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RESEARCH

Cytotoxic activity of TRPV4 antagonist RN-1734 in G-361 human melanoma cancer cell line

G-361 insan melanom kanseri hücre hattında TRPV4 antagonisti RN-1734'ün sitotoksik aktivitesi

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Abstract

Purpose: Intracellular calcium (Ca^{2+}) signaling plays a role in many cellular events, such as cell proliferation and differentiation, gene transcription, oxidative stress, the antioxidant system, and apoptosis. Transient receptor potential vanilloid 4 (TRPV4) channels are non-selective cation (Ca^{2+}) channels. The present study aims to investigate the cytotoxic activity of RN-1734, a transient receptor potential vanilloid 4 (TRPV4) antagonist, in the G361 human melanoma cancer cell line.

Materials and Methods: The effects of RN-1734 on G361 cell viability at concentrations of 1, 5, 25, 50, and 100 µM were measured using the 3-(4,5-dimethylthiazol-2-il)-2,5-diphenyltetrazolium bromide (MTT) method. Total antioxidant status (TAS) and total oxidant status (TOS) levels were determined using a ready-made commercial kit, after which oxidative stress index (OSI) values were calculated. To determine the apoptotic effects of RN-1734, Bcl-2, Bax, and p53 expression levels, caspase-3 and -8 activities were examined via quantitative real-time PCR analysis.

Results: G361 cell viability significantly decreased to 82.72, 72.81, 56.36, 39.16 and 18.96% in RN-1734 groups (1, 5, 25, 50 and 100 µM) compared to the control group (100.00%). At IC_{50} concentration (39.48 µM), RN-1734 application (3.35 mmol/g prot.-TAS, 45.87 µmol/g prot.-TOS, and 1501.97 AU-OSI) increased the TAS level (2.17

Öz

Amaç: Hücre içi kalsiyum (Ca2+) sinyali, hücre çoğalması ve farklılaşması, gen transkripsiyonu, oksidatif stres, antioksidan sistem ve apoptoz gibi birçok hücresel olayda rol oynar. Geçici reseptör potansiyeli vanilloid 4 (TRPV4) kanalları, seçici olmayan bir katyon (Ca^{2+}) kanalıdır. Bu çalışmada, TRPV4 antagonisti olan RN-1734'ün G361 insan melanom kanser hücre hattındaki sitotoksik aktivitesinin araştırılması amaçlandı.

Gereç ve Yöntem: RN-1734'ün 1, 5, 25, 50 ve 100 uM konsantrasyonlarında G361 hücre canlılığı üzerindeki etkisi 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolyum bromid (MTT) yöntemi ile ölçüldü. Total antioksidan kapasite (TAS) ve total oksidan kapasite (TOS) seviyeleri hazır ticari bir kit kullanılarak belirlendi ve sonrasında oksidatif stres indeksi (OSI) değeri hesaplandı. RN-1734'ün apoptotik etkilerini tespit etmek için Bcl-2, Bax ve p53 ekspresyon seviyeleri ile kaspaz 3 ve -8 aktiviteleri kantitatif real-time PCR yöntemi ile belirlendi.

Bulgular: G361 hücre canlılığı, kontrol grubuna (%100.00) kıyasla tüm RN-1734 gruplarında (1, 5, 25, 50 ve 100 µM) önemli ölçüde %82.72, 72.81, 56.36, 39.16 ve 18.96'ya düştü. Yine IC_{50} (39.48 µM), konsantrasyonunda RN-1734 uygulaması (3.35 mmol/g prot.-TAS, 45.87 µmol/g prot.-TOS, 1501.97 AU-OSI) kontrol grubuna gore TAS'I (2.17 mmol/g prot.) artırmış, TOS (55.41 µmol/g prot.) ve OSI'yi (3142.76 AU) azaltmıştır.

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mmol/g prot.) and decreased the TOS level (55.41 μmol/g prot.) and OSI value (3142.76 AU) compared to the control group.
Conclusion: Our findings show that RN-1734 may be a novel therapeutic approach to treating melanoma by decreasing the cell viability of G361 human melanoma cancer cells.
Keywords:. Cell viability, G361 human melanoma cancer cells, oxidative stress, RN-1734, TRPV4

Sonuç: Bulgularımız, RN-1734'ün, G361 insan melanom kanser hücrelerinin canlılığını azaltarak, melanom için yeni bir terapötik yaklaşım olabileceğini göstermektedir.

Anahtar kelimeler: Hücre canlılığı, G361 insan melanom kanser hücreleri, oksidatif stres, RN-1734, TRPV4

INTRODUCTION

Malignant melanoma is an aggressive skin cancer that usually occurs on the skin of individuals and develops from melanocytes[1]. Melanoma, whose incidence has been increasing for the last 34 years[2], accounts for approximately 4% of all cancers[3]. It is the most dangerous type of skin cancer, accounting for 80% of deaths due to it[4]. Genetic risk factors and ultraviolet exposure induce the development of melanoma[5]. The prognosis for melanoma is poor despite the availability of numerous treatment medications[6]. In previous studies, the levels of Bax[7] and p53 proteins[3], as well as caspase 3[3,7,8] and 8[7,8] activities, in the G-361 human melanoma cancer cell line[8] were found to be lower than in experimental groups administered dacarbazine[7], ethanolic Asiasari radix extract (ARE)[3], and Houttuynia cordata Thunb (HCT)[8]. Therefore, apoptosis was suppressed in these cells. Another study showed that intracellular calcium (Ca^{2+}), which is associated with cancer, plays a role in many cellular events such as cell proliferation, cell differentiation, gene transcription, oxidative stress, the antioxidant system, and apoptosis[9].

Transient receptor potential vanilloid 4 (TRPV4), a non-selective cation channel for Ca^{2+}, belongs to the group of transient receptor potential (TRP) cation channels. TRPV4 channels are widely expressed in body tissues such as the skin, lung, brain, urinary bladder, vascular endothelial cells, liver, testis, inner ear, pancreas, cornea, kidney, and heart and play a role in regulating the amount of intracellular Ca^{2+}[10]. In some studies, it has been observed that TRPV4 channel activity inhibits tumoral endothelial cell proliferation[11] and induces apoptosis in breast cancer cells[12]. Fang et al. found that inhibition of TRPV4 channels in human hepatocellular carcinoma cells suppresses cell proliferation, induces apoptosis, and reduces migration ability[13]. In addition, Çakır and Erden found that the administration of RN-1734, a TRPV4 antagonist, in human prostate (PC-3) and human breast (MCF-7) cancer cell lines caused DNA damage in cells and reduced cell viability[14].

Apoptosis, commonly known as programmed cell death, is a sequential order of cell death that occurs regularly to maintain a healthy balance between the rates of cell production and cell death[15,16]. Apoptosis is known to be induced via signaling pathways mediated by mitochondrial/cytochrome c and death receptors[17]. Genes such as Bax, Bak, Bid, p-53, p-21, and C-myc induce apoptosis, whereas Bcl-2, Bcl-xl, Bcl-w, Bfl-1, and Mcl-1 suppress apoptosis[18]. One cause of cancer is a reduction in apoptosis[19].

No studies have been found on the cell viability and apoptotic effects of RN-1734 in the G-361 human melanoma cancer cell line. The present study investigated the effects of RN-1734, a TRPV4 antagonist, on cell viability, oxidative stress, and apoptosis in the G-361 human melanoma cancer cell line.

The research's hypothesis is that RN-1734 will reduce cell viability by acting on oxidative stress and apoptosis in the G-361 human melanoma cancer cell line. Therefore, it will be mentioned in the literature that RN-1734 may be a new therapeutic approach for melanoma.

MATERIALS AND METHODS

Cell culture

Since this study is an in vitro study, there is no need for ethical approval. The G-361 human melanoma cancer cells (ATCC® CRL-1424) used in the present study were obtained from the Department of Biochemistry, Faculty of Medicine, Afyonkarahisar University of Health Sciences. All experimental applications were made in the Biochemistry Laboratory. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Capricorn Scientific, cp19-2782, Germany) containing a mixture of 10% Fetal Bovine Serum (FBS) (Gibco, heat-inactivated, 10500-064, Brazil), 1% L-glutamine

(Gibco 25030-024, Paisley, Scotland, UK) and 1% penicillin-streptomycin (Gibco 15140-122, Paisley, Scotland, UK) in an incubator containing 5% CO2 and set at 37°C. After the cells reached 70-80% confluence, they were removed from the flask with 0.25% trypsin-1 mM EDTA solution (Sigma, 59418C, Darmstadt, Germany). Cells obtained from the same passage were used in the study groups.

MTT cytotoxicity assay

To determine the effect of various concentrations (1, 5, 25, 50, and 100 µM) of RN-1734 (98% purity, Sigma, R0658, St. Louis, MO, USA) on the viability of G-361 cells via the 3- (4,5-dimethylthiazole)–2-il) - diphenyltetrazolium bromide (MTT) method, cells were inoculated into 96-well plates (Thermo Scientific ™ Nunc MicroWell, 167008, Tewksbury, MA, USA) with $20x10^3$ cells per well. Plates were incubated for one day at 37°C in a humidified environment with 5% CO_2. Later, RN-1734 at concentrations of 1, 5, 25, 50, and 100 µM and the only medium were applied to the wells, and the control group was left to incubate for 24 hours. For cell viability testing, MTT solution (Calbiochem, 298-93-1, Darmstadt, Germany) was prepared at 5 mg/mL in phosphate-buffered saline (PBS; pH 7.0), and 20 µl was added per well and incubated for 2.5 hours. After the incubation period, different doses of RN-1734 applied to the wells were withdrawn, and 200 µL DMSO was added. The formazan absorbance formed in the plates was read in a microplate reader (Bio Tek Epoch Spectrophotometer, Winooski, VT, USA) at a wavelength of 570 nm[20]. The average of the absorbance values read from the control wells containing cells and media was accepted as 100% viable cells. The percent viability values were calculated by proportioning the absorbance values read from the wells treated with different doses of RN-1734 to the control absorbance value[21]. This test was repeated three times. The IC_{50} value was calculated with the graph created from the MTT results in Microsoft Excel (2010).

Neutral red staining

With the neutral red staining method, cell viability is evaluated through lysosomal activity[22]. This dye accumulates in the lysosomes of living cells and stains them. For this, $150x10^3$ cells were put to each well of a 6-well plate, and the plates were then incubated for 24 hours at 37°C in a humidified environment containing 5% CO_2. The cells were then washed with 1000 µL/well (PBS; pH 7.0). Subsequently, RN-1734 in concentrations of 1, 5, 25, 50, and 100 µM was applied to the wells of the experimental group, and the only medium was applied to the wells of the control group, and the plate was again incubated in the oven for 24 hours. After the incubation period was completed, the medium in each well was removed. Cells were first detected with 4% paraformaldehyde for 15 min, as specified in the neutral red staining test procedure. After fixation, the cells were washed twice for two minutes with 1000 µL/well of distilled water. Then, 1000 µL/well of neutral red dye solution (Sangon Biotech, E607312, Shanghai, China) was applied to the wells and incubated at room temperature for 5 minutes. After incubation, the neutral red dye was removed, and the cells were washed twice for two minutes again with 1000 µL / well of distilled water. After the staining process was completed, each well was examined under an inverted microscope (Olympus, CKX53, Tokyo, Japan), and photographs were taken with a camera connected to the microscope (Olympus, DP74, Tokyo, Japan).

Cell lysate preparation

Cell lysates were prepared according to the following method for determining total protein, total oxidant status (TOS), and total antioxidant status (TAS) levels. Only the culture medium was applied to the cells in the control group, and the IC50 dose of 39.48 µM RN-1734 was applied to the cells in the experimental group. The cells were then incubated for 24 hours in a humidified atmosphere of 5% CO2 at 37°C. After this time, the cells were washed with phosphate-buffered saline (PBS; pH 7.0), followed by trypsin. Then centrifugation (Nüve, NF 800, Turkey) at 1000×g for 5 min at 4°C, cells were collected. Cells in ice-cold fresh lysis buffer (pH 8.0, 10 mM Tris - HCl, 20 mM EDTA, 1 mM dithiothreitol, pH 7.0, 50 mM HEPES, 1 mg/mL proteinase K) were centrifuged at 16000xg for 10 minutes at 4°C to remove cellular debris23. Cell lysates prepared after centrifugation were used immediately for analysis.

Bicinchoninic acid (BCA) assay

Bicinchoninic acid (BCA) analysis is a highly sensitive method used to determine total protein concentration. The macromolecular structure of the protein, the number of peptide bonds, and the presence of four specific amino acids (cystine, cysteine, tryptophan, and tyrosine) are necessary for

BCA color formation. Total protein determination was performed using a 562 nm wavelength microplate reader (Bio Tek Epoch Spectrophotometer, Winooski, VT, USA), as specified in a test procedure using a commercial smart BCA protein analysis kit (Intron Biotechnology, 21071, Gyeonggi, Korea), and protein concentrations were determined in g/mL.

Measurement of total antioxidant status (TAS)

TAS level analysis in cells is based on the reduction of by antioxidants dark blue-green-colored 2,2'-azino-3-ethylbenzothiazoline-6-sulphonic acid (ABTS) to colorless ABTS. TAS levels in cell lysates were read on a microplate reader (Bio Tek Epoch Spectrophotometer, Winooski, VT, USA) at 660 nm wavelength using a ready-made commercial kit (Rel Assay Diagnostics, RL0017, Gaziantep, Turkey) as specified in the test procedure, and the results were shown as mmol Trolox equivalents (equiv.)/g protein. Measurements of TAS levels in the cells were repeated three times.

Measurement of total oxidant status (TOS)

The analysis of TOS levels in cells is based on the oxidation of ferrous iron to ferric iron using oxidants in the cells. The color intensity of the complex formed by ferric iron with chromogen is directly proportional to the TOS level. TOS levels in cell lysates were read on a microplate reader (Bio Tek Epoch Spectrophotometer, Winooski, VT, USA) at 530 nm wavelength using a ready-made commercial kit (Rel Assay Diagnostics, RL0024, Gaziantep, Turkey) as specified in the test procedure, and the results were measured as μmol H_2O_2 equiv./g protein. Measurements of TOS levels in cells were repeated three times.

Determination of the oxidative stress index (OSI)

The ratio of TOS to TAS provides the OSI value, which is an indicator of the degree of oxidative stress. The OSI value is calculated by the formula below:

OSI (arbitrary unit)=TOS (μmol H_2O_2 equiv./g protein)/TAS (mmol Trolox equiv./g protein)×100[24].

Total RNA extraction and cDNA synthesis

The cells were lysed using the RNA synthesis kit (ABT 102-01-10, Ankara, Turkey) according to the manufacturer's instructions, followed by ethanol precipitation. All materials to be used during RNA isolation were prepared beforehand with suitable solutions (RNA away) to create an RNase-free environment. The amount and purity of the isolated RNAs were measured by nanodrop. RNAs with OD260 / 280 ratios in the range of 1.7-2.1 were used in cDNA synthesis[25]. All steps of the RNA isolation process were carried out on ice. Using the cDNA kit (Thermo Fisher Scientific, 4368814, Waltham, MA, USA) and 4 μL of 5 × RT primer, 10 ng of total RNA were transformed into cDNA. The 20 μL reactions were incubated in a thermocycler at 55°C for 15 minutes, 85°C for 5 minutes, and kept at 4°C.

Table 1. The gene primers sequences used for real-time PCR reactions (Sentebiolab, Türkiye).

Gene name	Forward primers (5' to 3')	Reverse primers (5' to 3')
B-actin	CACCCCAGCCATGTACGTTC	ACCATCGCTATCTGAGCAGC
P53	CTACAGTACTCCCCTGCCCT	CCGGAGTCCATCACGATGCCA
Bax	CGCCTCACTCACCATCTGGAA	CCTCAAGACCACTCTTCCCCA
Bcl-2	GAGGGGCTACGAGTGGGATGC	GGAGGAGAAGATGCCCGGTGC
Caspase -3	GGAAGCGAATCAATGGACTCTGGA	CCTGAGGTTTGCATCGAC
Caspase -8	AAGCTCTCCCCAAACTTGCTT	TGCATAAAAAGACCCCAGAGCA
Caspase -9	TACAGCTGTTCAGACTCTAGTA	AAATATGTCCTGGGGTAT

Quantitative real-time PCR analysis

Quantitative real-time PCR (QRT-PCR) analysis was performed using the BrightGreen Universal 2x qPCR kit (Abm, MasterMix-U, Canada). Ten μL of PCR reaction using 0.6 μL forward, 0.6 μL reverse, nuclease-free water, and other products in the kit was incubated at 95°C for 10 minutes in a 96-well plate, followed by 40 cycles at 95°C for 15 seconds each, and it was incubated at 60°C for 60 seconds. All reactions were carried out in triplicate. Primers were specific for each transcription analysis, and studies in the literature were referenced. The B-actin, Bax, Bcl-2, caspase-3, caspase-8, and caspase-9 primers are

presented in Table 1. The quantitative cycle (Cq) was defined as a fractional cycle in which the fluorescence of the sample crosses the defined threshold. The analysis of QRT-PCR data was calculated using the $2^{-\Delta\Delta Ct}$ method.

Table 2. Viability (%) of G-361 cells exposed to RN-1734 (0 µM- 100 µM).

RN-1734 concentrations (µM)	Cell viability (%) ($\bar{x} \pm S\bar{x}$)
Control (untreated)	100.00 ± 0.00^{a}
1	82.72 ± 2.73^{b}
5	72.81 ± 2.84^{bc}
25	56.36 ± 2.07^{c}
50	39.16 ± 2.08^{de}
100	18.96 ± 1.41^{e}
p	***

a,b,c,d,e Means within each grouping with different letter designations differ significantly. ***: $p < 0.001$, $\bar{x}$: mean, $S\bar{x}$: standard error of mean.

Statistical analysis

The SPSS 22.00 package program was used for the statistical analysis of the data obtained. Results are presented as mean±standard error. Using the Shapiro-Wilk test, the data was controlled to comply with the normal distribution. In MTT cytotoxicity analyses, the difference between groups was evaluated using Kruskall-Wallis variance analysis. The Mann-Whitney U test with Bonferroni correction was applied to determine which group or groups caused the difference. In TAS, TOS, and OSI levels and gene expression, analyses of the differences between groups that did not show normal distribution were performed using the Mann-Whitney U test, and the differences between groups with normal distribution were analyzed using the Student's t-test. Values of $p<0.05$ were deemed significant from the results obtained in statistical analysis[26].

RESULTS

Table 2 shows cell viability (%) in G-361 cells in the control group and the groups treated with RN-1734 at different concentrations. Cell viability (%) was statistically lower in the groups treated with 1, 5, 25, 50, and 100 µM RN-1734 than in the control group ($p < 0.001$). Moreover, cell viability (%) was lower in the 25 µM RN-1734 group than in the control and 1 µM RN-1734 groups ($p<0.001$), and was similar between the 1 µM RN-1734 group and the 5 µM RN-1734 group ($p>0.05$). Cell viability (%) was significantly lower in the group treated with 100 µM RN-1734 than in the control group and the 1, 5, and 25 µM RN-1734 groups; however, there was no statistically significant difference in cell viability between the 100 µM RN-1734 group and the 50 µM RN-1734 group ($p>0.05$) (Table 2).

Figure 1 shows cell viability in G-361 cells in the control group and the groups treated with RN-1734 at different concentrations. A neutral red dye accumulated in the lysosomes of living cells and stained them. Figure 1 shows that in G-361 cells (B-F) treated with RN-1734 at different concentrations, the stained cell density is lower than in the control group (A) treated with only a medium concentration.

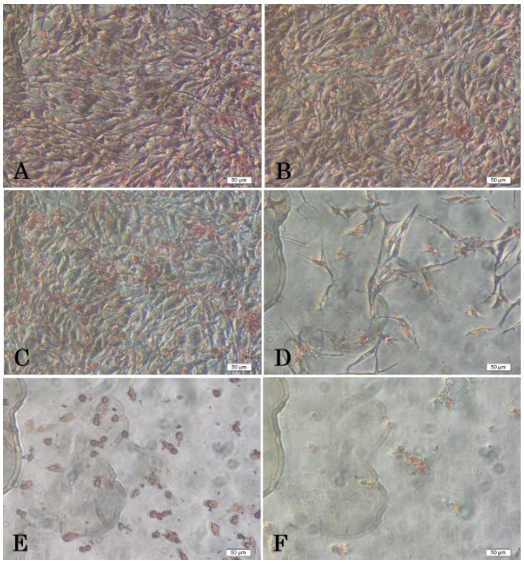

Figure 1. Inverted microscope results in the control group (A) and the groups treated with RN-1734 at different concentrations [1 µM (B), 5 µM (C), 25 µM (D), 50 µM (E) and 100 µM (F)] Neural red staining. Scale bars: 50 µm.

Table 3 shows the TAS and TOS levels, as well as the OSI value in the control group and the 39.48 µM (IC_{50}) RN-1734 groups. According to the statistical analysis, the TAS level was similar between the control group and the 39.48 µM (IC_{50}) RN-1734 groups ($p>0.05$). The TOS level ($p<0.01$) and OSI value ($p<0.05$) were higher in the group treated with 39.48 µM RN-1734 than in the control group (Table 3).

Table 3. The TAS and TOS levels as well as oxidative stress index in the control and 39.48 µM (IC_{50}) RN-1734 groups.

RN-1734 concentrations (µM)	TAS mmol/g prot ($\bar{x} \pm S\bar{x}$)	TOS µmol/g prot ($\bar{x} \pm S\bar{x}$)	OSI AU ($\bar{x} \pm S\bar{x}$)
Control (untreated)	2.17 ± 0.25	55.41 ± 3.50	3142.76 ± 358.69
39.48 (IC_{50})	3.35 ± 0.30	45.87 ± 4.01	1501.97 ± 161.45
p	**	*	***

TAS: Total oxidant status, TOS: Total antioxidant status, OSI: Oxidative stress index.
*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, $\bar{x}$: mean, $S\bar{x}$: standard error of mean.

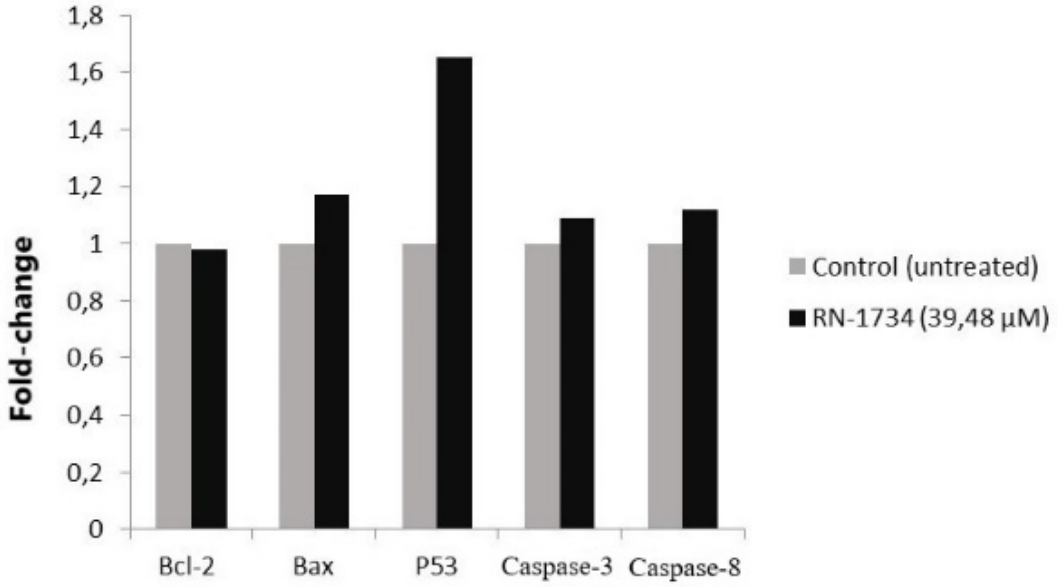

Figure 2. The relative expression levels of Bcl-2, Bax, and p53 proteins, and relative caspase-3, and -8 activities in the control and 39.48 µM (IC_{50}) RN-1734 groups.

The relative expression levels of Bcl-2, Bax, and p53 proteins in the control group and the 39.48 µM (IC_{50}) RN-1734 groups are shown in Figure 2. When the control and RN-1734 (IC_{50}) groups were compared statistically, the increases in the RN-1734 (IC_{50}) group were not significant in terms of the relative expression levels of Bax and p53 proteins associated with apoptosis ($p>0.05$). Additionally, there was no significant difference between the control and RN-1734 (IC_{50}) groups in terms of the relative expression levels of Bcl-2 proteins ($p>0.05$) (Figure 2).

The relative caspase-3 and -8 activities in the control and RN-1734 (IC_{50}) groups are shown in Figure 2. The relative caspase-3 and -8 activities were higher in the RN-1734 (IC_{50}) group than in the control group. However, the difference was not statistically significant ($p>0.05$) (Figure 2).

DISCUSSION

The survival and fate of all higher organisms depend on calcium (Ca^{2+}), a crucial second messenger in cells. Variations in the duration and amounts of intracellular Ca^{2+}, which may be temporary or sustained, are regulated by various Ca^{2+} channels, pumps, or exchangers. Several pathogenic diseases, including cancer, are linked to detrimental changes in

Ca^{2+} homeostasis and signaling[27]. Transient receptor potential vanilloid type 4 (TRPV4), which is an osmosensor and thermosensor, is a cation channel that is permeable to Ca^{2+} [28]. Studies have shown that the abnormal expression and activity of TRPV4 channels, which regulate intracellular Ca2+ amount, play a role in many types of cancer. These channels are also shown to be associated with proliferation, apoptosis, migration, angiogenesis, and metastasis in cancer cells[29,30]. TRPV4 is involved in the development of cancer, but its precise biological roles and underlying processes are still poorly understood[28].

Cakır and Erden conducted a study in which RN-1734 at concentrations of 50 and 100 μM in human prostate (PC-3) and human breast (MCF-7) cancer cell lines caused DNA damage in the cells and significantly reduced cell viability (%)[14]. Bahari et al. also found that the administration RN-1734 at concentrations of 1, 10, and 100 μM in the colorectal cancer cell line statistically decreased cell viability (%)[31]. It was determined that RN 1734 treatment with fatty acid amide hydrolase (FAAH) inhibitor PF-3845 for the human colon adenocarcinoma (Colo-205) cell line significantly reduced cell viability[32]. Moreover, the selective antagonist HC-067047 decreased cell proliferation and induced apoptosis in human hepatocellular carcinoma (HCC) cells by pharmacologically inhibiting TRPV4 channels[33]. In line with the results reported in the literature, the current investigation showed using MTT measurement and neutral staining that the cell viability (%) at all concentrations of RN-1734 (1, 5, 25, 50, and 100 μM) demonstrated considerable reduction compared to the control group. Thus, the effect of RN-1734 in reducing cell viability in the G-361 human melanoma cancer cell line was illustrated.

Lipids, nucleic acids, and proteins can suffer harm from reactive oxygen types (ROS) by having their functions changed. Oxidative stress develops when there is an imbalance between ROS generation and the antioxidative defense system. Oxidative stress causes many diseases, such as cancer. Antioxidant enzymes (SOD, CAT, and GSH-Px) defend cells from oxidative damage[34]. Cancer cells produce more ROS than in normal cells. Therefore, ROS production is associated with tumorigenesis[35]. Sander et al. found that the malondialdehyde (MDA) level in skin biopsies taken from patients with melanoma was higher than in the control group[36]. Studies have reported that the activity of TRPV4 channels can cause an intracellular Ca^{2+} increase, production of ROS and nitric oxide (NO), and decreased activities of catalase (CAT) and glutathione peroxidase (GSH-Px) in many cell types[37-39]. Wu et al. also found that blockade of the TRPV4 channel decreased ROS production and increased antioxidant activities (SOD, CAT, and GSH-Px)[40]. When activated TRPV4 in human embryonic kidney 293 (HEK293) cell lines was inhibited, reactive oxygen radicals, lipid peroxidation, Ca^{2+} concentration, and cell death and apoptosis were restored[41]. In the present study, it was determined that the application of RN-1734, which is a TRPV4 antagonist, significantly decreased the TOS level and oxidative stress index and increased the TAS level, as reported in the literature. Therefore, it can be thought that the inhibition of TRPV4 channels may suppress the growth of cancer by repressing oxidative stress.

Thoppil et al. and Peters et al. found that TRPV4 channel activity inhibited tumor endothelial cell proliferation and induced apoptosis in breast cancer cells[11,12]. In contrast, the inhibition of TRPV4 channels suppressed cell proliferation, induced apoptosis, and decreased migration ability in human hepatocellular carcinoma cells[13]. In a separate study, Liu et al. found that TRPV4 inhibition suppressed the growth of colon cancer cells by inducing apoptotic and autophagic cell death[42]. In this study, the inhibition of TRPV4 channels with RN-1734 application found no significant differences between the control and RN-1734 (IC_{50}) groups in terms of the relative expression levels of Bcl-2, Bax, and p53 proteins or the relative caspase-3 and -8 activities associated with apoptosis. This could be due to the use of IC_{50} dose (39.48 μM). RN-174 treatment at higher doses may induce apoptosis.

The limitation of our study is that the apoptosis results were not supported by western blot or flow cytometry analysis.

In conclusion, in vitro RN-1734 administration suppresses oxidative stress and decreases cell viability in human melanoma cancer cells. The effect of RN-1734 on reducing cell viability may be due to the induction of a cellular death mechanism other than apoptosis. To better understand the mechanisms of action of RN-1734, it is recommended to conduct both in vivo experiments and novel studies on different cancer cell lines, as well as to investigate inflammation markers, such as TNF-α and IL-1. The RN-1734 treatment may potentially lead to new treatment approaches for malignant melanoma.

Author Contributions: Concept/Design : ÖG, EB, EK; Data acquisition: EB, EK; Data analysis and interpretation: ÖG, MB; Drafting manuscript: ÖG; Critical revision of manuscript: ETE; Final approval and accountability: ÖG, EB, EK, ÖFL, MB, ETE; Technical or material support: ÖG, MB; Supervision: MB, ÖFL; Securing funding (if available): n/a.
Ethical Approval: Since G-361 human melanoma cancer cell line is used in our study, no Ethics Committee Approval Certificate is required.
Peer-review: Externally peer-reviewed.
Conflict of Interest: The authors state that they have no interests that conflict. There are no animal products used in the making of this article.
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