Inês de Andrade Matos Gonçalves Saraiva

Licenciada em Química Aplicada

Evaluation of the role of LRP1B in the response of human ovarian cancer cells to liposomal doxorubicin

Dissertação para obtenção do Grau de Mestre em
Bioquímica para a Saúde

Orientadora: Raquel T. Lima, PhD
Investigadora no Grupo Cancer Signaling and Metabolism I3S / IPATIMUP
Professora Afiliada da Faculdade de Medicina da Universidade do Porto

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Setembro 2018

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Faculdade de Ciências Médicas
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“Nothing you can know that isn’t known,
Nothing you can see that isn’t shown,
Nowhere you can be that isn’t where you’re meant to be,
It’s easy.”

John Lennon

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ABSTRACT

Improvements on the knowledge of cancer associated pathways, led to the development of several anticancer drugs and drug nanocarriers, which allow potentiating drug delivery and overcoming some limitations. In particular, liposomal drugs (including liposomal doxorubicin), arose as leading drug nanocarriers and are used in the clinic nowadays. Nevertheless, several patients do not respond to these drugs, causing the need to identify markers that influence cancer cells response to liposomal therapies.

The present work is based on LRP1B (Low-density Lipoprotein Receptor-related Protein 1B), which belongs to the Low-Density Lipoprotein Receptor superfamily, scavengers for multiple ligands and mediators of endocytosis. Although LRP1B has been described has a putative tumour suppressor, amongst the 10 most significantly deleted genes in human cancers, its role in cancer is not fully disclosed. LRP1B re-expression in several cancer cells reduced cell proliferation, colony formation and tumourigenicity *in vitro* and *in vivo*. LRP1B may also deplete soluble factors critical for tumour invasion/progression and influence the uptake of liposomal drugs. In a previous study, deletion/downregulation significantly correlated with resistance to liposomal doxorubicin in ovarian cancer patients.

The aim of this study was to further evaluate the effect of LRP1B expression in response of ovarian cancer cells to liposomal doxorubicin (frequently used in ovarian cancer therapy).

For this, the development of three ovarian cancer cell lines (A2780, OVCAR-4 and OVCAR-8) overexpressing LRP1B was attempted by transfection with a pCDNA3.0 vector expressing LRP1B. After confirming LRP1B overexpression, cells were treated with liposomal doxorubicin and their response analysed.

Overall, although results obtained still did not allow to fully conclude on the effect of LRP1B overexpression in the response to liposomal doxorubicin in the cell lines, the clones overexpressing LRP1B developed during this work represent an important tool to further studies on understanding the role of LRP1B in the response to liposomal drugs.

RESUMO

Avanços no conhecimento dos mecanismos associados ao cancro levaram ao desenvolvimento de vários fármacos e nanotransportadores que potenciam a entrega de fármacos ultrapassando algumas limitações. Em particular, os lipossomas são os principais incorporadores de fármacos usados atualmente na clínica. No entanto, vários pacientes não respondem a estes fármacos, sendo necessário identificar marcadores que influenciem a resposta das células tumorais às terapias lipossomais.

O presente trabalho baseou-se no LRP1B, pertencente à superfamília de recetores de lipoproteínas de baixa densidade, sequestradores de múltiplos ligandos e mediadores de endocitose. Embora tenha sido descrito como um potencial supressor tumoral, entre os 10 genes mais significativamente deletados em cancros humanos, o papel do LRP1B no cancro ainda não foi totalmente compreendido.

A re-expressão de LRP1B em várias linhas celulares tumorais promoveu a redução da proliferação celular, da formação de colónias e da tumorigenicidade *in vitro* e *in vivo*. O LRP1B poderá ainda depletar fatores importantes para a invasão/progressão tumoral e influenciar a internalização de fármacos. Um estudo anterior descreveu uma correlação significativa com a resistência à doxorubicina lipossomal em pacientes com cancro do ovário.

O objetivo deste estudo foi avaliar o papel da expressão de LRP1B na resposta doe células de cancro do ovário à doxorrubicina lipossomal (frequentemente usada nestes cancros).

Para isso, foram desenvolvidas linhas celulares de cancro do ovário com sobre-expressão de LRP1B, através de transfecção com um vector pCDNA3.0 expressando LRP1B. Após confirmação de (sobre)expressão de LRP1B, a resposta à doxorubicina liposomal foi analisada.

No geral, os resultados obtidos não permitem ainda concluir sobre o efeito da sobre-expressão do LRP1B na resposta das células tumorais à doxorrubicina lipossomal. No entanto, os clones (com sobre-expressão de LRP1B) desenvolvidos durante este trabalho serão uma ferramenta importante para estudos futuros com vista à compreensão do papel do LRP1B na resposta a fármacos lipossomais.

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INTRODUCTION

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1.1. Cancer, A Global Healthcare Problem

Cancer represents a major concern for global healthcare. Described as the second leading cause of death worldwide, it is estimated to account for approximately 9.6 million deaths in 2018[6]. According to the World Health Organization, 1 out of 6 deaths is caused by cancer[6].

The observed impact of this disease can be associated with hallmarks which are composed by biological capabilities achieved during the several phases of human cancers development. The hallmarks can include: resisting cell death, sustaining proliferative signaling, enabling replicative immortality, evading growth suppressors, activating invasion/metastasis and inducing angiogenesis[32]. Moreover, genome instability is either related to these hallmarks and it is able to generate genetic diversity allowing multiple functions of the hallmarks[30].

Due to the fact that global incidence and mortality of cancer have progressively been increasing[6], the development of new therapeutic strategies emerged as a crucial aim for research.

1.2. Anticancer Therapeutic Approaches

Cancer therapeutic approaches include chemotherapy, radiation and surgery which are often used in combination[30]. However, these methods of treatment are associated also with side effects regarding the damages caused not just at tumour tissues but either at normal tissues[30]. These powerful and aggressive methods of cancer treatment, such as cytotoxic doses of chemotherapy and also radiation of the total body, assuming non-bearable side-effects (i.e. pain, nausea), call for new strategies of treatment[23]. Furthermore, several cancer treatments are directly bond to low specificity alike cancer drugs represent rapidly clearance and biodegradation with target limiting[30].

A huge progress arose from the knowledge gathered by the development of molecular biology allowing identify cancer associated molecular pathways, biomarkers and molecular targets[32]. This has allowed to develop new approaches, particularly the development of new drugs, which include not only the cytotoxic conventional drugs as

well as drugs targeted for cancer molecules/pathways[32]. Despite the advance of this area of research, there are still major hurdles to overcome, not only in what concerns the development of new drugs (or improving existing ones) but also in understanding the way patients respond to treatment (both at short and longer periods of time). This would allow to optimize the ways (and the type) of drugs given to patients in order to increase their efficiency to reduce toxicity and associated morbidity as well as to optimize the use of expensive drugs.

1.2.1. Liposomes as Vehicle for Anticancer Drugs

The interest to increase the success of cancer therapy is focused not only in the development of new drugs but also strongly associated in increasing its efficiency, namely by improving their delivery to tumour cells[30].

For this, nanomedicine has brought advances providing better solutions for cancer therapy. These included the development of nanoparticles (ranging from 1-100 nm) with important characteristics such as: nanoscale sizes, high surface-to-volume ratios, optimal drug release profiles and adaptation to targeting modifications, making these vehicles an optimal choice to target the tumour tissue and stably release drugs[30]. Considering drug delivery mechanisms, nanocarriers have the ability to improve drug features prolonging their periods of body circulation (higher bioavailability), protecting the enclosed drug from rapid elimination, avoiding certain mechanisms of resistance to chemotherapeutic agents, reducing drug toxicity, easily crossing the blood brain barrier, changing the stability/distribution of drugs by itself charge and promoting specific types of endocytosis/cellular uptake through its modified surface with ligands[5,25]. Moreover, these drug vehicles may have two different types of targeting the tumour site: passive or active[30]. **"**Passive targeting" focus on the pharmacokinetics and biodistribution of the nanocarrier system without involving interactions between specific targeting ligands to the tumour target site. In addition, it relies in the "enhanced permeability and retention" (EPR) effect, potentiated by the leaky blood vasculature of tumour tissues, which allow this nano-vehicles to passively accumulate into those tissues[29]. On the other hand, in "active targeting", the surface of nanocarriers is functionalized using ligands (for example: transferrin, folic acid, enzymes, engineered antibodies, proteins) that may then

be attached with high specificity to cancer targets (including glycans or protein receptors)[30].

It is also relevant the fact that, nanoparticles may modify drug in solubility. Hydrophilic drug nanocarriers increase the bioavailability and promote a more efficient delivery[30].

Liposomes, as a vehicles for anticancer drugs, are considered to date the most successful drug-nanocarrier system with advantages in biological and technological fields (i.e. reducing drug toxicity)[5]. Interestingly, liposomal therapies represent already more than one-third (35%) of the submissions to FDA (Food and Drug Administration) related to drug treatment with nanomaterials[29].

Liposomes are small and spherical vesicles constituted by, at least, one outer lipid phospholipidic bilayer involving an internal aqueous compartment[25,29]. These vesicles may have different diameters and lipid bilayers being: unilamellar (composed by one lipid bilayer and having 50-250nm in diameter) or multilamellar (composed by various concentric lipid bilayers and with 1-5 μm of diameter)[29].

Anticancer agents (and other several drugs) inclusion occurs within the lipid bilayer(s) or into the aqueous compartment, depending on their hydrophobic and hydrophilic characteristics, respectively[25,29].

Regarding the already described passive or active targeting of tissues by nanocarriers, usually for liposomes the active mechanism is the preferred choice to overcome delivery restrictions and to reach a higher drug concentration on the tumour target site[5,29]. As for other nanocarriers, selectivity for the tumour site can be improved through the ability of liposomal surface of being modified[25]. Likewise, liposomes may have also some pharmacokinetics and pharmacodynamics disadvantages and may be recognized as foreign body by RES (reticuloendothelial system), consequently rapidly phagocyted[25]. Pegylation, inclusion of Polyethylene glycol (PEG; a hydrophilic and non-ionic polymer) is a commonly used strategy to overcome this issue. Therefore, this novel "stealth liposomes" emerged with improved stability and solubility, prolonging their time at bloodstream avoiding degradation[29,30]. These liposomes can afford higher drug passive accumulation in tumours (concentrations of 10-100 fold compared with free drug)[25].

However, there are still some toxic side effects which have been associated to liposomal drug administration, such as hypersensitivity reactions. Also, the high prices

associated to production as well as the low reproducibility in large-scale production may represent some of the problems in their use in the clinic[25,29].

Despite these problems, to date, several liposomal formulations have been studied and some of them approved to be used in the clinic, proving once more that these nano-vehicles may be highly relevant for anticancer strategies. It all started in 1995, when the a doxorubicin HCl liposomal injection was the first liposomal encapsulated anticancer drug approved for clinical trials, in acquired immune deficiency syndrome (AIDS)-related Kaposi's sarcoma[28]. Since then, several chemotherapeutic drugs have been incorporated, some of which are already given to patients (namely liposomal doxorubicin, for ovarian cancer treatment) (Figure 1). Others are emerging from new developments on this field including: non-pegylated liposomal doxorubicin, liposomal daunorubicin and liposomal cytarabine, among others.

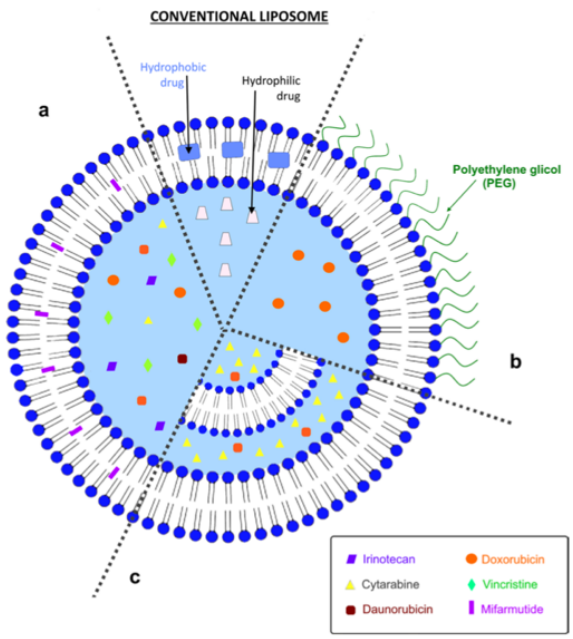


Figure 1. Representation of approved liposomal encapsulated anticancer drugs.

Hydrophilic drugs are enclosed in the central compartment of the conventional liposome (consisting in of a lipid bilayer enclosing an aqueous core), while hydrophobic ones are in enclosed in the bilayer. a) Conventional liposome incorporation of different anticancer drugs. b) PEGylated/stealth liposomes, coating liposome surface with a layer of polyethylene glycol (PEG) increases its stabilization. c) Bilamellar liposome. Liposomes may have more than one lipid bilayer. The represented approved liposomal formulation has two lipid bilayers and the two drugs (cytarabine and daunorubicin) are encapsulated in a 5:1 ratio, in its aqueous space. (Adapted from Sousa, I.; 2018)

1.2.1.1. Liposomal Doxorubicin, the first and the most studied anticancer liposomal drug

As previously referred, the first anticancer liposomal encapsulated drug approved by Food and Drug Administration (FDA) was doxorubicin (Doxil, a PEGylated liposomal doxorubicin), in 1995 as nanodrug, for the treatment of various cancer types including metastatic ovarian cancer[1,25].

Doxorubicin is a well-known anthracycline used as conventional chemotherapeutic treatments. It has two distinct mechanisms of action: i) disruption of topoisomerase II (mediator of DNA repair) by intercalation of DNA[25] and ii) generation of free radicals and damage cellular membranes, DNA and proteins[25]. Despite the accepted use of this drug in conventional chemotherapy approaches, as for many other chemotherapeutic drugs, its use presents problems to the patient, due to known side effects. In particular, for anthracyclines, such as doxorubicin, there is a risk of cumulative cardiac toxicity which may cause austere life-threatening heart problems (i.e. heart failure). Therefore, different strategies are being tried to minimize the risks associated to this drug, including: dose reduction, fractionated administration, protective agents implementation and, particularly, its encapsulation into liposomes[25].

The advantages observed on liposomal encapsulated doxorubicin were possible by promoting alterations in lipid bilayer composition and by "stealth" technology (process of pegylation) allowing to "upgrade" doxorubicin bioavailable[29]. PEGylated liposomal doxorubicin has a hydrophilic profile, so its excretion is completed continuously through the sweat. Consequent, palmar-plantar erythrodysesthesia (PPE), stomatitis and mucositis are some diseases that can be related to this doxorubicin formulation. Nevertheless, and most important, cardiotoxicity (and alopecia) are observed at lower rates comparing with the conventional doxorubicin[10].

1.2.1.2. Clinical applications of liposomal doxorubicin

Liposomal doxorubicin was approved for the first time with a phase III trial for epithelial ovarian carcinoma treatment[26], based on its efficacy and safety profile[11]. Other trials were carried out since, showing an increase efficacy of liposomal doxorubicin for ovarian cancer treatment when compared with other chemotherapy methods[21]. Liposomal

doxorubicin is commercialised under different names: Doxil or Caelyx (Johnson & Johnson – USA, or Janssen-Cilag-Europe, respectively), Evacet (The liposome Company Inc.) and LipoDox (SunPharma) or Myocet (Enzon), some of them depending on specific characteristics of these formulations. Its clinical use is already carried out in several cancers such as Kaposi's sarcoma, ovarian cancer and breast cancer[25].

1.3. Need to improve response to liposomal drugs: a possible role for LRP1B

Despite all the advances, there are still problems regarding the response of patients to liposomal drugs, which is not well-succeeded. Several strategies are being promoted to overcome this issue, not only by the development of new drug formulations and the study of novel targets, but particularly through the research on predictive biomarkers. These last ones may allow strategies in which patients may respond to a particular (liposomal) drug.

Although there is yet not enough data to conclude on the exact role of cancer biomarker as predictors of response to liposomal drugs, some studies (although some not directly) have suggested the role of possible biomarkers for liposomal drugs. These include: CA-125 (a well-known biomarker for epithelial ovarian cancer), antigen 125 (CA-125), shown to increase in patients treated with pegylated liposomal doxorubicin[24]. P-glycoprotein (a drug-efflux pump) whose expression at higher levels results in lower survival levels (poor overall survival) of epithelial ovarian cancer after the beginning of the drug treatment (poor progression-free survival)[20]; Topoisomerase alpha II (TOP2A) whose gene gain and protein overexpression was associated with high activity of pegylated liposomal doxorubicin[9] and also LRP1B gene, described in previous study, to contribute for the resistance to liposomal doxorubicin in high-grade serous ovarian cancer patients[8,29].

1.3.1. LRP1B - Low-density Lipoprotein Receptor - related Protein 1B

LRP1B gene was described for the first time in lung cancer cells, as a putative tumour suppressor gene, involved in important stages of tumour development such as progression, invasion and metastasis[17]. LRP1B is among the 10 most altered genes in human cancers, with several chromosomal, epigenetic and microRNA-mediated mechanisms as well as deletions and mutations being described as frequently inactivating this gene in different tumours (melanoma, urothelial, ovarian, breast and thyroid cancer)[4,27].

This gene codes for a transmembrane glycoprotein of 4,599 amino acids, of approximately 500 kDa (calculated mass without glycosylation) mostly expressed in thyroid, brain, skeletal muscle and testis[22]. LRP1B belongs to the Low density lipoprotein receptor proteins family which consists in the principal class of endocytic lipoprotein receptors[18].

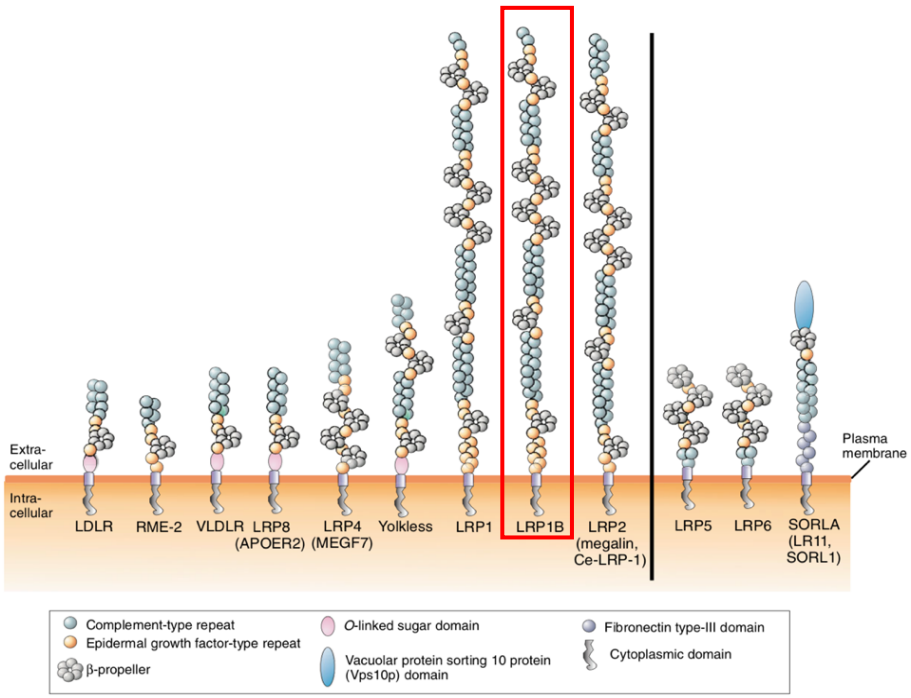


Figure 2. The structure of LRP1B and other LDLR family members.

(Adapted from Willow T.E., 2007)

From all the LDLR family, LRP1B is highly homologous to LRP1 (with 55% identity in the sequence of amino acids) and they have identical overall structure (Figure 3) both being composed by four putative ligand-binding domains (I, II, III, and IV from the N-terminus) and both having a cytoplasmic tail with five potential endocytosis motifs (two NPXY motifs, two di-leucine motifs, and one YXXL motif)[16]. However, some distinct characteristics are noticed between these two receptors such as: the average size of the introns (10 times larger on LRP1B) and the presence of an additional ligand-binding repeat in domain IV at LRP1B[16]. LRP1B has two additional exons, of which one (exon 68) codes for an additional repeat in the fourth ligand-binding repeat cluster and another (exon 90) encodes a 33-amino-acid sequence within the cytoplasmic tail that shows no homology to other known proteins.

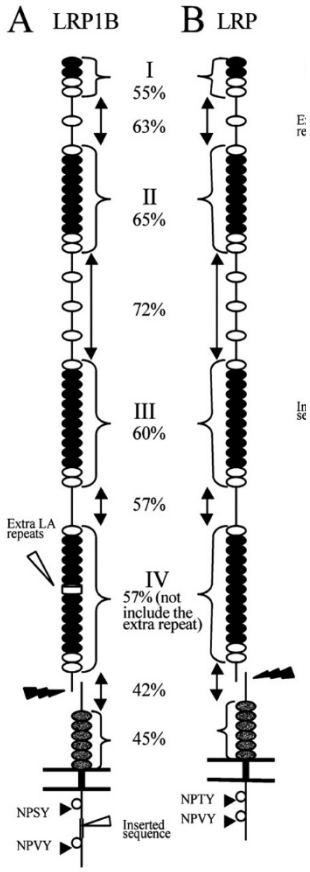


Figure 3. Comparison of LRP1b and LRP structure domains.

LRP and LRP1B are almost identical except for the two extra sequences in LRP1B: an extra ligand-binding repeat (repeat 6 in domain IV, amino acids 3513–3550) and an inserted sequence between the two NPXY motifs in the cytoplasmic tail (Adapted from Liu;2001)

A large number of different ligands have been described to be recognized by LRPs, including: lipoproteins, proteinases, proteinase-inhibitor complexes, extracellular matrix (ECM) proteins, bacterial toxins, viruses and other intracellular proteins (Figure 4)[13]. However, the major ligands of LRPs are proteinases and other molecules associated with the regulation of proteolytic activity. While some ligands bind directly to LRPs (namely serine proteinases; metalloproteinases), others only bind when complexed with their specific inhibitors, which is then able to be recognized by conformation change[13]. The similarity between LRP1B and LRP protein domain strongly affects similarity in their ligands[16].

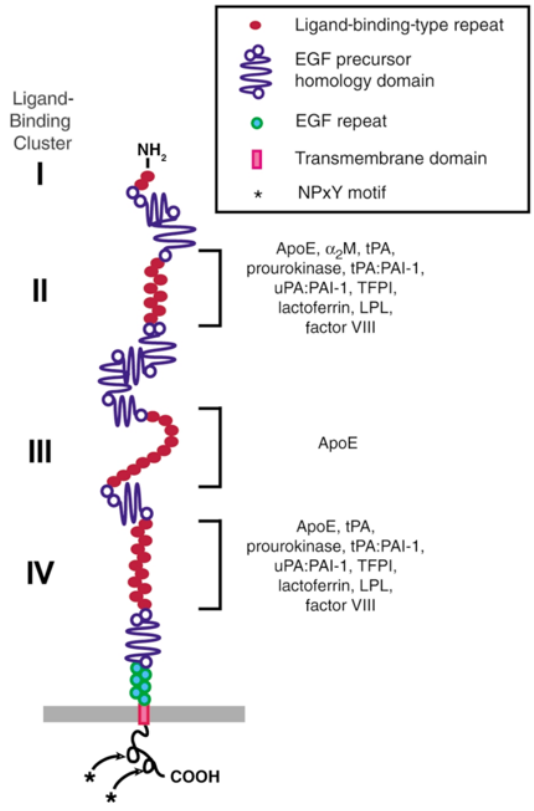


Figure 4. LRPs ligand binding to LRPs domains. (Adapted from Herz and Strickland, 2001).

Interestingly, since LRPs can regulate the levels of certain matrix metalloproteinase (MMP) family members and it can be responsible for their internalization, this molecule shows its important role as a receptor and able to remove

excessive extracellular proteolytic activity[2,12]. Furthermore, as shown in previous studies, the levels of LRPs considerably decrease in tumours, which can lead to an accumulation at the tumour sites of certain proteinases and enzymes responsible for some processes such as tumour progression and metastasis. The regulation of lipid-degrading lysosomal enzymes and its activator (sphingolipid activator protein - SAP) indirectly involves LRP. As a result of this, LRP is one of the receptors that mediate the transportation of SAP to lysosomes, contributing for the survival of the cells[13,14].

Despite the structural and functional similarities with LRP1, LRP1B has been described to have lower ligand internalization and degradation rates[16].

1.3.2. LRP1B possible role in cancer and in its liposomal response to drugs

As already referred, LRP1B is one of the most altered genes in human cancers. Several mechanisms have been described to inactivate its expression in tumors, including: deletions, mutations and also chromosomal, epigenetic and microRNA-mediated mechanisms. However, the role of altered LRP1B expression in cancer is not fully understood.

Previous studies have showed that LRPB re-expression in cancer cells decreased proliferation, colony formation, angiogenesis and invasive capacity. Due to LRP1B large size, most of the studies on LRP1B result from the overexpression in cell lines of its soluble ectodomains or from mini-receptors [comprising the ligand binding domain region IV, transmembrane segment and intracellular tail] allowing to mimic function of the full receptor[15]. This may be not the ideal strategy to study all LRP1B function but some of the obtained results have been validated by RNA interference. Also recently a study transfection, for the first time, of human tumor cells was achieved with a recombinant full length (murine) LRP1b confirming the suppressed tumor cell growth of lung cancer cells, previously found with mLRP1B[3]. Also, LRP1B down-regulation was related with the inhibition of growth, migration and metastasis in colon cancer cells[31].

In addition, it has been shown that LRP1B modulated cancer cells extracellular environment, affecting the invasive behavior of cancer cells. In a previous study showing a decrease in the highly invasive potential of cancer cells after incubation with

conditioned medium from LRP1B-overexpressing cells, analysis of the content of the LRP1B-medium, showed decreased amounts of several factors namely metalloproteinases, growth factors, cytokines and angiogenic factors[27].

Also the endocytic activity of LRP1 may interfere with drug internalization process via liposomes[7], thus having clinical implications. A previous study has raised the hypothesis that LRP1B contributes to emerge the resistance of liposomal-doxorubicin at high-grade serous ovarian cancer patients[8]. Decreased at LRP1B levels in ovarian cancer cells demonstrated an increase of the resistance of these cells to liposomal-doxorubicin treatment comparing with conventional doxorubicin therapy[8].

Overall, it is possible that LRP1B may affect response of cancer cells to liposomal drugs. Nevertheless, further studies are needed to confirm this hypothesis. This will be important to evaluate the role of LRP1B in the response to liposomal drugs and ultimately as potential biomarker of response.

AIM

The main aim of the present study was to evaluate the role of LRP1B in the response of human ovarian cancer cells to liposomal doxorubicin.

To achieve this, two specific aims were proposed:

- To develop human ovarian tumour cell lines overexpressing mLRP1B (LRP1B mini-receptor, which mimics LRP1B endocytic function)
- To analyse the effect of mLRP1B overexpression in the sensitivity human ovarian tumour cell lines to liposomal doxorubicin.

MATERIALS AND METHODS

Chapter II

2.1. Cell Culture

All procedures were performed at Telstar Bio-II-A/P laminar flux biological safety cabinet under aseptic conditions. The following cell lines, derived from human ovarian tumours, were used: A2780 (ovarian endometroid adenocarcinoma), Ovcar-4 (high grade ovarian serous adenocarcinoma) and Ovcar-8 (high grade ovarian serous adenocarcinoma). Cells were available at the lab, kindly given by the groups' collaborators. These adherent cell lines were routinely maintained in culture, in exponential growth, in RPMI-1640(Roswell Park Memorial Institute - 1640 medium with stable glutamine (Gibco/BRL – Invitrogen) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS; Gibco™), 1% (v/v) Penicillin/Streptomycin (Gibco™) and 0.5% (v/v) Fungizone (Amphotericin B, Gibco™) and kept at 37ºC in an incubator with humidified atmosphere of 37.0 ºC and 5% of CO2.

Cells were routinely observed under an inverted phase microscope (Olympus CK2) to assess cell confluency and to discard possible contamination problems. In addition, when images were needed to be acquired, a Nikon microscope with a camera incorporated was used.

2.1.1. Cell Subculture

At approximately 70% confluency, cells were divided according to the following procedure. Cell medium was removed and cells washed with 2 mL sterile Phosphate-Buffered Saline (PBS, pH 7.5; 137 mM NaCl; 2.7 mM KCl; 6.4 mM K2HPO4; 1mM NaHPO4) to remove cell debris and remaining medium (including FBS). TrypLE Express reagent (Gibco™) was added to cells (500µl for a 25 cm^2 tissue culture flask) which were then further incubated for 5 minutes within the 37.0 ºC incubator atmosphere to allow cells detachment. Cells were split according to the cell lines (1:5 for OVCAR-4, 1:20 for A2780/OVCAR-8) and new complete medium is added. As an example, to perform a 1:5 split of cells, 1:5 of the total suspension was transferred into a new flask and 4.5 ml of growth medium was added.

In case cells were used for experiments, cell density was determined. For this, 50 µl of cell suspension was diluted in 10 ml Isoton II diluent (Beckman Coulter) and cells were counted using a cell counter (Beckman Coulter). After determining cell density,

cells were diluted with growth medium to obtain cells suspension with the desired concentration.

2.1.2. Cell Cryopreservation

To maintain a stock of cells for posterior use, cell suspensions were centrifuged at 1,200 rpm for 5 minutes and the supernatant was discarded. Cells pellets were resuspended in 1 mL of freezing medium [10% (v/v) dimethyl sulfoxide (DMSO) in FBS)] and aliquoted into cryovials. Cryovials were then placed into an isopropanol containing cooling chamber (Mr. Frosty; Nalgene) at a -80 ºC freezer.

2.1.3. Cell Defrosting

Cells stored at cryovials were quickly thawed in a 37 ºC water bath after being removed from the freezer. Cells were then resuspended in pre-warmed complete growth medium and centrifuged at 1,200 rpm for 5 minutes. Supernatant was discarded and cells transferred to complete medium (to discard DMSO toxicity) into a cell culture flask.

2.1.4. Cell Genotyping

Cell lines authenticity was verified. For this, for each cell line DNA was extracted (according to the procedure described in section “DNA: extraction and quantification”) and genotyped in the i3S Genotyping Service using the POWERPLEX 16 HS kit (Promega).

2.2. Transfection of Cell Lines

pCDNA3.0 vector (Empty vector) and pCDNA3.0 containing mLRP1B mini-receptor CDNA were transfected into cell lines using a liposome based approached. For all cell lines, transfection with the Lipofectamine 3000 (Invitrogen) reagent was attempted following the manufacturer protocol. For this, cells were plated ($5x10^4$ cells/well) into 24 well plates in complete medium and allowed to adhere for 24h. The next day, medium

was changed to new complete medium (500 µL/well). Meanwhile, three epppendorfs were prepared. each with 1,5 µL of Lipofectamine 3000 transfection reagent in 25 µL of Opti-MEM I Reduced Serum Media (GIBCO). After vortexing 2-3 seconds, eppendorfs were maintained at room temperature. Additionally, another three eppendorfs were prepared with 25 µL of Opti-MEM: i) Opti-MEM only (to control for possible transfection toxicity of the procedure) reagent to the cells in study or ii) with 500 ng pCDNA 3.0 (Empty-vector) and iii) with 500ng of mLRP1B-pCDNA 3.0 (expressing mini-receptor for LRP1B which mimics full length LRP1B endocytic activity. These solutions were homogenised and allowed to stand for 10-15 minutes at room temperature. Each DNA dilution was then combined and mixed to the diluted lipofectamine transfection reagent and further incubated for 15 minutes at room temperature. The total volume (50 µL) was then added dropwise to the cells and incubated for 24 hours at 37 ºC and 5% CO_2, upon which the transfection medium was removed and replaced by new supplemented medium. Following 72 h, medium was discarded and G418 was added (200 µg/ml for OVCAR cells (-4a and -8) and 500µg/ml for A2780 cells) to allow the selection of pCDNA 3.0 expressing cells (both empty vector and mLRP1B transfected cells). Cells were kept under selection changing the medium (with G418) every 2-3 days. Blank cells (treated with medium only) were used as comparison to assess G418 effect and to assess LRP1B expression. Following one month, A2780 and OVAR-8 cells transfected with empty and LRP1B expressing vector which continued to growth under selection were used.

Regarding the OVCAR-4 cell line, other different transfection reagents such as Lipofectamine STEM (Invitrogen) and Fugene (Roche) were also tested, following the respective manufacturer's protocol and changing some parameters: i) different DNA/transfection reagent ratios; ii) presence *vs* absence of FBS during the first 4h of transfection; iii) different cell densities at transfection. While, the protocol for transfection with Lipofectamine STEM used was the same as the one previously described for Lipofectame 3000, FuGene, transfection assay was different: Briefly; three eppendorfs were prepared with 50µL of Opti-MEM each and also with 500 ng/µL of the DNA, as previously for Lipofectamine transfection. After a vortex of 2-3 seconds, 1,5 µL of FuGene transfection reagent (at room temperature) was added to these eppendorfs. The mixed solutions were allowed to rest for 15 minutes at room temperature. Then, 50 µL of these mixtures were added (dropwise) and well homogenized to three different wells of

the 24-wells plate. Cells were incubated for 24 hours at 37 ºC and 5% CO_2, upon which the transfection medium was removed and replaced by new complete medium. After this, cells were incubated again for 6 days at 37 ºC and 5% CO_2.

2.2.1. Isolation of Transfected Clones

To isolate clones of stable LRP1B overexpressing cells, transfected cells were diluted into 10 cells/mL in complete medium and plated (100 µL/well) in 96 well plates. Cells were incubated in cell culture incubator and wells carefully analysed for the presence of only one cell. Clones deriving from these cells were allowed to grow and were then expanded, first unto a a 24 well plate and then into tissue culture flask. All steps were carried out in complete medium with G418 (200 ug/mL for OVCAR-8 and 500 ug/mL for A2780).

2.2.2. Extraction of DNA

Extraction of DNA was carried directly from the cell pellets (after cell suspensions were centrifuged at 1,200 rpm for 5 minutes and supernatant was discarded) or from Trizol treated samples (as described later in this thesis in the section “RNA: extraction and quantification”). Initial procedures were therefore dependent on the initial samples. For cell pellets, cells were lysed with 300 µL cell lysis buffer (Citogen) and further vortexed (2-3 seconds). For the TRIZOLtreated samples (please see initial treatment in section “RNA: extraction and quantification”), 300 µL of 99,9% Ethanol (VWR) were added and samples centrifuge at 16,000 xg for 10 minutes at 4 ºC. After discarding ethanol, 300 µL cell lysis buffer (Citogen) were added and vortexed (2-3 seconds).

The following procedure was carried out for all samples.

Samples were incubated with Proteinase K (5 µL) at 55 ºC with agitation overnight, after which more Proteinase K (5 µL) was added and further incubated for 3-4 hours. After adding 100 µL of Protein Precipitation Solution (high-salt buffer, Citogen), samples were carefully homogenised and incubated at 4 ºC for10 minutes. Samples were then centrifuged at 16,000 xg, at 0 ºC for 10 minutes. The proteins precipitated and the DNA material was removed as supernatant for new eppendorfs with 500 µL of 99,9% of

Isopropanol (VWR) which recover purified DNA by previous precipitation procedure. The samples were well-mixed and allowed for rest at 4 ºC for 30 minutes. After a centrifugation of 16,000 xg, at 0 ºC for 10 minutes, the supernatant was removed and 500 µL of 70% Ethanol Reagent Grade (VWR) were added to efficiently precipitate the polymeric nucleic acids and to remove residues of short-chain and monomeric nucleic acid components (ribonucleotides) from RNase treatment still in solution. Samples were well homogenised and centrifuged at 16,000 xg, at 0 ºC for 5 minutes. After resuspending DNA in 21 µL of ultrapure water, DNA was quantified and using a NanoDrop UV-Vis spectrophotometer, which allows also to assess the presence protein or organic solvent (phenol). In the present study only samples presenting ratios higher than 1.8 for absorbance at 260/ 280 nm (A260/A280), were considered.

2.2.3. RNA Extraction

All the plastic material used was previously carefully cleaned with RNase-free solution. Cell pellets were resuspended in 1 mL of Trizol reagent (Invitrogen) and all the following protocol carried out at 4ºC (or stored at -80 ºC for later extraction). Chloroform (200 µL) was added to the samples and solutions were well homogenised and centrifuged at 16,000 xg at 4 ºC for 15 minutes. This allowed to obtain two phases and thus the separation of nucleic acids (remaining soluble in upper aqueous phase) from proteins. Proteins containing hydrophobic and hydrophilic regions in which hydrophobic cores interact with phenol (from Trizol reagent) results in precipitation of proteins to collect at the interface between the two phases (white flocculent). Additionally, the pH of phenol determinate the partitioning of DNA and RNA in the middle of the organic phase and the aqueous phase, acid phenol retains RNA in the aqueous phase and moves DNA into phenol phase (easily neutralized DNA phosphate groups). The RNA aqueous phase was transferred into new eppendorfs and 500 µL Isopropanol (VWR) were added to precipitate RNA. After centrifugation at 16,000 xg, 4 ºC for 10 minutes, isopropanol was discarded and RNA washed with 500 µL 70% Ethanol (PanReac, Applichem). Then samples were centrifuged again at 16,000 xg, 4 ºC for 10 minutes, ethanol was discarded, and RNA samples air-dried. After resuspending RNA in 21 µL of ultrapure water, RNA was quantified and using a NanoDrop UV-Vis spectrophotometer (using 1 OD260= 40µg

RNA) which allows also to assess the presence protein or organic solvent (phenol). In the present study with A 260 /A 280 ratios higher than 1.7 or lower 2.1.

The remaining samples (phase containing DNA and proteins) were stored at -80ºC and then used for DNA extraction, according to the previously described protocol ("DNA: extraction and quantification" section).

2.2.4. Analysis of mRNA Levels by Quantitative Real Time PCR

To analyse the levels of mRNA expression, previously extracted RNA (section "RNA: extraction and quantification") was first treated with DNase and then converted into cDNA as follows.

2.2.5. DNase Treatment

DNase treatment was carried out to remove residual genomic DNA from RNA samples. For this, 8 µl of total RNA (1µg RNA) was mixed with 1 µl of 10X Reaction Buffer with $MgCl_2$ (Thermo Scientific™) and then 1µl recombinant DNase I (which digests single- and double-stranded DNA; Thermo Scientific™) was added and incubated at 37°C on a thermocycler (Thermo Scientific™) for 30 minutes. To inhibit DNase activity and to further preserver cDNA, 1µl of EDTA (Thermo Scientific™) was added and samples were placed at 65 ºC for 10 minutes.

2.2.6. cDNA Synthesis

For synthesis of cDNA, 1µl of Random Hexamer Primers (mixture of oligonucleotides representing all hexamer sequences possible; Primer Sequence: 5′ – d (NNNNNN) –3′ N = G, A, T or C; Thermo Scientific™) was added to the RNA samples and incubated for 5 minutes at 65 ºC to allow primer annealing. Meanwhile, a master mix was prepared with 4 µl of RT Buffer 5x (250 mM Tris-HCl (pH 8.3 at 25°C), 375 mM KCl, 15 mM MgCl2, 50 mM DTT) (200 U/µL; Thermo Scientific™), 0.5 µl of RiboLock RNase Inhibitor (40 U/µL; Thermo Scientific™), 2 µl of dNTP mix (10 mM; Thermo Scientific™) and 0.5 µl of ultrapure water. After adding 6.5 µl of master mix to each

RNA- random primer mixed samples, 1 µl RevertAid Reverse Transcriptase (200 U/ µl; Thermo Scientific™) was added to the samples. A "-RT" (minus reverse transcriptase) control was included in which RT enzyme was not added to the samples. Finally, all the samples were placed in the thermocycler on the sequential temperature steps; at 25 ºC for 10 min for primer extension, followed the polymerization time at 42 ºC for 60 min for the annealing step and then 70 ºC for 10 min for enzyme denaturation. Samples were kept at 4 ºC.

2.2.7. Real Time PCR Analysis

For quantitative real time analysis, a probe-based technique was chosen with the VIC-TaqMan-primer/probes for LRP1B hybridization probes using TaqMan Gene Expression assay hs-00218582_m1 which had been previously optimized with in previous study of the group.[27] As housekeeping control primer/probes for TBP (TATA binding protein) were also used. For each reaction, (total volume of 10 µl), 9 µl master mix and 1 µl of cDNA were used. Reaction conditions were as follows: 50 cycles, each with 20 seconds at 55°C, 10 minutes at 95 ºC and 15 seconds at 95 ºC and finally 1 minute at 60 ºC. Reactions were run in triplicate in three independent experiments in a QuantStudio5 Real-Time PCR System (AppliedBiosystems). Non-template control (without cDNA) and "-RT" (using the -RT samples from section "cDNA Synthesis") were also included. The levels of mRNA LRP1B in the samples tested cancer cell lines were analysed using the following formula: $2^{-\Delta CT}$ (in which *CT* stands for "cycle threshold "and ΔCT = CT(LRP1B) – CT(TBP)).

2.3. Cell Treatments and Viability Analysis

2.3.1. Presto Blue Viability Assay

For the analysis of cell viability, the PrestoBlue™ (PB) was used. This assay allows to indirectly assess cell viability through the reducing power of living cells. Presto blue is a solution based in resazurin (permeable to the cells) that once modified by the reducing conditions of the cells environment and changes its colour (initially blue) into red colour.

For this, cells were plated in 100 µl complete medium into 96-well plates and incubated at 37ºC to adhere. Following 24h, 100 µl of fresh medium was added to each well and cells were further incubated for additional 24h, 48h or 72h. Cells were then washed three times with non-supplemented medium and then incubated with Presto Blue reagent solution (previously diluted to 10% in complete medium) for 45 min at 37ºC. Fluoresce signal was then analysed in multiplate reader Synergy HT Multi-Mode Microplate Reader (BioTek Instruments Inc.) using 560 nm as excitation wavelength and 590 nm as emission wavelength. Five technical replicates were analysed per sample. The average fluorescence values were determined for each condition (after removing the background values). Cell viability was determined as percentage in relation to blank cells (cells treated with medium only).

2.3.2. Drug sensitivity analysis

Cells (5 000 cells/well for A2780/OVCAR-8) were plated in 100 µl medium into 96-well plates and incubated for 24 h to adhere. Cells were then treated with five serial dilutions of doxorubicin (ranging from to 0.6 µg/ml) or liposomal doxorubicin (Caelyx, ranging from 0 to 75 µg/ml). Controls were also included consisting in: i) medium only (blank cells) and ii) medium containing equivalent volumes of drug vehicles (DMSO or H20, for doxorubicin and Caelyx, respectively). Five technical replicates were analysed per experiments (only three for controls). In addition, wells with medium or drug containing medium were included to remove background levels. Following 48h of incubation at cell culture incubator conditions (37 ºC; 5% CO_2), cell viability was determined using Presto Blue assay (as previously described in section “Cell viability

assays"). Dose-response curves were prepared and analysed at Excel Office (Microsoft) and GraphPadPrism 6.0 (GraphPad Software, Inc.).

2.4.Statistical Analysis

At least three independent experiments were carried out for each analysis, unless otherwise stated in the results section. Statistical analysis was carried out using the unpaired and paired Student's t-test using GraphPadPrism 6.0 (GraphPad Software, Inc.), differences were considered statistically significant whenever $P \leq 0.05$.

RESULTS AND DISCUSSION

Chapter III

3.1. Analysis of the Basal LRP1B mRNA Levels in Ovarian Cancer Cell Lines

As previously referred, the present study aimed at studying the role of LRP1B in the response of ovarian cancer cell lines to the treatment with liposomal doxorubicin.

Therefore, in this study A2780 (ovarian endometroid adenocarcinoma), Ovcar-4 (high grade ovarian serous adenocarcinoma) and Ovcar-8 (high grade ovarian serous adenocarcinoma) were used. OVCAR-8 cell line was already routinely cultured in the lab. A2780 and OVCAR-4 cell lines were also available at the lab but cryopreserved in frozen vials at -80ºC. At the beginning of this work, A2780 and OVCAR-4 cell lines were thawed and maintained in culture. To confirm their authenticity, DNA was extracted and typing processes were carried out at i3S Genotyping Service which allowed to simultaneously amplify and detect sixteen different loci [fifteen STR (short tandem repeats) and Amelogenin]. The information obtained from the i3S Genotyping service regarding the A2780 and OVCAR- 4 cells STR analysis (Table 1) was compared with reference published information available at ATCC – STR Database for human cell lines. Based on the homology of the STRs present in the cell lines when compared to the reference, the authenticity of the cell lines used was confirmed.

Table 1. Autosomal STR DNA profile of A2780 and OVCAR-8 cell lines – allele distribution (genotype) of 16 different loci of the DNA tested.

		A2780	**OVCAR-4**
STRs	**D3S1358**	15-16 (+14)	15
	TH01	6	9
	D21S11	28 (+27)	28-31 (+30)
	D18S51	16-17$^{(-)}$-18 (+15)	15
	Penta E	10-13	11
	D5S818	11-12	13
	D13S317	12-13	9
	D7S820	10	10-11
	D16S539	11-13 (+12)	11
	CSF1PO	10-11	10
	Penta D	8-9	12-14
	Amelogenin	XX	XX
	vWA	15-16	14-18
	D8S1179	15-16$^{(-)}$-17 (+14)(+18)	13
	TPOX	8-10	8
	FGA	19-23 (+18)(+22)	21

The levels of basal mRNA expression of LRP1B were then analysed by real-time quantitative PCR analysis. This methodology is considered to be the most sensitive among the methods of measuring gene expression, if the experiment is well-designed with the proper controls[33]. For the present work, a probe-based technique was chosen

with the hybridization TaqMan method. This strategy had been optimized and used in previous studies of the group[27].

The same protocol was followed in the same samples using specific VIC-TaqMan-primer/probes for the housekeeping gene (TBP) which allowed to normalize the quantity of LRP1B mRNA in each sample. After normalizing, results showed that A2780 cell line has the higher levels of LRP1B mRNA, comparing with OVCAR-4 and OVCAR-8 cells.

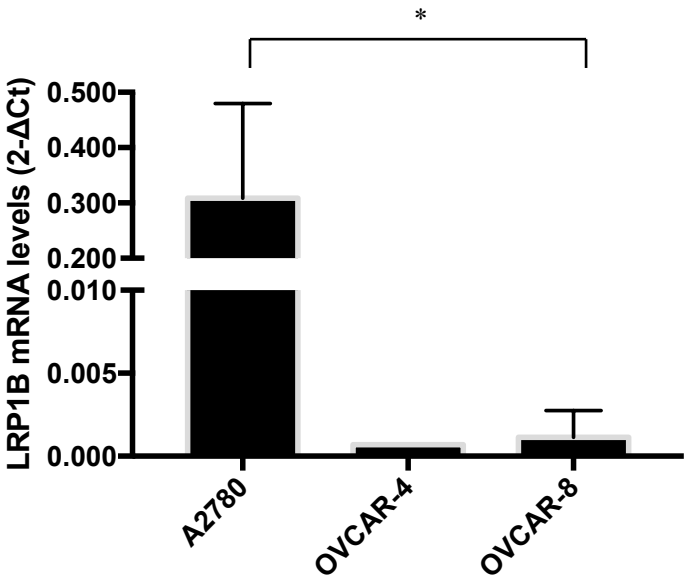


Figure 5. Relative levels of LRP1B mRNA in ovarian tumour cell lines.

Analysis of LRP1B mRNA expression levels were quantified by real-time-qPCR. The graph integrate data from four experiments for A2780 and OVCAR-8 cell lines and one experiment for OVCAR-4 cell line. Results are presented as mean ± STD. * indicates $p < 0.05$ = statistically significant.

3.2. Analysis of Response of the Ovarian Cancer Cells to Liposomal Doxorubicin

To assess the response of these cells to liposomal doxorubicin, PrestoBlue viability assay was performed allowing to indirectly assess cell viability through the reducing power of living cells. After treating cells for 48 hours with increasing concentrations of Caelyx (liposomal doxorubicin), a dose-response curve was obtained for each cell line.

Results showed that A2780 had increased sensitivity to Caelyx, when compared with the other two cell lines (Figure 6). In fact, the percentage of cell viability represented by A2780 in the first drug concentrations was lower than 50% when compared to the higher percentages (>60%) observed in the OVCAR (-4 and-8) cells.

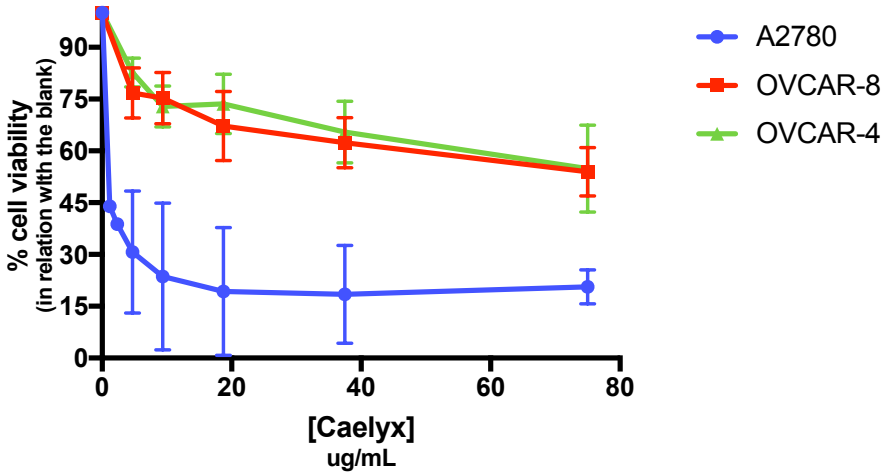


Figure 6. Dose-response curves for Caelyx in A2780, OVCAR-8 and OVCAR-4 cell lines.

Cells were treated for 48 h with increasing concentrations of Caelyx. Cell viability is expressed as percentage of cells in relation to blank (cells treated with medium only, 100%). Results are presented as mean ±STD, from at least 4 experiments. Statistically non-significant results.

When analysing the cell lines response to Caelyx at the light of the previously obtained results for LRP1B mRNA in the same cells, it is possible to observe that the cell line with increased LRP1B expression (A2780) was also the one that was more sensitive to Caleyx. This is in agreement with the hypothesis that LRP1B may contribute to the enhanced effect of liposomal doxorubicin as previously published in another study with high-grade serous cancer (HGSC)[8], in which lower mRNA expression levels of LRP1B showed reduced sensitivity to this drug.

3.2.1. Overexpression of LRP1B in Ovarian Cancer Cell Lines

To further assess the role of LRP1B in cell response to liposomal doxorubicin, LRP1B was overexpressed in the cell lines. For this, an LRP1B mini-receptor (mLRP1B) cloned into a pCDNA3.0 vector was used, as well as a pCDNA3.0 empty vector that was used as control. Transfections were carried out using a liposome-based reagent in the previously mentioned cell lines. A mLRP1B mini-receptor was used for two reasons: i) since this mini-receptor mimics the function and trafficking of LRP1B being composed by the entire cytoplasmatic tail, the trans-membrane region and extracellular sub-domain IV[19,27]; ii) due to the huge size of the LRP1B full lengths that does not allow for it successful transfection[19] into cell lines. In fact, previous studies demonstrated the instability of the full-length LRP1B cDNA after attempted the construction of a cell line stable expressing this full cDNA[19]. Additionally, even earlier reported studies of Liu *et al,* 2001 show that mini-receptors of several large cell surface receptors (LRP; Insulin-like growth factor receptor II) were successfully used for functional analysis. This genetic material was transferred into the cells by liposomal mechanisms in which the cargo of nucleic acid was released directly to the cytoplasm and the liposome was endocytosed by the cell, regarding the semi-permeability of the cell membrane.

In the present work, cells were transfected with Lipofectamine, a liposome-based reagent, and a vector (pCDNA3.0 mLRP1B or empty-vector) with a G418 resistance gene was introduced. Since transfection of the OVCAR-8 cell line with both these vectors (mLRP1B or empty-vector) had been previously carried out in the lab, in the present study, only the transfection and antibiotic selection of the cell lines A2780 and OVCAR-4 was carried out. A preliminary experiment assessed the increase in the levels of LRP1B in OVCAR-8 mLRP1B cells after more than 1 month in G418 selective pressure.

For A2780 cell line, it was possible to transfect cells with the vectors and obtain a pool of cells transfected and selected with G418 that initiated a regular cell proliferation and division in culture over the time. On the other hand, it was not possible to obtain OVCAR-4 transfected cells during the time of this study, although different strategies have been tested. Transfection was performed with three different transfection reagents and distinct concentration ratios of DNA. Additionally, cells were cultured with non-supplemented (only RPMI) and with supplemented growth medium (RPMI and

10%FBS). In some of these assays, the period of transfection was altered as the G418 selection was performed after 2-4 days or 4-7 days after the final step of transfection protocol. Despite all attempts, cells did not survive the selection process with G418, which indicated that transfection of OVCAR-4 cell line was not successful in this work.

The study proceeded using only the A2780 and Ovcar-8 transfected pools. The overexpression of LRP1B in the transfected cells was confirmed using quantitative real time PCR and determining the $2^{-\Delta Ct}$ which allows to calculate changes in gene expression as a relative fold difference among an experimental and calibrator sample. In A2780 cells, LRP1B expression was near fourfold higher in the mLRP1B transfected cells ($2^{-\Delta Ct}$ values of 4.17 ± 2.1) when compared to non-transfected cells ($2^{-\Delta Ct}$ values of 0.308 ± 0.17). Nevertheless, an increase was also observed in LRP1B mRNA levels in cells transfected with the empty vector ($2^{-\Delta Ct}$ values of 0.78 ± 0.6), although not comparable to the one observed with mLRP1B expression vector.

In what concerns the OVCAR-8 cell line, it was also observed an increase (almost for the double) in the LRP1B mRNA expression levels in the cells transfected with mLRP1B ($2^{-\Delta Ct}$ = 1.27 ± 0.4) when compared to the levels of wild-type cells or to the cells transfected with empty vector (in which LRP1B was almost undetected under the conditions used).

When comparing the two cell lines, it may be seem that transfection of A2780 was more successful in what concerns the amount of LRP1B levels obtained in relation to wild-type or empty-vector cells. Nevertheless, the fact that in OVCAR-8, the basal levels of LRP1B in wild type and empty vector cells is very low/ undetected does not allow to fully compare the increase in the LRP1B expression ratios between the two cell lines.

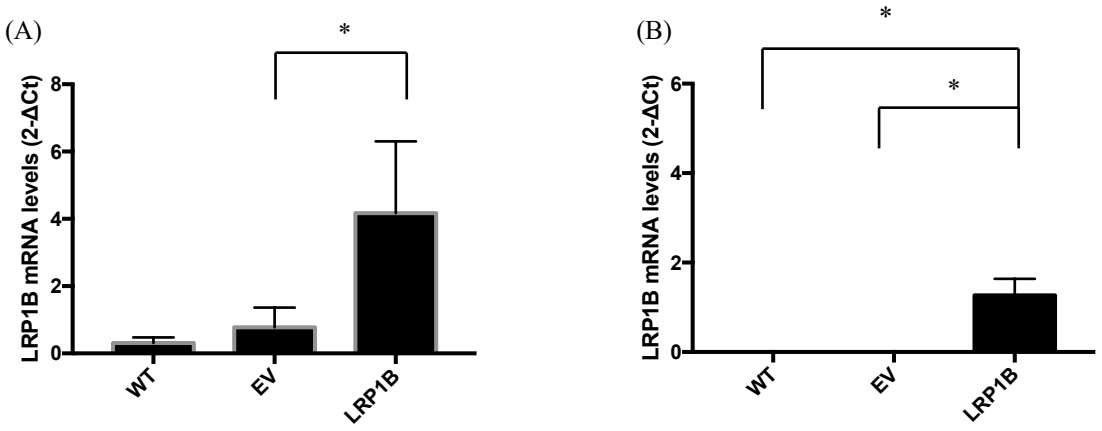


Figure 7. LRP1B mRNA levels in ovarian cancer cells transfected with mLRP1B expression vector.

A2780 (A), OVCAR-8 (B) cells were transfected with pcDNA3.0 (empty vector, EV) or with mLRP1B-pcDNA 3.0 (LRP1B). LRP1B levels were quantified by real-time-qPCR**.** Expression values were normalized to the endogenous control (HPO). Results are presented as mean ±STD of at least 3 independent experiments. * indicates $p< 0.05$ = statistically significant.

3.2.2. Cells Morphology and Cell Density Evaluation

By observing cells overexpressing LRP1B over time in culture, no clear alterations were noted in the cells morphology when compared to the blank or empty vector cells. This was true for both cell lines.

However, slight variations on cell density were observed: concerning A2780 cell line, cells transfected with mLRP1B and wild-type cells demonstrated a slightly increase on growth ratio than cells transfected with empty vector; regarding OVCAR-8 cell line, cells transfected with mLRP1B and empty vector showed either a slightly increase on growth ratio than wild-type cells.

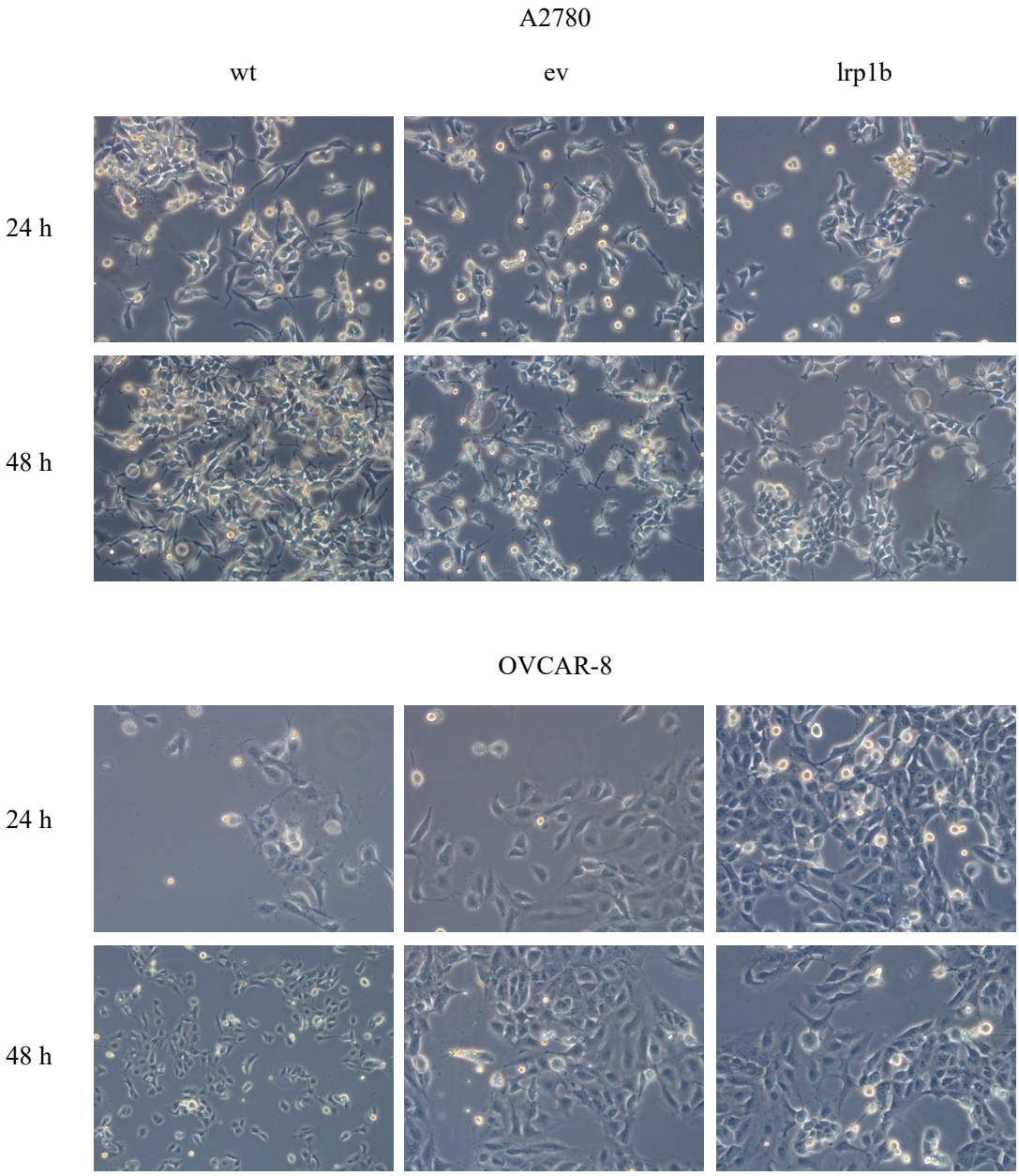


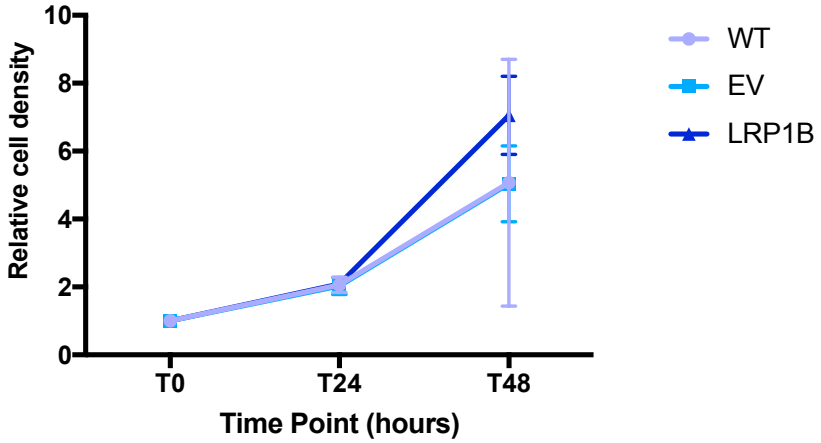
Figure 8. Cells were passaged into a new flask and maintained in culture. Representative images were taken to determine possible phenotype modifications. Images correspond to 20x of magnification.

Considering these observations which suggested a different growth rate between the cell lines, Presto-Blue viability assays were carried out at different time points (24h, 48h and 72h).

When analysing the growth curves obtained (Figure 9A/9B) for both cell lines, no differences were revealed, at similar cell density, over the time-points selected and among

the transfected and non-transfected cells. These results do not fit with what had been previously observed by microscopy, in which transfected cells seemed to grow more than the wild-type cells, although with the limitations underlying the accuracy and subjectivity of microscopy observation.

(A)



(B)

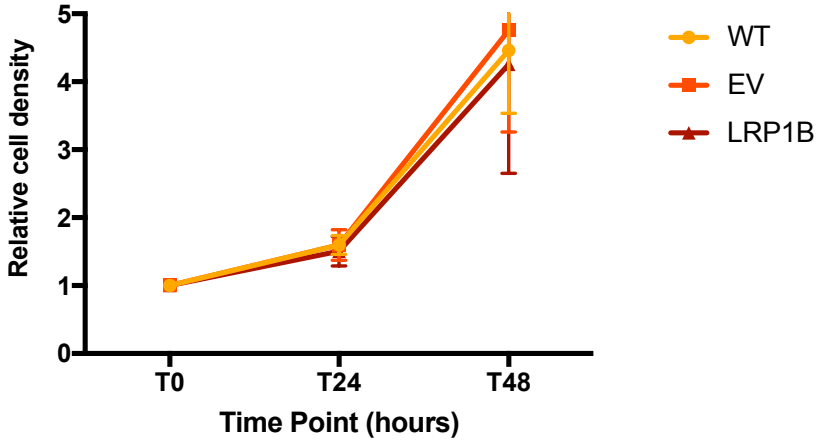


Figure 9. Cell density curves for A2780 (A), OVCAR-8 (B) WT cells and cells transfected with pcDNA3.0 (empty vector, EV) or with mLRP1B-pcDNA 3.0 (LRP1B).

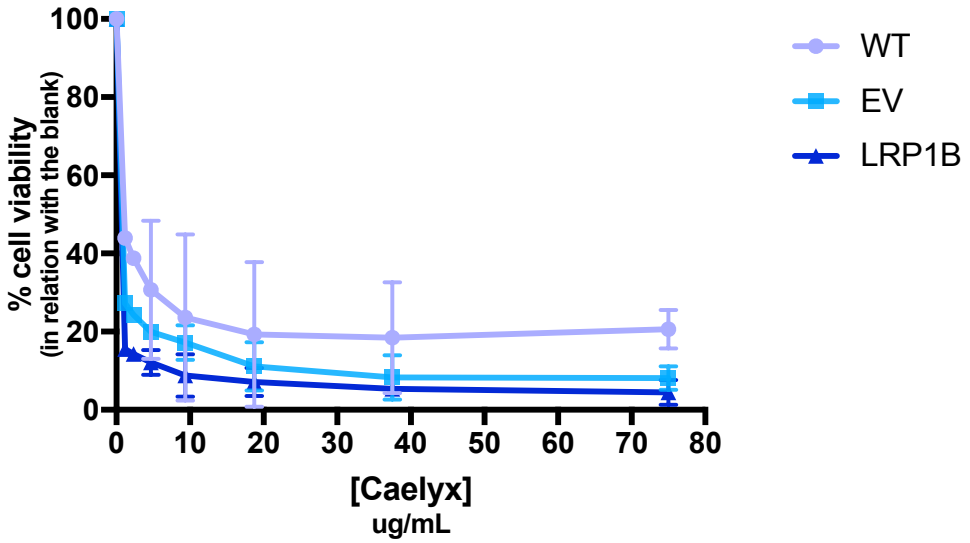
Cells density was analysed at 24h (T0), 48h (T24) and 72h (T48) in culture. Results were analysed in relation to the respective 0 hours (T0) and are presented as mean ± STD, from at least 4 experiments. Statistically non-significant results.

3.2.3. Response to LRP1B Expressing Cells to Liposomal Doxorubicin

In order to confirm that LRP1B affects the cell sensitivity to liposomal doxorubicin, cells response to liposomal doxorubicin according to LRP1B overexpression was analysed. For that, cell lines were analysed within 48h of drug treatment using the PrestoBlue viability. Moreover, the response to non-liposomal doxorubicin was also studied.

Analysing the response of A2780 cells (Figure 10A) to liposomal doxorubicin, it was possible to observe that wild-type cells showed to be less sensitive than the LRP1B transfected cells, as expected. Nevertheless, there was also a clear increase in the sensitivity of the cells transfected with the empty-vectors. This increase in the response is similar to the LRP1B transfected cells, although a (not statistically significant) slight difference may be observed at lower concentrations. As previously referred in the section 3, transfection of A2780 cells with empty vector also presented an increase in LRP1B mRNA levels. This may possible be related with the increase in sensitivity observed in this case. As expected, the response of the cells to free doxorubicin was similar in transfected cells lines (both with empty and LRP1B vector), although in both cell lines transfected cells were more sensitive than WT cells (probably related with effect in transfection).

(A)



(B)

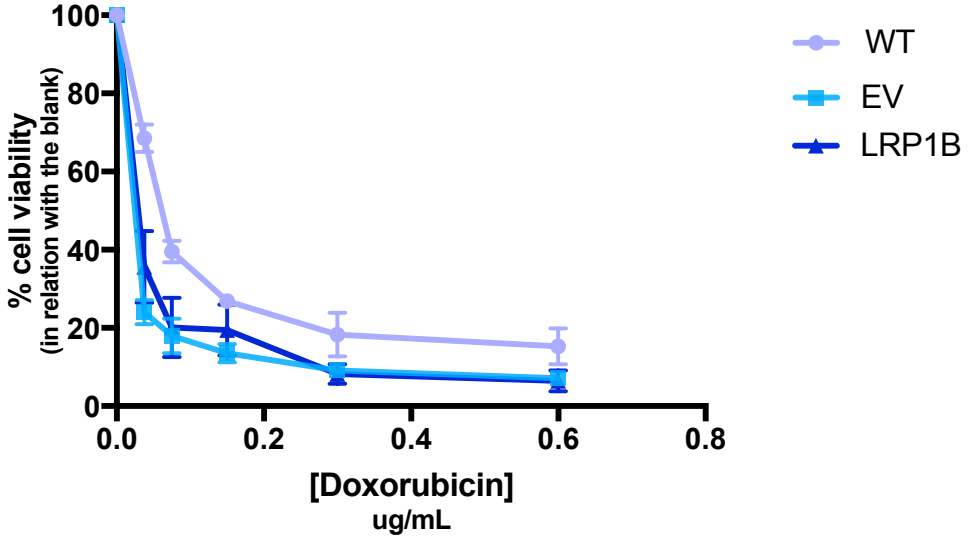


Figure 10. (A) Dose-response curves for Caelyx in A2780 cell line. (B) Dose-response curves for non-liposomal Doxorubicin in A2780 cell line.

Cells were treated for 48 h with increasing concentrations of Caelyx (A) or non-liposomal Doxorubicin (B). The growth inhibition effects were expressed in percentual terms, in relation to cells treated with medium only (, blank (100%). Results are presented as mean ± STD, from at least 4 experiments. Statistically non-significant results.

Regarding the response of Ovcar-8 cells, a similar dose-response was obtained for all cells. In particular cells transfected with mLRP1B did not demonstrate higher sensibility to liposomal doxorubicin. Likewise, response to non-liposomal doxorubicin was also the same.

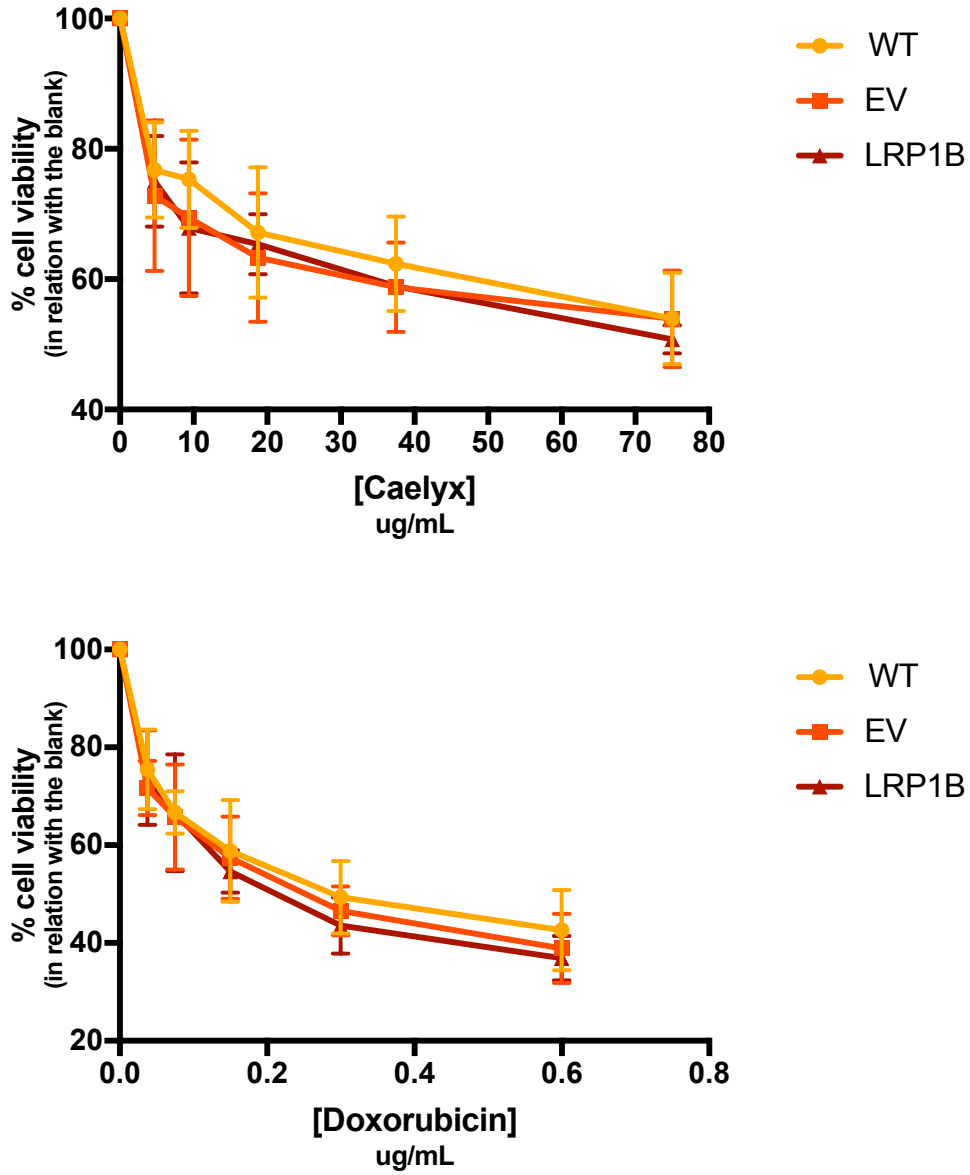


Figure 11. (A) Dose-response curves for Caelyx in OVCAR-8 cell lines. (B) Dose-response curves for non-liposomal Doxorubicin in OVCAR-8 cell line.

Cells were treated for 48 h with increasing concentrations of Caelyx (A) or non-liposomal Doxorubicin (B). The growth inhibition effects were expressed in percentual terms, in relation to treated with medium only , blank (100%). Results are presented as mean ± STD, from at least 4 experiments. Statistically non-significant results.

In summary, we observed distinct effects of mLRP1B overexpression in the response of A2780 and OVCAR-8 cells to liposomal doxorubicin. This can be related with the differential cell growth detected in A2780 cells transfected with mLRP1B (Figure 9A). However we must stress that the response of A2780 cells to free doxorubicin was similar in transfected cells lines (both with empty and LRP1B vector), indicating a specific increased sensitivity of mLRP1B transfected cells to liposomal drug delivery.

3.2.4. Isolation of Clones Overexpressing LRP1B

The previous results were obtained using a pool of transfected cells in culture. Since transfection efficiency and LRP1B levels may vary among the cells of the pool, it would be important to isolate clones in order to obtain a homogeneous LRP1B expression and thus assess more rigorously the influence of the LRP1B presence in the chemotherapeutic treatment experiments. For this, different clones were isolated, yet maintained in selection with G418. Cells were highly diluted and plated. Only clones resulting from a single cell, which were able to grow under G418 selection, were selected and the levels of LRP1B expression by real time PCR evaluated (Figure 12).

(A) (B)

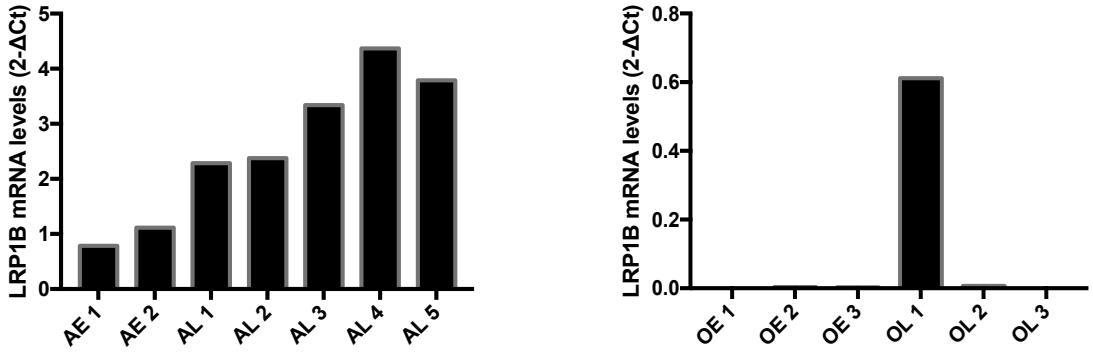


Figure 12. LRP1B mRNA levels in clones of ovarian cancer cells (A2780 (A); OVCAR-8 (B)) transfected with pcDNA3.0 (E - representing cells transfected with empty) or with mLRP1B-pcDNA 3.0 (L – representing cells transfected with mLRP1B). LRP1B levels were quantified by real-time-qPCR. Expression values were normalized to the endogenous control (HPO). Results are presented as mean ±SEM of at least 3 independent experiments. Statistically non-significant results.

Regarding A2780 cells, 7 clones were selected for analysis (2 with empty vector and 5 with mLRP1B). Overexpression of LRP1B levels was confirmed in all mLRP1B transfected clones, and the levels of expression (determined with $2^{-\Delta Ct}$ ranged from 2.3 to

4.1) are within the range of the LRP1B expression in the pool of cells previously analysed (Figure 7A). Considering the empty-vector transfected clones, the LRP1B expression levels obtained ($2^{-\Delta Ct}$ ranging from 0.78 to 1.11) were in accordance with the previous result acquired with the pool of transfected cells ($2^{-\Delta Ct}$ = 0.78 +/- 0.5). Also, according to the conclusions of the previous results, A2780 demonstrate to be higher sensitive to drug treatments and it is curious that with this cell line we observed a higher expression of the gene in several clones.

From the 6 selected clones of OVCAR-8 cells (3 from empty vector and 3 from LRP1B), only one of the mLRP1B transfected clones presented LRP1B overexpression. (Figure 12B)

Moreover, two of the OVCAR-8 mLRP1B clones show low levels of LRP1B expression (almost null). The low level of gene expression revealed by clones of OVCAR-8 transfected cells can be correlated with, for example, a poor transfection efficiency or silencing of the expression of the transgene by the cells. Additionally, the low sensitivity in the dose-response studies of OVCAR-8 cells (poll of cells) can be explained by this low levels of gene expression noticed in clonal cultures.

During the clone isolation procedure, other clones were obtained for both cell lines, nevertheless due to time limit constrains it was not possible to analyse and confirm the LRP1B levels in these clones.

Generally, we can hypothesise that the differential response found in A2780 and OVCAR-8 mLRP1B transfected pools of cells to liposomal doxorubicin, is directly related with the highly heterogeneous level of expression of LRP1B found at the single cell level (clones). Additional studies, testing the sensitivity of the selected single clones to liposomal doxorubicin will be necessary to disclose the role of LRP1B expression in response to the drug.

CONCLUSIONS

Chapter IV

Advances on the knowledge of cancer associated pathways and molecules, added to the biotechnological development as led novel cancer therapies to emerge. Not only numerous anticancer drugs exist (cytotoxic conventional drugs or targeted drugs, used alone or in combination) but also there are now available several drug carriers, in particular liposomes, which allow to overcome the chemotherapy limitations, improving bioavailability and stability of the drugs and reduce side effect.

Several liposomal anticancer drugs are being study with some of them being already used in the clinic. In particular, liposomal doxorubicin, the first liposomal anticancer drug described, is frequently used for the treatment of several cancer types including metastatic ovarian cancer. Nevertheless, a high number of patients do not respond to drugs that often cause toxic side effects and increased morbidity.

Therefore, there is the need to identify markers that influence the response of cancer cells to liposomal therapies increasing the efficiency of these drugs.

LRP1B, described as a putative tumour suppressor gene, is among the most altered genes in human cancers[4,17]. It belongs to the Low-Density Lipoprotein Receptor (LDLR) family and is involved in the endocytosis of several ligands and to be involved in signaling pathways associated with cell migration and differentiation. Importantly, LRP1B may also affect the internalization of drugs, through liposomes[7]. In a single study by Cowin *et al.* (2012), decreased *LRP1B* expression was shown to increase ovarian cancer cells resistance to liposomal therapy, describing LRP1B as playing a role in the resistance of high-grade serous ovarian cancer patient to liposomal doxorubicin.

The aim of the present study was to further evaluate the effect of LRP1B expression in ovarian cancer tumour cells response to liposomal doxorubicin (frequently used in ovarian cancer therapy).

To achieve this, three different human ovarian cancer cell lines were used: A2780, OVCAR-4 and OVCAR-8.

Comparison of the basal LRP1B levels of the cells was possible by analysing the respective mRNA by quantitative real time PCR. Results obtained showed that A2780 presented the higher levels of LRP1B, followed by OVCAR-8 and finally by OVCAR-4 (almost undetected). Although, ideally, the levels of protein should have been assessed, the lack of a reliable antibody for LRP1B did not allow it.

Importantly, when comparing the response of these cell lines to liposomal doxorubicin treatment a correlation with the levels of LRP1B was observed with A2780 (higher levels of LRP1B mRNA) being more sensitive to the drug than the other two cell lines (lower levels of LRP1B mRNA). This was in agreement with what had been previously described by Cowin *et al.* (2012) in which a decrease in the expression levels of LRP1B in ovarian cancer cells demonstrated an increase of the resistance by these cells to liposomal-doxorubicin treatment.

To evaluate the role of LRP1B in this response, a vector expressing LRP1B was transfected into cells lines. Instead of the total LRP1B (for which the construction of stable cell lines expressing the full-length is not possible[19]), a mini-receptor (mLRP1B, which mimics LRP1B endocytic activity) was used. This approach is commonly used for several large cell surface receptors (LRP; Insulin-like growth factor receptor II) and approved for functional analysis[19].

Although, mini-receptors do not represent all biological functions of the full-length form[3]**,** it was reported by a recent study a well-succeeded construction and expression of a transfection vector containing the 13.800 bp full-length murine LRP1B cDNA, in human tumour cells, confirming the same responses as the ones previously obtained with the mini-receptor[3]. Likewise, this reported construction can represent a powerful tool to disclose potential functions of LRP1B and its total biologic features[3].

Transfections carried out in this study were in general well-succeeded, with selection of A2780 and OVCAR-4 overexpressing LRP1B pools (as confirmed by real time PCR). The exception was the OVCAR-4 cell lines, which did not survive the selection treatments. Although a study by Cowin *et al.* (2012) had demonstrated positive results of transfections in the same cell line, with the same transfection reagent, in this study it was not possible to reproduce it.

A limitation of our study is related with the absence of protein expression analysis to further confirm the results obtained at the mRNA level in the present work, however finding specific antibodies that recognize the entire length, or even mLRP1B as a matter of fact, was not well-succeeded.

Analysing cell growth of the transfected cells it was possible to observe that A2780 overexpressing mLRP1b presents a faster growth than WT or EV cells. This differences

on growth ratios are also observed at the Cowin *et al.* (2012) studies. For the OVCAR-8 cell line no differences were observed.

Regarding the response to liposomal doxorubicin, A2780 LRP1B overexpressing cells seemed to be slightly more sensitive to liposomal treatment (at lower concentrations). OVCAR-8 over-expressing mLRP1B cells did not show differences in the response to Caelyx. Curiously, the only (although slight) difference was observed in the cell line in which LRP1B is higher at basal levels. Regarding free doxorubicin treatment the response of mLRP1B overexpressing cells (for both cell lines) did not demonstrate differences when compared with WT or EV pools.

With these results, we raised the hypothesis that transfection efficiency and LRP1B overexpression could have been not equally achieved in all cells lines (since we were using the pool of selected cells over time), therefore affecting the observed effect of LRP1B expression in the response to Caelyx. Consequently, isolation of clones of cell lines over-expressing mLRP1B was attempted by dilution of cells and their levels of LRP1B analysed. During the time of this work, it was possible to isolate and analyse some of these clones. Interestingly, all the A2780 clones selected from mLRP1B expression vector, overexpressed LRP1B while only 1 out of 3 clones isolated from OVCAR-8 mLRP1B vector showed an overexpression of LRP1B. Although the number of clones analysed for OVCAR-8 is low, it may probably already indicate that LRP1B is less expressed in the pool of cells initially analysed in the drug treatment assays. Future studies using these isolated clones (expressing higher levels of LRP1B) may have an impact on the response to liposomal treatment.

Overall, the results presented did not allow to fully conclude on effect of LRP1B in response of cancer cells to liposomal drugs. Nevertheless, the work developed during this period, particularly the clones obtained overexpressing LRP1B will be an important tool to the additional work regarding confirmation of previous studies. Moreover, additional studies involving assays of gene silencing (SiRNA/CRISPR) or experiments for apoptosis detection (TUNEL assay) can also represent future results to understand the function of LRP1B in response of cancer cells to therapy.

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Chapter V

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ANNEX I

LRP1B endocytic activity: possible implications in the uptake of liposomal anticancer drugs

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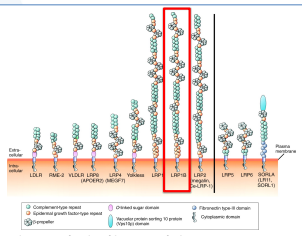
Background

The low-density Lipoprotein Receptor-related Protein 1B (LRP1B) belongs to the Low-Density Lipoprotein Receptor superfamily, scavengers for multiple ligands and major functional mediators of endocytosis [1]. Although LRP1B has been described has a putative tumour suppressor, being amongst the 10 most significantly deleted genes in human cancers [2], the exact role of LRP1B in cancer is still not fully disclosed.

LRP1B binds to several ligands, activating extracellular proteolytic cascades and regulating adhesion, motility and invasion. LRP1B re-expression in cancer reduced cell proliferation, colony formation and tumourigenicity *in vitro* and *in vivo*. We have previously presented an alternative/complementary role for LRP1B endocytic activity, as modulator of cancer secretome, through depletion of soluble factors critical for tumour invasion/progression [3].

LRP1B endocytic activity may have impact in the uptake of liposomal drugs [4]. LRP1B deletion/downregulation was shown to significantly correlate with resistance to liposomal doxorubicin in ovarian cancer patients [5].



Structural organization of members of the LDL receptor family.

The extracellular domains contain clusters of complement-type repeats that are the site of ligand binding. The cytoplasmic tails harbor recognition sites for adaptor proteins that are involved in protein trafficking and signal transduction.
The cytoplasmic domains are unique, indicating distinct cellular fates for ligands internalized by individual receptors.
APOER2, apolipoprotein E receptor 2; Ce, C. elegans; LDLR, low-density lipoprotein receptor; LRP, LDL receptor-related protein; MEGF7, multiple epidermal growth factor-type repeat containing protein 7; RME-2, receptor-mediated endocytosis-2; SORLA, sortilin-related receptor with A-type repeats; VLDLR, very low-density lipoprotein receptor. *Adapted from [6]*

AIM :

To develop human tumour cell lines (derived from thyroid, melanoma, urothelial and ovarian cancer) with LRP1B overexpression and analyse their response to liposomal doxorubicin.

Results

1. LRP1B mRNA expression in human tumor cell lines

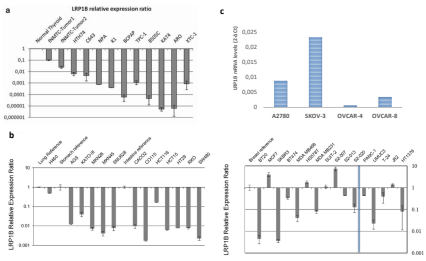


Figure 1. LRP1B expression levels in different cancer cell line models, analysed by real time RT-PCR. a) FNMTC tumors and thyroid cancer cell lines [3] **b)** Other cell line models and **c)** Ovarian cancer cell lines. Expression values were normalized to the endogenous control (HPO) and divided by the relative expression of normal thyroid sample, lung or breast reference as indicated (**a**,**b**) or values normalized to the endogenous control (TBP)(**c**).

2. Response of ovarian cancer cell lines to liposomal doxorubicin

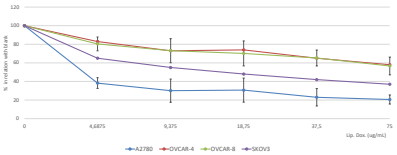


Figure 2. Response of ovarian cancer cell lines to liposomal doxorubicin, analysed with Presto Blue assay. Cells were treated for 48h with increasing concentrations of liposomal doxorubicin. Viability is expressed as % of control cells (with medium only). Results are expressed as mean ± STD of 3 independent experiments (except for SKOV-3). Vehicle solvent (H_2O) had no effect (*data not shown*).

➢ **Ovarian cancer cells with higher LRP1B expression were more sensitive to liposomal doxorubicin**

3. LRP1B overexpression in human tumor cell lines

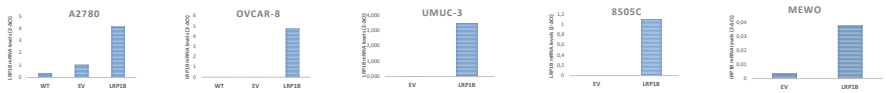


Figure 4. LRP1B expression levels of LRP1B-transfected cell line models, analyzed by real time RT-PCR. Melanoma (Mewo), anaplastic thyroid cancer (8505c), urothelial cancer (UMUC3) and ovarian cancer (A2780; OVCAR-8). Cells were transfected with pcDNA3.0- m*LRP1B* expressing vector (LRP1B) or with the empty vector (EV) and selected with antibiotic. Expression values were normalized to the endogenous control-TBP. Results are the mean of 3 independent experiments except 8505C and MEWO.

4. Response of LRP1B-overexpressing cells to liposomal doxorubicin

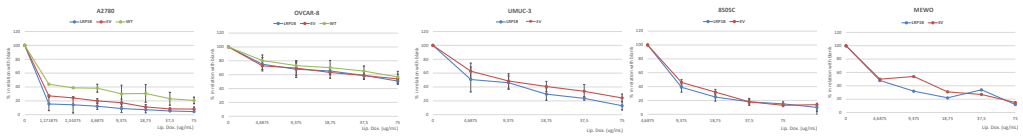


Figure 5. Response of cancer cell lines overexpressing LRP1B to liposomal doxorubicin, analysed with Presto Blue assay. Cells were treated for 48h with increasing concentrations of liposomal doxorubicin. Viability is expressed as % of respective non-treated cells. Results are expressed as mean ± STD of 3 independent experiments (except for MEWO). Vehicle solvent (H_2O) had no effect (*data not shown*).

Conclusion

Future studies will be carried out to validate the role of LRP1B's endocytic activity in the response of tumour cells to liposomal therapy and its use as a predictive marker to liposomal formulations of anticancer drugs.

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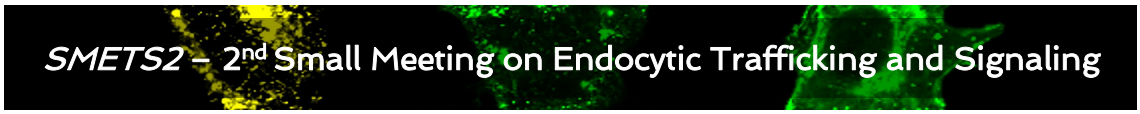

CERTIFICATE OF ATTENDANCE

This is to certify that Inês Saraiva,

has presented a poster untitled "*LRP1B endocytic activity: possible implications in the uptake of liposomal anticancer drugs*" in the 2nd Small Meeting on Endocytic Trafficking and Signaling held July 10-12, 2018 at the International Iberian Nanotechnology Laboratory (INL) in Braga, Portugal.

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George Diallinas |Greece
Peter Nagy |Hungary
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