Crucial Role of Mitochondrial Mechanisms in the Antineoplastic Effects of Desethylamiodarone

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1 Introduction

1.1 Cancer

Cancer, is the second leading cause of death worldwide following cardiovascular diseases. Approximately 1.9 million new cases and 600,000 deaths were recorded by 2022 in the United States, with 27.5 million new cases predicted by 2040 worldwide. Therefore, cancer is a serious health threat facing the humans. Unfortunately, cancer is a group of diseases where cells grow, change, and multiply out of control, at the tissue level, which makes it the major challenge for the specific diagnosis and treatment.

More than hundred types of cancer have been identified. In men, the most frequent types of cancer are prostate, lung, colon, urinary bladder, and melanoma cancer. In women, on the other hand, cancer prevalence is highest in breast, lung, colon, uterine corpus, and melanoma. Different factors are involved in the initiation of cancers including, internal factors like hormones, genetics, and immune system conditions. Or / and external factors mainly known as carcinogens such as, chemicals, asbestos, arsenic, radiations, tobacco, and others.

Generally, cancer is astatic disease characterized by multi-genetic disorder, it evolves overtime gaining multiple mutations. Those mutations include constant activation of oncogenes that are responsible for cell division and growth, or else, the deactivation of cancer suppressor genes leading to uncontrolled cell division. Additionally, cancer cell circumvents negative cell proliferation pathways, mainly apoptosis.

Cancer therapy usually starts with chemotherapy or surgery, radiation, targeted, and/or immunotherapy. Additionally, among the new therapeutic approaches, targeted and immunological therapies are more effective since the target proteins are identified. Withal, cancer drug resistance is still increasing.

1.1.1 Skin Cancer

Human skin is made up from 3 main layers: the epidermis, dermis, and hypodermis. Epidermis, the outermost layer, consists of different cell types including melanocytes and keratinocytes, any defect in those cell types can lead to skin cancer. Nearly half of all cancer patients in the United States were diagnosed with skin cancer, which makes it one of the most common types of cancer. Two main types of skin cancer: melanoma, which is the skin tumor of melanocytes, nearly 132,000 new cases yearly and it accounts for 4% of all skin cancer. However, 75% of total skin cancer deaths are caused by melanoma, which makes it the most lethal form of skin cancer, and one of the top six most common cancer-related mortality worldwide. On the other hand, non-melanoma skin cancer (NMSC), which is the keratinocyte cells' originated skin cancer. Almost 2-3 million new cases are reported yearly worldwide, which makes it one of the most diagnosed malignancies with 95% representation of all skin cancer cases.

1.1.2 Breast Cancer

Breast cancer (BC) refers to cancers initiated from breast tissue, usually from the lobules or the inner lining of milk ducts. Currently, BC is the most diagnosed cancer type among all cancer patients especially females. According to 2022 statistics in USA, BC is the 4th cause of cancer mortality worldwide in both genders, and the 2nd cause of cancer death among females. BC accounts for more than 2.2 million new cases and approximately 685,000 new deaths. Early-stage BC, which is limited to the breast or spread to the axillary lymph nodes, is curable. Whereas advanced BC, characterized by metastases and invading surrounding organs, is not curable, yet treatable in terms of symptoms control and life prolongation. Several risk factors might lead to BC including gender, smoking, radiations, genetic history, genetic mutations, age, chemicals, drugs among others. Two main types of BC, hormone receptor-positive (progesterone receptor (PR) or estrogen receptor (ER)-positive) and triple negative breast cancer (ER, PR, and human epidermal growth factor receptor-2 (HER2)-negative).

1.2 Cancer treatment

Recently, the main objective of cancer research and medical trials is to develop novel methods for cancer treatment. Thus, the choice of treatment and its progress depends on cancer type, location, size of the tumor, stage of progression, as well as the impact on non-tumor cells.

1.2.1 Chemotherapy

Chemotherapy is the most applied therapy after the diagnosis for cancer treatment before or after surgery. Chemotherapy is the use of any anticancer (cytotoxic) drug (not specific). Usually, chemotherapeutics target cell's DNA and/or processes important for cell division to kill rapid dividing cancer cells, including normal cells surrounding the tumor. Different groups of chemotherapeutics where classified depending on the modes of action including alkylating agents, topoisomerases, microtubule targeting agents, antimetabolites, and antibiotics.

Unfortunately, these drugs have serious side effects including hair loss, loss of appetite, increased chance of infections, bleeding, nervous complications, and fatigue. Consequently, new therapies need to counter these side effects. Thus, the current development and the use of MAbs or specific cell surface inhibitors are steps in that direction.

1.2.2 Targeted and Immunotherapies

Targeted therapy is the use of antibodies, small molecule inhibitors or oral drugs that somehow precise to cancer cells and have limited effects to normal cells. Immunotherapy, on the other hand, is based on the use of patient's own natural defense system -immune system- to fight off cancer. Nevertheless, no targeted therapy or chemotherapy is 100% effective since cancer cells evolve and gain resistance against the available drugs.

1.3 Cancer Drug Resistance

Cancer cells escape and resist therapy agents by different mechanisms which are in general called drug resistance. Two major types of resistance are known: the intrinsic and the acquired resistance. Intrinsic resistance, on the one hand, is the one that is already existed in cancer cells before therapy is started, like a mutation in certain genes. Acquired, also called secondary resistance, on the other hand is the process in which cancer cells gain anti-drug resistance due to the prolonged treatment with anticancer agents. This is the case where cancer cells show a positive response to therapies in the first stages of treatment.

1.3.1 Inflammation-Related Cancer Drug Resistance

Immune system plays an important role in cancer treatment; however, inflammation can do completely the opposite through increasing cancer progression and drug resistance. Growing evidence have shown that chemotherapy-induced inflammation is one of the reasons for chemo-resistance and metastasis in breast cancer. Hence, inflammation is considered as a key characteristic of cancer, especially chronic inflammation where the risk of malignancy is a typical sign. Indeed, cyclooxygenase-2 (COX-2) enzyme is one of the enzymes that leads to the transformation from acute to chronic inflammation upon treatment.

However, considering all mechanisms and factors for cancer drug resistance; apoptotic elusion plays a key role in cancer therapy resistance, since apoptotic evasion is one of the top hallmarks of cancer, especially intrinsic (mitochondrial) apoptosis.

1.4 Mitochondria

Mitochondria, known for centuries as the dynamic powerhouses of a cell, are considered one of the most important intercellular maternally inherited organelles of the cell. Mitochondria are membrane-bound organelles that contain two major membranes, outer and inner membrane separated by intermembrane matrix. The outer mitochondrial membrane (OMM) contains protein-based pores for ions and large molecules passaging. Whereas the inner membrane is more restricted compartment mainly for electron transport chain where adenosine triphosphate (ATP) is generated. In addition, mitochondrial matrix holds citric acid cycle that produces electrons for electron transport chain. To summarize, mitochondria are essential organelles for ATP production, metabolic signaling, redox homeostasis, as well as proliferation and apoptotic pathway control. However, metabolic alterations in mitochondria are linked with multiple diseases including cancer. Of note, mitochondria control the intrinsic apoptosis which is one of the most important pathways in the cell, especially for cancer cell. Since pro-apoptotic and anti-apoptotic proteins are downregulated and upregulated, respectively. Yet, cancer cells are more sensitive to apoptosis than normal cells. Hence, targeting mitochondria have become the novel strategies for anti-cancer drugs.

1.4.1 Mitochondrial Apoptotic Pathways

Apoptosis (programed cell death) is a highly regulated form of cell death to maintain a normal cell population, yet dysregulation in apoptotic pathways is carcinogenesis. Two main pathways of apoptosis: An intrinsic pathway, also called the mitochondrial apoptosis which involves different protein families including BCL-2, BH3-only proteins family, caspase-9, Akt, apoptotic inducing factor (AIF) and others. This type of apoptosis is induced by multiple signals such as DNA damage, loss of mitogens, increase reactive oxygen species (ROS) level, chemotherapeutic agents, cytochrome C (Cyt c), and calcium (Ca^{2+}) overload in mitochondria, among others. Extrinsic pathway, on the other hand, involves cell surface death receptor FAS-associated death domain protein (FADD), or tumor necrosis factor (TNF) receptor members, by external signals like drugs, hormones, and/or pathogen effectors. To activate the downstream caspase-8 and bid protein which can play a role in the crosstalk between the two apoptotic pathways. Accordingly, apoptosis of both pathways is initiated by the activation of down-stream of the executioner caspase-3.

1.5 Small Molecules in Therapy

In 2001, tyrosine kinase inhibitor (TKI) was the first small molecule to be approved by the US Food and Drug Administration (FDA). At present, 42 small anti-cancer molecules have been approved by the FDA. Thereby, small molecules are in a great interest and demand for cancer treatment, despite the huge use and challenges by macromolecules especially monoclonal antibodies. Due to the advantages of small molecules: costs, pharmacokinetic properties, drug storage and transportation, patient compliance, absorption method, and more importantly their mood of action which is signal transduction inhibition. Furthermore, cell membrane and blood brain barrier penetration of small molecules are easier than macromolecules, which are the limitation of antibodies use.

1.6 Desethylamiodarone (DEA)

Nowadays, small-molecule targeted drugs are in a great interest, due to their effectiveness, cost, storage, transportation, and pharmacokinetic properties. Thereby, mono-N-desethyl amiodarone (Desethylamiodarone-DEA), a small pharmacological active compound, is the major metabolite of the widely used antiarrhythmic drug, Amiodaron (AM). AM, (2-butyl-3benzofuranyl 4-[2-(diethylamino_-ethoxy]-3,5diiodophenyl-ketone hydrochloride), is an FDA approved class III antiarrhythmic drug for variety of cardiac diseases including ventricular and supraventricular arrhythmias. Usually, AM is given orally to patients with a recommended therapeutic range of <5.7 μM, where its half-life is in the range of 14-59 days. AM metabolism takes place in the liver and in the gut wall by an oxidase-dependent oxidative de-ethylation reaction catalyzed by cytochrome p450 3A family (CYP3A) to give DEA.

DEA has a similar electrophysiological effects as AM, where both work on the prolongation of action potential duration by blocking β-adrenergic receptors, sodium, and L-type calcium channels. Both AM and DEA are strongly bound to plasma proteins, However, DEA-serum level is higher than that of AM-serum level during long-term AM treatment 1.7-4.5 μM and 1.6-5.3 μM, respectively, as its elimination half-life is approximately 40 days. Moreover, in the context of treatment, DEA compared to AM is more toxic *in-vitro*, where AM's activity triggers necrotic cell death, while DEA activates apoptotic pathways. Due to DEA's lipophilic structure; it is highly accumulated (higher than AM) in skin, liver, lungs, myocardium, thyroid gland, pancreas, but not in adipose tissue. Different toxic side-effects limit long-term AM therapy including hepatic, dermatologic, cardiac, pulmonary, thyroid, ocular, etc. Due to the fact that DEA and AM are highly concentrated in the tissues after AM treatment, which can be hundred to more than thousand times higher than that of the plasma concentration. Thereby, based on the toxic effects of DEA and tissue accumulation properties, its potentiality in cancer therapy was proposed.

2 Objectives

Throughout history, cancer used to be primarily a disease of the minority, however, recently has become a leading cause of morbidity and mortality in the majority of human populations. Consequently, cancer research and treatment trials are constantly growing to design, identify, and describe targets, mechanisms, and pathways of different cancer types aiming for treatment. Indeed, in the light of these information provided by tons of cancer research, we aim to imply a DEA on multiple cancer types. The thesis is structured into two parts, which are closely related since they deal with experimental data about skin and breast cancer treatment.

Our aims:

- To establish DEA's potentiality in B16F10 metastatic melanoma cells including *in-vivo* lung metastasis formation, *in-vitro* cell viability, cell cycle arrest, apoptosis, Reactive Oxygen Species (ROS) formation, colony formation. And mitochondrial processes, including mitochondrial respiratory chain, mitochondrial permeability transition (mPT), and mitochondrial membrane potential ($\Delta\Psi m$).

- To study and compare the *in-vitro* cellular and mitochondrial effects of DEA on different BC cells. Including cell death, cell migration, mitochondrial respiratory chain, mitochondrial dynamics and fragmentation, mitochondrial membrane potential ($\Delta\Psi_m$). And possible TNBC DEA's resistance mechanism.

3 Results and Discussion 1.0

3.1 **Results** 1.0

3.1.1 Effect of DEA on the Viability of B16F10 Melanoma Cells

To get an overview of DEA's effect on B16F10 cells, a short-term assay was performed. We treated the cells for 3–12 h with 5 or 10 μM of DEA before determining their viability using the SRB assay. DEA had a statistically significant anti-proliferative and cell death-inducing effect on the cells in a time- and concentration-dependent manner. Therefore, the possible pathways contributing to DEA-induced cell death were further analyzed. Although at 5 μM and for up to 6 h of incubation, this effect did not reach statistical significance.

3.1.2 Effect of DEA on B16F10 Colony Formation

Colony formation assay utilizes lower drug concentrations and longer exposure times. Accordingly, we sought assess DEA's effect on colony forming ability of the B16F10 cells. We treated the cells with 0–3 μM DEA for 7 days then colonies of > 0.5 mm were counted. As we found, DEA significantly decreased number and size of the colonies even at the lowest concentration used. These data indicate that DEA can induce cell death and can inhibit colony formation at low micromolar concentrations.

3.1.3 Effect of DEA on Apoptosis Activation in B16F10 cell

Cell death could be of different mechanisms mainly necrosis or apoptosis. We were interested to investigate the mode of cell death induced by DEA. For that, flow cytometry with the Muse™ Annexin V & Dead Cell Assay was used. We observed that DEA increased the total apoptosis rate in a dose-dependent manner. We found a total apoptosis rate of 34.61 ± 2.17% for 5 and 73.71 ± 3.12% for 10 μM DEA in contrast to the control's 10.11 ± 1.97%. At the lower DEA concentration, rate of early apoptosis exceeded that of the late, however, at the higher concentration, late apoptosis predominated.

3.1.4 Effect of DEA on the Cell Cycle in B16F10 Cells

DEA limits the proliferation of B16F10 cells through inducing predominantly apoptotic cell death. However, the net result of cell death and cell division is cell proliferation. Thus, we performed cell cycle analysis to study the other aspect of proliferation. We found that the percentage of cells in G0/G1 phase significantly increased from 56.75 ± 1.73% (0 μM) stepwise to 63.9 ± 1.94% (5 μM) and 75.91 ± 2.67% (10 μM). At the same time there was a decrease in the percentage of S and G2/M phase cells. These data indicate that DEA at both concentrations induced cell cycle arrest in the G0/G1 phase that may contribute to its overall inhibitory effect on B16-F10 cell proliferation.

3.1.5 Effect of DEA on Outer Mitochondrial Membrane (OMM) Permeabilization

We assessed DEA's effect on OMM integrity by determining its effect on the expression, localization, and activation of Cyt c, Opa1, AIF, Bad, cleaved caspase-3, and Akt. To this end, we prepared whole-cell homogenate, and in parallel nuclear and cytoplasmic fractions from B16F10 melanoma cells treated with different concentrations of DEA for 6 h, and then subjected them to immunoblot analysis. At a concentration of 10 μM, DEA increased the steady-state level of Bad and decreased Bad phosphorylation in a concentration-dependent manner. Both effects shift the balance in the pro-apoptotic direction. Accordingly, DEA induces caspase-3 cleavage, as well as the release of Cyt c and Opa1 into the cytosol and nuclear translocation of AIF. In addition, 10 μM of DEA decreased Akt phosphorylation without affecting the steady-state level of the enzyme. These data indicated that DEA caused OMM permeabilization.

3.1.6 Effect of DEA on the $\Delta\Psi_m$

We used a membrane potential-dependent fluorescent dye, JC-1, which accumulates in the mitochondria due to its positive charge. Within 3 h, treatment with > 5 μM of DEA markedly depolarized the mitochondria in intact B16F10 melanoma cells.

3.1.7 Effect of DEA on Mitochondrial Fragmentation

Compromised ΔΨm results in mitochondrial fragmentation, raising the possibility that DEA treatment causes such an effect. To test this possibility, we performed confocal fluorescence microscopy on DEA-treated B16F10 melanoma cells transiently transfected with mitochondria-targeted red fluorescent protein (mtRFP) expressing vector. Treatment for 3 h with 10 μM of DEA resulted in mitochondrial fragmentation comparable to that caused by 25 μM of cisplatin. Although treatment with 5 μM of DEA tended to increase the mitochondrial fragmentation, the difference from the control did not reach the level of statistical significance.

3.1.8 Effect of DEA on the Energy Metabolism of B16F10 Melanoma Cells

Given that the DEA treatment depolarized ΔΨm, a major determinant of ATP synthesis, we next though to determine the effect of DEA treatment on the energy metabolism of B16F10 cells. We used the Seahorse XF Cell Mito Stress Test to monitor the cellular oxygen consumption rate (OCR), an indicator of mitochondrial respiration, and the extracellular acidification rate (ECAR), an indicator of aerobic glycolysis in live B16F10 melanoma cells. We treated cells with 5 or 10 μM of DEA for 3 h, and then monitored the OCR and ECAR for 75 min. The DEA treatment did not significantly affect basal respiration or proton leak, although 10 μM of DEA tended to decrease the former and increase the latter. By contrast, 10 μM of DEA did suppress the maximal respiration, ATP production, and coupling efficiency; the last of these indicates how tightly respiration is coupled to ATP synthesis. DEA decreased the non-mitochondrial oxygen consumption, and spare respiratory capacity, expressed either as the difference or ratio of maximal

and basal respiration, in a concentration-dependent manner. Although it did not affect the basal fermentative ATP synthesis, DEA decreased lactate accumulation after oligomycin administration in a concentration-dependent manner, indicating that the drug interfered with the glycolytic machinery as well as the mitochondrial respiratory chain.

3.1.9 Effect of DEA on mPT in Intact B16F10 Melanoma Cells

We measured mPT in intact live cells using a 96-well automated high-content fluorescence imaging system. Accordingly, we monitored the calcein fluorescence of melanoma cells treated with 5 or 10 μM of DEA or 1.5 mM of $CaCl_2$ (positive control) in the presence of acetoxymethylcalcein, $CoCl_2$, and A23187 with or without CsA for 3 h. As expected, 10 μM of DEA induced mPT that was CsA-independent. Also, elevated Ca^{2+}, the cellular uptake of which is facilitated by A23187, caused a massive mPT that was fully CsA-dependent.

3.1.10 Effect of DEA on Lung Metastasis Formation in an In-Vivo Model

To evaluate the effect of DEA on metastasis formation, we used an in-vivo lung metastasis model. Murine melanoma B16F10 cells were injected into the lateral tail vein of 6-weeks-old male C57BL/6 mice then they were divided into two groups (6 mice/group). Intraperitoneal administration of 25 mg/kg DEA or vehicle control was started 24 hours after tumor cell administration and was repeated every third day. Accordingly, DEA treatment diminished lungs mass index and the number of tumor nodules. Additionally, histopathological analysis was performed by an expert who was blind to the experiment on lung tissue sections after hematoxylin and eosin staining. There were melanoma cells with poliedric morphology with a great amount of melanin content as cytoplasm granules or in a perinuclear distribution. In addition, aberrant nodular proliferation in broncho-alveolar regions characteristic of epithelial melanoma was observed in all sections. However, there was a marked difference in tumor nodule pattern distribution and concentration between untreated and DEA treated animals. In the vehicle treated group, the nodules were of considerable size and were distributed in all part of the lung parenchyma. In contrast, in the DEA group, the tumor nodules were much smaller in size and were organized in a predominantly peripheral distribution. To quantify the morphological observations, image analysis with the Pannoramic Viewer Imaging System was performed on randomly selected sections (6 per lungs), and tumor area as the percentage of lung section area was calculated. As we found, the tumor area was significantly decreased in the lung tissue of DEA vs. vehicle treated animals. These results are in complete accordance with those of the macroscopic observations and indicate that DEA can inhibit melanoma tumor metastasis in-vivo.

3.2 Discussion 1.0

Melanoma (metstatic) is still the most lethal form of skin cancer and one of the top six most common cancer-related mortality worldwide. Unfortunately, melanoma is highly resistant to chemotherapy, most of chemotherapeutic agents have failed because of the patients' low response rates. Consequently, a major cause of this resistance in melanoma is related to a defect in the apoptotic signaling pathway, BCL-2. However, according to the literature, cytostatic agents induce G1 arrest and down regulation of BCL-2. The latter effect is the typical function of p53, which is found to be non-mutated wild type expressed P53 by B16F10 melanoma cells. In agreement with this view, DEA at low μM concentrations reduced the viability and proliferation of B16F10 cells by acting as an apoptosis stimulating factor. First by detecting phosphatidylserine through annexin V positive staining, upregulating P53, leading to cell cycle arrest in G0/G1 phase and Bad, also downregulating p-Bad. In a concentration- and time-dependent manner; DEA has significant anti-proliferative and pro-apoptotic effects on B16F10 cells *in-vitro*, which suggest its potentiality as an anti-cancer agent in melanoma treatment. Additionally, the resulting apoptosis likely involved mitochondrial mechanisms leading to Opa1, Cyt c, and AIF release from mitochondria.

In melanomas, cell cycle's G1 phase regulators cyclins type D and E, CDK4/6, and CDK inhibitors are suggested to act as a therapeutic target. For this reason, we determined the effect of DEA on the cell cycle as well as on the metastasis inducer markers cyclin D1 and CDK2, the tumor suppressor p53, and the cell cycle inhibitors p21 and p27. As we observed, in a concentration dependent manner, DEA reduced cell proliferation by increasing number of cells in the G0/G1 phase that was accompanied by reduced steady state levels of cyclin D1 and CDK2, and increased levels of p21, p27 and p53. Due to the fact that p53 gene is not mutated in B16F10 cells, the observed G1 phase arrest by DEA was likely mediated through p21, in a p53-dependent manner.

The proliferation rate and lung metastasis formation of melanoma are relatively high. In this case, we performed colony formation and invasive growth experiments to test the effect of DEA on melanoma's migration and colony formation abilities. Our findings showed that DEA decreased colony formation below 50% of the control at 2 μM DEA concentration during a seven-days exposure. Also, DEA at 5 μM concentration eliminated melanoma's invasive growth within 24 h treatment.

The balance between cell proliferation and apoptosis determines the rate of tumor progression. Therefore, Constant activation of intracellular pro-survival signaling cascades such as the phosphatidylinositol 3-kinase/Akt pathway has been showed to significantly enhance cancer progression. Akt, which is constitutively active in melanoma, stimulates cell proliferation and survival by repressing intrinsic apoptosis and increasing cell cycle advancement. Progression and malignancy of various tumors is often associated with the constitutive activation of Akt, which inactivates many proapoptotic proteins by phosphorylation such as Bad and caspase 9 at its Ser^{196}. The invasiveness of melanoma cells and their ability to form metastases may be related to the

frequently observed high basal activity of Akt in these cells. Our results showed that the high basal levels of active, phosphorylated Akt was decreased dose dependently by DEA treatment in B16F10 cells, a highly invasive variant of B16 murine melanoma. This decrease in the activation of Akt may contribute to the effects of DEA on both the cell cycle and apoptosis.

Mitochondria actively participate in all stages of cancer development including carcinogenesis, metastasis, survival, as well as therapy resistance. Importantly, OMMP is a significant factor in mitochondria-associated apoptosis, which is control by pro- and anti-apoptotic BCL-2 protein family. Typically, disrupting the balance between these proteins, downregulation of pro-apoptotic and upregulation of anti-apoptotic BCL-2, is the hallmark of cancer resistance to apoptosis. According to our findings, 6h of DEA treatment was able to induce OMMP through elevating the steady-state activation of the pro-apoptotic BCL-2 family member Bad and inhibiting its phosphorylation. The permeabilization of OMM then results in the release of Cyt c to the cytosol activating caspases-dependent apoptosis though activating caspase-3, as well as the release if AIF from the mitochondria to the nucleus, where it plays a role caspase-independent apoptosis by inducing chromatin condensation and DNA fragmentation. However, AIF release could be a result of mPT pore opening since AIF is an IMS protein.

mPT pore is located between the OMM and the IMM, upon opening it forms a non-specific passage to water and solutes of up to 1.5 kDa in size, which eventually leads to the release of IMS proteins such as EndoG, Cyt c, and AIF. In complete agreement with our results, AIF release was due to mPT pore opening, 10 μM of DEA results in CsA-independent mPT. Moreover, Ca^{2+}, which is a main cause of mPT pore opening induced CsA-dependent mPT which was more than the one induced by 10 μM of DEA. Of note, continues opening of mPT pores results in disrupting different mitochondrial processes including the loss of ΔΨm.

ΔΨm is essential for cell survival because its crucial role in ATP production, ROS generation, mitochondrial proteins transportation and network dynamics, and the regulation of apoptosis via IMS pro-apoptotic proteins release. Consequently, OMMP and mPT pore opening are indicator of ΔΨm depolarization. In this case, we showed that DEA treatment for 3 h was able to induce depolarization and partial loss of the ΔΨm. Nonetheless, intact ΔΨm directly affects mitochondrial dynamics, mainly mitochondrial fusion. Mitochondrial fusion and fission must be balanced, as below a certain ΔΨm threshold the fusion process will be disrupted. Accordingly, fusion of the IMM is controlled by L-OPA1 protein, when cleaved; it is released to the cytosol, in complete agreement with our findings, DEA treatment for 6 h was able to induce OPA1 cleavage and release to the cytosol indicating loss of fusion, which also contributes to the release of Cyt c from the mitochondrial, since huge amount of Cyt c is stored in cristae junction which is also controlled by L-OPA1. Furthermore, loss of fusion leads to mitochondrial fragmentation, which is a hallmark of mitochondrial apoptosis and quality control since the fragmented mitochondria are eliminated by mitophagy. In context of therapy, cisplatin a chemotherapeutic drug used for different types of malignancies, it induces mitochondrial fragmentation, however, its use is still

limited due to the nephrotoxicity side effect. To complete our view of this process, we performed mitochondrial fragmentation experiment, and so within 3 h DEA treatment induced mitochondrial fragmentation that was almost the same effect as the two folds difference in concentration of the positive control cisplatin. Considering that fusion requires intact ΔΨm, and DEA compromised ΔΨm, it seems that DEA promote mitochondrial fragmentation by impeding fusion, which needs further investigation to conclude withier its fission dependent or independent. The later effect might contribute to DEA's cytotoxic properties to limit *in-vivo* melanoma metastasis.

As mentioned above, intact ΔΨm plays a critical role in ATP production. Since cancer cells follow the Warburg effect, which explain the usage of glycolysis instead of OXPHOS for ATP production in cancer cells, metastatic cells have elevated level of mitochondrial ATP synthesis. Indeed, 10 μM concentration of DEA impeded both mitochondrial and glycolytic ATP synthesis pathways though reducing the fermentative glycolysis and non-mitochondrial oxygen consumption, as well as decreasing the maximal respiration and coupling efficiency. Based on these effects on B16F10 cell's energy metabolism, DEA exhibits promise as a cancer therapy candidate.

Withal, *in-vitro* cell culture experiments translate very poorly to human studies; therefore, the therapeutic dose must be determined *in-vivo.* Since metastasis is fundamental property of malignant cancer cells, by which a certain cancer spreads from the location at which a tumor first arises to distant locations in the body. Cancer recurrence by metastasis is responsible for about 90% of mortality in cancer patients, which makes it a main target for anti-cancer therapy. Metastasizing proceeds in a series of sequential steps including invasion, intravasation, survival and translocation in the circulation system, extravasation, and survival in a new organ. Within its limitations, the B16F10 lung metastasis model mimics the majority of this process's steps. Through the period of 16 days, several large tumor loci in the lungs were formed upon B16F10 injection into the tail vein of the animals. These loci represent near 30% of tissue area in the lung's sections. Both number and size of the tumor loci were reduced by DEA concluding that the drug has potentiality in reducing *in-vivo* metastasis formation. Of note, B16F10 have been selected for their metastasizing property, and therefore have low immunogenicity. Yet, the cells are of mouse tissue culture and not of human primary tumor origin. For that reason, further studies are needed preferably by using primary human melanomas to establish DEA's potentiality in the therapy of metastatic melanomas.

The data presented in this study provide *in-vitro* and *in-vivo* experimental evidence for DEA's potentiality in the therapy of metastatic melanomas. Based on the on the effects of DEA which indeed decreases ΔΨ; induces mitochondrial fragmentation; decreases maximal respiration, ATP production, coupling efficiency, glycolysis, and non-mitochondrial oxygen consumption; and induces CsA-independent mPT and OMM permeabilization. All these effects may account for the rapid (3–12h) cytotoxicity of the drug and may account for DEA's long-term (24–72h) cytotoxicity and ability to suppress *in-vivo* metastasis.

3.3 Results 2.0

3.3.1 DEA Induced Apoptotic Cell Death in BC Cell Lines

DEA demonstrated signs of anti-neoplastic potentiality in therapy-resistant cancer cell line B16F10. Accordingly, we assessed its effect on the cell death process of the TN 4T1 BC line in comparison with the HR+ MCF-7 line. We used flow cytometry with the Muse™ Annexin V & Dead Cell Assay kit to investigate the mode of DEA-induced cell death. We treated the cells with 5 and 10 μM DEA for 24 h before the flow cytometry analysis. In a concentration-dependent way, DEA induced early, then late apoptosis. At the lower DEA concentration, the rate of early apoptosis exceeded that of the late; however, at the higher drug concentration, late apoptosis predominated in both BC cell lines. However, we found a total apoptosis rate of 28.18 ± 6.34% for 5 and 56.05 ± 5.12% for 10 μM DEA in the 4T1 cell line in contrast to the apoptosis rates of 71.14 ± 6.39% for 5 and 78.94 ± 4.77% for 10 μM DEA observed in the MCF-7 cell line, indicating that MCF-7 cells were more sensitive toward DEA treatment than the 4T1 cells.

3.3.2 DEA Mitigated Invasive Growth of BC Cell Lines

Cell migration, an important aspect of cancer invasiveness, is often assessed by means of the wound-healing assay we inflicted a wound into semi-confluent monolayers of MCF-7 and 4T1 cells and treated them by 0, 5, or 10 μM DEA for 12 h. Cell migration of the TNBC cells was more intense, resulting in almost complete closing of the wound within 12 h, while the HR+ BC cell line achieved an about 40% healing during the same time. 10 μM DEA treatment completely prevented wound healing in the 4T1cell line, while it caused wound exacerbation due to killing cells at the wound edge in the MCF-7 cell line. At the concentration of 5 μM, DEA treatment induced a less pronounced effect on wound healing as it did at 10 μM. To supplement the wound-healing assay, we assessed the invasive growth of the MCF-7 and 4T1 cells using an xCelligence Real-Time Cell Analysis (RTCA) system. The cells were cultured in the presence of 0, 5, or 10 μM DEA for 24 h and the cell index proportional to invasive growth of the cells was monitored in real time. DEA decreased invasive growth in a concentration-dependent manner in both MCF-7 and 4T1 cells. Similarly to the results of the wound-healing experiment, MCF-7 was more sensitive to the drug than 4T1.

3.3.3 DEA Differentially Modulated Regulators and Markers of the Cell Death Process in the BC Cell Lines

To gain greater insight into the apoptotic pathways induced by DEA in BC cell lines, we analyzed Akt activation, protein levels of BCL-2 family members including pro-apoptotic Bad and Bax and antiapoptotic BCL-2, and the caspase-3 cleavage and caspase-3-mediated cleavage of PARP, which are all reporters of the mitochondrial apoptotic pathway. Akt phosphorylation at Ser^{473} decreased in a concentration-dependent manner, while the total Akt remained constant. This was accompanied by a significant decrease in the phosphorylation level of Bad at Ser^{136}, while the

overall level of Bad protein remained constant in both cell lines. We also found that DEA indeed resulted in a dose-dependent increase in the amount of p53 protein in 4T1 cells. We also measured a significant increase in Bax levels while BCL-2 levels decreased. Furthermore, we found that DEA treatment led to an increase in the amount of a 19 kDa caspase-3 cleavage intermediate as well as cleaved poly (ADP-ribose) polymerase (PARP). Taken together, these data demonstrate that cytotoxic effects of DEA on MCF-7 and 4T1 cells are due to the activation, at least partially, of two apoptotic pathways, the PI3K/Akt pathway and the mitochondrial pathway.

We also studied DEA's effect on COX-2 protein levels in the 4T1 and MCF-7cell lines. We detected considerable steady-state COX-2 levels in the TN BC cell line, while it was just above the detection limit in the HR+ one. Additionally, DEA increased the COX-2 level in a concentration-dependent way in the TN 4T1 cell line only.

3.3.4 DEA Caused the Loss of Mitochondrial Membrane Potential (ΔΨm)

We determined the effect of DEA on the ΔΨm of BC cells by using the positively charged fluorescent mitochondrial dye, JC-1. We treated the MCF-7 and 4T1 BC cell lines for 3 h with 0, 5, or 10 μM DEA, before loading them with JC-1 and taking fluorescent microscopy images. At the concentration of 10 μM, the drug significantly depolarized the mitochondria in both BC cell lines, while 5 μM DEA did not have a considerable effect on the ΔΨm during the 3 h treatment.

3.3.5 DEA Induced Mitochondrial Fragmentation in BC Cell Lines

We studied the effect of DEA on mitochondrial network dynamics by fluorescent microscopy after loading the cells with MitoTracker Red to visualize the mitochondria. The MCF-7 and 4T1 cells were treated with 0, 5, or 10 μM of DEA for 3 h before the assessment of mitochondrial fragmentation. DEA treatment caused mitochondrial fragmentation in both BC cell lines in a concentration-dependent manner. Recently, a link has been established between the proliferation of cancer cells and mitochondrial fragmentation. Accordingly, we performed immunoblot analysis of the proteins involved in the regulation of mitochondrial fusion and fission from homogenates of BC cells treated identically to the fragmentation experiment in separate plates. In both BC cell lines, the DEA treatment increased the steady-state level of fusion-associated protein OPA1, but it decreased MFN 1 and 2 in 4T1 cells. However, it increased the steady-state level of fission-associated proteins such as DRP1 and Fis1.

3.3.6 COX-2 Inhibition Potentiated DEA's Anti-Neoplastic Effect in the TN BC Cell Line

We treated both cell lines with 0–15 μM of DEA for 24 h in the presence and absence of 20 μM of the COX-2 inhibitor celecoxib before measuring the viability of the cells using the SRB assay. DEA reduced the viability of both BC lines in a time and concentration-dependent way. However, as expected, the viability loss caused by DEA treatment was higher for the MCF-7 than for the 4T1 cell line, indicating a higher treatment sensitivity for the former cell line. However,

when COX-2 was inhibited by celecoxib, DEA's effect on the viability of 4T1 cells was significantly more pronounced in contrast to MCF-7 cells, suggesting that COX-2 activation may have contributed to the resistance of 4T1 cells to DEA treatment.

3.3.7 Effect of DEA on MCF-7 and 4T1 Colony Formation

The effect of COX-2 inhibition on DEA's anti-neoplastic effect was tested using colony formation assay. We treated the MCF-7 and 4T1 cells with 0 to 2 μM DEA in the presence or absence of 5 μM celecoxib for 7 days before quantifying colony formation. Even at the concentration of 1 μM, DEA significantly suppressed colony formation in both cell lines. The TNBC line 4T1 demonstrated higher resistance against the treatment, since 2 μM DEA almost eradicated MCF-7 colony formation while it induced about a 50% decrease only in the formation of 4T1 colonies. However, as in the case of the viability study, 5 μM celecoxib augmented the effect of DEA on the 4T1 cells, and the combined treatment decreased colony formation in this cell line close to the level of the one observed in the MCF-7 line. In contrast, celecoxib did not affect DEA's effect on MCF-7 colony formation.

3.4 Discussion 2.0

Despite regular chemotherapy followed by a surgery, tumor cells frequently persist, and metastasis may develop. New therapeutic strategies are needed to improve the prognosis of patients with aggressive tumors including breast cancer, especially TNBC, since this cancer type is characterized with metastatic patterns, aggressiveness and poor prognosis (5% of all cancer-related deaths). This category of aggressive tumors has a balanced metabolism, where mitochondria is actively involved. Mitochondria participate in cancer development starting from carcinogenesis via tumor survival and therapy resistance to metastasis formation and most of cancer types rely on mitochondrial ATP synthesis for energy production. Therefore, drugs that significantly interfere with mitochondrial energy production and processes may have a therapeutic value in these tumors. DEA fulfill this principle based on its mitochondrial effects in B16F10 melanoma cells presented in part I. In the current study, we reported how low concentrations of DEA reduces the viability of the BC cell lines MCF-7 and 4T1 in a concentration- and time-dependent manner, through disrupting mitochondrial processes followed by apoptotic cell death. Of note, the mouse 4T1 was used rather than the human MDA-MB-231 TNBC cell line, because the therapeutic dose of DEA must be determined in future animal experiments, since *in-vitro* cell culture experiments translate very poorly to human studies.

Mitochondria play a significant role in cellular survival through many mechanisms, most importantly ATP production and intrinsic apoptosis. The latter depends on ATP as an energy source, while energy shortage induces $\Delta\Psi_m$ loss which enhances the release of pro-apoptotic IMS proteins to the cytosol resulting in apoptosis [33, 34]. Hence, DEA caused apoptotic cell death in both BC cell lines , which was determined by annexin V staining of the phosphatidylserine residues in the outer surface of the cell membrane, decrease in the BCL-2/Bax ratio, activation of caspase 3 and cleavage of PARP-1; all are hallmarks of apoptosis. In agreement with its TN phenotype, 4T1 cell line showed less sensitivity in response to DEA treatment than the HR+ MCF-7 cell line in these experiments.

TNBC's proliferation and metastases formation in the liver, brain, and lungs are much higher than other BC types. Accordingly, we found that 4T1 cells were less sensitive against DEA treatment than the MCF-7 cells in colony formation and invasive growth experiments as well. In addition, the results showed that DEA reduced colony formation below 50% of the control at a concentration of 2 μM during a seven-day exposure, and at 10 μM, it eliminated invasive growth during a 12 h treatment, suggesting that DEA's therapeutic concentration may not exceed the DEA concentrations, which were observed previously during human AM therapy.

One of the key roles in cellular survival is mitochondrial network dynamics regulation which requires a healthy $\Delta\Psi_m$. Since DEA decreased the $\Delta\Psi_m$ in both BC cell lines; mitochondrial dynamics would be disrupted and so mitochondrial biogenesis and quality control, which are essential for meeting the cellular energy and metabolic demands. Mitochondrial biogenesis is

represented through mitochondrial fusion and fission processes that are mediated by large GTPases; MFN 1, 2 and OPA1 for the fusion and Drp1 for the fission. The latter is regulated by phosphorylation and recruited to the mitochondria by Fis1. Balance of the fusion and fission processes is maintained by intracellular signaling, but the fusion is prevented when the $\Delta\Psi_m$ is low. Therefore, mitochondrial fragmentation is a common feature in many cancer types such as in breast, colon, and hepatocellular carcinomas. Fragmented mitochondria are more likely to be damaged and eliminated by mitophagy, leading for mitochondrial copy number reduction. Accordingly, DEA caused mitochondrial fragmentation in both BC cell lines that might contribute to its anti-cancer properties. Since DEA depolarized the $\Delta\Psi_m$, mitochondrial fragmentation caused by DEA treatment in BC cell lines seemed to be caused by promoting fission, as Drp1 expression increased accompanied by its decreased inhibitory phosphorylation. Compared to B16F10 melanoma cell line; it seems that mitochondrial fragmentation was caused by inhibiting fusion, which was supported by the release of OPA1 from mitochondria to the cytosol. It seems that DEA interacted with a yet to be identified regulatory element related to the mitochondria that induced mitochondrial fragmentation by shifting the balance of fusion and fission. Thus, DEA's target has to be localized to the mitochondria, since DEA induced mitochondrial fission in isolated, Percoll gradient-purified mitochondria.

Previously, we have established the effect of DEA on AKT pathway in B16F10 cells. It is well known that constant activation of AKT signaling pathway is important for cancer progression through promoting cell survival and inhibiting apoptosis. In TNBC, dysregulation of AKT signaling pathways is one of the most frequent oncogenic anomalies. We showed that AKT activation was decreased by DEA in both BC cell lines. Furthermore, phosphorylated AKT level, which is the baseline activation of AKT, in HR+ MCF-7 cell line was significantly lower than in TN 4T1 cells. Since AKT had strong mitochondria-protecting potential; the decreased activation of AKT together with the abovementioned compromised mitochondrial functions may account for the differential apoptosis-promoting effect of DEA among the BC cell lines investigated.

Inflammatory cells and factors are major components of the tumor microenvironment. COX-2 is one of the most significant immunomodulatory factors found in tumors, and the most studied anti-inflammatory target in cancer therapy since it is associated with large tumor size, lymph node metastasis, and poor differentiation. The use of COX-2 inhibitor celecoxib in metastatic BC therapy as monotherapy and combination with aromatase inhibitors, proved to be effective through reducing the size and the area of the tumor. Currently, COX-2 overexpression is linked with anti-cancer drug resistance in BC. Of note, COX-2 is highly inducible and can be rapidly upregulated in response to pro-inflammatory agents like cytokines, even though it is barely expressed in healthy tissues. Surprisingly, COX-2 was hardly detected in MCF-7 cell line and not affected by DEA treatment as well. However, in 4T1 TNBC cell line, COX-2 was expressed and even elevated when treated with different DEA concentrations. While the upregulation of COX-2 has been implicated in cancer therapy resistance, the latter result raised the possibility that DEA reduced its own anti-cancer effects in the 4T1 cell line, thus creating the difference between the two BC cell lines in

response to DEA treatment. Indeed, celecoxib enhanced DEA's effect on viability and colony formation of the 4T1 cells only. Recently, studies have shown activation of caspase-3 during radiotherapy-induced apoptosis, which resulted in prostaglandin E_2 production by COX-2, that is linked with treatment resistance. Accordingly, DEA treatment induced caspase-3 activation in both BC cell lines but upregulation of COX-2 expression in the 4T1 cell line only. It seems that the DEA-induced caspase 3-assisted mitochondrial apoptosis resulted in COX-2-mediated resistance in 4T1 cells, which expressed it. Consequently, the difference in COX-2 expression accounted for, at least partially, the differential anti-neoplastic effects of DEA in several cell lines. These results also suggest that co-treatment with COX-2 inhibitors can increase the efficacy of DEA and significantly reduce therapy resistance.

Our results show the ability of DEA treatment to induce predominant apoptotic cell death by modulating mitochondrial functions in BC cells. Regardless the low efficacy compared to HR+ BC cells, DEA exerted anti-neoplastic effects in 4T1 TNBC cells, too. Besides, the upregulation of COX-2 in DEA-treated 4T1 cells, as a DEA-resistant mechanism, was counteracted by inhibiting COX-2 enzymatic activity.

4 Conclusions

Cancer is the second leading cause of death worldwide, which makes it a major threat facing the humans. More than hundred types of cancer have been identified including skin and breast cancer. Accordingly, treatment choices are then considered based on the detected cancer type and stage. Designed anti-cancer treatments, for the majority of cancers especially the metastatic ones, are insufficient after the prolonged treatment period due to the resistance developed afterward. Other treatments were limited because of their toxic side effects. Here, Desethylamiodarone (DEA) is presented. DEA is the major metabolite of the well-known antiarrhythmic FDA approved drug, Amiodaron (AM).

In the first part of the study, a view of DEA's workflow was determined using *in-vitro* and *in-vivo* metastatic melanoma model. Since metastatic melanoma considered the most lethal form of skin cancer, and one of the top six most common cancer-related mortality worldwide. Consequently, DEA's main target was identified *in-vitro*, the mitochondrial mechanisms resulting in intrinsic apoptosis, furthermore, melanoma growth and metastasis were inhibited by DEA's treatment *in-vivo*. Considering the therapeutic concentrations of anti-cancer drugs; DEA's treatment is much lower in both *in-vitro* and *in-vivo*, which elevate the chance of being a promising candidate for metastatic tumors therapy.

Regarding anti-cancer drug resistance, a highly resistant cancer type, TNBC, was used along with treatable type, HB^+ BC, under DEA's low treatment concentrations. By comparing the two types, TNBC showed less sensitivity as DEA's treatment increases through elevating the inflammatory COX-2 protein expression. Thus, a combination of DEA and COX-2 inhibitor celecoxib was used, which results in synergetic effect led to almost the same DEA's effect in both BC types. Concluding, DEA could be combined with specific inhibitors for resistant-cancer treatment.

Since DEA is the major metabolite of AM, considering that within the suggested safety limits, the drug does not have therapy-restricting side effects, the safety concerns might not hinder the introduction of DEA into clinical studies.

5 Publications

- Publications related to the thesis

Ramadan, Fadi H.J, et al. “Involvement of Mitochondrial Mechanisms in the Cytostatic Effect of Desethylamiodarone in B16F10 Melanoma Cells.” International Journal of Molecular Sciences, vol. 21, no. 19, 2020, p. 7346., doi:10.3390/ijms21197346. Q1/D1. IF: 5,923

Bognar, Zita, et al. “Amiodarone’s Major Metabolite, Desethylamiodarone Inhibits Proliferation of B16-F10 Melanoma Cells and Limits Lung Metastasis Formation in an in Vivo Experimental Model.” PLOS ONE, vol. 15, no. 9, 2020, doi:10.1371/journal.pone.0239088. Q1. IF: 3.24

Gallyas, Ferenc, et al. “Involvement of Mitochondrial Mechanisms and Cyclooxygenase-2 Activation in the Effect of Desethylamiodarone on 4t1 Triple-Negative Breast Cancer Line.” International Journal of Molecular Sciences, vol. 23, no. 3, 2022, p. 1544., doi:10.3390/ijms23031544. Q1/D1. IF: 6.208

- Other publications

Szekeres, Zsolt, et al. “Clinical Study of Metabolic Parameters, Leptin and the SGLT2 Inhibitor Empagliflozin among Patients with Obesity and Type 2 Diabetes.” International Journal of Molecular Sciences, vol. 24, no. 5, 2023, p. 4405., doi:10.3390/ijms24054405. Q1/D1. IF: 6.208

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