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ORIGINAL ARTICLE

Paclitaxel induced apoptosis in breast cancer cells requires cell cycle transit but not Cdc2 activity

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Abstract
Purpose Paclitaxel (PTX) is a widely used chemotherapy agent and may cause cell death by apoptosis subsequent to microtubule (MT) disruption. In this paper, we have investigated whether cell cycle transit and or Cdc2 (Cdk1) activity is required for the apoptosis induced by PTX.
Methods Cell cycle was analyzed by flow cytometry, Cdc2 was assayed bio chemically. Cdc2 activity was decreased by siRNA and dominant negative (dn) Cdc2 expression. Cells were arrested by chemical or biological inhibitors in a G_1or S phase. Apoptosis was measured by DNA fragmentation and examination of nuclei by microscopy. JNK and AKT activations were assessed as well.
Results Cell cycle inhibition was highly effective in decreasing PTX induced apoptosis. MT morphology was not altered by these inhibitors. PTX induced JNK activity or AKT mediated BAD phosphorylation was unaffected by cell cycle inhibitors. Abrogation of Cdc 2 activity was without effect on PTX induced apoptosis.
Conclusions While cell cycle transit is required for PTX induced apoptosis; Cdc2 activity is not required.

Keywords Cell cycle · Paclitaxel · Cdc2 · Microtubules · Apoptosis

Abbreviations

Dn	Dominant negative
MTs	Microtubules
MTAs	MT disrupting agents
m.o.i	Multiplicity of infection
PTX	Paclitaxel
p.f.u	Plaque forming unit

Introduction

The majority of cells in solid tumors are in a G_0/G_1 phase. Chemotherapy agents that block DNA synthesis and induce apoptosis (perhaps through the DNA damage checkpoint) will not kill most tumor cells during treatment. This phenomenon will leave a large pool of cancer cells, including stem cells, available to enter the cell cycle and create expanding clones. Potentially, these cells and their clones may acquire drug resistance and other properties during their prolonged G_0/G_1 phase residence which gives rise to metastasis [1]. The development of agents, which can induce apoptosis of cells in G_0/G_1 phase, should be a priority. Drugs directed against membrane bound receptors such as HER 2, which mediate G_1 to S phase transition in response to growth factors, may possibly achieve this goal [1, 2].

In addition to their critical role in forming the mitotic spindle [3], MTs are essential structural compo-

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nents of normal and tumor cells [4]. They perform a variety of functions such as organelle movement, cell movement, and transport of macromolecules. The taxane, paclitaxel (PTX), and several other chemotherapy agents in the vinca alkaloid family disrupt MT function by stabilizing or depolymerizing MTs or by altering MT dynamics [5]. The therapeutic role of these MT disrupting agents (MTA) may be related to the disruption of the mitotic spindle. The toxicity of PTX in non-dividing cells (e.g. neurons) may result from the disruption of other functions of MTs [6]. These agents may inhibit the function of MTs in G_1 and induce apoptosis in these non-dividing cells in the solid tumors [5]. If this hypothesis is correct, then MTAs may be more efficient in decreasing tumor mass than agents that disrupt DNA synthesis.

Previous studies have shown that PTX induces apoptosis in a variety of cell types [7] including MCF-7 breast cancer cells [8–12]. Similarly, we demonstrated that PTX causes early and late phases of apoptosis in ovarian cancer cells, and only the early phase is dependent on the activity of the Ras/Rac/MEK/JNK/AP1 pathway [13]. Prolonged activation of JNK in response to PTX has been demonstrated by us [13] and others [14, 15]. When JNK is inhibited, cells still accumulate in G_2/M upon treatment with PTX [16]. In this study, we found that JNK activation is not sufficient to cause apoptosis. We demonstrated that Cdc2 (Cdk1) is not involved in PTX induced apoptosis, although it is known that the G_2/M block caused by PTX is associated with increased Cdc2 activity. Furthermore, our results show that apoptosis induction by PTX requires transit through the cell cycle. Collectively, these data suggest that PTX induces the spindle checkpoint, and some signal from the checkpoint acts distal to JNK to cause apoptosis. Our studies imply that the interactions of PTX with the MTs of the mitotic spindle rather than the MTs of cells in G_1 phase are the basis of the cytotoxicity of PTX.

Materials and methods

Reagents and antibodies

Cell culture media and antibiotics, histones, RNAse A, propidium iodide, PTX, hydroxyurea (HU), and other chemicals were from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise noted. ICI 182,780 was kindly supplied by Dr. Alan Wakeling at Zeneca Pharmaceuticals (Alderly Park, Cheshire, UK). Protein A/G beads, antibodies to Cdc2, Cdk2 (M2), total BAD (H-16), cyclin B1 (GNS1), JNK-1 (C-17), and survivin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-actin, anti-HA, and anti-tubulin (12CA5) were from Boehringer Mannheim (Indianapolis, IN, USA). Antibodies to phosphoBAD 112 and phosphoBAD136 were from Upstate Biotechnology (Lake Placid, NY, USA). [γ-^{32}P] ATP was from ICN (Irvine, CA, USA). FBS was from Summit Biotechnology (Fort Collins, CO, USA). Horseradish peroxidase-conjugated secondary antibodies were from Jackson Immunoresearch (West Grove, PA, USA), and roscovitine was from Calbiochem (La Jolla, CA, USA). RNAiFect was purchased from Qiagen (catalog no. 301605). Cdc2 siRNA was from Cell Signaling Technologies (catalog no. 6376), which was synthesized based on the sequence in [17]. Control oligonucleotide was from Qiagen.

Cell culture, transfections and UV irradiation

MCF-7 cells were a kind gift from Dr. R. P. Shiu and were maintained in Dulbecco's Modified Essential Medium (DMEM) supplemented with antibiotics and 5% fetal bovine serum (FBS). The plasmid vector for p16 (pBPSTRI-p16) was provided by Dr. G. Peters [18]. The plasmid vectors for $p27^{Kip1}$ [19] and dn Cdc2 were provided by Dr. M. Pagano and Dr. E. Harlow [20], respectively. The hemagglutinin (HA)-epitope tagged expression vector pSRα-HA-JNK1 was from Dr. M. Karin (University of California at San Diego). Transfections were carried out using Lipofectamine PLUS transfection reagent (Invitrogen, Bethesda, MD, USA). UV irradiation was performed by exposing cells to a germicidal ultraviolet lamp (254 nm, 38 W, 72-cm distance between plates and the UV lamp) in a tissue culture hood for 2 min. The UV dose was approximately 40 J/m^2.

Viral vectors and infections

Replication-defective adenoviral vector for expression of $p16^{INK4a}$ (Adv p16) was constructed in the laboratory of Dr. P. Seth [21]. Control adenovirus (Adbgal) was from Dr. J. Nevins [22]. The adenoviral vector expressing $p27^{Kip1}$ (Ad.p27) was derived similarly [23]. The adenoviral vector for expression of Cdk2 (Ad.Cdk2) was kindly provided by Dr. J. Nevins [22, 24]. Adenoviruses were propagated in 293 cells (ATCC) and viral lysates for use in experiments were tittered by a standard plaque assay. Cultures were infected an m.o.i. of 80 p.f.u./cell. After infection, the medium was replaced with normal growth medium and the cultures were incubated for an additional 48 h before treatment and harvest.

Hoechst staining

Following treatments cells were cytospun onto microscope slides and rehydrated for 10 min in PBS containing 1 mM CaCl and 0.5 mM MgCl. The slides were stained with 3 μg/ml Hoechst 33342 (Sigma) for 10 min, rinsed in PBS, and the cover slips were attached using DAKO fluorescent mounting medium. A minimum of 400 cells for each treatment was visually scored on an Olympus IMT-2 fluorescent microscope as either apoptotic or normal. The graphed results were derived from at least three independent experiments.

Tubulin staining

Cells grown on cover slips were washed with PBS, fixed for 10 min at room temperature in 3% paraformaldehyde, washed again with PBS, then permeabilized with PBS/1% BSA and 0.5% Triton X100 for 5 min. Cells were blocked for 30 min with PBS/10% goat serum/1% BSA and washed for 5 min in PBS. Cells were stained with anti-α-tubulin mouse monoclonal (236-10,501 Molecular Probes) at 1 μg/ml in PBS/1% BSA overnight at 4°C. Cover slips were washed three times for 5 min each with PBS, then incubated with Alexa Fluor 350 conjugated goat anti-mouse IgG (Molecular Probes) in PBS/1% BSA for 30 min at room temperature. The cover slips were again washed three times for 5 min in PBS and mounted on slides using DAKO fluorescent mounting medium.

Thymidine block

For synchronizing cells in S phase by a double thymidine block, cells were plated in regular growth medium at 10–20% confluence, and incubated for 1 day. Thymidine at 2 mM (Sigma) was added to the medium and the cells were incubated for 16 h. The cells were then washed with PBS and the medium replaced with medium containing 10 μM deoxycytidine (Sigma) but no added thymidine. After 9 h, the medium was washed out and the cells were again incubated with medium containing 2 mM thymidine for 16 h [25].

DNA fragmentation

DNA fragmentation was measured using the Cell Death Detection ELISA PLUS kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's protocol. Following treatment, both adherent and detached cells were removed and equal numbers of cells were centrifuged and lysed. Data are reported as fold increase of optical density over that of the control cells.

Flow cytometric analysis

Cells were harvested in PBS–EDTA, fixed in cold 70% ethanol, and stored at −20°C. Fixed cells were subsequently washed, treated with 100 μg/ml RNAse A, and stained with 50 μg/ml propidium iodide. Analysis of DNA content was performed in a Becton–Dickinson FAC Scan with a minimum of 10,000 events collected for analysis using Becton–Dickinson Cell Quest software. For flow cytometric analysis of transfected cultures, MCF-7 cells were transfected with pEGFPN1 (Clontech, Palo Alto, CA, USA) along with appropriate plasmids (1:5 mass ratio) and treated as described previously. Harvested cells were fixed in 0.5% formalin followed by ethanol fixation and propidium iodide staining. Cells were sorted based on expression of green fluorescent protein and DNA content was analyzed in these cells [26].

Western blot analysis

Cells were lysed as described previously. Briefly, following treatments, cell monolayers were washed in ice-cold PBS and lysed by addition of ice-cold NP-40 lysis buffer (20 mM Tris, pH 7.5, 250 mM NaCl, 0.5% NP-40, 0.1 mM EDTA, 1 mM $NaOV_4$, 10 mM NaF, 1 mM PMSF). Lysed Cells were collected in microcentrifuge tubes, and then centrifuged at 15,000 × *g* for 10 min at 4°C to remove cellular debris. The supernatant was aliquoted and frozen at −80°C for later use. Equal amounts of protein (50–100 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and membranes were incubated in blocking buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20, and 0.5% casein) for 10–15 min. Membranes were incubated with primary antibodies in blocking buffer overnight at 4°C. All primary antibodies were used at 0.5 μg/ml. Bound antibodies were detected using horseradish peroxidase-conjugated secondary antibodies and developed using ECL chemiluminescent immunodetection reagent (Amersham, Irvine, CA, USA).

Immune complex kinase assays

Kinase assays were performed essentially as described [26–28]. Equal amounts of lysate proteins prepared as described above (100–200 μg) were immunoprecipitated along with protein A/G agarose beads for 18 h in NP-40 lysis buffer. The immune precipitates were washed three times in lysis buffer, twice in kinase buffer (20 mM HEPES, pH 7.5, 20 mM $MgCl_2$), and resuspended in kinase buffer supplemented with 10 mM ATP, 1 mM DTT, 0.5 mM EGTA, 0.2 mM

$NaOV_4$, 5 μCi [γ-^{32}P] ATP and 400 μg/ml histones (type III-SS) or c-jun recombinant protein as recommended by the supplier (Santa Cruz). Kinase reactions were incubated for 1 h at room temperature. All kinase assays were stopped with 25 μl 2× SDS-PAGE sample buffer. Reaction products were separated on SDS-PAGE gels followed by autoradiography using Kodak XAR film.

Cdc2 siRNA transfection experiments

MCF-7 Cells were seeded on 6 or 24 well plates at 10% confluence to achieve 80% confluence at time of lysis. Cells were transfected for 72 h in DMEM phenol red containing medium supplemented with 2.5% FBS and penicillin/streptomycin. siRNA was added according to RNAiFect manufacturers protocol (Qiagen). For six well plates, 50 nM of siRNA was added to each well with 15 μl of transfection reagent; 24 well plates contained 25 nM of siRNA per well and 1.5 μl reagent. At 48 h post-transfection, cells were treated with 100 nM PTX or vehicle control DMSO for 24 h. Cells were lysed with buffer containing 0.5% NP-40 and Triton X-100, 250 mM NaCl and 1 mM NaF, PMSF, $NaVO_4$ and leupeptin (1 ng/μl). Cells for DNA Fragmentation ELISA experiments were lysed according to manufacture's protocol as described previously.

Statistics

All the experiments in this paper were repeated three to four times unless otherwise noted.

Statistics were calculated using Prism Graphpad software and are presented in the figure legends.

Results

Previous results from our laboratory demonstrated that the PTX induced G_2/M block at 24 h of treatment was unaffected by dn JNK, dnASK, dnRac, and dnJNKK. These same expression constructs decreased PTX induced apoptosis in ovarian cancer cells [16]. This observation implies a relationship between cell cycle phase, Cdc2 activation, and apoptosis. The following experiments explore the mechanism of apoptosis caused by PTX. We will determine whether cell cycle transit is essential for PTX induced apoptosis, investigate the role of Cdc2 in apoptosis, and the relationship of JNK activation by PTX to the cell cycle. Lastly, we will research the role of the AKT survival pathway in apoptosis caused by PTX.

Inhibition of G_1/S phase transition by chemical inhibitors lead to reduction of PTX induced apoptosis

In general (unless otherwise stated), cells were treated with 100 nM PTX for 24 h. At 48 h, ~50% of cells detach from the plate and make this time point unsuitable for biochemical analysis. At 24 h, the majority of cells were still attached. Similar results were obtained with apoptosis experiments with 25 nM PTX treatment for 48 h.

We have used the term G_2/M in this paper to define any state where the cells contain 4 × DNA as determined by flow cytometry, much like many other authors have done in the past. Since PTX blocks mitosis in many, but not all, cell types and leads to the formation of abnormal MT "bundles" (Fig. 1e), PTX does not arrest cells in a normal mitosis. As a method for cell cycle arrest, thymidine block, which arrests cells in early S phase, was utilized. The double thymidine block significantly decreased the PTX induced G_2/M accumulation (> 50%, $p < 0.01$) (Fig. 1a,f). The double thymidine block also prevented apoptosis in MCF-7 cells caused by PTX ($p < 0.01$) as measured by DNA fragmentation (Fig. 1b). In Fig. 1b right panel, apoptosis was measured morphologically by the staining of chromatin with the Hoechst reagent. These results also show that the early S phase arrest abrogated PTX induced apoptosis.

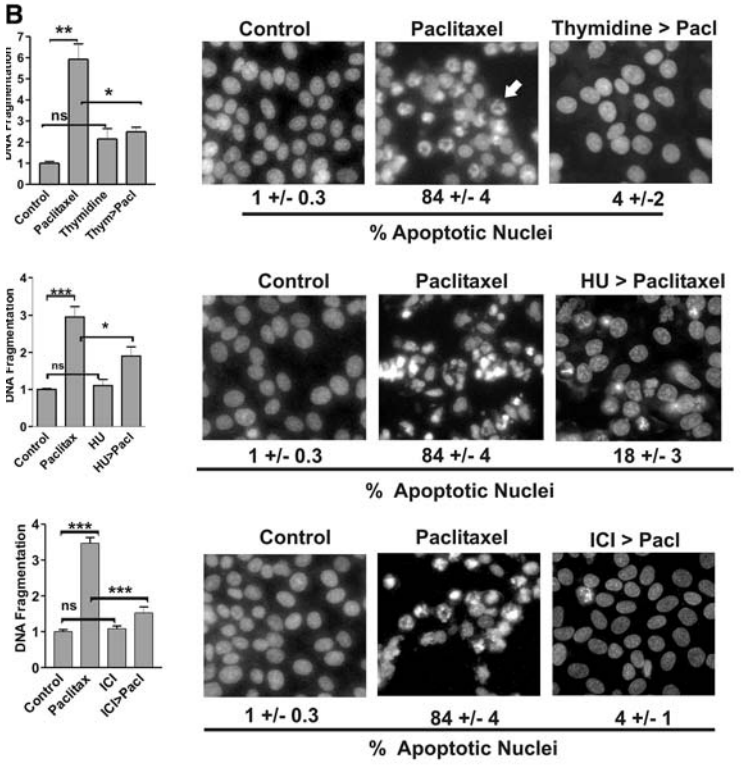
We used hydroxyurea as another chemical to inhibit progression through S phase. Asynchronous cells were treated with 1 mM HU for 24 h before treatment with PTX as in Fig. 1a,b. As shown in Fig. 1a,g, pretreatment with HU essentially eliminated ($p < 0.01$) the G_2/M block imposed by PTX, indicating again that arresting cells in S phase prevents the PTX induced G_2/M arrest. HU also reduced PTX induced apoptosis in a dose dependent manner (data not shown) with approximately 50% of reduction ($p < 0.05$) at 2 mM (Fig. 1b) without having a significant effect by itself. These data were confirmed by the Hoechst staining of nuclei (Fig. 1b), which demonstrates that HU inhibited the apoptotic effect of PTX about 80% ($p < 0.01$). MCF-7 cells are estrogen receptor (ER) positive cells whose proliferation even by growth factors in serum appears to require function of the ER. This was deduced by arrest in G_1 phase by antiestrogens such as ICI 182,780 (ICI) [29]. To prevent G_1 to S phase transition, we treated cells with ICI prior to PTX. As shown in Fig. 1a, treatment of cells with ICI for 48 h forced a G_1 block. This significantly inhibited a PTX induced G_2/M block by 50% ($p < 0.01$). ICI was also able to completely inhibit PTX induced apoptosis as measured by DNA fragmentation (Fig. 1b) and Hoechst staining

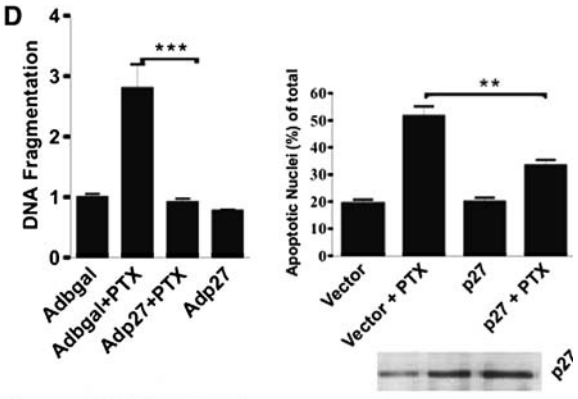
A

	%G1	SEM (+/-)	p value	%S	SEM (+/-)	p value	%G2/M	SEM (+/-)	p value
Control[1]	63.7	2.4		12	2.2		20	4.4	
Paclitaxel	7.4	4.3	<0.001[1]	6.8	1.0	>0.05[1]	79.7	7.1	<0.001[1]
HU	36.6	4.8	<0.001[1]	49.5	0.0	<0.01[1]	27	3.3	>0.05[1]
HU>Paclitaxel[2]	24.7	10.0	<0.001[1]	29.7	6.4	>0.05[1]	31.3	7.5	>0.05[1]
	-	-	>0.05[2]	-	-	<0.01[2]	-	-	<0.001[2]
Control[1]	55.6	6.1		21.9	2.2		20.5	4.8	
Paclitaxel	10.3	4.2	<0.001[1]	9.8	2.2	<0.05[1]	75.7	6.4	<0.001[1]
Thymidine	26	4.0	<0.001[1]	51.9	4.3	<0.001[1]	18.1	2.1	>0.05[1]
Thy>Paclitaxel[2]	16.3	4.2	<0.001[1]	45.5	3.8	<0.001[1]	32.3	2.8	>0.05[1]
	-	-	>0.05[2]	-	-	<0.001[2]	-	-	<0.001[2]
Control[1]	56.3	4.0		15.2	1.9		25.7	4.6	
Paclitaxel	5.7	1.7	<0.001[1]	7.8	2.3	>0.05[1]	80.9	5.0	<0.001[1]
ICI	75.4	3.2	<0.01[1]	4.4	0.9	>0.05[1]	10.4	2.9	>0.05[1]
ICI>Paclitaxel[2]	38	4.8	<0.01[1]	5.3	0.6	>0.05[1]	47.4	8.4	<0.05[1]
	-	-	<0.001[2]	-	-	>0.05[2]	-	-	<0.01[2]

C

	%G1	SEM (+/-)	%S	SEM (+/-)	%G2/M	SEM (+/-)	p value
Adbgal[1]	40.5	3.9	13.6	1.1	33.1	1.4	
Adbgal> Paclitaxel[12]	14	2.4	10.5	1.8	63.3	5.5	<0.001[1]
Adp27[1]	67.3	4.6	12.2	3.6	12.5	2.2	
Adp27> Paclitaxel[12]	37.8	4.8	12.6	4.2	46.6	5.8	<0.001[1]
	-	-	-	-	-	-	<0.01[2]





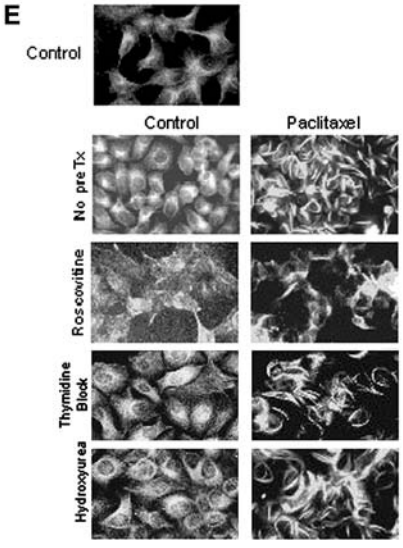


Fig. 1 Cell cycle inhibition inhibits PTX induced apoptosis. **a** Flow cytometric analysis of the effects of PTX, HU, double thymidine, and ICI on growing cells. MCF-7 cells were pretreated with 1 mM HU for 24 h before adding PTX for an additional 24 h. Double thymidine block was imposed on growing cells before PTX treatment for 24 h. Growing cells were treated with 20 nM ICI 182, 780 for 48 h before PTX treatment (24 h). Control cells were treated with vehicle where appropriate.[1] Indicates differences between controls and treatment. [2] Refers to differences between PTX alone and PTX with other chemicals treatments. **b** DNA fragmentation assays were performed using an ELISA kit as described in materials and methods. Statistical significances are $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. The *brackets indicate* comparisons made between pairs. *Right panels* show Hoechst stained nuclei of cells treated as in (**a**). The numbers indicate % apoptotic nuclei (±SE). The *arrow indicates* a typical apoptotic nucleus. The experiment was repeated three times for each treatment with similar results. **c** Flow cytometric analysis of $p27^{kip1}$ transduced cells ± *p* values: [1] for Adbgal versus Adbgal with PTX, or Adp27, or Adp27 with PTX, [2]for Adbgal with PTX versus Adp27 with PTX for % G_2/M values. **d** $p27^{kip1}$ was over expressed in MCF-7 cells, as described in methods, 48 h prior to PTX treatment. DNA fragmentation experiments (*left panel*) and Hoechst stained apoptotic nuclei right panel were assessed as described previously. The definition for *p* values is the same as in (**b**). Loading control for $p27^{kip1}$. **e** The characteristic bundling of bound MT is apparent in PTX treated MCF-7 cells, and in cells treated with 25 μM roscovitine, 2 mM thymidine, or 1 mM HU for 12 h followed by PTX treatment (24 h). *Left panel* shows cells treated with cell cycle inhibitors, *right panel* shows PTX only (*top*) or PTX with inhibitors. **f–h** Representative flow cytometric analyses of thymidine, HU, ICI treated cells ± PTX, respectively. **i** Similar analysis of thymidine treated Brady ovarian cancer cells

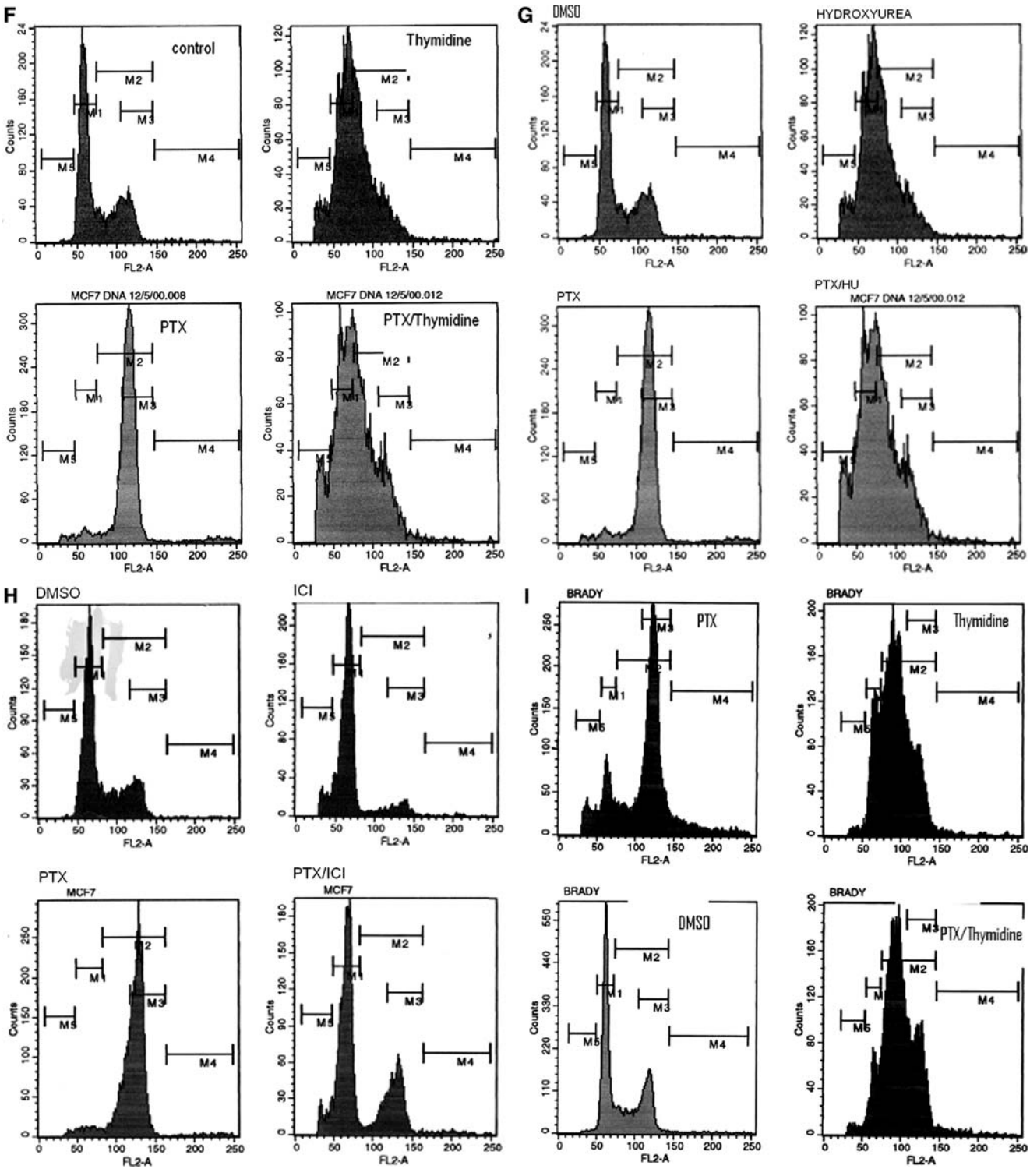


Fig. 1 continued

(Fig. 1b right panel). These results demonstrate the requirement of G_1 to S phase transition for PTX induction of apoptosis. In contrast, the ER agonist estradiol (E_2) had no reliable effect on PTX induced apoptosis under conditions of asynchronous growth (data not shown).

Asynchronously growing MCF-7 cells were pretreated with 25 μM roscovitine or DMSO and then

with PTX (100 nM) for 24 h. Flow cytometric analysis showed that roscovitine, an inhibitor of Cdk2 and 4 [26], altered the distribution of cells in the cell cycle by inducing a sub G_1 accumulation (~15%). However, roscovitine completely inhibited the effect of PTX on induction of a mitotic arrest and apoptosis as shown by DNA fragmentation and by Hoechst staining of apoptotic nuclei (data not shown). Similar results demonstrating that G_1/S transition is required for PTX induced apoptosis were obtained with ovarian cancer cells (Fig. 1i) as well as T47D breast cancer cells.

Biological inhibitors of G_1 to S phase transition abrogate PTX induced apoptosis

Since chemical inhibitors of Cdks may have effect on other non-Cdk kinases, which can regulate cell cycle transit, we sought to blockade G_1 to S phase transit by other means. Many studies have shown that over expression of $p27^{Kip1}$ and $p16^{INK4}$, which inhibit Cdk2 and Cdk4, will arrest cells in G_1. Previously, we have successfully used these constructs to arrest MCF-7 cells in G_1 [26, 30, 31]. Adenoviral $p27^{Kip1}$ and $p16^{INK4}$ were over expressed in >80% of cells prior to treatment with PTX. In Fig. 1c, cells expressing adenoviral $p27^{Kip1}$ and $p16^{INK4}$ (data not shown) and treated with PTX accumulated in G_1 phase ($p < 0.01$) and less in G_2/M phase ($p < 0.01$) compared to cells infected with control virus plus PTX. This is presumably due to the inhibition of G_1 transit by the Cdk inhibitors. Adenoviral $p27^{Kip1}$ completely abolished PTX induced apoptosis (Fig. 1d). GFP and $p27^{Kip1}$ expressing plasmids were co-expressed prior to PTX treatment (Materials and methods), and Hoechst stained nuclei were counted in the GFP positive (green) cells. As shown in Fig. 1d right panel, $p27^{Kip1}$ was able to significantly ($p < 0.01$) decrease apoptosis. Note that the transfection itself (irrespective of control or $p27^{Kip1}$ constructs) resulted in substantial apoptosis when compared to treatment with DMSO (compare the nuclei in Fig. 1d with those in Fig. 1b). This accounts for the smaller increase in apoptotic nuclei in PTX treated cells, and may have decreased the preventive effect of $p27^{Kip1}$ in assays for apoptosis. $p16^{INK4A}$ also inhibited PTX induced DNA fragmentation, but the results were not consistent to achieve statistical significance. These data agree with the chemical inhibitor data and demonstrate that blockade of the G_1 to S phase transition, or S phase progress, is essential for the ability of PTX to induce apoptosis in MCF-7 cells. The data on chemical and biological inhibitions of the cell cycle clearly establish that PTX can induce apoptosis only if the cell can traverse G_1 and *S* phases of the cell cycle.

Chemical inhibitors of cell cycle transit did not inhibit effects of PTX on MT morphology

The effect of PTX on MT morphology in intact cells has been described previously [5]. Since G_1 to S phase transit inhibitors profoundly inhibit PTX induced apoptosis, it was important to determine whether roscovitine, HU, or thymidine inhibited the interaction of PTX with MT in cells. Cells were grown on cover slips and treated with PTX with or without pretreatment with roscovitine, thymidine, or HU. Following fixation, cells were stained with anti α-tubulin antibody (materials and methods) and secondary antibody conjugated with Alex Fluor 350, and visualized by fluorescent microscopy. The photographs in Fig. 1e left panel show the control cells. PTX caused an aggregation of MT to "bundles" with strong localized staining (Fig. 1e right panels) as reported by other investigators [5]. None of the inhibitors of the cell cycle used above caused consistent changes in MT appearance (Fig. 1e left panels) when compared to control. In the presence of these inhibitors, PTX still caused the same "bundling" of MT (Fig. 1e right panels) as observed in cells treated with PTX alone. We concluded that the inability of PTX to induce apoptosis in the presence of cell cycle inhibitors does not result from a change in the interaction of PTX with MT.

Cell cycle inhibitor effects on Cdc2 activity in the presence of PTX

Previous work in our laboratory has demonstrated that inhibition of JNK in PTX treated ovarian cancer cells did not alter the accumulation of cells in G_2/M phase induced by PTX [16]. Since the accumulation of cells in G_2/M is associated with the activation of Cdk1 (Cdc2) and we demonstrated that cell cycle inhibitors can prevent PTX induced G_2/M block, we measured Cdc2 activity following treatment of cells with cell cycle inhibitors. Cdc2 activity was determined in cyclin B immunoprecipitates as described previously in proliferating cell populations [28]. As shown in Fig. 2a, PTX increased Cdc2 activity significantly following 24 h of treatment as expected from the accumulation of cells in G_2/M phases. Roscovitine did not affect Cdc2 activity by itself. However, roscovitine completely prevented the effect of PTX when cells were pretreated with it for 12 h. Similarly, when cells were pretreated with ICI for 24–48 h forcing a G_1 arrest, it completely abrogated the effect of PTX on Cdc2 (Fig. 2b). As expected, ICI was able to substantially inhibit the basal Cdc2 activity in proliferating MCF-7 cells. The quantity of Cdc2 protein was unchanged in these experiments. Taken

together, these results demonstrate that PTX does not directly activate Cdc2, and the activation is an indirect result of the accumulation of cells in G_2/M phases. In other experiments, we established that PTX does not change cyclin B or D1, p27 or Cdk2 protein levels (Fig. 3a, data not shown). Therefore, PTX does not appear to regulate these cell cycle related proteins, suggesting that PTX does not act directly on the cell cycle in MCF-7 or in ZR75 breast cancer cells (data not shown).

PTX induced apoptosis and Cdc2 activity

As shown in Fig. 2, PTX treatment consistently activated Cdc2 in cells, and the agents which blocked Cdc2 activation substantially, decreased PTX induced apoptosis. Some authors have suggested that the activation of Cdc2 may have a critical role in PTX induced apoptosis (see discussion); however, whether Cdc2 activity is required for PTX action and the role of Cdc2 in G_2/M associated apoptosis is controversial. To examine the role of Cdc2 in detail, we transfected cells with a dn construct of Cdc2 prior to PTX treatment for 18 h, and Cdc2 was assayed in cyclin B immunoprecipitates. In DMSO treated cells, there was a small amount of Cdc2 activity (Fig. 2c), which was unchanged by over expression of either wild type (wt) Cdc2 (data not shown) or by dnCdc2. However, PTX induced Cdc2 activity was drastically decreased by over expression of dnCdc2 (average 80% in three determinations) but not by wtCdc2 (data not shown). Next, we measured apoptosis in transfected cells by Hoechst staining of nuclei. Apoptosis was readily induced in MCF-7 cells in the absence of caspase-3 [32]. We counted over 1,000 cells/treatment, and PTX induced apoptosis was surprisingly unaffected by either wt or dnCdc2 expression constructs (Fig. 2d). The block in G2/M phases by PTX was not inhibited by dnCdc2 (Fig. 2e).

To further investigate the role of Cdc2, we used a commercially available siRNA to inhibit the production of Cdc2 protein selectively (the siRNA was expressed in > 60% cells as estimated by counting fluorescence emitted by the control oligonucleotide). In Fig. 3a, the specific Cdc2 siRNA, but not the control oligonucleotide, inhibited the expression of Cdc2 protein (data representative of three experiments). How-

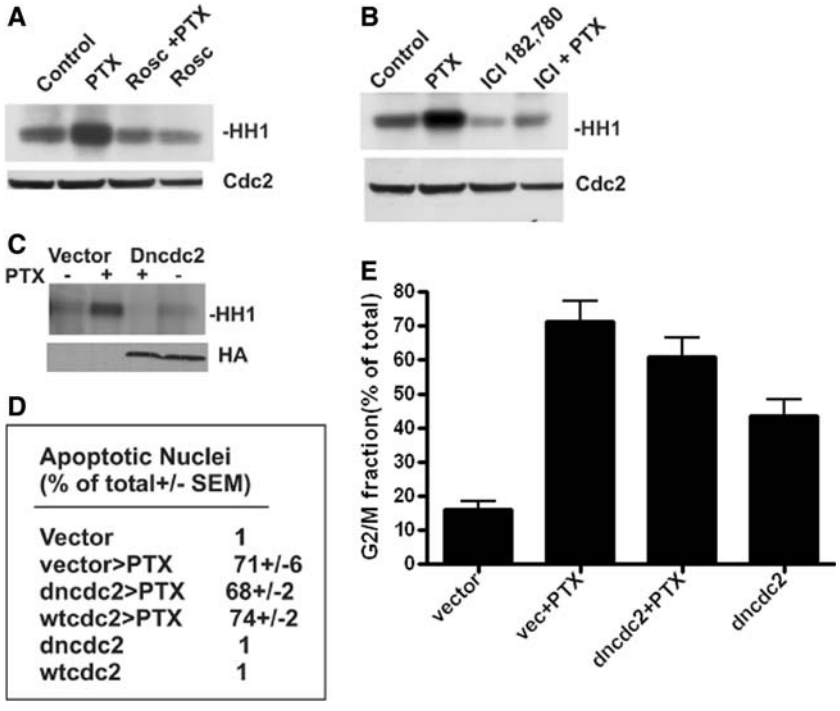


Fig. 2 Roscovitine and ICI 182,780 block PTX induced Cdc2 kinase activity and dnCdc2 do not inhibit PTX induced apoptosis. **a** MCF-7 cells were treated with 25 μM roscovitine for 12 h then 100 nM PTX was added for an additional 24 h. Cdc2 kinase activity was measured in cyclin B1 immunoprecipitates using histone H1 (HH1) as a substrate. **b** Cdc2 kinase activity was measured in MCF-7 cells treated with and without 20 nM ICI 182,780 for 48 h prior to 100 nM PTX addition for 24 h. Cdc2 in lysates were measured by western blot. **c** MCF-7 cells were transfected with HA tagged dnCdc2 or control expression constructs and incubated for 48 h prior to 100 nM PTX treatment for 24 h. Cdc2 *kinase assay* shows the effect of the over expression of dnCdc2 in cells treated with or without PTX. Expression of dnCdc2 is shown by a western blot for HA (**d**), or analyzed for the percentage of apoptotic nuclei by Hoechst staining. **e** Effect of dn Cdc2 on the G_2/M fraction

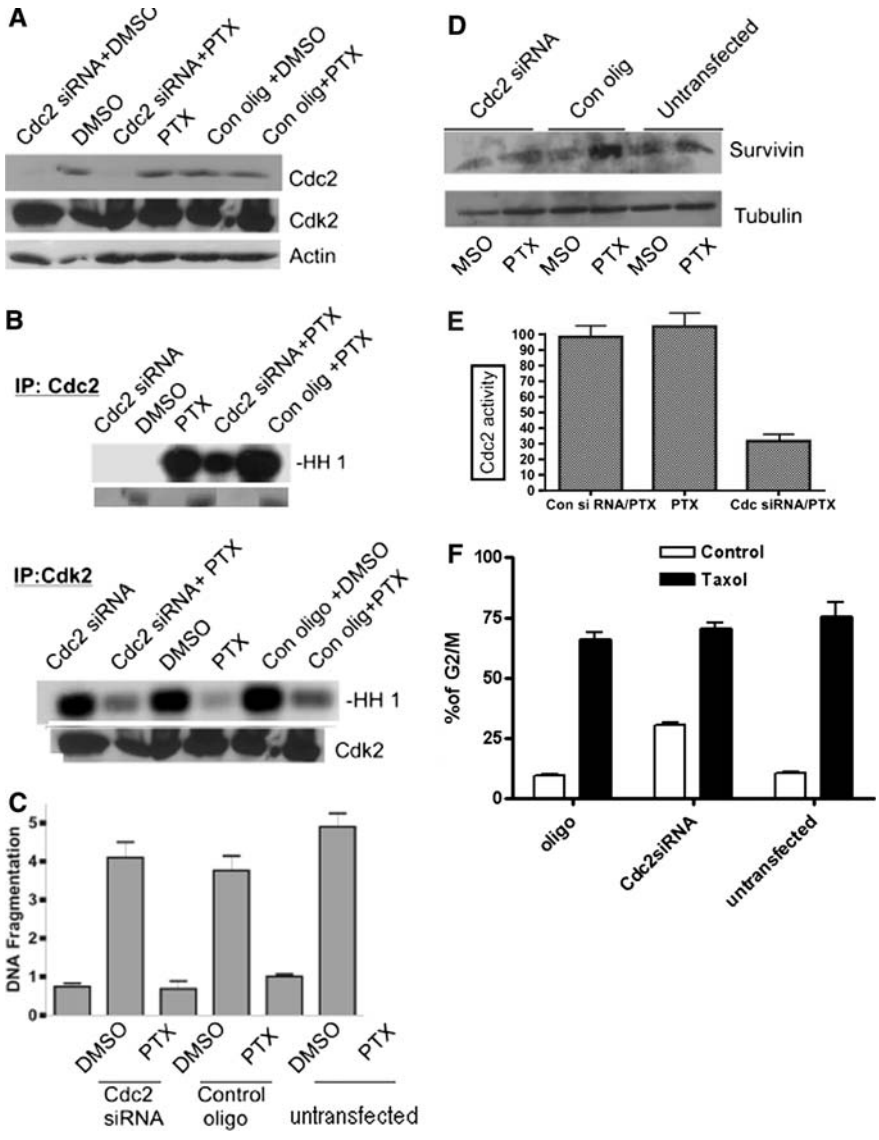


Fig. 3 Cdc2 siRNA decreases PTX effects on Cdc2 activity and survivin protein but not on apoptosis. **a** MCF-7 cells were transfected with Cdc2 siRNA or control oligonucleotide for 48 h and treated with PTX or DMSO for an additional 24 h. Western blot analyses for Cdc2, Cdk2 and actin. **b** In vitro kinase assay was performed with cyclin B or Cdk2 immunoprecipitates using histone H1 as a substrate. **c** Effects of Cdc2 siRNA on PTX induced apoptosis were measured by DNA fragmentation ELISA. **d** Cells, untransfected or transfected with Cdc2 siRNA, or control oligonucleotide were Western blotted for survivin and tubulin. **e** Cdc2 activity from three experiments as in (**b**) are expressed as mean ± SEM, with control siRNA treatment taken as 100%. **f** Effect of dnCdc2 siRNA on PTX induced accumulation of cells in G_2/M ($n = 3$) ($p < 0.01$ for control oligo versus Cdc2 siRNA)

ever, the siRNA had no effect on the closely related Cdk2 protein or actin expression. This data illustrates the specificity of Cdc2 siRNA. As expected, the Cdc2 siRNA inhibited PTX induced Cdc2 activity (Fig. 3f) by 60%, compared to the PTX treated untransfected cells. Also, the siRNA had little or no effect on Cdk2 activity with or without PTX (Fig. 3b lower). The increase in Cdk2 activity by PTX is presumably due to the accumulation of cells in G_2/M (we observed a approximate three fold increase in G_2/M fraction in Cdc2 si RNA treated cells under proliferating conditions, [Fig. 3e]). In Fig. 3c, we used similarly treated cells to assay for PTX induced apoptosis by DNA fragmentation. The Cdc2 siRNA did not decrease apoptosis induced by PTX.

In addition, survivin expression was assayed in cells treated with PTX and transfected with Cdc2 siRNA, control oligonucleotide, or in untransfected cells (Fig. 3d). Survivin protein is stabilized by active Cdc2. As shown in Fig. 3d, PTX increased survivin and this was decreased in cells transfected with Cdc2 siRNA. Given the essentially identical data from dnCdc2 as well as Cdc siRNA experiments, we conclude that Cdc2 activity is not required for PTX induced apoptosis.

Effect of chemical and biological cell cycle inhibitors on PTX induced JNK activity and BAD phosphorylation

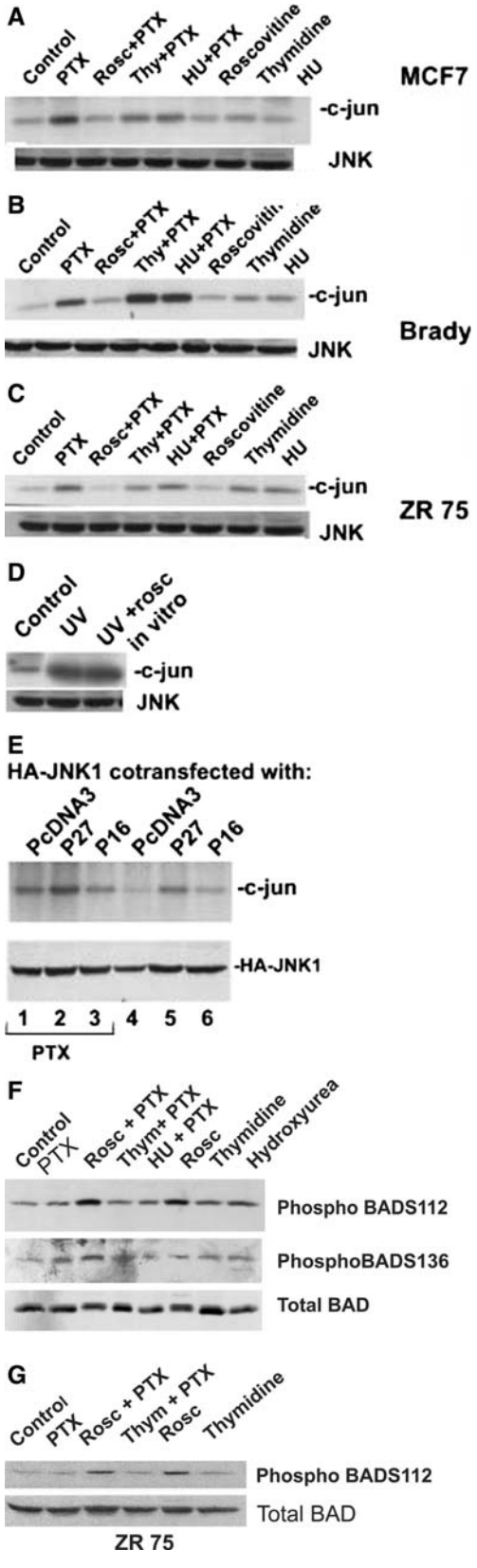
Previous work in our laboratory demonstrated that the Ras/Rac/MEKK/MEK/JNK pathway was activated by PTX, and that this pathway played an essential role only in the early phase of PTX induced apoptosis [33]. Therefore, we considered the possibility that the G_1/S

phase transit inhibitors described above might prevent the activation of JNK by PTX and block apoptosis. In order to test this hypothesis, proliferating cells were treated with PTX for four hours and JNK activation was assessed by kinase assay. We had previously demonstrated that JNK activation by PTX was optimal under these conditions [13]. To evaluate possible effects of chemical inhibitors, the cells were pretreated with roscovitine, double thymidine block, or HU. As shown in Fig. 4a–c, JNK activation by PTX was hardly affected by the double thymidine block or by HU treatment in two breast cancer lines (MCF-7 & ZR 75) and the ovarian cancer cell line (Brady). Neither of these agents modulates JNK activity by itself. While roscovitine did not change basal JNK activity, it was able to completely abrogate the effect of PTX. These results were consistently observed in three other experiments. None of these treatments altered the quantity of JNK1 protein in the cell lysates. To clarify the possibility that the effects of roscovitine are not due to carry over roscovitine inhibiting JNK in vitro, we added roscovitine directly into the UV induced cell lysates and assayed JNK. As seen in Fig. 4d, roscovitine had no effect on UV induced JNK activity when added in vitro, ruling out a direct inhibitory effect of roscovitine on JNK activity. Therefore, it appears that roscovitine was able to inhibit JNK activation by PTX through an unknown mechanism operative in intact cells. To establish further the inability of cell cycle inhibitors to prevent PTX induced JNK activity, we co-transfected HA-JNK1 with $p27^{Kip1}$ or $p16^{INK4}$ expression constructs into MCF-7 cells, and then assayed JNK1 activity in HA immunoprecipitates as previously reported [16]. In Fig. 4e (representative result from three experiments), neither $p16^{INK4}$ nor $p27^{Kip1}$ expression constructs prevented the activation of JNK1 by PTX, even though these inhibitors clearly arrested cells in G_1 (see above). Interestingly, we observed that over expression of $p27^{Kip1}$ appeared to activate JNK1, whereas $p16^{INK4}$ did not activate JNK1. $p27^{Kip1}$ may have an additive effect with PTX on JNK activity (Fig. 4e, compare lanes 2 and 5). Furthermore, neither of these constructs had any effect on HA- JNK1 expression. Taken together, the data from chemical as well as biological inhibitors demonstrate that activation of JNK by PTX is not affected by the inhibition of cell cycle transit. This suggests that the activation of JNK by PTX is unrelated to cell cycle transit and agrees with our previous observation on inhibition of the JNK pathway in PTX treated cells [16].

There is evidence that PTX alters BAD phosphorylation in some cell types [34–36]. Therefore, we determined whether PTX, in the presence or absence of cell cycle inhibitors, altered BAD phosphorylation in proliferating cells as described previously [32]. As shown in Fig. 4f, treatment with 100 nM PTX had no effect on serine 112 (S112) phosphorylation of BAD at 3 h. Treatment of cells with PTX (3 h) with or without HU pretreatment or double thymidine block did not alter the basal phosphorylation of BAD either on S112 or S136 residues. However, treatment with roscovitine (25 μM) for 18 h consistently increased S112 phosphorylation of BAD, but a more variable increase in phosphorylation at S136 of BAD was also observed. Essentially, identical results were obtained with ZR75 cells (Fig. 4g). The AKT and Erk/RSK pathways phosphorylate S136 and S112 residues of BAD, respectively; however, under our experimental conditions, we did not observe changes in the activation state of either AKT or Erk in MCF-7 cells by PTX or roscovitine treatments (data not shown), which confirms the results on BAD phosphorylation. Since these cells are proliferating, the basal activity of AKT and Erk is high, which may obscure effects of PTX or roscovitine on these two enzymes (as was reported previously by us for Erk activity in MCF-7 cells [13]). Therefore, we conclude that in these breast cancer cells, PTX does not regulate AKT or Erk activity or the phosphorylation status of BAD.

Discussion

As discussed in the introduction, MTAs may induce apoptosis in G_1 arrested cells and, therefore, be fundamentally different from DNA synthesis targeting agents in their mode of action. This possibility has not been critically explored in the literature and it is of importance to in vivo therapy, because most of the cells in a tumor are in G_1/G_0 phases of the cell cycle [1]. Therefore, we assessed the relationship between G_1/S phase transit and apoptosis induction by PTX. Our results demonstrate that PTX can induce apoptosis only when cells progress through the cell cycle and accumulate at G_2/M (Fig. 1). Some authors have suggested that PTX at low doses enforces a “mitotic arrest” [3, 7, 37–40]. By definition, a mitotic arrest is reversible as cells arrest to repair an abnormal mitosis, and there is evidence that cells treated with low doses of PTX proceed through M phase and accumulate in a G_1 state [41, 37]. At 100 nm of PTX, more than 80% of the MCF-7 cells are killed within 72 h (data not shown). Therefore, if there was a so called “mitotic arrest”, it is irreversible and precedes to inevitable apoptosis. It is possibly better to use the term mitotic block to describe the irreversible cell cycle block [7, 39]



observed in our studies. Whatever the exact term used, at 100 nM, PTX causes apoptosis in MCF-7 cells within 24–48 h. We have established apoptosis here, not only

Fig. 4 Effect of chemical and biological inhibitors on PTX induced JNK1 kinase activity and BAD phosphorylation. **a** MCF-7 cells were treated with 1 μM PTX for 4 h ± the indicated pretreatment: 25 μM roscovitine (rosc) for 12 h; thymidine block, or 1 mM HU for 24 h. JNK1 kinase activity was assayed using c-jun as a substrate. **b** and **c** Kinase assays using Brady cells and ZR75 cell lysates, respectively. **d** Stimulation of JNK by UV treatment. The third lane (UV + rosc in vitro) shows kinase activity of the same cell lysate as in the second lane (UV) with the addition of 25 μM roscovitine directly to the kinase reaction. **e** MCF-7 cells expressing both HA-JNK1 and $p27^{Kip1}$ or $p16^{INK4}$ constructs and pcDNA3, and treated with or without 1 μM PTX for 4 h beginning 48 h post-transfection. JNK1 kinase activity (*top*) was assayed in anti-HA immunoprecipitates. Western blot (*bottom*) of JNK1 levels is shown from the same immunoprecipitates. **f** and **g** Cultures were treated with 1 μM or 100 nM PTX for the indicated times and the lysates were Western blotted for phosphoBAD S112 or total BAD. Western blots in (**f**) show BAD phosphorylation (S112 and S136) and total BAD levels for cells treated with 100 nM PTX for 3 h alone or with the indicated pretreatment. **g** Similar experiment for ZR-75 cells. Representative result of three experiments

morphologically, but also by a quantitative method of measuring of DNA fragmentation. Similar results have been reported with 5-flurouracil, an S phase inhibitor [42]. Inhibition of G_1/S transit with biological reagents such as adenoviral $p27^{Kip1}$ markedly decreased apoptosis induction by PTX (Fig 1d) as well. Thus, by using well established biological inhibitors of Cdks, we were able to reproduce the results obtained with chemical inhibitors of the cell cycle. Therefore, the MTs that operate in the mitotic spindle and cytokinesis appear to be different from those that perform a variety of functions in G_0, G_1, and S phases of the cell cycle in their susceptibility to PTX.

Our results, importantly, indicate that microtubule disruption by itself is not an apoptotic stimulus. Instead, disruption of the mitotic spindle may lead to apoptosis resulting from the activation of the spindle assembly checkpoint [3]. Interestingly, a prolonged abnormal metaphase and associated increased Cdc2 activity which enforces the spindle assembly checkpoint has been shown to cause apoptosis [3]. The toxicity of PTX to rapidly dividing cells, therefore, is different from its toxicity to non-dividing cells, which can result in e.g. neurotoxicity, a well established toxicity of PTX. The JNK pathway, which is clearly a primary target of MTAs like PTX [13, 14], is activated in G_1 arrested breast and ovarian cancer cells (Fig. 4). These observations demonstrate that the prolonged activation of JNK by PTX [16] is not sufficient by itself to induce apoptosis in the absence of checkpoint activation. Inhibition of the G_1/S transition with chemical methods does not interfere with the PTX's ability to cause "bundling" of MTs (Fig. 1), reinforcing the notion that these agents do not modify PTX and MT interactions.

Our results strongly suggest that Cdc2 probably does not have a role in the apoptosis of MCF-7 cells induced by PTX. Cdc2 inhibition with two well established biological methods (dnCdc2 and Cdc2 siRNA) failed to block PTX induced apoptosis. The role of Cdc2 in apoptosis, in particular in the prolonged G_2/M arrest associated (due to PTX) apoptosis, remains controversial (reviewed in [43]). Some studies suggest that Cdks including Cdc2 are essential for apoptosis and that PTX may regulate Cdc2 and cyclin B protein [44–60]. However, our data do not support this hypothesis. Other authors have suggested that Cdk activation, in particular Cdc2, results in the inhibition of apoptosis [61–64]. Additionally, Cdk inhibitors such as $p57^{Kip2}$, $p27^{Kip\ 1}$, $p21^{Cip1}$, and $p15^{INK4}$ induce apoptosis [65–67], suggesting that Cdks prevent apoptosis.

One potential target for Cdc2 in apoptosis inhibition is survivin, an IAP protein family member [68–73]. Our data show an increase in survivin in PTX treated cells (Fig. 3) and a significant reduction of survivin when Cdc2 was chemically inhibited (data not shown). Also, Cdc2 siRNA completely inhibited the effect of PTX on survivin (Fig. 3). Altieri and co-workers have suggested that chemical inhibition of Cdc2 prevents survivin phosphorylation and its stabilization [69, 74]. Phosphorylated survivin is suggested to be essential for normal MT function at mitosis and destabilization of survivin could lead to apoptosis [74]. However, under conditions where PTX lead to apoptosis, it up regulated survivin (Fig. 3).

Despite numerous investigations, the role of JNK and p38 in PTX induced apoptosis is still uncertain [13, 14, 16, 75–80]. Cell cycle inhibition by HU, thymidine, $p27^{Kip1}$, or $p16^{INK4}$ did not influence the PTX induced JNK activity in three cell lines (Fig. 4). These results agree with our earlier work [13] that the effects of PTX on JNK are independent of G_1/S transit and G_2/M arrest; therefore, JNK activation and apoptosis in response to PTX are probably independent events. Previous studies by us [13] and others [14] show that PTX activates AP1 factor mediated transcription, which may result from nuclear JNK activation [16]. The function of AP1 in PTX induced apoptosis and its role in apoptosis following disruption of the mitotic spindle are questions that need to be answered. Interestingly, over expression of $p27^{Kip1}$ activated JNK irrespective of PTX treatment (Fig. 4e). Although the function of $p27^{Kip1}$ outside its traditional cell cycle role have been described [81, 82], we are not aware of any studies which examine a role for this Cdk inhibitor in relation to JNK activation, and this question needs to be addressed in the future [83].

Another pathway proposed to be modulated by PTX is the PI3K/AKT pathway [36, 84–89]. A proposed target of AKT relevant to apoptosis is the inhibitory phosphorylation of BAD at S136. Previously, we showed that BAD is important in the apoptosis in MCF-7 cells [32]. However, careful evaluation of BAD in asynchronously growing MCF-7 or ZR75 cells did not show any consistent changes in either BAD phosphorylation or levels following PTX treatment (Fig. 4f,g). Interestingly roscovotine increased BAD phosphorylation. Some reported changes in cell cycle regulators and signaling kinases could be an indirect result of cell cycle arrest and stress imposed by MT dysfunction, rather than a direct effect of PTX.

In conclusion, PTX is similar to other chemotherapeutic agents that require G_1/S phase transit to cause apoptosis. However, unlike S phase inhibitors, PTX inhibits spindle body formation and enforces the spindle assembly checkpoint in dividing cells. To induce apoptosis, a signal generated by the spindle checkpoint must act distal to the activation of JNK by PTX. Therefore, PTX is not toxic to tumor cells that are arrested in G_0/G_1 and is, thus, likely to leave the majority of cells in a tumor in a viable state since PTX is administered clinically in a treatment-recovery cycle [90]. Furthermore, after completion of treatment, these dormant cells may reestablish tumors. The toxicity that PTX causes in tumor cells (apoptosis) may be different from the PTX toxicity to differentiated G_0cells like neurons. This suggests that MTs in breast and ovarian cancer cells differ in sensitivity to PTX in G_0/G_1 phase compared to normal, differentiated G_0 phase cells.

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