Biochemical and Biophysical Research Communications 417 (2012) 237–244

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

p38 mitogen-activated protein kinase is a key regulator of 5-phenylselenyl- and 5-methylselenyl-methyl-2′-deoxyuridine-induced apoptosis in human HL-60 cells

Byeong Mo Kim [a,1], Kee-Ho Lee [a], In Seok Hong [b], Sung Hee Hong [a,*]

[a] Division of Radiation Cancer Research, Korea Institute of Radiological and Medical Sciences, Seoul 139-706, Republic of Korea
[b] Department of Chemistry, College of Natural Sciences, Kongju National University, Gongju, Chungnam 314-701, Republic of Korea

ARTICLE INFO

Article history:
Received 14 November 2011
Available online 28 November 2011

Keywords:
5-Phenylselenyl-methyl-2′-deoxyuridine (PhSe-T)
5-Methylselenyl-methyl-2′-deoxyuridine (MeSe-T)
Caspases
Inhibitors
p38 mitogen-activated protein kinase (p38 MAPK)
ROS

ABSTRACT

Two novel, modified thymidine nucleosides, 5-phenylselenyl-methyl-2′-deoxyuridine (PhSe-T) and 5-methylselenyl-methyl-2′-deoxyuridine (MeSe-T), trigger reactive oxygen species (ROS) generation and DNA damage and thereby induce caspase-mediated apoptosis in human HL-60 cells; however, the mechanism leading to caspase activation and apoptotic cell death remains unclear. Therefore, we investigated the signaling molecules involved in nucleoside derivative-induced caspase activation and apoptosis in HL-60 cells. PhSe-T/MeSe-T treatment activated two mitogen-activated protein kinases (MAPKs), extracellular-receptor kinase (ERK) and p38, and induced the phosphorylation of two downstream targets of p38, ATF-2 and MAPKAPK2. In addition, the selective p38 inhibitor SB203580 suppressed PhSe-T/MeSe-T-induced apoptosis and activation of caspase-3, -9, -8, and -2, whereas the jun amino-terminal kinase (JNK) inhibitor SP600125 and the ERK inhibitor PD98059 had no effect. SB203580 and an ROS scavenger, tiron, inhibited PhSe-T/MeSe-T-induced histone H2AX phosphorylation, which is a DNA damage marker. Moreover, tiron inhibited PhSe-T/MeSe-T-induced phosphorylation of p38 and enhanced p38 MAP kinase activity, indicating a role for ROS in PhSe-T/MeSe-T-induced p38 activation. Taken together, our results suggest that PhSe-T/MeSe-T-induced apoptosis is mediated by the p38 pathway and that p38 serves as a link between ROS generation and DNA damage/caspase activation in HL-60 cells.

1. Introduction

The nucleoside derivatives 5-phenylselenyl-methyl-2′-deoxy uridine (PhSe-T) and 5-methylselenyl-methyl-2′-deoxyuridine (MeSe-T) are selective DNA damaging agents that act via incorporation into DNA [1,2]. Recently, we demonstrated that PhSe-T and MeSe-T induce reactive oxygen species (ROS) generation and DNA damage, such as double-stranded and single-stranded breaks in human HL-60 cells, which activate the caspase cascade of apoptotic signals [3]. This activation was followed by increases in apoptotic nuclei and the sub-G1 fraction [3]. However, the regulatory proteins acting upstream of caspase activation in both PhSe-T- and MeSe-T-induced apoptosis remain unidentified.

The members of the mitogen-activated protein kinase (MAPK) family respond to extracellular stimuli and regulate various cellular activities. The MAPK family is composed of three subfamilies, including the extracellular-receptor kinases (ERKs), stress-activated protein kinases (SAPKs)/jun amino-terminal kinases (JNKs), and p38 MAPK. In general, ERKs are associated with growth and proliferation, whereas JNKs and p38 are involved in cell death, including apoptosis [4]. MAPKs may also play an important role in PhSe-T/MeSe-T-induced apoptosis in HL-60 cells.

MAPKs can be activated and exert their roles in response to stressors, such as DNA damage. In some cases, MAPKs can also contribute to anticancer agent-induced DNA damage as measured by the detection of γ-H2AX [5–7]. One of the potential stressors that induces DNA damage and activation of the MAPK pathway is oxidative stress due to ROS. Many anticancer agents induce ROS generation and trigger cancer cell apoptosis via the ROS–MAPK pathway. In particular, ROS can act as a second messenger by activating a diverse redox-sensitive signaling transduction cascade, including p38 MAPK and its subsequent target, the stress-related transcription factor activating transcription factor-2 (ATF-2) [8].

This study was designed to elucidate the molecular pathway that mediates PhSe-T/MeSe-T-induced apoptosis in human HL-60 cells. We found that both PhSe-T and MeSe-T activate the MAPK signaling pathway involving ERK and the p38 pathway; however, only the p38 pathway is necessary for nucleoside derivative-induced apoptosis. Our results also demonstrate that p38 plays an important role in ROS-mediated DNA damage, caspase cascade activation, and apoptosis in PhSe-T/MeSe-T treated HL-60 cells.

* Corresponding author. Address: Division of Radiation Cancer Research, Korea Institute of Radiological and Medical Sciences, 215-4, Gongneung-Dong, Nowon-Gu, Seoul 139-706, Republic of Korea. Fax: +82 2 970 2402.
E-mail address: gobrian@kcch.re.kr (S.H. Hong).
[1] Current address: Division of Gerontology, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA.

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doi:10.1016/j.bbrc.2011.11.092

2. Materials and methods

2.1. Cell culturing

The human leukemia cell line HL-60 was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were grown in RPMI 1640 medium (Gibco-BRL Life Technologies, Gaithersburg, MD) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco-BRL Life Technologies) at 37 °C in an atmosphere containing 5% CO_2.

2.2. Preparation and treatment of PhSe-T and MeSe-T

PhSe-T and MeSe-T were prepared as described previously [1,2]. A 10 mM stock solution of PhSe-T and MeSe-T was prepared by dissolving both nucleoside derivatives in distilled de-ionized water (DDW). The stock solution was then filtered through a 0.22 μM filter and stored at 4 °C until use. PhSe-T (150 μM) or MeSe-T (10 μM) was then added to the culture medium in the presence or absence of caspase inhibitors or MAPK signaling inhibitors.

2.3. Inhibition of caspases and MAPKs

HL-60 cells were pre-treated with the caspase-3 inhibitor z-DEVD-fmk, caspase-9 inhibitor z-LEHD-fmk, caspase-8 inhibitor z-IETD fmk, caspase-2 inhibitor z-VDVAD-fmk (all from Calbiochem, La Jolla, CA), or MAPK inhibitors, which included the p38 inhibitor SB203580, the JNK inhibitor SP600125, and the ERK inhibitor PD98059 (all from Biosource, Camarillo, CA), or vehicle [dimethyl sulfoxide (DMSO)] for 2 h, followed by exposure to PhSe-T or MeSe-T.

2.4. Western blot analysis

Cell lysates were prepared using RIPA lysis buffer and soluble protein concentrations were determined using the Bradford assay (Bio-Rad Laboratories, Hercules, CA). An aliquot containing 10 μg of protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (NEN; PerkinElmer, Wellesley, MA). The blots were blocked with 5% skim milk in Tris-buffered saline (TBS)-Tween 0.1% (TBS-T) and probed with specific primary antibodies, followed by HRP-conjugated secondary antibodies. The Western blot shown is representative of two experiments.

2.5. Nuclear staining with Hoechst 33342

After 40 h-treatment with PhSe-T or MeSe-T, cells were harvested, washed in ice-cold phosphate buffered saline (PBS), and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. After rinsing with PBS, the cells were incubated with 1 μg/mL Hoechst 33342 for 20 min at room temperature in the dark. The cells were visualized microscopically using fluorescence optics. Apoptotic nuclei were identified by the presence of condensed chromatin that was distinct from that in live or necrotic nuclei.

2.6. Caspase activity assay

Enzymatic activities of caspase-3, -8, and -2 were monitored in the untreated control cells and in cells treated with PhSe-T for 40 h or MeSe-T for 32 h. The Caspase-3/CPP32 Colorimetric Assay Kit, FLICE/Caspase-8 Colorimetric Assay Kit, and Caspase-2 Colorimetric Assay Kit (all from BioVision, Palo, Alto, CA) were used to determine the activities of caspase-3, -8, and -2, respectively. The activity levels in the cell lysates were determined by incubating the samples with the caspase-3, -8, or -2 synthetic peptide substrate DEVD-pNA (p-nitroanilide), IETD-pNA, or VDVAD-pNA, respectively. Cleavage of the substrates was followed spectrophotometrically and activity levels were calculated according to the manufacturer's instructions.

2.7. p38 activity assay

In vitro p38 kinase activity was measured using a p38 MAP kinase assay kit (Cell Signaling Technology), according to the manufacturer's protocol. Immobilized phospho-p38 MAPK (Thr180/Tyr182) monoclonal antibody (20 μl) was added to a 200 μl aliquot of cell lysate and incubated overnight at 4 °C with gentle rocking. The immunoprecipitated pellet was washed and resuspended in 50 μl kinase buffer supplemented with 200 μM ATP and 2 μg of ATF-2 fusion protein, then incubated for 30 min at 30 °C. The reaction mixture was separated by SDS–PAGE. Western blots were performed to detect phosphorylated ATF-2.

2.8. Immunostaining for γ-H2AX foci

After HL-60 cells were treated with PhSe-T or MeSe-T for 32 h in the absence or presence of the ROS scavenger tiron or the p38 inhibitor SB203580, they were fixed in 2% paraformaldehyde for 15 min 4 °C and then in 70% ethanol at −20 °C for 20 min. The fixed cells were permeabilized in PBS containing 100 mM Tris–HCl, 50 mM ethylenediamine tetra-acetic acid (EDTA), and 0.5% Triton X-100 for 15 min at room temperature. Cells were then incubated with primary antibody against phospho-H2AX (1:100) at 4 °C overnight and detected with AlexaFluor 594-conjugated anti-rabbit secondary antibody (Molecular Probes, Eugene, OR, USA). Slides were counterstained with DAPI and images were acquired on a fluorescence microscope (ECLIPSE E600; Nikon, Tokyo, Japan). Cells were classified as 'positive' when more than three H2AX foci per cell were observed.

2.9. Measurement of reactive oxygen species (ROS)

The level of intracellular ROS generation was detected using 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFDA; Molecular Probes). After HL-60 cells were exposed to PhSe-T or MeSe-T for 24 h in the absence or presence of a p38 MAPK inhibitor SB203580, the medium was discarded and culture medium containing 20 μM DCFDA was added under low-light conditions. The cells were incubated for 30 min at 37 °C and analyzed for fluorescence distribution using flow cytometry (FACSCalibur, Becton–Dickinson, CA, USA).

2.10. Statistical analysis

Statistical analyses were performed using SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL). The data are expressed as the mean ± standard error (SE) of three independent experiments. An analysis of variance (ANOVA) followed by Dunnett's multiple comparison *post hoc* test was used to assess differences between the two groups and a p-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Effects of PhSe-T/MeSe-T on MAPK pathways

MAPK signaling plays an important role in the mitogenic response and the induction of apoptosis in response to stress. MAPKs are also involved in activating the caspase cascade. For example,

some studies have indicated that p38 activates the caspase-8 and -9-mediated apoptotic pathways [9,10]. We analyzed the effects of PhSe-T/MeSe-T treatment on the Erk, p38, and JNK pathways. Western blotting revealed that both nucleoside derivative treatments increased the expression levels of phosphorylated mitogen-responsive Erk MAPK and stress-responsive p38 MAPK. In contrast, these derivatives did not affect JNK activation (Fig. 1A).

3.2. Effects of PhSe-T/MeSe-T on the c-Raf/MEK/ERK pathway and p38 MAPK activity

Because the protein kinases c-Raf and MEK1/2 specifically activate Erk MAP kinase [11,12], we investigated whether these kinases were activated during PhSe-T/MeSe-T exposure. Western blots revealed that the phosphorylation of c-Raf and MEK1/2 was significantly upregulated in nucleoside derivative-exposed cells, indicating that PhSe-T/MeSe-T affects the early events in the Erk signaling cascade (Fig. 1B). We also observed that these derivatives induced phosphorylation of ATF-2 and MAPKAPK-2. Because these two proteins are target substrates of active p38 [13], these results indicate that PhSe-T/MeSe-T can enhance p38 activity (Fig. 1C). Furthermore, *in vitro* kinase assays also indicated that both PhSe-T and MeSe-T enhanced the kinase activity of p38 (data not shown).

3.3. Suppression of PhSe-T/MeSe-T-induced apoptosis through inhibition of p38 MAPK

To examine the role of MAPKs in PhSe-T/MeSe-T-induced apoptosis, we pre-incubated HL-60 cells with selective MAPK inhibitors before exposure to PhSe-T and MeSe-T. We then performed Hoechst 33342 staining to examine the cells for the morphological characters of apoptosis. Pre-incubation with a selective inhibitor of p38, SB203580 (4 μM), significantly attenuated the PhSe-T/MeSe-T-induced increase in the number of apoptotic cells (Fig. 2A). However, pre-incubation with SP600125 (JNK inhibitor, 20 μM) or PD98059 (Erk inhibitor, 60 μM) did not inhibit PhSe-T/MeSe-T-induced apoptosis (Fig. 2A). Small interfering RNA (si-RNA) targeting p38, but not JNK or ERK, inhibits PhSe-T/MeSe-T-induced apoptosis (data not

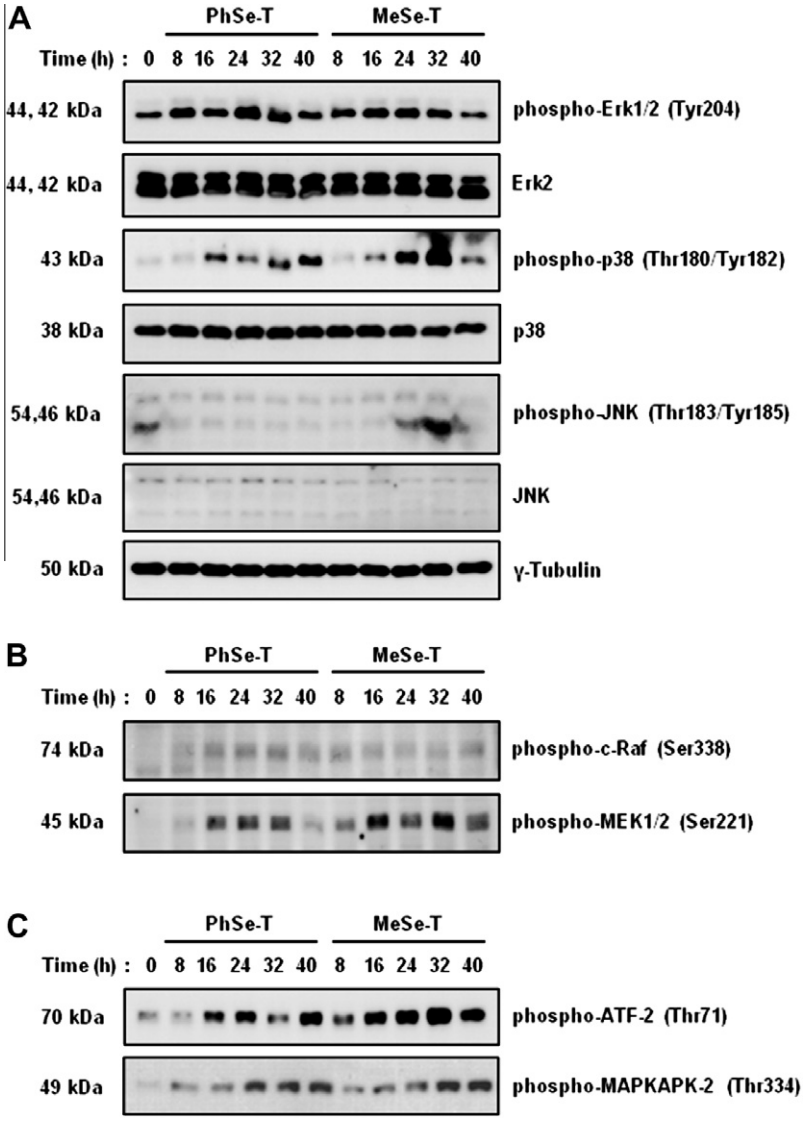

Fig. 1. Effects of PhSe-T and MeSe-T on various MAPKs. Human HL-60 cells were challenged with 150 μM of PhSe-T or 10 μM of MeSe-T for the indicated times, and protein expression was analyzed by Western blotting. (A) Time course of the effects of PhSe-T/MeSe-T on MAPK pathway components, including Erk, p38, and JNK. (B) PhSe-T/MeSe-T-mediated phosphorylation of Erk signaling cascade members, such as c-Raf and MEK1/2, were analyzed by phospho-specific antibodies. (C) The effects of PhSe-T and MeSe-T on p38 activity were monitored for the phosphorylated substrates of p38, ATF-2, and MAPKAPK-2 by phospho-specific antibodies.

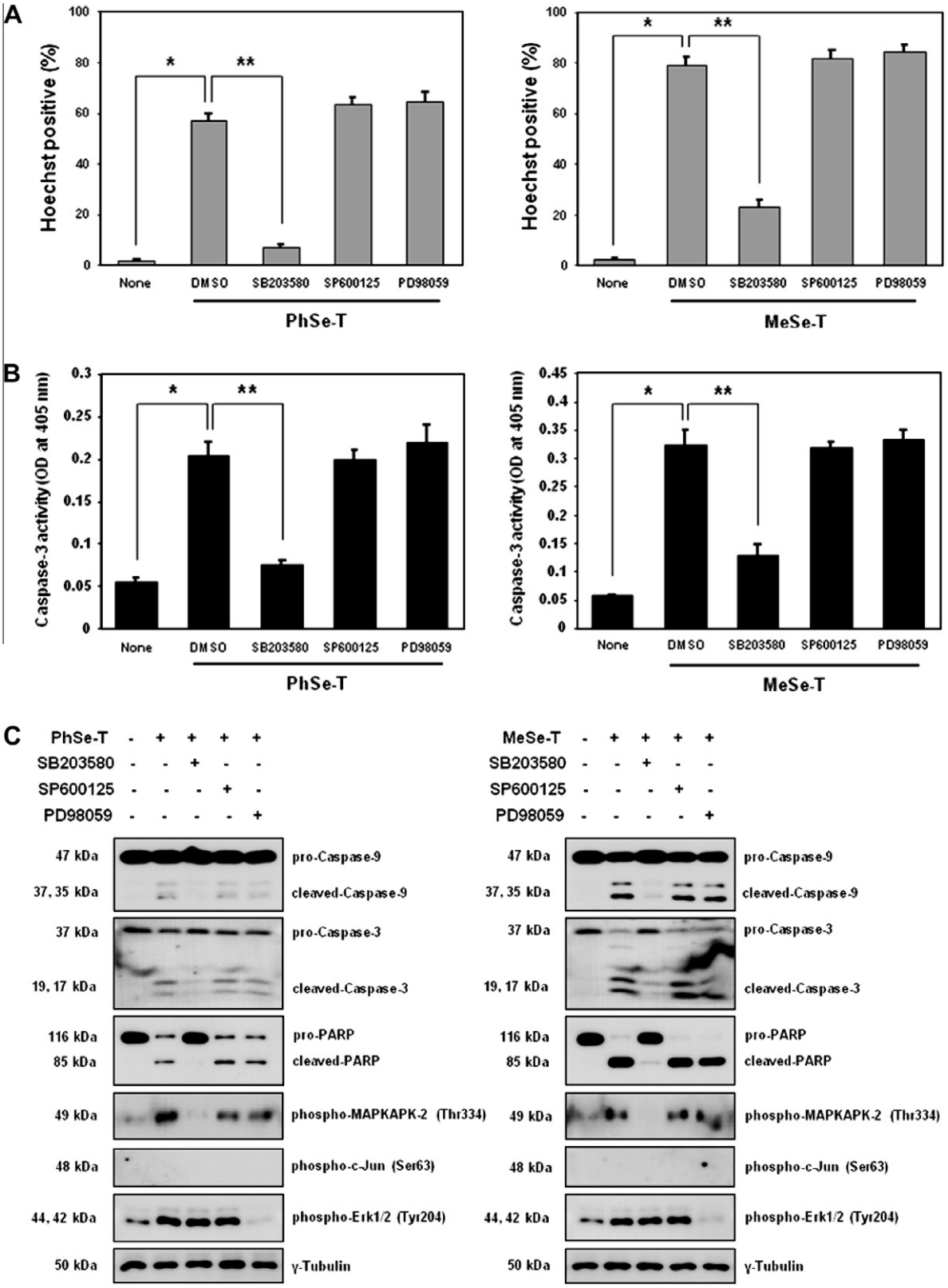

Fig. 2. The essential role of p38 MAPK in PhSe-T/MeSe-T-induced caspase-mediated apoptosis. (A–C) HL-60 cells were exposed to 150 μM of PhSe-T or 10 μM of MeSe-T for 40 h for Hoechst 33342 staining or for 40 h (for PhSe-T) or 32 h (for MeSe-T) for caspase-3 activity and Western blot analysis in the presence or absence of SB203580 (4 μM), SP600125 (20 μM), or PD98059 (60 μM). Apoptotic cells (A) were quantified after Hoechst 33342 staining and caspase-3 activity (B) was monitored via the detection of pNA (p-nitroanilide) liberated from the substrate DEVD-pNA. Each data point represents the mean ± standard error of three independent experiments (*$P < 0.05$ compared to the untreated control; **$P < 0.05$ compared to PhSe-T alone or MeSe-T alone; ANOVA/Dunnett's test). (C) Western blotting was used to analyze cleavage of caspase-9 and -3 and caspase-3-mediated PARP cleavage in PhSe-T/MeSe-T-treated cells. The efficacy of the MAPK inhibitors SB203580, SP600125, and PD98059 was confirmed by determining the phosphorylation levels of MAPKAPK-2, c-Jun, and Erk, respectively.

shown). These results indicate that the p38 pathway is important for the nucleoside derivative-induced apoptosis in HL-60 cells.

3.4. Suppression of PhSe-T/MeSe-T-induced caspase-3 activation and caspase-3-mediated PARP cleavage through inhibition of p38

Because caspase-3 is involved in PhSe-T/MeSe-T-induced apoptosis in HL-60 cells [3], we examined the role of MAPKs in derivative-induced caspase-3 activation. As shown in Fig. 2B, SB203580, but not SP600125 or PD98059, significantly suppressed PhSe-T/MeSe-T-induced increases in caspase-3 activity. To further verify the results shown in Fig. 2A and B, we also examined the effects of the MAPK inhibitor on the cleavage of caspase-9, -3, and PARP. Western blots demonstrated that SB203580, but not SP600125 or PD98059, abolished PhSe-T/MeSe-T-induced cleavage of caspase-9, caspase-3, and PARP, confirming the results shown in Fig. 2A and B (Fig. 2C). As controls, the phosphorylation of MAPKAPK-2 and c-Jun, which are direct substrates of p38 and JNK, respectively [13,14], and the phosphorylation of Erk1/2 were examined to assess the selectivity of the MAPK inhibitors at the concentrations used in this study. In all cases, each of the inhibitors tested selectively inhibited their corresponding MAPK substrate without altering the phosphorylation of the other MAPKs (Fig. 2C).

3.5. Suppression of PhSe-T/MeSe-T-induced activation of caspase-2 and caspase-8 through inhibition of p38

Based on our previous study, caspase-2 and caspase-8 are initiator caspases that play important roles in PhSe-T/MeSe-T-induced apoptosis and caspase-3 activation [3]. To examine the role of p38 in the nucleoside derivative-induced activation of caspase-2 and caspase-8, we pre-incubated HL-60 cells with the indicated concentrations of the p38 inhibitor SB203580 before exposure to PhSe-T and MeSe-T. The caspase activity assay showed that pre-incubation with SB203580 significantly attenuated PhSe-T/MeSe-T-induced activity of caspase-2 and caspase-8 (Fig. 3A). Small interfering RNA (si-RNA) targeting p38 also inhibited PhSe-T/MeSe-T-induced activity of caspase-2 and caspase-8 (data not shown). Western blot analysis demonstrated that the indicated concentrations of SB203580 abolished PhSe-T/MeSe-T-induced cleavage of caspase-2 and caspase-8, as well as cleavage of caspase-3 and caspase-9 (Fig. 3B). However, neither a caspase-2 inhibitor, z-VDVAD-fmk, nor a caspase-8 inhibitor, z-IETD-fmk, affected PhSe-T/MeSe-T-induced enhanced phosphorylation of p38 (Fig. 3C) and p38 kinase activity (Fig. 3D). These results indicate that p38 acts upstream of the caspase cascade in derivative-treated HL-60 cells.

3.6. Effects of ROS scavenging on PhSe-T/MeSe-T-induced DNA damage

Previously, we showed that ROS are critical regulators of PhSe-T/MeSe-T-induced apoptosis and caspase activation [3]. We also showed that PhSe-T/MeSe-T triggered DNA damage, such as double-stranded and single-stranded breaks [3]. Therefore, we examined whether ROS are involved in derivative-induced DNA damage. The indicated concentrations of tiron, which was found to be the most efficient ROS scavenger in PhSe-T/MeSe-T-treated cells in a previous study [3], significantly suppressed PhSe-T/MeSe-T-induced γ-H2AX foci, which is a DNA damage marker [7]. These results suggest that ROS are critical mediators in PhSe-T/MeSe-T-induced DNA damage (Fig. 4A).

3.7. Effects of p38 inhibition on PhSe-T/MeSe-T-induced DNA damage

To examine the roles of p38 in PhSe-T/MeSe-T-induced DNA damage, we determined the frequency of cell staining for γ-H2AX foci formation. As shown in Fig. 4B, the indicated concentrations of SB203580 significantly attenuated PhSe-T/MeSe-T-induced γ-H2AX foci. Western blot analysis using phospho-H2AX (Ser139) antibody also showed that SB203580 inhibits PhSe-T/MeSe-T-induced Ser139 phosphorylation of H2AX (Fig. 4B). These results suggest that p38, like ROS, is a critical regulator of DNA damage in PhSe-T/MeSe-T-treated cells.

3.8. The essential role of ROS in PhSe-T/MeSe-T-induced enhanced p38 activity

To examine the possible role of p38 in PhSe-T/MeSe-T-induced ROS generation, we pre-incubated HL-60 cells with the indicated concentrations of SB203580 and measured ROS generation. As shown in Fig. 4C, SB203580 did not suppress PhSe-T/MeSe-T-induced DCF fluorescence. Small interfering RNA (si-RNA) targeting p38 also did not suppress derivative-induced DCF fluorescence, thus confirming the results shown in Fig. 4C (data not shown). In contrast, tiron efficiently attenuated PhSe-T/MeSe-T-induced p38 kinase activity (Fig. 4D). Tiron also efficiently attenuated PhSe-T/MeSe-T-induced phosphorylation of p38 (data now shown). These results suggest that ROS are critical mediators of p38 activation in PhSe-T/MeSe-T-treated HL-60 cells.

4. Discussion

Recently, we reported that two novel thymidine nucleoside derivatives, PhSe-T and MeSe-T, induced apoptotic cell death in various human cancer cells [3]. We also observed that these derivatives triggered DNA damage, such as double-stranded breaks (DSBs), and induced caspase-2-dependent apoptosis. In addition, ROS are essential for PhSe-T/MeSe-T-induced apoptosis and caspase-2 activation [3]. In this study, we demonstrated a critical role for p38 MAPK and its link between ROS generation and caspase-mediated apoptosis in PhSe-T/MeSe-T-treated HL-60 cells.

First, we provided evidence that both PhSe-T and MeSe-T activated the MAPK signaling pathway. MAPK signaling is important for both the mitogenic response and the induction of apoptosis during a variety of stress responses. MAPKs are also implicated in activating the caspase cascade. For example, a recent study reported that p38-MAPK signaling is linked to the activation of caspase-8 and caspase-9 [9,10]. Our present results showed that both PhSe-T and MeSe-T induced a time-dependent increase in the phosphorylation of Erk and p38. Our data also showed that PhSe-T and MeSe-T induced an increase in the phosphorylation of ATF-2 and MAPKAPK-2, two downstream targets of p38 [13], indicating that these derivatives enhance p38 activity.

The roles of MAPKs in PhSe-T/MeSe-T-induced apoptosis were analyzed using the selective MAPK inhibitors SB203580, SP6 00125, and PD98059. Hoechst33342 staining, caspase-3 activity assays, and Western blot analysis of caspase-9, caspase-3, and PARP revealed that the p38 inhibitor SB203580, but not the JNK inhibitor or ERK inhibitor PD98059, reduced PhSe-T/MeSe-T-induced caspase-mediated apoptosis. We also found that the inhibition of p38 was associated with reduced activation of caspase-2 and caspase-8. These findings suggest that PhSe-T/MeSe-T-induced apoptosis and the activation of initiator caspases, such as caspase-2 and caspase-8, as well as effector caspases, such as caspase-3, in HL-60 cells involve the p38 pathway. However, the mechanism of p38-mediated caspase activation is still not well understood. We performed co-immunoprecipitation, but we could not identify any interaction between p38 MAPK and caspase-2 or caspase-8 in either the inactive or active forms (data not shown) under our experimental conditions. These results suggest that p38 does not react directly with these caspases, but rather induces other mole-

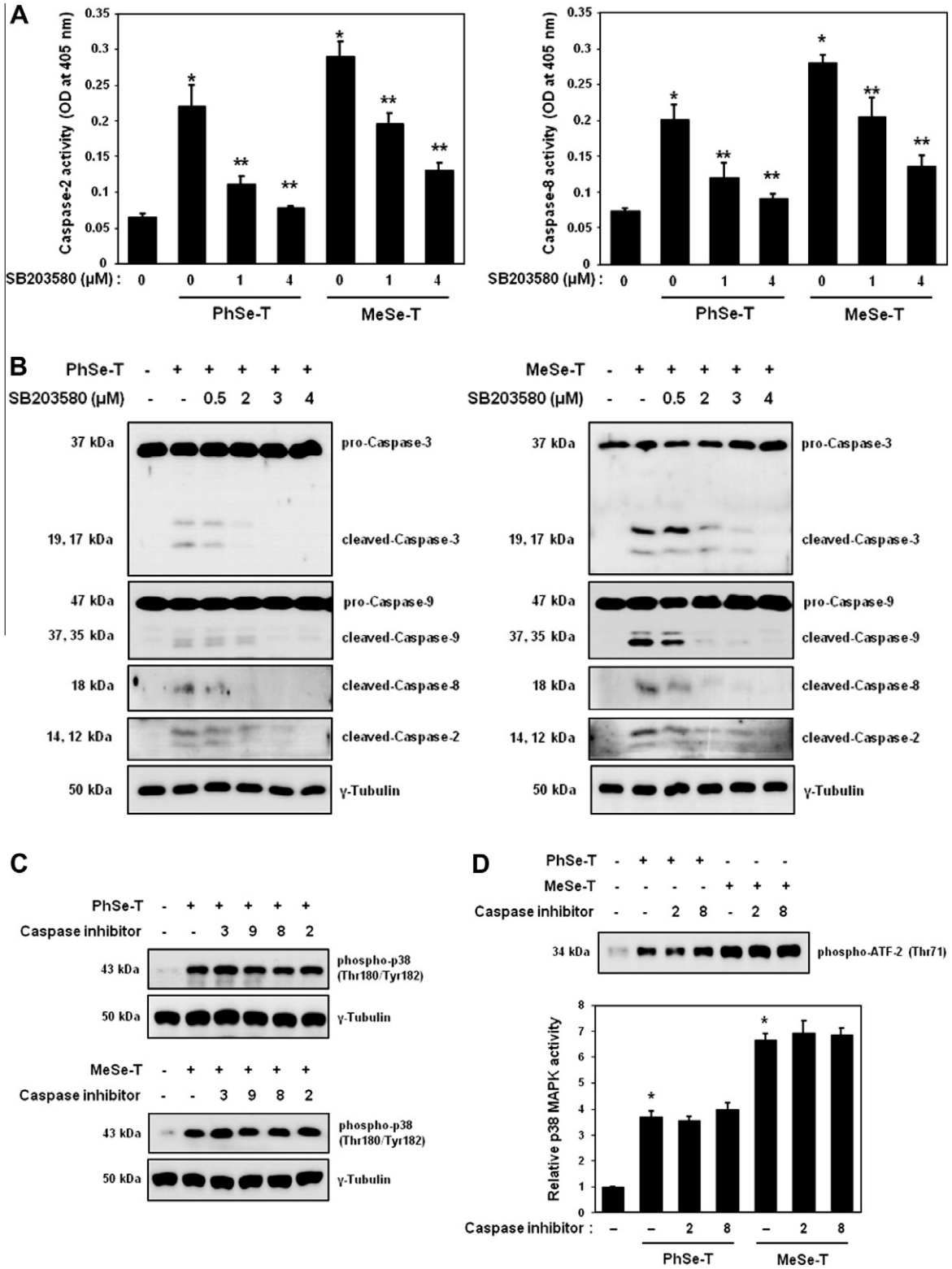

Fig. 3. The essential role of p38 MAPK in PhSe-T/MeSe-T-induced activation of initiator caspases, such as caspase-2 and caspase-8. (A and B) HL-60 cells were exposed to 150 μM of PhSe-T for 40 h or 10 μM of MeSe-T for 32 h in the presence or absence of the indicated concentrations of SB203580. (A) The activity of caspase-2 and caspase-8 was monitored *via* the detection of pNA liberated from the substrates VDVAD-pNA and IETD-pNA, respectively. Each data point represents the mean ± standard error of three independent experiments ($^{*}P < 0.05$ compared to the untreated control; $^{**}P < 0.05$ compared to PhSe-T alone or MeSe-T alone; ANOVA/Dunnett's test). (B) Western blotting was used to detect cleaved caspase-2 and cleaved caspase-8, as well as caspase-9 and caspase-3. (C and D) HL-60 cells were exposed to 150 μM of PhSe-T for 40 h and 10 μM of MeSe-T for 32 h in the presence or absence of caspase-2 inhibitor (z-VDVAD-fmk, 20 μM), caspase-8 inhibitor (z-IETD-fmk, 20 μM), caspase-9 inhibitor (z-LEHD-fmk, 20 μM), or caspase-3 inhibitor (z-DEVD-fmk, 20 μM). (C) Western blotting was used to detect phosphorylation of p38. (D) *In vitro* kinase activity of p38 was determined using ATF-2 as a substrate. ATF-2 phosphorylation was determined by Western blotting using phospho-specific ATF-2 (Thr71) antibody. Upper panel: the representative Western blots of p38-induced phosphorylation of ATF-2. Lower panel: densitometric quantitative analysis. Each data point represents the mean ± standard error of three independent experiments ($^{*}P < 0.05$ compared to the untreated control; ANOVA/Dunnett's test).

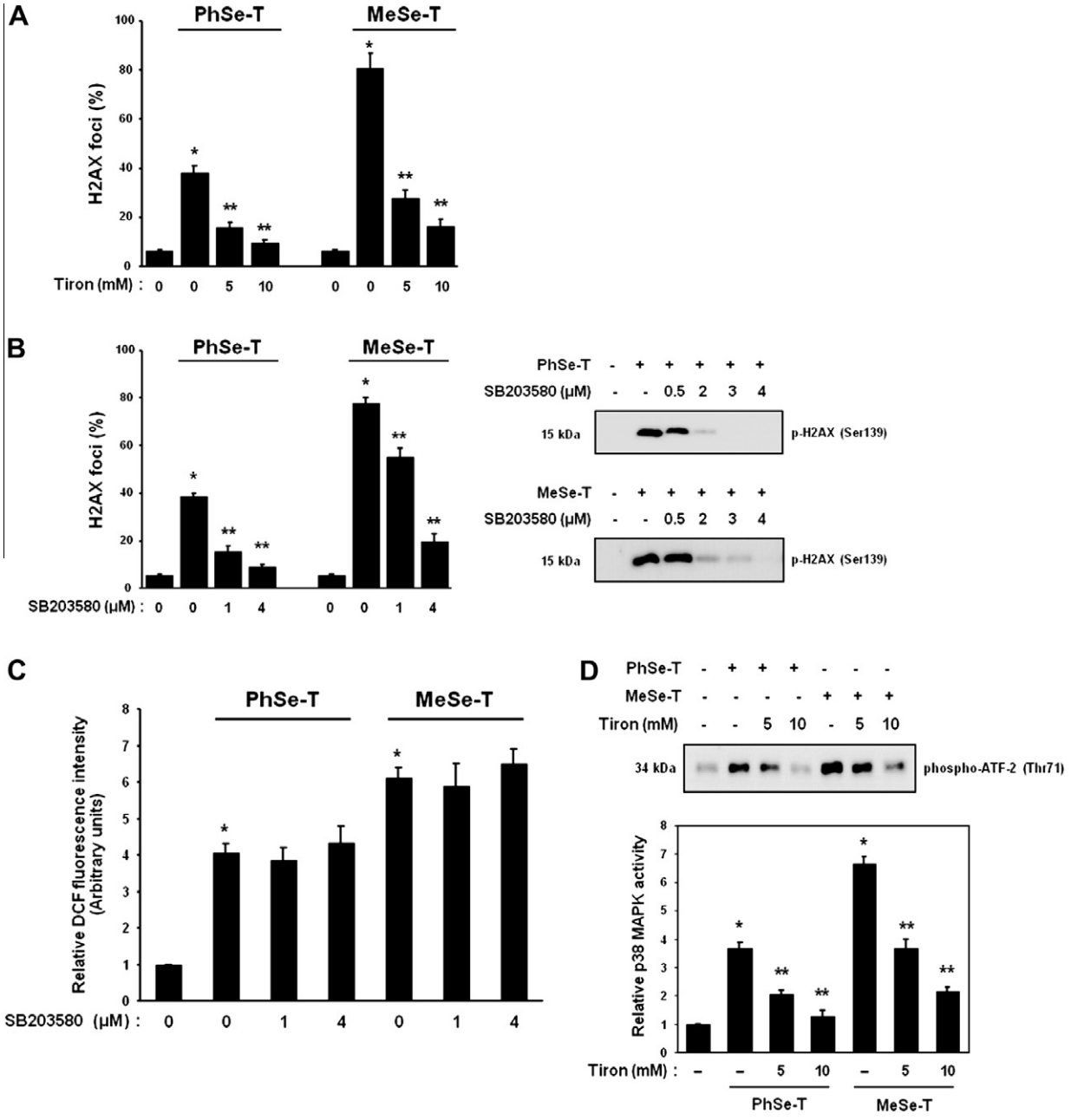

Fig. 4. Effects of an ROS scavenger on PhSe-T/MeSe-T-induced p38 activation. (A and B) HL-60 cells were exposed to 150 μM of PhSe-T or 10 μM of MeSe-T for 32 h for immunostaining for γ-H2AX in the presence or absence of the indicated concentrations of tiron (A) or SB203580 (B). Anti-phospho-H2AX (Ser139) antibody was used for immunostaining with AlexaFluor 594-conjugated secondary antibodies. The percentage of cells positive for γ-H2AX foci is shown. Each data point represents the mean ± standard error of three independent experiments (*$P < 0.05$ compared to the untreated control; **$P < 0.05$ compared to PhSe-T alone or MeSe-T alone; ANOVA/Dunnett's test). (B) For Western blot detection for H2AX phosphorylation at Ser139, HL-60 cells were exposed to 150 μM of PhSe-T for 40 h or 10 μM of MeSe-T for 32 h in the presence or absence of the indicated concentrations of SB203580. (C) HL-60 cells were challenged with 150 μM of PhSe-T or 10 μM of MeSe-T for 24 h in the presence or absence of the indicated concentrations of SB203580. To measure ROS, cells were harvested and incubated with the fluorescent probe DCFDA (20 μM). The fluorescence distribution of 10^4 cells was determined by flow cytometry. Each data point represents the mean ± standard error of three independent experiments (*$P < 0.05$ compared to the untreated control; ANOVA/Dunnett's test). (D) HL-60 cells were challenged with 150 μM of PhSe-T for 40 h or 10 μM of MeSe-T for 32 h in the presence or absence of the indicated concentrations of tiron. *In vitro* kinase activity of p38 was determined using ATF-2 as a substrate. ATF-2 phosphorylation was determined by Western blotting using phospho-specific ATF-2 (Thr71) antibody. Upper panel: representative Western blots of p38-induced phosphorylation of ATF-2. Lower panel: densitometric quantitative analysis. Each data point represents the mean ± standard error of three independent tests (*$P < 0.05$ compared to the untreated control; **$P < 0.05$ compared to PhSe-T alone or MeSe-T alone; ANOVA/Dunnett's test).

cules to activate caspases. Further research on the link between p38 and initiator caspases is needed.

Phosphorylation of H2AX is believed to be a sensitive molecular marker of DNA damage, such as DNA double-stranded breaks [7]. In addition, a recent finding suggests a novel function of H2AX in cellular apoptosis [15]. It was previously shown that p38, one of the required components of the DNA damage response to genotoxic stress, can phosphorylate H2AX directly *in vitro* and co-localize with H2AX *in vivo* [16]. In this study, we demonstrated that a p38 inhibitor, SB203580, and a ROS scavenger, tiron, reduced PhSe-T/MeSe-T-induced H2AX phosphorylation suggesting that there may be a novel signaling pathway involving p38/H2AX regulating apoptosis.

Finally, we showed that ROS generation was highly involved in PhSe-T/MeSe-T-induced p38 activation. Other studies have also

shown that p38 MAPK, a redox-sensitive kinase, is activated by ROS generation [8,17,18]. It is known that ROS can inactivate MAPK phosphatases (MKPs), which are known to dephosphorylate activated p38 by oxidizing critical residues in their phosphatase domain, thus leading to prolonged p38 activation [19,20], presenting a possible molecular mechanism by which ROS activate p38. Further studies are needed to address this issue in more detail.

Taken together, our results indicate that PhSe-T/MeSe-T-induced apoptosis of HL-60 cells involves p38 MAPK activation, as well as ROS generation and caspase activation. Our data also demonstrate that p38 is a link between ROS generation and caspase-mediated apoptosis in PhSe-T/MeSe-T-treated HL-60 cells.

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