

Antagonism of the transient receptor potential melastatin-2 channel leads to targeted antitumor effects in primary human malignant melanoma cells

SHELBY G. McKAMEY, LUKAS R. JIRA, CHRISTOPHER M. TWEED, STEVEN D. BLAKE,
DANIEL P. POWELL, AYAH T. DAGHISTANI and DAVID W. KOH

Department of Pharmaceutical and Biomedical Sciences, Ohio Northern University, Ada, OH 45810, USA

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Abstract. Melanoma continues to be the most aggressive and devastating form of skin cancer for which the development of novel therapies is required. The present study aimed to determine the effects of antagonism of the transient receptor potential melastatin-2 (TRPM2) ion channel in primary human malignant melanoma cells. TRPM2 antagonism via use of the antifungal agent, clotrimazole, led to decreases in cell proliferation, as well as dose-dependent increases in cell death in all melanoma cell lines investigated. The targeting of TRPM2 channels was verified using TRPM2 knockdown, where treatment with TRPM2 small-interfering RNA led to similar levels of cell death in all melanoma cell lines when compared with clotrimazole treatment. Minimal effects on proliferation and cell death were observed following antagonism or knockdown of TRPM2 in non-cancerous human keratinocytes. Moreover, characteristics of TRPM2 were explored in these melanoma cells and the results demonstrated that TRPM2, localized to the plasma membrane as a non-specific ion channel in non-cancerous cells, displayed a nuclear localization in all human melanoma cell lines analyzed. Additional characterization of these melanoma cell lines confirmed that each expressed one or more established multidrug resistance

genes. Results of the present study therefore indicated that antagonism of the TRPM2 channel led to antitumor effects in human melanoma cells, including those that are potentially unresponsive to current treatments due to the expression of drug resistance genes. The unique cellular localization of TRPM2 and the specificity of the antitumor effects elicited by TRPM2 antagonism suggested that TRPM2 possesses a unique role in melanoma cells. Collectively, the targeting of TRPM2 represents a potentially novel, efficacious and readily accessible treatment option for patients with melanoma.

Introduction

Melanoma is the most aggressive and debilitating form of skin cancer, as it progresses quickly and readily metastasizes (1). Moreover, the survival rate of patients with melanoma is markedly decreased as the condition progresses to more advanced stages, as the five-year survival rate in patients with localized tumors is >98%, but this survival rate decreases to <27% once tumors metastasize (2). Thus, prevention and early detection of melanoma are key for optimal patient outcomes. Within the last decade, the development of novel treatment options have significantly improved the survival rate of patients with early to mid-stage melanoma (3-5). Smaller, yet significant improvements were also seen in patients with advanced-stage disease; however, the overall survival rate remains low in this group (4,5). Therefore, due to the increased incidence of new cases diagnosed each year, the ongoing levels of mortality due to melanoma in both men and women, and the continued difficulty to treat patients with late-stage melanoma, further development of novel treatment options is required (2).

Disadvantages of current treatment options and the emergence of drug resistance have contributed to poor outcomes in patients with cancer (6,7). One current treatment option for melanoma is conventional chemotherapy. However, it is not a first-line treatment option and can cause debilitating adverse effects, such as hepatotoxicity and myelosuppression (8). Both immunotherapy and targeted therapy have improved treatment outcomes with greater efficacy and tolerability in patients with melanoma, when compared with conventional chemotherapy (5,9,10). However, limitations include a lack of response by numerous patients with melanoma, the inability

Correspondence to: Dr David W. Koh, Department of Pharmaceutical and Biomedical Sciences, Ohio Northern University, 525 South Main Street, Ada, OH 45810, USA
E-mail: d-koh@onu.edu

Abbreviations: AMG 9810, (2E)-N-(2,3-Dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-propenamide; AMTB, N-(3-Aminopropyl)-2-[(3-methylphenyl)methoxy]-N-(2-thienylmethyl)benzamide hydrochloride; MDR, multidrug resistance; MnSOD, manganese superoxide dismutase; TMZ, temozolomide; TRP, transient receptor potential; TRPM2, transient receptor potential melastatin-2

Key words: melanoma, skin cancer, TRP channels, TRPM2, antifungal agent, cell death, multidrug resistance, reverse transcription-quantitative PCR

to consistently treat patients with late-stage melanoma, and the cost of immunotherapy and targeted therapy treatment regimens, particularly if combination therapies are utilized (11-13). Moreover, levels of drug resistance contribute to poor treatment outcomes, as patients fail to adequately respond to the current available treatment options. This is due, in part, to cellular mechanisms that allow the cancer cell to overcome or avoid the anti-tumor effects of treatment. Such mechanisms include the evasion of cell death due to the ability to inhibit pro-apoptotic signaling, or the decreased expression of pro-cell death mediators, such as death receptors and apoptotic protease activating factor-1 (14,15). Another form of resistance in cancer cells is known as multidrug resistance (MDR). This involves the expression of drug-resistance proteins, which allows cancer cells to avoid exposure to cancer drugs (6,16-18). Three well-known drug resistance proteins are phosphorylated-glycoprotein 1 (MDR1) (19), multidrug resistance-associated protein 1 (MRP1) (20) and lung resistance-related protein (LRP) (21). These enable MDR in cancer cells, where they serve as specialized efflux pumps that expel chemotherapeutic drugs from cancer cells and thereby allow tumors to avoid the associated toxic effects (22). By doing so, MDR proteins promote the survival of cancer cells following treatment. Thus, it is paramount that novel and efficient treatment options are made available for patients with melanoma, including those that are more economical and accessible, and those that can overcome drug resistance.

The transient receptor potential (TRP) channels represent a superfamily of non-specific ion channels with numerous functions in healthy cells. They reside on the plasma membrane and through their ability to serve as ionophores, TRP channels exhibit numerous functions, including facilitation of vision and taste (23,24), detection of heat and pain sensations (24), enabling contraction of smooth muscle and migration of squamous cells (25,26), and the facilitation of cell death (27). Several types of TRP channels have been studied to determine their potential roles in cancer. One such channel is the second member of the TRP melastatin sub-family, TRP melastatin-2 (TRPM2) channel. TRPM2 is a calcium-permeable non-selective ion channel that is activated in response to oxidative stress (28). Activation of TRPM2 channels promotes a number of processes, including microglial activation (29), immune cell activation (30) and cell death (27). It has previously been reported that antagonism of TRPM2 channels in healthy cells was beneficial following oxidative injury, leading to increased cell survival (31,32). However, a paradoxical effect was observed in several cancer types, where TRPM2 antagonism led to decreased proliferation and increased cell death. These anti-tumor effects were observed in numerous cancers, such as breast, prostate and oral cancer (33-36).

The purpose of the present study was to determine the effects of TRPM2 antagonism in primary human malignant melanoma cells by analyzing the effects on proliferation and cell death. Moreover, the present study aimed to further characterize TRPM2 channels in these cells, in order to identify the potential differential role of TRPM2 channels in melanoma cells.

Materials and methods

Chemicals. (2E)-N-(2,3-Dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-propenamide (AMG 9810),

was prepared as a 1 mM stock solution in dimethyl sulfoxide (DMSO) for each experiment, and was purchased from Tocris Bioscience. N-(3-Aminopropyl)-2-[(3-methylphenyl)methoxy]-N-(2-thienylmethyl)benzamide hydrochloride (AMTB; 5 mM stock solution in water) was purchased from Santa Cruz Biotechnology, Inc. Clotrimazole (25 mM in DMSO) and temozolomide (TMZ; 10 mM in DMSO) were purchased from Sigma-Aldrich; Merck KGaA. ApoScreen Propidium Iodide (PI) and ApoScreen annexin V-fluorescein isothiocyanate (FITC) solutions were purchased from SouthernBiotech.

Cell lines and cell culture reagents. The primary human malignant melanoma cell line, SK-Mel-23, was obtained from the Memorial Sloan-Kettering Cancer Center. This cell line was previously established from a metastatic lymph node, the tissue of origin was a rectal tumor, and the genotype profile is wild-type, with respect to the GTPase N-RAS (N-RAS) and serine/threonine-protein kinase B-RAF (B-RAF) genes (37). The primary human malignant melanoma cell lines, UKRV-Mel-4 and UKRV-Mel-5 were both obtained from the European Searchable Tumor Line Database, directed by Dr Graham Pawalec at the University of Tübingen (Germany) (38). The UKRV-Mel-4 cell line was previously established from a liver metastasis and is wild-type for both N-RAS and B-RAF (39,40). The UKRV-Mel-5 cell line was previously established from a subcutaneous lesion and is also wild-type for N-RAS and B-RAF (40). All three melanoma cell lines were cultured in RPMI 1640 medium (GenDEPOT, LLC), supplemented with 10% fetal bovine serum (FBS; characterized; HyClone; Cytiva), 2 mM L-glutamine (Corning Inc.) and 100 U/ml penicillin/streptomycin (HyClone; Cytiva) at 37°C in 5% CO₂. The human keratinocyte cell line, HaCat, was purchased from AddexBio Technologies (cat. no. T0020001). This cell line was authenticated by the manufacturer using DNA STR profiling and the full statement of authentication can be accessed online (<https://www.addexbio.com/productdetail?pid=117>). The human embryonic kidney cell line, 293T, was purchased from the American Type Culture Collection (cat. no. CRL-3216). Both the HaCat and 293T cell lines were cultured in DMEM medium (GenDEPOT, LLC) supplemented with 10% FBS and 2 mM L-glutamine at 37°C in 5% CO₂.

Reagents. OptiMEM reduced serum medium and Lipofectamine® 2000 reagent were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). Protease inhibitor cocktail tablets (Complete Mini, EDTA-free) were purchased from Roche Diagnostics, GmbH. Primary antibodies utilized were polyclonal rabbit anti-human TRPM2 (cat. no. A300-414A; Bethyl Laboratories, Inc.), polyclonal rabbit anti-human β -actin (cat. no. 600-401-886; Rockland Immunochemicals Inc.), monoclonal mouse anti-human β -actin, clone AC-74 (cat. no. A5316; Sigma-Aldrich; Merck KGaA), polyclonal rabbit anti-human manganese superoxide dismutase (MnSOD; cat. no. 06-984; MilliporeSigma), monoclonal mouse anti-human lamin B2, clone LN43 (cat. no. MA1-06104; Thermo Fisher Scientific, Inc.), polyclonal rabbit anti-human MDR1 (cat. no. 13342S; Cell Signaling Technology, Inc.), polyclonal rabbit anti-human MRP1 (cat. no. 14685S; Cell

Signaling Technology, Inc.) and monoclonal mouse anti-human LRP, clone 1014 (cat. no. sc-23916; Santa Cruz Biotechnology, Inc). Two secondary antibodies, peroxidase-AffiniPure goat anti-rabbit IgG, Fc fragment specific (cat. no. 111-035-008) and peroxidase-AffiniPure rabbit anti-mouse IgG, Fc (γ) fragment specific (cat. no. 315-035-008) were purchased from Jackson ImmunoResearch Laboratories, Inc.

Cell culture. All cells were grown and maintained in RPMI 1640 or DMEM supplemented with 10% FBS, L-glutamine and 100 U/ml penicillin/100 μ g/ml streptomycin, and incubated at 37°C in 5% CO₂ for further experiments, as previously described. For transfection experiments, no antibiotics were utilized. Every 2 days in culture, cells were washed once with PBS (pH 7.2) and cultured in fresh growth medium. Cells were subcultured or harvested using 0.25% trypsin/0.02% EDTA (Quality Biological, Inc.).

Cell proliferation analyses. Cells were seeded in triplicate in 24-well cell culture plates in growth medium at a minimum seeding density of 1x10⁵ cells/ml. Cells were incubated at 37°C overnight and subsequently treated with 5-50 μ M clotrimazole, 1 μ M AMG 9810 or 5 μ M AMTB. Growth medium and treatments were replaced every 48 h. At time points 0, 1, 2, 3 and 6 days, growth medium was removed and cells were harvested using trypsinization. Cell numbers were then determined using a hemocytometer. All counts were performed in triplicate and all experiments were performed at least twice with similar results.

Cell death assays. For the quantification of cell death using flow cytometry, cells were treated with clotrimazole or TRPM2 small-interfering (si)RNA, harvested by trypsinization, washed with PBS and resuspended in annexin-binding buffer (10 mM HEPES; pH 7.4; 140 mM NaCl; 2.5 mM CaCl₂) to ~1x10⁶ cells/ml. For each 100 μ l of cell suspension, 4 μ l ApoScreen annexin V-FITC conjugate solution and 2 μ l 100 μ g/ml ApoScreen propidium iodide (PI) solution was added. Cells were subsequently maintained at room temperature for 15 min in the dark. Following this, 0.4 ml annexin-binding buffer (SouthernBiotech) was added and cell death was measured by quantifying the percentage of cells that exhibited annexin V-FITC (FL1 channel) and PI (FL3) fluorescence using a flow cytometer (BD Accuri model C6; BD Biosciences). Flow cytometry analysis was performed using BD Accuri C6 software (version 1.0.264.2; BD Biosciences). Total cell death was quantified by adding the percentage of cells detected in the upper left (PI), upper right (PI + annexin V-FITC) and lower right (annexin V-FITC) quadrants in the flow cytometry dot plots. A minimum of 1x10⁵ cells was analyzed for each cell death determination and all experiments were performed in triplicate.

For quantification of cell death using a WST-1 assay, cells were seeded in 96-well cell culture plates at a density of 50,000 cells/ml and incubated at 37°C. The following day, cells were treated with 5-50 μ M clotrimazole for 2 days. Following treatment, cell death was determined using the CytoScan WST-1 Cell Cytotoxicity Assay (Geno Technology, Inc.). This is a colorimetric assay based upon the reduction of WST-1, a tetrazolium salt, by cellular dehydrogenases (41). The

product, formazan, is detected at 440 nm and any decreased dehydrogenase activity is directly associated with the amount of cell death following treatment. The assay was initiated by the addition of 10 μ l WST-1/CEC assay dye to each well and the reaction was carried out for 30 min at 30°C. Detection was performed using a BioTek Synergy HT microplate reader using Gen5 software (version 1.05.11). All data points were assayed in triplicate and all experiments were performed twice with similar results.

Transfection. The silencing of TRPM2 using transfection was performed as previously described, using siRNA specific to nucleotides 4574-4594 (5'-AUAGAUCAGGAACUC CGUCUC-3') of human TRPM2 (33). This RNA oligo was purchased as duplexed RNA (Integrated DNA Technologies, Inc.), resuspended in RNase-free water at a final concentration of 40 μ M, and stored at -20°C. The following non-targeting oligo primers: Forward, 5'AGACAGAAGACAGAUAGGCtt-3' (sense) and reverse, 5' GCCUAUCUGUCUUCUGUCUtt-3' (antisense) were used for all negative controls (Integrated DNA Technologies, Inc.). For transfection, cells were plated in 24-well plates containing 0.5 ml medium per well without antibiotics. Transfections were performed when cells reached 40-60% confluence. The transfection protocol was initiated by adding duplex siRNA to 50 μ l OptiMEM, and adding 1 μ l Lipofectamine® 2000 to 50 μ l OptiMEM in a separate tube. The two solutions were mixed and maintained at room temperature for 20 min. The final concentration was 100 nM for both TRPM2 siRNA and the corresponding negative control. The mixtures were added to each well in drops, mixed and the cells were cultured for an additional 48 h at 37°C. Western blotting and cell death analyses were subsequently performed 48 h after transfections.

Whole cell lysate extraction. Transfected cells were harvested using trypsinization, centrifuged at 112 x g for 5 min at 4°C, and washed once with 0.2 ml ice-cold PBS. The pellet was resuspended in 0.2 ml lysis buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40 and protease inhibitors. Suspensions were placed on ice for 30 min and vortexed every 10 min. The cell lysates were cleared by centrifugation at 16,000 x g for 10 min at 4°C and the protein concentration for all sample lysates was immediately obtained using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Subsequently, 10X SDS-PAGE sample buffer [500 mM Tris-HCl (pH 6.8), 10% SDS, 25% glycerol, 0.05% bromophenol blue and 100 mM dithiothreitol] was added to the supernatants to reach a final concentration of 1X (50 mM Tris-HCl, pH, 6.8, 1% SDS, 2.5% glycerol, 0.005% bromophenol blue and 10 mM dithiothreitol). Samples were then heated for 2 min at 95°C.

Subcellular fractionations. Cells were grown in 60-mm tissue culture dishes at 37°C for 48 h (~2x10⁶ cells/dish) and harvested by scraping in ice-cold PBS. Cell suspensions were centrifuged 200 x g for 5 min at 4°C and the resulting cell pellets were fractionated using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, Inc.). Briefly, the cell pellets were washed with 1 ml ice-cold PBS and centrifuged at 500 x g for 3 min at 4°C. The supernatant

was removed, and the pellet was resuspended in 0.2 ml ice-cold CER I solution (a kit component) containing protease inhibitors. The cell suspension was vortexed for 15 sec, placed on ice for 10 min and 11 μ l CER II solution (a kit component) was added. The suspension was vortexed for a further 5 sec, placed on ice for 1 min and subsequently vortexed again. The resulting extract was centrifuged at 16,000 \times g for 5 min at 4°C and the supernatant (cytoplasmic fraction) was removed. The pellet was washed with PBS and centrifuged at 16,000 \times g for 5 min at 4°C then resuspended in 0.1 ml of ice-cold NER solution (a kit component). The suspension was vortexed and placed on ice for 10 min, and the vortexing/ice procedure was repeated three times for a total of 40 min. The resulting extract was centrifuged at 16,000 \times g at 4°C for 10 min and the supernatant (nuclear fraction) was removed. Protein concentrations for cytoplasmic and nuclear fractions were obtained using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Subsequently, 10X SDS-PAGE sample buffer [500 mM Tris-HCl (pH 6.8), 10% SDS, 25% glycerol, 0.05% bromophenol blue and 100 mM dithiothreitol] was added to each cytoplasmic and nuclear fraction to reach a final concentration of 1X [50 mM Tris-HCl (pH 6.8), 1% SDS, 2.5% glycerol, 0.005% bromophenol blue and 10 mM dithiothreitol]. Samples were then heated for 2 min at 95°C.

Western blotting. For each western blot analysis, total proteins were originally extracted using either: i) Lysis buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40 and protease inhibitors (for RNAi and MDR protein extracts), ii) CER I/CER II solution (Thermo Fisher Scientific, Inc.; for cytoplasmic fractionation extracts) or iii) NER solution (Thermo Fisher Scientific, Inc.; for nuclear fractionation extracts). Total proteins were subsequently added to 10X SDS sample buffer [500 mM Tris-HCl (pH 6.8), 10% SDS, 25% glycerol, 0.05% bromophenol blue and 100 mM dithiothreitol] to reach a final concentration of 1X [50 mM Tris-HCl (pH 6.8), 1% SDS, 2.5% glycerol, 0.005% bromophenol blue and 10 mM dithiothreitol]. Protein concentrations for all sample lysates were obtained using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Following protein determination, 40 μ g of whole cell lysates or cell fractionations were loaded into each lane on a 7.5% SDS-PAGE gel. Following separation, the proteins were transferred to 0.45- μ m nitrocellulose (Protran BA 85; GE Healthcare Bio-Sciences) by semi-dry transfer at 25V for 30 min using a Trans-blot SD apparatus (BioRad Laboratories, Inc.). Membranes were blocked with PBS containing 0.05% Tween-20 (PBST) and 5% milk at room temperature for 1 h, then incubated with the following primary antibodies: Anti-TRPM2 (1:1,000), polyclonal β -actin (1:2,000), monoclonal β -actin (loading control for RNAi experiments; 1:5,000), anti-MnSOD (1:1,000), anti-lamin B2 (1:1,000), anti-MDR1 (1:1,000), anti-MRP1 (1:1,000) and anti-LRP (1:1,000) in PBST + 5% milk overnight at 4°C with shaking. Membranes were subsequently washed with PBST three times and incubated with anti-rabbit (1:5,000) or anti-mouse (1:5,000) secondary antibodies for 1 h at room temperature. The membranes were washed as previously described, and chemiluminescence was induced using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.)

for immunodetection of β -actin, MnSOD, lamin B2, MDR1 and LRP. SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.) was utilized for immunodetection of TRPM2 and MRP1. Densitometric analysis was completed using Adobe Photoshop CSS Extended (version 12.1x64; Adobe Systems, Inc.). Chemiluminescent detection was achieved using a ChemiDoc XRS gel imaging system (Bio-Rad Laboratories, Inc.).

Reverse transcription-quantitative (RT-q)PCR. For gene expression analyses using RT-qPCR, relative quantification using the comparative $2^{-\Delta\Delta C_q}$ method was utilized (42). Total RNA for each cell line was isolated using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was obtained using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). Briefly, 10 μ l total RNA was added to 10 μ l reaction mix containing primers, dNTPs and MultiScribe Reverse Transcriptase (all included in the kit). The reaction was carried out in four steps: 25°C for 10 min, 37°C for 120 min, 85°C for 5 min followed maintenance at 4°C. For qPCR, each reaction mix (20 μ l total) contained 10 ng cDNA, 10 μ l PowerUp SYBR Green Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and 5 pmol of the following primer sets: MDR1 forward, 5'-TTG CTGCTTACATTCAGGTTTCA-3' and reverse, 5'-AGCCTA TCTCCTGTGCGATTA-3'; MRP1 forward, 5'-AGAACCTCA GTGTCGGGCAGCG-3' and reverse, 5'-TCGCATCTCTGT CTCTCCTGGG-3'; LRP forward, 5'-CCTCGAGATCCATTG TGCTGG-3' and reverse, 5'-CACAGGGTTGGCCACTGT GGA-3'. β -actin was amplified as an internal control using the following primer set, as previously reported (43): Forward, 5'-CACCAACTGGGACGACAT-3'; and reverse, 5'-ACAGCCTGGATAGCAACG-3'. Following initial denaturation at 94°C for 2 min, qPCR amplification was conducted using 40 cycles of 94°C for 30 sec, 57°C for 15 sec and 72°C for 30 sec in a Bio-Rad DNAEngine thermal cycler with Chromo4 Continuous Fluorescence Detector (Bio-Rad Laboratories, Inc.). Gene expression was analyzed using MJ Opticon Monitor Analysis software, version 3.1.32 (Bio-Rad Laboratories, Inc.). Each reaction was repeated in quadruplicate for each experiment. A total of four separate qPCR quantifications were completed for each gene analysis.

Statistical analysis. All error bars for cell death and growth quantifications represented the standard error of the mean (SEM). Statistical analyses were carried out using one-way ANOVA followed by Dunnett's post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. Statistical analyses were completed using Microsoft Excel, version 14.1.0. All experiments were performed a minimum of three times and similar results were obtained.

Results

Clotrimazole treatment causes decreased proliferation in human malignant melanoma cells. To determine the effects of TRPM2 inhibition on the proliferation of human melanoma cells, three primary human malignant melanoma cell lines were treated with the TRPM2 inhibitor, clotrimazole (44). Treatment with 30 μ M clotrimazole led to significant decreases

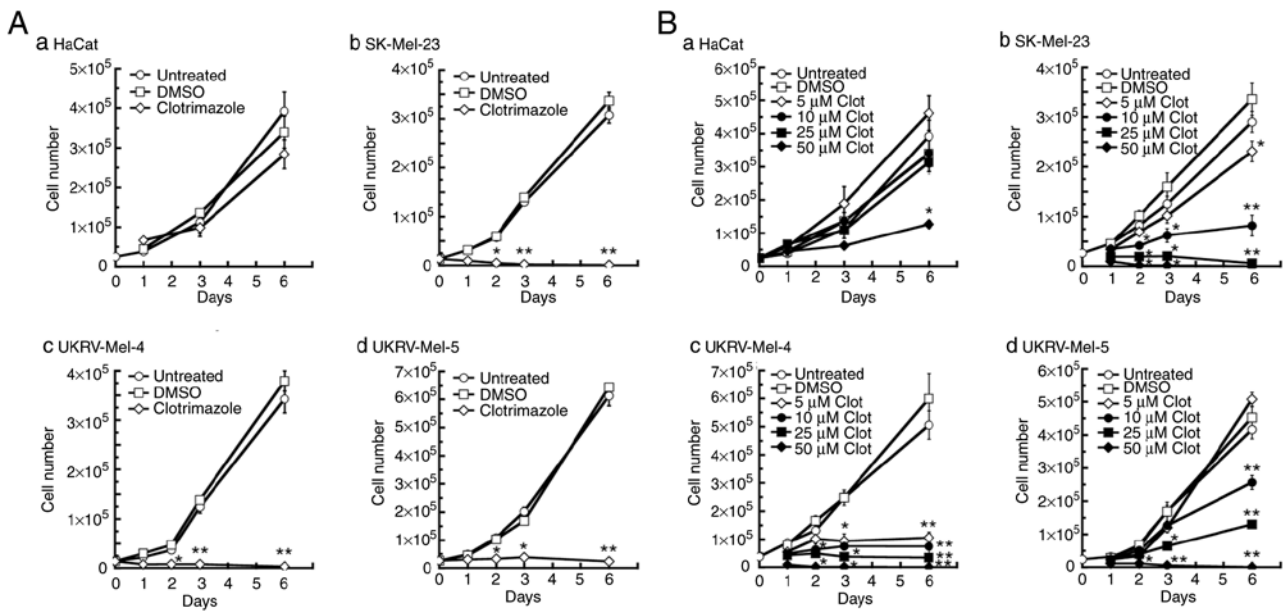


Figure 1. Effects of clotrimazole treatment on the proliferation of human malignant melanoma cells. (A) Treatment with the transient receptor potential melastatin-2 inhibitor, clotrimazole (30 μ M) was assessed for 1-6 days, after which proliferation was quantified in the non-cancerous human keratinocyte cell line (a) HaCat, and the primary malignant melanoma cell lines (b) SK-Mel-23, (c) UKRV-Mel-4 and (d) UKRV-Mel-5. (B) Quantification of proliferation following treatment with 5-50 μ M clotrimazole in (a) HaCat cells and the primary malignant melanoma cell lines (b) SK-Mel-23, (c) UKRV-Mel-4 and (d) UKRV-Mel-5. Untreated cells and cells treated with 0.1% DMSO acted as controls. * $P < 0.05$ and ** $P < 0.01$ vs. DMSO treatment; error bars represent the standard error of the mean. All experiments were performed at least three times with similar results. Clot, clotrimazole.

in proliferation after 2 days in all melanoma lines used, compared with the untreated and DMSO-treated controls (Fig. 1A-b-d). Further cell proliferation was not observed in the SK-Mel-23 and UKRV-Mel-4 melanoma cell lines after 2 days of treatment, compared with the positive growth observed in the same melanoma cell lines that were either untreated or treated with DMSO (Fig. 1A-b and -c). In the UKRV-Mel-5 melanoma line, cell proliferation was decreased by ~84% by day 3 of clotrimazole treatment (Fig. 1A-d); however, no further cell proliferation was observed subsequently. No significant decrease in cell proliferation was observed in human keratinocytes (HaCat cells) during 1-6 days of treatment with clotrimazole (Fig. 1A-a). These results demonstrated that clotrimazole treatment led to a notable decrease in cell proliferation in human malignant melanoma cells compared with HaCat cells.

Subsequently, the dose-dependent response of human melanoma proliferation to clotrimazole was investigated by treating each cell line with 5-50 μ M clotrimazole. Significant decreases in proliferation were observed in SK-Mel-23 and UKRV-Mel-4 cells following each dose of clotrimazole after 3 days of treatment (Fig. 1B-b and A-c). In the SK-Mel-23 cell line, in contrast with the DMSO-treated negative control groups, a 30% reduction in cell proliferation was observed following treatment with 5 μ M clotrimazole after 6 days of treatment, a 75% reduction was observed after 10 μ M treatment, and cell proliferation was completely inhibited following treatment with 25 μ M clotrimazole (Fig. 1B-b). In the UKRV-Mel-4 line, notable decreases in cell proliferation were observed at all doses by day 3 of treatment, compared with the untreated and DMSO-treated controls (Fig. 1B-c). By day 6, cell proliferation was decreased 85% following treatment with 5 μ M clotrimazole, 90% following treatment with 10 μ M clotrimazole, and

>98% following treatment with 25 μ M clotrimazole, compared with the DMSO-treated negative control groups (Fig. 1B-c). In the UKRV-Mel-5 line, a significant decrease in cell proliferation was observed after 3 days of treatment with 25 or 50 μ M clotrimazole, compared with the untreated and DMSO-treated groups after 3 days (Fig. 1B-d). By day 6 of treatment, cell proliferation was reduced by ~50% following treatment with 10 μ M clotrimazole and >80% following treatment with a dose of 25 μ M, compared with the cell growth observed in the corresponding DMSO-treated cells at day 6 (Fig. 1B-d). In all melanoma cell lines, cell proliferation was not observed following treatment with 50 μ M clotrimazole, compared with the maximal levels of cell growth observed in untreated and DMSO-treated cells. In HaCat cells, no significant effect on cell proliferation was observed following treatment with 5-25 μ M clotrimazole for 1-6 days. However, at the highest dose of clotrimazole (50 μ M), significant effects on cell proliferation were observed at days 3 and 6, with a ~60% decrease in cell proliferation by day 6. These results demonstrated that clotrimazole treatment led to dose-dependent decreases in cell proliferation in human malignant melanoma cells, with no significant effect on normal human keratinocytes within the 5-25 μ M dose range.

Clotrimazole causes increases in cell death in human primary malignant melanoma cells in a dose-dependent manner. To determine if the effects of clotrimazole on the primary human melanoma cell lines involved the induction of cell death, levels of cell death were determined in each cell line following 2 days treatment with 10-50 μ M clotrimazole using flow cytometry. The results of the present study demonstrated that clotrimazole treatment led to dose-dependent increases in cell death in each human melanoma line, when compared with similar treatments in noncancerous keratinocytes at each dose (Fig. 2A). While

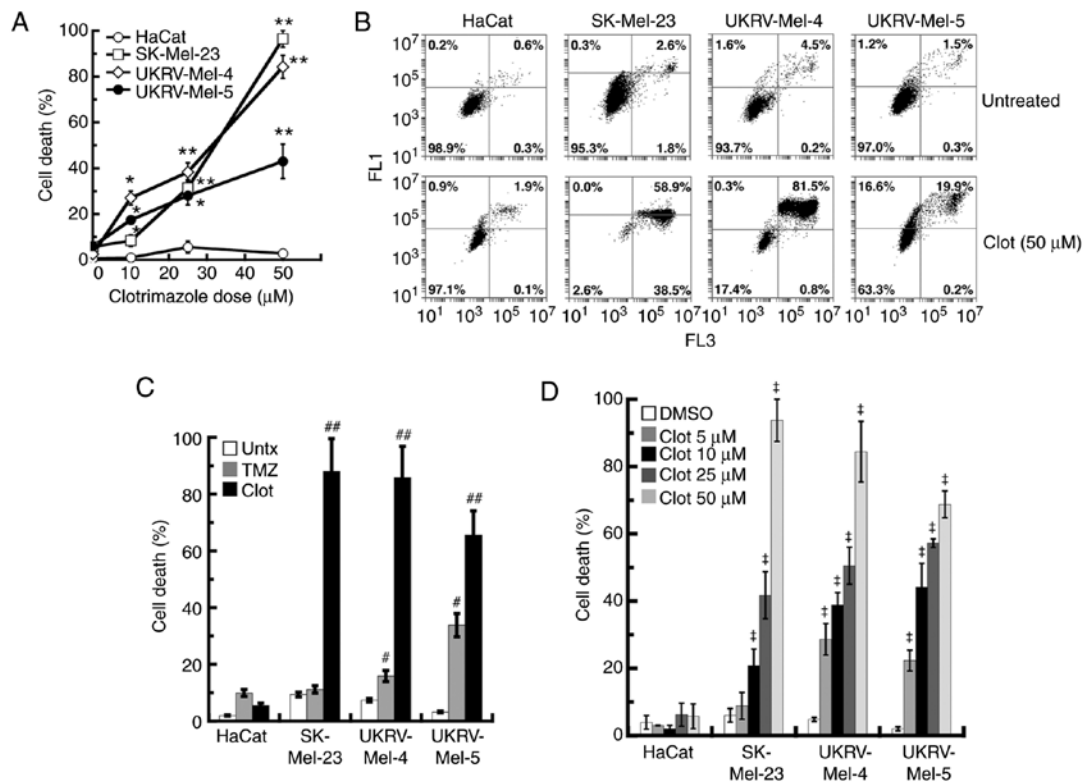


Figure 2. Quantification of cell death in human malignant melanoma cells following clotrimazole treatment. (A) Treatment of non-cancerous human keratinocytes (HaCat) and primary malignant melanoma cell lines (SK-Mel-23, UKRV-Mel-4 and UKRV-Mel-5) with 10-50 μ M clotrimazole for 2 days, followed by analysis of cell death using flow cytometry. (B) Flow cytometry analysis of cells treated with 50 μ M clotrimazole for 2 days. (C) Comparison between the levels of cell death in all cells treated with 10 μ M temozolomide or 50 μ M clotrimazole for 3 days. (D) Treatment of HaCat, SK-Mel-23, UKRV-Mel-4 and UKRV-Mel-5 cells with 5-50 μ M clotrimazole for 2 days and the subsequent analysis of cell death using the WST-1 assay. * $P < 0.05$ and ** $P < 0.01$ vs. HaCat; # $P < 0.05$ and ## $P < 0.01$ vs. untreated; † $P < 0.05$ vs. DMSO-treated. Error bars represent the standard error of the mean. Each experiment was performed at least three times with similar results. Untx, untreated; Clot, clotrimazole; TMZ, temozolomide.

statistically significant levels of cell death were observed in each human melanoma line following 10 μ M treatment, 25 μ M treatment led to increased levels of cell death (28% for UKRV-Mel-5, 31% for SK-Mel-23 and 38% for UKRV-Mel-4). Among all cell lines analyzed, the highest levels of cell death were observed following 50 μ M treatment of clotrimazole, where the levels of cell death were ~95% for the SK-Mel-23 line and 82% for the UKRV-Mel-4 line. Cell death in the UKRV-Mel-5 line was reduced compared with the SK-Mel-23 and UKRV-Mel-4 cell lines, following 50 μ M treatment of clotrimazole (42%), but significantly increased compared with 25 μ M treatment. Analysis of cell death following treatment with clotrimazole in each cell line indicated that the primary form of cell death induced was most likely apoptosis, due to dual PI and annexin V-FITC fluorescence detection in most of the human melanoma cells (Fig. 2B). No significant levels of cell death were observed in HaCat cells following 10-50 μ M clotrimazole treatment (Figs. 2A and B). These results demonstrated that clotrimazole caused dose-dependent increases in cell death in primary human malignant melanoma cells.

Each human melanoma cell line was further investigated by comparing the levels of cell death induced by clotrimazole with the levels induced by TMZ, a conventional chemotherapeutic treatment option previously utilized in patients with melanoma (8). Treatment with 10 μ M TMZ led to significant increases in cell death 3 days after treatment in the UKRV-Mel-4 and UKRV-Mel-5 melanoma lines, compared

with untreated cells in each group (Fig. 2C). The greater response was observed in the latter cell line, where 33% cell death was determined. However, when compared with 10 μ M TMZ treatment, 3 days of 50 μ M clotrimazole treatment caused increased levels of cell death in all melanoma lines, with the highest levels observed in the SK-Mel-23 line (88%) and UKRV-Mel-4 line (85%; Fig. 2C). While TMZ achieved an ~10% level of cell death in HaCat cells, cell death following clotrimazole treatment was markedly lower (4%; Fig. 2C). These results demonstrated that treatment with clotrimazole leads to increased levels of cell death in human melanoma cells compared with treatment with TMZ.

Levels of cell death induced by clotrimazole treatment were also investigated in the primary human melanoma lines using an alternative cell death assay. Following 2 days of treatment with clotrimazole, significant levels of cell death were observed using a WST-1 assay in SK-Mel-23 cells, where 25 μ M treatment caused 42% cell death and 50 μ M caused 94% cell death (Fig. 2D). This was comparable to the levels of cell death detected using flow cytometry (31% following 25 μ M treatment, 95% following 50 μ M treatment; Fig. 2A). In UKRV-Mel-4 cells, results of the WST-1 assay demonstrated 49% cell death after 25 μ M treatment and 84% after 50 μ M treatment (Fig. 2D). This was comparable to the levels observed using flow cytometry (38% after 25 μ M treatment, 83% after 50 μ M treatment; Fig. 2A). However, in UKRV-Mel-5 cells, the levels of cell death levels determined using the WST-1 assay were significantly increased, compared

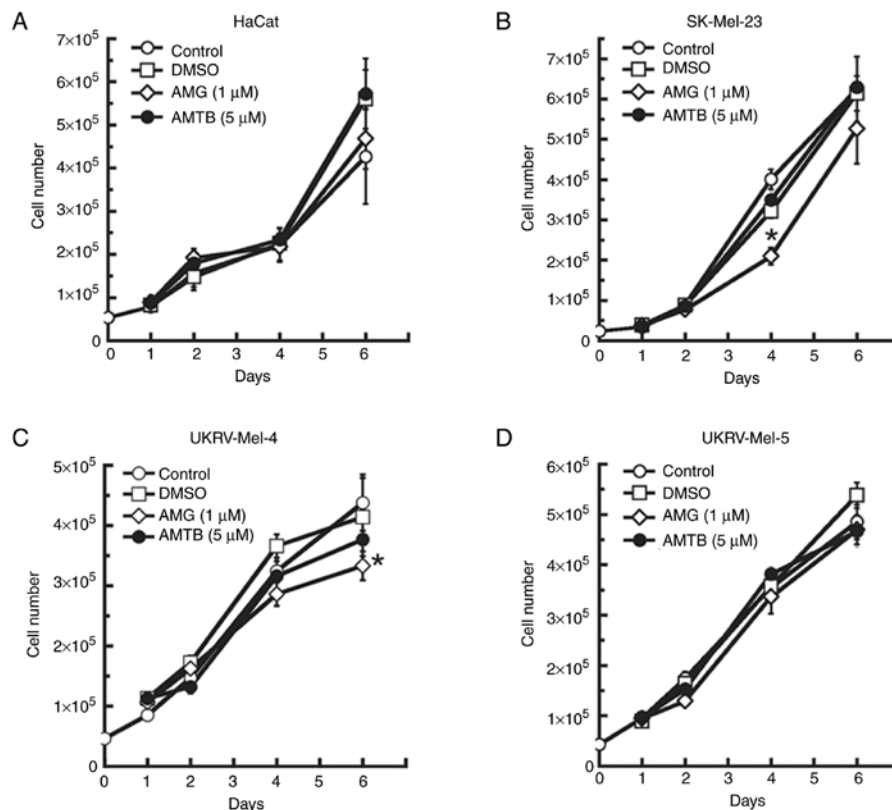


Figure 3. Effects of TRPV1 and TRPM8 antagonists on proliferation of human malignant melanoma cells. Treatment of (A) non-cancerous human keratinocytes (HaCat cells) and the primary malignant melanoma cell lines (B) SK-Mel-23, (C) UKRV-Mel-4 and (D) UKRV-Mel-5 for 1-6 days with 1 μ M of the TRPV1 inhibitor, AMG 9810 or 5 μ M of the TRPM8 inhibitor, AMTB. All cell counts were performed in triplicate and all experiments were performed at least three times with similar results. * $P < 0.05$ vs. DMSO-treated; error bars represent the standard error of the mean. TRP, transient receptor potential; TRPM8, TRP melastatin-8; TRPV1, TRP vanilloid-1; AMG 9810/AMG, (2E)-N-(2,3-Dihydro-1,4-benzodioxin-6-yl)-3-[4-(1,1-dimethylethyl)phenyl]-2-propenamide; AMTB, N-(3-Aminopropyl)-2-[(3-methylphenyl)methoxy]-N-(2-thienylmethyl)benzamide hydrochloride.

with those quantified using flow cytometry. More specifically, while cell death quantified using flow cytometry in these cells was 28% after 25 μ M treatment and 42% after 50 μ M treatment (Fig. 2A), results of the WST-1 assay demonstrated 57% cell death after 25 μ M and 68% after 50 μ M treatment (Fig. 2D). Minimal levels of cell death were observed following treatment with all doses of clotrimazole in HaCat cells, when compared with the levels of cell death observed in untreated HaCat cells (Fig. 2D). Collectively, these results demonstrated that clotrimazole treatment led to significant levels of cell death in human melanoma cells. Furthermore, the increased levels of UKRV-Mel-5 cell death detected using the WST-1 assay compared with the levels quantified using flow cytometry indicated that an additional cell death pathway, one that was not detected using flow cytometry, may have been induced.

Treatment with an antagonist of the TRP vanilloid-1 (TRPV1) or TRP melastatin-8 (TRPM8) channel exhibits minimal effects on the proliferation of human malignant melanoma cells. To determine whether effects similar to clotrimazole treatment may be induced by the antagonism of other TRP channels involved in skin function or skin cancer development, the human melanoma cell lines were treated with an antagonist of the TRPV1 channel. The TRPV1 channel is highly expressed in skin (45), and it was found to be overexpressed in several cancers, including squamous cell carcinoma and glioblastoma (46,47). In the present study, AMG 9810,

a competitive and selective TRPV1 channel antagonist was used. Following treatment with 1 μ M AMG 9810, which is higher than the IC_{50} value of 25 nM (48), no significant effect was observed in UKRV-Mel-4 or UKRV-Mel-5 cells up to 4 days of treatment (Fig. 3C and D). A total of 6 days of treatment led to a 25% reduction in cell proliferation in UKRV-Mel-4 cells (Fig. 3C), but no significant effect in UKRV-Mel-5 cells (Fig. 3D). In SK-Mel-23 cells, AMG 9810 exerted no significant effect on cell proliferation after 2 days of treatment, but exerted a 34% reduction in cell proliferation after 4 days of treatment (Fig. 3B). However, no significant effect was observed following 6 days of treatment. In human keratinocytes, AMG 9810 treatment for up to six days had no significant effect on cell proliferation (Fig. 3A). Collectively, these results indicated that AMG 9810, and thus, antagonism of TRPV1 channels, caused minimal overall effects on cell proliferation in the human malignant melanoma cell lines, as these effects were similar to the minimal effects observed on growth in each cell line after DMSO treatment. However, the reduction in proliferation of SK-Mel-23 cells after 3 days of treatment suggested a potential role of TRPV1 in these cells.

Subsequently, due to the involvement of the TRPM8 channel in skin cancer development (49,50), AMTB, a competitive and selective TRPM8 antagonist was used in the present study. Treatment of the human melanoma cell lines with a dose of 5 μ M AMTB, which is higher than the IC_{50} of 0.6 μ M (51), resulted in no significant effect on cell proliferation in any

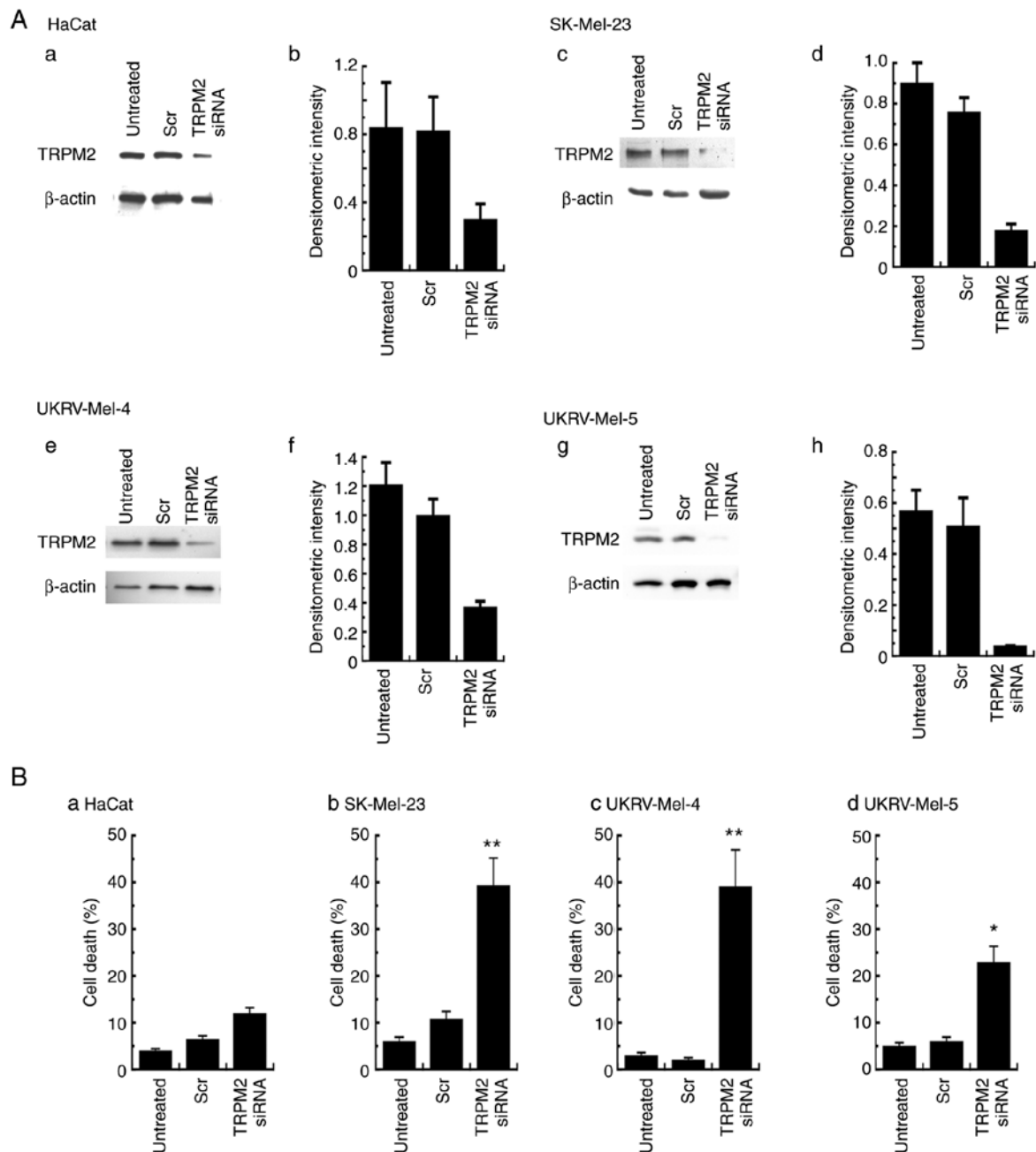


Figure 4. Cell death levels in human malignant melanoma cells following TRPM2 silencing. (A) TRPM2 protein expression levels were determined using western blot analysis in (a) non-cancerous human keratinocytes (HaCat) and primary malignant melanoma cell lines, (c) SK-Mel-23, (e) UKRV-Mel-4 and (g) UKRV-Mel-5 following TRPM2 silencing for 48 h. Quantification of transfection was performed using densitometric analysis of TRPM2 protein expression levels in (b) non-cancerous human keratinocytes (HaCat) and primary malignant melanoma cell lines, (d) SK-Mel-23, (f) UKRV-Mel-4 and (h) UKRV-Mel-5. β-actin was used as the internal control. Densitometry values represent TRPM2/β-actin ratios. Untreated cells and cells treated with non-silencing siRNA were used as negative controls. (B) Cell death levels analyzed in (a) HaCat cells and primary malignant melanoma cell lines, (b) SK-Mel-23, (c) UKRV-Mel-4 and (d) UKRV-Mel-5, 48 h after transfection with TRPM2 siRNA. All cell death analyses were run in duplicate and all experiments were performed at least three times with similar results. * $P < 0.05$ and ** $P < 0.01$ vs. non-silencing control transfections; error bars represent the standard error of the mean. TRPM2, transient receptor potential melastatin-2; siRNA, small-interfering RNA; Scr, non-silencing siRNA.

of the cell lines following 1-6 days of treatment as compared to untreated and DMSO-treated negative control groups (Fig. 3A-D). These results indicated that AMTB, and thus, antagonism of TRPM8 channels, did not adversely affect proliferation in the human malignant melanoma cell lines.

Knockdown of TRPM2 leads to increased death in primary human malignant melanoma cells. To determine whether the effects of clotrimazole were caused by the antagonism of

TRPM2, the effects of TRPM2 knockdown on the levels of cell death were determined. In all cell lines, TRPM2 siRNA was used to knockdown the expression levels of TRPM2, as previously described using breast cancer cells (33). TRPM2 expression levels were decreased in all cell lines, determined using western blot analysis (Fig. 4A-a, -c, -e and -g) and quantification of TRPM2 knockdown was determined using densitometry analysis as outlined in the Material and Methods section (Fig. 4A-b, -d, -f and -h). In SK-Mel-23 cells, knockdown

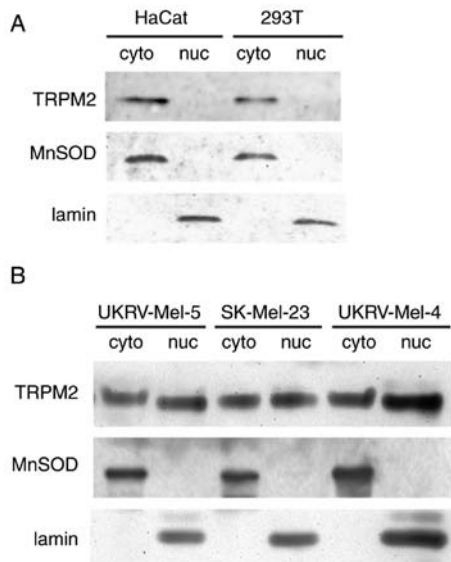


Figure 5. Cellular localization of TRPM2 in human malignant melanoma cells. (A) Non-cancerous human keratinocytes (HaCat) and human embryonic kidney cells (293T), and (B) human malignant melanoma cells UKRV-Mel-5, SK-Mel-23 and UKRV-Mel-4 were fractionated into subcellular fractions, and the cellular localization of TRPM2 was subsequently analyzed using western blot analysis. Western blot analysis of MnSOD and nuclear lamin B2 was performed to verify the successful fractionations into cytoplasmic and nuclear fractions, respectively. Fractionations and subsequent western blot analyses were performed twice with similar results. TRPM2, transient receptor potential melastatin-2; MnSOD, manganese superoxide dismutase; cyto, cytoplasmic; nuc, nuclear.

of TRPM2 expression levels by 80% (Fig. 4A-c and -d) led to ~38% cell death (Fig. 4B-b). TRPM2 knockdown of >70% in UKRV-Mel-4 cells (Fig. 4A-e and -f) led to similar levels of cell death (39%; Fig. 4B-c). For UKRV-Mel-5 cells, TRPM2 knockdown by 90% (Fig. 4A-g and -h) caused ~23% cell death (Fig. 4B-d). Minimal effects on cell death were observed in human keratinocytes, as 61% TRPM2 knockdown in HaCat cells (Fig. 4A-a and b) led to only 12% cell death (Fig. 4B-a). These results indicated that TRPM2 knockdown led to increased levels of cell death in human malignant melanoma cells, compared with the minimal levels of cell death observed in the cells transfected with non-silencing siRNA. Results of the present study also suggested that the specific inhibition of TRPM2 function in these cells may be a primary mechanism underlying the anti-tumor effects caused by treatment with clotrimazole.

TRPM2 exhibits nuclear localization in primary human malignant melanoma cells. To further characterize TRPM2 in human malignant melanoma cells, its cellular localization was investigated. Subcellular fractionation of each cell line was carried out to form both nuclear and cytoplasmic fractions. Subsequent western blot analysis determined that TRPM2, normally localized to the plasma membrane in healthy cells, exhibited a nuclear localization in all human malignant melanoma cell lines investigated (Fig. 5B). In comparison, this was in contrast to the results of the Western blot analysis of the normal human keratinocyte cell line, where after fractionation, TRPM2 localization was exclusively observed in the cytoplasmic fraction and not observed in the nuclear fraction

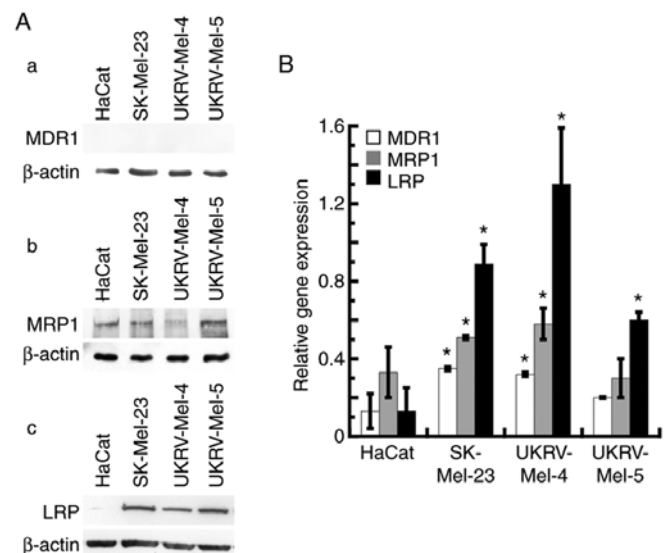


Figure 6. Expression levels of MDR genes in human malignant melanoma cells. (A) Non-cancerous human keratinocytes (HaCat) and the human malignant melanoma cells SK-Mel-23, UKRV-Mel-4 and UKRV-Mel-5 were analyzed using western blot analysis, for the detection of MDR genes, (a) MDR1, (b) MRP1 and (c) LRP. β-actin was used as the internal control. Western blot analysis was performed at least three times with similar results. (B) HaCat cells and human malignant melanoma cells (SK-Mel-23, UKRV-Mel-4 and UKRV-Mel-5) were analyzed using RT-qPCR to detect the gene expression levels of MDR1, MRP1 and LRP. All RT-qPCR assays were run in triplicate. Values represent four separate RT-qPCR experiments for each gene in each cell line, for a minimum of 16 RT-qPCR determinations. *P<0.05 vs. HaCat; error bars represent the standard error of the mean. RT-qPCR, reverse transcription-quantitative PCR; MDR, multidrug resistance; MRP1, multidrug resistance-associated protein 1; MDR1, phosphorylated-glycoprotein 1; LRP, lung resistance-related protein.

(Fig. 5A). Furthermore, additional comparison via analysis of an additional non-cancerous cell line, the human embryonic kidney cell line (293T), also revealed TRPM2 localization exclusively to the cytoplasmic fraction (Fig. 5A). These results confirmed that TRPM2 exhibits a cytoplasmic localization in noncancerous cells (HaCat and 293T), but in human malignant melanoma cells, TRPM2 exhibits a unique nuclear localization. This highlighted the potential alternative role of TRPM2 channels in melanoma cells.

Characterization of MDR protein expression levels in primary human malignant melanoma cells. Numerous patients with melanoma exhibit MDR; thus, the expression levels of three MDR proteins were investigated in all cell lines used in the present study. For each human primary malignant melanoma cell line used, expression levels of MDR1, MRP1 and LRP were analyzed using western blot analyses. Results of the present study demonstrated that MDR1 expression was not observed in the normal human keratinocyte cell line and the three human malignant melanoma lines (Fig. 6A-a); however, results of the RT-qPCR analysis revealed the reduced, yet significant, expression level of MDR1 mRNA in the SK-Mel-23 and UKRV-Mel-4 cell lines when compared with the expression levels of the other MDR genes (Fig. 6B). All human malignant melanoma cell lines, as well as the human keratinocyte cell line, exhibited expression of MRP1 (Fig. 6A-b and B). Moreover, each human melanoma cell line

exhibited significant levels of LRP expression via western blot analysis, but no significant expression levels were observed in human keratinocytes (Fig. 6A-c). Notably, results of the RT-qPCR analysis demonstrated that high levels of LRP mRNA were present in each human malignant melanoma cell line, with the highest level observed in the UKRV-Mel-4 line (Fig. 6B). These results further verified the expression of MRP1 and/or LRP in each human malignant melanoma cell line investigated. These findings are suggestive of the ability of TRPM2 antagonism to successfully treat melanoma tumors that exhibit MDR.

Discussion

Results of the present study demonstrated that clotrimazole may decrease the levels of proliferation and induce cell death in human malignant melanoma cells. Due to the minimal deleterious effects observed in healthy human keratinocytes (HaCat cells), there may be a high level of specificity of clotrimazole in melanoma cells. Clotrimazole is a well-established drug that has been used for decades as an imidazole antifungal agent (52). Its use as a fungicidal is based upon attaining blood levels of clotrimazole up to 20 $\mu\text{g/ml}$ in humans, and its normal oral dose is 10 mg up to five times daily (53). It is also currently utilized in chemotherapy regimens for prophylaxis of oral candidiasis at a dose of 10 mg three times daily (54). The doses utilized in the treatment of human melanoma cells in the present study ranged from 1.7-17 $\mu\text{g/ml}$. Thus, the doses utilized in the present study are comparable to those currently in use in chemotherapy regimens, and the *in vitro* levels utilized in the present study are comparable with the desired therapeutic blood levels of clotrimazole in treating oral mycoses. Moreover, therapeutic doses of clotrimazole in the potential treatment of melanoma may be obtainable.

As clotrimazole has previously been used, the potential side effects and toxicity profiles of clotrimazole are well-established, and overall, its use is well-tolerated (52). While most adverse effects experienced following clotrimazole administration occur after oral use, clotrimazole is still administered both orally and topically (52,55). Topical administration may be more advantageous in patients with melanoma, due to the ability to minimize adverse effects and the potential to readily treat primary tumors in the skin. Notably, an additional advantage is that clotrimazole is an economical drug that is available for purchase over-the-counter (52). Thus, the potential treatment regimens in patients with melanoma may cost significantly less than the cost of conventional chemotherapy, immunotherapy or targeted therapy. In the present study, TRPM2 was characterized as a potential target in the treatment of melanoma. Moreover, in addition to the benefits of utilizing clotrimazole to target TRPM2 for its anti-tumor effects, this potential treatment option may also be widely accessible, affordable and safe.

Clotrimazole is an antagonist of TRPM2 channels (44). As it may also exhibit interactions with other targets or cellular processes, inhibitors of other types of TRP channels were used in the present study, to determine if similar effects were induced. The lack of similar effects following this treatment demonstrated that antagonism of two other types of TRP channels was not the source of the anti-cancer effects. While

the results of the present study do not demonstrate any favorable effects in targeting these TRP channels in the human metastatic melanoma cell lines utilized, they do remain potential targets in melanoma. In A375 human melanoma cells, modulation of TRPV1 or TRPM8 channels led to increased cell death (56,57). Moreover, A375 cells harbor the BRAF V600E mutation (58), while the melanoma cell lines utilized in the present study were wild-type, and thus, do not exhibit this genotype. Collectively, the results indicated that successfully targeting TRP channels in melanoma may be dependent on the activation or inhibition of their ionophore function, or the presence or absence of mutations, where specific TRP channels can be targeted in different melanoma tumors based on their genotype.

To determine whether these effects were TRPM2-specific, gene silencing was carried out in the present study. Knockdown of TRPM2 led to notable levels of cell death in each human melanoma cell line investigated. These results indicated that the effects observed following clotrimazole treatment in human melanoma cells were primarily mediated by TRPM2 antagonism. However, other mechanisms may play a role, including the ability of clotrimazole to inhibit hexokinase and decrease overall glycolytic energy metabolism in melanoma cells (59,60). Nevertheless, the results of the present study and others previously reported (59,60) demonstrated a high level of specificity for clotrimazole in inhibiting proliferation and inducing cell death in cancer cells. Therefore, clotrimazole may act as a novel potential treatment option for patients with melanoma, primarily via TRPM2 antagonism, but also in addition to other contributing mechanisms.

TRPM2 channels have been identified as novel targets in several other types of cancer. These include breast cancer (33,34), head and neck cancer (36), acute myeloid leukemia (61), prostate cancer (35) and neuroblastoma (62). The anti-cancer effects of clotrimazole in melanoma were previously reported by Benzaquen *et al*, and the results of this study demonstrated that clotrimazole decreased Ca^{2+} gating (63). Subsequent studies also determined that clotrimazole functioned to decrease hexokinase and overall glycolytic activity in melanoma cells (59,60). As Hill *et al* (44) demonstrated that clotrimazole antagonized TRPM2 channels, the present study aimed to determine whether TRPM2 antagonism was the primary mechanism through which clotrimazole exerts its effects in human melanoma cells. TRPM2 silencing in the present study highlighted TRPM2 antagonism as a major contributor to the anti-cancer effects observed in human melanoma cells. However, results of the present study also demonstrated that TRPM2 played a unique role in melanoma due to its observed nuclear localization. Thus, the specificity of TRPM2 antagonism may be due to the prevention of its nuclear role in melanoma, and not its role as an ion channel at the plasma membrane. A similar nuclear localization of TRPM2 in other cancer cells, including breast (33), prostate (35) and oral cancer cells (36) indicated that a similar level of specificity may occur following treatment with TRPM2 antagonism.

Results of the present study also demonstrated that human melanoma cell lines express MDR proteins. MDR is present in melanoma and often involves overexpression of one or more of the following drug resistance genes: MRP1, MDR1 and LRP (6,16,17). These enable MDR in cancer cells, where

they remove chemotherapeutic drugs from the cell; thus, promoting survival following treatment due to the lack of active drugs at the tumor site. A previous study focused on analyzing the expression of MDR1 in melanoma cells, with the authors hypothesizing that additional MDR genes may exhibit clinical relevance in melanoma (64). The present study further characterized the expression of additional MDR genes in both melanoma and non-cancerous cells. Results of the present study demonstrated that treatment with clotrimazole led to increased cell death and decreased proliferation in primary human malignant melanoma cells that express both MRP1 and LRP proteins. The ability to successfully treat human melanoma cells that exhibit MDR enhances the therapeutic potential of TRPM2 antagonism, as the presence of MDR can lead to poor treatment outcomes in patients with melanoma (16,17). However, further studies using numerous human melanoma cell lines that exhibit MDR are required in order to definitively determine whether TRPM2 antagonism can be used to treat drug-resistant melanoma.

In conclusion, results of the present study demonstrated that TRPM2 antagonism led to decreased proliferation, as well as increased cell death, in human malignant melanoma cells exhibiting MDR. In addition, TRPM2 in these melanoma cells exhibited notable nuclear localization. Results of the present study further indicated that TRPM2 may act as a novel potential target in the treatment of melanoma. Based on the results of the present study, future studies should focus on elucidating the potential alternative role of TRPM2 in the nuclei of melanoma cells, and determining the overall susceptibility of melanoma cells that express MDR1, MRP1 or LRP to TRPM2 antagonism. Moreover, as the melanoma cell lines used in the present study were wild-type cell lines, future studies should also investigate the susceptibility of alternative melanoma cell lines to TRPM2 antagonism, that are also unresponsive to current treatment options, including those that exhibit N-RAS Q61R or B-RAF V600E mutations.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SGM performed proliferation assays, cell death assays, assisted in western blot analyses and prepared the manuscript.

LRJ performed RT-qPCR experiments and assisted in manuscript preparation. CMT performed proliferation assays, cell death assays, transfection and assisted in manuscript preparation. SDB performed proliferation assays, cell death assays and transfection. DPP performed proliferation and cell death assays. ATD performed RT-qPCR experiments. DWK directed all experiments, performed proliferation assays, cell death assays, western blot analysis, RT-qPCR and manuscript preparation. SGM, LRJ, and DWK confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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