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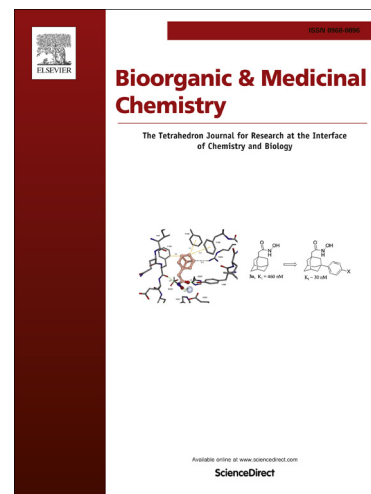
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New derivatives of lupane triterpenoids disturb breast cancer mitochondria and induce cell death

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List of Abbreviations

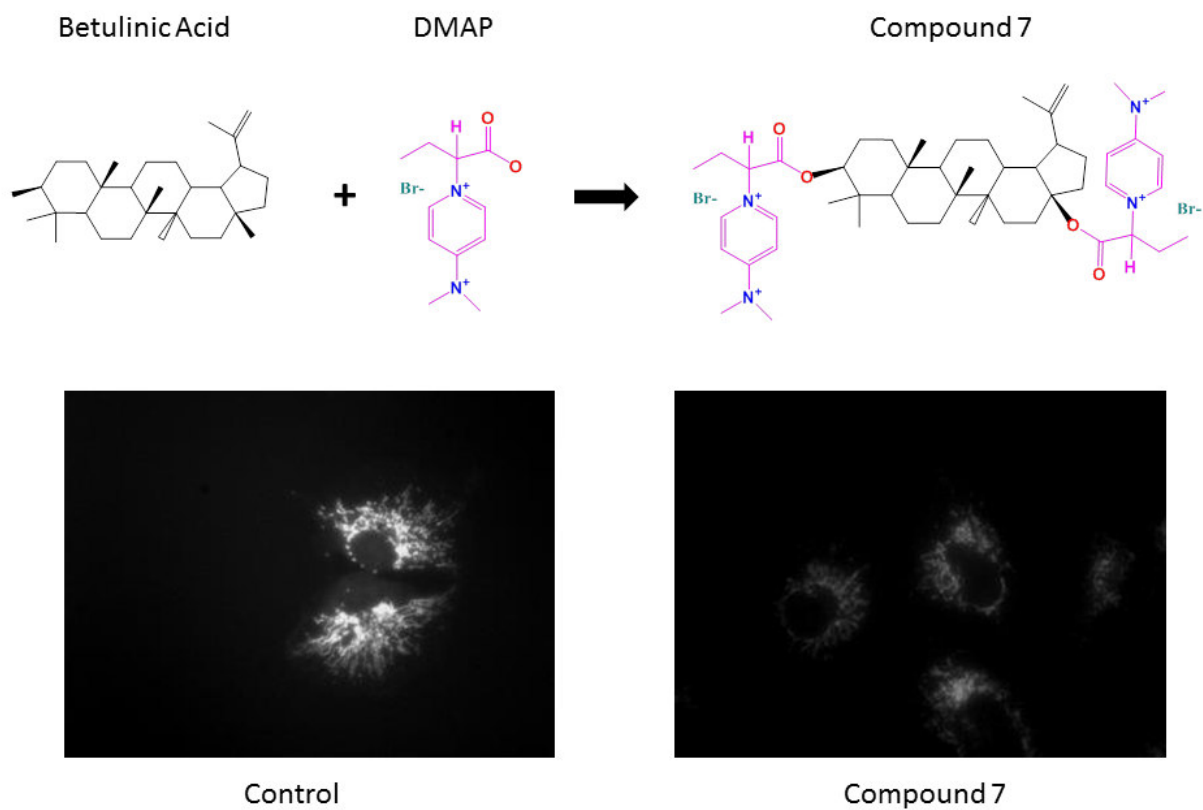
ADP – Adenosine diphosphate; ATP – Adenosine triphosphate; CsA – Cyclosporin A; DMSO – Dimethylsulphoxide; DMAP – dimethylaminopyridine; FCCP – Carbonyl cyanide p-trifluoromethoxyphenylhydrazone; MPT – Mitochondrial permeability transition; Log P – Partition coefficient; RCR – Respiratory control ratio; ROS – Reactive oxygen species; TMRM – Tetramethyl rhodamine methyl ester; TPP⁺ – Tetraphenylphosphonium cation

Abstract

Novel cationic dimethylaminopyridine derivatives of pentacyclic triterpenes were previously described to promote mitochondrial depolarization and cell death in breast and melanoma cell lines. The objective of this work was to further investigate in detail the mechanism of mitochondrial perturbations, correlating those effects with breast cancer cell responses to those same agents. Initially, a panel of tumor and non-tumor cell lines was grown in high-glucose or glucose-free glutamine-containing media, the later forcing cells to synthesize ATP by oxidative phosphorylation only. Cell proliferation, cell cycle, cell death and mitochondrial membrane polarization were evaluated. Inhibition of cell proliferation was observed, accompanied by an arrest in the G1- cell cycle phase, and importantly, by loss of mitochondrial membrane potential. On a later time-point, caspase-9 and 3 activation were observed, resulting in cell death. For the majority of test compounds, we determined that cell toxicity was augmented in the galactose media. To investigate direct evidences on mitochondria isolated rat liver mitochondria were used. The results showed that the compounds were strong inducers of the permeability transition pore. Confirming our previous results, this work shows that the novel DMAP derivatives strongly interact with mitochondria, resulting in pro-apoptotic signaling and cell death.

ACCEPTED MANUSCRIPT

Graphical Abstract



Keywords: bioenergetics; breast cancer; cell death; cytotoxicity; lupane triterpenoids derivatives; mitochondrial physiology

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1. Introduction

Plants produce natural products as a defense against flora pathogens, with some of those products having important applications for humans. Triterpenoids are a diverse group of phytochemicals, some of which presenting low biological toxicity and currently used in drug, cosmetic, dietary supplement and biocide industries, among others [25]. Triterpene molecules also have other clinically relevant properties [27], with the triterpenes betulin and betulinic acid in particular having received significant attention in this regard. These compounds are of interest because of the potential for industrial-level manufacturing capabilities; the bark of birch trees (*Betula papyrifera*), currently a waste product of paper-processing plants, can be used to make extracts containing 72.4% and 5.4% of these compounds, respectively [25].

Betulin and betulinic acid have a wide variety of potentially useful medical applications, including anti-inflammatory and anti-cancer [45], anti-bacterial [1] and anti-viral properties [22,36]. Regarding anti-cancer activity, Galgon et al. [17] showed that betulinic acid caused apoptosis in melanoma cells, later showing this activity to be specific for tumors derived from neuroectoderm [30]. Betulinic acid exhibits anti-cancer activity in many other malignancies as well, including leukemia, prostate, ovarian, breast, lung, and colon cancer [14]. Importantly, betulinic acid appears to display some degree of selectivity towards tumor cells, minimally affecting normal cells [16].

Betulinic acid triggers apoptosis in cancer cells by triggering mitochondrial outer membrane permeabilization [15], which is an effective approach since cancer cells frequently develop defective apoptotic signaling, thus becoming resistant to traditional therapies [3]. In spite of these properties, clinical trials of betulinic acid and related compounds have proven disappointing. Therefore, it is important to further investigate the structure-activity of betulinic acid derivatives, aimed at increasing the solubility of these compounds in water, reducing possible harmful side effects, and increasing the inhibition of tumor growth [25]. Toward this purpose, we have synthesized a number of semi-synthetic derivatives of lupane triterpenoids based on betulin and betulinic acid, and found that they display the ability to disrupt the mitochondrial membrane potential, resulting in the killing of melanoma and breast cancer cells [21] [7]. The present work investigates in detail the mechanism of action of some of these novel dimethylaminopyridine (DMAP) derivatives in breast cancer cells, when compared to normal mammary epithelial and stromal cells. Among the tested compounds, we have used those already evaluated in Holy et al. [21] in melanoma cells that showed to have some anti-tumoral activity, as well as a new group of compounds with slight modifications with the expectation of increasing their activity. Furthermore, we investigated the mechanism of action of these DMAP derivatives on isolated liver mitochondrial fractions in order to determine how they directly disrupt mitochondrial function. The results of this study indicate that in general the DMAP compounds tested were more toxic to mammary tumor cells than non-transformed lines, and that they were able to induce the mitochondrial permeability pore transition, resulting in pro-apoptotic effects, G1 arrest, and cell death.

2. Material and Methods

2.1. Reagents

A full list of reagents is described in the Online Supplementary Material (OSM).

2.2. Compounds

The DMAP compounds used were synthesized using betulin and betulinic acid as basic natural precursors at the National Resources Research Institute at University of Minnesota, Duluth, USA (Fig.1). Betulin, isolated from the extract of outer birch bark of *Betula papyifera* and Betulinic acid, were used as backbone for the DMAP derivatives. Protocols for isolation and synthesis were as described in Holy et al. [21]. Briefly, through intermediate acylation of corresponding hydroxy groups with bromoacids and chloroacids, the reaction mixtures were heated up to 65°C overnight and poured into ethyl ether dropwise under stirring. The precipitate was then filtered and washed twice with ethyl ether. All the compounds prepared were characterized by melting point, infrared (IR), nuclear magnetic resonance (¹H NMR and ¹³C NMR) and mass spectrometry (MS), as described in Holy et al. [21]. The compounds **4**, **5** and **6** correspond to compounds **4**, **11** and **12** respectively in the previous work by Holy et al. [21]. The remaining compounds, **1**, **2**, **3** and **7** are here tested for the first time.

2.3. Cell Culture

Human breast cancer cell lines MDA-MB-231 (92020424, ECACC, United Kingdom), MCF-7 (86012803, ECACC, United Kingdom) and HS578T (HTB-126, ATCC, Manassas, VA, USA) were used in this study. The normal human breast cell lines HS578Bst (HTB-125, ATCC, Manassas, VA, USA), MCF-12A (CRL-10782, ATCC, VA, USA) and the normal human fibroblast BJ cell line (CRL-2522, ATCC, Manassas, VA, USA) were used as non-tumor controls. Cells were cultured in high-glucose medium composed by Dulbecco's modified Eagle's medium (DMEM; D5648) supplemented with sodium pyruvate (0.11g/L), sodium bicarbonate (1.8g/L) and 10% Fetal bovine serum (FBS) and 1% of antibiotic penicillin-streptomycin 100x solution in 5% CO₂ atmosphere at 37°C. Cells were also cultured in galactose/glutamine medium, prepared from glucose-free DMEM (D5030) supplemented with galactose (1.8g/L), L-glutamine (0.584g/L), sodium pyruvate (0.11g/L), sodium bicarbonate (1.8g/L), 10% FBS and 1% of antibiotic penicillin-streptomycin 100x solution in 5% CO₂ atmosphere at 37°C.

2.4. Cell Proliferation Measurement by the Sulforhodamine B method

Cell proliferation measurements by using the sulforhodamine B assay were performed to evaluate the effect of all DMAP derivatives on the different cell lines, as previously described [38]. Compounds were added to cells in a 48-well plate 24 hours after cell seeding (20,000 cells/ml). Vehicle controls were also performed. Cells were fixed and total cell mass was determined as previously described [43]

2.5. Cell Cycle Analysis by flow cytometry

Cell cycle progression was analyzed by using flow cytometry [42]. Cells in log-phase growth were treated with different concentrations of the tested compounds for various lengths of time. Adherent and floating cells were then collected and fixed with cold 70% ethanol and stored overnight at -20°C . After washed and resuspended in PBS-T (132 mM NaCl, 4mM KCl; 1.2mM NaH_2PO_4 , plus 0.1% Tween) samples were incubated at 37°C in 0.5 ml PBS-T with $20\mu\text{g/ml}$ RNase for 45 min, and with Propidium Iodide at 37°C , for further 30 min. The percentage of cells in the different cell cycle phases was quantified using a flow cytometer (Becton-Dickenson FACScalibur), with Modfit LT software (Verity Software House, Topsham, ME, USA).

2.6. Live/Dead Assay by flow cytometry

After treatment with DMSO or selected triterpenoids (compounds **2**, **3**, **5** and **7**) at $1\mu\text{g/ml}$ for 24 and 48 hours, HS578T cells (initially seeded at 2.5×10^4 cells/ml in 6well-plates), were collected by trypsinization, centrifuged and resuspended in buffer solution (NaCl 120 mM, KCl 3.5 mM, KH_2PO_4 0.4 mM, HEPES 20 mM, NaHCO_3 5 mM, NaSO_4 1.2 mM and Glucose 15 mM). Cells were incubated for 15 min at 37°C with the Live-Dead kit ($1\mu\text{M}$ ethidium homodimer and $0.1\mu\text{M}$ calcein-AM; Invitrogen, Eugene, OR). A Becton-Dickenson FACScalibur flow cytometer was used to measure FL1 (calcein, green) and FL3 (ethidium homodimer, red) signals. To quantify red and green fluorescence, CellQuest software verion 5.1 package was used.

2.7. Caspases-like Activity Assay

Attached and floating cells were collected by trypsinization and pellets were saved after centrifugation, in lysis/assay buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10% Glycerol and 10mM DTT) and frozen at -20°C overnight. Protein quantification was determined by the Bradford method [26] using bovine serum albumin (BSA) as standard. To measure caspase 3 and 9-like activity, aliquots of cell extracts in ambar eppendorfs containing 25 mg (for caspase 3) or 50 mg (for caspase 9) were incubated in a reaction buffer containing 25 mM HEPES (pH 7.4), 10% sucrose; 10 mM DTT, 0.1% CHAPS and 100 mM caspase substrate (Ac-DEVD-pNA for caspase 3 or Ac-LEHD-pNA for caspase 9, purchased from Calbiochem, NJ, USA) for 2 h at 37°C . Caspase-like activities were determined by following the detection of the chromophore p-nitroanilide after cleavage of the labeled substrate Ac-DEVD-p-nitroanilide or AcLEHD-p-nitroanilide. The method was calibrated with known concentrations of p-nitroanilide, two hour later, at endpoint.

2.8. Mitochondrial Proteins Semi-quantification by Western blotting

For Western blot analyses, approximately 1.0×10^6 attached and floating cells were collected, including floating cells, resuspended and added to lysis buffer (20 mM HEPES/NaOH, pH 7.5, 250 mM sucrose, 10 mM KCl, 2 mM MgCl_2 , 1 mM EDTA) supplemented with 2 mM dithiothreitol (DTT), 100 mM phenylmethylsulfonyl fluoride (PMSF) and a protease inhibitor cocktail. Afterwards, extracts were stored at -80°C and later analyzed by Western blotting, as described in the OSM.

2.9. Vital Epifluorescence Microscopy

Images were obtained using a Nikon Eclipse TE2000U epifluorescence microscope. Cell lines were seeded in 6-well plates with a glass coverslip per well, at a density of $5\text{-}10 \times 10^4$ and allowed to attach for 24 hours. Cells were then treated with either the test compound solutions or the vehicle for the desired time. Thirty minutes prior the end of the treatment time, cells were incubated with TMRM (100 nM) in a buffer solution (NaCl 120mM, KCl 3.5mM, KH_2PO_4 0.4mM, HEPES 20mM, NaHCO_3 5mM, NaSO_4 1.2mM and glucose 15mM). The probe was maintained with cells during the imaging procedure in order to avoid extensive loss from mitochondria.

2.10. Animals

Male Wistar-Han rats (Charles River, Barcelona, Spain), 10 weeks old, were housed in our accredited animal colony (Laboratory Research Center, Faculty of Medicine of University of Coimbra). Animals were group-housed in type III-H cages (Tecniplast, Italy) and maintained in specific environmental requirements (22 °C, 45-65 % humidity, 15-20 changes/hour ventilation, 12 h artificial light/dark cycle, noise level < 55 dB) and with free access to standard rodent food (4RF21 GLP certificate, Mucedola, Italy) and acidified water (at pH 2.6 with HCl to avoid bacterial contamination). This research procedure was carried out in accordance with European Requirements for Vertebrate Animal Research and according to the ethical standards for animal manipulation at the Center for Neuroscience and Cell Biology, University of Coimbra, Portugal.

2.11. Isolation of Rat Liver Mitochondria

Mitochondria were isolated from the livers of male Wistar rats by conventional differential centrifugation [29]. Livers were harvested and homogenized with buffer medium containing 250 mM sucrose, 10 mM HEPES (pH 7.4), 1 mM EGTA and 0.1% BSA lipid-free. After obtaining a crude mitochondrial preparation, pellets were washed twice with 250mM sucrose and 10mM HEPES (pH 7.2). The protein content was determined by the biuret method, using BSA as a standard [20].

2.12. Measurement of Mitochondrial Oxygen Consumption

A Clark-type oxygen electrode connected to a suitable recorder in a 1 mL thermo-stated, water-jacketed and closed chamber was used to measure the oxygen consumption of isolated liver mitochondria as previously described [41]. Further experimental details can be found in the OSM.

2.13. Measurement of Mitochondrial Transmembrane Electric Potential

The mitochondrial transmembrane electropotential ($\Delta\Psi$) was monitored indirectly, through the activity of the lipophilic cation tetraphenylphosphonium (TPP^+), using a TPP^+ selective electrode also connected to a suitable recorder in a 1 mL thermo-stated, water-jacketed and open chamber as previously described [34]. Mitochondrial protein (1.5 mg) was suspended in reaction medium composed of 125 mM sucrose, 65 mM KCl, 5 mM KH_2PO_4 , 2.5 mM MgCl_2 and 5 mM HEPES (pH 7.4, 30°C), and supplemented with 3 μM TPP^+ . The compounds were added to

mitochondria for 1 min, followed by 10 mM succinate (plus 3 μ M rotenone). In order to initiate state 3 respiration, ADP (83.3 nmol/mg protein) was added. Assuming a Nernst distribution of the ion across the membrane electrode, the equation proposed by Kamo *et al* [23] without correction for passive membrane binding, was used to calculate the values for $\Delta\Psi$.

2.14. Induction of the Mitochondrial Permeability Transition Pore

The turbidity of the mitochondrial suspension was measured at 540nm in a JASCO V-560 spectrophotometer after a single pulse of calcium (40mM) as described in the OSM.

2.15. Statistics

Data was analyzed in GraphPad Prism 5.0 software (GraphPad Software, Inc.), all results being expressed as means \pm SEM, and analyzed by the student's T-test for comparison of two means, one-way ANOVA with Bonferroni or Dunnett multiple comparison post-test to compare groups with one independent variable; and two-way ANOVA for comparisons involving two independent variables. Values with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Breast cancer cell proliferation is inhibited by lupane triterpenoids

The compounds used in this study are shown in Fig. 1. To evaluate their effects on cell proliferation, a dose-response assay using the SRB assay was performed (Fig.2). Monolayer cultures of human breast cancer cell lines MDA-MB-231, HS578T and MCF-7, and the non-tumorigenic cell lines MCF-12A (human breast epithelial cells) and BJ (human foreskin fibroblasts) were incubated in high-glucose medium with different concentrations of the compounds (0.125 - 2 μ g/ml) for 1, 3 and 5 days.

Compounds **1** and **4** exhibited more toxicity toward normal cell lines than cancer cells, whereas compounds **5** and **6** had similar effects on the proliferation of all cells lines tested. Compound **5** was the most toxic against cancer cells, having a more powerful effect against MDA-MB-231 cells (Fig.2C).

Moreover, compound **2** was the less toxic molecule against the BJ cell line, not significantly affecting cell proliferation, and showing toxicity at the third day of treatment in all breast cancer cell lines. However, that same compound presented some toxicity on MCF-12A cells, already present in the first day of treatment. Compound **3** reduced preferentially the cell numbers of the HS578T cell line for lower concentrations, with little effect on the normal cell lines. Structurally, compounds **5**, **6** and **7** are very similar but compound **7** showed stronger effects in all cell lines tested, inhibiting cell proliferation on the first day of treatment for all cell lines except HS578T cells.

When comparing cancer cell lines, the SRB data showed that both HS578T and MCF-7 cells were the most susceptible to the test compounds. Since HS578T cells are more aggressive due to their invasive capacity [32], this cell line was chosen for the following studies. To test the ability of this cell line to recover from DMAP treatment, HS578T cells were pulse-treated with compounds for 6 hours, followed by drug wash out and culture in fresh medium. In general, the 6-hour pulse of drug treatment at the higher concentrations still resulted in an inhibition of

proliferation 5 days after wash out, with the exception of compound **7**, which showed stronger toxicity at the third day of treatment (Fig.2F).

Typically, cancer patients receive a combination of chemotherapeutic drugs in attempts to reduce tumor cell growth, which allows for lower doses of each drug to be used, thereby reducing side-effects on non-tumor cells [13]. Thus, we next tested whether a combination of DMAP compounds and a number of commonly or historically used chemotherapeutics, including Paclitaxel (Taxol), Doxorubicin and Cisplatin were effective in suppressing HS578T cell growth over 3 days of treatment (Fig.A1). The objective was to combine compound concentrations that individually would inhibit cell proliferation by about 25%, in order to facilitate the detection of additive or synergistic effects. No additive or synergistic effects were observed with doxorubicin or cisplatin; however, combinations with Taxol showed a significant reduction of cell proliferation, especially when compound **2**, **3** and **4** were used, suggesting an additive effect of the combination.

3.2. Lupane triterpenoids induce cell cycle arrest and cell death

To distinguish between the contributions of cell cycle arrest vs. cell death in the proliferation experiments, the effects of the test compounds on the HS578T cell cycle was analyzed by flow cytometry. For this assay, a single compound concentration (0.5 μ g/ml) was used, corresponding approximately to 50% growth inhibition for the HS578T cell line verified for 24 and 48 hours. All the triterpenoids showed a similar pattern of cell cycle arrest, with an increase in the percentage of cells in G0/G1 phase and a decrease in the percentage of cells in S phase. For compound **4**, a decrease in the percentage of cells in G2/M phase was also observed (Fig.3).

Since compounds **2**, **3**, **5** and **7** inhibited cell proliferation, in part due to cell cycle arrest, the next question was whether the same compounds could cause cell death. Several experimental endpoints were measured to assess the induction of HS578T cell death (Fig.4). The assays included caspase-3 and 9-like activity, semi-quantification by Western blotting of proteins involved in cell death and mitochondrial integrity, as well as the Live/Dead assay to quantitatively measure cell viability. Live/Dead assays demonstrate that all compounds at a concentration of 1 μ g/ml modestly increased cell death at the two time points studied, 24h and 48h (Fig.4A). All compounds decreased the percentage of live HS578T cells by 10-20%, with the exception of compound **5**, which had a milder effect. Caspase-like assay activity assays demonstrate that a significant increase in caspase-9-like activity occurs after 24 hours of treatment with 1 μ g/ml of compounds **3**, **5** and **7** (Fig. 4B). Compound **2** caused a small, non-statistically significant increase in caspase-9-like activity. Caspase-3-like activity was also increased in the presence of all tested compounds (Fig.4B).

To complement the present results, proteins associated with cell death, such as Bcl-2 family proteins [10], p53 [39] and Cyclophilin D [4] were quantified by western blotting. An increase of total p53 protein was detected by western blot after treatment of HS578T cells with compounds **2**, **3**, **5** and **7** (Fig.4D). Similarly, cyclophilin D protein was found largely increased after treatment with all compounds, while no significant increase was observed for the pro-apoptotic Bax protein, at least for the time point measured. Similarly, no differences were also obtained for the anti-apoptotic Bcl-2 protein after treatment with compounds **2**, **3**, **5** and **7** (Fig.A2).

3.3. Mitochondrial structure and function are altered by lupane triterpenoids

The previous results demonstrated that the new DMAP derivatives not only promoted cell cycle arrest, but also induced cell death. Since mitochondria are intrinsically involved in cell death, and following previous results from our group [21] [7], we next evaluated *in situ* mitochondrial effects of some of the selected compounds. To detect alterations in $\Delta\Psi$, tetramethylrhodamine methylester (TMRM) a mitochondrial membrane potential-dependent fluorescent probe was used. Mitochondrial membrane potential ($\Delta\Psi$) alterations were studied in HS578T breast cancer cells, and compared with the non-transformed cell lines MCF12A and BJ cells. In addition, the HS578Bst cell line was also included, which is a normal mammary myoepithelial cell line genetically matched to the HS578T cells [46]. Cells were labeled with TMRM and its mitochondrial accumulation was observed by epifluorescence microscopy. Non-tumor cells appeared to have a more elongated and diffuse polarized mitochondrial network, while the 578T cancer cells showed a more polarized mitochondrial network located in the perinuclear region (Fig.5A). Moreover, fluorescent microscopy images also suggested that the baseline intensity of TMRM mitochondrial fluorescence was higher for the 578T cells than for the normal cell lines (Fig.5B).

To test the effects of the DMAP compounds on mitochondrial polarization, compounds **2**, **3**, **5** and **7** were used and cells incubated with different concentrations (0.125 μ g/ml to 2 μ g/ml) for 6 hours. In general, the concentration of triterpenoids used appeared to preferentially affect the mitochondrial network in tumor breast cells (Fig.5A and B), leading to a thread-to-grain transition and loss of mitochondrial TMRM fluorescence intensity. The most toxic compound in terms of inducing mitochondrial depolarization appears to be **3**, affecting transversely all cell lines. Compounds **2** and **7** affected preferentially the mitochondrial network of HS578Bst and HS578T cell lines, but in a milder manner when compared with compound **3**. Compound **5** did not affect BJ or MCF-12A cells, a reduced effect on the HS578Bst cells, although promoting a decrease in TMRM fluorescence for the HS578T cell line.

3.4. Effects of lupane triterpenoids under forced mitochondrial oxidative phosphorylation conditions

To identify mitochondria as a direct target for DMAP derivatives, MDA-MB-231, MCF-7 and HS578T breast cancer cells were grown in a cell culture media which forces cells to rely solely on oxidative phosphorylation for ATP production. This glucose-free media contains galactose as carbon source and glutamine/pyruvate as mitochondrial substrates [28]. A dose-response SRB assay was used to evaluate compound toxicity. The test compounds significantly decreased MDA-MB-231 and MCF-7 cell number for all concentrations tested at the third and fifth days of treatment (Fig.6A and 6B, respectively, versus Fig.2), when comparing with results obtained with the high-glucose media. Surprisingly, the HS578T cell line, which was susceptible to the compounds under normal high-glucose media conditions, was now less affected than the other cell lines under these new conditions (Fig.6C). Still, compound **7** appeared to be the most toxic for that cell lines in all time points and concentrations tested.

3.5. Direct effects of lupane triterpenoids on mitochondrial oxidative phosphorylation – isolated mitochondrial fractions experiments

Our results show that some of the DMAP compounds tested act directly on mitochondria in MCF-7 and MDA-MB-231 cancer cells. To further understand how the different compounds interfere with mitochondrial function, isolated rat liver mitochondria were used to test their direct toxicity. By using isolated mitochondrial fractions, effects on other cellular structures can be excluded. Several parameters for mitochondrial function were evaluated including respiration, generation of $\Delta\Psi$ and induction of the MPT pore. Assays were performed by energizing mitochondria via complex II with succinate in the presence of rotenone (Table A1 in supplementary material). No effects on succinate-sustained respiration, regardless of the respiration state investigated were found after incubation with the compounds. The RCR and the ADP/O ratio of untreated fractions were indicative of well-coupled mitochondrial preparations and presented no alterations comparative to controls.

Regarding the effects of DMAP derivatives on mitochondrial $\Delta\Psi$, no effects were also observed for succinate-energized preparations when compounds **5** and **7** were analyzed (Table A2 in supplementary data). However, compounds **2** and **3** decreased the depolarization caused by ADP for concentrations above 1.3 $\mu\text{g}/\text{mg}$ and 0.7 $\mu\text{g}/\text{mg}$ of protein, respectively. Also, compound **3** decreased the $\Delta\Psi_{\text{max}}$ for concentrations higher than 0.3 $\mu\text{g}/\text{mg}$ of protein.

Although no significant alterations were observed when respiration and mitochondrial $\Delta\Psi$ were measured, assays on intact cells showed that mitochondrial function was disturbed and cell death was promoted. A mitochondrial process associated with cell death is the MPT, which is related with an increase of mitochondrial inner membrane permeability to ion and solutes [6]. We have measured this event by following variations in pseudo-absorbance of the mitochondrial suspension in the presence of calcium. The results showed that compounds **2**, **3** and **7** acted as MPT pore inducers *in vitro*. When compounds were pre-incubated with mitochondria and the MPT pore was induced with calcium, the amplitude of mitochondrial swelling increased in a dose-dependent manner (Fig. 7 A and B). For the highest concentration tested (1.33 $\mu\text{g}/\text{mg}$), swelling amplitude was five times higher than in the control groups (calcium-only). Surprisingly, compound **5** did not have any effect on MPT induction. The MPT pore-inducing effect of some of the compounds, occurring for concentrations where no respiration or $\Delta\Psi$ alterations were observed, was inhibited by cyclosporin A, a MPT pore desensitizer [8].

4. Discussion

An important strategy in cancer cell chemotherapy is the re-activation of apoptotic pathways that may have been silenced or modified in tumor cells. In this context, mitochondria are a promising target to increase tumor susceptibility to chemotherapy-mediated death [19]. One characteristic of some cancer cells is increased mitochondrial $\Delta\Psi$ when compared with normal cells [44], which can drive the accumulation of positively charged molecules [5]. New cationic derivatives of lupane triterpenoids were already described as potential mitocans against human cancers [7,21]. The present work extends previous observations by investigating whether DMAP derivatives from the same family of compounds promote mitochondrial dysfunction and lead to selective killing of breast cancer cells, represented in this work by the MCF-7, HS578T and MDA-MB-231 human cell lines. Normal breast epithelial

MCF-12A and myoepithelial HS578Bst cells, as well as normal foreskin (BJ) fibroblasts, were used as non-tumor controls. The data shows that MCF-7 (Fig.2D) and HS578T (Fig.2E) cells are the cell lines most susceptible to inhibition of proliferation by the DMAP derivatives. Compounds **4**, **5** and **6** were already tested in melanoma cell lines, in which compound **4** showed a weak effect, while compounds **5** and **6** were very active mitochondrial disturbers in melanoma cell models. In the present work, the target for anti-tumor activity was shifted to breast cancer. Together with previously analyzed compounds, we have tested four new derivatives. Interestingly, compound **5** did affect the usually resistant MDA-MB-231 cell line, which is a 'triple-negative' line (lacking overexpression of Her2/Neu, epidermal growth factor receptor, and estrogen receptor; [33]) as well as HS578T cells. Triple negative breast cancers are normally associated with a poor prognosis, and are therefore particularly important targets for more effective therapeutic strategies [33](Fig.2C). Compounds **5** and **6** had similar effect on all cells tested, which is not surprising since their chemical structure is identical, differing only by the presence of a different counterion (Cl^- or Br^- , respectively), suggesting that this alteration does not interfere with cell proliferation, as previously described [21]. Moreover, both compounds **1** and **2** had similar effects, although compound **2** had a stronger effect overall. This small difference may result from a higher accumulation in cells since compound **2** contains an extra nitrogen in the chemical structure, which may undergo protonation/deprotonation cycles and change the overall molecule charge. Furthermore, we investigated whether the toxicity of the DMAP molecules was persistent through pulse-exposure experiments (Fig.2F). The results show that the anti-proliferative effects of a short (6 hour) DMAP treatment are persistent over the time frame studied. Compound **7** showed the highest potency in this regard, with a strong persistent toxic effect for cell the concentrations tested. From these results we can roughly order the compounds based on strength of cell proliferation effects ($1 < 6 < 4 < 5 < 2 < 3 < 7$).

Of the well-known previously established chemotherapeutics tested in this study, only Taxol showed additive effects with the DMAP compounds. Taxol is a clinically important anti-tumor agent, which functions by suppressing microtubule dynamics [18]. Interestingly, the acquisition of Taxol resistance in some types of tumors is associated with mitochondrial biogenesis [35]. Since our results demonstrate that the DMAP derivatives so far tested characteristically profound effects on mitochondrial function, it is possible that they could be used to augment Taxol toxicity, or delay the onset of resistance to Taxol.

Overall, the data on this paper suggest that DMAP derivatives can induce cell cycle arrest and cell death. All the compounds tested demonstrated a similar cell cycle arrest pattern, with an increase of cells in G0/G1 phase, accompanied by a reduction in S-phase cells, being observed (Fig.3). This type of cell cycle block is normally associated with DNA damage, although other mechanisms can also cause such type of arrest. For instance, a down-regulation of cyclin A, cyclin D3 and CDK4, can contribute to G0/G1 phase block, resulting from ROS production [2]. It is also clear that in addition to cell cycle arrest, DMAP triterpenoids are also potent inducers of cell death. Notably, compounds **2**, **3**, **5** and **7** appeared to be more effectively trigger cell death in cancer cells than normal cells. The results confirm the ability of the triterpenoids to induce breast cancer cell death (Fig. 4), possibly by inducing mitochondrial perturbations. The marked increase in cyclophilin-D expression (Fig.4D) suggests that triterpenoids may sensitize the MPT pore for opening *in situ* through increasing intra-mitochondrial stress [4].

High accumulation of cations in mitochondria can lead to mitochondrial depolarization (Fig.5A) through several mechanisms, including induction of the MPT, charge-driven matrix depolarization or inhibition of the respiratory chain, disrupting mitochondrial function and structure, and promoting cell death [31]. Smaller molecules (compounds **7** and **3**) promoted a larger mitochondrial membrane depolarization in all cells when compared with longer ones (compounds **2** and **5**), preferentially depolarizing mitochondria in the breast cancer HS578T cell line. The different activity can be explained by the fact that longer side chain compounds maybe present an increased difficulty in permeating membranes or to be more susceptible to breakage.

To further investigate the possible primary role of mitochondria in the mechanism of toxicity of the novel triterpenoid compounds, the cancer cell lines MCF-7, MDA-MB-231 and HS578T were cultured in galactose/glutamine media in order to force cells to exclusively use oxidative phosphorylation (OXPHOS) for ATP production [28]. With this strategy, direct toxicity of the DMAP derivatives on mitochondria was determined. Under these conditions, cancer cells up-regulate glutaminolysis and Krebs cycle activity to supply reducing equivalents for the respiratory chain [11]. Our results showed that the DMAP compounds dramatically inhibited the proliferation of MCF-7 and MDA-MB-231 cancer cells for all concentrations tested under these conditions. However, HS578T cells were less affected under galactose/glutamine growth conditions, despite the fact that this cell line was strongly affected by the test compounds when growing in normal high-glucose media. One possible explanation is that the compounds may also present an inhibitory effect on glycolysis, although we cannot exclude other targets. Direct evidences of mitochondrial effects of the compounds **2**, **3**, **5** and **7** were obtained by using isolated rat liver mitochondria. Isolated mitochondrial fractions are considered a prime biosensor to test compound toxicity [37] and to predict drug safety in the pharmaceutical industry [12]. Strikingly, compounds **2**, **3** and **7** caused a dose-dependent cyclosporin-A-sensitive induction of the MPT pore (Fig. 7). This may imply that these compounds have a direct effect on a pore component, or act through the induction of oxidative stress, since no effects on $\Delta\Psi$ or respiration (Tables A1 and A2) were noticed for the same range of concentrations in the absence of calcium. Contrarily, compound **5** had no effect on MPT pore opening, which is interesting since this compound has a similar structure to compound **2**, differing by only two carbons. The difference may rely on the fact that compound **5** is a longer molecule, which may interfere with its ability to bind to its targets as efficiently as a shorter molecule. The MPT has been correlated with a series of deleterious effects to the cell, including mitochondrial depolarization, ATP depletion and cell death, which is often associated with necrotic cell death [24,40]. In fact, MPT pore induction, regardless of the exact mechanism, may well be one of the mechanisms by which some of the triterpenoids here described cause mitochondrial dysfunction and cell death.

5. Conclusion

From the present study, we can suggest that the new DMAP derivatives compounds are promoting cell death through directly targeting mitochondria of breast cancer cells, promoting ROS production, leading to typical apoptotic signaling including resulting from MPT induction and from p53 activation to downstream activation of caspases. The compounds that were more likely to have these attributes were those with smaller and more complex

structure (e.g. four nitrogen atoms coupled to a small side chain). We have also verified that the type of counter ion does not seem to influence the higher or lower activity of the compounds.

6. Conflict of Interest

Teresa L. Serafim, Filipa S. Carvalho, Telma C. Bernardo, Gonçalo C. Pereira, Edward Perkins, Jon Holy, Dmytro A. Krasutsky, Oksana N. Kolomitsyna, Pavel A. Krasutsky and Paulo J. Oliveira declare that they have no conflict of interest.

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8. Reference Section

- [1] S. Alakurtti, T. Mäkelä, S. Koskimies, J. Yli-Kauhaluoma, Pharmacological properties of the ubiquitous natural product betulin., *Eur. J. Pharm. Sci. Off. J. Eur. Fed. Pharm. Sci.* 29 (2006) 1–13.
- [2] J.J. An, K.J. Shi, W. Wei, F.Y. Hua, Y.L. Ci, Q. Jiang, et al., The ROS/JNK/ATF2 pathway mediates selenite-induced leukemia NB4 cell cycle arrest and apoptosis in vitro and in vivo., *Cell Death Dis.* 4 (2013) e973.
- [3] J.S. Armstrong, Mitochondrial medicine: pharmacological targeting of mitochondria in disease., *Br. J. Pharmacol.* 151 (2007) 1154–1165.
- [4] C.P. Baines, R.A. Kaiser, N.H. Purcell, N.S. Blair, H. Osinska, M.A. Hambleton, et al., Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death., *Nature.* 434 (2005) 658–62.
- [5] I.A. Barbosa, N.G. Machado, A.J. Skildum, P.M. Scott, P.J. Oliveira, Mitochondrial remodeling in cancer metabolism and survival: Potential for new therapies, *Biochim. Biophys. Acta - Rev. Cancer.* 1826 (2012) 238–254.
- [6] P. Bernardi, The mitochondrial permeability transition pore: a mystery solved?, *Front. Physiol.* 4 (2013) 95.
- [7] T.C. Bernardo, T. Cunha-Oliveira, T.L. Serafim, J. Holy, D. Krasutsky, O. Kolomitsyna, et al., Dimethylaminopyridine derivatives of lupane triterpenoids cause mitochondrial disruption and induce the permeability transition., *Bioorg. Med. Chem.* 21 (2013) 7239–7249.
- [8] K.M. Broekemeier, M.E. Dempsey, D.R. Pfeiffer, Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria., *J. Biol. Chem.* 264 (1989) 7826–7830.

- [9] B. CHANCE, G.R. WILLIAMS, G. HOLLUNGER, Inhibition of electron and energy transfer in mitochondria. III. Spectroscopic and respiratory effects of uncoupling agents., *J. Biol. Chem.* 238 (1963) 439–44.
- [10] D.T. Chao, S.J. Korsmeyer, BCL-2 family: regulators of cell death., *Annu. Rev. Immunol.* 16 (1998) 395–419.
- [11] C. V Dang, Glutaminolysis: supplying carbon or nitrogen or both for cancer cells?, *Cell Cycle.* 9 (2010) 3884–6.
- [12] J.A. Dykens, Y. Will, The significance of mitochondrial toxicity testing in drug development., *Drug Discov. Today.* 12 (2007) 777–85.
- [13] G. Filomeni, S. Cardaci, A.M. Da Costa Ferreira, G. Rotilio, M.R. Ciriolo, Metabolic oxidative stress elicited by the copper(II) complex [Cu(isaepy)₂] triggers apoptosis in SH-SY5Y cells through the induction of the AMP-activated protein kinase/p38MAPK/p53 signalling axis: evidence for a combined use with 3-bromopyruvate in neur, *Biochem. J.* 437 (2011) 443–53.
- [14] S. Fulda, Betulinic acid: a natural product with anticancer activity., *Mol. Nutr. Food Res.* 53 (2009) 140–146.
- [15] S. Fulda, G. Kroemer, Targeting mitochondrial apoptosis by betulinic acid in human cancers., *Drug Discov. Today.* 14 (2009) 885–890.
- [16] S. Fulda, C. Scaffidi, S.A. Susin, P.H. Kramer, G. Kroemer, M.E. Peter, et al., Activation of mitochondria and release of mitochondrial apoptogenic factors by betulinic acid, *J. Biol. Chem.* 273 (1998) 33942–33948.
- [17] T. Galgon, W. Wohlrab, B. Dräger, Betulinic acid induces apoptosis in skin cancer cells and differentiation in normal human keratinocytes., *Exp. Dermatol.* 14 (2005) 736–743.
- [18] A. Ganguly, H. Yang, F. Cabral, Class III β -tubulin counteracts the ability of paclitaxel to inhibit cell migration., *Oncotarget.* 2 (2011) 368–77.
- [19] V. Gogvadze, B. Zhivotovsky, S. Orrenius, The Warburg effect and mitochondrial stability in cancer cells., *Mol. Aspects Med.* 31 (2010) 60–74.
- [20] A.G. Gornall, C.J. Bardawill, M.M. David, Determination of serum proteins by means of the biuret reaction., *J. Biol. Chem.* 177 (1949) 751–766.
- [21] J. Holy, O. Kolomitsyna, D. Krasutsky, P.J. Oliveira, E. Perkins, P.A. Krasutsky, Dimethylaminopyridine derivatives of lupane triterpenoids are potent disruptors of mitochondrial structure and function., *Bioorg. Med. Chem.* 18 (2010) 6080–6088.
- [22] L. Huang, C.H. Chen, Molecular targets of anti-HIV-1 triterpenes., *Curr. Drug Targets Infect. Disord.* 2 (2002) 33–36.
- [23] N. Kamo, M. Muratsugu, R. Hongoh, Y. Kobatake, Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state., *J. Membr. Biol.* 49 (1979) 105–121.
- [24] K.W. Kinnally, P.M. Peixoto, S.-Y. Ryu, L.M. Dejean, Is mPTP the gatekeeper for necrosis, apoptosis, or both?, *Biochim. Biophys. Acta.* 1813 (2011) 616–22.

- [25] P.A. Krasutsky, Birch bark research and development., *Nat. Prod. Rep.* 23 (2006) 919–942.
- [26] N.J. Kruger, The Bradford Method for Protein Quantitation, in: J.M. Walker (Ed.), *Protein Protoc. Handb.*, Humana Press, 2002: pp. 15–21.
- [27] R.-J. Li, X. Ying, Y. Zhang, R.-J. Ju, X.-X. Wang, H.-J. Yao, et al., All-trans retinoic acid stealth liposomes prevent the relapse of breast cancer arising from the cancer stem cells., *J. Control. Release.* 149 (2011) 281–291.
- [28] L.D. Marroquin, J. Hynes, J.A. Dykens, J.D. Jamieson, Y. Will, Circumventing the Crabtree effect: replacing media glucose with galactose increases susceptibility of HepG2 cells to mitochondrial toxicants., *Toxicol. Sci.* 97 (2007) 539–47.
- [29] A.J.M. Moreno, T.L. Serafim, P.J. Oliveira, V.M.C. Madeira, Inhibition of mitochondrial bioenergetics by carbaryl is only evident for higher concentrations -- Relevance for carbaryl toxicity mechanisms., *Chemosphere.* 66 (2007) 404–11.
- [30] F.B. Mullauer, J.H. Kessler, J.P. Medema, Betulinic acid induces cytochrome c release and apoptosis in a Bax/Bak-independent, permeability transition pore dependent fashion., *Apoptosis an Int. J. Program. Cell Death.* 14 (2009) 191–202.
- [31] M.P. Murphy, Selective targeting of bioactive compounds to mitochondria., *Trends Biotechnol.* 15 (1997) 326–30.
- [32] B. Nawrocki Raby, M. Polette, C. Gilles, C. Clavel, K. Strumane, M. Matos, et al., Quantitative cell dispersion analysis: new test to measure tumor cell aggressiveness, *Int J Cancer.* 93 (2001) 644–652.
- [33] R.M. Neve, K. Chin, J. Fridlyand, J. Yeh, F.L. Baehner, T. Fevr, et al., A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes., *Cancer Cell.* 10 (2006) 515–527.
- [34] P.J. Oliveira, P.M. Coxito, A.P. Rolo, D.L. Santos, C.M. Palmeira, A.J. Moreno, Inhibitory effect of carvedilol in the high-conductance state of the mitochondrial permeability transition pore., *Eur. J. Pharmacol.* 412 (2001) 231–237.
- [35] G.A. Orr, P. Verdier-Pinard, H. McDaid, S.B. Horwitz, Mechanisms of Taxol resistance related to microtubules., *Oncogene.* 22 (2003) 7280–95.
- [36] N.I. Paylova, O. V Savinova, S.N. Nikolaeva, E.I. Boreko, O.B. Flekhter, Antiviral activity of betulin, betulinic and betulonic acids against some enveloped and non-enveloped viruses., *Fitoterapia.* 74 (2003) 489–492.
- [37] C. V Pereira, A.C. Moreira, S.P. Pereira, N.G. Machado, F.S. Carvalho, V.A. Sardão, et al., Investigating drug-induced mitochondrial toxicity: a biosensor to increase drug safety?, *Curr. Drug Saf.* 4 (2009) 34–54.
- [38] G.C. Pereira, A.F. Branco, J.A.C. Matos, S.L. Pereira, D. Parke, E.L. Perkins, et al., Mitochondrially targeted effects of berberine [Natural Yellow 18, 5,6-dihydro-9,10-dimethoxybenzo(g)-1,3-benzodioxolo(5,6-a) quinolizinium] on K1735-M2 mouse melanoma cells: comparison with direct effects on isolated mitochondrial fractions., *J. Pharmacol. Exp. Ther.* 323 (2007) 636–649.
- [39] E.C. Pietsch, S.M. Sykes, S.B. McMahon, M.E. Murphy, The p53 family and programmed cell death., *Oncogene.* 27 (2008) 6507–6521.

- [40] A. Rasola, P. Bernardi, Mitochondrial permeability transition in Ca(2+)-dependent apoptosis and necrosis., *Cell Calcium*. 50 (2011) 222–33.
- [41] T.L. Serafim, J. a C. Matos, V. a Sardão, G.C. Pereira, A.F. Branco, S.L. Pereira, et al., Sanguinarine cytotoxicity on mouse melanoma K1735-M2 cells--nuclear vs. mitochondrial effects., *Biochem. Pharmacol.* 76 (2008) 1459–75.
- [42] T.L. Serafim, P.J. Oliveira, V.A. Sardao, E. Perkins, D. Parke, J. Holy, Different concentrations of berberine result in distinct cellular localization patterns and cell cycle effects in a melanoma cell line., *Cancer Chemother. Pharmacol.* 61 (2008) 1007–1018.
- [43] P. Skehan, R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, et al., New colorimetric cytotoxicity assay for anticancer-drug screening., *J. Natl. Cancer Inst.* 82 (1990) 1107–1112.
- [44] J. Wang, C.-T. Yang, Y.-S. Kim, S.G. Sreerama, Q. Cao, Z.-B. Li, et al., ⁶⁴Cu-Labeled triphenylphosphonium and triphenylarsonium cations as highly tumor-selective imaging agents., *J. Med. Chem.* 50 (2007) 5057–69.
- [45] K. Yasukawa, M. Takido, T. Matsumoto, M. Takeuchi, S. Nakagawa, Sterol and triterpene derivatives from plants inhibit the effects of a tumor promoter, and sitosterol and betulinic acid inhibit tumor formation in mouse skin two-stage carcinogenesis., *Oncology*. 48 (1991) 72–76.
- [46] K. Yuan, N. Frolova, Y. Xie, D. Wang, L. Cook, Y.-J. Kwon, et al., Primary cilia are decreased in breast cancer: analysis of a collection of human breast cancer cell lines and tissues., *J. Histochem. Cytochem.* 58 (2010) 857–70.

9. Figures Legend

Fig.1- Chemical structure of the DMAP derivatives used in this study (1 mw 809.986; 2 mw 840.016; 3 mw 801.084; 4 mw 868.07; 5 mw 868.07; 6 mw 985.02 and 7 mw 985.02 in g/mol), compounds 4, 5 and 6 were described in Holy et al [21], while compounds 1, 2, 3 and 7 are new members of that same family. The additional groups added to betulinic acid structure are colored (or marked with asterisk in printed version).

Fig.2 – Effect of DMAP derivatives on tumor and non-tumor cell proliferation. A) normal BJ fibroblast cells, B) MCF-12A normal breast cells, C)MDA-MB-231 breast cancer cells, D) MCF-7 breast cancer cells and E) HS578T breast cancer cells. 1×10^4 cells/ml (breast cancer cells) and 2×10^4 cells/ml (non-tumor cells) were seeded in 48-well plates. Cells were grown in glucose medium and incubated during 1, 3 and 5 days with DMAP derivative concentrations ranging from 0.125 to $2 \mu\text{g/ml}$. The compounds tested are shown in Fig.1. Cell proliferation was determined by the SRB method and the results are expressed as percentage of control, which represents the density of cells without any drug in the respective time point, in order to normalize the growth rate between cell lines. F) the recovery of HS578T breast cancer cells after treatment with triterpenoids. HS578T cells were incubated with different triterpenoids at concentrations from 0.125 to $2 \mu\text{g/ml}$ for 6 hours, after which the cells were washed and fresh medium were added. The graphics represent the moment after washing. All experiments were performed in

five independent experiments and are represented as means \pm SEM, (*) $p < 0.05$ vs. control using two-way ANOVA followed by Bonferroni post-test.

Fig.3 – DMAP derivatives arrest HS578T cell cycle progression. Compounds (**1**, **2**, **3**, **4**, **5**, **6** and **7**) at 0.5 $\mu\text{g/ml}$ altered HS578T cell cycle after 24 and 48 hours of treatment. Flow cytometry assays showed that all the compounds lead to an increase of cell number in G1/G0 phase, while cells in S-phase decreased. Data are means \pm SEM of 5 separate experiments, (*) $p < 0.05$ vs. control, one-way ANOVA followed by Bonferroni correction for multiple comparisons.

Fig.4 – DMAP derivatives promote caspases-dependent cell death. HS578T breast cells were treated with compounds **2**, **3**, **5** and **7**, at 1 $\mu\text{g/ml}$ for 24 and 48 hours and A) the Live/Dead Kit was used to measure the number of live and dead cells by flow cytometry; B) caspase-like activity assay was used to estimate activation of caspase-9 and caspase-3 after 24 hours of treatment. Cell death-related proteins were measured by western blot, namely Pro-caspase-9 (C), cleaved caspase-3 (C), p53 (D) and Cyclophilin D (D). Data are means \pm SEM of 6 separate experiments, (*) $p < 0.05$ vs. control, one-way ANOVA followed by Dunnett multiple-comparisons post-test.

Fig.5 - Triterpenoids disturb mitochondrial polarization and morphology. Non-tumor BJ fibroblasts, MCF-12A and HS578Bst breast cells, and the breast cancer cell line HS578T were treated with vehicle or 0.25 $\mu\text{g/ml}$ of compounds **2**, **3**, **5** and **7** for 6 hours. A) Cells were labeled with TMRM, which accumulates in mitochondria due to the negative-inside $\Delta\Psi$, and were imaged by confocal microscopy in order to observe alterations regarding mitochondrial polarization. The images are representative of four independent preparations; scale bar represents 10 μm . B) Relative TMRM fluorescence standard deviation was measured in each cell line by using Image Processing and Analysis in Java (NIH). Data are means \pm SEM of 4 independent measurements, (*) $p < 0.05$ vs. control, one-way ANOVA followed by Bonferroni for multiple comparisons post-test.

Fig.6 - Effects of triterpenoids on breast cancer cells proliferation in glucose-free, galactose/glutamine media. Human breast cancer MDA-MB-231 cell line (A), MCF-7 breast cancer cells (B) and HS578T breast cancer cells (C) were cultured in galactose/glutamine medium, and incubated with different concentrations of triterpenoids, namely compounds **2**, **3**, **5** and **7** for 1, 3 and 5 days. Differences were found between toxicity in the high-glucose and galactose media (see Fig.2). Cell proliferation was accessed by SRB and the results are expressed as a percentage of the control. Data are means \pm SEM of 5 separate experiments, (*) $p < 0.05$ vs. control, two-way ANOVA followed by Bonferroni for multiple comparisons post-test.

Fig.7 - Effects of compound **2**, **3**, **5** and **7** on rat liver MPT pore induction. A) Representative recording of mitochondrial swelling assays, evaluated by measuring pseudo-absorbance at 540nm, as described in the Materials and Methods section. Swelling amplitude was calculated as the difference between the basal absorbance value and the absorbance read 600s after calcium addition (40 nmol/mg of protein). Mitochondria were energized with succinate in the presence of rotenone. Control (1), compound **5** (2), compound **7** (3), compound **2** (4), compound **3** (5), cyclosporin A (6; 1 μM). The compounds concentration varied from 0.07 $\mu\text{g/mg}$ to 1.33 $\mu\text{g/mg}$ of protein, which are the same as tested in cell cultures. B) Swelling amplitude data was calculated means \pm SEM of 6 different

preparations. (*) Significant difference compared to control ($p < 0.05$), (#) significant difference compared to cyclosporin A ($p < 0.05$), one-way ANOVA and Bonferroni for multiple comparisons post-test.

ACCEPTED MANUSCRIPT

Fig.1

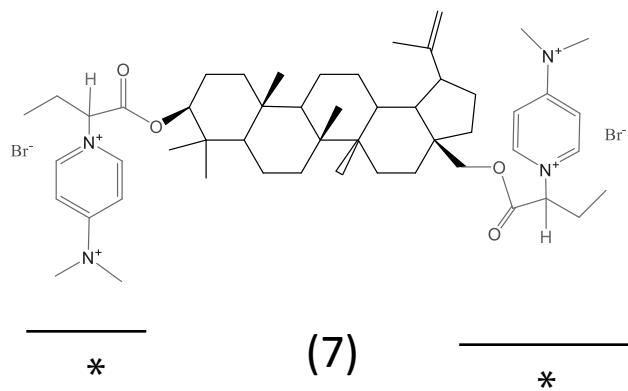
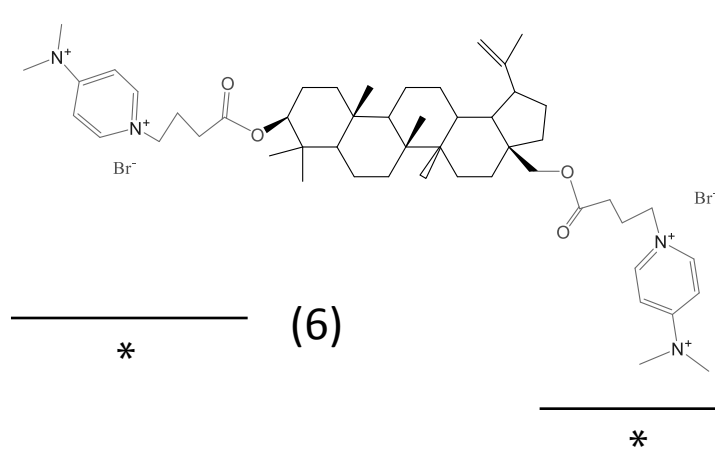
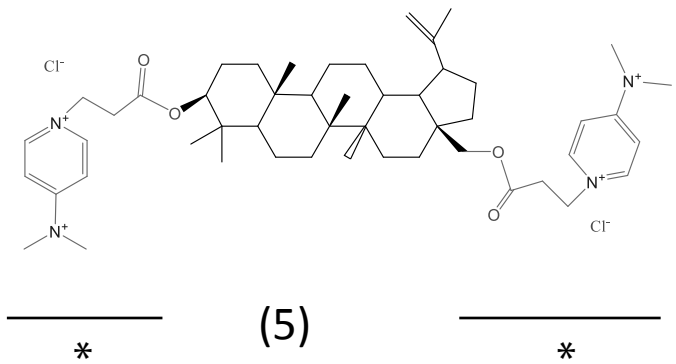
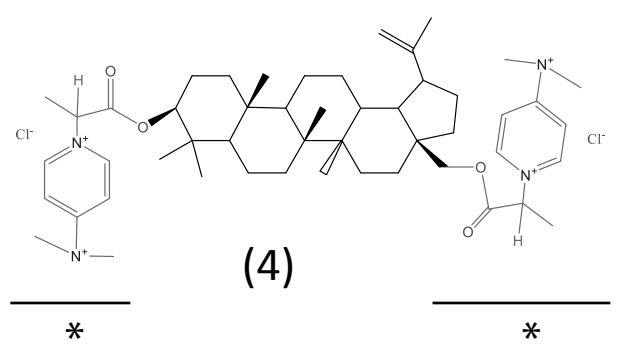
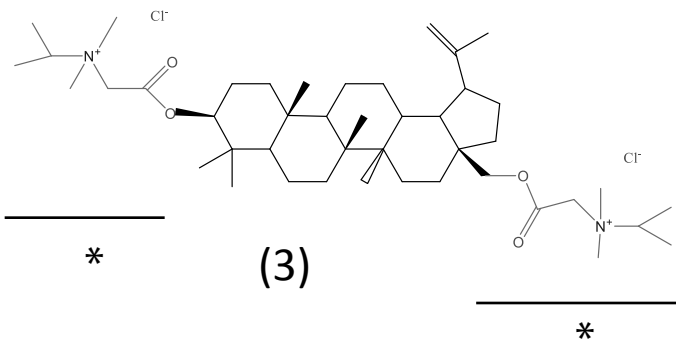
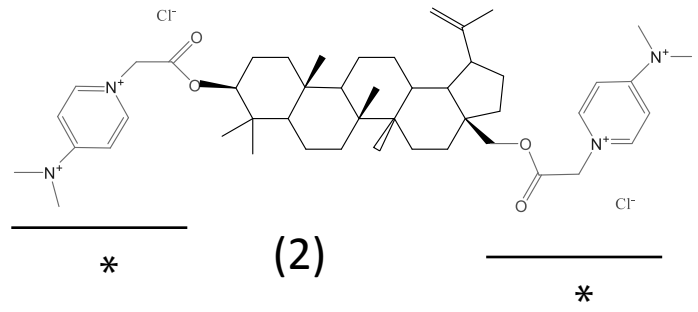
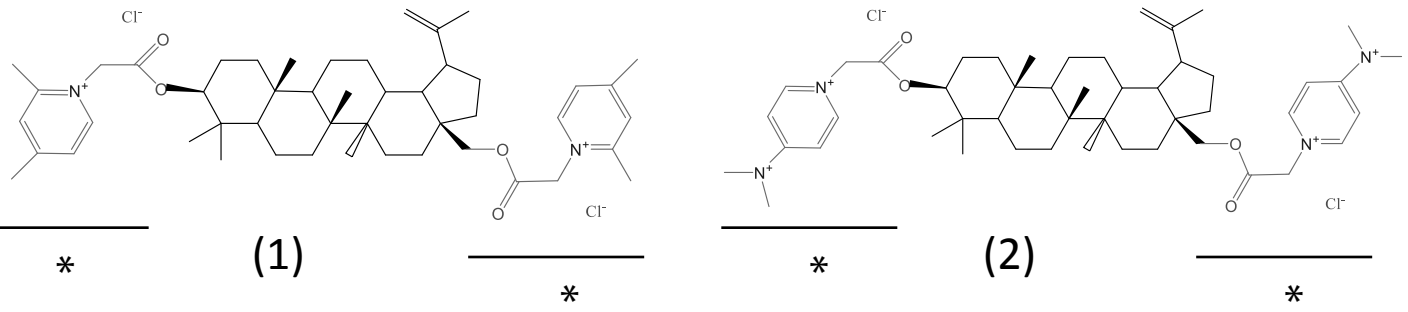


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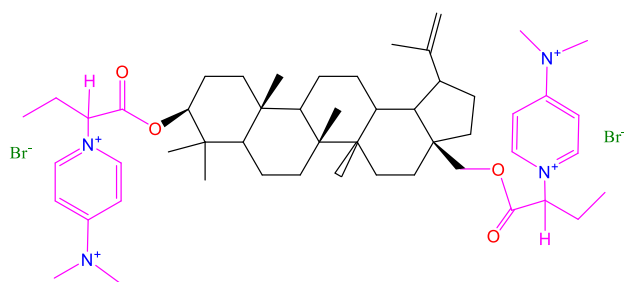
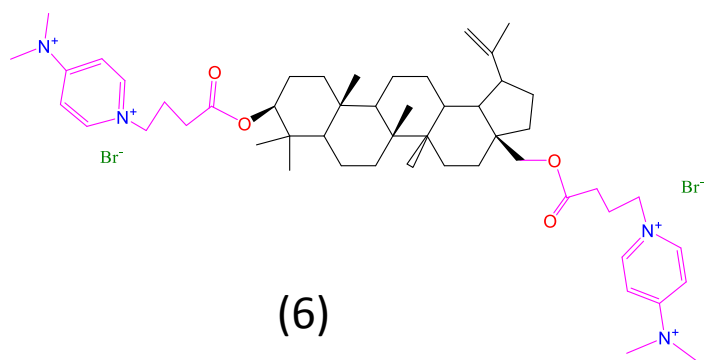
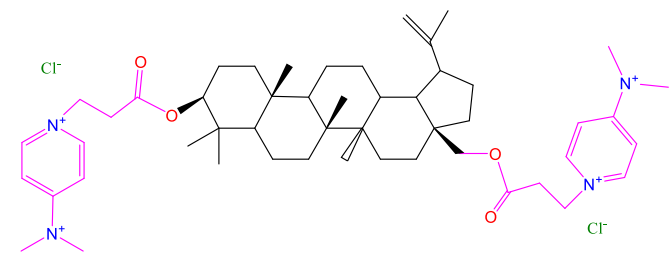
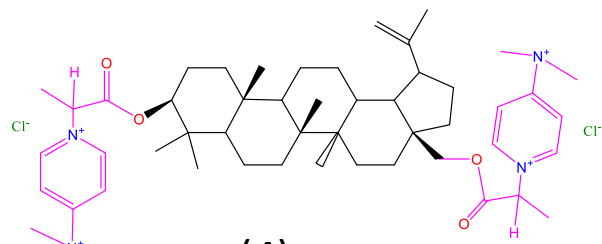
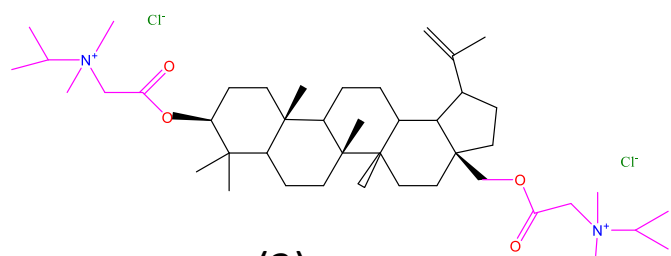
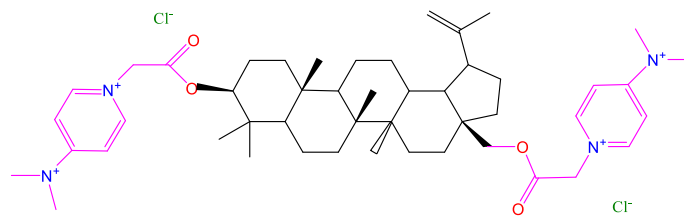
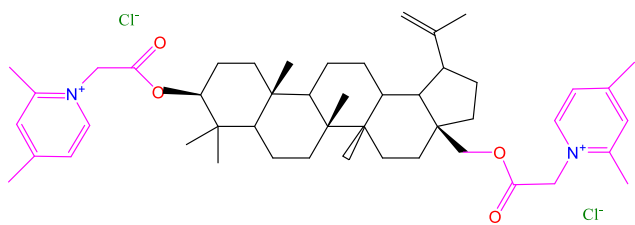


Fig.2A

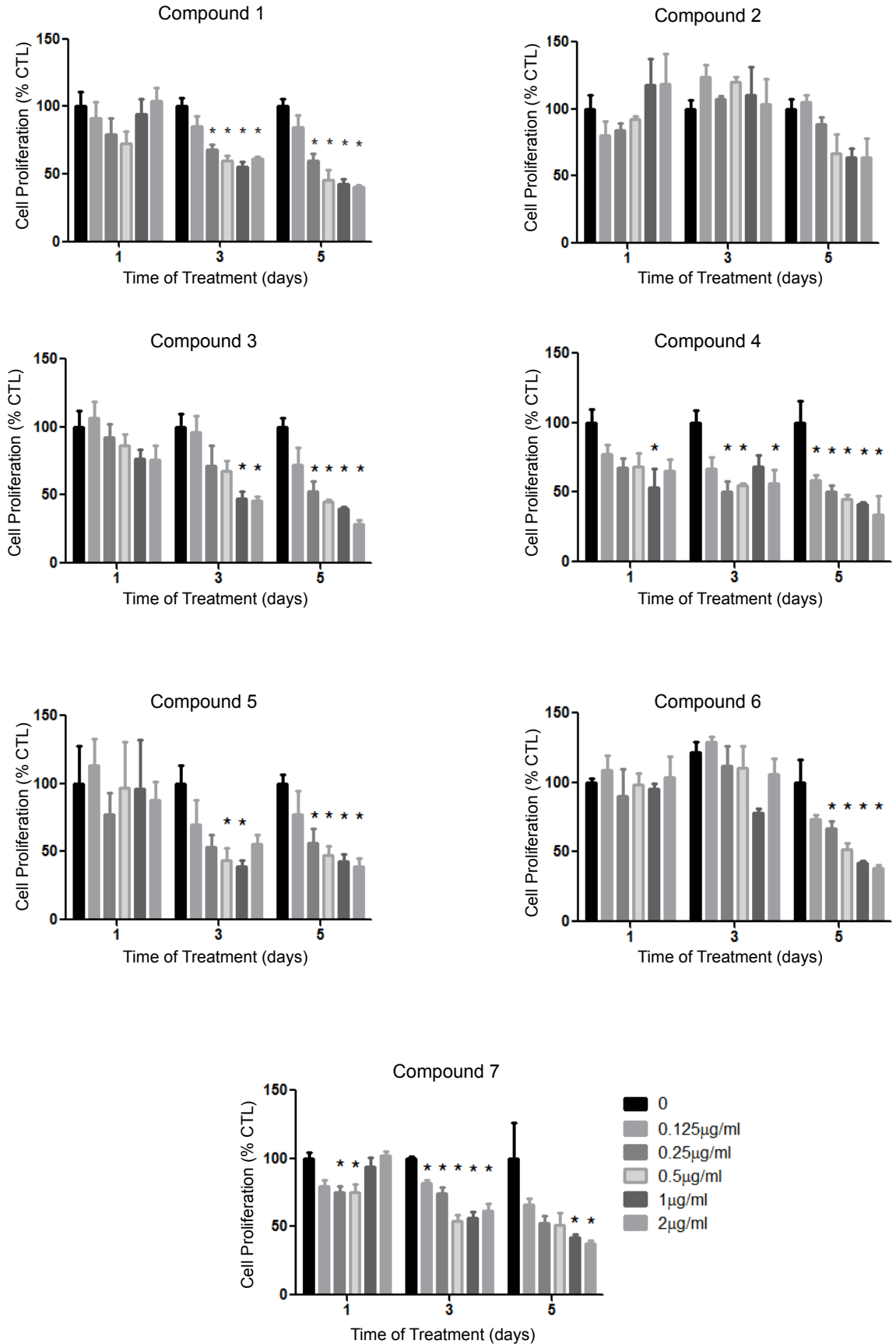


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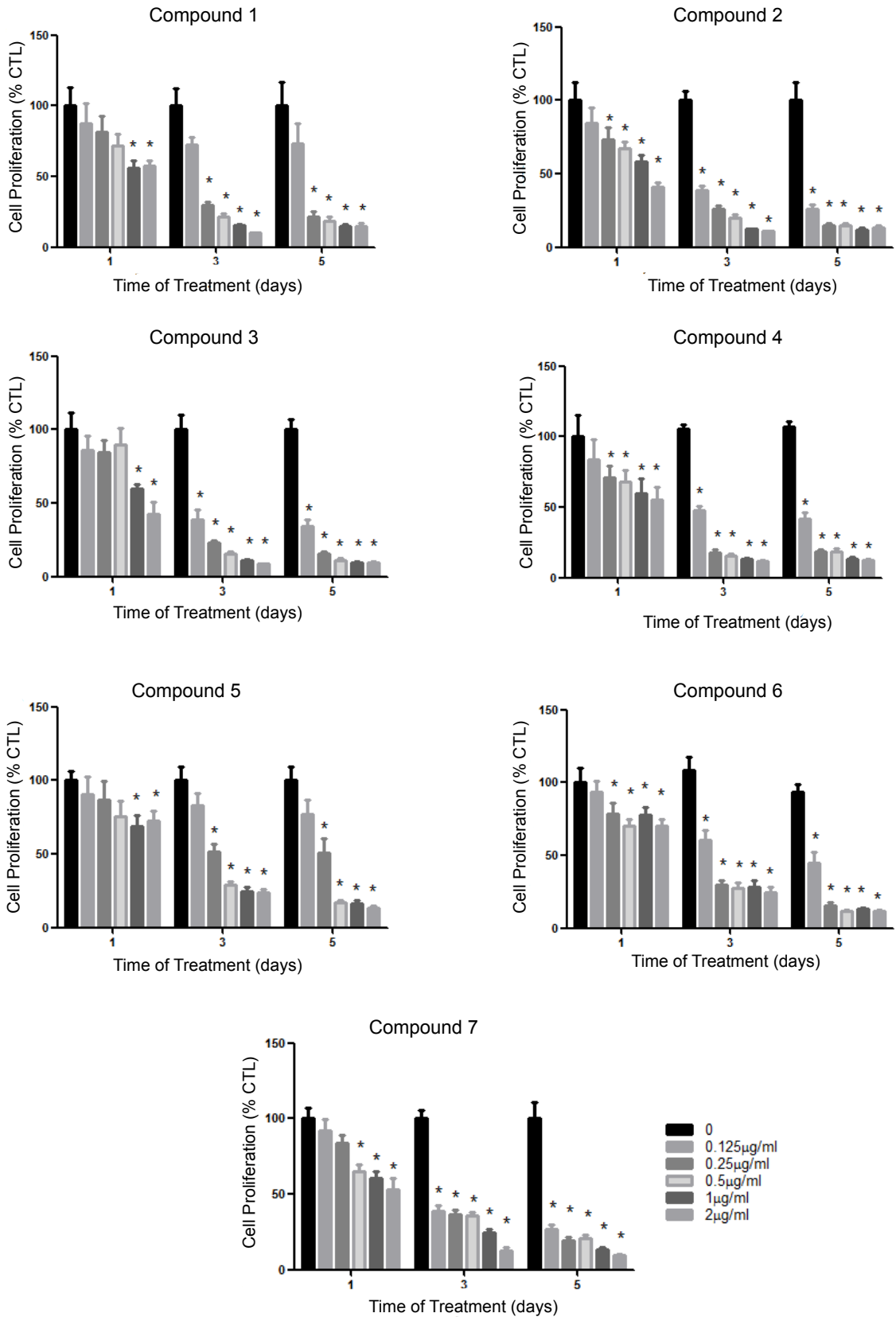


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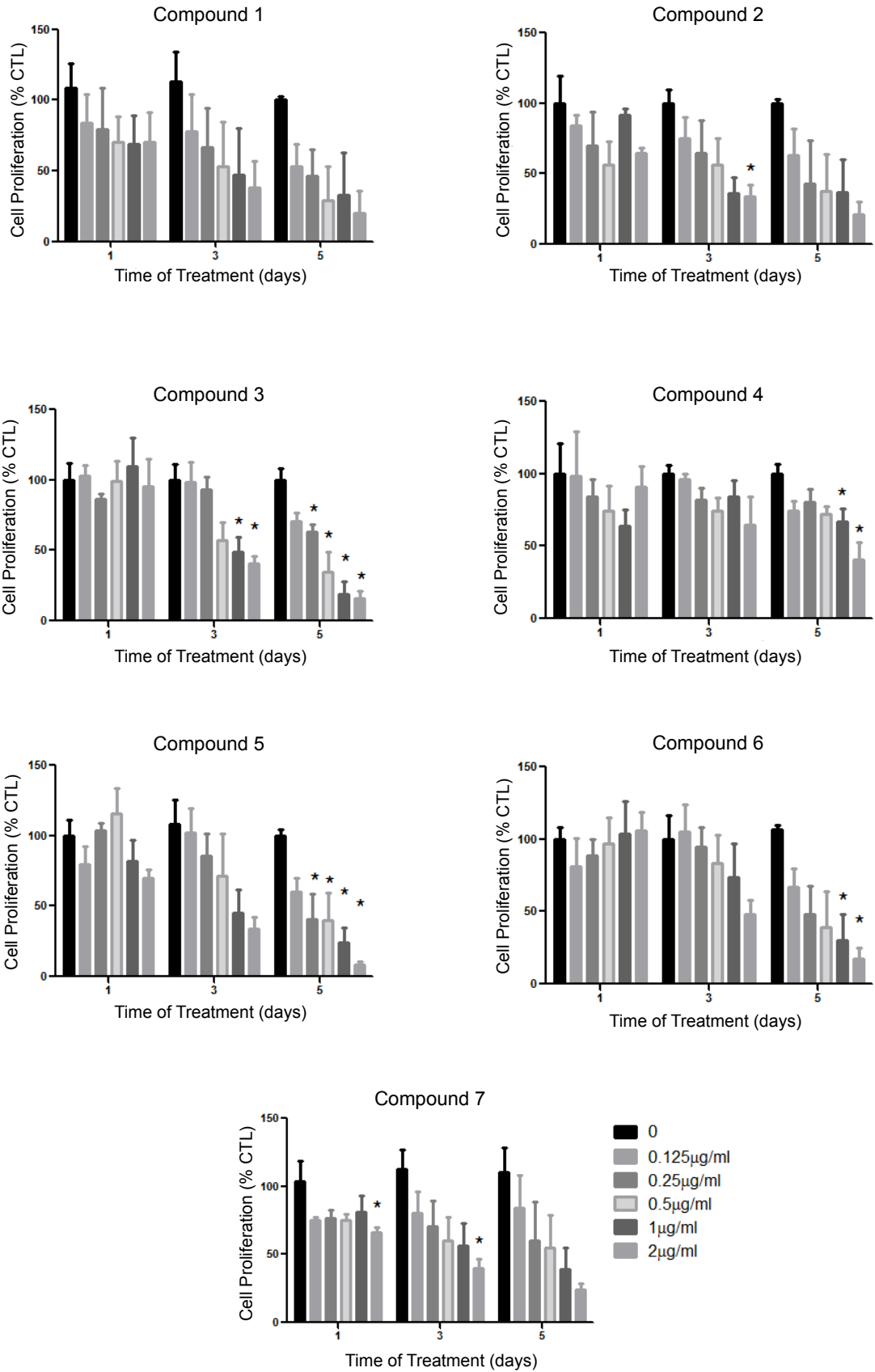


Fig.2D

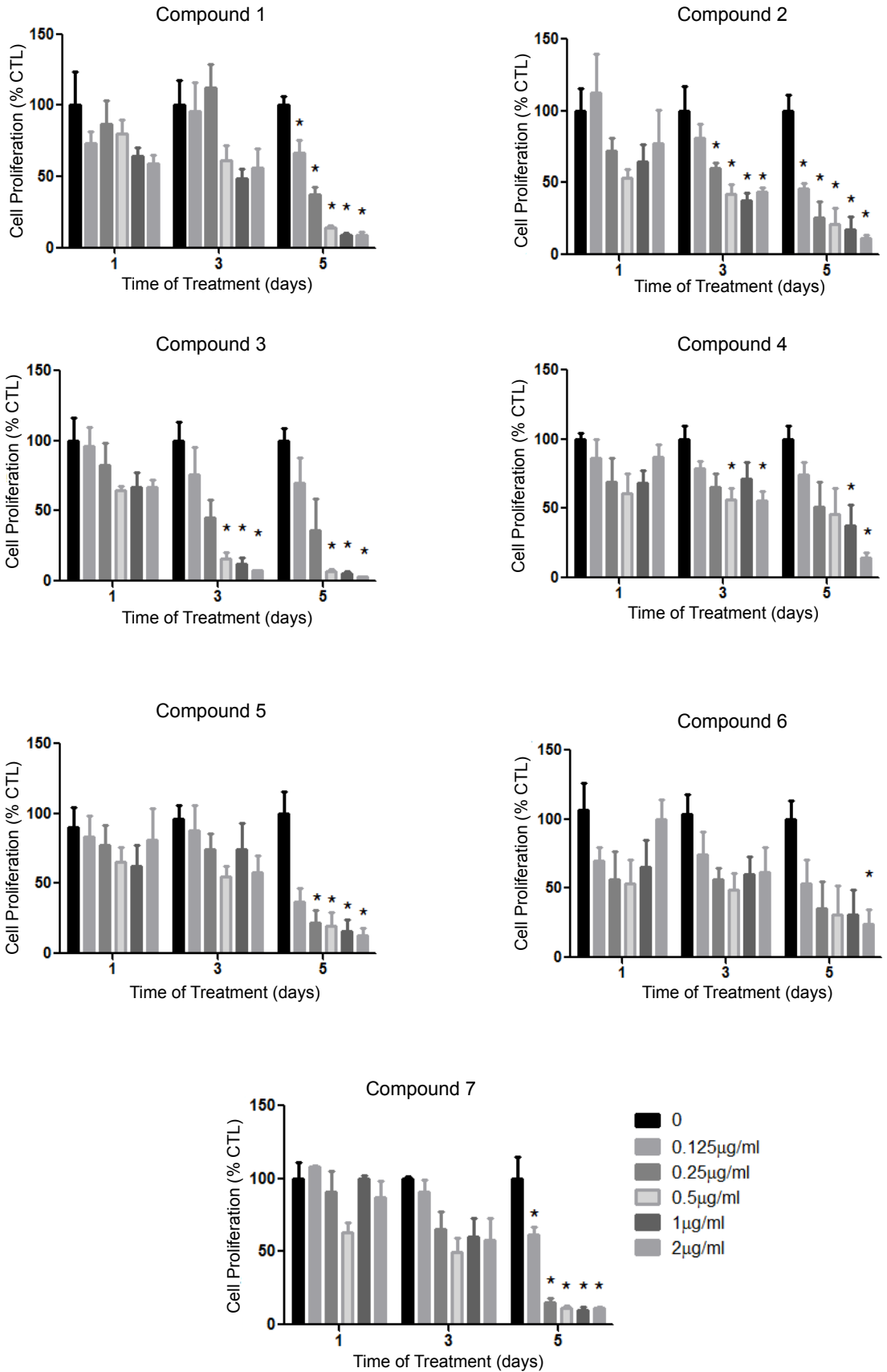


Fig.2E

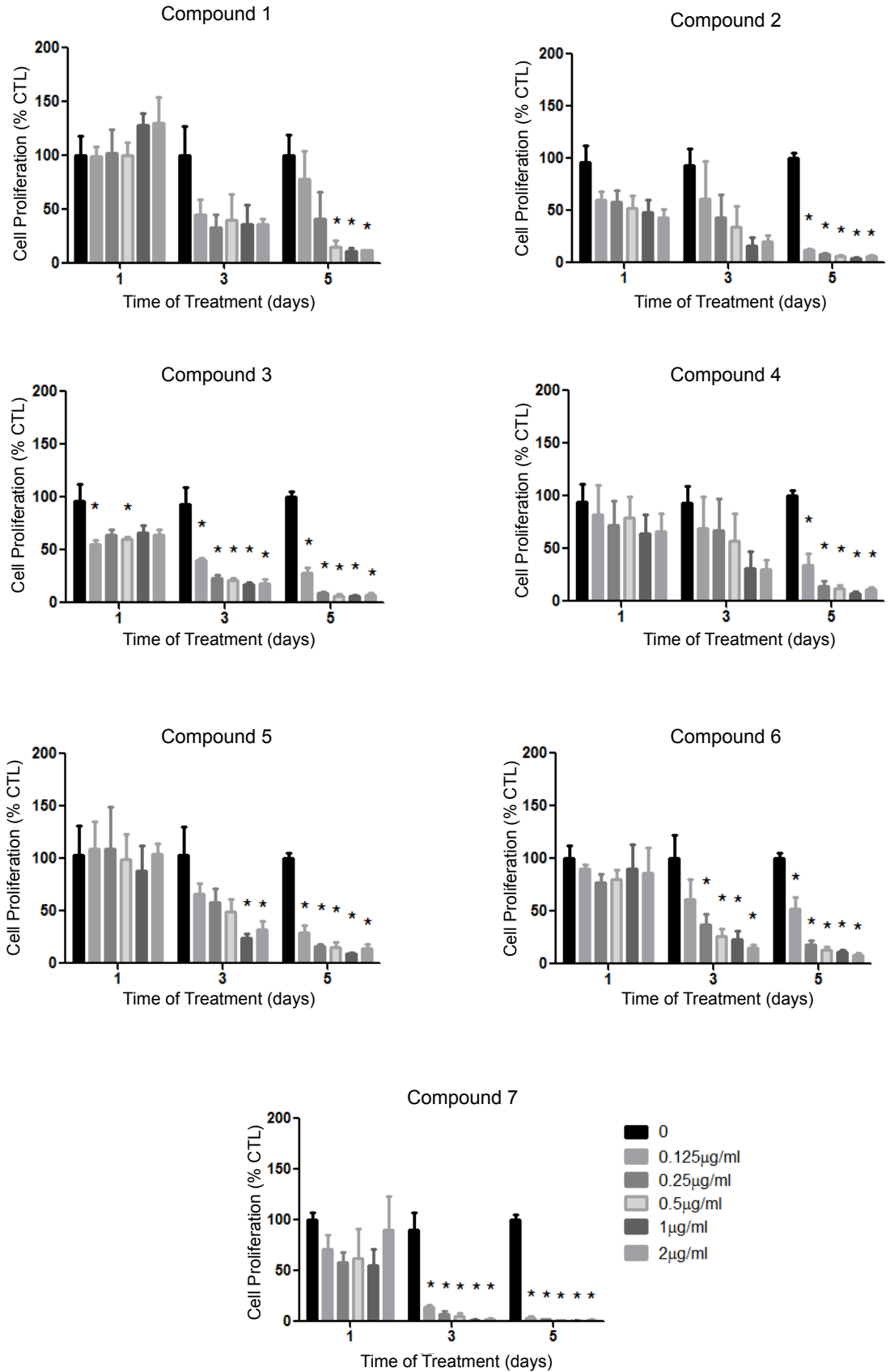


Fig.2F

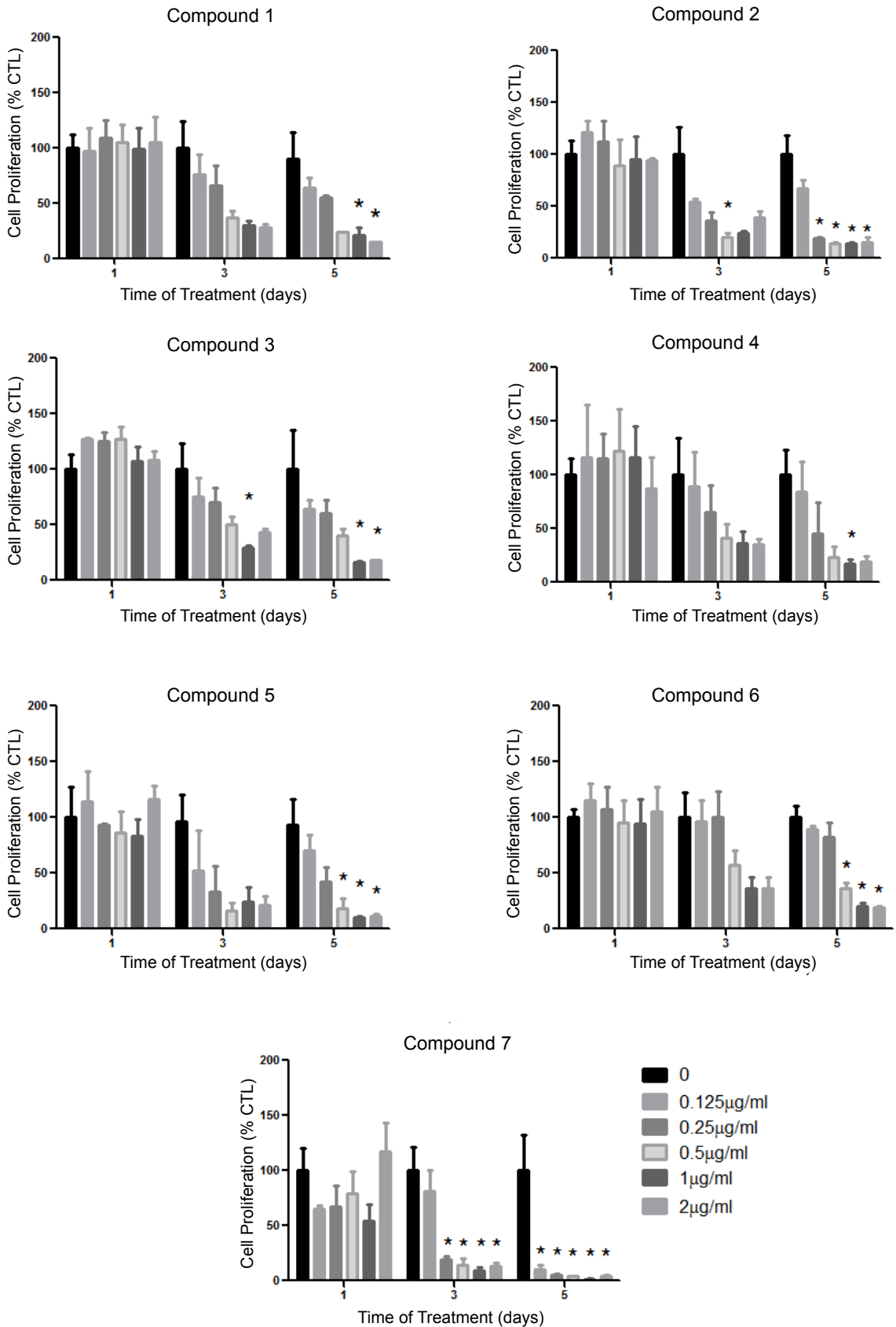
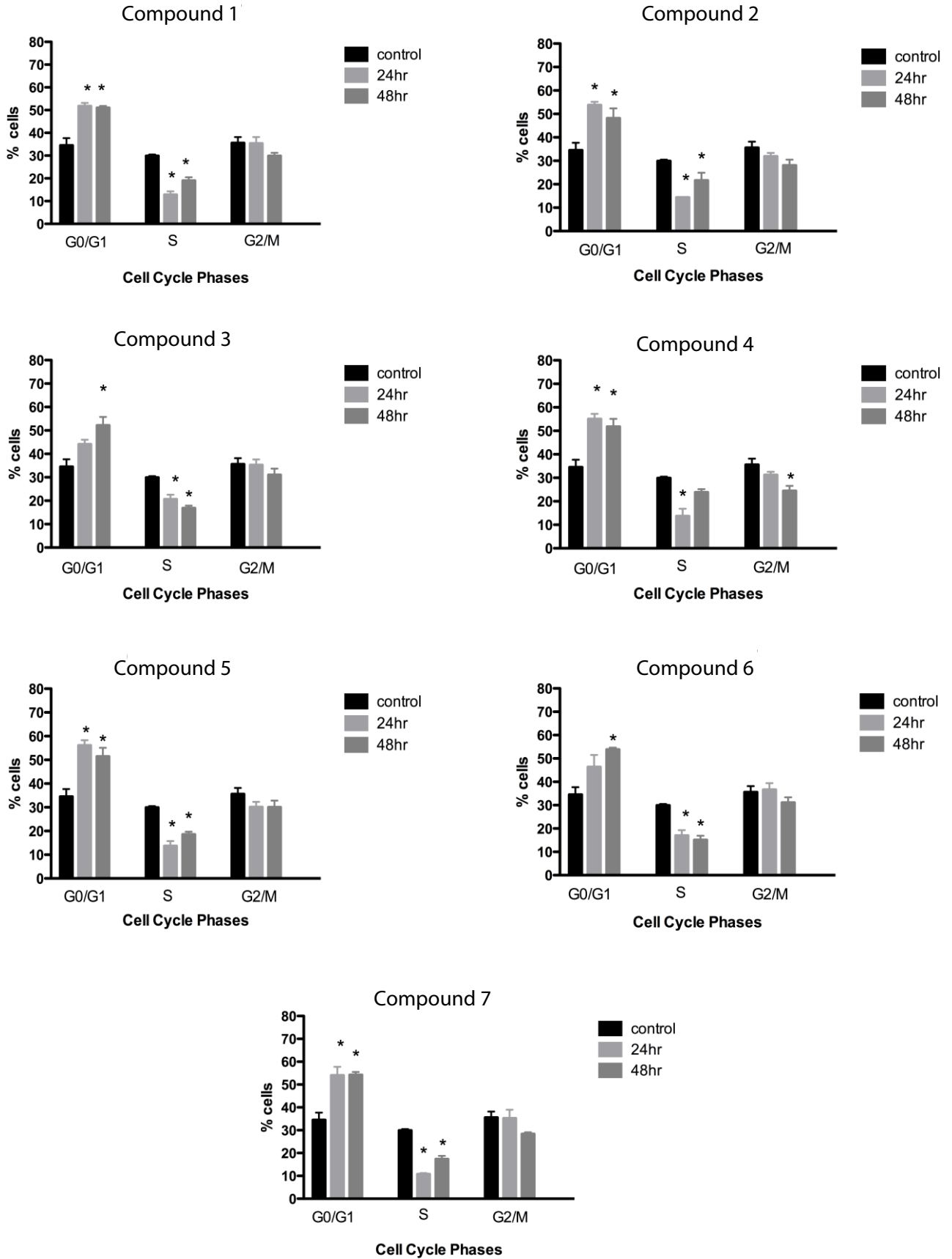
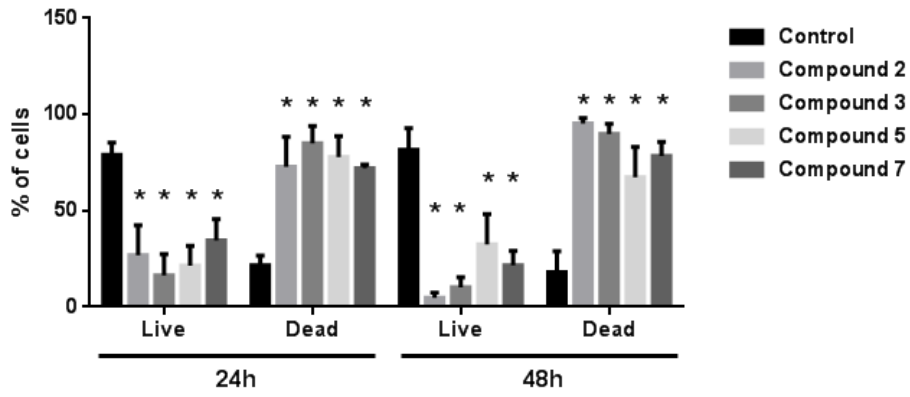


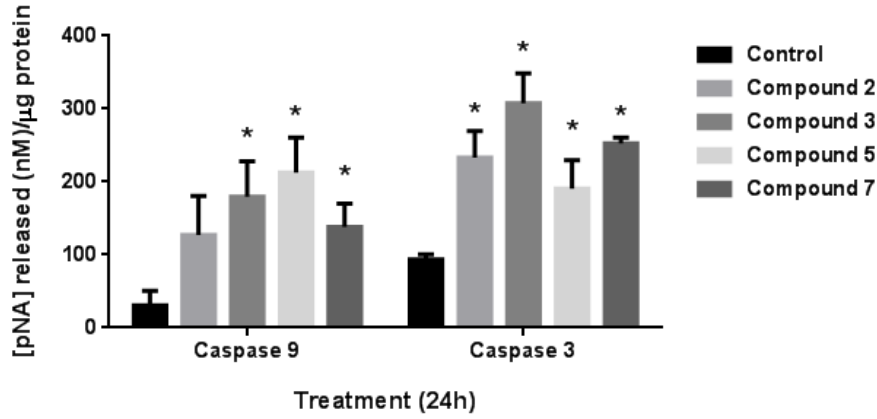
Fig.3



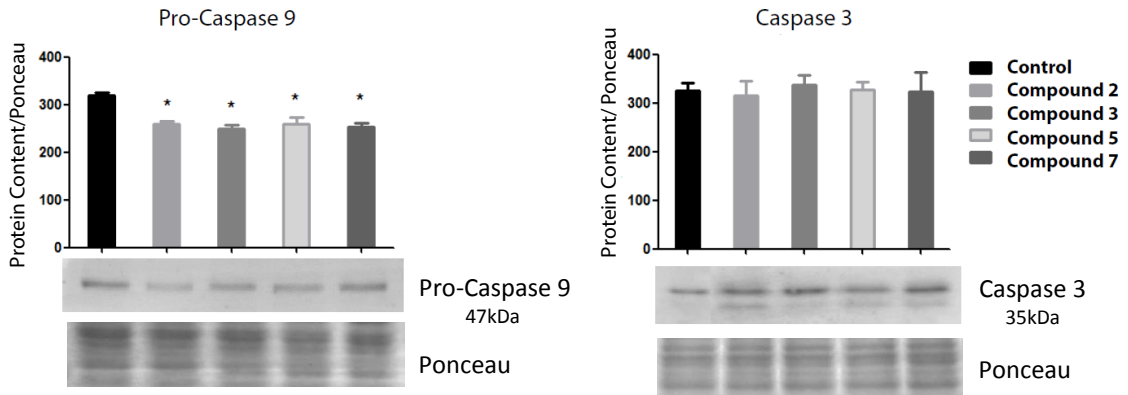
(A)



(B)



(C)



(D)

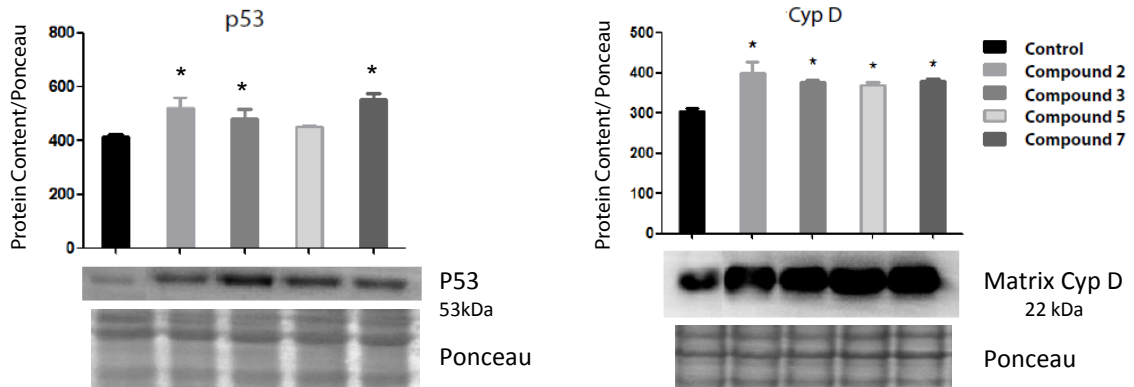


Fig.5A

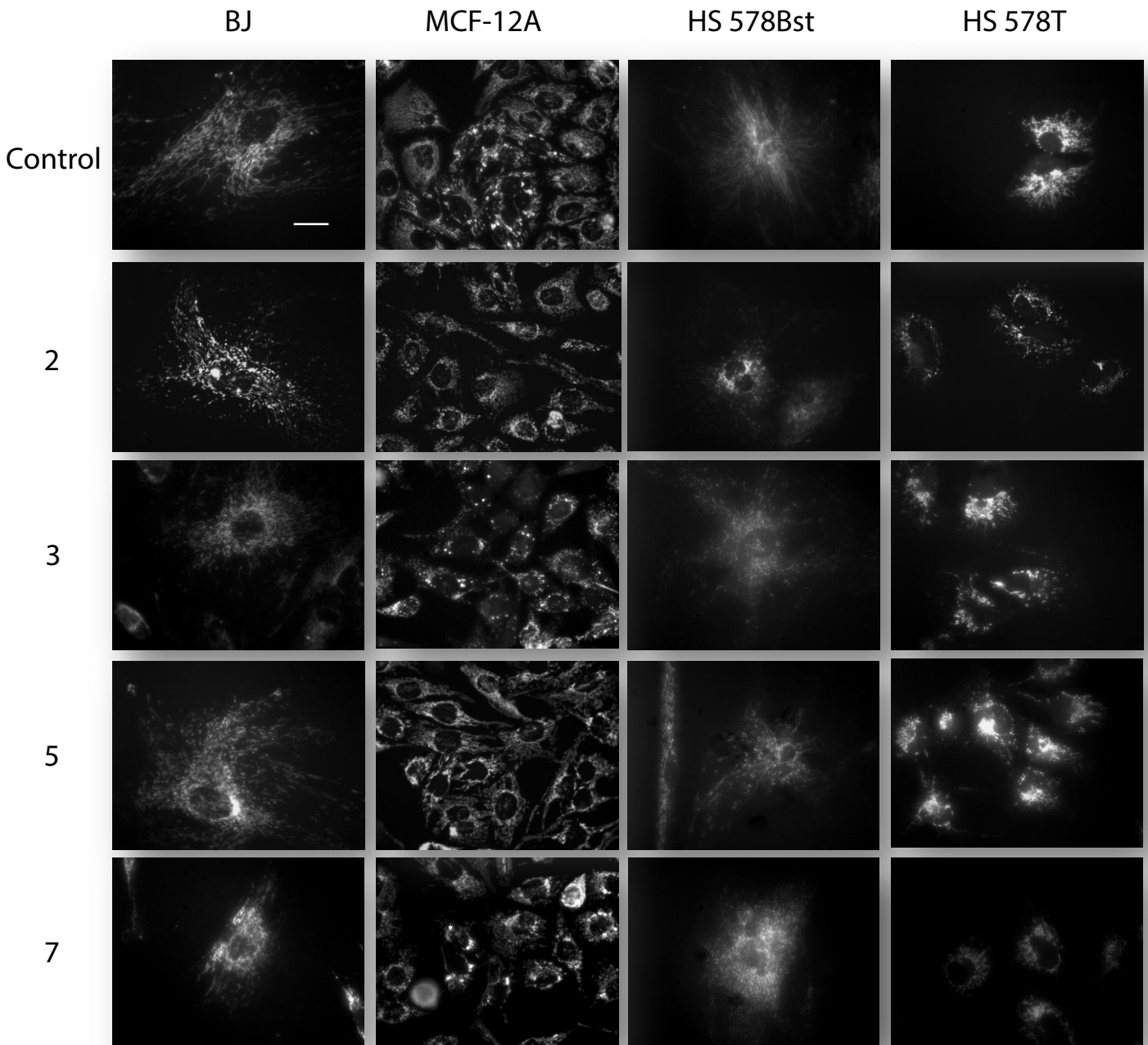
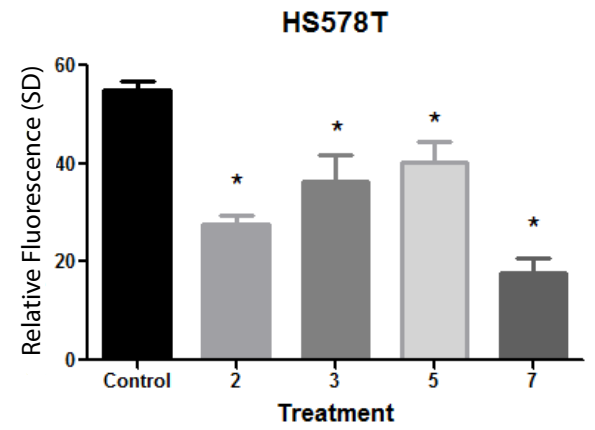
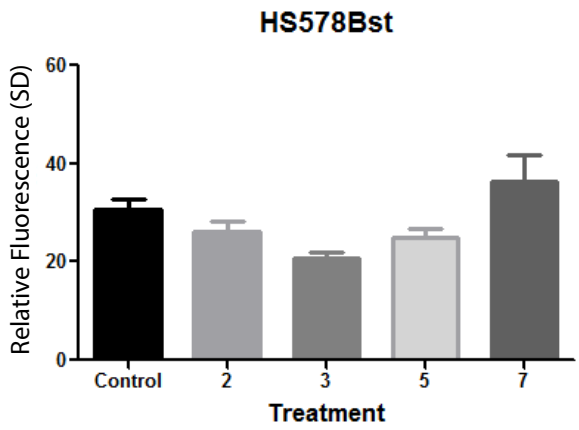
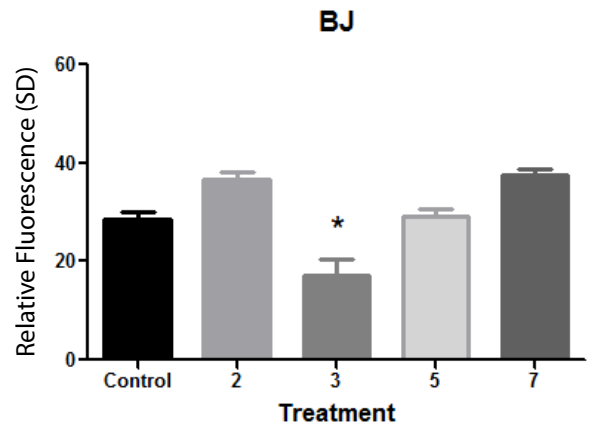
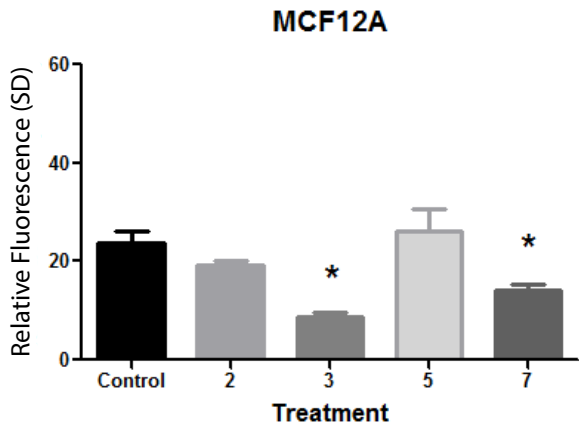
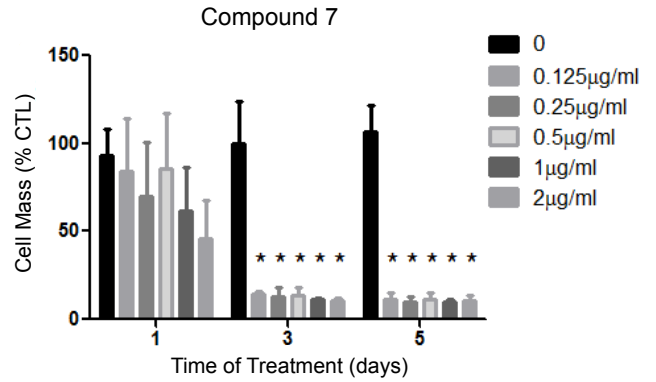
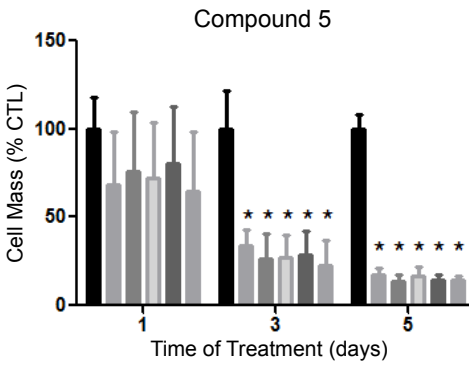
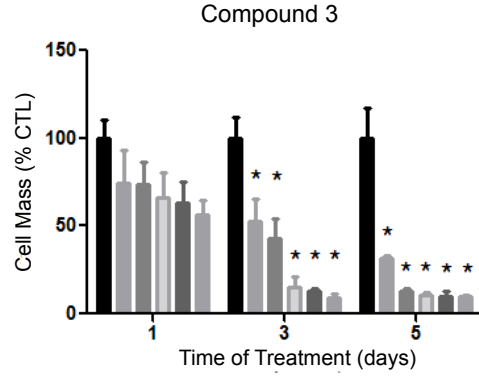
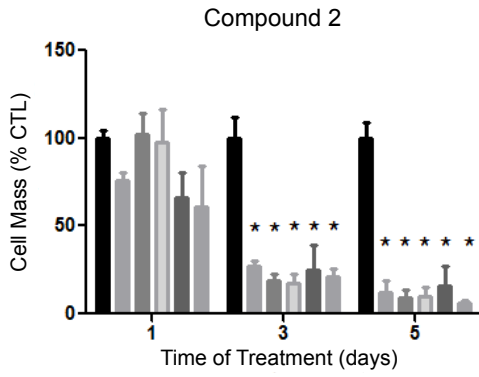


Fig.5B



(A)



(B)

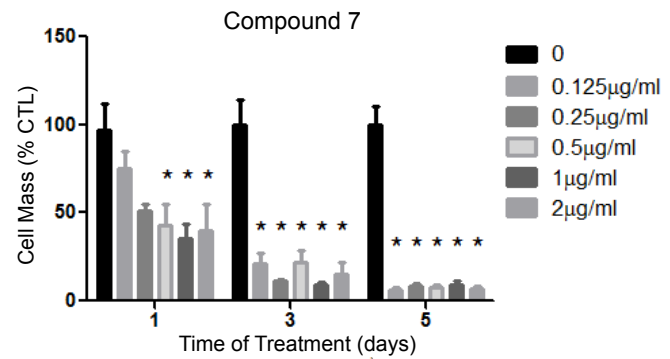
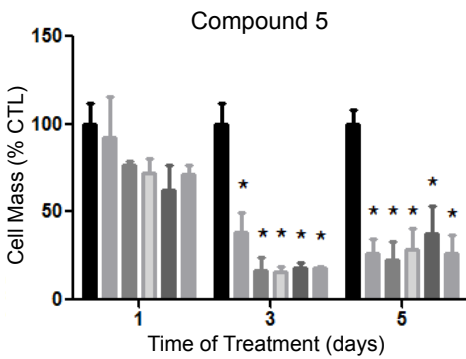
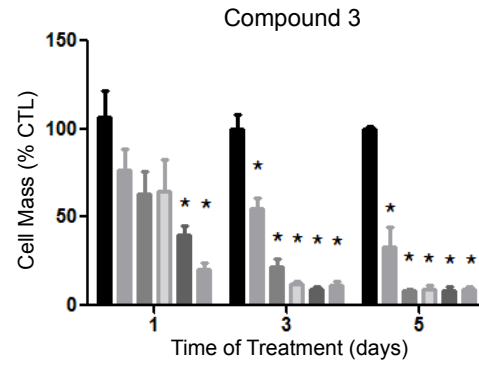
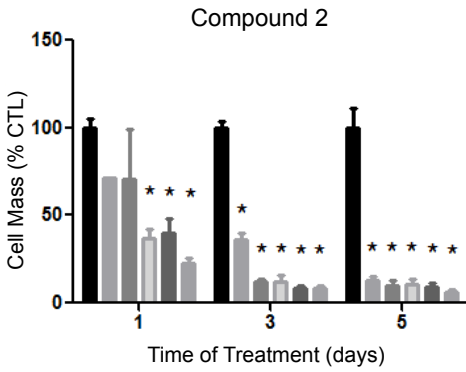
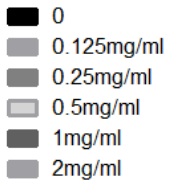
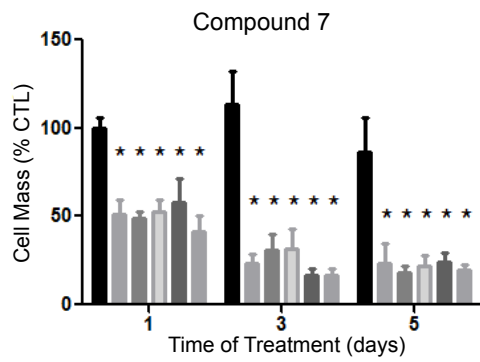
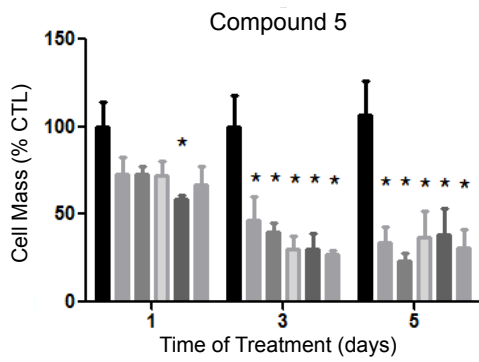
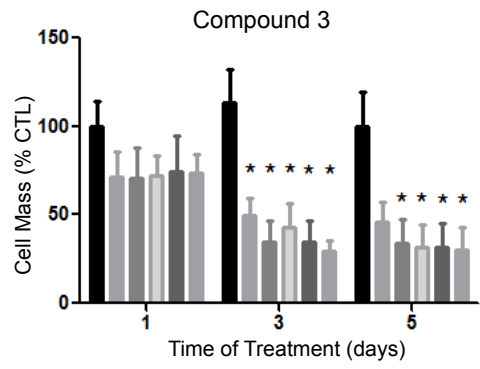
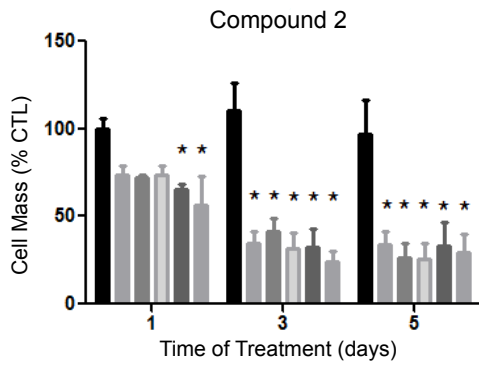
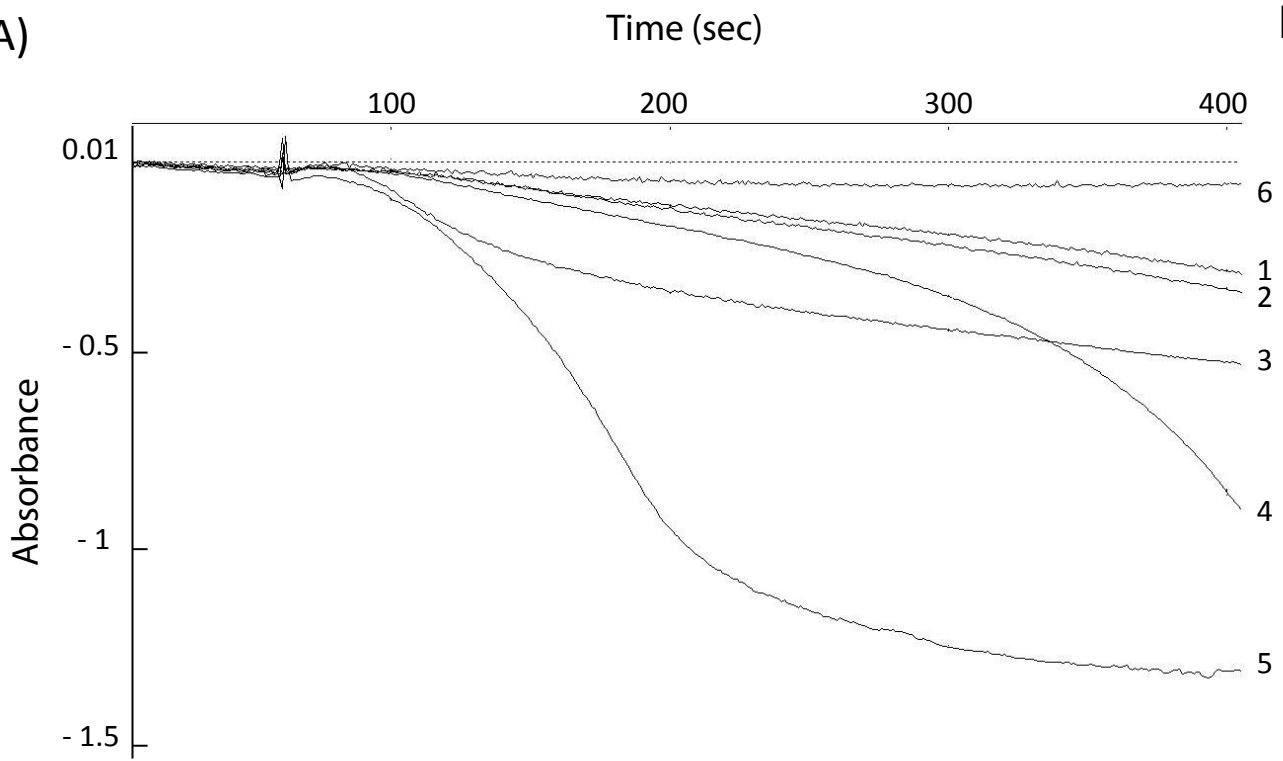


Fig.6C



(A)



(B)

