Mastic Oil from *Pistacia lentiscus var. chia* Inhibits Growth and Survival of Human K562 Leukemia Cells and Attenuates Angiogenesis

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**Abstract:** Mastic oil from *Pistacia lentiscus var. chia*, a natural plant extract traditionally used as a food additive, has been extensively studied for its antimicrobial activity attributed to the combination of its bioactive components. One of them, perillyl alcohol (POH), displays tumor chemopreventive, chemotherapeutic, and antiangiogenic properties. We investigated whether mastic oil would also suppress tumor cell growth and angiogenesis. We observed that mastic oil concentration and time dependently exerted an antiproliferative and proapoptotic effect on K562 human leukemia cells and inhibited the release of vascular endothelial growth factor (VEGF) from K562 and B16 mouse melanoma cells. Moreover, mastic oil caused a concentration-dependent inhibition of endothelial cell (EC) proliferation without affecting cell survival and a significant decrease of microvessel formation both in vitro and in vivo. Investigation of underlying mechanism(s) demonstrated that mastic oil reduced 1) in K562 cells the activation of extracellular signal–regulated kinases 1/2 (Erk1/2) known to control leukemia cell proliferation, survival, and VEGF secretion and 2) in EC the activation of RhoA, an essential regulator of neovessel organization. Overall, our results underscore that mastic oil, through its multiple effects on malignant cells and ECs, may be a useful natural dietary supplement for cancer prevention.

**Introduction**

Substantial evidence from epidemiological and laboratory studies supports that regular consumption of dietary phytochemicals is strongly associated with reduced risk of cancer (1–3). The essential oil and gum from the plant *Pistacia lentiscus (L.)* var. *chia* have been widely used as food and beverage flavoring additives and traditional medicines in the Mediterranean region since ancient times without any reported toxicity in humans. Numerous recent studies have demonstrated that they possess antimicrobial properties against a broad range of bacterial and fungal pathogens (4–7) and antioxidant activity as well (8). Lately, a hexane extract of mastic gum was demonstrated to induce apoptosis in HCT116 human colon cancer cells (9). Analysis of the chemical composition of mastic oil revealed that it is a complex mixture of volatile compounds, mainly terpenes (5,10), with established beneficial biological properties (11). The monoterpene perillyl alcohol (POH), which corresponds to 0.84% of mastic oil, is of great clinical interest due to its established chemopreventive and chemotherapeutic potential demonstrated in a variety of rodent tumor models (12,13), and it has currently advanced to phase I–II clinical trials (14,15). Recently, we have shown that POH, besides its antitumor effect, acts as an angiogenesis inhibitor (16).

Aberrant angiogenesis plays an important role in the progression not only of solid tumors but of hematological malignancies as well (17,18). In fact, increased angiogenesis in bone marrow is one of the characteristics of various types of leukemia (19). Vascular endothelial growth factors (VEGFs) essentially coordinate the angiogenic process by activating several signal pathways upon binding to the tyrosine kinase VEGF receptors (VEGFRs), largely restricted to the vascular endothelium (20,21). It has been recently shown that certain leukemia cells not only produce VEGF but also express functional VEGFRs resulting in an autocrine and paracrine loop for tumor growth and propagation (22,23). Therefore, the use of VEGF and VEGFR inhibitors for the treatment of leukemias seems particularly attractive because it may target not only the vascular environment but also the malignant cells per se.

It is currently widely accepted that health benefits associated with phytochemicals present in complex mixtures of nutrients are attributed to their additive and/or synergistic interactions rather than to the effect of single constituents (24).
This has also been indicated for mastic oil in a recent investigation correlating the antimicrobial activity with individual components or the mastic oil itself (25). The results from this study support that the observed activity of mastic oil was due to the combination of several compounds, including some of the trace elements, working synergistically. Based on these observations, we sought out to determine whether mastic oil would exhibit tumor and angiogenesis preventive properties. POH was also examined in parallel experiments as a reference compound. We demonstrated that mastic oil was able to inhibit cell proliferation and survival of human leukemia K562 cells and decrease VEGF release from K562 and mouse melanoma B16 cells. In addition, mastic oil attenuated endothelial cell (EC) proliferation and neovessel formation both in vitro on Matrigel and in vivo on the chicken embryo chorioallantoic membrane (CAM). Finally, mastic oil reduced the activation levels of extracellular signal–regulated kinases 1/2 (Erk1/2) in K562 cells and of RhoA small GTPase in ECs, thus modulating important signaling pathways in target cells.

Materials and Methods

Materials

Mastic oil was kindly donated by the Chios Mastic Growers Association. POH was from Fluka Chemie AG (Buchs, Switzerland). Microvascular ECs (MVECs) were obtained from Vec Technologies (Rensselaer, NY). K562 and B16 cell lines were originally obtained from the American Type Culture Collection (Manassas, VA). Tissue culture plastic ware was from Corning-Costar (Corning, NY). Cell culture media and supplements were obtained from Life Technologies (Paisley, UK). A VEGF enzyme-linked immunosorbent assay (ELISA) Duo set kit was provided by R&D Systems (Minneapolis, MN). The CyQUANT and EnzChek Caspase-3 Assay kits were purchased from Molecular Probes (Eugene, OR). Antibodies against Erk1/2 and RhoA were from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnology, respectively. GST-Rhotekin RBD agarose was purchased from Upstate (Lake Placid, NY). All other materials were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

K562 and B16 cells were maintained in RPMI-1640 and MVECs in Dulbecco’s Modified Eagle’s medium. Media were supplemented with 10% FCS, L-glutamine, and antibiotics (10 U/ml penicillin and 100 µg/ml streptomycin). Human umbilical vein ECs (HUVECs) were isolated as previously described (16) and cultured in M199 supplemented with 20% FCS, 200 µg/ml EC growth supplement, 5 µ/ml heparin sodium, L-glutamine, and antibiotics (as previously described). Cells were incubated in a humidified 37°C incubator containing 5% CO₂.

Cell Proliferation and Apoptosis Assays

For cell proliferation assays, endothelial (MVEC or HUVEC) or K562 cells were plated at 4 × 10³ or 10⁴ cells per well, respectively, in a 96-well plate. Twenty-four hours later, proliferating cells were treated with mastic oil (0.01–0.02% vol/vol), POH (0.5 mM), or dimethyl sulfoxide (DMSO) vehicle (0.1%); after 24–72 h, cell numbers were measured as described previously (16) using the CyQUANT Cell Proliferation assay kit. Briefly, cells were washed with phosphate-buffered saline, and then 200 µl of CyQUANT GR dye in cell lysis buffer was added to each well. This dye exhibits strong fluorescence enhancement when bound to nucleic acids. After 5 min of incubation at room temperature under light protection, the sample fluorescence was measured. A reference standard curve with ECs or K562 cells in the linear detection range (50–50,000 cells) was created for converting sample fluorescence to cell number.

Cell apoptosis was monitored by measuring caspase-3 activity as previously described (16). Briefly, 1 × 10⁶ K562 cells per well in 24-well plates or confluent ECs in 12-well plates were incubated with fresh media containing mastic oil (0.01–0.1% vol/vol), POH (1 mM), or DMSO vehicle (0.1%), or 10 µM cyclohexamide. After 24–72 h, the cells were collected and processed according to EnzChek Caspase-3 Assay kit #1 instructions. Results were normalized as relative fluorescence units per milligram of protein.

ELISA for VEGF Detection

K562 (1×10⁶) or B16 cells grown to confluence in 24-well plates were treated with fresh media containing mastic oil (0.01–0.1% vol/vol), POH (1 mM), or DMSO vehicle (0.1%) for 24–48 h. Conditioned media were then collected for ELISA analysis, whereas cells were solubilized and total protein was measured. VEGF ELISA was performed according to the manufacturer’s specifications. Results were normalized as picograms per milligram of protein.

Angiogenesis Assays

The Matrigel tube formation assay was performed as described in Ref. 26. Briefly, HUVECs (10⁴ cells per milliliter) were added onto Matrigel-coated wells in 96-well plates in the presence of mastic oil (0.01–0.04% vol/vol) or vehicle (0.1% DMSO) and incubated for 6 h. The cells were then fixed, and the length of tube-like structures was measured in the total area of the wells using Scion Image software (Scion Corp., Frederick, MD) as previously described (27).

The in vivo chicken embryo CAM angiogenesis model was used as previously described (27). In brief, Leghorn fertilized eggs were incubated for 4 days at 37°C, when a window was opened on the egg’s shell, exposing the CAM. The window was covered with tape, and the eggs were returned to the incubator. Different amounts of mastic oil (1 × 10⁻⁴ to 1×10⁻⁶) or vehicle (0.1% DMSO) were applied onto an area of 1 cm² (restricted by a plastic ring) of the CAM on Day 9 of embryo development. Forty-eight hours later, CAM was fixed in situ and
excised from the eggs, and pictures were taken through a stereo­scope equipped with a digital camera. The total length of the vessels was measured using the Scion Image software.

**Western Blotting**

K562 cells grown in 24-well plates (1 × 10^6) were treated with fresh media containing mastic oil (0.01–0.04% vol/vol) or vehicle (0.1% DMSO) for 5–30 min and then subjected to cell lysis and Western blotting. Briefly, cells were collected and solubilized with lysis buffer (50 mM Tris-HCl, pH 7.6; 150 mM NaCl; 50 mM NaF; 1 mM Na₃VO₄; 0.5% sodium deoxycholate; 1 mM ethylendiaminetetraacetic acid (EDTA); 0.1 mM ethylene glycol-bis(2-amionoethyl)ether)-NN,N,N',N' tetraacetic acid (EGTA); 1% Triton-X; 1% sodium dodecyl sulfate (SDS); 1 mM phenylmethanesulfonyl fluoride (PMSF); 10 µg/ml aprotinin; 5 µg/ml leupeptin; and 10 ng/ml pepstatin). Lysates containing equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on 12% gels, transferred to nitrocellulose membranes, and probed with primary antibodies that specifically recognize the phosphorylated or total form of Erk1/2. Immunoreactive bands were visualized by enhanced chemiluminescence detection.

**Determination of Active RhoA Levels**

Serum-starved confluent HUVECs in 10-cm dishes were stimulated by adding 10% FCS containing mastic oil (0.01–0.04% vol/vol), POH (0.5 mM), or vehicle (0.1% DMSO) for 2 min. RhoA activation was then determined by a pull-down assay using GST-Rhotekin RBD agarose beads essentially as described in Ref. 28. In brief, cells were solubilized with lysis buffer (25 mM HEPES, pH 7.5; 150 mM NaCl; 1% vol/vol Igepal CA-630; 10 mM MgCl₂; 1 mM EDTA; 10 % glycerol; 10 µg/ml leupeptin; 10 µg/ml aprotinin; 1 mM PMSF; and 1 mM sodium orthovanadate), and cell lysates containing equal amounts of protein were incubated with 30 µg GST-RBD agarose beads for 45 min at 4°C. Active RhoA bound to the beads was then eluted and analyzed in parallel with aliquots containing 20 µg protein of total cell lysates by 12% SDS-PAGE and Western blotting using anti-RhoA antibody.

**Data Analysis and Statistics**

Data are presented as mean ± SE of the indicated number of observations. Statistical comparisons between control and other groups were performed using the Mann-Whitney nonparametric test. Differences among means were considered significant when \( P < 0.05 \) from control.

**Results**

**Effects of Mastic Oil on Tumor Cells**

Mastic oil inhibits K562 cell growth and survival: To estimate the effect of the examined compounds on cell proliferation, cultures of dividing cells were treated for various time periods, and thereafter the cell number was measured. Mastic oil induced a concentration-dependent decrease in cell number compared with control treatment, indicating an inhibition in the proliferation of K562 cells. Following a 48-h incubation with 0.01 and 0.02% vol/vol mastic oil, the cell number was reduced to 91.9% and 68.5% of control samples, respectively (Fig 1A).

Moreover, treatment of cells with mastic oil (0.01–0.1% vol/vol) caused a concentration-dependent stimulation of caspase-3 activity in K562 cells after 72-h of incubation (Fig. 1B). It is interesting to note that the apoptotic effect of mastic oil and POH followed a similar time-response pattern while cyclohexamide (used as a positive control) caused an earlier induction of caspase-3 activity at 24–48 h of incubation (not shown).

Mastic oil down-regulates VEGF release from tumor cells: VEGF is produced by most tumor cells and appears to be one of the main mediators relevant to the proangiogenic tumor phenotype. Treatment of K562 cells with mastic oil (0.01–0.1% vol/vol) for 24–48 h caused a concentration- and time-dependent reduction of the secreted VEGF (Fig. 2A). A similar inhibition of VEGF protein production was also observed in a B16 mouse melanoma cell line in response to increasing concentration of mastic oil (Fig. 2B).

![Figure 1.](image-url) Mastic oil inhibits K562 (A) cell proliferation and (B) survival. (A) In a 96-well plate, 10⁴ cells per well were plated and 24 h later were treated with mastic oil (0.01–0.02% vol/vol), perillyl alcohol (POH, 0.5 mM), or vehicle. Cell numbers were measured after 48 h. (B) Cells (1 × 10⁶) grown in 24-well plates were treated with mastic oil (0.01–0.1% vol/vol), POH (1 mM), or vehicle and analyzed after 48–72 h for caspase-3 activity. Results are expressed as mean ± SE; \( n = 9–12 \); \(* P < 0.05 \) from vehicle.
Effect of Mastic Oil on ECs

**Mastic oil inhibits EC proliferation:** EC proliferation is a key component of normal and tumor-associated angiogenesis. As shown in Fig. 3, mastic oil efficiently reduced the number of growing MVECs and HUVECs in a concentration-dependent manner (0.01–0.02% vol/vol), indicating inhibition of cell proliferation. EC treatment with mastic oil (0.01–0.04% vol/vol) did not induce any significant change in caspase-3 activity, suggesting that mastic oil does not affect EC survival. Representative values (% of vehicle) from HUVEC incubated with 0.04% vol/vol mastic oil for 24, 48, and 72 h were 104.4 ± 3.7, 101.0 ± 8.5, and 96.4 ± 2.5, respectively.

**Mastic oil attenuates capillary-like organization of EC on Matrigel:** The effect of mastic oil on the morphological differentiation of ECs was studied in the Matrigel assay. As shown in Fig. 4A, vehicle-treated HUVECs grown on Matrigel developed tube-like networks. However, mastic oil inhibited the organization of the cells in structures mimicking vascular networks. Visual observations were confirmed by morphometric analysis (Fig. 4B) showing that mastic oil (0.01–0.04% vol/vol) induced a concentration-dependent reduction of the total length of the tube-like structures.

**Mastic oil inhibits neovascularization in the CAM:** The effect of mastic oil on the in vivo formation of new vessels was evaluated using the CAM system of angiogenesis. As demonstrated by morphometric analysis, mastic oil induced in the examined concentrations a small but statistically significant reduction in the number of CAM vessels under basal conditions (Fig. 5).

**Molecular Targets for Mastic Oil in Leukemia and Endothelial Cells**

To elucidate the signaling mechanism(s) through which mastic oil exerted its effects on leukemia cells, we examined by Western blotting the levels of phosphorylated Erk1/2 relative to total Erk1/2 following treatment of K562 cells with different concentrations of mastic oil (0.01–0.1% vol/vol) and for various incubation times (5–30 min). Mastic oil caused a concentration-dependent inhibition of Erk1/2 phosphorylation without affecting total levels of Erk1/2. This inhibition was reversible and reached a maximum within the first 10 min of incubation. It should be noted that K562 cells express higher levels of Erk1; a minor band for Erk2 could only be detected with the phosphospecific antibody.

To explore the signaling pathways that are affected by mastic oil in ECs, we induced serum-starved HUVECs with complete medium containing mastic oil (0.01–0.04% vol/vol) and then determined the levels of activated RhoA relative to total levels of RhoA. As shown in Fig. 7, mastic oil concentration dependently inhibited RhoA activation without changing the total levels of RhoA. POH exhibited a similar effect (not shown).

**Discussion**

The search for new effective cancer chemopreventive and chemotherapeutic agents, especially for those that have a nat-
ural origin and relatively low toxicity, has recently increased and become an important issue in public health–associated research policy (29). In the present study, we demonstrated for the first time the pleiotropic effects of mastic oil, a natural mixture of phytochemicals, on tumor cell growth and angiogenesis, and we investigated its potential molecular targets in tumor and ECs. POH, one of mastic oil’s bioactive components, was also included in our study because it pos-

Figure 4. Mastic oil attenuates endothelial cell (EC) differentiation on Matrigel. Human umbilical vein ECs (10^5 cells per milliliter) were added into Matrigel-coated wells in 96-well plates in the presence of mastic oil (0.01–0.04% vol/vol) or vehicle and incubated for 6 h. (A) Representative photomicrographs showing the formation of tube-like structures on Matrigel after vehicle or mastic oil (0.01% vol/vol) treatment. (B) The length of the tube network was measured in the total well area. Results are expressed as mean ± SE; n = 9; *P < 0.05 from vehicle.

Figure 5. Mastic oil inhibits angiogenesis on the chorioallantoic membrane (CAM). Mastic oil (1 × 10^-4) or vehicle was applied onto 1 cm^2 of the CAM on Day 9 and incubated for 48 h at 37°C. CAM was fixed and excised from the eggs, and the total length of the vessel network was measured using image analysis software. Results are expressed as mean ± SE; n ≥ 24; ***P < 0.001 from vehicle.

Figure 6. Mastic oil inhibits extracellular signal–regulated kinase 1/2 (Erk1/2) phosphorylation in K562 cells. Cells were grown in a 24-well plate at 1 × 10^6 per well. (A) A concentration-dependent effect was assessed after cell treatment with mastic oil (0.01–0.04% vol/vol) or vehicle (CTL) for 10 min and analysis of lysates by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting using antibodies specific for the phosphorylated (p-Erk1/2) or total Erk1/2. The p-Erk1/2/total Erk1/2 ratios for mastic oil–treated cells were 85.2 ± 2.2%, 72.3 ± 10.3%, and 60.4 ± 4.6% of the corresponding ratios of vehicle-treated cells (control) after incubation with 0.01, 0.02, and 0.04% mastic oil, respectively. (B) Time course effect was determined after treatment with 0.04% vol/vol mastic oil (MO) or vehicle (C) for the indicated time (min) and analysis as in (A). The p-Erk1/2/total Erk1/2 ratios for mastic oil–treated cells were 43.6 ± 1.9%, 24.1 ± 4.6%, 73.0 ± 4.1%, and 127.2 ± 29.7% of the corresponding ratios of vehicle-treated cells (control) after incubation for 5, 10, 15, and 30 min, respectively. Blots are representative of three independent experiments.
油处理的细胞分别为92.6 ± 6.0%, 80.4 ± 8.3%, 和38.3 ± 3.5%。

在Western印迹中，我们分析了总细胞裂解物中VEGF的表达水平。因此，VEGF通过刺激功能性VEGFRs促进基质金属蛋白酶的活化和细胞迁移(22,23)。K562细胞已被证明可产生VEGF，这是autocrine和paracrine机制，用于白血病细胞的生长(31)。VEGF的表达几乎不变，是由所有已建立的白血病细胞系以及白血病细胞和ECs相互影响的。

在ECs中，我们研究了mastic油对VEGF表达的影响。有趣的是，mastic油的活性可能在肿瘤细胞的信号转导中起着重要作用，包括对包括ECs在内的不同细胞类型。

Figure 7. Mastic oil inhibits RhoA activation in endothelial cells (ECs). Starved human umbilical vein ECs were serum stimulated in the presence of mastic oil (0.01–0.04% vol/vol) or vehicle (CTL) for 2 min. Cell lysates were incubated with GST-RBD beads, and eluted active RhoA samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting, in parallel with aliquots of total cell lysates, using anti-RhoA antibody. The active RhoA/total RhoA ratios for mastic oil–treated cells were 92.6 ± 6.0%, 80.4 ± 8.3%, and 38.3 ± 3.5% of the corresponding ratios of vehicle-treated cells (control) after incubation with 0.01, 0.02, and 0.04% mastic oil, respectively. Blots are representative of three independent experiments.

油具有抗肿瘤作用，被评估在人类慢性髓性白血病上。在类风湿性关节炎中，mastic油被用作化疗的抗肿瘤药物。此外，mastic油可能通过抑制肿瘤微环境中的血管生成来抑制肿瘤生长。

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As mentioned previously, POH was used in the present study as a reference compound. Comparison of the concentrations at which mastic oil and POH are effective (as determined herein and in Ref. 16) as well as consideration of the POH content of mastic oil (approximately 1%) suggest that mastic oil is more potent than POH in inhibiting tumor growth and angiogenesis. We speculate that this can be attributed to the additive and/or synergistic action of its bioactive constituents, as has been already shown for the chemopreventive effect of other mixtures of phytochemicals (24) and for the antimicrobial activity of mastic oil (25).

In conclusion, our results suggest that mastic oil is a tumor and angiogenesis inhibitor targeting components of critical signaling cascades in both leukemia and ECs. If present findings are extended to other malignancies and to in vivo tumor models, they can form the basis for establishing mastic oil as a naturally occurring chemopreventive and antiangiogenic agent.

Acknowledgements and Notes

This work was supported in part by a grant from the Greek General Secretariat of Research and Technology and The Chios Gum Mastic Growers Association and funds from the Thorax Foundation. Address correspondence to H. Loutrari, G.P. Livanos and M. Simou Laboratories, Evangelismos Hospital, Department of Critical Care and Pulmonary Services, Medical School, University of Athens, 3 Ploutarchou Street, Athens, Greece 10675. Phone: +30−210−7235521. FAX: +30−210−7239127. E-mail: elloutrar@med.uoa.gr.

Submitted 25 January 2006; accepted in final form 3 April 2006.

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