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RESEARCH

Menthol Inhibits the Proliferation and Motility of Prostate Cancer DU145 Cells

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Abstract In recent years, the transient receptor potential melastatin member 8 (TRPM8) channel has emerged as a promising prognostic marker and putative therapeutic target in prostate cancer. We have found that forced overexpression of TRPM8 in PC-3 cells can inhibit the cell proliferation and motility probably through the TRPM8 activation. In this study, we aimed to investigate whether activating the TRPM8 channel by its selective agonist menthol can inhibit the proliferation and motility of androgen-independent prostate cancer (AIPC) with remarkable expression of TRPM8. Menthol is a naturally occurring compound, which has been widely used in cosmetics and pharmaceutical products, and also as flavoring in food. DU145 cells are androgen-independent but have a remarkable expression of TRPM8. The demonstration of the existence of TRPM8 and the absence of TRPA1 in DU145 cells provided the foundation for the following experiments, because both TRPM8 and TRPA1 are molecular targets of menthol. The outcome of MTT assay indicated that menthol inhibited the cell growth ($p<0.01$). Cell cycle distribution and scratch assay analysis revealed that menthol induced cell cycle arrest at the G_0/G_1 phase ($p<0.01$). Furthermore, menthol inhibited the migration of DU145 cells by downregulating the focal-adhesion kinase. So it suggests that the activation of the existing TRPM8 channels may serve as a potential and pragmatic treatment for those AIPC with remarkable expression of TRPM8, and menthol is a useful compound for future development as an anticancer agent.

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Introduction

Prostate cancer (PCa) is a significant cause of morbidity and mortality in men [1]. It is well known that androgen-ablative therapy is an effective treatment for early-stage PCa [2, 3]. However, prostate cancers will evolve to be recurrent, incurable and androgen-independent if there is sufficient time to elapse [4, 5]. Therefore, it's imperative to develop better therapeutic strategies for androgen- independent prostate cancer (AIPC).

Recently transient receptor potential melastatin 8 (TRPM8) has emerged as a promising prognostic marker and putative therapeutic target in PCa. TRPM8 is a Ca^{2+}-permeable cation channel which plays a key role in calcium homoeostasis. It is acknowledged that Ca^{2+} plays an important role in cancer-related cell signaling pathways. Fluctuations in Ca^{2+} homeostasis may lead to an increase in cell proliferation [6, 7], and even may induce differentiation [8] and apoptosis [9–11]. Therefore, Ca^{2+} transport has emerged as a novel therapeutic target for cancers [12]. It has been observed that abnormal TRPM8 expression is associated with a cancerous phenotype including breast, lung, colon and prostate tissue [13]. Especially TRPM8 expresses abundantly in the prostate. It's evident that the TRPM8 expression increases in early-stage PCa [13], and then decreases as tumor progresses to late, invasive and androgen-independent stage [14].

Our former study has confirmed that the forced overexpression of TRPM8 in PC-3 cells, which express an extremely low level of TRPM8, can cause proliferation and motility inhibition [15]. So what if activating the TRPM8 channel by its selective agonist in AIPC with

remarkable expression of TRPM8? If the activation of TRPM8 does work, it may serve as a potential and pragmatic treatment for the AIPC with remarkable expression of TRPM8.To find out the answer for this question, menthol and DU145 cell line would be the best choice for this study.

Menthol is a naturally occurring compound, which has been widely used in cosmetics and pharmaceutical products, and also as flavoring in food [16]. It is considered to be Generally-Recognized-As-Safe and has been approved for over-the-counter external use in the concentration up to 16% [16]. The mechanisms by which menthol induces a cool sensation were unknown until TRPM8 was recognized as a molecular target of menthol [17, 18]. In addition, it was reported that menthol could cause a sustained increase of $[Ca^{2+}]_{cyt}$ in LNCaP cells and such effect can cause cell apoptosis [19].

Lots of studies have confirmed that the TRPM8 expression increases in androgen-dependent PCa, and decreases in AIPC. Being androgen-independent, DU145 cells are supposed to have a low level expression of TRPM8. However, the expression of TRPM8 in DU145 cells is remarkable and much higher than that in androgen-independent PC-3 cells [20]. Such character makes DU145 cells to be the best choice for our study.

So this study was designed to investigate the possible effects of menthol on the proliferation and motility of androgen-independent cancer DU145 cells.

Materials and Methods

Cell Culture

Human prostate cancer DU145 cells were purchased from American Type Collection (ATCC, Manassas, VA, USA) and were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 100 IU mL^{-1} penicillin G sodium, 100 μg mL^{-1} streptomycin sulphate and 10% foetal bovine serum (FBS) (Gibco BRL, Grand Island, NY, USA) in a humidified atmosphere containing 95% air and 5% CO_2, at 37°C.

RT-PCR

Extract total RNA from DU145 cells by using TRIzol reagent (Invitrogen). Reverse transcription was performed with a commercial kit in compliance with manufacturer's instruction (Invitrogen). Nested RT-PCR was performed to assay the expression of TRPM8 and TRPA1, with the conditions as follows: pre-heating (94°C/2 min), 35 cycles of denaturation (94°C/30 sec), annealing (50°C/30 sec), extension (72°C/60 sec) and 1 cycle of final extension (72°C/10 min). PCR primers used to assay the expression of TRPM8, TRPA1 and β-actin are as follows: 1) $TRPM8^{1st}$ PCR: TGT TTT GCC CAA GGA GGT GG (forward) and CAA CCA GTT TCC AGA CAA ACG (reverse); 2) $TRPM8^{2nd}$ PCR: ATG GGC AGC TGA AGC TTC (forward) and CTG CAG ATT CCG GTA CAC (reverse); 3) $TRPA1^{1st}$ PCR: TGG TGC ACA AAT AGA CCC AGT (forward) and TGG GCA CCT TTA GAG AGT AGC (reverse); 4) $TRPA1^{2nd}$ PCR: GAA GGT GCA CAG CCA TTC (forward) and AGA TGC AGA AGC AGT TGC (reverse); and 5) β-actin: TTA GTT GCG TTA CAC CCT TTC (forward) and GTC ACC TTC ACC GTT CCA GTT (reverse) [21].

Immunohistochemical Assay

Immunohistochemistry was performed by using Streptavdin–peroxidase–biotin (SP) method. DU145 cells were plated on 12-mm coverslips and incubated overnight before being fixed with 4% paraformaldehyde at room temperature for 20 min. After three washes in phosphate buffered solution (PBS), the cells were incubated in 0.5% Txiton X-100 at room temperature for 20 min and then washed in PBS thrice. The slides were immersed with 3% H_2O_2 for 10 min to block endogenous peroxidase. After antigen retrieval by microwave, newborn calf serum was added for blocking for 10 min, and then rabbit polyclonal anti-TRPM8 antibody (code: ACC-049, Alomone Labs, Jerusalem, Israel) and rabbit polyclonal anti-TRPA1 antibody(code: ACC-037, Alomone Labs, Jerusalem, Israel) (1:50) was added to incubate overnight (4°C) and anti-rabbit IgG (BOSTER, China, using the dilution: 1:200) to incubate for 20 min at room temperature. Then streptavidin–biotin–peroxidase solution (Sigma cat # S-5512) was used to incubate for 30 min and DAB (3, 3-diaminobenzidine , Gibco BRL, Grand Island, NY, USA) was added to chlorate for 15 min. Followed by hematoxylin staining, dehydration and hyalinization, the slip was covered. The positive staining in DU145 cells would show brown.

Cell Proliferation Assay

MTT assay applied properly can be used for viability studies as well as for cell proliferation measurements. For example, short period exposure of treatment factor can be used for cell viability and long run experiments for cell proliferation. So we applied MTT assay to examine the cell proliferation after 48 h exposure. Approximately 5,000 cells were plated in each well of a 96-well plate with the treatments as follows:0,25, 50, 75, 100 μM menthol and 20 μM BCTC pretreatment for 20 min before 100 μM menthol separately for 48 h. The MTT assay was performed according to the manufacturers' instructions (Sigma). The assay was quantitated by measuring the absorbance at 570 nm on micro-plate spectrophotometer (ASYS).

Cell Cycle and Cell Apoptosis were Detected by Flow Cytometry

Approximately 5×10^5 cells per well were incubated with 100 μM menthol for 24 h, 48 h and 72 h separately, and another well incubated without menthol as control for 72 h. Then they were digested with 0.25% trypsin (Gibco BRL, Grand Island, NY, USA). Cells were subsequently harvested and fixed by the addition of 70% ethanol (in PBS) at 4°C overnight and then re-suspended in PBS containing 40 μg mL^{-1} propidium iodide (PI), 100 μg mL^{-1} RNase and 0.1% Triton X-100 in a darkroom. After incubation at 37°C for 30 min, the cells were analyzed through flow cytometry (Becton-Dick-inson, San Jose, CA, USA) equipped with an argon ion laser at a wavelength of 488 nm. The cell cycle stage was then determined and analyzed.

For cell apoptosis analysis, cells were seeded in a 75 mm flask at 40–50% confluence. After receiving the same treatments as the cell cycle analysis, the cells were incubated in a binding buffer containing FITC-conjugated Annexin V and PI (Abcam, Cambridge, MA, USA) at room temperature for 5 min in the dark, according to the manufacturer's protocol. The percentages of apoptotic cells were then determined using flow cytometry.

Scratch Motility Assay

The wound was created with a standard 200 μL pipette tip [22] after cells were cultured as confluent monolayers for 24 h and then washed twice to remove the non-adherent cells. After the wound was created, cells were incubated with 0 μM menthol (control), 100 μM menthol and 20 μM BCTC pretreatment for 20 min before 100 μM menthol separately. Photos were taken by an inverted phase contrast microscope (Leica, Wetzlar, Germany) at the time the wound was created, and after 24 h and 48 h incubation respectively. This 48 h time interval was chosen for it is shorter than the DU145 cells' doubling time. We measured the distance between the borderlines by four different equidistant points in four independent fields from each sample to get a better estimate. The migration rate is expressed as a percentage of the control, and was calculated as the proportion of the mean distance between both borderlines caused by scratching to the distance that remained cell-free after re-

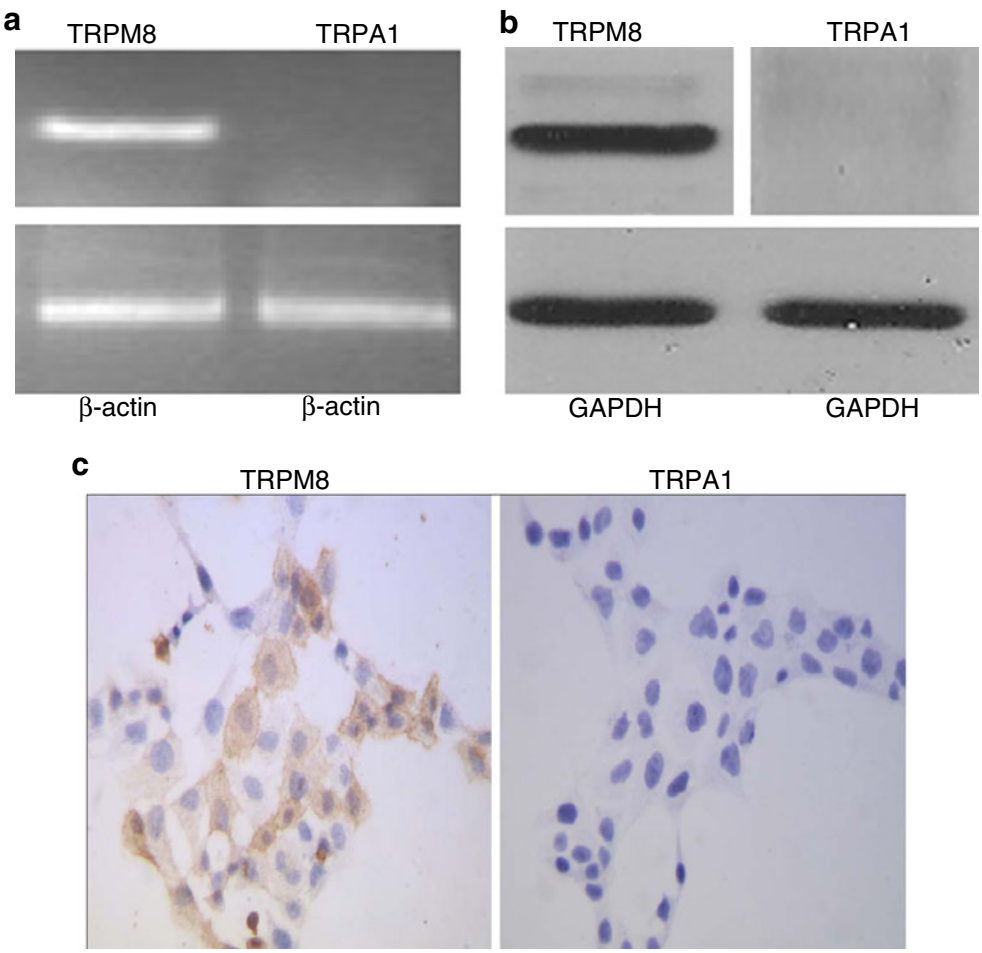

Fig. 1 The expression of TRPM8 and TRPA1 in DU145 cells. The expression of TRPM8 and TRPA1 was detected by (**a**) nested RT-PCR, (**b**) western blot and (**c**) immunohistochemical assay. The dilution of anti-TRPM8 antibody used in western blot was 1:500 and 1:50 in immunohistochemical assay The staining of TRPM8 is positive, presenting brown, while the staining of TRPA1 is negative (× 40)

growing. Two independent series of experiments were performed in quadruplicates.

Western Blot Assay

Cells with or without the treatment of 100 μM menthol incubation for 48 h were pooled and lysed in lysis buffer (50 mmol L^{-1} Tris–HCl pH 7.4, 5 mmol L^{-1} ethylenediaminetetraacetic acid, 1 mmol L^{-1} EGTA, 10 mmol L^{-1} 2-mercaptoethanol) containing protease inhibitors (5 μg mL^{-1} leupeptin, 5 μg mL^{-1} aprotinin, 10 μg mL^{-1} soybean trypsin inhibitor and 1 mmol L^{-1} phenylmethylsulphnyl fluoride) and were subsequently sonicated. After centrifugation at 12,000×g for 15 min to remove all organelles, the supernatant was recovered, and the total protein content was measured using a bicinchoninic acid (BCA) kit. The proteins were resolved in 10% SDS-PAGE with 40 μg per lane and were analyzed with anti-TRPM8-specific (code: ACC-049, Alomone labs, Jerusalem, Israel), anti-CDK2-specific (Neomarkers, Union City, CA, USA), anti-CDK4-specific (Neomarkers), anti-CDK6-specific (Neomarkers), anti-FAK-pY397-specific (Biosource, Camarillo, CA, USA) and anti-GAPDH-specific (Neomarkers) antibodies, as mentioned previously [10].

Statistical Analysis

The SPSS version 11.5 for Windows (SPSS, Chicago, IL, USA) was used for the statistical analysis. Data are presented as means ± SEM of the indicated number of experiments. Statistical analysis was performed with unpaired t-test. Significance was considered when $p<0.05$.

Results

The Expression of TRPM8 and TRPA1 in DU145 Cells

Through the experiments of nested RT-PCR, western blot assay and immunohistochemical assay, we examined the expression of TRPM8 and TRPA1 in DU145 cells, the two of which were established as molecular targets of menthol [23]. In the analysis of nested RT-PCR (Fig. 1a), DU145 cells expressed a high level of TRPM8 but no TRPA1, and the sequencing of PCR products that recuperated from the agarose gel confirmed the TRPM8 mRNA expression. Also the western blot assay and immunohistochemical assay verified this (Fig. 1b and c). Since the anti-TRPM8 antibody is polyclonal, there was a faint non-specific protein band over

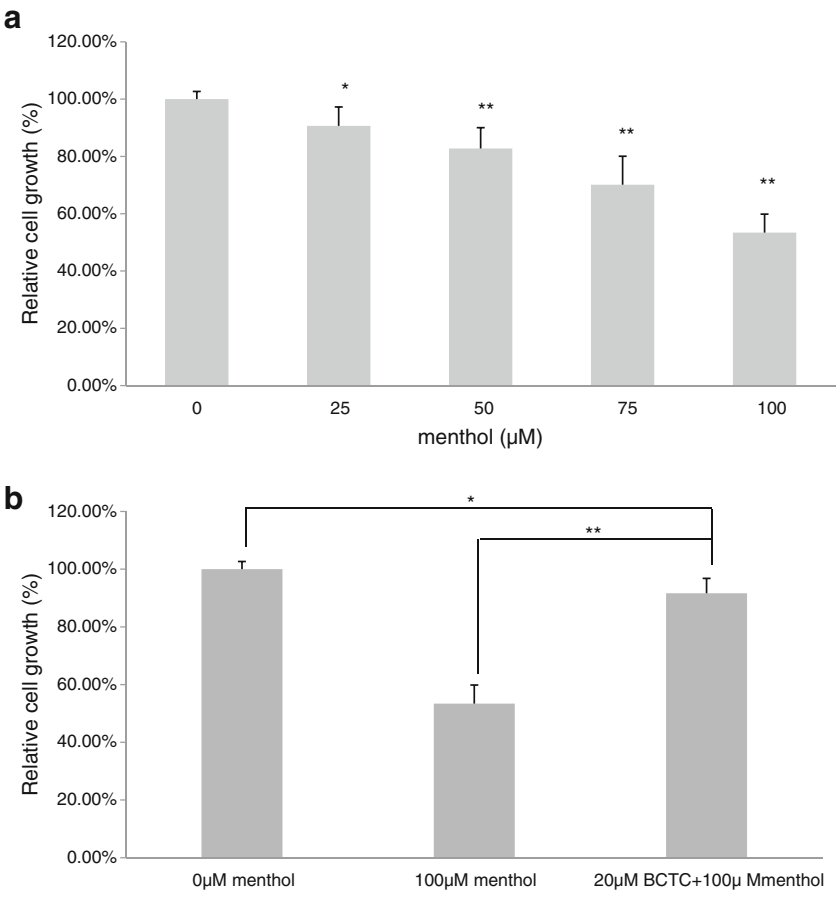

Fig. 2 Menthol inhibited DU145 cell growth and viability. **a** DU145 cells were treated with menthol at the concentrations of 0, 25, 50, 75,100 μM for 48 h before the assay. **b** DU145 cells were given different disposal (0 μM menthol, 100 μM menthol and 20 min BCTC pretreatment before 100 μM menthol) for 48 h before the assay. Cell growth is expressed as a relative value of control (0 μM menthol) which is set to 100%. Data were presented as the mean ± SEM of three independent experiments, $^{*}p<0.05$, $^{**}p<0.01$

the main one. In the immunohistochemical assay, the staining of TRPM8 was positive, presenting brown, while the staining of TRPA1 was negative.

Menthol Inhibits DU145 Cell Growth

Since the androgen-independent DU145 cells express a remarkable level of TRPM8, we performed the MTT assay to examine whether the TRPM8 activation can inhibit the cell proliferation. The results indicated that, compared to the non-menthol-treated cells, the population of cells under different concentrations of menthol (25, 50, 75, 100 μM) decreased (the results are presented as percentages with the latter as 100%) (100%±2.68% vs. 90.66%±6.60%, 82.78%±7.24%, 70.12%±9.96%, 53.41%±6.45%, respectively, $p<0.05$ for 25 μM and the rest $p<0.01$). Cell population fell down gradually in relation to the menthol concentration (Fig. 2a).To explore whether such phenomenon is resulted from the activation of TRPM8, we applied BCTC, the TRPM8 channel blocker. As shown in MTT assay (Fig. 2b), it's obvious that the population decrease of menthol-treated cells was corrected by 20 min BCTC (20 μM) pretreatment ($p<0.01$). With non-menthol-treated cells population as 100%, the menthol-treated cells with or without BCTC pretreatment can be expressed as percentages. The comparison of the three can be obtained as follows: 100%±2.68% vs. 53.41%±6.45% vs. 91.17%±5.12%.

Menthol Induces G_0/G_1 Arrest without Triggering Apoptosis in DU145 Cells

To understand the mechanisms of cell viability inhibition induced by menthol, we analyzed the cell cycle profile of DU145 cells using flow cytometry. It can be observed from the results that compared with control, there is a significant increase in G_0/G_1 phase after 24 h ($p<0.05$), 48 h and 72 h ($p<0.01$) 100 μM menthol treatment (49.12%±1.92% vs. 61.71%±2.70%, 77.65%±1.63%, 71.81%±2.46%, respectively, Fig. 3a and b). Menthol's effect on apoptosis was also investigated, but we found that it failed to induce cell apoptosis at the concentration of 100 μM (Fig. 3c and d).

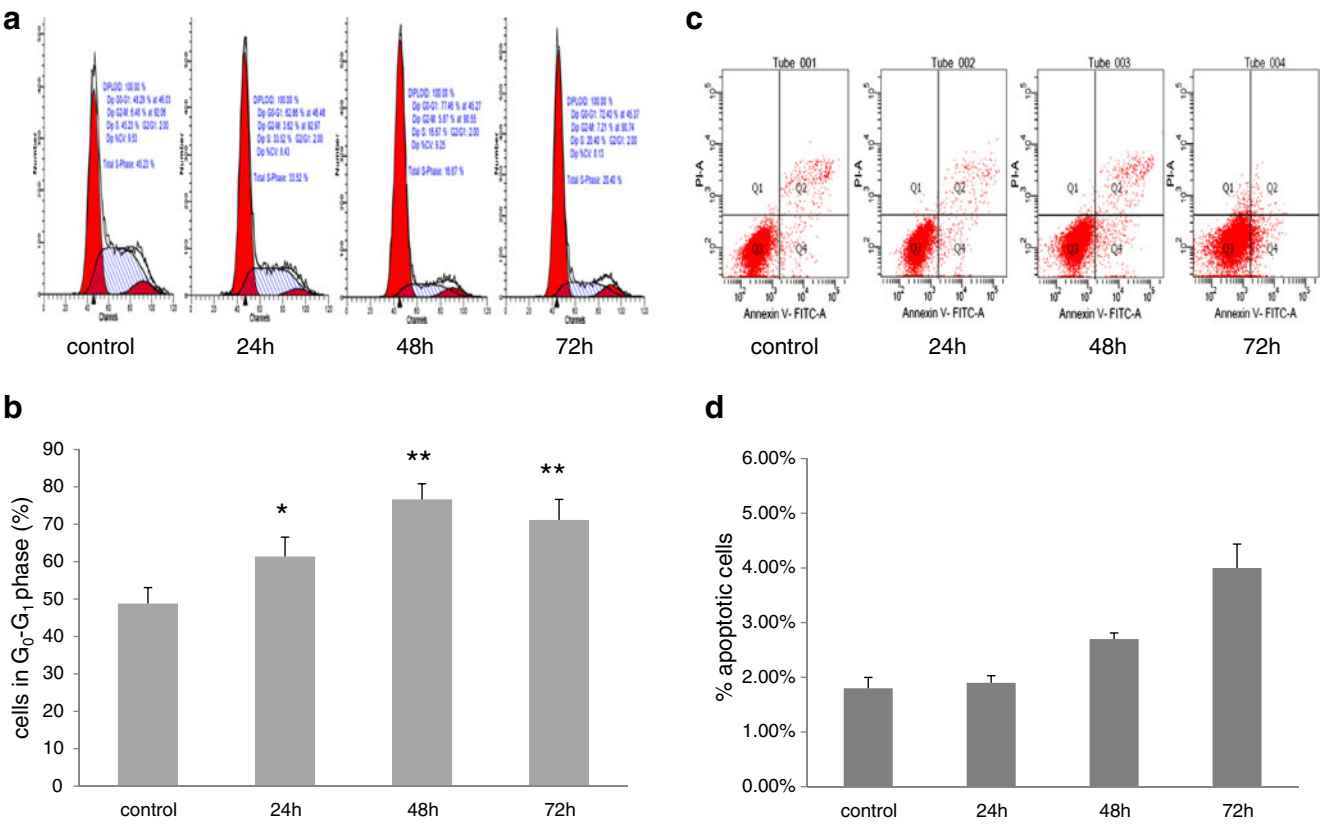

Fig. 3 Menthol induced G_0/G_1 phase arrest without triggering apoptosis in DU145 cells. **a** With the cells incubated without menthol for 72 h as control, the cells treated with 100 μM menthol were incubated for 24 h, 48 h and 72 h separately and then the cell cycle was analyzed with flow cytometry. **b** The percentage values for cells in G_0/G_1 phase were analyzed, and the final result was the mean value of three independent experiments, $^{*}p<0.05$, $^{**}p<0.01$. **c** After receiving the same treatments as in the cell cycle analysis, cells were subsequently harvested and fixed and then the cell apoptosis was analyzed with flow cytometry. **d** The percentages of cell apoptosis were analyzed, and the data were the mean value of three independent experiments

Menthol Induces DU145 Cell Motility Reduction

Cell proliferation and migration are anticancer agent development targets and also serve as indicators of malignance. Since menthol can induce G_0/G_1 phase arrest, we explored its effect on cell motility. As shown in the wound scratch assay (Fig. 4a), the migration of non-menthol-treated cells was obvious 24 h and 48 h after the scratch, when the confluent monolayer region gradually migrated to the cell-free wound area. On the contrary, the migration of menthol-treated cells was significantly reduced after 24 h and 48 h incubation. The migration rate is expressed as a percentage with the value for non-menthol-treated cells being 100% (58.62%±11.55% for 24 h, $p<0.05$ and 48.21%±11.11% for 48 h, $p<0.01$, Fig. 4b). Compared with the cells only treated with 100 μM menthol, the migration of the cells with 20 min BCTC pretreatment (20 μM) was partly corrected ($p<0.05$, Fig. 4c), and the migration rate is presented as a relative value of non-menthol-treated cells which is set to 100% (44.59%±18.18% and 75.68%±13.48%, Fig. 4d).

Menthol Downregulates the Expression of Cell Cycle- and Motility-related Proteins

To get a further understanding of the molecular mechanisms of menthol-induced cell cycle arrest and motility reduction in DU145 cells, we examined the expression of proteins associated with cell cycle and migration. CDK family is well known for its ability in cell cycle regulation, and FAK family is thought to play a key role in cell migration

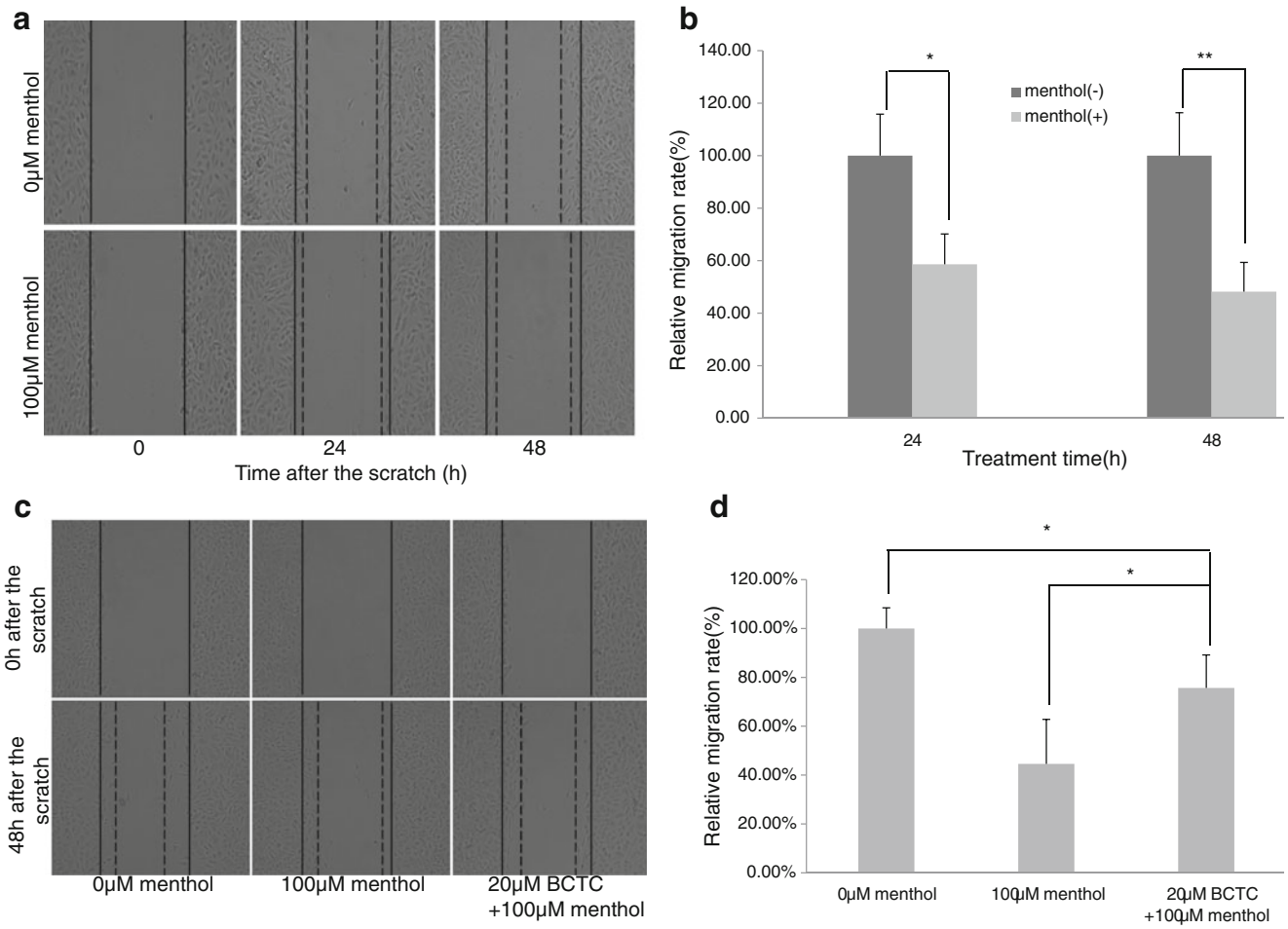

Fig. 4 Menthol inhibited the motility of DU145 cells. **a** Cells incubated in 6-well culture plates were divided into two groups to receive 0 μM and 100 μM menthol treatments separately after the wound was created. Photomicrographs for the representative views of motility assay were taken at 0, 24 h and 48 h after the scratch (original magnification: × 200). The solid lines represent the original borderlines at the time of the scratching, whereas the dashed lines represent the borderlines 24 h and 48 h after the scratching. **b** The distance between the solid and the dashed lines was measured and normalized relative to the control (value of cells treated with 0 μM menthol for 24 h or 48 h) from four independent experiments. **c** DU145 Cells were given different disposal (0 μM menthol, 100 μM menthol and 20 min BCTC pretreatment before 100 μM menthol) for 48 h after the wound was created. The motility of menthol-treated cells was partly corrected by BCTC. **d** The distance between the solid and the dashed lines was measured and normalized relative to the control (0 μM menthol). The data from four independent experiments were collected and the mean values were plotted with SEM, $^{*}p<0.05$, $^{**}p<0.01$

and be required for the invasion and metastasis of cancer cells. The western blot outcome revealed that menthol downregulated the expression of CDK2, CDK4 and CDK6 and consequently induced cell cycle arrest at the G_0/G_1 phase. And menthol also can downregulate the phospho-FAK without changing the non-phospho-FAK which may account for the cell motility inhibition (Fig. 5).

Discussion

TRPM8, which has recently emerged as an important factor in PCa, is a member of the melastatin subfamily of TRP channels and is activated by both cold temperatures and menthol. Although the number of reports on TRPM8 is growing rapidly, the exact role of TRPM8 in the prostate still remains unclear. Experiments using TRPM8 antagonist and siRNA [19, 24] suggest that the Ca^{2+} influx mediated by TRPM8 plays an essential role in cellular Ca^{2+} homoeostasis in LNCaP cells and is involved in cell survival. These results indicate that TRPM8 may be a potential drug target for the treatment of prostate cancer. Our former study has confirmed that overexpression of TRPM8 in PC-3 cells, which express an extremely low level of TRPM8, can induce the cell proliferation and motility inhibition.

For DU145 cells we demonstrate that there is a remarkable expression of TRPM8 by using nested RT-PCR, western blot and immunohistochemical assay. Being insensitive to androgen, DU145 cells are supposed to express a low level of TRPM8 like PC-3 cells. However, there is a remarkable expression of TRPM8 in DU145 cells. So what if activating the TRPM8 channel in DU145 cells by using its selective agonist? To explore this question, we applied menthol, the selective agonist for TRPM8. Menthol has been used since antiquity for medicinal purposes, and its use in dermatology is ubiquitous. The safety and toxicity of menthol in dermatology have been well studied. Menthol is considered a safe and effective topical OTC product according to the FDA. Concentrations of menthol up to 16% have been approved by the FDA for OTC external use, and their safety profile has been well established [16]. The animal experiments for menthol acute toxicity oral studies indicated that the LD50 in mice was 4.4 g/Kg [25]. In vitro toxicity studies, the IC_{50} of menthol was 0.32 mM for oxygen consumption in adult hamster adipocytes and 0.76 mM for inhibition of 'state 3' oxidation of malate/glutamate in rat liver mitochondria [26]. And it is also reported that the EC50 value of menthol on $[Ca^{2+}]_i$ in human melanoma cells was 286 μM, and the Hill coefficient was 1.46 [27]. However, except topical external use, there are still no reports on the safety and toxicity of menthol in the other route of administration like oral or injection in human.

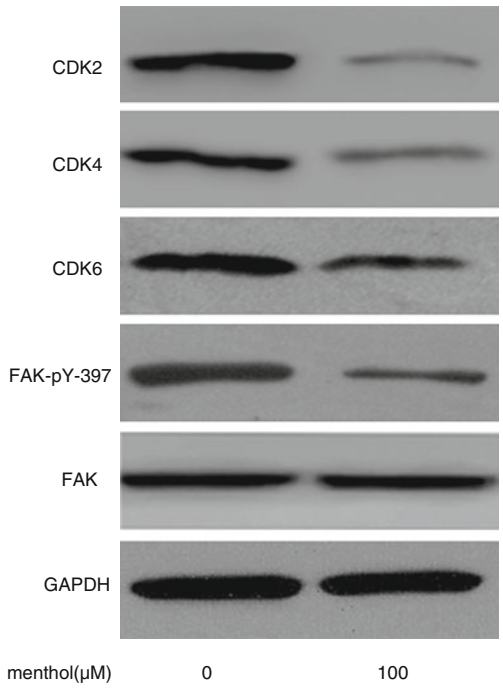

Fig. 5 Changes in the expression of CDK2, CDK4, CDK6, FAK-pY-397 and FAK. Proteins were extracted from cells received 0 μM and 100 μM menthol treated for 48 h, and then western blot was performed. The expression of GAPDH in the same samples was used as a loading control

Although it's well known that menthol can activate both TRPM8 and TRPA1, the involvement of TRPA1 in mediating the observed results can be excluded because TRPA1 was not found in DU145 cells. Through the MTT assay, we can see that 100 μM menthol caused a 53% suppression of DU145 proliferation. We found that the proliferation suppression can be partly corrected by 20 μM BCTC pretreatment, but there is statistical difference compared with non-menthol-treated cells ($p<0.05$). Such phenomenon may be caused by the blocking efficiency of BCTC or the low concentration we used.

Cell cycle deregulation and metastasis are important symbols of cancers. Deregulation of cell cycle is involved in uncontrolled proliferation of tumor cells [28], and metastasis is a biological phenomenon linked to the tumor cell's ability to migrate, seed and colonize distant sites. So it is well accepted that cell cycle- and metastasis-targeted therapy can be a potential treatment for prostate cancer.

Cell cycle checkpoints are important control mechanisms that ensure the proper execution of cell cycle events. In addition, cell cycle progression is precisely regulated by a series of CDKs [29]. In this study, we found that menthol could induce G_0/G_1 arrest without triggering cell death by downregulating the expression of CDK2, CDK4 and CDK6. Such effect is different from the CDK inhibitors which have entered cancer clinical trials, targeting the ATP-binding site of CDK molecules and inducing apoptosis [30]. Being different from the effect in LNCaP cells, menthol did not induce cell apoptosis in DU145 cells. Such difference may

be resulted from the difference of expression level and location of functional TRPM8 and the concentration of menthol. After all, LNCaP cells are androgen-dependent and express androgen receptor. Apart from the cell cycle arrest, menthol also reduced the motility of DU145 cells through the downregulation of phospho-FAK without changing the non-phospho-FAK, and FAK is an important regulator of cell migration [31]. Therefore, there are three findings suggesting that menthol is a useful compound for future development as an anticancer agent: (1) other CDK inhibitors elicit off-target responses to induce apoptosis; (2) menthol induces cell cycle arrest by downregulating the expression of CDKs without triggering apoptosis; (3) menthol reduces cell motility by downregulating the expression of phospho-FAK.

In summary, this study indicated that DU145 cells have a remarkable expression of TRPM8, and menthol has a negative effect on proliferation and motility of DU145 cells. Such effect can be partly corrected by BCTC. Therefore, our findings suggest that the activation of TRPM8 channel may serve as a potential and pragmatic treatment for patients in the late androgen-independent stage with high level expression of TRPM8, and menthol is a useful compound for future development as an anticancer agent.

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