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BUSULFAN-INDUCED SENESENCE IS DEPENDENT UPON ROS PRODUCTION UPSTREAM OF THE MAPK PATHWAY

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Abstract

Induction of cellular senescence is a common response of a normal cell to a DNA damaging agent, which may contribute to cancer chemotherapy- and ionizing radiation-induced normal tissue injury. The induction has been largely attributed to the activation of p53. However, the results from the present study suggest that busulfan (BU), an alkylating agent that causes DNA damage by cross-linking DNAs and DNA and proteins, induces senescence in normal human diploid WI38 fibroblasts through the extracellular signal-regulated kinase (Erk) and p38 mitogen-activated protein kinase (p38 MAPK) cascade independent of the p53-DNA damage pathway. The induction of WI38 cell senescence is initiated by a transient depletion of intracellular glutathione (GSH) and followed by a continuous increase in reactive oxygen species (ROS) production via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which leads to the activation of the Erk and p38 MAPK pathway. Incubation of WI38 cells with N-acetyl-cysteine (NAC) replenishes intracellular GSH; abrogates the increased production of ROS; ameliorates Erk and p38 MAPK activation; and attenuates senescence induction by BU. Thus, inhibition of senescence induction using a potent antioxidant or specific inhibitor of the Erk and p38 MAPK pathway has the potential to be developed as a mechanism-based strategy to ameliorate cancer therapy-induced normal tissue damage.

Keywords

Alkylating agent; senescence; reactive oxygen species; Erk; p38; p53

INTRODUCTION

Induction of senescence has been suggested to be an underlying cellular mechanism of organismal aging [1–3]. In addition, it can contribute to normal tissue injury induced by various cancer therapies [4–7]. This is because senescent cells, being permanently arrested, cannot proliferate to generate progeny that are normally required for the maintenance of normal tissue homeostasis and for the repair of damaged tissues [1]. Moreover, senescent cells secrete increased levels of pro-inflammatory cytokines, matrix metalloproteases, and epithelial growth factors that are known to participate in the pathogenesis of pulmonary fibrosis and other normal tissue injuries after chemotherapy and/or ionizing radiation [1]. However, the mechanisms by which radiation or cytotoxic chemotherapeutic agents induce senescence have not been well

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established, nor are effective strategies to prevent the induction of normal cell senescence available to ameliorate the injuries.

Using busulfan (BU) as a representative chemotherapeutic agent, we recently studied the mechanisms of action whereby BU induces cellular senescence in normal mouse bone marrow hematopoietic cells and human diploid fibroblasts (WI38 cells) [7,8]. BU is commonly used in the treatment of chronic myelogenous leukemia and for myeloablation prior to bone marrow transplantation. However, the therapeutic efficacy of BU is compromised by a wide range of normal tissue damage, such as bone marrow suppression, chronic lung fibrosis, liver veno-occlusive disorder (VOD), and hemorrhagic cystitis [9,10]. As a potent alkylating agent, BU can cause DNA damage by cross-linking DNAs and DNA and proteins [11,12]. The cross-links can be converted into DNA strand breaks that can subsequently activate the p53 pathway [8]. Therefore, it has been assumed that BU may induce senescence via the p53-DNA damage pathway. To our surprise, we have found that induction of hematopoietic cell senescence by BU is not associated with activation of p53 and up-regulation of p21^{Cip1/WAF1} (p21) [7]. In addition, down-regulation of p53 expression using p53 specific small interference RNA (siRNA) fails to inhibit BU-induced senescence in WI38 cells [8]. These findings suggest that BU may induce cellular senescence via a p53-independent mechanism. This suggestion is supported by the observations that induction of WI38 cell senescence by BU is associated with a prolonged activation of extracellular signal-regulated kinase (Erk) and p38 mitogen-activated protein kinase (p38 MAPK) and that inhibition of the Erk-p38 MAPK pathway with a specific Erk or p38 inhibitor can abrogate BU-induced senescence in WI38 cells [8].

Activation of the Erk-p38 MAPK pathway has been implicated in oncogenic stress-induced senescence as seen in cells ectopically transfected with Ras or Raf oncogene [13,14]. Activation of p38 also contributes to the induction of senescence in response to DNA damage and telomere shortening [15]. Thus, it has been suggested that Erk and p38 may play a central role in mediating senescence induction by various stimuli including BU [15]. Although our previous studies showed that BU can selectively induce WI38 cell senescence through the Erk-p38 MAPK cascade [8], the mechanism by which BU activates Erk and p38 has yet to be elucidated. Extensive research has shown that reactive oxygen species (ROS) are capable of activating various signal transduction pathways including the Erk-p38 MAPK cascade [16,17]. Oxidative stress is also a well-documented inducer of cellular senescence [16]. Several reports have shown that BU forms conjugates with glutathione (GSH) leading to GSH depletion and oxidative stress [18,19]. In addition, a recent study found that BU can inhibit thioredoxin reductase, which also plays an important role in regulation of oxidative stress in a cell [20]. Therefore, in the present study we attempted to determine whether BU can cause oxidative stress in WI38 cells and whether ROS act upstream of the Erk-p38 MAPK pathway for the induction of senescence by BU.

MATERIALS AND METHODS

Reagents

Selective inhibitors for p53 (α -PFT, α -pifithrin) [21], Erk (PD98059, 2'-amino-3'-methoxyflavone) [22], p38 (SB203580, 4-[4-fluorophenyl]-2-[4-methylsulfinylphenyl]-5-[4-pyridyl]1H-imidazole) [22], and c-Jun NH₂-terminal kinase or JNK (SP600125, 1,9-pyrazoloanthrone) [22] were purchased from Calbiochem (San Diego, CA). BU, N-acetylcysteine (NAC) and 5-bromo-2'-deoxyuridine (BrdU) were obtained from Sigma (St. Louis, MO). All the inhibitors for various oxidases [23], including diphenylene iodonium (DPI, a NADH/NADPH oxidase inhibitor), apocynin (a selective NADPH oxidase inhibitor), rotenone (an inhibitor of mitochondrial complex I), allopurinol (a xanthine oxidase inhibitor), indomethacin (a cyclooxygenase inhibitor), and ketoconazole (a cytochrome P450 oxygenase inhibitor) were obtained from Sigma. Antibodies against phosphorylated p53 (P-p53) and Erk

(P-Erk) and those against total p53, Erk, p38, JNK, and p21 were obtained from Cell Signaling (Beverly, MA); anti-phosphorylated p38 (P-p38) and JNK (P-JNK) from Promega (Madison, WI); anti- β -actin from Santa Cruz (Santa Cruz, CA); anti-p16^{Ink4a} (p16) from BD PharMingen (San Diego, CA); anti-BrdU from Sigma; 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, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin in a humidified incubator at 37 °C and 5% CO₂. Cells at early passages (<25) 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 BU (120 μ M) 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.

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 d. Untreated cells were used as control. Every 3 d the cells were trypsinized, counted and sub-cultured in a fresh medium at 1:5 and 1:2 dilutions for control untreated and BU-treated cells, respectively.

Senescence associated β -galactosidase (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. 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-treated cells was determined by measuring BrdU incorporation into DNA as previously described [8]. Images of BrdU and Hoechst 33342 nuclear staining 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, CO). To calculate the percentage of BrdU positive cells, a minimum of 1000 cells were counted in 8 random fields on a slide and the number of BrdU positive cells was divided by the total number of cells counted.

ROS Detection

Dihydrorhodamine (DHR) (Invitrogen) stock solution (10mM) was prepared in DMSO. The stock solution was diluted with a staining solution (5mM glucose, 1mM CaCl₂, 0.5mM MgSO₄, and 5 mg/ml BSA) to a final concentration of 1 μ g/ml. After BU treatment, cells were harvested by trypsinization, centrifuged at 1,200 x g for 5 min, and re-suspended in the staining solution containing 1 μ g/ml DHR. After cells were incubated with DHR for 30 min at 37 °C in the dark, the oxidation of DHR by ROS, which converts it to rhodamine 123 (R123), was determined by measuring the mean fluorescent intensity of R123 in a minimum of 20,000 cells using a FACS Caliber flow cytometer (Becton Dickinson, San Jose, CA) at λ_{ex} = 488 nm and λ_{em} = 525 nm [24].

GSH Detection

WI38 cells were plated in a 96-well plate at 50,000 cells/well. After overnight growth, they were treated with BU for various durations and then incubated with monochlorobimane (mBCI, 40 μ M) (Invitrogen) in a staining solution (5mM glucose, 1mM CaCl₂, 0.5mM MgSO₄, 5 mg/ml BSA) for 30 min at 37 °C in the dark. Although mBCI is a nonfluorescent probe, it forms a stable fluorescent adduct with GSH in a reaction catalyzed by the GSH S-transferases [25]. The mean fluorescent intensity of the fluorescent GSH-bimane adduct was measured using a Spectra Max Gemini XS fluorescent plate reader (Molecular Devices Corp., Sunnyvale, CA) at λ_{ex} =380 nm and λ_{em} =460 nm to detect GSH [25].

Western blot analysis

Lysates of WI38 cells were prepared and analyzed as previously described [8].

Statistical Analysis

The data were analyzed by analysis of variance (ANOVA). In the event that ANOVA 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 via the Erk-p38 MAPK pathway

Senescence is defined as a permanent cell cycle arrest accompanied by positive staining for SA- β -gal, a biomarker of senescent cells [26]. As shown in Fig. 1A, the growth of WI38 cells was permanently arrested after 24 h treatment with BU, while control untreated cells proliferated exponentially. The inhibition of WI38 cell growth by BU is not due to an induction of cell death (apoptosis/necrosis), but is attributed to the induction of senescence [8]. This suggestion is supported by the observation that cells briefly treated with BU exhibited a significant reduction in BrdU incorporation (BU: $3.6 \pm 1.5\%$ vs Ctrl: $45.1 \pm 4.7\%$, *p*<0.0001) and a drastic increase in SA- β -gal staining (BU: $75.4 \pm 6.6\%$ vs Ctrl: $9.7 \pm 4.9\%$, *p*<0.0001) as compared with control untreated cells at 11 d after the initial treatment (Figs. 1B & C).

To elucidate the mechanisms of action whereby BU induces senescence, we first evaluated activation of the two key pathways that have been implicated in senescence induction: (1) the p53-p21 pathway triggered by DNA damage and (2) the p16-Rb pathway activated by the MAPK cascades [1,3,15,27]. The activation of these pathways was determined by analysis of phosphorylation of p53, Erk, p38, and JNK and expression of p21 and p16 using Western blot. As shown in Figs. 1D & E, BU treatment incited a moderate p53 activation, but strong Erk, p38, and JNK phosphorylation, in a time-dependent manner. In addition, BU treatment elicited an immediate up-regulation of p21 expression, which subsided by day 11. In contrast, the increase in p16 expression occurred 3 d after BU treatment but was sustained for more than 11 d. These findings are in agreement with the suggestion that p21 plays an important role in the initiation of senescence, while p16 is required for the maintenance of senescence [28].

To further clarify the roles of the p53 pathway and the Erk-p38 MAPK cascade in BU-induced senescence, WI38 cells were pre-treated with α -PFT, PD98059, SB203580, and SP600125 prior to BU treatment to specifically inhibit p53, Erk, p38, and JNK, respectively [21,22]. The induction of senescence in WI38 cells by BU was analyzed by quantification of SA- β -gal staining and BrdU incorporation. It was found that inhibition of Erk or p38 abrogated BU-induced increase in SA- β -gal staining and reduction in BrdU incorporation and cell

proliferation in WI38 cells as compared with the cells treated with BU alone (Figs. 1F–H). In contrast, inhibition of p53 and JNK had no significant effect on these changes induced by BU ($p>0.05$). These findings indicate that BU induces WI38 cell senescence primarily via activation of the Erk-p38 MAPK pathway, whereas activation of p53 and JNK plays an insignificant role in its induction.

BU induces a transient reduction in GSH but a continuous increase in ROS production

The mechanism by which BU activates the Erk-p38 pathways is unknown. It has been demonstrated that ROS can function as a signal molecule to stimulate the Erk-p38 MAPK pathway [17]. BU has the ability to increase ROS production in part by depletion of intracellular GSH [18,19]. Therefore, we hypothesized that BU may activate Erk and p38 by inducing oxidative stress. To test this hypothesis, we first examined the effect of BU on the intracellular levels of GSH using mBCI which readily enters cells to form a fluorescent GSH-bimane adduct that can be measured fluorometrically [25]. As shown in Fig. 2A, within 30 min after BU treatment, the intracellular levels of GSH were reduced by 50%. This finding was subsequently confirmed by an enzymatic assay using glutathione reductase and 2-vinylpyridine [29]. The reduction in GSH sustained for about 2 h and then gradually lessened. Within 24 h of BU treatment, the levels of GSH were restored to control levels and maintained at the same levels for the duration of the experiment.

Corresponding to the immediate reduction in GSH, the intracellular levels of ROS increased 34% at 30 min after BU treatment and maintained for about 4 h (Fig. 2B). Thereafter, a further increase in ROS production occurred in a linear fashion in BU-treated cells, even though the levels of GSH had returned to normal levels in these cells 24 h after BU treatment. By day 11, the levels of ROS in BU-treated cells were about 3-fold greater than those in control cells. The continuous increase in the levels of ROS after BU treatment is likely attributable to an elevation in ROS production by the cells.

To determine the cellular source(s) of the ROS, BU-treated cells were harvested at day 11 after the treatment and the production of ROS by the cells was measured after a brief incubation with DPI, apocynin, allopurinol, indomethacin, ketoconazole, and rotenone to selectively inhibit NADH/NADPH oxidase, NADPH oxidase, xanthine oxidase, cyclooxygenase, cytochrome P450 oxygenase, and mitochondrial complex I, respectively [23]. As shown in Fig. 3, the increased production of ROS by BU-treated cells was suppressed by DPI and apocynin ($p<0.01$). In contrast, inhibition of xanthine oxidase with allopurinol potentiated the ROS production ($p<0.05$), whereas rotenone, indomethacin, and ketoconazole had no significant effects on BU-induced production of ROS ($p>0.05$). This finding suggests that NADPH oxidase is primarily responsible for BU-induced production of ROS in WI38 cells.

Effects of NAC on BU-induced oxidative stress, activation of the Erk-p38 MAPK pathway, and induction of senescence

NAC is commonly used as a supplemental substrate for the synthesis of GSH and a scavenger of ROS [30,31]. It has the ability to inhibit oxidative stress induced by various stimuli. Therefore, we examined if NAC could inhibit BU-induced oxidative stress, leading to an attenuated activation of the Erk-p38 MAPK pathway and reduced induction of senescence by BU in WI38 cells. First, we determined if incubation of WI38 cells with NAC would prevent GSH from depletion by BU. Since BU treatment induces an immediate and transient reduction in intracellular GSH and the reduction is the most pronounced at 30 min after the treatment, this 30 min time point was selected for the examination of the effect of NAC on BU-induced GSH reduction. It was found that BU treatment caused a 50% reduction in intracellular GSH (Fig. 4A). This reduction was completely inhibited by co-incubation with NAC. In addition, we examined the effects of NAC on BU-induced ROS production in WI38 cells. As previously

shown, BU treatment alone induced a nearly 3-fold increase over control in ROS production by day 11. This increase in ROS production was also abrogated by NAC treatment (Fig. 4B). These results demonstrate that NAC can effectively inhibit BU-induced oxidative stress in WI38 cells.

Next, we examined the effects of NAC on the activation of the Erk-p38 MAPK pathway and induction of senescence by BU. As shown in Figs. 3C & D, treatment of WI38 cells with BU for 3 d led to phosphorylation of p53, Erk, p38, and JNK as well as up-regulation of p21 and p16 expression, as previously shown. Treatment of the cells with NAC abrogated activation of Erk, p38, and JNK and induction of p21 and p16 by BU (Figs. 4C & D). It also inhibited BU-induced p53 phosphorylation but to a lesser degree than the inhibition of Erk, p38, and JNK activation by BU. Correspondingly, NAC-treated cells showed a significant reduction in SA- β -gal staining and an increase in BrdU incorporation and cell proliferation in response to BU treatment as compared to the cells treated with BU alone ($p < 0.001$ by Two-way ANOVA) (Figs. 4E, F & G). These findings are in agreement with our hypothesis that induction of ROS production and activation of the Erk-p38 pathway may act sequentially to mediate BU-induced cellular senescence in WI38 cells.

DISCUSSION

As an alkylating agent, BU is known to cause DNA damage by cross-linking DNAs and DNA and proteins [11,12]. Thus, the majority of its cytotoxicity and side effects have been attributed to BU-induced DNA damage that can subsequently activate the p53 pathway to induce either apoptosis or senescence in a cell-type dependent manner [8,11,32,33]. However, this view of mechanism of action of BU has been challenged by the observations that BU induces senescence, but not apoptosis, in normal mouse bone marrow hematopoietic cells independent of p53 activation and p21 up-regulation [7]. Moreover, down regulation of p53 expression using p53 siRNA or inhibition of p53 activity with a specific p53 inhibitor such as α -PFT fails to protect normal human diploid WI38 fibroblasts from undergoing senescence in response to BU treatment [8].

The lack of involvement of p53 in BU-induced senescence prompted us to hypothesize that activation of the Erk-p38 MAPK pathway may mediate the induction, because activation of the Erk-p38 MAPK pathway has also been implicated in the induction of cellular senescence [13,15,34,35]. Indeed, it was found that treatment of WI38 cells with BU activated Erk and p38 in a time-dependent manner and inhibition of Erk or p38 with a specific inhibitor significantly suppressed BU-induced WI38 cell senescence. Although BU also activated JNK in WI38 cells, inhibition of JNK had no significant effect on BU-induced senescence, indicating that JNK is not involved in the senescence induction by BU.

The activation of the Erk-p38 MAPK pathway is likely attributable to the oxidative stress induced by BU, because Erk and p38 are sensitive to ROS [16,17,31,36]. It has been shown that the major route of BU metabolism is through GSH-S-transferase catalyzed conjugation to GSH [18,19]. Therefore, treatment of cells with BU can lead to GSH depletion. By depleting GSH, BU can initiate oxidative stress. This hypothetical mode of action of BU is supported by the finding that BU treatment causes an immediate reduction in intracellular levels of GSH in association with an increase in ROS production. In addition, it has yet to be determined whether inhibition of thioredoxin reductase also contributes to BU-induced increase in ROS production as shown in a recent study [20]. However, the increased production of ROS persists, even though the levels of GSH return to normal within 24 h after BU treatment. This suggests that the early increase in the levels of ROS in the cells may be attributable to GSH depletion by BU, whereas the continuous rise in the levels of ROS after BU treatment is unlikely due to GSH depletion, but attributable to an elevated production of ROS by the cells. This suggestion

is confirmed by the finding that inhibition of NADPH oxidase with DPI or apocynin can significantly reduce BU-induced ROS production, whereas inhibition of mitochondrial complex I and several other oxidases has no significant effect on the production. However, the mechanisms by which BU increases NADPH oxidase production of ROS have yet to be determined. There are several reports indicating that NADPH oxidase is expressed at an increased level in various aging tissues [37,38]. Over expression of NADPH oxidase induces NIH 3T3 cell senescence [39]. These reported observations along with the findings presented in this study indicate that dysregulation of NADPH oxidase may contribute to the induction of cellular senescence and organismal aging, which deserves further investigation.

NAC is a potent antioxidant that can inhibit oxidative stress by directly scavenging ROS and replenishing GSH [31]. If ROS production mediates BU-induced senescence via activation of the Erk-p38 MAPK pathway, we would expect that NAC should have the ability to inhibit BU-induced senescence while suppressing the activity of the Erk-p38 pathway. This was confirmed by the study showing that incubation of WI38 cells with NAC prior to BU treatment inhibited oxidative stress; attenuated Erk and p38 MAPK activation; and finally, reduced senescence induction. Although inhibition of Erk or p38 also abrogates BU-induced senescence in WI38 cells, it has no significant effect on BU-induced increase in ROS production (data not shown). This suggests that ROS should act upstream of the Erk-p38 MAPK pathway to mediate BU-induced senescence in WI38 cells.

Senescence is a cellular damage characterized by permanent cell cycle arrest and failure to maintain normal cell reproductive function thereby leading to a compromised ability of normal cells to maintain the structural integrity of a tissue and to repair damaged tissues in response to cytotoxic cancer therapies [1–3]. Induction of normal cell senescence via ROS and the Erk-p38 pathway may not be unique to BU. Other chemotherapeutic agents can also induce oxidative stress. It has yet to be determined if these agents may cause normal tissue damage in part by induction of cellular senescence through the Erk-p38 cascade as BU. A better understanding of the mechanism by which chemotherapeutic agents induce senescence in normal cells would be beneficial in developing novel strategies to inhibit the detrimental side effects of cancer therapy on normal tissue. Example strategies may include antioxidant therapy and inhibition of the Erk-p38 pathway as shown in this study. Further studies using an animal model to validate the effectiveness of these strategies to reduce cancer therapy-induced normal tissue damage will be of great clinical significance.

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Abbreviations

SA-β-gal	senescence associated β-galactosidase
DHR	dihydrorodamine
mBCl	monochlorobimane
NAC	N-acetyl-cysteine
MAPK	mitogen-activated protein kinase

ERK	extracellular signal-regulated kinase
JNK	c-Jun NH ₂ -terminal kinase (also called stress-activated protein kinase, SAPK)
GSH	glutathione
BrdU	5-bromo-2'-deoxyuridine
ROS	reactive oxygen species
DPI	diphenylene iodonium
NAC	N-acetyl-cysteine

Fig 1. BU induces premature senescence in WI38 cells via the Erk and p38 MAPK pathway

A. WI38 cells were treated with BU (120 μ M) for 24 h and then washed with PBS to remove BU from the culture. The cells were cultured in fresh media for up to 11 d, during which a cell growth curve was generated. Cells that were not treated with BU were used as controls (Ctrl). A representative cell growth curve is shown. **B & C.** Cells were stained for SA- β -gal (**B**) or BrdU incorporation (**C**) at day 11 after BU treatment. Untreated cells were used as controls (Ctrl). The data are presented as mean $\pm$ SEM ($n = 6$). *** $p < 0.001$. **D & E.** The cells were untreated (Ctrl) or treated with BU for 24 h as previously described. Cell lysates were prepared immediately after the treatment (1 d) or after culture in fresh medium for various durations and assayed via Western blot for detection of phosphorylated p53 (P-p53), Erk (P-Erk), p38 (P-

p38), and JNK (P-JNK) and expression of p21 and p16. Blots for total p53, Erk, p38, and JNK and β -actin were used as loading controls. Similar results were observed in at least two additional experiments. **F–H.** WI38 cells were pretreated with vehicle (V), α -PFT (20 μ m), PD98059 (PD, 50 μ M), SB203580 (SB, 1 μ M), or SP600125 (SP, 20 μ M) 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 d by changing the medium with freshly made inhibitor every 3 d. Untreated WI38 cells served as a control (Ctrl). SA- β -gal staining (**F**) was quantified 11 d after treatment with BU as described before. BrdU incorporation (**G**) and cell proliferation (**H**) were determined 3 d and 5 d, respectively, after the inhibitors had been removed from the culture at day 11. The data are presented as mean $\pm$ SEM ($n = 4$). *a*, $p < 0.001$ vs. Ctrl; *b*, $p < 0.001$ vs. vehicle.

Fig 2. BU induces a transient reduction in GSH but causes a continuous increase in ROS production WI38 cells were treated with BU (120 μ M) for up to 24 h, and then washed with PBS to remove BU from the culture. The cells were then cultured for up to 11 d in a drug-free medium with changes of medium every 3 to 4 d of culture. Untreated cells served as control (Ctrl). **A.** The levels of intracellular GSH were determined by measuring the fluorescent intensity of the fluorescent GSH-bimane adduct after the cells were pulsed with mBCI (40 μ M) for 30 min. **B.** The levels of ROS were determined by measuring the mean fluorescent intensity of R123 in a minimum of 20,000 cells using a flow cytometer after the cells were harvested by trypsinization and then pulsed with DHR (1 μ g/ml) for 30 min. The data are presented as mean $\pm$ SEM (n $\geq$ 4). A one-way ANOVA analysis revealed that BU significantly reduced the

levels of GSH while increased the levels of ROS in a time-dependent manner ($p<0.0001$). *
 $p<0.001$.

Fig 3. Inhibition of NADPH oxidase suppresses BU-induced production of ROS

WI38 cells were treated with BU (120 μ M) for 24 h, and then washed with PBS to remove BU from the culture. The cells were then cultured for 11 d in a drug-free medium with changes of medium every 3 to 4 d of culture. The levels of ROS were determined by measuring the mean fluorescent intensity of R123 in a minimum of 20,000 cells using a flow cytometer after the cells were harvested by trypsinization and then incubated with vehicle (V), DPI (1 μ M), apocynin (Apo, 100 μ M), rotenone (Rot, 10 μ M), allopurinol (Allo, 100 μ M), indomethacin (Indo, 10 μ M), and ketoconazole (Keto, 10 μ M) for 30 min followed by the addition of DHR (1 μ g/ml) as described previously. Untreated cells served as control (Ctrl). The data are presented as mean $\pm$ SEM ($n \geq 4$). *a*, $p < 0.001$ vs. Ctrl; *b*, $p < 0.001$ vs. vehicle.

Fig 4. Effects of NAC on BU-induced oxidative stress, activation of the Erk-p38 MAPK pathway, and induction of senescence

WI-38 cells were pre-incubated with NAC (1mM) or medium. After 24 h incubation, they were either treated with BU (120 μ M) or untreated along with continuous incubation with NAC or medium for 24 h. Thereafter, BU was removed from the culture as described previously and the cells were continuously incubated with NAC or medium for various durations. **A & B.** The levels of GSH (**A**) were determined at 30 min and those of ROS (**B**) were determined at day 11 after BU treatment as described before. **C & D.** Cell lysates were prepared 3 d after BU treatment and assayed via Western blot for detection of phosphorylated p53 (P-p53), Erk (P-Erk), p38 (P-p38), and JNK (P-JNK) and expression of p21 and p16. Blots for total p53, Erk,

p38, and JNK and β -actin were used as loading controls. A representative blot is shown. **E–F.** SA- β -gal staining (**E**) was quantified 11 d after BU treatment as described before. BrdU incorporation (**F**) and cell proliferation (**G**) were determined 3 d and 5 d, respectively, after NAC had been removed from the culture at day 11. The data presented in Figs. A, B, E–G are mean $\pm$ SEM ($n > \text{or} = 3$). A two-way ANOVA analysis revealed that NAC significantly inhibited the effects of BU on the levels of GSH, ROS production, SA- β -gal staining, BrdU incorporation and cell proliferation in WI38 cells ($p < 0.05$).