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The Effects of Mitogen-Activated Protein Kinase Inhibitors or Small Interfering RNAs on Gallic Acid-Induced HeLa Cell Death in Relation to Reactive Oxygen Species and Glutathione

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Gallic acid (GA) is widely distributed in various plants and foods and has various biological properties including anticancer effects. In this study, we investigated the effects of mitogen-activated protein kinase (MAPK) [MAP 20 kinase or ERK kinase (MEK), c-Jun N-terminal kinase (JNK), or p38)] inhibitors or small interfering RNAs (siRNAs) on GA-induced HeLa cell death in relation to reactive oxygen species (ROS) and glutathione (GSH) levels. GA dose dependently inhibited the growth of HeLa cells via apoptosis and/or necrosis at 24 h, which was accompanied by the loss of mitochondrial membrane potential (MMP; $\Delta\Psi_m$). Treatment with 70 μM GA increased the ROS level including $O_2^{\bullet-}$ and significantly induced GSH depletion in HeLa cells. GA decreased the activity of extracellular signal-regulated kinase (ERK) at 24 h, whereas it increased that of JNK at the same time. While the MEK inhibitor or ERK siRNA did not affect cell growth and death in 70 μM GA-treated HeLa cells at 24 h, JNK and p38 inhibitors enhanced cell growth inhibition and death in these cells. Additionally, p38 siRNA administration augmented growth inhibition, death, and MMP ($\Delta\Psi_m$) loss in 70 μM GA-treated HeLa cells. In relation to ROS and GSH levels, JNK and p38 inhibitors increased ROS levels, and GSH-depleted cell numbers in GA-treated HeLa cells. Moreover, p38 siRNA increased $O_2^{\bullet-}$ levels and GSH depletion in GA-treated HeLa cells. Each MAPK inhibitor and siRNA differentially affected ROS and GSH levels in HeLa control cells. Conclusively, JNK and p38 inhibitors and p38 siRNA enhanced growth inhibition and cell death in GA-treated HeLa cells, which were to some extent related to GSH depletion and ROS levels, especially $O_2^{\bullet-}$.

KEYWORDS: Gallic acid; apoptosis; MAPK inhibitor; ROS; GSH

INTRODUCTION

Gallic acid (GA; 3,4,5-triphydroxyl-benzoic acid) as a polyhydroxylphenolic compound is widely distributed in various plants, fruits, and foods (*1*) and is very well absorbed in humans (*2*). Various biological activities of GA have been reported, including antibacterial (*3*), antiviral (*4*), and anti-inflammatory (*5*) activities. The major interest in GA is related to its antitumoral activity. In fact, the anticancer activity of GA has been reported in various cancer cells, such as prostate cancer (*6*), lung cancer (*7*−*9*), and gastric, colon, breast, cervical, and esophageal cancers (*10*).

Apoptosis induced by GA is associated with oxidative stresses derived from reactive oxygen species (ROS), mitochondrial dysfunction, and an increase in intracellular Ca^{2+} levels (*11*, *12*). Controversially, it has been suggested that GA can have both pro-oxidant and antioxidant properties depending on iron or H_2O_2 in medium and plasma (*13*, *14*). ROS including hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), and hydroxyl radical ($^{\bullet}OH$) have been implicated in the regulation of many important cellular events such as transcription factor activation, cell proliferation, and differentiation (*15*, *16*). Particularly, excessive production of ROS gives rise to the activation of events leading to death in many cell types (*17*, *18*).

The mitogen-activated protein kinases (MAPKs) as a large family of serine/threonine kinases mainly consist of three subfamilies: the extracellular signal regulated kinase (ERK1/2), the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), and the p38 (*19*). These MAPKs are major components of signaling pathways in cell proliferation, differentiation, and cell death (*20*). Each MAP kinase pathway has relatively different upstream activators and specific substrates (*21*). Numerous evidence demonstrates that JNK and p38 are strongly activated by ROS or by a mild oxidative shift of the intracellular thiol/disulfide redox state, leading to apoptosis (*22*−*24*). ROS also are known to regulate the activation of the ERK1/2-activating kinase (MEK) and ERK (*25*, *26*). In most instances, MEK and ERK activation have a pro-survival function rather than pro-apoptotic effects (*27*). Because different ROS levels and diverse functions of MAPKs by ROS may have opposite effects even in the same type of cells, the relationship between ROS and MAPKs in view of cell survival or cell death signaling needs further clarification.

Recently, we demonstrated that GA inhibited the growth of HeLa cervical cancer cells via apoptosis and/or necrosis (*28*). However, little is known about the relationships between GA and

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MAPK signaling in cancer cells. It is important to understand the underlying mechanism of GA toxicity in cancer cells in view of ROS and MAPK signaling. Therefore, in the present study, we investigated the effects of MAPK inhibitors or siRNAs on cell growth, cell death, ROS, and GSH levels in GA-treated HeLa cells.

MATERIALS AND METHODS

Cell Culture. The human cervix adenocarcinoma HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in a humidified incubator containing 5% CO_2 at 37 °C. HeLa cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin−streptomycin (GIBCO BRL, Grand Island, NY). Cells were routinely grown in 100 mm plastic tissue culture dishes (Nunc, Roskilde, Denmark).

Reagents. GA purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO) was dissolved in ethanol at 200 mM as a stock solution. MEK inhibitor (PD98059), JNK inhibitor (SP600125), and p38 inhibitor (SB202190) obtained from Calbiochem (San Diego, CA) were dissolved in DMSO (Sigma-Aldrich Chemical Co.) at 10 mM as a stock solution. Cells were pretreated with each MAPK inhibitor for 1 h before GA treatment. On the basis of the previous experiment (*29*), a 10 μM concentration of each MAPK inhibitor was used as an optimal dose in this experiment. Ethanol (0.2%) and DMSO (0.3%) were used as a control vehicle. All stock solutions were wrapped in foil and kept at −20 °C.

Growth Inhibition Assay. The growth inhibition effect of GA on HeLa cells was determined by measuring the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Chemical Co.) dye absorbance by living cells as previously described (*30*). In brief, cells 3×10^4 cells per well were seeded in 96-well microtiter plates (Nunc) for MTT assays. After exposure to the indicated doses of GA with or without a given MAPK inhibitor for 24 h, 20 μL of MTT solution (2 mg/mL in PBS) was added to each well of 96-well plates. The plates were incubated for 4 additional hours at 37 °C. Medium in plates was withdrawn using pipetting, and 200 μL of DMSO was added to each well to solubilize the formazan crystals. The optical density was measured at 570 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co, Sunnyvale, CA).

Western Blot Analysis. The expressions of proteins were evaluated using Western blot analysis, as previously described (*31*). In brief, 1×10^6 cells in 60 mm culture dish (Nunc) were incubated with or without 70 μM GA for 1 h. The cells were then washed in phosphate-buffered saline (PBS) and suspended in five volumes of lysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 200 mM KCl, 0.5 mM ethylenediaminetetraacetic acid, 0.5% NP40, 0.5 mM dithiothreitol, and 1% protease inhibitor cocktail). Lysates were then collected and stored at −20 °C until further use. Supernatant protein concentrations were determined using the Bradford method. Supernatant samples containing 40 μg of total protein were resolved by 12.5% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred to Immobilon-P PVDF membranes (Millipore, Billerica, MA) by electroblotting, and probed with anti-ERK, antiphospho-ERK, anti-JNK, antiphospho-JNK, anti-p38, antiphospho-p38 antibodies (Cell Signaling Technology, Danvers, MA), and anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Blots were developed using an ECL kit (Amersham, Arlington Heights, IL).

Annexin V/PI Staining. Apoptosis was determined by staining cells with annexin V-fluorescein isothiocyanate (FITC, PharMingen, San Diego, CA; Ex/Em = 488 nm/519 nm) and propidium iodide (PI; Sigma-Aldrich; Ex/Em = 488 nm/617 nm). In brief, 1×10^6 cells in 60 mm culture dish (Nunc) were incubated with 70 μM GA with or without a given MAPK inhibitor for 24 h. Cells were washed twice with cold PBS and then resuspended in 500 μL of binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. Five microliters of annexin V-FITC and PI (1 μg/mL) was then added to these cells, which were analyzed with a FACStar flow cytometer (Becton Dickinson). Viable cells were negative for both PI and annexin V; apoptotic cells were positive for annexin V and negative for PI, whereas late apoptotic dead cells displayed both high annexin V and PI labeling. Nonviable cells, which underwent necrosis, were positive for PI and negative for annexin V.

Measurement of MMP ($\Delta\Psi_m$). MMP ($\Delta\Psi_m$) levels were measured using rhodamine 123 fluorescent dye (Sigma-Aldrich Chemical Co.; Ex/Em = 485 nm/535 nm) as previously described (*32*). In brief, 1×10^6 cells in 60 mm culture dish (Nunc) were incubated with 70 μM GA with or without a given MAPK inhibitor for 24 h. Cells were washed twice with PBS and incubated with rhodamine 123 (0.1 μg/mL) at 37 °C for 30 min. Rhodamine 123 staining intensity was determined by flow cytometry (Becton Dickinson). An absence of rhodamine 123 from cells indicated the loss of MMP ($\Delta\Psi_m$) in cells. The MMP ($\Delta\Psi_m$) levels in viable cells [excluding MMP ($\Delta\Psi_m$) loss cells] were expressed as the mean fluorescence intensity (MFI), which was calculated by CellQuest software (Becton Dickinson).

Detection of Intracellular ROS. Intracellular ROS were detected by means of an oxidation-sensitive fluorescent probe dye, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Invitrogen Molecular Probes, OR; Ex/Em = 495 nm/529 nm) (*33*). As H_2DCFDA is poorly selective for $O_2^{\bullet-}$, dihydroethidium (DHE, Invitrogen Molecular Probes; Ex/Em = 518 nm/605 nm), which is highly selective for $O_2^{\bullet-}$, was used for its detection. In brief, 1×10^6 cells in 60 mm culture dish (Nunc) were incubated with 70 μM GA with or without a given MAPK inhibitor for 24 h. Cells were then washed in PBS and incubated with 20 μM H_2DCFDA or DHE at 37 °C for 30 min. DCF and DHE fluorescences were detected using a FACStar flow cytometer (Becton Dickinson). ROS and $O_2^{\bullet-}$ levels were expressed as MFI, which was calculated by CellQuest software (Becton Dickinson).

Detection of the Intracellular Glutathione (GSH). Cellular GSH levels were analyzed using 5-chloromethylfluorescein diacetate (CMFDA, Invitrogen Molecular Probes; Ex/Em = 522 nm/595 nm) as previously described (*33*). In brief, 1×10^6 cells in 60 mm culture dish (Nunc) were incubated with 70 μM GA with or without a given MAPK inhibitor for 24 h. Cells were then washed with PBS and incubated with 5 μM CMFDA at 37 °C for 30 min. The CMF fluorescence intensity was determined using a FACStar flow cytometer (Becton Dickinson). Negative CMF staining (GSH-depleted) cells were expressed as the percent of (−) CMF cells. The CMF levels in cells (excluding GSH-depleted cells) were expressed as MFI, which was calculated by CellQuest software (Becton Dickinson).

Transfection of Cells with MAPK siRNAs. Gene silencing of ERK, JNK, and p38 was performed as previously described (*34*). A nonspecific control siRNA duplex [5′-CCUACGCCACCAAUUUCGU(dTdT)-3′], ERK siRNA duplex [5′-CACCAUUCAAGUUCGACAU(dTdT)-3′], JNK siRNA duplex [5′-CUGGAUAUAGCUUUGAGAA(dTdT)-3′], and p38 siRNA duplex [5′-CAAAUUCUCCGAGGUCUAA (dTdT)-3′] were purchased from the Bioneer Corp. (Daejeon, South Korea). In brief, 2.5×10^5 cells in six-well plates (Nunc) were incubated in RPMI-1640 supplemented with 10% FBS. The next day, cells (approximately 30−40% confluence) in each well were transfected with the control or each MAPK siRNA [80 pmol in Opti-MEM (GIBCO BRL)] using LipofectAMINE 2000, according to the manufacturer's instructions (Invitrogen, Brandford, CT). One day later, cells were treated with or without 70 μM GA for additional 24 h. The transfected cells were collected and used for Western, MTT assay, annexin-FITC/PI staining, rhodamine 123 staining, ROS, and GSH level measurements.

Statistical Analysis. The results represent the mean of at least three independent experiments (mean ± SD). The data were analyzed using Instat software (GraphPad Prism4, San Diego, CA). The Student's *t* test or one-way analysis of variance (ANOVA) with posthoc analysis using Tukey's multiple comparison test was used for parametric data. Statistical significance was defined as $p < 0.05$.

RESULTS

Effects of MAPK Inhibitors on Cell Growth Inhibition and Death in GA-Treated HeLa Cells. We first examined the effect of GA on the growth inhibition of HeLa cells using MTT assays. After exposure to GA for 24 h, HeLa cell growth was dose dependently diminished with an IC_{50} of approximately 80 μM GA (**Figure 1A**). Next, the effects of MAPK inhibitors on cell growth inhibition and death in GA-treated HeLa cells were assessed. For this experiment, 70 μM GA was chosen as a suitable dose to differentiate the levels of cell growth inhibition or death in the presence or absence of each MAPK inhibitor. It has been

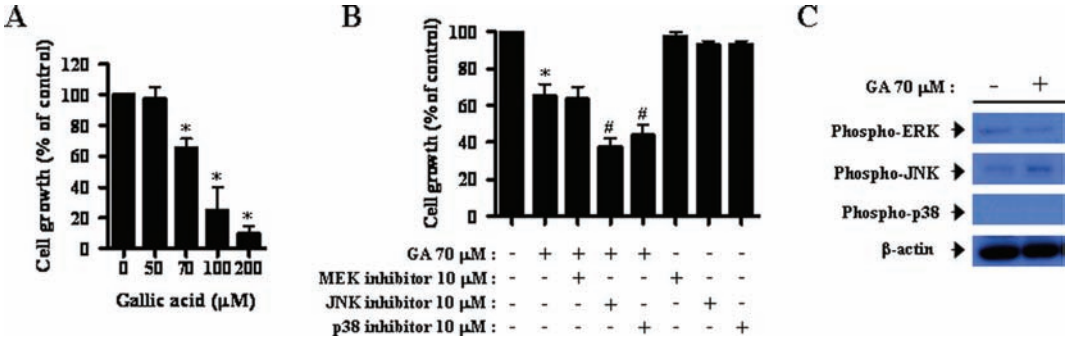

Figure 1. Effects of MAPK inhibitors on the growth of GA-treated HeLa cells. Exponentially growing cells were treated with GA for 24 h following 1 h of preincubation of 10 μM MEK, JNK, or p38 inhibitor. (**A** and **B**) Cell growths were assessed by MTT assays at 24 h. (**C**) The phosphorylation status of each MAPK was examined by Western blotting at 1 h. Forty microgram protein extracts were resolved by SDS-PAGE gel, transferred onto the PVDF membranes, and immunoblotted with the indicated antibodies against phospho-ERK, phospho-JNK, phospho-p38, and β-actin. $^{*}P < 0.05$ as compared with GA-untreated HeLa control group. $^{\#}P < 0.05$ as compared with cells treated with GA only.

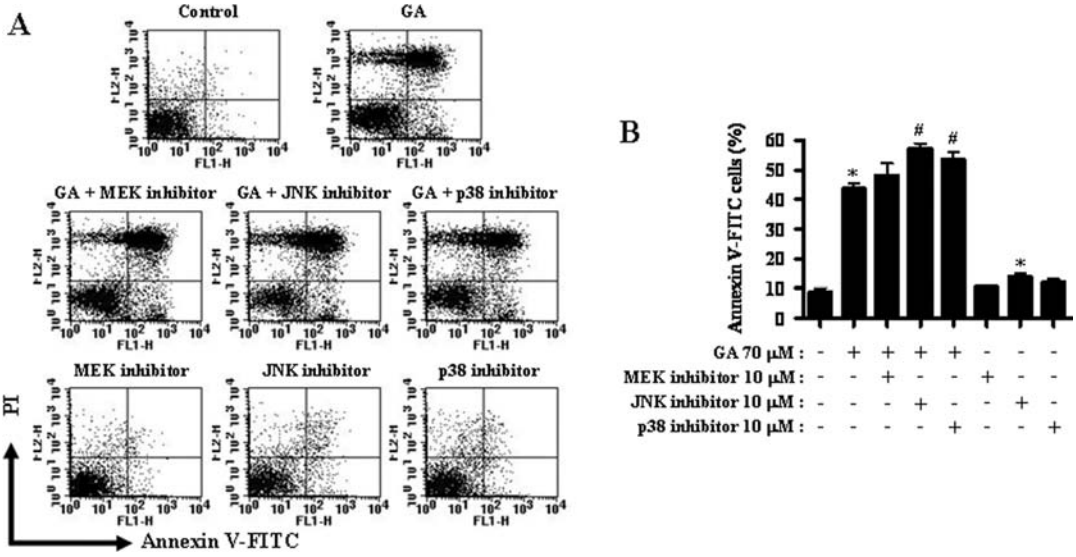

Figure 2. Effects of MAPK inhibitors on cell death in GA-treated HeLa cells. Exponentially growing cells were treated with 70 μM GA for 24 h following 1 h of preincubation of 10 μM MEK, JNK, or p38 inhibitor. (**A**) Annexin V-FITC and/or PI positive cells were measured with a FACStar flow cytometer. (**B**) Graph shows the percents of annexin V positive staining cells from **A**. $^{*}P < 0.05$ as compared with GA-untreated HeLa control group. $^{\#}P < 0.05$ as compared with cells treated with GA only.

demonstrated that high dose usages of MAPK inhibitors may decrease their specificity (*35*). In fact, a 20 μM concentration of each MAPK inhibitor significantly induced HeLa cell growth inhibition and death (data not shown). In contrast, a 1 or 5 μM concentration of MAPK inhibitors did not significantly affect cell growth inhibition and death in GA-treated HeLa cells (data not shown). Therefore, 10 μM MAPK inhibitors were used in the current experiments to evade the nonspecific inhibition of other kinases. As shown in **Figure 1B**, MEK inhibitor did not affect the growth inhibition of GA-treated HeLa cells, but JNK and p38 inhibitors significantly intensified the growth inhibition. JNK or p38 alone slightly inhibited the growth of HeLa control cells (**Figure 1B**). Because the phosphorylation status of specific sites on MAPKs indirectly indicates the quantity of their activities, the phosphorylation status of each MAPK in GA-treated HeLa cells was examined by Western blotting at the early time point of 1 h. GA decreased the phosphorylation of ERK but increased the phosphorylation of JNK (**Figure 1C**). Unfortunately, we did not detect the form of phosphorylated-p38 in HeLa cells regardless of GA treatment (**Figure 1C**).

GA significantly increased annexin V staining cells to about 35% as compared with GA-untreated HeLa control cells, implying that GA-induced HeLa cell death occurs via apoptosis (**Figure 2A**,**B**). GA also increased necrotic cell numbers in HeLa cells (annexin V negative and PI positive proportion cells) (**Figure 2A**). JNK and p38 inhibitors increased annexin V-FITC positive cells in GA-treated cells (**Figure 2A**,**B**). JNK inhibitor alone increased the number in GA-untreated HeLa control cells (**Figure 2B**).

Effects of MAPK Inhibitors on MMP ($\Delta\Psi_m$) in GA-Treated HeLa Cells. When MMP ($\Delta\Psi_m$) in GA-treated HeLa cells was assessed using a rhodamine 123 dye, GA significantly induced the loss of MMP ($\Delta\Psi_m$) at 24 h (**Figure 3A**,**B**). While MEK inhibitor did not change the level of MMP ($\Delta\Psi_m$) loss in GA-treated HeLa cells, JNK and p38 inhibitors intensified the MMP ($\Delta\Psi_m$) loss (**Figure 3A**,**B**). JNK inhibitor alone also triggered MMP ($\Delta\Psi_m$) loss in GA-untreated HeLa control cells (**Figure 3B**). In relation to MMP ($\Delta\Psi_m$) levels in HeLa cells except rhodamine 123 negative cells, GA reduced the MMP ($\Delta\Psi_m$) level to about 40% as compared with control cells (**Figure 3A**,**C**). Treatment with JNK and p38 inhibitors intensified the decreased MMP ($\Delta\Psi_m$) level in GA-treated HeLa cells (**Figure 3A**,**C**). In addition, each MAPK inhibitor alone reduced the basal MMP ($\Delta\Psi_m$) level in GA-untreated HeLa control cells (**Figure 3A**,**C**).

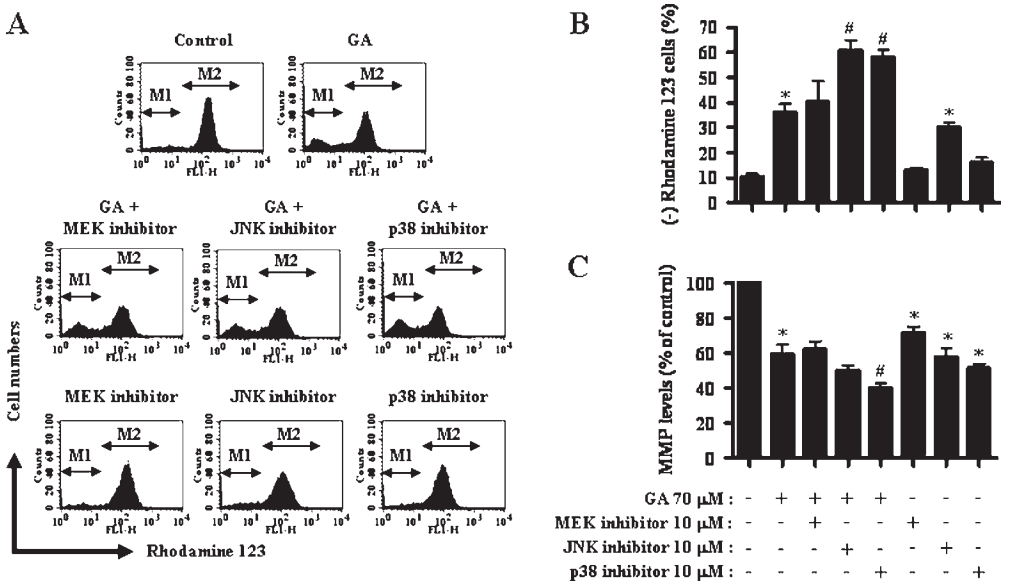

Figure 3. Effects of MAPK inhibitors on MMP ($\Delta\Psi_m$) in GA-treated HeLa cells. Exponentially growing cells were treated with 70 μM GA for 24 h following 1 h of preincubation of 10 μM MEK, JNK, or p38 inhibitor. (**A**) MMP ($\Delta\Psi_m$) in HeLa cells was measured with a FACStar flow cytometer. M1 regions indicate rhodamine 123 negative (MMP ($\Delta\Psi_m$) loss) cells. M2 regions indicate cells except rhodamine 123 negative (MMP ($\Delta\Psi_m$) loss) cells. (**B** and **C**) Graphs show the percents of rhodamine 123 negative (MMP ($\Delta\Psi_m$) loss) cells from M1 regions of **A** (**B**) and the percent of MMP ($\Delta\Psi_m$) levels from M2 regions of **A** (**C**). $^*P < 0.05$ as compared with GA-untreated HeLa control group. $^{\#}P < 0.05$ as compared with cells treated with GA only.

Effects of Each siRNA for MAPKs on Cell Growth Inhibition, Cell Death, and MMP ($\Delta\Psi_m$) in GA-Treated HeLa Cells. Although MAPK inhibitors used in this experiment are specific inhibitors for their target MAPKs, it is possible that these inhibitors may affect other kinases that indirectly influence each MAPK signaling. To directly address the roles of each MAPK signaling in GA-mediated HeLa cell growth inhibition and death, HeLa cells were transfected with either nontarget control siRNA or each MAPK siRNA. As shown in **Figure 4A**, the expressions of each MAPK in cells transfected with each MAPK siRNA were clearly decreased as compared with cells transfected with control siRNA. While ERK siRNA did not affect the growth inhibition of GA-treated HeLa cells, JNK and p38 siRNAs seemed to enhance the growth inhibition (**Figure 4B**). None of MAPK siRNAs altered HeLa control cell growth (**Figure 4B**). Furthermore, neither ERK nor JNK siRNAs changed the proportion of annexin V-FITC positive cells in GA-treated cells, and p38 siRNA significantly increased the positive cells in GA-treated or -untreated cells (**Figure 4C**). In relation to MMP ($\Delta\Psi_m$), none of ERK and JNK siRNA changed MMP ($\Delta\Psi_m$) loss cell number in GA-treated HeLa cells (**Figure 4D**). Transfection with p38 siRNA enhanced the MMP ($\Delta\Psi_m$) loss in GA-treated or -untreated cells (**Figure 4D**). In addition, none of each MAPK siRNA strongly changed MMP ($\Delta\Psi_m$) level in GA-treated HeLa cells (**Figure 4E**). JNK and p38 siRNA reduced the basal MMP ($\Delta\Psi_m$) level in GA-untreated HeLa control cells (**Figure 4E**).

Effects of MAPK Inhibitors on ROS and GSH Levels in GA-Treated HeLa Cells. Next, we determined whether the levels of intracellular ROS and GSH levels in GA-treated HeLa cells were changed by each MAPK inhibitor at 24 h. The intracellular ROS (DCF) level was increased in GA-treated cells (**Figure 5A**). All of the MAPK inhibitors increased ROS level in GA-treated cells, and those levels of MEK or p38 inhibitor-treated cells were significant (**Figure 5A**). Treatment with p38 inhibitor alone increased ROS level in GA-untreated HeLa control cells (**Figure 5A**). When we detected the intracellular $O_2^{\bullet-}$ levels in GA-treated HeLa cells, red fluorescence derived from DHE reflecting intracellular $O_2^{\bullet-}$ level was increased in these cells (**Figure 5B**). Both JNK and p38 inhibitors intensified the increased $O_2^{\bullet-}$ levels in GA-treated cells (**Figure 5B**). MEK and JNK inhibitors decreased basal $O_2^{\bullet-}$ levels in GA-untreated HeLa control cells, whereas p38 inhibitor increased the $O_2^{\bullet-}$ level (**Figure 5B**). In relation to GSH levels, GA increased GSH-depleted cell number to about 35% as compared with GA-untreated HeLa control cells (**Figure 6A**,**B**). JNK and p38 inhibitors increased GSH-depleted cell number in GA-treated HeLa cells, and JNK inhibitor alone also increased the number in HeLa control cells (**Figure 6A**,**B**). When CMF (GSH) levels were assessed, GA significantly increased the GSH level in HeLa cells (**Figure 6A**,**C**). All of the MAPK inhibitors significantly attenuated GSH levels in GA-treated HeLa cells (**Figure 6A**,**C**).

Effects of MAPK siRNAs on ROS and GSH Levels in GA-Treated HeLa Cells. Furthermore, it was determined whether MAPK siRNAs changed the levels of ROS and GSH in GA-treated HeLa cells at 24 h. As shown in **Figure 7A**, none of MAPK siRNAs significantly influenced ROS (DCF) level in GA-treated HeLa cells, and only ERK siRNA increased ROS level in HeLa control cells. While ERK and JNK siRNAs did not significantly change the $O_2^{\bullet-}$ level in GA-treated HeLa cells, p38 siRNA significantly augmented $O_2^{\bullet-}$ level in these cells (**Figure 7B**). In relation to GSH, ERK and JNK siRNA did not significantly affect GSH-depleted cell numbers in GA-treated HeLa cells (**Figure 7C**). Transfection with p38 siRNA strongly enhanced the proportion of GSH-depleted cells in GA-treated HeLa cells (**Figure 7C**). In addition, ERK siRNA among MAPK siRNAs seemed to increase GSH levels in GA-treated and-untreated HeLa cells (**Figure 7D**).

DISCUSSION

In the present study, we focused on evaluating the effects of MAPK inhibition by its inhibitors or siRNAs on GA-treated HeLa cells in relation to cell death, ROS, and GSH levels, since we have observed that GA inhibited the growth of HeLa cells. GA increased annexin V-FITC positive cells in HeLa cells, which indirectly implied that GA-induced HeLa cell death occurred via apoptosis. GA also to some extent triggered necrotic death in HeLa cells.

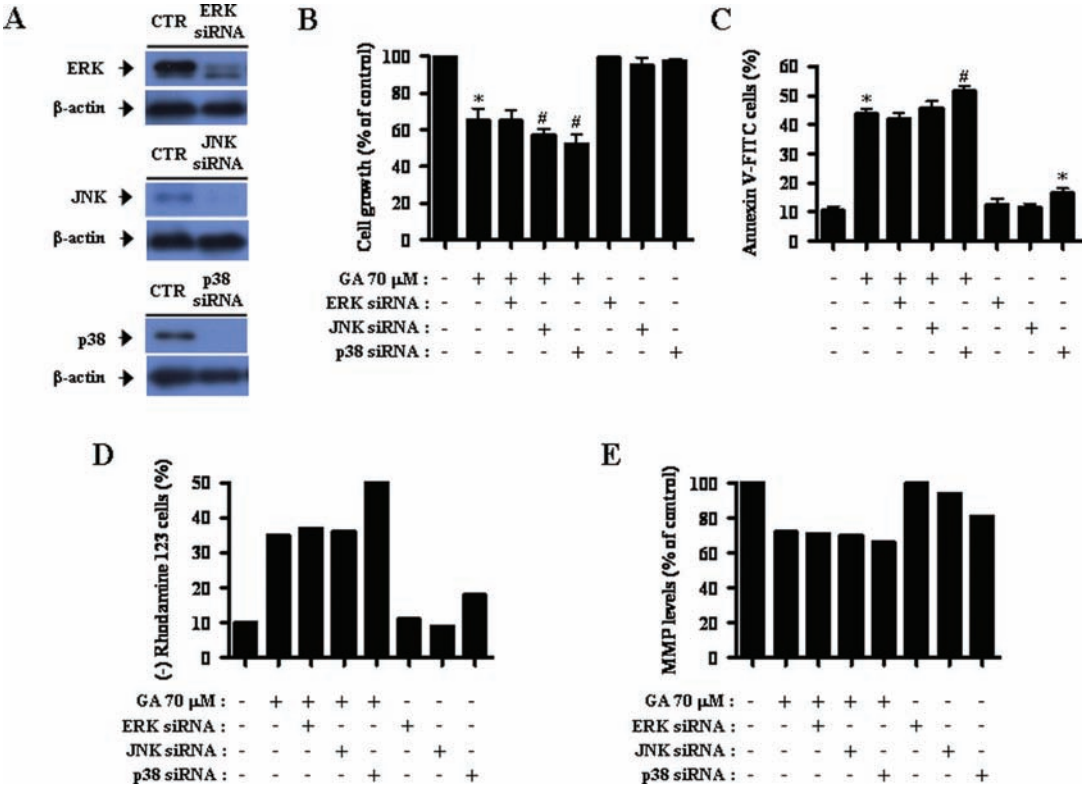

Figure 4. Effects of each siRNA for MAPKs on cell growth inhibition, death, and MMP ($\Delta\Psi_m$) in GA-treated HeLa cells. HeLa cells (approximately 30−40% confluence) were transfected with either nontarget control (CTR) siRNA or each MAPK siRNA. One day later, cells were treated with 70 μM GA for an additional 24 h. (**A**) The expression of each MAPK was examined by Western blotting. Forty microgram protein extracts were resolved by SDS-PAGE gel, transferred onto the PVDF membranes, and immunoblotted with the indicated antibodies against ERK, JNK, p38, and β-actin. (**B**) Cell growth was assessed by MTT assays. (**C**) Graph shows the percents of annexin V positive staining cells in HeLa cells, as measured with a FACStar flow cytometer. (**D** and **E**) Graphs show rhodamine 123 negative (MMP ($\Delta\Psi_m$) loss) cells (%) (**D**) and MMP ($\Delta\Psi_m$) levels (%) in HeLa cells (**E**). $^*P < 0.05$ as compared with GA-untreated HeLa control group. $^{\#}P < 0.05$ as compared with cells treated with GA only.

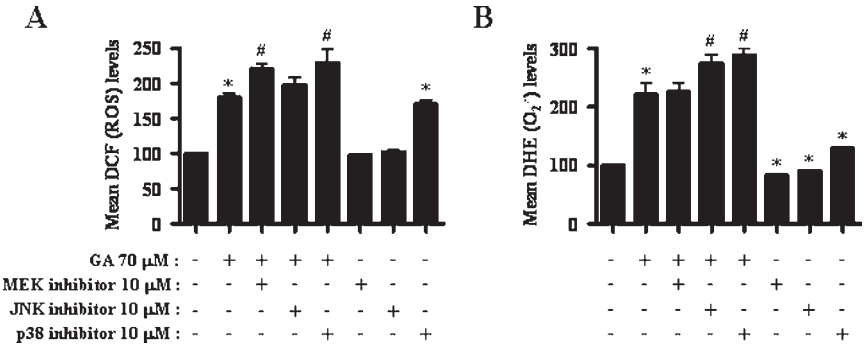

Figure 5. Effects of MAPK inhibitors on ROS levels in GA-treated HeLa cells. Exponentially growing cells were treated with 70 μM GA for 24 h following 1 h of preincubation of 10 μM MEK, JNK, or p38 inhibitor. ROS levels in HeLa cells were measured using a FACStar flow cytometer. (**A** and **B**) Graphs indicate DCF (ROS) levels (%) as compared with GA-untreated control cells (**A**) and DHE ($O_2^{\bullet-}$) levels (%) as compared with GA-untreated control cells (**B**). $^*P < 0.05$ as compared with GA-untreated HeLa control group. $^{\#}P < 0.05$ as compared with cells treated with GA only.

ERK activation has a pro-survival function rather than pro-apoptotic effects (*27*, *36*). Correspondingly, GA showing an apoptotic effect on HeLa cells decreased the activity of ERK. However, MEK inhibitor, which presumably decreased ERK activity, did not affect the growth inhibition and death in GA-treated HeLa cells. Furthermore, ERK siRNA also did not change the growth inhibition and death by GA. Therefore, these results suggested that ERK signaling was not tightly related to cell growth inhibition and death in GA-treated HeLa cells. However, we observed that MEK inhibitor attenuated growth inhibition and death in GA-treated Calu-6 lung cancer cells, whereas it enhanced growth inhibition and death in other lung cancer cell line, A549 (*37*). In addition, MEK inhibitor did not change growth inhibition and death in GA-treated primary human pulmonary fibroblast cells (*38*). Collectively, the anti- or pro-apoptotic effects of MEK inhibitor on GA-treated lung cells can be changed depending on cell types. Additionally, the phosphatidylinositol 3′ kinase/Akt constitutes an important pathway that regulates the signaling of multiple essential biological processes such as cell proliferation, survival, apoptosis, cell migration, and metabolism (*39*, *40*). Akt inhibitor [1−10 μM; Calbiochem (catalog no. 124005)] did not change cell growth inhibition and

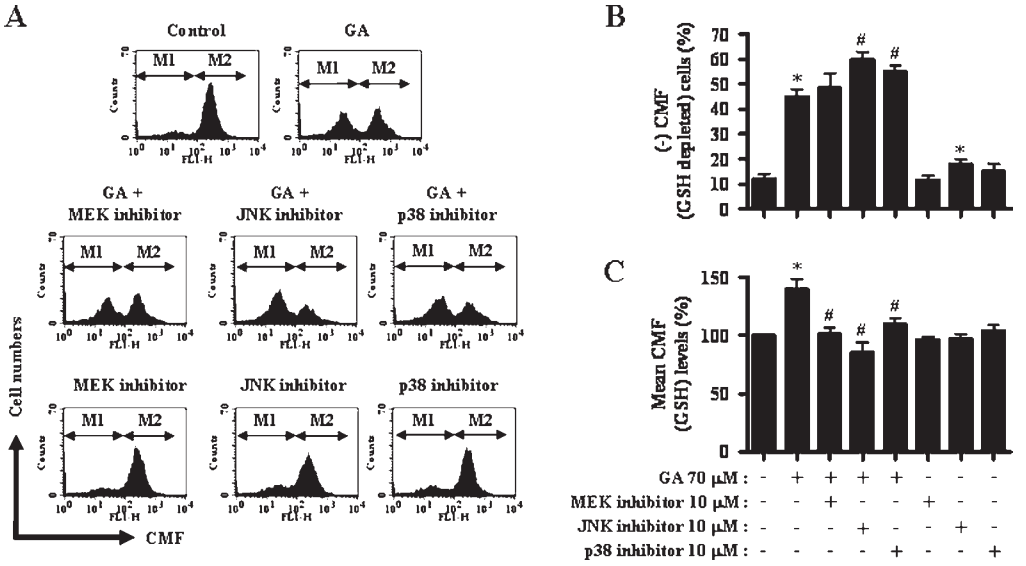

Figure 6. Effects of MAPK inhibitors on GSH levels in GA-treated HeLa cells. Exponentially growing cells were treated with 70 μM GA for 24 h following 1 h of preincubation of 10 μM MEK, JNK, or p38 inhibitor. GSH levels in HeLa cells were measured using a FACStar flow cytometer. (**A**) Histograms for CMF intensity in HeLa cells. (**B** and **C**) Graphs show the percents of (−) CMF (GSH-depleted) cells (M1 region in **A**) (**B**) and the percents of mean CMF (GSH) levels as compared with GA-untreated control cells (M2 region in **A**) (**C**). $^{*}P < 0.05$ as compared with GA-untreated HeLa control group. $^{\#}P < 0.05$ as compared with cells treated with GA only.

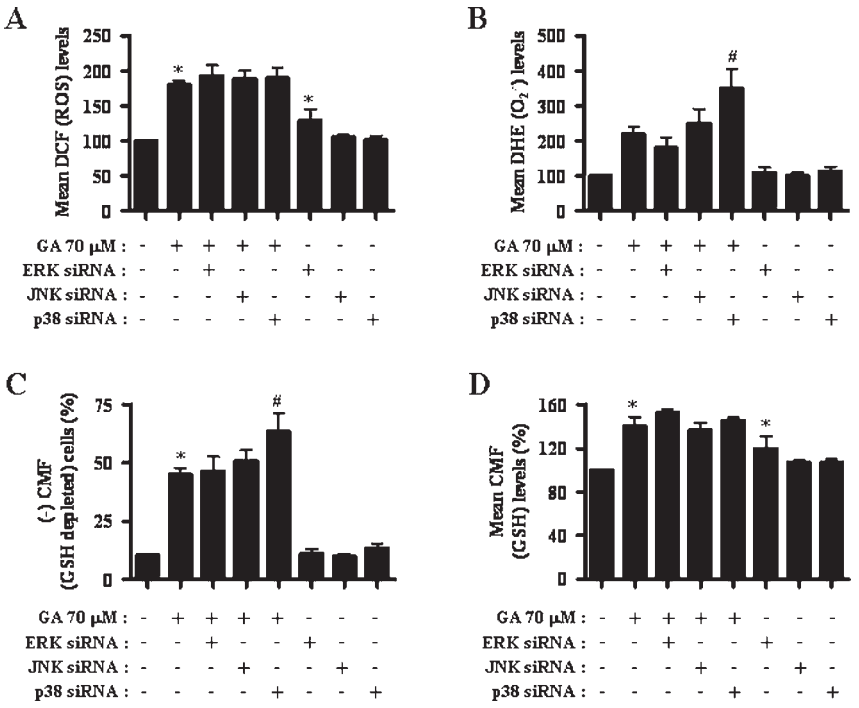

Figure 7. Effects of MAPK siRNAs on ROS and GSH levels in GA-treated HeLa cells. HeLa cells (approximately 30−40% confluence) were transfected with either nontarget control (CTR) siRNA or each MAPK siRNA. One day later, cells were treated with 70 μM GA for additional 24 h. (**A** and **B**) Graphs indicate DCF (ROS) levels (%) as compared with GA-untreated control cells (**A**) and DHE ($O_2^{\bullet-}$) levels (%) as compared with GA-untreated control cells (**B**). (**C** and **D**) Graphs show the percents of (−) CMF (GSH-depleted) cells (C) and the percents of mean CMF (GSH) levels as compared with GA-untreated control cells (**D**). $^{*}P < 0.05$ as compared with GA-untreated HeLa control group. $^{\#}P < 0.05$ as compared with cells treated with GA only.

death in GA-treated HeLa cells (data not shown). This result suggests that Akt signaling was not involved in GA-induced HeLa cell growth inhibition and death.

The activation of JNK or p38 generally leads to apoptosis (*22*−*24*). In fact, JNK inhibitor protects PC12 rat pheochromocytoma against GA-induced cell death (*41*). p38 inhibitor decreased the death of pyrogallol-induced calf pulmonary artery endothelial cells (*29*). Likewise, GA increased the activity of JNK in HeLa cells, but JNK inhibitor intensified both the growth inhibition and the apoptosis in GA-treated HeLa cells. Another JNK inhibitor [JNK inhibitor I, (L)-form from Calbiochem] also enhanced those in these cells (data not shown). In addition, JNK inhibitor or JNK inhibitor I alone induced cell death in HeLa control cells. Therefore, the prevention of JNK signaling by its inhibitor in GA-treated and -untreated HeLa cells seemed to act as a pro-apoptotic function. In contrast, JNK siRNA did not

affect cell death in GA-treated and -untreated HeLa cells, implying the possibility that the JNK inhibitors used in this experiment may enhance GA-induced HeLa cell death via affecting other kinases as well as JNK itself.

Interestingly, although the change of p38 activity was not observed in GA-treated HeLa cells, p38 inhibitor (SB202190) enhanced both the growth inhibition and the death in GA-treated HeLa cells. Furthermore, p38 siRNA also augmented both the growth inhibition and the death in these cells. These results suggested that the inhibition of p38 signaling is positively involved in HeLa cell death by GA. Recently, we demonstrated that p38 inhibitor intensified cell growth inhibition and cell death in GA-treated endothelial cells (*42*). On the other hand, p38 inhibitor (SB202190) did not significantly change cell growth inhibition and death in GA-treated normal lung cells (*38*) and Calu-6 and A549 lung cancer cells (*37*). Another p38 inhibitor (SB203580; 10 μM from Calbiochem) also did not affect both the growth inhibition and the death in GA-treated HeLa cells (data not shown). These results imply that p38 inhibitors differently affect cell growth and death in GA-treated HeLa cells, and their effects can be changed depending on cell types.

GA induced the loss of MMP ($\Delta\Psi_m$) in HeLa cells. While MEK inhibitor did not affect MMP ($\Delta\Psi_m$) loss in GA-treated HeLa cells, JNK and p38 inhibitors intensified the MMP ($\Delta\Psi_m$) loss. JNK alone significantly triggered MMP ($\Delta\Psi_m$) loss in HeLa control cells. These results were similar to annexin V assay results. Therefore, the cell death by GA and/or MAPK inhibitors seemed to be tightly correlated with the loss of MMP ($\Delta\Psi_m$). In addition, p38 siRNA showing an increased death effect in GA-treated or-untreated HeLa cells strongly augmented MMP ($\Delta\Psi_m$) loss in these cells. GA reduced MMP ($\Delta\Psi_m$) levels in viable HeLa cells. JNK and p38 inhibitors intensified the decreased MMP ($\Delta\Psi_m$) level in GA-treated HeLa cells. Because MTT reduction is considered to be a measurement of mitochondrial activity (*43*), the decreased effect of JNK or p38 inhibitor on MTT reduction in GA-treated HeLa cells is probably due to the decrease of MMP ($\Delta\Psi_m$) levels by these inhibitors. However, because each MAPK inhibitor alone reduced MMP ($\Delta\Psi_m$) level in GA-untreated HeLa control cells without cell growth inhibition, the changes of MMP ($\Delta\Psi_m$) levels did not seem to be fully associated with those of MTT reduction in HeLa cells. ERK but not JNK and p38 siRNAs reduced MMP ($\Delta\Psi_m$) levels in GA-untreated HeLa control cells, whereas all of these siRNAs did not alter the MMP ($\Delta\Psi_m$) level in GA-treated HeLa cells. The inhibitions of MAPK signaling by means of its inhibitors or siRNAs seemed to be slightly different in the maintenance of MMP ($\Delta\Psi_m$) levels in HeLa cells.

GA has been reported to have both pro-oxidant and antioxidant properties (*13*, *14*). Increasing evidence suggests that apoptosis induced by GA is associated with oxidative stresses derived from ROS (*12*, *44*). According to our results, the intracellular ROS (DCF) levels including $O_2^{\bullet-}$ were significantly increased in GA-treated HeLa cells. This result implies that GA plays a role as a pro-oxidant in HeLa cells. MEK inhibitor not showing effects on apoptosis and MMP ($\Delta\Psi_m$) loss in GA-treated HeLa cells increased the ROS (DCF) level but did not change the $O_2^{\bullet-}$ level. JNK inhibitor significantly increased the $O_2^{\bullet-}$ level but not the ROS (DCF) level in GA-treated cells. In addition, p38 inhibitor increased ROS levels including $O_2^{\bullet-}$ in these cells. Moreover, MAPK siRNAs did not affect ROS (DCF) levels in GA-treated HeLa cells. ERK and JNK siRNAs not showing effects on apoptosis in GA-treated HeLa cells did not significantly change the $O_2^{\bullet-}$ level in these cells, whereas p38 siRNA significantly augmented the $O_2^{\bullet-}$ level in these cells. These data indirectly suggested that the changes of ROS, especially $O_2^{\bullet-}$ by GA and each MAPK inhibitor or siRNA, are in part related to HeLa cell death. Interestingly, JNK inhibitor triggering cell death in HeLa control cells did not increase the ROS (DCF) level and even decreased the $O_2^{\bullet-}$ level. In contrast, p38 inhibitor strongly increased ROS including $O_2^{\bullet-}$ in HeLa control cells without the significant induction of apoptosis. These data imply that JNK or p38 inhibitor individually affects different ROS levels in HeLa control cells without influencing HeLa cell death. In addition, ERK siRNA increased ROS (DCF) level but not the $O_2^{\bullet-}$ level in HeLa control cells. JNK and p38 siRNAs did not affect ROS (DCF) levels including the $O_2^{\bullet-}$ level in HeLa control cells. These results imply that decreases in MAPK expressions by their siRNAs did not strongly influence ROS levels in HeLa control cells as compared with the inhibitions of MAPK signaling by its inhibitors.

GSH is a main nonproteic antioxidant in cells. It is able to eliminate the $O_2^{\bullet-}$ and provide electrons for enzymes such as GSH peroxidase, which reduce H_2O_2 to H_2O. It has been reported that the intracellular GSH content has a decisive effect on anticancer drug-induced apoptosis, indicating that apoptotic effects are inversely comparable to GSH content (*45*, *46*). Likewise, GA increased the number of GSH-depleted cells in HeLa cells. In addition, JNK or p38 inhibitor increased the numbers of GSH-depleted cells in GA-treated HeLa cells, and JNK inhibitor alone also increased the numbers in HeLa control cells. Furthermore, p38 siRNA showing an increase in GA-induced HeLa cell death significantly augmented GSH depletion in GA-treated HeLa cells. These results might be correlated with the results derived from annexin V and MMP ($\Delta\Psi_m$) assays. It is of note that the CMF (GSH) level in HeLa cells was increased by GA. Probably, the increased GSH level occurred against the increasing ROS level by GA, and some HeLa cells beyond their capacity to resist oxidative stresses (or ROS insults) would undergo cell death pathway. All of the MAPK inhibitors reduced the GSH level in GA-treated HeLa cells. These results imply that MAPK inhibitors are involved in the down-regulation of GSH levels in GA-treated HeLa cells, consequently influencing the proportions of GSH-depleted cells. However, none of MAPK siRNAs significantly altered the GSH level in GA-treated HeLa cells. Only ERK siRNA increased the GSH level in HeLa control cells. These results also implied that the down-regulation effects of MAPK expressions by their siRNAs are not perfectly interconnected with the inhibition effects of MAPK signaling by its inhibitors.

Conclusively, GA induced the growth inhibition and death of HeLa cells via the loss of MMP ($\Delta\Psi_m$), which was accompanied by increasing ROS level as well as triggering GSH depletion. JNK and p38 inhibitors and p38 siRNA enhanced growth inhibition and cell death in GA-treated HeLa cells, which were to some extent related to GSH depletion and ROS, especially $O_2^{\bullet-}$ levels. In addition, the inhibitions of MAPK signaling by its inhibitors and the down-regulation of MAPK expressions by their siRNAs partially showed different effects on cell growth, cell death, MMP ($\Delta\Psi_m$), ROS, and GSH levels in HeLa cells.

ABBREVIATIONS USED

GA, gallic acid; ROS, reactive oxygen species; MAPK, mitogen-activated protein kinase; MEK, MAP kinase or ERK kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; MMP ($\Delta\Psi_m$), mitochondrial membrane potential; FBS, fetal bovine serum; PI, propidium iodide; FITC, fluorescein isothiocyanate; H_2DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; GSH, glutathione; CMFDA, 5-chloromethylfluorescein diacetate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; siRNA, small interfering RNA.

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