Macrovipecetin, a C-type lectin from *Macrovipera lebetina* venom, inhibits proliferation and migration of SK-MEL-28 human melanoma cells and enhances their sensitivity to Cisplatin

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Abstract

Treatment failure of malignant melanoma is a major clinical problem. Cisplatin, a chemotherapeutic agent that generates DNA damage and induces cell apoptosis, is commonly used in the treatment of malignant melanoma. However, its efficacy is challenged by the resistance of melanoma cells to therapy. Therefore, the search for novel tumor inhibitors, that can overcome the resistance of malignant melanoma hold much promise. For this purpose, we purified Macrovipecetin, a novel heterodimeric C-type lectin, from *Macrovipera Lebetina* snake venom and investigated its anti-tumoral effect on its own or combined with cisplatin, in human SK-MEL-28 melanoma cells.

In our study, we found that Macrovipecetin significantly decreased SK-MEL-28 cell viability and it was 100 times more active compared to Cisplatin. Interestingly, when combined with the chemotherapeutic drug, Macrovipecetin enhances the sensitivity of SK-MEL-28 cells to the drug by increasing their apoptosis through activation of $ERK_{1/2}$, p38 and AKT kinases. Thus, cell death occurs through increased expression of the apoptosis inducing factor (AIF), the main molecular mediator of caspase-independent apoptosis,. Moreover, Macrovipecetin alone or

combined with cisplatin triggers the activation of NF-κB and induced the expression of TRADD together with the accumulation of p53, which in turn upregulated the apoptosis-related proteins Bax, Bim and Bad and down-regulated the anti-apoptotic Bcl-2 expression. Our results indicated that Macrovipecetin alone or combined with cisplatin acted as an antioxidant molecule by decreasing ROS levels in SK-MEL-28 melanoma cells. Interestingly, the protein alone or in combination to drug decreased the adhesion of SK-MEL-28 to poly-L-lysine, fibrinogen, fibronectin and vitronectin matrix through modulating the function and expression of the $\alpha v\beta 3$ integrin. The *in silico* study suggests that Macrovipecetin engages only its α chain to interact with a site overlapping the RGD motif binding site on this integrin. These treatments impaired also cell migration together with up-regulation of E-cadherin expression that is compatible with less invasive phenotype in these cells. Thus, our results suggest that Macrovipecetin protein may have therapeutic potential for chemotherapy-resistant melanoma as a natural compound that could be employed alone or in combination with chemotherapeutic drug cisplatin.

Key-words
Macrovipecetin- Snaclec - Cisplatin – anti-melanoma- cellular effectors.

INTRODUCTION

Melanoma, a malignancy from mutations in melanocytes, is the deadliest skin cancer. According to the World Health Organization (WHO), the global incidence of melanoma skin cancers continues to increase (Siegel et al., 2012). Approximately, 132 000 malignant melanomas occur each year (Gray-Schopfer et al., 2007) with only 5-year survival and high mortality rates for patients in the advanced stage (Sandru et al., 2014). The poor prognosis of melanoma is due to the strong resistance of melanoma cells to conventional chemotherapeutic drugs, including platinum-based drugs like cisplatin (Jiang et al., 2009; Ascierto et al., 2010). Cisplatin or cis-diaminedichloroplatine (II) is known for its ability to induce DNA damage and cell apoptosis (Siitonen et al, 2000; Galluzzi et al., 2014). However, this drug is not very effective in the treatment of melanoma cells. Defects in the apoptotic pathway has been proposed as one of the key factors responsible for melanoma resistance to Cisplatin (Grossman and Altieri, 2001; Shahzad et al., 2009). Some reports showed that this drug is clinically beneficial to melanoma patients when it is used in combination with other chemotherapeutic agents or after targeted therapy (Flaherty et al., 2014). Therefore, identifying new molecules that could render tumor cells more sensitive to Cisplatin is an alternative to improve actual drug effectiveness in the treatment of melanoma.

Snake venoms have been considered as an attractive option in the development of anticancer agents (Vyas et al., 2013).

The venom of *Macrovipera lebetina* snake is a complex mixture of active proteins or peptides and other components that activate or inhibit platelets, blood coagulation factors or other vascular components (Sarray et al., 2003). Non-enzymatic components from this snake venom that affect haemostasis include the snake C-type lectin-like family (snaclecs). Snaclecs are natural inhibitors of integrin-ligand interactions (Sarray et al., 2003; Jebali et al., 2014). They have various targets and biological activities and include many proteins that affect platelet functions and several tumor-associated cell activities, including adhesion, migration, invasion, metastasis and angiogenesis (Sarray et al., 2003; Jebali et al., 2014).

Based on their potential anti-cancer effects (Yau et al., 2015), further studies on these molecules may lead to the design more efficient drugs and might provide new therapeutic strategies to reduce side effects.

For this purpose, we isolated and characterized, a snaclec from *Macrovipera lebetina* snake venom which we designed Macrovipecetin. We investigated its anti-tumoral potential alone and

combined with Cisplatin in SK-MEL-28 cells in order to check whether it can enhance the Cisplatin efficacy on melanoma cells.

MATERIALS AND METHODS

Cells and reagents

SK-MEL-28 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Foster City, CA) containing 10% foetal bovine serum (FBS, Dutscher, France), penicillin (50 U/ml), and streptomycin sulphate (50 mg/ml) (Gibco BRL, Germany). They were routinely authenticated and tested for mycoplasma contamination as previously described (Hammouda et al., 2016). Cells were plated and allowed to adhere overnight in the culture medium before treatments. Antibodies against the following proteins were used for western blotting. Phospho-$ERK_{1/2}$ was obtained from Sigma (L'Isle d'Abeau, Chesnes, France). β-actin, Integrin β3, Integrin αv, TRADD, Cleaved PARP (cl PARP), Caspase 3, Caspase 8, E-cadherin, p53, Phospho p53 (Ser 15), Phospho-p38, Phospho-AKT, Phospho-NF-κB, Histone H1, Bax, Bcl-2, Bim, Bad and AIF were obtained from Cell Signaling Technology (Danvers, MA). Cisplatin or cis-diaminedichloroplatin (CDDP) was obtained from Ebewe Pharma (Austria). All other reagents used were analytical grade.

Purification of Macrovipecetin

Macrovipera lebetina venom was collected in ice-cold beakers from snakes in the serpentarium of Pasteur Institute, Tunis, Tunisia. The venom was lyophilized and stored at +4°C until use. Macrovipecetin was purified as previously described (Gasmi et al., 1994)

Separation of Macrovipecetin subunits

Macrovipecetin was reduced in 6 M guanidine-HCl, 0.5 M Tris-HCl, 2 mM EDTA, 1.4 μM DTT, pH 7.5 for 1 h at 37 °C. The subunits were alkylated with 4-vinylpyridine at a final concentration of 4 μM for 5 min. The reaction was stopped with DTT (14 μM final concentration). The reaction mixture was desalted and the Macrovipecetin subunits were purified by reverse phase chromatography on a C8 (5 μm, 4.6 x 250 mm) column (Beckman, USA). Solvents A and B were 0.1 % trifluoroacetic acid (TFA) (v/v) and 0.1 % TFA (v/v) in 100% (v/v) acetonitril, respectively. The subunits were eluted with a linear gradient of 10-80% B over 65 min and 80-100% over 10 min at a flow rate of 1 ml/min, monitored at 214 nm.

Determination of protein concentration

Protein concentration was determined by the BCA (Bicinchoninic acid protein) method (Assay Kit, Sigma) using bovine serum albumin (BSA) as standard.

Enzymatic and chemical digestions of Macrovipecetin subunits

Reduced and alkylated α subunit was digested with Asp-N or Lys-C proteases (Roche Diagnostics, Basle, Switzerland). Urea (3M final concentration) was added to the mixture to ensure solubility. The α subunit was also cleaved chemically at Asn-Gly bonds with 4.5 M LiOH in 6 M guanidine-HCl, 2 M hydoxylamine-HCl, PH 9.0. Peptides from cleavages were separated by reverse phase chromatography on a C8 column eluted with a linear gradient of 10-70% B in 60 min at 1 ml/min. Reduced and alkylated β subunit was digested with Lys-C or Asp-N proteases as above. Chemical cleavage after methionine was done by adding a solution of Cyanogen bromide (70 mg/ml) (CNBr, Sigma, St. Louis, MO) in 70 % formic acid to β subunit (10 μl/10 μg of protein) in the dark for 24 h at room temperature. The cleavage mixture was diluted with 10 volumes of water and concentrated in a Speed Vac. The peptides were purified as above.

Sequencing and mass spectral analysis of Macrovipecetin

N-terminal sequences of subunits and peptides from enzymatic and chemical digestions were obtained by Edman degradation with an Applied Biosystem 476 A liquid-phase sequencer equipped with on-line phenylthiohydantoin reverse HPLC using RP 18 column. The molecular masses of subunits were determined by Electrospray mass spectrometry on a Trio2/3000 ESI instrument (VG Biotech).

Cell viability

Cells were seeded in 96-well plates (1000 cells/well) and allowed to adhere overnight in the culture medium before treatments with various concentrations of Macrovipecetin, Cisplatin or the combination of both. After 24h and 72h of treatments, images of cells were taken by inverted microscopy. Cell viability was measured by colorimetric MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) assay. The absorbance at 540 nm was measured with a microplate reader (MULTISKAN, Labsystems) and cell viability was expressed as percentage of the viable cell number of the control (mock-treated cells).

Apoptosis assay

Apoptosis was performed by the Annexin V-phycoerythrin (PE)/7-amino-actinomycin D (7-AAD) apoptosis detection kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. Stained cells were analyzed on a BD FACScan II flow cytometer (BD Biosciences, San Jose, CA, USA) and with BD FACSDiva 6 software (BD Biosciences, San Jose, CA, USA). The quantification of cell death was evaluated as previously described (Hammouda et al., 2016). The results were provided in percentage of total cell number and the percentage of apoptotic cells (%) was calculated as follows: Early apoptotic cells (%) + late apoptotic cells (%).

Measurement of reactive oxygen species (ROS)

The intracellular level of ROS was determined using a cell-permeable fluorogenic probe, CMH2DCF-DA (Life Technologies, Oregon, USA). SK-MEL-28 cells were seeded in 96-well plates (2000 cells/well) and were treated with Macrovipecetin (0.1 µM), Cisplatin (3.75 µM) or a combination of both for 24h and 72h. After washing cells with PBS (1X), they were re-suspended in HBSS (GIBCO) and incubated with 10 µM of CMH2DCFDA at 37°C for 30 min in the dark. Fluorescence was detected with excitation and emission wavelength at 492 and 517 nm respectively.

Cell Fractionation

Cells were collected by centrifugation and washed in ice-cold PBS. Cell pellets were fractionated into nucleic and cytoplasmic proteins as previously described (Hammouda el al., 2016).

Western Blotting Analysis

Cells were lysed in 100 µl of Laemmli buffer. Protein concentrations were quantified by the Bicinchoninic acid assay (Sigma) with bovine albumin as a standard. 30 µg of whole cell lysates or nuclear extracts were subjected to SDS-PAGE and transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore). The immuno-reactive proteins were detected by the enhanced chemiluminescence detection system (ECL, Pierce, Rockford, USA).

Cell Adhesion Assay

Human melanoma SK-MEL-28 cells were exposed to the cell adhesion assay. Adhesion substrata were prepared by coating 96-well microtiter plates overnight at 4°C with poly-L-lysine (5 µg/ml), fibronectin (5 µg/ml), fibrinogen (5 µg/ml), vitronectin (5 µg/ml), collagen (5

μg/ml) and laminin (5 μg/ml). Nonspecific binding was blocked by incubating the wells with 1% BSA in PBS for 1h. SK-MEL-28 cells were obtained in single cell suspension after treatment with Versene. Harvested cells were washed twice with Dulbecco modified Eagle's medium (DMEM) containing 0.2% BSA (adhesion buffer) and re-suspended in the same medium. Cells were treated with 0.1 μM of Macrovipecetin or Cisplatin (3.75 μM) or the combination of both for 2 h. Then, mock and treated-cells were added to each well and were incubated at 37°C for 1 h. Unbound cells were removed by washing the wells three times with adhesion buffer. Thereafter, bound cells were fixed by 1% glutaraldehyde, stained with 0.1% of Crystal Violet and lysed with 1% of SDS. The absorbance was measured at 600 nm.

For adhesion assays using a blocking antibody, SK-MEL-28 cells were treated with 0.1 μM of Macrovipecetin or Cisplatin (3.75 μM) or the combination of both for 30 min at room temperature. Cells were then added to 96-well microtiter plates coated with monoclonal antibody LM609 anti-αvβ3 (10 μg/ml) (Millipore) and allowed to adhere. Adhesion assay was performed as above.

Wound Healing

Cells were seeded (2000 cells/well) in 6 multi-well plates. They were grown to ~90% confluency prior to wounding with sterile 200 μL pipette tips and washed twice with PBS (1X). After then, media were replaced by fresh ones supplemented with 0.1 μM of Macrovipecetin or Cisplatin (3.75 μM) or the combination of both or PBS as vehicle control. Wounds were photographed at zero time (T0). After 24 h and 72 h of treatments, cells were washed with PBS (1X), fixed with cold ethanol and stained with Giemsa. Images were acquired on an inverted microscope at T0 and after 24h and 72h of treatments. Data are presented as percentage of cell migration ± standard error of the mean (S.E.M.). The Percentage of cell migration was calculated using the following formula: Cell migration (%) = $[T_0 - T_t - T_{mock}]/[T_0 - T_{mock}] \times 100$ where T_0 is wound area at zero time, T_{mock} is wound area in PBS-treated control wells, and T_t is wound area in treatment wells.

Homology modeling

The 3D structure model of Macrovipecetin protein was obtained using comparative modeling with MODELLER program (Sali and Blundell, 1993). A template was identified using BLAST program to search the Protein Data Bank (PDB) for homologous sequences using both subunits α and β sequences of Macrovipecetin. The sequence of the identified template was aligned with the target sequence using a dynamic programming algorithm implemented in the EMBOSS

sequence analysis suite (Rice et al., 2000). The alignment serves to extract the spatial restraints from the template structure and to build the 3D model of Macrovipecetin using MODELLER (version 9.16). We build 20 models for Macrovipecetin starting from different random seeds. The overall energy quality was evaluated by calculating the DOPE score (Discrete Optimized Protein Energy) for each model (Shen and Sali, 2006). Stereochemical quality was assessed by establishing the Ramachandran Plot (Lovell et al., 2003).

Protein-Protein Docking

The selected model of Macrovipecetin was used to run a protein-protein docking against αvβ3 integrin. For the latter, we used a crystal structure of the integrin in complex with RGDF cyclic peptide (PDB code: 1L5G) (Xiong et al., 2002) from which we removed the segment 440-956 of chain A and 532-690 of chain B. Therefore, the receptor structure consists of the β propeller domain the β1 domain and the hybrid domain. The ClusPro server was used to run the docking which combines a fast Fourier transform sampling, a clustering stage and an energy minimization refinement using CHARMM (Kozakov et al., 2006; 2017; Comeau et al., 2004). The returned complexes were analyzed based on the Van der Waals, Electrostatics (VdW+Elec) combined terms.

Statistical analysis

The results were evaluated by Graph-pad Prism. The Data were presented by mean ± S.E.M values from four to six determinations in triplicate. The program used to analyse data was One-Way ANOVA and Two-Way ANOVA tests. Differences were considered to be statistically significant at $P < 0.05$.

RESULTS

Purification and characterization of Macrovipecetin from *Macrovipera lebetina* snake venom

Macrovipecetin was purified from *Macrovipera lebetina* venom by size exclusion chromatography on a Superose 12 preparative grade column, followed by ion-exchange chromatography on a Mono Q column and by reverse phase High Performance Liquid Chromatography (HPLC) on a C8 column (Gasmi et al, 1994). Macrovipecetin α and β subunits were separated after reduction and alkylation of the protein (**Fig. 1A**). The N-terminal sequence of 55 residues was clearly identified by Edman degradation for each subunit. The complete amino acid sequence of the reduced and alkylated subunit α chain was established following

two enzymatic digestions with endoproteinases Asp-N or Lys-C, and a chemical digestion with hydroxylamine at Asn-Gly bonds. The resulting peptides were separated using a C8 column (data not shown) and designated A, L and N, respectively. The reduced and alkylated β chain was digested with endoproteinases Asp-N or Lys-C and chemically with CNBr. The peptides from these digestions were separated as above and designated A', L' and M, respectively (data not shown).

The complete amino acid sequence of the Macrovipecetin subunits was established after sequencing and overlapping of peptides generated by a variety of selective enzymatic and chemical cleavage methods (**Fig. 1B**). Peptides are indicated below the sequence and numbered according to their order of elution. Thus, Macrovipecetin is a heterodimer composed of a α subunit of 131 residues and a β subunit of 127 residues. Each subunit contains 7 cysteines. The experimental masses of the α and β chains (15422.7 and 15031.7 Da, respectively) obtained by ElectroSpray ionisation, are effectively identical to the average masses calculated from their corresponding sequences. The two subunit sequences display a sequence identity of 52%.

Using the BLAST program, Macrovipecetin α and β subunits sequences were found to be similar to other snake C-type lectins, and the highest alignment scores were recorded with lebecetin (Sarray et al., 2003) and lebecin (Jebali et al., 2014) from the same venom. The alignment of sequences of their α and β subunits is shown in **Fig. 1C**. The α subunit of Macrovipecetin showed 58.8 % of sequence identity with the α chain of lebecin and 67.5 % with that of lebecetin (for the 40 determined amino-acids). The β subunit of Macrovipecetin showed 43.4% of sequence identity with the β chain of lebecin () and 58.5% with that of lebecetin (for the 41 determined amino-acids).

Macrovipecetin and/or Cisplatin inhibits cell viability of melanoma cells

We investigated whether Macrovipecetin alone or combined with the chemotherapeutic drug, Cisplatin, could affect the proliferation of human SK-MEL-28 melanoma cells. The cells were treated with increasing concentrations of Macrovipecetin or Cisplatin and cell viability was evaluated by the MTT assay. After 24 h of incubation, Cisplatin affected the viability of SK-MEL-28 cells slightly compared to Macrovipecetin that significantly decreased cell proliferation in a concentration-dependent manner to about 42 % at 0.1 μM with respect to the

mock-treated cells (**Fig. 2A and B**). This inhibitory effect was further enhanced after 72 h of treatment to about 50% and 72% at 0.1 µM and 1 µM of Macrovipecetin whereas Cisplatin inhibits about 11% and 63% of cell viability at 0.9 µM and 12.5 µM, respectively. The calculated IC_{50} values supported that Macrovipecetin (IC_{50}=0.1 µM) is 100 times more active in human SK-MEL-28 melanoma cells compared to the chemotherapeutic drug (with IC_{50}=9.5µM).

It is worth noting that Cisplatin at concentrations between 0.1 to 0.8 µM did not exhibit any antiproliferative effect on the SK-MEL-28 cells (data not shown). Interestingly, Macrovipecetin had a selective inhibitory effect since it did not affect the viability of non tumoral cells, NIH/3T3 Fibroblasts (**Fig. 3A**). For Cisplatin, the concentration of 3.75 µM had no cytotoxic effect on these cells. On the basis of these results, a Macrovipecetin concentration of 0.1 µM, giving approximately 50% inhibition of SK-MEL-28 cell proliferation, was selected for further experiments.

Since cisplatin-related toxicity is dose-dependent, we then investigated whether Macrovipecetin (0.1 µM) could improve the inhibitory effect of Cisplatin when used at a non-cytotoxic concentration (3.75 µM). For this purpose, we analyzed the effect of Macrovipecetin on the viability of SK-MEL-28 when it was combined with cisplatin. After 24 h, Cisplatin (3.75 µM) had a moderate inhibitory effect on SK-MEL-28 cell viability (9%) while treatment of cells with cisplatin (3.75 µM) combined with Macrovipecetin (0.1 µM) decreases the viability of SK-MEL-28 cells further (44% inhibition) associated with typical morphological changes. This effect was enhanced to about 62% with the combined treatment (**Fig. 3B**), and to about 26 % for the drug after 72h (**Fig. 3B**). Interestingly, the tested combination did not affect the viability of non-tumorigenic NIH-3T3 cells.

Macrovipecetin-Cisplatin treatment induces caspase-independent apoptosis through AIF

The typical morphological changes (irregular and shrunk shape) observed by microscopy (**Fig. 3C**) suggested that the decreased viability in Macrovipecetin-cisplatin-treated-SK-MEL-28 cells could be a result of cell apoptosis. Thus, the capacity of different treatments to induce apoptosis in SK-MEL-28 cells was assessed by annexin V binding assay and the percentage of apoptotic cells was quantified by flow cytometry.

Interestingly, even if Cisplatin (3.75 µM) did not induce apoptosis in SK-MEL-28 cells after 24 h, the percentage of apoptotic cells in response to Macrovipecetin and the combined treatment increased in a time-dependent manner reaching (17% and 23%) and (29% and 51%) ($P < 0.05$) of Annexin-positive cells after 24h and 72h, respectively compared to the mock-treated cells and positive control cells (staurosporine-treated cells). This result suggested that Macrovipecetin induced apoptotic cell death in melanoma cells and sensitized SK-MEL-28 cells to Cisplatin.

We then investigated the role of caspase activation in Macrovipecetin-Cisplatin-induced apoptosis in SK-MEL-28 cells by treating the cells with the pan-caspase inhibitor, z-VAD-fmk (20 µM) for 2 h before adding the protein (0.1 µM), the drug (3.75 µM) or the combination of both for 24 h and 72 h. Our results indicated that the inhibition of caspases did not prevent the apoptotic effect of Macrovipecetin and/or Cisplatin (**Fig. 4A**), while it diminished the cell death induced by the positive control, staurosporin. This result suggested that the induced effects in SK-MEL28 cells were independent of caspase activation.

In order to confirm that Macrovipecetin and/or Cisplatin induces caspase-independent apoptosis of SK-MEL-28 cells, we investigated the activation of caspases 3, 8 and the subsequent proteolytic cleavage of poly (ADP-ribose) polymerase (PARP) by western blotting. As expected, Macrovipecetin and/or Cisplatin did not induce the activation of caspases 8, 3 or the cleavage of the caspase 3 substrate, PARP in treated cells (**Fig. 4B)**.

The apoptosis of SK-MEL-28 cells is associated with an increase in apoptotic inducing factor (AIF) protein expression in the nucleus (**Fig. 4C)**. This result suggests that AIF translocates to the nucleus and induces the caspase-independent cell death program in response to Macrovipecetin protein on its own or combined with Cisplatin.

Macrovipecetin and/or Cisplatin treatment induces mitochondrial proteins involved in regulating cell death

Macrovipecetin combined with Cisplatin induced a caspase-independent cell death of SK-MEL-28 cells that could mainly be caused by mitochondria outer membrane permeabilization initiated by expression of Bcl-2-associated X protein (Bax), and prevented by expression of Bcl-2 during apoptosis (Hammouda et al., 2016; Moubarak et al., 2007).

Macrovipecetin alone (at 0.1 µM) or combined with cisplatin (at 3.75 µM) increases the expression of Bim and Bad proteins known to trigger Bax activation (**Fig. 4D**). Consistently, the increase in Bim and Bad expression was associated with an increase in Bax (**Fig. 4D**) and a decrease in the expression of the anti-apoptotic protein Bcl-2 (**Fig. 4D**). These results suggested that Macrovipecetin alone or combined to cisplatin activated the mitochondrial-mediated apoptotic pathway by changing the balance between proapoptotic and antiapoptotic proteins.

Macrovipecetin and/or Cisplatin treatment activates survival pathways in SK-MEL-28 melanoma cells

To further characterize whether Macrovipecetin and/or Cisplatin-inhibited cell proliferation is associated with alterations of survival pathways in melanoma cells, SK-MEL-28 cells were investigated for the activation of extracellular regulated kinase ($ERK_{1/2}$), p38 and AKT signaling pathway. Western blot analysis showed that after 24 h, treatment of cells with Macrovipecetin, Cisplatin or the combination of both induced a slight increase in the level of the phosphorylated form of $ERK_{1/2}$ that reached basal level when the protein was combined with the drug at a concentration of 3.75 µM. Increased $ERK_{1/2}$ phosphorylation was prolonged

after 72 h and only the combination of cisplatin at 3.75 μM and Macrovipecetin reduced the level of the phosphorylated form of this kinase without affecting its basal expression (**Fig. 5A)**. Macrovipecetin-cisplatin-mediated cell proliferation inhibition was also associated with the activation of p38 and AKT kinases that play a major role in regulating cell growth and death (Zhang and Liu., 2002; Bononi et al., 2011). Macrovipecetin, Cisplatin and the combined treatment decreased the phosphorylation of p38 in SK-MEL-28 cells after 24 h. In contrast, after 72 h, Macrovipecetin increased the phosphorylation of this kinase compared to the combination (at 3.75 μM) that inhibited the level of its phosphorylated form **(Fig. 5A)**.

Macrovipecetin alone or combined with Cisplatin slightly decreased the phosphorylation of AKT after 24 h of treatment. This effect was more pronounced after 72 h when the protein was combined to the drug at a concentration of 3.75 μM. These results suggested that $ERK_{1/2}$, p38 and AKT kinases are implicated in Macrovipecetin-Cisplatin related inhibition of SK-MEL-28 cell proliferation and showed that the activation of these kinases is time -dependent (**Fig. 5A)**.

The role of TRADD, NF-κB, and p53 in Macrovipecetin-Cisplatin related induction of apoptosis in SK-MEL-28 melanoma cells

In order to get more insight into the mechanisms of cell death induced by Macrovipecetin alone or combined with cisplatin, we investigated the role of TRADD, NF-κB, and p53 in SK-MEL-28 cells. Interestingly, we found that Macrovipecetin and the combined treatment induced TRADD expression, associated with an increase in the level of phosphorylated form of NF-κB along with increased p53 expression in SK-MEL-28 cells after 24 h and 72 h (**Fig. 5B**). The generated effects were enhanced in time-dependent manner.

Macrovipecetin and/or Cisplatin modulates ROS generation in SK-MEL-28 melanoma cells

It is well documented that reactive oxygen species (ROS) play a key role in tumor progression and cell death (Sreevalsan and Safe, 2013). We (Hammouda et al., 2016) and others previously reported the existence of a relationship between p53 and levels of ROS (Sablina et al., 2005).

Based on the fact that Macrovipecetin alone or combined with cisplatin induced p53 expression, we investigated whether these treatments modulated ROS production in SK-MEL-28 cells. Thus, after 24 h and 72 h, Macrovipecetin reduced the level of ROS compared to mock-treated cells. The combined treatment exerted a synergetic effect by causing more decrease in the amount of generated ROS ($P < 0.05$) (**Fig. 5C**).

Macrovipecetin and/or Cisplatin affected the attachment of SK-MEL-28 melanoma cells to various extracellular matrix proteins

Cell adhesion through the ECM requires cell–matrix interactions that are mainly mediated by integrins (Schwartz et al., 2010). Because snaclecs are natural inhibitors of integrin-ligand interactions (Marcinkiewicz et al., 2000; Du et al., 2001; Suzuki-Inoue et al., 2001; Eble et al., 2002; Sarray et al., 2007) and Macrovipecetin belongs to the same family of proteins, we investigated whether it could affect the attachment of SK-MEL-28 melanoma cells. Thus, we investigated the effect of Macrovipecetin alone or combined with cisplatin on the adhesion of human melanoma SK-MEL-28 cells to different purified ECM proteins as integrin ligands.

Macrovipecetin (at 0.1 µM) or Cisplatin (at 3.75 µM) reduced significantly the attachment of melanoma SK-MEL-28 cells to poly-L-lysine, fibronectin, vitronectin matrix and slightly affected their adhesion to fibrinogen. Furthermore, these cells were not able to adhere to collagen and laminin matrices. This result suggests that the SK-MEL-28 cells did not express a set of integrins subunits that could mediate adhesion to collagen and laminin matrices.

When SK-MEL-28 cells were treated with the combination of Macrovipecetin (0.1 µM) and Cisplatin (3.75 µM), the inhibitory effect on the adhesion of cells to different matrix proteins was enhanced in dose-dependent manner. It reached 99% of inhibition to poly-L-lysine, 92% to fibronectin, 95% to vitronectin, and 27% to fibrinogen compared to the protein and Cisplatin alone **(Fig. 6A**). The ability of Macrovipecetin, Cisplatin and the combined treatment to affect

the adhesion of SK-MEL-28 cells to the poly-L-lysine (used as an integrin-independent substratum), indicated that the generated effects are not restricted to the integrin family of adhesion receptors.

Since SK-MEL-28 cell adhesion to vitronectin and fibronectin is the most affected and αvβ3 integrin is knowm to be mediator of vitronectin binding to melanoma cells, this integrin is probably targeted by Macrovipecetin.

To verify if the protein could target the integrin αvβ3 in SK-MEL-28 cells, we performed adhesion assay using a function-blocking antibody raised against this integrin used as matrix. Interestingly**,** compared to mock-treated cells, Macrovipecetin and the combined treatment impaired the function of αvβ3 integrin in SK-MEL-28 cells by 92% and 96%, respectively while cisplatin reduced cell adhesion on anti-αvβ3 antibody by only 33%. Therefore, we checked whether Macrovipecetin on its own or combined to cisplatin by blocking the adhesive function of αvβ3 integrin could affect its expression level (**Fig. 6B)**. The western blot analysis revealed that Macrovipecetin alone or combined with cisplatin induced a reduction in αv and β3 protein levels in SK-MEL-28 cells (**Fig. 6C**). This effect was enhanced to reach a total abrogation of integrin β3 expression after 72 h of treatment with the combination of Cisplatin (3.75 µM) and the protein (0.1 µM).

In silico **study of Macrovipecetin interaction with αvβ3 integrin**

Model of 3-D structure of Macrovipecetin

The 3D model of Macrovipecetin was constructed by homology to the crystal structure of Bitiscetin (at a resolution of 2 Å; PDB code: 1JWI) in its heterodimeric state which its α and β subunits showed 55% and 54% of sequence identity with those of Macroviperacein, respectively. The Cα atoms structural deviation is of 0.1 Å, compared to the template structure. The Ramachandran plot shows the proportions of 97% (247 residues), 2.6% (7 residues) and 0.4% (one residue) of the φ/ψ dihedral angles situated respectively in the favored regions, the allowed regions and the outlier regions.

The two subunits of Macrovipecetin showed an α/β fold. The two subunits are joint together with one disulfide bond formed between Cys80(α)-Cys77(β) while 6 other interchain disulfide bridges are spread through the principal axis of each subunit (Cys2-Cys13, Cys31-Cys126, Cys101-Cys118 for subunit α and Cys98-Cys113, Cys32-Cys103, Cys4-Cys15 for the β subunit). Our model showed that the two subunits of Macrovipecetin are associated in a domain swap manner. The interface is formed between two equivalent long loops consisting of segments 74-100 and 70-97 for subunits α and β, respectively. Each of these loops harbors a cystein residue contributing in the formation of the interchain disufide bond (**Fig. 7A**).

Protein-Protein Docking

The five best complexes, according to the VdW+Elec scoring function, showed an interaction mode of Macrovipecetin with the outermost side of the propeller sheets side of the integrin αvβ3 which faces the interaction interface with the β1 domain. We judged, that these poses are functionally irrelevant to describe the inhibitory effect of Macrovipecetin. The 6th best solution showed that Macrovipecetin interacts with a site overlaping the RGD motif binding site on the integrin (**Fig. 7B**). In this mode the Macrovipecetin engages only its α chain in the interaction with αvβ3 integrin.

The snake protein occupies the RGD binding pocket using mainly three segments: 17-21, 61-66 and 119-122. The interaction surface measures 1995 Å^2. The propeller domain of the integrin interacts only with the 17-21 segment (FRLFK) while the β1 domain interacts with the two other segments. R18, K21, K62, K64 and K121 make a positively charged contour of the interface. They are in close distance with D218, D150, D336, D251, D127, D126 and D179 with the potential formation of salt bridge bonds. The center of the interface is occupied by F20 and Y65 hydrophobic residues (**Fig. 7C**).

Macrovipecetin-Cisplatin effect on SK-MEL-28 cell migration

As the migratory capability is one of the basic steps for melanoma cells to gain metastatic potential, we investigated the role of Macrovipecetin-Cisplatin on the migration of SK-MEL-28 cells using the wound-healing assay. Representative microscopic images of wounds at zero time, 24 h and 72 h post-treatment for mock, Macrovipecetin, Cisplatin and the combination of both are shown in **Fig. 8A**. Compared to mock-treated cells, Macrovipecetin alone or combined with Cisplatin reduced the SK-MEL-28 cell migration *in vitro* after 24h (**Fig. 8A**). In contrast,

treatment of SK-MEL-28 cells with Cisplatin caused a partial inhibition of cell migration. The anti-migratory effect was more pronounced after 72h of treatment (**Fig. 8A)**. SK-MEL-28 cell migration was inhibited by 0.1 µM Macrovipecetin (79% of inhibition) and by 3.75µM Cisplatin (65% of inhibition) treatments. Interestingly, inhibition of migration reached a total blockade after combining the Macrovipecetin to Cisplatin (3.75 µM) (**Fig. 8B)**. This result suggested that Macrovipecetin improves the Cisplatin efficacy through increasing its effect in impairing SK-MEL-28 cell migration.

Macrovipecetin -Cisplatin modulates the protein level of E-cadherin on SK-MEL-28 cells

In malignant melanoma, the acquisition of invasive behavior was related at least in part to deregulated expression of epithelial cell adhesion protein, E-cadherin (Gaggioli and Sahai., 2007, Hsu et al., 2002; Li et al., 2001, Putnam et al., 2009). We have then explored the effect of Macrovipecetin alone or combined with cisplatin on the expression of E-cadherin in SK-MEL-28 melanoma cells. Western blot analysis revealed that Macrovipecetin alone or combined with cisplatin increased the amounts of E-cadherin protein in SK-MEL-28 cells (**Fig. 8C**).

DISCUSSION

Snake venom is considered to be a rich source of naturally occurring inhibitors of integrins. Among them, the family of snake C-type lectin-like proteins is often endowed with anti-tumoral activity (Marcinkiewicz, 2013). Although, the beneficial effects of different proteins belonging to this family were highlighted in different cancer cell lines (Calderon et al., 2014), their underlying anti-tumor mechanisms are still incompletely understood.

In the present study, we focused not only on identifying a snaclec protein with antitumor activity but also improving the efficacy of a chemotherapeutic drug, Cisplatin known for its limited effectiveness in melanoma. Macrovipecetin is a heterodimeric snaclec composed of two (α- and β-) subunits that contain 131 and 127 residues, respectively.

These subunits are 52% identical with each other and both are highly similar to two other snaclecs lebecin (Jebali et al., 2014) and lebecetin (Sarray et al., 2003) purified from the venom of the same species and showed antitumoral effect on different cancer cell lines.

In melanoma, tumor progression begins, at least in part, when the balance between pro and anti-proliferative pathways is altered in favor of prolonged cell survival and resistance to treatment-induced apoptosis. Based on the fact that the resistance of cells to a DNA-damaging compound, Cisplatin restricts its clinical application in melanoma (Rünger et al., 2000; Helmbach et al., 2002), we investigated whether Macrovipecetin could improve the efficacy of Cisplatin in SK-MEL-28 cells.

Interestingly, Macrovipecetin exhibited anti-proliferative and pro-apoptotic activities against SK-MEL-28 cells. Our result is in agreement with several studies reporting that snaclecs promoted cell death in different tumour cell lines (Nunes et al., 2012; Aranda-Souza et al., 2014; Pathan et al. 2017). Our work showed that, the suppressive effects were significantly enhanced following treatment with a combination of Macrovipecetin and cisplatin while, no effect was observed on the non-tumorigenic NIH-3T3 cells.

Our result is in line with the study of Nelson et al., 2012 who reported synergistic effects of a phospholipase A2 from the venom of *Agkistrodon piscivorus piscivorus* with four chemotherapeutic agents (paclitaxel, methotrexate, actinomycin D and daunorubicin) that initiate apoptosis by different mechanisms (Nelson et al., 2012).

It is well documented that apoptosis regulation is triggered by two major signaling pathways. Intrinsic pathway induced by cellular and DNA damage and extrinsic pathway initiated by death ligands (Kiraz et al., 2016; Ashkenazi et al., 2017).

In response to anticancer chemotherapy, the activation of caspases can be initiated through activation of the extrinsic or intrinsic pathway (Larsen and Sorensen, 2016). Even if caspases, play a central role in many apoptotic mechanisms, several mediators such as AIF and

endonuclease G have been considered as the molecular effectors of caspase independent programmed cell death (Norberg et al., 2010; Delavallée et al., 2011). Macrovipecetin-Cisplatin-induced a caspase independent apoptosis of SK-MEL-28 cells was associated with an increase of AIF level that it is released from mitochondria upon outer membrane permeabilization and translocates into the nucleus to contribute to DNA fragmentation (Hammouda et al., 2016). TRADD, a Tumor necrosis factor receptor type 1-associated death domain protein (TNF-R1-associated protein), has been shown to be involved in chemotherapeutic drug-induced apoptosis (Rokudai et al., 2002).

TRADD, interacting with TRAF proteins, induces the transcription factor NF-κB that plays an important role in the regulation of anti-apoptotic (Micheau et al., 2001) and pro-apototic signaling (Fujioka et al., 2004). Chemotherapeutic drugs combined to inhibitors of NF-κB promote apoptosis of tumor cells (Godwin et al., 2013). However, NF-κB functions also as a proapoptotic factor by stabilizing p53 as a mechanism for initiating cell apoptosis (Fujioka et al., 2004). A link between induction of apoptosis, TRADD expression and NF-κB activation has been well documented (Hsu et al., 1995; Hiscott et al., 2001). In response to Macrovipecetin-Cisplatin treatment, NF-κB is up-regulated which in turn may activate p53-dependent apoptotic pathways (Fujioka et al., 2004). Subsequently and in case of irreparable damage, p53 induces pro-apototic Bcl-2 proteins such as Bax, Noxa, Puma, Bik and Bid. Moreover, to trigger mitochondrial apoptosis, p53 may also directly interact with Bcl-2 family members (Mathai et al., 2002; Schuler and Green, 2005). Thus, the combination of Macrovipecetin and cisplatin-induced p53 correlated with reduced protein expression of anti-apoptotic protein Bcl-2 which coincides with the increase in Bax, Bim and Bad expression in SK-MEL-28 cells.

Several studies highlighted a relationship between p53 and ROS production. Activation of p53 leads to inhibition of ROS levels through increasing the antioxidant enzymes (Sablina et al., 2005).

In this study, we propose Macrovipecetin-Cisplatin as a treatment inducing apoptosis through AIF protein and TRADD death receptor. This TNF-R1-associated death domain protein induces NF-κB phosphorylation that acted as a pro-apoptotic protein which in turn activates p53 expression. Moreover, p53, the most common tumor suppressor proceeds by decreasing ROS levels, up-regulating pro-apoptotic Bax and Bad proteins and inhibiting Bcl-2 that its function could be antagonized by the increase in Bim levels (Hemann and Lowe, 2006).

It is known that the mitogen-activated protein kinase (MAPK) $ERK_{1/2}$ and p38 pathway is constitutively activated in melanomas and mediates a number of cellular behaviors in response to extracellular stimuli (Meierjohann., 2017; Inamdar et al., 2011; Estrada et al., 2009). Albeit $ERK_{1/2}$ activation was usually associated with survival and cell proliferation but depending on stimuli and the cell types engaged, $ERK_{1/2}$ activation can mediate cell death. Indeed, some anticancer agents constitutively activate $ERK_{1/2}$ pathway leading to the apoptosis and cell cycle arrest via p53 participation and stabilization (Lv et al., 2013).

Our results suggested that Macrovipecetin-induced inhibition of SK-MEL-28 cell proliferation is through activating $ERK_{1/2}$ and p38 kinases. A such mechanism is highlighted in the previous study reported by our team (Zakraoui et al., 2017) where we demonstrated that Lebein, a snake venom disintegrin, suppresses human colon cancer cells proliferation and triggers the activation of $ERK_{1/2}$ pathway in these cells. In line with our work, Dai et al., 2005 reported that Gal-9, an animal lectin, induced the phosphorylation of the MAPK p38 and $ERK_{1/2}$ in monocyte-derived dendritic cells.

Interestingly, the combined Macrovipecetin-Cisplatin treatment inhibited p38 and $ERK_{1/2}$ phosphorylation. This result is in accordance with the study reported by Estrada et al., 2009

where they show that the inhibition of both $ERK_{1/2}$ and p38 activation was more effective in blocking the growth of melanoma cells than the inhibition of each kinase individually.

It has been established that increasing levels of phosphorylated form of AKT correlated with the progression of melanoma (Dai et al., 2005). In this study, Macrovipecetin combined with Cisplatin affected the viability of SK-MEL-28 cells that was associated with reduced phosphorylation level of AKT in the SK-MEL-28 cells. In contrast, Sarray et al., 2009 reported that a snaclec lebectin isolated from *Macrovipera lebetina* venom increases the phosphorylated form of AKT in IGR39 melanoma cells (Sarray et al., 2009).

Our study suggested that Macrovipecetin and cisplatin synergistically induce cell death through the regulation of the MAPK $ERK_{1/2}$, p38 kinases, AKT and NF-κB. Thus, the combination of Macrovipecetin and cisplatin may have therapeutic potential for chemotherapy-resistant melanoma cells and the effects induced are likely exerted through the regulation of these survival pathways.

Adhesion to the vasculature wall is one of several key steps that contribute to tumor progression (Okegawa et al., 2004). It is worth noting that SK-MEL-28 cells were not able to adhere to collagen and laminin matrices. This could be explained by the work of Nakano et al., 1999 reporting that the expression of the adhesion receptors for collagen and laminin, α2β1 and α2β3 integrins, are very low or undetectable in SK-MEL-28 cells (Nakano et al., 1999). Since the SK-MEL-28 cells express high levels of αvβ3 integrin on its cell surface (Seoane, et al. 2010; Vogetseder et al., 2013), we have explored the ability of Macrovipecetin alone or combined to Cisplatin to affect cell adhesion through modulating the function and expression of the αvβ3 integrin in these cells. The vitronectin receptor αvβ3 integrin, is one of the integrins that plays a role in the aggressiveness of melanoma cells (Ganguly et al., 2013). The Macrovipecetin-Cisplatin treatment decreased significantly cell adhesion to fibronectin and vitronectin in SK-MEL-28 cells through blocking the function of αvβ3 integrin.

Many studies have reported that C-type lectins glycoproteins interfere with integrins associated to the adhesion process (Marcinkiewicz et al., 2000; Sarray el al., 2007). BJcuL, a lectin purified from *Bothrops jararacussu* venom, induces apoptosis in human gastric carcinoma cells accompanied by inhibition of cell adhesion and actin cytoskeleton disassembly (Nolte et al, 2012). Lebecetin and lebectin, two snaclecs isolated from *Macrovipera lebetina* affected cell adhesion and migration by inhibiting $\alpha 5\beta 1$ and αv-containing integrins (Sarray et al., 2001; 2007). Lebecin, another snaclec from the same venom has anti-proliferative, anti-adhesive and anti-migratory properties against the breast cancer cell line MDA-MB231 (Jebali et al., 2014). In our work, we investigated *in silico* for the first time the interaction mode of the snaclec with an integrin; and we showed that Macrovipecetin engages only its α chain to interact with the αvβ3 integrin. The interface is situated far from the presumably interaction site with the von Willebrand Factor A1 Domain situated nearby the 74-100 loop which was described previously for some homologous proteins (Maita et al, 2003; Mizuno et al, 2001). Indeed Macroviperacetin interact with a site overlapping the RGD motif binding site on the αvβ3 integrin.

We suggested that Macrovipecetin, while being a snaclec that is known as integrin-binding molecule, could affect the function of some integrins related to its inhibitory effect on cell viability and adhesion. Indeed, the protein alone, the drug and the combined treatment also affected the adhesion of SK-MEL-28 cells to poly-L-lysine highlighting that this effect is not restricted to adhesion receptors.

In melanoma, the transition and progression from a benign melanocyte hyperplasia to dangerous disease is characterized by the acquisition of invasive behavior (Gaggioli and Sahai, 2007). One of the key regulators that control the interactions with keratinocytes and the behavior of normal melanocytes (Hsu et al., 2002; Li et al., 2001) is the E-cadherin protein, the loss of which coincides with promoted invasiveness of melanoma cells. Moreover, it has been shown that the up-regulation of E-cadherin expression in melanoma cells inhibited their

invasion into the dermis, induced keratinocyte-mediated growth control and decreased the expression of invasion-related adhesion receptors such as MCAM/MUC18 and αvβ3 integrin (McGary et al., 2002).

In this study, the combined treatment induced E-cadherin expression and inhibited αv and β3 integrins that play an important role in regulating several processes involved in the progression and metastasis of melanoma. However, lebectin a snaclec isolated from the same venom mediated cell–cell contact in IGR39 melanoma cells that express a N-cadherin/catenin but not an E-cadherin/catenin complex without affecting their expression levels (Sarray et al., 2009).

Since integrins play a central role in cancer research due to their link to many pathways which regulate different steps of tumor development and progression, we suggested that Macrovipecetin-Cisplatin treatment can restore E-cadherin expression by inhibiting cell adhesion and migration through decreasing the expression of αvβ3 integrins receptors.

CONCLUSION

In this study, we validated for the first time the effect of Macrovipecetin combined with Cisplatin drug in SK-MEL-28 melanoma cells. This effect was further associated with the modulation of cell effectors mediating proliferation, survival, adhesion and migration in melanoma cells. Thus, Macrovipecetin-cisplatin treatment inhibits proliferation through inhibiting the activation of $ERK_{1/2}$, p38 and AKT kinases.

This combined treatment triggers apoptosis by activating AIF and NF-*κ*B proteins through inducing TRADD expression. By acting as a proapoptotic protein, NF-*κ*B up-regulates p53 that regulates ROS levels and modulates the expression of proapototic proteins Bim, Bax and Bad and the anti-apoptotic Bcl-2 protein. Also, Macrovipecetin-Cisplatin treatment suppresses invasion by overexpressing E-cadherin and impairing the SK-MEL-28 cell adhesion through decreasing αv and β3 integrins. Finally, these data suggested that Macrovipecetin-Cisplatin treatment could be used as a chemo-sensitizer and/or therapeutic agent on melanoma cells.

Further investigations are needed to highlight if the *in vitro* anti-tumour effect of Macrovipecetin-Cisplatin in human SK-MEL-28 melanoma cells could be extended to the *in vivo* studies before proposing such combination as effective strategy for clinical melanoma treatment.

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FIGURE LEGENDS

Fig. 1. (A) Separation of Macrovipecetin subunits from reduced and alkylated protein on a C8 reverse phase column. Elution was performed by a linear gradient of 0.1 % TFA in 100 % acetonitrile. (**B**) Complete amino acid sequences of α and β Macrovipecetin subunits. Peptides

from digestions with Asp-N, Lys-C, CNBr and hydroxylamine are designated A, A', L, L.', M and N, respectively. (C) Amino acid sequence comparison between Macrovipecetin and two other snaclecs, Lebecin (Jebali et al., 2014) and Lebecetin (Sarray et al., 2003) from *Macrovipera lebetina* snake venom.

Fig 2. Macrovipecetin and Cisplatin inhibit the viability of human melanoma SK-MEL-28 **cells**. (**A)** SK-MEL-28 Cells were treated with a concentration range from 0.9 µM up to 12.5 µM of Cisplatin after 24h and 72h. (**B)** Cells were treated with a concentration range from 0.01 µM to 1µM of Macrovipecetin after 24h and 72h respectively. Cell viability was estimated by MTT assay. The absorbance was measured at 540 nm. ** $p < 0.01$ and *** $p < 0.005$ with respect to CN. Results represent the mean ± S.E.M of three independent experiments.

Fig 3. Macrovipecetin-Cisplatin treatment is endowed with selective activity in inhibiting the viability of SK-MEL-28 melanoma cells **(A and B).** Non-tumourigenic NIH-3T3 cells and SK-MEL-28 melanoma cells were cultured in 96-well plates and treated with 0.1 µM of Macrovipecetin, 3.75 µM of Cisplatin and the combined treatment of the both for 24 h and 72 h. Cell viability was measured by MTT assay. The absorbance was measured at 570 nm. **$p <$ 0.01 and ***$p < 0.005$ when compared to their respective CN. Assays were performed in triplicate **(C)** Microscopic observations demonstrating morphological changes in SK-MEL-28 after (24h and 72h) of Macrovipecetin-Cisplatin treatment.

Fig 4. The Macrovipecetin-Cisplatin treatment induced cell death in SK-MEL-28 melanoma cells. (**A**) Flow cytometry analysis using Annexin-V/7-AAD staining of Z-VAD-fmk (20 µM)-pretreated melanoma cells cultured in the absence (control) and the presence of Macrovipecetin and Cisplatin for 24 h and 72 h. Staurosporine (2 µM, Str) was used as a positive control of

apoptosis. ** $p < 0.01$ and *** $p < 0.005$ with respect to untreated controls. (**B)** Western blot of several apoptosis-related proteins after cell treatments with Macrovipecetin, cisplatin, and the combination of both for 24 h and 72 h. Staurosporine (2 μM, Str) was used as a positive control of apoptosis. β-actin was used as a control for equal loading. (**C**) AIF protein levels in nuclear extracts were detected by western blot using a specific antibody. **(D)** SK-MEL-28 cells were treated with Macrovipecetin, cisplatin, and the combination of both for 24 h and 72h. Levels of Bax, Bim, Bad and Bcl-2 proteins were analysed by western blot. The data shown are representative of three independent experiments.

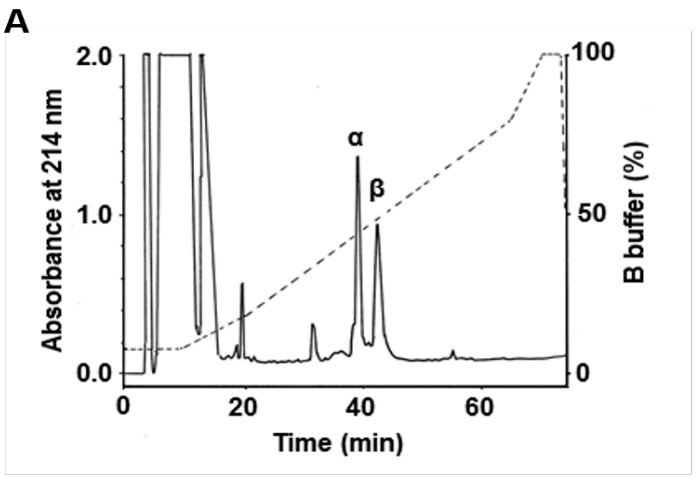
Fig. 5. Macrovipecetin-Cisplatin treatment modulates survival pathways and activates p53 that inhibits ROS generation in melanoma SK-MEL-28 cells. **(A)** $ERK_{1/2}$, p38 an AKT activations were monitored by western blot (WB) after 24 h and 72 h of the listed treatments. β-actin was used as a reference protein for equal loading. Individual experiments were performed in triplicate. **(B)** Protein extracts (30 μg) prepared and analysed by western blotting using specific antibodies after 24 h and 72 h. β-actin was used as a loading control **(C)** ROS production was measured with CMH2DCFDA staining after 24 h and 72 h of treatment with the same concentrations used previously. Detection of ROS was related to the quantity of Subsequent oxidation leading emitting fluorescence. The results were presented by histograms analysis. *** $p < 0.005$ with respect to mock-treated controls. Data are reported as the means ± S.E.M of three independent experiments.

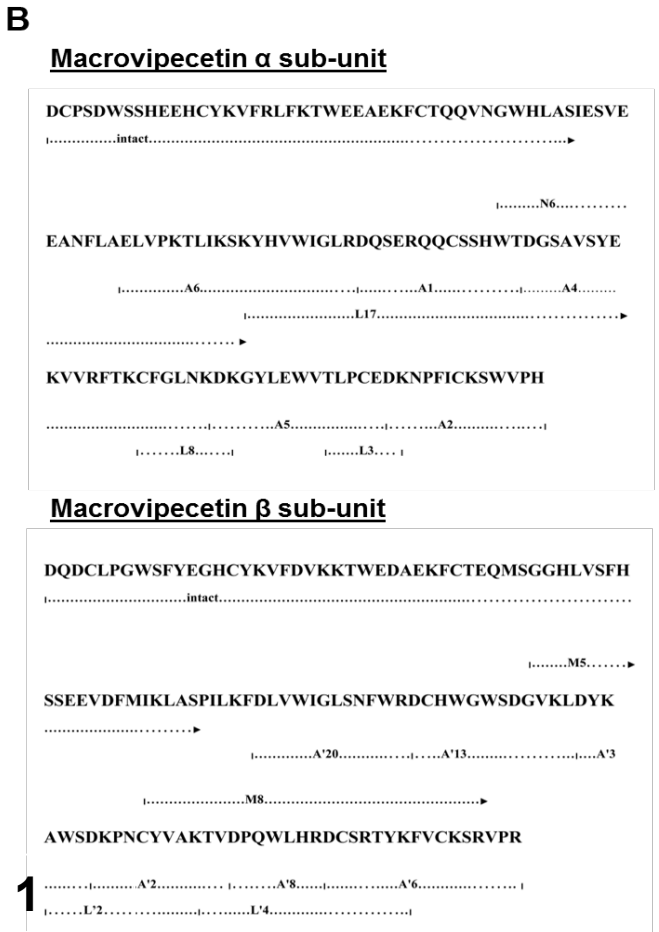
Fig. 6. Macrovipecetin-Cisplatin treatment affected SK-MEL-28 cell adhesion. **(A)** SK-MEL-28 cells were pretreated with PBS (vehicle) or with 0.1 μM of Macrovipecetin, Cisplatin (3.75 μM) and the combined treatment of the both for 2 h. Cells were then added to 96-well microtiter plates coated with different extracellular matrices; vitronectin (5 μg/ml), fibrinogen (5 μg/ml),

fibronectin (5 µg/ml), Laminin (5 µg/ml) and collagen type I (5 µg/ml), poly-L-lysine (5 µg/ml) and allowed to adhere for 1 h at 37 °C. Adherent cells were stained with crystal violet, solubilized by SDS and absorbance was measured at 600 nm. Data are reported as the means ± S.E.M of three separate experiments (*$p < 0.05$ with respect to control). **(B)** Adhesion of mock and treated-SK-MEL-28 melanoma cells with 0.1 µM of Macrovipecetin, Cisplatin (3.75 µM) and the combined treatment of the both for 30 min was measured on microtiter plates coated with antibodies (10 mg/ml) raised against αvβ3 integrin subunits as described in the materials and methods section. **(C)** αvβ3 integrin expression was detected by western blot after 24 h and 72 h of the indicated-treatments. β-actin was used as a loading control.

Fig 7. Homology model of Macrovipecetin and its interaction modes with αvβ3 integrin**. (A)** Macrovipecetin is a heterodimer protein with an α and a β subunits, associated together mainly with two long loops. Macrovipecetin contains a total of 7 disulfide bonds (orange shperes). **(B)** Macrovipecetin interacts with the binding site of the RGD motif on αvβ3 integrin. **(C)** The interaction surface of Macrovipecetin is colored in white showing that only residues from chain α are involved in the interaction.

Fig. 8. Macrovipecetin-Cisplatin treatment affected SK-MEL-28 cell migration. **(A)** Representative microscopic images of the wounds at zero time and 24 h and 72 h post-treatment comparing mock control and treatment wells. The data shown are from a representative experiment repeated three times. **(B)** Concentration response diagram of indicated-treatments and SK-MEL-28 percent cell migration. Data represent mean ± SEM of three independent experiments. *** $p < 0.005$ with respect to control. **(C)** E-Cadherin expression was detected by western blot after 24 h and 72 h of the indicated-treatments. β-actin was used as a loading control.





C

α sub-unit

```
                    10        20        30        40        50        60
                    |         |         |         |         |         |
Macrovipecetin  --DCPSDWSSHEEHCYKVFRLFKTWEEAEKVFCTQQVNGWHLASIESVEEANFLAELVPK
Lebecin         DQDCLPGWSFYEGGCYYVFDV-KTWEDAEK-FCQKQSNGKHLATIEWLGKANFVADLV--
Lebecetin       DQDCLPGWSSHEGHCYKVFNLDKTWEDAEK-FCTEQPSNGHLV----------------

                    70        80        90        100       110       120
                    |         |         |         |         |         |
Macrovipecetin  TLIKSKYHVWIGLRDQSERQQCSSHWTDGSAVSYEKVVRFTKCFGLNKDKGYLEWVTLPC
Lebecin         TLNSDPRLDWIGLRVEDKRQQCSSHWTDGSAVSYENVVHNTKCFGLDQKTGYRTWVALRC
Lebecetin       ------------------------------------------------------------

                    130
                    |
Macrovipecetin  EDKNPFICKSWVPH
Lebecin         ELAYHFIC-SRVPR
Lebecetin       --------------
```

β sub-unit

```
                    10        20        30        40        50        60
                    |         |         |         |         |         |
Macrovipecetin  DQDCLPGWSF-YEGHCYKVFDVKKTWEDAEKFCTEQMSGGHLVSFHSSEEVDFMIKLASP
Lebecin         --DCPSDWSS-DEEHCYYVFFLLFTWEEAAKFCTQQANGGHLVSIESVEEAEFVAQLISE
Lebecetin       ALNCASGWSGGYDQHCYKVFDIPPSWAADEKFCKQQTSGGHLV----------------

                    70        80        90        100       110       120
                    |         |         |         |         |         |
Macrovipecetin  ILKF--DLVWIGLSNFWRD--CHWGWSDGVKLDYKAWSDK--PNCY--VAKTVDPQWLHR
Lebecin         NIKTSADYVWIGLWNQRKAPYCVSKWTDGSSVIYKNVIERFIKNCFGLEKETNYRTWFNL
Lebecetin       ------------------------------------------------------------

                    130
                    |
Macrovipecetin  DCSRTYKFVCKSRVPR
Lebecin         SCGDDYPFVCKSPA--
Lebecetin       ----------------
```

Fig. 1

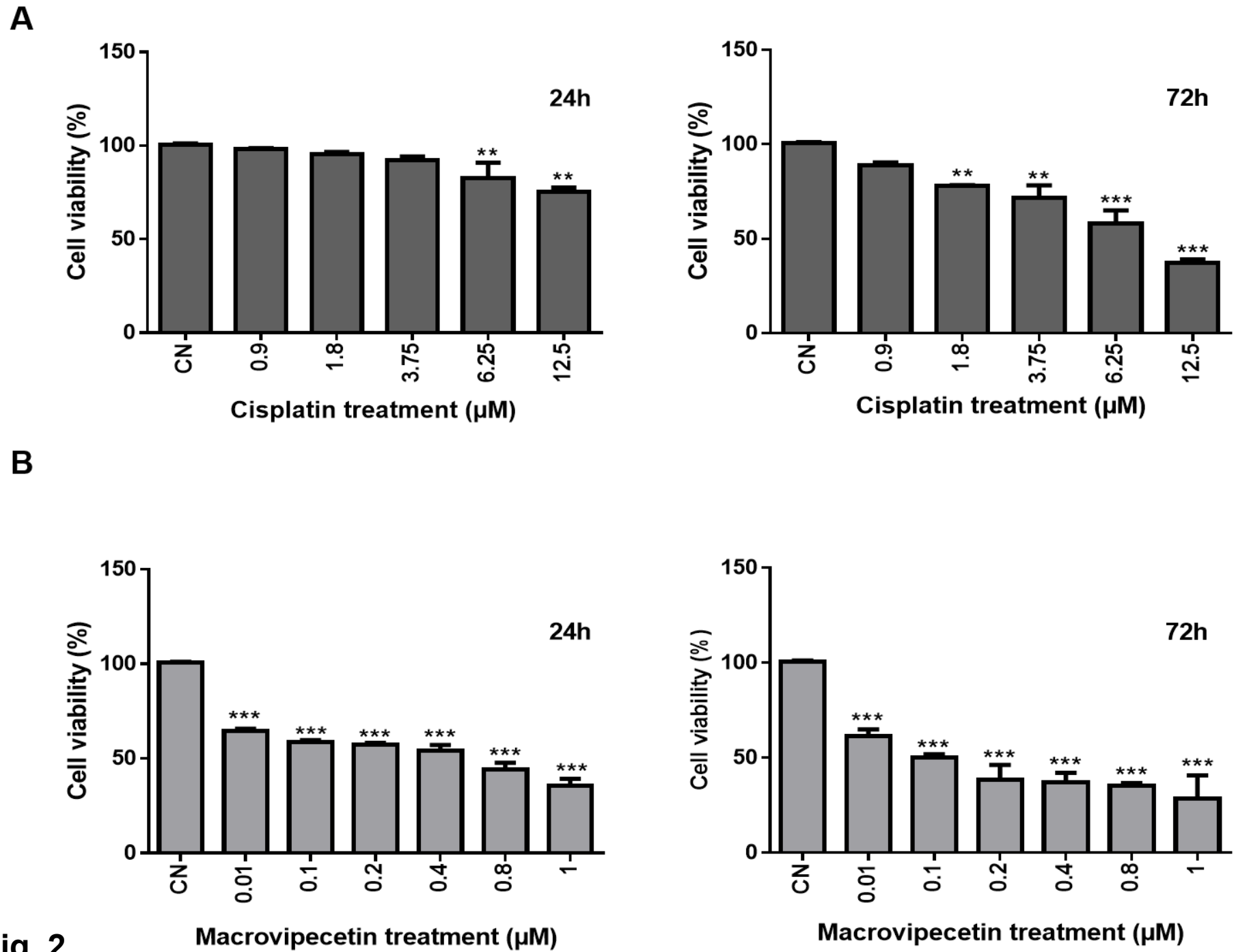

A
Cell viability (%)
150
100
50
0
24h
**
**
CN
0.9
1.8
3.75
6.25
12.5
Cisplatin treatment (μM)
72h
**
**
***
***
B
24h
***
***
***
***
***
***
0.01
0.1
0.2
0.4
0.8
1
Macrovipecetin treatment (μM)
72h

Fig. 2

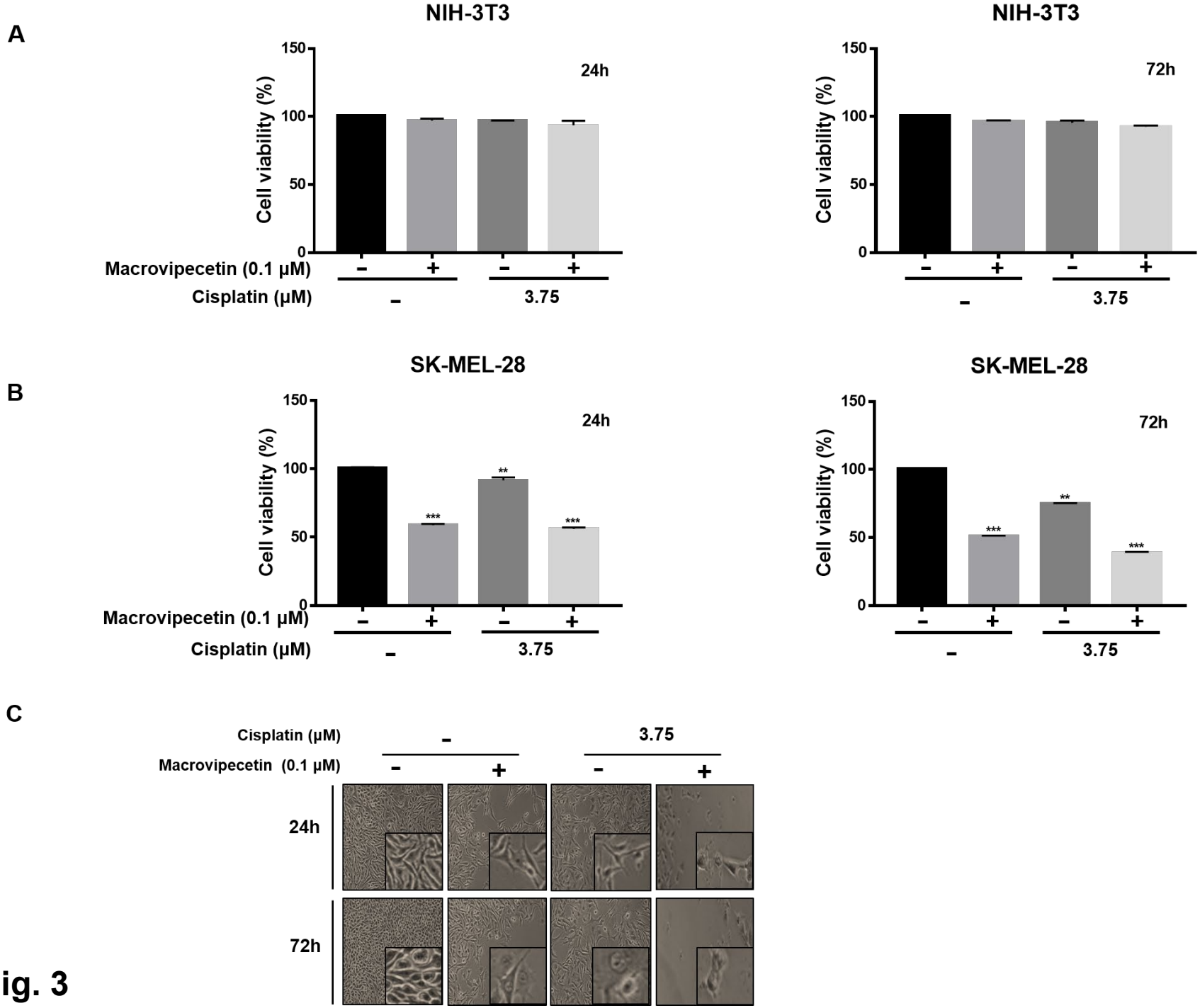

A
NIH-3T3
24h
Cell viability (%)
150
100
50
0
Macrovipecetin (0.1 μM)
Cisplatin (μM)
NIH-3T3
72h
B
SK-MEL-28
24h
SK-MEL-28
72h
***
**
C
Cisplatin (μM)
Macrovipecetin (0.1 μM)
3.75
24h
72h

Fig. 3

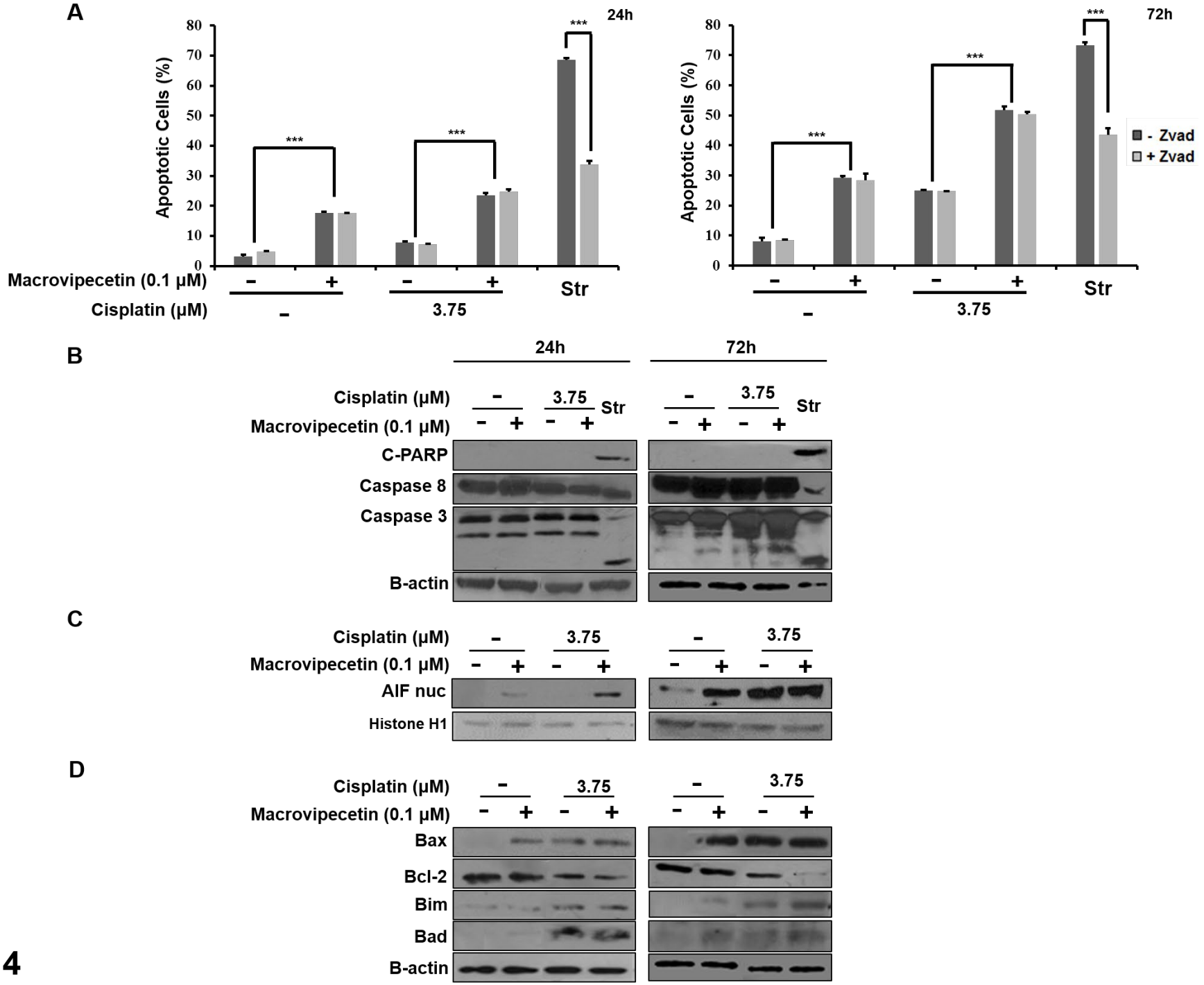

A
24h
72h
Apoptotic Cells (%)
***
- Zvad
+ Zvad
Macrovipecetin (0.1 μM)
Cisplatin (μM)
Str
3.75
B
C-PARP
Caspase 8
Caspase 3
B-actin
C
AIF nuc
Histone H1
D
Bax
Bcl-2
Bim
Bad
B-actin

Fig. 4

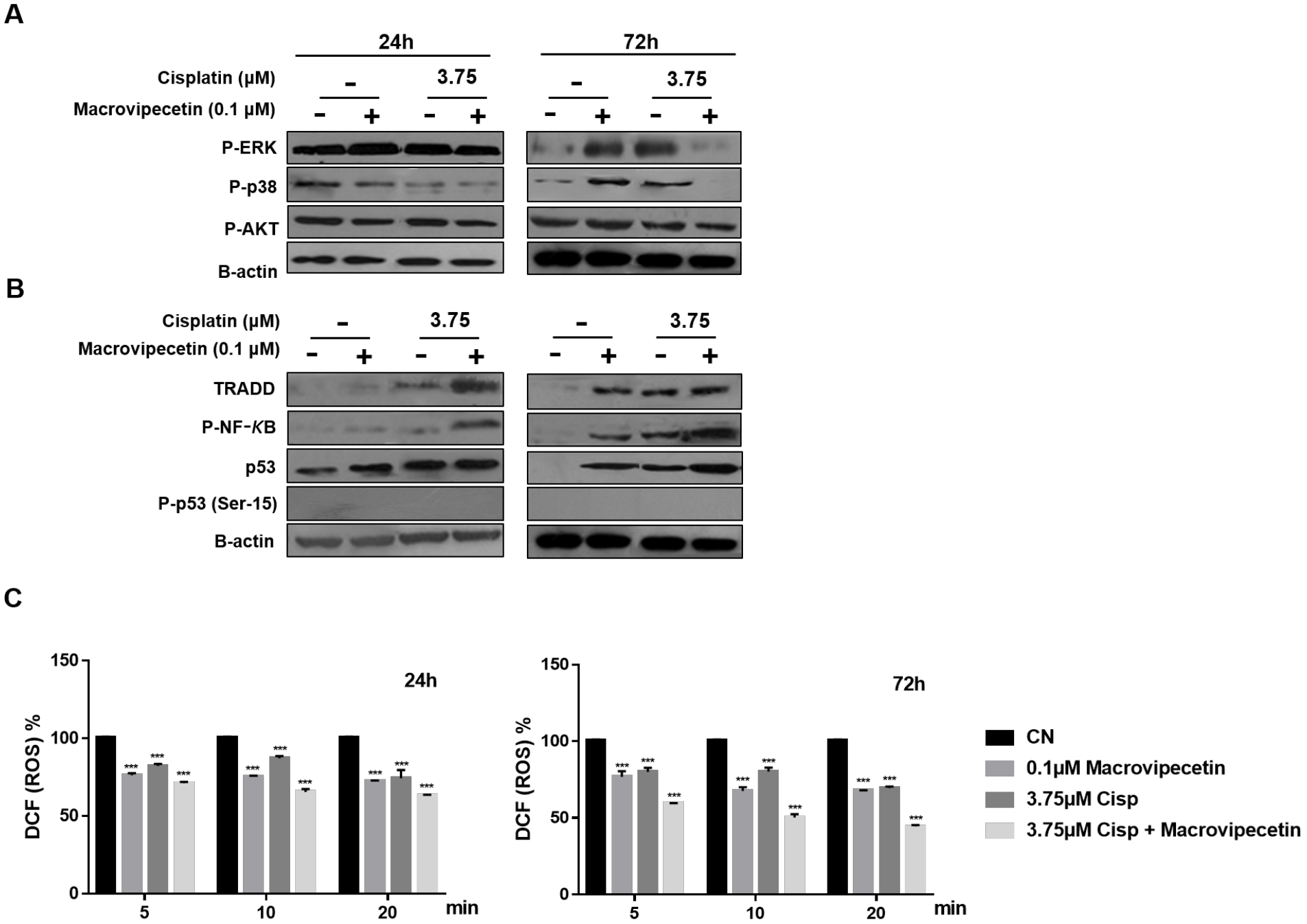

A
24h
72h
Cisplatin (μM)
-
3.75
Macrovipecetin (0.1 μM)
P-ERK
P-p38
P-AKT
B-actin
B
TRADD
P-NF-KB
p53
P-p53 (Ser-15)
C
DCF (ROS) %
150
100
50
0
5
10
20
min
CN
0.1μM Macrovipecetin
3.75μM Cisp
3.75μM Cisp + Macrovipecetin

Fig. 5

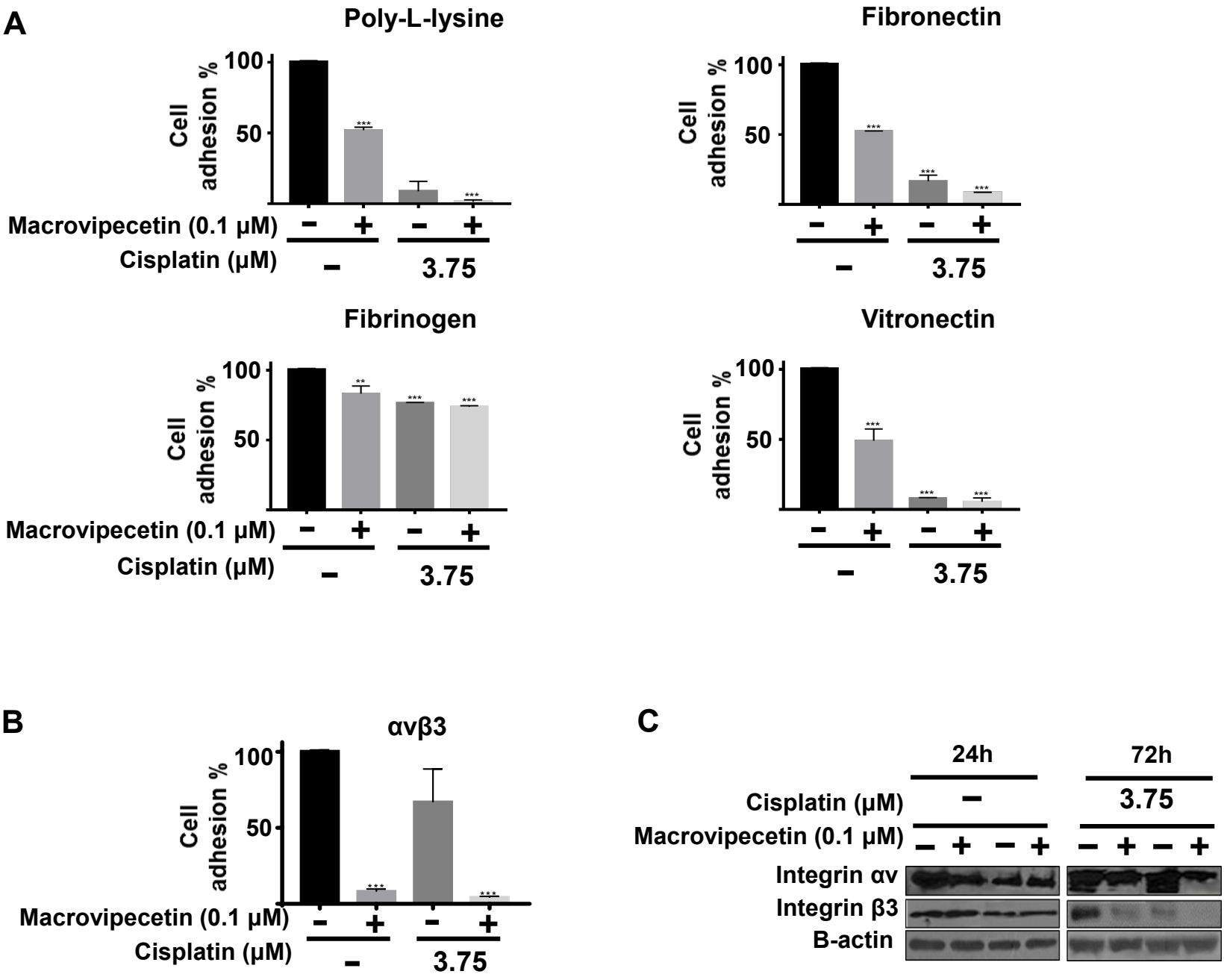

A
Poly-L-lysine
Fibronectin
Fibrinogen
Vitronectin
Cell adhesion %
100
50
Macrovipecetin (0.1 μM)
Cisplatin (μM)
3.75
B
αvβ3
C
24h
72h
Integrin αv
Integrin β3
B-actin

Fig. 6

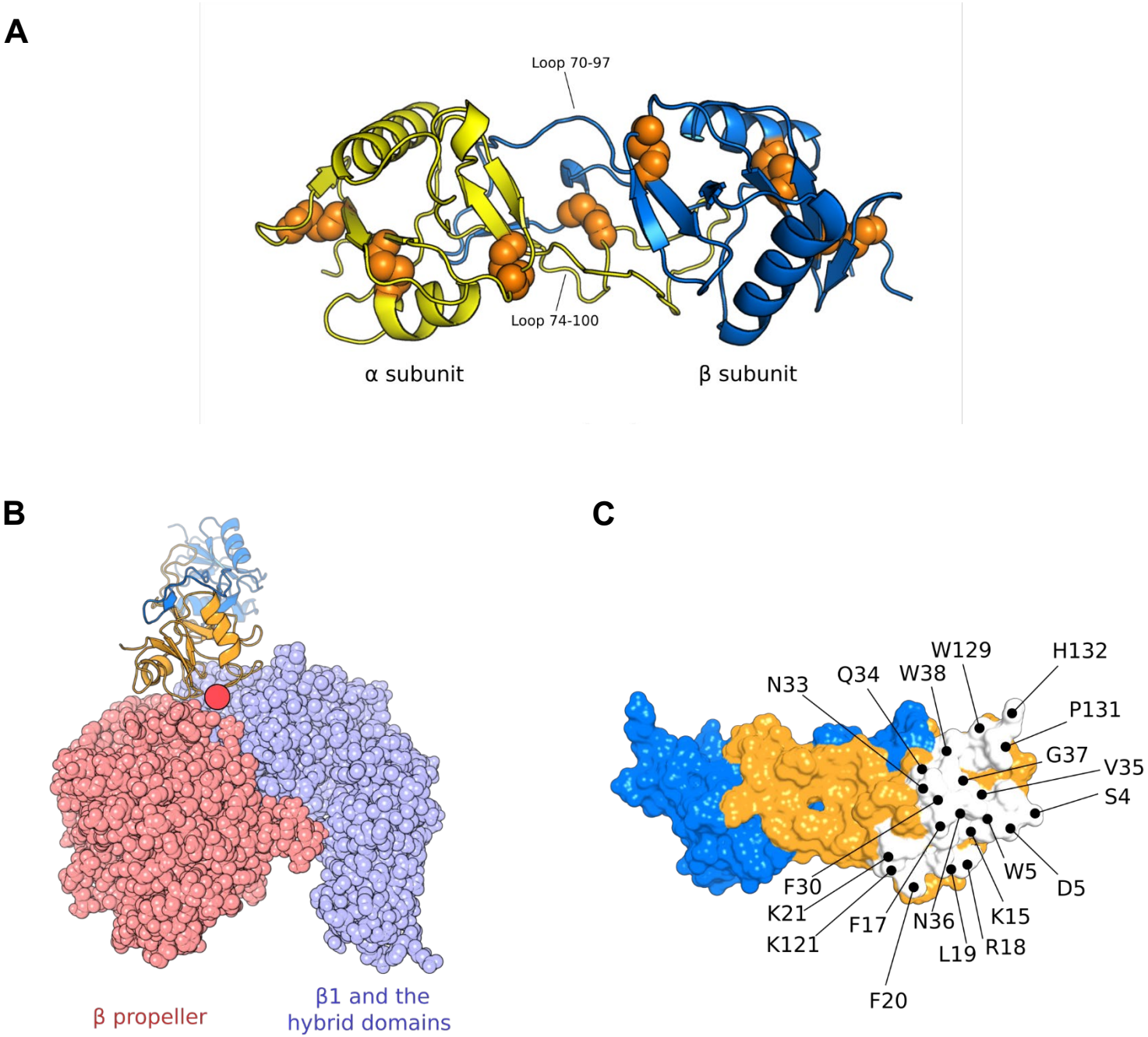

A
Loop 70-97
Loop 74-100
α subunit
β subunit
B
β propeller
β1 and the
hybrid domains
C
W129
H132
N33
Q34
W38
P131
G37
V35
S4
W5
D5
F30
K21
K121
F17
N36
K15
L19
R18
F20

Fig. 7

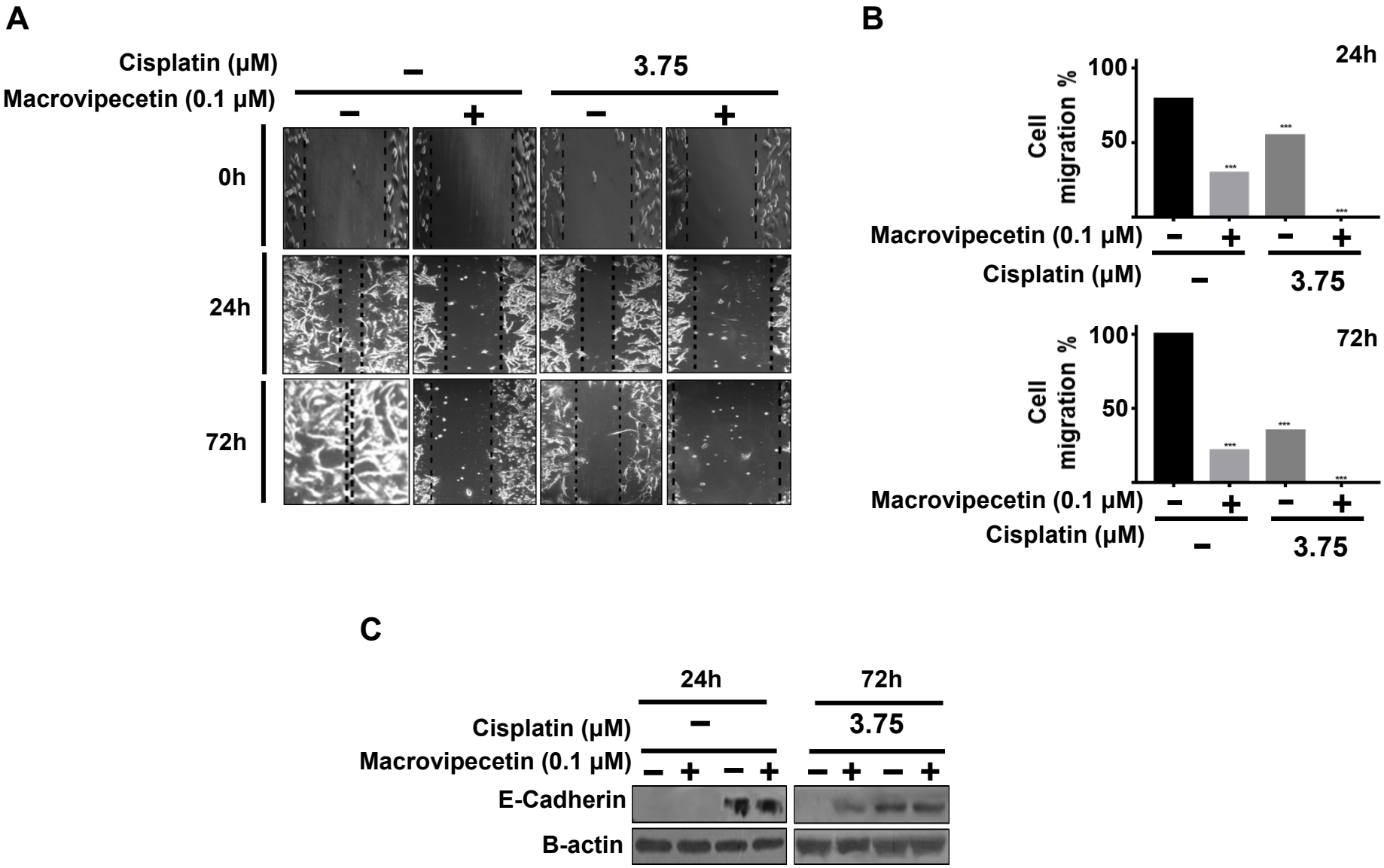

A
Cisplatin (μM)
Macrovipecetin (0.1 μM)
0h
24h
72h
B
24h
Cell migration %
Macrovipecetin (0.1 μM)
Cisplatin (μM)
72h
C
24h
72h
E-Cadherin
B-actin

Fig. 8