Anticancer Effects of Auranofin in Human Lung Cancer Cells Through Increasing Intracellular ROS Levels and GSH Depletion

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Research Article

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Abstract

Purpose Auranofin is known to inhibit thioredoxin reductase (TrxR) and has promising anticancer activity in several cancer types. However, at present, there is no clear explanation for the mechanism underlying the inhibitory effects of Auranofin on lung cancer cell growth. In this study, we evaluated the antigrowth effects of Auranofin in cells from various lung cancer cell lines with regard to cell death, reactive oxygen species (ROS), and glutathione (GSH) levels.

Methods Cell proliferation was assessed using the trypan blue staining cell counting. ROS levels including $O_2^{\cdot -}$, GSH levels, and MMP ($\Delta\Psi_m$) loss were measured using a flow cytometry. Apoptosis was determined with annexin V-PI staining assay and the change of apoptosis-related protein level was detected by western blotting. TrxR activity was evaluated using a thioredoxin reductase colorimetric assay kit.

Results Treatment with Auranofin inhibited cell proliferation and induced cell death based on cell number at 24 h in Calu-6, A549, SK-LU-1, NCI-H460, and NCI-H1299 cells. In addition, Auranofin led to increased ROS levels including $O_2^{\cdot -}$ and GSH depletion in these cells. Treatment with N-acetyl cysteine (NAC) attenuated the growth inhibition, mitochondrial membrane potential (MMP, $\Delta\Psi_m$) loss, and increased ROS levels and GSH depletion in Auranofin-treated Calu-6 and A549 cells. By contrast, L-buthionine sulfoximine (BSO) enhanced cell death, MMP ($\Delta\Psi_m$) loss, ROS production, and GSH depletion. Furthermore, the western blot analysis indicated that Auranofin-induced caspase-3 activation and poly (ADP ribose) polymerase (PARP) cleavage, both of which were prevented by pretreatment with NAC but enhanced by pretreatment with BSO in Calu-6 and A549 cells. Consistent with these changes, the decrease in TrxR activity caused by Auranofin was enhanced by preincubation with BSO and restored in response to the preincubation with NAC in both Calu-6 and A549 cells.

Conclusion Our present findings demonstrate that Auranofin-induced cell death is tightly related to oxidative stress in lung cancer cells.

Introduction

Lung cancer is the most frequent malignant tumor and the leading cause of cancer-related mortality and morbidity worldwide (Bray et al. 2018). Lung cancer, which arises from lung epithelial cells, can be histologically divided into two types: small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), accounting for 15% and 85% of all lung cancer, respectively. NSCLC is further categorized into three subtypes, namely lung adenocarcinoma (LUAD), lung squamous cell carcinoma (LUSC), and lung large cell carcinoma (LULC) (Blandin Knight et al. 2017; Watanabe et al. 2010). More than 40% of NSCLC cases are metastatic at diagnosis, and the 5-year overall survival for patients with distant metastatic NSCLC is < 5% (de Silva et al. 2020; Xu et al. 2013). There are various clinical cancer therapies such as surgical removal, chemotherapy, and radiation therapy for lung cancer treatment. Of them, the main primary therapy for lung cancer is molecular targeted agents (Nagasaka and Gadgeel 2018; Noreldeen et

al. 2020; Sharma et al. 2019). However, these agents often have a limited effect and treatment-associated toxicity that reduce their therapeutic efficacy (Becker et al. 2010; Wang et al. 2019). For this reason, new alternative therapeutic strategies for patients with lung cancer are critically required.

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), superoxide anions ($\text{O}_2^{\cdot-}$), and hydroxyl radicals ($\cdot\text{OH}$), are byproducts of normal cellular metabolism. These molecules play significant roles in many cellular events such as gene expression, cell differentiation, proliferation, survival, apoptosis, cognitive function, and immune response (Brieger et al. 2012; Davalli et al. 2016; Sena and Chandel 2012; Trachootham et al. 2009). However, excessive production of ROS can cause cell damage by oxidizing DNA, RNA, protein, and lipid that can lead to the pathogenesis of a diverse number of diseases including cancer (Nogueira and Hay 2013; Perillo et al. 2020). Higher levels of ROS in cancer cells are associated with tumorigenesis and are an indicative hallmark of cancer. Several studies have demonstrated that oxidative stress-mediated signaling pathways can affect cancer progression, differentiation, and apoptosis (Gao et al. 2020; Hua et al. 2016; Park 2017). Thus, ROS regulation may represent a critical target for the development of antitumor agents.

To maintain ROS homeostasis, an antioxidant defense system including glutathione (GSH) and thioredoxin (Trx) exists to reduce ROS levels in cells (Trachootham et al. 2009). The Trx system is composed of Trx and a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent Trx reductase (TrxR) (Lu and Holmgren 2014). Trx is a small (12 kDa) reduction-oxidation (redox) protein with a catalytically active dithiol site in a cysteine residue (Cys-Gly-Pro-Cys), which can reduce disulfides in target proteins. Oxidized Trx can then be reduced by the TrxR enzyme using NADPH to promote its activities (Arner and Holmgren 2000; Jia et al. 2019; Lu and Holmgren 2014). TrxR is a family of selenium-containing pyridine nucleotide-disulfide oxidoreductases (Selenius et al. 2010). Three isoforms of TrxR have been identified in mammals: cytosolic TrxR1, mitochondrial TrxR2, and thioredoxin glutathione reductase TrxR3 (Karlenius and Tonissen 2010). It has been suggested that TrxR completes pivotal roles in a wide range of biological and pathological processes including apoptosis, cancer, chronic inflammation, and autoimmune diseases (Arner and Holmgren 2000; Karlenius and Tonissen 2010; Mohammadi et al. 2019). However, elevated expression of Trx or TrxR is found in different types of tumors, such as hepatocellular, breast, gastric, oral, and lung cancers (Bhatia et al. 2016; Fernandes et al. 2009; Iwasawa et al. 2011; Lim et al. 2012; Zheng et al. 2018). In this regard, targeting the Trx system is a promising strategy for cancer therapy.

Auranofin, as a TrxR inhibitor, is an oral gold (Au)-containing phosphine compound that is approved by the United States Food and Drug Administration (FDA) for treatment of rheumatoid arthritis (Chaffman et al. 1984; Onodera et al. 2019). Although it was initially established as an anti-inflammatory drug, recent studies also revealed that Auranofin has potential therapeutic effects for various human diseases including cancer, neurodegenerative disorders, acquired immunodeficiency syndrome, and parasitic and bacterial infections (Madeira et al. 2012; Roder and Thomson 2015). The pharmacological effect of Auranofin is due to its high reactivity with cellular nucleophiles such as selenocysteine and cysteine, making Auranofin a potent inhibitor of TrxR (Rigobello et al. 2004; Zhang et al. 2019). In particular,

Auranofin exhibits anticarcinogenic activity that is closely related with mitochondrial dysfunction and ROS overproduction in human chronic leukemia and gastric cancer cells (Fiskus et al. 2014; Zou et al. 2015).

Although several studies have demonstrated that Auranofin exhibits potent anticancer activity in human cancer cells, its effect on human lung cancer cells and the underlying mechanisms of action in view of redox state changes still remain to be elucidated. In our previously published reports, we found that Auranofin inhibited cell growth through cell cycle arrest and cell death due to necrosis and caspase-dependent apoptosis in lung cancer cells (Cui et al. 2020). In this study, we investigated the antigrowth effects of Auranofin related to the ROS level and GSH depletion in cells from various lung cancer cell lines and evaluated the cellular effects of N-acetyl cysteine (NAC; a well-known antioxidant) and L-buthionine sulfoximine (BSO, an inhibitor of GSH synthesis) on Auranofin-induced cell death in Calu-6 and A549 cells.

Materials And Methods

Cell culture

Human pulmonary fibroblast (HPF) cells were obtained from Promo Cell GmbH (Heidelberg, Germany). The human lung adenocarcinoma (A549 and SK-LU-1), large cell carcinoma (NCI-H460 and NCI-H1299), and Calu-6 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were cultured in an RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY, USA). All cultures were maintained in an incubator containing 5% CO₂ at 37°C. Cells were grown in 100 mm plastic cell culture dishes (BD Falcon, Franklin Lakes, NJ, USA) and harvested with trypsin-EDTA (Gibco BRL). HPF cells were used between passages 4 and 5 (indicated as #5).

Reagents

Auranofin was purchased from Sigma-Aldrich and dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich.) as a 10 mM stock solution. NAC and BSO were also obtained from Sigma-Aldrich. NAC was dissolved in a 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.0) buffer, and BSO was dissolved in distilled water. Based on previous studies, cells were pretreated with 2 mM NAC or 10 µM BSO at 37°C for 1 h followed by treatment with Auranofin at 37°C for 24 h before assays were performed. DMSO (0.2%) was used as a control vehicle, and it did not affect cell growth or death. All stock solutions were wrapped in foil and kept at 4°C or – 20°C.

Cell proliferation assay

Viable and dead cell numbers were determined using the trypan blue staining method. In brief, 1 × 10⁶ cells per well were seeded into 60 mm culture dishes (BD Falcon) for cell counting. After exposure to the

indicated concentrations of Auranofin for 24 h, the cells then underwent trypan blue cell counting. For all experimental conditions, three replicates were used, and the experiment was performed at least twice.

Determination of intracellular ROS and $O_2^{\cdot-}$ levels

Intracellular ROS, such as H_2O_2 , $\cdot OH$, and $ONOO\cdot$, were measured using 2',7'-dichlorodihydrofluorescein diacetate dye [H_2DCFDA , Excitation/Emission (Ex/Em) = 495 nm/529 nm; Invitrogen Molecular Probes, Eugene, OR, USA]. DCF is poorly sensitive to $O_2^{\cdot-}$. By contrast, dihydroethidium dye (DHE, Ex/Em = 518 nm/605 nm; Invitrogen Molecular Probes) is a fluorogenic probe that is very selective for $O_2^{\cdot-}$ among ROS. In brief, 1×10^6 cells in 60 mm culture dishes (BD Falcon) were pretreated with 2 mM NAC or 10 μM BSO for 1 h and then treated with Auranofin for 24 h. Cells were then washed in phosphate-buffered saline (PBS) and incubated with 20 μM H_2DCFDA or 20 μM DHE at 37°C for 30 min. Mean DCF and DHE fluorescence values were detected using a FACStar flow cytometer (BD Sciences, Franklin Lakes, NJ). Mean DCF and DHE levels are expressed as percentages compared with control cells.

Detection of intracellular GSH

Cellular GSH levels were evaluated using 5-chloromethylfluorescein diacetate dye (CMFDA, Ex/Em = 522 nm/595 nm; Invitrogen Molecular Probes). In brief, 1×10^6 cells in 60 mm culture dishes (BD Falcon) were pretreated with 2 mM NAC or 10 μM BSO for 1 h and then treated with Auranofin for 24 h. Cells were washed with PBS and incubated with 5 μM CMFDA at 37°C for 30 min. CMF fluorescence intensity was determined using a FACStar flow cytometer (BD Sciences). Negative CMF stained (GSH-depleted) cells are expressed as a percentage of (-) CMF cells.

Detection of apoptosis

Apoptosis was identified by staining with annexin V-fluorescein isothiocyanate (FITC, Ex/Em = 488/519 nm; Life Technologies, Carlsbad, CA). Briefly, 1×10^6 cells in 60 mm culture dishes (BD Falcon) were pretreated with 2 mM NAC or 10 μM BSO for 1 h and then treated with Auranofin for 24 h. Cells were washed twice with cold PBS and then resuspended in 200 μL of a binding buffer (10 mM HEPES/NaOH, pH 7.4 and 140 mM NaCl; 2.5 mM $CaCl_2$) at a concentration of 5×10^5 cells/mL at 37°C for 30 min. Annexin V-FITC (2 μL) and propidium iodide (1 $\mu g/mL$) were added to the solution, and cells were then analyzed with a FACStar flow cytometer (BD Sciences).

Measurement of mitochondrial membrane potential (MMP; $\Delta\Psi_m$)

MMP ($\Delta\Psi_m$) was assessed using the fluorescent dye rhodamine 123 (Ex/Em = 485 nm/535 nm; Sigma-Aldrich), which is a cell-permeable cationic dye that preferentially enters into mitochondria because of their typical highly negative MMP ($\Delta\Psi_m$). Depolarization of MMP ($\Delta\Psi_m$) results in the loss of rhodamine 123 from mitochondria and reduces the dye's intracellular fluorescence intensity. In brief, 1×10^6 cells in 60 mm culture dishes (BD Falcon) were incubated with the designated doses of Auranofin for 24 h. Cells were washed twice with PBS and incubated with rhodamine 123 (0.1 mg/mL) at a concentration of 5×10^5 cells/mL at 37°C for 30 min. Rhodamine 123 staining intensities were determined by a FACStar flow cytometer (BD Sciences). The absence of rhodamine 123 from cells indicates a loss of MMP ($\Delta\Psi_m$).

Western blotting

Protein expression levels were evaluated by western blotting. Briefly, 1×10^6 cells in 60 mm culture dishes (BD Falcon) were pretreated with 2 mM NAC or 10 μ M BSO for 1 h and then treated with the indicated concentrations of Auranofin for 24 h. Cells were washed with PBS, and four volumes of a lysis buffer were added (Intron Biotechnology, Seongnam, Gyeonggi-do, Korea). Lysates were centrifuged at $13,500 \times g$ for 20 min at 4°C, and the protein concentration of supernatants was determined using the Bradford method using an assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples (30 μ g) were resolved by 8–15% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) by electroblotting. The membranes were probed with a primary poly (ADP ribose) polymerase (PARP), cleaved PARP, pro-caspase-3, or cleaved-caspase-3 antibody (Cell Signaling Technology, Danvers, MA, USA) at 1:1,000 dilution in 5% nonfat milk overnight at 4°C, and the membranes were then incubated with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:5,000 dilution for 2 h at room temperature. Finally, to assess comparable amounts of proteins in each lane, the membranes were stripped to detect GAPDH (Santa Cruz Biotechnology). Protein signals were developed using an EZ-Western Lumi Pico ECL solution kit (DoGen, Seoul, Korea) and imaged on an Amersham™ Imager 600 (GE Healthcare, Chicago, IL, USA).

TrxR activity assay

TrxR activity was determined using a fluorescent thioredoxin reductase colorimetric assay kit (Cayman Chemical, Ann Arbor, MI, USA). Briefly, 1×10^6 cells in 60 mm culture dishes (BD Falcon) were pretreated with 2 mM NAC or 10 μ M BSO for 1 h and then treated with the indicated concentrations of Auranofin for 24 h. Cells were homogenized in a cold radioimmunoprecipitation assay (RIPA) buffer (CureBio, Seoul, Korea) and centrifuged at $13,500 \times g$ for 20 min at 4°C. The supernatant was collected after centrifugation, and protein concentrations were then determined using the Bradford method with a Bio-Rad assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of total protein (30 μ g) were added to each well in a Nunc 96-well plate (Thermo Fisher Scientific, Waltham, MA, USA) with a master mix containing 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and NADPH. The fluorescence intensity of each

well was measured at 410 nm using a Synergy™ 2 spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). TrxR activity was calculated using a formula provided by the protocol.

Statistical analysis

The results are reported as the mean of at least two or three independent experiments (mean $\pm$ SD). Data were analyzed using InStat (GraphPad, San Diego, CA, USA). The Student's t-test or one-way analysis of variance with a post-hoc analysis using Tukey's multiple comparison test was used for the parametric data. Statistical significance was defined as $p < 0.05$.

Results

Effects of Auranofin on cell growth and death in lung cancer cells

The effect of Auranofin on the growth of cells from a normal lung cell line (HPF) and lung cancer cell lines (Calu-6, A549, SK-LU-1, NCI-H460, and NCI-H1299) was examined by counting the number of trypan blue positive- and negative-stained cells. Treatment with various concentrations of Auranofin for 24 h significantly reduced the population of viable cells and increased the number of dead cells in a dose-dependent manner (Fig. 1). The number of live (trypan blue negative-stained) cells was significantly decreased by Auranofin with a concentration of 3–5 μ M in HPF (#5) and Calu-6 cells (Figs. 1A and 1B), 4–5 μ M in A549 and SK-LU-1 cells (Figs. 1C and 1D), 2–5 μ M in NCI-H460 cells (Fig. 1E), and 0.5–3 μ M in NCI-H1299 cells (Fig. 1F). Correspondingly, the population of dead (trypan blue positive-stained) cells was statistically increased by Auranofin ranging from a concentration of 3–5 μ M in both HPF (#5) and NCI-H460 cells (Figs. 1A and 1E), 2–5 μ M in Calu-6 cells (Fig. 1B), 5 μ M only in A549 cells (Fig. 1C), 4–5 μ M in SK-LU-1 cells (Fig. 1D), and 1–3 μ M in NCI-H1299 cells (Fig. 1F). These results indicate that normal lung cells and cells from different lung cancer cell lines have differential sensitivity to Auranofin.

Effects of Auranofin on intracellular ROS levels in lung cancer cells

To assess intracellular ROS levels in Auranofin-treated normal lung cells and cells from lung cancer cell lines at 24 h, two fluorescent probe dyes (H_2DCFDA and DHE) were used to detect non-specific ROS and $O_2^{\cdot-}$ levels, respectively. We found that Auranofin affected ROS (DCF) and $O_2^{\cdot-}$ (DHE) levels depending on the treatment dose and cell type (Figs. 2 and 3). Among the tested concentrations of Auranofin, treatment with 4 μ M significantly increased the ROS level in HPF cells (#5), but 5 μ M Auranofin did not (Fig. 2A), whereas treatment with 3 μ M Auranofin led to a maximum ROS level in Calu-6 cells, yet 4–5 μ M Auranofin decreased the ROS level (Fig. 2B). In A549 and NCI-H460 cells, we found that all tested concentrations of Auranofin increased ROS levels, but these levels were not raised in a dose-dependent manner (Fig. 2C and 2E). ROS levels in 4 μ M Auranofin-treated A549 and 3 μ M Auranofin-treated NCI-H460 cells were the highest (Figs. 2C and 2D). Treatment with 4 μ M Auranofin led to a maximum level of ROS in SK-LU-1 cells, whereas 5 μ M Auranofin did not affect the ROS level (Fig. 2D). In NCI-H1299 cells, all tested concentrations of Auranofin generally decreased ROS levels, and the levels of 2–3 μ M Auranofin-treated cells were remarkably reduced compared to untreated cells (Fig. 2F). The level of red

fluorescence derived from DHE, which reflects $O_2^{\bullet-}$ accumulation, gradually increased in Auranofin-treated HPF (#5), Calu-6, A549, and NCI-H460 cells (Figs. 3A–3C and 3E). Significant increases in $O_2^{\bullet-}$ levels were observed in 5 μ M Auranofin-treated HPF cells (#5), 3–5 μ M Auranofin-treated Calu-6 cells, and 4–5 μ M Auranofin-treated A549 cells (Figs. 3A–3C). Among the tested concentrations, we found that 5 μ M Auranofin led to the highest level of $O_2^{\bullet-}$ in NCI-H460 cells, but it was not significant (Fig. 3E). Treatment with 1 μ M Auranofin indicated the highest $O_2^{\bullet-}$ level increase in SK-LU-1 and NCI-H1299 cells, but > 1 μ M Auranofin did not affect $O_2^{\bullet-}$ levels in cells from either cell line (Figs. 3D and 3F). Furthermore, 3 μ M Auranofin reduced the $O_2^{\bullet-}$ level in NCI-H1299 cells compared to that in control cells (Fig. 3F).

Effects of Auranofin on intracellular GSH depletion in lung cancer cells

Changes of intracellular GSH levels were analyzed using the fluorescent dye CMF. All tested doses of Auranofin increased the number of GSH-depleted cells in normal lung cells and cells from lung cancer cell lines compared with each group of control cells at 24 h. A dramatic increase in GSH-depleted cell number was observed in > 3 μ M Auranofin-treated HPF (#5) cells (Fig. 4A). Further, treatment with 4–5 μ M Auranofin significantly increased the percentage of GSH-depleted cells in Calu-6, A549, SK-LU-1, and NCI-H460 cells compared with that in control cells (Figs. 4B–4E). In NCI-H1299 cells, we found that relatively lower concentrations of 0.5–3 μ M Auranofin steadily elevated the number of GSH-depleted cells (Fig. 4F).

Effects of NAC and BSO on cell death, MMP ($\Delta\Psi_m$), the ROS level, and GSH depletion in Auranofin-treated Calu-6 and A549 cells

Because Auranofin is an inhibitor of TrxR that affects cellular redox status, we examined the effects of NAC (a well-known antioxidant) or BSO (an inhibitor of GSH synthesis) on cell death of Auranofin-treated Calu-6 and A549 cells. For this experiment, 3 μ M or 5 μ M Auranofin was used as a suitable concentration for Calu-6 or A549 cells, respectively. Treatment of Auranofin significantly increased the number of annexin V-FITC-positive cells at 24 h in Calu-6 and A549 cells. Further, NAC slightly prevented cell death, but BSO dramatically increased annexin V-FITC-positive cell numbers in cells from these two cell lines (Figs. 5A and 5E). Since cell death is closely associated with the collapse of MMP ($\Delta\Psi_m$) interruption, MMP ($\Delta\Psi_m$) in cells treated with Auranofin, NAC, and BSO was evaluated at 24 h using rhodamine 123. Treatment with 3 μ M and 5 μ M Auranofin significantly induced the loss of MMP ($\Delta\Psi_m$) in Calu-6 and A549 cells, respectively. NAC prevented the loss of MMP ($\Delta\Psi_m$) caused by Auranofin, whereas BSO significantly intensified this loss in these cells (Fig. 5B and 5F). Next, we investigated whether ROS levels in Auranofin-treated Calu-6 and A549 cells were changed by NAC or BSO. As expected, Auranofin significantly increased the level of $O_2^{\bullet-}$ in Calu-6 and A549 cells at 24 h, and the level of $O_2^{\bullet-}$ in Auranofin-treated Calu-6 and A549 cells was decreased by NAC (Fig. 5C and 5G). BSO dramatically augmented the increased $O_2^{\bullet-}$ level in Auranofin-treated Calu-6 cells (Fig. 5C), but it failed to enhance the level of $O_2^{\bullet-}$ in Auranofin-treated A549 cells (Fig. 5G). Regarding GSH depletion, Auranofin treatment alone led to an increase in the number of GSH-depleted Calu-6 and A549 cells. Treatment with NAC reduced the number

of GSH-depleted cells induced by Auranofin in Calu-6 and A549 cells, whereas BSO intensified the GSH depletion caused by Auranofin in these cells (Figs. 5D and 5H).

Effects of NAC and BSO on apoptosis-related protein levels in Calu-6 and A549 cells

To investigate the relationship between ROS accumulation and cell apoptosis, PARP degradation and cleavage of caspase-3 as apoptotic markers were measured by western blot analysis in Auranofin-treated Calu-6 and A549 cells under different redox states using NAC or BSO. As predicted, compared with the control group, activated caspase-3 and cleaved PARP were strongly expressed by treatment with Auranofin in Calu-6 and A549 cells. By contrast, the expression levels of caspase-3 and PARP were notably reduced in Auranofin-treated Calu-6 and A549 cells (Figs. 6A–6D). Furthermore, pretreatment with NAC and subsequent treatment with Auranofin increased the expression levels of intact caspase-3 and PARP compared to treatment with Auranofin alone in Calu-6 and A549 cells, whereas the cleavage of caspase-3 and degradation of PARP by Auranofin were prevented in the presence of NAC in these cells (Figs. 6A and 6C). In cells pretreated with BSO and then co-treated with Auranofin, the expression levels of pro-caspase-3 and PARP were further decreased compared to Auranofin treatment in the absence of BSO in Calu-6 and A549 cells (Figs. 6B and 6D). Activated caspase-3 in cells pretreated with BSO and co-treated with Auranofin was detected similarly to the changes observed in Auranofin-treated Calu-6 cells (Fig. 6B). In A549 cells treated with BSO and Auranofin, despite a diminishing level of pro-caspase-3, cleaved active caspase-3 fragments were not detected (Fig. 6D). In addition, cleaved PARP was observed in the combined treatment with BSO and Auranofin compared to controls in Calu-6 and A549 cells (Fig. 6B and 6D). These results suggest that apoptosis is a mechanism of Auranofin-mediated cell death, and ROS accumulation contributes to Auranofin-induced apoptosis in lung cancer cells.

Effects of NAC and BSO on TrxR activity in Auranofin-treated Calu-6 and A549 cells

Inhibition of TrxR activity by Auranofin leads to increased intracellular oxidative stress and induced cell death. As expected, after exposure to Auranofin for 24 h, we found that TrxR activity was dose-dependently decreased with concentrations $\geq$ IC₅₀ in Calu-6 and A549 cells (Figs. 7A and 7B). To further explore changes of TrxR activity in different redox states, we investigated TrxR activity in Auranofin with NAC- or BSO-treated Calu-6 and A549 cells. Low or high (IC₅₀) concentrations of Auranofin were also used to differentiate the alteration levels of TrxR activity in Calu-6 and A549 cells, respectively. Pretreatment of NAC prevented the decrease of TrxR activity by Auranofin in both Calu-6 and A549 cells, whereas BSO enhanced the reduction of TrxR activity by Auranofin in only Calu-6 cells (Figs. 7A and 7B).

Discussion

Gold compounds have long been used in medicine since ancient times, and their use can be dated to over 2,000 years ago (Ott 2009). Among gold-containing drugs, Auranofin is an oral FDA-approved antirheumatic drug introduced in 1985, and it has since been investigated for its potential repurposing to treat various other pathologies (Chaffman et al. 1984; Madeira et al. 2012; Onodera et al. 2019; Ott 2009;

Roder and Thomson 2015). In particular, Auranofin has been demonstrated to have potent antitumor effects in various types of cancer (Gandin et al. 2010; Onodera et al. 2019), and it is currently in clinical trials for the treatment of leukemia (Weir et al. 2012). Numerous studies have indicated that Auranofin inhibits TrxR activity and increases the intracellular ROS level (Kim et al. 2017; Onodera et al. 2019; You and Park 2016). As Auranofin has high affinity for protein thiol and selenol groups, it presumably binds to the active sites of TrxR, thereby inhibiting its activity (Di Sarra et al. 2013). Consequently, Auranofin-induced inhibition of TrxR leads to ROS accumulation and induces apoptosis and cell death. Based on our previously published reports, Auranofin inhibits cell growth and induces apoptosis in lung cancer cells (Cui et al. 2020). In this further study, we evaluated the antitumor effects of Auranofin on lung cancer cells in relation to the levels of ROS in more detail.

According to our results, Auranofin diminished cell proliferation and induced cell death in HPF normal lung cells and cells from five NSCLC cell lines: Calu-6, A549, SK-LU-1, NCI-H460, and NCI-H1299, in a dose-dependent manner. The susceptibility to Auranofin with an IC_{50} of 4–5 μM at 24 h in A549 (LUAD) and SK-LU-1 (LUAD) cells was lower than that found in Calu-6, NCI-H460 (LULC), and NCI-H1299 (LULC) cells. Although NCI-H460 and NCI-H1299 are both large cell carcinoma cell lines, NCI-H1299 cells were more sensitive to Auranofin with IC_{50} of 1–2 μM . This agent also inhibited normal HPF lung cell growth. However, Auranofin susceptibility of A549 and SK-LU-1 cells was higher than that of normal lung cells. This finding implies that Auranofin can be an effective antitumor agent for lung cancer, but before using it in a clinical trial, we must first consider the differential susceptibility of Auranofin depending on the type of lung cancer cell.

Intracellular ROS overproduction or antioxidant imbalance can lead to oxidative stress that finally damages cells (Ferrer et al. 2010; Uttara et al. 2009). The GSH and Trx systems are two important ROS scavenging pathways in cells to regulate redox homeostasis (Ortega et al. 2011; Powis et al. 2000). The Trx system is regarded as an antitumor target, and TrxR, which is a key component of the Trx system, is an important modulator of tumor development (Arner and Holmgren 2000; Jia et al. 2019; Lu and Holmgren 2014). Therefore, the inhibition of TrxR can induce cell death by causing oxidative stress. In our results, Auranofin increased ROS (DCF) levels including $O_2^{\bullet -}$ (DHE) levels at 24 h in normal HPF lung cells and in cells from five lung cancer cell lines. However, the alteration patterns of ROS and $O_2^{\bullet -}$ levels varied depending on the incubation concentration of Auranofin and cell type. In relatively high doses of Auranofin, ROS (DCF) levels such as H_2O_2 were decreased in all tested cells, and $O_2^{\bullet -}$ (DHE) levels were reduced in SK-LU-1 and NCI-H1299 cells. These patterns were also observed in A549 cells pretreated with BSO and co-treated with Auranofin. Because a high dose of Auranofin-induced cell death and MMP ($\Delta\Psi_m$) loss in lung cancer cells, it is possible that accumulated H_2O_2 conspicuously generates $O_2^{\bullet -}$ by mitochondrial damage, and H_2O_2 and $O_2^{\bullet -}$ are efficiently converted into toxic $\cdot OH$ through the Fenton reaction (Winterbourn 1995) to kill lung cancer cells. In addition, NAC, an antioxidant, attenuated Auranofin-induced MMP ($\Delta\Psi_m$) loss and $O_2^{\bullet -}$ levels in Calu-6 and A549 cells, whereas BSO, an inhibitor

of GSH synthesis, intensified Auranofin-mediated MMP ($\Delta\Psi_m$) loss in these cells but enhanced $O_2^{\cdot-}$ levels in only Auranofin-treated Calu-6 cells.

The GSH system utilizes NADPH as an electron donor like the Trx system. GSH is a non-protein antioxidant and prevents cells from damage caused by oxidative stress (Hayes and McLellan 1999; Ortega et al. 2011). The thiol group of cysteine in GSH supplies an electron to unstable molecules, and GSH itself is then oxidized. When GSH is converted into its oxidized form, it is reduced back by GSH reductase (Dunning et al. 2013; Iversen et al. 2010; Kumar et al. 2011). GSH is involved in many biological processes including cell survival, and its depletion augments cellular susceptibility to apoptosis (Franco et al. 2012; Ortega et al. 2011). Importantly, we found that Auranofin dose-dependently increased the number of GSH-depleted cells in normal HPF lung cells and cells from five lung cancer cell lines. Furthermore, NAC ameliorated Auranofin-provoked apoptosis and GSH depletion in Calu-6 and A549 cells. Thus, NAC plays a role as a precursor of GSH and an antioxidant in lung cancer cells, whereas BSO significantly accelerates an increase in apoptosis and GSH depletion in Auranofin-treated Calu-6 and A549 cells. These results demonstrate that an inhibition of GSH effectively enhances cell death in Auranofin-resistant lung cancer cells.

Apoptosis is controlled by extrinsic and intrinsic pathways, which are also known as the death receptor and mitochondrial pathways, respectively (Elmore 2007). A critical process in apoptosis is caspase activation. Caspases are a family of cysteine proteases that are divided into initiator caspases (caspase-2, -8, -9, and -10) and executioner caspases (caspase-3, -6, and -7) (Budihardjo et al. 1999; Elmore 2007; Fan et al. 2005). Caspase-3 activation is a crucial event in both pathways of apoptosis that leads to the downstream cleavage of various cytoplasmic or nuclear substrates including PARP, which causes degradation of the cytoskeleton and DNA fragmentation, ultimately resulting in apoptosis (Brauns et al. 2005; Choudhary et al. 2015; Cohen 1997). Similarly, in the present study, we found that caspase-3 activation and PARP degradation were induced by Auranofin in Calu-6 and A549 cells. Pretreatment with a ROS scavenger restored these changes in Calu-6 and A549 cells, whereas pretreatment with BSO intensified the decrease of pro-caspase-3 and PARP. These results indicate that Auranofin-mediated apoptosis may be a consequence of ROS-dependent damage.

Trx and TrxR are overexpressed in many cancer cells including lung cancer, and high levels of these proteins are potentially linked to cancer proliferation and chemotherapeutic resistance (Kim et al. 2005; Noike et al. 2008; Soini et al. 2001). For instance, it has been reported that the inhibition of Trx and TrxR increases the sensitivity of cancer cells to radiotherapy and anticancer agents in various cancer cells (Javvadi et al. 2010; Liang et al. 2014; Yokomizo et al. 1995; Zhang et al. 2017). Therefore, regulation of the Trx system can be a prime target for cancer therapy. Our previous results indicated that Auranofin dose-dependently reduced the activity of TrxR in Calu-6 and A549 cells, and, moreover, NAC prevented the observed decrease of TrxR activity by Auranofin in Calu-6 and A549 cells. By contrast, BSO bolstered the decrease in TrxR activity by Auranofin in Calu-6 cells but did not affect TrxR activity in Auranofin-treated A549 cells. These results also suggest that the TrxR inhibitor Auranofin may be an effective antitumor drug for patients with lung cancer.

In the present study, we mainly focused on the potential of Auranofin as an anticancer drug in lung cancer, and conclude that Auranofin-induced growth inhibition and death of lung cancer cells, which were accompanied by increasing ROS levels and GSH depletion. In addition, the changes of ROS and GSH levels by NAC or BSO affected cell growth inhibition and death in Auranofin-treated lung cancer cells. Our present findings provide useful information toward better understanding of the antitumor effects of Auranofin in lung cancer cells in relation to ROS and GSH levels.

Abbreviations

NSCLC, non-small-cell lung cancer; SCLC, small-cell lung cancer; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; LULC, lung large cell carcinoma; ROS, reactive oxygen species; GSH, glutathione; Trx, thioredoxin; TrxR, thioredoxin reductase; PARP, poly (ADP-ribose) polymerase; MMP, mitochondrial membrane potential ($\Delta\Psi_m$); FITC, fluorescein isothiocyanate; H₂DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; DHE, dihydroethidium; CMFDA, 5-chloromethylfluorescein diacetate; NAC, N-acetyl cysteine; BSO, L-buthionine sulfoximine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide; PVDF, polyvinylidene difluoride; NADPH, nicotinamide adenine dinucleotide phosphate; redox, reduction-oxidation; FDA, Food and Drug Administration; HPF, human pulmonary fibroblast; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; Ex/Em, Excitation/Emission; PBS, phosphate-buffered saline; RIPA, radioimmunoprecipitation assay; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid)

Declarations

Compliance with ethical standards

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Conflicts of interest

The authors do not have any conflicts of interest to declare.

Availability of data and materials

Data collected during the present study are available from the corresponding author upon reasonable request.

Ethics declarations

The material in this paper has not been published and is not under active consideration by another journal. The research was conducted in accordance with the declaration of Helsinki.

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Figures

Figure 1.

Figure 1

Effects of Auranofin on cell proliferation in lung cancer cells. Exponentially growing cells were incubated in the presence of the indicated concentrations of Auranofin for 24 h. Live and dead cell numbers were assessed using trypan blue staining cell counting. Graphs illustrate numbers of live and dead cells in normal HPF cells (A) and cells from the lung cancer cell lines: Calu-6 (B), A549 (C), SK-LU-1 (D), NCI-H460 (E), and NCI-H1299 (F). * p < 0.05 compared with Auranofin-untreated control cells.

Figure 2.

Figure 2

Effects of Auranofin on intracellular DCF (ROS) levels in lung cancer cells. Exponentially growing cells were incubated in the presence of the designated concentrations of Auranofin for 24 h. DCF (ROS) levels in cells were measured using a FACStar flow cytometer. Graphs illustrate DCF (ROS) levels (%) in normal HPF cells (A) and cells from the lung cancer cell lines: Calu-6 (B), A549 (C), SK-LU-1 (D), NCI-H460 (E), and NCI-H1299 (F), compared with each group of control cells. * $p < 0.05$ compared with the Auranofin-untreated control group.

Figure 3.

Figure 3

Effects of Auranofin on intracellular DHE (O₂•-) levels in lung cancer cells. Exponentially growing cells were incubated in the presence of the indicated concentrations of Auranofin for 24 h. DHE (O₂•-) levels in cells were measured using a FACStar flow cytometer. Graphs illustrate DHE (O₂•-) levels (%) in normal HPF cells (A) and cells from the lung cancer cell lines: Calu-6 (B), A549 (C), SK-LU-1 (D), NCI-H460 (E), and NCI-H1299 (F), compared with each group of control cells. * p < 0.05 compared with the Auranofin-untreated control group.

Figure 4.

Figure 4

Effects of Auranofin on intracellular GSH depletion in lung cancer cells. Exponentially growing cells were incubated in the presence of the indicated concentrations of Auranofin for 24 h. Intracellular GSH levels in cells were assessed using a FACStar flow cytometer. Graphs designate percentages of negative CMF (GSH-depleted) cells in normal HPF cells (A) and cells from the lung cancer cell lines: Calu-6 (B), A549 (C), SK-LU-1 (D), NCI-H460 (E), and NCI-H1299 (F). * $p < 0.05$ compared with the Auranofin-untreated control group.

Figure 5.

Figure 5

Effects of NAC and BSO on cell death, MMP ($\Delta\Psi_m$), DHE ($O_2^{\bullet-}$) levels, and GSH depletion in Auranofin-treated Calu-6 and A549 cells. Exponentially growing Calu-6 cells or A549 cells were incubated in the presence of the indicated concentrations of Auranofin for 24 h following 1 h preincubation of 2 mM NAC or 10 μ M BSO. Graphs illustrate the percentage of annexin V-FITC-stained cells (A and E) and rhodamine 123 negative MMP ($\Delta\Psi_m$) cells (B and F) in Calu-6 cells and A549 cells, respectively. Graphs indicate DHE ($O_2^{\bullet-}$) levels (%) compared with control cells in Calu-6 (C) and A549 (G) cells. Graphs designate the percentages of negative CMF (GSH-depleted) cells in Calu-6 (D) and A549 (H) cells. * $p < 0.05$ compared with Auranofin-untreated control cells. # $p < 0.05$ compared with cells treated with Auranofin only.

Figure 6.

Figure 6

Effects of NAC and BSO on apoptosis-related protein levels in Auranofin-treated Calu-6 and A549 cells. Exponentially growing Calu-6 cells or A549 cells were incubated in the presence of the indicated concentrations of Auranofin for 24 h following 1 h preincubation of 2 mM NAC or 10 μ M BSO. Whole-cell extracts were prepared, and equal amounts of lysates were then resolved through SDS-PAGE, transferred onto PVDF membranes, and immunoblotted with indicated antibodies against PARP, cleaved PARP, pro-caspase-3, cleaved-caspase-3, and GAPDH (A, B, C, and D, respectively). GAPDH detection was used to confirm equal protein loading.

Figure 7.

Figure 7

Effects of NAC and BSO on TrxR activity in Auranofin-treated Calu-6 and A549 cells. Exponentially growing Calu-6 cells or A549 cells were incubated in the presence of the indicated concentrations of Auranofin for 24 h following 1 h preincubation of 2 mM NAC or 10 μM BSO. TrxR activity was measured using the insulin reduction assay. The graphs indicate inhibition of TrxR activity by Auranofin or combination treatment with NAC or BSO in Calu-6 (A and B) and A549 (C and D) cells. * $p < 0.05$ compared with Auranofin-untreated control cells.