Decreased cyclin B1 expression contributes to G₂ delay in human brain tumor cells after treatment with camptothecin


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DNA damage produces delayed mitosis (G₂/M delay) in proliferating cells, and shortening the delay sensitizes human malignant glioma and medulloblastoma cells to cytotoxic chemotherapy. Although activation of the cyclin-dependent kinase CDC2 mediates G₂/M transition in all tumor cells studied to date, regulation of CDC2 varies between tumor types. Persistent hyperphosphorylation of kinase and reduced cyclin expression have been implicated as mediators of treatment-induced G₂ delay in different tumor models. To evaluate regulation of G₂/M transition in human brain tumors, we studied the expression and/or activity of CDC2 kinase and cyclins A and B1 in U-251 MG and DAOY medulloblastoma cells after their treatment with camptothecin (CPT). Synchronized cells were treated during S phase, then harvested at predetermined intervals for evaluation of cell cycle kinetics, cyclin kinase mRNA, and protein expression. CPT produced G₂ delay associated with decreased CDC2 kinase activity and cyclin B1 expression. Kinase activity was associated with CDC2 bound to cyclin B1, not cyclin A, in both cell lines. Cyclin A mRNA and protein expression were reduced after CPT treatment; however, decreased protein expression was short lived and moderate in the glioma and primitive neuroectodermal tumor/medulloblastoma cells, respectively. We conclude that G₂ delay is a common response of brain tumor cells to chemotherapy with topoisomerase I inhibitors and that a mechanism of this delay may be reduced expression of cyclin B1.

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Signals that regulate transition between cell cycle phases in proliferating cells represent potential targets for new cancer treatment strategies because they modulate cell responses to DNA injury as well as progression through the cell cycle (Johnson and Walker, 1999). Inhibition of cellular division by delaying the transition from the second gap phase of the cell cycle (G₂) to mitosis (M) is an important cellular response to DNA-damaging agents (Lock and Ross, 1990; McKenna et al., 1991; O’Connor et al., 1993). G₂ delay may be necessary to complete DNA repair before damaged, potentially lethal replicons are conferred to the next generation of cells through mitosis. Cytotoxicity of radiation and chemotherapy is inversely associated with the duration of radiation- or chemotherapy-induced G₂ delay in human lymphoma (O’Connor and Kohn, 1992) and transformed rat embryo cells (McKenna et al., 1991). Furthermore, pharmacologic abrogation of radiation- or drug-induced G₂ delay with caffeine, staurosporine, or pentoxifylline increases cell death in HeLa cells (Bernhard et al., 1994), human lymphoma cells.
O’Connor et al., 1993), and rat brain tumor cells 9-L and 9-L2 (Aida and Bodell, 1987). We recently published studies demonstrating the ability of caffeine and staurosporine to abbreviate chemotherapy-induced G2 delay in U-251 MG cells and DAOY medulloblastoma, 2 well-characterized human brain tumor cell lines. Shortening of G2 delay produced by the platinating agent, cisplatin, and the topoisomerase I inhibitor, CPT3 increased cytotoxicity and apoptosis (Janss et al., 1998b). However, doses of caffeine, staurosporine, and pentoxifylline, necessary to overcome G2 delay in vitro, would be lethal in vivo (Nehlig et al., 1992; Stewart et al., 1997). Strategies that selectively alter signals for commitment to mitosis and increase the cytotoxicity of currently available DNA-damaging agents need to be identified; however, it appears that regulation of G2 delay varies between tumor cell types.

G2 to M transition in eukaryotic cells is regulated by the cyclin-dependent kinase, CDC2 (synonyms cdki, p34CDC2; Nurse, 1994; Ohi and Goud, 1999). Kinase activity of CDC2 requires activating phosphorylation at threonine 161 (Desai et al., 1995; Gould and Nurse, 1989) and complex formation with either cyclin A or cyclin B1 protein (Pagano et al., 1992). Inhibitory phosphorylations at threonine 14 and tyrosine 15 positions are imposed by Wee1Hu (human analog to yeast wee1 kinase) or Myt1 (membrane-associated and tyrosine/threonine-specific CDC2 inhibitory kinase 1) (Liu et al., 1997, 1999). At G2/M transition, the inhibitory phosphorylations are removed from CDC2 by CDC25C phosphatase. CDC2 is inactivated as cyclin dissociates and is rapidly degraded during mitosis (Dunphy, 1994).

G2 delay induced by treatment with DNA-damaging agents may be regulated by different mechanisms in various cell lines. In HeLa cells, decreased CDC2 kinase activity after radiation is mediated by reduced expression of cyclin B1 protein (Kao et al., 1997) due to shortened cyclin B1 mRNA half-life (Maity et al., 1995). In contrast, treatment-induced G2 delay in human lymphoma, human leukemia, and Chinese hamster ovary cells results from persistent hyperphosphorylation, hence inactivity of CDC2 kinase (Lock, 1992; O’Connor et al., 1992; Shimizu et al., 1995). These mechanisms may be redundant or may differ between tumors. Thus, understanding regulation of G2/M transition in brain tumor cells is necessary if strategies that exploit abrogation of G2 delay are to be applied to increase efficacy of current therapy in neuro-oncology.

To evaluate regulation of chemotherapy-induced G2 delay in human brain tumor cells, we used CPT to study the impact of DNA-damaging therapy on cell cycle kinetics, activity and phosphorylation of CDC2 kinase, and expression of mitotic cyclin A and cyclin B1 in U-251 MG and DAOY primitive neuroectodermal tumor/medulloblastoma cells. CPT was chosen because of its documented toxicity to both U-251 MG and DAOY cells (Janss et al., 1998a, 1998b), its ability to produce prolonged G2 delay in these cells, and the observation that its cytotoxicity is increased by staurosporine, a pharmacological agent that shortens CPT-induced G2 delay (Janss et al., 1998b). Use of 2 different brain tumor cell lines permitted identification of cell-specific differences in G2/M regulation and may enable selection of target (targets) with broad activity as adjuvant therapy. CPT produced prolonged G2 delay associated with decreased CDC2 activity and cyclin B1 expression. Although cyclin A protein expression decreased after CPT treatment, the decrease was short lived and moderate in glioma and medulloblastoma cells, respectively. CDC2 existed in both inactive (hyperphosphorylated) and active (hypophosphorylated) forms throughout the cell cycle; shift of kinase to active form correlated with times of low cyclin expression rather than G2/M transition. These data suggest that reduced cyclin B1 expression contributes to chemotherapy-induced G2 delay in human brain tumors.

Materials and Methods

Cell Culture

Two human brain tumor cell lines were used: DAOY primitive neuroectodermal tumor/medulloblastoma and U-251 MG (Friedman et al., 1988). Both cell lines were grown in Richter’s zinc option medium (GIBCO BRL, Rockville, Md.) with 10% fetal calf serum (Sigma, St. Louis, Mo.) and 50,000 units streptomycin and penicillin per liter of medium and were harvested as previously described (Friedman et al., 1988).

Synchronization

Subconfluent cultures were harvested and plated at 6 × 104 cells/100-mm culture dish in the medium described above. A thymidine-aphidicolin block similar to that described by Heinz et al. (1983) was used to synchronize the cells at the G1/S boundary (Maity et al., 1996), then to release them from the G1 block. Cells were harvested for flow cytometric evaluation and extraction of RNA or protein at predetermined times throughout the cell cycle after release from G1 block. Additional plates were harvested for assessment of the toxicity of treatment in synchronized cells treated during S phase.

Treatment

After release from the aphidicolin block, cells were allowed to progress from G1 to S phase, then treated with CPT (100 nM, 1-h exposure; SmithKline Beecham Pharmaceuticals, Philadelphia, Penn.) or serum-free medium control (1-h exposure). U-251 MG and DAOY cells were exposed to CPT or serum-free medium 4 h after their release from aphidicolin to maximize treatment of tumor cells in S phase. (Three hours after release from aphidicolin, 70% [SE 2.3%] of U-251 MG cells were in S phase and 18.5% [SE 2.3%] DAOY cells were in S phase. Four hours after release from aphidicolin, 71% [SE 3.0%] U-251 MG cells and 62% [SE 7.0%] DAOY cells were in S phase.) Cells were incubated with CPT-100 nM in serum-free medium for 1 h at 37°C then rinsed and incubated in medium with 20% fetal calf serum. The dose of CPT was chosen because it approximates the ID50 (inhibitory dose resulting in 50% growth...
suppression) for unsynchronized DAOY and U-251 MG cells (Janss et al., 1998a). Control treatment consisted of 1-h exposure of cells to serum-free medium.

**Assay for Cytotoxicity in Synchronized Cells**

Cells were harvested and plated (300 cells per 10-mm petri dish). Six replicate plates were prepared for each treatment group (control, CPT). After 7 days, cells were stained and colonies were counted. Fractional survival was calculated by dividing mean colony count for the 6 replicate plates treated with CPT (100 nM, 1-h exposure during S phase) by the mean colony count for 6 replicate control plates (cells treated with serum-free medium for 1 h during S phase).

**Flow Cytometry**

Cells were harvested and immediately frozen in liquid nitrogen in 1 ml of 40-mM citrate buffer and were stored at −70°C. At time of analysis, cells were thawed and stained with propidium iodide as described by Vindelov and Christensen (1990). Flow analysis was performed on the Becton-Dickinson FACScan flow cytometer, and the data were interpreted using the CellFIT cell cycle analysis program (Version 2.01.2; Becton Dickinson, Mansfield, Mass.). At least 10,000 cells were used for each sample.

**Protein Extraction and CDC2 Kinase Activity**

For each time point, more than 100,000 synchronized cells were lysed with 300 µl of buffer, then frozen at −70°C (Prasad et al., 1995). Protein concentration was determined using the BCA™ protein assay kit (#23225; Pierce, Rockford, Ill.). Kinase activity was quantified with SignaTECT™ CDC2 Protein Kinase Assay System (Promega, Madison, Wis.) using (32P)ATP 10 µ Ci/µl. Details of the procedure can be found in the technical bulletin provided by the company. Briefly, reactions were performed with cell lysate (10 µg/5 µl volume), positive control (CDC2 kinase 5 U/µl, Cat. # V2891) or negative control (no substrate). Total reaction volume was 25 µl at 30°C for 10 min. Reaction was terminated using 100 µM olomoucine. Samples (15 µl) of each terminated reaction were spotted onto the SAM² Membrane, washed, dried, then separated and analyzed by measuring radioactivity in a scintillation counter.

**Immunoprecipitation**

To immunoprecipitate CDC2 bound to a specific cyclin, cell lysate (50 µg of protein) was added to 1 µg mouse antihuman cyclin B1 (Upstate Biotech, Lake Placid, N.Y.) or mouse antihuman cyclin A (Upstate Biotech) and lysis buffer to a final volume of 200 µl and incubated at 7°C. After 2 h, 20 ml 50% Protein A-Sepharose CL-4B (Amersham Corp., Arlington Heights, Ill.) was added. This mixture was washed with lysis buffer and centrifuged (1000 RPM, 2 min), and supernatant was stored at −70°C until CDC2 kinase activity could be determined using the assay described above.

**RNA Extraction and Northern Blot Analysis**

Total cellular RNA was extracted as previously described (Chirgwin et al., 1979). RNA was quantified by spectrophotometer at a wavelength of 260 nm. RNA (10 µg) was denatured with formalin and formamide, separated by electrophoresis on a 1% agarose gel, transferred to a Durulon membrane (Stratagene, La Jolla, Calif.), and cross-linked using a UV Stratalinker (Stratagene). Membranes were prehybridized with 5× saline-sodium citrate, 100 mg denatured herring sperm DNA, 4× Denhard’s solution (2% bovine serum albumin, 2% Ficoll, and 2% polyvinylpyrrolidone), then hybridized using a cyclin B1, cyclin A, and rpl23 probe. Radioactive probes were made from a 1-kb fragment of human cyclin B1 cDNA, a 0.281-kb fragment of human cyclin A cDNA, and the 0.6-kb DNA of the ribosomal protein rpl32, then labeled with (32P)DCPT using the random primer method as previously described (Maity et al., 1995).

**Immunoblotting for Cyclin B1, Cyclin A, and CDC2**

For each time point, cells were lysed and protein was quantified as described above. Samples consisting of 50 µg protein were separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels before transferring them to a nitrocellulose membrane (Bio-Rad, Hercules, Calif.). The membrane was probed with one of the following primary monoclonal antibodies: mouse antihuman cyclin B1 (dilution 1:1000; Upstate Biotech), mouse antihuman cyclin A (dilution 1:500; Upstate Biotech), or rabbit antihuman CDC2/cck1 (dilution 1:250; Calbiochem, San Diego, Calif.). Secondary antibody coupled to horseradish peroxidase (goat antimouse, or goat antirabbit; Amersham) was then used, and detection was performed by chemiluminescence (Amersham).

**Optical Densitometry and Statistical Analysis**

Durulon membranes containing mRNA were exposed with an intensifying screen using XAR-5 film at −70°C. Autoradiographs were digitized with a charge-coupled device camera, and densitometry was performed with NIH Image software (Version 1.53; National Institutes of Health, Bethesda, Md.). The relative cyclin mRNA level was calculated as the ratio of the intensity of cyclin B1 or cyclin A to the rpl23 band. Relative cyclin protein expression was quantified by subjecting exposed film to analysis using densitometry as per above. Relative cyclin protein expression per 50 µg protein was calculated as the ratio of the intensity of cyclin B1 or cyclin A to the intensity of peak expression of cyclin in samples not treated with CPT.

**Results**

**Cytotoxicity of Camptothecin in U-251 MG and DAOY Cells**

CPT treatment (100 nM, 1-h exposure during S phase) of synchronized DAOY and U-251 MG cells was compara-
ble, resulting in 6% and 9% clonogenic survival, respectively, relative to treatment with serum-free medium.

**Cell Cycle Kinetics in U-251 MG and DAOY Cells in the Absence and Presence of CPT**

Exposure to 100 nM CPT during S phase resulted in G2 delay in both U-251 MG and DAOY cells. Fig. 1 illustrates flow cytometry profiles of brain tumor cells at various times after release from G1 block. Four hours after release from G1/S interface, 66% to 69% control and CPT-treated glioma cells were in S phase (n = 4). Twelve hours after release, 58% to 71% of control U-251 MG cells had cycled around into G2, whereas only 7% to 18% of CPT-treated cells were in G1 and 55% to 72% remained in G2/M (for example, see Fig. 1A). There was a transient S phase delay after treatment with CPT. Note the persistence of S-phase cells 12 h, but not 16 h, after release from G1 as illustrated in Fig. 1A. In control U-251 MG cells, the percentage of cells in G2/M peaked at 8 to 12 h after release from G1, then declined as cells cycled into G1 phase. After CPT treatment, the percentage of U-251 MG cells in G2/M peaked at 18 h because cells persisted in G2 phase (Fig. 2A, top).

G1 arrest was also seen in DAOY medulloblastoma cells after treatment with CPT. More than 12 h after release from G1 block, 41% to 48% of cells treated with CPT were still in G2/M phase, but only 8% to 13% of control cells remained in G2/M, and 51 to 75% had cycled through to G1 at that time (see Fig. 1B). There was a transient S-phase delay after treatment with CPT. Note the persistence of S-phase cells 9 h, but not 12 h, after release from G1 as illustrated in Fig. 1B. The maximum percentage of untreated DAOY cells in G2/M occurred 12 to 15 h after release from G1 block. After CPT treatment, the peak percentage of DAOY cells in G2/M was delayed to 18 h because cells persisted in G2/M (Fig. 2B, top). Flow cytometry indicated that by 30 h after release from G1, U-251 MG and DAOY cells treated with CPT had begun to cycle around into G1.

**CDC2 Kinase Activity in U-251 MG and DAOY Cells in the Absence and Presence of CPT**

Peak CDC2 kinase activity in untreated U-251 MG and DAOY cells was 12 to 15 h and 12 h, respectively, coinciding with the peak percentage of cells in G2/M phase and anticipating decline of cells in G2/M phase as cells cycled around to G1 phase (Fig. 2). The magnitude of peak kinase activity was consistent within cell type, but differed between tumor cell type; peak activity was 0.0337 to 0.0519 and 0.0585 to 0.0633 pmol ATP/min/μg protein in U-251 MG and DAOY cells, respectively. Treatment with 100 nM CPT during S phase decreased CDC2 kinase activity in both brain tumor cell lines, which correlated with delayed entry into mitosis. Immunoprecipitation of protein with monoclonal antibodies for cyclin A or cyclin B1 indicated that CDC2 kinase activity in both cell lines was associated with cyclin B1, not cyclin A (data not shown).

Hyperphosphorylated (inactive) and hypophosphorylated (active) forms of CDC2 were detected throughout the cell cycle in both U-251 MG and DAOY cells (Fig. 2C). In treated control U-251 MG and DAOY cells, the shift to the hypophosphorylated form occurred at 15 and 20 h after release from G1 block, respectively, and persisted at subsequent sampling times, corresponding to times of decreased cyclin A and cyclin B1 expression. Treatment with CPT (100 nM for 1 h during S phase) did not reduce total CDC2 expression in either U-251 MG or DAOY cells. Shift to the active, hypophosphorylated CDC2 was seen between 12 and 15 h after release from G1 block despite persistence of G2 delay in both cell lines.
Peak cyclin B1 mRNA expression in U-251 MG and DAOY cells occurred 15 to 20 h after release from G
1 block in the absence of DNA damaging therapy, whereas peak protein expression occurred 12 to 15 h after release from G
1 block in control cells (Figs. 3, 5, 6). Treatment with CPT reduced cyclin B1 mRNA expression to levels below baseline, and this low level persisted at least 25 h after removal of CPT (30 h after release from G
1 block; Fig. 3). Peak cyclin B1 protein expression was reduced more than 50% by brief exposure to CPT in both U-251 MG and DAOY cells and remained below peak levels for up to 30 h after release from G
1 block (Fig. 3). In U-251 MG cells, CPT treatment reduced cyclin B1 protein expression (mean 46% of peak level in untreated cells, range 33%-60%). In DAOY cells, CPT treatment reduced cyclin B1 protein expression (mean 51% of peak level in untreated cells, range 32%-70%).
Cyclin A Expression in U-251 MG and DAOY Cells in the Absence and Presence of CPT

In the absence of CPT treatment, cyclin A mRNA expression peaked in U-251 MG and DAOY cells 12 and 20 h after release from G1 block, respectively (Figs. 4-6). Similarly, protein expression peaked 12 to 15 h after release from G1 block in the control U-251 MG and DAOY cells (Fig. 4). Treatment with CPT reduced cyclin A mRNA expression to baseline levels in U-251 MG and DAOY cells (Fig. 4). Expression of cyclin A mRNA remained low at least 25 h after removal of CPT (30 h after release from G1 block). Cyclin A protein expression was decreased by CPT treatment in both cell lines, but the pattern of decrease differed. Three hours after removal of CPT (8 h after release from G1 block), cyclin A protein expression in U-251 MG cells was 56% to 76% of that in untreated cells and remained low until 15 h after release from G1 block, when expression surpassed that of untreated cells (Fig. 4A). Cyclin A protein expression in DAOY cells was not depressed 3 h after removal of CPT and was minimally decreased 4 and 7 h later (12 and 15 h after release from G1 block; Fig. 4B).
Discussion

We recently reported that human malignant glioma and medulloblastoma cells exhibit increased sensitivity to cytotoxic agents when treatment-induced G₂ delay is abrogated (Janss et al., 1998b). High rates of treatment failure observed for patients with malignant gliomas and medulloblastomas (Packer et al., 1994; Walker and Strike, 1976) justify efforts to identify novel therapeutic strategies, including those that target G₂/M transition. In this report, we show that G₂ delay is a common response for U-251 MG glioma and DAOY medulloblastoma cells after treatment with DNA-injuring agent CPT and that the delay is associated with reduced CDC2 kinase activity. Reduction in CDC2 kinase activity in brain tumor cell lines did not correlate with a large shift of CDC2 to the hyperphosphorylated (inactive) form but rather with reduced expression of cyclin B1 mRNA and protein expression. Decrements in cyclin A protein expression that were associated with CPT-induced G₂ delay were transient or moderate in both glioma and medulloblastoma cells. Together, these findings suggest that cyclin B1 expression may be a rate-limiting mediator of treatment-induced G₂ delay in human brain tumor cells.

This is the first report of decreased cyclin B1 mRNA and protein expression associated with chemotherapy-induced G₂ delay in human brain tumor cell lines. Regulation of G₂ delay in U-251 malignant glioma cells and
DAOY medulloblastoma cells approximates that of HeLa cells rather than that of human lymphoma or leukemia cells. In synchronized HeLa cells, CPT and radiation-induced G2 delay was associated with decreased cyclin B1 mRNA and protein expression (Maity et al., 1996; Muschel et al., 1991). However, in HeLa cells, decreased cyclin B1 protein expression associated with CPT treatment was transient (Maity et al., 1996), whereas in brain tumor cells the decrease was prolonged. Tsao et al. (1992) documented both decreased cyclin B1 protein expression and CDC2 kinase activity in synchronized HeLa cells treated with CPT. The importance of cyclin B1 in treatment-induced G2 delay was supported by Kao et al. (1997), who reported that overexpression of cyclin B1 in HeLa cells abbreviated the radiation G2 delay, whereas depression of cyclin B1 expression using antisense oligonucleotides augmented the duration of G2.

In both U-251 MG and DAOY cells, treatment-induced G2 delay was associated with decreased CDC2 kinase activity. In some cell lines, the decrease is associated with persistent phosphorylation of CDC2 (Lock, 1992; O’Connor et al., 1992; Shimizu et al., 1995), whereas in others it is associated with decreased cyclin expression (Kao et al., 1997; Maity et al., 1995; Maity et al., 1996). Because both hyperphosphorylated (inactive) and hypophosphorylated (active) forms of CDC2 appeared throughout the cell cycle in synchronized brain tumor cells regardless of treatment, it is difficult to implicate persistent phosphorylation of CDC2 as the sole mediator of G2 delay in U-251 MG and DAOY cells. Incomplete synchronization of brain tumor cells may have contributed to distribution of CDC2 in different phosphorylation states throughout the cell cycle. However, it does not account for the persistence and even predominance of hypophosphorylated CDC2 during treatment-induced G2 delay.

Topoisomerase-I inhibitors are a class of anticancer agents that bind to DNA-topoisomerase-I complexes, stabilize single-strand DNA nicks, and facilitate double-strand breakage upon collision with the replication fork (Ryan et al., 1991), thereby interfering with DNA synthesis and cell proliferation. Efficacy of topoisomerase-I
inhibitors in treatment of brain tumors has been documented in in vivo and in vitro studies and in clinical trials (Slichenmyer et al., 1993). CPT, the prototypical topoisomerase-I inhibitor, was used to identify molecular events that contribute to $G_2$ delay in brain tumor cells because previous work had documented its cytotoxicity and ability to induce prolonged $G_2$ delay in U-251 MG and DAOY cells (Janss et al., 1998a, 1998b). Shortening CPT-induced cell cycle delay in DAOY cells by coadministration of staurosporine, a protein kinase C inhibitor, increased cytotoxicity and apoptotic cell death (Janss et al., 1998a). This is consistent with the report of Shao et al. (1997) that the staurosporine analog, UCN-01, which abrogates CPT-induced cell cycle delay, augments the cytotoxicity of CPT in human colon and breast carcinoma cell lines. Interestingly, they observed that S-phase delay and $G_2$ arrest of HT29 colon carcinoma cells after treatment with CPT were associated with increased expression of cyclin A and no alteration of cyclin B1 16 h after removal of CPT. Cyclin A binds to cyclin-dependent kinase 2 (Cdk2) and facilitates S-phase progression, and binds with CDC2 to facilitate G$_2$/M transition (Desai et al., 1995; Pagano et al., 1992). The authors postulated that accumulation of cyclin A after treatment with CPT reflects the drug’s interference with S phase after recruitment of cyclin A-Cdk2 complex.

Whereas synchronized malignant glioma and medulloblastoma cells, like HT29 cells, exhibited S-phase delay and $G_2$ arrest when exposed to CPT, cyclin A and cyclin B1 expression decreased at 16 h after removal of CPT. Discrepancies between our findings and those of Shao et al. (1997) may be due to use of different cell lines and/or technical differences (that is, treatment duration of 8 h in unsynchronized cells versus 1 h during S phase in synchronized cells). It is unlikely that the decreased cyclin B1 mRNA expression observed in malignant glioma and medulloblastoma cell lines after treatment with 100 nM CPT was due to a nonspecific effect of CPT on transcription. Although CPT is known to inhibit transcription at doses much higher than used in this study, this effect is dose dependent and reversible within 30 min following removal of the drug (Horwitz et al., 1971; Kessel, 1971). Furthermore, Maity et al. (1996) demonstrated that decreased cyclin B1 mRNA expression in synchronized HeLa cells after treatment with CPT (500 nM) is not due to generalized down-regulation of transcription. Unsynchronized U-251 malignant glioma cells are relatively resistant to the cytotoxic effects of CPT compared with DAOY medulloblastoma cells (Janss et al., 1998a). As discussed in the introduction, some authors postulate that the duration of treatment-induced $G_2$ delay correlates with treatment resistance. The data presented here

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**A U-251 MG cyclin protein expression**

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<th>Treatment Time (hours)</th>
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**B DAOY cyclin protein expression**

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Fig. 6. Western blots depicting expression of cyclin B1 and cyclin A in synchronized brain tumor cells with and without CPT treatment. A. U-251 malignant glioma cells. Time of cell harvest, in hours, after release from $G_1$ blockade is presented above each band. Treatment, either 100 nM CPT or serum-free medium for 1 h, during S phase is indicated above time of harvest. B. DAOY medulloblastoma cells.
do not address whether duration of CPT-induced G\textsubscript{2} delay contributes to relative resistance in human brain tumor cells, because both synchronized U-251 MG and DAOY cells exhibited prolonged G\textsubscript{2} delay and decreased cyclin B1 expression after exposure to CPT. U-251 MG cells may employ strategies of resistance to drug treatment not related to G\textsubscript{2} delay, or use of synchronized cells may have obscured the small difference in the relative toxicity of CPT in U-251 MG and DAOY cells.

U-251 MG and DAOY cell lines used in this study have a mutated P53 oncogene (Bigner et al., 1989; He et al., 1991). Agents that abrogate G\textsubscript{2} delay induced by DNA injury, such as staurosorine or UCN-01, increase cytotoxicity more effectively in cells without intact P53 (Bredel et al., 1999; Shao et al., 1997; Wang et al., 1996). This is likely due to the importance of intact P53 in blocking progression through G\textsubscript{2} after DNA damage (Cox and Lane, 1995). Cells that progress through G\textsubscript{2} despite DNA injury rely heavily on S-phase and G\textsubscript{2}-phase regulation to delay commitment to mitosis before repair can be completed. Failure to complete the repairs leads to accumulation of DNA damage and decreased cell viability. Thus, strategies that target regulation of G\textsubscript{2}/M progression to increase the efficacy of cancer treatments are likely to be effective in tumors with impaired regulation of G\textsubscript{2}, such as those having a mutated P53. Such strategies may include alteration of CDC2 activity by altering cyclin B1 expression (Innocente et al., 1999; Maity et al., 1995; Toyoshima et al., 1998), recruitment of recently identified regulatory kinases (Matsuoka et al., 1998; Peng et al., 1997), and/or suppression of transport proteins (Hermeking et al., 1997; Toyoshima et al., 1998). We would expect such strategies to have significant impact in neuro-oncology because mutations of the P53 gene are common in primary malignant brain tumors (Fults et al., 1992; Howng et al., 1996; Wu et al., 1993) and secondary malignancies of the CNS (Gafanovich et al., 1999). Although P53 mutations are rare in pediatric brain tumors, including medulloblastomas (Biegel et al., 1992; James et al., 1990), abnormalities of G\textsubscript{1} regulatory proteins other than P53 have been identified in brain tumors (He et al., 1995), and shortening treatment-induced G\textsubscript{2} delay produces modest increases in cytotoxicity in cells with intact P53 (Shao et al., 1997).

The findings of this report indicated that G\textsubscript{2}/M transition checkpoints, and specifically cyclin B1, represent promising targets for novel strategies to augment the cytotoxic effects of chemotherapy in human medulloblastomas and malignant gliomas.

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**References**


