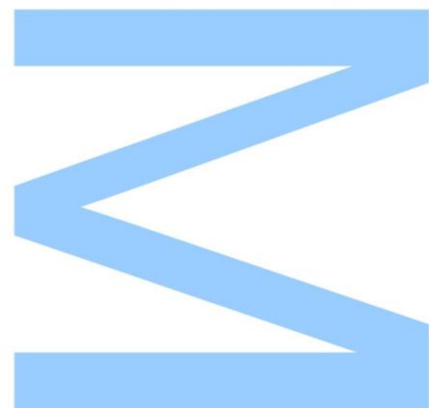
Evidence for a role of autophagy in the release of extracellular vesicles by tumour cells: possible implications for drug resistant cells with impaired autophagy



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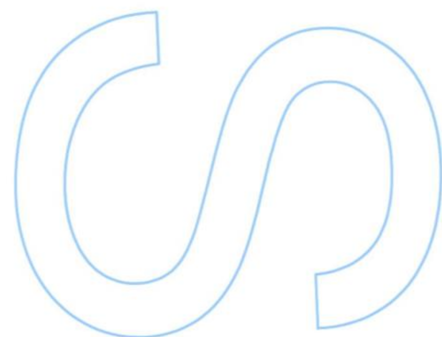

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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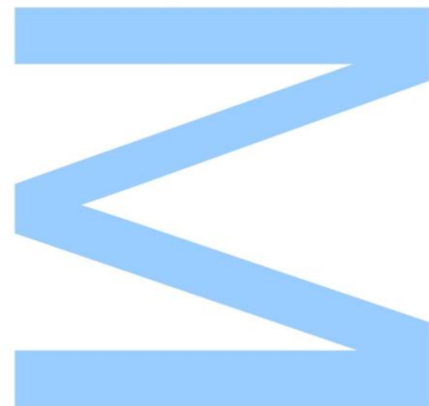

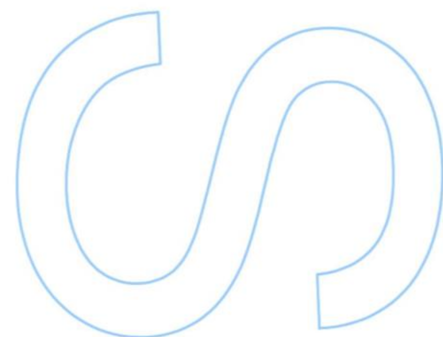

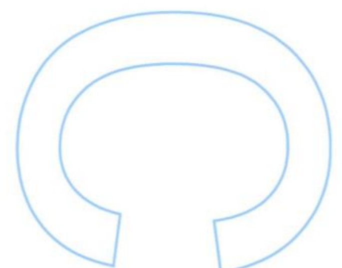

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"The man who moves a mountain begins by carrying away small stones."

-- Confucius, "The Analects"

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Abstract

Cancer is one of the most concerning public health issues, and cancer therapy is often hindered by multidrug resistance (MDR). Previous studies performed by this research group demonstrated that MDR tumour cells release larger EVs when compared to their drug-sensitive (DS) counterpart cells. In addition, it is accepted that EVs may be horizontally transferred from MDR to DS tumour cells, inducing a MDR phenotype in the recipient cells.

Therefore, the main aim of this work was to confirm the release of larger EVs by P-gp overexpressing MDR cells, when compared to their drug sensitive counterparts, and to further investigate the possible relationship between the release of EVs and cellular autophagy. In addition, due to the need to identify new molecules with dual anti-tumour and anti-P-gp activity, another aim was to test several quinazolinone derivatives for their potential effect as inhibitors of growth of tumour cell lines and as inhibitors of P-gp activity.

Two pairs of drug-sensitive and MDR counterpart cell lines were used: the leukemia (K562 – sensitive and K562Dox – resistant), and the non-small cell lung cancer (NCI-H460 – sensitive and RH460 – resistant) cell lines. EVs released by those cells (in the presence and absence of imipramine or 3-MA) were isolated and characterized regarding their size and content in EVs markers. The inhibition of the release of microvesicles using imipramine, had different effects in the two counterpart pairs of NCSLC and leukemia cell lines used. Moreover, 3-MA surprisingly led to an increase in the number of EVs shed by drug sensitive cells but not by the MDR NSCLC counterpart cells. To our knowledge, this is the first report of a different effect of 3-MA on the release of EVs by MDR or DS counterpart cells. Since 3-MA is an autophagy inhibitor, it was hypothesised that there could be differences in the autophagy levels between the counterpart cell lines which could be related to the verified difference in the release of EVs. Therefore, additional studies were conducted to assess autophagy levels between these counterpart cell lines. Data obtained suggests that the MDR cell line has an impaired autophagic flux, while the DS cell line has a functional autophagy, suggesting that there is a possible relation between autophagy and EVs release.

Regarding the tests of several quinazolinone derivatives, none of the compounds presented potent antitumour effect in the three cell lines studied, nor an inhibitory effect on the activity of P-gp.

Keywords: EVs; MDR; drug-resistance; P-gp; drug-efflux; 3-methyladenine; imipramine; autophagy; apoptosis; antitumour; drug screening; anti-P-gp activity

Resumo

O cancro é um dos problemas mais preocupantes a nível de saúde pública, sendo a sua terapia frequentemente dificultada pela resistência a múltiplos fármacos (MDR). Estudos realizados anteriormente neste grupo de investigação demonstraram que células tumorais MDR libertam vesículas extracelulares (EVs) de maiores dimensões, comparativamente às suas células homólogas sensíveis a fármacos (DS). Além disso, sabe-se que as EVs poderão ser transferidas horizontalmente das células tumorais MDR para as células tumorais DS, induzindo um fenótipo de MDR nas células recipientes.

Assim, o principal objetivo deste trabalho foi confirmar a libertação de EVs de maiores dimensões pelas células MDR que sobre-expressam a glicoproteína-P (P-gp), quando comparadas com as suas células homólogas sensíveis a fármacos. Pretendeu-se ainda investigar a possível relação entre a libertação de EVs e a autofagia celular. Adicionalmente, devido à necessidade de identificar novas moléculas com dupla atividade anti-tumoral e anti-P-gp, outro objectivo foi testar vários compostos derivados da quinazolinona quanto ao seu potencial efeito como inibidores do crescimento de linhas celulares tumorais e inibidores da actividade da P-gp.

Dois pares de linhas celulares homólogas, sensíveis a fármacos e MDR, foram utilizados: as linhas celulares leucémicas (K562 – sensíveis e K562Dox – resistentes), e a as linhas celulares de cancro do pulmão de não-pequenas células (NSCLC) (NCI-H460 – sensíveis e RH460 – resistentes). As EVs libertadas por estas células (na presença e ausência de imipramina ou 3-metiladenina, 3-MA) foram isoladas e caraterizadas relativamente ao seu tamanho e conteúdo em marcadores de EVs. A inibição da libertação de microvesículas, com imipramina, teve diferentes efeitos nos dois pares de linhas celulares homólogas de NCSLC e de leucemia. Além disso, o 3-MA surpreendentemente levou a um aumento do número de EVs libertadas pelas células NCSLC sensíveis a fármacos, mas não pelas suas células homólogas MDR. Que seja do nosso conhecimento, esta é a primeira vez que é reportado um efeito diferente do 3-MA na libertação de EVs pelas células MDR ou pelas suas células homólogas DS. Uma vez que o 3-MA é um inibidor da autofagia, levantou-se a hipótese de que poderiam haver diferenças entre os níveis de autofagia das linhas celulares homólogas, o que poderia estar relacionado com a diferença verificada na libertação de EVs. Assim, estudos adicionais foram realizados para analisar os níveis de autofagia entre as linhas celulares homólogas. Os resultados obtidos sugerem que a linha celular MDR tem o

fluxo autofágico comprometido, enquanto a linha celular DS tem autofagia funcional, sugerindo a existência de uma possível relação entre a autofagia e a libertação de EVs. No que diz respeito aos testes dos derivados de quinazolinonas, nenhum dos compostos apresentou potente efeito anti-tumoral nas três linhas celulares estudadas, nem efeito inibitório na atividade da P-gp.

Palavras-chave: EVs; MDR; resistência a fármacos; glicoproteína-P; efluxo de fármacos; 3-metiladenina; imipramina; autofagia; apoptose; antitumoral; pesquisa de compostos; atividade anti-P-gp

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List of Abbreviations (and Symbols)

3-MA	3-methyladenine
5-FU	5-fluorouracil
ABC	ATP-binding cassette
ADME	Absorption, Distribution, Metabolism, and Excretion
APAF1	Apoptotic protease-activating factor 1
Barkor	Beclin 1-associated autophagy-related key regulator
BCRP	Breast Cancer Resistance Protein
BER	Base Excision Repair
BH	Bcl-2 Homology
CDR	Cancer Drug Resistance
CML	Chronic Myelogenous Leukemia
CYP	Cytochrome P450
DLS	Dynamic Light Scattering
DMSO	Dimethyl sulfoxide
DS	Drug-sensitive
EBSS	Earle's Balanced Salt Solution
EMT	Epithelial-to-Mesenchymal Transition
ER	Endoplasmic Reticulum
ESCRT	Endosomal Sorting Complexes Required for Transport
EV	Extracellular Vesicle
FBS	Fetal Bovine Serum
FIP200	Focal adhesion kinase family interacting protein of 200 kDa
GI_{50}	Concentration that causes 50% inhibition of cell growth
GSH	Glutathione
GST	Glutathione transferase
HBSS	Hank's Balanced Salt Solution
HER2+	Human Epidermal growth factor Receptor 2-positive
HR	Homologous recombination
IAP	Inhibitor of apoptosis protein
IC_{50}	Inhibitory concentration of 50%
ILV	Intraluminal vesicle
lEV	large EV
lncRNA	long non-coding RNA

MDR	Multidrug resistance
MMR	Mismatch repair
MOMP	Mitochondrial Outer Membrane Permeabilization
MRP1	Multidrug resistance-associated protein 1
mTOR	mammalian Target of Rapamycin
MVB	Multivesicular body
NAT	Arylamine N-acetyltransferases
NBD	Nucleotide Binding Domain
ncRNA	non-coding RNA
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End Joining
NSCLC	Non-Small Cell Lung Cancer
NTA	Nanoparticle Tracking Analysis
PBS	Phosphate-Buffered Saline
PE	Phosphatidylethanolamine
P-gp	P-glycoprotein
PI3K	Phosphatidylinositol-3-phosphate kinase
PI3P	Phosphatidylinositol-3-phosphate
Rh123	Rhodamine-123
RT	Room Temperature
sEV	small EV
siRNA	Small interfering RNA
SLC	Solute Carrier
SMAC	Second Mitochondria-derived Activator of Caspase
sncRNA	small non-coding RNA
SQSTM1	Sequestosome 1 (also known as p62)
SRB	Sulforhodamine B
TCA	Trichloroacetic Acid
TfR	Transferrin Receptor
TMD	Transmembrane Domains
UGT	UDP-glucuronosyltransferase
ULK1	unc-51-like kinase 1
UVRAG	UV radiation-associated resistance gene
Vps34	Vesicular sorting protein 34
WB	Western Blotting

Introduction

1. Cancer and Therapies

Cancer is a major public health issue, with a substantial increase in the past decades, being the second leading cause of death in a global scale. In 2012, 14.1 million new cases have occurred [1], a number that is expected to increase in the following years.

Cancer develops when normal cells start to grow in an uncontrolled manner, due to sequential accumulation of defects in their DNA, leading to a stepwise process called carcinogenesis. Carcinogenesis can be seen as a process of micro-evolution, in which, by accumulative mutations, cells acquire a selective advantage [2]. This process is mediated by four steps: cell transformation, growth of transformed cells, local invasion and distant metastasis, ultimately giving rise to a malignant tumour. Eventually, this allows cancer cells to sustain chronic proliferation, evade growth suppressors, activate invasion and metastasis, enable replicative immortality, induce angiogenesis and resist to cell death [3].

Nowadays, tumours are known to be more than a simple isolated mass of proliferating cells, being not only composed by tumour cells, but also stromal cells. This stroma is part of what is known as tumour microenvironment, contributing to tumour growth and being responsible for certain hallmark capabilities. This microenvironment is thought to help in the selection of adaptive mutations and increased aggressiveness, since it is a harsh environment containing low levels of oxygen and nutrients [4]. Such knowledge gave rise to two new emerging hallmarks of cancer: the deregulation of cellular energetics and the capability to avoid immunological destruction, effectively supporting neoplastic proliferation [3].

The mutations that promote tumour growth generally occur in two types of genes: growth-promoting proto-oncogenes – encoding proteins involved in cell growth and proliferation; and growth-inhibiting tumour suppressor genes, which leads to a loss of function predisposing the cell to a cancer phenotype [3]. Due to the genomic instability and high rate of mutations that cancer cells present, their replication originates several cell subpopulations, each one with different phenotypes. This is one of the main reasons why cancer treatment can be extremely difficult. Interestingly, Bruce Alberts states in his book, Molecular Biology of the Cell, that "the difficulty of curing a cancer is similar to the difficulty of getting rid of weeds" [5].

Nowadays, there are several available cancer treatments, such as: surgical removal of the neoplastic mass, radiotherapy, conventional chemotherapy with cytotoxic drugs, targeted therapy, immunotherapy, and hormonal therapy. Since the development of conventional chemotherapy, more than 60 years ago, these drugs have been regarded as the main therapy for many tumours [6]. Chemotherapy consists in the use of antitumor drugs that kill all rapidly dividing cells. Several types of chemotherapeutic agents are used: i) alkylating agents and platinum-based antitumor compounds *(e.g.* cyclophosphamide and cisplatin); ii) topoisomerase inhibitors (*e.g.* camptothecin)*;* iii) anthracyclines (*e.g.* doxorubicin); iv) *vinca* alkaloids and taxanes (*e.g.* vinblastine and paclitaxel); v) purine and pyrimidine anti-metabolites (*e.g.* 6-mercaptopurine and 5-fluorouracil); and vi) folate antagonists (*e.g.* methotrexate) [7].

More recently, several targeted therapies have been developed, to overcome one of the biggest problems of conventional chemotherapy which is lack of specificity [8]. Targeted therapies, like conventional chemotherapies, are based on pharmacological compounds to inhibit cell growth, increase cell death, and ultimately restrain the spread of cancer. However, contrary to conventional chemotherapy, targeted therapies act on specific molecular targets involved in tumorigenesis, being capable of treating several cancers with less side effects. The main types of target therapies are the monoclonal antibodies and the small molecule inhibitors [6]. The monoclonal antibodies can act through several mechanisms, exerting their anti-cancer effects by recruiting and activating the immune system or by inhibiting specific ligand-receptor interactions essential for cell survival. A good example is the case of trantuzumab, a monoclonal antibody used to treat human epidermal growth factor receptor 2-positive (HER2+) breast cancers [9]. The small molecule drugs, on the other hand, block specific enzymes or receptors involved in cell proliferation. It is the case of imatinib, an inhibitor of BCR-ABL, a fusion protein present in the leukemia cells of chronic myelogenous leukemia (CML) patients [10].

Chemotherapy is still nowadays one of the most used cancer treatments. But regardless the treatment option (chemotherapy of molecular targeted therapy), its effectiveness may be limited due to drug resistance [11].

2. Multidrug Resistance (MDR) and mechanisms involved

Drug resistance is one of the major obstacles to the effectiveness of current cancer therapies, leading to recurrence, cancer dissemination and ultimately, death. It

can either be intrinsic, if the drug-resistance mechanisms were already present before treatment; or acquired, if it develops during treatment by therapy-induced adaptive responses, or positive selection of drug-resistant tumour subpopulations already present.

Several molecular mechanisms have been implicated in cancer drug resistance. These can act individually or synergistically, leading to what is known as multidrug resistance (MDR). A tumour cell with a MDR phenotype is resistant to a variety of structurally and mechanistically unrelated drugs. A plethora of mechanisms contribute to MDR: i) alterations in drug transport and metabolism; ii) alterations in drug targets; iii) resistance-promoting adaptive responses; iv) downstream resistance mechanisms, and lastly, v) the tumour microenvironment.

Besides the pharmacokinetic factors, such as drug absorption, distribution, metabolism and elimination (ADME), the major obstacle that a drug has to face in order to reach the intracellular compartment of a cell is the plasma membrane. The chemotherapeutic agents can enter the cell either by passive diffusion [12] or facilitated transport [13].

One type of secondary active transporters thought to be involved in anticancer drug uptake is the membrane-bound solute carriers (SLC). One evidence for this is the diminished response to methotrexate treatment in patients that present a decreased expression of the reduced-folate carrier (SLC19A1) [14]. This family of genes is composed by approximately 400 SLCs, that can be categorized in 52 different subfamilies [15]. Although the exact mechanisms of cellular uptake of chemotherapeutic agents are not known, as evidence suggests for SLC19A1, other SLCs may also be involved in their uptake. Thereby, the impairment of drug uptake can also be seen as a mechanism of resistance.

Another mechanism that decreases the intracellular drug concentration of chemotherapeutic agents is the overexpression of drug efflux pumps. Several ATP-dependent drug efflux pumps, including the ATP-binding cassette (ABC) transporters, are responsible for this mechanism of resistance, being thoroughly discussed in the subchapter 2.1. of this thesis. However, ABC transporters are not the only ones with a role in ATP-dependent drug efflux. Other transporters such as RLIP76, which mediates the export of GSH-conjugates [16, 17], and ATP7A/B [18], responsible for the export of platinum agents (*e.g.* cisplatin) have also been described as being involved in drug resistance.

Once they reach the intracellular compartment, some chemotherapeutic agents need to be metabolized into their active form. These are designated prodrugs. However,

drug inactivation by phase I and phase II drug metabolizing enzymes may also be a cause of drug resistance. These mechanisms work together to form the second line of cellular resistance against chemotherapeutic agents. One example of a drug that needs to be metabolized in order to be active is the capecitabine. Capecitabine is a fluoropyrimidine prodrug that is converted into the antimetabolite 5-fluorouracil (5-FU), by the enzyme thymidine phosphorylase [19]. However, the gene that encodes this enzyme is epigenetically regulated, and may be inactivated by methylation, thereby causing capecitabine resistance [20].

Moreover, phase I, also known as oxidative metabolism, involves the role of cytochrome P450 (CYP) enzymes, as well as epoxide hydrolases. CYPs are part of the superfamily of hemoproteins, which catalyse the monooxygenase reaction, leading to drug detoxification. These metabolites can then be conjugated by the phase II enzymes and effluxed by transporters across the plasma membrane. Phase II involves several enzymes, including the glutathione transferases (GST) [21], the UDP-glucuronosyltransferases (UGT) [22], sulfotransferases [23], and arylamine N-acetyltransferases (NAT) [24]. These are responsible for the transformation of the reactive compounds into hydrophilic nontoxic metabolite conjugates, which can also be effluxed by the ABC family transporters. Genetic polymorphisms in these genes have been correlated with the overall survival in cancer patients [25].

Drugs that evade the mechanisms of resistance described above can then reach the desired intracellular concentration.

Most chemotherapeutic drugs cause induction of DNA damage, either by acting directly in DNA, or indirectly, e.g. the topoisomerase inhibitors. They cause extensive DNA damage in proliferating cells, ultimately causing cell cycle arrest and, if the damage is extensive, cell death. However, cells have the capacity to monitor the integrity of their DNA, and to repair it in the case of DNA damage.

DNA repair can be performed by several different repair systems, according to the type of lesion. These systems are: the direct reversal of DNA damage by photolyase-, alkyltransferase-, and dioxygenase-mediated repair processes [26], the mismatch repair (MMR), the nucleotide excision repair (NER), the base excision repair (BER), the homologous recombination (HR), and the non-homologous end joining (NHEJ) [27]. Upregulation of these pathways in a cancer cell is responsible for some cases of cancer drug resistance, since the cancer cell is then able to successfully repair the damage induced by the chemotherapeutic agents.

Since the primary pharmacological purpose for the use of anticancer drugs is to promote cell death, the disruption of the apoptotic pathways, a hallmark of cancer [3], is

one of the biggest obstacles to the success of cancer treatment. Both apoptotic and non-apoptotic mechanisms like necrosis, senescence, autophagy, and mitotic catastrophe [28] can lead to cell death. Due to their relevance for the present work, both apoptosis and autophagy will be further discussed in the following sections.

Another molecular mechanism responsible for resistance of cancer cells to molecular targeted therapy is the alteration of drug targets. These alterations may be due to mutations or changes in the expression levels of the drug target. Indeed, increased expression of a drug target reduces the effectiveness of inhibitors of that target, since more target molecules must be inhibited for the drug to have a therapeutic effect. In addition, mutations in the binding-site of the molecular targeted drugs can also affect its capacity to bind to the target. An example of this is the case of resistance to inhibitors of BCR-ABL, an oncogenic kinase present in patients with chronic myeloid leukaemia (CML) [29]. The gold standard in the treatment of CML is imatinib, a highly specific inhibitor of BCR-ABL [30]. However, some patients relapsed after treatment with imatinib, due to a mutation in the residue T135 of BCR-ABL. Indeed, one single missense mutation in the kinase domain of BCR-ABL is able to hinder the binding of imatinib, without affecting the catalytic activity of the enzyme [31]. This type of residues are called gatekeepers. They are conserved residues present in the opening of the ATP-binding pocket of many kinases. Mutations in this sites are usually the cause of resistance to inhibitors of oncogenic kinases [32].

As previously mentioned, tumours are composed by a heterogeneous microenvironment, with an abnormal vasculature embedded in the extracellular matrix [33, 34]. Therefore it is expected that the tumour microenvironment can influence the treatment outcome [35]. Indeed, the tumour microenvironment provides a physical protection against cytotoxic agents. Tumour abnormal vasculature is characterized by a disorganized structure, being composed of dilated and convoluted blood vessels [36]. Together with an elevated interstitial fluid pressure, the microenvironment impedes the penetration of macromolecules into the tumour [37, 38]. This also causes a hypoxic environment due to the decreased blood flow, thus leading to the acidification of the extracellular medium [39]. This effect drastically decreases the efficacy of chemotherapy, since weakly basic drugs such as doxorubicin, mitroxantrone and vincristine are ionized in these conditions, thus hindering their entry into the cell [40]. Additionally, hypoxia also upregulates the antioxidant mechanisms, thus reducing the effectiveness of alkylating agents and platinum-based drugs [41, 42].

Together, all the aforementioned MDR mechanisms contribute to the reduction of the effectiveness of cancer treatment.

2.1. ABC transporters: P-glycoprotein (P-gp)

One of the main mechanisms responsible for MDR is drug-efflux caused by increased expression of membrane transporters, namely the ABC transporters. These transmembrane proteins regulate the flux of chemotherapeutic agents across the plasma membrane, using energy from the hydrolysis of ATP as the driving force. In total, there are 49 ABC transporters known to date, that can be divided into 7 different subfamilies, named from A to G [43].

These proteins are highly conserved and have a high sequence homology between all its members. They are typically composed by two nucleotide-binding domains (NBDs) and two transmembrane domains (TMDs), usually encoded by a single polypeptide [43].

ABC transporters have several physiological roles in the context of cell protection against xenobiotics. These transmembrane proteins are present in the blood–brain, blood–cerebrospinal fluid, and blood-testis barriers, as well as in placenta, where they regulate the permeability of xenobiotic compounds. ABC transporters are also found in the liver, in the gastrointestinal tract, and in the kidney, where their function is to excrete toxins to be eliminated, thereby protecting the organism [44].

The first ABC transporter to be discovered was the ABCB1 (**Figure 1**), more commonly known by P-glycoprotein (P-gp), back in 1976 (more than 40 years ago). It was described as promoting cell resistance against several structurally and mechanistically unrelated compounds [45].

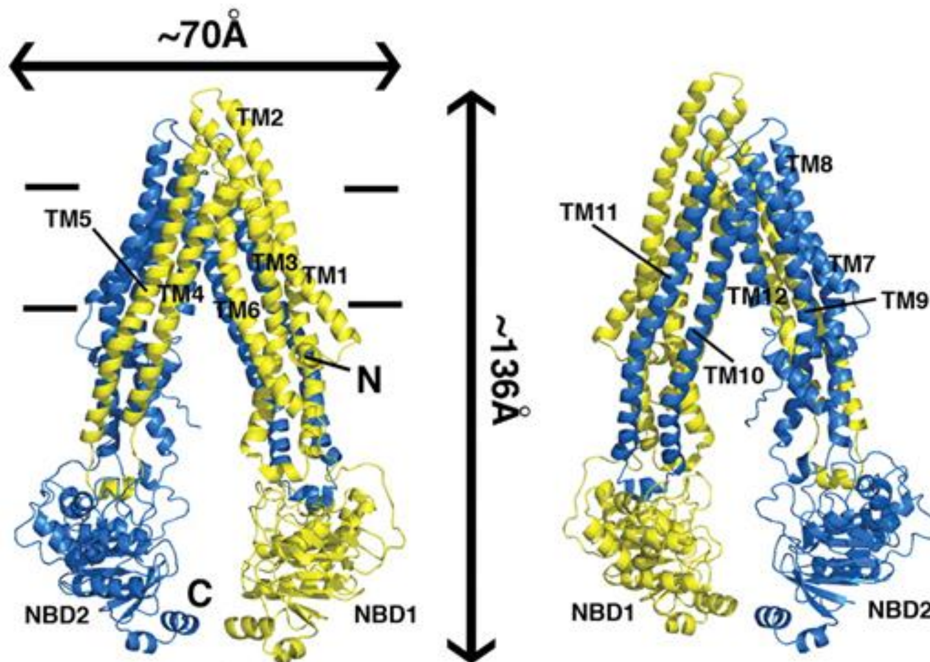

Figure 1 – Structure of P-gp. Front (left) and back (right) stereo views. Transmembrane (TM) domains 1-12 are labelled, as well as the nucleotide binding domains (NBD). The N- and C-terminal of the molecule, are respectively coloured with yellow and blue. Horizontal bars represent the approximate positioning of the plasma membrane. Adapted from [46].

Nowadays more ABC transporters have been associated with MDR. The three most extensively studied ones, regarding the phenomenon of MDR in cancer are the P-glycoprotein (P-gp; also known as multi-drug resistance protein 1 or ABCB1), the MDR-associated protein 1 (MRP1; also known as ABCC1) and the breast cancer resistance protein (BCRP; also known as ABCG2). They have overlapping substrate specificities, promoting the elimination of some of the most used cancer chemotherapeutic drugs, such as taxanes, topoisomerase inhibitors, and antimetabolites (*e.g.* 5-fluorouracil and methotrexate) [11]. However many attempts to translate this knowledge into clinical approved drugs to counteract this form of MDR have so far been unsuccessful [47].

Several studies have been made in order to address the genetic polymorphisms of ABC transporters, and their relationship with treatment outcome. In fact there is increasing evidence that there is a correlation between ABCB1 genotypes and clinical outcome [48].

2.2. Escape from apoptosis

Apoptosis is a conserved process of programmed cell death used by multicellular organisms to deal with unwanted cells [49]. During apoptotic cell death, cells undergo

morphological alterations which include cytoplasmic shrinkage and nuclear condensation, while keeping membrane integrity [50].

Apoptosis can be triggered both intrinsically or extrinsically, in both cases culminating in the activation of caspases, a class of cysteine aspartic acid-specific proteases, that are expressed as inactive zymogens [51]. These caspases can be divided in two main categories: the initiator caspases, which include caspases 1, 2, 4, 5, 8, 9, 10, 11, and 12; and the effector caspases, composed by caspases 3, 6, 7, and 14 [52]. Initiator caspases, such as caspase 9, are responsible for the cleavage of the inactive forms of the effector caspases, triggering a proteolytic cascade of events that lead to the cleavage of multiple crucial cellular target proteins ultimately leading to cell death [53].

However, for that cascade to start, the apoptosome, a large protein structure constituted by the apoptotic protease-activating factor 1 (APAF1) and cytochrome c, released from the mitochondria in response to death stimulus, must be formed to recruit and activate caspase 9 [53-55].

The mitochondrial outer membrane permeabilization (MOMP) is essential for the release of cytochrome c, being a decisive point in the cell commitment to apoptosis. MOMP is regulated by members of the BCL2-family, constituted by both pro- and anti-apoptotic proteins [56, 57]. The pro-apoptotic BAX-like subfamily of proteins, constituted by proteins like BAX, BAK, and BOK, are responsible for the formation of pores in the mitochondrial outer membrane. This is induced with the help of a structurally diverse group of proteins that have a single BCL-2 homology (BH) domain, designated BH3-only proteins (BIK, HRK, BIM, BAD, BID, PUMA, NOXA, and BMF). They are also pro-apoptotic proteins that facilitate the BAX/BAK-dependent release of cytochrome c from mitochondria. On other hand, the anti-apoptotic BCL-2 family members comprise BCL-2 and its close relatives (BCL-XL, MCL1, BCL2A1, BCL-W, and BCL-B), that prevent the oligomerization of BAX and/or BAK in mitochondrial outer membranes, impeding the release of cytochrome c from the mitochondria. Therefore, the balance between all the different types of BCL-2 family proteins contributes to determining if a cell undergoes apoptosis [51].

Another way to regulate apoptosis is through the inhibition of caspases. The proteins responsible for that function are the inhibitor of apoptosis proteins, also known as IAPs [58]. Therefore, for a cell to undergo apoptosis, IAPs must be inactivated. The IAP-binding proteins responsible for this are the second mitochondria-derived activator of caspase (SMAC, also known as DIABLO) and the mitochondrial serine protease HTRA2. These proteins are localized in the mitochondrial intermembrane space and are

released in the cytoplasm following MOMP, together with cytochrome c, binding to IAPs, thus promoting caspase activation [59-61].

However, cancer cells develop mechanisms to deregulate the apoptotic pathway, leading to escape from apoptosis, providing the cells with a survival advantage [62].

One example of this type of mechanisms is the increased expression of the anti-apoptotic Bcl-2 gene that has been identified in several cancers, reducing therapy efficacy [63]. The use of potent BH3 mimetic compounds, such as ABT-263, which bind to anti-apoptotic BCL-2 proteins, allow BAX and BAK to induce MOMP and thus apoptotic cell death [64].

Another way that cancer cells have to resist to apoptosis is through the inactivation of the tumour suppressor gene p53. The pro-apoptotic protein BAX and the BH3-only proteins PUMA and NOXA are known transcriptional targets of p53. When p53 is inactivated, these proteins will not be transcribed. Several therapeutic strategies, such as the use of mutant p53 reactivors, (*e.g.* PRIMA-1 and NSC319726) have been attempted in order to restore p53 function, ultimately inducing apoptosis [65-67]. However, none of these molecules has reached the clinic so far.

Another example of a mechanism associated with resistance to apoptosis in cancers is the overexpression of IAPs, which has been associated with poor prognosis [68, 69]. Furthermore, low levels of caspase-3 have also been frequently observed in cancer cells, being associated with chemoresistance [70, 71].

2.3. Alterations in autophagy

Autophagy is one of the major protein degradation systems, targeting long-lived macromolecular complexes and organelles. It is highly conserved, occurring constitutively in all eukaryotic cells, where it is responsible for the degradation and recycling of cytoplasmic material, therefore playing a vital role in the maintenance of cell homeostasis. It can be further activated in response to some stress conditions, such as starvation and oxidative stress, and also acts as a mode of cell death, the type II programmed cell death [72]. Autophagy, can be categorized in three different classes according to their mechanism: microautophagy, chaperone-mediated autophagy, and macroautophagy, hereupon only referred to as autophagy [73].

Autophagy (**Figure 2**) is a successive process that begins with the formation of the phagophore, a double membrane structure that can arise from multiple sources including the endoplasmic reticulum (ER), the mitochondrial outer membrane, and the

plasma membrane [72, 74]. Upon activation of the appropriate signalling pathways, inactivated mammalian target of rapamycin (mTOR) dissociates from the unc-51-like kinase 1 (ULK1) complex that is then activated, and becomes associated with the phagophore, allowing the maturation of the autophagosome. The ULK1 complex is constituted by serine/threonine-protein kinase ULK1, Atg13, Atg101, and FIP200 (focal adhesion kinase family interacting protein of 200 kDa) [75-77].

The maturation of the autophagosome starts with the association of the vesicular sorting protein 34 complex/phosphatidylinositol-3-phosphate kinase III (Vps34/PI3K class III) with the phagophore through the PI3-kinase p150 subunit, being then anchored to the phagophore membrane *via* myristic acid. In addition, Beclin-1 is also an essential part of the complex by binding to the anti-apoptotic protein BCL-2, that competes with Vps34 for its binding, thereby working as an inhibitor of autophagy [72]. The beclin-1-associated autophagy related key regulator (Barkor, also known as Atg14) and UV radiation-associated resistance gene (UVRAG) are also responsible for the stabilization of the Vps34 complex [78, 79]. The PI3P generated by this complex then attracts a number of components that results in two ubiquitin-like conjugations [72]. The first conjugation is the covalent linkage of Atg12 and Atg5, involving the E1-like enzyme Atg7 and the E2-like enzyme Atg10. The Atg12-Atg5 conjugate complexes with Atg16L and directly binds to the outer membrane of the phagophore [80, 81]. The second conjugation also involves the E1-like enzyme Atg7 to activate LC3-I. Previous to that activation, Atg4, a cysteine protease, cleaves LC3 revealing its C-terminal glycine, originating LC3-I. The activated LC3-I is transferred to the E2-like enzyme Atg3, that conjugates LC3-I with phosphatidylethanolamine (PE) to originate LC3-II, an autophagosomal lipoprotein present both in the exterior and lumen of the vesicle. The LC3-II present in the exterior of the autophagosome are cleaved and recycled by Atg4, while the luminal LC3-II is degraded by lysosomal proteases together with the rest of the cargo [72, 80]. Thus, LC3-II levels can be used as an index of autophagy [82]. Although its function is not known, it is clear that LC3-II is responsible for the recruitment of the cargo adaptor protein p62 (also known as sequestosome 1, SQSTM1), and therefore participates in cargo selection [83].

The degradation of cytosolic proteins, ER, peroxisomes and mitochondria is a selective process regulated in part by ubiquitination of candidate proteins, and mediated by p62, a multifunction protein that captures ubiquitinated targets and secures them to LC3-II in the autophagosome [83].

The autophagosome then fuses with the lysosome, forming the autolysosome, where all the cargo will be degraded, thereby recycling amino acids, fatty acids, and

nucleotides. This recycling is especially important when the cell is under nutrient deprivation, being considered a mechanism of survival [73].

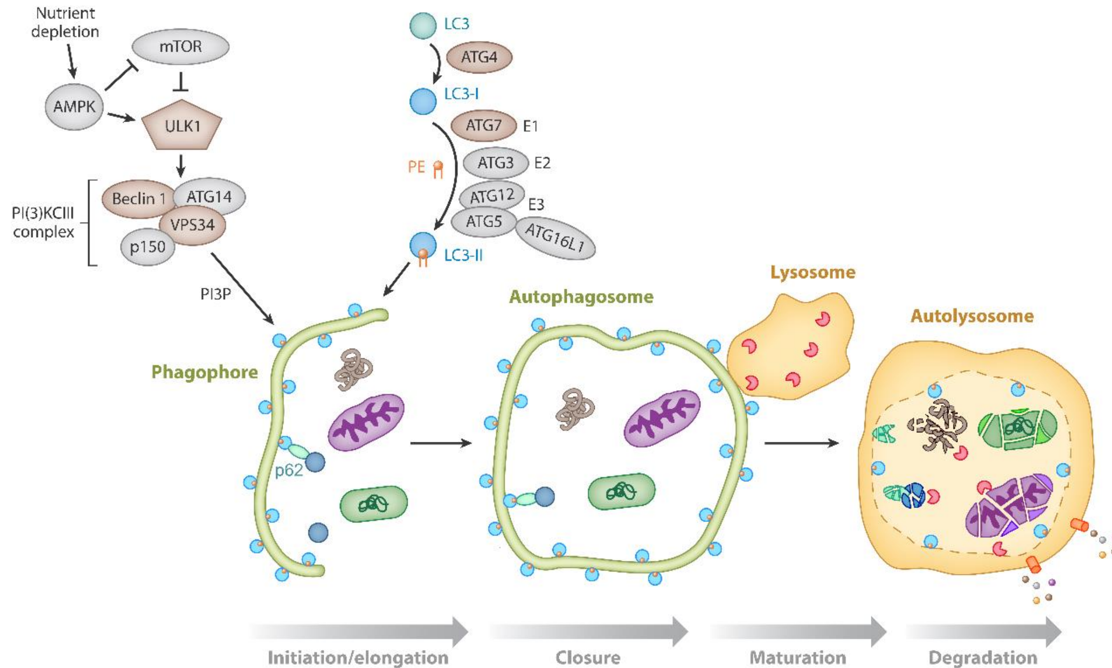

Figure 2 – Molecular mechanisms of autophagy. The several stages of autophagy are shown. Adapted from [84].

However, autophagy can have a dual role in MDR tumour cells, contributing differently to their outcome. Autophagy can lead to the development of MDR, and protect cancer cells from chemotherapeutic agents, while on the other hand it can work as a tumour suppressor, and kill MDR tumour cells by the induction of autophagic cell death. The pro-death and pro-survival roles of autophagy depend on the tumour type and the treatment used [84-86].

Autophagy protects MDR tumour cells against apoptosis, promoting resistance to chemotherapy treatment. In fact, enhanced autophagy levels were detected in patients with poor prognosis, suggesting a role for autophagy in the development of MDR [86].

Since MDR-promoted autophagy has been well documented, autophagy inhibition has been proposed as a strategy to enhance the efficacy of chemotherapeutic agents [86]. For example, the use of small interfering RNAs (siRNAs) that target *Atg12* or the use of chemical autophagy inhibitors such as chloroquine, successfully sensitized SGC7901/VCR cells (from a MDR cell line that was developed by prolonged exposure to vincristine) to chemotherapeutic agents [87]. This suggests that vincristine-based MDR is related to autophagy. In addition, autophagy also protects MDR cells against anthracyclines [88].

Beside its role as a pro-survival mechanism, autophagy can also promote cell death in apoptosis-deficient MDR tumour cells. Several compounds are being developed to promote autophagic cell death in these cells, thereby helping to overcome MDR [86].

3. Extracellular Vesicles (EVs): an overview

Extracellular vesicles (EVs) are a heterogeneous group of membrane-limited vesicles that can contain several cargoes from the donor cells such as, proteins, lipids, and nucleic acids, that can arise both through the endosomal pathway (exosomes) and by budding from the plasma membrane (microvesicles) [89, 90].

Initially it was thought that EVs were only a disposal mechanism of the cell to discard non-functional or unwanted cellular components [91, 92]. However, increasing evidence indicates that EVs have a much bigger role, being key players in intercellular communication, both in normal physiological processes, as well as in pathological progression [89]. A big contribution towards that conclusion was the discovery of RNA inside EVs [93, 94]. Indeed, RNA, microRNAs, proteins and other molecules from the donor cell, might be transferred between cells as a form of intercellular communication, due to the fact that EVs are membrane-limited vesicles thereby protecting these informative molecules from the harsh external environment.

EVs are of different sizes (**Figure 3**) and contain different cargoes, probably due to the fact that they can have different biogenesis. These different types of EVs can be released from a single cell at the same type, and their release may change according to the physiological or pathological state of the cell [90].

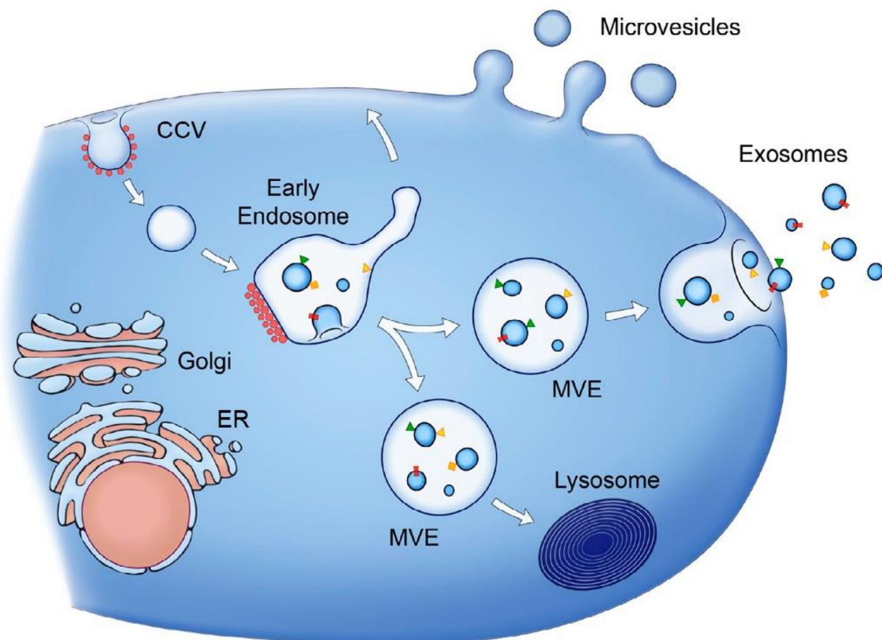

Figure 3 – EVs can be released from the cell in the form of microvesicles and exosomes, according to their biogenesis. Microvesicles bud directly from the plasma membrane, whereas exosomes have their origin in the endosomal pathway [95].

Their cargo composition comprises a multitude of different proteins, lipids, DNA, and several small ncRNAs (sncRNAs), such as microRNA, small nucleolar RNA, Y RNA, mitochondrial RNA, and vault RNA, as well as long ncRNAs and mRNA (although it is mostly fragmented). Proteins can either be present on the outside (*e.g.* transmembrane proteins) or inside (*e.g.* soluble proteins) of EVs. These may serve as markers of the biogenesis mechanisms of EVs [89, 96-98].

Beside their function as cell-cell communicators, EVs can also work in the elimination of unwanted molecules from cells, such as modified RNAs and amyloid proteins [99, 100]. Nevertheless, much research is still necessary on the role of EVs in intercellular communication and the mechanisms involved.

3.1. Differences in the size of EVs and their biogenesis

Several types of EVs have been characterized, and classified according to their different biogenesis pathways, resulting either in their release through the fusion of the multivesicular bodies (MVBs) with the plasma membrane, or via outward budding and fission of the plasma membrane. The two main types of EVs are the exosomes and the microvesicles [101].

Exosomes are small EVs, typically with a diameter smaller than 150 nm. They are derived from the endosomal system, and are formed as intraluminal vesicles (ILVs)

in the MVBs [89, 90]. The endosomal system comprises three different types of endosomes: the early endosomes, the late endosomes, and the recycling endosomes [102]. The early endosomes fuse with the endocytic vesicles, that can either recycle molecules to the plasma membrane via recycling endosomes, or maturate into late endosomes, making them competent for fusion with lysosomes, for degradation. The late endosomes accumulate ILVs that are formed by inward budding of the endosomal membrane. It is during this process that cytosolic proteins, nucleic acids, and lipids are sorted into what will be the exosomes. Late endosomes that contain several ILVs are them termed MVBs. These can either fuse with lysosome committing their content for degradation or fuse with the plasma membrane, thereby releasing the ILVs as exosomes into the extracellular medium [89].

The formation of ILVs within MVBs is then the first step in the biogenesis of exosomes. Several mechanisms have been described, but in all cases lipid curvature must be induced for the vesicles to be formed [90]. The recruitment of the endosomal sorting complex required for transport (ESCRT) machinery is fundamental. Four different ESCRT complexes have been identified (ESCRT-0, -I, -II, and -III) along with accessory proteins such as Alix, VPS4 and VTA-1, working in a sequential manner to bind what will be the exosome cargo and incorporate it into the ILVs [103]. ESCRT-0 recognizes ubiquitinated proteins on the outside of the endosomal membrane. Then, ESCRT-I is recruited to the endosome by protein-protein interaction with ESCRT-0, helping with cargo sorting. In addition, ESCRT-I interacts with ESCRT-II, and together they are capable of budding membranes into the endosome lumen. All these three ESCRT complexes are able to interact with ubiquitinated cargo. In contrast, ESCRT-III does not form a stable protein complex, being only transiently assembled on the endosomes. It is responsible for the fission of vesicles into the endosome lumen. It also recruits deubiquitinases, which mediate cargo deubiquitination, therefore recycling ubiquitin prior to vesicle formation. Finally, ESCRT-III is disassembled for recycling by the AAA-ATPase VPS4 [104].

An alternative pathway for exosome formation involves synthesis of ceramide, as the mechanism responsible for inducing vesicle curvature and budding [105]. A third mechanism involves the tetraspanin-mediated organization of specific proteins. Two tetraspanins that are thought to play a critical role in exosome formation are CD9 and CD63 [106].

Another type of EVs, which are formed by direct budding from the plasma membrane, are the microvesicles. They are larger vesicles, typically in the range of 200 to 500 nm; however, vesicles having 1 μm have been found. Their biogenesis is far less

defined than exosomes [89, 90]. Similar to exosome biogenesis, mechanisms that generate or alter the asymmetry of the plasma membrane with respect to lipids have been implied in microvesicles formation. These mechanisms include alterations in the activity of enzymes that transfer lipids from one side of the plasma membrane, to another [107, 108]. Alterations in the ceramide content of the outer leaflet of the plasma membrane via activation of acid sphingomyelinase were found to induce membrane curvature leading to microvesicle release [109-111]. The recruitment of the ESCRT machinery involved in the formation of ILVs in the MVBs can also lead to microvesicles release [90].

The isolation of the distinct subpopulations of EVs according to their biogenesis or molecular content is still a challenge [96, 112] leading to a confusion in the literature in the nomenclature of exosomes and microvesicles. Therefore, since the composition of the purified vesicle preparations is usually unclear (due to the fact that the isolation is based on size and density), the scientific community that works in the area has proposed the general use of the term “Extracellular Vesicles” (EVs) to refer to these vesicles.

3.2. EVs as mediators of intercellular transfer of MDR

Traditionally, intercellular communication consisted only in direct secretion of molecules from cells or in cell-cell interactions. However, in the recent years another mechanism of intercellular communication has been described, consisting in the incorporation of EVs in recipient cells, which had been released by other cells, designated as donor cells [95].

Despite their functions in normal physiology, the role of EVs in tumour progression was shown to be of extreme importance [113]. In fact, EVs have been described as vehicles of dissemination of cancer drug resistance (CDR), associated with promotion of immune escape, with epithelial-to-mesenchymal transition (EMT), and with metastasis [114].

Regarding the intercellular transfer of MDR by EVs, several mediators of MDR, such as drug-efflux pumps, microRNAs and long non-coding RNAs (lncRNAs) have been identified as being transferred by EVs, from donor drug-resistant tumour cells into recipient drug-sensitive cells [115].

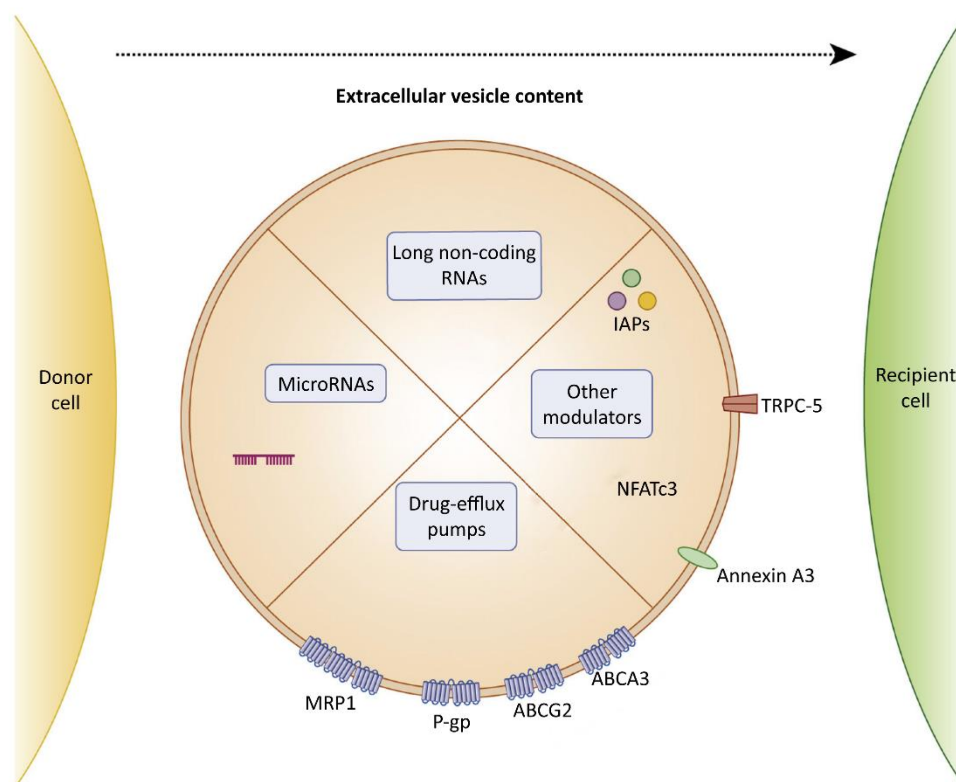

Figure 4 – The transference of a drug-resistant phenotype by EVs, known to be implicated in the therapy failure, is mediated by: drug-efflux pumps, microRNAs, and long noncoding RNAs. Adapted from Sousa, D. *et al* [115].

The presence of drug-efflux pumps in the EVs released by drug-resistant cells has been related with their capacity of sequestering drugs, thereby decreasing the effective intracellular drug concentration on the donor cells, leading to sublethal doses of these compounds and drug-resistance [116, 117]. Indeed, a study suggested that this is possible due to the inverted orientation of the P-gp in the EVs, thereby leading to an active influx of drugs to the lumen of EVs [116].

Several studies described that drug-efflux pumps are capable of being transferred from drug-resistant to drug-sensitive cells by EVs, leading to the development of a MDR phenotype in these recipient cells [118-120].

P-gp is one of the drug-efflux pumps transferred by EVs. This "non-genetic acquisition" of P-gp was detected for the first time in a neuroblastoma model, in which a drug-sensitive cell line presented functional P-gp following co-culture with a P-gp expressing drug-resistant counterpart cell line [121]. Bebawy *et al.* also verified that when drug-sensitive leukaemia cells were incubated with EVs isolated from their drug-resistant counterpart cells, the recipient cells showed the presence of functional P-gp [122]. The incubation period was of only 2h and 4h, revealing that P-gp present in these cells was originated from the EVs released by the drug-resistant cells, and not by the induction of

transcription and subsequent translation of the *MDR1* gene [122]. Other study using a drug-sensitive and its docetaxel-resistant counterpart cell line, demonstrated that the increase of P-gp levels in the recipient cells was proportional to the amount of co-cultured EVs from the drug-resistant cell line [123].

Additionally, other drug-efflux pumps were found to be transferred by EVs, such as MRP1 [124], or BCRP [125, 126]. However, their possible transfer between drug-resistant EVs and drug-sensitive cells still needs to be clarified.

Some RNAs responsible for drug resistance have been found inside EVs, namely microRNAs [89, 96, 97]. microRNAs are commonly deregulated during tumorigenesis and metastization, thereby affecting drug responses [127-133]. When present inside EVs, microRNAs become protected from the action of Rnases [134], being good markers of drug resistance [135, 136]. These microRNAs carried by EVs have been associated with the intercellular transfer of MDR [137], being capable of modulating transcripts in the recipient cells. This alteration of the transcriptional landscape of recipient cells caused by microRNAs present in EVs originated on MDR tumour cells, illustrates the implication of microRNAs carried in EVs in the dissemination of the MDR phenotype [138].

Similarly to microRNAs, lncRNAs may also be selectively packaged in EVs [139]; however, their contribution to the horizontal transfer of a MDR phenotype is still poorly understood. The transfer of functional mRNAs from donor to recipient cells, through EVs, has also been described [135].

In summary, the transference of a drug-resistance phenotype mediated by EVs contributes to therapy failure, and therefore more research is needed to identify ways to counteract this mechanism [115].

3.3. Modulators of EVs release by cells: 3-MA (an autophagy inhibitor) and imipramine

Due to the role of EVs as mediators of intercellular transfer of MDR, inhibition of their release has been a major goal. However, since EVs biogenic pathways are not fully known, the development of modulators of their release has been a big challenge. Nevertheless, some inhibitors of exosome and microvesicles release have been found.

Since the ceramide content of the membranes has been related with the membrane curvature, ultimately leading to ILVs formation in the MVBs and to

microvesicle release in the extracellular medium, inhibitors of sphingomyelinases have been developed [109] to inhibit the release of EVs.

Imipramine, an inhibitor of the enzyme acid sphingomyelinase can lead to the inhibition of microvesicles shedding from the plasma membrane, suggesting that this enzyme is necessary and sufficient for their release [109]. Acid sphingomyelinase is one of the key enzymes responsible for the production of ceramide, catalysing the hydrolysis of sphingomyelin into ceramide and phosphorylcholine, and consequently altering membrane fluidity, which will then lead to membrane blebbing and shedding [140].

On the other hand, the blebbing and budding of ILVs into MVBs, that will then be released in the extracellular medium as exosomes, results from the activation of neutral sphingomyelinase, as suggested by Trajkovic and her co-workers [141]. This indicates that different members of the sphingomyelinase family specifically control the release of distinct populations of EVs. In fact, when the Oli-neu cells, from a mouse oligodendroglial cell line that contains a large number of MVBs [142], were treated with GW4869, an inhibitor of the neutral sphingomyelinase, exosome release was markedly reduced [141].

Moreover, *Dardalhon et al.* described that the autophagy inhibitor 3-methyladenine (3-MA) also leads to a decrease in the release of exosomes [143]. This potentially indicated a connection between the release of EVs and autophagy modulation in MDR cells. However, the exact mechanisms that mediate this phenomenon are yet to be discovered.

4. The importance of compounds with dual-activity: antitumour (inducing apoptosis or autophagy) and anti-P-gp

In the recent years it has been widely recognised that drugs that act on more than one molecular target can have a superior efficacy in the treatment of complex diseases such as cancer. These multi-target drugs are also less prone to the problem of drug-resistance and present reduced side effects [144, 145].

This type of strategy has been applied in the treatment of complex diseases such as HIV [146], cancer [147], and several neurodegenerative diseases [148, 149].

Moreover, since MDR is a phenomenon broadly found in cancer cells, the discovery or design of compounds that are concomitantly P-gp inhibitors and also have an antitumoral activity would be of great advantage for the treatment of cancer [150].

4.1. Screening of compounds for antitumour and P-gp inhibitory activities

In the last 3-4 decades, more than 40 reviews about the structure, function and application of ABC transporter inhibitors have been published [151]. Despite all these studies, none of the potential inhibitors of P-gp tested in preclinical and clinical trials have shown an improvement in therapeutic efficacy. Therefore, new strategies, as the fallback to searching natural products, have arisen [152].

A group of compounds that showed potent P-gp inhibitory activity, in addition with an antitumout activity, are the natural alkaloids [151, 153]. Indeed, some natural alkaloids that emerged in the last two decades showed promising antitumoral activities, such as the quinazolinone alkaloids [154].

Quinazolinones belong to a family of heterocyclic nitrogenated compounds widely distributed in plants and microorganisms [155, 156]. Interest in these compounds has recently increased due to the broad spectrum of biological activities that have been described: antitumour, antibacterial, antifungal, anti-HIV, among others [154]. Their activity as dual inhibitors of P-gp and MRP1 has also been described [157], making the quinazolinones very interesting molecules for further studies. Therefore, it is necessary to unravel the antitumoral and P-gp inhibitory potential of derivatives of these compounds.

Aim of the work

The main aim of this work is to confirm the release of larger EVs by P-gp overexpressing MDR cells, when compared to their drug-sensitive counterparts. In addition, to investigate the possible relationship between the release of EVs and cellular autophagy. To accomplish these overall aims, the following specific objectives were established:

- Study the effect of specific chemical inhibitors of microvesicles (imipramine) or exosomes (3-methyladenine, 3-MA) in the release of EVs by drug-sensitive and their MDR counterpart cells;
- Compare the basal levels of autophagy of the drug-sensitive and their MDR counterpart cells;

In addition, due to the need to identify new molecules with dual antitumour and anti-P-gp activity, the following objective was also defined:

- Screen a small library of quinazolinone derivatives (synthesized by collaborators) for their potential effect as inhibitors of tumour cell growth and P-gp inhibition.

Materials and Methods

1. Cell culture and maintenance of a drug-resistant phenotype in the MDR cells

Two pairs of multidrug-resistant (MDR) and their counterpart drug-sensitive human tumour cell lines were used in the work presented in this thesis. The non-small cell lung cancer (NSCLC) cell line NCI-H460 (henceforth named H460 for a matter of simplicity), and its drug-resistant P-gp overexpressing counterpart cell line, RH460, were a kind gift of our group collaborator Dr. M. Pešić (Belgrade, Serbia) [158, 159]. The K562 chronic myelogenous leukaemia (CML) cell line was obtained from European Collection of Authenticated Cell Cultures (ECACC) and its counterpart cell line, K562Dox (that overexpresses P-gp), was a kind gift of Dr. J.P.Marie (Paris, France) [160, 161]. All cell lines were genotyped and routinely tested for possible mycoplasma contamination. In the case of the K562Dox cell line, to maintain constant P-gp expression levels throughout the experiments, 1 µM of doxorubicin (Sigma, USA) was added to the cells every two weeks, and all the experiments were performed 6 days after stimulation with doxorubicin.

For the screening experiments, three different human tumour cell lines were used: NCI-H460 (non-small cell lung cancer), kindly provided by the National Cancer Institute (NCI, NIH, Bethesda, MD, USA), HCT-15 (human colorectal adenocarcinoma) and MCF-7 (breast adenocarcinoma, both obtained from the European Collection of Authenticated Cell Cultures (ECACC, Salisbury, UK)).

All cells were grown at 37 °C in a humidified incubator with an atmosphere of 5% CO_2. The cell culture medium used was RPMI-1640 (with Ultraglutamine I and 25 mM HEPES; Lonza, Verviers, Belgium) supplemented with 5% fetal bovine serum (FBS, heat inactivated, Biowest, South America) for the Sulforhodamine B (SRB) assay, and 10% FBS for all the remaining experiments. All experiments were carried out with cells in exponential cell growth and having more than 90% viability.

Cell number and viability were determined using the trypan blue exclusion assay, which consists on the capacity of a blue dye (trypan blue) being taken up only by cells in which the membrane permeability is compromised (death cells). Cell suspension was then mixed in a 1:1 ratio with trypan blue solution (0,2%; Sigma-Aldrich) and loaded into a Neubauer chamber. The cells were counted under a bright-field inverted microscope and the cell density determined through the following formula: (number of viable cells × dilution factor × 10^4) / number of quadrants counted. The percentage of viability (i.e. the ratio of the number of viable cells to the total cell number times 100) was also determined.

2. Isolation of extracellular vesicles (EVs) by ultracentrifugation

Extracellular vesicles (EVs) were collected from both pairs of MDR and drug-sensitive counterpart cell lines (H460/RH460 and K562/K562Dox). In the case of the cell lines H460 and RH460 (adherent cells), 10 000 – 15 000 cells cm^{-2} were seeded and allowed to attach for at least 6 h before treatment. In the case of the cell lines K562 and K562Dox (suspension cells), the density seeded was 500 000 cells mL^{-1}, and cell treatment was performed immediately. In both cases, EVs-depleted complete medium, i.e. medium containing EVs-depleted FBS (by ultracentrifugation at 100 000 g overnight) was used. For cell treatment with the compounds 3-MA (3-methyladenine) or imipramine, at 0.5 mM and 20 µM respectively, cells were cultured for additional 72h following treatment. Controls with the vehicle of the compounds, DMSO for 3-MA and water for imipramine, were included in the experiments, as well as, a Blank treatment (cells without incubation with any solvent, just with complete medium).

The EVs were then isolated from the culture supernatant, by differential centrifugation. All the centrifugation steps were performed at 4 °C, and performed as follow: 5 min at 1200 rpm to pellet cells (just for the K562 and K562Dox cells), 10 min at 300 g to remove some cells that could have remained in suspension and 10 min at 2000 g to remove cellular debris and apoptotic bodies (centrifuge 5810R, Eppendorf, Germany); 30 min at 10 000 g to remove larger vesicles (high speed centrifuge Avanti J-25, Beckman Coulter) and 60 min at 100 000 g to pellet the desired EVs. The EVs were then rinsed once with phosphate-buffered saline (PBS) and centrifuged again at 100 000 g, for 60 min. EV pellets were then handled differently depending on the protocol to be followed, as indicated below.

3. Characterization of EVs

3.1. Dynamic Light Scattering (DLS)

The EVs size was measured by dynamic light scattering (DLS), using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd, UK). The EV pellet from each cell treatment was resuspended in 70 µL of PBS and added to a disposable polystyrene cuvette with a 10 mm path length. The measurement was performed at 25 °C using a laser beam with a wavelength of 633 nm, and a detection of back scattered light at an angle of 173°. The refractive index of the material was defined as 1.390 [162, 163], and the absorption as 0.010. The parameters for the dispersant (PBS) were 0.8882 cP for

the viscosity and a refractive index of 1.330. The attenuator used and the number of reads were determined automatically, and three measurements per sample were made. The results were generated by the Zetasizer software v7.12. and an average of three measurements represented as the number of particles (%) vs. size (nm) was obtained.

3.2. Nanoparticle Tracking Analysis (NTA)

Nanoparticle tracking analysis (NTA) was performed to determine the EVs concentration and size distribution, with the use of a NanoSight NS300 (Malvern Instruments Ltd, Malvern, UK) and the corresponding analytical software NTA 3.2.

All samples were diluted in PBS prior to the experiment, in a volume that ensured a concentration of EVs within the detection limits of the instrument (i.e. ~10^7 – 10^9 particles mL^{-1}).

All samples were analysed at camera levels of 13, with a detection threshold of 5. Automatic settings for the maximum jump distance and blur settings were used. Thus, prior to the video recording, 300-400 µL of sample were injected into the system with a constant flow of 40, achieved with the aid of a NanoSight syringe pump in conjunction with 1 mL syringes (Omnifix® 100 Solo, B. Braun, Germany). Then, at least three 30-seconds videos were captured for each sample and processed by the software. The data obtained, which corresponds to the mean concentration and the mode of the sizes of EVs, was then exported and analysed with the use of the Microsoft Office Excel software (Microsoft, USA).

3.3. Western Blotting (WB) for markers of EVs

The cells or EVs shed by those cells were washed in PBS and lysed in Winman's Buffer (1% NP-40, 0.1 M Tris-HCl pH 8.0, 0.15 M NaCl and 5 mM EDTA) with EDTA-free protease inhibitor cocktail (Roche) for 30 min at 4 °C. Total protein content of EVs or cell lysates was quantified using the DC Protein assay kit (Bio-Rad), a method based on the Lowry assay, and performed according to the manufacturer's protocol. After quantification, protein was loaded (5-8 µg from EVs or 20-25 µg from cells) and separated on a 12% Bis-Tris SDS-PAGE gel. Then, the proteins were electrophoretic transferred into nitrocellulose membranes (GE Healthcare, UK), and probed with the following primary antibodies: goat anti-Actin (1:2000; sc-1616 from Santa Cruz Biotechnology), mouse anti-P-gp (1:2000; P7965 from Sigma), mouse anti-syntenin-1

(1:200; sc-100336 from Santa Cruz Biotechnology), and mouse anti-cyt c (1:1000, sc-13560 from Santa Cruz Biotechnology). The secondary antibodies used were: goat anti-mouse IgG-HRP (sc-2031), or donkey anti-goat IgG-HRP (sc-2020) (all diluted at 1:2000 and from Santa Cruz Biotechnology). Signal was then detected using the ECL Western Blot Detection Reagents (GE Healthcare, UK), the Amersham Hyperfilm ECL (GE Healthcare, UK), and the Fujifilm FPM-100A X-ray film processor (Fujifilm, Japan). The films were digitalized in the Bio-Rad GS-800 densitometer (Bio-Rad, USA), and the intensity of the bands obtained in each film further analysed using the software Quantity One 1D Analysis (Bio-Rad, USA).

4. Verification of cellular autophagy and apoptosis levels

4.1. Cell treatments with 3-MA, rapamycin and serum starvation

For this experiment, the NSCLC cell line H460 and its MDR counterpart cell line, RH460, were used.

All cells were grown as stated in section 1, and the medium was supplemented with 10% FBS. All experiments were carried out with cells at the exponential growth phase, and having more than 90% viability. Cell number and viability were determined using the trypan blue exclusion assay.

Cells were seeded in 6-well plates at the density of 2×10^5 cells per well, with a final volume of 3 mL. Twenty-four hours later, 1 mL of complete medium containing the following compounds was added to each cell line: 3-MA (final concentration of 0.5 mM; an autophagy inhibitor) and rapamycin (final concentration of 100 nM; an autophagy inducer). The condition of serum starvation was also used to induce autophagy, and for that the complete medium was replaced with 4 mL of medium without serum. In this experiment, the following controls were also included: wells containing the cells grown in complete medium only (Blank) and cells grown with the highest concentration of DMSO used. Twenty-four hours after, cell lysates were collected, and the protein content quantified.

4.2. Western Blotting (WB) for autophagy- and apoptosis-related proteins

The protocol was performed as stated in section 3.3. After protein quantification, 20 µg of protein were loaded and separated on a 12% Bis-Tris SDS-PAGE gel. Then, the proteins were electrophoretic transferred into nitrocellulose membranes (GE Healthcare, UK), and probed with the following primary antibodies: goat anti-Actin (1:2000; sc-1616, Santa Cruz Biotechnology), rabbit anti-Vps34 (1:1000; 3811, Cell Signaling Technology), rabbit anti-p62 (1:2000; BML-PW9860, Enzo Life Sciences), rabbit anti-LC3B (1:1000; 2775, Cell Signaling Technology), rabbit anti-PARP (1:1000; sc-7150, Santa Cruz Biotechnology), and mouse anti-caspase 3 (1:500; 05-654, Upstate). The secondary antibodies used were: goat anti-rabbit IgG-HRP (sc-2004), goat anti-mouse IgG-HRP (sc-2031), or donkey anti-goat IgG-HRP (sc-2020) (all diluted at 1:2000 from Santa Cruz Biotechnology). Signal was then detected in the same manner as in section 3.3, and the intensity of the bands was further analysed using the software Quantity One 1D Analysis (Bio-Rad, USA).

5. Screening of tumour cell growth inhibition activity

5.1. Preparation of stock solutions of compounds to be screened

A total of 8 compounds, synthesised by the Group of Natural Products and Medicinal Chemistry of CIIMAR, Porto, were screened for tumour cell growth inhibitory activity.

The compounds screened were derived from Fiscalin B, a fungal metabolite with a pyrazino[2,1-*b*]quinazoline-3,6-dione core, which was reported to have significant biological activities, namely as chemoterapic [154]. The novel compounds were designated as compounds **4a** to **4d** and **5a** to **5d**. All compounds were reconstituted in sterile DMSO to a final stock concentration of 60 mM, and several aliquots were made and stored at -20 °C to avoid repeated freeze-thaw cycles. For the experiments, the compounds were freshly diluted in complete medium to the desired concentration.

5.2. Screening of compounds for tumour cell growth inhibitory activity using the Sulforhodamine B (SRB) assay

The Sulforhodamine B (SRB) colorimetric assay indirectly infers the cell growth by measuring the cellular protein content. The stoichiometric binding of the die SRB to the basic amino acid residues of the proteins, under mild acidic conditions, is directly proportional to the number of cells.

For this assay, cells were seeded in two 96-well plates, one designated T0 and the other T48 (which means 0 and 48 h after drug treatment). The NCI-H460 and MCF-7 cells were seeded at the density of 5×10^4 cells mL^{-1}, while the seeding concentration of the HCT-15 cells was 1×10^5 cells mL^{-1}. Wells containing medium only were used as a control (to determine the background absorbance). In both plates, all the three cell lines were seeded in duplicate.

Twenty-four hours later, the T0 plate was fixed with 10% (w/v) ice-cold trichloroacetic acid (TCA, Merck, Germany), for 1 hour at 4 °C. Thus, the initial cell concentration could be determined. On the other hand, the compounds were added to all cell lines of the T48 plate, in two-fold serial dilutions ranging from 150 µM to 9.375 µM, and incubated for 48 hours. Doxorubicin (Sigma, USA) was used as a positive control, within the same range of concentrations used for the compounds, except in the HCT15 cell line, where they ranged from 1500 nM to 93.75 nM.

Forty-eight hours later, the T48 plate was fixed as described above for the T0. Then both plates were washed 3 times with distilled water and allow to dry at RT overnight. After that, 50 µL of 0.4% w/v SRB (Sigma-Aldrich, USA) were added to each well, in both plates, and incubated for 30 min at RT. The SRB-stained cells were washed 3 times with 1% v/v acetic acid (Merck, Germany) and the plates were left to dry again, at RT. In the end, protein-bound SRB was solubilized by the addition of 100 µL of 10 mM Tris Base buffer. The plates were agitated to achieve complete dissolution of SRB in each well and the absorbance was measured in a microplate reader (Synergy™ Mx, BioTek Instruments Inc.) at 510 nm. The GI_{50} values (i.e. the concentration that causes 50% inhibition of cell growth) for each compound was determined using an excel datasheet as previously described [164].

5.3. Screening of compounds for drug-efflux inhibitory activity with the Rhodamine-123 (Rh123) accumulation assay

In order to determine the P-gp inhibitory potential of the synthesised compounds, the rhodamine-123 (Rh123) accumulation assay was performed. The K562 and K562Dox cells (seeded at density of 5×10^5 cells/mL) were incubated for 1 h at 37 °C in the presence of 20 μM of the synthesised compounds, and 1 μM of rhodamine-123 (Sigma, USA). Cells (K562Dox and K562) without incubation with any compound were used as negative controls. Additionally, K562Dox cells treated with the P-gp inhibitor verapamil (20 μM, Sigma, USA) were used as a positive control. After incubation, cells were washed and re-suspended in ice-cold PBS, and kept protected from light at 4 °C and immediately analysed in a BD Accuri™ C6 Flow Cytometer (BD Biosciences, USA). At least 10 000 events were counted and analysed per sample. Cells shown in forward scatter and side scatter were electronically gated and acquired through the FL1 channel. Data were analysed using the FlowJo software (version 7.6.1, Tree Star, Inc.) to determine the mean values of FL1 within the gate. The ratio of Rh123 accumulation in the cells was then calculated as (Mean FL1K562Dox + Compound − Mean FL1K562Dox)/Mean FL1K562Dox as previously described [150].

6. Statistical analysis

Statistical analyses were performed using either the GraphPad Prism 6 or the Microsoft Office Excel software. All assays were performed in triplicate unless specified, and the results are reported as mean ± SEM.

Results and discussion

Since the results obtained in this thesis focus on different aspects of MDR, they will be presented in three individual chapters corresponding to the results obtained for the three individual initial aims of the project:

Chapter I: Characterization of the EVs released by the counterpart pairs of drug-sensitive and MDR cell lines

Chapter II: Comparison of the capacity of H460 and RH460 cells to undergo autophagy and apoptosis

Chaper III: Screening of compounds for antitumour and anti-P-gp activity

CHAPTER I

Characterization of the EVs released by the counterpart pairs of drug-sensitive and MDR cell lines

1. Characterization of the EVs released by the counterpart pairs of drug-sensitive and MDR cell lines

Previous results obtained by our group demonstrated that MDR tumour cell lines shed more microvesicle-like EVs (large EVs) and less exosomes (small EVs), than their drug-sensitive counterpart cells [165]. Thus, to confirm the increased production of microvesicles by the MDR tumour cells (when compared to their drug-sensitive counterpart cells), the release of microvesicles was inhibited in both the drug-sensitive and MDR counterpart cells, with the use of a pharmacological inhibitor, imipramine [109]. In addition, the release of exosomes was also inhibited in both cell lines, with 3-MA [143]. With these experiments, it was intended to confirm that the population of EVs released by MDR tumour cells is indeed composed by more microvesicles than exosomes, with different biogenic pathways [89]. In addition, since MDR tumour cells are capable of horizontally transferring the resistant phenotype to drug-sensitive cells, it would be of utmost interest to selectively inhibit the release of EVs from the MDR tumour cells with a pharmacological inhibitor.

To perform this study, two models of counterpart cell lines were used: the non-small cell lung cancer (NSCLC) pair of cell lines, H460 and its MDR counterpart, RH460; and the BCR-ABL positive leukaemia pair of cell lines (obtained from a patient with chronic myeloid leukaemia), K562 and its MDR counterpart, K562Dox. The cell lines H460 and RH460 were treated with two chemical compounds, 3-MA and imipramine which, as stated above, are known modulators of EVs release [109, 143], while the cell lines K562 and K562Dox were only treated with imipramine due to time constrains. The EVs from these cell lines were obtained by several steps of differential centrifugation and characterized in terms of their size, using DLS and NTA. Whenever possible, the isolation of EVs was confirmed by detecting the presence of EV markers (by WB).

The results obtained with both inhibitors (imipramine and 3-MA) will be presented and discussed separately.

1.1. Effect of imipramine (microvesicles formation inhibitor) on EVs released by the counterpart pair of cell lines

In order to inhibit the release of microvesicles by the cells, imipramine was used. Imipramine is an inhibitor of the enzyme acid sphingomyelinase, responsible for the hydrolysis of sphingomyelin (a phospholipid abundant in the outer layer of the plasma membrane). Sphingomyelin hydrolysis results in an increased membrane fluidity [166, 167], thus inducing membrane destabilisation and causing membrane blebbing [168]. Therefore, imipramine causes a decrease in sphingomyelin hydrolysis, being responsible for a limitation of the blebbing of the plasma membrane and thus inhibiting the cellular release of microvesicles [143].

As shown in **Figure 5A**, according to the DLS analysis, imipramine (20 μM) had no effect on the number and size distribution of the EVs released by H460 cells. On other hand, in its MDR counterpart cell line, RH460, the percentage of EVs released by these cells decreased when cells were treated with imipramine. A confirmation that the analysed sample contained EVs was possible by confirming the presence of syntenin-1 (a marker of EVs) in all the vesicles analysed (**Figure 5B**). In addition, the absence of cytochrome c in the isolated vesicles confirmed that there was no contamination of the analysed EVs with cellular debris (**Figure 5B**). Moreover, higher levels of syntenin-1 were detected in the EVs isolated from the H460 cells, when compared with the EVs isolated from the MDR cells, RH460. Since syntenin-1 is an EV marker found primarily in populations of smaller EVs [112], the higher detection of this protein in EVs isolated from the H460 cells is a clear indication that this population of EVs is enriched in EVs of smaller sizes (*i.e.* exosomes).

Therefore, it was confirmed that the release of EVs by the RH460 cells was more affected by imipramine treatment (a microvesicle release inhibitor) than the release of EVs by the H460 cells.

Interestingly, the levels of syntenin-1 were increased in the EVs isolated from either H460 or RH460 cells following treatment with imipramine (**Figure 5B**), suggesting that imipramine treatment increased the release of exosomes in both cell lines. However, as mentioned above (**Figure 5A**), this was only detected in the RH460 cell line when the sizes of the vesicles were analysed by DLS.

Moreover, P-gp was only detected in the RH460 cell line, and not in H460 cells, corroborating the fact that RH460 cells present a MDR phenotype (**Figure 5B**). Although P-gp has been detected by members of our research group in the EVs shed by RH460 cells [165], under this experimental condition it was not possible to detect P-gp in the EVs, probably due to the small amount of protein loaded into the gel for Western blot analysis (5 µg).

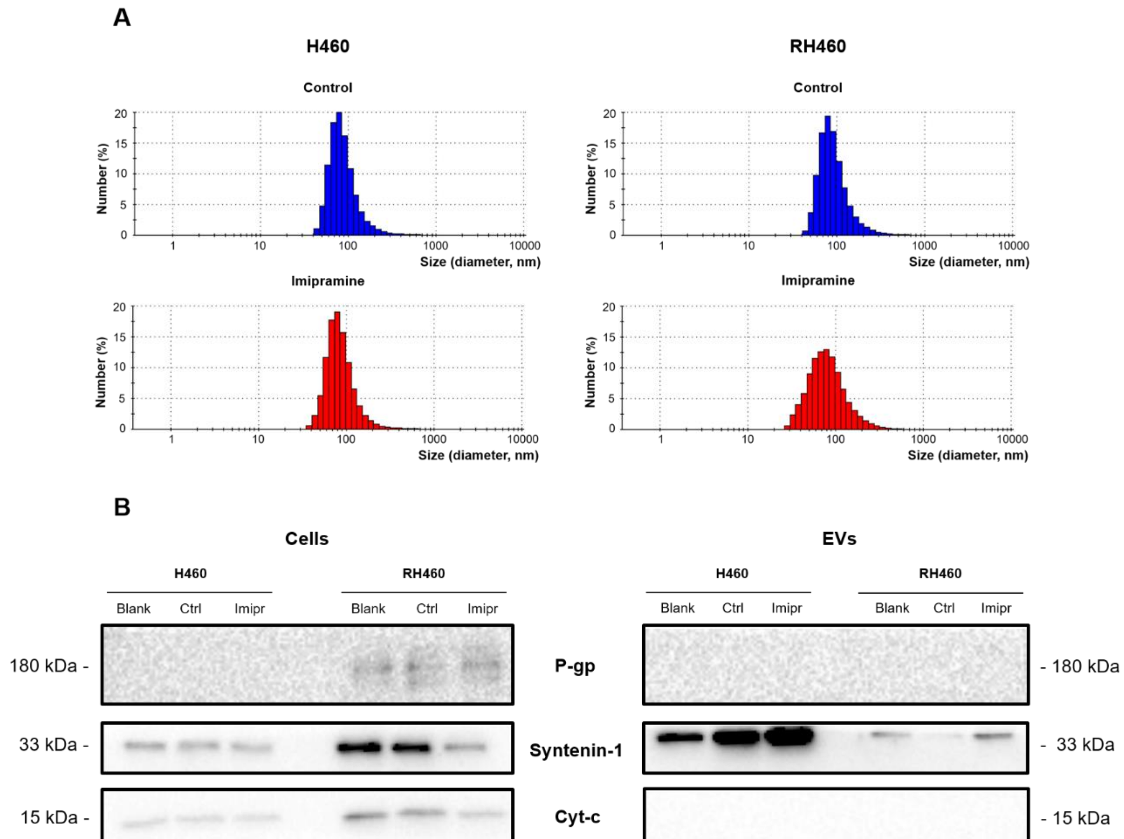

Figure 5 – Treatment with imipramine had no effect in the EVs released by the drug-sensitive H460 cells, but decreased the % of EVs released by its MDR counterpart cells, RH460. (A) Size distribution of EVs isolated from H460 (left panel) and RH460 cells (right panel), treated with water (control, blue) or with 20 µM imipramine (red), analysed by DLS. Results are the mean of 2 independent experiments and were generated with the Zetasizer software v7.12. **(B)** Western blotting analysis of the expression of EV markers in H460 and RH460 cells, and in the EVs released by those cells, with and without treatment with imipramine. Images are cropped blots representative from samples run under the same experimental conditions.

Regarding the results obtained from the leukemia pair of cell lines, the drug-sensitive cell line, K562, released more small-size vesicles when compared with its drug-resistant counterpart cell line, K562Dox. These results are in agreement with the results previously obtained by members of the research group [165]. In addition, imipramine treatment decreased the % of EVs shed by the K562 cells and had a similar effect on

both cell lines regarding a decrease in the size of EVs released by cells (i.e. shift of the DLS curves to the left, **Figure 6**).

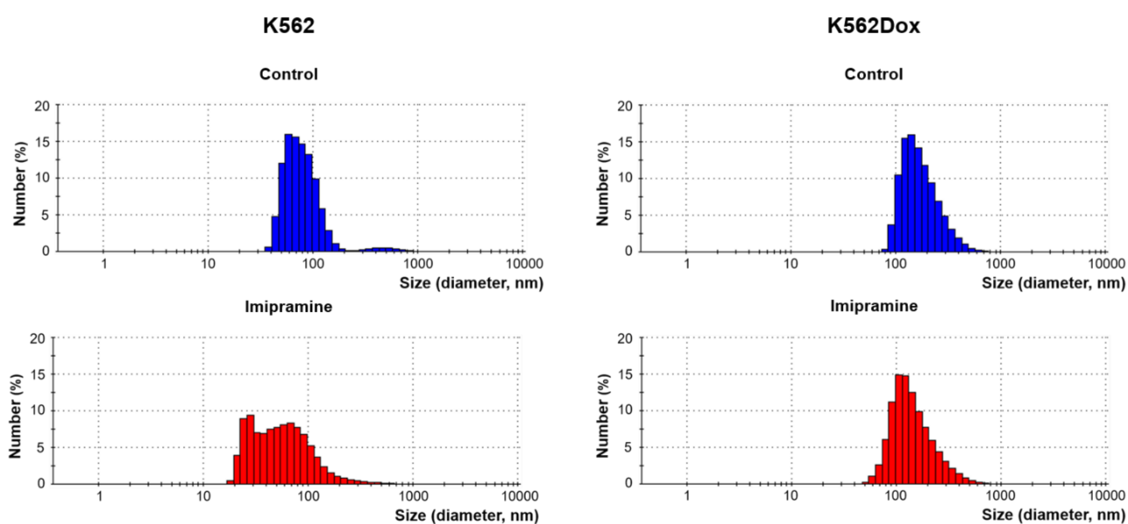

Figure 6 – Treatment with imipramine decreased the % of EVs released by the K562 cells and had no effect on its MDR counterpart cells, K562Dox. The figure refers to the size distribution of EVs isolated from K562 (left panel) and K562Dox cells (right panel), treated with water (control, blue) or with 20 μM imipramine (red), analysed by dynamic light scattering (DLS). Results are the mean of 2 independent experiments and were generated with the Zetasizer software v7.12.

These results suggest that the effect of imipramine was not very different between the 2 cell lines from this leukemic pair, although it was different between the cell lines from the NSCLC pair. The reason for this is unknown but could be related to the levels of P-gp expressed by the resistant counterparts of these two pairs of cell lines. In fact, it has been reported that imipramine is a substract of P-gp [18, 19] and therefore different levels of P-gp would implicate different effect of this drug in the treated cell lines. It is not known if the K562Dox cells express more P-gp than the RH460 cells and and it was not possible to verify this hypothesis since the amount of protein obtained from these vesicles was not enough to allow analysis by WB.

For this same reason (i.e. not enough protein available from the isolated vesicles), it was not possible to confirm by WB that the isolation of EVs was successful.

In summary, given the fact that imipramine is a P-gp substrate, it was concluded that it is not a good inhibitor to use with the objective of verifying the effect of a MVs inhibitor in these pairs of cell lines. However, since 3-MA is not a P-gp substract, it is expected that the results from the 3-MA treatment (section 1.2 bellow) will allow to take more conclusions.

1.2. Effect of 3-MA (endosomal trafficking inhibitor) on EVs released by the counterpart pair of cell lines

The 3-methyladenine (3-MA), a class III PI3K Vps34 inhibitor usually used to inhibit autophagy [169], was previously shown to cause a decrease in exosome release [143, 170]. Indeed, Dardalhon *et al.* found that upon incubation of rat reticulocytes with 3-MA, the detection of transferrin receptor (TfR), known to be released in exosomes during *in vitro* reticulocyte maturation [91], decreased in the extracellular medium, indicating that 3-MA decreased the release of exosomes [143]. Therefore, 3-MA was used in this study as an inhibitor of exosome release. Treatment of the NSCLC pair of cell lines (the sensitive H460 and its MDR counterpart, RH460) with 3-MA was performed in the same manner as described above for the studies with imipramine. Due to time constrains, it was not possible to carry out the 3-MA treatment on the leukemia cell lines, K562 and K562Dox.

The size distribution analysis (by DLS) of the isolated EVs showed that, when treated with 3-MA, both resistant and sensitive counterpart cell lines shed more EVs of smaller sizes when compared to the treatment with the vehicle (DMSO, solvent of the 3-MA). This effect was more noticeable in the EVs isolated from the sensitive (H460) cell line (**Figure 7A**).

The NTA analysis did not show a clear difference in the size of the EVs released by both cell lines following treatment with 3-MA (**Figure 7B**). Indeed, the modal size of the EVs shed by H460 control cells was of 112 nm versus 89 nm following 3-MA treatment; and the size of the EVs released by RH460 control cells was 134 nm versus 123 nm following 3-MA treatment.

However, surprisingly, the NTA data revealed that the concentration of EVs shed by H460 cells treated with 3-MA was highly increased when compared with the control treatment (DMSO) (**Figure 7B** and **C**). In fact, the number of EVs shed per cell was 31 times higher in the H460 cells treated with 3-MA versus the control. The increase in released EVs was of only 5 times in the RH460 cells compared to the control (**Figure 7D**).

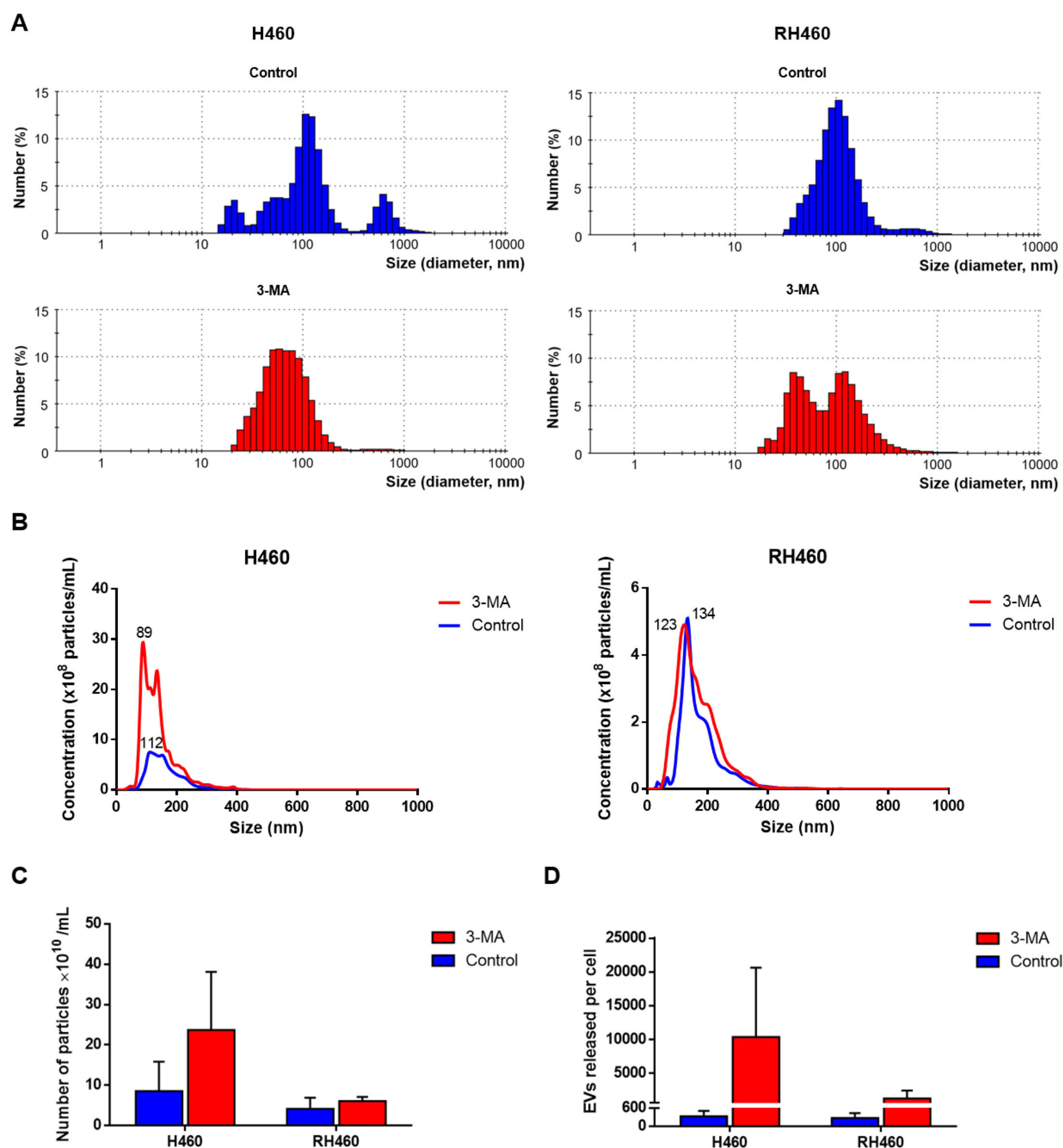

Figure 7 – 3-MA treatment did not greatly affect the size of the EVs released by either the sensitive (H460) or the MDR (RH460) cells but greatly increased the number of EVs released by the sensitive cells (H460). (A) Size distribution of EVs isolated from the H460 (left panel) and the RH460 cells (right panel), treated with the vehicle (DMSO, as control) or with 0.5 mM of 3-MA, analysed by dynamic light scattering (DLS). Results are the mean of 3 independent experiments and were generated by the Zetasizer software v7.12. **(B)** Particles concentration were determined by nanoparticle tracking analysis (NTA) in the populations of EVs isolated from the H460 cells (left panel) and RH460 cells (right panel), treated with DMSO (control) or 3-MA. The modal sizes were of 112 nm and 134 nm for H460 and RH460 control cells, and of 89 nm and 123 nm for H460 and RH460 cells treated with 3-MA, respectively. **(C)** Concentration of EVs released by H460 and RH460 cells, when treated with 3-MA vs control (DMSO). **(D)** EVs released per cell (H460 and RH460), when treated with 3-MA vs control (DMSO).

The observed differences in the number of EVs released by the sensitive and resistant cells following 3-MA treatment was not expected. A possible justification is related to the effect of 3-MA in cellular autophagy.

Indeed, other authors previously described that autophagy induction (by either starvation, rapamycin or LC3 overexpression in drug-sensitive cells) inhibits exosome release, suggesting that there is a correlation between autophagy and exosomes production. In fact, when autophagy is stimulated, multivesicular bodies (MVBs) are guided to the autophagic pathway, with a consequent decrease in the release of exosomes into the extracellular medium [171, 172].

On the contrary, the inhibition of autophagy in drug-sensitive cells, either pharmacologically or by silencing key players of the autophagic flux, causes an increase in the release of exosomes by cells. Indeed, the use of 3-MA (and other autophagic inhibitors) as inducer(s) of exosomes release has been patented by other authors [173].

This effect of 3-MA in exosomes release might be due to the fact that it causes blockage in the autophagosome maturation [174], which would culminate in the accumulation of cellular waste. It is possible that cells try to eliminate the accumulated waste (when there is autophagy inhibition) by exporting the waste towards the outside of the cell, doing so by increasing the release of exosomes. On other hand, when cells are under cellular starvation conditions, and the equilibrium is shifted towards more autophagic degradation, a reduced biogenesis of exosomes occurs. Under such conditions, the building blocks of the cell are already scarce and it is favourable to the cell to use them in its essential organelles, and not to release them into the extracellular medium, thereby causing a decrease in the exosomes released.

The fusion of MVBs with autophagic vacuoles when the cell is under starvation suggests that the balance between autophagy induction and exosome release might be regulated by the metabolic state of the cell. It has been shown that there are differences between the metabolism of drug-sensitive cancer cell lines and their counterpart MDR cells [175]. Therefore, these metabolic differences might explain why H460 cells released more EVs upon treatment with 3-MA, when compared to RH460 cells.

One possible justification would be that RH460 cells have impaired autophagy, thus producing more EVs than their drug-sensitive counterpart cells, under control conditions. If that was the case, treatment with 3-MA (an autophagy inhibitor) would not cause an increase in the number of EVs released by those cells, since the basal levels of autophagy were already low. In other words, a difference in the basal levels of autophagy between the resistant and the sensitive counterpart cell lines might explain

the observed difference in the amount of EVs released by the two cell lines upon treatment with 3-MA.

Of note, although other authors have previously shown that 3-MA increases the release of exosomes by cells, to our knowledge this is the first report that there is a difference between sensitive and MDR counterpart cells in the release of EVs following 3-MA treatment. Therefore, to confirm the above stated hypothesis, further work was carried out in order to compare the autophagic levels of the sensitive (H460) and resistant (RH460) cell lines, both under basal conditions and under conditions of induction of autophagy.

CHAPTER II

Comparison of the capacity of H460 and RH460 cells to undergo autophagy and apoptosis

2. Autophagic levels of H460 and RH460 cell lines

The basal expression of three known autophagic markers, Vps34, p62 and LC3, in normal cell growth conditions was studied.

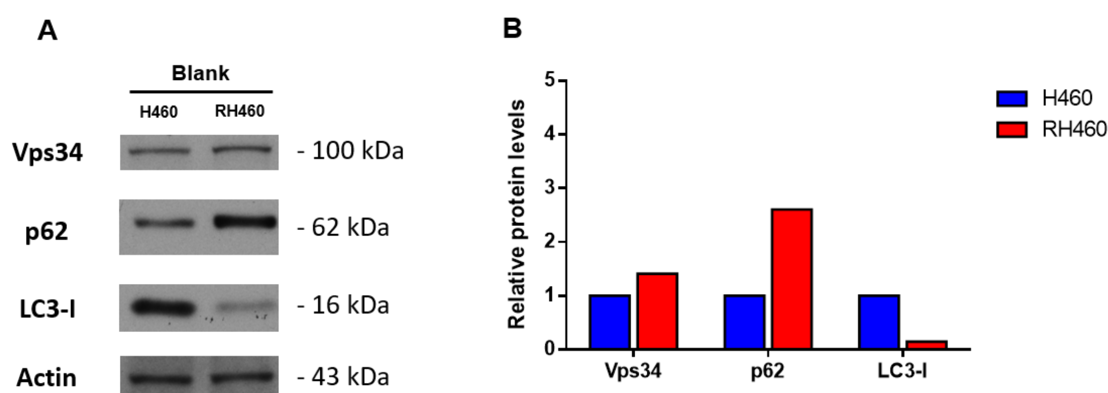

Figure 8 – Basal levels of autophagy were higher in sensitive (H460) cells in comparison with their MDR counterpart cells RH460. (A) Basal levels of autophagic markers were analysed by Western blot in both cell lines, H460 and RH460. Image refers to crop blots from samples run under the same experimental conditions and is representative of two independent experiments. Actin was used as loading control. **(B)** Densitometry analysis. Results are represented as relative protein expression levels of the MDR tumour cells (RH460) compared with their drug-sensitive cells (H460).

Vps34 is a class III PI3K involved in the formation step of the autophagosome, one of the first steps of the autophagic process [176]. The expression levels of Vps34 were very similar in both cell lines under basal conditions, as depicted in **Figure 8**. In addition, when cells are treated with the autophagic inhibitor 3-MA, there was a tendency for Vps34 levels to increase in the H460 cells, while remaining equal in the RH460 cells (**Figure 9**). However, this difference was very small and more experiments should be done to statistically prove it. Nevertheless, it is important to highlight that 3-MA is a competitive inhibitor that binds to the ATP binding pocket of the Vps34, thereby not having a direct impact in its expression [169]. Therefore, Vps34 levels are expected to

be maintained upon the use of 3-MA. Moreover, when autophagy was induced by either serum-starvation or rapamycin, the levels of Vps34 were approximately equal, except in the RH460 cells treated with rapamycin, in which there was an increase in the levels of Vps34. More experiments are needed to address the statistical significance of this tendency.

The p62 and LC3 have been well established in the literature as markers of the autophagic flux [82]. According to the literature, suppression of autophagy results in an increased intracellular accumulation of p62 as detectable by Western blots [177, 178]. The p62, also known as Sequestosome-1 (SQSTM1), is a multifunctional adaptor protein that acts as cargo receptor targeting specific poly-ubiquitinated substrates (*e.g.* protein aggregates) to autophagy, being also degraded during this process [73, 179]. The obtained results when comparing the sensitive with the MDR cell lines show that, under basal conditions, p62 levels are higher in the RH460 cell line (**Figure 8**), suggesting that the resistant cell line has lower basal levels of autophagy. Furthermore, when autophagy was induced by serum-starvation (*i.e.* with the use of RPMI-1640 medium without supplementation with FBS), the levels of p62 decreased pronouncedly in H460 cells but remained the same in RH460 cells (**Figure 9**). On other hand, rapamycin treatment decreased the expression levels of p62 in H460 cells only (slightly increasing p62 levels in the RH460 cells). Taken together, these results suggest that the MDR cell line might have a blockage in the autophagic flux. However, more studies need to be performed in order to confirm this hypothesis, such as treating the cells with late stage inhibitors of autophagy, e.g. hydroxychloroquine or bafilomycin A1. These endosomal acidification inhibitors increase the lysosomal pH, through inhibition of the Na^+ H^+ pumps at the lysosome, consequently inhibiting the degradation of the autolysosome content [82]. Therefore, in order to confirm that there is a blockage in the autophagic flux in the RH460 cells but not in the H460 cells, treatment with bafilomycin A1 or hydroxychloroquine should cause an increase in the accumulation of p62 and LC3-II in the H460 cells, but not in the RH460 cells. Future work will be carried out (by other elements of the research group) in order to confirm this hypothesis. The differences observed between the rapamycin- and the serum-starvation-induced levels of p62, may be due to the fact that the withdrawal of serum might not be enough to consistently induce autophagy. Therefore, future experiments should be performed using a solution that also lacks amino acids (such as the Hank's Balanced Salt Solution (HBSS) or Earle's Balanced Salt Solution (EBSS)) in order to induce autophagy.

LC3, also known as microtubule-associated protein 1A/1B-light chain 3, is a soluble protein ubiquitously expressed in all mammalian cells [180]. During autophagy LC3 is activated by Atg4 originating LC3-I, which is then conjugated to phosphatidylethanolamine (PE) forming LC3-phosphatidylethanolamine conjugate (LC3-II). LC3-II is then recruited to the membrane of the autophagosome being found on both internal and external surfaces of the autophagosome, where it plays a role in hemifusion of membranes, as well as, in selecting cargo for degradation by interacting with p62. Autophagosomes are then fused with lysosomes forming autolysosomes, where the intraluminal content is degraded by lysosomal hydrolases, together with LC3-II present in the inner membrane. Therefore, since the turnover of LC3-II reflects the autophagic activity of the cell, its detection by Western blot is also used for monitoring the autophagic flux together with p62 [82]. Nonetheless, in the present work only LC3-I was detected, even though the antibody used (Cell Signalling, #2775) is specific for the autophagy-related LC3B isoform and has stronger reactivity to the LC3-II. More optimizations (*e.g.* using another antibody or increasing the incubation time) will be carried out in the future, to detect LC3-II. Nevertheless, the results obtained with LC3-I were analysed. It was observed that the basal levels of LC3-I were lower in the RH460 cell line, suggesting an increase in autophagy in the RH460 cells (by inferring that a decrease in LC3-I will correspond to an increase in LC3-II), which is in contradiction to what was suggested by the p62 results. In addition, when autophagy was induced either by serum-starvation or rapamycin treatment, the levels of LC3-I decreased, mostly in the RH460 cells. In summary, more work is necessary to study the levels of LC3-II and allow taking conclusions on the autophagic flux of these cell lines.

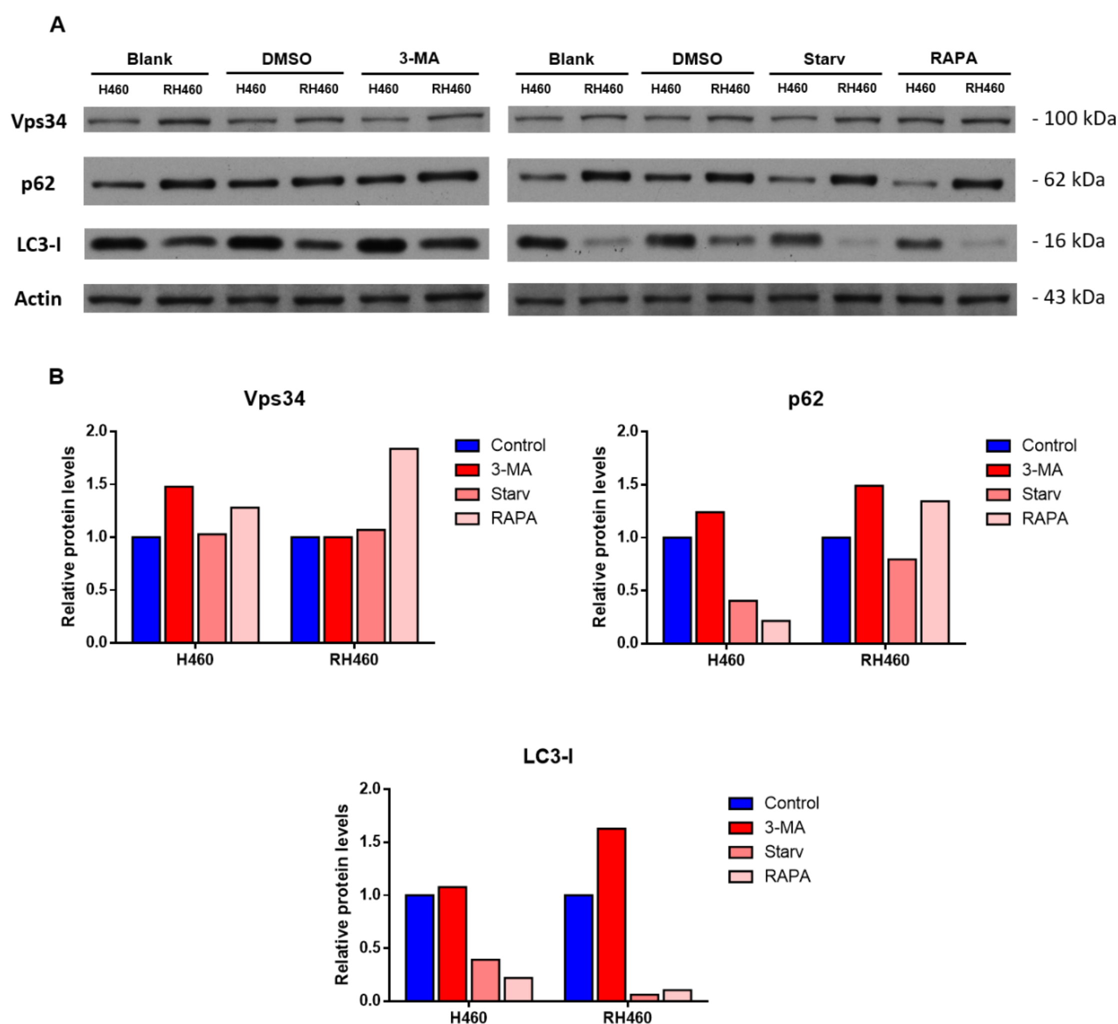

Figure 9 – Levels of the autophagic markers in H460 and RH460 cells, under basal conditions and conditions which modulate autophagy levels, suggest that autophagy might be impaired in the RH460 cell line. (A) Western blot analysis of the levels of the autophagy-related proteins Vps34, p62, and LC3-I in H460 and RH460 cell lines, following treatment with 0.5 mM 3-MA, 100 nM rapamycin (RAPA) and serum-starvation (Starv). Image refers to crop blots from samples run under the same experimental conditions and are representative of two independent experiments. Actin was used as loading control. **(B)** Results of the densitometry analysis performed are represented as relative protein expression levels of the different treatments in both cell lines, relatively to the control condition (DMSO).

Extensive studies indicate that autophagy plays a controversial role in cancer cells, either promoting their survival or causing their death [181, 182]. The double face of autophagy in tumour cell survival is complex, depending, to a great extent, on the type of tumour, stage of disease, and nature of the treatment. In addition, a blockage in autophagy could contribute to survival under drug treatment, i.e. could contribute to drug resistance [86]. In fact, Kim *et al.*, reported that induction of autophagy in H460 cells enhances the efficacy of radiation therapy *in vitro*, which suggests the potential of autophagy to overcome MDR [183]. Interestingly, there are also studies demonstrating

that autophagy is required to kill MDR tumour cells [184]. In conclusion, our preliminary results suggest that the RH460 cells have impaired autophagy when compared to the parental, H460 cells. One possible justification is that the impairment of the autophagic flux might be an additional mechanism of drug resistance of these cells (even though the cells are known to express P-gp as a main mechanism of drug resistance).

It is known that autophagy suppresses tumours by cooperating with apoptosis to cause cell death, and thus a blockage in autophagy could partially attenuate the apoptotic process [185-187]. Therefore, if RH460 cells have defective autophagy (as suggested by the above shown results) they might also have reduced capacity to undergo apoptosis. Therefore, further work was carried out intending to compare the capacity of sensitive (H460) resistant (RH460) cells to undergo apoptosis.

3. Apoptotic levels of the H460 and RH460 cell lines

Autophagy and apoptosis are two tightly regulated and interconnected biological mechanisms [188]. Wirawan et al. reported that caspases are able to cleave Beclin 1, thereby destroying its pro-autophagic activity [188]. Moreover, the C-terminal fragment resultant of the cleavage of Beclin 1 acquires a new function, being capable of amplifying mitochondria-mediated apoptosis [188]. These findings show evidence of an existing molecular cross-talk between autophagy and apoptosis, therefore making it relevant to compare the levels of apoptosis between the H460 and RH460 cell lines, in the context of this thesis.

The cleavage of PARP by caspases is considered a hallmark of apoptosis [189, 190]. The most frequent apoptotic pathway triggered by cellular treatment with drugs is the mitochondrial pathway, with activation of caspase-3 by cleavage of pro-caspase 3 [191, 192]. Once activated, caspase-3 is able to cleave several cellular substrates committing the cell to apoptotic death [193, 194]. Thereby, a comparison of the basal levels of expression of both PARP and caspase-3 was carried out in both cell lines (H460 and RH460). It was observed that the full-length PARP levels were similar between both cell lines. However, pro-caspase 3 levels were decreased in the RH460 cell line, suggesting that the levels of caspase 3 were increased in this resistant cell line (**Figure 10**). However, this analysis needs to be confirmed by repeating the experiment and carrying out statistical analysis. Unfortunately, the bands for cleaved PARP and caspase-3 were not detected in the Western blots. These results suggest that the levels of caspase-3 might be increased in the resistant cell line and therefore that the basal levels

of apoptosis might be increased in this cell line. This could be related to the lower levels of autophagy previously found in this cell line. Future work will confirm these results by looking at apoptosis levels in both cell lines with the Annexin V/PI assay.

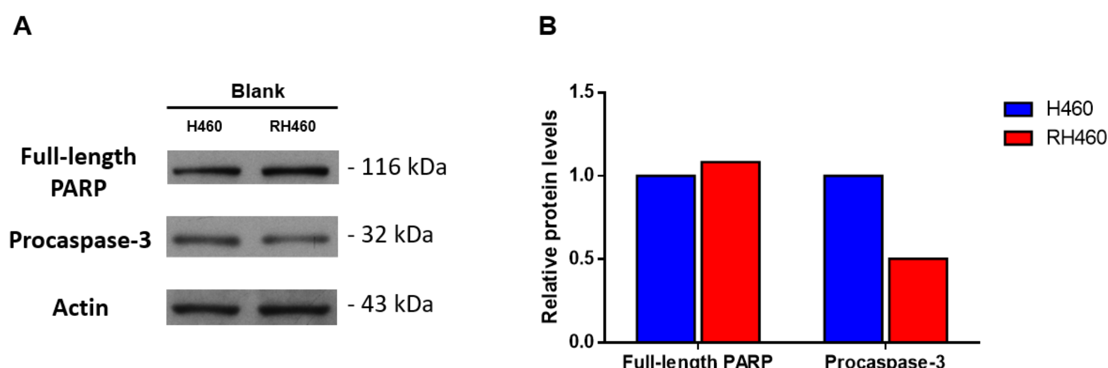

Figure 10 – Full-length PARP basal levels are identical between both cell lines, but pro-caspase 3 is less expressed in the RH460 cell line. (A) Basal levels of the apoptotic markers, PARP and pro-caspase 3 were analysed by Western blotting in both cell lines, H460 and RH460. Image refers to crop blots from samples run under the same experimental conditions. Actin was used as loading control. **(B)** Results of the densitometry analysis performed and are from one experiment only. These are represented as relative protein expression levels of the MDR tumour cells (RH460) when compared to the levels of the drug-sensitive counterpart cells (H460).

Results show that treatment with 3-MA caused a minor effect on both full-length PARP and pro-caspase 3 in both cell lines. Serum-starvation decreased the levels of full-length PARP and pro-caspase 3 in the H460 cell line, reducing them to approximately half, suggesting that caspase-3 was activated in this sensitive cell line. However, the effect was not as evident in the resistant cell line (particularly regarding the pro-caspase 3 levels, **Figure 11**). Rapamycin treatment did not have an effect on the levels of full-length PARP in the sensitive cell line while it increased the levels in the resistant cell line. However, rapamycin caused a similar decrease in pro-caspase-3 levels in both cell lines.

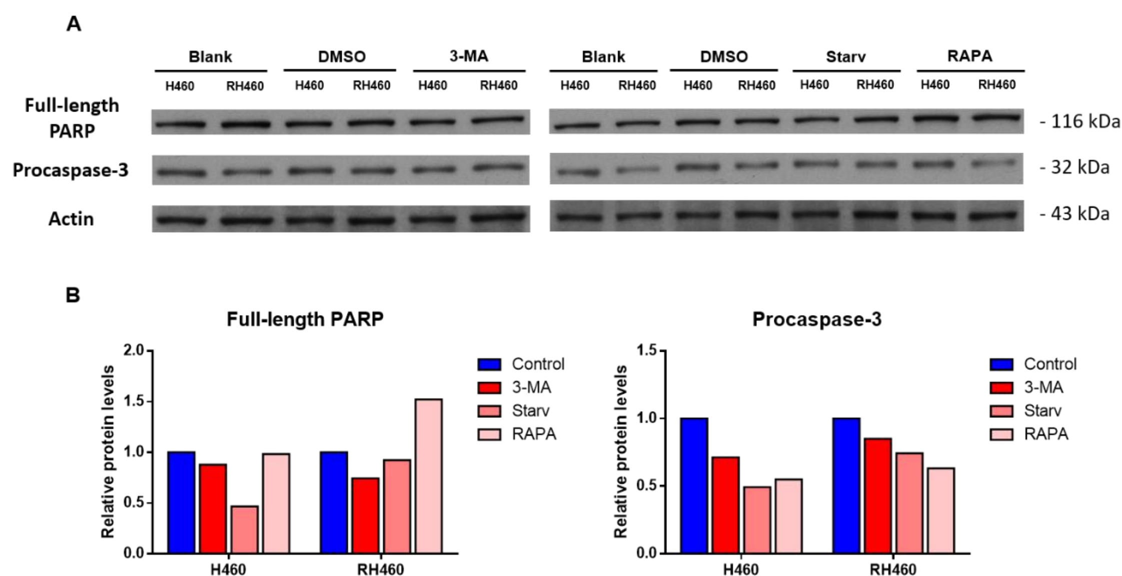

Figure 11 – Alterations in full-length PARP and pro-caspase 3 following administration of the compounds previously used to study autophagy of H460 and RH460 cell lines. (A) Apoptotic markers, PARP and caspase-3 were analysed by Western blot in both cell lines, H460 and RH460, following treatment with DMSO (control), 0.5 mM 3-MA, 100 nM rapamycin (RAPA) or serum-starvation (Starv). Only the full-length PARP and pro-caspase 3 were detected with the antibodies used. Image refers to crop blots from samples run under the same experimental conditions. Actin was used as loading control. **(B)** Results of the densitometry analysis performed are represented as relative protein expression levels in both cell lines, compared to the control treatment (DMSO). Results are represented and are from one experiment only.

Overall, there were no major differences found between the two counterpart cell lines regarding the levels of these apoptotic proteins following treatment with these modulators of autophagy, except for an unexpected increase in full-length PARP in the resistant cell line following rapamycin treatment. The reason for this increase is not known.

CHAPTER III

Screening of compounds for antitumour and anti-P-gp activity

4. Screening of compounds for antitumour and anti-P-gp activity

New bioactive compounds are discovered and isolated from natural sources daily, however their structure elucidation remains a challenge [154]. To save time and money, an alternative strategy consists in creating libraries of synthetic analogues of bioactive natural compounds. These compounds are then evaluated regarding their bioactive properties. In cancer research, their cytotoxic properties are usually evaluated in a panel of different cancer cell lines. Other bioactivities of the compounds may be studied.

This work intended to study the activity of compounds with potential as antitumour and also as inhibitors of P-gp from a small library synthesised by collaborators from CIIMAR. If potent, such compounds might have potential to inhibit the growth of human tumour cells and/or inhibit P-gp activity and therefore revert the MDR phenotype.

The studied library of compounds were fiscalin B (**4c**) and derivatives, containing a pyrazino[2,1-*b*]quinazoline-3,6-dione ring system, a key structural fragment present in metabolites isolated primarily from fungi [195]. One example is the peptide alkaloid *N*-acetylardeemin, a fungal metabolite isolated from *Aspergillus fischeri* (var. brasiliensis) that is a very potent inhibitor of MDR (IC_{50} = 5-30 μM). This compound is able to chemosensitize tumour cells against the effect of antitumor drugs, such as vinblastine, doxorubicin or paclitaxel, being even 10-fold more effectively than verapamil, while presenting low toxicity [196].

Therefore, taking into account the importance of these type of compounds, the pyrazino[2,1-*b*]quinazoline-3,6-dione fiscalin B (**4c**), a secondary metabolite initially isolated from the fungi *Neosartorya fischeri* and *Corynascus setosus*, and reported to have significant biological activities [197], was synthesised by our collaborators of CIIMAR. In addition, several other analogues (**4a**, **4b**, **4d**, **5a**, **5b**, **5c** and **5d**) were also synthesised and their potential antitumoral activity was assessed in the framework of this thesis. The IUPAC nomenclature and structure of the analogue compounds cannot be shown in this work for confidentiality reasons. Thus, the compounds were designated

according to the side chain that they possess at C1, being divided in groups **4** and **5.** They are also stereoisomers between themselves, meaning that **4a** is an enantiomer of **4d**, and **4b** is an enantiomer of **4c** while **5a** is an enantiomer of **5d**, and **5b** is an enantiomer of **5c**.

4.1. Antitumoral effect of the compounds

In order to test the cytotoxic potential of the synthesised compounds, the sulforhodamine B (SRB) colorimetric assay was carried out. The GI_{50} of each compound was determined in three different human tumour cell lines that represent models of the three most common cancers worldwide: non-small cell lung cancer (H460), colon adenocarcinoma (HCT15) and breast cancer (MCF7) [1]. The results are summarized in **Table 1** and **Annexes A** to **H**.

Table 1

GI_{50} concentrations of the synthesised compounds in the non-small cell lung cancer (H460), colorectal adenocarcinoma (HCT15) and breast cancer (MCF7) cell lines.

	GI_{50} (μM)		
	H460	**HCT15**	**MCF7**
4a	81.33 ± 1.55	40.33 ± 3.12	81.29 ± 2.34
4b	70.20 ± 3.15	38.15 ± 0.29	74.26 ± 2.43
4c	57.62 ± 2.08	31.78 ± 1.21	64.71 ± 1.05
4d	60.10 ± 2.61	33.30 ± 1.37	57.65 ± 3.62
5a	32.52 ± 4.24	48.18 ± 2.51	37.04 ± 2.09
5b	41.52 ± 2.52	51.94 ± 4.26	45.56 ± 2.29
5c	31.19 ± 3.01	43.63 ± 0.25	34.25 ± 2.53
5d	36.47 ± 3.98	47.00 ± 1.47	39.71 ± 3.60

Values were determined with the SRB assay and are the mean ± SEM of 3 independent experiments. Doxorubicin was used as a positive control, and its GI_{50} concentrations were the following: 23.02 ± 0.54 nM in H460 cells, 331.49 ± 39.44 nM in HCT15 cells and 19.67 ± 0.62 nM in MCF7 cells.

The synthesised compounds demonstrated moderate cytotoxic effects, presenting GI_{50} concentrations ranging from 30 to 80 μM. Noteworthy, some differences were obtained between the groups of compounds **4** and **5**. The cell lines H460 and MCF7 presented GI_{50} values higher for compounds from group **4**, while the GI_{50} values for the HCT15 cell line were higher for compounds from group **5**.

A compound is usually considered a good cytotoxic if its *in vitro* potency is in the nanomolar range, under the assumption that it has more efficacy and safety when used at lower doses, a principle known as "the nanomolar rule" [198]. Nevertheless, this rule has its own limitations, only taking into account the *in vitro* potency of the compounds and not other parameters that may contribute to its potential efficacy and toxicity, such as the physiochemical, pharmacokinetic and toxicological properties. Several antitumour compounds, such as alkylating agents, hormonal agents, antimetabolites, thalidomide, or valproic acid, which have milimolar potency, are good examples of this [199, 200]. On other hand, compounds that present potencies in the nanomolar range are not necessarily good for clinical use. For example, the cyclooxygenase-2 inhibitors have an in *vitro* efficacy in the nanomolar range (Celecoxib, $IC_{50} = 0.54 \pm 0.07$ μM; Valdecoxib, $IC_{50} = 0.65 \pm 0.06$ μM; Rofecoxib, $IC_{50} = 0.18 \pm 0.03$ μM [201]), but either failed in clinical trials or were proved to be unsafe [202-204]. Therefore, the compounds tested in this work only presented moderate cytotoxic activity but more studies should be carried out in order to fully assess their bioactive potential.

4.2. Effect of the compounds on P-gp activity

The compounds were also studied for their possible modulatory activity of P-gp, a drug efflux pump associated with drug resistance, since they resembled other compounds with published activity on MDR [196]. Thus, to determine P-gp activity of the compounds, an assay was used which measures the mean fluorescence intensity of cells treated concomitantly with rhodamine 123 (Rh123), a known substrate of the P-gp (that acts as its functional reporter). Rh123 is a cell-permeant, cationic, green-fluorescent dye that is sequestered in active mitochondria inside the cells. In the case of cells with a MDR phenotype due to P-gp overexpression, Rh123 is effluxed from cells. Thus, if a compound inhibits P-gp, then Rh123 will accumulate inside the cells and this may be detected by flow cytometry.

So, in order to analyse the possible P-gp inhibitory activity of the synthesised compounds, drug resistant cells (K562Dox) were treated concomitantly with Rh123 and

each of the compounds. The drug-sensitive cells (K562) were used as control. After incubation, cells were washed, and the fluorescence of Rh123 detected by flow cytometry. Since the K562Dox cell line overexpresses P-gp, it is possible to infer that differences in the accumulation pattern of Rh123 between treatments is due to the modulation of P-gp.

The results of the effect of the tested compounds (**4a**, **4b**, **4c**, **4d**, **5a**, **5b** and **5d**) on the Rh123 accumulation ratio are presented in **Figure 12**.

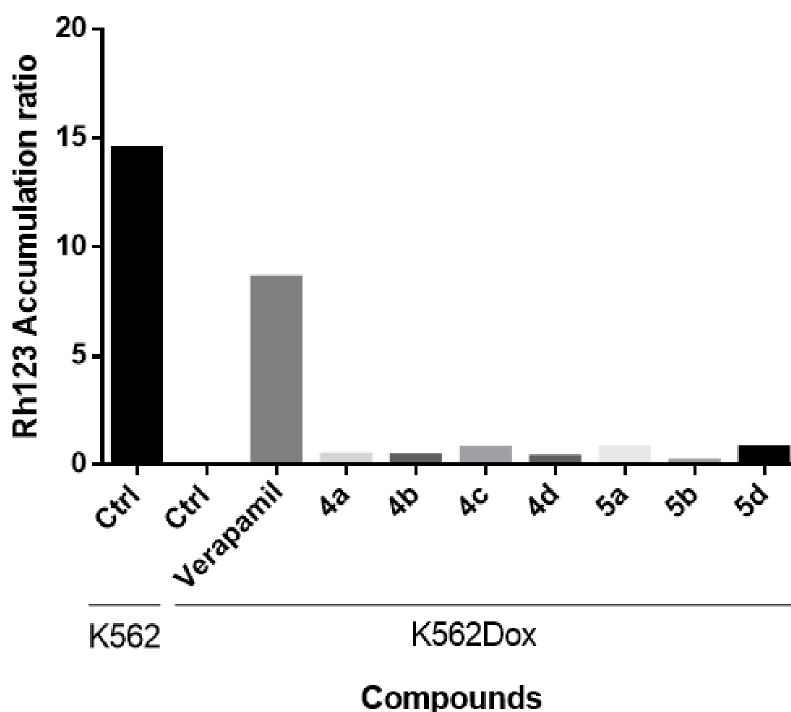

Figure 12 – The tested compounds did not show effect on the accumulation ratio of Rh123 in K562Dox cells. Cells were incubated for 1 h with the synthesised compounds (**4a**, **4b**, **4c**, **4d**, **5a**, **5b** and **5d**), at a final concentration of 20 μM. Verapamil (20 μM) was used as a positive control (a known P-gp inhibitor). The K562 cell line that does not overexpress P-gp (K562 Ctrl) was also used as control. The accumulation ratio in the untreated K562Dox cells was defined as zero (K562Dox Ctrl).

According to the **Figure 12**, none of the synthesised compounds presented an effect in the modulation of P-gp activity. The positive pharmacological control, verapamil (a known P-gp inhibitor [205]), significantly increased the accumulation of Rh123 in the K562Dox cells. In addition, the use of the drug-sensitive K562 cells (without P-gp overexpression) confirmed that the assay was well performed.

Conclusions and Future Perspectives

This project aimed to confirm the release of larger EVs by MDR tumour cells when compared to drug-sensitive counterpart cells, which was recently discovered by members of the research group that supervised this work. It is known that EVs may be horizontally transferred from MDR to drug-sensitive tumour cells, thus inducing a MDR phenotype in the recipient cells. Therefore, it is important to investigate possible differences in the EVs released by DS and MDR tumour cells, in order to find biomarkers or new molecular targets that might contribute to recognizing and overcoming MDR.

In this work it was found that inhibition of the release of microvesicles using imipramine, had different effects in the two counterpart pairs of NSCLC and leukemia cell lines used. The release of EVs by the RH460 cells was more affected by imipramine treatment than the release by the H460 cells. However, in the leukemia cell lines, the effect of imipramine was not very different between the MDR and DS cell lines. Since imipramine is a substrate of P-gp, this could possibly be related to different levels of P-gp expression by the resistant counterparts of these two pairs of cell lines. Therefore, in future experiments will be more appropriated to use an inhibitor that is not a substrate of P-gp.

Moreover, 3-MA, which was used in this work as an inhibitor of exosomes release, surprisingly led to an increase in the number of EVs shed by DS NSCLC cells. The effect of 3-MA on the release of EVs by cells has been previously described in the literature. However, to our knowledge, this is the first report of a different effect of 3-MA on the release of EVs by MDR or drug-sensitive counterpart cells. Since 3-MA is also an autophagy inhibitor, it was here hypothesised that the different effect of 3-MA on EVs release may be due to different basal levels of autophagy between these counterpart cell lines. Therefore, studies were conducted to investigate if there were differences in the basal levels of autophagy between the counterpart NSCLC cell lines. Results suggested that the MDR cell line (RH460) has an impaired autophagic flux, while the DS cell line (H460) has a functional autophagy. Therefore, it is possible that the autophagy process is related to the production of EVs and that since the basal levels of autophagy in the MDR cells are very low, then 3-MA will not induce the release of EVs in those cells. On the other hand, in the sensitive cells with efficient autophagy, treatment with 3-MA will reduce autophagy and cells will then increase the release of EVs, possibly as a way to eliminate cellular waste. This hypothesis is schematically represented in **Figure 13**. However, further work would be necessary to confirm this hypothesis.

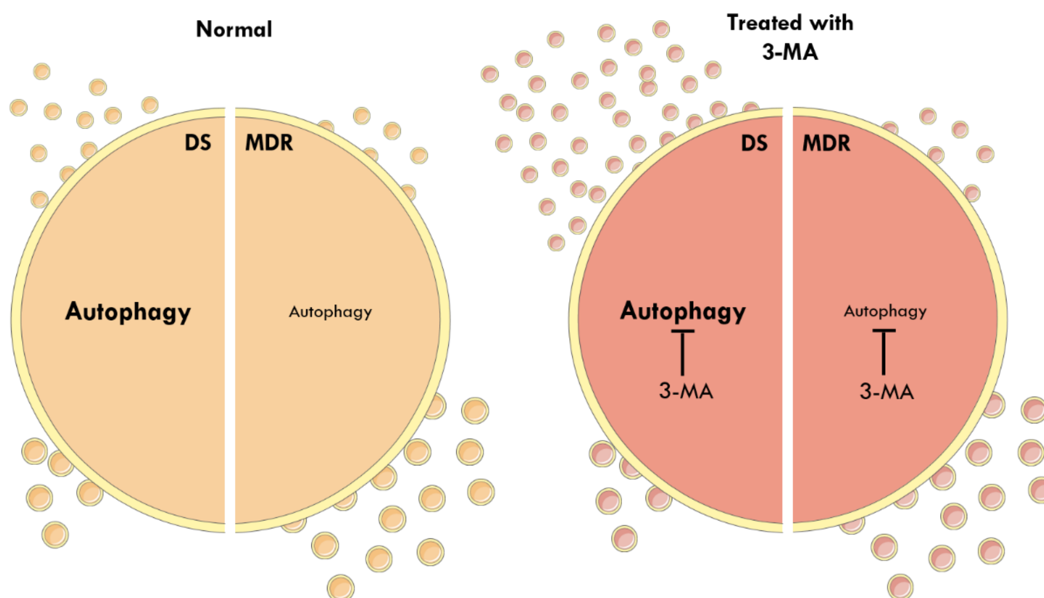

Figure 13 – Schematic representation of the hypothesis relating the release of EVs and autophagy levels. In this model, the MDR cell line has an impaired autophagic flux, while the DS cell line has a functional autophagy. Since the basal levels of autophagy in the MDR cells are very low, then 3-MA will not induce the release of EVs by those cells. In the sensitive cells with efficient autophagy, treatment with 3-MA will reduce autophagy and cells will then increase the release of EVs, possibly as a way to eliminate cellular waste.

The discovery of new molecules with antitumoral and P-gp inhibitory activity is essential, to inhibit the growth of tumour cells and to counteract the MDR phenotype. In this work several quinazolinone derivatives were tested regarding antitumoral and P-gp inhibitory activities. Results showed that none of the tested compounds presented potent antitumour effect in the three cell lines studied, having GI_{50} values higher than 30 μM. In addition, none of the compounds presented an inhibitory effect on the activity of P-gp.

In conclusion, the mechanisms behind the release of larger vesicles by MDR cells need to be further confirmed and more studies are required to further investigate these mechanisms. However, the results presented in this thesis suggest that there is a connection between autophagy and the release of EVs by cells. In addition, results also suggest that MDR cells with impaired autophagy do not release much more EVs to eliminate cellular waste, contrary to what happens in drug-sensitive cells with efficient autophagy.

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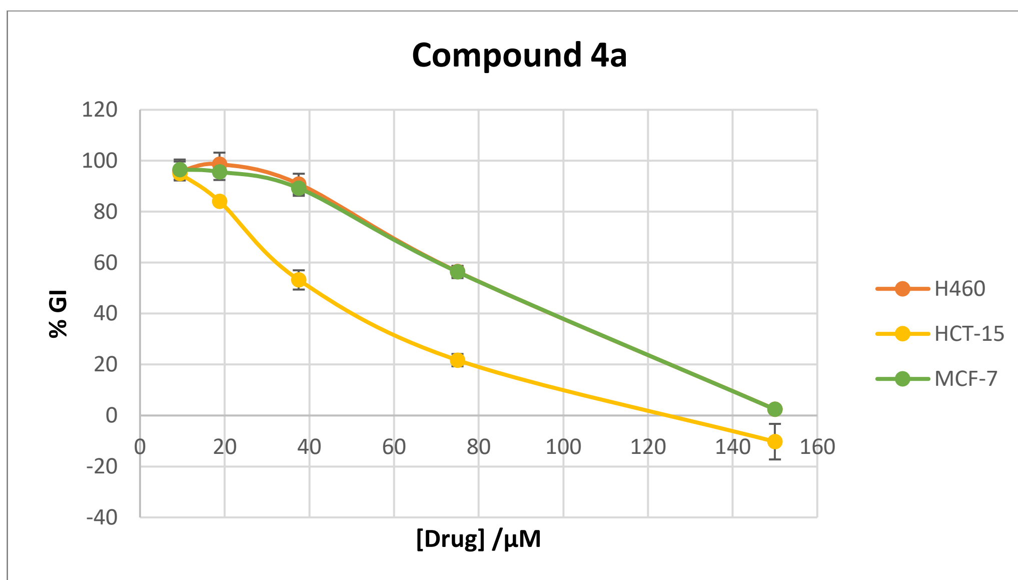
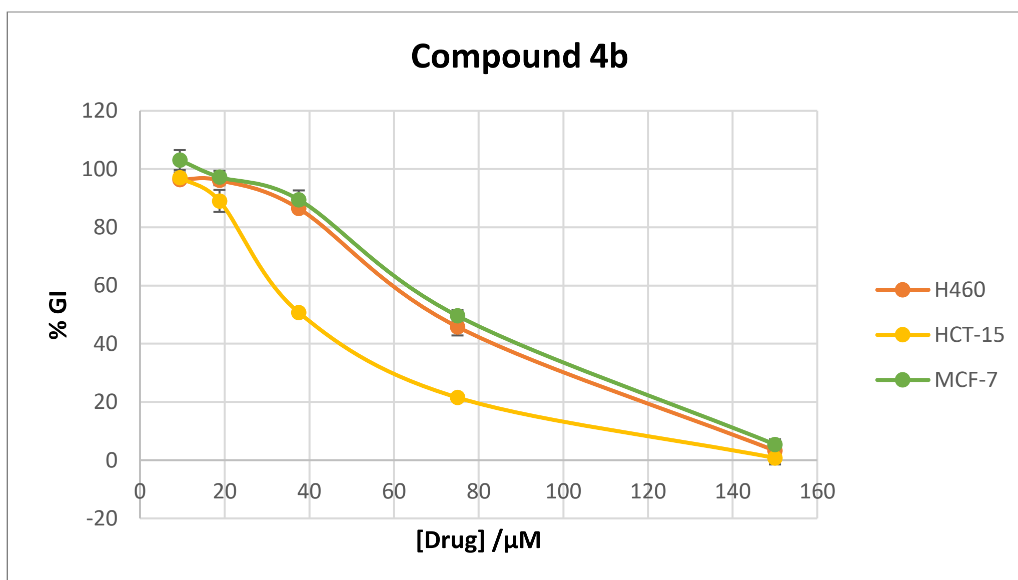
Annexes

Annex A



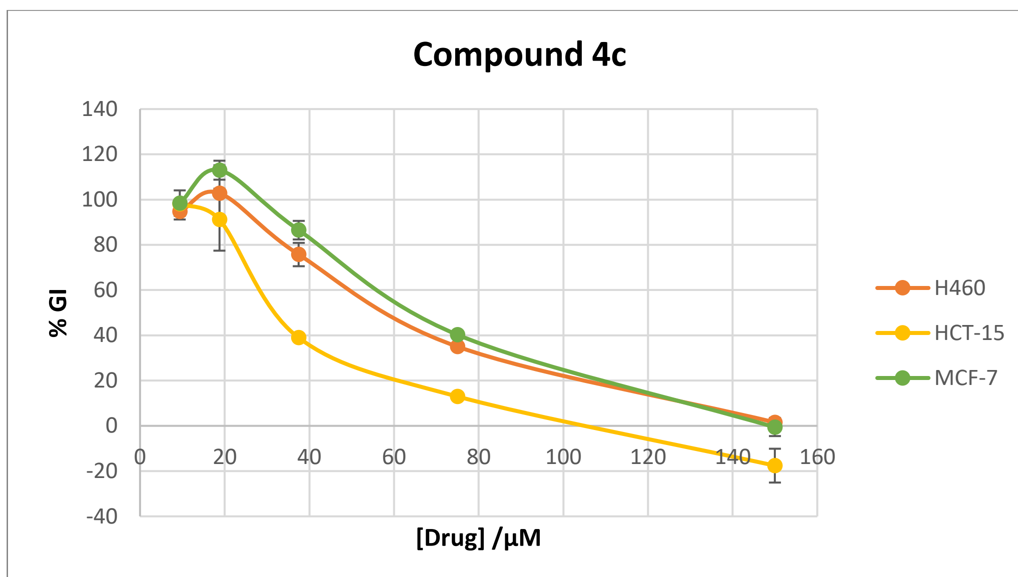
Supplementary Figure 1 – Dose response curves of H460 cells, HCT-15 cells and MCF-7 cells to compound 4a

Annex B



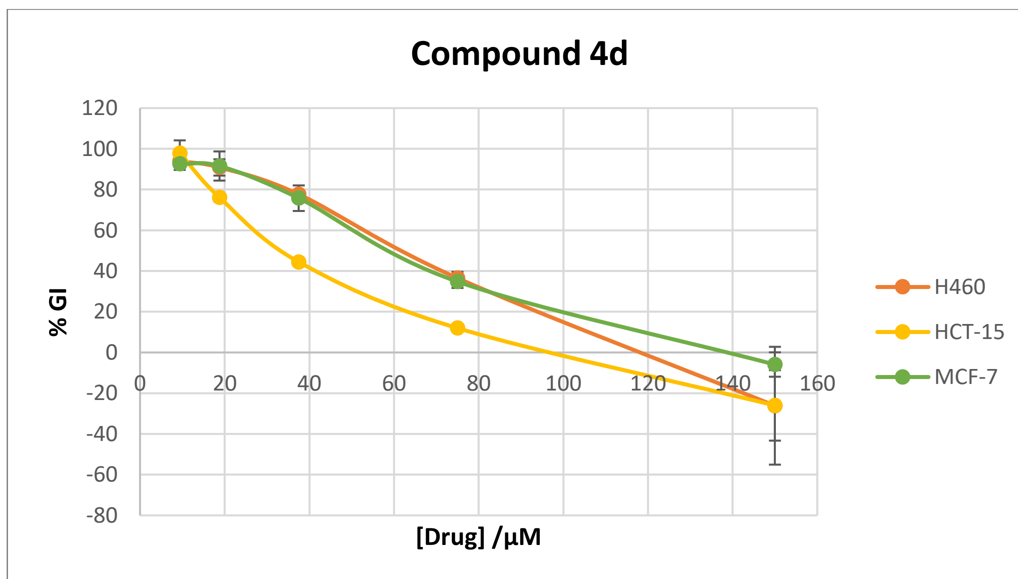
Supplementary Figure 2 – Dose response curves of H460 cells, HCT-15 cells and MCF-7 cells to compound 4b

Annex C



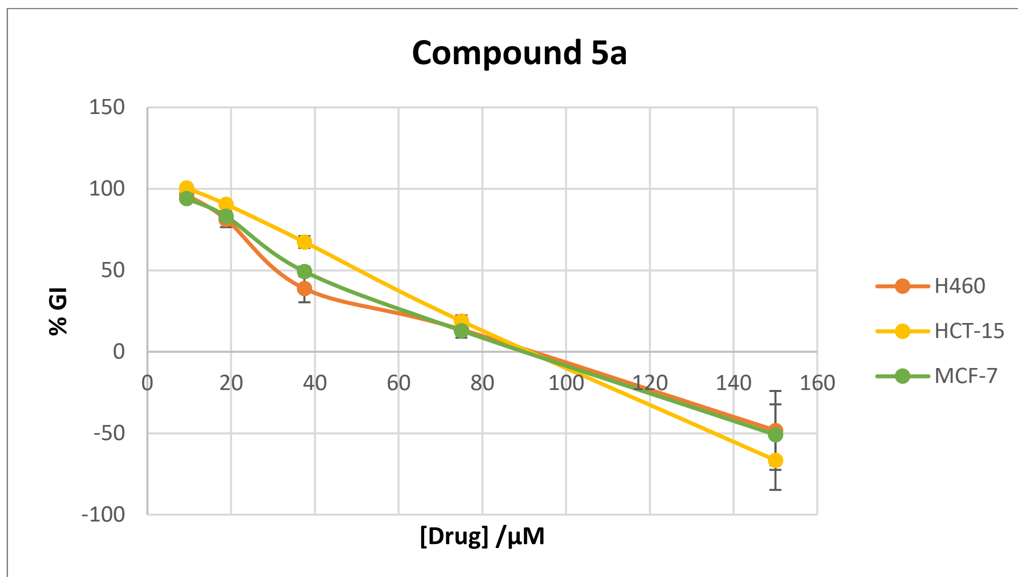
Supplementary Figure 3 – Dose response curves of H460 cells, HCT-15 cells and MCF-7 cells to compound 4c

Annex D



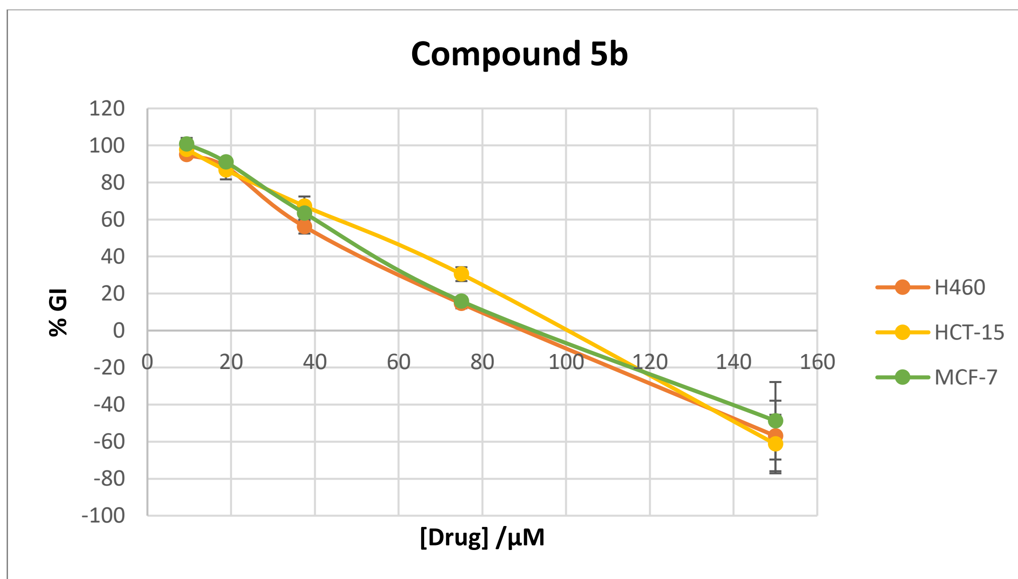
Supplementary Figure 4 – Dose response curves of H460 cells, HCT-15 cells and MCF-7 cells to compound 4d

Annex E



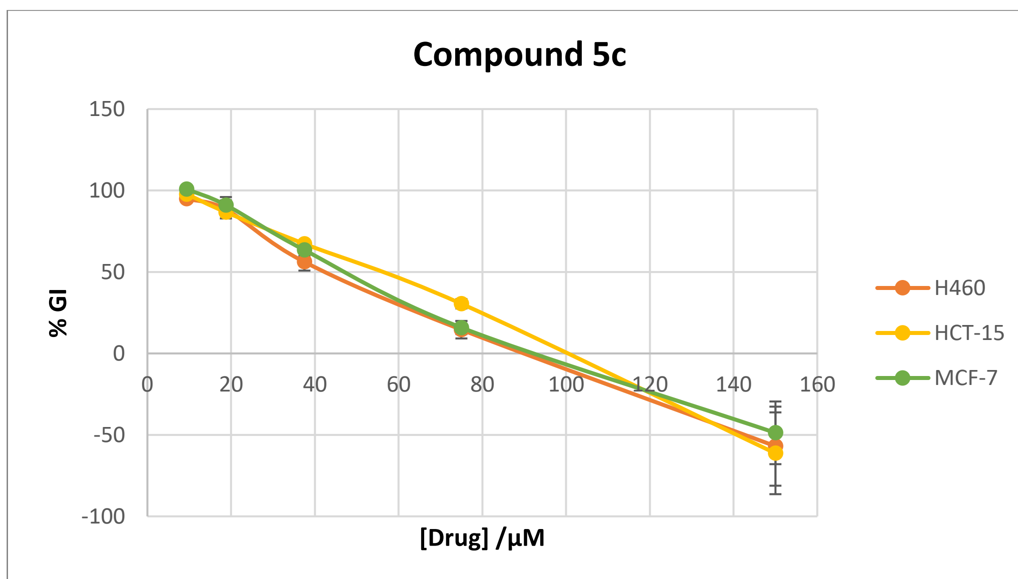
Supplementary Figure 5 – Dose response curves of H460 cells, HCT-15 cells and MCF-7 cells to compound 5a

Annex F



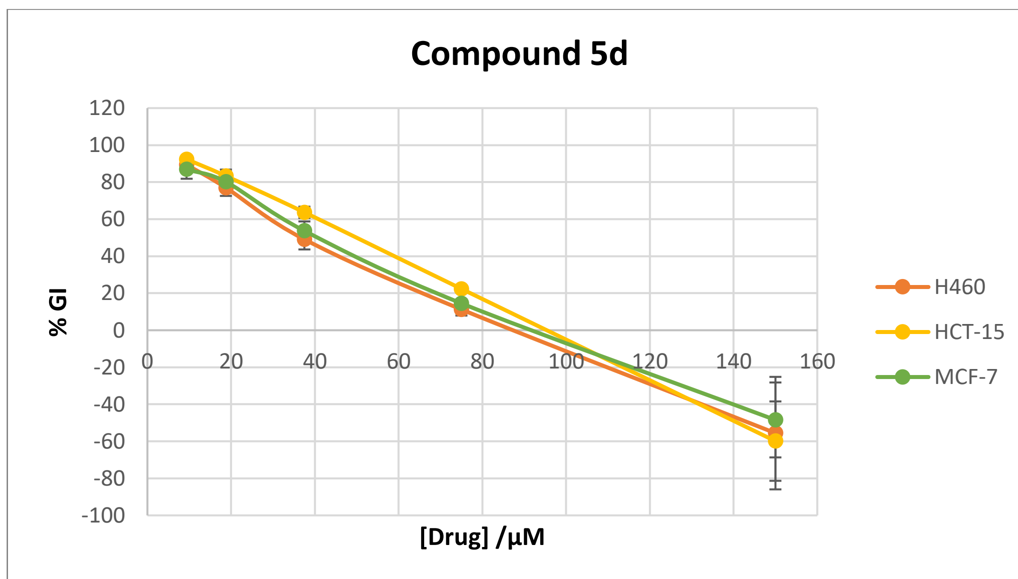
Supplementary Figure 6 – Dose response curves of H460 cells, HCT-15 cells and MCF-7 cells to compound 5b

Annex G



Supplementary Figure 7 – Dose response curves of H460 cells, HCT-15 cells and MCF-7 cells to compound 5c

Annex H



Supplementary Figure 8 – Dose response curves of H460 cells, HCT-15 cells and MCF-7 cells to compound 5d