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

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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.

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.[24] Triterpene molecules also have other clinically relevant properties,[26] 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.[24]

Betulin and betulinic acid have a wide variety of potentially useful medical applications, including anti-inflammatory and anti-cancer,[44] anti-bacterial[1] and anti-viral properties.[21,35] Regarding anti-cancer activity, Galgon et al.[16] showed that betulinic acid caused apoptosis in melanoma cells, later showing this activity to be specific for tumors derived from neuroectoderm.[29] Betulinic acid exhibits anti-cancer activity in many other malignancies as well, including leukemia, prostate, ovarian, breast, lung, and colon cancer.[13] Importantly, betulinic acid appears to display some degree of selectivity towards tumor cells, minimally affecting normal cells.[15]

Betulinic acid triggers apoptosis in cancer cells by triggering mitochondrial outer membrane permeabilization,[14] which is an effective approach since cancer cells frequently develop defective apoptotic signaling, thus becoming resistant to traditional

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; CsA, cyclosporin A; DMSO, dimethylsulfoxide; 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.

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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.[24] 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.[20,7] 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.[20] 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. Materials 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.[20] 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 (IV), nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) and mass spectrometry (MS), as described in Holy et al.[20] The compounds **4**, **5** and **6** correspond to compounds **4**, **11** and **12** respectively in the previous work by Holy et al.[20] 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 (0.11 g/L), sodium bicarbonate (1.8 g/L) and 10% fetal bovine serum (FBS) and 1% of antibiotic penicillin–streptomycin 100× solution in 5% CO_2 atmosphere at 37 °C. Cells were also cultured in galactose/glutamine medium, prepared from glucose-free DMEM (D5030) supplemented with galactose (1.8 g/L), L-glutamine (0.584 g/L), sodium pyruvate(0.11 g/L), sodium bicarbonate (1.8 g/L), 10% FBS and 1% of antibiotic penicillin–streptomycin 100× solution in 5% CO_2 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.[37] Compounds were added to cells in a 48-well plate 24 h after cell seeding (20,000 cells/mL). Vehicle controls were also performed. Cells were fixed and total cell mass was determined as previously described.[42]

2.5. Cell cycle analysis by flow cytometry

Cell cycle progression was analyzed by using flow cytometry.[41] 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, 4 mM KCl; 1.2 mM NaH_2PO_4, plus 0.1% Tween) samples were incubated at 37 °C in 0.5 mL PBS-T with 20 μg/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 μg/mL for 24 and 48 h, HS578T cells (initially seeded at 2.5×10^4 cells/mL in 6 well-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 μM ethidium homodimer and 0.1 μM calcein-AM; Invitrogen, Eugene, OR). A Becton-Dickenson FACSalibur flow cytometer was used to measure FL1 (calcein, green) and FL3 (ethidium homodimer, red) signals. To quantify red and green fluorescence, CellQuest software version 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 10 mM DTT) and frozen at −20 °C overnight. Protein quantification was determined by the Bradford method[25] using bovine serum albumin (BSA) as standard. To measure caspase 3 and 9-like activity, aliquots of cell extracts in amber 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