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On the nature of ceramide-mitochondria interactions -- dissection using comprehensive mitochondrial phenotyping

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Abstract

Sphingolipids are a unique class of lipids owing to their non-glycerol-containing backbone, ceramide, that is constructed from a long-chain aliphatic amino alcohol, sphinganine, to which a fatty acid is attached via an amide bond. Ceramide plays a star role in the initiation of apoptosis by virtue of its interactions with mitochondria, a control point for a downstream array of signaling cascades culminating in apoptosis. Many pathways converge on mitochondria to elicit mitochondrial outer membrane permeabilization (MOMP), a step that corrupts bioenergetic service. Although much is known regarding ceramides interaction with mitochondria and the ensuing cell signal transduction cascades, how ceramide impacts the elements of mitochondrial bioenergetic function is poorly understood. The objective of this review is to introduce the reader to sphingolipid metabolism, present a snapshot of mitochondrial respiration, elaborate on ceramides convergence on mitochondria and the upstream players that collaborate to elicit MOMP, and introduce a mitochondrial phenotyping platform that can be of utility in dissecting the fine-points of ceramide impact on cellular bioenergetics.

Keywords

Ceramide; Mitochondria; Bioenergetics; Sphingolipids; Cancer

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Abbreviations: Listed throughout, in order of appearance.

1. Introduction

Mitochondria play a principle role in orchestrating the intrinsic pathway of apoptosis. This cell death spiral can be elicited by cellular stimuli such as radiation, DNA-damaging anticancer drugs, and ER stress. The essential position that the sphingolipid (SL), ceramide exerts in mitochondrial-driven apoptosis has been the subject of extensive studies. It is well known that levels of ceramide increase in cells in response to extracellular insult. Several key SL-metabolizing enzymes are involved in increased ceramide production; this leads to apoptosis via intermediate interaction at the mitochondrial level. When blockers of SL enzymes involved in ceramide synthesis are introduced, apoptotic responses are counteracted, hence the keen connection between ceramide and programmed cell death. Mitochondrial outer membrane permeability (MOMP) is central for the successful apoptotic cascade in which the formation of ceramide channels is believed to participate. Although much is known regarding ceramide interaction with mitochondria and ensuing signaling cascades, how ceramide impacts the elements of mitochondrial bioenergetic function is poorly understood. Establishing the decisive molecular mechanisms by which cellular ceramides directly impinge on mitochondrial bioenergetics is open for exploration. Such studies, aided by an “omics” type of analysis, should be essential in identifying novel, mitochondrial-targeted therapeutics designed to combat diseases, including cancer.

2. Sphingolipids 101, an overview

To most, the mention of lipids recalls our recent blood work with levels of triglycerides, now termed triacylglycerols, and cholesterol, which is not a lipid. And of course, we know that lecithin, also known as phosphatidylcholine, is found in egg yolk and ice cream, for those of us who read labels. Moving on in complexity, many of us connect the term sphingolipids (SL) with sphingomyelin that we understand has something to do with myelin that has a lot to do with nerves and brain. Ceramide, on the other hand, is to many, a wonderous ingredient in hand cream, a skin-restoring moisturizer with considerable fame to the lay public. For the curious, however, how did science come up with the moniker, sphingolipid”?

SL’s were first identified in the 1870’s in extracts of brain, and because of their enigmatic nature they were named after the mythological creature, the Sphinx. Why enigmatic? Many brain SL’s are highly glycosylated and do not dissolve in traditional lipid solvents like chloroform, often used by the chemists of the day. Further adding to the enigma, these lipids frequently contain very long-chain fatty acids coupled as an amide bond that is not labile to mild alkaline treatment, as are the fatty acids in glycerolipids. So, there you have it, “sphinx-o-lipids”, a unique class of lipids with a cornucopia of amazing functions [1].

Sphingolipids comprise an enormous family of lipids found in animals, plants, fungi, and in some prokaryotic organisms and viruses. Appropriate in this 2020 pandemic, FTY720 (Fingolimod), a sphingomimetic drug that promotes SL receptor internalization and degradation, is in clinical trials for management of COVID-19 (NCT04280588 and NCT04276688—ClinicalTrials.gov) [2].

SL's do not contain the trihydroxy alcohol backbone, glycerol, common to glycerolipids (mono-, di-, and triglycerides, phospholipids) but instead harbor ceramide, a main feature in this review. Ceramide is constructed from sphingosine, an aliphatic amino alcohol to which a fatty acid is attached via an amide bond. Ceramide is the neutral lipid building block of sphingolipids or, if they contain sugars, of glycosphingolipids. These lipids not only serve structural roles in biomembranes, but they also have wide-ranging effects on signal transduction and the regulation of cell function. Chief among these actions is the potentiation of signaling cascades with emphasis on mitochondrial interactions that lead to apoptosis, the "tumor suppressor" property of ceramide.

The steps of ceramide biosynthesis, *de novo*, are illustrated in Fig. 1. In brief, the synthesis of ceramide is initiated by serine palmitoyltransferase, which uses serine and palmitoyl-CoA to generate the 'sphingoid' base, sphinganine. On towards the production of ceramide, sphinganine is acylated by dihydroceramide synthases, a sextet of enzymes that produce an astonishing array of chemically diverse dihydroceramides, via dihydroceramide synthase preferences for specific acyl-CoAs. This specificity produces dihydroceramides with carbon chain lengths ranging from C14 to C32, with varying degrees of saturation. Dihydroceramides are not the ceramides we usually read about or buy in hand lotion, until a desaturase step, catalyzed by dihydroceramide desaturase, completes ceramide production via insertion of a 4,5-*trans* double bond. Ceramide can also be produced by the action of specialized phospholipases, known as sphingomyelinases. These enzymes, which are classified according to their optimum pH and subcellular locations, cleave SM at the phosphodiester bond that is proximal to ceramide, producing ceramide and choline phosphate.

As nature would have it, many of the actions of ceramide are molecular species specific in biology ranging from cancer to diabetes to coronary artery disease to cystic fibrosis [3–11]. For example, work by Siddique et al, [12] illustrated that ceramide molecular species nuances focusing on the 4,5-*trans* double bond were required for cancer cell sensitivity to etoposide, a semisynthetic derivative of podophyllotoxin from the rhizome of the wild mandrake that is used in the treatment of Kaposi's and Ewing's sarcoma, lung and testicular cancer, and glioblastoma. Rudd and Devaraj [13] showed that the apoptotic impact of several ceramide species was dependent on acyl-chain saturation, and in aging where loss of skeletal muscle mass is widespread and unwelcome, CerS1 and 5 were identified as potential regulators of fiber size and strength [14].

Two additional points of ceramide generation merit mention. These are essentially the back reactions to ceramide kinase, catalyzed by ceramide 1-phosphate phosphatase, and glucosylceramide synthase, catalyzed by glucosylcerebrosidase (Fig. 2). So, what becomes of ceramide once generated? By and large, tumor cells can be protected from ceramide's apoptosis-inducing effects by two major constructive metabolic steps that produce a wide variety of SL's such as sphingomyelin (SM), glucosylceramide (GC), the precursor of higher cerebrosides, globosides, and gangliosides, and galactosylceramides, the precursors of sulfatides (Fig. 2). Of particular interest here is ceramide glycosylation, a voracious metabolic pathway utilized in multidrug resistant cancer cells to facilitate ceramide clearance [15–17]. Whereas ceramide is a powerful tumor suppressor, the glycosylated product,

GC, formed by the action of glucosylceramide synthase (GCS) (Ceramide: UDP-Glc Glucosyltransferase), is ineffectual in this realm. Upregulated ceramide glycosylation is an avenue through which cancer cells skirt the deleterious effects of ceramide [18–22]. Ceramide can also be converted to ceramide1-phosphate (C1-P) by ceramide kinase. C1-P plays various, novel roles in cell proliferation, wound healing, inflammation, and tumor cell metastasis [23–25]; signaling aspects of C1-P have recently been reviewed [26]. Also relevant is ceramide hydrolysis, specifically by acid ceramidase (AC), another sentinel enzyme regulator of cancer cell growth [27–32]. Similar with GCS, AC thwarts the tumor-killing properties of ceramide via hydrolysis, producing fatty acids and sphingosine, the latter a substrate for sphingosine kinase (SPHK), the enzyme catalyzing production of sphingosine 1-phosphate (S1-P), a cancer cell mitogen [33–35]. In summary, ceramide can be generated *de novo* or via sphingomyelin hydrolysis by a host of agents, anticancer drugs included [36], and ceramide's elimination can be hastened by hydrolysis or conversion to higher SL's. What little ceramide manages to hang around has, as we shall read, a tremendous impact on mitochondrial function.

3. Mitochondrial respiration in a nutshell

Mitochondria are double-membrane organelles ubiquitous to all mammalian cells, sans red blood cells. Across eukaryotic life, mitochondria are critically involved in generating and maintaining cellular energy charge. They accomplish this task through a series of energy transduction reactions that ultimately transduce the chemical potential energy of electron-rich carbon intermediates to ATP free energy (ΔG_{ATP}). Oxidation of carbon intermediates is carried out in the matrix space by a network of dehydrogenase enzymes that deliver electrons to the electron transport system (ETS) that upon electron transport through a series of multi-subunit protein complexes leads to O_2 reduction to generate H_2O. Driven by the redox potential energy span across the ETS (NADH/NAD+ ~ H2O/O2), three of the respiratory complexes pump protons across the inner-mitochondrial membrane to generate an electrochemical proton gradient known as the proton motive force (Δp). The mitochondrial proton motive force is in turn coupled to a variety of cellular processes that carry out essential cellular functions, including ATP synthesis (ATP synthase) and exchange (phosphorylation system; ATP synthase 'Complex V', adenine nucleotide translocase 'ANT'), as well as, NADPH generation (nicotinamide nucleotide transhydrogenase 'NNT'), calcium cycling, and metabolite exchange (Fig. 3).

Although best known for their role in cellular ATP generation through the process of oxidative phosphorylation (OXPHOS), it is now understood that mitochondria are central to a wide variety of cellular functions (e.g., reactive oxygen species production, calcium buffering, macromolecular synthesis). In addition, mitochondria are critically involved in cellular survival through regulation of the intrinsic apoptotic pathway (discussed below). Given that mitochondria are present in all cell types (RBCs excluded), the assumption has been that all mitochondria are alike, and that function declines due to disease or aging. However, new evidence is emerging that all mitochondria are not alike but, in fact, are unique in composition and function within each cell type [37,38], including cancer cells [39,40]. This raises the exciting possibility that identifying the unique bioenergetic signature(s) of cancer cells may hold the key to designing mitochondrial-targeted chemotherapeutics that

specifically target only those cancer cells. Related to this, mounting evidence indicates that in addition to alterations in ceramide handling, multi-drug resistance in cancer involves alterations in mitochondrial form and function that facilitates cancer cell survival [41,42].

4. Ceramide convergence on mitochondria— the end game

Mitochondria instigate the intrinsic apoptotic pathway, obviously initiated from within the cell; the consequences are executed downstream. It is important to understand that through ceramide, whether by intracellular generation [43] or exogenous administration [44,45], there exists the means to promote tumor cell death via a myriad of signal transduction pathways that converge on mitochondria to elicit release of proapoptotic proteins; a process which results in mitochondrial damage. Although the present work is focused on ceramide, note that Obeid and colleagues [46] presented an excellent recap dealing with the broader subject of SL's and mitochondria.

Regarding cancer and the intrinsic apoptotic pathway, resistance to mitochondrial apoptosis is a prominent feature of cancer cells; however, in some situations, ceramide can override this. As ceramide is highly insoluble, an energy barrier hinders its intracellular movement from the plasma membrane or other intracellular membranes in which it is produced. Therefore, how ceramide reaches mitochondria to modulate function is relevant and will be briefly discussed.

Upon generation in the plasma membrane, ceramide forms platforms that subsequently invaginate and fuse with mitochondria [47,48]. This event has been termed 'the kiss of death' in other words, *the end game*. Via this commute, ceramide can be transferred directly from the plasma membrane to the mitochondria, a function promoting mitochondrial ceramide accumulation and the induction of apoptosis [47,48]. Some studies indicate that endoplasmic reticulum (ER) membranes, called mitochondria-associated membranes, are physically and functionally allied with mitochondria to integrate several aspects of ER and mitochondrial function [49,50]. Experiments with isolated mitochondria have revealed that these mitochondria-associated membranes (known as ER-like membranes) can produce enough ceramide through ceramide synthases or the salvage pathway to transiently permeabilize the outer mitochondrial membrane [49]. Ceramide also reaches mitochondria through the localization of CerS (ceramide synthases), neutral SMase, and neutral ceramidase in (or associated with) mitochondria to orchestrate *in situ* ceramide production [51–54].

5. Ceramide and mitochondrial outer membrane permeabilization (MOMP) —same old, same old

The following summarizes the effects of ceramide on mitochondrial-governed apoptosis. These ideas have been the mainstay in the field; however, there is more to ceramide's impact than meets the eye. For example, what are the bioenergetic consequences of a "ceramide storm", a storm elicited either by ceramide-generating drugs or by administration of ceramide itself [55]. This question will be visited in a latter section entitled, Mitochondrial Diagnostics and deciphering the fine points of ceramide's impact —what we can learn.

Cancer cells typically display dysfunctional apoptotic pathways that in most cases arise through a defect in intrinsic pathway sensitivity. Therefore, the idea that mitochondria might serve as a target in cancer therapy [56–59] makes sense. A common cause of tumorigenesis lies in the imbalance between the proapoptotic (BAX and BAK) and the antiapoptotic (Bcl-2) members of the Bcl-2 family. Bcl-2 expression is linked to cell survival and resistance to chemotherapy [60,61], whereas Bcl-2 mutations suppress the normal functions of the proapoptotic proteins BAX and BAK [62]. Elevated ceramide levels can leverage MOMP, which is crucial for apoptotic signaling [63,64]. MOMP is closely regulated via interactions between the *for*- and *against*- apoptosis members of the Bcl-2 family [65], a delicate minuet that can elicit the "point of no return". Stress caused by elevated ceramide coax a dialogue between Bcl-2 family proteins that control activation of effector relatives, BAX and BAK. Some have shown that ceramide alone is not enough to induce MOMP [66,67], but instead ceramide and BAX are thought to synergistically permeabilize the outer mitochondrial membrane and induce apoptosis [66,67]. Ceramide-rich macrodomains and/or channels in the outer mitochondrial membrane are essential for BAX insertion, oligomerization, and pore formation in order to induce MOMP [68,69]. Short-chain ceramides can also induce MOMP [70]. MOMP promotes leakage of apoptotic proteins, like cytochrome c and SMAC (second mitochondria-derived activator of caspases), and the release of intermembrane space proteins with a molecular mass of less than ~60,000 Da [71–73]. SMAC-DIABLO triggers caspase 3 activation. Of note and on point, MOMP directly correlates with the levels of ceramide in the outer mitochondrial membrane [72]. Together, we see that ceramide upregulates mitochondrial proapoptotic proteins, which is a good thing, and at the same time directly targets mitochondria. This relationship between ceramide and mitochondria and the consequences of this interaction highlight the appeal of ceramide as a therapeutic agent [55,74,75] that can stymie cancer cell resistance to the intrinsic apoptotic pathway.

6. From plasma membrane to mitochondria—a concerto of ceramide-involved signaling

The translocation of BAX from 14-3-3 proteins, a family of regulatory molecules that bind a multitude of signaling elements [76,77] in the cytoplasm, to mitochondria requires JUN N-terminal kinase (JNK) activation [78,79]. Ceramide-mediated activation of p38 MAPK [80,81] or downregulation of AKT can elicit BAX translocation to mitochondria [81]. Ceramide can also contribute to MOMP via glycogen synthase kinase 3β (GSK3β) [82], by activation of protein phosphatase 2A (PP2A), via inactivation of AKT, and via activation of the endolysosomal protease cathepsin D [83–86] (Fig. 4). This leads to caspase 2 and caspase 8 activation, and the cleavage of BID to form tBID, which translocates to mitochondria [83,87–89]. Ceramide can also impact mitochondrial function by inducing translocation and activation of protein kinase Cδ (PKCδ) to mitochondria. This promotes cytochrome c release and caspase 9 activation, as demonstrated in a model of prostate cancer [90]. Bcl-2 [89,91], calcium overload [87,91], and cell death receptors [92] are also involved in inducing MOMP. Revisiting the omni-potent Bcl-2 family, ceramide can activate caspase 2 through the downregulation of Bcl-2 via activation of PP2A, which also leads to MOMP (Fig. 4). On the other hand, overexpression of Bcl-2 or low calcium can prevent ceramide-induced caspase 2 activation

and mitochondrial apoptosis [89,91,93]. In summary, ceramide-mitochondria interactions are a complex aspect of cancer biology that put mitochondria in the position of “hired gun”.

Neutral and acid sphingomyelinases, ceramide synthases, and ceramidases manufacture ceramide for the initiation of mitochondrial-directed apoptosis. These enzymes are often cell type-specific in cancer; some phenotypes might harbor suppressed ceramide synthase activity, whereas others, as with ceramide overproduction, might overexpress GCS to decrease ceramide levels. Overexpression of GCS is particularly relevant to multidrug-resistant cancer [36,94–96]. Moreover, the ablation of ceramide synthase 2, which catalyzes synthesis of very long acyl chain ceramides (C22-C24), was shown to produce chronic oxidative stress via disruption of Complex IV, thought by the authors to result from compensatory increases in C16 ceramide [97].

7. Mitochondrial Diagnostics and deciphering the fine points of ceramide’s impact —what we can learn.

Ceramide plays a premier role in the initiation of cell death by virtue of its interactions with mitochondria, a control point for a downstream array of signaling cascades culminating in apoptosis. Many pathways converge on mitochondria to elicit MOMP, a step that corrupts bioenergetic service. Although much is known regarding ceramides interaction with mitochondria and the ensuing cell signal transduction cascades, how ceramide impacts the elements of mitochondrial bioenergetic function is poorly understood. To begin to fill in this gap in knowledge, we leveraged a recently described mitochondrial diagnostics workflow [37]. The key technological advancement of this assay workflow is the utilization of the creatine kinase (CK) clamp that allows bioenergetic assays to be performed across physiological ATP free energies (ΔG_{ATP}). Such conditions mimic live cell thermodynamic energy constraints and in so doing provide a quantitative snapshot of mitochondrial OXPHOS kinetics, relative to total respiratory capacity. Exposure of energized mitochondria to more negative ΔG_{ATP} values (i.e, increasing ATP free energies) inversely impacts respiratory flux, as the build-up of ATP/ADP is transmitted throughout the entire energy transduction system to slow flux. By evaluating OXPHOS kinetics with a variety of distinct carbon substrate combinations, in combination with parallel assays designed to quantify total respiratory capacity, it becomes possible to assign a given change in bioenergetic flux to a specific energy transduction control node: 1. Matrix Dehydrogenases, 2. Electron transport system (ETS), 3. ATP synthesis.

To demonstrate the utility of our novel, mitochondrial phenotyping system, we tested the effect of SACLAC, an AC inhibitor [96,98] on mitochondrial function using a chemotherapy resistant leukemia cell model, HL-60/dnr (daunorubicin resistant) [96]. SACLAC blocks the hydrolysis of ceramide into its components, sphingosine, and fatty acid (see Fig. 2), and thus introduction should result in the buildup of intracellular ceramides. We anticipated that ceramide measurement data (mass spectroscopy) in response to inhibition of AC would allow understanding of how ceramide changes support response. As shown in Fig. 5, SACLAC exposure promoted phenomenal increases in nearly all ceramide and dihydroceramide molecular species in HL-60/dnr cells, a striking outcome. This model is

thus well suited for studying the impact of ceramide molecular species on mitochondrial function [3,99–102] and opens avenues for exploring novel dihydroceramides, players that deserve at least some attention [103–108].

The influence of the "dihydroceramide/ceramide storm" on cellular bioenergetic function is summarized in Fig. 6. Importantly, these 24 hr changes were observed before overt cytotoxicity ensued after a 72 hr exposure to SACLAC [96]. At the 24 hr timepoint, despite minimal changes in cell viability (Fig. 6A) and basal respiration (Fig. 6B & 6D, **'Basal'**), respiration stimulated by physiological ΔG_{ATP} was dramatically impaired in SACLAC exposed cells (Fig. 6B). Importantly, these changes were apparent using distinct carbon substrate combinations (pyruvate/malate and pyruvate/malate/octanoyl-carnitine/glutamate/succinate) and occurred despite no evidence of mitochondrial outer membrane permeabilization (i.e., no change in respiration induced in response to exogenous cytochrome C, 'Cyt C') (Fig. 6B). Quantification of OXPHOS impairment induced by SACLAC was done by plotting the relationship between mitochondrial respiration and ΔG_{ATP}, the slope of which represents global OXPHOS conductance (Fig. 6C). In this way, relative to vehicle control, SACLAC reduced OXPHOS conductance by ~ 2 -fold. In a parallel assay, SACLAC reduced maximal uncoupled respiration in substrate replete permeabilized cells, indicating overt disruptions in ETS capacity (Fig. 6D). The combination of impaired OXPHOS conductance and lowered respiratory capacity suggest a model whereby elevated dihydroceramide/ceramide, induced by SACLAC, impinges on the protein subunits that comprise the ETS. Taken together, these findings highlight the unique ability of comprehensive mitochondrial phenotyping to reveal nuanced crosstalk between cellular sphingolipids and mitochondrial bioenergetics and suggest that direct ETS disruption likely precedes MOMP in the context of elevated dihydroceramide/ceramide.

Interestingly, in earlier work Siskind et al [73] showed that C16-ceramide, but not dihydroceramide, formed large, stable channels in membranes, albeit the system employed planar membranes formed by the monolayer method. The same study also revealed that apoptotic and channel-forming activities were greatly reduced with dihydroceramide (C-18). Moving forward, elucidating the precise molecular mechanisms by which cellular ceramide species directly impinge on mitochondrial bioenergetics is a fruitful area of research, ripe for identifying novel, mitochondrial-targeted therapeutics designed to combat cancer.

8. Coda

Defining the actions of dihydroceramides, ceramides, and their prominent, biologically potent molecular species is paramount in unravelling the fine-points of the ceramide-mitochondrial union, as it is this bond that can be all-important in directing lucrative therapeutic direction. MOMP is fine; however, comprehensive mitochondrial phenotyping as described herein can pave the way to new discovery. Our findings highlight the unique ability of mitochondrial phenotyping to unmask exacting crosstalk between cellular sphingolipids and mitochondrial bioenergetics and suggest that direct ETS disruption likely precedes MOMP in the context of elevated ceramides. We curiously await work on specific ceramide molecular species, but if you are in the lipid field, there is always the question: *yeah*, but what about "solubility"?!

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Research highlights

- Introductory review on sphingolipid metabolism in the context of cancer.
- Brief discussion of the interaction between cellular sphingolipids and mitochondrial bioenergetics.
- Introduction to a mitochondrial phenotyping platform that can be of utility in dissecting the fine-points of ceramide impact on cellular bioenergetics.

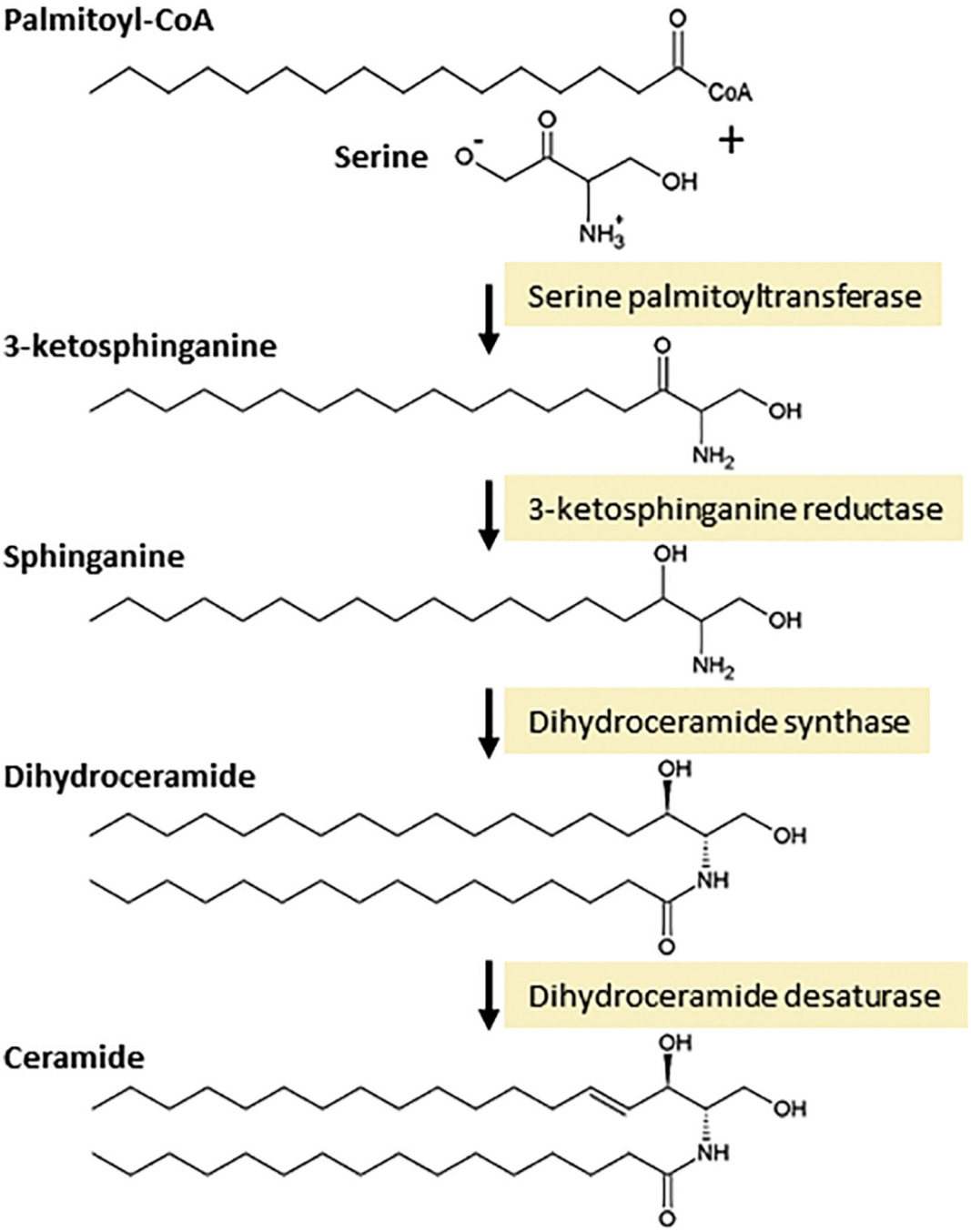

Fig. 1. Biosynthesis of ceramide by the *de novo* pathway.

Ceramide is composed of a sphingolipid base, sphinganine, joined by an amide bond to a fatty acid. Ceramide is synthesized in the endoplasmic reticulum. The *de novo* steps begin with the condensation of serine and palmitoyl-CoA, catalyzed by serine palmitoyltransferase. The product, 3-ketosphinganine, contains 18 carbons and is reduced to sphinganine by 3-ketosphinganine reductase. The next step generates ceramides saturated precursor, dihydroceramide, via the action of dihydroceramide synthase, of which there are several isoforms that ultimately give rise to a multitude of molecular species of ceramide

with distinct roles. Finally, although dihydroceramide is nearly identical in structure to ceramide, it lacks the 4, 5-*trans* double bond, which is inserted by dihydroceramide desaturase to form ceramide.

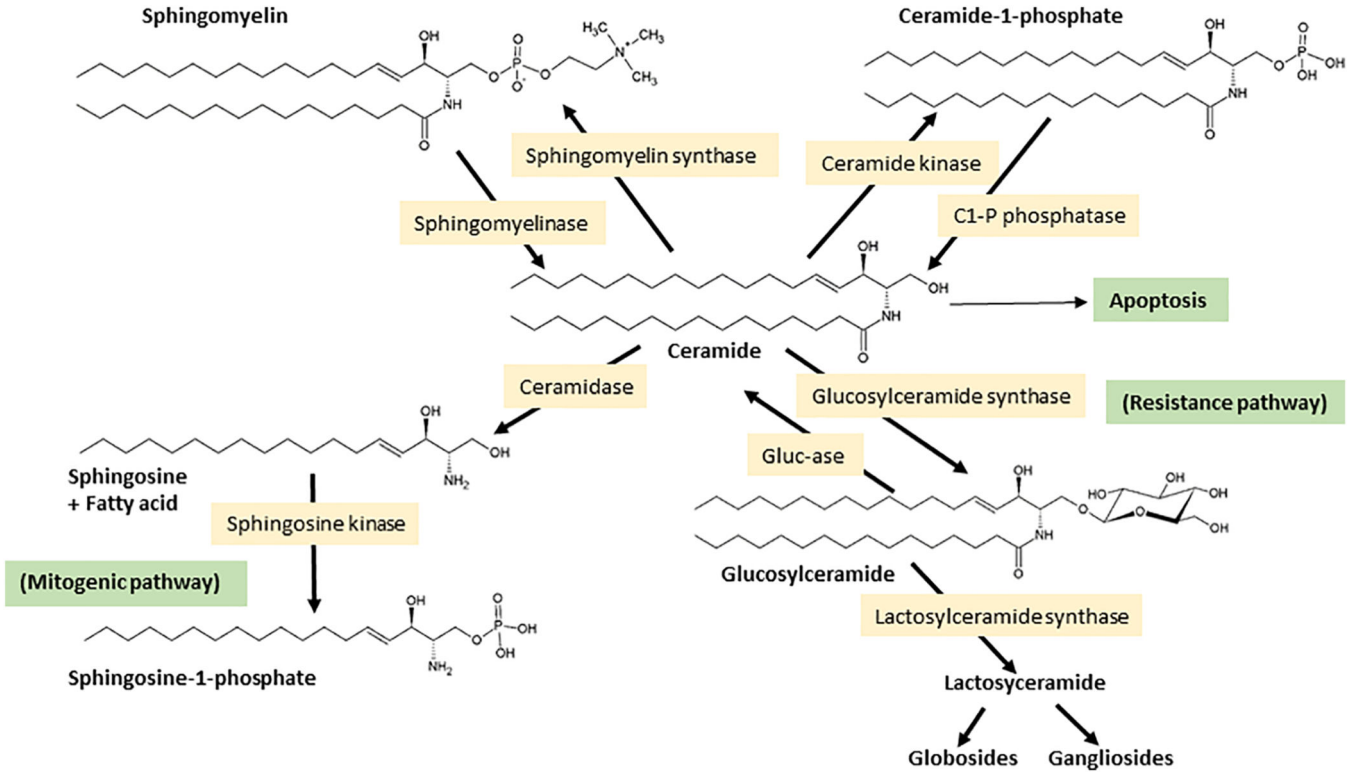

Fig. 2. Cellular fate of ceramide.

Ceramide can be produced by the action of specialized phospholipases, known as sphingomyelinases. Sphingomyelinases, which are characterized according to their optimum pH and subcellular locations, cleave sphingomyelin at the phosphodiester bond that is proximal to ceramide, producing ceramide and choline phosphate. Ceramide can also be generated by the action of ceramide 1-phosphate phosphatase (C1-P phosphatase) and via glucosylcerebrosidase (Gluc-ase). Once produced, ceramide can be hydrolyzed by ceramidase, glycosylated by glucosylceramide synthase (GCS), or phosphorylated by ceramide kinase producing ceramide 1-phosphate (C1-P). Strategic points in *de novo* synthesis and in subsequent ceramide metabolism can be activated or inhibited, providing useful avenues for studying ceramide-regulated events and for controlling cell fate. Enzyme inhibitors and P-glycoprotein (P-gp) antagonists are often used to amplify the induction of cell death by ceramide. Enzymes that “remove” ceramide either by destructive, ceramidase, or constructive metabolism, glucosylceramide synthase (GCS), can contribute to cancer cell growth.

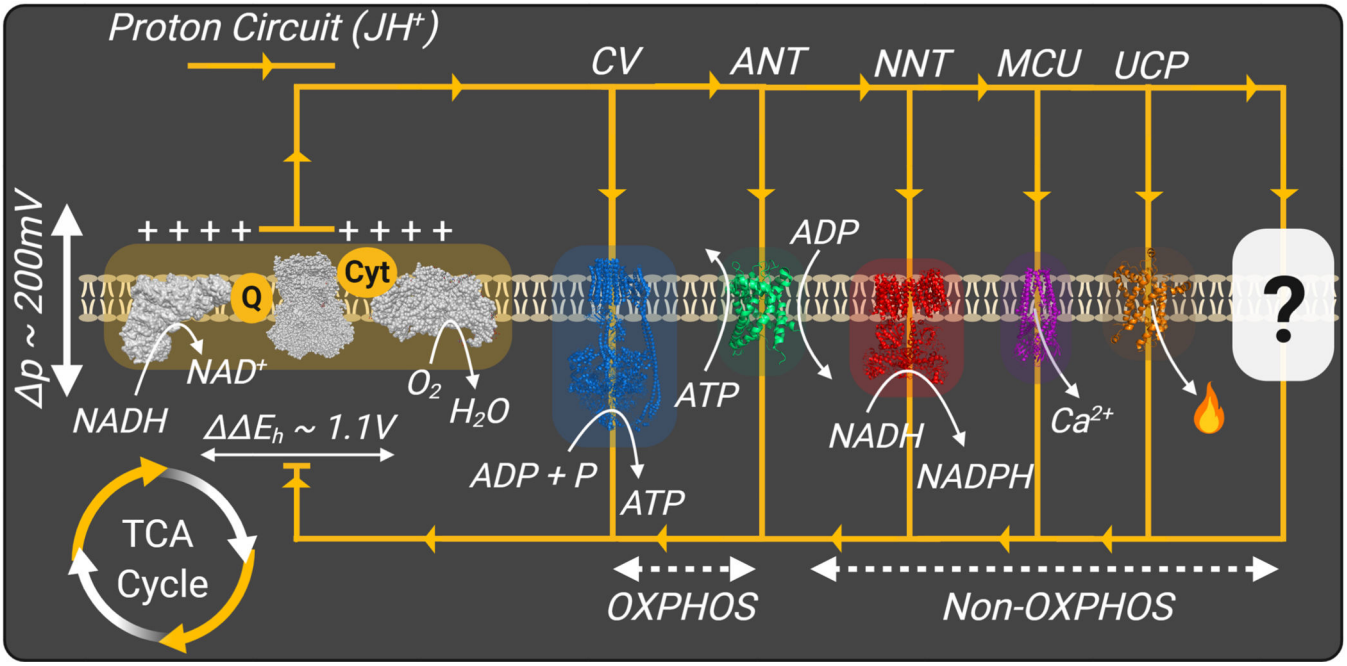

Fig. 3. Mitochondrial Energy Transduction.

Mitochondrial energy transfer mediated by the dehydrogenase network, ETS, and the phosphorylation system. Mitochondrial flux is depicted as a H^+ circuit conducted via various 'OXPHOS' and 'Non-OXPHOS' resistors; '?'–predicted 'Non-OXPHOS' resistors. Proton motive force (Δp), Complex V (CV), Adenine nucleotide translocase (ANT), Nicotinamide nucleotide transhydrogenase (NNT), mitochondrial calcium uniporter (MCU), Uncoupling protein (UCP).

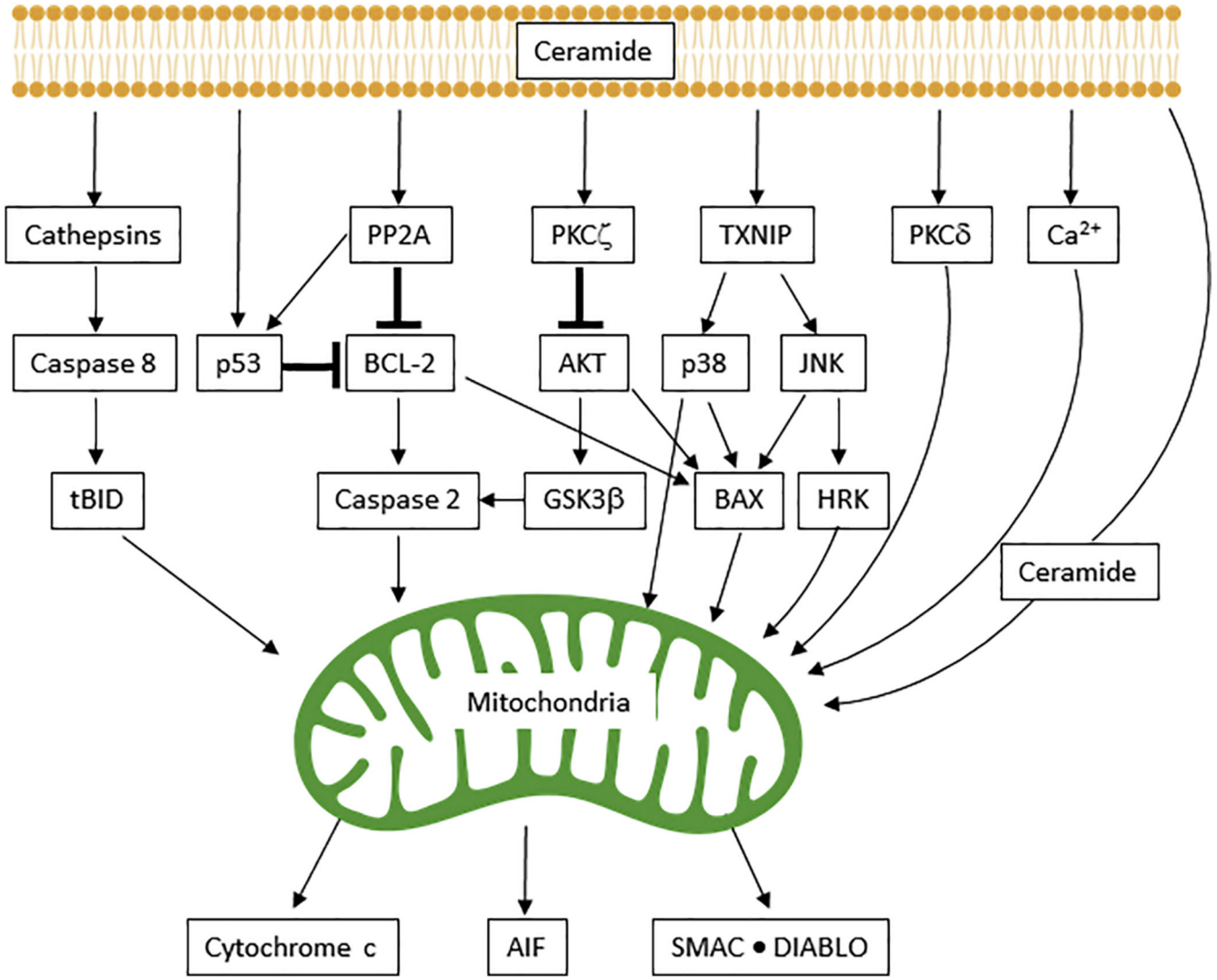

Fig. 4. Musicians in the intrinsic pathway of apoptosis—a large ensemble.

The intrinsic mitochondrial pathway of ceramide-assisted apoptosis is largely regulated by caspases and Bcl-2 family members. Crosstalk between signaling members is designated by arrows. Loss of mitochondrial outer membrane permeability (MOMP) subsequently leads to the release of proapoptotic proteins: cytochrome *c*, apoptosis-inducing factor (AIF), and second mitochondria-derived activator of caspase (SMAC)–direct inhibitor of apoptosis protein (IAP)-binding protein with low PI (DIABLO). GSK3β, glycogen synthase kinase 3β; HRK, harakiri; JNK, JUN N-terminal kinase; PKC, protein kinase C; PP2A, protein phosphatase 2A; TXNIP, thioredoxin interacting protein.

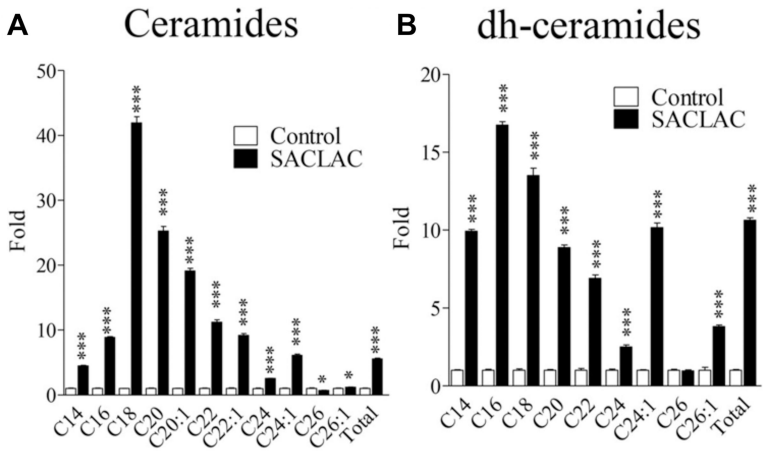

Fig. 5. Effect of SACLAC exposure on ceramide levels in drug-resistant leukemia cells.

HL-60/dnr cells (resistant to daunorubicin) (800,000/ml dnr-free, RPMI-1640 medium, 10% FBS) were exposed to 10 μM SACLAC (DMSO vehicle) or DMSO (control) for 24 hr. Cells were then harvested by centrifugation, washed three times in PBS, and subjected to lipidomic evaluation by LC/ESI/MS/MS. **A.** Ceramide molecular species. **B.** Dihydroceramide molecular species. Viability in SACLAC-treated cells was 80% at harvest. Data are expressed as fold change compared with untreated controls. Data from Kao L-P, et al., *J. Lipid Res.*, 60: 1590–1602, 2019, and reproduced with permission from the publisher.

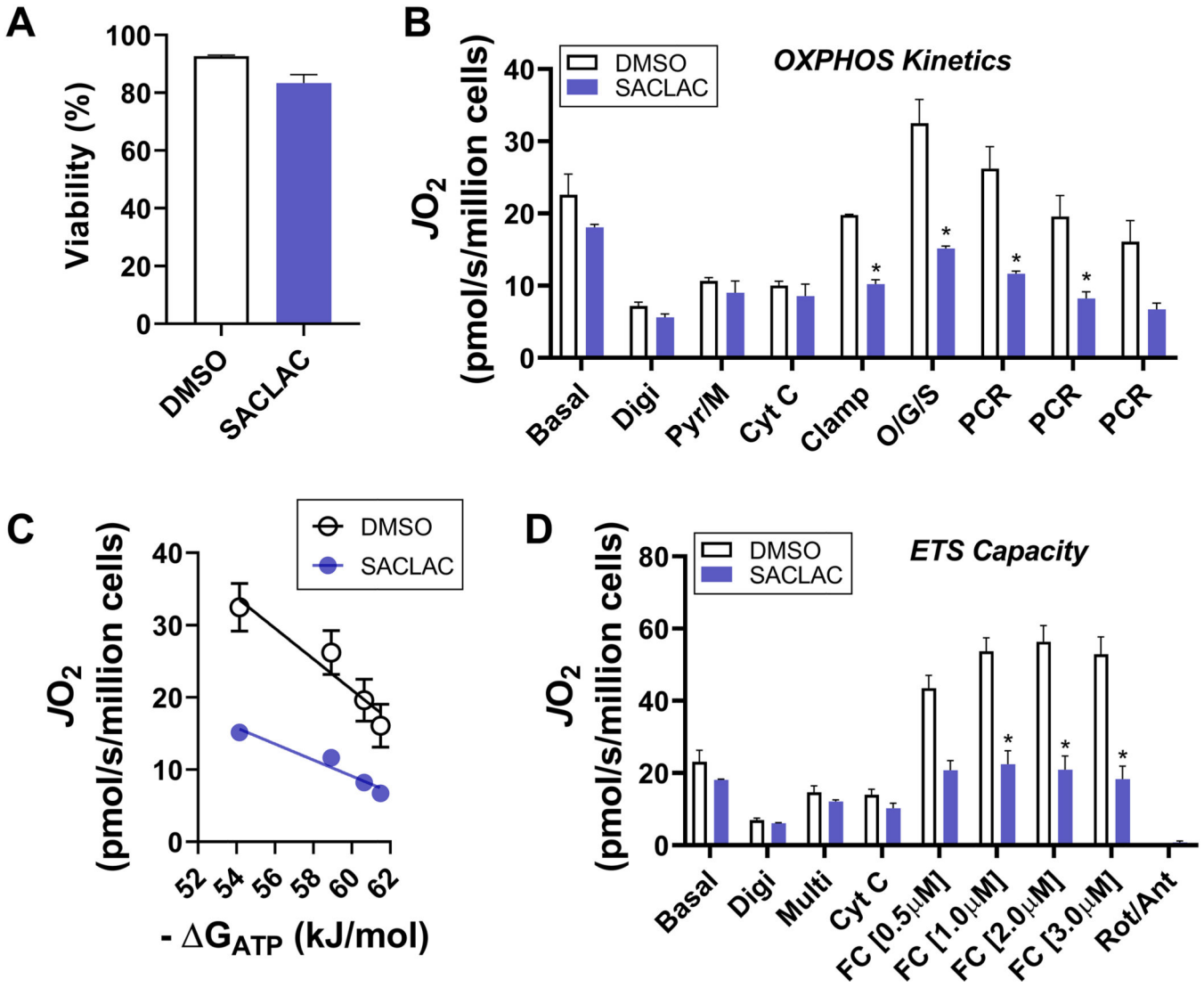

Fig. 6. Effect of SACLAC exposure on mitochondrial bioenergetics in drug-resistant leukemia.

HL-60/dnr cells (resistant to daunorubicin) (800,000/ml dnr-free, RPMI-1640 medium, 10% FBS) were exposed to 10 μM SACLAC (DMSO vehicle) or DMSO (control) for 24 hr. Cells were then harvested by centrifugation, washed three times in PBS, and subjected to bioenergetic characterization. For respiration experiments, cells were suspended in a potassium-based respiration buffer, permeabilized with digitonin (10μg/mL), and energized with various carbon substrates and respiratory stimuli/inhibitors. All data were normalized to live cell count. **A.** Cell viability. **B.** Respiration under basal conditions, as well as in response to digitonin (Digi, 10μg/mL), pyruvate/malate (Pyr/M, 5mM/1mM), cytochrome c (Cyt C, 10μM), CK clamp (CK 20U/mL; phosphocreatine, PCR, 1mM; ATP, 5mM), octanoyl-carnitine/glutamate/succinate (O/G/S, 0.2mM/5mM/5mM); and multiple PCR additions to titrate ATP free energy (ΔG_{ATP}) across a physiological span. **C.** Relationship between oxygen consumption (JO_2) and ATP free energy (ΔG_{ATP}). Calculation of ΔG_{ATP} done using the online resource https://dmpio.github.io/bioenergetic-calculators/ck_clamp/. D. Respiration under basal conditions, as well as in response to digitonin (Digi, 10μg/mL),

Pyr/M/O/G/S (Multi), cytochrome c (Cyt C, 10μM), and FCCP titration (0.5–3.0μM). Rotenone (0.5μM) and antimycin A (0.5μM) were added at the end to control from any non-mitochondrial respiration. Data are mean ± SEM, N=3/group, *P<0.05.