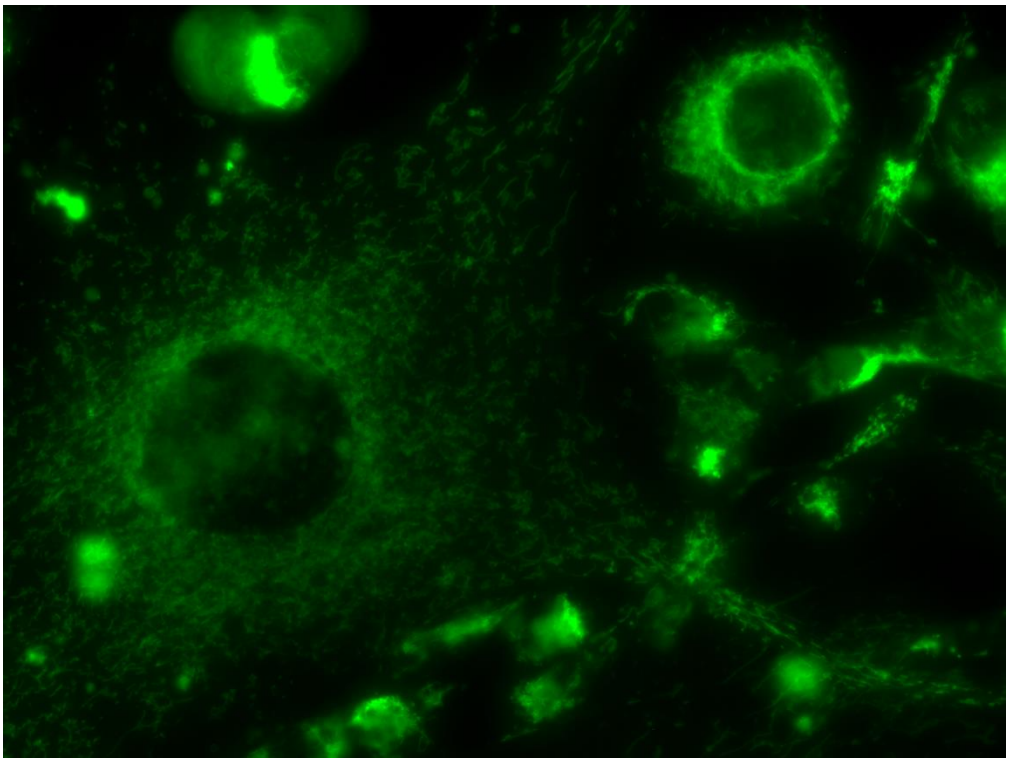
Manipulation of the Mitochondrial Network as a Therapeutic Strategy for Multidrug Resistant Triple-Negative Breast Cancer



Pseudònim: Mr. Mitochondria

Abstract

Triple negative breast cancer (TNBC) has the highest mortality rate within the first 3 to 5 years of diagnosis among all the breast cancer subtypes. Recent studies have shown that TNBC can acquire chemo-resistance and, as a result, multidrug resistance (MDR) is the major cause of therapeutic failure with TNBC [1]. Moreover, it has been shown that mitochondrial fusion is altered in MDR cancer cells, allowing them to resist apoptosis. In this research, MDR TNBC cells were treated with an anti-MFN2 peptide which inhibited mitochondrial fusion. The anti-MFN2 peptide was encapsulated in liposomes in order to achieve optimal delivery. These cells were then treated with BAM7, Shikonin and PTX drugs which induced cell death. The anti-MFN2 loaded liposomes were very effective in inhibiting mitochondrial fusion and sensitized MDR TNBC cells to chemotherapeutic treatments. In the near future, this treatment could potentially be used as a second line therapy in order to eradicate MDR cancers.

Keywords: Triple negative breast cancer; Multidrug resistance; Mitochondrial fusion; Anti-MFN2; Liposomes; Second line therapy

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El càncer de mama triple negatiu (TNBC) presenta una taxa de mortalitat molt alta en els primers 3-5 anys de diagnòstic comparat amb tots els subtipus de càncer de mama. Estudis recents han demostrat que el TNBC pot adquirir resistència a tractaments quimioteràpics i, com a resultat, aquesta resistència és la causa principal del fracàs terapèutic amb el TNBC [1]. A més, s'ha demostrat que els mitocondris en les cèl·lules canceroses amb resistència a la quimioteràpia tenen una distribució molt diferent, fet que podria provocar aquesta resistència a la mort cel·lular programada (apoptosis). En aquesta investigació, les cèl·lules TNBC resistents han estat tractades amb el pèptid anti-MFN2, inhibidor de la fusió mitocondrial, encapsulant-lo en liposomes per aconseguir un lliurament òptim del nostre tractament a les cèl·lules d'interès. Consegüentment, a aquestes cèl·lules se les ha tractat amb fàrmacs quimioteràpics com el BAM7, Shikonin i PTX que indueixen la mort cel·lular. Els resultats ens mostren que els liposomes carregats amb anti-MFN2 són molt eficaços per inhibir la fusió mitocondrial i per sensibilitzar les cèl·lules TNBC als

tractaments quimioteràpics. En un futur proper, aquest tractament podria ser utilitzat com a teràpia de segona línia per eradicar els càncers amb resistència a la quimioteràpia.

Paraules clau: Càncer de mama triple negatiu; Resistència a la quimioteràpia; Mitocondris; Anti-MFN2; Liposomes; Teràpia de segona línea.

Personal motivation

The adventure started about two years ago when I received a scholarship to enter one of the most renowned science programs in Catalonia for secondary school students, Joves i Ciència. It consists of spending three different summers doing research in laboratories with professional investigators who have been working on ground-breaking projects. This opportunity allowed me to get really deep into the world of science and start developing my skills as a scientist.

Last summer, I participated in the 2019 Research Science Institute (RSI) summer program, which gave me the opportunity to collaborate between and among leaders in the global community. RSI is one of the most prestigious international summer research programs in the world for high school students. Being able to participate at this highly considered program definitely made a switch in my life. I had the pleasure to work under the direction of Dr. Lara Milane and Dr. Amiji Mansoor at the Pharmaceutical Science lab in Northeastern University (Boston). I am very glad about the research I did for a few weeks. I have always been passionate about science and its procedures. Hypotheses, investigations, materials, methods, new techniques, results, analysis, errors, soldiering on, more results, conclusions... and new discoveries achieved! It was thrilling!

Since I was a little kid, I had always been intrigued by one of the most lethal diseases on Earth, cancer. I dreamed more than once that I was working in a lab trying to figure out how to cure this disease and save millions of lives. Those weeks back in July felt like my dream was coming true. The over-arching question that I was trying to answer is the following: Can we manipulate the mitochondrial network in cancer as to increase the effectiveness of chemotherapy? Currently, when I think of the amazing experience I had in Boston, I cannot avoid a big smile on my face.

As Stephen Hawking once said, science is not only a disciple of reason but also, one of romance and passion. Scientific research is my passion and one of my life goals is to contribute as much as I can to the scientific community in order to make the world a better place.

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Introduction

According to the Global Cancer Report issued by the World Health Organization (WHO), there are over 10 million new cases of cancer each year and over 7.9 million annual deaths from the disease [2]. Breast cancer is the most commonly occurring cancer in women and the second most common cancer overall. It is estimated that worldwide over 508000 women die every year due to breast cancer [23]. There are many different types of breast cancer. The type of tissue where your breast cancer arises determines how the cancer behaves and what treatments are most effective. Currently, one of the medical goals in breast cancer therapy is the early detection in order to improve its outcome and survival. In this research I have focused on triple-negative breast cancer (TNBC), which accounts for approximately 15 percent of breast cancers diagnosed worldwide, which amounts to almost 200,000 cases each year [24]. TNBC is one of the most aggressive forms of cancer and difficult to treat, especially as the cancer becomes drug resistant. Nowadays, the lack of targeted therapies for TNBC remains one of the biggest challenges in cancer therapy. Chemotherapeutic drugs continue to be the primordial treatment for patients with TNBC [25]. Recent studies have shown that drug resistant TNBC cells show high levels of mitochondrial fusion, which is the physical merging of the outer and the inner membrane of two distinct mitochondria, and this process results in blocking the programmed cell death [26]. Therefore, we hypothesize that mitochondrial hyperfusion is a common phenotype in multidrug resistant cancer cells and, if we inhibit this fusion, they will be sensitized to chemotherapeutic drugs that will kill the cells and eradicate the tumor. In summary, the goal of this project is to manipulate mitochondria in TNBC cells as a therapeutic approach.

Theoretical Background

Triple-negative breast cancer, a lethal disease

Triple-negative breast cancer (TNBC) is a breast cancer subtype that has a poor prognosis; within the first 3 to 5 years of diagnosis, the mortality rate is the highest of all the breast cancer subtypes [3, 4]. TNBC cells differ from other breast cancer subtypes in that they have a lack of estrogen receptors, progesterone receptors, and HER2 receptors [5]. Therefore, common treatments such as hormone therapy and specific drugs that target these receptors are ineffective, making TNBC a very aggressive disease and a tough clinical challenge [6]. For example, think of the cancer cell as if it was a house. In order to get inside the house and destroy the cancer cell 3 locks on the front door must be bypassed: estrogen (ER), progesterone (PR) and HER2. If the cancer cell tests positive for any of these 3 locks, which are known as receptors, then doctors have a few keys they can use to get inside the cell to destroy it. However, if you have TNBC, those locks are not there, the keys doctors usually use will not work. Moreover, it has been shown that the metastatic potential in triple-negative breast cancer is similar to that of other breast cancer subtypes, but TNBC tumors are associated with a shorter median time to relapse. In summary, TNBC has attracted more attention both clinically and experimentally because of its high-risk biological characteristics and lacking of effective treatment methods.

Chemotherapy remains the backbone of therapy for TNBC. However, recent studies have shown that TNBC can acquire chemoresistance [1]. Although many types of cancers are initially susceptible to chemotherapy, over time they can develop resistance through these and other mechanisms, such as DNA mutations and metabolic changes that promote drug inhibition and degradation. It has become evident that the development of TNBC chemoresistance is multifaceted and based on the elaborate interplay of the tumor microenvironment, drug efflux, cancer stem cells, and bulk tumor cells (**Fig. 1**). Moreover, TNBC's high heterogeneity, highlighted in the existence of several molecular signatures, presents a significant obstacle to

successful treatment [5]. Chemo-resistant cancers are known as multidrug resistant cancers (MDR). This concept was first considered when bacteria became resistant to certain antibiotics, but since then similar mechanisms have been found to occur in other diseases, including cancer.

It goes without saying that multidrug resistance (MDR) remains a major cause of therapeutic failure of TNBC and it necessitates further research and treatment development. Because improvements in targeted therapies are an urgent unmet medical need in the TNBC patient population, it is necessary to increase our understanding of the complexity of this disease at the genomic, molecular, and biological levels so that therapeutic targets might be identified, and more effective treatments developed. Resistance of cancer cells to treatment induced apoptosis is one of the biggest obstacles in cancer therapy that has to be overcome.

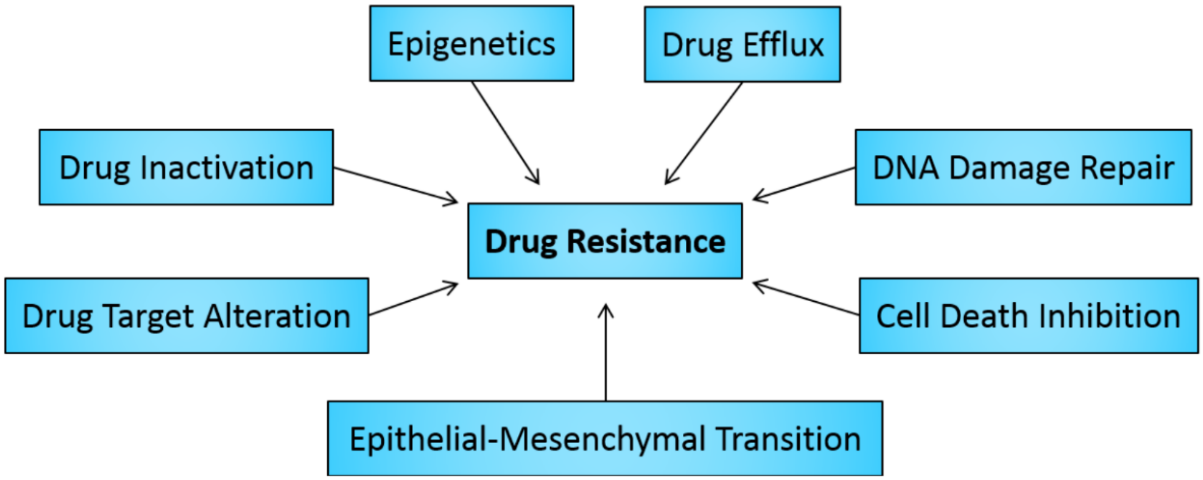

Figure 1: **Categories of mechanisms that can enable or promote direct or indirect drug resistance in human cancer cells.** These mechanisms can act independently or in combination and through various signal transduction pathway[27].

Hypoxia: multidrug resistance inducible factor

One of the factors that induces MDR to TNBC cells is tumor hypoxia [7]. The role of hypoxia in the phenomenon of MDR has been acknowledged for at least 60 years. Hypoxia is a common feature in intensively proliferating and expanding tumor tissues, where oxygen demand is surpassed by oxygen supply, and the distance between cells and the existing vasculature increases, hampering oxygen diffusion and creating an even more hypoxic environment (**Fig. 2**) [8]. While hypoxia is lethal for many cells, a subpopulation of tumor cells is able to not only adapt to hypoxic conditions but also become resistant to chemotherapy.

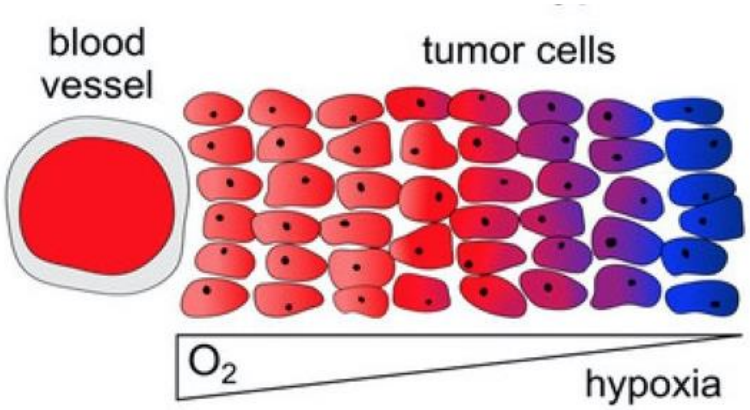

Figure 2: **Effect of hypoxia in a tumor microenvironment.** Blue cells are under hypoxic conditions as they cannot reach the blood stream and, thus, they live in a very low concentration of oxygen in that region of the tumor microenvironment. Figure adapted from Barbara Muz, et al. [8].

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Hypoxia confers treatment resistance of cancer cells by i) inducing the cell cycle arrest, ii) inhibiting apoptosis, iii) controlling mitochondrial activity and iv) acquiring chemoresistance by affecting drug delivery and drug efflux pump expression as well as by the lack of oxygen required for the cytotoxicity of the chemotherapeutics [8]. Furthermore, hypoxia regulates tumor vascularization, metabolism, cell survival, and cell death.

Oxygen concentration in humans ranges between approximately 9.5% O2 to 4.6% O2. When oxygen is abundant, normoxic (normal) cells are sensitive to chemotherapy due to “oxygen

fixation", which happens when oxygen molecules react with free radicals in DNA generated by ionizing radiation leading to irreversible DNA damage and killing the cell. However, oxygen level in hypoxic tumor tissues is poorer than the oxygenation of the respective normal tissues and on average it is between 1%–2% O_2 and below. In experimental settings, hypoxic conditions are considered when the cells are incubated in hypoxia between a few hours and as long as several weeks.

Cells irradiated in hypoxic conditions are resistant to death, due to decreased production of DNA radicals (which can be restored "chemical restitution") caused by reduced generation of ROS and decreased DNA damage. Hypoxia plays a crucial role in the process of cancer progression. And it is one of the main factors that induces MDR. It has been previously demonstrated that hypoxic cells are more aggressive and invasive with better ability to metastasize [9]. Therefore, novel therapeutic strategies for targeting and eradicating MDR TNBC cells are required.

Mitochondria, much more than the powerhouse of the cell

In most cell biology lectures in high school, students are always told that mitochondria are the "power-house" of the cell. Hence, not only are these organelles static cellular energy producers, but also crucial for many other cellular processes. Mitochondria are in charge of ATP production, thermal regulation, they contain and produce reactive oxygen species (ROS), as well as regulating the intrinsic apoptotic pathway. Moreover, they assist with intracellular and extracellular trafficking as well as cell movement, immunity, inflammation, stress response, and cell danger response. Furthermore, evolutionary tracking through mitochondrial DNA is crucial, as well as actively engaging with other organelles, performing diverse functions, profusely distributed, and maintaining cellular homeostasis.

Mitochondria morphological adaptations are crucial for many cellular processes such as cell cycle, immunity, apoptosis, and mitochondrial quality control. Mitochondria are highly dynamic organelles that undergo coordinated cycles of fission and fusion, referred to as 'mitochondrial dynamics', in

order to maintain their shape, distribution and size (**Fig. 3**). Recent studies have shown that mitochondrial activity can be altered in MDR cancer cells in order to resist apoptosis. Moreover, they can also adjust their bioenergetic and biosynthetic needs to support tumor initiating and transformation properties including proliferation, migration, and therapeutic resistance [10]. Dysregulations of mitochondrial dynamics with associated resistance to apoptosis plays a crucial role in several types of human cancer.

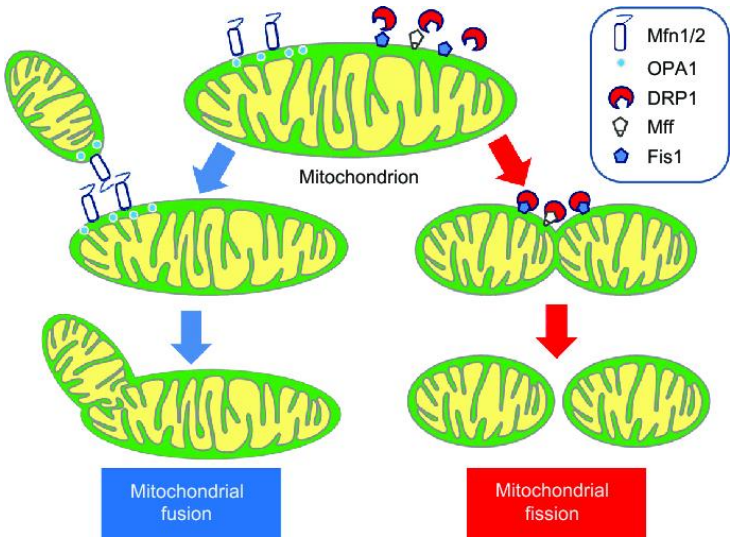

Figure 3: **Mitochondrial dynamics.** Mitochondrial fusion and fission occur as part of the mitochondrial dynamics. Mitofusins 1 and 2 (MFN1/2) play a crucial role in mitochondrial fusion while Dynamin Related Proteins 1 (DRP1) are in charge for splitting mitochondria apart [11].

Mitofusin 2 (MFN2) is an outer mitochondrial membrane GTPase that is critical for mitochondrial fusion, which in turn affects mitochondrial dynamics, distribution, quality control, and function [13]. MFN2 is localized at both the outer membrane of mitochondria and the endoplasmic reticulum and is particularly enriched at specialized contact regions known as mitochondria associated membranes (MAM). It has been shown that dysregulation of the mitochondrial network can have an effect on MFN2 proteins, provoking mitochondrial hyperfusion and a multidrug resistant phenotype (**Fig. 4**) [14]. Studying the mechanisms of mitochondrial function during tumorigenesis will be critical for the next generation of cancer therapeutics.

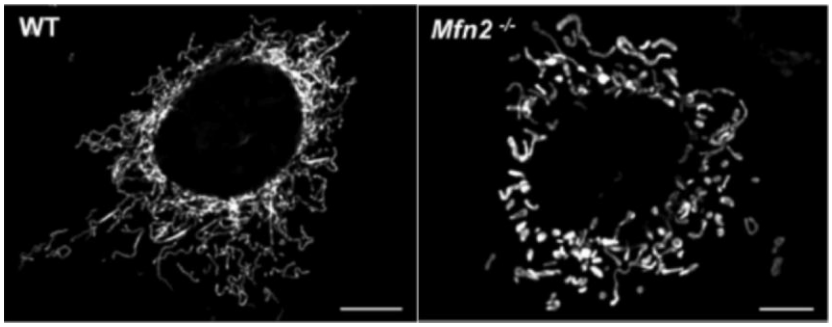

Figure 4: **Mitochondrial network hyperfusion.** Respresentative confocal microscopy images of wild type (WT) mitochondrial network which is disperse and thin whereas when MFN2 protein causes hyperfusion, enlarged structures of a variable size can be seen. Scale bar: 5 µm. Figure adapted from Gianluca Sgarbi, et al. [15].

A novel therapeutic strategy

A new therapeutic strategy combining traditional chemotherapy with an anti-MFN2 peptide (MFN2 inhibitor) was designed and tested in order to eradicate multidrug resistant TNBC cells. TNBC cells were treated in both normoxic and hypoxic conditions, using hypoxia as a tool for creating MDR cells. We hypothesized that mitochondrial hyperfusion is one of the main mechanisms that makes cells resistant to traditional chemotherapy treatments. Hence, inhibiting mitochondrial fusion will sensitize the cells to chemotherapy drugs such as BAM7, Shikonin or PTX. BAM7 results in functional BAX activation to the mitochondrial outer membrane, which activates the intrinsic apoptotic pathway and releases cytochrome 3 [16]. Shikonin induces necroptosis in cancer via regulating the expression of RIP1/RIP3 on the cell membrane (**Fig. 5**) [16]. PTX hyper-stabilizes microtubules structure, and, consequently, this destroys the cell's ability to use its cytoskeleton in a flexible manner, leading to cell death [16].

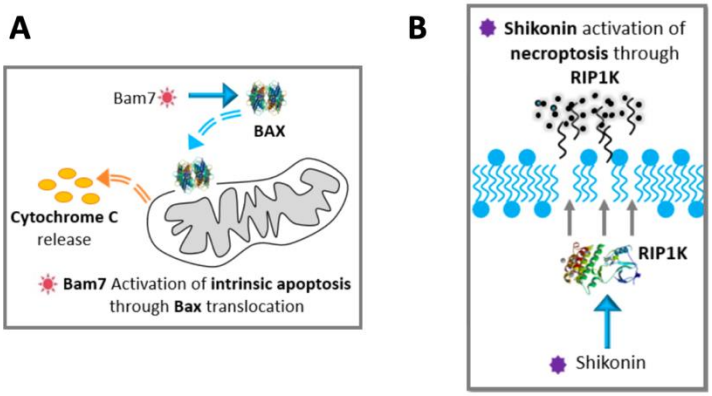

Figure 5: **BAM7 and Shikonin mechanisms of action.** A) Activation of the intrinsic apoptotic pathway through BAX activation. B) Shikonin activation of necroptosis through RIP1K.

In order to inhibit mitochondrial hyperfusion, anti-MFN2 peptide was used, which is a peptide that binds to the mitochondria membrane MFN2 proteins to prevent them from binding together [12]. Anti-MFN2 peptide is highly sensitive to enzymes that are present in the environment. Therefore, we encapsulated it in liposomes in order to achieve optimal delivery into cells. Liposomes are very stable spherical vesicles having at least one lipid bilayer that will protect anti-MFN2 from degrading (**Fig. 6**). Finally, in order to investigate changes in the mitochondrial network in response to drug treatments, an evaluation was performed using the Mitochondrial Network Analysis (MiNA) software.

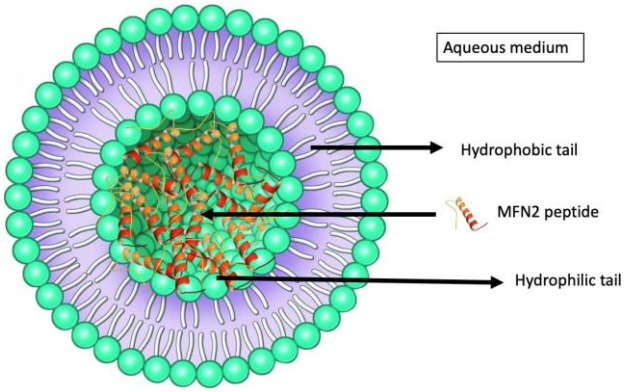

Figure 6: **Composition of our lipid nanoparticle.** Anti-MFN2 peptide is hydrophilic, hence, it was encapsulated in the hydrophilic tail of the peptide.

Materials and Methods

Cell culture

Human TNBC cells (MDA-MB-231) were maintained as instructed by distributor protocols and cultured in DMEM containing 4500 mg/L glucose, 4 mM l- glutamine and 1 mM sodium pyruvate and further supplemented with 10% fetal bovine serum, 2X MEM containing non-essential amino acids, and penicillin(50 I.U./mL)/streptomycin (50 g/mL) solution. All cell lines were grown within an incubator containing a humidified, 37°C, atmospheric O_2 and 5% CO_2 environment, unless otherwise indicated. For experiments involving hypoxia exposure, cells were placed within a humidified hypoxic chamber that was purged with 95% N2, 5% CO_2 gas mix until O_2 levels reached 0.5%. The chamber was sealed and kept within a humidified, 37°C, atmospheric O_2 and 5% CO_2 incubator.

Microscopy and MiNA analysis

To harvest TNBC cells, 5 mL of trypsin were added into T150 flasks where hu- man breast cancer cells were previously cultured. Once cells detached, they were transferred into 50 mL falcon tubes and mixed with 10 mL of media. A centrifugation at 2700 RPM was performed for 5 min. The pellet was resuspended in 10 mL of medium. 500 µL of the cell suspension were mixed with 20 mL of medium and distributed among 2 mL Petri dishes. 0.75 µL of 250 µM MitoTracker™ Green FM (Thermo Scientific) were added to each of the 2 mL dishes and were left for 45 min in order for the dye to stain mitochondria. Mitochondrial network images were obtained using BZ-X700 All-in-One Fluorescence Microscope (Keyence Corporation of America), they were then processed using Fiji software (ImageJ) in order to achieve different perspectives and pathways to identify and do previous MiNA analyses of the mitochondrial network in different cell treatments and conditions (**Fig. 7**). After pre-MiNA analysis, they were processed using the Mitochondrial Network Analysis (MiNA) toolset, composed by two ImageJ Macro Tools, one for batch analysis and another for processing a single image or region of interest (ROI). This allowed us to do semi-automated analysis

of mitochondrial networks for TNBC cells.

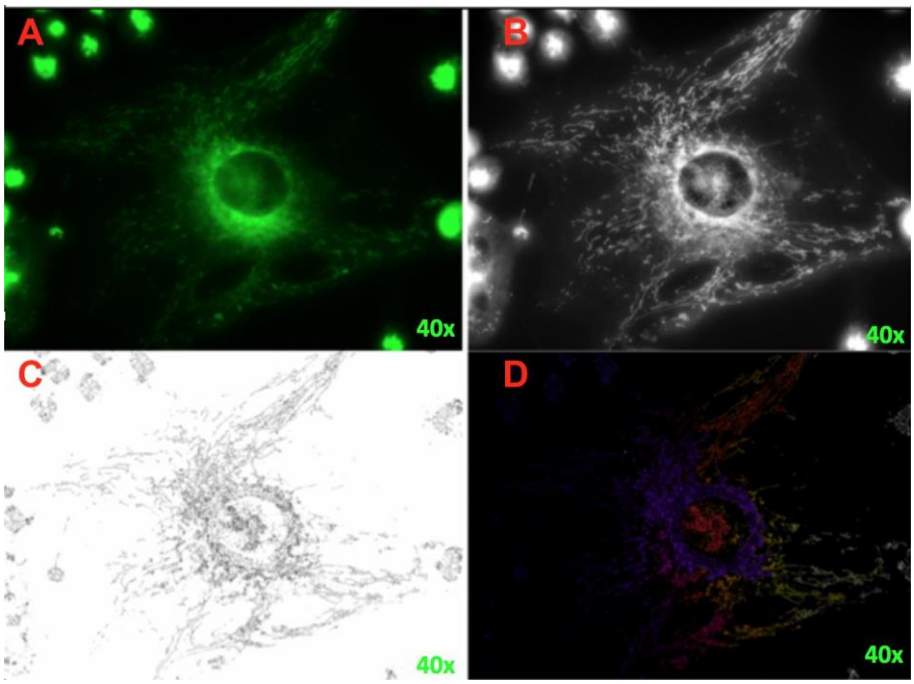

Figure 7: **Pre-MiNA analysis.** Image A) shows green fluorescent stained mitochondria. It was then processed and converted into a B) binary image C) skeletonized image and D) regional network connections image [17].

Liposome preparation

Liposomes were used for encapsulating our different samples of interest: 2xanti-MFN2 peptide (QDRLKFIDKQGELLAQDYKLR), 2x scrambled sequence (DFQRKALL- LYLDIERDKKGQQ), 2x anti-MFN2 with FITC fluorophore and 2x blank liposome preparations. In order to prepare the stock solution for anti-MFN2, scrambled sequence and anti-MFN2 + FITC were diluted at 1 mg of peptide in 200 µL and then 800 µL of PBS were added [18].

Lipid cake

The lipid film was prepared using DOTAP (1,2-dioleoyl-3-trimethylammonium- propane chloride salt) a cationic lipid, cholesterol (stabilizer), and DPPC (1,2- dipalmitoyl-sn-glycero-3-phosphocholine) a neutral lipid in a 5:3:5 molar ratio. For the lipid film, a stock concentration of each lipid was made in chloroform and 1 mL of each lipid was added to a Sigma-Aldrich ST/NS14/20 10 mL round bottom flask attached to a Rotavap (IKA works Inc. Wilmington, NC-28405, Model RV 10 C S99), and allowed to rotate at 100 rpm in a water bath at room temperature (RT).

Rehydration

Following chloroform evaporation, the thin lipid film at the bottom of the flask was dried overnight in vacuum to remove the solvent, and the film was subsequently hydrated with 1 mL of the previously prepared stock samples of anti- MFN2, scrambled sequence, FITC and blank (PBS and DI water), followed by additional vortexing for 1 min.

Freeze-thaw cycles

After rehydration of the lipid films, 4 of the samples were kept on ice for 2 min, vortexed, placed in a water bath at 37°C for 2 min, and vortexed again. Five such freeze-thaw cycles were performed. A second method was performed for the remaining samples in order to optimize our freeze-thaw cycles and evaluate which was the most efficient. After vortexing for 30 s, the flasks were attached to a Rotavap (IKA works Inc. Wilmington, NC-28405, Model RV 10 C S99), and allowed to rotate at 100 RPM in a water bath at 42°C for 5 min. Samples were frozen in liquid nitrogen and put into a 42°C hot bath. Five such cycles were completed.

Sonication

The liposomal preparation was then probe-sonicated for 5 min on ice. The mixture was centrifuged in a Beckman-Coulter ultracentrifuge at 20,000 RPM for 15 min at 4°C to separate the peptide-encapsulated liposomes from unencapsulated peptide.

Encapsulation efficiency

Encapsulation efficiency was determined with the use of an indirect method. In the indirect method, the amount of protein in the supernatant obtained after centrifugation was measured at 490 nm using a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific) for proteins and it was subtracted from the starting concentration of the peptide to get the total encapsulated peptide in liposomes.

Liposome sizing and Zeta potential

The particle size of liposomes was determined using a dynamic light scattering technique (Zetasizer Nanoseries ZS, Malvern Instruments). 1 µL of the liposomes preparation were diluted in 1 mL of DI water and placed in disposable cuvettes. Then, they were inserted into the measurement chamber, and allowed to equilibrate to 25°C for 5 min. After that, liposomes were sized. Z-potential values of the empty and loaded MLV were measured in order to investigate the effect of drug loading on liposomal surface charge. For this, liposome dispersions werediluted with DI water and Z-potential values were measured at 25°C, by laser Doppler spectroscopy (Zetasizer 5000 Malvern Instruments, UK).

Cell viability MTS assay

Cells were counted using a haemocytometer and 10,000 cells per well in the 96-well plates were plated. Each column of the different plates was treated with different conditions. After 48 h of

incubation with the different treatments, CellTiter 96® AQueous One Solution Reagent was thawed for 10 min in a water bath at 37ºC. 20 µl of CellTiter 96® AQueous One Solution Reagent were pipetted into each well of the 96-well assay plate containing the samples in 100 µl of culture medium. The plate then incubated at 37°C for 1 to 4 hours in a humidified, 5% CO2 atmosphere. The absorbance was recorded at 490 nm using Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek). The statistical analysis was per- formed with Gen5 2.0 software and graphs were then generated.

Protein analysis

Culture medium was carefully removed from adherent cells. 200 µL of M-PER Reagent were added to each of the wells of the 6-well plates. They were shaken gently using a Spindrive Orbital Shaker Platform (Bel-Art) and a Stirrer/Hot Plate (Corning) for 10 min. The lysate was collected and transferred to microcentrifuge tubes. Samples were centrifuged at 14,000 x g for 5-10 min to pellet the cell debris. The supernatant was transferred to a new tube for analysis. Protein con- centration was calculated using a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific).

Results

Liposome encapsulation, sizing and zeta potential

Blank liposomes, which were used as our negative control, had a low concentration of the peptide in the supernatant and an encapsulation efficiency of 0%, as expected (**Table 1**). The liposomes encapsulated with anti-MFN2 peptide had an average encapsulation efficiency of 75.25%. The freezing on ice method (MFN2 1) was more effective than the freezing in liquid nitrogen method (MFN2 2), with an encapsulation efficiency of 77.7%. Liposomes encapsulated with our scrambled sequence achieved an optimal encapsulation efficiency of 99.8% and 101.8%. Anti-MFN2 peptide with the FITC fluorophore showed negative values and more than 100% encapsulation efficiency.

Sample	Supernatant concentration (mg/ml)	Liposomes concentration (mg/ml)	Encapsulation efficiency %
Blank 1	0.012	0	0
Blank 2	0.021	0	0
MFN2 1	0.223	0.777	77.7
MFN2 2	0.272	0.728	72.8
Scramble 1	0.002	0.998	99.8
Scramble 2	-0.018	1.018	101.8
FITC 1	1.878	-0.878	-87.8
FITC 2	1.609	4.57	457

Table 1: **Encapsulation efficiency of anti-MFN2 peptide in liposomes** Encapsulation efficiency of anti-MFN2, the scrambled sequence and FITC were obtained by subtracting the supernatant concentration from the peptide initial concentration (1 mg/ml). Samples are labeled as 1) freezing on ice method and 2) freezing in liquid nitrogen method.

MFN2 loaded liposomes had an average size of 142.2 nm (**Fig. 8**). The three different samples showed an unimodal size distribution meaning that all of our vesicles were of a consistent size. A small peak of large vesicles appeared at around 5800 nm, probably representing liposome aggregates. The Z-potential was calculated in order to determine the liposomes' toxicity, which measured the charge difference between the surface of the vesicles and the colloidal suspension. Anti- MFN2 liposomes had a Z-potential of 22.2 mV, meaning they are biocompatible.

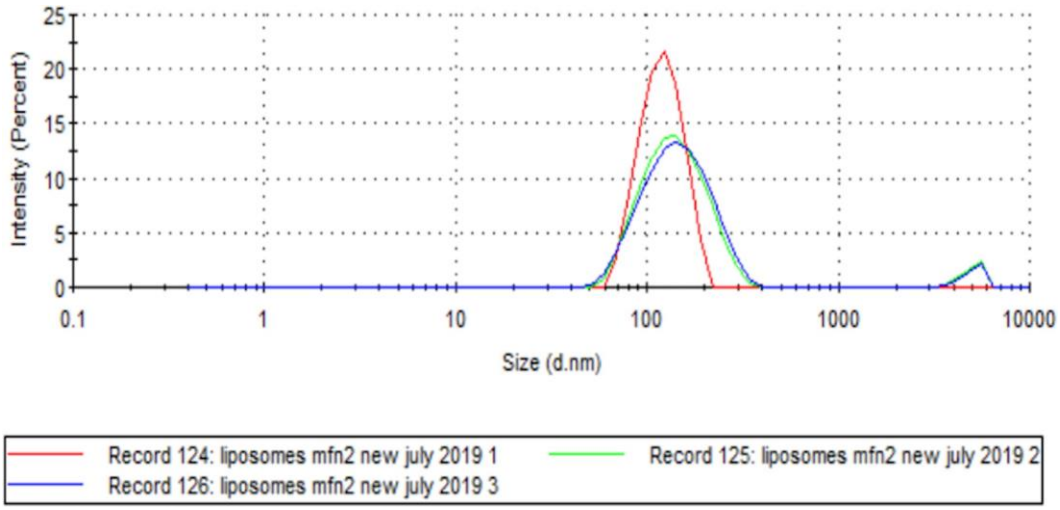

Figure 8: **Vesicles size distribution.** Three different runs were performed in order to determine the average size of our vesicle. Unimodal peaks appeared at 142.2 nm and smaller peaks arose at 5800 nm.

Pre-MiNA analysis

Mitochondria stained with MitoTracker Green are seen all around the nucleus creating the mitochondrial network **(Fig. 9)**. Between normoxic cells alone and normoxic cells with the peptide in solution there was not a significant difference. There were substantial network connections in both conditions. After 5 days in hypoxic conditions, cells showed a very strong staining in the endoplasmic reticulum. Hence, mitochondria were hyperfused to this organelle as a sign of MDR. After 3 days of hypoxia, cells treated with liposomes showed notable differences compared to untreated cells. Instead of seeing big networks like in the untreated cells, there were fewer aggregates and mitochondria were less fused together.

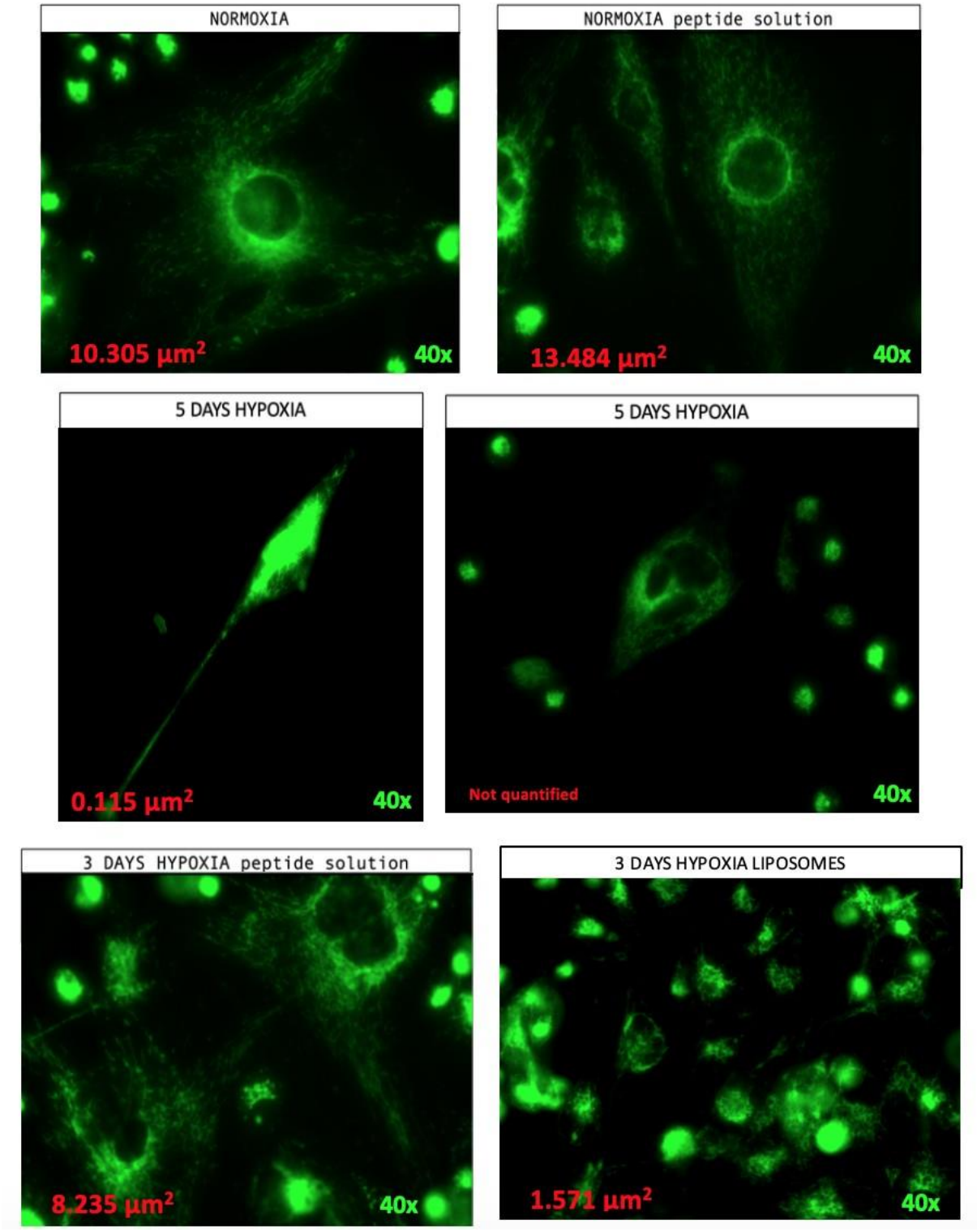

Figure 9: **Pre-MiNA imaging analysis** Mitochondrial network in different conditions: normoxia, 3 days hypoxia, 5 days hypoxia, anti-MFN2 peptide in solution and anti-MFN2 peptide in liposomes. Images show green fluorescent stained mitochondria. Values are showing network count to network size ratio μm^2 calculated using MiNA.

Mitochondrial Network Analysis

MiNA was used to calculate the network count to network size ratio (NCNS) which quantitatively measured the average number of networks per unit of network observed. The NCNS ratio in normoxic cells and normoxic cells with the peptide in solution were similar, as seen in pre-MiNA images. In fact, normoxic cells with the peptide in solution had a greater NCNS ratio **(Fig. 10)**. However, when comparing 3 days hypoxic cells with 3 days hypoxic cells with the peptide in solution, there was a slight decrease. 5 days hypoxic cells show a significant decrease due to mitochondrial hyperfusion with the endoplasmic reticulum (ER), creating large network connections as seen in pre-MiNA images. There was an extensive transformation from 3 days hypoxia to 5 days hypoxia. Due to hyperfusion with the ER, MiNA failed to quantitatively analyses the NCNS ratio in 5 days hypoxia. We were expecting a higher NCNS ratio than in 3 days hypoxia due to mitochondrial hyperfusion, which leads to a higher number of networks. Cells treated with liposomes in both normoxic and hypoxic conditions had a very low NCNS ratio compared to treatments with the peptide in solution, meaning that our anti-MFN2 in liposomes could effectively break mitochondrial networks.

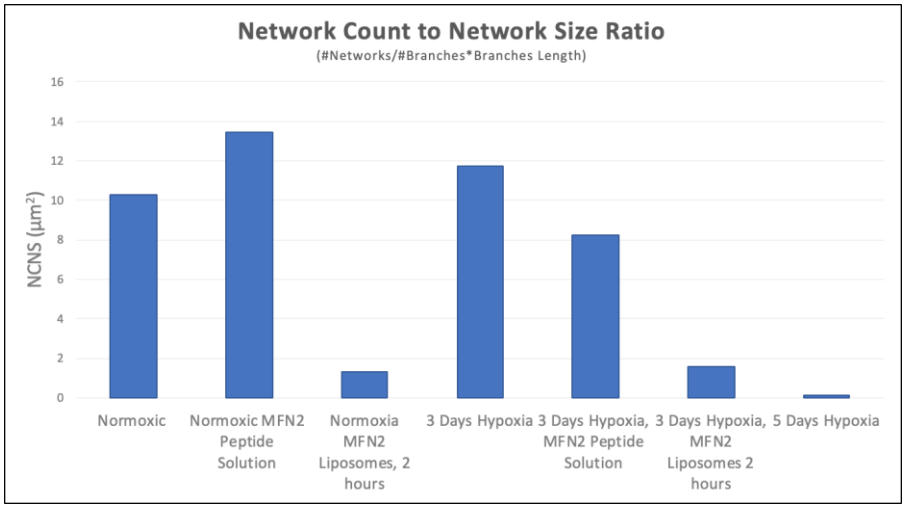

Figure 10: **MiNA analysis.** This graph shows the network count to network size ratio (NCNS) in TNBC cells that are exposed to different conditions. The NCNS ratio was calculated dividing the # networks by # branches * branches length.

MTS analysis

Dose response curves show that with increasing concentration, there is an increased effect of the drug **(Fig. 11)**. PTX was the most toxic drug among all the tested drugs. Hence, PTX is clinically used as a chemotherapy agent. BAM7 and Shikonin, the exploratory drugs, were less toxic. However, at a 100 µM concentration, all drugs had a cell viability of 60 % or less. Dose response curves show that normoxic cell viability is above hypoxic in every single drug we tested, meaning that normoxic cells are more resistant to chemotherapy drugs. IC50 values represent the concentration of a drug that is required for 50% inhibition in vitro [19]. A trend indicates that Shikonin is the less toxic drug, followed by BAM7 and PTX.

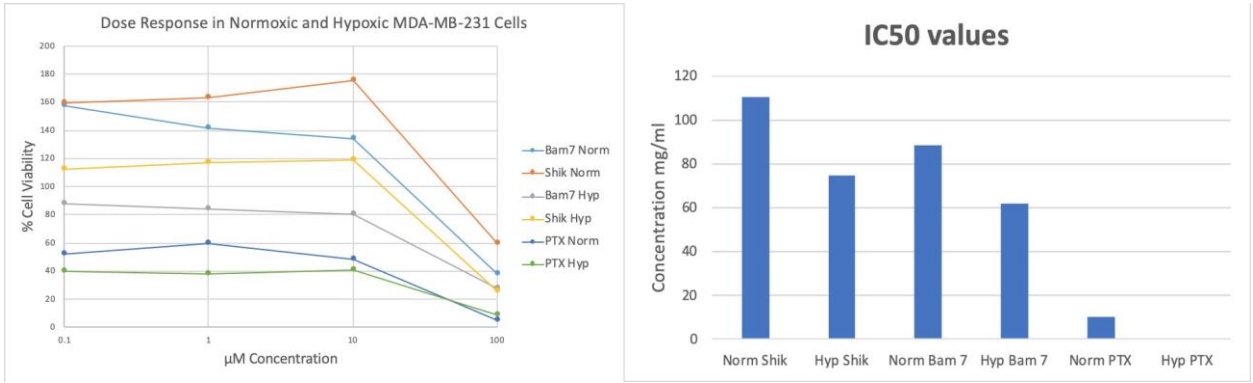

Figure 11: **Dose response curve and IC50 values after 72 h of treatment.** The dose response curve was used to measure the cell viability and to calculate the IC50 values.

Hypoxic cells were more resistant to drug treatments at 24 h than normoxic cells, as expected **(Fig. 12)**. Nonetheless, after 72h of treatment, normoxic cells became more resistant to drug treatments than hypoxic cells even though we were expecting hypoxic cells to have a higher cell viability due to their innate MDR.

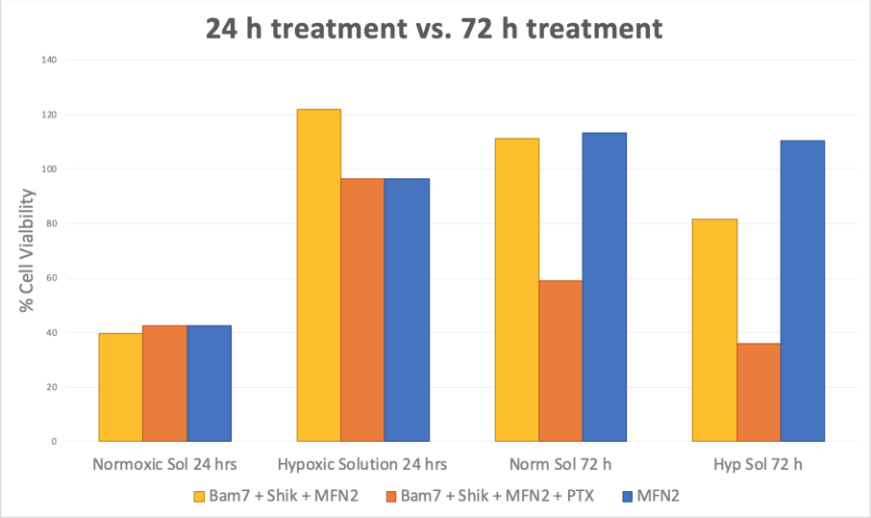

Figure 12: **Time effect on normoxic and hypoxic cells.** It is shown that changes in cell viability percentages occur at the different timepoints of treatment that were measured: 24 h and 72 h.

The anti-MFN2 peptide in liposomes sensitized cells to chemotherapy drugs, meaning that it inhibited mitochondrial hyperfusion. However, the scrambled sequence of anti-MFN2, which was used as a control, had an unexpected effect. It acted very similar as anti-MFN2 peptide in all the different conditions and combinations **(Fig. 13)**.

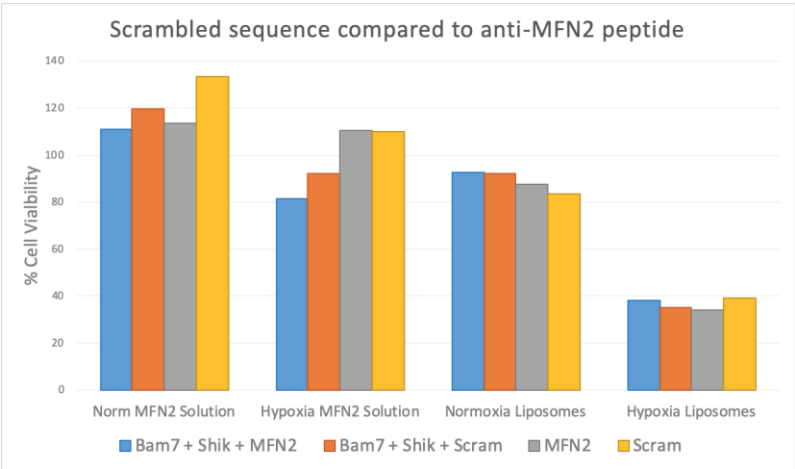

Figure 13: **Comparison of the anti-MFN2 peptide and the scrambled sequence.** This graph represents the cell viability of anti-MFN2 and the scrambled sequence in different treatment combinations and conditions, showing that they had similar effects.

The anti-MFN2 peptide loaded liposomes and the combination of our liposomes with BAM7, Shikonin and PTX had the greatest effect in hypoxic cells after 72 hours of treatment. Therefore, the therapeutic strategy we designed has been shown to be very effective. When treating hypoxic cells for 72 h with PTX 1 µM, there was a cell viability of 40 % as seen in the dose response curve **(Fig. 11)**.

However, when using our liposomes combined with the three drugs, it went down to 20 % **(Fig. 14)**. This confirms that anti-MFN2 sensitized cells to chemotherapy drugs.

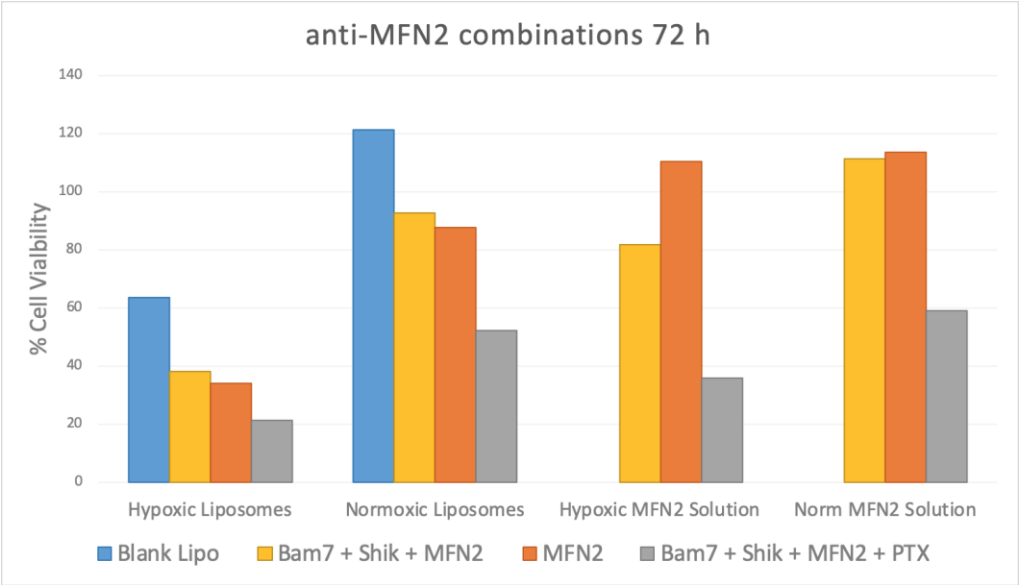

Figure 14: **Comparison of all the different combinations of the anti-MFN2 peptide.** This graph represents the cell viability of the different combinations we used with the anti-MFN2 peptide in order to achieve the most effective treatment.

Discussion

Our research seems promising in the goal to sensitize MDR TNBC to chemotherapeutic treatments. It was found that anti-MFN2 liposomes were very effective in inhibiting MFN2 from binding mitochondria together. Anti-MFN2 was able to break up the mitochondrial networks and sensitize cells to chemotherapy drugs. The combination involving PTX, Shikonin, Bam7 and the encapsulated anti-MFN2 was the most successful treatment, meaning that this therapy could be potentially used as a second line therapy for MDR cancer cells (**Fig. 14**).

The encapsulation efficiency in some liposomes with the scrambled sequence was over 100%. These values were obtained due to instrumentation errors. However, as they were not significant, we assumed an encapsulation efficiency of 100%. Anti-MFN2 with FITC liposomes had negative and out of orbitencapsulation efficiencies, meaning that the fluorophore probably interfered with the spectrophotometer.

Our liposomes had an average size of 142.2 nm, meaning that we got large unilamellar vesicles (100 nm - 400 nm) [20]. However, a peak appeared around 5800 nm **(Fig. 8)**. This is probably because the probe sonication method, which is used for splitting up the big clusters of vesicles, was not efficient enough. There- fore, this step of the liposome formation could be optimized by doing a membrane extrusion, for example. Extrusion is a technique where the liposome suspension is passed through a membrane filter of defined pore size [21]. A narrow size distribution of the liposomes was crucial for future applications. If the peaks were noisy and inconsistent, liposomes would encapsulate different volumes of the peptide, meaning that we would not be able to treat cells with specific dose concentrations. Liposomes were positively charged as they contained DOTAP, a cationic lipid. The Z-potential was used to determine the liposomes' electrokinetic potential. We found that that they could be used for cell delivery in humans and model organisms as they have a Z-potential between -25 mV to 25 mV.

Normoxic cells with the peptide in solution had a higher NCNS ratio than normoxic cells alone (**Fig.14**). This was probably because the anti-MFN2 peptide is easily degraded by enzymes present in the medium. This is why it had to be encapsulated in liposomes in order to achieve optimal cell delivery. In 5 days hypoxia, mitochondria hyperfused to the endoplasmic reticulum as a phenotype of an MDR cell transformation. MiNA failed to measure these networks due to the strong staining of MitoTracker Green. We were expecting higher NCNS ratios for the 5 days hypoxic cells than for the 3 days hypoxia. However, the MiNA software has its limitations after 3 days hypoxia. When using anti-MFN2 liposomes, mitochondrial networks were fragmented into little aggregates. There was a significant change in hypoxic cells treated with anti-MFN2 liposomes. This suggests that one of the phenotypes of innate MDR cells is mitochondrial hyperfusion.

We have come up with different hypotheses that could explain why normoxic cell viability increases and why hypoxic cell viability decreases after 72 h of treatment. On the one hand, it could be possible that hypoxic cells are more susceptible to the cell death system of BAM7, Shikonin and PTX than normoxic cells. Therefore, the liposomes formulation would not be effective as a first line treatment (normoxic cells) but it would have a very strong potential as a second line therapy (hypoxic cells). The hypoxic cells that were used for the MTS analysis were in 8 days hypoxia: 5 days before treatment and 3 days with treatment. Cells are usually grown and treated for 3 to 5 days to prevent excessive cell stress due to prolonged hypoxia. Hence, it could be that these cells were sensitized as they remained under stressful conditions for a longer period of time, going beyond the threshold for MDR cell sensitization. On the other hand, the rea- son why normoxic cells had a higher cell viability could be because of acquired MDR. Acquired MDR is one of the main reasons for chemotherapy failure, leading to the recurrence of malignant tumors [22]. Various mechanisms have been attributed to acquired MDR: enhanced drug efflux, increased DNA damage repair, altered drug metabolism. P-glycoprotein (P-gp) and other drug efflux transporters are considered to be critical in pumping anticancer drugs out of the cells and causing chemotherapy failure. We hypothesize that normoxic cells exposed to the chemotherapy drugs for 72 h acquired MDR through the enhanced drug efflux mechanism. The scrambled sequence, which was the positive control in the MTS assay, had unexpected effects. All the treatments involving the anti-MFN2

peptide and the scrambled sequence had very similar effects. It could be possible that the manufacturers that made the scrambled sequence did not scramble the anti-MFN2 properly. It cannot be considered that there was contamination in the scrambled sequence cells because as (**Fig. 13**) shows, it was tested in different combinations and conditions. Our scrambled sequence acted as a mitochondrial fusion inhibitor. Therefore, we might be on the way of a new MFN2 inhibitor peptide. However, further research needs to be done.

In the near future BAM7, Shikonin and PTX will be introduced inside of our liposomes in order to create a novel treatment to fight MDR tumors. Liposomes delivery efficiency will be tested to observe its effectiveness in inducing cell death. After clinical analysis, our treatment would be employed in the form of an injection in the region where the tumor is located in order for the liposomes to trigger anti-MFN2 and chemotherapy drugs. Future studies will look at treating the cells after they have been growing for 3 days of hypoxia and treated for 2 more days. Then, we will be able to answer the question of why normoxic cells have a higher cell viability than hypoxic cells. Follow up studies are going to do protein analysis to look at the markers of multidrug resistance and, therefore, get a better knowledge of the composition of MDR cells. Further research will get a new scrambled sequence from anti-MFN2 peptide and it will be tested in different treatments and conditions. Moreover, we will test whether we have discovered a new peptide that can inhibit mitochondrial hyperfusion and sensitize TNBC cells to chemotherapy agents or not. Finally, it is hoped that research on mitochondrial dynamics can be continued in other diseases, such as Alzheimer's disease, in the reverse direction of this project, where mitochondrial fusion will be promoted to preserve brain cells and prevent them from dying.

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Appendix

Glossary

Liposomes: A liposome is a spherical vesicle having at least one lipid bilayer. The liposome can be used as a vehicle for administration of nutrients and pharmaceutical drugs.

Mitochondrial fusion: Merging of the outer and the inner membrane of two distinct mitochondria

Prognosis: Prognosis is a medical term for predicting the likely or expected development of a disease, including whether the signs and symptoms will improve or worsen or remain stable over time

Hormone receptors: A hormone receptor is a receptor molecule that binds to a specific hormone. Hormone receptors are a wide family of proteins made up of receptors for thyroid and steroid hormones, retinoids and Vitamin D, and a variety of other receptors

DNA mutations: In biology, a mutation is the alteration of the nucleotide sequence of the genome of an organism, virus, or extrachromosomal DNA.

Tumor microenvironment: The tumor microenvironment is the environment around a tumor, including the surrounding blood vessels, immune cells, fibroblasts, signaling molecules and the extracellular matrix. The tumor and the surrounding microenvironment are closely related and interact constantly.

Efflux pumps: **Efflux** pumps are proteinaceous transporters localized in the cytoplasmic membrane of all kinds of cells. They are active transporters, meaning that they require a source of chemical energy to perform their function.

P-glycoprotein: P-glycoprotein 1 also known as multidrug resistance protein 1 is an important protein of the cell membrane that pumps many foreign substances out of cells.

Cancer stem cells: Cancer stem cells (CSCs) have the ability to give rise to all cell types found in a particular cancer sample. CSCs are therefore tumorigenic, perhaps in contrast to other non-tumorigenic cancer cells.

Bulk tumor cells: The total mass of all the cancer cells brought together in a specific region.

Epigenetics: In biology, epigenetics is the study of heritable phenotype changes that do not involve alterations in the DNA sequence.

Mitochondrial network: A group or system of interconnected mitochondria.

Vasculature: The blood vessels or arrangement of blood vessels in an organ or part.

Cytotoxicity: Cytotoxicity is the quality of being toxic to cells. Examples of toxic agents are an immune cell or some types of venom.

Hypoxic: Hypoxia is a condition in which the body or a region of the body is deprived of adequate oxygen supply at the tissue level.

Normoxic: Having a normal oxygen concentration; typically, 20-21% in the atmosphere

Oxygen fixation: radical-induced DNA damage can be permanently 'fixed' by molecular oxygen, rendering DNA damage irreparable

DNA radicals: radical damage to DNA can occur as a result of exposure to ionizing radiation

Apoptosis: Apoptosis is a form of programmed cell death that occurs in multicellular organisms. Biochemical events lead to characteristic cell changes and death.

Necroptosis: Necroptosis is a programmed form of necrosis, or inflammatory cell death. Conventionally, necrosis is associated with unprogrammed cell death resulting from cellular damage

Reactive oxygen species (ROS): Chemically reactive species containing oxygen. Examples include peroxides, superoxide, hydroxyl radical, singlet oxygen, and alpha oxygen.

Intrinsic apoptotic pathway: In the intrinsic pathway the cell kills itself because it senses cell stress.

Mitochondrial DNA (mtDNA): Mitochondrial DNA (mtDNA) is the physical embodiment of the mitochondrial **genome**, the sum total of genetic information encoded in the mitochondrion.

Intracellular/extracellular trafficking: tightly regulated process used by a variety of molecules to cross the membranes of living cells.

GTPase: GTPases are a large family of hydrolase enzymes that bind to the nucleotide guanosine triphosphate (GTP) and hydrolyze it to guanosine diphosphate (GDP)

Cytochrome 3: Cytochrome c is primarily known for its function in the mitochondria as a key participant in the life-supporting function of ATP synthesis. However, when a cell receives an apoptotic stimulus, cytochrome c is released into the cytosol and triggers programmed cell death through apoptosis.

Cytoskeleton: The cytoskeleton is a network of fibers forming the "infrastructure" of cells.

Microtubules: Microtubules are microscopic hollow tubes made of the proteins alpha and beta tubulin that are part of a cell's cytoskeleton, a network of protein filaments that extends throughout the cell, gives the cell shape, and keeps its organelles in place.

Supernatant: denoting the liquid lying above a solid residue after crystallization, precipitation, centrifugation, or other process.

Cell aggregates: The phenomenon by which dissociated cells intermixed in vitro tend to group themselves with cells of their own type.

Endoplasmic reticulum: The endoplasmic reticulum is a type of organelle found in eukaryotic cells that forms an interconnected network of flattened, membrane-enclosed sacs or tube-like structures

Biocompatible: Not harmful or toxic to living tissue.

Organelle: An organelle is a tiny cellular structure that performs specific functions within a cell

Peptide: A peptide is a short chain of amino acids. Typically, peptides are distinguished from proteins by their shorter length

Spectrophotometer: The spectrophotometer is an optical instrument for measuring the intensity of light relative to wavelength.

Acquired MDR: Drug resistance which is obtained over the time due to different external factors.

Innate MDR: Drug resistance which is inherent, and cells are born with this resistance already.

Diagnosis: Identification of the nature of an illness or other problem by examination of the symptoms.

Second line therapy: Treatment that is given when initial treatment (first-line therapy) doesn't work or stops working.

Alzheimer's disease: Alzheimer's disease is an irreversible, progressive brain disorder that slowly destroys memory and thinking skills and, eventually, the ability to carry out the simplest tasks.