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Repurposing L-Menthol for Systems Medicine and Cancer Therapeutics? L-Menthol Induces Apoptosis through Caspase 10 and by Suppressing HSP90

Uzma Faridi,† Sunita S. Dhawan,† Shaifali Pal, Sanchita Gupta, Ashutosh K. Shukla, Mahendra P. Darokar, Ashok Sharma, and Ajit K. Shasany

Abstract

The objective of the present study was to repurpose L-menthol, which is frequently used in oral health and topical formulations, for cancer therapeutics. In this article, we argue that monoterpenes such as L-menthol might offer veritable potentials in systems medicine, for example, as cheaper anti-cancer compounds. Other monoterpenes such as limonene, perillyl alcohol, and geraniol have been shown to induce apoptosis in various cancer cell lines, but their mechanisms of action are yet to be completely elucidated. Earlier, we showed that L-menthol modulates tubulin polymerization and apoptosis to inhibit cancer cell proliferation. In the present report, we used an apoptosis-related gene microarray in conjunction with proteomics analyses, as well as *in silico* interpretations, to study gene expression modulation in human adenocarcinoma Caco-2 cell line in response to L-menthol treatment. The microarray analysis identified caspase 10 as the important initiator caspase, instead of caspase 8. The proteomics analyses showed downregulation of HSP90 protein (also corroborated by its low transcript abundance), which in turn indicated inhibition of AKT-mediated survival pathway, release of pro-apoptotic factor BAD from BAD and BCLxL complex, besides regulation of other factors related to apoptosis. Based on the combined microarray, proteomics, and *in silico* data, a signaling pathway for L-menthol-induced apoptosis is being presented for the first time here. These data and literature analysis have significant implications for ''repurposing'' L-menthol beyond oral medicine, and in understanding the mode of action of plant-derived monoterpenes towards development of cheaper anticancer drugs in future.

Introduction

Animal studies have suggested the anti-carcinogenic properties of monoterpenes in the past. In this connection, menthol, a cyclic monoterpene, is frequently used in oral health and topical formulations. In this article, we argue that monoterpenes such as L-menthol might offer veritable potentials in systems medicine, for example, as cheaper anti-cancer compounds. Other monoterpenes such as limonene, perillyl alcohol, and geraniol have been shown to induce apoptosis in various cancer cell lines and to hold promise as a novel class of anticancer drugs (Loza-Tavera, 1999).

Limonene and perillyl alcohol have been suggested to play bioactivity against a wide variety of malignancies (Barthelman et al., 1998; Chen et al., 2015; Clark et al., 2003; Haag et al., 1992; Miller et al., 2015; Mills et al., 1995; Reddy et al., 1997; Stark et al., 1995; Stayrook et al., 1997). Perillyl alcohol selectively induces G_0/G_1 arrest (Sahin et al., 1999) and c-Myc-dependent apoptosis (Clark, 2006) and has a common mechanism of action as compared to geraniol and farnesol (Wiseman et al., 2007). D-Limonene induces apoptosis by inactivation of protein kinase B (AKT) in human colon cancer cells (Jia et al., 2013). In HL-60 cells, D-limonene and D-carvone are reported to induce apoptosis through caspase-8 activation (Yu et al., 2008). The anti-tumor activity and/or mechanism for 37 monoterpenes found in natural essential oils were reviewed by Sobral et al. (2014).

While these monoterpenes are reported to cause apoptosis, their detailed mechanisms of action are not known (Wiseman et al., 2007). Earlier, we reported apoptosis in human colon adenocarcinoma cells by L-menthol (Faridi et al., 2011) with enhanced tubulin polymerization. L-menthol is broadly utilized not only in oral hygiene products but also in confectionary, pharmaceuticals, cosmetics, pesticides, or as a

Biotechnology Division, CSIR–Central Institute of Medicinal and Aromatic Plants, Lucknow, U.P., India.
†Equal contribution as first authors.

flavoring agent. Positive modulation of tubulin polymerization by L-menthol underscores its destabilizing effect on microtubule assembly similar to that of taxol.

Since the apoptosis signaling pathway involved is yet to be delineated clearly, we conducted a microarray analysis with 368 targets/genes related to apoptosis/cell proliferation and with the help of *in silico* analysis and proteomics data we were able to propose a putative model. Perillyl alcohol is already in clinical trial for treatment of metastatic breast cancer (Bailey et al., 2008) and malignant gliomas (da Fonseca et al., 2008). As L-menthol is abundantly available from the essential oil of *Mentha* species, a detailed investigation of the mechanism of apoptotic signaling is very timely both for oral health and systems medicine, not to mention in developing or ''repurposing'' it towards a potentially cheaper anticancer therapy (Kale et al., 2015; Kato et al., 2015; McCabe et al., 2015).

In the present study, the combined use of standard experimental (Burnette, 1981; Faridi et al., 2011; Herrmann et al., 1994;) and *in silico* (Mi et al., 2013; Sturn et al., 2002) procedures has indicated that L-menthol induces apoptosis through caspase 10 and by suppressing the heat shock protein 90 (HSP90).

Materials and Methods

Cell line treatment

Caco-2 (ATCC HTB37, human colon adenocarcinoma) cells were obtained from National Centre for Cell Science (Pune, India), and treated with L-menthol at IC_{50} (12 mg/mL) for 48 h (Faridi et al., 2011). Taxol treatment was also carried out as described in our previous study (Faridi et al., 2011).

DNA fragmentation assay

L-menthol-treated cells were trypsinized and pelleted by centrifugation at 1600 *g* for 5 min. This was followed by washing with 500 μL 1x PBS and addition of 500 μL lysis buffer [1 mL Nonidet P-40, 4 ml EDTA (0.5 M) and 3.33 mL Tris-HCl (1.5 mM) were added and volume was made up to 100 mL] for 10 seconds. Post-treatment, the sample was centrifuged at 1600 *g* for 5 min and the supernatant was re-extracted with same amount of lysis buffer. To it, added 500 μL of SDS (1%) with 8 μL of RNase (5 μg/μL) and incubated at 56°C for 2 h. Then added 8 μL protinase K (2.5 μg/μL) and incubated for 2 h at 37°C. DNA was precipitated by adding 10% ammonium acetate and 2.5 volume of absolute ethanol followed by centrifugation at 8000 *g* for 5 min. The precipitated DNA was dissolved in gel loading buffer and observed on 1.5% agarose gel (Herrmann et al., 1994).

Microarray

An oligo-set for 368 apoptosis-related genes (Operon Technologies, USA) was spotted on slides (Genomic Solutions, USA). For each gene, there were 8 technical replicates/spots and the average intensity was calculated.

Labeling and purification for cDNA [synthesized from 10 μg total RNA isolated using TRIzol® (Invitrogen, USA) as per manufacturer's guidelines] were carried out using the ChipShot™ Indirect Labeling and Clean-Up System (Promega, USA). The eluted aminoallyl cDNA was conjugated with CyDye™ NHS Ester as per protocol and the eluted, purified, labeled cDNA was quantified for dye incorporation by measuring absorbance at 260, 550, and 650 nm. The labeled cDNA samples were vacuum dried and dissolved in 120 μL of salt-based hybridization buffer (Ocimum Biosolutions, India), followed by heating at 95°C for 5 min and cooling to room temperature.

Hybridization was carried out for 16 h at 65°C, followed by washing and image acquisition using a Genomic Solutions (USA) platform. The images were scanned with a Gene TAC™ UC 4 Microarray Scanner (Genomic Solutions Inc., a Harward Bioscience Company, MA). Signal intensities for each spot were quantified using Gene TAC Integrator (Version 4; Genomic Solutions). The observed fold change value provides an estimate of the level of differential expression of each gene. Total intensity normalization was performed globally, meaning that for each expression value the same transformation was applied independent of its intensity. Cy5 was used for labeling L-menthol-treated sample and Cy3 was used for labeling untreated sample.

The fold change was calculated as follows: Fold change (FC) value = (Cy5/Cy3) x Normalization factor (N), where N was calculated on the basis of global normalization. For analysis, the fold change value used for upregulated genes was the FC value itself (positive values), whereas for downregulated genes the negative reciprocal of the FC value was used for easier comprehension.

GENETAC Integrator version 4.0 software was used for the analysis (Genomic Solutions). Initially the raw ratios were calculated for both the dyes Cy3 (volume) and Cy 5 (volume) and if the value was less than 1, N fold was calculated by taking negative reciprocal of the raw ratios. The absolute values of all the N- fold changes were calculated. Normalization factor was determined by using reference sample or housekeeping genes within array. The foreground and background intensity values were recorded for every gene in the array using a fixed threshold boundary by Gene TAC Integrator software (Genomic Solutions).

Background intensity values were subtracted from the foreground and normalized using global normalization algorithm used by GeneTAC Integrator software. The resultant comma separated file with the background subtracted, globally normalized intensity ratio between the treated versus control (Cy5/Cy3) values were used for statistical analysis. Dunnett Multiple Comparisons Test (using ''Graph Pad InStat Version 3.01'') was performed by comparing the fold change values for all the genes in L-menthol-treated cell line with untreated cell line. Out of 330 genes, only 12 genes in the range of 0 to 2 (SFRP1, DAP13, ING1, HSPA5, MCL1, KIAA0720, MDM4, DFFB, TNFRSF10A, AKT3, TNFRSF14, and IGFALS) and 6 genes in the range of 0 to −2 (TRAF5, PDCD2, CNR2, SPTAN1, DAP3, and P53AIP1) fold expression were having no significant difference with $p > 0.05$ and the rest of the genes showed significant differences with $p < 0.01$.

The data from this microarray experiment were submitted to GEO (NCBI) under series accession number GSE53701 [associated sample GSM1299167].

Semi-quantitative RT-PCR for microarray data validation

Total RNA was isolated using TRIzol® (Invitrogen, USA) (as done previously for the microarray analysis) and analyzed

through ethidium bromide staining and Nanodrop ND1000 spectrophotometer. Four micrograms of DNaseI-treated total RNA was used for first strand cDNA synthesis using Thermoscript RT-PCR System (Invitrogen). Transcription factor 3C polypeptide 5 (*TF3C-epsilon*, NM_001122823.1) was amplified as control (Faridi et al., 2011). The primer sequences were designed using Gene Runner *version 3.05* (Hastings Software Inc., USA.) (Supplementary Table S1; supplementary material is available online at www.liebertpub.com/omi).

Proteomics analysis

Protein samples were isolated from L-menthol-treated and untreated cells and subjected to 2D electrophoresis as described earlier (Faridi et al., 2011). Differentially expressing spots were excised and MS spectra were analyzed through MALDI-TOF-TOF (Faridi et al., 2011). For Western blotting, the protein samples (40 μg each) were loaded on 12% SDS-polyacrylamide gel and electrophoresed for 45 min at 250 V. The proteins were transferred to a nitrocellulose membrane (Burnette, 1981). After complete transfer, the membrane was incubated in blocking buffer (Pierce Biotechnology, Rockford, USA) at room temperature, washed with PBS in 0.1% Tween 20 for 1 h and immune-reacted overnight at 4°C with anti-HSP90 antibody (Cell Signaling Technologies, Danvers, MA).

The membrane was washed four times with PBS with 0.1% Tween 20 (each time for 15 min.) and the HRP-conjugated anti-rabbit IgG (diluted 1:2,000) was applied to the membrane for 2 h at room temperature. Finally the membrane was washed with PBS/0.1% Tween 20 for 1 h and the signal was detected using 4-chloronaphthol as substrate (following the protocol of Thermo Scientific, USA).

In silico analysis

The role of selected genes in cancer-related pathways was visualized using PANTHER (Protein ANalysis THrough Evolutionary Relationship) (Mi et al., 2013). It was used to classify proteins/genes according to family and subfamily, molecular function, biological process, and pathway. The genes involved in apoptosis signaling pathway were clustered according to their expression values. Genesis 1.7.6 clustering tool (Sturn et al., 2002) was used for grouping and heat-map generation. KEGG Mapper (http://www.genome.jp/kegg/mapper.html) was used for visualization of the genes in apoptosis pathway based on their expression values.

Results

DNA fragmentation

DNA in cells undergoing apoptosis shows a typical ladder-like pattern due to fragmentation caused by caspase-activated DNase. The DNA isolated from L-menthol-treated cells was observed to be degraded as compared to the intact DNA from the untreated cells (Fig. 1). Paclitaxel/taxol (positive control) treatment showed complete DNA degradation.

Microarray analysis

Of the 368 genes, consistent data was obtained for 330 genes (Supplementary Table S2), whereby 146 genes demonstrated upregulation and 184 genes showed downregulation

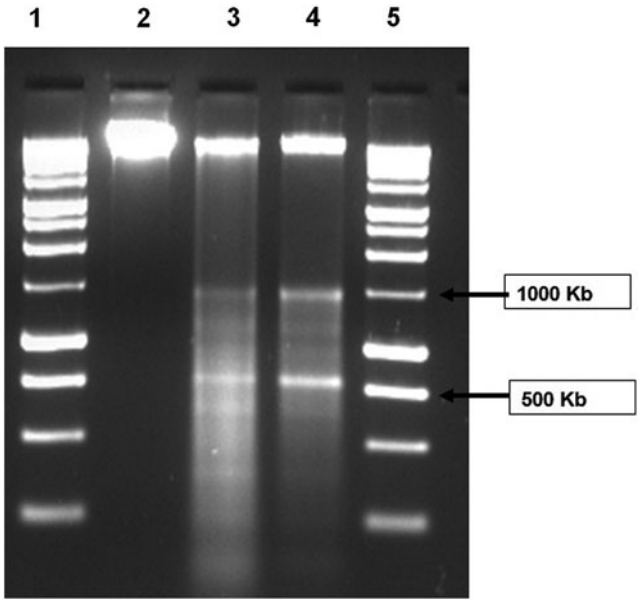

FIG. 1. DNA from Caco-2 cell lines. *Lanes 1 and 5*: molecular weight marker; *Lane 2*: DNA from untreated cell; *Lane 3*: DNA from paclitaxel-treated cell; and *Lane 4*: DNA from L-menthol-treated cell. DNA sample from L-menthol-treated cell shows similar degradation as in case of paclitaxel treatment.

(Fig. 2). The upregulated (above 3-fold) and downregulated (below −3-fold) genes are presented in Figure 3A and 3B, respectively.

The genes TNFSF 6, 7, and 9 and the receptors TNFRSF 7 and 9 were upregulated (> 2-fold) (Fig. 4A and 4B). Similarly, the genes for caspases CASP 3, 4, 7, 9, 10, and 14 were also upregulated (Fig. 5). Substantially higher expression of BCL-2 such as genes BCLG, BCL2L10, BCL3, BAD, BID, and BNIP2 was also observed (Fig. 6). Semi-quantitative RT-PCR validated the microarray data for some randomly selected genes (TNFSF9, TNFRSF7, CASP3, CASP7, CASP9, BID, and AKT1) (Fig. 7).

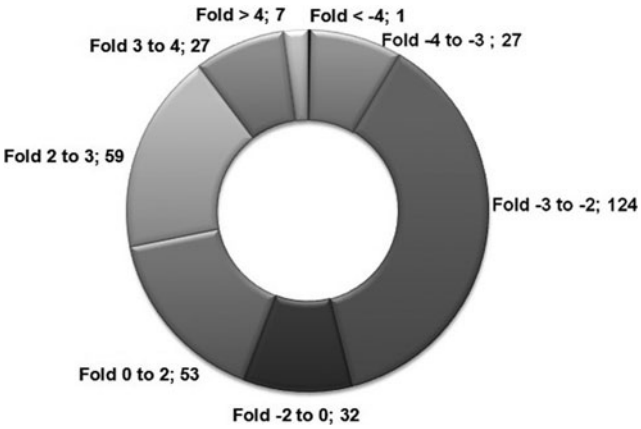

FIG. 2. Doughnut diagram representing the number of differentially expressed genes in L-menthol-treated Caco2 cells as compared to untreated control cells.

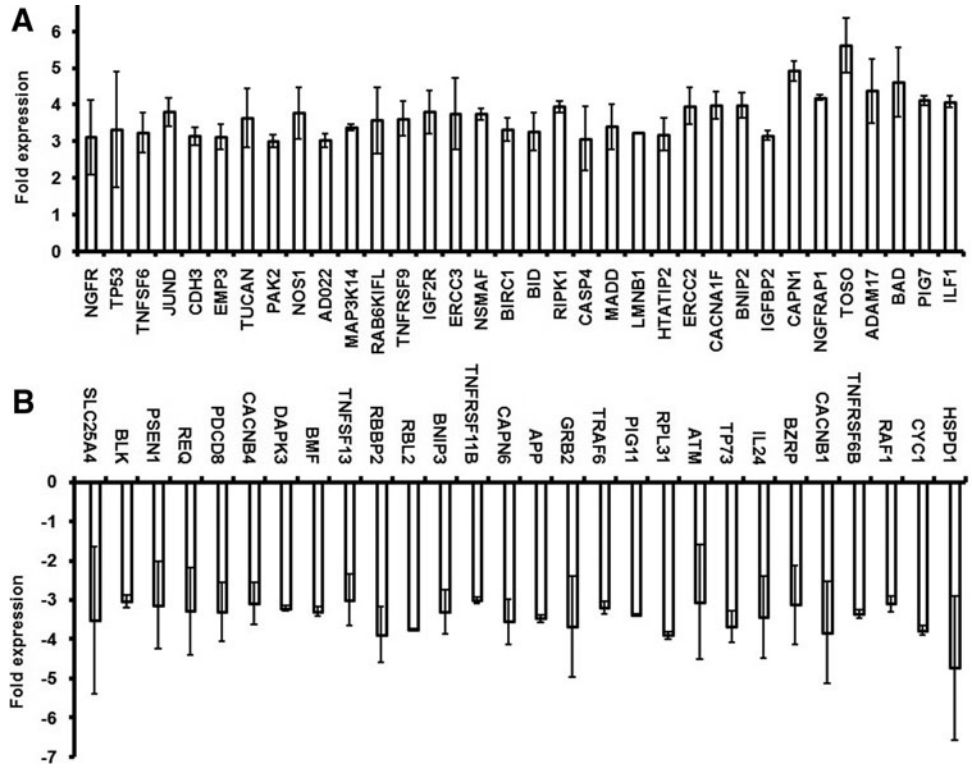

FIG. 3. Differentially expressed genes in L-menthol-treated Caco2 cells. **(A)** Genes upregulated (more than 3-fold); **(B)** Genes downregulated (less than −3-fold).

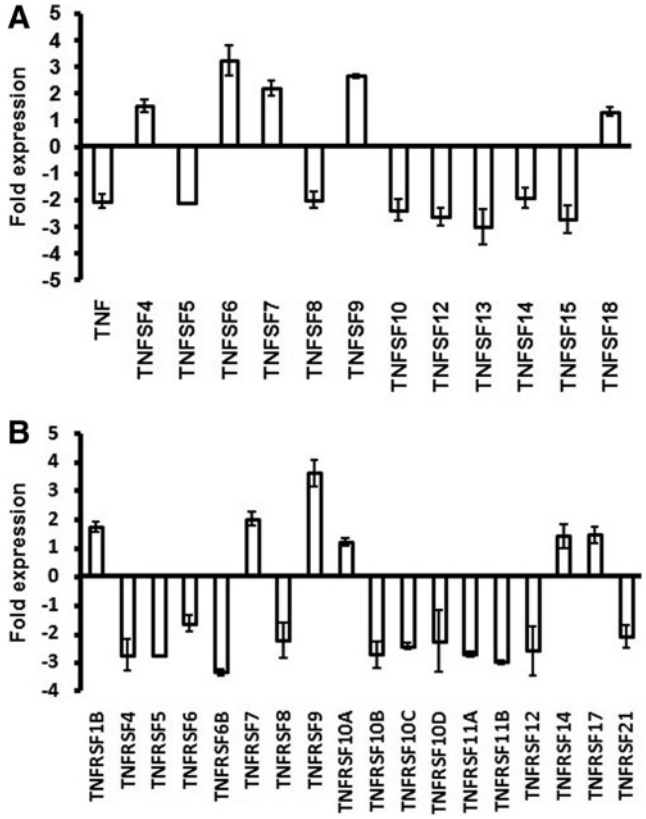

FIG. 4. TNF **(A)** and TNF receptor **(B)** genes showing up/down regulation in L-menthol-treated Caco2 cells.

Proteomics and expression analysis

In 2D electrophoresis and MS-MS analysis (Faridi et al., 2011), 7 proteins were conspicuously absent along with tubulin in case of L-menthol-treated cells (Supplementary Table S3). Absence/decrease in HSP90 expression was confirmed through Western blotting and its transcript abundance was also found to decrease in L-menthol-treated cells (Fig. 8).

Pathway map

The selected genes with their fold change values were analyzed for their role in different signaling pathways using PANTHER (Supplementary Fig. S1). Fifty-four genes were

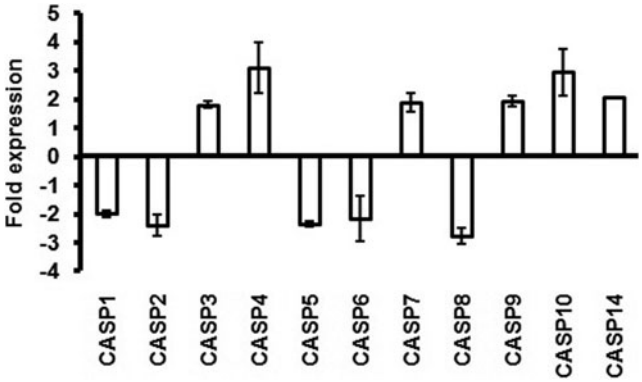

FIG. 5. Caspase family genes showing up/down regulation in L-menthol-treated Caco2 cells.

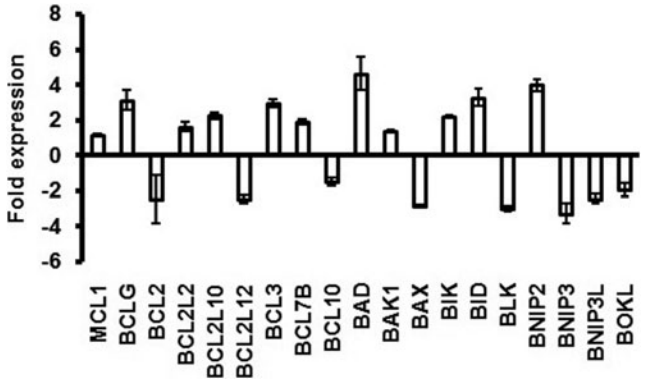

FIG. 6. BCL-2 family genes showing up/down regulation in L-menthol-treated Caco2 cells.

found associated with apoptosis signaling pathways and were analyzed through a heatmap (and arranged according to their fold change values) (Supplementary Fig. S2). The color range was light blue (downregulated) to orange (upregulated). The genes were visualized in accordance to their expression values in the apoptosis pathway map (Supplementary Fig. S3). Red color denoted upregulation, whereas green color indicated downregulation. Based on microarray, proteomics and *in silico* data, a signaling pathway for L-menthol-induced apoptosis was proposed (Fig. 9).

Discussion

Omics biotechnologies such as genomics, proteomics, and metabolomics allow us to repurpose drugs or examine the system scale impacts of molecular perturbations, be they induced by drugs, nutrition, xenobiotics, or changes in the environmental stressors (Dandara et al., 2014; Gong et al., 2014; Kale et al., 2015; Kato et al., 2015; McCabe et al., 2015; Mounayar et al., 2014; Sahu et al., 2014; Sitole et al., 2014; Zheng et al., 2014). Based on the combined microarray, proteomics, and *in silico* data, a signaling pathway for L-menthol-induced apoptosis is being presented for the first time here. To contextualize the present findings, we provide below the broader rationale and molecular background to consider repurposing L-menthol beyond applications in oral medicine, with a view to anti-cancer therapeutics and systems medicine.

Tumor necrosis factor superfamily (TNFSF) proteins govern many cellular functions including apoptosis (Ware, 2008). They bind to members of the tumor necrosis factor receptor superfamily (TNFRSF) and depending on the proteins involved, it triggers a series of chemical signals that instruct cells to grow/divide, self-destruct, or mature for specialized functions. In this analysis, *TNFSF7* and *TNFSF9* and their corresponding *TNFRSF*s (*TNFRSF7*, *TNFRSF9*) were upregulated in L-menthol-treated cells (Fig. 4). TNFSF7 expression is epigenetically downregulated during progression in breast cancer cells (Yu et al., 2010) and it is also associated with the tumor necrosis factor (TNF) ligand-receptor death pathway (Hengartner, 2000). There are reports on apoptosis induction by TSNSF7-CD27L (Kashii et al., 1999) and TNFRSF9-TNFSF9 (Seko et al., 2004; Ebmeyer et al., 2011) in tumor cells. Hence, in case of L-menthol-treated cells, the apoptosis signal was predicted to be initiated by TNFSF7-TNFRSF7 and TNFSF9-TNFRSF9.

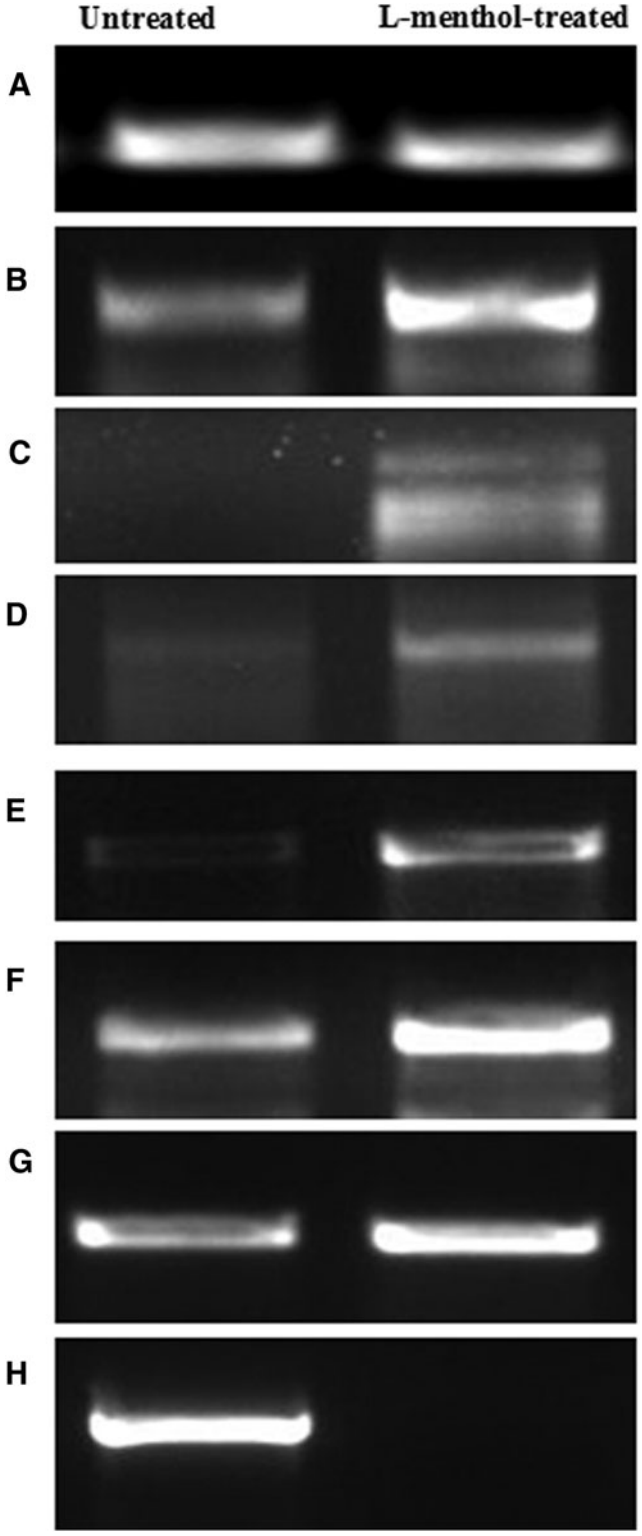

FIG. 7. Representative gel image depicting the semi-quantitative RT-PCR-based validation of microarray data for randomly selected differentially expressed genes in L-menthol-treated Caco2 cells. **(A)** Control (*TF3C-epsilon*); **(B)** *TNFSF9*; **(C)** *TNFRSF7*; **(D)** *Caspase 3*; **(E)** *Caspase 7*; **(F)** *Caspase 9*; **(G)** *BID*; **(H)** *AKT1*.

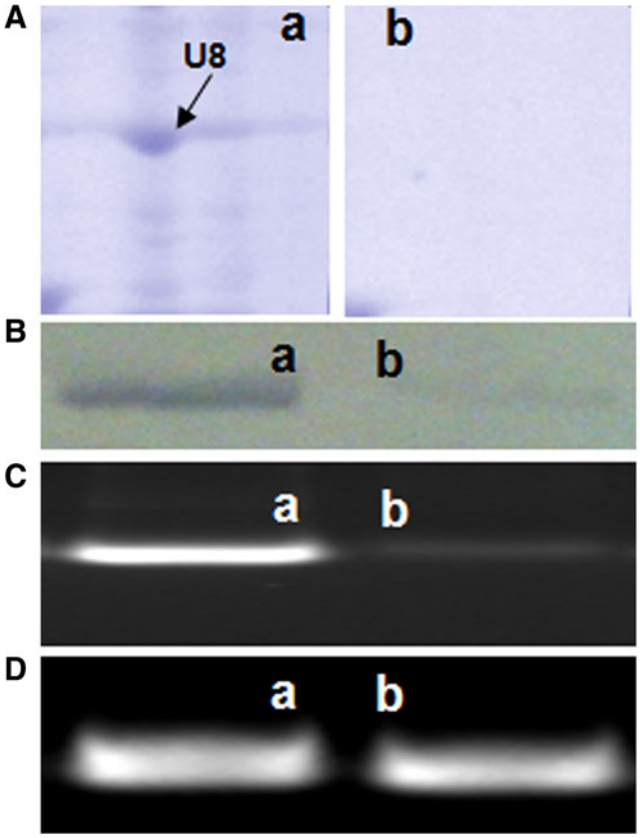

FIG. 8. HSP90 protein and gene expression in untreated **(a)** *vs* L-menthol-treated **(b)** Caco-2 cells. **(A)** Magnified view of the gel portion showing absence of the spot ''U8'' representing HSP90 protein in L-menthol-treated cells. **(B)** Western blot showing decrease in concentration of HSP90 in L-menthol-treated cells. **(C)** Semi-quantitative RT-PCR showing decrease in expression of *HSP90* in L-menthol-treated cells. **(D)** Semi-quantitative RT-PCR of control gene *TF3C-epsilon*.

Induction of apoptosis *via* death receptors typically activates an initiator caspase (CASP) such as CASP8 or CASP10, which in turn activate other caspases in a cascade leading to the activation of effector caspases, like CASP3 and CASP6, responsible for cleaving key proteins in cells undergoing apoptosis. CASP8 plays an obligatory role in apoptosis initiation, but the role of its structural relative, CASP10, remains to be investigated (Kischkel et al., 2001).

In this investigation, high expression of *CASP10* was obtained instead of *CASP8* (Fig. 5). *CASP10* is closely related in sequence to *CASP8* (Fernandes-Alnemri et al., 1996; Hu et al., 1997) and both are encoded on the same region of human chromosome 2, suggesting duplication of an ancestral gene. *In vitro* experiments have demonstrated the ability of CASP10 to process CASP3 and CASP7 (Fernandes-Alnemri et al., 1996), and CASP10 has also been implicated in apoptosis (Hu et al., 1997; Kischkel et al., 2001; Milhas et al., 2005; Wang et al., 2001). Thus, involvement of CASP10 rather than CASP8 in apoptosis signaling in L-menthol-treated cells seems more likely. Post-activation, CASP10 will activate downstream caspases including CASP3 and CASP7. Here L-menthol was found to induce the expression of genes for pro-apoptotic caspases 3, 7, 9, and 10 (Fig. 5).

Moving on to the next category, BCL-2 family proteins, having either pro- or anti-apoptotic activities, have been studied intensively owing to their importance in regulation of apoptosis, tumor genesis and cellular responses to anticancer therapy. In mammals, there are at least 12 core BCL-2 family proteins, having either three-dimensional structural similarity or a predicted secondary structure that is similar to BCL-2 (Youle and Strasser, 2008).

In this investigation, genes for many BCL-2 family proteins showed higher expression in L-menthol-treated cells (Fig. 6). Their role in induction of apoptosis has been described in detail in many earlier studies (Brocke-Heidrich et al., 2006; Chinnadurai et al., 2008; Guo et al., 2001; Hsu et al., 1997; Inohara et al., 1998; Luo et al., 2009; Valencia et al., 2007; Zhao et al., 2003).

A lot of interplay is also involved among various categories of proteins in effecting apoptosis. APAF1 is a key link in apoptosis (Soengas et al., 2001), which is associated with pro-caspase-9 and cytochrome c, forming the apoptosome complex and activating CASP9 that in turn cleaves and activates effector caspases (Li et al., 1997). BCL2L10 binds to this protein directly and prevents Bcl-xL (apoptosis inhibitor) binding to APAF1 (Inohara et al., 1998). In this analysis, higher expression of *APAF1* and *BCL2L10* indicated the occurrence of this pathway for apoptosis after L-menthol treatment (Supplementary Table S2).

Although it is well established that the high-affinity NGF receptor plays a pivotal role in cell survival, there are reports about low-affinity NGF receptor inducing apoptosis (Taglialatela et al., 1997). Resveratrol is known to induce apoptosis through a TP53-dependent pathway (Huang et al., 1999). Apoptosis enhancements by JUND (Li et al., 2002), EMP3 (Fumoto et al., 2009), AD022 (Zucchelli et al., 2009), RAB6KIFL (Hill et al., 2000), TNFRSF9 (Ebmeyer et al., 2011), IGFR (Wylie et al., 2003), NSMAF (O'Brien et al., 2003), BID (Zhao and Wang, 2004), RIPK1 (Wang et al., 2008), CASP4 (Hitomi et al., 2004), MADD (Schievella et al., 1997), HTATIP2 (Xiao et al., 2000), BNIP-2 (Valencia et al., 2007), and IGFBP2 (Frommer et al., 2006) are known. TNFSF6 initiates apoptosis by binding to its receptor FAS (TNFRSF6) (Castellano et al., 2006). However, in this investigation TNFRSF6 transcript was downregulated (Fig. 4).

The other important proteins showing more than 4-fold increase in transcript expression in response to L-menthol (Supplementary Table S2) and reported earlier to be regulating apoptosis are CAPN1 (Piñeiro et al., 2007), NGFRAP1 (Naderi et al., 2007), ADAM17 (Wang et al., 2011), BAD (Adachi et al., 2002), PIG7 (Matsumura et al., 2004), and ILF1 (Sadkowski et al., 2008). Taxol induces CASP3-independent apoptosis in NIH3T3 cells by a calpain (CAPN1)-mediated mechanism (Piñeiro et al., 2007). L-menthol has also been found to promote tubulin polymerization (Faridi et al., 2011) such as taxol and increases the expression of *CAPN1*.

Besides these, several other predominantly anti-apoptotic protein transcripts showing overexpression in response to L-menthol were CDH3 (Wheelock and Johnson, 2003), TUCAN (Pathan et al., 2001), NOS1 (Andoh et al., 2000), MAP3K14 (Liao et al., 2004), BIRC1 (Yin et al., 2008), and CACNA1F (Kotturi et al., 2003) (Supplementary Table S2). PAK2, although anti-apoptotic (Jakobi et al., 2001), can be activated *via* cleavage by caspases mediating cell death (Rudel and Bokoch, 1997; Rudel et al., 1998). The role of *DNASE1l3*

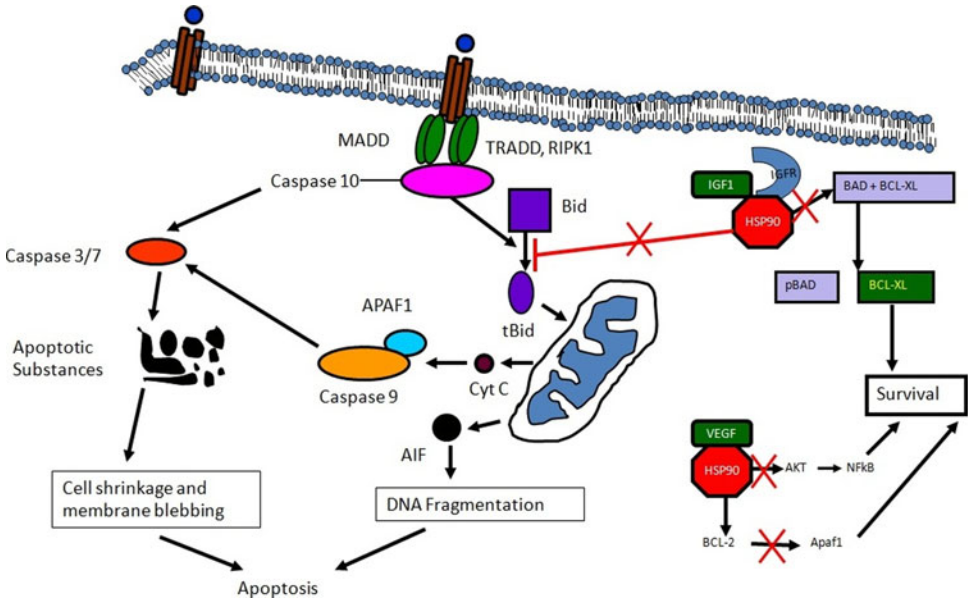

FIG. 9. Model predicting the possible mode of action of L-menthol. As described, the initial response is through Caspase 10 and followed by Caspase 3 and Caspase 7. Caspase 10 may also influence the activation of BID to tBID. This step is inhibited by HSP90, but due to lower expression in L-menthol-treated cell, the availability of HSP90 decreases. After release of cytochrome c from mitochondria, APAF1 and Caspase 9 induce Caspase 3 and Caspase 7 towards apoptosis. HSP90 may not be available in sufficient amount to trigger the AKT- and NFkB-mediated survival pathway. Similarly, BAD (a pro-apoptotic factor) is released from the complex ''BAD and BCL-xL'' due to low availability of HSP90.

(upregulated 2.11-fold) in chromatin cleavage during apoptosis has been proposed earlier (Napirei et al., 2009). Hence, the nucleotide excision repair enzyme excision repair cross-complementing group 2 and 3 genes (*ERCC2*, *ERCC3*) (Hsia et al., 2003; Justenhoven et al., 2004) are expected to overexpress both in cancer cells and during apoptosis. But the protein mTOSO (Song and Jacob, 2005) is critical in inhibiting CASP8 activation. Since *CASP8* is downregulated in this investigation (Fig. 5), high expression of *TOSO* in L-menthol-treated cells (Fig. 3) may not have any anti-apoptotic effect, although influence on other proteins cannot be ruled out.

Several genes downregulated in this investigation were involved with the survival pathway. SLC25A4 functions as a gated pore translocating ADP from the mitochondrial matrix into the cytoplasm (Napoli et al., 2001), whose downregulation leads to mitochondrial dysfunction. BCL-2 proteins are shown to interact and regulated by BH3 domain-containing proteins such as BLK (Zong et al., 2001). Presenilins (PSEN1 and 2) are required for maintenance of neural stem cells in the developing brain (Kim and Shen, 2008). Although the protein requiem (REQ) is described to be a regulator of apoptosis, its role in signaling is unclear (Wong et al., 2006). Apoptosis-inducing factor (AIF) has dual functions, a pro-apoptotic activity in the nucleus and an anti-apoptotic activity through oxidoreductase activity (Klein et al., 2002; Lipton and Bossy-Wetzel, 2002).

TNFSF13 is a NF-kappaB-activating receptor for TALL1 (Shu and Johnson, 2000). The other pro-apoptotic or anti-proliferative protein genes downregulated were *DAPK3* (Kawai et al., 1998), *BMF* (Puthalakath et al., 2001), *RBL2* (Dick, 2007), *RBBP2* (Chicas et al., 2012), *BNIP3* (Burton et al., 2013), *APP* (Takahashi et al., 2009), *PIG11* (Wu et al., 2009), *RPL31* (Su et al., 2012), *ATM* (Kim et al., 1999), *TP73* (Wang et al., 2013), and *IL24* (Nagakawa et al., 2012) (Supplementary Table S2). Similarly, anti-apoptotic/cell proliferation genes downregulated are *CACNB4* (Tadmouri et al., 2012), *TNFRSF11B* (Oliver et al., 2013), *CAPN6* (Rho et al., 2008), *GRB2* (Kraskouskaya et al., 2013), *TRAF6* (Wong et al., 1999), *BZRP* (Maaser et al., 2002), *TNFRSF6B* (Chen et al., 2010), *RAF1* (McPhillips et al., 2006), *CYC1* (Zhu et al., 2012), and *HSPD1* (Ghosh et al., 2008) (Supplementary Table S2).

In the proposed model, CASP10 and HSP90 have major roles (Fig. 9). HSP90 modulates tumor cell apoptosis and its inhibition disrupts multiple pathways essential for survival, proliferation, and metastasis of transformed cells, making it a promising target for developing cancer chemotherapeutics. It acts as an anti-apoptotic factor *via* several mechanisms (Arya et al., 2007) and also forms a cytosolic complex with APAF1, inhibiting cytochrome c-mediated APAF1 oligomerization and pro-caspase-9 activation (Pandey et al., 2000). Besides, HSP90 interacts with BID, a pro-apoptotic member of the Bcl-2 family, and prevents TNF-α-induced BID cleavage, which is involved in cytochrome c release (Zhao and Wang, 2004).

By keeping pro-apoptotic factors inert, HSP90 regulates anti-apoptotic proteins. It also modulates the stability of receptor-interacting protein, which functions as an anti-apoptotic protein by regulating NF-kB activity (Lewis et al., 2000). HSP90 regulates TNF-induced activation of IkB kinase (IKK) and NF-kB by interacting with the kinase domains of IKK and by forming a hetero-complex, containing NF-kB essential modulator and co-chaperone protein CDC37 (Chen et al., 2002). This protein also directly interacts with and maintains the activity of AKT by inhibiting its dephosphorylation, and functions together with AKT to inhibit the activity of pro-apoptotic kinase ASK1 (apoptosis signal-

regulating kinase 1) (Basso et al., 2002; Sato et al., 2000; Zhang et al., 2005). Inhibition of CASP8/CASP10 activation inhibits HSP90 cleavage and largely inhibits UV-B-induced apoptosis (Chen et al., 2009).

Conversely, downregulation of HSP90 by expression of its shRNA, which would be expected to relieve its inhibition of pro-apoptotic proteins and the protection of anti-apoptotic proteins, promotes cell death in response to UV-B irradiation (Chen et al., 2009). In this investigation, the effect of L-menthol on downregulation of HSP90 has been shown both at transcript and protein levels (Fig. 8). Further, *AKT1* and *AKT2* were downregulated and expression of *AKT3* was marginally positive (Supplementary Table S2). *GRB2* and *RAF1*, involved in the AKT pathway, were severely downregulated, indicating inhibition of the pathway (Supplementary Table S2). Apoptosis can also be inhibited by the activation of pro-survival signaling pathways, such as insulin-like growth factor (IGF)-I and its HSP90-dependent receptor (Nielsen et al., 2004). Activation of this pathway increases the expression of pro-survival factors, like BCL-2 and BCL-xL, and decreases the expression of pro-apoptotic factors, like BIM (Kooijman, 2006).

Furthermore, IGF-I receptor activation induces the phosphorylation of BAD and phosphorylated BAD dissociates from Bcl-xL, liberating its anti-apoptotic activity (Nielsen et al., 2004; Xu and Neckers, 2007). The mannose-6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R) encodes a multifunctional protein involved in lysosomal enzyme trafficking, fetal organogenesis, tumor suppression, and T cell-mediated immunity (Wylie et al., 2003). Perillyl alcohol treatment increases M6P/IGF2R (Belanger, 1998). IGFBP2-induced gene expression is of functional significance for proliferation, cell adhesion, cell migration, and apoptosis, and IGFBP2 can promote apoptosis in tumor cells independent of IGF (Frommer et al., 2006). In this investigation increase in expression of *BAD*, *IGF2R*, *IGFBP2,* and decrease in the expression of *BCL2* was observed in L-menthol-treated cells, indicating the inhibition of this pro-survival pathway (Supplementary Table S2).

The expression of another gene *VEGF* (vascular endothelial growth factor) believed to be inducing HSP90 expression decreased (-2.71-fold) in L-menthol-treated cells (Supplementary Table S2). Upon VEGF stimulation, HSP90 binds to BCL2 and APAF1, an effect mediated through VEGFR2 and involving the activation of the MAP kinase pathway. These actions of VEGF result in increased resistance to apoptosis (Dias et al., 2002). Also, high levels of expression of *BID* (Fig. 6), the two *TNFSF*s (*TNFSF7*, *TNFSF9*) and the corresponding *TNFRSF*s (*TNFRSF7*, *TNFRSF9*) (Fig. 4) in L-menthol-treated cells indicated their association in apoptosis signaling. *FADD* demonstrated negative expression, whereas *TRADD* showed positive expression indicating the involvement of TRADD (Supplementary Table S2).

MADD (3.4-fold) has been shown to be associated with the death domain of the type 1 TNF receptor through its own C-terminal death domain (Schievella et al., 1997). Some of the variants of this gene IG20 are known to activate CASP3 and CASP8 (Al-Zoubi et al., 2001), indicating the possibility of its involvement in apoptosis. In L-menthol-treated cells, receptor-associated protein kinase genes *RIPK1*, *RIPK2*, and *RIPK3* were upregulated (Supplementary Table S2). RIPK1 is critical for CASP8 activation induced by SMAC mimetic (Wang et al., 2008), but in this experiment CASP8 function is being apparently taken care by CASP10. It may be possible that upregulation of this gene by L-menthol induction may be aiding activation of CASP10.

Whereas the upregulation of different apoptosis-related genes indicates programmed cell death, simultaneous upregulation of anti-apoptotic genes indicates the cell's tendency to maintain a delicate balance between apoptosis and survival. A similar argument can be made to explain the simultaneous downregulation of anti-apoptotic and pro-apoptotic genes. The competition between these genes determines the cellular fate depending on the family member and isoform expressed in the specific cell type. Further future studies need to be carried out for more direct implication of the two target candidates in the apoptotic pathway. Blocking caspase 10 in order to confirm that the apoptotic pathway goes through it, as well as the addition of HSP90 to inhibit apoptosis will be the further experiments needed to be carried out. Another futuristic dimension would be to carry out the experiment on more and varied human cancer cell lines towards establishing the role of L-menthol in inducing apoptosis. However, the data and literature analysis in the present study have significant implications for ''repurposing'' L-menthol beyond oral medicine, and in understanding the mode of action of plant-derived monoterpenes towards development of cheaper anticancer drugs in future.

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AK Sha conceived the study. SSD, MPD, and AK Sha designed the wet lab experiments. UF, SSD and SP performed the wet lab experiments. SG and AS performed the *in silico* experiments and analysis. UF, SSD, MPD and AK Sha analyzed the wet lab data. AK Shu and AK Sha compiled the analyzed data and wrote the manuscript. All authors read and approved the final manuscript.

Author Disclosure Statement

The authors declare no conflicting financial interests.

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Address correspondence to:
Ajit K. Shasany, PhD
Biotechnology Division
CSIR-Central Institute of Medicinal and Aromatic Plants
P.O. CIMAP
Lucknow 226015 U.P.
India

E-mail: ak.shasany@cimap.res.in