Dimethylaminopyridine derivatives of lupane triterpenoids cause mitochondrial disruption and induce the permeability transition

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Abstract

Triterpenoids are a large class of naturally occurring compounds, and some potentially interesting as anticancer agents have been found to target mitochondria. The objective of the present work was to investigate the mechanisms of mitochondrial toxicity induced by novel dimethylaminopyridine (DMAP) derivatives of pentacyclic triterpenes, which were previously shown to inhibit the growth of melanoma cells in vitro. MCF-7, HS 578T and BJ cell lines, as well as isolated hepatic mitochondria, were used to investigate direct mitochondrial effects. On isolated mitochondrial hepatic fractions, respiratory parameters, mitochondrial transmembrane electric potential, induction of the mitochondrial permeability transition (MPT) pore and ion transport-dependent osmotic swelling were measured. Our results indicate that the DMAP triterpenoid derivatives lead to fragmentation and depolarization of the mitochondrial network in situ, and to inhibition of uncoupled respiration, induction of the permeability transition pore and depolarization of isolated hepatic mitochondria. The results show that mitochondrial toxicity is an important component of the biological interaction of DMAP derivatives, which can explain the effects observed in cancer cells.

1. Introduction

Triterpenoids are naturally occurring compounds with ubiquitous distribution, and whose biological activity in mammalian cells includes antiviral, antifungal, anti-inflammatory and antitumor effects. Several triterpenoids appear to exert their effects via disruption of mitochondrial function, namely by inducing the generation of reactive oxygen species, disruption of redox status, calcium deregulation, decrease in mitochondrial potential and cytochrome c release. These deleterious effects on mitochondria may be interesting from a chemotherapeutic perspective, as they could be of use in promoting apoptosis in malignant cells. For example, the triterpenoid betulinic acid selectively promotes apoptosis of melanoma cancer cells and also exhibits cytotoxic activity in glioma, ovarian carcinoma and cervical carcinoma cell lines. However, the use of triterpenoids remains quite limited due to their low solubility, high cost and insolvency in promoting apoptosis in malignant cells. For example, the triterpenoid betulinic acid selectively promotes apoptosis of melanoma cancer cells and also exhibits cytotoxic activity in glioma, ovarian carcinoma and cervical carcinoma cell lines. However, the use of triterpenoids remains quite limited due to their low solubility, high cost and insolvency. Hence, the need of new synthetic derivatives that may overcome these limitations.

Our research group has previously shown that a number of dimethylaminopyridine (DMAP) derivatives of lupane triterpenoids inhibited human melanoma cell proliferation and the extent of proliferation inhibition was correlated with the strength of mitochondrial depolarization. However, the mechanisms behind the mitochondrial effects were not completely elucidated. Here, we investigate in detail the effects of the same compounds on isolated hepatic mitochondrial fractions, including...
induction of the mitochondrial permeability transition pore (MPTP), in order to explain the mitochondrial alterations observed in melanoma\(^2\) and breast cancer cells (this work).

2. Materials and methods

2.1. General chemicals

MitoTracker Red CMXRos was obtained from Molecular Probes (Invitrogen, Eugene, OR, USA); Sulforhodamine B (SRB) was obtained from Sigma (St Louis, MO, USA). All other reagents and chemical compounds used were of the greatest degree of purity commercially available. In the preparation of every solution, ultra-pure distilled water, filtered by the Milli Q from a Millipore system, was always used in order to minimize the contamination with metal ions.

2.2. Synthesis and preparation of the compounds

Birch bark lupane triterpenoids betulin and betulinic acid have been chosen as basic natural precursors for synthesis of DMAP derivatives of the pentacyclic triterpenes: 28-(4'-dimethylaminopyridinium-1'-acetoxy)-3β-hydroxylup-20(29)-ene chloride (1); betulin 30-[4-(dimethylamino)pyridinium-1'-yl]-3β,28-di[4'-(dimethylaminopyridinium-1-yl acetoxy)] tribromide (2); lup-20-(29)-ene-3β-[(4'-dimethylaminopyridiniumacetoxy)chloride (3); betulin 3β,28-di[4'-(dimethylaminopyridinium-1-yl-acetoxy)] bromide (4) and betulinic acid 28-(4'-dimethylaminopyridinium-1'-yl) bromide (5) (Fig. 1), which correspond, respectively, to compounds 7, 5, 10, 14 and 1 in our previous study.\(^2\) Betulin with 99% purity was isolated from the extract of outer birch bark of *Betula papyrifera*—the North American commercial birch tree—and betulinic acid was then synthesized from betulin. The compounds were prepared as stock solutions in dimethylsulfoxide (DMSO), as described previously.\(^2\)

2.3. Composition of solutions

Phosphate buffered saline solution (PBS): 132.0 mM NaCl, 4.0 mM KCl; 1.2 mM NaH\(_2\)PO\(_4\). PBS Tween (PBST): PBS with 0.1% Tween 20. Trypan blue was used as a 0.04% (w/v) solution in PBS.

2.4. Animal handling

Eight-10 week male Wistar–Han rats were housed in our accredited animal colony (Laboratory Research Center, Faculty of Medicine, University of Coimbra, Portugal) in type III-H cages (Techniplast, Italy) and maintained in specific environmental requirements: 22 °C, 45–65% humidity, 15–20 changes/h ventilation, 12 h artificial light/dark cycle, noise level <55 dB. Rats had free access to standard rodent food (4RF21 GLP certificate, Mucedola, Italy) and water (acidified 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.

2.5. Cell culture

MCF-7 (HTB-22, ECACC, United Kingdom) and Hs 578T (HTB-125, ATCC, Manassas, VA, USA), breast cancer cell lines, as well as normal BJ fibroblasts (CRL-2522, ATCC, Manassas, VA, USA), were cultured in monolayers in Dulbecco's modified Eagle's medium (DMEM), supplemented with 1.8 g/l sodium bicarbonate, 10% fetal bovine serum, and 1% of penicillin–streptomycin, in 75 cm\(^2\) tissue culture flasks at 37 °C in a humidified atmosphere of 5% CO\(_2\). Cells were fed every 2–3 days, and sub-cultured once they reached 70–80% of confluence. BJ fibroblasts were used between passage 10 and 25.

![Figure 1. DMAP compounds synthesized from betulin and betulinic acid.](image-url)
2.6. Epifluorescence microscopy

For detection of morphological alterations including chromatin condensation and polarized mitochondrial network morphology, cells were seeded in six-well plates containing glass coverslips (final volume of 2 ml/well at the same density described in for cell proliferation studies) and allowed to attach for 24 h. The human breast cancer cell lines and the normal fibroblast line were then treated with the test compounds for 48 h. Thirty minutes prior to the end of the incubation time, the cultures were incubated with MitoTracker Red CMXRos (7.3 nM) at 37 °C in the dark, washed with cold PBS, and fixed with ice cold absolute methanol overnight at −20 °C. The cells were then gently rinsed three times with PBST, for 5 min in the dark, at room temperature. Glass coverslips were removed from the wells and placed on glass slides with a drop of mounting medium. The images were obtained using a 63× objective in a Zeiss Axioskop 2 Plus microscope.

2.7. Isolation of rat hepatic mitochondria

Mitochondria were isolated from the livers of male Wistar rats by conventional differential centrifugation. Rats were killed by decapitation and the livers were harvested, minced and washed in ice-cold buffer medium containing 250 mM sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.2), 1 mM EGTA, and 0.1% lipid-free bovine serum albumin (BSA). Tissue fragments were quickly homogenized with a motor-driven Teflon Potter homogenizer in the presence of ice-cold isolation medium (7 g/50 ml). Hepatic homogenate was centrifuged at 800 g for 10 min (Sorvall RC6 centrifuge) at 4 °C and mitochondria were recovered from the supernatant by centrifugation at 10,000g for 10 min. The mitochondrial pellet was resuspended using a paintbrush and centrifuged twice at 10,000g for 10 min before obtaining a final mitochondrial suspension. EGTA and BSA were omitted from the final washing medium, which was adjusted to pH 7.2. Protein content was determined by the biuret method, using BSA as a standard.

2.8. Measurement of mitochondrial oxygen consumption

Oxygen consumption of isolated hepatic mitochondria was polarographically monitored with a Clark-type oxygen electrode connected to a suitable recorder in a 1 ml temperature-controlled, water-jacketed, and closed chamber with constant magnetic stirring. The reactions were carried out at 30 °C in 1 ml of standard respiratory medium with 1 mg of hepatic mitochondria. Mitochondrial respiratory medium comprised 130 mM sucrose, 50 mM KCl, 2.5 mM MgCl2, 5 mM KH2PO4, 0.1 mM EGTA and 5 mM HEPES (pH 7.2, 30 °C), and supplemented with 3 μM TPP+. Triterpenoid derivative compounds were added to mitochondria for 1 min, followed by 5 mM glutamate/2.5 mM malate or 5 mM succinate plus 3 μM rotenone. In order to initiate state 3 respiration, ADP (125 nmol/mg protein) was added. Addition of valinomycin (0.2 μg) at the end of all experiments led to a complete collapse of ΔΨm and allowed evaluating if test compounds interfered with the electrode. Assuming a Nernst distribution of the ion across the membrane electrode, the equation proposed by Kamo et al. yielded the values for transmembrane electric potential.

2.10. Effects of the compounds on mitochondrial permeability transition: evaluation of calcium-induced ΔΨm depolarization

The phenomenon of the mitochondrial permeability transition (MPT) occurs when a large amount of Ca2+ is accumulated by mitochondria in the presence of an inducing agent such as phosphate (P), leading to mitochondrial depolarization caused by the formation of pores, possibly formed by ATP synthase dimers. The ΔΨm fluctuations associated with the uptake of calcium and the induction of the MPT pore were followed with a TPP−-selective electrode (as described above), in an open thermostated water-jacketed reaction chamber with magnetic stirring, at 30 °C. Mitochondria (1 mg) were suspended in 1 ml of reaction medium consisting of 200 mM sucrose, 10 mM tris-3-(N-morpholino)propanesulfonic acid (MOPS) (pH 7.2), 10 μM EGTA, 1 mM KH2PO4, 3 μM rotenone, supplemented with 3 μM TPP+. Mitochondria were incubated with the test compounds for 1 min, to guarantee their total internalization, and then energized with 5 mM succinate. Two pulses of 30–60 nmol calcium (CaCl2) were added to the reaction medium in the assays. As a control, mitochondrial preparations were pre-incubated with cyclosporin A (1 μM), a specific MPT pore desensitizer, in the presence of the highest concentration of the tested compounds that induced mitochondrial swelling. As a positive control, FCCP (25 × 10−3 nmol) was incubated with mitochondrial suspension before calcium addition, in order to induce a small reduction in ΔΨm.

2.11. Effects of the compounds on MPT: measurement of calcium-induced mitochondrial swelling

The induction of the MPT pore in isolated mitochondrial fractions leads to mitochondrial swelling, which can be estimated by changes in light scattering of the hepatic mitochondrial suspension. The turbidity of the mitochondrial suspension was measured at 540 nm in a Lambda 45 UV/VIS Spectrometer (Perkin Elmer Inc., Boston, MA, USA). Mitochondrial protein 0.5 mg/ml (final volume of 2 ml) was incubated for 1 min at 30 °C in reaction medium (see previous section) in the presence of the triterpenoid compounds under study. Mitochondrial swelling was induced by adding CaCl2 to the system. As a control, 1 μM CsA was incubated with the mitochondrial preparation in the presence of the highest con-
centration of the test compound observed to induce mitochondrial swelling. Mitochondrial swelling rates were calculated starting 2 min after calcium addition and measured during 1 min.

2.12. Osmotic swelling experiments

Passive proton and potassium permeability of the mitochondrial inner membrane in the presence or absence of the triterpenoids derivative compounds was estimated by measuring the swelling of non-respiring mitochondria in isosmotic media containing NH₄NO₃, KSCN or KCH₃COO. Rat liver mitochondria (1 mg) were incubated at 25 °C in 2 ml ionic medium constituted by 135 mM of NH₄NO₃, KSCN or KCH₃COO, 10 mM HEPES and 0.1 mM EDTA supplemented with 2 µM rotenone. Valinomycin (1 µg) was used in order to increase the permeability to potassium, while FCCP (1 µM) was used to increase permeability to protons. Triterpenoid compounds were tested for the maximum concentration used in other experiments (6 µg/ml for each compound, for 48 h). Thirty minutes before the end of incubation period cells were incubated with MitoTracker Red (7.3 nM). Decreases in turbidity were measured at 540 nm in a Lambda 45 UV/VIS Spectrometer (Perkin Elmer, Inc., Boston, MA, USA), in temperature-controlled chambers.

2.13. Statistical analysis

Data was loaded to GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA) and all results are expressed as means ± standard error of the mean (SEM) and evaluated by one-way ANOVA followed by Bonferroni multiple comparison tests. Values with p < 0.05 were considered as statistically significant.

3. Results

3.1. Mitochondrial depolarization caused by DMAP triterpenoid derivatives on breast cancer lines, and BJ fibroblasts

In order to investigate whether the five DMAP triterpenoid derivatives previously tested by our group in melanoma cells also depolarize mitochondria in breast cancer cell lines, we exposed MCF-7 and the estrogen-insensitive Hs 578T breast cancer cell lines to the compounds (Fig. 2). The triterpenoid derivatives showed a visible effect on mitochondrial polarization in any of the cell lines in study, for the concentration and time exposure chosen. In turn, treatment with compounds (2), (3) and (4) resulted in mitochondrial depolarization for these concentrations in normal BJ fibroblasts and in both breast cancer cell lines (Fig. 2) after 48 h of incubation. Thus, all DMAP triterpenoid derivatives tested, except compound (5), lead to fragmentation and depolarization of the mitochondrial network in situ, in breast cancer cell lines and some of them also disrupted mitochondria in BJ fibroblasts.

3.2. Effects of DMAP triterpenoid derivatives on isolated hepatic mitochondria: evaluation of the mitochondrial oxygen consumption

To test whether DMAP triterpenoid derivatives interfere directly with mitochondrial function, we tested these compounds in isolated rat liver mitochondria, a biological model used by pharmaceutical companies as a reliable biosensor for drug-induced toxicity. To study mitochondrial respiratory parameters, both glutamate/malate (substrates for complex I) and succinate (substrate for complex II) were used for mitochondrial energization in the absence and presence of increasing concentrations (3 and 6 µg/mg of protein) of the compounds (Fig. 3). The triterpenoid derivative compound (1) (Fig. 3A) seemed to exert direct effects in ATP synthase Fo subunit (for some concentrations) since the increases observed in state 2 and state 4 were not visible when this specific subunit was inhibited by oligomycin. A decrease in ADP/O ratio further confirms this result (Fig. 3A). The triterpenoid derivatives (2), (3) and (5) did not interfere with any mitochondrial respiration parameter at the selected concentrations (Fig. 3B, C, E), except for the RCR parameter that was decreased in the presence of the higher concentration of compounds (2) and (5). Compound (4), which was the most powerful mitochondrial respiration inhibitor, acted in the respiratory chain in both glutamate-malate and succinate-energized mitochondria, as suggested by the decrease in FCCP-induced respiration (Fig. 3D). Compound (4) also increased passive flux of protons through the mitochondrial inner membrane, as shown by the increase in mitochondrial respiration when ATP synthase Fo subunit was blocked by oligomycin (state oligomycin) (Fig. 3D). State 2, state 3 and state 4 respiratory parameters confirmed that com-
Figure 3. Effects of the triterpenoid derivatives (1–5) on mitochondrial respiratory parameters with 10 mM glutamate/5 mM malate (left panels) or 5 mM succinate (right panels) as substrates. Mitochondria were incubated in 1 ml respiration medium (see Section 2). ADP (187.5 nmol) was added to induce state 3 respiration. Oligomycin (1 μg) and FCCP (1 μM) were added to the system in order to inhibit passive flux through the ATP synthase and to uncouple respiration, respectively. The RCR was calculated as the ratio between state 3 and state 4 respiration. The ADP/O ratio was calculated as the number of nmol ADP phosphorylated by n atoms of O consumed during ADP phosphorylation. Data were expressed as % of control and represent means ± SEM of three different preparations. *p < 0.05 versus control. Control values for Complex I: State 2 = 19.0 ± 4.1 natsoms O/min/mg protein; State 3 = 124.1 ± 20.2 natsoms O/min/mg protein; State 4 = 18.0 ± 5.2 natsoms O/min/mg protein; State oligomycin = 9.3 ± 2.9 natsoms O/min/mg protein; ADP/O = 2.9 ± 0.1; RCR = 9.3 ± 2.0 (mean ± SEM, n = 3). Control values for Complex II: State 2 = 22.3 ± 5.5 natsoms O/min/mg protein; State 3 = 131.8 ± 8.5 natsoms O/min/mg protein; State 4 = 18.3 ± 3.3 natsoms O/min/mg protein; State oligomycin = 8.3 ± 2.5; natsoms O/0 min/mg protein; State FCCP = 158.2 ± 25.2; ADP/O = 2.0 ± 0.2; RCR = 6.6 ± 2.1 (means ± SEM, n = 3).
bound (4) acts as a powerful inhibitor of mitochondrial function (Fig. 3D). The values obtained for the RCR ratio of the control group were of 9.3 ± 2.0 for complex I and 6.6 ± 2.1 for complex II, showing that our mitochondrial preparations were well coupled, which was further confirmed by the ADP/O ratio values of 2.9 ± 0.1 for complex I and 2.0 ± 0.2 for complex II.

3.3. Effects of DMAP triterpenoid derivatives on isolated hepatic mitochondria: Evaluation of the ΔΨ_m fluctuations

To investigate the effect of triterpenoid derivatives on ΔΨ_m generation, both glutamate/malate (substrates for complex I) and succinate (substrate for complex II) were used to energize mitochondrial preparations. The same range of concentrations used in mitochondrial respiratory parameters was again used (3 and 6 μg/mg of protein). Typical recordings for the effect of each one of the tested compounds on ΔΨ_m are shown in Figure 4 and quantitative data are presented in Supplementary Table 1. The ΔΨ_m was slightly decreased for increasing concentrations of triterpenoid derivative (1), when compared to the control. For the remaining compounds, there was a dose-dependent immediate ΔΨ_m depolarization and an increase in lag phase, for both glutamate/malate and succinate-energized mitochondria.

3.4. DMAP triterpenoid derivatives induce the MPT on isolated hepatic mitochondria

MPT pore opening can be accompanied by an increase in mitochondrial internal volume (mitochondrial swelling) and by a decrease in ΔΨ_m. The two phenomena can be followed experimentally by measuring the changes in the suspension absorbance at 540 nm and by using a TPP⁺ selective electrode, respectively. In the present work, MPT pore opening was induced by Ca²⁺ in a phosphate-buffer medium and measured in the absence and in the presence of the DMAP triterpenoid derivatives. Fig. 5 shows typical recordings of the effect of the tested compounds on calcium-induced MPT pore opening followed by measuring ΔΨ_m fluctuations. Increasing concentrations of compounds (3) and (5) caused calcium-induced ΔΨ_m dissipation, but compounds (1), (2) and (4) had the more pronounced effects. An additional control with FCCP was performed in order to investigate if the MPT pore induction could be due to the small decrease in ΔΨ_m, caused by the compounds. The results show that the MPT-inducing effect of the test compounds was not caused by a depolarizing effect. Similarly to what happened in ΔΨ_m experiments, mitochondrial swelling was most drastically observed for increasing concentrations of compounds (1), (2) and (4), with compounds (3) and (5) having a milder effect (Fig. 6). Both approaches demonstrated that the effect of the tested compounds in MPT pore induction was dose-dependent. The MPT pore desensitizer, CsA, prevented both mitochondrial swelling (Fig. 6) and ΔΨ_m dissipation (Fig. 5), confirming the involvement of MPT pore induction.

3.5. DMAP compounds do not cause osmotic swelling of mitochondria

The osmotic swelling assay in non-energized rat liver mitochondria in the presence of different ionophores is a standard experimental protocol to study. To investigate the capacity of DMAP triterpenoids to trigger mitochondrial swelling as a result of an increased permeability of the inner membrane to ions, different isosmotic reaction media were used with non-respiring mitochondria (Fig. 7). The effect of DMAP triterpenoids on proton permeability was examined using a NH₄NO₃ based medium. In this case (Fig. 7A), nitrate is freely permeable, while ammonia crosses the membrane in the unprotonated form (NH₃). The net result of the influx of NO₃⁻ and NH₃ is that electroneutrality (and swelling) can only be achieved by the influx of protons. In these experiments, the control FCCP caused the largest increase in mitochondrial swelling, as opposed to DMAP compounds, which did not cause any increase in proton permeability. Moreover, in contrast to the ionophore valinomycin, DMAP compounds did not promote electrochemical equilibrium, as determined by mitochondrial swelling due to the influx of potassium and thiocyanate in an isosmotic medium containing KSCN (Fig. 7B). Finally, the effect of DMAP triterpenoids was tested in mitochondria incubated in an isosmotic KCH₃COO medium (Fig. 7C). Acetate, in its neutral protonated form, crosses the mitochondrial inner membrane. The membrane impermeability to potassium was overcome with valinomycin. Mitochondrial swelling, indicative of influx of both acetate and potassium, was only achieved when FCCP was added to promote the efflux of protons and maintain the electroneutrality of the system. The compounds did not cause any noticeable effect on proton permeability.

4. Discussion

The increased resistance to apoptosis induction is a common feature in many cancers. Since mitochondria occupy a strategic position between bioenergetic/biosynthetic metabolism and cell death regulation, these organelles emerged as idealized targets for cancer therapy. Thus, compounds that directly affect mitochondrial function and trigger apoptosis are considered as potential anti-cancer agents. Triterpenoids are a class of natural occurring compounds with ubiquitous distribution, and whose anticancer activity was previously documented and observed to be dependent on apoptosis induction via direct mitochondrial alterations. Betulinic acid is one of such natural compounds that display a notable level of discrimination in promoting apoptosis in some cancer cell lines such as melanoma, glioma and ovarian carcinoma. Although extracted from natural sources in large amounts, the use of these triterpenoids in the same form as existing in nature remains quite limited due to their low solubility, high pH in solution and high molecular weight. The design of new synthetic derivatives of these compounds, taking advantage of quantitative structure–activity relationships (QSARs), could help producing more active and selective compounds to overcome these limitations. With this in mind, DMAP derivatives of lupane triterpenoids were synthesized based on birch bark lupane triterpenoids betulin and betulinic acid. Our research group has previously tested a number of DMAP derivatives on human melanoma cell lines. These novel compounds induced mitochondrial fragmentation and depolarization, along with an inhibition of cell proliferation. The potency of their effects was correlated with the number, position, and orientation of the DMAP groups. Overall, the extent of proliferation inhibition mirrored the effectiveness of mitochondrial disruption. The present work is a follow-up to this previous study, aiming at understanding in more detail the mechanisms behind the mitochondrial toxicity observed. We have selected a panel of 5 different compounds from the original tested in order to be representative of the strength of mitochondrial effects. DMAP derivatives (2) and (5) were catalogues as weak mitochondrial perturbants, whereas compounds (1) and (3) were considered of intermediate strength. Finally, compound (4) was considered by Holy et al. as a strong mitochondrial perturbant. All the DMAP triterpenoid derivatives in this study have a hydrophobic central region composed by four cyclohexane rings and one cyclopentane ring which corresponds to betulinic acid (Fig. 1). Polar groups were added to the structure backbone in order to provide an increased amphiphilic character to molecules. This ring-like structure gives a planar geometry to molecules and provides affinity to hydrocarbon-chain of fatty acids of phospholip-
These polar groups are protonated at physiological pH which means that the compounds are likely to be positively charged in the physiological environment and preferentially interact with anionic membrane lipids. Once inserted in plasma membrane the orientation and localization of DMAP groups promotes their rapid diffusion towards the cytosol. It is expected that once in cytosol, the DMAP derivatives translocate to mitochondria driven by the negative charge in the matrix.

In our previous study, we reported mitochondrial structure and function alterations in the presence of these triterpenoid derivatives in melanoma cells. In the present study, we used epifluorescence microscopy to investigate in situ mitochondrial effects in the breast cancer cell lines MCF7 (estrogen-sensitive) and Hs 578T (estrogen-insensitive), compared to a non-tumoral cell line. This allowed confirming that the previous observations were not cell/tumor-type specific. The fluorescent probe MitoTracker Red was used not only to detect alterations in mitochondrial membrane polarization but also to give insights into the morphology of the polarized network. Compound (5) did not present any significant effect on mitochondrial morphology or polarization (Fig. 2).

Figure 4. Representative recordings of the effect of all triterpenoid derivatives on ΔΨm. Hepatic mitochondria (1 mg) were incubated in 1 ml of standard reaction medium as described in Section 2. Mitochondria were energized by adding 5 mM glutamate/2.5 mM malate (A) or 5 mM succinate with 3 μM rotenone. (B) ADP (125 nmol) was added to initiate state 3 and valinomycin (Val.) 0.2 μg was added to the system in order to confirm if triterpenoid compounds interfere with the TPP⁺ electrode. DMAP compounds 3 μg/mg protein and 6 μg/mg protein were pre-incubated with 1 mg of protein for 1 min prior the ADP addition. Quantitative data are presented in Supplementary Table 1.
whereas compounds (2) (3) and (4) proved to be potent disruptors of mitochondrial function, in all cell lines (Fig. 2). Compound (1) induced fragmentation and depolarization of the mitochondrial network in the two cancer cell lines (Fig. 2).

Since mitochondrial structure and function was compromised in breast cancer, and also in melanoma cell lines after incubation with the triterpenoid derivatives, we investigated whether the compounds exerted direct effects on isolated rat hepatic mitochondria, in order to gain more mechanistic insights into their effects. Although normal hepatic and cancer cell mitochondria present some structural and functional differences, we believe that sufficient similarities exist to justify the use of isolated hepatic mitochondria as models to study the interactions of tested compounds with mitochondria. Isolated mitochondrial fractions have been previously used as a biological model by pharmaceutical companies as a sensitive and reliable biosensor for drug-induced toxicity.\(^\text{[16]}\)

Our results show that compound (1) appeared to have an effect in the phosphorylative system, as suggested by the decrease in the ADP/O ratio (Fig. 3A). Compounds (5), (3) and (2) did not elicit any alteration in respiratory parameters, with the exception of a RCR decrease observed for compounds (5) and (3) (Fig. 3E, C, B). The results also suggest that some of the compounds studied may present mixed effects including inhibition of the respiratory chain and uncoupling, the latter being suggested, for example, by the immediate depolarization observed upon addition of the compounds to the mitochondrial suspension (Fig. 4). Compound (4) induced multiple levels of mitochondrial toxicity, appearing to have a protonophoretic/uncoupler effect and at the same time to inhibit the respiratory chain, as seen by a decrease in FCCP-induced maximal respiration. Both effects may have contributed to the increase in phosphorylative lag phase and to the reduction in the ADP/O ratio (Fig. 3D). The increase in the lag phase observed in the presence of some of the compounds can be partly explained by \(\Delta \Psi_m\) depolarization induced by the compound itself, due to its positive charge. However, the depolarization induced by compound (4) can also be linked to a direct effect on the respiratory chain, as well as to increased permeabilization to protons (Fig. 3D).

Two distinct experiments (mitochondrial swelling and \(\Delta \Psi_m\) fluctuations) demonstrated that increasing concentrations of compounds (1), (2) and (4) induced the MPT pore (Fig. 6). The results obtained for compounds (1) and (2) are very interesting, since both compounds induced the MPT pore at concentrations that did not present marked toxic effects on mitochondrial respiration (Fig. 3A and B). The maintenance of mitochondrial integrity is extremely important because apoptosis is an ATP-dependent process.\(^\text{[21]}\) With these results, it is predictable that DMAP derivatives (1) and (2) may induce cell death through a MPT pore-related mechanism. This phenomenon is considered very important, not only in the crossroad between apoptosis and necrosis, but also in organ dysfunction associated with different pathologies.\(^\text{[22]}\) Confirming cell experiments and mitochondrial oxygen consumption, compound (5) did not present any effect in MPT pore for the concentrations and time points tested (Fig. 7). Together, these results...
present convincing evidence that mitochondrial effects underlie the toxicity of these agents. Although compound (3) induced depolarization and fragmentation of the mitochondrial network in cancer cell lines (Fig. 2), this compound showed a very small degree of toxicity on mitochondrial respiratory parameters (Fig. 4C) and had a marginal effect, if any, in the induction of the MPT pore, at least when evaluating mitochondrial swelling (Fig. 6) for the concentrations tested, which suggests that compound (3) may exert its activity on cancer cells independently of direct mitochondrial effects. The fact that mitochondrial depolarization occurs with the addition of compound (3) (Fig. 5), implies that the compound is accumulated by mitochondria but no toxicity results from that.

Another relevant observation is that mitochondrial depolarization was visible after 48 h of treatment in cancer cells whereas in isolated mitochondrial fractions, a depolarizing effect, either resulting from the addition of the compound itself, or from other effects on the respiratory chain or on the MPT itself, was visible after some seconds/minutes. This may result from the fact that barriers to mitochondria/compound interaction are present in the intact cell. Several factors, including compound binding to media serum and passage and/or passive binding to plasma/organellar membranes, may lead to a delay in the accumulation of critical compound concentrations in mitochondria. Also, for our own experience, measurement of mitochondrial $\Delta$Ψ$_m$ based on epiflu-
orescent microscopy imaging with the fluorescent probe Mito-Tracker Red in intact cells is not as sensitive as measuring the same parameter with a TPP+ electrode in isolated mitochondrial fractions, in which a small depolarization of 5–10 mV can be measured.

An interesting result came from osmotic swelling experiments where none of the compounds appeared to increase the inner mitochondrial membrane permeability to potassium or protons (Fig. 7). Although the latter result disagrees with the hypothetical protonophoretic activity that we proposed to explain part of the depolarizing effects of the compounds, there may be a simple explanation for this. Since osmotic experiments are made with non-energized mitochondria, there is no driving force for the charge-driven accumulation of compounds in mitochondrial membranes, thus greatly limiting their effect.

We can also speculate that the activity of DMAP triterpenoid derivatives may depend on the DMAP group position. Although compounds (1) and (3) are theoretically similar (Fig. 1) having the same number of positive charges at physiological pH and differing only in the DMAP position groups, their effects are notably distinct. As expected for compound (5), the lower affinity to lipid membranes and low positive net charge5 was reflected by the absence of activity in the models tested, which confirms that promising compounds must have an amphiphilic character and be positively charged to exert their biological activity on organelles with the highest negative potential inside, such as mitochondria. The present work also serves as a framework for the development of derivatives able to distinguish cancer from non-cancer cells based on their native mitochondrial ΔΨm.

5. Conclusion

In general, the present work corroborates the idea that DMAP triterpenoid derivatives are promising in cancer therapy. The experiments with isolated mitochondria demonstrate that some of these agents can directly induce MPT pore in concentrations that did not interfere with normal mitochondrial metabolism, suggest-
ing that this may be a valid mechanism that explains their toxicity. Global analysis of the results show that, despite a single exception, toxicity on isolated mitochondrial fractions correlates well with in situ cell mitochondrial toxicity. Further assays are clearly needed to explore the mechanisms of mitochondrial toxicity of the test compounds in more detail, since the borderline between a desired pharmacological effect (i.e., disruption of mitochondrial function in cancer cells) and a toxic side-effect (mitochondrial toxicity in non-target organs) is often very blurry.

6. Conflict of interest
The authors declare they have no conflict of interests.

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Supplementary data
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References and notes