RAPID COMMUNICATION

Proteomics indicates modulation of tubulin polymerization by L-menthol inhibiting human epithelial colorectal adenocarcinoma cell proliferation

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Menthol is a naturally occurring cyclic monoterpene used in oral hygiene products, confectionary, pharmaceuticals, cosmetics, pesticides, and as a flavoring agent. In the present study, we analyzed the differentially expressing proteome in L-menthol-treated Caco-2 cell line as it was found to inhibit cell proliferation. Interestingly, free tubulin proteins were observed to be limited after menthol treatment. Semiquantitative RT-PCR with α-tubulin primers showed no change in the level of RNA expression in menthol-treated cell line. However, tubulin polymerization assay with menthol indicated a trend similar to taxol in promoting microtubule assembly. Further, physical counting of apoptotic nuclei and active caspase-3 assays confirmed onset of apoptosis though the rate was slower as compared with that of taxol treatment. This study is the first report of a monoterpene L-menthol modulating tubulin polymerization and apoptosis to inhibit cancer cell proliferation.

Keywords:
Apoptosis / Caco-2 / Cell biology / Menthol / 2-DE/MALDI-TOF-MS / Tubulin polymerization

Monoterpenes have been hypothesized to act as both cytostatic and cell differentiating agents [1]. They have several effects on the mevalonate-lipid metabolic pathway including the inhibition of protein isoprenylation [2–3], the inhibition of ubiquinone synthesis, and the blockage of the conversion of lathosterol to cholesterol [4]. Terpenoids have different modes of action like effect on oxidative stress, carcinogenesis, and cardiovascular diseases [5]. Perillyl alcohol (*p*-mentha,1,7-diene-6-ol or 4-isopropenyl-cyclohexenecarbinol) induces apoptosis in tumor cells without affecting normal cells and can revert the tumor cells back to a differentiated state [6]. Microtubules of *Arabidopsis* seedlings were disrupted after exposure to citral in the gaseous phase as polymerization of microtubules in vitro was inhibited in its presence [7]. In the present study, we analyzed the effect of L-menthol, an abundant monoterpene present in the essential oil of the plant *Mentha arvensis*, on the human epithelial colorectal adenocarcinoma (Caco-2) cells through a proteomics approach and identified surprising absence of major protein tubulin in menthol-treated cells. In addition, L-menthol was found to inhibit cell proliferation. Positive modulation of tubulin polymerization by L-menthol was shown, indicating the destabilizing effect of the compound on microtubule assembly as observed in case of the anti-cancer compound taxol obtained from the bark of *Taxus wallichiana*. The study bears significance as L-menthol is used routinely in food, beverages, and cosmetics.

Caco-2 (ATCC HTB37, human colon adenocarcinoma) cells were obtained from National Centre for Cell Science, Pune, India, and were grown in Dulbecco's-modified eagle medium (DMEM) supplemented with 10% FBS. Undifferentiated cells were passaged upon reaching 80% confluence, at a split ratio of 1:4 using 0.25% trypsin in PBS. About

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Colour Online: See the article online to view Fig. 1 in colour.

4×10^3 cells/well were incubated with DMEM media overnight (18 h) and treated with the test compound for 48 h. In vitro growth inhibition activity of L-menthol against Caco-2 cells was performed by MTT assay [8] in triplicate. For isolation of RNA/protein, overnight grown cells were incubated with or without L-menthol/paclitaxel (or taxol) dissolved in 100% DMSO at IC50 (12 μg/mL for L-menthol and 0.0084 μg/mL for taxol) in 5% CO_2 incubator for 48 h. Always, control flasks were also set with only DMSO. The total cell count was measured with hemocytometer (Sigma-Aldrich, USA). Taxol and L-menthol were obtained from Sigma-Aldrich.

Cell pellets were washed in cold PBS, resuspended in PBS containing protease inhibitors cocktail (Sigma-Aldrich), and subjected to freeze–thaw cycles followed by sonication in ice for four 30 s pulses. The lysate was centrifuged at 15 000 rpm for 30 min at 4°C and supernatant was collected. Protein content of the solutions was estimated by Lowry's method [9] and equal amount of protein samples (1.5 mg) isolated from control (without any treatment), DMSO-treated, and menthol-treated cultures were analyzed. Protein was precipitated in 10% w/v cold trichloroacetic acid in acetone containing 20 mM DTT and washed thrice with acetone to eliminate nucleic acid and salts. 2-DE was performed as described previously [10] with isoelectric focusing within pH 3–7 because maximum protein spots were concentrated within this range. Gel images obtained from control, DMSO-, and menthol-treated cells were analyzed to identify the differentially expressed protein spots from the gels using HT Analyser 2D Software *v 2004* (Genomic Solution, USA) and excised by hand. The 2-D pattern of DMSO-treated culture protein was similar to control protein without any treatment. In-gel digestion of proteins, purification of peptides from plugs, and spotting onto MALDI sample plate were carried out following the protocol described earlier [11]. MS and MS/MS spectrum were acquired from MALDI-TOF/TOF Mass Spectrometer (Applied Biosystems 4700 Proteomics Analyzer, Framingham, MA, USA) and database searching for protein identification was performed according to our earlier standardized protocol [10, 12]. The criteria used to accept identifications for PMF included the probabilistic protein score-based confidence interval percentage, the extent of sequence coverage, the number of peptides matched, and whether any *Homo sapiens* protein appeared as top candidates during the first search when applying no restriction to the species of origin. Identification criteria with MS/MS data were set to peptide count more than two and confidence interval percentage for the best ion score above 95. Of the 33 protein spots for which quality data were obtained, 29 spots were found to be having differential expression. All the seven (U1–U7) spots picked from 50 to 60 kDa range, present in the control but conspicuously absent in the menthol-treated sample, were found to be the isoforms of α/β-tubulins (Table 1, Fig. 1A).

Semiquantitative RT-PCR was carried out using total RNA (Invitrogen, USA ThermoScript RT-PCR kit) and α-tubulin (5′ATATGTGGCCAGAGGGAAGT3′ and 5′GG CTGTGTTTGTAGACTTGG3′, 625 bp fragment)-specific primers. A general transcription factor 3C polypeptide 5

Table 1. MALDI-MS and MS/MS analysis of differentially expressed proteins of Caco-2 cell line in response to menthol treatment

Spot no.	Control Caco-2	Menthol-treated Caco-2	Protein name	Species	Accession no.[a)]	Mr/p*I*[b)]	PM/MS/CI%[c)] (MS)	PM/MIS/CI% (MS-MS)[d)]
U1	+	–	Tubulin α-ubiquitous chain (α-tubulin ubiquitous) (Tubulin K-α-1)	*H. sapiens*	TBAK_HUMAN (P68363)	50.8/ 4.94	16/158/100	–
U2	+	–	Tubulin β-2 chain (OK/SW-cl.56)	*H. sapiens*	TBB2_HUMAN (P07437)	50.1/ 4.78	24/280/100	–
U3	+	–	Tubulin α-ubiquitous chain (α-tubulin ubiquitous) (Tubulin K-α-1)	*H. sapiens*	TBAK_HUMAN (P68363)	50.8/ 4.94	–	6/169/100
U4	+	–	Tubulin β-2 chain (OK/SW-cl.56)	*H. sapiens*	TBB2_HUMAN (P07437)	50.1/ 4.78	23/260/100	15/562/100
U5	+	–	Tubulin α-ubiquitous chain (α-tubulin ubiquitous) (Tubulin K-α-1)	*H. sapiens*	TBAK_HUMAN (P68363)	50.8/ 4.94	14/101/100	
U6	+	–	Tubulin α-6 chain (α-tubulin 6)	*H. sapiens*	TBA6_HUMAN (Q9BQE3)	50.5/ 4.96	9/64/99.5	
U7	+	–	Tubulin α-3 chain (α-tubulin 3) (Tubulin B-α-1)	*H. sapiens*	TBA3_HUMAN (Q71U36)	50.8/ 4.94	16/158/100	

"+" or "–" indicates the presence or absence/decreased concentration of protein spots.
a) Accession numbers according to NCBI and Swiss-Prot accession number.
b) Experimental Mr and p*I*.
c) Number of peptides matched/MASCOT protein score/confidence interval percentage for MS analysis.
d) Number of peptides matched/MASCOT ion score/confidence interval percentage for MS-MS analysis.

(TF3C-ε, Q9Y5Q8, 5′CTTTGGGAGTACTTTGTGGG3′, and 5′AGGAAAAGAGAGCAGGCCTC3′, 1480 bp fragment) was amplified as control. The α-tubulin-specific primers did not indicate any difference in transcript abundance between untreated and treated RNA samples (Fig. 1B). Tubulin polymerization in the presence of L-menthol (IC25, IC50, and IC75) and taxol (IC50) was carried out using the ''assay kit'' following the manufacture's guidelines (Cytoskeleton, Denver, CO, USA) and the polymerization was measured by the change in absorbance at 340 nm every 1 min for 60 min at 37°C (Fig. 1C). Elongated cell morphology was observed when treated with L-menthol and taxol, compared with normal Caco-2 cells (Fig. 2A) at the initial stage. Occurrence of apoptosis upon menthol treatment was analyzed and compared with taxol with comparable pattern. In DAPI staining [13], 90–100% apoptotic nuclei could be observed 96 h after treatment (Fig. 2B). Active caspase-3 staining (caspase-3 activation assays Kit, BD Biosciences, USA) indicated the presence of active caspase-3 when treated with menthol (Fig. 2C).

Menthol is a naturally occurring cyclic monoterpene with increasing use in different hygiene products [14, 15] and is considered a safe and effective topical OTC product according to the FDA. It has no cytotoxic effect on human HeLa cells in vitro at concentrations of 1, 10, and 100 μg/mL [16] but induces cell death at supra-millimolar concentrations in PC-3 cells independent of Ca^{2+} influx pathways and increases a phosphorylated form of c-jun N-terminal kinase (JNK) in PC-3 cells through TRPM8-independent mechanisms [17]. Against murine leukemia WEHI-3 cells, menthol

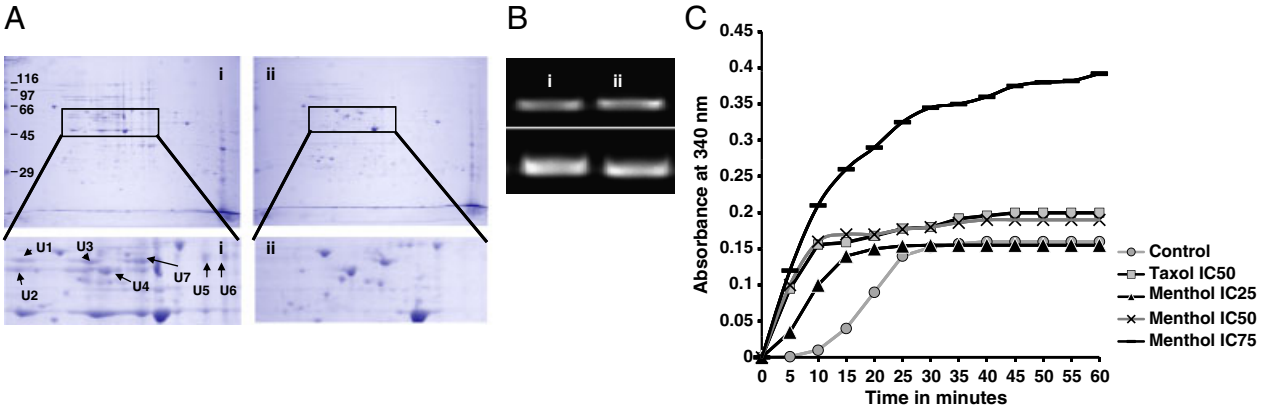

Figure 1. (A) 2-D gels for soluble protein isolated and analyzed from untreated (i) and L-menthol-treated (ii) Caco-2 cell line. Magnified view of the gel portions from which random spots were taken for MS and MS-MS analysis is also shown below the original gel. (B) Semi-quantitative RT-PCR showing expression in untreated (lane i) and menthol-treated (lane ii) Caco-2 cell lines. Upper lanes indicate amplification by α-tubulin-specific primers and lower lanes indicate amplification with TF3C-ε-specific primers. (C) Tubulin polymerization assay showing the polymerization of tubulin in the presence of IC25, IC50, and IC75 concentration of L-menthol compared with controls (the absence of any compound and the presence of IC50 concentration of taxol).

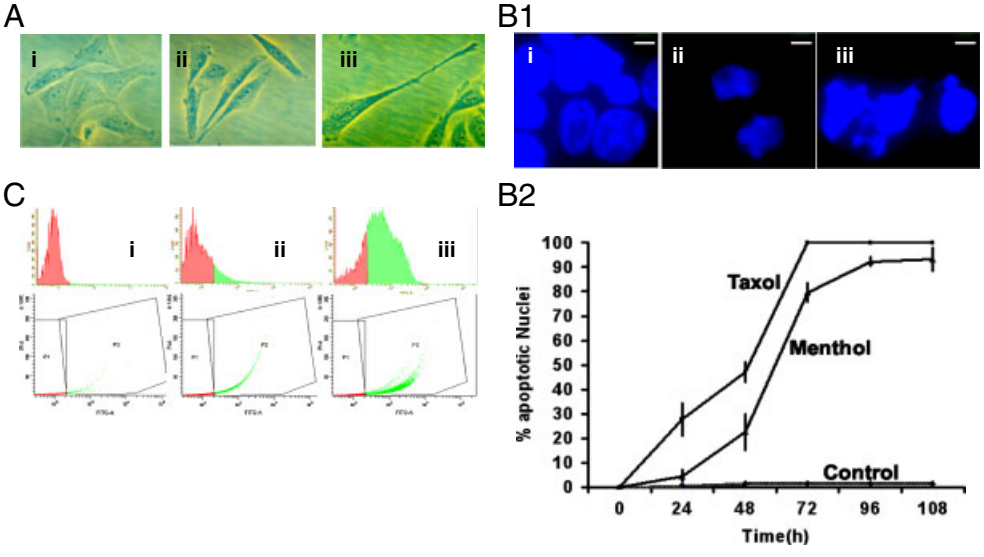

Figure 2. (A). Caco-2 cell lines (i) untreated, (ii) L-menthol-treated, and (iii) taxol-treated at IC50 concentrations (10 × 100 magnification). Menthol- and taxol-treated cells show elongation before entering into apoptosis. (B1) Apoptotic nuclei from (i) untreated, (ii) L-menthol-treated cells and (iii) taxol-treated cells. (B2) Percentage of apoptotic nuclei at different time points. (C) Active caspase-3 marker and cell count in flow cytometer; (i) untreated, (ii) L-menthol-treated (12 μg/mL), and (iii) taxol-treated cells (1 μg/mL).

induced cytotoxicity in vitro in a dose-dependent manner [18]. Menthol is also implicated in cell death via the TRPM8 channel in the human bladder cancer cell line T24 [19]. Recently, menthol is reported to increase human glioblastoma intracellular Ca^{2+}, BK channel activity, and cell migration [20]. However, glioblastoma is one of the deadliest forms of cancer, in part because of its highly invasive nature. The tumor suppressor PTEN is frequently mutated in glioblastoma and is known to contribute to the invasive phenotype [21]. Hence, it is a matter of further experimentation to explore the stimulation for migration and motility of the malignant cells in vivo.

In the present investigation, the onset of apoptosis was confirmed through DAPI staining and active caspase-3 assays. Due to absence/undetected concentration of tubulin in menthol-treated cell line (Fig. 1A, Table 1), initially it was believed that the expression of tubulin genes was suppressed. However, in semiquantitive RT-PCR, the mRNA expression for α-tubulin in treated RNA was comparable to the control level (Fig. 1B), indicating that the absence of tubulins in menthol-treated cells was caused at the protein level. Gu et al. [22] also reported that taxol, promoting the microtubule assembly, does not decrease the α-tubulin mRNA content in adrenal chromaffin cells in culture. Tubulins (α,β-tubulin heterodimer) are the components of microtubules and important members of the cell cytoskeleton. Phytomolecules like taxol act as promoter of calf brain microtubule assembly in vitro, in contrast to plant products such as colchicine and podophyllotoxin, which inhibit assembly. Taxol decreases the lag time for microtubule assembly and shifts the equilibrium for assembly in favour of the microtubule, thereby decreasing the critical concentration of tubulin required for assembly and polymerization [23, 24]. When the tubulin polymerization was tested, menthol showed a trend parallel to taxol (Fig. 1C), indicating the possibility of promoting microtubule assembly. Though both menthol and taxol demonstrated similar response to tubulin, still the concentration required for menthol was very high (IC50 = 12 μg/mL) as compared with taxol (IC50 = 0.0084 μg/mL).

By modulating cell cycle in gastric cancer cells, menthol inhibits the proliferation of the cell in a dose- and time-dependent manner, represses topoisomerase I, II a, and III β, but promotes the levels of NF-κB gene expression [25]. Izumi et al. [26] demonstrated geraniol (a monoterpene) as a potent inducer of apoptosis-like cell death in the cultured shoot primordia of the plant *Matricaria chamomilla*. Although, at this stage, a menthol-derived chemo-preventive agent does not seem to be a near feasibility. Nevertheless, the current investigation has yielded a novel mechanism for the action of menthol in promoting tubulin polymerization and ultimately leading to cell death. A variety of dietary monoterpenes have been shown to be effective in the chemoprevention and chemotherapy of cancer. Monoterpenes also possess many characteristics of ideal chemopreventive agents, namely, efficacious antitumor activity, commercial availability, low cost, oral bioavailability, and low toxicity, making it feasible to consider them for human cancer chemoprevention testing [27]. L-Menthol is a natural monoterpene present abundantly in the essential oil of *M. arvensis* and *M. piperita*, and consumed world wide in different forms. Though this is the first report on the effect of L-menthol on tubulin, delineation of the detailed mechanism of action of menthol inhibiting the human colon adenocarcinoma and improvement of the molecule through semisynthesis may lead to better candidate for chemoprevention or therapy in future.

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