Activation of p34\textsuperscript{cdc2} Coincident with Taxol-induced Apoptosis

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Abstract
Toxicity elicited by the antitumor compound taxol has been linked to irreversible tubulin polymerization, cell cycle block at mitosis, and cell death from apoptosis. We have used pulsed drug exposure of synchronized populations to identify two points, one in transition from G\textsubscript{0} to G\textsubscript{1} and the other at G\textsubscript{2}/M of cell cycle, that are most sensitive to taxol-induced cell killing. By analyzing these lesions separately, we have differentiated events related to mitotic block from those that may contribute to apoptosis. The taxol lesion forms rapidly and stably in transition or mitotic cells, because secondary washes to remove residual drug will decrease cytotoxicity except for cells in these populations. Both G\textsubscript{2}/M cells and G\textsubscript{0}/G\textsubscript{1} transition cells synchronously initiated apoptotic DNA fragmentation within 20 h of pulsed taxol treatment, indicating that a sustained mitotic block is not requisite to initiate cell death. Apoptosis was inhibited by cyclohexamide and by 2-aminopurine and sodium orthovanadate; thus, cell cycle progression appeared requisite for cell death. Taxol treatment of G\textsubscript{0}/G\textsubscript{1} or G\textsubscript{2}/M cells clearly leads to a block of mitosis followed by a perturbation of tyrosine phosphoprotein regulation; however, protein tyrosine phosphorylation correlated with mitotic block rather than time after drug exposure. Conversely, p34\textsuperscript{cdc2} kinase activation does not occur at mitotic block but rather 20 h after drug exposure and coincident with DNA fragmentation. Taken together, these results suggest that mitotic block may not be a sufficient signal for taxol-induced apoptosis and that the taxol lesion initiates apoptosis via a phosphoregulation pathway possibly involving the p34\textsuperscript{cdc2} kinase.

Introduction
The antimicrotubule agent taxol has shown efficacy in the treatment of ovarian and metastatic breast cancers (1) and malignant melanoma (2). Particularly encouraging is its utility in advanced ovarian cancers that are refractory to other forms of chemotherapy (1, 3). Taxol stabilizes microtubule formation (4), and continuous treatment prevents completion of mitosis, resulting in cell cycle blockage in G\textsubscript{2}/M (5). Other effects of taxol are not readily explained by mitotic block, and in addition to forming multiple mitotic asters in G\textsubscript{2}/M (6), taxol may also impact the nonmitotic stages of the cell cycle, preventing quiescent cells from reentering the cell cycle (7) and promoting tubulin bundling throughout the cell cycle. Interestingly, tubulin bundling throughout the cell cycle rather than mitotic astor formation appears to correlate with tumor cell toxicity (6). In the macrophage, taxol activates microtubule-associated protein kinase (8), increases tumor necrosis factor production, and down-regulates levels of tumor necrosis factor receptor (9). Increased protein phosphorylation and up-regulation of lipopolysaccharide-inducible genes has also been reported (10). However, these observations do not account for the preferential effect of the drug on transformed cells as compared to normal proliferating cells.

As with the majority of anticancer agents, cell death from taxol appears to result from apoptosis (11), an active cell process characterized by changes in cell morphology and cytoskeleton, chromatin condensation, blebbing, and activation of endogenous nucleases that results in degradation of nuclear DNA (reviewed in Refs. 12 and 13). Kung et al. (14) have proposed that defects in feedback control and cell cycle checkpoints may confer the differential sensitivity of transformed cells to anticancer agents (14). DNA damage of normal cells results in arrest at cell cycle checkpoints to facilitate repair, but arrest may not occur in transformed cells. The tumor suppressor gene p53, in concert with the cyclin-dependent kinases, is implicated in these checkpoint arrests (15), but a role in growth arrest following tubulin damage has not been reported. Conversely, inappropriate activation of p34\textsuperscript{cdc2} kinase has been implicated in the apoptosis of lymphoma cells after fragmentin-2 treatment; and the DNA fragmentation induced by this treatment was reduced in cells bearing a temperature-sensitive mutation in p34\textsuperscript{cdc2} (16). Lack of feedback control in transformed cells and subsequent lack of cell cycle arrest after damage may uncouple cell cycle regulators from cell division, and this may serve as a signal for active cell death (reviewed in Refs. 17 and 18). This effect is well described in DNA damage, but the correlation has not been demonstrated for agents that perturb microtubule structure.

To examine the role of cell cycle progression in taxol-induced cell death, we have used brief drug exposure to define two points of high taxol sensitivity, one in transition to proliferation (G\textsubscript{0} to G\textsubscript{1}) and the other in cells in mitosis, and have separately examined processes that ensue after taxol treatment of each population. Taxol-induced lesions occurring at either G\textsubscript{2}/M or at G\textsubscript{0}/G\textsubscript{1} lead to synchronous DNA fragmentation within 20 h of drug exposure, suggesting that a fixed duration of mitotic block is not related to induction of cell death. Since cell death is reduced by inhibition of protein synthesis and by inhibitors of both protein kinase and phosphatase, we examined the continuation of cell cycle-regulated processes after drug treatment. Taxol treatment gives rise to changes in protein tyrosine phosphorylation that correlate with entry into a mitotic block but not with time of drug exposure. Conversely, p34\textsuperscript{cdc2} kinase activation does not correlate with
mitosis block but rather with time of drug exposure and onset of DNA fragmentation.

Results
Maximum Sensitivity Occurs during G0 to G1 Transition and during Mitosis: Transition from Quiescence to Proliferation. To clearly define points of taxol sensitivity as cells begin to proliferate and progress through the cell cycle and to establish appropriate drug levels for cytotoxicity experiments, we first examined the taxol sensitivities of cells in transition from quiescence (G0) to the proliferative state. Populations of quiescent IMR90 cells, responsive to contact inhibition and serum deprivation (19), were exposed to a titration of taxol for a 1-h period at progressive times after stimulation to proliferate. An aliquot of control cells not treated with taxol was removed for determination of DNA synthesis and cell cycle position at each time point. Equal numbers of cells from each time point were exposed to serial dilutions of taxol for 1 h; the drug was removed, and cells were washed three times with complete media, followed by reculture in fresh complete media. At 72 h post drug exposure, the percentage of viable cells remaining was determined by XTT2 reduction assay (20). IC50S were determined from the dose-response curve (average of triplicate experiments) for each population and are shown together with a profile of control cell DNA synthesis in Fig. 1A. Compared to resting cells, taxol sensitivity transiently increased at 8 h after restimulation, was minimal at onset of DNA synthesis (t = 16 h), and increased again as cells progressed through the cell cycle (Fig. 1A). Maximum sensitivity occurred at 42 h post stimulation, with the highest percentage of cells in mitosis. These changes were highly reproducible in multiple experiments; however, synchrony of cells diminished after onset of S, as judged by FACS (data not shown) and the decline in control DNA synthesis.

Toxicity through the Cell Cycle. To confirm increased sensitivity in G0-G1 transition and assess sensitivity at progressive stages of the cell division cycle, taxol toxicity was examined in synchronous HeLa S3 cell populations synchronized by release from a double dThD block (21). DNA synthesis in non-taxol treated cells, determined by [3H]dThD incorporation (Fig. 1, O), and flow cytometry were monitored in parallel with taxol treatment of samples taken every 2 h for a complete cell division cycle, S through subsequent S. Flow cytometry for DNA content of control populations shown in Fig. 1, B and C, is shown in the left panel of Fig. 2, (control). Cells released from dThD block rapidly reenter the cell cycle; DNA synthesis began immediately after release and for the second cell division cycle at 16 h after release. Cells were >95% G2 at 8 h post release. Cell division occurred at ~10 h post dThD release, and cells were 94% G1 by 12 h (by Beckman-Dickinson CELL-FIT program analysis).

Cells treated with taxol at translation (t = 0 h) are more sensitive (IC50 20 nm) than cells at the onset of DNA synthesis in the next cell cycle (IC50 32 nm at t = 14 h). Maximum sensitivity (IC50 16 nm) occurs at t = 8 h (mito-

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2 The abbreviations used are: XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfopheno-yl)-5-[phenylamino] carbonyl)-2H-tetrazolium hydroxide; IC50 and IC25, 50 and 25% inhibitory concentrations, respectively; dThD, thymidine; FACS, fluorescence-activated cell sorting; CHX, cycloheximide; 2-AP, 2-amino-purine; SOS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; NP-40, Nonidet P-40.
Fig. 2. Taxol-induced DNA fragmentation. HeLa S1 cells were positioned at G1/S by double dThd block, released and treated with taxol, or released for 8 h to progress to late G2/M and treated with taxol (5X IC50). At progressive times after dThd release, aliquots of non-taxol-treated control or taxol-treated cells were harvested for flow cytometry to determine DNA content or for gel electrophoresis to detect DNA fragmentation. CONTROL, FACS profiles of cell DNA content for nontaxol-treated cells after dThd release shows normal cell cycle progression with cell division at ~10 h. No cell division occurs in (A) cells treated with taxol at G1/S (0 h); B, cells treated at G2 (8 h); or C, cells treated at G2/M with taxol and CHX. Fragmented DNA appears as a DNA signal of less than G1 content in taxol-treated cells. X-axis, relative cell number; Y-axis, propidium iodide fluorescence intensity for DNA content. Note that indicated times are h after dThd release. DNA was extracted from taxol-treated cells, resolved by agarose gel electrophoresis, and visualized by ethidium bromide-staining. The onset of DNA fragmentation, shown for cells treated with taxol at transition G1/S, G2, or taxol treatment in G2 (C), occurs with similar kinetics. Cells treated with taxol plus CHX at G2/M are shown in (D). Times indicated are h after taxol exposure.
sis), and this sensitivity is rapidly reversed upon cell division ($t = 10$ h). The difference in sensitivity to taxol in the first and second cell division $G_1$/S phases suggest that transition into the cell cycle rather than position at the first S boundary increased drug sensitivity. The effect of $G_2$/M transition in HeLa is similar to the increased sensitivity following serum stimulation of quiescent cells ($t = 8$ h in Fig. 1A) and, consistent with reports that continuous taxol exposure, affects the ability of quiescent cells to enter the cell cycle as well as proliferating cells to initiate cell division (7).

**Taxol Toxicity Is Not Reversible in Transition or $G_2$/M Cells.** The significance of these hypersensitive points in transition and $G_2$/M cells was further examined by measuring the reduction in toxicity following successive washings to remove free drug. The above experiment was repeated in triplicate, excepting that cells were washed with fresh media 8 or 12 h after the initial removal of the drug. Fig. 1C shows that an additional wash at 12 h post taxol removal significantly increased the IC$_{50}$ of $G_1$/S populations ($t = 12–16$ h) from 30 nM to 95 nM at $t = 12$. In contrast, similar washes of either cells treated immediately after dThD release ($t = 0$ h) or cells treated at $G_2$/M had no effect on cell survival (IC$_{50}$ 18 and 10 nM at $G_2$/M for the control and second wash populations, respectively). This suggests that, despite the brief exposure, a stable taxol lesion is formed in both transition and mitotic cells such that additional washing does not reduce toxicity. At other phases of the cell cycle, cells may need to progress to mitosis to be affected by residual drug and are thus preserved by additional washings. The interval between first and second washings was not critical, and a second wash done at 8 h post drug removal gave similar results.

**Mitotic Block Is Not Sufficient to Induce DNA Fragmentation.** Based on prior reports of taxol induced apoptosis (11) and observed sensitivity of $G_1$/G, and M cells, we examined the kinetics of chromosomal DNA fragmentation in response to taxol treatment in transition cells or in mitotic cells. Cells were treated with taxol immediately after release from dThD block ($G_2$/G) or allowed to progress 8 h to late $G_2$ prior to treatment. Each population was treated with five times its respective IC$_{50}$, a dose sufficient to give no detectable surviving cells at 72 h post exposure. At progressive times after release from dThD block, the cellular DNA content of taxol-treated cells was analyzed by FACS, and the DNA extracted from the same population was resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. Fig. 2, control, shows the FACS analysis for DNA content of untreated cells after dThD release. In non-taxol-treated control cells, mitosis occurred at $\sim 10$ h post dThD release and is complete by 12 h; subsequent DNA synthesis initiated by 16 h. To examine the effect of inhibiting protein synthesis on the induction of DNA fragmentation by taxol, cells were released from dThD block, allowed to progress 8 h to $G_1$, and then treated with taxol or with taxol and CHX (50 µg/ml). FACS analysis for DNA content in taxol at $G_1$, $G_2$, or taxol + CHX at $G_2$-treated cells are shown in Fig. 2, A, B, and C, respectively. No conversion from $G_2$ to $G_1$ is seen in any taxol treatments.

The fragmentation pattern of the DNA extracted from these cells as a function of time after taxol exposure is shown in Fig. 2, D, E, and F, respectively. Fig. 2D shows the DNA fragmentation pattern of transition cells treated with taxol at release from dThD block. DNA fragmentation became evident 20 h after treatment of either transition cells (Fig. 2D) or mitotic cells (Fig. 2E and seen as an increase in the DNA of $<$G$_1$ content and reduced G$_2$ DNA in Fig. 2B FACS profiles). This result is surprising as transition cells require an additional 8 h to reach mitotic block, indicating that onset of DNA fragmentation is a function of time after exposure, rather than length of sustained arrest in mitosis. DNA fragmentation is substantially reduced, but not eliminated, in mitotic cells treated with CHX coincident with taxol (Fig. 2, C and F), suggesting that nascent protein synthesis may significantly contribute to apoptosis. The CHX experiment could not be paralleled in transition cells because inhibition of protein synthesis prevents S cells from progressing to mitosis.

To test the effect of blocking cell cycle progression during taxol treatment, cells were synchronized by double dThD block, released for 12 h to allow entry into the subsequent $G_1$ phase, blocked with dThD to prevent entry into S, and treated with taxol. Cells were harvested for DNA fragmen-
tation at 6-h intervals thereafter. Fig. 3 shows that the levels of taxol-induced DNA fragmentation are reduced in cells maintained in thymidine block. However, maintaining dTHD block beyond 18 h results in a significant loss in cell viability and DNA integrity (Fig. 3). It is interesting to note that cells maintained in dTHD for >20 h appear necrotic and lack the chromosomal condensation and vacuoles formed in typical apoptotic cells. Likewise, DNA from these cells appeared as smears on gels rather than the discretely fragmented DNA ladder seen in apoptotic cells (Fig. 3).

Inhibitors of Kinase and Phosphatase Activity Protect from Taxol Toxicity. To determine if the changes in the protein phosphorylation state are relevant to activation of cell death, cytotoxicity assays were performed on mid-log proliferating cells as described under "Materials and Methods" with taxol in combination with 2-aminopurine, a known inhibitor of interferon-induced protein kinase activity (22, 23), or in combination with sodium orthovanadate, a general inhibitor of protein phosphatase activity (24). The results of two-dimensional drug titrations, represented as isobolograms (25), are shown in Fig. 4A for taxol and 2-AP (IC50) and Fig. 4B for taxol and sodium orthovanadate (IC50). Studies using orthovanadate were calculated using IC50-S due to the high toxicity of this inhibitor alone. Data shown in Fig. 4 are representative of triplicate experiments.

By way of example, isobolograms of taxol and 2-AP combination IC50-S falling along a line connecting the IC50 of each drug alone would indicate a simply additive or noninteractive effect. Drugs acting in synergy to effect cell death will have combination IC50-S falling below this line (less than the predicted levels of each drug), and conversely, antagonistic drugs in combinations require greater than the additive IC50 for each drug to achieve the same cell killing. Fig. 4 shows that secondary treatment with inhibitors of either protein phosphatase or protein kinase activity gave greater than predicted combined IC50 or IC50-S or a decrease in the effective cell killing by taxol. Predictably, these combined treatments also resulted in a reduced level of DNA fragmentation (data not shown). Thus, protein phosphorylation state may affect onset of apoptosis.

Tyrosine Phosphorylation Occurs in Response to Mitotic Block. It has been reported previously that taxol treatment induces the phosphorylation of proteins including microtubule-associated protein kinase in macrophages (8) and increased protein phosphorylation and up-regulation of lipopolysaccharide-inducible genes in other cells (10). On this basis, we compared the levels of tyrosine phosphoproteins in synchronous populations treated with taxol, at transition or at mitosis, with levels in untreated populations. Lysates were made at 4-h intervals from untreated cells (Fig. 5C) or cells treated with taxol directly at release (Fig. 5A) or 8 h after release (Fig. 5B) from dTHD block. One hundred µg of total cell protein from each treatment were resolved by SDS-PAGE, transferred to polyvinylidene difluoride paper, and probed with anti-phosphotyrosine antibodies. The major protein detected, approximately Mr 150,000, reached maximal phosphorylation at 16 h post release and declined thereafter, independent of the time of taxol exposure. A second protein, approximately Mr 60,000, reached maximal phosphorylation at 24 h post release and was then dephosphorylated. These results were the same in each treatment despite an 8-h difference in exposure time, and both were markedly different from the phosphorylation seen in untreated cells after, but not prior to, mitosis (t = 10 h). Thus, changes from normal tyrosine phosphorylation appear to issue from mitosis rather than from time of drug exposure and may constitute a response to mitotic block rather than a signal for apoptosis.

p34cdc2 Hyperactivation Occurs in Response to Drug Treatment. In normal progression through the cell division cycle, p34cdc2 kinase is cyclically activated in G2, as phosphorylation of substrate proteins are requisite for cell division, and is inactivated prior to progression into subsequent anaphase (26). Phosphorylation of histone H1 by p34cdc2 in vivo results in chromosomal condensation prior to mitosis and in vitro; this provides a simple assessment of p34cdc2 activation (27). Inappropriate p34cdc2 activation has been implicated in apoptosis (16), and we asked how p34cdc2 activation was modulated by taxol block. Cells were positioned at G1/S by release from a dTHD block and exposed to taxol immediately or 8 h into cell cycle progression, just prior to mitosis. At progressive times after block release, cytosol was harvested, and p34cdc2 was immunoprecipitated from equal amounts of cytosolic protein and assessed for histone H1 kinase activity as described under "Materials and Methods." Fig. 6A shows the DNA synthesis profile of untreated control and taxol-treated cells progressing from dTHD release. Upon release, taxol-treated cells complete DNA synthesis and become blocked at mitosis; no subsequent DNA synthesis is observed. Similarly, no subsequent DNA synthesis was seen in G2/M cells treated with taxol. H1 kinase activities in extracts of untreated cells and those of the two treatments are shown in Fig. 6, C, D, and E, and corresponding quantitation of these activities is shown in Fig. 6B. H1 kinase activity in control populations (Fig. 6C) was maximal at 10–12 h, declined after cell division, and then reached a maximum again 32 h post release, reflecting cyclical activation of p34cdc2 at each mitoses. In cells treated with taxol at dTHD release (Fig. 6D), no activity was seen at 12 h but became apparent at 16 h and was maximal at 28 h post release. When taxol exposure was delayed for 8 h after dTHD release (Fig. 6E), minor activation could be observed at 12 h. Activation began again at 32 h and
Treatment of cells with either 2-AP or sodium orthovanado-
date provided partial protection from taxol toxicity, and at
their respective IC50 or IC25 levels (Fig. 4), resulted in a loss
of p34\textsuperscript{ck2} activity in cell extracts (data not shown). General
inhibitors of protein kinase and phosphatase do not clearly
link p34\textsuperscript{ck2} activity with DNA fragmentation because these
inhibitors have multiple effects on cell metabolism. How-
ever, modulating p34\textsuperscript{ck2} activity has been shown to have
a direct effect on apoptosis induced by CTL proteins (16)
and is consistent with our results showing inappropriate
activation. These results differ from prior reports on the
effect of taxol (14, 28), and possible reasons for this are
outlined under “Discussion.” Attempts at H1 kinase and
tyrosine phosphorylation experiments using normal IMR90
cells have not been informative because cells were not
sufficiently synchronous at mitosis to provide definitive
results.

Taxol Sensitivity Is Increased after Transformation. In
the course of these experiments, a significant (>100-fold)
difference was seen in sensitivity of normal IMR90 com-
pared to HeLa cells and prompted us to ask if sensitivity was
cell type or transformation related. The dose-response to
taxol of normal IMR90 cells, HeLa cells, and IMR90 cells
transformed with SV40 is shown in Fig. 7. Transformation of
IMR90 cells by SV40 resulted in a 140-fold increase in
sensitivity from an IC50 of 7 µM to one of 50 nm. This
latter value is comparable to that seen with HeLa S3 cells (IC50,
30 nm). Cell division time for passage 22 IMR90, SV40-IMR90,
and HeLa S3 cells are 41, 39, and 26 h, respectively. Thus, a change in cell division time does not appear
to account for the change in sensitivity to taxol.
Consistent with findings with HeLa S3 cells, SV40-trans-
formed IMR90 cells following release from dThD block and
treatment with taxol showed changes in tyrosine phosphor-
ylation after mitotic block compared to untreated cells and
induction of p34\textsuperscript{ck2} at 20 h post taxol exposure (data not shown). Results with normal IMR90 cells are less clear due
to an inability to obtain synchronous populations after entry
into S. The correlation between taxol sensitivity and func-
tion of known tumor suppressor genes in these cells is
currently under investigation.

Discussion
In synchronous cell populations, we have identified two
points of taxol sensitivity, one during transition to the pro-
liferative state and the other at G2/M of proliferating cells.
By analyzing these lesions separately, we have begun to
differentiate events related to mitotic block from those that
may contribute to apoptosis. Stage-specific sensitivity is not
reversed by secondary washes of cells exposed to taxol at
these particular points, suggesting that nonreversible taxol
lesions form rapidly in the context of a transition or mitotic
cell. Increased sensitivity observed in quiescent, normal
cells entering G1, or in dThD-released transformed cells is
most likely due to transition from quiescence into the cell
cycle, because proliferating K562 cells partitioned into pro-
gressive stages of the cell cycle by centrifugal elutriation
showed only one point of high sensitivity, consistent with a
single lesion at mitosis (IC50, 20 nm in late G1, compared
to 87 nm for early G1, cells; data not shown). In either transition
cells or in mitotic populations, treatment with taxol resulted
in initiation of DNA fragmentation, an early marker in
apoptosis, with similar kinetics at ~20 h post drug
exposure. This indicates that a sustained block at mitosis was not

\[ \text{TAXOL} \]

\[ \begin{array}{c}
0 4 8 12 16 20 24 28 32 36 40 44 \\
0 4 8 12 16 20 24 28 32 36 40 44 \\
0 4 8 12 16 20 24 28 32 36 40 44 \\
\end{array} \]

\[ \begin{array}{c}
\text{TAXOL} \\
\text{CONTROL} \\
\text{TIME POST-RELEASE (h)} \\
\end{array} \]

\[ \begin{array}{c}
-205 \\
-96 \\
-68 \\
-43 \\
\end{array} \]

Fig. 5. Tyrosine phosphoproteins in synchronous cells treated with taxol.
HeLa S3 cells were treated with 100 nm taxol immediately after dThD release
(G1/S); (A); or allowed to progress 8 h to G2/M before taxol treatment (B); or
left untreated (C). At the indicated times after release, cell lysates were
prepared, and antiphosphotyrosine Western analysis was performed on
equal amounts of cell lysate as described in “Materials and Methods.” Major
differences in protein tyrosine phosphorylation are apparent only after mito-
totic block occurs (~12 h) and are independent of the time of drug exposure.
Cell division occurs at ~10 h in untreated cells.

became maximal at 40 h post dThD release. The minor
activity at 12 h may indicate that a small population of cells
has entered mitosis at the time of drug treatment. However,
under these conditions, p34\textsuperscript{ck2} kinase activation does not
appear coincident with mitotic block but rather begins 20 h
after drug treatment and is coincident with onset of DNA
fragmentation. Thus, delaying taxol treatment by 8 h delays
p34\textsuperscript{ck2} activation by an equivalent time. This occurs de-
spite the apparent equivalent position of these populations
in the cell cycle.
required to induce cell death. DNA fragmentation is reduced by maintaining a dThd block during taxol treatment. Cytotoxicity and DNA fragmentation can be inhibited by coincident treatment of mitotic cells with taxol and high levels of CHX (Fig. 2, E and F), indicating that active protein synthesis may be required for apoptosis.

Apoptosis is also inhibited by 2-AP and sodium orthovanadate. Although 2-AP is a demonstrated inhibitor of protein kinase activity (22, 23), it has numerous effects on cell metabolism, including bypass of multiple cell cycle checkpoints following DNA damage in BHK cells (29). Therefore, we expected sensitization when 2-AP was combined with taxol. However, treatment with 2-amino purine or inhibition of dephosphorylation by sodium orthovanadate both antagonized taxol-induced cell death (Fig. 4), suggesting that checkpoints recognizing DNA damage may not recognize the taxol lesion and that continued phosphorylation and histone H1 phosphorylation by p34<sup>chk2</sup> kinase after taxol treatment of transition or mitotic cells. Synchronous populations exposed to taxol at G<sub>2</sub>/M transition or exposed at the G<sub>2</sub>/M phase showed no difference from each other in the pattern of tyrosine phosphorylation. Furthermore, differences compared to untreated control did not become apparent until after cells had undergone a mitotic block. After mitotic block, major changes in tyrosine phosphorylation were identical, despite taxol exposure times differing by 8 h, suggesting that this phosphorylation was a response to mitotic block rather than a signal for apoptosis. In contrast, p34<sup>chk2</sup> kinase activation does not appear to

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\[\text{Fig. 7. Taxol cytotoxicity of normal and SV40-transformed IMR90 and HeLa S3 cells. Cytotoxicity of IMR90 fibroblast (■), SV40 transformed IMR90 (●), and HeLa S3 (○) cells in response to treatment with taxol. Mid-log proliferating cells were exposed to drug for 1 h and recultured in complete media; the percentage survival was determined after 72 h as described in "Materials and Methods." IC}_{50} 7 µM, 50 µM, and 30 µM for IMR90, SV40-IMR90, and HeLa S3 cells, respectively. IC}_{50} for all prior figures were determined from similar plots. Points, mean of triplicate determinations; bars, SD.}\n
\[\text{Fig. 6. p34<sup>chk2</sup> kinase activity after taxol treatment. A. DNA synthesis profile of non-taxol-treated HeLa cells released from dThd block (■) compared to cells treated with taxol immediately after dThd release (G<sub>2</sub>/S; ○). Equivalent results (no subsequent DNA synthesis after 12 h) were obtained with cells treated with taxol 8 h post-dThd release (data not shown). Equal amounts of cytosol, prepared from these cells, were immunoprecipitated with anti-p34<sup>chka</sup>, and the precipitates were assayed for p34<sup>chka</sup> kinase activity using histone H1 as a substrate as described in "Materials and Methods." }\]
Toxoplasm has been shown to elicit tumor cell killing by activating a cell death program. As this program is also induced by natural factors such as tumor necrosis factor (37) or removal of interleukin 3 (38), it is unclear how signals as disparate as growth regulators and cytotoxics activate a common process. One possibility is that both types of treatment separate cell cycle events that are temporally linked and that this dissociation may serve as a trigger. This may occur more readily with defective damage recognition and cell cycle checkpoints found in transformed cells and thus may provide a significant target in antitumor intervention (17).

Materials and Methods

Cell Culture. HeLa S3 cells were grown as spinner cultures at 37°C with 6% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 2 mM L-glutamine (complete media). IMR90, normal human diploid fetal lung fibroblast cells, and SV40-transformed IMR90 cells were obtained from N. I. A. Aging Cell Repository (Camden, NJ) and maintained in Dulbecco’s modified Eagle’s complete medium. Cytotoxicity experiments of normal cells were initiated at ~30% confluency to permit continued growth and were terminated at 72 h post exposure. Media and supplements were obtained from GIBCO Laboratories (Grand Island, NY) and from Bethesda Research Laboratories, Inc. (Gaithersburg, MD).

DNA Synthesis. Cellular DNA synthesis was monitored in parallel cultures without drug treatment for each experimental time point. Five × 10^4 cells were plated in 150 μl of complete media into wells of a 96-well microtiter plate. Assays were done in triplicate. Cells were pulse labeled with [methyl-3H]dThD (6.7 Ci/mmol; Dupont NEN, Boston, MA), 0.5 μCi/well, for 30 min (1 h for IMR90) at 37°C prior to harvest. Cells were lysed onto glass fiber filters using a Tomtec cell harvester, washed with dH2O, and air dried. Scintillation fluid was added, and the filters were then counted in a Betaplate scintillation counter (LKB Wallac).

G0 to G1 Transition by Serum Deprivation and Restimulation. IMR90 fibroblast cells at 22 population doubling passages were grown to confluency in replicate 225-cm² flasks. Complete media were replaced with media containing 0.1% serum for 96 h to bring about quiescence. Cells were stimulated to synchronously proliferate by replating at one-third density in complete media (19). The timing of each population’s serum deprivation, replating, and stimulation was staggered to allow drug treatment and cytotoxicity assays to be done simultaneously. Cells were replated for drug treatment and DNA synthesis assays at progressive time intervals after serum restoration. Cellular DNA synthesis at each time point was monitored in parallel 96-well microtiter plates by [3H]dThD incorporation.

Cell Staging by Metabolic Block. HeLa S3 cells in logarithmic growth were maintained between 2–5 × 10^5 per ml in spinner flasks and synchronized by double dThD block (21) using 2.5 mM dThD for 18 h, a 10-h release in fresh media, followed by a second 18-h block prior to release. To release from block, cells were centrifuged at 200 × g for 10 min, rinsed with media, and resuspended in complete media at 37°C for the times indicated prior to drug treatment and DNA synthesis assay. The timing of blocks and releases was staggered to allow DNA synthesis and cytotoxicity assays of all populations to be done simultaneously. To verify cell cycle progression, an aliquot of each population was fixed in 70% methanol in phosphate-buffered saline on ice for 18 h. Cells were pelleted, and the pellet was incubated with propidium iodide/RNase according to the Becton-Dickinson protocol (Becton-Dickinson Instruments,
Mountain View, CA) for 30 min. The stained cells were analyzed for DNA content on a fluorescence-activated cell sorter (FACScan; Becton-Dickinson Instruments) using CellFIT cell cycle analysis software.

Drug Titration and Cytotoxicity Assays. Cell viability for all assays was determined in triplicate for each drug level at 72 h post drug exposure by XTT/phenazine methosulfate viable cell dye assay. The assay quantitates reduction of tetrazolium in intact mitochondria to a measurable color product (20). All IC50s are the average of triplicate dose-response determinations. Taxol (Paclitaxel; Bristol-Myers Squibb) was made as a 10 mM stock in dimethyl sulfoxide. At designated times after release from growth block, the cell number was determined, and cells were diluted into 96-well tissue culture plates in 150 µl complete media. HeLa S3 cells were plated at 6 × 103 cells/well and IMR90 at 9 × 103 cells/well. Fifty µl of 4X drug dilution in complete media were added to triplicate wells, and plates were incubated for 1 h at 37°C. For washing, cells were centrifuged at 200 × g for 5 min to one side of the microtiter well, and the medium was aspirated from the opposite side. Cells were washed twice with and replated in 150 µl of complete media. This method retained >95% of the initial cells independent of cell attachment. In double washout experiments, this procedure was repeated 8 or 12 h after the initial drug removal.

DNA Extraction and Gel Electrophoresis. During timed experiments, aliquots of 2 × 106 cells were centrifuged at 200 × g, the media was removed by aspiration, and the cell pellets were resuspended in 300 µl digestion buffer [100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25 mM EDTA, 0.5% SDS, and 0.1 mg/ml proteinase K] and incubated for 12 h at 50°C. The DNA was extracted once with phenol:chloroform, once with chloroform, and then precipitated with 0.5 volume of ammonium acetate and 2 volumes of ethanol at −70°C for 12 h. Pelleted DNA was resuspended in 200 µl TE buffer for 4 h at 37°C and quantified by measuring A260. Fifteen µg were applied to a 1.3% agarose gel in TBE buffer (0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA) and resolved at 20 mA constant current. The resultant DNA ladder was visualized by ethidium bromide staining.

Protein Extraction and Phosphotyrosine Western Analysis. HeLa S3 cells were synchronized by double dThD block as described above. Taxol was added immediately at release (G1/S) and in a second population, taxol was added at 8 h post release (G2). At 4-h intervals, 1 × 106 cells were harvested from each population and washed; then the cell pellets were frozen at −70°C. The pellets were subsequently solubilized in NP-40 lysis buffer [50 mM Tris-HCl (pH 7.5), 1% NP-40, 2 mM EDTA, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 5 mM sodium orthovanadate, 100 µM sodium molybdate, and 100X protease inhibitors (39)] for 30 min at 4°C. Lysates were centrifuged at 15,000 × g for 15 min; supernatants were transferred to clean tubes, and the protein concentration was quantitated using the BCA protein assay (Pierce). Equal quantities (100 µg) of lysate combined with 2X Laemmli reducing sample buffer (40) were boiled for 10 min, resolved on SDS-PAGE gradient gel 5%–16% (Novex), and transferred via electroblot to polyvinylidene difluoride Immobilon paper (Millipore). The blot was blocked in 5% bovine serum albumin and 1% ovalbumin containing 500 µM sodium orthovanadate, washed, and blotted with polyclonal rabbit antibody to phosphotyrosine (Zymed 1:2000 in 5% bovine serum albumin and 500 µM sodium orthovanadate) for 1 h. The blot was rinsed twice with TNT (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.3% Tween-20) and then washed once for 15 min and three times for 5 min. The blot was incubated in 125I-protein-A (ICN Radiochemicals) and 0.5 mM/mI in blocking buffer for 1 h, rinsed twice with TNT, and then washed every 10 min for 1 h. The blot was dried, and phosphotyrosine proteins were visualized by autoradiography or quantitated on a Phosphorimager (Molecular Dynamics). p34cdc2 Histone H1 Kinase Assay. Histone H1 kinase assay was performed as described previously (27). One hundred µg of lysate were combined with 1 µg of anti-p34cdc2 antibody (Oncogene Sciences) for 1 h at 4°C. Immune complexes were then precipitated with 50 µl of protein-A Sepharose CL-4B (Pharmacia; 50% slurry) for 1 h at 4°C, washed four times with NP-40 lysis buffer by centrifugation and resuspension, and then twice with 50 µM Tris-HCl (pH 8.0). The kinase assay was carried out by combining the washed Sepharose in 45 µl kinase buffer [50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, and 1 mM dithiothreitol], 5 µCi of [γ-32P]ATP, 5000 C/l M (Amberson; specific activity 50 Ci/mM) and Histone H1 (Sigma type V) was added to a final concentration of 15 µg ml−1. The reaction was allowed to proceed for 20 min at 37°C and was terminated by the addition of 30 µl Laemmli sample buffer and boiling for 10 min. Products were resolved by 8–16% gradient SDS-PAGE (Novex). The histone H1 (M, 30,000) was located by Coomassie blue staining of molecular weight marker in parallel lanes, visualized by autoradiography and quantitated by scanning densitometry (Molecular Dynamics).

Two-Dimensional Drug Titration. Taxol was made as a 10 mM stock in dimethyl sulfoxide. 2-AP nitrate salt (Sigma, St. Louis, MO) was prepared as a 100 mM stock in 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.2). Sodium orthovanadate was made as a 500 mM stock in dH2O. Mid-log proliferating HeLa cells were plated at 3 × 105 cells/well in 150 µl complete media in 96-well tissue culture plates. Fifty µl of 4X taxol concentration were added to each well across the plate in 2-fold titration and incubated for 1 h at 37°C. Plates were centrifuged at 200 × g for 5 min; the medium was aspirated, and the cells were washed twice with and replated in 100 µl of complete media. A 2X concentration of 2-AP (or sodium orthovanadate) was added as 100 µl to each well in 3-fold titration down the plate and incubated at 37°C. Plates with 2-AP were centrifuged after 12 h incubation, washed three times, and recultured in complete media. The centrifugation-wash method retained >95% of the original cells independent of cell attachment to the plastic. Plates with sodium orthovanadate were exposed continuously for 72 h. Cell viability was determined 72 h post taxol exposure by XTT/phenazine methosulfate viable cell dye assay (20). Inhibitory concentrations of drugs in combination were plotted as isobolograms as described by Greco et al. (25).

References
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