, which can exhibit opposing functions, either supporting cell survival or promoting apoptosis, depending on the specific prevailing circumstances and conditions [12].

Assessment of the importance and role of specific death and survival pathways in anticancer drug action is compounded by lack of standardization, with different studies conducted under widely different conditions. In order to gain a better understanding of the role of MAPK and p53 pathways in the response of tumor cells to chemotherapy, we set out to directly compare the effects of three structurally and functionally distinct antitumor agents in a well-characterized cell line, KB-3 carcinoma cells. The results, described herein, are both revealing and surprising, and provide novel insight into the roles that the JNK, ERK, and p53 signaling pathways play in the fate of cells exposed to different types of chemotherapeutic drugs.

2. Materials and methods

2.1. Materials

Antibodies to JNK1, JNK2, ERK1/2 and actin were obtained from Santa Cruz Biotechnology; antibodies to p21 and cytochrome *c* were from Pharmingen; antibody to PARP was from Calbiochem; antibody to p53 was from NeoMarkers; antibody to c-Jun was from Transduction Laboratories; and phosphospecific antibodies for c-Jun (Ser63) and ERK1/2, as well as the fusion protein GST-c-Jun (1–79), were from Cell Signaling. The caspase 3 substrate (DEVD-AMC) and the JNK inhibitor SP600125 were obtained from Biomol. SP600125 was

also provided as a kind gift of Celgene, Signal Research Division. The MEK inhibitor U0126 was from Promega and the p53 chemical inhibitor pifithrin- α was from Alexis Biochemicals. Vinblastine, doxorubicin, and etoposide (VP-16) and other chemicals were obtained from Sigma Chemical Co.

2.2. Cell culture and treatment

The KB-3 cell line, which expresses a low level of wild-type p53, was kindly provided by Dr. M.M. Gottesman (National Cancer Institute). Stock solutions of drugs and other compounds were made in DMSO, the final concentration of which did not exceed 0.2% in the medium, with controls receiving vehicle alone. Cells were treated at about 70% maximum density; cells that became non-adherent after treatment were combined with those that remained adherent.

2.3. Subcellular fractionation and immunoblotting

Whole cell extracts were made as described previously [13]. Cytosolic and mitochondria-enriched extracts were prepared by lysing cells in 20 mM HEPES, pH 7.4, 2 mM EDTA, 2 mM EGTA, 2 mM DTT, and 10 mM Na_3VO_4 . After passage through a 20-gauge syringe needle, the lysate was centrifuged (1000 g, 10 min) and the resulting supernatant was centrifuged again (100,000 g, 1 hr) to obtain a cytosolic fraction (supernatant) and a particulate fraction (pellet) enriched in mitochondria. The pellet was suspended in lysis buffer containing 0.5% Triton X-100. Immunoblotting was performed as described previously using 50 μg protein per lane [13].

2.4. Caspase 3 assay

Caspase 3 activity was measured with DEVD-AMC as substrate as described previously [13], with results expressed as mean \pm SD ($N = 3$).

2.5. JNK assay

JNK activity was measured by immunocomplex assay, with GST-c-Jun and $[\gamma^{32}\text{P}]\text{ATP}$ as substrates, as described previously [14]. Quantitation of ^{32}P -labeled substrate was performed by phosphorimager analysis as described [15].

2.6. Other techniques

AP-1 transcriptional activity was measured using a standard luciferase system as described in detail previously [15]. The viability of KB-3 cells after drug treatment was measured by standard MTT assay, with each condition in triplicate, and results expressed as percent control or OD units (mean \pm SD). Drug treatment time varied from 48 to

96 hr as indicated in individual experiments. To evaluate the effects of the p53 inhibitor pifithrin- α on drug-induced cell death, cells were treated with 20 μ M pifithrin- α for 1 hr prior to the addition of either vinblastine, doxorubicin, or VP-16. After 48 hr, cells were stained with trypan blue, and those excluding or including the dye were scored as alive or dead cells, respectively. Two hundred cells were scored for each condition, and results presented as percentage of dead cells (mean \pm SD, $N = 4$).

3. Results

3.1. Kinetics and characteristics of apoptosis induction

In order to directly compare possible involvement of the JNK, ERK, and p53 pathways in the apoptotic actions of the three drugs chosen for study (vinblastine, doxorubicin, and VP-16), we first performed standard MTT assays, to ascertain IC_{50} values. Thus, KB-3 cells were treated with increasing concentrations of the three drugs and viable cell mass relative to control determined after 96 hr. The

concentration required to reduce KB-3 viable cell mass by 50% of the control under these conditions was 0.6 nM for vinblastine, 20 nM for doxorubicin, and 300 nM for VP-16 (data not shown). For the experiments reported here, the following concentrations, equivalent to $IC_{50} \times 50$, were used: 30 nM vinblastine; 1 μ M doxorubicin; and 15 μ M VP-16. KB-3 cells were next treated with the drugs for time periods of 0–48 hr and extracts analyzed for PARP cleavage and caspase 3 activity, two well-established hallmarks of apoptosis. PARP cleavage was first evident at about 24 hr of treatment with vinblastine or VP-16, and at about 28 hr with doxorubicin, confirming that each drug induced apoptosis with a similar kinetic profile (Fig. 1A). Additionally, each drug caused activation of caspase 3, with increased activity occurring mainly during the second day of treatment (Fig. 1B), consistent with the PARP cleavage data. However, while the kinetics of caspase 3 activation was similar, the relative magnitude varied, with VP-16 > doxorubicin > vinblastine. Finally, each drug also caused a time-dependent release of mitochondrial cytochrome *c* to the cytosol, first apparent at the earliest time-point examined of 12 hr (Fig. 1C).

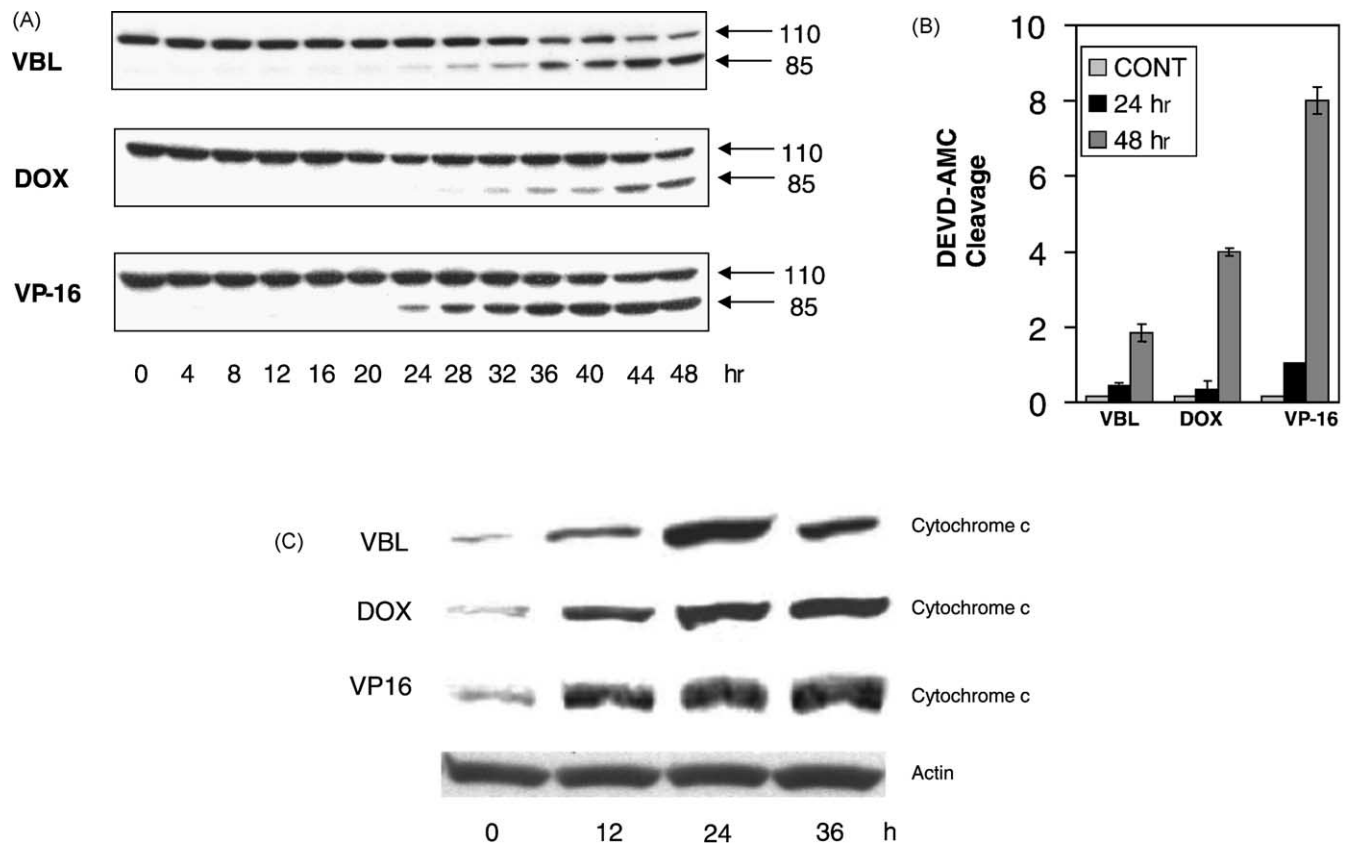


Fig. 1. Kinetics of PARP cleavage, caspase 3 activation, and cytosolic cytochrome *c* accumulation in response to chemotherapeutic drugs. KB-3 cells were treated with 30 nM vinblastine (VBL), 1 μ M doxorubicin (DOX), or 15 μ M VP-16 for the times indicated. (A) Cell extracts were subjected to immunoblotting for PARP, with uncleaved (110 kDa) and cleaved (85 kDa) species indicated. (B) Caspase 3 activity with DEVD-AMC as substrate, expressed as fluorescent intensity units with background subtracted, was determined as described in Section 2. Results are presented as mean \pm SD of three independent determinations. (C) Cytosolic extracts were subjected to immunoblotting for cytochrome *c*. The samples generated from cells treated with vinblastine were also subjected to immunoblotting for actin as a loading control; similar results were found for the other sample sets.

3.2. Kinetics of JNK activation

We had previously reported that vinblastine, doxorubicin, and VP-16 activated JNK in KB-3 cells [14]. We now sought to compare the effects on JNK of drug concentrations, equivalent to $IC_{50} \times 50$ values, and establish a relationship to the kinetics of apoptotic induction. Cells were treated for periods up to 48 hr, as in Fig. 1A, and JNK activity determined by immunocomplex assay with GST-c-Jun as substrate (Fig. 2). Activation of JNK by vinblastine was clearly biphasic, with an initial peak representing about 10-fold activation at 8 hr, and a second peak of slightly lower activity at 32–40 hr. JNK activation in response to doxorubicin also peaked at about 8 hr, with sustained activity up to 48 hr. The pattern of activation in response to VP-16 was more complex, with an initial peak at 24 hr and a second peak at 40–48 hr. The overall patterns

of activation observed were highly reproducible and similar profiles and extents of JNK activity were found in two repeats of these experiments. Similar results were also obtained using an independent “pull-down” assay, where glutathione beads with bound GST-c-Jun substrate were used to capture JNK from the cell extracts (data not shown).

Immunoblotting of cell extracts with antibodies against JNK1 and JNK2 was performed to examine JNK protein expression after drug treatment. As shown in Fig. 2C, JNK2 (p54) and JNK1 (p46) levels remained fairly constant under these conditions, although after prolonged drug treatment of 48 hr, some apparent cleavage of JNK2 had occurred. These results indicate that drug-mediated JNK activation is not a result of increased JNK protein expression. Actin was also used as a loading control and the level of expression was similarly unchanged after drug treatment.

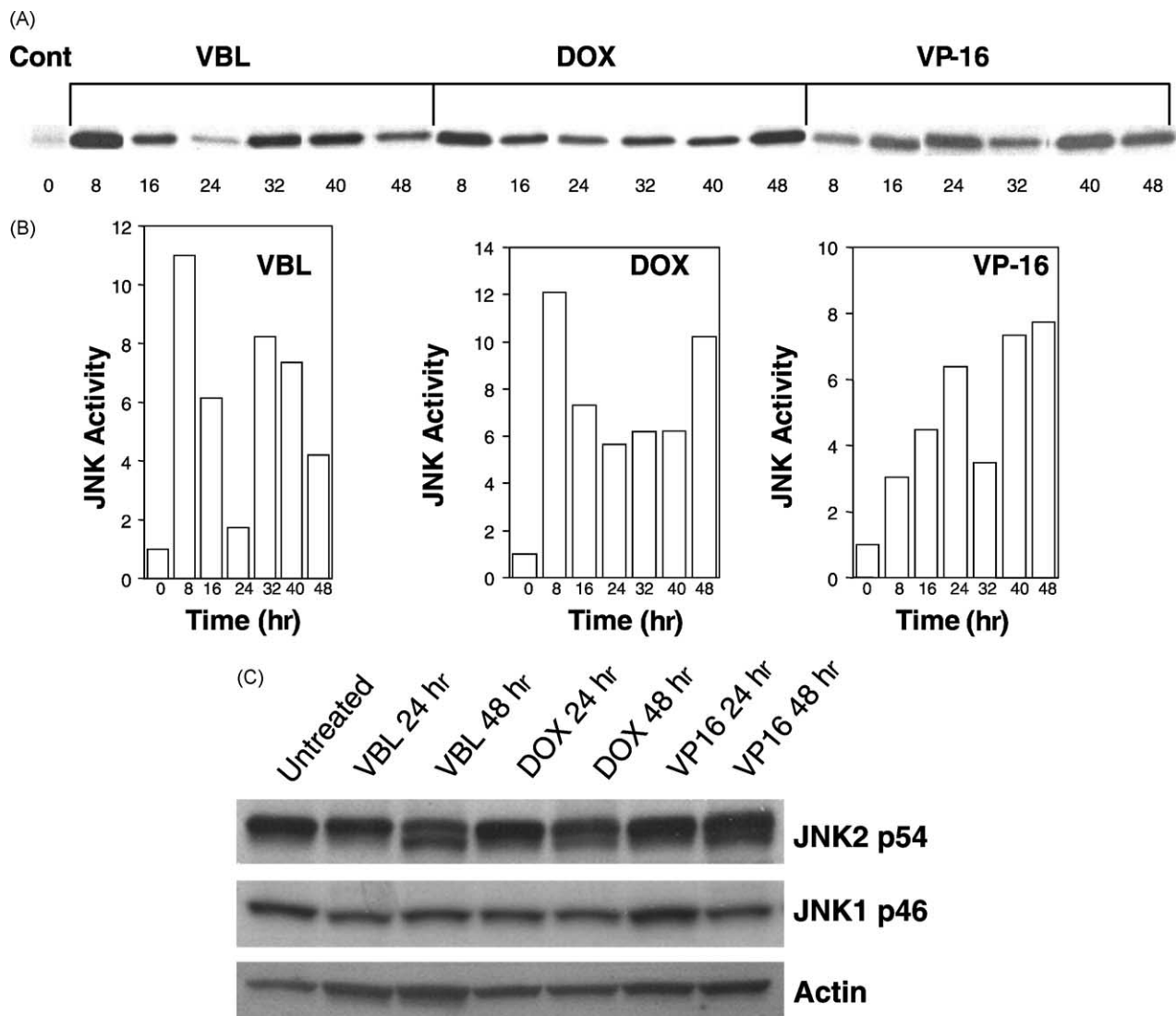


Fig. 2. Drug-induced JNK activation. KB-3 cells were treated with 30 nM vinblastine (VBL), 1 μ M doxorubicin (DOX), or 15 μ M VP-16 for the times indicated, and cell extracts subjected to JNK immunocomplex assay, as described in Section 2. (A) Autoradiograph of phosphorylated GST-c-Jun substrate; treatment times (in hr) indicated. (B) Quantitation of results by phosphorimager analysis. Results shown are representative of three independent experiments. (C) Cell extracts from control and drug-treated cells were subjected to immunoblotting for JNK1, JNK2, and actin, as indicated.

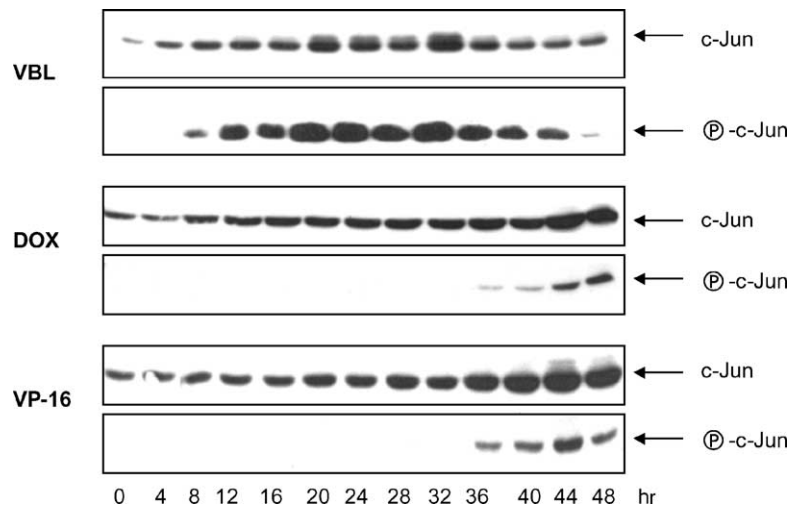


Fig. 3. Differential effect of drugs on c-Jun phosphorylation and expression. KB-3 cells were treated with 30 nM vinblastine (VBL), 1 μM doxorubicin (DOX), or 15 μM VP-16 for the times indicated. Cell extracts were prepared and subjected to immunoblotting with a phosphorylation-independent c-Jun antibody or an antibody recognizing c-Jun phosphorylated at Ser63 (P-c-Jun).

3.3. Differential effects on c-Jun phosphorylation/AP-1 activation

One of the main functions of JNK is to phosphorylate the amino-terminus of c-Jun, with subsequent AP-1 transcriptional activation [16]. Indeed, c-Jun phosphorylation is widely regarded as an inevitable consequence of JNK activation. KB-3 cells were treated with vinblastine, doxorubicin, or VP-16, as in Fig. 1A, and c-Jun amino-terminal phosphorylation examined by immunoblotting using a phospho-specific (Ser63) c-Jun antibody (Fig. 3). In response to vinblastine, c-Jun phosphorylation was observed at early time points, reached a broad maximum at 20–32 hr, and declined to near undetectable levels by 48 hr. c-Jun expression, assessed with a highly specific

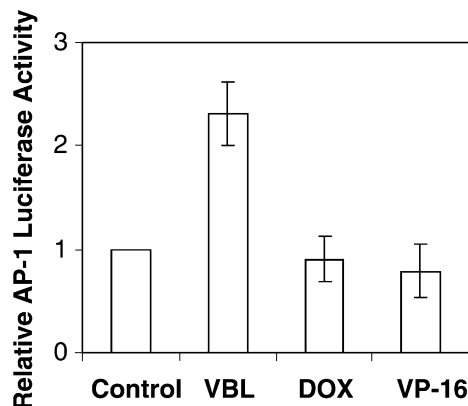


Fig. 4. Effect of drugs on AP-1 transcriptional activity. KB-3 cells were transiently transfected with a firefly luciferase reporter gene under control of two copies of an AP-1 site (TRE2-Luc) and with control Renilla luciferase under control of a constitutive promoter (TK-Luc). Cells were then untreated or treated for 24 hr with 30 nM vinblastine, 1 μM doxorubicin, or 15 μM VP-16, and luciferase activities determined. Results (mean ± SD, N = 3) are expressed as average relative firefly luciferase activity normalized to Renilla activity.

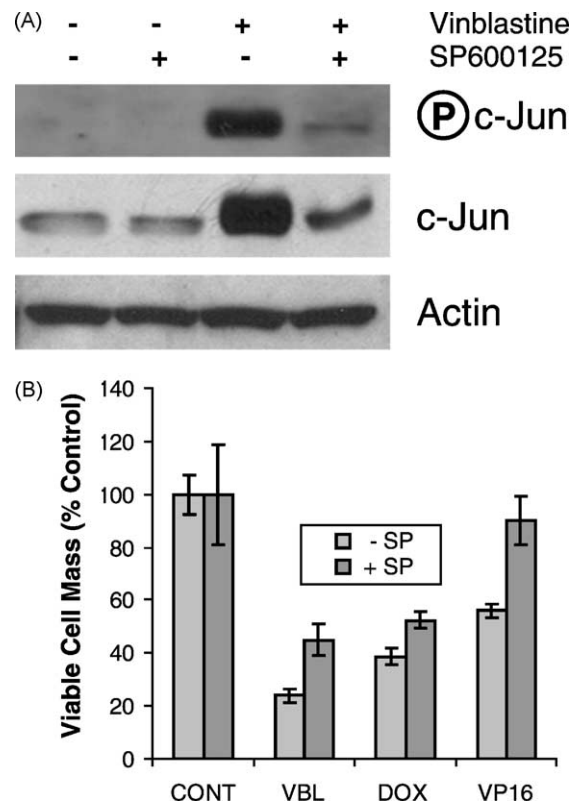


Fig. 5. Inhibition of JNK by SP600125 and effect of JNK inhibition on cell viability after drug treatment. (A) KB-3 cells were untreated or treated for 24 hr with 30 nM vinblastine (VBL), 20 μM SP600125, or both, with the JNK inhibitor added 1 hr prior to VBL, as indicated. Cell extracts were prepared and subjected to immunoblotting for c-Jun phosphorylated at Ser63 (P-c-Jun); c-Jun expression, using a phosphorylation-independent antibody; and actin. (B) KB-3 cells were treated with 20 μM SP600125 (+SP) or vehicle (–SP) for 1 hr, and then further treated with vehicle (control, CONT) or either 30 nM vinblastine (VBL), 1 μM doxorubicin (DOX) or 15 μM VP-16 for 48 hr. Viable cell mass was determined by MTT assay, as described in Section 2. Results are expressed as mean ± SD (N = 6). Viable cell mass after drug treatment was highly significantly different in the presence vs. absence of SP600125 in each case ($P \leq 0.001$ by two-tailed t test).

phosphorylation-independent c-Jun antibody, showed a similar profile. A parallel increase in phosphorylation and expression of c-Jun is expected, due to a positive feedback loop where activated c-Jun induces expression of its own gene, together with increased stability of the phosphorylated protein [17,18]. The kinetics of vinblastine-induced c-Jun phosphorylation paralleled the kinetics of JNK activation to a close degree, although c-Jun phosphorylation was more sustained and not as markedly biphasic. In stark contrast, in doxorubicin-treated cells, c-Jun phosphorylation was only detectable after prolonged treatment times of 36–48 hr (Fig. 3), and thus occurred well after JNK activation, and furthermore was subsequent to apoptosis induction shown in Fig. 1. This delayed phosphorylation of c-Jun was accompanied by an increase in c-Jun expression, and c-Jun expression also increased at earlier time-points, but in the absence of detectable phosphorylation. Results for VP-16 were essentially identical to those with doxorubicin, with detectable phosphorylation of c-Jun only occurring after prolonged treatment (Fig. 3).

Amino-terminal phosphorylation of c-Jun is accompanied by AP-1 transcriptional activation. Therefore, AP-1 activation was measured using a luciferase reporter system as described in Section 2, after 24 hr treatment of cells with the drugs. An increase in AP-1-dependent luciferase activity was observed after vinblastine treatment, but AP-1 activity remained at basal levels after treatment with doxorubicin or VP-16 (Fig. 4). Thus, doxorubicin and VP-16 fail to induce c-Jun phosphorylation or AP-1 activation despite JNK activation.

3.4. Effect of JNK inhibition on drug cytotoxicity

In order to determine the role of JNK activation in drug-induced cytotoxicity, we utilized the newly available JNK inhibitor, SP600125 [19]. SP600125 was an effective inhibitor in this system, and at a concentration of 20 μ M almost completely inhibited drug-induced JNK activation, as determined by inhibition of vinblastine-induced c-Jun phosphorylation and expression (Fig. 5A). To determine the effect of JNK inhibition on drug cytotoxicity, cells were pretreated with SP600125 or vehicle for 1 hr, and then either untreated or treated with vinblastine, doxorubicin, or VP-16, and cell viability assays performed. As shown in Fig. 5B, SP600125 protected cells from each of the three drugs, with cell viability highly significantly increased in the presence of the JNK inhibitor. It is evident that SP600125 partially protected cells from vinblastine and doxorubicin and almost completely abrogated VP-16-induced lethality under these conditions. These results indicate that JNK plays a destructive role in the mechanism of action of each of the three drugs, and may be particularly relevant to VP-16-induced cell death.

3.5. Effects of drugs on ERK signaling and consequences of ERK inhibition

JNK and ERK often play opposing roles, with one pathway antagonizing the other. We had previously shown that ERK was constitutively activated in KB-3 cells, and that vinblastine treatment caused a time-dependent

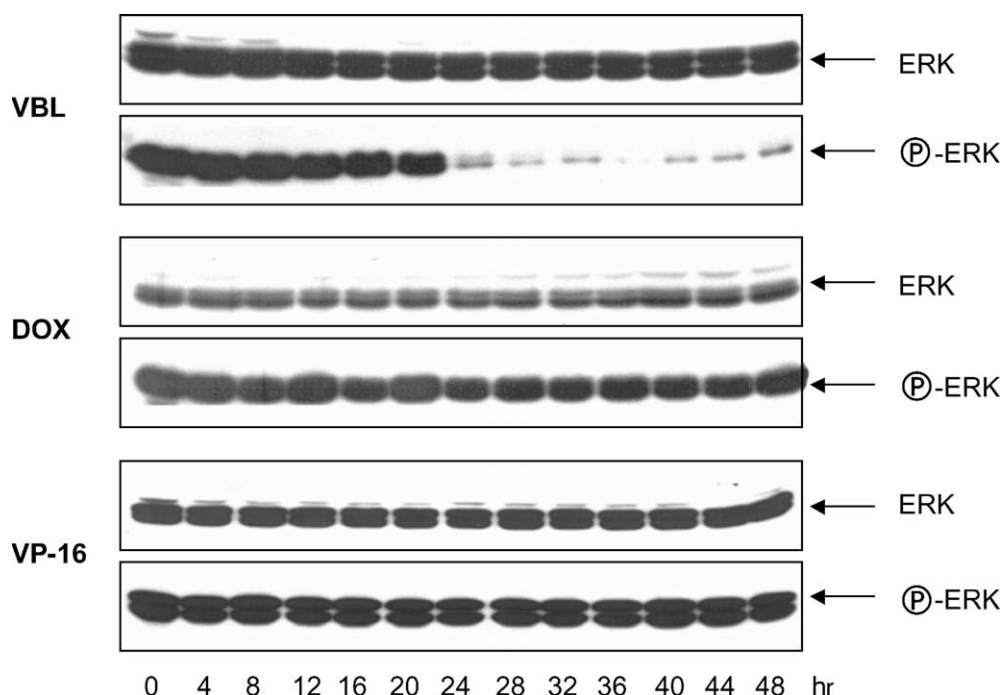


Fig. 6. Effect of drugs on ERK activation. KB-3 cells were treated with 30 nM vinblastine (VBL), 1 μ M doxorubicin (DOX), or 15 μ M VP-16 for the times indicated. Cell extracts were prepared and subjected to immunoblotting with a phosphorylation-independent ERK1/2 antibody or an antibody recognizing phosphorylated, activated ERK1/2 (P-ERK).

decrease in ERK activity, in parallel with JNK activation [20]. In order to demonstrate whether ERK inactivation accompanied drug-induced JNK activation in KB-3 cells, the effects of the chemotherapeutic drugs on ERK activity were examined. Phospho-ERK levels declined with vinblastine treatment, but remained fairly constant after treatment with doxorubicin or VP-16 (Fig. 6). In all cases, total ERK levels remained relatively unchanged. Thus, vinblastine, but not doxorubicin or VP-16, promoted inactivation of ERK, and in the case of the latter two drugs, apoptosis was induced and JNK activated without evident alteration in the status of ERK.

Because activated ERK may promote cell survival [21], it was of interest to determine whether ERK inhibition modulated the apoptotic response to drug treatment. Therefore, cells were pretreated with the MEK inhibitor U0126 [22], and then treated with vinblastine, doxorubicin, or VP-16. For these experiments, drug concentrations were lowered to the IC_{50} values, so that possible additive effects of U0126 on drug cytotoxicity could be evaluated. First, the ability of the MEK inhibitor U0126 to inhibit ERK activation was examined. Cells were pretreated with 20 μ M U0126 and then treated with vinblastine, doxorubicin, or VP-16, and after 48 hr, cell extracts were prepared and subjected to immunoblotting with the phospho-ERK antibody. As shown in Fig. 7A, U0126 was very effective, inhibiting ERK activity both in control and drug-treated cells. It should be noted that 0.6 nM vinblastine only induces ERK inactivation after prolonged treatment (>72 hr), as opposed to 30 nM vinblastine which more rapidly inactivates ERK (Fig. 6). Thus, under the experimental conditions of Fig. 7A, ERK remains partially active upon treatment with vinblastine alone.

To determine the effect of ERK inhibition on drug cytotoxicity, standard MTT assays were conducted under identical conditions. The results are presented in Fig. 7B. Inhibition of ERK by U0126 had no effect on the viability of control cells. However, U0126 strongly potentiated cytotoxicity induced by vinblastine or doxorubicin, but had no effect on VP-16-treated cells.

3.6. Effect of drugs on p53 expression and consequences of p53 inhibition

The possible role of p53 in apoptosis induced by the chemotherapeutic drugs was evaluated by examining p53 levels after drug treatment, and by the use of pifithrin- α , a chemical inhibitor of p53 function [23]. KB-3 cells express low levels of wild-type p53 [15]. Vinblastine induced a time-dependent decrease in p53 protein expression, to levels that were almost undetectable. Similarly, the p53 target and cyclin kinase inhibitor, p21, was detectable in control cells, and vinblastine caused a reduction to near non-detectable levels at most time-points (Fig. 8). In contrast, both doxorubicin and VP-16 induced p53 expression, and in parallel p21 expression, with the greatest

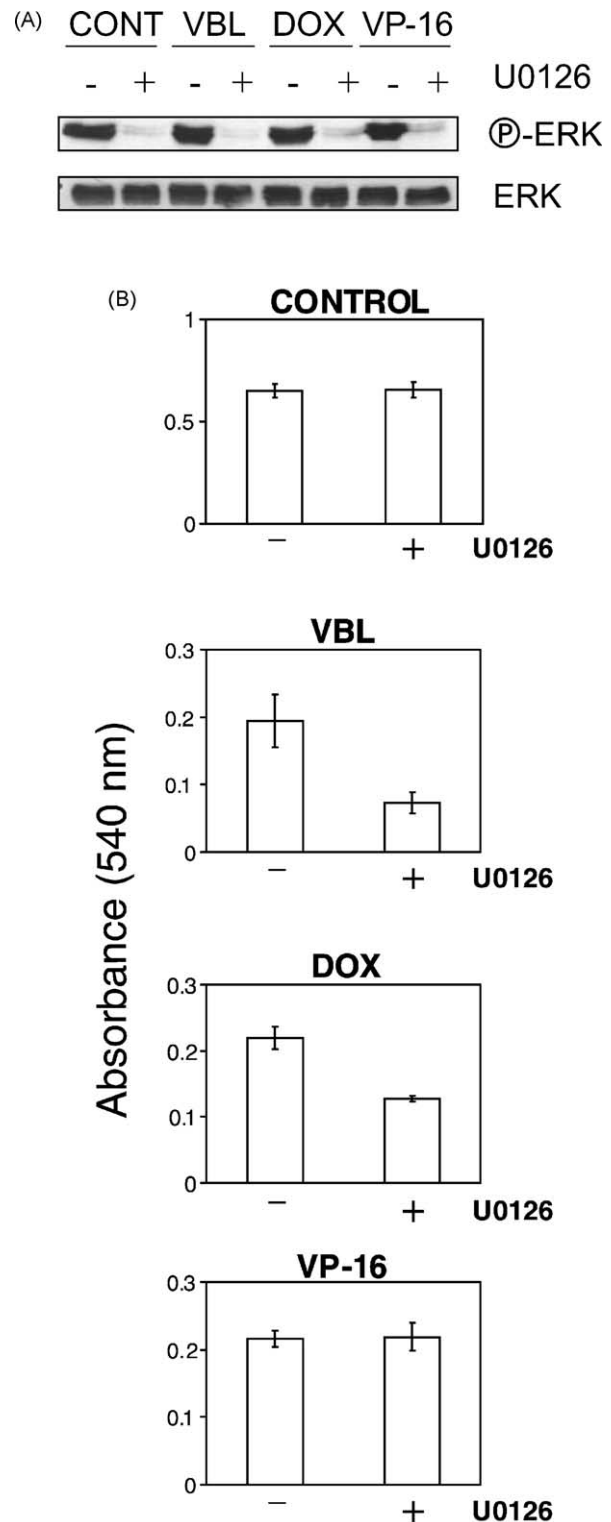


Fig. 7. Inhibition of ERK by U0126 and effect of ERK inhibition on cell viability after drug treatment. (A) KB-3 cells were untreated or treated with 0.6 nM vinblastine (VBL), 20 nM doxorubicin (DOX), or 300 nM VP-16, in the presence or absence of 20 μ M U0126, for 48 hr, with the MEK inhibitor added 1 hr prior to drug addition. Cell extracts were prepared and subjected to immunoblotting with a phosphorylation-independent ERK1/2 antibody or an antibody recognizing phosphorylated, activated ERK1/2 (P-ERK). (B) KB-3 cells were treated for 72 hr as in (A), and relative cell viability determined by MTT assay, as described in Section 2. Results are expressed as mean \pm SD (N = 3).

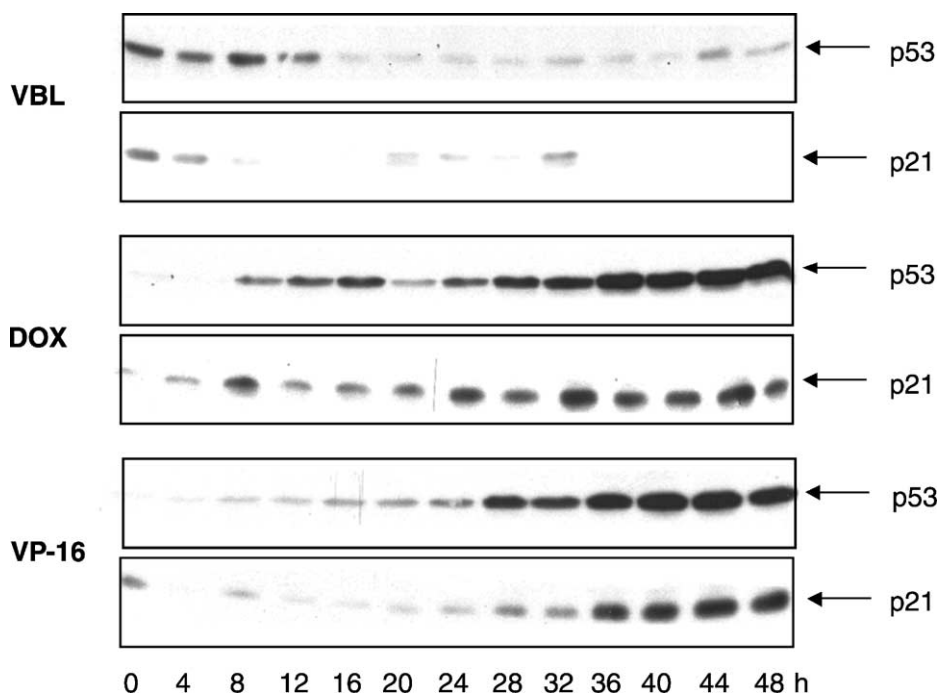


Fig. 8. Effect of drugs on p53 and p21. KB-3 cells were treated with 30 nM vinblastine (VBL), 1 μ M doxorubicin (DOX), or 15 μ M VP-16 for the times indicated, cell extracts were prepared, and subjected to immunoblotting with antibodies to p53 or p21 as indicated. Note that the (uppermost) p53 immunoblot after VBL treatment was deliberately overexposed during processing of the ECL image relative to those after DOX or VP-16 treatment, to allow the reduction in p53 expression to be visualized.

increase occurring at 24–48 hr. (Note that the p53 immunoblot after vinblastine treatment was deliberately overexposed relative to the two others shown, to allow the reduction in p53 expression to be visualized.) In order to determine the possible role of p53 in apoptosis, cells were pretreated with pifithrin- α , and then with the chemotherapeutic drugs, and cell viability determined (Fig. 9). Pifithrin- α increased the extent of cell death induced by vinblastine, but decreased the extent of cell death induced by doxorubicin or VP-16. These results suggest that p53 plays opposite roles in cell death induced by the microtubule inhibitor compared to that induced by the two DNA damaging drugs.

4. Discussion

In this study, we examined and compared the roles of JNK, ERK, and p53 pathways in the mechanism of action of three important chemotherapeutic agents, namely vinblastine, doxorubicin, and VP-16. In order to make comparisons under similar conditions, we used a concentration of each drug that was equivalent to 50 times the IC_{50} value determined from standard MTT assays. Because the MTT assay measures the number of viable cells after incubation, IC_{50} values derived from this assay can be influenced by effects of drugs on both proliferation and cell death. Thus, equivalent drug concentrations based on the MTT assay may not be equi-apoptotic. However, under the conditions

used in this study, each drug induced apoptotic cell death with closely similar kinetics. Thus, the kinetics of PARP cleavage and of caspase 3 activation was similar for each drug, as was the kinetics of cytochrome *c* release. The finding that all three drugs promoted cytochrome *c* release suggests an involvement of the mitochondrial apoptotic pathway in cell death induced by these agents. However, this does not rule out possible involvement of other apoptotic pathways, including death receptor signaling, in the process. For example, vinblastine treatment of KB-3 cells leads to increased expression of TNF- α mRNA [15], which may also play a role in apoptosis induction by this agent. Interestingly, caspase 3 was activated to different extents by the drugs (Fig. 1B), despite the fact that PARP cleavage was similar, or in the case of vinblastine, somewhat more pronounced after 48 hr treatment (Fig. 1A). These results suggest that other or additional caspases may also be involved in PARP cleavage. Caspase 3 is only one of several effector caspases [24] and a more detailed examination of the spectrum of caspases activated will be required to clarify the role of individual caspases in drug-induced apoptosis.

JNK activation was a relatively early event in the response of KB-3 cells to each of the chemotherapeutic drugs (Fig. 2). Although the patterns of JNK activation were complex, the maximum extent of activation was similar for each drug, ranging from 8- to 12-fold over basal. In the case of vinblastine treatment, JNK activation was temporally associated with c-Jun phosphorylation.

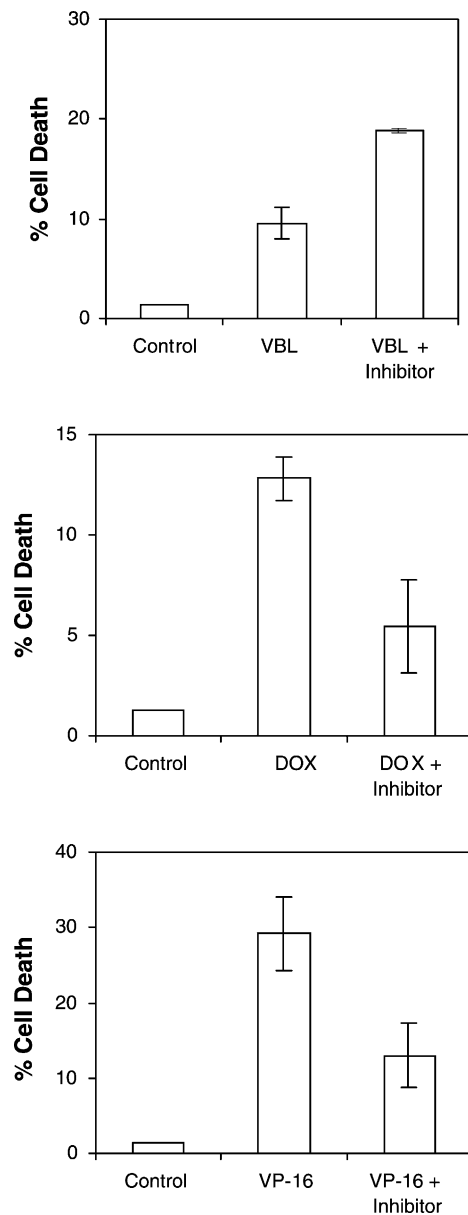


Fig. 9. Effect of the p53 inhibitor, pifithrin- α , on drug-induced cell death. KB-3 cells were treated with 20 μ M pifithrin- α for 1 hr prior to the addition of either 30 nM vinblastine (VBL), 1 μ M doxorubicin (DOX), or 15 μ M VP-16. After 48 hr, cells were stained with trypan blue, and those excluding or including the dye were scored as alive or dead cells, respectively. Two hundred cells were scored for each condition, and results presented as percentage of dead cells (mean \pm SD, $N = 4$).

However, JNK activation in response to doxorubicin or VP-16 was not associated with c-Jun phosphorylation. Based on the relative kinetics of the events examined, c-Jun appears to be a primary target of vinblastine-activated JNK, but not of JNK activated by the other two drugs. Indeed, the time-course of c-Jun phosphorylation in response to these latter drugs, relative to PARP cleavage and caspase 3 activation, suggests that c-Jun phosphorylation is a secondary, post-apoptotic event, and perhaps a consequence of apoptosis. To our knowledge, this is the first report of JNK activation in the absence of c-Jun phosphorylation. The finding that

c-Jun is not an obligate substrate of JNK and that JNK can signal in the absence of c-Jun/AP-1 activation has important implications for understanding JNK function. Furthermore, in cases where c-Jun is not the primary target, attempts to block the pathway at the level of c-Jun itself may have a neutral effect, and findings from such studies should be interpreted with caution. Other substrates may be targeted by JNK activated by doxorubicin and VP-16. However, while many potential JNK substrates have been described [16], the ones pertinent in this context await identification.

Inhibition of JNK signaling by the specific inhibitor SP600125 protected cells from the cytotoxic effects of vinblastine, doxorubicin and VP-16. These results indicate that JNK signaling plays a destructive role in each case. However, the mechanism of pro-apoptotic signaling may be different in each case because only vinblastine activated c-Jun/AP-1 whereas the other drugs failed to do so. These findings suggest that JNK may mediate its effects through multiple mechanisms that may differ depending on the stimulus.

While all three drugs activated JNK, only vinblastine modulated ERK, with a decrease in activity, whereas in response to doxorubicin or VP-16, ERK was maintained in an active state. These results show that JNK and ERK are not necessarily reciprocally regulated. However, based on results with the MEK inhibitor U0126, ERK appeared to play different roles. Thus, ERK inhibition potentiated vinblastine and doxorubicin-mediated cytotoxicity, whereas inhibition of ERK did not influence cell killing by VP-16. These results suggest that ERK plays a protective role in cells treated with vinblastine or doxorubicin but a neutral role in cells treated with VP-16. These findings are in agreement with other reports where the role of ERK differs, from pro-apoptotic, to pro-survival, to neutral, and appears to depend on a host of parameters including the cell type, drug dose, and the status of other signal transduction pathways [8]. However, in many cases MEK inhibitors enhance the apoptotic actions of anticancer drugs, including apoptosis induced by paclitaxel [25,26], vinblastine [27; this study], and cisplatin [10,28,29], suggesting a strong potential for MEK inhibitors as chemosensitizers in specific cancer therapies [9].

Another important mediator of apoptosis is the tumor suppressor p53. Wild-type p53 is well known to induce either cell cycle arrest or apoptosis in cells that undergo DNA damage, acting in a crucial G1 checkpoint [12]. Doxorubicin and VP-16, both of which cause DNA damage, among other perturbations, both induced p53 expression in KB-3 cells. Importantly, inhibition of p53 partially protected the cells from these agents, suggesting the mechanism is in part p53-dependent. In contrast, vinblastine caused downregulation of p53, and prior inhibition of p53 enhanced vinblastine-induced cell death. These results suggest that p53 protects cells from microtubule inhibition. This is in agreement with evidence from paclitaxel-treated p53 knockout cells. In the absence of

p53, cells with damaged spindles adapt without dividing into a G1-like state followed by resynthesis of DNA [30]. Thus, p53 functions in a post-mitotic checkpoint, arresting cells that have exited mitotic arrest after spindle disruption, blocking S phase reentry, and protecting cells from subsequent cell death. Chemical inhibition of p53 would be expected to be akin to p53 ablation, and thus enhance the toxicity of microtubule inhibitors, as we found (Fig. 9). These results suggest that p53 inhibitors may have potential as chemosensitizers for microtubule active drugs. Interestingly, a major function of c-Jun is in transcriptional downregulation of p53 [31]. Our data are consistent with such a model. Thus, vinblastine activates c-Jun/AP-1, and p53 is downregulated; c-Jun is likely directly responsible for p53 downregulation because the latter event is blocked in cells expressing dominant-negative c-Jun [15]. These events may be important to promote cell cycle reentry and subsequent apoptosis after aberrant mitotic exit of vinblastine-treated cells, as discussed above. In contrast, because p53 induction may be required for doxorubicin and VP-16 induced cell death, it is logical that c-Jun/AP-1 is not activated by these drugs, because c-Jun, as a negative regulator, would oppose p53 function. Separation of JNK activation from c-Jun/AP-1 induction in the case of these DNA-damaging drugs may be critical to allow p53 to perform a proapoptotic function. Finally, p53 is a potential substrate of JNK [32], and may act as a JNK target in doxorubicin- and VP-16-treated cells. This possibility is currently under investigation.

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References

- [1] Kaufmann SH, Earnshaw WC. Induction of apoptosis by cancer chemotherapy. *Exp Cell Res* 2000;256:42–9.
- [2] Johnstone RW, Ruefli AA, Lowe SW. Apoptosis: a link between cancer genetics and chemotherapy. *Cell* 2002;108:153–64.
- [3] Costantini P, Jacotot E, Decaudin D, Kroemer G. Mitochondrion as a novel target of anticancer chemotherapy. *J Natl Cancer Inst* 2000;92:1042–53.
- [4] Petak I, Houghton JA. Shared pathways: death receptors and cytotoxic drugs in cancer therapy. *Pathol Oncol Res* 2001;7:95–106.
- [5] Makin G. Targeting apoptosis in cancer chemotherapy. *Expert Opin Ther Targets* 2002;6:73–84.
- [6] Mayo MW, Baldwin AS. The transcription factor NF-kappaB: control of oncogenesis and cancer therapy resistance. *Biochim Biophys Acta* 2000;1470:M55–62.
- [7] Widmann C, Gibson S, Jarpe MB, Johnson GL. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. *Physiol Rev* 1999;79:143–80.
- [8] Fan M, Chambers TC. Role of mitogen-activated protein kinases in the response of tumor cells to chemotherapy. *Drug Resistance Updates* 2001;5:253–67.
- [9] Dent P, Grant S. Pharmacologic interruption of the mitogen-activated extracellular-regulated kinase/mitogen-activated protein kinase signal transduction pathway: potential role in promoting cytotoxic drug action. *Clin Cancer Res* 2001;7:775–83.
- [10] Persons DL, Yazlovitskaya EM, Cui W, Pelling JC. 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* 1999;5:1007–14.
- [11] Wang X, Martindale JL, Holbrook NJ. Requirement for ERK activation in cisplatin-induced apoptosis. *J Biol Chem* 2000;275:39435–43.
- [12] Amundson SA, Myers TG, Fornace Jr AJ. Roles for p53 in growth arrest and apoptosis: putting on the brakes after genotoxic stress. *Oncogene* 1998;17:3287–99.
- [13] Berry A, Goodwin M, Moran CL, Chambers TC. AP-1 activation and altered AP-1 composition in association with increased phosphorylation and expression of specific Jun and Fos family proteins induced by vinblastine in KB-3 cells. *Biochem Pharmacol* 2001;62:581–91.
- [14] Osborn MT, Chambers TC. Role of the stress-activated/c-Jun NH₂-terminal protein kinase pathway in the cellular response to adriamycin and other chemotherapeutic drugs. *J Biol Chem* 1996;271:30950–5.
- [15] Fan M, Goodwin ME, Birrer MJ, Chambers TC. The c-Jun NH₂-terminal protein kinase/AP-1 pathway is required for efficient apoptosis induced by vinblastine. *Cancer Res* 2001;61:4450–8.
- [16] Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell* 2000;103:239–52.
- [17] Karin M. The regulation of AP-1 activity by mitogen-activated protein kinases. *J Biol Chem* 1995;270:16483–6.
- [18] Fuchs SY, Fried VA, Ronai Z. Stress-activated kinases regulate protein stability. *Oncogene* 1998;17:1483–90.
- [19] Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Anderson DW. *Proc Natl Acad Sci USA* 2001;98:13681–6.
- [20] Stone AA, Chambers TC. Microtubule inhibitors elicit differential effects on MAP kinase (JNK, ERK, and p38) signaling pathways in human KB-3 carcinoma cells. *Exp Cell Res* 2000;254:110–9.
- [21] Bonni A, Brunet A, West AE, Datta SR, Takasu MA, Greenberg ME. Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science* 1999;286:1358–62.
- [22] Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J Biol Chem* 1998;273:18623–32.
- [23] Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV, Gudkov AV. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* 1999;285:1733–7.
- [24] Earnshaw WC, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem* 1999;68:383–424.
- [25] MacKeigan JP, Collins TS, Ting JP. MEK inhibition enhances paclitaxel-induced tumor apoptosis. *J Biol Chem* 2000;275:38953–6.
- [26] McDaid HM, Horwitz SB. Selective potentiation of paclitaxel (taxol)-induced cell death by mitogen-activated protein kinase inhibition in human cancer cell lines. *Mol Pharmacol* 2001;60:290–301.
- [27] Stadheim TA, Xiao H, Eastman A. Inhibition of extracellular signal-regulated kinase (ERK) mediates cell cycle phase independent apoptosis in vinblastine-treated ML-1 cells. *Cancer Res* 2001;61:1533–40.
- [28] Hayakawa J, Ohmichi M, Kurachi H, Ikegami H, Kimura A, Matsuoka T, Jikihara H, Mercola D, Murata Y. Inhibition of extracellular signal-regulated protein kinase or c-Jun N-terminal protein kinase cascade, differentially activated by cisplatin, sensitizes human ovarian cancer cell line. *J Biol Chem* 1999;274:31648–54.

- [29] Mandic A, Viktorsson K, Heiden T, Hansson J, Shoshan MC. The MEK1 inhibitor PD98059 sensitizes C8161 melanoma cells to cisplatin-induced apoptosis. *Melanoma Res* 2001;11:11–9.
- [30] Lanni JS, Jacks T. Characterization of the p53-dependent postmitotic checkpoint following spindle disruption. *Mol Cell Biol* 1998;18:1055–64.
- [31] Schreiber M, Kolbus A, Piu F, Szabowski A, Mohle-Steinlein U, Tian J, Karin M, Angel P, Wagner EF. Control of cell cycle progression by c-Jun is p53 dependent. *Genes Dev* 1999;13:607–19.
- [32] Hu MC, Qiu WR, Wang YP. JNK1, JNK2 and JNK3 are p53 N-terminal serine 34 kinases. *Oncogene* 1997;15:2277–87.