”VICTOR BABEȘ” UNIVERSITY
OF MEDICINE AND PHARMACY TIMIŞOARA

FACULTY OF MEDICINE
DEPARTMENT OF FUNCTIONAL SCIENCES

MITROI ALINA MARIA

ABSTRACT

PHARMACOLOGICAL MODULATION OF MITOCHONDRIAL FUNCTION IN BLOOD CELLS

Scientific coordinator:

PROF. MUNTEAN MIRELA-DANINA, MD, PhD

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Key words: platelets, PMBCs, HepG2 cells, mitochondrial dysfunction, high-resolution respirometry, ATP, amiodarone, desethylamiodarone, sotalol, cell-permeable succinate, ibuprofen, APAP

I. BACKGROUND & RESEARCH OBJECTIVES

Cardiovascular disorders are the leading cause of mortality globally. As the population ages and additional risk factors for arrhythmias are discovered, it is expected that the prevalence of arrhythmias will increase. Amiodarone, a class III antiarrhythmic drug (according to Vaughan-Williams' classification) is one of the widely prescribed drugs, mostly due to its effectiveness in the treatment of supraventricular and ventricular arrhythmia. Besides its higher efficacy as compared to the majority of the other antiarrhythmics, amiodarone demonstrates a minimal negative inotropic activity together with a low incidence of ventricular proarrhythmic effects, rendering it a valuable therapy in patients with heart failure. However, it is well knownn that the usage of amiodarone may lead to several adverse reactions. In the absence of contraindications, sotalol, a non-cardioselective beta-blocker, also belonging to the class III antiarrhythmic drugs, may serve as a potential substitute for amiodarone due to its more advantageous adverse-event profile.

Although the specific mechanisms underlying human amiodarone-induced toxicity are not completely elucidated, a growing body of evidence indicates that altered mitochondrial bioenergetics together with oxidative stress play an essential role.

The importance of mitochondrial dysfunction as a primary pathophysiological mechanism in nearly all cardiovascular pathologies is currently widely acknowledged, making these organelles viable targets for novel therapeutic strategies; yet no drugs are available for clinical use so far. Cell-permeable succinate prodrugs have been designed to support oxidative phosphorylation in the setting of drug-induced toxicity or in various acute and chronic pathologies characterized mainly by a defective activity of complex I of the electron transport system (ETS) at the inner mitochondrial membrane. These prodrugs designed to have increased cellular membrane permeability, are able to provide the ETS with succinate, the substrate of complex II, thus allowing to recover/improve the electron transport impaired by disease and/or drug toxicity.

Acetaminophen (N-acetyl-p-aminophenol, APAP) and ibuprofen are two of the most widely used over-the-counter drugs and mitochondrial dysfunction has emerged as an important pathomechanism in the toxicity of both drugs. However, despite several of studies conducted in animal models, there is a paucity of information concerning the role of mitochondria in the toxicity of APAP and ibuprofen in humans. Regrettably, the COVID-19 pandemic amplified the rate of self-medication, resulting in a higher probability of mitochondrial damage from overdose and/or drug interactions. This is especially relevant in the elderly population, whose comorbidities make rather challenging to possibility to prevent simultaneous use of multiple drugs with associated mitochondrial liabilities, in addition to the age-related decrease of mitochondrial function.

Blood cells isolated from the peripheral blood have been increasingly used in research as alternatives to tissue biopsies for assessing mitochondrial bioenergetics in humans. In the past decade, isolated platelets have particularly emerged as a source of viable mitochondria for the study of respiratory mitochondrial dys/function in various acute and chronic pathologies. Similarly, drug-induced mitochondrial toxicity has been investigated in these cells by assessing the dose-dependent effect of several drugs on mitochondrial respiration and how mitochondriotropic compounds could counteract it.

The aim of the PhD study was to characterize the effects of commonly used antiarrhythmic and analgesic drugs (two of each class), along with the investigation of the capability of a cell-permeable succinate compound, NV118 (generously provided by Abliva AB

through the kindness of Prof. Dr. Eskil Elmer of Lund University, Sweden) to counteract drug toxicity, on mitochondrial respiratory function in several cell types.

The research within the doctoral study was carried out at the Centre for Translational Research and Systems Medicine from the Faculty of Medicine at ”Victor Babes” University of Medicine and Pharmacy of Timisoara, and at the Centre for Mitochondrial Medicine from the Lund University, Sweden, respectively, based on the approvals of the Ethics Committees of both universities (as mentioned in the two original articles).

The research objectives were as follows:

1. Characterization of the acute concentration-dependent effects of amiodarone, its metabolite, desethylamiodarone (DEA) and sotalol on mitochondrial respiration in isolated human platelets.
2. Characterization of the acute concentration-dependent effects of amiodarone on mitochondrial respiration in isolated human peripheral blood mononuclear cells (PBMCs) and in HepG2 cells, a human liver cell line.
3. Assessment of the potential of a cell-permeable succinate prodrug, NV118, to alleviate the drug-induced impairment of platelet respiration.
4. Characterization of the acute dose-dependent effects of APAP and ibuprofen on mitochondrial respiration in platelets isolated from healthy blood donors-derived buffy coat.

II. RESULTS OF THE DOCTORAL RESEARCH included in the Special Part of the thesis:

1. Amiodarone But Not Sotalol Induced Dose-Dependent Mitochondrial Respiratory Dysfunction in Human Platelets

The effects of amiodarone or sotalol on mitochondrial respiration were firstly assessed in intact human platelets exposed to increasing concentrations (15–240 µM) of these drugs and compared to the equivalent volume of solvent (DMSO). By measuring mitochondrial oxygen consumption, it was demonstrated that amiodarone, but not sotalol, induced a significant concentration-dependent inhibition of mitochondrial respiration when applied in the highest concentration (Figure 1A). To identify the mechanism underlying the concentration-dependent ETS dysfunction elicited by antiarrhythmic drugs, mitochondrial respiration was further analyzed in digitonin-permeabilized platelets in the presence of three selected concentrations (60, 120 and 240 µM) of the drugs. Amiodarone elicited a significant progressive inhibition of OXPHOS capacity starting at the lowest tested concentration (60 µM) (Figure 1B), by decreasing both NADH-linked OXPHOS (Figure 1C) and succinate-linked OXPHOS (Figure 1D), with the latter showing a more pronounced drop. ET capacity or the maximal noncoupled respiration, which is a measure of the maximal electron transport (ET) capacity of the ETS was also dose-dependently reduced by amiodarone, but not by sotalol (Figure 1E).

Amiodarone also affected the ATP production by mildly elevating the non-phosphorylating (LEAK) respiration (Figure 1F). Interestingly, the uncoupling effect peaked at 60 µM and fade away with the increase in drug’s concentration.

In order to additionally (indirectly) validate the detrimental effect of amiodarone on ATP generation, two ratios were computed: P-L control efficiency (Figure 1G) and E-L coupling

efficiency (Figure 1H). While both ratios were concentration-dependently reduced by amiodarone, the first one showed a significant decrease even at the lowest concentration tested (60 µM).

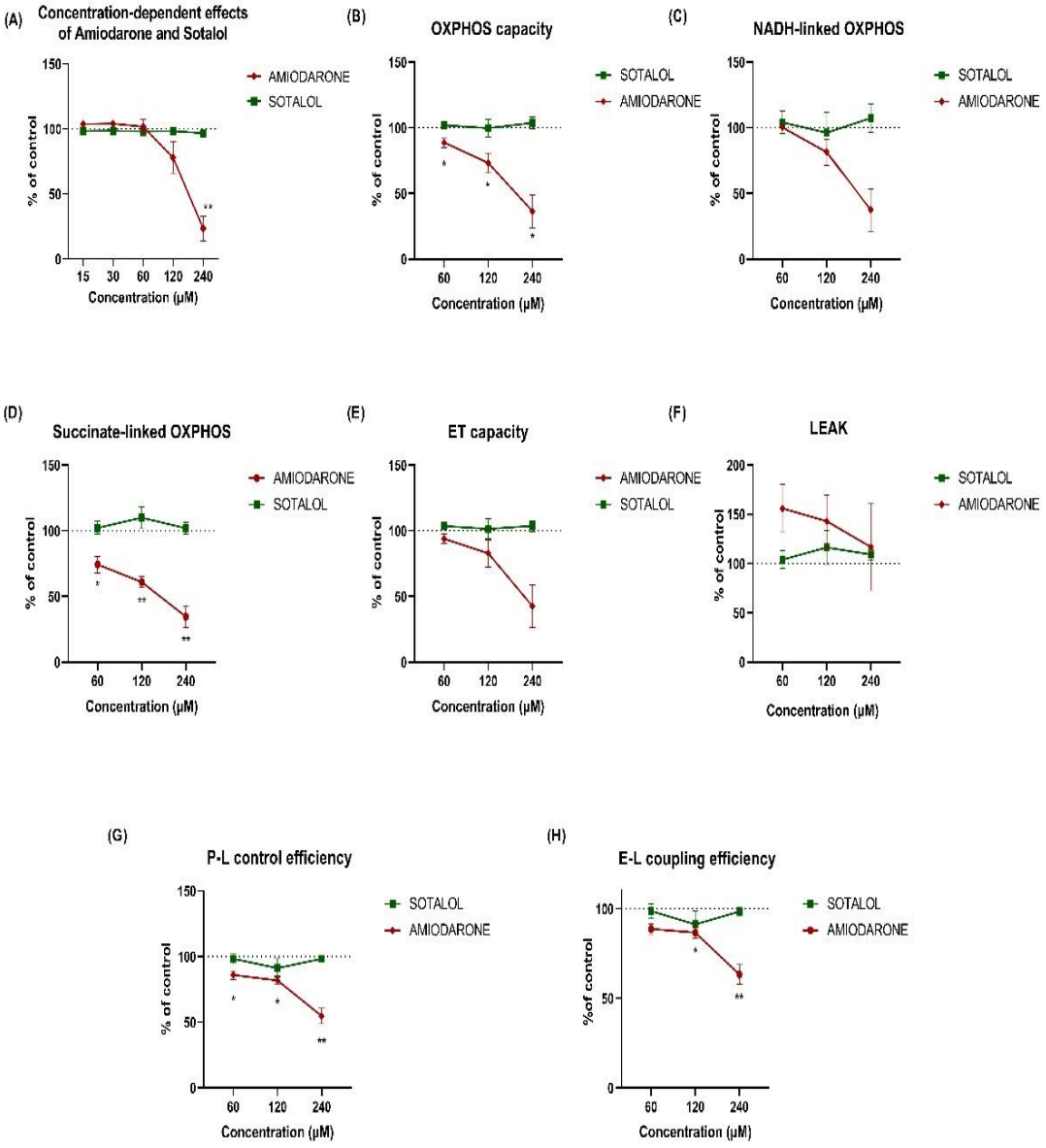

Figure 1. The acute effects of amiodarone and sotalol on mitochondrial respiration of isolated human platelets.

2. Amiodarone Metabolite, Desethylamiodarone (DEA), Induced Mitochondrial Respiratory Dysfunction in Human Platelets

Amiodarone has the potential to induce mitochondrial dysfunction both directly and indirectly through the build-up of its metabolite, desethylamiodarone (DEA). In order to investigate the effects of DEA on mitochondrial oxygen consumption, the same parameters of high-resolution respirometry were analyzed as those used for amiodarone.

When compared to the methanol control, exposure to the highest DEA dose (60 µM) lowered the analyzed parameters as stated below: the OXPHOS capacity (Figure 2A) to 31%±4.1 of control, NADH-linked OXPHOS (Figure 2B) to 20%±5.6 of control, succinate-linked OXPHOS (Figure 2C), to 41.3%±13.1 of control, ET capacity (Figure 2D) to 36.1%±6.5 of control, LEAK respiration (Figure 2E) to 62.2%±3.6, E-L coupling efficiency (Figure 2F) to 53.9%±5.4, but no statistical significance was achieved. In contrast, the P-L control efficiency (Figure 2G) was significantly decreased by DEA (60 µM) in a concentration dependent manner to 37%±13.7 of control ($p<0.05$).

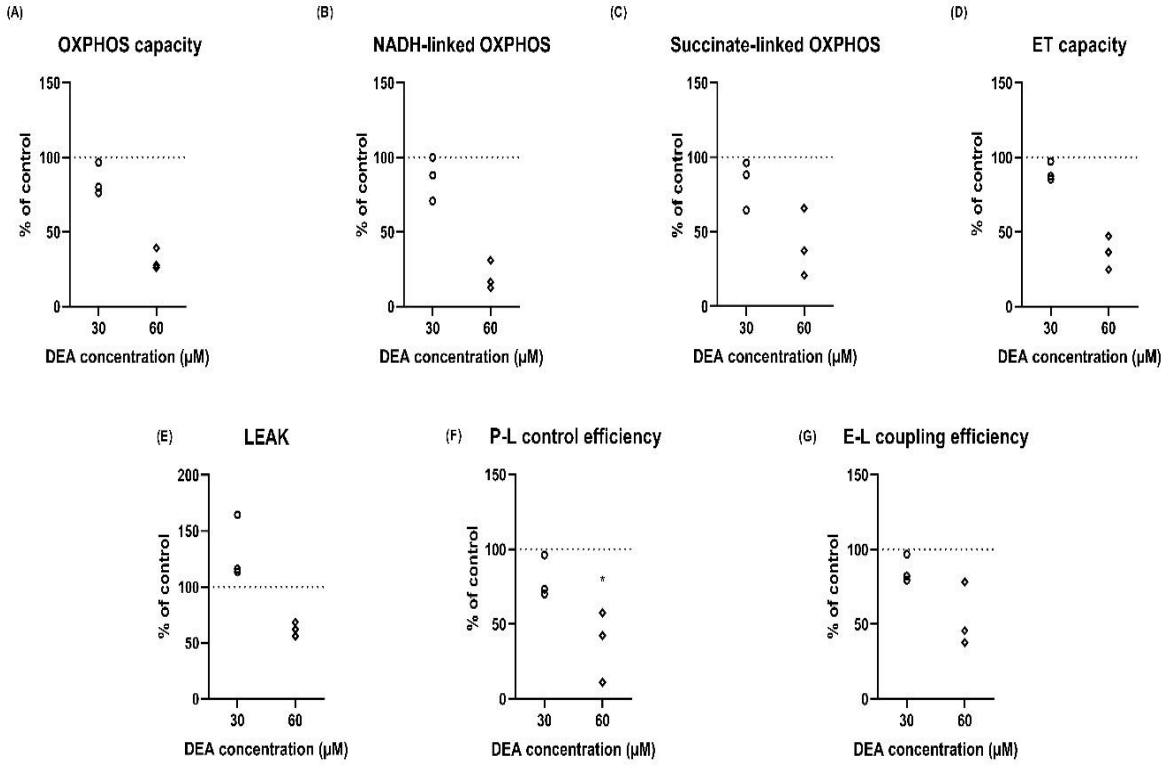

Figure 2. The acute effects of DEA on the mitochondrial respiration in isolated human platelets.

3. Amiodarone Induced Mitochondrial Respiratory Dysfunction and ATP Depletion in Intact Peripheral Blood Mononucelar Cells (PBMC)

To ascertain if the results observed in platelets subjected to amiodarone can be repeated in other blood cells that are more metabolically active, additional assessment of mitochondrial respiration was performed in peripheral blood mononuclear cells (PBMC). As amiodarone induced detrimental effects on total OXPHOS capacity and NADH-linked OXPHOS, the effects of

amiodarone and rotenone (the latter, used as a positive control for complex I inhibition) on mitochondrial oxygen consumption and cellular ATP content were investigated.

Incubation of isolated PBMCs with 100 µM amiodarone or 2 µM rotenone resulted in a time-dependent reduction of mitochondrial oxygen consumption (Figure 3A) and ATP levels (Figure 3B). At 25 minutes of exposure, amiodarone significantly lowered mitochondrial respiration in human PBMCs, but this effect did not exceed the decrease caused by rotenone ($p < 0.0001$). At variance, the drop in the ATP content elicited by amiodarone which excedeed the one induced by rotenone, suggesting additional toxicity beyond mitochondrial toxicity ($p < 0.01$).

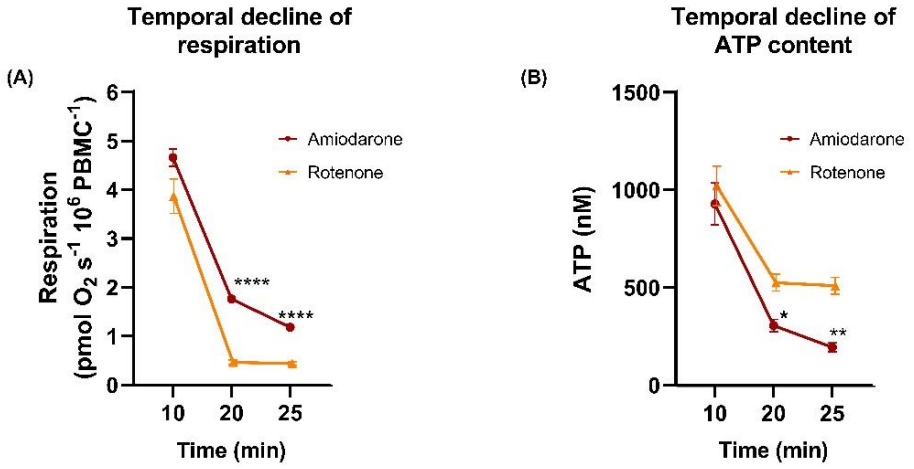

Figure 3. Time-dependent effects of amiodarone on the mitochondrial respiration and ATP levels in PBMCs.

In intact PBMCs the concentration-dependent effect of amiodarone (15-240 µM) on mitochondrial respiration (Figure 4A) and ATP content (Figure 4B), respectively was also assessed. Amiodarone induced a significant impairment of respiration starting from a concentration of 60 µM ($p<0.0001$), whereas the ATP content was already significantly decreased at a concentration of 30 µM ($p<0.0001$).

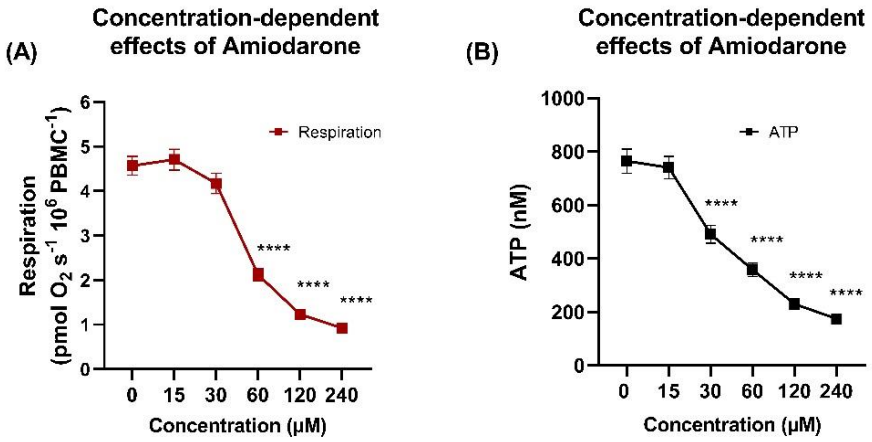

Figure 4. Concentration-dependent effects of amiodarone on mitochondrial respiration and ATP levels in PBMC.

4. Cell-Permeable Succinate NV118 Alleviated the Amiodarone-Induced Acute Respiratory Mitochondrial Dysfunction in Isolated Human Platelets

Since amiodarone induced mitochondrial respiratory impairment by decreasing the activity of both NADH and succinate-linked pathways (with a higher toxicity in case of the latter), the extent to which this dual respiratory dysfunction can be ameliorated by the cell-permeable succinate (NV118) in platelets acutely challenged with the highest concentration of amiodarone (120 µM) was assessed. As an additional measure of control, mitochondrial oxygen consumption was also evaluated in platelets subjected solely to DMSO, the vehicle for both amiodarone and NV118.

Administration of the succinate prodrug to the amiodarone-treated platelets led to an increase in the ET capacity (Figure 5A) which surpassed the control levels (21.3±1.8, vs 10±1.3)($p < 0.05$) and also in the rates of coupled respiration (Figure 5B), a measure of ATP generating respiration which were comparable to those of the control samples (7±0.5 vs 4.4±0.4). To investigate whether the beneficial effects mentioned above may be explained by an increase in succinate-supported respiration (Figure 5C), oxygen consumption was further evaluated after administration of rotenone, the complex I inhibitor. Succinate-linked respiration increased from 0.5±0.1 in amiodarone-exposed platelets to 4.2±0.3 in platelets receiving NV118 ($p<0.01$).

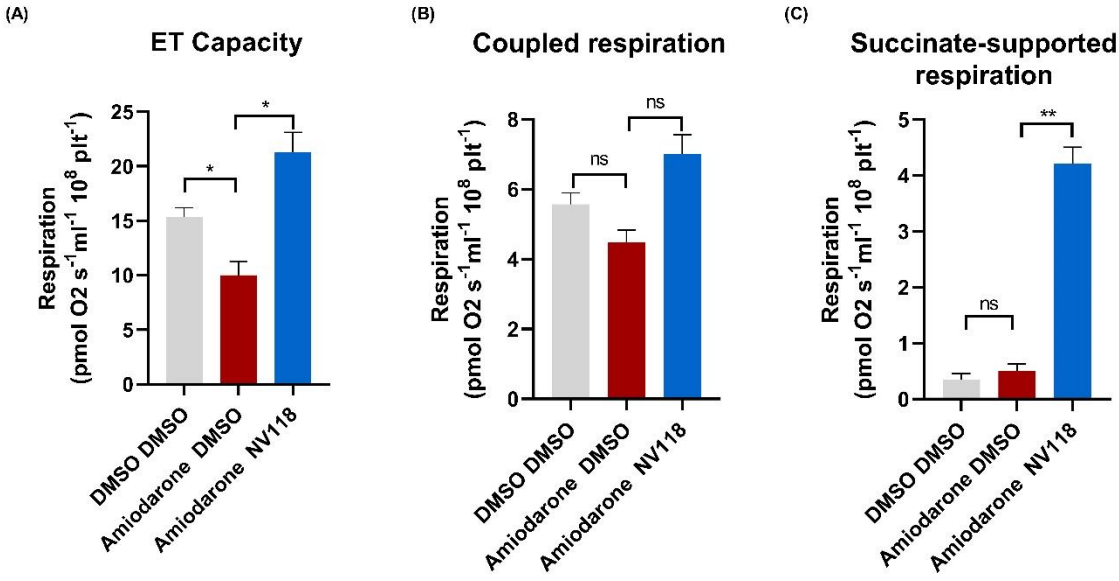

Figure 5. NV118 alleviated the amiodarone-induced respiration impairment in intact human platelets.

5. Cell-Permeable Succinate NV118 Alleviated the Amiodarone-Induced Acute Respiratory Mitochondrial Dysfunction in HepG2 Cells

Given the well known amiodarone hepatotoxicity side-effect, additional studies were performed in order to determine if the beneficial effects of NV118 occur in a liver-derived human cell line (HepG2 cells).

A concentration-dependent respiratory impairment was determined by amiodarone, following exposure to increasing concentrations of the drug in intact HepG2 cells (Figure 6A). Comparably to the results acquired in human platelets, NV118 improved the ET capacity (Figure 6B) of amiodarone-exposed HepG2 cells from 70.6±5.2 to 101±7.4, by enhancing the succinate-supported respiration (Figure 6D) from 2.4±1 to 18±2.1 ($p<0.05$). Coupled respiration (Figure 6C) was also improved in the presence of NV118, with an oxygen consumption increasing from 15.6 ±1.4 to 26.7±2.9 ($p < 0.05$).

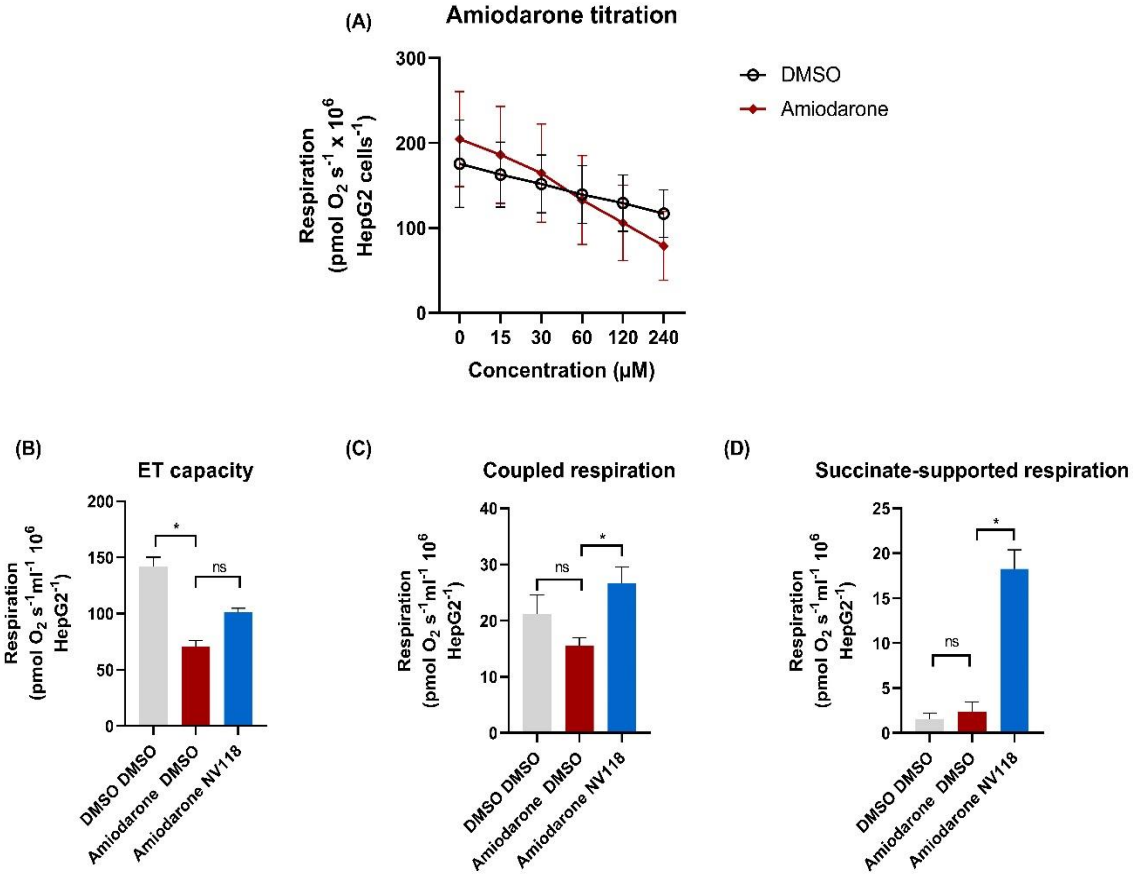

Figure 6. NV118 alleviated amiodarone-induced respiration impairment in HepG2 cells.

6. APAP Induced Impairment of Mitochondrial Respiration in Human Platelets Isolated From Blood Donors Buffy Coat

The concentration-dependent effect of APAP on mitochondrial respiration was initially investigated in intact platelets (vs the equivalent amount of solvent, DMSO). APAP elicited a significant inhibition of mitochondrial respiration already at 4 mM (Figure 7A).

In order to gather further insights into the mechanisms behind the ETS dysfunction determined by APAP, mitochondrial respiration was investigated in permeabilized platelets incubated for 10 minutes with the highest previously tested concentration (10 mM) of APAP. In the presence of

APAP, both NADH-linked OXPHOS (Figure 7B) and OXPHOS capacity (Figure 7C) were significantly reduced, while the non-phosphorylating LEAK respiration remained unaffected (Figure 7D). ET capacity (Figure 7E) indicating the maximal uncoupled respiration was also altered by APAP in a comparable manner, although without reaching statistical significance. The succinate-linked ET capacity (Figure 7F), measured after the addition of rotenone to induce complex I inhibition also remained unaffected in the presence of APAP.

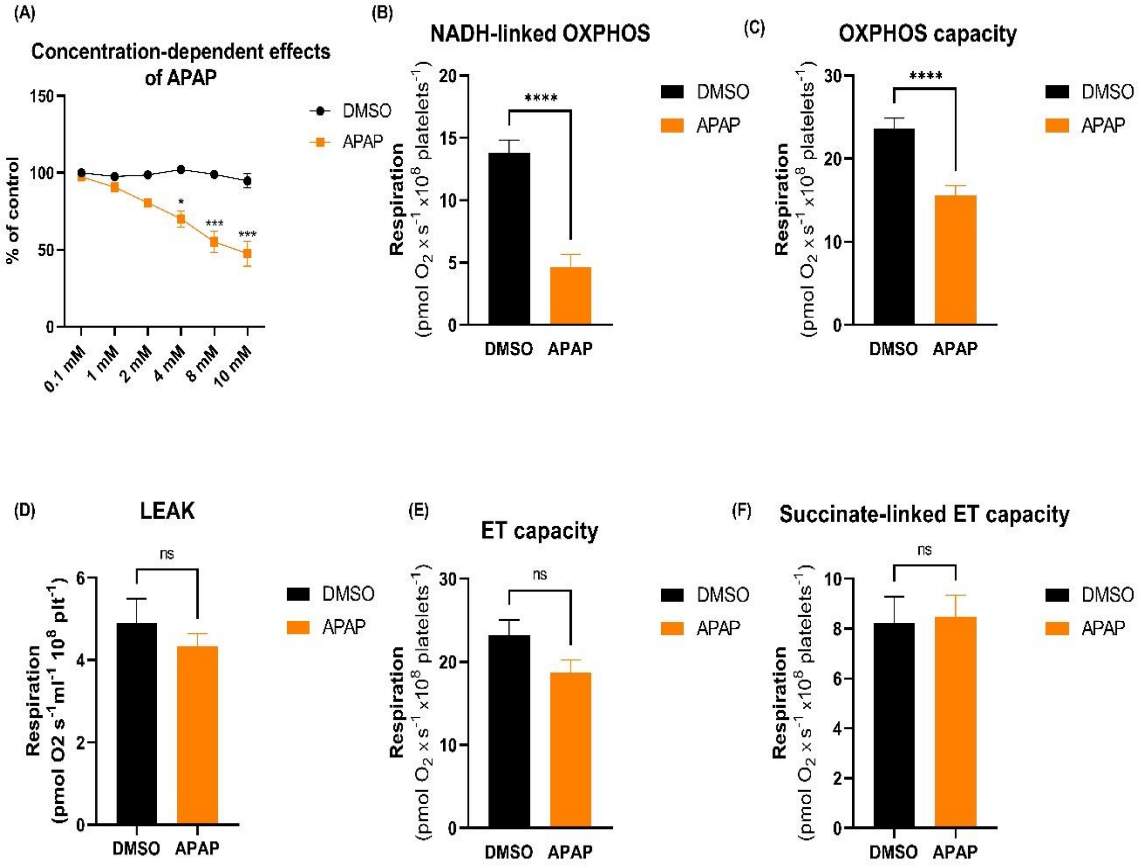

Figure 7. The acute effects of APAP on the mitochondrial respiration in buffy coat platelets.

7. Ibuprofen Induced Mitochondrial Respiratory Dysfunction in Human Platelets Isolated from Blood Donors Buffy Coat

The evaluation of mitochondrial oxygen consumption in intact human platelets derived from buffy coat was performed by applying gradually higher concentrations of ibuprofen, reaching up to 3 mM. As compared to the DMSO control, ibuprofen elicited a significant and progressive drop in mitochondrial oxygen consumption, starting at a concentration of 1.5mM ($p < 0.05$), that resulted in severe respiratory inhibition when the maximal concentration was reached ($p < 0.0001$) (Figure 8A).

To localize the defect underlying ibuprofen-induced ETS disruption, mitochondrial respiration was assessed in digitonin-permeabilized platelets exposed to three concentrations of

ibuprofen (1, 1.5, and 2 mM). The rationale for evaluating three different drug concentrations (with no effect, moderate, and significant detrimental effect, in intact platelets) was due to the fact that, contrary to APAP, there was no information in the literature about the effects of ibuprofen on platelet respiration.

Ibuprofen induced a concentration-dependent decrease of both NADH-linked OXPHOS (Figure 8B) and OXPHOS capacity (Figure 8C), attaining statistical significance at the concentration of 2 mM vs control ($p < 0.05$). LEAK respiration (Figure 8D) demonstrated an upward-trending pattern following exposure to lower doses of ibuprofen, with the maximum increase being noticed at 1.5 mM, compared to the control. ET capacity (Figure 8E), was found to be significantly decreased by each of the three analyzed concentrations of ibuprofen.

Two ratios were calculated to investigate (indirectly) the influence of ibuprofen on ATP generation: P-L control efficiency (Figure 8F) and E-L coupling efficiency (Figure 8G), both being significantly lowered in the presence of ibuprofen in a concentration dependent manner.

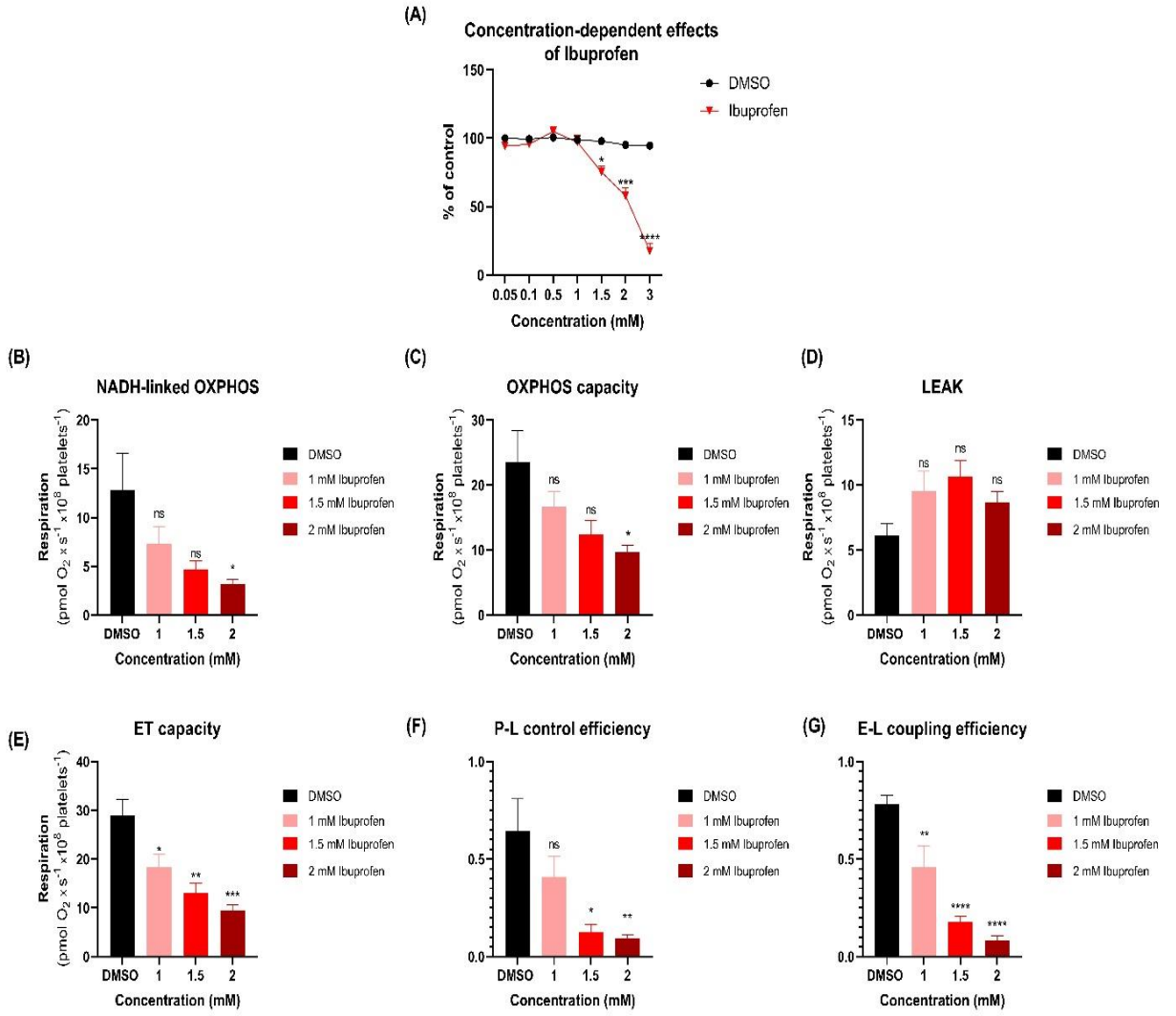

Figure 8. The acute effects of ibuprofen on the mitochondrial respiration in buffy coat platelets.

IX. CONCLUSIONS

1. Amiodarone determined a dose-dependent reduction of OXPHOS capacity in isolated human platelets, mainly by decreasing succinate-supported respiration.

2. Amiodarone elicited a reduction of ATP generating efficiency in human platelets via the association of two mechanisms: direct inhibition of electron transport and uncoupling (increased LEAK respiration).

3. Desethylamiodarone (DEA) elicited a concentration-dependent inhibition of mitochondrial respiration in isolated platelets, suggesting that amiodarone may induce mitochondrial toxicity both direct and indirect, via the accumulation of its metabolite.

4. Amiodarone induced a time-dependent inhibition of respiration and lowered ATP content of PBMCs. The latter effect depassed the one of rotenone (a classic inhibitor of respiration), indicating that the deleterious effects of the drug may extend beyond mitochondrial dysfunction.

5. Sotalol did not impaired the mitochondrial respiration in isolated human platelets.

7. A mitochondriotrop compound, cell-permeable succinate, significantly improved coupled respiration in amiodarone-induced mitochondrial dysfunction in both isolated platelets and the HepG2 cells, and may represent an effective therapeutic approach to counteract amiodarone toxicity.

9. Platelets isolated from healthy blood donor-derived buffy coat are a readily accessible population of cells that can be regularly utilized in research addressing the mitochondrial toxicity of drugs (isolated or in combination).

10. In platelets isolated from buffy coat, both acetaminophen and ibuprofen decreased mitochondrial oxygen consumption in a concentration dependent manner, mainly by inhibiting complex I supported (NADH-linked) respiration; this observation points to the benefits of using the cell permeable succinate prodrugs in counteracting the putative drug-induced mitochondrial toxicity (by supporting complex II-related respiration).

X. ORIGINAL CONTRIBUTIONS

- Characterization of the dose-dependent amiodarone-, DEA- and sotalol-induced impairment of mitochondrial respiration in isolated human platelets.
- Characterization of amiodarone-induced mitochondrial respiratory dysfunction in PBMCs and HepG2 cells.
- Demonstration of the amiodarone direct suppressive effect of cellular ATP production.
- Demonstration of the beneficial role of a cell-permeable succinate in alleviating the deleterious effects of amiodarone on mitochondrial respiration in two cell types.
- Characterization of the acetaminophen- and ibuprofen-induced mitochondrial respiratory dysfunction in platelets isolated from blood donor-derived buffy coat, according to a technique implemented for the first time in Laboratory for Mitochondria Studies from the Centre for Translational Research and Systems Medicine where I was affiliated during my doctoral studies.

XI. FUTURE RESEARCH DIRECTIONS

- To characterize the effects on mitochondrial respiration of acute exposure to amiodarone in combination with other drugs reported in the literature to be able to induce mitochondrial dysfunction (statins, metformin), which are commonly used in cardiometabolic pathology.
- To characterize the effects on mitochondrial respiration of acute exposure to amiodarone in combination with the most frequently used OTCs pain relievers.
- To assess the efficacy of other cell-permeable succinates prodrugs in improving mitochondrial respiration in the presence of combined drug-induced toxicity and diseased-related mitochondrial dysfunction.

XII. SCIENTIFIC PUBLICATIONS

1. **Bețiu AM**, Chamkha I, Gustafsson E, Meijer E, Avram VF, Åsander Frostner E, Ehinger JK, Petrescu L, Muntean DM, Elmér E. *Cell- Permeable Succinate Rescues Mitochondrial Respiration in Cellular Models of Amiodarone Toxicity*. **International Journal of Molecular Sciences 2021**; 22(21):11786. **ISI Journal (FI – 6.208)**
2. **Bețiu AM**, Noveanu L, Hâncu IM, Lascu A, Petrescu L, Maack C, Elmér E, Muntean DM. *Mitochondrial Effects of Common Cardiovascular Medications: The Good, the Bad and the Mixed*. **International Journal of Molecular Sciences 2022**; 23(21):13653. doi: 10.3390/ijms232113653. **ISI Journal (FI – 5.6)**
3. **Bețiu AM**, Lighezan R, Avram VF, Muntean DM, Elmér E, Danina M, Petrescu L. *Dose-Dependent Effects of Acetaminophen and Ibuprofen on Mitochondrial Respiration of Human Platelets*. **Mol Cell Bioch 2023** Jul 24. doi: 10.1007/s11010-023-04814-z. **ISI Journal (FI – 4.3)**