Bcl-2 family proteins and cytoskeleton changes involved in DM-1 cytotoxic effect on melanoma cells

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Abstract

Melanoma is one of the most aggressive types of skin cancer and its incidence rate is still increasing. All existing treatments are minimally effective. Consequently, new therapeutic agents for melanoma treatment should be developed. The DM-1 compound is a curcumin analog that possesses several curcumin characteristics, such as antiproliferative, antitumor and antimetastatic properties. The aim of this study was to evaluate the different signaling pathways involved in the cytotoxic effect of DM-1 on melanoma cells.

The apoptotic process and cytoskeletal changes were evaluated by immunoblotting and immunofluorescence, respectively, in melanoma cells.

After DM-1 treatment, SK-MEL-5 melanoma cells showed actin filaments disorganization with spicules formation throughout the cytoskeleton and significant reduction of focal adhesion as well as they were present only at cell extremities, conferring a poor connection between the cell and the substrate. Besides this, there was significant filopodium retraction and loss of typical cytoskeleton scaffold. These modifications contributed to cell detachment followed by cell death.

Furthermore, DM-1-induced apoptosis was triggered by multiple Bcl-2 proteins involved in both the extrinsic and the intrinsic apoptotic pathways. SK-MEL-5 cells showed a death mechanism mainly by Bcl-2/Bax ratio decrease, whereas A375 cells presented apoptosis induction by Mcl-1 and Bcl-xL down-regulation. In SK-MEL-5 and A375 melanoma cells, there were a significant increase in the active form of caspase 9 and the inactive form of the effector caspase 3 was decreased in both cell lines. Expression of cleaved Parp was increased after DM-1 treatment in these melanoma cell lines, demonstrating

that the apoptotic process occurred**.** Altogether, these data elucidate the cellular and molecular mechanisms involved in the cytotoxicity induced by the antitumor agent DM-1 in melanoma cells.

Keywords: Melanoma, DM-1, Curcumin analog, Apoptosis, Bcl-2 proteins, cell adhesion

Introduction

Melanoma is one of the most aggressive types of skin cancer and its incidence rate is still increasing. Until now, no agent has been shown to improve survival over supportive care and treatment guidelines recommend that patients with metastatic disease enter into clinical trials [1].

Dacarbazine has been the standard therapy for metastatic melanoma for many years [2], but only around 10% of patients have objective tumor response and median overall survival is little more than 6 months [3,4]. This unsatisfactory treatment outcome encourages additional studies on novel therapeutic molecules, delivery systems and combination therapies for melanoma [5].

Curcumin has long been known as a chemo-preventive and chemotherapeutic agent and *in vivo* studies with curcumin have demonstrated decreased tumorigenesis in many organs [6], including antiproliferative effects in melanoma [7]. Because of its lack of toxicity, there has been increasing interest in further studies with curcumin [8,9]. Unfortunately, its low potency and poor absorption undermine its clinical potential [10].

DM-1 is a curcumin analog and has previously been studied in animal models, such as melanoma and Ehrlich ascites tumor in mice. This compound is a powerful antitumor agent with both antimetastatic and antiproliferative activities. Interestingly, its pharmacological activity is restricted to tumor tissue, with minimal side effects on the normal surrounding tissue [11,12]. This may be explained by the high selectivity that DM-1 shows for cancerous cells without significant cytotoxic effect on normal cells [13].

Most of the currently used chemotherapeutic drugs exert its antitumor effect by activating a programmed cell death called apoptosis. This process can be triggered by many different stimuli, such as DNA damage, serum deprivation or incorrect cell-extracellular matrix (ECM) attachment, being the latter known as Anoikis [14]. The apoptotic process can be carried out by the extrinsic and/or the intrinsic pathways. The key regulator proteins of the intrinsic pathway are the Bcl-2 family proteins. Once the pro-apoptotic proteins are released from the complexes that form with the anti-apoptotic Bcl-2 proteins, the mitochondrial membrane outer permeabilization occurs, leading to the efflux of several apoptogenic factors to the cytosol [15]. This induces the activation of specific proteinases called caspases and, subsequently, the cleavage of many key proteins provoking the cell death [16]. In this work we try to elucidate the molecular mechanisms that participate in the apoptotic process induced by DM-1 in human melanoma cell lines as well as analyze some morphological changes due to alterations in cytoskeletal structures related to cellular adhesion and migration.

Materials and methods

Cell lines and culture conditions

Tumor cell lines of human melanoma (SK-MEL-5 and A375) were purchased from the American Type Culture Collection (Manassas, VA). These cells were grown with DMEM (Biological Industries, Beit Haemek, Israel) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Carlsbad, CA), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine all from Biological Industries. Cells were grown at 37 ºC in a 5% CO_2 atmosphere.

DM-1: Sodium 4-[5-(4-hydroxy-3-methoxyphenyl)-3- oxo-penta-1,4-dienyl]-2-methoxy-phenolate

Dried sodium ethanolate (0.01 mol) was mixed with 1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one [17] (0.01 mol; 3.26 g) in a 1:1 molar ratio and stirred at room temperature under anhydrous reaction conditions, followed by solvent rotoevaporation until solidification. The compound $C_{19}H_{17}O_5Na$ has 348 of molecular weight (Fig.1A). Results of the structural characterization of the isolated compound were the same as described previously by our group [18,19].

Cell viability assay (MTT assay)

Cell viability was determined using MTT assay [20]. Cells were plated in triplicate wells (1 x 10^5 cells/well) in 100 µL of growth medium in 96-well plates and treated with increasing concentrations of DM-1 (1-100 µM) or drug diluent (DMSO). After 24 h incubation with DM-1, 10 µL of MTT (Sigma Chemical Co., St. Louis, MO) was added to each well for an additional 4 h. The blue MTT formazan precipitate was then dissolved in 100 µL of isopropanol: 1N HCl (24:1). The absorbance at 570 nm was measured on a multiwell plate reader. Cell viability was expressed as a percentage of control and IC_{25} or IC_{50} represents the concentration of drug causing respectively, 25% or 50%, inhibition of the increase in absorbance compared with control cells. Data are shown as the mean ± standard deviation (s.d.) of three independent experiments.

Morphological changes and filopodium retraction analysis by optical microscopy

The human melanoma cells SKMEL-5 (2 x 10^5 cells/mL) were cultured 24 h with DMEM supplemented with 10% FBS before treatment. Cells were treated with DM-1 at 75 µM (IC_{50} value) for 90 min. During this period, temperature and CO_2 cell culture conditions were maintained and phase contrast images were obtained from cell cultures with phase contrast microscope (Zeiss ApoTome) (Carl Zeiss, Jena, Germany) at time 0 and 90 min of DM-1 treatment for the analysis of filopodium retraction and cell morphology changes.

Cytoskeleton analysis by fluorescence microscopy

Cells adhere to the substrate through the focal contacts establishing therewith. By immunofluorescence assay, using vinculin as a focal contacts marker, we analyzed the DM-1 capacity in inducing loss of these contacts and, hence, the cell de-adhesion.

SK-MEL-5 melanoma cells (2 x 10^5 cells/mL) were grown in a 24-well plate containing glass coverslips (20 x 20 mm). After treatment with DMSO

(control) or DM-1 (IC_{50}) for 90 min, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, before 0.5% Triton-x-100 permeabilization. Then, cells were incubated with 1% albumin in phosphate buffered saline (PBS) (w/v) for 15 min, and afterwards they were incubated with primary antibodies: anti-vinculin (1:400 dilution) (Sigma Chemical Co.) for 30 min at 37 ºC. Secondary antibody Cy^3 goat anti-mouse (Jackson ImmunoResearch, Inc.) was used at 1:50 dilution. Simultaneously to secondary antibody incubation, cells were incubated with Bodipy Fl Phallacidin (Invitrogen, Carlsbad, CA) at 1:50 dilution for 30 min at 37ºC for actin staining. After fixing cells with Mowiol (Sigma–Aldrich Chemical Co, St Louis, MO), samples were dried at 4ºC protected from light. Images were obtained using a Nikon eclipse E800 microscopy.

Western blot

SK-MEL-5 and A375 melanoma cells (2 x 10^5 cells/mL) were exposed to DM-1 IC_{25} (75 and 65 μM DM-1, respectively) for 3, 6 or 24 h. They were then washed with PBS prior to the addition of lysis buffer (0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 50 mM NaF, 40 mM β-glycerophosphate, 200 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, serin and cystein protease inhibitor cocktail (Roche 11836170001)). After that, 40 μg protein extracts were separated by SDS-PAGE on a 10-15% polyacrylamide gel and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Blots were blocked in 5% dry milk diluted in TBS-T (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 h and then incubated overnight with primary antibodies. Antibodies were obtained from the following sources: anti-caspase 3 (Cat#sc-1225), anti-Bcl-2 (B-cell lymphoma 2) (Cat#sc-492), anti- Mcl-1 (Induced myeloid leukemia cell differentiation protein) (Cat#sc-69840), anti-Bak (Bcl-2 homologous antagonist/killer) (Cat#sc-1035) and anti-Bcl-xL (B-cell lymphoma-extra large)(Cat#sc-634) were from Santa Cruz Biotechnologies (Santa Cruz, CA); anti-caspase 9 (Cat#9501), anti-Parp (Poly ADP ribose polymerase)(Cat#9542), anti-Bax (Bcl-2-associated X protein)(Cat#2772) and anti-Bid (BH3 interacting domain death agonist) (Cat#2002) were from Cell Signaling Technology (New England Biolabs, Hertfordshire, UK); anti-Vinculin (Cat#9131, Sigma Chemical Co.). All primary antibodies were used according to the manufacturer's instructions. Antibody binding was detected with the appropriated secondary antibodies conjugated to horseradish peroxidase, and signals were detected using enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK). Protein bands were quantified with the image analysis software program MacBiophotonics Image-J 1.43m. Results were presented as normalized fold change respect control. Normalization has been done using vinculin as a loading control.

Statistical analysis

Results are expressed as mean ± s.d. Data were analyzed using one-way analysis of variance (ANOVA) and significant mean differences were determined using multiple comparisons by the TUKEY-KRAMER test at the $p<0.05$ level. Significant differences between the control and treated groups are indicated by ***$p<0.001$, **$p<0.01$ and *$p<0.05$.

Results

Effect of DM-1 on cell viability

The antiproliferative effect of DM-1 (Fig 1A) was determined using the MTT reduction assay in human melanoma cell lines. Fig. 1B shows DM-1 effects on cell viability after 24 h of drug exposure, ranging from 0 to 100 μM of this compound. Cell viability was significantly decreased in both tumor cells. In SK-MEL-5 melanoma cells, IC_{25} and IC_{50} values were 36 and 75 μM, respectively. In A375 melanoma cells, IC_{25} and IC_{50} values were 37 and 65 μM, respectively. Altogether, DM-1 shows a significant cytotoxicity in human melanoma cells.

Cytoskeleton changes and reduction in the number of focal adhesions after DM-1 treatment

Cellular morphological changes after DM-1 treatment were observed. To evaluate whether anchorage-dependent cell detaching from the extracellular matrix was participating in DM-1 cytotoxicity, cytoskeleton reorganization and number of focal adhesions were analyzed. Cytoskeleton of non-treated SK-MEL-5 melanoma cells presented aligned actin filaments, clearly defined and organized. The focal adhesions, which are the dynamic link between the cytoskeleton and ECM proteins, showed up in large number and spread throughout the cell in non-treated cells (Fig. 2A). After DM-1 treatment, SK-MEL-5 melanoma cells showed actin filaments disorganization with spicules formation throughout the cytoskeleton and significant reduction of focal adhesion as well as they were present only at cell extremities, conferring a poor connection between the cell and the substrate (Fig. 2B).

Filopodia are thin, actin-rich plasma-membrane protrusions involved in cell migration [21]. Filopodium retraction in SK-MEL-5 melanoma cells was evaluated after DM-1 treatment for 90 min (Fig. 3). There was significant filopodium retraction and loss of typical cytoskeleton scaffold. The control group did not present notably differences after 90 min of diluent treatment. All in all, these results indicate that cells start to detach after 90 min of DM-1 treatment and this may be a signal to activate cell death.

Bcl-2 family of proteins are involved in DM-1-induced apoptosis

To elucidate what molecular mechanisms were participating in DM-1-induced apoptosis, Bcl-2 family proteins were analyzed. The protein expression level of anti-apoptotic proteins (Bcl-2, Mcl-1, Bcl-xL and Bid) and pro-apoptotic proteins (Bax and Bak) was studied by immunoblotting assays in two human melanoma cell lines, SK-MEL-5 and A375. As we can observe in Fig 4, most of the anti-apoptotic proteins decreased their levels upon DM-1 exposure whilst the pro-apoptotic members increased or remain stable.

The ratio among the anti and the pro-apoptotic family members is critical to regulate the apoptotic process. To establish whether DM-1 regulated the Bcl-2/Bax ratio, we measured the levels of both proteins in lysates of SK-MEL-5 and A375 melanoma cells, treated with or without IC_{25} value (75 and 65 μM DM-1, respectively) for 24 h. We found that DM-1 increased the levels of Bax (pro-apoptotic) and generally decreased the levels of Bcl-2 (anti-apoptotic) at all

treatment times (3, 6 and 24 h) in SK-MEL-5 cell line. This effect was observed only after 24 h of DM-1 treatment in A375 cell line. Consequently, the Bcl-2/Bax ratio was greatly reduced in all SK-MEL-5 samples treated with DM-1 compared to control cells and only in the last time point for A375 cell line (Fig 5 A and B).

On the other hand, Mcl-1 was down-regulated at all treatment times in A375 melanoma cell line, but this effect was not observed in SK-MEL-5 melanoma cell line, that showed Mcl-1 decrease only after 24 h of DM-1 treatment (Fig. 5 C). DM-1 did not affect expression of pro-apoptotic member Bak in both melanoma cell lines so the ratio Mcl-1/Bak was also decreased (Fig 5 D).

Furthermore, only A375 melanoma cell line had Bcl-xL down-regulation after 24 h of DM-1 treatment. This anti-apoptotic protein was not modified in SK-MEL-5 melanoma cell line (Fig. 5 E).

Finally, the BH3-only protein Bid (inactive form) was decreased mainly in A375 melanoma cells treated with DM-1. SK-MEL-5 melanoma cells only presented Bid decrease after 24 h of DM-1 treatment (Fig. 5 F). Anyway, this demonstrates that the extrinsic apoptotic pathway is activated at different points in each cell line.

Altogether, these results confirm the participation of the pro-apoptotic Bcl-2 protein members in DM-1-induced apoptosis.

Caspase activation and Parp degradation after DM-1 treatment

The apoptotic process was also analyzed by caspase activation (Fig. 6). In SK-MEL-5 melanoma cells, the inactive form of the initiating caspase 9 (pro-caspase 9) was reduced from 3 h of DM-1 treatment with a high increase in the active form (cleaved caspase 9) after 24 h. A375 melanoma cells showed no notable proform decrease, however, after 24 h there was a significant increase in the active form of caspase 9.

Keeping on with the apoptotic pathway, the inactive form of the effector caspase 3 was decreased in both cell lines, and in SK-MEL-5 melanoma cells this reduction was started after 24 h. On the other hand, in A375 melanoma cells, caspase 3 proform decrease was remarkable from 3 h to 24 h of DM-1 treatment.

Protein expression levels of cleaved Parp, a caspase substrate, were increased after DM-1 treatment in both melanoma cell lines. The higher increase of this marker was found after 24 h of DM-1 treatment, demonstrating that the apoptotic process has occurred.

Discussion

The curcumin analog called DM-1 is a potent antitumor agent with proved antiproliferative and antimetastatic activities in animal models [11,12]. In this study we present the molecular mechanisms involved in its antitumor activity, analyzing in detail the type of cell death induced by DM-1.

Cell growth, death and survival responses are the result of the integration of numerous chemical and biophysical signals from the cell's surrounding environment. Interactions between the cell and the ECM are a major source of these environmental signals and ECM provides structural support by serving as a scaffold for cells [22]. The survival of adherent cells depends on an uninterrupted connection with the components of the ECM, such as laminin and

fibronectin [23]. The ability of cells to adhere to the ECM is a critical determinant of cytoskeletal organization and thus of cellular morphology [24]. Focal adhesions are specialized organelles that provide structural and functional continuity between the cytoskeleton and the underlying ECM [25] and are essential for survival of anchorage-dependent cells [26,27,28]. When ECM-integrin interactions are disrupted, cells undergo apoptotic cell death. This adhesion dependent cell death is termed *anoikis* [29]. DM-1 induced actin filaments disorganization with cytoskeleton disarray in SK-MEL-5 melanoma cells. These factors, in combination with significant loss of cell-substrate focal adhesions, may induce cell detachment followed by death. Similarly, prostate tumor cells treated with curcumin also showed effects on actin filaments with impaired cytoskeletal dynamics [30].

It has been proved that curcumin rapidly arrests cell movements through the alteration of microfilament organization and function, which are critical for cell adhesion [31]. Filopodia and lamellipodia are cellular structures that possess the critical elements to assemble integrin-dependent adhesions. The responsible mechanisms for focal adhesions formation are not well understood, but probably depend on the physical state of the underlying actin scaffold, integrin-receptor spacing as well as biochemical signals [32]. Filopodia play an essential role during cell migration, cell-cell or cell-matrix adhesion, by initiating contacts and conveying signals to the cell cortex. Because their dynamics depend on a discrete number of actin filaments, filopodia provide a model of choice to study elementary events linked to adhesion and downstream signaling [33].

SK-MEL-5 melanoma cells treated with DM-1 showed significantly cytoskeleton changes as filopodium retraction and loss of original scaffold. These modifications contributed to cell detachment followed by cell death. These effects in tumor cells treated with curcumin are triggered mainly by down-regulation of focal adhesion kinase (FAK) co-related to detachment-dependent apoptosis (*anoikis*) [34] or by integrin α6β4 inhibition which is correlated with tumor cell proliferation and anchorage-independent growth. This integrin mobilizes lamellipodia and filopodia, besides it interacts with growth factor receptors, leading to interaction with the actin cytoskeleton [35].

At the molecular level, pro-apoptotic members of the Bcl-2 family, Bax and Bak, play crucial but predominantly functionally redundant roles in the mitochondria-dependent apoptosis pathway induced by numerous apoptotic stimuli downstream from BH3-only proteins [36]. Pro-survival protein Mcl-1 is considered to preferentially restrict the activation of Bak but not Bax [37,38], whilst Bcl-2 inactivates Bax. Previous publications reported that some apoptotic stimulus induced apoptosis via a Bak-dependent pathway by accelerating the degradation of Mcl-1 [37,39,40,41].

Curcumin is known to induce the up-regulation of pro-apoptotic proteins of the Bcl-2 family and the down-regulation of anti-apoptotic proteins Bcl-2 and Bcl-xL in melanoma cells [42]. In this work, apoptosis was evaluated by anti-apoptotic and pro-apoptotic protein expression levels in two different melanoma cell lines. SK-MEL-5 melanoma cells treated with DM-1 showed Bcl-2/Bax ratio decrease after 3, 6 and 24 h of treatment, but expression of Mcl-1 and inactive Bid was diminished only after 24 h. Another curcumin analog, dimethoxycurcumin, induces Bcl-2 down-regulation and Bax up-regulation in breast tumor cells [43].

In SK-MEL-5 cell line, Bak protein expression was not modified, but Bax protein expression was significantly increased after DM-1 treatment. Activated pro-apoptotic proteins like Bid and Bax cause the release of the apoptogenic cytochrome-c from the mitochondrial membrane, leading to caspase cascade activation [44]. DM-1 also induces cytochrome-c release and electric mitochondrial membrane potential decrease in B16F10 melanoma cells after 6 h of treatment [13].

A375 melanoma cells showed some alterations in response to DM-1 treatment in comparison to SK-MEL-5 cells. A375 showed Bcl-2 decrease only after 24 h of DM-1 treatment, without Bax and Bak modifications at all studied times. The main mechanism of action was Mcl-1 and inactive Bid decrease at 3, 6 and 24 h and Bcl-xL decrease only after 24 h, resulting in mitochondria-mediated cell death, suggesting that apoptosis in SK-MEL-5 melanoma cells treated with DM-1 was not correlated with down-regulation of Bcl-xL. This is also the case of 5-fluouracil or paclitaxel treatment where the down-regulation of Bcl-XL is not observed in the apoptosis induced [45].

It is known that curcumin and curcumin analogs possess pro-apoptotic activity in many tumor cells as it was shown by cleaved caspase 3 increase in colon cancer [46] and breast cancer [12] or Parp cleavage in osteosarcoma cells [47]. Both melanoma cell lines showed cleavage of pro-caspase 9, pro-caspase 3 (inactive form) and Parp (caspase substrate), as well as cleaved caspase 9 and cleaved Parp. These results were time-dependent and they were more relevant after 24 h of DM-1 treatment.

Some of the differences in DM-1 treatment response among several melanoma cell lines could be co-related to several factors such as the origin of tumor tissue, different protein expression and mutations. SK-MEL-5 melanoma cells are derived from a metastatic site in axillary node [48], whereas A375 melanoma cells are derived from primary site in the skin [49]. For instance, A375 melanoma cells express wild-type p53 [50] and, although SK-MEL-5 melanoma cells do not exhibit any p53 mutations, the absence of p21Waf1 expression suggests functionally aberrant p53 [51]. Moreover, Placzek and coworkers showed a significantly up-regulation of Bcl-2 protein in several SK-MEL melanoma cell lines [52] that could explain the Bcl-2 target in SK-MEL-5 by DM-1 treatment. These particularities may explain the different pathways in cell death in these both tumor cells.

Furhermore, it was proven that DM-1 compound induces cell cycle arrest by cyclin D1 and Ki67 decrease and apoptosis by free radicals production, TNF-R1 and cleaved caspase-8 increase, as well mitochondrial electrical potential decrease. Additionally, apoptosis was also confirmed by apoptotic bodies in human melanoma cell line. DM-1 antitumor therapy in vivo showed tumor burden decrease with DM-1 monotherapy or in combination with Dacarbazine, besides survival rate increase [13].

In conclusion, this study demonstrates that low concentrations of DM-1 induce apoptosis triggered by multiple Bcl-2 proteins involved in both the extrinsic and the intrinsic apoptotic pathways. SK-MEL-5 cells showed a death mechanism mainly by Bcl-2/Bax ratio decrease, whereas A375 cells presented apoptosis induction by Mcl-1 and Bcl-xL down-regulation, suggesting multiple molecular mechanisms involved in melanoma treatment by DM-1. Furthermore, DM-1 treatment also induces changes in morphology and loss of cell-substrate contact, which may suggest cell death by Anoikis.

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Conflict of interest: none

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Figure legends

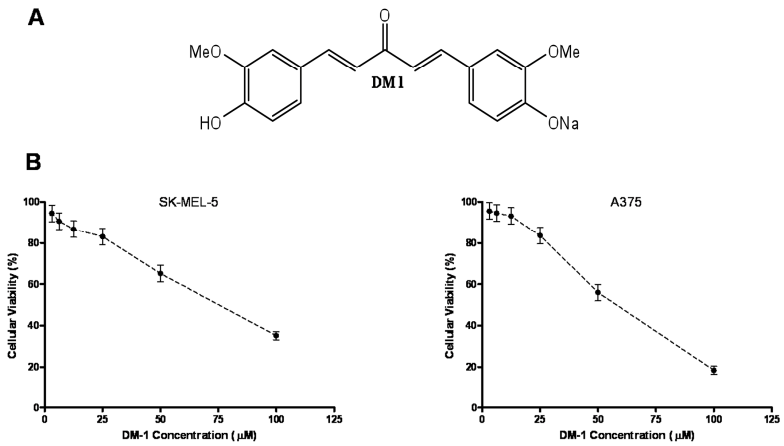

Figure 1 – (A) Structure of DM-1; (B) DM-1 effect on cell viability after 24 h of drug exposure (from 3 to 100 µM) was analyzed by MTT assay. The linear regression curve and IC_{50} were calculated using Graph Pad Prism Instat 3 software. The IC_{50} values estimated were 75 and 65 µM for SK-MEL-5 and A375 human melanoma cells, respectively.

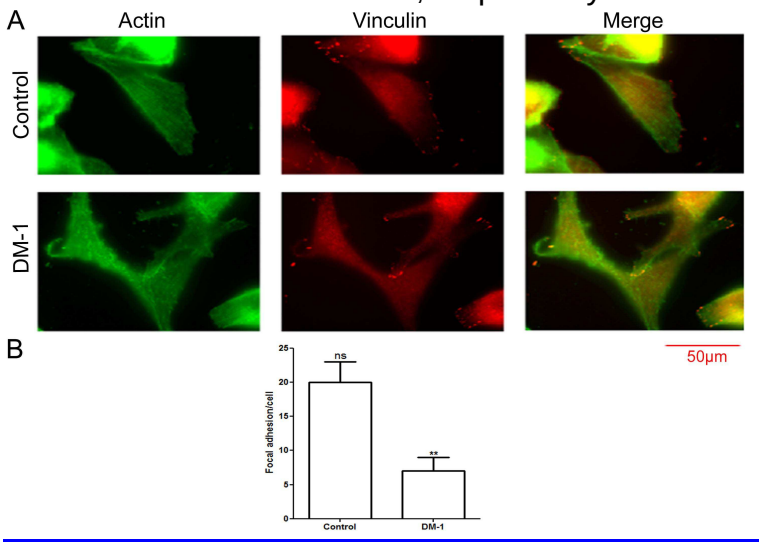

Figure 2 – Cytoskeleton changes after DM-1 treatment analyze by fluorescence microscopy. (A) SK-MEL-5 melanoma cells were treated with DM-1 at 75 µM for 90 min. Actin filaments are shown in green and focal adhesions (vinculin) in red. (B) Decrease of the number of focal adhesions is shown as a percentage respectively to control cells. The values are expressed as mean ± s.d. Significance are indicated by **p<0.01.

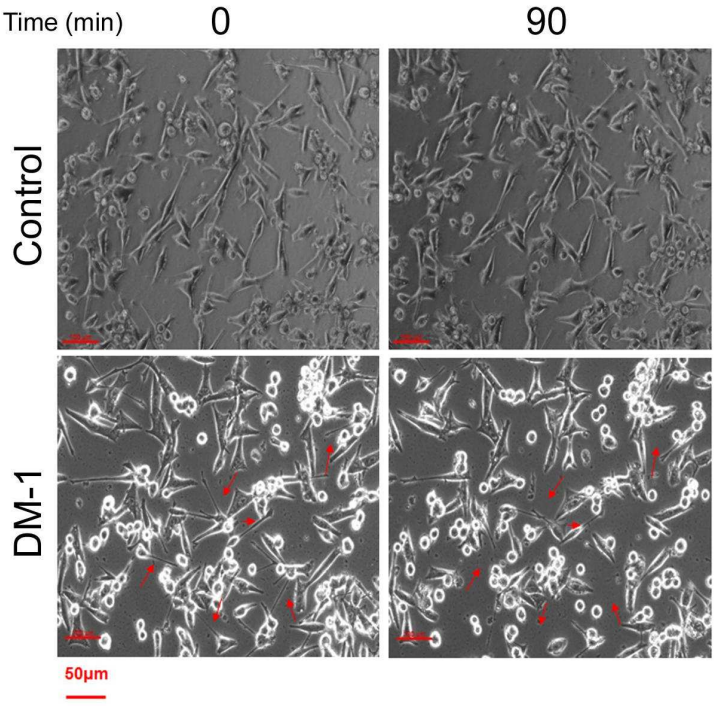

Figure 3 – DM-1 induced morphological changes in SK-MEL-5 melanoma cells. Filopodium retraction (red arrows) were monitorized for 90 min after DM-1 treatment at 75 µM. Images were obtained with phase contrast microscope (Zeiss Apoptome).

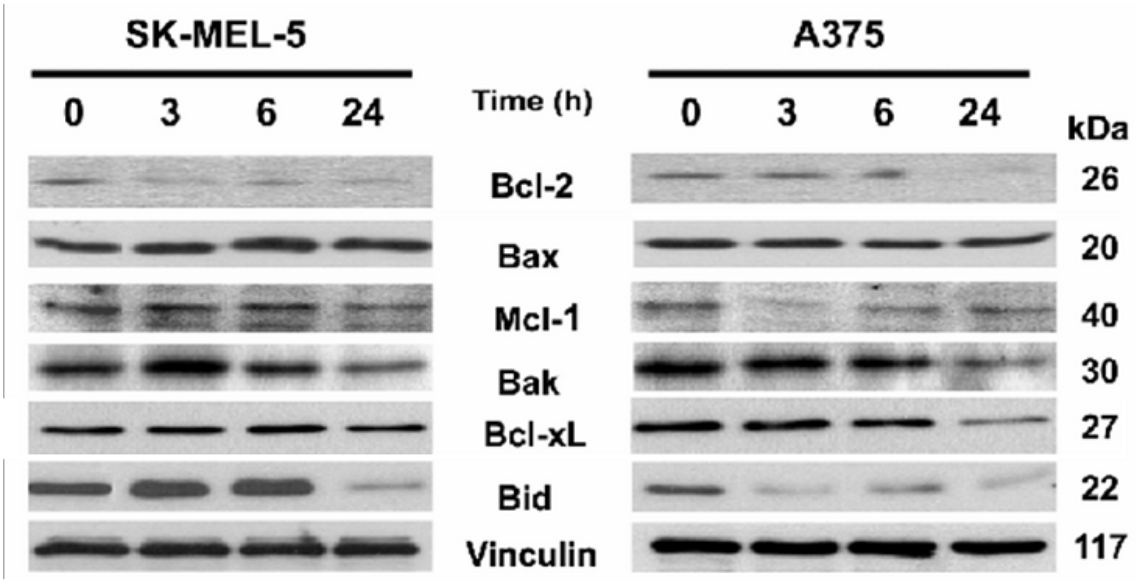

Figure 4 – Involvement of anti and proapoptotic members in SK-MEL-5 and A375 melanoma cell death. Total extracts were used to analyzed Bcl-2, Bcl-xL, Mcl-1, Bak, Bax and Bid protein levels after DM-1 treatment at 3, 6 and 24 h. Vinculin was used as a loading control.

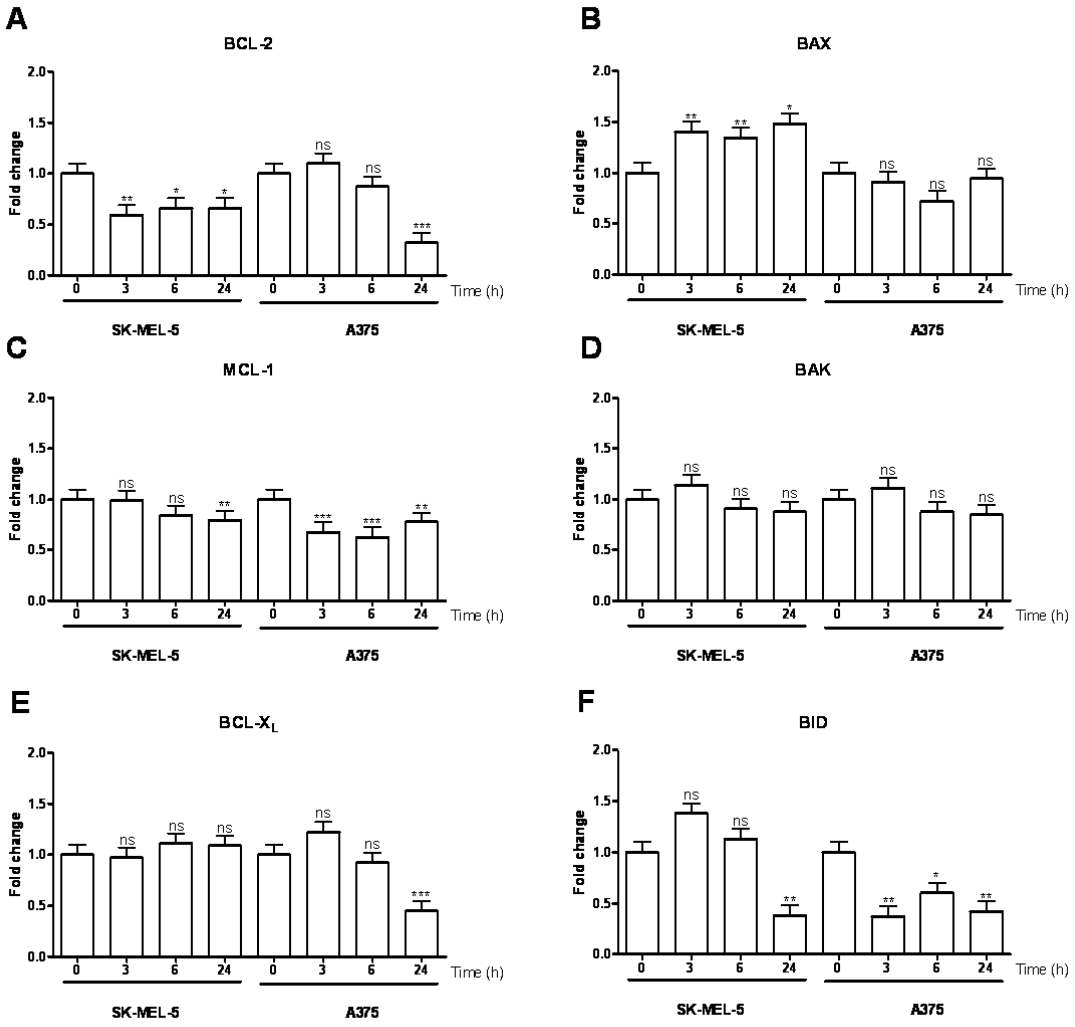

Figure 5 – Effect of DM-1 treatment on anti and pro-apoptotic members Expression in SK-MEL-5 and A375 melanoma cells. The cells were treated for 3, 6, and 24 h with respectively DM-1 IC_{50} and fold changes of protein expression with respect to control cells were determined by quantitative densitometry using Image J software. The columns (mean ± s.d) are mean of triplicate experiments, normalized by using vinculin expression as loading control.

ns: not significant compared to control.

Significance are indicated by *p<0.05; **p<0.01; ***p<0.001.

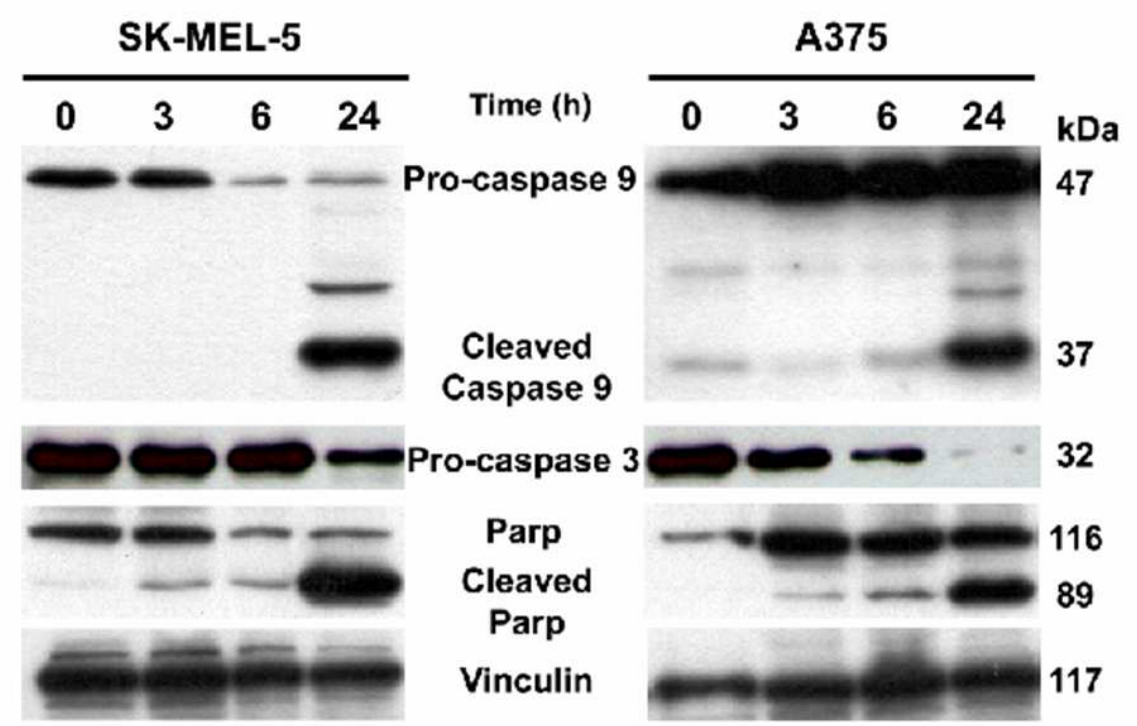

Figure 6 – Caspase activation and Parp degradation in SK-MEL-5 and A375 melanoma cells after DM-1 exposure. Total extracts were used to analyzed the inactive proform of caspases 9 and 3, the active form of caspase 9, Parp and cleaved Parp protein levels after DM-1 treatment at 3, 6 and 24 h. Vinculin was used as a loading control.