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Organelle-targeted Radioconjugates for Auger Therapy of Prostate Cancer

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II

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"One day, in retrospect, the years of struggle will strike you as the most beautiful"

– Sigmund Freud

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Abstract

Prostate cancer (PCa) remains a major public health problem since it is one of the most diagnosed cancer in men worldwide and one of the leading causes of cancer-related deaths. Thus, there is a huge demand for novel diagnostic and therapeutic strategies. Recently, targeted radionuclide therapy (TRT) has emerged as an efficient tool for cancer treatment. TRT strategies deliver ionizing radiation to tumors in a targeted manner, reducing off-target damage. In PCa, targeting prostate-specific membrane antigen (PSMA), which is overexpressed in prostatic cancer, has shown enormous potential.

TRT using Auger electrons (AE) has shown greater potential to treat small size cancers and metastases, because of their high level of cytotoxicity to tumor cells, high linear energy transfer (LET) and short-range biological effectiveness. Moreover, organelles such as mitochondria are emerging as potential targets for radiotherapy-based therapies. Therefore, the AugerTher project aims to study dual-targeted metal complexes based on AE-emitting radionuclides carrying a PMSA ligand ($PSMA_{617}$) for selective uptake by PCa cells, and pharmacophores with a well-recognized affinity for the nucleus or the mitochondria. By initiating the targeting of mitochondria, this project expects to change the radiation biology paradigm, which sustains that the biological effects of radiation are associated to nuclear damage.

In the present study, we assessed the effects of $PSMA_{617}$ alone and ^{nat}In-DOTAGA-AO on the viability and cellular metabolism of PCa, using four *in vitro* cell models (cell lines PNT2, LNCaP, PC3-FLU and PC3-PIP). Our findings suggests that the $PSMA_{617}$ alone did not induce toxicity to the cells, but was accompanied by small alterations in mitochondrial and glycolytic functions, regardless the cell phenotype (normal or cancerous). Interestingly, we found that cells that do not express PSMA were more sensitive to $PSMA_{617}$. Moreover, the alterations observed, although non-significant, suggest that treatment with $PSMA_{617}$ in cells expressing PSMA may lead to the internalization of PSMA protein leading to a decrease in cell metabolism which at long-term can lead to cell proliferation and migration inhibition. On the other hand, all the cells internalized the cold-surrogate ^{nat}In-DOTAGA-AO within the nucleus and

mitochondria which leads to alterations in mitochondrial and glycolytic function, mitochondrial membrane potential (MMP), and mitochondrial ROS production. Moreover, although ^{nat}In-DOTAGA-AO did not induce cytotoxicity in our *in vitro* models, it seems to revert the cancerous phenotype.

Therefore, this study represents an important preliminary work for a major project, and supports the development of AE-emitting radiocomplexes functionalized with targeting ligands such as PSMA$_{617}$ and mitochondria-directed ligand to target specific PCa cells' organelles.

Resumo

O cancro da próstata continua a ser um dos maiores problemas de saúde publica sendo um dos cancros mais diagnosticados em homens a nível mundial e é uma das principais causas de morte relacionada ao cancro. Assim, a procura de novas estratégias diagnosticas e terapêuticas é elevada. Recentemente, a terapia com radionuclídeo direcionado (TRT) tem surgido como uma eficiente ferramenta para o tratamento de cancro. As estratégias de TRT direcionam radiação ionizante para os tumores, reduzindo assim danos para alem do alvo. O Antígeno de Membrana Específico da Próstata (PSMA) que tem elevada expressão no cancro da próstata, em PCa o seu direcionamento tem mostrado um enorme potencial, tanto para diagnostico, como para tratamento.

A utilização de eletrões Auger (AE) em TRT tem demonstrado uma grande potencial para tratar cancros e metástases de pequenas dimensões devido ao seu elevado nível de citoxicidade em células tumorais, à alta transferência linear de energia (LET) e à eficácia biológica de curto alcance. Alem disto, os organelos e as biomoléculas como a mitocôndria tem surgindo como potenciais alvos para terapias à base de radioterapia. Portanto, o projeto AugerTher tem como objetivo estudar complexos metálicos de direcionamento duplo baseados em radionuclídeos que emitam AE cuja estrutura é funcionalizada com um ligante PMSA (PSMA$_{617}$) de modo que a que este complexo seja capturado seletivamente por as células PCa, e pretende ainda estudar os utilizar moléculas com alta afinidade com o núcleo e a mitocôndria para alvejar especificamente esses organelos. Assim, este projeto espera mudar o paradigma da biologia da radiação, que sustenta que os efeitos biológicos da radiação estão associados ao dano nuclear.

Neste estudo, nós avaliamos o efeito biológico do tratamento com PSMA$_{617}$ isolado e de ^{nat}In-DOTAGA-AO tanto na viabilidade das células, bem como no seu metabolismo celular, utilizando quatro modelos *in vitro* (PNT2, LNCaP, PC3-FLU and PC3-PIP). Os nossos resultados sugerem que a resposta das células a PSMA$_{617}$ isoladamente não induz toxicidade celular, mas ocorrem pequenas alterações na função mitocondrial e glicolítica, independentemente do fenótipo celular (normal ou cancerígena). Curiosamente, os resultados sugerem que as células que não expressam PSMA são mais

sensíveis ao $PSMA_{617}$. Além disto, os resultados, embora não significativos, sugerem que o tratamento com $PSMA_{617}$ em células que expressam PSMA pode levar à internalização da proteína contribuindo para a diminuição do metabolismo celular que a longo prazo pode levar à diminuição da proliferação celular e inibição da migração. Por outro lado, todas as células internalizaram o composto frio ^{nat}In-DOTAGA-AO tanto no núcleo como na mitocôndria, levando a alterações das funções mitocondrial e glicolítica, do potencial de membrana mitocondrial (MMP) e da produção de ROS a nível mitocondrial. Para além disto, apesar do ^{nat}In-DOTAGA-AO não induzir citotoxicidade nos modelos *in vitro* estudados, as células cancerígenas parecem mudar o seu comportamento, tornando-se mais semelhantes às células normais da próstata (PNT2)

Portanto, este estudo representa um importante trabalho preliminar para um projeto maior, apoiando o desenvolvimento de radiocomplexos emissores de AE direcionados às células alvo através do ligando $PSMA_{617}$ e funcionalizados com grupos de direcionamento para organelos, nomeadamente as mitocôndrias.

Palavras-chave: Cancro da próstata; Radiofármacos; Mitocôndria; Metabolismo

List of Acronyms and Abbreviations

AA	Antimycin A
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AO	Acridine orange
APS	Ammonium persulfate
ATP	Adenosine triphosphate
AUC	Area under curve
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
DNA	Deoxyribonucleic acid
DRP1	Dynamin-related protein-1
ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
FADH$_2$	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
FIS1	Fission protein 1
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
HRP	Horseradish peroxidase
KEAP1	Kelch-like ECH-associated protein 1
MFN1	Mitofusin 1
MFN2	Mitofusin 2
MMP	Mitochondrial membrane potential
mtDNA	Mitochondrial DNA
mtROS	Mitochondrial reactive oxygen species
NADH	Nicotinamide adenine nucleotide (reduced form)
NADPH	Nicotinamide adenine nucleotide phosphate (reduced form)
OCR	Oxygen consumption rate
OPA1	Optic atrophy-1
OXPHOS	Oxidative phosphorylation
PBS	Phosphate-buffered saline
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1α
PINK1	PTEN-induced kinase 1
PLL	Poly-L-lysine
PMSF	Phenylmethanesulfonyl fluoride
PVDF	Polyvinylidene difluoride
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SDS	Sodium dodecyl sulphate

SDS-Page	Sodium dodecyl sulphate ??
SEM	Standard error of mean
SRB	Sulforhodamine B
TBS-T	Tris-buffered saline tween
TCA	Trichloroacetic acid
TEMED	N, N,N´,N´-tetramethylethylenediamide
TMRM	Tetramethylrhodamine methyl ester
TPP⁺	Triphenylphosphonium cation
2-DG	2-Deoxy-glucose

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1. Introduction

1.1. Prostate cancer: incidence, mortality, diagnosis, staging and current treatment options

Prostate cancer (PCa) is a major public health problem worldwide (Carlsson et al. 2020). With 1.4 million estimated new cases and more than 375,000 deaths in 2020, PCa is the second most incident cancer in men and the fifth most mortal. More specifically, the estimated PCa diagnosed cases represent 14.1% of all new cancer cases in male individuals and 7,3% in both males and females. Regarding mortality, PCa deaths represent 6.8% and 3.8% of all cancer-related deaths, considering males and both sexes, respectively. The patterns of incidence and mortality vary by region, with the highest incidence rates registered in Northern and Western Europe, with an incidence ratio of 83.4 and 77.6 per 100,000 men, respectively, while the Caribbean region and Middle Africa present the highest mortality rates, with 27.9 and 24.8 per 100,000 men (GLOBOCAN, Sung et al. 2021). So far, little is known about the etiology of the disease, and the established risk factors are aging, family history, and certain genetic mutations (e.g., BRCA1 and BRCA2). Patterns of incidence and mortality across the world are likely modulated by lifestyle and environmental factors, since smoking, excess body weight, and nutritional imbalance might increase the risk of PCa incidence (Rebbeck et al. 2013). Of note, the development and access to the health system also contribute to differences in the international incidence and mortality patterns. In high-income countries, rates of incidence are higher, due to greater diagnosis and monitoring of the disease. However, despite the higher incidence rate, there is a lower mortality rate, reflecting earlier screening and more advanced treatment. In poor-income countries, limited access to the health system, as well as the unavailability of more sophisticated diagnostic and treatment tools, are reflected in low incidence rate and higher mortality rates (Tsodikov et al. 2017; Jemal et al. 2020).

Prostate malignant transformation is a multistep process that initiates as prostatic intraepithelial neoplasia and is followed by localized prostate cancer, advanced prostate adenocarcinoma with local invasion and, finally, metastatic PCa (Wang G et al. 2018). An early-stage PCa is considered a localized tumor without identifiable metastases (Sebesta

et al. 2017; Litwin et al. 2017; Janiczek et al. 2017), and an advanced-stage PCa includes patients diagnosed with recurrent tumors or with identifiable metastases distant from the primary tumor, most frequently in the lymph nodes or bones (Litwin et al. 2017; Janiczek et al. 2017).

A determinant factor in the mortality rate is diagnosis as an early diagnosis is crucial to improve survival outcome. There are several PCa screening modalities, including digital rectal examination (DRE), trans-rectal ultrasound (TRUS), assessment of serum prostate-specific antigen (PSA) blood levels (also known as the PSA test), and histopathological or cytological examination of specimens from the gland (Surasi et al. 2020). These techniques vary in terms of sensitivity and capacity to detect PCa at different stages of the disease. For instance, although the PSA test is widely used for PCa screening, successfully detecting malignant disease at all stages, it has poor specificity, with only 30% to 40% of positive results confirmed by biopsy analysis (Uno H. et al. 2021). The PSA test assesses PSA levels in the blood which, are standardized for a certain age range, but the variation of PSA levels can change due to factors other than cancer, namely due to urine infection. Sometimes, false positives continue to be monitored over the years, namely by performing DRE and TRUS at regular intervals, which is associated with increasing pain and suffering for the patients, who may suffer from infections and hematuria. There may also create psychological distress associated with fear of a diagnosis confirmation (Dudith et al. 2021). To increase accuracy, diagnosis is often made using a combination of screening modalities (Popa et al. 2017).

Proper staging of PCa is also essential to establishing the most appropriate patient-specific treatment (Bouchelouche et al. 2010). In 1974, Gleason and Mellinger defined a grading system, which came to be known as the Gleason grading system, based on histological patterns of prostate adenocarcinoma (Gleason and Mellinger 1974). This grading system which has been refined over the years, is the most used grading system. It allows the sorting of patients into a five-grade stratification scheme according to the results of the screening trials. More specifically, clinicians consider PSA blood levels and the Tumor, Node, Metastasis (TNM) staging of the cancer, the latter assessing the size of the tumor and how far it has grown. Grade 1 (Gleason score ≤ 6) corresponds to the less aggressive tumor, with cells looking similar to normal prostate cells, and is

expectable to grow slowly. Grade 5 (Gleason score 9 or 10) is the most aggressive stage, with cells looking very abnormal, and is a cancer likely to grow quickly. Between these extreme grades, there are three grades with 6 < Gleason score < 9. Available treatments for PCa include surgery, radiation therapy, standard chemotherapy (also known as traditional chemotherapy), immunotherapy, and androgen deprivation therapy. For early-stage PCa, active surveillance is often recommended, as these tumors are unlikely to grow quickly, but radiation therapy or radical prostatectomy may be considered. In advanced-stage PCa, the initial treatment options are prostatectomy and radiation therapy combined with different approaches of androgen deprivation therapy or immunotherapy and chemotherapy, if the initial options failure. (Bekelman et al. 2018; Chen et al. 2016). As can be appreciated, radiation therapy is an option to manage all PCa stages, either as monotherapy or combined with the other available treatments. Unfortunately, despite the available treatment options, advanced stages of the disease are still associated with high mortality. Thus, innovative treatments are clearly needed (Czerwinska el at 2020).

1.2. Radiation therapy: general mechanism of action and available modalities

Radiation therapy (also called radiotherapy) has been part of the multidisciplinary management of PCa for a long time, sparing patients the adverse effects of surgical intervention. This therapy uses high-energy particles or waves, such as x-rays, gamma rays, electron beams, or protons, to damage and eliminate malignant cells. The emitted particles have enough energy to ionize (i.e., induce gain of loss of electrons) atoms and molecules and, as such, are described as ionizing radiation (IR) Direct interaction of IR with cell structures and biomolecules, most notably DNA, induces chemical and biological changes that ultimately lead to cellular dysfunction and often death. In addition, IR exposure promotes the radiolysis of water, generating the hydroxyl radical ($^{\bullet}OH$) and hydrogen radical ($H^{\bullet}$). These radical species promote a cascade of reactions that produces superoxide ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), and other secondary reactive oxygen species (ROS) (Filomeni et al. 2015; Plante 2021). In addition, IR stimulates the activity of inducible nitric oxide synthases (NOS), resulting in nitric oxide

($^\bullet$NO) production. Even though $^\bullet$NO is inactive towards most cellular constituents, it reacts with O2$^{\bullet-}$, generating the peroxynitrite anion (ONOO$^-$). This highly reactive radical interacts with several species producing other reactive nitrogen species (RNS), namely peroxynitrous acid (ONOOH), nitrogen dioxide (NO2$^\bullet$), and dinitrogen trioxide (N$_2$O$_3$), (Verigos et al. 2020; Zhao et al. 2021). Besides exogenous ROS/RNS, there are endogenous ROS/RNS sources, including the mitochondrial electron transport chain (ETC) (Zhao et al. 2019; Cheng et al. 2021), NADPH oxidase (NOX) and NOS. Although, at certain levels, ROS and RNS may play a role in cell signaling pathways crucial for maintaining cell homeostasis, exacerbate levels of reactive species imbalance the cellular redox status, resulting in several deleterious effects within cells (Plante 2021; Zhao et al. 2021).Specifically, ROS and RNS induce damage to proteins, fatty acids, and nucleotides, compromising the normal function of cellular organelles such as nucleus, mitochondria, cell membrane and lysosomes (Yang et al. 2021; Lei et al. 2020).

Cumulative ROS/RNS within cells damage nucleic acids, enhancing genomic instability because of deleterious alterations to bases and sugars, DNA strand cross-linking, single- and double-strand breaks (SSBs/DSBs), and DNA clustering (Ragunathan et al. 2020; Zhao et al 2021; Srinivas et al. 2019). DNA strand breaks occur randomly in the DNA backbone by O2$\bullet$- direct interaction and through activation of nucleic acid-binding enzymes. Those interactions include depurination and depyrimidinations of DNA bases, such as the oxidation of deoxyguanosine (dG) to 8-hydroxyguanine (8-oxodG), one of the major products of DNA oxidation, and a well-known DNA damage hallmark (Srinivas et al. 2019). Upon DNA insult, cells immediately trigger intrinsic mechanisms of defense, the DNA damage response (DDR), activating an extensive protein network. Briefly, the damage site is recognized by the protein kinase ataxia telangiectasia mutated (ATM) and other sensor proteins, and through activation of the ATM/p53 pathway, the cell undergoes apoptosis or cell cycle arrest (Ku et al. 2019; Srinivas et al. 2019).

Lipids are another class of biomolecules targeted by IR. Radiation induces extensive damage to the lipid bilayer of biological membranes, through lipid peroxidation, in particular the peroxidation of polyunsaturated fatty acids (PUFAs). Subsequently, there is an increase in membrane permeability and disruption of ion gradients, compromising

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all processes dependent on cell membrane integrity (Su et al. 2019; Bai et al. 2019). The oxidative degradation of lipids generates peroxyl radicals, such as lipid hydroperoxides (LOOH) and PUFAs fragments (malondialdehyde (MDA), acrolein, and 4-hydroxy-2-nonenal (HNE)). In turn, increased levels of peroxyl radicals disrupt the cellular redox status, compromising several intracellular processes (Tsikas 2017; Kajarabille et al. 2019).

As briefly mentioned before IR-generated ROS/RNS also target proteins, modulating protein expression, inducing post-translation modifications (PTMs), and altering protein activity (Niforou K et al. 2014). Protein expression is mainly modulated by IR-induced epigenetic modifications (DNA methylation, histone methylation and acetylation) that change the gene expression pattern. (McDonagh 2017). PTMs induced by IR include oxidation, carbonylation, phosphorylation, acetylation, and glycosylation. Those modifications promote conformational changes, affecting protein structure and function, which subsequently alter protein interaction and protein trafficking resulting in deep impact on numerous signaling pathways and cellular processes (Niforou et al. 2014; McDonagh 2017).

As reported, cells are very susceptible to ROS and RNS and the damage sustained can be lethal to the cell. Therefore, cells have a complex defense system against excessive ROS/RNS levels, which is quickly activated upon IR exposure. This system includes a network of ROS/RNS-scavenging molecules and detoxifying enzymes that neutralize free radicals (Einor et al. 2016; Slimen et al. 2014). This detoxification is mainly carried out by superoxide dismutases (SODs), with an important contribution from several catalases activity such as peroxiredoxins (Prx) and glutathione peroxidases (GPxs). In addition, other scavenging molecules including glutathione (GSH), ascorbate (vitamin C), melatonin, lipoic acid, ubiquinone (coenzyme Q10), and vitamin E, play a crucial role in maintaining cellular redox status (Slimen et al. 2014). In response to IR-induced increased levels of oxidative stress, cells quickly modulate the expression of antioxidant enzymes through the activation of transcriptional factors such as nuclear factor (erythroid-derived 2)-like 2 and nuclear factor kappa-light- chain-enhancer of activated B cells (NF- B) (Slimen et al. 2014).

Taking it in consideration, it is mandatory to ensure a balanced oxidative stress status, and therapies based on radiation need to ensure an accurate energy dosimetry to minimize the extremely deleterious effects of IR. The two classic modalities of radiation therapy available are external beam radiation therapy (EBRT) and brachytherapy (BT) (internal radiation) (Podder et al. 2018). In EBRT, the prostate gland is irradiated from outside the body with high-energy beams, such as X-ray, photons or protons which are generated by a linear accelerator. The major challenge associated with this modality is to spare the neighbouring healthy tissue from the radiation, ultimately preventing severe adverse effects, including urinary, bowel and sexual dysfunction, as well as secondary cancers (Brawley et al. 2018; Lumen et al. 2013). BT allows the delivery of localized high doses of radiation to small volumes, ensuring high cytotoxicity to damaged tissues, while sparing healthy tissue (Hannoun-Lèvi et al. 2017; Zaorsky et al. 2017). There are different BT approaches available, including the low-dose-rate (LDR) BT, in which a radioactive seed implanted into the prostate gland delivers a steady dose of radiation over the organ, during a period that might vary from hours to days to months or even permanently. This modality is usually used as monotherapy to manage low-risk disease patients and the most common radioisotopes used are iodine-125 (125I), palladium-103 (103Pd) and cesium-131 (131Cs) (Dutta S et al. 2018; Butler et al. 2016). Another BT approach is the high-dose-rate (HDR) modality, which involves delivering radiation in short bursts through thin tubes that are temporarily inserted into the prostate gland (Hannoun-Lèvi et al. 2017; Crook J et al. 2020). As monotherapy, BT and EBRT are mostly used to manage early-stage PCa with localized tumor. For advanced states of the disease, EBRT and BT are usually used in combination or combined with androgen deprivation therapy (Gay et al. 2018).

In addition to these main radiation therapy modalities, over time new radiation-based therapies have been studied (Gay et al. 2018). With the advancement of technology, these novel modalities take advantage of high-quality imaging tools, robotic assistance, and better delivery systems (Podder et al. 2018) for both cancer screening and treatment. Over the last years, one of the novel emerging approaches is the targeted radionuclide therapy (TRT) (Filippi et al. 2020).

1.3. Targeted radionuclide therapy

TRT is a theragnostic modality based on the use of a radioactive nucleus attached to a targeting vector to deliver cytotoxic dosed of ionizing radiation to cancer cells (Gill et al. 2017) for both cancer imaging and treatment purposes (Filippi et al. 2020; Hindie et al. 2018). A key aspect of this modality is the use of specific ligands that interact with target biomolecules present in a diseased tissue (Filippi et al. 2020). This feature ensures the deposition of large amounts of energy in a narrow location, allowing it to reach the malignant cells and avoid damaging the surrounding healthy tissue (Sapienza et al. 2019). The cytotoxic effect is guaranteed by energetic emissions whose magnitude depends on the radionuclide, which can emit α-particles, ß-particles, or Auger electrons (AE) (Figure 1) (Czerwinska et al. 2020; Gill et al. 2017). The emitted energy originates from decaying radioactive atoms, each of which has its own features, namely chemistry, half-life, decay properties, tissue range, and availability (Czerwinska et al. 2020).
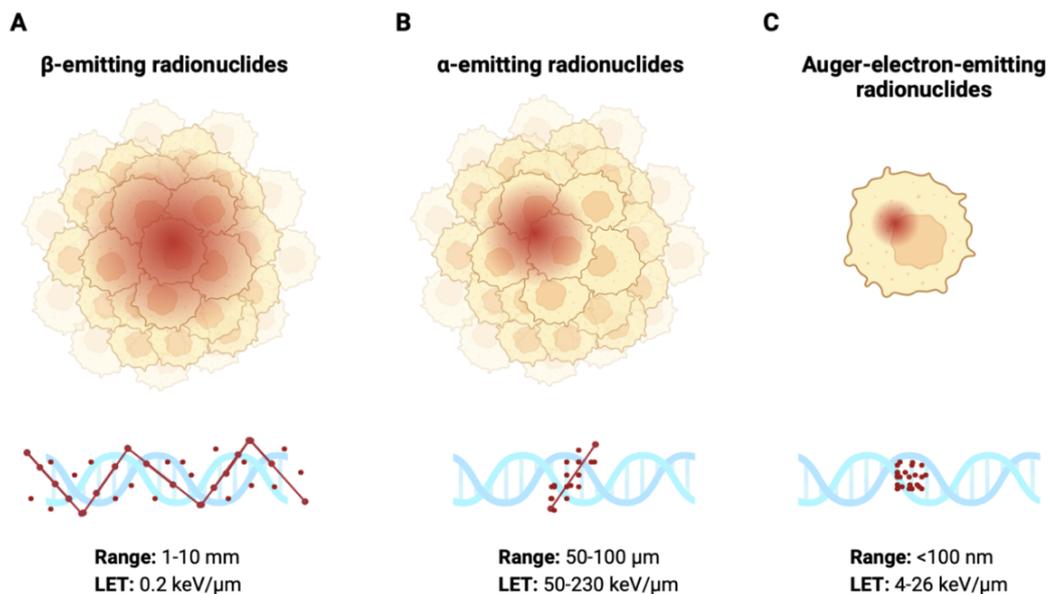
Figure 1. Representation of different features of radionuclides emitting β-particles, α-particles, and AE, including tissue-penetration, LET and density of ionizing events. (A) β-emitting radionuclides. (B) α-emitting radionuclides. (C) AE-emitting radionuclides. Figure 1 was created with BioRender. LET, linear energy transfer.

1.3.1. ß-emitting radionuclides

ß-emitting radionuclides generate ß-particles during the decayment of the nucleus, which variable energy. In general, ß-particles have a relatively long range (1–10 mm) and low linear energy transfer (LET) (0.2keV/µm) (Czerwinska et al. 2020). Consequently, this energy penetrates large masses of tissue and can hit not only large tumors but also their neighbouring healthy tissue (Czerwinska et al. 2020; Sadremomtaz et al. 2019). The cytotoxic effect of the ß-particles is associated with water radiolysis, resulting in SSBs or DSBs (Tafreshi et al. 2019). The ß-emitting radionuclides, iodine-131 (^{131}I), copper-67 (^{67}Cu), lutetium-177 (^{177}Lu), and yttrium-90 (^{90}Y) are the most investigated for the diagnostic and treatment of cancer, including PCa (Sadremomtaz et al. 2019). Westcott et al. describe the development and US Food and Drug Administration (FDA) approval of two commercially available 90Y attached to two different types of microspheres that ensure the selective radionuclide targeting (Westcott et al. 2016). According to Dash et al. 2014, and Das et al. 2015, ^{177}Lu is a ß-emitting radionuclide with enormous potential in nuclear medicine theragnostic, as it has a long half-life ensuring a diagnostic imaging advantage. This property has already been studied *in vivo*, with successful clinical tracking long after its administration (Dash et al. 2014: Das et al. 2015).

1.3.2. α-emitting radionuclides

Radiotherapy based on α-emitting radionuclides is an interesting alternative to radiotherapy based on ß-particles emitters (Marcu et al. 2018). α-Particles are ^{4}He nuclei, which have a larger size than other subatomic particles emitted from decaying radionuclides. Due to its size, its whole generated energy is deposited in a small area as these particles have a low penetrance capacity (50–100 µm) (Parker et al. 2018). The radioactive nuclei decaying generates daughter nuclei that are often themselves α-emitters leading to a decay cascade which contributes significantly to a greater energy deposition (Czerwinska et al. 2020; Parker et al. 2018). This high LET radiation (50–230keV/µm) has therefore a huge potential to handle small tumors, micro-metastases, and for the treatment of hematological malignancies (Marcu et al. 2018; Jurcic et al.

2019). These α-particle agents interact directly with DNA macromolecules inducing lethal DNA DSB ensuring a high relative biological effectiveness. So far, several α-emitting radionuclides approaches have been studied, with different radioisotopes, including astatine-211 (^{211}At), radium-223 (^{223}Ra), thorium-227 (^{227}Th), and actinium-225 (^{225}Ac) (Czerwinska et al. 2020). In 2013, ^{223}Ra dichloride injections were approved by FDA for the treatment of patients with castration-resistant prostate cancer, with bone and lymph nodes metastasis, excluding visceral metastases (Das et al. 2015). Previously, the ALSYMPCA trial (phase III) showed an overall survival benefit with the addition of ^{223}Ra to standard care, and after FDA approval, many other studies focusing on ^{223}Ra have been performed to shift the paradigm of PCa treatment (Hoskin et al. 2014; Parker et al. 2013; Heidegger et al. 2018).

1.3.3. AE-emitting radionuclides

AE are emitted by decaying radionuclides by electron capture and/ or internal conversion (Ramogida et al. 2013). These processes create a vacancy in the inner electron shell which is subsequently filled by electrons from higher energy shells triggering a cascade of radiative and non-radiative transitions (Czerwinska et al. 2020; Ramogida et al. 2013; Rosenkranz et al. 2020; Paillas et al. 2016). While radiative transitions may result in emission of X-ray, **γ**-rays, and ß-particles, non-radiative transitions mainly correspond to the emission of AE, and Coster-Kronig and super Coster-Kronig electrons, collectively referred to as AEs (Knapp et al. 2016; Paillas et al. 2016). These are extremely low-energy electrons, with very limited tissue penetration (nanometers scale), generating high LET radiation (4–26 keV/μm) (Czerwinska et al. 2020; Ramogida et al. 2013; Falzone et al. 2015; Kiess et al. 2015). The extremely localized deposition of energy allows targeting single cells or even subcellular organelles, ensuring high cytotoxicity, while lowering induced damage to surrounding tissue (Rosenkranz et al. 2020; Falzone et al. 2015; Sobolev et al. 2018; Costa et al. 2021). Importantly, the toxicity of AE is induced mainly through two mechanisms: 1) direct DNA damage by high LET radiation, generating SSB and DSB; or 2) indirect DNA damage by ROS generated during water radiolysis (Tafreshi et al. 2019; Ku et al. 2019; Rosenkranz

et al. 2020; Gudkov et al. 2016). Moreover, AE toxicity depends on the proximity of the decaying site to the target due to its low energy and high LET radiation. Several approaches have been followed, employing different delivery molecules, to ensure greater proximity between the radionuclide and the target and thus increase the biological effectiveness (Czerwinska et al. 2020; Kiess et al. 2015; Ku et al. 2019).

1.4. Targeting strategies

The relative biological effectiveness of radionuclide therapy depends on the specific targeting, which is achieved by intrinsic targeting properties of some radionuclides or by conjugating the radionuclide to a delivery molecule or vector (Ramogida et al. 2013; Dekempeneer et al. 2016). The delivery molecules currently used include antibodies, antibody fragments, peptides, small molecules, liposomes, dextrans, and microspheres (Dash et al. 2015; Ramogida et al. 2013). To choose the most suitable delivery system, it is necessary to consider the type of cancer and its features, namely its morphology and physiology (Dekempeneer et al. 2016). It is also necessary to bear in mind the properties of the delivery molecule that determine its pharmacokinetics (bioavailability, biospecificity, metabolism, excretion, and interaction on-target and off-target) to ensure maximum biological effectiveness of the modality. Emerging TRT approaches have been focused on a ligand-receptor mechanism., in which delivery molecules correspond to a ligand that targets and binds to a protein, the receptor. The success of this mechanism depends 1) on the affinity and specificity of the interaction between the ligand and the receptor, and 2) on the properties of the receptor, which is usually overexpressed in the malignant cells, allowing to selectively target the cancer cells, reducing the off-target damage (Ramogida et al. 2013; Dekempeneer et al. 2016). Specifically, for PCa, prostate-specific membrane antigen (PSMA) has shown enormous potential as target for imaging diagnosis and treatment purpose (Vaz et al. 2020).

1.4.1. Prostate-specific membrane antigen

PSMA, also known as glutamate carboxypeptidase II or folate hydrolase, is a 100–120 kDa protein with glutamate-carboxypeptidase and folate hydrolase I activity, playing a role in cell migration, cell survival, and proliferation (Emmett et al. 2017). This type II transmembrane glycoprotein, which is anchored at the prostate epithelial cell surface, is constituted by an intracellular domain with 18 amino acids, a transmembrane domain with 24 amino acids, and an extensive extracellular domain with 707 amino acids (O`driscoll et al. 2016). Upon binding to a ligand on the membrane surface, PSMA undergoes an internalization process via the endosome. This feature makes PSMA an attractive molecular target for cancer diagnostic imaging and treatment, as it has been shown to allow the internalization of PSMA-labelled radionuclides, ensuring a more efficient deposition of the pharmacophore within the cells (Emmett et al. 2017). PSMA is markedly overexpressed in prostatic cancer cells compared to other tissues, including normal prostatic tissue (Uijen et al. 2021). Moreover, high levels of PSMA expression are correlated with higher PCa malignancy and aggressiveness (Uijen et al. 2021; Chang 2004; Rahbar et al. 2018).

Despite its name, PSMA is also expressed in other normal tissues, such as brain, kidneys, small intestine, and salivary gland, playing different roles depending on the tissue. In the brain, its glutamate-carboxypeptidase regulates the release of key neurotransmitters by cleaving the neurotransmitter N-acetyl-L-aspartyl-L-glutamate (NAAG). Intestinal PSMA, on the other hand, displays folate hydrolase activity, which is characterized by the cleavage of polyglutamated peptides, playing a role in cellular uptake of dietary folate (Conway et al. 2006; Conway et al. 2016). Interestingly, PSMA seems to be universally upregulated in the neovasculature of solid tumors, such as those of the bladder, kidney, lung, and pancreas, while it is not expressed in the normal vasculature of tumors (Tumedei et al. 2021 Wang et al. 2015). This observation suggests that PSMA is a key player in tumor angiogenesis, which is an essential step in cancer invasion and in the development of metastasis (Conway et al. 2016; Wang et al. 2015; Russo et al. 2012). However, the underlying mechanism is not clear. Conway and colleagues suggested that PSMA acts downstream of metalloproteinase-2 (MMP-2),

generating a proteolytic cascade that promotes angiogenesis. Laminin, the predominant component of the extracellular matrix, is digested by MMP-2/PSMA into small laminin peptides that enhance endothelial cell adhesion and migration through the activation of integrins and adhesion kinases (Conway et al. 2006; Conway et al. 2016).

1.4.2. Subcellular targeting of targeted radionuclide therapy

The main target of different radiotherapy modalities, including TRT, is nuclear DNA. In fact, for many years, the central dogma of radiation biology sustains that the induction of genotoxicity was the responsible for biological effects of radiation. Currently, radiation-based therapies target other organelles, including mitochondria, lysosomes, and cell membranes (Chen et al. 2020), as it is believed that the distribution of IR in specific organelles could increase cytotoxicity and should also allow the use of lower radiation doses to reduce IR-associated adverse effects (Bavelaar et al. 2018). A study conducted by Maucksch et al., in 2016, an AE-emitting radionuclide, ^{99m}Tc, was tested with three different ligands that ensured radiation deposition in the cytosol, plasma membrane and nucleus. The different subcellular distributions demonstrated alterations in the induced cytotoxicity which was not exclusively determined by DNA DSBs. The authors speculated that the observed differences in cytotoxicity were due to differential mitochondrial accumulation (Maucksch et al. 2016). This evidence highlights the importance of targeting extra-nuclear targets. In particular, mitochondrial DNA (mtDNA) has enormous potential as a target for novel radionuclide therapy modalities, as it is known to be extremely radiosensitive, but this potential has not yet been deeply explored. The interest in targeting mitochondria has increased recently due to their recognized role in tumorigenesis (Poole et al. 2021; Srinivasan et al 2017; Guo L 2021). Therefore, mitochondria are an excellent target for new therapies, especially targeted AE-emitting radionuclide therapy since it has high LET radiation with little tissue penetration, ensuring the deposition of IR in such small organelle (Badrinath et al. 2018; Czerwinska et al. 2020).

1.5. Mitochondria and cancer

In 2000, Hanahan and Weinberg proposed six hallmarks of cancer: sustaining proliferative signaling; evading growth suppressors; activating invasion and metastasis; enabling replicative immortality; inducing angiogenesis; resisting cell death. These are common features shared by most cancer cells that are acquired during the process of malignant transformation (Hanahan et al. 2000). Later, in 2011, the same authors suggested adding two emerging hallmarks: deregulating cellular energetics; avoiding immune destruction. The first one relies on reprogramming cellular metabolism to support malignant transformation and neoplastic proliferation. The second includes the capacity of cancer cells to avoid being destructed by the immune system. Moreover, the authors suggested genome instability and mutation, and tumor-promoting inflammation as two enabling characteristics, i.e., characteristics that do not cause cancer themselves, but sustain the malignant transition (Hanahan et al. 2011). The first emerging hallmark, deregulating cellular energetics, includes alterations in cell bioenergetics through modifications in nutrient uptake and metabolic pathways leading to cell survival and proliferation (Hanahan et al. 2011).

Mammalian cells use two main processes to obtain energy, lactic acid glycolysis, and aerobic cellular respiration. Glycolysis occurs in the cytosol in which glucose is metabolized to pyruvate, generating two net molecules of ATP and two NADH molecules (Ashton et al. 2018). Subsequently, pyruvate may be reduced by lactate dehydrogenase, producing lactate (lactic acid fermentation), or may be converted to acetyl-CoA, which is fully oxidized in the tricarboxylic acid (TCA) cycle generating metabolic energy (ATP, indirectly through GTP) and reducing power (NADH, and complex II-bound $FADH_2$). NADH and $FADH_2$ act as electron transporters, feeding the ETC with electrons, in a process called oxidative phosphorylation (OXPHOS), which occurs mainly in mitochondria in an oxygen-dependent manner. The electrons flow in the inner mitochondrial membrane (IMM) along four multiprotein ETC complexes (complex I (NADH: UQ oxireductase), II (succinate: UQ oxireductase), III (cytochrome bc_1 complex), and IV (cytochrome c)), creates an electron flux that allows the pumping of protons from the mitochondrial matrix to the intermembrane space, producing a high proton

gradient. These protons return to the mitochondrial matrix through the ATP synthase, leading to ATP formation by ADP phosphorylation. When arriving at complex IV, the electrons that flow along with the ETC are reduced to oxygen, the final electron acceptor, generating water (Payen et al. 2020). Despite the higher energy efficiency of respiration, compared to glycolysis, in 1923, Otto Warburg found evidence that cancer cells rely heavily on the latter to generate ATP. Initially, it was thought that the mitochondria of cancer cells were dysfunctional and therefore the cells had to rely on fermentation. Later, it was discovered that cancer cells produce ATP via glycolysis even though they have fully functional mitochondria and with oxygen available in which the acid lactic fermentation favors the cancer cells proliferation in the hypoxia microenvironment (Payen et al., 2020; San-Millán & Brooks, 2017; Xu et al., 2015)). This shift in cellular energetics is known as the Warburg effect or aerobic glycolysis and it is now known that it does not occur in all cancer cells, being dependent on cell type and type of cancer (Ashton et al. 2018; Xu et al. 2015; Wallace et al 2012).

Despite their role in the cellular energy metabolism, making this organelle known as the "powerhouse of the cell", mitochondria are involved in many other cellular processes, including cell death, oxidative stress regulation and signaling (Hou et al. 2018). Moreover, cancer cells alter several signaling pathways to promote their malignant transformation and to induce cell proliferation and survival. The oncogenic signaling pathways have a direct influence on mitochondrial functioning, which is altered to favor cancer cells (Genovese et al. 2020; Vyas et al. 2016).

1.5.1. Mitochondrial dynamics

Mitochondria is a very well-regulated organelle, which function can be modulated according to the cell host demand through biogenesis or turnover processes. This regulation depends on many factors, including cellular metabolic state, oxidative stress, temperature, and microenvironment (Jornayvaz et al. 2010). Mitochondrial biogenesis can be defined as the growth and division of mitochondria which could vary in number, size, and mass, when there is an increase in the need for their functions to maintain cell homeostasis (Bhatti et al. 2017. Mitophagy is a form of organelle-specific

autophagy selective for mitochondria turnover, ensuring a healthy mitochondrial population (Bernardini et al. 2017). Importantly, dysregulated mitochondrial function and dynamics are strictly associated with tumorigenesis and oncogenic signaling pathways (Popov et al. 2020; Maycotte et al. 2017).

Mitochondrial biogenesis is regulated by both nuclear and mitochondrial genomes, which encode mitochondrial proteins. The master regulator of the process is the peroxisome proliferator-activated receptor-y (PPAR-y) coactivator α (PGC-1α), a nuclear transcriptional coactivator that interacts with the nuclear receptor PPAR-y, regulating multiple transcription factors (Bhatti et al. 2017; Popov LD et al. 2020). LeBleu et al. performed a set of experiments where they found evidence of cancer cells dependent on PGC-1α-mediated mitochondrial biogenesis (Lebleu et al. 2014). PGC-1α has been shown to play a key role in metastatic potential, ensuring cancer cell growth in an anchorage-independent manner during their transit to target organs of metastasis (Popov et al. 2020; Lebleu et al. 2014). Moreover, LeBleu et al. observed that the suppression of PGC-1 resulted in impaired mitochondrial biogenesis and noticed a decrease in the frequency of metastases (Lebleu et al. 2014). In addition, according to Tan et al., PGC-1α-mediated tumor suppression is due to the induction of intrinsic apoptotic pathway in colorectal and ovarian epithelial carcinoma cells and through induction of mitophagy in breast cancer (Tan et al. 2016).

The major mitophagy induction pathways are the PTEN-induced putative kinase 1 (PINK1)/Parkin pathway, and the adenovirus E1B 19 kDa-interacting protein 3 (BNIP3L)/NIP3-like protein X (NIX) pathway. These pathways are activated upon mitochondrial membrane depolarization resulting from the lack of reducing equivalents, hypoxia, and impaired electron transport (Bernardini et al. 2017; Vara-Perez et al. 2019). In normal conditions, PINK1 is translocated to the IMM in a voltage-dependent manner, where this kinase is cleaved by the mitochondrial protease PARL. Upon depolarization, PINK1 is translocated and accumulates in the outer mitochondrial membrane (OMM), where it phosphorylates Parkin. Consequently, several mitochondrial proteins are recruited and the mitophagy machinery is activated. Under hypoxia conditions, the hypoxia-inducible factor 1 (HIF-1) induces the expression of BNIP3L and NIX, which

contain an LC3-interacting domain that promotes activation of the mitophagy machinery (Ferro et al. 2020).

The relationship between mitophagy and cancer is not very clear. Mitophagy can be both pro- and anti-tumorigenic and its role and influence depend on cell type and cancer stage (Vara-Perez et al. 2019). In the early stages, mitophagy acts as a tumor suppressor, because the misregulation of mitophagy results in the release of mitochondrial signals and there is an increase in oxidative stress that induces a favorable microenvironment for tumor growth. In advanced-stage cancer, mitophagy is pro-tumorigenic, as it promotes tumor adaptation to the microenvironment, ensuring cancer survival (Vara-Perez et al. 2019; Ferro et al. 2020).

The metabolic state of a cell can be associated with the morphology and size of the mitochondrial network. Depending on the cell's physiological demands, mitochondria can alter their shape and size through fusion and fission processes (Maycotte et al., 2017). In mammalian cells, optic atrophy-1 (OPA1), mitofusin 1 (MFN1), and mitofusin 2 (MFN2) are three key proteins in fusion processes, and, on the other hand, the fission process is mediated by dynamin-related protein-1 (DRP1) and fission protein 1 (FIS1) (Maycotte P et al. 2017; Barbosa et al. 2012; Srinivasan et al. 2017; Dai et al. 2019). Mitochondrial exist as either fused, tubular networks or as fragmented granules and the increase in mitochondria fusion gives a more elongated shape to mitochondria forming an extensive tubular network that is associated with an increase in OXPHOS capacity (Srinivasan et al. 2017). Multiple studies have revealed that dysregulation in mitochondrial fusion and fission processes is associated with several diseases including cancer (Srinivasan et al. 2017; Dai et al. 2019). The malignant imbalance is related to decreased mitochondrial fusion and increased fission resulting in a fragmented mitochondrial network. In many cancers, there is an enhanced DRP1 activity and downregulation of MFN2. Moreover, studies associate the high levels of DRP1 with the migratory phenotype, suggesting, once again, that mitochondria and their dynamics play a crucial role in metastasis (Dai et al. 2019).

1.5.2. Cell death

Apoptosis, a form of programmed cell death, is an essential mechanism to control cell proliferation and maintain tissue homeostasis, as it eliminates damaged and harmful cells, as well as superfluous cells (Goldar et al. 2015). This well-regulated mechanism has two major pathways: the extrinsic pathway (death receptor-mediated pathway) and the intrinsic pathway (mitochondrial-mediated pathway (Goldar et al. 2015; Ke et al. 2016). The first starts with the binding of an external signal such as tumor necrosis factor (TNF), Fas ligand (Fas-L), and TNF-related apoptosis-inducing ligand (TRAIL) to the extracellular domain of so-called death receptors. This ligand-receptor interaction activates a signaling cascade inside the cell, activating pro-caspase-8. During the process, the death-inducing signaling complex (DISC) is formed, which leads to auto-catalytic activation of pro-caspase-8. Subsequently, caspase-8 activates effector caspases that induce cell death by damaging the nucleus and other intracellular structures (Goldar et al. 2015; Ke et al. 2016). The second pathway is mediated by several internal stimuli such as DNA damage, oxidative stress, and increased cytosolic Ca^{2+} levels, which are recognized by intracellular proteins, leading to outer mitochondrial membrane permeabilization. Consequently, several apoptogenic factors are released, including cytochrome *c*, apoptosis-inducing factor (AIF), second mitochondria-derived activator of caspase (Smac)/ direct inhibitor of apoptosis protein (IAP) binding protein with low PI (DIABLO), and endonuclease G. Cytochrome *c* interacts with and activates the apoptosis protease activating factor (Apaf-1), forming a multi-protein complex known as apoptosome, which recruits and actives caspase-9, which is the effector caspase responsible for inducing cell death. The other apoptogenic factor also has a crucial role as Smac/DIABLO interacts with IAPs, facilitating caspase activation. AIF and endonuclease G is involved in other processes which result in cell death in a caspase-independent pathway. OMM permeabilization is mainly mediated by the B-cell lymphoma 2 protein (BCL-2) family, which can be divided into three different groups in mammalian cells. The BCL-2 family includes proteins with a pro-apoptotic role (e.g., Bac, Bak, and Bok), anti-apoptotic action (e.g., Bcl-2, Bcl-XL, Bcl-w, Mcl-L, and A1), and include proteins Bcl-2 homology (BH) domain-only such as Bim, Bad Bid, Puma, and Noxa

which interact both with anti-apoptotic proteins inhibiting their action and with pro-apoptotic proteins stimulating their activity (Goldar et al. 2015; Ke et al. 2016; Maximchik et al. 2016).

Interestingly, cancer cells can alter the apoptotic pathways by transcriptional, translational, and post-translational mechanisms, leading to an advantage gain against apoptosis and thus ensuring their survival. This capacity to avoid death is one of the most important features contributing to cancer resistance against current therapies (Maximchik et al. 2016).

1.5.3. Mitochondrial DNA and ionizing radiation effects

Mitochondria have their own genome, and the mtDNA of mammalian cells, in particular, is formed by a closed circle of double-stranded DNA, which contains 16,659 bases that encode 37 genes for rRNA, tRNA, and 13 proteins, in humans (Azzam et al. 2012; Yan et al. 2019). These 13 polypeptides are core elements of the OXPHOS complexes, being essential for OXOPHOS activity, as well as for all mitochondrial dynamics (Yan et al. 2019; Sharma et al. 2019). mtDNA is located in the mitochondrial matrix, being close to ETC, which is the main ROS-generator endogenous system (Fuhrmann et al. 2017). Importantly, since mtDNA lacks histones to regulate its structure, and effective repair systems, it is particularly susceptible to damage induced by IR and imbalances in the cellular redox status (Nissanka el al. 2018). Consequently, mtDNA is prone to numerous mutations, responsible for mitochondrial dysfunction, contributing to the aging process and is highly associated with several human diseases such as Alzheimer's disease, Parkinson's disease, and cancer (Yan et al. 2019; Nissanka et al 2018; Sharma et al. 2019).

Altogether, mitochondria is a central cell organelle playing an essential role in energy metabolism, control of stress response, cell signaling, and is a hub for biosynthetic processes. Importantly, it is an extremely radiosensitive organelle, making mitochondria a potential target for novel radiotherapy-based therapies, reenforcing the shift in the paradigm of radiation biology to take in consideration the potential of other radiosensitive organelles, besides the nucleus.

1.6. Objectives

AugerTher project aim to synthesize dual-targeted (PCa and organelle) stable metal complexes containing DOTA chelators carrying a PSMA-binding motif (PSMA$_{617}$) to ensure a specific internalization in PCa cells. These complexes also contain a DNA intercalator or a mitochondriotropic group (tri-phenyl-phosphonium (TPP)), to further direct the pharmacophore to the nucleus or mitochondria, respectively, enhancing their anticancer effects. This innovative strategy can lead to a shift in the radiation biology paradigm, as TRT based on AE-emitting radionuclides targeting mitochondria is almost unexplored, despite their well-recognized radiosensitivity and association to tumorigeneses (McCann et al. 2021).

Particularly, in the present study we performed preliminary work for AugerTher project. Specifically, we aim to assess the biological effect of PSMA$_{617}$ and the cold surrogate ^{nat}In-DOTAGA-AO, the first synthesized compounds in the AugerTher project, in four *in vitro* cell models. Specifically, we evaluated the impact of both compounds on cellular metabolism by assessing 1) mitochondrial respiration, 2) mitochondrial membrane potential, and 3) mitochondrial superoxide levels, and 4) glycolytic function Mitochondria-related factors were evaluated in normal prostate cells (PNT2) compared with malignant PCa cells (LNCaP). PC3-PIP cells, which express the human PSMA protein, were also used to assess the role/influence of PSMA in PCa cancer cells metabolism and compared to PC3-FLU cells (respective control).

2. Material and Methods

2.1. Novel compounds

PSMA$_{617}$ (Figure 2A) and the cold surrogate ^{nat}In-DOTAGA-AO (Figure 2B) were synthesized by a multidisciplinary team led by Doctor António Paulo, from the Center for Nuclear Sciences and Technologies (C^{2}TN), from Instituto Superior Técnico of the University of Lisboa.
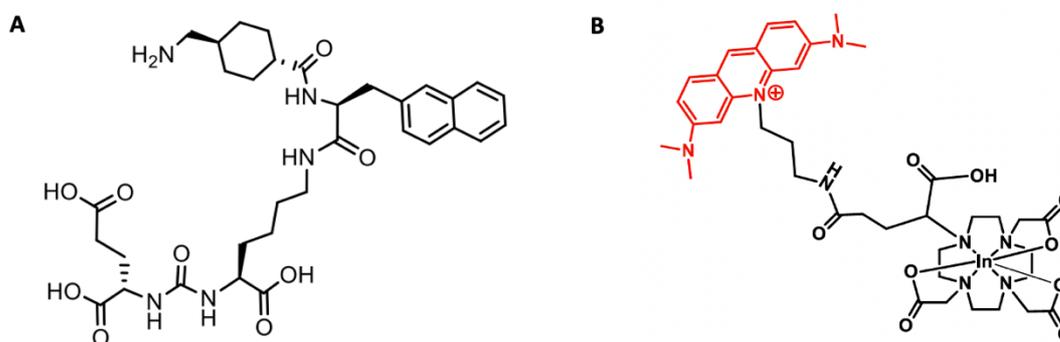
Figure 2. Chemical structure of the compounds under investigation in the present study. (A) PSMA617 with 655.74 g/mol molecular weight and (B) natIN-DOTAGA-AO with 893.71 g/mol molecular weight.

2.2. Cell culture

In the present study, four different cell lines were used as *in vitro* models, PNT2, LNCaP, PC3-FLU, and PC3-PIP. The cell line PNT2, derived from normal prostate epithelium immortalized with SV40, was the control of the malignant LNCaP cells derived from a lymph node metastatic prostate carcinoma. PC3 cells are a prostate cancer cell line derived from bone metastasis that do not express PSMA. In particular, PC3-PIP cells were generated by transduction using VSV-G pseudotyped lentiviral vector expressing human PSMA; and isogenic PSMA negative PC3-FLU cells. All cell lines were cultured in RPMI-1640 medium containing 2 g/L sodium bicarbonate and 10% fetal bovine serum (FBS). For the culture of PC3-PIP and PC3-FLU cells, and the medium was further supplemented with 20 μg/mL puromycin dihydrochloride (A11138-03, Gibco^{TM}), for selection and maintenance of cells with VSV-G pseudotyped lentiviral vector incorporated. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO$_2$

/95% air and were passage every 3–4 days, at 80–90% confluence. For subculturing, the medium was removed, and the monolayers were rinsed with phosphate-buffered saline 1x (PBS). Cells were individualized and detached from the substrate using a solution of 0.05% trypsin/EDTA(1x) (25300-062, Gibco^{TM}) at 37 °C for ca. 5 minutes. Fresh medium was added to inactivate trypsin and the cell suspension was centrifuged at 150 x g for 5 minutes. Next, cells were split into a new 25 cm^2 flask (10062-872, VWR®) in a 1:10 to 1:20 ratio. LNCaP cells were cultured in flasks coated with Poly- L -Lysine (PLL) to improve attachment to the flasks. In addition, the seeding density for this cell line was twice the seeding density used with the other cell lines. Cells were tested for mycoplasm by the MYCOPLASMA CHECK protocol (GATC-Biotech).

2.3. Preparation of total cell extracts

To assess the impact of ^{nat}In-DOTAGA-AO on mitochondrial protein expression, cells were seeded on a pre-coated 6 multi-well plate (10062-892, VWR®) with PLL. PNT2, PC3-PIP and PC3-FLU were seeded with a cellular density of 1.0 x 10^4 cells/cm^2, and LNCaP were seeded with a cellular density of 2.0x10^4 cells/cm^2. After 24 hours in culture, cells were treated or not with 50 μM ^{nat}In-DOTAGA-AO and incubated at 37 °C, in a 5% CO$_2$ atmosphere for 72 hours. Then, cells were harvested with trypsinization and isolated by centrifugation at 150 rpm for 5 minutes. Pellets were stored at -80 °C until assayed.

For protein extraction, cell pellets were resuspended in RIPA buffer supplemented with 0.5 mM PMSF, 20 mM NaF, 10 mM NAM, 5 mM sodium butyrate, 0.5% DOC, and PIC (diluted 1:400 (v/v)). Next, cellular suspensions were sonicated, and their protein content was assessed using the Bradford method, using the Bio-Rad Protein Assay kit (5000001, Bio-Rad) and Bovine Serum Albumin (BSA) as standard. First, the working reagent was diluted in ultrapure water (1:3) as suggested by the kit's protocol. The reaction was initiated by adding 120 μL of working reagent to 1 μL of sample and 79 μL of water in a micro test flat-base 96-well plate (82.1581, SARSTEDT AG). After 15 minutes of incubation at room temperature and in the dark, absorbance was read at 595 nm, using a BioTEK ® Cytation3^{TM} (BioTek).

2.4. Western blotting

After total protein quantification, samples were denatured with sample buffer 6x at 95 °C for 5 minutes. Then, 18 to 25 µg of protein was loaded into the wells of an 8% dodecyl sulfate (SDS-polyacrylamide gel (SDS-PAGE) and separated by electrophoresis at a constant voltage of 80 V, for 90 minutes, at room temperature using a PowerPacTM Basic Power Supply (Bio-Rad). In each gel, a molecular weight standard (Precision Plus Protein Dual Color Standards, Bio-Rad) was loaded to allow for molecular weight estimation. Next, proteins were transferred to a pre-activated (5 seconds in 100% methanol followed by 15 minutes in water and 15 minutes in TBS-T) polyvinylidene difluoride membrane (PVDF, 0.45 um, Millipore) using the Trans-Blot® TurboTM Transfer System (Bio-Rad) at a constant voltage of 0,75V, for 2 hours. Once protein transfer was complete, the membrane was incubated with a blocking solution (5% non-fat dry milk), at room temperature, for 1 hour. Afterwards, membranes were incubated overnight and at 4 °C with a primary antibody (Table 1), under continuous stirring. On the following day, membranes were washed three times with TBS-T, 5 minutes each time, and were incubated with the respective Horseradish Peroxidase (HRP)-conjugated secondary antibody (Table 1) at room temperature, for 1 hour. Both primary and secondary antibodies were prepared in 1% non-fat dry milk in TBS-T. Finally, membranes were washed again three times with TBS-T and incubated with ClarityTM Western ECL Substrate detection kit (Bio-Rad). Chemi-fluorescence was detected using the VWR® Gel Documentation System Imager Chemi 5QE. Densitometry analysis was performed using the ImageLab software (Bio-Rad).

Table 1. Antibodies used in western blotting analysis.

Protein	MW (kDa)	Host Species	Dilution	Gel (%)	Catalog Number	Manufacturer
β-Actin	43	Mouse (monoclonal)	1:10,000	8	MAB1501	Cell Signaling
PSMA	100	Mouse (monoclonal)	1:1000	8	sc-514444	Santa Cruz
Anti-Mouse	–	Horse	1:10000	8	7076	Cell Signaling

2.5. Resazurin assay

The resazurin assay is commonly used as an indirect measure of cytotoxicity/cell proliferation by measuring the dehydrogenase activity of cell cultures. In this technique, the blue non-fluorescent resazurin is reduced to resofurin through the dehydrogenases activity of viable cells. The resulting resofurin is a pink-fluorescent metabolite detected by spectrofluorometers (Prabst et al. 2017; Silva et al. 2016).

Cells were seeded on a pre-coated 96 multi-well plate (10062-900, VWR®) at a density of 1.0×10^4 cells/cm^2 for PNT2, PC3-PIP and PC3-FLU and 2.0×10^4 cells/cm^2 for LNCaP. After 24 hours of incubation, PSMA$_{617}$ or ^{nat}In-DOTAGA-AO were directly added to the culture medium, to final concentrations of 0.1; 1; 10; and 50 μM. Resazurin reduction was evaluated at 0 h (time zero), 48, and 72 hours after PSMA$_{617}$ addition. For ^{nat}In-DOTAGA-AO, the resazurin assay was performed after 0 hours, 24 hours, 48 hours, and 72 hours after the addition of the compound. For each timepoint, the medium of the respective wells was removed and substituted by 10 μg/mL solution of resazurin prepared in microscopy medium (0.1 M NaCl; 20 mM NaHCO$_3$; 6 mM KH$_2$PO$_4$; 5 mM KCl; 0.9 mM CaCl$_2$; 5 mM Na$_2$HPO$_4$; 0.4 mM MgSO$_4$; 10 mM glucose; 10% FBS; pH= 7.2)). Cells were then incubated at 37 °C and in a 5% CO$_2$ atmosphere for 120 minutes. The amount of produced resofurin was measured by fluorescence at an excitation wavelength of 540 nm and emission wavelength of 590 nm, using the BioTEK ®

Cytation3^{TM} system (BioTek). Afterwards, wells were washed with PBS 1x and allowed to dry until the end of the whole experiment, at which time cells were fixated overnight with 1% (v/v) acetic acid, at -20 °C. Subsequently, the Sulforhodamine B (SRB) assay (Silva et al. 2016) was performed as described below (section 2.6).

2.6. Sulforhodamine B assay

SRB is a colorimetric assay that provides an estimate of cell mass based on the measurement of cellular protein content. This assay employs the sodium salt of SRB, which is a red fluorescent dye that binds basic amino acid residues under mild acidic conditions (Silva et al. 2016; van Tonder et al. 2015). In this work, SRB assay was used both to complement the cytotoxicity/cell proliferation evaluation, but also for normalization purposes.

Firstly, the fixation solution (1% (v/v) acetic acid or methanol or 60% (w/v) trichloroacetic acid (TCA) was discarded and the plate was left to dry at 37 °C. Further, 100 μL of 0.05 % SRB in acetic acid were added to each well and the plate was incubated at 37 °C for 1 hour. Then, the SRB solution was removed, and the plate was washed with 1% (v/v) acetic acid. When dried, 250 μL of 10 mM Tris pH 10.5 were added to the plates that subsequently were shaken for 15 minutes. Finally, absorbance was read at 540 nm, using the BioTEK ® Cytation3^{TM} (BioTek) system.

2.7. Real-time cell metabolic analysis

To assess the impact of PSMA$_{617}$ and ^{nat}In-DOTAGA-AO on metabolism, the Agilent Seahorse XF Cell Mito Stress Test Kit and the Agilent Seahorse XF Glycolysis Stress Test Kit were performed. The Seahorse XFe96 Analyzer (Seahorse Bioscience, Billerica, MA, USA) measures in real-time the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), which can be taken as a measure of the bioenergetic status of the cultures under analysis. ORC and ECAR are key indicators of the energy metabolism of cells providing information about mitochondrial respiration, lactic acid fermentation, and ATP production (Divakaruni et al. 2014).

In the Agilent Seahorse XF Cell Mito Stress Test, different modulators of respiration are sequentially injected during the assay to monitor mitochondrial function. The injected compounds include oligomycin, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), rotenone, and antimycin A. The first compound injected is oligomycin, an ATP synthase inhibitor (F_o subunit of complex V), which leads to a decrease in the electron flow through the ETC, ultimately decreasing the mitochondrial respiration. The second compound injected is FCCP, which is an uncoupler that promotes the dissipation of the electrochemical proton gradient, thus disrupting the mitochondrial membrane potential. As a result of both injections, the electron flow through ETC is inhibited and the oxygen consumption by complex IV is maximal. Finally, a mix of rotenone and antimycin is injected, which are complex I and complex III inhibitors, respectively. At this point, OXPHOS is completely inactivated, and the measured OCR results from non-mitochondrial processes (Figure 3). This assay allows for the calculation of different mitochondrial function parameters, including basal, maximal, ATP production-linked, and proton leak-linked respiration, as well as spare respiration capacity, coupling efficiency, and non-mitochondrial respiration (Table 2).
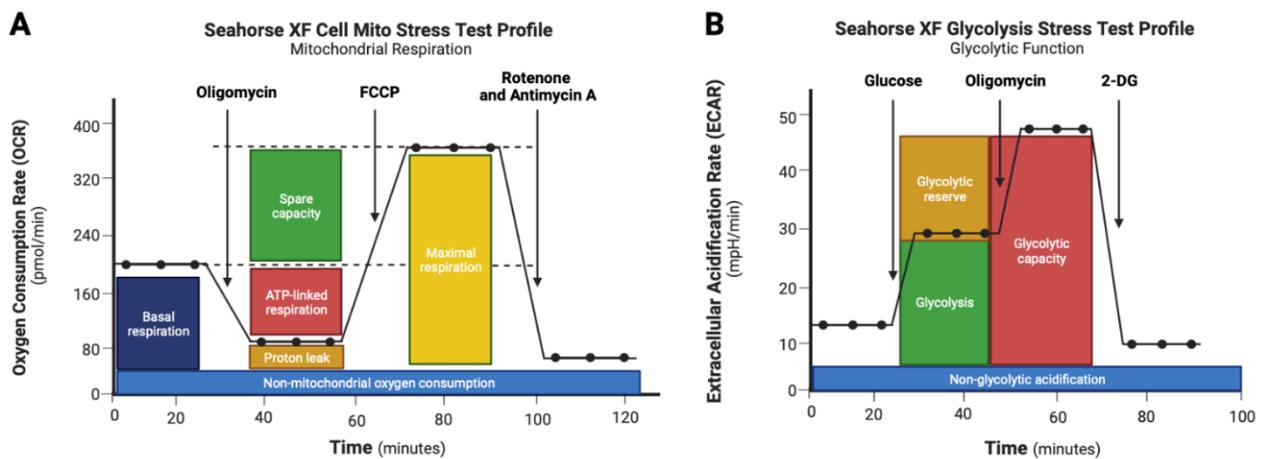
Figure 3. Seahorse XF profile and respective parameters of the performed. (A)Seahorse XF Cell Mito Stress Test Profile. (B) Seahorse XF Glycolysis Stress Test Profile. Figure 3 was created with BioRender. FCCP, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone; 2-DG, 2-deoxy-glucose.

Table 2. Key parameters of mitochondrial respiration and formulas used in their evaluation when performing the Agilent Seahorse XF Cell Mito Stress Test

Parameter	Indicator	Formula
Basal Respiration	Cellular energy demand under baseline conditions	(Late rate measurement before 1st injection) $-$ (Non-mitochondrial respiration rate)
ATP Production	Mitochondrial ATP produced mitochondria to meet the energy needs of the cell	(Late rate measurement before oligomycin injection) $-$ (Minimum rate measurement after oligomycin injection)
H^+ (Proton) Leak	Mitochondrial damage, or mechanism to regulate the mitochondrial ATP production	(Minimum rate measurement after oligomycin injection) $-$ (Non-mitochondrial respiration)
Maximal Respiration	The maximum rate of respiration that the cell can achieve	(Maximum rate measurement after FCCP injection) $-$ (Non-mitochondrial respiration)
Spare respiration capacity	Cell fitness or flexibility	(Maximal respiration) $-$ (Basal respiration)
Non-mitochondrial respiration	Oxygen consumption by other cellular enzymes	Minimum rate measurement after rotenone and antimycin A injection

The Agilent Seahorse XF Glycolysis Stress Test Kit monitors the glycolytic function of cells by measuring the extracellular medium acidification, reported as ECAR. During lactic acid, pyruvate generated from glucose by glycoslysis is converted to lactate, with all steps occurring in the cytosol. This results in an extrusion of protons to the extracellular medium and its consequent acidification (Adeva-Andany et al. 2014). In this assay, cells are incubated in a glycolysis stress test medium without glucose or pyruvate. Further, glucose, oligomycin, and 2-deoxy-glucose (2-DG) are injected. The injected glucose is metabolized in glycolysis, with some of the pyruvate formed converted to

lactate production, acidifying the extracellular medium. Next, oligomycin is added, shifting the production of energy to the glycolytic pathway, resulting in ECAR augment, revealing the maximal cellular glycolytic capacity. Finally, 2-DG, which is a glucose analogue, inhibits the glycolytic pathway by competitive binding to glucose hexokinase. As result, ECAR decreases confirming, that the acidification of the medium was due to glycolysis function. This assay allows the assessment of glycolysis, glycolytic capacity, glycolytic reserve, and non-glycolytic acidification (Table 3).

Table 3. Key parameters of glycolytic function and respective formulas used in their evaluation when performing the Agilent Seahorse XF Cell Glycolytic Stress Test

Parameter	Indicator	Formula
Glycolysis	Glycolytic function after addition of saturating amounts of glucose	(Maximum rate measurement before oligomycin injection) − (Las rate measurement before glucose injection)
Glycolytic capacity	Maximal glycolytic capacity after shutting down OXPHOS	(Maximum rate measurement after oligomycin injection) − (Last rate measurement before glucose injection)
Glycolytic reserve	Glycolytic response to an energetic demand	(Glycolytic capacity) − (Glycolysis)
Non-glycolytic acidification	Other sources of extracellular acidification	Last rate measurement prior to glucose injection

In these assays, two different concentrations were tested (10 μM and 50 μM, for both PSMA$_{617}$ and ^{nat}In-DOTAGA-AO), as well as two different times of exposure (6 and 72 hours). Firstly, cells were seeded on an Agilent Seahorse XF96 Cell Culture Microplate (101085-004, Agilent) pre-coated with PLL at a density of 1.5 x 10^4 cells/cm^2 for PNT2, PC3-PIP and PC3-FLU cells and and 3.0 x 10^4 cells/cm^2 for LNCaP cells, in 80 μL of culture medium. On the following day, 70 μL solutions of PSMA$_{617}$ and ^{nat}In-DOTAGA-AO prepared in culture medium were added directly to the cells in culture, to final concentrations of 10 μM and 50 μM. Non-treated cultures received 70 μL of culture medium. Further, cells were incubated at 37 °C and in a 5% CO$_2$ atmosphere for 72 hours

exposed to the compounds. Six hours previously to the assay, the culture medium was discarded, and 150 μL of 10 μM and 50 μM of both PSMA$_{617}$ and ^{nat}In-DOTAGA-AO were added. On the day before the assay, the XFe96 sensor cartridge was hydrated overnight and at 37 °C in with Seahorse XF Calibrant in a non-CO$_2$ incubator.

On the day of the experiment, the assay medium was prepared by supplementing the Seahorse RPMI medium with 10 mM glucose and 2 mM glutamine, for the Mito stress test assay, or with 2 mM glutamine, for the glycolysis stress test. Next, the medium pH was adjusted to 7.4 and warmed to 37 °C in a water bath, before usage. Microplates containing cells were washed 3 times with the warmed assay medium and then 175 μL of assay medium were added to each well of the cell culture microplate, which was then incubated at 37 °C in a non-CO$_2$ incubator for 45 minutes. Further, the sensor cartridge ports were loaded with 25 μL of the different modulators, previously prepared in the assay medium. The concentrations of these solutions were such as to obtain the following final concentrations: for Mito stress test port A – oligomycin (2 μM); port B – FCCP (0.2 μM for PNT2, and 0.5 μM for PC3-PIP, PC3-FLU, and LNCaP); port C – rotenone + antimycin A (2 μM). For glycolysis stress test port A – glucose (10 mM); port B – oligomycin (2μM); port C – 2-DG (10 mM). During the assay, the modulators were injected automatically, port by port.

At the end of the experiment, 50 μL of TCA 60% were added to each well, to a final concentration of 10% TCA to fixate cells, at 4 º C overnight. Further, an SRB assay was performed, as previously described, to normalize the data. SRB absorbance values were inserted on the Agilent Seahorse Wave Desktop Software (Agilent) to obtain OCR and ECAR values normalized to cell mass.

2.8. Evaluation of ^{nat}In-DOTAGA-AO internalization and localization

To assess ^{nat}In-DOTAGA-AO internalization and co-localization, confocal images were obtained using a Plan-Apochromat/1.4NA 63× lens on an Axio Observer.Z1 confocal microscope (Zeiss Microscopy, Germany) with Zeiss LSM 710 software. FIJI (ImageJ, National Institute of Health, USA) was used for image analysis. ^{nat}In-DOTAGA-AO internalization was assessed by analyzing compound fluorescence and co-

localization within nucleus and mitochondria analysis were achieved using Macros in Fiji designed by Dr. Jorge Valero. Briefly, image background was normalized using the function Subtract Background, included in Fiji. Mitochondria-targeting tetramethylrhodamine methyl ester (TMRM) images were extracted to grayscale. FindFoci function was then used to allow the identification of peak intensity regions (Herbert et al. 2014) to show mitochondria-specific fluorescence. A threshold was applied to optimally resolve individual mitochondria. Mitochondrial outlines were traced through the Analyze Particles function. To obtain information about protein co-localization with mitochondria, a selection of mitochondrial ROIs was done, and the respective protein Integrated Density inside the ROIs was considered.

2.9. Assessment of mitochondrial membrane potential

The red fluorescent TMRM dye was used to measure the mitochondrial membrane potential (MMP). TMRM is a lipophilic cationic dye that accumulates within active mitochondria with intact MMP (Creed et al. 2019; Chazotte 2011).

Cells seeded at a density of 1.0×10^4 cells/cm^2 (for PNT2, PC3-PIP and PC3-FLU cells) and 2.0×10^4 cells/cm^2 (for LNCaP cels) on a μ-Slide 8 Well ibiTreat (80826, iIbidi®) pre-coated with PLL were treated for 72 h 24 hours post-seeding with 10 or 50 μM [nat]In-DOTAGA-AO. Control cultures received the same volume of the addition vehicle. On the day of the experiment, cells were washed with microscopy medium and incubated for 30 minutes with a solution of 100 nM TMRM and 1 μg/mL Hoechst 33342, prepared in microscopy medium. Confocal images were acquired as described in section 2.8.

2.10. Measurement of mitochondrial superoxide levels

MitoSOX^{TM} (M36002, Invitrogen^{TM}) was used to assess mitochondrial O_2^- production. MitoSOX^{TM} is a fluorogenic dye that specifically targets mitochondria in live cells. It is quickly oxidized by O_2^-, but not by other ROS or RNS. The resulting product emits highly red fluorescence that can be detect by spectrofluorometers or for live-cell imaging (Kalyanaraman B 2020; Kauffman M et al. 2016).

Cells were seeded on a 96-well black/clear bottom polystyrene microplate (3603, Corning®) pre-coated with PLL. PNT2, PC3-PIP and PC3-FLU were seeded with a cellular density of 1.0×10^4 cells/cm^2, and LNCaP were seeded with a cellular density of 2.0×10^4 cells/cm^2. 24 hours after seeding, cells were treated with 10 μM and 50 μM ^{nat}In-DOTAGA-AO and incubated at 37 °C and in a 5% CO_2 atmosphere for 72 hours. Then, the culture medium was discarded and MitoSOX^{TM} 5 μM prepared in pre-heated microscopy medium were added to each well. The microplate containing cells was incubated at 37 °C in a non-CO_2 incubator for 45 minutes. Next, the microplate was washed once with microscopy medium and 100 μL of the same medium was added to each well. The fluorescence of the produced metabolite was measured using a BioTEK® Cytation3^{TM} system (BioTek), at an excitation wavelength of 510 nm and emission wavelength of 580 nm, for 20 minutes. Finally, cells were fixed with 1% (v/v) acetic acid in methanol, at -20 °C overnight. Subsequently, an SRB assay was performed to normalize the data to cell mass.

2.11. Statistical analysis

Data were expressed as the mean ± SEM of the number of experiments or elements (neuritis or mitochondria) indicated in the figure legends. The normal distribution of each population was analyzed, and all experimental groups were considered non-parametric. Thus, comparisons among multiple groups (relative to control or to treatment) were performed by non-parametric one-way analysis of variance (ANOVA) followed by the Kruskal–Wallis Multiple Comparison post-hoc test. Mann–Whitney U test was also performed for comparison between two populations, as described in figure legends. Significance was defined as $p < 0.05$.

3. Results

3.1. Cell lines characterization: PSMA expression levels

PSMA is markedly overexpressed in prostatic cancer cells compared to other tissues, including normal prostatic tissue (Uijen et al 2021). Moreover, high levels of PSMA expression are correlated with higher PCa malignancy and aggressiveness (Uijen et al 2021; Chang 2004; Rahbar et al 2018). Since AugerTher project aims to synthetize novel dual-targeted TRT compounds with specific targeting to PSMA, we initially characterized the in vitro models used in this study by assessing PSMA levels (Figure 4).

Results evidenced that PNT2, a normal prostate epithelial cell line, does not express PSMA, as expected. In contrast, LNCaP cells, which derived from a lymph node metastatic prostate carcinoma, expressed PSMA as shown in Figure XB. Importantly, beside the 100 kDa band corresponding to PSMA, we were also able to detect a band at approximately 200 kDa corresponding to PSMA dimers as shown by Liu et al 2014 (Liu et al 2014). PC3 cells are a prostate cancer cell line derived from bone metastasis that do not express PSMA. In contrast, PC3-PIP, transduced with human PSMA overexpress the protein. In addition to the expression of 100 kDa PSMA, PC3-PIP cells also express 200 kDa PSMA and lower levels of PSMA glycoforms (Liu et al 2014, Yuan et al 2022). Comparing the cell lines that express the protein of interest, PC3-PIP have a higher expression of PSMA than LNCaP.
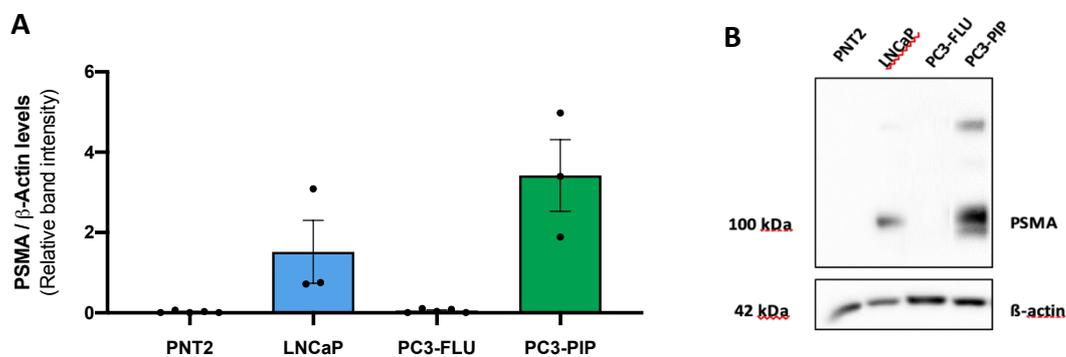
Figure 4. Characterization of the cell lines: expression of PSMA in PNT2, LNCaP, PC3-FLU and PC3-PIP. **(A)** Levels of PSMA protein normalized for ß-actin protein levels used as loading control. **(B)** Representative Western Blotting analysis of PSMA and ß-actin labelling. Data are the mean ± SEM of 3-5 independent experiments.

3.2. Effect of PSMA$_{617}$ compound in cell viability and metabolism

3.2.1. PSMA$_{617}$ did not induce cell mass or metabolic activity alterations

PSMA$_{617}$ was the initial compound to be synthesized in the context of this project. This ligand is intended to be present in the structure of synthesized compounds in the future to be used as a tag to PCa cells which overexpress PSMA protein. Therefore, we assessed the cytotoxicity of PSMA$_{617}$, itself, in the four cell lines used in this study, using the SRB and the resazurin assays to evaluate cell mass and metabolic activity, respectively (Figure X). Cells were treated with 0.1, 1, 10, or 50 μM PSMA$_{617}$ or vehicle for 48 and 72 hours. Treatment with 250 μM H$_2$O$_2$ showing high cytotoxicity as described (Xiang et al 2016), was used as positive control to induce cell death. Data evidenced that none of the four concentrations of PSMA$_{617}$ tested affect significantly neither the cell mass, neither the metabolic activity. Importantly, treatment with H$_2$O$_2$ decreases significantly both parameters as expected.

To evaluate if the changes in metabolic activity were related with changes in cell mass, we replotted results from graphics i) and ii) into a correlation graphic iii) for each cell line. Results show a positive correlation between both parameters in PNT2 ($\rho = 0.97$; $r^2 = 0.94$), LNCaP ($\rho = 0.91$; $r^2 = 0.83$), PC3-FLU ($\rho = 0.97$; $r^2 = 0.94$), and PC3-PIP ($\rho = 0.96$; $r^2 = 0.92$), indicating that the change observed in the metabolic activity are the result of an increase in cell mass and very likely due to cell proliferation.
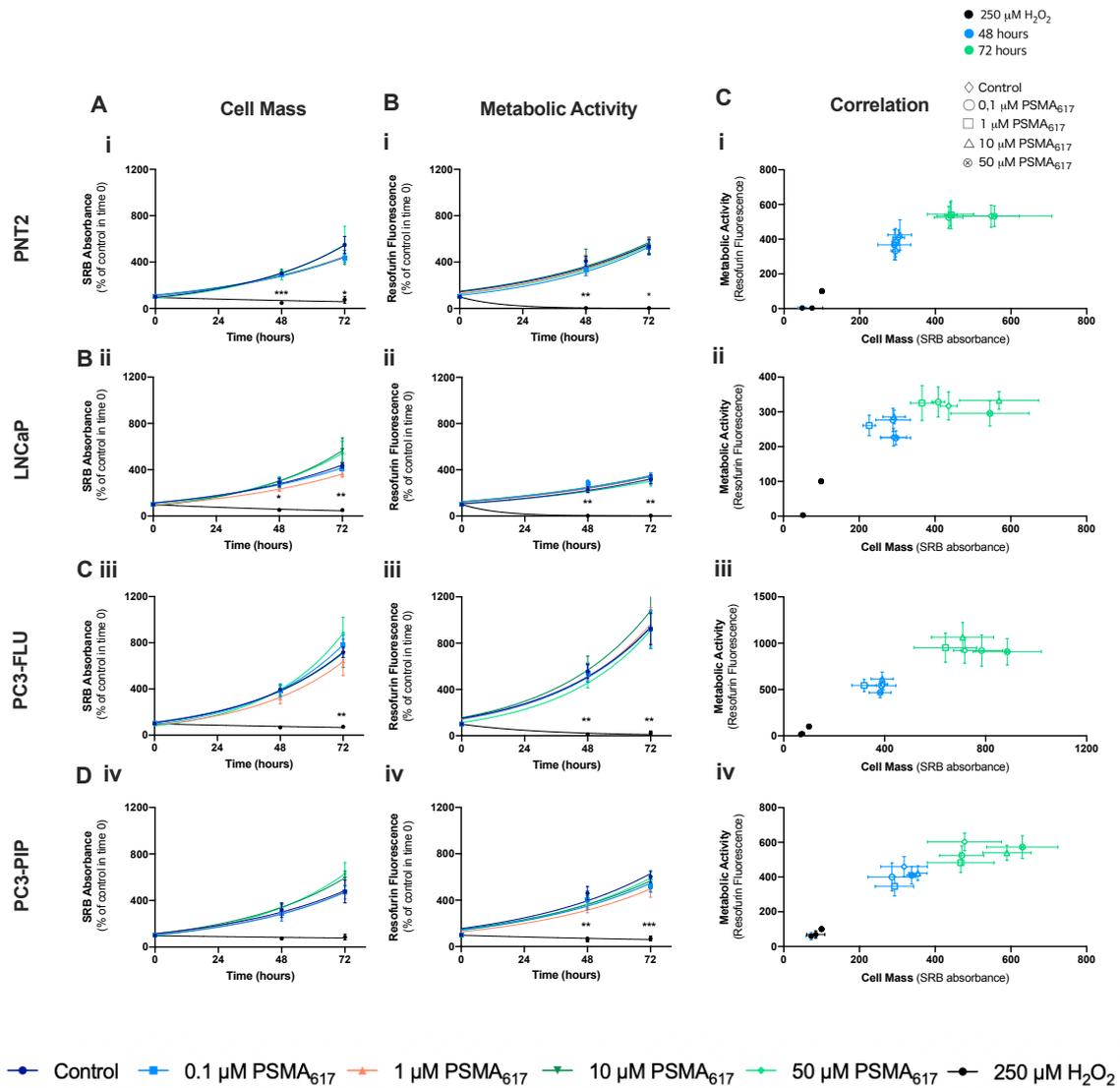
Figure 5. Cytotoxic effect of PSMA$_{617}$ on PNT2, LNCaP, PC3-FLU and PC3-PIP cells. **(A)** The sulforhodamine B (SRB) assay was used to measure cell mass of untreated cells (control) and cells treated with 0,1 µM; 1 µM; 10 µM and 50 µM PSMA$_{617}$ for 0 (time 0), 48, and 72 hours. Data are the mean ± SEM of 4 independent experiments in triplicate and expressed as percentage of the time 0 value. The different conditions were compared to the control by a two-way ANOVA (* p< 0.05; ** p<0.01; *** p<0.001). **(B)** The metabolic activity of untreated cells (control) and cells treated with 0,1 µM; 1 µM; 10 µM and 50 µM PSMA$_{617}$ for 0, 48, and 72 hours was measured by resazurin assay. Data are the mean ± SEM of 4–6 independent experiments in triplicate and expressed as percentage of the respective control. The different conditions were compared to the control by a two-way ANOVA (* p < 0.05; ** p<0.01; *** p<0.001). **(C)** The values of SRB and resazurin assay were re-plotted to assess the correlation between cell mass and metabolic activity. The Pearson correlation coefficients (ρ) are: 0.97 for PNT2; for 0.91 LNCaP; 0.97 for PC3-FLU; 0.96 for PC3-PIP.

3.2.2. Long-term PSMA$_{617}$ exposure affect mitochondrial respiration of PCa cells

PSMA is a transmembrane protein that internalize upon interaction with a ligand (Emmett et al 2017). Thus, we hypothesize that the interaction of PSMA617 with PSMA protein might lead to the internalization of both and that once in the cell, PSMA$_{617}$ could induce changes in mitochondrial function. Although no differences were noticed in the metabolic activity of the cells (Figure 5), we sought to understand the impact of PSMA$_{617}$ on mitochondrial respiration using the Agilent Seahorse XF Cell Mito Stress Test Kit. Moreover, considering the Warburg effect and the capacity of cancer cells to remodel their metabolism (Ashton et al 2018; Xu et al 2015), we evaluated the impact of short-term (6 hours, Figure 6) and long-term (72 hours; Figure 7) incubation with PSMA617 in PNT2, normal prostate cells, and LNCaP, PCa cells. As PSMA$_{617}$ did not induce cytotoxicity (Figure 5), we used 10 and 50 μM, the two highest concentrations of compound, for this experiment.

Data suggested that exposure to PSMA$_{617}$ for 6 hours did not induce significant alterations in the mitochondrial function of PNT2 and LNCaP cells. Thus, we do not see significant differences between control and treated cells regarding basal (Figure 6D), maximal (Figure 6E), ATP production-linked (Figure 6F), proton leak-linked (Figure 6G), spare respiratory capacity (Figure 6H), coupling efficiency (Figure 6I) and non-mitochondrial respiration (Figure 6J). However, LNCaP cells present higher OCR values over time (Figure 6A), when compared to PNT2 cells, which on the opposite, present higher ECAR values (Figure 6B). This observation is supported by the energy phenotype profile (Figure 6C) which evidence that LNCaP cells as more aerobic, using mitochondrial respiration predominantly, while PNT2 cells appear to be more glycolytic. Importantly, PNT2 cells exposed to both 10 and 50 μM PSMA$_{617}$ showed a more energetic profile, both metabolic pathways, glycolysis and OXPHOS, are used to produce energy (Figure 6, A-C evidencing that although PSMA$_{617}$ does not induce significant changes in mitochondrial respiration-related parameters, in PNT2 cells it induces a small shift in the energetic profile.

A Oxygen consumption rate

B Extracellular acidification rate

C Energy phenotype profile

D Basal respiration

E Maximal respiration

F ATP production-linked respiration

G Proton leak-linked respiration

H Spare respiration capacity

I Coupling efficiency

J Non-mitochondrial respiration
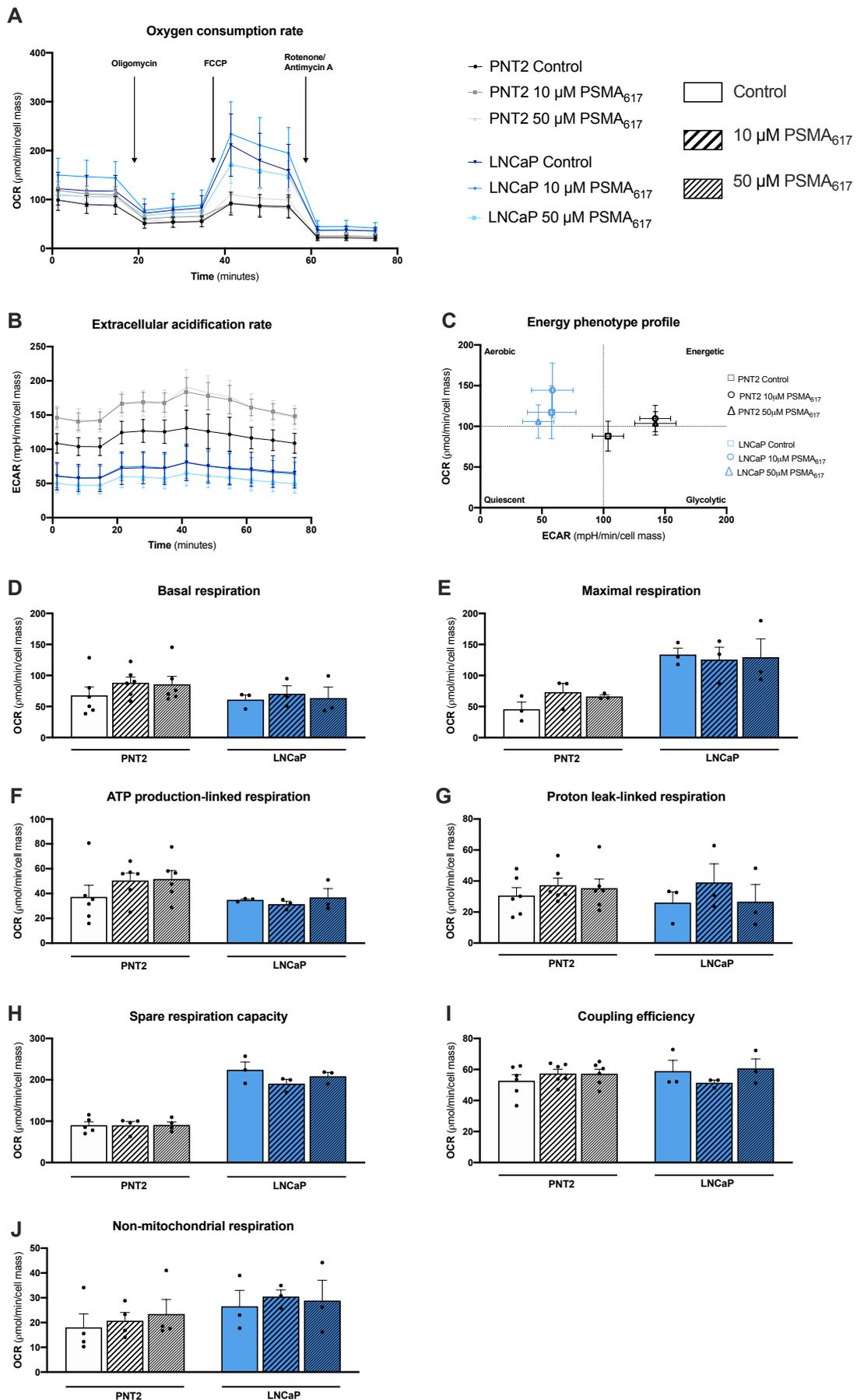
Figure 6. Impact of short-term exposure to PSMA$_{617}$ on mitochondrial oxygen consumption of PNT2 and LNCaP cells. The Seahorse XF^{e}96 Extracellular Flux Analyzer was used to measure Oxygen Consumption Rate (OCR) expressed as pmol/min **(A)**, and Extracellular Acidification Rate (ECAR) expressed in mpH//min **(B)**, both normalized per cell mass of cells untreated (control) and treated with 10 µM and 50 µM PSMA$_{617}$ for 6 hours. **(C)** The values of the last OCR and ECAR rate measured before first injection were displayed as a scatter plot to assess the energetic profile. Several respiratory parameters were evaluated: **(D)** basal OCR; **(E)** maximal OCR; **(F)** ATP production-linked OCR **(G)** proton leak-linked OCR; **(H)** spare respiration capacity OCR; **(I)** coupling efficiency OCR; and **(J)** non-mitochondrial OCR. Data are the mean ± SEM of 4-6 independent experiments in triplicate. The different conditions were compared to the control by Kruskal-Walli's followed by Dunn´s post-hoc test.

Results depicted in Figure 7 evidence that although no significant changes were observed in most of the mitochondrial respiration parameters assessed with the Mito Stress test, the spare capacity of 10 µM PSMA$_{617}$-treated LNCaP cells was significantly decreased when compared to control cells (Figure 7H). Moreover, long-term exposure to PSMA$_{617}$ appear to induce a shift in the energetic profile of LNCaP cells. Thus, tumoral cells LNCaP slightly shift their metabolism to a quiescent profile when treated with PSMA$_{617}$ for 72 hours. Importantly, the switch from glycolytic to energetic profile observed in non-tumoral PNT2 cells after a short-term exposure to the compound (Figure 6C) is maintained at 72 hours (Figure 7C).

Altogether, our results suggest that although PSMA$_{617}$ did not induce changes in cell proliferation (Figure 5), it affected the metabolic profile of tumoral cells by inducing a shift to a more quiescent profile, which could be of interest in the context of cancer therapeutic.

A Oxygen consumption rate

Oligomycin · FCCP · Rotenone/Antimycin A

PNT2 Control
PNT2 10 µM PSMA$_{617}$
PNT2 50 µM PSMA$_{617}$

LNCaP Control
LNCaP 10 µM PSMA$_{617}$
LNCaP 50 µM PSMA$_{617}$

Control
10 µM PSMA$_{617}$
50 µM PSMA$_{617}$

B Extracellular acidification rate

C Energy phenotype profile

Aerobic · Energetic · Quiescent · Glycolytic

PNT2 Control
PNT2 10µM PSMA$_{617}$
PNT2 50µM PSMA$_{617}$

LNCaP Control
LNCaP 10µM PSMA$_{617}$
LNCaP 50µM PSMA$_{617}$

D Basal respiration

E Maximal respiration

F ATP production-linked respiration

G Proton leak-linked respiration

H Spare respiration capacity

I Coupling efficiency

J Non-mitochondrial respiration

Figure 7. **Impact of long-term exposure to PSMA$_{617}$ on mitochondrial oxygen consumption of PNT2 and LNCaP cells.** The Seahorse XF^{e}96 Extracellular Flux Analyzer was used to measure Oxygen Consumption Rate (OCR) expressed as pmol/min **(A)**, and Extracellular Acidification Rate (ECAR) expressed in mpH//min **(B)**, both normalized per cell mass of cells untreated (control) and treated with 10 μM and 50 μM PSMA$_{617}$ for 72 hours. **(C)** The values of the last OCR and ECAR rate measured before first injection were displayed as a scatter plot to assess the energetic profile. Several respiratory parameters were evaluated: **(D)** basal OCR; **(E)** maximal OCR; **(F)** ATP production-linked OCR **(G)** proton leak-linked OCR; **(H)** spare respiration capacity OCR; **(I)** coupling efficiency OCR; and **(J)** non-mitochondrial OCR. Data are the mean ± SEM of 3-6 independent experiments in triplicate. The different conditions were compared to the control by Kruskal-Walli's followed by Dunn´s post-hoc test. Significant differences in LNCaP cells are marked by * ($p < 0.05$).

3.2.3. Short- and long-term exposure to PSMA$_{617}$ increases the glycolytic function of normal prostate PNT2 cells

Since we observed some slight changes in the energetic profile of cells treated with PSMA$_{617}$, we also assessed the glycolytic function of the cells through the Agilent Seahorse XF Glycolysis Stress Test Kit, after both short- (Figure 8) and long-term exposure (Figure 9). This assay allows the determination of ECAR, but also glycolytic parameters based on extracellular acidification.

Results depicted in Figure 8A evidence an increase in ECAR values over time in PNT2 cells treated with PSMA$_{617}$ for 6 hours. Of note, this effect was dependent of the concentration of compound. The glycolytic parameters calculated from the Figure 8A are plotted in B-E and show that PNT2 cells treated with 50 μM PSMA$_{617}$ present a significant increase in glycolysis (Figure 8B), glycolytic capacity (Figure 8C) and glycolytic reserve (Figure 8D). Treatment with 10 μM PSMA$_{617}$ also induce an increase in those parameters, although only changes in glycolytic reserve appears to be statistically significant. No changes were observed regarding the non-glycolytic acidification. On the other hand, tumoral cell line LNCaP glycolytic pathway is not affected by short-term exposure to both 10 and 50 μM PSMA$_{617}$.

Results obtained after 72 hours treatment evidence a similar increase in the ECAR value of both 10 and 50 μM treated cells when compared to respective control (Figure 9A). Moreover, although changes in glycolytic pathway-associated parameters were not statistically significant, results showed an increase in glycolysis (Figure 9B), glycolytic capacity (Figure 9C) and glycolytic reserve (Figure 9D) in PNT2 cells treated

with PSMA$_{617}$. LNCaP cells did not suffer significant alterations in any of the glycolytic parameters under long-term treatment with 10 or 50 μM PSMA$_{617}$ (Figure 9, B-E).

Altogether, our results indicate that PSMA$_{617}$ alone already induce changes in normal prostate cells (PNT2) increasing the contribution of the glycolytic pathway to energy production. However, it has no effect on PCa cells (LNCaP).
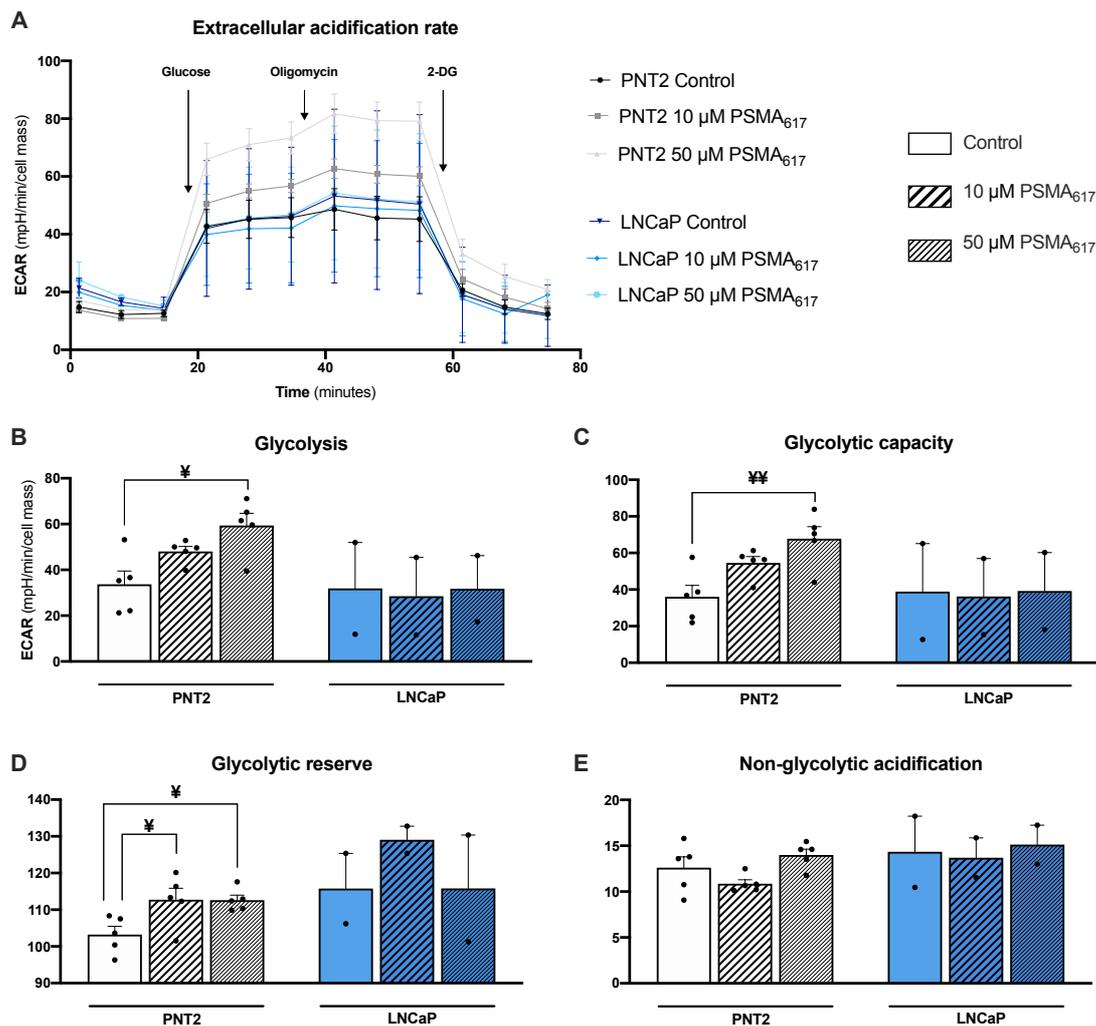
Figure 8. **Impact of short-term exposure to PSMA$_{617}$ on glycolytic function of PNT2 and LNCaP cells.** The Seahorse XF^{e}96 Extracellular Flux Analyzer was used to measure Extracellular Acidification Rate (ECAR) expressed in mpH//min **(A)**, normalized per cell mass of cells untreated (control) and treated with 10 μM and 50 μM PSMA$_{617}$ for 6 hours. Several glycolytic parameters were evaluated: **(B)** glycolysis ECAR; **(C)** glycolysis capacity ECAR; **(D)** glycolytic reserve ECAR **(E)** and non-glycolytic acidification ECAR. Data are the mean ± SEM of 2 or 3 independent experiments in triplicate. The different conditions were compared to the control by Kruskal-Walli's followed by Dunn's post-hoc test. Significant differences in PNT2 cells are marked by ¥ ($p < 0.05$) and ¥¥ ($p < 0.01$).

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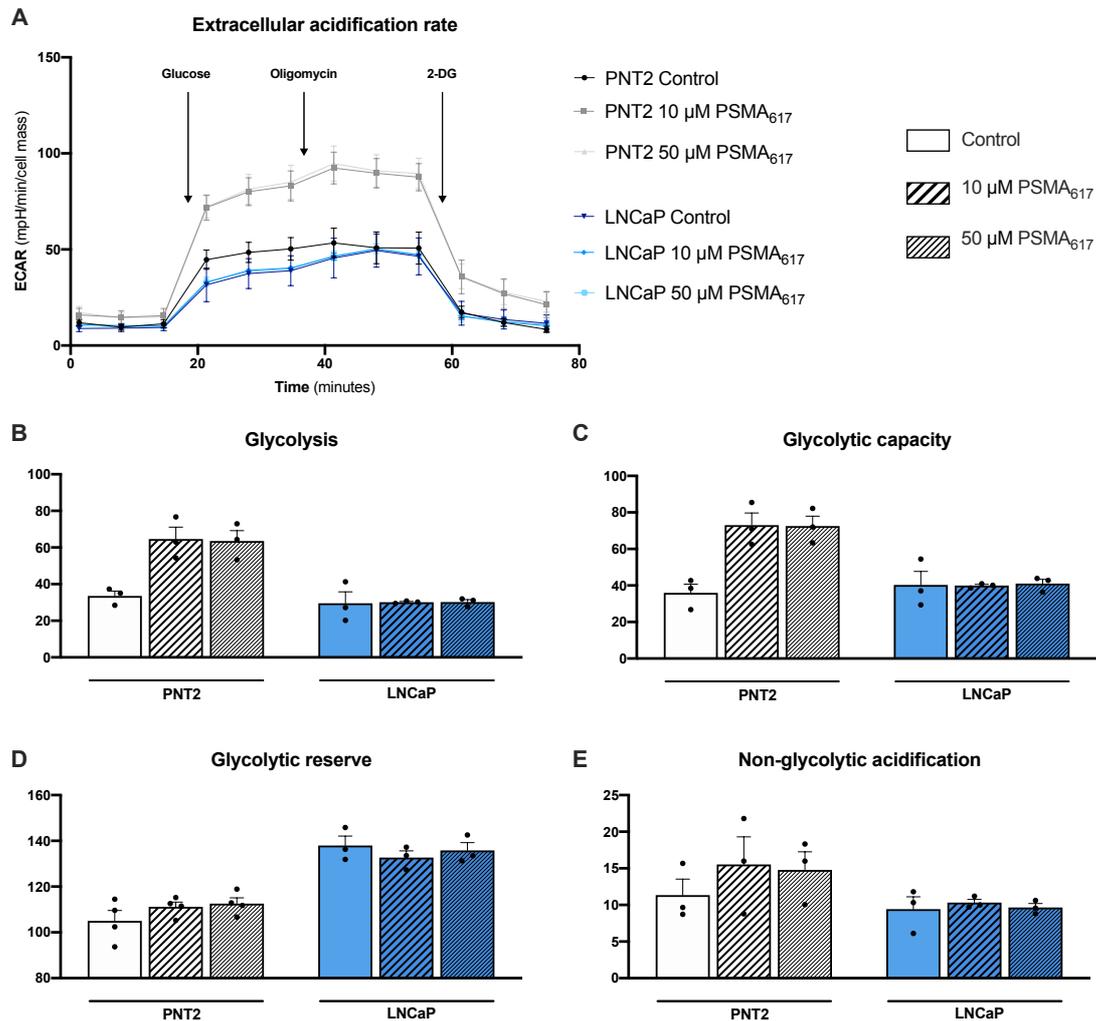
Figure 9. Impact of long-term exposure to PSMA$_{617}$ on glycolytic function of PNT2 and LNCaP cells. The Seahorse XF^{e}96 Extracellular Flux Analyzer was used to measure Extracellular Acidification Rate (ECAR) expressed in mpH//min **(A)**, normalized per cell mass of cells untreated (control) and treated with 10 μM and 50 μM PSMA$_{617}$ for 72 hours. Several glycolytic parameters were evaluated: **(B)** glycolysis ECAR; **(C)** glycolysis capacity ECAR; **(D)** glycolytic reserve ECAR **(E)** and non-glycolytic acidification ECAR. Data are the mean ± SEM of 3 independent experiments in triplicate. The different conditions were compared to the control by Kruskal-Walli's followed by Dunn´s post-hoc test.

3.2.4 Exposure to PSMA$_{617}$ has no effect on PC3-PIP cells but induce an energetic switch in PC3-FLU cells

PSMA$_{617}$ has already been shown to have high PSMA cellular specificity and fast pharmacokinetics (Czerwinska et al 2020). Results obtained above (Figure 6 – 9) shows almost no significant effects of PSMA$_{617}$ on tumoral cells LNCaP. Thus, we assessed the same parameters on a tumoral cell line overexpressing PSMA protein, PC3-PIP, and

compared to its isogenic control, PC3-FLU. As previously, we evaluated the impact of short-term (6 hours; Figure 10) and long-term (72 hours; Figure 11) incubation with PSMA$_{617}$ (10 or 50 μM) in PC3-FLU tumoral cells (non-expressing PSMA) and in PC3-PIP tumoral cells overexpressing PSMA.

Results in Figure 10A and B show that there are no significant differences in OCR and ECAR values between PC3-FLU and PC3-PIP cells treated for 6 hours and their respective control. This result is supported by Figure 10C which show that both cell lines, regardless of the treatment, have an aerobic energetic profile, producing energy mainly through OXPHOS. Looking at mitochondrial respiration-related parameters, PC3-FLU cells presented a significant concentration-dependent increase in basal respiration when treated with PSMA$_{617}$ for 6 hours (Figure 10D), while other parameters remain unchanged. No effects were detected in the PC3-PIP cell line.

Figure 11 depicted the same parameters but after a long-term incubation with the compound (72 hours). Data evidenced that the long-term treatment with PSMA$_{617}$ induced a shift in the energetic profile of PC3-FLU cells to a more energetic profile, producing energy through both OXPHOS and glycolysis (Figure 11C), in a concentration-dependent manner. This shift is also represented in the ECAR values over time which are increased in PC3-FLU treated cells, comparing to control cells (Figure 11B). Concomitantly, PC3-FLU presented a dose-dependent increase in basal respiration (Figure 11D) and ATP production (Figure 11F) as well as in maximal respiration (Figure 11E) although this last is not statistically significant. PC3-PIP cells treated with the lower concentration of PSMA$_{617}$ (10 μM) only present a decrease in spare respiratory capacity (Figure 11H) which is probably related with the non-significant increase in the same condition of basal respiration (Figure 11B).

Altogether, our results suggest that the tumoral cell line PC3-PIP, overexpressing the PSMA protein, is not sensitive to PSMA$_{617}$; while, interestingly, the cell line non-expressing the PSMA protein shows a concentration-dependent response to the compound.
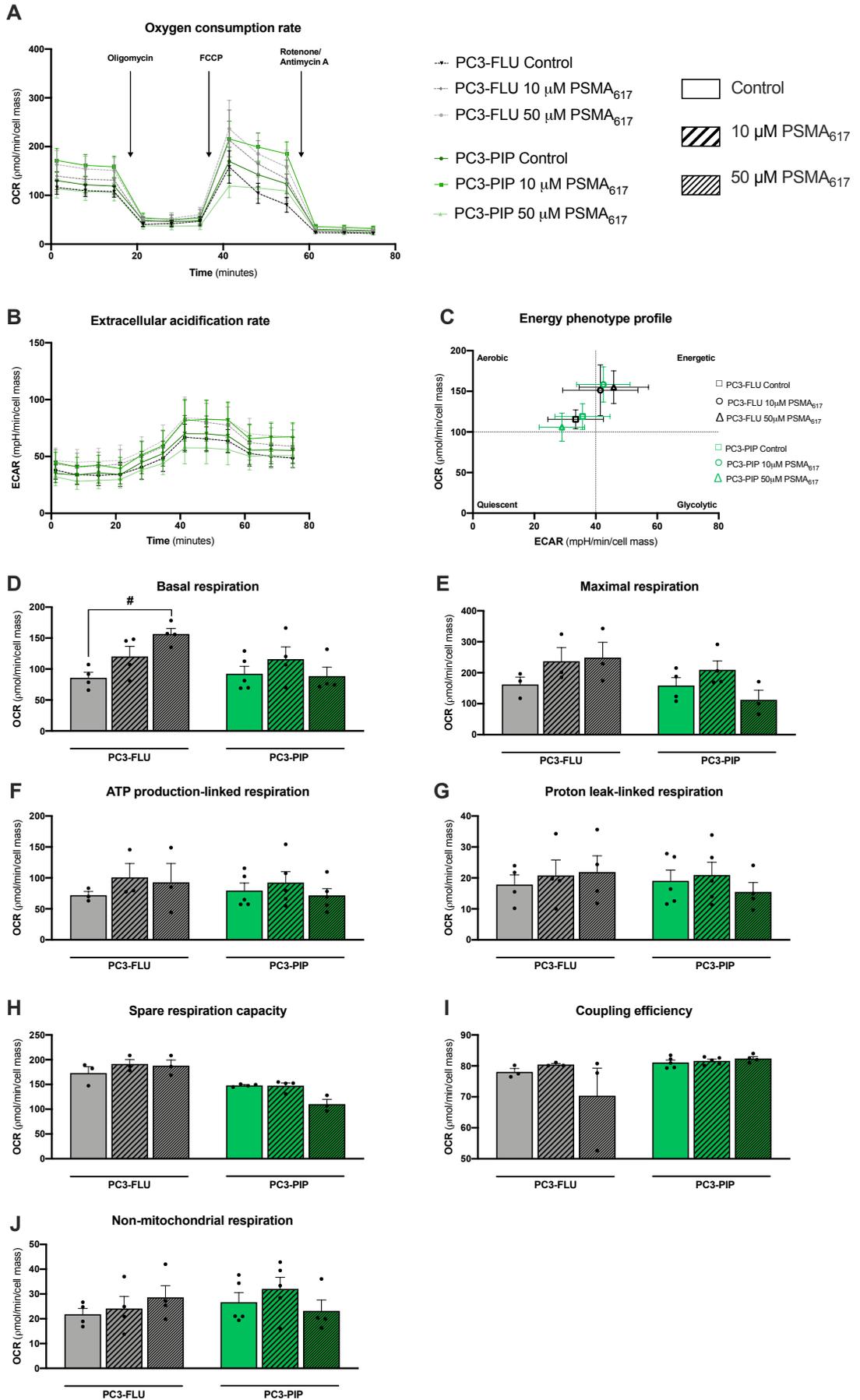
Figure 10. Impact of short-term exposure to PSMA$_{617}$ on mitochondrial oxygen consumption of PC3-FLU and PC3-PIP cells. The Seahorse XF^{e}96 Extracellular Flux Analyzer was used to measure Oxygen Consumption Rate (OCR) expressed as pmol/min **(A)**, and Extracellular Acidification Rate (ECAR) expressed in mpH//min **(B)**, both normalized per cell mass of cells untreated (control) and treated with 10 µM and 50 µM PSMA$_{617}$ for 6 hours. **(C)** The values of the last OCR and ECAR rate measured before first injection were displayed as a scatter plot to assess the energetic profile. Several respiratory parameters were evaluated: **(D)** basal OCR; **(E)** maximal OCR; **(F)** ATP production-linked OCR **(G)** proton leak-linked OCR; **(H)** spare respiration capacity OCR; **(I)** coupling efficiency OCR; and **(J)** non-mitochondrial OCR. Data are the mean ± SEM of 3-6 independent experiments in triplicate. The different conditions were compared to the control by Kruskal-Walli's followed by Dunn´s post-hoc test. Significant differences in PC3-FLU cells are marked by # ($p < 0.05$).

3.2.5 PSMA$_{617}$ treatment did not induce alterations on the glycolytic function of PC3-FLU and PC3-PIP cells

The previous results suggest that PC3-FLU cells become more energetic after treatment with PSMA$_{617,}$ starting to produce energy also through the glycolytic pathway and not predominantly through OXPHOS. Thus, we assessed the glycolytic function of PC3-FLU and PC3-PIP under PSMA$_{617}$ treatment and sought to understand the influence of PSMA expression in the cellular response to treatment. Thus, we performed the Glycolytic Stress assay on both PC3-FLU and PC3-PIP treated with PSMA$_{617}$ (10 and 50 µM) for 6 hours to evaluate the short-term exposure (Figure 12) and for 72 hours to evaluate the long-term exposure to the compound (Figure 13). Results evidence that the glycolytic rate of the cells is not significantly affected by PSMA$_{617}$ treatment.

A Oxygen consumption rate

B Extracellular acidification rate

C Energy phenotype profile

D Basal respiration

E Maximal respiration

F ATP production-linked respiration

G Proton leak-linked respiration

H Spare respiration capacity

I Coupling efficiency

J Non-mitochondrial respiration

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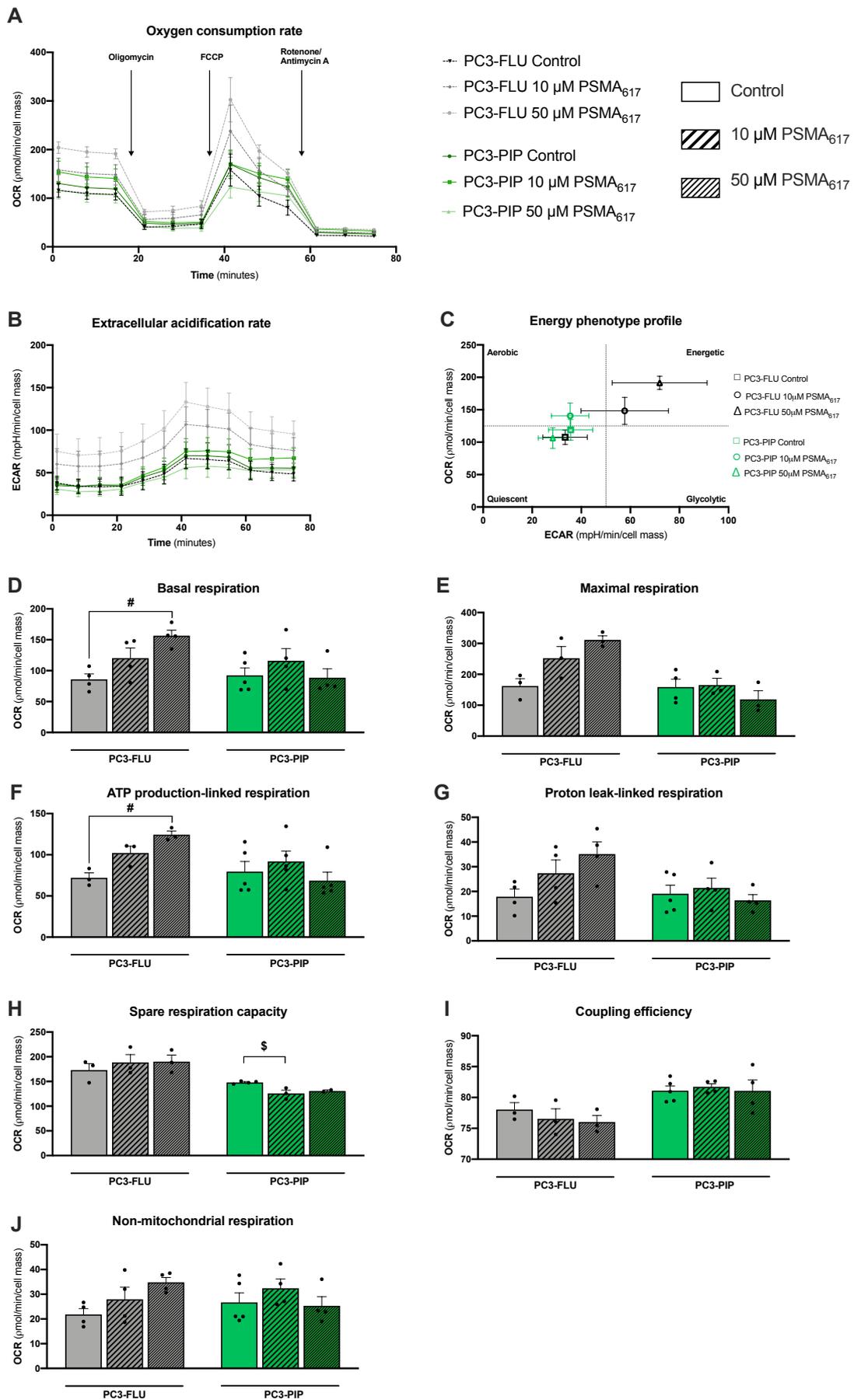
Figure 11. Impact of long-term exposure to PSMA$_{617}$ on mitochondrial oxygen consumption of PC3-FLU and PC3-PIP cells. The Seahorse XF^{e}96 Extracellular Flux Analyzer was used to measure Oxygen Consumption Rate (OCR) expressed as ρmol/min **(A)**, and Extracellular Acidification Rate (ECAR) expressed in mpH//min **(B)**, both normalized per cell mass of cells untreated (control) and treated with 10 μM and 50 μM PSMA$_{617}$ for 72 hours. **(C)** The values of the last OCR and ECAR rate measured before first injection were displayed as a scatter plot to assess the energetic profile. Several respiratory parameters were evaluated: **(D)** basal OCR; **(E)** maximal OCR; **(F)** ATP production-linked OCR **(G)** proton leak-linked OCR; **(H)** spare respiration capacity OCR; **(I)** coupling efficiency OCR; and **(J)** non-mitochondrial OCR. Data are the mean ± SEM of 3-5 independent experiments in triplicate. The different conditions were compared to the control by Kruskal-Walli's followed by Dunn´s post-hoc test. Significant differences in PC3-FLU cells are marked by # ($p < 0.05$). Significant differences in PC3-PIP cells are marked by $ ($p < 0.05$).

Figure 12. Impact of short-term exposure to PSMA$_{617}$ on glycolytic function of PC3-FLU and PC3-PIP cells. The Seahorse XF^{e}96 Extracellular Flux Analyzer was used to measure Extracellular Acidification Rate (ECAR) expressed in mpH//min **(A)**, normalized per cell mass of cells untreated (control) and treated with 10 μM and 50 μM PSMA$_{617}$ for 6 hours. Several glycolytic parameters were evaluated: **(B)** glycolysis ECAR; **(C)** glycolysis capacity ECAR; **(D)** glycolytic reserve ECAR **(E)** and non-glycolytic acidification ECAR. Data are the mean ± SEM of 3 independent experiments in triplicate. The different conditions were compared to the control by Kruskal-Walli's followed by Dunn´s post-hoc test.

Figure 13. Impact of long-term exposure to PSMA$_{617}$ on glycolytic function of PC3-FLU and PC3-PIP cells. The Seahorse XF^{e}96 Extracellular Flux Analyzer was used to measure Extracellular Acidification Rate (ECAR) expressed in mpH//min **(A)**, normalized per cell mass of cells untreated (control) and treated with 10 μM and 50 μM PSMA$_{617}$ for 72 hours. Several glycolytic parameters were evaluated: **(B)** glycolysis ECAR; **(C)** glycolysis capacity ECAR; **(D)** glycolytic reserve ECAR **(E)** and non-glycolytic acidification ECAR. Data are represented as the mean ± SEM of 2 or 3 independent experiments in triplicate The different conditions were compared to the control by Kruskal-Walli's followed by Dunn´s post-hoc test.

3.3 Effects of ^{nat}In-DOTAGA-AO compound in cell viability and metabolism

3.3.1 ^{nat}In-DOTAGA-AO internalizes and co-localizes with the nucleus and mitochondria

In the present study we performed preliminary work for AugerTher project that aim to develop a novel dual-targeting TRT modality based on AE-emitting radionuclide. Thus, we evaluate the cellular effects of ^{nat}In-DOTAGA-AO on four *in vitro* models This cold surrogate is constituted by a native form of ^{111}In isotope chelated with DOTAGA and functionalized with an AO attached ligand. The AO intercalate with nucleic acids, ensuring the targeting of nuclear DNA or mtDNA (Baskic et al 2006; Agorastos et al 2007), with any specific cell type target. Firstly, we assessed the internalization and co-localization of ^{nat}In-DOTAGA-AO within nucleus and mitochondria by analyzing the compound fluorescence using confocal microscopy and Fiji software (Figure 14). ^{nat}In-DOTAGA-AO successfully internalizes into PNT2, LNCaP and PC3-PIP cells, and is distributed within the nucleus (Figure 14B) and mitochondria (Figure 14C). Data evidence no significant difference between the compound co-localization in the nucleus of normal prostate PNT2 cells and LNCaP or PC3-PIP tumor cells (Figure 14B). We also observed accumulation of ^{nat}In-DOTAGA-AO within PC3-FLU nucleus (data not shown) but have not evaluated its accumulatio in mitochondria. Moreover, ^{nat}In-DOTAGA-AO internalization is significantly higher in PCa LNCaP cells compared to normal prostate PNT2 cells, while no changes are observed in PC3-PIP cells compared to PNT2 (Figure 14C). In addition, data suggest that LNCaP are the cells with more compound internalization within both nucleus and mitochondria. Interestingly, PC3-PIP appears to accumulate more ^{nat}In-DOTAGA-AO within the mitochondria than in the nucleus.

Overall, data evidence that ^{nat}In-DOTAGA-AO internalize in both tumoral and non-tumoral prostate cells, in both mitochondria and nucleus. In addition, LNCaP cells show higher internalization of ^{nat}In-DOTAGA-AO.
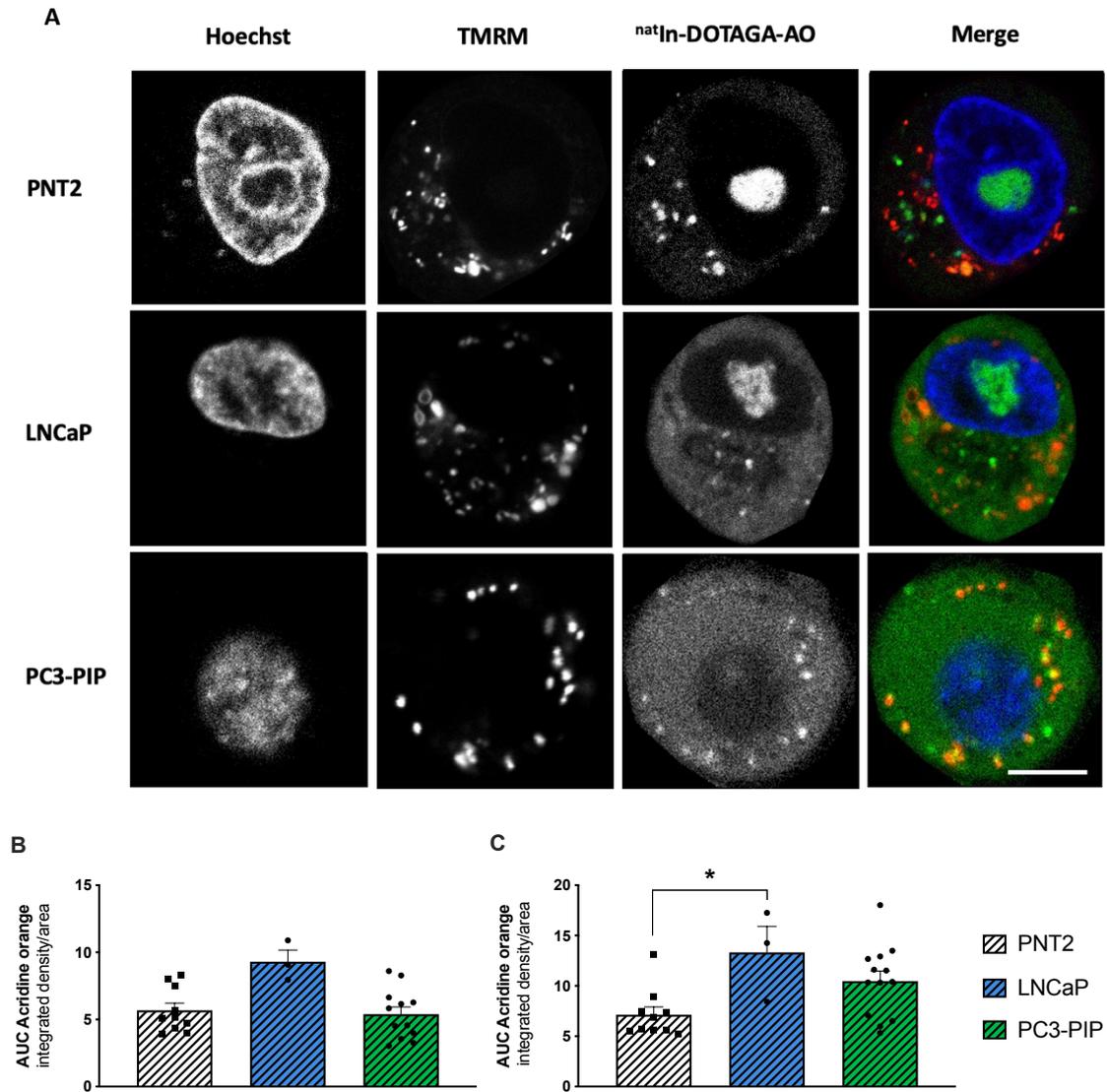
Figure 14. ^{nat}In-DOTAGA-AO internalization and co-localization within the nucleus and mitochondria. (A) Representative confocal images of PNT2, LNCaP, and PC3-PIP cells treated with ^{nat}In-DOTAGA-AO for 72 hours obtained using TMRM and Hoechst for mitochondrial and nuclei labeling, respectively. Scale bar = 10 μm. (B-C) Area under the curve (AUC) of single cell integrated density per area (from threshold 1-21) in the nucleus (B) and in the mitochondria (C). Data are the mean ± SEM for 10 PNT2 cells, 3 LNCaP cells, and 13 PC3-PIP cells, obtained from one single experiment. LNCaP and PC3-PIP treated cells were compared to the PNT2 treated cells by a one-way ANOVA followed by Kruskal-Walli's post-test. Significant differences in LNCaP cells are marked by * (p < 0.05).

3.3.2 natIn-DOTAGA-AO did not induce cell mass or metabolic activity alterations

We further evaluate the cytotoxicity of natIn-DOTAGA-AO in the four cell lines used in this study. Cells were treated or not (control) with 0.1, 1, 10, or 50 μM natIn-DOTAGA-AO for 24, 48 and 72 hours and the cell mass (Figure 15i) and metabolic activity (Figure 15ii) were evaluated through SRB and rezasurin assays, respectively. Data evidence that none of the four concentrations of natIn-DOTAGA-AO affect neither the cell mass, neither the metabolic activity. To assess if the changes in metabolic activity were related with changes in cell mass, we replotted results from graphics i) and ii) into a correlation graphic iii) for each cell line. Results show a positive correlation between both parameters in PNT2 (ρ = 0.94; r^2 = 0.88), LNCaP (ρ = 0.87; r^2 = 0.76), PC3-FLU (ρ = 0.98; r^2 = 0.96) and PC3-PIP (ρ = 0.97; r^2 = 0.94), indicating that the change observed in the metabolic activity are the result of an increase in cell mass and very likely due to cell proliferation.
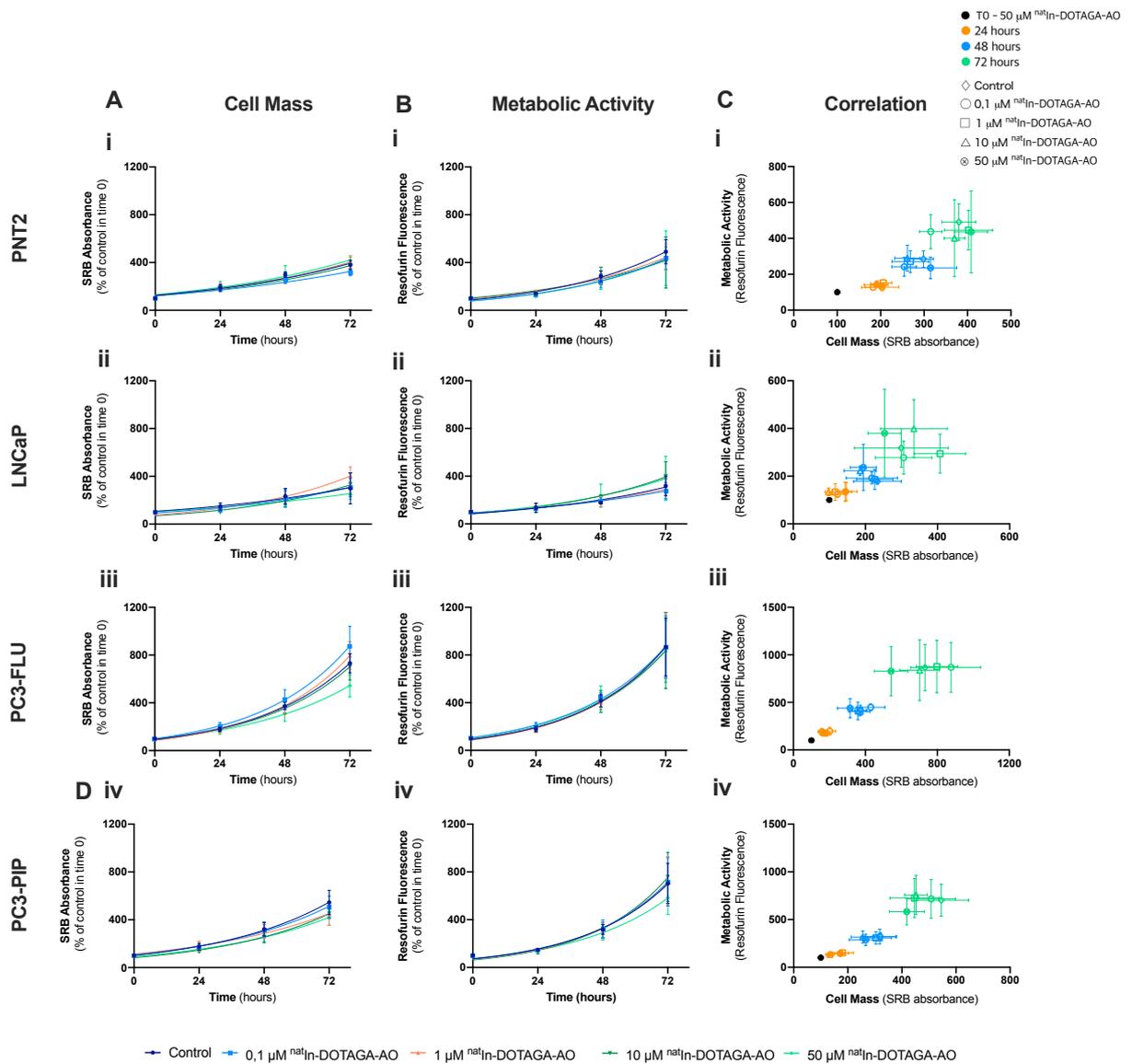
Figure 15. Cytotoxic effect of ^{nat}In-DOTAGA-AO on PNT2, LNCaP, PC3-FLU and PC3-PIP cells. **(A)** The sulforhodamine B (SRB) assay was used to measure cell mass of untreated cells (control) and cells treated with 0,1 μM; 1 μM; 10 μM and 50 μM ^{nat}In-DOTAGA-AO for 0 (time 0), 24, 48, and 72 hours. Data are the mean ± SEM of 4 independent experiments in triplicate and expressed as percentage of the time 0 value. The different conditions were compared to the control by a two-way ANOVA. **(B)** The metabolic activity of untreated cells (control) and cells treated with 0,1 μM; 1 μM; 10 μM and 50 μM $PSMA_{617}$ for 0, 24, 48, and 72 hours was measured by resazurin assay. Data are represented as the mean ± SEM of 4–6 independent experiments in triplicate and expressed as percentage of the respective control. The different conditions were compared to the control by a two-way ANOVA. **(C)** The values of SRB and resazurin assay were re-plotted to assess the correlation between cell mass and metabolic activity. The Pearson correlation coefficients are: 0.94 for PNT2; 0.87 for LNCaP; 0.98 for PC3-FLU; 0.97 for PC3-PIP.

3.3.2 Long-term exposure to ^{nat}In-DOTAGA-AO impaired mitochondrial function of non-tumoral and tumoral cells

As for PSMA$_{617}$, we also sought to understand the impact of short- (6 hours) and long-term (72 hours) exposure to ^{nat}In-DOTAGA-AO on mitochondrial function of normal prostate cells and PCa cells. Thus, mitochondrial respiration was assessed in PNT2 and LNCaP cells in the absence or the presence of 10 or 50 µM ^{nat}In-DOTAGA-AO using the the Agilent Seahorse XF Cell Mito Stress Test Kit.

Data presented in Figure 16 show that PNT2 cells treated for 6 hours with 10 µM ^{nat}In-DOTAGA-AO present significantly higher levels of basal (Figure 16D) and maximal respiration (Figure 16E), concomitantly with increased ATP production-linked respiration although not significant. Curiously, the treatment with 50 µM ^{nat}In-DOTAGA-AO did not induce significant changes in mitochondrial respiration except in coupling efficiency (Figure 16I). Results suggest that the mitochondrial function of LNCaP was not affected by the treatment with ^{nat}In-DOTAGA-AO in our conditions. Importantly and similar to what happened with PSMA$_{617}$, PNT2 cells treated with ^{nat}In-DOTAGA-AO show a slight shift in the energetic profile increasing ECAR values overtime (Figure 16C).

Regarding long-term exposure, results depicted in Figure 17 evidence that PNT2 cells responded to long-term exposure ^{nat}In-DOTAGA-AO in the opposite way to short-term exposure. Thus, PNT2 cells treated with 50 µM ^{nat}In-DOTAGA-AO showed a significantly lower basal (Figure 17D), maximal (Figure 17E), ATP production-linked (Figure 17F), and proton leak-linked respiration (Figure 17G). Moreover, although the effects of 10 µM ^{nat}In-DOTAGA-AO are not significant, the compound seems to have a concentration-dependent effect. Treatment of PNT2 cells with 10 µM ^{nat}In-DOTAGA-AO also increases the coupling efficiency. These changes translate into a shift to quiescent PNT2 cells (Figure 17C). Interestingly, long-term exposure to ^{nat}In-DOTAGA-AO significantly affected the mitochondrial respiration of PCa cells, LNCaP. Thus, LNCaP presented a significant decrease in basal (Figure 17D), maximal (Figure 17E), ATP production-linked (Figure 17F), and proton leak-linked respiration (Figure 17G), as well as decreased spare respiration capacity (Figure 17H). Moreover, tumoral cells LNCaP treated with 50 µM ^{nat}In-DOTAGA-AO for 72 hours also presented a shift in their

energetic profile from aerobic to quiescent (Figure 17C), suggesting that the compound could have a beneficial effect to decrease cell metabolic activity although we did not observe any effect on cell proliferation at the same time point.

A Oxygen Consumption Rate

Oligomycin / FCCP / Rotenone/Antimycin A

PNT2 Control
PNT2 10 μM natIn-DOTAGA-AO
PNT2 50 μM natIn-DOTAGA-AO

LNCaP Control
LNCaP 10 μM natIn-DOTAGA-AO
LNCaP 50 μM natIn-DOTAGA-AO

Control
10 μM natIn-DOTAGA-AO
50 μM natIn-DOTAGA-AO

B Extracellular acidification rate

C Energy phenotype profile

Aerobic / Energetic / Quiescent / Glycolytic

PNT2 Control
PNT2 10μM natIn-DOTAAGA-AO
PNT2 50μM natIn-DOTAAGA-AO

LNCaP Control
LNCaP 10μM natIn-DOTAAGA-AO
LNCaP 50μM natIn-DOTAAGA-AO

D Basal respiration

E Maximal respiration

F ATP production-linked respiration

G Proton leak-linked respiration

H Spare respiration capacity

I Coupling efficiency

J Non-mitochondrial respiration

Figure 16. Impact of short-term exposure to ^{nat}In-DOTAGA-AO on mitochondrial oxygen consumption of PNT2 and LNCaP cells. The Seahorse XF^{e}96 Extracellular Flux Analyzer was used to measure Oxygen Consumption Rate (OCR) expressed as ρmol/min **(A)**, and Extracellular Acidification Rate (ECAR) expressed in mpH//min **(B)**, both normalized per cell mass of cells untreated (control) and treated with 10 μM and 50 μM ^{nat}In-DOTAGA-AO for 6 hours. **(C)** The values of the last OCR and ECAR rate measured before first injection were displayed as a scatter plot to assess the energetic profile. Several respiratory parameters were evaluated: **(D)** basal OCR; **(E)** maximal OCR; **(F)** ATP production-linked OCR **(G)** proton leak-linked OCR; **(H)** spare respiration capacity OCR; **(I)** coupling efficiency OCR; and **(J)** non-mitochondrial OCR. Data are represented as the mean ± SEM of 3-6 independent experiments in triplicate. The different conditions were compared to the control by Kruskal-Walli's followed by Dunn´s post-hoc test. Significant differences in PNT2 cells are marked by ¥ ($p < 0.05$).

A — Oxygen Consumption Rate

Oligomycin, FCCP, Rotenone/Antimycin A

OCR (pmol/min/cell mass) vs Time (minutes)

- PNT2 Control
- PNT2 10 μM ^{nat}In-DOTAGA-AO
- PNT2 50 μM ^{nat}In-DOTAGA-AO
- LNCaP Control
- LNCaP 10 μM ^{nat}In-DOTAGA-AO
- LNCaP 50 μM ^{nat}In-DOTAGA-AO

Control
10 μM ^{nat}In-DOTAGA-AO
50 μM ^{nat}In-DOTAGA-AO

B — Extracellular acidification rate

ECAR (mpH/min/cell mass) vs Time (minutes)

C — Energy phenotype profile

OCR (pmol/min/cell mass) vs ECAR (mpH/min/cell mass)

Aerobic, Energetic, Quiescent, Glycolytic

- □ PNT2 Control
- ○ PNT2 10μM ^{nat}In-DOTAAGA-AO
- △ PNT2 50μM ^{nat}In-DOTAAGA-AO
- □ LNCaP Control
- ○ LNCaP 10μM ^{nat}In-DOTAAGA-AO
- △ LNCaP 50μM ^{nat}In-DOTAAGA-AO

D — Basal respiration

OCR (pmol/min/cell mass) — PNT2 (¥¥), LNCaP (**)

E — Maximal respiration

OCR (pmol/min/cell mass) — PNT2 (¥), LNCaP (**)

F — ATP production-linked respiration

OCR (pmol/min/cell mass) — PNT2 (¥), LNCaP (**)

G — Proton leak-linked respiration

OCR (pmol/min/cell mass) — PNT2 (¥¥), LNCaP (**)

H — Spare respiration capacity

OCR (pmol/min/cell mass) — PNT2, LNCaP (**)

I — Coupling efficiency

OCR (pmol/min/cell mass) — PNT2 (¥), LNCaP

J — Non-mitochondrial respiration

OCR (pmol/min/cell mass) — PNT2, LNCaP

Figure 17. Impact of long-term exposure to ^{nat}In-DOTAGA-AO on mitochondrial oxygen consumption of PNT2 and LNCaP cells. The Seahorse XF^{e}96 Extracellular Flux Analyzer was used to measure Oxygen Consumption Rate (OCR) expressed as pmol/min **(A)**, and Extracellular Acidification Rate (ECAR) expressed in mpH//min **(B)**, both normalized per cell mass of cells untreated (control) and treated with 10 μM and 50 μM ^{nat}In-DOTAGA-AO for 72 hours. **(C)** The values of the last OCR and ECAR rate measured before first injection were displayed as a scatter plot to assess the energetic profile. Several respiratory parameters were evaluated: **(D)** basal OCR; **(E)** maximal OCR; **(F)** ATP production-linked OCR **(G)** proton leak-linked OCR; **(H)** spare respiration capacity OCR; **(I)** coupling efficiency OCR; and **(J)** non-mitochondrial OCR. Data are represented as the mean ± SEM of 3-6 independent experiments in triplicate. The different conditions were compared to the control by Kruskal-Walli's followed by Dunn´s post-hoc test. Significant differences in PNT2 cells are marked by ¥ ($p < 0.05$), ¥¥ ($p < 0.01$). Significant differences in LNCaP cells are marked by * ($p < 0.05$), ** ($p < 0.01$).

3.3.3 ^{nat}In-DOTAGA-AO increases the glycolytic function of PNT2 cells

To better understand how exposure to ^{nat}In-DOTAGA-AO may affect the metabolism of normal prostate cells and PCa cells, we proceeded with the Glycolytic stress test to assess the glycolytic rate of cells. Thus, PNT2 and LNCaP cells were treated with ^{nat}In-DOTAGA-AO (10 and 50 μM) for 6 hours (Figure 18) or 72 hours (Figure 19).

Results depicted in Figure 14 show that PNT2 cells treated with ^{nat}In-DOTAGA-AO have higher ECAR values over time compared to respective control. This alteration reflects in the glycolytic parameters in graphics B-D. Thus, we observed a significant increase in glycolysis (Figure 18B), glycolysis capacity (Figure 18C), and glycolytic reserve (Figure 18D) of PNT2 cells treated with 10 μM ^{nat}In-DOTAGA-AO. These same parameters are also increased in PNT2 cells treated with 50 μM ^{nat}In-DOTAGA-AO, although not significant. In contrast, the treatment with ^{nat}In-DOTAGA-AO did not induce significant alterations in the glycolytic function of LNCaP cancer cells, although we observed a slight tendency to decrease glycolytic parameters under 50 μM ^{nat}In-DOTAGA-AO treatment. Of note, experiments performed with LNCaP are preliminary and have a lower sample size (2 independent experiments), for which the variability is elevated. Thus, more assays are needed with this experimental condition.

Data represented in Figure 15 show the glycolytic activity of PNT2 and LNCaP cells after a long-term incubation with ^{nat}In-DOTAGA-AO. Results evidenced that the effect observed after 6 hours incubation with the compound is maintained after 72 hours. Thus, normal prostate PNT2 cells, 10 μM ^{nat}In-DOTAGA-AO for 72 hours induced a significant increase in glycolysis (Figure 15B), and glycolytic capacity (Figure 19C). Of

note, PCa LNCaP cells treated with 50 µM natIn-DOTAGA-AO evidence a slight decrease in some glycolysis parameters, namely glycolysis capacity (Figure 19C) and glycolytic reserve (Figure 19D).

Altogether, data suggest that natIn-DOTAGA-AO has an impact on the glycolytic rate of normal prostate PNT2 cells. In addition, although preliminary, our results reveal a promising effect of 50 µM natIn-DOTAGA-AO in tumoral-cells by decreasing some glycolysis parameters.
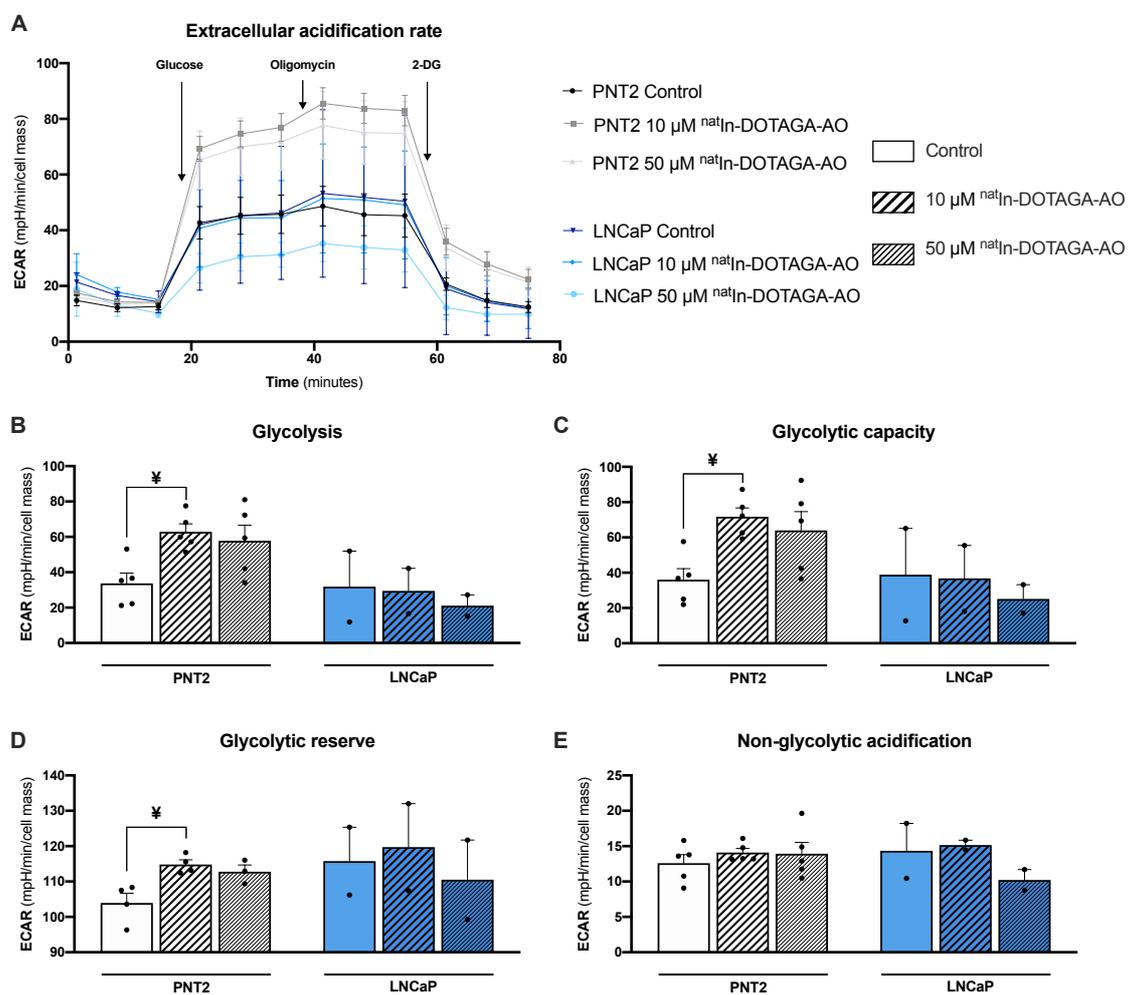
Figure 18. **Impact of short-term exposure to natIn-DOTAGA-AO on glycolytic function of PNT2 and LNCaP cells.** The Seahorse XFe96 Extracellular Flux Analyzer was used to measure Extracellular Acidification Rate (ECAR) expressed in mpH//min **(A)**, normalized per cell mass of cells untreated (control) and treated with 10 µM and 50 µM natIn-DOTAGA-AO for 6 hours. Several glycolytic parameters were evaluated: **(B)** glycolysis ECAR; **(C)** glycolysis capacity ECAR; **(D)** glycolytic reserve ECAR **(E)** and non-glycolytic acidification ECAR. Data are represented as the mean ± SEM of 2 or 3 independent experiments in triplicate. The different conditions were compared to the control by Kruskal-Walli's followed by Dunn's post-hoc test. Significant differences in PNT2 cells are marked by ¥ ($p < 0.05$).

Figure 19. Impact of long-term exposure to ^{nat}In-DOTAGA-AO on glycolytic function of PNT2 and LNCaP cells. The Seahorse XF^{e}96 Extracellular Flux Analyzer was used to measure Extracellular Acidification Rate (ECAR) expressed in mpH//min **(A)**, normalized per cell mass of cells untreated (control) and treated with 10 μM and 50 μM ^{nat}In-DOTAGA-AO for 72 hours. Several glycolytic parameters were evaluated: **(B)** glycolysis ECAR; **(C)** glycolysis capacity ECAR; **(D)** glycolytic reserve ECAR **(E)** and non-glycolytic acidification ECAR. Data are represented as the mean ± SEM of 2 or 3 independent experiments in triplicate. The different conditions were compared to the control by Kruskal-Walli's followed by Dunn's post-hoc test. Significant differences in PNT2 cells are marked by ¥ ($p < 0.05$).

3.3.4. Short-term ^{nat}In-DOTAGA-AO treatment increased basal and ATP production-linked respiration of PC3-FLU cells while long-term exposure to 50 μM ^{nat}In-DOTAGA-AO decreases mitochondrial function of cells overexpressing PSMA

Although the compound ^{nat}In-DOTAGA-AO does not have a tag to target the PSMA protein, we evaluated its potential off-target effects on the mitochondrial respiration of tumoral cells PC3-FLU (no PSMA expression) and PC3-PIP (PSMA

overexpression) cells. Once again, the impact of short- (Figure 16) and long-term (Figure 17) ^{nat}In-DOTAGA-AO (10 or 50 μM) treatment were assessed through the Mito stress assay.

Data depicted in Figure 20 indicate that PC3-FLU cells treated with both 10 μM and 50 μM evidenced a slight increase in basal (Figure 20D), maximal (Figure 20E), ATP production-linked (Figure 20F, $p < 0.05$ for 50 μM vs control), and proton leak-linked respiration (Figure 20G) as well as non-mitochondrial respiration (Figure 20J). On the other hand, treatment of PC3-PIP has no effect on mitochondrial respiration.

Then, we evaluated the long-term exposure to ^{nat}In-DOTAGA-AO, treating PC3-FLU and PC3-PIP cells with ^{nat}In-DOTAGA-AO (10 and 50 μM) for 72 hours. The results obtained are shown in Figure 21. Overall, ^{nat}In-DOTAGA-AO treatment appears to decrease mitochondrial respiration of treated cells. Contrary to what was observed in PC3-FLU cells treated with ^{nat}In-DOTAGA-AO for 6 hours the OCR values appear to decrease, with increasing the exposure time In addition, although there are no statistically significant results, all the parameters of mitochondrial respiration of PC3-FLU cells seem to be affected by ^{nat}In-DOTAGA-AO, with a strong decrease in basal (Figure 21D), maximal (Figure 21E), and ATP production-linked respiration (Figure 21F). Additionally, proton leak-linked respiration (Figure 21G), spare respiratory capacity (Figure 21H), coupling efficiency (Figure 21I) and non-mitochondrial respiration (Figure 21J) were slightly decrease. Interestingly, the long-term treatment of PC3-PIP cells significantly affect it mitochondrial respiration in a concentration-dependent manner and induce a switch from an energetic profile to a glycolytic one (Figure 21C). Thus, 50 μM ^{nat}In-DOTAGA-AO significantly decreased basal (Figure 21D), maximal (Figure 21E), ATP production-linked (Figure 21F), and proton leak-linked respiration (Figure 21G), as well as coupling efficiency (Figure 21I) in tumoral PC3-PIP cells.

Therefore, overall data suggest that ^{nat}In-DOTAGA-AO negatively affects the mitochondrial activity of PC3-PIP cells in a concentration- and time-dependent manner. Furthermore, data shows a shift in the metabolism of both PC3-FLU and PC3-PIP cells treated with 50 μM ^{nat}In-DOTAGA-AO, becoming more glycolytic mainly by reducing OXPHOS-dependent respiration.

A Oxygen Consumption Rate

Oligomycin   FCCP   Rotenone/Antimycin A

- PC3-FLU Control
- PC3-FLU 10 μM ^{nat}In-DOTAAGA-AO
- PC3-FLU 50 μM ^{nat}In-DOTAAGA-AO
- PC3-PIP Control
- PC3-PIP 10 μM ^{nat}In-DOTAGA-AO
- PC3-PIP 50 μM ^{nat}In-DOTAAGA-AO

Control
10 μM ^{nat}In-DOTAGA-AO
50 μM ^{nat}In-DOTAGA-AO

B Extracellular acidification rate

C Energy phenotype profile

Aerobic   Energetic

Quiescent   Glycolytic

□ PC3-FLU Control
○ PC3-FLU 10μM ^{nat}In-DOTAGA-AO
Δ PC3-FLU 50μM ^{nat}In-DOTAGA-AO

□ PC3-PIP Control
○ PC3-PIP 10μM ^{nat}In-DOTAGA-AO
Δ PC3-PIP 50μM ^{nat}In-DOTAGA-AO

D Basal respiration

E Maximal respiration

F ATP production-linked respiration

G Proton leak-linked respiration

H Spare respiration capacity

I Coupling efficiency

J Non-mitochondrial respiration

Figure 20. Impact of short-term exposure to ^{nat}In-DOTAGA-AO on mitochondrial oxygen consumption of PC3-FLU and PC3-PIP cells. The Seahorse XF^{e}96 Extracellular Flux Analyzer was used to measure Oxygen Consumption Rate (OCR) expressed as pmol/min **(A)**, and Extracellular Acidification Rate (ECAR) expressed in mpH//min **(B)**, both normalized per cell mass of cells untreated (control) and treated with 10 µM and 50 µM ^{nat}In-DOTAGA-AO for 6 hours. **(C)** The values of the last OCR and ECAR rate measured before first injection were displayed as a scatter plot to assess the energetic profile. Several respiratory parameters were evaluated: **(D)** basal OCR; **(E)** maximal OCR; **(F)** ATP production-linked OCR **(G)** proton leak-linked OCR; **(H)** spare respiration capacity OCR; **(I)** coupling efficiency OCR; and **(J)** non-mitochondrial OCR. Data are represented as the mean ± SEM of 3-6 independent experiments in triplicate. The different conditions were compared to the control by Kruskal-Walli's followed by Dunn´s post-hoc test. Significant differences in PC3-FLU cells are marked by # ($p < 0.05$).

3.3.5 Long-term ^{nat}In-DOTAGA-AO treatment increases PC3-FLU cells glycolytic reserve

Considering the switch in energetic profile in tumoral PC3-FLU and PC3-PIP cells observed in Figure 21, we performed the Glycolytic stress test on both cells under exposure to ^{nat}In-DOTAGA-AO. Figure 22 evidence no effect of 6 hours incubation with ^{nat}In-DOTAGA-AO in both PC3-FLU and PC3-PIP cells while Figure 23 show a significant increase of the glycolytic reserve of PC3-FLU cells after 72 hours treatment with 50 µM ^{nat}In-DOTAGA (Figure 23D). Thus, results suggest that changes in glycolytic profile obtained from the Mito Stress assay are not directly related with major changes in the glycolytic pathway but rather to changes in the OXPHOS respiration.

A PC3-PIP vs PC3-FLU - Oxygen Consumption Rate 72 hours

B Extracellular acidification rate

C Energy phenotype profile

D Basal respiration

E Maximal respiration

F ATP production-linked respiration

G Proton leak-linked respiration

H Spare respiration capacity

I Coupling efficiency
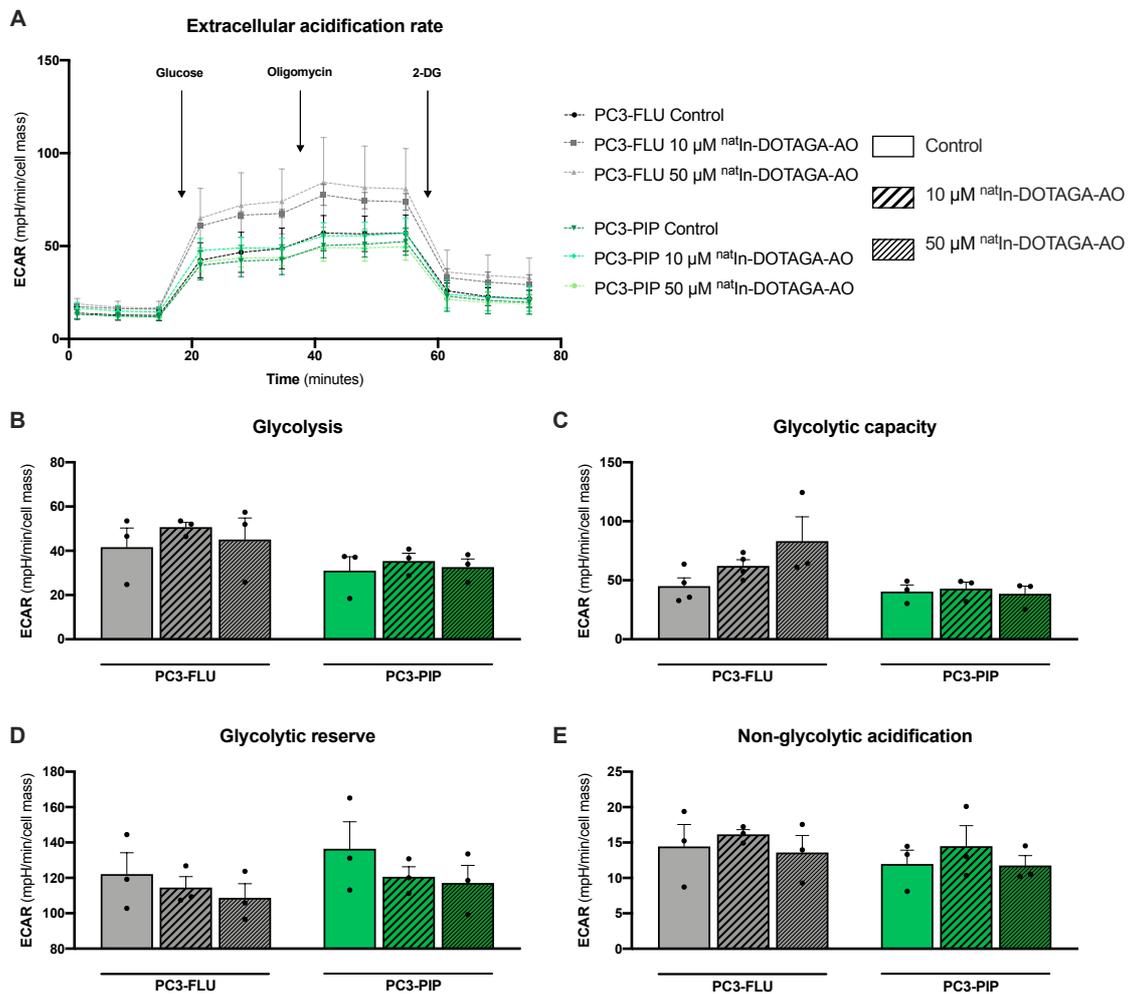
J Non-mitochondrial respiration
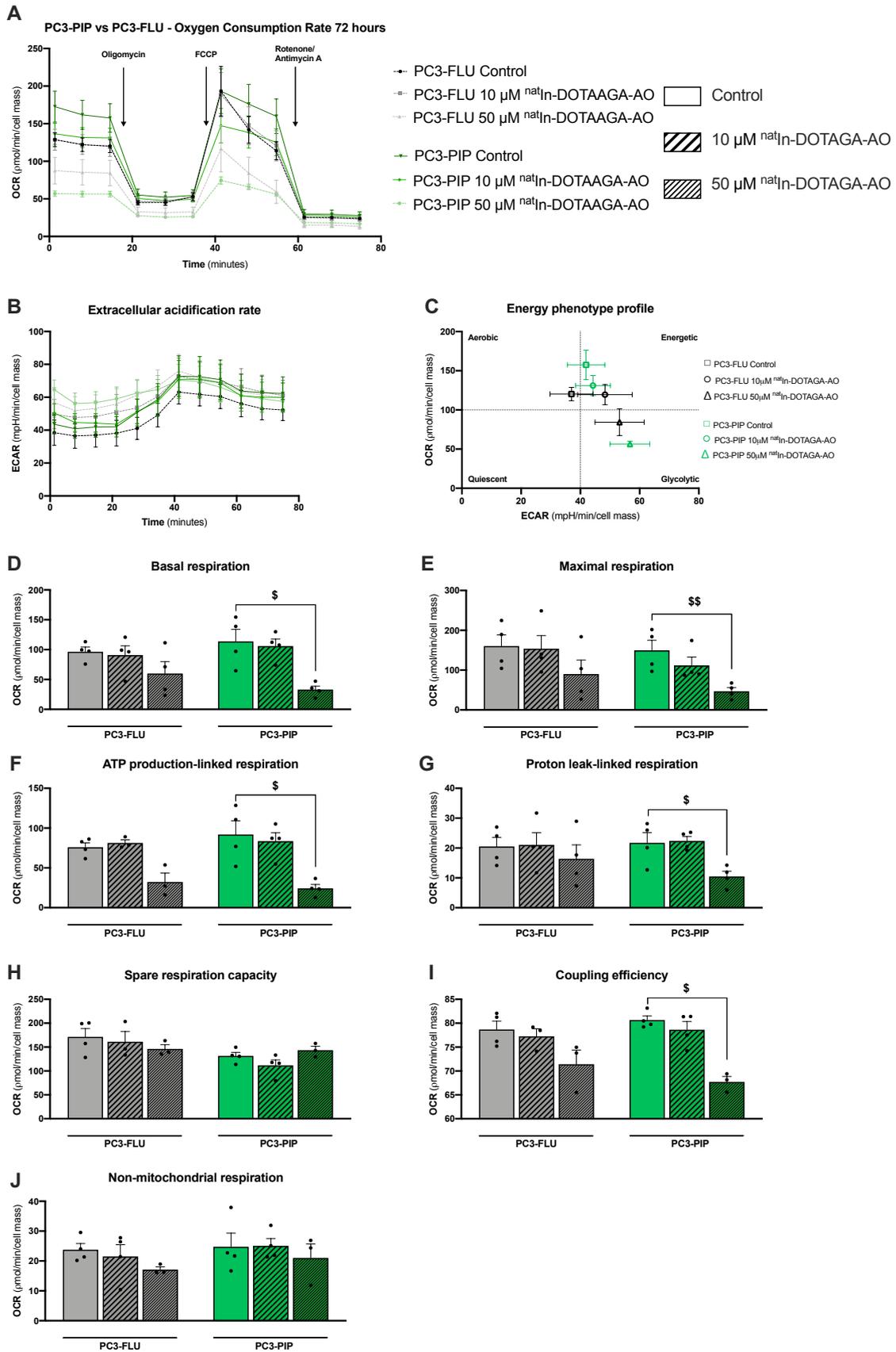
Figure 21. **Impact of long-term exposure to ^{nat}In-DOTAGA-AO on mitochondrial oxygen consumption of PC3-FLU and PC3-PIP cells.** The Seahorse XF^{e}96 Extracellular Flux Analyzer was used to measure Oxygen Consumption Rate (OCR) expressed as pmol/min **(A)**, and Extracellular Acidification Rate (ECAR) expressed in mpH//min **(B)**, both normalized per cell mass of cells untreated (control) and treated with 10 µM and 50 µM ^{nat}In-DOTAGA-AO for 72 hours. **(C)** The values of the last OCR and ECAR rate measured before first injection were displayed as a scatter plot to assess the energetic profile. Several respiratory parameters were evaluated: **(D)** basal OCR; **(E)** maximal OCR; **(F)** ATP production-linked OCR **(G)** proton leak-linked OCR; **(H)** spare respiration capacity OCR; **(I)** coupling efficiency OCR; and **(J)** non-mitochondrial OCR. Data are represented as the mean ± SEM of 3-6 independent experiments in triplicate. The different conditions were compared to the control by Kruskal-Walli's followed by Dunn´s post-hoc test. Significant differences in PC3-PIP cells are marked by $ ($p < 0.05$), $$ ($p < 0.01$).

Figure 22. **Impact of short-term exposure to ^{nat}In-DOTAGA-AO on glycolytic function of PC3-FLU and PC3-PIP cells.** The Seahorse XF^{e}96 Extracellular Flux Analyzer was used to measure Extracellular Acidification Rate (ECAR) expressed in mpH//min **(A)**, normalized per cell mass of cells untreated (control) and treated with 10 µM and 50 µM ^{nat}In-DOTAGA-AO for 6 hours. Several glycolytic parameters were evaluated: **(B)** glycolysis ECAR; **(C)** glycolysis capacity ECAR; **(D)** glycolytic reserve ECAR **(E)** and non-glycolytic acidification ECAR. Data are represented as the mean ± SEM of 2 or 3 independent experiments in triplicate. The different conditions were compared to the control by Kruskal-Walli's followed by Dunn´s post-hoc test.

Figure 23. Impact of short-term exposure to ^{nat}In-DOTAGA-AO on glycolytic function of PC3-FLU and PC3-PIP cells. The Seahorse XF^{e}96 Extracellular Flux Analyzer was used to measure Extracellular Acidification Rate (ECAR) expressed in mpH//min **(A)**, normalized per cell mass of cells untreated (control) and treated with 10 µM and 50 µM ^{nat}In-DOTAGA-AO for 72 hours. Several glycolytic parameters were evaluated: **(B)** glycolysis ECAR; **(C)** glycolysis capacity ECAR; **(D)** glycolytic reserve ECAR **(E)** and non-glycolytic acidification ECAR. Data are represented as the mean ± SEM of 2 or 3 independent experiments in triplicate. The different conditions were compared to the control by Kruskal-Walli's followed by Dunn´s post-hoc test. Significant differences in PC3-FLU cells are marked by # ($p < 0.05$).

3.3.6. ^{nat}In-DOTAGA-AO decreases the mitochondrial membrane potential of PNT2 cells while it increases the mitochondrial membrane potential of PC3-PIP cells

To uncover the differences in the mitochondrial function observed above, mitochondrial membrane potential was indirectly assessed using TMRM dye by confocal imaging after treatment with 50 µM ^{nat}In-DOTAGA-AO for 72 hours (Figure 24) Interestingly, data evidence that the compound induces a significant decrease in the

mitochondrial membrane potential of normal prostate PNT2 cells, and, in contrast, a significant increase in tumoral PC3-PIP cells. No differences were found in PCa LNCaP cells treated with μM ^{nat}In-DOTAGA-AO for 72 hours.

Figure 24. Impact of ^{nat}In-DOTAGA-AO treatment on mitochondrial network area and mitochondrial membrane potential. *(A)* Representative confocal images of PNT2, LNCaP, and PC3-PIP cells treated or not (control) with ^{nat}In-DOTAGA-AO for 72 hours obtained using TMRM and Hoechst for mitochondrial and nuclei labeling, respectively. Scale bar = 10 μm. (B) Area under the curve of single cell integrated density per area (from threshold 1-254). Data are expressed as mean ± SEM for 5 untreated PNT2 cells and 10 treate PNT2 cells; 8 untreated LNCaP cells and 3 treated LNCaP cells; 10 untreated PC3-PIP cells and 13 treated PC3-PIP cells, obtained from one single experiment. Each treated cells condition of each cell line was compared to the respective untreated cells condition. Statistical analysis was performed using the non-parametric test Mann-Whitney. Significant differences in PNT2 cells are marked by ¥¥¥ (p < 0.001). Significant differences in PC3-PIP cells are marked by $$$$ (p < 0.0001).
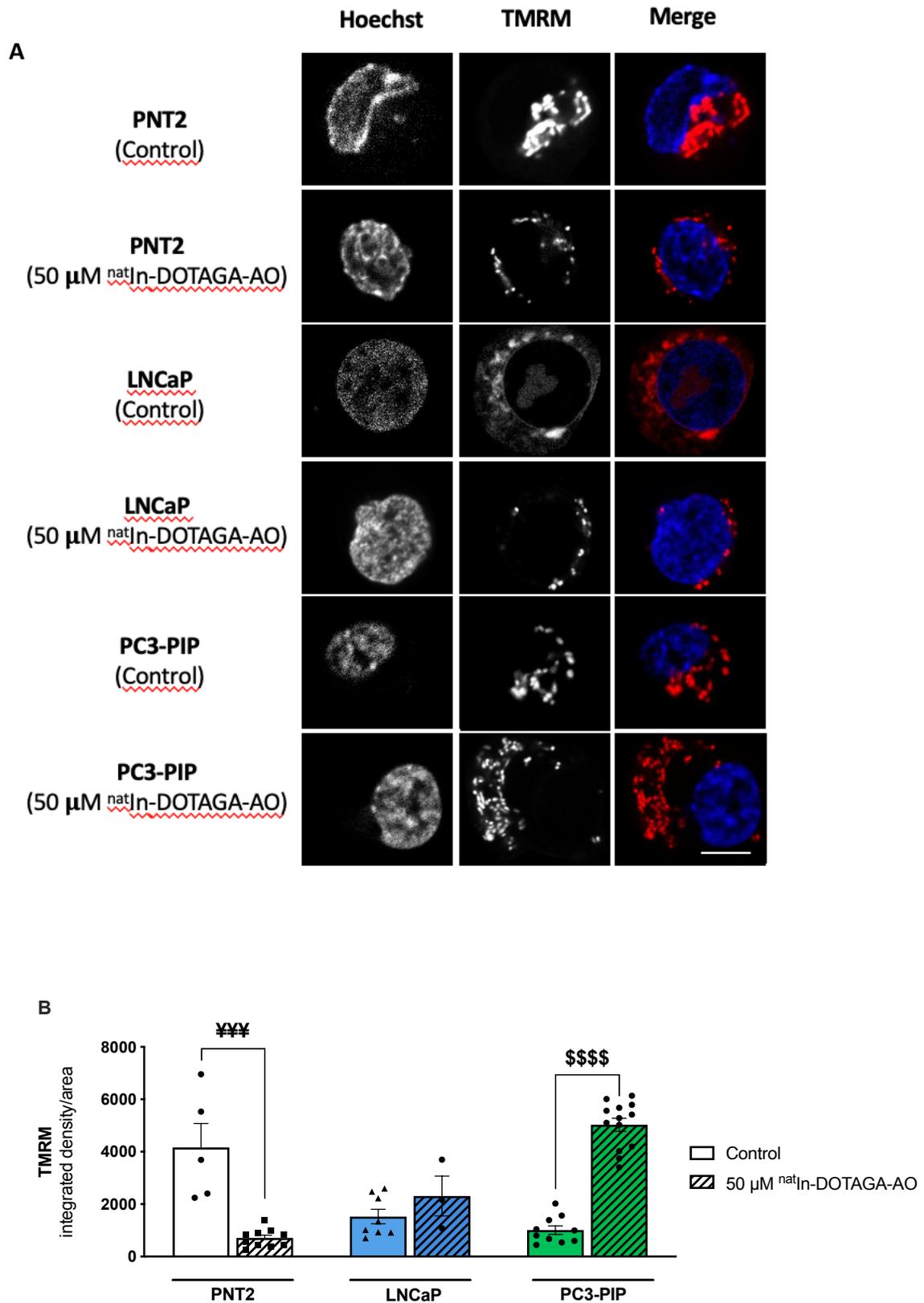
3.3.7 ^{nat}In-DOTAGA-AO treatment increased mitochondrial reactive oxygen species levels in LNCaP cells

Since our results evidence that long-term treatment with ^{nat}In-DOTAGA-AO induces a significant decrease in the mitochondrial respiration in both tumoral cell lines LNCaP and PC3-PIP cells, we evaluate if changes in mitochondrial respiration could be related with changes in mitochondrial superoxide levels. For that purpose, cells were treated with ^{nat}In-DOTAGA-AO (10 and 50 μM) for 72 hours and the mitochondrial superoxide anion levels were quantified using MitoSOX (Figure 25 and 26).

Graphics of the fluorescence variation along time (Figure 25 and 26, i) evidenced no major detectable changes during the 20 minutes of acquisition. Thus, we replotted the basal value of fluorescence for each condition on graphics ii of Figure 25 and 26. Normal prostate cells PNT2 show no significant difference in superoxide levels (Figure 25Ai), although there is a tendency to an increase. In tumoral cells, ^{nat}In-DOTAGA-AO induced a concentration-dependent significant increase in the levels of superoxide as depicted in Figure 25Bii (LNCaP), Figure 26 A and B ii (PC3-FLU and PC3-PIP, respectively). Therefore, results suggest that long-term exposure to 50 μM ^{nat}In-DOTAGA induce an increase in non-tumoral and tumoral cell lines.
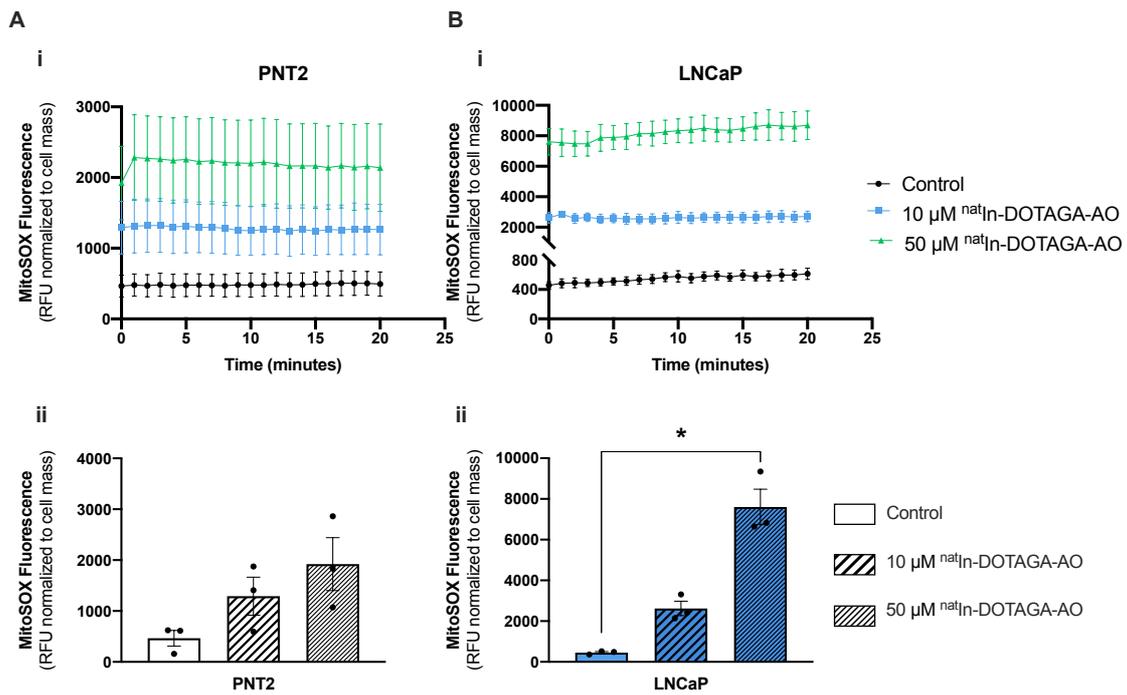
Figure 25. Impact of ^natIn-DOTAGA-AO treatment on mitochondrial superoxide anion production on PNT2 and LNCaP cells. (A) The fluorescent report molecule (MitoSOX) was used to measure the mitochondrial superoxide anion levels (over time) of PNT2 and LNCaP cells untreated (control) and treated with 10 µM and 50 µM ^natIn-DOTAGA-AO for 72 hours. **(B)** Basal mitochondrial superoxide anion levels. Data are represented as the mean ± SEM of 3 independent experiments in triplicate. The different conditions were compared to the control by Kruskal-Walli's followed by Dunn´s post-hoc test. Significant differences in LNCaP cells are marked by * ($p < 0.05$).
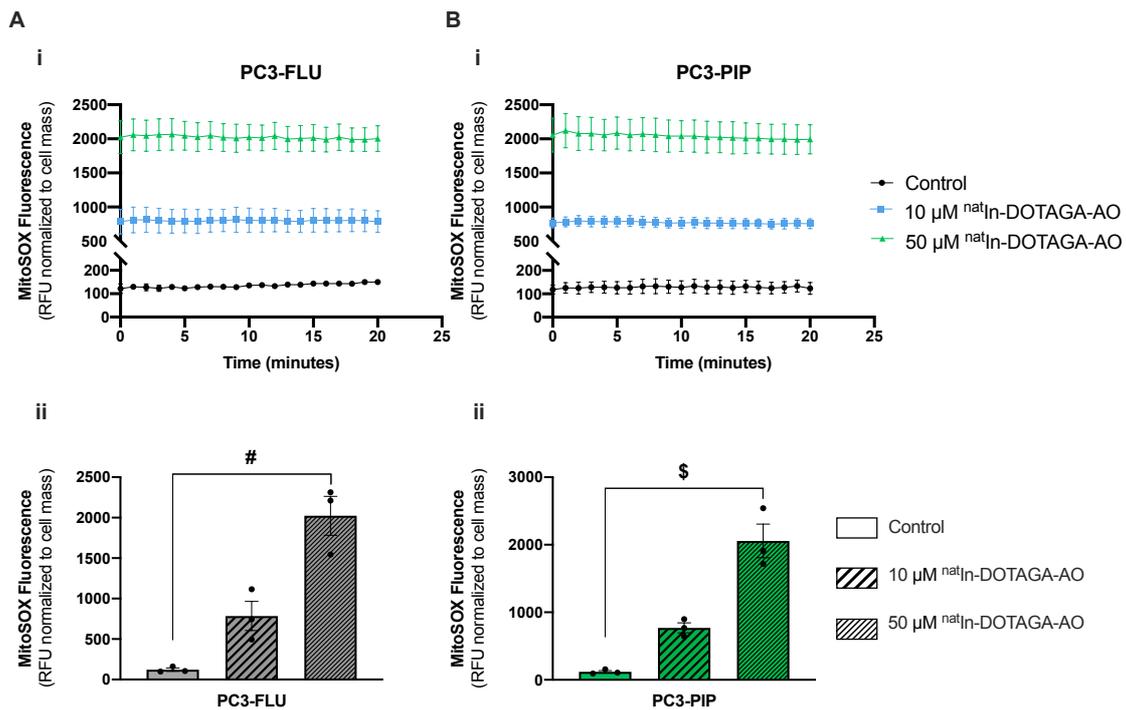
Figure 26. Impact of ^{nat}In-DOTAGA-AO treatment on mitochondrial superoxide anion production on PC3-FLU and PC3-PIP cells. (A) The fluorescent report molecule (MitoSOX) was used to measure the mitochondrial superoxide anion levels (over time) of PC3-FLU and PC3-PIP cells untreated (control) and treated with 10 µM and 50 µM ^{nat}In-DOTAGA-AO for 72 hours. **(B)** Basal mitochondrial superoxide anion levels. Data are represented as the mean ± SEM of 3 independent experiments in triplicate. The different conditions were compared to the control by a one-way ANOVA followed by Kruskal-Walli's post-test. Significant differences in PC3-FLU cells are marked by # ($p < 0.05$). Significant differences in PC3-PIP cells are marked by $ ($p < 0.05$).

4. Discussion

During the last decades, TRT has emerged as an efficient intervention for cancer treatment (Pouget et al. 2015; Chang T et al. 2020). TRT strategies involves the use of radiocomplexes comprising a high-affinity PCa cell-targeting ligand attached to therapeutic and diagnostic radionuclides which emit charged particles during decaying process. The specific targeting is achieved by intrinsic targeting properties of some radionuclides or by conjugating the radionuclide to a delivery molecule or vector, which specifically targets to biomarkers present in malignant tissue (Ramogida et al. 2013; Dekempeneer et al. 2016). For PCa, targeting PSMA has shown enormous potential since it is a transmembrane protein markedly overexpress on the cell surface of prostate malignant tissue (Uijen et al. 2021; Chang S 2004; Rahbar et al. 2018). Indeed, PSMA is an excellent target for both diagnosis and treatment of PCa, and it has been focused on several studies (Haberkorn et al. 2016; Muller et al. 2019). The most common strategies to target PSMA are an antibody-based modality that uses antibodies as targeting vectors, and approaches that use small-molecule enzyme inhibitors or binding agents (Haberkorn et al. 2016). Considering the antibody based TRT targeting to PSMA, several preclinical studies have been performed using different murine monoclonal antibodies (mAb), including mu7E11, muJ591, muJ533 and muE99, and humanized antibody huJ591 (Czerwinska et al. 2020). Of note, 7E11 mAb conjugated with capromab peptide (CYT-365) radiolabeled with the γ-emitter [111]In ([111]In-7E11/CYT-365), was the first radioimmunoconjugate designed and approved by the FDA for PCa patients (Kahn et al. 1994), followed by others (Czerwinska et al. 2020; Dev et al. 1996; do Pazo et al. 2021)

In 2000, the first α-emitting radionuclides-based therapy targeting PSMA, the radioconjugate [213]Bi-J591, was tested by McDevitt and colleagues *in vitro* (LNCaP cells) and in an *in vivo* model. The promising results of this first agent triggered new studies with antibodies conjugated with α-emitting radionuclides (Czerwinska et al. 2020; McDevitt et al. 200). Thus, in May 2021, Telix Pharmaceuticals started the phase III trial of a radiopharmaceutical targeting PSMA, TLX591 (^{177}Lu-DOTArosopatamab) (do Pazo et al. 2021). As previously mentioned, another strategy to target PSMA is using small-

molecule enzyme inhibitors or binding agents. So far, only three structures have been synthesized to target PSMA successfully: PSMA-11, PSMA$_{617}$ and PSMAI&T. These compounds are based on the same binding moiety that binds the enzymatic pocket of PSMA, inhibiting the glutamate carboxypeptidase activity of this protein (Miyahira et al 2018). In recent years, these compounds labeled with different radionuclides, namely α-, ß-, and AE-emitting radionuclides, have been used in numerous clinical studies (Czerwinska et al. 2020). In general, PSMA-11, PSMA$_{617}$ and PSMAI&T demonstrated high PSMA cellular specificity and fast pharmacokinetics, however, adverse effects have also been reported due to accumulated radioactivity in various off-target organs (Czerwinska et al. 2020). Additionally, in the last yeasrs, Novartis carries out three different phases III trial, one of them using ^{177}Lu-PSMA$_{617}$ [NCT 03511664] (do Pazo et al. 2021), which was recently approved by FDA for the treatment of PSMA-positive metastatic castration-resistant PCa (Novartis, March 2022)

In the AugerTher project, we aimed to develop novel TRT strategies based on AE-radionuclide emitters attached to PSMA$_{617}$ to ensure a specific internalization by PCa cells. The characterization of our cells evidenced that PNT2, a normal prostate epithelial cell line, did not express PSMA. Same results were observed with PC3-FLU cells which do not express PSMA. In contrast, LNCaP, derived from a lymph node metastatic prostate carcinoma, and PC3-PIP, transduced to overexpress PSMA showed PSMA positive labelling by Western blotting. Moreover, besides the 100 kDa band, which corresponds to PSMA, our Western blotting also detected a slight band at 200 kDa, as well as positive bands between 100 and 200 kDa. Liu et al. 2014 hypothesized that the labelling detected by Western blotting after incubation with an anti-PSMA antibody could be either from the cell surface PSMA or the vesicle PSMA, which is internalized into endosomes. Their data suggest that PSMA can be enriched in endosomes, exhibiting a higher content of glycosylation and partial proteolysis in comparison to cellular PSMA. (Liu T et al. 2014) In this sense, Yuan et al. 2022 evidenced that PSMA structure has numerous sites susceptible to glycosylation and identified several PSMA glycoforms in prostate human cell lines and prostate tissue, which, they believe, could be associated with different prostate cancer staging (Yuan W et al. 2022), an information that could be used to improve PCa diagnosis. Thus, the different bands that we observed only on

PSMA-expressing cells are probably labelling PSMA and reflect different cell phenotypes.

In our study, we initially assessed the cellular effects of the synthesized PSMA$_{617}$ compound and verified no toxicity in the used models. Indeed, we did not register any changes neither in cell mass nor in metabolic activity of cells treated with PSMA$_{617}$ for 72 hours. Additionally, we assessed the impact of short- and long-term exposure to PSMA$_{617}$ on the metabolism of normal prostate cells (PNT2) and PCa cells (LNCaP) through the Seahorse XFe96 Analyzer using the Mito stress test and Glycolytic stress test to assess mitochondrial- or glycolytic-associated parameters, respectively. Although we found no differences in the evaluated mitochondrial parameters in PNT2 cells, PSMA$_{617}$ treatment increased glycolysis, glycolytic capacity, and glycolytic reserve of cells treated with 50 μM PSMA$_{617}$ for 6 hours. Moreover, curiously, long-term treatment with PSMA$_{617}$ (10 and 50 μM) did not the glycolytic parameters of PNT2 cells, suggesting that these cells are sensitive to the treatment in a time-independent manner. Furthermore, our data showed that treatment with PSMA$_{617}$ in LNCaP cells only induced a decreased spare respiration capacity in cells treated with 10 μM PSMA$_{617}$ for 72 hours. In the parameters associated with glycolysis, neither the short- nor the long-term treatment induced significant changes in LNCaP cells. Additionally, data showed that normal prostate PNT2 cells produce energy predominantly through the glycolytic pathway and the PCa LNCaP are more aerobic cells, using mitochondrial respiration as main source of energy. In 1923, Otto Warburg found evidence that cancer cells rely heavily on glycolysis to generate ATP. Initially, it was thought that the mitochondria of cancer cells were dysfunctional and therefore the cells opted for the glycolytic pathway. However, it was later discovered that cancer cells produce ATP via glycolysis even though they have fully functional mitochondria and oxygen available, in which acid lactic fermentation favors the cancer cells proliferation in the hypoxia microenvironment (Payen et al., 2020; San-Millán & Brooks, 2017; Xu et al., 2015)). This shift in cellular energetics is known as the Warburg effect and it is not common to all cancer cells, being dependent on cell type and type of cancer (Ashton et al. 2018; Xu et al. 2015; Wallace et al 2012). Importantly, in the 1940s, Huggins and colleagues (Barron and Hugging et al. 1944) characterized prostate metabolism using isolated dog and rabbit prostate tissue and found that

normal prostate tissue relies on the glycolytic pathway instead of OXPHOS even under aerobic conditions. Following studies evidenced that peripheral prostatic epithelium has an androgen receptor-mediated metabolic program that favors the production, accumulation, and secretion of citrate into the prostatic fluid (Putluri et al. 2011; Bader and McGuire 2020). Outstanding, the citrate synthesis relies on the condensation of aspartate-derived oxaloacetate and glucose-derived acetyl-CoA in the mitochondrial matrix. In addition, zinc-mediated aconitase 2 inhibition prevents citrate oxidation, resulting in citrate production, which is coupled with a mandatory physiological truncation of the TCA cycle. Consequently, normal prostate tissue uses the glycolytic pathway to produce energy because it cannot produce reducing equivalents in the TCA cycle to power the ETC chain (Mycielska et al. 2009; Costello and Franklin 2016; Bader and McGuire 2020). In contrast, prostate adenocarcinoma relies on OXPHOS to produce energy. During the malignant transformation of normal prostate tissue, the physiological truncation of the TCA is abolished. Once again, the metabolic reprogramming is predominantly under androgen receptor control, which when activated drives to clinical progression of PCa by lipogenesis and OXPHOS enhancement (Costello and Franklin 2016; Bader and McGuire 2020). Moreover, in prostate adenocarcinoma, mitochondrial aconitase 2 is activated because of zinc depletion. Consequently, citrate is oxidized in the TCA cycle or exported to the cytoplasm for use as de novo lipogenic substrate (Costello and Franklin 2016; Bader and McGuire 2020; Mycielska et al. 2009). Thus, our results are in accordance with the literature. In fact, in basal or control conditions, normal prostate PNT2 cells produce energy mainly through the glycolytic pathway while the PCa LNCaP are more aerobic cells, using mitochondrial respiration as predominant source of energy.

PNT2 cells do not express PSMA, however, they are sensitive to PSMA$_{617}$. We sought to find whether the presence or absence of PSMA could play a role in the metabolic response of tumoral cells to PSMA$_{617}$ treatment. Thus, we used two tumoral cell lines derived from the PC3 cell line which do not express PSMA: the PC3-PIP cells, generated by transduction using VSV-G pseudotyped lentiviral vector expressing human PSMA to overexpress PSMA; and isogenic PSMA negative cells PC3-FLU. Data evidence that the tumoral cell line PC3-PIP is not sensitive to PSMA$_{617}$, while the cell line PC3-FLU

shows a concentration-dependent response to the compound, similarly to what happened with the LNCaP and PNT2 cells, respectively. These results suggest that cell lines without PSMA are more sensitive to $PSMA_{617}$ than cells expressing the PSMA protein which is surprising. Moreover, we found that untreated (control) PC3-FLU and PC3-PIP cells have both a predominantly aerobic metabolism, using OXPHOS as the main energy source. Curiously, $PSMA_{617}$ treatment induce a metabolic shift only in PC3-FLU cells which became more energetic (producing energy by both OXPHOS and glycolysis) in a concentration-dependent manner. The analysis of the glycolytic function of PC3-FLU and PC3-PIP cells treated with $PSMA_{617}$ evidenced no alterations, which is not surprising since PCa cells rely on OXPHOS to produce energy. Thus, our data suggest that the response of cells to $PSMA_{617}$ treatment is independent of whether cell has a normal or cancerous phenotype. Moreover, cells without PSMA protein seems to be more sensitive to $PSMA_{617}$ than cells expressing the protein.

The function of PSMA within the cell remains unclear (Emmett et al. 2017; Uijen et al. 2021). Moreover, so far, we do not know if there is any interaction between $PSMA_{617}$ compound and PSMA protein as well as if the compound is internalized. Xi Hong and colleagues performed metabonomic and transcriptomic analysis in PSMA-knockdown LNCaP and 22rv1 cell lines to assess the effect of PSMA on PCa cells transcription and modulation of metabolism. Interestingly, their results evidenced that PSMA knockdown induces metabolic disorder and abnormal transcription promoting the biosynthesis of arginine and proline in PCa and leading to cell proliferation and migration inhibition (Hong et al. 2022). Despite our study does not assess $PSMA_{617}$ – PSMA interaction or internalization of either the protein or the compound or the complex, we were able to observe in the case of PC3-PIP cells a tendency to decrease mitochondrial respiration-related parameters and a slight shift to a more quiescent energetic profile after long-term treatment with 50 μM $PSMA_{617}$. This result, although non-significant, suggest that treatment with $PSMA_{617}$ in cells expressing PSMA may lead to the internalization of PSMA protein leading to a decrease in cell metabolism which at long-term can lead to cell proliferation and migration inhibition.

In comparison to α- and ß-particles, AE generate low energy with subcellular range (< 100 nm), yielding high LET (4-26 keV) resulting in high radiotoxicity when

located close to radiosensitive targets with limited non-specific radiotoxicity to healthy neighboring cells (Czerwinska et al. 2020; Costa et al. 2021). Therefore, AE-emitting radionuclides are interesting alternative to the α- and ß-emitting radionuclides, and promising appealing tools for the treatment of micro metastasis and circulating tumor cells (Costa et al. 2021). Among therapeutic AE-emitters, several studies evidence ^{125}I and ^{111}In as potential radionuclides to consider for clinical translation (Bodei et al. 2003; Unverricht-Yeboah et al. 2020; Costa et al. 2021). So far, ^{111}In has been evaluated in both breast cancer and neuroendocrine tumors (Othman et al. 2020). Moreover, there is a commercially available ^{111}In radionuclide TRT (^{111}In-Pentetrotide; Phe-D-octreotide) which have an attached octreaotide analog for somatostatin receptor specific targeting (Knapp et al. 2016). The ^{111}In-Pentetrotide internalization by octreaotide analog – somatostatin receptor mediation and subsequent therapeutic effectiveness of intracellular AE emission has been demonstrated in both animal tumor models (de Jong et al. 1998) and human tumors *in vivo* (Krenning et al. 1996). Also, AugerTher project aim to develop novel dual-targeting ^{111}In radionuclides complexes with high-affinity to PCa (ensured by $PSMA_{617}$), and their organelles, namely the nucleus and mitochondria. Therefore, in the present study we performed preliminary work with the [nat]In-DOTAGA-AO. In this cold surrogate the native form of Indium ([nat]In) was stabilized by DOTAGA chelator and functionalized with AO, a fluorescent and cell-permeable probe commonly used for nuclear cell staining which intercalates DNA and RNA (Baskic et al. 2006; Agorastos et al. 2007). The binding of these probe to double-strand DNA interferes with important biological processes, namely DNA synthesis and gene transcription and translation (Nafisi et al. 2006). We observed a successful internalization of the compound in all the cell lines. Data evidenced a nuclear accumulation of [nat]In-DOTAGA-AO for the four cell lines, and we confirmed the mitochondrial accumulation in PNT2, LNCaP and PC3-PIP cells (PC3-FLU have not been tested). Of note, PCa LNCaP cells presented higher internalization of the compound, and seem to accumulate more compound in the mitochondria than in the nucleus.

When looking at [nat]In-DOTAGA-AO toxicity, data suggested that the compound did not induce cytotoxicity in PNT2, LNCaP, PC3-FLU and PC3-PIP cells. Moreover, data showed that short-term [nat]In-DOTAGA-AO treatment (10 μM) significantly increased

basal and maximal respiration of PNT2 cells, and although without significance, the respiration associated to ATP production. Curiously, treatment with 50 μM for the same period of exposure, only increased the coupling efficiency of LNCaP. In contrast, long-term treatment with 50 μM [nat]In-DOTAGA-AO treatment significantly increased basal and maximal respiration, as well as ATP production-linked and proton leak-linked respiration of PNT2 cells. Treatment with 10 μM [nat]In-DOTAGA-AO significantly increased the cellular coupling efficiency. Moreover, data showed that some of the evaluated glycolytic parameters were increased in PNT2 treated cells. In detail, short-term treatment with 10 μM [nat]In-DOTAGA-AO increased glycolysis, glycolytic capacity, and glycolytic reserve. Also, although no significant the same parameters seem to be slightly increased in PNT2 cells treated with 50 μM [nat]In-DOTAGA-AO for 6 hours. Long-term treatment induced an increased glycolysis and glycolytic capacity of PNT2 cells treated with 10 μM [nat]In-DOTAGA-AO. Interestingly, our Mito stress test data suggested a significant reduction in mitochondrial function of PNT2 cells, in a concentration-dependent manner, shifting the cells to a quiescent profile.

Furthermore, data showed that, although short-term [nat]In-DOTAGA-AO treatment (10 and 50 μM) did not induce alterations in the mitochondrial function of LNCaP cells, increasing the exposure time induced some cellular response of LNCaP. Indeed, long-term treatment with 50 μM markedly decreased basal, maximal, ATP production-linked, and proton leak-linked respiration, as well as the spare respiration capacity of LNCaP cells. In contrast, the evaluated glycolytic parameters were not altered in LNCaP treated cells. Interestingly, taken together, data evidenced a more quiescent profile of LNCaP, in a time- and concentration-dependent manner.

Regarding tumoral PC3 cells, PC3-FLU showed a slight increase in mitochondrial respiration-related parameters (basal and ATP production-linked respiration) after short-term treatment with [nat]In-DOTAGA-AO. Although long-term treatment did not induce significant alterations in PC3-FLU cells, data suggested a slightly decreased in all the assessed mitochondrial parameters in PC3-FLU cells treated with 50 μM [nat]In-DOTAGA-AO, similarly to results observed for non-tumoral PNT2 cells, also lacking PSMA protein. In PC3-PIP, which overexpress PSMA, data clearly showed that [nat]In-DOTAGA-AO negatively affects the mitochondrial respiration in a concentration- and time-

dependent manner. In detail, our data suggested that any mitochondrial parameter changed in PC3-PIP treated cells for 6 hours, and in contrast, long-term treatment with 50 μM natIn-DOTAGA-AO markedly decreased basal, maximal, ATP production-linked, and proton leak-linked respiration, as well as PC3-PIP coupling efficiency. Furthermore, Mito Stress assay evidenced a shift to a more glycolytic profile in treated PC3-PIP, was not proven by our Glycolytic Stress test since we did not find changes to support this hypothesis. Indeed, we only reported an increased glycolytic reserve of PC3-FLU cells treated with 50 μM natIn-DOTAGA-AO for 72 hours. Curiously, although the natIn-DOTAGA-AO compound is not expected to interact with PSMA protein, we reported similar changes in both non-tumoral and tumoral cells not expressing PSMA, suggesting that PSMA expression may affect the cellular response to the compound, but little is known in this regard. Importantly, the comparison of our data evidence that values of basal and maximal respiration, as well as mitochondrial respiration-associated ATP production and proton leak from tumoral cells (LNCaP, PC3-PIP and PC3-Flu) treated with 50 μM natIn-DOTAGA-AO, decrease to reach values similar to those of untreated non-tumoral PNT2 cells. PNT2 cells, which are predominantly glycolytic have a low mitochondrial function compared to tumoral cells, which undergo a switch to aerobic profile during malignant transformation becoming more aerobic (Costello and Franklin 2016; Bader and McGuire 2020). Thus, data suggest that 50 μM natIn-DOTAGA-AO treatment induce the tumoral cells to adopt a less aerobic energetic profile, similar to the glycolytic normal prostate cells.

The OXPHOS involves an electron flux that allows the pumping of protons from the mitochondrial matrix to the intermembrane space, producing a proton gradient. These protons return to the mitochondrial matrix through the ATP synthase, leading to ATP formation by ADP phosphorylation (Payen et al. 2020. Indeed, the basal mitochondrial respiration refers to hydrolyzed ATP in order to meet cellular demand. Moreover, maximal mitochondrial respiration is assessed by uncoupling the mitochondria respiration, which means that at this point, respiration is only limited by substrate oxidation (Sotolongo et al. 2020) Our findings, in general, suggested that natIn-DOTAGA-AO induced a decreased basal respiration in our cell lines, which means they are demanding less energy. Interestingly, this data agrees with our results that

suggested a shift in the energetic profile, with PNT2, LNCaP and PC3-PIP becoming quiescent (i.e., cells are not very energetic via either OXPHOS or glycolytic pathway). Moreover, our data reported significant decrease in maximal respiration of treated cells, suggesting that the limiting factor of in mitochondrial respiration of treated cells is the substrate oxidation in the ETC. Furthermore, this implies a reduction or even blockage of the pumping of protons from the mitochondrial matrix to the intermembrane space, so that the membrane potential does not favor the return of protons back to the mitochondrial matrix. Therefore, it is expected that consequently, there is a decrease in proton leak. Indeed, our data showed decreased proton leak-lined respiration with treated cells. Concomitantly, and consequently, there is a decreased in ATP production, as we observer measuring the ATP production-linked respiration. Taken together, our findings suggested that [nat]In-DOTAGA-AO may be uncoupling mitochondrial respiration by interfering/inhibiting ETC complexes. Since [nat]In-DOTAGA-AO binds mtDNA interfering with DNA synthesis and gene transcription and translation (Nafisi et al. 2006), it may be affecting the expression of mitochondrial proteins encoded by the mitochondrial genome, with essential function in mitochondrial processes, namely proteins from the ETC complexes.

Although our data are not conclusive, targeting OXPHOS elements, namely complexes or ATP synthase, is not something new. Indeed, several studies propose that inhibition of ETC (Yang Q et al. 2021; Basit et al. 2017) and inhibition of ATP synthase (Fiorillo et al. 2021) are potential strategies for the treatment of cancer. Yang and colleagues showed that the targeting of ETC complexes I and III limits the proliferation of liver cancer cells in cell lines, liver organoids, and xenografts *in vivo*. This restriction is associated to reduced ATP production, increased ROS levels, and induction of apoptosis (Yang Q et al. 2021). Also, using a panel of BRAF[V600E] melanoma cell lines, Basit and colleagues report that ETC complex I inhibition induce a dose-dependently decrease cell viability, and significantly increase the production of ROS, and reduction in cellular ATP levels (Basit et al. 2017). Moreover, Fiorillo and his team, found that Bedaquiline, an FDA-approved antibiotic which binds directly to ATP synthase gamma-subunit (ATP5F1C), induces mitochondrial ATP depletion in a time- and -concentration-

dependent manner, inhibiting spontaneous metastasis *in vivo*, without significant toxicity in MCD10A human cells *in vitro* or chicken embryos *in vivo* (Fiorillo et al. 2021).

Importantly, as we have been seen, a fundamental aspect for the proper functioning of mitochondria and respiration it the balance between in both the flow of electrons and the exchange of protons Thus, MMP reflects the functional mitochondrial status, and therefore, small changes in MMP values have a significant effect on mitochondrial energetics (DeHart et al. 2018; Distelmaier et al. 2008). Moreover, dysfunctional mitochondria and impaired respiration induce ROS production, namely mitochondrial superoxide, which result from electron leakage of ETC chain and their transfer to molecular oxygen (Fuhrmann et al. 2017). Although low levels of mitochondrial ROS may play a role in essential cell signaling pathways for maintaining cell homeostasis, exacerbated mitochondrial ROS production induce several deleterious effects, leading to mitochondrial dysfunction and ultimately cell death (Amorim et al. 2022). Importantly, mitochondrial membrane depolarization and increased ROS production, among other death signals, induce the release of cytochrome C, and other pro-apoptotic proteins (e.g., AIF, Smac and endonuclease G) as well, activating downstream the effector caspase 9 to carry out the apoptotic pathway (Burke P 2017; Zaib et al. 2022). Taking this in account, and in an attempt to better understand how [nat]In-DOTAGA-AO could be affecting our *in vitro* models, especially in terms of mitochondrial response, we assessed the MMP of PNT2, LNCaP and PC3-PIP treated cells (50 μM [nat]In-DOTAGA-AO; 72 hours) using TMRM dye by confocal imaging. Moreover, mitochondrial superoxide levels of PNT2, LNCaP, PC3-FLU, and PC3-PIP cells treated with [nat]In-DOTAGA-AO (10 and 50 μM; 72 hours) were assessed using MitoSOX. Our results evidenced that while the MMP of treated PNT2 cells decreased markedly compared to untreated cells (control), the MMP of PC3-PIP cells increases dramatically. Moreover, the MMP of treated LNCaP cells slightly increases although not statistically significant. Furthermore, data indicate that long-term exposure to 50 μM [nat]In-DOTAGA induces an increase in non-tumoral and tumoral cell lines mitochondrial ROS, with emphasis on the LNCaP cells for which presented much higher levels of mitochondrial ROS when compared to the other cell lines, in these conditions.

Importantly, data clearly indicated that the MMP of untreated normal prostate PNT2 cells is higher than the MMP of untreated tumoral cells, LNCaP and PC3-PIP. Moreover, 50 μM ^{nat}In-DOTAGA-AO in PC3-PIP and LNCaP cells increases the MMP values to values similar to untreated PNT2 cells. As we have seen with mitochondrial respiration parameters ^{nat}In-DOTAGA-AO treatment appears to induce a behavioral change in tumoral cells, which seem to adopt a cellular response similar to the non-tumoral counterparts.

Although cell death is associated with membrane depolarization with subsequent release of cytochrome c and activation of the apoptotic pathway (Burke P 2017; Zaib et al. 2022), hyperpolarization, as our data showed for tumoral LNCaP and PC3-PIP cells, can also lead to the same endpoint, cell deth. Interestingly, Hsiao et al. 2019 assessed the anti-cancer properties of honokiol, an active component of *Magnolia officinalis,* against bladder cancer; and found that honokiol-induced apoptosis is mediated by ROS accumulation and transient hyperpolarization of the mitochondria membrane (Hsiao et al. 2019). However, our results were not accompanied by decrease cell proliferation. Literature also suggest that mitochondrial hyperpolarization may occur due to a metabolic shift, from an aerobic energetic profile to a predominantly glycolytic metabolism that results in lactate production and subsequent cytosol acidification (Nguyen et al. 2019; Bhat et al. 2015); however, our results do not report evidence significative changes in glycolytic function of LNCaP or PC3-PIP cells. Importantly, the maximum duration of our treatments were 72 hours, and therefore, those events might occur in a different timeline, as the cellular response and adaptation to treatment may take longer.

5. Conclusion and future perspectives

The AugerTher project aim to synthetize dual-targeted (PCa and organelle) metal complexes attached to a PSMA ligand ($PSMA_{617}$) to ensure a specific internalization in PCa cells. Moreover, the structure of the compounds would be functionalized with other ligands, a DNA intercalator (AO) or a mitochondriotropic group (TPP^+) to direct the pharmacophore to the nucleus or mitochondria, respectively.

In the present study, we aim to assess whether $PSMA_{617}$ alone and ^{nat}In-DOTAGA-AO could induce some effects on viability and cellular metabolism of PCa. Our findings suggests that the response of cells to $PSMA_{617}$ alone do not induce toxicity to the cells but are accompanied by small alterations in mitochondrial and glycolytic function. Furthermore, results suggest that $PSMA_{617}$ treatment is independent of whether cell has a normal or cancerous phenotype. Interestingly, we evidence that $PSMA_{617}$ alone induce changes in cells that do not express PSMA. Moreover, results, although non-significant, suggest that treatment with $PSMA_{617}$ in cells expressing PSMA may lead to the internalization of PSMA protein leading to a decrease in cell metabolism which at long-term can lead to cell proliferation and migration inhibition. On the other hand, ^{nat}In-DOTAGA-AO which internalizes within cell nucleus and mitochondria do not induce toxicity in our *in vitro* models. However, ^{nat}In-DOTAGA-AO induce changes in mitochondrial and glycolytic function, MMP, and mitochondrial ROS levels which tend to revert cancerous phenotype. Thus, our study evidence that ^{nat}In-DOTAGA-AO, without being fully functionalized have a significant impact on cells, supporting the development of this compound functionalized with the ligands $PSMA_{617}$ and TPP^+ to target PCa cells and their mitochondria, respectively; as well as the radioactive isotope of ^{111}In instead of the native form.

This work is the first steps of a major project and a necessary preliminary work to support the production of an anti-cancer therapy. We expect to further assess whether the $PSMA_{617}$ interacts with PSMA protein with subsequent internalization, using for instance a labelled $PSMA_{617}$ to assess the distribution and co-localization of the

compound through confocal microscopy. Although the treatment of cells with ^{nat}In-DOTAGA-AO for 72 hours revealed no toxicity, we reported several effects on cells, namely in terms of remodeled cell bioenergetics. Therefore, it would be important to increase treatment time in cell viability assays. Moreover, it would be interesting to assess the effects reported in this study in more detail. Due to the decreased mitochondrial function and it related parameters, we should assess the mitochondrial content and mitochondrial network morphology through confocal imaging, using Hoechst and TMRM for nuclei and mitochondria labeling, respectively. Additionally, it would be interesting to evaluate the expression levels of proteins related to mitochondrial dynamics and bioenergetic remodeling namely fusion proteins (e.g., MFN1, MFN2 and OPA1) and fission proteins (e.g., Drp1 and Fis1), by western blotting or immunocytochemistry. The process of mitophagy could also be assessed using live imaging and a plasmid construct (mitoKeima). Finally, we could assess the protein expression levels of endogenous ROS/RNS-scavengers such as SOD, Prx, GPxs catalase, by western blotting.

The future perspectives of AugerTher project include synthetize compounds initially with reactive isotopes, such as ^{nat}In (in the future with AE-emitting radionuclides as ^{111}In), functionalized with PSMA$_{617}$ and TPP^{+} to ensure targeting to PCa cells and their mitochondria, respectively. Further, it will be necessary to assess the biological effects of these new/re-structured compounds in 2D (e.g., PNT2, LNCaP, PC3, DU145, 22rv1 cell lines) and 3D multicellular spheroids *in vitro* models, and in *in vivo* models such as C57BL/6J-congenic Pten^{flox}.

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