Int J Acad Med Pharm, 2020; 2(2); 130-139
International Journal of Academic Medicine and Pharmacy
journal homepage: www.academicmed.org

Erlotinib and Melatonin Treatment Reduce Proliferation of the Neural Tumor Cells (SH-SY5Y) by Mediating TRPV1 Channel Function

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Article info

Alındı: 01.04.2020
Revize form alındı: 10.04.2020
Kabul: 20.05.2020
Online yayım: 05.06.2020

Keywords

Erlotinib
Melatonin
Neuroblastoma
Oxidative Stress
TRPV1

Abstract

Research Article

Neuroblastoma, which is caused by the precursors of the sympathetic nervous system, is the most commonly encountered extracranial solid tumor in the pediatric age group. TRPV1 channels, which are a subchannel of the TRP channel family in the cell membrane, are channel permeable to calcium that are known to have increased expression based on increased free radicals. They were reported to lead to apoptosis based on increased amount of intracellular free calcium when they are excessively stimulated. With this study, we purposed to determine the effect levels of erlotinib and melatonin on neuroblastoma cells through TRPV1 channels. SH-SY5Y neuroblastoma cells were used in the study. The cell series were divided into 7 main groups as Control, Erlotinib (ERLT) (5 µM, 24 hours), MEL (1 mM, 24 hours), ERLT+CPZ, MEL+CPZ, ERLT+MEL and ERLT+MEL+CPZ. TRPV1 channel activation in all groups was achieved by capsaicin. The calcium signals, intracellular reactive oxygen types, caspase-3 and -9 and mitochondrial membrane depolarization levels were analyzed in the groups, and the groups were compared to each other. Cytosolic calcium concentration, apoptosis, mitochondrial membrane depolarization caspase-3 and caspase-9 activation, and reactive oxygen species levels were importantly higher in comparison to the control group in the ERLT ($p<0.001$), ERLT+MEL ($p<0.001$) and MEL ($p<0.05$; $p<0.001$) groups. In the comparison of the groups that used TRPV1 channel blocker (ERLT+CPZ, ERLT+MEL+CPZ, MEL+CPZ) and the groups that did not use channel blocker (ERLT, ERLT+MEL, MEL), all values were lower in the group that used the blocker ($p<0.05$). Using melatonin and erlotinib together in neuroblastoma tumor cells increased apoptotic pathway activity through TRPV1 channels.

INTRODUCTION

TRP channels are transmembrane ion channels that are permeable to Ca^{2+} ions which were found for the first time in the photoreceptor cells of the Drosophila species of fruit flies. In mammals, the TRP ion channel family consists of 6 subfamilies as Ankyrin (TRPA), Canonical (TRPC), Melastatin (TRPM), Polycystin (TRPP) Mucolipin (TRPML) and Vanilloid (TRPV). These are divided into a total of 29 sub-types based on their specific types of bonding[1,2]. The TRPV subfamily consists of 6 sub-types. Although TRPV channels usually allow cation transition in general, they have a very important role in keeping intracellular calcium concentrations in balance as they have high selectivity for Ca^{2+} ions[1]. While TRPV1 channels have been reported to act as nociceptors, they mainly act as chemo-sensors that interact with chemical compounds. TRPV1 channels are activated by natural vanilloids found in some plants such as vanillin, vanillic acid and capsaicin (Capsn), and it may be stated that they are physically sensitive against pressure, pH, temperature (43°C<) and some chemical and biological molecule[2]. TRPV is a channel that is permeable to Ca^{+2} which is induced by oxidative stress and Capsn and inhibited by capsazepine (CPZ)[5]. TRPV1 channels, which have been reported to have higher expression levels based on the increases in the free radicals in their environment, were stated to lead to fatal cell damages in repeated activations, increased oxidative stress based on the increases in free calcium in the cytosol and eventually programmed cell death (apoptosis)[4].

Previous studies showed that TRP channels change the intracellular calcium concentrations and have an effect in the regulation of release of Ca^{2+} from several organelles in cells[6]. Considering the difference in the Ca^{2+} concentrations in the extracellular fluid outside the cell (1.2 millimolar) and the intracellular fluid (80-100 nanomolar), it is believed that ion transition from the TRP channels that affect the Ca^{2+} concentration in the cytosol has great importance for the cell to perform its vital functions. Increase intracellular levels of Ca^{2+} lead to increases in mitochondrial depolarization, emergence of irreversible changes in the intracellular structural components due to oxidative stress and eventually apoptosis[5]. Intracellular Ca^{2+} concentrations vary in cases such as proliferation of

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http://dx.doi.org/10.29228/jamp.43598

cancer cells, apoptosis and abnormal differentiation[6].

Several drugs that are used to treat cancer affect several other receptors in addition to their target receptor, and this may lead to increases or decreases in these receptors' activities, tolerance development or side effects. The epidermal growth factor receptor (EGFR) is a tyrosine kinase receptor that is expressed excessively in several epithelioid cell cancers and a potential target for oncologic medication[7]. Studies have clearly shown that excessive expression of EGFR supports tumor formation. The clinical response to EGFR inhibitors is variable. EGFR inhibitors which are aimed at EGFR as an important target for several types of cancer are being used for the treatment of various cancers today[8]. One of these inhibitors, Erlotinib (ERLT) is a quinazoline derivative that selectively and reversibly inhibits the tyrosine kinase activity of EGFR. The effects of ERLT, which is clinically preferred in lung and breast cancer patients, on neuroblastoma have been investigated in various cell culture studies[7,9].

Melatonin (N-acetyl-5-methoxytryptamine) (MEL), which is a hormone of the pineal gland, plays important roles in a very broad variety of biological processes including oxidative stress.[11] Some studies even showed that MEL's antioxidant potential may avoid cell death in both physiological and pathological conditions[12]. Moreover, it was reported that MEL has oncostatic effects in different types of cancer[13] . While it is known that MEL shows antitumoral effects by various activities such as migration inhibition and apoptosis induction in cancer cells and activation of anti-oxidation, the molecular mechanisms behind the effects that it mediates are not yet completely known[14] .

In this study, we aimed to reveal the role of TRPV1 channels that are known to be sensitive against oxidative stress in the treatment process by usage of ERLT and MEL separately and in combination on SH-SY5Y neuroblastoma cell cultures, as well as the activity of these drugs on TRPV1 channels.

MATERİALS and METHODS

Cell culture

SH-SY5Y (Human Neuroblastoma cell line) was obtained from American Type Culture Collection (ATCC) (Manassas, VA). Cells were cultured in HAM'S F12 and Dulbecco's Modified Eagle Mediums 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 have reached 75–85% confluence, cell were incubated with the chemical compounds described in groups section. Cells were examined daily for evidence of contamination. After chemical treatments, washed cells were detached with %0.25 Trypsin–EDTA from T25 flasks then added 4 ml fresh medium into the per flasks and the cell suspensions were collected from the flasks by means of a recharged automatic pipette and transferred into the 15 ml falcon tubes. After that, cells were centrifuged (100G, 5 min) then removed the supernatants and centrifugation was repeated by adding fresh medium into the sterile falcon tubes for was-hing the cells and ready for use in experiments.

Reagents

Caspase-3 (AC-DEVD-AMC) and Caspase 9 (AC-LEHD-AMC) substrates were obtained from Enzo (Lausen, Switzerland). DMEM, HAMS F12, Trypsin–EDTA, Fetal Bovine Serum and penicillin-streptomycine and Dimethyl sulfoxide, Dihydrorhodamine-123 (DHR 123) were obtained from Sigma Aldrich (St. Louis, MO), Fura 2 (AM) calcium florescent dye was bought from Calbiochem (Darmstadt, Germany). APOPercentage assay with releasing buffer were purchased from Biocolor (Belfast, Northern Ireland). Pluronic® F-127 was obtained from Biovision (San Francisco, USA). A 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).

Groups

The study was planned as 7 main groups below,

Group 1 (Control): Non of the study drugs were used and SH-SY5Y cells were kept in a flask containing the same cell culture condition.

Group 2 (ERLT): SH-SY5Y cells were incubated with 5 µM Erlotinib for 24 h.[15]

Group 3 (ERLT+CPZ): SH-SY5Y cells were incubated with 5 µM Erlotinib for 24 h and then incubated with Capsazepine (CPZ, 0.1 mM, 30 min).

Group 4 (ERLT+MEL): SH-SY5Y cells were incubated with 5 µM Erlotinib and 1 mM melatonin for 24 h.

Group 5 (ERLT+MEL+CPZ): SH-SY5Y cells were

incubated with 5 µM Erlotinib and 1 mM melatonin for 24 h and then incubated with Capsazepine (CPZ, 0.1 mM, 30 min).

Group 6 (MEL): SH-SY5Y cells were incubated with 1 mM Melatonin for 24 h.[16]

Group 7 (MEL+CPZ): SH-SY5Y cells were incubated with 1 mM Melatonin for 24 h and then incubated with Capsazepine (CPZ, 0.1 mM, 30 min).

In CPZ incubated groups, SH-SY5Y cells were also blocked by TRPV1 blocker CPZ (0.1 mM, 30 min) before related analysis in the existence of 1.2 mM calcium in extracellular environment. For all experiments (except for calcium signaling), the cells were further treated with capsaicin (Capsn, 0.1 mM, 10 min) for activation of TRPV1 channel before related analysis. During calcium signaling analysis (Fura-2/AM), cells were stimulated on 20th cycles with 0.1 mM Capsn in the existence of 1.2 mM calcium and calcium free buffer in extracellular environment.

Fura-2 loading and measurement of intracellular calcium

SH-SY5Y 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 after cell culture treatments,. 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 (cycle interval: 3 sec; gain: 120) in response to agonists (Capsn, 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 to according to method of Martinez et al.[17]

Intracellular ROS production measurement

Dihydrorhodamine-123 (DHR-123) which is a non-fluorescent, non-charged dye and it can easily pass the cell membranes where it is oxidized to cationic rhodamine 123 (Rh-123) which localizes in the mitochondria and exhibits green fluorescence and it was sequestered by mitochondria. The cells (10^6 cells/ml for per group) were incubated with DHR 123 (2 µm) at 37 °C for 30 min.[18] The Rh-123 fluorescence intensities were measurein in a fluorescence multiplate reader (Synergy^{TM} H1, Biotek, USA). Excitation and emission wavelengths of the analyses were set 488 nm and 543 nm, respectively. Data were shown as fold change experimental to control.

Assay for apoptosis level, Caspase 3 & 9 activities

Apoptotic analyses were performed according to the manufacturer instruction using with the APOPercentage^{TM} assay (Biocolor Ltd., Belfast, Northern Ireland). The APOPercentage^{TM} which is a dye-uptake assay that stains only the apoptotic cells with a red dye. When the apoptotic cell membrane lost its asymmetry, the APOPercentage dye is bonded phosphatidyl serine lipids actively and pass through the membrane and localized into the cells, staining apoptotic cells red, thus allowing the detection of apoptosis by a multiplate reader as previously described, elsewhere.[5] Measurement of apoptosis levels was performed by spectrophotometry at 550 nm (Synergy^{TM} H1, Biotek, USA). Data were shown as fold change experimental to control.

Caspase 3 and caspase 9 activitiy determinations were based on methods previously reported (33,34). Human neuroblastoma cancer cells were sonicated and cell lysates were incubated with 2 ml of substrate solution with Caspase 3 substrate (AC-DEVD-AMC) and caspase 9 substrate (AC-LEHD-AMC) for 1 h at 37°C. Caspase cleavages were measured with the Synergy^{TM} H1 plate reader (Biotek, USA) with 360 nm (excitation) and 460 nm (emission) wavelengths. Data were quantitied as fluorescence (units/mg protein) and presented as fold change over the pretreatment 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 quantitied at 485 nm excitation wavelength and 535 nm emission wavelength, and the red signal, at the excitation wavelength of 540 nm and the emission wavelength of 590 nm. Fluorescence changes were measured using a fluorescence spectrophotometer (Synergy^{TM} H1, Biotek, USA).[19] Data are presented as emission ratios (590/535) and fold change over experimental and control pretreatment level.

Statistical analyses

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

RESULTS

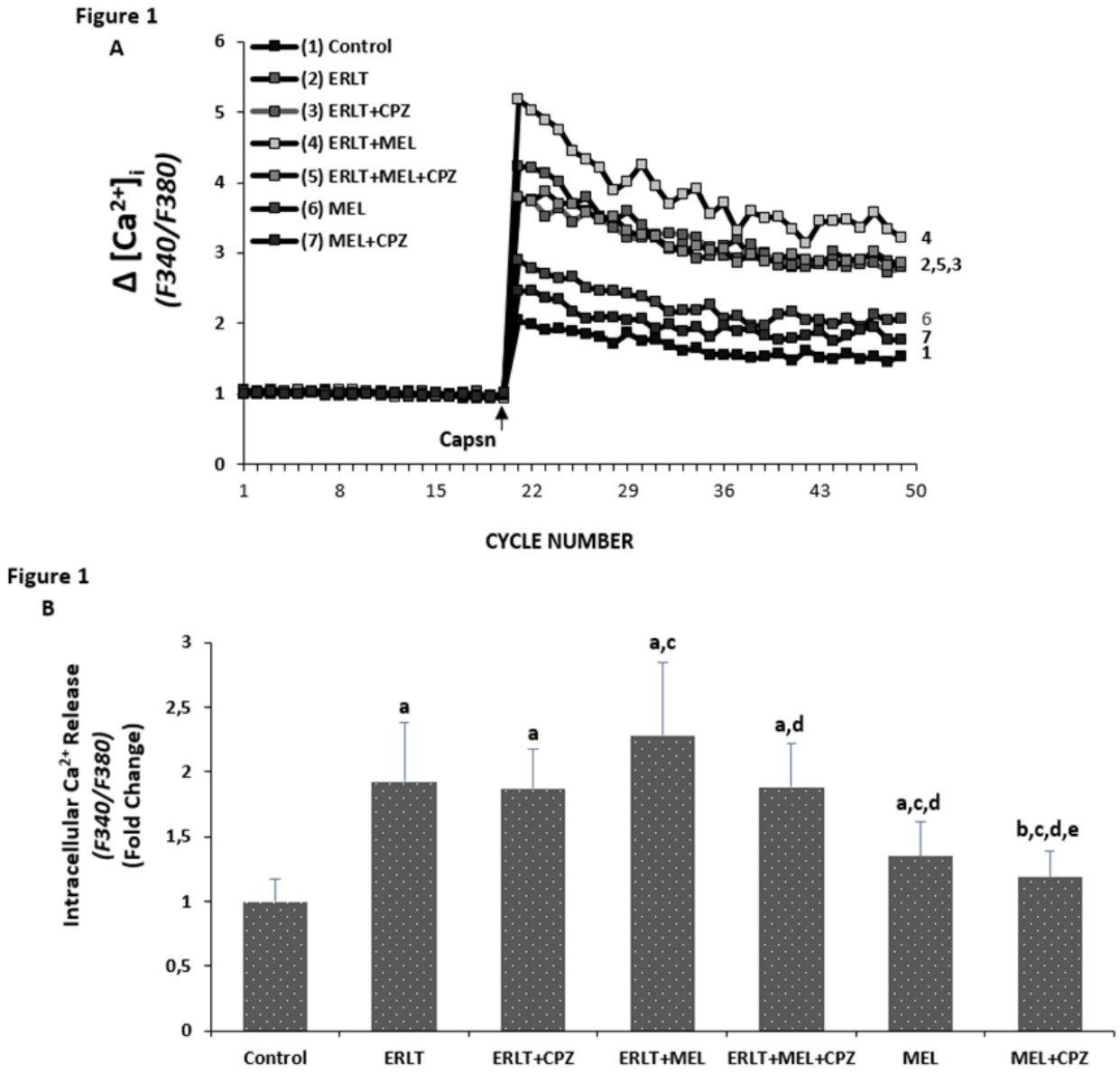

Figure 1 A/B. The effect of Erlotinib (ERLT, 5 µM, 24 h) and Melatonin (MEL, 1 mM, 24 h) on intracellular calcium levels (Fig. 1A) and calcium ion release (Fig. 1B) in SH-SY5Y cells. Cells are stimulated by Capsaicin (Capsn 0.1 mM and on 20th cycle) but they were inhibited by CPZ (0.1 mM for 30 min) (mean ± SD and n=3). $^{a}p<0.001$ and $^{b}p<0.05$ vs control, $^{c}p<0.001$ vs ERLT group, $^{d}p<0.001$ vs ERLT+MEL group and $^{e}p<0.05$ vs MEL group.

Effects of erlotinib and melatonin on cytosolic calcium levels in neuroblastoma cells

The effect of erlotinib and melatonin administrations on cytosolic calcium levels in SH-SY5Y are shown in Figure 1A/B. The TRP Vanilloid 1 channel stimulator (capsaicin) and blocker (capsazepine) were used to evaluate intracellular Ca^{2+} increase through TRPV1 channels in SH-SY5Y cells. As shown in figure 1b, the cytosolic Ca^{2+} release in SH-SY5Y was less in control than erlotinib ($p<0.001$), erlotinib+melatonin ($p<0.001$) and melatonin ($p<0.001$) groups. The Ca^{2+} level was higher in the erlotinib+melatonin group compare to erlotinib ($p<0.001$) and melatonin ($p<0.001$) groups. There was no statistical difference between erlotinib and erlotinib+Cpz groups, but when the erlotinib+melatonin group and erlotinib+melatonin+Cpz groups were compared, the erlotinib+melatonin+capsazepine group was found to be less ($p<0.001$). Compared to melatonin and melatonin+capsazepine, melatonin group was markedly higher in the cytosolic Ca^{2+} concentration level ($p<0.05$).

Results of ROS, Mitochondrial Depolarisation and Caspase 3 - Caspase 9 Values in Neuroblastoma Cells

Figure 2

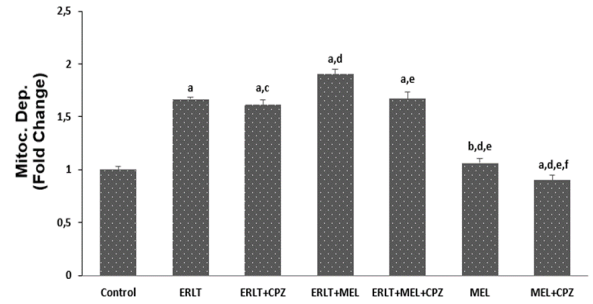

Figure 2. The effect of Erlotinib (ERLT, 5 µM, 24 h) and Melatonin (MEL, 1 mM, 24 h) on Mitochondrial Depolarization levels in SH-SY5Y cells. Cells are stimulated by Capsaicin (Capsn 0.1 mM for 10 min) but they were inhibited by CPZ (0.1 mM for 30 min). (mean ± SD and n=10). [a]$p<0.001$ and [b]$p<0.05$ vs control, [c]$p<0.05$ and [d]$p<0.001$ vs ERLT group, [e]$p<0.001$ vs ERLT+MEL group and [f]$p<0.001$ vs MEL group.

Figure 3

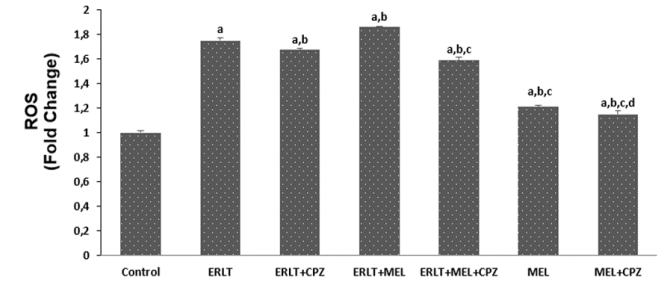

Figure 3. The effect of Erlotinib (ERLT, 5 µM, 24 h) and Melatonin (MEL, 1 mM, 24 h) on reactive oxygene species levels in SH-SY5Y cells. Cells are stimulated by Capsaicin (Capsn, 0.1 mM for 10 min) but they were inhibited by CPZ (0.1 mM for 30 min). (mean ± SD and n=10) [a]$p<0.001$ vs control, [b]$p<0.001$ vs ERLT group, [c]$p<0.001$ vs ERLT+MEL group and [d]$p<0.05$ vs MEL group.

Erlotinib and melatonin were administered separately and together to investigate the levels of mitochondrial depolarization (Figure 2) and reactive oxygen species (Figure 3). It was concluded that administration of erlotinib and melatonin with TRPV1 channel stimulator (Capsn) in SH-SY5Y cells significantly increased degree of mitochondrial depolarization and reactive oxygen species levels compared to the control group ($p<0.001$) but use of TRPV1 channel inhibitor (CPZ) in erlotinib + capsazepine, erlotinib + melatonin + capsazepine and melatonin + capsazepine groups, significantly decreased levels of mitochondrial depolarization and reactive oxygen levels ($p<0.001$; $p<0.05$) compared to erlotinib. erlotinib+melatonin and melatonin. It was found that mitochondrial depolarization and reactive oxygen species levels was statistically higher in the erlotinib+melatonin group compared to erlotinib group ($p<0.001$) and melatonin groups ($p<0.001$).

Effects of erlotinib and melatonin administrations on caspase 3 and caspase 9 levels are shown in Figure 4 A/B. The caspase 3 & 9 values were greater in the erlotinib, erlotinib+melatonin and melatonin groups than in the control ($p<0.001$). The values were lower in the erlotinib and melatonin groups than in the erlotinib+melatonin group ($p<0.001$). Also the caspase 3 and 9 values were lower in the erlotinib+capsazepine, erlotinib+melatonin+capsazepine and melatonin+capsazepine groups when compared with the erlotinib, erlotinib+melatonin and melatonin groups respectively ($p<0.001$, $p<0.05$, $p<0.001$).

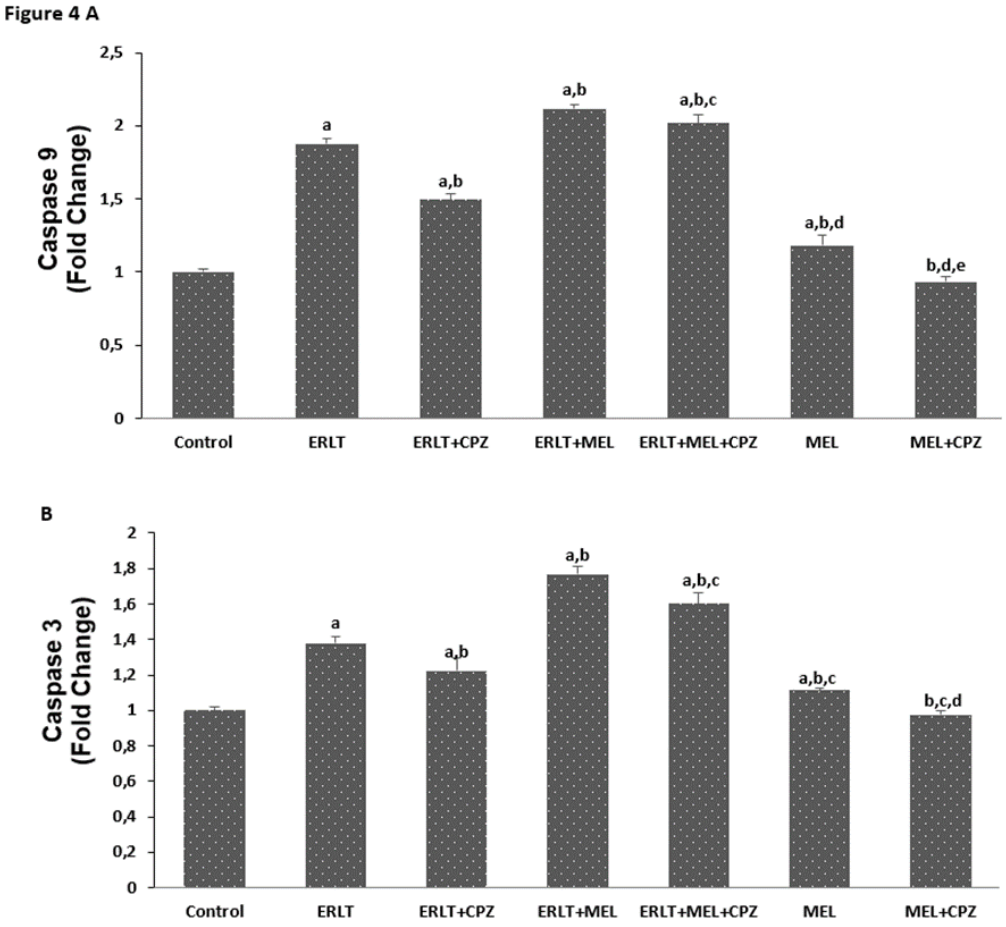

Figure 4 A/B. The effect of Erlotinib (ERLT, 5 µM, 24 h) and Melatonin (MEL, 1 mM, 24 h) on caspase 9 (A) and caspase 3 (B) levels in SH-SY5Y cells. Cells are stimulated by Capsaicin (Capsn, 0.1 mM for 10 min) but they were inhibited by CPZ (0.1 mM for 30 min). (mean ± SD and n=10). Caspase 9: [a]p<0.001 vs control, [b]p<0.001 vs ERLT group, [c]p<0.05 and [d]p<0.001 vs ERLT+MEL group and [e]p<0.001 vs MEL group. Caspase 3: [a]p<0.001 vs control, [b]p<0.001 vs ERLT group, [c]p<0.001 vs ERLT+MEL group and [d]p<0.001 vs MEL group.

Effects of erlotinib and melatonin on apoptosis levels in neuroblastoma cells

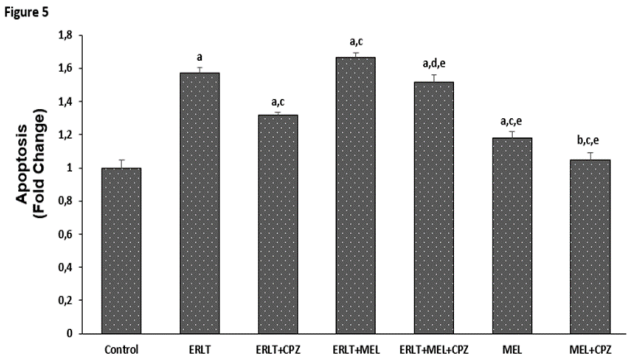

Figure 5: The effect of Erlotinib (ERLT, 5 µM, 24 h) and Melatonin (MEL, 1 mM, 24 h) on apoptosis levels in SH-SY5Y cells. Cells are stimulated by Capsaicin (Capsn 0.1 mM for 10 min) but they were inhibited by CPZ (0.1 mM for 30 min). (mean ± SD and n=10). [a]p<0.001 and [b]p<0.05 vs control, [c]p<0.001 and [d]p<0.05 vs ERLT group, [e]p<0.001 vs ERLT+MEL group and [f]p<0.001 vs MEL group.

Effects of erlotinib and melatonin administrations on apoptosis levels are shown in Figure 5. The apoptosis values were greater in the erlotinib, erlotinib+melatonin and melatonin groups than in the control (p<0.001). The apoptosis values were lower in the erlotinib group and melatonin group than in the erlotinib+melatonin group (p<0.001). Also the values were lower in the erlotinib+ capsazepine, erlotinib+melatonin+capsazepine and melatonin+capsazepine groups when compared with the erlotinib, erlotinib+melatonin and melatonin groups respectively (p<0.00, p<0.05, p<0.001).

DISCUSSION

Neuroblastoma is the most common malignity in infancy and the most frequently encountered extracranial solid tumor in the pediatric age group that is caused by the precursors of the sympathetic nervous system. Long-term survival rates for the high-risk group are only about 30-40%. On the other hand, in the low-risk and medium-risk groups, survival rates are higher

than 90% in the case of early staging and appropriate treatment. The clinical course for neuroblastoma patients is highly heterogenous in terms of localized tumors, spontaneous regression, early metastasis, rapid progression and treatment resistance. Tumors in the neuroblastoma category provide one of the best models to investigate the relationships between their genetic/molecular characteristics and morphological symptoms. In oncogenesis, a better understanding of the involved molecular alterations, molecular pathways that function irregularly or incorrectly or molecules that are excessively expressed is highly important in terms of developing therapeutic agents for fixing these problems. Promising treatment methods include sensitive medication approaches that maximize damage on neuroblastoma cells and minimize normal cell toxicity. Autophagia is the main regulator catabolic mechanism that is responsible for preserving the homeostasis in the cell. It is active on the basal level in all types of cells. During chemotherapy, it may be induced to a high extent due to the abundance of the stress stimulants caused by anticancer drugs. Autophagia is an effective mechanism that may prevent the success of treatment by promoting therapeutic resistance against the medication in tumor cells. An increasing number of reports on neuroblastoma has shown the intense activation of cytoprotective autophagia in drug treatment. In addition to this, the balance between autophagia and apoptosis is crucially important in determining the eventual fate of the neuroblastoma cells that are treated[20].

One of the TRP channels that are known to play and important role and overexpression in the development of several diseases is TRPV1 channels[21]. It was reported that apoptosis is induced by stimulation of TRPV1 channels in several types of cancer cells such as osteosarcoma, colon, glioma and pancreas cancer cells, and the growth of cancer cells is prevented without damaging the normal cells[22]. Similar effects of TRPV1 channels were also demonstrated on neuroblastoma cells[23]. It was stated that the expression of TRPV1 channels increases based on the increases in free radicals in their environment, and in the case of repeated activation, this leads to fatal cell damages, increased oxidative stress due to the increased concentration of free calcium in the cytosol, and eventually, apoptosis[4].

ERLT, which is an EGFR inhibitor, has been determined to be effective on several tumor cell cultures. In tests with children with refractory neuroblastoma and studies on xenografts by using ERLT, partial response or stable disease activity was observed[7,9]. A study that was conducted to understand the underlying anticancer mechanisms of ERLT in non-human small cell lung cancer cell series (A549) found that, in increasing in-vitro concentrations, it increased ROS production, showed proapoptotic effects and suppressed cell growth and reproduction to a significant extent. As expected, it was determined that ERLT showed its effects by activating the c-Jun N-terminal kinase (JNK) signal pathway that proapoptotic ROSs depend on. Caspase-3 was activated with the phosphorylated JNK signal pathway induced by ERLT. The entirety of the proapoptotic effect of ERLT was reversed by applying N-acetylcysteine, which is an ROS scavenger.[24] A study with non-small cell lung cancer cells treated with ERLT determined that it selectively inhibited the oxidative metabolism and disrupted the energy homeostasis[25]. A study on human hepatocarcinoma cells stated that ERLT increases oxidative stress.[26] Again, a study on rats determined that chronic ERLT usage increased oxidative stress in the heart tissue[27]. ERLT is also known to increase oxidative stress by increasing VEGF production by mechanisms that involve CYP1A2 cytochrome P450[26]. Likewise, in our study, it was observed that, when the cytosolic calcium ion levels were lower in the ERLT+CPZ group where the inhibitor was used for TRPV1 which is known to be induced by oxidative stress in comparison to the ERLT control group. Similarly, when the ERLT+CPZ and MEL+CPZ groups were compared to the ERLT and MEL groups, lower calcium ion levels were found in the groups where the channel inhibitor was used. This proves the effects of ERLT on TRPV1 channels, which control the transition of Ca^{2+} ions into the cell. Again, the finding that apoptosis levels were higher in the extracellular ERLT and ERLT+MEL application groups in comparison to the groups where the channel inhibitor was used showed that TRPV1 channels increased apoptosis in tumor cells.

TRPV1 is the first membrane receptor which has a tumor-suppressing effect associated with the down-regulation of another membrane receptor[8]. A study on intestinal epithelial cells determined a feedback loop between TRPV1 and EGFR, which is a known mediator in epithelium proliferation. If was found that TRPV1 was expressed by intestinal epithelial cells and intrinsically activated after EGFR stimulation. In a study on multiple intestinal neoplasm in mice, TRPV1 deficiency increased adenoma formation and treatment with EGFR kinase

inhibitor reversed the tumorigenic effect and found a functional relationship between TRPV1 and EGFR in intestinal epithelial cells[28]. In our study, when we used ERLT, an EGFR inhibitor, on neuroblastoma cells, we observed that the TRPV1 channel was activated due to oxidative stress, it increased Ca^{+2} ion transition, and this triggered apoptosis in the cell.

Several studies on SH-SY5Y neuroblastoma cell cultures showed that using Capsn, which is a TRPV1 channel antagonist increased intracellular Ca^{+2} concentrations, and CPZ, another TRPV1 channel antagonist, decreased intracellular Ca^{+2} concentrations[3,11]. In our study, we determined that there were higher Ca^{+2} levels when all groups were induced by Capsn and lower levels when they were induced by CPZ.

MEL, which has been known for a long time and is a pineal gland hormone, is a natural antioxidant. It was found that MEL's ability to neutralize the OH radical is five times higher than that of glutathione, and its peroxide radical elimination ability is two times higher than that of vitamin E. It was determined that MEL stimulates antioxidant enzymes like glutathione peroxidase, prevents lipid peroxidation and protects the brain tissue from oxygen-related free radicals[29]. But, as reported in some studies, it is known that melatonin can undertake the proapoptotic role as depending on dose[30]. Likewise, in our study, it was observed that apoptosis levels were higher in the ERLT+MEL group in comparison to the ERLT group, and the groups where the TRPV1 channel inhibitor was used had lower levels of apoptosis. This made us think using ERLT and MEL in combination together revealed the effectiveness of TRPV1 channels in higher apoptosis levels, and combined treatment of ERLT and melatonin may increase the therapeutic effects of anticancer drugs. In this study, MEL showed a synergetic effect with ERLT by increasing the apoptosis of SH-SY5Y cancer cells.

A previous study found that, in non-small cell lung cancer (H358 and H441) cell lines, ERLT increased the activity of separated procaspase-9 and caspase-3 and led to cell death and apoptosis to a significant extent[31]. It was found that treatment of non-small cell lung cancer A549, H838, H460 and HCC827 cell series with ERLT caused a dose-dependent increase in the caspase-9a/9b ratio[32]. Another study that was carried out on the same cells determined that caspase-9 activation increased with ERLT.[33] A study on melanoma cell lines ERLT and IL-24 gene therapy increased caspase-3 and caspase-9 activity and induced apoptosis[34]. In our study, when the ERLT group and the ERLT+MEL group were compared based on their ROS, mitochondrial depolarization, caspase-3 and results, the ERLT+MEL group was found to have higher values, while there was no significant difference between these groups based on their caspase-9 values. In the study, ERLT-related apoptosis and caspase-3 activity were increased even further by MEL in the SH-SY5Y.

Consequently, we may state that, in SH-SY5Y neuroblastoma cells, ERLT affected not only the inhibition of EGFR receptors but also TRPV1 channels indirectly. When combined with MEL in neuroblastoma cells, ERLT and MEL combination leads to higher levels of programmed cell death. Using melatonin and ERLT as a combination may be a useful way of improving the therapeutic effects of chemotherapy on SH-SY5Y neuroblastoma cells. MEL may be an adjuvant drug as dose-dependent that is used in neuroblastoma treatment in the future.

Study limitations

We could not be able to perform electrophysiological analysis by patch clamp and evaluate the TRPV1 channel expressions by western blot in neuroblastoma cells.

Disclosures

There are no conflicts of interest concerning this article.

Abbreviations

TRPV1	Transient Receptor Potential Vanilloid 1
ROS	Reactive oxygen species
ERLT	Erlotinib
MEL	Melatonin
Capsn	Capsaicin
CPZ	Capsazepine
TRP	Transient Receptor Potential
RNS	Reactive nitrogen species
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
JC-1	5,50,6,60-tetrachloro-1,10,3,30-tetraethylbenzimi-dazolylcarbocyanine iodide
BSA	Bovine serum albumin
DHR-123	Dihydrorhodamine-123
Rh-123	rhodamine-123
$[Ca^{2+}]_i$	intracellular free calcium concentration

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