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Induction of G_2/M arrest and apoptosis by sesquiterpene lactones in human melanoma cell lines

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ABSTRACT

Malignant melanoma is a highly aggressive tumor which frequently resists chemotherapy, therefore, the search for new agents for its treatment is of great importance. In this study, we purified the sesquiterpene lactones (SLs), Tomentosin and Inuviscolide from *Inula viscosa* (*Compositae*) leaves and studied their anti-cancer potency against human melanoma cell lines in order to develop new agents for melanoma treatment. SLs inhibited the proliferation of three human melanoma cell lines: SK-28, 624 mel and 1363 mel in a dose-dependent manner. We further investigated SLs mechanism of action using SK-28 as a representative cell line model. SLs caused cell-cycle arrest at G_2/M, accompanied by the appearance of a sub-G0 fraction, indicative of apoptotic cell death. Induction of apoptosis was further confirmed by changes in membrane phospholipids, changes in mitochondrial membrane potential ($\Delta\Psi$) and by detection of Caspase-3 activity. Rapid inhibitory phosphorylation of Cdc2 (Thr14 and Tyr15) was seen early after treatment, followed by a later decrease in the expression level of both Cyclin b1 and Cdc2. Induction of p53 and $p21^{waf1}$ proteins and phosphorylation of p53 at Ser15 were also detected early after treatment. The anti-apoptotic proteins, p65 subunit of nuclear factor κB (NF-κB), and Survivin were reduced in a dose-dependent manner. Taken together, these changes partially explain the ability of the SLs to induce G_2/M arrest and apoptosis. Induction of apoptosis by Tomentosin and Inuviscolide in human aggressive melanoma cell lines has high pharmacological value and implies that SLs might be developed as new agents for melanoma treatment.

1. Introduction

Plants of the *Compositae* family produce a wide array of sesquiterpenoid compounds, especially sesquiterpene lactones (SLs), as their main secondary metabolites [1,2]. SLs have been identified as the active constituents of several medicinal plants used in traditional medicine, with a wide spectrum of biological activities including, anti-inflammatory and fungicidal properties [3,4]. Recent studies attempted to reveal the anti-cancer activity and the chemotherapeutic applications of SLs [5,6]. The bioactive functional group in the SLs is believed to be the highly electrophilic α-β-unsaturated carbonyl

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Abbreviations: ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia-Rad3-related; MDM2, murine double minute; NF-κB, nuclear factor κB; SLs, sesquiterpenoid lactones; IV, *Inula viscosa* water extract; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PI, propidium iodide; PS, phosphatidylserine; SSBs, single-strand breaks; DSBs, double-strand breaks.

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structures, such as α-methylene-γ-lactone, which is capable of interacting rapidly with the nucleophilic sites of biological molecules in a Michael-type addition [6]. Alkylation of cysteine sulfhydryl groups in proteins by SLs was suggested to disrupt the function of various macromolecules [6]. Furthermore, DNA was also suggested to be a target molecule of SLs; thus, DNA alkylation represents a potential molecular basis of cytotoxicity for these compounds [7]. Nevertheless, the exact cellular target(s) and the molecular mechanisms relevant to SLs antitumor activity *in vitro* or *in vivo* have not yet been elucidated [8].

The present study focused on two SLs isolated from *Inula viscosa* (*Compositae*), Inuviscolide and Tomentosin. *I. viscosa* is known in folk medical tradition for its various properties including antipyretic, anti-inflammatory, antiseptic and antimicrobial [9,10]. Inuviscolide was reported to be the main anti-inflammatory sesquiterpenoid contained within *I. viscosa* and has proved effective against skin diseases [11]. It also exhibits antimicrobial activities [12] and cytotoxicity to some cancer cell lines [1]. Tomentosin possesses fungistatic [13] and antimalarial activities [14]. Although these compounds were described previously, their anti-cancer efficacy had not been evaluated.

Malignant melanoma is one of the most virulent forms of cancer, and advanced melanoma has a very poor prognosis. Therefore, the discovery of better anti-cancer agents against melanoma is an urgent goal. Melanocytes, the natural photoprotectors of the skin, secrete melanin and protect neighboring epidermal cells from DNA damaging agents, such as UV light [15]. Therefore, it is not surprising that the very poor survival seen in melanoma patients is mainly due to the notorious resistance of melanoma to radiation or chemotherapy. In cancer, drug resistance is mainly attributed to improved DNA repair, reduced drug accumulation and/or defects in the apoptotic pathways [16]. In contrast to many other aggressive and chemoresistant cancers, p53 mutations are rarely observed in melanoma. The basis of drug resistance in melanoma is most often dysregulation of apoptosis that is manifested in over-expression of survival proteins such as Survivin, down regulation of proteins involves in apoptosis such as Apaf-1, or high expression of the anti-apoptotic protein, Bcl-2 [16,17].

The main goals of the present study were to demonstrate the anti-carcinogenic effect of Tomentosin and Inuviscolide purified from a water extract of *I. viscosa* (IV) and to delineate the mechanisms involved in their anti-cancer properties. Our model systems included the human melanoma cell lines SK-28, 624 mel and 1363 mel. In the present study, we demonstrated that the effect of the two SLs, Tomentosin and Inuviscolide on melanoma cell growth involves interference with cell-cycle progression and induction of apoptosis. Their mode of action and the signaling pathways leading to their profound biological effect were studied in detail.

2. Materials and methods

2.1. Extraction and isolation procedure

Fresh leaves of *I. viscosa* were collected from a field at Bar-Ilan University. The leaves were dried for 2 days in the sun, and for another 4 days at room temperature. The dried leaves were homogenized in distilled water 1:8 (w/v). The homogenate was collected, filtered through Whatman No. 1 filter paper and centrifuged at 20,000 × *g* for 10 min. The supernatant was frozen in liquid nitrogen and dried in a lyophilizer (0.07 mbar, −48 °C). The resulting powdered extract was designated IV watery extract. The powder was further extracted in acetonitrile overnight, filtered, and evaporated under reduced pressure. The acetonitrile extract was separated by thin layer chromatography (TLC) into two main SLs components: Tomentosin (Rf = 0.88) and Inuviscolide (Rf = 0.725) (silica gel 60 F_{254} plates, Merck Eurolab SA, Strasbourg, France) using the solvent system benzene/methanol (3:1). The SLs, Inuviscolide and Tomentosin (Fig. 1A) were identified by full analysis of the ^{1}H and ^{13}C NMR spectra and by comparison with published data, respectively [18,19]. The purity of the SLs was greater than 80%. The SLs were delivered to the cells in ethanol solution. They were mixed with the medium and then the medium was added to cells. Control cultures were similarly treated with respective amounts of ethanol. Final concentration of ethanol in the culture medium did not exceed 0.2% for any treatment (v/v).

2.2. Cell culture

SK-28 melanoma cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). The two melanoma cell lines, 624 mel and 1363 mel were obtained from the Surgery Branch of the National Cancer Institute. All three cell lines were cultured in RPMI 1640 medium (Biological Industries, Inc., Kibbutz Beit Haemek, Israel), supplemented with 10% fetal calf serum (FCS), 1% penicillin–streptomycin–nystatin, and 0.2% amphotericin. The cells were maintained at 37 °C and 5% CO_2 in a humid environment.

2.3. Cell proliferation and viability assay

The effect of SLs on cell proliferation was measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, MO) assay, based on the ability of live cells to cleave the tetrazolium ring in active mitochondria to a product molecule that absorbs at 570 nm. SK-28, 624 mel and 1363 mel cells (5×10^3) were grown in RPMI-10% FCS on a 96-well microtiter plate and incubated with the SLs, Tomentosin or Inuviscolide (9–36 μM). After 24 h, the medium was replaced with 130 μl of fresh RPMI-1640 complete media and 20 μl of MTT reagent (5 mg/1 ml PBS) added to each well. The cells were incubated at 37 °C for an additional 2 h. Subsequently, the cells were lysed by the addition of 100 μl *N*,*N*-dimethyl formamid solution (50% final concentration of *N*,*N*-dimethyl formamid and 20% sodium dodecyl sulphate, pH 4.7) to each well, and incubated for 7 h. Absorption at 570 nm for each well was assessed using an ELISA reader. IC_{50} values were calculated from cell viability curves (MTT assay, Fig. 1B) and defined as the concentration resulting in 50% inhibition in cell survival as compared to untreated cells.

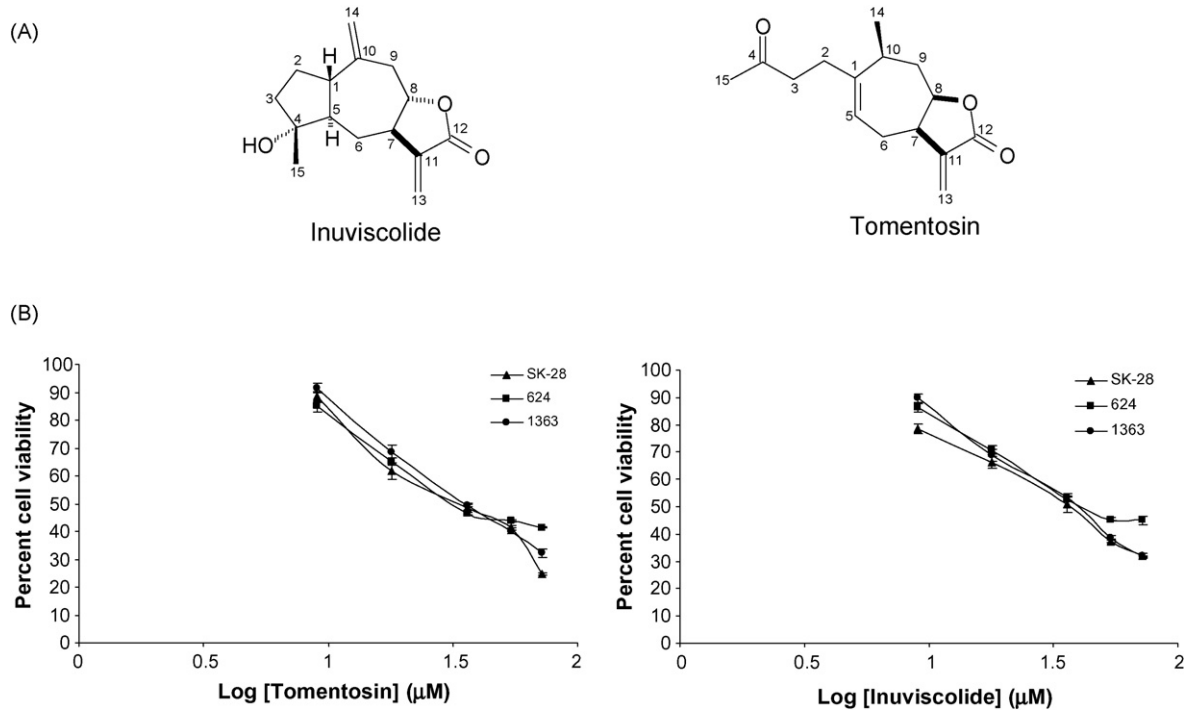

Fig. 1 – Reduced cell viability following treatment with the SLs Tomentosin or Inuviscolide. (A) Chemical structure of Tomentosin and Inuviscolide (Mw = 278) purified from *Inula viscosa*, as described in Section 2. (B) Cell viability of SK-28, 1363 mel or 624 mel melanoma cells was measured by MTT assay following treatment with the SLs (9–72 μM) for 24 h. Values represent percent of living cells relative to the control (untreated cells), against the log of the drug concentration, from three independent experiments ± S.D.

2.4. *Flow cytometry*

SK-28 cells (7×10^5) were seeded in 100 mm culture dishes, and allowed to attach overnight. The medium was replaced with fresh complete medium containing the desired concentration of the SLs (Tomentosin, 36 and 54 μM; Inuviscolide, 36–72 μM). Cells were incubated for 4, 24 or 48 h at 37 °C, washed with PBS and centrifuged at 300 × *g*. Both the cells growing as a monolayer (harvested by scraping) and those floating in the medium, were collected. The pellet was stained with 50 μg/ml propidium iodide (PI) for 15 min, and analyzed using a flow cytometer.

2.5. *Annexin V/PI flow cytometric staining technique*

Apoptosis was detected with an Annexin V-FITC kit purchased from MBL Co., Ltd. (Watertown, MA, USA), according to the manufacturer's instructions. SK-28 cells (6×10^5) were seeded in 100 mm culture dishes, and allowed to attach overnight. The cells were treated with the SLs (Tomentosin, 54 μM; Inuviscolide, 72 μM) for 48 h, and then were collected and washed with ice-cold PBS. To detect early and late apoptosis, both adherent and floating cells were harvested together. The washed cell pellet was resuspended in ice-cold binding buffer containing FITC-conjugated annexin V and PI. The sample was incubated for 15 min in the dark before analysis by flow cytometer.

2.6. *JC-1 mitochondrial membrane potential detection assay*

The fluorescent cationic dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide (JC-1) (Sigma, MO, USA) was used for *in situ* detection of mitochondrial membrane transition events in live cells, to provide an early indication of the initiation of cellular apoptosis. JC-1 is internalized as a monomer in the cytosol (green fluorescence and emission wavelength 530 nm) and also accumulates as aggregates in mitochondria with negative inner membrane potential (red fluorescence and emission wavelength 590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. For this assay, cells were treated with the SLs (0, 36 and 54 μM) and maintained at 37 °C in a humidified 5% CO_2 atmosphere for 24 h. Cells were washed with PBS and centrifuged at 300 × *g*. The pellet was resuspended in 500 μl PBS, and 2 μl of 1 mg/ml JC-1 reagent was added for 20 min at 37 °C in the dark. Cells were washed with PBS and analyzed for apoptosis using a flow cytometer. Analysis was performed using Cell Quest software (BD Biosciences, San Jose, CA, USA).

2.7. *Caspase-3 activity*

Caspase-3 activity was measured using the colorimetric assay (Bachem) with Ac-DEVD-*p*-nitroanilide (pNA) as substrate,

according to the manufacturer's protocols. SK-28 cells (6.5×10^5) were plated in 100 mm culture dishes and grown for 24 h. Various concentrations of the SLs (36–72 μM) were added in RPMI-1640 complete media for 24 h. The medium was collected and the cells were washed with cold PBS. The cells were scraped and then washed twice by centrifugation at $500 \times g$ for 5 min at 4 °C. The cell pellet was resuspended in lysis buffer supplemented with phosphatase inhibitors and incubated for 1 h at 4 °C. After centrifugation at $14{,}000 \times g$ for 40 min at 4 °C, the supernatant (total cell lysate) was collected and 100 μg protein was transferred to a well of 96-well microtiter plate. The reagent mixture consisting of 140 μl reaction buffer (100 mM Hepes pH 7.5, 20% glycerol, 0.5 mM EDTA pH 8, and 5 mM dithiothreitol in water) and 10 μl substrate (117 μM) was added to 50 μl of the supernatant and incubated at 37 °C for 6 h. The rate of Caspase-3 enzymatic hydrolysis was measured by release of pNA from Caspase substrate (405 nm).

2.8. Western blot analysis

SK-28 cells (7×10^5) were treated with the SLs (18–54 μM) for 2, 4 or 24 h. At the end of the incubation period, 30 μg of cytoplasmic protein extracts, prepared as described previously [20], was resolved on 12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (1% nonfat dry milk/1% Tween 20 in PBS) for 1 h at room temperature, and then incubated with appropriate monoclonal primary antibodies (human reactive anti-Cdc2, anti-Survivin from Santa Cruz Biotechnology, anti-Cyclin b1 from Biosource, and anti-$p21^{waf1}$ from BD Biosciences) or polyclonal primary antibodies (human reactive anti-phospho-Cdc2 (Thr14 and Tyr15) from Santa Cruz, anti-p53 from Biolegend, anti-phospho-p53 (Ser15) from cell signaling and anti-NF-κB (p65/RelA) from Delta Biolabs) in blocking buffer overnight at 4 °C. The blot was then incubated with appropriate secondary horseradish peroxidase-conjugated antibody and detected by chemiluminescence and autoradiography using X-ray film. α-Actin was detected on the same membrane and used as a loading control. When necessary, quantification of α-actin normalized immunoblotting was carried out by densitometry. In caffeine–SL combination studies, cells were treated with caffeine (5 mM) 15 min prior to SL treatment.

2.9. Statistical analysis

All experiments were performed at least three times. Statistical analysis was performed using Student's *t*-test and statistical significance was expressed as $^{*}p < 0.05$, $^{**}p < 0.001$.

3. Results

3.1. Characterization and identification of SL compounds from Inula viscosa extract

We previously observed that a water extract of *I. Viscosa* (IV) has anti-cancer potential, as it induced changes in cell-cycle phase distribution and apoptotic cell death in various cell lines of human and mouse origin (data not shown). Notably, we found that B16 melanoma cells were more sensitive to IV treatment than 3T3 fibroblast cells (a non-cancerous cell line) with respect to inhibition of cell proliferation and induction of apoptotic death (data not shown). Importantly, these preliminary results suggest that the growth inhibitory effect of IV might be more specific to cancer cells, a pivotal requirement if this material is to be used as a potential chemotherapeutic drug. These preliminary results encouraged us to purify the active compounds in IV watery extract. The compounds were separated using TLC and their structures were determined by NMR spectroscopy as described in Section 2. The compounds isolated were identified as the SLs Tomentosin and Inuviscolide (Fig. 1A). This study focused on elucidating the biological activities of these two SLs tested in parallel against human melanoma cell lines.

3.2. SLs inhibit melanoma cell proliferation by cell-cycle arrest at G_2/M and by induction of apoptosis

To evaluate the effect of SLs on human melanoma cell viability, we used three different cell lines: SK-28, 1363 mel and 624 mel (Fig. 1B). The cells were cultured with different concentrations of the SLs (9–72 μM) on a 96-well plate for 24 h, and cell viability was measured by MTT assay. Dose response curves of cell viability of Tomentosin and Inuviscolide, expressed as a percentage of untreated control value against the log of the drug concentration, have been determined for all three cell lines. IC_{50} values have been calculated from the curves (Table 1). Treating the cells with either Tomentosin or Inuviscolide resulted in a dose-dependent inhibition of cellular proliferation, with no significant changes between the three cell lines. The IC_{50} mean values of Tomentosin in the three cell lines tested ranged from 31.6 ± 1.2 μM to 35.3 ± 1.4 μM and the IC_{50} values of Inuviscolide ranged from 37 ± 2.1 μM to 41.1 ± 3.8 μM. Furthermore, preliminary test showed that SLs treatment-induced apoptosis similarly in all three cell lines tested (data shown in Appendix A). For that reason, we focused in our further research on SK-28 as a representative cell line model, using SL concentrations close to their IC_{50} values. SK-28 is an aggressive human melanoma cell line which contains a very complex karyotype with deletions, translocations and atypical chromosome number.

To elucidate the mechanism(s) responsible for the SL-induced inhibition of cell proliferation, we first examined whether inhibition of cell viability by the SLs was associated

Table 1 – IC_{50} values for inhibition of human melanoma cell viability by Tomentosin and Inuviscolide

	IC_{50} (μM)		
	SK-28	624 mel	1363 mel
Tomentosin	33.6 ± 1.8	31.6 ± 1.2	35.3 ± 1.4
Inuviscolide	37 ± 2.1	41.1 ± 3.8	39 ± 3.1

Inhibition of the proliferation of SK-28, 624 mel, or 1363 mel melanoma cell lines was measured by MTT assay following 24 h of treatment with the two SLs. IC_{50} values were calculated from the logarithmic curves as shown in Fig. 1B. Data represent IC_{50} mean values ± S.D. from three different experiments.

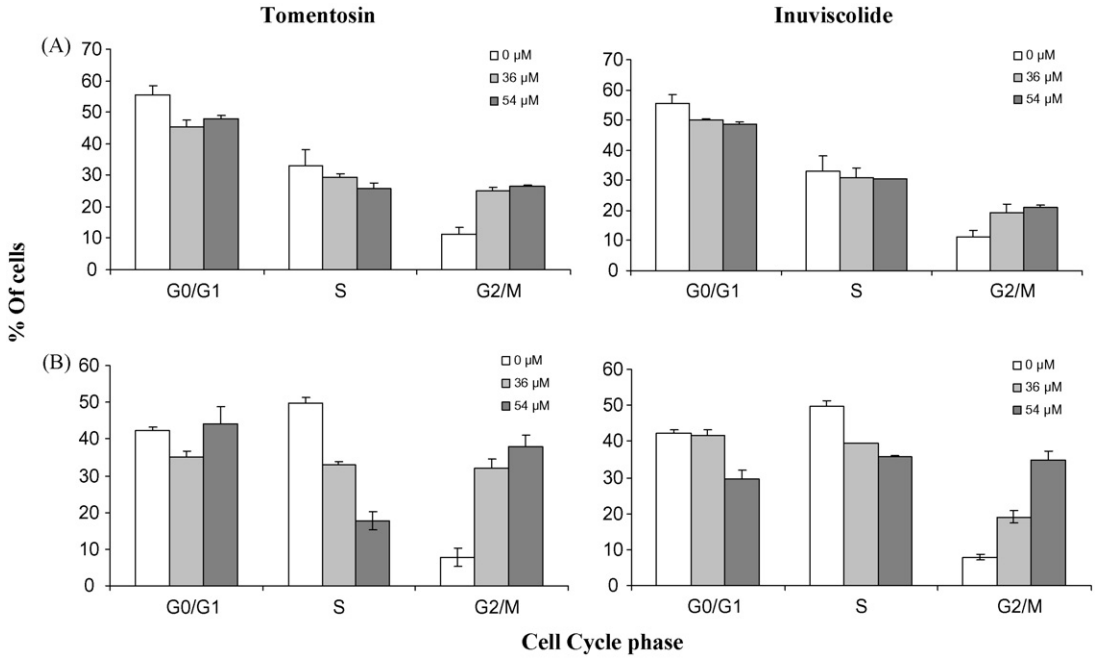

Fig. 2 – Treatment of SK-28 melanoma cells with SLs led to profound G_2/M arrest. Representative flow cytometric graphs of cells treated with SLs (36 and 54 μM) for 4 h (A) or 24 h (B). Control cells were treated with ethanol alone. Similar results were obtained in three independent experiments.

with changes in cell-cycle progression. A dose-dependent accumulation of cells at the G_2/M phase was seen as early as after 4 h treatment with each of the SLs (36 and 54 μM) (Fig. 2A). This G_2/M accumulation was strengthened following 24 h treatment (Fig. 2B). We could not detect apoptotic cells at the sub-G0 fraction (subdiploid DNA content) at either time point (data not shown). In order to determine the outcome of G_2/M arrest, we followed cell-cycle distribution after treatment for 48 h. At this time point, Tomentosin or Inuviscolide (36 μM) treatment led to a ~3-fold increase in the percentage of cells in G_2/M; higher doses of Tomentosin or Inuviscolide (54 μM and 72 μM, respectively) induced a profound sub-G0 peak at the expense of cells accumulated in the G_2/M phase (Fig. 3A). Such a sub-G0 peak could also be observed following treatment with low doses of SLs for extended periods of time (data not shown). To confirm the possibility that prolonged treatment with SLs induces apoptosis, we measured the induction of apoptosis by the SL compounds using a double staining method employing FITC-conjugated Annexin V and PI.

One characteristic of apoptosis is the loss of plasma-membrane polarity, accompanied by translocation of phosphatidylserine (PS) from the inner to outer membrane leaflets, thereby exposing PS to the external environment. The phospholipid-binding protein, Annexin V, binds to cells with externally exposed PS [21], while PI staining occurs only after loss of membrane integrity. The dual staining distinguish between unaffected cells (unlabelled; quadrant 3), early apoptotic cells (Annexin V positive; quadrant 4), and late apoptotic cells (Annexin V positive, PI positive; quadrant 2). Treatment of SK-28 cells for 48 h with 54 μM Tomentosin or 72 μM Inuviscolide resulted in ~70% of the cells exhibiting markers of early apoptosis (Fig. 3B). Taken together, these results suggest that SL-treated cells resolved G_2/M arrest by induction of apoptosis.

3.3. *SL-induced apoptotic cell death involves changes in mitochondrial membrane potential and Caspase-3 activation*

Although sub-G0 cells were not detected after 24 h treatment with SLs, a small number of Annexin V positive cells were observed at this time point (data not shown). For this reason, we attempted to determine whether other features of apoptosis were already apparent after treatment for 24 h. To this end, we measured the effect of SLs on mitochondrial membrane potential ($\Delta\Psi$) using JC-1, a fluorescent cationic dye exhibiting potential-dependent accumulation in mitochondria. Collapse of mitochondrial membrane potential is an early event in the apoptotic process [22]. In healthy, non-apoptotic cells, JC-1 exists as monomers in the cytosol (FL1 positive; green) and also accumulates as aggregates in the mitochondria (FL2 positive; red). In apoptotic or necrotic cells, JC-1 dye remained in its monomeric form, since the loss in $\Delta\Psi$ does not enable accumulation of the dye in the mitochondria. Thus, apoptotic cells emit relatively more green fluorescence (present in figure as R2). As shown in Fig. 4A, treatment of SK-28 cells with SLs for 24 h decreased the mitochondrial membrane potential in a dose-dependent manner, as indicated by a decrease in the red/green fluorescence ratio. The green signal increased from 17% in untreated cells to 63% in cells treated with 54 μM Tomentosin or 37% in cells treated with 54 μM Inuviscolide.

Another apoptotic characteristic is the activation of specific cysteine proteases, known as Caspases, which play an

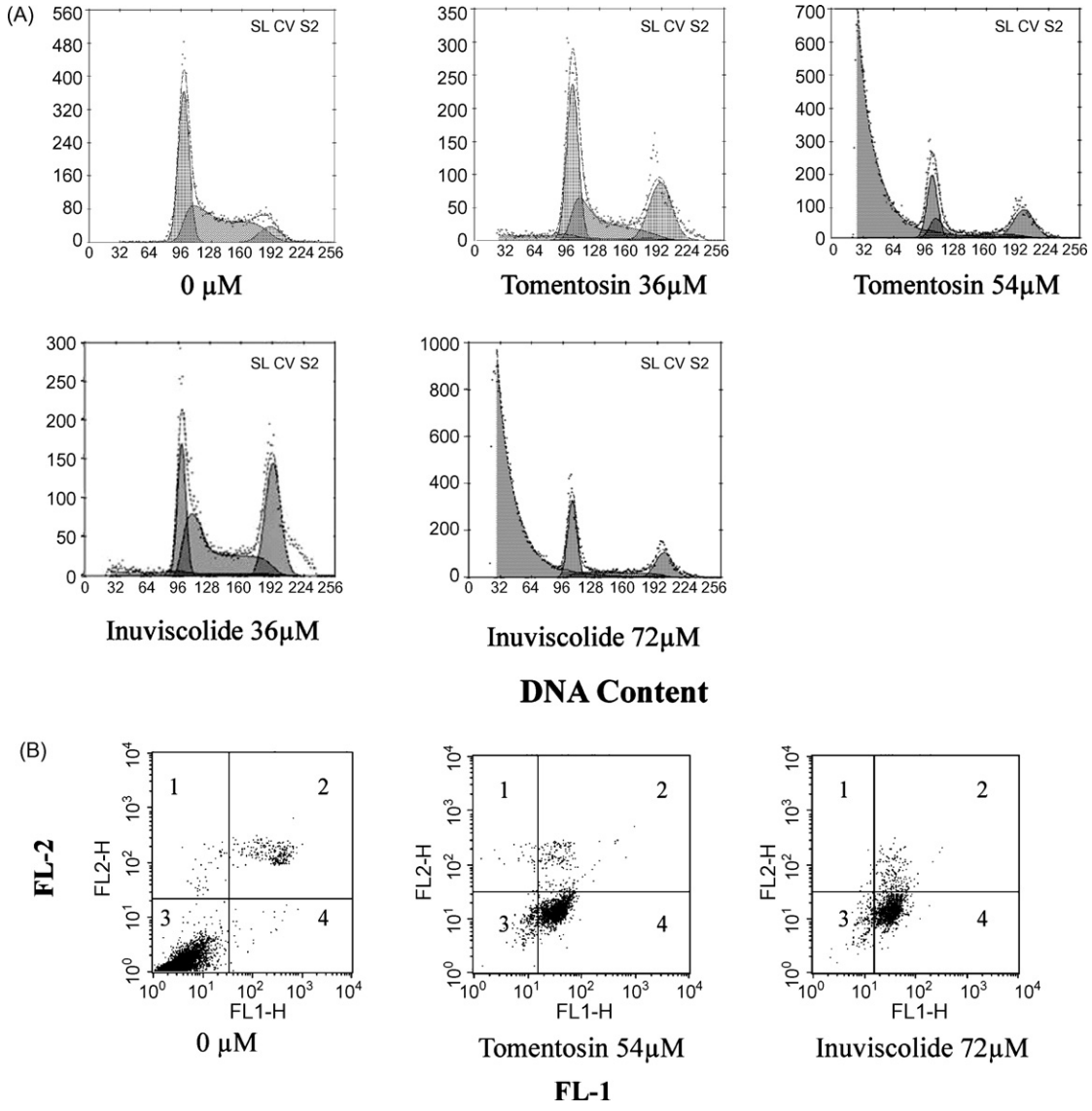

Fig. 3 – SK-28 cells treated with the SLs for 48 h exhibited G_2/M arrest and apoptosis. Representative flow cytometric histograms of (A) cell-cycle distribution following treatment with Tomentosin (36 and 54 μM), or Inuviscolide (36 and 72 μM) and (B) induction of apoptosis, measured by Annexin V assay following treatment with Tomentosin 54 μM or Inuviscolide 72 μM. The horizontal (FL1) and vertical (FL2) axes represent labeling with Annexin V and PI, respectively. Control cells were treated with ethanol alone. Similar results were obtained in three independent experiments.

important role in executing apoptotic cell death. Caspases act in a cascade initiated by 'regulator' Caspases, ultimately leading to the cleavage of substrates by 'effector' Caspases that produce the characteristic features of apoptosis [23]. Therefore, we investigated whether Caspase-3, which is the main 'effector' Caspase in the apoptotic pathway, was activated in SK-28 cells following treatment with SLs. Caspase-3 activation was measured by monitoring cleavage of its substrate Ac-DEVD-pNA. We observed a net dose-dependent elevation of Caspase-3 enzymatic activity in cells treated for 24 h with SLs, with Tomentosin having a stronger effect than Inuviscolide (Fig. 4B).

These results support the cell-cycle analysis and demonstrate that although the immediate effect of SL treatment in melanoma cell lines is G_2/M arrest, the final outcome is apoptotic cell death. When considering the general resistance of melanoma cells to apoptosis [16,17], these results emphasize the potential of SLs as new agents for treatment of melanoma.

3.4. *SL-induced G_2/M arrest involves Cdc2 (Tyr15 and Thr14) phosphorylation following a decrease in Cdc2/Cyclin b1 expression*

Having established that the SLs induce cell-cycle arrest, we attempted to characterize, at the molecular level, the mechanisms by which this effect is achieved. To this end, we investigated the effects of the SLs in SK-28 cells on key regulators of the G_2 to M transition, Cdc2 (Cdk1, p34) and Cyclin b1 using Western blot analysis. Treatment for 24 h with

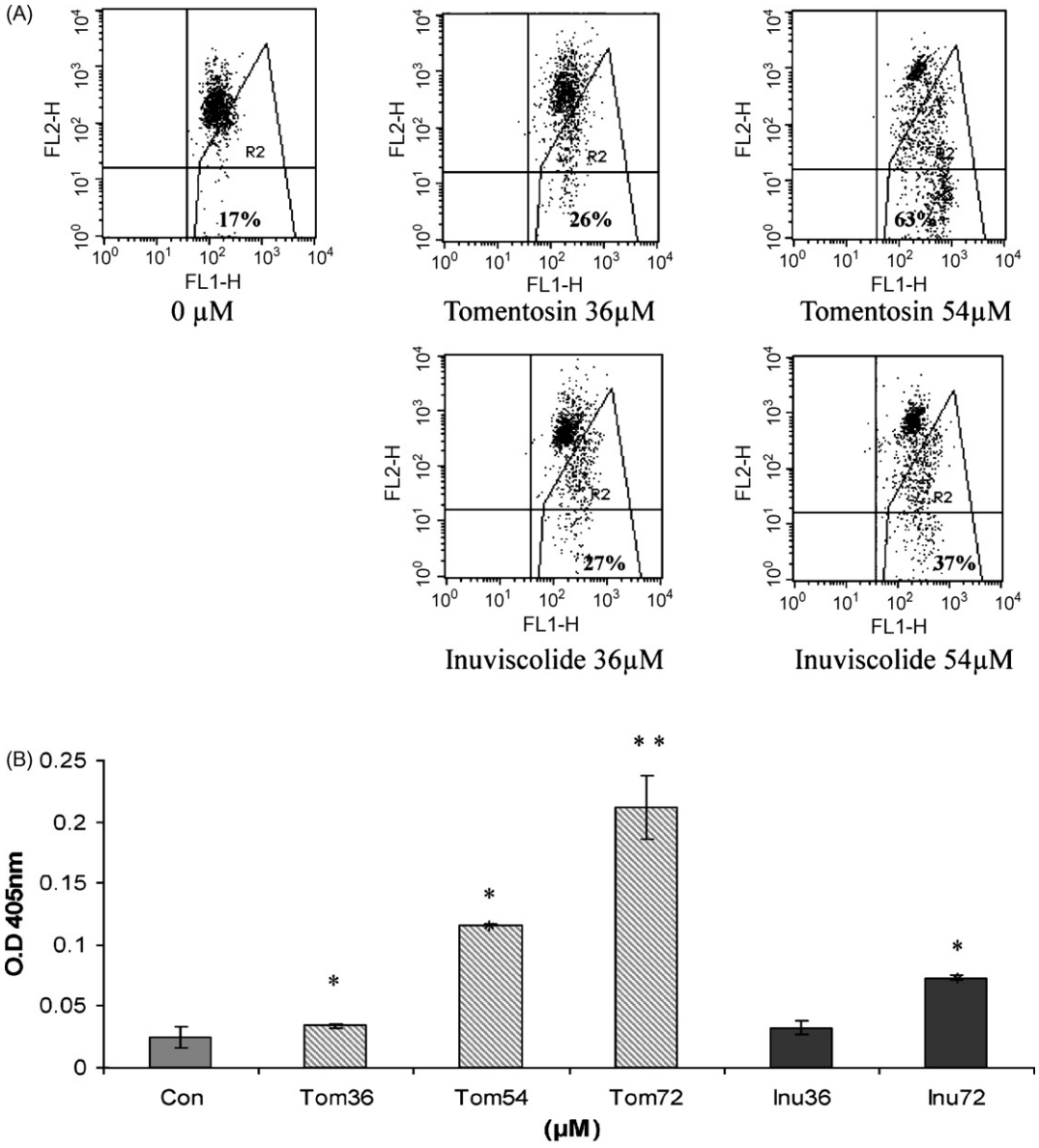

Fig. 4 – Tomentosin (Tom) or Inuviscolide (Inu)-induced apoptosis in SK-28 cells. Induction of apoptosis following 24 h treatment with SLs was assessed by (A) measurement of mitochondrial membrane potential, using JC-1 reagent, and (B) measurement of Caspase-3 activity. (A) Cells were treated with the SLs (36 and 54 μM) for 24 h and analyzed on a FACScan cytometer. Dot plots of red (FL2) vs. green fluorescence (FL1) show live cells with intact mitochondrial membrane potential and apoptotic cells with lost mitochondrial potential, respectively. Similar results were obtained in three independent experiments. (B) Cells were incubated with the SLs (36–72 μM) for 24 h. Cells were then lysed and measured for Caspase-3 activity. Enzymatic activity was determined by cleavage of pNA from Ac-DEVD-pNA (405 nm). Untreated cells incubated with substrate in lysis buffer were used as control. Data represent mean ± S.D. from four different experiments (*$p < 0.05$; **$p < 0.001$).

the SLs, caused reduced protein expression of both Cdc2 (Cdk1, p34) and Cyclin b1 (Fig. 5A and C, respectively). Treatment for 4 h had no effect on Cdc2 protein levels, and only a slight increase in Cyclin b1 levels was observed. Since cell-cycle entry into the mitotic phase is initiated by dephosphorylation of the two inhibitory residues, Thr14 and Tyr15, of Cdc2, we determined the phophorylation status of these residues using a phosphorylation-specific antibody. Although 4 h treatment had no effect on the absolute protein level of Cdc2, a dose-dependent increase in Cdc2 phosphorylation (Thr14, Tyr15) was seen, indicating that the enzymatic activity of Cdc2 was inhibited (Fig. 5B). These results might explain the G_2/M arrest already detected after 4 h. At 24 h after treatment, there was a decrease in Cdc2 phosphorylation, probably due to decreased expression or increased degradation of Cdc2 itself. The reduced expression of both Cdc2 and Cyclin b1 at 24 h and the increased inhibitory phosphorylation of Cdc2 at 4 h, suggest that these proteins are mediators of the G_2/M arrest induced by SLs.

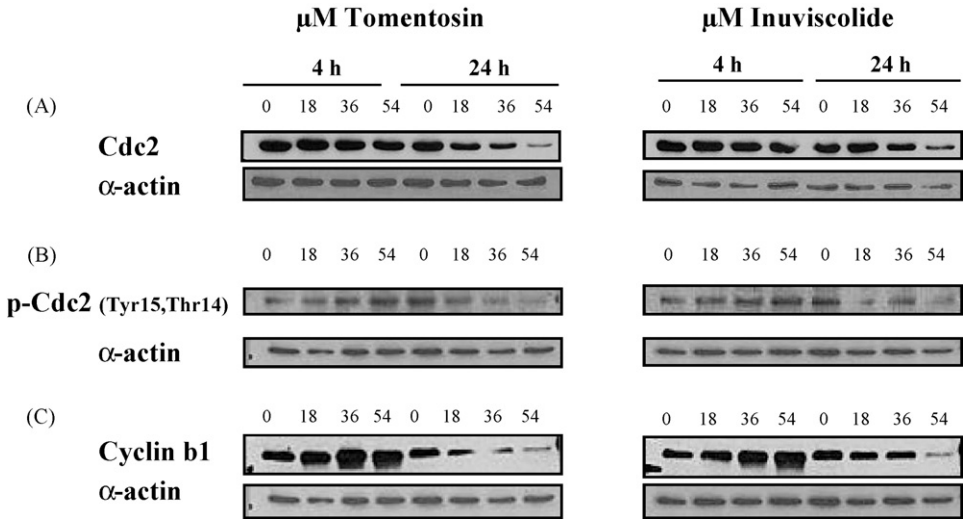

Fig. 5 – Tomentosin or Inuviscolide-induced changes in G_2/M regulatory proteins in SK-28 cells. Cells were treated with either ethanol (control) or with increasing concentrations of SLs (18–54 μM) for 4 or 24 h. After treatment, total cell lysates were prepared and 30 μg protein was subjected to SDS-PAGE, followed by immunoblot analysis. The expressions of Cdc2, phospho-Cdc2 (Tyr15, Thr14), and Cyclin b1 were measured (A–C, respectively) and equal loading was confirmed by measuring α-actin levels. Results are representative of five independent experiments.

3.5. *SL-induced p53 activation involves Ser15 phosphorylation and $p21^{waf1}$ induction*

The rapid phosphorylation of Cdc2 following treatment with the SLs, which is a typical response of cells to DNA damaging agents [24], led us to speculate that SLs might cause DNA damage. p53 is a tumor suppressor protein that modulates both cell cycle and apoptosis in response to DNA damaging agents. Since SK-28 cells express wild-type p53 [25], we examined possible changes in protein expression induced by SL treatment. Indeed, p53 expression level increased after 2 h incubation with the SLs (36, 54 μM, Fig. 6A). Phosphorylation of p53 at residue Ser15, plays a role in stabilizing p53 and enhancing its trans-activation capacity [26]. Thus, 4 h treatment with either Tomentosin or

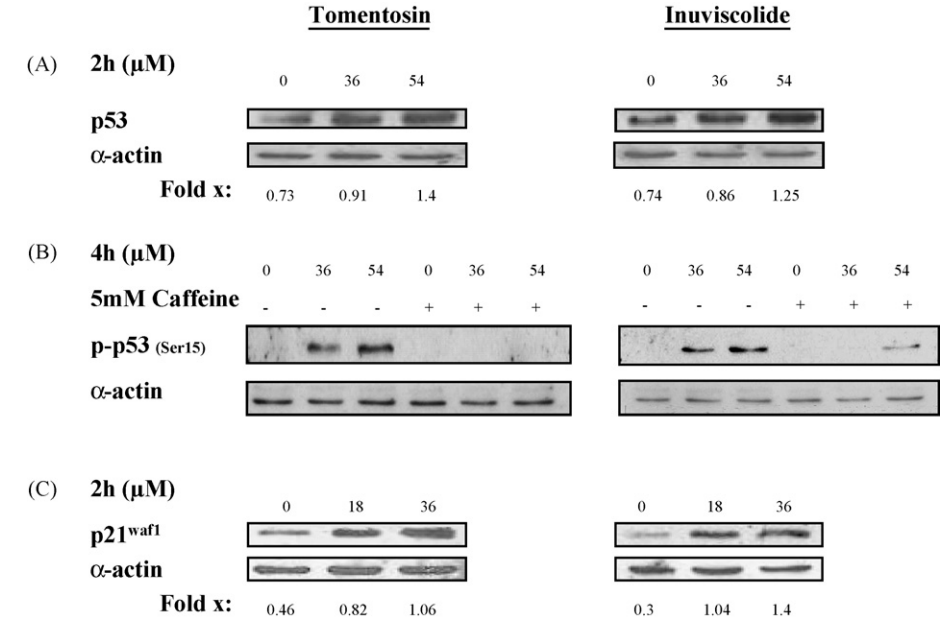

Fig. 6 – Tomentosin and Inuviscolide increased (A) p53 (B) phospho-p53 (Ser15) and (C) $p21^{waf1}$ protein expression in SK-28 cells. Cells were treated with ethanol (control) or with SLs (18–54 μM) for 2 (A and C) or 4 (B) h. After treatment, total cell lysates were prepared and 30 μg protein was subjected to SDS-PAGE, followed by immunoblot analysis. Membranes were probed with anti-p53, anti-phospho-p53 (Ser15), anti-$p21^{waf1}$ and α-actin antibodies. For (A) and (B), quantification of protein levels by α-actin-normalized densitometry is shown (Fold x). In caffeine-treated cells, caffeine (5 mM) was added to cells 15 min before SLs. Results are representative of four independent experiments.

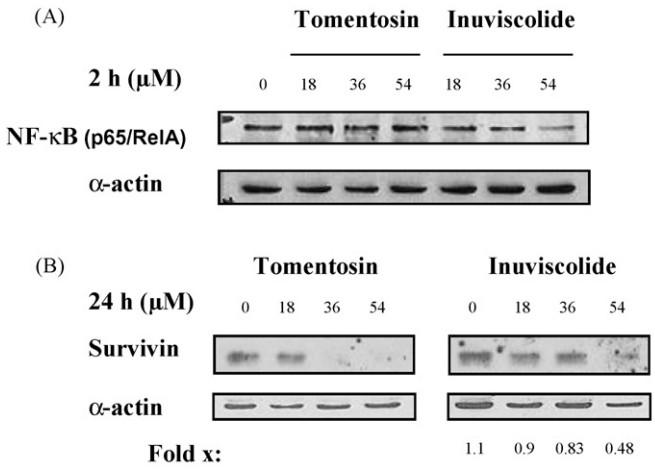

Fig. 7 – Tomentosin and Inuviscolide reduced protein expression of the survival proteins NF-κB (p65/RelA) (A) or Survivin (B) in SK-28 cells. Cells were treated with ethanol (control) or with SLs (18–54 μM) for 2 h (A) or 24 h (B). After treatment, total cell lysates were prepared and 30 μg protein was subjected to SDS-PAGE, followed by immunoblot analysis. Equal loading was confirmed by measuring α-actin levels. For (B) Inuviscolide, quantification of protein levels by α-actin-normalized densitometry is shown (Fold *x*). Results shown are representative of four independent experiments.

Inuviscolide exhibited a clear concentration-dependent elevation in the phosphorylation of p53 at Ser15, while no phosphorylation was detected in control cells (Fig. 6B). This phosphorylation was already seen after 1 h incubation with the SLs (data not shown). One of the target genes of p53 is $p21^{waf1}$, a cyclin-dependent kinase inhibitor (CDKI), which inhibits Cyclin b1/Cdc2 activity [27]. In order to verify the transactivation activity of p53 following treatment with the SLs, we measured $p21^{waf1}$ levels in SK-28 cells following 2 h treatment with the SLs; our results demonstrate an elevation in $p21^{waf1}$ expression (Fig. 6C). Thus, we suggest that $p21^{waf1}$ elevation and the increased Cdc2 phosphorylation (Thr14, Tyr15) can both contribute to Cdc2 inhibition, leading to the observed early accumulation of cells in G_2/M phase.

Phosphorylation of p53 at Ser15 is usually catalyzed by the protein kinases, ataxia telangiectasia mutated (ATM) and ataxia telangiectasia-Rad3-related (ATR) [28,29]. Caffeine has been demonstrated to inhibit the activity of these kinases [30]. Thus, in order to gain preliminary data concerning the possible connection between the observed SL-induced p53 phosphorylation and activation of the kinases ATM/ATR in SK-28 cells, we treated the cells with 5 mM caffeine prior to SL treatment. Caffeine-treated cells exhibited a significant decrease in Ser15 phosphorylation (Fig. 6B), suggesting that ATM/ATR may be involved in activation of p53 by SLs.

3.6. *Effect of SLs on the expression of the survival proteins, NF-κB and Survivin*

NF-κB and Survivin are two anti-apoptotic proteins known to be up-regulated in melanoma. The increased level of these proteins is thought to contribute to the resistance of malignant melanocytes to apoptotic death, as induced by common chemotherapeutic drugs [31–33]. NF-κB is a nuclear transcription factor that is important for cell survival and is known to be a molecular target of many SLs [34]. In mammalian cells, NF-κB is a homo- or heterodimer containing five different DNA-binding subunits. Most frequently NF-κB is present as a heterodimer composed of the p50 and p65/RelA subunits [35]. We investigated the effects of Tomentosin and Inuviscolide (18–54 μM) on the expression of the p65/RelA subunit of NF-κB, which is necessary for the transactivation activity of NF-κB. Inuviscolide application for 2 h, induced a dose-dependent decrease in p65/RelA protein levels. However, Tomentosin applied to cells for 2 h did not change the expression of p65/RelA (Fig. 7A). Longer incubation with Tomentosin also did not change p65/RelA levels (data not shown). Survivin, a member of the 'inhibitor of apoptosis proteins' (IAP) family, is a bifunctional protein that regulates cell division and suppresses apoptosis [24]. Treating SK-28 cells with either Tomentosin or Inuviscolide for 24 h resulted in decreased Survivin expression, which was more profound following treatment with Tomentosin versus Inuviscolide (Fig. 7B). The decreased protein level of the two anti-apoptotic proteins, Survivin (by both SLs) and p65/RelA (by Inuviscolide) might contribute to the observed apoptosis following SLs treatment.

4. Discussion

Melanoma is the most aggressive form of skin cancer and is resistant to all current modalities of cancer therapy. Drug resistance in melanoma is associated with defects in the apoptotic program [16,17,36]. To combat the problem of chemoresistance, alternative agents are needed for the treatment of this tumor. Natural products are an important source of potential chemotherapeutic agents [37,38]. Recently, we elucidated the potential of cucurbitacin glucosides extracted from *Citrullus colocynthis* for the treatment of breast cancer, leading to G_2/M arrest and impairment of actin filament organization [20]. In the present study, we isolated two active compounds from *I. Viscosa* leaves, the SLs

Tomentosin and Inuviscolide. Although many SL compounds were suggested as potential anti-cancer agents, the cellular target relevant to their antitumor activity and their mechanism of action has not been described [8].

Tomentosin and Inuviscolide inhibited cell viability of three different human melanoma cell lines: SK-28, 624 mel and 1363 mel, indicating that these SLs have a broad spectrum of activity against human melanoma cells. We further investigated SLs mechanism of action, using the aggressive human SK-28 melanoma cell line as a representative model.

Cell-cycle analysis revealed that inhibition of cell viability by SLs resulted from cell-cycle arrest at the G_2/M phase, accompanied by an increase in sub-G0 fraction, indicating apoptotic cell death. G_2/M arrest was shown early after treatment, while apoptotic cell death was a later event. Treatment for 48 h with high doses of these SLs or treatment with low doses for an extended time period led to a decrease in the proportion of cells in the G_2/M phase at the expense of an increased sub-G0 phase, suggesting that programmed cell death ultimately follows G_2/M arrest. The induction of apoptosis by the SLs, as indicated by the sub-G0 peak, was further confirmed by other apoptotic features, detected as early as 24 h after treatment including cell-surface Annexin V binding, which measured the appearance of phosphatidylserine on the external plasma membrane, changes in mitochondrial membrane potential, measured by JC-1, and Caspase-3 activity. Taken together, these results suggest that cells begin apoptosis 24 h after treatment, and are manifested as fragmented apoptotic bodies (sub-G0 fraction) only 24 h later. These data emphasize that SL treatment leads to unavoidable apoptotic cell death. In order to elucidate the mechanism by which SLs induce G_2/M arrest, we examined changes in the expression of key proteins that are involved in regulating the cell cycle. The Cdc2/Cyclin b1 complex is the key enzyme regulating the G_2 to M transition, and is controlled by phosphorylation at various sites. This enzyme is maintained in an inactive form when phosphorylated by Wee1 kinase at residues Thr14 and Tyr15 in its ATP-binding domain [39]. Dephosphorylation of these sites late in G_2 activates the Cyclin b1/Cdc2 complex and triggers the initiation of mitosis. In mammalian cells, phosphorylation at these inhibitory sites of Cdc2 is stabilized in response to DNA damage [39], which delays mitosis in these cells [40]. We monitored Cdc2 status at various time points after treatment with the SLs, and observed an elevation in the inhibitory phosphorylation of Cdc2 (p-Cdc2) early after treatment, at a time when the total level of Cdc2 was still unchanged. At a later time point, a reduction in the protein level of Cdc2 was documented. Cyclin b1 protein levels showed a different kinetic behavior, with an increase in protein level shortly after initiation of treatment and a sharp reduction after 24 h. The increased levels of Cyclin b1 at early time points may represent the rapid accumulation of cells in G_2, when Cyclin b1 is preferentially expressed [41]. In summary, our observations of early elevation of Cdc2 phosphorylation followed by the reduction in Cyclin b1 and Cdc2 expression, suggest that SL-induced G_2/M phase arrest in SK-28 melanoma cells is mediated by inhibition of Cdc2 activity.

The above molecular changes closely resemble changes seen in cells subjected to DNA damage. The tumor suppressor protein, p53, can be activated by DNA damage, hypoxia, or aberrant oncogene expression to promote arrest at cell-cycle checkpoints, DNA repair, cellular senescence or apoptosis. Phophorylation of p53 at residue Ser15 is responsible for both enhanced transactivation activity and the dissociation of its inhibitor, murine double minute (MDM2), thereby preventing p53 degradation [42]. Indeed, p53 protein level was elevated early after treatment with either Tomentosin or Inuviscolide. In addition, we demonstrated phosphorylation of p53 at Ser15 early after treatment with both SLs. Activated p53 can alter the pattern of gene expression by activating or reducing the transcription of many genes that mediate its downstream functions [43]. One of the transcription targets of p53 is $p21^{waf1}$, a cyclin-dependent kinase inhibitor (CDKI). $p21^{waf1}$ binds to and inhibits the activity of the Cyclin b1/Cdc2 complex to cause arrest in the G_2 phase of the cell cycle [44]. In order to verify that the observed p53 induction and phosphorylation has biological significance in our cellular system, we measured $p21^{waf1}$ expression and found that cells treated with SLs express significantly higher levels of $p21^{waf1}$ compared to untreated cells. Under pathological conditions, p53 can modulate the activity of the Cdc2/Cyclin b1 complex via promoter repression, culminating in reduced protein expression [43]. Indeed, reduced expression of Cdc2 and Cyclin b1 was detected 24 h following treatment with the SLs. Taken together, we speculate that p53 activation is likely to contribute to the regulation of Cdc2 and cell-cycle changes observed after SL treatment.

ATM and ATR are known to phosphorylate p53 at Ser15 in response to DNA damage [28,29]. The increased phosphorylation of p53 at Ser15 following treatment with the SLs, became almost undetectable in cells pre-treated with caffeine, an ATM/ATR inhibitor. The findings presented here suggest that ATM and/or ATR might be involved in the signaling pathway initiated by Tomentosin or by Inuviscolide in melanoma cells.

The SL family of compounds is known as alkylating agents which can also alkylate the DNA [7]. It has been reported that various antitumor SLs-induced single-strand breaks (SSBs) in cellular DNA in a dose- and time-dependent manner. In addition, it was suggested that there is a correlation between DNA damage and the ability of SLs to inhibit cellular growth [45]. These previous reports, together with our results exhibiting that SLs induced early increase in the expression of p53, $p21^{waf1}$ and increased phosphorylation of Cdc2 (Thr14, Tyr15), and p53 (Ser15), strongly support the hypothesis that SLs might cause DNA damage. The DNA damage response could initiate a signaling cascade, leading to cell-cycle arrest and eventually, to apoptotic cell death. Some of the SSBs lesions typical to alkylating agents essentially require DNA replication to trigger apoptosis. These SSBs lead to collapse of replication forks, therefore converted into double-strand breaks (DSBs) that activate ATM and ATR, which trigger apoptosis via p53 [46]. It is possible that both SLs induce SSBs in DNA that are converted to DSBs during replication and therefore activate cell-cycle arrest at G_2/M. Elucidating ATM/ATR involvement and the possible mechanism of SLs-inducing DNA damage response will be the goal of our future research.

Chemoresistance is a major problem when treating melanoma. Many chemotherapeutic agents cause cell-cycle arrest but not apoptosis. Growth arrest allows cells to repair their DNA and limits the effectiveness of chemotherapy [44]. Thus, agents that can override tumor resistance toward

apoptosis are of great pharmacologic value [47]. Since the agents described here cause massive apoptotic death, we wished to better understand the mechanisms by which this critical effect is achieved. To this end, we followed the effects of the SLs on the expression of the main survival proteins, NF-κB and Survivin. These proteins are known to be constitutively active in melanoma, and contribute to the resistance of melanoma to apoptosis-inducing chemotherapeutic agents [31–33].

NF-κB, a pro-inflammatory nuclear transcription factor, regulates genes important for tumor invasion, metastasis, angiogenesis, and chemoresistance [48]. Exposure of cancer cells to anticancer drugs can induce the activation of the NF-κB pathway, leading to the expression of anti-apoptotic genes [49] and resistance to apoptosis [50]. NF-κB is a molecular target of many SLs [32], which influence NF-κB by directly alkylating specific cysteine residues (especially Cys^{38}) on the p65/RelA subunit of NF-κB, thereby preventing DNA binding of active NF-κB [51]. In this study, we found that Inuviscolide decreased the protein level of p65/RelA, suggesting that the apoptotic effect of Inuviscolide may be partly mediated via reduction of NF-κB signaling. The mechanism by which Inuviscolide decreased the protein level of p65/RelA is, as yet, unknown. However, because removal of chemically damaged proteins would be necessary to promote recovery from protein damage, it is tempting to speculate that the protein might be alkylated by Inuvisclolide and then targeted for degradation. Because the decrease in NF-κB protein levels has a major importance in reducing chemoresistance of cancer cells, checking the mechanism responsible for this decrease will be the goal of our future research. Treatment with Tomentosin did not alter p65/RelA expression levels. However, inhibition of NF-κB activity was not measured; therefore it is still possible that Tomentosin might inhibit NF-κB activity.

Survivin is a member of the 'inhibitor of apoptosis protein' (IAP) family. Its anti-apoptotic function is attributed to its inhibition of the terminal effectors Caspases-3 and -7, and its interference with Caspase-9 activity and processing [27]. Survivin is over-expressed in the majority of human tumors including melanoma, but is undetectable or found at very low levels in terminally differentiated adult tissue [52]. The expression of Survivin is dramatically increased upon malignant cell transformation [53]. Inhibition of Survivin can promote spontaneous apoptosis in tumor cells and can also enhance the efficacy of chemotherapy [54]. Grossman et al. [33] reported that targeting Survivin in melanoma cell lines triggered spontaneous apoptosis and increases Caspase-3 protease activity. Interestingly, we observed that Survivin levels decreased following 24 h of treatment with both SLs. Since treatment with Tomentosin resulted in both an extremely sharp decrease of Survivin expression even at low concentrations, and significant elevation in Caspase-3 activity, we suggest that the reduction in Survivin levels following SL treatment might contribute to the observed elevation of Caspase-3 activity. Taken together, our observations of decreased Survivin protein levels in response to SLs treatment, and decreased NF-κB (p65/RelA) induced by Inuviscolide in melanoma cells might explain the efficacy of these SLs in induction of apoptosis. This is highly significant with respect to the pharmacological value of these compounds, since it suggests that SLs might sensitize cancer cells toward apoptosis either alone or in combination with other chemotherapeutic agents, especially in cancers resistant to apoptosis, such as melanoma.

The two SLs examined in this study shared a common mechanism of action with a little difference between them. While Inuviscolide decreased NF-κB (p65/RelA) expression, Tomentosin was a more potent inhibitor of Survivin and induced higher Caspase-3 activity. The difference between these two SLs may be explained by differences in functional groups and by differences in molecular geometry [6]. However, because compounds that are closely related chemically sometimes differ greatly in their activity [55], we are unable to attribute the functional differences observed to specific structural features.

In summary, this study demonstrates the cytotoxic activities of the SLs Tomentosin and Inuviscolide against melanoma cell lines. SLs may also represent potential chemotherapeutic agents against other malignancies that exhibit resistance to regular chemotherapy. Some of the mechanisms by which SLs mediate G_2/M arrest and apoptosis are outlined in Fig. 8.

An important issue concerning cytotoxic therapeutic agents is the balance between efficacy of tumor eradication and nonspecific cytotoxicity. Since reports from literature exhibit a range of toxicity for different SLs [55], it is important to study the cytotoxicity of Tomentosin and Inuviscolide if these compounds are to be developed into new agents for cancer treatment. However, new strategies to deal with

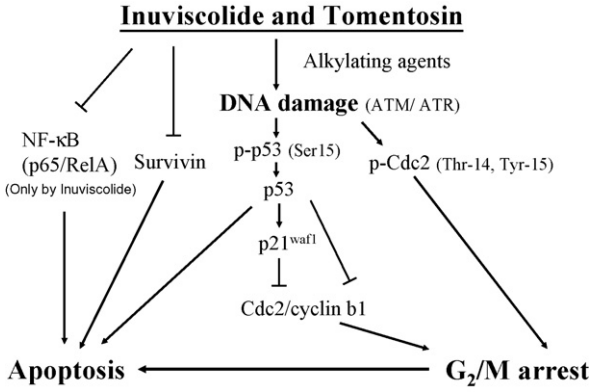

Fig. 8 – Proposed mechanism for the SLs Inuviscolide and Tomentosin-mediated G_2/M arrest and apoptosis. As alkylating agents, SLs might cause DNA damage which activates the kinases ATM/R. This activation possibly resulted in early phosphorylation of p53 (Ser15) and Cdc2 (Thr14, Tyr15). Phosphorylated p53 cause p53 to accumulate in cells and to transactivate $p21^{waf1}$. $p21^{waf1}$ elevation and Cdc2 phosphorylation contribute to Cdc2 inhibition leading to an early G_2/M arrest. Activated p53 also can decrease the protein level of Cdc2/Cyclin b1 (via repression of their promoters). This late effect contributes to elongation of the G_2/M arrest, ultimately resulting in apoptosis. Decreased level of Survivin (by both SLs) and of p65/RelA subunit of NF-κB (only by Inuviscolide) contribute to the apoptotic effect. Taken together, these overall changes lead to G_2/M arrest followed by apoptosis.

toxicity of chemotherapeutic drugs such as allowing efficient and selective delivery of the drugs to cancer cells are now being developed. Our future studies will focus on this issue, as well as on further elucidating the molecular mechanism of these molecules, in order to promote the development of SLs as potential chemotherapeutic agents to treat melanoma.

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Appendix A

See Fig. A1.

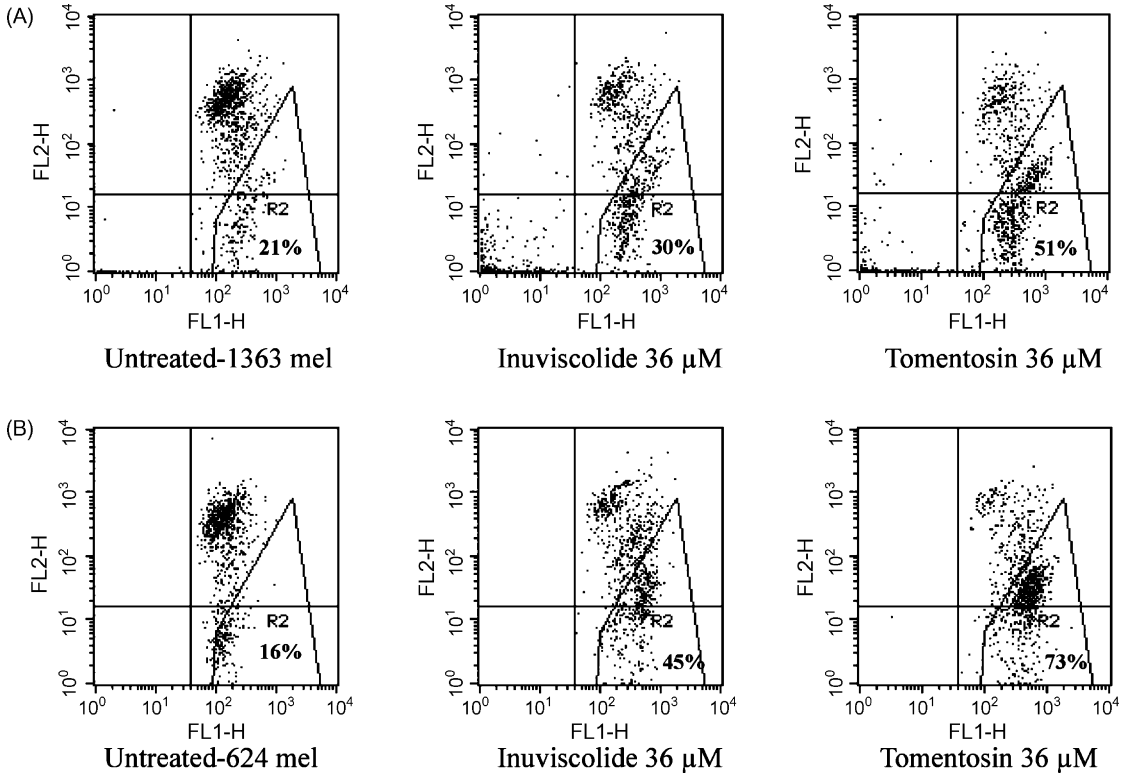

Fig. A1 – Tomentosin or Inuviscolide-induced apoptosis in 1363 mel (A) and 624 mel (B) melanoma cells. Induction of apoptosis following 24 h treatment with SLs was assessed by measurement of mitochondrial membrane potential, using JC-1 reagent. Cells were treated with the SLs (36 μM) for 24 h and analyzed on a FACScan cytometer. Dot plots of red (FL2) vs. green fluorescence (FL1) show live cells with intact mitochondrial membrane potential and apoptotic cells with lost mitochondrial potential, respectively. Similar results were obtained in three independent experiments.

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