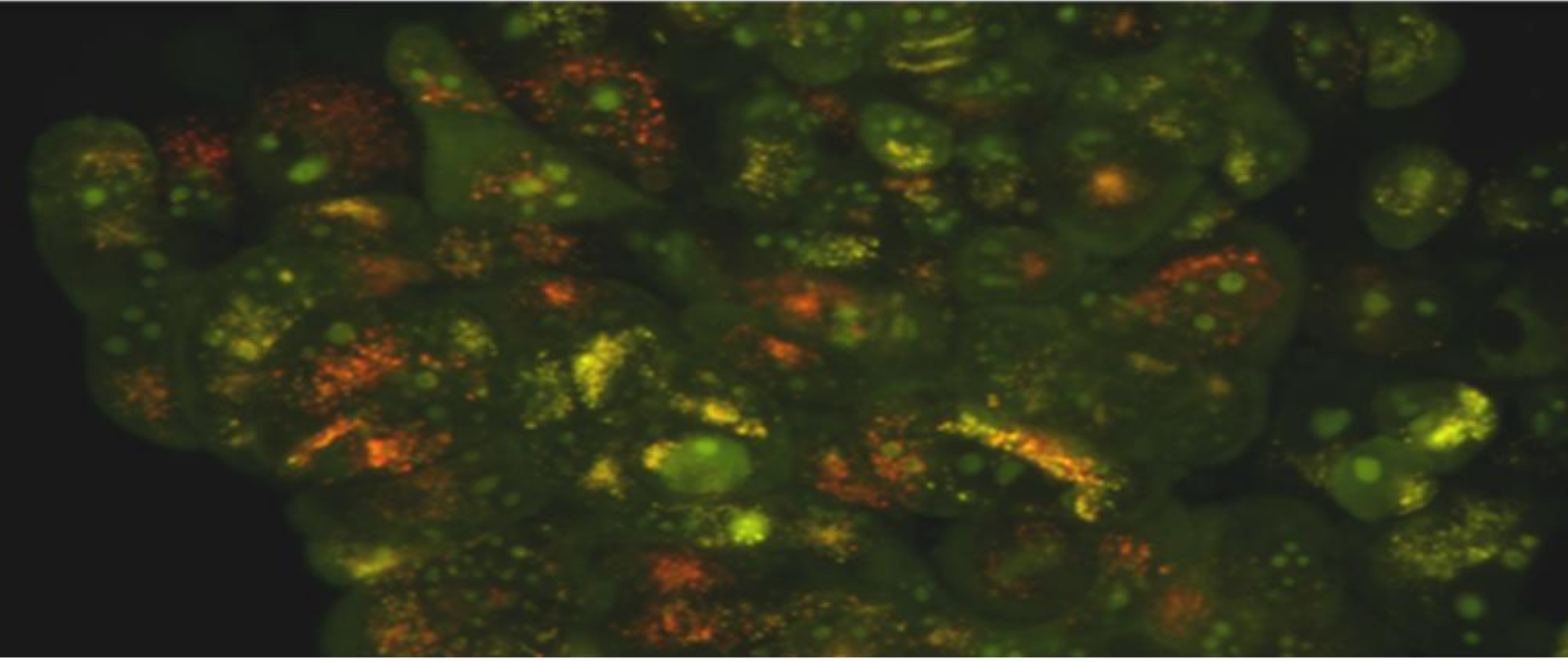

Aromatase inhibitors in breast cancer: Signaling pathways involved in exemestane-acquired resistance

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.
O Presidente do Júri,

Porto, _____/_____/_____

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Abstract

The use of aromatase inhibitors (AI's) is one of the therapeutic approaches for estrogen-receptor positive (ER^+) breast cancer, being Exemestane (Exe) the third-generation steroidal AI used in clinic. Besides its therapeutic success, acquired resistance may develop causing tumor relapse. Thus, it is important to search for new strategies to surpass Exe-acquired resistance. It was already reported that autophagy may be implicated in Exe-acquired resistance. Moreover, PI3K/AKT is considered a major pathway in endocrine resistance. Therefore, using an AI-resistant breast cancer cell line (LTEDaro) it was investigated the roles of autophagy and of the PI3K/AKT survival pathway in Exe-resistance process. In that way, the effects of two different pan-PI3K inhibitors, Wortmannin (WT) and LY294002 (LY), and of one autophagic inhibitor, Spautin-1 (SP), in Exe-treated LTEDaro cells were studied. Our results demonstrate that the combination of Exemestane with LY, WT or SP induced a reduction in LTEDaro cell viability. Moreover, in Exe-treated LTEDaro cells, WT, LY and SP caused cell cycle arrest in different cell cycle phases. Furthermore, all the compounds in combination with Exe induced apoptosis through different pathways in a ROS-independent manner. LY activates apoptosis through the mitochondrial pathway, while SP caused apoptosis recruiting extrinsic pathway players. Curiously, WT induced apoptosis through the cross talk between the intrinsic and the extrinsic pathway. In addition, all the inhibitors reduced Exe-induced autophagy, as well as, the activation of the survival pathway, PI3K/AKT. Thus, by modulating the survival pathways and autophagy it may be possible to sensitize acquired-resistant breast cancer cells to Exe therapy. This work provides new insights in breast cancer therapy by elucidating the mechanisms and targets involved in Exe-acquired resistance.

Keywords: breast cancer, aromatase inhibitors, Exemestane, endocrine resistance

Resumo

O uso de inibidores da aromatase (AI's) é uma das abordagens terapêuticas para o cancro da mama recetor de estrogénio positivo (ER^+), sendo o Exemestano (Exe) o AI esteroide da terceira geração usado na clínica. Apesar do seu sucesso terapêutico, ocorrem situações de resistência causando reincidência do tumor. Assim, é importante procurar novas estratégias para ultrapassar a resistência adquirida ao Exe. Foi previamente descrito o envolvimento da autofagia na resistência adquirida ao Exe. Para além disto, a via PI3K/AKT é considerada uma das principais na resistência endócrina. Assim, foi investigado o papel da autofagia e da via de sobrevivência PI3K/AKT no processo de resistência ao Exe usando uma linha celular de cancro da mama resistente aos AI's (LTEDaro). Foram estudados os efeitos de dois inibidores pan-PI3K, a Wortmanina (WT) e o LY294002 (LY), e de um inibidor autofágico, a Spautina-1 (SP), nas células LTEDaro tratadas com Exe. Os resultados demonstraram que a combinação de Exe com LY, WT ou SP induz uma redução da viabilidade celular das células LTEDaro. Para além disso, em células LTEDaro tratadas com Exe, a WT, o LY e a SP causaram uma retenção do ciclo celular em diferentes fases do ciclo. Todos os compostos induziram apoptose através de diferentes vias de uma forma independente de ROS. O LY induziu apoptose pela via mitocondrial, enquanto que a SP causou apoptose ao recrutar elementos da via extrínseca. Curiosamente, a WT induziu apoptose através de um *cross talk* entre a via intrínseca e extrínseca. Todos os inibidores reduziram a autofagia induzida pelo Exe, bem como, a ativação da via de sobrevivência, PI3K/AKT. Assim, ao modular as vias de sobrevivência e a autofagia, pode ser possível sensibilizar células de cancro da mama resistentes à terapia com Exe. Este trabalho traz novos conhecimentos à terapia de cancro da mama ao elucidar os mecanismos e alvos envolvidos na resistência adquirida ao Exemestano.

Palavras-Chave: cancro da mama, inibidores da aromatase, exemestano, resistência endócrina

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Abbreviations List

AF-1 - Activation Function-1

AF-2 - Activation Function-2

AG - Aminoglutethimide

AI(s) - Aromatase Inhibitor(s)

AKT - Protein Kinase B

AO - Acridine Orange

AO^+ - Acridine Orange positive

AO^- - Acridine Orange negative

AP-1 - Activator Protein 1

AR – Androgen Receptor

AREG – Amphiregulin

Atg 4 - Autophagy-related 4

ATM - Ataxia Telangiectasia Mutated

AVO(s) - Acid Vesicular Organelle(s)

Bcl-2 - B-cell lymphoma-2

BRAC1 - Breast Cancer 1, early onset

BRAC2 - Breast Cancer 2, early onset

cAMP - Cyclic Adenosine Monophosphate

CCND1 - Cyclin D1 gene

CFBS - Charcoal heat-inactivated Fetal Bovine Serum

cGMP - Cyclic Guanosine Monophosphate

CHEK2 - Checkpoint Kinase 2

CoA - Co-Activator

CoR - Co-Repressor

CPR - NADPH-Cytochrome P450 Reductase

CYP - Cytochrome P450

CYP19 - Aromatase

DBD - DNA-binding Domain

DCFH2-DA - 2',7'-Dichlorodihydrofluorescein Diacetate

DHEA - Dehydroepiandrosterone

DMSO - Dimethylsulfoxide

DR - Dimerization Domain

E1 - Estrone

E2 - Estradiol/17β-Estradiol

E3 - Estriol

EDTA - Ethylenediaminetetraacetic Acid

EGF - Epidermal Growth Factor

EGFR - Epidermal Growth Factor Receptor

ER(s) - Estrogen Receptor(s)

ER^+ - Estrogen Receptor Positive

ER^- - Estrogen Receptor Negative

ERE(s) - Estrogen response element(s)

ERK - Extracellular Signal-regulated Kinase

Exe - Exemestane

FBS - Fetal Bovine Serum

FDA - U.S Food and Drug Administration

FL-1/2/3 - Fluorescence Channels

FOXO3A - Forkhead box O3A

FSC - Forward Scatter

GFR(s) - Growth Factor Receptor(s)

GnRHa - Gonadatropin-releasing Hormone agonist

GPCR(s) - G Protein-coupled Receptor(s)

GPR30 - G protein-coupled estrogen receptor 30

17-βHE - 17β-hydroxy-6-methylenandrosta-1,4-dien-3-one

HDAC - Histone Deacetylase

HER2 - Human Epidermal Growth Factor Receptor 2

17β-HSD - 17β-Hydroxysteroid Dehydrogenase type 2

HSP - Heat Shock Protein

IGF1 - Insulin-like Growth Factor 1

IGF1R - Insulin-like Growth Factor 1 Receptor

IRS 1/2 – Insulin Receptor Substrate 1/2

LBD - Ligand-binding Domain

LC3 - Microtubule-associated protein 1A/1B-light chain 3

LDH - Lactate Dehydrogenase

LTEDaro - Long-term Estrogen Deprivation Cell Line overexpressing aromatase

LY - LY294002

3-MA - 3-methyladenine

MAPK - Mitogen-activated Protein Kinase

MCF-7 - Estrogen receptor-positive breast cancer cell line

MCF-7aro - Estrogen receptor-positive breast cancer cell line overexpressing aromatase

MEM - Minimum Essential Medium

MFI - Mean Fluorescence Intensity

mTOR - Mammalian Target Of Rapamycin

mTORC1 - Mammalian Target Of Rapamycin Complex 1

mTORC2 - Mammalian Target Of Rapamycin Complex 2

MTT - Tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5- difenyltetrazolium

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NF-κB - Nuclear Factor-kappa B

SRC-1/2/3 - Steroid Receptor Co-activator 1/2/3

4-OH-A - 4-Hidroxyandrostenedione

PBS - Phosphate-buffered saline

PI - Propidium Iodide

PI3K - Phosphoinositide 3-kinase

PKA/PKC – Protein Kinase A/C

PLC - Phospholipase C

PMA - Phorbol 12-Myristate 13-Acetate

PR - Progesterone Receptor

PTEN - Phosphatase and Tensin homolog

ROS - Reactive Oxygen Species
SERD(s) - Selective Estrogen Receptor Downregulator(s)
SERM(s) - Selective Estrogen Receptor Modulator(s)
shRNA – Small Hairpin Ribonucleic Acid
S6K1 - Ribosomal protein S6 kinase beta-1
SP - Spautin-1
SP-1 - Specificity protein 1
SQSTM1 - p62/Sequestosome 1
SSC- Side Scatter
STS – Staurosporine
RLU - Relative Luminescence Units
RNA – Ribonucleic Acid
RNAi - Ribonucleic acid interference
T – Testosterone
TBS - Tris-buffered saline
TF(s) - Transcription Factor(s)
Vps 34 - Vacuolar Protein Sorting 34
WB - Western Blot
WT - Wortmannin

CHAPTER I - Introduction

1. Breast cancer

Breast cancer is the most common cancer in women worldwide (Fig. 1) and the second cause of cancer death in females [1]. The breast is composed by lobes which contain lobules, and ducts. The lobules end in several tiny bulbs that can produce milk. Lobes, lobules and bulbs are linked by thin tubes called ducts. Usually, breast tumors either begin in the cells of lobules or of ducts. Less commonly, breast cancer can begin in the stromal tissues, which include the adipose and fibrous connective tissues of breast. The most common type of breast cancer is ductal carcinoma.

Fig. 1 - The incidence of new diagnoses per 100,000 women per year (adapted from [2]).

Breast cancer is not a single disease, but it represents a heterogeneous disease classified into several subtypes [3]. It is well established that these subtypes have unique prognosis and differ in their responsiveness to chemoprevention and chemotherapy [4]. Approximately 60% of premenopausal and 75% of postmenopausal breast cancer patients have estrogen receptor positive carcinomas, ER^+. These carcinomas are estrogen dependent, which means that they rely on the mitogenic effects of estrogen to drive carcinogenesis [5, 6]. Collectively, the ER^+ tumors are designated luminal cancers. These are sub-classified into luminal A, [ER^+, PR^+, $HER2^-$], and luminal B, [ER^+, PR^+, $HER2^+$], subtypes based on their human epidermal growth factor receptor 2 (HER2) status and proliferation rates [3]. Among the ER^+ breast cancers, the luminal B tumors are associated with a significantly worse prognosis than

luminal A subtype [7]. The difference in the outcome between these two subtypes is due to variations in the response to anti-estrogen therapy [8].

The ER^- breast cancers are sub-classified as human epidermal growth factor receptor 2 positive, [ER^-, PR^-, $HER2^+$], and basal-like (triple negative) [3]. In 15% to 20% of breast cancers the HER2 is overexpressed and has been associated with poor prognosis [9].The triple negative breast cancers, [ER^-, PR^-, $HER2^-$], constitute 10% of breast cancers. With a poor clinical outcome, this subtype does not have an approved targeted therapy [3, 10, 11]. The receptors expression, or lack of it, is a determining factor for tumor growth and tumor recurrence, since these factors vary among the different types of breast cancer.

1.1 Risk Factors and Treatments

As breast cancer is more frequently found in postmenopausal women, the age of the patient is considered one of the majors risk factors [2]. In fact, around 75% of breast cancer cases are present in postmenopausal women [2]. However, the risk factor more associated with breast cancer is inheritance of an inactivating mutation in one of the breast cancer genes: Breast Cancer 1, early onset (BRCA1), Breast Cancer 2, early onset (BRCA2), Checkpoint kinase 2 (CHEK2), p53 and Ataxia Telangiectasia Mutated (ATM). Mutations in these genes contribute to around 5% of breast cancer cases [2]. Other factors that are associated to an increased risk of breast cancer are: dietary habits, which may lead to obesity, occurring more frequently in the developed countries; high levels of endogenous estrogens, absence of lactation, late menopause, hormonal replacement therapy, oral contraception and high circulating levels of insulin-like growth factor-1 (IGF-1). Exercise is thought to reduce this risk [2].

Different types of treatment are available for patients with breast cancer. The standard therapies are: surgery, radiation therapy, chemotherapy, endocrine therapy and the targeted therapy [12]. Amongst the standard treatment the surgery is the most common. Chemotherapy uses drugs to limit the growth of tumors, either by killing the cancer cells or by stopping them from dividing. Chemotherapy may be given before the surgery, as neoadjuvant therapy, to reduce the tumor, or after the surgery, as adjuvant therapy to kill the remaining cancer cells, and therefore, lower the risk of cancer recurrence [12]. Radiation therapy uses high energy x-rays or other types of radiation to kill, or at least prevent the growth of tumors. The way the radiation therapy is given depends on the type and stage of the cancer. Hormone therapy is a treatment that prevents, or blocks, the action of hormones involved in cancer growth, and can be

performed by ovarian function ablation, to stop the production of estrogens, or administration of drugs, like Tamoxifen or Aromatase Inhibitors (AI's). Nevertheless, Tamoxifen's treatment may increase the chance of developing endometrial cancer. Aromatase Inhibitors are given to postmenopausal patients, as well as in pre-menopausal women whose ovarian function is suppressed. Other targeted therapy uses substances like the monoclonal antibody, Trastuzumab (blockade of ligand binding to HER2), tyrosine-kinase inhibitors, like Lapatinib (blocks the transduction of HER2 signals), and cyclin-dependent kinases inhibitors like Palbociclib (blocks the activity of the cyclin-dependent kinases) that are used in postmenopausal women's whose cancer was not treated with AI's [13].

2. Estrogen biosynthesis

Estrogens are essential for the normal physiology and normal woman reproduction. The leading role of estrogens is the maturation and growth of the sexual organs, being also responsible for the secondary sex characteristics during puberty. Estrogens act in the mammary gland promoting the mammary development through stimulation of stroma and ductal growth and accumulation of adipose tissue. Besides the reproductive system, estrogens also have pivotal roles in the musculoskeletal system, cardiovascular system and brain.

Estrogens like every steroidal hormone are cholesterol-derived (Fig. 2). The estrogens in women are Estrone (E1), Estradiol (E2) and Estriol (E3). E2 is the main estrogen in women at fertile age. In contrast, E1 is the principal estrogen in postmenopausal women, while E3 is the main estrogen during pregnancy.

Estrogens are derived from androstenedione, which is either converted directly to estrogens, namely, E1 or E2, or to Testosterone and then to E2. Reduction of Androstenedione to Testosterone requires 17β-Hydroxysteroid Dehydrogenase type 2 (17β-HSD). Androstenedione can also undergo a three-step A-ring aromatization to E1 catalyzed by aromatase, that belongs to the cytochrome P450 family's monooxygenases [14]. This enzyme also converts Testosterone into Estradiol (17β-Estradiol). Interconversion of E2 and E1 requires other Hydroxysteroid Dehydrogenases [12, 15, 16]. This interconversion is necessary since the E1, contrary to E2, does not activate the ER [17].

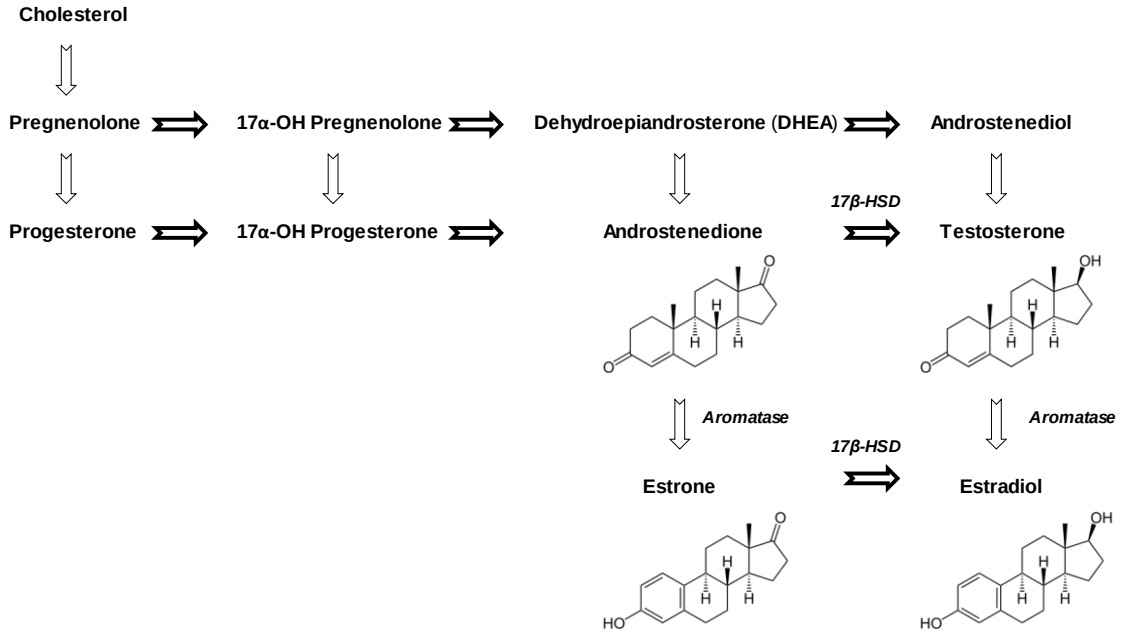


Fig. 2 - The estrogen biosynthetic pathway.

Ovaries are the main organ for estrogen synthesis in premenopausal women. When women reach menopause ovarian biosynthesis ceases. In postmenopausal women the plasma estrogens levels are present in low concentration and are derived from the androgens produced by the adrenal glands. These molecules are then converted to estrogens in other organs and tissues, like liver, muscle, connective tissue and skin [14, 17].

In the 70's the first indications of extraglandular aromatase activity in both men and women were discovered, countering the dogma that steroid hormones were only synthesized in endocrine glands such as ovaries, testis and adrenal [14]. Although the circulating androgens, in postmenopausal women, are from adrenal origin, the ovary seems to provide a minor contribution to circulating testosterone levels [15].

2.1 Aromatase

Human aromatase is an enzyme of the cytochrome P450 family and is the product of the *CYP19A1* gene on chromosome 15q21.1. It consists of a heme group and a polypeptide chain of 503 amino-acid residues (58 kDa) [18]. Aromatase structure was only determined in 2009 by Gosh, being its structure composed by twelve α-helix and ten β-sheets (Fig. 3) [19]. The catalytic complex of human aromatase is a heterodimer constituted by aromatase and by a flavoprotein, the NADPH-cytochrome

P450 reductase (CPR). The catalytic portion of aromatase contains the heme group and the binding site for steroids, like androstenedione [18].

Aromatase is the only enzyme in vertebrates known to catalyze the biosynthesis of estrogens from their androgenic precursors [20]. This enzyme uses with high specificity androstenedione, testosterone, and 16α-hydroxytestosterone, all with the same androgen backbone, converting them to Estrone (E1), 17β-Estradiol (E2), and Estriol (E3), respectively, though it has higher affinity to androstenedione.

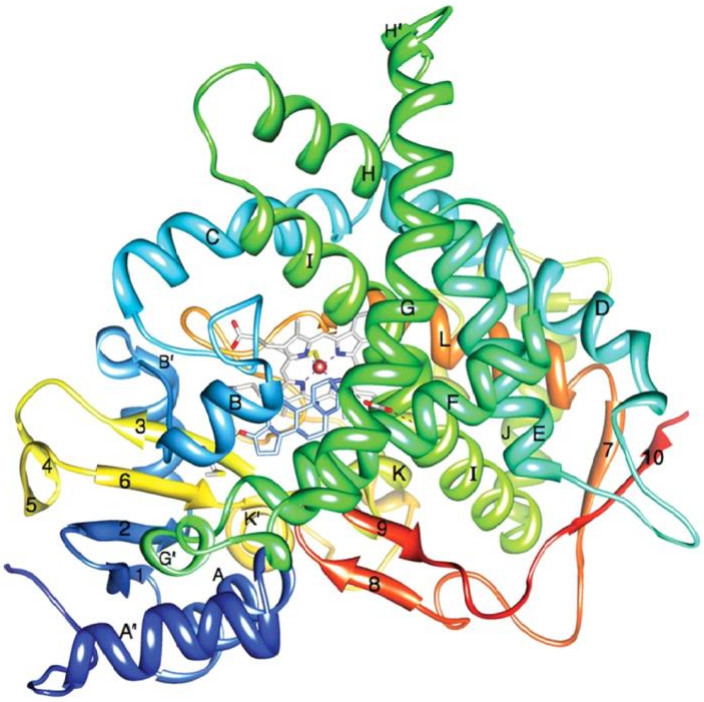


Fig. 3 - General structure of Aromatase. Ribbon diagram showing the overall structure. The N terminus, starting at residue 45 (dark blue) and the C terminus ending at residue 496 (red). The α-helices are labeled from A to L and β-strands are numbered from 1 to 10. The heme group, the bound Androstenedione molecule at the active site and its polar interactions are shown. (adapted from [18]).

The conversion of androgens to estrogens, by the aromatization of the A-ring (Fig. 4), is a three-step process, each requiring one molecule of O_2 and NADPH, coupling with its redox partner CPR for the transfer of electrons. The last step is exclusively catalyzed by aromatase (CYP19) [20].

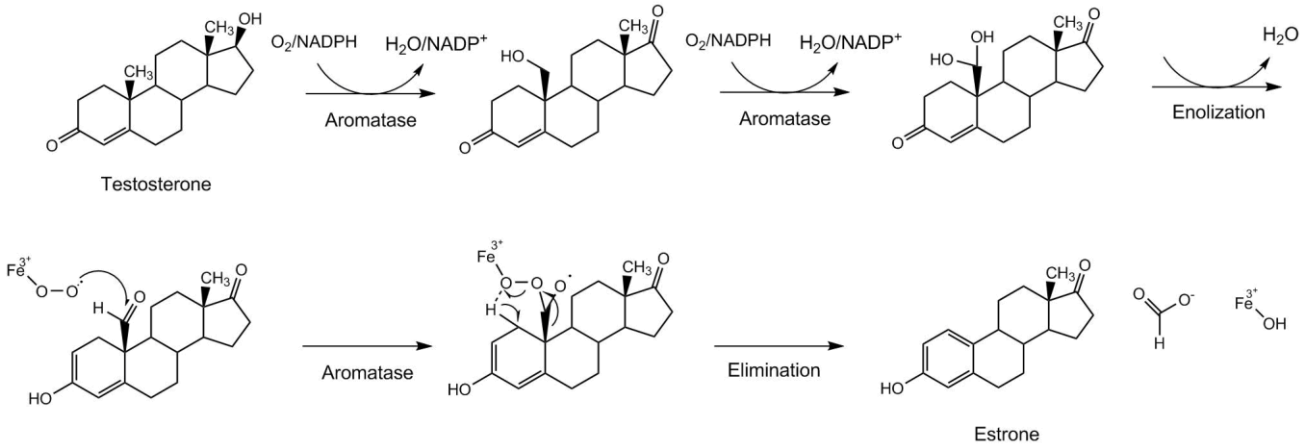


Fig. 4 - Biosynthesis of estrogens by aromatase. Aromatization mechanism of the A-ring.

3. Estrogen receptor signaling pathways

Estrogen receptors (ER) are nuclear receptors that act as transcription factors involved in the regulation of many physiological processes in humans [21]. The ER has two isoforms, ERα and ERβ, with a 56% homology [22]. ERα is expressed in ovary, uterus and mammary gland and is located in chromosome 6, while the ERβ is mainly expressed in the ovary and in the reproductive system of males, and is located in the chromosome 14 [23]. Estrogens exert their activity through binding to the estrogen receptors (ER), ERα and ERβ.

ERα has been recently connected to the promotion of proliferation and survival of breast cancer cells, consequently, it is considered the subtype necessary to the majority of estrogenic responses, while ERβ presents growth inhibitory properties [24, 25]. ERα is capable to form a heterodimer with ERβ, which has a similar binding affinity to DNA as the ERα homodimer, but a lower level of transcriptional activity [26]. When the two subtypes are co-expressed in the cell, ERβ can antagonize ERα-dependent transcription, since its expression alters the recruitment patterns of transcription factors involved in the genomic ERα-dependent responses. Moreover, expression of ERβ increases the proteolytic degradation of ERα [27].

Both ERs contain a DNA-binding domain (DBD), a dimerization region (DR), a ligand-binding domain (LBD), and two transactivation domains, one located near the N-terminus, Activation Function-1 (AF-1), and other near the C-terminus, Activation Function-2 (AF-2). Despite the high sequence homology in the DBD they are not

redundant genes, since they have different expression patterns and functions (Fig. 5) [24].

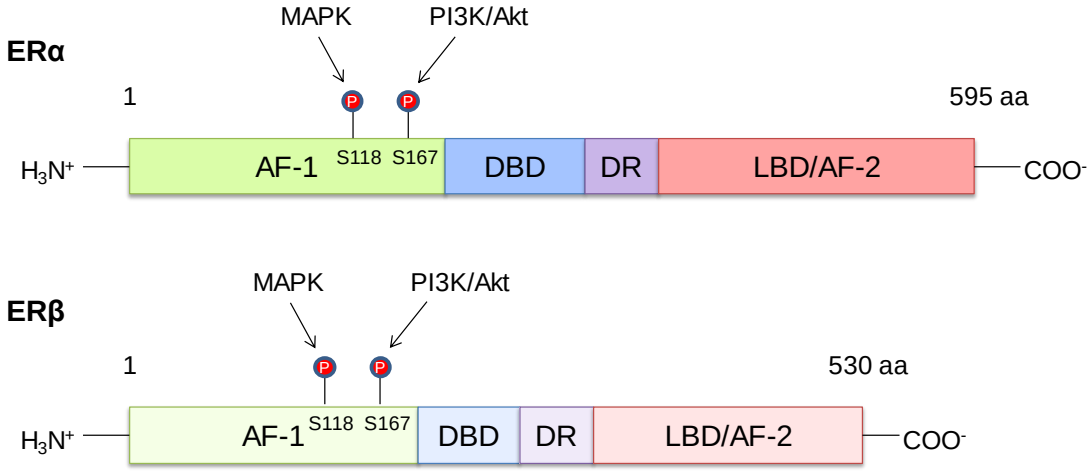


Fig. 5 – Structure of ERα and ERβ. Both receptors have four functional domains, harboring a DNA-binding domain (DBD), a ligand-binding domain (LBD) and two transactivation domains (AF-1 and AF-2) and a dimerization domain (DR). The location of the phosphorylation sites at AF-1 are indicated. AKT, serine/threonine specific-protein kinase family encoded by the AKT genes; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase.

ER is mainly functional in the nucleus, where it activates transcription of ER-regulated genes. This transcription is dependent on binding of E2 that helps to stabilize the ER binding to DNA. Within the cytosol, ER is bound, through LBD (AF-2) to chaperone proteins such as Heat Shock Protein 90 (HSP90) and HSP70 [24, 28, 29]. Upon binding of E2 to the LBD of ER, the receptor undergoes conformational changes like dissociation from the HSP, ER dimerization, translocation into the nucleus and formation of a hydrophobic domain, exposing the AFs to which co-activators (Co-A), such as steroid receptor co-activator (SRC-1, 2 or 3), or co-repressors (Co-R), such as nuclear receptor co-repressor 2 (SMRT), bind [21, 29, 30].

ER can act either by the genomic pathway or the non-genomic pathway. In the genomic pathway, ER forms a dimer upon binding of E2 (Fig. 6). Then the dimer translocates into the nucleus and binds the estrogen responsive element (ERE) in the promoter region to initiate the “classical” transcriptional activation or repression. The ER can also interact with other transcription factors such as activator protein 1 (AP-1) and specificity protein 1 (SP-1) to bind DNA indirectly, leading to activation or repression of target genes located at alternative responsive elements. This process is known as “non-classic” or “ERE-independent” genomic action. In addition to these two different genomic pathways, a third genomic mechanism exists. This involves ligand-independent ER activation by phosphorylation, via stress related kinases (p38 mitogen-

activated protein kinase (MAPK), Extracellular signal-regulated kinase 1/2 (Erk 1/2) MAPK, Phosphoinositide 3-kinase (PI3K) /Protein kinase B (AKT)), at the AF-1 domain. ER and its co-activators can be phosphorylated, whether the E2 is bound to the ER or not, through the genomic or non-genomic mechanism, which may lead to endocrine resistance [30-34].

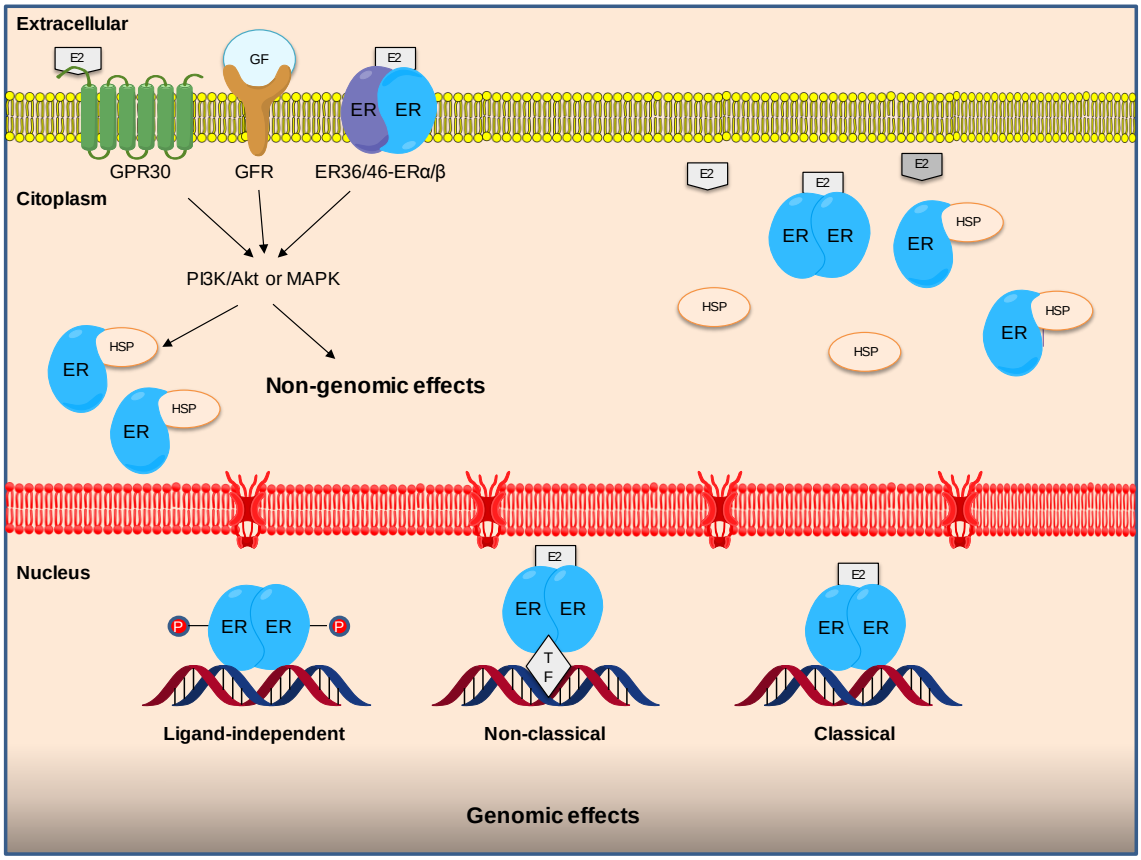


Fig. 6 - Mechanisms involved in estrogen signaling. Estradiol (E2) binding to estrogen receptors (ER) induces the dissociation of the receptor from Heat Shock Proteins (Hsp), the dimerization, and translocation to the nucleus. Nuclear E_2-ER complexes bind directly to Estrogen Response Elements (ERE) (classical) or interact with other transcription factors (TF) in target gene promoters (non-classical). Growth factors (GF) and E2, through GPR30 or ER variants-ERα/β dimers, activate protein-kinase cascades. These active kinases can indirectly lead to phosphorylation (P) and activation of nuclear ERs at EREs, even in the absence of E2, or lead to non-genomic events directly.

In addition to the well-established transcriptional effects of E2 mediated by ER, there are rapid effects that do not rely on transcriptional activity and are triggered by E2 binding to growth factor receptors and other membrane receptors, like G protein-coupled estrogen receptor 30 (GPR30) [35] and ER variants [36, 37]. These pathways are known as non-genomic. The ER variants, such as 36 kDa and 46 kDa ER, localized at lipid rafts, are important in this non-genomic pathway [24]. They can dimerize with ERα and ERβ activating various kinase signaling pathways like

Phospholipase C (PLC)/Protein Kinase C (PKC), Ras/Raf/MAPK and cyclic adenosine monophosphate (cAMP)/Protein kinase A (PKA) [38]. GPR 30 functions as an ER, since it senses E2, and can activate EGFR [39]. These signal transduction pathways may connect non-genomic actions of estrogens to genomic responses. This is due to the fact that many transcription factors are regulated by protein-kinase phosphorylations. The AF-2 of the ER is only activated by ligand binding of E2, however the AF-1 site is activated by phosphorylation at several residues like S167 that is phosphorylated by PI3K/AKT [32] and S118 that is phosphorylated by the Ras-MAPK [33] signaling cascade. These two residues are crucial for the activation of ER and mediate ER binding to co-activators. Nevertheless, S118 phosphorylation increases hypersensibility to E2, leading to ligand-independent activation of ER, a process that may result in the development of AI resistance [33, 40, 41].

4. ER^+ breast cancer endocrine therapy

Endocrine therapy is complementary to surgery in the majority of patients, since approximately 65% of breast cancer patients are ER^+. This therapy has become the standard adjuvant treatment for ER^+ breast cancer patients since the 1970. There are many approaches: ovarian function suppression; selective estrogen receptor modulators (SERMs); selective estrogen receptors down-regulators (SERDs); aromatase inhibitors (AI's) or a combination of two or more treatments, that have the purpose to reduce, as much as possible, the serum and tissue levels of estrogen [1].

Ovarian function suppression consists mainly in the surgical oophorectomy (ovarian ablation) ceasing the estrogen production in ovaries in pre-menopausal women. Ovarian function can also be suppressed by administration of gonadatropin-releasing hormone agonists (GnRHa), simultaneously to chemotherapy. GnRHa have reversible action and could have the potential to preserve ovarian function. Therefore, ovarian function suppression treatment in addition with AI's could be a valid alternative option for pre-menopausal women with high risk of recurrence [1, 42, 43].

4.1 SERMs and SERDs

As referred before, estrogen and their receptors are key regulators in hormone-dependent breast cancer progression. Therefore, drugs that target selectively the ER, like Fulvestrant (SERD) and Tamoxifen (SERM) are important to achieve the blockade of the ER signaling pathways (Fig. 7). Tamoxifen is a non-steroidal molecule that modulates the activation of ER since it can act both as a partial agonist, in the liver, uterus and bone, and as an antagonist in the breast and brain [32]. In breast cancer cells, Tamoxifen, via its metabolites 4-hydroxiTamoxifen (4-OHT) and endoxifen, binds competitively to ER and recruits co-repressors, blocking ER-related gene transcription [1, 44]. Although the reduction in mortality and reduced risk of recurrence, Tamoxifen presents some age-related side effects, such as increased risk of thromboembolic disease and a 3-fold increased risk of endometrial cancer [1, 45, 46]. Moreover, some patients can develop resistance to Tamoxifen.

Due to the adverse effects of Tamoxifen, other SERMs, like Raloxifene and Toremifene have been developed. Toremifene is similar to Tamoxifen in terms of tolerability and efficacy and also exhibits less vascular and endometrial side effects. However, is cross-resistant with Tamoxifen and has less benefit action in the bone. On other hand, Raloxifene is less toxic and less effective than Tamoxifen, being currently used in the prevention of osteoporosis in post-menopausal women [1, 47].

Selective estrogen down-regulators, like Fulvestrant, are capable of causing down-regulation and degradation of the ER preventing proliferation of estrogen-dependent breast cancer cells. Fulvestrant, or ICI 182,780, is a pure ER antagonist that blocks and degrades ER. It has a hundred-fold higher affinity to ER than Tamoxifen. Contrary to Tamoxifen, Fulvestrant exhibits only anti-estrogen effects and lacks endometrial adverse effects [1, 48]. It is used as a second-line therapy, in patients where the tumor has developed resistance to SERMs and AI's [49, 50].

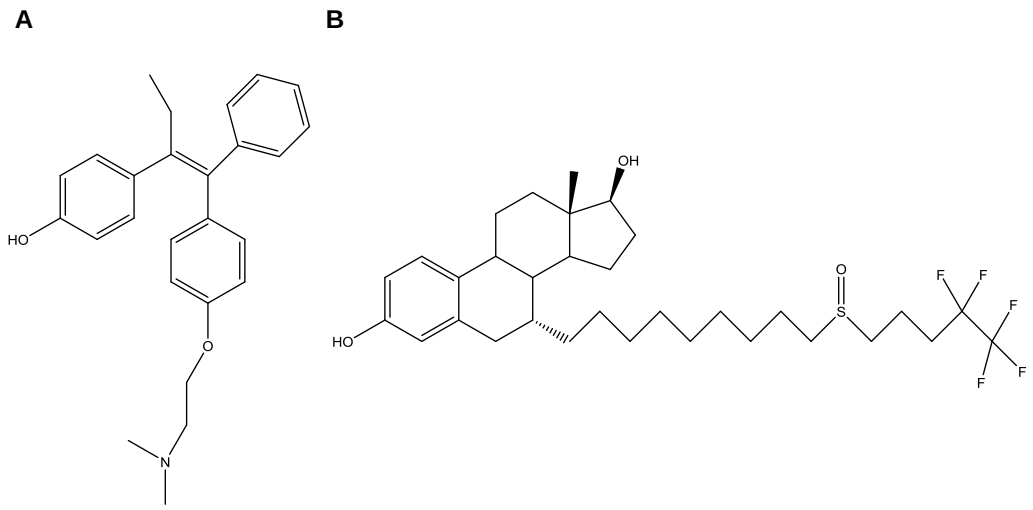


Fig. 7– Chemical structures of: **(A)** Tamoxifen, **(B)** Fulvestrant.

4.2 Aromatase Inhibitors (AI's)

Other effective treatment is based on the inhibition of the last step of estrogen biosynthesis, by blocking the aromatase enzyme. Aromatase inhibitors (AI's) are molecules that block aromatase activity, reducing the levels of circulating estrogens and are mainly used in post-menopausal women. In pre-menopausal women, where estrogen is produced in the ovaries, this therapy results in transient reduction of estrogens that will activate the hypothalamus and pituitary axis. This induces an increase in gonadotropin secretion and, consequently, stimulation of ovaries with increased estrogen production [51].

AI's are typically described as first-, second-, or third-generation according to their development order. Current AI's can be classified into two subtypes, namely steroidal (Type I) and non-steroidal (Type II). Steroidal AI's mimics the structure of the aromatase substrate, androstenedione, therefore binding to the substrate-binding pocket of the enzyme. Its reactive intermediate then binds covalently to the enzyme, resulting in an irreversible inactivation of aromatase. These AI's are also known as *suicidal inhibitors* because the enzyme is inactivated and ultimately lead to aromatase degradation. In contrast, type II AI's bind non-covalently to the heme moiety of aromatase and saturate the binding site. Unlike the Type I inhibition, type II inhibition is reversible by competitive inhibition of androgens [14, 51]. Remarkably, these two classes for aromatase inhibitors are not totally cross-resistant, and patients failing to respond to one class, still have 25% chance of achieving clinical benefit from the other class [52]

AI's have demonstrated to be superior to other hormonal agents, like Tamoxifen, and, consequently, are now established as first-line treatments in endocrine therapy [26, 51]. Aromatase inhibitors are associated with decreased risk of endometrium cancer remission, vaginal bleeding and thromboembolic events than Tamoxifen. Nevertheless, AI's decrease circulating estrogen levels, resulting in accelerated bone loss and increased risk of fractures. Although Exemestane (Exe) does not protect bone like Tamoxifen, it does not accelerate postmenopausal bone loss due to its androgenic effect. On other hand, AI's also present other side effects like musculoskeletal disorders, arthralgia and myalgia. Thus, it is necessary a careful look on patients subjected to this type of therapy given the long-term effects of these inhibitors in the bones and cardiovascular system [52-54].

The first-generation AI, Aminogluthetimide (AG) (Fig. 8), was initially used as an anti-epileptic drug, however, it was first directed for the treatment of breast cancer in late 1970s. Although the AG, a non-steroidal (Type II) AI, showed aromatase inhibition and therapeutic benefits, it lacked specificity and potency, which often produced side effects, like the inhibition of various cytochrome P450 enzymes. More specifically, the inhibition of CYP11, an enzyme responsible for the conversion of cholesterol to pregnolone, led to cortisol supplementation in combination with AG [26, 51, 52].

Subsequently, second-generation specific AI's were developed during the 1980s and 1990s (Fig. 8). This generation includes the non-steroidal imidazole derivate, Fadrozole, and the first steroidal (Type I) AI, Formestane, also known as 4-hidroxyandrostenedione (4-OH-A). Fadrozole is more selective and potent than AG, but has inhibitory effects in the cytochrome P450 enzymes involved in aldosterone, progesterone, and corticosterone biosynthesis. On the other hand, Formestane was the first effective and well tolerated AI used in clinic. However, its intramuscular administration confined its use [54].

Third-generation AI's were developed in the beginning of 1990s and are more effective than the previous generations. It includes two non-steroidal AI's derived from triazole, namely Anastrozole and Letrozole, and a steroidal AI, Exe (Fig. 8). These are largely used as first-line drugs in breast cancer hormone-dependent treatment in post-menopausal women, since they act specifically in aromatase without suppressing adrenal function [52].

4.2.1 Non-steroidal third-generation AI's (Letrozole and Anastrozole)

Anastrozole and Letrozole act as competitive inhibitors. Both are potent AI's *in vivo*, showing aromatase suppression superior to 97% at clinical doses [26, 55]. In fact, Anastrozole decreases estrogen levels by 80% in patients [56]. This reduction occurs without detectable changes in other steroid hormones. Although Anastrozole shows high aromatase suppression, Letrozole has higher inhibitiory activity [57]. Anastrozole also shows a high antitumor activity in patients with breast cancer and is a good chemoprotective agent [51, 52]. Clinically, Letrozole causes tumor remission in women with breast cancer resistant to other endocrine therapies and chemotherapy [52]. Currently, in ER^+ postmenopausal women, Anastrozole is used as first- and second-line treatment of advanced breast cancer and, along with Letrozole, as adjuvant treatment for early breast cancer.

4.2.2. Steroidal third-generation AI (Exemestane)

Exe, also known as Aromasin®, is the only steroidal AI currently used in clinic. Its structure is derived from aromatase natural substrate, androstenedione. Exe is a *suicide inhibitor* causing aromatase proteasome-mediated degradation and blocking estrogen biosynthesis until the *de novo* synthesis of aromatase, leading to a persistent inhibitory effect even after clearance. With this unique mechanism, Exe reduces aromatase half-life in 50% [58] and presents a total body aromatase inhibition around 98% [59, 60]. Exe's metabolism occurs through CYP3A4, being the main Exe metabolite the 17β-hydroxy-6-methylenandrosta-1,4-dien-3-one (17-βHE) [61]. This AI reveals maximum decrease in estrogen plasma levels, without affecting the plasma levels of other steroidal hormones [57], after 3 to 7 days upon administration. Due to its highly specific mechanism, Exe is highly selective and does not affect other enzymes in the steroids biosynthetic pathway [57, 62]. Exe is considerably more effective than Tamoxifen in preventing breast cancer remission [53, 63].

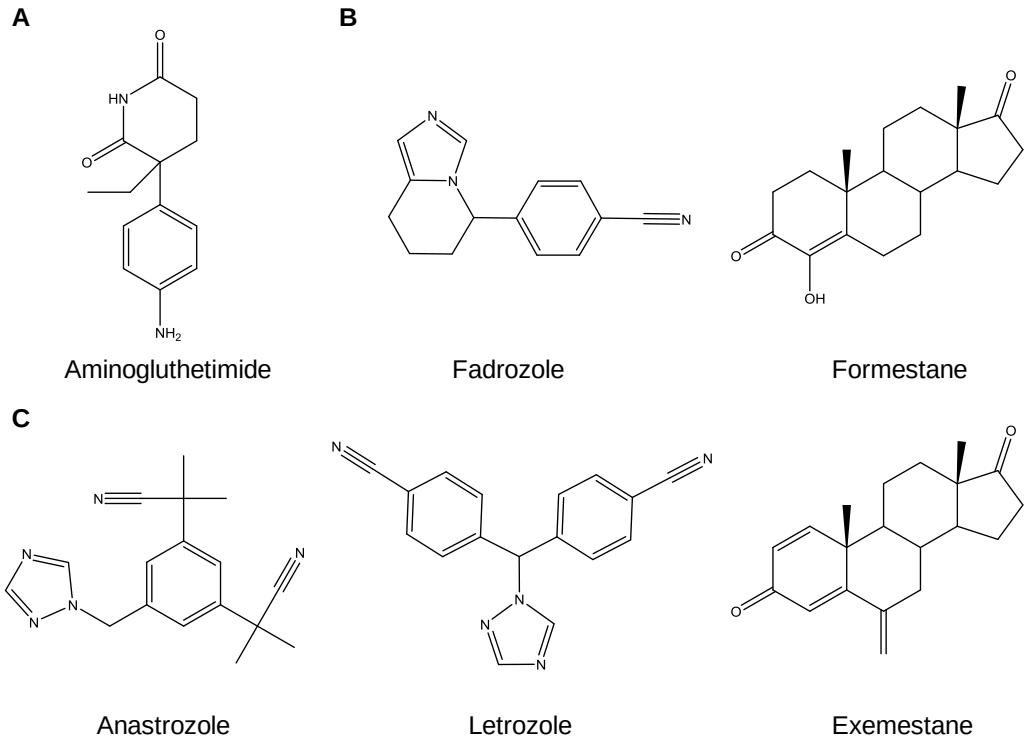


Fig. 8 - Chemical structures of the AI's used in clinic: first-generation **(A)**, second-generation **(B)** and third-generation **(C)**.

5. Breast cancer acquired-resistance to AI's

Since it was first observed that tumors can regress following oophorectomy, estrogen deprivation has been an important treatment for estrogen-dependent (ER^+) breast cancers [64]. Therefore, all endocrine therapies are centered on the disruption of the estrogen signaling pathway, either by interfering with the receptor itself or by depleting its ligand. However, despite the potency of AI's therapy, over 20% of patients with early-stage disease suffer a relapse. Metastatic breast cancer continues to be an incurable disease, thus, the goal of the treatment is to convert this disease into a chronic condition that can be controlled with well-tolerated therapies. Although the vast majority of patients with metastatic breast cancer experience initial benefit from the endocrine therapy, subsequent disease progression often occurs [65].

Resistance to aromatase inhibitors can be divided into two main types, namely: *de novo* or intrinsic resistance and acquired resistance. In *de novo* resistance the primary ER^+ breast cancer cells never respond adequately to endocrine therapy. While, in acquired-resistance it develops after a long period of exposure to the endocrine therapy. Once the patients develop acquired-resistance to AI's, the response to

endocrine therapy is ceased and chemotherapy is necessary [66]. Still, the clinical distinction between the two types of resistance is not well determined, however, the mechanisms underlying these two types are likely to overlap [65].

5.1 Intrinsic Resistance

In intrinsic resistance, where there is an initial lack of efficacy of AI's in primary tumors, there is a Ki67 continued expression despite the AI treatment. This indicates an estrogen-independent proliferation in the majority of cases, possibly through several polymorphisms in the aromatase gene CYP19 [67-69], which are associated with an increased risk of disease recurrence and death [65]. Recently, p53 accumulation is being described as a biomarker associated with AI's-resistance [70]. Furthermore, intrinsic resistance to AI's may not be due to defects in aromatase nor in ER, as shown in studies conducted in two cell lines that mimic intrinsic resistance: HER2-aro and AKT-aro cells [71]. These express decreased levels of a proliferation marker and ER-regulated genes with AI treatment, but, the overall cellular response remained unaffected, as previously reported by Miller et al [72]. Thus, altered expression of co-regulators may also help to explain the phenotypes in some resistant cells [73]. Furthermore, overexpression of HER2 or AKT in ER^+ breast cancer cells results in resistance to AI treatment [24]. In fact, several randomized trials reported beneficial effects on the combination of Trastuzumab (HER2 inhibitor) or lapatinib (HER2 and EGFR inhibitor) with AI's (Letrozole or Anastrozole) [74-77].

Very recently, the role of miRNAs in endocrine resistance started being studied [78]. It was reported that overexpression of microRNA (miR)-125b and silencing of miR-424 is sufficient to confer resistance to Letrozole and Anastrozole by activation of the PI3K/AKT/mTor pathway [79]. Nevertheless, other study demonstrated that a high-baseline miR-155 is correlated with poor response to Anastrozole [80]. However, little is yet known about this type of resistance to AI's. It is, although, a major field of research interest, since it may be able to help early identification of poor responders to endocrine therapy.

5.2 Acquired resistance

Acquired-resistance is seen in ER-expressing breast cancers, which initially respond to endocrine therapy, indicating that the loss of ER expression is not the main mechanism for acquired-resistance and that ER may still play a role in resistance to endocrine therapy. Thus, mutations in the *ESR1* gene (encodes ERα), more specifically in the ligand-binding domain (LBD), have been described as a mechanism of acquired-resistance since the early 1990s [81]. Mutations in this gene are rare in primary tumors treatment-naïve [82, 83]. However, they appear with high frequency in metastatic tumors, especially in those tumors that have progressed despite AI treatment [84-86]. *ESR1* mutations allow estrogen-independent ERα activation and cell proliferation [65, 84-88]. *ESR1* chromosomal translocations is another proposed mechanism. Several in-frame fusion genes that preserve the first exons, including the DBD domain, are spliced in-frame into the C-terminus of other genes that are capable of inducing estradiol-independent growth and ER-regulated gene transcription, reducing endocrine sensibility [86, 89, 90] *ESR1* amplification, and thus, ER overexpression is also a documented mechanism of resistance. However, in this case, exposure to estrogen leads to apoptosis through a mechanism that remains under investigation [91]. On the other hand, the role of ERβ in resistance remains unclear and, therefore, it will not be taken into account on this chapter, being ERα simply designed as ER.

5.2.1 Growth factors receptors expression and aberrant activation

In the past decade, several studies have described multiple aberrant pathways and adaptive changes that are deregulated due to the prolonged estrogen deprivation and ER signaling disruption [66] (Fig. 9). The aberrant activation of growth factors receptors, such as fibroblast growth factor receptor 1 (FGFR1) [92, 93], insulin-like growth factor 1 receptor (IGF1R) [94, 95] and human epidermal growth factor receptor 2 (HER2) [96, 97], and their associated downstream signaling components, including MAPKs [98, 99] and PI3K pathways [100, 101], have been associated with acquired-resistance mechanisms because they allow breast cancer cells to bypass estrogen deprivation. Several studies have shown that HER2 overexpression modulates ER expression and activity, through a MAPK-dependent phosphorylation of ER in S118 [96, 97]. In these cases, resistance could be overcome with the treatment with Fulvestrant [102, 103]. However, a sustained overexpression of HER2 may lead to loss

of ER expression, as a mechanism of resistance, bypassing, in these cases, the beneficial effects of Fulvestrant and rendering these cells ligand- and ER-independent [104, 105]. Similar observations were described regarding ER expression and PI3K pathway activation. The AKT downstream effector, p70S6K, was the responsible for the ER phosphorylation on S167 and activation of ER [30, 32, 106], and consequently loss of ER expression [107]. The latter can be through prevention of nuclear localization of the transcription factor forkhead box O3A (FOXO3A), and, thus, of binding to the ESR1 promoter. Thus, inhibition of PI3K induces ER expression through FOXO3A binding to ESR1 [108-110].

Components of the PI3K-AKT-mTOR pathway are often altered in breast cancer and mutation in the α-catalytic subunit of PI3KCA, which encodes p110α, is the most frequent genetic abnormality in luminal-type breast cancer [111-114]. Nevertheless, loss of phosphatase and tensin homolog (PTEN) expression is also associated with a continuous activation of PI3K pathway [114-116]. Activation of the PI3K pathway has been shown to regulate ER expression, and vice-versa. ER has the ability to bind to the regulatory subunit of PI3K, p85, activating the catalytic subunit, p110, and, thus, activating the pathway [117]. The NOTCH pathway was also found to be overactive in endocrine resistant cells. The transcription factor Pre-B-Cell Leukemia Homeobox 1 (PBX1), target gene of the NOTCH pathway, is required for growth in the endocrine therapy-resistant cells [118]. Therefore, targeting growth factor receptors may seem a reasonable approach to delay resistance to AI's. For example, co-targeting HER2 and ER signaling in an ER^+ breast cancer cell model resistant to Letrozole, LTLT-Ca, has proved to be very effective [119]. Moreover, inhibition of mammalian target of rapamycin (mTOR), by everolimus, appears to be very effective in ER^+ patients with advanced disease that progress during, or relapse after, non-steroidal AI therapy, as shown in the large BOLERO-2 trial [120]. Nevertheless, some details have to be taken into account in the BOLERO-2 trial, since there is a lack of statistically significant survival benefit from the combination of everolimus and Exe [121]. A negative feedback loop downstream of PI3K/AKT/mTOR pathway may limit mTOR inhibitors effectiveness [122-124]. When mTOR is activated it phosphorylates and activates ribosomal protein S6 kinase beta-1 (S6K1), a kinase that phosphorylates and destabilizes Insulin Receptor Substrate 1 (IRS-1) and Insulin Receptor Substrate 2 (IRS-2), disrupting IGF1R signaling. When mTOR inhibiton is accomplished there is a reduction in S6K1 activity. This allows IRS-1 and IRS-2 expression that leads to an enhanced activation of IGF1R-dependent AKT activity, counteracting the anti-tumor effectiveness of mTOR blockade. In addition, a positive feedback loop involving the mTORC2 complex exists and leads to an increased AKT signaling [125], that, consequently, results in ER

phosphorylation on Ser 167 preventing the effects of the combination therapy. The BKM120 (buparlisib), a PI3K inhibitor, was shown to have an anti-tumor efficacy in combination with Letrozole [126]. Other inhibitors are also currently being tested in phase I and II clinical trials, more specifically AKT (AZD5363) and dual AKT/mTOR inhibitors (XL765).

5.2.2 Androgen receptor involvement

Despite the importance of GFR and downstream kinase pathways, a receptor has recently come to the spotlight regarding acquired-resistance to AI's. The androgen receptor (AR) is a steroid receptor similar to ER. Although, AR and ER have opposite effects in AI-sensitive breast cancer cells [127], AR plays a different role in AI's-resistant cells fate. AR overexpression, induced by a decreased ER activity, enhances ER transcriptional activity through a cooperation between AR and ER, via PI3K pathway [128-130]. Moreover, an androgen-metabolite with androgenic and estrogenic activities can also activate ER in androgen abundant conditions [131].

5.2.3 Cell-cycle related mechanisms

Cell-cycle-regulators have been associated with acquired-resistance to AI's. It has already been documented that the cyclin D1 encoding gene, CCND1, is commonly amplified in breast cancer [132]. Cyclin D1 is a promoter of G1-to-S progression by forming a complex with cyclin-dependent kinase 4/6 (CDK4/6), thus, inactivating the retinoblastoma protein. In a study conducted by Lundgren, it was found that patients with CCND1 amplification have an increased risk of tumor recurrence in response to Anastrozole [133]. Very recently, it was approved by U.S Food and Drug Administration (FDA) the combination of palbociclib, a CDK 4/6 inhibitor, with Letrozole in ER^+ postmenopausal women due to its high efficacy [134-136]. Moreover, the involvement of aurora kinases in resistance to third-generation AI's was recently described, especially for Exe resistance [137]. Aurora kinases are Ser/Thr kinases involved in cell proliferation through the control of chromatin segregation, thus, promoting cell cycle progression through mitosis.

5.2.4 Autophagy-related mechanisms

Recently, it has been reported the occurrence of autophagy in breast cancer hormone-dependent resistance. Autophagy is a cellular degradation process initiated in response to stress or nutrient deprivation, which attempts to restore metabolic homeostasis through the catabolic lysis of aggregated proteins, unfolded/misfolfed proteins or damaged cellular organelles. In mammalian cells, autophagosome degradation is driven by p62/sequestosome-1 (SQSTM1), which binds directly to ubiquitinated proteins and microtubule-associated protein-1 light chain 3 (LC3), linking ubiquitinated proteins to the autophagic machinery. Formation of the autophagosome double membrane requires the actions of vacuolar protein sorting 34 (Vps34), p150, autophagy-related 4 (Atg4) and beclin-1. Binding of Bcl-2 to beclin-1 inhibits the binding and activation of Vps34, decreases Vps34 mediated activation of PI3K class III and prevents autophagosome formation. Autophagy is also directly regulated by the PI3K class I pathway, since mTOR activation inhibits autophagy initiation [138]. This correlation is important, especially where it is commonly observed alterations/mutations in the PI3KI pathway in a wide range of cancers [3].

In cancer, autophagy can act either as tumor suppressor or as tumor promoter. Studies *in vitro* have shown that blocking autophagosome formation via 3-methyladenine (3-MA) or beclin-1 ribonucleic acid interference (RNAi) enchanced cell death, when combined with a Tamoxifen metabolite or with Exe, suggesting a pro-survival role of autophagy in anti-estrogen therapies [139, 140]. In fact, Inhibition of autophagy through beclin-1 small hairpin RNA (shRNA) or 3-MA treatment partially restores anti-estrogen therapy effectiveness in models of Fulvestrant-resistance and Tamoxifen-cross resistance cancer cells [3]. Moreover, this increased responsiveness of resistant breast cancer cells requires the inhibition of both Bcl-2 and beclin-1, highlighting that inhibition of autophagy pathway, coupled with Bcl-2 inactivation, is more detrimental to anti-estrogen resistant breast cancer cells survival than the inhibition of either pathway alone [3].

Despite these approaches, several others strategies are being currently studied in clinic and began to show some promise, like the use of Histone Deacetylase (HDAC) inhibitors [119, 141-143], among others [144-147].

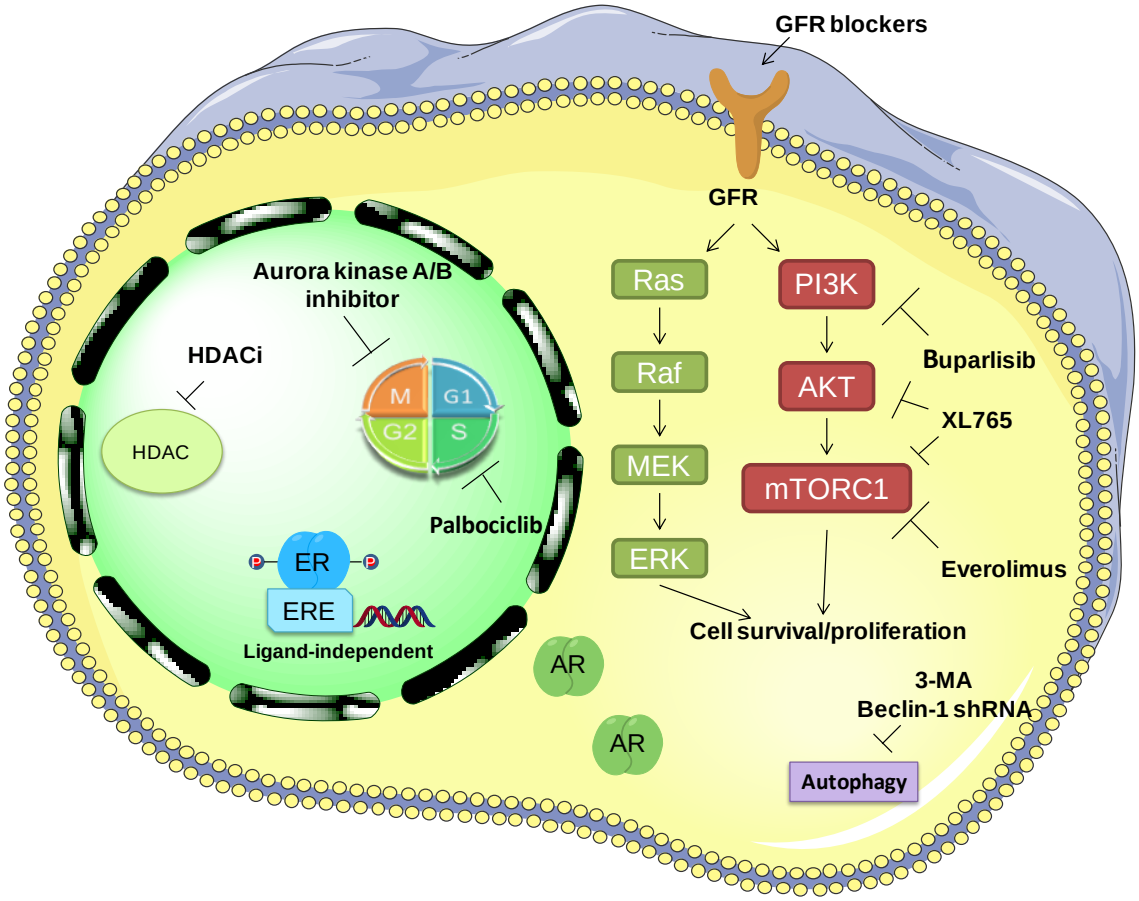


Fig. 9 - Summary of pivotal players and treatments in AI's-acquired resistance. AKT, protein kinase B; AR, Androgen Receptor; ER, Estrogen Receptor; ERE, Estrogen Responsive Element; GFR, Growth Factor Receptor; HDAC, Histone Deacetylase; mTORC1, mammalian Target Of Rapamycin Complex 1; PI3K, Phosphoinositide 3-kinase.

5.3 Exemestane-acquired resistance

Several mechanisms for Exe-acquired resistance have been described (Table 1). Contrary to non-steroidal AI resistant cells, in Exe-acquired resistance, the cancer cells are hormone-dependent [5, 144]. It has been suggested that Exe resistance results from its weak estrogen-like activity, this is, its capacity to activate the ER, since it is seen a high level of amphiregulin (AREG) expression [145]. AREG expression is up-regulated by estrogens [148, 149], thus, it was suggested that Exe may induce AREG up-regulation in a similar way to estrogens, through an ER-dependent manner [145]. AREG is an epidermal growth factor (EGF)-like growth factor that binds to and activates EGFR, which, ultimately, leads to tumor proliferation through MAPK pathway [144, 145]. MAPK inhibition led to a suppression of tumor proliferation in Exe-resistant cancer cell lines. Moreover, aurora kinase A and B have also been associated with

Exe-resistance as they were found to be up-regulated in Exe-resistant cancer cells [137]. Besides, in the BOLERO-2 clinical trial, the combination of everolimus and Exe seem to reverse Exe-resistance [120]. Panobinostat, a HDAC inhibitor, also prevents Exe-resistant cancer cells proliferation, through cell cycle arrest and apoptosis [142]. This study also suggested an involvement of the transcription factor Nuclear Factor-kappa B (NF-kB), since evidences indicated its over-expression in AI's-resistant cells. Similarly, the use of the HDAC inhibitor, entinostat, shows equally promising results [143].

Autophagy is another potential mechanism associated with Exe resistance, described previously by this group. In this case autophagy appears to act as a pro-survival mechanism and the combination of the autophagic inhibitor, 3-methyladenine (3-MA), with Exe resensitized resistant breast cancer cells [150]. Moreover, other recent work described a role of the complex INrf2 (Keap1):Nrf2 in Exe-resistant cell lines. This complex induces the expression of cytoprotective genes when activated by reactive oxygen species (ROS). It was reported a down-regulation of the INfr2 that, consequently, led to an up-regulation of Nfr2, reducing Exe efficacy and, eventually, development of resistance. [144]. Inhibition of Nrf2 was able to resensitize Exe resistant cells [146].

Table 1 - Mechanisms of Exemestane-acquired resistance.

Alteration	***Mechanism of resistance***	***References***
AREG overexpression	Increased MAPK pathway activity	[145]
PI3K/AKT/mTOR overexpression	Decrease expression and Ligand-independent activation of ER	[120]
Aurora kinase A/B up-regulation	Promotion of cell cycle progression	[137]
HDAC aberrant activity	NF-kB expression	[142, 143]
Pro-survival autophagy	Increase cell resistence	[150]
INfr2 down-regulation	Expression of cytoprotective genes	[144, 146]

In conclusion, the understanding of ER^+ breast cancers biology has allowed new approaches to treatment, some of which have now entered clinical practice with the objective to overcome acquired-resistance to AI's. The ultimate goal will be an accurate molecular profiling of patients with ER^+ breast cancer, which would allow personalized and refined treatment strategies, though it still remains in a far future.

6. Aims

Despite the therapeutic success of aromatase inhibitors used in clinic, acquired resistance may develop causing tumor relapse. Thus, it emerges the need to search for new targets and strategies to surpass AI's-acquired resistance. Previous results from the research group where this project is being developed have demonstrated that pro-survival autophagy occurs in Exemestane-treated LTEDaro cells, and that, the PI3K inhibitor, 3-MA, sensitized these cells to Exemestane.

The main objective of this Master Thesis is to contribute to the elucidation of the role of autophagy in Exemestane-acquired resistance. To do this, the effects of two pan-PI3K inhibitors and of a specific autophagic inhibitor in cell fate, autophagic flow and in the phosphorylation state of the main survival pathways will be studied in the LTEDaro cell line, a cell line that mimics the late stage of AI's acquired-resistance.

This work may provide new insights on the mechanisms underlying Exe-acquired resistance and help to discover new possible therapeutic targets.

CHAPTER II - Materials and methods

1. Materials

Eagle's minimum essential medium (MEM), fetal bovine serum (FBS), L-glutamine, antibiotic-antiycotic (10 000 units/mL penicillin G sodium, 10 000 mg/mL streptomycin sulphate and 25 mg/ml amphotericin B), Geneticin (G418), sodium pyruvate and trypsin were supplied by Gibco Invitrogen Co. (PAI'sley, Scotland, UK). Testosterone (T), trypan blue, ethylenediaminetetraacetic acid (EDTA), dimethylsulfoxide (DMSO), tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-difenyltetrazolium (MTT)], propidium iodide (PI), Triton X-100, DNase-free RNase A, staurosporine (STS), activated charcoal, dextran, 2´,7´-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), phorbol 12-myristate 13-acetate (PMA), acridine orange (AO), LY294002 (LY), Wortmannin (WT), Spautin-1 (SP) and the protease inhibitor cocktail were from Sigma-Aldrich Co. (Saint Louis, USA). Z-VAD-FMK was from BD Biosciences Pharmingen (San Diego, CA, USA). Exemestane (Exe) was from Sequoia Research Products Ltd (Pangbourne, UK). Cyto-Tox 96 nonradioactive cytotoxicity assay kit, Caspase-Glo® 9, Caspase-Glo® 8 and Caspase-Glo® 3/7 luminometric assays were from Promega Corporation (Madison, WI, USA). Bradford assay reagent was from Bio-Rad (Laboratories Melville, NY, USA). Chemiluminescent substrate Super Signal West Pico was from Pierce (Rockford, USA). Rabbit polyclonal β-tubulin, rabbit polyclonal p-AKT 1/2/3 (Ser 473), rabbit polyclonal AKT 1/2/3, rabbit polyclonal p-mTOR (Ser 2448), rabbit polyclonal mTOR, rabbit anti-goat IgG, goat anti-rabbit IgG and broad range molecular weight markers were from Santa Cruz Biothecnology (CA, USA). Rabbit monoclonal PI3K p85α was from Abcam (Cambridge, UK). Rabbit polyclonal LC3 I/II was from Cell Signaling (Danvers, MA, USA).

2. Cell culture

The hormone-dependent ER^+ aromatase-overexpressing AI's-sensitive human breast cancer cell line, MCF-7aro, was obtained by stable transfection with human placental aromatase gene and Geneticin selection [151], and kindly provided by Dr. Shiuan Chen (Beckman Research Institute, City of Hope, Duarte, CA, USA). Cells were maintained in Eagle's minimum essential medium (MEM) with phenol-red supplemented with Earle's salts, 1 mmol/L sodium pyruvate, 1% penicillin–streptomycin-amphotericin B, 700 ng/ml Geneticin (G418) and 10% heat-inactivated

FBS. Three days before the experiment MCF-7aro cells were cultured in complete MEM without phenol-red and with 5% pre-treated charcoal heat-inactivated fetal bovine serum (CFBS) to avoid estrogenic interferences from phenol-red and FBS [152]. For the experiments, in the control, and in the different treatments, MCF-7aro cells were incubated with 1 nM of Testosterone (T), which is an aromatase substrate and a proliferation inducing agent.

The hormone-independent ER^+ aromatase-overexpressing AI's-resistant human breast cancer cell line, LTEDaro (*Long-Term Estrogen Deprivation*), was obtained by prolonged culture (six months) of the parental cells, MCF-7aro, in steroid-free medium [6, 144], and was also kindly provided by Dr. Shiuan Chen. Cells were maintained in MEM without phenol-red, with 10% of CFBS, 1% of sodium pyruvate (1 mmol/L), 1% penicillin-streptomycin-amphotericin B and 700 ng/ml Geneticin (G418). Assays were performed in the same medium, and cells without treatment were considered as control. These cells mimic the late-stage of AI's-acquired resistance [153].

Cells were incubated in 5% CO_2 at 37ºC and after reaching a 70-80% confluence, were successively sub-cultured to new culture flasks. For this, cells were detached using 2.5% trypsin/ 1 mM EDTA for 2 minutes, collected to centrifuge tubes containing culture medium with FBS/CFBS to inactivate trypsin/EDTA action and centrifuged at 260 x g for 5 minutes at 4ºC. Supernatant was rejected and the pellet was resuspended in culture medium. After trypan blue staining, cells were counted in Neubauer chambers, and seeded. Culture medium and treatments were refreshed every three days.

3. Charcoal heat-inactivated fetal bovine serum preparation

The FBS was inactivated for 1 hour at 56ºC. In order to remove steroids from the medium, FBS was incubated with activated charcoal for 24 hours at room temperature. After incubation, a series of successive centrifugations were performed during 15 minutes at 4000 x g. In-between centrifugations, supernatant was filtered to eliminate charcoal particles. Centrifugations were performed until CFBS was clear from charcoal. After the final centrifugation, supernatant was filtered by a vacuum filter system of 0.22 µm pore, aliquoted and kept at -20ºC.

4. Cell viability analysis

In order to evaluate the effects of the different treatments in MCF-7aro and LTEDaro cell viability, tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and Lactate dehydrogenase (LDH) release assays were performed.

The MTT colorimetric assay relies on the metabolic activity of mitochondria, since the tetrazolium salt (yellow dye) is reduced by mitochondrial reductases into a blue precipitate, formazan (blue dye), in viable cells. MCF-7aro and LTEDaro cells were cultured in 96-well plates with a cell density of 2 x 10^4 cells/mL, for 3 days experiments, and 1 x 10^4 cells/mL, for 6 days experiments, in MEM without phenol-red containing 5% CFBS and 1nM of T (MCF-7aro), or in MEM without phenol-red containing 10% CFBS, respectively. After 24 hours, cells were treated with the different combinations of the inhibitors, 1-15 μM LY, 0.01-2.5 μM WT, 0.1-10 μM SP, with or without 1-15 μM of Exe. After treatment, cells were incubated with MTT (0.5 mg/mL added to each well) for 2.5 hours at 37ºC in 5% CO_2. Then, DMSO:isopropanol mixture (3:1) was added to stop the reaction and cells were left for 15 minutes under agitation, to dissolve the formazan crystals. After this, formazan was spectrophotometrically quantified at 540 nm.

In order to evaluate the cytotoxic effects of the compounds, LDH release assay was performed in LTEDaro cells. LDH is a commonly used biomarker for cytolysis, since it is a cytosolic enzyme that is release into the culture medium after membrane disruption. This assay relies on the catalytic activity of LDH to convert a tetrazolium salt to a red formazan product. LDH activity was performed through the use of Cyto-Tox 96 nonradioactive cytotoxicity assay kit, according to manufacturer's protocol. Absorbance was read at 450 nm in BioTek Power Wave XS.

All the assays were performed in triplicate in three independent experiments and results were expressed as relative percentage (for MTT) or as absolute value (for LDH) of the untreated control cells.

5. Cell cycle analysis

To study the anti-proliferative effects of the compounds, cell cycle analysis was performed in LTEDaro cells by flow cytometry. Cells were cultured in 6-well plates at a cellular density of 7 x 10^5 cells/mL (3 days) or 3.5 x 10^5 cells/mL (6 days) in MEM without phenol-red containing 10% CFBS. After 24 hours, cells were treated with the different combinations of the inhibitors, 5 μM LY, 0.1 μM WT or 0.5 μM SP, with or without 10 μM of Exe. Untreated cells were considered as controls. After the treatments, non-adherent and adherent cells harvested by 2.5% trypsin/ 1mM EDTA treatment were transferred to centrifuge tubes containing 1mL of culture medium with 10% CFBS. Cells were then centrifuged at 260 x g for 5 minutes at 4ºC. Supernatant was rejected and cells were fixed in 0.1 % PBS and in 70% cold ethanol and stored at 4ºC for 24 hours. Fixed cells were centrifuged as referred, washed two times with PBS and stained with 0.4 mL of a DNA staining solution (5 μg/mL propidium iodide (PI), 0.1% Triton X-100 and 200 μg/ml DNase-free RNase A in PBS), overnight at 4ºC. Triton-X100 permeates the fixed cells membrane allowing PI to reach the nucleus; PI is a fluorescent dye that intercalates nucleic acids, enabling DNA content determination. DNase-free RNase A degrades RNA present in the samples to avoid its interference with the analysis. Flow cytometric analysis was performed based on the acquisition of 40 000 events/cells in BD Accuri™ C6 cytometer (San Jose, CA, U.S.A), equipped with BD Accuri™ C6 analysis software. The forward scatter (FSC) and side scatter (SSC) detectors and the three fluorescence channels (FL-1 (green), FL-2 and FL-3 (red)) were set on a linear scale. Debris, cell doublets and aggregates were gated out using a two parameter plot of FL-2-Area versus FL-2-Width of PI fluorescence. The results were indicated by the percentage of cells in the different cell cycle phases. The anti-proliferative effects of each treatment were indicated by the percentage of cells in G_0/G_1, S and G_2/M phases of the cell cycle. All assays were performed in triplicate and in three independent experiments.

6. Apoptosis analysis

In order to study apoptosis, a type of programmed cell death, it was evaluated the activities of caspase-7, -8 and -9 as well as reactive oxygen species (ROS) formation. Caspases are proteases enzymes involved in apoptosis promotion.

Caspase-7 is an executioner caspase, while caspase-8 and -9 are initiator caspases with the capacity of activate caspase-7.

Caspases activities were measured in treated LTEDaro cells after 3 days by a luminescent assay kit. These assays rely on the use of a caspase-specific substrate, which is cleaved, releasing a substrate for luciferase. Cells were cultured in white-walled 96-well plates with a cellular density of 2 x 10^4 cells/mL. After 24 hours, cells were treated with the different inhibitors, 5 μM LY, 0.1 μM WT or 0.5 μM SP, with or without 10 μM of Exe. After the incubation period, Caspase-Glo® 9, Caspase-Glo® 8 or Caspase-Glo® 3/7 luminescence kits were used according to manufacturer's instructions. Untreated cells were used as control. As positive controls, cells were treated with staurosporin (STS) at 10 μM and as negative control cells were treated with Exe plus LY and then incubated with Z-VAD-FMK (50 μM), a pan-caspase inhibitor, for 3 hours. Luminescence, presented as relative light units (RLU), was measured in a 96-well microplate luminometer (Synergy HT, BioTek, USA) after 1 hour and 30 minutes of incubation. It is noteworthy that MCF-7aro, the parental cells of LTEDaro, are caspase-3 deficient [154], therefore, in LTEDaro cells the Caspase-Glo® 3/7 will only evaluate caspase-7 activity.

ROS formation occurs after mitochondrial damage and may be associated to apoptosis. In order to evaluate the levels of intracellular ROS it was used the 2´,7´-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) method. $DCFH_2$-DA is a lipophilic non-fluorescent compound that cross cell membrane and is oxidized, by ROS, giving rise to the fluorescent compound 2´,7´-dichlorofluorescein (DCF) [155]. LTEDaro cells were cultured in black-walled 96-well plates with a cellular density of 2 x 10^4 cells/mL. Cells were treated with the different inhibitors, 5 μM LY, 0.1 μM WT or 0.5 μM SP, with or without 10 μM of Exe and after 24 hours of treatment, cells were incubated with $DCFH_2$-DA (50 μM), for 1 hour, at 37ºC. As positive controls, cells were treated with phorbol 12-myristate 13-acetate (PMA) at 25 ng/mL during 3 hours, prior to the addition of $DCFH_2$-DA. Untreated cell were considered as controls. Fluorescence, present as mean fluorescence intensity (MFI), was measured using an excitation wavelength of 480 nm and an emission filter at 530 nm, in a 96-well microplate luminometer (Synergy HT, BioTek, USA). All the assays were performed in triplicate in three independent experiments.

7. Detection of acidic vesicular organelles

Acidic vesicular organelles (AVOs) are suggestive of autophagy. Acridine orange (AO) was used to evaluate and quantify the formation of AVOs, by flow cytometry and fluorescent microscopy. AO is an acidotropic fluorescent dye that stains DNA and cytoplasm bright green (AO^-), while, in the presence of acidic compartments, such as lysosomes and autolysosomes, stains bright red (AO^+).

For flow cytometry, LTEDaro cells were cultured in 6-well plates with a cellular density of 3.5 x 10^5 cells/mL. After 24 hours, cells were treated with the different inhibitors, 5 μM LY, 0.1 μM WT or 0.5 μM SP, with or without 10 μM of Exe. After 6 days, adherent and non-adherent cells were harvested by 2.5% trypsin/ 1mM EDTA treatment and transferred to separate centrifuge tubes containing 1mL of culture medium with 10% CFBS. Cells were then centrifuged at 260 x g for 5 minutes at 4ºC. Supernatant was rejected and the pellet was resuspended in PBS, centrifuged and incubated with AO (0.5 μg/mL) for 15 minutes, at 37ºC. Then, cells were centrifuged, washed with PBS between centrifugations. Cells treated with H_2O_2 (0.1 mM) during 16 hours, prior to the end of the experiment, were considered as positive controls and untreated cells were considered as controls. Flow cytometric analysis was performed based on the acquisition of 40 000 events/cells in BD Accuri™ C6 cytometer (San Jose, CA, U.S.A), equipped with BD Accuri™ C6 analysis software. Green (510-530 nm) and red (>650 nm) fluorescence emission with blue (488 nm) excitation light was measured with detectors for fluorescence channels FL-1 and FL-3. The FSC and SSC detectors as well as FL-1 and FL-3 channels were set on a linear scale. Debris, cell doublets, aggregates and negatively stained cells were gated out using a two parameter plot of FL-1 versus FL-3 and FSC versus SSC. The results were indicated by the percentage of AO^- (FL-1 positive/FL-3 negative) and AO^+ cells (FL-1 positive/FL-3 positive). All assays were performed in triplicate and in three independent experiments.

For fluorescence microscopy, LTEDaro cells were cultured in 24-well plates with coverslips at a cellular density of 1 x 10^5 cells/mL, for 6 days. After 24 hours, cells were treated with the different inhibitors, 5 μM LY, 0.1 μM WT or 0.5 μM SP, with or without 10 μM of Exe. After the incubation time cells were stained with AO (0.1 μg/mL) during 15 minutes and the coverslips were mounted with PBS and observed under a fluorescent microscope (Eclipse Ci, Nikon, Japan) equipped with a 490 nm band-pass blue excitation filters and a 515 nm long-pass barrier filter. Images were processed by Nikon NIS Elements image software.

8. Western blot analysis

In order to quantify PI3K expression, AKT phosphorylation, mTOR phosphorylation and LC3 turnover, LTEDaro cells were cultured in 6-well plates at a cellular density of 7 x 10^5, for 3 days, and of 3.5 x 10^5, for 6 days. After 24 hours, cells were treated with the different inhibitors, with or without 10 μM of Exe. After the incubation, the culture medium was removed and the cells were lysed with cold TNTE lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 0.3% Triton X-100 and 5 mM EDTA), pH 7.4, containing 1% of protease inhibitors cocktail. Then, cells were collected, and the cell lysates were centrifuged at 18 800 x g for 10 minutes at 4ºC. The protein concentrations in the supernatant were assessed by Bradford assay. A total of 50 μg of protein per sample was subjected to 4-20% (for LC3 samples) or 10% SDS polyacrylamide gels and transferred to nitrocellulose membranes in 25 mM Tris-HCl, 250 mM glycine and 20% methanol. The membranes were blocked for 1 hour, with 5% non-fat milk in TBS/Tween® 20, and incubated with the different primary antibodies, rabbit polyclonal p-AKT 1/2/3 (1:200), rabbit polyclonal p-mTOR (1:200), rabbit monoclonal PI3K p85α (1:1000) and rabbit polyclonal LC3 I/II (1:200) in blocking solution overnight at 4ºC. Secondary antibodies were then incubated for 1 hour, after previous washes with TBS/Tween® 20. Membranes were exposed to chemiluminescent substrate Super Signal West Pico and immunoreactive bands were visualized by ChemiDoc™ Touch Imaging System (BioRad, Laboratories Melville, NY, USA). Membranes were further stripped and incubated with rabbit polyclonal β-tubulin (1:500), rabbit polyclonal AKT 1/2/3 (1:200) or rabbit polyclonal mTOR (1:200) followed by incubation with the respective secondary antibodies (1:2000), to control loading variations. Untreated cells were used as control. All assays were performed in triplicate in three independent experiments.

9. Statistical analysis

Statistical analysis of data was performed using analysis of variance (ANOVA) test followed by Bonferroni test for multiple comparisons in Graphpad Prism 7 software. Values of $p < 0.05$ were considered statistically significant. The data presented were expressed as mean ± SEM (standard error of the mean).

CHAPTER III - Results

1. Cell viability in ER^+ sensitive and resistant breast cancer cells

It has been described that autophagy may be involved in resistance to AI's. Thus, inhibition of autophagy may contribute to re-sensitize resistant cells to AI's therapy. In order to validate this hypothesis, the effects of two PI3K inhibitors, LY294002 (LY) and Wortmannin (WT), and of a specific autophagic inhibitor, Spautin-1 (SP), in cell viability, were evaluated, in MCF-7aro cells, an hormone-dependent ER^+ sensitive breast cancer cell line, and in LTEDaro cells, an hormone-independent ER^+ resistant breast cancer cell line, by MTT assays. To select suitable concentrations for the following studies and based on the concentration ranges described in the literature, MCF-7aro and LTEDaro cells were cultured, respectively, with or without testosterone and with the inhibitors at different concentrations (1 – 15 μM for LY, 0.01 – 2.5 μM for WT and 0.1 – 10 μM for SP) during different times of incubation (3 and 6 days). MCF-7aro cells treated only with testosterone (T) or untreated LTEDaro cells were considered as controls and represent 100% of cell viability.

The results allowed the selection of concentrations that did not exhibit statistically significant differences comparing to control at the two different incubation times (3 and 6 days). For LY was 1 μM in MCF-7aro cells and 5 μM in LTEDaro cells; for WT the selected concentrations were 1 μM in MCF-7aro and 0.1 μM in LTEDaro and for SP it was 0.5 μM for both cell lines (Fig.10).

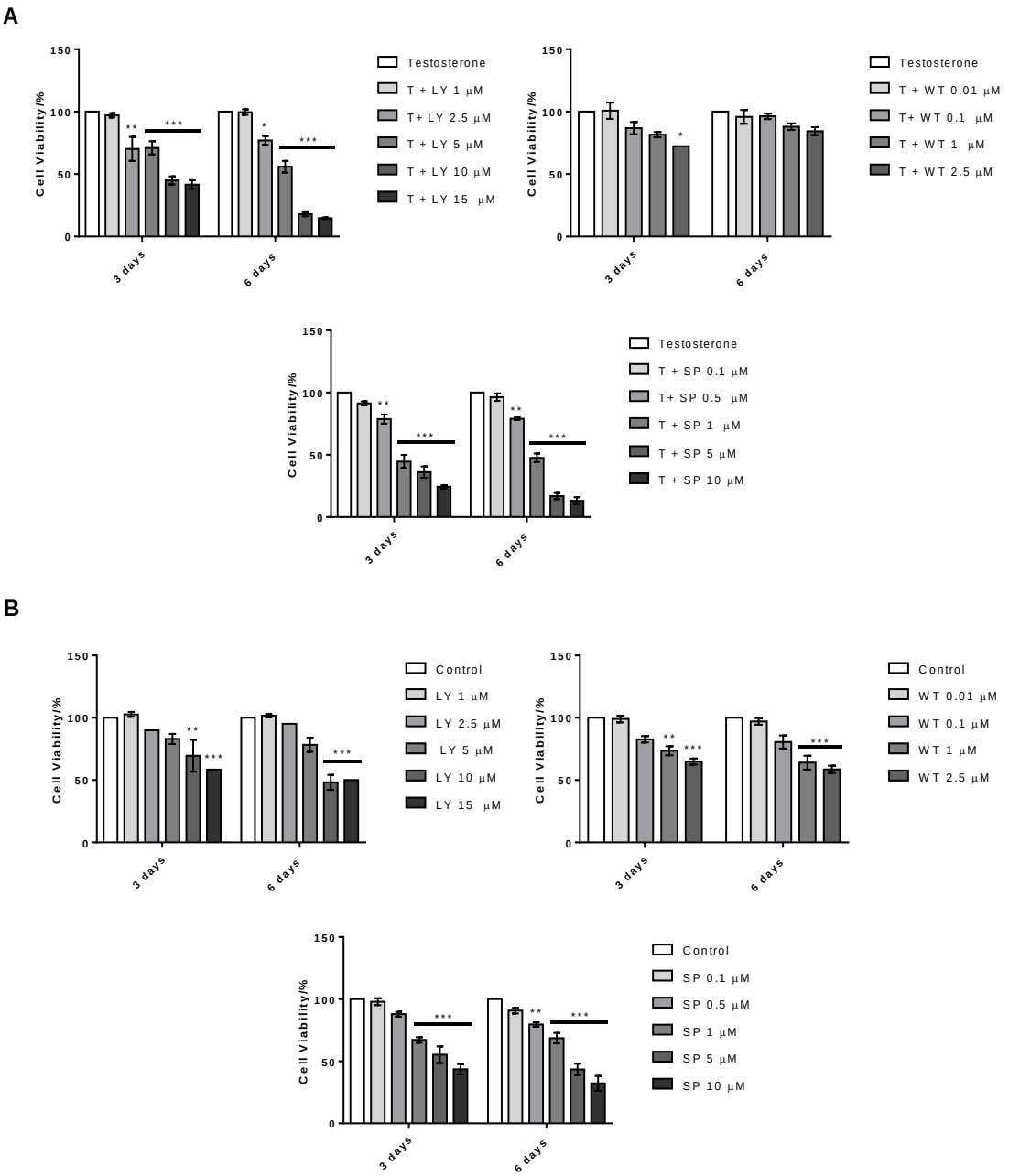


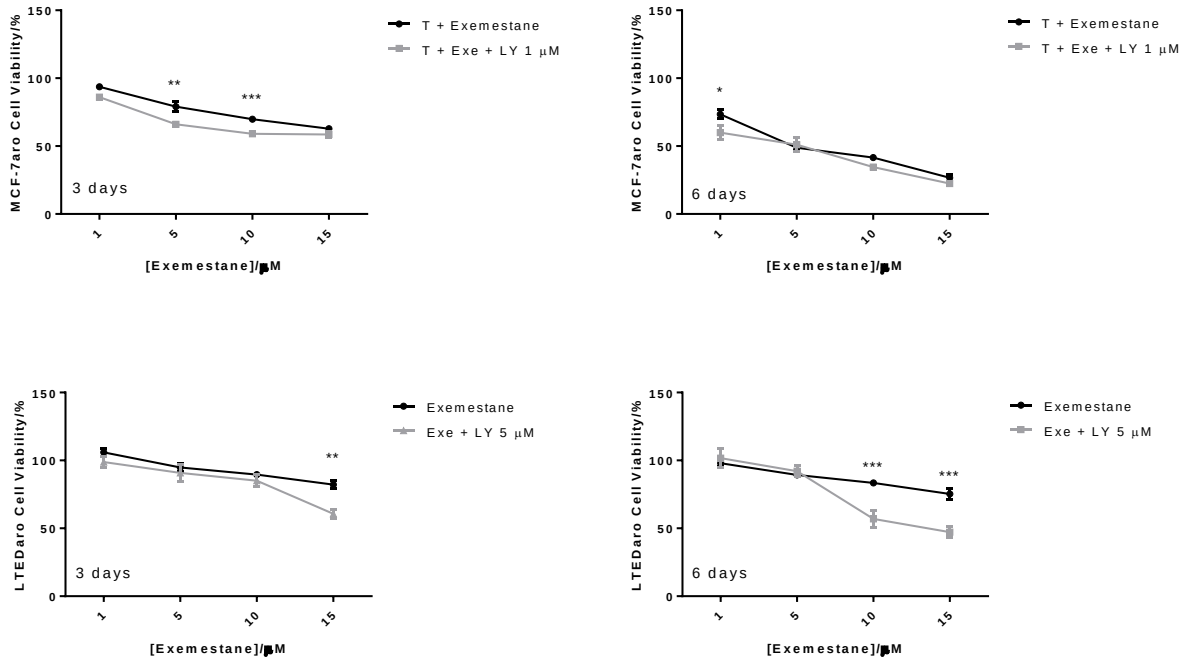
Fig. 10 - Effects of the PI3K and autophagic inhibitors on cell viability. **(A)** Comparison of the effects of the different inhibitors in MCF-7aro cells. (**B)** Comparison of the effects of the different inhibitors in LTEDaro cells. MCF-7aro cells cultured with testosterone (T) were considered as control at 3 or 6 days of incubation. LTEDaro cells were treated with different concentrations of compounds during 3 or 6 days. Untreated LTEDaro cells were considered as control. Results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the inhibitors and control are denoted by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

In order to explore the signaling pathways that may be involved in Exe-acquired resistance it was evaluated the effects of the inhibitors in combination with the AI, Exemestane (Exe), in the sensitive and resistant cell lines, for 3 and 6 days.

In relation to the PI3K inhibitor, LY, in MCF-7-aro cells and when combined with Exe, it was observed a significant decrease, in cell viability when compared to Exe alone after 3 days of incubation (Fig. 11). However, at 6 days, there were no statistically significant differences between Exe and LY in combination with Exe. In LTEDaro cells LY with Exe at higher concentrations than 10 μM induced a significant ($p<0.001$) decrease in cell viability, when compared to Exe. On the other hand, WT had no significant effects in Exe-treated MCF-7aro cells, while it decreased significantly the viability of Exe-treated LTEDaro cells after 6 days of treatment. The SP had an apparent protective role in the Exe-treated sensitive cell line, since in these cells it prevents the reduction of cell viability induced by Exe alone. On the contrary, SP significantly decreased the viability of Exe-treated resistant cells in a dose and time-dependent manner.

Overall, all the inhibitors sensitize LTEDaro cells to Exe in a time-dependent manner.

A



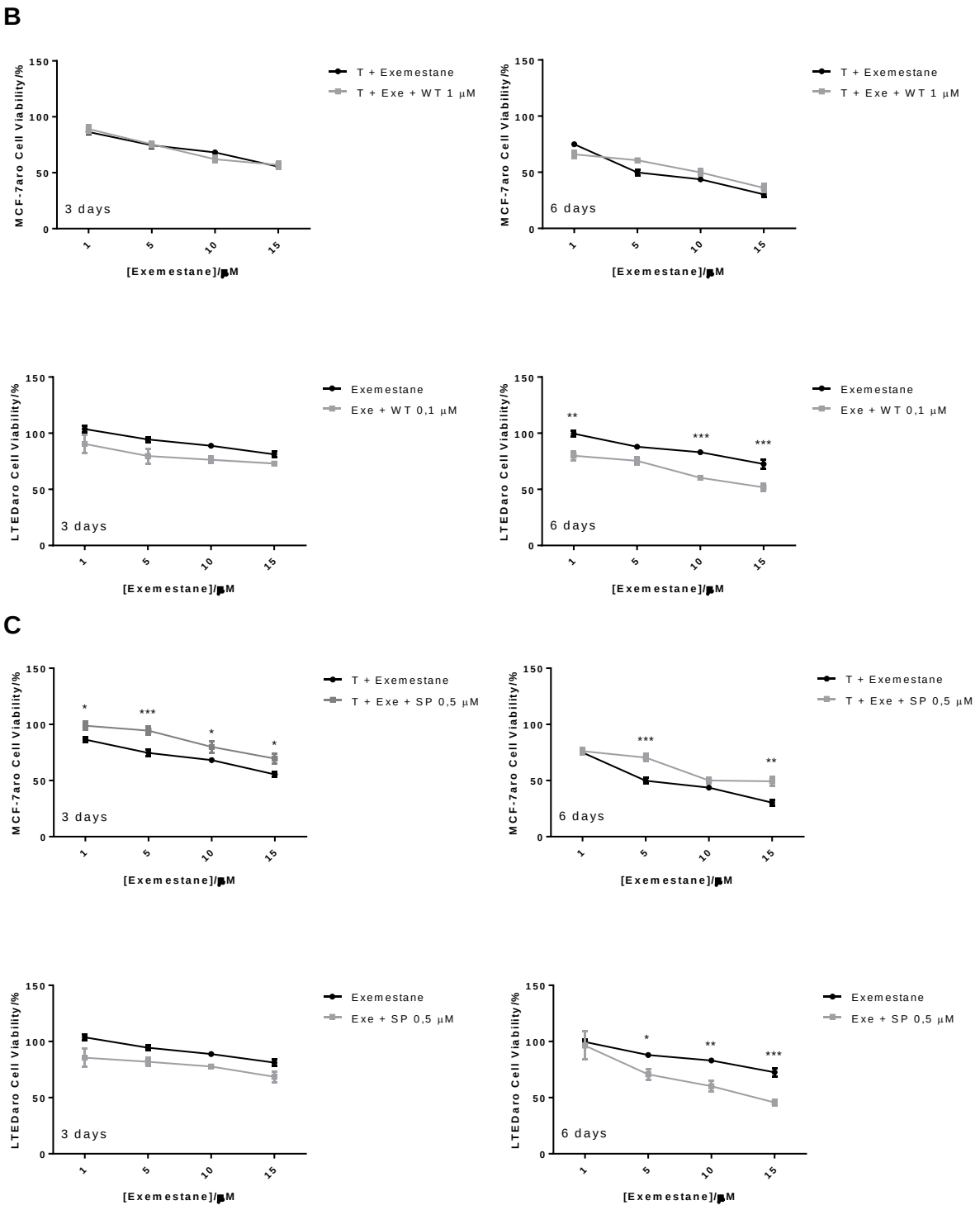


Fig. 11 - Effects of the combination of Exemestane with PI3K and autophagic inhibitors on the viability of MCF-7aro and LTEDaro, evaluated by MTT assay. (**A)** Effects of the combination of LY with Exemestane. **(B)** Effects of the combination of WT and Exemestane. **(C)** Effects of the combination of SP with Exemestane. MCF-7aro and LTEDaro cells were treated with different concentrations of inhibitors during 3 or 6 days. MCF-7aro cells cultured with testosterone (T) were considered as control. Untreated LTEDaro cells were considered as control. Results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between Exemestane alone and in combination with the inhibitors are denoted by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

2. LDH activity assay in LTEDaro

Through the determination of LDH activity in the cell culture medium, it was possible to evaluate if the decrease in LTEDaro cell viability, induced by the different treatments, was due to cytotoxicity caused by cell membrane lysis. Untreated LTEDaro cells were considered as control and represent the basal value of LDH release. As shown in the figure 12, in the resistant cell line, only LY in combination with the highest dose of Exe induced alterations in cell membrane permeability ($p<0.01$). For this reason, in the subsequent studies only Exe at 10 μM was used in combination with the inhibitors.

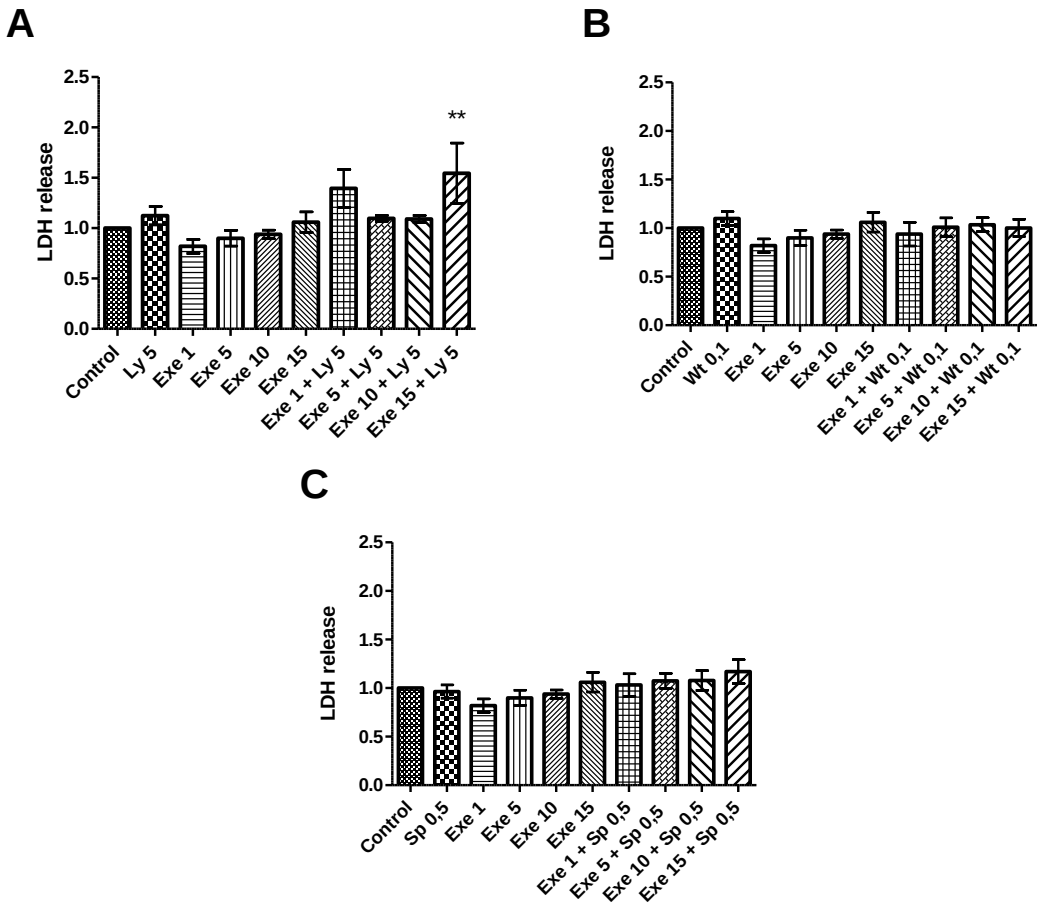


Fig. 12 - Effects of Exemestane alone or in combination with LY **(A)**, WT **(B)** or SP **(C)** in LTEDaro cells, evaluated by LDH release assay. LTEDaro cells were treated with different concentrations of compounds during 3 days. Untreated LTEDaro cells were considered as control. Results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between control and the treatments are denoted by ** ($p < 0.01$).

3. Cell proliferation studies

In order to better understand if the reduction in LTEDaro cell viability, observed by MTT assay, was due to anti-proliferative effects, it was studied by flow cytometry analysis the cell cycle progression. Cell cycle analysis was performed after Propidium Iodide (PI) staining, both for 3 and 6 days of incubation. PI is a fluorescent dye that intercalates DNA, allowing the determination of its content within cells.

Results have demonstrated that Exe, after 3 and 6 days, does not affect cell cycle by itself, since no significant differences were observed between Exe and control. However, the combination of Exe with the pan-PI3K inhibitors disturbs cell cycle progression in a similar way, after 3 days of treatment. As observed in table 2 and figure 13, in Exe-treated LTEDaro cells, LY and WT promoted an increase of 5.84% ($p<0.001$) and 6.10% ($p<0.001$), respectively, in G_0/G_1 phase, accompanied by a decrease of 3.54% ($p<0.01$) and 3.78% ($p<0.01$), respectively, in G_2/M phase, when compared to Exe. Curiously, both PI3K inhibitors caused no significant difference in S phase when compared to Exe alone. Strikingly, and on the contrary, the combination of SP with Exe induced a decrease of 2.92% in G_0/G_1 phase, accompanied by an increase of 4.81% ($p<0.001$) in the G_2/M phase, when compared to Exe alone, without causing, as well as the other inhibitors, significant differences in the S phase.

Table 2 - Effects of the different treatments on cell cycle distribution in LTEDaro cells for 3 days of incubation.

Cell cycle	G_0/G_1	S	G_2/M
Control	74.33 ± 1.11	7.99 ± 0.51	17.70 ± 0.79
Exemestane 10 µM	77.48 ± 0.46	5.47 ± 0.65	16.72 ± 0.52
LY 5 µM	77.23 ± 0.80	6.08 ± 0.58	16.14 ± 0.73
Exe + LY 5 µM	83.32 ± 0.97 *** ###	2.81 ± 0.18 *	13.18 ± 0.76 * ##
WT 0.1 µM	75.03 ± 0.58	6.36 ± 0.41	17.40 ± 0.29
Exe + WT 0.1 µM	83.58 ± 0.83 *** ###	2.80 ± 0.34 *	12.94 ± 0.76 *** ##
SP 0.5 µM	76.86 ± 0.77	5.51 ± 0.76	16.35 ± 0.75
Exe + SP 0.5 µM	74.56 ± 0.77 #	3.55 ± 0.15	21.53 ± 0.76 *** ###

Cells were treated with different concentrations of compounds during 3 days and analysed by flow cytometry after PI staining. Cells without treatment, or treated only with the inhibitors, were considered as control. The data represents the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the controls and treated cells are denoted by * ($p < 0.05$) and *** ($p < 0.001$). Significant differences between Exemestane alone and in combination with the inhibitors are denoted by # ($p < 0.05$), ## ($p < 0.01$) and ### ($p < 0.001$).

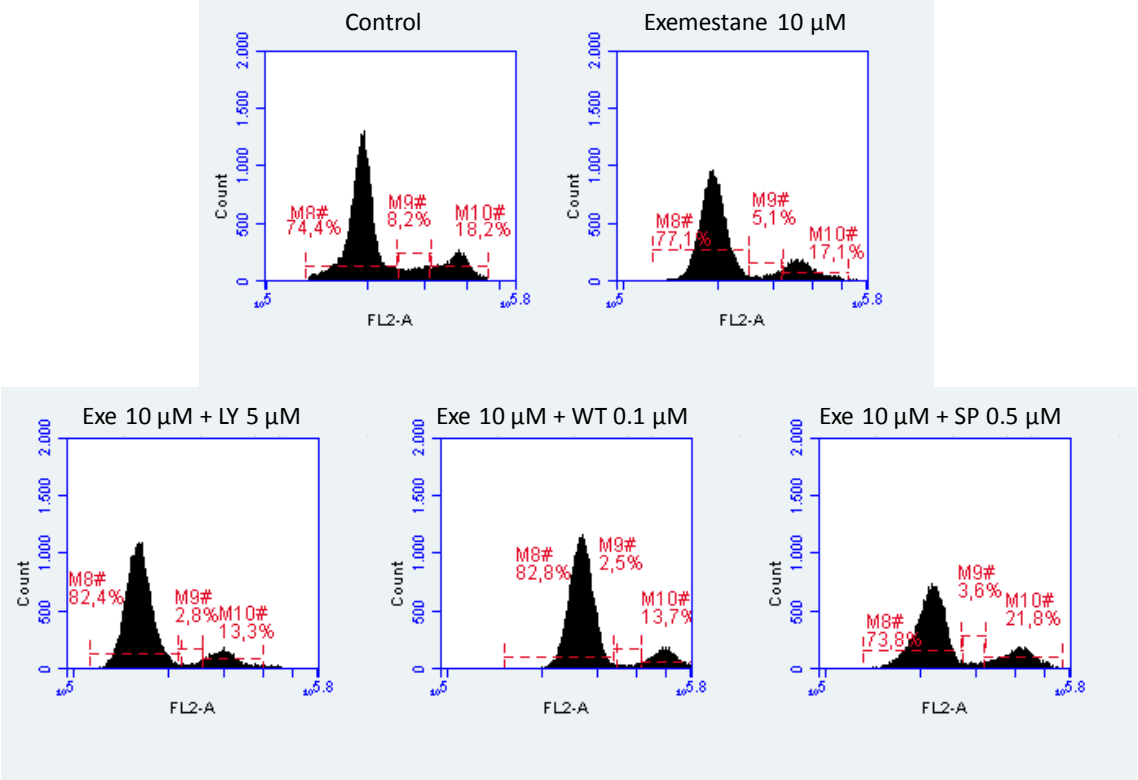


Fig. 13 – A representative histogram of the effects of Exemestane alone and in combination with each inhibitor on cell cycle progression in LTEDaro cells after 3 days of incubation. To study cell cycle progression, cells were analysed by flow cytometry after PI staining. Untreated cells were considered as control**.** The histograms of cell cycle are representative of one independent assay, being M8 gate the corresponding to G_0/G_1 phase, M9 gate to S phase and M10 gate to G_2/M phase.

However, after 6 days of incubation, LY and WT did not induce a G_0/G_1 phase arrest in Exe-treated cells, as seen in table 3 and figure 14. Only WT caused a significant increase of 3.29% ($p<0.05$) in G_2/M phase, in relation to Exe alone. SP induced similar effects on the deregulation of cell cycle when combined with Exe, through a significant decrease in G_0/G_1 phase, 8.24% ($p<0.001$), and a more accentuated increase in the G2/M phase, 10.71% ($p<0.001$) when compared to Exe alone.

Table 3 - Effects of the different treatments on cell cycle distribution in LTEDaro cells for 6 days of incubation.

Cell cycle	G_0/G_1	S	G_2/M
Control	78.93 ± 1.08	7.37 ± 0.47	14.71 ± 0.77
Exemestane 10 µM	78.77 ± 0.79	4.69 ± 0.20	15.81 ± 0.84
LY 5 µM	78.26 ± 0.87	5.92 ± 0.50	16.32 ± 0.47
Exe + LY 5 µM	80.07 ± 0.25	2.85 ± 0.26 *	17.80 ± 0.53
WT 0.1 µM	77.56 ± 0.36	5.71 ± 0.39	17.02 ± 0.51
Exe + WT 0.1 µM	78.36 ± 1.41	3.11 ± 0.18	19.10 ± 1.51 #
SP 0.5 µM	77.71 ± 0.76	5.88 ± 0.17	17.53 ± 0.95
Exe + SP 0.5 µM	70.53 ± 1.19 *** ###	3.26 ± 0.23	26.52 ± 1.04 *** ###

Cells were treated with different concentrations of compounds during 6 days and analysed by flow cytometry after PI staining. Cells without treatment, or treated only with the inhibitors, were considered as control. The data represents the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the controls and treated cells are denoted by * ($p < 0.05$) and *** ($p < 0.001$). Significant differences between Exemestane alone and in combination with the inhibitors are denoted by # ($p < 0.05$) and ### ($p < 0.001$).

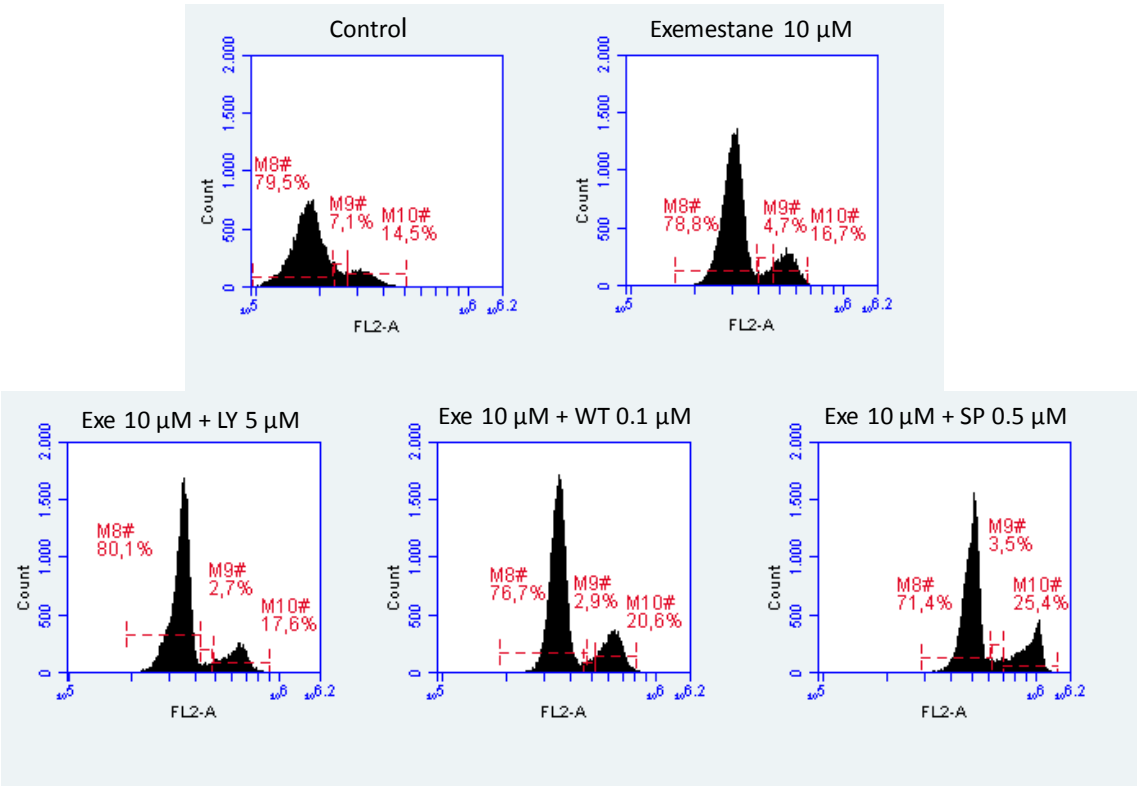


Fig. 14 - A representative histogram of the effects of Exemestane alone and in combination with each inhibitor on cell cycle progression in LTEDaro cells after 6 days of incubation. To study cell cycle progression, cells were analysed by flow cytometry after PI staining. Untreated cells were considered as control**.** The histograms of cell cycle are representative of one independent assay, being M8 gate the corresponding to G_0/G_1 phase, M9 gate to S phase and M10 gate to G_2/M phase.

4. Cell death mechanisms

Several types of cell death mechanisms can be associated to the decrease in LTEDaro cell viability induced by the treatments. In order to study the involvement of apoptosis, it was evaluated the activity of caspases -7, -8 and -9. Caspases are enzymes involved in cellular processes such as programmed cell death. Caspase-7 is an executioner caspase of apoptosis, while caspase-8 and -9 are initiator caspases with the function to activate executioner caspases, through the extrinsic and intrinsic pathway, respectively.

LTEDaro cells were cultured and exposed to the different treatments for 3 days (Fig. 15). As expected, Exe did not cause any caspase activation, since these cells are AI-resistant. On the contrary, all the inhibitors, in combination with Exe, induced an increase in caspase-7 activity, suggesting an apoptotic process of cell death. However, while LY caused an increase in caspase-9 activity, WT induced an increase in caspase-9 and -8 activities and SP only caused an increment in caspase-8 activity, when compared to Exe. The increase of caspase-7 activity for LY in combination with Exe was 23.4% ($p<0.01$); for WT in combination with Exe was 25.2% ($p<0.001$) while for SP plus Exe it was 13.9% ($p<0.05$), when compared to Exe alone. The increment of caspase-9 activity for Exe in combination with LY was 38.3% ($p<0.001$), and with WT was 53.8% ($p<0.001$). Lastly, WT and SP in combination with Exe increased caspase-8 activity by 87.0% ($p<0.001$) and 31.5% ($p<0.01$), respectively, in comparison with Exe.

As reactive oxygen species (ROS) may be produced by damaged mitochondria and lead to apoptosis. ROS formation was also studied. It was observed that none of the different treatments promoted an increase in intracellular ROS when compared to Exe (Fig. 15).

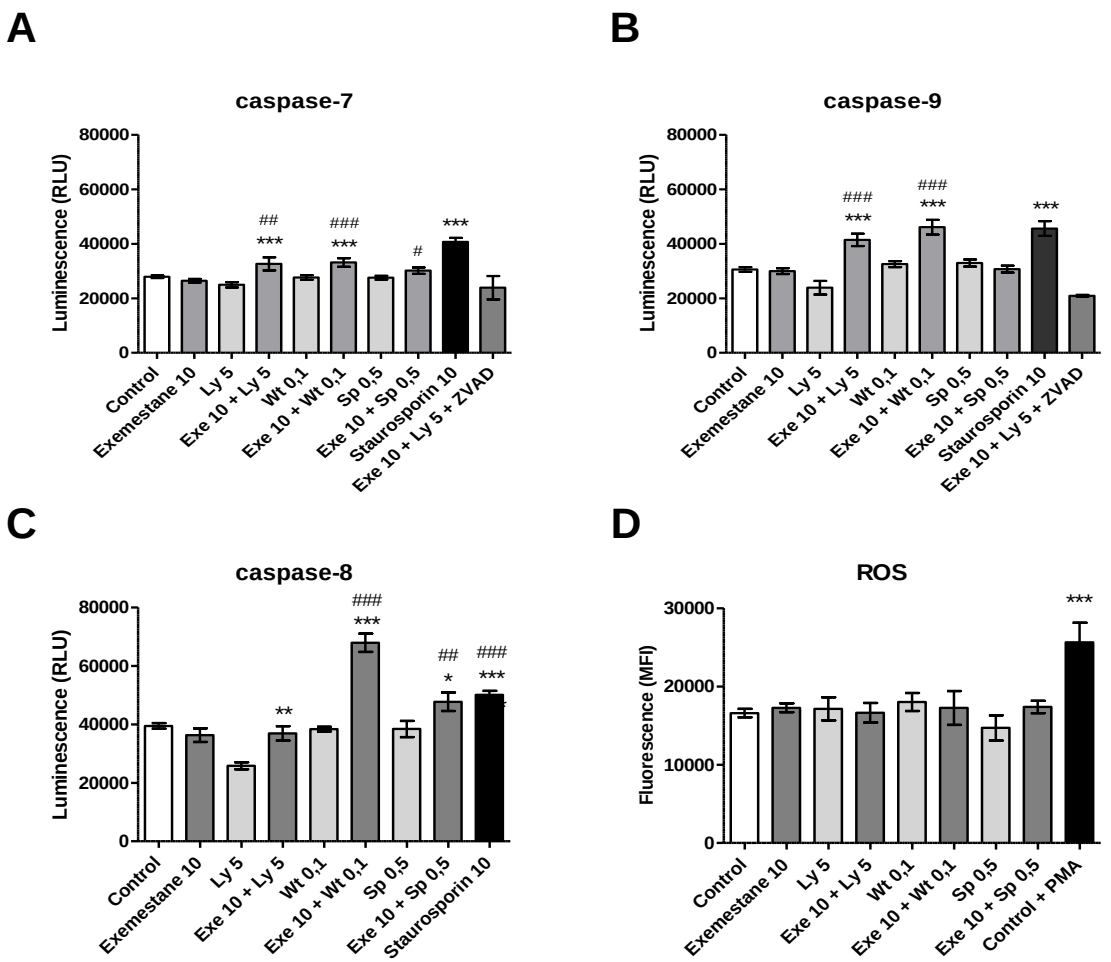


Fig. 15 - Effects of the different treatments in caspases-7 **(A)**, -9 **(B)** and -8 **(C)** activation and ROS production **(D)**. LTEDaro cells were treated with the different combinations for 3 days. Untreated cells, or treated only with the inhibitors, were considered as control. Cells treated with staurosporin were considered as positive controls and with Z-VAD-FMK as negative control, for caspases activation. Cells treated with PMA were considered as positive control for ROS formation. The results are presented as relative luminescence units (RLU), for caspases activation, and as mean fluorescence intensity (MFI), for ROS generation. Results represent the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the controls and treated cells are denoted by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). Significant differences between Exemestane alone and in combination with the inhibitors are denoted by # ($p < 0.05$), ## ($p < 0.01$) and ### ($p < 0.001$).

5. Autophagic studies

Acidic vesicular organelles (AVOs) are suggestive of autophagy occurrence. These vesicles are detected by an acidotropic dye, the acridine orange (AO) fluorescent dye. This stain marks DNA and cytoplasm bright green (AO^-), while, in acidic compartments, such as lysosomes and autolysosomes, AO is protonated and accumulates, forming aggregates that fluorescence bright yellow/orange/red (AO^+).

Acidic vesicular organelles formation was firstly studied by fluorescence microscopy (Fig. 16). LTEDaro cells were treated with the different combinations of compounds during 6 days. The results showed that cells treated with Exe exhibit more

yellow/orange/red fluorescence than the control. On the other hand, the combination of Exe with LY or WT appears to slightly reduce AVO-related fluorescence. Nevertheless, it was observed that each inhibitor by itself seems to induce the presence of yellow/orange/red fluorescence, suggesting AVOs formation. To confirm these results, the levels of AVOs were quantified by flow cytometry, after AO staining, in the same conditions.

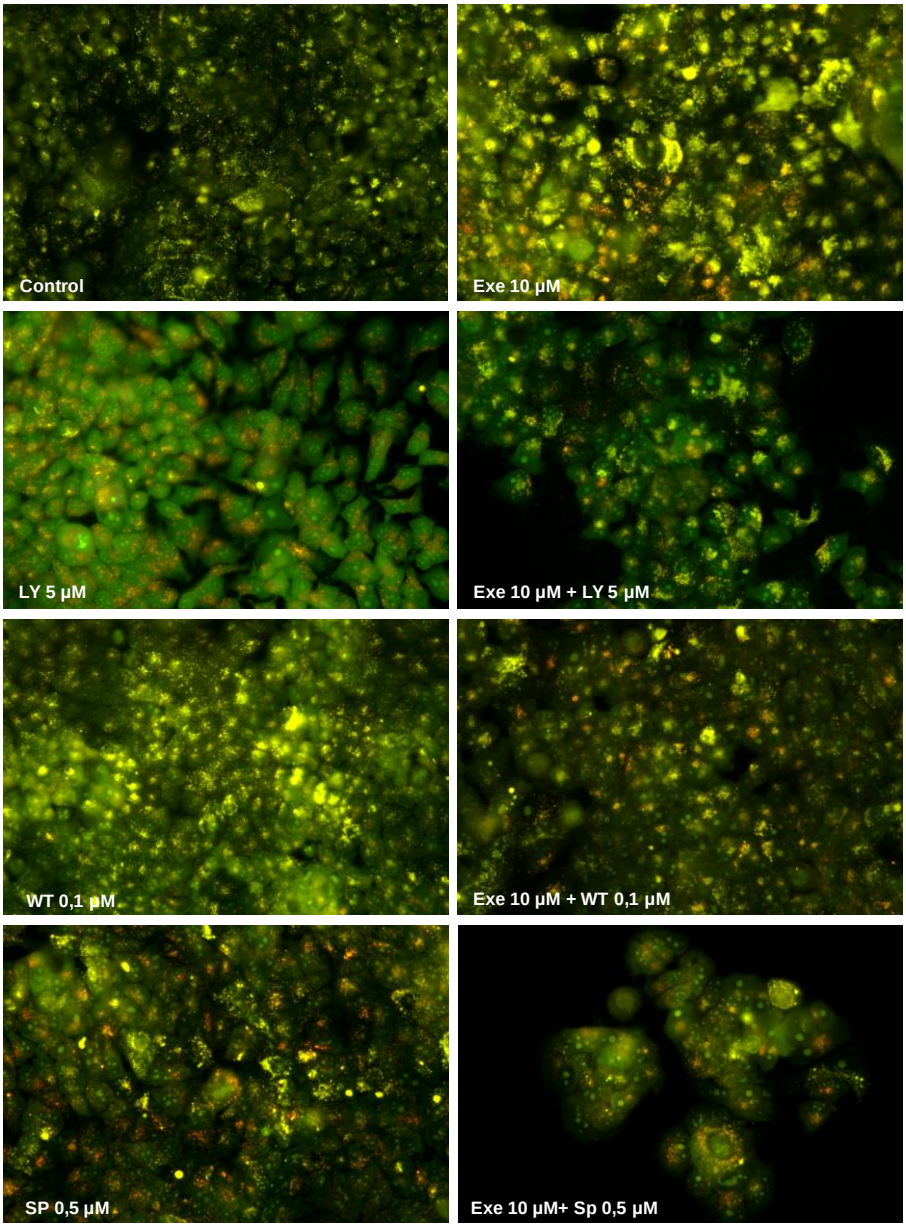


Fig. 16 - Effects of Exemestane and inhibitors, both alone or in combination, on AVOs formation in LTEDaro cells, analyzed by fluorescence microscopy, after 6 days of treatment. The presence of AVOs was detected by acridine orange staining and are stained with yellow to orange/red fluorescence (Original magnification x 400).

By flow cytometry analysis, it was observed that Exe significantly increases AO^+ population ($p<0.001$), by itself, which confirms that Exe increases AVOs production (table 4 and fig. 17). Moreover, the combination of LY and Exe significantly ($p<0.05$) increased total AO^+ marked cells, when compared to Exe alone. Nevertheless, comparing the ratio between Exe + LY/ LY (2.12) and Exe/ Control (5.21), it was verified a decrease in the Exe-induced AVOs formation. On the contrary, the combination of Exe with WT or with SP decreased significantly the total AO^+ marked cells. In fact, the comparison of the ratios between Exe + WT/ WT (2.12) or Exe + SP/ SP (1.81) and Exe/ Control (5.21) demonstrated a reduction in the Exe-induced AVOs formation. In the figure 17, increments on AO^+-marked cells are represented by a displacement of the labeled cells to right in the FL-3 fluorescence channel (FL-3 positive).

Table 4 - Effects of the different treatments on AVOs formation in LTEDaro cells for 6 days of incubation.

AVOs formation	AO^-	AO^+
Control	93.41 ± 0.47	6.78 ± 0.46
Exemestane 10 µM	65.09 ± 1.23 ***	35.33 ± 0.83 *** (5.21)
LY 5 µM	81.00 ± 1.96	19.60 ± 1.95
Exe + LY 5 µM	59.75 ± 2.51 ***	41.57 ± 2.70 *** # (2.12)
WT 0.1 µM	89.48 ± 1.37	10.89 ± 1.41
Exe + WT 0.1 µM	74.78 ± 1.40 *** ###	23.14 ± 1.52 *** ### (2.12)
SP 0.5 µM	84.80 ± 1.42	15.50 ± 1.31
Exe + SP 0.5 µM	72.59 ± 1.06 *** #	28.07 ± 1.04 *** # (1.81)
H_2O_2 50 µM	74.49 ± 2.35 ***	26.25 ± 2.41 ***

Cells were treated with different concentrations of compounds during 6 days and analysed by flow cytometry after AO staining. Cells without treatment, or treated only with the inhibitors, were considered as control. The data represents the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the controls and treated cells are denoted by *** ($p < 0.001$). Significant differences between Exemestane alone and in combination with the inhibitors are denoted by # ($p < 0.05$) and ### ($p < 0.001$).

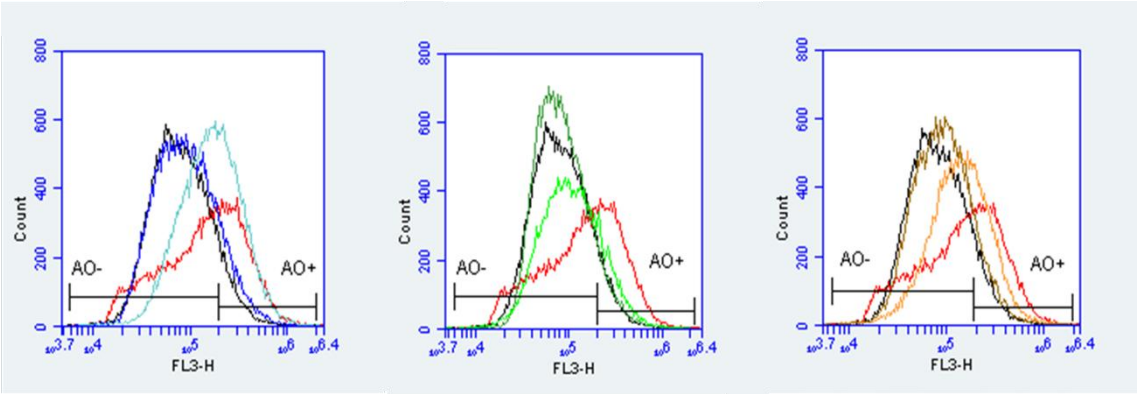


Fig. 17 - A representative histogram of the effects of Exemestane, of each inhibitor alone and of the combination of exemestane with inhibitors on AVOs formation, in LTEDaro cells, after 6 days of incubation. To study AVOs formation, cells were analysed by flow cytometry after AO staining. Untreated cells or treated only with the inhibitors were considered as control**.** The histograms of AO staining are representative of one independent assay. L Control (black), Exemestane 10 µM (red), LY 5 µM (dark blue), Exe 10 µM + LY 5 µM (light blue), WT 0.1 µM (dark green), Exe 10 µM + WT 0.1 µM (light green), SP 0.5 µM (brown), Exe 10 µM + SP 0.5 µM (orange).

Furthermore, it was also evaluated by Western Blot the conversion of LC3-I to LC3-II (Fig. 18), a biomarker of the autophagic process. Preliminary results suggest an increase in LC3 turnover (LC3 II/I ratio) on LTEDaro cells treated with Exe alone after 3 days. Moreover, a decrease in LC3 turnover for the combination of WT and SP with Exe, when compared to Exe alone, was also apparent, suggesting a decrease in autophagy.

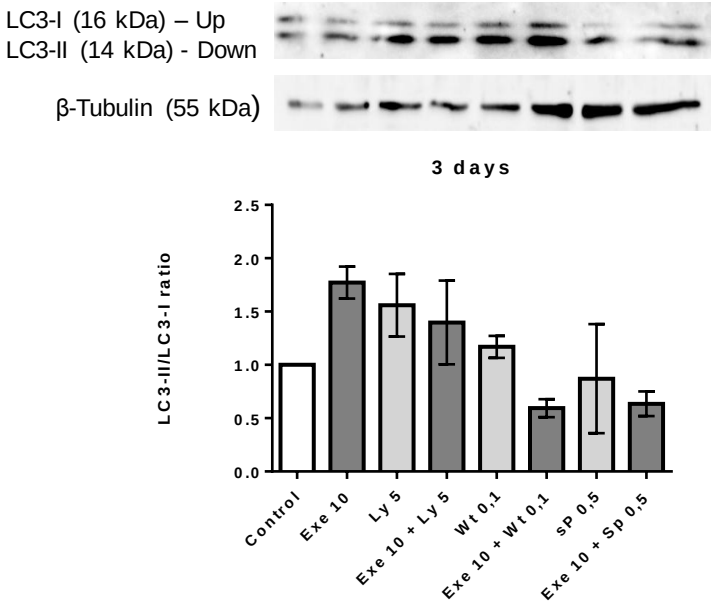


Fig. 18 - Effects of the different treatments on LC3 turnover (LC3 II/I ratio) by WB and densitometry analysis. LTEDaro cells were treated with different combinations during 3 days. Cells without treatment were considered as control.

6. Survival pathways analysis

In order to understand the underlying signaling pathways that led to the previous results, it was evaluated, by western blot, the activation of the PI3K-AKT-mTOR pathway, which is implicated in the regulation of autophagy, through expression of PI3K and phosphorylation of AKT and mTOR. By densitometric analysis (Fig. 19) it was verified that Exe did not significantly interfere with PI3K expression. However, the Exe combinations with all the inhibitors decreased, in a significant manner, the PI3K expression when compared to Exe alone, after 3 and 6 days of incubation.

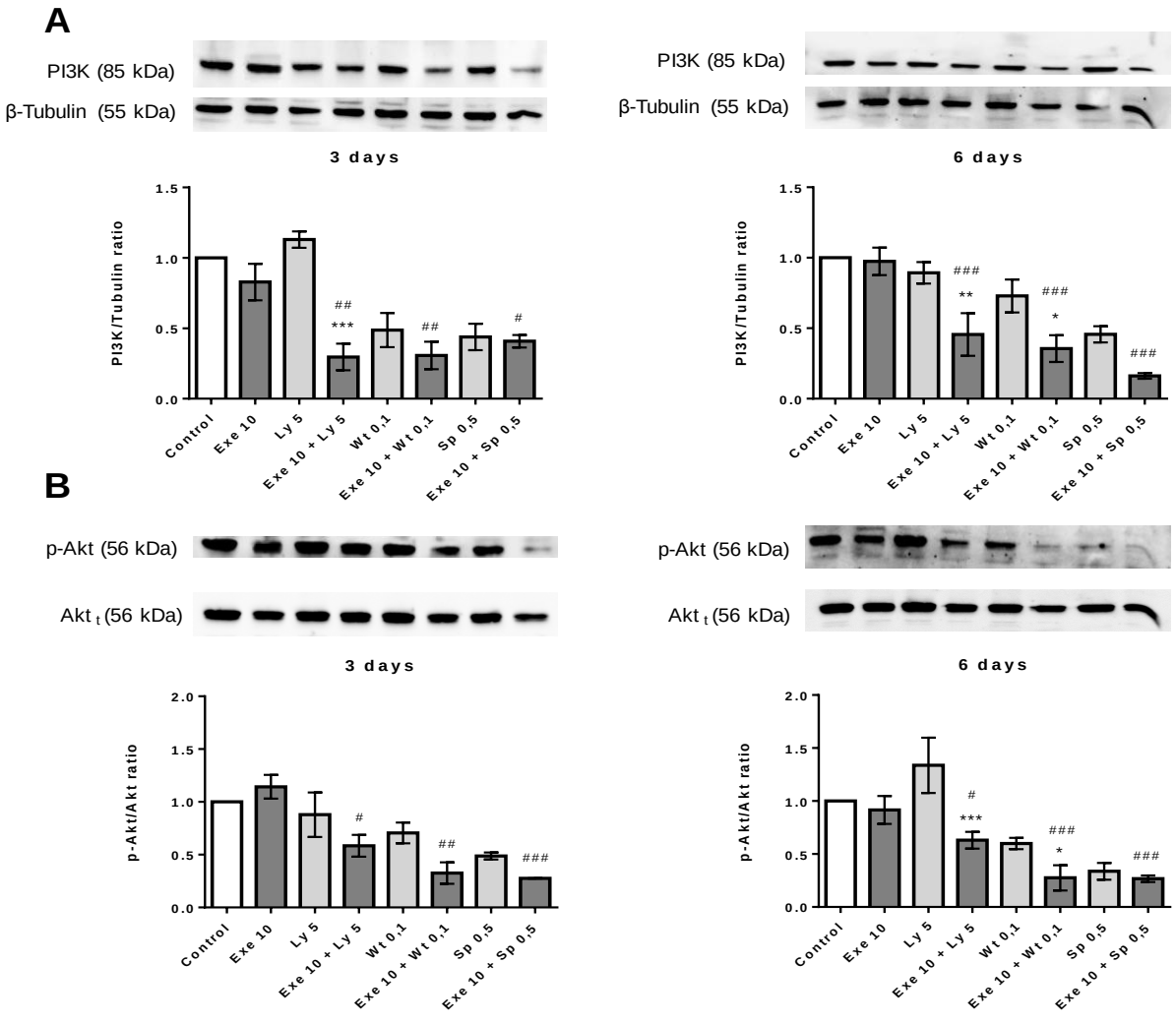


Fig. 19 - Effects of the different treatments on the PI3K/AKT pathway. **(A)** Effects on the PI3K expression and **(B)** on AKT phosphorylation, after 3 or 6 days. Untreated cells were considered as control. Results are the mean ± SEM of three independent experiments. Significant differences between the controls and treated cells are denoted by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). Significant differences between Exemestane alone and in combination with the inhibitors are denoted by # ($p < 0.05$), ## ($p < 0.01$) and ### ($p < 0.001$).

As expected, Exe alone did not decrease AKT phosphorylation (Fig. 19). However, a significant ($p<0.05$; $p<0.01$; $p<0.001$) decrease in AKT phosphorylation was observed for Exe in combination with all the inhibitors when compared to Exe alone.

In order to verify if the alterations in phosphorylated AKT would reflect in mTOR activity, a downstream kinase of PI3K/AKT pathway and a known regulator of autophagy, the mTOR phosphorylation was addressed by WB and densitometric analysis (Fig. 20). The preliminary results suggest that there is no alteration in mTOR phosphorylation, after 3 days of incubation, despite the decrease in AKT phosphorylation.

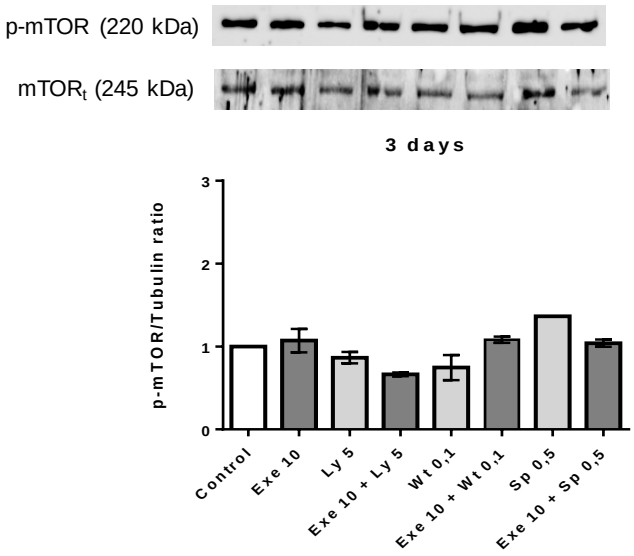


Fig. 20 - Effects of the different treatments on mTOR phosphorylation. LTEDaro cells were treated with different combinations during 3 days. Cells without treatment were considered as control.

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CHAPTER IV - Discussion

Exemestane (Exe) is the third-generation steroidal AI used in clinic to treat ER^+ breast cancer. Despite, the therapeutic success of Exe, AI's-acquired resistance may develop causing tumor re-growth. In that way, it is important to understand the mechanisms underlying AI's-acquired resistance in order to search for new strategies to surpass AI's-acquired-resistance.

The aim of this work was understand of the role of autophagy in AI's-acquired resistance as it was previously demonstrated by our group that autophagic inhibition sensitized AI's-resistant cancer cells [150]. Thus, and to explain the underlying pathways that may be involved in Exe-resistance, two pan-PI3K inhibitors, LY294002 (LY) and Wortmannin (WT), and a specific autophagic inhibitor, Spautin-1 (SP), were used in combination with Exe. LY and WT have been described as autophagic inhibitors [156-158] due to inhibition of PI3K class III, an autophagic promoter, despite their effects on PI3K class I. The latter is involved in PI3K/AKT/mTOR pathway that regulates autophagy [159]. Moreover, SP was also studied due to its role on autophagic inhibition through promotion of beclin-1 degradation [160, 161].

The results showed that the combination of all the inhibitors with Exe is capable of re-sensitize the resistant LTEDaro cells to the AI. Thus, in order to unravel the reasons behind the decrease in cell viability, cell cycle progression and cell death analysis were performed.

It was verified that all the inhibitors have anti-proliferative properties since in Exe-treated LTEDaro cells they disrupted the cell cycle progression. At 3 days of incubation, the combination of Exe with LY and WT induced G_0/G_1 cell cycle arrest, while, the combination with SP induced a G_2/M arrest, when compared to Exe alone. However, for longer periods of treatment, there was a shift of behavior for the PI3K inhibitors, since the combination with LY did not affect cell cycle progression, while the combination with WT induced a G_2/M arrest, in comparison to Exe. On the other hand, the combination with SP accentuated its effect on G_2/M arrest. This arrest in the G_2/M cell cycle phase has been associated with increased apoptosis and cytotoxicity [162].

To understand if the reduction in LTEDaro cell viability is also a consequence of an apoptotic mechanism, it was studied caspase activities. It was observed that all the inhibitors combined with Exe increased caspase-7 activity in comparison with Exe, confirming the occurrence of apoptosis. Moreover, LY and WT in combination with Exe induced an increase in caspase-9 activity, while WT, as well as the combination of SP with Exe, also induced an increment in caspase-8 activity. These findings suggest that, in Exe-treated LTEDaro cells, LY induced apoptosis through the intrinsic pathway, while, SP induced apoptosis through the extrinsic pathway. Curiously, the induction of apoptosis with the combination of Exe plus WT involved a cross talk between the

intrinsic and extrinsic apoptotic pathways. In addition, it was also observed that none of the treatments increased intracellular ROS production, suggesting that the activation of apoptosis is ROS-independent.

To verify the effects of the different treatments on autophagy, it was performed a fluorescence microscopy analysis and flow cytometry studies to determine the formation of AVOs. Firstly, the effects on autophagy were studied qualitatively through fluorescence microscopy after AO staining, and it was demonstrated that none of the combinations completely inhibited autophagy. Moreover, Exe effectively promoted autophagy since Exe-treated LTEDaro cells presented orange/red fluorescence associated with AVOs formation. Through flow cytometry, it was further quantified the levels of AVOs formation and results have shown that the combination of Exe with LY, WT or with SP effectively decreased the Exe-induced autophagy. Exe increased AVOs formation by a 5.21 fold, however when combined with the PI3K inhibitors or with SP, Exe only increased AVOs production by 2.12 and 1.81 fold, respectively. These results demonstrated that the induction of autophagy by Exe was inhibited by the combination with the different inhibitors. To confirm these findings, it was also studied the expression of the main biomarker of autophagy, the LC3-II. As described earlier, LC3-I is converted to LC3-II when autophagy is stimulated, therefore, by comparing the ratios of LC3-II/I among the different treatments it was possible to validate the previous results. Although, it is a preliminary result, it was verified that Exe alone increased LC3-II/I ratio, which corroborates the induction of autophagy by this AI. On the other hand, a decrease in LC3-II/I ratio was observed for the combination of Exe with LY, WT, or with SP, which indicates a decrease in autophagy. These findings were similar to the ones observed by flow cytometry and confirms that the combination of Exe with the inhibitors reduces AVOs production and LC3 turnover, suggesting that they have autophagic inhibition properties.

The effects of these combinations on PI3K/AKT cell survival pathway were also evaluated, since LY and WT are PI3K inhibitors and this pathway, not only regulates autophagy, is also overexpressed in AI's-acquired resistance [98-101].

In fact, PI3K expression and AKT phosphorylation were not altered for Exe but were diminished for Exe in combination with the different inhibitors, confirming the already described inhibition of PI3K pathway by LY and WT and revealing a non-expected role for SP. However, preliminary results suggested that mTORC1 phosphorylation, at 3 days, was not altered by the different treatments, as it would be expected from AKT decreased phosphorylation. mTORC1 is a down-stream effector of AKT, and a described regulator of autophagy, that integrates multiple signals of several different signaling pathways. However, our findings regarding mTORC1

phosphorylation may be explained through various feedback mechanisms [122, 124, 125]. Therefore, in order to understand the regulation of autophagy by mTOR, since the different treatments decreased PI3K/AKT pathway activation and also inhibited autophagy, further studies must be performed.

Despite, the PI3K inhibitors re-sensitize LTEDaro cells to Exe and act on the same target, their effects appear to be induced by different mechanisms. The PI3K inhibitors effectively inhibit autophagy, apparently not through a decrease in mTOR activity, that could be associated with the PI3K class I inhibition, but through a direct effect on PI3K class III. LY re-sentitized Exe-resistant cells through inhibition of Exe-induced autophagy, while maintaining autophagy levels elevated, possibly by promoting a switch in autophagy role from pro-survival to apoptosis [163, 164]; and/or through inhibition of the survival pathway, PI3K/AKT, without affecting mTOR activity. On the other hand, WT re-sensitized Exe-treated LTEDaro cells through a decrease in Exe-induced autophagy by inhibiting autophagy levels and/or, as LY, through survival pathways inhibition. These mechanisms promote different caspases recruitment. Both LY and WT induced caspase-9, and consequently, caspase-7 activation, which can be explained by the decrease on AKT activation, a kinase that inhibits the loss of mitochondrial potential, trough Bad and caspase-9 phosphorylation [165, 166]. As noted before, contrary to LY, WT increased caspase-8 activity. This difference may be a consequence of the only divergent point on the evaluated mechanisms of sensitization, since, between the two inhibitors, the general autophagy levels are different as WT effectively inhibit autophagy while LY maintains autophagy levels elevated. In fact, it has been reported, and also observed for Exe in sensitive cells [140], that in cytoprotective autophagy, active caspase-8 is sequestered in autophagosomes and degraded by lysosomes [167]. On the other hand, as WT caused a more pronounced inhibition of AKT than LY, the activation of caspase-8 by WT may be due to the decrease in a FOXO transcription factor AKT-mediated phosphorylation, promoting the expression of Fas-ligand, which culminates in caspase-8 activation [108, 168]. Nevertheless, caspase-8 can also induce caspase-9 activation and vice-versa, by mechanisms not fully understood [169]. In fact, it was already described that both inhibitors induced apoptosis through activation of caspase-9 and/or caspase-8, depending on the cell model used [170, 171]. The results also indicate that the increase in caspase-9 activity is not due to intracellular ROS production. Furthermore, the different behaviors between these two inhibitors at the cell cycle progression may be explained by AKT activity. AKT is a regulator of cell cycle, through phosphorylation of p21 or p27. At 3 days of incubation, LY-mediated inhibition of AKT may be sufficient to inhibit p21 or p27 phosphorylation, leading to the G_0/G_1 arrest mechanisms already

described [172, 173]. However, as LY is a reversible PI3K inhibitor, for longer periods of incubation, its effects on AKT phosphorylation may not be sufficient and consequently, the effects on the cell cycle are not evident. On the other hand, WT initially promoted a G_0/G_1 arrest, possibly through the same mechanisms as LY, but at 6 days of incubation, it shifts to G_2/M arrest, which has been associated with apoptosis as mentioned earlier. This change of behavior may, therefore, be due to an over-activation of apoptosis, since the inhibition of autophagy may induce the activation of apoptosis.

Lastly, SP sensitized LTEDaro cells to Exe through inhibition of autophagy and/or through its inhibitory effects on the survival pathway, PI3K/AKT. SP induced caspase-7 and caspase-8 activation, strengthening the relation between autophagy inhibition and the latter, and between decreased AKT phosphorylation and caspase-8 activation. In addition, there was an arrest in G_2/M cell cycle phase that is also related to apoptosis. As SP is not described as a PI3K/AKT inhibitor, the inhibition of these pathways could be a non-reported direct effect or a consequence of an unknown mechanism, and so more studies need to be performed.

The results obtained in this work suggest that the decrease in the Exe-induced autophagy and/or in the survival pathways activation were important for LTEDaro sensitization. The most potent inhibitor was WT. In fact, the combination of WT with the non-steroidal AI, Anastrozole, has already been reported to have beneficial effects in a cell model of AI-resistance similar to the LTEDaro [174]. Thus, by modulating PI3K/AKT pathway and autophagy it may be possible to re-sensitize acquired-resistant breast cancer cells to Exe therapy. Nevertheless, more studies need to be performed in order to clarify the role of the pro-survival autophagy in Exe-acquired-resistance. Studies regarding MAPK pathways should also be performed, since Erk 1/2 overexpression was already described in Exe-acquired resistance [145].

This work provides new insights in the mechanisms involved in Exe-acquired resistance and, consequently, new targets that together with aromatase inhibition through Exe may improve breast cancer therapy, overcoming acquired-resistance.

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CHAPTER V – Annexes

U.PORTO

Certifica se que Tiago Augusto parricipou no IJUP'16 – 9º *Encontro de Jovens Investigadores da Universidade do Porto*, que decorreu nos dias 17, 18 e 19 de fevereiro de 2016, na Fundação da Juventude – Palácio das Artes, tendo apresentado a comunicação oral com o título "PI3K/Akt and autophagy pathways as possible targets in breast cancer endocrine resistance to exemestane".

Pela Comissão Organizadora

(A Vice-Reitora, Profª. Doutora Maria João Ramos)

2ND ASPIC INTERNATIONAL CONGRESS

IPO-PORTO 28-29 APRIL 2016

CERTIFICATE OF ATTENDANCE

This is to certify that ***Tiago Augusto*** has attended the 2nd ASPIC International Congress, held in Porto, Portugal 28-29 April, 2016.

The 2nd ASPIC International Congress is accredited by the **European Accreditation for Continuing Medical Education** (EACCME) to provide the following CME activity for medical specialists. The EACCME is an institution of the European Union of Medical Specialists (UEMS), www.uems.net.

The 2nd ASPIC International Congress is designated for a maximum of 11 hours of European external CME credits. Each medical specialist should claim only those hours of credit that he/she actually spent in the educational activity.

The above mentioned participant has earned 11 ECMEC's (European Continuous Medical Education Credit) at this event. The EACCME credit system is based on 1 ECMEC per hour with a maximum of 3 ECMECs for half a day and 6 ECMECs for a full–day event.

European Accreditation is granted by the EACCME in order to allow participants who attended the above-mentioned activity to validate their credits in their own country.

Through an agreement between the European Union of Medical Specialists and the American Medical Association, physicians may convert EACCME credits to an equivalent number of AMA PRA Category 1 Credits™. Information on the process to convert EACCME credits to AMA credits can be found at www.ama-assn.org/go/internationalcme.

Live educational activities, occurring outside of Canada, recognized by the UEMS-EACCME for ECMEC credits are deemed to be Accredited Group Learning Activities (Section 1) as defined by the Maintenance of Certification Program of The Royal College of Physicians and Surgeons of Canada.

Leonor David, MD, PhD

President of ASPIC