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 up-regulated survivin expression (3.5–15-fold) within 4 h without G2/M arrest. Lengthening the treatment of cells (48 h) 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 effective than 3000 nM. As a result, 3 nM 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 phosphatidylidyinositol 3-kinase/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 counteract taxol-induced apoptosis. Targeting this survival pathway may result in novel approaches for cancer therapies.

Survivin is a recently characterized novel member of the inhibitor of apoptosis (IAP) family. It is undetectable in most normal adult tissues but highly expressed in cancer. Survivin expression has been shown to be associated with drug resistance, 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 therapies.

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, can confer resistance to taxol. This is because P-glycoprotein 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 resulting 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 upon 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 because of 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 up-regulated survivin expression without apparent arrest of cells into G2/M phase. Lengthening the treatment of cells (48 h) 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 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 the effects of taxol 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 a time 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 P-glycoprotein and multidrug-resistant protein-1 expression, were maintained in RPMI 1640 medium containing 10% fetal bovine serum (MediaTech CellGro, Herndon, VA), penicillin (100 units/ml), and streptomycin (0.1 μg/ml) (Invitrogen) in a humidified atmosphere incubator with 5% CO2 at 37 °C. Cells were routinely subcultured twice weekly.

Reagents—Taxol, anti-β-actin, goat peroxidase-conjugated anti-rabbit IgG and fluorescein isothiocyanate-conjugated anti-rabbit IgG were purchased from Sigma. Anti-survivin (FL-142), anti-Erk (K-23), and anti-Akt (H-136) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-Akt (Ser-473) and phospho-p44/42 MAPK (Thr-202/Tyr-204) antibodies were purchased from Cell Signaling Technology (Beverly, MA). LY294002, PD98059, U0126, and dual anti-Akt antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). FuGENE 6 transfection reagent was bought from Roche Diagnostics (Indianapolis, IN). Oligotransfectamine reagent was purchased from Invitrogen. CellGro, Herndon, VA), penicillin (100 units/ml), and streptomycin (0.1 μg/ml) (Invitrogen) in a humidified atmosphere incubator with 5% CO2 at 37 °C. Cells were routinely subcultured twice weekly.

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Western Blot—Cells with and without taxol treatment were washed with phosphate-buffered saline (PBS: 50 mM phosphate, pH 7.4, 100 mM NaCl, and 10 mM KCl) and lysed on ice for 30 min in PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml phenylmethylsulfonyl fluoride, and 20 μM leupeptin. After the lysates were cleared by centrifugation at 15,000 × g for 20 min 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× SDS sample loading buffer for 5 min at 95 °C, separated on 10–15% SDS-PAGE gels, and electrotransferred to Immobilon-P membranes (Millipore, Bedford, MA) using semidry electroblotter 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, 0.15% 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 secondary anti-IgG antibody (1:5000) at room temperature for 1 h with constant shaking. After the membranes were blocked with 5% skimmed milk in TBS-T buffer containing the appropriate secondary anti-IgG antibody (1:5000) at room temperature for 1 h 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 secondary anti-IgG antibody (1:5000) at room temperature for 1 h with constant shaking.

Trypan blue exclusion staining for determination of cell viability—Cells were counted by trypan blue exclusion staining and resuspended in PBS buffer. A small sample of the cell suspension was diluted in 4% (w/v) trypan blue (one sample at a time because viable cells absorb trypan blue or take up trypan blue 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 ×100 magnification, respectively. Each sample cell 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 number/total cell number × 100.

Immunofluorescence Microscopy—Cells were seeded on the round glass coverslips coated with 2% gelatin (Sigma) in 12-well plates. At different intervals after 3 nM taxol treatment as described above, medium was removed and cells were washed once with PBS and fixed overnight with 4% paraformaldehyde in PBS at 4 °C. Cells were then permeabilized and incubated in PBS containing 1% BSA and rabbit anti-survivin antibody (1:500) for 60 min at 37 °C followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:200) for 30 min at 37 °C. The cells then were analyzed for immunofluorescence intensities by flow cytometry (FACScan, BD Biosciences) from 10,000 events/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) in 12-well plates. At different intervals after 3 nM taxol treatment as described above, medium was removed and cells were washed once with PBS and then fixed with 4% paraformaldehyde in PBS at 4 °C. Cells were then permeabilized and blocked for 30 min in PBS containing 1% 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 min at 37 °C followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG (1:200) for 30 min at 37 °C. The cells then were stained with 4',6-diamidino-2-phenylindole at a final concentration of 0.5 μg/ml in H2O for 10 min at room temperature. The resultant glass coverslips containing cells were mounted on glass slides with GelMount™ solution (Biomedia Corp., Foster City, CA). The cells were analyzed under a Zeiss Axiovert 100M digital fluorescence microscope. Images were captured using Zeiss LSM510, version 2.8 and processed with Photoshop Elements software.
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 under “Experimental Procedures.” A, cell cycle distribution was determined by propidium iodide (PI) staining and flow cytometry after taxol treatment at various time points and shown as a histogram. Each bar represents the mean ± S.D. 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 h 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.

**SIRNA Preparation**—A human survivin mRNA-specific RNA oligonucleotides with 3′-TT overhangs were chemically synthesized and purified by high pressure liquid chromatography (Xeragon, Huntsville, AL) as follows: SRI-2F (5′-GCG CCU GCA CCC CGG AGC G110TT*) and SRI-2R (110CGC UCC GGG GUG CAG GCG C 92TT). 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, and 2 mM MgAc2, pH 7.4). After denaturation at 90 °C for 1 min, the mixture (Sri-2) was annealed at 37 °C for 60 min 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 Vitro Transfection with siRNAs**—Cells were transfected with survivin siRNAs using the Oligofectamine reagent following the manufacturer’s instruction. 1 day prior to transfection, 5 × 10⁴ MCF-7 cells/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 of DMEM containing 6 μl of 20 μM siRNA with 15 μl of DMEM containing 3 μl of Oligofectamine reagents. Before transfection, the medium in 6-well plates was replaced with serum-free DMEM medium (800 μl/well). The transfection mixture was added to the 6-well plate within 20–40 min after mixture preparation in a total volume of 990 μl/well. The transfected cells were incubated at 37 °C for 4 h, and then 500 μl of DMEM medium containing 30% fetal bovine serum was added. Cells were treated with and without 3 and 30 nM taxol 24 h after transfection with siRNA or control siRNA as described above. For Western blot, cells were harvested 8 h after taxol treatment. For flow cytometry analysis of sub-G1 DNA content, cells were harvested 24 and 48 h after taxol treatment and analyzed by propidium iodide staining and flow cytometry as described above. The percentage of dead cells (sub-G1 DNA contents) was plotted as a histogram. All of the 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 FuGENE 6 transfection reagent (Roche Diagnostics), and luciferase activities were measured using a dual luciferase reporter assay system (Promega) according to the respective manufacturers’ recommendations. Cells were seeded in 24-well plates 1 day prior to transfection. On the following day, the transfection solution for each well was prepared by sequentially adding 1 μl of FuGENE 6 transfection reagent and 0.4 μg of plasmid DNA in a 1.5-ml tube containing 50 μl of serum-free DMEM medium. The DNA FuGENE 6 mixture was incubated at room temperature for 20–30 min and then added onto cells at −50% confluence in each well containing complete medium. The transfected cells were treated with 3 and/or 30 nM taxol 24 h after transfection for the 30-h time point, and then taxol (3 and/or 30 nM in final concentration) was added to the transfected cells at other time points at 4 (26-h time point), 8 (22-h time point), and 22 h (8-h time point) after the first taxol treatment to permit the simultaneous harvesting of the cells from all of the time points. Cells were washed with PBS and lysed in 200 μl of 1× passive lysis buffer (Promega) per well 8 h after the last taxol treatment. After incubation of the plate on ice for 45 min, the lysate was transferred to a 1.5-ml Eppendorf tube by scraping with a rubber policeman. Cellular debris was pelleted by centrifugation at 15,000 × g for 10 min at 4 °C. 20 μl of cell lysates/well was used to measure Firefly and Renilla luciferase activity using a luminometer by adding 20 μl of 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 h increases survivin expression to 1.2–4-fold 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 h or more) significantly attenuated survivin induction in comparison with the early times (24 h, Fig. 1A). The high induction of survivin at 24 h 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 h and that extending treatment times beyond 24 h 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 probably 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-h 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 h or more (Fig. 2A). We next examined individual cells by survivin immunofluorescence microscopy after taxol treatment for 4 h. 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 for 4 h. 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 for 4 h. 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 for 4 h. 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 for 4 h. 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 for 4 h. The results indicated that interphase cells showed increased immunoreactivity with anti-survivin antibody in comparison to no taxol treatment control.

Low Concentrations (3–30 nM) of Taxol Effectively Induced Survivin Expression but Were Ineffective for Apoptosis Induction—Unexpectedly, the induction of survivin expression by
siRNA significantly increased taxol-induced cell death. The modulation infolds after normalization to the lyzed by Western blot 8 h after taxol treatment. The relative survivin cated 24 h after siRNA transfection.

![Image](59x300 to 303x737)

**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 after siRNA transfection. B, survivin expression was analyzed by Western blot 8 h after taxol treatment. The relative survivin modulation infolds after normalization to the β-actin internal control was indicated. Alternatively, 24 and 48 h after taxol treatment, apoptotic cells were analyzed by propidium iodide (PI) DNA staining and flow cytometry. C, the sub-G1 DNA contents were plated as histogram. Each bar is the mean ± S.D. from a representative experiment in triplicate.

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) 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), propidium iodide 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 up-regulate 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**—We examined whether inhibition of taxol-mediated PI3K/Akt signaling by the PI3K inhibitor, LY294002, diminished taxol-mediated survivin induction. Taxol-induced up-regulation of survivin was significantly decreased by the 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 containing 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 h after transfection with taxol for 0, 8, 22, and 30 h as indicated. Cells were then lysed, and luciferase activities were measured using the dual luciferase re-

**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 under "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.
porter 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 h as indicated 24 h 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). These 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 was associated with up-regulation 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.

**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 h 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. C, cell death was photographed and quantitated under phase-contrast microscopy 48 h after taxol treatment. The quantitated data are shown as a histogram. The bars represent the mean ± S.D. from four independent microscopic fields of a representative expression.
and that the rapid up-regulation 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 h 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. 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 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 h and increased again at 24 h after taxol treatment (Fig. 5), although the significance of this phenomenon is not clear and 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 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 h and increased again at 24 h after taxol treatment (Fig. 5), although the significance of this phenomenon is not clear and warrants further investigation.

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as caspase-3 and -7 by survivin is controversial (1). Whereas some reports showed that survivin did not inhibit effector caspases (41, 42), others showed that it did (43, 44). Interestingly, a very recent study showed that survivin interacts with mitochondria-released Smac/DIABLO initiated by taxol treatment (45). This study demonstrates in a cell-free cytosol system that survivin inhibited caspase-9 and -7 activation only in the presence of X chromosome-linked IAP. They proposed that the interaction of survivin with Smac/DIABLO would release X chromosome-linked IAP 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 induced apoptosis induced by taxol. However, to support their model, it is important to demonstrate that survivin has significant higher affinity to Smac/DIABLO than X chromosome-linked IAP 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 construct is responsible for the effect of taxol on transcriptional up-regulation of survivin (Fig. 7B). Consistent with this observation, previous reports show 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−cycl1.2 (containing 267 bp of survivin proximal promoter) (31) failed to detect the induction of survivin promoter activity by taxol. Moreover, we found that taxol down-regulated 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 show that the phosphorylation of survivin on threonine at position 94 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 h 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 h 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 underway 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.
