ISSN 0026-8933, Molecular Biology, 2025, Vol. 59, No. 5, pp. 784–794. © Pleiades Publishing, Ltd., 2025.
ISSN 0026-8933, Molecular Biology, 2025. © Pleiades Publishing, Ltd., 2025.

CELL MOLECULAR BIOLOGY

UDC 616_006

Chrysin Boosts the Cell Death and Anticancer Actions of Doxorubicin by Stimulating the TRPM2 Channel in Glioblastoma Cells

Y. Yazğan[a,*] and K. Yıldızhan[b]

[a] *Department of Biophysics, Faculty of Medicine, Kastamonu University, Kastamonu, Türkiye*
[b] *Department of Biophysics, Faculty of Medicine, Van Yuzuncu Yil University, Van, Türkiye*
**e-mail: yener8275@hotmail.com*

Received February 28, 2025; revised April 16, 2025; accepted April 29, 2025

Abstract—The prognosis for glioblastoma multiforme (GBM), the adult brain and spinal cord tumour with the poorest prognosis, has not improved enough with current therapies. Investigating potential molecular routes of tumour growth and metastasis and novel therapeutic methods are, therefore, obviously necessary. Although some natural substances have been shown to increase intracellular Ca^{2+} concentration and ROS generation by activating TRPM2 channel and potentiate the anticancer effects of doxorubicin (DOX), and chrysin (CHR) has been shown to enhance the anticancer effect of DOX, its anticancer effect through TRPM2-mediated Ca^{2+} homeostasis and ROS generation in glioblastoma tumour cells (DBTRG) has not been investigated. This work aimed to clarify the molecular processes behind the synergistic chemotherapeutic action of DOX and CHR in DBTRG cells. For this purpose, we investigated the stimulatory role of CHR on DOX-induced cell death via TRPM2 channel activation in DBTRG cells. DOX treatment caused cell cytotoxicity, increased apoptosis (caspase 3 and 9), ROS, lipid peroxidation levels, and decreased cell viability and antioxidant levels. The combination of CHR and DOX made the treatment even more effective. The effects in cells were reduced with treatments with 2APB, a TRPM2 antagonist. In conclusion, the increase in ROS and cell death levels mediated by TRPM2 activation in DOX-induced DBTRG cells was further enhanced by CHR treatment. The combination of CHR and DOX can be used as a successful agent in the treatment of glioblastoma tumours.

Keywords: chrysin, doxorubicin, glioblastoma, oxidative stress, TRPM2
DOI: 10.1134/S0026893325600710

INTRODUCTION

With a median survival of around 15 months, glioblastoma multiform (GBM) is the brain and spinal cord tumour with the poorest prognosis in adults [1]. There is an obvious need for new strategic approaches to the investigation and treatment of this disease because the dismal prognosis persists despite present therapeutic techniques. Several research studies have examined the physiopathological causes of malignancies, and several molecular pathways have been postulated to treat tumours, including GBM, more successfully [2–4]. The mechanisms of this stimulation are still unclear, even though GBM is known to be produced by overstimulation of glial cells in the central nervous system [4]. Glial cell carcinogenesis has been linked to pathological processes such as oxidative stress, elevated reactive oxygen species (ROS), inflammation, and endogenous antioxidant depletion [5]. Numerous physiological activities, such as cell motility, secretory functions, and apoptosis, depend on cytosolic free Ca^{2+} content variations in all cells [6, 7]. Nevertheless, in all cells, including GBM, an overabundance of Ca^{2+} can pathologically activate calcium signalling pathways, resulting in mitochondrial depolarisation, increased oxidative stress and ROS, activation of procaspases and DNA damage [8, 9]. When some cation channels are stimulated physiologically or pathologically, the concentration of free Ca^{2+} in the cytosol rises [10]. Thus, these channels can be activated or inhibited to interfere with calcium-dependent pathogenic processes. Under conditions of severe oxidative stress and inflammation, stimulating Ca^{2+} influx through Ca^{2}-permeable cation channels may represent a promising alternative therapeutic strategy for GBM tumours.

Effective therapeutic options may be offered by combining medications that damage DNA and produce oxidative stress and inflammation, like doxorubicin (DOX), with substances that control the activation of these channels [11, 12]. It has been discovered that DOX raises intracellular Ca^{2+} levels, causes oxidative stress and increases ROS levels [13, 14]. According to some research, DOX-induced elevations in intracellular Ca^{2+} trigger the release of procaspases

and apoptosis [14, 15]. Furthermore, it has been proposed that the sensitivity of DOX to destroy tumour cells is also influenced by the rise in intracellular ROS and inflammatory markers [15, 16]. Determining whether Ca^{2+}-permeable TRPM2 channels mediate the Ca^{2+}-dependent antitumor actions of DOX is crucial. Mammalian cells include 28 members of the transient receptor potential (TRP) superfamily, including TRP melastatin-2 (TRPM2) cation channels, a subset of Ca^{2+}-permeable cation channels [8, 9, 17]. TRPM2 channels are activated by ADP-ribose (ADPR), which is generated in the nucleus due to DNA damage. Furthermore, oxidative stress activates TRPM2 channels, while 2-aminoethoxydiphenyl borate (2APB) inhibits them [4, 18].

Researchers are looking at the impact of mixing some natural substances that have been demonstrated to have anti-cancer qualities with the current conventional therapies for various malignancies, including GBM [3, 10, 19]. Research is being conducted to identify the molecular pathways that mediate the actions of these natural chemicals, which are believed to help avoid drug resistance and minimise the adverse effects of chemotherapy [3, 4]. To improve the effectiveness and lower the toxicity of DOX, several studies have been done on the combination of DOX therapy with natural substances [14, 20]. Flavonoids are considered a versatile source for discovering and developing anticancer agents. Based on its chemical structure, chrysin (CHR) is a potential phytochemical that belongs to the flavonoid class. While its synthetic equivalents are also used for medicinal purposes, it is naturally found in honey, propolis, passion fruit, mushrooms, and other plant sources. It has been used extensively to treat various degenerative diseases and has anti-inflammatory and cytotoxic properties. Its structural variety in ring A and lack of oxygenation in rings B and C are responsible for its anti-oxidant and disease-preventive properties [21]. It has many properties such as antiallergic, hepatoprotective, neuroprotective, anticancer, antidiabetic, antibacterial, antihypertensive, vasodilator, anxiolytic, antiviral, antiestrogenic, anti-aging, anticonvulsant, antioxidant, and anti-inflammatory effects. It increases mesenchymal stem cell proliferation [21–23]. CHR has the potential to be used in clinical and therapeutic settings to counteract the physiological and metabolic impacts of ageing. It possesses cytotoxic and anti-inflammatory properties and is frequently used to treat several degenerative disorders [24]. CHR exhibits anti-inflammatory, antioxidant, and antiallergic properties [25], along with key biochemical and pharmacological attributes associated with cancer prevention [26]. It functions as an inhibitor of cell proliferation and tumor angiogenesis in vivo [27], and promotes tumor cell death in vitro [28]. In a study by Zheng et al., CHR encapsulated in a nanopolymer with DOX enhanced chemotherapeutic efficacy, which was reported to induce apoptosis in human hepatocellular carcinoma (HepG2) cells [29]. In a study by Zheng et al., CHR increased the efficacy of DOX by altering cell cycle distribution and induced apoptosis in MCF-7 breast cancer cells. Nanostructured lipid carriers increased the efficacy of CHR in sensitising cancer cells to DOX, a chemotherapy agent [30]. Certain natural substances have been demonstrated to activate TRPM2 channels, which increase intracellular Ca^{2+} concentration and ROS generation, augmenting DOX's anticancer effects [2, 31]. Furthermore, although CHR has been demonstrated to enhance DOX's anticancer action [32, 33], its anticancer impact through TRPM2-mediated Ca^{2+} homeostasis and ROS generation in glioblastoma cells has not been studied.

This study aimed to determine the anticancer effects of CHR and DOX treatment in DBTRG cells and the role of the TRPM2 channel in this mechanism.

MATERIALS AND METHODS

Chemicals

CHR (C80105-25G) and 2APB (Cat; D9754) were purchased from Sigma Aldrich C. (USA). DOX (Cat; T1020) was purchased by TargetMol (Target Molecule Corp., USA). Caspase 3 (REF; DZE201120970), Caspase 9 (REF; DZE201120969), and ROS (REF; DZE201129433) ELISA kits were purchased from Sun. Red Biotech Comp (SRB) Ltd (China). TRPM2 (Cat. no; abx551423) ELISA kits and Poly ADP ribose polymerase 1 (Cat. no; abx250333) ELISA kits were purchased from Abbexa Ltd (UK). Total Antioxidant/Oxidant Capacity (TAS-Pro No.: RL0017 and TOS-Pro No.: RL0024) ELISA kit was purchased from RelAssay (Türkiye).

Cell Culture

In this study, cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution. Once the cultures reached approximately 80–90% confluency, they were subcultured and allocated into five experimental groups. This procedure was repeated six times for each group. The cells were incubated in T25 flasks under standard conditions (5% CO_2, 95% air, at 37°C). A 0.25% trypsin-EDTA solution was employed during incubation to detach the adherent cells from the flask surface.

Experimental Groups

The DBTRG cells were divided into five groups. The experiments were repeated 6 times for each group mentioned below.

Control (CON): DBTRG tumour cells in this group were not treated with any treatment and were incubated (48 h).

CHR: DBTRG tumour cells in this group were kept in cell culture conditions for 24 h without any treatment and then incubated with CHR (30 μM) for 24 h as described in previous studies [34].

DOX: DBTRG tumour cells in this group were kept in cell culture conditions for 24 h without any treatment and then incubated with DOX (1 μM) for 24 h as described in previous studies [35].

CHR + DOX: The DBTRG tumour cells in this group were kept with CHR (30 μM) in the cell culture medium for 24 h, and then they were treated with DOX (1 μM) further for 24h.

2APB + DOX: The DBTRG tumour cells in this group were kept with CHR (30 μM) in the cell culture medium for 24h, and then they were treated with TRPM2 channel antagonist 2APB (100 μM) [4] and DOX (1 μM) further for 24 h.

Cell Homogenate Preparation Steps

In accordance with the manufacturer's protocol, cells from each experimental group were transferred into individual Eppendorf tubes and subjected to centrifugation at 1000 rpm for 20 min. Following centrifugation, the supernatant was carefully aspirated, and the resulting cell pellets were resuspended in PBS to achieve a final concentration of 1×10^6 cells/mL. Cellular lysis was induced through repeated freeze-thaw cycles. Subsequently, the lysates were centrifuged at 4°C at 3000 rpm for 10 min to separate cytoplasmic contents. The supernatant obtained was collected using an automated pipetting system and transferred into sterile microtubes for subsequent analyses.

Cell Viability Assay in DBTRG Cells

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8, Abbkine, Cat# KTA1020), a sensitive and reproducible colourimetric assay. To evaluate the potential effect of chrysin against doxorubicin (DOX)-induced cytotoxicity in human glioblastoma cells, CCK-8 analysis was conducted. Absorbance values, indicative of viable cell numbers, were measured at 450 nm using the BioTek ELx808™ microplate reader, in accordance with the manufacturer's instructions. The assay was independently performed in triplicate for each experimental group, and results were expressed as a percentage relative to the untreated control group.

Total Antioxidant/Oxidant Status Levels in DBTRG Cells

The supernatant fractions obtained from the samples were utilized to assess total antioxidant status (TAS) and total oxidant status (TOS), following the assay procedures outlined in the respective kit protocols. Initially, each sample was combined with Reagent 1, and absorbance was recorded using an ELISA microplate reader (TAS at 660 nm; TOS at 530 nm) after incubation. Subsequently, Reagent 2 was added, and a second absorbance reading was taken at the same wavelengths. For TAS quantification, a Trolox-equivalent standard curve (1 mmol/L) provided by the kit was used. In TOS analysis, hydrogen peroxide served as the reference standard, with results expressed as micromolar H_2O_2 equivalents per litre (μmol H_2O_2 eq/L). The oxidative stress index (OSI), representing the extent of oxidative imbalance, was computed as the ratio of TOS to TAS expressed as a percentage. For calculations, the resulting unit of TAS, mmol Trolox eq/L, was converted to μmol Trolox eq/L, and the OSI value was calculated using the following formula: OSI = [TOS(μM H_2O_2 eq/L)/TAS(μmol Trolox eq /L)] × 100 [13].

Measurement of Biochemical Parameters in DBTRG Cells by ELISA Kits

The concentrations of TRPM2, PARP-1, Caspase, and ROS in the supernatants of the cells were quantified using commercially available ELISA kits, following the protocols provided by the manufacturers. Briefly, supernatants and standards were added to 96-well microplates and incubated at 37°C for 60 min. After the initial incubation, wells were washed according to the kit guidelines, followed by the addition of chromogenic substrates. Plates were then incubated again at 37°C for 15 min. Finally, a stop solution was applied to terminate the reactions, and absorbance was measured using an ELISA reader (BioTek EL808™) at appropriate wavelengths [36].

Glutathione, Glutathione Peroxidase, and Lipid Peroxidation Levels in DBTRG Cells

Levels of glutathione (GSH), glutathione peroxidase (G-Px), and lipid peroxidation (LipPx) in human glioblastoma cells were analyzed using a V-730 UV spectrophotometer (Japan). For lipid peroxidation assays, cell samples were diluted at a 1 : 9 ratio (2.25 mL) with thiobarbituric acid (TBARS) reagent. A blank was prepared using 0.25 mL phosphate buffer mixed with the same volume ratio of TBARS. Samples and blanks were incubated in a boiling water bath, subsequently cooled, and centrifuged at 3500 rpm. The upper pink-colored phase was measured at 532 nm in a 1 cm pathlength cuvette against the blank.

For GSH quantification, 10% trichloroacetic acid (TCA) and Tris-II buffer were used. A total of 0.1 mL of cell homogenate was mixed with 0.4 mL TCA in an Eppendorf tube and centrifuged. From the resulting supernatant, 0.4 mL was transferred to a glass tube, followed by adding 2.0 mL Tris-II buffer and 0.1 mL

DTNB reagent. The absorbance was recorded at 412 nm.

G-Px activity was determined using Tris-I buffer, GSH solution, cumene hydroperoxide (CHPO), 10% TCA, Tris-II buffer, and DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)]. In this assay, 0.5 mL of cell homogenate was combined with 0.3 mL Tris-I HCl and 0.1 mL CHPO, followed by 0.1 mL of GSH. After a 10 min incubation at room temperature, 1.0 mL TCA was added, and the mixture was centrifuged. Subsequently, 0.1 mL of the supernatant was mixed with 2.0 mL Tris-II buffer and 0.1 mL DTNB, and the absorbance was measured at 412 nm.

Statistical Analysis

Statistical analyses were conducted using SPSS software (version 17.0, USA). Results were presented as mean values and standard deviations (mean ± SD). Group comparisons were assessed using one-way analysis of variance (ANOVA), followed by Tukey's post-hoc test for multiple comparisons. A p-value of < 0.05 was accepted as the threshold for statistical significance.

RESULTS

Cell Viability, Oxidant, and Antioxidant Levels in DBTRG Cells

We examined whether CHR was harmful or protective against DOX therapy in DBTRG cells. Cell viability and TAS levels in DBTRG cells decreased significantly with CHR and DOX treatment. 24 h after CHR treatment, cell viability and TAS levels decreased further after 24 h of DOX (CHR + DOX group) treatment (Fig. 1). A significant decrease in Cell viability (Fig. 1a) and TAS (Fig. 1c) levels was observed in the CHR and DOX-treated groups compared to the CON group ($p \leq 0.001$). A significant increase in TOS (Fig. 1b) and OSI (Fig. 1d) levels was observed in the CHR and DOX-treated groups compared to the CON group ($p \leq 0.001$). CHR and DOX treatment decreased cell viability rate (Fig. 1a) and TAS (Fig. 1c) levels compared to the CON group ($p \leq 0.001$) but decreased further after CHR and DOX co-treatment (CHR + DOX group) compared to the CON group. In parallel with the results of cell viability rate and TAS levels, TOS (Fig. 1b) and OSI (Fig. 1d) levels were increased in the CHR and DOX groups compared to the CON group ($p \leq 0.001$) but increased further in the CHR+DOX groups with the effect of CHR treatment. Furthermore, the TRPM2 channel blocker 2APB was used to show whether TRPM2 channels affect cell viability and oxidant/antioxidant balance in DBTRG cells. There was a significant increase in cell viability (Fig. 1a) and TAS (Fig. 1c) levels and a substantial decrease in TOS (Fig. 1b) and OSI (Fig. 1d) levels in TRPM2 channel blocker 2APB (2APB + DOX) treated groups compared to the combined treatment group (CHR + DOX). These results indicate that 2APB, a TRPM2 channel blocker, affects cell viability and oxidant/antioxidant balance in DBTRG tumour cells.

Glutathione, Glutathione Peroxidase, and Lipid Peroxidation Levels in DBTRG Cells

Antioxidants scavenge ROS. In many cells, the primary function of thiol redox system members like GSH and G-Px is to scavenge ROS [37]. After observing an increase in ROS levels, we have considered that decreased GSH concentration and G-Px activity may induce the activation of the TRPM2 channel in the DBTRG tumour cells. GSH and G-Px levels in DBTRG cells decreased significantly with CHR and DOX treatment. 24 h after CHR treatment, GSH and G-Px levels further reduced after 24 h of DOX (CHR+DOX group) treatment (Fig. 2). A significant decrease in GSH (Fig. 2a), and G-Px (Fig. 2b) levels was observed in the CHR and DOX-treated groups compared to the CON group ($p \leq 0.001$). A significant increase in LipPx (Fig. 2c) levels was observed in the CHR and DOX-treated groups compared to the CON group ($p \leq 0.001$). CHR and DOX treatment decreased GSH (Fig. 2a) and G-Px (Fig. 2b) levels compared to the CON group ($p \leq 0.001$) but decreased further after CHR and DOX co-treatment (CHR + DOX group) compared to the CON group. In parallel with the results of GSH and G-Px levels, LipPx (Fig. 2c) levels were increased in the CHR and DOX group compared to the CON group ($p \leq 0.001$) but increased further in the CHR+DOX groups with the effect of CHR treatment. Furthermore, TRPM2 channel blocker 2APB was used to show whether TRPM2 channels affect oxidant/antioxidant balance in DBTRG cancer cells. There was a significant increase in GSH (Fig. 2a) and G-Px (Fig. 2b) levels and a substantial decrease in LipPx (Fig. 2c) levels in TRPM2 channel blocker 2APB (2APB + DOX) treated groups compared to the combined treatment group (CHR + DOX). These results indicate that 2APB, a TRPM2 channel blocker, affects oxidant/antioxidant balance in DBTRG tumour cells. Our data showed that CHR treatment could further enhance the effect of DOX-induced cell death. These reduced-GSH and G-Px results confirmed the glutathione depletion effect through excessive oxidative stress mediated by TRPM2 channel activation in the DBTRG tumour cells.

Caspase and ROS Levels in DBTRG Cells

The activation of TRPM2 channels in cells leads to ROS activation in the mitochondria, resulting in cell death and apoptosis [19]. In this context, we evaluated ROS and caspase status in cancer cells. ROS and caspase levels in DBTRG cells increased significantly with CHR and DOX treatment. 24 h after CHR treat-

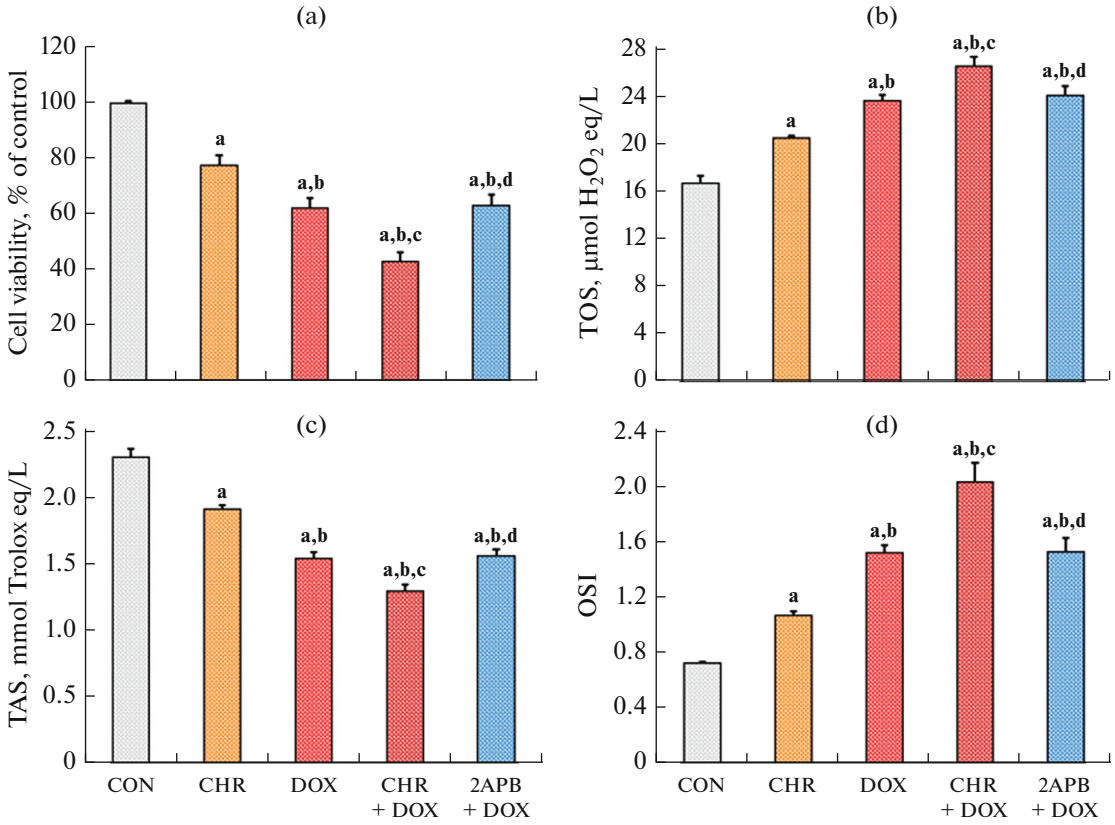

Fig. 1. Effect of CHR on Cell viability (a), TOS (b), TAS (c), and OSI (d) levels in human glioblastoma tumour cells after DOX-induced cytotoxicity (mean ± SD) ($^a p \le 0.001$ vs. CON group; $^b p \le 0.001$ vs. CHR group; $^c p \le 0.001$ vs. DOX group; $^d p \le 0.001$ vs. CHR + DOX group).

ment, ROS and caspase levels increased further after 24 h of DOX (CHR+DOX group) treatment (Fig. 3). A significant increase in ROS (Fig. 3a), Casp-3 (Fig. 3b), and Casp-9 (Fig. 3c) levels was observed in the CHR and DOX-treated groups compared to the CON group ($p \le 0.001$). The combination of CHR and DOX (CHR + DOX group) treatment made the treatment even more effective. This data showed that the effect of DOX-induced ROS and caspase could be further enhanced by CHR treatment in DBTRG cells. Furthermore, TRPM2 channel blocker 2APB was used to show whether TRPM2 channels affect caspase and ROS levels in DBTRG cells. There was a significant decrease in ROS (Fig. 3a), Casp-3 (Fig. 3b), and Casp-9 (Fig. 3c) levels in the TRPM2 channel blocker 2APB (2APB+DOX group) treated groups compared to the combined treatment group (CHR+DOX). These results indicate that TRPM2 channels affect caspase and ROS levels.

TRPM2 Cation Channel and PARP-1 Levels in DBTRG Cells

TRPM2 and PARP-1 expression levels were analyzed, and data are shown in Fig. 4. Expression levels of the TRPM2 cation (Fig. 4a) channel and PARP-1 (Fig. 4b) in the cells were increased by CHR and DOX treatment ($p \le 0.001$). The expression level of TRPM2 and PARP-1 was further increased in the CHR+DOX group ($p \le 0.001$). There was a significant decrease in TRPM2 (Fig. 4a) and PARP-1 (Fig. 4b) levels in the TRPM2 channel blocker 2APB (2APB + DOX) treated groups compared to the combined treatment group (CHR + DOX). In DBTRG tumour cells, CHR and DOX initiate a pro-oxidant response that stimulates ROS and LipPx by inhibiting GSH and G-Px. The DOX and CHR, TRPM2 activation-mediated excessive Ca^{2+} influx cause apoptosis and cell death via the activations of caspase pathways such as Casp-3 and Casp-9 in the DBTRG cell.

DISCUSSION

Drug resistance during therapy, adverse effects in non-tumour tissues, and the ineffectiveness of chemotherapeutic medications in treating a variety of tumours, including GBM, are all growing challenges [19, 38]. For the first time, we evaluated the effects of CHR therapy both by itself and in conjunction with DOX on the TRPM2-channel-mediated signalling pathway in GBM tumour cells. According to our findings, DOX and CHR worked together to promote

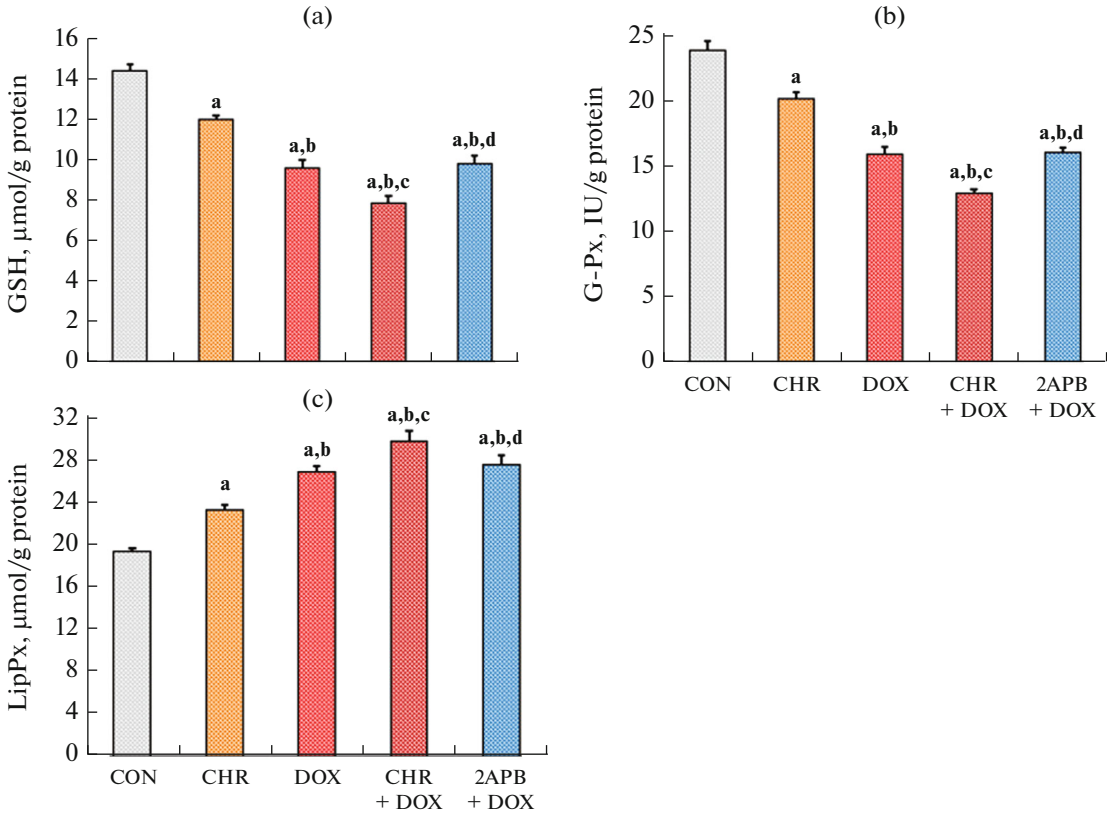

Fig. 2. Effect of CHR on GSH (a), G-Px (b), and LipPx (c) levels in human glioblastoma tumour cells after DOX-induced cytotoxicity (mean ± SD) ([a]$p \le 0.001$ vs CON group; [b]$p \le 0.001$ vs CHR group; [c] $p \le 0.001$ vs DOX group; [d] $p \le 0.001$ vs CHR + DOX group).

excessive Ca^{2+} influx, which resulted in oxidative stress, an increase in ROS, and the activation of TRPM2 by ROS (the Nudix box domain of the TRPM2 channel is sensitive to ROS). Reduced cytosolic GSH and G-Px levels and increased cytosolic ROS generation resulted from the rise in cytosolic Ca^{2+}. Furthermore, through the activation of caspase pathways, including Casp-3 and Casp-9, increased Ca^{2+} influx caused glioblastoma tumor cells to undergo apoptosis and cell death (Fig. 5). As shown in Fig. 5, we found that CHR and DOX act synergistically on glioblastoma cells through the molecular pathways.

The viability of DBTRG cells was observed to be reduced by DOX and CHR+DOX treatments in this investigation; the combined treatment group saw a more cytotoxic impact (Fig. 1a). Increases in ROS, caspases, and LipPx levels may arise from the activation of the TRPM2 channel, which raises cytosolic Ca^{2+} and causes cell death [39, 40]. Our findings suggested that these channels may drive tumour cell death. In contrast, DOX and CHR treatments enhanced ROS (Fig. 3a); they simultaneously elevated TRPM2 expression levels (Fig. 4a). Furthermore, we discovered that the combination treatment group (2APB+DOX group) had higher cell survival than the CHR+DOX group when 2APB blocked TRPM2 channels (Fig. 1a). These findings supported our theory that DOX and CHR's deadly effects on DBTRG cancer cells are mediated via TRPM2. DNA damage can result from elevated ROS [8, 9]. DNA damage leads to the release of ADP-ribose via the activation of PARP-1 and, consequently, to the stimulation of TRPM2 [41, 42].

Furthermore, the TRPM2 channel's Nudix box domain is sensitive to ROS, and elevated ROS causes the TRPM2 channel to become active [10]. The molecular mechanisms of DOX and CHR's antitumor actions remain unclear, even though they are known to generate ROS and damage DNA in tumour cells [43, 44]. The use of CHR in cancer treatment has attracted a lot of attention, and most research has shown that it can stop tumour development by causing tumour cells to undergo apoptosis via several biochemical pathways [45, 46]. Several studies reported that one possible molecular mechanism by which CHR exerts its antitumour activity is by increasing intracellular Ca^{2+} [47, 48]. CHR has been shown to increase ROS and ROS-activated Ca^{2+} entry with a pro-oxidant impact, resulting in Ca^{2+}-dependent

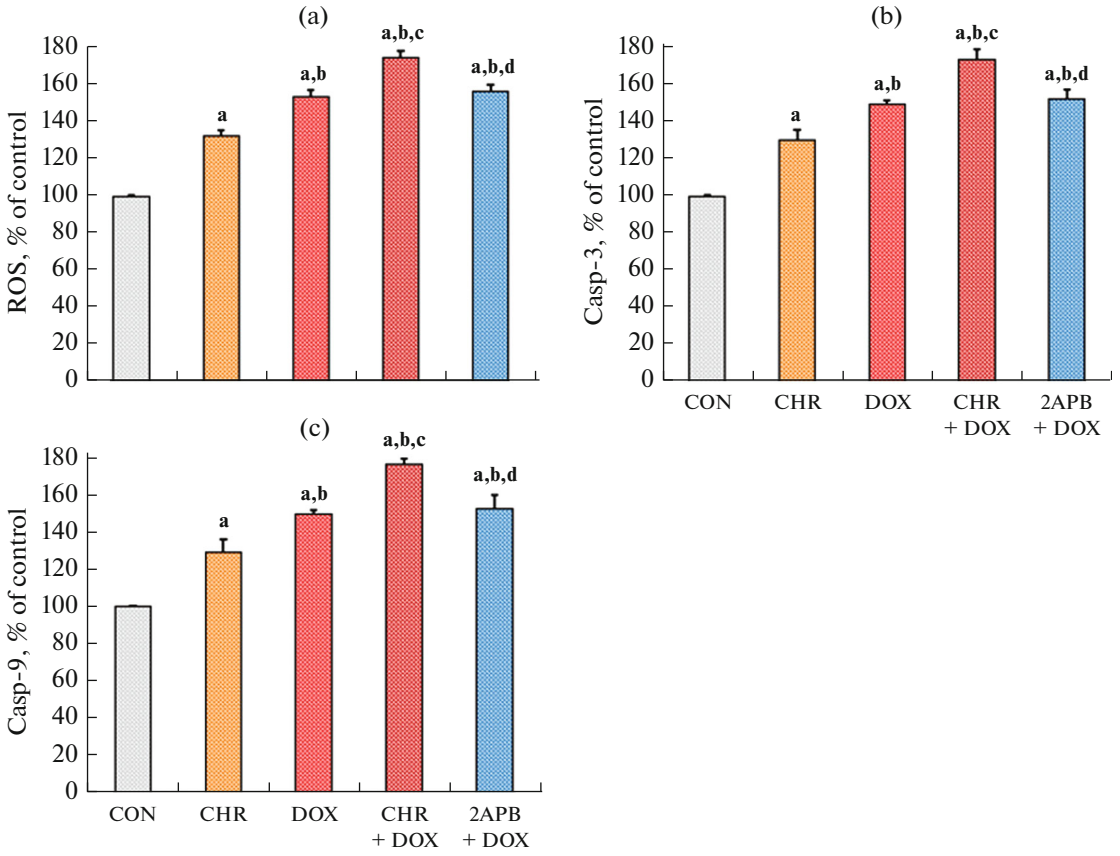

Fig. 3. Effect of CHR on ROS (a), Casp-3 (b), and Casp-9 (c) levels in human glioblastoma tumour cells after DOX-induced cytotoxicity (mean ± SD) ($^{a}p \le 0.001$ vs. CON group; $^{b}p \le 0.001$ vs. CHR group; $^{c}p \le 0.001$ vs. DOX group; $^{d}p \le 0.001$ vs. CHR + DOX group).

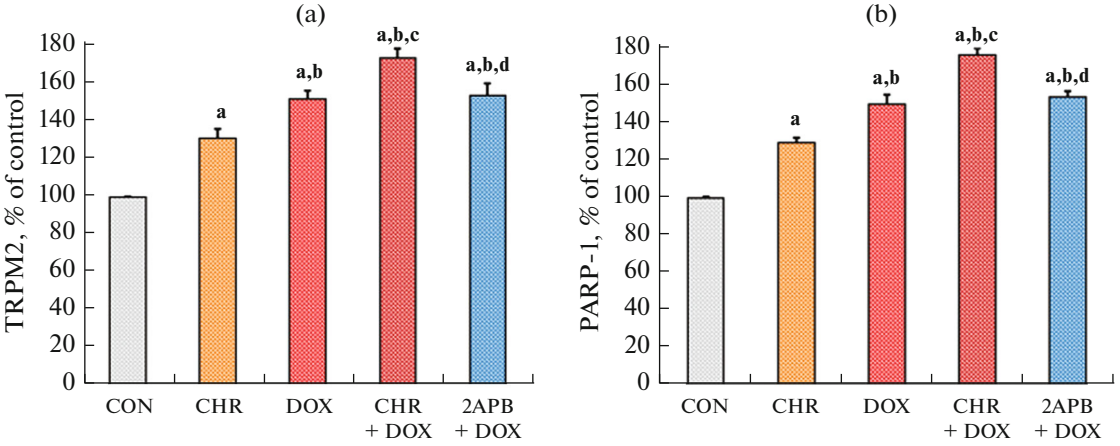

Fig. 4. Effect of CHR on TRPM2 (a) and PARP-1 (b) levels in human glioblastoma tumour cells after DOX-induced cytotoxicity (mean ± SD) ($^{a}p \le 0.001$ vs. CON group; $^{b}p \le 0.001$ vs. CHR group; $^{c}p \le 0.001$ vs. DOX group; $^{d}p \le 0.001$ vs. CHR+DOX group).

lethal effects [48]. Since TRPM2 channels are known to drive intracellular Ca^{2+} elevation, which is crucial for tumor cell death, CHR might increase DOX's antitumor efficacy by activating these channels. This work has shown that whereas TRPM2 inhibition caused a reduction in the 2APB + DOX group, ROS, PARP-1, and TRPM2 expressions—all of which had been elevated by DOX treatment—were further elevated by CHR (Figs. 3a, 3d and 3e). Therefore, in this study, we showed for the first time that one of the

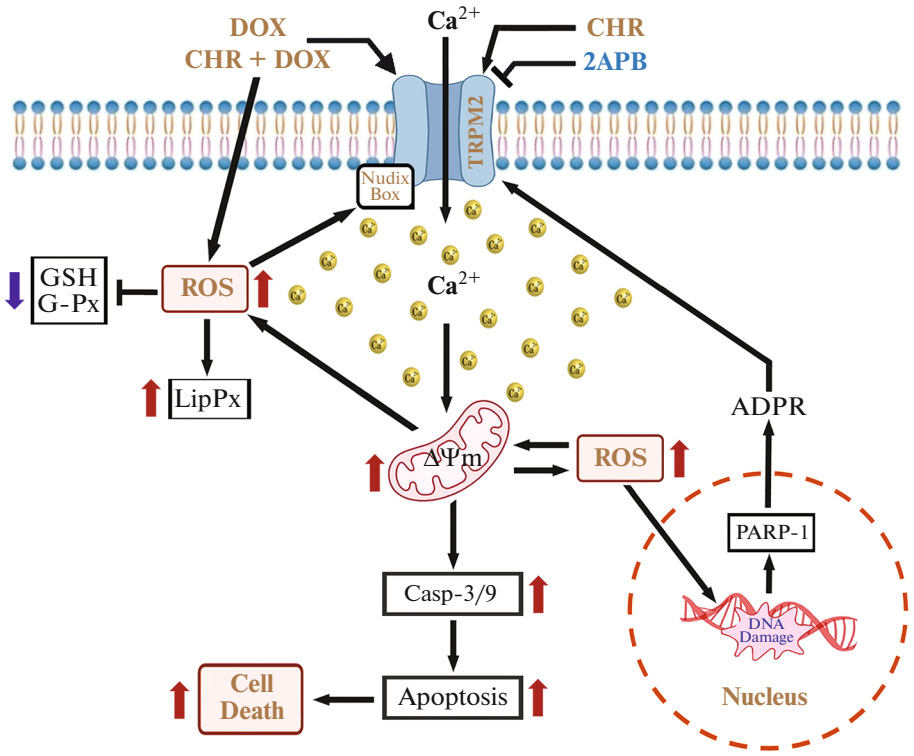

Fig. 5. Damage mechanism in human glioblastoma tumor cells due to excessive ROS increase caused by alteration of TRPM2 channel activation by DOX and CHR. CHR and DOX treatments cause DBTRG cells to generate excessive intracellular ROS. It is shown that ROS and DNA damage (via PARP-1 activation) induce ADP-ribose to stimulate TRPM2. Cytosolic Ca^{2+} increase via TRPM2 activation further increases, leading to an increase in ΔΨm. In turn, the increase in ΔΨm leads to excessive cytosolic ROS production, mitochondrial oxidative stress, and a decrease in cytosolic GSH and G-Px levels. Although nonspecific TRPM2 blockers (2APB) limit TRPM2 activation in cells, PARP1 activation (via DNA damage) induced ADP-ribose (ADPR), intracellular and mitochondrial ROS, leading to TRPM2 induction in cells. Induction of Casp-3 and Casp-9 via mitochondrial Ca^{2+} accumulation in cells, in turn, increases DOX- and CHR-induced ΔΨm and cell death. In addition to this mechanism, TRPM2 activation may be a response to the increase in ROS production caused by CHR and DOX, as well as the decrease in GSH levels and activation of lipid peroxidation, which may further increase oxidative stress and apoptotic effects.

molecular pathways of the anti-tumour effects of CHR and DOX might be the ROS-induced PARP1/ADPR/TRPM2 pathway in DBTRG tumour cells.

Research has demonstrated that in GBM cell lines, TRPM2 activation increases caspase activity and apoptotic levels [4, 10]. Supporting these findings, a similar study reported that TRPM2 inhibition decreased the antitumour effect of cisplatin chemotherapy, and another study reported that targeting the TRPM2 channel promoted cell death in T-cell leukaemia [39, 49]. Our findings demonstrated that suppression of TRPM2 with 2APB (in the 2APB + DOX group) decreased caspases (Casp-3 and Casp-9), whose expression rose with DOX in DBTRG cancer cells (Figs. 3b and 3c). Thus, our findings and other recent research showed that by triggering apoptosis, TRPM2 activation might inhibit the proliferation of tumor cells. The CHR + DOX group had a drop in cell viability and increased apoptosis, while the CHR combination therapy further elevated the levels of Casp-3 and Casp-9 (Figs. 1a, 3b, and 3c). According to recent research, combining chemotherapy with some antioxidants, like curcumin, eicosapentaenoic acid, and selenium, has a synergistic impact on cancer cell lines, including DBTRG, and strengthens anticancer actions by activating TRPM2 [19, 50]. Our research also showed that by activating the TRPM2 channel more than the DOX therapy, the combination CHR+DOX treatment enhanced caspase activation. This implies that during cancer treatment, natural substances like CHR may be able to inhibit tumour development and promote cell death by activating the TRPM2 channel.

The TRPM2 channel is a crucial oxidative stress sensor in the body involved in several pathological processes [51]. Moreover, oxidative stress serves as an activator of TRPM2 channels, whereas their activity can be suppressed by 2-aminoethoxydiphenyl borate (2-APB) [4, 18]. Increased cytosolic free Ca^{2+} concentration from TRPM2 stimulation causes excessive ROS production and a drop in cytosolic GSH and G-Px [52]. Overproduction of ROS and depletion of GSH damage DNA and release ADPR, which acti-

vates TRPM2 and causes excessive entrance of Ca^{2+} into the cell (Fig. 4). In this work, we examined how CHR affected DOX-induced oxidative stress and apoptosis in DBTRG cancer cells, as well as how TRPM2 and 2APB contributed to these outcomes. We observed that the decrease in GSH and G-Px levels and the increase in LipPx levels induced by CHR and DOX were associated with reduced cell viability (Figs. 1a, 2a, 2b, and 2c). CHR + DOX combo treatment significantly exacerbated lipid peroxidation (increased LipPx) and antioxidant depletion. According to recent research, CHR and DOX exhibit pro-oxidant properties that raise ROS levels and lipid peroxidation while lowering antioxidant enzyme activity [32, 53, 54]. Additionally, we discovered in this work that the administration of CHR and DOX-induced the production of ROS and disrupted the oxidant/antioxidant balance in cancer cells (in the treatment groups, TAS reduced while TOS and OSI index rose). Consequently, our findings demonstrated that CHR had a pro-oxidant impact that increased DOX's anti-tumor effectiveness. It is commonly recognised that oxidative stress activates TRPM2 channels [10, 50]. Our results, in line with the literature, indicated that TRPM2 channels were more strongly expressed in the treatment groups and that the OSI index value rose as GSH and G-Px levels decreased. In contrast to the CHR + DOX group, we demonstrated that 2APB treatment blocked TRPM2 channels in the 2APB + DOX group, increasing antioxidant capacity and reducing oxidative parameters. Therefore, we have shown that the anticancer impact of CHR and DOX treatments is mediated by oxidative stress-induced activation of TRPM2 channels. This effect was more substantial in the combined treatment group.

CONCLUSIONS

In conclusion, CHR was observed to have anticancer effects in DBTRG cancer cells. It was determined that using CHR and DOX further reduced cell viability in DBTRG cells. It was observed that TRPM2 channel activation in DBTRG cells in DOX and CHR treatments played an essential role in this damage mechanism. With the extensive studies to be conducted, CHR treatment and TRPM2 channel activation regulation will offer critical therapeutic approaches in producing anticancer drugs for GBM patients.

ABBREVIATIONS AND NOTATION

CHR	chrysin
DMEM	Dulbecco's Modified Eagle Medium
DOX	doxorubicin
DBTRG	human glioblastoma tumour cells
FBS	fetal bovine serum
GBM	glioblastoma multiforme
GSH	glutathione
G-Px	glutathione peroxidase
LipPx	lipid peroxidation levels
PARP-1	poly ADP ribose polymerase-1
TRPM2	transient receptor potential melastatin 2
TAS	total antioxidant status
TOS	total oxidant status
2APB	2-aminoethoxydiphenyl borate

AUTHOR CONTRIBUTION

Supervision: YY. Study design: YY, KY. Literature search: YY, KY. Data collection: YY, KY. Data interpretation: YY, KY. Statistical analysis: YY. Manuscript preparation: YY. All authors reviewed the manuscript.

FUNDING

This work was supported by ongoing institutional funding. No additional grants to carry out or direct this particular research were obtained.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This research used cells propagated through commercially available cell culture. Ethics committee approval is not required in this study. The study was conducted following the international declaration, guidelines, etc.

CONFLICT OF INTEREST

The authors of this work declare that they have no conflicts of interest.

REFERENCES

1. Shakya S., Gromovsky A.D., Hale J.S., Knudsen A.M., Prager B., Wallace L.C., Penalva L.O., Brown H.A., Kristensen B.W., Rich J.N. 2021. Altered lipid metabolism marks glioblastoma stem and non-stem cells in separate tumor niches. *Acta Neuropathol. Commun.* **9** (1), 101.
2. Chen S.-J., Hoffman N.E., Shanmughapriya S., Bao L., Keefer K., Conrad K., Merali S., Takahashi Y., Abraham T., Hirschler-Laszkiewicz I. 2014. A splice variant of the human ion channel TRPM2 modulates neuroblastoma tumor growth through hypoxia-inducible factor (HIF)-1/2α. *J. Biol. Chem.* **289** (52), 36284–36302.
3. Ramalho M.J., Alves B., Andrade S., Lima J., Loureiro J.A., Pereira M.C. 2024. Folic-acid-conjugated poly (lactic-co-glycolic acid) nanoparticles loaded with gallic acid induce glioblastoma cell death by reactive-oxygen-species-induced stress. *Polymers.* **16** (15), 2161.
4. Akyuva Y., Nazıroğlu M. 2023. Silver nanoparticles potentiate antitumor and oxidant actions of cisplatin via

the stimulation of TRPM2 channel in glioblastoma tumor cells. *Chem. Biol. Interact.* **369**, 110261.

5. Hsu S.-S., Chou C.-T., Liao W.-C., Shieh P., Kuo D.-H., Kuo C.-C., Jan C.-R., Liang W.-Z. 2016. The effect of gallic acid on cytotoxicity, Ca2+ homeostasis and ROS production in DBTRG-05MG human glioblastoma cells and CTX TNA2 rat astrocytes. *Chem. Biol. Interact.* **252**, 61–73.
6. Yazğan Y., Nazıroğlu M. 2021. Involvement of TRPM2 in the neurobiology of experimental migraine: Focus on oxidative stress and apoptosis. *Mol. Neurobiol.* **58** (11), 5581–5601.
7. Naziroglu M., Oz A., Yildizhan K. 2020. Selenium and neurological diseases: Focus on peripheral pain and TRP channels. *Curr. Neuropharmacol.* **18** (6), 501–517.
8. Akpınar O., Özşimşek A., Güzel M., Nazıroğlu M. 2020. *Clostridium botulinum* neurotoxin A induces apoptosis and mitochondrial oxidative stress via activation of TRPM2 channel signaling pathway in neuroblastoma and glioblastoma tumor cells. *J. Recept. Signal Transduct. Res.* **40** (6), 620–632.
9. Hajnóczky G., Csordás G., Das S., Garcia-Perez C., Saotome M., Roy S.S., Yi M. 2006. Mitochondrial calcium signalling and cell death: Approaches for assessing the role of mitochondrial Ca2+ uptake in apoptosis. *Cell Calcium*. **40** (5–6), 553–560.
10. Öcal Ö., Nazıroğlu M. 2022. Eicosapentaenoic acid enhanced apoptotic and oxidant effects of cisplatin via activation of TRPM2 channel in brain tumor cells. *Chem. Biol. Interact.* **359**, 109914.
11. Aye K.T., Wattanapongpitak S., Supawat B., Kothan S., Udomtanakunchai C., Tima S., Pan J., Tungjai M. 2021. Gallic acid enhances pirarubicin-induced anticancer in living K562 and K562/Dox leukemia cancer cells through cellular energetic state impairment and P-glycoprotein inhibition. *Oncol. Rep.* **46** (4), 227.
12. Yıldızhan K., Huyut Z., Altındağ F. 2023. Involvement of TRPM2 channel on doxorubicin-induced experimental cardiotoxicity model: Protective role of selenium. *Biol. Trace Elem. Res.* **201** (5), 2458–2469.
13. Yazğan Y., Yazğan B. 2024. gossypin regulated doxorubicin-ınduced oxidative stress and ınflammation in H9c2 cardiomyocyte cells. *Med. Rec.* **6** (1), 44–49.
14. Han Z., Yang J., Wang P., Bian F., and Jia J. 2023. Oxidative stress induction by narasin augments doxorubicin's efficacy in osteosarcoma. *BMC Pharmacol. Toxicol.* **24** (1), 56.
15. Yıldızhan K., Huyut Z., Altındağ F., Ahlatcı A. 2023. Effect of selenium against doxorubicin-induced oxidative stress, inflammation, and apoptosis in the brain of rats: Role of TRPM2 channel. *Ind. J. Biochem. Biophys.* **60** (3), 177–185.
16. Punia R., Raina K., Agarwal R., Singh R.P. 2017. Acacetin enhances the therapeutic efficacy of doxorubicin in non-small-cell lung carcinoma cells. *PLoS One.* **12** (8), e0182870.
17. Bayir M.H., Yıldızhan K., Altındağ F. 2023. Effect of hesperidin on sciatic nerve damage in STZ-ınduced diabetic neuropathy: Modulation of TRPM2 channel. *Neurotoxic. Res.* **41** (6), 638–647.
18. Yıldızhan K., Nazıroğlu M. 2023. NMDA receptor activation stimulates hypoxia-ınduced TRPM2 channel activation, mitochondrial oxidative stress, and apoptosis in neuronal cell line: Modular role of memantine. *Brain Res.* **1803**, 148232.
19. Ertilav K., Nazıroğlu M. 2023. Honey bee venom melittin increases the oxidant activity of cisplatin and kills human glioblastoma cells by stimulating the TRPM2 channel. *Toxicon*. **222**, 106993.
20. Qu Y.n., Gao R., Wei X., Sun X., Yang K., Shi H., Gao Y., Hu S., Wang Y., Yang J.e. 2022. Gasdermin D mediates endoplasmic reticulum stress via FAM134B to regulate cardiomyocyte autophagy and apoptosis in doxorubicin-induced cardiotoxicity. *Cell Death Dis.* **13** (10), 901.
21. Naz S., Imran M., Rauf A., Orhan I.E., Shariati M.A., Shahbaz M., Qaisrani T.B., Shah Z.A., Plygun S., Heydari M. 2019. Chrysin: Pharmacological and therapeutic properties. *Life Sci.* **235**, 116797.
22. Huo J., Zhang M., Wang X., Zou D. 2021. Chrysin induces osteogenic differentiation of human dental pulp stem cells. *Exp. Cell Res.* **400** (2), 112466.
23. Kasala E.R., Bodduluru L.N., Madana R.M., Gogoi R., Barua C.C. 2015. Chemopreventive and therapeutic potential of chrysin in cancer: Mechanistic perspectives. *Toxicol. Lett.* **233** (2), 214–225.
24. Ayna A., Sağ S., Bayav İ., Darendelioğlu E. 2025. Chrysin protects neuronal cells against carboplatin exposure-induced apoptosis and oxidative damage. *J. Cell. Neurosci. Oxid. Stress*. **17** (1), 1237–1244.
25. Zeinali M., Rezaee S.A., Hosseinzadeh H. 2017. An overview on immunoregulatory and anti-inflammatory properties of chrysin and flavonoids substances. *Biomed. Pharmacother.* **92**, 998–1009.
26. Middleton E., Jr., Kandaswami C., Theoharides T.C. 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol. Rev.* **52** (4), 673–751.
27. Ren J., Cheng H., Xin W.Q., Chen X., Hu K. 2012. Induction of apoptosis by 7-piperazinethylchrysin in HCT-116 human colon cancer cells. *Oncol. Rep.* **28** (5), 1719–1726.
28. Bahadori M., Baharara J., Amini E. 2016. Anticancer properties of chrysin on colon cancer cells, in vitro and in vivo with modulation of caspase-3,-9, Bax and Sall4. *Iran. J. Biotechnol.* **14** (3), 177.
29. Zheng H., Li S., Pu Y., Lai Y., He B., Gu Z. 2014. Nanoparticles generated by PEG-Chrysin conjugates for efficient anticancer drug delivery. *Eur. J. Pharm. Biopharm.* **87** (3), 454–460.
30. Sabzichi M., Mohammadian J., Bazzaz R., Pirouzpanah M.B., Shaaker M., Hamishehkar H., Chavoshi H., Salehi R., Samadi N. 2017. Chrysin loaded nanostructured lipid carriers (NLCs) triggers apoptosis in MCF-7 cancer cells by inhibiting the Nrf2 pathway. *Process Biochem.* **60**, 84–91.
31. Cheung J.Y., Miller B.A. 2017. Transient receptor potential–Melastatin Channel Family Member 2: friend or foe. *Trans. Am. Clin. Climatol. Assoc.* **128**, 308.
32. Maruhashi R., Eguchi H., Akizuki R., Hamada S., Furuta T., Matsunaga T., Endo S., Ichihara K., Ikari A. 2019. Chrysin enhances anticancer drug-induced toxic-

ity mediated by the reduction of claudin-1 and 11 expression in a spheroid culture model of lung squamous cell carcinoma cells. *Sci. Rep*. **9** (1), 13753.

33. Al-Oudat B.A., Alqudah M.A., Audat S.A., Al-Balas Q.A., El-Elimat T., Hassan M.A., Frhat I.N., Azaizeh M.M. 2019. Design, synthesis, and biologic evaluation of novel chrysin derivatives as cytotoxic agents and caspase-3/7 activators. *Drug Des. Dev. Ther.* **13**, 423–433.

34. Wang J., Wang H., Sun K., Wang X., Pan H., Zhu J., Ji X., Li X. 2018. Chrysin suppresses proliferation, migration, and invasion in glioblastoma cell lines via mediating the ERK/Nrf2 signaling pathway. *Drug Des. Dev. Ther*. **12**, 721–733.

35. Maszczyk M., Banach K., Karkoszka M., Rzepka Z., Rok J., Beberok A., Wrześniok D. 2022. Chemosensitization of U-87 MG glioblastoma cells by neobavaisoflavone towards doxorubicin and etoposide. *Int. J. Mol. Sci*. **23** (10), 5621.

36. Yazğan B., Yazğan Y. 2022. Potent antioxidant alpha lipoic acid reduces STZ-induced oxidative stress and apoptosis levels in the erythrocytes and brain cells of diabetic rats. *J. Cell. Neurosci. Oxid. Stress*. **14** (2), 1085–1094.

37. Nahar K., Hasanuzzaman M., Fujita M. 2016. Physiological roles of glutathione in conferring abiotic stress tolerance to plants. *Abiotic Stress Response in Plants*. 155–184.

38. Krakstad C., Chekenya M. 2010. Survival signalling and apoptosis resistance in glioblastomas: Opportunities for targeted therapeutics. *Mol. Cancer*. **9**, 135.

39. Gökçe Kütük S., Gökçe G., Kütük M., Gürses Cila H.E., Nazıroğlu M. 2019. Curcumin enhances cisplatin-induced human laryngeal squamous cancer cell death through activation of TRPM2 channel and mitochondrial oxidative stress. *Sci. Rep*. **9** (1), 17784.

40. Çınar R., Yıldızhan K. 2025. Curcumin protects against MPP+-induced neurotoxicity in SH-SY5Y cells by modulating the TRPV4 channel. *Mol. Biol. Rep.* **52** (1), 255.

41. Akyuva Y., Nazıroğlu M., Yıldızhan K. 2021. Selenium prevents interferon-gamma induced activation of TRPM2 channel and inhibits inflammation, mitochondrial oxidative stress, and apoptosis in microglia. *Metab. Brain Dis*. **36**, 285–298.

42. Neves K.B., Alves-Lopes R., Montezano A.C., Touyz R.M. 2023. Role of PARP and TRPM2 in VEGF inhibitor-induced vascular dysfunction. *J. Am. Heart Assoc*. **12** (4), e027769.

43. Xu J., Zhang Z., Hu H., Yang Y., Xiao C., Xi L., Lu J., Tian S., Zhao H. 2024. Synergistic antitumor effects of Peiminine and Doxorubicin on breast cancer through enhancing DNA damage via ZEB1. *Biomed. Pharmacother*. **173**, 116353.

44. Songbo M., Lang H., Xinyong C., Bin X., Ping Z., Liang S. 2019. Oxidative stress injury in doxorubicin-induced cardiotoxicity. *Toxicol. Lett*. **307**, 41–48.

45. Raina R., Afroze N., Sundaram M.K., Haque S., Bajbouj K., Hamad M., Hussain A. 2021. Chrysin inhibits propagation of HeLa cells by attenuating cell survival and inducing apoptotic pathways. *Eur. Rev. Med. Pharmacol. Sci*. **25** (5), 2206–2220.

46. Salari N., Faraji F., Jafarpour S., Faraji F., Rasoulpoor S., Dokaneheifard S., Mohammadi M. 2022. Anti-cancer activity of chrysin in cancer therapy: A systematic review. *Indian J. Surg. Oncol*. **13** (4), 681–690.

47. Bae Y., Lee S., Kim S.-H. 2011. Chrysin suppresses mast cell-mediated allergic inflammation: Involvement of calcium, caspase-1 and nuclear factor-κB. *Toxicol. Appl. Pharmacol*. **254** (1), 56–64.

48. Lim W., Ryu S., Bazer F.W., Kim S.M., Song G. 2018. Chrysin attenuates progression of ovarian cancer cells by regulating signaling cascades and mitochondrial dysfunction. *J. Cell. Physiol*. **233** (4), 3129–3140.

49. Klumpp D., Misovic M., Szteyn K., Shumilina E., Rudner J., Huber S.M. 2016. Targeting TRPM2 channels impairs radiation-induced cell cycle arrest and fosters cell death of T cell leukemia cells in a Bcl-2-dependent manner. *Oxid. Med. Cell. Longevity*. 2016 (1), 8026702.

50. McKamey S.G., Jira L.R., Tweed C.M., Blake S.D., Powell D.P., Daghistani A.T., Koh D.W. 2022. Antagonism of the transient receptor potential melastatin-2 channel leads to targeted antitumor effects in primary human malignant melanoma cells. *Int. J. Oncol*. **60** (4), 43.

51. Yağcı T., Çınar R., Altıner H.İ., Dündar R., Yıldızhan K. 2025. The role of TRPM2 channel in doxorubicin-induced cell damage in laryngeal squamous cancer cells. *Dokl. Biochem. Biophys*. **520**, 123–129.

52. Wang L., Negro R., Wu H. 2020. TRPM2, linking oxidative stress and Ca2+ permeation to NLRP3 inflammasome activation. *Curr. Opin. Immunol*. **62**, 131–135.

53. Popov A., Klimovich A., Styshova O., Tsybulsky A., Hushpulian D., Osipyants A., Khristichenko A., Kazakov S., Ahuja M., Kaidery N. 2022. Probable mechanisms of Doxorubicin antitumor activity enhancement by ginsenoside Rh2. *Molecules*. **27** (3), 628.

54. Noole V., Krishna T., Godeshala S., Meraji S., Rege K., Reddy C.K., Kedika B. 2022. Synthesis and biological evaluation of new 1, 2, 3-triazole derivatives of the chrysin flavonoid as anticancer agents. *Anticancer Agents Med. Chem*. **22** (1), 160–168.