

Exploring the apoptotic potential of Prunus spinosa Trigno extract in BRAF- mutated melanoma cells

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Abstract

Melanoma is one of the most aggressive forms of human neoplasm due to its ability to invade and metastasize. The aim of this work is to examine the effect of our patented compound *Prunus spinosa* Trigno + Nutraceutical Activator Complex (PsT + NAC®) on primary (WM115) and metastatic (WM266-4), malignant (A375) human melanoma cell lines. Data evidence that PsT + NAC® induced on all melanoma cell lines, particularly on WM266-4 metastatic cells, a dose- and time-dependent reduction in cell viability. Persistent morphological changes indicative of cell death were observed, remaining irreversible even after treatment recovery. Cell cycle analysis revealed arrest in the G2/M phase for WM115 primary cells, and in the G1 phase, at lower concentration, for WM266-4 metastatic and A375 malignant cells. As the treatment concentration increased, all melanoma cell lines showed an increase in the sub-G1 population, which is associated with apoptosis. Western blotting analysis revealed that lower concentrations of PsT + NAC® elicited a protective autophagic response, while higher concentrations triggered caspase-dependent apoptosis. These results demonstrate the efficacy of PsT + NAC® in inhibiting the growth of BRAF-mutated melanoma cells.

Introduction

Cutaneous melanoma is one of the most aggressive tumors due to the uncontrolled growth of melanocytes and is characterized by high heterogeneity and the ability to metastasize to distant organs [1, 2]. Although melanoma mortality has decreased, the overall incidence has increased, especially in males and older groups, representing a significant public health problem [3]. Furthermore, the prognosis of patients with advanced melanoma remained inauspicious and fatal: in particular, individuals with lymphnode involvement and distant metastases showed a 5-year survival rate of between 5 and 19% [4].

Melanoma cells have grown, spread, acquired drug resistance and escaped immunosurveillance due to their characteristic high heterogeneity, involving not only tumor tissue but also the surrounding microenvironment [5].

Today, for all these reasons, there is no suitable standard therapy for patients with disseminated disease. The most administered treatments (immunotherapy and targeted therapy) have taken advantage of the biological characteristics and genetic background, represented mainly by BRAF mutations [6]. A mutation in the BRAF gene, particularly the V600E mutation, leads to a substitution of valine (V) by glutamic acid (E) at position 600, resulting in the continuous activation of the B-Raf protein which is part of the RAS/MAPK signaling pathway regulating cell growth and division. This hyperactivation induced uncontrolled cell proliferation, contributing to the development and progression of melanoma [7]. The low response rate to chemotherapy, as well as immunotherapy and targeted therapy, significantly hampers the efficacy of drug treatments, due to the emergence of the resistance phenomenon.

Apoptosis resistance was probably the main cause of chemotherapy drug resistance in melanoma [8]. However, the tumor microenvironment, the characteristics of the tumor cells themselves (intrinsic

resistance), and the function of the patient's native immune cells have also been implicated [9]. Drug resistance in melanoma has also been mediated by the induction of autophagy, a highly conserved and programmed cell degradation process for damaged organelles and proteins through lysosomal machinery [10].

Currently, due to the significant challenges posed by the multidrug resistance phenomenon, multiple side effects and the high costs of conventional therapies for the treatment of melanoma, researchers are seeking to accelerate the study and evaluation of new safe and effective compounds for the treatment of this malignancy [11]. This has led to an increased interest in nutraceuticals, natural substances derived from food sources and nutrients abundant in fruits, plant-derived foods and beverages, for their numerous biological activities (antioxidant, ROS scavenger, anti-inflammatory, antiproliferative) beneficial to human health, which could prove beneficial in the treatment of melanoma [12, 13].

Many molecular mechanisms have been activated through which natural substances exert their anti-melanoma effects. Resveratrol and curcumin have been shown to induce apoptosis in melanoma cells by upregulating pro-apoptotic proteins (e.g., Bax) and downregulating anti-apoptotic proteins (e.g., Bcl-2) [14]. These compounds have also demonstrated anti-inflammatory properties by inhibiting the expression of cytokines and key pro-inflammatory enzymes such as COX-2, reducing tumor-promoting inflammatory microenvironment [14]. Resveratrol exerted anti-angiogenic effects by decreasing levels of vascular endothelial growth factor (VEGF), a key regulator of tumor angiogenesis. This inhibition may limit the supply of essential nutrients and oxygen to the tumor, preventing its growth and proliferation [14]. Curcumin suppressed the NF- κ B signaling pathway, which is often activated in cancer cells and plays a critical role in promoting inflammation and cell survival. Similarly, flavonoids can disrupt the Wnt/ β -catenin signaling cascade which is involved in the growth and metastasis of melanoma cells [15].

Many dietary polyphenols, including epigallocatechin-3-gallate (EGCG) and genistein, inhibited melanoma cell proliferation by disrupting key signaling pathways such as MAPK/ERK, PI3K/AKT, and mTOR, which are often dysregulated in melanoma [16].

It has been shown that dietary antioxidants can enhance the functionality of immune cells, thereby improving their ability to recognize and eliminate tumor cells. This immunomodulatory effect could play a crucial role in increasing the effectiveness of immune surveillance and tumor eradication processes [17].

The focus of this study was on *Prunus spinosa* Trigno (PsT) fruit extracts combined with a nutraceutical activator complex (NAC) consisting of amino acids, vitamins and mineral salt mixtures patented by our research group [18]. *Prunus spinosa* Trigno ecotype, commonly known as blackthorn, is a perennial, thorny shrub belonging to the *Rosaceae* family that grows on the banks of the Trigno river in the Molise region of Italy. It was rich in natural bioactive compounds, including flavonoids and anthocyanins, which confer various health properties and contribute positively to human health. The presence of these compounds has underlined the therapeutic potential of *Prunus spinosa* in the prevention and management of various diseases [19].

Our previous study showed that PsT plus NAC® has a cytotoxic effect on several human tumor cell lines: human colon carcinoma cells (HCT116 and SW480), cervical carcinoma cells (HeLa) and human bronchoalveolar adenocarcinoma cells (A549) [20]. Given the absence of adverse effects on normal cells, as small intestinal epithelial cells (IEC-6) and gingival fibroblasts, studies continued on colon carcinoma cells, both on a two-dimensional (2D) and three-dimensional (3D) model, with the aim of better investigating the mechanism of action [21]. We showed that the product PsT + NAC® was effective alone and in combination with conventional chemotherapy drugs, as 5-FU, significantly reducing autophagy-mediated resistance and inducing apoptotic cell death [22].

In our work we therefore wanted to verify whether our patented *Prunus spinosa* Trigno ecotype (PsT) extract had antiproliferative activity on primary (WM115) and metastatic (WM266-4), and malignant (A375) human melanoma cell lines, since there are no experimental studies on the effect of polyphenol-rich fruit extracts on these cell lines.

The antiproliferative effect of this natural extract was carried out by means of the MTT assay followed by the observation of the morphological changes using phase contrast light microscope. The experimental design continued with the evaluation of cell cycle alterations and apoptosis induction on melanoma cell lines by flow cytometry. Finally, to further our understanding of the mechanism of action of PsT extract, we analyzed the expression of some key proteins involved in important molecular pathways (apoptosis and autophagy) by western blotting.

Materials and Methods

Plant material

Prunus spinosa Trigno ecotype (PsT) is a native plant of Molise that grows in the district of Bagnoli del Trigno (Molise Region, Italy, latitude 41°42' N, longitude 14°27' E, altitude 650 m a.s.l.). We always used the same extract obtained from fully mature blackthorn fruits of *Prunus sp.* harvested in their balsamic period and not different extracts coming from fruits of different collections. The extraction process was conducted by the Agriculture, Environment and Food Department of the University of Molise. After being collected, the fruits are cleaned and then used, both fresh and dried in a ventilated oven until completely dehydrated. The extraction is generally carried out by the maceration of the plant material in ethyl alcohol at 60° for 40 days, stirring occasionally. After, the solvent is separated, and the material is pressed to recover all the liquid which is left to settle for a day and then filtered. The concentration of the *Prunus spinosa* hydroalcoholic solution obtained is 86 mg/mL (PsT 86 mg/mL). Chemical composition of *Prunus spinosa* Trigno ecotype extract (phenolic acid, flavone/ol, and anthocyanin) was performed by HPLC-DAD-ESI/MS analysis and it was presented in a previous publication [20]. We used lot number PS161213-SI deposited at National Institute of Health, Rome, Italy. The hydroalcoholic solution (PsT 86 mg/mL) was diluted with a complex blend of amino acids, vitamins and minerals, called nutraceutical activator complex (NAC), and patented as Trigno M (Inventors: Stefania Meschini and Franco Mastrodonato, Italian Patent N°RM2015A 000133, 4 January 2015) [18].

Cell cultures

Primary (WM115) and metastatic (WM266-4) human melanoma cell lines were kindly provided by Istituto Dermopatico dell'Immacolata (IDI) of Rome, Italy; malignant human melanoma cell line (A375) was purchased from American Type Culture Collection (ATCC, CRL-1619).

WM115 and WM266-4 cell lines grown in MEM medium (Euroclone, Carmlington, UK, ECB2071L) with the addition of 10% fetal bovine serum (FBS, Hyclone Laboratories, USA), 1% non-essential amino acids, 1% of L-glutamine, 1% penicillin (50 U/mL)-streptomycin (50 µg/mL, Euroclone), and 1% sodium pyruvate (Euroclone, ECM0542D), while A375 cell line in RPMI 1640 medium (Euroclone, ECB9006) supplemented with 10% FBS (Hyclone), 1% non-essential amino acids (Euroclone, ECB3054D) 1% L-glutamine (Euroclone, ECB3000D) and 1% penicillin (50 U/mL)-streptomycin (50 µg/mL) (Euroclone, ECB3001D). All of them grown in a humidified atmosphere at 37°C, 5% CO₂.

Normal Human Epidermal Melanocytes (NHEM)

Normal human epidermal melanocytes (NHEM), obtained from PromoCell, are primary human melanocytes isolated from the epidermis of juvenile foreskin. These cells are cultured in a specialized serum-free melanocyte growth medium and supplement mix C-39420 (PromoCell, Heidelberg, Germany, Cat.No. C-14040).

Cell Treatments

The experiments were performed treating human melanoma cell lines with different concentrations of PsT (0.2–10 mg/mL) obtained after the progressive dilution of PsT 86 mg/ml hydroalcoholic solution with a complex blend of amino acids, vitamins and minerals, called nutraceutical activator complex (NAC) (Italian Patent N°RM2015A 000133, 4 January 2015). To perform positive control of apoptosis induction, the cells were treated with Staurosporine (STS, 1 µM, Sigma-Aldrich, Saint Louis, MO, USA) [23].

MTT Assay

3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT, Sigma Aldrich, Saint Louis, MO, USA, M2128) was used to evaluate the effect of different concentrations of PsT plus NAC[®] for 24 and 48 h on melanoma cells and melanocytes on cell viability. Furthermore, this test was used to evaluate the contribution of the vehicle (hydroalcoholic solution) to the toxicity of PsT plus NAC[®] on the melanoma lines using the percentage of alcohol corresponding to that used for the concentrations 2–10 mg/mL. At the end of exposure, cell medium was removed from untreated and treated cells that were then washed with phosphate buffer saline (PBS, Sigma Aldrich, P4417) and incubated with 1 mg/mL MTT solution for 2 h at 37°C. After removing the MTT solution, the samples were lysed by dimethyl sulfoxide (DMSO, Sigma Aldrich, D5879) and analyzed with a microplate reader (Bio-Rad, Hercules, CA, USA) at 570 nm.

The optical density values of the different wells are used to calculate the percentage of viable cells according to the following formula: (Absorbance means value of the treated sample/absorbance mean value of the control sample) ×100.

Phase contrast microscopy

A phase contrast optical microscope (ECLIPSE Ti2 light microscope, Nikon Europe, Amsterdam, Holland) connected to a digital camera was used to observe the effect of different concentrations of PsT plus NAC® on the morphology of melanoma cell lines for 24 h and 48 h. Moreover, to verify that the damage was not reversible, a medium change was carried out (“recovery after drug free medium”) and then cells were observed after 24 h of recovery and photographed.

Annexin V-FITC/PI Assay

Annexin V-fluorescein isothiocyanate (FITC)/Propidium iodide (PI) staining was used to verify whether melanoma cells, following different PsT plus NAC® treatments, have undergone cell death by apoptosis after 24 h. Subsequently, cells were detached, and each suspension was resuspended in binding buffer solution (1 X), labeled with Annexin V-FITC (1 µg/mL) and propidium iodide (1 µg/mL) for 5 min at room temperature (Medical and Biological Laboratories Co., Ltd, Japan, Cod. 4696). Samples were analyzed with a BDLSRII flow cytometer (Becton, Dickinson & Company, Franklin Lakes, NJ, USA) equipped with a 5 mW, 488 nm, air-cooled argon ion laser and a Kimmon HeCd 325 nm laser. Fluorescence signals were collected with a 530 nm bandpass filter for FITC, and a 575 nm bandpass filter for PI. For each sample, 10,000 events were acquired in logarithmic mode. For quantitative analysis, the FACS Diva Software was used to calculate the percentage of cells positive for fluorescent signals.

Cell cycle analysis

To verify the effect of PsT plus NAC® on the different phases of the cell cycle, we measured DNA content on melanoma cells by flow cytometry. After 24 h of treatment, samples were fixed with a 70% ethanol solution; after washing in PBS, they were incubated with Ribonuclease A (0.1 mg/mL; RNase, Sigma-Aldrich) and propidium iodide (40 µg/mL) and analyzed by flow cytometry after 30 min. For each sample, 10,000 events were acquired in linear mode; the FACS Diva Software was used to calculate the percentage of cells in each cell cycle phase.

Western blotting

The total cell lysate was obtained by suspending the cell pellets in RIPA buffer containing 50 mmol/L Tris-HCl, pH 7.5, 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 100 mmol/L NaCl, 1 mmol/L Na₃VO₄, 1% Nonidet P-40, 10 g/mL leupeptin, 5 g/mL aprotin and 10 g/mL phenyl-methylsulfonyl fluoride in the presence of standard protease and phosphatase inhibitors at + 4°C, according to standard

procedures. The protein content of the supernatant was determined by spectrophotometer following the "Bio-Rad protein assay" (Bio-Rad Laboratories, Munich, Germany). Equal protein content of total cell lysates (30µg) was resolved on 10 or 12% SDS-PAGE and electrically transferred onto poly(vinylidene difluoride) membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with TBS-T (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.02% Tween-20) containing 5% skimmed milk (Bio-Rad Laboratories), for 1 h at room temperature, and then incubated overnight at 4°C with antigen-specific antibodies diluted in TBS-T + 5% milk or 5% BSA: MAbs: anti-Caspase-9 (Cell Signaling Technology, Inc., Beverly, MA, USA; #9508S; dil. 1:1000); anti-mTOR (Cell Signaling Technology, Inc., Beverly, MA, USA; #4517S; dil. 1:1000); pAb: anti-p-Akt (Cell Signaling Technology; #9271S; dil. 1:1000); anti-LC3 (Novus Biological, #NB100-2220; 1:1000); anti-Akt (Santa Cruz; # sc-8312; dil. 1:1000); anti-Caspase-3 (Cell Signaling; #9661S; dil. 1:1000); anti-p-mTOR (Cell Signaling Technology, Inc.; #2971S; dil. 1:1000); anti-SQSTM1/p62 (Sigma-Aldrich; #P0067; dil. 1:1000); primary antibodies were used. After three washes in TBS-T, immune complexes were detected with horseradish-peroxidase-conjugated species-specific secondary antibodies (Jackson Laboratory, Bar Harbor, ME, USA). Membranes were developed using ECL detection reagents (Millipore Corporation, Billerica, MA, USA). Reactive bands were detected by the ChemiDocMP system (Bio-Rad Laboratories Inc.) and signal quantification was performed using the IMAGE LAB software (Bio-Rad). The arbitrary units obtained were used to calculate the relative increase/decrease in bands. To ensure the presence of equal amounts of protein, the membranes were re-probed with anti-GAPDH (Santa Cruz; #sc-32233; dil. 1:1000).

Statistical analysis

The results obtained from three independent experiments were expressed as mean $\pm$ standard deviation. One-way analysis of variance (ANOVA) and Bonferroni post hoc analysis were applied to reveal differences between all samples, using the GraphPad Prism 5 software (GraphPad, San Diego, CA, USA). The alpha level was set at $p < 0.05$; each figure legend specifies symbols and significance.

Results

The effect of PsT plus NAC® on human melanoma cell lines and normal human epidermal melanocytes (NHEM)

To investigate the effects of PsT plus NAC® on cell viability, MTT tests were performed on primary (WM115) and metastatic (WM266-4), and malignant (A375) human melanoma cell lines after 24 and 48 h of treatment with different concentrations (0.2–10 mg/mL) (Fig. 1). Treatments with low concentrations of PsT plus NAC® (0.2 and 0.5 mg/mL) for 24 h did not affect the viability of all melanoma cell lines, which started to decrease with the 1 mg/mL concentration. A clear and significant reduction in the viability of WM115 and WM266-4 cells was observed after treatment with PsT 4 mg/mL plus NAC® (cell viability of approximately 60% and 70%, respectively Fig. 1a and 1d), whereas that of A375 cells was approximately 45% (Fig. 1g).

The MTT data showed that the effect of PsT + NAC® was dose-dependent and at the higher concentration (PsT 10 mg/mL) the percentage of viable cells was comparable with that induced by staurosporine (STS, 1 μ M) in all cell lines. Furthermore, the effect of the PsT plus NAC® was time-dependent, as shown in Fig. 1b, e, h.

To demonstrate that the hydroalcoholic component of our PsT extract (60% ethanol solution) was not responsible for the observed cytotoxic effect, we also used the vehicle alone corresponding to chosen concentrations (V-PsT 2mg/mL, 4 mg/mL, 5 mg/mL, 6 mg/mL, 10 mg/mL plus NAC®). The results, shown in Fig. 1c, f and i, showed that the cell viability, after treatment with vehicle alone, was comparable to that of control sample, and it was always higher than the viability of the respective PsT concentrations. Data from lowest concentrations (0.2-1 mg/mL) were not reported due to the ineffectiveness of PsT plus NAC® at these concentrations.

The effect of different concentrations of *Prunus spinosa* Trigno (PsT) extract was also studied by the MTT assay on normal human epidermal melanocytes (NHEM) after treatment with PsT (2–10 mg/mL) plus NAC® for 24 h (Fig. 1j) and for 48 h (Fig. 1k). We observed that PsT plus NAC®, at the same concentrations used on tumor cell lines, did not induce a cytotoxic effect. Lower concentrations (PsT 0.2-1 mg/mL plus NAC®) were omitted, as they were not effective on melanoma cells.

Treatment with PsT plus NAC® induced morphological changes in melanoma cell lines

The effect of PsT plus NAC® on human melanoma cell lines was confirmed by observations under a phase-contrast light microscope (Fig. 2). After treatment with PsT 2 mg/mL plus NAC® (Fig. 2d, e and f), all melanoma cell lines showed similar shape and density to their respective control cells (respectively Fig. 2a, b and c). The effect on cellular morphology appeared after treatment with PsT 4 mg/mL plus NAC® for 24 h of exposure (Fig. 2g, h and i) and was even more pronounced with PsT 6 mg/mL plus NAC® (dose-dependence). In particular, WM115 and WM266-4 cells were increasingly elongated and highly rounded (Fig. 2j and k), while A375 were floated completely in the growth medium (Fig. 2l) compared to control cells (respectively Fig. 2a, b and c).

Observations after 48h of treatment confirmed that the effect of PsT plus NAC® was time-dependent (Supplementary Fig. 1).

Finally, we performed a “recovery in drug free medium” experiment to assess whether the effects induced by PsT plus NAC® were irreversible: in all three cell lines there was no recovery of damage, even at the lowest concentration of PsT (2 mg/mL) (Fig. 3). The cells showed a distressed phenotype, although initially adherent, did not proliferate and detached after 48h. All this suggests that treatment with PsT plus NAC® causes irreversible death.

PsT plus NAC® was leading to changes in the cell cycle in human melanoma cells

We analyzed the disruption of the cell cycle by flow cytometry (Fig. 4). After 24 h of treatment with PsT 2 mg/mL plus NAC® there were no significant differences in the distribution of WM115 cells in the cell cycle phases (Fig. 4d) compared to the distribution of the control (Fig. 4a). After treatment with PsT 4 mg/mL plus NAC®, however, the percentage of cells in the G2/M phase increased (about 40%, Fig. 4g) compared to the control (Fig. 4a). Treatment with PsT 6 mg/mL plus NAC® induced an increase in pre-G1 phase cells (about 23%, Fig. 4j), visible as the cell population to the left of the G1 peak, indicating cells characterized by a reduced amount of DNA that underwent fragmentation during the apoptotic process.

WM266-4 cells, on the other hand, showed an increase in cells in the G1 phase (approximately 95%, Fig. 4e) compared to the control (85%, Fig. 4b) after treatment with 2 mg/mL PsT plus NAC®. Then, already starting with PsT 4 mg/mL plus NAC® and following with the concentration of 6 mg/mL a peak of cells in the pre-G1 phase (17.1% and 24.3% respectively, Fig. 4h and k) and the accumulation of cells in the G2/M phase (11.4% and 19.5% respectively, Fig. 4h and k) can be observed.

The same trend occurred in A375 cells, where PsT 2 mg/mL increased cells in G1 phase (83.5%, Fig. 4f) compared to the control where the population was about 65% (Fig. 4c). Finally, at concentrations 4 and 6 mg/mL there was a dose-dependent increase in pre-G1 phase cells (17.5% and 28% respectively) and G2/M phase cells (15.8% and 28.3% respectively, Fig. 4i and l).

PsT plus NAC® induced apoptosis in human melanoma cell lines

The use of Staurosporine as positive control, in all the experiments performed, seems to indicate that PsT plus NAC® treatment triggered cell death by apoptosis. To verify this, surface exposure of phosphatidylserine, an early event of the apoptotic process, was assessed using the Annexin V/FITC-PI assay by flow cytometry (Fig. 5). After treatment of WM115 cells with 2 mg/mL PsT plus NAC® for 24 h, 15.2% apoptotic cells were evident (Fig. 5d) compared to 7% of control cells (Fig. 5a). The apoptotic fraction increased in a dose-dependent manner with PsT 4 and 6 mg/mL (Fig. 5g and j), reaching approximately 60% with the 6 mg/mL PsT plus NAC®. In the WM266-4 cell line, an increase in the fraction of apoptotic cells (about 16% comparable to the control sample, Fig. 5b) was not found after treatment with PsT 2 mg/mL plus NAC® (Fig. 5e), a value which increased in a dose-dependent manner with concentrations of 4 mg/mL and 6 mg/mL (respectively 39.8% and 72.6%) (Fig. 5h and k). A375 cells also showed a significant increase in the fraction of apoptotic cells after treatment with PsT 4 mg/mL plus NAC® (about 49%, Fig. 5i), and after PsT 6 mg/mL (about 63%, Fig. 5l). These analyses suggested that PsT plus NAC® induced apoptotic cell death in a dose-dependent manner. The effect was more pronounced in the metastatic cell line WM266-4.

PsT 6 mg/mL concentration showed high cellular toxicity, so the mechanisms inducing this change at the PsT 2 and 4mg/mL concentrations were analyzed. As Annexin V assay, Western-blot analysis confirmed an apoptotic effect of PsT plus NAC®. After treatment with PsT concentrations, changes in some proteins regulating the apoptosis process were observed (Fig. 5m-p). Indeed, a dose-dependent reduction of the anti-apoptotic Bcl-2 protein was observed after PsT plus NAC® treatment in WM115 cells respect to control. In the cell lines WM266-4 and A375, an initial increase in Bcl-2 was observed at PsT 2 mg/mL plus NAC®, followed by a reduction in the protein at the higher concentration (PsT 4 mg/mL). In the A375 cell line, this reduction was greater than in the control (Fig. 5m). Conversely, under the same conditions, the pro-apoptotic protein Bax dose-dependent increase occurred. Particularly, in the A375 cell line this increase was statistically significant (Fig. 5n). Caspase-9 and -3 also confirmed these findings. Treatment with PsT plus NAC® induced a dose-dependent increase in the expression of active caspase-9 in WM266-4 and A375 cell lines, which was statistically

significant only in WM266-4 (Fig. 5o). Finally, analysis of caspase-3 showed that only PsT 4 mg/mL plus NAC® induced the expression of the active form of caspase-3 (Fig. 5p).

PsT plus NAC® induced changes in PI3K/AKT/mTOR signaling pathway and autophagy process in human melanoma cell lines

Since autophagy can act as cancer survival mechanism, we investigated whether treatment with PsT plus NAC® could affect this process. In particular, we asked whether PsT could influence the PI3K/AKT/mTOR signaling pathway, the most important autophagy regulator. The levels of AKT and phospho-AKT proteins (Fig. 6a and b) were determined by Western blot analysis. We found that the amount of AKT (Fig. 6a), although it shows a slight increase after treatment with PsT 2 mg/mL, significantly decreased with PsT 4 mg/mL only in the WM115 cell line, whereas it remained constant in the WM266-4 line compared to untreated cells. In the A375 cell line, AKT showed a partial increase after treatment with PsT 2 mg/mL and then a decrease after treatment with PsT 4 mg/mL compared to untreated cells. Furthermore, AKT phosphorylation analysis (Fig. 6b) showed that PsT plus NAC® treatment caused a significant dose-dependent increase in phosphorylated protein in all cell lines compared to untreated cells. It is known that phosphorylation of AKT can promote the phosphorylation of mTOR, the main suppressor of autophagy. We therefore investigated whether PsT plus NAC® treatment could affect both molecular pathways. The amount of mTOR protein and its phosphorylated form (p-mTOR) increased, although not significantly, in all three cell lines after PsT plus NAC® treatment in a dose-dependent manner compared to untreated cells (Fig. 6c and d).

We also examined the expression levels of some autophagy-related proteins, LC3 and p62, to confirm the effects of PsT plus NAC® treatment on autophagy process. As shown in Fig. 6f, LC3 II levels were increased after treatment with PsT plus NAC®, particularly in the WM266-4 cell line ($p \leq 0.05$), compared to untreated cells; the increase was dose-dependent. The amount of p62 increased in a dose-dependent manner, after treatment with PsT plus NAC® compared to untreated cells, although it is statistically

significant only in the WM266-4 cell line (Fig. 6g). These results show that PsT plus NAC® has an effect on the PI3K/AKT/mTOR signaling pathway through the inhibition of the autophagic process.

Discussion

The antioxidant and antitumor activity of the natural product PsT plus NAC®, whose peculiar composition is specified in Italian Patent N°RM2015A 000133, 4 January 2015, is well known in literature [20, 24]. The PsT plant extract is characterized by the presence of numerous bioactive compounds: flavones, flavonols, phenolic acids and anthocyanins, all components known for their antioxidant and antiproliferative activities [25]. The efficacy of this extract on different tumor cell lines as human colon, cervical and lung carcinoma are well demonstrated [20, 21, 24]. Moreover, we demonstrated that the combination of PsT plus NAC® and 5-FU was efficacy on HCT116 colon cancer spheroids reducing autophagy and increasing apoptosis [22].

The aim of our study was to evaluate antiproliferative effects of PsT plus NAC® on melanoma cell lines. We focused our interest on melanoma, because it is one of the most aggressive tumors, with a progressively increasing worldwide incidence and resistance to multiple pharmacological treatments. We chose primary (WM115) and metastatic (WM266-4) human melanoma cells, both obtained from the same patient, and another malignant human melanoma cell line (A375). There are no scientific studies demonstrating the effect of fruit extract containing polyphenols on the melanoma lines WM115, WM 266-4 and A375. The WM115 cell line originates from the primary tumor and the WM266-4 cell line from individual lymph node metastases, with a flat, small mesenchymal morphology; both cell lines have the specific V600D (Val600Asp) mutation at codon 600 in the BRAF gene [26]. While the A375 cell line has BRAF V600E mutation [27].

Firstly, MTT data on melanoma cells treated with PsT plus NAC® at different concentrations and exposure times (24 and 48 hours) indicated that at low concentrations (0.2–0.5 mg/mL) no effect was observed on all three lines, whereas increasing the PsT plus NAC® concentration and exposure time resulted in a reduction of cell viability (dose and time-dependent) (Fig. 1). At higher concentrations (PsT 5–10 mg/mL) the effect on both cell lines (WM266-4 and A375) was more pronounced.

Phase contrast light microscopic observations confirmed MTT data: PsT plus NAC® was effective on human melanoma cells, inducing morphological changes. This effect was dose and time dependent (Fig. 2 and Supplementary Fig. 1) and irreversible (Fig. 3).

As PsT was a hydroalcoholic extract (60% ethanol solution), treatments with the vehicle alone were performed to demonstrate the effect of the alcoholic component (Fig. 1c, f and i).

Analysis of PsT plus NAC® treatments on normal human epidermal melanocytes demonstrated that this compound was not cytotoxic (Fig. 1j and k), thereby reinforcing the results obtained on melanoma cells and supporting further studies to elucidate the mechanism of action of PsT plus NAC® on metastatic and non-metastatic human melanoma lines.

Recent work examined the activity of polyphenols against melanoma upregulating cell death and downregulating cell proliferation, invasion, angiogenesis and metastasis [14]. As phenolic acids, flavonoids and anthocyanins were the main constituents of the PsT extract, we verified if PsT plus NAC® could have an effect on melanoma cell lines by inducing apoptosis, as suggested by MTT results obtained after treatment staurosporine, a known inducer of apoptosis [23] (Fig. 1).

To verify the induction of apoptosis by PsT plus NAC®, we performed a quantitative analysis using the Annexin V assay by flow cytometry. Since the changes in the necrotic fraction in all three cell lines were less than the increase of the apoptotic fraction, we can infer that treatment with the highest concentrations of PsT plus NAC® extract induced cell death by apoptosis (Fig. 5). The apoptotic fraction was higher in the metastatic WM266-4 cells (about 73%) than WM115 and A375 cells (about 63%).

At the lowest concentration (2 mg/mL) we can assume that PsT plus NAC® extract can slow down cell growth, as also suggested by light microscopy observations (Fig. 2). Therefore, we analyzed the perturbations of the cell cycle phases by flow cytometry (Fig. 4). Analyses showed that at low doses PsT plus NAC® induced a G1 phase blockade on the melanoma cell lines WM266-4 and A375, whereas at higher doses (4 and 6 mg/mL) a G2/M phase arrest occurred on WM115 cells and the appearance of the preG1 population on all three cell lines, confirming the induction of apoptosis.

The molecular pathway involved was studied by western blotting analysis. We assessed the inner mitochondrial membrane-associated protein Bcl-2 expression, which can block the induction of programmed cell death [28], and the expression of the pro-apoptotic protein Bax, which permeabilizes the outer mitochondrial membrane [29]. The dose-dependent decrease in the anti-apoptotic protein Bcl-2 and the increase in the pro-apoptotic protein Bax confirmed the initiation of apoptosis (Fig. 5).

Subsequently, proteolytic cascade of caspases, which represents the execution event of intrinsic apoptosis, was analyzed through the expression of caspase-9 and caspase-3. It is known that caspase-9 acts by uncoupling mitochondria to increase ROS content, while caspase-3 inhibits ROS production which is essential for efficient intrinsic apoptosis [30]. From the data obtained, a marked increase in Caspase-9 and Caspase-3 expressions was observed for all three cell lines with increasing PsT concentration, confirming the induction of apoptosis (Fig. 5).

The PI3K/AKT signaling pathway is an important pathway in cancer cells. Constitutive activation of the PI3K/AKT/mTOR signaling pathway has been demonstrated in several human tumors. This pathway is essential for the regulation of tumor cell growth, metastasis and apoptosis [31]. In addition, this is closely related to autophagy regulation [32]. Indeed, numerous studies have shown that autophagy is regulated by the AKT/mTOR signaling pathway, which is a regulator of tumor progression [33].

It has been observed that autophagy is activated in cancer cells, enabling them to survive [34]. To understand whether PsT plus NAC® could influence the autophagic mechanism, a known cellular defense process by which the cell responds to drug treatment, the AKT/mTOR signaling pathway and the expression of certain autophagy-related proteins such as LC3-II and p62 were analyzed [35]. It is known

that phosphorylation of AKT can promote the phosphorylation of mTOR, the main suppressor of autophagy [36]. The complex mechanism of autophagy activation involves the cytoplasmic form of the microtubule-associated protein light chain 3 (LC3-I, 16 kDa) which is activated, transferred and converted to the phosphatidylethanolamine (PE)-conjugated form, LC3-II (14 kDa), which is membrane-associated and finally recruited to autophagosomes [37].

Furthermore, p62 is a marker of autophagic flux and its levels are inversely correlated with autophagic activity [38]. Basal autophagy under normal conditions has relatively low levels of p62, because it is degraded with its target by the lysosomal system [39]. Western Blotting results suggested that after treatment with PsT 2 mg/mL plus NAC®, melanoma cells activated autophagy as a defense mechanism (as evidenced by increased levels of p-AKT, p-mTOR and LC3-II, Fig. 6). However, an increase in p62 levels is also observed in this study. This suggests that we are not witnessing an increase in autophagic flux, but rather a blockade of vacuole degradation. By increasing the concentration to 4 mg/mL, the expression levels of p62 and LC3-II proteins increased in all cell lines, but much more so in WM266-4 cells. We hypothesized that the higher sensitivity of metastatic WM266-4 cell line may be due to the lower constitutive levels of autophagy, as evidenced by the lower basal expression of LC3-II compared to WM115 cells (Fig. 6) and by previous molecular characterization studies of both cell lines [26].

At higher concentrations of PsT plus NAC®, the increased expression of p62 and LC3-II expression suggests that the protective autophagic response was blocked, the vacuoles remained within the cells and were no longer degraded and, as a result, the cells died by apoptosis [40]. This hypothesis is also supported by the level of apoptosis found under the same conditions (Fig. 5).

Overall, our results indicate that treatment with PsT + NAC® induces a profound change in the balance between autophagy and apoptosis in the melanoma cell lines examined. All three tumor lines appear to respond positively to treatment with PsT + NAC®, indicating a possible use in combination with tumor therapies.

Conclusions

Melanoma is a highly invasive and metastatic skin tumor characterized by drug resistance, and it continues to attract research interest. In this study, we demonstrated that PsT plus NAC® induced apoptotic cell death by inhibiting autophagy of human primary and metastatic melanoma cell lines. This effect was particularly evident on WM266-4 metastatic cells, encouraging further studies on other drug-resistant and BRAF-mutant tumor cells.

Declarations

Financial interests

The authors have no relevant financial or non-financial interests to disclose.

Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

Ethics approval

N/A

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Author Contribution

Conceptualization, A.D.P and M.C.; validation, M.C., S.M; formal analysis, A.D.P, C.C., and R.V.; investigation, A.D.P, A.D.N. and C.C.; resources, S.M.; writing—original draft preparation, A.D.P, R.V., and M.C.; writing—review and editing, M.C. and S.M.; visualization, A.D.P and R.V.; supervision, S.M.; project administration, M.C.; funding acquisition, S.M. All authors have read and agreed to the published version of the manuscript.

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Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

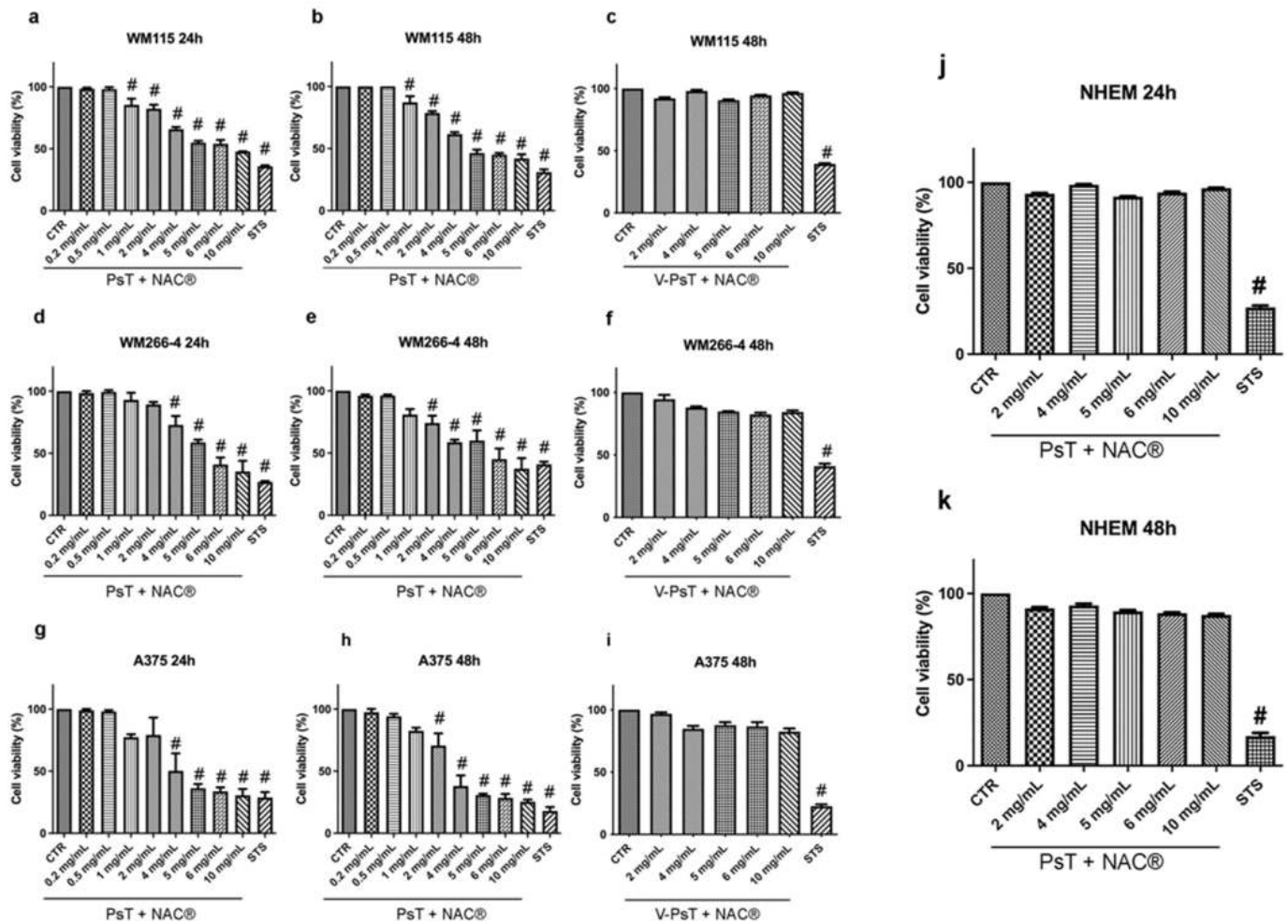


Figure 1

Effect of PsT plus NAC[®] and ethanol vehicle on human melanoma cell lines: WM115 (a-c), WM266-4 (d-f), A375 (g-i), and normal human epidermal melanocytes (NHEM) (j,k). Cells were treated with different concentrations of PsT plus NAC[®]: 0.2 mg/mL, 0.5 mg/mL, 1 mg/mL, 2 mg/mL, 4 mg/mL, 5 mg/mL, 6 mg/mL, 10 mg/mL for 24 and 48 hours. The results show the efficacy of dose- and time-dependent treatments on melanoma cell lines, and the ineffectiveness of treatments on normal cells. The concentrations of 60% ethanol dilutions (V=vehicle) used were equivalent to PsT plus NAC[®] treatments and did not affect cell survival. Staurosporine (STS, 1 μ M) was used as a positive control. Cell viability was assessed by the MTT assay, performed for three independent experiments, each sixfold. One way analysis of variance (ANOVA) was applied. # = significant differences compared to control cells ($p < 0.05$)

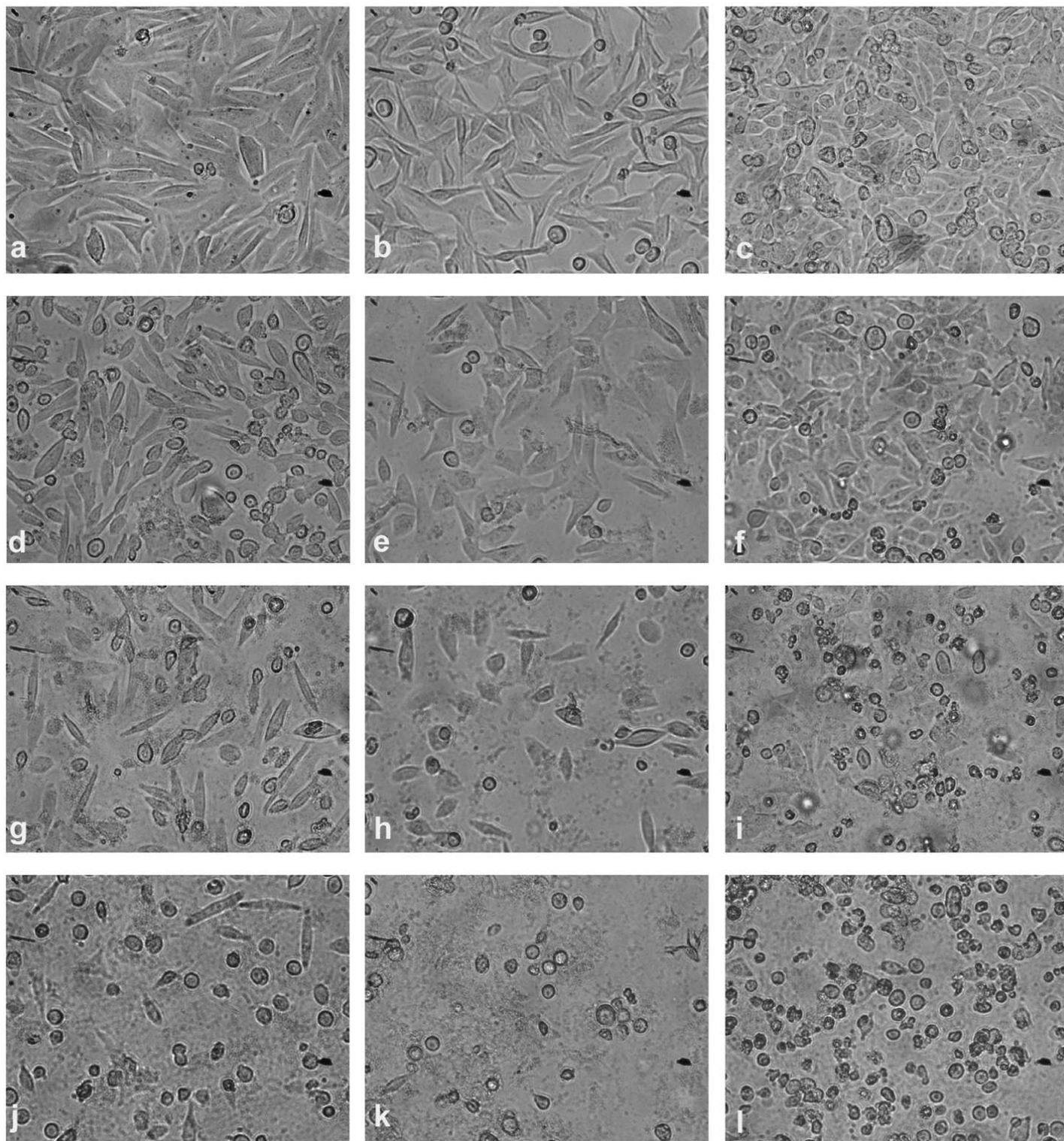


Figure 2

Phase contrast microscopic observations of WM115 (left column), WM266-4 (central column) and A375 (right column) cells after 24 h of treatment. Control (a, b, c); PsT 2 mg/mL plus NAC® (d, e, f); PsT 4 mg/mL plus NAC® (g, h, i); PsT 6 mg/mL plus NAC® (j, k, l). Images were acquired with a 20x objective

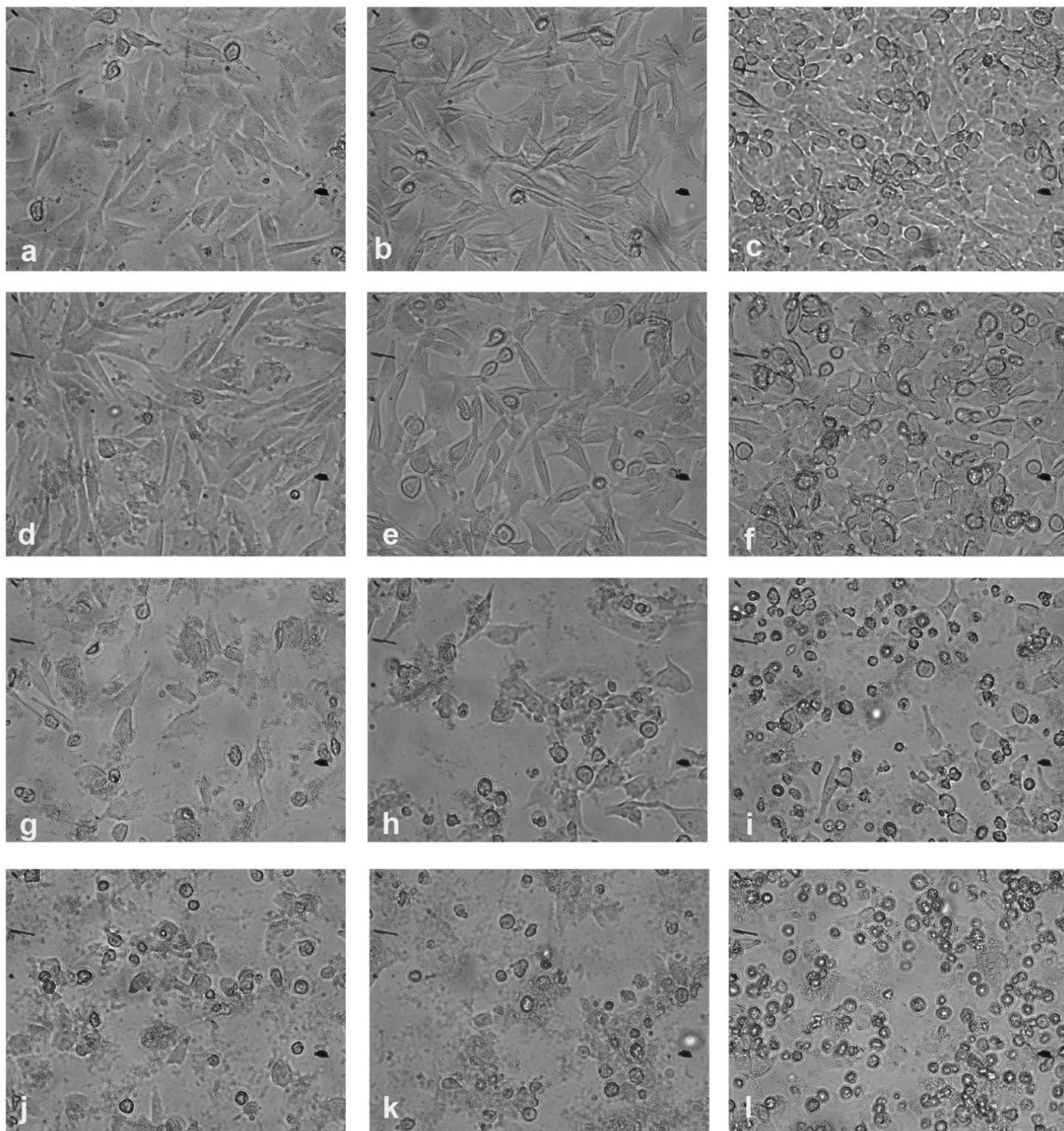


Figure 3

Phase contrast light microscopic observations of recovery at 24 h after treatment ("Recovery in drug free medium"). Cell lines: WM115 (left column), WM266-4 (middle column), and A375 (right column). Control (a, b, c); PsT 2 mg/mL plus NAC[®] (d, e, f); PsT 4 mg/mL plus NAC[®] (g, h, i); PsT 6 mg/mL plus NAC[®] (j, k, l). Images were acquired with a 20x objective

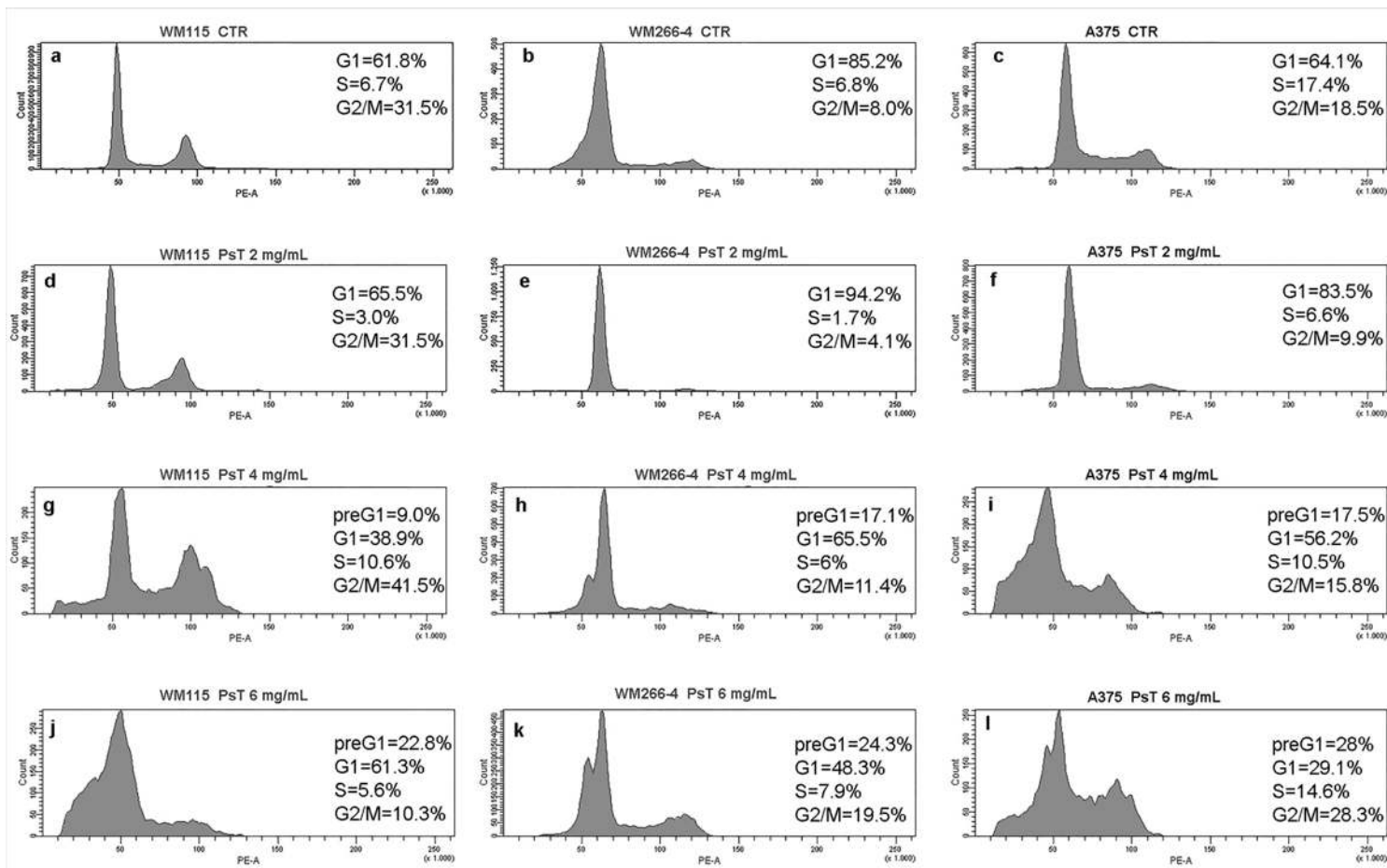


Figure 4

Analysis of cell cycle phase distribution after treatment with PsT plus NAC[®] on three human melanoma cell lines by DNA labeling with propidium iodide. WM115 control, CTR (a); WM266-4 control, CTR (b); A375 control, CTR (c). All cell lines were treated with different concentrations of PsT plus NAC[®]: 2 mg/mL, WM115 (d), WM266-4 (e), A375 (f); 4 mg/mL, WM115 (g), WM266-4 (h), A375 (i); 6 mg/mL, WM115 (j), WM266-4 (k), A375 (l) for 24 h. Histograms are representative of three independent experiments

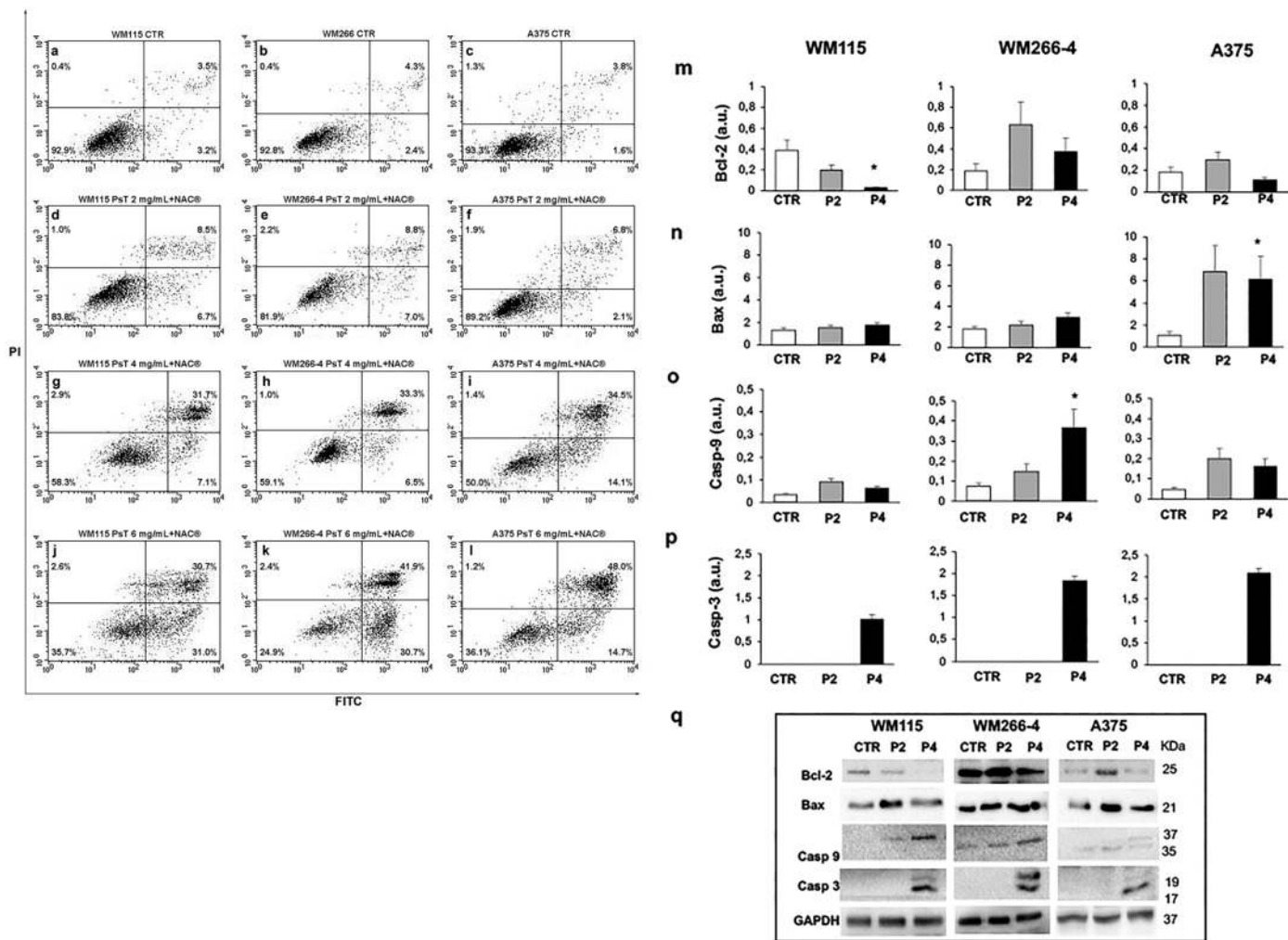


Figure 5

Flow cytometric and western blot analyses of cell death induced by PsT plus NAC[®] in three human melanoma cell lines. WM115 control, CTR (a); WM266-4 control, CTR (b); A375 control, CTR (c); treatment with PsT 2 mg/mL, WM115 (d), WM266-4 (e), A375 (f); with 4 mg/mL WM115 (g), WM266-4 (h), A375 (i); and 6 mg/mL WM115 (j), WM266-4 (k), A375 (l) for 24 h. The analysis was assessed by Annexin V-FITC and propidium iodide assay. Dot plots are representative of three independent experiments. Western blot analyses of cell death induced by PsT plus NAC[®] for 24 h on three human melanoma cell lines. Left panels WM115; middle panels WM266-4 and right panels A375; treatments with PsT 2 mg/mL and 4 mg/mL plus NAC[®], shown in the panel as P2 and P4, respectively. Protein level of Bcl-2 (m) is reduced at PsT 4mg/mL in WM115 and WM266-4 cell lines respect to control, while the levels of Bax (n), active caspase-9 (o) and active caspase-3 (p) increase in a dose-dependent manner after PsT plus NAC[®] treatments. (q) Representative Western blot images for each protein analyzed. The data represent three independent experiments. Data are presented as the mean $\pm$ SD. * $p \leq 0.05$ compared with control

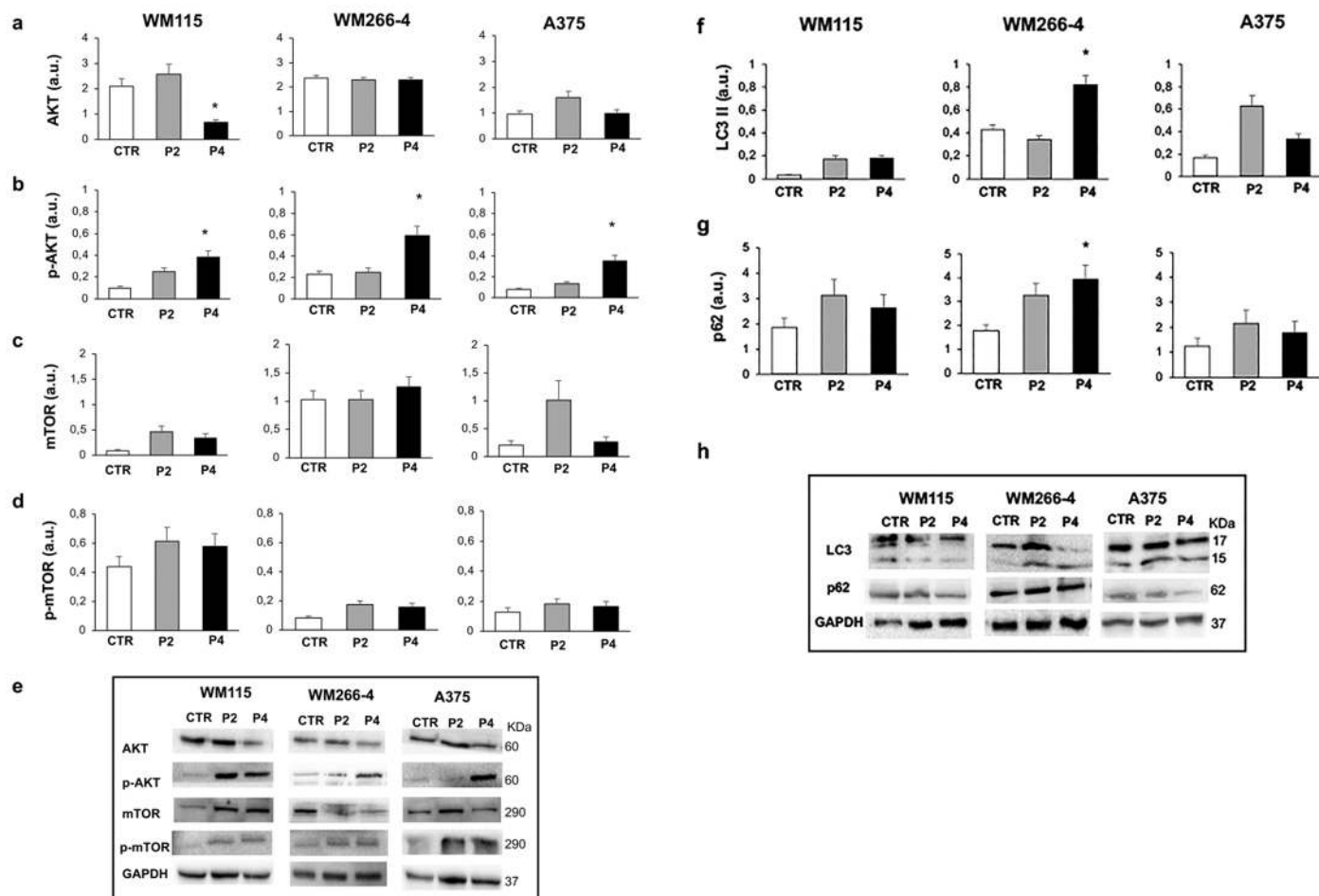


Figure 6

Western blot analysis of PI3K/AKT/mTOR and LC3/p62 signaling pathway induced by PsT plus NAC[®] in three human melanoma cell lines. Left panels WM115; middle panels WM266-4 and right panels A375; treatment with PsT 2 mg/mL and 4 mg/mL plus NAC[®], shown as P2 and P4 respectively, for 24 h. AKT protein levels (a) was reduced at PsT 4 mg/mL plus NAC[®] only in WM115 cell line. p-AKT (b) mTOR (c) and p-mTOR (d) levels increased, in a dose-dependent manner, in all cell lines after treatment with PsT plus NAC[®] at 24h. Protein levels of LC3 II (f) and p62 (g) increased in all cell lines, in a dose-dependent manner, after PsT plus NAC[®] treatment for 24h. (e, h) Representative Western blot images for each protein analyzed. Data represent three independent experiments. Data are presented as the mean $\pm$ SD. * $p \leq 0.05$ compared with control

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