G2 Arrest in Response to Topoisomerase II Inhibitors: The Role of p53

Brad Clifford, Milos Beljin, George R. Stark, and William R. Taylor

Department of Molecular Biology, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195

ABSTRACT

We have previously found that the overexpression of p53 causes G2 arrest and represses the synthesis of cyclin-dependent kinase 1 and cyclin B1, two proteins required for cells to traverse from G2 into M. G2 arrest occurs in response to DNA damage caused by a variety of agents and treatments. Here, we investigate the role of p53 in the G2 arrest that occurs in response to the topoisomerase inhibitors etoposide and merbarone. In HT1080 cells expressing a dominant-negative form of p53, treatment with etoposide still caused G2 arrest, but the arrest could be overcome by additional treatment with caffeine, which inhibits the damage-responsive kinases ataxia telangietasia mutated (ATM) and atn and rad3-related (ATR). However, caffeine could not overcome etoposide-induced G2 arrest in HT1080 cells with functional p53. We conclude that etoposide activates two pathways, one of which depends on p53 and the other of which is sensitive to caffeine, and that either pathway is sufficient to activate G2 arrest. Etoposide inhibits topoisomerase II by trapping the enzyme in a complex with cleaved DNA. Inhibition of topoisomerase II with merbarone, which does not stabilize a cleavage complex, causes G2 arrest by a checkpoint that monitors the decatenation of chromatin. We find that caffeine can abrogate merbarone-induced G2 arrest even in cells with functional p53, indicating that p53 does not contribute to the deconcatenation-sensitive response. Thus, p53 has a differential role in effecting G2 arrest in response to topoisomerase II inhibitors, depending upon the mechanisms of action of the inhibitors tested.

INTRODUCTION

p53 suppresses tumor formation by inducing transient or permanent cell cycle arrest or apoptosis in response to DNA damage or other stresses (reviewed in Ref. 1). The level of p53 protein is rapidly elevated, and the protein is activated in response to ionizing or UV radiation, DNA damaging chemicals, nucleotide deprivation, hypoxia, or activated oncogenes (1, 2). High levels of activated p53 drive the transcription of a large number of genes that mediate its biological functions. Phosphorylation of p53 in response to stress is essential for its accumulation and activation, and p53 is also acetylated in response to stress (reviewed in Ref. 3). These modifications enable p53 to bind to DNA and activate transcription. Loss of cell cycle control upon inactivation of p53 contributes to the genomic instability that drives the emergence and progression of tumors (1). When overexpressed, p53 blocks the cell cycle at G1 and G2 (reviewed in Ref. 4). p53 also stabilizes the S-phase arrest induced in cells treated with PALA3, an inhibitor of de novo pyrimidine nucleotide biosynthesis (5). The cell cycle responses to DNA damage are more complicated because of the activation of p53-independent pathways. For example, the loss of p53 eliminates G1 arrest in response to DNA damage, but cells lacking p53 still arrest rapidly in G2, showing that even if p53 contributes to G2 arrest in response to DNA damage, p53-independent pathways also play an important role (6, 7). A major p53-independent pathway for G2 arrest is controlled by the related ATM and ATR protein kinases (8, 9). ATM is mutated in the tumor susceptibility syndrome ataxia telangietasia (10) and, along with ATR, phosphorylates and activates the serine kinases CHK1 and CHK2 (11–13), which phosphorylate the phosphatase CDC25, causing it to be sequestered in the cytoplasm bound to a protein of the 14-3-3 family (14–18). In the cytoplasm, CDC25 cannot dephosphorylate CDK1, the CDK that drives cells from G2 into mitosis. Dephosphorylation of CDK1 by CDC25 is normally required for the activation of CDK1 at the G2-M boundary (19, 20). Although human colon tumors lacking p53 can still initiate a G2 arrest, they are unable to remain arrested in G2 and eventually enter mitosis (21). Also, G2 arrest in response to DNA damage can be abrogated by caffeine, probably because of its inhibition of both ATM and ATR (22–25). However, the effect of caffeine appears to require the loss of p53 function (26, 27), suggesting that p53 plays a role in the long-term maintenance of G2 arrest that occurs in response to DNA damage. The mechanism by which p53 maintains G2 arrest has been attributed to transcriptional induction of the p21, gadd45, and 14-3-3 protein genes (reviewed in Ref. 4). GADD45 contributes to G2 arrest, especially in response to UV radiation, by dissociating CDK1 from its essential subunit cyclin B1, thus inactivating the complex (28–30). 14-3-3 proteins sequesters CDK1/cyclin B1 in the cytoplasm, where it is unable to induce entry into mitosis (31, 32). p21 binds directly to CDKs, including CDK1, and inhibits them (33). However, the binding of p21 to CDK1 is relatively inefficient (34), p21 may inhibit CDK1 by blocking its phosphorylation on threonine 161, a modification required for activity (35). Our work has uncovered an additional mechanism by which p53 maintains G2 arrest, involving the induction of p21, which inhibits the phosphorylation of the Rb family members p130 and p107 by CDKs, allowing p130 and p107 to repress the transcription of a large number of genes required for transit through G2 and M (4). We now describe the role of p53 in stabilizing G2 arrest in response to the topoisomerase inhibitors etoposide and merbarone. We find that the down-regulation of CDK1 and cyclin B1 is a major mechanism used by p53 to stabilize G2 arrest. Without this protection, cells are prone to enter mitosis with damaged DNA, which contributes to genomic instability and can trigger cell death.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Cells were grown in a humidified atmosphere containing 10% CO2 in DMEM (Life Technologies, Inc., Grand Island, NY) supplemented with antibiotics and 10% fetal bovine serum (Life Technologies, Inc.). Ht1080 is a human fibrosarcoma-derived cell line containing wild-type p53 (our unpublished observations). HT1080 cells were infected with a retrovirus expressing GSE56, a dominant-negative COOH-terminal fragment of p53 (36), to produce a pool of infected GSE cells. A parallel culture of HT1080 cells was infected with the empty LXSX virus to produce a pool of infected WT cells. Cell cycle
content was determined by FACScanning of propidium iodide-stained nuclei (37). The FACS data were analyzed by using Cellfit software (Becton Dickinson). Cells were treated with 10 μM etoposide (Bristol-Myers Squibb, Princeton, NJ) and from 2 to 10 mM caffeine (Sigma, St. Louis, MO). Merbarone was used at 50 μM and obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute was dissolved in DMSO. The LD₅₀ of etoposide and merbarone were determined using a colony assay after 16 h of drug treatment. Immunofluorescence was carried out as we have described previously (38). Images were quantitated with Metamorph software. Background fluorescence was determined using cells stained in the same manner except that the primary antibody was not added.

**Western Analysis**

Extracts were prepared by lysing cells in radioimunoprecipitation assay buffer, which contains 10 mM TRIS (pH 7.4), 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 5 mM NaF, 1 mM NaVO₄, 1 mM phenyl-methanesulfonyl fluoride, 2 μg/ml aprotinin, 25 μg/ml leupeptin, 5 μg/ml peptatin A, and 1 mM DTT. Extracts containing equal quantities of proteins, determined by the Bradford method (Bio-Rad, Hercules, CA), were separated by SDS-PAGE (12.5% acrylamide) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were probed with monoclonal antibodies specific for p53 (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA), CDK1 (17; Santa Cruz Biotechnology), cyclin B1 (GNS1; Santa Cruz Biotechnology), p21 (C-19; Santa Cruz Biotechnology), and rabbit polyclonal antibodies specific for serine 10-phosphorylated histone H3 (Upstate Biotechnology, Inc., Waltham, MA) and serine 15-phosphorylated p53 (Cell Signaling, Beverly, MA). Bound antibodies were detected with goat-antimouse or goat-antirabbit antibody conjugated to horseradish peroxidase (Hoffman-La Roche, Basel, Switzerland) using enhanced chemiluminescence (Dupont, Wilmington, DE; Ref. 37). For quantitation, films were scanned and intensities determined using NIH Image software.

**Determining the Number of Mitotic Cells**

**Two-Parameter FACS.** The analysis was carried out essentially as described previously (38, 39). Cells harvested by trypsinization (including floating cells) were washed once with PBS, resuspended in 0.4 ml of PBS, and fixed by adding them dropwise to 1 ml of 100% ethanol at −20°C. The cells were resuspended in 0.1% BSA (w/v), 0.02% NaN₃ (w/v) in PBS, incubated at room temperature for 30 min, and stained with an antiserum against histone H3 phosphorylated at serine 10, after which the cells were washed once with the same buffer and stained with a goat-antirabbit antibody labeled with FITC (Sigma). The cells were washed, counterstained with a solution containing propidium iodide (50 μg/ml), and RNase (2 units/ml) and analyzed by two-parameter FACScanning. Twenty thousand cells were analyzed for each condition. To determine the effect of caffeine and p53 on rapid G2 arrest in response to DNA damage, cells were pretreated or not with caffeine for 16 h. Etoposide was added, and the number of cells in mitosis 1 h later was determined by FACScanning. The percentage of cells in mitosis was calculated by comparison to cells that were treated or not with caffeine but not with etoposide.

**Metaphase Spreads.** Many cells treated with merbarone and caffeine entered mitosis, allowing the quantitation of mitosis by direct counting. Metaphase spreads were prepared as described previously (40). Briefly, floating and attached cells were collected as above, incubated in 75 mM KCl for 15 min at 37°C, fixed in fresh methanol:acetic acid::3:1 (v/v) for 20 min on ice, and dropped from a distance of 1 meter onto glass slides. The slides were dried in air, and the DNA was stained with 5 μg/ml Hoechst 33342 (Molecular Probes, Eugene, OR) for 10 min at room temperature. Cells with condensed chromosomes were scored as mitotic.

**Time-Lapse Analysis of Mitosis**

Time-lapse microscopy used the Metamorph Imaging software program to capture a series of individual frames every 20 min (Fig. 1) or every 10 min (Fig. 3) to generate a movie. Digital images were captured using a Photometrics CoolSnap HQ camera. Cells were maintained on an inverted Leica DM IRB microscope with a warming stage heated to 37°C and enclosed in a humidified environmental chamber containing 5% CO₂ at 37°C. An automated shutter system was used to ensure that the cells were exposed to light only while images were captured —10 ms for phase contrast images and ~300 ms for fluorescent images. In Fig. 1, a cell was scored mitotic if it entered metaphase as characterized by loss of almost all contact with the substratum, resulting in the formation of a smooth, round cell (38).

**Reporter Constructs**

cdk1 and cyclin B1 promoter activities were determined by measuring luciferase activity in pools of WT or GSE cells stably transfected with constructs containing regions of the cdk1 or cyclin B1 promoter driving the expression of luciferase (40). Luciferase activity in extracts of stable pools was corrected for total protein concentrations in each lysate, determined by the Bradford method. Luciferase activity in transient transfections was corrected for transfection efficiency, determined by cotransfection of a plasmid constitutively expressing β-galactosidase.

**RESULTS**

**Caffeine Abrogates the G₂ Checkpoint to Induce Mitosis in Cells Lacking p53.** Caffeine inhibits ATM and ATR, both of which have been implicated in turning off CDK1 activity by modulating its phosphorylation state in response to DNA damage (22–24). On the basis of our previous studies, we hypothesized that p53 may block the effects of caffeine by eliminating proteins required for G₂ and M (4, 40, 41). The earlier studies with caffeine relied on FACScanning of DNA content to estimate cell cycle position, and we investigated these effects in more detail (26, 27). HT1080 cells, which contain wild-type p53, were infected with a retrovirus to express GSE56, a COOH-terminal fragment of p53 that acts as a dominant negative. Pools of cells infected with GSE56 (GSE cells) and pools of cells infected with the empty parental LXSN virus (WT cells) were used. In GSE cells, caffeine could overcome the cell cycle arrest induced by etoposide. Direct microscopic examination of WT or GSE cells exposed to...
etoposide for 1 or 2 days revealed very few mitotic cells (data not shown). However, when caffeine was added to etoposide-treated GSE cultures, mitotic cells were evident (data not shown). Time-lapse microscopy revealed that WT cells remained arrested when treated with etoposide and caffeine, and very few cells entered mitosis (Fig. 1). However, up to 18% of the GSE cells entered mitosis when treated with etoposide and caffeine. GSE cells did not enter mitosis until 10 h after adding caffeine, and entry into mitosis continued at a similar rate (~1%/h) until the end of the experiment (Fig. 1).

We used a biochemical assay to confirm that the cells scored by time-lapse analysis were indeed mitotic. High levels of Histone H3, phosphorylated on serine 10 are only observed in mitosis (42, 43). Treatment of either WT or GSE cells with etoposide for 24 h caused a major reduction in the amount of phosphorylated histone H3 (pS10-H3), consistent with the absence of mitotic cells (Fig. 1). Forty h after adding etoposide, the level of pS10-H3 was increased in both cell types, suggesting that some cells had escaped the arrest and had entered mitosis, although more pS10-H3 was evident in GSE cells than in WT cells (Fig. 1). Thus, the loss of p53 appears to reduce the stability of arrest. Exposure of either WT or GSE cells to caffeine for 8 h did not reverse the loss of pS10-H3 observed after 24 h of exposure to etoposide (Fig. 1). However, treatment of GSE cells with caffeine for 16 h caused a large increase in the level of pS10-H3, unlike WT cells in which the phosphorylation of histone H3 was still suppressed. These results confirm that caffeine can override the cell cycle arrest induced by etoposide in cells lacking p53, causing them to enter mitosis.

GSE Cells Cannot Complete Mitosis and Die in Response to Etoposide and Caffeine. FACScanning for DNA content indicated that the cell cycle profiles of untreated WT and GSE cells was similar (Fig. 2). Treatment of WT cells with etoposide for either 24 or 48 h caused them to accumulate mostly in S phase but also in G1 and G2-M (Fig. 2). GSE cells accumulated in S and G2-M in response to etoposide (Fig. 2). The lack of G1 accumulation in GSE cells suggests that WT cells retain a p53-dependent G1 arrest in response to DNA damage. Surprisingly, treatment with caffeine had very little effect on the number of etoposide-treated WT or GSE cells with a 4N (G2-M) DNA content (Fig. 2).

Because GSE cells enter mitosis when treated with caffeine, we expected that the number of cells with a 4N content of DNA would decrease and the number of cells with a 2N content of DNA would increase. However, there are several reasons that caffeine does not change these two parameters in GSE cells. Using GSE cells expressing fluorescently tagged histone H2B, we observed that the mitosis induced by caffeine in the presence of etoposide was approximately four times longer than mitosis that occurs in the absence of treatment (Fig. 3A). Also, some of the GSE cells that entered mitosis after treatment with etoposide and caffeine did not complete mitosis, and reentered interphase without dividing (Taylor and Stark, unpublished data; Fig. 3B). Thus, these cells retain a 4N DNA content. Our time-lapse analysis also revealed that ~66% of the GSE cells that entered mitosis when treated with etoposide and caffeine died before completing mitosis; an example is shown in Fig. 3C. This phenomenon may explain why the number of G1 cells does not increase. Also, caffeine caused a decrease in the number of WT and GSE cells in S phase, perhaps by abrogating S-phase checkpoints. This effect may contribute to the increase in G2-M cells observed in cells treated with caffeine. FACScanning of propidium iodide-stained nuclei indicated that ~15.5% of WT cells and 7.7% of GSE cells contained a sub-G1 content of DNA, indicative of dead cells, after treatment with etoposide, suggesting that p53 contributes to the killing of HT1080 cells by etoposide. Caffeine increased the number of dead WT cells by only 1.1-fold but increased the death of GSE cells treated with etoposide by 2.9-fold. Thus, caffeine enhances the killing of HT1080-derived cells to etoposide, but only after loss of p53 function.

Low Levels of Caffeine Abrogate the G2 Checkpoint but Do not Reverse the Activation of p53 by Etoposide. As described above, HT1080 cells containing wild-type p53 do not escape G2 arrest when exposed to 2 mm caffeine. Caffeine inhibits the protein kinases ATM and ATR, both of which can phosphorylate serine 15 of p53, contributing to its activation (22–24, 44–46).

Fig. 2. DNA content of cells treated with etoposide and caffeine. FACScanning was used to measure DNA content and estimate cell cycle distribution. Cells were treated with etoposide for a total of 40 h and with caffeine for 16 h before fixation. The experiment shown is representative of the three independent experiments performed.

Fig. 3. Cells lacking p53 enter a catastrophic mitosis when treated with etoposide and caffeine. GSE cells were stably transfected with H2B-GFP, and time-lapse fluorescence microscopy was used to visualize the chromatin. A, the average length of mitosis of GSE cells entering mitosis in the presence of etoposide and caffeine, determined by fluorescence microscopy. B, the average length of mitosis is the interval from the start of chromatin condensation to the point at which chromatin became decondensed. C, an example of a GSE cell that died while attempting mitosis.

ROLE OF p53 IN G2 CHECKPOINT CONTROL

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show that p53 was still active in the presence of 2 mM caffeine because it keeps cells from entering mitosis. To test this point, we analyzed the phosphorylation of p53 on serine 15 by the Western technique. Caffeine did not reduce the phosphorylation of p53 at serine 15 when added after cells were treated with etoposide (Fig. 4). Also, the accumulation of p53 that occurred in response to etoposide was not blocked by caffeine (Fig. 4). Thus, under the conditions used, caffeine did not efficiently reverse the phosphorylation of p53 on serine 15 or the accumulation of p53 in response to etoposide. GSE cells contain high levels of p53, as expected from the formation of mixed tetramers of endogenous p53 and the COOH-terminal GSE56 fragment, which contains the tetramerization domain (Fig. 4; Ref. 36). The normal degradation of p53 requires the formation of full tetramers (47). The p53 that accumulated in GSE cells was found to be phosphorylated on serine 15 in untreated samples as well as in samples exposed to etoposide (Fig. 4). We analyzed the level of p21, a transcriptional target of p53, to obtain an additional measure of p53 activity. Treatment of WT cells with etoposide caused an increase in p21 that was not affected by the addition of caffeine, again suggesting that p53 remains active under these conditions (Fig. 4). The expression of p21 was very low in GSE cells, confirming that p53 function was inhibited.

Effect of Pretreatment with Caffeine on the Activation of p53 by Etoposide and G2 Arrest. The persistence of phosphorylated p53 in cells treated with caffeine may be attributable to enhanced stability of p53 that is phosphorylated on serine 15. However, when cells were pretreated for 16 h with caffeine followed by exposure to etoposide, p53 was still phosphorylated on serine 15 (Fig. 5A). p53 became phosphorylated on serine 15 within 1 h of treatment with etoposide, and pretreatment with caffeine reduced only slightly the phosphorylation of p53 (Fig. 5A). GSE cells contain high levels of p53 phosphorylated on serine 15 in the absence or presence of caffeine (Fig. 5A). Very similar results were obtained when WT and GSE cells were pretreated with caffeine for 3 h, followed by etoposide for various times (Fig. 5B). Thus, the phosphorylation of serine 15 of p53 is induced by etoposide even in WT cells pretreated with caffeine at a concentration that allows GSE cells to bypass G2 arrest. We also observed that higher concentrations of caffeine (10 mM) significantly reduced the phosphorylation of p53 on serine 15 in WT cells (Fig. 5C), consistent with the ability of caffeine to inhibit ATM and ATR, suggesting that high levels of caffeine are needed to block the phosphorylation of p53 in response to etoposide.

Once cells have arrested in response to etoposide, p53 helps to maintain the arrest. However, the cell cycle is rapidly blocked in response to DNA damage. This initial event may depend on the blockade to dephosphorylation of tyrosine 15 and threonine 14 of CDK1 as a result of the inactivation of CDC25. We used FACS analysis for pS10-H3 to quantitate the number of mitotic cells (39). Similar numbers of WT (37 ± 7%) and GSE (50 ± 7%) cells were in mitosis 1 h after adding etoposide, suggesting that the rapid G2 arrest is similar in both cell types. A total of 59 ± 19% of WT cells that were pretreated with caffeine for 16 h before adding etoposide was in mitosis 1 h after adding etoposide. Even more, GSE cells (68 ± 12%) pretreated with 2 mM caffeine were still in mitosis, and when compared with WT cells not pretreated with caffeine, this difference was statistically significant (P = 0.05). Thus, p53-dependent pathways and caffeine-sensitive pathways both contribute to the rapid G2 arrest that occurs in response to etoposide. However, the effect of p53 on the initiation of G2 arrest is relatively small compared with its effect on the maintenance of G2 arrest.

Down-Regulation of CDK1 and Cyclin B1 Correlates with Stable G2 Arrest. WT cells treated with etoposide had reduced levels of both CDK1 and cyclin B1 and exposure to caffeine after adding etoposide did not overcome the down-regulation of these proteins (Fig. 4). Cells lacking p53 expressed abundant levels of CDK1 and cyclin B1 after exposure to etoposide (Fig. 4). Consistent with these results, WT cells transfected with reporter constructs for either cdk1 or cyclin B1 showed a reduction in promoter activity when treated with etoposide, whereas neither reporter was repressed in GSE cells (Fig. 6). Caffeine partially abrogated the repression by etoposide of the cyclin B1 promoter in WT but had no effect on the repression of the cdk1 promoter (Fig. 6). Thus, once p53 has repressed the cdk1 and cyclin B1 promoters, caffeine cannot efficiently reverse the effect,
consistent with the continued phosphorylation of p53 on serine 15 and induction of p21 in the presence of 2 mM caffeine. The persistence of CDK1 and cyclin B1 in GSE cells correlates with their entry into mitosis upon treatment with caffeine.

G2 Arrest Induced by Different Topoisomerase II Inhibitors Is Differentially Controlled by p53. Etoposide inhibits topoisomerase II-stabilizing complexes of the enzyme covalently attached to cleaved DNA (48). In contrast, merbarone is a catalytic inhibitor that inhibits the cleavage of DNA by topoisomerase II but does not inhibit binding of the enzyme to DNA (49). Although catalytic inhibitors of topoisomerase II do not create DNA damage as an integral part of their mechanism of action, they still cause G2 arrest at a checkpoint that blocks entry into M when chromosomes are not decatenated (50, 51). The effects of merbarone provide an opportunity to determine whether p53 contributes to this checkpoint. First, we determined the amount of DNA damage using immunofluorescence to detect histone H2A.X phosphorylated on serine 139 (pS139-H2A.X), which is present after DNA is damaged (52). Cells treated with merbarone for 4 h contained ~3.9-fold more pS139-H2A.X than untreated cells, and cells treated with etoposide ~8.3-fold more pS139-H2A.X than untreated cells (Fig. 7). In different nuclei, we detected either diffuse or punctate staining after treatment with either etoposide or merbarone (data not shown). Interestingly, WT cells are much more sensitive to etoposide (LD50 = 0.16 μM) than merbarone (LD50 = 9.1 μM), although both drugs induced similar levels of pS139-H2A.X staining.

Merbarone caused a G2 arrest in both WT and GSE cells (Fig. 8A). Interestingly, caffeine abrogated merbarone-induced G2 arrest and caused both WT and GSE cells to enter mitosis with similar efficiencies (Fig. 8B). Thus, under our conditions, p53 does not contribute to the decatenation checkpoint. Merbarone did cause p53 phosphorylated on serine 15 to accumulate, but the amount was lower than in cells treated with etoposide (Fig. 8C). p21 was induced by merbarone but to a slightly lower level than by etoposide. Importantly, merbarone did not suppress CDK1 (Fig. 8C). Thus, inhibition of topoisomerase II by a catalytic inhibitor can induce p53, but CDK1 persists, which may contribute to the failure of the cells to remain arrested when exposed to caffeine. The lower levels of p53 activation in response to merbarone may be attributable to lower levels of genotoxic stress, as determined by staining for p139H2A.X.

DISCUSSION

Caffeine was shown to abrogate G2 arrest in response to DNA damage two decades ago (25). A probable explanation, uncovered recently, involves the inhibition by caffeine of the ATM and ATR kinases, which transduce the DNA damage signal to CDK1 to block entry into mitosis (22–24). Before ATM and ATR were identified as targets of caffeine, several groups reported that caffeine abrogated G2 arrest only in cells lacking p53 function, suggesting that p53 contributes to G2 arrest in a manner that cannot be overcome with caffeine (26, 27). A role for p53 in G2 arrest in response to DNA damage is consistent with our work showing that overexpression of p53 in the absence of exogenous stress can cause G2 arrest, in part, by repressing
suggesting that caffeine only abrogated G2 arrest in cells lacking p53 of cell cycle regulators such as CDK1. Our observations with caffeine, independent pathways involving ATM and ATR block the activation needed to enter mitosis but remain arrested only because p53-sensitive pathways also help to maintain G2 arrest in response to etoposide. If GSE cells were to lack any mechanism to maintain the arrest, the reduction in pS10-H3 after DNA damage would only be transient.

Caffeine Does not Reverse the Activation of p53 by Etoposide. Serines 15 and 37 of p53 can be phosphorylated by the protein kinases ATM and ATR and both serines do become phosphorylated in response to DNA damage (44–46). Phosphorylation of serines 15, 37, and other NH$_2$-terminal residues of p53 mediate its accumulation after DNA damage by blocking the binding of MDM2, which normally accelerates the degradation of p53 (3). Caffeine blocks not only the phosphorylation of serine 15 but also the accumulation of p53 in response to ionizing radiation, probably because of the inhibition of ATM and ATR (23). In contrast, caffeine did not completely block the phosphorylation of serine 15 or the accumulation of p53 in response to UV radiation (23). This observation suggests that caffeine-insensitive pathways for the activation of p53 are induced by UV radiation.

We observed that the phosphorylation of p53 at serine 15 was not eliminated when 2 mM caffeine were added either before or after adding etoposide. Caffeine at 2 mM did not substantially affect the induction of p21 by p53. Consistent with a role for p21 in the p53-dependent repression of $cdk1$ transcription (41), this level of caffeine also did not alleviate the repression of $cdk1$ by p53. Similarly, p53-dependent repression of the $cyclin\,B1$ promoter and of the levels of $cyclin\,B1$ protein by etoposide was not reversed by subsequent addition of 2 mM caffeine. The persistence of $cyclin\,B1$ and Cdk1 correlates with the ability of 2 mM caffeine to induce mitosis in the HT1080 human tumor cell line. These results also show that, under conditions where caffeine does not eliminate the phosphorylation of p53 at serine 15, that p53 prevented etoposide-treated cells from entering mitosis. Pretreating cells with 10 mM caffeine did reduce the phosphorylation of serine 15 of p53, suggesting that 2 mM caffeine abrogates G2 arrest but may not completely inhibit ATM and ATR in cells exposed to etoposide.

The inability to suppress Cdk1 may also underlie our observations with merbarone, which did not efficiently suppress Cdk1 levels and induced a G2 arrest that could be abrogated by caffeine whether p53 was present or not. Merbarone may not induce the down-regulation of Cdk1 because of its relatively weak activation of p53. Interestingly, merbarone did induce the phosphorylation of histone H2A.X on serine 139, an event that occurs in response to DNA damage. We also observed punctate staining of the phosphorylated H2A.X in response to merbarone, reminiscent of the staining pattern induced by DNA damaging agents (54). Individual foci of staining may represent areas of the genome containing DNA damage.

Unlike merbarone, etoposide stabilizes the cleavage complex of topoisomerase II and DNA, presumably creating more DNA damage. Consistent with this comparison, we observed ~2-fold higher levels of H2A.X phosphorylation in cells treated with etoposide than with merbarone. However, etoposide was ~50-fold more toxic than merbarone. One possible explanation for the much larger difference in toxicity than phosphorylation of H2A.X is that the damage caused by...
merbarone is easier to repair than that caused by etoposide. Both types of damage may induce the phosphorylation of histone H2A.X, but only etoposide-induced damage might be highly toxic. Thus, different foci that are positive for p139H2A.X may differ drastically in their ability to be repaired and their ultimate contribution to cell death. As of yet, we have no direct evidence for this explanation, and others are possible.

Previous work with catalytic inhibitors of topoisomerase II have suggested that because these agents do not directly create DNA damage, some other signal such as catenated chromatin, must be responsible for G2 arrest. In these studies, merbarone was not used, however, our results suggest that merbarone can also trigger the decatenation checkpoint because it inhibits topoisomerase II without directly causing DNA damage, and it causes G2 arrest, which can be abrogated with caffeine. Our studies with merbarone also suggest that p53 does not contribute to the decatenation checkpoint under the conditions studied.

Mitosis in Cells Exposed to Etoposide Is Associated with Pro-longed Metaphase and Death. The cells lacking p53 function that entered mitosis when treated with etoposide and caffeine spent more time in mitosis and completed mitosis to produce viable daughter cells infrequently: ~66% of the cells that entered mitosis died before reaching telophase. Consistent with this observation, caffeine increased the number of dead cells with a sub-G1 content observed after exposure to etoposide but only if p53 was absent. These results underscore the importance of inappropriate entry into mitosis in the cytotoxic effect of combined exposure to etoposide and caffeine. Caffeine has been shown to sensitize p53-mutant cells and not p53-wild-type cells to a variety of DNA damaging agents (26, 27, 53, 55).


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