Induction of survivin expression by taxol (paclitaxel) is an early event which is independent of taxol-mediated G2/M arrest*

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Running Title: G2/M arrest-independent survivin induction by taxol

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ABSTRACT

Survivin is a novel anti-apoptotic protein that is highly expressed in cancer but is undetectable in most normal adult tissues. It was reported that taxol-mediated mitotic arrest of cancer cells is associated with survivin induction, which preserves a survival pathway and results in resistance to taxol. In this study, we provide new evidence that induction of survivin by taxol is an early event and is independent of taxol-mediated G2/M arrest. Taxol treatment of MCF-7 cells rapidly upregulated survivin expression (3.5-15 fold) within 4 hours without G2/M arrest. Lengthening the treatment of cells (48 hours) with taxol resulted in decreased survivin expression in comparison with early times following taxol treatment although G2/M cells were significantly increased at later times. Interestingly, 3 nM taxol induces survivin as effectively as 300 nM and more effectively than 3000 nM. As a result, 3nM taxol is ineffective at inducing cell death. However, inhibition of taxol-mediated survivin induction by small interfering RNA significantly increased taxol-mediated cell death. Taxol rapidly activated the PI3K/Akt and MAPK pathways. Inhibition of these pathways diminished survivin induction and sensitized cells to taxol-mediated cell death. A cis-acting DNA element upstream of –1430 in the survivin pLuc-2840 construct is at least partially responsible for taxol-mediated survivin induction. Together, these data show, for the first time, that taxol-mediated induction of survivin is an early event and independent of taxol-mediated G2/M arrest. This appears to be a new mechanism for cancer cells to evade taxol-induced apoptosis. Targeting this survival pathway may result in novel approaches for cancer therapeutics.
INTRODUCTION

Survivin is a recently characterized novel member of the IAP¹ (inhibitor of apoptosis) protein family. It is undetectable in most normal adult tissues but highly expressed in cancer. Survivin expression has been shown to be associated with carcinogenesis, cancer progression, poor prognosis, drug resistance and short patient survival (1), and that inhibition of survivin expression and/or function in tumor cells by survivin antisense or dominant-negative mutants triggers apoptosis (2-6) as well as a defect in cell division (7,8). Thus, survivin is considered an exciting target for cancer prevention and therapeutics.

Taxol (paclitaxel) is one of the most active cancer chemotherapeutic agents. It is effective against a variety of human tumors, including ovarian, breast, and non-small-cell lung tumors, as well as head and neck carcinomas (9-13). However, its effectiveness is often limited because many tumors display taxol resistance. Cancer cells can acquire resistance to taxol by at least two different mechanisms (14). Overexpression of the multidrug resistance 1(MDR1) gene, which encodes P-glycoprotein (Pgp), can confer resistance to taxol. This is because Pgp functions as a xenobiotic pump that pumps taxol as well as many other chemotherapeutic agents out of cells (15). The other is that tubulin mutations, which result in alterations in either the assembly or stability of microtubules, can lead to taxol resistance (16,17). However, taxol resistance resulted from apoptotic blockade has not been well studied.

Taxol treatment induces mitotic arrest through taxol-induced polymerization and stabilization of microtubules (18-22), and it induces cell death by apoptosis or necrosis dependent of drug concentration (23-26). On the other hand, it has been demonstrated that
survivin expression is cell cycle-regulated with a robust increase in the G2/M phase of cell cycle (2,27). Presumably, cells treated with taxol should show an increased survivin expression due to G2/M arrest. Consistent with this notion, it was reported that taxol-induced microtubule stabilization and mitotic arrest increase survivin expression, which engenders a cell survival pathway to counteract taxol-induced apoptosis (28). However, it is not clear whether this mitotic survival pathway is the only means involving survivin by which cancer cells counteract taxol-induced apoptosis following drug treatment. Here, we report that induction of survivin by taxol is an early event and is independent of taxol-mediated G2/M arrest. We found that taxol treatment of MCF-7 breast cancer cells rapidly upregulated survivin expression without apparent arrest of cells into G2/M phase. Lengthening the treatment of cells (48 hours) with taxol resulted in decreased survivin expression in comparison with early times following taxol treatment although the percentage of cells in G2/M phase was significantly increased at later times. Consistent with the observation that 3 nM taxol induced survivin as effectively as 300 nM and more effectively than 3000 nM of taxol, 3 nM taxol are ineffective for apoptotic induction in these cells. However, inhibition of taxol-mediated induction of survivin by small interfering/inhibitory RNA (siRNA) significantly increased taxol-mediated cell death. Mechanistic studies indicated that taxol rapidly activated the PI3K/Akt and Erk MAPK pathways, and inhibition of PI3K/Akt signaling by Ly294002 or Erk MAPK signaling by U0126/PD98059 diminished survivin induction by taxol and sensitized cells to taxol-induced cell death. Survivin promoter-luciferase reporter assays revealed that early taxol-mediated induction of survivin is at least in part transcriptionally regulated and that the cis-acting DNA element mediating taxol’s effects on survivin promoter activity is located
upstream of –1430 in the pLuc-2840 construct. Together, these data show, for the first time, that induction of survivin by taxol is an early event following drug exposure and is independent of taxol-mediated G2/M arrest. This appears to be a new mechanism by which cancer cells evade taxol-induced apoptosis. Targeting this novel survival pathway may have potential in cancer therapeutic applications.
EXPERIMENTAL PROCEDURES

Cell culture – MCF-7 cells, a breast cancer cell line without Pgp and MRP-1 expression, were maintained in RPMI 1640 containing 10% fetal bovine serum (FBS) (MediaTech CellGro, Herndon, VA), penicillin (100 units/ml) and streptomycin (0.1 µg/ml) (Invitrogen Co., Grand Island, NY) in a humidified atmosphere incubator with 5% CO₂ at 37°C. Cells were routinely sub-cultured twice weekly.

Reagents – Taxol (paclitaxel), anti-β-actin, Goat Peroxidase-conjugated anti-rabbit IgG and FITC-conjugated anti-rabbit IgG were purchased from Sigma (St. Louis, MO). Anti-survivin (FL-142), anti-Erk (K-23) and anti-Akt (H-136) antibodies were purchased from Santa Cruz Biotechnology Co (Santa Cruz, CA). Phospho-Akt (Ser473) and phospho-p44/42 MAP kinase (Thr202/Tyr204) antibodies were purchased from Cell Signaling Technology (Beverly, MA). LY294002, PD98059, U0126 and Dual Luciferase Report Assay System were purchased from Promega (Madison, WI). Oligotransfectamine™ reagent was purchased from Invitrogen (Carlsbad, CA). FuGEN-6 Transfection Reagent was bought from Roche Diagnostics (Indianapolis, IN).

Treatment – Taxol stock solutions were 4 mM in DMSO and stored at -20°C. Just prior to taxol treatment, the stock solution was freshly diluted with DMSO (3 μM, 30 μM, 300 μM and 3000 μM) and then further diluted in RPMI 1640 to final concentrations of 3 nM, 30 nM, 300 nM and 3000 nM, respectively. In the concentration-dependent experiments, MCF-7 cells were treated with 3, 30, 300 and 3000 nM taxol for 8 hours. In the time-dependent experiments, MCF-7 cells were treated with 30 nM taxol and harvested at 4 h, 8 h, 16 h, 24 h and 48 h after taxol treatment. In the sub-cellular localization experiments, MCF-7 cells were treated with 3 nM taxol. For all experiments, the control group was
treated with the same amount DMSO. In experiments for exploration of signaling pathways and cell death, MCF-7 cells were treated with and without taxol (3 and 30 nM) in the presence and absence of various concentrations of PI3K (LY294002) or MEK (PD98059 and U0126) inhibitors. Survivin expression was analyzed by Western blot described below after treatment for 8 hours. Cell morphological changes were microscopically photographed after treatment for 48 hours.

*Western blot* – Cells with and without treatment were washed with phosphate-buffered saline (PBS: 50 mM phosphate [pH 7.4], 100 mM NaCl, 10 mM KCl) and lysed on ice for 30 minutes in PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10 µg/ml phenylmethyl sulfonyl fluoride, and 20 µM leupeptin. After the lysates were cleared by centrifugation at 15,000 g for 20 minutes at 4°C, the total protein was determined using Bio-Rad protein assay solution. Up to 75 µg of total protein was denatured in 2 X SDS sample loading buffer for 5 minutes at 95°C, separated on 10-15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gels, and electrotransferred to Immobilon-P membranes (Millipore, Bedford, MA) using semi-dry electrophoretic transfer. After the nonspecific binding sites on the membranes were blocked with 5% skimmed milk or bovine serum albumin (BSA) in TBS-T [20 mM Tris-HCl (pH 7.5), 0.137 M NaCl, and 0.01% Tween 20] for 3 h at room temperature with constant shaking, the membranes were incubated in TBS-T containing the relevant primary antibody (1: 500-1000) and 5 % BSA overnight at 4°C. After washing with TBS-T for three times, the membrane was incubated in 5% skimmed milk in TBS-T buffer containing the appropriate second anti-IgG antibody (1:5000) at room temperature for 1 h with constant shaking. The phosphorylation or expression of the target protein was detected by the ECL protein
detection kit (Amersham, Arlington Heights, IL) following the manufacturer’s instructions and visualized by autoradiography with the Kodak X-Omat film. For normalization of protein loading, the same membranes were stripped with stripping buffer (100 mM 2-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5 mM Tris-HCl pH 6.7) and used for Western blot by the same procedure with a monoclonal antibody against β-actin (1:1000 dilution) and/or with the polyclonal antibodies against the relevant total protein.

**Trypan blue exclusion staining for determination of cell viability** – Cells to be counted were collected by trypsinization/centrifugation and resuspended in PBS buffer. A small sample of the cell suspension was diluted in 0.4% (w/v) trypan blue (one sample a time since viable cells absorb trypan blue over time as well). A cover glass was centered over the hemacytometer chambers and one chamber was filled with the cell dilution using a Pasteur pipette. Stained (dead) and unstained (viable) cells were counted in each of the four corner and central squares under an inverted microscope using 100X magnification, respectively. Each cell sample was counted in this way for three times. The percentage of cell viabilities in each sample was calculated with the formula of “% viability = total viable cell numbers/total cell numbers x 100”.

**Propidium iodide staining and flow cytometry** – At various time intervals after taxol treatment (30 nM) as described above, the cells were harvested by trypsinization and washed with PBS. Cells (~1x10^6) were resuspended in 5 ml 70% ethanol. After the initial fixation, cells were suspended in 0.5 ml PBS containing 25 µg/ml propidium iodide (PI), 0.2% Triton X-100 and 40 µg/ml RNase A and incubated for at least 30 minutes at 4°C. Then cells were analyzed for immunofluorescence intensities by flow cytometry (FACScan, Becton Dickinson, San Jose, CA) from 10,000 events per sample. Data from
flow cytometry were analyzed using WinList software (Verity Software House Inc., Topsham, ME). For each time point, triplicate assays were performed.

**Immunofluorescence microscopy** – Cells were seeded on the round glass coverslips coated with 2% gelatin (Sigma, MO) in 12-well plates. At different intervals after 3 nM taxol treatment as described above, medium was removed, cells were washed once with PBS and fixed overnight with 4% paraformaldehyde in PBS at 4°C. Cells were then permeabilized and blocked for 30 minutes in PBS containing 2% BSA and 0.2% Triton X-100. For survivin and nuclear DNA double staining, the blocked cells were first incubated in PBS containing 1% BSA and rabbit anti-survivin antibody (1:500) for 60 minutes at 37°C followed by FITC-conjugated anti-rabbit IgG (1:200) for 30 minutes at 37°C. The nuclear DNA was then stained with DAPI (4',6-diamidino-2-phenylindole) at a final concentration of 0.5 µg/ml in H2O for 10 minutes at room temperature. The resultant glass coverslips containing cells were mounted on glass slides with Gel/Mount™ solution (Biomedia corp. CA). The cells were analyzed under a Zeiss Axiovert 100 M digital fluorescence microscope. Images were captured using Zeiss LSM510 v2.8 and processed with Photoshop Element software.

**SiRNA preparation** – A human survivin mRNA-specific RNA oligonucleotides with 3'-TT overhangs were chemically synthesized and purified by HPLC (Xeragon, Huntsville, AL): SRi-2F (92 GCG CCU GCA CCC CGG AGC G110 TT*) and SRi-2R (110 CGC UCC GGG GUG CAG GCG C 92 TT). Equal moles of SRi-2F/SRi-2R (designated SRi-2) were mixed together to a final concentration of 20 µM in annealing buffer (100 mM KAc, 30 mM HEPES-KOH, 2 mM MgAc₂, pH 7.4). After denaturation at 90°C for 1 minute, the mixture (Sri-2) was annealed at 37°C for 60 minutes and stored at −80 °C for transfection
experiments. A scramble RNA duplex (designated scraSRi) was also prepared same as above for a negative control in this study. The scramble sequence [5’CAG UCG CGU UUG CGA CUG GTT (forward chain) and 5’CCA GUC GCA AAC GCG ACU GTT (reverse chain)] was not present in mammalian cells by BLAST search at NCBI.

*In vitro transfection with siRNAs* – Cells were transfected with survivin siRNAs using the Oligofectamine™ reagent (Invitrogen) following the manufacturer’s instruction. Briefly, one day prior to transfection, 5 x 10⁴ MCF-7 cells per well were seeded in six-well plates (corresponding to a density of 40% at the time of transfection) without antibiotics. The transfection mixture was prepared by mixing 175 µl DMEM containing 6 µl 20 µM siRNA with 15 µl DMEM containing 3 µl Oligofectamine™ reagents. Before transfection, the medium in 6-well plates was replaced with serum-free DMEM medium (800 µl per well). The transfection mixture was added to the 6-well plate within 20-40 minutes after mixture preparation in a total volume of 990 µl per well. The transfected cells were incubated at 37°C for 4 hours, and then 500 µl of DMEM medium containing 30% FBS was added. Cells were treated with and without 3nM and 30nM taxol 24 hours after transfection with siRNA or control siRNA as described above. For Western blot, cells were harvested 8 hours after taxol treatment. For flow cytometry analysis of sub-G1 DNA content, cells were harvested 24 and 48 hours after taxol treatment and analyzed by PI staining and flow cytometry as described above. The percentage of dead cells (sub-G1 DNA contents) was plotted as a histogram. All transfection experiments were performed at triplicate for each experiment.

*Luciferase reporter assay* – MCF-7 cells were transfected with survivin promoter-luciferase constructs pLuc-6270, which contains a 6.3-kb survivin promoter sequence (29),
using the FuGEN 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN) and luciferase activities were measured using a dual Luciferase Reporter Assay System (Promega) according to the respective manufacturers’ recommendations. Briefly, cells were seeded in 24-well plates one day prior to transfection. On the following day, the transfection solution for each well was prepared by sequentially adding 1 µl of FuGEN 6 Transfection Reagent and 0.4 µg of plasmid DNAs in a 1.5 ml tube containing 50 µl of serum-free DMEM medium. The DNA FuGEN 6 mixture was incubated at room temperature for 20-30 minutes and then added onto cells at ~50% confluence in each well containing complete medium. The transfected cells were treated with 3 nM and/or 30 nM taxol 24 hours after transfection for the 30-hour time point, and then taxol (3 nM and/or 30 nM in final concentration) was added to the transfected cells at other time points at 4 (26-hour time point), 8 (22-hour time point), and 22 hours (8-hour time point) after the first taxol treatment to permit the simultaneous harvesting of the cells from all time points. Cells were washed with PBS and lysed in 200 µl of 1x passive lysis buffer (Promega) per well 8 hours after the last taxol treatment. After incubation of the plate on ice for 45 minutes, the lysate was transferred to 1.5 ml eppendorf tube by scraping with a rubber policeman. Cellular debris was pelleted by centrifugation at 15,000g for 10 min at 4°C. Twenty µl of cell lysate per well was used to measure Firefly and Renilla luciferase activity using a Luminometer by adding 20 µl luciferase assay reagent. Luciferase activity was normalized to Renilla luciferase activities as arbitrary units and plotted as a histogram from a representative experiment in triplicate.
RESULTS

Induction of survivin by taxol is an early event and is independent of taxol-mediated G2/M arrest – It was previously shown that taxol (2-200 nM) treatment of HeLa cells for 48 hours increases survivin expression to 1.2 - 4 folds in taxol-mediated G2/M arrest (28). To closely investigate the relationship between taxol-mediated G2/M arrest and survivin induction, MCF-7 cells were treated with taxol at various concentrations and times as shown (Fig. 1A), and survivin expression was analyzed by Western blot. To our surprise, lengthening taxol treatments (48 hours or more) significantly attenuated survivin induction in comparison with the early times (24 hours, Fig. 1A). The high induction of survivin at 24 hours suggested that induction of survivin expression might occur at even earlier times. Consistent with this notion, Western blot experiments to test early time points after taxol treatment indicated that taxol-mediated survivin induction is as early as 4 hours, and that extending treatment times beyond 24 hours diminished the effect of taxol on survivin induction in comparison with earlier time points (Fig. 1B). This result strongly suggested that induction of survivin by taxol is likely independent of taxol-mediated G2/M arrest. To confirm this possibility, we determined cell cycle distribution by propidium iodide staining and flow cytometry after taxol treatment. As shown (Fig. 2A), there is no apparent G2/M cell population increase at the 4 and 8-hour times of taxol treatment in comparison with the no taxol control. A significant increase of G2/M cell population was observed only after taxol treatment for 16 hours or more (Fig. 2A). Next, we examined individual cells by survivin immunofluorescence microscopy after taxol treatment for 4 hours. The results indicated that interphase cells showed increased immunoreactivity with anti-survivin antibody in comparison to no taxol treatment control. A representative experiment from the
immunofluorescence microscopy study is shown in Fig. 2B. The G2/M phase-independent induction of survivin expression by taxol was further confirmed by monitoring the expression of cyclin B1 (a G2/M phase marker) after taxol treatment. There was no change in cyclin B1 expression 4 hours after taxol treatment (Fig. 2C). Interestingly, the cyclin B1 protein is almost undetectable 48 hours after taxol treatment (Fig. 2C), suggesting that the cells were synchronized in later anaphase.

Low concentrations (3-30 nM) of taxol effectively induced survivin expression but were ineffective for apoptosis induction – Unexpectedly, induction of survivin expression by taxol at low concentrations (3 nM) was as effective as or more effective than high concentrations (3000 nM) (Fig. 3A). Consistent with the observation that high expression of survivin increases cell viability and engenders drug resistance (28,30,31), low concentrations (3-30 nM) of taxol were ineffective for induction of cell death in comparison with high concentrations of the drug (Fig. 3B).

Inhibition of taxol-mediated induction of survivin expression by siRNA sensitized taxol-induced cell death – We employed a survivin siRNA approach [(32), and Ling & Li, submitted] for inhibition of taxol-mediated survivin induction to determine the effect of survivin inhibition on taxol-induced cell death. Fig. 4A shows the structures of siRNAs used in this study. Consistent with the result that survivin siRNA significantly inhibited the taxol-mediated induction of survivin in MCF-7 cells (Fig. 4B), PI staining and flow cytometry analysis indicated that a combination of siRNA-targeting-survivin and a low concentration of taxol treatment strikingly increased cell death (the sub-G1 DNA content increase) in comparison with either treatment alone (Fig. 4C). This observation suggests
that survivin expression plays a critical role in cell viability and that induction of survivin by taxol is a potential drug resistance factor leading to cell survival.

*Taxol treatment rapidly enhanced Akt and Erk1/2 phosphorylation in MCF-7 cells* – It has been shown that activation of the Akt survival pathway can upregulate survivin expression (33,34) and inhibition of Akt and Erk1/2 activation can block growth factor-mediated induction of survivin (35). To delineate the underlying mechanism by which taxol rapidly induced survivin expression, the activation of Akt and Erk1/2 after taxol treatment was examined in MCF-7 cells. Interestingly, the phosphorylation of both Akt and Erk1/2 were rapidly and strongly increased after taxol treatment of these cells (Fig. 5).

*Inhibition of taxol-mediated PI3K/Akt signaling by LY294002 or MEK/Erk signaling by U0126/PD98059 diminished taxol-mediated survivin induction and enhanced cell death* – Next, we examined whether inhibition of taxol-mediated PI3K/Akt signaling by the PI3K inhibitor, LY294002, diminished taxol-mediated survivin induction. Taxol-induced upregulation of survivin was significantly decreased by addition of LY294002 (Fig. 6A). Similarly, taxol-mediated induction of survivin was strongly inhibited in the presence of MEK inhibitors, U0126 or PD98059 (Fig. 6B). To explore whether inhibition of taxol-mediated Akt and Erk1/2 activation would enhance taxol-mediated apoptosis, we examined the combination of taxol treatment with either PI3K inhibitor or MEK inhibitor on cell death. The results indicated that their combination significantly increased the effectiveness of taxol to initiate cell death (Fig. 6C).

*Taxol-mediated induction of survivin expression is at least in part transcriptionally regulated, and a cis-acting DNA element upstream of –1430 in pLuc-2840 is responsible for this event* – To explore whether taxol-mediated induction of survivin expression is
transcriptionally regulated, the survivin promoter-luciferase construct pLuc-6270 [contains a 6.3-kb survivin promoter sequence (29)] was utilized in promoter-reporter assay experiments. MCF-7 cells were transfected by pLuc-6270 and treated 24 hours after transfection with taxol for 0, 8, 22 and 30 hours as indicated. Cells were then lysed, and luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega) and normalized to Renilla luciferase activities. Consistent with the results from Western blot (Figs. 1 and 3A), taxol increased the pLuc-6270 luciferase activity after treatment (Fig. 7A). To locate the cis-acting DNA element responsible for the effect of taxol on survivin promoter activity, a series of survivin promoter-luciferase constructs containing different length of survivin promoter sequences (29) were transfected into MCF-7 cells and treated with 30 nM taxol for 0, 8 and 26 hours as indicated 24 hours after transfection (Fig. 7B). As shown, a strong induction of luciferase activity was found in the pLuc-2840 construct but not in pLuc-1430c or smaller constructs (Fig. 7B). This data strongly indicated that the cis-acting DNA element responsible for the effect of taxol on induction of survivin promoter activity is upstream of –1430 in the pLuc-2840 construct.
DISCUSSION

It was previously demonstrated that taxol-mediated mitotic arrest in HeLa cells is associated with upregulation of survivin expression (up to 4 fold), which engenders a survivin-dependent survival pathway (28). This finding appears to be consistent with the previous observation that the expression of survivin is cell cycle-dependent with a robust increase at the G2/M phase of cell cycle (2,27). However, in this study, we concluded that taxol-mediated induction of survivin is a very early event and is independent of taxol-induced mitotic arrest, and that the rapid upregulation of survivin by taxol generates survival signaling that blocks taxol-induced cell death. This conclusion was supported by several observations. First, induction of survivin by taxol occurred as short as 4 hours without increases of G2/M cell population analyzed in flow cytometry. This was confirmed both by survivin immunofluorescence microscopy studies to inspect individual cells and by Western blot to monitor the G2/M indicator cyclin B1 expression after taxol treatment. Second, survivin induction by taxol was significantly diminished after a full mitotic arrest following longer treatment (48 hours or more). Third, inhibition of taxol-mediated induction of survivin by survivin-specific siRNA significantly increased taxol-mediated cell death. Finally, consistent with the finding in this paper that taxol treatment activated Akt and MAPK signaling, suppression of taxol-mediated Akt and Erk activation by pharmacological inhibitors decreased taxol-mediated survivin induction and increased taxol-induced cell death.

To our knowledge, this is the first report showing that taxol can induce a G2/M phase-independent induction of survivin, which is essential for cell survival and resistance to taxol-induced cell death. This finding extended our current vision of the mechanistic
function of survivin to the conservation of a cell survival pathway during taxol treatment (5,28,31) and provided new insight into the mechanisms by which cancer cells activate cell survival pathways and initiate taxol resistance. It was reported that taxol induces prolonged activation of the Ras/MEK/ERK pathway independently of activating the programmed cell death machinery in human esophageal squamous cancer cell lines (36) but the significance of the ERK activation during taxol treatment was not elucidated in their study. While our finding that taxol treatment rapidly activates Erk1/2 in MCF-7 breast cancer cells is consistent with the earlier observation, we extended those studies and demonstrated that ablation of taxol-activated MEK/Erk signaling by pharmacological MEK inhibitors, PD98059 and U0126 reduces taxol-mediated survivin induction and enhances taxol-induced cell death in MCF-7 cells. These findings also provide an explanation for previous observation that U937 leukemia cells treated with taxol followed by PD98059 exhibited a significant increase in cytochrome c release, caspase activation, poly ADP-ribose polymerase (PARP) cleavage, and apoptosis (37). In the present study, the role of survivin in taxol-mediated cell survival was also independently confirmed by directly targeting survivin with siRNA [(32), and Ling & Li, submitted]. That is, inhibition of taxol-induced survivin induction by siRNA increased taxol-induced cell death. In addition, in this report, we also found that taxol treatment rapidly activates Akt signaling in addition to ERK signaling, and inhibition of taxol-induced PI3K/Akt signaling by PI3K inhibitor Ly294002 also decreases taxol-mediated survivin induction and enhances cell death. This finding further extended the current understanding of taxol-mediated cell survival (taxol resistance) and apoptosis. Interestingly, we have observed that taxol-activated Akt phosphorylation was significantly reduced at 12 hours and increase again at 24 hours after
taxol treatment (Fig. 5) although the significance of this phenomenon is not clear but warrants further investigation.

The most significant finding in our report is the observation that induction of survivin expression by taxol is an early event and is independent of taxol-mediated microtubule stabilization and mitotic arrest. Moreover, this finding is consistent with and supported by a number of previous reports. It was shown that taxol could induce inflammatory and apoptotic gene expression that is independent of microtubule stabilization (38), and that abrogation of ERK signaling by MEK inhibitor PD98059 induced a cell cycle-independent apoptosis in vinblastine-treated human ML-1 myeloid leukemia cells (39). In this regard, one explanation to our finding that inhibition of taxol-mediated survivin induction increased cell death might be that loss of survivin shifts the balance of expression/activation of anti- and pro-apoptotic factors toward initiation of apoptosis.

Regardless, studies of the underlying mechanism by which induction of survivin by taxol prior to mitosis preserves a cell survival pathway may have significant consequences for cancer therapeutics and are on the way in our laboratory.

A recent report demonstrated that in taxol-treated MCF-7 breast cancer cells, although mitochondrial cytochrome c release and caspase-9 cleavage were detectable by immunoblotting, assays of both cytosol and nuclei failed to demonstrate the presence of caspase activities that cleaved the synthetic caspase substrates (40). One possibility for the lack of correlation between caspase activation (itself cleavage) and caspase activity is that the rapidly taxol-induced survivin expression found in this study may directly interact with taxol-activated caspases to neutralize caspase activities. However, direct evidence for survivin to inhibit activated caspases is currently lacking. In addition, direct inhibition of
effector caspases, such as caspase-3 and 7 by survivin is controversial (1). While some reports showed that survivin did not inhibit effector caspases (41,42), others showed it did so (43,44). Interestingly, a very recent study showed that survivin interacts with mitochondria-released Smac/DIABLO initiated by taxol treatment (45). These authors demonstrated in a cell-free cytosol system that survivin inhibited caspase-9 and 7 activation only in the presence of XIAP. They proposed that the interaction of survivin with Smac/DIABLO would release XIAP from Smac to inhibit caspase activation. This is a plausible alternative model to support our finding in this report that inhibition of taxol-mediated rapid induction of survivin by siRNA or by pharmacological inhibitors increased apoptosis induced by taxol. However, to support their model, it is important to demonstrate that survivin has significant higher affinity to Smac/DIABLO than XIAP in the presence and/or absence of taxol treatment.

The last issue we would like to mention is the potential transcriptional mechanism that is involved in taxol-mediated induction of survivin expression. Transfection of a series of survivin promoter-luciferase constructs containing different lengths of survivin promoter sequences (29) into MCF-7 cells followed by luciferase assays after taxol treatment indicated that a cis-acting DNA element upstream of –1430 in the pLuc-2840 construct is responsible for the effect of taxol on transcriptional upregulation of survivin (Fig. 7B). Consistent with this observation, previous reports showed that transfection of HeLa cells with the survivin promoter-luciferase construct pLuc-1430c (28), or of MCF-7 cells with the survivin minimal promoter-luciferase construct pLuc-cyc1.2 (containing 267 bp survivin proximal promoter) (31), failed to detect the induction of survivin promoter activity by taxol. Moreover, we found that taxol downregulate survivin promoter activities
when using the pLuc-1430c construct or the shorter survivin-promoter constructs (Fig. 7B). However, the promoter activity increase (1.5-3 fold) induced by taxol does not fully account for the levels of induction of survivin protein by taxol found in Western blots (3-15 fold) in this study. This suggests that a potential post-transcriptional mechanism may also be involved in taxol-mediated survivin induction. Previous studies showed that the phosphorylation of survivin on threonine at position 34 by CDC2 kinase stabilizes survivin protein (28). However, this mechanism is unlikely for the results reported here because survivin protein was increased after taxol treatment as short as 4 hours without a corresponding increase of the cyclin B1 and the G2/M cell population (Fig. 2) and taxol-mediated induction of survivin protein decreased after a taxol-mediated G2/M arrest for 48 hours in comparison with the early time points following taxol treatment. It was shown that CDC2 kinase has its maximum activity at this point (28). Therefore, further investigation of alternative mechanisms is on the way in our laboratory.

In summary, although it is known that taxol treatment induces cells to arrest at mitosis (18), we have found that taxol-mediated induction of survivin occurs earlier in the cell cycle and is independent of taxol-mediated mitotic arrest. The rapid induction of survivin by taxol appeared to increase cell viability and resistance to apoptosis induced by taxol. Targeting this survival pathway may result in novel approaches for cancer therapeutics.
REFERENCES


FOOTNOTE

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1The abbreviations used are: IAP, inhibitor of apoptosis; Taxol, paclitaxel; MDR1, multidrug resistance 1, Pgp, P-glycoprotein; MRP-1, multidrug resistant protein-1; siRNA, small interfering RNA; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PI, propidium iodide; FITC, fluorescein isothiocyanate; DAPI, 4', 6-diamidino-2-phenylindole; FBS, fetal bovine serum; SD, standard deviation.
FIGURE LEGENDS

Fig. 1. **Rapid induction of survivin expression by taxol treatment in MCF-7 breast cancer cells.** Cells were treated with taxol in the concentrations and times as shown, and survivin expression was analyzed by Western blot (A and B) as described in "Experimental procedures". The relative fold increase of survivin protein after normalization to β-actin internal control (monitoring total protein loading in each lane) and the percentage of cell viability determined by trypan blue exclusion staining for each treatment are indicated.

Fig. 2. **Effects of taxol on cell cycle distribution, and the expression of survivin and cyclin B1 in MCF-7 cells.** Cells were analyzed by flow cytometry and immunofluorescence as described in the "Experimental Procedures". A. Cell cycle distribution was determined by PI staining and flow cytometry after taxol treatment at various time points and shown as a histogram. Each bar represents the mean ± SD from a representative experiment in triplicate. Cell viability determined by trypan blue exclusion in each time point is shown in Fig. 1B. B. Rapid induction of survivin expression by taxol was determined by immunofluorescence microscopy. As shown, survivin expression in non-mitotic cells was significantly increased 4 hours after taxol treatment in comparison with no taxol treatment control. C. Time course of induction of cyclin B1 expression after taxol treatment. The relative fold increase of cyclin B1 protein after normalization to β-actin internal control is indicated.

Fig. 3. **Induction of survivin by low concentrations of taxol is as effective as or more effective than high concentrations but the low concentration of taxol is ineffective for induction of cell death.** MCF-7 cells were treated with different taxol concentrations as indicated. Cells were either lysed at 8 hours for Western blot (A) or photographed at 24
hours (B) after taxol treatment as described in "Experimental Procedures". A. Concentration-associated induction of survivin by taxol. The relative survivin increase in folds after normalization to β-actin internal control and the percentage of cell viability are indicated. B. Concentration-dependent induction of cell death by taxol. The quantitated results are shown as a histogram. Each bar is the mean ± SD from four independent microscopic fields of a representative experiment.

Fig. 4. Inhibition of taxol-mediated survivin induction by siRNA significantly increased taxol-induced cell death. The structures of survivin siRNA (SRI-2) and control siRNA (scraSRi) are shown in A. MCF-7 cells were treated with and without taxol as indicated 24 hours after siRNA transfection. Survivin expression was analyzed by Western blot 8 hours after taxol treatment (B). The relative survivin modulation in folds after normalization to the β-actin internal control was indicated. Alternatively, 24 and 48 hours after taxol treatment, apoptotic cells were analyzed by PI DNA staining and flow cytometry. The sub-G1 DNA contents were platted as histogram (C). Each bar is the mean ± SD from a representative experiment in triplicate.

Fig. 5. Rapid phosphorylation of Akt and Erk1/2 in MCF-7 cells after taxol treatment. Cells were treated with taxol as indicated and analyzed for phosphorylation of Akt and Erk1/2 by Western blot using phospho-specific Akt and Erk1/2 antibodies as described in "Experimental Procedures". A. Taxol-mediated phosphorylation of Akt in MCF-7 cells. B. Taxol-mediated phosphorylation of Erk in MCF-7 cells. The percentage of cell viability in each time point is indicated at the bottom.

Fig. 6. Pharmacological PI3K and MEK inhibitors diminished taxol-mediated survivin induction and increased taxol-induced cell death. MCF-7 cells were treated
with and without taxol in the presence and absence of PI3K or MEK inhibitors as indicated. Survivin expression (A and B) was analyzed by Western blot 8 hours after taxol treatment. The relative survivin modulation in folds after normalization to the β-actin and the percentage of viable cells in each condition are indicated at the bottom. Cell death was photographed and quantitated under phase-contrast microscopy 48 hours after taxol treatment (C). The quantitated data are shown as a histogram. The bars represent the mean ± SD from four independent microscopic fields of a representative expression.

Fig. 7. **Identification of the cis-acting DNA elements responsible for the induction of survivin promoter activity by taxol.** MCF-7 cells were transfected with the survivin promoter-luciferase construct pLuc-6270 (A) or with a complete set of survivin promoter-luciferase constructs (B) as shown, and treated with taxol at the indicated time points 24 hours after transfection. Cells were lysed and luciferase activities were measured using the Dual Luciferase Reporter Assay System as described in "Experimental Procedures". A. Taxol treatment increased survivin promoter activities. B. The cis-acting DNA element upstream of –1430 in the pLuc-2840 construct is responsible for the effect of taxol on survivin promoter activity (see text in detail). Each column/bar represents the relative survivin promoter (luciferase) activity and is the mean ± SD from a representative experiment in triplicate.
Fig. 1.

A. Taxol:  
<table>
<thead>
<tr>
<th>Time (day)</th>
<th>3000 nM</th>
<th>300 nM</th>
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<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
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<td>6</td>
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β-Actin-35KD:  
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<th>Cell viability (%)</th>
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<td>4</td>
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<tr>
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B. Taxol: 30 nM  
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<th>16</th>
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<th>48</th>
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<table>
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<tbody>
<tr>
<td>3</td>
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</tr>
<tr>
<td>9</td>
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<tr>
<td>12</td>
</tr>
<tr>
<td>15</td>
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β-Actin-35KD:  
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<td>~96</td>
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<td>~91</td>
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Fig. 2.

**A. MCF-7 Cells**

Effect of taxol (30nM) on cell cycle distribution over the times.

- G1%
- S%
- G2/M%

**B.**

<table>
<thead>
<tr>
<th></th>
<th>DAPI</th>
<th>Survivin</th>
<th>Overlay</th>
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<td>Control</td>
<td><img src="image1" alt="Control DAPI" /></td>
<td><img src="image2" alt="Control Survivin" /></td>
<td><img src="image3" alt="Control Overlay" /></td>
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<tr>
<td>3nM taxol (4 hours)</td>
<td><img src="image4" alt="3nM taxol DAPI" /></td>
<td><img src="image5" alt="3nM taxol Survivin" /></td>
<td><img src="image6" alt="3nM taxol Overlay" /></td>
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</table>

**C.**

<table>
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<tr>
<th>Time (h):</th>
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<th>36</th>
<th>48</th>
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<td></td>
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<tr>
<td>Fold increase:</td>
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<td>.02</td>
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<tr>
<td>β-actin</td>
<td></td>
<td></td>
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Fig. 3.

A. Taxol(nM): 0 3 30 300 3000

<table>
<thead>
<tr>
<th>Survivin-15KD</th>
<th>Fold increase</th>
<th>8 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 15 13.5 14.8 8.5</td>
<td></td>
</tr>
</tbody>
</table>

β-Actin-35KD

Cell viability(%): ~100 ~99 ~99 ~99 ~97

B. Control (24 h) Taxol 3nM (24 h) Taxol 3000nM (24 h)

![Microscopy images of cell cultures]

![Graph of cell death percentage]

Percentage of cell death 24 hours after

- Control
- Taxol 3nM
- Taxol 3000nM
Fig. 4.

A. MCF-7 cells

<table>
<thead>
<tr>
<th>Reagent</th>
<th>survival</th>
<th>fold modulation</th>
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</thead>
<tbody>
<tr>
<td>scraSRi</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>SRL-2</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>taxol (3nM)</td>
<td>52</td>
<td>52</td>
</tr>
<tr>
<td>taxol/scraSRi</td>
<td>4.5</td>
<td>4.5</td>
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<tr>
<td>SRL-2</td>
<td>5</td>
<td>5</td>
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</table>

B. MCF-7 Cells

C. MCF-7 cells

Relative percentage of cell death (sub-G1 DNA contents)

- scraSRi + 3nM
- SRL-2 + 3nM
- scraSRi + 30nM
- SRL-2 + 30nM
- scraSRi
- SRL-2

0 hours | 24 hours | 48 hours
Fig. 5

![Image of Fig. 5 showing a time course experiment with Taxol (30 mM) treatment on cell viability and protein expression over 48 hours.]

Fig. 6.

A. 8 hours

<table>
<thead>
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<th>Ly294002 (μM)</th>
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<th>25</th>
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<tbody>
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<td>-</td>
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<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Survivin</td>
<td>-</td>
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<td>5</td>
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<tr>
<td>Fold increase</td>
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<td>β-Actin</td>
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B. 8 hours

<table>
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<tr>
<td>Taxol (nM)</td>
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<tr>
<td>Survivin</td>
<td>-</td>
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<td>1</td>
<td>0.8</td>
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<td>Fold increase</td>
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<td>0.6</td>
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<td>0.6</td>
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<tr>
<td>β-Actin</td>
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<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>
Fig. 6

C.  

No kinase inhibitors

LY294002

U0126

LY294002 + U0126

Percentage of cell death 48 hours after various treatment
Fig. 7.

A.

B.
Induction of survivin expression by taxol (paclitaxel) is an early event which is independent of taxol-mediated G2/M arrest
Xiang Ling, Ralph J. Bernacki, Michael G. Brattain and Fengzhi Li

J. Biol. Chem. published online January 13, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M310947200

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