Benzylguanine induces cellular senescence and decreased relaxation of vascular smooth muscle in rat aorta

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ABSTRACT

Cellular senescence is defined by a condition of permanent cessation of growth, accompanied by distinct morphological and physiological alterations. This phenomenon is closely associated with DNA damage. Recently, our department identified that the inhibition of the DNA repair protein O6-methylguanine-DNA methyltransferase (MGMT) by O-6-benzylguanine (BG) induces cellular senescence in vascular smooth muscle cells (VSMCs). Consequently, we have resolved to validate these findings in an animal model. The objective of this study is to examine the impact of BG on cellular senescence in vascular smooth muscle and to assess its influence on the relaxation response. Wistar rats were administered BG by gavage with a dose of 12 mg/kg (2 doses per 24 hours). After 12 weeks, animals were sacrificed, the aorta was removed, cleaned, and samples were prepared for Western blotting, and small aortic rings were prepared for vascular response measurement. Senescent markers p27Kip1, p21 Cipl were significantly upregulated after BG

treatment, which confirms that MGMT inhibition leads to cellular senescence. We did not observe any statistically significant changes in the relative expression of protein p53 and ERK1/2. This means that the expression of cyclin-dependent kinase inhibitors is regulated independently of the cell cycle regulator p53 and the ERK1/2 branch of the MAPK signaling pathway. Furthermore, we observed significantly decreased relaxation response of aortic smooth muscle to sodium nitroprusside, which confirms known adverse effects of cellular senescence on the vascular system. To conclude, we found that treatment with BG triggered an increase in the relative expression of p27, p21, and PCNA compared to healthy aortic tissues, acting as mediators and markers of senescence. Our findings have provided new insights into the signaling of cellular senescence in a model of MGMT inhibition.

Keywords: cellular senescence, aortic smooth muscle, stress, benzylguanine.

1. Introduction

Cellular senescence (CS) is defined as a state of irreversible cell division arrest, which is accompanied by changes in cellular morphology, functionality, chromatin architecture, and gene expression, as well as alterations in the profile of secreted factors. This phenomenon is triggered by various stressors, including exposure to genotoxic agents, nutrient scarcity, hypoxia, mitochondrial dysfunction, and the activation of oncogenes (1). Furthermore, CS can be initiated by a diverse array of stress-inducing factors, which encompass damaging environmental and internal stimuli, aberrant cellular development, oxidative stress, and autophagy-related elements, among others (2). Based on the nature of the stressors involved, researchers have categorized CS into three distinct types: (i) replicative senescence, (ii) premature or stress-induced senescence, and (iii) oncogene-induced senescence (3).

Currently, a substantial body of research has highlighted the importance of cellular senescence, alongside cell death, in the context of responses to cancer radiotherapy and chemotherapy (3). Traditionally, senescence, which is defined by the cessation of cellular proliferation, has been regarded as a beneficial consequence of cancer treatment, with no adverse implications for the patient. However, emerging studies have demonstrated that senescent cells are not necessarily in a state of permanent growth arrest; rather, they may re-enter the cell cycle, thereby posing a potential risk to patients. This is particularly concerning as these cells frequently exhibit resistance to the therapies from which they have escaped (4).

Cellular senescence (CS) is an essential biological process that facilitates the repair of DNA damage, thereby maintaining the integrity of the cellular genome (5). This process is governed by complex signal transduction mechanisms, notably the P16Ink4a/Rb pathway and the P19Arf/P53/P21Cip1 pathway, which play pivotal roles in the regulation of the cell cycle. The P16Ink4a/Rb pathway, which encompasses the Ink4 family of cyclin-dependent kinase inhibitors, is recognized as the principal pathway that induces senescence in cells, particularly in response to stressors in epithelial cells (5). Conversely, senescent cells exhibiting telomere damage predominantly activate the P19Arf/P53/P21Cip1 pathway, particularly in murine models (5).

In human cells, both pathways are co-regulated. The P16 protein, a CDK inhibitor, plays a crucial role in senescence by inhibiting CDK4/6 and preventing the phosphorylation of the retinoblastoma protein Rb, thereby halting cell proliferation and inducing senescence (6). The P53 protein, a tumor suppressor, is upregulated in senescent cells and is activated by the P19Arf protein in response to DNA damage. P53, in turn, induces the expression of P21Cip1, which acts as a CDK inhibitor to inhibit further RB phosphorylation and cell cycle progression (6). The intricate interplay between these pathways underscores the complexity of CS regulation. CS can be induced by blocking in

the G1 phase. Stress on P21Cip1 can lead to direct cell growth arrest. Cells regulated solely by the P53/P21Cip1 pathway may resume growth after this pathway is deactivated, whereas senescent cells opt to remain permanently or temporarily stagnant (6). Extracellular signal-regulated protein kinase (ERK)1/2 is a member of the mitogen-activated protein kinase (MAPK) family, characterized by its involvement in cascade signaling mechanisms. It plays a crucial role in various signal transduction pathways and influences the activity of transcription factors, including activator protein-1, proto-oncogene c-Fos (c-Fos), and ETS domain Elk-1 (Elk1) (7). The majority of research has concentrated on its regulatory influence on cellular growth and differentiation; however, preceding studies have shown that ERK1/2 promotes cell senescence (8).

O6-methylguanine-DNA methyltransferase (MGMT) is transcriptionally upregulated in response to DNA damage, playing a pivotal role in the repair of mutagenic and cytotoxic O6-alkylguanine lesions that arise from exposure to carcinogens or cytostatic agents. Inhibitors of MGMT have a wide range of applications in biochemical and physiological research (9). O6-Benzylguanine (O6-BG), a synthetic derivative of guanine, functions as an antineoplastic agent by inhibiting the enzyme O6-alkylguanine-DNA alkyltransferase, thereby interfering with DNA repair mechanisms. In clinical practice, O6-BG has been administered alongside the alkylating agent temozolomide for the treatment of glioblastoma (10). However, this combination has been found to be excessively toxic and has not demonstrated significant therapeutic benefits. The aim of this study is to illustrate that the inhibition of MGMT by O6-BG can induce cellular senescence (CS). To this end, we investigated markers associated with CS and cellular proliferation, as well as evaluated the effects on the characteristics of vascular smooth muscle cells (VSMCs).

2. Materials and Methods

2.1. Animals

The male Wistar rats with average weight of 250-300g and age of 2-3 months were prepared from Animal House of Comenius University in Bratislava, Bratislava, Slovakia. The animals were housed under controlled light and temperature conditions, and their diet was composed of standard rodent chow and water *ad libitum*.

2.2. Aortic tissue

Rats were anaesthetized via administrating 3% isoflurane-oxygen mixture. Aorta was excised and, put in a cell culture dish with cold DMEM (Gibco, Germany) and perivascular tissue and excess blood were removed under aseptic conditions, and aorta was cut longitudinally. Entire aorta had been transferred to a second petri dish and washed several times with fresh media. By gentle scraping of the intimate layer with forceps the endothelial cells were removed. With preparation medium, remaining intimate cell was washed off and the vessel was transferred to a new petri dish again.

2.3. In-vitro aortic tissues preparation

In this study, Wistar rats were orally administered BG at a dosage of 12 mg/kg twice within a 24-hour period (10). Following administration, the animals were euthanized (ketamine/xylazine 100/10 mg/kg)), and their aortas were extracted and cleaned. A protein assay using the Pierce BCA method, which involves a colorimetric reaction, was conducted to determine protein concentration. Absorbance measurements were taken at a wavelength of 562 nm using a spectrophotometer. Subsequently, the samples were diluted to a uniform concentration, treated with Laemmli sample buffer, and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

2.4. The relaxation response of aortic smooth muscle to sodium nitroprusside (SNP)

The aorta was dissected and readied for the functional assessment of smooth relaxation in response to sodium nitroprusside. To eliminate the potential influence of the endothelium in this study, an inhibitor of endothelial NO synthesis (eNOS), specifically L-NAME, was employed. This approach was chosen to focus solely on the role of the aortic smooth muscle, excluding the endothelium from the experimental observations (11).

2.5. Western blot

In the beginning, following the addition of protein extraction mixture (0.2 ml, Sigma-Aldrich, USA). They were treated with SDS-PAGE 5X buffer for 10 minutes. The samples were separated on a 12% SDS-PAGE gel and transferred onto a polyvinylidene fluoride membrane. Subsequently, the proteins were subjected to a blocking procedure for a duration of 60 minutes utilizing a 5% solution of bovine serum albumin (Sigma-Aldrich, USA) and stored at 4 °C in the presence of monoclonal antibodies (Abcam, USA) specific to P27, P21, P53, PERK1/2, ERK, PERK/ERK, and PCNA at dilution scale 1:1000 in 1% BSA in TBST. After performing three washes with TBST, the membranes were incubated at 21°C with the secondary antibody (goat anti-mouse/rabbit, diluted 1:1000) for a duration of 120 minutes. Subsequently, the membranes underwent enhanced chemiluminescence and were analyzed utilizing a gel imaging system (Bio-Rad, USA).

2.6. Statistical analysis

In order to improve the reliability of the findings, all *in vitro* experiments were replicated three times. The gathered data were analyzed using analysis of variance (ANOVA) with SPSS software (Version 26.0), and a post hoc test was subsequently conducted. A significance threshold of $P < 0.05$ was established to denote a statistically significant difference.

3. Results

3.1. The relaxation response of aortic smooth muscle to SNP

To investigate senescence, we utilized mouse-derived aortic tissues and assessed the expression of specific proteins and signaling pathways that serve as indicators of accumulated DNA damage and senescence induction resulting from this inhibition. Our findings were compared with those from a control group of healthy cells of the same type. Due to the effects of BG, we observed a significantly reduced relaxation response of the aortic smooth muscle to SNP compared to the control group. Consequently, the inhibition of MGMT resulted in damage to the aortic smooth muscle (Figure 1).

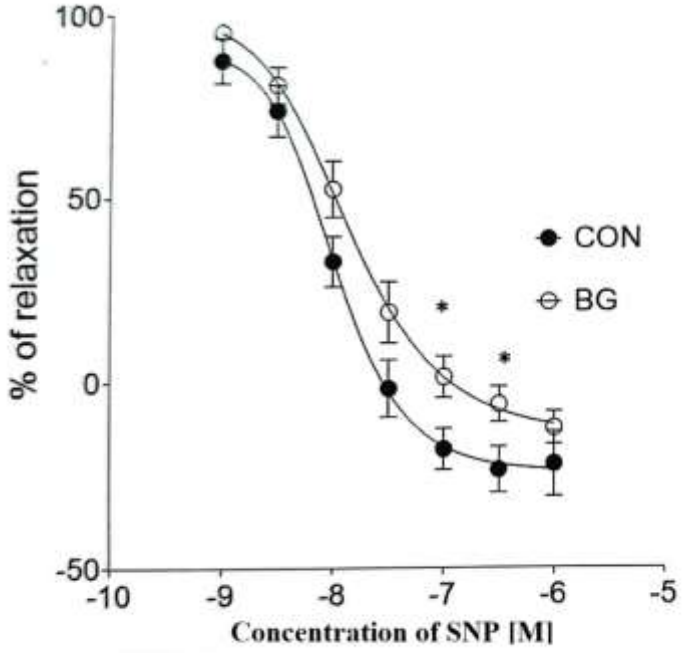

Figure 1. Aortic smooth muscle relaxation using sodium nitroprusside. (BG) a group administered with O-(6)-benzylguanine. (C) control group. * p<0,05 vs CON

3.2. Western blotting for protein expression level

The western blot analysis showed that the expression protein level of senescent markers p27, p21, and PCNA was significantly upregulated after BG treatment ($p<0.05$) (Figure2). On the other hand, he western blot analysis exhibited the relative expression of protein p53, ERK1/2, ERK, and pERK/ERK was not significantly change after BG treatment (Figure 3).

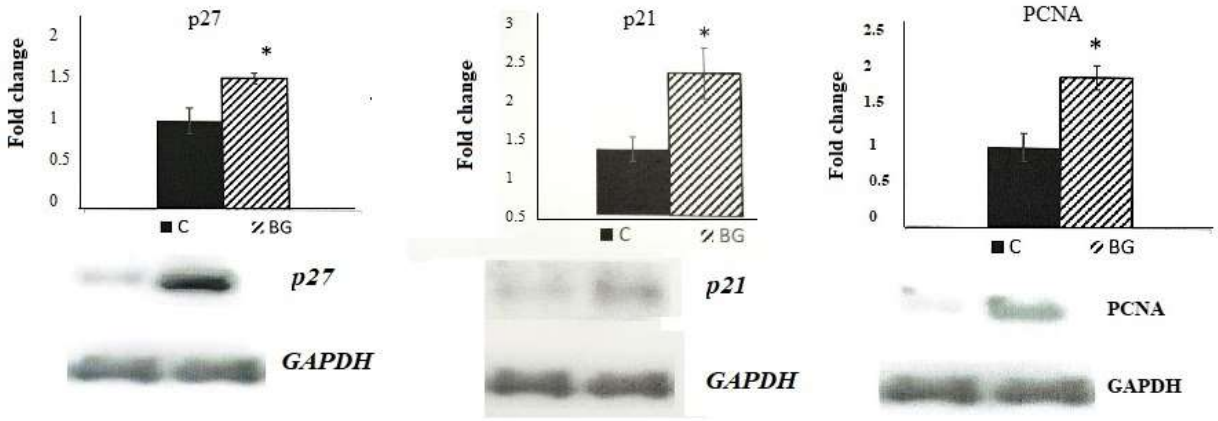

Figure 2. Protein expression level of p21, p27, PCNA, and p-ERK1/2 in aortic tissues with O-6-benzylguanine (BG)-mediated MGMT (O6-methylguanine-DNA methyltransferase) inhibition by Western blot. * $p<0,05$ vs CON

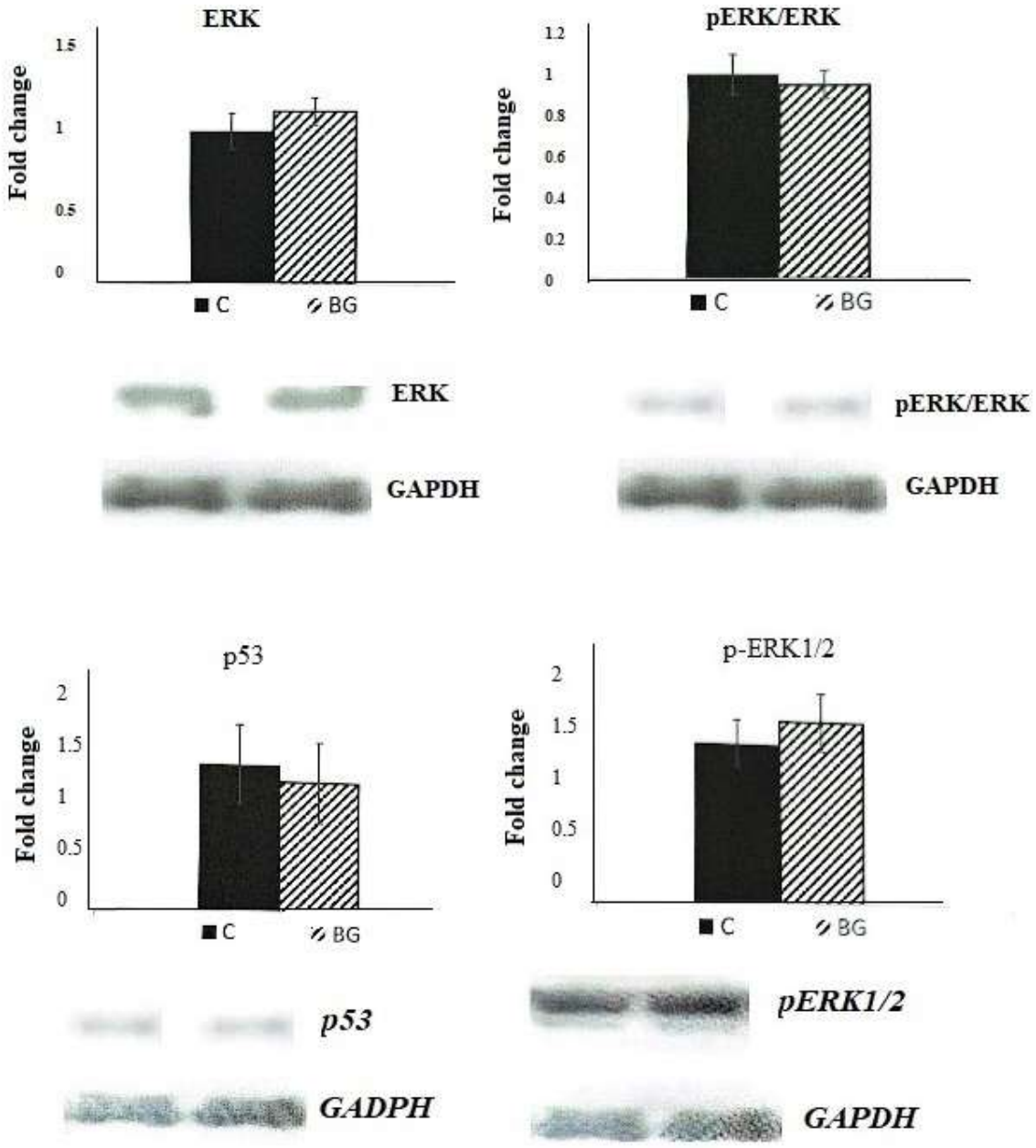

Figure 3. Protein expression level of ERK, pERK/ERK, p53, and pERK1/2 in aortic tissues with O-6-benzylguanine (BG)-mediated MGMT (O6-methylguanine-DNA methyltransferase) inhibition by Western blot.

4. Discussion

Cellular senescence can be triggered by various stimuli and is regulated by a number of coordinating or independent mechanisms, many of which remain poorly understood. In this study, we aimed to investigate whether MGMT inhibition induces senescence, examining senescence markers and potential alterations in proliferation pathways to determine if cellular senescence in this model (MGMT inhibition) adversely affects the relaxation response of VSMCs (9). To achieve this, we conducted preliminary research within the context of cellular senescence, focusing on the effects of O°-benzylguanine (BG)-mediated MGMT inhibition in aortic tissues by observing senescence markers and vascular relaxation using antibodies to assess changes in the relative protein expression of associated signaling cascades through Western blot analysis.

Concerning p53 expression, we observed that no significant changes occurred after BG treatment. Konduri et al. have shown that O'-benzylguanine's inhibition of MGMT expression contributes to tumor growth suppression and the induction of apoptosis (12). In a similar study, tumor growth was more effectively inhibited when combined with gemcitabine, and this inhibition was followed by the activation of the senescence markers p53 and p21 (13). This indicates that a variety of coexisting factors are influencing the effects of MGMT inhibition on senescence and proliferation. As previously described, senescence often serves as a protective barrier that prevents the transmission of DNA damage to daughter cells during mitosis. Our findings suggest that senescence may result from MGMT's lack of effective repair mechanisms, leading to chronic DNA damage. Although sprevious dtudies showed that the multiple downregulated repair genes could contribute to cancer development, however, some studies indicated that the loss of MGMT correlates with a concomitant loss of MLH1 in numerous gastric cancer specimens, with this loss accelerating as the tumor progresses (13, 14). These changes can be carcinogenic, suggesting that

SIPS is activated by the senescent cascade. Since MGMT deficiency alone is not a sufficient carcinogenic factor, BG may have induced additional stressors that contribute synergistically to DNA damage (14).

Concerning p21 expression, we observed that it is significantly upregulated following BG treatment. P21Clip is a cell cycle inhibitor, and its overexpression can arrest cell cycle progression during the transitions from G1/S and G2/M phases (15). P53 is a major transcriptional activator of p21Clip, and any factor that inhibits p53 function could downregulate p21Clip (15). Based on the measured p53 expression after BG treatment, we hypothesize that BG treatment does not operate as an environmental stressor that may activate ATM and ATR (16). These agents are responsible for the phosphorylation and activation of the p53 signaling cascade. Therefore, our results suggest that p21Clip induction occurs through an independent mechanism (16). According to Jeesun Kim et al., the intracellular accumulation of reactive oxygen species (ROS) is correlated with the action of ataxia-telangiectasia mutated (ATM) protein, specifically the activation of p21 and the mediation of cellular senescence (17). Consequently, we speculate that treatment with BG has resulted in an increased intracellular accumulation of ROS, likely by influencing mitochondrial function and triggering a senescence response. P21 is also believed to mediate inflammatory responses, excluding stress responses. The induction of senescence is followed by the development of an inflammatory phenotype, which may be linked to the upregulation of p21. Additionally, Chk2-dependent senescence was observed in tumor cells with p53 deficiencies, and it relied on the upregulation of p21 (18). This indicates a novel p53-independent mechanism that leads to p21 accumulation and senescence (18).

Our findings indicate that the relative expression of p27 significantly increased following BG treatment, suggesting that the upregulation of p27 may occur through numerous and complex pathways. This protein is expressed under strong transcriptional control and plays a critical role in regulating the G1/S transition (19). Disruption of the transcriptional balance of p27Kip, particularly through overexpression, inhibits progression to the S phase, potentially initiating cellular senescence to some extent (19). It has been proposed that the ERK pathway is crucial for entry into the S phase and overlaps with p27Kip, leading to its downregulation. However, our findings demonstrated simultaneous activation of p27Kip without a significant change in ERK activity. In the short term, ERK signals promote proliferation, while prolonged expression signals cellular senescence (19). Villanueva et al. (2007) indicated that the effect of ERK on p27Kip is mediated by SCFSkp2 and E3 ligase, which post-transcriptionally regulates p27Kip levels via ubiquitin-mediated proteolysis during the late G1/S phase (20). This process could potentially interfere with BG treatment, allowing for the overexpression of p27Kip. Moreover, according to the previous studies, SCFSkp2 exhibits low affinity for a mutant form of p27 (p27T187A), thereby indirectly disrupting the downregulation of ERK-mediated p27Kip (20, 21). Since the upregulation of ERK in our experiment does not correlate with the downregulation of p27Kip, we hypothesized that treatment with BG may have triggered a mutation in p27Kip1 that regulatory proteins cannot recognize (21). In fact, several other signaling pathways can regulate p27, and we observed only a fragment of these pathways in our results. Such a situation could be complemented by potential oncogenic upregulation, which subsequently leads to senescence and cell cycle arrest, aiming to limit the possible DNA manipulation by SCFSkp2. This level of regulation might present a promising therapeutic target for p27Kip regulation in cases where the overexpression of this protein appears to be ERK-independent (22). However, T187-mediated degradation of p27Kip is

believed to be independent of both SCFSkp2 and ERK. Oncogenic stress leading to increased oncogene activation may negatively affect the reduction of Thr187-dependent p27Kip, thereby maintaining the protein's physiological levels (22). In its unphosphorylated form, p27Kip demonstrates affinity for CDK, particularly CDK2. In our study, the activation of p27Kip may be linked to a possible BG effect on the Thr187 subunit, resulting in a loss of function (marking p27Kip for degradation) and disruption of its physiological activity. Depending on the pathological condition, the Thr187 substrate may serve as a pharmacological target for induction or suppression. For example, the upregulation of p27Kip appears to stimulate senescence, which may be beneficial in various diseases associated with DNA damage propagation (potentially related to BG), while preventing p27Kip phosphorylation by specific oncogene kinases (23) may reduce tumor resistance to drugs.

According to our findings, the effect of BG significantly reduced the relaxation response of aortic smooth muscle to SNP compared to the control group. Besse et al. report for the first time that 50 minutes of hypoxia leads to an impairment of phenylephrine (PE)-induced contraction and SNP-induced relaxation. They demonstrated that hypoxic stress causes greater endothelial injury in senescent aorta and decreases transient hypoxic contraction, without aggravating late hypoxic contraction (24). Lönnrot et al. have shown that aging is linked to a reduced nitroprusside relaxation response (25). Interpreting our results further, we observed no changes in the induction of ERK1/2. The Ras-Raf-MEK-ERK1/2 pathway consists of a protein chain that extends from the cell membrane to the nucleus, functioning as a signal propagator. This pathway regulates feedback mechanisms and amplifies signals. The role of ERK1/2 is to phosphorylate adjacent proteins, and it is involved in cell proliferation, differentiation, the construction of cytoskeletons, and possibly the promotion of cellular senescence (27). MEK1/2 functions upstream of ERK1/2 and plays a

crucial role as an activator of ERK1/2 (27). The MEK1/ERK complex tends to stimulate cell proliferation, while the MEK2/ERK complex is associated with the arrest of the cell cycle at the G1/S phase (26). Enhanced expression of the upstream regulator MEK1 may promote senescence, potentially depending on the cell type. Research on ERK1/2 has demonstrated its dual function; its activation can either trigger forced mitogenesis or lead to the arrest of the G1/S cell cycle (28, 29). The cell's fate following MEK activation and subsequent stimulation of ERK1/2 depends on the integrity of key senescence regulators such as p16 and p53, which likely drive the affected cell toward senescence rather than excessive proliferation (26). Since stress-induced premature senescence (SIPS) often occurs as a result of DNA damage, we may infer that BG has negatively impacted genomic integrity, likely due to the increased incidence of guanine methylation resulting from MGMT inhibition (30) . Considering the dual function of ERK1/2 and the fact that the cells underwent SIPS rather than forced mitogenesis, we speculate that the integrity of senescence regulators remained intact and that the activity of their signaling cascades was not adversely affected by BG treatment. A mutation or other disturbance may have induced cells carrying BG-induced damage, thereby increasing the risk of carcinogenesis. These findings support the idea that bioactive compounds may activate and upregulate the senescence markers p21 and p27. This is confirmed by experiments involving the microtubule-stabilizing agent discodermolide, which has been shown to induce senescence-associated phenotypic changes (SIPS) as a result of p21 and p27 overexpression (31).

Since MEK/ERK functional uncoupling occurs in response to specific stimulating factors, such as cytokines or Toll-like receptor ligands in human neutrophils (32), it is likely that interventions aimed at modulating inflammation and/or senescence should focus on the suppression or induction of these factors, respectively. However, the effects of BG treatment on the expression of

senescence markers in aortic tissue suggest a specific mechanism underlying BG's role in inhibiting senescence. In our study, we observed an enhanced induction of the PCNA signaling cascade. Numerous regulatory proteins that control the cell cycle influence its progression through interactions with PCNA. PCNA interacts with cyclin-CDK complexes and the CDK inhibitor p21. Additionally, PCNA serves as an auxiliary factor for DNA polymerases and is crucial for DNA replication both in vitro and in vivo. P21 interacts with PCNA and inhibits DNA replication when present in cells. Hwang et al. demonstrated that sublethal doses of H2O2 reduced the levels of proliferating cell nuclear antigen (PCNA) in normal cells, such as primary human dermal fibroblasts and IMR-90 cells, without affecting the activity of cyclin-dependent kinase 2 (CDK2). This reduction resulted in cell cycle arrest and subsequent senescence. PCNA, a DNA polymerase accessory protein and regulator of the G1/S transition, is a senescence factor that is activated by H2O2 (33, 34).

To conclude, we found that treatment with BG actually triggered an increase in the relative expression of p27, p21, and PCNA compared to healthy aortic tissues, acting as mediators and markers of senescence. This increase was not observed in p53 and ERK1/2, suggesting that BG treatment induces senescence through an independent pathway. All the evaluated proteins and signaling cascades play crucial roles in maintaining cellular homeostasis, regulating the cell cycle, proliferation, differentiation, and responding to stressful stimuli. These pathways interact with senescence mediation and directly or indirectly influence this process. Cellular reactions to chemical stimuli vary depending on the specific drug, the cell system, and the mutations involved, as well as pre-existing conditions and post-treatment factors. Our findings provide insights into the harmful stimuli and the activated pathways related to cellular responses to BG. This information can be contextualized within the framework of manipulating senescence and

highlights the role of BG as a potential senescence-inducing agent, which may be utilized in novel therapeutic approaches. A better understanding of cellular senescence and its complex mechanisms will enhance therapies for various senescence-related diseases, such as cancer and cardiovascular diseases, both of which pose significant health concerns in the developed world and present challenges for the scientific community.

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Author Contributions

NAK and MPV designed and supervised the study and writing the draft; NAK do experiments, obtained data, review and edited the manuscript; all authors agreed the final version to be published.

Ethics

The animal Research Ethics Committee of the Faculty of Pharmacy, Comenius University in Bratislava, Bratislava, Slovakia approved the animal experimental protocols (No. 14011914104).

Conflict of interest

The authors declare no conflict of interest in this study.

Findings

None.

Data Availability

No datasets were produced or examined in the course of the present study.

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