A p34^{cdc2} survival checkpoint in cancer

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Summary

A checkpoint surveying the entry into mitosis responds to defects in spindle microtubule assembly/stability. This has been used to trigger apoptosis in cancer cells, but how the spindle checkpoint couples to the cell survival machinery has remained elusive. Here, we report that microtubule stabilization engenders a survival pathway that depends on elevated activity of p34^{cdc2} kinase and increased expression of the apoptosis inhibitor and mitotic regulator, survivin. Pharmacologic, genetic, or molecular ablation of p34^{cdc2} kinase after microtubule stabilization resulted in massive apoptosis independent of p53, suppression of tumor growth, and indefinite survival without toxicity in mice. By ablating this survival checkpoint, inhibitors of p34^{cdc2} kinase could safely improve the efficacy of microtubule-stabilizing agents used to treat common cancers.

Introduction

Checkpoints act as surveillance mechanisms to ensure proper timing of the cell division cycle (Sherr, 1996). At mitosis, the assembly of a bipolar spindle is vital to the preservation of genetic fidelity between daughter cells, and is monitored by a checkpoint (Rudner and Murray, 1996) that senses microtubule defects (Andersen, 2000) or aberrant kinetochore attachment (Nicklas, 1997). Activation of the spindle checkpoint by mitotic stresses causes a prolonged arrest of cell division that may eventually lead to apoptosis or programmed cell death (Hengartner, 2000). This strategy has been exploited for anticancer therapy, and agents that perturb microtubule dynamics or interfere with microtubule assembly (Sorger et al., 1997) have shown efficacy in the management of common human tumors (Rowinsky and Donehower, 1995).

Among the regulators of apoptosis that may affect the cell death/viability balance of dividing cells, interest has recently focused on the Inhibitor of Apoptosis (IAP) (Deveraux and Reed, 1999) protein and mitotic regulator, survivin (Altieri, 2001). Expressed during cell division in a cell cycle-dependent manner and localized to various components of the mitotic apparatus, survivin has been implicated in both regulation of spindle microtubule function and preservation of cell viability (Altieri, 2001; Reed and Bischoff, 2000). A critical requisite for survivin function was identified in the phosphorylation on Thr^{34} (O'Connor et al., 2000) by the main mitotic kinase, p34^{cdc2}-cyclin B1 (Pines, 1999). Accordingly, expression of nonphosphorylatable survivin Thr^{34}→Ala prevented phosphorylation of endogenous survivin, resulted in apoptosis of various cancer cell types (O'Connor et al., 2000), and suppressed tumor growth in vivo (Grossman et al., 2001b; Mesri et al., 2001), suggesting that this phosphorylation step may provide a suitable target for anticancer therapy. Although the role of p34^{cdc2}-cyclin B1 as a universal mitotic switch is well established (Pines, 1999), its potential contribution to cell death/survival during spindle checkpoint activation has remained controversial (Ongkeko et al., 1995; Shi et al., 1994).

Here we report that elevated p34^{cdc2} kinase activity during spindle checkpoint activation results in increased survivin expression and cancer cell viability. Conversely, sequential ablation of p34^{cdc2} kinase activity in mitotically arrested cells removed this survival mechanism, caused massive apoptosis, and dramatically enhanced the anticancer activity of a common microtubule poison, i.e., taxol, in vivo.

Results

Regulation of survivin expression during spindle checkpoint activation

Treatment of cervical carcinoma HeLa cells with increasing concentrations of microtubule modifiers taxol or vincristine resulted in dose-dependent and uniform cell cycle arrest with G2/M DNA content, as visualized by propidium iodide staining and flow cytometry (Figure 1A). Spindle checkpoint activation by microtubule poisons was also associated with a 2- to 5-fold increase

SIGNIFICANCE

Protein kinase inhibitors are currently explored as anticancer drugs for their ability to disrupt signaling pathways controlling cell proliferation and cell survival. However, concerns of specificity, influence of cellular context, and an incomplete understanding of molecular targets have limited the use of kinase antagonists in cancer therapy. Here, we show that suppression of p34^{cdc2} kinase in tumor cells treated with taxol eliminates a critical survival pathway. This results in escape from taxol-imposed mitotic block, massive apoptosis, and sustained inhibition of tumor growth in vivo. Taxanes are first-line treatment for common human tumors, and the sequential combination with an antagonist of p34^{cdc2} kinase may rationally enhance their therapeutic efficacy.

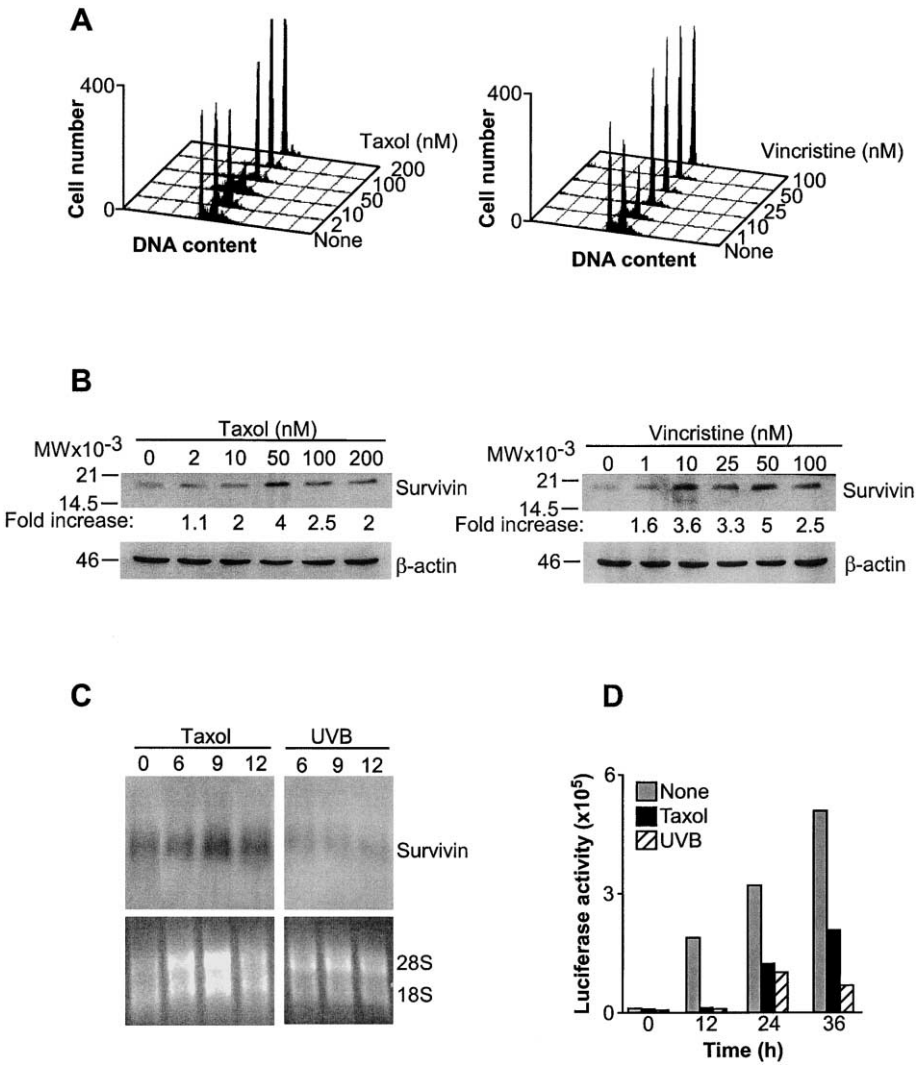

Figure 1. Effect of spindle checkpoint activation on survivin expression

A: Cell cycle analysis. HeLa cells were treated with the indicated increasing concentrations of taxol (left panel) or vincristine (right panel), harvested after 48 hr, and analyzed for DNA content by propidium iodide staining and flow cytometry.

B: HeLa cells treated with the indicated concentrations of taxol or vincristine were harvested after 48 hr incubation and analyzed for expression of survivin or β-actin by Western blotting. Numbers indicate fold increase by densitometry normalized to β-actin levels.

C: Northern hybridization. HeLa cells treated with 0.2 μM taxol or 50 J/m^2 UVB were harvested at the indicated time intervals (0–12 hr) and total RNA was hybridized with a α-^{32}P-dCTP-labeled survivin probe followed by autoradiography. The position of ribosomal RNA bands in ethidium bromide-stained gels is shown.

D: Promoter activity. HeLa cells transfected with a minimal survivin promoter construct (pLuc-1430) upstream of a luciferase reporter gene, were treated with vehicle (None), taxol (2 μM), or UVB (50 J/m^2) and harvested at the indicated time intervals, and luciferase activity was determined in a luminometer. Values were normalized to β-galactosidase expression.

in survivin expression in HeLa cells, as visualized by Western blotting (Figure 1B). To determine whether taxol increased survivin levels via de novo gene expression, Northern hybridization experiments were carried out. Treatment of HeLa cells with taxol did not result in significant changes in survivin RNA levels (Figure 1C). In contrast, DNA damage-induced G2/M arrest by exposure to 50 J/m^2 UVB was associated with loss of endogenous survivin RNA expression, as visualized by Northern blotting (Figure 1C). Next, we transfected HeLa cells with a minimal survivin promoter upstream of a luciferase reporter gene. In untreated cultures, there was a time-dependent increase in luciferase activity directed by the minimal survivin promoter, in agreement with previous observations (Li et al., 1998). In contrast, taxol or UVB treatments significantly inhibited survivin promoter activity at all time intervals tested (Figure 1D).

Effect of targeting p34^{cdc2} kinase activity on cell cycle progression and apoptosis

Mitotic arrest induced by spindle poisons is typically associated with elevated p34^{cdc2} kinase activity due to persistence of cyclin B1 (Hagtig et al., 1998). The possibility that phosphorylation on Thr^{34} by p34^{cdc2} (O'Connor et al., 2000) may contribute to increased survivin expression during spindle checkpoint activation was investigated. To target p34^{cdc2} kinase, we first used a pharmacologic approach with a panel of cyclin-dependent kinase (CDK) inhibitors (Sausville, 2002). Taxol treatment of HeLa cells resulted in mitotic arrest with elevated p34^{cdc2} kinase activity, which remained sustained for a 32 hr culture, as determined by MPM-2 phosphoepitope expression (Vandre and Borisy, 1989) (Figure 2A). Sequential addition of the 2,6,9 trisubstitute purine analog of the CDK ATP binding site, Purvalanol A (Purv.A) (Gray et al., 1998), to taxol-treated cells suppressed MPM-2 expression at the earliest time point tested (16 hr), and throughout a 32 hr culture (Figure 2A). In contrast, MPM-2 detection was partially inhibited by CDK inhibitors alsterpaullone, flavopiridol, or olomoucine by 16 hr, and completely suppressed by 32 hr (Figure 2A). When analyzed for DNA content, sequential addition of Purv.A or the other CDK inhibitors to taxol-treated HeLa cells resulted in escape from the mitotic block and prominent appearance of apoptosis (Figure 2B, see below). For high affinity and improved specificity for p34^{cdc2} as compared to other CDKs (Gray et al., 1998), we used Purv.A in subsequent experiments of pharmacologic inhibition of p34^{cdc2} kinase. To further investigate the ability of Purv.A to suppress p34^{cdc2} activity in

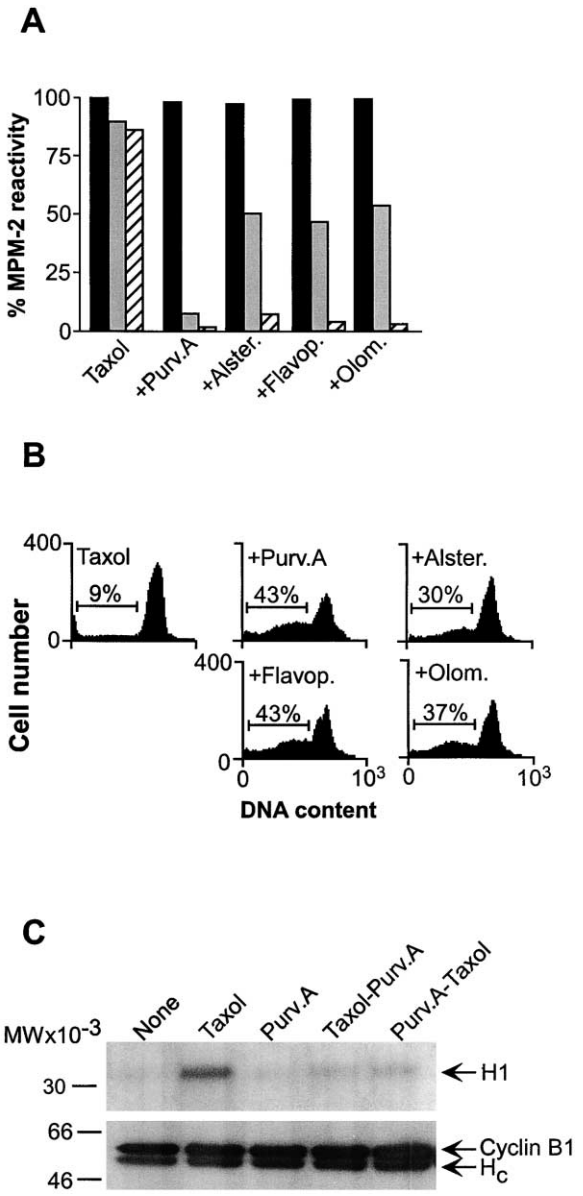

Figure 2. Effect of CDK inhibitors on cell cycle progression and kinase activity

A: MPM-2 mitotic phosphoepitope expression. HeLa cells were treated with taxol (0.2 μM for 16 hr, black bars) followed by CDK inhibitors Purvalanol A (Purv.A, 10 μM), alsterpaullone (Alster., 20 μM), flavopiridol (Flavop., 250 nM), or olomoucine (Olom., 400 μM) for additional 16 hr (gray bars) or 32 hr (striped bars). Cells were harvested and analyzed for MPM-2 mitotic phosphoepitope expression by flow cytometry. Data are expressed as % of MPM-2$^+$ cells in the entire cell population analyzed by propidium iodide staining.

B: DNA content analysis. HeLa cells were treated with 0.2 μM taxol for 16 hr followed by the various CDK inhibitors as described in **A**. Cells were harvested after an additional 16 hr culture and analyzed for DNA content by propidium iodide staining and flow cytometry. The percentage of hypodiploid cells with sub-G1 (apoptotic) DNA content is indicated.

C: Kinase assay. Cyclin B1 was immunoprecipitated from HeLa cells treated with vehicle (None), taxol (0.2 μM for 16 hr), Purv.A (10 μM for 16 hr), or the indicated combinations as described in **A**. The immune complexes were incubated with histone H1 in a kinase buffer containing γ-^{32}P ATP for 30 min at 30°C before analysis by autoradiography (top panel). Samples were analyzed by Western blotting with an antibody to cyclin B1 to confirm comparable immunoprecipitation of the CDK1 complex under the various conditions tested (bottom panel).

vivo, the p34^{cdc2}-cyclin B1 complex was immunoprecipitated from taxol-treated cultures in the presence or absence of Purv.A and analyzed for phosphorylation of histone H1 in a kinase assay. Taxol treatment resulted in increased H1 phosphorylation by p34^{cdc2}-cyclin B1, as compared with control cultures (Figure 2C). In contrast, sequential addition of Purv.A after taxol treatment suppressed p34^{cdc2} phosphorylation of H1 to background levels of untreated cells (Figure 2C).

Requirement of p34^{cdc2} phosphorylation for survivin stability

Next, we asked if inhibition of p34^{cdc2} kinase by Purv.A affected mitotic phosphorylation of survivin on Thr^{34}. Although high concentrations of Purv.A may conceivably affect Cdk2 kinase activity (Gray et al., 1998), this is inconsequential for survivin function, as recombinant Cdk-2-cyclin E did not phosphorylate survivin on Thr^{34} or other residues in a kinase assay, and did not associate with survivin, as visualized by coimmunoprecipitation (O'Connor et al., 2000). Sequential addition of Purv.A to taxol-treated HeLa cells suppressed survivin phosphorylation on Thr^{34} (O'Connor et al., 2000), as visualized by Western blotting of survivin immunoprecipitates with a Thr^{34}-phospho-specific antibody (Figure 3A). Loss of survivin phosphorylation on Thr^{34} was also associated with inhibition of increased survivin expression after taxol treatment, as visualized by Western blotting (Figure 3B). In contrast, Purv.A alone, or the reverse sequence of Purv.A followed by taxol, did not affect survivin levels in HeLa cells (Figure 3B). Because increase in survivin expression after spindle checkpoint activation did not involve changes in RNA levels or promoter activity, we asked whether Thr^{34} phosphorylation by p34^{cdc2} influenced survivin stability. The half-life ($t_{1/2}$) of endogenous survivin has been determined in recent studies (Zhao et al., 2000). To ascertain a role of Thr^{34} phosphorylation in this process, HeLa cells were transfected with HA-tagged wild-type survivin or nonphosphorylatable HA-survivin(T34A), metabolically labeled with ^{35}S-methionine and ^{35}S-cysteine for 5 hr, chased with complete medium, and sequentially immunoprecipitated with an antibody to HA. In these experiments, ^{35}S-labeled wild-type survivin was progressively reduced over time with an estimated $t_{1/2}$ of ~240 min (Figure 3C). In contrast, ^{35}S-labeled survivin(T34A) exhibited significantly accelerated clearance, with an apparent $t_{1/2}$ of ~90 min (Figure 3C).

We next used a genetic approach to probe the role of p34^{cdc2} kinase on survivin expression. For these experiments, we took advantage of HT2-19 cells carrying an inactivated allele of p34^{cdc2} and a second allele that is conditionally silenced upon removal of IPTG (Itzhaki et al., 1997). In the presence of IPTG (p34$^{cdc2\ +/-}$), HT2-19 cells expressed p34^{cdc2} and endogenous survivin, as visualized by Western blotting (Figure 3D). A 48 hr culture in the absence of IPTG (p34$^{cdc2\ -/-}$) resulted in nearly complete loss of both p34^{cdc2} and survivin expression (Figure 3D), consistent with the requirement of p34^{cdc2} kinase activity in survivin stability. Taxol treatment resulted in increased survivin levels in IPTG$^+$ HT2-19 cells, whereas only a minimally detectable survivin band was observed in the absence of IPTG (Figure 3D). This may reflect a residual population of mitotic cells exhibiting p34^{cdc2} kinase activity, as determined by the presence of detectable MPM-2-expressing HT2-19 cells after taxol treatment (Figure 3D).

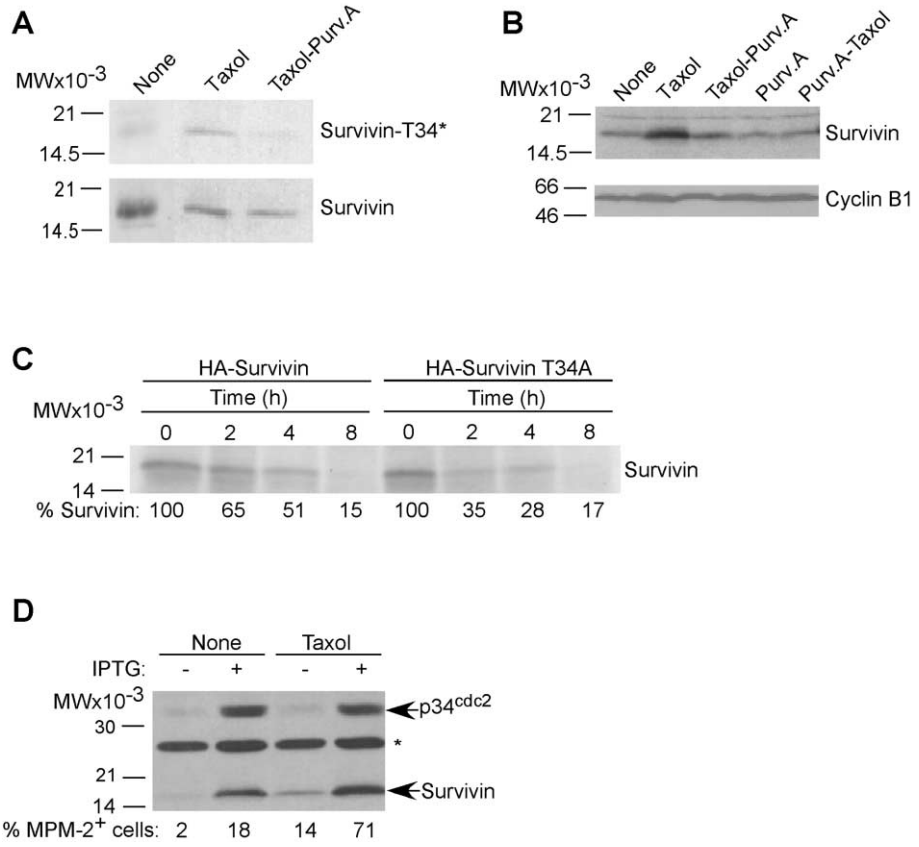

Figure 3. Requirement of Thr[34] phosphorylation by p34[cdc2] for survivin stability

A: Inhibition of survivin phosphorylation on Thr[34] by Purv.A. HeLa cells were incubated with vehicle (None), taxol (0.2 μM for 16 hr), or the sequential combination of taxol (0.2 μM for 16 hr) followed by Purv.A (20 μM for 16 hr). Cells were immunoprecipitated with an antibody to survivin and the immune complexes were analyzed by Western blotting with a T34-phospho-specific antibody (Survivin-T34*) or an antibody to survivin (Survivin).

B: Effect of Purv.A on survivin expression. HeLa cells were left untreated (None) or treated with taxol (0.2 μM), taxol (0.2 μM for 16 hr) followed by Purv.A (20 μM for 16 hr), or in reverse combination, and analyzed for expression of survivin or cyclin B1 by Western blotting.

C: Regulation of survivin turnover by Thr[34] phosphorylation. HeLa cells were transfected with HA-survivin or HA-survivin(T34A) by lipofectamine, metabolically labeled with 300 μCi/ml of ^{35}S-methionine/^{35}S-cysteine in methionine- and cysteine-free medium for 5 hr, chased with complete medium, and immunoprecipitated at the indicated time intervals with an antibody to HA. Bands were revealed by autoradiography and quantified by densitometry.

D: Conditional ablation of p34[cdc2] gene expression. HT2-19 cells in the presence (p34$^{cdc2\ +/-}$) or absence (p34$^{cdc2\ -/-}$) of IPTG for 24 hr were treated with vehicle or taxol (0.2 μM) for additional 24 hr, and analyzed for expression of p34[cdc2] or survivin by Western blotting. *, nonspecific band. The percentage of MPM-2$^+$ cells in the presence or absence of taxol is indicated.

Sequential ablation of p34[cdc2] kinase activity after spindle checkpoint activation induces massive apoptosis

Treatment of HeLa cells with 0.2 μM taxol resulted in homogeneous mitotic arrest, negligible apoptosis for up to 32 hr (see below), and maximal MPM-2 phosphoepitope expression (Figure 4A). Incubation with Purv.A alone also resulted in uniform mitotic arrest with loss of MPM-2 expression, and undetectable levels of apoptosis (Figure 4A). In contrast, sequential addition of Purv.A to taxol-arrested HeLa cells resulted in escape from the mitotic block and appearance of a large population with hypodiploid, i.e., apoptotic, DNA content, by propidium iodide staining and flow cytometry (Figure 4A). Purv.A induction of apoptosis after taxol treatment was concentration- and time-dependent. Maximal induction of cell death was observed after a 16 hr treatment of HeLa cells with taxol (0.2 μM) followed by an additional 16 hr exposure to 10–20 μM Purv.A (Figure 4B). At this time interval, ~80% of the HeLa cell population exhibited hypodiploid (apoptotic) DNA content, as opposed to 16% or 7% cell death in cultures treated with taxol or Purv.A alone, respectively (Figure 4A). Similar results were obtained with breast carcinoma MCF-7 cells carrying wild-type p53 (not shown). Induction of apoptosis by the taxol-Purv.A sequential combination was also strictly sequence-dependent and drug-specific. Treatment of HeLa cells with the reverse combination of Purv.A for 16 hr followed by taxol (0.2 μM) for an additional 16 hr resulted in a sustained mitotic arrest with undetectable MPM-2 expression (not shown), but negligible apoptosis throughout a 32 hr culture (Figure 4C). Similarly, substituting Purv.A with the DNA damaging agent, adriamycin (100 nM), or the nucleoside analog 5-fluorouracile (5-FU, 500 μM) after taxol treatment did not result in enhanced apoptosis, as compared with single agent treatment (Figure 4C, inset). Lastly, induction of apoptosis by taxol-Purv.A was observed in prostate carcinoma PC3 cells carrying mutated p53, whereas taxol or Purv.A alone, or the reverse combination of Purv.A-taxol, was ineffective (Figure 4D). Consistent with the role of p34[cdc2] phosphorylation in survivin stability, taxol treatment caused increased survivin expression in PC3 cells, in a reaction reversed by sequential addition of Purv.A, as visualized by Western blotting (Figure 4D, inset).

Requirement for p34[cdc2] kinase in survivin cytoprotection

We next used a molecular approach to target p34[cdc2] kinase activity during spindle checkpoint activation. For these experiments, we transfected HeLa cells with a kinase-dead p34[cdc2] (D146N) dominant negative mutant (van den Heuvel and Harlow, 1993), which was previously characterized for suppression of survivin phosphorylation on Thr[34], in vivo (O'Connor et al., 2000) Transfection of HeLa cells with p34[cdc2] (D146N) resulted in prominent expression of p34[cdc2]-reactive material, as visualized by Western blotting (Figure 7A, inset), with negligible apoptosis by DNA content analysis (Figure 5A), and in agreement with published data (van den Heuvel and Harlow, 1993). In contrast, expression of p34[cdc2] (D146N) in taxol-treated HeLa cells re-

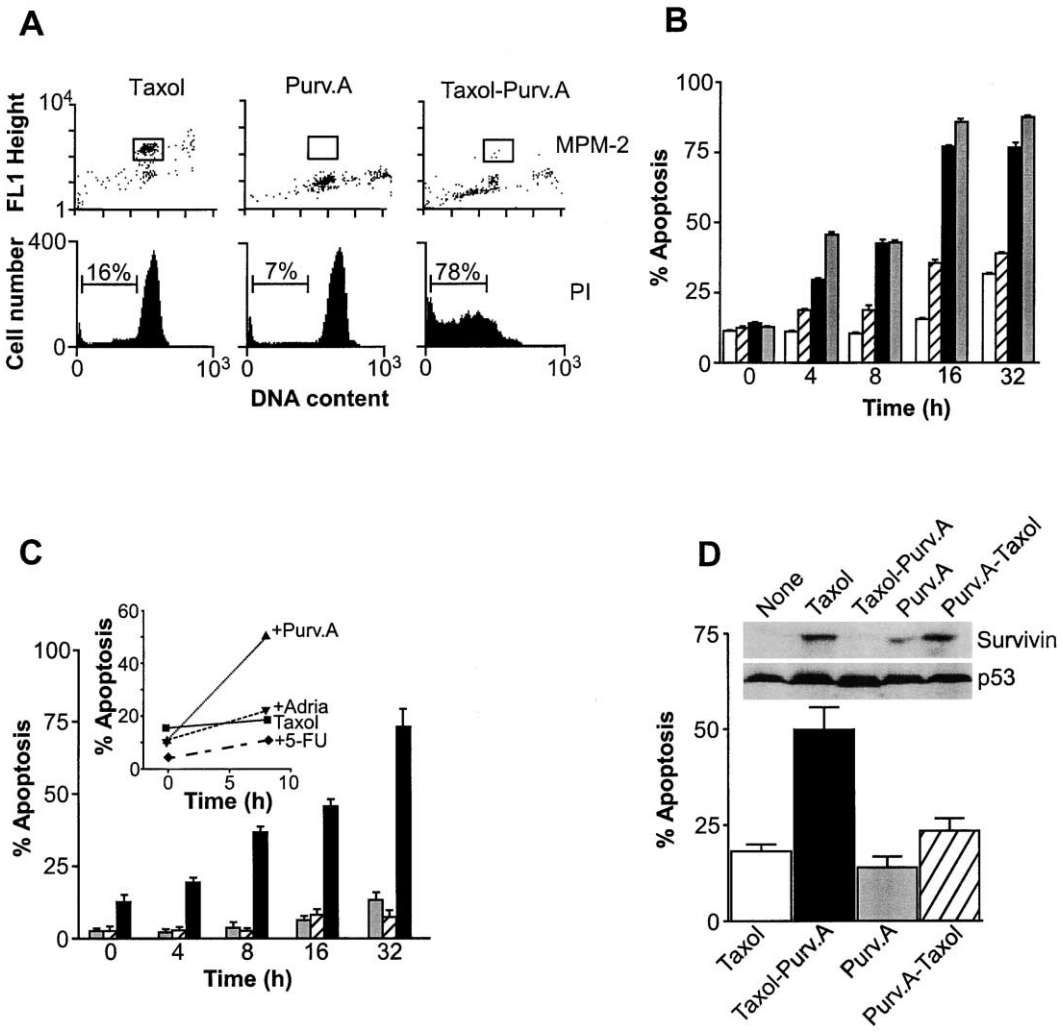

Figure 4. Induction of apoptosis by sequential ablation of $p34^{cdc2}$ kinase after spindle checkpoint activation

A: DNA content analysis and kinase activity. HeLa cells were treated with taxol (0.2 μM for 16 hr), Purv.A (10 μM for 16 hr), or the sequential combination of taxol (0.2 μM for 16 hr) followed by Purv.A (10 μM for additional 16 hr), and analyzed for MPM-2 expression (top panel, MPM-2) or DNA content by propidium iodide staining and flow cytometry (bottom panel, PI). A box contains the MPM-2^+ cell population under the various conditions tested. The percentage of hypodiploid cells with sub-G1 DNA content is indicated.

B: Time course of apoptosis and concentration dependence. HeLa cells incubated with 0.2 μM taxol for 16 hr (white bars) were sequentially treated with 1 μM (striped bars), 10 μM (black bars) or 20 μM (gray bars) Purv.A. Cells were harvested at the indicated time intervals and analyzed for hypodiploid (apoptotic) DNA content by propidium iodide staining and flow cytometry.

C: Sequence specificity. HeLa cells treated with Purv.A (20 μM, gray bars), Purv.A-taxol (0.2 μM) in reverse combination (striped bars), or taxol-Purv.A (black bars) were analyzed for induction of apoptosis as in **B**. **Inset:** Drug specificity. HeLa cells were treated with taxol (0.2 μM) or the sequential combination of taxol-Purv.A (+Purv.A, 10 μM), taxol-adriamycin (+Adria, 100 nM), or taxol-5-Fluorouracil (+5-FU, 500 μM) and analyzed for induction of apoptosis as in **B**.

D: p53 independence. Prostate carcinoma PC3 cells were treated with the indicated combinations, harvested after 16 hr, and analyzed for induction of apoptosis as in **B**. **Inset:** Modulation of survivin expression. PC3 cells were left untreated (None) or incubated with the various indicated combinations, and analyzed for expression of survivin or p53 by Western blotting.

sulted in massive induction of apoptosis as compared with control cultures transfected with pcDNA3 vector (Figure 5A).

Finally, we investigated a potential modulation of taxol-induced apoptosis in $p34^{cdc2}$ conditional knockout cells. In the presence of a functional $p34^{cdc2}$ allele ($p34^{cdc2\ +/-}$), HT2-19 cells exhibited negligible apoptosis, and responded to taxol with homogeneous mitotic arrest (Figure 5B). Upon IPTG withdrawal, $p34^{cdc2\ -/-}$ HT2-19 cells also exhibited mitotic arrest and a modest increase in the fraction with hypodiploid (apoptotic) DNA content, in agreement with published observations (Itzhaki et al., 1997). Under these experimental conditions, and in agreement with the data of pharmacologic (Purv.A) or molecular ($p34^{cdc2}$ DN expression) interference with $p34^{cdc2}$ activity, taxol treatment of $IPTG^-$ HT2-19 cells ($p34^{cdc2\ -/-}$) resulted in escape from the mitotic block, and significantly increased apoptosis, as compared with untreated $IPTG^-$ cultures (Figure 5B). Upon prolonged exposure to taxol-Purv.A (72–96 hr), a fraction of HT2-19 cells with 8N and 16N DNA content was also observed (not shown), suggestive of DNA endoreduplication and polyploidy (Itzhaki et al., 1997). To determine if expression of survivin

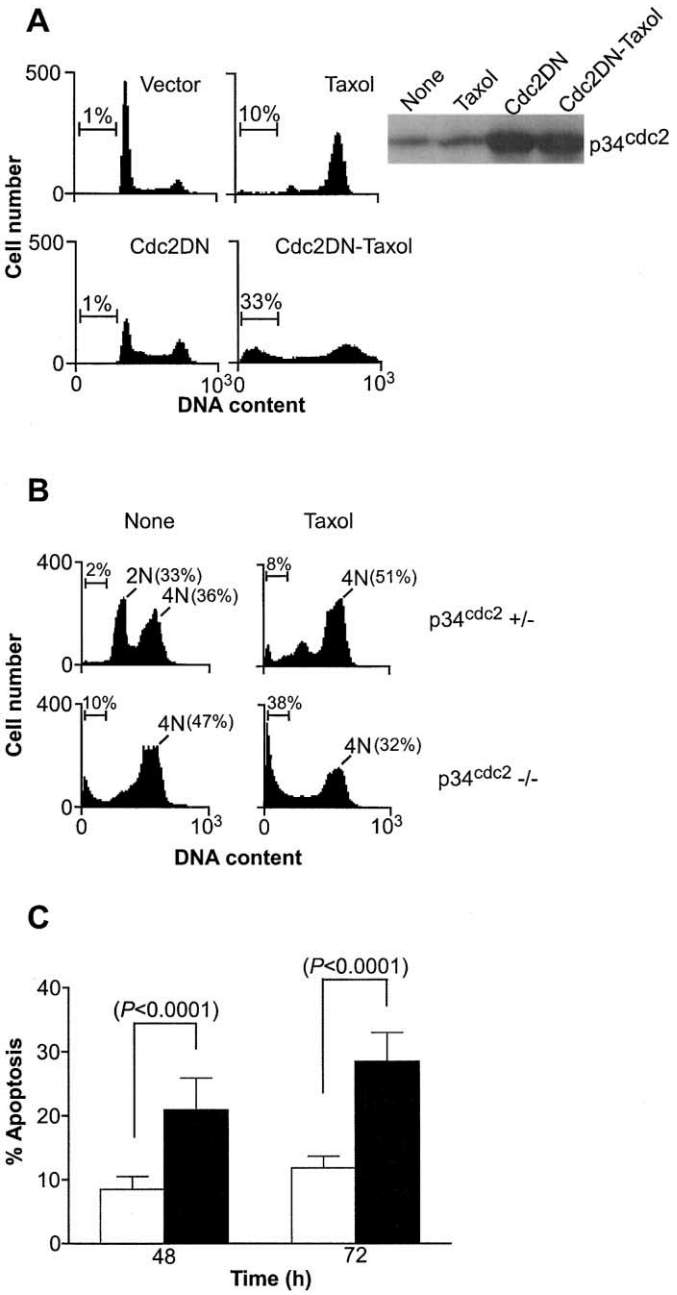

Figure 5. Requirement of p34^{cdc2} for metaphase viability checkpoint

A: Molecular targeting of p34^{cdc2} kinase. HeLa cells were transfected with pcDNA3 (vector) or kinase-dead p34^{cdc2} (D146N) dominant negative mutant (Cdc2DN) in the presence or absence of taxol (0.2 μM), and analyzed for DNA content by propidium iodide staining and flow cytometry. The percentage of cells with hypodiploid (apoptotic) DNA content is indicated per each condition tested. **Inset:** Expression of p34^{cdc2}. Control HeLa cells or cultures transfected with kinase-dead dominant negative p34^{cdc2} (Cdc2DN) in the presence or absence of taxol were analyzed with an antibody to p34^{cdc2} by Western blotting.

B: Genetic ablation of p34^{cdc2}. HT2-19 cells in the presence (p34$^{cdc2\ +/-}$) or absence (p34$^{cdc2\ -/-}$) of IPTG were simultaneously treated with vehicle or taxol (0.2 μM for 24 hr) and analyzed for induction of apoptosis by propidium iodide staining and flow cytometry. The percentage of cells with hypodiploid (apoptotic), 2N, or 4N DNA content is indicated.

C: Expression of survivin reverses apoptosis induced by ablation of p34^{cdc2}. HT2-19 cells in the absence of IPTG (p34$^{cdc2\ -/-}$) were infected with pAd-GFP (black bars) or pAd-Survivin (white bars) and analyzed for induction of apoptosis at the indicated time intervals by propidium iodide staining and flow cytometry. The percentage of apoptosis in IPTG$^-$ HT2-19 cells in the absence of viral transduction was 17.4% (48 hr) and 24% (72 hr). For both panels, data are representative of two independent determinations.

was sufficient to rescue p34$^{cdc2\ -/-}$ cells from apoptosis, we used replication-deficient adenoviruses encoding wild-type survivin (pAd-Survivin) or a reporter marker (pAd-GFP) characterized in previous studies (Mesri et al., 2001). Infection of HT2-19 cells with pAd-Survivin or pAd-GFP resulted in comparable levels of GFP expression in >95% of the transduced cell population, as visualized by fluorescence microscopy (not shown). Consistent with the data presented above, loss of p34^{cdc2} resulted in time-dependent induction of apoptosis in IPTG$^-$ HT2-19 cells (Itzhaki et al., 1997), which was not affected by infection with pAd-GFP (Figure 5C). In contrast, adenoviral expression of survivin suppressed apoptosis in p34$^{cdc2\ -/-}$ cells to background levels of untreated, IPTG$^+$ cultures (Figure 5C).

Taxol-Purv.A treatment activates mitochondrial-dependent apoptosis

To identify the apoptotic pathway induced by loss of survivin phosphorylation on Thr^{34} during spindle checkpoint activation, we first looked at the profile of caspase activation under these experimental conditions. Exposure of HeLa cells to taxol-Purv.A sequential treatment was associated with proteolytic processing of the ~46 kDa mitochondrial initiator caspase-9 (Wang, 2001) to a ~37 kDa active fragment (Figure 6A). In contrast, treatment with Purv.A or taxol alone did not result in significant caspase-9 cleavage (Figure 6A). Next, we used cell lines genetically deficient in selected apoptosome proteins. Treatment of wild-type mouse embryonic fibroblasts (MEFs) expressing endogenous survivin (not shown) with the sequential combination of taxol-Purv.A resulted in induction of apoptosis, whereas single agent treatment was ineffective (Figure 6B). In striking contrast, apoptosis induced by taxol-Purv.A was completely ablated in MEFs deficient in caspase-9 (Hakem et al., 1998) or Apaf-1 (Yoshida et al., 1998) (Figure 6B), the critical components of the apoptosome in mitochondrial-mediated apoptosis (Wang, 2001).

p34^{cdc2} cytoprotection by survivin requires a stabilized microtubule environment

To determine a potential role of microtubule integrity in p34^{cdc2}/survivin cytoprotection, we used the microtubule-depolymerizing agent, vincristine. Treatment of HeLa cells with vincristine resulted in a sustained mitotic (prometaphase) arrest with elevated p34^{cdc2} kinase activity (Figure 7A), and increased survivin expression, as visualized by Western blotting (Figure 1B). Sequential addition of Purv.A to vincristine-treated cells suppressed p34^{cdc2} kinase activity at the earliest time point tested of 8 hr, by loss of MPM-2 mitotic phosphoepitope expression (Figure 7A). Similar results were obtained in a kinase assay, in which p34^{cdc2} immunoprecipitated from vincristine-treated cells phosphorylated histone H1 in a reaction entirely suppressed by treatment with Purv.A in vivo (Figure 7B). However, at striking variance with taxol, sequential addition of Purv.A to vincristine-treated cells did not result in induction of apoptosis, which remained unchanged at background levels throughout a 32 hr culture (Figure 7C), irrespective of loss of survivin expression,

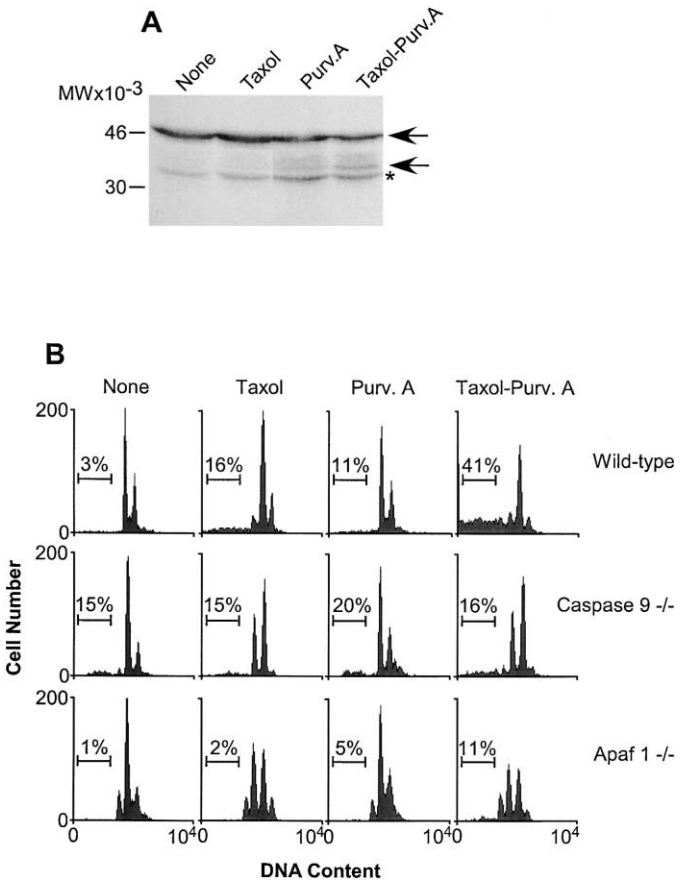

Figure 6. Suppression of p34^{cdc2} kinase after spindle checkpoint activation induces mitochondrial-dependent apoptosis

A: Caspase-9 cleavage. HeLa cells were left untreated (None) or treated with taxol (0.2 μM), Purv.A (20 μM), or the sequential combination taxol-Purv.A for 16 hr, and analyzed for caspase-9 cleavage by Western blotting. Arrows indicate the position of ~46 kDa proform and ~37 kDa active form of caspase-9, respectively. *, nonspecific band.

B: Analysis of apoptosome-deficient MEFs. Wild-type MEFs or MEFs isolated from caspase-9 or Apaf-1 knockout mice were incubated with vehicle (none), taxol (0.2 μM for 16 hr), Purv.A (20 μM for 16 hr), or taxol-Purv.A in sequential combination. Cells were harvested and analyzed for DNA content by propidium iodide staining and flow cytometry. The percentage of hypodiploid cells is indicated. Data are from a representative experiment of at least two independent determinations.

as visualized by Western blotting (Figure 7C, inset). After a prolonged 96 hr culture, vincristine induced a comparable level of apoptosis in HeLa cells with or without Purv.A (not shown).

Sequential taxol-Purv.A treatment as a novel anticancer regimen

To investigate the potential efficacy of taxol-purv.A sequential treatment in vivo, we used a breast cancer xenograft model extensively characterized in recent studies of survivin targeting (Mesri et al., 2001). Subcutaneous injection of MCF-7 cells in immunocompromised CB-17 SCID mice gave rise to exponentially growing tumors (Figure 8A), in agreement with previous observations (Mesri et al., 2001). Treatment with vehicle, taxol alone (2.5 or 5 mg/kg), or Purv.A alone (20 mg/kg) did not affect the kinetics of tumor growth, and resulted in loss of all animals by 21–23 days (Figures 8A–8C). In contrast, sequential administration of taxol followed by Purv.A suppressed tumor growth in a concentration-dependent manner (Figure 8A). Consistent with the data of sequence specificity in vitro (Figure 4C), the reverse combination of Purv.A followed by taxol did not reduce tumor growth, and actually enhanced tumor expansion (not shown). In survival curves, the taxol-Purv.A sequential treatment resulted in significantly improved animal survival even after suspension of treatment, as compared with single-agent therapy alone (Figure 8B; $p < 0.0008$ for 5 mg/kg taxol). In addition, the continued administration of taxol-Purv.A sequential treatment resulted in indefinite survival of all animals (Figure 8B). To determine if tumors retained sensitivity to the taxol-Purv.A sequential regimen, animals were given one taxol-Purv.A cycle, followed by a 6 day interruption before readministration of treatment. In these animals, MCF-7 tumors doubled in size during the suspension of treatment (Figure 8C). However, reintroduction of taxol-Purv.A sequential therapy suppressed additional tumor growth and afforded long-term survival of all treated animals (Figure 8C). None of the animals in the various treatment groups exhibited signs of systemic toxicity throughout the different treatment cycles.

Discussion

In this study, we have shown that survival signals emanating from the mitotic apparatus can preserve the viability of cancer cells in response to microtubule poisons (Sorger et al., 1997). Critical requirements of this pathway included elevated levels of p34^{cdc2} kinase activity, which in turn promoted increased expression of the apoptosis inhibitor and mitotic regulator, survivin (Altieri, 2001), and the presence of stabilized microtubules (Yvon et al., 1999). Conversely, pharmacologic, genetic or molecular ablation of p34^{cdc2} kinase in mitotically arrested cells resulted in escape from the mitotic block, loss of survivin expression, massive activation of mitochondrial-mediated apoptosis independent of p53, and strong antitumor efficacy without toxicity, in vivo.

A potential role of CDK activity, and p34^{cdc2} in particular, in apoptosis has been extensively debated. Although unscheduled CDK activity has been associated with cell death (Shi et al., 1994; Wang and Walsh, 1996) and caspase activation (Harvey et al., 2000), the significance of elevated CDK activity during spindle checkpoint activation, and its potential participation in apoptosis, has remained uncertain. In fact, recent genetic evidence suggests that endogenous inhibitors of p34^{cdc2} kinase may trigger apoptosis and function as tumor suppressors. Accordingly, disruption of the human homolog of the *Drosophila lats* gene resulted in deregulated p34^{cdc2} kinase activity, increased cell proliferation, and tumor formation (Tao et al., 1999). In addition, loss of heterozygosity of the human *LATS1* locus has been demonstrated in human tumors (Theile et al., 1996), and forced expression of human *LATS1* suppressed p34^{cdc2} kinase activity and caused mitotic arrest and apoptosis (Xia et al., 2002). A role of p34^{cdc2} kinase activity in preserving cell viability was also suggested by conditional knockout studies, in which deletion of both p34^{cdc2} alleles caused apoptosis and DNA endoreduplication (Itzhaki et al., 1997).

In agreement with earlier predictions (Ongkeko et al., 1995), the data presented here are consistent with a model of p34^{cdc2}-dependent cytoprotection at mitosis using survivin (Altieri, 2001) as one of the critical downstream effector genes. This fits well

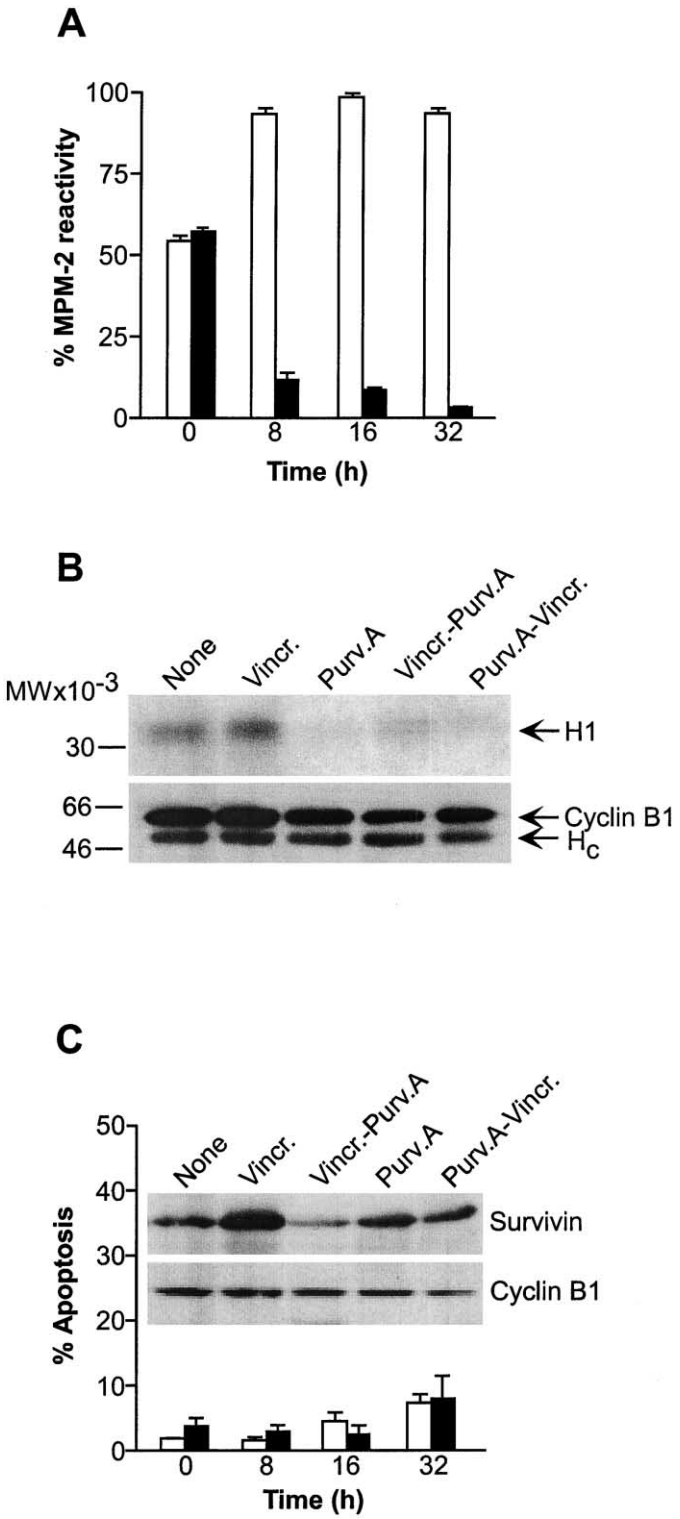

Figure 7. Requirement of stabilized microtubules for metaphase viability checkpoint

A: MPM-2 activity. HeLa cells were treated with vincristine (100 nM for 16 hr, white bars) or vincristine followed by Purv.A (20 μM, black bars), and analyzed for MPM-2 mitotic phosphoepitope expression at the indicated time intervals.

B: Kinase assay. HeLa cells treated with vincristine, Purv.A, or the indicated combinations as described in **A** were immunoprecipitated with an antibody to cyclin B1 and the immune complexes were mixed with histone H1 and γ-^{32}P-ATP followed by autoradiography (top panel). Samples were analyzed by Western blotting with an antibody to cyclin B1 to confirm equal immunoprecipitation of the CDK1 complex under the various conditions tested (bottom panel).

C: Determination of apoptosis. HeLa cells were treated with vincristine (100 nM for 16 hr, white bars) or vincristine followed by Purv.A (20 μM, white bars), and analyzed for DNA content at the indicated time intervals. **Inset:** modulation of survivin expression. HeLa cells left untreated (None) or treated with the various indicated combinations were analyzed for expression of survivin or cyclin B1 by Western blotting.

with the cell cycle-dependent expression of survivin at mitosis (Li et al., 1998), its physical association with p34^{cdc2}, and the phosphorylation on Thr^{34} by p34^{cdc2}-cyclin B1 as a requirement for survivin cytoprotection (O'Connor et al., 2000). A preferential survivin-cyclin B1 relationship was independently recognized in recent gene profiling studies of large cell non-Hodgkin's lymphoma, and correlated with a more aggressive disease phenotype (Kuttler et al., 2002). As shown here, one of the mechanisms by which p34^{cdc2} phosphorylation may regulate survivin is by promoting its increased stability at mitosis. This may cooperate with the cell cycle periodicity of *survivin* gene transcription at G2/M (Li et al., 1998) to achieve maximal levels of survivin expression at cell division. Phosphorylation of cell death regulators has been previously implicated in modulation of protein stability, and phosphorylation of Bcl-2 on MAP kinase sites Thr^{56}, Thr^{74}, and Ser^{87} (Breitschopf et al., 2000), or p53 on Ser^{15} and Ser^{20} (Hirao et al., 2000), counteracts ubiquitin-dependent protein degradation. Proteasome-dependent destruction plays a critical role in regulating the expression of IAP family proteins (Yang et al., 2000), including survivin (Zhao et al., 2000), and may contribute, at least under certain circumstances, to their cytoprotective function(s) (Huang et al., 2000). In this context, interference with survivin phosphorylation on Thr^{34} by dominant negative mutant (O'Connor et al., 2000), or by targeting p34^{cdc2} (this study), is expected to destabilize survivin levels at mitosis favoring accelerated clearance and induction of apoptosis.

Consistent with this model, loss of survivin expression by antisense targeting has been invariably associated with spontaneous apoptosis (Li et al., 1999), enhancement of chemotherapy-induced cell death (Olie et al., 2000), and antitumor activity in vivo (Kanwar et al., 2001). The mechanism(s) by which elevated levels of survivin are required to preserve an antiapoptotic threshold at mitosis has been debated (Altieri, 2001), and, unlike other IAP proteins (Deveraux and Reed, 1999), may not involve direct inhibition of caspase-3 activity (Verdecia et al., 2000). Here, apoptosis induced by destabilization of survivin in taxol-Purv.A-treated cells resulted in caspase-9 cleavage, and was abolished in cells genetically deficient in caspase-9 (Hakem et al., 1998) or Apaf-1 (Yoshida et al., 1998), the critical components of the apoptosome in mitochondrial-mediated cell death (Wang, 2001). These data are in agreement with recent observations that dominant negative interference with survivin phosphorylation on Thr^{34} resulted in cytochrome c release and caspase-9 activation (Mesri et al., 2001), and transgenic animals expressing survivin were protected against UVB- (mitochondrial), but not Fas (death receptor)-induced apoptosis (Grossman et al., 2001a). The ability of survivin to selectively regulate mitochondrial apoptosis may also explain the lack of evolutionary conservation of this cytoprotective pathway in *C. elegans*, where the mitochondria does not participate in cell death (Wang,

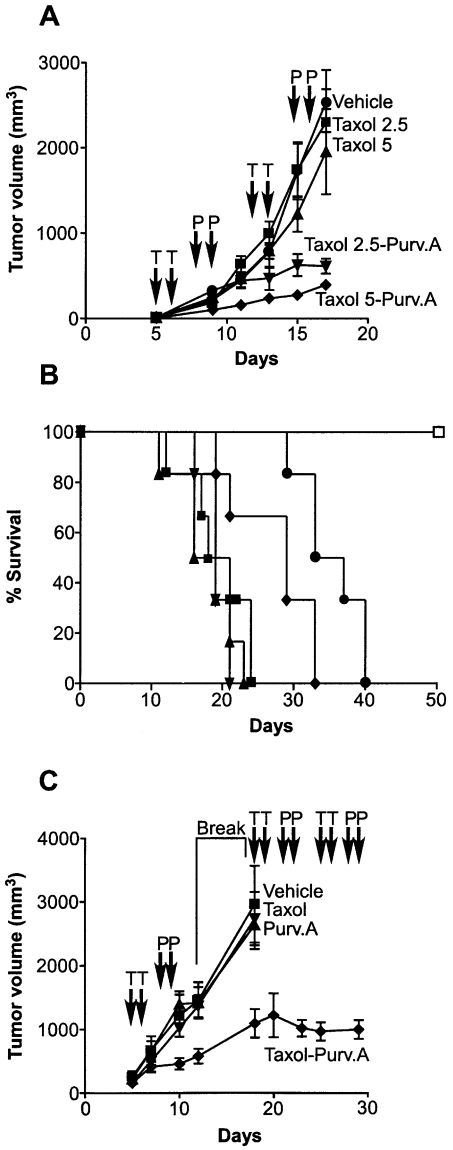

Figure 8. Timed ablation of the metaphase viability checkpoint as a novel antitumor treatment in vivo

A: MCF-7 cells (2.5×10^6) were injected subcutaneously in the flank of immunocompromised CB17 SCID mice, and grown as superficial tumors for 5 d (~50–75 mm^3) before initiation of treatment. Animals (6/group) were injected i.p. with vehicle, taxol alone (T, 2.5 or 5 mg/kg), or the sequential combination of taxol-Purv.A at the indicated intervals (arrows), followed by a day with no treatment. For single agent treatment, vehicle was given in place of taxol or Purv.A with the same schedule. Each cycle was separated by 2 days without treatment. Tumor volume was measured in three dimensions with a caliper.

B: Survival. The experimental conditions are as in **A**. Treatment was terminated on day 16 for all groups except for group □, where sequential administration of taxol-Purv.A was continued as in **A**. For all groups, tumor size was monitored at the indicated time intervals, and animals with tumor burden >3000 mm^3 were sacrificed (Survival). ■, vehicle; ▲, taxol alone (2.5 mg/kg); ▼, taxol alone (5 mg/kg); ◆, taxol (2.5 mg/kg)-Purv.A; ●, taxol (5 mg/kg)-Purv.A; □, taxol (2.5 mg/kg)-Purv.A continuous treatment.

C: Retained sensitivity to treatment. The experimental conditions are as in **A**. In the Purv.A alone group, animals were injected i.p. with 20 mg/kg Purv.A with the indicated schedule. Sequential treatment with taxol (5 mg/kg) followed by Purv.A (20 mg/kg) was suspended for the indicated time interval (Break, 6 days) and readministered on day18 with the same schedule as in **A**. All animals treated with vehicle, taxol alone (5 mg/kg), or Purv.A alone (20 mg/kg) reached tumor burdens >3000 mm^3 on day 20 and were sacrificed.

2001), and a potential survivin homolog, BIR 1, acts exclusively as a mitotic regulator (Speliotes et al., 2000).

An important requirement of $p34^{cdc2}$/survivin cytoprotection was the integrity of microtubules, thus indicating that the cellular response to spindle checkpoint activation may vary depending on the status of microtubule dynamics (Sorger et al., 1997). The fact that loss of survivin resulted in apoptosis only under conditions of microtubule stabilization may reflect a tightly timed window of survivin cytoprotection at cell division, which may coincide with the assembly of a bipolar metaphase spindle. In this context, survivin cytoprotection has been shown to require polymerized microtubules (Li et al., 1998), and overexpression of survivin blocked taxol-induced, but not vincristine-induced, cell death (Li et al., 1998). This suggests a role of a specialized "microtubule environment" in $p34^{cdc2}$/survivin cytoprotection, potentially recruiting other cell death effectors/regulators, i.e., caspase-9 (O'Connor et al., 2000). A microtubule environment required for survivin cytoprotection is consistent with the predominant localization of survivin to mitotic spindle microtubules (Fortugno et al., 2002), anticipated previously (Li et al., 1998), and its role in microtubule stability (Giodini et al., 2002; Tran et al., 2002) and spindle checkpoint function (Kallio et al., 2001).

When tested in a xenograft breast cancer model in mice, sequential ablation of $p34^{cdc2}$ kinase activity after microtubule stabilization converted a low-dose, noneffective taxol regimen into a highly curative treatment that suppressed tumor growth and afforded indefinite survival of all treated animals. Kinase inhibitors have recently emerged as promising anticancer agents (Griffin, 2001), and a flavone CDK inhibitor, flavopiridol, is currently explored as an anticancer agent (Sausville, 2002). The concept of sequential therapy provides a rational approach to improve the efficacy of anticancer regimens, and, consistent with the data presented here, sequential administration of flavopiridol following taxol treatment enhanced apoptosis in vitro (Motwani et al., 1999), and is being considered as a therapeutic regimen for cancer trials (Schwartz et al., 2002). However, flavopiridol functions as a broad inhibitor of various CDKs as well as non-cell cycle regulated kinases, and its anticancer and proapoptotic properties may be unrelated to $p34^{cdc2}$ inhibition and reflect global suppression of gene transcription by targeting the CDK9/cyclin T1 complex (Sausville, 2002). This is consistent with the ability of flavopiridol and the other CDK inhibitors tested here to induce apoptosis even in the absence of other chemotherapeutic treatments (our unpublished data). In contrast, when used alone, the 2,6,9 trisubstitute purine analog Purv.A, previously characterized as a more selective inhibitor of $p34^{cdc2}$ (Gray et al., 1998), caused a sustained mitotic block without accompanied apoptosis. This may contribute to a high degree of specificity of the taxol-Purv.A sequential treatment in vivo, and the latter may explain the lack of systemic toxicity of this regimen in mice. As shown here, the proapoptotic efficacy of taxol-Purv.A was strictly sequence- and drug-dependent, and was not observed by substituting Purv.A with a DNA damaging agent (adriamycin) or a nucleoside analog (5-FU). Importantly, the reverse combination of Purv.A followed by taxol not only failed to promote apoptosis, but also reduced the modest induction of cell death mediated by low-dose taxol, and paradoxically enhanced tumor growth in vivo. This is reminiscent of previous observations in which inhibition of $p34^{cdc2}$ kinase by olomoucine (Ye et al., 2001), or via by upregulation of $p21^{Cip1/Waf1}$ (Yu et al., 1998) actually attenuated apoptosis induced by microtubule

poisons. One potential explanation that may reconcile previous discrepancies as to the requirement of $p34^{cdc2}$ in cell death is that premature suppression of $p34^{cdc2}$ kinase may arrest mitotic progression before cells require the enhanced antiapoptotic environment afforded by $p34^{cdc2}$/survivin.

In summary, these data suggest that survival signals engendered by $p34^{cdc2}$ activity, increased survivin levels, and a stabilized microtubule environment may preserve the viability of cancer cells during a protracted mitotic arrest. Consistent with the exquisite sensitivity of cancer cells to manipulation of the survivin pathway (Altieri, 2001), timed and sequence-specific ablation of $p34^{cdc2}$ kinase after taxol treatment may destabilize survivin levels, remove this survival checkpoint, and promote strong anticancer activity in vitro and in vivo. This may provide a rational approach to lower the therapeutic concentrations of taxane-based chemotherapy (Rowinsky and Donehower, 1995) and improve the treatment of common human cancers.

Experimental procedures

Cell cultures and antibodies

Breast carcinoma MCF-7, prostate carcinoma PC3, and cervical carcinoma HeLa cells (American Type Culture Collection, Manassas, VA) were maintained in culture according to the supplier's recommendations. HT2-19 cells with conditional inactivation of the $p34^{cdc2}$ gene were described previously (Itzhaki et al., 1997). In this cell line, removal of IPTG from the culture medium results in inactivation of the second $p34^{cdc2}$ allele (Itzhaki et al., 1997). Transformed mouse embryonic fibroblasts (MEFs) isolated from caspase-9 (Hakem et al., 1998) or Apaf-1 (Yoshida et al., 1998) -deficient mice were described previously. A replication-deficient adenovirus encoding wild-type survivin (pAd-Survivin) or control GFP (pAd-GFP) was generated using the pAd-Easy system, as described previously (Mesri et al., 2001), and propagated in HEK293 cells with purification by CsCl banding. With this protocol, no replication-competent adenovirus particles are generated (Mesri et al., 2001). For viral transduction, HT2-19 cells in the absence of IPTG were infected with pAd-GFP or pAd-Survivin at multiplicity of infection (m.o.i.) of 50, and analyzed after a 48–72 hr culture for changes in cell cycle progression and apoptosis. Antibodies to $p34^{cdc2}$ or cyclin B1 were obtained from Pharmingen (San Diego, CA) or Santa Cruz (Santa Cruz, CA), respectively, and used in previous experiments (O'Connor et al., 2000). Antibodies to β-actin or caspase-9 were obtained from Sigma or Transduction Laboratories (Beverly, MA), respectively. A rabbit polyclonal antibody to survivin was from NOVUS Biologicals (Littleton, CO), and was characterized in recent studies (Fortugno et al., 2002). An affinity-purified antibody to Thr^{34}-phosphorylated survivin (α-survivinT34*) was characterized previously (O'Connor et al., 2000).

Modulation of survivin expression during checkpoint activation

For time-course experiments of spindle checkpoint activation, HeLa cells were treated with increasing concentrations of the microtubule-stabilizing agent taxol (2–200 nM, Sigma Chemical Co., St. Louis, MO), or the microtubule-depolymerizing drug, vincristine (1–100 nM, Sigma) for 48 hr at 37°C. Cells were harvested and analyzed for DNA content by propidium iodide staining and flow cytometry or modulation of survivin expression by Western blotting, as described (O'Connor et al., 2000). For Northern hybridization experiments, HeLa cells were exposed to 0.2 μM taxol or 50 J/m^2 UVB and harvested at increasing time intervals after treatment (0–12 hr), and total RNA was hybridized with a α-^{32}P-dCTP-labeled survivin probe with detection of radioactive bands by autoradiography, as described (Li et al., 1998). In some experiments, HeLa cells were transfected by lipofectamine with a minimal survivin promoter construct upstream of a luciferase reporter gene (pLuc-cyc1.2), as described (Li et al., 1998). Cells were treated with taxol (2 μM) or UVB (50 J/m^2), and analyzed for changes in luciferase activity at increasing time intervals (12–36 hr) in a luminometer. Luciferase values were normalized to β-galactosidase expression. For analysis of survivin stability, HeLa cells were transfected by lipofectamine with HA-tagged wild-type survivin or nonphosphorylatable survivin(T34A), as described previously (O'Connor et al., 2000). For pulse-chase experiments, cells were metabolically labeled with 300 μCi/ml of ^{35}S-methionine/^{35}S-cysteine (Amersham Biosciences) in methionine- and cysteine-free medium for 5 hr at 37°C, chased in complete medium, and immunoprecipitated with an antibody to HA at increasing time intervals (0–8 hr) after labeling. Radioactive bands were detected by autoradiography and quantified by densitometry.

Pharmacologic, genetic, and molecular targeting of $p34^{cdc2}$ kinase activity

Pharmacologic suppression of $p34^{cdc2}$ kinase was carried out with inhibitors of cyclin-dependent kinases (CDKs). In a first series of experiments, HeLa cells were incubated with taxol (0.2 μM) or vincristine (100 nM) for 16 hr at 37°C, followed by addition of Purvalanol A (Purv.A, 1–10 μM), alsterpaullone (20 μM), flavopiridol (250 nM), or olomoucine (400 mM) for increasing time intervals (4–32 hr) at 37°C. At the end of each time interval, cells were harvested and analyzed for $p34^{cdc2}$ kinase activity and DNA content analysis (see below). In all other experiments, HeLa, PC3, or MCF-7 cells or wild-type MEFs or MEFs isolated from caspase-9- or Apaf-1-deficient mice were incubated with 0.2 μM taxol for 16 hr and sequentially treated with 20 μM Purv.A for an additional 16 hr before analysis of $p34^{cdc2}$ kinase activity or DNA content (see below). For sequence- and treatment-specificity, HeLa cells were incubated with Purv.A (10–20 μM) for 16 hr followed by taxol (0.2 μM) for 16 hr, or treated with taxol (0.2 μM for 16 hr) followed by the DNA-damaging agent adriamycin (100 nM, Sigma) or the nucleoside analog 5-fluorouracil (500 μM, Sigma) for 8 hr at 37°C before DNA content analysis. To determine the effect of pharmacologic inhibition of $p34^{cdc2}$ kinase activity on survivin phosphorylation on Thr^{34}, endogenous survivin was immunoprecipitated from HeLa cells treated with taxol or the sequential combination of taxol-Purv.A, as described (O'Connor et al., 2000). The immune complexes were separated by SDS gel electrophoresis and analyzed by Western blotting with an antibody to survivin or an affinity-purified Thr^{34}-phospho-specific antibody (α-survivinT34*) described previously (O'Connor et al., 2000). For genetic ablation of $p34^{cdc2}$, HT2-19 cells were washed and cultivated in the absence of IPTG for 48–72 hr before DNA content analysis (see below) or expression of survivin or $p34^{cdc2}$ by Western blotting. In some experiments, taxol (0.2 μM) was added to HT2-19 cells simultaneously with the withdrawal of IPTG, and cells were processed for DNA content analysis or MPM-2 mitotic phosphoepitope expression after a 48 hr culture at 37°C. For molecular targeting of $p34^{cdc2}$, HeLa cells were transfected with control plasmid pcDNA3 or a kinase-dead $p34^{cdc2}$ (D146N) dominant negative mutant (van den Heuvel and Harlow, 1993) by lipofectamine. Transfected cells were simultaneously treated with taxol (0.2 μM) so that maximal expression of $p34^{cdc2}$ DN (24 hr) would occur in the mitotically arrested cell population following spindle checkpoint activation. Cells under the various conditions tested were analyzed for protein expression with an antibody to $p34^{cdc2}$ by Western blotting, or for DNA content by propidium iodide staining and flow cytometry.

Determination of $p34^{cdc2}$ kinase activity

For detection of mitotic proteins expressing the MPM-2 phosphoepitope (Vandre and Borisy, 1989), HeLa cells (1–2 × 10^5 cells/60 mm dish) were treated with taxol (0.2 μM) or vincristine (100 nM) for increasing time intervals (8–36 hr) at 37°C. Cells were fixed in 70% ethanol and labeled with MPM-2 antibody (6 μg/ml; Upstate Biotechnology, Lake Placid, NY) followed by addition of goat anti-mouse FITC (Boehringer Mannheim) for 1 hr at 22°C in the presence of 5 μg/ml propidium iodide containing 50 μg/ml RNase A. Samples were analyzed on a FACScan (Becton Dickinson, Mountain View, CA), using CellQuest software. Data were expressed as % MPM-2 positive cells in the entire population. For kinase assays, the $p34^{cdc2}$-cyclin B1 complex was immunoprecipitated with an antibody to cyclin B1 (6.6 μg/ml) from taxol- or vincristine-treated HeLa cells in the presence or absence of Purv.A. The immune complexes were mixed with histone H1 as a substrate (1 μg) in kinase buffer containing 20 μCi γ-^{32}P-ATP for 30 min at 30°C, as described (O'Connor et al., 2000), with detection of radioactive bands by autoradiography. Membranes were analyzed for comparable immunoprecipitation of $p34^{cdc2}$-cyclin B1 by Western blotting with an antibody to cyclin B1.

Determination of apoptosis

Changes in apoptosis in cultures treated with the microtubule poisons or CDK inhibitors, alone or in sequential combination, were monitored by DNA content analysis by propidium iodide staining and flow cytometry, as described (Li et al., 1999). In other experiments, HeLa cells were detergent-

solubilized and analyzed for proteolytic processing of ~46 kDa proform caspase-9, by Western blotting (O'Connor et al., 2000).

Breast cancer xenograft model

All experiments involving animals were approved by the institutional animal care and use committee. Six- to eight-week-old female CB17 SCID/beige mice (Taconic Farms, Germantown, NY) were injected s.c. into the flanks with 2.5×10^6 exponentially growing MCF-7 cells in 250 μl of sterile PBS (pH 7.4). Tumor growth was confined to local masses and did not affect animal survival over a 4 month observation period. Tumors became palpable (~75 mm^3) within 5 days of tumor cell injection, after which groups of six animals were randomized and assigned to different treatment groups. Animals were injected i.p. with taxol alone (2.5 or 5 mg/kg), Purv.A alone (20 mg/kg), or the sequential combination of taxol-Purv.A for two consecutive days each divided by a day with no treatment. For single agent treatment, vehicle was given in place of taxol or Purv.A with the same schedule. Each complete cycle was separated by 2 days without treatment. Tumor volume was measured in the three dimensions with a caliper. Animals with tumor burden >3000 mm^3 were sacrificed (Survival).

Statistical analysis

All in vitro experiments were repeated at least three times. For in vivo studies, each X value (time) shows the fraction of animals still alive calculated using the product limit or Kaplan-Meier method. The survival curves were compared using the log-rank test. This test generates a p value testing the null hypothesis that the survival curves are identical in the overall populations.

Acknowledgments

We thank Drs. Tak W. Mak for generously providing the caspase-9- and Apaf-1-deficient MEFs, Alan Sartorelli for advice, Nathanael Gray and Peter Schultz for discussion regarding Purvalanol, and Thomas Manes for help with HT2-19 cells. This work was supported by NIH grants CA78810, CA90917, and HL54131.

Received: March 15, 2002
Revised: June 11, 2002

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