

RESEARCH ARTICLE

Dose-Dependent Cytotoxic Effects of Menthol on Human Malignant Melanoma A-375 Cells: Correlation with TRPM8 Transcript Expression

Teeratas Kijpornyongpan¹, Amornpun Sereemasapun², Chanpen Chanchao^{1*}

Abstract

Background: Transient receptor potential melastatin 8 (TRPM8), a principle membrane receptor involved in calcium ion influx and cell signal transduction, has been found to be up-regulated in some cancer types, including melanomas. Efficiency of menthol, an agonist of TRPM8, in killing melanoma cancer cells has been reported previously, but the mechanisms remain unclear. We here determined whether *in vitro* cytotoxic effects of menthol on A-375 human malignant melanoma cells might be related to TRPM8 transcript expression. **Materials and Methods:** The PrestoBlue[®] cell viability assay was used to assess the *in vitro* cytotoxic effect of menthol after 24h of treatment. RT-PCR was used to quantify TRPM8 transcript expression levels in normal and menthol-treated cells. Cell morphology was observed under inverted phase contrast light microscopy. **Results:** TRPM8 transcript expression was found at low levels in A-375 cells and down-regulated in a potentially dose-dependent manner by menthol. Menthol exerted *in vitro* cytotoxic effects on A-375 cells with an IC₅₀ value of 11.8 μM, which was at least as effective as 5-fluorouracil (IC₅₀=120 μM), a commonly applied chemotherapeutic drug. Menthol showed no dose-dependent cytotoxicity on HeLa cells, a TRPM8 non-expressing cell line. **Conclusions:** The cytotoxic effects on A-375 cells caused by menthol might be related to reduction of the TRPM8 transcript level. This suggests that menthol might activate TRPM8 to increase cytosolic Ca²⁺ levels, which leads to cytosolic Ca²⁺ imbalance and triggers cell death.

Keywords: A-375 melanoma cells - Ca²⁺ balance - cytotoxicity - menthol - TRPM8

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Introduction

Although melanomas are less common than other skin cancers, they are one of the most fatally severe types of skin cancer causing an estimated 75% of all skin cancer related deaths, and their incidence is steadily increasing worldwide. According to the American Cancer Society estimation (Jemal et al., 2008; Society, 2012), the number of new melanoma cases in just the USA increased by 22% from 62,480 in 2008 to 76,250 in 2012, and this included 8% increase in the number of fatalities from 8,420 to 9,180. The disease is caused by transformation of melanocytes, the melanin-producing cells, into melanoma cells, which rapidly proliferate and become metastatic (Liu et al., 2006). Surgical removal of the melanoma is used to cure the disease in early stages when still small and not spread with good success, but this is ineffective once the cancer cells have started to metastasize. Several traditional non-surgical methods, such as radiotherapy and/or chemotherapy, are not successful enough because of melanoma resistance (Yamamura et al., 2008) and

intolerance of patients towards side effects of these treatments. Therefore, the development of alternative therapies is of interest for potentially more effective melanoma cancer treatments.

Transient receptor potential melastatin 8 (TRPM8), a membrane receptor involved in calcium ion influx and cell signal transduction (Behrendt et al., 2004; Bautista et al., 2007), is over-expressed in melanoma cells and some other types of cancer, such as prostate, breast and colorectal cancers (Zhang and Barritt, 2006; Prevarskaya et al., 2007; Johnson et al., 2009; Faridi et al., 2011), although in untransformed cells TRPM8 is mainly found in sensory neurons and some epithelial cells, and plays a role in cooling sensation (Bautista et al., 2007; Sabnis et al., 2008; Banner et al., 2011). Although there is a suggestion that TRPM8 is related to tumorigenesis and cancer cell physiology (Zhang and Barritt, 2006; Prevarskaya et al., 2007a; 2007b), there is only limited evidence to support this idea. Therefore, further studies in the correlation of TRPM8 and cancer are required to develop TRPM8 agonists as an alternative approach for cancer therapy in

¹Department of Biology, Faculty of Science, ²Department of Anatomy, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand *For correspondence: chanpen.c@chula.ac.th

the future.

Menthol, a volatile cyclic terpene compound (chemical formula $C_{10}H_{20}O$) that is used in many pharmaceutical drugs such as chest rubs, analgesic balm, nose drop and spray, cough drops and lotion, is one of the major agonists of TRPM8. Several reports show that menthol triggers an increase in cytosolic Ca^{2+} levels (Behrendt et al., 2004; Yamamura et al., 2008; Kim et al., 2009), leading to downstream Ca^{2+} -dependent cell signaling pathways. With respect to its bioactivity, some studies have recently reported on the efficiency of menthol in killing cancer cells such as gastric, leukemia, prostate, colorectal adenocarcinoma and skin (melanoma) cancers (Lin et al., 2005; Lu et al., 2006; Yamamura et al., 2008; Kim et al., 2009; Lu et al., 2009; Faridi et al., 2011), but few studies have pointed out a mechanism. Rather, it is still controversial as to whether the process involves or is related to TRPM8 (Yamamura et al., 2008; Kim et al., 2009).

Here, using the TRPM8 expressing A-375 human malignant melanoma cell line as an *in vitro* model, the effect of menthol on A-375 cell death was evaluated in direct comparison with the efficiency of 5-fluorouracil (5-FU), a typical chemotherapeutic drug, and also the TRPM8 negative HeLa cell line.

Materials and Methods

Chemicals

Menthol, N-(4-t-Butylphenyl)-4-(3-chloropyridin-2-yl) tetrahydropyridazine-1(2H)-carboxamide (BCTC) and Dulbecco modified Eagle's medium (DMEM) were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) and Penicillin-Streptomycin antibiotics were purchased from Gibco (Eggenstein, Germany). PrestoBlue[®] and Trizol's reagents were from Invitrogen (Carlsbad, CA). Reagents for reverse transcription and PCR were from Roche Applied Science (Mannheim, Germany) and Promega (Fitchburg, UW), respectively.

Menthol preparation

Research-grade menthol was dissolved in 20% (v/v) ethanol to a stock concentration of 2.5 mM and subsequently diluted into a working solution with the desired menthol concentration and a constant final ethanol concentration of 2.5% (v/v).

Cell culture

Human malignant melanoma (A-375) and human cervical malignant (HeLa) cell lines were cultured in DMEM supplemented with 10% (v/v) FBS, 50 units/mL penicillin and 50 μ g/mL streptomycin at 37°C in a humidified 5% (v/v) CO_2 atmosphere.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was performed as a two-stage reaction. Total RNA was extracted from around 2×10^5 cells using the Trizol[®] reagent, and then in the first stage converted into cDNA using Transcriptor First Strand cDNA synthesis Kit (Roche, Mannheim, Germany) with oligodT primers, as

per the manufacturer's instructions. In the second stage, specific oligonucleotide primers from Sabnis et al. (2008) were used for TRPM8 cDNA amplification via PCR to assay the human TRPM8 transcript levels (expected amplicon size 593 bp). A pair of oligonucleotide primers designed to be specific for the GAPDH gene (sense: 5'-GAGGGGCCATCCACAGTCTTC-3' and antisense: 5'-CATCACCATCTTCCAGGAGCG-3') was used as a positive control (expected amplicon size 357 bp). The PCR condition was 35 cycles of 95°C for 30 s, 63°C (TRPM8) or 58°C (GAPDH) for 45s and 72°C for 45s. PCR amplicons were detected following resolution by 2% (w/v) agarose gel electrophoresis and visualized by UV-transillumination after ethidium bromide staining.

PrestoBlue[®] cell viability assay

To measure the cytotoxic effect of menthol, A-375 or HeLa cells were seeded at 5,000 cells per well (in 45 μ L DMEM) in a 96-well plate and incubated for 12h. Then, ethanol (solvent) alone (negative control) or containing menthol at various concentrations to give the indicated final menthol concentration and 2.5% (v/v) ethanol was added and incubated for 24h. The cell viability was then determined using the PrestoBlue[®] reagent, using a microplate reader with an excitation and emission wavelength of 560 and 590 nm, respectively. Finally, the data was converted into the relative cell viability (%) from the absorbance of cells in each treatment relative to that in the solvent only control group (set as 100%). Unpaired t-test was used to compare data between each treatment and the negative control.

Cell morphology

Cells were seeded at 50,000 cells per well in 500 μ L DMEM in a 24-well plate and incubated for 12h. After that, ethanol (solvent) alone (negative control) or containing menthol at various concentrations to give the indicated final menthol concentration and 2.5% (v/v) ethanol was added and incubated for 24h. The cell morphology change was then visualized and photographed under an inverted phase contrast microscope at 200 \times magnification.

Results

TRPM8 transcript expression in A-375 Cells

RT-PCR was used to determine if TRPM8 was still expressed in the human malignant melanoma A-375 cell line used in this study. Amplification of the GAPDH transcript (357 bp) was used as a positive control. TRPM8 amplicons (597 bp) were clearly detected, albeit at a ~6-fold lower expression level than that for GAPDH amplicons (Figure 1).

In Vitro cytotoxic effect of menthol on the A-375 cell line

The cell viability of A-375 cells treated with different levels of menthol decreased in a dose-dependent manner from 100% to $42.5 \pm 10.3\%$ at increasing menthol levels from 0-200 μ M, with a derived IC_{50} value of 11.8 μ M (Figure 2A). A menthol concentration from 20 μ M upwards caused a significant reduction in the A-375 cell viability ($p < 0.0001$) compared to that of the solvent (2.5% (v/v) ethanol) only control treatment.

In direct comparison, the corresponding *in vitro* cytotoxicity of the traditional anti-cancer drug 5-fluorouracil (5-FU), at its previously reported IC₅₀ concentration of 120 μM (Sun et al., 2006), was not statistically significantly different from the cell viability obtained at a menthol concentration of 20 μM (Figure 2B), suggesting that menthol might be some six-fold more active but, regardless, at any rate may be an alternative treatment agent for melanoma cancers. This is relevant given that menthol is a FDA GRAS (generally regarded as safe) compound, with an estimated safe dosage by oral administration of at least 200 μg/kg body weight (LD₅₀ ~192-2900 mg/kg) and with no known serious side effects, in contrast to those associated with systemic *in vivo* administration of 5-FU.

A-375 cell morphology after menthol treatment

To further characterize the *in vitro* cytotoxic effect of menthol on A-375 cells, the cell morphology following menthol treatment was observed in order to roughly determine the type of cell death. From the inverted phase contrast microscopy (Figure 3), it was found that following menthol treatment the A-375 cells detached

from the substratum and appeared abnormal with a rounded shape, compared to those in the control group which were attached and spindle-shaped. Additionally, some cells shrank with a ruptured membrane, suggesting apoptotic cell death. The proportion of abnormal and dead cells increased with increasing menthol concentrations.

TRPM8 transcript expression in menthol-treated A-375 cells

To clarify if the A-375 cell death caused by exposure to menthol was related to changes in the TRPM8 receptor, the effect of menthol on the TRPM8 transcript expression level was tested. A-375 cells were treated by menthol at a low (10 μM) and high (100 μM) concentration for 24h and then the total RNA was extracted and used for two-stage RT-PCR evaluation of the TRPM8 transcript expression level relative to that of GAPDH. The result showed a slight and marked (and so an apparent dose-dependent) reduction of TRPM8 transcript expression levels with 10 μM and 100 μM menthol, respectively, while GAPDH transcript expression levels remained essentially constant (Figure 4).

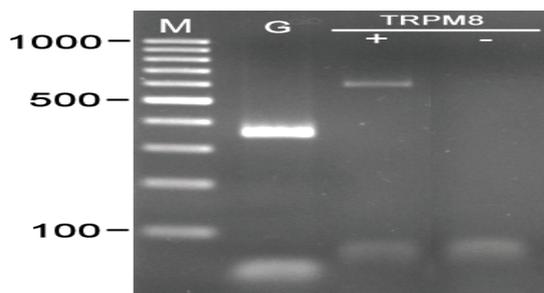


Figure 1. Expression of Transient Receptor Potential Subfamily Melastatin8 (TRPM8) Transcripts in the Human Malignant Melanoma A-375 Cell Line. Total mRNA from A-375 cells was extracted and converted into cDNA. Then, cDNA was separately PCR-amplified for GAPDH (357 bp) as a positive control (abbreviated as G), and TRPM8 (597 bp,+and-represent with and without inclusion of the reverse transcriptase). DNA markers are shown on the left. Image shown is representative of three independent repeats

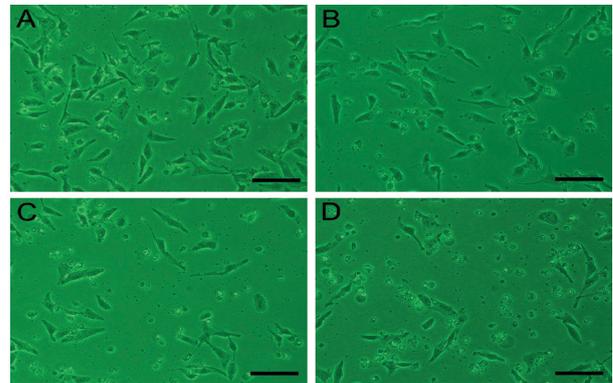


Figure 3. *In vitro* Effect of Menthol on the A-375 Cell Line Morphology. Cells were treated with solvent (2.5% (v/v) ethanol) containing menthol at (A) 0 μM (control), (B) 10 μM, (C) 20 μM, and (D) 50 μM for 24h. Cell morphology was visualized and photographed under inverted phase contrast microscopy at 200 x magnification. Bar=100 μm. Images shown are representative of those seen from at least 2 such fields of view per sample and 3 independent samples

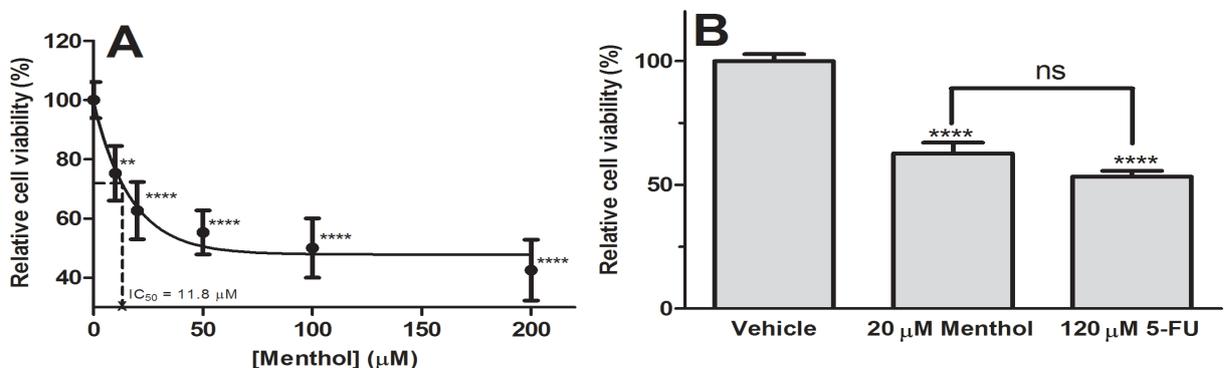


Figure 2. *In vitro* Cytotoxicity of Menthol on the A-375 Cell Line. Cells were treated by menthol at different concentrations or solvent alone (2.5% (v/v) ethanol) for 24h. The cell viability was calculated relative to that in the solvent only control group (set to 100%). (A) Dose-dependent cytotoxicity of menthol on A-375 cells with an IC₅₀ value of 11.8 μM. (B) 20 μM menthol treatment showed no statistical difference in cytotoxicity compared to 5-fluorouracil (5-FU) at its IC₅₀ concentration (120 μM). Data are shown as the mean±1 SD and are derived from 4 independent repeats. Means that are significantly different from the control are indicated as *, ** and **** for p<0.05, p<0.01 and p<0.0001, respectively

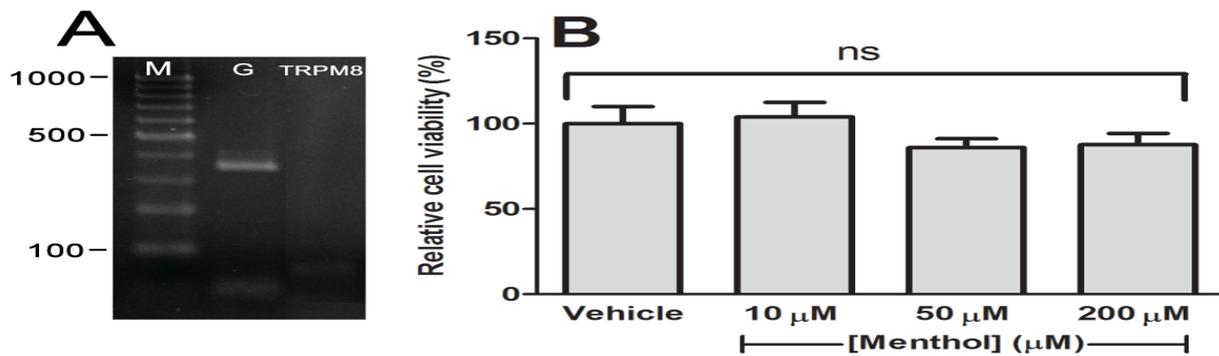


Figure 5. *In vitro* cytotoxicity of the HeLa cell by menthol. (A) Representative (from 3 independent reactions) RT-PCR-derived TRPM8 transcript expression level with that for GAPDH (+) as a template control. (B) The relative cell viability (compared to the solvent only control cells set as 100%) of HeLa cells after menthol treatment at the indicated final dose for 24 h. Data are shown as the mean \pm 1 SD and are derived from 4 independent repeats. Means that are significantly different from the control are indicated as *, ** and **** for $p < 0.05$, $p < 0.01$ and $p < 0.0001$, respectively, and ns=not significant ($p > 0.05$)

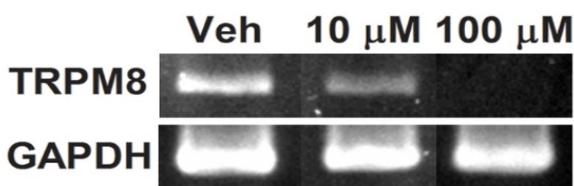


Figure 4. Effect of *in vitro* Menthol Treatment on TRPM8 Transcript Expression in the A-375 Cell Line. The RT-PCR-derived transcript levels of TRPM8 and GAPDH (positive control) in cells treated with solvent (2.5% (v/v) ethanol) alone (negative control) or containing 10 μM or 100 μM menthol for 24h

However, the quantitative TRPM8 transcript expression level relative to that of the GAPDH transcripts could not be precisely quantified due to the large variation in their expression levels between each experiment.

In vitro cytotoxic effect of menthol on hela cells

In addition, the *in vitro* effect of menthol on the HeLa cell line, which does not express TRPM8 transcripts (Expression Atlas, EMBL-EBI), was evaluated. RT-PCR analysis confirmed the absence of detectable levels of TRPM8 transcripts in the HeLa cell line used in this study (Figure 5A). However, menthol treatment only slightly affected the cell viability of the HeLa cell line even at 100 μM, numerically reducing the cell viability to 87% which was not statistically significant (Figure 5B). This correlates with the absence of TRPM8 transcript expression in the HeLa cells, suggesting that the action of menthol might occur via the TRPM8 receptor pathway.

Discussion

TRPM8 is a major menthol receptor, the expression of which is mainly found in sensory neurons, and is involved in regulating Ca^{2+} influx and cooling sensation (Bautista et al., 2007). It is also found to be expressed in many types of cancer cells, such as prostate, breast, pancreas and melanoma skin cancers (Zhang and Barritt, 2006; Prevarskaya et al., 2007; Liu and Qin, 2011). Here, TRPM8 transcript expression in the A-375 malignant melanoma cell line was confirmed (Figure 1). Combined

with the fact that menthol is one of the principle agonists of TRPM8, some reports have suggested that menthol is responsible for TRPM8-mediated cancer cell death. However, neither the correlation nor the mechanism has been confirmed. Thus, we evaluated the cell viability and TRPM8 transcript expression levels in A-375 cells exposed *in vitro* to menthol.

Menthol induced a dose-dependent cytotoxic effect on the A-375 cell line (Figure 2A). Although a similar result has previously been reported on the G-361 malignant melanoma cell line (Yamamura et al., 2008), the IC_{50} value of menthol on the A-375 line reported here was over 20-fold lower, suggesting a significant difference in the menthol sensitivity between the two cell lines. Several diverse studies have reported that menthol elicits an increase in the cytosolic calcium concentration (Behrendt et al., 2004; Yamamura et al., 2008; Kim et al., 2009; Latorre et al., 2011; Liu and Qin, 2011), and so it has been suggested that the menthol-triggered increased cytosolic Ca^{2+} by TRPM8 action in A-375 cells might cause an ion imbalance and lead to cell death, similar to the prostate cancer cell model (Zhang and Barritt, 2006; Liu and Qin, 2011). Additionally, compared with 5-FU treatment, menthol was at least as effective as 5-FU in the *in vitro* cytotoxicity of the A-375 cell line, with an IC_{50} for menthol of 11.8 μM (Figure 2B) compared to 120 μM for 5-FU. However, the use of systemic 5-FU administration in chemotherapy has the serious drawback of multiple and sometimes limiting side effects to the patients, such as mucositis, myelosuppression, neurotoxicity and arrhythmia (Grem, 2000), whilst menthol is a FDA GRAS compound with no known serious side effects following oral administration. Thus, menthol may have potential as an alternative drug for the therapeutic treatment of certain cancer types, subject to not inducing rapid resistance.

A-375 cell shrinkage with ruptured membranes was observed after menthol treatment (Figure 3). This morphological change is in agreement with several publications suggesting that menthol leads to apoptotic cell death in prostate cancer and colon adenocarcinoma cells (Zhang and Barritt, 2006; Prevarskaya et al., 2007; Faridi et al., 2011; Liu and Qin, 2011), but it does not establish this. In contrast, other reports have suggested that

cell cycle arrest, but not apoptosis, is the major cause for a decreased number of cancer cells (Kim et al., 2009; Yang et al., 2009; Yee et al., 2010; Wang et al., 2012). Therefore, further work is required such as TUNEL, Annexin V and DNA degradation assays, to elucidate the mechanism(s) of the observed reduction in viable cell numbers and the changed cellular morphology elicited by menthol.

Although variable, like in prostate cancer cell model (Zhang and Barritt, 2006), the TRPM8 transcript expression level in A-375 cells was slightly and markedly down-regulated by 10 and 100 μ M menthol, respectively (Figure 4), suggesting a possible dose-dependent response. In pancreatic adenocarcinoma, TRPM8 expression was found to be required for cell proliferation, where siRNA-mediated gene knockdown of TRPM8 caused a decline in the number of cancer cells (Yee et al., 2010). The menthol-induced decreased TRPM8 transcript expression level in A-375 melanoma cells may then be linked to the reduced cell proliferation and increased cell death, although this, the protein expression level and functional level of the receptors, and any correlation to the decreased cell viability, all require confirmation. In another study, the induction of TRPM8 overexpression in PC-3 prostate cancer cells also had a negative effect on the cell proliferation and migration (Yang et al., 2009). Thus, TRPM8 might play a role in intracellular Ca^{2+} balance, where increased cytosolic Ca^{2+} induced by menthol might cause an ion imbalance and lead to cell death (Zhang and Barritt, 2006; Prevarskaya et al., 2007; Liu and Qin, 2011), with the reduced TRPM8 transcript levels being a response in order to try to reduce the TRPM8-mediated Ca^{2+} influx and regain Ca^{2+} homeostasis in the cancer cell.

BCTC, a TRPM8 antagonist, has been used as a receptor blocker to confirm the function of TRPM8 in several studies (Behrendt et al., 2004; Cho et al., 2010; Wang et al., 2012). Here, when BCTC pretreatment (1 μ M) was used 30 min before menthol treatment, no reduction in the loss of cell viability was detected following menthol exposure (100 μ M), but rather BCTC induced A-375 cell death itself (Supplementary file 1). This result agrees with an earlier report that TRPM8 antagonists reduced the proliferation of prostate cancer cells (Valero et al., 2012). Hence, TRPM8 might play a role in the Ca^{2+} balance of specific intracellular calcium pools, and that either excess activation or inhibition of TRPM8 results in cytosolic Ca^{2+} imbalance and causes a reduced cell proliferation and, if not corrected, induced cell death.

The TRPM8 negative HeLa cell line was used to indirectly assess if the action of menthol requires or involves the TRPM8 receptor. In accord with the observation that these HeLa cells lacked detectable TRPM8 transcript expression (Figure 5A), they also showed no dose-dependent cytotoxic effect of menthol (Figure 5B). However, it cannot be confirmed whether menthol action is just cell type-specific and so other TRPM8 expressing and non-expressing cell types, combined with comparison of the effect of menthol on normal and cancer cell types, should be evaluated.

In conclusion, the obtained data in this research supports the potential role of TRPM8 in the mechanism of menthol against A-375 malignant melanoma cell

proliferation/cytotoxicity. Clearly, menthol had a cytotoxic effect on A-375 cells with an IC_{50} value of 11.8 μ M, and was at least as effective as 5-FU treatment. Also, menthol caused a down-regulation of TRPM8 transcript expression levels, suggesting a potential involvement of menthol with this receptor pathway. Future research should investigate the mechanism in-depth, examining menthol efficacy and its side-effects prior to any proposed clinical trials. However, TRPM8 may be a potential alternative therapeutic agent for melanoma and other TRPM8-expressing cancer types.

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