SPANDIDOS PUBLICATIONS

The effects of buthionine sulfoximine, diethyldithiocarbamate or 3-amino-1,2,4-triazole on propyl gallate-treated HeLa cells in relation to cell growth, reactive oxygen species and glutathione

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Abstract. Propyl gallate (PG) as a synthetic antioxidant is widely used in processed food and medicinal preparations. It also exerts a variety of effects on tissue and cell functions. In the present study, we investigated the effects of L-buthionine sulfoximine (BSO, an inhibitor of GSH synthesis), diethyldithiocarbamate (DDC, an inhibitor of Cu/Zn-SOD) or 3-amino-1,2,4-Triazole (AT, an inhibitor of catalase) on PG-treated HeLa cells in relation to cell growth, reactive oxygen species (ROS) and glutathione (GSH). Treatment with PG induced growth inhibition, the loss of mitochondrial membrane potential [MMP ($\Delta\Psi_m$)] and apoptosis in HeLa cells. ROS levels including $O_2^{\bullet-}$ were increased or decreased in PG-treated HeLa cells depending on the incubation times. PG caused depletion in GSH content in HeLa cells. While BSO enhanced the growth inhibition of PG-treated HeLa cells at 4 h, DDC and AT did not. All the agents down-regulated MMP ($\Delta\Psi_m$) levels in PG-treated cells. Although BSO, DDC or AT slightly increased ROS or $O_2^{\bullet-}$ levels in PG-treated cells at 1 h, these enhancements of ROS did not intensify apoptosis in these cells. In addition, BSO, DDC or AT slightly reduced GSH level in PG-treated HeLa cells at 1 h, but this reduction did not affect cell death of HeLa. Furthermore, PG induced a G1 phase arrest of the cell cycle. BSO, DDC or AT significantly inhibited the G1 phase arrest in PG-treated cells. Conclusively, the changes of ROS and GSH levels by BSO, DDC or AT in PG-treated HeLa cells did not strongly affect the cell growth and death.

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Abbreviations: PG, propyl gallate; ROS, reactive oxygen species; MMP ($\Delta\Psi_m$), mitochondrial membrane potential; NADPH, nicotine adenine diphosphate; XO, xanthine oxidase; SOD, superoxide dismutase; FBS, fetal bovine serum; PBS, phosphate buffer saline; 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; PI, propidium iodide; DDC, diethyldithiocarbamate; BSO, L-buthionine sulfoximine; AT, 3-amino-1,2,4-triazole

Key words: propyl gallate, apoptosis, HeLa, glutathione, reactive oxygen species

Introduction

Propyl gallate [PG, 3,4,5-trihydroxybenzoic acid propyl ester (Fig. 1A)] is used as a synthetic antioxidant in processed food, cosmetics and food packing materials, to prevent rancidity and spoilage. PG is also used to preserve and stabilize medicinal preparations on the US Food and Drug Administration list (1). Because of its prevalent usage, the potential toxicity of PG has been investigated *in vivo* (2,3) and *in vitro*, to assess various toxicological properties, i.e., mutagenicity (4) and cytogenetic effects (5). Despite the assumed low toxicity of PG, it exerts a variety of effects on tissue and cell functions. Several studies demonstrate the benefits of PG as an antioxidant (3,6,7) and an anti-inflammatory agent (8). For instance, PG is an efficient protector of liver cells from lipid peroxidation by oxygen radicals (3). PG also has protective effects against oxidative DNA damage using 8-oxoguanine formation as a marker (7). In contrast, it is reported that PG has prooxidant properties (9,10). PG is cytotoxic to isolated rat hepatocytes because it impairs mitochondria, leading to ATP depletion (11). PG inhibits growth of microorganisms by inhibiting respiration and nucleic acid synthesis (12). Controversially, the effects of PG on carcinogenesis and mutagenesis can be both enhancing and suppressing (4,13). Antioxidative and cytoprotective properties of PG may change to prooxidative, cytotoxic and genotoxic properties in the presence of Cu(II) (14). Therefore, in order to clarify the discrepancy between the different effects of PG, further studies need to be performed to re-evaluate its function and safety on cells and tissues.

Reactive oxygen species (ROS) include hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$). These molecules have recently been implicated in regulating many important cellular events, including transcription factor activation, gene expression, differentiation and cell proliferation (15,16). ROS are formed as by-products of mitochondrial respiration or the action of oxidases, including nicotine adenine diphosphate (NADPH) oxidase, xanthine oxidase (XO) and

certain arachidonic acid oxygenases (17). A change in the redox state of a tissue implies a change in ROS generation or metabolism. The principal metabolic pathways include superoxide dismutase (SOD), which is expressed as extracellular, intracellular and mitochondrial isoforms. These isoforms metabolize $O_2^{\bullet-}$ to H_2O_2. Further metabolism by peroxidases, which include catalase and glutathione (GSH) peroxidase, yields O_2 and H_2O (18). GSH is the main non-protein antioxidant in the cell and provides electrons for enzymes such as glutathione peroxidase, which reduce H_2O_2 to H_2O. GSH has been shown to be crucial for cell proliferation, cell cycle progression and apoptosis (19,20) and is known to protect cells from toxic insult by detoxifying toxic metabolites of drugs and ROS (21). Although cells possess antioxidant systems to control their redox state, which is important for their survival, excessive production of ROS can be induced and gives rise to the activation of events that lead to death or survival in different cell types (22-24).

Little is known about the relationship among PG, ROS and GSH in cancer cells. Because we recently observed that PG treatment inhibited the growth of HeLa cells via caspase-dependent apoptosis (unpublished data), in the present study, we investigated the effects of L-buthionine sulfoximine [BSO, an inhibitor of GSH synthesis (25)], diethyldithiocarbamate [DDC, an inhibitor of Cu/Zn-SOD (26)] or 3-amino-1,2,4-Triazole [AT, an inhibitor of catalase (27)] on PG-treated HeLa cells in relation to cell growth, ROS and GSH.

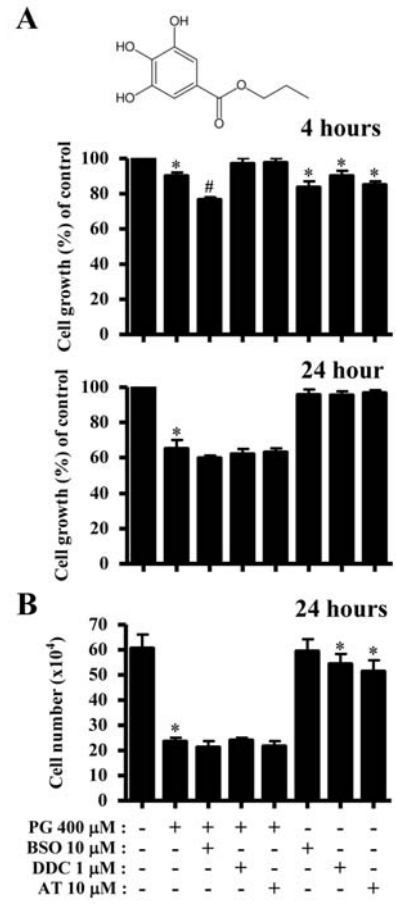

Figure 1. Effects of BSO, DDC or AT on the growth of PG-treated HeLa cells. The structure of propyl gallate (PG) is shown (A). Exponentially-growing cells were treated with 400 μM PG for 4 or 24 h following 30 min pre-incubation of 10 μM BSO, 1 μM DDC or 10 μM AT. Cell growth (A) and cell number (B) were assessed by an MTT assay and trypan blue exclusion cell counting, respectively. *P<0.05 compared with the control group. $^{\#}$P<0.05 compared with cells treated with PG only. Data are presented as means ± SD of three replicates.

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) and harvested with a solution of trypsin-EDTA while in a logarithmic phase of growth. Cells were maintained in these culture conditions for all experiments.

Reagents. PG was purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO). PG was dissolved in ethanol at 200 mM as a stock solution. L-buthionine sulfoximine (BSO), diethyldithiocarbamate (DDC) and 3-amino-1,2,4-triazole (AT) were also obtained from Sigma. These agents were dissolved in water or ethanol at 100 mM as a stock solution. Cells were pretreated with BSO, DDC or AT for 30 min prior to treatment with PG. Ethanol was used as a control vehicle. All stock solutions were wrapped in foil and kept at 4˚C or -20˚C.

Cell growth assay. The effect of drugs on HeLa cell growth was determined by trypan blue exclusion cell counting or measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) dye absorbance of living cells as previously described (28). In brief, cells ($2x10^5$ cells per well) were seeded in 24-well plates (Nunc, Roskilde, Denmark) for cell counting, and cells ($2x10^4$ cells per well) were seeded in 96-well microtiter plates for an MTT assay. After exposure to 400 μM PG with or without BSO, DDC or AT for 4 or 24 h, cells in 24- or 96-well plates were collected with trypsin digestion for trypan blue exclusion cell counting or were used for the MTT assay. Twenty microliters of MTT (Sigma) solution (2 mg/ml in PBS) were added to each well of 96-well plates. The plates were incubated for 4 h at 37˚C. MTT solution in the medium was aspirated off and 200 μl DMSO were added to each well to solubilize the formazan crystals formed in viable cells. Optical density was measured at 570 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co, Sunnyvale, CA). Each plate contained multiple wells at a given experimental condition and multiple control wells. This procedure was replicated for 2-4 plates per condition.

Cell cycle and sub-G1 analysis. Cell cycle distributions and sub-G1 cells were determined by propidium iodide (PI, Sigma-Aldrich; Ex/Em = 488 nm/617 nm) staining as previously described (29). PI is a fluorescent biomolecule that can be used to stain DNA. In brief, $1x10^6$ cells were

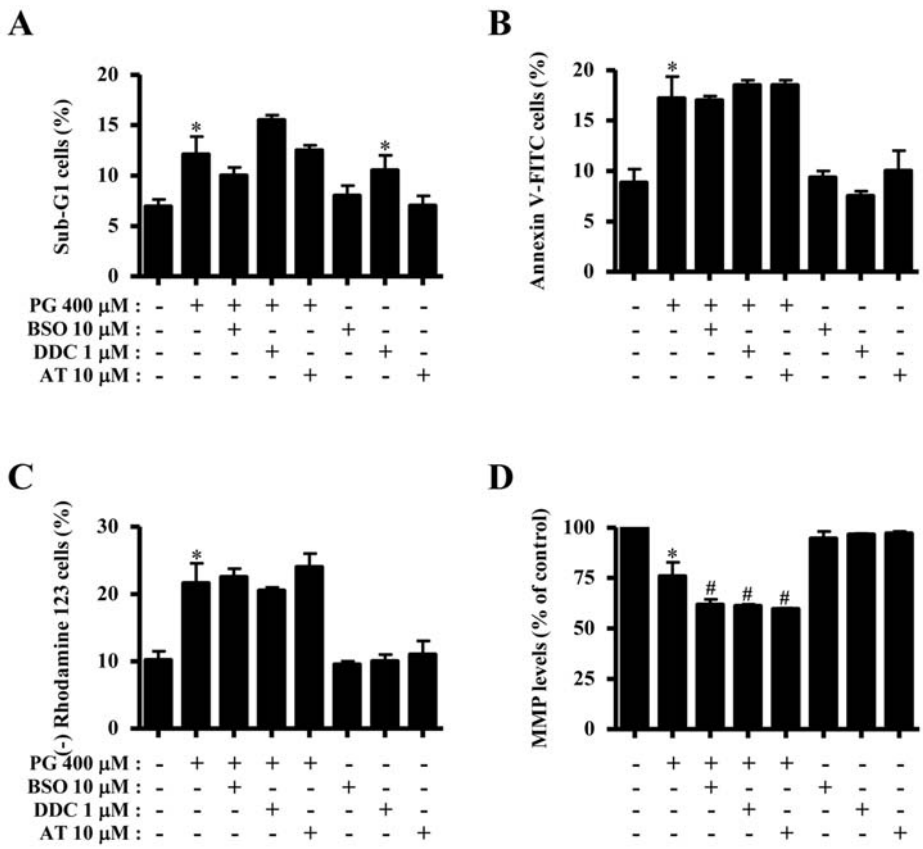

Figure 2. Effects of BSO, DDC or AT on apoptosis and MMP ($\Delta\Psi_m$) in PG-treated HeLa cells. Exponentially-growing cells were treated with 400 μM PG for 24 h following 30 min pre-incubation of 10 μM BSO, 1 μM DDC or 10 μM AT. Graphs show the percents of sub-G1 cells (A), annexin V staining cells (B), Rhodamine 123 negative [MMP ($\Delta\Psi_m$) loss] cells (C) and MMP ($\Delta\Psi_m$) levels (D) in HeLa cells, as measured with a FACStar flow cytometer. *P<0.05 compared with the control group. #P<0.05 compared with cells treated with PG only. Data are presented as means ± SD of three replicates.

incubated with the indicated amounts of PG with or without BSO, DDC or AT for 24 h. Cells were then washed with PBS and fixed in 70% ethanol. Cells were washed again with PBS, then incubated with PI (10 μg/ml) with simultaneous RNase treatment at 37˚C for 30 min. Cell DNA contents were measured using a FACStar flow cytometer (Becton-Dickinson, San Jose, CA) and analyzed using lysis II and CellFIT software (Becton-Dickinson) or ModFit software (Verity Software House, Inc., ME).

Annexin V staining. Apoptosis was determined by staining cells with annexin V-fluorescein isothiocyanate (FITC) (Ex/Em = 488 nm/519 nm) as previously described (30). In brief, 1x10^6 cells were incubated with the indicated amounts of PG with or without BSO, DDC or AT 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, 2.5 mM $CaCl_2$) at a concentration of 1x10^6 cells/ml. Five microliters of annexin V-FITC (PharMingen, San Diego, CA) was then added to these cells, which were analyzed with a FACStar flow cytometer (Becton-Dickinson).

Measurement of mitochondrial membrane potential (MMP) ($\Delta\Psi_m$). The MMP ($\Delta\Psi_m$) was monitored using the Rhodamine 123 fluorescent dye (Ex/Em = 485/535 nm), which preferentially enters mitochondria based on their highly negative membrane potential ($\Delta\Psi_m$), as previously described (31). Depolarization of MMP ($\Delta\Psi_m$) results in the loss of Rhodamine 123 and a decrease in intracellular fluorescence level. In brief, 1x10^6 cells were incubated with 400 μM PG with or without BSO, DDC or AT for 24 h. Cells were washed twice with PBS and incubated with Rhodamine 123 (0.1 μg/ml; Sigma) at 37˚C for 30 min. Rhodamine 123 staining intensity was determined by flow cytometry. Rhodamine 123 negative cells indicate the loss of MMP ($\Delta\Psi_m$) in HeLa cells. MMP ($\Delta\Psi_m$) levels in cells except MMP ($\Delta\Psi_m$) loss cells were expressed as mean fluorescence intensity (MFI), which was calculated by CellQuest software.

Determination of intracellular ROS and $O_2^{\bullet-}$ levels. Intracellular ROS such as H_2O_2, $^{\bullet}OH$ and $ONOO^{\bullet}$ were detected by means of an oxidation-sensitive fluorescent probe dye, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen Molecular Probes, Eugene, OR) (32,33). H_2DCFDA was deacetylated intracellularly by non-specific esterase, which was further oxidized by cellular peroxides, yielding 2,7-dichlorofluorescein (DCF), a fluorescent compound (Ex/Em = 495/529 nm). DCF is poorly selective for superoxide anion radical ($O_2^{\bullet-}$). In contrast, dihydroethidium (DHE) (Ex/Em = 518/605 nm) (Invitrogen Molecular Probes) is a fluorogenic probe that is highly selective for $O_2^{\bullet-}$ among ROS. DHE is cell-permeable and reacts with superoxide anion to form ethidium, which in turn intercalates in deoxyri-

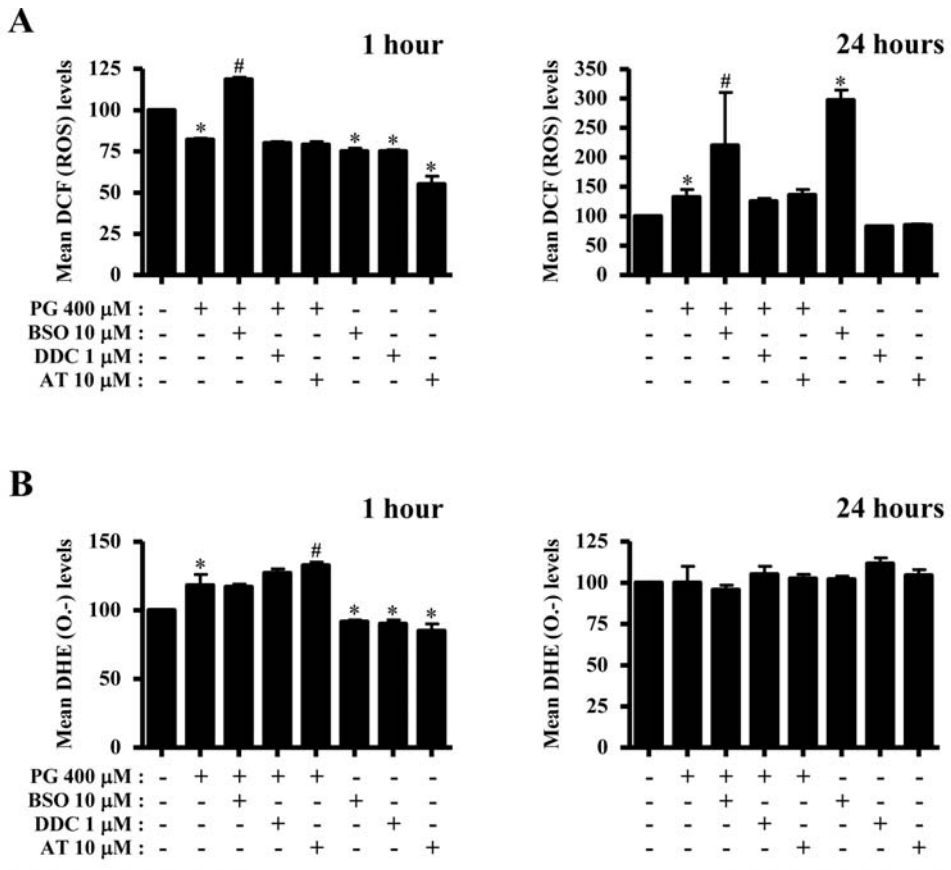

Figure 3. Effects of BSO, DDC or AT on ROS levels in PG-treated HeLa cells. Exponentially-growing cells were treated with 400 μM PG for 1 or 24 h following 30 min pre-incubation of 10 μM BSO, 1 μM DDC or 10 μM AT. ROS levels in HeLa cells were measured using a FACStar flow cytometer. Graphs indicate DCF (ROS) levels (%) compared with HeLa control cells at 1 or 24 h (A) and also indicate DHE ($O_2^{\bullet-}$) levels (%) compared with HeLa control cells at 1 and 24 h (B). *P<0.05 compared with the control group. #P<0.05 compared with cells treated with PG only. Data are presented as means ± SD of three replicates.

bonucleic acid, thereby exhibiting a red fluorescence. In brief, 1x10^6 cells were incubated with 400 μM PG with or without BSO, DDC or AT for 1 or 24 h. Cells were then washed in PBS and incubated with 20 μM H_2DCFDA or DHE at 37˚C for 30 min according to the instructions of the manufacturer. DCF and DHE fluorescences were detected using a FACStar flow cytometer (Becton-Dickinson). ROS and $O_2^{\bullet-}$ levels were expressed as mean fluorescence intensity (MFI), which was calculated by CellQuest software.

Detection of the intracellular glutathione (GSH). Cellular GSH levels were analyzed using 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes) (Ex/Em = 522/595 nm) as previously described (33). In brief, 1x10^6 cells were incubated with the indicated amounts of PG with or without BSO, DDC or AT for 1 or 24 h. Cells were then washed with PBS and incubated with 5 μM CMFDA at 37˚C for 30 min according to the instructions of the manufacturer. Cytoplasmic esterases convert non-fluorescent CMFDA to fluorescent 5-chloromethylfluorescein, which can then react with GSH. 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. CMF levels in cells except (-) CMF cells were expressed as mean fluorescence intensity (MFI), which was calculated by CellQuest software.

Statistical analysis. The results shown in Figures represent the mean of at least two independent experiments; bar, 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 post hoc analysis using Tukey's multiple comparison test was used for parametric data. The statistical significance was defined as p<0.05.

Results

Effects of BSO, DDC or AT on cell growth, apoptosis and mitochondrial membrane potential (MMP) ($\Delta\Psi_m$) in PG-treated HeLa cells. We examined the effect of BSO, DDC or AT on the growth of PG-treated HeLa cells using the MTT assay and trypan blue cell counting at 4 or 24 h because PG treatment inhibited the growth of HeLa cells via caspase-dependent apoptosis (Fig. 1 and unpublished data). For this experiment, we chose the suitable dose of 400 μM PG, which inhibited the growth of HeLa cells about 40% using an MTT assay at 24 h (Fig. 1A) and about 60% using trypan blue cell counting at this time (Fig. 1B). We also determined the optimal doses of 10 μM BSO, 1 μM DDC and 10 μM AT, concentrations of which did not strongly affect the growth of HeLa control cells at 24 h (Fig. 1). Treatment with BSO intensified the growth inhibition of PG-treated HeLa cells at the short time period of 4 h (Fig. 1A). BSO, DDC or AT

ibited the growth of HeLa control cells at this time. At 24 h, treatment with BSO, DDC or AT slightly increased the growth inhibition of PG-treated HeLa cells (Fig. 1A). None of BSO, DDC or AT significantly affected cell numbers in PG-treated HeLa cells, but DDC and AT reduced the number of HeLa control cells at 24 h (Fig. 1B).

We observed that 400 μM PG induced apoptosis in HeLa cells about 8% compared with HeLa control cells, as evidenced by sub-G1 cells and annexin V staining cells (Fig. 2A and B). Apoptosis by PG was accompanied by the activation of caspases (unpublished data). We determined whether treatment with BSO, DDC or AT affect PG-induced apoptosis at 24 h. As shown in Fig. 2A, DDC slightly increased the number of sub-G1 cells in PG-treated and -untreated HeLa cells. Neither BSO nor AT affect that of sub-G1 cells in HeLa cells (Fig. 2A). In addition, none of these drugs affected the number of annexin V-FITC positive cells in PG-treated or PG-untreated cells (Fig. 2B).

Apoptosis is closely related to the collapse of MMP ($\Delta\Psi_m$) (34). Therefore, we determined the loss of MMP ($\Delta\Psi_m$) in PG-treated HeLa cells using a Rhodamine 123 dye. As expected, the loss of MMP ($\Delta\Psi_m$) was observed in PG-treated cells at 24 h (Fig. 2C). None of these drugs significantly changed the loss in PG-treated or -untreated cells (Fig. 2C). In relation to MMP ($\Delta\Psi_m$) levels in HeLa cells except Rhodamine 123 negative cells, treatment with PG reduced the MMP ($\Delta\Psi_m$) level about 25% (Fig. 2D). Treatment with BSO, DDC or AT additionally decreased the MMP ($\Delta\Psi_m$) level in PG-treated HeLa cells, but not in HeLa control cells (Fig. 2D).

Effects of BSO, DDC or AT on ROS levels in PG-treated HeLa cells. Next, to determine whether the levels of ROS in PG-treated HeLa cells were changed by treatment with BSO, DDC or AT, we assessed ROS levels in cells using various fluorescence dyes at the short time period of 1 h or the long time period of 24 h (Fig. 3). Intracellular ROS (DCF) level such as H_2O_2 was decreased in PG-treated cells at 1 h (Fig. 3A) whereas ROS level was increased in these cells at 24 h (Fig. 3A). Treatment with BSO increased ROS level in PG-treated HeLa cells at 1 or 24 h, but DDC and AT did not affect the level of ROS in these cells (Fig. 3A). Interestingly, BSO, DDC and AT alone decreased ROS level in HeLa control cells at 1 h whereas BSO markedly increased ROS level at 24 h (Fig. 3A).

When we detected the intracellular $O_2^{\bullet-}$ levels in PG-treated HeLa cells, red fluorescence derived from DHE reflecting intracellular $O_2^{\bullet-}$ level was increased in these cells at 1 h (Fig. 3B). Treatment with DDC or AT slightly intensified the increased $O_2^{\bullet-}$ level in PG-treated cells at 1 h (Fig. 3B). However, treatment with BSO, DDC or AT decreased $O_2^{\bullet-}$ level in HeLa control cells at 1 h (Fig. 3B). At 24 h, $O_2^{\bullet-}$ level in HeLa cells was not significantly changed by treatment with PG, BSO, DDC or AT (Fig. 3B).

Effects of BSO, DDC or AT on GSH levels in PG-treated HeLa cells. Cellular GSH is crucial for regulation of cell proliferation, cell cycle progression and apoptosis (19,20). Therefore, we analyzed the changes of GSH levels in HeLa cells by using CMF fluorescence at 1 or 24 h (Fig. 4). Treatment with PG depleted the intracellular GSH content at 24 h in HeLa cells about 9% compared with the HeLa control cells (Fig. 4A), but not at 1 h (data not shown). Treatment with BSO, DDC or AT did not significantly affect GSH depletion in PG-treated HeLa cells (Fig. 4A). When CMF (GSH) levels in HeLa cells were assessed, PG did not change GSH level at 1 h (Fig. 4B). BSO, DDC or AT slightly reduced GSH level in PG-treated and -untreated HeLa cells at 1 h (Fig. 4B). At 24 h, PG treatment significantly increased GSH levels in HeLa cells, and none of drugs changed the GSH level in PG-treated or -untreated HeLa cells (Fig. 4B).

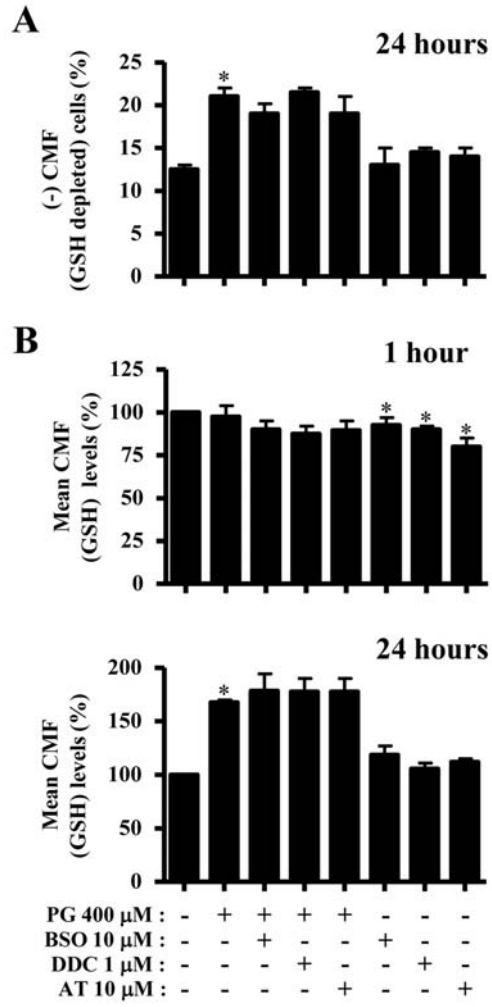

Figure 4. Effects of BSO, DDC or AT on GSH levels in PG-treated HeLa cells. Exponentially-growing cells were treated with 400 μM PG for 1 or 24 h following 30 min pre-incubation of 10 μM BSO, 1 μM DDC or 10 μM AT. GSH levels in HeLa cells were measured using a FACStar flow cytometer. Graphs indicate (-) CMF (GSH depleted) cells (%) at 24 h of incubation time (A). Other graphs indicate mean CMF (GSH) levels (%) compared with HeLa control at 1 or 24 h (B). *P<0.05 compared with the control group. #P<0.05 compared with cells treated with PG only. Data are presented as means ± SD of duplicates.

Effects of BSO, DDC or AT on the cell cycle distribution in PG-treated HeLa cells. We examined the effect of PG on the cell cycle distribution in HeLa cells, except sub-G1 DNA content cells, in the presence or absence of BSO, DDC or AT. As shown in Fig. 5, DNA flow cytometric analysis indicated

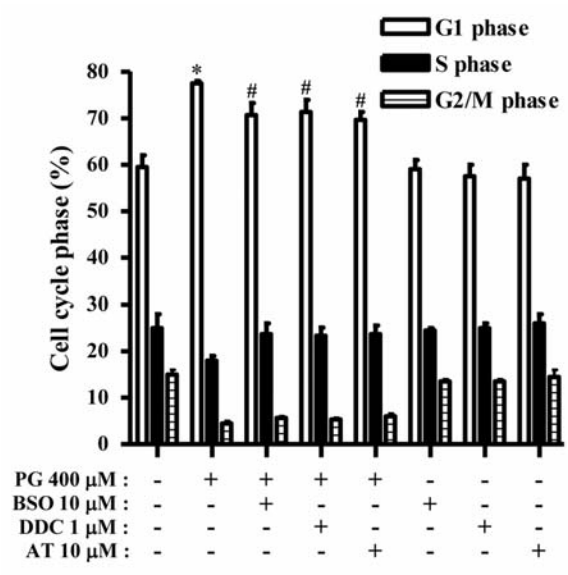

Figure 5. Effects of BSO, DDC or AT on the cell cycle distribution in PG-treated HeLa cells. Exponentially-growing cells were treated with 400 μM PG for 24 h following 30 min pre-incubation of 10 μM BSO, 1 μM DDC or 10 μM AT. The changes of cell cycle phase distribution were assessed by DNA flow cytometric analysis. *P<0.05 compared with the control group. $^{\#}$P<0.05 compared with cells treated with PG only. Data are presented as means ± SD of duplicates.

that treatment with PG significantly induced a G1 phase arrest of the cell cycle at 24 h. Treatment with BSO, DDC or AT significantly reduced the number of cells at the G1 phase in PG-treated HeLa cells (Fig. 5).

Discussion

In the present study, we focused on evaluating the effects of BSO, DDC or AT on PG-treated HeLa cells in relation to cell growth, changes of ROS and GSH levels, since we have observed that PG inhibited the growth of HeLa cells. We also observed that PG induced apoptosis in HeLa cells, as evidenced by sub-G1 cells and annexin V staining cells. Treatment with BSO, DDC or AT slightly intensified the growth inhibition of PG-treated HeLa cells at 24 h, as measured by the MTT assay. At 4 h, especially BSO intensified the growth inhibition of PG-treated HeLa cells, and treatment with BSO, DDC or AT alone inhibited the growth of HeLa control cells. These results suggest that the effects of BSO, DDC or AT on HeLa cell growth depend on the incubation times. None of BSO, DDC or AT significantly affected cell numbers in PG-treated HeLa cells, but DDC and AT reduced the number of HeLa control cells at 24 h. In addition, none of BSO, DDC or AT significantly increased the number of sub-G1 cells in PG-treated HeLa cells. None of these drugs affected the number of annexin V-FITC positive cells in PG-treated or -untreated cells. Collectively, these results suggest that co-treatment with PG and BSO, DDC or AT do not synergistically affect growth inhibition and apoptosis in HeLa cells. With regard to the MMP ($\Delta\Psi_m$), PG treatment induced the loss of MMP ($\Delta\Psi_m$) and reduced the MMP ($\Delta\Psi_m$) level in HeLa cells. None of BSO, DDC or AT significantly changed the level of MMP ($\Delta\Psi_m$) loss in PG-treated or -untreated cells. These results imply that the loss of MMP ($\Delta\Psi_m$) following treatment with PG leads to apoptosis, and BSO, DDC and AT did not directly trigger the loss of MMP ($\Delta\Psi_m$). Furthermore, because MTT reduction is considered to be a measure of mitochondrial activity (35), the slightly decreased effect of BSO, DDC and AT on MTT reduction in PG-treated HeLa cells is probably due to the down-regulation of MMP ($\Delta\Psi_m$) levels by these agents. In addition, PG significantly induced a G1 phase arrest of the cell cycle at 24 h. Treatment with BSO, DDC or AT significantly reduced the number of cells at the G1 phase in PG-treated HeLa cells. This result suggests that the preventive effect of BSO, DDC or AT on a G1 phase arrest in PG-treated HeLa cells is not directly related to HeLa cell growth.

PG can play a role as an antioxidant (3,6,7,36) or a prooxidant (9,10). Interestingly, our data showed that PG reduced ROS (DCF) levels for the short incubation time of 1 h, but significantly increased the levels for the long incubation time of 24 h. In contrast, PG increased $O_2^{\bullet-}$ (DHE) levels for 1 h, but this increase did not last for 24 h. These data indicate that the intracellular ROS including $O_2^{\bullet-}$ were increased (pro-oxidant) or decreased (anti-oxidant) in PG-treated HeLa cells depending on the incubation times. A change in the redox state of cell or tissue implies a change in ROS generation or metabolism (18). SOD, which catalyzes the dismutation of $O_2^{\bullet-}$ into H_2O_2 and molecular oxygen, is one of the most important antioxidative enzymes. Catalase then metabolizes H_2O_2 to O_2 and H_2O. Treatment with 400 μM PG increased the activities of SOD and catalase in HeLa cells at 24 h (unpublished data). Therefore, the increased ROS levels by PG did not result from the decreased activities of both SOD and catalase, but probably from the strong generation of ROS by other oxidases such as NADPH oxidase, XO and certain arachidonic acid oxygenases. Because Nakagawa *et al* suggests that PG mediates its toxicity by uncoupling the oxidative phosphorylation in mitochondrial respiration using isolated hepatocytes (37), it is also possible that PG can directly generate $O_2^{\bullet-}$ in HeLa cells via impairing mitochondrial function. Since apoptosis in PG-treated cells was accompanied by an increase in ROS levels at 24 h, this result suggests that the changes of ROS levels by PG are at least in part related to apoptotic cell death of HeLa.

When we determined whether the levels of intracellular ROS in PG-treated HeLa cells were changed by treatment with BSO, DDC or AT, treatment with BSO increased ROS level in PG-treated HeLa cells at 1 or 24 h. Treatment with DDC or AT slightly intensified the increased $O_2^{\bullet-}$ level in PG-treated cells at 1 h. However, these enhancements of ROS did not intensify apoptosis in PG-treated cells. Therefore, the exact role of ROS in PG-induced cell death needs to be defined further. In general, the inhibition of SOD and catalase by DDC and AT in cells leads to an increase in ROS. However, our results demonstrated that treatment with DDC or AT did not increase ROS levels including $O_2^{\bullet-}$ in HeLa control cells. Instead, both of them reduced ROS levels in HeLa control cells at 1 h. When exposed to DDC for the long time of 72 h, HeLa cells showed the increased level of $O_2^{\bullet-}$ (data not shown). In addition, we observed an increased level of ROS in AT-treated Calu-6 lung cancer cells (unpublished

These results suggest that the effects of DDC and AT and catalase activities depend on cell types and incubation times.

GSH directly eliminates $O_2^{\bullet-}$ and provides 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 comparative to GSH content (38,39). Likewise, when PG depleted intracellular GSH content in HeLa cells for 24 h, these cells showed apoptotic phenomenon. It is of note that CMF (GSH) level in HeLa cells was increased for 24 h. Probably, an increase in GSH content happened to reduce the increasing ROS by PG. Thus, cells beyond their capacity to resist ROS would be immediately dead. Treatment with BSO, DDC or AT did not significantly affect GSH depletion in PG-treated HeLa cells. This result is probably correlated to the fact that BSO, DDC and AT did not affect growth inhibition and apoptosis in PG-treated HeLa cells. It is of note that BSO as a GSH synthesis inhibitor did not enhance the GSH depletion in PG-treated HeLa cells. However, we observed that BSO enhanced the GSH depletion and apoptosis in antimycin A-treated Calu-6 lung cells (unpublished data). It is possible that the effect of BSO on GSH depletion is dependent on cell types. Concerning GSH levels following treatment with BSO, DDC or AT, all of them slightly reduced GSH level in PG-treated and -untreated HeLa cells at 1 h, but not 24 h. These results suggest that the agents differently influence GSH levels in HeLa cells depending on the incubation times. Particularly, BSO has an effect on GSH level as well as ROS level in PG-treated HeLa cells at the early time of 1 h. The precise role of the time-dependent changes of ROS level by these drugs needs to be defined further.

Conclusively, our data demonstrated that the changes of ROS and GSH levels by BSO, DDC or AT in PG-treated HeLa cells did not strongly affect the cell growth and death.

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