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Review | Revisión

The mitochondrial uncoupling as a promising pharmacological target against cancer

[El desacoplamiento mitocondrial como prometedor blanco farmacológico]

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Abstract

Context: Mitochondria represent a key intracellular signalling hub that is emerging as important determinants of numerous aspects of cancer development and progression. In this sense, the organelle constitutes a promising target for the development of novel anticancer agents. Despite the negative history, mitochondrial uncoupling has recently proposed as a pharmacological target against cancer, but little is known about the mechanisms involved.

Aims: To demonstrate the role of mitochondria in tumor formation and to describe how targeting of mitochondria uncoupling can be beneficial in the therapy of these diseases, which affect a large human population.

Methods: The data for this systematic review were collected from three popular databases including PubMed, Google Scholar, and Scopus and included in the search terms the words "mitochondrial uncoupling" and "cancer". The influence of mitochondrial uncoupling on cells physiology that could lead to cancer cells death, invasion and metastasis was critically appraised from relevant researches. The anticancer effects of small molecules mitochondrial uncouplers and their anticancer mechanisms were also discussed. The present dataset finally included 201 published articles.

Results: It was found that the mitochondrial uncoupling-mediated responses possibly involved in the anti-cancer/anti-metastatic effects include Ca^{2+} homeostasis and bioenergetics disruption, mitochondrial membrane potential dissipation, reactive oxygen species generation, mitochondrial dynamics alteration, and gene expression modulation.

Conclusions: Overall, this critical review suggests that mitochondrial uncoupling could be an interesting pharmacological target to be considered in the design and synthesis of novel anti-cancer compounds with optimal physic-chemical and biopharmaceutical properties and improved safety profiles.

Keywords: cancer; mitochondria; mitochondrial uncoupling, systematic review.

Resumen

Contexto: Las mitocondrias representan un centro de señalización intracelular clave que emerge como determinante en numerosos aspectos del desarrollo y la progresión del cáncer. Así, el orgánulo constituye un blanco terapéutico prometedor para el desarrollo de nuevos agentes anticancerosos. A pesar de su historia negativa, el desacoplamiento mitocondrial se ha propuesto como un blanco farmacológico contra el cáncer, pero se sabe poco acerca de los mecanismos involucrados.

Objetivos: Demostrar el papel de las mitocondrias en la formación de tumores y describir cómo el desacoplamiento puede ser beneficioso en el tratamiento de esta enfermedad.

Métodos: Los datos para esta revisión se colectaron desde tres bases de datos, PubMed, Google Scholar y Scopus, e incluyó en los términos de búsqueda las palabras "desacoplamiento mitocondrial" y "cáncer". La influencia del desacoplamiento mitocondrial en la fisiología tumoral que podría conducir a la muerte, invasión y metástasis de las células cancerosas se evaluaron críticamente. También se discutieron los efectos anticancerígenos de algunos desacopladores mitocondriales y sus mecanismos de acción. El presente conjunto de datos finalmente incluyó 201 artículos publicados.

Resultados: Las respuestas mediadas por desacoplamiento mitocondrial involucradas en los efectos anticancerosos/antimetastásicos incluyen la alteración de la homeostasis del Ca^{2+} y la bioenergética mitocondrial, la disipación del potencial de la membrana, la generación de especies reactivas de oxígeno, y la alteración de la dinámica mitocondrial y la expresión génica.

Conclusiones: Esta revisión crítica sugiere que el desacoplamiento mitocondrial podría ser un blanco farmacológico interesante a considerar en el diseño y síntesis de nuevos compuestos anticancerígenos con propiedades físico-químicas y biofarmacéuticas óptimas y perfiles de seguridad mejorados.

Palabras Clave: cáncer; mitocondria; desacoplamiento mitocondrial, revisión sistemática.

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INTRODUCTION

Cancer is the second leading cause of death in Cuba reaching about 24 % of all registered death, which represents more than 24000 deceased people per year (Bess, 2018). It is an increasing cause of death in the developing world; in the United States, it is also the second cause of death (Arbiser et al., 2017). These numbers are just a few examples indicating an unsolved medical crisis.

After almost a century of many advances, cancer research has generated a rich and complex body of information revealing cancer to be a disease involving dynamic changes in the genome (Hanahan and Weinberg, 2000). However, cancer is not only a genetic but also a metabolic disease (Seyfried and Shelton, 2010). The cancer cells must undergo several transformations in its metabolic activity in order to survive and to meet the special needs of rapid and uncontrolled proliferation. Hence, a profound knowledge of cancer cell metabolism is invaluable in the comprehension of this pathology, as originally was proposed by Warburg (Warburg, 1923; 1925; 1927; 1956; López-Lázaro, 2008; Ferreira, 2010). On the other hand, mitochondria are ubiquitous intracellular organelles that regulate important cellular functions including not only bioenergetics metabolism but Ca^{2+} homeostasis and apoptosis. Given this central role, it is not surprising that cancer cells often exhibit some type of mitochondrial dysfunction, including mitochondrial DNA mutations, alterations in energy metabolism, elevated reactive oxygen species (ROS) generation, and increased mitochondrial membrane potential (Modica-Napolitano and Singh, 2004; Seyfried and Shelton, 2010; Boland et al., 2013). These changes suggest that targeting mitochondria for tumor treatment may lead to a breakthrough in the management of malignancies (Gogvadze et al., 2008; 2009; Pathania et al., 2009; Neuzil et al., 2013; Wen et al., 2013; Cui et al., 2017). In fact, it has been proposed a classification of anti-cancer agents that act via mitochondrial destabilization (mitocans, an acronym for mitochondria and cancer), based on the site of action of the individual agents from the surface of the mitochondrial outer membrane to the mitochondrial matrix (Neuzil et al., 2013).

Since 1974 the studies of the mechanism of uncoupling and the mitochondrial components involved in this process were done in order to gain information regarding the details of energy coupling (Hanstein and Hatefi, 1974). Uncoupler compounds like 2,4-dinitrophenol, sodium azide, pentachlorophenol, carbonylcyanide m-chlorophenylhydrazone and 5-chloro-3-t- butyl-2'-chloro-4'- nitrosalicylanilide (Hanstein and Hatefi, 1974), were tested in those days, but any or very few applications were evaluated. Later, in 1999, the uncouplers were associated with an enhanced signal of cell death (Linsinger et al., 1999), and nowadays they constitute an emergent prospect for therapy in different areas as neuroprotection, diabetes, hypertriglyceridemia, fatty liver disease, and cancer (Korde et al., 2005; Han et al., 2008; Pardo-Andreu et al., 2011a; 2011b; Perry et al., 2013; Reis et al., 2014; Figarola et al., 2015; Giralt and Villarroya, 2016; Marín-Prida et al., 2017). On the contrary, the natural occurring uncoupling proteins (UCPs), overexpressed in many cancers (Valle et al., 2010; Donadelli et al., 2015), serve as an important way for reducing oxidative stress promoting adaptation of high proliferative cancer cells and chemoresistance (Baffy, 2010; Baffy et al., 2011). Additionally, UCP2 has been identified as a metabolic tool that makes aerobic cells become glycolytic and use glutamate or fatty acids for their need of mitochondrial energy production, which is rather similar to the metabolic changes seen in cancer cells (Baffy, 2017). In consequence, mitochondrial recoupling mediated by selective inhibition of UCP2 may hinder the benefits of metabolic reprogramming in cancer cells and could be a therapeutic strategy for cancer treatment (Baffy et al., 2011). Therefore, how cancer cells would respond to the induction of extrinsic uncoupling mediated by exogenous agents? The current paper represents a state of the art review of the available knowledge on the exogenous mitochondrial uncoupling and its therapeutic potential to treat and overcome cancer. This review paper also constitutes the first attempt (to the best of our

knowledge) to systematize and organize the experimental data acquired in the last years concerning the above-mentioned topic.

METHODOLOGY

This systematic literature review was made in PubMed, Google Scholar and Scopus databases and included in the search terms the words "mitochondrial uncoupling" and "cancer". The search yielded almost 700 articles, of which 201 were selected for review based mainly on their directed relation to the topic under revision. The influence of mitochondrial uncoupling on cells physiology that eventually could lead to cancer cells death, invasion and metastasis was critically appraised from relevant researches. The anticancer effects of small molecules mitochondrial uncouplers and their anticancer mechanisms were also discussed in the review. The present dataset finally included 201 published articles.

RESULTS AND DISCUSSION

The mitochondrial role on cellular metabolism

The current understanding of ATP synthesis in mitochondria is derived from the chemiosmotic hypothesis introduced by Peter Mitchell in 1961 (Mitchell, 1961; Nelson and Cox, 2008). The mitochondria integrate molecular pathways of energy production, by oxidizing substrate derived from our dietary carbohydrates and fats (β-oxidation) with oxygen, to generate heat and adenosine 5´-triphosphate (ATP). Reducing equivalents generated by the tricarboxylic acid (TCA) cycle or by β-oxidation of fatty acids provide the electrons that are transported along the electron transfer complexes I–IV of the inner mitochondrial membrane (IMM). Two electrons donated from NADH to complex I (NADH dehydrogenase) or from succinate to complex II (succinate dehydrogenase, SDH) are passed sequentially to ubiquinone (coenzyme Q or CoQ) to give ubisemiquinone ($CoQH^{\bullet}$) and then ubiquinol ($CoQH_2$). Ubiquinol transfers its electrons to complex III (ubiquinol: cytochrome c oxidoreductase), which transfers them to cytochrome c. From cytochrome c, the electrons flow to complex IV (cytochrome c oxidase, COX) and finally to ½ O_2 to give H_2O. Each of these electron transport chain (ETC) complexes incorporates multiple electron carriers. Complexes I, II, and III encompass several iron-sulfur (Fe-S) centers, whereas complexes III and IV encompass the b + c_1 and a + a_3 cytochromes, respectively. The energy released by the flow of electrons through the ETC is used to pump protons out of the IMM through complexes I ($4H^+/2e^-$), III ($4H^+/2e^-$), and IV ($2H^+/2e^-$). This creates an electrochemical gradient capacitance across the IMM ($\Delta P = \Delta\Psi + \Delta pH$). The potential energy stored in ΔP is coupled to ATP synthesis by complex V (ATP synthase). Re-entry of protons to the mitochondrial matrix drives the ATP synthase (complex V) that converts adenosine 5´-diphosphate (ADP) to ATP (see Fig. 1). To complete the process, matrix ATP is then exchanged for cytosolic ADP by the adenine nucleotide translocator (ANT).

Major mitochondrial functions such as protein and metabolite transport, oxidative phosphorylation, ion homeostasis, or the initiation and execution of apoptosis critically depend on ΔΨ (Solaini et al., 2011). If mitochondrial respiration is impaired, ATP synthase and ANT may work in reverse mode fueled by mitochondrial ATP uptake and hydrolysis to sustain ΔΨ by pumping protons out of the matrix (Chevrollier et al., 2011).

The mitochondrial role in apoptosis and redox regulation

It is now clear that mitochondria are not only the site of energy metabolism but also play key roles in redox regulation and apoptosis. The mechanism of apoptosis involves mainly two signaling pathways, including the mitochondrial pathway and the cell death receptor pathway (Ashkenazi and Dixit, 1998; Budihardjo et al., 1999; Shi, 2002). The key element in the mitochondrial pathway is the efflux of cytochrome c from mitochondria to cytosol, where it subsequently forms a complex (apoptosome) with Apaf-1 and caspase-9, leading to the activation of the caspase-3 (Mehmet, 2000). The cell death receptor pathway is characterized by binding cell death ligands and cell

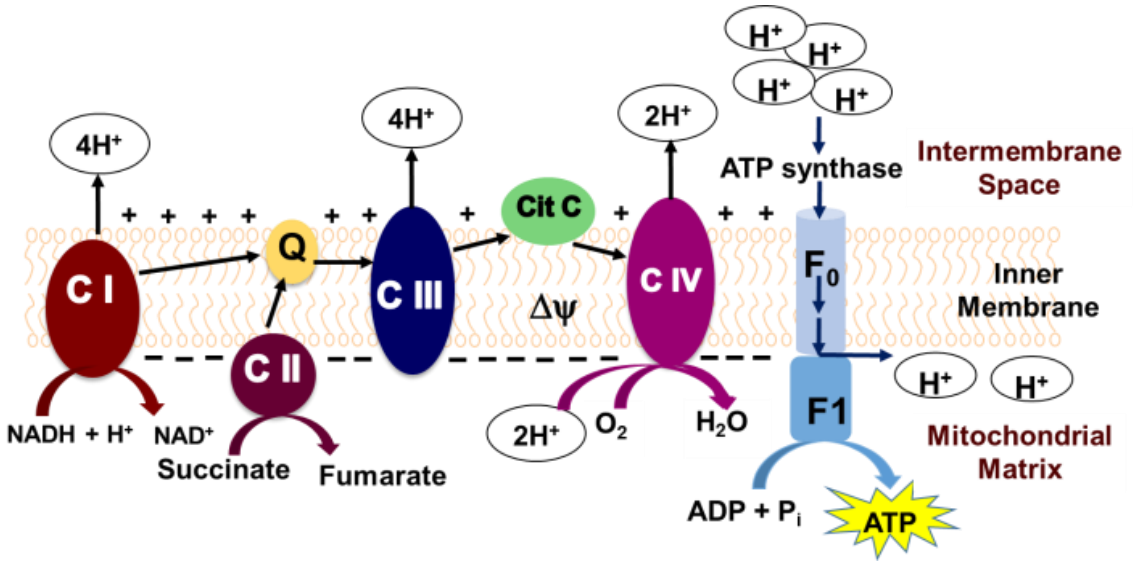


Figure 1. Mitochondrial oxidative phosphorylation.

Substrate-derived electrons flow through complexes I–IV of the electron transport chain embedded in the mitochondrial inner membrane. The energy of this process is used for pumping protons (H^+) from the matrix into the intermembrane space. The resulting proton gradient sustains the mitochondrial membrane potential (ΔΨ), which drives the ATP synthesis.

death receptors, and subsequently activates caspase-8 and caspase-3 (Hengartner, 2000; Liu et al., 2004). Caspase-3 is an executioner caspase, which activation can systematically dismantle cells by cleaving key proteins such as PARP.

As an important collateral event of mitochondrial electron transport, the mitochondria generate reactive oxygen species (ROS), like superoxide ($O_2^{\cdot-}$), through the escape of electrons at complex I from iron-sulfur groups, at complex II from flavin-containing proteins, and at complex III from the ubiquinone cycle (Fridovich, 1997). Unobstructed electron flux gives the electrons less time to reside at sites where superoxide is generated, while higher ΔΨ due to excess substrate supply or mismatched ATP synthase activity results in a longer half-life of ETC intermediates, adding to the risk of ROS generation (Casteilla et al., 2001). The process of $O_2^{\cdot-}$ generation by ETC seems to be highly regulated, and ROS can function both adversely and beneficially. Mitochondrial ROS generation is required for the regulation of many cellular processes like those effectuated by H_2O_2 on cell cycle, stress response, energy metabolism, redox balance, and oncogenic transformation (Hamanaka and Chandel, 2010). Under normal conditions, cells maintain their redox balance through the generation and elimination of ROS using different antioxidant systems (Nicholls, 2009; Hamanaka and Chandel, 2010; Martin, 2010). A large body of evidence indicates that mitochondrial oxidative imbalance is responsible for the development and progression of a series of abnormalities such as aging, diabetes, inflammatory diseases, hypertension, neurodegenerative and ischemia-related diseases, as well as cancer (Nicholls, 2009; Hamanaka and Chandel, 2010; Martin, 2010; Dikalov, 2011).

Role of uncoupling proteins as antioxidant defense

Superoxide production is very sensitive to changes in ΔΨ and mitochondrial ROS levels can be effectively controlled by the rate of proton reentry (Brand, 1990). A considerable amount of protons may bypass the ATP synthase pathway and leak back to the mitochondrial matrix. This seemingly wasteful dissipation of the proton-motive force as heat energy is termed mitochondrial uncoupling (uncoupling of the mitochondrial electron transport from ADP phosphorylation) (Brand,

1990). This event is mediated partly by UCPs, which decrease ΔΨ with subsequent protection from the ETC-derived generation of ROS (Esteves and Brand, 2005) and represents the first line of antioxidant defense aimed at resolving mismatched outward and inward proton fluxes. Based on its broad tissue distribution, UCP2 has been under particular scrutiny as a potential system-wide regulator of mitochondrial oxidative stress relevant to diverse physiological and pathological processes, including obesity, neurodegenerative diseases, aging and cancer (Brand and Esteves, 2005; Nubel and Ricquier, 2006; Baffy, 2010). UCP2-mediated proton leak requires activation by superoxide and lipid peroxidation derivatives (Echtay et al., 2002; Brand et al., 2004). Thus, UCP2 may be considered as a sensor and suppressor of mitochondrial ROS, with increasing functional impact at increasing levels of oxidative stress.

Alterations of the mitochondrial bioenergetics in cancer cells

One of the first studies on the energy metabolism of a tumor was carried out by Otto Warburg (1923). This scientist noted in tumor slices that cancer cells were performing lactic fermentation, even in aerobic conditions (Warburg, 1924). Lactic fermentation occurs exclusively in the cytosol. The glucose ($C_6H_{12}O_6$) is initially converted to pyruvate ($C_3H_5O_3$) through glycolysis. Pyruvate is subsequently reduced to lactate ($C_3H_7O_3$), which is excreted into the bloodstream. Warburg was also the first describing that tumor masses present a deficient blood supply of glucose and oxygen, being the percentage of consumed glucose higher than in normal tissues (Warburg, 1927). Cells belonging to different tissues have distinct capacities to capture glucose and subsequently, diverse ability to utilize it in glycolysis and importantly, in other metabolic pathways.

Pentose phosphate pathway (PPP) is another important route for glucose consumption that may lead to the production of lactate, via replenishment of glycolytic intermediates (Nelson and Cox, 2008). Starting from glucose 6-phosphate, NADPH and ribose 5-phosphate are produced, something that is not attainable neither by glycolysis nor by complete glucose oxidation. NADPH is essential to maintain the high rate of fatty acids synthesis observed in cultured tumor cells (Lazo, 1981) and it is an important co-factor for the antioxidant machinery against ROS (Ahmad, 2005). On the other hand, ribose 5-phosphate is the precursor of nucleic acids bases and its biosynthesis is a constant demand for rapidly proliferating cells (Ramos-Montoya, 2006). A physiological consequence of increased lactate production is the diminution of interstitial pH (Warburg, 1927). Lactate production and consequent induced acidosis in healthy tissues is a significant way of tumor invasion. It enhances malignant progression by reducing cell adherence and providing spaces through each cell can move (Stern, 2002). Besides, augmentation of lactate inhibits pyruvate dehydrogenase, promoting the glycolytic pathway. However, lactic acid may not account alone for tumor acidosis, another mechanism of lactate-independent extracellular acidification in cancer cells are mediated by the extrusion of protons via surface F_1F_0 ATPase (Kroemer and Pouyssegur, 2008).

An anaerobic medium provokes an enhanced proliferation in tumor cells, through downregulation of p53, a tumor suppressor gene (Schmaltz, 1998). Solid tumors present high levels of hypoxia-inducible factor HIF-1. It is a heterodimeric protein responsible for sensing oxygen levels, modulating cellular responses to hypoxia, which induces the overexpression of glycolytic enzymes, lactate dehydrogenase, and carbonic anhydrase IX. The last one is a membrane-bound ectoenzyme that converts bicarbonate to CO_2 in response to low extracellular pH, whilst inhibiting pyruvate dehydrogenase (Semenza, 2009). However, it was proposed, many years ago, that the repeated exposure of cancer cells to periods of hypoxia rendered them more aggressive and resistant, while continued hypoxia per se could ultimately kill them (Warburg, 1956). Short periods of hypoxia are sufficient to stabilize HIF-1, while long hypoxic periods actually decrease its stability (Berra, 2001). Furthermore, hypoxia-reoxygenation cycles enhance tumor cells survival and proliferation through ROS-activated proteins produced in reoxygenation phase, inhibition of apoptotic proteins

and increased resistance of endothelial cells that are associated with neoplastic ones (Toffoli and Michiels, 2008). Additionally, it was demonstrated that targeted depletion of mitochondrial DNA that encodes several ETC components weaken cancer cells (Schulze and Harris, 2012). The extent of utilization of the Warburg phenotype possibly depends on the stage of malignancy, as is evidenced by the "reversion of Warburg effect" observed in metastatic cells (Sotgia et al., 2012). Thus, oxidative phosphorylation is an important physiological parameter to maintain the survival and cell proliferation, and a relevant therapeutic target.

Role of uncoupling proteins in cancer cells

Progressive changes in ROS production are observed during malignant transformation and progression (Szatrowski and Nathan, 1991; Toyokuni et al., 1995). It is realized by driving DNA damage and genomic instability, and by activating signaling networks, like receptor tyrosine kinases (RTKs) and protein tyrosine kinases (PTKs), redox-sensitive transcription factors, like HIF-1 and nuclear factor-erythroid 2-related factor 2 (NRF2), that promote tumor cell proliferation, survival, angiogenesis, altered metabolism, and invasiveness (Liu, 2006; Figueira et al., 2013). It has been noted a decrease in ROS production in the mitochondrial matrix, which could be explained as a result of the metabolic shift from oxidative phosphorylation to glycolysis (Figueira et al., 2013). Alternatively, but not mutually exclusively, since tumor cells actively produce high levels of ROS and are continuously exposed to endogenous oxidants, they also develop mechanisms to protect themselves from intrinsic oxidative stress. Higher levels of ROS-scavenging enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx) and peroxiredoxin (Prx), and antioxidant molecules have been observed in tumors compared with normal tissues (Janssen et al., 2000; Hu et al., 2005). Such adaptation contributes to malignant progression and resistance to anticancer therapy (Schumacker, 2006; Wu, 2006; Landriscina et al., 2009). Similarly, UCP2 is overexpressed in several chemoresistant cancer cell lines (Ayyasamy et al., 2011; Donadelli et al., 2015). UCP2 reduce $\Delta\Psi$ in this scenario (compensating the hyperpolarized cancer cells mitochondrial membranes) and decrease ROS production (Donadelli et al., 2015), forming part of a feedback mechanism that shunts excessive ROS production (Echtay et al., 2002). Suppression of UCP2 (siRNA) results in greater ROS levels and the induction of apoptosis in cancer cells (Deng et al., 2012). Many anti-cancer drugs exert their effect by generating ROS and UCP2 inhibition potentiates their effect (Derdak et al., 2008) undermining the cancer cell's ROS mitigation apparatus. Genipin is a UCP2 inhibitor, which sensitizes cancer cells to cytotoxic agents and induces apoptosis via increased ROS (Kim et al., 2005; Hong and Kim, 2007; Baffy, 2010; Mailloux et al., 2010). Additionally, UCP2 exports pyruvate, oxaloacetate, and related C_4 compounds from mitochondria, denying them to aerobic respiration and helping the switch to aerobic glycolysis (Ayyasamy et al., 2011; Vozza et al., 2014). The above mentioned UCP2 role in cancer cells could give two different therapeutic approaches: the reduction in its activity, which could hyperpolarize $\Delta\Psi$, increasing ROS; or increasing its expression/activity, which could depolarize $\Delta\Psi$ leading to apoptosis. Treatment with low doses of uncoupler compounds can replicate both options in a dose dependent manner. It has been reported that low doses of the exogenous uncoupler FCCP can prevent ROS and apoptosis, while higher doses produce cell death (Derdak et al., 2008).

Chemical uncouplers as potential anti-cancer drugs

Pharmacological use of synthetic and naturally occurring uncouplers, with the potential to compromise mitochondrial functions affecting cellular bioenergetics and metabolism, have lately shown promising anticancer effects. Exogenous protonophoric uncouplers, similar to UCPs, transport protons across the inner mitochondrial membrane and dissipate the proton motive force as heat. One problem in cancer therapy is the accumulation of DNA mutations, which confer drug resistance. It is a problem for drugs that target or rely on DNA encoded proteins. The cancer cells develop DNA mutations, which confer a change in the protein

structure and the drug can no longer interact with it in the same way. Protonophores (protein-independent uncouplers that transport protons across the IMM), and other kinds of uncouplers, do not interact with or rely on proteins to collapse ΔΨ and to kill cancer cells.

In general, mitochondrial uncouplers dissipate the transmembrane proton gradient required for the coupling between electron transport and oxidative phosphorylation (OxPhos). In consequence, they stimulate mitochondrial respiration, increasing the oxygen consumption rate and decreasing ΔΨ and ATP levels. These effects are common in many structurally divergent uncoupling compounds, even with different uncoupling mechanisms (see Table 1). Protonophoric uncouplers translocate protons across the IMM avoiding its pass through the complex V. The protonated uncoupling compound (weak acid or basis) may diffuse (due to its high log P value) from the mitochondrial intermembrane space (high H^+ concentration) into the matrix (relatively low H^+ concentration), dissociate and diffuse back in its ionized form to the intermembrane space where it may be protonated again repeating the cycle. Non-protonophoric uncoupling compounds activate proton leak through protein complexes, such as the ANT or UCPs, or by affecting IMM permeability promoting perturbations on the lipid membrane organization.

It is noteworthy that cancer cells death mediated by uncouplers is initially associated to a mitochondrial membrane potential dissipation and decreased mitochondrial ATP levels, suggesting that the cytotoxic action of these compounds involves mitochondrial impairment (Pardo-Andreu et al., 2011a; 2011b; Reis et al., 2014; Marín-Prida et al., 2017), a well-known event in the mechanisms that lead to apoptosis (Arciuch et al., 2012). Table 1 shows a representative (not exhaustive) sample of anti-cancer uncoupling compounds and some of its biological effects.

Anti-cancer activity of the classical mitochondrial uncouplers 2,4-dinitrophenol (DNP), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), and carbonyl cyanide m-chlorophenyl hydrazine (CCCP)

The uncoupling potency of new compounds under investigation is frequently compared with the classic protonophoric uncouplers DNP, CCCP, and FCCP. For this reason, it is important to know the mitochondria-related mechanisms of cell death induction triggered by these classic compounds, in order to establish appropriate conclusions of the uncoupling efficiency of the new ones.

Table 1. Representative examples of anti-cancer uncoupling compounds and its biological effects.

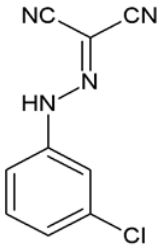
Uncoupling compounds	Structure and log P	Biological effects	References
CCCP	 Log P = 3.4	- Decreases ΔΨ - Decreases ATP. - Apoptosis. - Decreases Ca^{2+} uptake and promotes Ca^{2+} release. - Promotes mitochondrial swelling - Increases membrane fluidity - Reduces the basal rate of ROS generation. - Decreases HIF-1 transcription.	(Graaf et al., 2004; Pardo-Andreu et al., 2011a; 2011b; Hsu et al., 2013)

Table 1. Representative examples of anti-cancer uncoupling compounds and its biological effects (continued...)

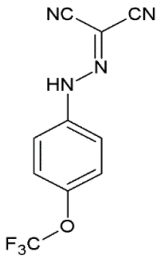
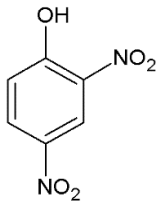
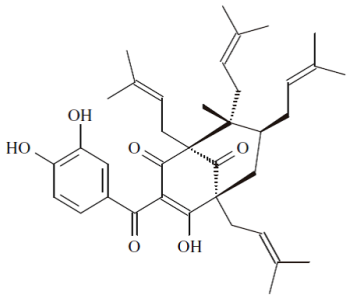
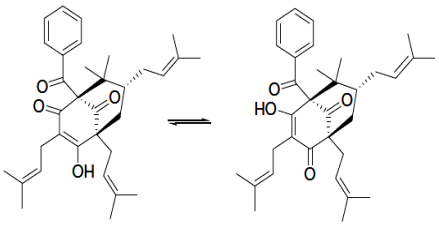
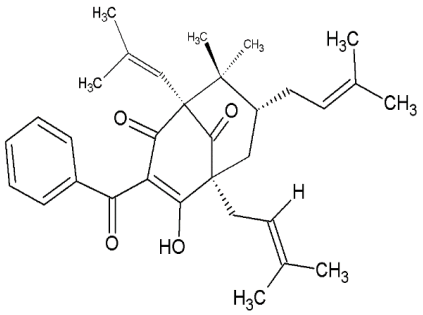
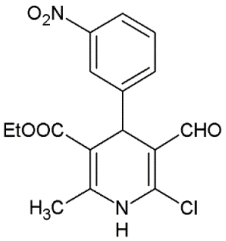
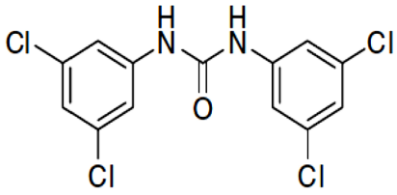
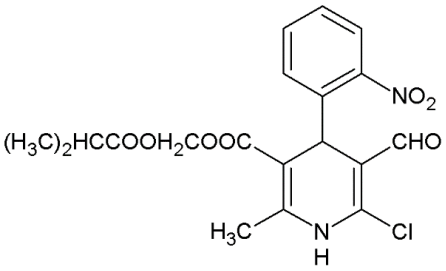
Uncoupling compounds	Structure and log P	Biological effects	References
FCCP	 Log P = 3.7	- Decreases ΔΨ - Decreases ATP. - Apoptosis. - Decreases HIF-1 transcription. - Sensitizes mitochondria to Ca^{2+}-induced MPT. - Activates the AMPK-mTOR signaling pathways. - Induces cell cycle arrest.	(Kuruvilla et al., 2003; Thomas and Kim, 2007; Han et al., 2009; Figarola et al., 2015
2, 4-Dinitrophenol	 Log P = 1.7	- Decreases ΔΨ - Decreases ATP. - Apoptosis. - G1 phase arrest. - Increases $O_2^{\cdot-}$ and H_2O_2. - Depletes the intracellular GSH content.	(Han et al., 2008)
Guttiferone-A	 Log P = 10.4	- Decreases ΔΨ - Decreases ATP. - Apoptosis. - Decreases Ca^{2+} uptake and promotes Ca^{2+} release. - Promotes mitochondrial swelling - Increases ROS levels. - Decreases NADPH levels. - Increases membrane fluidity.	(Pardo-Andreu et al., 2011a)
Nemorosone	 Log P = 8.3	- Decreases ΔΨ - Decreases ATP. - Apoptosis. - Decreases Ca^{2+} uptake and promotes Ca^{2+} release - Promotes mitochondrial swelling (protonophoric action). - Reduces the basal rate of ROS generation	(Pardo-Andreu et al., 2011b)

Table 1. Representative examples of anti-cancer uncoupling compounds and its biological effects (continued...)

Uncoupling compounds	Structure and log P	Biological effects	References
Clusianone	 Log P = 9.1	- Decreases ΔΨ - Decreases ATP. - Apoptosis. - Decreases Ca^{2+} uptake and promotes Ca^{2+} release - Promotes mitochondrial swelling (protonophoric action) - Decreases the rate of mitochondrial ROS generation. - Decreases NADPH levels.	(Reis et al., 2014)
VE-3N	 Log P = 3.1	- Decreases ΔΨ - Decreases ATP. - Apoptosis. - Decreases Ca^{2+} uptake and promotes Ca^{2+} release - Promotes mitochondrial swelling (protonophoric action) - Increases membrane fluidity.	(Marín-Prida et al., 2017)
SR4	 Log P = 5	- Decreases ΔΨ - Apoptosis. - Increases ROS levels. - Promotes mitochondrial swelling (protonophoric action). - Increases extracellular acidification rates. - Decreases ATP. - Activates the AMPK-mTOR signaling pathways. - Induces cell cycle arrest.	(Figarola et al., 2015)
VdiE-2N	 Log P = 3.4	- Decreases ΔΨ - Decreases ATP. - Apoptosis - Increases the number of mitochondria (mitochondrial fission). - Reduces invasion in metastatic cell line. - Affects angiogenesis signaling. - Reduces the volume of xenograft tumor. - Affects genetic regulation.	(Goto et al., 2018)

It has been demonstrated that the classical mitochondrial uncouplers induce apoptosis in several cell types (Marton et al., 1997; Dispersyn et al., 1999; Linsinger et al., 1999; Armstrong et al., 2001; Graaf et al., 2004; Han et al., 2008; Han et al., 2009). DNP and FCCP, for example, arrest the cell cycle at the G1 phase, dissipate mitochondrial membrane potential and induce apoptosis in Calu-6 cells (Han et al., 2008; 2009). DNP- and FCCP-induced cell cycle arrest has been associated with induction of p27 and reduction of CDKs and cyclin proteins (Han et al., 2008; 2009). CCCP-induced apoptosis is mediated by both its direct action on mitochondria (Graaf et al., 2004) and a Fas-mediated death signaling (Linsinger et al., 1999). Since mitochondrial uncoupling may cause membrane permeabilization, apoptosis induction by uncouplers may be via cytochrome c release from mitochondria into cytosol followed by activation of caspases.

The DNP treatment on Calu-6 (lung cancer cells) showed that the uncoupler primarily gives damage to the mitochondria of target cells, and then make progress to next step of apoptosis such as is indicated for the phosphatidylserine exposure. The loss of mitochondrial membrane potential following treatment with DNP activated the caspases (via cytochrome c efflux) and consequently induced apoptosis. Besides, DNP increased intracellular H_2O_2 and O_{2^-}, which is also responsible for apoptosis induction in Calu-6 cells. Treatment with DNP also reduced the GSH content. The caspases inhibitors prevented partly the depletion of GSH content, supporting that intracellular GSH level is a decisive factor in DNP-induced cell death. On the other hand, at lower doses, DNP induced G_1 phase arrest mediated by the decrease of Cdks (cyclin-dependent kinases) supplying, cyclin proteins and an increase of p27 (a member of Cdk inhibitors) (Han et al., 2008).

Anti-cancer activity of nemorosone, clusianone, guttiferone-A and Cuban propolis on HepG2

Nemorosone is a naturally occurring polycyclic polyprenylated acylphloroglucinol that has been receiving increasing attention due to its potent *in vitro* anti-cancer action. We demonstrated the toxic effect of nemorosone (1–25 µM) on HepG2 cells by means of the MTT assay, as well as early mitochondrial membrane potential dissipation and ATP depletion in this cancer cell line. In mitochondria isolated from rat liver, nemorosone (50–500 nM) displayed a protonophoric uncoupling activity, showing a potency comparable to CCCP. Nemorosone enhanced the succinate-supported state 4 respiration rate, dissipated mitochondrial membrane potential, released Ca^{2+} from Ca^{2+}-loaded mitochondria, decreased Ca^{2+} uptake and depleted ATP. The protonophoric property of nemorosone was attested by the induction of mitochondrial swelling in hyposmotic K^+-acetate medium in the presence of valinomycin. In addition, uncoupling concentrations of nemorosone in the presence of Ca^{2+} plus ruthenium red induced the mitochondrial permeability transition process. We showed for the first time that this molecule is a new potent protonophoric mitochondrial uncoupler and this property is potentially involved in its toxicity on cancer cells (Pardo-Andreu et al., 2011a). Consequently, we found that Brown Cuban propolis, which is a natural reservoir of nemorosone, also showed important cytotoxic and uncoupling activities on HepG2 cells (Pardo-Andreu et al., 2015).

Clusianone is a regioisomer analog of nemorosone. Their structural differences occur at the position of the benzoyl group in the bicyclo ring; the benzyl group is at the C_3 and C_1 position for clusianone and nemorosone, respectively. This characteristic of clusianone favors the formation of an intramolecular hydrogen bond involving the hydroxyl group at the C_4 position. We found that the cytotoxic and uncoupling actions of clusianone were appreciably less than those of nemorosone, likely due to the presence of an intra-molecular hydrogen bond with the juxtaposed carbonyl group at the C_{15} position that limited its protonophoric action. Therefore, clusianone is capable of pharmacologically increasing the leakage of protons from the mitochondria and with favorable cytotoxicity in relation to nemorosone (Reis et al., 2014).

On the other side, guttiferone A (GA), a structurally related compound, have a quite different

uncoupling mechanism. In isolated rat-liver mitochondria, we found that this molecule promoted membrane fluidity increase, cyclosporine A/EGTA-insensitive membrane permeabilization, uncoupling (membrane potential dissipation/state 4 respiration rate increase), Ca^{2+} efflux, ATP depletion, NAD(P)H depletion/oxidation and ROS levels increase. All effects in cells, except mitochondrial membrane potential dissipation, as well as NADPH depletion/oxidation and permeabilization in isolated mitochondria, were partially prevented by the NAD(P)H regenerating substrate isocitrate. The results suggest the following sequence of events: 1) GA interaction with mitochondrial membrane promoting its permeabilization; 2) mitochondrial membrane potential dissipation; 3) NAD(P)H oxidation/depletion due to inability of membrane potential-sensitive $NADP^+$ transhydrogenase of sustaining its reduced state; 4) ROS accumulation inside mitochondria and cells; 5) additional mitochondrial membrane permeabilization due to ROS; and 6) ATP depletion. These GA actions are potentially implicated in its well-documented anti-cancer property (Pardo-Andreu et al., 2011a).

The mitochondrial permeability transition (MPT) could be the common event potentially implicated in nemorosone, clusianone, GA, and propolis toxicities on cancer cells. It has been proposed that the drop of mitochondrial membrane potential due to uncoupling increases the probability of the permeability transition pore (PTP) opening due to its voltage-sensitive property (Bernardi, 1992; Petronilli et al., 1993; 1994). At low membrane potentials the NAD(P)H transhydrogenase cannot sustain high levels of mitochondrial reducing power, thus favoring Ca^{2+}-induced ROS accumulation, thiol cross-linkage and MPT (Vercesi, 1987). Therefore, due to its uncoupling property under specific conditions such as those observed in the presence of ruthenium red (Ca^{2+} retention), nemorosone could sensitize mitochondria to Ca^{2+}-induced MPT. Indeed, 4,6-dinitro-o-cresol- or FCCP-induced uncoupling of Ca^{2+}-loaded mitochondria treated with ruthenium red caused a non-specific cyclosporine A-sensitive mitochondrial membrane permeabilization (Bernardi, 1992; Petronilli et al., 1994; Castilho et al., 1997).

Anti-cancer activity of SR4

SR4 is a novel compound with promising therapeutic potential against leukemia, melanoma, hepatocarcinoma and lung cancer (Figarola et al., 2012; 2015; Singhal et al., 2012; 2013). SR4-induced cell death in HepG2 cells in relation to mitochondrial and bioenergetics dysfunction, probably as upstream signals for its anticancer properties based on indirect AMPK (AMP-dependent kinase) activation (Figarola and Rahba, 2013). The decrease in cellular ATP production by SR4 triggers the activation of AMPK. The uncoupling of OxPhos also affects mTOR (mammalian Target of Rapamycin) signaling. An association of AMPK and mTOR signaling pathways in cancer growth and proliferation is well documented (Inoki et al., 2012; Chen et al., 2014) and as expected, these uncoupling mediated effects modulated a number of key genes associated with proliferation and cell metabolism. Other effects as induction of MPT, ROS generation (independent of MPT) and cell cycle arrest were also exerted by SR4 (Figarola and Rahba, 2013).

Anti-cancer activity of VE-3N and VdiE-2N

1,4-Dihydropyridines (DHPs) constitute a valuable class of calcium channel blocking agents, commonly used for the treatment of cardiovascular diseases (Triggle, 2003). Scientists have recently proved that these compounds may represent a new class of multidrug resistance (MDR) reversal agents for the treatment of different types of cancer (Kawase et al., 2002), but their intrinsic cytotoxic mechanisms remain unclear. It was recently observed that VE-3N [ethyl 6-chloro-5-formyl-2-methyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3-carboxylate] induced mitochondrial membrane potential dissipation, ATP depletion, annexin V/propidium iodide double labelling, and Hoechst staining; events indicating apoptosis induction on HepG2 cells. In isolated rat liver mitochondria, VE-3N promoted mitochondrial uncoupling by exerting protonophoric actions and by

increasing membrane fluidity. These results indicate that mitochondrial uncoupling is potentially involved in the VE-3N cytotoxic actions towards HepG2 cells (Marín-Prida et al., 2017). On the other side, its structural derivative VdiE-2N ((isobutyryloxy)methyl 6-chloro- 5-formyl-1,4-dihydro-2-methyl-4-(2-nitrophenyl)pyridine-3-carboxylate)) demonstrated anti-cancer activity in head and neck squamous cell carcinoma cell lines (HN13, HN12, HN6, and CAL27) without a direct uncoupling effect on mitochondria. Nevertheless, the organelles seem to be involved since in HN13 cells, VdiE-2N dissipated mitochondrial membrane potential, altered the mitochondria size, shape, and number in such a way that induced mitochondrial fission and apoptosis in a concentration-dependent manner, and reduced their invasion capacity. Treatment of mice bearing xenograft tumors with VdiE-2N significantly diminished proliferation of cancer cells (Goto et al., 2018). The consistency of the *in vitro* and *in vivo* anticancer effect observed, associated with the novelty of their chemical structure, point out to both DHPs as lead compounds for the design of new, more selective, and less toxic anticancer agents (Goto et al., 2018).

The mitochondrial uncouplers exert anti-cancer activity modulating several physiological pathways

The uncoupler discussed before modulates several physiological pathways like calcium homeostasis, ROS suppression/generation, mitochondrial dynamic, genes expression, etc. These pathways are modified in cancer cells and play a key role in cancer cell progression and invasiveness. Therefore, modulating them by exogenous uncoupling could be a plausible therapeutic alternative to overcome cancer that deserves further researches.

Modulation of calcium homeostasis

The calcium cellular homeostasis depends on intracellular organelles like ER and mitochondria. They are such tightly interconnected that carry out an interorganellar Ca^{2+} signaling called "ER-mitochondrial Ca^{2+} communication" (ER-Mt) (White, 2017). The ER-localized inositol 1,4,5-trisphosphate receptor (IP3R) delivers Ca^{2+} to the mitochondria (Rizzuto et al., 1998; 2012). Different structural elements link both membranes (Csordás et al., 2006; Brito and Scorrano, 2008) , and the interactions between proteins bond the ER release and mitochondrial uptake machinery (Szabadkai et al., 2006; Betz et al., 2013). To reach the intermembrane space, Ca^{2+} moves across the outer mitochondrial membrane through the voltage-dependent anion channel (VDAC) (Rapizzi et al., 2002; Colombini, 2012) and to get to the matrix, Ca^{2+} moves across the IMM through the mitochondrial Ca^{2+} uniporter (MCU) (Baughman et al., 2011; Stefani et al., 2011).

That high regulated interorganellar Ca^{2+} homeostasis could be modulated by exogenous uncoupling since the Ca^{2+} transport into mitochondria is fully depend on $\Delta\Psi$ (Nicholls and Chalmers, 2004). In this sense, nemorosone, clusianone, guttiferone-A and VE-3N, due to their uncoupling effects, promoted calcium release from Ca^{2+}-loaded mitochondrial and inhibited the mitochondrial calcium uptake in HePG2 cells (Pardo-Andreu et al., 2011a; 2011b; Reis et al., 2014; Marín-Prida et al., 2017). Another study on calcium and CCCP showed that resting LNCaP prostatic cancer cells responded to CCCP by a biphasic increase in intracellular calcium concentration (Vaur et al., 2000). CCCP releases Ca^{2+} from mitochondrial and non-mitochondrial stores, like ER and more generally IP_3 (inositol 1,4,5-trisphosphate)-sensitive Ca^{2+}-pools, followed by an influx of extracellular Ca^{2+} through the plasma membrane (Vaur et al., 2000). Such Ca^{2+} deregulation by uncoupling could affect a number of important cellular pathways that could be therapeutically harnessed to fight cancer.

Regulation of tumor cell invasion and metastasis

Metastasis is exerted by an arrangement of events known as the invasion-metastatic cascade (Hanahan and Weinberg, 2000; 2011; Talmadge and Fidler, 2010). Ca^{2+} signaling mediated by ER-Mt plays a key role at several points in this cascade. Cancer causes transcriptional and functional changes that often affect key regulators of cytoplasmic Ca^{2+} and alter the ER-Mt (Prevarskaya et

al., 2011; Hoth, 2016). The cancer cells, in order to drive cell migration, rise mitochondrial calcium uptake by increasing different physiological parameter as VDAC expression (or VDAC1 cancer isoform) (Shoshan-Barmatz et al., 2015), type 3 IP_3R cancer isoform (Kang et al., 2010; Shibao et al., 2010) and MCU expression (Hall et al., 2014; Tang et al., 2015; Tosatto et al., 2016). This promotion of calcium uptake is functionally linked to other mitochondrial events like bioenergetics, mitochondrial dynamic and ROS elevation; being all cells motility effectors. The diminution in mitochondrial Ca^{2+} level induced by uncouplers constitutes a promising alternative to stop cell migration. Uncouplers can directly target VDAC, IP_3R and MCU at the same time and may circumvent any adaptive response of tumor cells. In this sense, we recently observed that VdiE-2N elicited an antimetastatic activity, in close association with a mitochondrial membrane potential dissipation (Goto et al., 2018).

Modulation of calcium homeostasis and disruption of membrane lipid organization could reverse the multidrug resistance

The modulation of calcium homeostasis could be related to the reversion of multidrug resistance (MDR) activity. MDR is defined as the resistance of tumor cells to the cytotoxic action of multiple structurally and functionally divergent chemotherapeutic agents (Ueda et al., 1987; Miri and Mehdipour, 2008). Such resistance is considered to be one of the major reasons for the failure of chemotherapy for the majority of cancer patients. Over 90% of patients with metastatic cancer develop MDR (Radadiya et al., 2014). Although the mechanisms of MDR appear to be complex, a major factor contributing to drug resistance in cancer is the classical MDR or ATP-Binding Cassette (ABC)-transporters-mediated MDR. P-glycoprotein (P-gp) mediated MDR is one of the most common causes of the limited effectiveness of chemotherapy (Hamada and Tsuruo, 1986; Rosenberg et al., 2005; Perrin et al., 2007). P-gp, a plasmatic membrane protein, acts as a pump and mediates the efflux of wide classes of chemotherapeutic drugs. A relationship between processes controlled by intracellular calcium homeostasis and P-gp-mediated multidrug resistance has been reported (Sulová et al., 2009). The radioactive $^{45}Ca^{2+}$ uptake was measured in P-gp negative and positive cells in order to determine if there is a link between calcium and P-gp mediated MDR (Sulova et al., 2005). The results showed a greater calcium entrance into P-gp-positive than into P-gp-negative cells and were consistent with another studio made in P-gp-positive MCF-7 cells compared with the negative counterparts (Mestdagh et al., 1994). Furthermore, differences in the contents of several proteins involved in calcium homeostasis associated with P-gp over-expression was observed (Seres et al., 2008). For example, it has been suggested that calmodulin (a calcium signaling regulator protein) inhibitors may reduce the activity of calmodulin kinase II and HIF-1 in a calcium-dependent way, causing a decrease in P-gp expression (Riganti et al., 2009a; 2009b; Sulová et al., 2009). Moreover, calnexin (Ca^{2+}dependent molecular chaperone of the endoplasmic reticulum) is involved in P-gp synthesis in the endoplasmic reticulum (ER) and just a structurally mature P-gp molecule is able to escape to the association with calnexin (Loo et al., 2004). The interaction of calnexin and the non-mature protein molecule depends on the calcium content in the ER (Sulová et al., 2009).

It has been widely documented that DHPs have reversal effects on MDR (Tsuruo et al., 1983; Cornwell et al., 1987; Fedeli et al., 1989; Tanabe et al., 1998), meanly mediated by P-gp inhibition (Shekari et al., 2015). DHP is a pharmacophore (see Fig. 2) with a wide range of pharmacological effects according to its substituents. Since VE-3N has similar structure respect to other MDR reverser as nifedipine (Fedeli et al., 1989), and it interferes with calcium homeostasis, it could be expected an anti-MDR action of this molecule, which is now assessed by our group. Furthermore, VE-3N and guttiferone-A diminished the levels of the mitochondrial membrane fluorescence anisotropy, which reflects the increased mobility of the fluorescence label DPH into membranes and indicates disruption of the membrane structural order. This perturbation of the mitochondrial membrane or-

ganization could affect the plasmatic membrane as well (Marín-Prida et al., 2017), and consequently the P-gp action. All these results might also offer new light into potential effects of the uncoupler compounds against classical MDR activity but required additional studies.

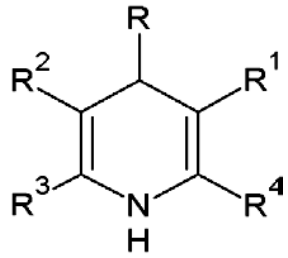


Figure 2. Basic structure of 1,4-dyhidropiridyne derivatives.

Bioenergetics and disruption of calcium homeostasis

Mitochondrial Ca^{2+} activates several Ca^{2+}-dependent enzymes involved in the TCA cycle affecting the ATP synthesis (Jouaville et al., 1999; Cárdenas et al., 2010; Glancy and Balaban, 2012;). It has been proved that the Warburg effect is not absolute and glycolysis typically accounts for no more than 60% of total cellular ATP production in most cancer cells (Busk et al., 2008). Hence, the inhibition of calcium mitochondrial uptake may generate a significant affectation of TCA cycle and could induce cell death or stop the cellular proliferation. It was demonstrated that blocking IP_3Rs in cancer cells inhibited the oxidative phosphorylation and the tumor growth (*in vivo*) (Cárdenas et al., 2016).

Modulation of genes expression

Uncoupling by SR4 affected a number of genes (1,329 up-regulated and 2,815 down-regulated after 24 h of SR4 treatment) involved in apoptosis, cell cycle regulation, and mitochondria and energy metabolism (Figarola et al., 2015). Especially, a number of key genes associated with the downstream effects of AMPK activation and mTOR inhibition were modulated by SR4 including those for decreased lipid and cholesterol synthesis, inhibition of protein synthesis and gluconeogenesis and induction of autophagy/mitophagy. Knockdown of AMPK significantly prevented the cell cycle arrest and uncoupler-mediated apoptosis (Figarola et al., 2015). Similar effects on cell cycle and proliferation have been found in DNP and FCCP treatments (Kuruvilla et al., 2003; Han et al., 2008; Hsu et al., 2013). For example, treatments with CCCP reduced both the protein level and transactivation activity of HIF-1 in HepG2 cells under normoxia or hypoxia, in such a way that treatments with AMPK inhibitor or knock-down of AMPK, partially rescued the mitochondrial dysfunction-repressed HIF-1 expression (Hsu et al., 2013). Additionally, SR4 leads to diversion from OxPhos to glycolytic phenotype, as is evidenced by the immediate increase in extracellular acidification rates upon the uncoupler exposure, down-regulation of numerous OxPhos genes, transcriptional up-regulation of several key glycolytic genes with simultaneous down-regulation of gluconeogenic genes and down-regulation of isoleucine, leucine and valine degradation (substrates to TCA cycle). These metabolic shifts disrupt the aforementioned existent equilibrium between Oxphos and glycolysis, which is required for survival and cell proliferation (Figarola et al., 2015).

ROS modulation

Many chemotherapeutic drugs acting on mitochondria increase organellar ROS levels and promote apoptosis (Pelicano et al., 2003; Trachootham et al., 2006; 2009). When ROS production is limited within the constraints of the cellular redox system (because the cells must maintain moderate ROS to sustain cellular signaling) an acute and substantial ROS increase may have a much more immediate effect and commit the cells to undergo apoptosis (Trachootham et al., 2009).

Two important questions emerge: 1- Are uncouplers ROS inhibitors or inducers? 2-How are ROS generated by uncouplers? In order to address the first question, it can be seen in Table 1 that nemorosone and clusianone slightly reduced the basal rate of ROS generation by the organelle; the relatively more oxidized state of the respiratory chain components of mitochondria undergoing uncoupling makes them less prone to transfer electrons to oxygen (Pardo-Andreu et al., 2011b; Reis et al., 2014). On the other side, guttiferone-A, also

a natural uncoupler, increased ROS levels in isolated rat liver mitochondria probably by its ability to disrupt mitochondrial membranes. NAD(P)H is the major source of reducing equivalents for the antioxidant systems glutathione peroxidase/reductase and thioredoxine peroxidase/reductase. The reduced levels of NAD(P)H in the mitochondrial matrix are controlled by the membrane potential-sensitive $NADP^+$ transhydrogenase (Hoek and Rydstrom, 1988). It is believed that under conditions of low mitochondrial membrane potential, which can be promoted by uncouplers, this enzyme is unable to sustain the reduced levels of NAD(P)H. In fact, nemorosone, clusianone and gutifferone-A induced a depletion/oxidation of NAD(P)H in rat liver mitochondria, but only guttiferone-A promoted an increase in ROS generation. This compound induced mitochondrial membrane potential dissipation that renders transhydrogenase unable to sustain the reduced state of NAD(P)H and allows mitochondria to accumulate ROS. We believe that not only the impairment of NAD(P)H generation, but also its draining through a damaged membrane together with other endogenous antioxidant defenses may contribute to ROS generation/accumulation by uncoupling. In the field of the second question concerning the mechanism of ROS modulation by uncouplers, Figarola et al. (2015) stated that mitochondrial NAD(P)H mediated superoxide production can be ruled out because it was not observed SR4 to induce oxidation of NAD(P)H in isolated liver mitochondria. They demonstrated that pretreatment with 6-KCH (a mitochondria re-coupler agent) significantly prevented ROS generation by SR4, and it suggests that the ROS increase is directly associated with the uncoupling mechanism. They made an RNA-seq analysis and observed that SR4 significantly down-regulated the expression of various components of the mitochondrial ETC, primarily complex I genes as well as an important ROS scavenger SCARA3 (scavenger receptor class A, member) (Figarola et al., 2015). Another potential mechanism is the MPT, which promotes the draining of endogenous antioxidant molecules and reducing equivalents (NAD(P)H) hindering the organelles ability to neutralize ROS. However, uncouplers as SR4 generate ROS even with MPT blockers like cyclosporine A (Figarola et al., 2015). Moreover, because macromolecules within the mitochondria are more inclined to ROS-induced damage due to their close proximity to the source of ROS, the damage exerted by ROS on mitochondrial components may lead to a higher degree of mitochondrial dysfunction and, in turn, to higher ROS production, leading to a vicious cycle of ROS amplification (Figueira et al., 2013).

The aforementioned data suggest the absence of a unique or general explanation for ROS generation by mitochondrial uncoupling, and it implicates that each uncoupler compound, even with the same mechanism of action, could have different effects on ROS production. The modulation of Oxphos genes, MTP opening dependence, NADPH depletion/oxidation, oxidized state of ETC complexes, uncoupling power evidenced by the degree of depolarization, the ability to disrupt mitochondrial membranes should be all considered to explain ROS modulation by uncouplers.

Alteration of mitochondrial dynamics

Mitochondria are dynamic organelles that constantly undergo fusion and fission processes, collectively termed "mitochondrial dynamics". The balance between fission and fusion is required for proper mitochondrial functions due to its involvement in the maintenance of mitochondrial DNA, segregation of damaged mitochondria by mitophagy, distribution and movement of mitochondria within the cell, as well as regulation of mitochondrial morphology (Bereiter-Hahn and Voth, 1994; Twig et al., 2008). Mitochondria form physically interconnected networks that may represent an efficient system to deliver energy, or channel calcium between different areas of the cell (Giorgi et al., 2000; Skulachev, 2001). A decrease in connectivity occurs under conditions that compromise mitochondrial function, such as treatment with mitochondrial toxins (Legros et al., 2002). These shifts from highly branched to fragmented morphologies of mitochondria are most likely regulated by the rates of fission and fusion events (Karbowski and Youle, 2003; Youle and Bliek, 2012). Mitochondrial fission produces small spher-

ical mitochondria morphology and fragmentation, whereas fusion produces tubular or elongated-shaped mitochondria resulting in an increase in mitochondrial connectivity (Sesaki and Jensen, 1999; Karbowski and Youle, 2003; Youle and Bliek, 2012). Mitochondrial fusion is regulated by mitofusins (Mfn1 and Mfn2) and optic atrophy 1 (OPA1) (Chen et al., 2003; Cipolat et al., 2004), while fission is mediated by dynamin-related protein 1 (Drp1; also known as DLP-1), mitochondrial fission 1 protein (Fis-1) and mitochondrial fission factor (Mff) (Smirnova et al., 2001; James et al., 2003; Gandre-Babbe and Bliek, 2008; Otera et al., 2010). Recent studies indicate that mitochondrial fission/fusion machinery actively participates in the process of apoptosis since dysregulation of mitochondrial dynamics leads to mitochondrial fragmentation (Karbowski and Youle, 2003; Arnoult et al., 2005a; 2005b). In this sense, it has been proposed that mitochondrial fragmentation can occur without activation of apoptosis, but apoptosis may not be able to occur without activation of the mitochondrial scission machinery (Karbowski and Youle, 2003). Another work shows that mitochondrial fusion can inhibit apoptosis, whereas mitochondrial fission can promote the latter (Li et al., 2010).

Not much is known about the role of mitochondrial dynamics in cell proliferation and apoptosis in cancer cells. One study conducted in human lung cancer cell lines exhibited an imbalance of Drp-1/Mfn-2 expression, which promoted a state of mitochondrial fission (Rehman et al., 2012). In this work was found that in lung tumor tissue samples from patients there was an increase in Drp-1 and decrease in Mfn-2 in comparison to the adjacent healthy lung (Rehman et al., 2012). Accordingly, it was studied the effects produced by restoration of the mitochondrial network formation by overexpression of Mfn-2, Drp-1 inhibition, or Drp-1 knockdown, and the result was a pronounced reduction of cancer cell proliferation and an increase in spontaneous apoptosis. Also, in a xenotransplantation model, Mfn-2 gene therapy or Drp-1 inhibition could regress the tumor growth. Thus, this study proposed that impaired fusion and enhanced fission contribute fundamentally to the proliferation/apoptosis imbalance in cancer (Rehman et al., 2012).

On the contrary, VdiE-2N-induced mitochondrial fission (previously discussed) was related to its cytotoxic effect (Goto et al., 2018). Considering previous results (Karbowski and Youle, 2003; Rehman et al., 2012) we can conclude that cancer cells may require basal levels of fission but stress-induced activation of fission may be detrimental, promoting excessive mitochondrial fission and apoptosis. Hence, weighing up these information, an equilibrium state may be suggested, in which, on one hand, a treatment with Drp-1 inhibitors could reverse the tumor growth by invaliding tumor's capacity of adaptability, but on the other hand, the exerted stress (increasing the pDRP1 levels) by compounds like VdiE-2N could contribute to desired apoptosis induction by augmenting disadvantageous fission for cancer cells.

Two ways to modulate fission in cancer cells by uncoupling may be proposed, based in some reported observations. The first is through oxidative stress, which induces transient changes in mitochondrial morphology as well as fragmentation of the mitochondrial network (Jendrach et al., 2008; Fan et al., 2010; Wu et al., 2011). With prolonged and persistent cellular oxidative damage, interconnected tubular mitochondrial networks are reorganized as small punctate spheres (fragmentation) due to extensive fission, resulting in apoptosis (Hiu-Ling et al., 2017). The other way is mediated by calcium. Mitochondrial fission is a process promoted by mitochondrial Ca^{2+} accumulation (Hom et al., 2007; Chang et al., 2011). Evidence that MCU plays a role in fission comes from the observation that fission is inhibited by the pharmacological block of the MCU (Liang et al., 2014; Zhao et al., 2015). Mitochondrial Ca^{2+} might influence fission by regulating the activity of Drp1. The ability of Drp1 to promote fission is dependent on phosphorylation at serine 616 (S616) and dephosphorylation of serine 637 (S637) (Cribbs and Strack, 2007; Chang and Blackstone, 2007). Cytoplasmic Ca^{2+} signaling is known to regulate the phosphorylation status of Drp1 through calcineurin-dependent dephosphorylation of S637 (Cribbs and Strack, 2007; Cereghetti et al., 2008),

and blocking the MCU suppress fission by decreasing Drp1 phosphorylation at S616 (Zheng et al., 2017). Drp1 is widely associated with tumor invasion and metastatic potential (Rehman et al., 2012; Zhao et al., 2013; Ferreira-da-Silva et al., 2015; Kashatus et al., 2015), and increased S616 is found in breast cancer and lymph node metastases (Zhao et al., 2013). Although speculative, it is possible that the increased mitochondrial Ca^{2+} uptake in cancer cells linked to invasion and metastasis could be reverted by uncouplers (White, 2017), that inhibit mitochondrial Ca^{2+} uptake and promote Ca^{2+} release and mitochondrial fission.

Integration of the uncoupling compounds effects

Fig. 3 shows an integrated scheme of the effects mediated by mitochondrial uncouplers on cancer cells. There are three interrelated primary effects: (i) the increase in the oxygen consumption rate; (ii) the dissipation of the transmembrane proton gradient (or proton motive force); and (iii) the drop of $\Delta\psi$ due to the entrance of non-associated complex V protons into the matrix. The $\Delta\psi$ drop leads to MPT and apoptosis by cytochrome c release. $\Delta\psi$ fall also affects the calcium homeostasis, which inhibits cell proliferation and metastasis through the decrease in mitochondrial calcium target VDAC, IP_3R, and MCU. This calcium homeostasis modulation could affect the tricarboxylic acids cycle (reducing ATP synthesis) and reverse the MDR by decreasing the expression/activity of P-gp. The uncouplers may both decrease or increase ROS at mitochondrial level. ROS induction promotes apoptosis and may be mediated by MPT induction. Similarly, mitochondrial fission may be promoted or hindered. A fission promotion could be detrimental for tumor cells and may cause apoptosis, and its inhibition could decrease the invasiveness capacity of cancer cells. The ATP levels diminution induces an AMP/ATP imbalance, which modulates the AMPK/mTOR pathway that in turn may drive the expression of key genes involved in apoptosis, cell cycle regulation, and mitochondria and energy metabolism. The latter effect is potentiated by the disruption of calcium homeostasis.

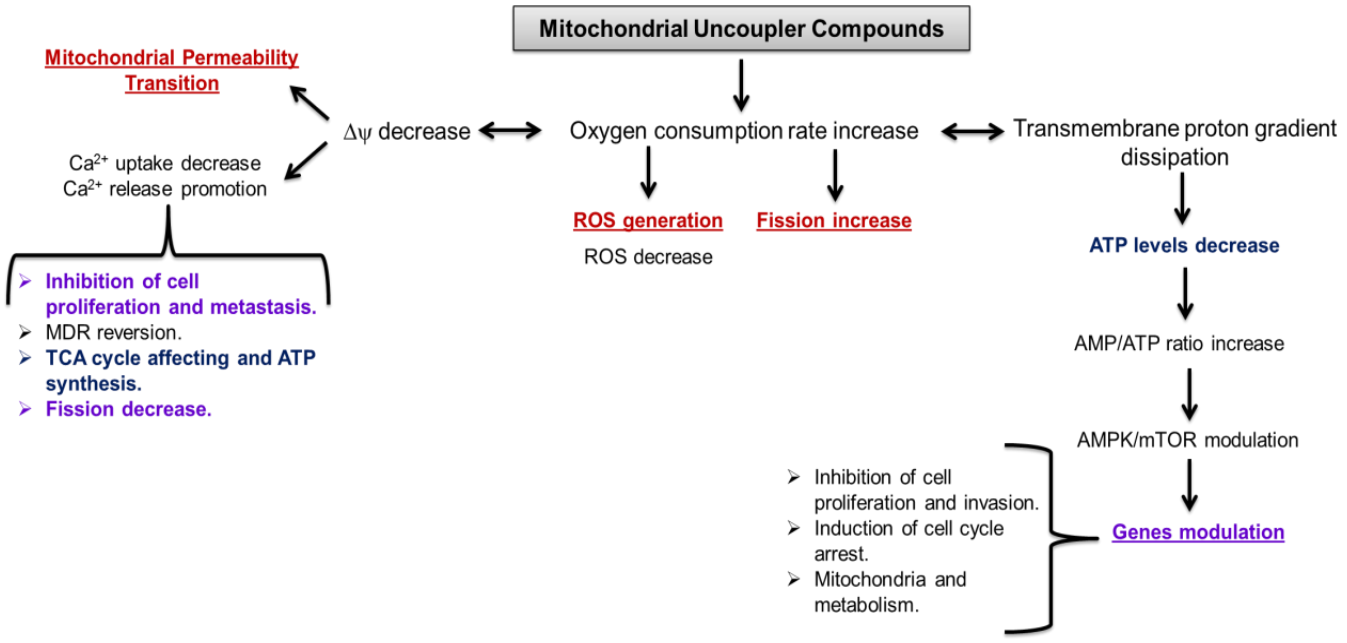


Figure 3. General effects exerted by mitochondrial uncoupler compounds.

Bold type and colored words indicate interrelated events according to the color. Underlining words designate the uncoupling-induced effects that directly trigger apoptosis. The two-way arrow shows fundamental and interconnected events originated by the same cause.

Limitations. Uncoupler compounds could selectively kill cancer cells

Mitochondrial uncoupling seems to be an unspecific toxic mechanism affecting both normal or cancerous cells. 2,4-dinitrophenol (DNP) and other nitrophenols have long been known to be toxic at high concentrations, an effect that appears essentially related to interference with cellular energy metabolism due to uncoupling of mitochondrial oxidative phosphorylation. The toxicity of these compounds has raised a general reluctance to use and develop mitochondrial uncouplers as therapeutics. Nevertheless, uncouplers seem to accumulate in cancer cells because a more hyperpolarized $\Delta\psi$ than in normal cells; uncouplers would be selectively targeted to, and accumulated by, cancer cells mitochondria. The more aggressive cancer, the more hyperpolarized its $\Delta\psi$ (Heerdt et al., 2005; 2006) and the more it will be targeted by the uncouplers.

Additionally, in spite of extracellular acidification in cancer cells its cytoplasm is and needs to be, neutral like normal cells (Casey et al., 2010; Damaghi et al., 2013). Tumors are acidic, normal tissue is neutral (Gerweck and Seetharaman, 1996; Gerweck et al., 2006; Damaghi et al., 2013). The more aggressive the cancer is, the more acidic its tumor environment (McCarty and Whitaker, 2010). So, cancer cells, unlike normal cells, must maintain their intracellular pH above their extracellular acidity. The protonophoric activity leads to cytoplasm acidification undermine this homeostasis and could kill, selectively, cancer cells. Besides, these acidic compartments can act as sinks for the accumulation of high lipophilic weak bases and acids molecules that could pass through the plasma and mitochondrial membranes (Hurwitz et al., 1997; Luciani et al., 2004).

Table 2 shows a comparison of the cytotoxic action of three uncouplers on non-cancer and cancer cells lines. As it can see, there are great differences in the values of cytotoxicity between non-cancer and cancer cells. Especially, the values on primary culture cells (a more accurate cellular model) confirm the relatively minor toxic effects of the tested uncouplers on normal cells lines.

Future perspectives

Recent data indicate that mitochondria in cancer not only represent mere effectors of apoptosis but also have a more complex role in oncogenesis and oncosuppression. The use of mitochondrial uncouplers should be considered further for cancer prevention, particularly in high-risk patient populations, and for retreating drug-resistance in cancer patients that classical therapy has failed. As such, understanding the compartment-specific role of mitochondrial uncoupling in cancer pathogenesis may be the key to unlocking new anticancer therapies and preventing the onset of drug resistance in cancer patients.

Table 2. Cytotoxic effects of nemorosone, SR4 and VdiE-2N on hepatocarcinoma HepG2 cells, human embryonic kidney HEK293 cells (non-cancer cells line), primary mouse hepatocytes (non-cancer cells line), head (HN13) and neck (HN12) cancer cells, and primary cultures of fibroblasts derived from human oral health mucosa OHMF (non-cancer cells line).

Uncoupler/ cell lines	HepG2	HEK293	Primary mouse hepatocytes	HN13/HN12	OHMF
Nemorosone (25 μM)	75% cell death	10% cell death	---	---	---
SR4	IC_{50} = 3.5 μM	---	IC_{50} > 20 μM	---	---
VdiE-2N	---	IC_{50} > 50 μM	---	IC_{50} = 7.55/ 20.87 μM	IC_{50} > 50 μM

The IC_{50} is the concentration that inhibits 50% of the cellular proliferation. See Table 1 for references.

CONCLUSIONS

It has been discussed here a wealth of mechanisms by which uncoupler compounds may exert its well-documented cytotoxicity on cancer cells. This is the first time in which these mechanisms are displayed systematically and with detailed explanations of the interrelation of the several effects mediated by uncoupling. Given the day to day enormous progress in the oncological field, but at the same time, the lack of drugs or biological mechanisms that increase the survival of cancer patients or reverse the disease, new anticancer strategies need to be investigated. Mitochondrial uncoupling seems to be an interesting pharmacological target to be considered in the design and synthesis of novel anti-cancer compounds.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTION:

Contribution	Fernandez Acosta R	Piñeros O	Pardo Andreu GL
Concepts or ideas	x	x	x
Design	x		x
Definition of intellectual content	x	x	x
Literature search	x	x	x
Experimental studies	x		x
Data acquisition	x		x
Data analysis	x		x
Statistical analysis			
Manuscript preparation	x	x	x
Manuscript editing		x	x
Manuscript review	x	x	x

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