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Research Paper

Sequence Dependent Exposure of Mammary Carcinoma Cells to Taxotere® and the MEK1/2 Inhibitor U0126 Causes Enhanced Cell Killing In Vitro

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KEY WORDS

Taxane, Taxotere, Laulimalide, MEK1/2 inhibitor, survival, clonogenic, MTT

ABBREVIATION

MAPK	mitogen activated protein kinase
ERL	extracellular regulated kinase

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ABSTRACT

Taxol (paclitaxel) and Taxotere (docetaxel) are considered as two of the most important anti-cancer chemotherapy drugs. The cytotoxic action of these drugs has been linked to their ability to inhibit microtubule depolymerization, causing growth arrest and subsequent cell death. Studies by a number of laboratories have also linked suppression of mitogen activated protein kinase (MAPK) signaling to enhanced Taxol toxicity. The present study examined the interactions of the semi-synthetic taxane Taxotere with MEK1/2 inhibitors in epithelial tumor cells. Concurrent treatment of MDA-MB-231 mammary and DU145 prostate carcinoma cells with Taxotere and MEK1/2 inhibitor resulted in protection from the anti-proliferative effects of Taxotere in MTT assays. In contrast, in MCF-7 mammary cells, concurrent Taxotere and MEK1/2 inhibitor treatment weakly enhanced the anti-proliferative effects of the taxane. Sequential treatment of MDA-MB-231 and MCF-7 cells with Taxotere followed by MEK1/2 inhibitor also enhanced the anti-proliferative effects of the taxane in MTT assays. However, no enhancement was observed in DU145 or PC-3 cells. Colony formation assays, including isobologram analyses, provided a more definitive demonstration that MCF-7 and MDA-MB-231 cells were sensitized to the toxic effects of Taxotere by U0126. Similar data were observed using Laulimalide, which binds to tubulin at a different site to Taxotere. The enhancement in Taxotere anti-proliferative effects by U0126 correlated with increased cell killing, 48-72h after treatment of cells that was blocked by inhibition of caspase 9, but not caspase 8, function. This observation was associated with prolonged suppression of ERK1/2 and AKT activity, without alteration in either p38 or JNK1/2 activity. Collectively these findings demonstrate that sequential administration of Taxotere followed by MEK1/2 inhibition can lead to increased cell death and loss of reproductive capacity in some, but not all, human tumor cells.

INTRODUCTION

Paclitaxel (Taxol) and docetaxel (Taxotere) are members of the taxane class of anti-neoplastic agents that exhibit activity against solid tumors, both alone and in combination with other cytotoxic agents.[1-3] In contrast to microtubule disaggregating agents, Taxol, Taxotere and Laulimalide induce microtubular stabilization, leading to arrest of cells in G_2/M phase of the cell cycle and ultimately, apoptosis.[4-7] The mechanism(s) by which taxanes trigger the cell death process are not fully understood, but have been variously attributed to dysregulation of signal transduction pathways,[8,9] induction of cell cycle perturbations,[10] or inactivation of the anti-apoptotic protein Bcl-2.[11]

Mitogen-activated protein kinase (MAPK) pathways provide a mechanism by which signals are transduced from the cell surface to the nucleus, leading to activation of genes involved in the control of cell cycle progression, differentiation and cell death. Four major subfamilies have been identified: c-Jun N-terminal kinases (JNK1/2/3), extracellular signal-related kinases (p42/44 MAPK; ERK1/2), the p38 MAPK cascade and the "big MAPK" ERK5 pathway. Of these, the JNK and p38 pathways are activated by a variety of noxious stimuli, including inflammatory cytokines, endotoxins, changes in osmolarity, and ionizing and UV radiation.[12,13] In contrast, the ERK1/2 and ERK5 signaling pathways are responsive to growth factors, and have been implicated in cell proliferation and differentiation.[14-16] In general, it is believed that activation of the JNK and p38 cascades promotes apoptosis,[17] whereas activation of ERK1/2 and ERK5 exerts a cytoprotective effect.[18-21] Efforts to understand the functional role of the ERK1/2 pathway in various cellular functions have been facilitated by the recent development of several specific pharmacologic inhibitors of the MAPK kinase (MEK1/2), including PD98059, U0126 and PD184352.[22-24] The latter compound has attracted considerable interest in view of its capacity to inhibit the ERK1/2 pathway when administered in vivo.

The relationship between taxane-mediated lethality and signaling by the ERK1/2 pathway is still unclear. Previous studies have shown that activation of ERK1/2 is required for entry into and progression through G_2/M phase as well as for the proper functioning of the mitotic spindle apparatus.[25-27] Thus, alterations in the ERK1/2 cascade may influence the response of cell agents, such as Taxol and Taxotere that trigger G_2/M arrest. However, attempts to define the relationship between ERK1/2 signaling and taxane-related lethality are complicated by conflicting reports that Taxol and Taxotere exposure can induce, in a cell type dependent fashion, either an increase,[28] a decrease,[29] or no change[30] in ERK1/2 activity. Some groups have reported that administration of the PD98059 before and concurrently with Taxol reduced taxane lethality.[31] Our group also recently examined the schedule-dependent effects of pharmacologic MEK1/2 inhibitors on the response of p53 null human leukemic cells to Taxol and discovered that subsequent, but not prior, exposure of Taxol-treated leukemic cells to various MEK1/2 inhibitors potentiated mitochondrial damage, caspase activation and apoptosis.

Taxotere is a semi-synthetic taxane first described by Ringel and Horwitz in 1991.[32] It has been shown to have a greater potency than Taxol both in vitro and in vivo, however the interaction of this taxane with inhibitors of the MEK1/2-ERK1/2 pathway have not been investigated.[1,4] In addition, certain cell types are known to be resistant to Taxol and Taxotere via expression of P-glycoprotein or M40 human beta-tubulin gene mutation.[7] Our data demonstrated that MEK1/2 inhibitors enhanced cell killing by Taxotere and Laulimalide in a sequence dependent manner in mammary carcinoma cells that expressed either wild type or mutant p53 molecules: exposure of cells to Taxotere or Laulimalide followed by treatment with MEK1/2 inhibitor enhanced Taxotere-mediated lethality.

MATERIALS AND METHODS

Materials. Phospho-p44/42 MAP kinase antibody (1:1000, rabbit polyclonal, NEB, Beverly, MA), p44/42 MAP kinase antibody (1:1000, rabbit polyclonal, NEB), phospho-JNK1/2 antibody (1:1000, rabbit polyclonal, NEB), anti-caspase-9 (1:1000, rabbit polyclonal, Pharmingen). The selective MEK1/2 inhibitor U0126 and Caspase inhibitors (LEHD, IETD) were supplied by Calbiochem (San Diego, CA) as powder, dissolved in sterile DMSO, and stored frozen under light-protected conditions at -20°C. In all experiments, the final concentration of DMSO or ethanol did not exceed 0.1%. Western immunoblotting was performed using the Amersham Enhanced Chemi-Luminescence (E.C.L.) system (Bucks, England). For additional information see references 8 and 33.

Methods

Culture of carcinoma cells. Asynchronous carcinoma cells MCF7 ($p53^+$,RB^+,ER^+); MDA-MB-231 ($p53^-$,RB^+,ER^-); DU145 ($p53^-$,RB^-,AR^-); LNCaP ($p53^+$, RB^+, AR^+), A498 renal cell carcinoma cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% (v/v) fetal calf serum at 37°C in 95% (v/v) air / 5% (v/v) CO_2. Cells were plated at a density 3.2 x 10^4 cells/cm^2 plate area and grown for 24h prior to further experimentation.

Exposure of cells to Taxotere/Laulimalide, U0126 and cell homogenization. Cells were cultured in DMEM + 5% (v/v) fetal calf serum as above. Taxotere treatment was from a 1 mM stock solution and the maximal concentration of vehicle (ethanol) in media was 0.02% (v/v). U0126 treatment was from a 100 mM stock solution and the maximal concentration of vehicle (DMSO) in media was 0.02% (v/v). Treatment was performed in one of two ways: either cells were treated with Taxotere/Laulimalide (0.25 nM; 1.0 nM) for 6h followed by removal of Taxotere/Laulimalide and incubation for an additional 18h with U0126 (3 μM) or cells were treated with Taxotere/Laulimalide and U0126 at the same time for 24h. After all drug treatments (24h) cells were washed free of drugs and incubated for specified times followed by aspiration of media and snap freezing at -70°C on dry ice.

SDS poly-acrylamide gel electrophoresis (SDS PAGE) and Western blotting. Cells were drug treated and at specified time points/treatments media aspirated and the plates snap frozen. Cells were lysed with 100 μl 5X SDS PAGE sample buffer (10% (w/v) SDS), diluted to 250 μl with distilled water, and placed in a 100°C dry bath for 15 min. One hundred microliter aliquots of each time point were subjected to SDS PAGE on 10% (w/v) acrylamide gels. Gels were transferred to nitrocellulose by the Method of Towbin and Western blotting using specific antibodies performed as indicated. Blots were developed using Enhanced Chemi-Luminescence (Amersham) using Fuji RX x-ray film. Blots were digitally scanned using Adobe Photoshop, their color removed, and Figures created in Microsoft PowerPoint.

MTT assay for cell growth. Cells were grown in 12 well plates and 24h after plating treated with Taxotere/Laulimalide and U0126 as indicated. Cells were cultured for a further 96h. A 5 mg/ml stock solution of MTT reagent (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue) was prepared in DMEM. For assay of mitochondrial dehydrogenase function, the MTT stock solution is diluted 1:10 in fresh media (DMEM + 10% fetal calf serum) and 1 ml of this solution is added to each aspirated well of a 12 well plate. Cells are incubated for a further 3h at 37°C. MTT is converted into an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes. After 3h, media is aspirated and cells lysed with 1 ml DMSO, releasing the purple product from the cells. Cells are incubated for a further 10 min at 37°C with gentle shaking. Absorbance readings at 540 nM are determined using a computer controlled micro-plate analyzer. The relationship between cell number and MTT absorbance/mitochondrial enzyme activity was linear over the range of 10^6 cells.

Colony forming (clonogenic) assay. Single cell suspensions of cells were plated in Linbro® plates at densities of 250 cells/well, 500 cells/well and 750 cells/well 24h prior to experimentation. Cells were treated with Taxotere/Laulimalide and MEK1/2 inhibitor as indicated. Colony formation was defined as a colony of 50 cells or greater, 10–14 days after plating. Isobologram analyses were performed as described.[8] Briefly, cells were treated with different concentrations of Taxotere and U0126 at a fixed drug:drug ratio (for the studies in this manuscript, two fixed ratios were chosen; 1:12.5 and 1:3). Colony formation was determined and the data processed to determine the Combination Index (C.I.) under each condition. A C.I. of less than one indicates a synergistic interaction.

Data analysis. Comparison of the effects of treatments was done using one-way analysis of variance and a two-tailed t-test. Differences with a p-value of <0.05 were considered statistically significant. Experiments shown, except where indicated, are the means of multiple individual points from multiple separate experiments (±SEM). Statistical analyses were made using SigmaPlot and SigmaStat.

RESULTS

Initial studies examined the in vitro proliferation of DU145 (prostate: AR negative, p53 mutant, RB mutant), MDA-MB-231 (mammary: ER negative, p53 mutant, RB wild type) and MCF-7 (mammary: ER positive, p53 wild type, RB wild type) carcinoma cells. These cell lines exhibited a linear relationship between cell number and absorbance as measured by manual cell counting and MTT assays (data not shown).

The interaction of the semi-synthetic taxane, Taxotere, with the MEK1/2 inhibitor U0126 was then examined in these cell lines. Treatment was performed in one of two ways: either cells were treated with Taxotere (0.25 nM; 1.0 nM) for 6h followed by removal of Taxotere and incubation for an additional 18h with U0126 (3 μM) or cells were treated with Taxotere and U0126 at the same time for 24h. Cells were washed free from all drugs at 24h and cell numbers were determined 96h after the initiation of Taxotere treatment. Data are corrected for the anti-proliferative/toxic effects of U0126. Taxotere reduced the proliferation of MDA-MB-231 and DU145 cells in a dose-dependent fashion (Fig. 1 and Fig. 2). Sequential treatment of MDA-MB-231 cells with Taxotere followed by U0126 enhanced the anti-proliferative effect of Taxotere (*$p < 0.05$, n=3 ± SEM) (Fig. 1A). This effect, however, was not observed in DU145 cells. (Fig. 2A) In contrast, concurrent treatment of

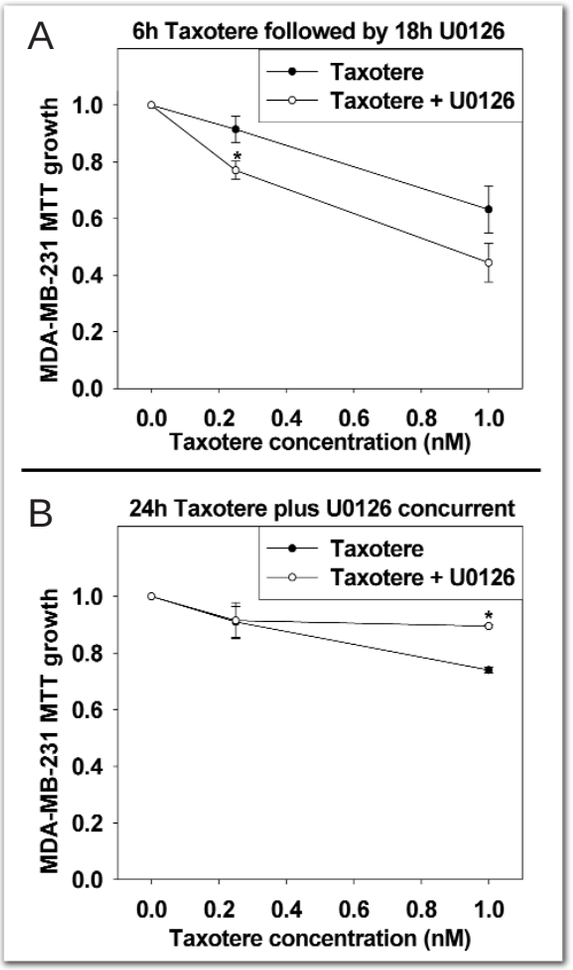

Figure 1. Sequence-dependent enhancement in the anti-proliferative effects of Taxotere by U0126 in MDA-MB-231 cells. MDA-MB-231 cells were plated and grown in 12 well plates and 24h after plating treated with Taxotere and U0126 as indicated. Treatment was performed in one of two ways: either (A) cells were treated with Taxotere (0.25 nM; 1.0 nM) for 6h followed by removal of Taxotere and incubation for an additional 18h with U0126 (3 μM) or (B). cells were treated with Taxotere and U0126 concurrent for 24h. All drugs were removed 24h after the start of treatment(s). Cell number was determined 96h after the initiation of treatment. Data are corrected for the anti-proliferative/toxic effects of U0126. Open circles: Taxotere + U0126, Closed circles: Taxotere. (A) (±SEM, n = 3): * $p < 0.05$ less than corresponding value not treated with U0126; (B) (±SEM, n = 3): * $p < 0.05$ greater than corresponding value not treated with U0126).

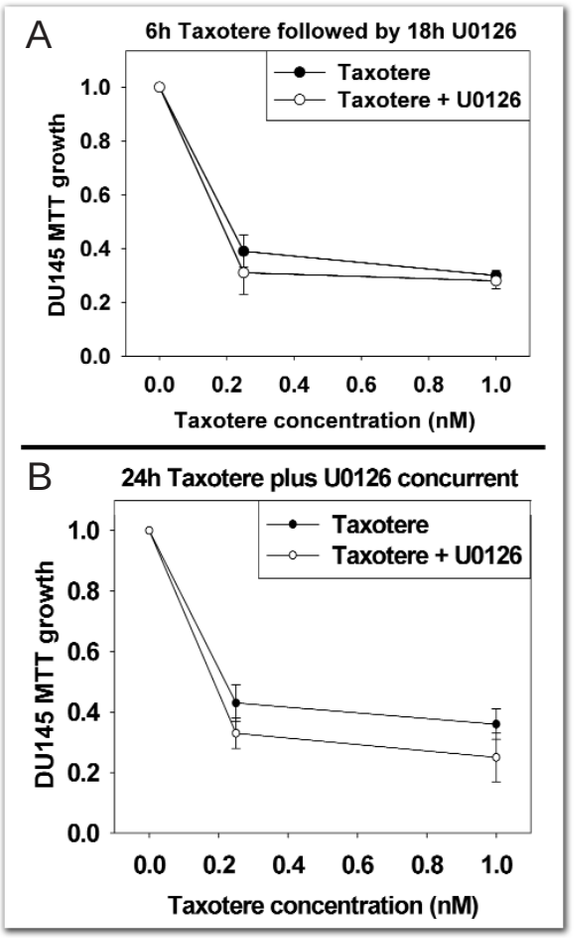

Figure 2. U0126 does not enhance the anti-proliferative effects of Taxotere in DU145 prostate carcinoma cells. DU145 cells were plated and grown in 12 well plates and 24h after plating treated with Taxotere and U0126 as indicated. Treatment was performed in one of two ways: either (A). cells were treated with Taxotere (0.25 nM; 1.0 nM) for 6h followed by removal of Taxotere and incubation for an additional 18h with U0126 (3 μM) or (B). cells were treated with Taxotere and U0126 concurrent for 24h. All drugs were removed 24h after the start of treatment(s). Cell number was determined 96h after the initiation of treatment. Data are corrected for the anti-proliferative/toxic effects of U0126. Open circles: Taxotere+U0126, Closed circles: Taxotere.

MDA-MB-231 cells with Taxotere and U0126 resulted in less growth suppression than observed with Taxotere alone (*$p < 0.05$, n = 3 ±SEM) (Fig. 1B).

Additional studies then examined the impact of Taxotere and U0126 in MCF-7 (mammary: ER positive, p53 wild type, RB wild type) carcinoma cells. MCF-7 cells are derived from a differentiated metastatic mammary carcinoma. Despite expression of wild type p53, which would predict a strong apoptotic response to toxic agents, these cells have been reported to be relatively refractory to chemotherapeutic agents due to a deletion of the gene expressing the executioner pro-caspase, pro-caspase 3. Sequential treatment of MCF-7 cells with Taxotere followed by U0126 resulted in an enhancement of the anti-proliferative effect of Taxotere (Fig. 3). However, concurrent treatment of MCF-7 cells with Taxotere and U0126 resulted in reduced growth suppression compared to Taxotere alone in MTT assays, (Fig. 3) which is similar to our observations in MDA-MB-231 cells (Fig. 1).

The alteration in cell numbers in Taxotere and U0126 treated MCF-7 cells were quantitated in terms of cell viability. As single agents, both Taxotere and U0126 weakly increased cell killing 72h after drug treatment (Figs. 4A and 4B). In contrast, the sequential combination of Taxotere and U0126 caused a greater than additive increase in cell killing that was significant 72h after combined drug treatment. Death responses are believed, in general, to be mediated by either the extrinsic death receptor pathway via pro-caspase 8, or the intrinsic mitochondrial pathway via pro-caspase 9. To determine whether the enhancement in Taxotere lethality was dependent on signaling by caspase 8 or caspase 9, cells were incubated with either the caspase 8 inhibitor IETD or the caspase 9 inhibitor LEHD, and cell growth determined as in Figures 1–3. The caspase 9 inhibitor LEHD, but not the caspase 8 inhibitor IETD, abolished the enhancement in Taxotere lethality by U0126 (Figure 4C, data not shown).

In parallel studies, the phosphorylation/activity of ERK1/2, JNK1/2, p38α/β, and Akt were determined over this time course. Taxotere weakly activated both ERK1/2 and Akt, 6h after drug treatment (Fig. 5). Removal of Taxotere from the culture media resulted in a slow decline in ERK1/2 activity back to basal levels. Exposure of cells to U0126, regardless of Taxotere exposure, suppressed ERK1/2 phosphorylation and following Taxotere

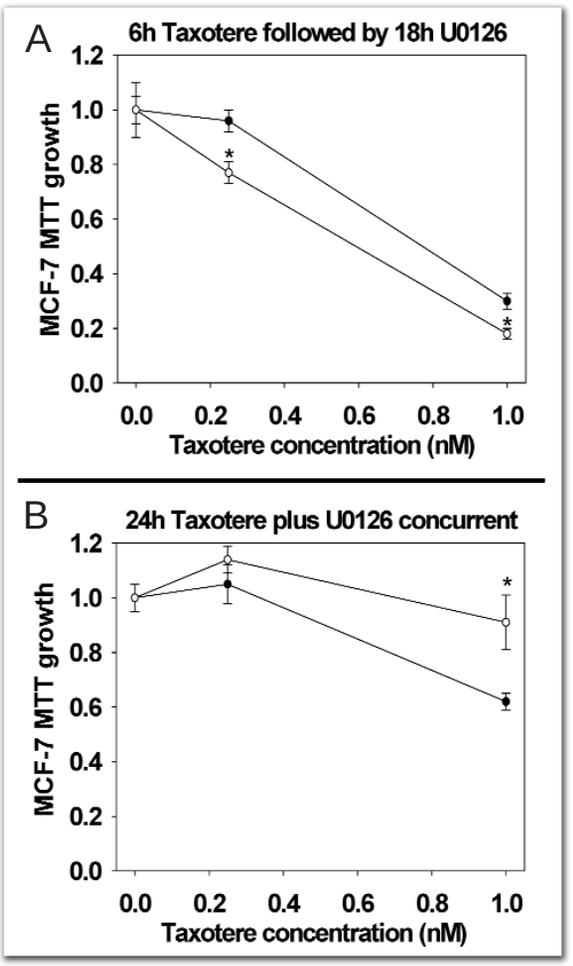

Figure 3. Sequence-dependent enhancement in the anti-proliferative effects of Taxotere by U0126 in MCF-7 cells. MCF-7 cells were plated and grown in 12 well plates and 24h after plating treated with Taxotere and U0126 as indicated. Treatment was performed in one of two ways: either (A). cells were treated with Taxotere (0.25 nM; 1.0 nM) for 6h followed by removal of Taxotere and incubation for an additional 18h with U0126 (3 μM) or (B). cells were treated with Taxotere and U0126 concurrent for 24h. All drugs were removed 24h after the start of treatment(s). Cell number was determined 96h after the initiation of treatment. Data are corrected for the anti-proliferative/toxic effects of U0126. Open circles: Taxotere+U0126, Closed circles: Taxotere. (A) (±SEM, n = 3): * $p < 0.05$ less than corresponding value not treated with U0126; (B) (±SEM, n = 3): * $p < 0.05$ greater than corresponding value not treated with U0126).

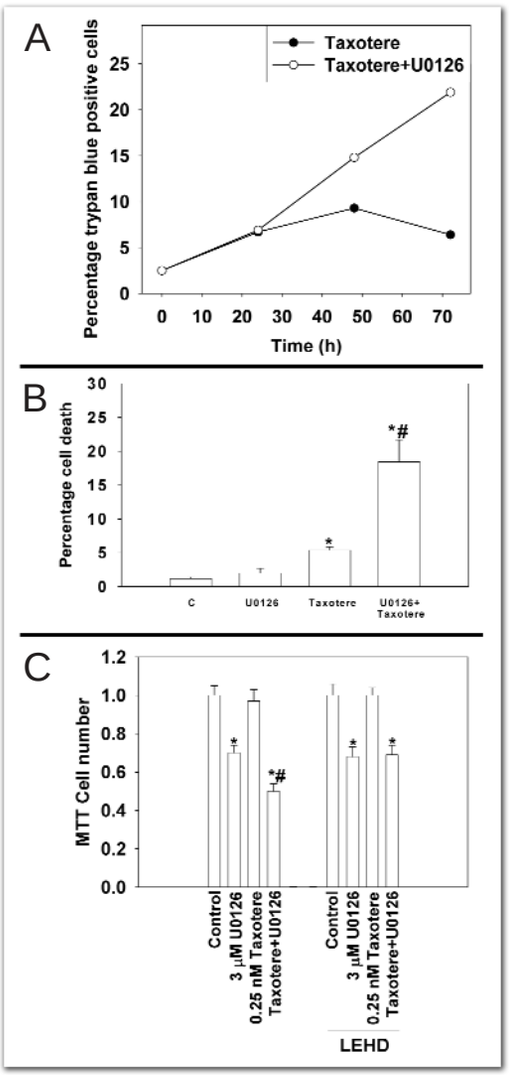

Figure 4. Sequence-dependent enhancement in cell killing by Taxotere and U0126 in MCF-7 cells. MCF-7 cells were plated and grown in 60 mm dishes and 24h after plating treated with Taxotere and U0126 Cells were treated with Taxotere (0.25 nM) for 6h followed by removal of Taxotere and incubation for an additional 18h with U0126 (3 μM). (A). Cell survival from a representative experiment (n = 2) over a time course, as assessed by trypan blue inclusion. Data are corrected for the effect of vehicle alone or U0126 alone on cell viability at each time point. (B). Cell survival was determined Hoescht staining assay and morphological analyses of stained nuclei 72h after the initiation of treatment. (C). Cells were incubated with either vehicle, IETD (25 μM) or LEHD (25 μM). Inhibitors were replenished every 24h. MTT assays were performed as described in Methods and Figures1–4. (B) (±SEM, n = 3): *$p < 0.05$ greater than control value, # $p < 0.05$ greater than corresponding value not treated with U0126; (C) (±SEM, n = 3): *$p < 0.05$ less than control value, # $p < 0.05$ less than corresponding value not treated with U0126).

treatment, and enhanced Akt activity. At 72h after combined treatment, ERK1/2 and Akt activity were almost undetectable, and this correlated with the enhancement in Taxotere-induced cell death. Surprisingly, based on data using one to two orders of magnitude higher concentrations of taxane,[8] no alteration of JNK1/2 or p38 signaling was observed to correlate with the enhancement in Taxotere lethality by U0126 (data not shown).

In vitro colony formation assays are, in general, considered to be a better approximation of in vivo drug action than MTT assays. Thus additional studies were performed to determine the impact of Taxotere and U0126 on the colony forming ability of DU145, MDA-MB-231, MCF-7, LNCaP and A498 cells. In general agreement with findings using MTT assays, Taxotere and U0126 did not interact to enhance cell killing in DU145 cells (data not shown).

Sequence dependent exposure of MDA-MB-231 cells to Taxotere followed by U0126 reduced colony forming ability (Fig. 6A). The effect was statistically significant using 1.0 nM Taxotere (*$p < 0.05$, n = 3 ± SEM) and approaching significance at 0.25 nM Taxotere ($p < 0.08$). The enhancement effect at both 0.25 nM and 1.0 nM Taxotere was ~2-fold. Surprisingly, concurrent treatment of MDA-MB-231 cells with Taxotere and U0126 using 0.25 nM Taxotere also significantly reduced colony forming below that observed with Taxotere alone (*$p < 0.05$, n = 3 ± SEM), although this effect was weak (Fig. 6B).

Sequence dependent exposure of MCF-7 cells to Taxotere and U0126 resulted in a large decrease in colony formation compared to either agent alone (*$p < 0.01$, n = 3 ± SEM) (Fig. 7A). The enhancement effect at 0.25

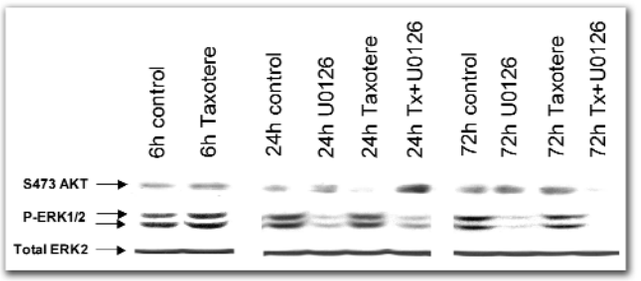

Figure 5. Modulation of ERK1/2 and AKT activity by Taxotere and U0126 in MCF-7 cells. MCF-7 cells were plated and grown in 100 mm dishes and 24h after plating treated with Taxotere and U0126, cells were treated with Taxotere (1.0 nM) for 6h followed by removal of Taxotere and incubation for an additional 18h with U0126 (3 μM). At various times after the start of drug treatment, cells were isolated. Cell lysates were subjected to SDS PAGE and immunoblotting to determine the phosphorylation status of ERK1/2 and AKT, and p38 and JNK1/2 (data not shown). Taxotere weakly activated Akt and ERK1/2 6h after treatment. U0126 did not alter Akt activity and Taxotere reduced Akt activity at 24h whereas Taxotere combined with U0126 caused a compensatory activation of Akt. Combined treatment of cells with Taxotere and U0126 suppressed ERK1/2 and Akt activity 72h after treatment.

nM was ~6-fold and at 1.0 nM was ~3-fold. In contrast to findings using MTT assays, MCF-7 cells were sensitized to the toxic effects of concurrent Taxotere and U0126 treatment (*$p < 0.05$, n = 3 ± SEM) (Fig. 7B). The enhancing effect was ~3-fold at 0.25 nM and ~1.3-fold at 1 nM. Similar data to that obtained in MCF-7 cells treated with Taxotere and U0126 were also obtained in LNCaP prostate and A498 renal carcinoma cells: taxotere (0.25 nM) lethality, corrected for U0126 anti-proliferative effects was enhanced from a non-significant 3% to a significant 26 ± 4% ($p < 0.05$) by U0126 in LNCaP prostate cancer cells, and was enhanced from a non-significant 2% to a significant 38 ± 7% ($p < 0.05$) by U0126 in A498 renal carcinoma cells. To confirm that the interaction between Taxotere and U0126 was synergistic, isobologram studies were performed at two fixed ratios in MCF-7 cells (based on previous findings using 0.25 nM and 1.0 nM Taxotere and 3 μM U0126). These studies demonstrated that the interaction between Taxotere and U0126 was synergistic, as judged by the Combination Index values that were significantly less than 1.0 (Fig. 8).

The microtubule-stabilizing effects of Taxotere was compared to another microtubule-stabilizing agent that binds to and stabilizes microtubules via interactions at another binding site, and we performed colony formation assays to investigate whether the tubulin-binding drug Laulimalide could interact with MEK1/2 inhibitors to enhance cell killing.[7,34] MCF-7 cells were treated with Laulimalide for 6h (0.25 nM) followed by U0126 (1μM). At this low concentration and for the period of time incubated, Laulimalide had a negligible effect on cell survival. However, when MCF-7 cells were treated with Laulimalide followed by U0126, corrected for U0126 anti-proliferative effects, a significant 23% ± 4% ($p < 0.05$) reduction in cell survival was observed.

DISCUSSION

These studies were performed to examine the interaction between Taxotere (docetaxel) and MEK1/2 inhibitors in cell lines derived from solid tumors. The present studies demonstrate that in mammary carcinoma cells, subsequent, but not concurrent exposure of Taxotere-treated cells to a pharmacologic MEK1/2 inhibitor (U0126) potentiates taxane-induced cell killing. Similar data were also obtained in LNCaP prostate and A498 renal carcinoma cells. Furthermore, U0126 also enhanced MCF-7 cell killing by the drug Laulimalide, which binds to tubulin at a different site to Taxotere.

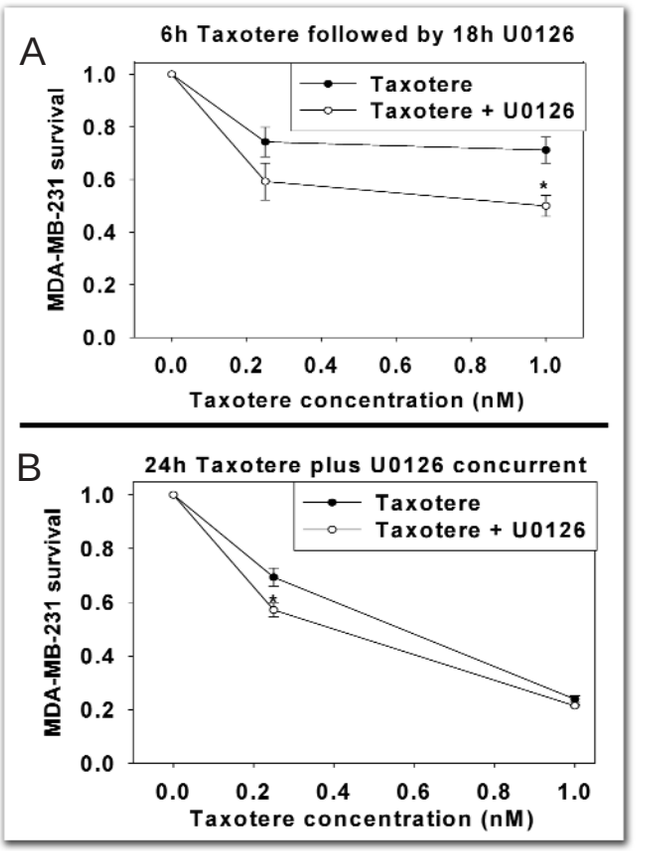

Figure 6. Sequence-dependent enhancement in clonogenic cell killing by Taxotere and U0126 in MDA-MB-231 cells. MDA-MB-231 cells were plated and grown in Linbro plates, and 24h after plating treated with Taxotere and U0126 as indicated. Treatment was performed in one of two ways: either (A). cells were treated with Taxotere (0.25 nM; 1.0 nM) for 6h followed by removal of Taxotere and incubation for an additional 18h with U0126 (3 μM) or (B). cells were treated with Taxotere and U0126 concurrent for 24h. All drugs were removed 24h after the start of treatment(s). Colony formation was determined 10-14 days after the initiation of treatment as in the Methods. Data are corrected for the anti-proliferative/toxic effects of U0126. Open circles: Taxotere + U0126; Closed circles: Taxotere. (±SEM, n = 3): *less than corresponding value not treated with U0126).

However, the relationship between ERK1/2 activity and Taxotere exposure is complex and may be cell-type-specific as the survival of either DU145 or PC-3 prostate carcinoma cells (Yacoub and Dent, Unpublished observations) were not altered by this drug combination.

Previously, treatment of MCF-7 and KB-3 cells with paclitaxel has been shown to cause either an increase,[28] no change,[30] or a decline in ERK1/2 activity.[29] MEK1/2 inhibitors have been shown to exert either no effect on paclitaxel toxicity,[28] or attenuate lethal actions of the taxane.[31] Our findings using Taxotere are, in part, consistent with those of MacKeigan et al, who recently demonstrated that coadministration of MEK1/2 inhibitors with Taxol for 24 h led to a marked increase in cytotoxicity in lung (H157) and ovarian (OVCA194) cancer cells.*35* Collectively, these findings suggest that scheduling and cell type may play a key role in determining the net effect of MEK1/2 inhibition on taxane toxicity.

Based on evidence linking Taxol- and Taxotere-mediated toxicity to cell cycle dysregulation, it seems a reasonable hypothesis that MEK1/2 inhibitors might promote taxane cytotoxicity. For example,

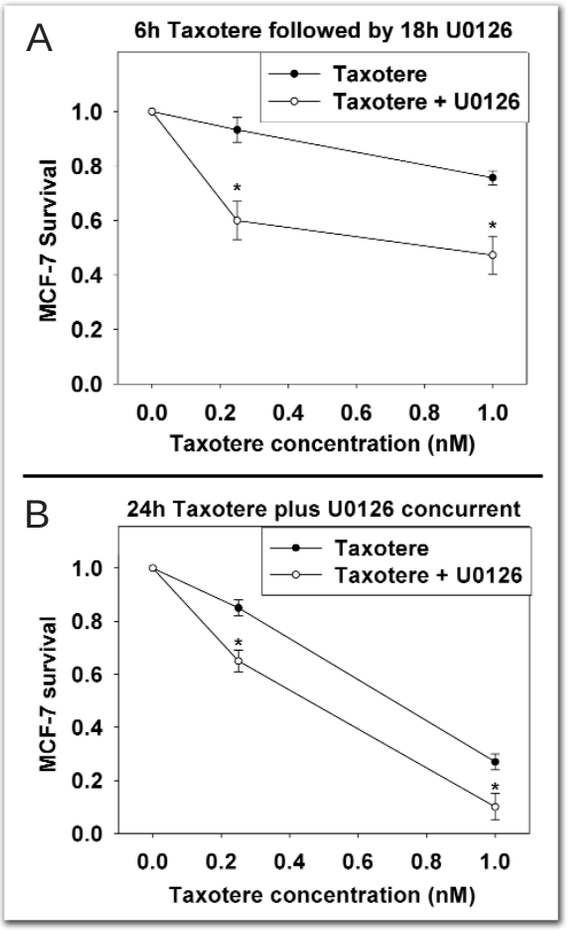

Figure 7. Sequence-dependent enhancement in clonogenic cell killing by Taxotere and U0126 in MCF-7 cells. MCF-7 cells were plated and grown in Linbro plates, and 24h after plating treated with Taxotere and U0126 as indicated. Treatment was performed in one of two ways: either (A) cells were treated with Taxotere (0.25 nM; 1.0 nM) for 6h followed by removal of Taxotere and incubation for an additional 18h with U0126 (3 μM) or (B) cells were treated with Taxotere and U0126 concurrent for 24h. All drugs were removed 24h after the start of treatment(s). Colony formation was determined 10–14 days after the initiation of treatment as in the Methods. Data are corrected for the anti-proliferative/toxic effects of U0126. Open circles: Taxotere + U0126, Closed circles: Taxotere. (± SEM, n = 3): *p < 0.05 less than corresponding value not treated with U0126).

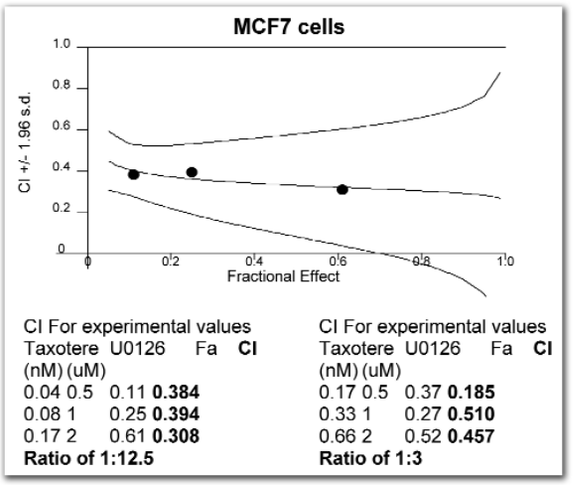

Figure 8. Taxotere and U0126 interact in a synergistic manner to enhance cell killing in MCF-7 mammary carcinoma cells. MCF-7 cells were plated and grown in 12 well plates and 24h after plating treated with Taxotere followed by U0126 as indicated. Sequential drug treatment was performed at two Taxotere: U0126 drug ratios. All drugs were removed 24h after the start of treatment(s). Colony formation was determined 10-14 days after the initiation of treatment as in the Methods. Data are corrected for the anti-proliferative/toxic effects of U0126.

exposure of cells to Taxol has been shown to induce dephosphorylation of the cyclin-dependent kinase $p34^{cdc2}$,[10] unscheduled activation of which is associated with cell death.8 Furthermore, ERK1/2 signaling has been implicated in the G_2/M transition, normal microtubular function, and the mitotic spindle checkpoint.[25,26] In recent studies by Yu et al, however, subsequent exposure of Taxol-treated cells to MEK1/2 inhibitor did not lead to further activation of $p34^{cdc2}$, although it did reduce the mitotic index while reciprocally increasing apoptosis.[8] These findings raise the possibility that disruption of the mitotic spindle assembly apparatus by MEK1/2 inhibitors may amplify the lethal consequences of microtubule stabilization induced by prior taxane treatment. Conversely, interference with the G_2/M transition (e.g., by prior MEK1/2 inhibitor exposure) might attenuate the lethal effects of subsequently administered paclitaxel. This concept is compatible with recent findings indicating that MEK1/2 inhibitors block the transition from G_2 to mitosis in cells previously induced to arrest by nocodazole.[36]

The present results indicate that the proximal cause of enhanced apoptosis in MCF-7 cells exposed to paclitaxel and MEK1/2 inhibitors is cytochrome c release, as judged by the ability of the caspase 9 inhibitor LEHD to block enhanced apoptosis in tumor cells. In contrast to malignant hematopoietic cells, significant increases in cell killing were not observed in carcinoma cells until 48–72 h after Taxotere/U0126 exposure, suggesting that reduced expression of cytoprotective proteins or cell cycle dysregulation requires a greater length of time to stimulate cell death in epithelial tumor cells.[8,33]

In our studies, enhanced cell killing did not correlate with enhanced JNK or p38 activity. Earlier studies have implicated JNK activation in paclitaxel-mediated lethality,[37] a phenomenon linked to Bcl-2 phosphorylation/inactivation.[11] Moreover, the concept that perturbations in stress and cytoprotective signaling pathways contribute to the lethal actions of other microtubule-active agents (e.g., vinblastine) has been proposed.[38,39] However, the observations that subsequent exposure of Taxotere-treated cells to U0126 resulted in little increase in phospho-JNK expression, and the ability of PD98059 to increase paclitaxel toxicity in dominant negative c-Jun-expressing U937 myeloid leukemic cells argues against a primary role for JNK in enhanced cell killing. The relationship between p38 MAPK activation and paclitaxel lethality, as in the case of the other MAPKs, seems to vary with cell type. For example, paclitaxel activated p38 MAPK in MCF-7 cells,[28] reduced basal activity in KB-3 carcinoma cells,[29] and exerted no effect in HL-60 cells.[40] In a similar manner to JNK activation, exposure of Taxotere-pretreated MCF-7 cells to U0126 produced no alteration in p38 MAPK activation. The findings in the present manuscript showing a lack of JNK and p38 activation may be related to the lower doses of taxane, which were significantly below the IC50 for Taxotere in many tumor cells, used in the present study, particularly when compared to the 1–2 higher orders of magnitude taxane used in many other studies.

Several studies by our group have shown that inhibition of one protective signaling pathway can lead to a compensatory upregulation of parallel signaling pathways.[33,41] In the present study, Taxotere enhanced the activity of both ERK1/2 and Akt in MCF-7 cells. Inhibition of MEK1/2 reduced ERK1/2 activity in the presence or absence of Taxotere. In contrast, while Akt activity was unaltered by U0126 alone, Taxotere + U0126 resulted in increased Akt activity 24h after exposure. The activity of both ERK1/2 and Akt in 72h cells treated with Taxotere + U0126 was almost abolished. As enhanced Akt activity has also been associated with taxane resistance, it is possible that cell killing by Taxotere + U0126 could be further enhanced by agents that will block the compensatory upregulation of Akt survival signaling.

In conclusion, our data using colony formation assays demonstrates that MEK1/2 inhibitors potently enhance Taxotere cell killing in MCF-7 cells and to a lesser extent also in MDA-MB-231, LNCaP and A498 cells. However DU145 and PC-3 cells were refractory to the drug combination. The potency of the enhanced killing effect appears to be sequence dependent, with the most profound cytotoxicity being observed when cells are treated with Taxotere followed by MEK1/2 inhibitor. Based on our findings, studies using MTT assays to determine the interactions between taxanes and MEK1/2 inhibitors in epithelial cells appear to be less sensitive than colony formation assays and may not fully reflect the true interactions of either or both agents on cell biology.

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