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Bifunctional DNA Alkylator 1,3-Bis(2-chloroethyl)-1-nitrosourea Activates the ATR-Chk1 Pathway Independently of the Mismatch Repair Pathway

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ABSTRACT

The presence of DNA damage initiates signaling through the ataxia-telangiectasia mutated kinase (ATM) and the ATM- and the Rad3-related kinase (ATR), which phosphorylate, thus activating, the checkpoint kinases (Chk) 1 and 2, which leads to cell cycle arrest. The bifunctional DNA alkylator 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) is cytotoxic primarily by inducing DNA monoadducts and ultimately, interstrand cross-links, which block DNA replication. In this study, we investigated the activation of the ATR-Chk1 pathway in response to BCNU treatment and the dependence of this response on the DNA mismatch repair (MMR) capacity. Medulloblastoma cells were exposed to low and moderate doses of BCNU, and the effects on this DNA damage signaling pathway were examined. In response to BCNU, Chk1 was found to be phosphorylated at serine 345 and exhibited increased kinase activity. Caffeine and wortmannin, which are broad-spectrum inhibitors of ATM and ATR, reduced this phosphorylation. Cell cycle analysis further revealed an accumulation of cells in the S phase in response to BCNU, an effect that was attenuated by caffeine. Small interfering RNA knockdown of ATR also reduced Chk1 phosphorylation after exposure to BCNU. However, knockdown of ATM had no effect on the observed Chk1 phosphorylation, suggesting that ATR was primarily responsible for Chk1 activation. Analysis of Chk1 activation in cells deficient in MMR proteins MutLα or MutSα indicated that the DNA damage response induced by BCNU was independent of the MMR apparatus. This MMR-independent activation seems to be the result of DNA interstrand cross-link formation.

The bifunctional DNA alkylating agent 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) has been an important component of multiple agent adjuvant chemotherapy regimens in the treatment of brain tumors. Because of its high lipophilicity, it has the capacity to cross the blood-brain barrier, essential for any brain tumor therapy. The primary cytotoxic effects of BCNU result from alkylation at the O^6 position of guanine in DNA, and the subsequent conversion of these monoadducts into DNA interstrand cross-links between the N^1-position of guanine in one strand and the N^3 position of cytosine in the opposite strand of DNA (Fischhaber et al., 1999). Monoadducts are also formed as a result of alkylation at the N^7 and N^1 positions of guanine, as well as at the phosphotriester backbone.

The ataxia-telangiectasia-mutated kinase (ATM) and the ATM- and Rad3-related kinase (ATR) are members of the phosphatidylinositol-3-kinase-related kinase (PIKK) family (Abraham, 2004). Checkpoint kinase 1 (Chk1) is a major effector of normal S-phase progression and is also involved in G_2/M-phase checkpoint signaling in response to DNA damage (Bartek and Lukas, 2003). ATM and, mainly, ATR have been shown to be upstream activators of Chk1 in response to various types of genotoxic stress (Abraham, 2004). The DNA damage response to DNA methylating agents of the unimolecular nucleophilic substitution (S_N1) type, like temozolomide, has been relatively well defined (Caporali et al., 2004; Stojic et al., 2004a,b, 2005). At low doses, these agents activate the ATR-Chk1 pathway in a manner that is dependent upon the presence of an intact DNA mismatch repair (MMR) apparatus. However, at high doses, this activation becomes independent of the MMR system. It has been further established that the DNA adduct O^6-methylguanine (O^6-MeG) provides the major signal for the elicited DNA damage signaling response (Yoshioka et al., 2006).

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ABBREVIATIONS: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; ATM, ataxia-telangiectasia-mutated kinase; ATR, ATM- and Rad3-related kinase; PIKK, phosphatidylinositol-3-kinase-related kinase; MMR, DNA mismatch repair; O^6-MeG, O^6-methylguanine; O^6-BG, O^6-benzylguanine; AGT, O^6-alkylylguanine DNA alkyltransferase; siRNA, small interfering RNA; S_N1, unimolecular nucleophilic substitution.

The formation of DNA interstrand cross-links produces a physical block to both replication and transcription, because the two DNA strands are unable to unwind, which accounts for the high toxicity of antitumor agents that produce these lesions. Because of this replication block, it is not surprising that cells are unable to progress through S phase. For agents such as photoactivated psoralens and mitomycin C, S-phase arrest is not merely a result of mechanical stalling of replication forks but is caused by the activation of the S-phase checkpoint (Joerges et al., 2003; Mladenov et al., 2007). The S-phase arrest is regulated by the ATR kinase, which mediates arrest through two parallel pathways, one involving Chk1 and the other involving DNA repair proteins NBS1-FANCD2 (Fanconi anemia, complementation group D2), actively effecting cell cycle arrest at the S phase (Pichierri and Rosselli, 2004).

Although the mechanisms whereby BCNU exerts its cytotoxicity are relatively well defined, the DNA damage signaling pathway that is activated in response to BCNU treatment remains largely unelucidated. As a bifunctional DNA alkylating agent, BCNU inflicts two major types of DNA damage upon the cell: DNA monoadducts and interstrand cross-links. We hypothesized that, unlike temozolomide, low doses of BCNU could activate the ATR-Chk1 pathway in a manner that is independent of the MMR apparatus because DNA interstrand cross-links induced by BCNU present a block to the replicating DNA forks. In this study, we have evaluated the effect of BCNU on the ATR-Chk1 pathway and its dependence on the MMR system in MMR-proficient or -deficient medulloblastoma or rhabdomyosarcoma cells. We have demonstrated that Chk1 is activated largely through ATR and that this activation is independent of DNA MMR proteins and likely to be triggered by DNA interstrand cross-links.

Materials and Methods

Chemicals and Reagents. BCNU (Sigma, St. Louis, MO), temozolomide (Schering-Plough, Kenilworth, NJ), O^6-benzylguanine (O^6-BG) (kindly supplied by Dr. Robert C. Moschel, National Cancer Institute, Bethesda, MD) and wortmannin were dissolved in dimethyl sulfoxide and freshly prepared each time before use. Caffeine (Sigma) was dissolved in phosphate-buffered saline.

Cell Lines and Limiting Dilution Assay. The medulloblastoma cell lines D341 MED, D341 MED (BR), D283 MED, D487 MED, and Daoy and the rhabdomyosarcoma cell lines TE-671 and TE-671 (OTR) were maintained in improved minimal essential medium, zinc option (Richter's modification) (Invitrogen, Carlsbad, CA) supplemented with 20% fetal bovine serum (Invitrogen). Human cervical carcinoma cell line HeLa was maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum. The cytotoxicity of BCNU against D341 MED cells was determined by the limiting dilution assay as described previously (Friedman et al., 1992).

Drug Treatments. For drug treatments, logarithmically growing cells were used and incubated with various concentrations of BCNU, temozolomide, caffeine, or wortmannin for the time periods indicated followed by washing in fresh medium and subsequent incubation. Where indicated, cells were pulsed with 100 μM O^6-BG for 10 min before treatment with BCNU or temozolomide and maintained in 10 μM O^6-BG.

***O*^{6}-Alkylylguanine DNA Alkyltransferase Activity Assay.** The activity of AGT was measured as described previously (Dolan et al., 1990). A ^{3}H-methylated DNA substrate, prepared by reaction of calf thymus DNA with [*methyl*-^{3}H]nitrosourea, was incubated with cell-free extract for 30 min at 37°C. The DNA was precipitated by adding ice-cold 0.25 M perchloric acid and then incubated with 500 μl of 0.1 M HCl at 70°C for 30 min. The modified bases were separated by reversed-phase high-performance liquid chromatography and quantified by scintillation counting. The enzyme activity was defined as femtomoles of O^6-[*methyl*-^{3}H]guanine removed from ^{3}H-methylated DNA per milligram of extracted protein.

Immunoblotting Studies. Whole-cell extracts for immunoblot analyses were prepared as described previously (Stommel and Wahl, 2004). The following antibodies were used for immunoblotting studies: anti-AGT antibody (Kamiya Biochemical Co., Seattle, WA), anti-MSH2 antibody (Calbiochem, La Jolla, CA), anti-MSH6 antibody (BD Biosciences, San Jose, CA), anti-MLH1 antibody (Calbiochem), anti-PMS2 antibody (Calbiochem), anti-ATR antibody (GeneTex, San Antonio, TX), anti-ATM antibody (Gene Tex), anti-Chk1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-phospho-Chk1 (Ser345) antibody (Cell Signaling Technology, Danvers, MA). Immunoblotting studies were carried out as described previously (Schild-Poulter et al., 2003).

Checkpoint Kinase Activity Assay. D341 MED or Daoy cells were treated with 20 μM BCNU and, 24 h after treatment, whole-cell extracts were prepared as described previously (Stommel and Wahl, 2004) and immunoprecipitations using anti-Chk1 antibody (Santa Cruz Biotechnology) were carried out as previously depicted (Schild-Poulter et al., 2003). Equal amounts of total protein (500 μg) were immunoprecipitated using the indicated antibodies. Assay for the activities of immunoprecipitated Chk1 was performed using K-LISA Checkpoint Activity Kit as instructed by the manufacturer (Calbiochem).

Small Interfering RNA Transfection Assays. The siRNA sequences for Chk1 (Zhao and Piwnica-Worms, 2001) and for ATM and ATR (Zhang et al., 2005) were described previously and supplied by Dharmacon (Lafayette, CO). The siRNA sequence for nonspecific control was 5′-AUGAACGUGAAUUGCUCAAUU-3′ (Dharmacon). The siRNA sequences for MLH1 were a pool of three target-specific siRNA sequences (Santa Cruz Biotechnology). For transfection experiments, logarithmically growing D341 MED or Daoy cells were plated in 60-mm dishes at a density of 1×10^6 cells per dish in Opti-MEM (Invitrogen). After 24 h, cells were transfected with the indicated siRNA duplexes at a final concentration of 67 nM by using Oligofectamine (Invitrogen). Cells were typically used for experiments after 48 to 72 h of siRNA treatment.

Cell-Cycle Analysis. Treated cells were washed once with phosphate-buffered saline, trypsinized, and washed again in phosphate-buffered saline with 2% fetal bovine serum and fixed in ice-cold ethanol for at least 1 h at −20°C, washed, and stained with propidium iodide (30 μg/ml) and treated with RNase (0.6 mg/ml) in phosphate-buffered saline plus 0.5% (v/v) Tween 20 and 2% fetal bovine serum. Stained cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) using Cellquest software, and the ModFit program (Verity Software House Inc., Topsham, ME) was used to analyze the cell-cycle profiles.

Results

Bifunctional DNA Alkylator BCNU Activates Chk1 in Human Medulloblastomas. D341 MED was established in our laboratory from a cerebellar medulloblastoma and is defective in DNA MMR (Bacolod et al., 2004). Our previous study established that high doses of BCNU (100–600 μM) caused extensive lethalities in D341 MED cells (Bacolod et al., 2002). In the current study, we performed limiting dilution assays on D341 MED cells with low doses of BCNU from 5 to 20 μM. We established that BCNU was cytotoxic to D341 MED cells with an LD_{50} of approximately 12 μM (Fig. 1A).

Chk1 is activated in response to various types of DNA

damage and undergoes phosphorylation at serine 317 or 345 (Zhao and Piwnica-Worms, 2001). To investigate whether DNA damage induced by low doses of BCNU could activate Chk1, we exposed D341 MED cells to BCNU over a 100-fold range of concentration from 0.2 μM to 20 μM. Then, 16 to 24 h after drug exposure, cell lysates were prepared and immunoblotted with phospho-specific antibody against phosphoserine 345 of human Chk1. We detected phosphorylated Chk1 in D341 MED cells exposed to BCNU at doses as low as 2 μM (Fig. 1B), indicating that the kinase was activated. We then examined the time course of Chk1 activation by BCNU in D341 MED cells and found that, after exposure to BCNU, the phosphorylated form of Chk1 appeared as early as 4 h, peaked around 16 to 24 h, and was almost undetectable by 48 h (Fig. 1C).

We extended our observations to three other medulloblastoma cell lines, D283 MED, Daoy, and D487 MED and detected phosphorylation of Chk1 at serine 345 in all three cell lines treated with 20 μM BCNU and in Daoy and D487 MED cells treated with 2 μM BCNU (Fig. 1D). We further examined Chk1 kinase activity in D341 MED or Daoy cells 24 h after treatment with 20 μM BCNU. The results showed that the activity of Chk1 in D341 MED cells treated with BCNU was 200 ± 43% over that of controls and that in Daoy cells treated with BCNU was 127 ± 8% over that of controls. These findings together demonstrate that, in medulloblastoma cells, Chk1 is activated in response to low levels of DNA damage induced by BCNU.

***O*^{6}-BG Potentiates Chk1 Activation by BCNU.** Similar to the DNA adduct O^6-MeG formed from S_N1-type methylators, O^6-chloroethylguanine and O^6-hydroxyethylguanine formed in DNA treated by BCNU can be dealkylated by the DNA repair protein AGT (Pegg, 2000). O^6-BG is a low-molecular-weight substrate of AGT and inactivates the protein (Dolan et al., 1990; Pegg, 2000), and pretreatment of cells expressing AGT with O^6-BG sensitizes them to BCNU. The level of AGT protein expression varies in different medulloblastoma cell lines. D341 MED, Daoy, and D487 MED cells all expressed AGT, in contrast to D283 MED cells, which did not (Fig. 2A). D341 MED (BR) is a BCNU-resistant subline of D341 MED. It was generated in our laboratory by treating D341 MED cells with escalating doses of BCNU over time and its increased BCNU resistance is due to a very high level of AGT. AGT activity assays showed that D487 MED, D341 MED, and D341 MED (BR) exhibited an AGT activity of 870 ± 80, 93 ± 13, and 1530 ± 100 fmol/mg of protein, respectively.

D341 MED (BR) and D487 MED cells were exposed to 100 μM O^6-BG for 10 min before treatment with 20 μM BCNU and maintained in 10 μM O^6-BG, a treatment protocol that reduces AGT activities essentially to zero (Bacolod et al., 2002). Chk1 phosphorylation was not observed after treatment with BCNU alone in the high-AGT-expressing D341 MED (BR) cells (Fig. 2B). When AGT was inactivated by O^6-BG in these cells, Chk1 phosphorylation was notably increased. In D487 MED cells, without O^6-BG treatment, we observed a low level of Chk1 phosphorylation after BCNU treatment; however, maximal phosphorylation was observed only when the cellular AGT had been inactivated by O^6-BG. These results demonstrate that the modulation of AGT by O^6-BG potentiates Chk1 activation by BCNU, thus indicating that alkylation at the O^6 position of guanine or the subsequently formed DNA interstrand cross-links are the primary BCNU-induced DNA lesions that trigger Chk1 activation.

ATR Is an Upstream Activator of Chk1 in Response to BCNU. ATM and ATR play overlapping roles in activating Chk1 in response to genotoxic stress, ATR being the major upstream activator of Chk1. Caffeine, a broad-spectrum inhibitor of the PIKK family, has been shown to inhibit the activities of both ATM and ATR (Hall-Jackson et al., 1999; Sarkaria et al., 1999). Wortmannin is another broad-spec-

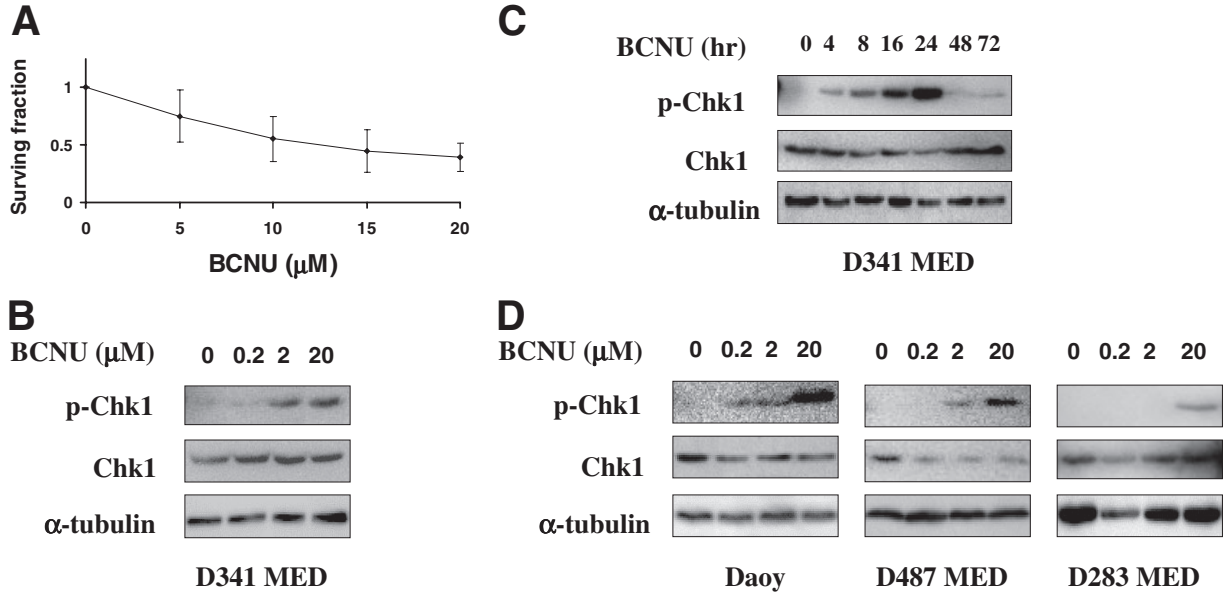

Fig. 1. BCNU induces phosphorylation of Chk1. A, toxicity of low doses of BCNU to D341 MED cells was determined by limiting dilution assay. Each data point represents the average of three independent experiments; bars, ± S.D. B to D, Chk1 is phosphorylated at serine 345 after DNA damage by BCNU. D341 MED cells were exposed to 0.2 to 20 μM BCNU for 16 h (B) or 20 μM BCNU for the indicated periods (C). D, Daoy, D487 MED, or D283 MED cells were exposed to 0.2 to 20 μM BCNU for 16 h. Cell lysates were immunoblotted with anti-phospho-Chk1 antibody (serine 345) (p-Chk1) and anti-Chk1 antibody.

trum inhibitor of the PIKK family, with ATR being least sensitive to its inhibition (Abraham, 2004). To identify the upstream activator(s) of Chk1 in response to BCNU, we explored the cotreatment of D341 MED cells with caffeine or wortmannin and BCNU. D341 MED cells were exposed to 20 μM BCNU with or without 10 mM caffeine, and cells were collected after 6 to 8 h and examined for Chk1 phosphorylation. The results showed that phosphorylated Chk1 was markedly reduced in BCNU-treated D341 MED cells by the addition of caffeine (Fig. 3A). A similar result was observed when cells were treated with wortmannin (Fig. 3B). These results demonstrate that Chk1 activation by BCNU is sensitive to inhibition of the PIKK family.

As caffeine and wortmannin are broad-spectrum inhibitors of PIKK, we transfected D341 MED or Daoy cells with siRNA against ATM, ATR, or scrambled RNA for 48 to 72 h followed by exposure to 20 μM BCNU for 4 to 6 h. Immunoblotting studies with anti-ATR or anti-ATM antibody showed that siRNA against ATR or ATM effectively reduced the level of ATR or ATM in D341 MED cells (Fig. 3C). In these cells, we observed levels of phosphorylated Chk1 induced by BCNU in untransfected cells that were similar to levels in cells transfected with scrambled RNA or siRNA against ATM (Fig. 3C). There was a noticeable reduction in the phosphorylation of Chk1 induced by BCNU in D341 MED cells transfected with siRNA against ATR. Likewise, phosphorylation of Chk1 was markedly reduced in Daoy cells transfected with siRNA against ATR compared with controls or those transfected with scrambled RNA or siRNA against ATM (Fig. 3D). These findings indicate that the phosphorylation of Chk1 is dependent upon ATR, which functions upstream of Chk1 in response to the DNA damage caused by BCNU.

Chk1 Activation by BCNU Is Independent of MMR. In the current study, we established that low doses of BCNU activated the ATR-Chk1 pathway in human medulloblastoma cell lines. Daoy cells were previously shown to have functional MMR, whereas D341 MED cells were MMR-deficient (Bacolod et al., 2004). D283 MED was also defective in MMR activity, a consequence of deficiency in the MMR polypeptides MLH1 and PMS2 (Dong et al., 1999). Immunoblotting of the cell lysates from D341 MED, Daoy, D487 MED, and D283 MED with antibodies against the MMR proteins MutLα and MutSα revealed the two subunits of MutSα (MSH2 and MSH6) to be present in all four cell lines and the two subunits of MutLα (MLH1 and PMS2) to be present only in Daoy and D487 MED (Fig. 4A). No MLH1 and low levels of PMS2 were detected in D341 MED and D283 MED cells, which accounts for the lack of MMR activity observed in these two cell lines.

To determine the MMR dependence of BCNU activation of Chk1, we treated MutLα-deficient D283 MED cells and MutLα-proficient Daoy cells with 20 μM BCNU or 20 μM temozolomide. We observed Chk1 activation by BCNU in both D283 MED and Daoy cells (Fig. 4B). As expected, Chk1 activation by temozolomide was present in MutLα-proficient Daoy cells, but was undetectable in MutLα-deficient D283 cells, which confirms that DNA monoadducts formed from exposure to temozolomide do not activate Chk1 in a MutLα-deficient background. TE-671 (OTR) is a temozolomide-resistant subclone of the rhabdomyosarcoma cell line TE-671 that was established in our laboratory. Immunoblotting studies with anti-MSH2 and MSH6 antibodies revealed a lack of expression of MutSα in TE-671 (OTR) (Fig. 4C). Because both cell lines express high levels of AGT (S. Johnson, unpublished results), we pretreated the paired rhabdomyosarcoma cell lines TE-671 and TE-671 (OTR) with 100 μM O^6-BG for 10 min before exposure to 25 μM O^6-BG with 20 μM BCNU. Chk1 phosphorylation was observed after BCNU treatment in both MutSα-proficient TE-671 and MutSα-deficient TE-671 (OTR) cells, whereas temozolomide induced the phosphorylation of Chk1 only in MutSα-proficient TE-671 cells (Fig. 4D).

Because D283 MED and Daoy cells are different cell lines with different genetic backgrounds and MMR-deficient TE-

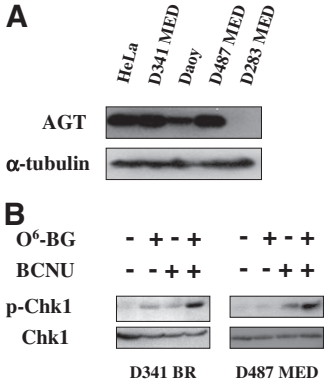

Fig. 2. O^6-BG potentiates the phosphorylation of Chk1. A, AGT expression in D341 MED, Daoy, and D487 MED. Cell lysates were immunoblotted with anti-AGT antibody, HeLa cell lysates were used as positive control for AGT and α-tubulin was used as a loading control. B, cotreatment with BCNU and O^6-BG increases Chk1 phosphorylation. D341 MED (BR) and D487 MED were treated with 20 μM BCNU alone or in combination with 10 μM O^6-BG. Cell lysates were immunoblotted with anti-phospho-Chk1 antibody (serine 345) and anti-Chk1 antibody.

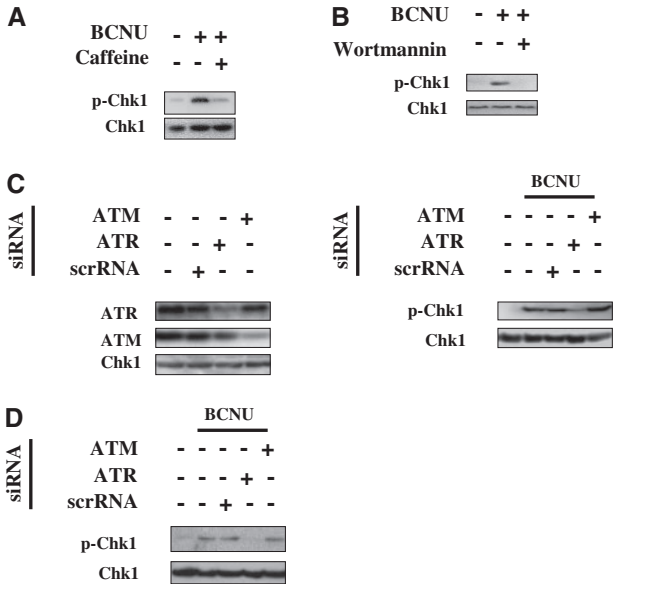

Fig. 3. ATR is involved in the activation of Chk1. D341 MED was treated with 20 μM BCNU alone or in combination with 10 mM caffeine (A) or 30 μM wortmannin (B), and cell lysates were prepared 6 h after treatment and immunoblotted with anti-phospho-Chk1 antibody (serine 345) and anti-Chk1 antibody. D341 MED cells (C) or Daoy cells (D) were transfected with siRNA against ATR, ATM, or a scrambled RNA sequence (scrRNA) and treated with 20 μM BCNU for 4 to 6 h at approximately 48 to 72 h after transfection. The cell lysates were immunoblotted with anti-ATR antibody, anti-ATM antibody, anti-phospho-Chk1 antibody (serine 345), and anti-Chk1 antibody.

671 (OTR) cell line, although a subline of MMR-proficient TE-671 cell line, may have additional genetic variations from its parental cell line, we further confirmed the dependence upon MMR of the activation of the ATR-Chk1 pathway by BCNU by transfecting MMR-proficient Daoy cells with siRNA against the MMR protein MLH1 or with scrambled RNA. The results indicated that siRNA against MLH1 effectively reduced the level of the MMR protein, whereas scrambled RNA exerted no noticeable effect on the level of the protein (Fig. 4, E and F). The level of Chk1 phosphorylation in response to BCNU in Daoy cells transfected with siRNA against MLH1 showed no difference from that in the untreated Daoy cells or the cells transfected with scrambled RNA (Fig. 4F), suggesting that BCNU signaling through the ATR-Chk1 pathway is not dependent upon the MMR apparatus. On the other hand, there was a noticeable reduction in the level of Chk1 phosphorylation in response to temozolomide in Daoy cells transfected with siRNA against MLH1 than that in the untreated Daoy cells or the cells transfected with scrambled RNA, suggesting the dependence upon a functional MMR apparatus of the ATR-Chk1 signaling elicited by temozolomide. Altogether, the above findings suggest that DNA interstrand cross-links induced by BCNU contribute to the activation of the ATR-Chk1 pathway in an MMR-independent manner, whereas O^6-MeG or O^6-ethylguanine adducts require the presence of an intact MMR apparatus to activate the ATR-Chk1 pathway.

BCNU Induces an Accumulation of Medulloblastoma Cells in the S Phase. *S_N1*-type DNA methylators have been shown to induce a prolonged cell cycle arrest at the second G_2/M phase (Caporali et al., 2004). To assess cell cycle perturbations caused by BCNU, we investigated the effects of low doses of BCNU on cell-cycle progression in medulloblastoma cells. D341 MED cells were exposed to 20 μM BCNU and at 4 to 72 h after treatment were processed for cell cycle analysis by flow cytometry. The results showed that the percentage of cells in the S phase increased from 27.54% before treatment to a peak of 49.21% 24 h after treatment (Fig. 5A). We also observed an increase in the number of cells in the S phase in other medulloblastoma cell lines 24 h after BCNU treatment: D283 MED (31 $\pm$ 3%, control group; 42 $\pm$ 2%, BCNU group), D487 MED (34 $\pm$ 1%, control group; 44 $\pm$ 2%, BCNU group), and Daoy (52 $\pm$ 1%, control group; 82 $\pm$ 1%, BCNU group). These findings indicated that BCNU induced an accumulation of medulloblastoma cells in the S phase, suggesting an activation of an intra-S-phase checkpoint.

Because Chk1 phosphorylation in response to BCNU was inhibited by caffeine, we further assessed the effects of caffeine on BCNU-induced cell cycle perturbations. D341 MED cells were treated with 20 μM BCNU alone or in combination with 3, 5, or 10 mM caffeine. The percentage of D341 MED cells in the S phase was 41% in the BCNU group and 25% in the control group (Fig. 5B). Caffeine at 3, 5, or 10 mM decreased BCNU-treated D341 MED cells in the S phase to 34, 27, and 27%, respectively. These data suggest that the S phase block resulting from BCNU treatment is attenuated by caffeine, a broad inhibitor of PIKK kinases.

Discussion

The DNA damage signaling response to BCNU has been largely undefined. In the current study, we addressed this by

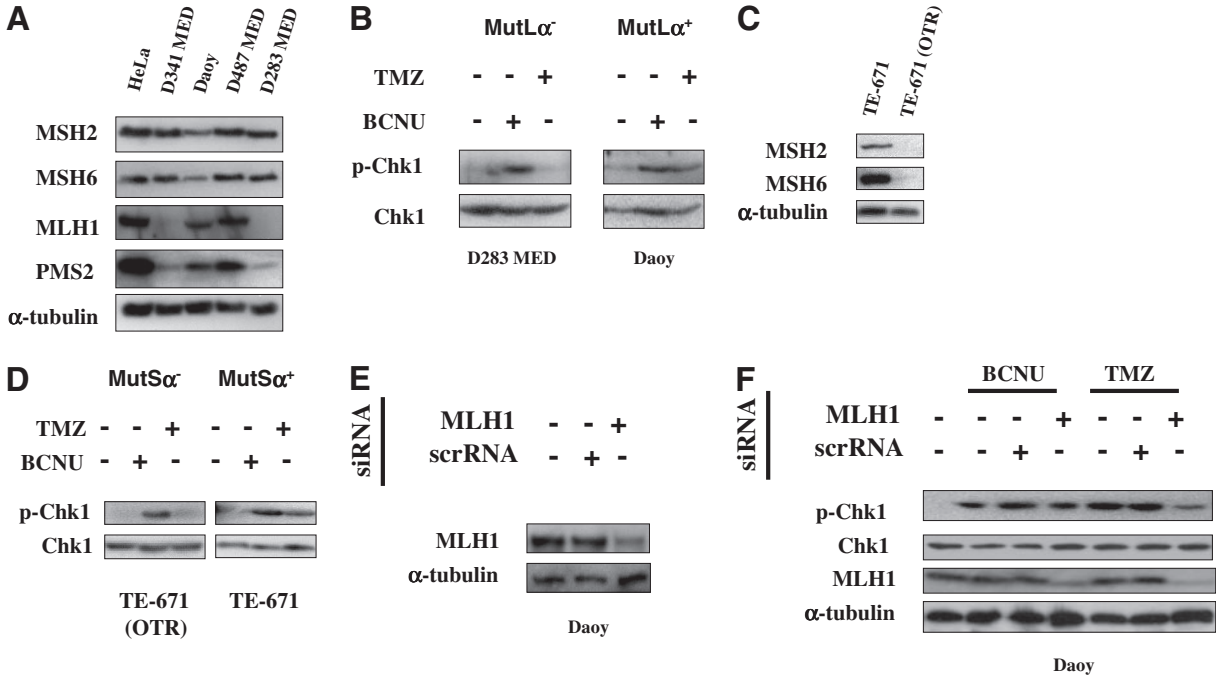

Fig. 4. BCNU-induced activation of Chk1 is independent of MMR proteins. Cell lysates from HeLa, D341 MED, Daoy, D487 MED, and D283 MED (A) or from TE-671 and TE-671 (OTR) (C) were immunoblotted with anti-MSH2, MSH6, MLH1, and PMS2 antibodies. α-Tubulin was used as a marker for loading control, and HeLa cell lysates were used as the positive control for the MMR proteins. D283 MED and Daoy cells (B) or TE-671 and TE-671 (OTR) (D) were treated with 20 μM BCNU or temozolomide (TMZ), and cell lysates were immunoblotted with anti-phospho-Chk1 antibody (serine 345) and anti-Chk1 antibody. E, Daoy cells were transfected with siRNA against MLH1 or scrambled RNA. The cell lysates were immunoblotted with anti-MLH1 and α-tubulin antibodies. α-tubulin was used as a marker for loading control. F, Daoy cells were transfected as in E. Transfected and untransfected cells were treated with 20 μM BCNU or temozolomide. The cell lysates were immunoblotted with antibodies against phospho-Chk1 (serine 345), Chk1, MLH1, and α-tubulin. α-tubulin was used as a marker for loading control.

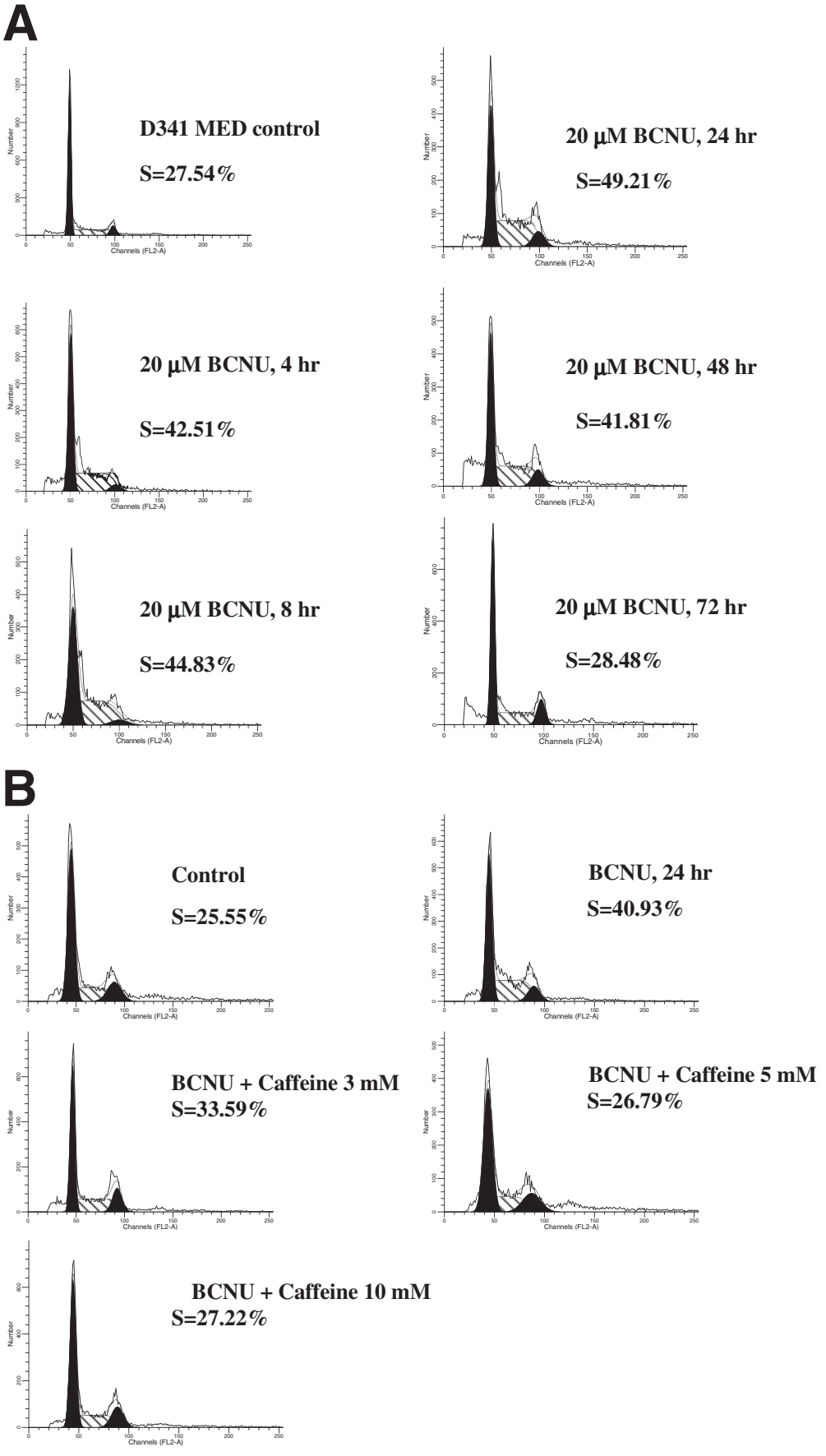

Fig. 5. Cell cycle perturbations caused by BCNU. A, D341 MED cells were treated with 20 μM BCNU for 1 h, and the cells were harvested and processed for flow cytometric analysis of DNA content at the indicated time points. The cell cycle profiles shown were representative of at least three independent experiments. B, D341 MED cells were treated with 20 μM BCNU alone or in combination with 3, 5, or 10 mM caffeine and processed for flow cytometry 24 h after treatment. The cell cycle profiles shown are representative of at least three independent experiments. Solid areas, G_1 phase or G_2/M phase; diagonal lines, S phase.

examining the DNA damage checkpoint response induced by the bifunctional DNA alkylator BCNU. We established that DNA interstrand cross-links induced by BCNU activate the ATR-Chk1 pathway. We further demonstrated that, unlike *S_N1*-type DNA methylators, the activation of the ATR-Chk1 pathway by BCNU is independent of the MMR system. Furthermore, our data indicate that, in response to DNA damage induced by low doses of BCNU, there is an activation of intra-S-phase checkpoint.

Chk1 is a major effector of normal S-phase progression, and ATR, a pivotal upstream regulator of Chk1, activates Chk1 by phosphorylating it at serine 317 and 345 in response to DNA damage (Zhao and Piwnica-Worms, 2001; Bartek and Lukas, 2003). *S_N1*-type DNA methylators, such as temozolomide or *N*-methyl-*N*′-nitro-*N*-nitroso-guanidine, signal through ATR, which phosphorylates, thus activating, the downstream effector kinase Chk1 and triggering a cell cycle arrest at the second G_2/M checkpoint (Caporali et al., 2004; Stojic et al., 2004a; Adamson et al., 2005). The *O^6*-MeG DNA adducts formed from exposure to *S_N1*-type methylators provide the major signal for the activation of the ATR-Chk1 pathway (Yoshioka et al., 2006) and are also the major elicitor of apoptotic responses in cells (Hickman and Samson, 2004). The activation of the ATR-Chk1 pathway and the G_2/M checkpoint by low doses of *S_N1*-type DNA methylators and the triggered apoptotic responses hinge upon a functional MMR apparatus (Hickman and Samson, 2004; Stojic et al., 2004b). A recent study has also shown that whereas temozolomide-induced apoptosis was dependent upon the presence of MMR proteins, the cell death induced by the DNA bifunctional alkylator 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea showed no such dependence on MMR proteins (Takagi et al., 2008).

BCNU induces two major types of genotoxic damage to the cell: DNA monoadducts and DNA interstrand cross-links. The *O^6*-ethylguanine adducts produced by BCNU could activate the ATR-Chk1 pathway in a fashion similar to that demonstrated by *O^6*-MeG. We also observed Chk1 phosphorylation in cells treated with the monofunctional alkylator ethylnitrosourea only if they were MMR-competent (B. Cui, unpublished results). The ATR-Chk1 DNA damage signaling pathway has been shown to be activated by DNA lesions such as those caused by ultraviolet light that stall replication forks (Stiff et al., 2006), and it has also been observed to be activated by other agents, including photoactivated psoralens and mitomycin C, that induce DNA interstrand cross-links; however, these types of activation are independent of DNA MMR (Akkari et al., 2000; Pichierri and Rosselli, 2004). Although psoralens, mitomycin C, BCNU, and phosphoramide mustard all produce DNA interstrand cross-links, the physical structures of these interstrand cross-links are quite dissimilar. It would seem likely that the stalled replication forks, rather than recognition of the DNA interstrand cross-links, result in the MMR-independent activation of the ATR-Chk1 pathway.

Chk1 has been implicated in the control of the intra-S-phase checkpoint (Zhao et al., 2002; Robinson et al., 2006), and ATR is a key member of the intra-S-phase checkpoint pathway in response to genotoxic stress (Cliby et al., 2002). DNA damage detected during S phase rapidly slows down S-phase progression and ongoing DNA synthesis. DNA damage may be readily repaired by homologous recombination in the S phase because replication intermediates are perfect substrates for recombination repair (Johnson and Jasin, 2000). DNA interstrand cross-links induced by photoactivated psoralens have been shown to elicit the activation of S-phase checkpoint (Akkari et al., 2000; Pichierri and Rosselli, 2004), and these cross-links are repaired in the context of DNA replication during S phase (Cipak et al., 2006). BCNU has been shown to induce sister chromatid exchanges, and there has been good correlation between cytotoxicity and induction of sister chromatid exchanges for bifunctional nitrosoureas (Biegel et al., 1982). In the current study, we have shown that BCNU induced a dramatic accumulation of medulloblastoma cells in the S phase, suggesting the activation of an intra-S-phase checkpoint. These observations together suggest that DNA interstrand cross-links induced by BCNU contribute to the activation of an intra-S-phase checkpoint, which slows down S-phase progression to allow repair of DNA damage.

However, there have been conflicting results about the effect of BCNU or other bifunctional nitrosoureas on cell cycle checkpoint activation. Another bifunctional DNA alkylator, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, was found to induce a dramatic accumulation of HeLa cells in the S phase 24 h after exposure to 25 μM concentrations of the drug and a marked accumulation of a PMS2-deficient subclone of HeLa cells in the S phase 24 h after exposure to 12.5 μM BCNU (Aquilina et al., 1999), suggesting that the activation of S-phase checkpoint by the bifunctional nitrosourea is independent of the MMR apparatus. On the other hand, BCNU with *O^6*-BG was shown to elicit a prolonged cell cycle arrest at the G_2/M checkpoint in two MMR-deficient cell lines, HCT116 and HCT 15 (Yan et al., 2005). This study used a high dose of BCNU (50 μM). At low doses of *S_N1* methylators, activation of the ATR-Chk1 pathway occurs only in MMR proficient cells. In contrast, high doses of *S_N1*methylators have been shown to trigger an MMR-independent activation of the ATR-Chk1 pathway and G_2/M checkpoint as observed by others (Stojic et al., 2005).

From the results of this study and the observations by others, we propose the following model for DNA damage signaling response to the bifunctional DNA alkylator BCNU. In both MMR-proficient tumors and MMR-deficient tumors, the BCNU-induced stalled replication forks that result from DNA interstrand cross-links signal to the upstream activator ATR to phosphorylate Chk1 and induce the activation of an intra-S-phase checkpoint. Thus, S-phase progression is halted so that DNA repair can proceed. If DNA repair cannot be effected, the cells are committed to die. One important implication arising from these observations is that pretreatment with S-phase-specific inhibitors may potentiate BCNU-induced cytotoxicity. In this regard, the broad-spectrum PIKK inhibitor caffeine was shown to effect a 10-fold potentiation of BCNU cytotoxicity in both BCNU-sensitive and BCNU-resistant cells (Aida and Bodell, 1987).

The effectiveness of BCNU has been hampered by the emergence of resistance in tumors during treatment. Besides high levels of AGT activities in tumors that affect the clinical response of gliomas to BCNU (Esteller et al., 2000), there are other unknown mechanisms of resistance at work (Silber et al., 1992; Bacolod et al., 2002, 2004). Recent study has shown that BCNU-resistant cancer cells may be sensitized to temozolomide as a result of the development of enhanced MMR

capacity (Yamauchi et al., 2008). Further investigations will be required to provide insight into the repair mechanisms of DNA interstrand cross-links and their contribution to BCNU resistance. The next crucial question to be resolved is the determination of what proteins activate ATR in response to DNA interstrand cross-links, the elucidation of which would provide potential novel molecular targets for drug development.

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