

(-)-Menthol Inhibits WEHI-3 Leukemia Cells *In Vitro* and *In Vivo*

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Abstract. (-)-Menthol ([1-*a*]-5-methyl-2-[1-methylethyl]-cyclohexanol), is a widely used flavoring ingredient in mouthwash, foods, toothpaste and cigarettes. The studies reported here revealed that (-)-menthol induced cytotoxicity against murine leukemia WEHI-3 cells *in vitro* in a dose-dependent manner. The effects of (-)-menthol on WEHI-3 cells *in vivo* (BALB/c mice) were also examined, and it was observed that the Mac-3 and CD11b markers were decreased, indicating inhibition of differentiation of the precursor of macrophage and granulocyte. The weights of liver and spleen samples from mice treated with (-)-menthol were found to be decreased compared to untreated animals.

(-)-Menthol is an aromatic compound used as a flavoring ingredient in a large number of products (1). (-)-Menthol inhibits the growth of rat liver epithelial tumor cells (2) and acts as a potent chemopreventive agent during DMBA initiation of rat mammary tumors (3). (-)-Menthol has been shown to be toxic in animals (4, 5), but in humans, it is considered to be safe, with a small cardio-accelerating effect (6). At high doses (-)-menthol may exert a depressant effect on the central nervous system in rodents (5, 7). It is possible that mentholated cigarette smoking increases the risk for lung cancer (8). It was also reported that the acute toxicity of (-)-menthol is low (5) and negative in a range of genotoxicity tests (9, 10), and as an inhibitor of P450 2A6-mediated coumarin 7-hydroxylase and the human liver microsomal oxidation of nicotine (11).

Our previous studies have demonstrated that (-)-menthol inhibits N-acetyltransferase activity (12). The distribution and metabolism of 2-aminofluorene in various tissues of Sprague-Dawley rats were affected by (-)-menthol after oral treatment (13). Recently, (-)-menthol was also found to inhibit the DNA topoisomerase I, II alpha and beta and to promote NF-kappaB expression in human gastric cancer SNU-5 cells (14). Additionally, (-)-menthol induced human promyelocytic leukemia HL-60 cell death through the Ca²⁺ release from the endoplasmic reticulum (15). In the present study the effects of (-)-menthol on leukemia cells *in vitro* and *in vivo* were examined.

Materials and Methods

Materials and reagents. RPMI 1640, fetal bovine serum, penicillin-streptomycin and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). (-)-Menthol and olive oil were obtained from Sigma (MO, USA).

Murine leukemia cell line (WEHI-3). The murine myelomonocytic leukemia cell line (WEHI-3) was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). The cells were grown into 75-cm³ tissue culture flasks and 37°C under a humidified 5% CO₂ atmosphere in RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin-streptomycin (10,000 U/ml penicillin and 10 mg/ml streptomycin) and 1% glutamine.

Male BALB/c mice. Male BALB/c mice, approximately 22-28 g, were obtained at the age of 8 weeks from the Laboratory Animal Center, National Taiwan University College of Medicine (Taipei, Taiwan), and were kept in the "Animal Center of China Medical University" for 2 weeks before the experiments.

In vitro studies

Assay for cytotoxic activity. Approximately 2x10⁵ cells (WEHI-3) were incubated in 12-well plates containing medium for 24 h without (control) or with various concentrations (0, 25, 50, 75 and 100 µM) of (-)-menthol. The cells were harvested and washed with PBS before PI staining. The percentage of viable WEHI-3 cells was

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determined by trypan blue exclusion and flow cytometry as described previously (16-19).

In vivo studies

(*-*)-Menthol treatment. Sixty BALB/c mice were divided into 5 groups. Groups I, II and III contained 10 animals, and Groups IV and V contained 15 each. Group I served as control, Group II was treated with PBS, Group III was injected with WEHI-3 cells as a positive control, Group IV was injected with WEHI-3 cells and treated with 1 mg/kg (*-*-menthol in olive oil, and Group V was injected with WEHI-3 cells and treated with 10 mg/kg (*-*-menthol in olive oil. The doses were administered to all animals orally and daily for up to 3 weeks. The animals were then weighed and sacrificed.

Blood samples and immunofluorescence staining. Blood was collected (about 1 mL) from all animals at the end of the experiments. These samples were treated with ammonium chloride to lyse the red blood cells, and then centrifuged at 1500 rpm at 4°C for 15 min. The isolated white blood cells were counted and examined for cell markers using flow cytometric analysis. The cells were stained with anti-Mac-3, CD11b, CD3, CD14 and CD19 antibodies (PharMingen), and then with the second fluorescent antibody before being analyzed to determine the cell marker levels using flow cytometry (FACS Calibur™, Becton Dickinson, NJ, USA) as described previously (18, 19).

Tissues samples (liver and spleen). Each animal was weighed before blood was sampled. The liver and spleen samples were obtained, weighed individually and used for histopathology (18).

Histopathology. Tissue samples (spleen and liver) of each animal fixed in 4% formaldehyde and embedded in paraffin. Sections of 5 mm were stained with hematoxylin and eosin according to standard procedures, as described previously (18).

Statistics. The results were expressed as mean±SD and the differences between groups were analyzed by one-way ANOVA. **P*<0.05 was considered significant.

Results

(*-*)-Menthol-induced cytotoxicity in WEHI-3 cells. The percentage of viable WEHI-3 cells treated with or without (*-*-menthol was determined by trypan blue exclusion and flow cytometric analysis, and the results are presented in Figure 1. The data indicate that (*-*-menthol decreased the percentage of viable cells in a dose-dependent manner (Figure 1).

The effects of (*-*-menthol on the whole blood cell surface markers in mice injected WEHI-3 cells. The data from flow cytometric analysis, indicating cell markers of white blood cells from BALB/c mice after treatment with or without (*-*-menthol, are presented in Figure 2. Both doses of (*-*-menthol induced a significant difference in Mac-3, CD11b and CD19 compared to the WEHI-3-only treated groups (*p*<0.05). However, neither dose of (*-*-menthol significantly affected the levels of CD3 compared to controls (*p*<0.05).

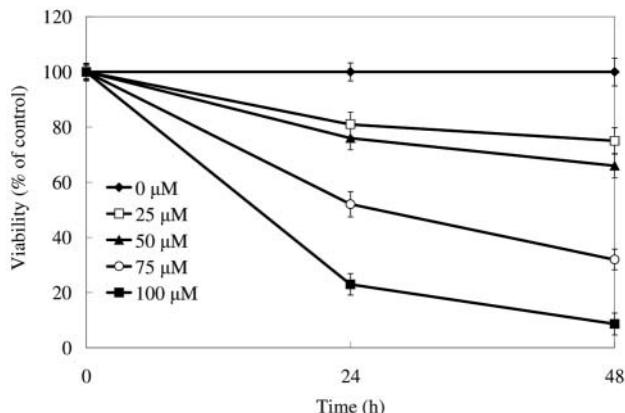


Figure 1. Viability of WEHI-3 cells after (*-*-menthol treatment for 24 h. WEHI-3 cells (2×10^5 cells/well; 12-well plates) were plated in RPMI 1640 medium + 10% FBS with various concentrations of (*-*-menthol for 24 h. The cells were then collected by centrifugation and the viable cells were determined by trypan blue exclusion and flow cytometry as described in the Materials and Methods. Each point is the mean±S.D. of three experiments. **p*<0.05.

The effects of (*-*-menthol on the weight and histopathology of liver. Liver tissues were isolated from animals of each group, photographed, weighed and histopathologically examined. Representative results are shown in Figure 3. (*-*-Menthol affected the morphology and the weight of the liver. Furthermore, based on histopathological examination, the liver demonstrated a pattern ranging from minimal histopathological change to scanty small neoplastic cell nests present in the sinusoid with apoptosis.

The effects of (*-*-menthol on the weight and histopathology of spleen. Spleen tissues were isolated from animals of each group, photographed, weighed and histopathologically examined. Representative results are presented in Figure 4. (*-*-Menthol affected the morphology and the weight of the spleen. The results also indicated that spleen tissues either displayed markedly decreased numbers of neoplastic cells or the cells were hard to find in the red pulp. The number of megakaryocytes also increased, based on the results of the histopathological examination.

Discussion

The *in vivo* model of mice injected *i.p.* with WEHI-3 has been well established (20). Murine host systems are often used for experimental tumor therapy due to the low cost, the ease with which cancer production is established and to the widely accepted experimental end-points (21, 22). Finally, murine monomyelocytic WEHI-3 leukemia cells were originally derived from the BALB/c mouse (22) and served as an ideal system for the study of potential therapeutic

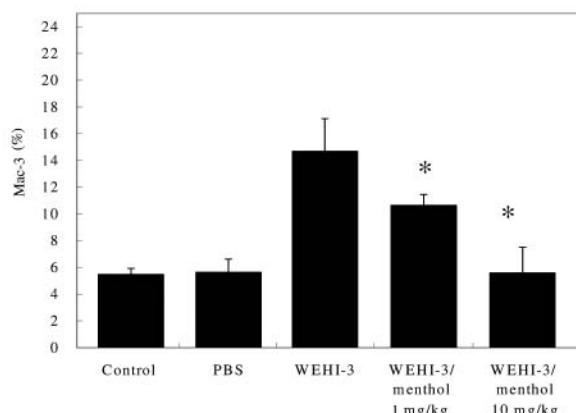
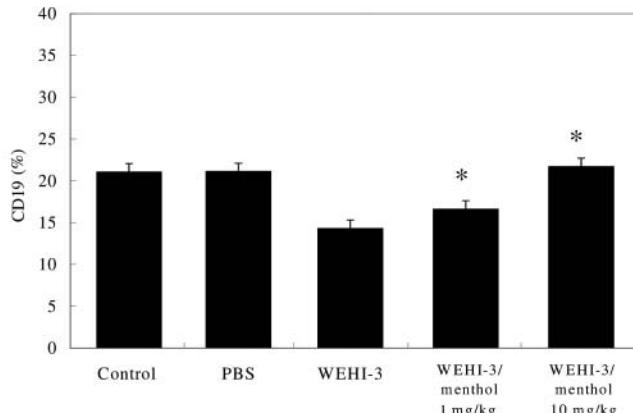
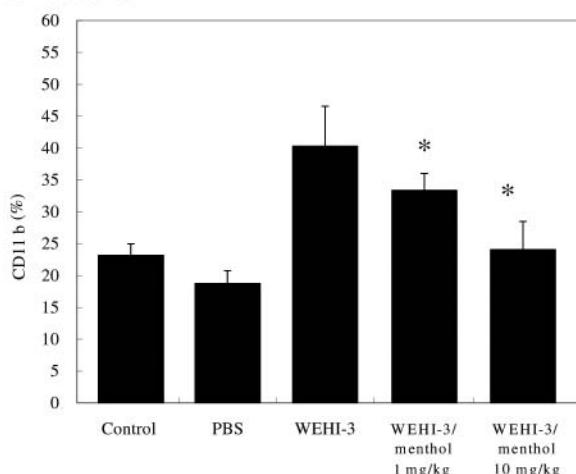
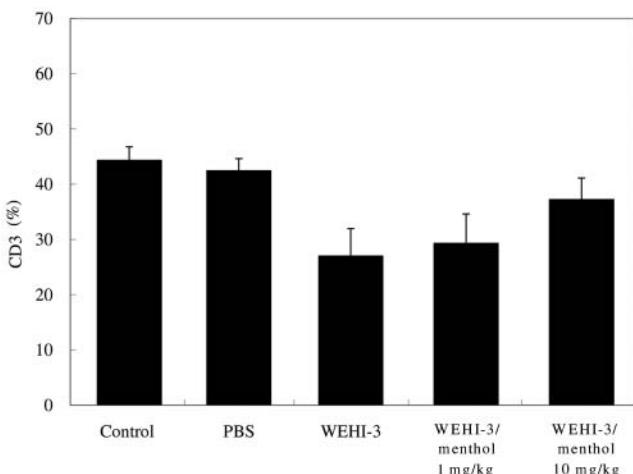
A Mac-3**C CD19****B CD11b****D CD3**

Figure 2. Cell marker levels as of white blood cells from BALB/c mice after treatment with (-)-menthol. The animals were injected with WEHI-3 cells (1×10^6 cells/100 μ L) in PBS for 3 weeks and were then treated with or without (-)-menthol for 3 weeks. Blood was collected and analyzed for cell marker (A: Mac-3; B: CD11b; C: CD19; D: CD3) by flow cytometry as described in the Materials and Methods. Each point is the mean \pm S.D. of three experiments. * $p < 0.05$.

drugs, such as ATRA, aclacinomycin A, IL-6, G-CSF and vitamin D₃, which could induce *in vitro* differentiation of WEHI-3 in monocytic and granulocytic lineages (23-27).

(-)-Menthol was found to induce cell death of HL-60 cells (18), raising the possibility that (-)-menthol could affect leukemia cells *in vivo*. In the present study, the cytotoxicity of (-)-menthol in WEHI-3 cells was examined. It was found that the viability of WEHI-3 decreased after treatment with (-)-menthol in a dose-dependent manner.

The effects of (-)-menthol *in vivo* on WEHI-3 tumor cells in BALB/c mice were also examined. The results demonstrated that (-)-menthol statistically decreased the weights of the liver and spleen in the examined animals and also decreased the percentage of MAC-3 and CD11b cells in the blood.

(-)-Menthol inhibited leukemia-related spleen growth. A notable characteristic of this model is the elevation of peripheral monocytes and granulocytes with immature morphology, as well as enlarged and infiltrated spleens compared to normal counterpart. Our experiments also indicated that spleen and liver sizes decreased in the (-)-menthol-treated groups. The mechanism of action of (-)-menthol should be the subject of further investigation.

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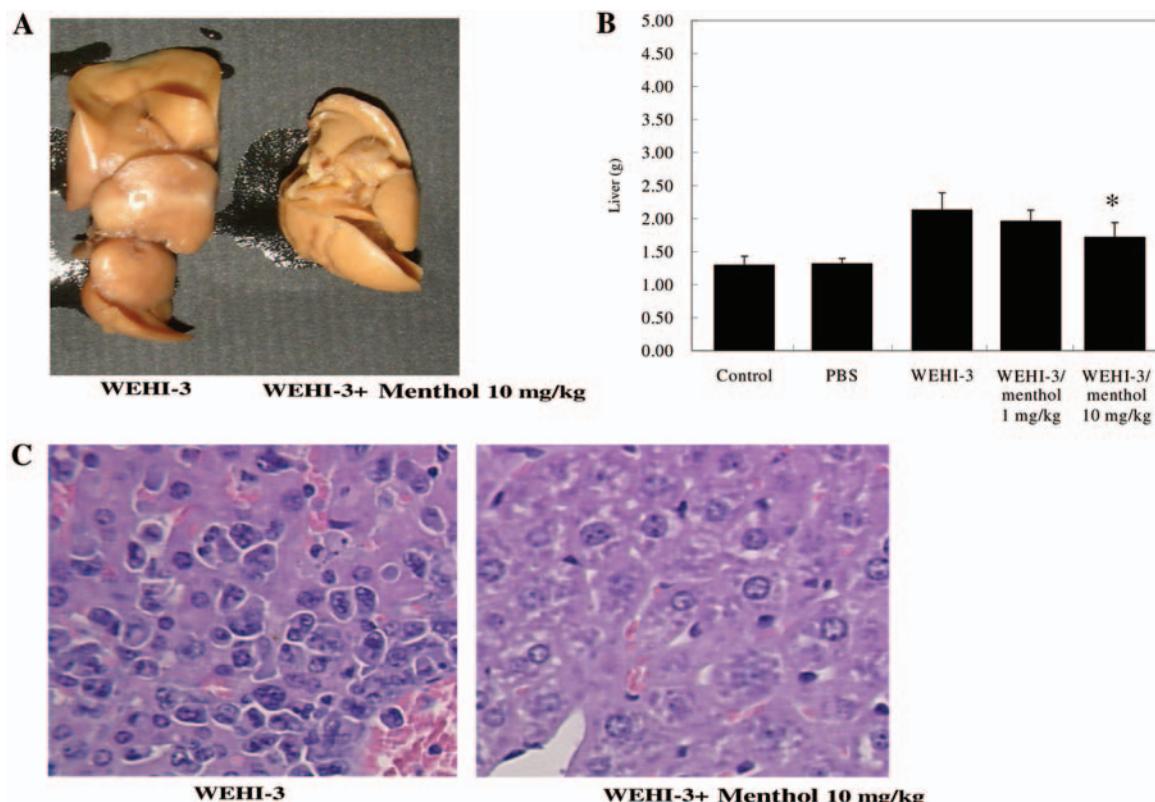


Figure 3. The effects of (-)-menthol on the morphology, weight and histopathology of the liver. Livers from each animal of each group were excised, photographed (A), weighed (B) and histopathologically examined (C) as described in Materials and Methods.

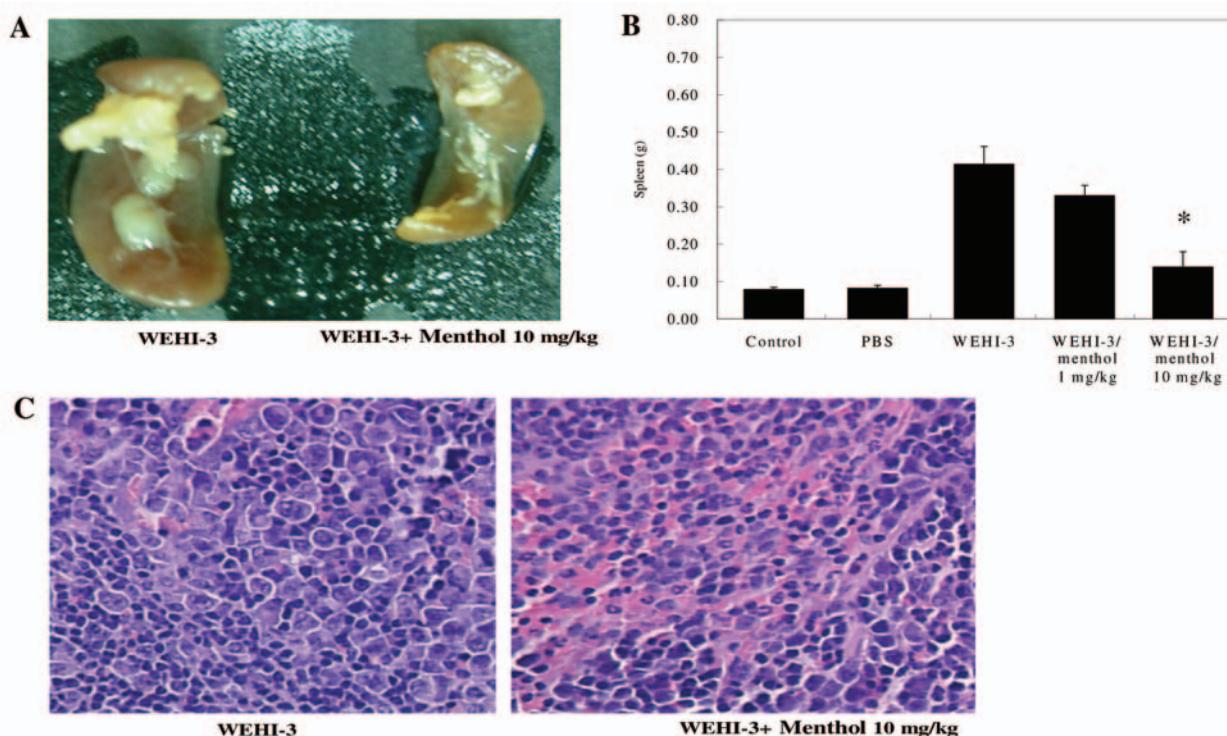


Figure 4. The effects of (-)-menthol on the morphology, weight and histopathology of the spleen. Spleens from each animal of each group were excised, photographed (A), weighed (B) and histopathologically examined (C) as described in Materials and Methods.

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