

A Mastic Gum Extract Induces Suppression of Growth of Human Colorectal Tumor Xenografts in Immunodeficient Mice

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Abstract. *Background:* We recently reported that ethanol and hexane extracts of the plant product, mastic gum (MG), contain constituents which can induce p53- and p21-independent G₁-phase arrest followed by apoptosis of human HCT116 colon cancer cells in vitro. Herein, we extended these studies to investigate the in vivo anticancer activity of the hexane extract of MG (He-MG) against human colon tumor. The in vivo anticancer activity of He-MG was assessed in a human colon cancer/immunodeficient mouse model. *Materials and Methods:* Control and HCT116 tumor bearing SCID mice were injected intraperitoneally with He-MG at different administration schedules and doses ranging from 100 to 220 mg/kg body weight and tumor growth (size) was monitored. *Results:* He-MG administered at a dose of 200 mg/kg administered daily for 4 consecutive days (followed by 3 days without treatment) inhibited tumor growth by approximately 35% in the absence of toxicity (side-effects) after 35 days. *Conclusion:* He-MG was found to possess antitumor activity against human colorectal cancer under the experimental conditions of this study. The extent of suppression and toxicity by a specific He-MG dose depends on the schedule of administration.

Progression of colorectal cancer from benign colorectal adenoma to malignant carcinoma requires a period of time as a result of the accumulation of variety molecular alterations (1-4). Therefore, it is highly desirable to identify and/or develop agents that can inhibit or suppress cancer progression. In this context, several plant products have exhibited chemopreventative activity against colorectal

cancer in vitro and in vivo, including buckwheat protein (5), resveratrol analogs (6, 7), linoleic acid conjugates (8), curcumin (9), green tea (10) and grape seed (11) extracts, and juice or freeze-dried powder from Brussels sprouts (12), by targeting various molecular and cellular mechanisms.

The plant *Pistacia lentiscus* L. var. *chia* grows exclusively on the island of Chios, Greece, and produces a resin known as mastic gum (hereafter termed MG), which has been used for a variety of gastric ailments in the Mediterranean and Middle East countries for at least 3,000 years. MG has been rediscovered for its antimicrobial effects, particularly with regard to its positive effects on the gastrointestinal environment as well as against strains of *Helicobacter pylori*, *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Porphyromonas gingivalis* (13-17). The chemical composition of the resins extracted from the insect galls on *Pistacia lentiscus* and the mastic oil has been analyzed, and a few constituents have been isolated and identified in various fractions (15, 17-19).

We recently showed that treatment of HCT116 cells with a hexane extract of MG (He-MG) can induce arrest at the G₁-phase of the cell cycle followed by detachment of the cells from the substrate and subsequent apoptosis as assessed by flow cytometry analysis and monitoring of the presence of activated caspases-3, -8, -9 and PARP degradation (20). Moreover, the pan-caspase inhibitor Z-VAD-fmk did not prevent cell detachment, but it did prevent apoptosis of the attached cells, indicating that the process of cell detachment, but not apoptosis, is independent of caspase activation in He-MG-treated HCT116 cells (20). Utilizing isogenic HCT116 cell clones, which are either incapable of activating caspase-8 (HCT116/DN.FADD cells), the hallmark event of death receptor-dependent apoptosis, or inhibiting release of cytochrome *c* and subsequent activation of caspase-9 (HCT116/Bcl-2 cells), the hallmark event of mitochondrion-dependent apoptosis (21-24), we investigated which classical apoptotic mechanism is activated in He-MG-treated cells. Our studies indicated that He-MG induced apoptosis not

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only by activation of caspases -8 and -9, but also by other mechanisms that remain to be identified. This hypothesis is further supported by findings that MG components can induce p53- and p21-independent G1-arrest and subsequent apoptosis of HCT116 cells (25). Furthermore, it was recently demonstrated that MG constituents can inhibit the expression of the androgen receptor at the transcriptional (*i.e.* mRNA) and translational (*i.e.* protein) levels in an *in vitro* model using the androgen-responsive prostate cancer cell line, LNCaP (26).

In this report, we present experimental findings to demonstrate that He-MG is capable of significantly suppressing growth of HCT116 tumors xenografted in immunodeficient SCID mice. To our knowledge, this is the first report to demonstrate that MG possesses anticancer activity *in vivo*.

Materials and Methods

Materials and reagents. Dry resin of MG was obtained from the Chios Gum Mastic Growers Association (Athens, Greece). The hexane extract of MG, He-MG, was prepared as described elsewhere (20). Dimethylsulfoxide (DMSO), Tween-20, -40, -80, and normal saline were purchased from Sigma-Aldrich Hellas (Athens, Greece). For intraperitoneal (*i.p.*) administration, solubilization of the highly viscous He-MG in various cocktails was attempted as described elsewhere (27). Complete solubilization of He-MG was accomplished by heating in a water-bath at 45-50°C for 1 h in a vehicle containing 5% Tween-40/1% DMSO/94% normal saline. Fresh He-MG in vehicle was always prepared immediately prior to administration. The final preparation of He-MG in vehicle was tested for toxicity in naïve mice. Cell culture medium, RPMI, bovine serum, trypsin and antibiotics were purchased from Sigma-Aldrich. Disposable plasticware for cell culturing and experimentation was from NUNC (Wiesbaden, Germany).

Cell lines. Human colon carcinoma HCT116 cells, obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) were previously shown to be susceptible to apoptosis induced by methanol and hexane extracts of MG *in vitro* (20, 25). The cells were propagated in RPMI medium containing 5% fetal calf serum and antibiotics (penicillin, streptomycin) in 5% CO₂ atmosphere in a 37°C-incubator. For injections, HCT116 cells growing attached at the exponential phase were detached by gentle trypsinization, suspended in RPMI and injected into mice.

Immunodeficient mice and tumor measurement. Immunodeficient SCID (NOD.CB17Prkdc^{scid}) mice were purchased from Charles River/Jackson Labs (L'Arbresle, France). The mouse colony was raised and maintained in a pathogen-free environment in type IIL cages. Male mice, 6-8 weeks old, were used in the studies described here. For developing xenografts the British model of bilateral axillary inoculations followed as described elsewhere (27). 18 mice received inoculums (200 µl) of 10⁶ HCT116 cells per inoculum according to the British system, and then randomly divided into groups of six mice/group. When the average tumor size reached approximately 100 mm³, in about six days post cell inoculation (pi), a group of mice received no further treatment (control #1) another group received the

vehicle alone (control #2), and a third group of mice received treatment with He-MG in vehicle. All administrations were done intraperitoneally (*i.p.*). Tumor volume was determined with the aid of a calliper applying the equation $a \times b^2/2$, where a and b were two largest dimensions, respectively (28, 29). The size of tumors was recorded every three to four days. Apart from tumor volume, the following parameters were also calculated: % $\Delta T/\Delta C$, where, $\Delta T = T - \Delta_0$ and $\Delta C = C - \Delta_0$ (Δ_0 is the average tumor volume at the beginning of the treatment, T and C are the tumor volumes at a specified day for treated and control (untreated) tumors, respectively), number of tumor-free animals, number of drug-related deaths, median days to achieve a defined tumor volume. The optimal % $\Delta T/\Delta C$ value was used as a measure of drug activity. In general, the $\Delta T/\Delta C$ value in percent is used as an indication of antitumor effectiveness, and a value of $\Delta T/\Delta C \leq 42\%$ is considered as showing significant antitumor activity by the Division of Cancer Treatment, NCI, NIH (27). Losses of weight, neurological disorders, behavioural and dietary changes, were also recorded as markers of side-effects. The experiment was terminated when tumors of untreated animals reached a size of approximately 1,000 mm³.

Handling and experimentation of animals were according to Greek laws (2015/92), guidelines of the European Union and European Council (86/609 and ETS123, respectively) and Compliance with Standards for Human Care and Use of Laboratory Animals, NIH, USA (Assurance No. A5736-01).

Statistical analysis. Significant differences in tumor volume were determined by the Student's *t*-test using the SPSS for Windows (release 11.0.0, SPSS Inc., USA) software package. A difference was considered significant if $p < 0.05$.

Results

He-MG suppresses human tumor growth in mice. To initially evaluate the efficacy of He-MG administered into mice, we applied the "1,100 mg/kg rule" (27). A total amount of 1,100 mg He-MG was administered in mice for five consecutive days, that is, at a dose of 220 mg He-MG/kg of body weight/day, and tumor size was measured as described under Materials and Methods every 3-4 days and recorded as a function of time in days as. The experiment was terminated at about 28 days pi of cells. At this time point the tumor size of the untreated control mice (Figure 1A) reached an average size of 1,000 mm³. A similar average tumor size was measured in mice that received the vehicle alone (results not shown) while however in the same period of time, the He-MG-treated animals carried tumors of approximately 700 mm³ (Figure 1B). Subsequently, the measurements shown in Figures 1A and 1B were utilized to calculate the % $\Delta T/\Delta C$. Using this administration schedule He-MG exerted a moderate suppressing effect on tumor growth for the administration schedule applied, without any significant side-effects. Thus, these results prompted us to extend the study.

In these subsequent studies, the inoculated mice were again randomly divided into four groups and the treatment with He-MG was initiated nine days post-inoculation, that is, the first day that all tumors were measurable with the use of a calliper.

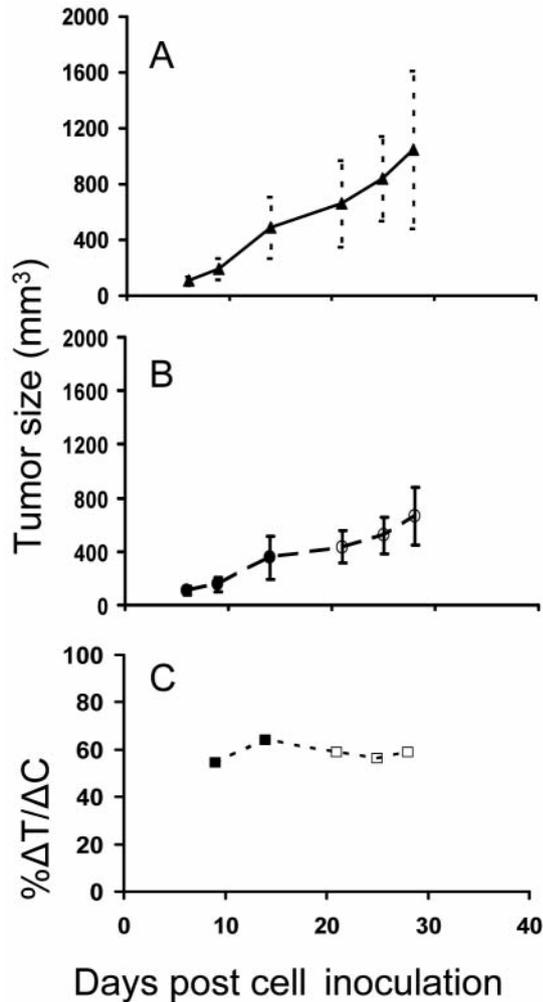


Figure 1. Size and $\% \Delta T/\Delta C$ of HCT116 tumor growth in mice after one-cycle of He-MG treatment. Measurements of tumor size in A, control (untreated) mice and B, in He-MG-treated mice. C, $\% \Delta T/\Delta C$, where T and C are measurements from graphs A and B, at a given day. Open symbols indicate a statistically significant difference at $p < 0.05$. Each point in A and B represents the average size-measurements of 8-12 tumors (from 4-6 mice). Vertical bars, SD.

Two treatment schedules were applied. One schedule (T-5/2) consisted of five consecutive days of He-MG administration followed by two consecutive days without treatment, whereas the second schedule (T-4/3) consisted of four consecutive days of He-MG administration followed by three consecutive days without treatment. Two doses, 100 mg He-MG/kg and 200 mg He-MG/kg of body weight, were tested for each schedule. Control animals bearing HCT116 tumors received no He-MG treatment. The T-5/2 and T-4/3 treatments were repeated in cycles until the control animals developed tumors to an average size of 1,500 mm³, at which point they were euthanized. A dose of 100 mg He-MG/kg had negligible or no effect on tumor growth regardless of the schedule applied

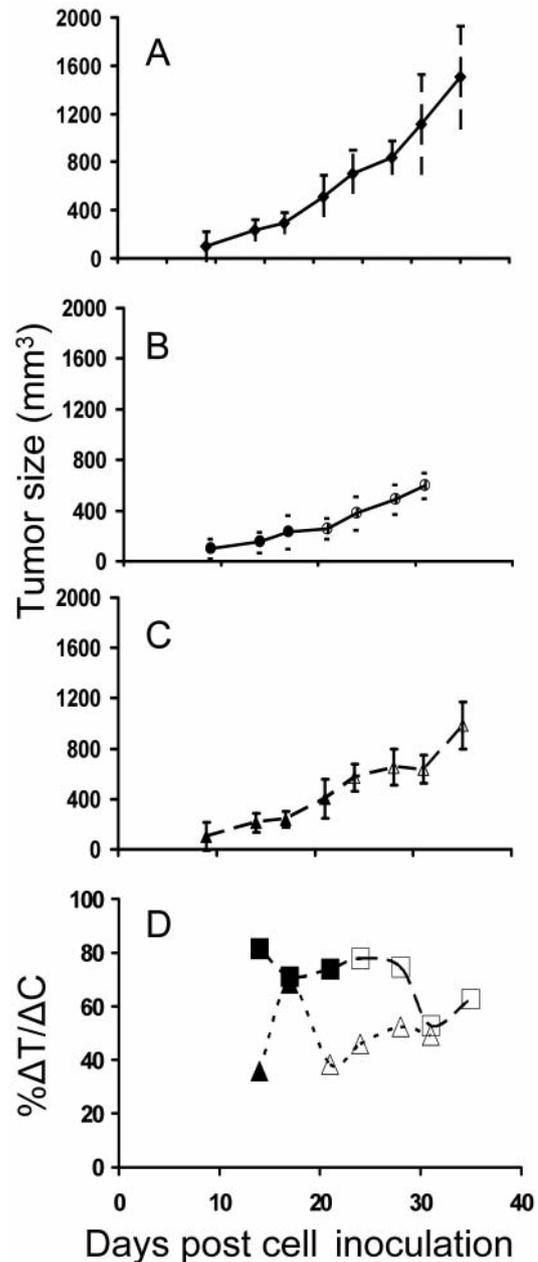


Figure 2. Suppression of HCT116 tumor growth in SCID mice treated with He-MG in cycles. Eighteen animals, each carrying two tumors, were randomly divided into three groups: A, mice with tumors, but not receiving further treatment; B, receiving T-5/2 treatment; and C, receiving T-4/3 treatment. The tumors were measured at the indicated days post inoculation, and average sizes and SD values (vertical bars) were plotted vs time (in days). The $\% \Delta T/\Delta C$ values for the T-5/2 and T-4/3 treatments are shown in graph D. He-MG was administered ip in a cocktail of DMSO/Tween-40/normal saline at 200 mg/kg of body weight. Open symbols indicate a statistically significant difference at $p < 0.05$.

(data not shown). However, the dose of 200 mg He-MG/kg MG extract resulted in tumor growth suppression dependent on the schedule. Figure 2B shows that the T-5/2 treatment

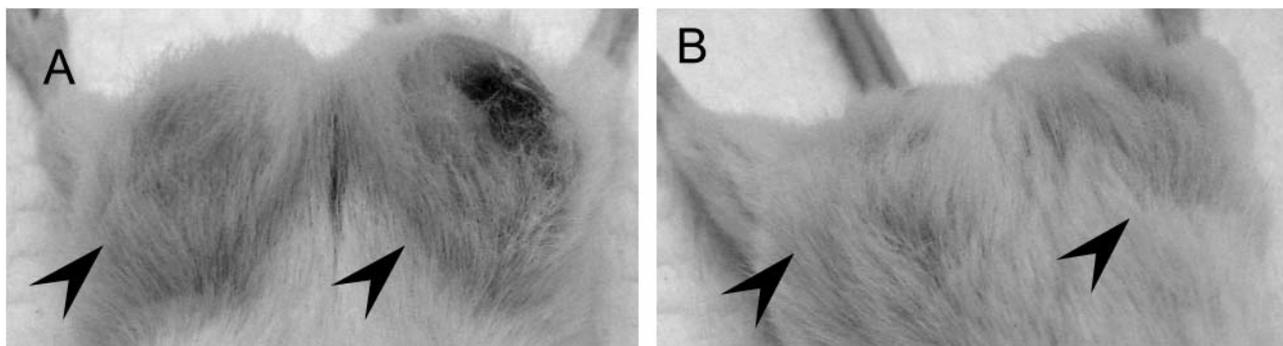


Figure 3. Direct observations of mice with tumors, with and without He-MG treatment. Mice, with a similar size of HCT116 tumors at day 9 post inoculation, received no further treatment (panel A) or T-4/3 treatment with 200 mg/kg of He-MG (panel B). The mice were sacrificed and photographed at day 31 post inoculation. Arrowheads point to tumor sites.

extensively suppressed tumor growth. However this dose was found to be highly toxic also as one mouse was found dead at day 28 pi and three additional dead mice were found on day 31 pi, with no mouse remaining alive at day 35 pi. As it can be seen in Figure 2D, a statistically significant $\Delta T/\Delta C$ (38%, $p < 0.05$), according to NCI standards ($\leq 42\%$) was recorded with this schedule at day 21 pi. In contrast, the T-4/3 treatment resulted in less dramatic tumor growth suppression (Figure 2C) than that observed after the T-5/2 treatment, but all mice were alive at the end of the experiment. In conclusion, in the untreated control mice, the tumors reached an average size of 1,500 mm³ in 35 days (Figure 2A), whereas after T-4/3 treatment the average tumor size reached 950 mm³ in 35 days ($p < 0.05$), that is, the T-4/3 treatment with He-MG resulted in tumor growth suppression of about 35%. The best $\Delta T/\Delta C$ for animals treated with the 4/3-T schedule was ~53% ($p < 0.05$) and was obtained on day 31 of the treatment, suggesting tumor growth suppression. The extensive tumor growth suppression can also be seen in Figure 3, which shows a control mouse, *i.e.* mouse that did not receive He-MG treatment (panel A), and a mouse that received T-4/3 treatment for 31 days (panel B).

Discussion

There has been an extensive search for phytochemicals and micronutrients that can reduce the risk of cancer development and suppress or cause tumor growth regression [see reviews (30-33)]. In this regard, we recently demonstrated that ethanol and hexane extracts of MG possess anticancer activities *in vitro* as manifested by their abilities to arrest human HCT116 colorectal cancer cells at the G₁-phase of the cell cycle and induce apoptosis independent of the expression of the proteins, p53, p21, and Bcl-2 (20, 25). Furthermore, it has been reported that whole MG dissolved in DMSO can inhibit the expression and function of the androgen-receptor (AR) in the human prostate cancer cells,

LNCaP, and in turn, the expression of AR-regulated genes, including prostate-specific antigen, human kallikrein-2 and prostate-specific transcription factor, NKX3.1 (26).

In this study, we used various protocols of dosing and scheduling to demonstrate the ability of the He-MG extract to extensively suppress growth of human colorectal cancer xenografts in immunodeficient SCID mice. It appears that the antitumor effectiveness and toxicity for normal tissues (*i.e.* side-effects) depend on both the He-MG dose administered and the administration schedule. In general, lower anticancer (*i.e.* tumor-suppressing) activity is accompanied by less toxicity in protocols that include He-MG administration in cycles. Under the experimental conditions we used, the schedule T-4/3 was more adequate for the dose of 200 mg He-MG/kg of body weight. However, determination of the ideal protocol parameters, *i.e.* “fine-tuning” of the treatment, will certainly result in a more dramatic suppression of growth of HCT116 colorectal tumor xenografts in SCID mice and a lowering of or elimination of toxicity. At any rate, He-MG can undoubtedly suppress human colon tumor growth in the *in vivo* model we used in this study. It is also possible that using the same administration protocols, He-MG can induce suppression or regression of other types of human tumors grown as xenografts in SCID mice. Such studies are in progress and the results will indicate whether the antitumor activity of He-MG can be exhibited against diverse types of human tumors.

MG constituents in the ethanol and hexane extracts have not been identified yet and, therefore, we do not know the extent of the overlapping compositions of these extracts. We also do not know whether or in which quantities, the ethanol and hexane MG extracts contain components (phytochemicals) which have already been identified in other products derived from the same plant (15, 17-19). In this regard, the major constituents of MG which have been identified include α -pinene (40%), β -pinene (1.5%), β -myrcene (9%), limonene (1.0%) and β -caryophyllene

(5%); therefore, other constituents account for 43.5% of the total components (15). We have obtained the major constituents from commercial sources and individually tested them for anticancer activity but no such activity was observed (data not shown). It is likely that the anticancer activity of mastic gum is attributed to a combination of two or more major and/or minor constituents rather than to one specific constituent. Relevant to this suggestion is a similar conclusion that the antibacterial activity of mastic oil is due to a cocktail of constituents including some of the trace elements (15).

As a conclusion, the hexane extract of mastic gum used in our study demonstrated a capability to delay the growth of colorectal tumors developed from HCT116 cells xenografted into SCID mice under the experimental conditions tested. The effectiveness however and the side-effects of the hexane extract were closely related to the dose and the administration schedule applied. At any rate, the observations described in this study warrant further investigations on the anticancer activities of mastic gum.

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