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BUSULFAN SELECTIVELY INDUCES CELLULAR SENESCENCE BUT NOT APOPTOSIS IN WI38 FIBROBLASTS VIA A P53-INDEPENDENT BUT ERK-P38 MAPK-DEPENDENT MECHANISM

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Running title: Erk and p38 MAPKs mediate busulfan-induced senescence

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Abbreviations: SA-β-gal, senescence associated β-galactosidase; siRNA, small interference RNA; CDK, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated or extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase (also called stress-activated protein kinase, SAPK); MKK, mitogen-activated protein kinase kinase; α-PFT, α-pifithrin; PD98059, 2'-amino-3'-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; SP600125, 1,9-pyrazoloanthrone; H2AX, histone 2AX; γH2AX, phosphorylated histone 2AX; DNA DSBs, DNA double strand breaks; Rb, retinoblastoma; BrdU, 5-bromo-2'-deoxyuridine.

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ABSTRACT

Busulfan (BU) is a unique alkylating agent that primarily targets slowly proliferating or non-proliferating cells in the body, leading to various normal tissue damage while killing leukemia cells. However, the mechanism(s) of action whereby BU injures normal cells has not been well defined and therefore, was investigated in the present study by using the normal human diploid WI38 fibroblasts as a model system. We found that WI38 fibroblasts incubated with BU (from 7.5 to 120μM) for 24 h underwent senescence but not apoptosis in a dose-independent manner, whereas cells incubated with 80μM and 20μM etoposide (Etop) committed to apoptosis and senescence, respectively. The induction of WI38 cell senescence by Etop was associated with p53 activation and could be attenuated by down-regulation of p53 using α-pifithrin (α-PFT) or p53 small interference RNA (siRNA). In contrast, WI38 cell senescence induced by BU was associated with prolonged activation of extracellular signal-regulated kinase (Erk), p38 mitogen-activated protein kinase (p38) and c-Jun NH_2-terminal kinase (JNK), and could be suppressed by the inhibition of Erk and/or p38 with PD98059 and/or SB203580, respectively. However, inhibition of p53 with α-PFT or p53 siRNA or JNK with SP600125 failed to protect WI38 cells from BU-induced senescence. These findings suggest that BU is a distinctive chemotherapeutic agent that can selectively induce normal human fibroblast senescence through the Erk and p38 pathways.

INTRODUCTION

Busulfan (BU) is a bifunctional alkylating agent that has been widely used for the treatment of patients with chronic myelogenous leukemia and in conditioning regimens prior to bone marrow transplantation (Buggia et al., 1994; Down and Ploemacher, 1993). Unlike other commonly used chemotherapeutic agents, BU is unique because it primarily targets slowly proliferating or non-proliferating cells. As such, it causes various normal tissue injuries while killing leukemia cells. The common side effects associated with BU treatment include severe myelosuppression, hepatic venoocclusive diseases, pulmonary fibrosis, hemorrhagic cystitis, sterility and cataracts, which limit its therapeutic efficacy as an anti-tumor agent (Bishop and Wassom, 1986; Morgan et al., 1991). However, the mechanism(s) of action by which BU injures normal tissues has not been well established.

As an alkylating agent, it is anticipated that BU can cause DNA alkylation leading to DNA-DNA and DNA-protein cross-links (Iwamoto et al., 2004; Mertins et al., 2004). Subsequently, these cross-links may trigger DNA damage responses and cause apoptosis or cell death through the p53 pathway. However, this mode of action was not supported by our recent studies (Meng et al., 2003a), where we found that, unlike other chemotherapeutic agents and ionizing radiation, BU does not induce significant apoptosis but senescence, in one of its primary targets, e.g. hematopoietic stem cells (HSCs) independent of p53 activation (Meng et al., 2003b). This finding is consistent with the previous observation showing that no significant increase in apoptosis was detected in bone marrow biopsies from chronic myelogenous leukemia patients receiving BU chemotherapy (Thiele et al., 1997). However, it is not known if induction of senescence is a specific cellular response of HSCs to BU or if it can occur in other normal cells when the cells are treated with BU. Nor have the molecular pathways mediating BU-induced

cellular senescence been defined. Therefore, in the present study we used the normal human diploid WI38 fibroblasts as a model system to determine whether BU treatment causes apoptosis or senescence in WI38 cells and to elucidate the molecular mechanisms by which BU induces cellular senescence, because extensive knowledge about senescence pathways has been well delineated using this cell line (Serrano et al., 1997; Iwasa et al., 2003; Beausejour et al., 2003).

The results from our studies show that the response of WI38 cells to BU treatment is similar to that of HSCs. Both cell types undergo senescence but not apoptosis after exposure to BU in a dose-independent manner. In contrast, exposure of WI38 cells to a high dose of etopside (Etop) induces apoptosis and to a low dose of Etop causes senescence, a typical cellular response to treatment with a cytotoxic chemotherapeutic agent (Han et al., 2002; Rebbaa et al., 2003; Zheng et al., 2004; Roninson, 2002; Roninson et al., 2001; Robles and Adami, 1998; Robles et al., 1999). Further investigations reveal that BU induces WI38 senescence independent of p53 as seen in HSCs (Meng et al., 2003b), whereas Etop relies on the p53 pathway to induce WI38 cell senescence. However, BU treatment strongly induced extracellular signal-regulated kinase (Erk) activation followed by sequential activation of p38 mitogen-activated protein kinase (p38 MAPK or p38) and c-Jun NH_2-terminal kinase (JNK) and up-regulation of $p16^{Ink4a}$ (p16). Inhibition of Erk and/or p38, but not that of JNK, significantly attenuated the BU-induced increase in senescence associated β-galactosidase (SA-β-gal) staining, up-regulation of p16 and development of senescent morphology, even though neither inhibitor immediately released the cells from BU-induced growth inhibition. Upon the removal of Erk and/or p38 inhibitors from the WI38 cells that had been treated with BU, the cells proceeded to cellular proliferation and 5-bromo-2'-deoxyuridine (BrdU) incorporation, indicating that suppression of Erk and/or p38 inhibits BU-induced senescence. To our knowledge, this is the first study demonstrating that BU

is a distinctive chemotherapeutic agent that can selectively induce cellular senescence in normal human fibroblasts through the Erk and p38 MAPK pathways.

MATERIALS AND METHODS

Reagents: Selective inhibitors for p53 (α-pifithrin, α-PFT), Erk (PD98059, 2'-amino-3'-methoxyflavone), p38 (SB203580, 4-[4-fluorophenyl]-2-[4-methylsulfinylphenyl]-5-[4-pyridyl]1H-imidazole) and JNK (SP600125, 1,9-pyrazoloanthrone) were purchased from Calbiochem (San Diego, CA). BU, Etop and BrdU were obtained from Sigma (St. Louis, MO). Antibodies against phosphorylated p53 and Erk and those against total p53, Erk, p38, JNK and $p21^{Cip1/WAF1}$ (p21) were obtained from Cell Signaling (Beverly, MA); anti-phosphorylated p38 and JNK from Promega (Madison, WI); anti-β-actin from Santa Cruz (Santa Cruz, CA); anti-p16 from BD PharMingen (San Diego, CA); anti-BrdU from Sigma; anti-γH2AX from Upstate (Lake Placid, NY); goat anti-rabbit IgG-HRP, rabbit anti-goat IgG-HRP, Texas Red Dye-conjugated goat anti-mouse IgG and FITC-conjugated anti-mouse IgG from Jackson ImmunoResearch (West Grove, PA).

Cell Culture: WI38 cells (human embryonic lung diploid fibroblasts) originally obtained from ATCC (Manassas, VA) were cultured in minimum essential medium (MEM; from Invitrogen, Carlsbad, AC) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator at 37 °C and 5% CO_2. Cells at early passages (<25 passages) were used in all experiments to avoid complications of replicative senescence as WI38 cells have a mean lifespan about 45-60 population doublings. For the induction of senescence, cells at about 70% confluence were exposed to varying concentrations of BU (7.5-

120μM) or Etop (20 or 80μM) for 24 h. The cells were washed once with PBS to remove drug and re-cultured in fresh medium for various durations as indicated in each experiments.

siRNA treatment: WI38 cells were transfected with 20μM scrambled sequence (siCONTROL Non-Targeting siRNA #1) or p53 siRNA (siGENOME SMART pool reagent for Human TP53) (Dharmacon, Chicago, Il) using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Two days after the transfection, they were exposed to either BU (120μM) or Etop (20μM) as described above.

Analysis of cell proliferation: WI38 cells were seeded in wells of a 24-well plate at 60,000 cells/well and cultured overnight. They were treated with BU for 24 h as described before, after which the drug was removed and the cells were cultured in fresh medium for up to 11 days. Every 3 days the cells were trypsinized, counted and sub-cultured again in a fresh medium at 1:5 and 1:2 dilutions for control untreated and BU- or Etop-treated cells, respectively.

Cell cycle analysis: Cell cycle analysis was down using propidium iodide staining and a flow cytometer as previously described (Wang et al., 2004).

Analysis of activated caspase 3: WI38 cells were cultured on chamber slides to sub-confluence and then exposed to BU or Etop for 24 h. Activation of caspase 3 was determined using a kit containing the SR-DEVD-FMK-FLICA reagent (Invitrogen) per the manufacturer's instructions. The cells were viewed using an Olympus BX61 fluorescent microscope. Images were captured with a Hamamatsu C4742-95 digital camera (Hamamatsu Photonics, Japan). Captured images were processed using Side Book 4.0 Digital Microscopy software (Intelligent Imaging Innovations, Denver, CA) and displayed using Adobe Photoshop version 6.0 (Adobe Systems, San Jose, CA).

SA-β-gal staining: SA-β-gal activity was determined using a SA-β-gal staining kit from Cell Signaling according to the manufacturer's instructions. Senescent cells were identified as blue-stained cells by standard light microscopy and a minimum of 1,000 cells was counted in 10 random fields to determine the percentage of SA-β-gal positive cells.

BrdU incorporation assay: DNA synthesis in control untreated and BU- or Etop-treated cells was determined by measuring BrdU incorporation into DNA as previously described (Wang et al., 2004). Images of BrdU and Hoechst 33342 nuclear staining were captured and processed similarly as to these described above for the analysis of activated caspase 3. To calculate the percentage of BrdU positive cells, a minimum of 1000 cells was counted in 8 random fields on a slide and the number of BrdU positive cells was divided by the total number of cells counted.

Analysis of DNA double strand breaks (DNA DSBs): WI38 cells were cultured on chamber slides to sub-confluence and then exposed to BU or Etop for up to 24 h. For determination of DNA DSBs, cells were fixed with ice cold 95% ethanol/5% acetic acid for 5 min, blocked with 3% BSA in TBS for 30 min, incubated with 2μg/ml of anti-phosphorylated histone 2AX (γH2AX) (at Ser 139) antibody (Upstate) for 1 h and washed three times with TBS. FITC-conjugated goat anti-mouse secondary antibody was then added and the cells were incubated in the dark for 1.5 h, rinsed with TBS and counterstained with Hoechst 33342 for nuclear detection as described previously. The total number of γH2AX foci and number of γH2AX positive cells were quantified for each treatment by counting a minimum of 1000 cells from 8 randomly chosen fields and expressed as a percentage of γH2AX positive cells and average γH2AX foci per cell. The number of γH2AX foci per cell closely correlates to the number of DNA DSBs induced in a cell (Rogakou et al., 1998; Rogakou et al., 1999).

Western blot analysis: Lysates of WI38 cells were prepared and analyzed as previously described (Wang et al., 2004).

Statistical Analysis: The data were analyzed by analysis of variance. In the event that analysis of variance justified post hoc comparisons between group means, these were conducted using the Student-Newman-Keuls test for multiple comparisons. For experiments in which only single experimental and control groups were used, group differences were examined by unpaired Student's *t* test. Differences were considered significant at $p < 0.05$. All of these analyses were done using GraphPad Prism from GraphPad Software, Inc. (San Diego, CA).

RESULTS

BU induces WI38 cell senescence but not apoptosis

Exposure of WI38 cells to BU for 24 h resulted in a dose-dependent inhibition of cell proliferation (Fig. 1A). The inhibition was permanent since the growth of BU-treated cells remained inhibited even though the drug had been removed from the culture for up to 11 days. The growth inhibition was not due to an induction of apoptosis, since no change in caspase 3 activation (Fig. 1B) and sub-G0/1 cells (Fig. 1C) was observed in WI38 cells that had been incubated with either a high dose (120μM) or a low dose (30μM) of BU. Instead, the cells were arrested in the G2/M phase and proceeded to senescence evidenced by the decrease in BrdU incorporation, appearance of senescent morphology and increase in SA-β-gal staining (Figs. 1C & D). In contrast, WI38 cells underwent apoptosis after exposure to a high dose of Etop (80μM) (Fig. 1B and data not shown) and senescence to a low dose of Etop (20μM), respectively (Figs. 1B-D). These findings indicate that the lack of apoptotic response to BU is not due to an inherited deficiency of the apoptotic machinery in WI38 cells, but suggest that that BU is a

distinctive chemotherapeutic agent that can selectively induce cellular senescence but not apoptosis in WI38 cells.

BU induces senescence in WI38 cells via a p53-independent mechanism

Induction of senescence by a chemotherapeutic agent has been largely attributed to the induction of DNA damage via the p53 pathway (Roninson et al., 2001). BU is an alkylating agent that can cause DNA-DNA and DNA-protein cross-links (Iwamoto et al., 2004; Mertins et al., 2004). However, whether the cross-links can lead to DNA strand breaks and the subsequent activation of p53 has not been examined. Histone 2AX (H2AX) phosphorylation is a well-recognized marker for DNA DSBs (Rogakou et al., 1998; Rogakou et al., 1999). By examining γH2AX using immunohistochemistry, we investigated the induction of DNA DSBs in WI38 cells that were exposed to 120μM BU or 20μM Etop. As shown in Fig. 2, treatment of WI38 cells with either BU or Etop resulted in a time-dependent induction of H2AX phosphorylation, which peaked around 8 h and completely resolved at 24 h after the treatment. However, 8 h after the drug treatment the percentage of WI38 cells stained positive for γH2AX (BU: 35.3% vs. Etop: 74.1%) and average number of γH2AX foci detected in each cell (BU: 5.1 vs. Etop: 14.7) were significantly lower in BU-treated cells than in Etop-treated cells ($p<0.01$), indicating that BU is a less potent inducer of DNA DSBs compared to Etop. Consistent with this finding, exposure of WI38 cells to Etop induced a more rapid and robust activation of p53 than BU (Fig. 3A). Specifically, only moderate levels of p53 phosphorylation were detected in BU-treated cells 24 h after BU treatment, whereas high levels of p53 phosphorylation were found in Etop-treated cells within 2 h of Etop treatment. However, both BU and Etop treatment induced the expression of p21 and p16 and the induction of these cyclin-dependent kinase (CDK) inhibitors has been

implicated in the initiation and maintenance of cellular senescence (Sharpless and DePinho, 1999; Campisi, 2005).

Next, we investigated if BU induces WI38 fibroblast senescence via the p53 pathway. WI38 cells were treated with either BU (120μM) or Etop (20μM) in the presence or absence of 20μM α-PFT (a selective p53 inhibitor) (Komarov et al., 1999) for 24 h, upon which the drug was removed and the cells were cultured continuously with α-PFT for up to 11 days. WI38 cells treated with BU or Etop alone underwent senescence, as the cells became permanently growth arrested and exhibited a significant reduction in BrdU incorporation and increase in SA-β-gal staining at 7 and 11 days after BU or Etop treatment (Figs. 3B-D). The presence of α-PFT in the medium was able to almost abrogate Etop-induced inhibition of cell proliferation and BrdU incorporation and increase in SA-β-gal staining in WI38 cells, but it had minimal effect on these induced by BU. This finding suggests that Etop induces WI38 cell senescence in part via the p53 pathway, whereas BU induces senescence by a p53-independent mechanism. This suggestion was further supported by the study utilizing p53 small interference RNA (siRNA) that has the ability to specifically down-regulate the expression of p53. As shown in Fig. 4, transfection of WI38 cells with p53 siRNA, but not that with a scrambled sequence siRNA, reduced the expression of p53 as well as its phosphorylation by BU and Etop (Fig. 4A). In addition, p53 siRNA transfection significantly attenuated Etop-induced increase in SA-β-gal staining and reduction in BrdU incorporation in WI38 cells but had no significant effect on these induced by BU compared to scrambled siRNA-transfected cells.

BU induces senescence in WI38 cells via the Erk and p38 MAPK pathways

Activation of the MAPK pathways has also been implicated in the induction of cellular senescence, particularly in response to stress (Haq et al., 2002; Deng et al., 2004; Wang et al., 2002; Iwasa et al., 2003). The lack of p53 involvement in BU-induced senescence prompted us to examine if BU induces WI38 cell senescence via the MAPK pathways. To test this hypothesis, we first examined if BU activates various MAPKs by analysis of Erk, p38 and JNK phosphorylation using Western blotting. As shown in Fig. 5, BU treatment elicited an immediate activation of Erk, while the increases in p38 and JNK phosphorylation occurred 3 days after BU treatment. The activation of all three MAPKs was sustained for up to 11 days after BU treatment.

To determine if activation of the MAPKs mediates BU-induced senescence in WI38 cells, we selectively inhibited Erk, p38 and JNK with their specific inhibitors PD98059, SB203580 and SP600125, respectively (Davies et al., 2000). The data presented in Fig. 6A showed that activation of each of these MAPKs by BU was specifically inhibited by their respective inhibitor. In addition, inhibition of Erk by PD98059 also suppressed BU-induced phosphorylation of p38 and JNK; SB203580 attenuated the activation of both p38 and JNK; and SP600125 only inhibited JNK, indicating that BU may cause activation of Erk, p38 and JNK in a cascading fashion as shown in other studies (Deng et al., 2004; Serrano et al., 1997; Zhu et al., 1998). Subsequently, we examined if inhibition of individual MAKPs attenuates BU-induced senescence. For this study, WI38 cells were incubated with BU for 24 h in the presence or absence of PD98059, SB203580, PD98059 plus SB203580, or SP600125, after which BU was removed and SA-β-gal staining was performed after the cells were cultured for 7 and 11 days with the inhibitor(s) that was added to the initial cultures with BU. The results showed that inhibition of Erk and/or p38 with PD98059 and/or SB203580 significantly inhibited the BU-induced increase in SA-β-gal staining (Fig. 6B) and prevented the development of senescent

morphology in WI38 cells (data not shown), as compared to the cells treated with BU alone. However, inhibition of JNK had no such effect. Correspondingly, inhibition of Erk and/or p38 but not that of JNK attenuated the induction of p16 by BU, but none of these inhibitors had any significant effect on BU-induced p21 expression (Fig. 6A). These findings suggest that activation of Erk and p38 mediates BU-induced WI38 cell senescence, probably via up-regulation of p16. Although JNK is also activated by BU, it is not critical to the induction of senescence (Fig. 6B).

However, to our surprise the inhibition of Erk and/or p38 did not immediately release BU-treated cells from growth inhibition (data not shown), even though the cells were not senescent judged by the lack of SA-β-gal staining (Fig. 6B), no increase in p16 expression (Fig. 6A) and completely normal cell morphology (data not shown). We postulate that this paradoxical phenomenon is due to the intimate involvement of the MAPK signaling in normal cell proliferation (Kyriakis and Avruch, 2001). A prolonged inhibition of Erk and/or p38 along with a brief exposure to BU may cause a temporary cell cycle arrest, possibly mediated by p21 as it was up-regulated in these cells (Fig. 6A). As such, the release of the cells from Erk and/or p38 inhibition should allow the cells to resume proliferation if the cells were not senescent. To test this hypothesis, WI38 cells were cultured with BU in the presence or absence of PD98059, SB203580, PD98059 plus SB203580, or SP600125 for 24 h. After the removal of BU, the cells were cultured with these inhibitors for 11 days. At this time, none of the cells incorporated BrdU even though only the cells treated with BU alone or BU plus SP600125 showed senescent morphology and SA-β-gal staining. Upon releasing the cells from the inhibition by culturing the cells in an inhibitor-free medium, the cells treated with BU and PD98059, SB203580, or PD98059 plus SB203580 started to grow again (Fig. 6C). Three days after the release, these cells showed normal levels of DNA synthesis measured by BrdU incorporation assay (Fig. 6D).

However, the cells treated with BU alone or BU plus SP600125 remained growth inhibited and were unable to incorporate BrdU after SP600125 had been removed from the culture. These observations reaffirm that activation of Erk and p38 but not that of JNK plays an important role in mediating BU-induced senescence in WI38 fibroblasts.

DISCUSSION

Treatment of normal human fibroblast cells and certain types of tumor cells (such as colon cancer cells and neuroblastoma cells) with a cytotoxic chemotherapeutic agent in general induces apoptosis or senescence in a dose-dependent manner (Han et al., 2002; Rebbaa et al., 2003; Zheng et al., 2004; Roninson, 2002; Roninson et al., 2001; Robles and Adami, 1998; Robles et al., 1999). Low dose treatment usually induces premature senescence while high dose treatment causes apoptosis as shown in WI38 cells treated with Etop. However, BU is unique as it can selectively induce WI38 cell senescence but not apoptosis in a dose-independent fashion. This observation is in agreement with our previous finding demonstrating that exposure of mouse HSCs to ionizing radiation (4 Gy), Etop (2μM) and camptothecin (2μM) induced apoptosis, whereas exposure of the cells to BU (up to 100μM) failed to induced HSC apoptosis but effectively inhibited their hematopoietic function (Meng et al., 2003a). Further studies demonstrate that BU inhibits HSCs primarily by induction of senescence. These findings suggest that induction of senescence may be a unique response of normal cells to BU, since induction of apoptosis was observed in various leukemia cells after BU treatment (Hassan et al., 2001; Schwarz et al., 1999).

Two major pathways have been implicated in the induction of senescence (Campisi, 2005; Serrano and Blasco, 2001; Sharpless and DePinho, 1999; Shay and Wright, 2000;

Marcotte and Wang, 2002): the p53-p21 pathway triggered by DNA damage or telomere shortening and the p16- retinoblastoma (Rb) pathway activated by the Ras-Raf-MEK-Erk-p38 cascade (Campisi, 2005; Serrano and Blasco, 2001; Sharpless and DePinho, 1999; Shay and Wright, 2000; Marcotte and Wang, 2002). As an alkylating agent, BU can cause DNA damage by cross-linking DNAs and DNA and proteins (Buggia et al., 1994; Iwamoto et al., 2004; Mertins et al., 2004). The cross-links may be converted into DNA strand breaks that can subsequently activate the p53 pathway to induce senescence. To our knowledge, this is the first report to demonstrate that BU treatment induces DNA DSBs, evidenced by the increase in γH2AX staining (Rogakou et al., 1998; Rogakou et al., 1999). However, the percentage of the cells stained positive for γH2AX and number of γH2AX foci detected in each cell were significantly lower in BU-treated cells than in Etop-treated cells, indicating that BU induces fewer DNA DSBs than Etop. Moreover, the induction of DNA DSBs by BU failed to elicit an immediate activation of p53, whereas a rapid and robust activation of p53 was observed in Etop-treated cells. A moderate level of p53 phosphorylation was found in WI38 cells at 3 days after BU treatment. However, this activation may not be attributable to DNA damage since at that time γH2AX was no longer detectable in the cells. Instead, the activation of p53 may be triggered by p38 that was also activated in the cells by that time and has the ability to phosphorylate p53 (Bulavin et al., 1999; She et al., 2000). Even this moderately delayed activation of p53 seems to play an insignificant role in BU-induced cellular senescence, since inhibition of p53 activity by a p53 specific inhibitor (α-PFT) or down-regulation of p53 expression using siRNA had no significant effect on BU-induced senescence in WI38 cells. In contrast, inhibition of p53 activity by the same means was able to greatly reduce the induction of senescence by Etop. This finding suggests that BU may induce cellular senescence via a p53-

independent mechanism. This suggestion is in agreement with our previous observation that the induction of HSC senescence by BU was not associated with an elevation in p53 activity and p21 expression (Meng et al., 2003b).

Alternatively, BU may induce WI38 cell senescence by activation of the MAPK pathways. Three major MAPK pathways have been implicated in the regulation of cellular senescence in vertebrates (Haq et al., 2002; Deng et al., 2004; Wang et al., 2002; Iwasa et al., 2003). All of them were sequentially activated in WI38 cells by BU: an immediate activation of Erk followed by the activation of p38 and JNK 3 days after BU treatment. The activation of p38 and JNK was down-stream of Erk, as inhibition of Erk prevented p38 and JNK phosphorylation. However, the delayed activation of p38 and JNK by BU suggests that p38 and JNK are not direct targets of Erk but rather activated indirectly by Erk through a yet to be defined mechanism (Haq, et al., 2002; Deng et al., 2004; Iwasa et al., 2003). A similar sequential activation of Erk and p38 was also observed in the cells ectopically transfected with Ras and Raf oncogenes that induce premature senescence in fibroblasts (Deng et al., 2004; Serrano et al., 1997; Zhu et al., 1998). Therefore, we further examined if specific inhibition of Erk, p38 and/or JNK can attenuate BU-induced senescence in WI38 cells. The results showed that inhibition of Erk and/or p38, but not that of JNK, significantly suppressed BU-induced WI38 cell senescence. These findings are in agreement with previous observations that Ras-induced fibroblast senescence is mediated by the cascading activation of the Erk and p38 pathways and that constitutive activation of p38 induces fibroblast senescence (Deng et al., 2004; Serrano et al., 1997; Zhu et al., 1998). However, inhibition of Erk and/or p38 did not immediately restore cell division after BU treatment. The cell proliferation resumed only after the removal of Erk and/or p38 inhibition, suggesting that continuous inhibition of Erk and/or p38 may prevent BU-treated cells from re-entering the cell

cycle even though Erk and/or p38 inhibition effectively suppresses BU-induced senescence in the cells. In addition, this phenomenon suggests that the induction of temporary cell cycle arrest and senescence by BU in WI38 cells may be mediated by different mechanisms. The induction of temporary cell cycle arrest is likely mediated by up-regulation of p21, probably in an Erk-p38-independent manner; whereas the induction of senescence may be attributable to the increased expression of p16 resulting from Erk and/or p38 activation. This suggestion is supported by the finding that BU-treated WI38 cells had normal cell morphology and lack of SA-β-gal staining and p16 up-regulation under continuous Erk and/or p38 inhibition, but showed a slight increase in p21 expression. Upon release from Erk and p38 inhibition, the cells proceeded to synthesize DNA and undergo cell division. A similar finding was also observed in ataxia telangiectoasia fibroblasts (Naka et al., 2004). It was shown that in ataxia telangiectoasia fibroblasts the temporary cell growth arrest induced by ionizing radiation or H_2O_2 was mediated by the activation of p53 and induction of p21, while activation of the Erk-p38 pathways and subsequent up-regulation of p16 led to the induction of senescence in the cells (Naka et al., 2004).

The mechanisms by which BU activates the Erk-p38 pathways have yet to be elucidated. The activation of Erk and p38 may result from cross-linking of plasma membrane proteins due to protein alkylation by BU (Buggia et al., 1994; Iwamoto et al., 2004). Alternatively, BU could activate p38 by increasing oxidative stress due to depletion of intracellular glutathione (DeLeve and Wang, 2000; Hassan et al., 2002). When p38 is activated by BU treatment, it can up-regulate the expression of p16 to induce cell cycle arrest and cellular senescence (Iwasa et al., 2003; Deng et al., 2004; Haq et al., 2002; Wang et al., 2002). In addition, activation of p38 can activate p53 or inactivation of cdc25 phosphatase (Bulavin et al., 1999; Bulavin et al., 2001; Wang et al., 2000; She et al., 2000). Activation of p53 in turn induces p21. Induction of p21 and inactivation

of cdc25 phosphatase can cause G1 and G2/M cell cycle arrest, respectively (Bulavin et al., 2001; Wang et al., 2000). It has yet to be determined whether induction of p21 and inactivation of cdc25 phosphatase play any role in BU-induced cellular senescence in WI38 cells.

In summary, the data presented in this study demonstrate that BU is a distinctive chemotherapeutic agent. It can selectively induce cellular senescence in normal cells, but apoptosis in leukemia cells as shown in previous studies (Hassan et al., 2001; Schwarz et al., 1999). The induction of normal cell senescence is independent of BU-induced DNA damage and p53 activation but relies on the Erk and p38 MAPK pathways. Induction of senescence may contribute to BU-induced normal tissue damage in several different ways. First, when a cell, especially if it is a stem cell such as HSC, become senescence, they cannot proliferate to generate progeny that are normally required for the maintenance of normal tissue homeostasis and for the repair of damage tissues by a cytotoxic chemotherapeutic agent. Moreover, senescent cells secret increased levels of pro-inflammatory cytokines, matrix metalloproteinases, and epithelial growth factors that are known to participate in the pathogenesis of pulmonary fibrosis and other normal tissue injuries (Campisi, 2005). Therefore, a better understanding of the mechanism by which BU and other alkylating agents induce normal cell injury would be beneficial to the effort of developing new therapeutics that could be more effective in depleting leukemia cells while limiting its toxicity to normal tissues.

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Footnotes:

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FIGURE LEGENDS

Fig. 1. BU induces WI38 cell senescence but not apoptosis in a dose-dependent manner. WI38 cells were treated with various concentrations of BU or Etop for 24 h and then cultured in drug-free media. Untreated WI38 cells served as a control. **A.** The number of cells was counted regularly for 11 days and data are presented as mean ± SD of triplicates to establish cell growth curves. **B.** After 24 h incubation in fresh media, activated caspase 3 was detected using the SR-DEVD-FMK-FLICA reagent (Green) and nuclei were counterstained with Hoechst (Blue). **C.** After 7 days of culture in drug-free media, untreated (Control), BU-treated and Etop-treated cells were fixed in 70% ethanol and stained with PI and then assayed by flow cytometry to establish a cell cycle profile (top panel); assayed for BrdU (Red) incorporation by immunohistochemistry and counterstained with Hoechst (Blue) (second panel); visualized under a phase contrast microscope to show changes in cell morphology (third panel); or imaged via bright field microscopy after SA-β-gal staining (bottom panel). **D.** SA-β-gal positive cells were quantified for each treatment after counting a minimum of 1000 cells from 10 randomly chosen fields and the data are presented as mean ± SEM (n = 3). * $p < 0.01$ vs. control.

Fig. 2. BU and Etop induce DNA DSBs in WI-38 cells. WI38 cells were treated with either BU or Etop for 0-24 h. After treatment, cells were fixed and stained with anti-γH2AX antibody (Green) to detect DNA DSBs. Cells were counterstained with Hoechst (Blue) for nuclear detection. Cells exposed to ionizing radiation (IR, 10 Gy) served as a positive control. Merged images of γH2AX and Hoechst staining are presented to demonstrate that H2AX phosphorylation occurred exclusively at nuclear sites after treatment. **A**. Representative images observed via fluorescent microscopy are shown. **B** & **C.** Eight h after BU or Etop treatment, the

number of γH2AX-positive cells and total number of γH2AX foci were quantified for each treatment by counting a minimum of 1000 cells from 8 randomly chosen fields and expressed as a percentage of γH2AX positive cells and average γH2AX foci per cell. The data are presented as mean ± SEM (n = 3). *a*, $p < 0.05$ to 0.0001 vs. control; *b*, $p < 0.05$ to 0.01 vs. IR or Etop.

Fig. 3. BU and Etop induce WI38 cell senescence in a p53-independent and p53-dependent manner, respectively. **A.** Cell lysates were prepared from WI38 cells that were treated with BU or Etop for 0, 0.5, 1, 2, 4, 8, 16, or 24 h or treated with either drug for 24 h and then cultured in fresh media for 3, 5, 7, or 11 days. Cell lysates from untreated WI38 cells served as a control (C). Western blot analyses were used to detect the levels of phosphorylated p53 (P-p53) and p53, p21 and p16 in the cell lysates. Actin was utilized as a loading control. Similar results were observed in at least two additional experiments. **B.** WI38 cells were cultured with BU or Etop in the presence or absence of α-PFT (20 μM). After 24 h of incubation, BU and Etop were removed and then the cells were continuously cultured with α-PFT for up to 11 days by changing the medium with freshly made α-PFT every 3 days. Periodically, the cells were counted and data are presented as mean ± SD of triplicates to generate cell growth curves. **C** & **D.** WI38 cells were treated similarly as described in **B** but stained for SA-β-gal or assayed for BrdU incorporation, respectively, 7 and 11 days after treatment with BU or Etop and quantified as described before. The data are presented as mean ± SEM (n = 4). A two-way ANOVA analysis revealed that α-PFT significantly attenuated the inhibitory effect of Etop on WI-38 cell growth and BrdU incorporation and Etop-induced increase in SA-β-gal staining ($p < 0.01$) but had no significant effects on those induced by BU ($p > 0.05$).

Fig. 4. Down-regulation of p53 by siRNA has no effect on senescence induced by BU but inhibits that induced by Etop. **A.** WI38 cells were pre-incubated with 20μM of either scrambled sequence (SCR) or p53 siRNA for 48 h and then either untreated (Control) or treated with BU (120 μM) or Etop (20 μM) for 24 h. The levels of phosphorylated p53 (P-p53) and total p53 were measured by Western blots. Actin was utilized as a loading control. **B** & **C.** WI38 cell were treated as described in **A** but cultured for additional 7 days after the removal of BU or Etop from the media. SA-β-gal staining and BrdU incorporation were assayed as described before. The data are presented as mean ± SEM (n = 6 for **B** and 4 for **C**). A two-way ANOVA analysis revealed that p53 siRNA significantly attenuated the effects of Etop ($p< 0.05$ or 0.01, respectively) but not those of BU on BrdU incorporation and SA-β-gal staining in WI38 cells ($p> 0.05$).

Fig. 5. BU activates Erk, p38 and JNK MAPKs in WI38 cells. Cell lysates were prepared from WI38 cells that were treated with BU (120μM) for 0, 0.5, 1, 2, 4, 8, 16, or 24 h or treated with BU (120 μM) for 24 h and then cultured in fresh media for 3, 5, 7, or 11 days. Cell lysates from untreated WI38 cells served as a control (C). Western blot analyses were used to detect the levels of phosphorylated (P-Erk, P-p38 and P-JNK) and total Erk, p38 and JNK in the cell lysates. Similar results were observed in at least two additional experiments.

Fig. 6. Inhibition of Erk and p38 but not that of JNK attenuates BU-induced senescence in WI38 cells. WI38 cells were pretreated with vehicle (V), SB203580 (SB, 1μM), PD98059 (PD, 50μM), a combination of SB + PD, or SP600125 (SP, 20μM) for 30 min, after which they were incubated with BU. After 24 h incubation, BU was removed and then the cells were continuously

cultured with these inhibitors for up to 11 days by changing the medium with freshly made inhibitor(s) every 3 days. Untreated WI38 cells served as a control (C). **A.** A lysate was made for each treatment 3 days after BU treatment and assayed by Western blot for total and phosphorylated-Erk, p38 and JNK and for p16, p21 and actin. **B.** SA-β-gal staining was quantified 7 and 11 days after treatment with BU as described before. **C** & **D.** WI38 cells were released from Erk, p38 or JNK inhibition 7 days after BU treatment by culturing the cells in fresh medium containing no inhibitor(s). Cell proliferation was determined 1, 3, 5 and 7 days after the release of the cells from Erk, p38 or JNK inhibition and BrdU incorporation was determined 3 days after the release. The data are presented as mean ± SEM (n = 4). A one-way ANOVA analysis revealed that PD, SB and PD/SB significantly attenuated the effects of BU ($p < 0.01$) on BrdU incorporation and SA-β-gal staining in WI38 cells whereas SP had no significant effect on the changes induced by BU ($p > 0.05$).

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Fig. 1

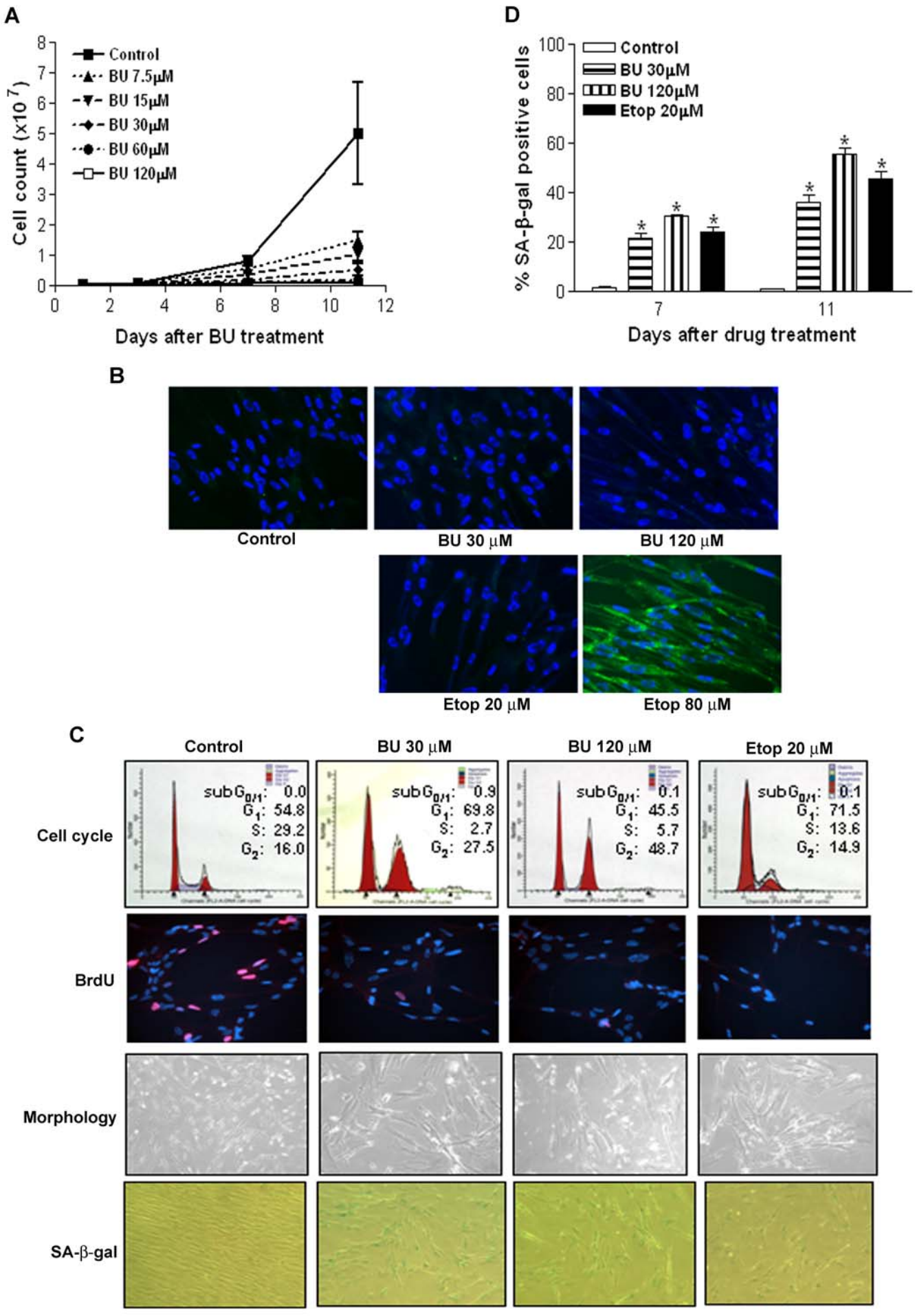

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Fig. 2

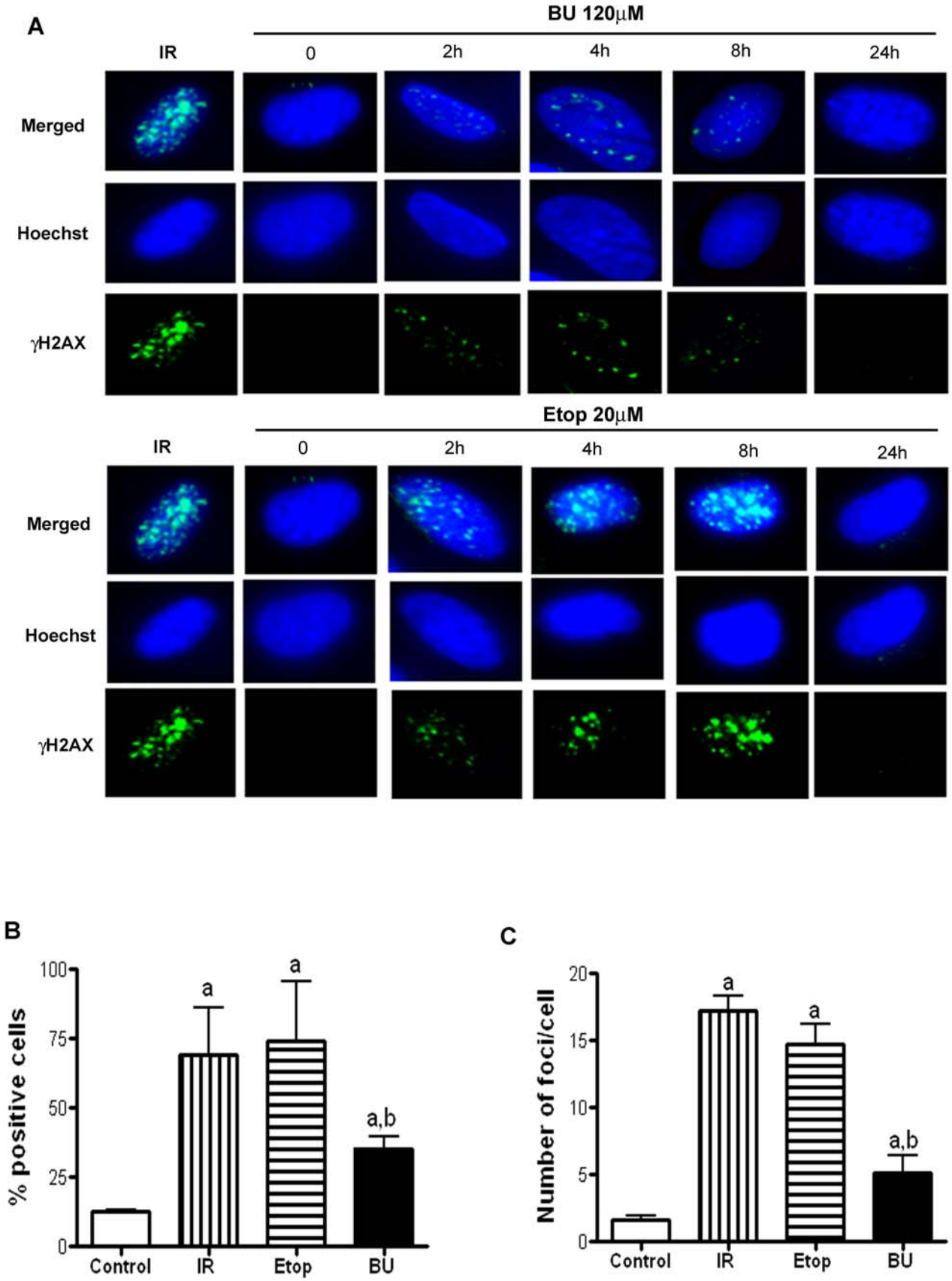

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Fig. 3

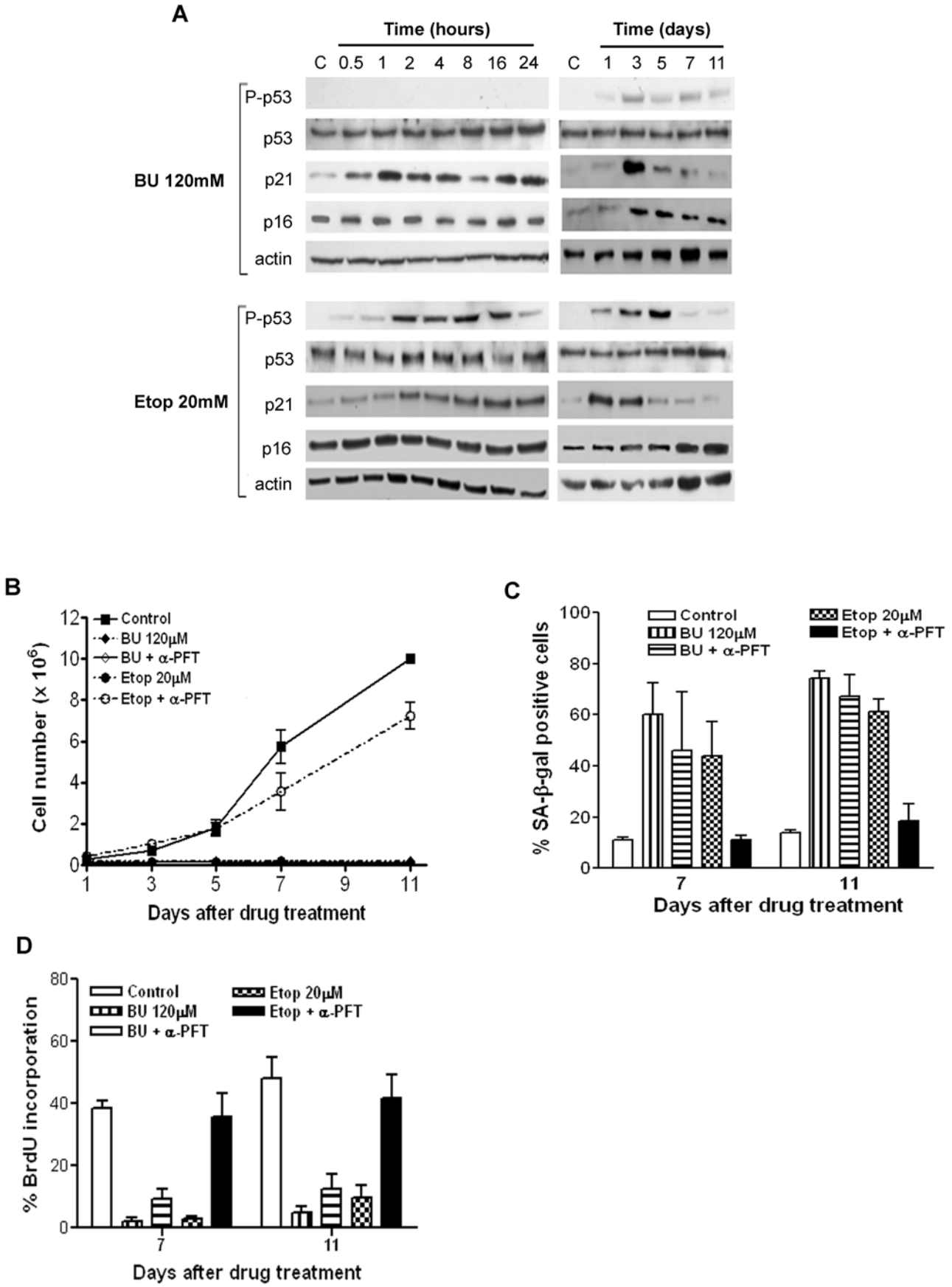

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Fig. 4

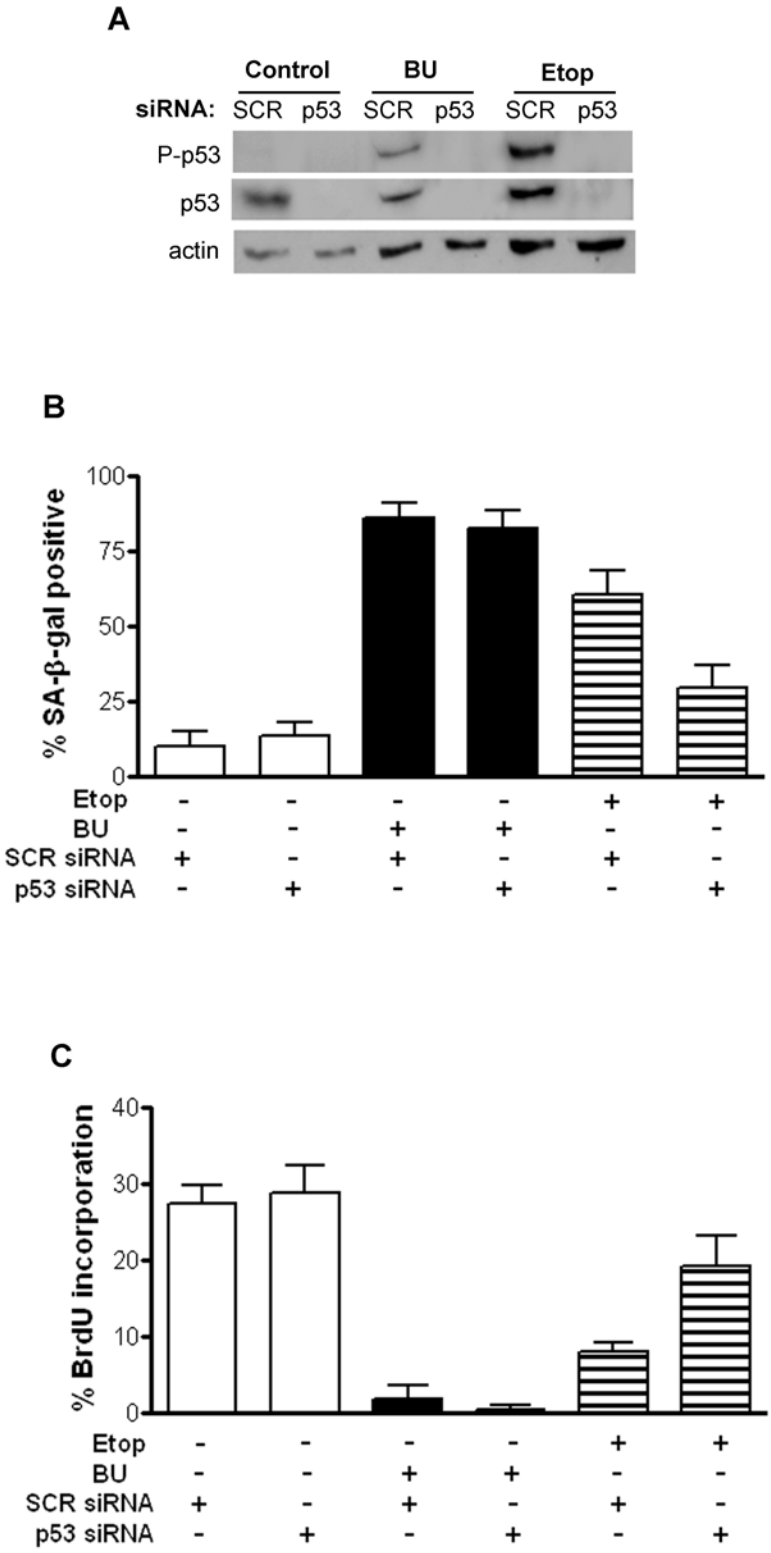

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Fig. 5

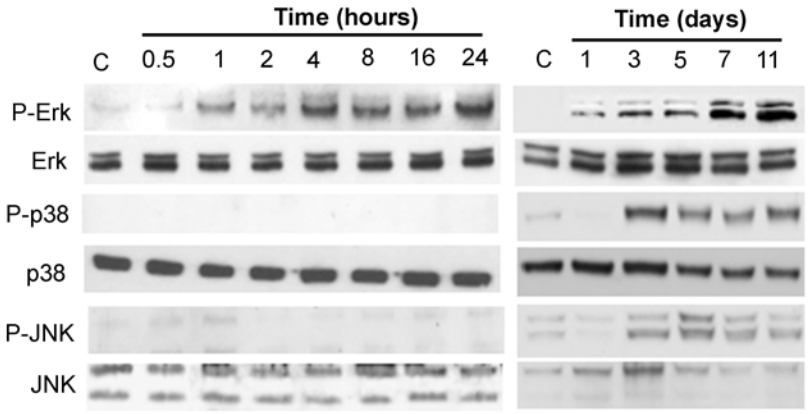

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Fig. 6