Carolina Correia de Figueiredo Abreu Pereira
Licenciada em Biologia Celular e Molecular

Nanomedicines, a Strategy to Evade Multidrug Resistance

Dissertação para a obtenção de grau de Mestre
em Genética Molecular e Biomedicina

Orientador: Doutora Mafalda Videira, Professora Auxiliar, Faculdade de Farmácia da Universidade de Lisboa
Co-orientador: Doutor João Gonçalves, Professor Associado, Faculdade de Farmácia da Universidade de Lisboa

Júri:

Presidente: Prof. Doutora Ilda Maria Barros dos Santos Gomes Sanches
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Agradecimentos

A realização desta tese só foi possível com o apoio e paciência de muitas pessoas. Mas gostaria de começar por si: Muito, mas mesmo muito obrigada pelo apoio, ajuda, carinho e paciência que demonstrou ao longo deste ano tão atribulado Professora Mafalda Videira, nunca irei esquecer o que fez por mim.

O meu profundo agradecimento à Professora Alexandra Brito, por me ter ensinado e apoiado com a Imunocitoquímica, pela disponibilidade e ajuda que me deu ao longo do meu percurso na Faculdade de Farmácia. Gostaria de agradecer também ao Professor Jorge Victor por todas as dicas e apoio. À Carmo pela ajuda com o HPLC. Agradeço à Marta Giria pela ajuda com a síntese de cDNA, sem a qual não seria possível realizar o "real-time" PCR.

Agradeço também ao Adriano do grupo da Professora Madalena Pimentel por me ensinar a técnica e demonstrar o Western Blot e SDS-PAGE. À Carolina Cunha e à Cátia Gomes do grupo da Professora Dora Brites por ajudarem com a extracção de RNA e qPCR, e pela paciência quando as coisas só pareciam correr mal. Ao Pedro Rodrigues do grupo da Professora Cecília Rodrigues, por me ajudar a arranjar uma outra forma de quantificação de RNA quando o "nanodrop" avariou, muito obrigada.

Gostaria ainda de agradecer à Ibane Abasolo pelas imagens de internalização facultadas.

À Lucília e a todas as pessoas sempre simpáticas que andam pelo pavilhão E, por toda ajuda e apoio.

Não posso deixar de agradecer à Fundação para a Ciência e Tecnologia pelo financiamento do projecto PTDC/SAU-FAR/120453/2010, onde se integrou o meu trabalho.

À minha família, namorado e amigos, por toda a paciência que gastaram comigo, compreensão e apoio, um beijinho grande a todos.

Resta-me desejar que o presente trabalho, que, naturalmente, só a mim compromete, não desvalorize todo o apoio que tive para o realizar.

Resumo

A multirresistência a fármacos é um dos maiores problemas que enfrentamos actualmente no combate ao cancro. Dos mecanismos envolvidos, destaca-se a acção da glicoproteína-P, uma proteína transmembranar responsável pelo efluxo dos fármacos diminuindo a sua biodisponibilidade. Um outro mecanismo de destaque envolve a acção do factor de transcrição Twist, que regula vias envolvidas na multirresistência, nomeadamente por estar envolvido na regulação do gene da glicoproteína-P. Com este estudo pretende-se verificar se as nanopartículas formuladas com paclitaxel possuem a capacidade de evitar estes mecanismos de multirresistência e se, após funcionalização com um anticorpo específico de receptores de células estaminais (anti-CD44v6) têm o seu efeito de selectividade para estas células aumentado. Para tal foi necessário preparar as nanopartículas de lípido sólido e testar numa linha celular de cancro de mama (MDA-MB-436), de modo a verificar a ausência de efeito tóxico de nanopartículas sem fármaco, o efeito farmacológico das nanopartículas com fármaco relativamente aquele que é atribuído ao fármaco livre. Verificou-se que as nanopartículas têm a capacidade de escapar aos mecanismos de multirresistência contrariamente ao observado para o fármaco livre, no entanto a presença do anticorpo à superfície não demonstrou qualquer vantagem em termos farmacológicos *in vitro*. Este facto permite-nos concluir que as nanopartículas propostas são eficazes contra os mecanismos de multirresistência estudados.

Palavras-chave: Multirresistência, Nanopartículas de Lípido Sólido, Glicoproteína-P, Twist

Abstract

Multidrug resistance is a major problems associated with cancer chemotherapy. Efflux transports is one of the numerous mechanisms involved in multidrug resistance. P-glycoprotein is a transmembrane protein, responsible for drug efflux, which decreases drugs intracellular bioavailability, consequently decreasing their efficacy against cancer. Cancer growth and dissemination depends on the expression of transcriptional factors such as, Twist. Among other features, this protein is related with cells chemoresistance possible by regulation of multidrug resistance pathways including the P-glycoprotein expression. The herein study proposes to demonstrate if paclitaxel entrapped nanoparticles is an effective system in evading multidrug resistance mechanisms and if functionalization of a specific antibody against cancer stem cells receptors (anti-CD44v6) has the capability to target selectively these cells increasing nanoparticles efficacy. Therefore solid lipid nanoparticles were prepared and a breast cancer cell line (MDA-MB-436) was exposed to them in order to assess unloaded nanoparticles cytotoxic effects, increased pharmacologic efficacy of loaded nanoparticles relative to the free drug and their ability to evade multidrug resistance. The proposed solid lipid nanoparticles system proved to be capable of efficiently evading multidrug resistance mechanisms; however no improvement was added when these nanoparticles were functionalized with the antibody in the *in vitro* studies. However, the nanoparticles system is effective against multidrug resistance mechanisms.

Keywords: Multidrug Resistance, Solid Lipid Nanoparticles, P-glycoprotein, Twist

Contents

Indexes of Figures

Indexes of Tables

List of abbreviations, acronyms and symbols

ALDH1 – Aldehyde Dehydrogenase1

Bcl-2 – B cell lymphoma-2

bp – base pairs

CSC – Cancer Stem Cells

ECM – Extracellular Matrix

EMT – Epithelial-Mesenchymal Transition

HA – Hyaluronic Acid

MDR – Multidrug Resistance

MMP – Matrix Metalloproteinases

PEG-PE – Polyethylene glycol-Phosphatidylethanolamine

P-gp – P-glycoprotein

Ptx – Paclitaxel

ROS – Reactive Oxygen Species

SLN – Solid Lipid Nanoparticles

SC – Stem Cells

TGFβ – Transforming Growth Factor β

TSP-1 – Thrombospondin-1

VEGF – Vascular Endothelial Growth Factor

1. Introduction

1.1 Cancer: epidemiologic scenario

Despite the massive resources that have been devoted to find a cure for cancer, the 14.1million new cancer cases and 8.2 million cancer deaths registered in 2012 shown that this disease is still one of the most critical unmet clinical needs (Ferlay et al., 2015). According to Ferlay et al. (2013) this heterogeneous disease accounts for 355.7 new cases with a mortality rate of 168.0 estimates for both genders per 100000 inhabitants in Europe for 2012, excepting melanoma skin cancers (Ferlay et al., 2013). Portugal with 429.7 (male), 263.0 (female) new cases and a mortality rate of 202.0 (male), 103.5 (female) present a disappointing situation. Particularly, Breast cancer has an incidence of 94.2 and a mortality rate of 23.1 in Europe, while in Portugal these values are respectively 85.6 and 18.4 (Ferlay, et al., 2013). This epidemiological data demonstrates that cancer is a common and, unfortunately deadly disease, stressing at the same time that the current clinical approach is failing. Therefore, new solutions are needed and more important, new strategies should been devised.

1.2 Hallmarks of Cancer

Hanahan and Weinberg (Hanahan & Weinberg, 2000, 2011) described six hallmarks in cancer which are the main physiological alterations in tumorigenesis: self-sufficiency in growth factors; insensitivity to antigrowth factors; death evasion; limitless replicative potential; angiogenesis; and tissue invasion and metastasis. Multidrug resistance (MDR), whereby cancer cells become resistant to the cytotoxic effects of various, structurally and mechanistically unrelated, chemotherapeutic agents can be considered another cancer cell hallmark.

MDR is generally owed to the reduction of drug intracellular accumulation, which occurs through membrane transporters. These have a critical role in a number of mechanisms underlying drug absorption, distribution and elimination processes, having a relevant impact on whole body pharmacokinetics and, consequently, on the drug's efficacy/safety. One major mechanism by which MDR occurs is through ATP-binging efflux transporters, such as P-glycoprotein (P-gp) (Nooter & Herweijer, 1991; M. Videira, Reis, & Brito, 2014).

Self-sufficiency in growth factors

When shifting from a quiescent to a proliferative state, normal cells growth factors are required, whereas malignant cells have oncogenes that mimic those growth factors and stimulate cell proliferation. Usually, external soluble growth factors follow a heterotypic signalling pathway, from

one cell to another in the vicinity, mainly through transmembrane receptors (Witsch, Sela, & Yarden, 2010). Cell number, architecture and function have to be preserved for homeostasis in normal tissue. Regrettably, cancer cells can acquire the capability to perform autocrine stimulation; producing growth factors and therefore controlling their own growth and development, to a further extent than normal homeostasis. Fundamentally, malignant cells can stimulate growth factors secretion in the stroma cells, through delivery of surface proteins and release of soluble factors (growth factors and cytokines) that will in a feed-back mechanism, respond to the cell growth factor receptors enhancement (Diaz-Cano, 2012; Hanahan & Weinberg, 2000, 2011). Constitutive activation of growth factor receptors downstream pathways represents another mechanism to circumvent normal mechanisms of cell regulation. For example, in cancer receptor tyrosine kinases that regulate cell growth, proliferation and survival through growth factors signal transduction, are constitutively activated (Hanahan & Weinberg, 2011; Niederst & Engelman, 2013).

Insensitivity to antigrowth factors

In normal tissue a number of signals, such as extracellular matrix (ECM) soluble antigrowth factors or immobilized inhibitors, modulate cell quiescent state and homeostasis. Malignant cells can escape these controlled events using tumour suppressor proteins, for instance, through the loss of retinoblastoma-associated protein, which is involved in the cell cycle regulation based on extracellular inhibitory growth factors and intracellular signals balance (Hanahan & Weinberg, 2000). Transforming growth factor β (TGFβ), is an antigrowth factor when inhibiting cell cycle progression, through the induction of cyclin-dependent kinase inhibitors (Ikushima & Miyazono, 2010). Nonetheless, a recent study has demonstrated that metastatic potential can be stimulated by TGFβ in response to contact between platelets and circulating tumour cells in bloodstream. TGFβ and NF-κB signalling pathways activation, induces cell epithelial-mesenchymal transition (EMT) and invasive behaviour, thus enabling cell extravasation and metastasis dissemination (Labelle, Begum, & Hynes, 2011)

Death evasion

Programmed cell death, apoptosis, is a biological tool used by cells in response to irreparable damages, which are detected in cell cycle checkpoints in normal cells. DNA damage is generally detected by p53, a tumour suppressor protein, allowing DNA repair or if necessary inducing apoptosis, by stimulating pro-apoptotic members from the B cell lymphoma-2 (Bcl-2) family such as, Bax (Moll, Marchenko, & Zhang, 2006).

On the other hand, unregulated cancer cells can evade apoptosis, continuing to proliferate. Apoptosis is a response to different signals, that includes various steps: protein cleavage, disruption of cellular membrane, breakdown of cytoplasmic and nuclear skeletons, cytosol extrusion, chromosome

degradation, nuclear fragmentation with further apoptotic vesicles formation, which are engulfed by neighbour cells (Hanahan & Weinberg, 2000; Radogna, Dicato, & Diederich, 2015). When *TP53* is lost or mutated, which happens in 42% of all cancers, malignant cells can acquire apoptotic resistance (Hanahan & Weinberg, 2000; Kim, 2015). Caspases mediation, both in the intrinsic and extrinsic apoptotic pathways, has been highlighted (Radogna, et al., 2015). The extrinsic pathway consists in activation of caspases through ligand-receptor interaction of tumour necrosis factor superfamily and its receptor. For example, Fas and Fas-receptor, specifically Fas-associated death domain cleaves pro-caspase 8, activating caspase 8, and consequently activating caspase cascades leading to apoptosis. The intrinsic pathway occurs when, DNA damage, oxidative stress or lack of growth factors is detected. In this scenario, caspase cascades are activated due to mitochondrial outer membrane permeabilization, that releases cytochrome c and other proteins after induction of pro-apoptotic members of Bcl-2 family, Bak and Bax, embedded in the mitochondrial outer membrane, and inactivation of inhibitory effects of anti-apoptotic members of Bcl-2 family, such as Bcl-2 and BclXL (Hanahan & Weinberg, 2011; Radogna, et al., 2015). Overexpression of oncogenic proteins instead of raising proliferation rate might lead to senescence and even apoptosis (Hanahan & Weinberg, 2011)

By suffering necrosis as cell death, malignant progression is promoted instead of being stopped (Hanahan & Weinberg, 2011). Collapsing Necrotic cells exposes their content to the extracellular microenvironment, releasing pro-inflammatory signals which attract immune system cells. However, since immune cells also have growth factors and ECM modification enzymes, they can promote angiogenesis, cancer cell proliferation and invasiveness, by releasing reactive oxygen species (ROS) that enhances genetic evolution of neighbour cancer cells (Hanahan & Weinberg, 2011).

In addition, cancer cell autophagy protects them from death, when some cytotoxic drugs, radiotherapy and nutrient deficiency induce organelles injury. Autophagy is a recycling process of organelles for biosynthesis and energy metabolism through autophagosomes that enclose organelles and fuse with lysosomes. Normal cells also undergo this mechanism at low levels, however under stressful conditions, mainly due to nutrient deficiency, it can be highly induced (Hanahan & Weinberg, 2011).

Limitless replicative potential

Chromosome edges, telomeres, have multiple repeats of short sequences with 6 base pairs (bp), after each replicative cycle, roughly 50-100 bp of telomeric DNA is lost. This happens because DNA polymerases are not capable of polymerising chromosomes 3' ends, leading to chromosome edges without protection. In cancer cells there are two mechanisms to surpass this limiting problem; upregulation of telomerase, enzyme responsible for adding the 6 bp sequences, or changes of recombinant sequences between chromosomes (Blackburn, 2005; Hanahan & Weinberg, 2000). In the first case, mutations in β-catenin, from Wnt/β-catenin pathway which is involved in pluripotency regulation in stem cells, induce *Tert*, which encodes for a protein subunit of telomerase, promoting

telomere stabilization (Hoffmeyer et al., 2012). More impressive is the capability that crossing-over sequences have to generate dicentric chromosomes during crises (Counter et al., 1992).

Angiogenesis

Defined as new blood vessel formation and growth, in normal tissues, angiogenesis occurs during organogenesis, being highly controlled and transient. Cells need oxygen and nutrients that come from bloodstream, therefore capillary vessels have to be at least 100μm from cells (Hanahan & Weinberg, 2000). Tumour central regions are hypoxic areas that lead to the overexpression of the transcriptional factor, Hypoxia Inducible Factor, which, among others, is responsible for the vascular endothelial growth factor (VEGF) family proteins. This event promotes tumour angiogenesis necessary for progression, survival and adjustment to oxygen and nutrient limited environments. Efforts are being made in order to control tumour angiogenesis through cancer treatment. For example as reviewed, bevacizumab is a specific antibody against VEGF and is in clinical trials for advanced metastatic cancers chemotherapy and cytokine therapy (Carmeliet & Jain, 2011).

TGFβ overexpression can upregulate VEGF-A and fibroblast growth factor family members supporting angiogenesis (Ikushima & Miyazono, 2010). As mentioned before, immune system inflammatory cells can induce angiogenesis as well as help to protect vessels from drugs that target endothelial cell signalling (Hanahan & Weinberg, 2000, 2011).

Tissue invasion and metastasis

Some cancer cells can relocate from the primary tumour and colonize new sites where nutrients and space are not restricted. This is possible because proteins involved in cell-to-cell adhesion and integrins are modified to ensure invasive and metastatic capabilities. During the moment cells move from primary tumour site to metastatic site the microenvironment changes, so adaptation to metastatic site is necessary. This is possible due to integrins and proteases that degrade ECM being that these proteases can be associated to transmembrane receptors or integrins (Hanahan & Weinberg, 2000).

Recently, different studies have stressed that metastasis formation and migration is a process involving local invasion, intravasation (bloodstream entrance), circulation survival, extravasation (exit from bloodstream) and colonization. During this process many genes are involved, which are grouped into three classes: metastasis initiation genes, those who can transform cells to invade the surrounding tissue, attract stroma, enable cancer cells dispersion, stimulate EMT and promote ECM degradation, among other functions, for example transcription factor Twist; proto-oncogenes, they are important during circulation survival, extravasation and survival in parenchyma, such as matrix metalloproteinases (MMP); and metastasis virulence genes, essential for metastasis colonization, depend on the primary tumour site and metastatic site, cells that have origin in breast cancer

expressing parathyroid hormone-related protein and interleukin 11 can colonize bone (Nguyen, Bos, & Massague, 2009; Valastyan & Weinberg, 2011).

1.3 Mammary adenocarcinoma: the role of CSC

Approximately one million cases of breast cancer are diagnosed each year. Unlike patients with estrogen receptor/progesterone receptor (ER/PgR+) and/or human epidermal growth factor receptor 2 (HER2) overexpressing disease, with positive responses to endocrine or HER2-targeted therapies, options for Triple-negative breast cancer patients are limited to cytotoxic chemotherapy (Tarasewicz et al., 2014). Disease recurrence owing the cells chemoresistence contributes to the poor prognosis and to outcomes associated with these malignancies including aggressive metastatic relapse.

Stem cells (SC) are recognized by their ability to differentiate in cells of specific tissues (Reya, Morrison, Clarke, & Weissman, 2001). Likewise, cancer stem cells (CSC) can self-renew and differentiate being capable of originating new tumours and more importantly disease recurrence which have been related with these cells sub-population within the tumour owing its resistance to chemotherapy (Hanahan & Weinberg, 2011). It has been observed that breast SC overexpress components of Hedgehog signalling pathway, that upon activation, triggers SC self-renewal and proliferation. This biological event is regulated through the balance between a specific inhibitor, cyclopamine, and protein Bmi-1, which promotes SC self-renewal and proliferation. Hedgehog activation increases Bmi-1 expression, while small interfering RNA (siRNA) downregulation decreases Bmi-1, inhibiting Hedgehog signalling pathway effects mediated by Bmi-1. In overexpressing Bmi-1 cells, tumour initiation was observed *in vivo* upon SC implantation in a mouse model, proving that deregulation of Hedgehog signalling pathway might promote CSC or progenitor cells clonal expansion (Liu et al., 2006).

Breast CSC expressing $ESA^{+}CD44^{+}CD24^{-/low}$ sub-population have demonstrated the ability to form mammospheres, a highly malignant characteristic, and were correlated with tumour dissemination and recurrence due to chemotherapy resistance (Bane et al., 2013; Fillmore & Kuperwasser, 2008; Guler et al., 2012; Harrison et al., 2010; Li et al., 2008). In fact, tumorigenic properties have been demonstrated in a breast CSC subset $ESA^{+}CD44^{+}CD24^{-/low}$, these cells are capable of extensive proliferations contrasting with the mainstream population that have restricted proliferative potential (Al-Hajj, Wicha, Benito-Hernandez, Morrison, & Clarke, 2003).

Tumour initiation with only 20 cells was observed for $CD44^{+}CD24^{-/low}$ CSC with $ALDH1^{+}$ phenotype. Ginestier and colleagues identified aldehyde dehydrogenase 1 (ALDH1), an enzyme that oxides intracellular aldehydes, as another SC and a CSC marker (Ginestier et al., 2007). In another study, a population of CSC $ALDH1^{+}$ in inflammatory breast cancer proved to be invasive, mediating

metastasis dissemination and the ability to contribute to mimic the primary tumour heterogeneity in the host-tissue (Charafe-Jauffret et al., 2010). CSC self-renewal and differentiation in early stages of carcinogenesis ($ALDH1^+$) was shown to be regulated by PTEN knockdown, a tumour suppressor gene, with consequent PI3K/Akt pathway activation and further mediation of Wnt/β-catenin signalling pathway (Korkaya et al., 2009). The consequence to this stimulation is the loss of cell epithelial phenotype, the so-called EMT, a crucial step for tumour malignancy.

As extensively stressed in the literature, to elicit invasion and migration to host-tissues, malignant cells undergo the EMT (Cannito et al., 2008). This event of transformed human mammary epithelial cells, is dependent on the ectopic expression of two transcription factors Twist or Snail, also responsible for the cells ability of self-renew and formation of mammospheres containing stem cells. Importantly, these cells phenotype, as expected, is $CD44^+CD24^{-/low}$ (Mani et al., 2008). Accordantly, an *in vitro* study with $CD44^{low}CD24^+$ human mammary epithelial cells, demonstrated that these cells could originate $CD44^+CD24^{-/low}$ cancer cells through EMT induction, presenting at the same time an aberrant activation of Ras/MAPK pathway (Morel et al., 2008).

1.3.1 Breast Cancer Molecular Insights

The acquisition of the mesenchymal phenotype has implications in patient's prognostics. At this point of the disease disappointing outcomes are usually obtained and therefore high mortality rates occur. The question that underlines this situation is: could we face advanced cancer stages as a mutation disease? From our point of view, suitable new therapeutic approaches might benefit from a deep knowledge on cell biology rather than tissue related pathology. Genes, transcription factors and proteins act in cooperation to allow cell specialization. Among them, P-gp/CD44/Twist cross talk has raised our attention.

Membrane receptors, either related to growth factors or efflux pump proteins are among the most powerful tools used by cells to progress and survive.

(i) The transmembrane glycoprotein (P-gp), encoded by the *MDR1* gene (also known by *ABCB1*) (Nooter & Herweijer, 1991; Szakacs, Paterson, Ludwig, Booth-Genthe, & Gottesman, 2006; M. Videira, et al., 2014), which is the most broad ATP-dependent drug efflux transporter responsible for one major clinical drawback: tumours MDR (Nooter & Herweijer, 1991; M. Videira, et al., 2014). P-gp over-expression in malignant cells results in a significant decrease in the intracellular drug accumulation of many chemotherapeutic agents limiting their effectiveness, eliciting chemoresistance and promoting tumour recurrence (Szakacs, et al., 2006) (Table 1.1). Unfortunately, drugs used in oncology not only increase its activity as ATP-dependent drug efflux transporter but more important, drugs, such as paclitaxel (Ptx), enhance *MDR1* promoter activity by increasing Y-box binding protein-

1 interaction with *MDR1* Y-box motive. Y-box binding protein-1 has been proved to be located in nucleus when P-gp is overexpressed within breast cancer patients that have demonstrated to be resistant to protocols with Ptx (Fujita et al., 2005).

(ii) As for the upregulation of the extracellular CD44 fraction, its interaction with hyaluronic acid (HA), a major ECM constituent, is responsible for proliferation, differentiation and adhesion in SC. Our group (Horta et al., 2015) already revised this glycoprotein relevance in tumour growth and dissemination. Therefore therapeutic strategies targeting CD44 isoforms and interfering with CD44-HA interaction might be an interesting approach against CSC (Horta, et al., 2015). Supporting these findings, Ni and colleagues demonstrated that downregulation of the CD44 variant 6 (CD44v6) is responsible for the impairment in the PI3K/Akt/mTOR axis and Wnt/β-catenin signalling proteins, confirming its involvement in sphere formation, EMT, chemo- and radioresistance, cell adhesion and invasion (Ni et al., 2014).

(iii) When it comes to the transcription factors, Twist has a more complex role, being involved in malignant cells survival mechanisms, EMT, migration, invasion and chemotherapy resistance (Ansieau et al., 2008; Hong et al., 2011; X. Wang et al., 2004). This transcriptional factor is involved in EMT through E-cadherin downregulation, mesenchymal markers upregulation, both critical events for the malignancy acquisition and loss of epithelial phenotype, with further activation of cell pathways involved in tumour progression, (Vesuna, van Diest, Chen, & Raman, 2008; Yang et al., 2004). Along with the effect in E-cadherin expression, Twist upregulates Akt2, both contributing to cells malignancy, thus migration, invasion and survival of breast cancer invasive cells (Cheng et al., 2007; Pereira, Horta, Mateus, & Videira, 2015).

Later on, it was observed that Twist-1 phosphorylation in Ser42 by Akt is involved in cell survival and growth upon DNA damage, through independent downregulation of p53 (Vichalkovski, Gresko, Hess, Restuccia, & Hemmings, 2010). Moreover, p53 and retinoblastoma-associated protein inhibition dictated by Twist intracellular overexpression, suppresses cell checkpoint mechanisms, triggering cell transformation and promoting EMT (Ansieau, et al., 2008).

As the thorough understanding of each player is deeply important there are a number of aspects resulting from their interaction:

- Decreasing P-gp, owing the presence of an inhibitor, alters CD44 membrane localization, decreasing cell migration. Colone and colleagues also verified that CD44 and P-gp blockage affected cell invasiveness proving their role in cell migration and for MAPK signalling pathway (Colone et al., 2008). CD44 and P-gp co-localization was observed in lipid rafts, and its interaction leads to increased expression of MMP, namely MMP-9, and proteolysis activity (Figure 1.1). On the contrary, in cell lines with low expression of P-gp, CD44 cell migration and invasion do not dependent on these two

players (Colone, et al., 2008). A correlation has been proved between *CD44s* and *MDR1* expression in *CD44s* negative cells where *MDR1* expression was induced after *CD44s* transfection. Also siRNA against *MDR1* decreases *MDR1* expression and cell migration, confirming how CD44/P-gp interaction affects cell migration (Miletti-Gonzalez et al., 2005). MMP-9 and P-gp association, in a resistant MCF-7 cell line promoted MMP-9 release in the ECM and MDR phenotype conferred by P-gp (Karroum et al., 2010). This MMP also interacts with CD44, MMP-9 co-localizes with CD44 aggregates induced by HA and co-immunoprecipitate. CD44 overexpression inhibits MMP-9 similarly to its specific inhibitor (Yu & Stamenkovic, 1999).

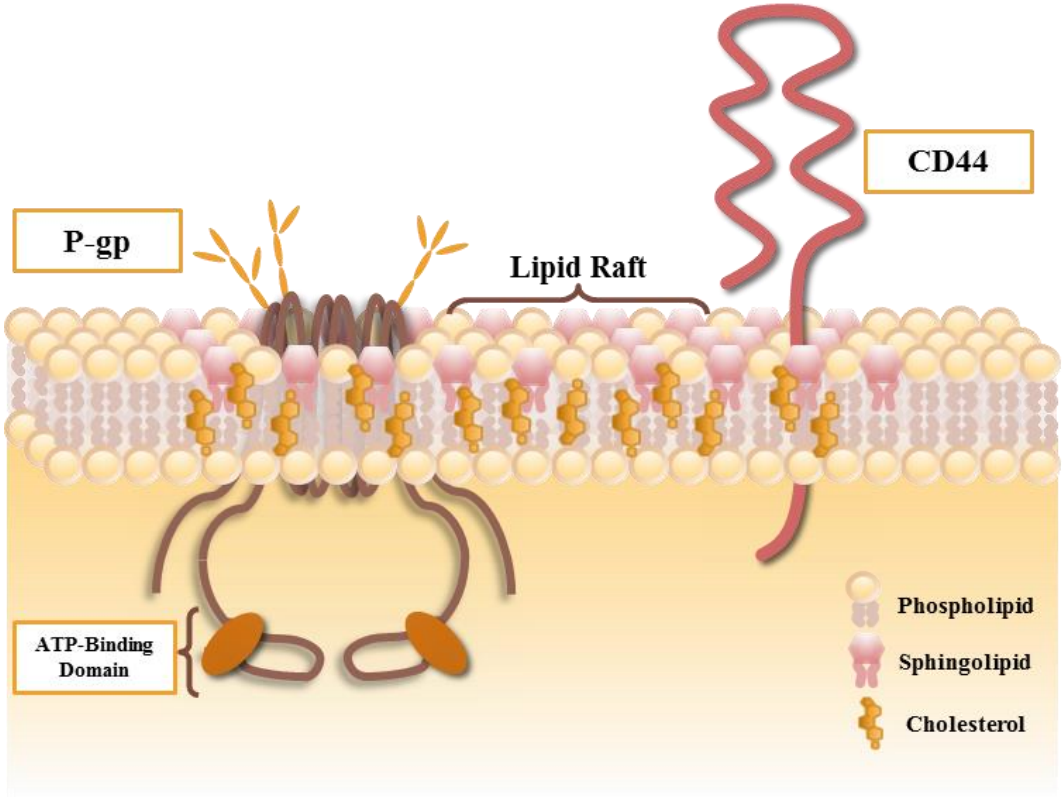

Figure 1.1 – Membrane co-localization of P-gp and CD44 in lipid rafts, schematic presentation.

- CD44 overexpression upregulates *TWIST*, whereas CD44 inhibition downregulates it (Spaeth et al., 2013). Furthermore, Wang and colleagues, using Ptx sensitive cells, confirmed its role as proto-oncogene. Cells transfected with the gene that encodes for the Twist protein were converted into Ptx resistant cells, apparently through interference with the p53 pathway (X. Wang, et al., 2004). Coincidently, Akt2 seems to be also involved in Ptx resistance mediated by Twist overexpression (Cheng, et al., 2007). Shiota and colleagues demonstrated that Twist might upregulate P-gp through Y-box binding protein-1. This protein was proven to be transcriptionally dependent on Twist and was

also involved in paclitaxel resistance (Shiota et al., 2009). A schematic diagram of the possible axis formed by these proteins is depicted in Figure 1.2.

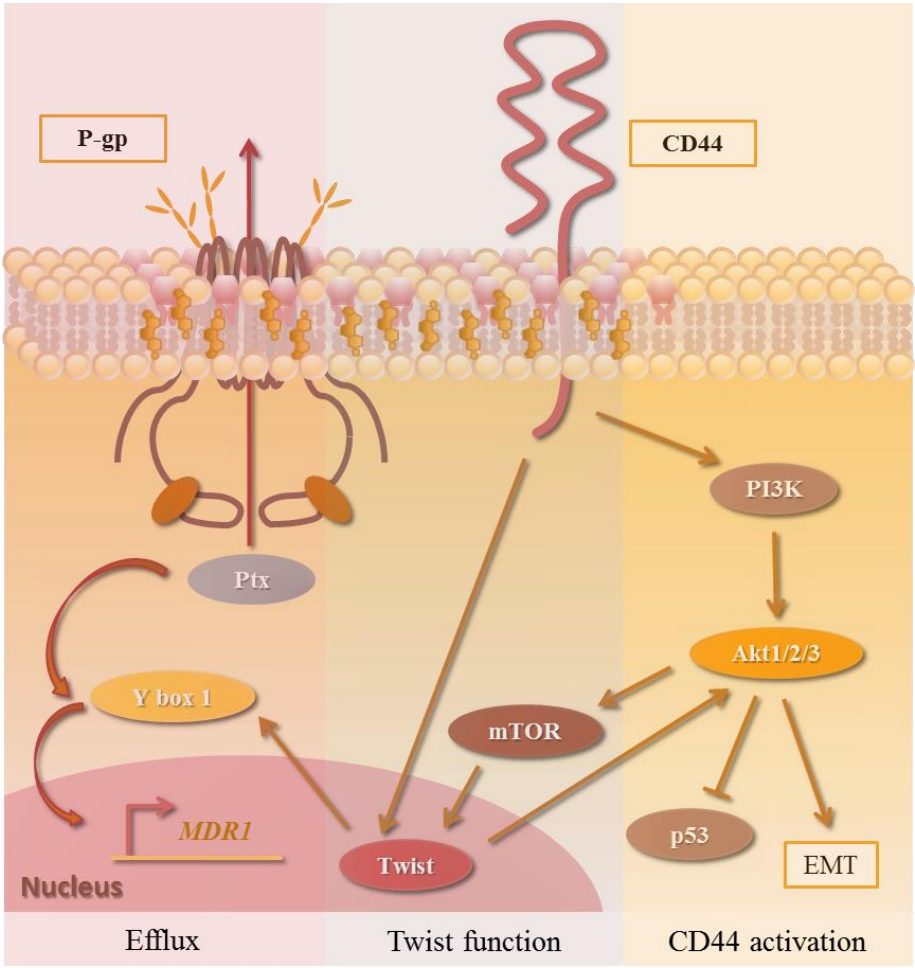

Figure 1.2 – Cross talk between P-gp/CD44/Twist pathways, schematic presentation based on literature information. CD44 overexpression in cancer cells increases PI3K/Akt/mTOR axis leading to p53 blockage while EMT is triggered. It is also related with Twist induction, a crucial transcription factor that induces Akt and increases *MDR1* transcription through the Y-box binging protein-1. Unfortunately, the protein Twist can also be stimulated by the intracellular presence of drugs, such as Ptx, which increases its nuclear translocation enhancing *MDR1* transcription, leading to a greater efflux transport.

1.4 Today therapeutic protocols

Low-solubility, high-permeability molecules make up nearly 90% of all the active pharmaceutical ingredients used in oncology (M. Videira, et al., 2014). Along with the chemical nature they share another feature: efflux proteins are required for drug internalization and ultimately, responsible for cancer cells chemoresistance (Table 1.1).

Table 1.1 – Some examples of current clinical chemotherapeutics and respective cancer target that are P-gp substrates and owing this mechanism, able to trigger tumour chemoresistance.

Chemotherapeutic	Line of action (adapted from Medlineplus)	Reference
Actinomycin D	Wilms' tumour and rhabdomyosarcoma. Testicular cancer, Ewing's sarcoma and gestational trophoblastic tumours. Ovarian cancer.	(Szakacs, et al., 2006)
Daunorubicin	Certain types of acute myeloid leukaemia Certain types of acute lymphocytic leukaemia.	
Docetaxel	Certain types of breast, lung, prostate, stomach, and head and neck cancers. Ovarian cancer.	
Doxorubicin	Certain types of bladder, breast, lung, stomach, and ovarian cancer. Hodgkin's lymphoma and non-Hodgkin's lymphoma and certain types of leukaemia, including acute lymphoblastic leukaemia and acute myeloid leukaemia. Thyroid cancer and certain types of soft tissue or bone sarcomas. Neuroblastoma and Wilms' tumour. Cancer of the uterus, endometrium and cervix; prostate cancer; pancreatic cancer; adrenocortical cancer; liver cancer; Kaposi's sarcoma; Ewing's sarcoma; mesothelioma; multiple myeloma and chronic lymphoblastic leukaemia.	(Szakacs, et al., 2006; Ueda, Cardarelli, Gottesman, & Pastan, 1987)
Epirubicin	Breast cancer after surgery.	(Szakacs, et al., 2006)
Etoposide	Certain types of lung cancer. Certain types of ovarian cancer.	
Imatinib	Leukaemia and other cancers of the blood cells. Gastrointestinal stromal tumours, dermatofibrosarcoma protuberans when the tumour cannot be removed surgically.	
Irinotecan	Colon or rectal cancer. Small cell lung cancer.	
Methotrexate	Cancers in uterus, breast cancer, lung cancer, certain cancers of the head and neck, certain types of lymphoma, and leukaemia.	
Mitoxantrone	Certain types of leukaemia. Non-Hodgkin's lymphoma.	
Paclitaxel	Breast cancer. Ovarian cancer, lung cancer and Kaposi's sarcoma. Cancer of the head and neck, oesophagus, bladder, endometrium, and cervix.	
Teniposide	Acute lymphocytic leukaemia after treatment with other medications.	
Vinblastine	Hodgkin's lymphoma and non-Hodgkin's lymphoma. Breast cancer after treatment with other medications. Gestational trophoblastic tumours after surgery or treatment with other medications. Bladder cancer, certain types of lung cancer, Kaposi's sarcoma, and certain brain tumours.	(Szakacs, et al., 2006; Ueda, et al., 1987)
Vincristine	Leukaemia, including acute myeloid leukaemia and acute lymphoblastic leukaemia, Hodgkin's lymphoma and non-Hodgkin's lymphoma. Wilms tumour, neuroblastoma and rhabdomyosarcoma. Certain types of brain tumours, certain types of lung cancer, multiple myeloma, chronic lymphocytic leukaemia, Kaposi's sarcoma, Ewings sarcoma and gestational trophoblastic tumours.	(Szakacs, et al., 2006)

MDR events in clinical practice, mainly through P-gp, have been verified with anti-cancer drugs over the past few years. A study revealed that a doxorubicin resistant cancer cell line has this chemotherapeutic in the cytoplasm, whereas in a doxorubicin sensitive cancer cell line it is in nucleus, which happens due to P-gp drug efflux transport, being the main mechanism involved in doxorubicin resistance (Bao et al., 2012). Hembruff and colleagues verified that Ptx resistance acquisition in MCF-7 breast cancer cell line occurs with a given concentration threshold, 3.7nM Ptx, Ptx intravenous administration in current cancer treatment has a concentration of 10μM (M. Videira, Almeida, & Fabra, 2012), P-gp expression increases upon Ptx induction, but this is not the only mechanism of Ptx resistance, as P-gp inhibitors cannot re-establish Ptx sensitivity to resistant cells (Hembruff et al., 2008).

Sensitivity to Ptx and doxorubicin was verified when siRNA was used against P-gp mRNA (Wu, Hait, & Yang, 2003). Huang and colleagues demonstrated that Ptx is a P-gp substrate, and also in cells treated com siRNA against P-gp the IC_{50} value was lower (Huang et al., 2004). Sensitivity to Ptx and intracellular accumulation increases after treatment with a P-gp inhibitor (Guo et al., 2004; Kemper et al., 2003).

Ptx is a low molecular weight compound that has the capability to inhibit cell division through stabilization of microtubule polymerization (Schiff, Fant, & Horwitz, 1979). It was extracted from *Taxus brevifolia* tree in United States of America, its target is β-tubulin and it is responsible for the stabilizing microtubule polymerisation and avoiding disassembly, Ptx leads to defective cell division due to anomalous mitotic assembly and chromosome segregation (Zhang, Yang, Wang, & Dong, 2014). Stops cell cycle in G_0/G_1 and G_2/M or induces cell death, reviewed in (McGrogan, Gilmartin, Carney, & McCann, 2008). It was reported that Ptx in *Trypanosoma cruzi*, parasite responsible for Chagas disease, inhibited replication after treatment (Baum et al., 1981). In Leukaemia cell lines, Ptx induced microtubule bundles and was associated with cytotoxic effects (Rowinsky, Donehower, Jones, & Tucker, 1988). Ptx has been used as a chemotherapeutic drug against lung, ovarian, breast, head and neck cancer, among others (Crown & O'Leary, 2000).

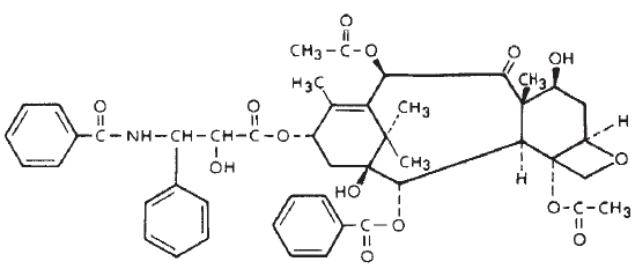

Figure 1.3 – Ptx molecular structure (Schiff, et al., 1979).

1.5 Nanomedicines

Strategies to evade MDR of chemotherapeutics recognized by P-gp are needed. Biopharmaceutics Classification System (BCS) Class II and IV drugs, such as Ptx and Dox, incorporation in particulate systems such liposomes, polymeric nanoparticles and Solid Lipid Nanoparticles (SLN), in particular; will be one possibility, improving the drug's poor transport properties across biological membranes promoting higher intracellular drug exposure.

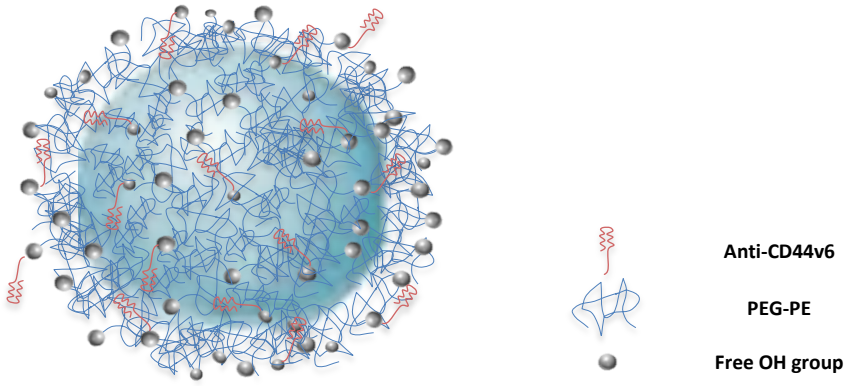

Figure 1.4 – Proposed nanoparticle, schematic structure of a nanoparticle with PEG-PE and CD44v6 antibody bounding its N-terminal to PEG-PE's free OH-group.

Studies have been evidencing that nanomedicines really are a means to evade P-gp. Emulsifying wax nanoparticles were used to entrap Ptx; those have lower IC_{50} value than free Ptx, being a consequence of the lack of interaction between Ptx and P-gp (Koziara, Lockman, Allen, & Mumper, 2004). These nanoparticles also have higher cytotoxicity in resistant cell lines and appear to enter cells through endocytosis or pinocytosis (Koziara, Whisman, Tseng, & Mumper, 2006). Another study by Mittapalli and colleagues using CD44 as mediated receptor for small HA-Ptx nanoconjugates to be up taken into brain metastasis of breast cancer, have proved the crucial role of this membrane receptor and shown that these nanoconjugates can bypass P-gp efflux pump. Therefore Ptx concentration increases inside cancer cells, arresting cell cycle in G_2/M phase, inducing cytotoxicity and apoptosis (Mittapalli et al., 2013).

Besides evading P-gp nanomedicines have other advantages, as discussed by Schleich and colleagues, who proposed a system that simultaneously performs targeting imaging, drug delivery and real-time monitoring of therapeutic response. This system consists in polymeric nanoparticles based on Poly Lactic-co-Glycolic Acid (PLGA) co-encapsulated with Ptx and a magnetic resonance imaging contrast

agent (superparamagnetic iron oxide) that delayed tumour growth and increases mean survival rates *in vivo* (Schleich et al., 2013).

Nanoparticles size between 100-200nm is important for long circulation and cellular internalization pathway, with a diffuse extravasation. The pore cut-off in diverse tumours, including breast cancer, ranges between 380 and 780nm (Hobbs et al., 1998). The study by Martins and colleagues suggests that SLN with 130-160nm are internalized by clathrin-mediated endocytosis, since inhibition of this pathway reduces SLN internalization (Martins et al., 2012). Clathrin-mediated endocytosis was previously observed for microspheres with a mean diameter of 200nm (Rejman, Oberle, Zuhorn, & Hoekstra, 2004).

SLN is a lipid-based system that along with other nanomedicines was devised to transport low soluble drugs, protecting them from biological degradation. This nanoparticles selectivity towards specific cells can be enhanced by surface decoration with ligands that bind specifically to cells membrane receptors. Conceptually, SLN main advantaged are related with the passive diffusion due to the enhanced permeability and retention (EPR) that typically occurs with nanoparticles in disease tissues. Nevertheless, through the use of target moieties cell selectivity within the tissues and organs is achieved, decreasing drastically the adverse effects commonly registered with cytotoxic drugs. Finally, SLN biocompatibility and submicrometric size contributes for this nanomedicines suitability to be used through all the administration routes (Wong, Bendayan, Rauth, Li, & Wu, 2007).

1.6 Objectives

Lipophilic drugs permeability and intracellular accumulation are the main problems associated with Ptx administration. Intracellular drug accumulation within tumour cells is reduced through MDR process, mainly due to the overexpression of P-gp (Nooter & Herweijer, 1991; M. Videira, et al., 2014).

New drug delivery systems have been developed in order to overcome these problems. Colloidal systems improve drug permeability and accomplish greater therapeutic responses (Ma & Mumper, 2013). Specifically, SLN are capable of lipophilic drugs nanoentrapment and have higher physiological stability, decreasing its toxicity; otherwise there is a nanoentrapped limit where drugs can recrystallize losing their effect (Shahbazi & Santos, 2013). Knowing that cellular uptake of SLN is through endocytosis (Martins, et al., 2012), Ptx nanoentrapped in the lipid matrix might be a strategy to reduce P-gp efflux transport of Ptx, increasing intracellular drug bioavailability, promoting efficacy and minimum side effects *in vivo* (Gratton et al., 2008; Sahay, Alakhova, & Kabanov, 2010)

Besides drug nanoentrapment, SLN surface can be coated, more precisely with PEG-PE and functionalized with antibody anti-CD44v6, enhancing their target specificity and improving their pharmacokinetic behaviour (Onoue, Yamada, & Chan, 2014).

The SLN herein formulated are based on the ICH Pharmaceutical Development guidelines (ICH Q8 (R2)) (ICHQ8(R2), 2009), with acceptance limits previously defined by our group: a mean size (MS) lower than 250nm, a polydispersity index (PI) lower than 0.250, a drug entrapment efficiency (EE%) higher than 90% and the absence of significant changes in these parameters after storage at room temperature up to 30 days.

Knowing that P-gp role in the occurrence of MDR in tumour cells confirms that resistance to a broad spectrum of hydrophobic compounds is due to a reduced intracellular drug accumulation. In this project the correlation between SLN-Ptx-anti-CD44 cell uptake and the evasion of the drug efflux mechanism “blinding” P-gp membrane activity will be evaluated. Owing Twist importance in breast cancer resistance and progression, we will also study SLN-Ptx capability to release the drug and to interfere with Twist intracellular expression after internalization.

2. Materials and Methods

2.1 SLN formulations

Plain SLN, SLN-PTX and PEGylated SLN formulations:

SLN were prepared by the combination of two immiscible liquids: a melted lipid and a hot aqueous phase, through an emulsification/solidification technique. The melted lipid was vigorously dispersed in an aqueous phase followed by a solidification step in order to obtain a monodisperse particle system. In order to obtain Ptx nanoentrapped in these SLN, Ptx was dissolved in ethanol; this solution was dissolved into the melted lipid forming a drug lipid phase. Afterwards, the drug lipid phase was also dispersed in an aqueous phase followed by solidification. According to Mafalda Videira's previous work (M. A. Videira, Arranja, & Gouveia, 2013; M. A. M. Videira, 2009).

Plain SLN are formulated in sterile conditions, with 1.5% or 3% of Precirol® ATO5 lipid ((glyceryl palmito-stearate), melting point of 64ºC, were kindly offered by Gattefossé (France)) mixed with an aqueous solution of Tween 80 (Sigma, Portugal) at 25% (surfactant/lipid). Lipid must be melt down before formulation, for that it should be heated at 80ºC, Tween 80 solution must also be heated to avoid lipid precipitation. Silverson® Machine (England) is used to perform the formulation, at approximately 10rpm, during 10min at 80ºC.

SLN-Ptx are formulated similarly to plain SLN, with an additional step: Ptx solution is mixed with melted lipid.

For SLN PEGylation, either plain or with Ptx, a PEG-PE 2000 dissolved in chloroform (10mg/mL) (Avanti Polar Lipids, Inc (USA)) film is prepared the day before SLN formulation. This film formation consists in hot (50ºC) solvent evaporation under vacuum to obtain a polymer-based thin film, using 1400µL of this solution with constant rotation at 250rpm. After film formation it rests overnight at room temperature. When SLN are formulated, 7mL are used to rehydrate PEG-PE's film.

All formulations must rest for 72h at room temperature to stabilize.

Antibody anti-CD44 functionalization:

After formulation and SLN PEGylation the antibody against the target proposed by this study, antibody anti-CD44v6 (Life Technologies, Portugal) needed to be functionalized. For that propose, 3µL of anti-CD44v6 was added to 3mL of formulation with a final concentration of 0.5µg/mL. This functionalization was performed for 30min at 300rpm. The free OH groups in PEG-PE were expected

to bind the N-terminal group of anti-CD44v6. To assess if this chemical bound did not affect the protein structure, a SDS-PAGE was performed.

2.2 SLN characterization

SLN particle size, size homogeneity and stability:

Unwanted microparticles presence, PI and particle MS were evaluate with laser diffraction spectrometry and photon correlation spectroscopy. Surface charge was measured through the zeta potential assessed by Differential Scanning Calorimetry analysis. Particle MS and size homogeneity were determined through Z-averaged and PI, respectively, using Malvern Zetasizer S® with 600µL of SLN formulation diluted in MiliQ water (1:10). Stability was assessed through Zeta-potential using Malvern Zetasizer Z® with 760µL of SLN formulation diluted in MiliQ water (1:10).

Microscopic observation of SLN was used to detect insoluble drug crystals.

Assessment of drug entrapment:

After drug incorporation on SLN, the EE% was determined, by drug extraction by centrifugal ultrafiltration and high-performance liquid chromatography (HPLC) quantification (M. A. Videira, et al., 2013; M. A. M. Videira, 2009). The amount of Ptx entrapped in SLN was assessed through HPLC (HPLC Hewlett Packard Series 1050; software Hewlett Packard ChemStation for LC), with absorption peak at 227nm. A reverse-phase column (Nova-Pak® C-18 60A, 3.9x150mm, 4µm; Waters®) was used. Sample preparation method is through centrifugal ultrafiltration (Nanosep 100K, Omega, Pall Life Siences; VWR, Portugal) and ultrafiltration column wash with ethanol for entrapped drug recovery. HPLC conditions: flow rate was 1.2mL/min, mobile phase was acetonitrile:MiliQ water (45:55, v/v) and the injection volume was 20µL. This method shows satisfactory linearity between 5 and 100µg/mL ($y=0.6733x$; $R^2=0.9967$). To assess EE% the following equation was used:

$$EE(\%) = \frac{[Ptx_e]}{[Ptx_i]} \times 100\% = \frac{[Ptx_i] - [Ptx_s]}{[Ptx_i]} \times 100\%$$

$[Ptx_e]$ stands for Ptx concentration entrapped into lipid nanoparticles

$[Ptx_i]$ known initial concentration

$[Ptx_s]$ stands for Ptx concentration in collected supernatant, measured against a calibration curve.

2.3 Protein integrity evaluation

Protein integrity upon the preparation process was evaluated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):

The protein separation must be performed at 12% acrylamide gel, ideal for 20-150kDa proteins (according to manufacturer's instructions). The first step is resolving gel preparation, with 6.1mL of MiliQ water, 4.5mL of Resolving Buffer 4x (VWR, Portugal), 7.2mL of 30% acrylamide/bisacrylamide solution (VWR, Portugal), 180µL of 10% APS (VWR, Portugal) and 18µL of TEMED (VWR, Portugal). After adding APS and TEMED in hotte, the gel must be mounted dropping at least 5mL of this preparation, and then the empty space must be field with ethanol in order to have a flat surface. Resolving gel takes 45-60min to polymerize, after 30min the 4% acrylamide stacking gel can be prepared, with 6.1mL of MiliQ water, 2.5mL of Stacking Buffer 4x (VWR, Portugal), 1.3mL of 30% acrylamide/bisacrylamide solution, 50µL of 10% APS and 10µL of TEMED. It takes 30-45min to polymerise.

While gel is polymerizing samples can be prepared, selecting the amount of sample needed and adding loading dye 5x (1:5). Loading dye 5x solution (all products were from VWR, Portugal) prepared according the following recipe:

- 2.5mL Tris-HCL (pH 6.8);
- 1g SDS;
- 5.75mL Glycerol (87%);
- 2,5mL EDTA (500mM, pH 8);
- 10mg Bromophenol Blue;
- 0.5mL β-mercaptoethanol.

Samples have to be boiled using dry bath to denature proteins, in order to be separated according to their molecular weight and not by their charge. Electrophoretic separation using Tris-Glycine-SDS 1x (VWR, Portugal) running buffer is performed under 130V for at least 1:30h (Mini-PROTEAN® Tetra Vertical Electrophoresis Cell and PowerPac™ Basic Power Supply, Bio-Rad, Portugal).

Coomassie Brilliant Blue's revelation:

Acrylamide gels after electrophoresis are submersed in Coomassie Brilliant Blue (VWR, Portugal) for approximately 20min with agitation. This solution is discarded and a decolouration solution is added for more 20min, again this new solution is discarded and another amount is added for an overnight incubation, also with agitation. Decolouration solution recipe (all solutions were from VWR, Portugal):

- 20% of methanol;

- 10% acetic acid;
- MiliQ water.

2.4 Cell culture and cell viability assays

A triple-negative breast cancer cell line MDA-MB-436 was chosen; owing the presence of $CD44^{+}CD24^{-/low}$ phenotype clusters (Sheridan et al., 2006; Tarasewicz, et al., 2014). MDA-MB-436 cells (ATCC, USA) were maintained in Advanced DMEM with 10% FBS, 1% L-Glutamine and 1% penicillin and streptavidin (purchase from Life Technologies, Portugal). Cells (10000cells/well) were plated in 200µL per well in 96-well plates. One day (24h) after seeding, SLN were added in four replicates per concentration, 24h after incubation the medium was replaced leaving 25µL in the bottom and adding 100µL of fresh medium. Cell viability was measured after 24h with 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (VWR, Portugal), incubating with 12.5µL MTT reagent for 3h, then clearing the wells, adding 125µL of DMSO (VWR, Portugal) and reading with FLOUstar Omega MicroPlate Reader at 570nm.

2.5 Immunocytochemistry

Cells (100000cells/well) were plated in 500µL per well onto glass coverslips in 24-well plates 24h before treatments. Incubations were performed with a sub therapeutic concentration of Ptx (0.1µM, i.e. 8.53ng/mL) for 1h, 3h, 6h and 24h, after that cells were fixed with 4% paraformaldehyde for 20min at room temperature, washed 3 times for 5min each with PBS 1x, storage at 4ºC in 500µL PBS 1x or use immediately. Permeabilization was with 0.5% Triton X-100 in PBS 1x for 30min at room temperature, washed 3 times with PBS 1x, the blocking was performed with 3% BSA in PBS 1x for 1h, the coverslip was rinsed 3 times in PBS 1x and incubated overnight at 4ºC with 30µL/coverslip at the ideal dilution of each monoclonal anti-P-gp (Merck Millipore, Spain) or anti-Twist (kindly offered by our partners in Spain) antibodies, and then incubated for 1h at room temperature with Alexa-flour 459 rabbit anti-mouse antibody (Life Technologies, Portugal). Cells were counterstained with 50µL/coverslip from a solution with 1:1000 DAPI:PBS 1x (Life Technologies, Portugal). Coverslips were then mounted with DPX mounting medium (VWR, Portugal). All solutions were freshly prepared and antibodies proper dilution was assessed for each antibody. This protocol was based on the previously used in Professor Alexandra Brito's laboratory (Mariano et al., 2013).

Photographs acquisition was obtained with confocal microscope with a camera (AxioScop.A1 coupled with Zeiss AxioCam HR) and images were treated with ZEN software.

2.6 mRNA extraction and qPCR

To quantify *TWIST* and *MDR1* mRNA, cells (400000cells/well) were plated in 3mL per well onto 6-well plates 24h before treatments. Incubations were performed with a sub therapeutic concentration of Ptx (0.1µM, i.e. 8.53ng/mL) at different time points (1h, 3h and 6h), for each time point eight conditions were necessary, Table 2.1. Total RNA was isolated from MDA-MB-436 using TRIzol ® reagent (Life Technologies, Portugal), with procedure adapted from manufacturer's protocol. For reverse transcriptase (RT)–PCR, cDNA was synthesized by the Bioline SensiFAST™ cDNA Synthesis Kit (Citomed, Portugal) according to the manufacturer's protocol. The cDNA was then amplified by quantitative PCR (qPCR) with Bioline SensiFAST™ SYBR Hi-ROX Kit (Citomed, Portugal), according to manufacturer's protocol, but instead of 20µL, final volume per well was 10µL; and qPCR conditions were as follows: 2min at 50ºC, 2min at 95ºC, 40 cycles at 95ºC for 5s and 62ºC for 30s, with melting curve analysis. Preliminary experiments were conducted to assess the best cDNA concentration to perform qPCR, using 200ng/mL of each sample. β-actin specific primers: Forward 5' CATGTACGTTGCTATCCAGGC 3' and Reverse 5' CTCCTTAATGTCACGCACGAT 3' (PrimerBank ID 4501885a1). *TWIST* specific primers: Forward 5' GTCCGCAGTCTTACGAGGAG 3' and Reverse 5' GCTTGAGGGTCTGAATCTTGCT 3' (PrimerBank ID 4507741a1). *MDR1* specific primers: Forward 5' GCAGTAGCTGAAGAGGTCTTGG 3' and Reverse 5' CCCATACCAGAAGGCCAGAGC 3'. (LifeTechnologies, Portugal)

Table 2.1 – Conditions for qPCR analysis. Control condition and corresponding test condition.

Control condition	Testing condition
Untreated cells	Ptx
P_3	P_3-Ptx
P_3-PEG	P_3-Ptx-PEG
P_3-PEG^{Ab}	P_3-Ptx-PEG^{Ab}

Relative fold change determination:

$$\Delta ct = (gene\ of\ interest\ ct) - (\beta - actin\ ct), \text{ for each condition}$$

$$\Delta\Delta ct = (testing\ \Delta ct) - (control\ \Delta ct)$$

$$Relative\ Fold\ Change = 2^{-\Delta\Delta ct}$$

3. Results

3.1 Lipid nanoparticles: SLN

Drug entrapment in the lipid matrix was obtained according to method patented by our group (M. A. M. Videira, 2009). The drug-loaded and unloaded nanoparticles were then PEGylated by an in-house developed method; the film re-hydration method. To assemble the ligand, in order for the nanoparticles to achieve cell receptor affinity, the PEGylated particles were incubated with a solution of CD44v6 let to incubate for 30min at room temperature. As the quantitative composition has been shown to produce significant differences in the final product characteristics, two concentrations of lipids were used. The final nanoparticles and the correspondent intermediates were obtained within the submicrometric size (Figure 3.1). Prepared samples and the corresponded abbreviation are indicated in the Table 3.1.

Table 3.1 – Formulation name list. This list represents the formulation name based on its composition.

Formulation composition	Abbreviation
Free Ptx	Ptx
SLN with 3% lipid and 82.5µg/mL of Ptx	P_3-Ptx
SLN with 3% lipid, 82.5µg/mL of Ptx and 2mg/mL of PEG-PE	P_3-Ptx-PEG
SLN with 3% lipid, 82.5µg/mL of Ptx, 2mg/mL of PEG-PE and 0.5µg/mL of anti-CD44v6	P_3-Ptx-PEG^{Ab}
Plain SLN with 3% lipid	P_3
Plain SLN with 3% lipid and 2mg/mL of PEG-PE	P_3-PEG
Plain SLN with 3% lipid, 2mg/mL of PEG-PE and anti-CD44v6 at 0.5µg/mL	P_3-PEG^{Ab}
SLN with 1.5% lipid and 40µg/mL of Ptx	P_1.5-Ptx
SLN with 1.5% lipid, 40µg/mL of Ptx and 2mg/mL of PEG-PE	P_1.5-Ptx-PEG
Plain SLN with 1.5% lipid	P_1.5
Plain SLN with 1.5% lipid and 2mg/mL of PEG-PE	P_1.5-PEG

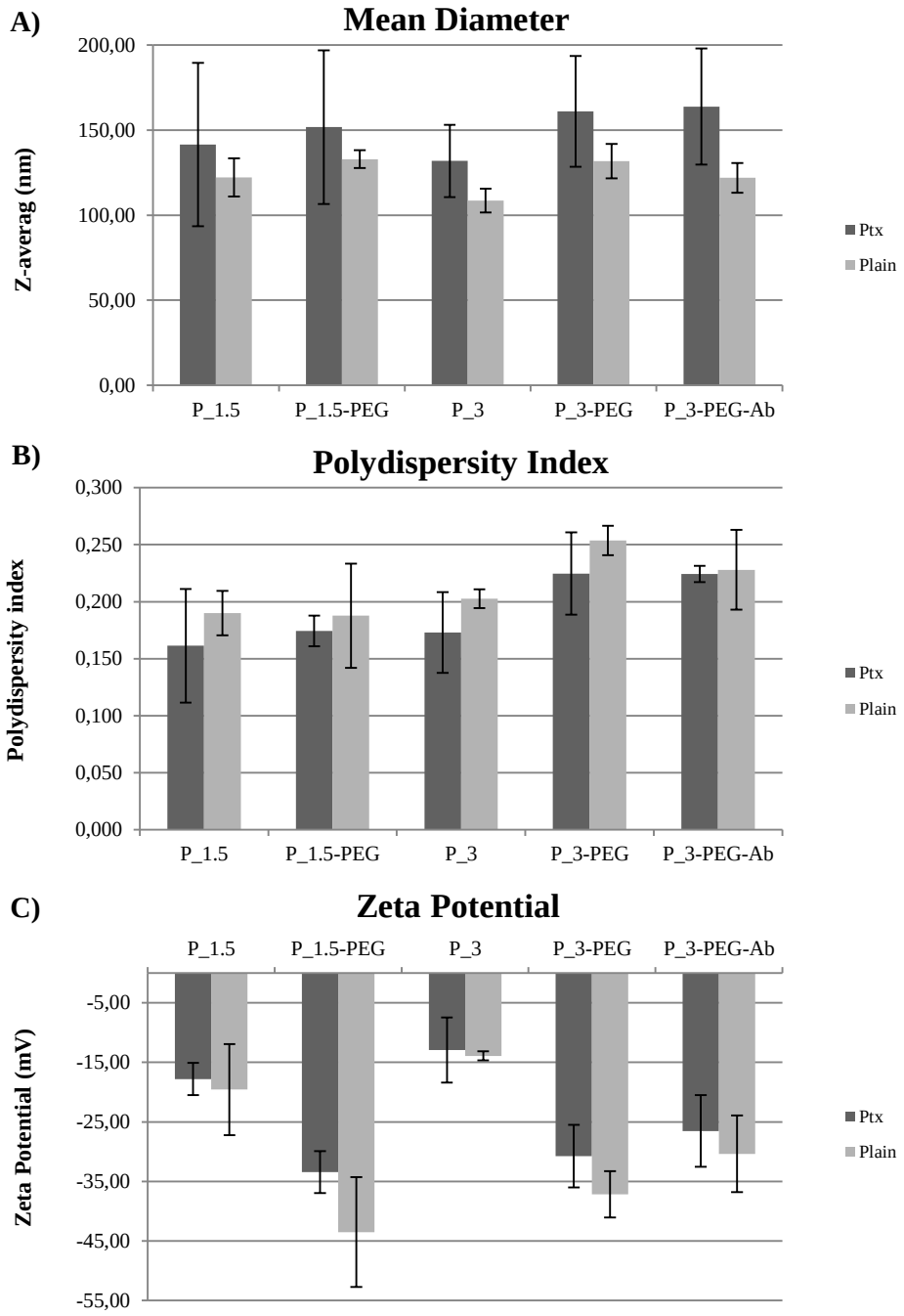

Figure 3.1 – Effect of the drug entrapment in the SLN MS values (A), PI (B) and Zeta Potential (C). Samples characterization was performed in coated and uncoated SLNs after and before functionalization with anti-CD44v6. Bars represent mean values with respective standard deviation (n=3). All prepared samples present MS and PI values within the required values, lower than 250nm and less than 0.250 of particles polydispersion. Negative Zeta values are in accordance with our group previous work (M. A. Videira, et al., 2013) and variations in this value confirms differences at the nanoparticles surface. PEGylation has an impact in the particles charge towards more negative values.

3.1.1 PEGylated nanoparticles functionalization with CD44v6 antibody

To achieve an efficient target binding, CSC^{CD44+} selectivity and enhanced nanoparticles internalization, the anti-CD44v6 peptide was used for PEGylated nanoparticles surface functionalization. Only P_3-Ptx-PEG nanoparticles were functionalized, since those nanoparticles can incorporate higher amounts of Ptx (82.5µg/mL whereas P_1.5-Ptx-PEG can incorporate 40 µg/mL). The system P_3-Ptx-PEG^{Ab} was obtained by the adsorption letting the OH from the PEG-PE shell to interact with the N-terminal from the antibody anti-CD44v6. Process mild conditions, are not expected to compromise the peptide structure. To evaluate the formulation effect on the peptide integrity the SDS-PAGE technique was used (Figure 3.2).

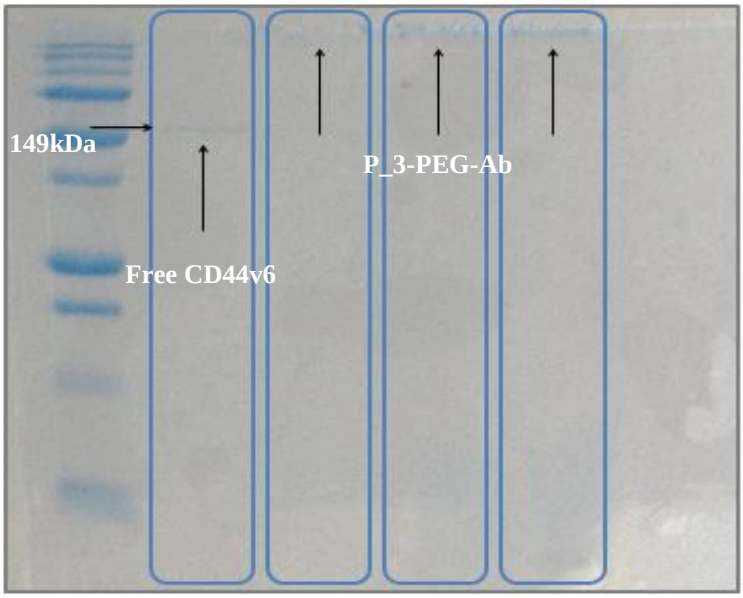

Figure 3.2 – SDS-PAGE of SLN functionalized with antibody anti-CD44v6. P_3-PEG^{Ab} and free anti-CD44v6 were subjected to electrophoresis in a 12% acrylamide gel to assess if protein integrity was preserved after anti-CD44v6 functionalization to PEGylated SLN. Based on the molecular weight of free anti-CD44v6 compared to the peptide extracted by ultrafiltration/centrifugation, it is possible to conclude that the protein integrity is maintained upon P_3-PEG^{Ab} preparation. Distinct bands of P_3-PEG^{Ab} at different dilutions were retained in the stacking gel fraction (Columns 2, 3 and 4). Lack of migration, although the protein is present indicates that the formulation do have the protein in its surface confirming the alteration on the Zeta Value observed for this formulation. Moreover, the absence of more lanes in these columns proves both that the protein is fixed to the nanoparticles and therefore no separation occurs and, more important, that all the protein is adsorbed in the nanoparticles surface and thus no free protein appears.

3.2 *In vitro* studies

Cell response to nanoparticulate systems were conducted in human breast cell lines. The studies were divided in (i) internalization studies, (ii) nanoparticles biocompatibility and, (iii) therapeutic efficacy.

(i) To assess a possible correlation between the cellular uptake and the nanoparticles surface, coated and uncoated SLNs, respectively, P_3-Ptx and P_3-Ptx-PEG, were compared with the functionalized ones P_3-Ptx-PEG^{Ab} .The internalization rate was evaluated using two cell lines: MDA-MB-231 and MCF-7 to disclose the influence of the cell line. Drug-loaded samples were used to determine the contribution of the coating and that from the functionalization (Figure 3.3).

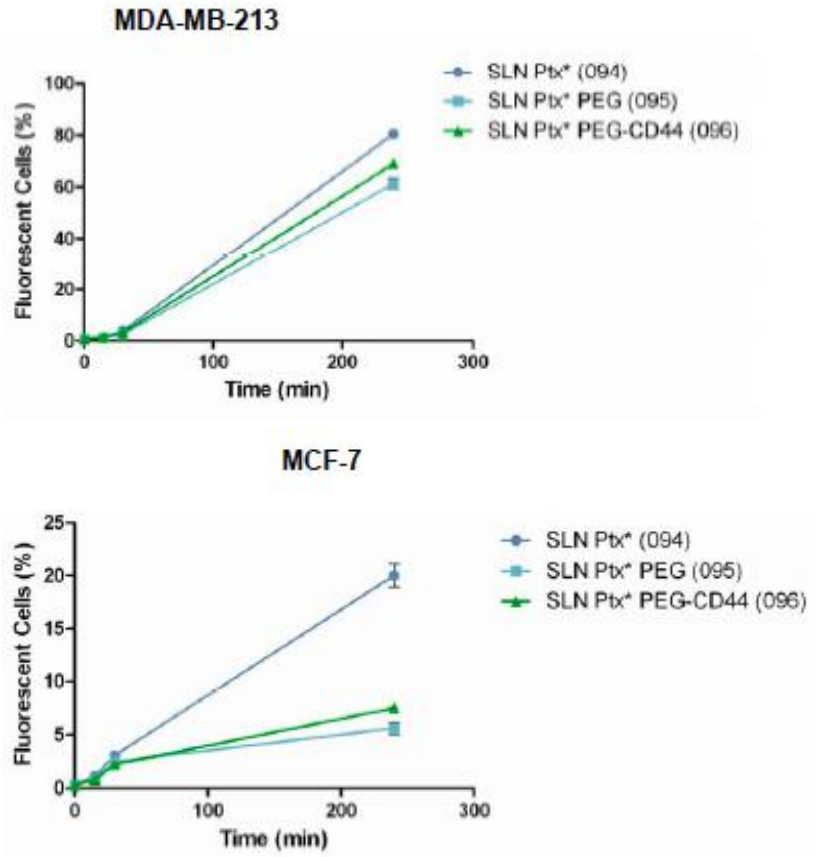

Figure 3.3 – Internalization of fluorescent Ptx (Ptx*) in an invasive (MDA-MB-231) and less invasive (MCF-7) breast cell lines. P_3-Ptx, P_3-Ptx-PEG and P_3-Ptx-PEG^{Ab}, herein as SLN Ptx*, SLN Ptx* PEG and SLN Ptx* PEG-CD44, internalization was not improved by the CD44v6 surface functionalization (our group partners in Barcelona unpublished work). Non PEGylated SLN, shown a more severe internalization rate and this effect is marked in the less invasive cell line.

(ii) and (iii) were conducted in MDA-MB-436 highly Twist expressing cells after 24h of cell exposure to the tested samples (Table 3.1). The apparent higher toxicity observed after cell uptake of unloaded SLN's, coated and functionalized, was attributed mainly to the possible impairment of the Endolysosomal cell mechanisms owing the massive intracellular presence of SLN. PEG-PE coating seems to slow-down nanoparticles cell internalization contributing for a more controlled lipid digestion (Figure 3.4). As for the therapeutic efficacy, the most compelling difference was observed between the free drug and the drug-loaded nanoparticles was the resulting IC_{50} (Table 3.2). When comparing drug-loaded SLN with and without PEG-PE coating differences in the level of effect are not so expressive and, might also be attributed to differences in the uptake rate as depicted in Figure 3.5. However, and although there was also a decrease in the cell uptake of the P_3-Ptx-PEG^{Ab} this system has not improve the drug efficacy as demonstrated by the IC_{50} value. For this nanoparticulate system, no differences were found concerning the free drug.

Table 3.2 – IC50 values for free Ptx and unloaded and loaded formulations, after and before coating and functionalization. These values were based on cellular viability (Figure 3.4 and 3.5) and were calculated according to a mathematical model previous developed by our group (M. A. Videira, et al., 2013).

Ptx	**P_3**	**P_3-PEG**	**P_3-PEG^{Ab}**	**P_3-Ptx**	**P_3-Ptx-PEG**	**P_3-Ptx-PEG^{Ab}**
2.20	4.48	5.35	2.92	1.48	1.51	2.23

Although a competitive binding could be achieved through the presence of the CD44 ligand at the SLN surface, the observed increase in toxicity together with the fact that this functionalization adds no improvement to the nanoparticles therapeutic efficacy was a disappointing and tough result to explain.

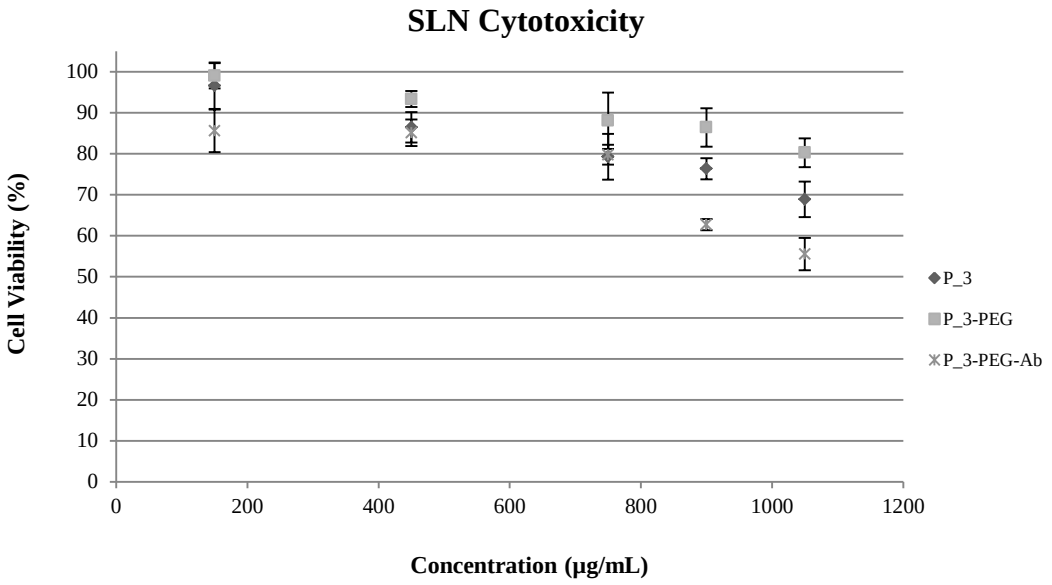

Figure 3.4 – SLN Cytotoxicity of plain nanoparticles. Cellular viability assays through MTT with P_3, P_3-PEG and P_3-PEG^{Ab} was performed in order to assess if nanoparticles without drugs are capable of inducing cell death, even with higher lipid concentrations it seems that the proposed nanoparticulate system is not cytotoxic.

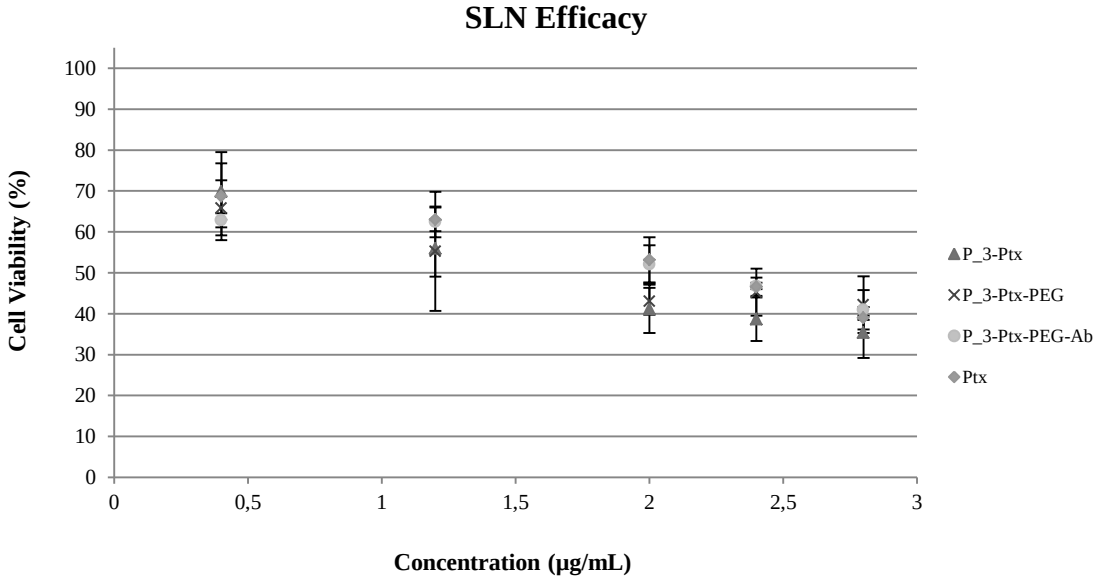

Figure 3.5 – SLN Efficacy of Ptx nanoentrapped nanoparticles. Cellular viability assays through MTT with P_3-Ptx, P_3-Ptx-PEG, P_3-Ptx-PEG-Ab and free Ptx, was performed to assess nanoentrapped Ptx efficacy in comparison to free Ptx. Higher Ptx concentrations lead to lower cellular viability, P_3-Ptx and P_3-Ptx-PEG have the lowest values. P_3-Ptx-PEG^{Ab} has a different action and an efficacy similar to free Ptx, this indicates that functionalization of SLN does not improves their efficacy, otherwise decreases it.

3.2.1 P-gp membrane expression

As postulated by the literature, sensitivity of drug-resistant cells is related with the P-gp expression. This protein expression and localization upon incubation with nanoparticles was evaluated by immunocytochemistry analysis and compared with the expression obtained when cells are incubated with a P-gp subtract, Ptx (Figures 3.6, 3.7, 3.8 and 3.9).

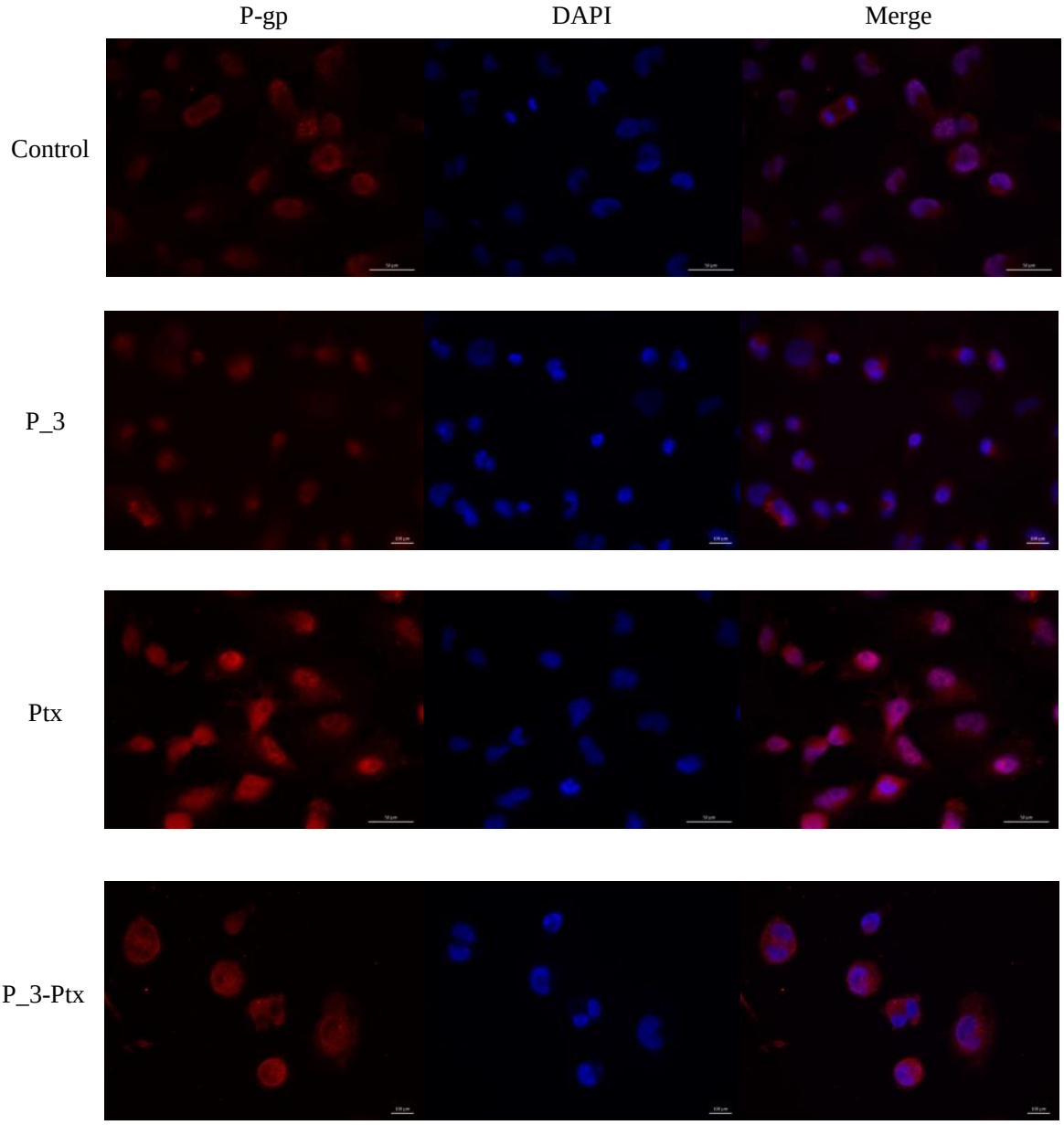

Figure 3.6 –Immunocytochemistry for P-gp follow 1h of cell contact with free medium, P_3, free Ptx, and P_3-Ptx. Exposition time 1100ms, scale bar 50µm.

Fluorescent micrographs confirm that nanoparticles are internalized by endocytosis as can be visualized by the slight P-gp expression in the formed vesicles following nanoparticles internalization. This expression is however similar to that obtained in the control (cells with no treatment).

Impressively, upon 1h incubation time, cells in contact with the free Ptx revealed high protein expression whereas almost no staining was observed in the Ptx-loaded SLN. Lower expression indicates less P-gp in membrane meaning that even if the protein were able to recognize its substrate in the cytosol, the efflux will be less expressive. But, it appears that the major event is the fact that nanomedicines can evade P-gp/drug recognition maintaining its expression in the basal levels avoiding therefore, the cascade events triggered by its activity and promoting drug accumulation in the cytosol (Figure 3.6).

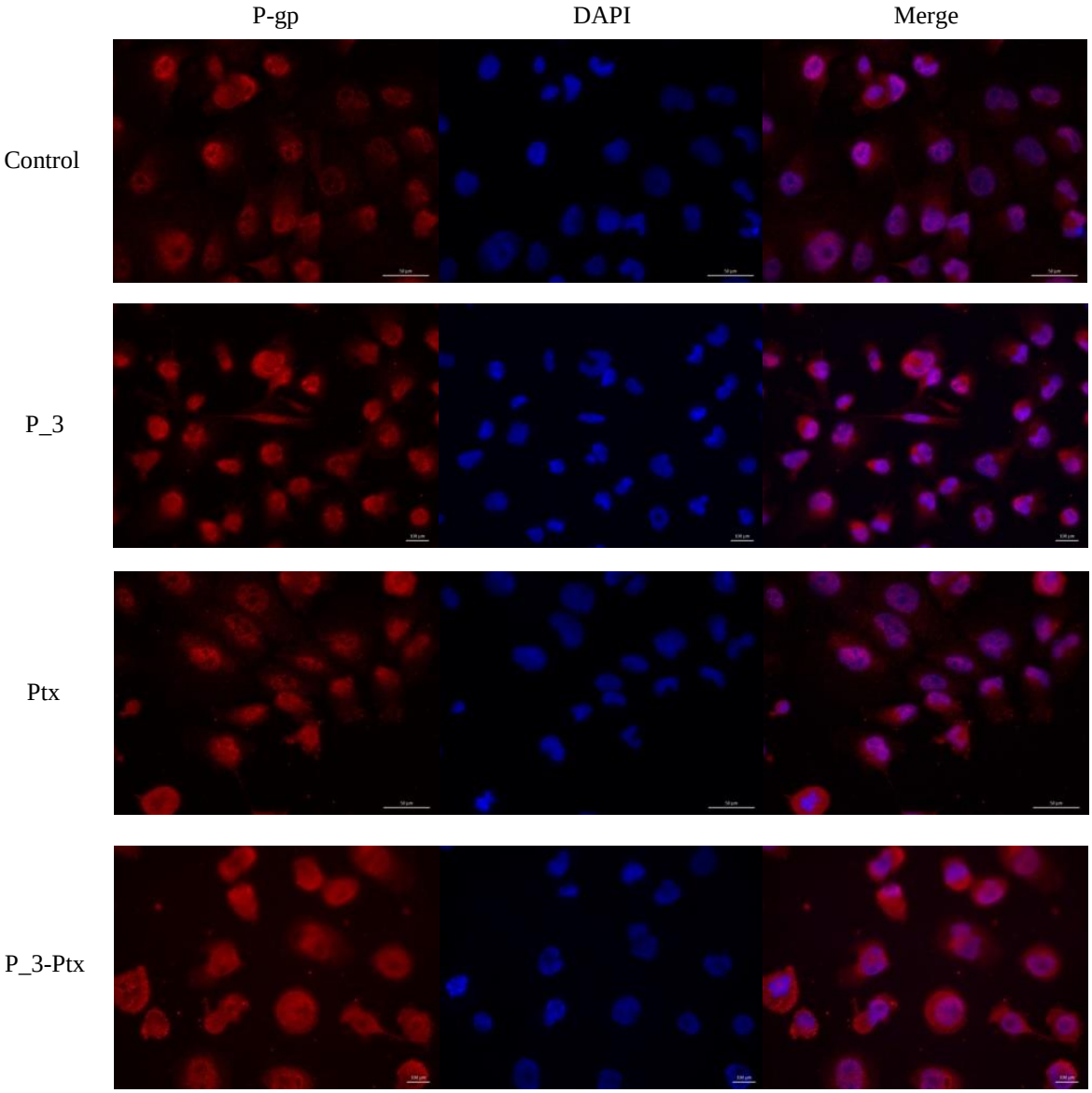

Figure 3.7 – Immunocytochemistry for P-gp follow 3h of cell contact with free medium, P_3, free Ptx, and P_3-Ptx. Exposition time 1100ms, scale bar 50µm.

Images of cells exposed to the samples for 3h indicates that P-gp expression in cells incubated with free drug remains. However, at this point the protein staining appears to be independent from the tested sample (Figure 3.7).

PEGylated SLN observations following 1h of incubation are similar to the expression observed with the control and cells incubated with P_3 and P_3-Ptx (Figure 3.6). However, P-gp response to the presence of P_3-Ptx-PEG suggested that the drug-loaded PEGylated SLN seems to originate a small increase in its expression, a scenario that has been reinforced by the functionalized PEGylated P_3-Ptx-PEG^{Ab} (Figure 3.8).

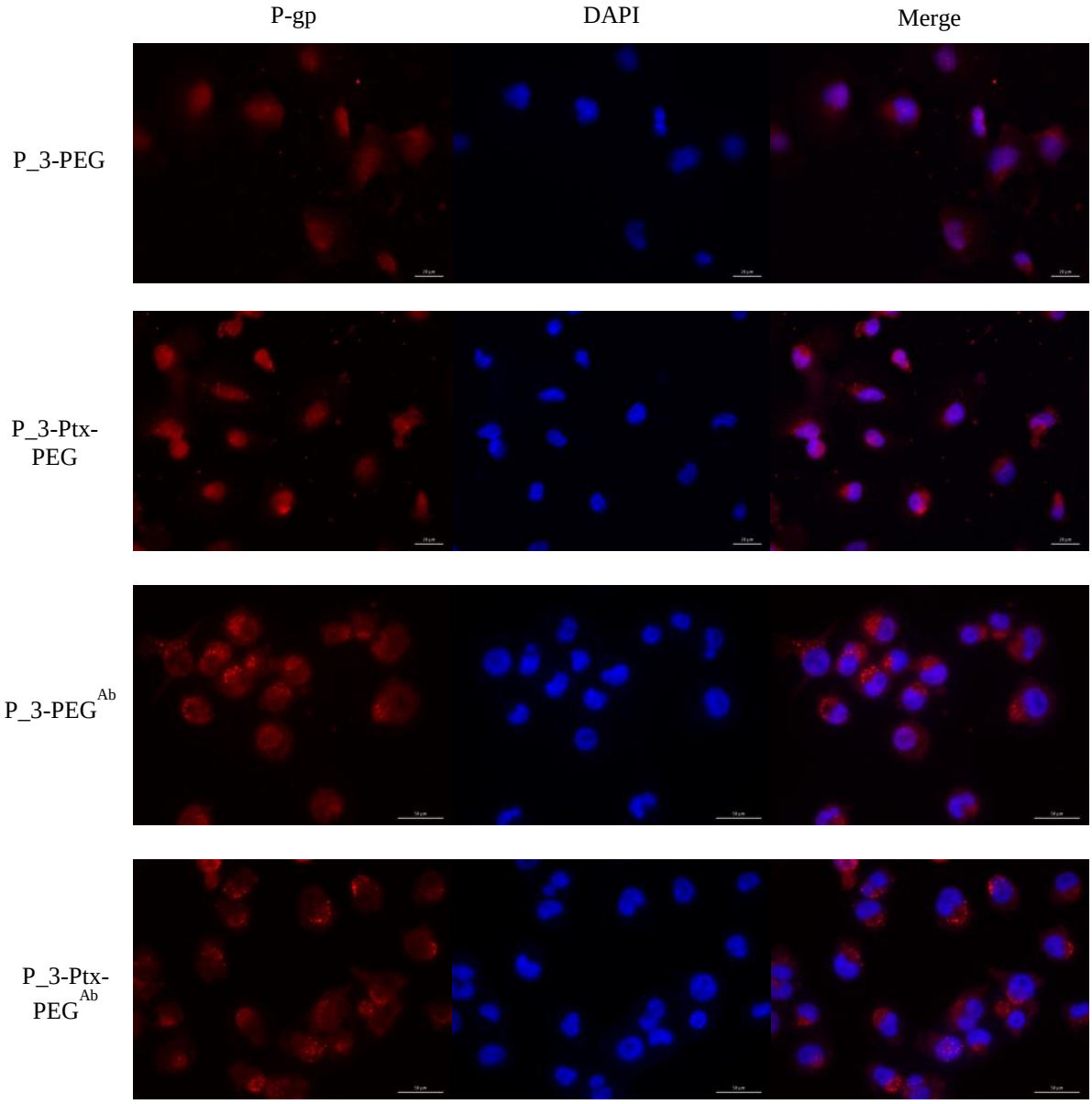

Figure 3.8 – Immunocytochemistry for P-gp follow 1h cell contact with P_3-PEG, P_3-Ptx-PEG, P_3-PEG^{Ab} and P_3-Ptx-PEG^{Ab}. Exposition time 1100ms, scale bar 50µm.

As P_3-Ptx-PEG^{Ab} internalization demands the formation of lipid rafts and therefore the recruitment of the membrane P-gp, justifying the protein staining in cells incubated with these samples (with and without the drug) is more or less logical. Similarities between, P_3, P_3-Ptx and P_3-PEG, for both 1h and 3h of incubation (Figure 3.9), indicate that this systems cell internalization might otherwise use

the clathrin-mediated endocytosis, with just a minor expression of P-gp (Figures 3.6 and 3.8). Conversely, the modest signal displayed by P_3-Ptx-PEG is thought to be related with the photo acquisition rather than a direct effect of this system.

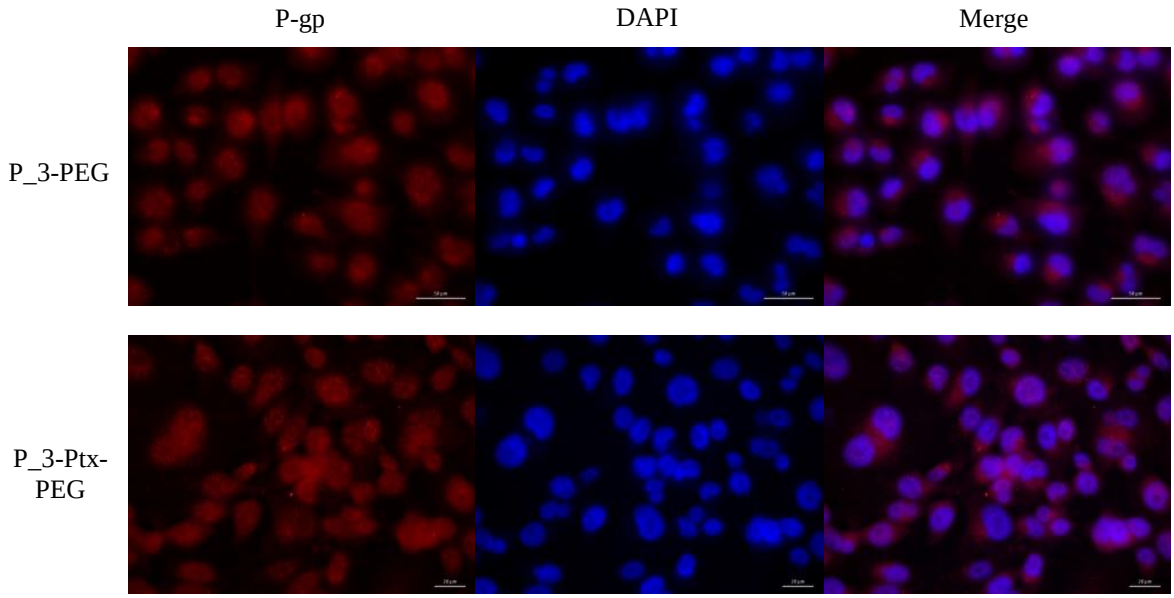

Figure 3.9 – Immunocytochemistry for P-gp follow 3h cell contact with P_3-PEG and P_3-Ptx-PEG. Exposition time 1100ms, scale bar 50µm.

3.2.2 Twist protein expression

Twist overexpression was observed in cancer cells resistant to Ptx. Therefore, Twist expression was also assessed with immunocytochemistry upon incubation with nanoparticles and free Ptx in order to evaluate the influence of the nanoparticulate system and compare it with the free drug in this protein expression (Figures 3.10, 3.11 and 3.12).

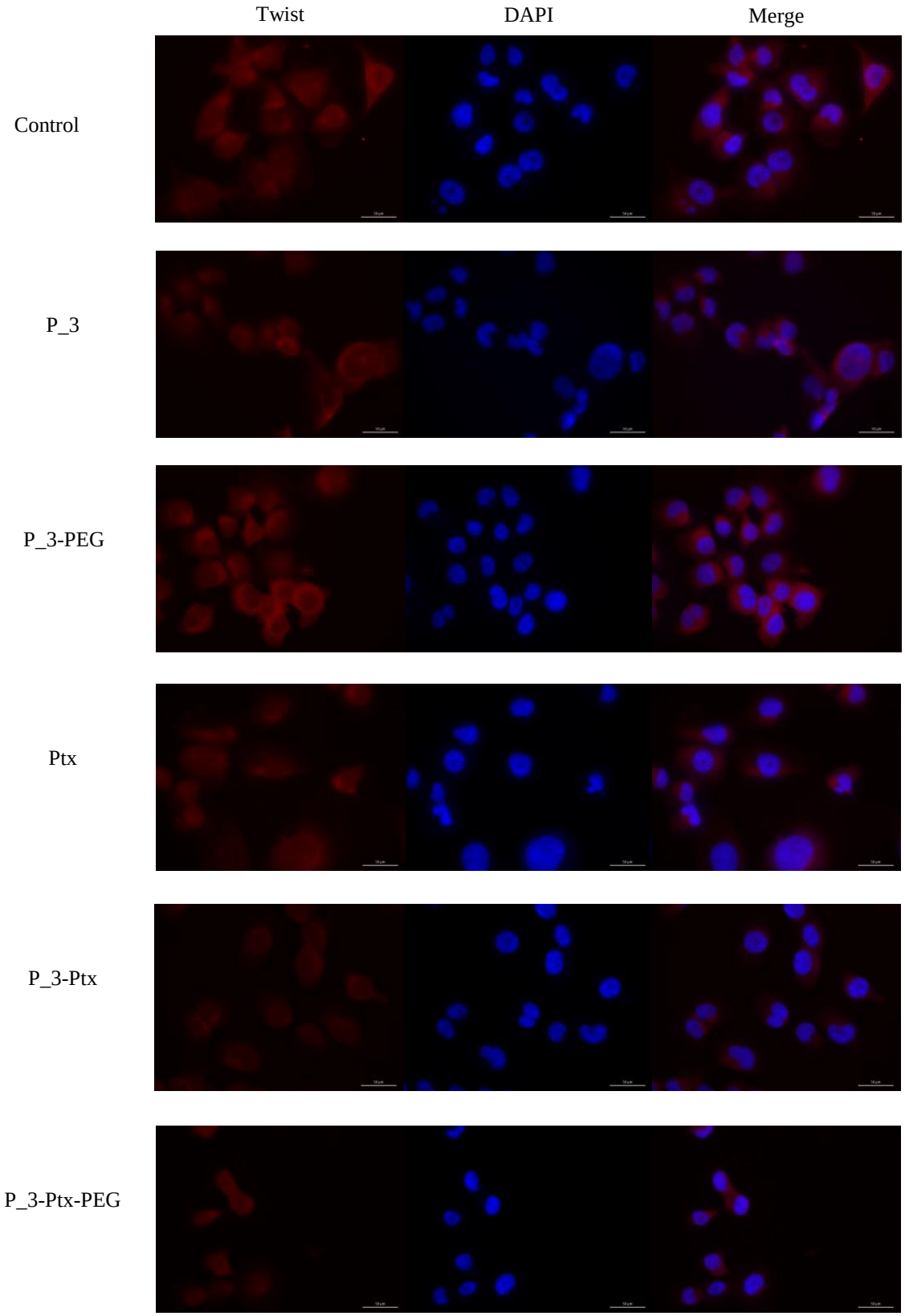

Figure 3.10 –Immunocytochemistry for Twist follow 1h cell contact with free medium, P_3, P_3-PEG, free Ptx, P_3-Ptx and P_3-Ptx-PEG. Exposition time 2800ms, scale bar 50µm.

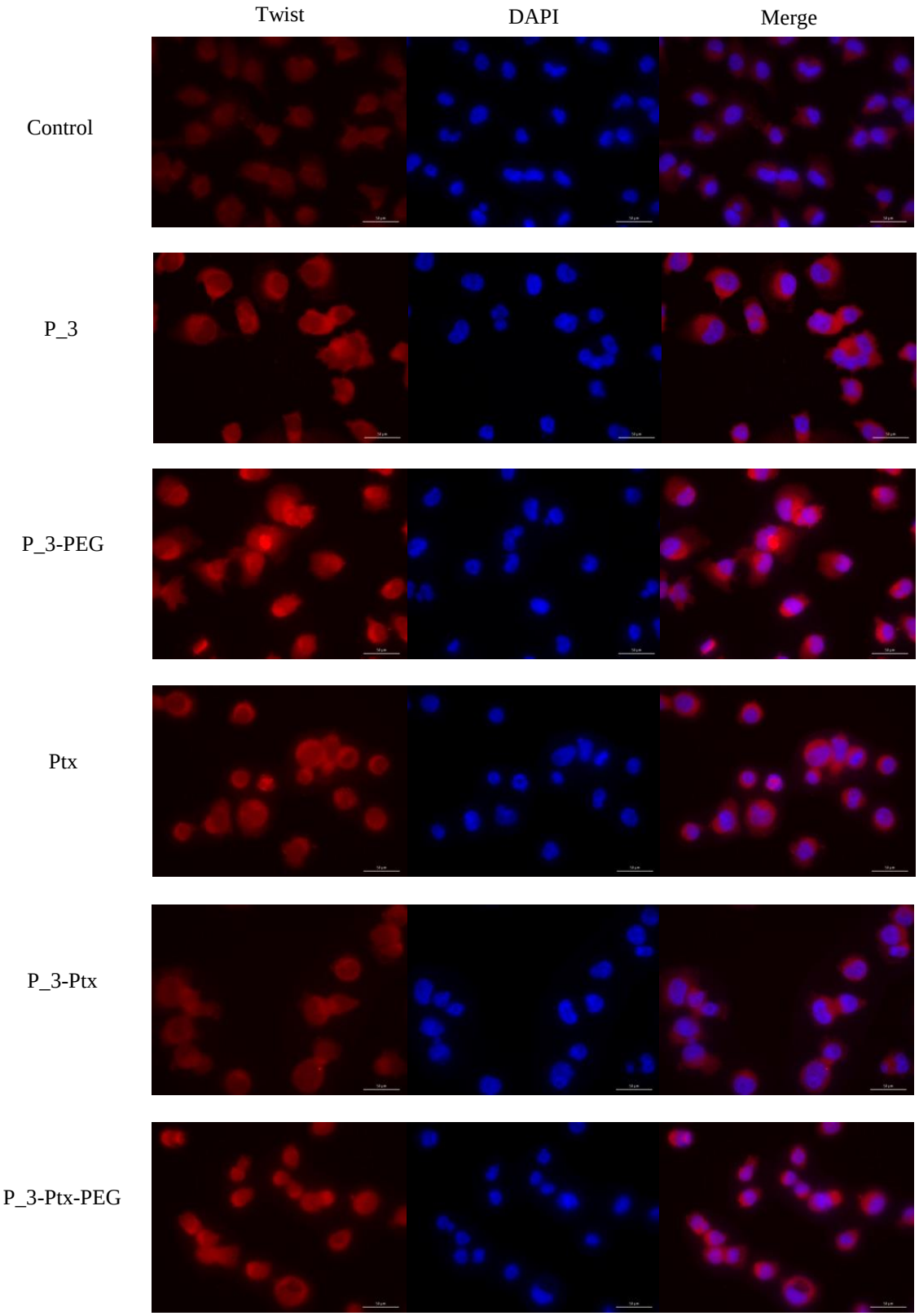

Figure 3.11 – Immunocytochemistry for Twist follow 6h cell contact with free medium, P_3, P_3-PEG, free Ptx, P_3-Ptx and P_3-Ptx-PEG. Exposition time 2800ms, scale bar 50µm.

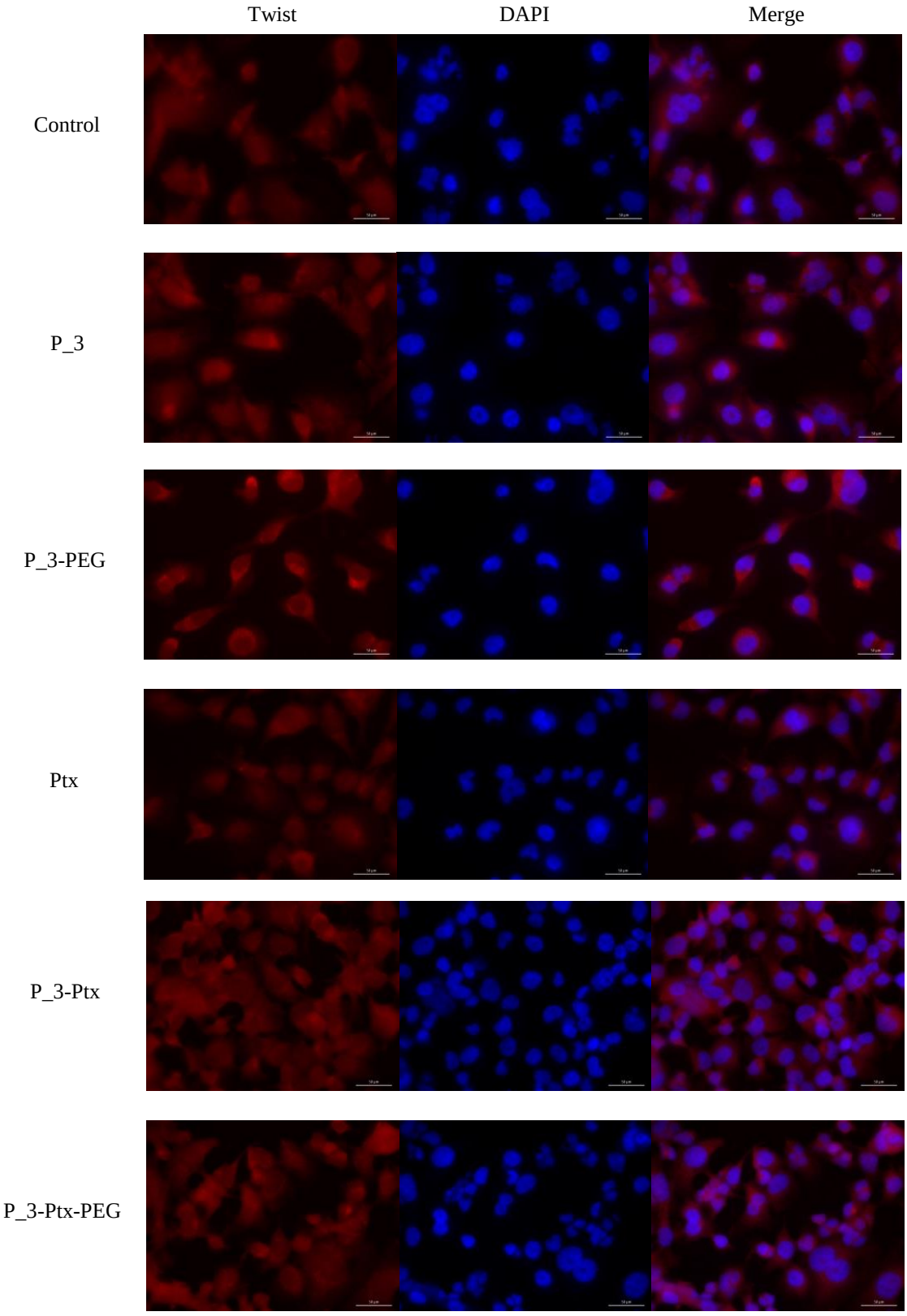

Figure 3.12 – Immunocytochemistry for Twist follow 24h cell contact with free medium, P_3, P_3-PEG, free Ptx, P_3-Ptx and P_3-Ptx-PEG. Exposition time 2800ms, scale bar 50µm.

As can be seen in the control cells for all range of times point, the protein basal expression is moderate, which is difficult to evaluate differences in its expression. Twist staining in fluorescent micrographs obtained for cell exposed to samples for 1h demonstrates that only samples containing Ptx lead to a moderate decrease in the fluorescence (Figure 3.10). Two reasons can be raised, first whatever the entrance path, intracellular Ptx do interact with Twist pathway. By being a transcriptional factor, alterations in the protein expression might take longer than 1h, being therefore another reason for this low variation. In fact, for 6h of cell exposition the bioavailability fostered by the drug-loaded clathrin-mediated endocytosis, a decrease in Twist staining indicates that cytosolic accumulation of Ptx is interfering with malignant cell cascades involving Twist upregulation (Figure 3.11). Confirmation of this important effect of the nanoentrapped drug after cell internalization is depicted in Figure 3.12. At this time point, cells treated with nanoentrapped drug do present a high level of cell death, although for this study a sub-therapeutic dose was used, maintaining the Twist downregulation effect. This scenario, allow us to conclude that cell sensitivity to the drug was not impaired.

On the contrary, the effect of the free Ptx on the cells was erratic through the time. Although it seems to interfere with the protein expression upon 1h, micrographs corresponding to cells with 6h contact shown an opposite effect with an increase in protein expression. This pattern could describe a cell response to the presence of the free drug and more important, to the observed increase in P-gp expression on time (data not show). No significant differences were found in cells treated for 24h. Nevertheless, cell sensitivity to the drug display a different pattern compared with the nanoentrapped drug, since a possible increase in cell resistance could explain the absence of cell death, contrasting with the large number of apoptotic bodies observed in the cells incubated with P_3-Ptx-PEG and P_3-Ptx (Figure 3.11).

3.2.3 *MDR1* and *TWIST* mRNA expression

To ascertain *MDR1* and *TWIST* expression in the breast cancer cells, mRNA expression was determined in the MDA-MB-436 cell line. *MDR1* expression increases after treatment with Ptx (1h and 3h) and decreases after treatment with P_3-Ptx, P_3-Ptx-PEG and P_3-Ptx-PEG^{Ab} (Figure 3.13A). *TWIST* expression decreases after treatment in most of the cases but increases when cells are treated with free Ptx (3h and 6h) (Figure 3.13B). This effect reflects a different intracellular pattern resulting from the cytosolic drug accumulation.

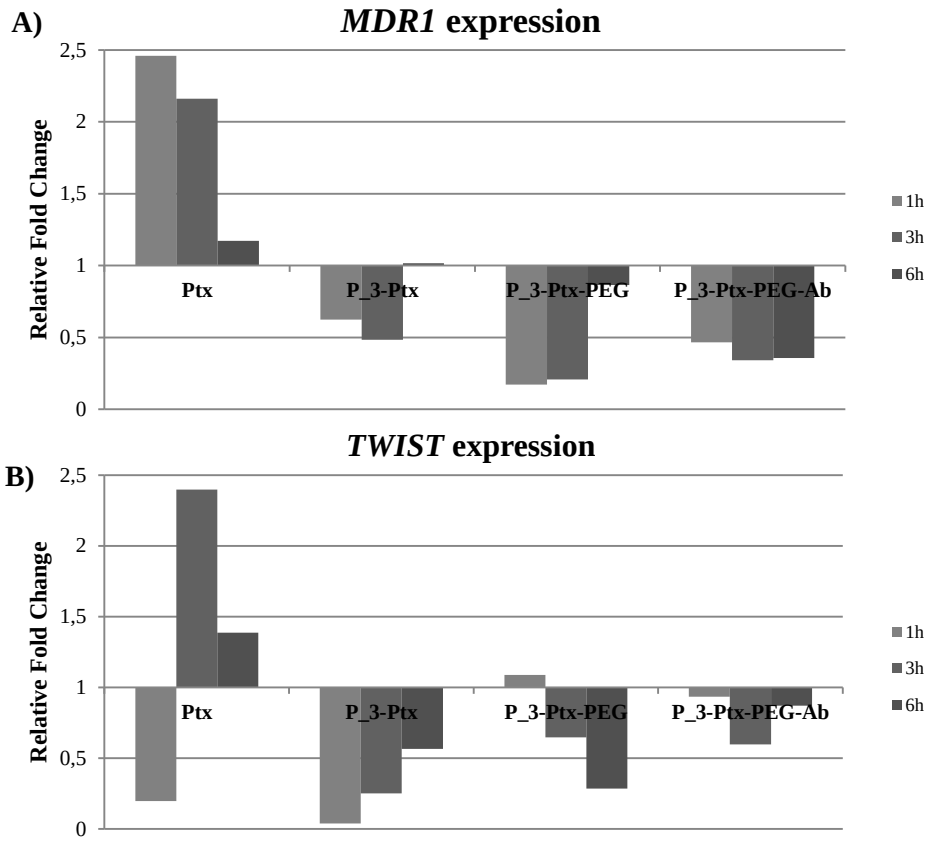

Figure 3.13 – Expression of mRNA through qPCR after 1h, 3h and 6h incubation with free and nanoentrapped Ptx. A) *MDR1* expression. B) *TWIST* expression. Three independent experiments were carried out, each with duplicates. All conditions were normalized to a housekeeping gene amplification, β-actin gene.

The recognition of Twist significance in tumour growth and progression, either promoting chemoresitance to anti-cancer drugs modulating P-gp expression, or being upregulated by the transmembrane CD44 protein, which trigger several events related to cell malignancy, stresses the outmost importance of the results herein described allowing us to anticipate an advantage of this system.

4. Discussion

4.1 Lipid nanoparticles: SLN

Relative effectiveness of today medicines can be overcome by the concept of Nanomedicines. This has special relevance in oncology were patients might benefit from higher pharmacologic action with less adverse reactions. Among other obstacles, decreasing particles size to the nanometre size is a technological challenge. Different processes were used to prepare nanoparticles and in particular lipid-based, such as, liposomes, SLN, nanoemulsions and others (Wong, et al., 2007).

Herein the hot-emulsifying method was used to produce the intended SLN both for characterization purposes and for cell response studies. SLN able to incorporate BCS class II anticancer drug was obtained without compromising drug activity and the target moiety structure. The final systems, P_3-PEG^{Ab} and P_3-Ptx-PEG^{Ab}, as well as the corresponded intermediates, have shown MS and PI within the proposed values: lower than 250nm (being between 108.6nm +/-7.0 and 163.8nm +/-34.1) for MS and lower than 0.250 for PI (in publication). MS values and nanoparticles stability has determined the adequate system to be selected to perform *in vitro* studies. Having in consideration that nanoparticles MS ranging from 100-200nm, will be ideal for *in vivo* intravenous administration studies (Hobbs, et al., 1998), the focus of this study was indeed to evaluate the *in vitro* performance of the P_3-Ptx-PEG^{Ab}. This was achieved by comparing this system with the intermediates and ultimately, to prove our hypothesis, with the free drug.

At the technological level, variations in the nanoparticles physic-chemical properties owing differences in the formulation should occur. Therefore, MS enhancement with the increase in the lipid concentration is an expected response (M. A. Videira, et al., 2013). Differences in MS value between the unloaded and drug-loaded SLN are also in accordance with our previous observation and is explained by the drug presence.

More interesting results were however determined by the nanoparticles PEGylation. From the SLN-Ptx with the solution of PEG-PE assemble, through the film hydration process results: a central core correspondent to the P_3-Ptx with a shell of PEG-PE. The P_3-Ptx-PEG displays higher MS values compared to the intermediate P_3-Ptx, which can be easily explained by the presence of a cloud around the nanoparticles. However the PI values for this PEGylated formulation are comparable to the non-PEGylated one, proving that the system was stabilized by the presence of this amphiphilic polymer. Neither one of the PEGylated formulations, with and without anti-CD44v6, have promoted significant changes in the PI value.

At this level, whereas tumour vessels extravasation of these particles is diffuse and have widespread circulation time, they are large enough for being uptake by the liver and too small to be filtrated in the spleen (Hobbs, et al., 1998; Petros & DeSimone, 2010).

Altering the nanoparticles surface by the presence of a hydrophilic "shell" and further inclusion of a peptide has effects on the surface charge value. Structural information can be derived from the shift for more negative values observed in the PEGylated formulations (P_1.5-PEG, P_1.5-Ptx-PEG, P_3-PEG and P_3-Ptx-PEG). Whereas values for non-PEGylated SLN were around -13.0mV +/-5.0 and -19.6mV +/7.7 (P_1.5, P_1.5-Ptx, P_3 and P_3-Ptx), values of -30.8mV +/-5.3 to -43.5mV +/-9.2 were determined in the PEGylated SLN, confirming the arrangement of the PEG-PE molecules at the nanoparticles surface. A slight increase towards more positive values, -26.5mV +/-6.0 and -30.4mV +/-6.5 displayed by P_3-PEG^{Ab} and P_3-Ptx-PEG^{Ab} suggests another structural change, meaning that the peptide insertion in the PEG shell has occurs successfully contributing to the attenuation in the final particles Zeta potential.

Finally, differences encountered in drug-loaded SLN relative to the plain particles raised a question. If the drug is entrapped in the lipid matrix, nanoparticles surface should not differ. The observed variations might result from drug molecules partial entrapped between the inter matrix and its surface (M. Videira, et al., 2012).

Zeta potential is the electric potential at the lair where liquid bounds to the solid surface, this indicates particles surface charge and is used to evaluate colloidal suspensions or emulsions stability, a zeta potential higher then 30mV and lower then -30mV indicate stable systems (Heurtault, Saulnier, Pech, Proust, & Benoit, 2003). Nevertheless, drug-loaded SLN with surface charge around -20mV have proved to be stable for 2 years (M. A. Videira, et al., 2013).

PEGylated SLN would be the chosen nanoparticles to proceed to *in vivo* studies. Besides their stability, MS and PI, PEG coating protects against opsonisation, premature cellular uptake and against macrophages phagocytosis; also avoids nanoparticles aggregation and interaction with blood proteins, which consequently contributes to extend SLN circulation time (Duan & Li, 2013; M. Wang & Thanou, 2010). This was also observed with Ptx PEGylated liposomes which have a slow release rate, due to EPR effect, Ptx PEGylated liposomes are addressed to tumour location, once there, resistant cells expressing P-gp up-take these liposomes releasing Ptx to the interstitial space. Ptx then enters in neighbour vascular endothelial cells inducing their apoptosis and consequently disturbing angiogenesis (Yoshizawa, Ogawara, Kimura, & Higaki, 2014).

Antibody anti-CD44v6 SLN functionalization was expected to preserve its structural integrity, thus SDS-PAGE was performed in order to verify this assumption (Figure 3.2). Comparing the free anti-

CD44v6 movement athwart the acrylamide gel and the retention of the P_3-PEG^{Ab} after sample denaturation, we can infer that structure was maintained after functionalization.

4.2 *In vitro* studies

In this study *MDR1* expression increases after treatment with free Ptx (1h, 3h and 6h) (Figure 3.13A), which is in accordance with literature, since Ptx induction of *MDR1* expression has been demonstrated previously (Fujita, et al., 2005; Hembruff, et al., 2008). Nanoentrapped Ptx has a different behaviour decreasing *MDR1* expression, which confirms our hypothesis that SLN were internalized via clathrin-mediated endocytosis (Martins, et al., 2012) evade P-gp MDR, thus increasing intracellular drug bioavailability and efficacy (Gratton, et al., 2008; Sahay, et al., 2010). Nevertheless, we should not exclude the contribution of another endocytic pathway, such as receptor-mediated endocytosis through caveolin (Bareford & Swaan, 2007), using CD44 as mediated receptor (Long, Huang, Wu, Shackleford, & Jong, 2012). This pathway may involve different mechanisms of internalization and different intracellular trafficking, which could explain the disparity in the IC_{50} value for P_3-Ptx-PEG^{Ab} and the internalization studies. Nevertheless, P_3-Ptx-PEG^{Ab} influence was identical to P_3-Ptx and P_3-Ptx_PEG in *MDR1* and *TWIST* expression. Intracellular uptake of P_3-Ptx-PEG^{Ab}, 1h after treatment, seems to recruit P-gp to the membrane (Figure 3.7), which was not confirmed by an increase in cells *MDR1* expression (Figure 3.12 A).

Nanoparticles that are internalized through clathrin-mediated endocytosis are transported to lysosomal a compartment (Bareford & Swaan, 2007; Gratton, et al., 2008), which was confirmed previously by our group (Figure 6.1, annex). Whereas those internalized through caveolin-mediated endocytosis are delivered into caveosomes that sort the particles to the Golgi apparatus and Endoplasmic Reticulum (Bareford & Swaan, 2007; Gratton, et al., 2008). SLN functionalized with anti-CD44v6 endocytic pathway was not yet defined, but it is known that CD44 is involved in caveolae lipid rafts and has a very important role as receptor in mediated endocytosis (Long, et al., 2012). As reviewed before, receptor-mediated endocytosis dependent on caveolin occurs through accumulation of ligand-receptor in caveolae lipid rafts, leading to caveosomes formation and these can suffer transcytosis to the opposite membrane domain or be transported to the endoplasmic reticulum (Bareford & Swaan, 2007).

Twist is involved in Ptx resistance (Cheng, et al., 2007; X. Wang, et al., 2004) and by knowing that, it seems reasonable that this drug would increase *TWIST* expression as a cellular mechanism to maintain cell survival. Increasing P-gp expression, by increasing its promoter activity, Y-box binding protein-1 (Shiota, et al., 2009) described in the introduction section. Therefore *TWIST* expression increases with free Ptx (3h and 6h) treatment, Figure 3.13B. Similarly to *MDR1* expression, nanoentrapped Ptx downregulates *TWIST*, through a mechanism which needs to be elucidated. It is possible that by

increasing drug bioavailability, Ptx could upregulate proteins involved in Twist downregulation increasing apoptosis (Figure 3.12, 24h P_3-Ptx and 24h P_3-Ptx-PEG). Data from cellular viability studies confirm its aspect (Figure 3.5; Table 3.2), namely lower IC_{50} values obtained with nanoentrapped Ptx stressing this system higher efficacy when compared with free Ptx. Our findings are in agreement with other studies (Danhier et al., 2009; Koziara, et al., 2004). SLN as a delivery vehicle will increase efficacy with lower drug concentrations, which would be an advantage in chemotherapy.

Immunocytochemistry results for P-gp are in concordance with qPCR results for *MDR1*, as demonstrated in Figure 3.6 P-gp increases after 1h with free Ptx treatment also observed for qPCR (Figure 3.13A) with decrease in mRNA expression for nanoentrapped Ptx being coherent with few changes in protein expression through immunocytochemistry. After 3h treatment P-gp maintained its expression relatively to control (Figure 3.8), whereas mRNA increases for cells exposed to free Ptx and decreases in cells treated with nanoentrapped Ptx. The mRNA expression observed in cells incubated for 6h solves this inconsistency. In this case, as expected, a decrease in P-gp is observed when the sample is the nanoentrapped Ptx.

Accordingly, expression of Twist decreases after 1h treatment with free Ptx, P_3-Ptx and P_3-Ptx-PEG (Figure 3.10), being in accordance with Figure 3.13B for all conditions except for P_3-Ptx-PEG, which seems to have no differences in mRNA expression. Supporting this data is the observation that 6h treatment with free Ptx increases Twist (Figure 3.11) and mRNA levels are not very upraise (approximately 1.4 fold). P_3-Ptx and P_3-Ptx-PEG slightly decreases Twist being in accordance with mRNA levels. To better understand and corroborate these immunocytochemistry and qPCR results, Western blot analyses will have to be performed, both for P-gp and Twist, in order to have real protein quantification.

Functionalization of anti-CD44v6 was performed to assist nanoparticles cell internalization. Surprisingly, P_3-Ptx-PEG^{Ab} did not show any improvement in the internalization rate when compared with P_3-Ptx-PEG. Decreasing MS means a huge increase in the surface area, therefore the number of nanoparticles used in each test could explain this drawback. Although, at least from a theoretical point of view, this nanoparticles will recruit the membrane receptor, the amount of nanoparticles in the ECM might have saturated this receptor, leading to the use of alternative entrance mechanisms; clathrin-mediated endocytosis.

Interestingly, internalization studies performed in two cell lines (MDA-MB-231MCF-7), no statistical differences were observed when comparing P_3-Ptx-PEG^{Ab} (SLN Ptx* PEG-CD44) with P_3-Ptx-PEG (SLN Ptx* PEG), confirming that in terms of internalization this step is pointless. Nevertheless, P_3-Ptx-PEG^{Ab} is expected to have an important biological role, once membrane CD44 receptors saturation will impair cell invasiveness. Moreover, because P-gp was involved in the P_3-Ptx-PEG^{Ab}

internalization and cell death was observed, we can assume that this system does diffuse selectively to cells with CD44 overexpression, been therefore suitable for CSC targeting.

What is important to emphasize is the fact that P_3-Ptx (SLN Ptx*) are rapidly taken up by the cells, using the non-mediated endocytic pathways, being more evident in the less invasive cell line MCF-7 (Figure 3.3) where CD44 overexpression is lower than the observed in the more malignant MDA-MB-231. Summarizing, not only the functionalization did not improve the nanoparticles cell internalization, but also, it seems to act as a barrier.

Evasion of P-gp was expected to leverage P_3-Ptx-PEG^{Ab} intracellular bioavailability and therefore improve Ptx therapeutic efficacy. Again, the obtained results are far away from this expectation. IC_{50} values comparable to those obtained with the free drug might be correlated with the entrance "barrier" limiting therefore the intracellular drug availability. This is more critical when compared with the formulations P_3-Ptx and P_3-Ptx_PEG, both with higher internalization rates and lower IC_{50}. Being evident that the nanoparticles physic matrix is protecting the drug and "blinding" the membrane efflux transporters.

Taken together these observations provide crucial information on the herein proposed system. As a pharmaceutical nanocarrier, P_3-Ptx-PEG^{Ab} provides a combinatory drug-antibody system able to target tumour cell different events. It was also clear that the system can potentially overcome a broad spectrum of membrane efflux transporters, not through the interaction with this ABC transporter, but mainly because it is a constitutive feature of the nanomedicines its suitability to be internalized by endocytosis. This change drastically the conventional pharmacokinetic concept in drug administration, mainly because it is an intracellular drug delivery, but not less expressive, because nanoparticles have the ability to accumulate in the lesion tissue targeting the abnormal cells and preserving healthy cells and tissues.

4.3 Conclusions

In the present study it was observed that the proposed nanomedicines are capable of "blinding" P-gp and downregulating Twist, two very important cellular mechanisms involved in MDR. Since one of the major problems in current therapeutic protocols against cancer is MDR through P-gp, our system could lighten up some hope on cancer drug development field. SLN can entrap not only chemotherapeutics, but also other clinically relevant drugs which are P-gp substrates, increasing their intracellular bioavailability by evading P-gp. Ultimately a combinatory approach would be possible allowing to improve cancer patients survival.

One of the main objectives was not fulfilled; antibody anti-CD44v6 functionalization had not the expected results *in vitro*. However we can still think on *in vivo* studies for these nanoparticles, mainly due to its targeting possibilities.

Theoretically, the decrease of Twist and CD44 receptor saturation, owing to P-gp evasion and CD44 internalization should decrease Ptx resistance and cellular malignancy. SLN with a specific targeting and a chemotherapeutic drug as the main objective of this work suggests a new combinatory therapy against CD44 overexpressing cancer cells.

4.3 Future perspectives

The proposed system, a pharmacological approached for CD44 targeting is expected to suppress or inhibit tumour growth efficiently *in vivo*. Extensive angiogenesis, aberrant vascular morphology and higher production of permeability mediators, are some of the main tumour characteristics involved in EPR that allows SLN passive targeting. Coupling anti-CD44v6 to SLN increases cancer $CD44^+$ cells targeting becoming an active system, therefore being a more specialized one.

It will be interesting to understand which internalization route is used by these nanoparticles, and if it is caveolin receptor-mediated endocytosis. More studies will have to be performed in order to verify if there are inhibition in CD44 intracellular signalling pathways, which would be an advantage since this membrane receptor is responsible for many features associated with cancer progression and invasiveness.

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6. Annexes

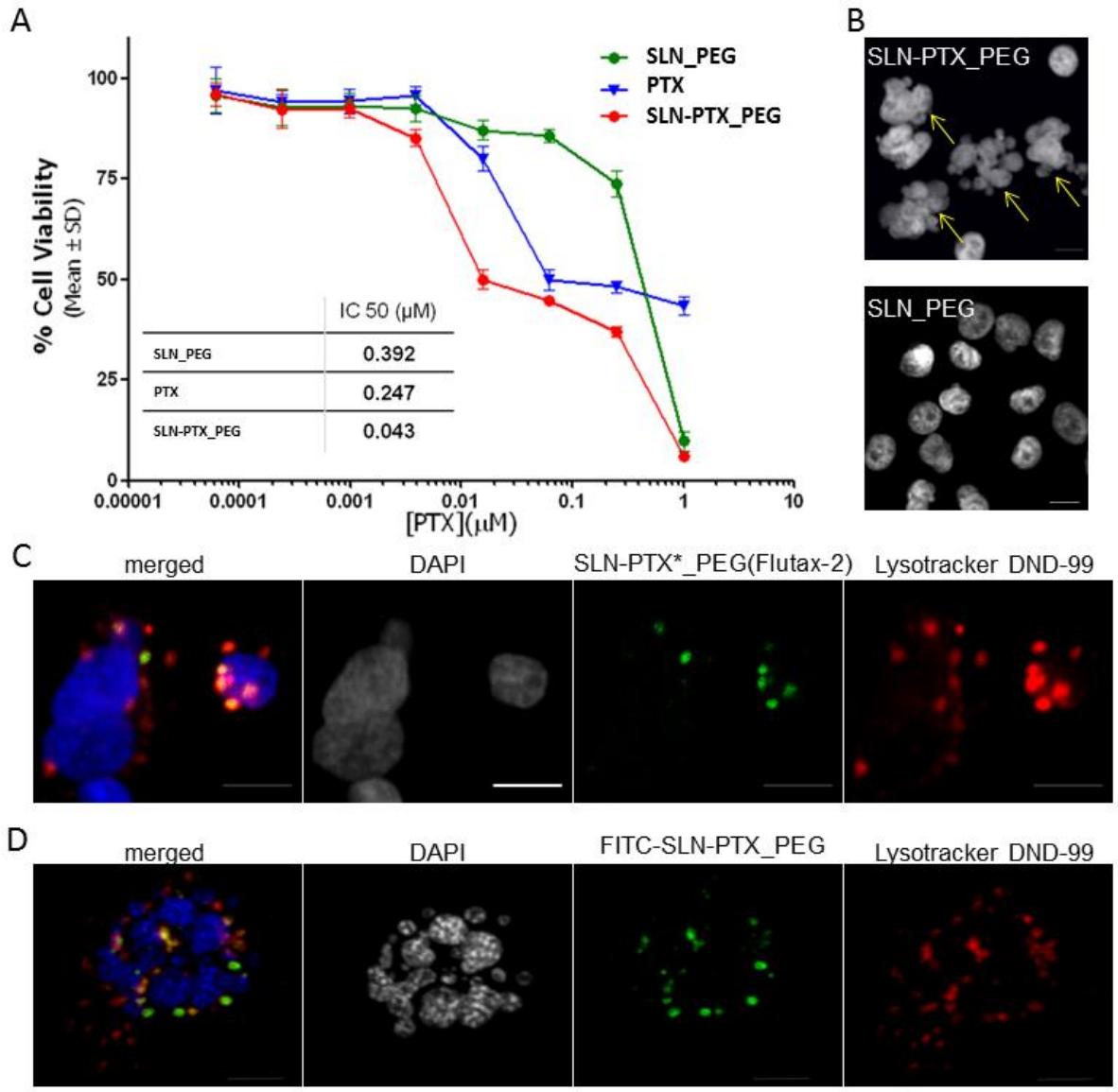

Figure 6.1 – *In vitro* therapeutic activity of free PTX and PTX loaded into PEGylated SLN. MDA-MB-231 cell viability curves and IC_{50} table represent the mean of at least three independent experiments with 6 technical replicates (A). The cytotoxic effect of FITC-SLN-PTX_PEG was confirmed by staining cell nucleus with DAPI and visualization of apoptotic bodies' formation (B). The lysosomal co-localization of Flutax2 delivered by PEGylated SLN (SLN-PTX*_PEG) after 2h of incubation (C) and the presence of FITC-SLN-PTX_PEG in the lysosomes after incubation over-night (D) was observed (Unpublished work from our group).