Bruna Kreutzer

Polymeric Micelles to improve Salinomycin delivery to cancer cells

Dissertação de Mestrado em Biotecnologia Farmacêutica, orientada pelo Professor Doutor Luís Almeida e pela Doutora Mafalda Marques Videira e apresentada à Faculdade de Farmácia da Universidade de Coimbra

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// RESUMO

Introdução: A abordagem de utilizar nano medicina para tratar dentre outras doenças, o cancro vem sendo utilizada e estudada cada vez mais para ultrapassar as barreiras encontradas na terapia convencional. A utilização do polímero anfifílico não tóxico como o Pluronic F127 para a formação de nano partículas traz grandes vantagens por formar micelas de substâncias biologicamente ativas com tamanhos de 10 a 100 nm que tem como objetivo entregar fármacos pouco solúveis as células, além de terem a habilidade de entrar passivamente via aumento de permeabilidade e retenção das células tumorais aumentando assim a biodisponibilidade e acúmulo do fármaco nas células de interesse, diminuindo efeitos adversos causados por quimioterápicos. A Salinomicina (SAL) é um antibiótico isolado de *Streptomyces albus* que recentemente mostrou ser um novo candidato ao tratamento de cancro por inibir seletivamente células estaminais presentes no cancro responsáveis pelo ressurgimento da doença e resistência aos fármacos quimioterápicos.

Objetivos: O objetivo principal deste estudo é o design de micelas poliméricas (PM) para distribuição de SAL para células cancerígenas e sua caracterização em termos de diâmetro médio (MD), carga de superfície (ZP) e índice de poli dispersão (PDI). A capacidade de encapsulaçao de MP SAL é determinada e sua eficácia in vitro em relação ao modelo NSCLC foi avaliada e comparada com a atividade livre do fármaco.

Métodos: A formação de micelas foi feita pela técnica de reidratação de filme, onde o solvente orgânico contendo uma mistura de fármaco e polímero é evaporado a fim de formar um filme polimérico. Esse filme então é reidratado com água e agitado e há a formação de micelas. Ensaios *in vitro* como MTT, wound healing e imunocitoquímica foram realizados em triplicata com cultura celular em placas de 96 e 24 poços seguidos de tratamentos com fármaco livre (SAL), fármaco encapsulado (PM SAL) em micelas bem como micelas vazias (Plain PM) em diferentes tempos de incubação. Para a imunocitoquímica, anticorpos anti Pg-p e Vimentina foram utilizados a fim de observar no microscópio de fluorescência os níveis de expressão destas proteínas.

Resultados: A caracterização das micelas confirmou que o polímero utilizado foi capaz de formar micelas com tamanhos e índice de poli dispersão esperados (<100nm e < 0,4 respectivamente) já o potencial zeta foi diretamente alterado a medida que a concentração de polímero aumentava, ou seja, quanto maior a concentração de polímero mais próximo da neutralidade a carga de superfície. Para os resultados de MTT, SAL em sua forma não encapsulada mostrou ter efeito dose e tempo dependente em células de cancro de pulmão

após 48 horas e além disso, mostrou ser mais eficaz do que em sua forma encapsulada.
Nos ensaios de invasão celular (wound healing) SAL e PM SAL mostraram efeitos semelhantes na retenção de migração celular após 24 horas. Nos resultados de imunocitoquímica, SAL e PM SAL mostraram resultados muito semelhantes em inibir a expressão de Pg-p. Por fim, quanto a expressão de vimentina, pode-se observar que quando a concentração de fármaco foi aumentada para 20 μM, houve uma diminuição mais acentuada da expressão de vimentina nas células tratadas com PM SAL.

Conclusão: As micelas poliméricas apresentaram as características propostas para a entrega de SAL. Nos ensaios de viabilidade celular, não demonstraram ter efeito citotóxico mais acentuados do que em sua forma livre, porém as PM SAL resistiram ao processo biológico e sua eficácia foi demonstrada na inibição da migração celular, na evasão da Pg-p e inibição da vimentina. Isto indica que as PM SAL podem ser um eficiente coadjuvante no tratamento de NSCLC. Estudos posteriores serão necessários mais para entender melhor a internalização das micelas assim como se faz necessária a funcionalização com anticorpos para que as micelas sejam direcionadas para as células de interesse.

Palavras chave: *cancro de pulmão, salinomicina, nano medicina, micelas poliméricas, células estaminais tumorais.*

ABSTRACT

Introduction: The approach of using nano medicine to treat several diseases, for cancer has been increasingly used and studied to overcome the barriers found in conventional therapy. The use of the non-toxic amphiphilic polymer such as Pluronic F127 for the formation of nano particles brings great advantages by forming micelles of biologically active substances with sizes from 10 to 100 nm. This technique aims to deliver poorly soluble drugs to the cells, in addition to achieve the ability to passively enter via enhanced permeability and retention (EPR) of tumour cells thereby increasing the bioavailability and accumulation of the drug in the cells of interest, decreasing adverse effects caused by chemotherapeutics. Salinomycin (SAL) is an isolated *Streptomyces albus* antibiotic that has recently been shown to be a new candidate for cancer treatment by selectively inhibits cancer stem cells responsible for disease resurgence and resistance to chemotherapeutic drugs.

Objectives: The main goal of this study is the design of polymeric micelles (PM) for SAL delivery to cancer cells and their characterization in terms of medium diameter (MD), surface charge (ZP) and poly dispersion index (PDI). MP SAL loading capacity is determined and it's in vitro in effect against NSCLC model was assessed and compared with the free drug activity.

Methods: The preparation of micelles was done by the film rehydration technique, where the organic solvent containing a mixture of drug and polymer is evaporated to form a polymer film. This film is then rehydrated with water and vortex to form micelles. In vitro assays such as MTT, wound healing and Immunocytochemistry were performed in triplicate with cell culture in 96 and 24 plates followed by treatments with free drug (SAL), encapsulated drug (PM SAL) in micelles and empty micelles (Plain PM) in different incubation time points. For immunocytochemistry, anti-Pg-p and vimentin antibodies were used to observe the levels of expression of these proteins in the fluorescence microscope.

Results: The micelles characterization confirmed that the polymer was able to form micelles with expected MD and PDI (<100nm and <0.4 respectively), ZP was directly altered as a function on the polymer concentration. For the MTT results, SAL in its non-encapsulated form has shown to have dose and time dependent effect on lung cancer cells after 48 hours and furthermore proved to be more effective than in its encapsulated form. The wound healing results SAL and PM SAL had similar effects on retention of cell migration after 24 hours. In the results of immunocytochemistry, SAL and PM SAL seems to have very

similar results in inhibiting Pg-p expression. Finally, as for Vimentin expression, it can be seen that, when the drug concentration was increased to 20 μM, there was more marked decrease in Vimentin expression in the cells treated with PM SAL.

Conclusion: The polymeric micelles have presented the characteristics proposed for SAL delivery. In the cell viability assays, PM SAL were not shown to have more marked cytotoxic effect than in their free form, but PM SALs resisted the biological process and their efficacy was demonstrated in inhibition of cell migration, Pg-p evasion and inhibition of vimentin expression. This indicates that PM SAL can be an efficient adjunct in the treatment of NSCLC. Further studies will be needed for better understanding of the micelles internalization process, also the functionalization with antibodies is necessary to directing the micelles to the cells of interest.

Key words: *lung cancer, salinomycin, nanomedicines, polymeric micelle, cancer stem cells.*

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LIST OF ABBREVIANTIONS

ALDH: Aldehyde Dehydrogenase

BSA: Bovine Serum Albumin

CCD: Central Composite Design

CMC: Critical Micelle Concentration

CMT: Critical Micelle Temperature

CQA: Critical Quality Attributes

CSC: Cancer Stem Cells

CXCL1: Chemokine (C-X-C motif) ligand 1

DLS: Dynamic Light Scattering

DMSO: Dimethyl sulfoxide

DNP: Dinitrophenyl Hydrazyne

DOX: Doxorubicin

DTX: Docetaxel

ECM: Extracellular Matrix

EE: Encapsulation Efficiency

EGF: Epithelial Growth Factor

EGFR: Epidermal Growth Factor Receptor

EMT: Epithelial Mesenchymal Transition

EPR: Enhanced Permeability And Retention

ETO: Etoposide

FGF: Fibroblast Growth Factor

GTP: Guanosine Triphosphate

HGF: Hepatocyte Growth Factor

HIF-1: Hypoxia-Inducible Factor 1

ICH: International Council for Harmonisation

IL-1β: Interleukin 1-beta

IL-6: Interleukin 6

IL-8: Interleukin 8

KRAS: Kirsten Rat Sarcoma Viral Oncogene Homolog

M.D: Medium diameter

MDR: Multidrug Resistance

MET: Mesenchymal Epithelial Transition

MMPs: Matrix Metalloproteinases

MPS: Mononuclear Phagocytic System

MTT: 3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MTX: Metotrexate

nm: Nanometers

nmt: No more than

NP: Nanoparticle

NSCLC: Non Small Cell Lung Cancer

PBS: Phosphate Buffer Saline

PDI: polydispersion index, V; Polydispersion index

PEG: Polyethylene glycol

PEO: Poly ethylene Oxide

Pg-p: P-glycoprotein

PM: Polymeric Micelle

PPO: Poly propylene oxide

PTX: Paclitaxel

QbD: Quality by Design

ROS: Reactive Oxygen Species

SAL: Salinomycin

SCC: Small Cell Cancer

SCLC: Small Cell Lung Cancer

TCA: Trichloroacetic Acid

TF: Transcription Factor

TGFβ: Transforming Growth Factor-Beta

TKI: Tyrosine Kinase Inhibitor

TPP: Target Product Profile

VEGF: Vascular Endothelial Growth Factor

VGFR: Vascular Growth Factor Receptor

ZP: zeta potential

PART I

INTRODUCTION

1. Lung Cancer Overview

Approximately 1,8 million of new cases of lung cancer are diagnostics every year, the high mortality (1,6 million), low survival rates (18% after 5 years) and late diagnosis put lung cancer on the top of the deadliest type of cancer worldwide (Molina *et al.*, 2008).

Despite lung cancer affect both genders, the incidence in men is higher than in women. In 2018 American Cancer Society predicts 234,30 the new cases of lung cancer in US (121,680 will affect men and 112,350 will affect women). The incidence rates can also vary by age, generally lung cancer occurs in older people with 65 years or more while the diagnosis in people younger than 45 years is less common (Nascimento *et al.*, 2015).

Lung cancer generally comprises two main types: a smaller percentage (20-25%) of the lung cancer cases is Small Cell Lung Cancer (SCLC), a highly malignant tumour derived from the cell that exhibit neuroendocrine characteristic. The majority (80-85%) of lung cancers diagnosed is Non-small cell lung (NSCLC) which is divided in other 3 subtypes: Adenocarcinoma; Squamous cell carcinoma; Large cell carcinoma. Despite these subtypes are grouped as NSCLC, they start from different lung cells (Cruz, Dela, Tanoue e Matthay, 2011).

Adenocarcinoma is the most type of NSCLC accounting for 40%. It develops in secretory epithelial gland cells, as a slow growth tumour greater are the chances with beforehand diagnostic to avoid the metastasis. Its prevalence affects younger and women people mainly current or former smokers although its prevalence on non-smokers is highly common (Yanaihara *et al.*, 2006).

Squamous cell lung carcinoma represents about 30% of NSCLC. It usually occurs in the flat squamous cells that are presented in the central part or inside the main airway of the lung. Its occurrence is strongly linked to a smoke history and exposure to the tobacco smoke, radon gas or asbestos. The constant flow of fluids through the lungs allows a quicker metastatization to nearby areas such as neck, chest wall and oesophagus (Hammerman *et al.*, 2012).

Large cell or undifferentiated carcinoma is the less common type of NSCLC, but its fast growth characteristic makes its treatment difficult. Its occurrence begins in large or small cells anywhere in the lung, however its more often found in the periphery (Zhou *et al.*, 2011).

The acquisition of lung cancer is highly associated with the use or the exposure to smoke, however, as the smoking rates decrease worldwide, the numbers of lung cancer in non-smokers (people that consume less than 100 cigarettes in a life time) increase. Also, other

factors are related to the appearance of cancer including genetic factors that contribute to the susceptibility of lung cancer suggesting a hereditary base to disease development (Molina *et al.*, 2008). Dietary factors such as low concentrations of antioxidants that possesses protective effect against lung cancer e.g. vitamin A, C and E have been related to the development of lung cancer. The obstruction of the airflow and the presence of other non-malignant lung diseases have been shown to increase the risk for lung cancer such as the presence of chronic inflammation. Smoking environment exposal, air pollution, occupational carcinogens are other examples of risk factors for lung cancer (Cruz, Dela *et al.*, 2011).

1.1 Non-Small Cell Lung Cancer: Molecular Insights

The pathology of NSCLC can be defined at a molecular level by current mutations that occur in multiple oncogenes in all subtypes of NSCLC. The tumorigenesis of lung cancer relates to the activation of growth promoting proteins or the inactivation of tumour suppressor genes. These alterations may happen at the level of gene silencing through methylation, DNA sequence changes, DNA amplification or deletion and gain or losses of chromosomes (Massion e Carbone, 2003). The understanding of these molecular alterations in lung cancer, have a potential impact on the diagnosis, prognostic and the treatment choice. Specific molecular alterations have been defined more precisely in adenocarcinomas since it's the most common type of NSCLC, however, the interest of molecular profile in SCC has been increasing in order to discover new therapeutic targets (Collisson *et al.*, 2014).

AKT1, ALK, BRAF, EGFR, HER2, KRAS, MEK1, MET, NRAS, PIK3CA, RET and ROS are known examples of oncogenes to be important in the lung cancer pathogenesis. As KRAS and EGFR mutations are more prevalent found in NSCLC, they will be summarized below (Collisson *et al.*, 2014).

KRAS is a proto oncogene member of the RAS family (including NRAS and HRAS) that encode a G-protein that plays a role of controlling signal transduction pathways responsible for cell proliferation, differentiation and survival. When RAS is in active form (RAS-GTP), a bunch of downstream pathways are activated such as RAS/RAF/MEK/MAPK and PI3K/AKT (Kutkowska, Porebska e Rapak, 2017).

Mutation on KRAS gene consists in single amino acid substitution most located in codons 12, 13 and 61 (Ettinger *et al.*, 2017; Riely *et al.*, 2008). It is found on 30% of lung adenocarcinomas being the most common type of mutation in NSCLC while HRAS and NRAS are rarely found. Even though this mutation is in tumours of both current and non-smokers, its presence in non-smokers is less common, western and male population are also

more likely to have KRAS mutation than Asian. Tumours that present mutation on KRAS hardly present other type of mutations, which means that it is a non-overlapping mutation with other oncogenes found in NSCLC (Cooper *et al.*, 2013).

EGFR is a transmembrane cell surface receptor member of the Erb family of tyrosine kinase, it is present on normal epithelial, mesenchymal and neurogenic tissues, however, its overexpression is related to the NSCLC pathology. EGFR possesses the tyrosine kinase domain through the membrane where one domain is placed in the inner part of the membrane while the extracellular portion allows a ligand binding. The attachment of an specific ligand allows the phosphorylation and dimerization of EGFR and consequently it will trigger the activation of signal pathways cascades such as PI3K/AKT/mTOR , RAS/RAF/MAPK and JAK/STAT that regulates cell proliferation, cell growth, neovascularization, invasion and metastasis (Bethune *et al.*, 2010; Lynch *et al.*, 2004).

About 35% of NSCLC present EGFR mutations, has been more prevalent in Asian, female and non-smokers' population. As KRAS, the EGFR mutation does not overlap with other mutations found in NSCLC and its presence is reported to a poor prognosis, reduce survival, frequent lymph nodes metastasis and poor chemo sensitivity (Ettinger *et al.*, 2017; Meyer zu Schwabedissen *et al.*, 2006; Paez *et al.*, 2004).

The EGFR mutation generally occurs within the exon 18-21of tyrosine kinase domain, 90% is a short in-frame deletion on the exon 19 or point mutations on the exon 21. As a result, these mutations increase the signal transduction activity promoting the hyper activation of pro survival and antiapoptotic signalling pathways (Bethune *et al.*, 2010; Pao *et al.*, 2004).

Table 1 Frequency of gene mutations involved in NSCLC.

Gene	Alteration	Frequency
AKT 1	Mutation	1%
ALK	Rearrangement	3–7%
BRAF	Mutation	1–3%
DDR 2	Mutation	~4%
EGFR	Mutation	10–35%
FGFR 1	Amplification	20%
HER 2	Mutation	2–4%
KRAS	Mutation	15–25%
MEK 1	Mutation	1%
MET	Amplification	2–4%
NRAS	Mutation	1%
PIK3CA	Mutation	1–3%
PTEN	Mutation	4–8%
RET	Rearrangement	1%
ROS 1	Rearrangement	1%

EGFR and KRAS accounts for the majority mutated genes in NSCLC (Lovly, Horn e Pao, 2016)**.**

Even though chemotherapy is an palliative option, it has a poor clinical response in advanced lung cancer, so the need for more effective and specific target therapy becomes necessary (Chen, J.-Y. *et al.*, 2015).

In 2003 Gefitinib (Iressa; AstraZeneca) was the first oral TKI approved by FDA in US followed by Erlotinib (Tarceva; OSI Pharmaceuticals) approval in 2004. These two small molecules are widely used as second or third line therapy in patients with metastatic NSCLC that failed at least one regimen of chemotherapy or as first line treatment in chemotherapy-naive patients. In 2013, Afatinib (Giotrif; Boehringer Ingelheim) was approved as second generation of EGFR TKI for first line treatment for patients that possesses exon 19 deletion or exon 21 substitution or as second line treatment for squamous cell carcinoma that conventional chemotherapy failed. Compared to conventional chemotherapy, TKIs showed to be effective in improving the progression free, response rate and quality life in patients that presented mutant EGFR (Burotto *et al.*, 2015; Ettinger *et al.*, 2017; Keating, 2014; Nurwidya, Takahashi e Takahashi, 2016).

Besides most patients experience a progression of disease after 9 to 13 months with TKI therapy, about 60% will eventually develop resistance to these drugs. EGFR T790M is a known mutation associated with acquired resistance after the initial response to sensitizing EGFR TKI. Osimertinib (Tagrisso; AstraZeneca) is the third generation of TKI therapy recommended for both EGFR sensitizing mutation or specific T790M mutation (Ettinger *et*

al., 2017).

Target therapy also includes other mutations presented in NSCLC for example, Crizotinib, Ceritinib, Alectinib and Brigatinib are available and act on ALK rearrangement, Crizotinib also act in ROS 1 rearrangement. Patients who has BRAF and MEK mutations are recommended to use an BRAF inhibitor such as Dabrafenib and Trametinib that attack MEK related proteins (Ettinger *et al.*, 2017; McKeage, 2015).

Monoclonal antibodies are also available for the NSCLC treatment inhibiting angiogenesis by attacking VEGF and VGFR. Some examples of monoclonal antibodies are Bevacizumab and Ramucirumab (Ettinger *et al.*, 2017; Ruiz-Ceja e Chirino, 2017).

Despite the progress on the development of drugs in targeting specific mutations, KRAS mutation in NSCLC lack of target therapy approval. Even though potential therapeutic approaches have been studied including direct inhibition of KRAS protein, inhibition of KRAS regulators, alteration of KRAS membrane localization and inhibition of effector molecules downstream of mutant KRAS, initial KRAS testing remains unclear (Tomasini *et al.*, 2016).

2. Epithelial Mesenchymal Transition

Originally known as epithelial mesenchymal transformation by Elizabeth Hay in 1980 when she first observed epithelial cells acquiring mesenchymal phenotypes in a study of embryonic development. Although EMT is a normal and silent process in health tissues to promote embryogenesis, it also occurs in pathological conditions such as fibrosis, chronic inflammation, wound healing and cancer diseases becoming an unpredictable event and contribute for dissemination of the tumour named metastasis (Hay, 1995).

The term Epithelial Mesenchymal Transition (EMT) started to be used more commonly when the transition of cells phenotype demonstrated to be a reversible process, meaning that the opposite can also occur, especially in cancer metastasis, when mesenchymal cells reach a suitable location to colonize other tissue and become epithelial cells, is called Mesenchymal- Epithelial Transition (MET) (Serrano-Gomez, Maziveyi e Alahari, 2016).

Epithelial cells display an apical and basal polarity and cells are joined together to each other by a complex of proteins called cell junctions which consists in tight junctions, adherens junctions, desmosomes, hemidesmosome and gap junctions. These complex allow the formation of a cohesive sheet of cells and anchor the epithelium in the basement membrane (Kalluri e Weinberg, 2009).

Dramatic changes on the signal pathways responsible for organization of cytoskeleton, cell shape and gene programming expression lead to the deconstruction and the degradation of

the cell junctions and epithelial cells lose the apical-basal polarity and the ability to interact and adhere to the basal membrane and cells nearby, assuming a mesenchymal cell phenotype, these changes allow epithelial cells to create a motility mechanism and leave the original tissue leading to the invasion to the extracellular matrix (ECM) and migration to other tissues enhancing the invasiveness and resistance to apoptosis (Kalluri e Weinberg, 2009; Lamouille, Xu e Derynck, 2014).

Such degradation of cell junctions is observed by the decrease of expression of certain proteins such as occludin and claudin, localized on the apical tight junctions, desmoplakin and plakofilin related to the degradation of desmosome and E-cadherin which is expressed in epithelial cells and its downregulation is a hallmark for the EMT in the destabilization of the adherens junctions. As the downregulation of proteins happens in EMT, important proteins considered as mesenchymal biomarkers in the EMT are upregulated for example N-cadherin, fibronectin and specially Vimentin (Lamouille *et al.*, 2014).

Vimentin is more often found in the cytoplasm of normal mesenchymal cells, it is responsible for the maintenance of cell and tissue integrity, however, as mentioned above, dramatic changes occur in epithelial cell shape during the EMT while at the same time, initiate the expression of Vimentin. Therefore, its overexpression in epithelial cells has been related in many types of cancers, including NSCLC (Satelli e Li, 2011).

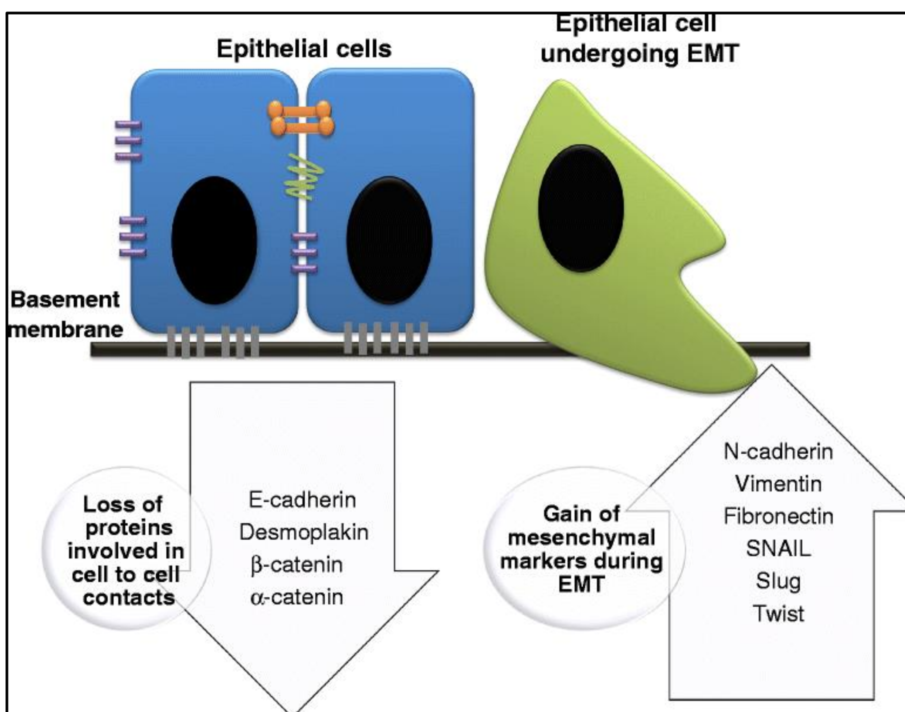


Figura 1 Epithelial cells undergoing EMT.

Loss of the proteins expression and the apical-basal polarity is a marker for EMT process in order to acquire mesenchymal phenotype with the gain of mesenchymal markers. Vimentin expression correlates with mesenchymal cell shape and motility behaviour (Serrano-Gomez *et al.*, 2016)**.**

Growth factors such as EGF, HGF, FGF and TGF-β are EMT inducers, they are secreted by transformed epithelial cells and bind to tyrosine kinase receptors to induce the EMT and allow invasive and migratory state. Transcription factors such as snail, slug, ZEB family and TWIST are EMT regulators and also have their expression increased. They act directly or indirectly as repressors of the E-cadherin expression binding to the E-cadherin promoter repressing its transcription. These EMT-TF are also controlled by microRNAs specifically members of miR-200 family (Karlsson *et al.*, 2017; Kopp, Hermawan, Oak, Herrmann, *et al.*, 2014).

2.1 Cancer Progression

Malignant cells are derived from epithelial tissues which in normal and early tumour state epithelial cells express cytokeratin and E-cadherin remaining the epithelial phenotype and lack of migratory capability. These properties differ from early to advanced tumours state due to the acquisition of mesenchymal phenotype, motility and invasive ability that are directly correlated to cancer progression (Sun, Qin e Zhong, 2016).

Many studies have been demonstrating that EMT is implicated in metastasis and associated with the progression of tumours, malignance level and high mortality rate on patients (Heerboth *et al.*, 2015; Sun *et al.*, 2016).

This association is demonstrated by the increased degradation of ECM by Matrix Metalloproteinases (MMPs) responsible for important cellular events such as the induction of tumour spread and cell invasion. MMPs are capable of cleaving cell surface proteins and degrade components of ECM, for example, E- cadherin as important substrate of MMPs helps to separate tissues into individual cells and induce signalling for EMT (Heerboth *et al.*, 2015; Son e Moon, 2010).

The increase of MMPs in tumour environment induces EMT in epithelial cells leading to an increase in the levels of MMPs expression which cause the degradation of ECM facilitating cell invasion. Because of this, the increase of MMPs has been suggested as marker for tumour malignancy in many types of cancer including lung cancer, for example, the overexpression of MMP-7, 28 induce EMT in A549 lung cancer cells (Son e Moon, 2010).

As mentioned earlier, specially, Vimentin plays recognized essential role in the regulation of EMT and signalling pathways involved in tumour progression. Moreover, its overexpression is related to the poor prognosis and high malignancy of cancer (Lazarova e Bordonaro, 2016).

The relation of Vimentin with invasiveness and metastasis is known by the influence that the protein possesses in affecting cell structure and motility signalling pathways.
In PI3K/AKT, signalling pathway often upregulated in cancer, AKT1 binds to and phosphorylates Vimentin preventing its proteolysis induced by caspase. This leads to an increase of the motility and invasiveness. Also, the activation of the PI3K signalling induce an increase of Vimentin phosphorylation promoting efficient cell migration (Kidd, Shumaker e Ridge, 2014). The up regulated Vimentin in EMT has been reported to interact with protein Scrib, which is involved in cell migration, protecting it from proteasomal degradation, promoting protein stabilization and thus, leading to an increase of invasiveness ability (Satelli e Li, 2011) By virtue of the strongly association of EMT and cancer progression, vimentin has been recognized as a marker for EMT (Satelli e Li, 2011).
Chronic inflammatory microenvironment has been related to the expression of tumour initiators proteins. Additionally carcinoma cells can reach high malignancy when the EMT program is associated with inflammatory signals. There are evidences that shows that specific cytokines present in the tumour microenvironment contributes for the induction of EMT. For example, RAS oncoproteins can induce cancer cells to produce IL-8 and CXCL1 which are proinflamatory cytokines and chemokines abundant in the tumour microenvironment, therefore the RAS- induced inflammatory tumour microenvironment highly contributes for the cancer progression through EMT activation. Association between tissue inflammation in carcinoma and EMT activation also includes cytokines IL-1β and IL-6. The presence of hypoxia in carcinoma cells in vitro has been demonstrated to have impact on the EMT induction through a dependent activation of HIF-1 pathway (Sato *et al.*, 2016; Shibue, Tsukasa e Weinberg, 2017).
After cancer cells migrate through the blood or lymphatic system from the primary tissue to invade distant tissues, in order to successfully complete the metastasis, it need to be anchored, settle and grow on the new tumour site. This cell anchorage occurs on the MET process, when the proteins responsible for attaching cells are re expressed. Although the EMT activation is well understood, the reverse process MET remain unclear, however, there are hypothesis that could explain it, for example, the reduction of EMT-TF that lead to the deactivation of previously active EMT, or the activation of MET is due from selectively pathways that are not involved with TF involved on the EMT (Heerboth *et al.*, 2015; Shibue, Tsukasa e Weinberg, 2017).

2.2 EMT and Cancer Stem Cells features

Recent evidences suggest that cells derived from EMT exhibit Cancer Stem Cells (CSC) like properties. These cells are identified in many studies as a minority subpopulations of cells within the tumour that self-renew and differentiate into heterogeneous cancer cells with altered molecular and cellular phenotypes (Bao *et al.*, 2013; Dean, Fojo e Bates, 2005).

CSC represent up to 1% of cells in the tumour microenvironment and contribute for the recurrence and progression of cancer due to the ability to initiate tumours and survive to the exposure to chemotherapy acquiring resistance to conventional treatments, for that, these cells are also important in the prognosis as they are found in most of the malignant tumours (Bao *et al.*, 2013; Scheel *et al.*, 2011).

One of the reasons that CSC are resistant to anti-cancer drugs and radiotherapy is that recent studies discovered that CSC possesses high levels of ABC transporter expression which lead to the drug export of the cell and as consequence, the conventional therapy is not effective on these cells. Studies have shown that he down regulation of EMT inducing transcriptional factors reduce the ABC transporters making cells more sensitive to anti-cancer drugs (Zhang, Yang *et al.*, 2012).

Taken the fact that CSC has the ability to seed new tumours, the EMT activation is necessary for the entrance of these cells and disseminate them in order to colonize metastatic colonies in distant tissues (Bao *et al.*, 2013; He *et al.*, 2013).

One explanation of how EMT activation mechanism enable the formation of CSC came from a study in human mammary HMLER cells demonstrated that demonstrated that autocrine loops of TGFβ and Wnt-β pathways occurs when proteins are secreted by carcinoma cells when EMT is activated. These signalling pathways play an important role on the induction of stemness and maintenance of stem cells features suggesting that its blockage could be efficient in preventing the formation of CSC (Scheel *et al.*, 2011; Shibue, T. e Weinberg, 2009; Shibue, Tsukasa e Weinberg, 2017).

3. Salinomycin

In a search for new antibiotics in 1974, Miyazaki and colleagues isolated for the first time, from a strain of *Streptomyces albus* (strain no 80614) a new biological substance that would become a member of the mono carboxylic polyether antibiotics, which was termed as Salinomycin (Miyazaki *et al.*, 1974). Such discovered demonstrated antimicrobial activity against Gram positive bacterium (*Bacillus subtilis, Staphylococcus aureus, Micrococcus flavus*, *Sarcina lutea, Mycobacterium spp.*) including also activity against *Filamentous funghi*, and some parasites (*Plamodium falciparum, Eimeria spp.*). Because of that, SAL has been widely used for the past 30 years as veterinarian medicine to prevent coccidiosis and fed ruminants and pigs in order to improve the nutrients absorption (Miyazaki *et al.*, 1974; Naujokat e Steinhart, 2012).

As antibiotic SAL´s mechanism of action is very clear and understood, it has selectively preference for binding alkali ions (especially potassium) and form complexes. These complexes interact with the lipid barrier of biological membranes for example the mitochondrial membrane and decrease the ion permeability in order to carry the cations, by passive diffusion, to inside the cell allowing the intracellular ions accumulation to toxic levels (Fuchs *et al.*, 2009; Mitani *et al.*, 1976; Story e Doube, 2004).

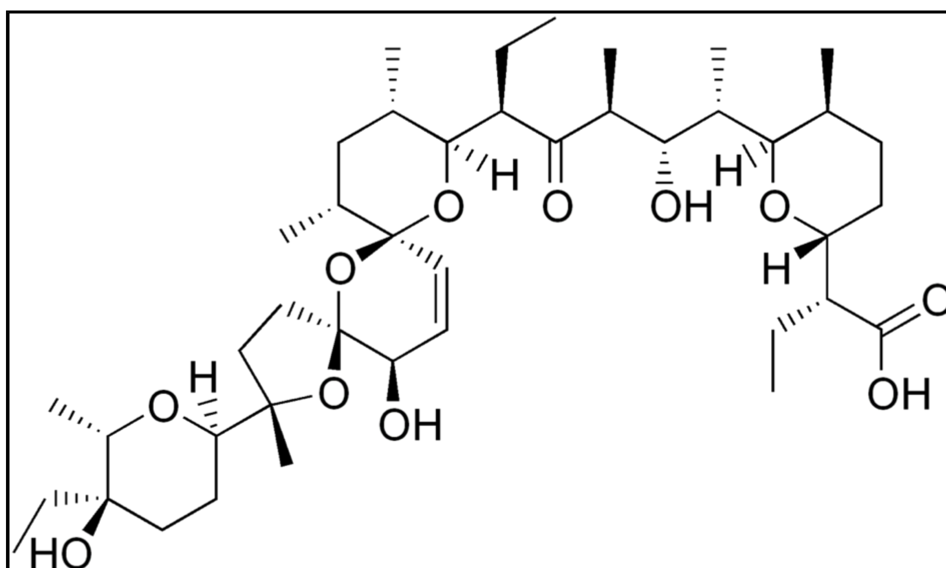


Figura 2 Chemical structure of the carboxylic ionophor salinomycin.
Molecular formula $C_{42}H_{70}O_{11}$ M.W 751Da.

3.1 Salinomycin as selective target of Cancer stem cells like

The use of SAL is not limited to being only an antibiotic, it was also reported that it could alter proliferation and apoptosis in tumors and has been demonstrated to have great ability to kill Cancer Stem Cells like (CSC), identified in many studies as a minority subpopulations of cells within the tumor that contribute for the recurrence and progression of cancer due to the ability to survive to the exposure to chemotherapy and acquiring resistance to conventional treatments (Dean *et al.*, 2005; He *et al.*, 2013).

In 2009, Gupta *et al* took in consideration studies that had shown that the induction of Epithelial- Mesenchymal Transition (EMT) in normal and cancerous cells results in cells with stem like properties, the group led a screening process to find selectively agents that could specifically kill CSC like using shRNA to inhibit the gene that code for E-cadherin in order to increase the proportion of CSC in HMLER breast cancer cells. The results demonstrated that the induced EMT $HMLER^{shecad}$ cells when treated with paclitaxel and doxorubicin showed high resistance in both neoplasic and normal cells and when these cells were treated with SAL a reduction of 20-fold on the proportion of $CD44^{high}$ / $CD24^{low}$ was observed (Ghuwalewala *et al.*, 2016; Gupta *et al.*, 2009).

SAL has also shown to be effective in reducing the viability of CSC that are naturally in HMLER breast cancer cells and not only in EMT induced cells, showing a reduction of 75-fold in the proportion of CSC naturally high in HMLER cells (Gupta *et al.*, 2009).

Furthermore, Gupta (2009) demonstrated that SAL resulted to be more effective in decreasing the tumour seeding in 100-fold more than paclitaxel (PTX) when HMELER and 4TI cancer cells were pretreated with SAL and PTX and then injected in mice, indicating that CSC that naturally exists in breast cancer cells are sensitive to SAL and resistant to PTX (Gupta *et al.*, 2009).

Another study, gastric cancer cells with $ALDH^{high}$, which represent to be CSC features, showed resistance to conventional drug therapy 5-FU and Cisplatin but when treated with SAL , it demonstrated to be 4 fold more sensitive to SAL than cells $ALDH^{low}$ suggesting that SAL can effectively kill CSC (Zhi *et al.*, 2011)

3.2 Salinomycin effects in cancer cells: apoptosis and growth inhibition

After SAL became known as a selector to kill CSC like, other studies began to show that SAL could induce apoptosis in different type of cancer cells such as breast cancer, prostate cancer, colorectal cancer, hepatocellular carcinoma, head and neck cancer, Burkitt´s

lymphoma, squamous carcinoma, uterine sarcoma, nasopharyngeal carcinoma, endometrial cancer, tongue carcinoma, larynx cancer, osteosarcoma, human ovarian cancer, pancreatic cancer, gastric cancer, several types of leukemia and lung cancer. Such results reinforce the use of SAL in cancer therapy. Studies comprising some of these cells will be summarized below to better understand the mechanism that SAL act in apoptosis and growth inhibition (Fuchs *et al.*, 2009; Nencioni *et al.*, 2005).

In a study using several types of Leukemia CD4+ cells, Fuchs *et al* demonstrated that in a dose dependent experiment, SAL induced apoptosis either in cells expressing p53 like Molt-4 CD4+ and in cells that fail to express p53 protein and shows resistance to cytostatic drugs and proteasome inhibitors as the Jurkat cells, suggesting that SAL can act in a different manner to induce apoptosis independent of p53 pathway (Nencioni *et al.*, 2005).

In Namalwa Burkitt lymphoma cells which express high resistance to apoptosis, SAL was also effective in inducing apoptosis, showing that can overcome the resistance caused by the proteolytic activity of 26S proteasome (Fuchs *et al.*, 2008, 2009; Naujokat, Fuchs e Berges, 2007).

Fuchs (2008) also demonstrated that SAL´s apoptosis effect is efficient in MES-SA/Dx5 uterine sarcoma cells resistant to anticancer drugs due to the P-gp efflux pump that is responsible for eliminating drugs and small molecules from the cytosolic compartment. This result goes according to an experiment made by Riccioni *et al.* (2010) where SAL acts as a potent inhibitor of multidrug resistance in MDR cancer cells overexpressing P-gp.

SAL was also identified to be responsible for the inhibition of Wnt/β signaling cascade by blocking the Wnt induced LRP6 phosphorylation and promoting the degradation of LRP6 protein in HEK 293 cells. The effect that SAL showed to interfere on the Wnt/β signaling pathway was also demonstrated in human hepatocellular carcinoma *in vitro* and *in vivo* (Lu, D. *et al.*, 2011; Wang *et al.*, 2012).

Other drugs used in chemotherapy such as Doxorubicin (DOX) and Etoposide (ETO) could have their effect potentialized when administered in combination with SAL, which was the case of the study held in China where the drugs showed to have potential to sensitize breast cells and uterine sarcoma cells and increase the DNA damage caused by the two drugs above mentioned, SAL also showed to decrease even more the cell viability when cancer cells were co-treated with DOX/SAL or ETO/SAL. However, the combination of DOX/ SAL showed to be more efficient than ETO/SAL suggesting that the effect of sensitize cells act in different pathways that could be either the increase of direct DNA damage or reducing the levels of p21 via proteasome activity (Kim, J. H. *et al.*, 2011).

The increase of cell inhibition and the induction of apoptosis by SAL was demonstrated by Wang (2012) in androgen dependent and independent prostate cancer cells, it was also demonstrated that SAL was able to activate caspase 3 causing decrease expression levels of antiapoptotic protein Blc2 and increase the pro apoptotic expression of Bax protein where Bax was translocated to the mitochondrial membrane and cytochrome C was released to the cytosol, the disruption of the mitochondrial membrane was also led by the ability that SAL showed to promote the elevation of oxidative stress through the accumulation of intracellular reactive oxygen species (ROS) levels. Two types of Non-Small Cell Lung Cancer Cells (LNM35 and A549) shown cell viability decreased, growth inhibition and migration repressed in a dose and time dependent manner (Kim, K. Y. *et al.*, 2011; Wang *et al.*, 2012).

4. Nanomedicines

The use of nanotechnology combined with medicine comprises the term nanomedicine. Despite nanomedicine is a wide area of study, this thesis will focus on the use of nanoparticles as drug delivery.

Nanoparticles (NP) defined as submicron (<1000 μm) particles, is a versatile approach where the interest of pharmaceutical research has increasing due to the possibility to use several organic or not organic materials to create a bunch of types of nano carriers that differ from each other in chemical properties, shape or sizes and have their own advantages. The most common types of NP include liposomes, solid lipid nanoparticles, quantum dots, metal nanoparticles, polymeric micelles (Torchilin, V. P., 2007; Torchilin, Vladimir P., 2007).

The nanomedicine approach allowed the improvement on the treatment of various diseases but it has been widely studied for the treatment of cancer due to the unique properties that nano carriers possesses such as the ability to delivery hydrophobic drugs, prolong the circulation in the blood system and increase the drug concentration around the tumour via the EPR effect. This enhance of drug efficacy and the reduction of the toxic side effects have become the main goal to achieve on the production of formulation at a nanoscale level to greatly overcome the limitations of the current chemotherapy, for example, liposomes encapsulated DOX decrease cardiac toxicity of the cytotoxic drug and albumin- stabilized PTX allows higher tolerated drug doses in patients (Park, 2013; Torchilin, Vladimir P., 2007).

The use of these various types of NP allowed the researches to modulate the surface in order to optimize not only drug delivery but also vaccines, proteins and biological molecules and achieve different areas of interest. Examples of such modulations are the conjugation of NP with antibodies, the combination of temperature sensitive polymers (to deliver insulin

for instance) (Arranja *et al.*, 2016; Wu *et al.*, 2017).

In order to be effective against cancerous cells, nanoencapsulated drugs need to be released in the cytosol. For this, NP cell uptake occurs by several mechanisms which differ according to their physicochemical properties such as composition, size, shape, charge surface, surface functionalization and surface hydrophobicity or hydrophilicity.

The entrapment and cellular uptake of nanoparticles by the mononuclear phagocytic system (MPS) present in the liver and spleen is one of the major obstacles in the drug delivery. This problem can be solved by the surface modulations with poly (ethylene glycol) (PEG), for example, that inhibit the recognition and phagocytosis by the MPS prolonging presence in the circulation system and improving the transport of NP by preventing opsonization and premature cellular uptake (Arranja *et al.*, 2016; Jong, De e Borm, 2008).

Cell uptake mainly occur through endocytosis where there are modification of the membrane and invagination of NP occurs and forms endocytic vesicles that are transported to intracellular trafficking system such as endosome or lysosome where particles are degraded (Treuel, Jiang e Nienhaus, 2013).

To better understand the mechanism of how NP is internalized by the cell, different endocytic pathways such as the Clathrin and Caveolin mediated endocytosis and Clatrin/Caveolae independent endocytosis are studied. However, particles ranging 20 nm demonstrated to have the cell internalization facilitate without the endocytic system (Jong, De e Borm, 2008).

Nanotechnology is also attractive for cancer therapy due to the possibility to use as a combination therapy as the NP can encapsulate more than one pharmacological agent. This approach offers synergetic effects promoting more efficacy of the therapy and limit the drug resistance, for instance, the combination of cisplatin pro drug and siRNA *in vivo* and the use of siRNA targeting two genes in human are example of demonstrated synergetic effect (Bertrand *et al.*, 2014).

Table 2 Different types of nanoparticles in use for cancer therapy. FDA has approved some while others are in clinical trials (Drugs Approved for Lung Cancer - National Cancer Institute, 2016; Ruiz-Ceja e Chirino, 2017).

Name / Manufactor	Cancer type	Nanoparticle type	Drug	Year
Oncaspar / Enzon Pharmaceuticals	Acute lymphoblastic leukemia	PEG conjugated	L-asparaginase	1994
	Karposi sarcoma			1995
Doxil / Janssen	Ovatian	Liposome	Doxorrubicin	2005
	Multiple myeloma			2008
DaunoXome / Galen	Karposi sarcoma	Liposomes	Daunorubicin	1996
Ontak / Eisai inc	Cutaneous T cell lymphoma	Protein conjugated	IL-2 and Diphiteria toxin	1999
Myocet / Elan sopherion therapeutics	Breast	Liposomes	Doxorrubicin	2000
Eligard / Tolmar	Prostate	Polymer PLGH	Leuprolide acetate	2002
	Breast			2005
Abraxane / Celgene	NSCLC	Albumin conjugated	Paclitaxel	2012
	Pancreatic			2013
Genexol PM / Samyang biopharm	Breast, NSCLC	Polymeric micelle	Paclitaxel	2007
Marqibo / Talon therapeutics	Acute lymphoblastic leukemia	Liposomes	Vincristine	2012
Onivyde / Merrimack	Pancreatic	Liposomes	Irinotecan	2015
LEP-ETU / Neopharma	Ovarian, Breast, Lung	Liposomes	Paclitaxel	clinical phase I/II
Aroplatin / Antigenics	Colorectal	Liposomes	cisplatin analog	clinical phase I/II
OSI-211 / OSI pharmaceutical	Ovarian, Lung	Liposomes	lurotecan	clinical phase II
SPI-77 / Alza pharmaceutical	Head / Neck, Lung	Liposomes	cisplatin	clinical phase III
EndoTAG-1 / Medigene	Breast, Pancreatic	Liposomes	Paclitaxel	clinical phase II
Thermodox / Celsion	Hepatocellular carcinoma	Liposomes	Doxorrubicin	clinical phase III
Lipoplatin / Regulon	Pancreatic, Head/Neck	Liposomes	Cisplatin	clinical phase III
Aurimmune / Cytimmune sciences	Head /Neck	Gold	TNF-α	clinical phase II
Paclical / Oasmia pharmaceutical AB	Ovarian	Micelles	Paclitaxel	clinical phase III
Nektar-102 / Nektar therapeutics	Breast, Colorectal	PEGylated Liposome	Irinotecan	clinical phase III
NKTR 105 / Nektar therapeutics	Solid tumors	PEG conjugated	Docetaxel	clinical phase I

4.1 Polymeric Micelles

Among many types of nanoparticles mentioned above, the work produced in this thesis made use of one named polymeric micelles (PM).

PM is an organic and colloidal dispersion that consist in the conjugation of water-soluble polymers with phospholipids or long chain fatty acids and other surfactants. (Singh *et al.*, 2017). This micellar structure property provides great advantages as it can solubilize poorly water soluble drugs increasing their bioavailability and due to their small size (generally 5 to 100 nm) they are able to accumulate in the leaky vasculature that cancer possess generally by passive targeting or EPR. It is also a versatile system as it can be easily modified by attaching ligands and the easily reproductively in large amounts is a great advantage for pharmaceutical research (Rao e Geckeler, 2011).

The micellar structure belongs to a group of amphiphilic molecules that in water solution exist separately at low concentration and as the concentration is increased they form aggregates and spontaneously self-assemble into micelles. The formation of micelles is dependent of the critical micelle concentration (CMC) and temperature (CMT), at below the CMC, polymers exist as monomers while the CMC and achieved the free energy in the system is decreased due to the removal of hydrophobic segments from the aqueous solution and the hydrogen bonds network in water are re stablished while Van der Walls forces resulted from the additional gain of energy bonds the hydrophobic segment (Torchilin, V. P., 2007).

The formed micelles are characterized by having a hydrophilic corona structure in which that is the hydrophilic head tale responsible to confer stability to the system and also is able to attach water soluble agents while the core corresponds to the hydrophobic inner tale and has the ability to encapsulate non water soluble molecules (Jong, De e Borm, 2008; Torchilin, V. P., 2007).

The hydrophilic segment in the outer layer of micelles that is exposed to the environment consists of non-reacted components with blood and tissues, this allows the protection of the loaded drug against the opsonisation by proteins and the identification by macrophages in the liver, this will permit the long circulation in the blood system (Torchilin, Vladimir P., 2007).

The hydrophobicity of amphiphilic polymers can vary by being arranged in di block polymers (A-B) or tri block polymers (A-B-C) with alternating hydrophobicity. Several polymers are used as hydrophilic segments in the micellar structure including PEG, poly (N-vinyl pyrrolidone) (PVP), poly (N- isopropyl acrylamide (pNIPAN), poly (vinyl alcohol) (PVA) and others. Many Micelles also possesses polymer in the inner layer as hydrophobic segment such as poly (latic acid) (PLA), poly (lysine) (PLL) and poly (beta-amino ester) and poly (propylene oxide) (PPO) and some others (Zhang, Yifei, Huang e Li, 2014).

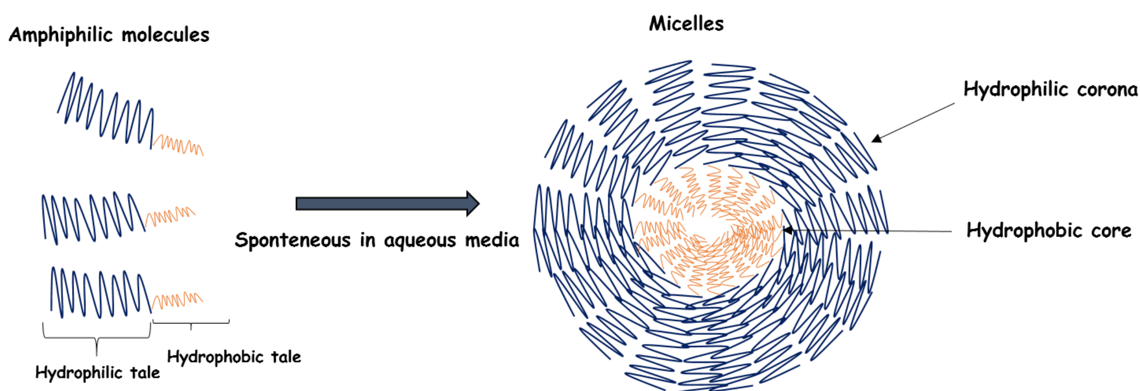


Figura 3 General micelle structure.

Scheme demonstrates the micellization process, polymers contains a hydrophobic and hydrophilic tale self-assembly into micelle in aqueous solution.

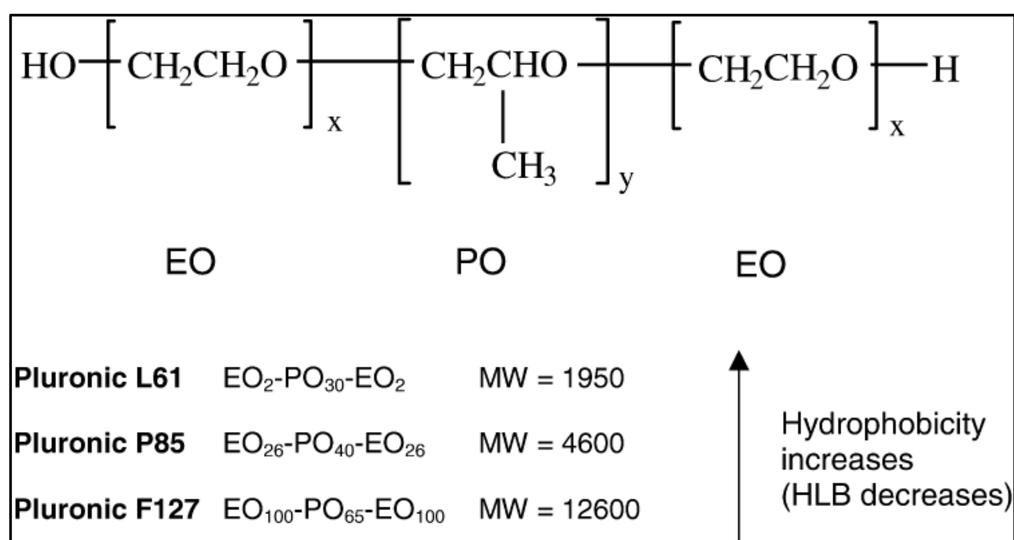
4.1.1 Pluronic

Pluronic, also known as Poloxamer is an amphiphilic non-ionic polymer, it is characterized by containing distinct hydrophobicity/ hydrophilicity balance (HBL). Alternating hydrophilic chains of poly (ethylene Oxide) (PEO) and one hydrophobic chain of poly (propylene Oxide) (PPO) forms a tri block structure $PEO_x - PPO_y - PEO_x$ where the hydrophobicity and hydrophilicity and so as the size of the polymer can vary according to the numbers of PEO/PPO units. The copolymer synthesis is made by the sequential polymerization of PPO and PEO with the presence of an alkaline catalyst followed by the growth of the PPO in the middle of the PEO at the both extremities (Kabanov *et al.*, 2002).

The self-assembly into micelle in aqueous solution make Pluronic an interesting and easy approach to encapsulate poor soluble drugs. In fact, the use of many Pluronic has been approved for medical use including the engineering of formulations. Studies have been made for drug delivery using these block copolymers to form PM. Some examples can include: PTX-Loaded PM, where it was used a mixture of Pluronic (P123 and P105) copolymers in order to enhance the time circulation in the blood and also modify the PTX bio distribution, Pluronic F123 loaded Docetaxel (DTX), mixed micelles of (P F127 and P105) to encapsulate Metotrexate (MTX), and others (Chen, Y. *et al.*, 2013; Liu, Z. *et al.*, 2011; Wei *et al.*, 2009).

In this thesis, Pluronic F127 has been chosen to form the loaded PM due to its biocompatibility and approval by FDA and also to be known to improve the stability and EE% of micelles.

A.



B.

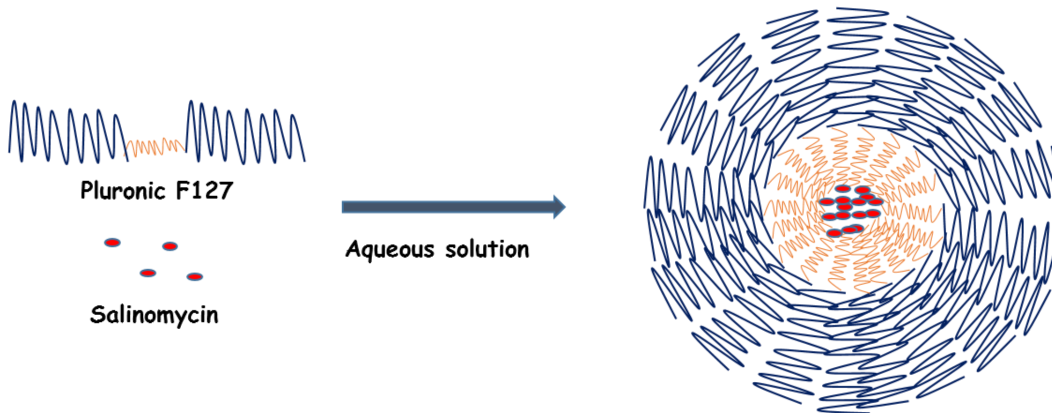


Figura 4 Tri block copolymer Pluronic structure.

(A) Pluronic consist in two hydrophilic blocks alternating with one hydrophobic block, Molecular weight varies according to the number of PEO/PPO units. (B) Scheme demonstrate Pluronic F127 encapsulating hydrophobic drug (e.g. SAL).

5. Objective

Despite lung cancer ranks the highest in terms of incidence and mortality, most of the conventional therapies fails to cure Lung cancer. The reasons to explain this failure is associated to the lack of anticancer drugs that are able to overcome processes responsible for metastasis, recurrence of cancer and resistance to drugs, such as the existence of CSC and the mesenchymal cells in the EMT process. Also, the poor solubility of most of chemotherapy drugs hampers the intracellular accumulation and the non-specific target of drugs promotes various side effects.

The main aim of this study was to use the tri-block polymer Pluronic F127 to create a PM loading SAL and observe its potential anticancer drug in NSCLC A549 cell line. In particular, the following specific aims pursuit.

- Understanding the formulation variables by using the Quality by Design approach;
- Physicochemical characterization of PM such as the M.D, PDI, ZP and EE%;
- Compare the effect of free SAL vs Loaded PM in terms of cell viability and migration;
- Access the expression of proteins markers for MDR such as P-gp and the presence of mesenchymal cells such as Vimentin.

PART 2

EXPERIMENTS AND ACHIEVEMENTS

6. MATERIALS AND METHODS

6.1 Materials

For cell culture and cell experiments, A549 cell line was obtained from American Type Culture Collection (ATCC), Dulbecco´s modified eagle medium, Trypsin EDTA, PBS and L-glutamine were provided from Lonza, Fetal bovine serum (FBS) were purchased from Biowest, 3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Dimethyl sulfoxide (DMSO) were from AppliChem.

Primary antibodies Pg-p and Vimentin, second antibodies Alexa fluor 594, TRITC and DAPI were purchased from Life technologies.

Salinomycin 25mg and Pluronic F127 were purchased from Sigma-Aldrich, Methanol, Trichloroacetic acid (TCA), 2, 4 Dinitrophenylhydrazine (DNP), MiliQwater, Distyrene Plasticizer Xylene (DPX), Triton X-100 were analytical grade.

6.2 Quality by Design approach for the development of Polymeric Micelles.

In order to gain additional understanding and to emphasise the development and preparation process of the proposed drug product candidate, a Quality by Design (QbD) approach for the pharmaceutical drug development was chosen with the objective to ensure that a product of quality will be obtained. For such propose it is necessary in the first place to define the Target Product Profile (TPP). In order to achieve the desired characteristics of the final product which includes: a) the ability to deliver a suitable/therapeutic dose of drug; (b) overcome the biological/physiological barriers and poor bioavailability; and (c) suitable general quality related characteristics and in particular the physical-chemical stability.

The next step is the identification of the drug product attributes that potentially play a critical role in the overall product quality. These are known as Critical Quality Attributes (CQA) which is expected to be in the appropriate range for the proposed formulation. In this case, the Mean Diameter (M.D), size distribution (PDI), surface charge (Zeta Potential) and entrapment efficiency (EE %) were considered to be the primary CQA's. The acceptance criteria for these CQA were: MD: (nmt) 100nm; PDI: (nmt) 0.4; EE%: as close as to 100% as possible; ZP: as far to the neutral as possible.

After defining the desired characteristics of the product, a sequential multivariate Design of experiments (DoE) was developed as it reveals the relationship between input factors (both

formula and process related) and output responses and allow us to quantify the interaction of the variables.

Two sets of DoE were defined and conducted. The first was a screening fractional factorial design with three formula variables, namely SAL concentration, the Polymer concentration and the Water volume used in the hydration step (Table 3). This 2-level, 3-variable incomplete factorial design, with centre replication originated 9 different formulations.

The second set of DoE, a CCD (central composite design) intended to optimize the formulation was conducted after discarding the factor with the least impact shown in the previous screening design. This DoE originated 11 formulations with 3 centre replications using an enlarged range of concentrations with the two remaining variables namely SAL concentration and Polymer Concentration (Table 4).

Table 3 Screening of Critical Quality Attributes.

Design matrix				Experimental matrix		
Sample	Salinomycin	Polymer	Water	Salinomycin µg/mL	Polymer mg/mL	Water µg
PM SAL 1.0	-1	-1	-1	10	5	3000
PM SAL BC	-1	1	1	10	50	5000
PM SAL AB	1	1	-1	100	50	3000
PM SAL AC	1	-1	1	100	5	5000
CENTER	0	0	0	55	27,5	4000
CENTER	0	0	0	55	27,5	4000
PM SAL 1.1	-1	-1	0	10	5	4000
PM SAL A	-1	0	0	10	27,5	4000
PM SAL B	0	-1	0	55	5	4000

Screening has a fractional factorial design comprising the three variable factors involved in the polymeric micelles engineering. In the table, (1) and (-1) represents the largest and smallest values defined. The minimum value (-1) determined was 10µg/mL (SAL), 5mg/mL (POL) and 3000µg (WATER); the maximum (1) value determined was 100µg/mL (SAL), 50mg/mL (POL) and 5000µg (WATER) and (0) represents the mid points between the + and – levels.

Table 4 Design of experiments.

Design matrix			Experimental matrix	
Sample	Salinomycin	Polymer	Salinomycin µg/mL	Polymer mg/mL
PM SAL 1	-1	-1	37,8	11,59
PM SAL 2	-1	1	37,8	43,41
PM SAL 3	1	-1	172,2	11,6
PM SAL 4	1	1	172,2	43,4
PM SAL 5	0	-1,414	105,0	5,0
PM SAL 6	0	1,414	105,0	50,0
PM SAL 7	-1,414	0	10,0	27,5
PM SAL 8	1,414	0	200,0	27,5
PM SAL 9	0	0	105,0	27,5
PM SAL 10	0	0	105,0	27,5
PM SAL 11	0	0	105,0	27,5

This Experimental design comprises the two remaining variable factors with a broader range of drug and polymer concentrations: the minimum value (-1,414) determined was 10µg/mL (SAL)

and 5mg/mL (POL); the maximum value determined was 200μg/mL (SAL) and 50mg/mL (POL); (1),(-1) represents the ratio between the largest and smallest values; and (0) represents the mid points between the + and – levels.

6.3 Polymeric Micelles engineering

Polymeric micelles were prepared using the physical method of thin film hydration. This technique consists basically in 4 important steps: (a) the mixture of polymer and drug in an ICH Q3c class 2 or 3 organic solvent; (b) solvent evaporation under certain conditions; (c) hydration with an aqueous solution and (d) vigorous mechanical agitation.

For the preparation of micelles used in the screening and DoE, in sterile conditions, different amounts of the tri block amphiphilic copolymer Pluronic F 127 was weighted in a round bottom flask and dissolved in 5 milliliters of methanol, then different volumes of SAL (1mg/mL in methanol) was added to the dissolved polymer. The round bottom flask containing the mixture was placed in the rotary evaporation under vacuum at 50°C for 30 and the formed thin film was to left overnight at room temperature to eliminate any trace of solvent. Afterwards, the film was hydrated with MiliQ water at 60°C and vortex for 2 minutes in order to form self-assembly micelles.

The same procedure was followed to prepare the plain micelles (without drug).

6.4 Nanoparticles physicochemical properties characterization

Particles M.D and PDI were evaluate by dynamic light scattering (DLS) using Malvern Zetasizer S instrument and ZP was measured by laser doppler electrophoresis through Malvern Zetasizer Z. Formulations were not diluted and were analyzed in triplicate.

In order to evaluate the EE%, the extraction of free drug from the PM were performed by the centrifugation at 6000 RPM for 10 minutes using Nanosep® centrifugal device with a molecular weight cutoff 3kDa.

A pre column derivatization process needed to be performed as SAL does not possess significant UV absorbance. For that, the free drug extracted in the supernatant was diluted in methanol and treated with an aqueous solution of (TCA 500mg/mL) for 10 minutes at room temperature. After that, a methanolic solution of 2, 4 DNP (1mg/mL) was added and the sample was heated at 55°C for 20 minutes. After the sample cooled, free drug could be evaluated using High Performance Liquid Chromatography (HPLC Hewlett Packard Series 1050 Software HP ChemStation for LC) with a reverse-phase column (Nova-Pak® C-18 60A, 3.9x150mm, 4μm; Waters®) at the absorption peak at 392nm. Mobile phase consisted in

Methanol / aqueous Acetic acid (90:10) with a flow rate of 1mg/mL and volume injection of 100μL.

The following equation was used in order to obtain the EE%

$$\mathrm{EE\%\%} = \frac{[SAL_i] - [SAL_S]}{[SAL_I]} \times 10$$

$[SAL_i]$ Stands for known initial concentration of SAL

$[SAL_s]$ Stands for concentration of SAL found in supernatant measured against calibration curve

6.5 Cell culture and cell viability assays

Lung Cancer cells A549 were maintained in DMEM media supplemented with 10% of fetal bovine serum and 1% L-glutamine. Cells in a density of $5x10^3$ /well were seeded in 180μL of media without serum in 96 well plate. 24 hours after seeding, the media was replaced with non-supplemented media and increased concentrations of PM Sal formulations and free drug were added to cells in 6 replicates. After 24 and 48 hours of incubation, free medium containing 0,5mg/mL of 3-(4,5- Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were added to each well and placed in the incubator for 3:30 hours, after that, the medium was removed and the crystals of formazan were dissolved with DMSO and absorbance could be measured with FLUOstarOmega microplate reader at 570nm. The half inhibitory concentration (IC50) values were obtained based on the cell viability assay and measured with GraphPad prism software.

6.6 Wound healing assay

For the migration experiment, $1x10^4$ of A549 cells were seeded with supplemented medium in 24 well plates. The next day, the media was removed and a scratch was made using a pipette point of 20μL followed by washing the wells with PBS 1x to remove non-attached cells. New supplemented media was added to the wells containing 10μM of SAL and incubated for 24 hours. The migration could be observed under a microscope with a coupled camera for the acquisition and treatment of photos with Motic image plus software.

6.7 Immunocytochemistry

For the antibody staining, A549 cells were seeded in 24 well plates with coverslips at a density of $1x10^4$/ well and left 24 hours in the incubator in order to left the cells attach at the bottom. For the Pg-p experiment, cells were treated with free drug and PM SAL formulation at desired concentrations for 1 hour and 24 hours for Vimentin experiment. The medium was removed and cells were fixed with 4% paraformaldehyde for 20 minutes at room temperature and washed 3 times with PBS (1x). In order to permeate cells, 500µl of 0,5% Triton solution was added to the wells for 30 minutes and washed 3 times with PBS. For the blocking step and primary antibody incubation, 500µl of blocking solution (BSA) was added to the wells and left at room temperature for 1 hour. After removing the BSA, coverslips were washed again with PBS (1x) and placed over a humidified chamber containing 30µl of Pg-p or Vimentin antibody at the ideal dilution and left overnight at 4°C.The next day was followed by immersing the coverslips in PBS (1x), rinsed 3 times and placed on 30µl of the second antibody solution for 1 hour at room temperature and protected from direct light. Coverslips were again rinsed 3 times with PBS (1x) and DAPI solution (diluted 1:1000 in PBS (1x)) was added for 2 minutes at room temperature in order to have the nucleus staining. Coverslips were then mounted with DPX mounting medium and observed under the confocal microscope AxioScop.A1 coupled with Zeiss AxioCam HR and images were treated with ZEN software. To evaluate the protein expression ImageJ software was used.

PART 3

RESULTS

7. Polymeric Micelles Characterization

The two sets of the Design of experiments were characterized as M.D, PDI, ZP and EE%.

7.1 Screening

It is possible to observe in the table that particles possesses the expected results as defined criteria for MD (<100nm). PDI values are in the accepted range for moderate dispersion (defined criteria for PDI lower than 0,4). ZP display a directly correlation on the polymer concentration and surface charge, as the polymer concentration is increased, the surface charge tends to be closer to neutral giving an indication of the stability of the colloidal system (Table 5).

Table 5 Characterization of M.D, PDI, ZP and EE% of formulations from the screening.

Sample	Size (nm)	PDI	Z.P (mV)	EE. %
PM SAL 1.0	130,7	0,30	-24,8	99
PM SAL BC	29,7	0,19	-4,0	98
PM SAL AB	24,0	0,16	-4,6	87,7
PM SAL AC	39,3	0,20	-21,2	88,9
CENTER	38,1	0,18	-7,62	92,2
CENTER	37,6	0,16	-8	91,4
PM SAL 1.1	29,7	0,16	-24,0	98,4
PM SAL A	23,2	0,14	-8,0	99
PM SAL B	34,9	0,18	-23,4	95,3

The values obtained on the characterization of nanoparticles are within the criteria previously defined. The similar results presented in the CENTER formulations (concentrations: 55µg/mL of SAL, 27,5mg/ml of POL and 4000µg of water) demonstrate the accuracy of the characterization method. PM SAL 1.0 (10µg/mL of SAL, 5 mg/mL of POL and 3000µg of water) presented the highest M.D, PDI and ZP. The EE% were better on those formulations with lower SAL concentration namely PM SAL 1.0; PM SAL BC; PM SAL 1.1; PM SAL A.

The data obtained from the sample (PM SAL 1.0) where the M.D and PDI are out of the proposed range may be related to the presence of aggregates caused by the low polymer concentration.

The lowest concentration of drug displayed by the matrix design (10µg/mL) renders the highly encapsulation efficiency by the micellar system. A decrease of EE% was observed on those formulations loading higher concentration. Please refer to table 3 for more information related to concentrations used on formulations.

7.1.1 Statistical Analysis

In relation to the impact of the three variables on the final formulation physic-chemical properties, it is possible to exclude the Water as a critical factor on the process. As it was observed, the final formulation parameters such as M.D PDI, ZP and EE% are not influenced by the volume of the hydration solvent (Equation 1-4). Identically, M.D and PDI are independent on the range of SAL and Polymer concentration studied (Equation (2) and (4)). On the contrary, ZP is dependent on the [POL] (Equation (3), Figure 5(A)).

The entrapment parameter is dependent on the concentration of SAL, demonstrating that the increase of drug concentration lead to lower EE% values (Figure 5(B)).

However, the concentration of water and polymer did not affect the EE% (Figure 5(C) and (D)).

The models fitted to the independent variables, based on the screening data are depicted below (bold values represent the significant factors).

Equation (1) $EE\% = \mathbf{93.18(\pm 1.13)} - \mathbf{5.21(\pm 1.35)S} - 0.6(\pm 1.4)P - 0.2(\pm 1.6)W.$

Equation (2) $Size(nm) = \mathbf{37.8(\pm 30.1)} - 11.1(\pm 36.0)S - 17.3(\pm 36.0)P - 23.1(\pm 42.2)W.$

Equation (3) $Z(mV) = \mathbf{-11.6(\pm 3.4)} + 0.3(\pm 4.1)S + \mathbf{10.2(\pm 4.1)P} + 1.1(\pm 4.8)W.$

Equation (4) $PDI = \mathbf{0.204(\pm 0.068)} - 0.0239(\pm 0.081)S - 0.039(\pm 0.081)P - 0.034(\pm 0.095)W.$

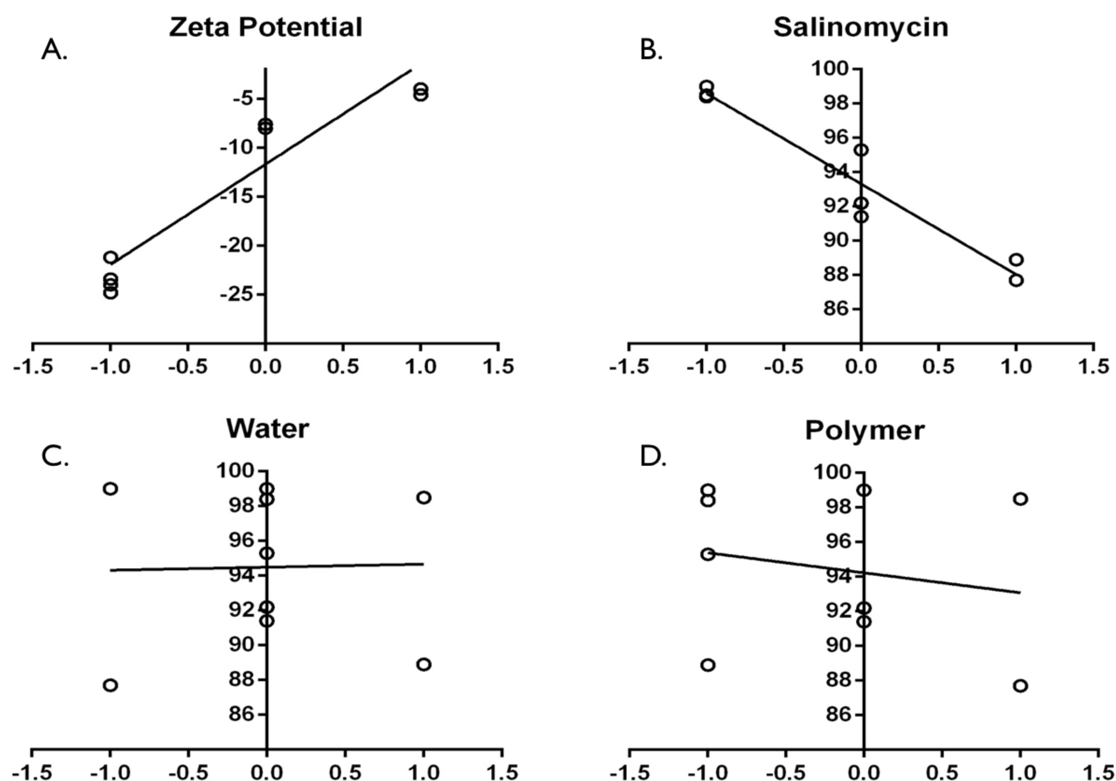


Figura 5 Influence of critical quality parameters of screening

(A) Display the ZP as function on the polymer concentration where the X axis refers to the range of formulations on the fractional factorial design and Y axis refers to the obtained ZP values. The interaction of these variables is observed as the polymer concentrations increase (from -1 to 1) the ZP values tend to be closer to neutrality. (B) EE% as a function of SAL concentration. From formulations with lower (-1) to the higher drug concentration (1) the EE% seems to diminish as the drug concentration is increased on the system. (C) Display the EE% as a function of water hydration volume and (D) display the EE% as a function of the polymer concentration. Constant factors response demonstrates low interaction of these variables.

7.2 Design of experiment

After the first screening a second DoE with the two remaining critical parameters that comprised broader POL and SAL concentrations was conducted. The objective was to confirm the results obtained in the screening such as M.D, PDI, ZP and EE%. (Table 6).

Table 6 Data obtained for M.D, PDI, ZP and EE% of formulations from the DoE.

Sample	Size (nm)	PDI	Z.P (mV)	EE. %
PM SAL 1	27,58	0,24	-10	92,5
PM SAL 2	22,11	0,15	-3	60,5
PM SAL 3	24,97	0,15	-13	82,3
PM SAL 4	23,63	0,13	-4	87
PM SAL 5	32,12	0,16	-16,5	80,9
PM SAL 6	21,1	0,16	-2,63	90,8
PM SAL 7	23,67	0,17	-2,84	94,8
PM SAL 8	23,41	0,12	-5,81	86,4
PM SAL 9	24,39	0,2	-4,31	85,4
PM SAL 10	24,6	0,2	-4,9	89,8
PM SAL 11	23,87	0,16	-6,65	93,6

As previously, the M.D and PDI data shown to be in the acceptance range for particles size. The interaction of ZP and polymer concentration obtained from the screening and statistical analysis was confirmed on this DoE. Concentrations of formulations with enhanced EE%: PM SAL 7 (10 μg/mL of SAL and 27,5mg/mL of polymer) PM SAL 1 (37,8μg/mL of SAL and 11,59mg/mL of polymer) and PM SAL 6 (105μg/mL of SAL and 50mg/mL of polymer).

As it concerns the EE%, it was possible to observe from the statistical analysis and from this DoE that formulations that contain the minimum concentration of SAL defined in the matrix design (10μg/mL) had EE% closer to 100%. It is understandable that micelles with low polymer concentrations do not effectively encapsulate high concentrations of drug, however, as the polymer concentrations are increased in the system, the number of formed micelles also expands, and nevertheless, this seems not to enhance the EE%. Please refer to table 4 for more information related to concentrations used on formulations.

7.2.1 Characterization of Plain PM and PM SAL

On the comparison of Plain PM and PM SAL, slightly differences were observed related to the M.D, PDI and ZP for most of the samples, this indicates that either high or low EE% do not substantially affect the measured parameters (Figure 6 (A), (B) and (C).

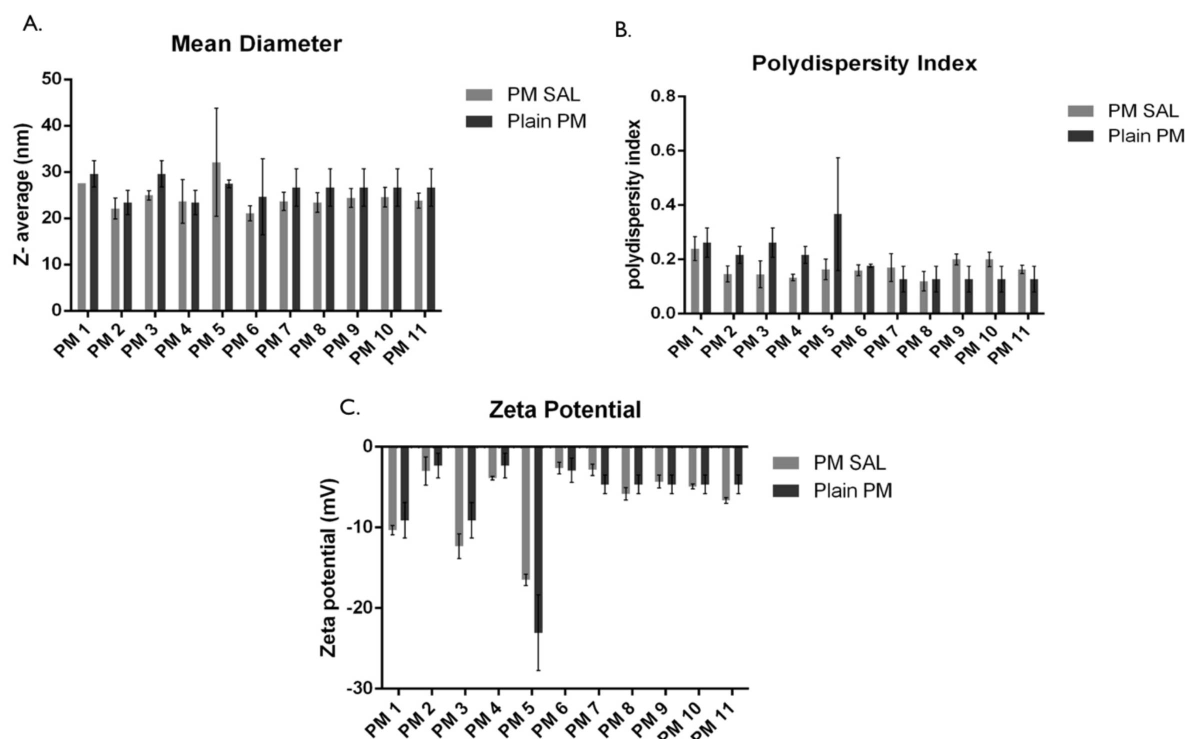


Figura 6 M.D, PDI and ZP of PM SAL and Plain PM.

In general, comparing M.D, PDI and ZP of Plain PM and PM SAL, no relevant differences were observed, the minor changes are considered normal due the variation testing equipment. All results are presented as means ± SD n=3.

Formulation PM SAL 5 displayed slightly high values of M.D when compared to the Plain PM (Figure 6 (A)), however, on the PDI measure, the opposite occurred, Plain PM 5 displayed higher values than the loaded nanoparticle (Figure 6 (B)). Similar results were obtained on the statistical analysis and the DoE, so as a conclusion, these differences may be attributed to the low polymer concentration (5mg/mL). Moreover, the presence of free monomers confers high dispersion contributing for the system lower stability since PDI can indicate the presence of agglomerates.

7.3 Optimal Formulation

According to the data obtained from the screening, formulation PM SAL AC (100μg/mL of SAL and 5mg/mL of polymer) has stood out from the others since it possesses the expected properties of micelles such as M.D, PDI and ZP. This formulation also had the higher concentration of SAL which was needed to conduct serial dilutions used in the *in vitro* experiments. Then, in the second DoE, the formulation PM SAL 5 (100μg/mL of SAL and 5mg/mL of polymer) displayed similar data previously obtained. In order to avoid future

instability owing low polymer concentration. PM SAL 5 give rise to the final formulation selected as PM SAL 12 to be used in the *in vitro* studies.

Tabela 7 Formulation for in vitro studies.

Sample	Size. (nm)	PDI	Z.P (mV)	EE %
PM SAL 12	26	0,22	-10,7	97,9

This formulation was prepared by extrapolating concentrations used in previous experimental designs with final concentration of 10mg/ml of Pluronic F127 and 200µg/ml of SAL.

Data obtained from the characterization of this optimum formulation has shown M.D, PDI, ZP and EE% were within the parameters required for micelle internalization and drug delivery.

7.4 *In vitro* Studies

Studies *in vitro* were performed in order to obtain the cytotoxic effects of the formulation, such as the free drug and Plain PM.

7.4.1 SAL Cytotoxic Effect

The antiproliferative effect of SAL was evaluated through MTT assay. The A549 cell lines were treated with increased concentrations for 24 and 48 hours. The ratio of viable cells compared with controls (non treated cells) are displayed in figures (7 (A)) and (7 (B)).

At 24 hours, it seems that SAL only has effect on the highest concentration (100µM) while the others are similar to control having low effect on the cell viability, however at 48 hours, the viable cells considerably decrease from the lowest concentration to the highest. (0,1 – 100 µM). This data implied that SAL may act in a dose and time dependent manner.

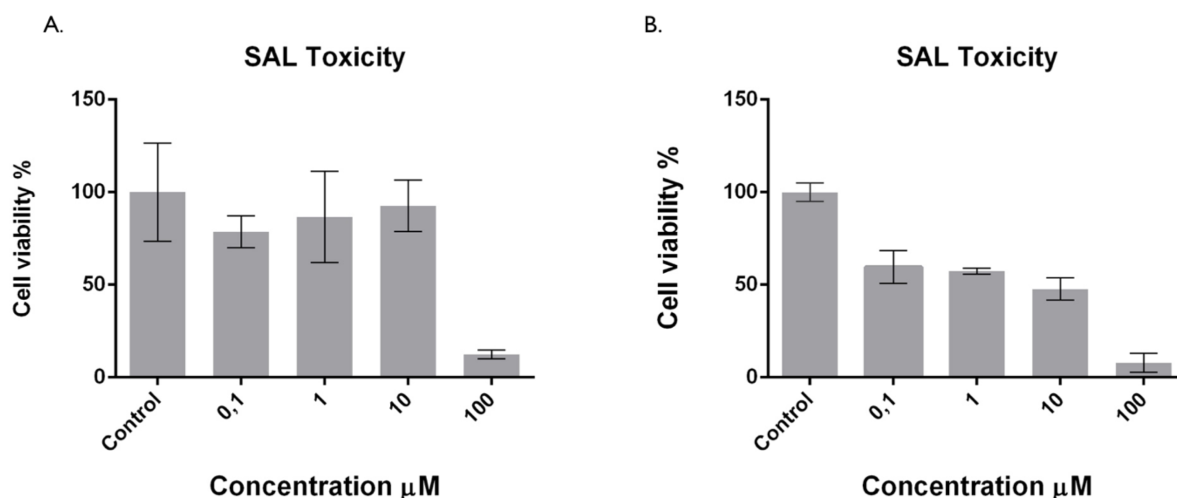


Figura 7 Cytotoxic effect of SAL in a model of human lung cancer.

Bars represent a decrease in cell viability after 24 and 48 hours respectively. All values are expressed as mean ± SD n=6.

7.4.2 PM and PM SAL Cytotoxic effect

SAL effect on A549 cell viability, Plain PM and PM SAL were assessed through MTT assay. As observed at the figure (8 (A)), polymer did not seem to induce cell death which is in accordance with the literature.(Taha *et al.*, 2014). PM SAL was tested in order to evaluate the effect of SAL loaded in PM at 48 hours. As shown in (Figure 8 (B) and (C)) PM SAL did not have the desired effect on cell viability as it was expected this formulation would increase cell death and as consequence the IC 50 would be lower over the free drug. The possible reasons for this result will be discussed later on this thesis.

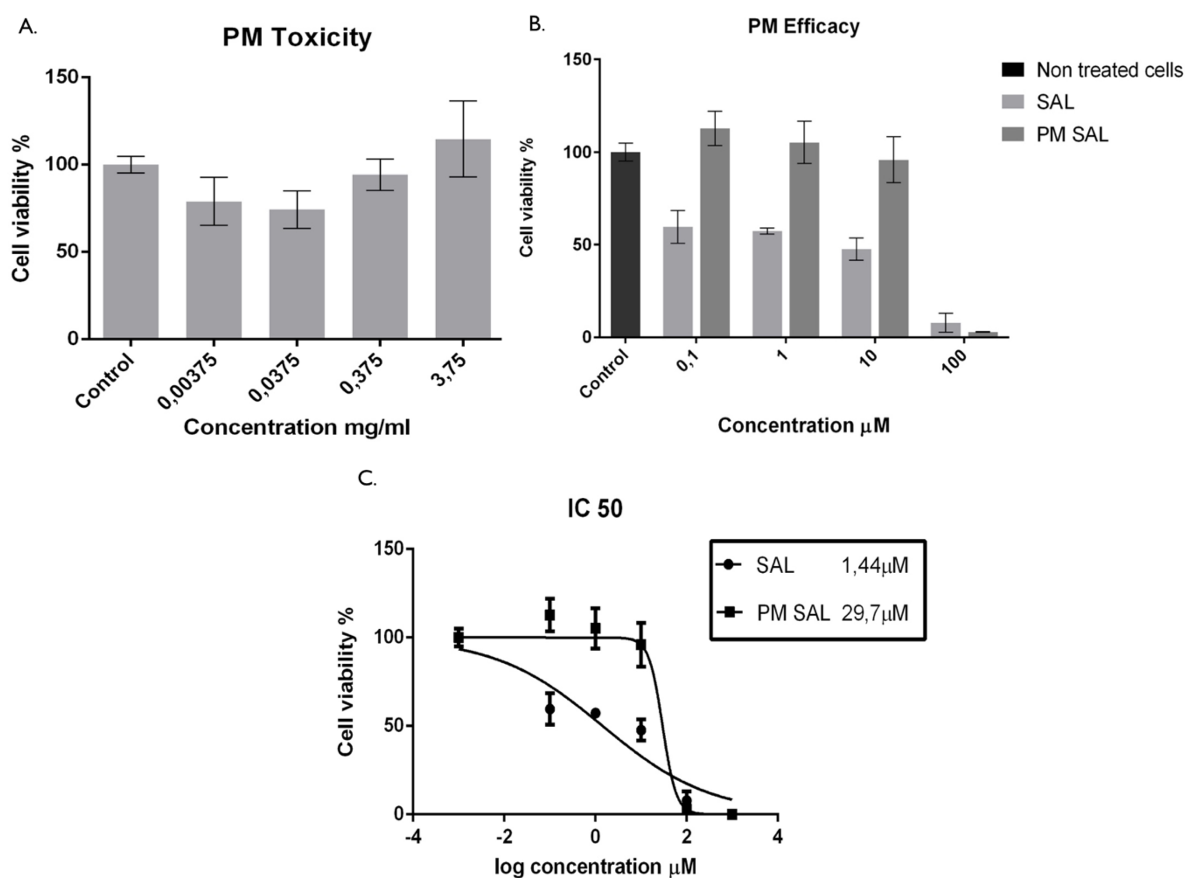


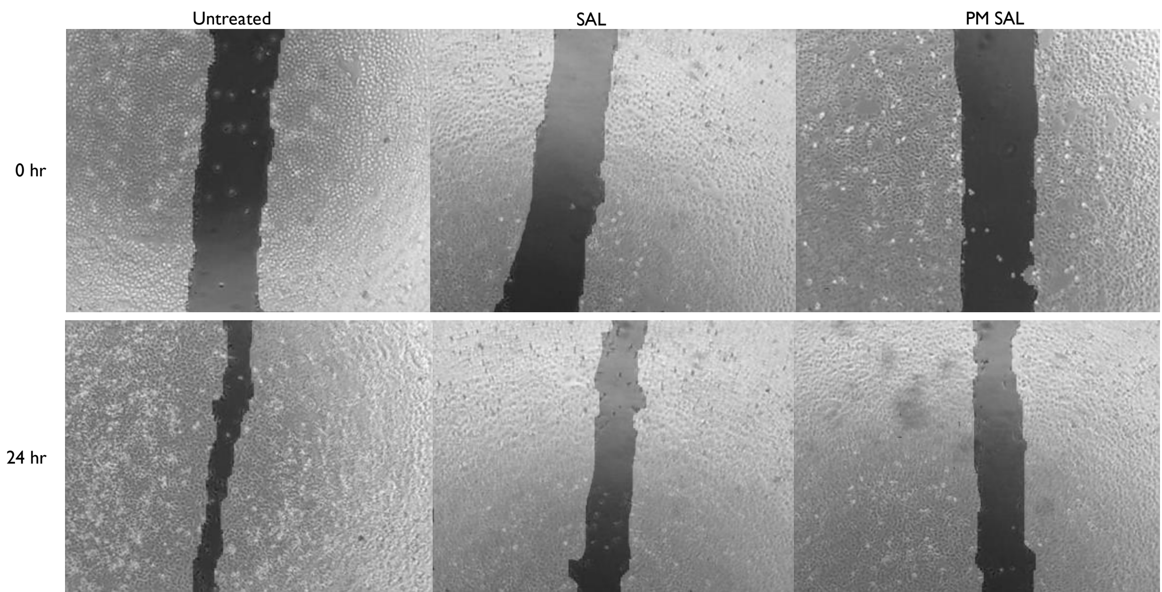
Figura 8 Comparison of SAL and PM SAL efficacy in a model of human lung cancer.

(A) Besides the minor cell variabilities, polymer has shown not to influence cell death. (B) Decrease in cell viability is observed more sharply in cells treated with SAL. (C) Slope was much higher in cells treated with free SAL. All values are expressed as mean ± SD n=6.

7.5 Wound healing assay

The migration assay was assessed in order to compare if SAL and PM SAL are capable of retarding the invasive behaviour of A549 cells. Images were collected after treatment with SAL and PM SAL at 10µM of SAL (loaded vs free) at 0 and 24 hours (Figure 9 (A)) and the value of the open scratch was calculated (Figure 9 (B)). After 24 hours, it is possible to observe that the scratch open area decrease 3 fold for untreated cells 1,5 fold for cells treated with SAL and 1,7 fold for cells treated with PM SAL compared to initial time. This results demonstrate that A459 cells are highly invasive and SAL and PM SAL showed to have the expected effect in retarding the cell migration.

A.



B.

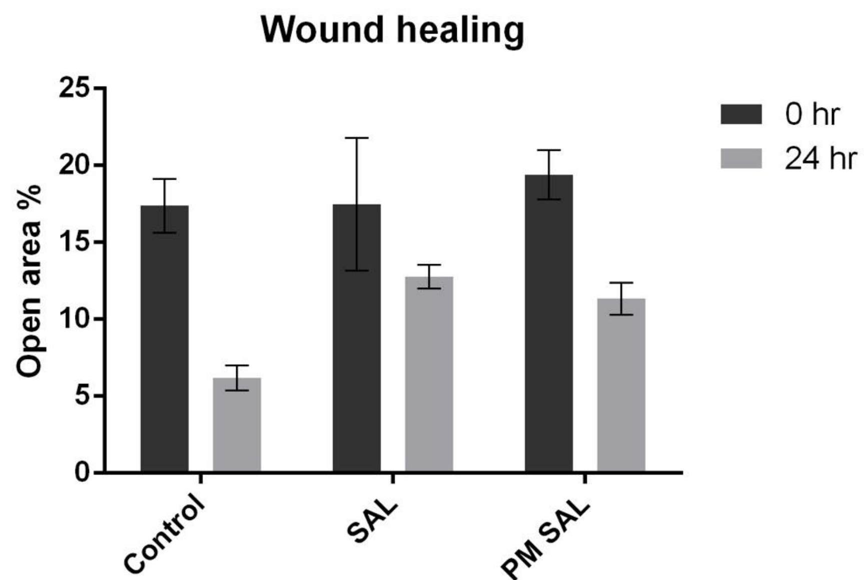


Figura 9 Migration assay in a model of human cell lung cancer.

(A) Migration of cells observed after 24 of treatment with SAL and PM SAL at 10μM. (B) bars represent the difference on the open area scratch at 0 hr. and 24 hr. The invasiveness observed in the untreated cells can be an indicative that this cell line possesses overexpression of Vimentin. All values are expressed as mean ± SD n=3.

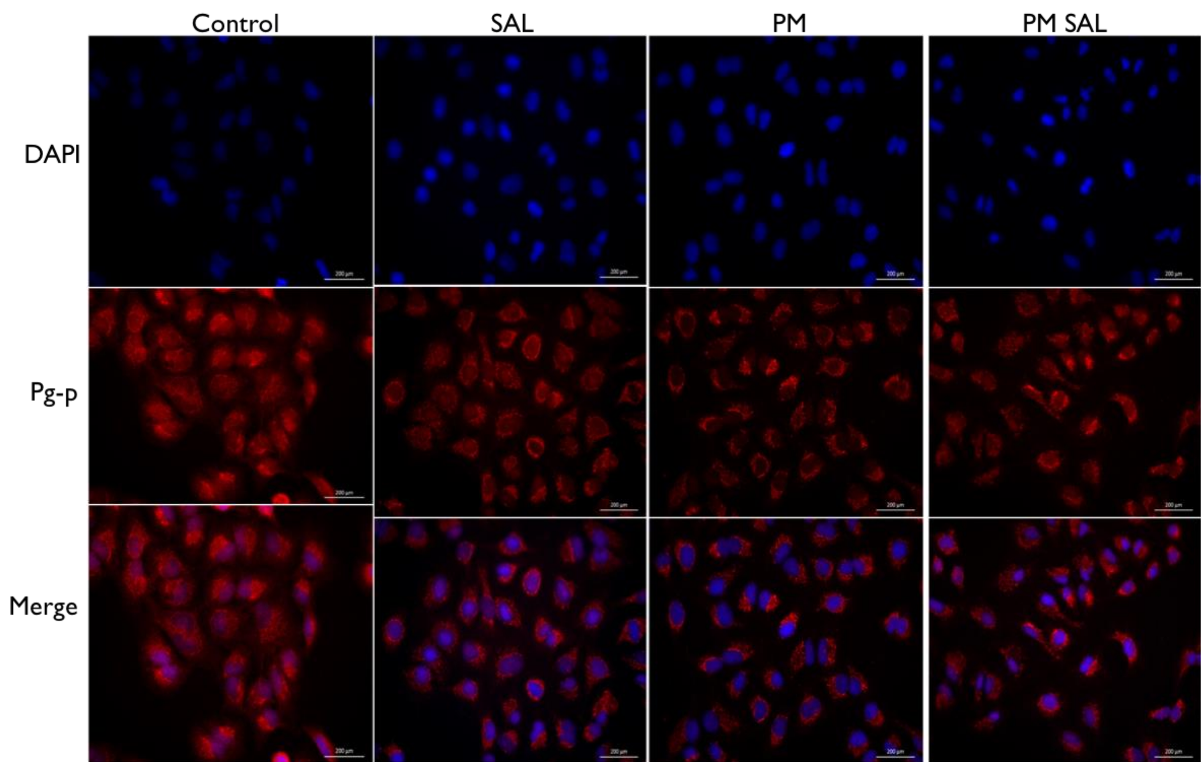
7.6 Immunocytochemistry

Immunocytochemistry assay was performed in order to study the effect of PM SAL and SAL on the P-gp and Vimentin proteins.

7.6.1 P-glycotprotein expression

The P-gp transmembrane protein is largely known as one of the players involved in the multidrug resistance mechanisms. Encoded by the ABCB1 gene Pg-p is an ABC transporter of substrates across extra and intracellular membranes. The efflux pump effect interferes with drug bioavailability influencing on the pharmacokinetics and pharmacodynamics of cytotoxic drugs reducing their efficacy conferring resistance to chemotherapy treatment.
The objective of this Immunocytochemistry experiment was to evaluate and compare if SAL and PM SAL act as a P-gp substrate and observe the effect on the expression of this transmembrane protein after 30 minutes of contact with A549 cells (Figure 10 (A)). As shown in the graph (Figure 10 (B)) at 30 minutes of incubation, the fluorescence intensity of cells treated with SAL and PM SAL have similar behaviour on the Pg-p expression compared to the untreated cells. In fact, it is possible to observe that the fluorescence levels of Pg-p similarly diminished in all the conditions (free drug, PM SAL and Plain PM). These results could indicate that PM SAL is able to evade the efflux pump mechanism of cell and provide accumulation of drug in the cytosol.

A.



B.

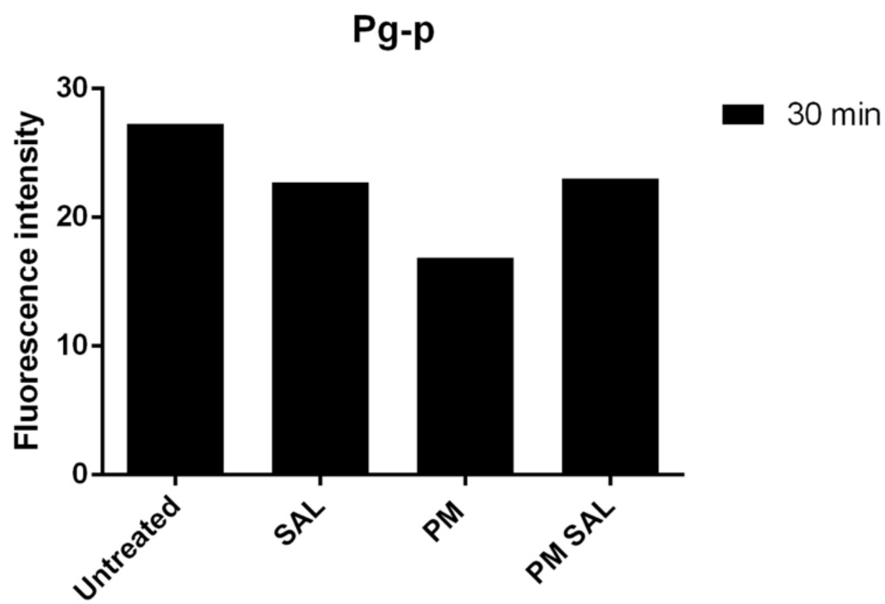


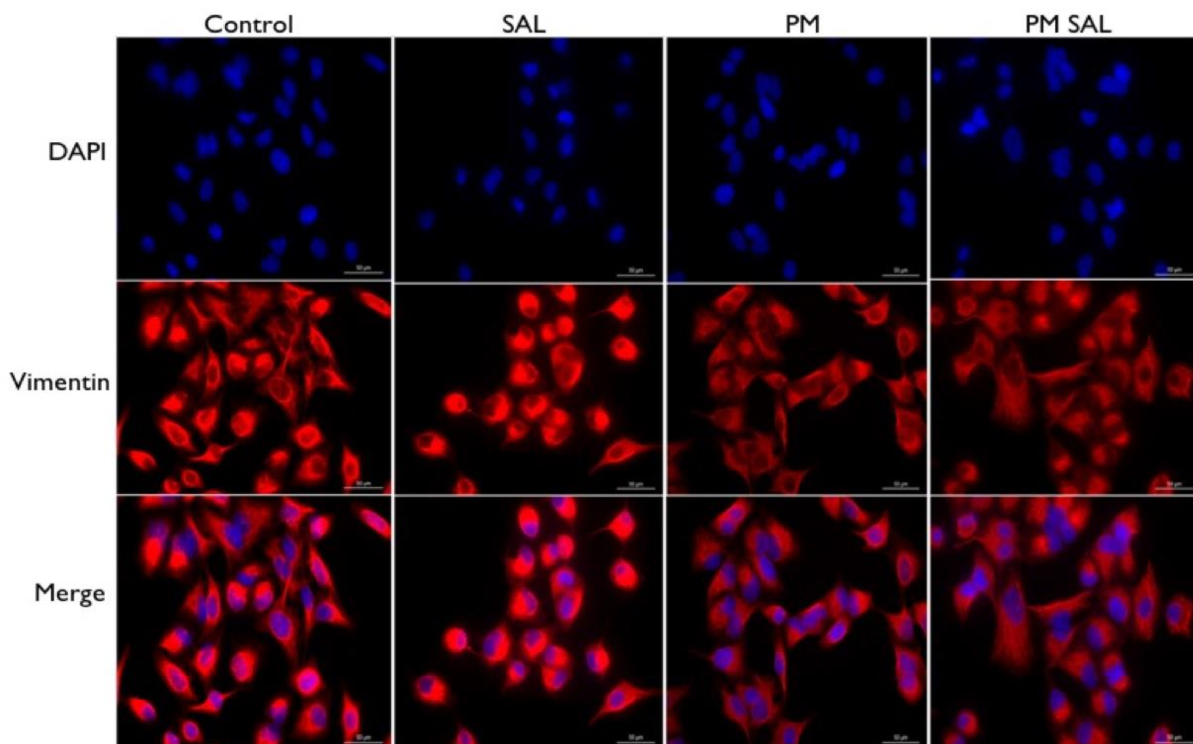
Figure 10 Immunocytochemistry for PG-P antibody in a model of human cell lung cancer.

Images and bars represent Pg-p staining and fluorescence intensity after 30 minutes of treatment with SAL and PM SAL at 10μL. Dilution used for PG-P 1:200 and 1:500 for Alexa Fluor 594.

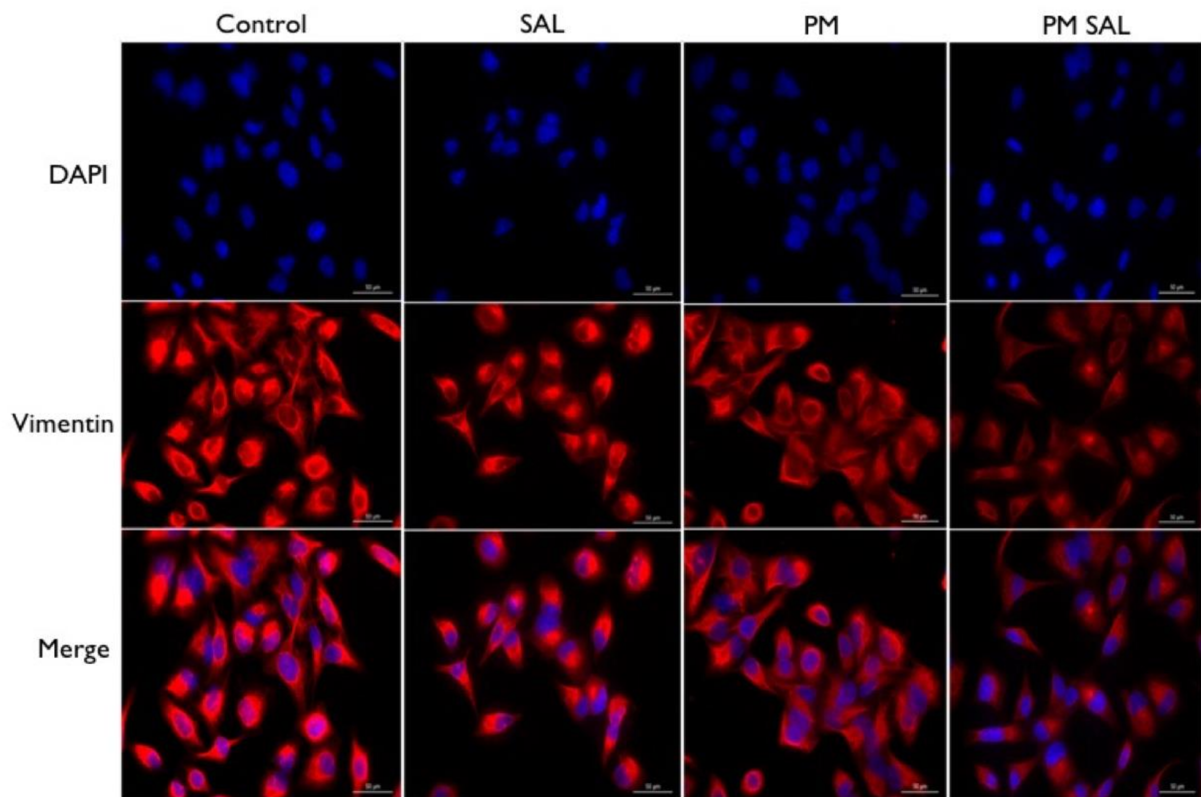
7.6.2 Vimentin expression

One of the expected effects of SAL is the inhibition of the EMT in malignant tumours and Vimentin protein is highly overexpressed. As we have seen in migration experiment that SAL is capable to repress the migration, this immunocytochemistry was assessed in order to evaluate the influence of SAL and PM SAL when A549 cells are treated with 10μM and 20μM for 24 hours (Figure 11 (A) and (B)). At concentrations of 10μM is possible to observe that free SAL and PM SAL are capable to decrease the overexpression of Vimentin, however, this expression is even more diminished for cells incubated with 20μM of drug concentration (Figure 11(C)). PM SAL appears to be more effective than free SAL, these results indicate that PM SAL was effectively captured by cells promoting the inhibition of Vimentin expression.

A.



B.



C.

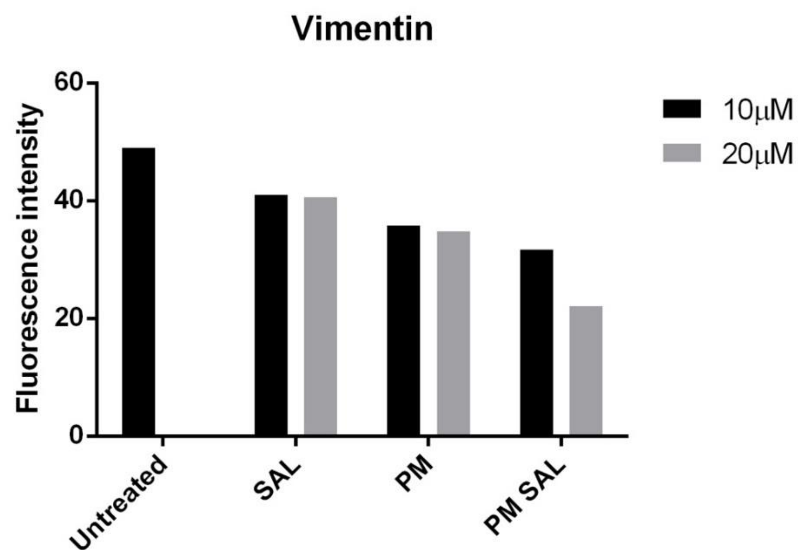


Figure 11 Immunocytochemistry for Vimentin antibody in a model of human cell lung cancer.

(A) Images represent vimentin staining after 24 hours of treatment with SAL and PM SAL at 10μM. (B) Image represent vimentin staining after 30 minutes of treatment with SAL and PM SAL at 20μM. (C) Bars represent the fluorescence intensity for the conditions used after 24 hours. Marked decrease has been observed by more concentrated PM SAL. Dilution used for Vimentin 1:100 and 1:500 for TRITC.

PART 4

DISCUSSION

8. Design of polymeric micelles

Nanomedicines as drug delivery have been largely used to treat various diseases, most relevant in oncology field. The approach of creating a particle in the nanometers size able to deliver one or multiple drugs to a specific target aiming to increase the pharmacologic action and avoid the undesirable side effects is challenging but it is an exciting progress that medicine has given to patients with cancer (Torchilin, V. P., 2007). There are many processes to construct nanoparticles using different materials and techniques. In this work the FDA approved tri-block polymer Pluronic F127 has been chosen in order to form polymeric micelles and the thin film hydration method was used to prepare it due to the simplicity and practicability (Ai *et al.*, 2014).

On this thesis we aimed to focus on the preparation of an ideal formulation in order to evaluate the performance *in vitro* of PM loaded with SAL. This was observed comparing loaded polymeric micelles with free SAL in a model of lung cancer cells.

From the statistical analysis of the screening it was possible to conclude some aspects from the variables used to construct the micelles. The particles surface charge on the formulations (Table 5) demonstrated to be influenced by the polymer concentration therefore, particles with lowest polymer concentration have more negative surface charge (Figure 5 (A)). ZP is an important parameter to evaluate the interaction with local environment and determinates in vivo stability and clearance. If the particles have a large negative or positive ZP they will repel each other stabilizing electrically while low ZP tend to coagulate or flocculate compromising the stability (Lu, G. W. e Gao, 2010).

In polymeric micelles the polymer concentration is an important parameter on the micellization process, since it is necessary that the concentration used is above the CMC, otherwise, free monomers will be dispersed in the system modifying its stability (Shaarani, Hamid e Mohd Kaus, 2017).

The EE% has shown an interesting result. Besides the high values obtained in general (Table 5), the dependency of the EE% on the initial drug concentration is observed. It was verified that when the concentration of SAL is increased on the formulation, micelles seems to have reached the maximum of its potential of loading and therefore the drug entrapment decrease (Figure 5 (B)).

Water volume did not affect any of the parameters studied (Table 5 and Figure 5(C)) indicating not to be a critical factor, because of that, this variable was excluded from the second set of DoE. The polymer concentration also demonstrated not to affect the EE%

values (Figure 5 (D)). These results were expected to be confirmed in the next set of experiments.

On the second DoE, similar results were obtained from the screening; e.g: M.D less than 100 nm (20 to 35 nm) and PDI equal or lower than 0.3, these results are within the proposed values for Pluronic F127 micelles (Table 6). It was also observed that M.D is not influenced by the drug incorporated inside the micelle (Figure 6 (A)). In fact, loaded micelles showed to be slightly smaller than plain micelles, this small difference on this parameter is not relevant since it is acceptable that some variations occur on the physical chemical properties. The small size of micelles is important on the drug delivery because they are able to easily penetrate and accumulate on tissues trough the EPR effect. In addition, they are suitable for intravenous administration and are able to avoid the recognition and the uptake by RES (Meng *et al.*, 2017; Sotoudegan *et al.*, 2016; Torchilin, V. P., 2007; Torchilin, Vladimir P, 2001).

The fact that formulations PM SAL 1.0 from the screening (Table 5) and Plain PM 5 (Figure 6 (B)) presented higher M.D and PDI respectively could be explained by the dilution of disperse system related to the low concentration of polymer, this could contribute for a poor stability in aqueous medium since there is not enough equilibrium between monomers and micelles (Torchilin, Vladimir P, 2001).

Similar results of EE% on the screening and DoE were obtained, however, the uncertainty related to the analytical method respectively the sample preparation may have influenced the accuracy of the results.

The choice of a formulation to conduct the *in vitro* studies (PM SAL 12) was made using the data obtained from the screening and DoE by the extrapolation from of the PM SAL AC and PM SAL 5. Furthermore, the M.D, PDI and Z.P showed to be similar to the parental formulations (Table 7).

9. *In vitro* studies

In this study, the cytotoxic effect of free SAL has shown to have a dose and time dependent profile and cell death was observed after 48 hours of treatment (Figure 7 (B)) in lung cancer cells, this result is in accordance with the literature (Arafat *et al.*, 2013). These cytotoxic effect on killing CSC is based molecular interactions, including the downregulation of the expression of oncogenes such as MYC and ERG (Ketola *et al.*, 2012).

The results of cell viability assay comparing encapsulated drug versus free drug did not complied with the expectancy (Figure 8 (A), (B) and (C)).

Similar results were obtained in a study using MCF-7 cells and the most likely reason is that SAL as free drug has been reported to be selective for CSC which in the heterogeneous microenvironment of the tumour represents the minority (Zhang, Yang *et al.*, 2012). As such, the non-functionalized PM SAL were most likely internalized by tumour cells and thus, not causing as much effect on CSC. Even though there are controversies about CSC markers in lung cancer, studies show that in NSCLC the minimum residual disease population with CSC like properties express CD133 and CD44, and so far, these surface markers are the most known CSC marker in this type of cancer (Alamgeer *et al.*, 2013).

The modification of PM surface by the attachment of these antibodies on the hydrophilic shell could be an alternative strategy to overcome the non-specific delivery of micelles and promote better cellular uptake. The direct target of CSC might lead to a greater efficacy of PM SAL in A549 cells. Moreover, the need of ALDH protein expression studies and isolation of CSC are essential in order to understand the effect of SAL and PM SAL on these cells (Li *et al.*, 2017).

SAL is reported in several studies to have anti invasive and anti-migratory effect in many types of cancerous cells including breast, ovarian and lung cancer (Kopp, Hermawan, Oak, Ulaganathan, *et al.*, 2014).

The wound healing assay is and effective way to confirm this migratory effect. The thin scratch displayed after 24 hours of the wound in non-treated cells proves that they possess invasiveness and migratory properties. SAL and PM SAL has demonstrated to effectively repress the invasive and migratory phenotype (Figure 9 (A)). The obtained anti invasive effect of SAL is in accordance with previous studies that demonstrated its ability to inhibit cell migration in vitro in a model of prostate and lung cancer and in vivo against metastasis from breast cancer. Furthermore, Arafat *et al.* (2013) demonstrated that SAL selectively impaired cell invasion of LNM35 and A549 cell lines in a time and dose dependent without significant cell death. These results demonstrate that PM SAL has played its part in ensuring the efficacy of the drug and thus not losing its effectiveness.

As mentioned in the literature, cancer and CSC naturally express high levels of Pg-p on its surface, the enhanced levels of this ABC transporter block the drug accumulation inside the tumour by the ejection of anti-cancer drugs. This efflux pump system interferes with the pharmacological efficacy and mediates drug resistance in many tumours allowing the cell survival and tumour regrowth after chemotherapy (Amin, 2013).

In a study using MDR cell lines it was observed that SAL act as Pg-p inhibitor and could restore the drug sensitivity of resistant cells, this contribute for its pharmacological activity as anti-cancer drug (Riccioni *et al.*, 2010).

In fact, the results obtained here in (Figure 10 (A) and B)) has shown a decrease of the Pg-p after 30 minutes of drug contact with A549 cells. Studies has demonstrated that SAL is able to overcome the ABC transporters and decrease the drug efflux pump expression and activity (Hermawan, Wagner e Roidl, 2016; Naujokat e Steinhart, 2012). This inhibitory effect may be due to a conformational change that the protein undergoes, since this effect is caused by other Pg-p inhibiting drugs such as cyclosporine, ivermectin and voacamin (Riccioni *et al.*, 2010). Even though loaded nanoparticles had similar results with free drug on decreasing the expression levels of Pg-p, this event was expected since one of the benefits of nanoparticles is to evade the efflux pump by the Pg-p non recognition avoiding the drug extrusion and promote drug accumulation in the cytosol (Zhang, S. *et al.*, 2009).

Finally, Vimentin expression had a markedly decrease when exposed to a more concentrated PM SAL (Figure 11 (B) and (C)). The low expression of Vimentin proves that encapsulated and free SAL are in accordance to studies that suggested that this drug act as inhibitor of invasiveness and migration that occur in EMT process (Liu, C.-Y. *et al.*, 2015).

The anti-migratory effect of SAL can be explained by several studies, for example, SAL and Metformine has shown to inhibit migration and EMT through the inhibition of EMT inducing effect of TGFβ. It was also demonstrated that SAL could block Wnt/β catenin signalling pathway affecting the EMT (Dong e Hu, 2017).

In summary, the results provide evidence that nanoparticles are an effective system to deliver SAL, whereas the drug did not degrade thus maintaining its activity as anti-migratory, inhibition of Pg-p and Vimentin.

PART 5

CONCLUSION AND FUTURE PERSPECTIVES

10. Conclusion

Polymeric micelles demonstrated to have the proposed properties for a successful drug delivery such as the encapsulation of large amount of drug in a small size nanoparticle which will promote cell internalization while surface charge will confer systemic stability.

In the present study it was observed that the new anti-cancer drug candidate is capable of killing malignant lung cancer cells and inhibit the EMT process by repressing cell invasion, these became more evident by the migration assay results and by the downregulation of Vimentin, a marker for mesenchymal cells and it is great responsible for invasion and migration of tumours.

In theory, PM SAL should have similar or greater effect compared to free drug. This was not observed on the viability assay, but great results were successfully obtained on the wound healing and immunocytochemistry assays where loaded micelles effectively inhibited the migration and reduced the Pg-p and Vimentin expression.

When SAL is encapsulated by micelles it is able to evade the efflux pump by blinding and diminishing levels of Pg-p which is responsible for the drug resistance being the major problem of cancer therapy. As polymeric micelles promote drug accumulation in the cytosol, the use of encapsulated SAL could be an alternative to restore drug sensitivity caused by ABC transporters, respectively Pg-p.

Migration retarding in combination with low expression of Vimentin caused by encapsulated drug, prove that this approach can effectively reduce cancer metastasis.

These results are very important to prove that malignancy of tumours and resistance acquired by ABC transporter can be reduced by SAL.

In general, the purpose of this thesis was successfully achieved, from the encapsulation of SAL to its *in vitro* results. Encapsulated SAL has demonstrated to overcome the biological barriers and more importantly was able to preserve the drug activity. Polymeric micellar system has demonstrated to be a great drug delivery candidate for hydrophobic drugs due to the high entrapment efficiency and the propitious size to be accumulated in tumour tissue by cells via EPR. Moreover, SAL and PM SAL can be helpful on the therapy of cancer and act as a supporting treatment along with other anti-cancer drugs already in use as the studies carried out by Kim *et al* (2011) that had shown that the combination of SAL, DOX and ETO had their effect potentialized in sensitizing breast and uterine sarcoma cells.

11. Future perspectives

More studies on the proposed system engineering need to be performed in order to validate and optimize the method. Also it will be extremely useful to study the internalization pathway and understand if caveolin or clathrin mediated endocytosis are involved in the internalization process.

An interesting approach would be the combinatory therapy since studies demonstrated that polymeric micelles are able to encapsulate not only one anticancer drug but could deliver other relevant therapeutic agents that would increase the pharmacological efficacy and reach different sites in cancer cells.

The functionalization of micelles by attaching specific antibodies that are highly expressed in CSC would increase the cell targeting and this could further improve the nanoparticles efficiency.

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