Anthraquinones Danthron and Quinizarin Exert Antiproliferative and Antimetastatic Activity on Murine B16-F10 Melanoma Cells

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Abstract. *The anticancer potential of anthraquinones danthron and quinizarin on highly metastatic B16-F10 melanoma murine cells was investigated. Several parameters related to cell proliferation and differentiation, i.e. cell number, intracellular content of polyamines, transglutaminase (EC 2.3.2.13) activity and melanin synthesis were evaluated. Tumor cell growth was strongly reduced after treatment with danthron and quinizarin, as well as polyamine intracellular levels. Moreover, the induction of differentiation in treated cells was demonstrated by the increase of transglutaminase activity. In vitro analysis of adhesion, wound healing migration and invasion showed a remarkable antimetastatic activity of the anthraquinones. The overall results suggest that danthron and quinizarin possess significant antineoplastic properties, probably exerted through the induction of intracellular transglutaminase activity.*

Polyamines putrescine (PUT), spermidine (SPD), and spermine (SPM) are polycations naturally occurring in both prokaryotic and eukaryotic organisms (1), where they exert a variety of biological functions, such as protein synthesis and cell division control (2), protein and nucleic acid stabilization and ion channel control (3). In addition, polyamine synthesis is rapidly induced when cells are stimulated to grow. Therefore, polyamines have been indicated as potential targets for therapeutic interventions (4). Polyamine biosynthesis is regulated by ornithine decarboxylase (ODC; EC 4.1.1.17), whereas catabolism occurs *via* acetylation by (SPD/SPM) *N*1-acetyltransferase (SSAT; EC 2.3.1.57) (5), oxidative degradation by polyamine oxidase (PAO; EC 1.5.3.11) and protein transamidation by transglutaminase (TG; EC 2.3.2.13). TG, a multifunctional enzyme involved in several biological processes (6), including cell differentiation (7), catalyzes the covalent cross-linking of polyamines to specific glutamine residues in proteins and appears to modulate cellular polyamine availability (8, 9). Most of the natural compounds acting as differentiation inducers in cancer cells exert significantly less cytotoxicity compared to standard treatments. Moreover, it is known that induction of differentiation in tumor cells increases the intracellular level of protein-polyamine derivatives due to TG activation (10). Differentiative natural compounds are distributed among the different classes of plant secondary metabolites, mostly phenolic compounds, characterized by an aromatic ring with one or more hydroxyl substituents. Anthraquinones are the most important quinone derivatives of anthracene (11), which belong to the family of polycyclic aromatic hydrocarbons. Their effects appear to be due to their planar structure, which allows DNA intercalation and topoisomerase II inhibition (12). Hydroxyanthraquinones are the most important group of the family, in particular 1,8-dihydroxy-anthraquinones are the most studied compounds for their known pharmacological activity. Danthron (DA), or crysazin (Figure 1A), is an hydroxyanthraquinone (1,8-dihydroxy-9,10-anthraquinone) mostly present in *Rheum palmatum* L. (11). Recent studies has been shown that DA reduced membrane peroxidation and neuronal damage induced by glutathione depletion, and counteracted the neurotoxicity of β-amyloid (13). DA also exerted anticancer activity by inducing apoptosis in human brain cancer cells through mitochondria-related and caspase-related pathways (14). Quinizarin (QZ) (Figure 1B) is a synthetic hydroxyanthraquinone (1,4-dihydroxy-9,10-anthraquinone), also found in traces in *Cassia obtusifolia* L. seeds (15), which seemed to induce frameshift mutation in Ames assay (16). Compelling data from different studies, epidemiological investigations and human clinical trials evidenced the important role of anthraquinones in cancer chemoprevention and therapy (17, 18). Nevertheless, few data are present in

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literature on the antineoplastic effects of DA and QZ in some types of tumor cells (19). The aim of this work was to analyze the antineoplastic action of DA and QZ on B16-F10 mouse melanoma cells, focusing the study on the possible stimulation of tumor cell differentiation through the induction of TG activity. Furthermore, the antimetastatic capability of DA and QZ were investigated through specific *in vitro* migration, adhesion and invasion assays.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered 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 Gibco (Grand Island, NY, USA). [^{14}C]-Methylamine (46.6 mCi/mmol) was purchased from Amersham International (Bucks, UK). Acetonitrile, tetrahydrofuran and all solvents came from Mallinckrodt Baker (Milan, Italy). DA, QZ, Matrigel (MG), sodium citrate, Tris, *o*-phthaldehyde (OPA), β-mercaptoethanol, ethylenediaminotetraacetic acid (EDTA), PUT, SPD, SPM, diaminoctane (DAO), trichloroacetic acid (TCA), perchloric acid (PCA) and all reagents were from Sigma Chemicals (St. Louis, MO, USA).

Cell cultures. 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 (20). Cell were cultured in DMEM with 10% FCS, 200 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin. Before each treatment, the cell line was starved for 24 hours to rule out possible interferences with cell growth due to serum components and promote synchronization.

Proliferation assay. Murine B16-F10 melanoma cells were seeded and grown in 35 mm dishes in DMEM supplemented as reported above, and treated with DA and QZ (at 10 μM final concentration) for 24, 48 and 72 h. Control cells were incubated with 0.1% methanol final concentration. Cells were harvested and counted with a Neubauer modified chamber, after trypan blue staining for cytotoxicity evaluation.

Determination of intracellular polyamine levels. Culture cell lysates were deproteinized with PCA, supernatants were derivatized with OPA and injected into the HPLC (AKTABASIC 10, Amersham Pharmacia Biotech., Milan, Italy). Reverse-phase separations were conducted at room temperature in a LC-18 Supelcosyl column (150 mm ×4.6 mm, 3 μm) (Supelco, Milan, Italy). The derivatives were separated on two mobile phases: A, 95% 350 mM sodium citrate, pH 4.0, 5% tetrahydrofuran and B, 45% 350 mM sodium citrate, pH 4.0, 40% acetonitrile, 15% tetrahydrofuran. Elution consisted of a linear gradient from 50% to 100% of buffer B in 5 min, then an isocratic elution for 15 min, at a flow rate of 0.9 ml/min. Detection was accomplished using a spectrofluorimeter (Jasco FP-1520; Easton, MD, USA). Fluorescence detection was set at λ_{ex} 330 nm and λ_{em} 445 nm.

TG activity assay and melanin determination. TG assay was performed by treating B16-F10 cells with 10 μM DA and QZ in the presence of [^{14}C]-methylamine (46.6 mCi/mmol, 0.5 μl/ml DMEM). Cells were then harvested, counted, washed twice in PBS. Cell proteins were precipitated in 10% TCA, washed extensively, solubilized in 0.1 N NaOH at 37˚C and measured for radioactivity, according to the method of Chung and Folk (21). Determination of intracellular melanin content in both control and DA- and QZ-treated cells was performed as previously described (22).

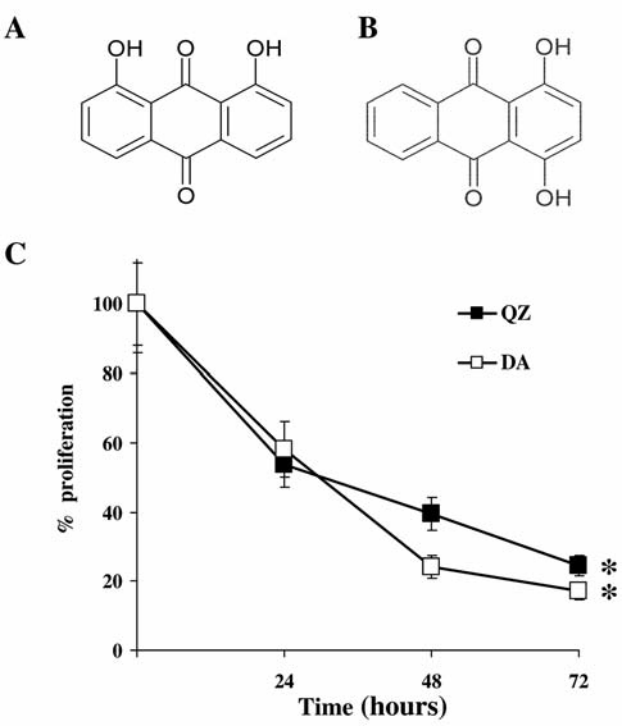

Figure 1. *Chemical structure of (A) 1,8-dihydroxy-9,10-anthraquinone (DA) and (B) 1,4-dihydroxy-9,10-anthraquinone (QZ). C: Proliferation curve of murine B16-F10 melanoma cells treated with 10 μM DA and QZ for 24, 48 and 72 hours. Control cells were incubated with methanol 0.1% only. Each point represent the mean of three different determinations±SD; *p<0.05, compared to the control.*

Adhesion, wound healing migration 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 DA- or QZ-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.

Wound healing migration was performed by growing cells to confluency in 12-well plates and wounds were made with a sterile plastic tip. Cells were further incubated for 24 h with or without DA or QZ in D-MEM (without FCS) and photographed under microscope at the time 0 point and after 24 h. The number of treated migrating cells was quantified with Image J software (NIH-http://rsb.info.nih.gov/ij/) and expressed as a percentage of the control (100%).

Invasion assay was carried out in a Boyden Chamber as previously described (23), using polycarbonate polyvinylpyrrolidone-free membranes (8 μm pore size; Neuroprobe, Cabin John, MD, USA). The number of invasive cells was evaluated by means of Image J software.

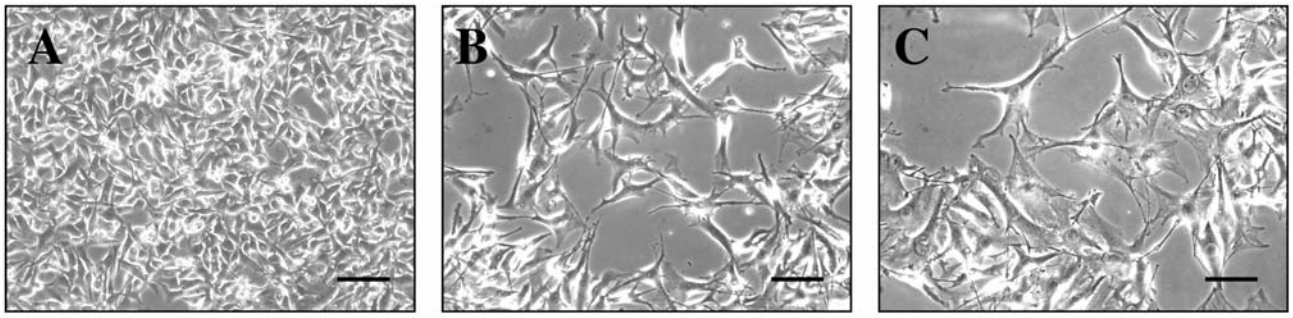

Figure 2. *Microscopic appearance of untreated (A), 10 μM DA-treated (B) and 10 μM QZ-treated (C) B16-F10 melanoma cells after 72 h of exposure. Typical morphological signs of melanocyte differentiation appear upon treatment (dendritic shape). Scale bar: 2 μm.*

Table I. *Effect of 10 mM DA and 10 mM QZ treatment treatment of murine B16-F10 melanoma cells on intracellular polyamine content.*

	nmol/mg protein		
	PUT	SPD	SPM
Control	2.33±0.23	26.61±2.66	40.22±4.02
DA 48h	2.07±0.20	31.81±3.18*	31.71±3.17*
DA 72h	2.08±0.20	24.31±2.43	22.76±2.27*
QZ 48h	2.25±0.22	33.52±3.35*	40.74±4.07
QZ 72h	2.27±0.22	16.03±1.20*	26.80±2.68*

PUT, Putrescine; SPM, spermine; SPD, spermidine. Each point represents the mean±SD of three different determinations. *$p<0.05$ compared to the control.

Table II. *Effect of 10 mM DA and 10 mM QZ on transglutaminase activity and melanin content of murine B16-F10 melanoma cells.*

	TG activity (%)	mg Melanin/mg protein
Control	100	13.63±0.68
DA 48h	151.5±7.6*	13.94±0.69
DA 72h	212.3±10.6*	15.05±0.75
QZ 48h	133.8±6.7*	14.93±0.74
QZ 72h	172.9±8.6*	29.45±1.47*

TG, Transglutaminase. Each point represents the mean±SD of three different determinations, *$p<0.05$ compared to the control.

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

Effect of anthraquinones on B16-F10 melanoma cell growth. Treatments of B16-F10 melanoma cells with 10 μM DA and QZ affected cell proliferation; in fact the treatments reduced cell growth, with respect to the control, by 41.9% and 46.4%, respectively, after 24 h, by 75.9% and 60.6% after 48 h, and by 83% and 75.6% after 72 h (Figure 1C). Neither treatment caused cell injury, as assayed by the trypan blue exclusion test. In fact, cells were still 90-100% viable after treatment (data not shown).

Intracellular polyamine levels in anthraquinone-treated B16-F10 melanoma cells. Polyamine intracellular concentration was determined after 48 and 72 h of cell treatment with 10 μM DA and QZ (Table I). PUT concentration was unchanged over time for all samples. On the contrary, the SPD level increased after 48 h by 19.5% in DA-treated cells, and returned to the control values after 72 h. In QZ-treated cells, SPD increased by 25.9% after 48 h and decreased by 39.8% after 72 h, compared to the control. SPM content significantly decreased in DA-treated cells by 21.2% after 48 h and by 43.4% after 72 h, with respect to control. After QZ treatment, the SPM level was unchanged after 48 h and decreased by 33.4%, with respect to the control, after 72 h.

TG activity and melanin content. TG activity determination represents one of the methods to evaluate cell differentiation, since it is considered a differentiative biomarker. The effects of 10 μM DA and QZ in the modulation of TG activity are shown in Table I. Treatment with DA significantly induced, compared to the control, an enhancement in TG activity by 51.5% after 48 h and by 112.3% after 72 h of exposure. Similarly, QZ increased TG activity by 33.8% after 48 h and by 72.9% after 72 h of treatment. Cell treatment with the anthraquinones did not exert significant changes in melanin content (Table II), except for QZ, which induced a 2-fold increase of the pigment content, with respect to the control, after 72 h of incubation. The enhanced differentiation of treated cells was confirmed by microscopic observations, in fact cells had a starry-dendritic morphology (Figure 2).

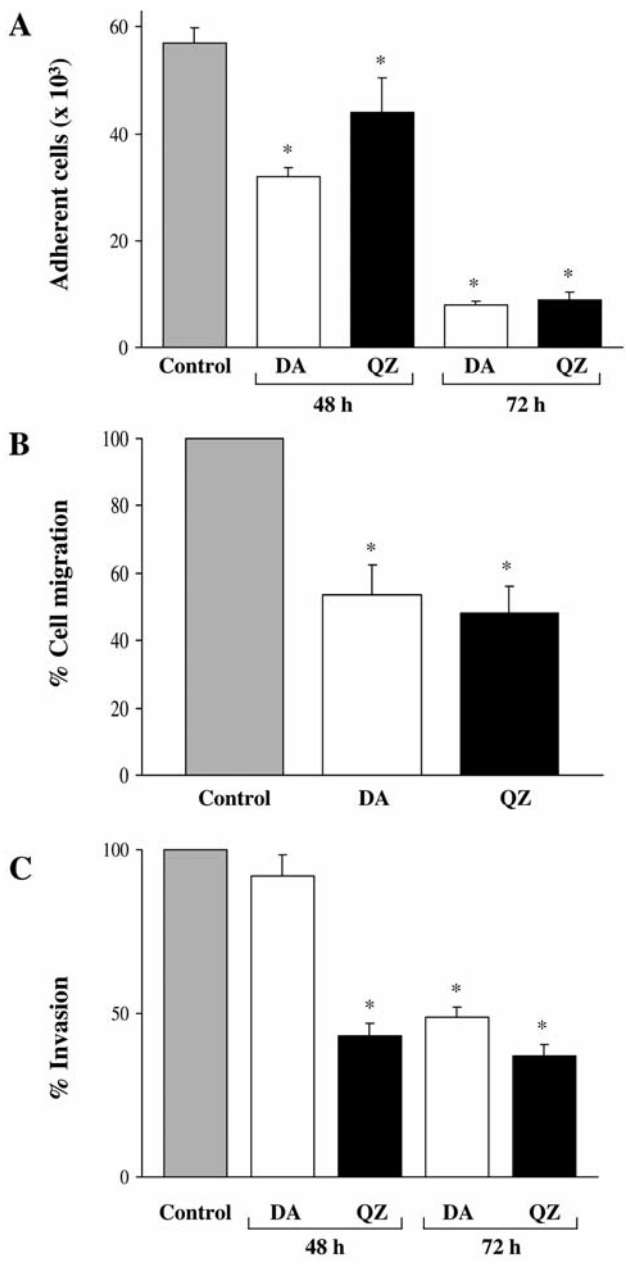

Figure 3. *A: Effect of 10 μM DA and QZ on in vitro adhesion to Matrigel of B16-F10 melanoma cells. B: Wound healing migration assay of B16-F10 melanoma cells treated with 10 μM DA and QZ after 24 h of exposure. Values were calculated as a percentage of the control, expressed as 100%. C: Effect of 10 μM DA and QZ on in vitro invasion of B16-F10 melanoma cells through Matrigel-coated filters, according to the Boyden chamber technique. Data are expressed as the mean±SD of three different determinations; *p<0.05, compared to the control.*

Effect of DA and QZ on B16-F10 cell adhesion, migration and invasion. The possible antimetastatic activity of DA and QZ was evaluated. Figure 3A shows the adhesion pattern on the MG layer of B16-F10 cells untreated and treated with the anthraquinones. The number of adherent cells decreased in a time-dependent manner for both molecules. In fact, DA treatment caused a reduction of adhesion, compared to the control, by 43.9% after 48 h and 86% after 72 h. Similarly, QZ caused a reduction of the adhesive capacity by 22.8% after 48 h and 84.2% after 72 h. Migration ability (Figure 3B) was reduced approximately by 50%, with respect to control, after exposure with both molecules. In the invasion experiments (Figure 3C), the densitometric analysis performed on the MG-coated porous filters invaded by B16-F10 cells showed that the invasive power of tumor cells incubated with DA was unchanged after 48 h and decreased, with respect to the control, by 51% after 72 h of exposure. QZ treatment caused a reduction of the invasive ability by 57% after 48 h and by 63% after 72 h of exposure.

Discussion

Anthraquinones are an important group of bioactive components found in many species of medicinal plants. In addition to their antifungal and antibacterial effects, they also show potential protective effects for the gastrointestinal and renal systems (24, 25), and many data demonstrated the promising action of anthraquinones as anticancer agents (11). To extend knowledge of the antineoplastic role of anthraquinones, we analyzed the effect of DA and QZ on the B16-10 melanoma cell line. We found that DA and QZ shared similar antiproliferative activity, without significant cytotoxic effects. The proliferation data were confirmed by the reduction of a typical proliferation marker, the intracellular polyamine content (26). Furthermore, this paper is the first report on the effect of these two anthraquinones on TG activity, one of the markers of cell differentiation. The reduced B16-F10 melanoma cell growth induced by the compounds was paralleled with a significant increase of TG activity, even though melanin content increased only after 72 h treatment with QZ. Moreover, the potential antimetastatic role of DA and QZ has been clearly demonstrated, as shown by the reduction of the adhesion, migration and invasion ability of B16-F10 melanoma cells treated with the two anthraquinones.

In conclusion, the present work demonstrated that DA and QZ interfere with melanoma cell growth, most likely through the induction of tumor cell differentiation, and exerted strong antimetastatic activity. The experimental use of anthraquinones as antineoplastic agents has been supported by a variety of studies but the mechanisms underlying the action of DA and QZ have not clarified yet. Indeed, few papers have been published on the antiproliferative role of DA, which seems to induce apoptosis in human brain cancer (14), and data published on the antineoplastic action of QZ are lacking. Therefore, the goal of this work was to provide more information about the anticancer effects of

anthraquinones. Although the complimentary or antagonistic actions of various anthraquinones and the cross-communications among the various pathways are yet to be elucidated, this report appears to offer encouraging evidence of the *in vitro* antitumor effect of DA and QZ. The strategy of enhancing the efficacy of anticancer therapy by increasing TG activity and altering the differentiation state of tumor cells (27), without significant side-effects, is a rather novel approach which warrants further investigation (28).

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