Inhibition of cell proliferation, migration and invasion of B16-F10 melanoma cells by $\alpha$-mangostin

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ABSTRACT

In this study, we have evaluated the potential antineoplastic effects of $\alpha$-mangostin ($\alpha$-M), the most representative xanthone in Garcinia mangostana pericarp, on melanoma cell lines. This xanthone markedly inhibits the proliferation of high-metastatic B16-F10 melanoma cells. Furthermore, by deeply analyzing which steps in the metastatic process are influenced by xanthone it was observed that $\alpha$-M strongly interferes with homotypic aggregation, adhesion, plasticity and invasion ability of B16-F10 cells, probably by the observed reduction of metalloproteinase-9 activity. The antiproliferative and antimetastatic properties of $\alpha$-M have been established in human SK-MEL-28 and A375 melanoma cells. In order to identify pathways potentially involved in the antineoplastic properties of $\alpha$-M, a comparative mass spectrometry proteomic approach was employed. These findings may improve our understanding of the molecular mechanisms underlying the anti-cancer effects of $\alpha$-M on melanoma.

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1. Introduction

Melanoma is the most aggressive form of skin cancer, and its incidence is increasing at a rate greater than any other form of cancer; further, it remains one of the most difficult cancers to treat [1]. Therefore, the development of effective therapy or at least as an adjuvant of classical chemotherapy is still required. Mangosteen (Garcinia mangostana L.) is a tropical evergreen tree. Different parts of mangosteen, mainly fruits, bark and roots have been used for centuries in Southeast Asia [2]. The major bioactive secondary metabolites of G. mangostana are xanthone derivatives and they are present mostly in the pericarp of the fruit [3]. Xanthones is a class of polyphenolic compounds with a skeleton of a xanthene-9-one [4]. The most studied xanthones are $\alpha$-, $\beta$- and $\gamma$-mangostins, garcinone E, mangostenones and gartanin. The antioxidant, anti-inflammatory, antibacterial and antiviral activities of xanthones are described [5]. The antineoplastic activity of $\alpha$-mangostin ($\alpha$-M; 1,3,6-trihydroxy-7-methoxy-2,8-diprenyl-9H-xanthen-9-one), the most representative xanthone in mangosteen pericarp (78% content), has been established on human hepatoma [6], breast cancer [7] and pancreatic cancer [8]. In particular $\alpha$-M was reported to have antitumor effects on human melanoma tumor cells previously [9]; however, the details of the mechanism remain unclear. Metastasis of cancer cells involves multistep processes and various cyto-physiological changes, including adhesion and invasion. Several lines of evidence suggest that tissue transglutaminase (TG; EC 2.3.2.13), an enzymes that modify proteins post-translationally in a calcium-dependent manner, leading to the formation of covalent $\epsilon$-$\gamma$-glutamyllysine linkages, has a potential role in the various stages of the metastatic cascade [10]. The aim of this study was to elucidate the antineoplastic effects of $\alpha$-M on B16-F10 mouse melanoma cells, human SK-MEL-28 and A375 melanoma cells, to find new evidences on the various stages of metastatic process involved. In particular, the antimetastatic potential, the effects on cell proliferation and the possible induction of tumor cell differentiation exerted by $\alpha$-M have been investigated.

2. Materials and methods

2.1. Cell line and reagents

B16-F10 highly metastatic murine melanoma cells, SK-MEL-28 and A375 human melanoma cells were propagated under standard conditions. http://dx.doi.org/10.1016/j.bbrc.2014.07.031 0006-291X/© 2014 Published by Elsevier Inc.
culture conditions. Dulbecco’s Modified Eagle Medium (D-MEM), Roswell Park Memorial Institute medium (RPMI), Foetal Calf Serum (FCS) and all cell culture media were obtained from Gibco (Grand Island, NY, USA). [14C]-methylamine (46.6 mCi/mmol) was purchased from Amersham International (Bucks, UK). Matrigel (MG), α-M, ethylenediaminetetraacetic acid (EDTA), synthetic melanin and all reagents were from Sigma Chemicals (St. Louis, MO, USA). Eight µm-sized porous filters were purchased from Millipore S.p.A. (Milano, Italy).

2.2. Cell cultures, proliferation studies and cell cycle analysis

Melanoma cells were grown in D-MEM (B16-F10 and A375) or RPMI (SK-MEL-28) supplemented with 10% FCS, 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. All cell cultures were incubated at 37 °C in a humidified atmosphere of 5% CO2. For treatments, α-M was dissolved in dimethylsulfoxide (DMSO). For proliferation assay, cells were seeded and grown in 35 mm dishes and treated with α-M (5, 10 and 15 µM) for 48 h. Cells were harvested and counted with a Neubauer modified chamber. For cytotoxicity evaluation, cells were tested by the Trypan Blue (0.25%) exclusion test. Cell cycle distribution was determined by flow cytometric analysis of propidium iodide-labelled cells as previously described [11].

2.3. Evaluation of melanoma cell differentiation

B16-F10 cells are a good model for melanoma differentiation. For melanin content evaluation, melanotic B16-F10 and lightly pigmented SK-MEL-28 cells were cultured in the presence of α-M for 48 h, then detached, washed twice in phosphate buffer saline and collected by centrifugation and counted. Melanin was extracted from cells with 1 ml of 1 M NaOH at 60 °C for 1 h. Melanin content was determined by absorption at 475 nm, using the standard curve obtained from a solution of synthetic melanin (0–100 µg/ml).

Transglutaminase (TG) activity assay was performed on B16-F10 cell lysates using [14C]-methylamine (46.6 mCi/mmol) as substrate, as previously described [12]. Radiolabelled amine incorporation into cell proteins was measured with a scintillation substrate, as previously described [12]. Radiolabelled amine incorporation into cell proteins was measured with a scintillation substrate, as previously described [12]. Radiolabelled amine incorporation into cell proteins was measured with a scintillation substrate, as previously described [12].

Determination of intracellular protoporphyrin IX (PpIX) concentration was performed as previously described [13].

2.4. Aggregation and adhesion assays

For aggregation assay, melanoma cells were cultured in the presence of α-M for 48 h, then detached and resuspended in 0.1% bovine serum albumin (BSA) in D-MEM. Viable cells (2 × 103) were transferred into a 24-well culture plate. The plate was gently shaken at 37 °C for 90 min and measurement of cell aggregates was determined under light microscope.

The adhesion assay was performed on 24-well plates coated with 10 µg/well of MG by using a modified adhesion assay already published [14].

2.5. Invasion assays and gelatin zymography

For 3D invasion assay, cells (4 × 103) were mixed with an equal volume of MG and plated on a 12-well culture plate, and incubated for 4 h at 37 °C to allow gel formation. After incubation, the number of cellular processes per cell was evaluated under light microscope.

Invasion assay was performed in a modified Boyden chamber as previously described [12] using NIH-3T3 murine fibroblast-conditioned medium (for B16-F10) or FCS (SK-MEL-28 and A375) as chemoattractant. MG-coated filters were placed between the two compartments. Chambers were incubated in a humidified 5% CO2 atmosphere for 6 h at 37 °C. The filter was fixed by 100% ethanol, stained with Giemsa solution (5%). Eight µm-sized porous filters were purchased from Millipore S.p.A. (Milano, Italy).

2.6. Mass spectrometry and protein identification

Gel-based proteome analysis of α-M-treated B16-F10 cells was performed. Cell lysates were prepared as previously described [16]. Proteins from collected cells were run in a 4–15% Mini-PROTEAN® TGX™ pre-cast polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA). For protein identification, the total gel lane was cut, proteins were reduced, alkylated and digested overnight with bovine trypsin sequencing grade (Roche Applied Science, Monza, IT). The peptide mixtures were analyzed by nano-reversed-phase liquid chromatography tandem mass spectrometry (RP-LC-MS/MS) using an HPLC Ultimate 3000 (DIONEX, Sunnyvale, CA) connected on line with a linear Ion Trap (LTQ, Thermoelectron, San Jose, CA). Peptides were desalted in a trap column (Acclaim PepMap 100 C18, LC Packings, DIONEX) and then separated in a reverse phase column, a 10 cm long fused silica capillary (Silica Tips FS 360–75–8, New Objective, Woburn, MA), slurry-packed in-house with 5 µm, 200 A pore size C18 resin (Michrom BioResources, CA). Data acquisition and analysis was performed as previously described [16–17]. Data were searched with 1.5 Da and 1 Da tolerance respectively for precursor and fragment ions. A peptide was considered legitimately identified when it achieved cross correlation scores of 1.5 for [M + H]+1, 2.0 for [M + 2H]2+, 2.5 for [M + 3H]3+, and a peptide probability cut-off for randomized identification of p < 0.001.

2.7. Bioinformatics analysis

Unique proteins identified by proteomic analysis were further analyzed using The Database Identified for Annotation, Visualization and Integrated Discovery (DAVID, ver 6.7) software (http://david.abcc.ncifcrf.gov). DAVID provides a comprehensive set of functional annotation tools for investigators to understand biological meaning behind a large list of proteins [18]. DAVID functional annotation cluster analysis was performed on the list of proteins of B16-F10 control cells and treated with 15 µM α-M for 48 h. Only those terms with a p-value < 0.005 were selected for DAVID analysis. The Gene Ontology (GO) terms of biological process and SP_PIR. Keywords in the Functional Categories section in DAVID were used.

2.8. Statistical analysis

All data represented at least three independent experiments and were expressed as mean ± S.D., unless otherwise indicated. Statistical comparisons were made using two tail Student’s t-test. p-values of 0.05 or less were considered to represent a statistically significant difference.

3. Results

3.1. Effects of α-M on melanoma cell proliferation

In this study, we first assayed the antiproliferative effects of α-M on B16-F10 for 48 h. As shown in Fig. 1A, α-M revealed dose-dependent inhibitory effect on the growth of melanoma cells.

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3.2. Effects on cell cycle

In order to elucidate the inhibition mode of B16-F10 cell growth by α-M, we measured DNA content using flow-cytometry after PI staining of nuclei. As shown in Fig. 1B, the proportion of subG0/G1 cell population increased after α-M exposure, especially at 15 μM treatment. A slight increase in G0/G1 phase has been observed after 24 h of drug exposure.

3.3. Induction of melanoma cell differentiation after α-M treatment

We investigated the potential effects of α-M on the differentiation of B16 cells. As shown in Table 2, treatment of B16-F10 with 15 μM α-M increased the amount of melanin, with respect to the control, by about 2-fold. Similar results were found for TG activity. Increase of intracellular PpIX, a compound synthesized during heme biosynthesis, is considered a differentiation marker [19]. The effect of α-M on intracellular accumulation of PpIX in B16-F10 cells is shown in Table 2, where the concentration of intracellular PpIX significantly increased by 66.2% and 77.9% in 10 and 15 μM α-M-treated cells respectively.

The induction of differentiation in 15 μM α-M-treated cells was confirmed by light microscopic observations. As known, cytoplasmic protrusions are recognized as a morphological indicator of melanocyte/melanoma cell differentiation. Indeed, the morphology of B16-F10 cells incubated with 15 μM α-M was markedly changed with respect to the control (Fig. 1C). No differences in morphology and in melanin content (SK-MEL-28) were found in human melanoma cells (data not shown).

3.4. α-M strongly interferes with metastatic potential of melanoma cells

Loss of tumor cell homotypic aggregation represents one of the early steps of the metastatic spread. To determine whether α-M would affect the cell–cell adherence behavior of B16-F10, cell aggregation assay was performed. We found that α-M significantly increased the ability of B16 cells to form cell aggregates (Fig. 2A and B). In particular, α-M treatment enhanced the amount of cell aggregates in a dose-dependent manner (by about 3-fold after 15 μM α-M treatment). The interaction of tumor cells with basement membrane proteins plays a pivotal role in tumor cell metastatization. Fig. 2B illustrates also the adhesion pattern of B16-F10 cells over MG-coated substrates. As shown, xanthone reduces, with respect to the control, the adhesion pattern of cells, by 31.5% and 88.8% after 10 and 15 μM α-M. The reduction of the plasticity in α-M-treated cells was established by the 3D-invasion assay. B16-F10 cells responded to α-M incubation in a dose-dependent manner by markedly reducing the branching-process formation (Fig. 2C and D). Next, we examined the invasive potential of B16 cells. The number of treated cells that were able to cross the MG-coated membrane was reduced by the administration of α-M at several concentrations. The reduction of invasion obtained following treatment of 5, 10 and 15 μM α-M was of 57.2%, 64.2% and 79.6% respectively (Fig. 2D and E).

MMP-9 is involved in extracellular matrix degradation and in several types of human cancers, its expression is abnormally elevated and has been associated with poor prognosis [20]. The evaluation of the integrated optical density of MMP-9 showed that its activity was reduced, with respect to the control, by about 59% and 63% after 10 and 15 μM α-M treatments respectively (Fig. 2F).

Table 1 illustrates the adhesion and invasion patterns of SK-MEL-28 and A375 melanoma cells. As shown, 15 μM α-M reduces significantly, with respect to the control, the adhesion in A375 cells (by about 70%). The invasion was reducing in both cell lines by 38% and 28% in SK-MEL-28 and A375 cells, respectively.
3.5. Functional analysis of B16-F10 proteome after α-M treatment

With the aim of improving the understanding of anti-cancer molecular mechanisms of α-M on melanoma cells we carried out a gel-based mass spectrometry analysis of the proteome of the treated B16-F10 cell line and data were compared with the control (Supplementary Table 1).

As shown in Fig. 3A, 569 proteins were common for both groups, 154 proteins were found only in control cells and 140 proteins in α-M-treated cells. The unique proteins were characterized by DAVID according to their biological processes and molecular function. As shown in pie charts, cell cycle (in control cells) and cytoskeleton and cellular component reorganization (in treated cells) are the discriminating biological processes between the two data sets of unique proteins (Fig. 3B). Performing functional annotation cluster by DAVID in the same data set, functions associated with endoplasmic reticulum (ER) and Golgi regulation, ubiquitination, actin binding, protein biosynthesis, GTP-binding and

### Table 1
Effects of α-M on proliferation, adhesion and invasion abilities of human SK-MEL-28 and A375 melanoma cells.

<table>
<thead>
<tr>
<th>Group</th>
<th>Proliferation (%)</th>
<th>Adhesion (%)</th>
<th>Invasion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B16-F10/SK-MEL-28/A375 (Control)</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>B16-F10 (15 μM α-M)</td>
<td>21.7 ± 2.2</td>
<td>11.2 ± 1.3</td>
<td>20.4 ± 1.9</td>
</tr>
<tr>
<td>SK-MEL-28 (15 μM α-M)</td>
<td>67.8 ± 12.8</td>
<td>86.5 ± 9.1</td>
<td>62.3 ± 13.7</td>
</tr>
<tr>
<td>A375 (15 μM α-M)</td>
<td>54.5 ± 2.3</td>
<td>30.2 ± 9.9</td>
<td>71.9 ± 19.7</td>
</tr>
</tbody>
</table>

Data are mean ± SD from three independent determinations.

* p < 0.05.

** p < 0.01.

*** p < 0.005 were considered to be statistically significant (vs control group).

### Table 2
Effect of α-M on melanin content, transglutaminase (TG) activity and protoporphyrin IX (PpIX) accumulation in B16-F10 cells.

<table>
<thead>
<tr>
<th>Melanin content (μg melanin/10⁶ cells)</th>
<th>TG activity (%)</th>
<th>PpIX accumulation (a.u. fluorescence/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Control)</td>
<td>1.52 ± 0.51</td>
<td>100</td>
</tr>
<tr>
<td>5 μM α-M</td>
<td>1.83 ± 0.58</td>
<td>93.5 ± 14.3</td>
</tr>
<tr>
<td>10 μM α-M</td>
<td>2.01 ± 0.46</td>
<td>94.1 ± 14.1</td>
</tr>
<tr>
<td>15 μM α-M</td>
<td>3.25 ± 0.72</td>
<td>223 ± 20.5</td>
</tr>
</tbody>
</table>

TG activity in control cells was 1280 CPM/mg protein. Data are mean ± SD from three independent determinations.

* p < 0.05 was considered to be statistically significant.
The pericarp of mangosteen is rich in xanthones, which exhibit potent antioxidant properties and have been proposed for prevention and treatment of many cancers [21]. Besides polyphenols, it has been suggested that melanoma might be a suitable target for treatment with differentiation-inducing agents [21]. The induction of terminal differentiation and growth arrest of cancer cells [24]. Melanoma is well known as a chemotherapy-resistant cancer, and it has been suggested that melanoma might be a suitable target for therapy with differentiation-inducing agents [21]. The induction of functional differentiation in B16-F10 melanoma cells occurs mainly after 15 µM α-M treatment. In fact an increase in the activity of TG, a marker of terminal differentiation [25], was observed. However, the effects of xanthones from mangosteen on melanogenesis seem controversial. Hamid and colleagues [26] have demonstrated the melanogenic potential of mangosteen leaf extract in B16-F1 melanoma cells. Conversely, mangostenone F was found to inhibit melanin production [27]. Since terminal differentiation is a part of apoptotic process [28] we also investigate the effect of α-M on cell cycle distribution. We found that α-M first arrests B16 cells in G0/G1 phase (24 h) and thereafter induces an increase in subG1 cell population (48 h). An additional observable parameter of melanoma cell differentiation is morphological change [29]. B16-F10 cells are usually polygonal but extension of dendritic processes is observed after induction of differentiation [30]. Surprisingly, cells treated with α-M displayed a non-classical differentiate phenotype and acquired an elongated morphology, hence this absence of a marked dendritogenesis requires further investigations. In the present study, we observed also that α-M has a strong antimetastatic potential, as shown by the reduction of homotypic aggregation, adhesion, shape fickleness and invasion of B16-F10 melanoma cells. This suggests the possibility to use α-M as new interesting drug to interfere with the complex balance among extra-cellular matrix components, angiogenesis and tumor cells adhesions, as suggested [14]. These data might depend, relatively to that observed for the highest dose of α-M, on the activation of the trans-samidating form of TG [10]. As last evidence, we observed that α-M is able to induce a reduction of MMP-9 activity, considered a prognostic marker in some human tumours [31]. Moreover, it has been demonstrated that TG inhibits significantly MMP-9 activity and expression [32]. The antimetastatic potential of α-M was established also in two human melanoma cell lines. In particular, we observed a marked reduction of the adhesion of A375 following treatment with α-M. Our data confirmed the antimetastatic effects of α-M on melanoma cells as previously reported [9]. Both human cell lines, while showing signs of reduction of malignancy, do not seem to differentiate.

The functional and metabolic differences between control and 15 µM α-M-treated B16 cells were investigated by proteomic analysis. The data revealed that in untreated cells there is a major prevalence of protein involved in cell cycle progression, as expected in proliferating cells. On the contrary, we found that α-M promote the induction of protein involved in cellular reorganization, probably after the induction of terminal differentiation and apoptosis. Interestingly, DAVID analysis of unique proteins revealed a possible correlation between functional categories found in α-M-treated B16...
cells and TG. In fact, TG is a multifunctional enzyme with both protein cross-linking (transamidating) and GTP-hydrolyzing and -binding activities [33]. Moreover, GTP binding is crucial for transamidating activity of tissue TG [34]. The presence of proteins related to cytoskeleton and actin (well-known substrate of TG) reorganization, from a part might explain the differences in cellular morphology and on the other hand might elucidate the progression of apoptosis and the inhibition of cellular plasticity and invasion [35]. The potential role of TG in the Golgi and vesicles network is also known [36]. Although randomized controlled trials of human participants are lacking, basic science and epidemiologic studies both show promising benefits of many natural products in chemoprevention for melanoma through dietary nutrients.

Disclosure of interest
The authors declare that they have no conflicts of interest concerning this article.

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Appendix A. Supplementary data
Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.07.031.

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