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MEK Inhibition Enhances Paclitaxel-induced Tumor Apoptosis*

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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 whether 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 four times more than the additive value of the two drugs alone. Enhanced apoptosis was verified by the terminal transferase-mediated dUTP nick end labeling assay, by an enzyme-linked immunosorbent 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.

Paclitaxel is a promising frontline chemotherapy in the treatment of patients with ovarian, breast, and nonsmall cell lung carcinomas (1, 2). Paclitaxel is isolated from the bark of the pacific yew (*Taxus brevifolia*) and functions by binding and stabilizing microtubules (3). 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 (4), 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[1] 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 (5, 6). 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 (7–9). The role of the downstream ERK MAP kinase in paclitaxel-induced tumor apoptosis is also not entirely clear (10–14).

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 (15, 16); (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 (17–19); and (iii) MEK is critical in transforming cells, leading to tumor survival and proliferation (20, 21). 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 2 h of paclitaxel (Sigma) treatment, cells were washed, harvested with lysis buffer, and centrifuged at 4 °C (10). Endogenous JNK was immunoprecipitated with anti-JNK antibody (Santa Cruz Biotechnology) and protein A-agarose beads for 2 h at 4 °C. Immunoprecipitates were collected by centrifugation (2,500 rpm) at 4 °C. Immunoprecipitated JNK was mixed with 5 μg of glutathione *S*-transferase-c-Jun and 10 μCi of [γ-^{32}P]ATP and incubated for 30 min

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[1] The abbreviations used are: ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; MEK, MAP kinase kinase; dnMEK, dominant-negative MEK; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; PI, propidium iodide; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP nick end-labeling; CMV, cytomegalovirus; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

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 h and treated simultaneously with the indicated concentrations of paclitaxel with or without 10 μM U0126 (Promega). After 15 min of treatment, cells were lysed in 1× PBS, 1% Trition X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 mM Na_3VO_4, 10 μM leupeptin, and 10 μM 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 Molecular Biochemicals). Briefly, cells were plated at 5×10^3 cells/well in 96-well microtiter plates for 24 h. The cells were treated for 20–24 h with the indicated doses of paclitaxel and U0126. Following lysis, the samples were centrifuged and 20 μl of the supernatant transferred to a streptavidin-coated microtiter plate as described (10). Anti-histone biotin and anti-DNA peroxidase antibodies were added to each well, and the plate was incubated at room temperature for 2 h. After three washes with incubation buffer, the peroxidase substrate was added to each well. Following a 15-min incubation, the plates were read at 405 nm in a microplate reader. The data in this report are expressed as -fold increase in optical density as compared with control treated cells.

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

TUNEL Assay—Cells were split at a density of 3×10^4 cells/well in a four-well chamber slide (Lab-Tek). Following a 36-h incubation, the cells were treated with 10 nM paclitaxel in the presence or absence of 10 μM U0126 for 20 h. Following treatment, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min. Cells were washed twice more with PBS and permeabilized with 0.2% Triton X-100 for 5 min. After two more washes, each slide was covered with equilibration buffer (Roche Molecular Biochemicals) for 10 more min. The buffer was then aspirated, and the slides were incubated with TdT buffer at 37 °C for 1 h. The reaction was stopped with 2× SSC, and the slides were viewed with an immunofluorescence microscope.

RESULTS AND DISCUSSION

The effect of paclitaxel on JNK and ERK activities is shown in Fig. 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. 1*A*). A basal level of ERK was also detected, and low doses of paclitaxel activated endogenous ERK1 and ERK2 (Fig. 1*B*). 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 (22); 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 that detects DNA-histone fragmentation. The combination of paclitaxel plus U0126 enhanced cell death (Fig. 2*A*). 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 four times more cell death than paclitaxel alone, and eight times more cell death than U0126 alone. A similar trend was observed in OVCA194 cells.

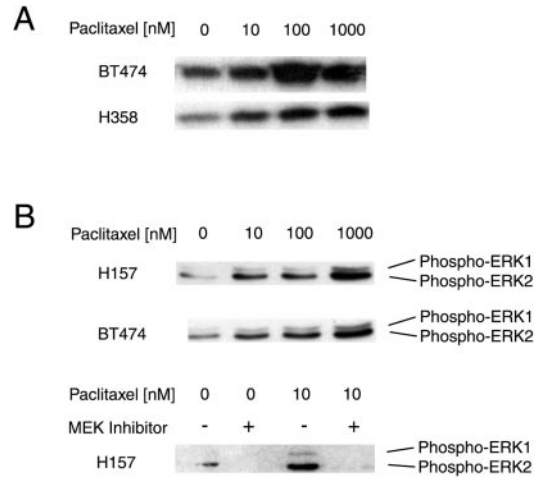

FIG. 1. **Effects of paclitaxel and MEK inhibitor on MAP kinases.** *A*, paclitaxel-activated endogenous JNK. Human breast (*BT474*) and lung (*H358*) carcinoma cell lines were treated with the indicated concentrations of paclitaxel for 2 h, and JNK kinase activity was assayed as described under "Experimental 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 h and treated with the indicated concentrations of paclitaxel for 15 min. 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 h and treated simultaneously with paclitaxel with and without 10 μM U0126 for 15 min.

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. 2*B*).

To control for pharmacologic specificity, two additional experiments were performed. First, a second MEK inhibitor PD98059 was used and produced similar data (Fig. 2*C*), 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 (50 and 250 nM) paclitaxel enhanced apoptosis over the pCMV empty vector control (Fig. 2*D*).

Table I 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 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 G_1, while 10 nM paclitaxel produced a dramatic G_2 block (Fig. 3A). The percentage of control treated cells in G_2-M was 13%, which increased to 75% after treatment with 10 nM paclitaxel for 24 h. Seventeen percentage of the cells underwent apoptosis in the presence of paclitaxel, while a negligible increase in cell death was detected in the presence of U0126

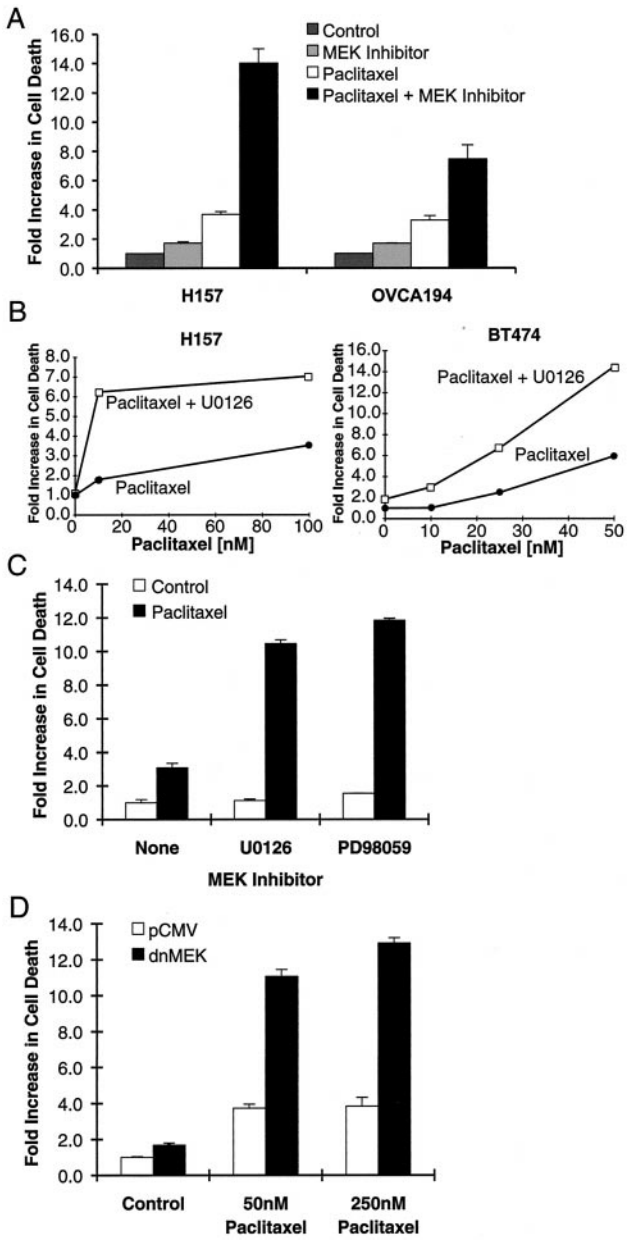

FIG. 2. **Analysis of paclitaxel and MEK inhibitor on carcinoma cell death.** *A*, paclitaxel and U0126 caused enhanced carcinoma cell death. H157 lung and OVCA194 ovarian carcinoma cells were treated with 1 μM paclitaxel, 10 μM MEK inhibitor U0126, or a combination of paclitaxel and MEK inhibitor. Twenty-four hours later, a cell death ELISA that measures cell death by DNA-histone release was performed as described under "Experimental Procedures." *B*, low dose, nanomolar range of paclitaxel and 10 μM 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 10 μM 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 10 μM U0126 or 50 μM PD98059 for 20 h and analyzed by the cell death ELISA. *D*, dnMEK and paclitaxel caused enhanced cell death. H157 cells were transfected with 100 ng of pCMV vector control or dnMEK (34). After 24 h, cells were treated with the indicated amount of paclitaxel for 24 h and cell death assayed by ELISA.

when compared with the control (6% compared with 4%). In contrast, the combination of paclitaxel and U0126 substantially increased cell death as evidence by accumulation of a sub-G_1 population that has <2 N DNA (Fig. 3*A*) and represents dead cells. These results further support the ELISA result that low doses of paclitaxel and U0126 enhanced tumor cell death.

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 24 h. Apoptosis was analyzed by cell death ELISA that measures DNA-histone release, and data are expressed as absorbance $[A_{405\ nm} - A_{490\ nm}] \times 100$.

	10 μM U0126	1 μM paclitaxel	Combination treatment		Fold increase over additive[b]
			Expected[a]	Observed	
H157	3.0	13.0	16.0	63.7	4.0
OVCA194	3.1	8.7	11.8	30.1	2.5

[a] (Mean cell death of U0126) + (mean cell death of paclitaxel).
[b] (Observed combination)/(expected combination).

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–vi*, Fig. 3*B*) 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. 3*B*). Phase-contrast photomicrographs of H157 cells revealed changes in morphology and cell membrane blebbing, which are characteristics of apoptosis (*panel ix*, Fig. 3*B*). These results further indicate that paclitaxel and U0126 enhance apoptosis.

In the last 2 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 (10–13). JNK/SAPK activation is primarily a stress response, long proposed to be a determining factor in cell cycle arrest and apoptosis (23). 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 (24–26). 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 (10–13).

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 (7, 9, 27–29), while other studies have found paclitaxel sensitivity varies with p53 status (30–32). 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 (33). 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

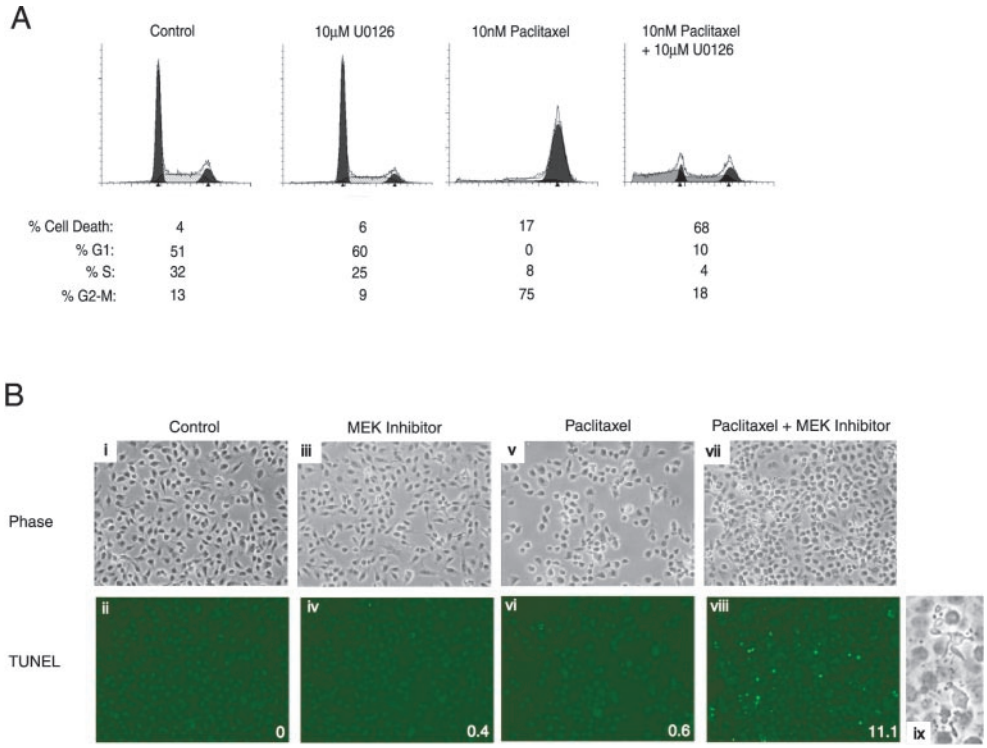

FIG. 3. **Paclitaxel and MEK inhibitor U0126 block cell cycle progression and cause enhanced cell death.** *A*, BT474 breast carcinoma cells were treated for 24 h before staining with propidium iodide and analyzed by flow cytometry as described under "Experimental Procedures." Histograms of control (Me^2SO-treated) cells exhibited normal cell cycle progression. Ten μM U0126 induced G_1 growth arrest, 10 nM paclitaxel induced G_2 growth arrest, and 10 nM paclitaxel plus 10 μM U0126 caused enhanced death by an accumulation of sub-G_1 dead cells. The percentage 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 10 nM paclitaxel and/or 10 μM U0126 as indicated. After 16 h, the slides were incubated and TUNEL stained. The phase contrast photomicrographs (*panels i*, *iii*, *v*, and *vii*) and the corresponding immunofluorescence photomicrographs (*panels ii*, *iv*, *vi*, and *viii*) of cells undergoing apoptosis are shown. The number in the *lower right* of each panel represents the percentage of TUNEL-positive cells.

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 percentage of cancer patients.

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