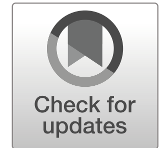
Child's Nervous System (2020) 36:1283–1292
https://doi.org/10.1007/s00381-020-04567-w

ORIGINAL ARTICLE



Selenium enhances TRPA1 channel-mediated activity of temozolomide in SH-SY5Y neuroblastoma cells

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Received: 23 August 2019 / Accepted: 28 February 2020 / Published online: 7 March 2020
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Abstract

Purpose Neuroblastoma is a malignant solid tumor that originates from the sympathetic nervous system in early childhood. Temozolomide is used for treatment in high-risk groups with low treatment response of neuroblastomas. TRPA1 channels in neuroblastoma cells are calcium permeable channels that can be activated by reactive oxygen species (ROT). In this study, we aimed to evaluate the level of activity of temozolomide and selenium in neuroblastoma cells via TRPA1 channels.

Method Seven main groups were formed using SH-SY5Y neuroblastoma cells. The control was divided into temozolomide (TMZ) (100 μM, 24 h), TMZ+SEL+AP18, SEL (sodium selenite, 100 μM, 24 h), and SEL+AP18 groups. Intergroup calcium signaling, intracellular reactive oxygen species, caspase-3 and caspase-9, and mitochondrial depolarization analyses were performed by channel activation with TRPA1 agonist cinnamaldehyde in all groups.

Results Cytosolic calcium concentration, apoptosis, caspase-3 and caspase-9 activation, mitochondrial membrane depolarization, and ROT levels were higher in TMZ ($p < 0.001$), TMZ+SEL ($p < 0.001$), and SEL ($p < 0.05$) groups than the control group. TRPA1 was lower in TTMZ+AP18, TMZ+SEL+AP18, and SEL+AP18 groups with channel blockers than respectively TMZ, TMZ+SEL, and SEL groups without channel blockers ($p < 0.05$).

Conclusion The use of selenium with temozolomide increased the apoptotic efficacy of temozolomide via TRPA1 channels on tumor cells.

Keywords Temozolomide · Neuroblastoma · Oxidative stress · TRPA1 · SH-SY5Y · Selenium

Introduction

Neuroblastoma originating from out-of-control primitive cells of the sympathetic nervous system is the most common solid tumor in early childhood after central nervous system tumors. Neuroblastoma is considered low, medium, and high risk depending on the response to treatment. The high-risk group is characterized by N-myc gene amplification on the second chromosome, metastases, and recurrent masses that cannot be resected. The long-term survival rate of this group of patients is only 30–40%. Nearly 20% of high-risk neuroblastoma patients are advanced stage at the time of diagnosis and are refractory to standard treatments. The 5-year survival rate of the relapse and refractory group patients is approximately 20%. Treatment planning of these patients is difficult due to disease heterogeneity, treatment resistance, and organ toxicity. Therefore, many studies have been carried out to better understand neuroblastoma biology and to discover tumor-specific molecular anomalies and targets to treat high-risk patients. In this patient group, chemotherapy drugs and other treatment methods may need to be used in combination [1–3].

The efficacy of temozolomide, a DNA alkylating agent of the imidazotetrazine family, is recognized in studies of relapse or refractory neuroblastoma patients [2–4]. Recently, the efficacy of many drugs combined with TMZ on neuroblastoma has been investigated [2, 5–7].

Selenium (Se), a part of the glutathione peroxidase enzyme, has been reported to reduce hydrogen peroxides and various lipid hydroperoxides to harmless products, reducing intracellular reactive oxygen species and preventing apoptosis [8, 9]. However, selenium and various selenium compounds have been shown to have apoptosis-inducing and anti-

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proliferative effects at nontoxic doses. Therefore, they are considered potential chemopreventive and chemotherapeutic agents for cancer [10, 11].

Drugs stimulate the production of reactive oxygen species (ROS) resulting in the activation of cation channels that allow Ca^{2+} ion transitions into the cell, such as transient receptor potential (TRP) channels [12–17]. Increased cytosolic calcium and ROS levels induce apoptosis by increasing oxidative stress [12].

TRP channels are channels that are highly selective to oxidative stress mediators such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) and have non-selective permeability to cations such as calcium, sodium, and potassium [14–20]. It has been found that TRP channels play an important role in the pathophysiology of neuroblastoma and many other neurodegenerative diseases as do drugs used indirectly through these channels [21, 22]. TRP ankyrin 1 (TRPA1) channels, one of the six TRP channels, are sensitive to acute and inflammatory pain signals and play a role in the etiology and progression of diseases [21, 23]. TRPA1 is activated by endogenous and environmental irritants under oxidative stress conditions and causes calcium ions to accumulate in the cytosol [14, 24, 25].

In this study, we aimed to investigate the effect of the combined administration of selenium and temozolomide on neuroblastoma cell culture via TRPA1 channels.

Methods

Cell culture

Human neuroblastoma cell line (SH-SY5Y) was obtained from American Type Culture Collection (ATCC) (Manassas, VA). Cells were cultured in Ham's F12 and Dulbecco's modified eagle mediums at a 1:1 ratio containing 10% FBS (fetal bovine serum) (Fisher Scientific) and 1% pen./strep. antibiotic combination in 8–10 flasks (filter cap, sterile, 5 ml, 25 cm^2). Cells were incubated in T25 flasks at 37 °C at 5% CO_2 in a humidified incubator. After cells reached 75–85% confluence, they were incubated with the chemical compounds described in the section on groups. Cells were examined daily for evidence of contamination. After chemical treatments, washed cells were detached with 0.25% Trypsin–EDTA from T25 flasks then 4 ml fresh medium was added per flask. Cell suspensions were collected using a recharged automatic pipette and transferred into the 15-ml falcon tubes. Cells were centrifuged (100*g*, 5 min) then the supernatants were removed and centrifugation was repeated by adding fresh medium into the sterile falcon tubes to wash the cells and make them ready for use in experiments.

Reagents

DMEM, Ham's F12, Trypsin–EDTA, AP18, fetal bovine serum, penicillin-streptomycin, dimethyl sulfoxide, Dihydrorhodamine-123 (DHR 123), and cinnamaldehyde were obtained from Sigma Aldrich (St. Louis, MO). Fura 2 (AM) calcium fluorescent dye was purchased from Calbiochem (Darmstadt, Germany). MTT (3-(4,5-Dimethylthiazol-2-yl) -2,5-Diphenyltetrazolium Bromide) dye was obtained from Thermo Fischer (Waltham, MA USA). Caspase-3 (AC-DEVD-AMC) and caspase-9 (AC-LEHD-AMC) substrates were obtained from Enzo (Lausanne, Switzerland). APOPercentage assay with releasing buffer was purchased from Biocolor (Belfast, Northern Ireland). Mitochondrial stain 5,50, 6,60-tetrachloro-1,10,3,30-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) and Probenecid were obtained from Santa Cruz (Dallas, Texas, USA). Pluronic® F-127 was obtained from Biovision (San Francisco, USA).

Groups:

The study was planned as seven main groups:

Group 1 (control): None of the study drugs was used and cells were preserved in a flask with the same cell culture conditions.
Group 2 (TMZ): Cells were incubated with 100 μM temozolomide (TMZ) for 24 h [26].
Group 3 (TMZ+AP18): Cells were incubated with 100 μm temozolomide for 24 h and then incubated with AP18 (TRPA1 channels antagonist, 0.1 mM, 30 min).
Group 4 (TMZ+SEL): Cells were incubated with 100 μm temozolomide for 24 h and then incubated with 100 μM selenium (sodium selenite) for 24 h.
Group 5 (TMZ+SEL+AP18): Cells were incubated with 100 μm temozolomide for 24 h, with 100 μM selenium (sodium selenite) for 24 h and with AP18 (TRPA1 channels antagonist, 0.1 mM) for 30 min.
Group 6 (SEL): Cells were incubated with 100 μM selenium (sodium selenite) for 24 h [11].
Group 7 (SEL+AP18): Cells were incubated with 100 μM selenium (sodium selenite) for 24 h and then with AP18 (TRPA1 channels antagonist, 0.1 mM) for 30 min.

The cells were further treated with cinnamaldehyde (Cinn, 0.1 mM, 10 min) for activation of the TRPA1 channel before related analysis in the presence of normal extracellular calcium (1.2 mM) for MTT, ROS, caspase-3 and caspase-9, and apoptosis level analyses. During calcium signaling analysis (Fura 2 AM), cells were stimulated on 20 cycles by 0.1 mM Cinn in the same extracellular calcium conditions.

Fura-2 loading and measurement of intracellular calcium

After cell culture treatments, neuroblastoma cells were incubated with HEPES-buffered saline (HBS; 5 mM KCl, 145 mM NaCl, 10 mM D-glucose, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM HEPES and 0.1% (w/v) bovine serum albumin (BSA); pH 7.4) containing 5 μM fura-2 AM and 0.05% (w/v) Pluronic F-127 for 1 h at 37 °C in the dark. The loaded cells were washed twice with HBS and covered with 1000 μL of HBS supplemented with 2.5 mM probenecid for at least 20 min at 37 °C in the dark to allow for Fura-2 AM de-esterification. Cells were seeded in clear flat-bottom black 96-well culture trays (Grainer Cell Star, Life Sciences, USA) at a density of 3×10^4 cells/per well. Fluorescence emission intensity at 510 nm was determined in individual wells using a plate reader equipped with an automated injection system (Synergy TM H1, Biotek, USA) at alternating excitation wavelengths of 340 and 380 nm every 3 s for 50 acquisition cycles in response to agonists (Cinn, 0.1 mM) added with the automated injector. $[Ca^{2+}]_i$ in cells was expressed as the average emission at 510 nm in individual wells in response to excitation at 340/380 nm normalized to initial fluorescence emission obtained during the first 20 cycles. Measurement of $[Ca^{2+}]$i including staining process modification was performed according to the method of Martinez et al. [27].

Intracellular ROS production measurement

Dihydrorhodamine-123 (DHR-123) is a non-fluorescent, non-charged dye that can easily pass through cell membranes where it is oxidized to cationic rhodamine 123 (Rh-123), which localizes in the mitochondria and exhibits green fluorescence as it is sequestered by mitochondria. The cells (10^6 cells/ml per group) were incubated with DHR 123 (2 μm) at 37 °C for 30 min [28]. The Rh-123 fluorescence intensities were measured in a fluorescence multiplate reader (Synergy™ H1, Biotek, USA). Excitation and emission wavelengths of the analyses were set at 488 nm and 543 nm, respectively. Data were shown as fold change experimental to control.

Apoptosis assay

All apoptotic analyses were performed according to the manufacturer's instructions using the APOPercentage™ assay (Biocolor Ltd., Belfast, Northern Ireland). The APOPercentage™ is a dye-uptake assay that stains only the apoptotic cells with a red dye. When the apoptotic cell membrane loses its asymmetry, the APOPercentage dye is bonded to phosphatidylserine lipids actively, passes through the membranes and is localized into the cells, staining apoptotic cells red, thus allowing the detection of apoptosis by a multiplate reader as previously described [29]. Measurement of apoptosis levels was performed by spectrophotometry at 550 nm (Synergy™ H1, Biotek, USA). Data were shown as fold change experimental to control.

Assay for caspase-3 and caspase-9 activities

Determining caspase-3 and caspase-9 activities was based on methods previously reported [29, 30]. Human neuroblastoma cancer cells were sonicated and cell lysates were incubated with 2 ml of substrate solution with caspase-3 (AC-DEVD-AMC) and caspase-9 substrates (AC-LEHD-AMC) for 1 h at 37 °C. Caspase cleavages were measured with the Synergy™ H1 plate reader (Biotek, USA) with 360 nm (excitation) and 460 nm (emission) wavelengths. Data were quantified as fluorescence (units/mg protein) and presented as fold change over the pre-treatment level (experimental/control).

Mitochondrial membrane potential (JC-1) analyses

The neuroblastoma cells were incubated with JC-1 (1 μM concentration of 5,50, 6,60-tetrachloro-1,10,3,30-tetraethylbenzimi-dazolylcarbocyanine iodide) at 37 °C for 15 min. The green JC-1 signal was quantified at 485 nm excitation wavelength and 535 nm emission wavelength and the red signal at the excitation wavelength of 540 nm and emission wavelength of 590 nm. Fluorescence changes were measured with a fluorescence spectrophotometer (Synergy™ H1, Biotek, USA) [30, 31]. Data were presented as emission ratios (590/535) and fold change over the pre-treatment level (experimental/control).

Cell viability (MTT) assay

Cell viability analysis was evaluated by the MTT assay previously described [32]. Optical density was quantified in an automatic microplate reader (Synergy™ H1, Biotek, USA) at 490 nm (test wavelength) and 650 nm (reference wavelength) to nullify the effect of cell debris. The data are presented as fold change (experimental/control) over the pre-treatment level.

Statistical analyses

All results were presented as means ± standard deviation (SD). Significant values in the groups were assessed with one-way ANOVA. Statistical analyses were calculated using GraphPad Prism version 7.04 for Windows (GraphPad Software, San Diego California, USA). $p < 0.05$ was considered significant.

Results

Effects of TMZ and SEL on 340/380 calcium concentration

The calcium signaling results shown as line plots and columns are presented in Fig. 1a, b. TRPA1 antagonist AP18 and agonist Cinn were used for the evaluation of the activities of SEL and TMZ in SH-SY5Y cell culture. When intracellular calcium ion levels were examined, it was observed that they increased significantly in the temozolomide group compared with the control ($p < 0.001$). Intracellular Ca^{2+} levels decreased in the temozolomide+AP18 group using AP18, a TRPA1 channel inhibitor, compared with the TMZ group ($p < 0.001$). Also, when the TMZ+SEL group was compared with the TMZ group, intracellular calcium levels were determined to be statistically higher in the TMZ+SEL group than the TMZ group ($p < 0.05$). When the SEL group and control group were compared, the calcium level was determined to be statistically higher in the SEL group ($p < 0.001$). It was also determined that the calcium level was lower in the SEL+AP18 group compared with the SEL group ($p < 0.05$).

Effects of TMZ and SEL on reactive oxygen species and apoptosis levels

The ROS levels and apoptotic activities of TRPA1 channel mediators SEL and TMZ in SH-SY5Y cell culture are presented in Figs. 2 and 3, respectively. When the ROS and apoptotic levels were examined, they were determined to statistically increase in the temozolomide group compared with the control ($p < 0.001$). The ROS and apoptosis levels were determined to significantly decrease in the temozolomide+AP18 group with TRPA1 channel inhibitor AP18 compared with the TMZ group ($p < 0.001$). In addition, when the TMZ+SEL group and the TMZ group were compared, the apoptotic levels were determined to be statistically higher in the TMZ+SEL group compared with the TMZ group ($p < 0.001$). When the SEL group and the control group were compared and the ROS and apoptosis levels were examined, they were determined to be statistically higher in the SEL group ($p < 0.001$). The ROS and apoptosis levels were determined to decrease in the SEL+AP18 group compared with the SEL group ($p < 0.001$).

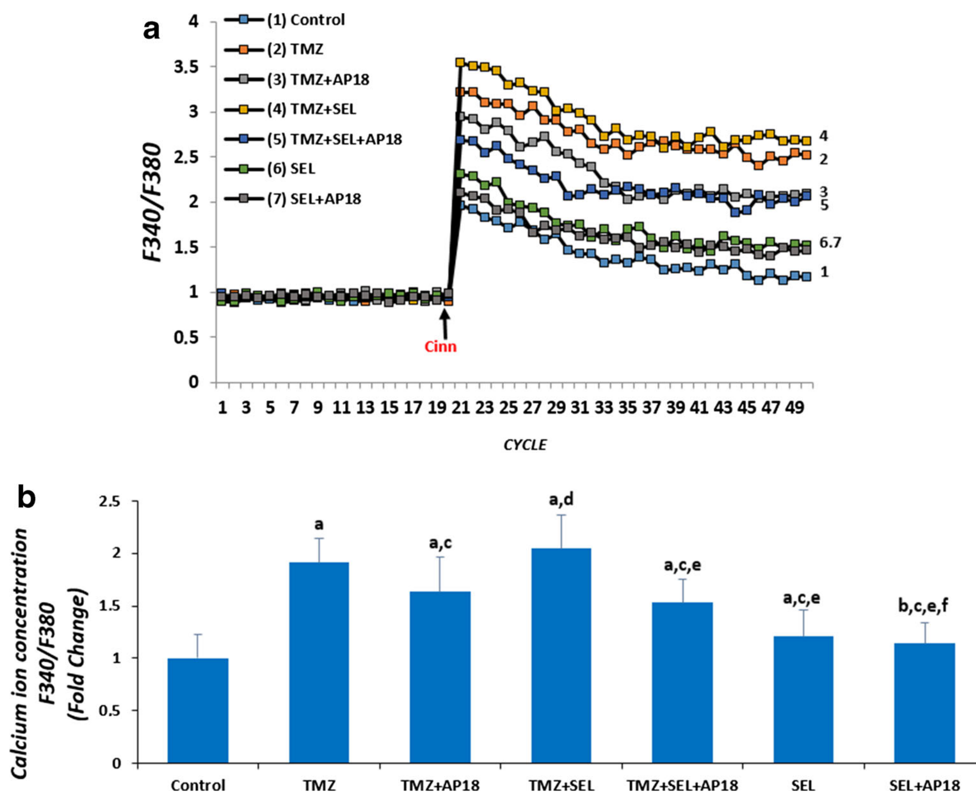

Fig. 1 The effect of temozolomide (TMZ, 100 μM, 24 h) and sodium selenite (SEL, 100 μM, 24 h) on intracellular calcium levels (**a**) and calcium ion concentrations (**b**) in SHSY5Y cells. Cells are stimulated by cinnamaldehyde (Cinn 0.1 mM and on 20th cycle) but they were inhibited by AP18 (0.1 mM for 30 min). (mean ± SD and $n = 3$). $^{a}p < 0.001$ and $^{b}p < 0.05$ vs control, $^{c}p < 0.001$ and $^{d}p < 0.05$ vs TMZ group, $^{e}p < 0.001$ vs TMZ+SEL group, and $^{f}p < 0.05$ vs SEL group

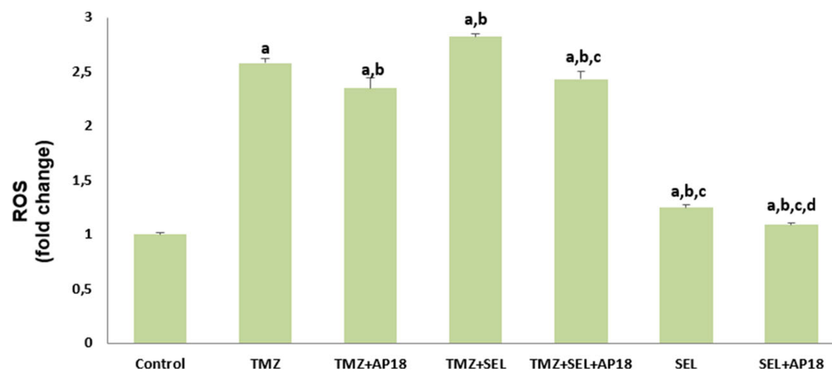

Fig. 2 The effect of temozolomide (TMZ, 100 μM, 24 h) and sodium selenite (SEL, 100 μM, 24 h) on reactive oxygen species levels in SHSY5Y Cells. Cells are stimulated by cinnamaldehyde (Cinn, 0.1 mM for 10 min) but they were inhibited by AP18 (0.1 mM for 30 min) (mean ± SD and $n = 10$). $^{a}p < 0.001$ vs control, $^{b}p < 0.001$ vs TMZ group, $^{c}p < 0.001$ vs TMZ+SEL group, and $^{d}p < 0.001$ vs SEL group

Effect of TMZ and SEL on mitochondrial membrane potential levels

The mitochondrial membrane potential levels are presented in Fig. 4. When the mitochondrial membrane potential levels were examined, they were determined to statistically increase in the TMZ and SEL group compared with the control group ($p < 0.001$). When the TMZ+SEL group and the TMZ group were compared, the mitochondrial membrane potential was determined to be statistically higher in the TMZ+SEL group ($p < 0.001$). When the SEL group and the control group were compared, the mitochondrial membrane potential was determined to be statistically higher in the SEL group ($p < 0.001$). When the TMZ, TMZ+SEL, and SEL groups and the TMZ+AP18, TMZ+SEL+AP18, and SEL+AP18 groups were compared, the mitochondrial membrane potential levels of inhibitor groups were found to be statistically lower ($p < 0.001$ and $p < 0.05$).

Effect of TMZ and SEL on caspase-9 and caspase-3 activities

Caspase-9 and caspase-3 levels are presented in Fig. 5 a and b, respectively). It was determined that caspase-3 and caspase-9 levels increased statistically in the TMZ and SEL group compared with the control ($p < 0.001$). When the TMZ group and the TMZ+SEL group were compared, they were observed to statistically decrease in the TMZ+SEL group ($p < 0.001$). When the SEL group and the control group were compared, caspase-3 and caspase-9 levels were determined to be statistically higher in the SEL group ($p < 0.001$). When the TMZ and TMZ+AP18, TMZ+SEL and TMZ+SEL+AP18, and SEL and SEL+AP18 groups were compared, caspase-3 and caspase-9 levels were determined to be statistically lower in the groups in which an inhibitor was used ($p < 0.001$).

Effect of TMZ and SEL on MTT (cell viability) levels

Cell viability levels are presented in Fig. 6. When the SEL group and the control were compared, it was determined that the cell viability levels were statistically lower in the SEL group ($p < 0.001$). When the TMZ+SEL and the TMZ group were compared, the cell viability levels were statistically lower in the TMZ+SEL group ($p < 0.001$). In groups in which the same chemicals were used, a statistically significant decrease was observed between groups where a channel inhibitor was applied and groups without channel inhibitors ($p < 0.001$).

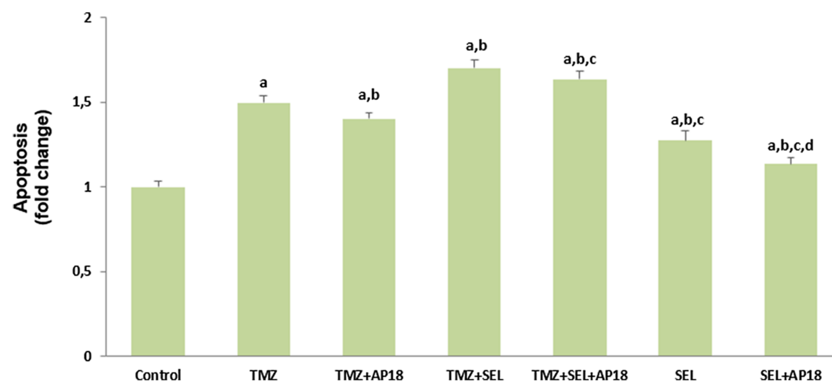

Fig. 3 The effect of temozolomide (TMZ, 100 μM, 24 h) and sodium selenite (SEL, 100 μM, 24 h) on apoptosis levels in SHSY5Y Cells. Cells are stimulated by cinnamaldehyde (Cinn, 0.1 mM for 10 min) but they were inhibited by AP18 (0.1 mM for 30 min) (mean ± SD and $n = 10$). $^{a}p < 0.001$ vs control, $^{b}p < 0.001$ vs TMZ group, $^{c}p < 0.001$ vs TMZ+SEL group, and $^{d}p < 0.001$ vs SEL group

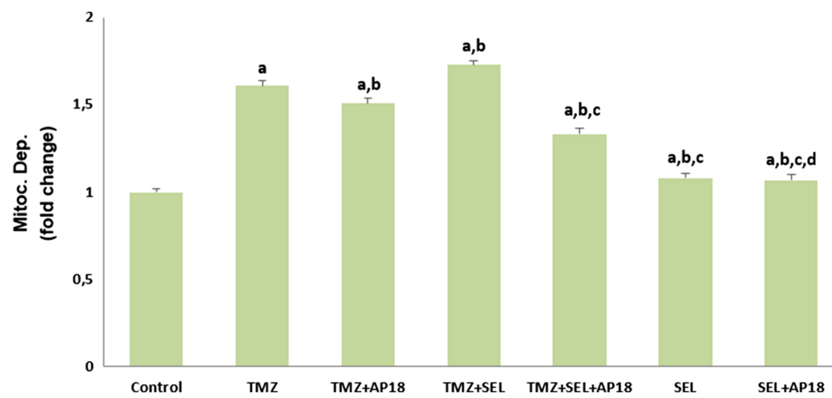

Fig. 4 The effect of temozolomide (TMZ, 100 μM, 24 h) and sodium selenite (SEL, 100 μM, 24 h) on mitochondrial depolarization levels in SHSY5Y Cells. Cells are stimulated by cinnamaldehyde (Cinn, 0.1 mM for 10 min) but they were inhibited by AP18 (0.1 mM for 30 min) (mean ± SD and $n = 10$). [a]p <0 .001 vs control, [b]$p < 0.001$ vs TMZ group, [c]$p < 0.001$ vs TMZ+SEL group, and [d]$p < 0.05$ vs SEL group

Discussion

Neuroblastoma, an embryonic tumor originating from the sympathetic nervous system, is evaluated as low, medium, and high risk according to resistance to treatment. When early staging and appropriate treatment are applied in the low- and medium-risk groups the survival rate can reach 90%, while the long-term survival in the high-risk group is reduced due to relapse and refractory patients [1, 3, 33]. Systemic chemotherapy, radiotherapy, molecularly targeted therapy, immunotherapy, and combinations thereof are used in the treatment of high-risk neuroblastoma patients. Successful results have been reported of combined treatments of temozolomide, one of the drugs used in chemotherapy. While the response rate to treatment of children with recurrent neuroblastoma is 15%, it is reported that 53% of patients using a temozolomide/

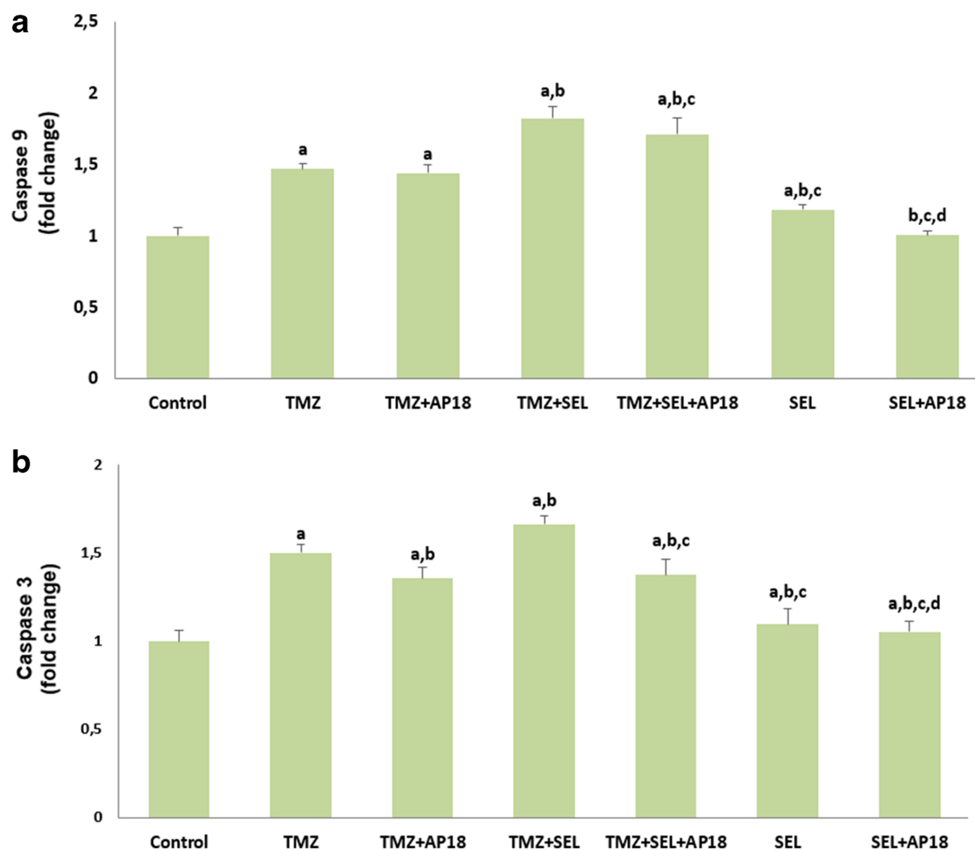

Fig. 5 The effect of temozolomide (TMZ, 100 μM, 24 h) and sodium selenite (SEL, 100 μM, 24 h) on caspase-9 (**a**) and caspase-3 (**b**) levels in SHSY5Y cells. Cells are stimulated by cinnamaldehyde (Cinn, 0.1 mM for 10 min) but they were inhibited by AP18 (0.1 mM for 30 min) (mean ± SD and $n = 10$). [a]$p < 0.001$ vs control, [b]$p < 0.001$ vs TMZ group, [c]$p < 0.001$ vs TMZ+SEL group, and [d]$p < 0.001$ vs SEL group

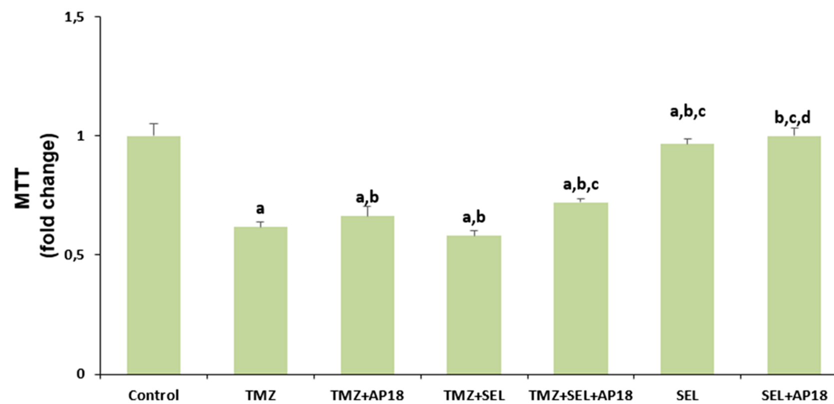

Fig. 6 The effect of temozolomide (TMZ, 100 μM, 24 h) and sodium selenite (SEL, 100 μM, 24 h) on cell viability levels in SHSY5Y Cells. Cells are stimulated by cinnamaldehyde (Cinn, 0.1 mM for 10 min) but they were inhibited by AP18 (0.1 mM for 30 min) (mean ± SD and n = 10). [a]$p < 0.001$ vs control, [b]$p < 0.001$ vs TMZ group, [c]$p < 0.001$ vs TMZ+SEL group, and [d]$p < 0.001$ vs SEL group

irinotecan combination became stable. Currently, a variety of combinations of temozolomide have been studied by targeting aurora A kinase, angiogenesis, chemoimmunotherapy in patients with refractory neuroblastoma [2]. The SHSY-5Y cell line is frequently used as a neuroblastoma tumor model [5, 21, 30]. We aimed to evaluate the effect of temozolomide in combination with selenium via TRPA1 channels in SHSY-5Y neuroblastoma cells.

There are six subtypes of transient receptor potential (TRP) channels in mammals: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystic kidney disease), TRPML (mucolipin), and TRPA (ankyrin) [34]. Abnormal expression of TRP channels has been shown in many studies on different types of cancer and the relationship between cancer surveillance and expression of TRP channels has been demonstrated [35]. The effects of TRP channels on invasion, proliferation, diffusion, and tumor vascularization of cancer cells have been reported [36]. Although there are studies investigating the role of TRP channels in neuroblastoma in the literature, there are limited numbers of studies examining the effects of chemotherapeutic agents on TRP channels in neuroblastoma. In studies on TRP channels in neuroblastoma cells, overexpression of TRPM8, TRPV1, TRPM2, TRPC1, and TRPA1 channels in neuroblastoma cells has been shown [21, 30, 37, 38]. It has been reported that when the TRPA1 channel is activated in neuroblasting cells, it increases intracellular Ca^{2+} levels and plays an important role in all stages of apoptosis. [21, 39]. In our study, the presence of TRPA1 channels in neuroblastoma cells and apoptosis played an important role in the whole molecular pathway.

In a 2015 study by Çıtışlı et al. on neuroblastoma cells using temozolomide, it was reported that 24 and 48 h 1 mM temozolomide incubation caused 50% Ic-50 (Ic-fifty) cell viability [40]. However, the same Ic-50 ratio was reached with 0.1 mM/24 h temozolomide in our study.

In a 2010 study conducted by Maraldi et al. investigating the efficacy of selenium on neuroblastoma, they found that the decrease in cell viability was higher compared with the prostate and kidney cell lines of SKNBE neuroblastoma cell lines treated with low concentrations (0.1 micromol/24 h) of sodium selenite. In addition, they showed that the use of selenium at this dose increased caspase activation in SKNBE neuroblastoma cell lines, induced apoptosis, and increased ROS/RNS ratios despite increased glutathione activity [41]. In a study on fish liver cells in vitro, it was found that selenium, which is known to be dose-dependent toxic, increased intracellular ROS and apoptosis and reduced cell viability. This increase in apoptosis was found to be related to the caspase-3/7 ratio. It has been found that exposure of hepatocytes to different doses of non-toxic selenium causes oxidative damage by inducing the imbalance of intracellular glutathione (GSH) redoxin [11]. Our study also showed that when compared with the selenium and TMZ+SEL group, the TMZ and the control group, caspase-3 and caspase-9 levels, MMT, and apoptosis were significantly increased.

In the study conducted by Cheng Y et al. in T98G, LN229, U251, and U87MG glioblastoma cell lines, it was found that 100 micromol/48 h temozolomide incubation decreased cell viability, and the application of temozolomide 150 and 200 μmol for the same period further reduced cell viability in the same cell cultures. In the same study, it was found that temozolomide+selenium incubation (15 mk/kg) was more effective ($p < 0.001$) in the brain tumor model in mice compared with the temozolomide group without selenium [42]. In the 2013 Nabissi M. et al. study, it was reported that administration of temozolomide (400 micromol/24 h) in glioblastoma cells reduced cell viability through TRPV2 channels [43]. In our study, when TMZ was used with SEL, it was found that TMZ increased significantly compared with the use of TMZ alone. Our study showed that when TMZ is used together in neuroblastoma cells, the anticancer activity of SEL increases and its effect on TRPA1 channels contributes to this situation as in previous studies.

Oxidative stress defense against reactive oxygen species (ROS) accumulated during tumor formation is associated with cancer cell survival. In a study investigating the effects of TRPA1 channels on oxidative stress in cancer cells, TRPA1 was said to increase resistance to ROS-producing chemotherapies. Inhibition of TRPA1 suppressed xenograft tumor growth and increased chemosensitivity in contrast to our study. In addition, TRPA1 channels did not have any effect on decreasing intracellular ROS levels. However, oxidative stress defense mechanisms containing TRPA1 that can be used in cancer treatments have been shown to increase intracellular Ca^{+2} levels in response to ROS increase [44].

Intracellular calcium ions (Ca^{2+}) serve as secondary messengers that regulate gene transcription, cell proliferation, migration, and death. The increase in intracellular Ca^{2+} and ROS levels triggers the molecular cascade of apoptosis and this leads to changes in nucleic acid, protein, and lipid in the cell structure due to oxidative stress and increase in mitochondrial depolarization [31–45]. The amount of intracellular Ca^{2+} in cancer cells varies. This variability has been shown to increase in tumor initiation, cancer invasion, progression, apoptosis, angiogenesis, and abnormal differentiation [46]. In cancer cells, TRP channels are effective in regulating intracellular calcium content by directly changing Ca^{2+} permeability and affecting cell organelles [47, 48]. In our study, as a result of the application of TMZ on neuroblastoma cell cultures, it was observed that intracellular calcium levels increased statistically significantly with the use of TRPA1 channel stimulators (SCS) in cancer cells compared with the control group and intracellular Ca^{2+} levels decreased statistically significantly with the use of TRPA1 channel inhibitor (AP18).

In the present study, temozolomide was applied alone or in combination with selenium to neuroblastoma cell culture (SHSY5Y). The effects of TMZ and selenium on indirect apoptotic activity mediated by TRPA1 channels, intracellular calcium, intracellular reactive oxygen species, caspase-3 and caspase-9, and mitochondrial depolarization levels were investigated. All groups were compared with the control by applying cinnamaldehyde as a stimulator for TRPA1 channel activation/inhibition and AP18 as a specific channel inhibitor. As a result of the experimental measurements, according to the control group, the application of 100 micromolar TMZ for 24 h in neuroblastoma cells caused TRPA1 channel activation, which is known to be indirectly susceptible to oxidative stress, resulting in a significant increase in intracellular calcium levels and apoptosis levels and a decrease in cell viability. In the groups treated with TRPA1 channel inhibition, these effects were significantly reduced. In general, there was a statistically significant increase in apoptosis levels compared with the control group. The statistical difference between the use of TRPA1 activator and inhibitor in TMZ and TMZ+SEL groups describes the indirect efficacy of these drugs on TRPA1 channel function.

In our study, we examined the activity of the use of TMZ and selenium in neuroblastoma tumor cells, its effects on TRPA1 channels, and the roles of these channels in the apoptosis process. As a result, it was determined that temozolomide was directly or indirectly associated with the apoptotic effect mediated by TRPA1 channel activation. It is recognized that TRPA1 channels play a crucial role in the development of apoptosis by causing an increase in intracellular Ca^{2+} levels and mitochondrial depolarization. Selenium treatment after TMZ increased TRPA1 channel activation and cell death.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author declares that no conflict of interest exists. The authors have no conflicts of interest to disclose.

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No part of this work has been previously published.

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