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Inhibition of Extracellular Signal-regulated Kinase (ERK) Mediates Cell Cycle Phase Independent Apoptosis in Vinblastine-treated ML-1 Cells[1]

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ABSTRACT

Chemotherapeutic agents induce alterations in intracellular signal transduction cascades that culminate in the initiation of the apoptotic program. Here, the relationship between the mitogen-activated protein kinase (MAPK) response and apoptosis in ML-1 cells treated with vinblastine and paclitaxel was investigated. We show that these compounds elicit different effects on MAPKs with vinblastine, but not paclitaxel, increasing both c-Jun-NH_2-terminal kinase (JNK) and p38 activity. However, vinblastine and paclitaxel both induced apoptosis with similar kinetics, suggesting that increased JNK and p38 activity is not required for apoptosis that is induced by microtubule interfering agents. Strikingly, the abrogation of extracellular signal-regulated kinase (ERK)-signaling by the MAPK/ERK kinase (MEK)1/2 inhibitor PD098059 in combination with vinblastine robustly induced apoptosis in ML-1 cells at a rate much faster than treatment with vinblastine alone and occurred at all phases of the cell cycle. This apoptotic induction was attributed to JNK activation because: (*a*) non-JNK-activating concentrations of vinblastine failed to increase apoptosis in the presence of PD098059; (*b*) apoptosis induced by paclitaxel, which did not activate JNK, was not potentiated by PD098059; and (*c*) transduction of an inhibitor of JNK activity partially suppressed both JNK activity and apoptosis induced by vinblastine plus PD098059. Additionally, we found that the activation of JNK by vinblastine occurred upstream of effector caspase activation because treatment with a pan-specific caspase inhibitor (valine-alanine-aspartate-fluoromethylketone) resulted in complete abrogation of apoptosis with no effect on MAPK signaling. Taken together, these data suggest that inhibition of the MEK→ERK signal transduction cascade alleviates cell cycle dependence for vinblastine-induced apoptosis by a mechanism that requires JNK activation.

INTRODUCTION

MIAs[4] are clinically important chemotherapeutic drugs. *Vinca* alkaloids such as vinblastine target microtubule dynamics by binding to tubulin monomers and dimers. Micromolar concentrations of vinblastine bind in a low affinity state along the microtubule resulting in the depolymerization of microtubules, whereas nanomolar concentrations of vinblastine suppress the dynamic instability of microtubules by binding to the ends of microtubules (1, 2). In contrast, paclitaxel stabilizes microtubules by preventing their depolymerization. Biological consequences of interfering with microtubule dynamics include G_2-M phase arrest, inhibition of cell proliferation, and apoptosis (3).

Apoptosis is a form of cell death that culminates in the activation of caspases and nucleases that serve to degrade protein and genomic DNA within the cell. Most cancer chemotherapeutic agents have been reported to induce apoptosis. However, the signal transduction mechanisms that regulate apoptosis have yet to be clearly defined. In the last few years, a large body of evidence has implicated the MAPK family of proline-directed serine/threonine kinases in the regulation of apoptosis. Three MAPK family members have been characterized thus far. Each MAPK is activated through a similar but selective pathway of kinases. MAPK kinase kinases become phosphorylated and activated in response to a stimulus. MAPK kinase kinases then phosphorylate a specific MAPK kinase that, in turn, phosphorylates its specific MAPK. ERKs (or $p42/44^{MAPK}$) are phosphorylated by the sequential activation of RAF1 and MEK1/2 in response to growth factors and mitogens and induce either proliferation or differentiation (4, 5). Phosphorylation of JNK (or SAPK) occurs in response to the selective activation of a MAPK kinase kinase such as MEKK1 (6), ASK1 (7) or MLKs (8) followed by phosphorylation of either MKK4 (6) or MKK7 (9). This pathway is stimulated by environmental and chemical stress as well as by exposure to cytokines, and it appears to play a role in the induction of apoptosis (10, 11). $p38^{MAPK}$ is activated by hyperosmolarity and environmental stress and regulates pro- or anti-apoptotic effects in a stimulus and cell-type specific manner (12, 13). $p38^{MAPK}$ is phosphorylated by either MKK3 (14) or MKK6 (15, 16).

Several studies have reported on the ability of MIAs to increase the activity of JNK (17–21). Moreover, many of the agents that increase JNK activity also increase p38 activation. The precise role that MAPKs play in the regulation of MIA-induced apoptosis is still unclear. In the present study, we examined the effect of vinblastine and paclitaxel on MAPK activity and apoptosis in ML-1 cells. Vinblastine, but not paclitaxel, activated JNK and p38, whereas both agents induced apoptosis by 24 h. Apoptosis induced by vinblastine was markedly enhanced by PD098059 (a MEK1/2 inhibitor) but only at concentrations of vinblastine that increased JNK activity. We determined further that the potentiation of apoptosis by the vinblastine plus PD098059 combination depended upon JNK activity, because suppression of JNK signaling was associated with an attenuation of apoptosis. These results suggest that the selective abrogation of survival signaling with the concomitant activation of proapoptotic signaling pathways markedly affects the induction of apoptosis and may provide a useful rationale for using *Vinca* alkaloids in the clinic.

MATERIALS AND METHODS

Materials

Vinblastine and paclitaxel were purchased from Sigma Chemical Co. (St. Louis, MO). PD098059 and SB203580 were obtained from Calbiochem (Carlsbad, CA). Phospho-specific rabbit polyclonal antibodies to p42/44 ERK, $p38^{MAPK}$, c-Jun (Ser-63), JNK, and MEK1 were purchased from New England Biolabs (Beverly, MA). Rabbit polyclonal antibodies to ERK1 (also detects ERK2), JNK1 (also detects JNK2), $p38^{MAPK}$, and c-Jun were from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal antibody to D4-GDI was prepared in this laboratory (22). Unless otherwise indicated, all other reagents were purchased from Sigma Chemical Co.

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[4] The abbreviations used are: MIA, microtubule interfering agent; ERK, extracellular signal regulated kinase; JBD, JNK binding domain; JIP-1, JNK interacting protein-1; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; SAPK, stress-activated protein kinase; zVAD-fmk, valine-alanine-aspartate-fluoromethylketone.

Cloning, Expression, and Purification of TAT-JBD

Cloning of TAT-JBD. The JBD (amino acids 134–202) of JIP-1 was isolated from a mouse thymus cDNA library (Stratagene, La Jolla, CA) using a nested PCR method. Briefly, a pair of outer primers were used to amplify bp 313–935 of the cDNA: forward primer 5′-TGCAGTGCAAAGACACCCTG; reverse primer 5′-TGGTAGTGGATTCGGTCTCG. The product was reamplified with inner primers to obtain the product bp 516–744: forward primer 5′-CCCAAAGCGGAGTCCAACCA; reverse primer, which includes a *Bam*HI site 5′-CAGTCGGATCCTTAAGGCGTCTGTTCTCCTGTCT. The 241-bp product, which also contains an endogenous *Bam*HI site downstream of the forward primer, was digested with *Bam*HI and subcloned into pET15b (Novagen, Madison, WI). The insert was sequenced to confirm that it matched the murine cDNA sequence. The insert was then excised with *Xho*I and *Eco*RI and subcloned into pTAT-HA (kind gift from S. Dowdy, Washington University, St. Louis, MO). pTAT-HA encodes 6 histidine residues and then a hemagluttinin epitope tag and an 11-amino acid sequence from the HIV TAT protein that is sufficient to mediate transmembrane passage of the fusion protein (23).

Expression and Purification of TAT-JBD. *Escherichia coli* BL21-pLys bacteria transformed with TAT-JBD were grown at 30°C with a 6-h induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside. Cells were pelleted by centrifugation at 5,000 rpm at 4°C for 5 min, washed in ice cold PBS (pH 7.2), and resuspended in PBS containing 1 mM phenylmethylsulfonyl fluoride. The cells were then sonicated at 4 × 20-s pulses on ice before centrifugation at 10,000 rpm at 4°C for 10 min. The extract was loaded onto a 3-ml His-bind column (Novagen, Madison, WI) equilibrated in PBS containing 30 mM imidazole and then washed with 50 ml of PBS/30 mM imidazole. TAT-JBD was eluted with 10 ml of PBS/500 mM imidazole and desalted on a PD-10 column (Amersham Pharmacia Biotech, Piscataway, NJ). The purity of TAT-JBD was verified by Coomassie Brilliant Blue staining as well as by immunoblot analysis using an anti-hemagluttinin monoclonal antibody and enhanced chemiluminescence (Amersham Pharmacia Biotech).

Cell Culture and Treatment

Human myeloid leukemia ML-1 cells were passaged in RPMI 1640 containing 7.5% fetal bovine serum and incubated at 37°C in 5% CO_2/95% humidified air. In experiments using signal transduction inhibitors, cells (1×10^6/ml) were treated with the inhibitor(s) or the appropriate vehicle control 30 min before the addition of vinblastine or paclitaxel. Inhibitors were left in the culture medium for the duration of the experiment. DMSO concentrations in the media never exceeded 0.2%.

Cell Cycle Analysis

Cells were washed in PBS, resuspended in 70% ice-cold ethanol, and stored at −20°C overnight. After rehydration by washing in PBS, cells were suspended in PBS containing 100 μg/ml propidium iodide and 1 mg/ml heat-inactivated pancreatic RNase A and incubated at 37°C for 30 min. DNA content was then measured by flow cytometry. Alternatively, cells were fixed in 2% paraformaldehyde for 30 min, rehydrated in PBS and then fixed in 70% ice-cold ethanol before DNA content analysis.

Chromatin Condensation

Cells were incubated with 2 μg/ml Hoechst 33342 for 20 min at 37°C. An aliquot of cells was transferred to a microscope slide and fitted with a coverslip, and DNA was visualized with a fluorescent microscope. Cells exhibiting condensed chromatin and fragmented nuclei were scored as apoptotic. At least 200 cells were scored from each group, and data were expressed as the percentage of cells with condensed chromatin.

Immunoblot Analysis

Cells were lysed in ice cold lysis buffer [20 mM HEPES (pH 7.9), 25% glycerol, 0.5% NP40, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM Na_2EDTA, 0.5 mM 2-mercaptoethanol, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 50 μM Na_3VO_4, and 1 mM NaF] for 20 min at 4°C. Cell lysates were then mixed with Laemmli sample buffer and boiled for 5 min. Proteins were subsequently separated by SDS-PAGE (12%) and transferred to polyvinylidene difluoride membrane (Millipore). Membranes were blocked with 5% nonfat milk in Tris-buffered saline, 0.05% Tween 20 and then probed with the appropriate primary antibody overnight. Subsequently, membranes were washed in Tris-buffered saline, 0.05% Tween 20 and then incubated with secondary antibody conjugated to horseradish-peroxidase. Proteins were visualized by enhanced chemiluminescence.

Statistical Analysis

Where indicated, a one-way ANOVA was performed and the Newman-Keuls test was used to test for significance.

RESULTS

Effects of Vinblastine and Paclitaxel on Apoptosis and MAPK Signaling. Many forms of cellular stress, including treatment with anticancer drugs, have been shown to modulate MAPK signaling pathways and induce apoptosis. However, the role of these signaling pathways in cell death has not been fully established, and contradictory evidence exists. The initial experiments performed here were designed to compare the effects of vinblastine and paclitaxel on MAPK activation and apoptosis in the ML-1 leukemia cell line. The activities of ERK, JNK, and $p38^{MAPK}$ were examined by immunoblot analysis with phospho-specific antibodies that recognize the active form of each kinase. A 3-h incubation with vinblastine induced a dose-dependent increase in phospho-JNK and phospho-p38 with no demonstrable effect on phospho-ERK (Fig. 1*A*). Immunoblot analysis using antibodies recognizing the total level of each MAPK protein (that is, nonphosphorylated plus phosphorylated forms) revealed no change in total protein levels, suggesting that the increases in phospho-specific immunoreactivity were attributable to changes in the phosphorylation status of existing proteins. In contrast to the ability of vinblastine to increase stress signaling, paclitaxel failed to elevate phospho-JNK or phospho-$p38^{MAPK}$ and also had no affect on the levels of phospho-ERK.

The same concentration ranges of vinblastine and paclitaxel were also compared for their ability to induce apoptosis. Vinblastine at concentrations of 0.0022–2.2 μM increased apoptosis in ML-1 cells by 24 h, with additional increase by 48 h (Fig. 1*B*). Interestingly, the higher concentrations of vinblastine that potently activated JNK seemed less apoptotic than the lower concentrations at the later time point. Paclitaxel induced apoptosis with similar kinetics and efficacy, except that the higher concentrations were the more potent inducers of apoptosis (Fig. 1*C*). The fact that these concentrations of paclitaxel did not elevate phospho-JNK or phospho-$p38^{MAPK}$ suggests that these signaling pathways are not required for paclitaxel-induced apoptosis.

Inhibition of the ERK Pathway Enhances Apoptosis Induced by Vinblastine but not by Paclitaxel. It has recently been suggested that ERK is an important effector in a pathway that mediates cell survival (24–26). Therefore, we investigated whether the inhibition of the ERK signaling pathway would affect MIA-induced apoptosis. Under normal serum-containing conditions, ML-1 cells displayed basal levels of phospho-ERK (Fig. 1*A*). PD098059, a selective inhibitor of MEK1/2 (27), was used to inhibit the ERK pathway. ML-1 cells were incubated with vinblastine or paclitaxel, with or without PD098059, and then assayed for apoptotic chromatin condensation. In contrast to the gradual accumulation of apoptotic cells induced by

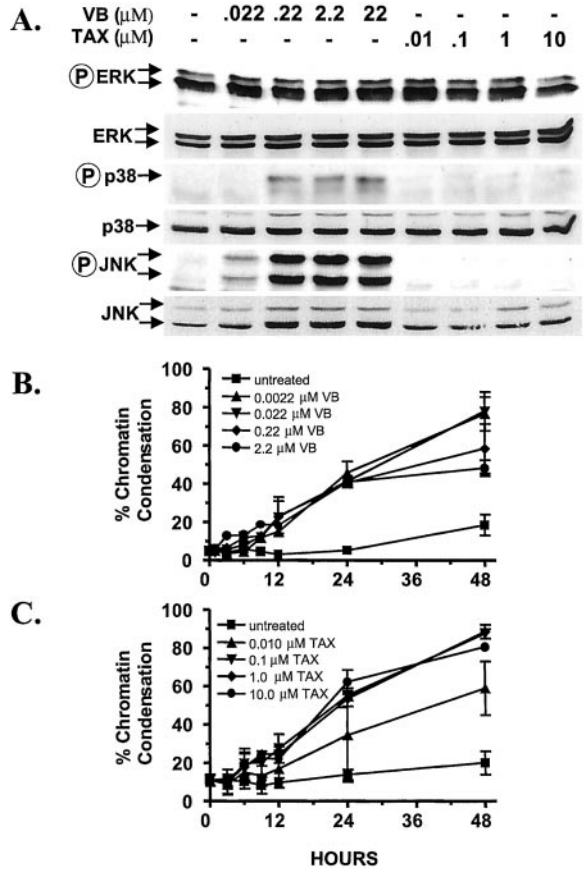

Fig. 1. Effects of vinblastine and paclitaxel on MAPK activity and apoptosis. *A*, ML-1 cells were treated with vinblastine (*VB*) or paclitaxel (*TAX*) for 3 h, and lysates were prepared. The indicated proteins were measured by immunoblotting. ML-1 cells were treated with the indicated concentrations of VB (*B*) or TAX (*C*) and apoptotic cells were scored at the indicated times by staining with Hoechst 33342. Results are expressed as a percentage of apoptosis and reported as the average ± SE of at least three independent experiments.

vinblastine alone, cells incubated with vinblastine plus PD098059 underwent apoptosis rapidly, with the majority of the cells apoptotic by 3 h (Fig. 2*A*). However, PD098059 failed to enhance the apoptosis induced by paclitaxel. When used alone, PD098059 was not toxic to ML-1 cells.

To examine further the effect of PD098059 on MIA-induced apoptosis, we performed immunoblot analysis of D4-GDI, a protein cleaved by caspases during apoptosis (22). Cell lysates were immunoblotted with an antibody that recognizes the full-length (M_r 26,000) and caspase 3-cleaved (M_r 22,000) form of D4-GDI. The combination of PD098059 plus vinblastine revealed D4-GDI cleavage by 3 h of treatment whereas the cleavage was not evident until 24 h in cells incubated with only vinblastine (Fig. 2*B*). Cleavage of D4-GDI was not apparent until 9 h of exposure to paclitaxel and was not altered by coincubation with PD098059 (Fig. 2*B*, *bottom*).

Enhancement of Vinblastine-induced Apoptosis by PD098059 Correlates with JNK Activation. The results in Fig. 1 suggested that JNK activation was not required for the induction of apoptosis by MIAs. However, because JNK was activated by vinblastine, we postulated that the increased phospho-JNK might be contributing to the enhanced apoptosis induced by the combination of vinblastine and PD098059. Cells were incubated with a range of concentrations of vinblastine for 24 h and phospho-JNK immunoreactivity was measured. In concordance with Fig. 1*A*, phospho-JNK levels exhibited a dose-dependent increase in response to vinblastine (Fig. 3*A*). The frequency of apoptosis was then assessed across this dose range. When cells were exposed to both PD098059 and vinblastine, only the concentrations of vinblastine capable of elevating phospho-JNK displayed a potentiated apoptotic response (Fig. 3*B*). PD098509 alone did not increase the incidence of apoptosis or induce phospho-JNK. This suggests that ERK activity may provide an antiapoptotic signal in the presence of activated JNK. Interestingly, at lower concentrations of vinblastine that failed to increase phospho-JNK, the addition of PD098059 appeared to reduce the incidence of apoptosis. This was confirmed by analysis of D4-GDI cleavage where non-JNK activating

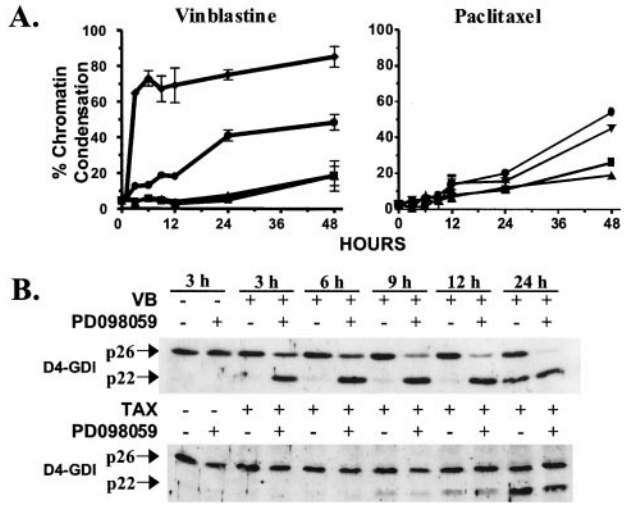

Fig. 2. Effects of PD098059 on vinblastine- and paclitaxel-induced apoptosis. *A*, ML-1 cells were treated with 50 μM PD098059 or vehicle (0.1% DMSO) for 30 min before the addition of 2.2 μM vinblastine (*VB*) or 100 nM paclitaxel (*TAX*). Apoptotic cells were scored at the indicated times by staining with Hoechst 33342. Results are expressed as a percentage of apoptosis and reported as the average ± SE of at least three independent experiments. ▲, untreated; ■, PD098059; ●, VB or TAX; ♦, VB + PD098059; ▼, TAX + PD098059. *B*, lysates were prepared at the indicated times, and immunoblot analysis was performed using the anti-D4-GDI antibody. Cleavage of the M_r 26,000 D4-GDI protein to the M_r 22,000 product (*arrows*) is indicative of caspase activation.

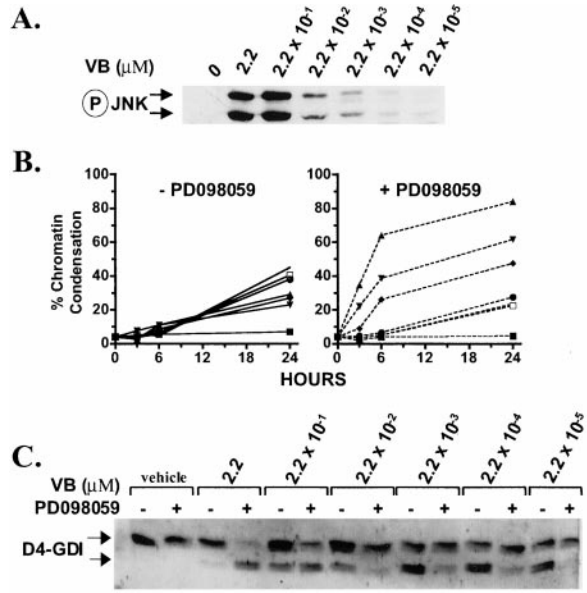

Fig. 3. PD098059-mediated potentiation of vinblastine-induced apoptosis is dose-dependent. *A*, ML-1 cells were treated with vinblastine (2.2×10^{-5}–2.2 μM) for 24 h, and lysates were prepared. Phospho-JNK protein levels were measured by immunoblot analysis. *B*, ML-1 cells were incubated with vinblastine (2.2×10^{-6}–2.2 μM) with (*right*) or without (*left*) 50 μM PD098059 and scored for apoptosis by Hoechst 33342 staining at the indicated times. (■, untreated; ∗, 2.2×10^{-5} μM; □, 2.2×10^{-4} μM; ●, 2.2×10^{-3} μM; ♦, 2.2×10^{-2} μM; ▼, 2.2×10^{-1} μM; ▲, 2.2 μM.) *C*, lysates were prepared and analyzed for D4-GDI cleavage.

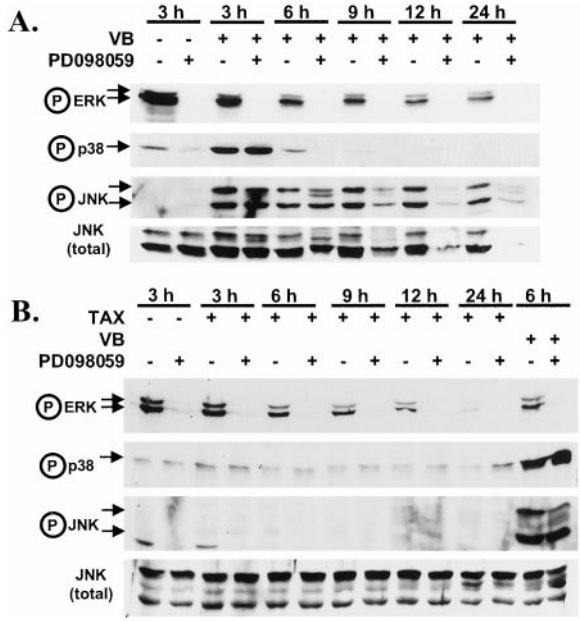

Fig. 4. Time-dependent effects of PD098059 in combination with vinblastine or paclitaxel. ML-1 cells were treated with 2.2 μM vinblastine (*VB*; *A*), or 100 nM paclitaxel (*TAX*; *B*) with or without 50 μM PD098059. Lysates were prepared at the indicated times, and proteins were measured by immunoblotting.

concentrations of vinblastine in combination with PD098059 produced less cleavage than vinblastine alone (Fig. 3*C*).

To study further the role of the various MAPK activities in apoptosis, proteins were analyzed at 3-h intervals up to 24 h (Fig. 4). Both vinblastine and paclitaxel caused a decrease in phospho-ERK over this time frame. The loss of phospho-ERK was not attributable to the loss of total protein, because total ERK immunoreactivity remained essentially constant (data not shown). In contrast, vinblastine markedly elevated phospho-JNK after 3 h, and this remained elevated throughout the 24-h period (Fig. 4*A*). Phospho-p38^{MAPK} was also increased by vinblastine at 3 h but then declined after 6 h (Fig. 4*A*). The increase in phospho-JNK and phospho-p38^{MAPK} were also seen as early as 1 h of vinblastine exposure (data not shown). Paclitaxel exposure for 24 h failed to increase either phospho-JNK or phospho-p38^{MAPK} (Fig. 4*B*).

Incubation of ML-1 cells with PD098059 caused complete suppression of phospho-ERK levels in both vinblastine- and paclitaxel-treated cells at every time point tested (Fig. 4). The addition of PD098059 had no impact on the rapid increase of phospho-JNK or phospho-p38^{MAPK} induced by vinblastine (Fig. 4*A*). However, the inclusion of PD098059 led to the appearance of a new phospho-JNK band between the p46^{MAPK} and p54^{MAPK} isoforms (Fig. 4*A*). This band is also evident upon analyzing the total-JNK immunoblot and seems to be attributable to cleavage of the p54 isoform (see below). At the 6-h time point and beyond, the addition of PD098059 led to the loss of both phospho-JNK and total-JNK, which may be the result of proteolysis occurring during death induced by this drug combination. Interestingly, although paclitaxel did not induce JNK activation in this model, some cleavage of the JNK p54^{MAPK} isoform was still observed after a 24-h treatment, which correlated with the appearance of some apoptosis at this time point (Fig. 4*B*). These results support the idea that the PD098059-enhancement of vinblastine-induced apoptosis is attributable to selective inhibition of the ERK signaling pathway in the presence of signaling through JNK or p38^{MAPK}.

Suppression of JNK Signaling Attenuates Vinblastine-plus-PD098059-induced Apoptosis. Although commercially available chemical inhibitors exist for both the ERK and p38^{MAPK} pathways, no such inhibitor exists yet for the JNK pathway. Whereas the expression of dominant-negative proteins of the JNK signaling pathway has been achieved with success through the use of transfection methodologies, ML-1 cells are refractory to the introduction of transfected DNA. Therefore, to directly test the role of JNK activity in the potentiation of apoptosis induced by the vinblastine and PD098509 combination, we used a fusion protein containing amino acids 134–202 of the JIP-1 protein (hereafter referred to as JBD). JIP-1 is a scaffold protein that binds to the JNK signaling module containing the proteins HPK, MKK7, and JNK1 (28). Furthermore, it has been demonstrated that transfection of JIP-1 into cells inhibits JNK-mediated c-Jun phosphorylation and restores cell viability (29–31). We cloned the region of the protein consisting of the JNK-binding domain (32) downstream of an 11-amino acid sequence of the HIV TAT protein that is sufficient to transduce across biological membranes (23). ML-1 cells were treated with the TAT-JBD fusion protein 1 h before the addition of PD098059 plus vinblastine, and JNK-mediated c-Jun phosphorylation was assessed. A 1-h exposure of ML-1 cells to vinblastine increased phosphorylation of c-Jun at serine 63 (Fig. 5*A*). Pretreatment with the TAT-JBD fusion protein produced a small but reproducible decrease in c-Jun phosphorylation relative to total c-Jun protein levels at 1 h (Fig. 5*A*). Interestingly, at later time points (3 h and beyond) TAT-JBD had no observable effect on the status of vinblastine-induced c-Jun phosphorylation. These results suggest that TAT-JBD is only able to partially inhibit JNK activity and, thereby, only transiently inhibit c-Jun phosphorylation. We excluded the possibility that the inhibitory effect of TAT-JBD on c-Jun phosphorylation was a nonspecific effect mediated by the TAT moiety, because pretreating cells with a different TAT-fusion protein did not inhibit vinblastine-induced c-Jun phosphorylation (data not shown).

Because TAT-JBD was able to negatively affect JNK activity, we hypothesized that this fusion protein might also abrogate apoptosis induced by vinblastine plus PD098059. ML-1 cells were treated with TAT-JBD for 1 h before the addition of PD098059 and/or vinblastine

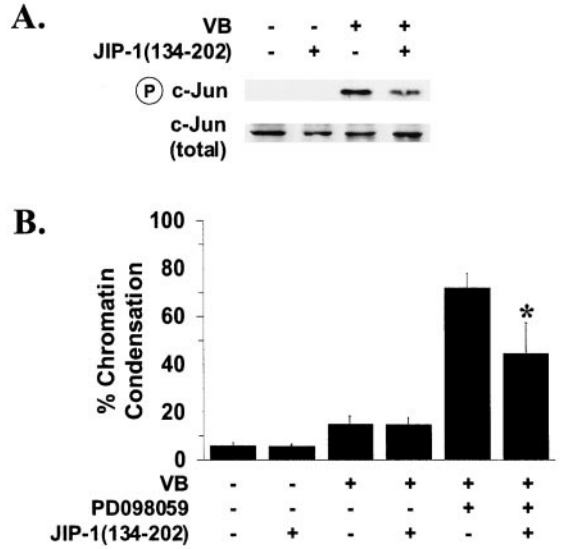

Fig. 5. TAT-JBD suppresses JNK activity and apoptosis. *A*, ML-1 cells were treated with 1 μM TAT-JBD or PBS vehicle for 1 h and then by a 1-h exposure to 2.2 μM vinblastine (*VB*). Lysates were prepared and analyzed by immunoblot analysis using antibodies against phosphorylated c-Jun (Ser-63; *top blot*) or total c-Jun (*bottom blot*). *B*, ML-1 cells were treated for 1 h with 1 μM TAT-JBD or PBS vehicle and then 2.2 μM vinblastine (*VB*) for 3 h. Where indicated, PD098059 (or DMSO vehicle) was added with VB. Cells were then stained with Hoechst 33342, and cells with condensed chromatin were scored as apoptotic. Data represents the average ± SE of at least three independent experiments. *, a significant difference ($P < 0.01$) between PD + VB and TAT-JBD + PD + VB.

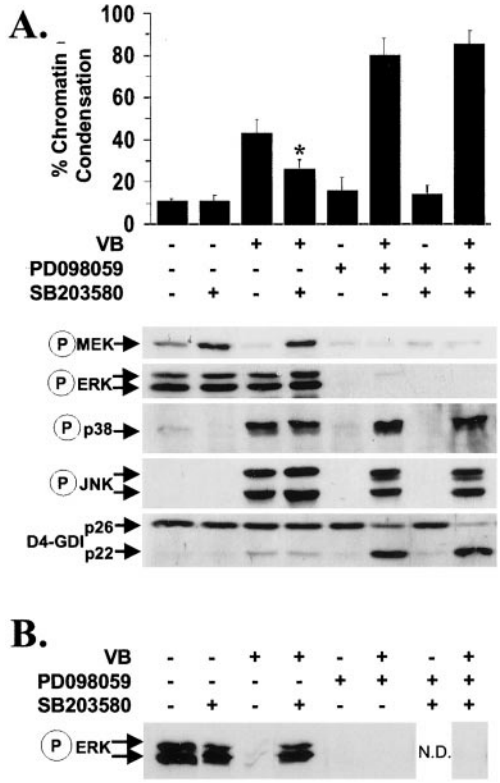

Fig. 6. Effects of SB203580 on MAPK activity and apoptosis. *A*, ML-1 cells were pretreated with 50 μM PD098059 or vehicle (0.1% DMSO) as indicated for 15 min and then with 20 μM SB203580 or vehicle (0.1% DMSO) for 30 min. Subsequently, cells were treated with or without 2.2 μM vinblastine (*VB*) for 18 h and then scored for apoptosis. Data represent the average ± SE from at least three independent experiments. *, a significant difference ($P < 0.05$) between VB alone and SB203580 + VB. ML-1 cells were treated as above for 3 h, and proteins were measured by immunoblotting. *B*, lysates from ML-1 cells were harvested after a 24-h exposure to VB with or without pretreatment with 20 μM SB203580, and immunoblot analysis was performed. The *lanes* have been reordered for clarity of presentation, but all derive from the same blot and experiment (*N. D.*, not determined).

for 3 h, and then DNA was stained for apoptotic chromatin condensation. We observed a significant reduction of apoptosis induced by vinblastine plus PD098059 in cells pretreated with TAT-JBD compared with a PBS vehicle (44.8 ± 12.7% *versus* 72.0 ± 5.8%; $P < 0.01$; Fig. 5*B*). However, at later time points (6 h and after) TAT-JBD was less effective at inhibiting apoptosis induced by vinblastine plus PD098059 and had no effect on apoptosis induced by vinblastine alone. Therefore, these data show that the transient inhibition of JNK activity correlates with the transient suppression of apoptosis, suggesting that JNK is required for apoptosis in this model.

The Role of p38^{MAPK} in Vinblastine-mediated Apoptosis. We next assessed the importance of p38^{MAPK} in the apoptotic response. The pyridinyl imidazole SB203580 selectively inhibits p38^{MAPK} activity by competitive inhibition at the ATP binding site (33); as such it did not prevent the phosphorylation of p38^{MAPK} (Fig. 6). Apoptosis induced by an 18-h incubation with vinblastine alone was inhibited ~40% by SB203580 (43.5 ± 6.2% *versus* 26.0 ± 4.6%, respectively; $P < 0.05$; Fig. 6*A*). However, apoptosis induced by vinblastine plus PD098059 was not inhibited by SB203580 (80.3 ± 7.9% *versus* 85.4 ± 6.5%, respectively). To determine whether SB203580 had antiapoptotic activity at earlier times, we measured D4-GDI cleavage after a 3-h incubation with vinblastine alone or in combinations including SB203580 and PD098059. As seen in Fig. 6*A*, SB203580 did not protect against D4-GDI cleavage in cells treated with the vinblastine-plus-PD098059 combination, nor did it prevent cleavage of JNK p54^{MAPK}.

In an effort to understand the biochemical basis for these observations, the phosphorylation status of the MAPKs was analyzed. Vinblastine-induced phosphorylation of JNK and p38 was not inhibited by 20 μM SB203580 (Fig. 6). SB203580 has been reported to increase the phosphorylation of ERK through a mechanism that still remains unclear (34, 35). We did not detect an increase in phospho-ERK after a 3-h treatment with SB203580. However, at 24 h, SB203580 clearly blocked the vinblastine-mediated decrease of phospho-ERK (Fig. 6*B*). This observation suggested that the ability of SB203580 to attenuate vinblastine-induced apoptosis may be attributable to the agonistic effects of SB203580 on phospho-ERK. The addition of PD098059 inhibited phospho-ERK as expected, and this was not prevented by SB203580 (Fig. 6*B*). These results provide additional confirmation that apoptosis is inversely correlated with the phosphorylation of ERK. The observation that PD098059 abolished the protective effect of SB203580 suggested that the ERK agonistic stimulus occurred through activation of the upstream events in the RAF→MEK→ERK pathway rather than through the inhibition of an ERK phosphatase. ERK is phosphorylated by MEK, and SB203580 was also found to increase phospho-MEK (Fig. 6*A*). Hence, it appears that a cross-talk mechanism may exist between the ERK and p38 pathways.

Caspase Inhibition Prevents Apoptosis but not MAPK Signaling. Because vinblastine, either with or without the addition of PD098059, induced apoptosis, we tested the ability of the panspecific caspase inhibitor zVAD-fmk to block apoptotic chromatin condensation and caspase dependent proteolysis (Fig. 7). ML-1 cells incubated with zVAD-fmk were resistant to apoptosis induced by vinblastine (35.1 ± 3.8% *versus* 5.5 ± 1.5%, respectively; $P < 0.01$) as well as by the combination of PD098059 and vinblastine (85.8 ± 4.9 *versus* 16.5 ± 5.1, respectively; $P < 0.001$). Treatment with zVAD-fmk also prevented apoptotic processing of D4-GDI as well as the cleavage of phospho-JNK p54^{MAPK} (Fig. 7). However, incubation with zVAD-fmk had no effect on the increase of phospho-JNK or phospho-p38, nor did it inhibit the ability of vinblastine to decrease phospho-ERK (Fig. 7 and data not shown). These results demonstrate that the

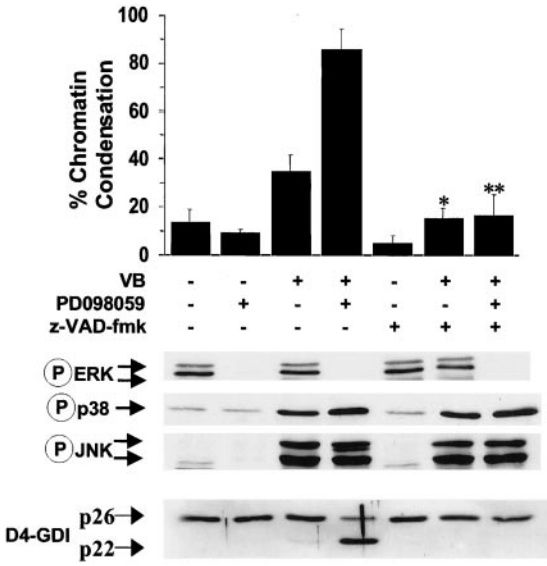

Fig. 7. Caspase inhibition blocks vinblastine- and PD098059-induced apoptosis. ML-1 cells were treated with 42 μM zVAD-fmk for 15 min and then by a 30-min incubation with 50 μM PD098059 before the addition of 2.2 μM vinblastine (*VB*). Apoptotic cells were scored by nuclear staining with Hoechst 33342 after an 18-h exposure to 2.2 μM VB. Data represent the average ± SE from three separate experiments. *, a significant difference ($P < 0.01$) between VB alone and zVAD-fmk + VB; **, a significant difference ($P < 0.001$) between PD098059 + VB and zVAD-fmk + PD098059 + VB. Cell lysates were also prepared after a 3-h exposure to VB and/or inhibitors, and immunoblot analysis was performed on the indicated proteins.

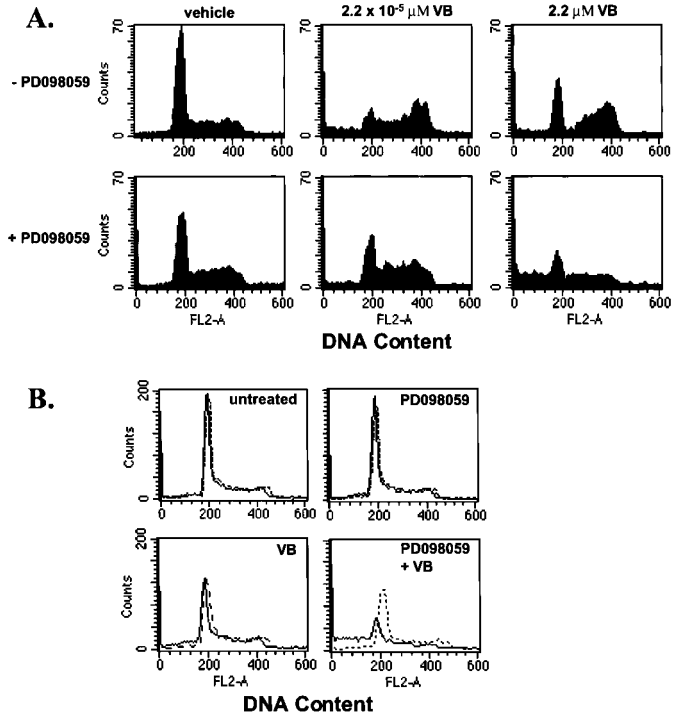

Fig. 8. Apoptosis induced by PD098059 plus vinblastine is cell-cycle independent. *A*, ML-1 cells were treated with (*bottom row*) or without (*top row*) 50 μM PD098059 for 30 min and then by vinblastine (*VB*) for 24 h. Cells were fixed in 70% ethanol, stained with propidium iodide, and DNA content was measured by flow cytometry. *B*, cells were treated with PD098059 and then by 2.2 μM VB for 6 h. Cells were then fixed in 70% ethanol (*solid line*) or in 2% paraformaldehyde/PBS and then 70% ethanol fixation (*dotted line*). Cells were stained with propidium iodide, and DNA content was measured by flow cytometry.

activation of JNK and p38 are not a consequence of apoptosis, and are therefore consistent with the hypothesis that they are upstream regulators of the apoptotic process.

Perturbation of Cell Cycle by Vinblastine Plus PD098059. All of the concentrations of vinblastine used here (as low as 2.2×10^{-11} M) caused accumulation of cells in the G_2-M phase of the cell cycle by 24 h and the appearance of sub-G_1 DNA content consistent with a low level of apoptosis (Fig. 8*A*). However, a pronounced G_1 arrest in addition to a G_2-M block was observed with vinblastine concentrations that activated JNK. At these concentrations of vinblastine, the addition of PD098059 caused a marked increase in cells with sub-G_1 DNA content. At low concentrations of vinblastine, where PD098059 slightly antagonized vinblastine-induced apoptosis (Fig. 3), there was an accumulation of cells in G_1 and S phases rather than at G_2-M (Fig. 8*A*). These data suggest that the protection afforded by PD098059 at low concentrations of vinblastine can be attributed to an alteration of the cell cycle. Specifically, PD098059 prevented ML-1 cells from reaching mitosis, where vinblastine is usually expected to elicit its cytotoxicity.

Considering that apoptosis occurred within 3 h in cells treated with high doses of vinblastine plus PD098059, it appeared unlikely that these cells could have reached mitosis before dying. To pursue this notion further, cells were harvested and fixed in either 70% ethanol or 2% paraformaldehyde. Fixation in 70% ethanol permeabilizes cells, facilitating the release of small DNA fragments and thus eliciting the typical sub-G_1 population of cells characteristic of apoptosis. In contrast, cell fixation in paraformaldehyde retains DNA within the cell. By comparison of the two profiles, it is possible to determine the phase of the cell cycle that the cells were in at the time they underwent apoptosis. In nonapoptotic cells, the two methods of fixation produced identical cell cycle profiles (Fig. 8*B*). After a 6-h incubation with vinblastine, there was little accumulation of cells in G_2-M and only a slight increase in the sub-G_1 population. The addition of PD098059 caused a marked increase in the sub-G_1 population. In the paraformaldehyde-fixed cells, it is evident that there was a dramatic decrease in the number of cells in the G_1 phase as well as a decrease in all other phases of the cell cycle. Accordingly, the cells are undergoing apoptosis in a cell cycle-independent manner and do not have to reach mitosis first.

DISCUSSION

It has been several years since it was suggested that apoptosis might be enhanced by the disruption of survival-associated MAPK signal transduction (24). This offers an attractive hypothesis for the rational design of therapeutic chemicals that could be used in conjunction with current chemotherapeutic agents. Indeed, many agents in clinical development are inhibitors of receptor tyrosine kinases (36, 37) or intracellular signal transduction pathways such as MEK (38). Furthermore, pharmacological inhibition of MEK1 has shown the potential to increase the cytotoxic index of various agents such as ara-C (39).

In this report, we have characterized the microtubule interfering agents vinblastine and paclitaxel with respect to their effects on MAPK signaling and apoptosis in ML-1 human leukemia cells. Growing evidence suggests that JNK activation serves as an important upstream event in the decision to undergo apoptosis (11, 25). The data presented here show that little apoptosis is induced by MIAs until after 12 h of treatment. This is consistent with the notion that MIAs cause apoptosis by interfering with the progression of cells through the M phase of the cell cycle. Additionally, vinblastine was more potent than paclitaxel in inducing apoptosis in ML-1 cells, as evidenced by the findings that all concentrations of vinblastine tested were apoptotic (as low as 2.2×10^{-11} M), whereas paclitaxel-induced apoptosis was dose-dependent in the nanomolar range (Fig. 1).

Although vinblastine and paclitaxel induced apoptosis with similar kinetics, their effects on the MAPKs were markedly different. Although both vinblastine and paclitaxel caused a time-dependent decrease in phospho-ERK immunoreactivity, only vinblastine increased the levels of the stress-associated MAPKs JNK and p38. The effect of vinblastine on JNK and p38 was dose-dependent, yet doses of vinblastine that did not elevate phospho-JNK still induced apoptosis. These results suggest that the effects of vinblastine on MAPK phosphorylation status is dissociable from apoptosis. The finding that paclitaxel did not elevate phospho-JNK levels in ML-1 cells is consistent with a report using Jurkat and HEK293 cells (40) but not other reports using OVCA 420 (17), RPMI-1788 B lymphoblasts (41), and ovarian carcinoma BR cells (42). These results indicate that the activation of JNK by paclitaxel is cell-type specific.

Treating cells concomitantly with PD098059 and vinblastine increased the percentage of cells undergoing apoptosis and markedly accelerated the kinetics of apoptosis. The increased kinetics of apoptosis did not correlate with an increased accumulation of cells in G_2-M, suggesting that PD098059 converted vinblastine into a non-cell cycle-specific toxin. This was confirmed by flow cytometric analysis in which apoptotic cells were observed at all phases of the cell cycle, most notably the G_1 phase (Fig. 8). Intriguingly, the combination of paclitaxel and PD098059 was no more apoptotic than paclitaxel alone. This observation is in agreement with a previous report indicating that PD098059 does not affect paclitaxel-mediated cytotoxicity in HL-60 cells (43). Moreover, this lack of potentiation of apoptosis was analogous to that observed when PD098059 was combined with low concentrations of vinblastine that did not activate JNK. It has been suggested that low concentrations of vinblastine ($<$100 nM) act by

stabilizing microtubule assembly (1, 2). This may explain the similarities between paclitaxel and low concentrations of vinblastine with respect to intracellular signaling and apoptosis. These data suggest that an increase in phospho-JNK immunoreactivity as mediated by vinblastine is required for the potentiation of apoptosis by PD098059.

To test the involvement of JNK in the potentiation of apoptosis, we used a cell-permeable peptide-inhibitor of JNK activity that consisted of the JNK-binding domain of JIP-1 fused to a cell-permeable TAT-derived peptide. We found that pretreatment of cells with the inhibitor suppressed the ability of JNK to phosphorylate c-Jun and attenuated apoptosis induced by vinblastine and PD098059 (Fig. 5). Although, these studies did not obtain a complete abrogation of JNK activity and apoptosis, they are consistent with a recent report showing that murine embryonic fibroblasts deleted for both JNK1 and JNK2 were resistant to UV-induced apoptosis (44). Furthermore, others have reported that transfected JIP-1 is capable of attenuating JNK activity and apoptosis in a variety of models (29–31). The use of the $p38^{MAPK}$ selective inhibitor SB203580 did not inhibit apoptosis mediated by the combination of vinblastine and PD098059, suggesting that vinblastine-induced $p38^{MAPK}$ phosphorylation is dispensable for apoptosis induced by this combination. The fact that JNK appears to be involved in the apoptotic program induced by the combination of vinblastine and PD098059, but not by vinblastine or paclitaxel alone, is an interesting one. Perhaps the rapid increase in apoptosis mediated by PD098509 plus vinblastine involves an alternate pattern of signaling cascades to those signals required for apoptosis after G_2-M arrest. Both of these pathways appear to involve mitochondrial dysfunction and caspase activity because both were inhibited in cells overexpressing Bcl-X_L[5] and by the pan-selective caspase inhibitor, zVAD-fmk (Fig. 7).

Our data indicate that the effects of vinblastine on MAPK phosphorylation appear to be upstream events, and not consequences of apoptosis, because zVAD-fmk had no effect on the phosphorylation status of any of the proteins analyzed (Fig. 7). An exception to this was the finding that both the total and the phosphorylated forms of p54 JNK were cleaved under conditions that were also found to cleave the known caspase substrate D4-GDI. This cleavage of JNK was completely inhibited by zVAD-fmk. These data suggest that in this drug-induced model of apoptotic induction, the cleavage of p54 JNK appears to occur subsequent to mitochondrial perturbations and cytochrome *c* release and may therefore be a consequential event in the execution phase of apoptosis. However, we cannot rule out that in other models of apoptosis, such as those involving engagement of Fas or the tumor necrosis factor receptor, cleavage of JNK by other caspases such as caspase 8 or 10 mediates an important regulatory event in the initiation of apoptosis. Support for this notion comes from studies indicating that caspase 8, in addition to caspase 3, cleaves MEKK1, causing alterations in cellular distribution and differential biological responses (45, 46). Efforts to characterize the cleavage of p54 JNK are currently underway.

The use of traditional chemotherapy agents in the management of cancer has not been successful in effecting a cure for most cancers. Therefore, the development of novel agents with selectivity against critical targets necessary for the control of apoptosis may provide an attractive rationale for combination therapy including conventional chemotherapeutic agents. Different cell lines are expected to depend on various signaling pathways for survival. We have found that other cell lines, including HL-60, are also sensitized to this combination of vinblastine and PD098059. However, this is not true of all cell lines. For example, the U937 and Jurkat cell lines are not sensitized by MEK inhibition despite activation of JNK;[6] therefore, it will be important to define the signaling pathways critical for survival in a given cell type. Our data provide a model whereby a cell cycle-specific agent such as vinblastine can be converted into a non-cell cycle-specific agent through pharmacological modulation of the MEK→ERK signal transduction pathway. This is of particular relevance in the clinical setting where tumor cells may be eradicated more effectively when cell cycle dependence is circumvented.

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[5] Unpublished observations.
[6] Unpublished observations.

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