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ORIGINAL ARTICLE

Aloin enhances cisplatin antineoplastic activity in B16-F10 melanoma cells by transglutaminase-induced differentiation

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Abstract Aloin, a natural anthracycline from aloe plant, is a hydroxyanthraquinone derivative shown to have antitumor properties. This study demonstrated that aloin exerted inhibition of cell proliferation, adhesion and invasion abilities of B16-F10 melanoma cells under non-cytotoxic concentrations. Furthermore, aloin induced melanoma cell differentiation through the enhancement of melanogenesis and transglutaminase activity. To improve the growth-inhibiting effect of anticancer agents, we found that the combined treatment of cells with aloin and low doses of cisplatin increases the antiproliferative activity of aloin. The results suggest that aloin possesses antineoplastic and antimetastatic properties, exerted likely through the induction of melanoma cell differentiation.

Keywords Anthraquinone · Differentiation · Melanogenesis · Invasion · Metastasis

Introduction

Melanoma is rapidly increasing in incidence throughout the world. Melanoma patients generally undergo surgical therapy, chemotherapy, radiotherapy, or a combination of these treatments. The foremost goal of chemotherapy is to destroy presumed undetectable micro-metastases and prolong overall survival (El-Sawy et al. 2002). Cisplatin (CDDP), dacarbazine and 5-fluorouracil represent the most common drugs used in cancer chemotherapy (Tawbi and Buch 2010), as well as anthracyclines (i.e. doxorubicin), whose major biological effect is cytostatic action, transforming into cytotoxicity (Skladanowski and Konopa 1993). Moreover, recent evidences have implicated BRAF mutations in the pathogenesis of melanoma. Indeed, BRAF inhibitors are now undergoing clinical trials (Cantwell-Dorris et al. 2011). Although the effects of chemotherapeutic treatments are significant, it is a fact that most patients suffer from side-effects. Thus, the search for novel chemotherapeutic and chemopreventing drugs with less toxicity, particularly from natural sources, is of great importance. Some literature data reported important anticancer effects of phytochemicals linked to the reduction of polyamine production and to the induction of tumor cell differentiation, through the activation of intracellular transglutaminase (TG; EC 2.3.2.13) (Facchiano et al. 2006; Lentini et al. 2010a). Polyamines putrescine (PUT), spermidine (SPD) and spermine (SPM) may be covalently conjugated to glutaminyl residues of polypeptides through a transamidation reaction catalyzed by TG (Folk 1980). Transglutaminases are a widely distributed and peculiar group of enzymes that catalyze the post-translational modification of proteins by the formation of isopeptide bonds. This may occur either through protein cross-linking via ε-(γ-glutamyl)lysine bonds or through the incorporation of primary amines (i.e. polyamines) into peptide-bound glutamine residues (Folk and Finlayson 1977). Increasing evidences showed a direct correlation between TG activation, reduction of cancer growth, and stimulation of melanoma cell differentiation (Lentini et al. 2009), where the

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induction of differentiation was also assessed by the increase of melanin synthesis, considered as typical differentiation marker, as well as the enhancement of tyrosinase (TYR, EC 1.14.18.1) activity. This enzyme catalyzes the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA), the oxidation of L-DOPA to dopaquinone, and the oxidation of 5,6-dihydroxyindole to indole-quinone (Hearing and Tsukamoto 1991). Then, the involvement of TG was also extended to tumor cell metastatic spread, an event depending on tumor cell adhesion to basement membranes and to the synthesis of matrix metalloproteinases (MMPs) (Baker et al. 2002), as investigated recently in vivo (Di Giacomo et al. 2009). Many studies have indicated the anticancer activity of several well-studied secondary metabolites from aloe plant exudate, which is commonly used in food products, pharmaceuticals and cosmetics, due to its beneficial properties (Korkina et al. 2003). Biologically active substrates in aloe include anthraquinones, anthrone derivatives, polysaccharides and chromones (Tom 2004). Aloin (AL; 10-glucopyranosyl-1,8-dihydroxy-3-hydroxymethyl-9(10H)-anthracenone), a naturally occurring anthracycline phytoanalog, is the principal constituent (15–40%) of the aloe plant juice obtained from leaf exudates of *Aloe ferox* Mill. and *Aloe vera* L. (*A. barbadensis* Mill.) (van Wyk et al. 1995). AL (Fig. 1) is characterized as the C-glycoside of an anthraquinone known as aloe-emodin (Reynold 1985). Biological effects of AL have been examined in a limited number of studies. Recent reports pointed out a potential cytotoxic effect of AL through the induction of S-phase cell cycle arrest and apoptosis in human epithelial type breast and ovarian tumor cell lines (Esmat et al. 2006) and in human uterine carcinoma HeLaS3 cells (Nićiforović et al. 2007). Moreover, AL increased the alcohol oxidation rate (Chung et al. 1996) and inhibited azoxymethane-induced DNA adduct formation in rats (Shimpo et al. 2003). To extend the knowledge on the anticancer role of anthraquinones, the purpose of our work is to investigate the possible role of TG on the antiproliferative, differentiative and antimetastatic properties of AL on the B16-F10 murine melanoma cell line. Moreover, the antineoplastic effects of the combined treatment of melanoma cells with AL and CDDP were investigated.

Materials and methods

Materials

Dulbecco's modified Eagle's 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)

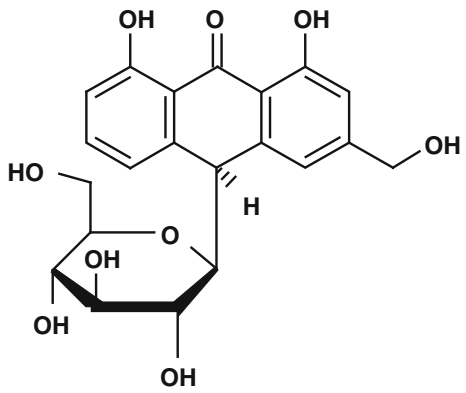

Fig. 1 Chemical structure of aloin (10-glucopyranosyl-1,8-dihydroxy-3-hydroxymethyl-9(10H)-anthracenone)

was from Gibco (Grand Island, NY, USA). [^{14}C]-methylamine (46.6 mCi/mmol) and [^{3}H]-PUT (40.35 Ci/mmol) were purchased from Amersham International (Bucks, UK). All solvents came from Mallinckrodt Baker (Milan, Italy). AL, CDDP, Matrigel (MG), sodium citrate, phenylmethylsulfonylfluoride (PMSF), Tris, dimethylcasein, guinea pig liver TG (GPL-TG), L-DOPA, ethylenediaminotetraacetic acid (EDTA), trichloroacetic acid (TCA), perchloric acid (PCA), *o*-phtalaldehyde (OPA), and all reagents were from Sigma Chemicals (St. Louis, MO, USA).

Cell cultures and proliferation assay

Highly metastatic murine B16-F10 melanoma cell line was purchased from the Division of Cancer Treatment, Tumor Repository NIH (Frederick, MD, USA) and propagated under standard culture conditions. Cells were cultured in DMEM containing 10% FCS, 0.05% L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) and maintained at 37°C with 5% CO_2 in a humidified atmosphere. Before each treatment, the cell line was starved for 24 h to rule out possible interferences with cell growth due to serum components and promote synchronization. For proliferation studies, B16-F10 melanoma cells were seeded and grown in 35 mm dishes in DMEM supplemented as reported above, and treated with AL (at 25, 50, 100 and 500 μM final concentration) for 24, 48 and 72 h. For combined therapy cells were treated with CDDP (at 0.1, 1 and 2 μM final concentration) with or without AL (500 μM) for 48 h. Cells were harvested and counted with a Neubauer modified chamber, after Trypan Blue staining for cytotoxicity evaluation.

Determination of intracellular polyamine levels

Determination of polyamines in culture cell lysates was performed by an high-performance liquid chromatography (HPLC) method with an AKTABASIC 10 HPLC apparatus

(Amersham Pharmacia Biotech., Milan, Italy), as previously reported (Baldini et al. 2006). Briefly, the separation was performed using a pre-column derivatization. Cell lysates were deproteinized with PCA, centrifuged (14,000*g* for 15 min), and supernatants filtered. The samples were derivatized with OPA (1:1). The OPA reagent was prepared dissolving 4 mg in 1 ml of methanol, adding 4.74 ml of borate buffer (1 M, pH 9.5) and 10 µl of β-mercaptoethanol. 100 µl of mixture was injected into the HPLC. The reverse-phase separations were conducted at room temperature with a LC-18 Supelcosyl column (Supelco, Milan, Italy). Detection was accomplished using a spectrofluorimeter (Jasco FP–1520, Easton, MD). Fluorescence detector was set at λ_{ex} 330 nm and λ_{em} 445 nm.

Transglutaminase activity assays

For intracellular TG activity (in vivo TG assay), cells plated on 100-mm petri dishes (1×10^6) were grown in the presence of [^{14}C]-methylamine (0.5 µl/ml DMEM) either in the absence or in the presence of 0.5 mM AL. After 48- and 72-h incubation, cells were detached by scraping, washed three times with PBS, and proteins precipitated in 10% TCA, washed extensively and solubilized in 0.1 N NaOH at 37°C. Radiolabeled amine incorporation into cell protein was measured with a scintillation counter (Beckman LS–5000TD, Fullerton, CA, USA).

The possible interference of AL on TG activity was investigated by an in vitro TG assay. GPL-TG (4 µg) was incubated in the presence of AL (500 µM final concentration) in assay buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2.5 mM $CaCl_2$, 15 mM dithiotreitol, 0.5 mg dimethylcasein and 2.5 mM [^{3}H]-PUT. The mixture, with a final volume of 250 µl, was incubated at 37°C for 1 h. Reaction was stopped with 10% TCA, proteins were precipitated, centrifuged and solubilized with 0,1 N NaOH at 37°C. Radiolabelled PUT incorporation into dimethylcasein was measured as described above.

Cellular tyrosinase activity and melanin content

Tyrosinase activity on B16-F10 cells was assessed by measuring the rate of L-DOPA oxidation (Yokozawa and Kim 2007). Cells were plated in 6-well dishes and treated with 500 µM AL. Cells were lysed with 50 mM sodium phosphate buffer (pH 6.8) containing 1% Triton X-100 and PMSF (0.1 mM) and frozen at −80°C for 30 min. Cell lysate was thawed, mixed, and centrifuged. Eighty (80) µl of supernatant and 20 µl of L-DOPA were placed in a 96-well plate, and the adsorbance at 490 nm was monitored at 37°C in a microplate reader every 10 min for 1 h. Determination of intracellular melanin content in both control and AL-treated cells was performed as previously described (Lotan and Lotan 1980).

Adhesion and invasion assays

The adhesion assays were performed on 24-well plates coated with 50 µg MG. Unbound surfaces were blocked with 3% BSA in DMEM for 30 min at 37°C and then aspirated prior to the addition of cells. Control and AL-treated cells were harvested and resuspended in 0.02% BSA in DMEM. A total of 8×10^5 cells/well were incubated for 1 h at 37°C. Cells were detached with trypsin/EDTA and counted.

Branching process (cytoplasmic protrusions) formation was assessed by 3D-invasion technique. For this assay, cells (4×10^5) 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. The number of cellular processes per cell was evaluated under light microscope.

Invasion assay was performed in a modified Boyden chamber (Albini et al. 1987). Briefly, a total of 1×10^6 B16 cells (resuspended in serum-free medium) were added to the upper compartment, and NIH-3T3 murine fibroblast-conditioned medium as chemoattractant was added to the lower chamber. MG-coated 10-mm-thick polyvinylpyrrolidone-free polycarbonate filters (8 µm pore size) were placed between the two compartments. Chambers were incubated in a humidified 5% CO_2 atmosphere for 24 h at 37°C, and nonmigrated cells were gently wiped away from the upper surface of the filter. The filter was fixed by 100% ethanol, stained with Toluidine Blue solution and then mounted on a glass slide. The number of invasive cells was evaluated by means of Image J software (National Institutes of Health, Bethesda, Maryland, USA; http://rsb.info.nih.gov/ij/) and results expressed as percentage with respect of the control (100%). MMPs activity was assessed by gelatin zymography in B16-F10 melanoma cell-conditioned media, after treatment with 500 µM AL according to Troeberg and Nagase's method (2003).

Statistical analysis

All experiments were repeated three times, and the results are expressed as the mean ± SD of three different determinations. Data were analyzed by Student's *t* test and differences were considered highly significant when $p < 0.05$.

Results

Aloin affects melanoma cell growth and spermine levels

In order to investigate whether AL could affect proliferation of murine B16-F10 melanoma cells, a series of increasing concentrations of the anthraquinone was used. Cell number

was evaluated after 24, 48 and 72 h of treatment. As shown in Fig. 2a, treatments with 25 and 50 μM AL did not alter B16-F10 cell growth. On the contrary, cell proliferation was significantly decreased by 34.9% and by 82.8% after 72 h, using 100 μM and 500 μM AL, respectively. For this reason, all the experiments were carried out using 500 μM AL. All treatments did not cause cell injury, as assessed by the Trypan Blue exclusion test. In fact, cells were still 95–100% viable after 72 h of incubation (data not shown).

Polyamine levels were detected during the treatment of B16-F10 cells with 500 μM AL for the time selected. The results are shown in Fig. 2b. Since data of 24 h of incubation were similar to the control they are not reported. PUT was undetectable, and SPD levels were unchanged with respect to the control for all the time of incubation, whereas SPM levels were significantly decreased by 39.4 and 58.9%, after 48 and 72 h, respectively.

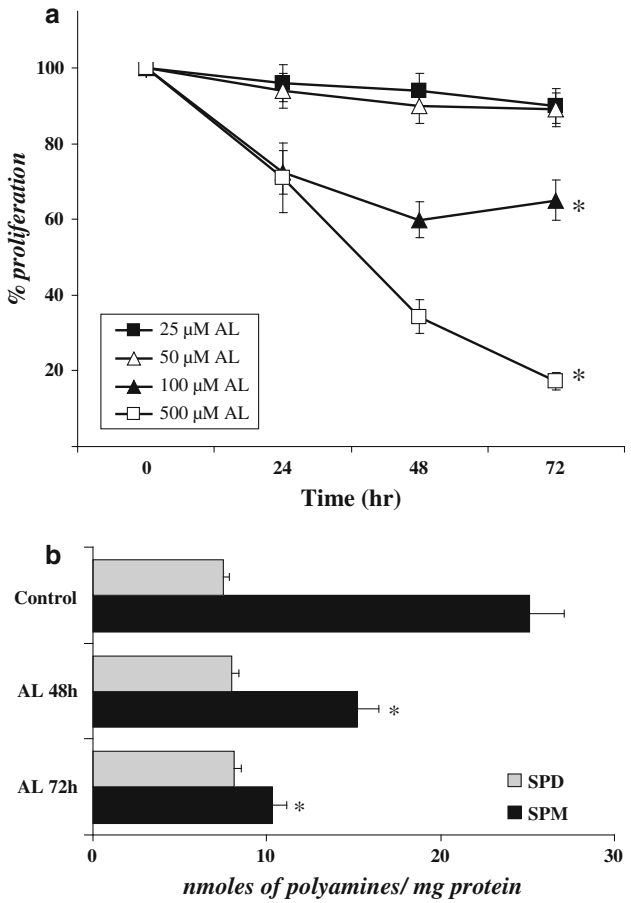

Fig. 2 Effects of aloin on B16-F10 melanoma cell proliferation and polyamine levels. **a** Proliferation curve of melanoma cells treated with 25, 50, 100 and 500 μM AL for 24, 48 and 72 h. Control cells were incubated with methanol 0.1% only. **b** AL reduces the intracellular levels of spermine (SPM) with no changes in spermidine (SPD) content. *The symbols* and *bars* represent the mean ± SD of three experiments carried out in duplicates (statistical significance versus control: $*p < 0.01$)

Aloin induces transglutaminase activity

Since TG and TYR activities and melanin synthesis are commonly considered differentiation markers for melanocytes, we checked if the antiproliferative activity of AL could be related to the stimulation of differentiation in the B16-F10 melanoma cell line. Treatment with AL significantly induced, compared with the control, an enhancement in TG activity by about 213% after 48 h and by 23% after 72 h of exposure (Fig. 3a).

Moroever, 500 μM AL direct in vitro incubation did not have effects on GPL-TG activity (data not shown). The enhanced differentiation induced by AL treatment was visible at microscopical level, where treated cells exhibited a starry-dendritic morphology (Fig. 3b).

Effect of aloin on melanogenesis

We analyzed the stimulation of TYR activity in AL-treated B16 melanoma cells, according to the procedure described above. Figure 3c shows that TYR activity was increased, with respect to the control, in cells treated with AL for 48 and 72 h. Changes of the intracellular levels of melanin in tumor cells incubated with AL at different times are shown in Fig. 3d. The amount of the pigment increased after 72 h only, by 58%, with respect to the control.

Aloin interferes with adhesion and invasion ability

The antimetastatic activity of AL was studied evaluating the adhesion and invasion pattern of 500 μM AL-treated B16-F10 cells. The adhesion assay (Fig. 4a) showed a decrease of the number of adherent cells (80% after 72 h), compared with the control. The reduction of plasticity in AL-treated cells was assessed by the 3D-invasion assay. As shown in Fig. 4b, B16-F10 cells responded to AL incubation by reducing markedly the branching-process formation. Similarly, invasion (Fig. 4c), estimated by the Boyden chamber assay, was impaired. In fact, the computerized analysis performed on MG-coated porous filters (Fig. 4d) showed a decrease of the invasive power of AL-treated B16-F10 cells, with respect to the control, by 64.2% after 72 h of treatment. In order to quantify the activity of secretory MMPs in B16-F10 cells treated with anthraquinone, we performed a gelatin-zymographic analysis. AL caused no changes in MMPs activity (data not shown).

Aloin sensitizes melanoma cells to cisplatin

The antiproliferative effect of AL, in combination with CDDP, was examined in B16-F10 cells after 48 h of exposure. As shown in Fig. 5, 2 μM CDDP treatment caused similar antiproliferative effects to 500 μM AL.

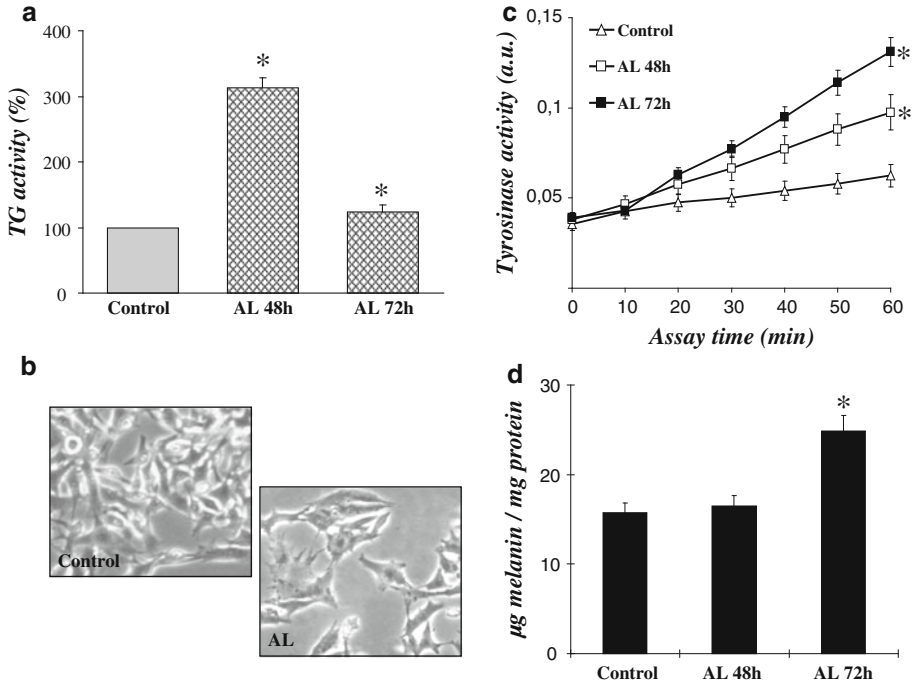

Fig. 3 Induction of differentiation in aloin-treated B16-F10 melanoma cells. **a** Determination of transglutaminase (TG) activity in B16-F10 melanoma cells treated with 500 µM AL, evaluated as incorporation of [^{14}C]-methylamine into cell protein. Results are expressed as percentage of the control value (100%). **b** Light microscopical appearance of untreated and 500 µM AL-treated B16-F10 melanoma cells after 72 h of exposure. Typical morphological signs of melanocyte differentiation appear upon AL treatment (dendritic shape; original magnification: ×200). **c** Cellular tyrosinase activity in B16-F10 cells after 48 and 72 h treatment with 500 µM AL. Results are expressed as percentage of control. **d** Determination of melanin content in melanoma cells after 48 h and 72 h treatment with 500 µM AL. *The bars* represent the means ± SD of at three separate experiments (statistical significance versus control: $*p < 0.01$)

Nevertheless, CDDP-induced cytotoxicity was higher than that caused by AL. Furthermore, AL in combination with 0.1 µM or 1 µM CDDP potentiated its antiproliferative ability, and no significant increase in cell toxicity occurred.

Discussion

Aloe plant has been used in Chinese medicine for a long time. Clarification of the mechanisms of action of the components of herbs may be important for developing their applications. Since aloe products are popular for the treatment of a number of disorders, establishing the mechanism of AL antineoplastic activity adds valuable data for consideration and may also be relevant for identifying potential therapeutic options in cancer. The results presented here show that AL induces differentiation and inhibits the invasive potential in B16-F10 melanoma cells, similarly to that found for aloe-emodin (Tabolacci et al. 2010). In particular we established that AL shows antiproliferative activity, without significant cytotoxic effects.

Polyamines have long been associated with tumor cell growth, and specific oncogenes and tumor-suppressor genes regulate their metabolism. Inhibition of polyamine synthesis has proven to be generally ineffective as an anticancer strategy in clinical trials, but it is a potent cancer chemoprevention strategy in preclinical studies. Clinical trials, with well-defined goals, are now underway to evaluate the chemopreventive efficacy of inhibitors of polyamine synthesis in a range of tissues (Gerner and Meyskens 2004). Since polyamines and their diamine precursor PUT are naturally occurring polycationic alkylamines that are essential for eukaryotic cell growth, we considered these biogenic amines as marker of cell proliferation (Seiler 2003). Therefore, our melanoma cell proliferation data have been strengthened by the reduction of SPM.

Furthermore, this paper is the first report on the effect of this anthraquinone on cellular TG activity, one of the markers of cell differentiation (Ozpolat et al. 2001). Moreover, we demonstrated that the increase in enzyme activity is not due to a direct interaction between AL and GPL-TG. The increase in TG activity induced by AL was paralleled with a significant increase in TYR activity, even though melanin content increased only after 72 h. Morphological evidences of cell differentiation in AL-treated B16-F10 melanoma cells were highlighted by typical

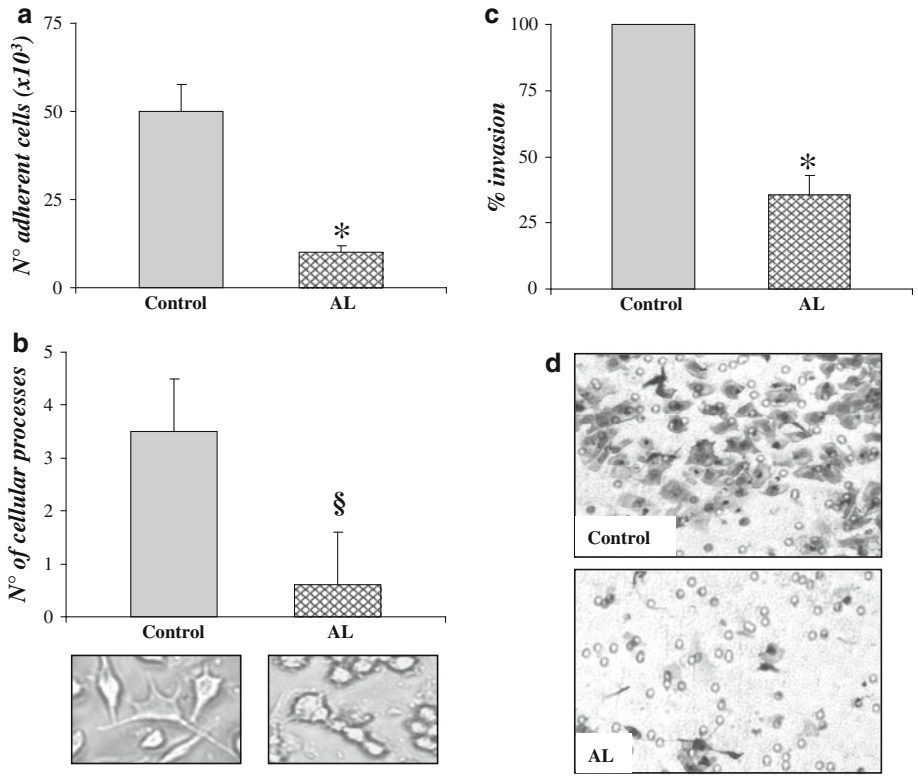

Fig. 4 Aloin reduces adhesion and invasion ability and branching formation. **a** Effect of 500 μM AL on cell adhesion to MG-coated substrates. **b** Quantitation of the average number of processes per cell was performed in AL-treated B16-F10 cells cultured for 4 h in a three-dimensional MG. Representative fields of untreated and 500 μM AL-treated cells after 72 h of exposure were photographed at ×400(original magnification). **c** Antiinvasive effect of treatment of murine, using the Boyden Chamber technique. Quantitative evaluation of the invasive ability of B16-F10 melanoma cells treated with 500 μM AL after 72 h of exposure. Data are expressed as percentage of the control (100%). **d** Representative micrographs show migrated cells to the lower surface of the filter in the Boyden chamber invasion assay (original magnification: ×200). Data are plotted as the mean ± SD (statistical significance versus control: $^{*}p < 0.01$, $^{\S}p < 0.05$)

melanocyte differentiation marker (Riley 1992) such as starry-dendritic cell morphology. Moreover, the potential antimetastatic role of AL has been clearly demonstrated, as shown by the reduction of the adhesion, shape fickleness and invasion ability of B16-F10 melanoma cells treated with the compound. Since other anthraquinones have demonstrated similar antineoplastic effects on B16 melanoma cells, i.e. danthron and quinizarin (Rossi et al. 2010) or aloe-emodin (Tabolacci et al. 2010), it is noteworthy to consider AL (the glycosilated form of aloe-emodin) as a good tool for both in vitro and in vivo experimentation, due to its higher water solubility and in vivo bioavailability with respect to aloe-emodin. Throughout history, natural products have afforded a rich source of compounds that have found many applications in medicine, pharmacy and biology. Within the sphere of cancer, a number of important new molecules, obtained from natural sources, display marked differentiative properties on tumor cells (Balasubramanian and Eckert 2007; Sánchez et al. 2009). 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, typical of the organ where they were originated. Although this would not eradicate cancer, the present study suggests that this kind of approach may be combined with the more conventional cytotoxic chemotherapy to interfere with cancer progression. In this work, we have demonstrated that the use of low-concentration CDDP, combined with AL, exerts greater antiproliferative action on B16-F10 melanoma cells than the single treatments and exhibits a lesser cytotoxicity than CDDP used alone at high concentrations. It is feasible that the proposed differentiative role of AL in the control of tumor cell growth may be exerted by the well-known antioxidant activity of this anthracycline phytoanalogue (Nićiforović et al. 2007).

Chemoprevention of melanoma through diet and dietary supplements will continue to be a hotly debated topic for many years. Despite the controversy surrounding the field of integrative medicines and nutritional supplements, a growing body of evidence supports the use of some of these dietary supplements in melanoma chemoprevention

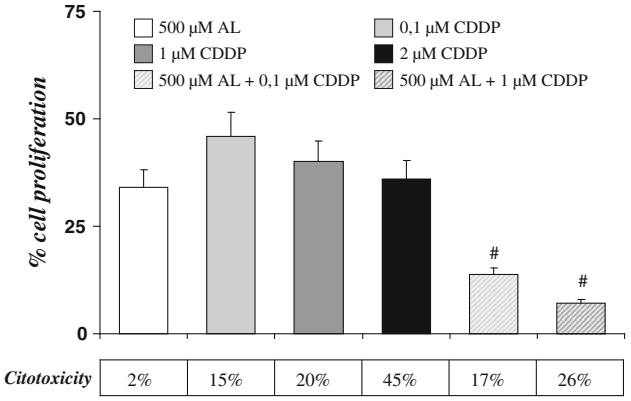

Fig. 5 Proliferation rate of murine B16-F10 melanoma cells treated with 500 μM AL and CDDP (0.1, 1 and 2 μM), alone or in combination, after 48 h of treatment. Data are expressed as % proliferation with respect to the control (100%). Cytotoxicity was evaluated by Trypan Blue dye exclusion assay. #Statistical significance ($p < 0.005$)

(Lentini et al. 2010b). 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, also in combination with common cytotoxic drugs. Future studies, hopefully, will yield concrete answers and clarify the role of commonly available dietary nutrients in melanoma chemoprevention.

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