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RESEARCH PAPER

Effects of natural and novel synthetic jasmonates in experimental metastatic melanoma

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Background and purpose: No current treatment reliably affects the course of metastatic melanoma. Consequently, novel approaches to the control of metastasis are actively sought. The overall goal of the present study was to identify new anti-metastatic agents active against melanoma cells.
Experimental approach: Two directions were taken: 1. To determine whether the natural plant hormone methyl jasmonate, which kills cancer cells selectively, can suppress the characteristic metastatic behavior of B16-F10 melanoma cells; 2. To synthesize and identify novel jasmonate derivatives with better cytotoxic and anti-metastatic activities than methyl jasmonate.
Key results: We found that methyl jasmonate suppressed B16-F10 cell motility and inhibited the development of experimental lung metastases of these cells. Furthermore, methyl jasmonate suppressed the motility of a sub-clone of these cells over-expressing P-glycoprotein and exhibiting multidrug resistance. The synthetic derivative Compound I (5,7,9,10-tetrabromo derivative of methyl jasmonate, the most active derivative) had greater cytotoxic potency (IC_{50}, 0.04mM) than methyl jasmonate (IC_{50}, 2.6mM). Compound I prevented B16-F10 cell adhesion efficiently and inhibited the development of lung metastases at a much lower dose than methyl jasmonate.
Conclusions and Implications: Natural and synthetic jasmonates have anti-metastatic actions. Further development of these agents for the suppression of metastasis in melanoma and other types of cancer is warranted.
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Keywords: jasmonate; metastasis; melanoma; cancer; motility; *P*-glycoprotein

Abbreviations: CLL, chronic lymphocytic leukemia; LPF, lipofundin; MJ, methyl jasmonate; PBL, peripheral blood lymphocytes

Introduction

Members of the plant stress hormones family of jasmonates, and some of their synthetic derivatives, exhibit anti-cancer activity *in vitro* and *in vivo*. Our initial report (Fingrut and Flescher, 2002) indicated that jasmonates suppressed cellular proliferation and induced death in various human and mouse cancer cell lines, including breast, prostate, melanoma, lymphoblastic leukemia and lymphoma cells. Jasmonates were also found to suppress the proliferation or induce death in various other cancer cells including lung and myeloid leukemia cells (Ishii *et al*., 2004; Kim *et al*., 2004; Samaila *et al*., 2004). Furthermore, jasmonates increased the life span of EL-4 lymphoma-bearing mice (Fingrut and Flescher, 2002) and exhibited selective cytotoxicity towards cancer cells while sparing normal blood lymphocytes, even when the latter were part of a mixed population of leukemic and normal cells drawn from the blood of chronic lymphocytic leukemia (CLL) patients (Fingrut and Flescher, 2002; Flescher, 2005, 2007).

Invasion and metastasis are the most insidious and life-threatening aspects of cancer. Once the neoplasm becomes invasive, it can disseminate via the lymphatics and/or vascular channels. The latter are induced through tumor-stimulated lymphangiogenesis, angiogenesis and other perturbations of the local microenvironment. Invasion and metastases kill hosts through two processes: local invasion and distant organ colonization and injury. The most significant turning point in cancer is the establishment of distant metastases. The patient can no longer be cured by local therapy alone at this point. The patient with metastatic disease most commonly succumbs to injury caused by cancer dissemination (Liotta and Kohn, 2003). At the cellular level, cancer cells move within tissues during invasion and metastasis by their own motility and control of the migration of cancer cells is an important problem in tumor treatment (Yamazaki *et al*., 2005).

Cutaneous melanoma is rapidly increasing in incidence throughout the world. Melanoma now accounts for approxi-

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mately 4% of all cancers diagnosed in the United States. The prognosis for patients with melanoma is directly related to the depth of invasion of the primary lesion and, in part, to initial treatment. When diagnosed early in the course of the disease, melanoma is readily cured by simple wide surgical excision. However, once melanoma metastasizes, no treatment currently available reliably affects the course of disease (Morton *et al*., 2003). This lack of appropriate treatment is mainly due to the resistance displayed by melanoma cells towards various chemotherapeutic drugs (Rockman and Schadendorf, 2003). At the time of autopsy, the lung, liver, brain and lymph nodes are the most common sites of metastasis. Patients with pulmonary metastases have a median survival period of 8–10 months (Morton *et al*., 2003). Here, we investigated whether jasmonates have anti-metastatic activities in the B16 melanoma model. The final disposition of the B16 melanoma cells after intravenous (i.v.) injection in the syngeneic C57BL mice has been documented, providing a fast and convenient method for metastasis research (Fidler, 1970). In the earliest post-injection time points, the majority of cells find themselves in the pulmonary tissue, but some are also localized in other organs. After 14 days, only the lungs contain B16 cells, now seen as tumor nodules and none of the other tissues show the establishment of tumors (Fidler, 1970).

We evaluated the anti-metastatic potential of the naturally occurring jasmonate, – methyl jasmonate (MJ). Furthermore, synthetic halogenated derivatives of the natural MJ exhibit stronger activities in plant systems (Kiyota *et al*., 1996). Consequently, we synthesized a series of halogenated MJ derivatives, screened them for superior cytotoxic activities, and chose the most potent one for evaluation of its potential anti-metastatic activity.

We found that the natural jasmonate, MJ, exhibits anti-metastatic activities *in vitro* and *in vivo*. MJ suppresses the growth of melanoma in the lungs. Furthermore, MJ is also active against melanoma cells with high expression of *P*-glycoprotein. Finally, we identified a synthetic derivative of MJ with superior anti-metastatic actions against melanoma cells.

Methods

Synthesis of novel halogenated MJ derivatives (Figure 1)
All compounds were purified by silica-gel vacuum–liquid–chromatography, eluting the compounds with hexane/ethyl acetate mixtures of rising polarity. Yields are of purified material after chromatography (at least 95% pure). All compounds were dissolved in absolute ethanol to give a stock solution of 167 mM. Further dilutions were performed in culture medium supplemented with up to 0.6% ethanol.

Compound Ia–b 5,7,9,10-tetrabromo derivatives of MJ. Reacting 1 g MJ (4.46 mmol) with excess bromine (3.83 g, 21.1 mmol) in 20 ml CCl_4 at $-10^{\circ}C$ for 1 h afforded two tetrabromo derivatives (532 mg, 22%). The two diastereomers could be separated by crystallization from ethanol. That is, Ia stayed in the mother liquor whereas Ib crystallized out. An X-ray diffraction analysis of Ib established its stereochemistry as depicted in Figure 1. According to the NMR data the difference between Ia and Ib is in the stereochemistry of C-9 (δ 52.9 p.p.m day, vs 51.6 day, respectively) and C-10 (δ 61.9 p.p.m. day, vs 62.0 day, respectively), Ia being the 9S*, 10R* isomer and Ib the 9R*, 10S* one. HR-EI-MS found: 458.8800 (M–Br), $C_{13}H_{18}Br_3O_3$ requires 458.8809. Compound I consists of Ia and Ib 1:1 mixture, (w/w).

Compound IIa–c 9,10-dibromo derivatives of MJ. Short (5 min) bromination of MJ (1 g, 4.46 mmol, with 158 mg, 0.99 mmol Br_2) at $-10^{\circ}C$ afforded three compounds. Two, IIa and IIb, are the expected dibromo derivatives (432 mg, 25%) with the 9R*,10S* and 9S*, 10R* configurations (C-NMR δ 55.8 p.p.m. day, 57.8 and 60.7 day, 62.0 day for C-9 and C-10, respectively) and a third compound IIc with an unexpected structure. The structure of the latter, depicted in Figure 1, was elucidated by NMR analysis (δ 119.7 p.p.m. s C-6, 82.5 day C-9, 59.7 day C-10). Compound IIc (390 mg, 26%) is assumed to be obtained from the initially obtained bromonium ion, by attack of the vicinal 6-oxo atom. The obtained lactol transfers in methanol to the methoxy acetal derivative. HR-EI-MS found: 381.9769 (M), $C_{13}H_{20}Br_2O_3$ requires 381.9779. Compound II consists of IIa, IIb and IIc, 1:1:1 mixture (w/w).

Compound IIIa–c 9,10-dibromo derivative of 7-epi cucurbate. - Short, 5 min, bromination of methyl 7-epi cucurabate (60 mg, 0.268 mmol) in 10 ml MeOH at $-10^{\circ}C$ (under the same conditions as for Compound II) afforded three compounds. Two, IIIa and IIIb, the expected dibromo derivatives with the 9R*, 10S* and 9S*, 10R* configurations (disappearance of the double bond in the NMR spectra and appearance of peaks at δ 58.7 day, 58.9 day and 62.2 day, 63.1 day for C-9 and C-10, respectively) and a third compound, IIIc, the 6-dehydroxy analog of IIc (Figure 1). HR-EI-MS found: 383.9942 (M), $C_{13}H_{22}Br_2O_3$ requires 383.9936. Compound III consists of IIIa, IIIb and IIIa, 3:3:1:mixture (w/w).

Compound IV 9-methoxy-10-iodo derivative of MJ. MJ (111 mg, 0.49 mmol) in 5 ml methanol at $0^{\circ}C$ was treated with I_2 (2.28 mmol, 580 mg in methanol, 10 ml) for 30 min. The solution was then kept at room temperature for additional 48 h, in the dark, before work-up (to afford 42 mg, 22%). The structure of IV was established by MS, 1D and 2D NMR experiments to be a single stereoisomer of the 9-methoxy-10-iodo derivative of 3(7)-dehydro MJ (Figure 1) (C-NMR δ 81.4 p.p.m. day, 44.6 day for C-9 and C-10, respectively). HR-EI-MS found: 380.0482 (M), $C_{14}H_{21}IO_4$ requires 380.0485. IV was obtained in 22% yield.

Compound V 9,10-difluoro derivatives of MJ. Fluorination of 2.1 g MJ (9.37 mmol) in a mixture of $CFCl_3/CHCl_3$/ethanol (4:4:1) (500 ml) at $-75^{\circ}C$ by 3% fluorine gas in nitrogen afforded two diastereomeric 9S*.10S*.- and 9R*, 10R* - difluoro derivatives of MJ. HR-EI-MS found: 262.1382 (M), $C_{13}H_{20}F_2O_3$ requires 262.1380. V was obtained as a 1:1 mixture of two diastereomers.

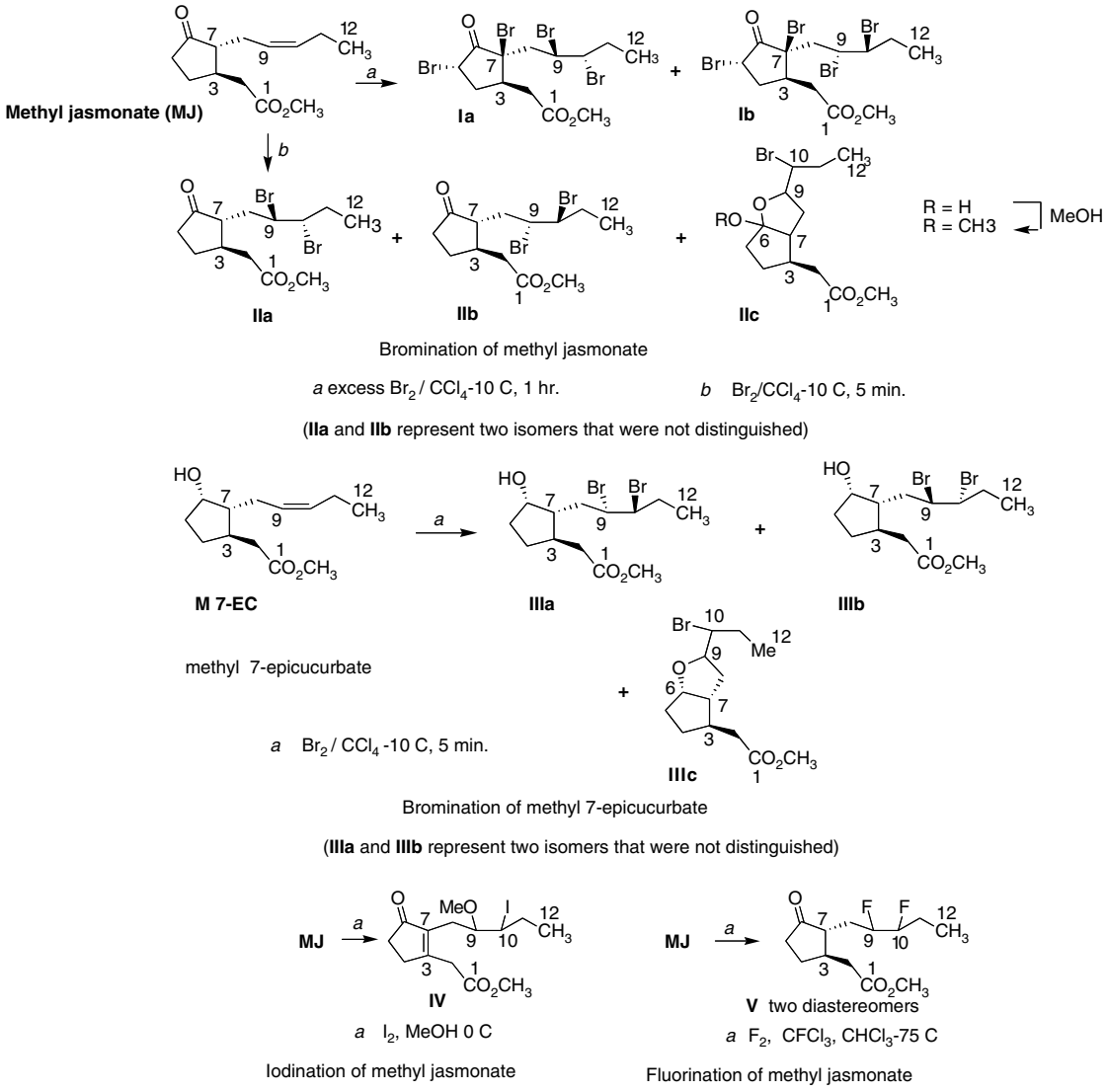

Figure 1 Synthesis and structure of halogenated jasmonate derivatives.

NMR spectra were taken on a Bruker Avance 500 MHz instrument in $CDCl_3$ using standrd Bruker pulse programs. Mass spectra were measured on a Fison, Autospec Q instrument in the EI mode.

Cell lines

B16-F10 murine melanoma cells, Molt-4 human lymphoblastic leukemia cells, MCF7 human mammary adenocarcinoma cells and MIA PaCa-2 human pancreas carcinoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). D122 murine lung carcinoma cells were kindly provided by Professor Yona Keisari, Department of Human Microbiology, Sackler Faculty of Medicine, Tel-Aviv University, Israel. B16 COL/R (COL/R), a highly metastatic variant of B16-F10, expressing high levels of *P*-glycoprotein, was sub-cloned as described (Nordenberg *et al*., 1994). B16-F10 and D122 cells were maintained in Dulbecco's modified Eagle medium (Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1 mM sodium pyruvate, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 1 × nonessential amino acids (all purchased from Biological Industries, Israel). COL/R cells were maintained in the same medium supplemented with 80 ng ml^{-1} colchicine. Molt-4, MCF7 and MIA PaCa-2, cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mM glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

All cells were maintained in a humidified atmosphere with 5% CO_2 at 37°C. The cell lines were found negative for mycoplasma infection as revealed by VenorGem mycoplasma detection kit 25T (Minerva Biolabs, Berlin, Germany).

Preparation of lymphocytes from the peripheral blood

Mononuclear cells from venous blood of healthy donors were prepared by Ficoll–Paque (Phamacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. The resultant mononuclear cell preparation was allowed to adhere to plastic dishes to remove macrophages. The

non-adherent peripheral blood lymphocytes (PBL) were used further in the experiments (Fingrut and Flescher, 2002).

Cytotoxicity assay
The XTT cell viability assay (Biological Industries, Beit Haemek, Israel) was employed. Experiments were performed in 96-well plates. Upon completion of a given experiment, 50 μl of XTT reaction solution (sodium 3′-(1-(phenyl-aminocarbonyl)-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate and *N*-methyl dibenzopyrazine methyl sulfate; mixed at 50:1) was added to each well for 1 h at 37°C. Optical density was measured at 490 nm by the VERSAmax tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA). Optical density is directly proportional to the number of living cells in the culture. Cytotoxicity (%) was calculated in the following way: ((OD of control cells–OD of drug-treated cells)/OD of control cells) $\times$ 100. The final concentration of ethanol in cultures, which did not exceed 0.6%, did not exhibit any cytotoxicity.

Determination of ATP levels
The CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) was employed. Experiments were performed in opaque-walled 96-wells plates. Upon completion of a given experiment, plates were equilibrated to room temperature. A total of 50 μl of CellTiter-Glo reagent containing luciferin and luciferase were added to each well and the plates were mixed on an orbital shaker for 2 min to induce cell lysis. In the presence of ATP, Mg^{2+} and molecular oxygen, mono-oxidation of luciferin is catalyzed by luciferase, generating a luminescence signal. Cells were then incubated for 10 min at room temperature to stabilize the luminescence signal. Luminescence was recorded using Kodak digital science – Image station 440 CD. Luminescence is directly proportional to ATP concentration. ATP depletion (% of control) was calculated in the following way: ((luminescence of control cells−luminescence of drug-treated cells)/luminescence of control cells) $\times$ 100. The final concentration of ethanol in cultures, which did not exceed 0.6%, did not exhibit any effect on the cellular ATP levels.

Migration assay
The wound healing assay was employed (Katerinaki *et al*., 2006). B16-F10 and COL/R cells were seeded at a density of 0.5×10^6 cells/well in six-well culture plates and allowed to form a confluent monolayer. The layer of cells was then scraped with a P200 pipette tip to create a wound of $\sim$1 mm width. Cells were then treated with MJ, compound I or ethanol (vehicle) as indicated. Untreated cells served as control. Images of the wounds were captured at $t=0$ and 22 h at $\times$40 magnification and the wound area was determined using the Scion Image for windows alpha 4.0.3.2 software. The ability of the cells to close the wound, that is, their motility, was evaluated by determining the healed area. Percentage of healed area was calculated in the following way: (wound area at $t=0$−wound area at $t=22$)/wound area at $t=0$) $\times$ 100. The plates were marked to ensure consistent photo-documentation. The final concentration of ethanol in cultures, which did not exceed 0.2%, did not exhibit any effect on cell migration.

Determination of P-*glycoprotein levels*
Western blot analysis was employed to determine P-glycoprotein levels. Whole-cell lysates of 6×10^6 B16-F10 or B16 COL/R cells were extracted with RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM ethylene diaminetetraacetic acid (EDTA), 1% nonidet P 40 (NP-40), 0.5% deoxycholic acid (DOC), 0.1% sodium dodecyl sulfate (SDS), 1 mM sodium orthovanadate, 0.02 mM leupeptin, 0.13 TIU ml^{-1} aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF)). Protein samples (120 μg of whole-cell lysate) were separated by electrophoresis on 10% SDS gel. The proteins were electroblotted onto a nitrocellulose membrane and probed using a specific antibody against *P*-glycoprotein (1:100, Alexis, Grunberg, Germany). *P*-glycoprotein-antibody complexes were stained with HRP-conjugated antibody (1:10 000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Actin levels were used as a loading control. Goat polyclonal anti-actin antibody was used as a primary antibody (1:500, Santa Cruz Biotechnology, CA, USA). Actin-antibody complexes were stained with HRP-conjugated antibody (1:2000, Santa Cruz Biotechnology, CA, USA). Enhanced chemiluminescence (ECL) reagent was added, and the blots were exposed to ECL film (Eastman Kodak, Rochester, NY, USA).

Adhesion assay
Adhesion assay was performed as described previously (Liu *et al*., 2006). B16-F10 cells (at 4×10^4 cells per well) were seeded in 96-well plates and allowed to adhere for 1 h at 37°C, in the presence or absence of compound I at the indicated concentrations or in the presence of the vehicle (ethanol, 0.2%). The wells were washed twice with PBS to remove unattached cells and adherent cells were detected using the XTT cell viability assay. The final concentration of ethanol in cultures, which did not exceed 0.2%, did not exhibit any effect on cell adhesion.

In vivo *study*
C57BL male mice (7- to 8-week-old, weighing 25 ± 3 g) were obtained from the breeding colony of Tel-Aviv University, Israel. Animal care and experimentation were carried out in accordance with Tel-Aviv University guidelines and approved by the institutional animal use and care committee. Mice were kept in cages in a constant 12/12 h light/dark cycle, in a temperature (23°C)-controlled environment, and allowed food and water *ad libitum* during the experiments.

B16-F10 cells (2×10^5) were inoculated i.v. into the tail vein of C57BL mice in 100 μl PBS to produce tumor growth in lungs. MJ at 40 or 75 mg kg^{-1} or compound I at 20 mg kg^{-1} was administered i.v. to animals 5 days a week, once daily, starting 3 days after cell inoculation, until day 21. These doses of MJ and compound I were found to be well tolerated by animals in our preliminary experiments (data not shown). MJ was dissolved in a lipid formulation – lipofundin (LPF)

(B Braun Melsungen, Melsungen, Germany). Compound I was initially dissolved in a small volume of DMSO and then in LPF, such that the concentration of DMSO did not exceed a final blood concentration of 0.14%. Control mice were treated with the LPF alone in experiments with MJ, or with LPF containing the equivalent percent of DMSO in experiments with Compound I. The mice were killed on day 21 after cell inoculation. Their lungs were removed, photographed and weighed. Tumor nodules were only observed in the lungs. Additionally, melanin content was measured in some of the experiments.

Melanin assay

Melanin quantification in tissue was performed according to Yoshikawa *et al.* (2001). Each lung was homogenized with double-distilled H_2O (1:8 (w/v)) using glass/glass homogenizer. A 400 μl sample of the homogenate was centrifuged at 10 000 *g* for 5 min. The pellets were de-lipidized with chloroform:methanol (2:1) and treated with 1 ml of buffer Tris-HCl 0.01 M containing 0.5% SDS, 0.01 M EDTA and 300 $\mu g\,ml^{-1}$ proteinase K (pH 7.8) for 2 h at 56°C. After centrifugation at 10 000 *g* for 5 min, the pellet was dissolved in 500 μl of 0.2 N NaOH. Melanin content was measured at 400 nm with the VERSAmax tunable microplate reader (Molecular Devices, Sunnyvale, CA, USA). Synthetic melanin was used as a standard.

Statistical analysis

Results are presented as mean $\pm$ s.d. of *n* independent experiments. Statistical significance was assessed using analysis of variance (ANOVA). Subsequent pairwise comparisons of specific means were assessed using the Tukey–Kramer test. $P<0.05$ was considered statistically significant.

Materials

MJ (methyl 3-oxo-2-(2-pentenyl) cyclopentaneacetic acid), EDTA, Tris-HCl, SDS, synthetic melanin, colchicine, NP-40, DOC, sodium orthovanadate, leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF) and vinblastine sulfate were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Proteinase K, absolute ethanol, chloroform and methanol were purchased from Merck, Darmstadt, Germany and doxorubicin was purchased from Pharmacia S.p.A, Milan, Italy. MJ was dissolved in absolute ethanol to give a stock solution of 500 mM. Vinblastine was dissolved in DMSO to give a stock solution of 10 $mg\,ml^{-1}$. Further dilutions of MJ and cytotoxic drugs were performed in culture medium supplemented with up to 0.6% absolute ethanol.

Results

Anti-metastatic effects of MJ in B16-F10 melanoma cells

As the naturally occurring jasmonate MJ has been shown to exhibit anti-cancer effects, we decided to evaluate its ability to suppress cancer metastasis in a melanoma model. For the *in vitro* studies, we identified sub-toxic concentrations of MJ in order to specifically study its anti-metastatic action. To that end, we performed a dose–response analysis of MJ treatment of B16-F10 metastatic melanoma cells. We evaluated both cytotoxicity and ATP levels, as we have shown that MJ can induce a fall in the ATP levels of lymphoma cells (Fingrut *et al.*, 2005). As can be seen in Figure 2, we identified a range of concentrations (up to 1 mM) at which MJ induces no more than 10% cytotoxicity and fall in ATP levels. The ability of MJ, at these concentrations, to suppress the motility of the B16-F10 cells was assessed in a migration assay. Our results (Figure 3) showed that MJ is capable of interfering with cell motility at concentrations that neither reduce cell number nor affect the cell ATP stores.

In light of the above findings, we evaluated the potential anti-metastatic effect of MJ in an *in vivo* model, where

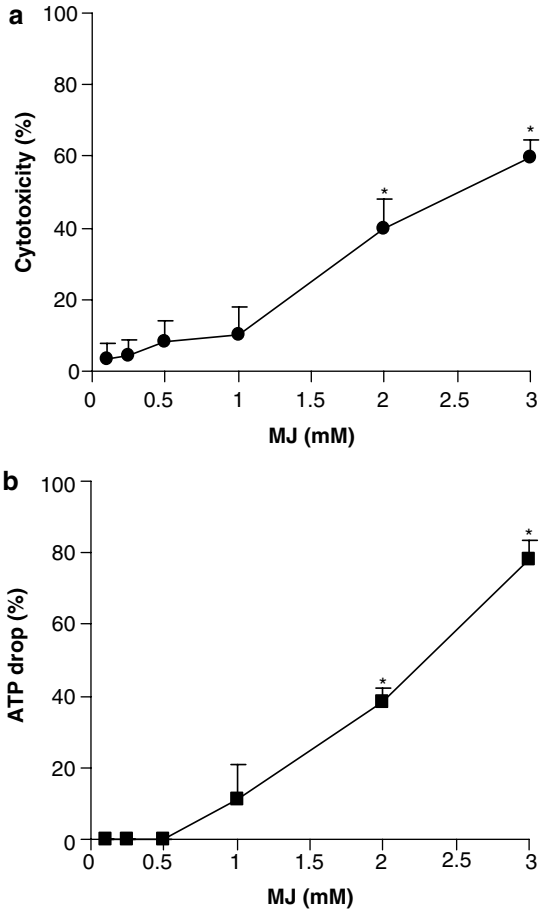

Figure 2 MJ induces cytotoxicity and ATP depletion in B16-F10 cells. (**a**) B16-F10 cells (at 5×10^3 cells per well) were seeded in 96-well plates and allowed to adhere overnight. MJ at the indicated concentrations was added for 24 h. Cell viability was determined by the XTT cell proliferation assay. Cytotoxicity is calculated as % of control untreated cells (mean $\pm$ s.d., $n=3$). $P<0.05$ by ANOVA comparing the different MJ-concentrations. (**b**) B16-F10 cells (at 3×10^3 cells per well) were seeded in 96-well opaque-walled plates and allowed to adhere overnight. MJ at the indicated concentrations was added for 24 h. ATP levels were determined by a luciferase based assay. ATP depletion is calculated as % of control untreated cells (mean $\pm$ s.d., $n=3$). $P<0.05$ by ANOVA comparing the different MJ-concentrations; *$P<0.05$ vs control.

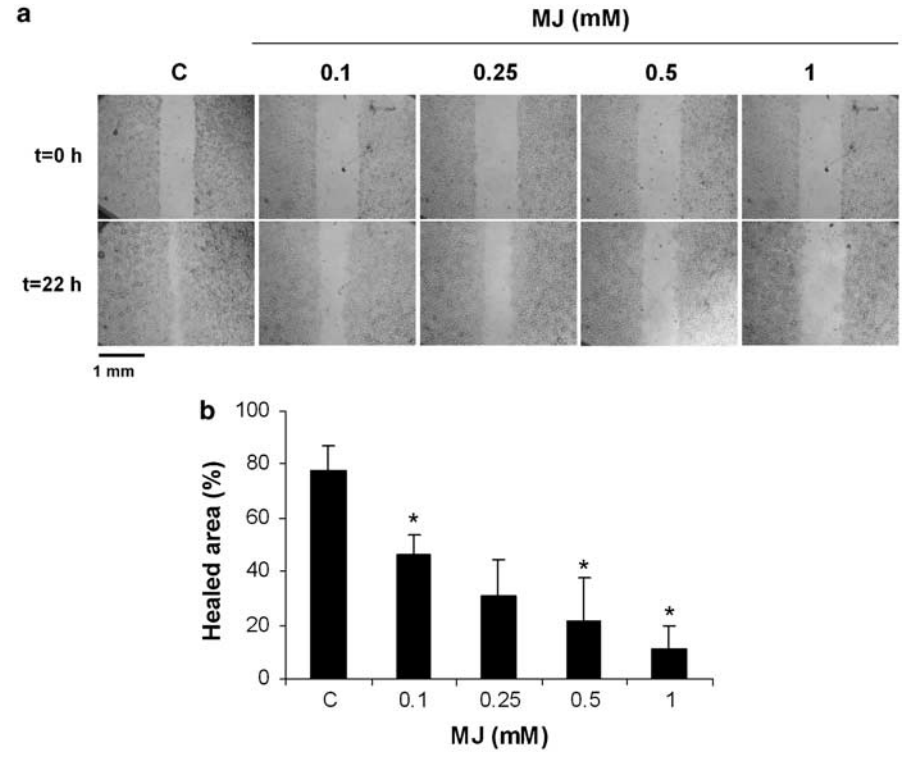

Figure 3 MJ inhibited the migration of B16-F10 cells. B16-F10 cells (at 0.5×10^6 cells per well) were seeded in six-well plates and allowed to reach a confluent monolayer. A scratch wound was created in the cell monolayer and MJ at the indicated concentrations was added for 22 h. (**a**) Images of the wounds were taken at $t=0$ and $t=22$ h. As the concentrations of MJ increased the motility of the cells decreased, that is, a greater portion of the scratched area remained uncovered. (**b**) The ability of the cells to close the wound was evaluated by determining the healed area at $t=22$ h. Healed area is calculated as % of wound area at $t=0$ (mean $\pm$ s.d., $n=3$). $P<0.05$ comparing all experimental groups by ANOVA. *$P<0.05$ in a pairwise comparison with control untreated cells by the Tukey–Kramer test.

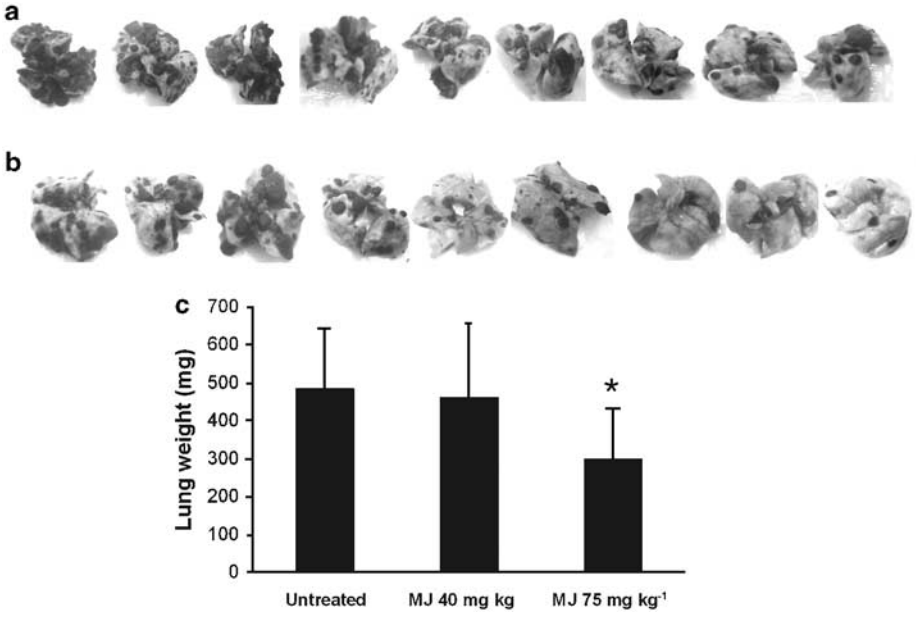

Figure 4 MJ inhibited experimental melanoma metastasis to the lungs of C57BL mice. Mice were injected i.v. with 2×10^5 B16-F10 melanoma cells. Treatment with 40 or 75 mg kg^{-1} MJ, given i.v., started on day 3 after cell inoculation and lasted until day 21, when mice were killed. Lungs were photographed and weighed. The experiment was performed twice. (**a**) Appearance of representative lungs from untreated C57BL mice on day 21 after B16-F10 cells i.v. injection. (**b**) Appearance of representative lungs from C57BL mice treated with 75 mg kg^{-1} MJ, on day 21 after B16-F10 cell i.v. injection. (**c**) MJ significantly decreases the lung weight of B16-F10 melanoma-bearing mice treated with 75 mg kg^{-1} MJ, (mean $\pm$ s.d.; $n=19$). $P<0.05$ comparing all experimental groups. *$P<0.05$ comparing pairwise 75 mg kg^{-1} MJ with untreated mice. The weight of a lung taken from a healthy mouse is about 200 mg.

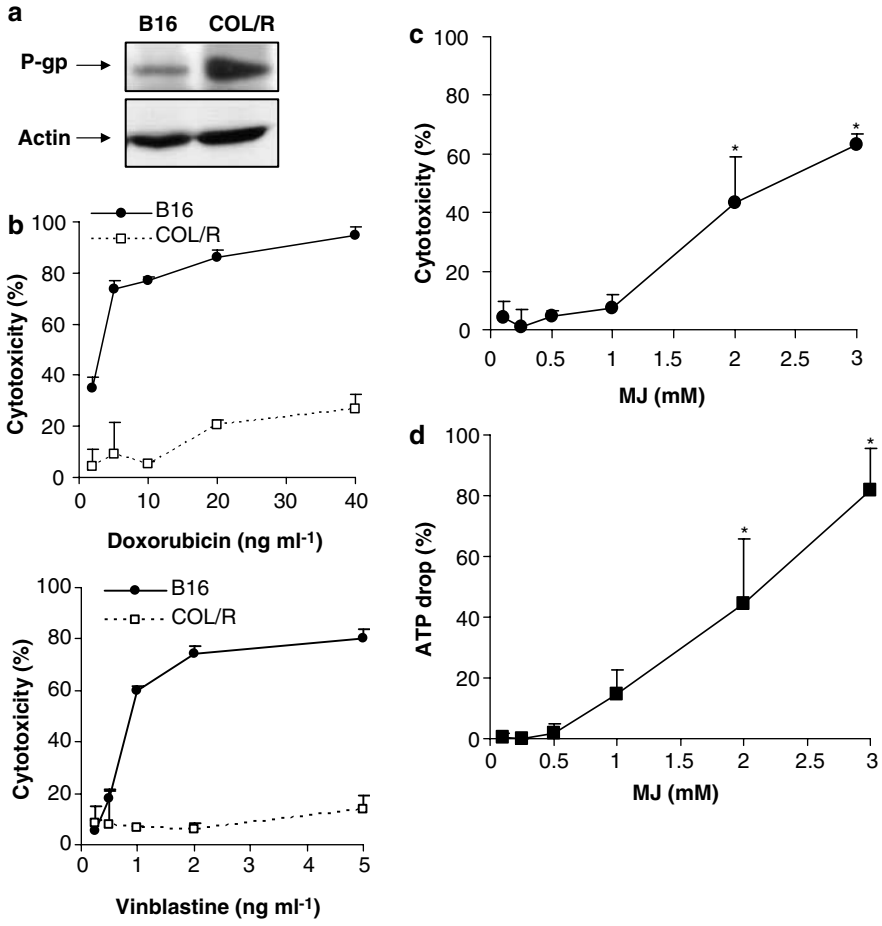

Figure 5 MJ induced death and ATP depletion in the highly metastatic and *P*-glycoprotein (*P*-gp) expressing B16 COL/R cells. (**a**) For analysis of *P*-gp levels, whole-cell lysates of B16-F10 (B16) or B16 COL/R (COL/R) cells were prepared and proteins were separated by SDS-PAGE, followed by immunoblotting using a specific antibody against *P*-gp. Actin levels were used as a loading control. (**b**) B16-F10 (B16) and B16 COL/R (COL/R) cells (at 1×10^3 cells per well) were seeded in 96-well plates and allowed to adhere overnight. Doxorubicin and vinblastine at the indicated concentrations were added for 72 h. Cell viability was determined by the XTT cell proliferation assay. Cytotoxicity is calculated as % of control untreated cells (mean$\pm$s.d., $n=3$). $P<0.05$ by ANOVA comparing B16 with COL/R cells for doxorubicin and vinblastine. (**c**) Similar to (**b**) with B16 COL/R cells (5×10^3 cells per well) and MJ added for 24 h (mean$\pm$s.d., $n=3$). $P<0.05$ by ANOVA comparing the different MJ-concentrations. (**d**) B16 COL/R cells (at 3×10^3 cells per well) were seeded in 96-well opaque-walled plates and allowed to adhere overnight. MJ at the indicated concentrations was added for 24 h. ATP levels were determined by a luciferase based assay. ATP depletion is calculated as % of control untreated cells (mean$\pm$s.d., $n=3$). $P<0.05$ by ANOVA comparing the different MJ-concentrations; *$P<0.05$ vs control.

melanoma cells colonize the lungs. As can be seen in Figure 4, MJ reduced significantly the level of melanoma growth in the lungs of treated mice, at a dose of 75 mg kg^{-1} but not at 40 mg kg^{-1}. The 75 mg kg^{-1} dose is the highest that can be given i.v. without observing any overt toxicity.

Anti-metastatic effects of MJ in B16 COL/R highly metastatic drug-resistant melanoma cells

B16 COL/R cells are a variant of B16-F10 cells which exhibit more pronounced metastatic spread to lungs as well as higher cell motility, in comparison to the parental cells (Staroselsky *et al*., 1996). Furthermore, the B16 COL/R cells exhibit multidrug resistance. Consequently, we evaluated the effect of MJ on these cells given the high clinical relevance of their specific characteristics. We confirmed the drug-resistant nature of the B16 COL/R cells (Figure 5a and b) and determined their sensitivity to MJ in assays measuring cytotoxicity and ATP depletion (Figure 5c and d). B16 COL/R cells are highly resistant to doxorubicin and vinblastine. On the other hand, comparing the results in Figures 5c and d, with those in Figure 2, the drug-resistant cells respond to MJ in a manner very similar to that of the drug-sensitive, parental B16-F10 cells. The IC_{50} of MJ in the parental cells is 2.6 ± 0.1 mM and it is 2.05 ± 0.2 mM in the drug-resistant cells. Thus, our results suggest that MJ affects B16 melanoma cells independently of their drug-resistance status. Accordingly, MJ inhibited the motility of B16 COL/R cells dose-dependently at concentrations that are not cytotoxic and do not decrease ATP levels (Figures 5 and 6).

Selection of a highly active halogenated MJ derivative

As halogenated derivatives of MJ exhibit superior activity in plant systems (Kiyota *et al*., 1996), we synthesized five MJ

derivatives containing Br, I or F atoms (Figure 1). The initial screening was performed with Molt-4 leukemic cells which are very sensitive to MJ (Fingrut and Flescher, 2002) and it was found that each of the novel derivatives had a lower IC_{50} than MJ (Table 1). Nevertheless, Compound I which contains four Br atoms is by far the most active derivative with an IC_{50} that is approximately 55 times lower than that of MJ. Compound I is a mixture of two isomers Ia and Ib at a 1:1 ratio (as described in Methods). It is interesting to note that both isomers were equally cytotoxic towards Molt-4 cells (data not shown). Next, we compared the cytotoxicity of MJ and Compound I in B16-F10 cells as well as three carcinoma cell lines of breast, pancreas and lung origin. In all cases, Compound I was clearly more active than MJ (Table 2). For instance, The IC_{50} of Compound I in B16-F10 cells is about 62 times lower than that of MJ. Finally, as found for MJ (Fingrut and Flescher, 2002), Compound I exhibits preferential cytotoxicity towards cancer cells, its IC_{50} with PBL being 5–27 higher than those with the different cancer cell lines. We, therefore, selected Compound I to evaluate its anti-metastatic potential.

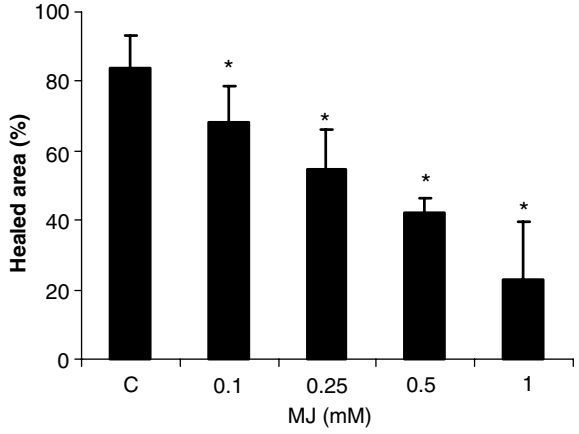

Figure 6 MJ inhibited the migration of the highly metastatic B16 COL/R cells. Migration of B16 COL/R cells was measured as described in the legend for Figure 3. The ability of the cells to close the wound was evaluated by determining the healed area at $t=22$ h. Healed area is calculated as % of wound area at $t=0$ (mean$\pm$s.d., $n=3$). $P<0.05$ comparing all experimental groups by ANOVA. *$P<0.05$ in a pairwise comparison with control untreated cells by the Tukey–Kramer test.

Table 1 Halogenated derivatives of MJ were cytotoxic in Molt-4 leukemic cells

Compound	*IC_{50} (mM)*
MJ	0.5$\pm$0.03
I	0.009$\pm$0.00048*
II	0.22$\pm$0.01*
III	0.23$\pm$0.03*
IV	0.29$\pm$0.0096
V	0.39$\pm$0.03

Abbreviation: MJ, methyl jsasmonate.
Molt-4 cells (at 2×10^4 per well) were seeded in 96-well plates and MJ derivatives were added at concentrations of 0.001–1 mM for 24 h at 37°C. Cell viability was determined by the XTT cell proliferation assay. IC_{50} represents the concentration of an MJ derivative that induces death in 50% of the cells (mean$\pm$s.d., $n=3$). $P<0.05$ comparing all compounds by ANOVA; *$P<0.05$, comparing pairwise compounds I, II and III with MJ by the Tukey–Kramer test.

Anti-metastatic effects of Compound I in B16-F10 melanoma cells
For the evaluation of the anti-metastatic potential of Compound I *in vitro*, we first identified concentrations under which it would not be cytotoxic towards B16-F10 cells. Figure 7a shows the cytotoxicity dose–response analysis. As a result, we tested the activity of Compound I, at concentrations up to 0.01 mM only, in the 22 h migration assay. As can be seen in Figure 7d, Compound I did not affect cell motility at these sub-toxic concentrations. Consequently, we employed a different assay reflecting another fundamental attribute of invasive behavior, cell adherence. As this assay lasts for only 1 h, we re-evaluated the cytotoxic and ATP-depleting effects of Compound I over this shorter time and showed that concentrations up to 0.1 mM were non-toxic under these conditions (Figure 7b and c). We carried out the adhesion assay accordingly and found that Compound I inhibited adhesion, dose-dependently, in B16-F10 cells (Figure 7e).

Compound I was evaluated *in vivo* measuring melanoma growth in the lungs. As can be seen in Figure 8a and b, Compound I administered i.v. suppressed the colonization of lungs by B16-F10 cells. This was determined both by lung weight (Figure 8c) as well as by the quantity of melanin in the lungs (Figure 8d). It should be noted that the level of melanin in normal lungs, as measured by us, was about 20 μg. We found a strong positive correlation between lung weight and melanin content in B16-F10-bearing mice (Figure 8e). This suggests that the increased weight of tumor-containing lungs reflects mostly the presence of melanoma cells. The dose of Compound I was 20 mg kg^{-1}, the highest dose causing no overt toxicity, as determined in preliminary experiments. The effects of Compound I on the development of melanoma growth in the lungs were similar to those of MJ (Figure 4).

Table 2 Compound I is more cytotoxic than MJ in a range of cancer cell lines

Cell line	*IC_{50} (mM)*	
	MJ	*Compound I*
B16-F10	2.6$\pm$0.1	0.042$\pm$0.002*
MCF7	1.5$\pm$0.06	0.015$\pm$0.00057*
MIA PaCa-2	1.4$\pm$0.09	0.009$\pm$0.0011*
D122	1.8$\pm$0.18	0.05$\pm$0.0074*
PBL	>3	0.25$\pm$0.015*

[a]Abbreviations: MJ, methyl jasmonate; PBL, peripheral blood lymphocytes.
B16-F10, MCF7, MIA PaCa-2 and D122 cells (at 5×10^3 per well) were seeded in 96-well plates. Normal lymphocytes (PBL) were pre-treated with PHA (0.8 μg ml^{-1}) and TPA (5 ng ml^{-1}) for 24 h, to induce cell proliferation, and then seeded in 96-well plates (at 1.2×10^5 per well). MJ and Compound I were added at concentrations of 0.001–3 mM for 24 h at 37°C. Cell viability was determined by the XTT cell proliferation assay. IC_{50} represents the concentration of an MJ derivative that induces death in 50% of the cells (mean$\pm$s.d, $n=3$). $P<0.05$ comparing MJ and compound I in all cancer cells lines by ANOVA; *$P<0.05$, comparing pairwise MJ with compound I in each cancer cell line by the Tukey–Kramer test.

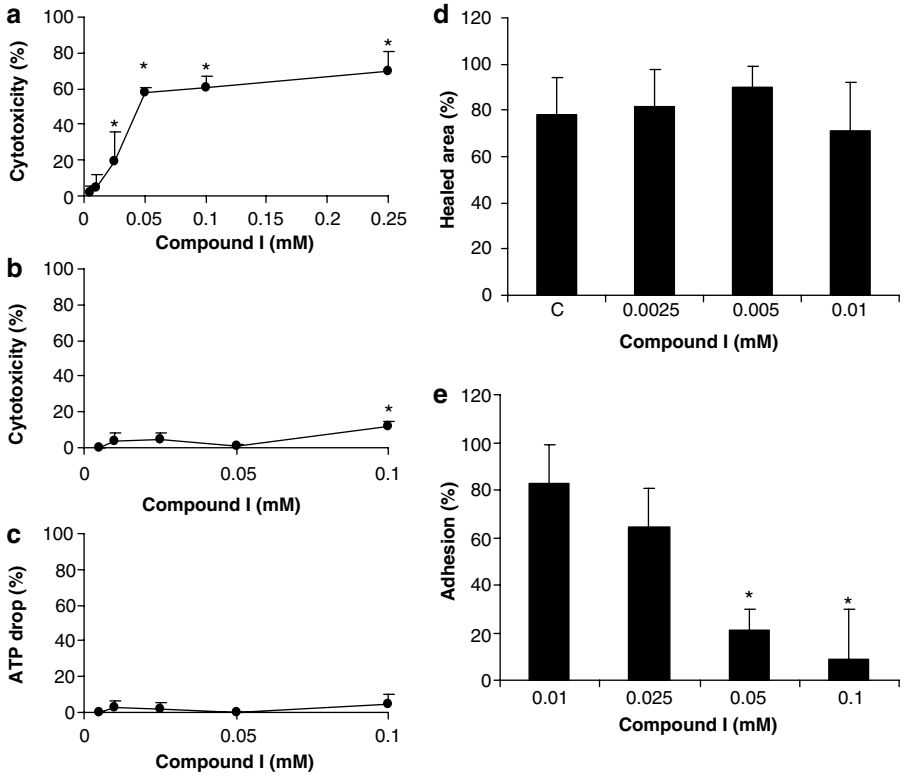

Figure 7 Compound I induced death and inhibits the adhesion of B16-F10 cells. (**a**) and (**b**) B16-F10 cells (at 5×10^3 cells per well) were seeded in 96-well plates and allowed to adhere overnight (**a**) or treated immediately (**b**) with Compound I at the indicated concentrations for 24 h (**a**) or 1 h (**b**). Cell viability was determined by the XTT cell proliferation assay. Cytotoxicity is calculated as % of control untreated cells (mean $\pm$ s.d., $n=3$). $P<0.05$ by ANOVA for both (**a**) and (**b**). comparing the different Compound I concentrations;. *$P<0.05$ vs control. (**c**) B16-F10 cells (at 3×10^3 cells per well) were seeded in 96-well opaque-walled plates. Compound I at the indicated concentrations was added for 1 h. ATP levels were determined by a luciferase based assay. ATP depletion is calculated as % of control untreated cells (mean $\pm$ s.d., $n=3$). Comparing the various Compound I concentrations by ANOVA did not yield a significant difference. (**d**) Migration of B16-F10 cells was determined as described in the legend for Figure 3. Compound I at the indicated concentrations was added for 24 h at 37°C. The ability of the cells to close the wound was evaluated by determining the healed area at $t=22$ h. Healed area is calculated as % of wound area at $t=0$ (mean $\pm$ s.d., $n=3$). Comparing the various Compound I concentrations by ANOVA did not yield a significant difference. (**e**) B16-F10 cells (at 4×10^4 cells per well) were seeded in 96-well plates and treated with Compound I at the indicated concentrations for 1 h at 37°C. Non-adherent cells were removed by washing and cell adhesion was evaluated using the XTT assay. Adherence is calculated as % of control untreated cells (mean $\pm$ s.d., $n=3$); $P<0.05$ comparing all experimental groups by ANOVA. *$P<0.05$ in a pairwise comparison with control untreated cells by the Tukey-Kramer test.

Discussion

Natural jasmonates, most importantly MJ, possess cytotoxic activity against various types of cancer cells. Given the critical role cancer spread plays in the clinical development of neoplastic growth, our overall goal in this project was to evaluate potential anti-metastatic activities of jasmonates. Focusing on melanoma cells and their ability to develop tumors in the lungs, we evaluated the anti-metastatic potential of MJ *in vitro* and *in vivo*. We found that MJ can inhibit melanoma cell migration and suppress the development of melanoma growth in murine lungs. It was also found that MJ interferes with melanoma cell function irrespective of the level of *P*-glycoprotein the cells express. In addition, we synthesized and screened a series of novel synthetic halogenated jasmonates. We identified a synthetic brominated jasmonate with exceptional anti-cancer effects, much more potent than MJ, and established its anti-metastatic potential.

Jasmonates are plant stress hormones (Howe, 2004) and salicylate is another plant stress hormone (Delaney, 2004) with documented anti-cancer activities. Indeed, the acetylated derivative of salicylate, known as aspirin, has been shown by many groups including ours to induce suppression of proliferation and death in cancer cells (Ordan *et al.*, 2003). Similar to MJ, salicylate and aspirin inhibited invasiveness of cervical and prostate cancer cells (Murono *et al.*, 2000; Lloyd *et al.*, 2003). It thus appears that plant stress hormones share not only a cytotoxic potential against cancer cells but also the ability to suppress various cellular functions in those cells, such as cell motility, essential to the metastatic process. This supports the suggestion that plant stress hormones are a promising group of plant-derived anti-cancer agents.

Cell motility is an essential component of the metastatic phenotype. However, one must consider the possibility that the anti-metastatic effect of MJ results in part from its cytotoxic effect on B16 F-10 melanoma cells.

The potential effect of the ethanol vehicle on the *in vitro* metastasis-related assays also deserves consideration. Our motility and adhesion assays were performed in the presence of 0.2% ethanol. Migration of B16-F10 cells is actually increased by 1% ethanol (Silberman *et al.*, 1990). Never-

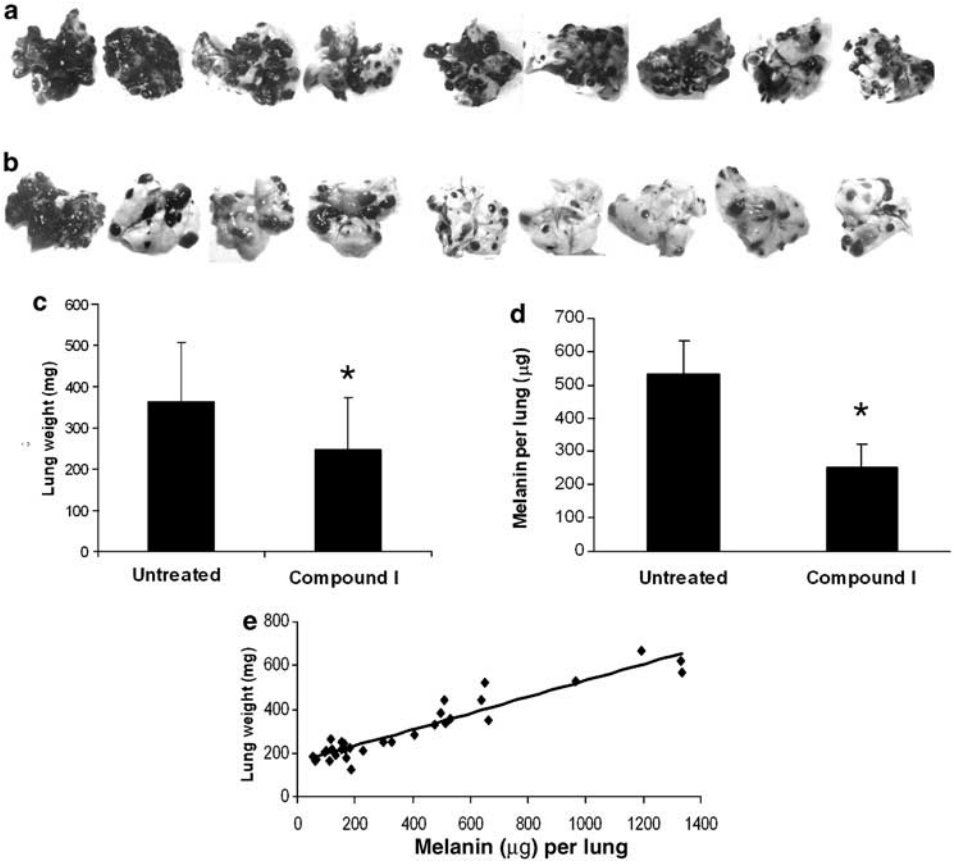

Figure 8 Compound I inhibited experimental melanoma metastasis to the lungs of C57BL mice. The mice were injected i.v. with 2×10^5 B16-F10 melanoma cells. Treatment with 20 mg kg^{-1} compound I, given i.v., started on day 3 after cell inoculation and lasted until day 21 when mice were killed. Lungs were photographed and weighed. The experiment was performed twice. Melanin measurement was performed as described in Methods. (**a**) Appearance of representative lungs from untreated C57BL mice on day 21 after B16 F10 cell i.v. injection. (**b**) Appearance of representative lungs from Compound I-treated C57BL mice on day 21 after B16-F10 cells i.v. injection. (**c**) Compound I significantly decreases the lung weight of B16-F10 melanoma-bearing mice (mean $\pm$ s.d.; $n = 16$). *$P < 0.05$ vs untreated. (**d**) Melanin content in lungs of B16-F10 melanoma-bearing mice is significantly reduced in compound I-treated mice versus untreated (mean $\pm$ s.d.). *$P < 0.05$ vs untreated. (**e**) Correlation between lung weight and melanin content in B16 melanoma-bearing mice ($R^2 = 0.9$).

theless, at the concentration used by us we did not observe any effect of ethanol.

Cancer cell drug resistance can be mediated via multiple mechanisms. A major one is the overexpression of an efflux pump called *P*-glycoprotein, encoded by the MDR1 gene (Flescher and Rotem, 2002; Tsuruo *et al.*, 2003). This pump is capable of reducing the effective intracellular concentrations of chemotherapeutic drugs belonging to many different chemical families, a phenomenon termed multidrug resistance. These include taxanes, anthracyclins, vinca alakaloids and epipodophylotoxins (Bodo *et al.*, 2003). Various types of cancers are affected by *P*-glycoprotein-mediated resistance, including melanoma cells (Molinari *et al.*, 2005). Furthermore, drug resistance and enhanced metastatic potential have been reported to be associated in melanoma cells (Staroselsky *et al.*, 1996; Molinari *et al.*, 2005). Thus, we tested the possibility, and indeed found, that MJ suppresses the motility of melanoma cells expressing high levels of P-glycoprotein. This suggests that jasmonates may be useful against multidrug-resistant tumors.

In the course of evaluating the anti-metastatic potential of jasmonates, we synthesized a series of halogenated jasmonate derivatives and selected from among them Compound I, exhibiting the highest cytotoxic activity. The tetrabromo derivative of MJ (Compound I) is the most bulky one of the various derivatives, suggesting that large bromine groups give a better adjustment to a putative binding site. Compound I was administered at 20 mg kg^{-1} whereas MJ was administered at 75 mg kg^{-1} (40 mg kg^{-1} proved ineffective, Figure 4). Also, the molecular weight of Compound I is 538, vs 224 of MJ. Thus, Compound I appeared to be much more potent than MJ *in vivo* as it generated a similar suppressive effect on tumor growth at about nine times lower doses in molar terms.

As compound I inhibits cell adhesion, one might expect it to be effective *in vivo* only if administered before lung colonization. Given the anti-metastatic effects of compound I *in vivo*, it is plausible that the mechanism of action of compound I involves additional effects to the anti-adhesive ones. For instance, compound I may downregulate the expression of proteolytic enzymes instrumental in the tissue invasion process (Hofmann *et al.*, 2005, Fontijn *et al.*, 2006). Interestingly, halogenated jasmonates which possess enhanced activity in plants (Zhao *et al.*, 2004; Qian *et al.*,

2005), exhibited also an unexpectedly greater activity against cancer cells, a radically different cellular system. Our results demonstrate that chemical manipulation of the jasmonate molecule can yield highly potent anti-metastatic compounds.

5-(3,3-Dimethyl-1-triazenyl)imidazole-4-carboxamide (DTIC) is the chemotherapy of choice for metastatic melanoma with a response rate of 16% (Tsao *et al.,* 2004). Immunotherapy with either IL-2 or interferon has demonstrated response rates of 10–15% in appropriately selected patients (Atallah and Flaherty, 2005). The current state of melanoma chemotherapy does not seem to be significantly different from the situation of 20 or 30 years ago. To date, no single drug, combination chemotherapy in addition to a hormonal or biotherapy compound, has demonstrated an overall survival benefit in a randomized clinical trial (Mandara *et al.*, 2006). With these dire facts as a background, we propose that jasmonates are novel and promising anti-metastatic agents for the treatment of malignant melanoma.

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Conflict of interest

This work was supported by a grant from Sepal Pharma, a company engaged in the development of jasmonates as anti-cancer agents. This support has not influenced the data shown in this paper nor the decision to publish

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