MEK inhibition enhances paclitaxel-induced tumor apoptosis

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Running Title: MEK inhibition enhances paclitaxel apoptosis

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The abbreviations used are: NSCLC, non-small cell lung carcinoma; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; JNK, e-Jun N-terminal kinase; SAPK, stress-activated protein kinase; MEK, MAP kinase kinase; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PI, propidium iodide; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP nick end-labeling; CMV, cytomegalovirus; DMSO, dimethyl sulphoxide.

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SUMMARY

The anti-cancer drug paclitaxel (Taxol) alters microtubule assembly and activates pro-apoptotic signaling pathways. Previously, we and others found that paclitaxel activates endogenous JNK in tumor cells, and the activation of JNK contributes to tumor cell apoptosis. Here we find that paclitaxel activates the prosurvival MEK/ERK pathway, which conversely may compromise the efficacy of paclitaxel. Hence, a combination treatment of paclitaxel and MEK inhibitors was pursued to determine if this treatment could lead to enhanced apoptosis. The inhibition of MEK/ERK with a pharmacologic inhibitor, U0126 together with paclitaxel resulted in a dramatic enhancement of apoptosis that is 4X more than the additive value of the two drugs alone. Enhanced apoptosis was verified by the terminal transferase-mediated dUTP nick end-labeling (TUNEL) assay, by an ELISA assay for histone-associated DNA fragments, and by flow cytometric analysis for DNA content. Specificity of the pharmacologic inhibitor was confirmed by the use of (a) a second MEK/ERK inhibitor, and (b) a transdominant-negative MEK. Enhanced apoptosis was verified in breast, ovarian, and lung tumor cell lines, suggesting this effect is not cell-type specific. This is the first report of enhanced apoptosis detected in the presence of paclitaxel and MEK inhibition and suggests a new anticancer strategy.
INTRODUCTION

Paclitaxel is a promising frontline chemotherapy in the treatment of patients with ovarian, breast, and non-small cell lung carcinomas (NSCLC) (1-3). Paclitaxel is isolated from the bark of the pacific yew (Taxus brevifolia), and functions by binding and stabilizing microtubules (4). Binding of paclitaxel to microtubules blocks normal cell cycle progression during the merger of mitotic metaphase and anaphase. This prevents chromosome segregation, leading to tumor cell death.

Combination therapy of paclitaxel and Herceptin, an anti-Her2-neu antibody, has produced impressive responses among breast cancer patients (5,6), although this combination is obviously limited to Her2-neu+ tumors. Combination therapy with other drugs, preferably via a rational molecular basis that is widely applicable to many tumor types, is essential for improved cancer treatment. A combination of paclitaxel with reagents that activate additional apoptotic signals, or inhibit survival signals may provide a rational molecular basis for novel chemotherapeutic strategies.

A rational molecular target is the ERK mitogen-activated protein (MAP) kinase pathway that may serve as an opposing force to Jun N-terminal kinase (JNK/SAPK). Previous reports have shown that JNK/SAPK leads to cell death, while MEK activation contributes to cell differentiation, proliferation and survival (7,8). Activated Raf-1, a serine-threonine kinase, initiates the signaling cascade through MEK, which in turn phosphorylates a second serine-threonine kinase ERK. ERK phosphorylates additional kinases and specific transcription factors, such as Elk-1 and c-Fos, which are important in cell proliferation. However, the link between Raf-1 and ERK activation and paclitaxel-induced cell death is not straightforward. Several studies have shown that at a low
concentration of the drug, paclitaxel-mediated apoptosis is attributed to activated Raf-1 (9-11). The role of the downstream ERK MAP kinase in paclitaxel-induced tumor apoptosis is also not entirely clear (12-17).

In this report, we tested the combined effects of paclitaxel and inhibitors of MEK1/2 kinase on tumor cell apoptosis. The specificity of MEK1/2 inhibition was achieved by using two different MEK inhibitors, and by the additional use of transdominant-negative mutants, which inhibit MEK/ERK activation. The reasons for selecting MEK1/2 as the target are: (i) MEK is activated in many tumors (18-20); (ii) small molecule-based MEK inhibitors are readily available and a recent report has described a novel MEK inhibitor that exhibited in vivo efficacy in mice (21-23); and (iii) MEK is critical in transforming cells, leading to tumor survival and proliferation (24,25).

In the present study, we show that paclitaxel increases MEK1/2 activity. The combined treatment of paclitaxel plus MEK1/2 inhibition leads to enhanced cell death in breast, ovarian, and lung tumor lines.
EXPERIMENTAL PROCEDURES

JNK kinase assay- Following two hours of paclitaxel (Sigma) treatment, cells were washed twice with ice-cold PBS, harvested with lysis buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM PMSF) and centrifuged (12,500 rpm) at 4°C. Endogenous JNK was immunoprecipitated with anti-JNK antibody (Santa Cruz Biotechnology) and protein A-agarose beads for 2 hours at 4°C. Immunoprecipitates were collected by centrifugation (2,500 rpm) at 4°C. The precipitates were washed twice with lysis buffer and twice with kinase buffer. Immunoprecipitated JNK was mixed with 5µg GST-c-Jun and 10µCi [γ-32P]ATP and incubated for 30 min at 30°C. The reactions were terminated with SDS sample buffer and resolved on a 10% SDS-PAGE gel.

Immunoblot analysis- H157 human lung carcinoma cells were serum starved for 16 hours and treated simultaneously with the indicated concentrations of paclitaxel with or without 10µM U0126 (Promega). After 15 minutes of treatment, cells were lysed in 1x PBS, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1mM PMSF, 1mM Na3VO4, 10µM leupeptin and 10µM pepstatin at 4°C. Cell lysates were separated with SDS-PAGE gels, transferred to nitrocellulose membranes, and subjected to immunoblot analysis with anti-ERK monoclonal antibody for phosphorylated ERK1/2 (Santa Cruz Biotechnology).

Cell death ELISA- Manufacturer’s instructions were followed for the Cell Death Detection ELISA PLUS (Roche Biochemicals). Briefly, cells were plated at 5 x 10^3 cells/well in 96-well microtiter plates for 24 hours. The cells were treated for 20-24 hours with
indicated doses of paclitaxel and U0126. After treatment, the plates were centrifuged (200 x g) for 10 minutes. The supernatant was discarded, lysis buffer was added to each well, and samples were incubated at room temperature for 30 minutes. Following lysis, the samples were centrifuged and 20µl of the supernatant transferred to a streptavidin-coated microtiter plate. Anti-histone biotin and anti-DNA peroxidase antibodies were added to each well and the plate was incubated at room temperature for 2 hours. After three washes with incubation buffer, the peroxidase substrate was added to each well. Following a fifteen-minute incubation, the plates were read at 405nm in a microplate reader. The data in this report are expressed as fold increase in optical density as compared to control treated cells.

Cell cycle analysis- Adherent and detached cells were collected with trypsin and centrifuged at 200 x g. Cells were resuspended at 2 x 10^6 cells/ml in PBS and fixed in ice cold 70% ethanol for 2 hours. Fixed cells were centrifuged at 200 x g and each sample resuspended in propidium iodide (PI) stain buffer (0.1% Triton X-100, 200µg DNase-free RNase A, 20µg PI) in PBS for 30 minutes. After staining, samples were analyzed using a FACScan (Becton Dickinson) and ModFit LT (Verity Software).

TUNEL assay- Cells were split at a density of 3 x 10^4 cells/well in a 4-well chamber slide (Lab-Tek). Following a 36-hour incubation, the cells were treated with 10nM paclitaxel in the presence or absence of 10µM U0126 for 20 hours. Following treatment, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10 minutes. Cells were washed twice more with PBS and permeabilized with 0.2% Triton X-100 for 5
minutes. After two more washes, each slide was covered with equilibration buffer (Boehringer Mannheim) for 10 more minutes. The buffer was then aspirated and the slides were incubated with TdT buffer at 37°C for one hour. The reaction was stopped with 2x SSC and the slides were viewed with an immunofluorescence microscope. For each section, TUNEL-positive and -negative nuclei were counted and results reported as the percentage TUNEL-positive cells.

RESULTS AND DISCUSSION

The effect of paclitaxel on JNK and ERK activities is shown in Figure 1. Basal JNK activity was detected, and this activity was significantly enhanced by treatment with low, nanomolar doses of paclitaxel in human lung and breast carcinoma cell lines (Fig. 1A). A basal level of ERK was also detected, and low doses of paclitaxel activated endogenous ERK1 and ERK2 (Fig. 1B). The MEK inhibitor, U0126, completely blocked ERK activation by paclitaxel. The activation of JNK in this scenario has been previously found to contribute to apoptosis, while the role of paclitaxel-induced ERK has not been studied. In other systems, ERK generally plays a critical role in cell proliferation and growth (26,27); thus, it was reasoned that ERK activation by paclitaxel might enhance cell proliferation and compromise the efficacy of this drug. A logical approach is to use pharmacologic blockers of MEK to inhibit paclitaxel-induced ERK activation and its downstream effects.

To test this hypothesis, a combination of paclitaxel and a potent MEK1 inhibitor, U0126, was used to treat a variety of human carcinoma lines, and cell death was measured by the cell death detection ELISA assay that detects DNA-histone fragmentation. The
combination of paclitaxel plus U0126 enhanced cell death (Fig. 2A). The fold increase in apoptosis was calculated by comparing the ELISA optical density readings of treated samples, with the value of the untreated control as 1.0. In H157 cells, paclitaxel and U0126 combined caused 4X more cell death than paclitaxel alone, and 8X more cell death than U0126 alone. A similar trend was observed in OVCA194 cells.

The potential use of low-dose chemotherapy is important because lower dosages are more attainable during cancer therapy and likely to cause less toxicity in patients. We performed a dose-response analysis to assess the minimal concentration of paclitaxel, which when combined with U0126, causes enhanced cell death. Low-doses of paclitaxel, starting at the 10 nM range, combined with U0126 cause enhanced cell death in both BT474 breast and H157 lung carcinoma cells (Fig. 2B).

To control for pharmacologic specificity, two additional experiments were performed. First, a second MEK inhibitor PD98059 was used and produced similar data (Fig. 2C), providing additional evidence that the MEK enzyme is the target. However, pharmacologic approaches have their limitations, because the specificity of the drug can always be questioned. To provide further evidence for the effects of MEK inhibition, a dominant-negative MEK (dnMEK) mutant was introduced into H157 cells. Expression of dnMEK in the presence of low-dose (50nM and 250nM) paclitaxel enhanced apoptosis over the pCMV empty vector control (Fig. 2D).

Table 1 summarizes enhanced apoptosis observed with paclitaxel and U0126. In H157 and OVCA194 cells, the combination treatment produced 4.0 and 2.5-fold enhancement of apoptosis over the expected additive effect. This enhancement was
achieved with relative low-dosages (1 µM paclitaxel, 10 µM of U0126) of these two drugs. This trend was also observed with the breast carcinoma BT474 (not shown).

To examine the mechanism of U0126 and paclitaxel induced cell death, their effects on cell cycle progression was studied. The BT474 breast carcinoma cells were treated with paclitaxel and/or U0126, and cell cycle progression was analyzed by incubating the cells with propidium iodide, which allowed the analysis of DNA content by flow cytometry. U0126 arrested BT474 cells in G1, while 10nM paclitaxel produced a dramatic G2 block (Fig. 3A). The percentage of control treated cells in G2-M was 13%, which increased to 75% after treatment with 10nM paclitaxel for 24 hours. Seventeen percent of the cells underwent apoptosis in the presence of paclitaxel, while a negligible increase in cell death was detected in the presence of U0126 when compared to the control (6% compared to 4%). In contrast, the combination of paclitaxel and U0126 substantially increased cell death as evidence by accumulation of a sub-G1 population that has < 2N DNA (Fig. 3A) and represents dead cells. These results further support the ELISA result that low-doses of paclitaxel and U0126 enhanced tumor cell death.

To assess if the cell death observed above represents apoptosis, a TUNEL assay was performed with paclitaxel, U0126, or a combination of the two drugs. Singly, paclitaxel and U0126 caused little apoptosis (0.6 and 0.4%, see panels i to vi, Fig. 3B) as measured by the number of TUNEL positive cells. When cells were treated with both, there was a dramatic increase in the number of TUNEL positive cells to 11.1% (panels vii and viii, Fig. 3B). Phase-contrast photomicrographs of H157 cells revealed changes in morphology and cell membrane blebbing, which are characteristics of apoptosis (panel ix, Fig. 3B). These results further indicate that paclitaxel and U0126 enhance apoptosis.
In the last two years, we and others have reported that paclitaxel affects MAP kinases. The best documented is the activation of JNK/SAPK by paclitaxel, which has been found in a variety of tumor cell lines (12-16). JNK/SAPK activation is primarily a stress response, long proposed to be a determining factor in cell cycle arrest and apoptosis (28). Studies of hippocampal neuronal cells show that these cells do not undergo apoptosis when a JNK subgroup (jnk1, jnk2, or jnk3) is mutated. Very recently, the use of mice lacking functional JNK provides strong evidence that JNK is important in causing apoptosis (29-32). Most relevant to this present study, JNK activation by paclitaxel directly contributes to apoptosis, as transdominant-negative JNK/SAPK significantly blocked paclitaxel-induced cell death (12-16,33).

Extensive research has identified potential mechanisms of paclitaxel-induced cell death; most prominent is the effect on BCL-2 family members and p53. Several reports indicate that paclitaxel causes the phosphorylation and inactivation of BCL-2 and its family members (9,11,34-37), while other studies have found paclitaxel sensitivity varies with p53 status (38-41). Additionally, a link between JNK and BCL-2 was found, where JNK mediated BCL-2 phosphorylation and the inactivation of JNK inhibited paclitaxel-induced BCL-2 phosphorylation (42). This establishes the important roles of BCL-2 and JNK family members in paclitaxel-induced apoptosis, although other cell-death and cell survival pathways are likely to either enhance or intercede with this cytotoxicity. One of the findings described here is that paclitaxel also enhances the activation of the MEK/ERK pathway, which is expected to increase cell proliferation and survival, and may compromise the efficacy of paclitaxel in cancer treatment.
Based on a molecular approach, this report describes a novel discovery that treatment with paclitaxel combined with the inhibition of MEK1/2 lead to enhanced apoptosis of lung, ovarian, and breast carcinoma cell lines. Two pharmacologic agents, paclitaxel and U0126, respectively caused JNK activation that promotes apoptosis, and MEK inhibition, which leads to cell cycle arrest. The two combined resulted in an impressive enhancement of tumor cell killing.

In summary, these findings illustrate the power of molecular and rational drug targeting. Paclitaxel and MEK inhibitor combination therapy may allow the use of lower drug doses, likely leading to lowered toxicity and enhanced tumor killing in vivo. The implications of these findings are broad for the potential clinical usage of paclitaxel plus MEK inhibitors by: (1) improving the response rate and (2) expanding the usefulness of paclitaxel in the treatment of resistant tumors that affects a large percent of cancer patients.

Acknowledgements- Channing Der kindly provided the MEK constructs. We thank Drs. Albert Baldwin, Lee Graves, Brian Martin, Debra Taxman, Hank VanDeventer and Beverly Mitchell for helpful comments and discussions. This work was supported by NIH grants AI 41751 and AI45580, and a grant from the Lineberger Comprehensive Cancer Center.
REFERENCES


Fig. 1. Effects of paclitaxel and MEK inhibitor on MAP kinases. (A) Paclitaxel activated endogenous JNK. Human breast (BT474) and lung (H358) carcinoma cell lines were treated with the indicated concentrations of paclitaxel for 2 hours, and JNK kinase activity was assayed as described in the Experimental Procedures. (B) Paclitaxel activated endogenous ERK and activation is reversed by U0126. H157, a human lung carcinoma, and BT474 cells were serum starved for 16 hours and treated with the indicated concentrations of paclitaxel for 15 minutes. Cell lysates were subjected to immunoblot analysis with anti-ERK antibody for phosphorylated ERK1/2. The MEK inhibitor U0126 blocked ERK activation by paclitaxel (lower panel). H157 cells were serum starved for 16 hours, and treated simultaneously with paclitaxel with and without 10µM U0126 for 15 minutes.

Fig. 2. Analysis of paclitaxel and MEK inhibitor on carcinoma cell death.

(A) Paclitaxel and U0126 caused enhanced carcinoma cell death. H157 lung and OVCA194 ovarian carcinoma cells were treated with 1µM paclitaxel, 10µM MEK inhibitor U0126, or a combination of paclitaxel and MEK inhibitor. Twenty-four hours later, a cell death ELISA that measures cell death by DNA-histone release was performed as described. (B) Low-dose, nanomolar range of paclitaxel and 10µM U0126 caused enhanced killing. BT474 breast carcinoma and H157 lung carcinoma cells were treated with the indicated concentrations of paclitaxel in the presence or absence of 10µM U0126, and fold increase in cell death was measured by ELISA. (C) A second MEK inhibitor, PD98059, and paclitaxel caused enhanced cell death. H157 lung carcinoma cells were treated with either 10µM U0126 or 50µM PD98059 for 20 hours, and
analyzed by the cell death ELISA. (D) Dominant-negative MEK (dnMEK) and paclitaxel caused enhanced cell death. H157 cells were transfected with 100ng pCMV vector control or dnMEK (43). After 24 hours, cells were treated with the indicated amount of paclitaxel for 24 hours and cell death assayed by ELISA.

**Fig. 3. Paclitaxel and MEK inhibitor U0126 block cell cycle progression and cause enhanced cell death.** (A) BT474 breast carcinoma cells were treated for 24 hours before staining with propidium iodide and analyzed by flow cytometry as described. Histograms of control (DMSO-treated) cells exhibited normal cell cycle progression. Ten µM U0126 induced G1 growth arrest, 10nM paclitaxel induced G2 growth arrest, and 10nM paclitaxel plus 10µM U0126 caused enhanced death by an accumulation of sub-G1 dead cells. The percent of cells in each phase of the cell cycle are shown below the histograms. (B) Paclitaxel and U0126 cause enhanced apoptosis. H157 cells were grown on coverslips and treated with 10nM paclitaxel and/or 10µM U0126 as indicated. After 16 hours, the slides were incubated and TUNEL stained. The phase contrast photomicrographs (panels i, iii, v, vii) and the corresponding immunofluorescence photomicrographs (panels ii, iv, vi, viii) of cells undergoing apoptosis are shown. The number in the lower right of each panel represents the percentage of TUNEL positive cells.
Table I  
Combination treatment with paclitaxel and MEK inhibitor U0126
H157 and OVCA194 cells were treated with the indicated concentrations of U0126 and paclitaxel for twenty-four hours. Apoptosis was analyzed by cell death ELISA that measures DNA-histone release, and data is expressed as absorbance \([A_{405nm} - A_{490nm}] \times 100\).

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\(^a\) (Mean cell death of U0126) + (mean cell death of paclitaxel).
\(^b\) (Observed combination) / (expected combination).
Figure 1

A

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Figure 2

A

Fold Increase in Cell Death

Control
MEK Inhibitor
Paclitaxel
Paclitaxel + MEK Inhibitor

H157
OVCA194

B

H157
BT474

Fold Increase in Cell Death

Paclitaxel + U0126
Paclitaxel

Fold Increase in Cell Death

Paclitaxel + U0126
Paclitaxel

C

Fold Increase in Cell Death

Control
Paclitaxel

None
U0126
PD98059

MEK Inhibitor

D

Fold Increase in Cell Death

pCMV
dnMEK

Control
50nM Paclitaxel
250nM Paclitaxel
Figure 3

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J. Biol. Chem. published online October 18, 2000

Access the most updated version of this article at doi: 10.1074/jbc.C00684200

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