



Aloe-emodin exerts a potent anticancer and immunomodulatory activity on BRAF-mutated human melanoma cells



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ABSTRACT

Aim of this study was to extend the knowledge on the antineoplastic effect of aloe-emodin (AE), a natural hydroxyanthraquinone compound, both in metastatic human melanoma cell lines and in primary stem-like cells (melanospheres). Treatment with AE caused reduction of cell proliferation and induction of SK-MEL-28 and A375 cells differentiation, characterized by a marked increase of transamidating activity of transglutaminase whose expression remained unmodified. *In vitro* antimetastatic property of AE was evaluated by adhesion and Boyden chamber invasion assays. The effect of AE on melanoma cytokines/chemokines production was determined by a multiplex assay: interestingly AE showed an immunomodulatory activity through GM-CSF and IFN- γ production. We report also that AE significantly reduced the proliferation, stemness and invasive potential of melanospheres. Moreover, AE treatment significantly enhanced dabrafenib (a BRAF inhibitor) antiproliferative activity in BRAF mutant cell lines. Our results confirm that AE possesses remarkable antineoplastic properties against melanoma cells, indicating this anthraquinone as a promising agent for differentiation therapy of cancer, or as adjuvant in chemotherapy and targeted therapy. Further, its mechanisms of action support a potential efficacy of AE treatment to counteract resistance of BRAF-mutated melanoma cells to target therapy.

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1. Introduction

Metastatic melanoma is a deadly disease with a 3-year survival rate of 10–15% and with limited treatment options. Dacarbazine (DTIC), cisplatin (CDDP), methotrexate (MTX) and 5-fluorouracil represent the most common drugs used in cancer chemotherapy (Tawbi and Buch, 2010), as well as anthracyclines (i.e. doxorubicin) (Skladanowski and Konopa, 1993). Furthermore, recent data highlighted the role of BRAF (a serine/threonine protein kinase activating the MAP kinase/ERK-signaling pathway) mutations in the pathogenesis of melanoma, triggering the use of BRAF inhibitors in clinical trials (Cantwell-Dorris et al., 2011). In fact, two BRAF V600 specific inhibitors, vemurafenib (Shaw and Nathan, 2013) and dabrafenib (GSK2118436; DAB) (Trinh et al., 2014), were recently approved for treatment of melanoma. Although these targeted agents cause objective responses and clinical benefit, resistance invariably develops. Moreover, many studies

support the presence and involvement of cancer stem-like cells (CSCs) in tumor initiation and progression (Yu et al., 2012), as well as in chemo-resistance and therapeutic failure of anti-tumor strategies (Eramo et al., 2010; Chen et al., 2012). Malignant melanoma-initiating cells (MICs), like other CSCs, may be responsible for the generation and perpetuation of a continuously-growing tumor (Regad, 2013). Therefore, novel therapies directed toward MICs represent a promising anticancer strategy.

A tumor's ability to stimulate or evade an immune response depends on its antigenicity and other immunomodulatory factors produced either by the tumor or tumor microenvironment. Compared to other malignancies, melanoma is a highly immunogenic tumor (Blankenstein et al., 2012; Shimanovsky et al., 2013). Since cancers possess several strategies to evade immune surveillance, the application of immunotherapy is of a great importance (Weber, 2011). For example, interleukin(IL)-12 and interferon- γ (IFN- γ) are among the best characterized and most potent antitumor cytokines (Cocco et al., 2009; Balachandran and Adams, 2013).

Although the effects of chemotherapeutic treatments are significant, side effects are noteworthy. Thus, the search for novel

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therapeutical, chemopreventing and/or adjuvant drugs with lower toxicity, particularly from natural sources, is of great interest. Several studies showed the anticancer activity of secondary metabolites from aloe plant exudate, which contains polyphenols, mainly 1,8-dihydroxyanthraquinones (Huang et al., 2007). Aloe-emodin (AE; 1,8-dihydroxy-3-(hydroxymethyl)anthracene-9,10-dione) is a bioactive compound mostly present in *Aloe barbadensis* Miller (*Aloe vera* L.) leaves. AE exerts antiproliferative activity in cancer cells (Guo et al., 2007; Lai et al., 2007; Harlev et al., 2012; Huang et al., 2013). We previously reported important anticancer effects of AE linked to the induction of tumor cell differentiation, probably through the activation of intracellular tissue transglutaminase (TG2; EC 2.3.2.13) (Tabolacci et al., 2010). Transglutaminases (TGs) are a widely distributed group of enzymes with several known enzymatic activities. The most studied and characterized catalyzes the post-translational modification of proteins by the formation of isopeptide bonds and covalent conjugation of polyamines into proteins (transamidating enzymatic activity) (Nurminskaya and Belkin, 2012). The purpose of this work was to extend the knowledge on the anticancer role of AE (Huang et al., 2007) also against human melanoma, investigating its antiproliferative, differentiative, immunomodulatory and antimetastatic properties. Moreover, the effects of AE on MICs and its possible use for sensitizing to DAB were investigated.

2. Materials and methods

2.1. Chemicals and reagents

Roswell Park Memorial Institute medium (RPMI-1640), Dulbecco's Modified Eagle Medium (DMEM), phosphate buffer saline (PBS), glutamine, penicillin (10,000 UI/ml) and streptomycin (10,000 µg/ml) were from Eurobio Laboratoires (Le Ulis Cedex, France). Fetal calf serum (FCS) was from HyClone (South Logan, UT, USA). [¹⁴C]-methylamine (46.6 mCi/mmol) was purchased from Amersham International (Bucks, UK). DAB was from Selleck Chemicals (Houston, TX, USA). Matrigel (MG) was from Becton Dickinson (Oxford, UK). All solvents were purchased from Mallinckrodt Baker (Milan, Italy). Granulocyte/macrophage colony-stimulating factor (GM-CSF) was from R&D Systems (Minneapolis, MN, USA). AE, CDDP, MTX, DTIC, IFN-γ, dimethyl sulfoxide (DMSO) and all other reagents were from Sigma Chemicals (St. Louis, MO, USA).

2.2. Cell cultures and proliferation assay

All cell lines (A375, M14 and SK-MEL-110) were cultured in DMEM except for SK-MEL-28 cells that were cultured in RPMI-1640. Cell culture medium was supplemented with 10% FCS, 0.05% L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) and maintained at 37 °C with 5% CO₂ in a humidified atmosphere. A surrogate of melanoma-initiating cell line (Mel1, melanospheres) was cultured as previously described (Sette et al., 2013). AE, CDDP, MTX, DTIC and DAB were dissolved in DMSO. For proliferation studies, melanoma cells were seeded and grown in 35 mm dishes in culture medium supplemented as reported above, and treated with AE for 24, 48 and 72 h. For combined treatments with chemotherapeutics, SK-MEL-28 cells were incubated for 48 h with CDDP, DTIC and MTX, with or without 30 µM AE. Cells were harvested and counted with a Neubauer modified chamber, after Trypan Blue staining for cytotoxicity evaluation.

2.3. Intracellular localization of aloe-emodin

SK-MEL-28 cells were plated on coverslips and cultured for 24 h. After 10 min or 24, 48 and 72 h of treatment with 30 µM AE, culture medium was removed, and the cells on coverslips were

visualized (unfixed) by a fluorescence microscope (DM 4000, Leica, Germany) equipped with a high resolution digital camera (DFC350FX, Leica). The excitation wavelength was 456 nm.

2.4. Flow cytometry

For cell cycle assay 1×10^5 cells were harvested and fixed in 80% cold ethanol. Fixed cells were washed and incubated with 200 µg/ml ribonuclease A (RNase A, Life Technologies) for 30 min at 37 °C and 50 µg/ml propidium iodide (PI) as described (Facchiano et al., 2001). Samples were analyzed with a FACScanto Becton Dickinson Instrument (Becton Dickinson, CA, USA) and FACS Diva software (5.0.3 version). Apoptotic cells were analyzed by Annexin V conjugated with fluorescein isothiocyanate (FITC) combined with 7-Aminoactinomycin D (7AAD), using flow cytometry as described (Li et al., 2009). The CD44 variant isoform 6 (CD44v6) is a functional marker involved in cancer migration and invasion (Orlan-Rousseau, 2010). CD44v6 is restricted to melanospheres and it is considered not expressed in terminal-differentiated cells or in melanocytes. The expression of CD44v6 on the surface of Mel1 cells was evaluated by flow cytometry as previously described (Sette et al., 2013). Briefly, melanospheres were washed and incubated 45 min with phycoerythrin (PE)-conjugated anti-CD44v6 (R&D system, Minneapolis, MN) or PE-conjugated mouse antibodies (isotypic control; Molecular Probes, Eugene, OR), washed again, suspended in PBS containing 7AAD (10 µg/ml) and analyzed with a FACScan as above mentioned.

2.5. Determination of melanin content, intracellular protoporphyrin IX concentration and transglutaminase transamidating activity

Determination of intracellular melanin content in both control and AE-treated cells was performed as previously described (Lotan and Lotan, 1980). For this experiment, lightly pigmented melanoma cells SK-MEL-28 were grown in standard complete growth medium supplemented with 0.05% L-tyrosine to promote melanin formation. Intracellular protoporphyrin IX (PpIX) concentration was determined as previously described (Tabolacci et al., 2010). For intracellular TG activity, melanoma cells plated on 100-mm Petri dishes (1×10^6) were grown in the presence of [¹⁴C]-methylamine (0.5 µl/ml complete culture medium) either in the absence or in the presence of 30 µM AE. Radiolabelled amine incorporation into cell protein was measured with a scintillation counter (Beckman LS-5000TD, Fullerton, CA, USA) and transamidating activity expressed as reported elsewhere (Tabolacci et al., 2011).

2.6. Cell extract preparation and Western blot analysis

Postnuclear cell lysates were prepared as described (Facchiano et al., 2013). The protein concentration was then measured by Bradford's procedure (Bio-Rad protein assay kit, Biorad Laboratories, Hercules, CA). Western blot analysis was performed as previously described (Facchiano et al., 2013). Briefly, whole cell lysate (60 µg per well) separated on 4–15% Mini-PROTEAN[®] TGX[™] pre-cast polyacrylamide gel Bio-Rad, blotted onto Nitrocellulose using Trans-Blot[®] Turbo[™] Transfer Starter System was probed with mouse monoclonal anti-transglutaminase 2 [CUB 7402] (1:400, Abcam Ltd., Cambridge, UK), rabbit polyclonal anti-Akt (1:500; Cat#7985-R Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-phosphorylated-Akt (1:500; Cat#8312 Santa Cruz Biotechnology) or monoclonal mouse IgM anti-actin (mAbGEa) from Novus Biologicals (Littleton, CO) (1:4000). HRP-conjugated goat anti-mouse IgG secondary antibodies (1:10,000 dilution; Zymax, CA, USA) and HRP-conjugated goat anti-rabbit IgG secondary antibodies (1:10,000; Cat#2004 Santa Cruz

Biotechnology) were used, followed by visualization with a Clarity Western ECL Substrate Kit (Bio-Rad). Chemiluminescence was revealed by means of FluorChem System (Cell Biosciences, CA, USA) and digitalized images were used for densitometric quantification.

2.7. Adhesion and invasion assays

The adhesion assays were performed on 24-well plates coated with MG (50 µg/well) as previously reported (Tabolacci et al., 2010). Briefly, after MG polymerization, 80 µl of cell suspension (1×10^6 cells/ml) of control and AE-treated SK-MEL-28 and A375 cells were added to each well. The plates were incubated for 1 h at 37 °C. The adherent cells were detached with trypsin/EDTA and counted. The same procedure was carried out for Mel1 cells. Invasion assay was performed in a modified Boyden chamber assay. Briefly, a total of 1×10^6 SK-MEL-28 and A375 cells (resuspended in serum-free medium) were added to the upper compartment and FCS (100%) as chemoattractant was added to the lower chamber. Mel1 cells were resuspended in a serum free-medium without growth factors. Complete medium (i.e. with 20 ng/ml EGF and 10 ng/ml bFGF) was used as chemo attractant for Mel1 cells. MG-coated polyvinylpyrrolidone-free polycarbonate filters (8 µm pore size) were used between the two compartments. Chambers were incubated in a humidified 5% CO₂ atmosphere for 6 h at 37 °C, and non-migrated cells were gently wiped away from the upper surface of the filter. The filter was fixed by 3.7% formaldehyde in PBS for 10 min, stained with Giemsa solution (5%) and then mounted on a glass slide. The number of invasive cells was evaluated by light microscopical counting and results expressed as percentage with respect of the control (100%).

2.8. Evaluation of cytokines and bioinformatic analysis

SK-MEL-28 and A375 cultures, after of AE incubation, were analyzed by Bio-Plex cytokine assays (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's protocol. Supernatants were analyzed for IL-2, IL-12, GM-CSF and IFN-γ. Kits for cytokines were obtained from Bio-Rad Laboratories. Samples were prepared according to the immunometric reactions protocol using a Bio-Plex Pro™ Wash Station (Bio-Rad). The quantification was carried out on 50 µl of sample making use of a Bio-Plex Array Reader (Bio-Plex 200 System) and related Bio-Plex Manager™ (Version 6.1) software (Bio-Rad). A comparative search was performed on the NCBI Gene Expression Omnibus web resource (GEO, <http://www.ncbi.nlm.nih.gov/gds>) in order to assess the RNA expression of cytokines receptors in SK-MEL-28 and A375 cell lines. In particular, the dataset GDS1314, (<http://www.ncbi.nlm.nih.gov/sites/GDSbrowser?acc=GDS1314>) was used. This dataset show the expression profiling of Mel JUSO, A375, 607B, 518A2, and SK-MEL-28 human malignant melanoma cell lines. Through the dataset analysis of SK-MEL-28 and A375 cell lines, the IL-2 receptor (IL-2R), IL-12 receptor beta 1 (IL-12RB1), CSF 2 receptor alpha (CSF2RA), IFN-γ receptor 1 (IFNGR1) and IFNGR2 gene expression profile were achieved.

We used the Protein Atlas online immunohistochemistry resource (<http://www.proteinatlas.org>) for expression levels of cytokine receptors in melanoma tumor sections (Uhlén et al., 2005; Berglund et al., 2008).

2.9. Statistical analysis

All experiments were repeated three times, and the results were expressed as the mean ± S.D. Statistical significance of differences among group means were assessed by an unpaired student's *t* test, two tails, for single comparisons and by analysis of variance for multiple comparisons. Values of $P < 0.05$ were

considered significant. The Prism software (Graph Pad Inc., La Jolla, CA) were used.

3. Results

3.1. Aloe-emodin affects melanoma cell growth

In order to investigate whether AE could affect proliferation of human SK-MEL-28 melanoma cells, increasing concentrations (5, 10, 20, 30 and 60 µM) of the anthraquinone were used. As shown in Fig. 1A, AE decreased tumor cell growth in a concentration-dependent manner. The lowest dose of AE (5 µM) did not affect cell proliferation. No significant changes in cell viability (assessed by the Trypan Blue exclusion test) were found; in fact cells were still 90–100% viable after 72 h of incubation, except for the highest (60 µM) AE concentration (data not shown) where AE sometimes showed solubility problems. Since anthraquinones are fluorescent molecules, we decide to investigate the intracellular AE distribution in cultured SK-MEL-28 by fluorescence microscopy (Fig. 1B). The fluorescence signal of AE was detectable already after 10 min (data not shown) mostly in the cytoplasm. After a longer exposure, AE was also found in the nucleus and as vesicles of increased diameters. Moreover, DNA content and complexity was evaluated using flow cytometry after PI staining of nuclei. As shown in Fig. 1C, treatment with AE resulted in a time-dependent increase in the distribution of cells at G2/M and S phases. Similar results were previously reported in A375 cell line (Radovic et al., 2012). Therefore, the antiproliferative effects of 30 µM AE was evaluated also in A375 melanoma cells (Fig. 1D). Moreover, we found that 30 µM AE impairs cell proliferation in a time-dependent manner (Supplementary Fig. 1A) in two additional human melanoma cells, SK-MEL-110 and M14.

3.2. Effects of aloe-emodin on differentiative state, adhesion and invasion abilities of melanoma cells

To investigate whether antiproliferative activity of AE could be related to the stimulation of differentiation in melanoma cell lines, possible changes of some differentiative parameters, i.e. TG2 activity and melanin synthesis, commonly considered melanocyte differentiation markers, and protoporphyrin IX (PpIX) accumulation (Ickowicz Schwartz et al., 2004) were considered. Melanin synthesis in SK-MEL-28 cells was increased with respect to the control, by about 50% after 48 h of AE treatment, and 2-fold after 72 h (Fig. 2A). PpIX amount was significantly increased (Fig. 2B), compared to controls, after 48 h and 72 h of anthraquinone exposure by about 2- and 10-fold in SK-MEL-28 and by about 1- and 6-fold in A375 cells, respectively. TG2 activity in AE-treated SK-MEL-28 cells (Fig. 2C) was increased by 3.5 fold after 48 h and by 2-fold after 72 h. In A375 cells too (Fig. 2C), the enzyme activity showed a time-dependent increase. Similar increase in TG2 activity was found in SK-MEL-110 and M14 human melanoma cells (Supplementary Fig. 1B). The expression of TG2 was determined in SK-MEL-28 melanoma cells treated with AE (Fig. 2D). To confirm these data, the whole SK-MEL-28 cell lysates were subject to Western blot analysis using antibodies which detect the activated or phosphorylated forms of traditional signaling proteins activated by TG2, such as Akt, components of the canonical PI3-kinase signaling cascade (Verma and Mehta, 2007). Again, Akt and p-Akt levels were unaffected by AE treatments (Fig. 2D). The enhancement of differentiation was also confirmed at morphological level. As known, cytoplasmic protrusions are recognized as a morphological indicator of melanocyte/melanoma cell differentiation (Bartolini et al., 2005). A375 are usually polygonal cells. Indeed, the morphology of A375 cells incubated with AE was markedly changed under AE treatment with respect to control (Fig. 2E). Since TG2 plays a pivotal role in metastatic

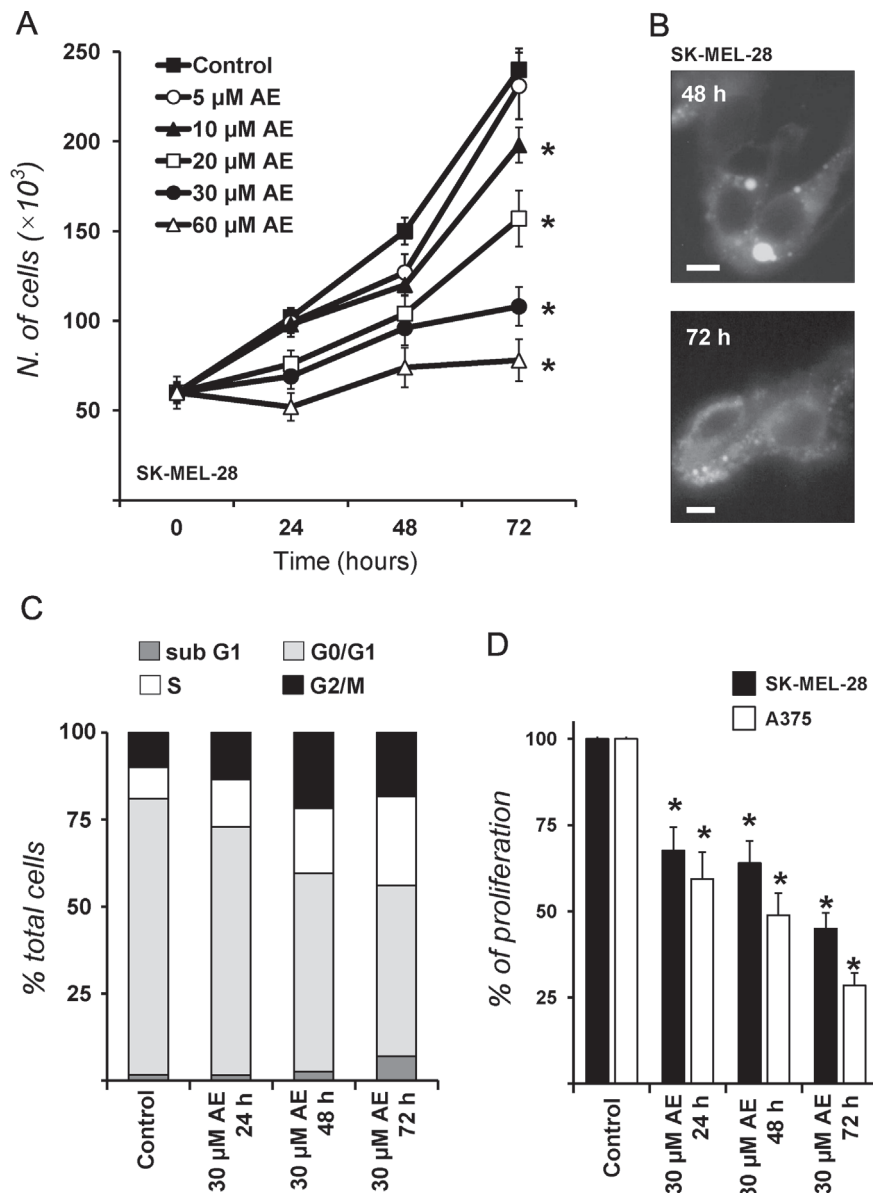


Fig. 1. Aloe-emodin (AE) inhibits the proliferation of melanoma cells. (A) Cell growth curve of SK-MEL-28 cells treated with 5, 10, 20, 30 and 60 μM AE for 24, 48 and 72 h. Control cells were incubated with DMSO 0.1% only. The data represent the mean \pm S.D. of three experiments carried out in duplicates (statistical significance versus control: $*P < 0.05$). (B) Distribution of AE in SK-MEL-28 cells treated with 30 μM AE for indicated times. The distribution of AE, a green autofluorescence compound, was examined using a fluorescence microscope. Representative photographs are shown (scale bar: 10 μm). (C) AE induces cell cycle arrest in S-G2/M phases in SK-MEL-28 cells. Flow cytometric analysis of the cellular DNA content was performed after propidium iodide staining (statistical significance versus control: $P < 0.05$). (D) Cell proliferation of SK-MEL-28 and A375 cells treated with 30 μM AE for 24, 48 and 72 h (statistical significance versus control: $*P < 0.05$).

cascade (Tabolacci et al., 2012), the effects of AE on metastatic potential of SK-MEL-28 and A375 cells were analyzed. Tumor metastasis occurs through a complex series of events. Cancer cells exhibit a variety of properties, including altered adhesiveness and invasive ability, to complete the metastatic process. Since tumor cell adhesion to basement membrane is considered as an essential step for cancer metastasis, we examined the adhesion capacity of SK-MEL-28 and A375 melanoma cells on MG-coated surface. As shown in Fig. 2F, AE significantly reduces with respect to the control, the adhesion pattern of SK-MEL-28 (23% and 52% decrease after 48 and 72 h of AE treatment) and A375 (64% and 87% decrease after 48 and 72 h of AE treatment) melanoma cells. Similarly, invasion (Fig. 2G), estimated by the Boyden chamber assay, was impaired. In fact, AE-treatment strongly decreased the number of SK-MEL-28 and A375 treated cells that were able to cross the MG-coated filters.

3.3. Immunomodulatory effects of aloe-emodin

The connection between cancer and inflammation is well known (Alatrash et al., 2013; Kolb et al., 2014). To measure the effect of AE on cytokines profile of SK-MEL-28 and A375 melanoma cells, we took advantage of the sensitive Bio-Plex system. A set of 4 cytokines was analyzed. As shown in Fig. 3, the levels of IL-2, IL-12, GM-CSF and IFN- γ are increased after AE treatment. Since malignant melanoma cells produce different cytokines with autocrine effects, after bioinformatic analysis (GEO) of the expression of receptors of studied cytokines (Supplementary Fig. 2A), the effects of GM-CSF and IFN- γ on SK-MEL-28 cell proliferation were investigated. In fact, GEO analysis revealed high CSF2RA, IFNGR1 and IFNGR2 gene expression in SK-MEL-28 cells. Moreover, as shown in Supplementary Fig. 2B, the presence of indicated cytokine receptors in melanoma samples were validated using the

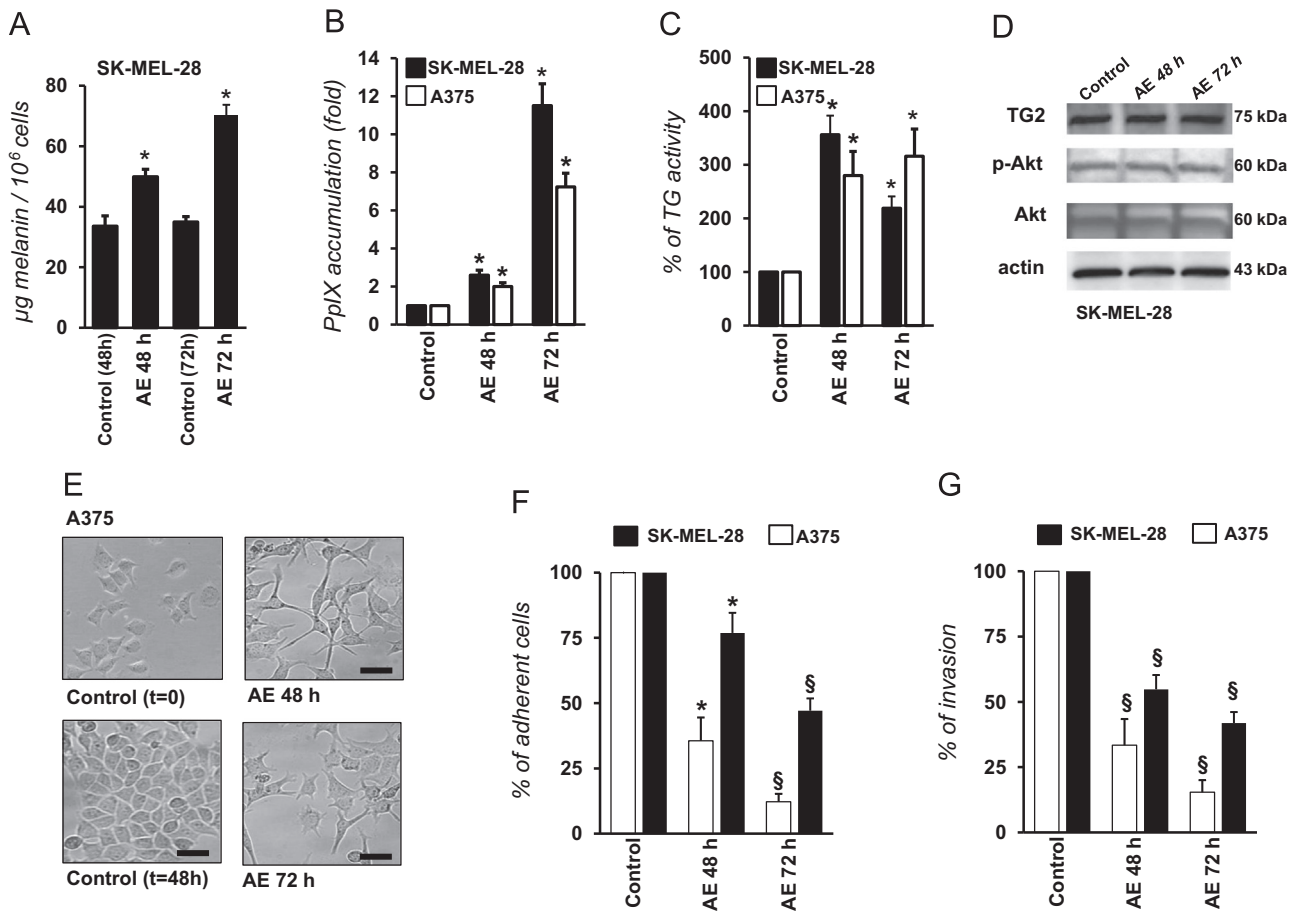


Fig. 2. Induction of differentiation and antimetastatic effects of 30 μ M aloe-emodin (AE) on human melanoma cells. (A) Determination of melanin content in SK-MEL-28 cells. The melanin content was expressed as μ g of melanin/10⁶ cells. (B) Evaluation of protoporphyrin IX (PpIX) accumulation. (C) Determination of transglutaminase (TG) transamidating activity. (D) Western blot analysis of SK-MEL-28 cell lysates showing TG2, Akt and p-Akt protein expression. Equal amounts of proteins from cell lysates were loaded. (E) Light microscopical appearance of untreated and 30 μ M AE-treated A375 melanoma cells after 48 and 72 h of exposure. Typical morphological signs of melanocyte differentiation appear upon AE treatment (dendritic shape; scale bar: 40 μ m). Aloe-emodin (AE) reduces adhesion and invasion ability of SK-MEL-28 and A375 melanoma cells. (F) Effect of 30 μ M AE on *in vitro* adhesion to MG. (G) AE (30 μ M) inhibits the *in vitro* invasion of melanoma cells (Boyden Chamber technique). Data are plotted as the mean \pm S.D. (statistical significance versus control: * P < 0.05; \S P < 0.01).

protein atlas immunohistochemistry database (Uhlén et al., 2005). In particular, CSF2RA is high expressed in melanoma with respect to other tumor samples (<http://www.proteinatlas.org>). We found that GM-CSF alone did not affect cell proliferation, whereas the combination of AE and the lowest concentration of GM-CSF potentiated its antiproliferative ability (Supplementary Fig. 2C) similarly to that found in combination with lowest dose of IFN- γ . However, IFN- γ decreased the proliferation of melanoma cells in a dose-dependent manner (Supplementary Fig. 2D).

3.4. Aloe-emodin interferes with the growth and metastatic potential of melanospheres

The CSCs model assumes that only a fraction of cells within a tumor has the capacity to initiate and sustain tumor growth (Eramo et al., 2008). Hence, new drugs that specifically target CSCs (i.e. MICs) have a very important therapeutic potential. To this aim, we investigated the effects of AE on Mel1 melanospheres, a MICs surrogate. Treatment of Mel1 cells with 10 μ M AE affected cell proliferation; in fact it reduced cell growth by about 32% after 48 h and 50% after 72 h (Fig. 4A). To further elucidate the mechanism of growth inhibition by AE, melanospheres were analyzed for cell cycle distribution. In contrast to that found in commercially available cell lines, a significant increase in sub-G1 cells was observed (Fig. 4B). Flow cytometric analysis showed that AE-treated Mel1 cells exhibited a progressive increase in Annexin V⁺/7AAD⁻

staining and Annexin V⁺/PI⁺. These results indicate that AE induced early apoptosis and late apoptosis/necrosis in a time-dependent manner (Fig. 4C). The expression of CD44v6, an antigen associated with MICs (Sette et al., 2013) was then investigated. In fact, melanospheres expressed high levels of this stem cell-related marker, while treatment with AE reduced the percentage of positive cell by 21% (Fig. 4D). The possible antimetastatic activity of AE was also evaluated. The number of adherent cells was unchanged after treatment (data not shown). On the contrary, the invasion experiments showed a decrease of the invasive potential of AE-treated Mel1 cells, with respect to the control, by 28% after 48 h of exposure (Fig. 4E).

3.5. Aloe-emodin potentiates the effect of dabrafenib on melanoma cell lines and melanospheres

SK-MEL-28 cells are generally considered a weakly sensitive or resistant cell line toward many chemotherapeutic drugs (Fuggetta et al., 2004; Chen et al., 2009; Thangasamy et al., 2010). Further, since they were isolated from a BRAF mutated melanoma, they represent also an optimal cellular model to investigate the effects of BRAF inhibitors and potentially the mechanisms of resistance. The antiproliferative effect of AE, in combination with CDDP, DTIC and MTX was examined. We found that the combination of AE and the lowest concentrations of the chemotherapeutic drugs potentiated their antiproliferative ability (Supplementary Fig. 3). Since SK-MEL-28, A375

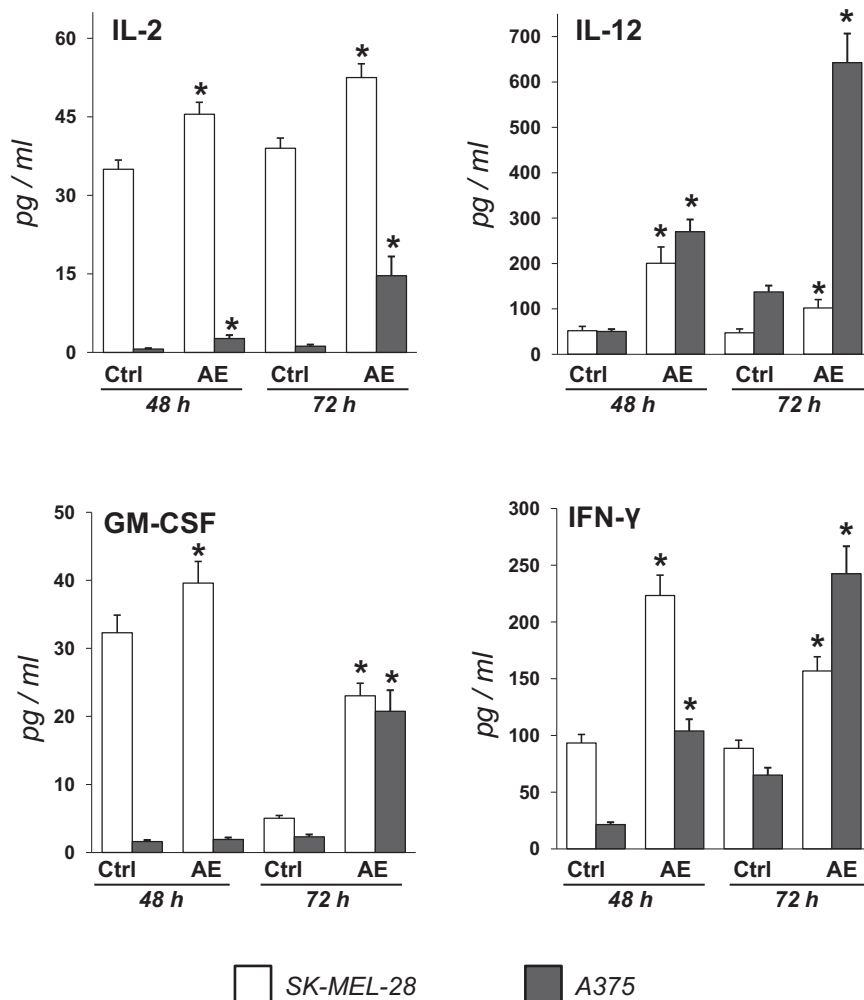


Fig. 3. Immunomodulatory effects of aloe-emodin (AE) on SK-MEL-28 and A375 melanoma cells. Analysis of levels of the indicated cytokines, after 48 or 72 h of AE exposure, was performed using a multiplex flow immunoassay. The data presented in each point are the average \pm S.D. of three different determinations (statistical significance versus control: * $p < 0.05$).

(Pal et al., 2014) and Mel1 cells (Sette et al., 2013) are BRAF-mutated (B-Raf^{V600E}) cell lines, we wanted to evaluate the possibility of a synergistic of AE and DAB, a well-known BRAF inhibitor. To this aim, AE was tested alone or in combination with DAB on cell lines and melanospheres. As shown in Fig. 5A, AE potentiated the antiproliferative effects of DAB (0.1–1 μ M) in SK-MEL-28 cells. We found similar effects on DAB-sensitive A375 melanoma cells (Fig. 5B). The effects of DAB on cell cycle and induction of apoptosis were analyzed in A375 and SK-MEL-28 cells. While the level of sub-G1 and G0/G1 cells are comparable in the two cells lines treated with DAB, the effects of combined treatments differ between SK-MEL-28 and A375 cell lines (Supplementary Fig. 4). Interestingly, AE and DAB alone revealed comparable activity on Mel1 cells, whereas a slight but significant ($P < 0.005$) increase in antiproliferative activity appeared when drugs were used in combination (Fig. 5C).

4. Discussion

The incidence of melanoma has increased over the past several decades. The role of diet and nutrition, such as aloe products, in cancer prevention has recently become a very popular subject (Chen et al., 2014). The results presented here show that AE induces differentiation and inhibits invasive potential in human melanoma cells, similarly to that found for murine B16-F10 cells

(Tabolacci et al., 2010). Adhesion and invasion are important characteristics of cancer cell metastasis. We demonstrated that AE significantly inhibits both human melanoma cell invasion and adhesion to reconstituted based membrane (MG). The observed reduction of adhesiveness and invasiveness was associated to an increase of the differentiative state of SK-MEL-28 and A375 cells, as shown by the AE-mediated enhancement of intracellular TG2 activity, a known cell differentiation marker (Ozpolat et al., 2001), as well as by the increase of melanin content and PpIX accumulation. Several studies demonstrated that the increased expression of TG2 in cancer cells was linked to increased drug resistance, metastasis and poor patient survival (Verma and Mehta, 2007; Budillon et al., 2013). We found that TG2 expression was unaffected by the anthraquinone treatment. This result is particularly interesting because, while the expression of TG2 is a negative event for tumor progression, the increase of its transamidating activity was associated to a positive effect in cancer cell growth and metastatic potential control (Barnes et al., 1985; Tabolacci et al., 2012). The immune system plays an important role in the surveillance and initial control of tumor growth and diffusion. The outcome of the tumor immuno-surveillance is dependent from many factors (Sivendran et al., 2014) including the dynamic of cytokine secretion (Wilmott et al., 2014). Cytokines released by melanoma cells may contribute to modulate and regulate the ongoing immune response against tumor cells. In this light, we

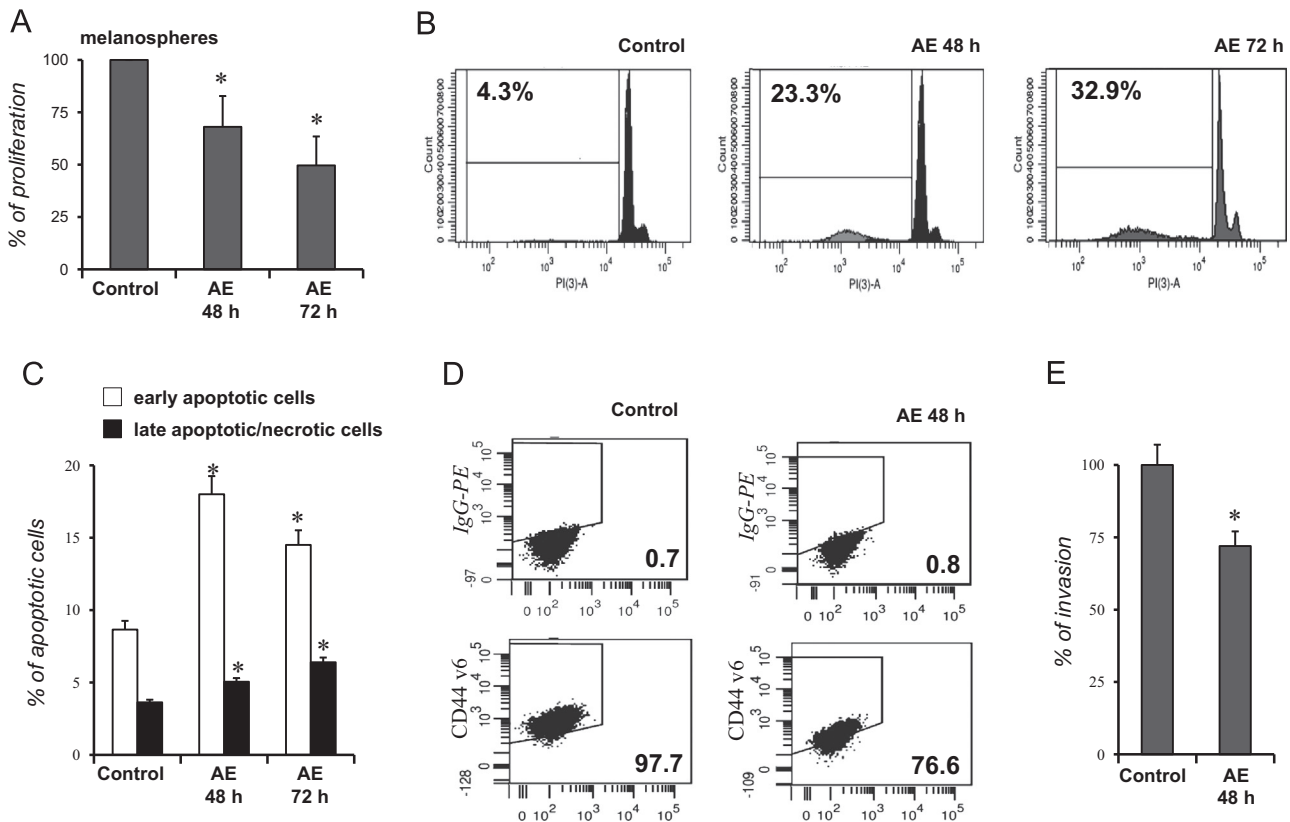


Fig. 4. Antineoplastic activity of aloe-emodin (AE) on Mel1 melanoma stem-like cells (melanospheres). (A) Antiproliferative activity of 10 μ M AE in Mel1 cells treated for 48 and 72 h. (B) AE induces apoptosis in Mel1 cells. Cells were treated with AE at the indicated times, and DNA content and fragmentation was assessed via propidium iodide staining through flow cytometry. Percentages of subG1 stage (apoptotic cells) of the cell cycle are indicated. (C) Apoptosis was also detected by Annexin V/7AAD staining assay. The percentages of early and late apoptotic/necrotic cells were measured. (D) FACS analysis of CD44 variant 6 (CD44v6), a stem cell-associated antigen, in melanospheres. Isotype control antibody was used as negative control (upper panels). Numbers indicate the percentage of positive cells. (E) Anti-invasive effect of 10 μ M AE treatment on Mel1 cells, using the Boyden Chamber technique. Data are expressed as percentage of the control (100%) and represent the means \pm S.D. of three separate experiments (statistical significance versus control: * $p < 0.05$).

investigated the influence of AE treatment of SK-MEL-28 and A375 cells in terms of cytokines/chemokines secretion. Interestingly, AE treatment seems stimulate cytokines with anticancer properties. Among these cytokines, IL-2, IL-12 and IFN- γ are of particular interest. IL-12 exhibits its antitumor activity by stimulating IFN- γ secretion and promoting T helper (Th)1 cells differentiation (Colombo and Trinchieri, 2002). This positive connection between IL-12 and IFN- γ is maintained in AE-treated melanoma cells. IL-2 is a growth factor for antigen-stimulated T lymphocytes and it has been used in the treatment of malignant melanoma and renal carcinoma (Sonpavde and Choueiri, 2012). Since systemic administration of IL-2 or IL-12, in cancer immunotherapy, induces severe side effects, the local stimulation of these cytokine by AE in the tumor microenvironment appears particularly promising for future anticancer strategies. This hypothesis is corroborated by our previous work, in which we demonstrated that AE is able to induce the production of anticancer cytokines, such as IL-12, also in U937 leukemia cells (Tabolacci et al., 2011). Interestingly, it has been demonstrated that the serum levels of IL-12 and IFN- γ in TG2 $^{-/-}$ mice are significantly decreased (Falasca et al., 2005), as well as in dendritic cells after the inhibition of TG2 cross-linking activity (Matic et al., 2010). Another cytokine with anticancer activity, GM-CSF, was stimulated by AE. In fact, it has been demonstrated that GM-CSF in combination with IL-12 increases the efficacy of melanoma cell vaccines (Miguel et al., 2013). Malignant tumors escape from exogenous regulatory mechanisms by expressing autocrine growth factors and cytokines. Autocrine growth factors produced by melanoma cells stimulate proliferation of the producing cell itself (Lázár-Molnár et al., 2000). After analysis of expression of

cytokines receptors by GEO, we focused our attention on GM-CSF and IFN- γ , demonstrating a possible autocrine effect in addition to paracrine one. In particular, we found that GM-CSF and IFN- γ affect SK-MEL-28 proliferation and that AE sensitizes this cell line to low doses of these cytokines/chemokines. Interestingly, the expression of CSFR2A seems to be very high in melanoma samples (<http://www.proteinatlas.org>). Moreover, since our data are supported by previous reports that emphasize the use of IFN- γ in cancer immunotherapy (Windbichler et al., 2000) and GM-CSF as adjuvant in melanoma therapy (Grotz et al., 2014), the stimulation of these cytokines through natural molecules and dietary compounds with autocrine and paracrine effects opens new intriguing possibilities. The antiproliferative effects of AE were then compared to those exerted by CDDP, DTIC and MTX, as conventional antineoplastics. Here, we have demonstrated that the combined treatment of SK-MEL-28 melanoma cells (a typical drug-resistant cell line) with AE and low concentrations of chemotherapeutic drugs enhanced the antiproliferative activity of the anthraquinone. It noteworthy that the cytotoxicity was lower than that found when using the single chemotherapeutic drug at the highest concentrations. In any case, even if the ability of anthraquinones to enhance the chemosensitivity was reported (Li et al., 2009; Zhang et al., 2013), the use of AE in combination with chemotherapy agents in melanoma deserves additional investigation. Although chemotherapy is one of the main approaches for cancer treatment, its effectiveness is limited by intrinsic or acquired drug resistance. Recent reports emphasize that tumors may contain a high degree of molecular and/or cellular heterogeneity and drug resistances may arise through therapy-induced selection of a resistant

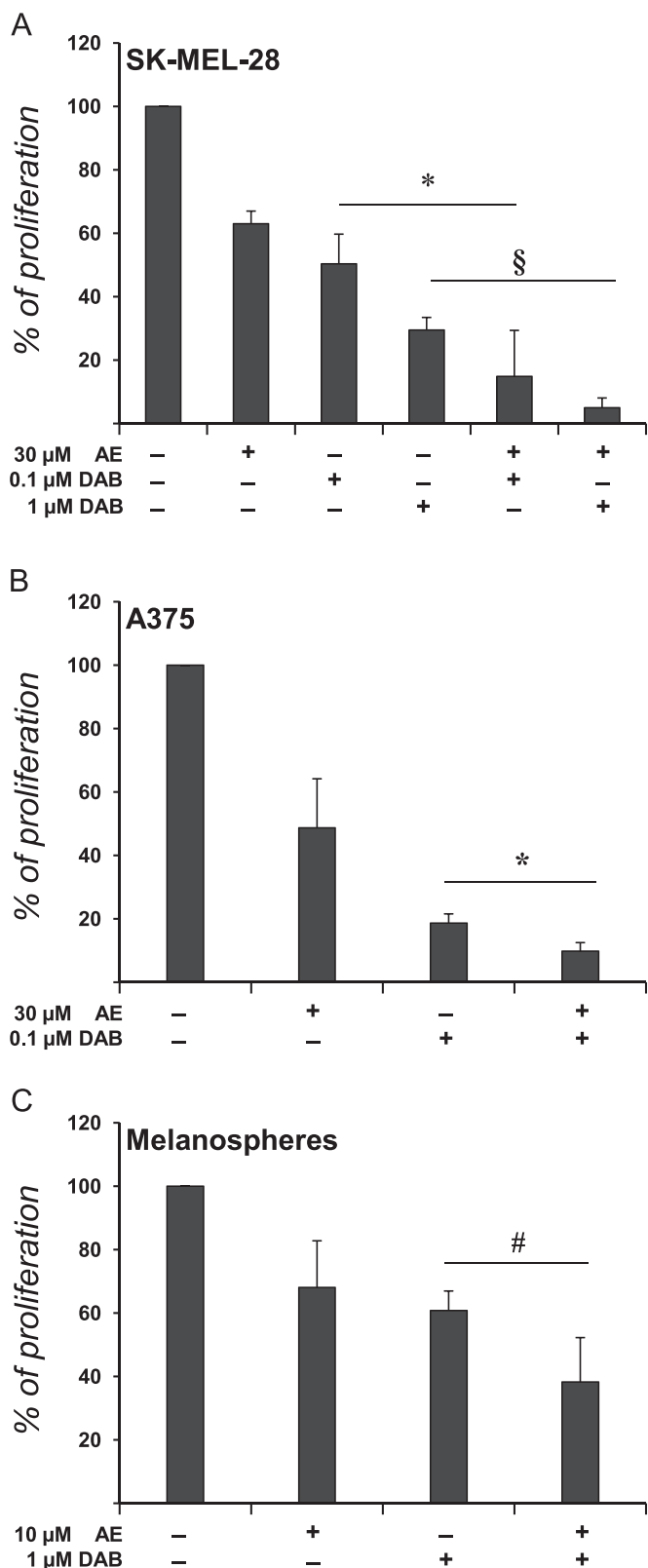


Fig. 5. Growth inhibitory effect of 30 μM aloe-emodin (AE) alone and in combination with the BRAF inhibitor dabrafenib (DAB), on SK-MEL-28 (A), A375 (B) and human melanoma Mel1 stem-like (C) cells. Data are reported as the mean ± S.D. (statistical significance: * $p < 0.05$, § $p < 0.01$, # $p < 0.005$).

subpopulation of CSCs present in the original tumor (Holohan et al., 2013; Cojoc et al., 2015). Interestingly, several natural molecules have been demonstrated to have anticancer stem cell

activity (Sotiropoulou et al., 2014). Our results show for the first time that AE efficiently inhibits cell viability and likely induce apoptosis in melanoma stem-like cells, although further studies to elucidate the mechanisms of such effects are required. In particular, our data reveal that AE treatment is sufficient to reduce CD44v6, a CSC surface marker (Todaro et al., 2014), as well as invasion ability of melanospheres. On the basis of our results, it is reasonable to believe that AE is a good drug candidate to target MICs. More detailed characterization of AE effects on melanospheres deserves further investigation (ongoing studies).

During the last few years several targeted therapies have proved effective against metastatic melanoma. These recent advances were facilitated by an improved understanding of the driving oncogenic mutations in melanoma, particularly alterations in the mitogen-activated protein kinase (MAPK) pathway (including BRAF and NRAS mutations), which contribute to the uncontrolled proliferation of cancer cells. DAB is a reversible and potent ATP-competitive inhibitor that selectively inhibits the BRAF^{V600E} kinase (Gibney and Zager, 2013). Although DAB is effective as a single-agent treatment, resistance appears in most patients that thereafter have a poor clinical outcome. For this reason it has been suggested that combination therapy might expand the efficacy of targeted drugs (Tentori et al., 2013). In this work we demonstrated that treatment with AE potentiate the effects of DAB on human melanoma cells and melanospheres. This study represents the first report indicating a synergistic effect of the combination of natural drug with DAB, a conventional BRAF inhibitor, suggests new interesting and effective therapeutic approaches. However, the ability of natural compounds to potentiate BRAF inhibitors activity needs further investigations.

5. Conclusion

The application of differentiation therapy seeks to reverse the loss of the differentiated state and forces cancer cells to resume a more mature phenotype, allowing them to regain the morphology and function of mature cells (Cruz and Matushansky, 2012). The present study suggests that this approach may be combined with the more conventional cytotoxic chemotherapy to interfere more effectively with cancer progression. In this study we also demonstrated the possible use of a differentiative molecule as immunomodulatory agent. Tumor persistence or recurrence is probably due to heterogeneous tumor–host interactions within the tumor microenvironment as well as to an inadequate immune response towards tumor antigens. Both factors may be effectively influenced by AE. In conclusion, our data suggest that AE by itself or in combination with the conventional chemotherapeutic or targeted regimens can be a new effective therapeutic strategy to prevent the emergence of cancer resistant cells by reducing the CSCs.

Declaration of interest

The authors report no conflicts of interest.

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Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ejphar.2015.05.057>.

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