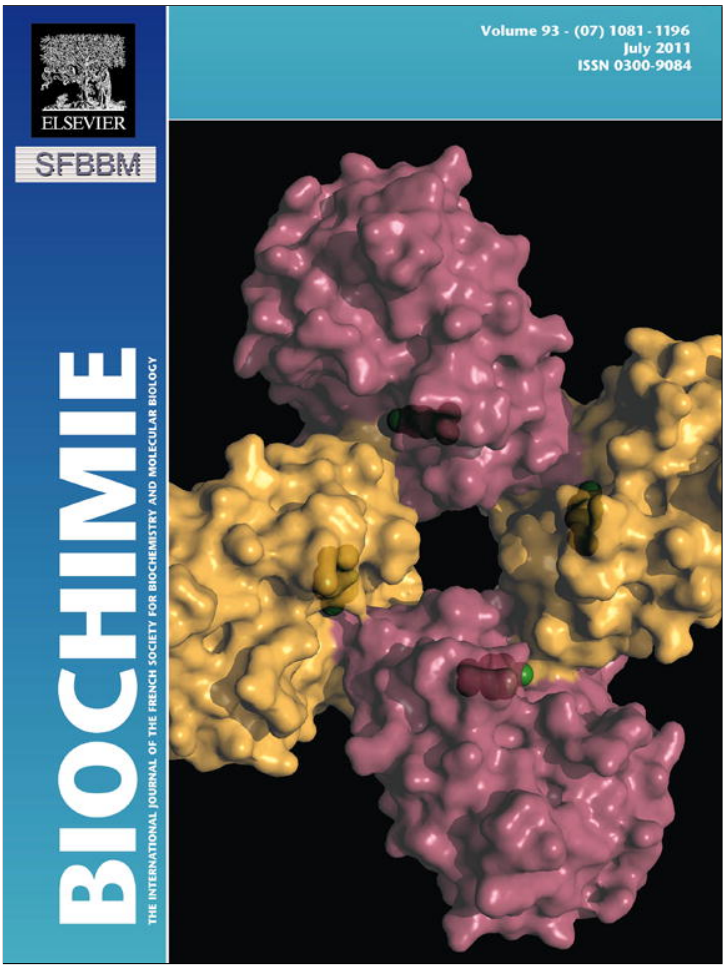

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Research paper

Selective toxicity of glycyrrhetinic acid against tumorigenic *r*/*m* HM-SFME-1 cells is potentially attributed to downregulation of glutathione

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ABSTRACT

Natural products from plants are expected to play significant roles in creating new, safe and improved chemopreventive and therapeutic antitumor agents. Selectivity is also an important issue in cancer prevention and therapy. The present study was designed to extend our previous study on the c-Ha-*ras* and c-*myc*-induced tumor cell-selective antiproliferative effects of a licorice component, glycyrrhetinic acid (GA). An *in silico* ligand-receptor docking simulation revealed that GA acts as an 11β-hydroxysteroid dehydrogenase type 2 inhibitor. GA disrupted the redox balance in tumor cells through upregulation of reactive oxygen species and downregulation of glutathione (GSH). The GA-induced GSH reduction and cytotoxicity were enhanced by an inhibitor of GSH, L-buthionine-[*S*,*R*]-sulfoximine. N-acetyl-L-cysteine, an antioxidant and precursor of GSH, restored the GA-induced GSH reduction and cytotoxicity in tumor cells. Taken together, these data highlighting the downregulation of GSH by GA and the efficacy of GSH in ameliorating GA-mediated cytotoxicity support the notion that GSH is involved in the selective toxicity of GA toward tumor cells.

1. Introduction

In antitumor therapy, there are some clinically available agents to help alleviate aggressive and resistant cancer burdens. Adriamycin is a potent and broad-spectrum antitumor agent used in the treatment of a variety of cancers, including leukemia, lymphoma, breast cancer, lung cancer and ovarian cancer [1]. The mechanism underlying the antitumor activity of adriamycin involves inhibition of topoisomerase II and DNA synthesis [2]. Manumycin A (MA) is a potent farnesyltransferase inhibitor that was originally identified as an effective tumoricidal agent against several cancers, such as myeloma [3], pancreatic tumors [4], hepatocellular carcinoma [5] and thyroid carcinoma [6]. Although these clinically available antitumor agents can be very effective, their selectivity against tumor cells can be very poor in some cases, which leads to damage to normal cells and serious side effects. Their antitumor effects can also vary among cells, and their effects and selectivity against central nervous system (CNS) tumorigenic cells still need to be explored. Consequently, the development of more effective and safer agents has recently been required for chemoprevention and therapy of cancers. Natural products from plants and their synthetic derivatives are expected to play important roles in creating new and improved antitumor agents [7]. Consistent with this notion, several natural compounds from plants, such as the green tea polyphenol epigallocatechin gallate [8] and apple ursolic acid (UA) [9], are being studied as antitumor agents. A licorice component, glycyrrhetinic acid (GA), has also been reported to have tumor cell-selective toxicity against CNS tumorigenic progenitor cells [10–12].

Serum-free mouse embryo (SFME) cells, which were established by Loo et al. [13], were originally derived from a 16-day-old whole Balb/c mouse embryo, and are maintained in a serum-free culture medium. These cells do not undergo growth crisis, maintain their diploid karyotype for extended passages and are non-tumorigenic *in vivo*. Consequently, they are non-transformed, behave as primary cultures, have a finite lifespan and display the characteristics of CNS progenitor cells [14,15]. SFME cells have been cotransfected with the human c-Ha-*ras* and mouse c-*myc* genes, and the resulting cells were designated *ras*/*myc* SFME cells [16]. Although SFME cells are non-tumorigenic *in vivo* and require epidermal growth factor (EGF) for their survival, growth and

Abbreviations: ASE-Dock, alpha sphere and excluded volume-based ligand-protein docking; BSO, L-buthionine-[*S*,*R*]-sulfoximine; CNS, central nervous system; DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorodihydrofluorescein-diacetate; EGF, epidermal growth factor; GA, glycyrrhetinic acid; GSH, glutathione; LBS, ligand-binding site; MA, manumycin A; NAC, N-acetyl-L-cysteine; *r*/*m* HM-SFME-1, human c-Ha-*ras* and mouse c-*myc*-cotransfected highly metastatic serum-free mouse embryo-1; ROS, reactive oxygen species; SFME, serum-free mouse embryo; UA, ursolic acid; 11βHSD2, 11β-hydroxysteroid dehydrogenase type 2.

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proliferation [14,15], *ras*/*myc* SFME cells are tumorigenic and do not require any growth factors such as EGF [16]. Another line of SFME-derived tumorigenic cells are human c-Ha-*ras* and mouse c-*myc*-cotransfected highly metastatic SFME-1 (*r*/*m* HM-SFME-1) cells, which were established by selecting *ras*/*myc* SFME cells that only metastasize to the lungs of Balb/c mice [17]. Elucidation of the characteristics and behaviors of normal and tumorigenic SFME cells could be of great importance in the field of medicinal plant studies for cancer prevention and therapy, because simple comparisons of these cells may contribute to our understanding of the behavioral differences between normal cells and tumor cells in the CNS in their responses to antitumor agents.

11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) has been reported to be expressed in various tumors and is associated with colonic [18] and pituitary [19] adenomas, and breast [20,21] and colorectal [22] cancers. Our previous study revealed that GA, an 11βHSD2 inhibitor, showed selective toxicity toward CNS-derived tumor cells [11]. Meanwhile, apoptosis induction in astrocytoma cells has been ascribed to reactive oxygen species (ROS) accumulation in response to acidic triterpenoids [7] and tumor cells with intrinsic oxidative stress can be preferentially affected through ROS-mediated mechanisms [23]. In the present study, normal SFME cells and tumorigenic *r*/*m* HM-SFME-1 cells were treated with GA and the clinically available antitumor agents adriamycin and MA, and the levels of ROS and glutathione (GSH) were measured for evaluation of the redox balance. Based on the data, a possible mechanism involving GSH that underlies the survival of normal cells and the death of tumor cells following exposure to GA is discussed. To the best of our knowledge, there are no previous reports suggesting the involvement of GSH regulation in the selective toxicity of GA toward tumor cells.

2. Materials and methods

2.1. Reagents

Adriamycin was obtained from Kyowa Hakko Industry Co. Ltd. (Tokyo, Japan). GA and MA were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). L-buthionine-[*S*,*R*]-sulfoximine (BSO) and N-acetyl-L-cysteine (NAC) were from Sigma–Aldrich Chemical Co. (St. Louis, MO).

2.2. Cell lines and cell culture

SFME cells were a gift from Dr. S. Shirahata (Kyushu University, Fukuoka, Japan) and *r*/*m* HM-SFME-1 cells were taken from our cell stocks [17]. The basal nutrient medium was a 1:1 mixture of DME and nutrient mixture F-12 Ham (DME/F-12) [24,25] containing 15 mM HEPES (pH 7.4), 1.2 g/l sodium bicarbonate, 10 nM sodium selenite and 10 μg/ml gentamicin, supplemented with insulin (10 μg/ml), transferrin (25 μg/ml) and EGF (50 ng/ml). Cell passages were accomplished by rapid trypsinization with 0.2% crude trypsin and 1 mM ethylenediaminetetraacetate in phosphate-buffered saline without calcium or magnesium, followed by dilution in the culture medium at room temperature. The medium containing the collected cells was centrifuged at 250 × *g* at 4 °C for 7 min and the supernatant was removed. The cells were suspended in the culture medium without the supplements, plated at 1×10^5 cells/dish and cultured again in the medium with the supplements. The cells were maintained in DME/F-12 supplemented with insulin, transferrin and EGF in 60-mm diameter dishes precoated with bovine fibronectin (Biomedical Technologies, Cambridge, MA) in a humidified atmosphere containing 20% O_2 and 5% CO_2 at 37 °C.

2.3. Measurement of cell proliferation

SFME and *r*/*m* HM-SFME-1 cells cultured continuously in the serum-free medium were detached from stock dishes (the stock SFME and *r*/*m* HM-SFME-1 cultures had undergone 60 to 120 population doublings) by trypsinization. The obtained cells were diluted, centrifuged, resuspended, plated at 1×10^4 cells/well in 96-well microplates and cultured in the culture medium. After reaching half-confluency at 48 h, the cells were treated with various concentrations of adriamycin (0.1–100 μM), MA (0.625–5 μM) or GA (2.5–20 μM). The cells were also treated with 0.5 mM BSO and 50 mM NAC. For the BSO and NAC experiments, the agents were added to the medium at 8 h and 1 h prior to GA exposure, respectively. After culture for a further 24 h, the cell numbers were determined by a standard colorimetric MTT assay utilizing the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [26].

2.4. Assay for ROS and GSH

2′,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA) can be deacylated to the nonfluorescent compound DCFH within cells and oxidized to the fluorescent compound 2′,7′-dichlorofluorescein (DCF) by a variety of ROS [27]. At half-confluency, the cells were treated with adriamycin, MA or GA at 0–2× the IC_{50} values for cell proliferation, and ROS production was measured using an OxiSelect™ ROS Assay Kit (Cell Biolabs Inc., San Diego, CA) every 10 min for 1 h. The IC_{50} values for SFME cell proliferation were 0.8 μM for adriamycin, 4.5 μM for MA and 18 μM for GA, while those for *r*/*m* HM-SFME-1 cell proliferation were 64.0 μM for adriamycin, 2.4 μM for MA and 7.3 μM for GA. DCF fluorescence was measured with 480-nm excitation and 530-nm emission. For cellular GSH analysis, the normal and tumor cells were treated with adriamycin, MA or GA in a range of 0–2× the IC_{50} values for cell proliferation at half-confluency and incubated for 8 or 24 h. The cells were also treated with 0.5 mM BSO and 50 mM NAC. For the BSO and NAC experiments, the agents were added to the medium at 8 h and 1 h prior to GA exposure, respectively. GSH production was analyzed using a Glutathione Assay Kit (BioVision, Mountain View, CA). GSH reacts with *o*-phthalaldehyde and the generated fluorescence was measured with 340-nm excitation and 420-nm emission. Each value was expressed as picomoles (pM)/10^3 cells.

2.5. Alpha sphere and excluded volume-based ligand-protein docking (ASE-Dock)

Since no molecular model for 11βHSD2 was available, 11βHSD1 (PDB code: 3HFG) was selected as a template for the 3D structure modeling of 11βHSD2 (NCBI reference sequence: NM_008289.2) because of its good crystal structure resolution (2.3 Å) and because its information was the most recent (from 2009) among the reported 11βHSD1 models. For construction of the 11βHSD2 model, 100 independent models of the target proteins were built using a Boltzmann-weighted randomized modeling procedure in the Molecular Operating Environment 2009.10 (MOE; Chemical Computing Group Inc., Montreal, Canada), which was adapted from reports by Levitt [28] and Fechteler et al. [29]. The intermediate models were evaluated using a residue packing quality function that was sensitive to the degrees to which the non-polar side-chain groups were buried and the hydrogen-bonding opportunities were satisfied. The 11βHSD2 model with the best packing quality function and full energy minimization was selected for further analyses. The secondary structures of the 11βHSD2 model exhibited a central 6-stranded all-parallel β-sheet sandwich-like structure, flanked on both sides by 3-helices, which are in agreement with the 11βHSD1

model. Hydrophobic or hydrophilic alpha spheres, which were created by the Site Finder module of the MOE, were utilized to define potential ligand-binding sites (LBSs).

Analyses of the ligand-protein interactions between the ligands (corticosterone: PubChem CID 5753; adriamycin: PubChem CID 31703; GA: PubChem CID 10114; and MA: PubChem CID 5387246) and the 11βHSD2 model were performed with ASE-Dock in the MOE [30]. In the ASE-Dock module, ligand atoms have alpha spheres within 1 Å. Based on this property, concave models were created and ligand atoms from a large number of conformations generated by superimposition with these points were evaluated and scored by the maximum overlap with the alpha spheres and the minimum overlap with the receptor atoms. The scoring function used by ASE-Dock is based on ligand-protein interaction energies and the score is expressed as a U_{total} value. The ligand conformations were subjected to energy minimization using the MMF94S force field [31], and 500 conformations were generated using the default systematic search parameters. Five thousand poses per conformation were randomly placed onto the alpha spheres located within the LBS in 11βHSD2. From the resulting 500,000 poses, the 200 poses with the lowest U_{total} values were selected for further optimization with the MMF94S force field. During the refinement step, the ligands were free to move within the binding pocket.

2.6. Statistical analysis

Experiments were performed in triplicate and repeated at least three to six times. The values are given as means ± SD. Ordinary or repeated-measures analysis of variance (ANOVA) followed by Student's *t*-test, Dunnett's test or Tukey–Kramer's multiple comparison test was used to evaluate the statistical significance of

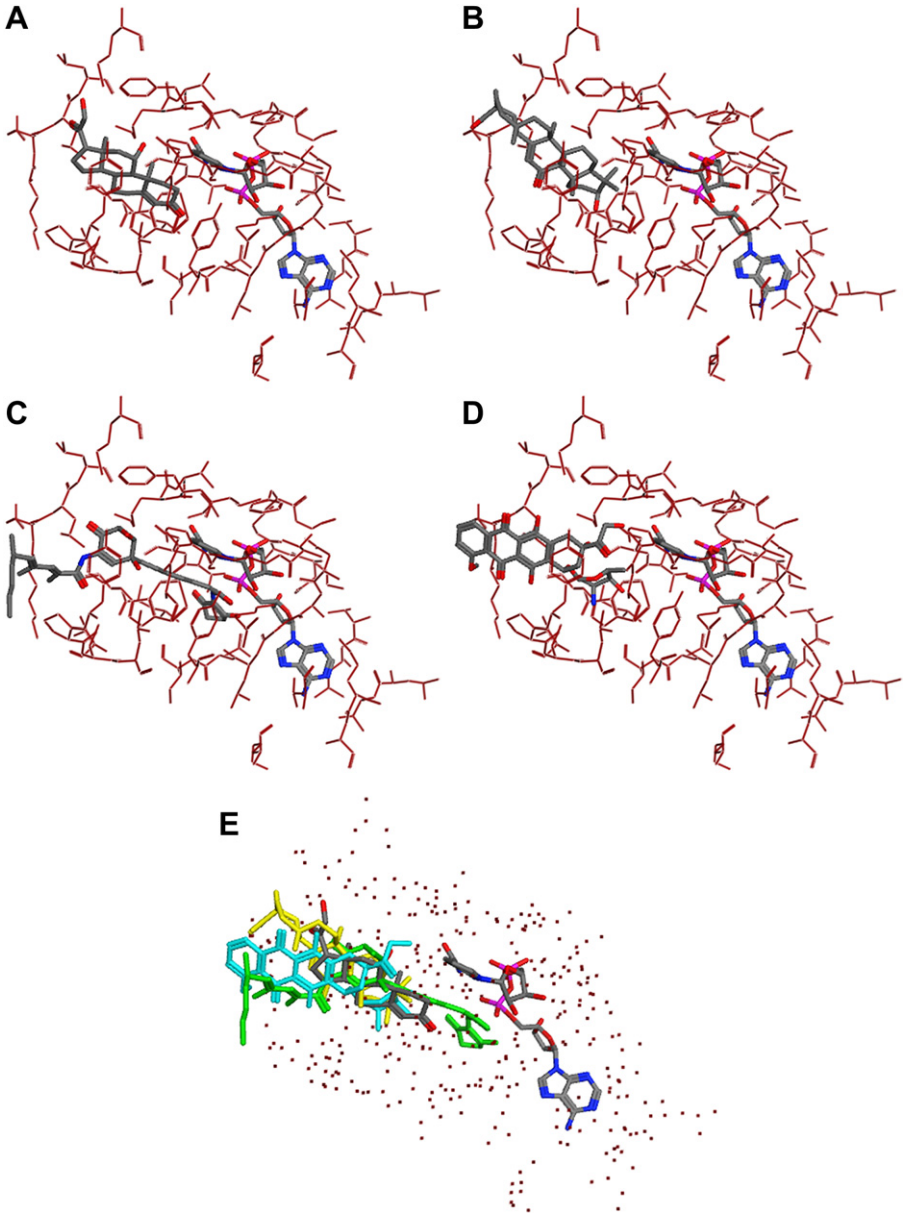

Fig. 1. GA and corticosterone have similar binding orientations to the LBS in the 11βHSD2 model. (A) Corticosterone at the LBS. (B) GA at the LBS. (C) MA at the LBS. (D) Adriamycin at the LBS. (E) Superimposition of the ligand molecules. Element: corticosterone; yellow: GA; green: MA; sky blue: adriamycin. The red lines (A–D) and red dots (E) are amino acid residues in the LBS. NAD^+ is also shown in (A–E). Blue: nitrogen; gray: carbon; purple: phosphorus; red: oxygen. The ASE-Dock reveals that GA and corticosterone exhibit similar binding orientations to the LBS in the 11βHSD2 model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

differences between groups. IC_{50} values were obtained using Prism 4.0 (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. GA and corticosterone have similar binding orientations to the LBS in the 11βHSD2 model

The ASE-Dock revealed that in addition to corticosterone (as the substrate of 11βHSD2; Fig. 1A and E: element), GA (Fig. 1B and E, yellow), MA (Fig. 1C and E, green) and adriamycin (Fig. 1D and E, sky blue) were able to bind to the LBS in the 11βHSD2 model. GA and corticosterone exhibited similar binding orientations to the LBS in the 11βHSD2 model.

3.2. Clinically available antitumor agents and GA upregulate ROS in tumor cells

Our previous studies revealed that cytoskeletal disruption could be one of the factors that contribute to the selective toxicity of GA toward tumor cells [10,11]. However, we consider that the antitumor effects of GA cannot be explained by a single factor, and instead would involve a wide range of factors or processes that induce tumor cell toxicity. Therefore, we treated SFME and *r*/*m* HM-SFME-1 cells with adriamycin, MA or GA, and analyzed the ROS production in these cells to investigate the possible correlation between ROS production and the selective toxicity against tumor cells. Although the ROS production in the adriamycin-, MA- and GA-treated tumor cells was always higher than that in the corresponding normal cells, the differences were not significant (Fig. 2A and B).

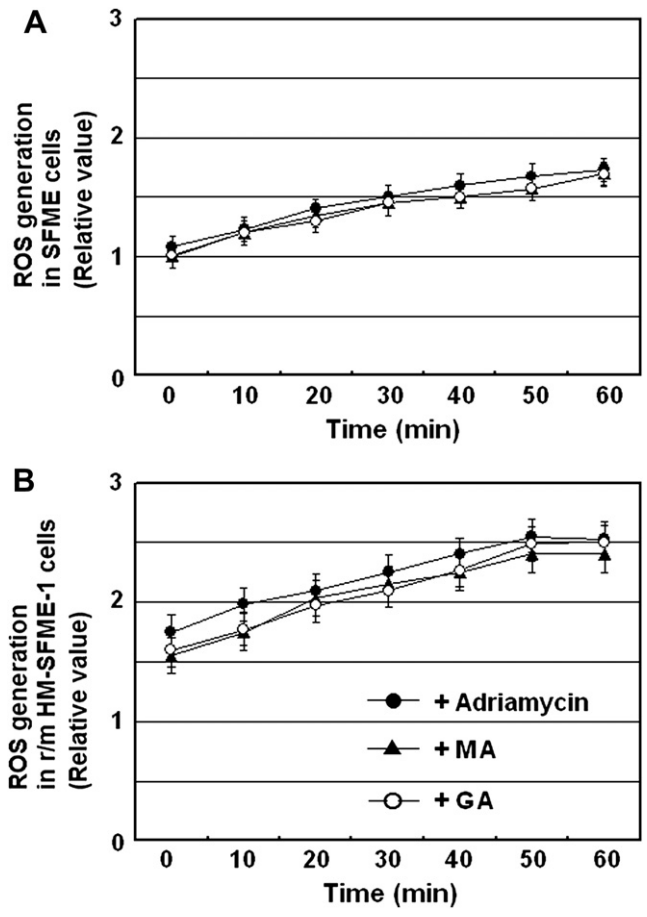

Fig. 2. ROS production levels in normal and tumor cells treated with the clinically available antitumor agents and GA. (A, B) At half-confluency, normal SFME cells (A) and tumorigenic *r*/*m* HM-SFME-1 cells (B) were treated with adriamycin, MA or GA at the IC_{50} values for cell proliferation, and ROS production was measured every 10 min for 1 h. The IC_{50} values for SFME cell proliferation were 0.8 μM for adriamycin, 4.5 μM for MA and 18 μM for GA while those for *r*/*m* HM-SFME-1 cell proliferation were 64.0 μM for adriamycin, 2.4 μM for MA and 7.3 μM for GA. The ROS production levels in the adriamycin-, MA- and GA-treated tumor cells (B) are always higher than those in the corresponding normal cells (A), but the differences are not significant. This trend was also found when the cells were treated with each compound at 0.5× IC_{50} and 2× IC_{50} (data not shown). Each point is the mean ± SD ($n = 18$) of three independent experiments.

3.3. Selective toxicity of GA against tumor cells can be attributed to downregulation of GSH

Although no significant differences in ROS production between the normal and tumor cells was found after treatments with adriamycin, MA and GA (Fig. 2A and B), it is well established that

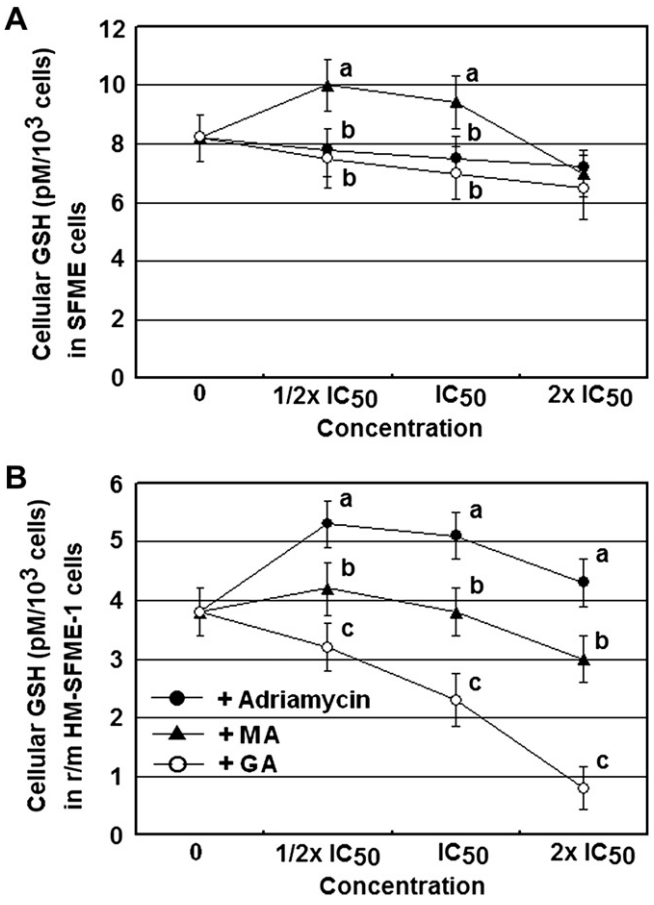

Fig. 3. The selective toxicity against tumor cells can be attributed to downregulation of GSH. (A, B) The GSH levels were analyzed in SFME cells (A) and *r*/*m* HM-SFME-1 cells (B) treated with adriamycin, MA or GA at 0.5×, 1× and 2× the IC_{50} values for cell proliferation for 8 h to investigate the possible correlation between the GSH levels and the selective toxicity toward tumor cells. The cytotoxic effects of GA against the tumor cells at 2× IC_{50} were so strong that we adopted the treatment time of 8 h for this experiment. (A) The decrease in GSH in the normal cells after treatment with GA is not very marked compared with those after the treatments with adriamycin and MA. (B) There is a significant difference in the tumor cells, in which GA is very effective in diminishing GSH in a dose-dependent manner (Pearson correlation coefficient, $r = -0.99849$). The GSH levels in the tumor cells treated with adriamycin, MA and GA are always lower than those in the corresponding normal cells. Each value (*y*-axis) is expressed as picomoles (pM)/10^3 cells. Each point is the mean ± SD ($n = 18$) of three independent experiments. The letter for each treatment indicates a significant difference by Tukey–Kramer's test ($p < 0.05$).

disruption of the redox balance by synthetic triterpenoids through ROS accumulation and GSH reduction contributes to the initiation of apoptotic cascades in tumor cells [32]. Therefore, the GSH levels in the adriamycin-, MA- and GA-treated SFME and *r*/*m* HM-SFME-1 cells were evaluated to examine whether there was a correlation between the GSH levels and the selective toxicity against tumor cells. In the normal cells, the decrease in GSH after treatment with GA was not very marked compared with those after the treatments with adriamycin and MA (Fig. 3A). In contrast, there was a great difference in the tumor cells, in which GA was very effective in diminishing GSH in a dose-dependent manner (Pearson correlation coefficient, $r = -0.99849$; Fig. 3B). The GSH levels in the tumor cells treated with each compound were always lower than those in the corresponding normal cells at all concentrations.

3.4. BSO further decreases GA-downregulated GSH and inhibits tumor cell growth, while NAC increases GA-downregulated GSH and attenuates GA-induced tumor cell cytotoxicity

As shown in Fig. 3B, the selective toxicity of GA against the tumor cells can possibly be attributed to downregulation of GSH. To further examine the involvement of GSH in the GA-induced cytotoxicity, the effects of BSO and NAC on GSH and cell growth were analyzed. BSO, a GSH biosynthesis inhibitor, downregulated GSH while NAC, the GSH precursor, upregulated GSH in both normal and tumor cells (Fig. 4A and B). However, the GSH decrease mediated by GA was only found in the tumor cells, and the GA-induced GSH decrease was intensified by BSO and restored by NAC (Fig. 4B). Although GA did not inhibit the growth of the normal cells (Fig. 5A), it exhibited strong cytotoxicity toward the tumor cells (Fig. 5B). Furthermore, the GA-induced tumor cell toxicity was promoted by BSO and attenuated by NAC (Fig. 5B).

4. Discussion

Triterpenoids are widely distributed throughout the vegetable kingdom and are known to be the major components of many medicinal plants used in Asian countries. The biological and pharmacological roles of triterpenoids have attracted great attention for their analgesic, antiinflammatory, antitumor, hepatoprotective and immunomodulatory effects [7]. We have studied certain triterpenoids as multifunctional agents for the prevention and treatment of cancer, and have recently analyzed normal and tumor cells from the same lineage in the CNS and found that UA exhibited selective toxicity toward the tumor cells [9]. We also found that the selectivity of GA against the tumor cells was more potent than that of the

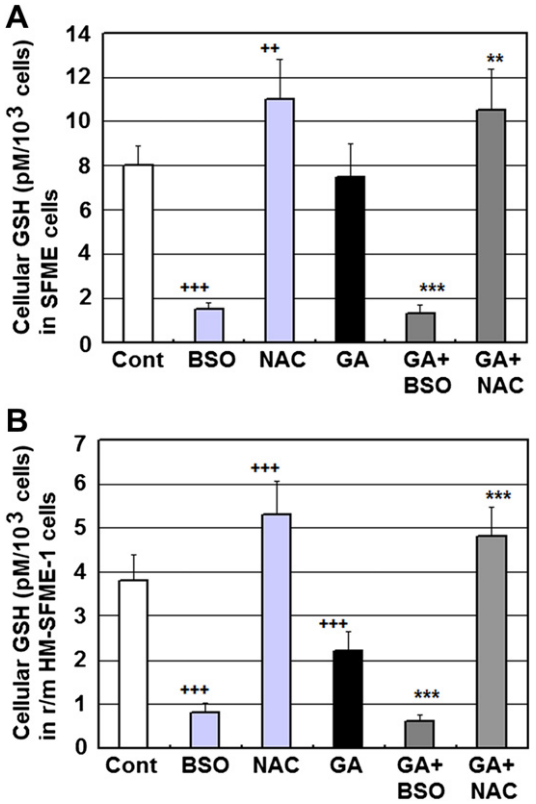

Fig. 4. BSO downregulates and NAC upregulates GSH in normal and tumor cells. (A, B) SFME cells (A) and *r*/*m* HM-SFME-1 cells (B) were treated with 0.5 mM BSO, 50 mM NAC or 10 μM GA at half-confluency and cultured for 24 h. The cells were also treated with GA plus BSO and GA plus NAC. BSO or NAC was added to the medium at 8 h or 1 h prior to GA exposure, respectively, and the treated cells were cultured for 24 h. (A) GA has no effects on GSH in the normal cells. BSO downregulates and NAC upregulates GSH in the normal cells. (B) GA downregulates GSH in the tumor cells. BSO further downregulates and NAC recovers the GA-mediated GSH downregulation in the tumor cells. Each value (*y*-axis) is expressed as picomoles (pM)/10^3 cells. Each point is the mean ± SD ($n = 18$) of three independent experiments. $^{++}p < 0.01$ and $^{+++}p < 0.001$ for control vs. BSO, NAC or GA treatment by Dunnett's test. $^{***}p < 0.001$ for GA vs. GA plus BSO or GA plus NAC treatment by Dunnett's test.

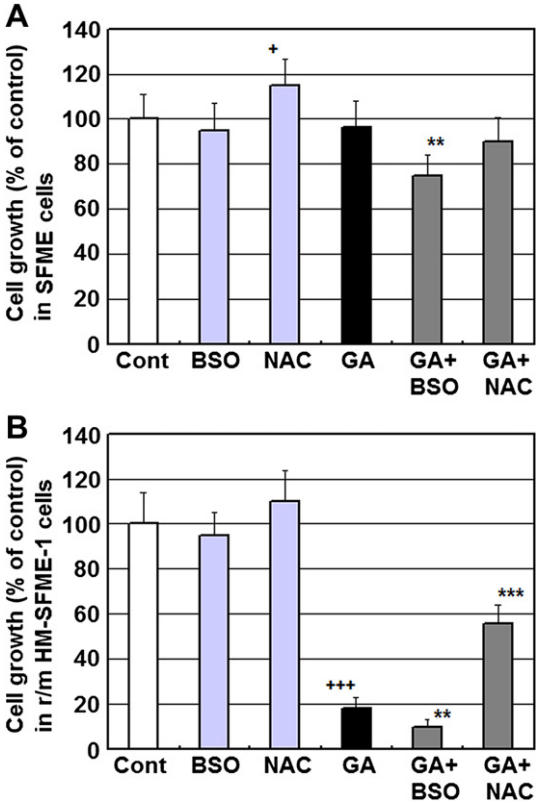

Fig. 5. GA is capable of affecting tumor cells without impeding normal cell growth, and BSO further promotes and NAC partially recovers the GA-downregulated cell growth in tumor cells. (A, B) SFME cells (A) and *r*/*m* HM-SFME-1 cells (B) were treated with 0.5 mM BSO, 50 mM NAC or 10 μM GA at half-confluency and cultured for 24 h. The cells were also treated with GA plus BSO and GA plus NAC. BSO or NAC was added to the medium at 8 h or 1 h prior to GA exposure, respectively, and the treated cells were cultured for 24 h. (A) GA has no effects on the normal cell growth. (B) GA inhibits the tumor cell growth. BSO further promotes and NAC partially recovers the GA-downregulated tumor cell growth. Each point is the mean ± SD ($n = 18$) of three independent experiments. $^{+}p < 0.05$ and $^{+++}p < 0.001$ for control vs. BSO, NAC or GA treatment by Dunnett's test. $^{**}p < 0.01$ and $^{***}p < 0.001$ for GA vs. GA plus BSO or GA plus NAC treatment by Dunnett's test.

clinically available antitumor agents adriamycin and MA [10–12]. Although these agents act through different mechanisms, they share one mechanism, namely the induction of ROS [33,34]. GSH contributes to cell survival through an increased ability to eliminate ROS after exposure to clinically available antitumor agents [33]. The thiol group appearing in a variety of proteins or nonproteins, such as GSH, undergoes reversible thiol–disulfide interactions to mediate oxidant-induced stress [35]. GSH is the most prevalent nonprotein thiol in human cells, was the first to be recognized for its antioxidant role and plays a very important role in determining the sensitivity of tumor cells to different antitumor agents. In general, high intracellular GSH levels diminish cell toxicity while low intracellular GSH levels enhance cell toxicity [36]. Depending on the cell type, GSH may be involved in the CD95 death-inducing signaling complex [37] and/or in the mitochondria-related [38,39] apoptotic pathways.

11βHSD2 is a NAD^+-requiring enzyme that shows dehydrogenase activity toward endogenous glucocorticoids, such as corticosterone [18,40,41]. It has also been reported to be associated with various cancers [18–22], and inhibition of 11βHSD2 by GA was found to prevent tumor growth and metastasis [42]. In the present study, the ASE-Dock module showed that corticosterone (as the substrate of 11βHSD2) and GA had similar binding orientations to the LBS in the 11βHSD2 model, suggesting that GA acts as a competitive inhibitor of corticosterone binding to 11βHSD2. Although *in vitro* GA-mediated inhibitory effects on 11βHSD2 in tumor cells have been reported [42], to the best of our knowledge, this is the first *in silico* analysis to show the GA-induced inhibition of 11βHSD2. Our data clearly revealed much higher ROS production levels and much lower GSH levels in the tumor cells compared with the normal cells. In the tumor cells, which had higher ROS levels and were already under oxidative stress, addition of GA, which would further elevate the ROS production, could lead to tumor cell apoptosis. Moreover, the decrease in GSH mediated by GA creates a redox imbalance that would lower the antioxidant capacity of the cells, and could contribute to the death of the tumor cells. In a previous study, the GSH precursor NAC restored the 2-chloroethyl ethyl sulfide (CEES)-induced reduction in GSH in JB6 and HaCaT cells and showed both protective and therapeutic effects against CEES cytotoxicity [43]. Furthermore, BSO, which reduces cellular GSH levels, increased the cytotoxicity of CEES toward both JB6 and HaCaT cells [43]. In the present study, the GA-induced GSH reduction and tumor cell cytotoxicity were enhanced by BSO, an inhibitor of GSH biosynthesis. We also found that NAC, an antioxidant and precursor of GSH, restored the GA-induced GSH reduction and cytotoxicity in the tumor cells. Taken together, these findings highlighting the efficacy of GSH in ameliorating GA-mediated cytotoxicity support the notion that GSH is involved in the GA-induced selective toxicity against tumor cells.

Selectivity is an important issue for cancer prevention and therapy, and an ideal antitumor agent should be toxic toward malignant cells with minimal toxicity toward normal cells. However, there are only limited numbers of such agents currently available for clinical use [23]. Gleevec is an example of such an agent [44], but mutations and overexpression of its target molecules often lead to drug resistance owing to multiple genetic and epigenetic alterations in tumor cells [45,46]. Therefore, instead of targeting specific oncogenic molecules, it may be possible to exploit the biochemical alterations in tumor cells for the development of antitumor agents. Elevated ROS generation is common after such alterations, and tumor cells with intrinsic oxidative stress can be preferentially killed through ROS-mediated mechanisms [23]. In fact, this was the case in the present study, since ROS production was enhanced in the GA-treated tumor cells. Moreover, tumor cells in advanced disease stages usually exhibit genetic instability and metabolic malfunction, and are often resistant to conventional anticancer drugs [23]. Therefore, the development of more effective and safer antitumor agents from natural products has been expected for better antitumor chemoprevention and therapy [7], and the use of a natural plant component with selective toxicity toward tumor cells could be a breakthrough that meets this expectation. However, only a few studies, such as those on β-phenylethyl isothiocyanate [23] and lupeol [47], have reported selective toxicity toward tumor cells. In the present study, we have demonstrated that GA, a licorice compound, has tumor cell-selective toxicity through ROS upregulation and GSH downregulation. To the best of our knowledge, no other detailed studies have described these characteristics of GA. Our preliminary experiments also revealed selective toxicity of GA toward the less metastatic *ras*/*myc* SFME cells but the effects were not as significant as those on the highly metastatic *r*/*m* HM-SFME-1 cells (data not shown). Further studies on these two transformed cell lines can be expected to promote a new paradigm for the antimetastatic function of a redox imbalance.

To date, we have found that downregulation of Ras [12] and cytoskeletal disruption [10,11] could be involved in the selective toxicity against tumor cells. In the present study, we found that a redox imbalance through downregulation of GSH is highly likely to be one of the factors that contribute to the selective tumor cell toxicity of GA. Our findings that the selective toxicity of GA against tumor cells can be attributed to decreased levels of GSH and consequently to decreased elimination of drug-induced ROS may have important implications for chemoprevention and therapy of cancer.

Conflict of interest

The authors declare no conflicts of interest related to this work.

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