

Doxorubicin and Gallic Acid Induce Oxidative Stress-Induced Cell Death in Laryngeal Cancer Cells via the TRPM2 Channel

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ABSTRACT

Laryngeal squamous cell carcinoma is one of the most common fatal cancers. The chemotherapeutic agent doxorubicin (DOX) has limited efficacy due to frequent side effects and the development of drug resistance during treatment. Therefore, we aimed to investigate whether Gallic acid (GA) has a synergistic effect on the chemotherapeutic effects of DOX and the mechanisms of its action. In order to do this, we looked at how GA stimulates the death of HEP-2 laryngeal cancer cells caused by DOX through the activation of TRPM2 channels. For the study, HEP-2 cells were divided into four groups: Control, GA, DOX, and GA+DOX. Cell viability, antioxidant and oxidant enzyme activity levels, inflammation markers, intracellular ROS levels, apoptosis markers, PARP-1 and TRPM2 expression values were evaluated. DOX treatment caused cytotoxic effects in laryngeal cancer cells and increased apoptosis markers, intracellular ROS, inflammation markers, oxidant enzyme activity levels, PARP-1 and TRPM2 values, while decreasing cell viability and antioxidant enzyme activity values. The therapy was significantly more successful when GA and DOX were used together. In summary, this study discovered that TRPM2 activation caused the synergistic impact of GA+DOX combination therapy on cancer cell death.

Keywords: Doxorubicin, Gallic acid, Oxidative stress, HEP-2 cell, PARP-1, TRPM2 channel

Introduction

Head and neck cancers, including laryngeal cancer, are among the most common fatal cancers worldwide (1). Chemotherapy is important in cancer treatment, but drug resistance and side effects are major problems (2). For this reason, anticancer agents derived from natural sources are being investigated for laryngeal and other cancers, either as adjuncts to current standard treatments (3). HEP-2 cell lines utilized in cancer research are human laryngeal carcinoma cells (4,5).

Doxorubicin (DOX) is an anthracycline chemotherapy drug widely used to treat various types of cancer (3). Anthracyclines induce the formation of reactive oxygen species (ROS) (6), causing oxidative stress and an increase in intracellular calcium (7). The increase in intracellular Ca²⁺ leads to the release of cellular procaspases and apoptosis of cancer cells (8,9). In

addition, an increase in inflammatory markers has been suggested as one of the chemotherapeutic mechanisms of action of DOX (8). However, its clinical use is limited by its lack of tumour specificity, dose-dependent toxicity and serious side effects such as drug resistance. Many studies have been conducted on adding natural compounds to the treatment to increase the efficacy of DOX and reduce its toxicity (10,11). Gallic Acid (GA) has been reported to have anti-cancer activity without damaging normal cells (12,13). Nevertheless, no research has been done on how GA affects DOX-induced drug resistance in HEP-2 cells. Antitumor action is the most notable of GA's many biological activities, which also include antibacterial, antioxidant, and anti-inflammatory qualities (14).

Excessive Ca²⁺ entry is also necessary for tumour cells to undergo apoptosis (9). This pathway is one of the possible mechanisms of chemotherapy, as

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Ca²⁺ overload to the cell can activate cell death pathways (8). The transient receptor potential (TRP) superfamily is a recently discovered family of channels that are often pathologically activated and mediate the influx of cations, particularly Ca²⁺, into the cell (15). ADP ribose (ADPR) and/or oxidative stress can activate the TRPM2 cation channel, which belongs to the TRP melastatin (TRPM) subfamily (16,17). Through an increase in intracellular Ca²⁺ concentration and ROS generation, activated TRPM2 may mediate anti-tumor actions. Some natural compounds have been shown to enhance the anti-cancer effects of DOX by activating various TRPM2 channels (9). Therefore, GA may have a similar potentiating impact on laryngeal cancer cells treated with DOX.

We suggest that GA may potentiate the effects of DOX by increasing Ca²⁺ entry and ROS production via TRPM2 in tumour cells. Therefore, the present study was undertaken to investigate the effect of GA on the anticancer activity in HEp-2 cells and to elucidate the underlying potential mechanisms.

Materials and Methods

Cell Culture: The Şap Institute in Ankara, Turkey, provided the HEp-2 laryngeal cancer cells. The cells were kept in DMEM supplemented with 1% penicillin/streptomycin solution and 10% heat-inactivated FBS. After the cells reached 80-85% confluence, they were passaged and split into four groups. This process was then done six times for each group. An incubator was used to cultivate the cells in T25 culture flasks. Doxorubicin (Cat; T1020, Target Molecule Corp., USA) and Gallic acid (Sigma Aldrich, USA) cytotoxic effects on the sample supernatants were assessed.

Experimental Groups: The laryngeal cancer cell line was divided into four groups; Cells in the control (CON, n;6) group were incubated for 48 hours without any treatment. Cells in the gallic acid group (GA, n;6) were maintained under conditions of cell culture without any treatment for the first 24 hours and then incubated (in a laminar airflow cabinet) with 100 µM GA for 24 hours as outlined in earlier studies (18). Cells in the doxorubicin group (DOX, n;6) were maintained under conditions of cell culture without any treatment for the first 24 hours and then incubated (in a laminar airflow cabinet) with 1 µM DOX for 24 hours as outlined in earlier studies (8). Cells in the doxorubicin and gallic acid treated group (GA+DOX, n;6) were kept under

cell culture conditions by 100 µM GA treatment for the first 24 hours and then, in accordance with earlier research, incubated with 1 µM DOX for 24 hours.

Cell Culture Passaging Protocol and Homogenate Preparation Steps: Cells were placed in cell culture medium for trypsinization, and then cells were washed with PBS. After adding 1 mL of trypsin to the cells, the flask was incubated in CO₂ for 3 min at 5 % CO₂ and 37 °C. After that, the flask was taken out of the incubator, checked for single cell suspensions using an inverted microscope, and tapped twice to make sure all of the cells had fully separated from the flask surface. The trypsinization was subsequently stopped by adding 1 mL of DMEM. After pipetting the cells back into suspension, they were put in a 15 mL tube and centrifuged at 1000 rpm for 10 min. Two mL of DMEM were added to the pellet following the aspiration of the supernatant. Cells for each group were moved into distinct eppendorf tubes and centrifuged for 10 min at 1000 rpm in accordance with the kit's instructions. The underlying cell pellets were diluted in PBS to a concentration of 1x10⁶ cells/ml after the supernatants were extracted using a pipette. Freeze-thaw cycles were used to lyse the cell structure and extract the cytoplasmic components. After centrifugation (3000 rpm, 10 min), the supernatants were removed using an automated pipette and put into Eppendorf tubes so they could be examined.

Analyses

Cell Viability Analysis in HEp-2 Cells: The CCK-8 test (Cat no; KTA1020, Abbkine) was a straightforward and trustworthy technique for assessing cell viability. In 96-well plates, laryngeal squamous cell carcinoma cells were plated at a density of 1 × 10⁶ cells/well. after a specific amount of time spent receiving gallic acid and DOX therapy. The cell viability ratio of the groups was determined by reading at 450 nm using BioTek ELx808™ device. To ascertain the cell viability rate in each group, the experiment was conducted three times. The results were presented as a percentage in relation to the control group (% of control).

Antioxidant and Oxidant Analysis in HEp-2 Cells: The commercial total antioxidant status (TAS) assay kit (Rel.Assay, Türkiye, Pro No: RL0017) was used to assess the samples' supernatants' TAS. This test demonstrates how the free radical ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) is oxidized by hydrogen peroxide, which results in the stability and loss of ABTS's dark blue color. The

color intensity reduces further with increasing antioxidant content. This was accomplished by mixing 300 μ L of reagent-1 with 18 μ L of water, ethanol sample extracts, normal water, or distilled water. Following 30 seconds, the absorbance (A1) was determined using spectrophotometry at 660 nm using a Bio Tek EL808™ spectrophotometer. After adding 45 μ L of reagent-2 to the mixture, the absorbance (A2) was measured at 660 nm and 37 °C 5 min later. Every sample's data was computed for TAS analysis using the kit's standard, which is equal to 1 mmol/L of Trolox.

Furthermore, the total oxidant status (TOS) commercial assay kit (Rel.Assay, Türkiye, Pro No: RL0024) was used to evaluate the oxidant status of the supernatants of the samples. The oxidation of Fe^{2+} to Fe^{3+} , which combines with xylenol orange to generate a purple complex that can be detected by spectrophotometry, provides the basis for this evaluation. This test involved mixing 300 μ L of reagent-1 with 45 μ L of sample or standard, and then measuring the absorbance (A1) spectrophotometrically at 530 nm after 30 s. The mixture was incubated at 37 °C for 5 min after adding 15 μ L of reagent-2, and the absorbance (A2) was then measured. Micromolar hydrogen peroxide equivalents per litre ($\mu\text{mol H}_2\text{O}_2$ equivalents/L) are the findings of the TOS analysis test, which was calibrated using hydrogen peroxide.

The oxidative stress index (OSI), which measures the level of oxidative stress, was defined as the percentage ratio of the TOS to the TAS. The OSI value was computed using the following formula after the resultant unit of TAS, mmol Trolox eq/L, was changed to $\mu\text{mol Trolox eq/L}$ for computations: $\text{OSI} = [\text{TOS } (\mu\text{M H}_2\text{O}_2 \text{ eq/L}) / \text{TAS } (\mu\text{mol Trolox eq/L})] \times 100$.

Biochemical Analysis in HEP-2 Cells: The ELISA commercial kits (Sun.Red Biotech China) were used to measure the amounts of TRPM2, PARP-1, Caspase (Casp-3 and Casp-9), ROS, and inflammation markers ($\text{TNF } \alpha$, IL 1 β , and IL-6) in the cell supernatants that were collected. The procedures established by the companies for commercial kits were taken into consideration while performing these analyses. The absorbance values were measured using an ELISA spectrophotometer (Bio Tek EL808™).

The Determinations of GSH, GSH-Px, and MDA Levels in HEP-2 Cells: Using a V-730 UV spectrophotometer (Japan), the amounts of lipid peroxidation (malondialdehyde (MDA)) in the frozen HEP-2 Laryngeal cancer cell samples were determined at 532 nm. The compounds that are reactive to the thiobarbituric acid reaction were

measured in the MDA studies by comparing the absorbance to the standard curve of MDA equivalents that are generated when 1,1,3,3-tetramethoxypropane hydrolyzes in the presence of acid. To test GSH, the frozen cell samples were precipitated with 50% trichloroacetic acid and centrifuged at 600 $\times$ g for 5 min. The reaction mixture contained cell supernatant (0.5), 10 μM 5,5-dithiobis-2-nitrobenzoic acid (0.1 mL), and Tris-II buffer (2.0 mL and 0.2 mM, pH 8.9). The solution was allowed to come to room temperature for 5 min before being measured at 412 nm using a spectrophotometer (V-730 UV). Spectrophotometry was used to measure the frozen cell samples' GSH-Px activity at 412 nm and 37°C. One milligram of cumene hydroperoxide was used in the analyses to stimulate GSH-Px. To represent the data as expressed per gram of protein, the protein content of the cell homogenate samples was determined using Lowry's reagent. The units of measurement for MDA and GSH are $\mu\text{mol per gram of protein}$. The unit of measurement for GSH-Px activity is IU per gram of protein.

Statistical Analysis: SPSS software (version 17.0, USA) was used to analyze the data, and all data were presented as mean $\pm$ standard deviation (SD). A one-way ANOVA and Post-hoc Tukey test were employed to assess all data, demonstrating statistically significant differences between groups. Statistical significance was established at a value of $p \leq 0.05$.

Results

GA and DOX Treatments Reduced Antioxidant and Cell Viability Levels in Laryngeal Cancer Cells: We looked at how GA and DOX affected laryngeal cancer cells, both singly and in combination. DOX therapy dramatically reduced the TAS levels and cell viability of laryngeal cancer cells. After GA and DOX combined therapy, TAS and cell viability values were further decreased (Figure 1). Cell viability (Figure 1A) and TAS (Figure 1C) values were considerably lower in the DOX-treated groups than in the CON group ($p \leq 0.001$). The DOX-treated groups showed significantly higher values of OSI (Figure 1D) and TOS (Figure 1B) than the CON group ($p \leq 0.001$). In comparison to the CON group, DOX treatment reduced the cell viability rate (Figure 1A) and TAS (Figure 1C) values ($p \leq 0.001$); however, the decline was greater following GA+DOX treatment than in the CON group. Alongside the cell survival rate and TAS results, the DOX group had higher values of TOS

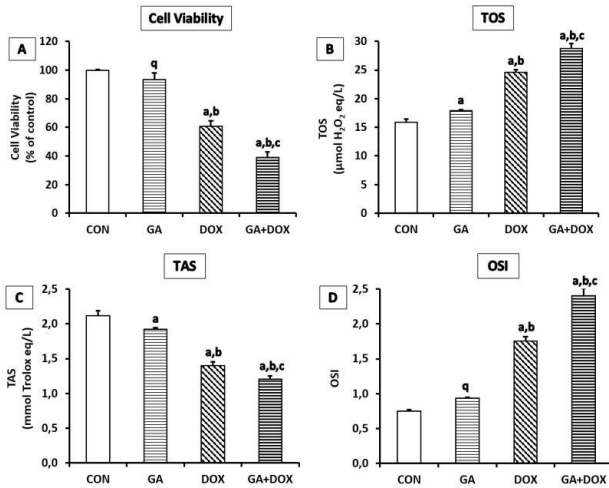


Fig. 1. Effect of GA on Cell viability (A), TOS (B), TAS (C), and OSI (D) levels in laryngeal cancer cells after DOX-induced cytotoxicity (mean $\pm$ SD) ($^a p \leq 0.001$, $^q p \leq 0.05$ vs CON group; $^b p \leq 0.001$ vs GA group; $^c p \leq 0.001$ vs DOX group)

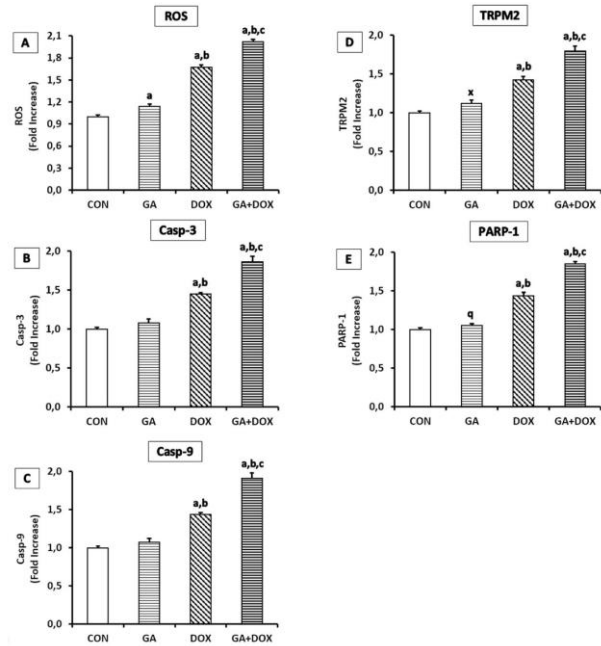


Fig. 3. Effect of GA on ROS (A), Casp-3 (B), Casp-9 (C), TRPM2 (D), and PARP-1 (E) levels in laryngeal cancer cells after DOX-induced cytotoxicity (mean $\pm$ SD) ($^a p \leq 0.001$, $^x p \leq 0.01$, $^q p \leq 0.05$ vs CON group; $^b p \leq 0.001$ vs GA group; $^c p \leq 0.001$ vs DOX group)

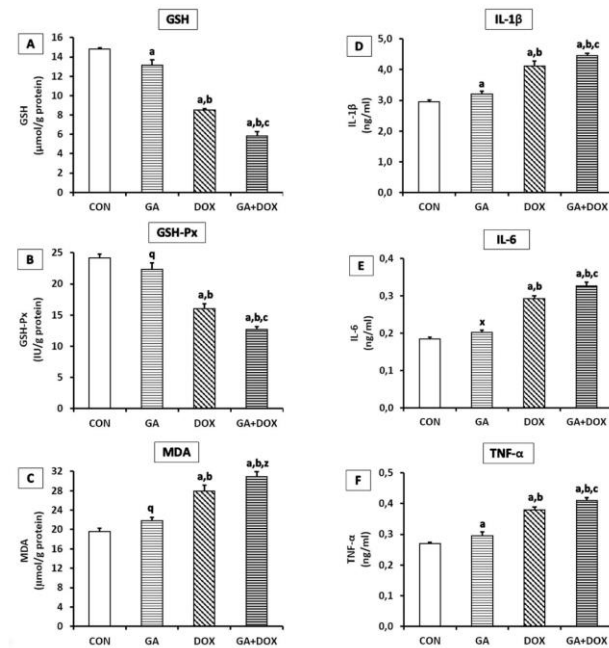


Fig. 2. Effect of GA on GSH (A), GSH-Px (B), MDA (C), IL-1 β (D), IL-6 (E), and TNF- α (F) levels in laryngeal cancer cells after DOX-induced cytotoxicity (mean $\pm$ SD) ($^a p \leq 0.001$, $^x p \leq 0.01$, $^q p \leq 0.05$ vs CON group; $^b p \leq 0.001$ vs GA group; $^c p \leq 0.001$, $^z p \leq 0.01$ vs DOX group)

(Figure 1B) and OSI (Figure 1D) than the CON group ($p \leq 0.001$), while the GA+DOX groups had even higher values as a result of GA treatment.

GA and DOX Treatments Decreased Glutathione Levels and Increased Lipid Peroxidation Levels in Laryngeal Cancer Cells: Antioxidants scavenge reactive oxygen

species. GSH and GSH-Px, two components of the thiol redox system, are essential for scavenging ROS in a variety of cells (19). In healthy cells, GA promotes GSH-Px activity and reduced-GSH concentration. Increased oxidative stress plays a major role in the activation of the TRPM2 channel and GSH depletion. After seeing a rise in ROS values, we hypothesized that a decrease in GSH values and GSH-Px activity would trigger the activation of the TRPM2 channel in HEP-2 cells. DOX therapy dramatically reduced the GSH-Px and GSH values of HEP-2 cells. After GA and DOX combined treatment, GSH-Px and GSH values were further reduced (Figure 2). GSH (Figure 2A) and GSH-Px (Figure 2B) values were notably lower in the DOX-treated groups than in the CON group ($p \leq 0.001$). The DOX-treated groups showed significantly higher levels of MDA (Figure 2C) than the CON group ($p \leq 0.001$). In comparison to the CON group, DOX treatment reduced the GSH (Figure 2A) and GSH-Px (Figure 2B) levels ($p \leq 0.001$); however, the decline was greater following GA+DOX treatment than in the CON group. Alongside the GSH-Px and GSH levels results, the DOX group had higher levels of MDA (Figure 2C) than the CON group ($p \leq 0.001$), while the GA+DOX groups had even higher levels as a result of GA treatment. According to our research, GA therapy may

intensify the effects of DOX-induced cell death. The glutathione depletion effect caused by severe oxidative stress mediated by TRPM2 channel activation in the laryngeal cancer cells was validated by these GSH-Px and reduced-GSH data.

GA and DOX Treatments Increased Inflammation Levels in Laryngeal Cancer Cells: DOX therapy dramatically increased the inflammation marker values of HEP-2 cells. After GA and DOX combined treatment, inflammation marker levels were further increased (Figure 2). The DOX-treated groups had substantially greater levels of TNF- α (Figure 2F), IL-1 β (Figure 2D), and IL-6 (Figure 2E) than the CON group ($p \leq 0.001$). The therapy was significantly more successful when GA and DOX were used together. This study showed that GA therapy might further improve the effect of DOX-induced inflammation and cell death in HEP-2 cells.

GA and DOX Treatments Increased Reactive Oxygen Species and Caspase Levels in Laryngeal Cancer Cells: It is commonly recognised that cell death and apoptosis are the outcomes of elevated mitochondrial ROS activation brought on by TRPM2 activation in cancer cells. Here, we assessed the levels of ROS and caspase in cancer cells. When laryngeal cancer cells were treated with DOX, their levels of ROS and caspase dramatically rose. After GA and DOX combined treatment, ROS and caspase levels were further increased (Figure 3). The DOX-treated groups showed significantly higher levels of ROS (Figure 3A), Casp-3 (Figure 3B), and Casp-9 (Figure 3C) than the CON group ($p \leq 0.001$). The therapy was significantly more successful when GA and DOX were used together. This study showed that GA treatment might further boost the impact of DOX-induced caspase and ROS in human laryngeal carcinoma cells.

GA and DOX Treatments Increased PARP-1 and TRPM2 Levels in Laryngeal Cancer Cells: It is commonly recognized that a number of causes may cause cell death. Among these well-known variables, oxidative stress and excessive Ca^{2+} entry are two of the most significant. Several cation channels allow Ca^{2+} to flow across the cell membrane. The TRP superfamily is one of these cation channels. Oxidative stress activates several members of the TRP family subgroup, including TRPV1, TRPA1, and TRPM2 (20), showing that the HEP-2 cell line exhibits a high degree of expression of the TRPM2 cation channel (4). Additionally, PARP-1 activation produces DNA damage-induced ADPR in the cell nucleus, which

reacts when ADPR attaches to the enzymatic nudix C domain of the TRPM2 channel (21). TRPM2 and PARP-1 expression levels were examined in this context; Figure 3 displays the TRPM2 channel and PARP-1 expression levels. DOX treatment raised the cells' expression levels of PARP-1 (Figure 3E) and the TRPM2 cation (Figure 3D) channel ($p \leq 0.001$). After GA and DOX combined treatment, PARP-1 and TRPM2 levels were further increased ($p \leq 0.001$). Our findings show that this mechanism involves the activation of TRPM2 channels.

Discussion

This study aimed to investigate the importance of increased ROS and cell death in the anti-tumour activity of DOX, whether the TRPM2-mediated cellular pathway is involved in this increase and whether combination therapy with GA would produce a synergy in anti-tumour activity. Recent studies have reported that DNA damage caused by increased ROS activates TRPM2 through PARP-1 activation/ADPR production (4,21). This study shows that DOX causes TRPM2 activation in laryngeal cancer cells, mediated by a rise in ROS and, thus, an increase in cytosolic Ca^{2+} . We also found that increased cytosolic Ca^{2+} causes mitochondrial membrane depolarization ($\Delta\Psi_m$), excessive ROS production, oxidant/antioxidant balance disruption, and increased cytokine levels. In addition, our results showed that $\Delta\Psi_m$ induced apoptosis and cell death in HEP-2 cells via activation of caspases. These molecular pathways, which have been investigated in a limited number of studies in the literature, are consistent with our results (4,8).

In this study, we demonstrated for the first time that the anti-tumour activity of DOX-treated HEP-2 cells via the ROS-induced PARP1/ADPR/TRPM2 pathway can be enhanced by GA. There has been a lot of interest in studying the application of GA in cancer treatment since it can stop tumor development by causing tumor apoptosis (22). GA has been reported to potentiate antitumor activity by increasing ROS and ROS-activated Ca^{2+} entry with a pro-oxidant effect in various cancers. One of the possible molecular pathways of the antitumor activity of GA is that it increases intracellular calcium (23). Given the importance of intracellular Ca^{2+} elevation for tumor cell apoptosis, this effect of GA may have a synergistic effect when combined with DOX in cancer treatment. In addition, the increase in intracellular

Ca^{2+} may be mediated by the activation of TRPM2 channels.

Our study's findings demonstrated that GA treatment increased the effect of DOX on HEP-2 cell viability (Figure 1A). Cell death in cancerous tissues has been attributed to a number of molecular pathways. Excessive ROS generation and elevated intracellular free Ca^{2+} are significant processes investigated for this goal (24,25). We thus examined the channels' expression levels and hypothesized that the rise in intracellular Ca^{2+} and ROS in HEP-2 cells may be mediated by TRPM2 channels. According to both *in vitro* and *in vivo* research, the HEP-2 cell line has significant expression of the TRPM2 cation channel, and tumor development is inhibited by activating these channels (4). TRPM2 inhibition has been reported to reverse the increased ROS and cell death caused by DOX, leading to tumor growth (26,27). In addition, a recent study has shown that targeting TRPM2 promotes cell death in T-cell leukemia (28). As TRPM2 activity can prevent tumor cell growth, its potential as a new therapeutic target is increasing. TRPM2 expression is activated by oxidative stress and PARP-1 (29). Our findings indicated that the expression levels of PARP-1 (PARP-1 that activates this channel, causing ADPR production) and TRPM2 were much higher in the GA+DOX and DOX groups. The combined therapy group saw a significantly larger rise (Figure 3D, 3E). Therefore, we discovered that in the DOX and GA+DOX groups, cell viability declined as TRPM2 and PARP-1 levels rose, indicating the involvement of this route in tumor cell death (Figure 1A, 3D, and 3E).

According to reports, oxidative stress is another significant activator of TRPM2, and both oxidative stress and PARP-1 promote TRPM2 expression in cancer cells (30). Because of the importance of TRPM2 channels activated by increased ROS and oxidative stress in tumor cell death, through elevated oxidative stress in laryngeal cancer cells, we examined the impact of GA on DOX-induced TRPM2 expression levels. We discovered that the rise in TRPM2 levels in the DOX and GA+DOX groups coincided with the rise in ROS in both groups (Figure 3A). We also observed that DOX treatment exerted anti-tumor activity through a pro-oxidant effect and that GA further enhanced this effect. TAS dropped, the OSI index rose, and cell viability also dropped in the DOX and GA+DOX groups, although TOS rose in those groups (Figure 1). Recent research has demonstrated that GA

stimulates calcium channels and has a pro-oxidant impact (31,32). DOX has been reported to decrease the activity of antioxidant enzymes and increase ROS levels and lipid peroxidation (26,33). According to reports, patients with laryngeal cancer had lower GSH levels and GSH-Px activity, and DOX therapy further reduced these levels (8). This study discovered that GSH-Px and GSH values were reduced in DOX- and GA-treated HEP-2 cells (Figure 2A, 2B). The DOX-induced decrease in GSH-Px activity and GSH values was further stimulated by GA treatment. This decrease is due to the excessive use of antioxidant enzymes, which may be due to the scavenging of ROS. Likewise, the DOX and combination therapy groups showed a substantial rise in MDA levels (Figure 2C). Consequently, our findings showed that GA worked in concert with DOX to provide anti-tumor action through a pro-oxidant impact. The DOX-treated groups (GA+DOX; it was greater in the combined treatment group) in this study showed a drop in GSH and GSH-Px levels and an increase in TRPM2 and ROS levels. Therefore, we discovered that TRPM2 channel activation was brought on by elevated ROS and antioxidant depletion in laryngeal cancer cells, and that GA therapy further exacerbated this effect.

DOX treatment has been reported to activate pro-apoptotic proteins, causing excessive ROS production due to mitochondrial depolarization (3). We looked into the consequences of DOX and GA on ROS increase and caspase activation in HEP-2 cells and whether TRPM2 mediates these effects. It is well known that both normal and malignant cells' mitochondrial membranes depolarize when intracellular Ca^{2+} concentration rises pathologically through TRPM2, which raises ROS (9). In HEP-2 cells, DOX administration led to increased intracellular ROS generation, casp-3 and casp-9 activation brought on by mitochondrial depolarization, and enhanced cell death (Figure 3A, 3B, 3C and 1A). GA+DOX treatment further enhanced these changes. Furthermore, cell viability dropped in these groups even while TRPM2 expression rose, suggesting that the combination therapy had greater anti-tumor action. Our study showed that GA and DOX treatments in HEP-2 cells increased TRPM2, PARP-1, casp-3 and casp-9 expression levels, ROS production, and caspase and MDA levels while decreasing GSH-Px and GSH values. By increasing ROS generation, caspase activation, and TRPM2 channel expression in laryngeal cancer cells, our data demonstrated that GA and DOX treatments decreased tumour cell viability,

demonstrating the significance of these pathways for anti-tumour effectiveness. In addition, combined treatment (GA+DOX) further increased TRPM2 activation and ROS production while decreasing cell viability and antioxidant capacity. The current findings are the first to identify the anti-tumour impact of GA in HEP-2 tumour cells, which is mediated via activation of the PARP-1/TRPM2 pathway.

DOX not only adds base pairs to DNA but also inhibits processes such as replication, transcription, and the activity of topoisomerase II. This inhibition leads to a reduction in both DNA and RNA synthesis. Additionally, DOX induces the production of ROS, which create oxidative damage, resulting in the fragmentation or degradation of cancer cell DNA. One of the primary ways anticancer drugs achieve cytotoxicity in cancer cells is through apoptosis. Mitochondria, which are the largest producers of ROS, play a significant role in cell death pathways by causing DNA damage (34,35). A recognized anti-tumour action of DOX is increased production of pro-inflammatory signaling molecules (36). Previous reports have shown a significant increase in TNF- α , IL-1 β and IL-6 levels with DOX treatment (36). Overproduction of inflammatory proteins may result from DOX and GA treatment-induced increases in ROS in HEP-2 cells. Furthermore, increased production of inflammatory proteins may lead to overactivation of TRPM2 channels (37). In order to ascertain this, we looked at how DOX and GA affected shifts in these markers' levels and TRPM2 channel expression brought on by elevated ROS generation. We observed a significant increase in IL-1 β , IL-6 and TNF- α levels in laryngeal cancer cells (Figure 2D, 2E, 2F). The GA+DOX group had a larger rise. According to these findings, GA therapy may intensify the effects of DOX-induced inflammation and cell death in cancer cells. These findings therefore show that the anti-tumour effectiveness of combination DOX and GA therapy is influenced by the overproduction of inflammatory proteins.

Finally, this study demonstrated that GA improves the anti-tumour activity of DOX by regulating the PARP-1/ADPR/TRPM2 pathway activated by increased ROS through its extraordinary bioactivities, such as apoptotic and anti-tumour effects.

The study's findings demonstrate that GA-induced ROS causes tumour cells' mitochondria to depolarize and activate the PARP-1/TRPM2 pathway, which in turn reduces cell viability. Significantly, activation of the PARP-1/TRPM2

pathway mediated the chemotherapeutic effect of DOX, whereas the combination with GA enhanced this effect. These results suggest that targeting the PARP-1/TRPM2 axis and redox-dependent signaling may represent a promising therapeutic strategy to improve the efficacy of conventional chemotherapy in laryngeal cancer. From a translational perspective, the GA-DOX combination could potentially reduce required drug doses and limit systemic toxicity while maintaining anticancer potency. Future research should therefore include in vivo validation of this synergistic mechanism, pharmacokinetic and safety profiling of GA-DOX co-administration, and exploration of its applicability in other oxidative stress-related malignancies.

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