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Evaluation of the cytotoxic effect of novel liposomal formulations in triple-negative breast cancer cell lines

Mariana Ribeiro Natalino

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Evaluation of the cytotoxic effect of novel liposomal formulations in triple-negative breast cancer cell lines

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Mariana Ribeiro Natalino
Faculty of Medicine, University of Porto

SUPERVISOR
M. Helena Vasconcelos, PhD
Cancer Drug Resistance Group - Institute of Molecular Pathology and Immunology of the University of Porto, Institute for Research and Innovation in Health of the University of Porto and Faculty of Pharmacy of the University of Porto

CO-SUPERVISOR
Sara Alves, PhD
Cancer Drug Resistance Group - Institute of Molecular Pathology and Immunology of the University of Porto, Molecular Microbiology Group - Institute of Cellular and Molecular Biology of the University of Porto and Institute for Research and Innovation in Health of the University of Porto

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Evaluation of the cytotoxic effect of novel liposomal formulations in triple-negative breast cancer cell lines

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AUTOR:
Mariana Ribeiro Natalino

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CORREIO ELETRÓNICO: mr.natalino20@gmail.com
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"Para ser grande, sê inteiro: nada
Teu exagera ou exclui
Sê todo em cada coisa. Põe o quanto és
No mínimo que fazes
Assim em cada lago a lua toda
Brilha, porque alta vive."

Fernando Pessoa

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Abbreviations List

A

ALDH	Aldehyde Dehydrogenase
AMPK	AMP-Activated Protein Kinase

B

Baf. A1	Bafilomycin. A1
BC	Breast Cancer
BL	Basal-like

C

C6-Cer	C6-Cer
Cer	Ceramide
CSC	Cancer Stem Cells

D

DEAB	Diethylaminobenzaldehyde
Doxorubicin	DRX

E

EGFR	Epidermal Growth Factor Receptor
EMA	European Medicines Evaluation Agency
EMT	Epithelial-Mesenchymal Transition
EPR	Enhanced Permeability and Retention
ER	Estrogen Receptor

F

FACS	Fluorescent Activated Cell Sorting
FBS	Fetal Bovine Serum
FDA	US Food and Drug Administration

G

GCS	Glucosylceramide synthase
GST	Glutathione-S-Transferase

H

HBS	Hepes buffered saline solution
HBSS	Hank's Balanced Salt Solution
HER-2	Human Epidermal Growth Factor Receptor 2

I

IM	Immunomodulatory

L

LAR	Luminal Androgen Receptor

M

M	Mesenchymal
MDR	Multi-Drug Resistance
MSL	Mesenchymal Stem-Like

N

NCL	Nucleolin
NP	Nanoparticle
O/N	Over-night
OD	Optical Density

P

P-gp	P-glycoprotein
P/S	Penicillin/Streptomycin
PARP	Poly(ADP-ribose) Polymerase
PR	Progesterone Receptor

R

RT	Room Temperature

T

TBS-T	Tris-Buffered Solution w/ Tween 20
TCA	Trichloroacetic Acid
TIC	Tumor Initiation Cell
TNBC	Triple-negative Breast Cancer

W

WHO	World Health Organization

Abstract

Triple-negative breast cancer (TNBC) is characterized by the absence of estrogen/progesterone receptors and of the epidermal growth factor receptor 2 (HER-2), currently used in targeted therapies for breast cancer (BC). Thus, chemotherapy is the only therapeutic option available for TNBC patients, excluding surgery, and most patients relapse in the first two years after diagnosis. Relapse is often associated with the presence of cancer stem cells (CSCs), a particular sub-population of cells within the tumor, with intrinsic drug resistance and capacity to re-populate the tumor after treatment. Given this problem, Nuno Fonseca *et al.* developed a lipossomal formulation (designated as pF3DC11) targeting cell-surface nucleolin (NCL, a promising therapeutic target for CSCs and TNBC) and encapsulating both doxorubicin (DRX) and C6-Ceramide (C6-Cer) - a combination previously proven advantageous in overcoming DRX-induced drug resistance.

The purpose of this project was to verify the cytotoxic effects of the NCL-targeted and non-targeted lipossomal formulations, developed by our collaborators, in TNBC cell lines and compare their effect with that of the commercially available Caelyx®. The first specific aim of this project was to determine the effect of these liposomal formulations in the cell growth inhibition and viable cell number of two TNBC cell lines: MDA-MB-468 and Hs578T. The second aim was to determine if the apoptotic and autophagic pathways were involved in the cytotoxic effect of those formulations in the TNBC cell lines. The third aim was to evaluate if these formulations affected the cell growth of the breast non-tumorigenic cell line, MCF12A. Furthermore, the last and forth aim was to isolate putative CSCs from the Hs578T cell line and compare the effect of these novel formulations, compared with Caelyx®, in sorted putative CSCs and non-CSCs sub-populations from TNBC cells.

The results obtained indicate that, at the concentrations and time-points studied, NCL-targeted and non-targeted formulations were more cytotoxic than Caelyx® in both TNBC cell lines, but surprisingly also in the non-tumorigenic cell line, being NCL-targeted formulation the most cytotoxic. Additionally, both novel formulations induced apoptosis to a greater extent than Caelyx® while partially blocking the autophagic flux, unlike Caelyx®. Putative CSC and non-CSC were successfully isolated from the Hs578T cell line and both novel formulations appear to be more cytotoxic than Caelyx® for the non-CSC sub-

population. Moreover, the NCL-targeted formulation was the most effective in the putative CSCs.

Altogether these results reinforce the potential of this NCL-targeted formulation, encapsulating C6-Cer and DRX, as a therapeutic tool in the treatment of TNBC and possible eradication of its CSCs.

Given that the concentrations used in the present work were high, and the non-tumorigenic breast cell line is not the ideal model to study toxicity, future work is required to confirm the safety of these formulations, in proper animal models and using physiological concentrations.

Keywords: Triple-negative breast cancer; Cell lines; Cancer stem cells; Drug Resistance; Liposomes; Nucleolin; Doxorubicin; C6-Ceramide; Cytotoxicity; Apoptosis and Autophagy.

Resumo

O cancro da mama triplo negativo (*Triple-negative breast cancer,* TNBC) é caracterizado pela ausência de expressão de recetores hormonais, para o estrogénio e progesterona, e do recetor de tirosina cinase HER-2. Por não expressar estes recetores, alvos de terapia dirigida, os doentes com TNBC têm como única opção terapêutica, para além da cirurgia, a quimioterapia. Infelizmente, a maioria destes doentes recai nos primeiros dois anos após o diagnóstico. Estas recaídas foram recentemente associadas à presença de uma pequena fração de células no tumor, designadas por células estaminais cancerígenas (*Cancer Stem Cells*, CSCs). Estas células possuem resistência intrínseca a fármacos antineoplásicos assim como uma capacidade única de se renovarem e diferenciarem, dando origem a um novo tumor após o tratamento. Perante este problema, Nuno Fonseca *et al.,* desenvolveram uma formulação lipossomal (designado como pF3DC11) tendo como alvo uma proteína expressa à superfície das células cancerígenas, a nucleolina (NCL), um promissor alvo terapêutico para o TNBC e CSCs. Esta nova formulação encapsula tanto doxorrubicina (DRX) como C6-ceramida (C6-Cer), uma combinação que previamente demonstrou potencial para ultrapassar o problema de desenvolvimento de resistência à DRX.

O objetivo deste projeto foi verificar os efeitos citotóxicos das formulações que encapsulam DRX e C6-Cer, com e sem o alvo para a NCL, desenvolvidas pelos nossos colaboradores, e comparar o seu efeito com uma formulação lipossomal já utilizada na prática clínica, a Caelyx®. O primeiro objetivo específico deste projeto foi determinar o efeito das três formulações, mencionadas anteriormente, na inibição do crescimento e número de célula viáveis de duas linhas de TNBC: MDA-MB-468 e Hs578T. O segundo objetivo foi determinar se o efeito citotóxico destas formulações estava relacionado com as vias da apoptose ou autofagia. O terceiro objetivo específico foi verificar se as formulações afetavam o crescimento de uma linha celular não tumoral da mama, MCF12A. Por fim, o último objetivo desta dissertação foi isolar CSCs e non-CSC putativas a partir da

linha celular Hs578T e avaliar o efeito citotóxico das novas formulações, comparando com a Caelyx®, em ambas as subpopulações.

Os resultados obtidos indicam que, nas concentrações e tempos estudados, as novas formulações que encapsulam DRX e C6-Cer, com e sem alvo para a NCL, são mais citotóxicas para ambas as linhas tumorais que a Caelyx®. Surpreendentemente, a mesma tendência foi verificada na linha celular não tumoral, sendo a formulação direcionada para a nucleolina foi a mais citotóxica para todas as linhas celulares. Além disso, ambas as novas formulações induziram maiores níveis de apoptose nas linhas tumorais do que a Caelyx® assim como causaram um bloqueio parcial no fluxo autofágico das mesmas, ao contrário da Caelyx®.

Para além disso, foi possível isolar com sucesso CSCs e non-CSC putativas, a partir da linha Hs578T. As novas formulações aparentam ser mais citotóxicas do que a Caelyx® para as non-CSC. Adicionalmente, a formulação dirigida à NCL aparenta ser a mais citotóxica para a subpopulação de CSC putativas.

Em suma, estes resultados reforçam o potencial desta formulação direcionada à NCL, encapsulando C6-Cer e DRX, como uma ferramenta terapêutica no tratamento de TNBC e possível erradicação das suas CSCs.

Uma vez que as concentrações usadas neste trabalho foram altas e que a linha celular não tumorigénica de mama não é o modelo ideal para estudar toxicidade, em estudos futuros será necessário confirmar a segurança destas formulações, usando modelos animais adequados e concentrações fisiológicas.

Palavras-chave: Cancro da mama triplo negativo; Linhas celulares; Células estaminais cancerígenas; Resistência à terapia; Lipossomas; Nucleolina; Doxorrubicina; C6-Ceramida; Citotoxicidade; Apoptose e Autofagia.

I

Introduction

1. Breast cancer

1.1. Epidemiology and risk factors

According to the World Health Organization (WHO), cancer is defined as the generic term for a multitude of diseases, characterized by the growth of abnormal cells that have acquired the capacity to invade other tissues[1]. The WHO estimated that in 2018 alone 18,1 million new cases would be diagnosed, and that 9,6 million people would die from cancer - illustrating why cancer is a major public health issue[2]. Thus, the scientific community has been dedicating a great amount of effort to better comprehend and tackle cancer. Breast cancer (BC) is a particular type of cancer that originates from the epithelial cells of the breast. BC is the most commonly diagnosed type of cancer in women, as shown in **Figure 1**. Moreover, based on demographic studies, both mortality and incidence rates of this particular type of cancer are expected to increase in the future[2].

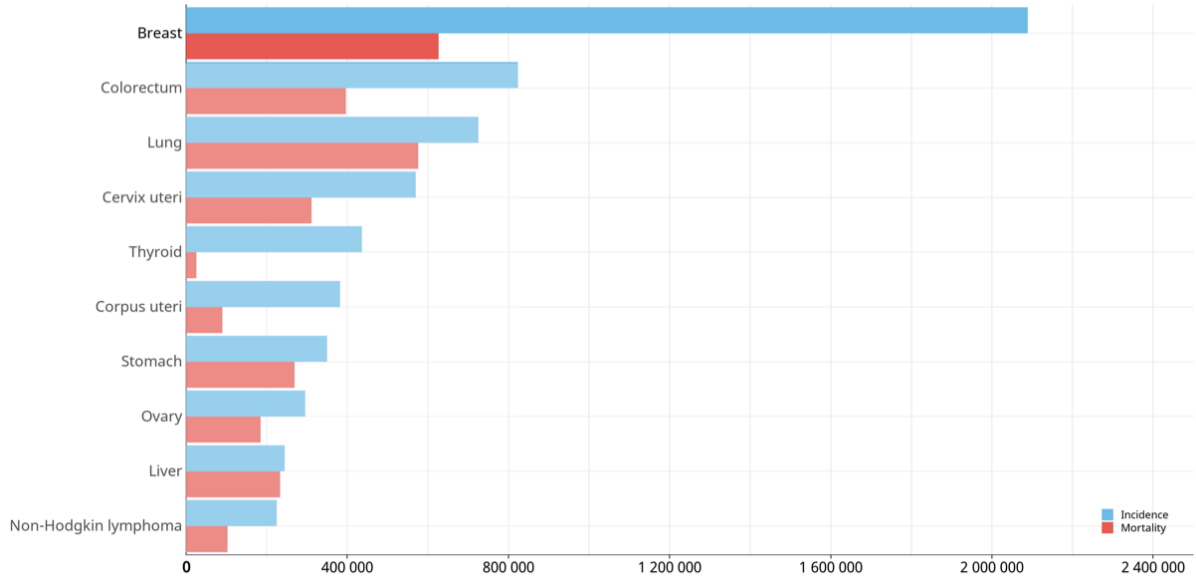

Figure 1. Estimated number of cancer incident cases and deaths in females, worldwide, in 2018. Adapted from: GLOBOCAN 2018

The differences in the incidence and mortality of BC worldwide are mostly related to the different risk factors, access to screening programs that allow early diagnosis and effective treatments[3]. The main risk factor for BC is the person's sex, since females have a significantly

higher (150 times more) risk of developing BC, than males[4]. Age is also an important risk factor for BC, as in all cancers. BC incidence is also associated with hormone, in particular estrogen. Direct evidence of this correlation between breast tumorigenesis and exposure to estrogens was inferred initially in mouse and cell line models[5]. Indirect evidence relates to the increase in BC associated with early menarche, late first birth and late menopause. Interestingly, breast-feeding is associated with a reduced risk of BC because it leads to the final differentiation of the terminal-ductepithelium[4,6]. Family history of BC is another relevant risk factor, with increased or decreased risk based on the proximity of the relative. Family BC accounts for approximately 5-10% of all BC cases and is due to inherited mutations in genes related to cancer susceptibility. *BRAC1* and *BRAC2* are two of most relevant genes that, when mutated, increase the probability of developing BC.[7] Therefore, considering some risk factors such as age or family history, proper screening is most important to reduce mortality rates. Indeed, data from the year 2000 showed a 46% reduction of BC mortality rates due to screening programs alone[8].

1.2. Heterogeneity in breast cancer: Intrinsic molecular subtypes

BC is characterized by its heterogeneity, being considered a multitude of diseases with origin in the malignant transformation of the epithelial cells that surround the milk duct. The heterogeneity of BC has long been related to different histopathological features and clinical outcomes among patients[9]. More recently, the histopathological classification of tumors has been complemented with molecular classifications. In fact, BC is one of the few cancers in which molecular characterization had a significant impact in the design of more individualized therapeutics and an improvement in the clinical outcome of patients[10].

This new classification of BC started with the work of Perou et al., in 2000, which consisted in the analyzes of gene expression patterns of breast tissue, using microarray technology, and BC classifications according to the expression of key genes. At the time, the first major division of BC was made according to the expression of the estrogen receptor (ER), with tumors being divided in ER positive (ER+) and ER negative (ER-). The ER+ tumors have an expression pattern similar to the breast luminal cells, therefore they are classified as the Luminal subtype. Another key gene, ERBB2, encoding for the Human Epidermal Growth Factor Receptor 2 (HER-2), is found over-expressed in tumors associated with a specific expression pattern. Another

subtype is the basal-like (BL), presenting expression patterns similar to basal epithelial cells, with high presence of basal markers such as keratins 5/6 and 17. Finally, the authors defined the normal-like subtype, which expresses basal markers and genes characteristic of adipocytes. Based on these results, the abovementioned authors proposed four molecular classifications in which BC could be categorized: ER^+ (luminal), HER-2 enriched, basal-like and normal-like[11]. However, it is important to note that the normal-like classification is still controversial, since some authors state that this subtype is an artifact derived from normal tissue contamination when the microarrays were performed[12,13].

This classification was later updated by Sørlie, T. *et. al*, when the authors related the different molecular subtypes with the different clinical outcomes of advanced BC patients that had been uniformly treated. Furthermore, these authors refined the previous subtypes, dividing the luminal (ER^+) into two different subtypes: Luminal A and B. Regarding prognosis, the BL subtype presented the worse prognosis and tumors overexpressing the ER were the ones with the most favorable outcome for the patients[14].

The **Luminal** subtype is the most commonly diagnosed subtype of BC and includes all tumors that have an expression pattern similar to epithelial luminal cells and overexpress hormonal receptors for estrogen and/or progesterone (PR). In 2001, this subtype was divided into Luminal A, which accounts for 50-60% of all BCs and Luminal B representing 15-20%[15]. The major biological difference between these two subtypes is their proliferation signature measured by the expression of proliferation-related genes such as *CCNB1*, *MKI67* and *MYBL2*, which are found more present in the Luminal B subtype. The Ki67 protein is an established proliferation marker, used in clinical routine and can also be useful in the distinction of Luminal A and B. Moreover, a great majority of luminal B tumors also present the overexpression of HER-2. These differences translate into a worse prognosis for luminal B subtype than for Luminal A[16].

The **HER-2** enriched subtype, as mentioned above, is characterized by a specific gene expression pattern often associated with the overexpression/amplification of the *ERBB2* gene[17]. This subtype accounts for 20-25% of all BCs and has a very aggressive phenotype that leads to poor prognosis[18]. However, in the past two decades, the prognosis of patients with this subtype of BC has significantly improved due to the development of a specific monoclonal antibody, trastuzumab, designed to interfere with the HER-2 function[19].

Another major subtype, accounting for 10-25% of all diagnosed BCs, is the **basal-like** BC. Apart from the expression of the basal markers mentioned above, these tumors often express the epidermal growth factor receptor (EGFR or HER-1)[20]. Nowadays, the BL subtype is often designated as triple-negative breast cancer (TNBC) because the great majority of BL tumors also lack the expression of the three key biomarkers: ER, PR and HER-2. Although there is a great overlap between these subtypes, not all BL tumors are triple-negative and not all triple-negative tumors have the gene expression profile associated with the basal subtype[21].

More recently, a new molecular subtype was described, the **claudin-low** subtype[22]. This particular subtype is characterized by the low expression of cell-cell adhesion molecules, namely claudins 3, 4 and 7. The expression patterns are very similar with the basal-like subtype, more specifically regarding the low expression of HER-2 and luminal-related genes[23]. This subtype is correlated with poor prognosis and high tumor grades. Interestingly, claudin-low have a greater fraction of tumor-initiating cells (TICS, also known as cancer stem cells) compared with the other subtypes and also a high expression of genes associated with the epithelial-mesenchymal transition (EMT) [24].

All the subtypes described above are considered intrinsic subtypes that can only be assessed through gene expression analysis. In the clinical context, this methodology is not feasible for all tumor samples. Thus, clinicopathological criteria, similar to what was proposed by Cheang et al.[25], are used in the current clinical practice to identify the different subtypes. In sum, immunohistochemistry is used to identify the presence of the key biomarkers: ER, PR and HER-2. Based on those markers the clinician can distinguish, roughly, between luminal (A or B), basal-like and HER-2 enriched. In addition, the Ki67 proliferation marker can be used to distinguish Luminal A from Luminal B, when the tumor doesn't present HER-2 expression[26].

1.3. Triple-negative breast cancer

The great majority of BCs (85 – 90%) is positive for either hormone receptors (ER and/or PR) or the HER-2. The remaining 10-15% of BCs lack the expression of these three receptors, being designated as "triple-negative breast cancer" (TNBC). These tumors are often more aggressive than the receptor-positive ones, presenting larger sizes, higher histological grades and lymph node involvement at the time of diagnosis[27]. Despite the high rate of response to neoadjuvant chemotherapy, the patients with TNBC are more likely to have distant metastasis

and a prognosis than patients with the other subtypes. In fact, only 30% of the patients diagnosed with the metastatic form of TNBC survive past 5 years after diagnosis and eventually the great majority dies from this disease[28].

This sub-group of BC is very heterogenous and, although most of the cases present the gene expression patterns characteristic of the BL subtype, different triple-negative tumors were found to have expression patterns similar to Luminal A/B, HER-2 enriched, claudin-low and even normal-like BC[23]. Unlike ER or HER-2 positive tumors, TNBCs does not present the expression of a driver oncogene that can be successfully targeted, thus highlighting the need for a better understanding of this subtype in order to develop new therapeutic strategies[29].

Lehmann *et al.* analyzed the gene expression patterns of tumors from a cohort of 578 TNBC patients and identified, for the first time, six novel subtypes within TNBC: Basal-like (1 and 2) (BL-1/2), Immunomodulatory (IM), Mesenchymal [normal (M) or stem-like (MSL)] and luminal androgen receptor (LAR). Moreover, the authors performed the same analysis in human BC cell lines to identify the subtype they represented[30]. More recently, the same team suggested that the MSL and IM subtypes were possible artifacts due to contamination by stroma and lymphocytic cells. Therefore, nowadays, only four subtypes of TNBC are considered: BL-1, BL-2, M and LAR[31]. Masuda *et al*. also validated this classification in a cohort of 140 TNBC patients, demonstrating the prognostic value of the different subtypes[32].

The **BL** subtype in TNBC is very similar to the general BL subtype described by Perou *et al*. BL-1 and BL-2 differ in the expression of key genes involved in growth factor signaling pathways, such as EGFR and Wnt/ß-catenin, which are found more present in the BL-2 subtype. The BL-1 is characterized by the high expression of DNA damage and cell cycle control genes. Both subtypes present the expression of basal markers and a high rate of proliferation, with high levels of Ki67, a known proliferation marker. Interestingly, the MDA-MB-468 cell line, used in this dissertation, was characterized by the authors as representative of the BL-1 subtype. The **M** subtype is characterized by the high expression of genes related with cell motility, differentiation and growth pathways. Interestingly, the majority of M tumors are associated with a downregulation of claudin, overlapping with the gene expression pattern observed in the claudin-low subtype[30,31]. Curiously, the Hs578T cell line used in this dissertation, previously categorized as claudin-low[33], was also characterized as being representative of the M subtype. Finally, the **LAR** subtype was identified by having an enrichment in hormone-related pathways and expression of the androgen receptor[30].

The combined new molecular-based classification of BC is depictured in **Figure 2.** This new molecular characterization of the TNBC subtypes has opened new possibilities for a more direct and targeted therapy. For example, the BL subtype, which is more proliferative, could be more susceptible to chemotherapeutic drugs that target high proliferating cells, when compared to the other subtypes[34]. Thus, the proper identification of these TNBC subtypes will have a great impact in the clinic, namely by contributing to deciding the appropriate line of treatment for each TNBC patient.

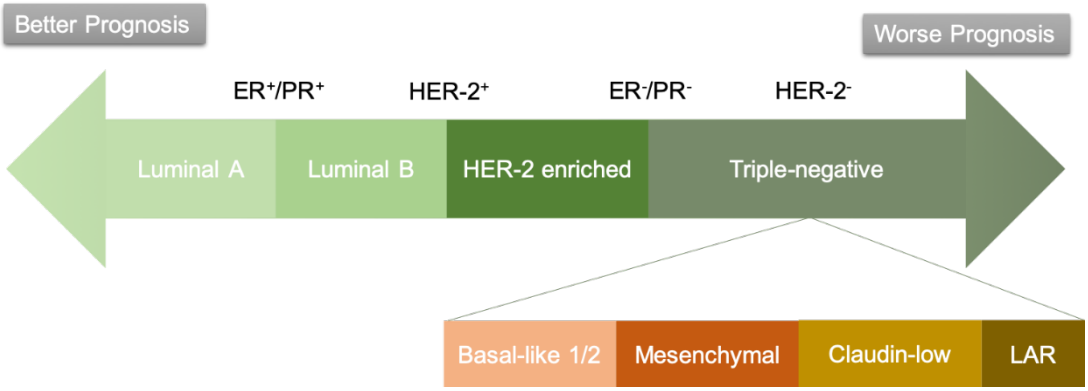

Figure 2. Schematic summary of the combined molecular classifications of breast cancer.

2. Breast cancer stem cells

2.1. Cancer stem cells: an overview

Stem cells can be simply defined as cells with the ability of constant self-renewal and preserved ability to differentiate into other types of cells. In adults, there are tenuous populations of these cells which are thought to be in a quiescent state until their progeny is needed to replace any aging or damaged cells[35]. The self-renewal capacity of healthy stem cells is similar to the one observed in some cancer cells, with several pathways being activated in both cancer and healthy stem cells[36]. One of those pathways is antiapoptotic and caused by the increased expression of the anti-apoptotic protein, Bcl-2[37]. Moreover, the activation of

pathways such as the Notch, Wnt signaling and Sonic hedgehog was also confirmed in both stem and cancer cells[38]. However, it is important to distinguish self-renewal from high proliferation. The self-renewal term implies that either a cell divides symmetrically, leading to a progeny with the same capacity of self-renewal, or asymmetrically giving rise to one stem cell and to another more differentiated cell, which lost the ability for self-renewal[39]. Therefore, it is possible that, in a given liquid or solid tumor, a small population of tumorigenic cells with self-renewal capacity is responsible for sustaining the malignant tumor growth through differentiation, similarly to the role of healthy stem cells. These cells were designated as cancer stem cells (CSC). In 1994, Lapidot *et al.* provided the first experimental evidence for the existence of CSCs in acute myeloid leukemia. These cells had low frequency in the tumor and could be identified by specific surface markers. Furthermore, these authors proved that only the cells expressing these specific markers could initiate tumor development *in vivo,* with only a few amount of cells, unlike the bulk of tumor cells[40]. Later, Clark *et al*. proposed a definition of CSCs as a distinct population, that can be isolated from tumors, with unique self-renewal capacity and able to repopulate a tumor in the long-term[39].

In 1976, Peter Nowell proposed for the first time a pioneer perspective on the origin of cancer, the "clonal evolution model". This author claimed that, like in the Darwinian model for the evolution of species, cancer also evolves following the principle of natural selection. Hence, according to this model, cancer appears as a result of an evolutionary process driven by sequential alterations and mutations, starting from a modified single-cell of origin (as a clonal event). This cell would then divide and acquire genetic variability, originating different sub-clones. The selective pressure on the tumour environment (including from therapy) would eventually result in the survival of the most resistant and suitable sub-clones[41]. More recently, with the discovery of the CSCs, a new model for the origin of cancer was proposed, the **cancer stem cell model**. This model defends that CSCs are responsible for the development and progression of many cancers[36]. However, it does not imply that CSCs are the cells-of-origin of all cancers. Instead, this model suggests that, like in normal tissues, cancer is organized in a hierarchal manner with CSCs in the top. The CSCs would divide asymmetrically, differentiating into phenotypically distinctive cells that form the bulk of the tumor. This model has been proven in leukemias, breast, brain and colon cancer[42]. Remarkably, the clonal evolution and CSC models are not mutually exclusive but instead they can complement each other, as

represented in **Figure 3**. Some authors believe that there are different sub-clones of CSCs that also undergo selective pressure.

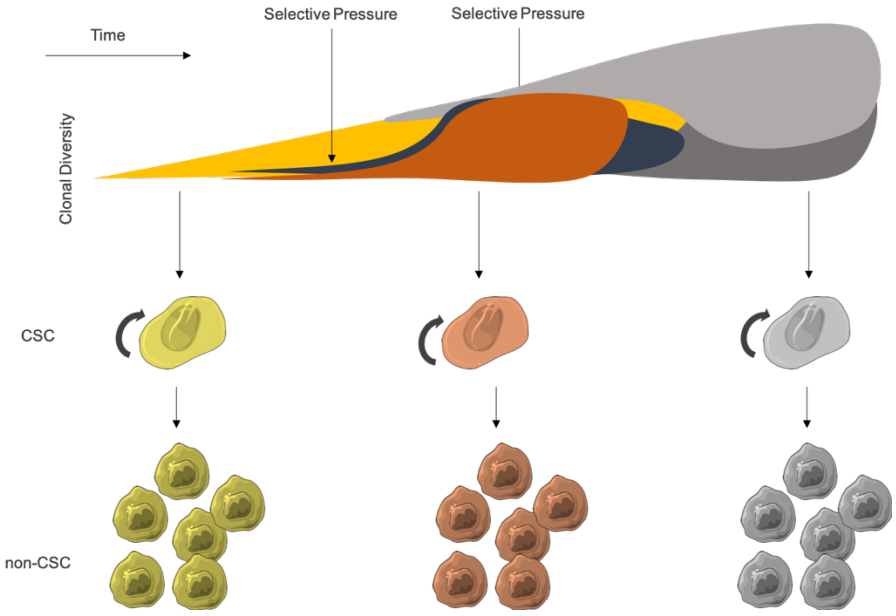

Figure 3. Unification of the clonal model and the cancer stem cell model of cancer evolution. Based on:[42]

2.2. Breast cancer stem cells

In 2003, Al-Hajj *et al*. published a landmark paper in the field of BC and CSC. Following the steps of Lapidot and collegues[40], this study described, for the first time, a group of cell-surface markers that could prospectively identify cells with an enhanced ability to form tumors in immunocompromised mice – an important characteristic of CSCs. These cells, sorted by high surface expression of CD44 and low expression of CD24 ($CD44^{+/high}/CD24^{-/low}$), had a tumor inducing capability 10 to 50-fold higher than unsorted cells. Moreover, the authors also identified EpCAM (ESA/CD326) as a potential biomarker to isolate breast cancer stem cells[43]. More recently, other authors have confirmed the potential of these markers to select CSCs[44,45].

Surprisingly, a year after the observations of Al-Hajj *et al*., a clinical study reported that the presence of CD44 and CD24 in BC had no correlation with tumor grade, subtype or size[46]. However, if the combination of these markers is specific of CSCs and these cells are a rare sub-population, immunohistochemistry is probably not the most sensitive technique to evaluate

their prognostic value. Hence, Liu *et al*. later compared the gene expression pattern of breast CSCs with normal cells from the breast epithelium, obtaining a cluster of genes differentially expressed which they designated by "*invasive gene signature"*. The authors than evaluated the association of this signature with gene expression data from almost 600 breast tumors, discovering that this expression pattern (the "*invasive gene signature"*) correlated with poor overall and metastasis-free survival[47], supporting the CSC model in BC. Moreover, the high expression of CD44 was also correlated with the upregulation of mRNA expression of known stem-cell markers, and also correlated with poor prognosis[44], thus sustaining the previous observations. Interestingly, an enrichment of the sub-population $CD44^{high}/CD24^{low}$ was found in the BL subtype, whereas the $CD44^{low}/CD24^{high}$ sub-population was more enriched in the luminal subtype[48]. Together these results demonstrate the clinical relevance of CD44 and CD24 expression in BC.

In 2007, Ginestier and colleagues identified another prominent biomarker of CSCs, the increase in aldehyde dehydrogenase (ALDH) activity. This marker alone was able to identify a fraction of tumor cells with the ability to form a tumor in immunocompromised mice and recapitulate tumor heterogeneity. Moreover, the expression of this protein was detected by immunostaining on several breast tumors correlating with other histopathological characteristics such as high tumor grade, HER-2 positivity, and Ki67 status [49]. The $ALDH^{+}$ marker, in combination with both $CD44^{high}/CD24^{low}$ or just with $CD44^{high}$, has proven to be more successful in selecting cells with the highest tumorigenic capacity. In fact, isolated cells with $ALDH^{+}/CD44^{high}/CD24^{low}$ presented increased stem-like properties when compared to the $CD44^{high}/CD24^{low}$ sub-population[50]. The enrichment in the expression of genes related with the stem phenotype in tumor cells positive for ALDH activity was observed and this expression pattern correlated with the one found in TNBC patients, being a predictor of poor prognosis for TNBC patients[51]. These data indicated that $ALDH^{+}$ could be a very effective marker for the isolation of CSCs from triple-negative tumors and cell lines.

2.3. CSC in drug resistance

A successful cancer therapy aims to eradicate all tumorigenic cells, leading to a complete response of the patient and tumor eradication. However, in several cancer cases, even after a complete pathological response to therapy, some patients relapse and the "new" tumor is,

most of the times, more aggressive than the original. According to the clonal evolution model, cancer therapeutics exert external selective pressure on the tumor cells, which leads to the selection of cells that are more resistant to therapy, contributing to treatment failure and disease progression[52]. This process is often designated as chemotherapy-induced clonal evolution[53].

Together, the CSC and the clonal evolution models offer a clear hypothesis on tumor acquirement of drug resistance and patients' relapse. Recent studies indicate that CSC are more resistance to chemotherapy, especially to drugs which target highly proliferative cells, such as doxorubicin (DRX)[54]. There are several mechanisms of drug resistance described in CSCs, with particular emphasis on their capability of activation of EMT[55], overexpression of drug-efflux pumps[56], such as the P-glycoprotein (P-gp), increased expression and activity of detoxifying proteins such as ALDH[57], CSC dormancy[58], among several other mechanisms. As mentioned above, common chemotherapeutic agents would easily target and eventually kill the bulk of the tumor cells in the majority of cancers. However, this CSC sub-population with intrinsic drug resistance would likely survive treatment and promote the re-appearance of the tumor due to their plasticity and capacity to recapitulate alone the tumor heterogeneity[59]. Thus, new therapeutic strategies should be developed in order to target this sub-population of CSCs to assure complete tumor extinction. However, therapies shouldn't be focused only on targeting CSCs, but rather on targeting both CSCs and the bulk of tumor cells. Indeed, recent evidence suggests that non-CSCs, in the presence of a harmful stimuli such as chemotherapy, can induce EMT and become more undifferentiated with a phenotype similar to the one of CSCs, thus becoming more resistance[60].

Crocker *et al*. demonstrated that breast CSCs cells isolated with the markers $ALDH^{+}/CD44^{high}$ were significantly less sensitive to chemotherapy than the $ALDH^{-}/CD44^{low}$ sub-population. This reduction in drug sensitivity was followed by an increase in the expression of a drug efflux pump, P-gp, and of the detoxifying enzyme, glutathione-S-transferase (GST). Interestingly, when the ALDH activity was inhibited the CSCs became significantly more sensitive to both chemo and radiotherapy. These results indicate that ALDH plays a crucial role in treatment response. Moreover, these authors showed that cells isolated based on these two well-established breast CSC markers, displayed a multidrug-resistance (MDR) phenotype [61].

3. Therapeutics in breast cancer

According to the American Cancer Society, there are two main types of treatment for BC: local and systemic. Local treatments are surgery or radiation, with surgery being the most common first procedure upon diagnosis. The systemic treatment includes chemotherapy, hormone therapy, targeted therapy and the more recent and innovative immunotherapy[62].

3.1. Current targeted therapies in breast cancer

The ER is considered a biomarker for hormonal therapy. In fact, almost all BC patients who are positive for this marker benefit from endocrine therapy, such as tamoxifen[63] or aromatase inhibitors[64]. The systemic adjuvant treatment with hormone therapy increases the overall survival of BC patients. In the case of tamoxifen, treatment with this molecule can decrease death or recurrence by 30% in BC patients positive for the ER[8]. The HER-2$^+$ BC used to have one of the poorest prognoses until the approval in 1998 of trastuzumab, a first-line treatment used in combination with the chemotherapeutic drug paclitaxel, for metastatic HER-2$^+$ BC. Trastuzumab is a specific monoclonal antibody that interferes with the HER-2 receptor[19]. The clinical approval of this antibody was a breakthrough in the treatment of HER2-enriched BCs, by providing various benefits with adjuvant or neoadjuvant chemotherapy[65]. However, not every tumor with the intrinsic HER2-enriched molecular subtype benefits from this therapy, since not all tumors that fit into this category present the overexpression of HER-2[23]. More recently, another monoclonal antibody, Pertuzumab, was approved by the FDA to target HER-2$^+$ BC in combination with trastuzumab and docetaxel, as neoadjuvant treatment[66].

As mentioned in section 1.1, family BC represents 5 to 10% of all BC cases and can be due to inherited mutations in the *BRAC1* or *BRAC2* genes, presenting deficiencies in the homologous recombination DNA repair pathway. However, these mutations aren't always inherited and can be acquired during tumor development. In 2014, the US Food and Drug Administration (FDA) approved, for the first time, a specific treatment for BC with mutations in the BRCA gene, the Olaparib. This small molecule is an inhibitor of poly(ADP-ribose) polymerase (PARP), a crucial protein for DNA repair in BRAC-deficient cancers[67].

3.2. Challenges in TNBC therapy: a possible link with the presence of CSCs

TNBC is currently the most challenging subtype of BC, mainly due to the absence of HER-2 or hormone receptors, which leads to the inefficiency of the targeted therapies currently clinically-available for the other subtypes. Hence, chemotherapy remains (apart from surgery) the pillar treatment for TNBC patients. Most patients with TNBC relapse within the first two years from diagnosis and less than 30% of the patients survive 5 years past the diagnosis[28]. These recurrent tumors are often drug-resistant and associated with poor prognosis[68]. Moreover, resistance to therapy is believed to cause treatment failure in over 90% of patients with metastatic cancer[69]. There are several resistance mechanisms described for TNBC which are, in part, similar to the ones observed in CSCs, such as the overexpression of drug efflux pumps, alterations in genes associated with apoptosis, mutations in DNA repair proteins among others[70].

Indeed, the presence of CSCs has been associated with the high relapse rates of TNBC. For example, in 2013 Bhola *et al.* demonstrated, through gene expression analysis, that primary TNBCs treated with paclitaxel display a gene expression pattern more similar to the one observed in CSCs, when compared with untreated tumors, thus suggesting that treatment may induce an enrichment in the CSC subpopulation[71]. Samanta *et al*. further validated these observations in TNBC cell lines[72].

Altogether, these results emphasize the need to develop new therapeutic strategies for TNBC, that can eradicate not only the majority of the tumor cells but also the less frequent CSC sub-populations, most thought to be responsible for relapse and drug resistance.

4. Nanomedicine in cancer therapy

4.1. Nanomedicine in cancer: an overview

The systemic delivery of different therapies to cancer patients has led to a significant reduction in cancer related mortality over the last century[73]. However, there are two major concerns related with systemic administration of therapeutic agents: lack of tumor-specific delivery and toxic side-effects in healthy cells and tissues[74]. Nonetheless, recent advances in the field of nanotechnology and biomaterials allowed to overcome some of those concerns,

through the incorporation of therapeutic agents into particles with sizes ranging from 1 to 1000 nanometers.

Nanomedicine-based approaches for cancer treatment offer several advantages when compared with the systemic delivery of drugs. Regarding the tumor-specificity, nanoparticles (NPs) can be more specific by allowing either passive or active targeting of the tumor (**Figure 4**). Passive targeting is possible due to key differences in the tumor vasculature when compared with the vasculature of healthy tissues. Indeed, the tumor endothelial cells are separated by much larger junctions than the ones from healthy endothelial vases[75]Given the size of NPs, they can easily pass through these junctions, thus preferentially entering the tumor-site than healthy tissues. On the contrary, targeted delivery is achieved by conjugating to the surface of the formulations a targeted element with high affinity to another molecule expressed in the surface of tumor cells, thus providing increased nanoparticle-cell surface intractions[76]. Moreover, the nanoformulations not only selectively extravasate into tumor tissues (due to the abnormal tumor vasculature) but they also have more difficulty in exiting the tumor site due to poor lymphatic drainage. The combination of this two effects is designated as "enhanced permeability and retention (EPR) effect" and leads to an accumulation of NPs in the tumor and while having low levels in the plasma or healthy organs[75]. Furthermore, both the EPR effect and targeted delivery help reducing side-effects and unwanted cytotoxicity. Besides the EPR effect, drug delivery using nanoformulations, such as liposomes, has several advantages when compared with conventional chemotherapy, such as: protection of the NP's cargo from degradation; ability to have a large amount of targeting ligands at their surface; simultaneous delivery of more than one therapeutic agent; controlled and adjustable release rate of the NPs' cargo and bypass of some drug resistance mechanisms such as the drug efflux pumps[77]. Overall, nanoformulations present a promising therapeutic tool to fight cancer.

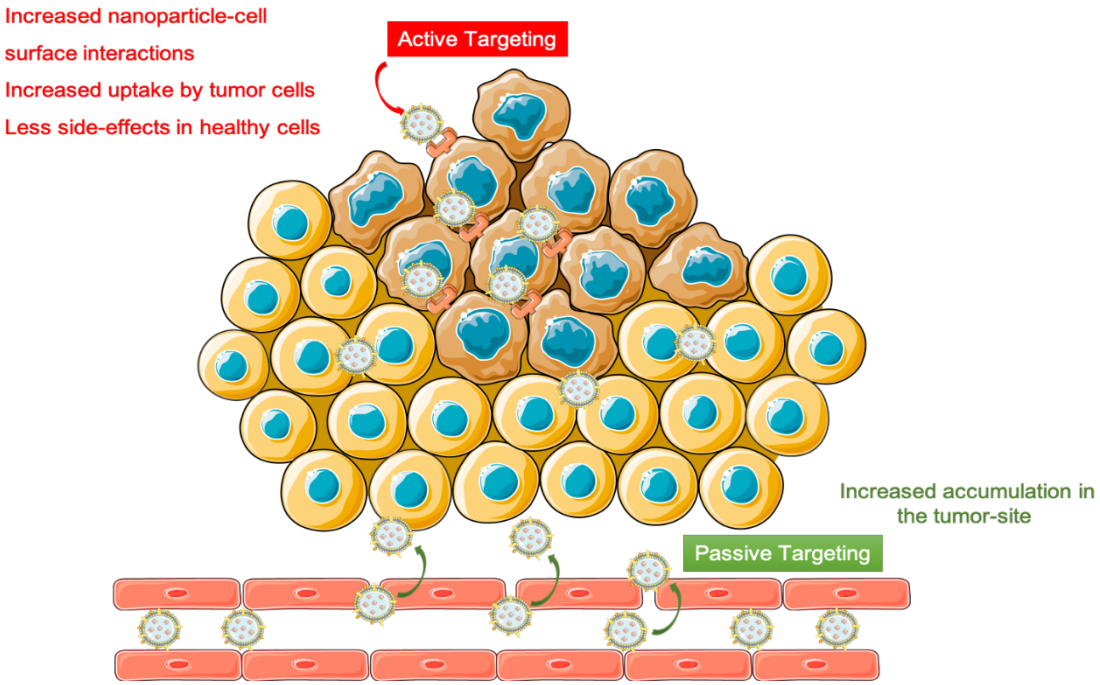

Figure 4. Schematic representation of passive and active targeting of tumor cells by nanoparticles, and their respective advantages.

4.2. Nanomedicine in breast cancer: the importance of targeted nanoformulations

In the past 20 years, the FDA has approved the use of nine different formulations to treat cancer. In the case of BC, the liposomal formulations Doxil® (Caelyx® in Europe) and Abraxane® are the most successful nanoformulations used in BC treatment[78].

Abraxane® was created in an attempt to overcome the poor solubility of paclitaxel, which targets high proliferating cells by suppressing cell division. This agent was approved for the treatment of pretreated metastatic BC[79]. In fact, a recent phase III clinical trial for metastatic TNBC patients treated with the encapsulated form Abraxane® showed improved overall and progression-free survival, in comparison with patients treated with free paclitaxel[80].

Doxil®/Caelyx® was the first "nano-drug" approved by FDA or by the European Medicines Evaluation Agency (EMA), respectively, changing the landscape of cancer treatment. Doxil® is a PEGylated liposomal formulation that encapsulates free doxorubicin (DRX). This NP was developed in order to reduce DRX-induced cardiotoxicity[81]. Doxil® was very successful, allowing the administration of a much higher cumulative dose than the one that could be administered with free DRX, leading to a significant reduction in cardiotoxicity[82]. However,

despite the proven clinical superiority of encapsulated DRX, unique side-effects emerge with its use such as stomatitis and palmar–plantar erythrodysesthesia[83].

One of the major challenges in cancer therapy is to reduce unwanted side-effects. This could be possible by actively targeting tumor cells using target-conjugated formulations such as nanoparticles or liposomes. Active targeting leads to increased cellular uptake by cancer cells while minimizing uptake by healthy cells[77]. Moreover, and as mentioned before, the high concentration of ligands at the surface of these formulations increases the interaction with cancer cells thus increasing the probability of internalization by these cells[84]. As previously mentioned, the acquisition of MDR is a serious concern in cancer treatment often leading to tumor relapse and progression. Recent evidence obtained in mouse models showed that drug-delivery with targeted-formulations may represent a promising approach to tackle the MDR phenotype[85,86]. In the particular case of BC, Lee *et al*. remarkably demonstrated the potential of targeted-liposomal formulations encapsulating DRX, in reversing the resistant phenotype of xenografted MCF-7 (BC cell line) models *in vivo*[87]. In the year 2000, Dorit *et al.* hypothesized that the reason behind this ability of targeted-NPs to overcome drug resistance was due to the fact that their cargo entered cells by endocytosis, thus bypassing the drug-efflux capacity of P-gp. Nonetheless, despite all the advantages of targeted-NPs, to this date only one was approved for cancer treatment, in particular for cutaneous T-Cell lymphoma, the Ontak (Eisai)[88].

As described previously in section 3.2, there are no approved targeted therapies specific for TNBC[89]. [89] and there is an increasing demand to find new therapeutic tools to tackle this BC subtype, in particular when these tumors present resistance to the currently available therapeutic options. Given the above-described advantages of targeted-formulations for cancer treatment, one can hypothesize that this therapeutic strategy could offer significant improvements in the management of TNBC. Thus, it is of the most importance to find the right target to tackle in TNBC.

5. Targeting Cell-Surface Nucleolin

5.1. The Role of Nucleolin

The nucleolus is a structure within the nucleus mainly responsible for the transcription of ribosomal RNA and ribosome assembly. More recently, the increased size of the nucleolus has been used as a marker of the proliferation rate in tumor cells[90]. This increase was also later associated with the deregulation of two specific proteins: nucleophosmin and nucleolin[91]. Nucleolin (NCL) is the most abundant protein in the nucleolus, playing an important role in the transcription of ribosomal RNA, ribosomal assembly and in the coordination between the nucleolus and proliferation pathways[92]. Although it was first described as a nucleolar protein, NCL has different sub-cellular localizations and roles involved in cancer. Nucleoplasmic NCL controls and regulates oncogene expression. The role of nucleoplasmic nucleolin in the tumorigenesis of BC was demonstrated by Pichiorri *et al.* These authors showed that not only NCL was overexpressed in breast tumor cell lines, it was also responsible for regulating the expression of microRNAs involved in BC progression and drug resistance. Moreover, they also described a reduction in the aggressiveness of the cell lines when NCL was inhibited by guanosine-rich aptamers[93]. NCL was also shown to be present in the cytoplasm having an anti-apoptotic role in cancer cells by protecting the Bcl-xl transcript from degradation[94].

Cell-surface nucleolin was first described in 1990 by Semenkovich *et al*.[95]and since then the role of this protein at the surface of cancer cells has been widely studied. In the tumor context, cell-surface NCL has also shown an anti-apoptotic role by interacting with the Fas receptor, which is a crucial element of the extrinsic pathway of apoptotic induction[96]. Moreover, cell-surface NCL also interacts with the Ras protein and with the EGFR, promoting the stabilization of the EGFR and thus contributing to an increase of tumor growth *in vivo*[97].

Furthermore, NCL is also located at the surface of endothelial cells and plays an important role in tumor angiogenesis through mediation of anti-angiogenic and anti-tumoral endostatin[98]. In a comprehensive study, Destouches *et al.* demonstrated that the specific inhibition of cell-surface NCL using a pseudo-peptide (HB-19) suppressed both tumor growth and neoangiogenesis *in vitro* and *in vivo*. More specifically, these authors showed that the inhibition of cell-surface NCL led to a remarkable reduction in the ability of several cancer cell lines of breast, prostate, glioblastoma and melanoma to form colonies *in vitro*. Additionally,

the administration of this peptide in ectopic xenograft models inhibited tumor growth and, in some cases, completely eliminated the tumors. Another interesting evidence from this work is that this inhibition of cell-surface NCL does not interfere with the NCL found in other subcellular locations, thus indicating that targeting this cell-surface protein could have reduced toxicity, especially in healthy cells[99].

5.2. Nucleolin as a potential target for TNBC

Interestingly, the great majority of available anti-NCL tools were evaluated using TNBC models, both *in vivo* and *in vitro*, and demonstrated great cytotoxicity against this particular BC[100]. For example, the work described by Destouches *et al.* was preformed using the TNBC cell line MDA-MB-231 and the BC metastatic cell line, MDA-MB-435 for both *in vitro* and *in vivo* evaluation of a cell-surface NCL inhibitor, showing promising results[99]. The high levels of cell-surface NCL on the MDA-MB-231 cell line were validated by other authors[93,101]. The levels of NCL mRNA expression were also associated with an overall poor prognosis in TNBC patients[102]. These evidence suggest the potential of targeting cell-surface NCL for cancer therapy, not only for inhibiting its function but also as a target for active delivery of NPs.

Building from the current literature on TNBC and NCL, Nuno Fonseca *et al.* developed a new liposomal formulation aiming to target cell-surface NCL in TNBC models. The authors validated cell-surface NCL as a possible marker of CSC in the TNBC context, by demonstrating a correlation between the levels of NCL and known pluripotency/stem markers such as NANOG and OCT4. Interestingly, sorted MDA-MB-231 cells based on high expression of cell-surface NCL demonstrated a similar phenotype as the ones sorted using the well-established breast CSC markers, $ALDH^{+}/CD44^{high}$. These results further validate cell-surface NCL as a promising target not only for TNBC but also for the much needed eradication of the CSC sub-population[103].

6. Novel liposomal formulation targeting cell-surface nucleolin

6.1. F3-peptide targeting nucleolin

The novel liposomal formulation developed by Nuno Fonseca *et al.* contains a functionalized peptide (the F3 peptide) at its' surface, to target NCL. This peptide was first described by Porkka *et al.* in a study in which the authors used phage-displayed cDNA libraries to identify peptides that had specific and selective binding to tumor cells and vasculature, thus driving the phage to home at their surface. For this reason, the F3 peptide is designated by tumor-homing peptide[104]. One year after the discovery of this peptide, Christian *et al.* demonstrated that the binding molecule for the F3 peptide was the cell-surface NCL and that this binding promoted the internalization of F3 into the nucleus, thus confirming the "shuttle" function of NCL[105]. To validate the previously observed affinity of the peptide to TNBC cells, Moura *et al.* evaluated the binding-affinity of liposomes functionalized with the F3 peptide to the surface NCL on TNBC cell lines, namely MDA-MB-231 and Hs578T. They observed a significant increase in cellular association of the targeted liposome compared with the non-targeted one. Moreover, they demonstrated that the internalization of the functionalized liposomes was through an energy dependent mechanism, probably by the endocytic pathway[106].

6.2. Simultaneous delivery of DRX and C6-Cer

DRX is a chemotherapeutic drug widely used as systemic treatment in several cancers. This drug is an anthracycline that intercalates with DNA and interacts with topoisomerase II impairing its function thus blocking the progression of cell cycle. Hence, this drug affects primarily cells with high proliferation rates, as is the case of the majority of cancer cells[107].

As mentioned above, the development of resistance to therapy presents one of the biggest challenges in cancer treatment, and the resistance to DRX is no exception. In order to gain a better understanding of the mechanisms behind the resistance to DRX in BC, AbuHammad and Zihlif analyzed the differences in gene expression between a pair of sensitive and Dox-resistant cancer cell lines. Interestingly, one protein found to be overexpressed in MDR cell lines, and more specifically in metastatic BC, is the glucosylceramide synthase (GCS).

When GCS is up-regulated, it decreases the endogenous levels of ceramide (Cer)[108]. However, an increase in endogenous Cer leads to the up-regulation of GCS, creating a positive feed-back loop[109].

Cer is a small lipidic molecule that plays a role in several signal pathways inside the cell, particularly as a secondary messenger. The levels of endogenous Cer increase in response to several stimuli such as treatment with cytotoxic drugs like DRX, contributing to cell cycle arrest and subconsequent induction of apoptosis[110]. C Ji *et al*. revealed that one of the mechanisms by which DRX induces cell death is through the activation of the AMP-activated protein kinase (AMPK) and that Cer enhances this activation of AMPK, leading to a chemo-sensitizing effect[111]. . Lucci *et al.* described for the first time an association between Cer, in particular C6-Ceramide (C6-Cer), and resistance to DRX, in the MCF7 BC cell line. These authors compared the increase in Cer levels after treatment with DRX in a pair of sensitive and DRX-resistant BC cell lines. Interestingly, they found that the DRX-resistant cell line did not respond to the treatment with an increase in C6-Cer, unlike its sensitive counterpart[112]. This relation between Cer and DRX sensitivity was later elucidate by the work of Liu *et al.* DRX increases the endogenous levels of C6-Cer, further increasing the expression of GCS. Since high levels of GCS reduce the levels of C6-Cer, this secondary messenger is not able to promote apoptosis. This feed-back cycle can contribute to the survival of DRX-treated cells[113]. To break this cycle, exogenous C6-Cer could be delivered simultaneously with DRX, to compensate the DRX-induced depletion of endogenous C6-Cer by the GCS.

With this in mind, Fonseca *et al.*, developed liposomal formulations functionalized with the F3 peptide encapsulating defined amounts of DRX an C6-Cer, designated by pF3DC11 (**Figure 5**)[114]. This liposomal formulation may present a promising alternative to the commercially available Caelyx® because they target the tumor cells both passively (mainly through the EPR effect) and actively (through the internalization of the functionalized F3 peptide with affinity for the NCL expressed at the surface of tumor tissue and vasculature). By encapsulation of both DRX and C6-Cer, these formulations may be more effective in avoiding the development of a DRX-resistant phenotype, thus preventing tumor relapse. The results initially obtained by the authors were promising. They demonstrated that the targeted formulation was more cytotoxic to a TNBC cell line, MDA-MB-231, when compared with a non-targeted formulation or a liposomal formulation encapsulating only DRX, with a cytotoxicity over 90% following a 4-hour treatment period[114]. In a more recent study, Fonseca *et al.* also

showed that the expression of NCL correlated with the stem phenotype in the MDA-MB-231 cell line and, due to this expression by the *putative* CSCs, the authors were able to specifically target this sub-population as well as the bulk of tumor cells, rendering 100% of cell death in both sub-populations following a 24h-treatment period with pF3DC11 at a concentration of ~10 µM[103].

7. Rationale of the project

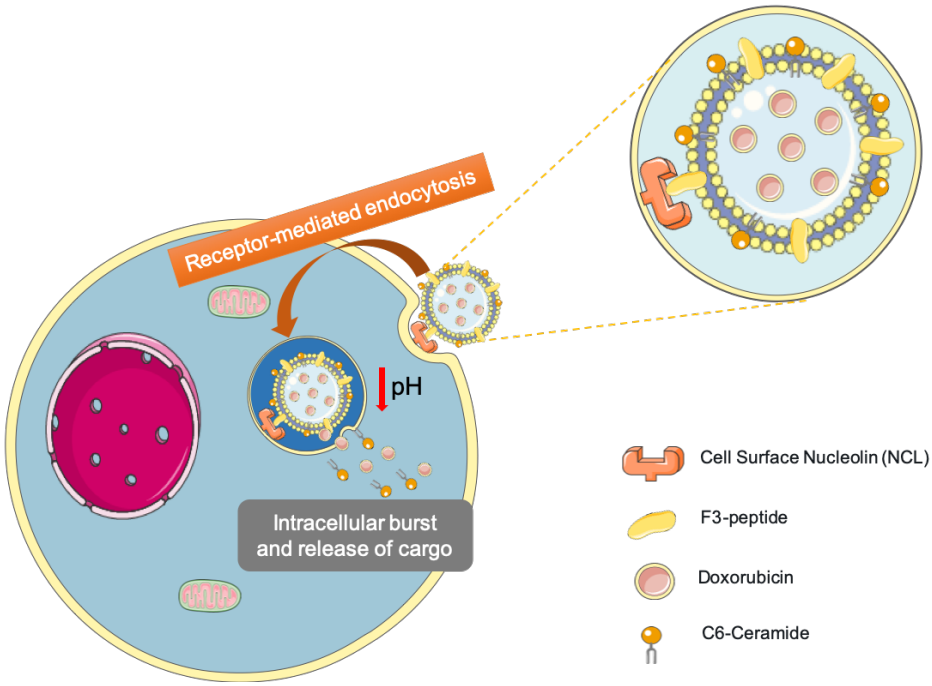

Figure 5. Schematic representation of the F3-targeted liposomal formulation and it's internalization.

The triple-negative subtype is the most challenging subtype of BC. Being chemotherapy the only therapeutic option available, most TNBC patients relapse within the first two years after diagnosis and less than 30% of the patients survive 5 years past the diagnosis[28]. These recurrent tumors are often drug-resistant. The high rate of relapse has been associated with the presence of a sub-population of cells, the CSCs, that could survive the conventional treatments and repopulate the tumor after treatment. Thus, there is an urgent need to find new therapeutic tools for TNBC that can eradicate both the normal tumor cells and the CSC sub-population.

. One of the main advantages of these therapies is the possibility to simultaneous deliver more than one therapeutic agent, which can increase the efficacy of the treatment, e.g. by

simultaneous delivery of DRX and C6-Cer. Another advantage is the possibility of adding targeting molecules to the surface of NPs/liposomes, increasing the specificity of the drug delivery and reducing side-effects. However, currently there are no approved targeted therapies for TNBC, mainly due to the heterogenous nature of these tumors.

In this regard, the work of Nuno Fonseca and colleagues[103,114], from the Center for Neurosciences and Cell Biology from the University of Coimbra, Portugal, has opened encouraging possibilities in the field of nanomedicine for TNBC, with the development of a liposomal formulation that targets cell-surface NCL and encapsulates both DRX and C6-Cer. However, to our knowledge, their data regarding the cytotoxicity of the formulation was only obtained in two cell lines (one from TNBC and the other from melanoma). Thus, there is still a lack of evidence regarding the cytotoxic effect of this liposomal formulation in other cell lines representative of TNBC. Moreover, the mechanism by which the combination of DRX and C6-Cer induces cytotoxicity is poorly understood in TNBC cell line models and needs to be elucidated. Finally, in order to validate the concept that cell-surface NCL is a promising target for both CSCs and non-CSCs, the CSCs should be isolated from TNBC cell lines and the mentioned NCL-targeted formulation should have increased cytotoxicity towards this sub-population than a control non-targeted formulation.

8. Main aim and specific objectives

The main aim of this dissertation was to assess the cytotoxic effects of the F3-targeted and non-targeted liposomal formulations containing equal amounts of C6-Cer and DRX, developed and quantified by our collaborators Nuno Fonseca *et al.*[114] (from CNC, University of Coimbra), and compare their effect with that of the commercially available Caelyx® in TNBC cell lines. Another aim was to compare the effect of these formulations in sorted putative CSC and non-CSCs sub-populations from TNBC cells.

The specific aims of this dissertation were to determine the effect of pDC11 (non-targeted), pF3DC11 (targeted) and Caelyx® liposomal formulations in the:

1. Cell growth inhibition and cell viability of the TNBC cell lines Hs578T and MDA-MB-468
2. Induction of apoptosis and/or autophagy pathways

3. Cell growth inhibition of the non-tumorigenic breast cell line MCF12A
4. Cell growth inhibition effect of sorted *putative* CSCs and non-CSCs from the Hs578T cell line

In order to achieve the first aim, cells were treated with different concentrations of the formulations during different short periods of time and then allowed to grow for further 96h in complete medium (without formulations). Growth inhibition effects were determined using the SRB assay and reduction in the number of viable cells using the trypan blue assay.

Regarding the second aim, to verify if the novel formulations activate the apoptotic and/or autophagic pathways, the levels of apoptotic markers such as caspase 3 and cleaved PARP-1 were monitored by Western blot. Autophagy induction was also assessed by Western blot, by monitoring the levels of LC3-II in the presence and absence of an autophagosome/lysosome fusion inhibitor.

To achieve the third aim, the MCF12A cell line was incubated with the three formulations for 1 or 4 hours and then allowed to grow for an additional 120 hours (without formulations). The cell growth inhibition effect was then determined with the Sulforhodamine B assay.

Finally, in order to achieve the fourth aim, cells with high expression/activity of two previously validated mammary CSC markers, ALDH and CD44, were sorted from the whole population of the Hs578T cell line by fluorescent activated cell sorting (FACS). These sorted *putative* CSCs and non-CSCs were incubated with the three formulations for 1 hour and then allowed to grow for an additional 96 hours (without formulations). The growth inhibition effect of the formulations in these sub-populations was determined with the Sulforhodamine B assay.

II

Methods and Materials

1. Cell lines and culture conditions

To achieve the aims proposed in the present study, three cell lines derived from human breast with different genetic backgrounds (listed in **Table 1**) were used: Hs578T (derived from carcinoma), MDA-MB-468 (derived from metastatic adenocarcinoma) and MCF12-A (derived from non-tumorigenic mammary gland) cell lines. All cell lines were kindly provided by Prof. João Nuno Moreira (CNC, University of Coimbra). Hs578T and MDA-MB-468 cells were cultured in RPMI 1640 with L-Glutamine (Lonza) supplemented with 1% of HEPES (1M, Lonza), 1% of penicillin/streptomycin (P/S) (100X, Corning) and fetal bovine serum (FBS) (Biowest). The concentration of FBS used was 5% for the SRB assay and 10% for the remaining assays. MCF12A cells were cultured in RPMI 1640 with L-Glutamine (Lonza) supplemented with 1% of HEPES (1M, Lonza), 1% of P/S (100X, Corning), 5 % of FBS, 500 mg/mL of hydrocortisone (Sigma) and 20 mg/mL of human epidermal growth factor (R&D systems). All cell lines were routinely cultured in 25 cm^2 tissue culture flasks, maintained in a humidified incubator with 5% CO_2 at 37^0C and subcultured when 80% confluence was reached, by treatment with Gibco™ TrypLE Express Enzyme (1X, Thermo Fisher Scientific). Cell phenotype was frequently monitored by light microscopy using the Leica DMI1 (Leica Microsystems).

The presence of mycoplasma was excluded using the VenorGeM® Advance Mycoplasma Detection Kit (Minerva Biolabs) by the Cell Culture and Genotyping facility of i3S, Porto. All the cell lines were also genotyped beforehand, by the Genomics Core facility of i3S using the PowerPlex® 16 HS System.

Table 1. Cell lines used in this study

CELL LINES	MOLECULAR SUB-TYPE	IMMUNOPROFILE	MORPHOLOGY	ORIGIN
HS578T	Claudin-low/ Mesenchymal	ER^-; PR^-; HER-2 negative	Epithelial/Mesenchymal	invasive ductal carcinoma
MDA-MB-468	Basal-like 1	ER^-; PR^-; HER-2 negative; EGFR+	Epithelial	adenocarcinoma
MCF12A	Basal-like	ER^-; PR^-; HER-2 negative	Epithelial	fibrocystic disease (non-tumorigenic)

ER, estrogen receptor; PR, progesterone receptor; EGFR, epidermal growth factor receptor; -, not present; HER-2 negative, not overexpressing the epidermal growth factor 2.

2. Liposomal Formulations

The liposomal formulations used in this study were: pDC11 (non-targeted), pF3DC11 (nucleolin-targeted) and Caelyx® (commercially available). All formulations were kindly provided by Prof. João Nuno Moreira from CNC, University of Coimbra. The synthesis of the pDC11 and pF3DC11 formulations and their quantification (based on the amount of DRX encapsulated, as described in[114]) was performed by the team of Prof. João Nuno Moreira at CNC, University of Coimbra.

3. Viability and cytotoxic assays

3.1. Trypan Blue Exclusion Assay

To assess the effect of the liposomal formulations in the viability of cells, the trypan blue exclusion assay was performed. This assay is based on the principle that the trypan blue dye is excluded from viable cells with intact cell membranes and can only penetrate non-viable cells, where the integrity of the membrane is compromised. Hence, this dye is used to determine cell concentration and viability[115]. Briefly, cells were incubated with the formulations or drugs at the chosen concentrations for 24h. After the treatments, cell

suspensions were mixed with 0.2% (v/v) Trypan blue (Sigma Aldrich) at a 1:1 ratio and loaded into a Neubauer chamber. Cells were then counted using a bright-field inverted microscope and the cell concentration and % of viable cells were calculated according to the following equations:

$$Cell\ concentration\ (cells/mL) = \frac{Number\ of\ viable\ cells \times Dilution\ Factor \times 10^4}{Number\ of\ Neubauer\ chamber\ quadrants\ analysed}$$

$$Cell\ viability\ (\%) = \frac{Number\ of\ viable\ treated\ cells}{Number\ of\ viable\ untreated\ cells} \times 100$$

3.2. Sulforhodamine B assay

To assess the growth inhibition effect of the liposomal formulations, the SRB assay was performed. This assay is based on the ability of SRB, a bright-pink aminoxanthene dye, to bind to the protein content of cells previously fixed. Consequently, it indirectly determines cell density based on the amount of protein mass, measured by spectrophotometry[116].

3.2.1. Cell number titration

To determine the optimal concentration of MDA-MB-468 cells to seed in a 96-well plate for the SRB assay, linearity range between cell number and absorbance was calculated. Cells ranging from 2,5x10^4/ml to 4,0x10^5 cells/mL were seeded in a 96-well plate**.** After 24h, 100 µL of fresh medium was added and the cells were allowed to grow for an additional 96 hours. The SRB assay was then performed as described by Vichai *et al.*[116]. Briefly, cells were fixed with ice-cold 10% (w/v) trichloroacetic acid (TCA, Merck) for at least 1 hour at 4^0C, washed 3x with distilled water and air-dried over-night (O/N). Cells were then stained with 0,4% (w/v) SRB solution (Sigma Aldrich) for 30 minutes at room temperature (RT) in the dark and then washed 3x with 1% v/v acetic acid (Merck) to remove excess and unbound dye. Air-dried cells were incubated with 10 mM Tris-Base (Sigma Aldrich) for 5 minutes in an orbital shaker, to solubilize the protein-bounded SRB. The optical density (OD) of each well was measured at 510 nm in a microplate reader (Synergy™ Mx, BioTek Instruments, Inc.) and analyzed using the Gen5™ software. The OD measured was plotted against the

correspondent cell concentration. The $1.5x10^5$ cells/mL concentration was selected for the other assays, because it was the highest concentration in the linear range of the plot, representing the highest cell density before reaching the saturation point.

For the Hs578T and MCF12A cell lines, the optimal cell concentration was previously determined to be $2x10^4$ cells/mL and $5x10^4$ cells/mL, respectively, by members of the Cancer Drug Resistance group, i3S.

3.2.2. Cytotoxic effect of the liposomal formulations in breast cancer cells

Dose-response curves of the liposomal formulations were obtained, in order to evaluate their cytotoxic effect in the TNBC cell lines. Hs578T ($2x10^4$ cells/mL) and MDA-MB-468 ($1.5x10^5$ cells/mL) cells were seeded in 96-well plates as depicted in **Figure 6** (one plate for each cell line and time-point). At 24h after seeding, cells were incubated with 1, 2, 5, 7.5 or 10 µM of Caelyx®, pDC11 or pF3DC11 for 1 hour or 4 hours. Suspensions were observed for the pF3DC11 formulation, under a brightfield inverted microscope. The treatment medium was then replaced with fresh medium and cells were allowed to grow for another 96 hours. The SRB assay was then performed as described in section 3.2.1. Untreated cells and cells treated with the vehicle HBS (Hepes buffered saline solution, Nzytech), were used as a negative control. The % of cell growth was calculated by subtracting the OD_{510} of treated cells (the T96h plate) with the OD_{510} of cells fixed at the time of the treatments (the T0h plate or no-growth control plate) using the formulas described by Vichai *et al.*[116]. Results are indicated as % of cell growth relative to the control from the T96h plate.

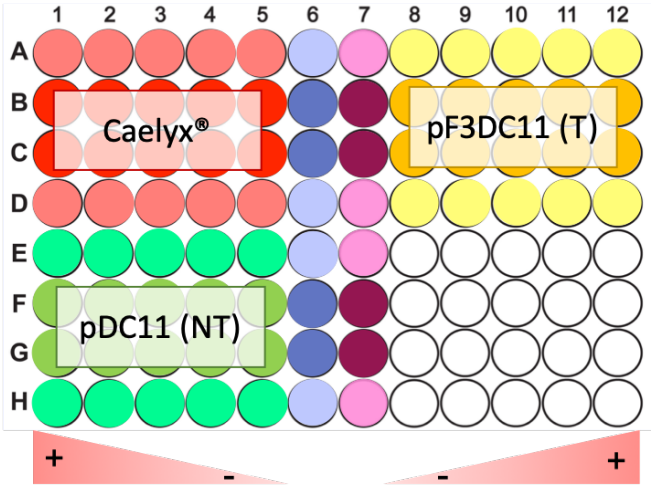

Figure 6. Schematic representation of the 96-well plate preparation.
The lighter colors represent the medium alone or with the formulations/vehicle and the darker colors represent the presence of cells. The different formulations were added to the wells with medium and well with cells, in five different concentrations (from column 1 to 5 and 8 to 12: 10, 7.5, 5, 2 and 1 µM, respectively). Column 6 (light and dark blue colour) represents cells treated with the vehicle, as a control.

3.2.3. Cytotoxic effect of the liposomal formulations in a non-tumorigenic cell line

To ascertain the cytotoxic effect of the liposomal formulations in the non-tumorigenic MCF12A cell line, the SRB assay was performed as described in section 3.2.1 with the following important alterations: cells were seeded at $5x10^4$ cells/mL and incubated with the highest concentration of the formulations tested – 10 µM, for 1hour or 4 hours. After this treatment, cells were allowed to grow for an additional 120 hours (time optimized according to their doubling time, to allow enough time for some degree of cell division to occur).

4. Assessment of apoptosis and autophagy induction by the liposomal formulations

To assess the effect of the formulations in the activation of apoptosis and autophagy in these TNBC cell lines, Hs578T ($2x10^4$ cells/mL) and MDA-MB-468 ($1.5x10^5$ cells/mL) cells were seeded in 6-well plates. After 24h, cells were incubated with 10 µM Caelyx®, pDC11 or pF3DC11 for further 24h, to allow a significant apoptotic and/or autophagic response. After these treatments, adherent and suspended cells were collected and further used to

assess the % of viable cells (as described in section 3.1) and to evaluate the levels of proteins involved in apoptosis and autophagy pathways, by Western blot. Treatment with 4 µM of free DRX (Sigma Aldrich) for 24h was used as a positive control for the induction of apoptosis. Incubation in Hanks balanced salt solution (HBSS, Thermo Fisher Scientific) for 24 hours was used as a positive control for autophagy induction. Bafilomycin A1 (Baf. A1, Invivogen) was added at 20 nM, to inhibit lysosomal degradation in order to analyze the accumulation of LC3-II by Western blot.

4.1. Western Blot

To evaluate the expression levels of apoptotic and autophagic markers by Western Blot, total proteins were extracted in 30 µL of Winman's buffer (1% NP-40, 0.1 M Tris-HCl pH 8.8, 5 M NaCl and 5 mM EDTA) with EDTA-free protease inhibitor cocktail (Roche) for 30 minutes (with agitation) at 4^0C. Afterwards, the protein lysate was precipitated by centrifugation at 13300 rpm, for 10 minutes at 4^0C (Micro Star 17R, VWR) and stored at -20^0C until use.

The quantification of the protein lysates was performed using Bio-Rad DC™ Protein Assay Kit, according to the manufactures instructions and using BSA as protein standart for the calibration curve. Then, 20 µg of protein lysates were separated on a 12% SDS-PAGE gel for 20 minutes at 80V followed by 1h30 at 100 V. Afterwards, the protein lysates were transferred to a nitrocellulose membrane (GE healthcare), in a Mini Trans-Blot® Cell (Bio-Rad) wet transfer system, for 1h30 at 80V. Membranes were stained with Ponceau and blocked in 5% (w/v) non-fat dry milk (Molica) in TBS-T [tris-buffered saline solution with 0,1 % of Tween-20 (Promega)], for at least 30 minutes in an orbital shaker at RT. After blocking, membranes were incubated with the primary antibody O/N in an orbital shaker at 4^0C, washed three times with TBS-T for 5 minutes and incubated with the secondary antibody for 1h in an orbital shaker at RT. The membranes were then washed again as previously described and peroxidase activity was revealed in Amersham Hyperfilm ECL, using the ECL detection reagents (GE Healthcare and Bio-Rad). Primary antibodies used were anti-actin (1:1000, Santa Cruz Biotechnology sc-1616), anti-GAPDH (1:500, sc-32233), anti-PARP-1 (1:500, sc-7150), anti-caspase-3 (1:1000, sc-7272), anti-cleaved caspase 3 (1:100, sc-56053), anti-LC3B (1:2000, L7543, Sigma-Aldrich). Peroxidase-coupled secondary

antibodies used were anti-mouse (1:2000, NA931V, GE), anti-rabbit (1:2000, NA934V, GE) and anti-goat (1:2000, sc-2354). Immunoblots were quantified using the Image Lab 6.0.1 software (Bio-Rad).

5. Cell growth inhibition effect in sorted putative CSCs from the Hs578T cell line

To study the cytotoxic effect of the liposomal formulations in the putative CSCs and non-CSCs sorted from the breast Hs578T cancer cell line, these two sub-populations were isolated by fluorescence activated cell sorting (FACS) based on ALDH (aldehyde dehydrogenase enzyme) activity and the expression of the cell surface marker CD44. These markers have previously been validated for the isolation of cancer cells with the stem phenotype [43,61,117]. The protocol used was previously optimized for the Hs578T cell line (unpublished results from the Cancer Drug Resistance group, i3S.)

5.1. Isolation of putative CSCs from the Hs578T cell line

Hs578T CSCs were sorted using the ALDEFLUOR™ assay kit (Stemcell Tech, BC) according to the manufacturers' instructions. Briefly, Hs578T cells were grown in 175 cm^2 culture flasks until > 80% confluency. Cells were then detached using Versene 1X (Thermo Fisher Scientific), which is a non-enzymatic solution, and resuspended in fresh medium. Cells were washed with ALDEFLUOR™ assay buffer and collected by centrifugation. Pelleted cells were then resuspended in ALDEFLUOR™ assay buffer and divided into different tubes: $2x10^4$ cells for each control and $3x10^5$ cells for the assay. The assay was performed in quadruplicates to increase the number of CSCs retrieved. The control conditions were an unstained (only cells) and controls with single-stains for every label used, namely: single-stain for 7-AAD (viability dye), anti-CD44 conjugated with APC and for the ALDH substrate. The ALDH substrate (BODIPY™-aminoacetaldehyde – BAAA) was added alone or in combination with diethylaminobenzaldehyde (DEAB), a selective inhibitor of the ALDH isoenzymes. Cells under all conditions were incubated at 37^0C for 45 minutes. After that, cells were washed with assay buffer and incubated with the antibody allophycocyanin (APC)-conjugated anti-CD44 (1:10, BD Biosciences) for 20 minutes in the dark, at 4^0C. The cells were then washed again with assay buffer and filtered through a cell strainer. Cells were stained with 1 μg/mL

of the viability dye 7-actinomycin-D (7-AAD, Sigma-Aldrich) to exclude dying/dead cells from the sorting. The sorting was performed using the FACS Aria II (BD, Biosciences) with the support of the Translational Cytometry Unity of i3S, Porto.

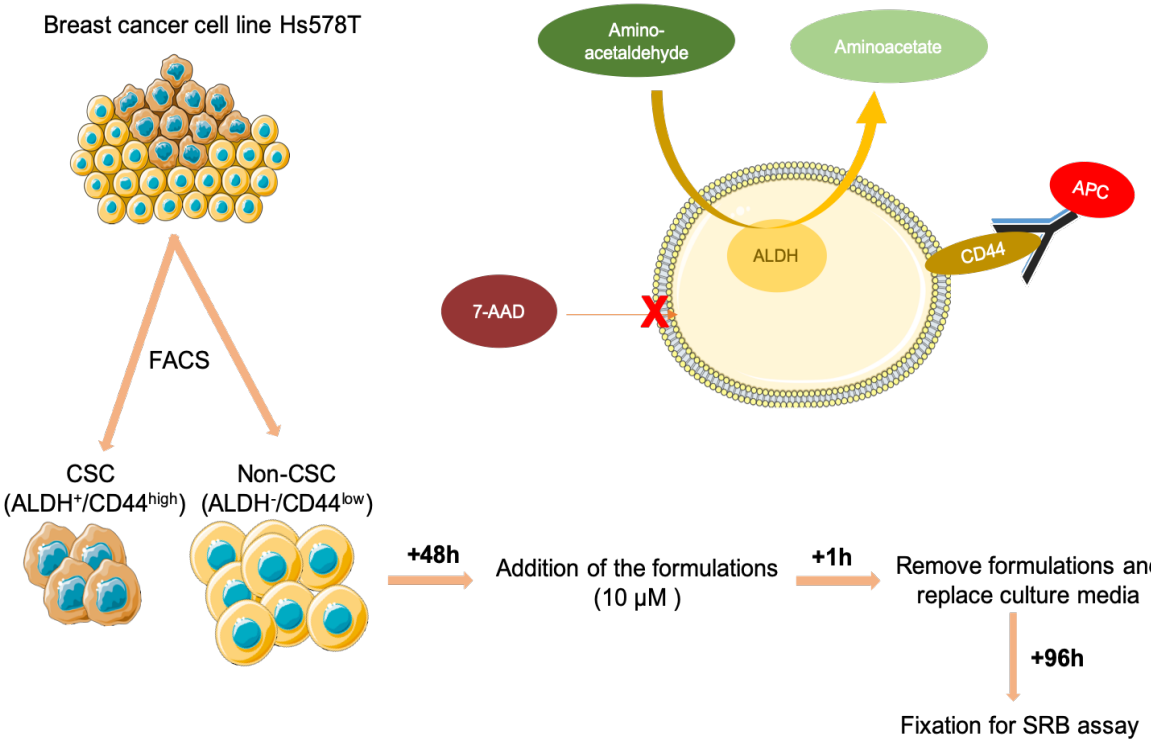

Figure 7. Schematic representation of the markers used for the isolation of putative CSCs and the post-sorting workflow.

5.2. Cytotoxic effect of the liposomal formulations in sorted putative CSCs and non-CSCs sub-populations

To assess the cytotoxic effect of the liposomal formulations in Hs578T putative CSCs and non-CSCs, sorted $ALDH^{+}/CD44^{high}$ (CSCs) and $ALDH^{-}/CD44^{low}$ (non-CSCs) populations were seeded in 96-well plates at a final concentration of $1x10^{5}$ cells/mL, and allowed to recover for 48h in medium supplemented with 20% FBS. Given 48h after seeding, cells were treated with 10 µM of each formulation for 1 hour according to the workflow represented in **Figure 4**. The incubation medium was replaced with fresh medium supplemented with 10% FBS and cells were allowed to grow for a further 96h before being processed for the SRB assay

as described in section 3.2.1. Due to the low number of sorted CSCs, it wasn't possible to perform the no-growth (or T0h) control plate in order to calculate the decrease in cell growth after plating cells at time=0h. Thus, results are indicated as % of cell growth relative to the control from the T96h plate, without subtraction of the OD from the T0h plate.

6. Statistical Analysis

All results are expressed as means ± SEM. Statistical analyses were performed using the One-way or Two-way ANOVA with the recommended Turkey's correction by GraphPad Prism 7.0 software. $p<0.05$ was considered statistically significant.

III

Results and Discussion

1. Cytotoxic effect of the liposomal formulations in the breast cancer cell lines Hs578T and MDA-MB-468

1.1. Different effect of the nucleolin-targeted formulation compared with the non-targeted one and Caelyx®

To confirm the different cytotoxic effects of the novel formulations, pDC11 (non-targeted) and pF3DC11 (NCL-targeted), encapsulating both DRX and C6-Cer, two cell lines representative of TNBC were used, Hs578T and MDA-MB-468. These cell lines were incubated with five increasing concentrations of pDC11 or pF3DC11, and Caelyx® (as a positive control), which is a commercial liposomal formulation encapsulating only DRX. Curiously, suspensions were observed under a microscope, on the both cell lines when treated with the pF3DC11 formulation. The percentage inhibition of cell growth was determined using the SRB assay, which infers cell density based on protein content.

As shown in **Figure 1A,** in the Hs578T cell line, the pF3DC11 was able to more significantly reduce cell than Caelyx® or pDC11, at concentrations above 2.5 µM, and was the only formulation that induced cell death (with % of cell growth relative to control reaching negative values).

In the MDA-MB-468 cell line (**Fig. 1B**) all concentrations tested of pDC11 and pF3DC11 formulations were significantly more efficient than Caelyx® in decreasing cell growth, following 1 hour or 4 hours of treatment period (**upper** and **lower panels**, respectively). Furthermore, both pDC11 and pF3DC11 appeared to induce cell death to a similar extent, for concentrations above 7.5 µM, independently of the treatment period. Cell death can be inferred in this assay when there is less protein content at the end of the experiment (time =96h) than at the beginning (at the no growth-control, time=0h), in which case the % of cell growth relative to control is negative (i.e. cell number at time=96h is lower than at time=0h).

Overall, the NCL-targeted formulation showed a greater effect in reducing cell growth than the non-targeted one or Caelyx®, in both cell lines, in concentrations equal or superior than 2.5 µM. Furthermore, at concentrations superior to 5 µM, the pF3DC11 appears to induce cell death in both cell lines.

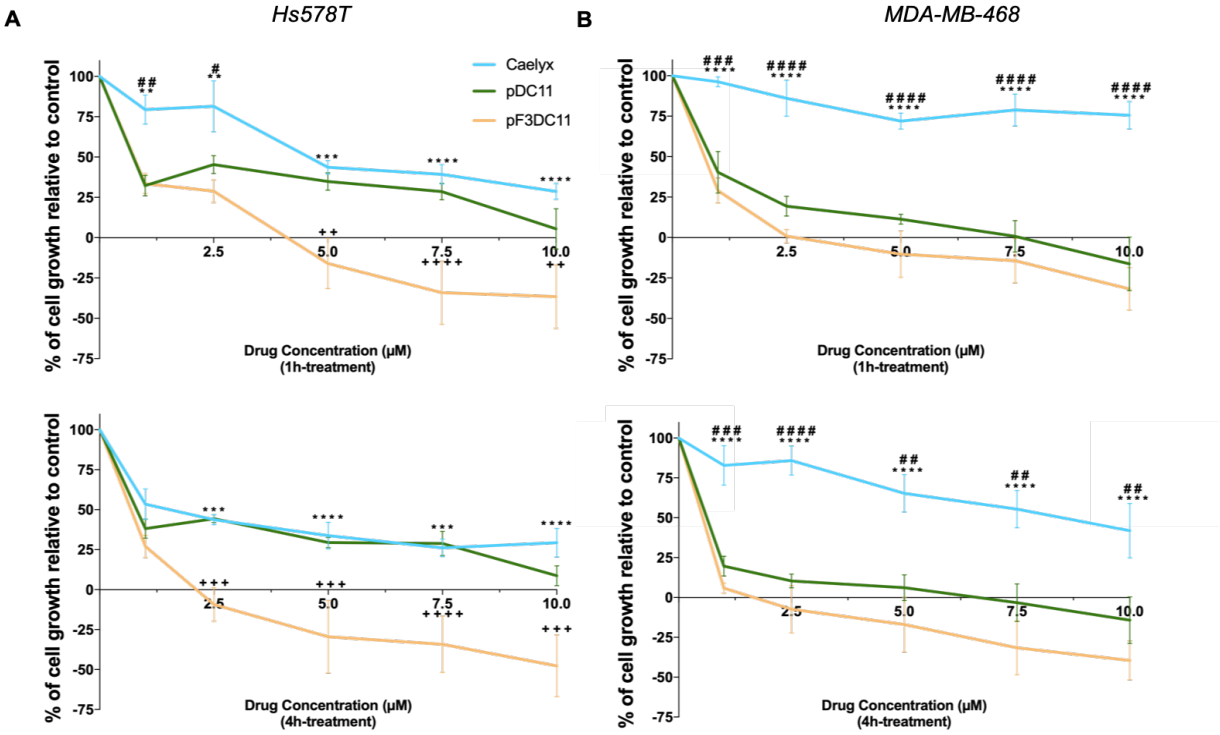

Figure 8. The liposomal formulations have cytotoxic effect in the in Hs578T and MDA-MB-468 cell lines.
The cell growth inhibition of **(A)** Hs578T and **(B)** MDA-MB-468 cell lines was analyzed with the SRB assay, 96h after treatment. Treatment period was for 1 hour (**upper panels**) or 4 hours (**lower panels**) with 5 defined concentrations of Caelyx® (blue), pDC11 (green) and pF3DC11 (orange): 1, 2.5, 5, 7.5 and 10 µM. The results are presented as percentage (%) of cell growth relative to untreated cells (control). The OD_{510} of the no-growth control (at time 0h) was subtracted to the final OD_{510} for each condition. Data are expressed as mean ± SEM from at least three independent experiments. *p<0,05; **p<0,001; ***p<0,0001; ****p<0,00001 comparing between formulations. *Caelyx® vs. pF3DC11; #Caelyx® vs. pDC11; +pF3DC11 vs. pDC11. Two-way ANOVA followed by Turkey's test.

Regarding the reduced cytoxicity of Caelyx® when compared with both novel formulations, two non-mutually exclusive hypothesis are suggested, to explain these results. The first is that the increased cytotoxic effect of the novel formulations is due to the simultaneous intracellular delivery of C6-Cer and DRX. This hypothesis is sustained by the findings of Ji *et al*. who demonstrated, in the MCF7 BC cell line, that the simultaneous treatment with exogenous C6-Cer and DRX led to greater levels of DRX-induced apoptosis and consequently decreased cell viability when compared with treatment with DRX

alone[111]. The other hypothesis is related with different rates of internalization and cargo release, due to the constitution of the liposomal formulations. Although pDC11, pF3DC11 and Caelyx® are all PEGylated liposomal formulations, the first two, unlike Caelyx®, are pH-sensitive. This is particularly relevant considering the internalization of liposomes through the endocytic pathway. They are internalized by the early and then late endosomes, which have a lower pH than the cytoplasm. This drop in pH leads to the destabilization of pH-sensitive liposomes which in turn results in a further destabilization of endosomal membranes and consequently in an early release of their cargo into the cytoplasm before reaching the lysosomes[118,119]. Hence, it is expected that pH-sensitive liposomes, like pDC11 and pF3DC11, deliver their cargo more effectively to the cytoplasm than the non pH-sensitive Caelyx®, increasing their cytotoxic effects. Combined, the simultaneous delivery of DXR and C6-ceramid and the increase in intracellular delivery due to the liposomes' constitution, may explain the differences observed in the cytotoxicity between the novel formulations and Caelyx®.

Moreover, pF3DC11 was the most cytotoxic formulation in both cell lines, probably due to the active targeting provided by the F3 peptide, thus highlighting the importance of active targeting that promotes a receptor-mediated endocytosis of the formulations, proven to be more efficient than non-specific endocytosis[120]. Additionally, different genetic backgrounds may affect the efficiency of NCL-targeted formulations, since our data show that pF3DC11 was more cytotoxic in the Hs578T cell line than in the MDA-MB-468 cell line, when compared with the non-targeted formulation. This difference may be due to a distinct expression of cell-surface NCL by the two cell lines. This hypothesis is being confirmed by our collaborators from this project (at CNC, Coimbra).

Finally, the concentration of 10 µM was selected for the following assays since, at this concentration, both NCL-targeted and non-targeted formulations have the highest cytotoxicity on both TNBC cell lines. Furthermore, this concentration provided a significant difference in effect between the targeted and non-targeted formulations, in the Hs578T cell line, allowing to study this difference in effect provided by these two formulations.

1.1. Decrease in cell viability following a 24h treatment period with nucleolin-targeted formulation, non-targeted formulation and Caelyx®

After evaluating the cytotoxic effect of the liposomal formulations, the effect on cell viability after a longer (24h) treatment period was determined, using the trypan blue exclusion assay. This method allows the determination of the viable cell number by excluding non-viable cells, which are permeable to the trypan blue dye. Briefly, both cell lines were incubated for 24h with 10 µM of the formulations or with 4 µM of free DRX, used as a positive control (known to reduce cell viability and induce apoptosis in the two cell lines)[121,122].

As shown in **Figure 9A,** both pDC11 and pF3DC11 caused a stronger decrease than Caelyx® in the viable cell number (which was statistically significant in the case of pF3DC11) in the Hs578T cell line. Additionally, free DRX (at a lower concentration, 4 µM) caused a stronger reduction in viable cell number than Caelyx®. For the MDA-MB-468 cell line (**Fig. 9B)** both pDC11 and pF3DC11 caused a stronger and statistically significant reduction in viable cell number than Caelyx®. In this cell line, treatment with free DRX led to a higher reduction in viable cell number than treatment with any of the tested formulations at 10 µM (statistically significant in the case of pDC11 and Caelyx®).

These results are in accordance with the cytotoxic effect of pDC11 and pF3DC11 observed in both cell lines (**Fig. 8**), with the NCL-targeted formulation being the most effective in decreasing the relative viable cell number.

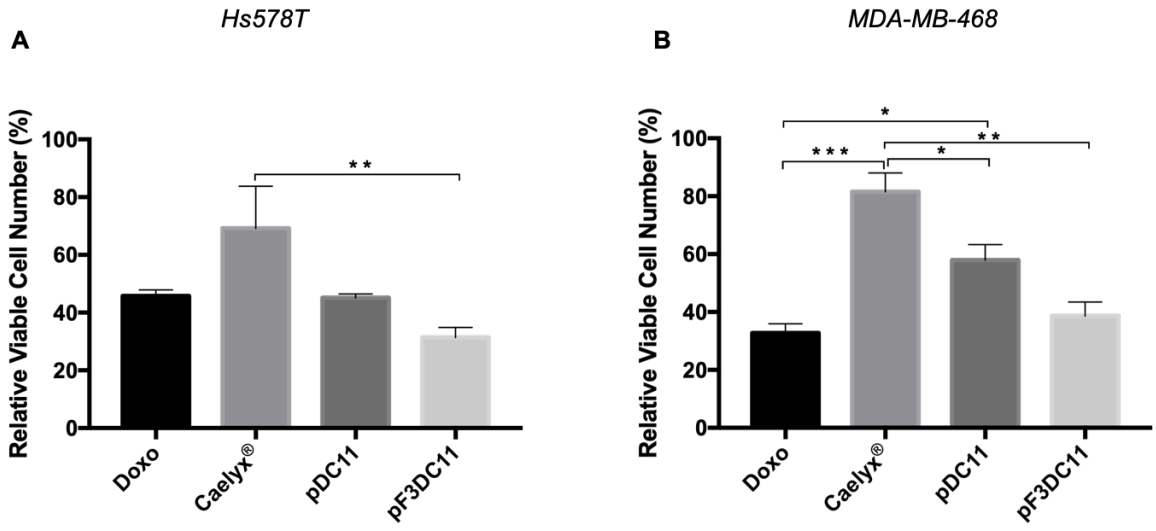

Figure 9. Targeted and non-targeted liposomal formulations decrease viable cell number of Hs578T and MDA-MB-468 cell lines.

Viable cell number of **(A)** Hs578T and **(B)** MDA-MB-468 cells determined using the trypan blue exclusion assay. Cells were incubated with 10 µM with Caelyx®, pDC11 or pF3DC11 for 24h. Additionally, 4 µM of free DRX (Doxo) was used as a positive control. Data are expressed as mean ± SEM from three independent experiments (n=3). *****p<0,05; ******p<0,001; *******p<0,0001. One-way ANOVA followed by Turkey's test.

2. Induction of apoptosis and autophagy by the liposomal formulations

2.1. Apoptosis

Apoptotic cell death is a very tightly regulated and hierarchical process. After an apoptotic stimulus, cells trigger the release of several proteins leading to a series of biochemical events that ultimately result in the proteolytic activation of procaspases, as the main executioners of apoptosis. Caspase-3 is one of the most important effectors of apoptosis and, together with caspase-7, cleaves PARP-1, which is another well-known marker of apoptosis. Therefore, high levels of cleaved PARP-1 and procaspase-3 are considered strong markers for cells dying by apoptosis[123]. Analyzing these markers allows assessment of apoptosis when other more specific assays (such as the Annexin V/PI staining and analyses by Flow Cytometry) cannot be executed due to autofluorescence of the tested formulations or drugs, as in the case of the present work. Indeed, since DXR is autofluorescent, analysis of apoptosis with the more specific and sensitive Annexin V/PI assay could not be performed.

Therefore, to understand if the decreased viable cell number of Hs578T and MDA-MB-468 cells in response to the liposomal formulations was due to the induction of apoptosis, the cleavage of these two markers of apoptosis - PARP-1 and procaspase-3 - was monitored by Western Blot after a 24 hours treatment with 10 μM of pDC11, pF3DC11 or Caelyx®. These results suggest that pDC11 and pF3DC11, containing both DRX and C6-Cer, promoted the cleavage of PARP-1 to a higher extent than Caelyx®, in both Hs578T and MDA-MB-468 cell lines, as shown by the Western Blot analysis and respective quantification (**Fig. 10A, 3B**). In accordance, increased levels of cleaved procaspase-3 were also observed following treatment with the pDC11 or pF3DC11 formulations in both cell lines (**Fig. 10C, 10D).** The cells were also treated during the same period of time with 4 μM of free DRX, as a positive control for the induction of apoptosis. Free DRX appeared to induce higher levels of both cleaved PARP-1 and caspase-3 than encapsulated DRX, Caelyx®. This difference was more significant in the MDA-MB-468 cell line (**Fig. 10B, 10D**).

The differences observed both in viable cell number (**Fig. 9**) and in the levels of cleaved PARP-1 and caspase-3 (**Fig. 10C, 10D**) between free (Doxo) or encapsulated DRX in Caelyx® can be explained by the different intracellular distribution of DRX once it reaches the cells. Indeed, Seynhaeve *et al*. demonstrated, in a melanoma cell line model, that DRX uptake to the nucleus was significantly higher (~ 7-fold) when the cells were treated for 24h with free DRX than with the a Caelyx® analogous, Doxil®. Interestingly, the accumulation of DRX in the cytoplasm was higher in the treatment with Doxil®, which suggests that this delivery system leads to a primary release of DRX in the cytoplasm, taking a longer time to reach the nucleus than free DRX. Since DRX exerts its cytotoxic role in the nucleus, such differences in intracellular distribution may justify the here observed increase in cytotoxic effect and induction of apoptosis by free DRX (Doxo) than by encapsulated DRX (Caelyx®), in these two cell lines and for this particular time-point.

In addition, the apparent higher sensitivity of the MDA-MB-468 cell line to free DRX, when compared with the Hs578T cell line, could be explained by the molecular intrinsic subtype that these cells lines represent. The MDA-MB-468 cell line has the gene expression pattern of a BL-1 subtype of TNBC, whereas the Hs578T cell line is representative of the M subtype (described in chapter I, section 1.3). The BL-1 subtype is more enriched in genes that promote proliferation than the M subtype, therefore it is expected that MDA-MB-468

cells would be more sensitive to drugs that interfere with cell cycle progression, as is the case of DRX.

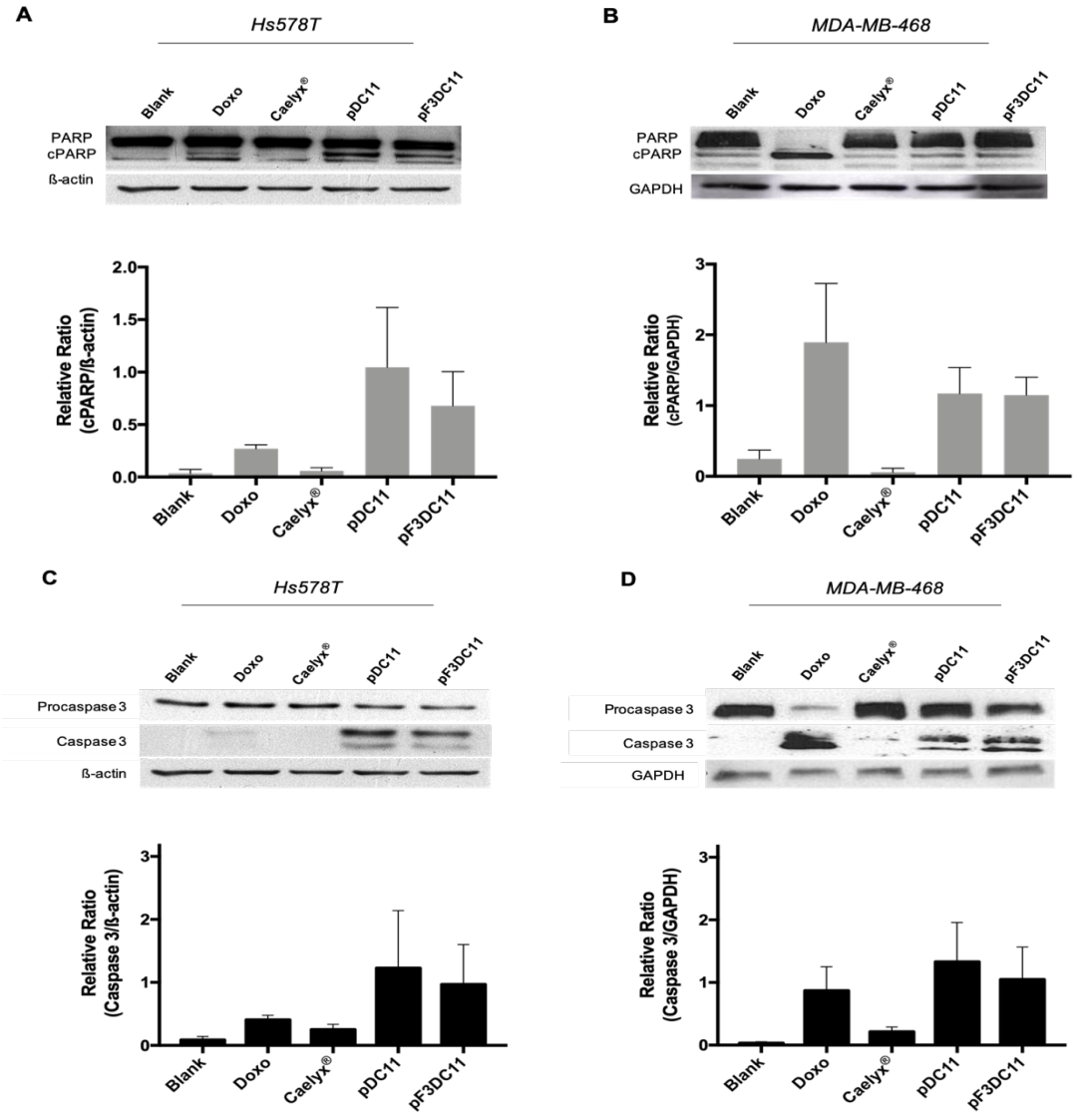

Figure 10. Targeted and non-targeted liposomal formulations induce the cleavage of PARP-1 and procaspase-3.

Representative Western Blots and respective quantification graphs of **(A, B)** cleaved PARP (cPARP) and **(C, D)** caspase 3 protein levels in the Hs578T and MDA-MB-468 cell lines. Cells were maintained in complete medium (Blank) or continuously incubated with 10µM of Caelyx®, pDC11 or pF3DC11 for 24h. Treatment with 4 µM of free DRX (Doxo) was used as a positive control for the induction of apoptosis. GAPDH and ß-actin were used as a loading control for the MDA-MB-468 and Hs578T cell lines, respectively. Protein/loading control ratios were determined using the *Image Lab* software. Western Blots are representative of three independent experiments (n=3) and quantification of the blots is expressed as the means ± SEM from three independent experiments.

Regarding the effect of combining DRX and C6-Cer in apoptosis, Ji *et al*. described that one of the mechanisms by which DRX induced cell death, and more specifically apoptosis, was through the activation of the AMP-activated protein kinase (AMPK). More importantly, these authors showed that C6-Cer dramatically increased DRX-induced apoptosis in several cell lines, by enhancing the activation of AMPK leading to a chemo-sensitizing effect[111]. Hence, the results here presented support the hypothesis that Cer might enhance DRX-induced apoptosis- To our knowledge, this is the first demonstration of the effect of this drug combination in the induction of apoptosis in these two TNBC cell lines. Notwithstanding, more assays would be required to confirm this hypothesis, namely by Flow Cytometry with a modified version of the above mentioned Annexin V/PI assay and cell cycle analysis following incubation with PI.

2.2. Autophagy

Induction of apoptosis is known as the first defense against damaged cells. Autophagy was previously thought to be a pro-survival mechanism, but recent studies suggest that in some tumor types autophagy could play an inverse role, promoting tumor development and survival to chemotherapy. Therefore, in cells that depend on high autophagy levels to survive, the total or partial inhibition of autophagy may increase the efficacy of specific therapies[124].

To determine if the liposomal formulations caused alterations in the autophagic flux, the levels of LC3-II protein (a well know marker for measuring alterations in the autophagic flux) were monitored. During the formation of the autophagosomes, the soluble LC3 protein (LC3-I) is lipidated and converted into LC3-II, which remains bound to the outer and inner membrane of the autophagosomes until fusion with the lysosome occurs, where the LC3-II bound to the inner membrane is degraded and the LC3-II bound to the outer membrane is recycled to cytosol[125]. Thus, the processes that contribute to alterations in LC3-II levels are the synthesis of new LC3, the degradation of LC3-II in the lysosome and the deconjugation from the membrane and conversion into LC3-I[126]. To correctly measure changes in the autophagic flux, the current guidelines on the field state that one should measure the differences in LC3-II levels in the absence and presence of lysosomal inhibitors. The inhibitor used in this work was Baf. A1. This inhibitor blocks the acidification

of the lysosomes by inhibiting the vacuolar H+ ATPase, therefore inhibiting the degradation of the cytoplasmatic contents delivered to the lysosome through autophagosomes, including proteins linked to the inner membrane of the autophagosome. Consequently, LC3-II accumulates in the lysosome instead of being degraded in the course of the autophagic pathway. Therefore, in order to see if autophagy is induced (or not) by a given treatment, the levels of LC3-II protein in the absence of Baf. A1 should be subtracted from the levels of LC3-II protein in the presence of Baf. A1; This analysis is sufficient to prove that there is (or not) an increase of the autophagic flux following a specific treatment, when compared with untreated or controls conditions[126].

Representative Western Blots (**Fig.11, upper panels**) and respective quantifications (**Fig.11, lower panels**) show that untreated Hs578T and MDA-MB-468 cells have an efficient autophagic flux, as shown by the increased accumulation of LC3-II upon inhibition of lysosomal degradation with Baf. A1. This is in accordance with previously observed studies in these cell lines[127,128]. Treatment with 10 μM of pDC11 or pF3DC11 for a 24 hours period induced the accumulation of LC3-II in the absence Baf.A1. However, the level of LC3-II did not increase further in the presence of Baf.A1, in comparison with the same condition in untreated cells. This result suggests that the liposomal formulations caused a partially block of the autophagic flux, given the observed decrease in LC3-II accumulation, measured by the subtraction of LC3-II levels without Baf.A1 treatment from the LC3-II with Baf.A1 treatment (**Fig.11, lower panels**).

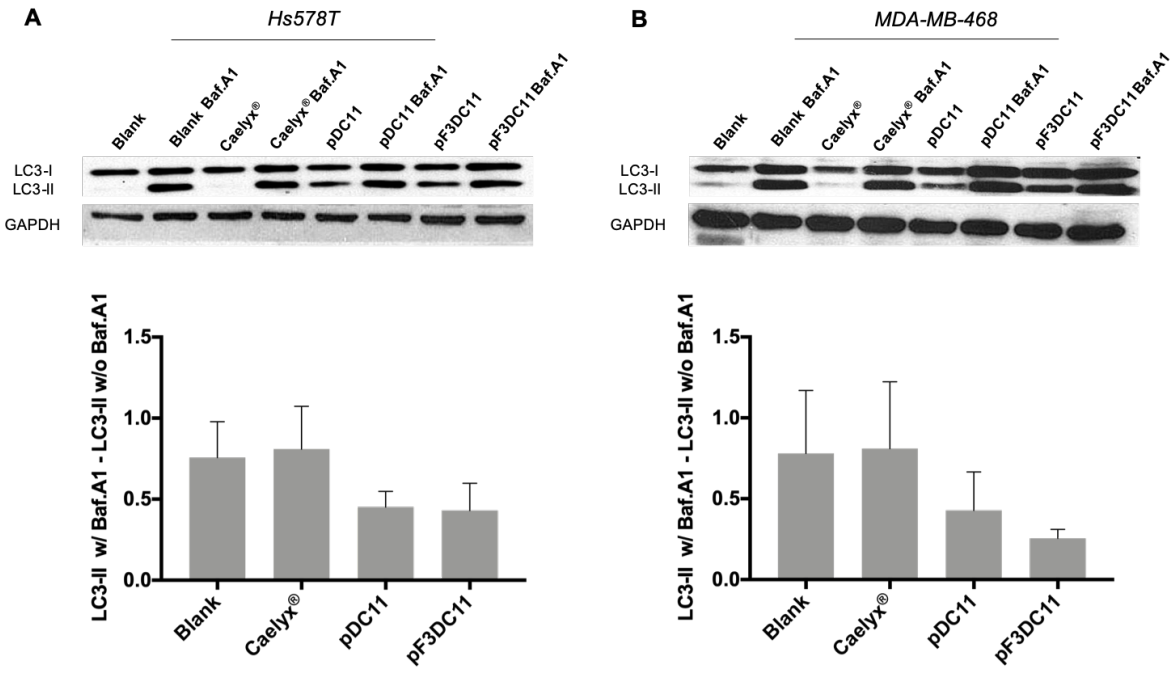

Figure 11. Targeted and non-targeted formulations partially block the autophagic flux.

Western Blot analysis of LC3-II protein levels in **(A)** Hs578T and **(B)** MDA-MB-468 cell lines. Cells were maintained for 24h in complete medium (Blank) or continuously incubated with 10µM of Caelyx®, pDC11 or pF3DC11 in the presence or absence of 20 nM Baf.A1. GAPDH was used as a loading control. LC3-II/GAPDH ratios were determined using the *Image Lab* software and used to measure the autophagic flux by subtracting the normalized LC3-II levels in the absence of Baf.A1 from the corresponding levels obtained in the presence of Baf.A1. Western Blots are representative of three independent experiments (n=3) and quantification of the blots is expressed as the means ± SEM from three independent experiments.

Moreover, both pDC11 and pF3DC11 blocked the autophagic flux to a similar extent, whereas cells incubated with Caelyx® appear to have an efficient autophagic flux, similar to the observed in untreated cells (Blank) (**Fig. 11, lower panels**). Since all liposomal formulations were unable to further increase the autophagic flux in comparison with untreated cells, the ability of both cell lines to induce autophagy in response to starvation - an autophagic stimuli (positive control) - was determined. Thus, Hs578T and MDA-MB-468 cells were starved, using HBSS medium for 24h, and the levels of LC3-II protein were measured in the presence or absence of Baf.A1. Contrary to the expected, neither of the cell lines induced autophagy in response to starvation, as shown by the decrease in the levels of LC3-II protein upon inhibition of lysosomal degradation with Baf.A1 (**Fig.12**). These results suggest that these cell lines are not good cellular models to explore the role of autophagy in the cytotoxicity of the formulations, since they counteract autophagy

induction, even in response to starvation which is a positive control commonly used for autophagy induction[129].

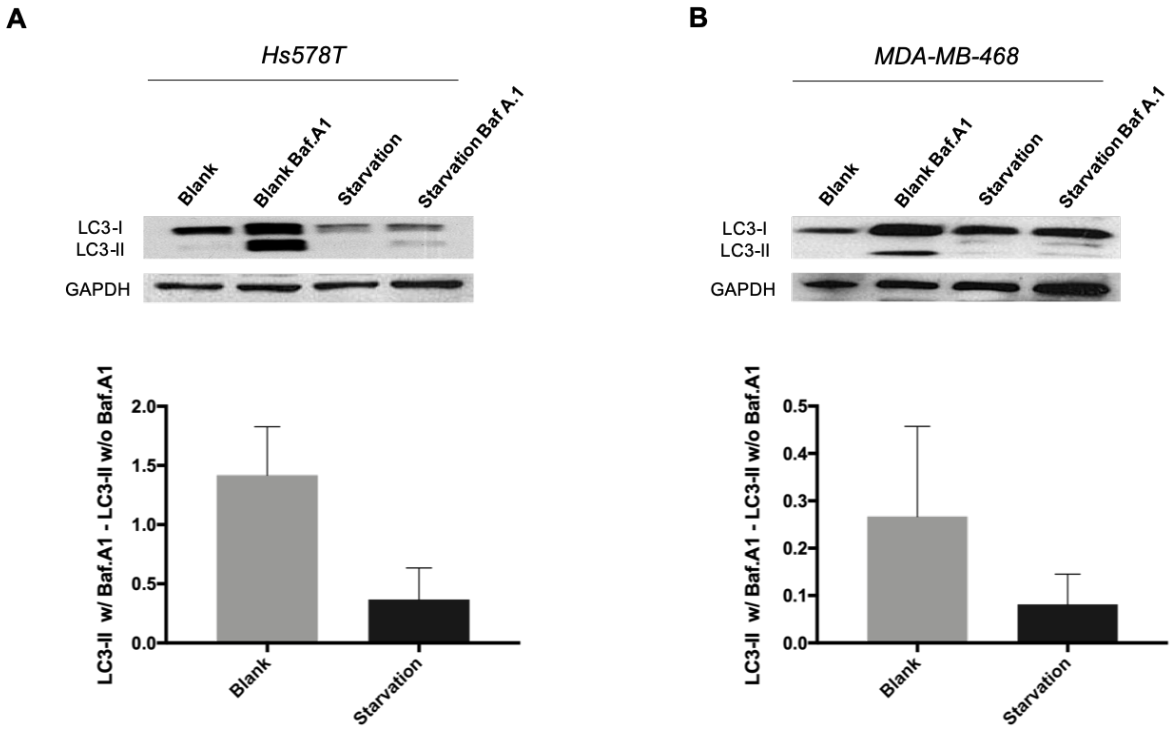

Figure 12. Starvation does not induce the autophagic flux of Hs578T and MDA-MB-468 cell lines.
Western Blot analysis of LC3-II protein levels in **(A)** Hs578T and **(B)** MDA-MB-468 cell lines. Cells were maintained for 24h in complete medium (Blank) or in HBSS (Starvation) in the presence or absence of 20 nM Baf.A1. GAPDH was used as a loading control. LC3-II/GAPDH ratios were quantified using the *Image Lab* software and used to determine the autophagic flux ratio by subtracting the normalized LC3-II levels in the absence of Baf.A1 from the corresponding levels obtained in the presence of Baf.A1. Western Blots are representative of three independent experiments and quantification of the blots is expressed as the means ± SEM from three independent experiments (n=3).

3. Cytotoxic effect in a non-tumorigenic breast cell line

MCF12A cells, derived from a reduction mammoplasty, are characterized by the absence of hormone receptors and an expression of epithelial and mesenchymal markers[130]. To determine if the formulations affected the growth of these cells, the non-tumorigenic MCF12A cell line was treated with 10 µM of each liposomal formulation for 1 hour (**Fig. 13A**) or 4 hours (**Fig. 13B**), and then further incubated for 120h (without formulations) before performing the SRB assay. The concentration used in this assay (10 µM) was the same used in most of the remaining assays presented in this dissertations (in which a high

cytotoxic effect in cancer cell lines was verified, with a stronger effect being verified with the targeted than the non-targeted formulation) and in other studies[103] However, the here tested concentrations are not likely to be representative of physiological concentrations. In fact, given the high concentrations tested in the present work, all formulations caused toxicity in this cell line, including the FDA approved Caelyx®. Nonetheless, results showed that both pDC11 and pF3DC11 were more cytotoxic than Caelyx® (used as a control), as can be seen by the higher decrease in cell growth, at both time-points studied. In addition, the targeted formulation (pF3DC11) was significantly more cytotoxic than both the non-targeted formulation (pDC11) and Caelyx®, inducing cell death of this cell line (depicted by the relative cell growth values lower than zero).

These results are not conclusive regarding possible toxicity of the formulations to non-tumor breast cells, since as mentioned above the concentration used in this assay was high (10 µM) and probably is not representative of physiological concentrations. In addition, this cell line is not an ideal model to evaluate toxicity since the cells are not completely normal cells. Therefore, conclusions should not be taken based only on this cellular model and further assays are required, particularly *in vivo* toxicity assays carried out in proper animal models and with physiological concentrations, in order to confirm the safety of the novel formulations.

Since the cytotoxic effect was lower when reducing the treatment period from 4 to 1 hour, this particular treatment period was selected for further experiments.

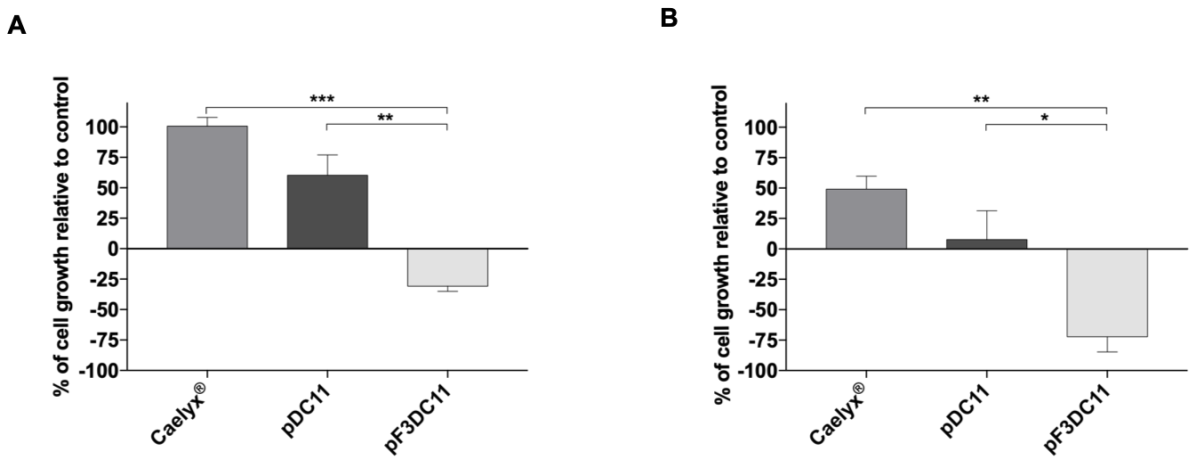

Figure 13. The liposomal formulations (10 µM) inhibit cell growth in a non-tumorigenic cell line.

The cell growth of MCF-12A cells was analyzed with the SRB assay, 120h after **(A)** 1 hour or **(B)** 4 hours of treatment period with 10 µM of Caelyx®, pDC11 or pF3DC11. Results are presented as percentage (%) of cell

growth relative to untreated cells (control). The OD_{510} of the no-growth control (at time=0h) was subtracted to the final OD_{510} for each condition. Data are expressed as mean ± SEM from three independent experiments (n=3). *****p<0,05; ******p<0,001; *** p<0,0001. One-way ANOVA followed by Turkey's test.

4. Cell growth inhibition effect in sorted *putative* CSCs and non-CSCs from the Hs578T cell line

4.1. Isolation of putative CSCs from the HS578T cell line

The cancer stem cell model is based on the existence of a small subset of cells, within a tumor, that are responsible for sustaining tumorigenesis and generating heterogeneity through differentiation[131]. This sub-population is resistant to both chemotherapy and radiotherapy, playing an important role in tumor recurrence, through several resistance mechanisms such as the induction of EMT or the expression of drug efflux pumps and detoxifying proteins (such as ALDH), among others[54]. Thus, the development of new therapeutic tools that can target not only the bulk of the tumor but also this sub-population is of the most importance.

Thus, in order to assess if the novel liposomal formulations, particularly the one with the target for NCL (pF3DC11), were able to specifically target putative CSCs sorted from the Hs578T cell line, cell sorting was performed in order to separate the putative CSCs from the non-CSCs.

The markers selected for the isolation of the putative CSCs were the high activity of ALDH enzyme and high expression of CD44 receptor. Briefly, the putative CSC sub-population was isolated by FACS using ALDH activity as the primary sorting criteria and $CD44^{high}$ as the secondary criteria. The cell population negative for ALDH activity was gated on **P4** using the ALDH inhibitor, DEAB, as control **(Fig. 14A)**. In the upper panel of **Figure 14B** the representative gating of both positive (**P5**) and negative (**P4**) ALDH sub-populations from the whole cells' population is depictured. For the three independent experiments performed, the average amount of cells positive for ALDH in this sub-population was 0.75 ± 0.27 %, hence, the great majority of Hs578T cells were negative for this marker. Regarding the expression of CD44, on average 85% of all cells that were positive for also presented high expression of CD44 – **P8** (**Fig. 14B, lower right panel**).

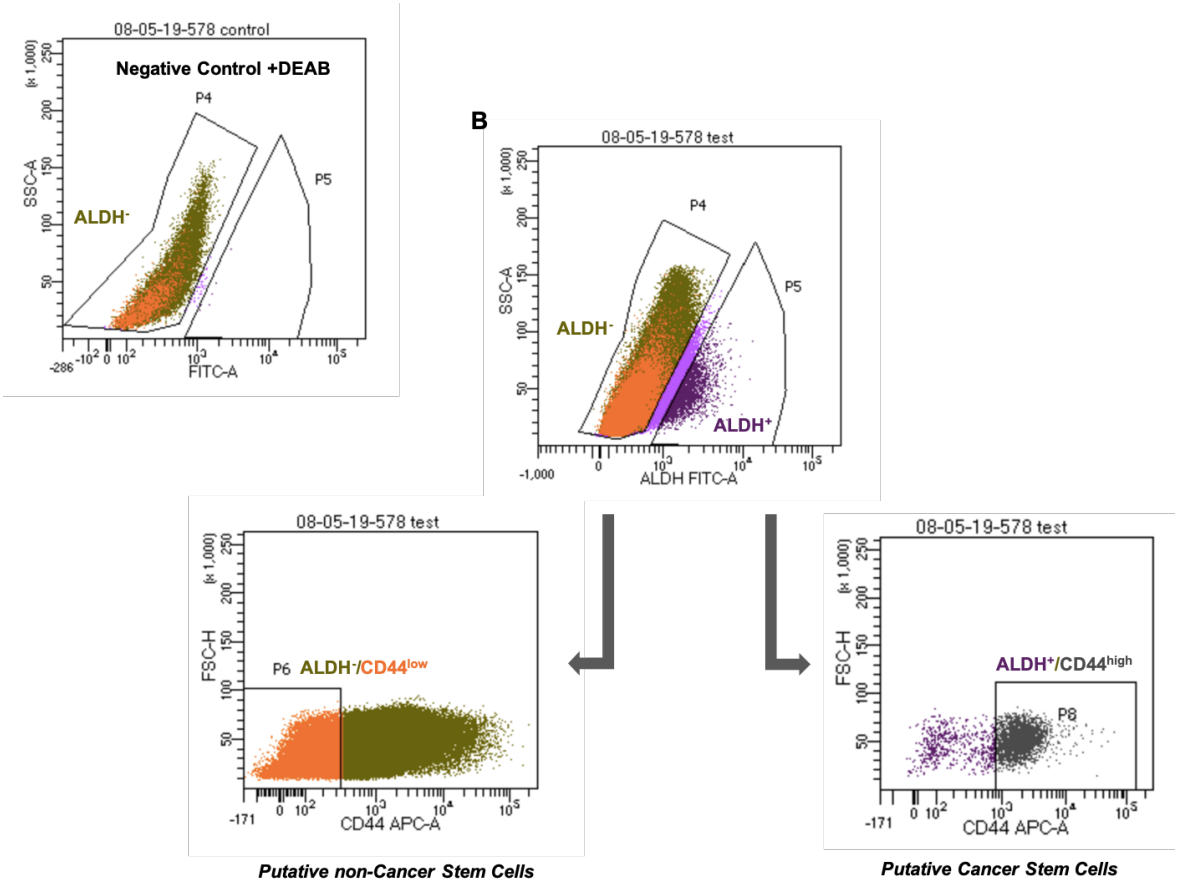

Figure 14. Strategy for isolation of putative CSCs and non-CSCs from the Hs578T cell line.
The ALDEFLUOR™ assay was performed in the Hs578T cell line in combination with labelling for CD44 with APC-conjugated anti-CD44 antibody. Single fluorescent markers were used to perform the required compensations. **(A)** The DEAB reagent, inhibitor of ALDH, was used as a negative control to determine the ALDH negative sub-population region - **P4**. **(B)** The **P5** sub-population represents cells positive for ALDH activity. The **P6** sub-population represents sorted cells that did not present ALDH activity and have low expression of membrane CD44 – $ALDH^-/CD44^{low}$ – the putative non-CSCs. The **P8** sub-population represents the sorted cells that presented both ALDH activity and high expression of membrane CD44 – $ALDH^+/CD44^{high}$ - the putative CSCs. The data shown in the dot plots is from one independent experiment but is representative of 3 independent experiments.

On the other hand, only about 6.5 % of all cells that were negative for ALDH had low expression of membrane CD44 – **P6** (**Fig. 14B, lower left panel**). Curiously, Phi *et al.* described that the average population positive for ALDH activity in this cell line was ~ 3% which is 4-fold higher than the percentage obtained in this work[54]. This reduction observed in the present dissertation maybe due to the gap left between the gates **P4** and **P5**, when performing the cell sorting, necessary to ensure the complete separation of the two sub-populations and to guarantee high purity of the sorted populations.

Regarding the isolated putative CSCs, the average percentage of cells sorted from the whole population in the three experiments was 0.65±0.2%, which is similar to the % of $ALDH^{high}$ cells.

4.2. Cytotoxic effect of the liposomal formulations in sorted $ALDH^{+}/CD44^{high}$ and $ALDH^{-}/CD44^{low}$ sub-populations.

The two distinct sub-populations sorted from Hs578T cells were $ALDH^{+}/CD44^{high}$ and $ALDH^{-}/CD44^{low}$, the putative CSCs and non-CSCs, respectively. In order to determine and compare the effect of the formulations in the growth of these two sub-populations of cells, they were incubated with 10 µM of pDC11, pF3DC11 or Caelyx® for 1 hour before continuing with the SRB assay which was terminated 96h later. Results showed that pF3DC11 is more effective than Caelyx® in reducing the growth of both cell sub-populations (**Fig. 15**). Nevertheless, pDC11 was nearly equally effective as Caelyx® in the putative CSC population, whereas it was more effective than Caelyx® in the non-CSC population.

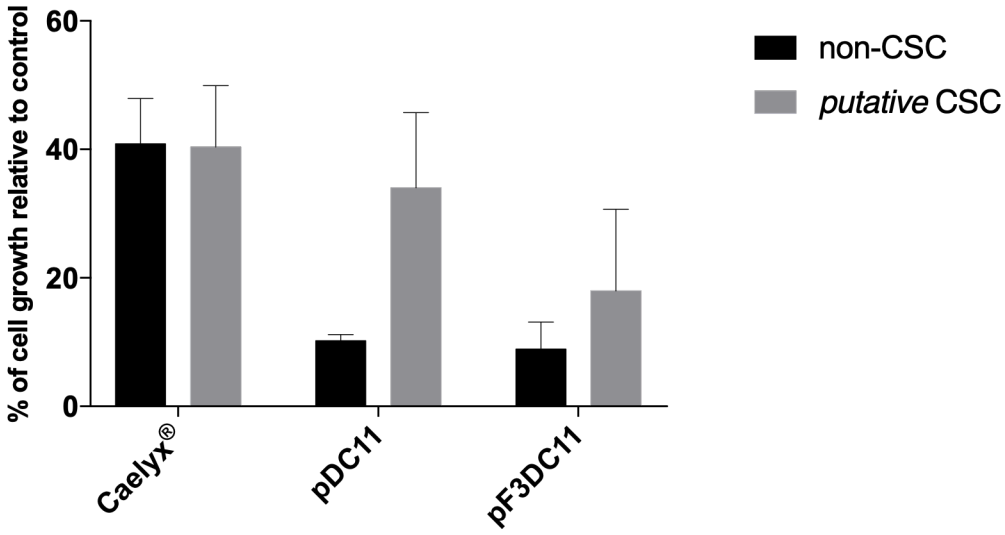

Figure 15. Growth inhibition effect of the formulations in sorted putative CSCs and non-CSCs sub-populations of Hs578T cells.

$ALDH^{+}/CD44^{high}$ and $ALDH^{-}/CD44^{low}$ sub-populations of Hs578T cells were sorted and incubated with 10 µM of Caelyx®, pDC11 or pF3DC11 for 1h. Later, 96h after this treatment, cell growth was determined with the SRB assay. Results are presented as percentage (%) of cell growth relative to untreated cells (control). Data are expressed as mean ± SEM from three independent experiments (n=3).

Moreover, the putative CSCs appear to be less sensitive to the treatment with pDC11 or pF3DC11 when compared with the non-CSCs. Interestingly, no difference was observed in the effect of Caelyx® between these two sub-populations.

The decreased sensitivity of putative CSCs to the novel formulations in comparison with the non-CSCs is in agreement with the current literature, given their resistance to chemotherapeutic drugs.

Most interestingly, it is possible to observe an increased cytotoxic effect of the NCL-targeted formulation in the putative CSC sub-population, when compared with the non-targeted formulation. However, this trend is not observable in the non-CSCs, in which both targeted and non-targeted formulations appear to have the same effect. This observed difference in the cytotoxic effect of the targeted and non-targeted formulations, in the putative CSC sub-population, supports the findings of Fonseca *et al.* describing NCL as a surface marker specific of CSCs that allowed the targeting of these cells in another TNBC cell line[103].

IV

Conclusion

1. Concluding remarks

Currently, TNBC is the most challenging subtype of BC regarding treatment of the disease. Triple-negative tumors frequently acquire resistance to conventional chemotherapy, causing high relapse and mortality rates in patients[28]. The poor prognosis of TNBC can be related with the presence of a niche of CSCs within the tumor. With this in mind, Nuno Fonseca *et al.* developed a novel liposomal formulation with the purpose of eradicating both CSCs and non-CSCs from TNBC. This formulation actively targets cell-surface NCL, found overexpressed at the surface of MDA-MB-231 TNBC cell line and in their sorted CSCs[103]. Moreover, this formulation encapsulates defined amounts of DRX and C6-Cer, whose combined delivery has shown a significant effect in inducing cytotoxicity of tumor cells and helping to overcome DRX-induced resistance[113]. Despite the remarkable work of these authors, there is still the need to assess the cytotoxic potential of this formulation in other TNBC cell lines and in non-tumorigenic cell line models. Additionally, the mechanism by which the combination of DRX and C6-Cer induces cytotoxicity is poorly understood in TNBC cell line models and needs to be elucidated. Finally, the effect of these formulations in putative CSC and non-CSC sub-populations, sorted from a TNBC cell line, should be studied.

The results presented in this dissertation demonstrate that regarding the cell growth inhibition effect determined with the SRB assay, both targeted and non-targeted formulations (encapsulating DRX and C6-Cer), were more cytotoxic to both cell lines than Caelyx® (containing only DRX), in almost every concentration and in the three treatment periods tested. Furthermore, the NCL-targeted formulation was more cytotoxic than the non-targeted one, for both cell lines and in the majority of the concentrations tested. This observation further suggests that targeting cell-surface NCL may be relevant in the TNBC context, as previously suggested by our collaborating team that produced these formulations[103]. Regarding the trypan blue exclusion assay, the results obtained showed the same trend, supporting the conclusion that both targeted and non-targeted formulations are more cytotoxic than Caelyx® to the here studied tumor cell lines, in the concentration and treatment periods tested. These differences were more accentuated in

the MDA-MB-468 cell line, reaching statistical significance when comparing the reduction in the number of viable cells caused by Caelyx® or by the novel formulations.

Based on the expression levels of caspase-3 and PARP-1, it was verified that both novel formulations (targeted and non-targeted), were able to induce apoptosis to a greater extent than Caelyx® in both TNBC cell lines. The differences observed between the targeted and non-targeted formulations in cytotoxicity (measured by the SRB and trypan blue assays), were not reflected by differences in the capacity to induce apoptosis. Interestingly, when treated with free DRX, the MDA-MB-468 cell line which is representative of the BL-1 subtype showed greater expression of apoptotic markers than the Hs578T cell line which is representative of the M subtype. Thus, the possible clinical relevance of the TNBC subtypes regarding response to free DRX was also demonstrated here.

Concerning alterations in autophagy, based on the levels of the LC3-II marker, it was possible to conclude that both targeted and non-targeted formulations induced a partial block in the autophagic flux, unlike Caelyx®. This partial block in combination with the induction of apoptosis offers a possible explanation for the differences observed regarding cytotoxicity, between the novel formulations and Caelyx®.

The effects of both targeted and non-targeted novel formulations, compared with Caelyx® (as a control), on the growth of the non-tumorigenic cell line MCF12A were determined, thus fulfilling the third aim of this dissertation. It was observed that the targeted formulation was significantly more cytotoxic to this cell line than both the non-targeted one or Caelyx®, inducing cell death in this cell line. Nonetheless, these results are not conclusive regarding possible toxicity of the formulations to non-tumor breast cells, since the concentration used in this dissertation is probably not representative of physiological concentrations and this cell line is not an ideal model to evaluate toxicity. Therefore, further assays are required in order to confirm the safety of the novel formulations.

Cells presenting activity of ALDH and expressing high levels of CD44 (putative CSCs) were successfully sorted from the whole population of the Hs578T cell line, as well as cells without activity of this enzyme and low surface expression of CD44 (putative non-CSCs). The cytotoxic effect of the targeted and non-targeted novel formulations, and of Caelyx® as a control, was assessed on both sub-populations. Results showed that pF3DC11 was more effective than Caelyx® in reducing the growth of both cell sub-populations and that

pDC11 was nearly equally effective as Caelyx® in the putative CSC population, whereas it was more effective than Caelyx® in the non-CSC population. These results are in accordance with previous observations regarding the CSCs intrinsic resistant phenotype[54]. Although the cytotoxic effect of both targeted and non-targeted formulations was very similar in the non-CSCs sub-population, the targeted formulation was more cytotoxic to the CSC sub-population than the non-targeted one. Hence, these results suggest that targeting cell-surface NCL increases the cytotoxicity of the here studied liposomal formulations in putative CSCs, corroborating the previous observations by Nuno Fonseca *et al*[103].

In conclusion, the results obtained in the context of this dissertation demonstrate that the simultaneous delivery of C6-Cer and DRX results in a more accentuated cytotoxic effect to the here studied TNBC cell lines than Caelyx®, associated with induction of apoptosis and alterations in the autophagic pathway. Despite the cytotoxicity verified in the non-tumorigenic cell line, at the high concentration tested, targeting cell-surface NCL with the F3 peptide increased the cytotoxicity to the whole population of the two TNBC cell lines here studied and also to the putative CSCs isolated from the Hs578T cell line.

2. Future perspectives

In the present dissertation, the cytotoxicity of the novel formulations was assessed *in vitro* in two TNBC cell lines cultured in monolayer. Future work should include the validation of the cytotoxic effect in 3D cell culture, since the 3D model is physiologically more relevant and is considered the best *in vitro* approach to determine cytotoxicity of therapeutic agents[132].

Regarding the induction of apoptosis by the novel formulations, the expression of other apoptotic markers such as Bcl-2 family members and other caspases should be evaluated. Concerning the study of the role of autophagy in the cytotoxicity of these liposomal formulations, another TNBC cell line more responsive to an autophagic stimuli like starvation, should be used in further assays.

To further evaluate the possible toxicity of this formulation in non-tumorigenic cells, the first approach could be to use another breast non-tumorigenic cell line (e.g., MCF10A) and then to use proper animal models to conduct *in vivo* work under physiological concentrations.

Finally, regarding the effect of the novel formulations in putative CSCs and non-CSCs, the markers used to isolate putative CSCs from the Hs578T cell line should be validated *in vivo,* by assessing the capacity of these putative CSCs to form heterogeneous tumors in immunocompromised mice. Additionally, the expression of known pluripotency markers such as NANOG and OCT-4 should be determined in the sorted putative CSCs and non-CSCs, to further confirm the stem phenotype.

V

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