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Aromatase inhibitors in breast cancer: Drug discovery and strategies to overcome acquired resistance

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TV. Augusto, C**.** Amaral, CL. Varela, F. Bernardo, E. Tavares-da-Silva, FFM. Roleira, S. Costa, N. Teixeira, G. Correia-da-Silva, *Effects of new C6-substituted steroidal aromatase inhibitors in hormone-sensitive breast cancer cells: Cell death mechanisms and modulation of estrogen and androgen receptors*. J Steroid Biochem Mol Biol. 2019 Dec;195:105486. doi: 10.1016/j.jsbmb.2019

TV. Augusto, SC. Cunha, C. Amaral, JO. Fernandes, E. Tavares-da-Silva, FFM. Roleira, N. Teixeira, G. Correia-da-Silva, *A novel GC-MS methodology to evaluate aromatase activity in human placental microsomes: a comparative study with the standard radiometric assay*. Anal Bioanal Chem. 2019 Oct;411(26):7005-7013. doi: 10.1007/s00216-019-02076-7.

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TV. **Augusto**, C. Amaral, N. Teixeira, G. Correia-da-Silva, *The involvement of autophagy in the acquired resistance to third generation aromatase inhibitors,* Free Radical Biology and Medicine 120 (2018) S45–S166, DOI: 10.1016/j.freeradbiomed.2018.04.388

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O começo de todas as ciências é o espanto de as coisas serem o que são

- Aristóteles

Abstract

Breast cancer is the leading cause of cancer death in women worldwide. Estrogen receptor-positive (ER^+) breast carcinoma is the most common subtype, corresponding to 60% of premenopausal and 75% of postmenopausal cases. Currently, the third-generation of aromatase inhibitors (AIs), comprising the non-steroidal Anastrozole and Letrozole and the steroidal Exemestane, are considered the first-line endocrine therapy for postmenopausal women. Despite their clinical success, the development of resistance has become a major drawback. However, in clinical practice, cross-resistance between AIs is not observable, suggesting distinct molecular mechanisms of action despite sharing aromatase as the biological target. Thus, the search for novel potent AIs, with higher efficacy and fewer side effects, as well as the identification of mechanisms associated with AI-resistance are imperative. This thesis focuses both on the discovery of new potent steroidal AIs and elucidation of biological mechanisms induced by AIs in sensitive and resistant breast cancer cells.

The biochemical and biological evaluation of eight newly synthesized molecules originated through modifications in the A- and B-rings of androstenedione backbone, was performed. Specifically, anti-aromatase activity, anti-proliferative effects, and mechanisms of action were explored in the MCF-7aro ER^+ breast cancer cell line that overexpresses aromatase. The results show that the simultaneous presence of a C-3-carbonyl group and a methyl- or allyl- aliphatic chain at C-6α was important to improve aromatase inhibition. Moreover, it was demonstrated the superiority of the methyl group over the allyl substitution in the C-6α position. All compounds induced cell cycle arrest and apoptotic cell death. In addition, they also presented single, dual, or triple dependency on aromatase, estrogen receptor α (ERα) and/or androgen receptor (AR).

Concomitantly, since the assessment of aromatase activity still relies on expensive, hazardous, and non-environmentally friendly radiometric assays, a new, accurate, sensitive, and safer gas chromatography-mass spectrometry (GC-MS) methodology was developed. The anti-aromatase activity was measured by determining the percentage of conversion of androstenedione into estrone (ratio estrone/androstenedione). This GC-MS method presented good linearity, accuracy, extraction efficiency, and intra-day and inter-day precision. Moreover, IC_{50} values for AIs in clinical use were very similar between both methodologies.

In addition, the mechanisms of action of the AIs used in clinical practice were explored in MCF-7aro cells. This study revealed that the non-steroidal AIs induce senescence, while Exemestane blocks the induction of senescence by promoting cytoprotective autophagy. It

was also demonstrated that in a hormone-enriched environment, the non-steroidal AIs upregulate ERα without inducing receptor activation, whereas Exemestane downregulates ERα but maintains its activation. AR was also upregulated by all AIs blocking ERα signaling. However, under hormone-depleted conditions, a crosstalk between AR and ERα, enhancing estrogenic signaling, was evident in Exemestane-treated cells. Thus, this indicates that Exemestane modulates both receptors, while Anastrozole and Letrozole act only as pure AIs.

Moreover, since PI3K is pivotal for AI-acquired resistance, either through activation of the PI3K/AKT/mTORC1 survival pathway or induction of cytoprotective autophagy, the anti-tumoral effects of PI3K inhibition, in combination with AIs were evaluated on AI-resistant cell models derived from MCF-7aro cells. In particular, the long-term estrogen-deprived cell line (LTEDaro) and AI-specific resistant cells (AnaR, LetR and ExeR) were used in these studies. Contrary to Exemestane, Anastrozole and Letrozole do not promote autophagy in sensitive and resistant breast cancer cells. On the other hand, the combinations of AIs with BYL-719, a PI3K class I inhibitor, decreased cell viability by different mechanisms. In addition, both the efficacy of BYL-719 in AnaR, LetR and ExeR cell lines and the importance of concomitantly targeting PI3K and ERα were clearly demonstrated.

In conclusion, this thesis contributed to the search of new potent steroid AIs, through the biological and biochemical characterization of new molecules and development of a new method to evaluate anti-aromatase activities of new compounds. This work also unveiled different mechanisms of action induced by AIs, which may explain the lack of cross-resistance observed in clinical practice, while suggesting that only Exemestane-treated patients might benefit from anti-AR therapies. In addition, the weak estrogen-like activity of Exemestane may hinder its anti-tumoral efficacy as second-line therapy. This work may support the effectiveness of the combination of BYL-719 with Letrozole or Exemestane in advanced ER^+ breast cancer observed in clinical trials (NCT01791478, NCT01870505). In addition, the concomitant inhibition of PI3K and ERα highlights the importance of both targets in AI-resistance.

Keywords: Hormone-dependent breast cancer, Aromatase inhibitors, Acquired-resistance, Estrogen receptor, Androgen receptor

Resumo

O cancro da mama é a principal causa de morte por cancro em mulheres em todo o mundo. O cancro da mama recetor de estrogénio-positivo (ER^+) é o subtipo mais comum, correspondendo a 60 e 75% dos casos em mulheres pré- e pós-menopáusicas, respetivamente. Atualmente, os inibidores da aromatase (AIs) de terceira geração, os não-esteróides Anastrozole e Letrozole e o esteróide Exemestano, são considerados uma terapia endócrina de primeira-linha para mulheres em pós-menopausa. Apesar do seu sucesso clínico, o desenvolvimento de resistência tornou-se a sua principal desvantagem. Contudo, não é observada resistência cruzada entre os AIs na clínica, sugerindo que, apesar de partilharem o mesmo alvo biológico, possuem mecanismos de ação moleculares distintos. Assim, é essencial a procura de AIs novos e potentes, com maior eficácia e menos efeitos secundários, bem como a identificação dos mecanismos associados à resistência. Esta dissertação foca-se na descoberta de AIs esteróides e na elucidação dos mecanismos biológicos induzidos pelos AIs em células de cancro da mama sensíveis e resistentes a estes inibidores.

Foi realizada a avaliação bioquímica e biológica de oito moléculas novas, originadas por modificações nos anéis A e B da estrutura da androstenediona. Especificamente, a atividade anti-aromatásica, os efeitos anti-proliferativos e os mecanismos de ação foram explorados numa linha celular de cancro da mama ER^+ que sobre-expressa a aromatase, MCF-7aro. Os resultados demonstraram que a presença simultânea de um grupo carbonilo em C-3 e de uma cadeia alifática, metilo ou alilo, na posição C-6α é importante para melhorar a inibição da aromatase. Foi ainda observada superioridade do grupo metilo em relação ao grupo alilo, na posição C-6α. Todos os compostos induziram paragem do ciclo celular e morte por apoptose. Estes compostos também demonstraram uma dependência singular, dupla ou tripla na aromatase, recetor de estrogénio α (ERα) e/ou recetor de androgénio (AR).

Uma vez que a avaliação da atividade da aromatase ainda depende de ensaios radiométricos caros, potencialmente perigosos e não ecológicos, foi desenvolvido um novo método de cromatografia gasosa-espectometria de massa (GC-MS), exato, sensível e seguro. A atividade anti-aromatásica foi quantificada através da determinação, em percentagem, da conversão de androstenediona em estrona (razão estrona/androstenediona). Este método de GC-MS apresenta boa linearidade, exatidão, eficiência de extração assim como boa precisão intra- e inter-dias. Os valores de IC_{50} obtidos para os AIs usados na clínica foram similares entre as duas metodologias.

Os mecanismos de ação dos AIs usados na clínica foram explorados nas células MCF-7aro. Este estudo revelou que os AIs não-esteróides induzem senescência, enquanto o

Exemestano bloqueia a indução de senescência ao promover uma autofagia citoprotetora. Foi também demonstrado que, num ambiente hormonal enriquecido, os AIs não-esteróides aumentam os níveis de ERα, sem aumentar a sua ativação, enquanto o Exemestano diminui os níveis de ERα, mas mantém a sua ativação. Os níveis de AR também aumentaram em resposta a todos os AIs, bloqueando, assim, a sinalização do ERα. No entanto, sob condições de depleção hormonal, foi evidente uma cooperação entre o AR e o ERα em células tratadas com Exemestano, o que induziu um aumento da sinalização estrogénica. Assim, estes resultados indicam que o Exemestano modula ambos os recetores, ao contrário do Anastrozole e do Letrozole que atuam apenas como AIs puros.

Uma vez que a PI3K desempenha um papel fulcral na resistência adquirida aos AIs, quer seja através da ativação da via de sobrevivência PI3K/AKT/mTORC1 ou indução de autofagia citoprotetora, foram avaliados os efeitos anti-tumorais da inibição da PI3K em combinação com AIs, em modelos celulares resistentes aos AIs derivados das MCF-7aro. Neste estudo foi usada a linha celular LTEDaro, gerada através de uma privação de estrogénios a longo prazo, e as linhas celulares resistentes especificamente a cada AI (AnaR, LetR e ExeR). Contrariamente ao Exemestano, o Anastrozole e o Letrozole não promovem autofagia em células de cancro da mama sensíveis e resistentes. Por outro lado, as combinações dos AIs com o BYL-719, um inibidor da PI3K, diminuíram a viabilidade celular através de diferentes mecanismos. Foi ainda demonstrada a eficácia do BYL-719 nas linhas celulares AnaR, LetR e ExeR e a relevância de inibir concomitantemente a PI3K e o ERα.

Em suma, esta tese contribuiu para a procura de AIs esteróides novos e potentes, através da caracterização bioquímica e biológica de novas moléculas, assim como para o desenvolvimento de um método para avaliar a atividade anti-aromatásica de novos compostos. Este estudo também revelou diferentes mecanismos de ação induzidos pelos AIs, o que pode explicar a falta de resistência cruzada observada na clínica, ao mesmo tempo que sugere que apenas doentes tratados com Exemestano poderão beneficiar de terapias anti-AR. A fraca atividade estrogénica do Exemestano poderá afetar negativamente a sua eficácia anti-tumoral enquanto terapia de segunda-linha. Este trabalho pode ainda apoiar a eficácia da combinação do BYL-719 com o Letrozole ou o Exemestano, em cancro da mama ER^+ avançado, verificada em ensaios clínicos (NCT01791478, NCT01870505). Além disso, a inibição concomitante da PI3K e do ERα destaca a importância destes alvos na resistência aos AIs.

Palavras-chave: Cancro da mama hormono-dependente, Inibidores da aromatase, Resistência adquirida, Recetor de esrogénio, Recetor de androgénio

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Abbreviation list

ABC	Advanced breast cancer
AF-1	Ligand-independent transactivation domain
AF-2	Ligand-dependent transactivation domain
AG	Aminoglutethimide
AIs	Aromatase Inhibitors
AO	Acridine orange
AO^+	Acridine orange-positive
AO^-	Acridine orange-negative
AP1	Activator protein 1
AR	Androgen receptor
ARE	Androgen-responsive elements
AREG	Amphiregulin
ASIR	Age-standardized incidence rates
AVOs	Acidic vesicle organelles
CA	Co-activators
CAFs	Cancer-associated fibroblasts
cAMP	Cyclic adenosine monophosphate
CBP	CREB-bindind protein
CCDC170	Coiled-coil domain containing 170
CDKs	Cyclin-dependent kinases
CDX	Casodex
CPR	NADPH-cytochrome P450 reductase
CR	Co-repressors
CREB	cAMP-responsive element binding protein
CYP11	Mitochondrial cholesterol side-chain cleavage enzyme
CYP19	Aromatase
DBD	DNA-binding domain
DCIS	Ductal carcinoma *in situ*
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DHT-$^{13}C_3$	Dihydrotestosterone-2,3,4-$^{13}C_3$,
DLLME	Dispersive liquid-liquid microextraction
EGFRs	Epidermal growth factor receptors
ER	Estrogen receptor
ERα	Estrogen receptor α
ERβ	Estrogen receptor β

ER$^+$	Estrogen receptor-positive
ER$^-$	Estrogen receptor-negative
ERE	Estrogen-responsive elements
E1	Estrone
E2	Estradiol
E3	Estriol
FAD	Flavin adenine
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FMN	Flavin mononucleotide
Gab1	GRB2-associated binding protein 1
β-gal	β-galactosidase
GATA-3	Transacting T-cell-specific transcription factor
GC-MS	Gas chromatography-mass spectrometry
GFRs	Growth factor receptors
GPER1	G-protein-coupled estrogen receptor 1
GRP78	Glucose-regulated protein
HDACs	Histone deacetylases
HER2	Human epidermal growth factor receptor 2
HER2$^+$	Human epidermal growth factor receptor 2-positive
HER2$^-$	Human epidermal growth factor receptor 2-negative
HSDs	Hydroxysteroid dehydrogenases
HSPs	Heat shock protein
ICI 182,780	Fulvestrant
IDC	Infiltrating Ductal
IGFs	Insulin-like growth factors
IGFBP	Insulin-like growth factor binding proteins
IGF-IR	Insulin-like growth factor receptor 1
ILs	Interleukins
IRSs	Insulin receptor substrates
LBD	Ligand-binding domain
LCIS	Lobular carcinoma *in situ*
LC-MS	Liquid chromatography-mass spectrometry
mTORC1	Mammalian target of rapamycin C1
NADPH	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor κ-B
OS	Overall survival

P450c17	17α-monooxygenase
PA	Pre-adaptation
PARP	Poly (ADP-ribose) polymerase
PDCD4	Programmed cell death 4
PDX	Patient derived xenografts
PHBs	Prohibitins
PFS	Progression-free survival
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PR	Progesterone receptor
PR^+	Progesterone receptor-positive
PR^-	Progesterone receptor-negative
ROS	Reactive oxygen species
SAR	Structure-activity relationship
SERCAs	Selective estrogen receptor covalent antagonists
SERDs	Selective estrogen receptor down-regulators
SERMs	Selective estrogen receptor modulators
SGK3	Serum- and glucocorticoid-inducible kinase 3
SIM	Selective ion monitoring
SMRT	Silencing mediator of retinoic acid and thyroid hormone
SP1	Specificity protein 1
SRCs	Steroid receptor co-activators
stAR	Steroidogenic acute regulatory protein
STS	Estrone sulfatase
T	Testosterone
TF	Transcription factor
TNFα	Tumor necrosis factor α
UPR	Unfolded protein response
4-OH-tam	4-Hydroxytamoxifen
6-HME	6-Hydroxymethylexemestane
17β-HE	17β-Hydroexemestane
$\Delta\Psi m$	Mitochondrial membrane potential

Chapter I

Introduction

1. Overview

Breast cancer is the most common cancer in women worldwide and the main cause of cancer death in women. Clinically, breast cancers are classified in stages 0 to IV. In stage 0, cells localized within the mammary ducts or lobules do not have the capacity to invade, being classified as non-invasive/localized breast cancer. Stages I, II, and III are used to describe regionally invasive breast carcinomas, depending on tumor size, number and location of nearby lymph nodes invaded. Stage IV is known as advanced or metastatic breast cancer that have spread beyond the breast to other organs, such as lungs, brain, or bones. The survival rate of breast cancer patients depends on the stage of disease at diagnosis. The 5-year survival rate for early breast cancers is 99%, while, for regional tumors the 5-year survival is 86% (1). Metastatic breast cancer only presents a 5-year survival of 27%, highlighting the importance of early detection for a good prognosis.

Histologically, breast cancer is divided into *in situ* and invasive. Localized carcinomas are subdivided into ductal (Ductal Carcinoma *in situ* – DCIS) or lobular (Lobular Carcinoma *in situ* - LCIS), being the former the most common (2). Invasive carcinomas are also subdivided into infiltrating ductal (IDC), invasive lobular, ductal/lobular, mucinous, tubular, medullary and papillary. Among all breast carcinomas, IDC corresponds to 80% of the cases (3).

The molecular classification is based on the presence of three major proteins, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). According to the molecular status of the tumors, they are divided into luminal A ($ER^+/PR^+/HER2^-$; $ER^-/PR^+/HER2^-$; $ER^+/PR^-/HER2^-$), luminal B ($ER^+/PR^+/HER2^+$; $ER^-/PR^+/HER2^+$; $ER^+/PR^-/HER2^+$), HER2-enriched ($ER^-/PR^-/HER2^+$), and triple-negative ($ER^-/PR^-/HER2^-$) (4). Luminal A is the most common subtype of breast cancer (73%), followed by triple negative (12%), luminal B (11%) and HER2-enriched (4%) (5). The luminal A is characterized by low aggressiveness, being associated with low grade tumors, presenting the best prognosis of all the subtypes (6-8). The luminal B is associated with high tumor grade, HER2 expression, and high expression of the proliferation marker Ki67, being therefore associated with a worse prognosis than its counterpart (6-8). The HER2-enriched subtype is defined by lack of ER and PR expression and overexpression of HER2 being usually associated with high tumor grade and high aggressiveness and presenting a poor prognosis (6-8). Triple-negative tumors present a lack of ER, PR and HER2 expression being highly aggressive cancers with poor prognosis (9, 10). Contrary to other subtypes where there is no difference according to menopausal status, this subtype is more common in premenopausal women and shows the lowest 5-year survival rate (5, 9).

1.1 Incidence and risk factors

Breast cancer is still a life-disrupting disease for millions of women worldwide. In 2020, it was reported 7,041 estimated new breast cancer cases and 1,864 estimated number of breast cancer deaths in Portugal (11), exhibiting a trend for an increase in incidence (12). Breast cancer presents the highest incidence in women, corresponding to approximately 30% of all the diagnosed cancers in the latest years (11, 13-15). The incidence is higher in North America and European countries, than in Asian and African countries (11). The incidence of this disease has been rising over the years, approximately 0.5% per year, mainly due to increases in null parities and obesity (1, 16). Despite the decrease in mortality observed in recent years, due to early diagnosis and advances in treatments, breast cancer is the leading cause of cancer mortality (15.5%) in women worldwide (11). Luminal A cases are more frequent in adult white women than in other races, basal-like are more common in black women than in white women and HER2-enriched neoplasms incidence does not vary according to race (17).

Epidemiologic studies have been investigating the risk factors associated with this disease. Most of them are well known, such as gender, since male breast cancer only accounts for 1% of total cases both in incidence and in deaths (14, 18), and increasing age, since 69.1% of diagnosed breast cancers occur in women aged 50 years or above (16). In fact, the estimated truncated age-standardized incidence rate is higher for postmenopausal women (> 50 years) than for premenopausal women worldwide (Figure 1) (16).

However, other aspects need to be considered, such as the genetic burden, which can modulate the risk of developing the disease. More specifically, the presence in the family of a first-degree relative with the pathology is known to increase the risk of other family members (19, 20).

Genetic polymorphisms (21, 22), mutations in DNA-machinery repair genes (23, 24), such as deleterious *BRCA* gene mutations, responsible for about 20% of the hereditary cases (25), and mutations in tumor suppressor genes, such as *TP53* and *PTEN* (24), are also correlated with in an higher risk of developing breast cancer. Moreover, the association between *BRCA* mutations and triple-negative tumors has been described (9). Life-style risk factors can also help to modulate the development of this tumor. Obesity and high body fat, for instance, are closely associated with breast cancer development in postmenopausal women but no association was verified for younger women (26-29). Accumulation of body fat significantly promotes the production of estrogens and also leads to hyperinsulinemia and insulin resistance, promoting breast cancer (29, 30).

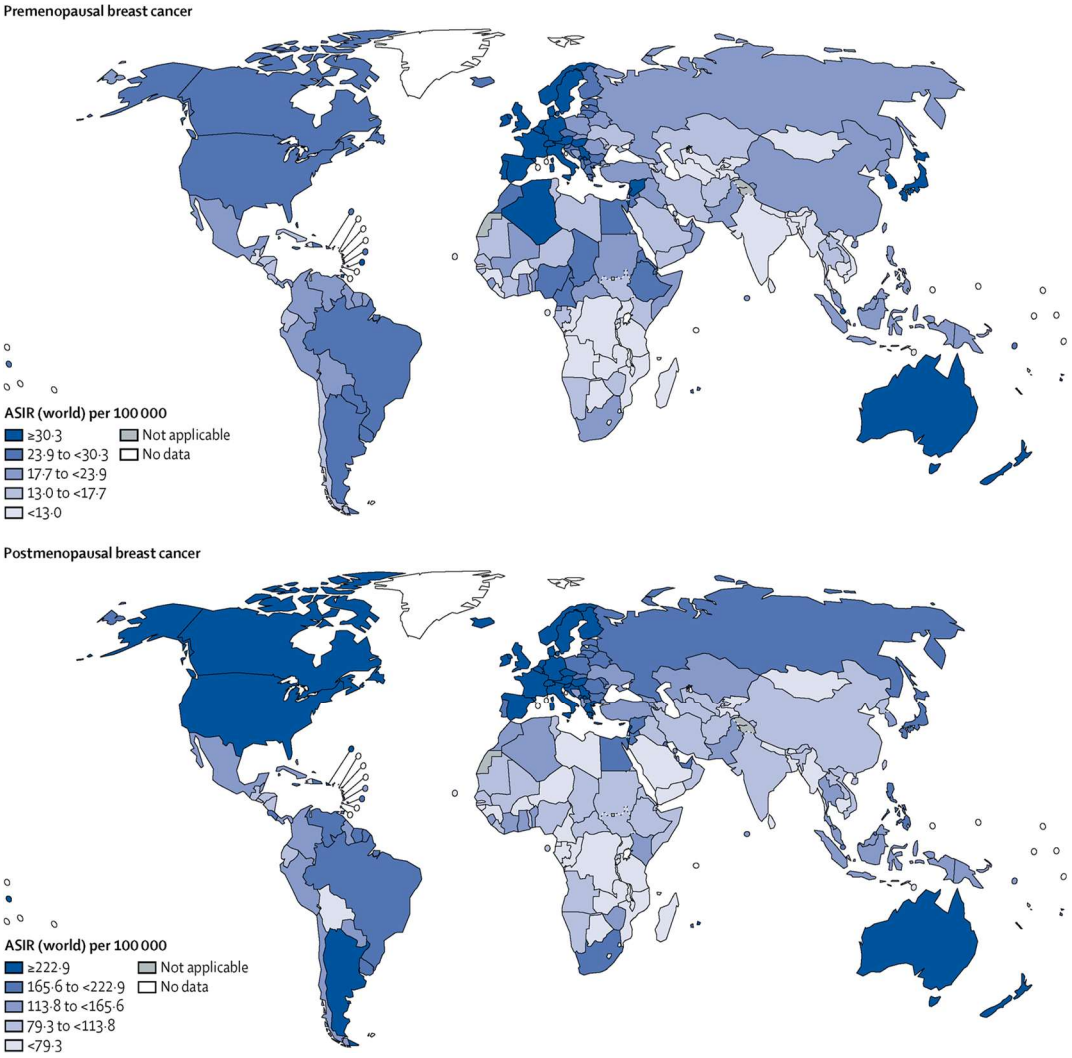

Figure 1. Estimated truncated age-standardized incidence rates (ASIRs) for female breast cancer in 2018. The upper map shows the incidence for premenopausal women (< 50 years) and the lower map for postmenopausal women (>50 years). Adapted from (16).

In accordance with these observations, physical activity is associated with a decreased risk in women, mainly due to its effects on body composition and hormonal status, and also to a possible delay of menarche in younger women (31-34). Other modifiable factors known to increase the risk of breast cancer are alcohol and tobacco consumption (35-38). The development of breast tumors is also associated with reproductive risk factors. Nevertheless, in recent years, it has been reported that these risk factors are differentially associated with each subtype of breast cancer. Early age at menarche or menopause at an older age are also associated with augmented breast cancer risk due to the increased years of exposure to sexual hormones (39). In addition, pregnancy appears to reduce the risk of developing luminal ER^+ breast cancer by 25% (40). It is observed a higher risk of ER^+ tumors for nulliparity (41) and older women in their first full-term pregnancy than for women who had pregnancies at earlier ages (40, 42), with the protective role of gestation being increased with each parity (41). Further supporting the role of reproduction, longer

breastfeeding displays a decreased risk of luminal and triple-negative breast cancer, possibly due to differentiation of mammary cells (40, 41, 43, 44). Nevertheless, pregnancy and short breastfeeding has also been linked to increased risk for ER^- and triple-negative tumors (40, 41, 45, 46). Despite the socioeconomic factors, the race seems to play a role in determinining cancer aggressiveness, with black women showing an increased tendency to have more aggressive subtypes, such as ER^- and triple-negative (47, 48). This may be associated with an increase in risk factors associated with these subtypes, such as younger age at first full-term pregnancy and high waist-to-hip ratio (49). Increased risk is also related to several other factors, such as hormone replacement therapy (50, 51) and high breast density (52-54).

2. Estrogen biosynthesis

Estrogens are the main sex hormones in females, being responsible for the development of secondary sexual traits through the maturation of sexual organs. The role of these hormones is especially relevant in the mammary gland, where they promote the accumulation of adipose tissue and ductal and stromal growth. In premenopausal women estrogens are synthesized in the gonads, mainly in granulosa cells of the ovaries. In postmenopausal women, gonadal synthesis of estrogens ceases, and the circulating precursor steroids, from adrenal glands, are converted in estrogens in extragonadal sites, such as adipose tissue, breast tissue, skin, brain and endometrium. The change in menopausal status is observed by the decreased levels of circulating estrogens in the plasma. However, the levels of estrogens in breast cancer tissue are higher than in non-malignant breast tissues and, in postmenopausal women, higher than in plasma (55-57).

Estrogens are synthesized *de novo* from cholesterol (Figure 2). This process begins with the conversion of cholesterol in pregnenolone through the action of the steroidogenic acute regulatory protein (stAR). This transport protein mediates the mitochondrial accumulation of cholesterol and of the mitochondrial cholesterol side-chain cleavage enzyme (CYP11). Pregnenolone is then converted to a pregnenolone derivate, through the 17α-hydroxylase activity of steroid 17α-monooxygenase (P450c17), and finally, to the androgenic dehydroepiandrosterone (DHEA), through the 17,20-lyase activity of the P450c17. DHEA is then converted either to androstenediol by the family of 17β-hydroxysteroid dehydrogenases (17β-HSD-1, -7 and -12), or to androstenedione by the 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (3β-HSD-1). Pregnenolone can be alternatively converted to progesterone, by the (3β-HSD-1), which is further converted to a progesterone derivative and, finally, to androstenedione. Both androstenediol and androstenedione are converted in testosterone (T), one of the main androgens, through the action of 3β-HSD-1 and 17β-

HSD-5, respectively. The two androgens, androstenedione and testosterone, are the building blocks of the main estrogens in women, estrone (E1) and 17β-estradiol (estradiol, E2), respectively. The conversion of androgens to estrogens, considered the the rate-limiting step of steroidogenesis, is catalyzed by aromatase, also known as CYP19, an enzyme of the cytochrome P450 (58, 59). E1 and E2 can be interconverted mainly by enzymes of the 17β-HSD family, 17β-HSD-1, -7 and -12 for the conversion of E1 to E2, and 17β-HSD-2 for the conversion of E2 to E1 (60). Aromatase shows a higher affinity for androstenedione than for testosterone (61). Thus, plasma levels of E1 are higher than E2 in postmenopausal women (62, 63). The majority of the newly synthesized estrone is converted into estrone sulfate, by the estrone sulfotransferase. This molecule is biologically inactive due to its low affinity to ER and presents a half-life in blood much higher than its unconjugated form (57). In postmenopausal women, estrone sulfate is readily converted into estrone via estrone sulfatase (STS), in normal and malignant breast tissues (57, 64). Nevertheless, the role of STS activity in the regulation of estrogen levels in breast tumors is debatable (56, 57). However, a demand for potent STS inhibitors still exists.

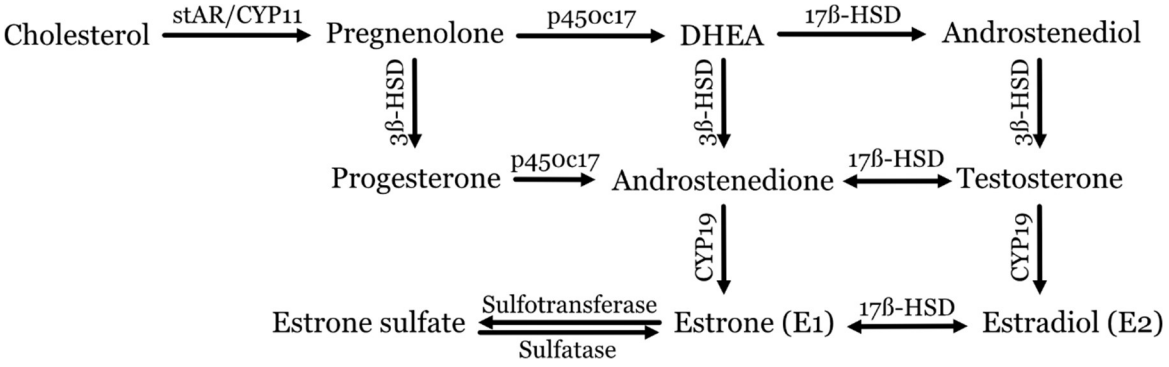

Figure 2. Estrogen biosynthesis pathway.

Physiologically, estrogens are represented by three steroids, E1, E2, and estriol (E3) in women. E1 is the primary estrogen produced in postmenopausal women through the conversion of circulating androstenedione in the adipose tissue and skin (65). E2 is the most biologically active estrogen, due to its highest affinity for ER when compared to E1 and E3 (66). In premenopausal women it is the main estrogen produced in the ovaries. E2 is also the major mediator of tumor growth and proliferation in luminal breast cancer. In fact, E2 concentrations are higher in tumor than in normal breast tissue, contrary to E1 (64). Contrariwise, tissue E2 levels are independent of menopausal status in normal breasts (64). In addition, the differences between tumor and plasma levels are higher for E2 than for E1 (64). On the other hand, E3 is primarily produced during pregnancy, being synthesized by the placenta (65).

One of the mechanisms responsible for the increased levels of E2 in tumor cells is its uptake from the plasma and binding to ER (56, 62, 67). In addition, the plasma levels of E2 seem to be highly correlated with estrogen-related genes expression (68). Nowadays, estrogen blockage relies on systemic approaches (56).

3. Aromatase

Aromatase, as mentioned before, is a 58 kDa enzyme responsible for the conversion of androgens in estrogens (Figure 3). In addition, it is the only enzyme known to perform an aromatization reaction in vertebrates (69). Human aromatase is coded by the *CYP19A1* gene (123 kb) on the 15q21.1 chromosome and belongs to the cytochrome P450 family, being localized in the endoplasmic reticulum membrane (70, 71). Aromatase structure consists of an heme group and 503 amino-acid long polypeptide chain, organized in twelve α-helix (A to L) and ten β-sheets (1 to 10) (72).

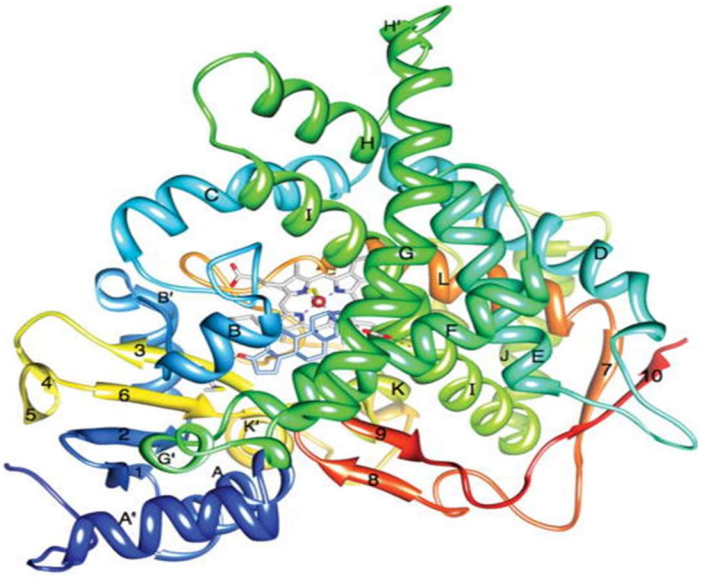

Figure 3. Aromatase structure. Ribbon diagram showing the overall structure. The N-terminus, starting at residue 45 (dark blue) and the C-terminus ending at residue 496 (red). The α-helices are labeled from A to L and β-strands are numbered from 1 to 10. The heme group, the bound androstenedione molecule at the active site and its polar interactions are shown. Adapted from (70).

Contrary to other cytochrome P450 enzymes, the aromatase structure is stable with a rigid core (73). Its catalytic complex is an heterodimer formed by aromatase, containing an heme group, and the nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase (CPR) (70). CPR is formed by three electron transfer domains: NADP-binding domain, flavin adenine dinucleotide (FAD)-binding domain and flavin mononucleotide (FMN)-binding domain (74). The active site is highly androgen-specific, mainly due to the tight packing of hydrophobic residues against the steroid backbone of androgens (72). The residues that constitute the active site are I305, A306, D309, and T310 from the I-helix,

F221 and W224 from the F-helix, I133, F134 from the B–C loop, V370, L372 and V373 from the K-helix-β3 loop, M374 from β3 and L477 and S478 from the β8 to β9 loop (70). In order to synthetize estrogens, aromatase utilizes electrons from CPR. The electrons are transferred, successively, from NADPH to the FAD and FMN domains, and, finally, to the heme group (75). Aromatase catalyzes three hydroxylation reactions, two C19-methyl hydroxilations and an aromatization of the A-ring of androgens, being the latter exclusive to aromatase, each requiring one molecule of O_2 and one of NADPH (69, 70) (Figure 4).

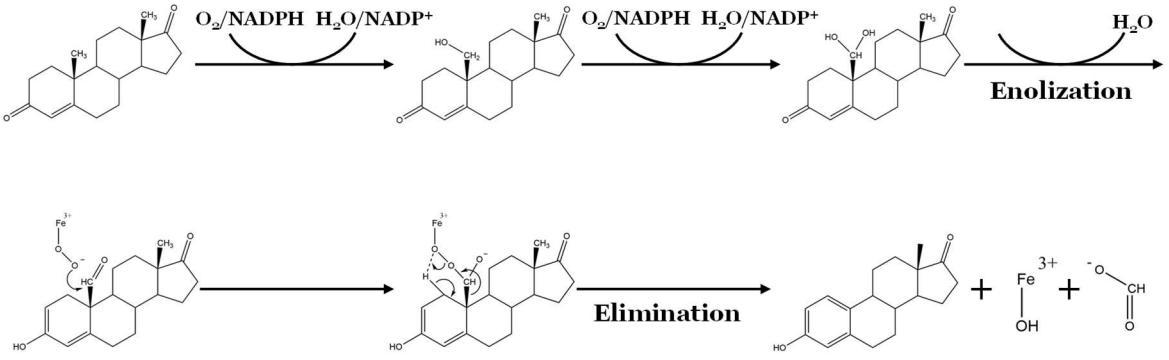

Figure 4. Aromatization mechanism.

Aromatase expression is under strict regulation. In premenopausal women it is mainly expressed in the ovaries and in the placenta during pregnancy, while, in postmenopausal women, it is mainly expressed in adipose fibroblast cells. Aromatase deficiency is a rare disorder that leads to ambiguous genitalia at birth, high levels of androgens and undetectable levels of estrogens, along with a lack of acquirement of secondary sex traits in women (76-78).

In the aromatase gene, there are 10 exons, the non-coding exons I and the nine coding exons II-X. The exons I are expressed in a tissue-specific manner due to their specific promoters regulated through different mechanisms (71, 79). In normal breast stromal and adipose tissue, aromatase expression is controlled by the promoter I.4, which is regulated by interleukin (IL)-6, IL-11, tumor necrosis factor α (TNFα) and glucocorticoids (80-82). However, tumor cells express more aromatase when compared to normal cells, via the I.3 and II promoters (60, 82-84). Through the release of IL-6 and TNFα (85, 86), tumor cells block the maturation of fibroblasts to adipocytes, being these fibroblasts responsible for 80% to 90% of aromatase expression in adipose tissue (65, 80, 87). Moreover, through the segregation of prostaglandin E2 (PGE2), which will act via cyclic adenosine monophosphate (cAMP) pathway to switch aromatase expression regulation from the I.4 promoter to the I.3 and II promoters (81, 86, 88-90), the tumor cells increase aromatase expression in the fibroblasts. Due to the pro-angiogenic tumor microenvironment, the breast endothelial cells

also become a relevant source of estrogens (91). All these alterations in aromatase expression contribute to the intratumoral synthesis of estrogens (92-94).

The radiometric evaluation of aromatase activity is the reference method in human cancer cells and human placental microsomes (95-102). This methodology was developed in 1974 (103) and relies on the release of tritiated water during the enzymatic reaction upon incubation with the substrate [1β-^{3}H]-androstenedione. The tritiated water is then quantified by a scintillator counter. Despite the high sensitivity of this method, it is expensive, not environmental friendly and poses a health hazard. Therefore, there is a need to develop new methodologies capable of circumventing these drawbacks. Alternative liquid-chromatography mass spectrometry (LC-MS) (104) and gas-chromatography mass spectrometry (GC-MS) (105) methodologies have been developed. However, the lack of validation, the high quantities of human placental microsomes/supersomes and of substrate used, or not using the natural substrate of the enzyme are some of the characteristics that need to be improved.

4. Estrogen signaling

Estrogens mediate their effects through binding to their transducer, ER. ERs belong to the nuclear receptor's superfamily, which, once activated, act as transcription factors promoting the expression of several genes involved in many physiological functions (106, 107). ER has two isoforms in mammals, ERα (595 aminoacids, 66 kDa) and ERβ (530 aminoacids, 59 kDa), that share 56% homology and are coded by different genes, *ESR1* on chromosome 6 (6q25.1) and *ESR2* on chromosome 14 (14q23.2), respectively (108). *ESR1* mutations occur more frequently in refractory tumors than in primary tumors (109). ERα was first cloned in 1986, from the MCF-7 breast cancer cell line (110), and ERβ was cloned in 1996, from rat prostate and ovaries (111). These two isoforms are differentially expressed, ERα is found in reproductive organs, such as uterus and ovaries, in breast, kidney, bones, white adipose tissue, and liver. In contrast, ERβ is expressed in the ovaries, central nervous system, cardiovascular system, lungs, prostate, colon, kidney, and immune system (108). Both ERs form homodimers in the active form. ERβ homodimers were reported to be less active than ERα dimers (112, 113). ERα is also capable of dimerizing with ERβ, reducing its transcriptional activity (114). Moreover, these receptors are not ambiguous in function since ERα is associated with tumorigenesis (95, 115, 116) and ERβ with anti-tumor signaling (117-122). In fact, when both receptors are expressed, ERβ has the ability to prevent ERα activity (122-125). This is in part explained by the substantial overlap of the binding sites of these receptors (122, 126). Based on the roles of each receptor, their expression in normal and tumoral breast epithelial cells is also altered. For instance, ERα is less expressed than ERβ

in normal epithelial mammary cells (127, 128). In cancerous cells the ratio is inverse, with increased ERα and decreased ERβ expression (128-130). Despite all the data, the clinical benefit of ERβ expression is dubious (119, 122, 125, 131-134). Nevertheless, the ratio between ERα:ERβ seems to be a good predictor of anti-estrogen therapy responsiveness (135, 136).

Structurally, both ERs have six domains (A through F), while functionally, they have two transactivation domains, the ligand-independent AF-1 (near the N-terminus) and ligand-dependent AF-2 (near the C-terminus), a DNA-binding domain (DBD), a dimerization region and a ligand-binding domain (LBD) (71). Both receptors share a 16% homology in the amino-terminal A/B domain. This is the largest domain and contains AF-1, which is involved in gene transcription by interacting with transcription co-regulators and does not require estrogen binding to be activated (108, 137). Instead, this domain contains several activation phosphorylation sites, such as serine 118 (S118) (138-141) and serine 167 (S167) (142, 143). This domain is also much shorter in ERβ (144). The C domain, which contains the DBD, is highly conserved in both receptors (97% similarity), explaining the overlap on binding sites. The DBD recognizes and binds ER-specific DNA motifs, known as estrogen-responsive elements (ERE), through two zinc fingers and a stretch of aminoacids called C-terminal extension. These zinc fingers also contribute to ER dimerization (145-147). The D domain has 36% of similarity between the two receptors (148), contains part of the C-terminal extension and is responsible for the nuclear localization of ER. Moreover, this domain also modulates ER interactions, through allosteric regulation, and, consequently, its activity (149-151). The E/F domains contain the LBD and AF-2, involved in estrogen recognition and binding. This is achieved through eleven α-helix that form a high-affinity and highly conserved pocket for estrogens, despite the 59 % homology of LBD as a whole (149) (Figure 5).

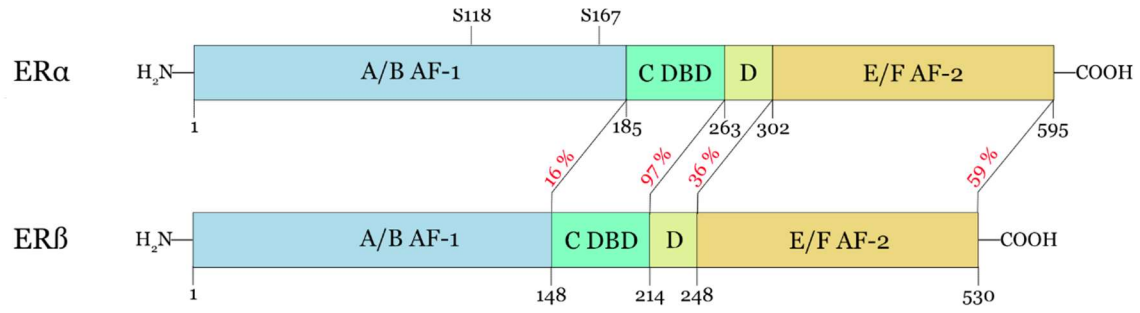

Figure 5. Structure of ERα and ERβ. The six domains are represented (A through F). These domains harbor a DNA-binding domain (DBD) and two transactivation domains (AF-1 and AF-2). Phosphorylation sites in the AF-1 domain are indicated (S118 and S167). The homologies between the domains in both receptors are indicated in red.

Regardless the high similarity in estrogen-binding pocket, that differ in only 2 aminoacids (152), this difference was sufficient for the development of selective ligands (153). The binding of estrogen, reallocates the α-helix 12, a component of the AF-2, allowing ER activation. A sequence in α-helix 11 also has a role in ER dimerization and nuclear localization (137).

The ER has several variants, resultant from mRNA alternative splicing. ERα has three different main variants, ERαΔ3, that lacks part of the DBD, but binds to E2 (154-156), ERα36, which lacks AF-1 and AF-2 (157), and ERα46 without the AF-1 (158). ERαΔ3 is highly expressed in breast tumors and acts as a dominant negative regulator of ERα (155, 159, 160). ERα36 is linked with carcinogenesis and aggressiveness, as well as, a poor therapeutic response, being, therefore, associated with a poor prognosis in breast cancer (157, 161-167). This isoform is localized at the plasma membrane (168-170), being expressed even in ER^- tumors (163, 171). Contrary to ERα36, ERα46 has anti-tumoral functions in breast cancer and is also localized in the plasma membrane (172-175). For ERβ, four variants are reported, ERβ2, ERβ3, ERβ4, and ERβ5, that have a different C-terminus and do not bind estrogens (176). However, ERβ3 expression in the breast is in most cases undetectable (177, 178). The exact role of these variants is still debatable and may vary according to ERα status. ERβ2 has been shown to dimerize and repress ERα transcriptional activity in breast cancer (179, 180). Several studies have reported this variant as a marker of good prognosis (180-184); however, others have attributed a poor prognosis to its expression (185, 186). ERβ4 has been shown to induce malignant transformation of a normal breast cancer cell line (186). ERβ5 has a protective role in breast cancer patients (181, 184), nevertheless, its role in promoting aggressiveness in triple negative tumors and as a marker of poor prognosis in HER2-enriched and triple negative breast cancer patients has also been reported (186, 187).

4.1 Genomic pathway

ER monomers are found in the cytosol bound to chaperone proteins, such as heat shock protein 70 (HSP70) and HSP90, whose function is to stabilize these monomers in a hormone-binding inactive state and to aid in the dimerization of ERα/ERα or ERα/ERβ (188-193). HSP90 function is essential for the regulation of ER levels since it allows unliganded ER degradation by the ubiquine-proteasome pathway, ER dimerization and also modulates ER transcriptional activity (191, 194-198). Upon ligand binding, the ER suffers conformational changes, promoting the dissociation from HSP90, dimerization and exposure of AF-1 and AF-2. The dimer translocates to the nucleus and binds ERE sequences within the promoter/enhancer regions of the target genes (Figure 6). Here the exposed AF

domains will interact with nuclear co-activators (CAs) or nuclear co-repressors (CRs) to modulate *classical* gene transcription (71). Deregulation of these co-regulators is associated with breast carcinogenesis (199). Moreover, the mapping of these co-regulators was mainly performed for ERα rather than ERβ (200). ERα co-activators are spread across multiple families, such as the steroid receptor coactivator (SRC)/p160 family, Src-1, -2 and -3 (201) and the histone acetyltransferase cAMP responsive element binding protein (CREB)-binding protein (CBP)/p300 (202), among others (203). Examples of co-repressors are prohibitin 1 and 2 (PHB and PHB2) (204), silencing mediator of retinoic acid and thyroid hormone receptors (SMRT), and N-CoR (205), among others (203). The recruitment of different co-regulators is modulated by different ligands (206, 207). Strikingly, the expression of these co-regulators is generally regulated by ER-dependent transcription, through feedback mechanisms (203).

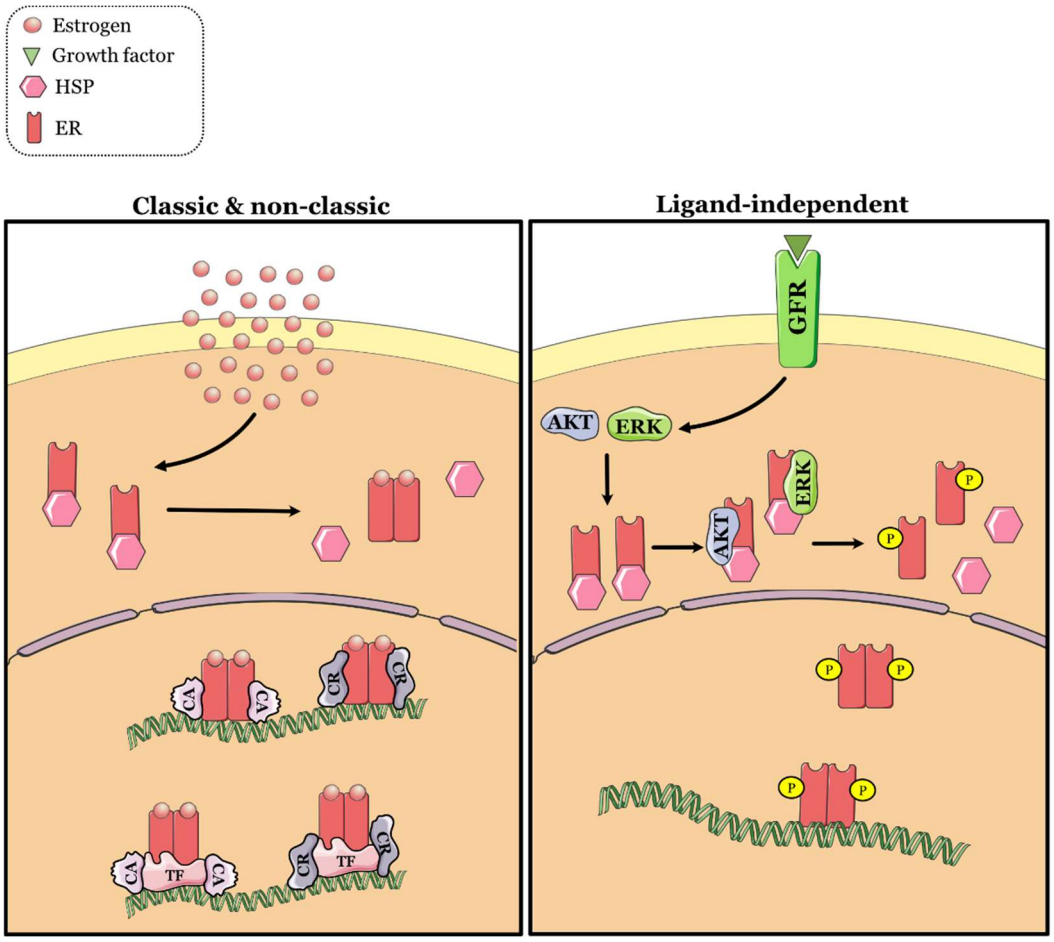

Figure 6. Genomic estrogen signaling. Estrogen binding to estrogen receptor (ER) induces its dissociation from heat-shock proteins (HSP), followed by dimerization and translocation to the nucleus. Nuclear ER-estrogen complexes bind directly to DNA, together with nuclear co-activators (CA) and co-repressors (CR), on estrogen-responsive elements (ERE) sequences (classic) or recruit other transcription factors (TF) in order to modulate different sets of genes (non-classic). ER can also be activated through phosphorylations in the AF-1 domain, promoted by growth factor receptors (GFRs), via the PI3K/AKT and MAPK/ERK pathways, in estrogen-depleted conditions (ligand-independent).

Posttranslational modifications of these co-regulators can also modulate their activity (208, 209). The ER can also regulate the transcription of genes that do not contain ERE sequences in what is known as *non-classical* transcription. In this case, ER interacts with other transcription factors, such as activator protein 1 (AP1), specificity protein 1 (SP1), nuclear factor κ-B (NFκB), FoxO family, and transacting T-cell-specific transcription factor (GATA-3), modulating the transcription of different genes (210-216).

The ligand-independent ER activation consists in the third genomic mechanism. The ER is activated through phosphorylation of the AF-1 domain, via growth factor receptors (GFRs) signaling pathways. The ER can be phosphorylated at S167 by the PI3K/AKT pathway (143), and at S118 by the MAPK/ERK pathway (140, 141). The phosphorylation of these sites, through kinases of the GFR pathways, can mediate the binding of ER to co-regulators (115, 203, 217, 218). These phosphorylations may promote the development of resistance to anti-estrogen therapies (219-221). It has also been reported a S305 phosphorylation that is associated with a poor outcome in patients treated with anti-estrogens (221-223).

4.2 Non-genomic pathway

The non-genomic pathway is mediated through binding of estrogens to cell membrane receptors to elicit faster responses than in the genomic pathways, through the activation of the Insulin-like growth factor receptor 1 (IGF-IR), epidermal growth factor receptors (EGFR) (224, 225), and of second messengers (226) (Figure 7). This signaling leads to the activation of several pathways, such as the phospholipase C (PLC)/ Protein kinase C (PKC), Ras/Raf/MAPK, PI3K/AKT, and cAMP/Protein kinase A (PKA), modulation of several transcription factors and, even, of ER (227). Indirectly it regulates gene transcription and promotes a convergence between the genomic and non-genomic pathways. This signaling is attributed to ER variants (228, 229), to membrane-bound G-protein-coupled estrogen receptor (GPER1) (230-235), and even, to membrane-bound ERα (236-238). The association of ER and its variants to the plasma membrane, through caveolin-1, seems to be mediated by palmitoylation of the receptor (169, 239). Moreover, the expression of GPER1 is encoded by the *GPER* gene and is exclusively membrane-bound. This receptor was also associated with IGF-IR and EGFR signaling (229, 240-242). GPER1 is also expressed independently of ERα in breast tumors and has different binding affinities for estrogens, when compared to ERα (231, 243-245). The activation of this receptor has been associated with carcinogenesis (246-252), aggressiveness (253) and anti-estrogen therapy resistance (254-256). In addition, *GPER* knockdown was associated with decreased proliferation in triple negative breast cancer cells (257). A recent study described an increased expression

of *GPER* in breast cancer stem cells when compared to non-stem cells (258). Moreover, GPER1 signaling abrogation led to a decrease survival of stem cells.

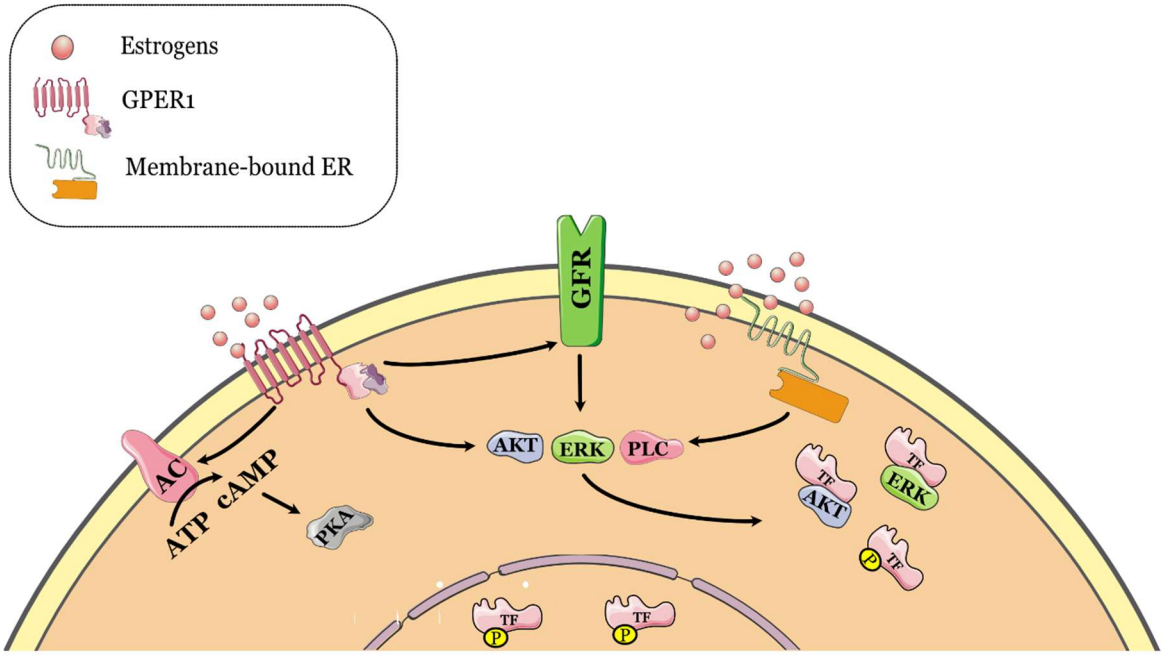

Figure 7. Non-genomic estrogen signaling. Estrogen binding to cell membrane receptors, such as the G-protein coupled receptor 1 (GPER1) and membrane-bound ER, elicit rapid responses. These responses include activation of growth factor receptors (GFRs), activation of the PI3K/AKT and MAPK/ERK pathways, of phospholipase C (PLC) and of adenyl cyclase (AC)/Protein kinase A (PKA), which can lead to the modulation of several transcription factors (TF). cAMP – Cyclic AMP; GFR – Growth factor receptor.

5. Endocrine therapy

As mentioned before, ER^+ tumors strongly depend on estrogens for growth (259). In addition, ER^+ breast cancer account for 60% of premenopausal and 75% of postmenopausal cases (260). Therefore, estrogen biosynthesis suppression or estrogen signaling blockade are the main therapies for these specific tumors. Endocrine therapy is the preferred adjuvant option for early and advanced luminal A breast cancers. Several drugs are used to achieve estrogen signaling blockade, such as Tamoxifen, a selective estrogen receptor modulator (SERM), and Fulvestrant (ICI 182,780), a selective estrogen receptor downregulator (SERD); or aromatase inhibitors (AIs), that block estrogen biosynthesis (260).

5.1 Selective Estrogen Receptor Modulators

SERMs are molecules that, depending on the target tissue, present partial agonistic or antagonistic activity (261, 262). These dual effects result from the mechanisms of action of these molecules, as SERMs compete with estrogens for ER binding and modulate its activity by recruiting co-regulators (Figure 8). In the breast, SERMs act as antagonists of the ER

(262, 263). SERMs are divided into different classes: triphenylethylenes, as Tamoxifen, benzothiophenes, such as Raloxifene and Arzoxifene, phenylindoles, such as Bazedoxifene and Pipindoxifene, and tetrahydronaphthalenes, as Lasofoxifene. Some of these SERMs were also proposed to decrease breast cancer risk in postmenopausal women (264-266) and to prevent or treat osteoporosis (267).

Tamoxifen is the most recognizable oral SERM in clinical use for breast cancer treatment. This first-generation triphenylethylene SERM showed great efficacy in clinical trials for advanced breast cancer in the 1970s (268, 269). However, Tamoxifen presented poor anti-proliferative activity *in vitro* when compared to the *in vivo* studies (270-272). This discrepancy was later explained by the products of Tamoxifen metabolization, 4-Hydroxytamoxifen (4-OH-tam) and Endoxifen, through the enzymes of the cytochrome P450, cytochrome P450 2D6 (CYP2D6), cytochrome P450 3A (CYP3A) and cytochrome P450 2C (CYP2C) (273-277). In fact, both metabolites present a higher affinity for ER than Tamoxifen (278-280). Besides that, several clinical trials confirmed the efficacy of Tamoxifen in adjuvant therapy (281-286) and in the prevention of ER^+ breast cancer (287-289). Nevertheless, it induces severe adverse effects, such as increased risk of endometrial cancer (281, 289, 290), and more manageable side effects, like hot flashes, sexual disfunction, blood clots, and thromboembolic events, that were outweighed by the clinical benefit (260, 291-293). Currently, for premenopausal women with early breast cancer, Tamoxifen is the drug of choice, with a possible switch to AIs when patients enter in a menopausal state within 5 years of Tamoxifen treatment (293, 294). In postmenopausal women with early breast cancer both Tamoxifen and AIs are the standard options (293, 294).

In advanced breast cancer, Tamoxifen is an option for premenopausal women who had ovarian function suppression while in postmenopausal women, it depends on previous endocrine treatment (295, 296). Nevertheless, it is estimated that 30% of ER^+ tumors are inherently resistant to Tamoxifen and that 40% will develop resistance (297). Tamoxifen-resistant tumors are often cross-resistant to other SERMs (267, 298).

The Tamoxifen metabolite, Endoxifen, has been shown to be more potent than Tamoxifen in pre-clinical studies, with the ability to degrade ERα (273, 274, 299, 300). This metabolite presented high anti-tumor activity, in AI-sensitive and AI-resistant cell lines, without leading to the development of resistance (301) and was also reported as having an anti-aromatase activity (302).

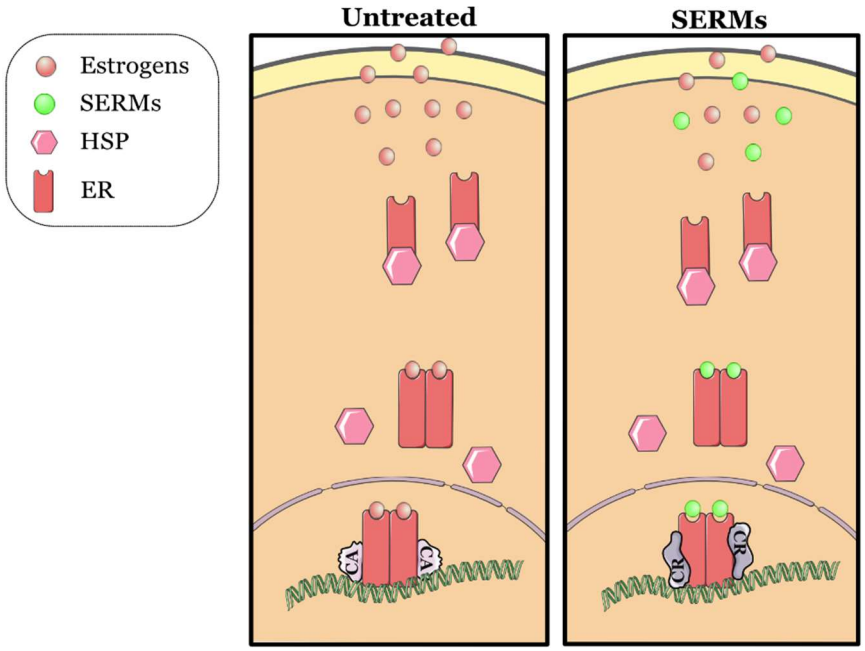

Figure 8. Mechanism of action of selective estrogen receptor modulators. In untreated breast cancer cells, estrogens bind to the estrogen receptor (ER), leading to the dissociation of heat-shock proteins (HSPs), dimerization, translocation to the nucleus and recruitment of co-activators (CA) to promote gene transcription. In treated cells, selective estrogen receptor modulators (SERMs) bind to ER, and, in the nucleus, SERM-bound ER recruits co-repressors (CR) blocking gene transcription.

A phase I clinical trial (NCT01327781) reported manageable side effects and promising antitumor activity in AI- and Tamoxifen-resistant patients (303). The study of Endoxifen as an effective option for the treatment of breast cancer continues in ongoing clinical trials (NCT02311933, NCT01327781). In addition, another secondary Tamoxifen metabolite and a direct metabolite of Endoxifen, Norendoxifen, was shown to have a selective and potent AI activity (304).

Toremifene, also a first-generation triphenylethylene SERM, like Tamoxifen, was approved in 1997 for ER^+ advanced breast cancer treatment due to a similar efficacy to Tamoxifen and a slightly different toxicity profile (305-307). Nowadays, it is considered a valid alternative to Tamoxifen and can be combined with CYP2D6 inhibitors for the ER^+ advanced breast cancer, in the USA (295). Recently, the combination of Bazedoxifene, a third-generation SERM used for the treatment of postmenopausal osteoporosis, with cyclin-dependent kinase (CDK) 4/6 inhibitors for advanced breast cancer, previously exposed to endocrine therapy, is being studied (NCT02448771).

5.2 Selective Estrogen Receptor Downregulators

SERDs bind to ER and induce its degradation (Figure 9) by destabilizing the α-helix 12 (308, 309). Therefore, as ER signaling is mitigated, these compounds do not present tissue-specific effects and are referred to as pure anti-estrogens. ICI 182,780 is the most recognized

SERD in clinical use. ICI 182,780 has good efficacy both *in vitro* and *in vivo* (310, 311). However, despite the lack of endometrial side effects observable for Tamoxifen, its intramuscular administration limits its use. ICI 182,780 binds to monomeric ER, preventing its dimerization and promoting ER degradation by the ubiquitin-proteasome pathway (147, 312-315). Curiously, although ER degradation is a defining characteristic, it is not imperative for its antagonistic activity (316). Several studies have also described that this drug bypasses Tamoxifen-resistance as well as, in some cases, AI-resistance (317, 318). Nowadays, ICI 182,780 is not recommended for adjuvant therapy in early breast cancer. Instead, depending on prior exposure to endocrine treatment and the time since the end of adjuvant endocrine treatment, ICI 182,780 alone or in combination with non-steroidal AIs is accepted as first-line therapy for ER$^+$ advanced breast cancer (295, 296).

Moreover, ICI 182,780 is recommended, either alone or in combination with CDK 4/6 inhibitors or with the mammalian target of rapamycin C1 (mTORC1) inhibitor, Everolimus, for the treatment of ER$^+$ advanced breast cancer previously exposed to endocrine therapy (295, 296, 319). Nevertheless, ER-independent mechanisms of cell growth may lead to the development of ICI 182,780-resistance (320).

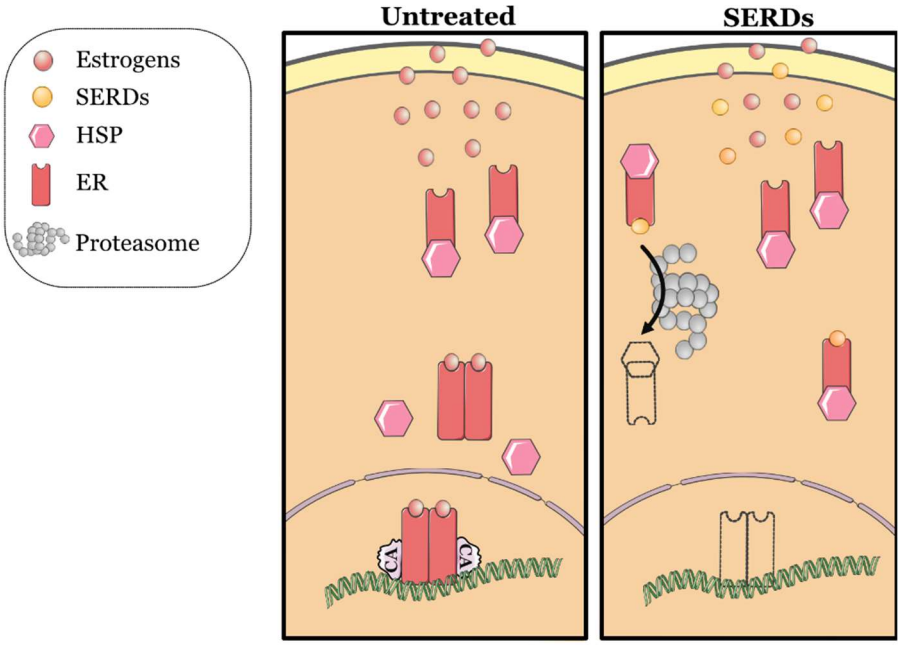

Figure 9. Mechanism of action of selective estrogen receptor downregulators. In untreated breast cancer cells, estrogens bind to the estrogen receptor (ER), leading to the dissociation of heat-shock proteins (HSPs), translocation to the nucleus and recruitment of co-activators (CA) to promote gene transcription. In treated cells, selective estrogen receptor downregulators (SERDs) bind to ER and promote its degradation by the proteasome.

The administration route of ICI 182,780 by intramuscular injection prompted the search for orally available SERDs. Although some SERDs studies have been discontinued, AZD9496, RAD1901, and GDC-0927 showed good anti-tumor activity in ER$^+$ breast cancer

models (321-328). In addition, the results from clinical trials (NCT02248090, NCT02338349, NCT 02316509) reported an acceptable safety and tolerability profile, for AZD9496, RAD1901 and GDC-0927, in pretreated ER^+ advanced breast cancer patients (329, 330). Currently, other clinical trials (NCT02650817, NCT03778931, NCT03332797) are studying RAD1901 and GDC-0927 to treat ER^+ advanced breast cancer.

5.3 Aromatase inhibitors

Aromatase inhibitors block the conversion of androgens to estrogens catalyzed by aromatase (Figure 10). AIs are categorized in first-, second- and third-generation, according to their development. Furthermore, they are also classified as steroidal (Type I) or non-steroidal (Type II). The steroidal AIs use the core structure of androstenedione as a molecular basis. Therefore, steroidal AIs enter the substrate-binding pocket of aromatase, and their reactive intermediates covalently bind aromatase resulting in an irreversible inhibition. These inhibitors are often called *suicidal inhibitors* since aromatase is inhibited by its own catalysis. Non-steroidal AIs saturate the available binding-sites of the heme group of aromatase by non-covalent binding. Therefore, these AIs act through a reversible competitive inhibition, competing with androgens (55, 331).

The first generation of AIs was marked by aminoglutethimide (AG), the first described non-steroidal AI. AG was first directed for breast cancer treatment, in the 1970s, since it effectively decreased estrogen levels. However, it also inhibited CYP11 due to the lack of specificity. This was bypassed by cortisol supplementation, nevertheless, these side effects led to its discontinuation (55, 82, 331).

Specific AIs were developed in the second generation during the decades of 1980 and 1990. This generation is mainly composed of two inhibitors, the non-steroidal imidazole derivative, Fadrozole, and the steroidal, 4-hydroxy-androstenedione (Formestane). Fadrozole revealed to be more potent than AG, however, it also inhibited enzymes involved in aldosterone, progesterone and corticosterone biosynthesis (332-334). Formestane was the first AI used in clinic, since it was well tolerated and revealed good efficacy, even, in Tamoxifen-resistant tumors. Nevertheless, due to low bioavailability when administered orally, its intramuscular administration hindered its use (82, 332, 335-337).

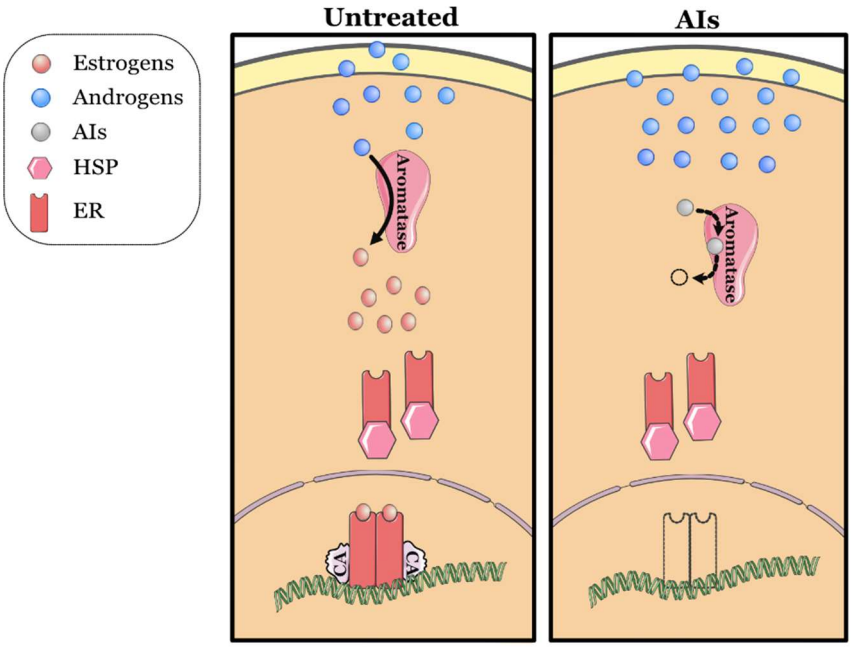

Figure 10. Mechanism of action of aromatase inhibitors. In untreated breast cancer cells, estrogens bind to the estrogen receptor (ER), leading to the dissociation of heat-shock proteins (HSPs), translocation to the nucleus and recruitment of co-activators (CA) to promote gene transcription. In treated cells, aromatase inhibitors (AIs) block the conversion of androgens to estrogens by inhibiting aromatase. The ER is not activated due to the lack of estrogens. CA – Co-activator.

Three orally administered AIs define the third generation, the non-steroidal, Anastrozole and Letrozole, and the steroidal, Exemestane (Figure 11). These three AIs inhibit aromatization by over 97% (338-341), decreasing estrogen levels in plasma by over 90% (63, 340, 342, 343). This generation presented higher efficacy and less severe side effects than Tamoxifen, such as endometrial and thromboembolic events, and increased efficacy and tolerability over ICI 182,780 (260). However, third-generation AIs are associated with arthralgia, musculoskeletal pain, cardiovascular events and sexual dysfunction. The use of these AIs also led to increased bone fractures, due to bone mineral loss induced by estrogen suppression, but this side effect is bypassed by the use of bisphosphonates (260).

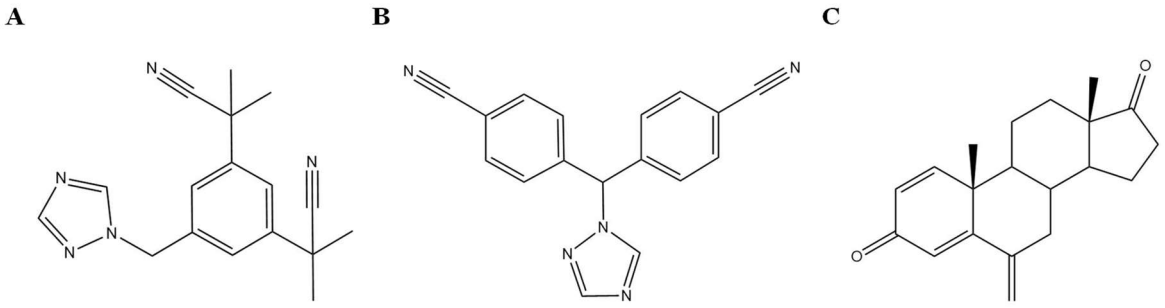

Figure 11. Chemical structure of the third-generation aromatase inhibitors. (A) Anastrozole. (B) Letrozole. (C) Exemestane.

Due to this superiority over Tamoxifen, in terms of efficacy and tolerability, AIs have become a standard therapeutic choice in breast cancer treatment. In fact, recent clinical

trials have been assessing the potential of AIs in breast cancer prevention. A follow-up study of high-risk postmenopausal patients treated with Anastrozole has reported an overall 49% reduction in the risk of breast cancer development, when compared to placebo (344). Another shorter follow-up study reported a decrease in breast cancer risk for postmenopausal patients treated with Exemestane, when compared to placebo (345). Taking together this data, Anastrozole and Exemestane, along with Tamoxifen and Raloxifene, are now also considered for breast cancer prevention in high-risk patients in the USA (346). However, in Europe only Tamoxifen is used, but risk-reducing surgery is preferred (347).

Currently, non-steroidal AIs are considered as extended therapy, after 5-years Tamoxifen, in premenopausal women, or first-line for premenopausal women with a high risk of recurrence after ovarian function suppression. For postmenopausal women with early-stage breast cancer, AIs are first-line therapy (293, 294). In advanced breast cancer, AIs are considered one of the first-line options depending on previous endocrine therapy. In addition, their combination with CDK 4/6 inhibitors or with Everolimus, in treatment-naïve or pretreated patients, is also a therapeutic option (295, 296). The combination of AIs with ICI 182,780 is also an option in USA (295). The prolongation of treatment with AIs, as with any anti-estrogen therapy, may lead to the development of resistance, however, since AIs do not present cross-resistance, a tumor that progressed on non-steroidal AIs is still eligible for steroidal AIs therapy (348).

5.3.1 Non-steroidal Aromatase Inhibitors

Anastrozole and Letrozole are derived from antifungal drugs that presented activity against cytochrome P450 enzymes, therefore, possible cross-reaction of these triazole-derived AIs with CYP1A2, CYP2C9, CYP3A4 and CYP2A6, were already described (349, 350). Nevertheless, Anastrozole seems to be more specific than Letrozole (351). Both AIs present very low IC_{50}, in human placental microsomes, with values of 15 nM for Anastrozole (352) and under 12 nM for Letrozole (353).

Anastrozole is rapidly absorbed, when administered orally at the recommended daily dose (1 mg), reaching maximum plasma concentrations within 2 hours, in fasting conditions (354). In the therapeutic range, 40% is bound to plasma proteins. Anastrozole is primarily metabolized in the liver, through oxidation, via CYP3A4, CYP3A5, and CYP2C8, glucuronidation via UGT1A4, or N-dealkylation into inactive metabolites, such as hydroxyanastrozole, Anastrozole glucuronide, and triazole, respectively (355-357). Its half-life is between 40 and 50 hours, with less than 10% excreted unaltered, and 60% excreted

as metabolites through urine (354, 358, 359). Maximum estrogen suppression is verified 3 to 4 days after the first administration (351).

Letrozole presents very high bioavailability (99.9%) and reaches maximum plasma concentration within 2 hours, when administered orally at the recommended daily dose (2.5 mg) (360, 361). Around 60% is bound to plasma proteins, mainly to albumin (360). Letrozole is metabolized in the liver, by CYP2A6, into a ketone analog metabolite that is further metabolized, by CYP3A4 and CYP2A6, into the inactive carbinol (362). This metabolite can suffer glucuronidation, by UGT2B7, into carbinol-gluc, the major metabolite (363, 364). The half-life of this AI is 40 hours in healthy volunteers, and 80 hours, in breast cancer patients (365, 366). This difference is attributed to decreased metabolic clearance in cancer patients (366). Around 90% of excretion occurs through urine in the glucuronidated form (365, 366). Maximum estrogen suppression is verified 2 to 3 days after first administration (367). Letrozole shows superiority over Anastrozole in decreasing estrogen levels both in breast cancer tissue and plasma (63, 342).

Letrozole is associated with changes in the lipid profile, more specifically, augmented cholesterol, increasing the risk of cardiovascular diseases (351, 368). The *in vitro* effects of these AIs in breast cancer cells have been described. Both AIs decrease the proliferation of tumor cells and induce cell cycle arrest at the G_0/G_1 phase (369, 370). This was accompanied by upregulation of p53 and p21, at the mRNA and protein levels, and by cyclin D1 and c-MYC mRNA downregulation. In addition, Letrozole treatment also induces apoptosis, through decreased Bcl-xL and BCL-2 levels, increased Bax, caspase-9, and caspase-6 levels and cleavage of Poly (ADP-ribose) polymerase (PARP) (370, 371). On the other hand, Anastrozole treatment increases p27 levels and caspase-8 expression, decreases Bcl-xL expression, and leads to activation of the MAPK pathway (371, 372).

5.3.2 Steroidal Aromatase Inhibitors

Exemestane is the only steroidal AI currently in clinical use. Its structure is derived from androstenedione and is considered a *suicidal inhibitor*. Because of this feature, Exemestane is highly selective for aromatase (351). In human placental microsomes, this AI presents an IC_{50} around 50 nM (69, 373, 374). Exemestane is administered orally, at the recommended daily dose of 25 mg, and rapidly absorbed, reaching maximum plasma concentrations within 2 hours (375). Around 90% is bound to plasma proteins, such as albumin and α_1-acid glycoprotein (376). Exemestane is metabolized, in the liver, by several enzymes of the cytochrome P450, such as CYP1A1/2, CYP4A11, CYP3A4/5, CYP2B6, CYP2A6, CYP2C8, CYP2C9, CYP2C19, and CYP2D6, and by aldoketoreductases (376-379). This

biotransformation originates several metabolites, such as 6-hydroxymethylexemestane (6-HME) and 17*β*-hydroexemestane (17β-HE) (377). Moreover, 17β-HE can be further glucurinated, by UGT2B17, into 17β-HE-Gluc, or metabolized by CYP3A4/5 into an inactive metabolite known as MIII (380, 381). Nevertheless, a recent study reported a novel pathway of metabolization through glutathione conjugation, originating cysteine conjugates of Exemestane and 17β-HE, detectable in plasma and urine (382). Thus, differences in the metabolism of this AI might explain different clinical outcomes observed through inter-patient variability. Moreover, CYP3A1 is widely expressed in the gastrointestinal tract, a first-passage metabolism site, decreasing Exemestane bioavailability (361). This AI presents a half-life of around 24 hours and is excreted mainly through urine (361, 376). Maximum estrogen suppression occurs between 3 to 7 days after the first dose, and effects persist at least 5 days after single-dose administration due to its tight aromatase binding (361, 376).

In addition to long-lasting aromatase inhibition, Exemestane destabilizes the enzyme, decreasing aromatase levels through proteasomal degradation, reducing aromatase half-life by 50% (383). *In vitro*, Exemestane is associated with decreased proliferation of ER^+ breast cancer cells, inducing cell cycle arrest at G_0/G_1, for short periods, and at G_2/M for longer periods. Moreover, apoptosis induction through the intrinsic pathway was also detected, with increased reactive oxygen species (ROS) levels (384). Cytoprotective autophagy was also reported (384). However, for lower doses, a weak estrogen-like activity is also described for this AI (384, 385). The biological *in vitro* effects of the metabolites have also been studied. The main metabolites, 17β-HE and 6-HME, inhibited aromatase and decreased the proliferation of ER^+ breast cancer cells, by apoptosis and cell cycle arrest, but also induced autophagy. Curiously, these metabolites also activated caspase-8, an effect not observed with Exemestane (386). Furthermore, 17β-HE can induce androgenic-like actions (387). In fact, Exemestane has been associated with high intratumoral androgenic activity possibly due to 17β-HE levels (385, 388). However, it induces AR overexpression/activation in ER^+ breast cancer cell lines that are AI-sensitive and AI-resistant (389). Another feature of Exemestane is that it does not impact bone mineral density as the non-steroidal AIs (390-394). Moreover, adjuvant Exemestane treatment is associated with a reduction in the risk of recurrence and better prognosis in postmenopausal women when compared to adjuvant Tamoxifen or Tamoxifen followed by Exemestane (395).

6. Mechanisms of resistance to Aromatase inhibitors

Estrogen deprivation, through AIs, has become a gold-standard in clinical practice for the treatment of ER^+ breast cancer. However, despite their clinical efficacy, 20% of patients with early-stage disease are unresponsive (283), and some patients with metastatic disease

relapse after showing benefit from AIs therapy. Two distinct types of resistance are described in clinic: primary/*de novo* and secondary/acquired (296). Primary resistance is defined by a relapse within the first two years of endocrine therapy or progression of disease within the first 6 months of first-line endocrine therapy for advanced breast cancer. Acquired resistance is defined as a relapse after the first 2 years of adjuvant endocrine therapy, relapse within a year of completing adjuvant endocrine therapy, or progression of disease after the first 6 months of initiating endocrine therapy for advanced breast cancer. Notwithstanding the clinical definition, molecularly, the line between one and the other is blurred, and the mechanisms are likely to overlap (260).

Recently, the development of resistance was studied through estrogen withdrawal in a breast cancer cell line (396). In this study, no features of resistance were found in treatment-naïve cells. Nevertheless, a small population of plastic cells, with a high expression of the stem-cell marker CD44, exhibited a pre-adaptation (PA) phenotype that allowed an increased survival rate in estrogen-depleted conditions, with features of aggressiveness and reduced ER activity. This PA phenotype was characterized by a signature of acute endocrine therapy, resembling starvation, in an estrogen-rich environment. Moreover, these cells were genetically different from the resistant cells. Ultimately, this study suggested that during endocrine therapy, the PA phenotype acts as a bottleneck and that the cells with this phenotype enter a quiescence/senescent state, in which they can stay for years and reprogramme themselves to a fully AI-resistant phenotype. Thus, this study concludes that AIs resistance does not result from a selection of pre-treatment resistant cells but from several cell adaptations that occur over a large period. Quiescence acts as an adaptation mechanism for MCF-7 cells in response to estrogen withdrawal, leading to the development of resistance (396). Confirming this claim, senescence has been described as a response to the lack of estrogen signaling, due to anti-estrogens treatment, as Tamoxifen and ICI 182,780, in ER^+ breast cancer cell lines (397-400). The quiescent/senescent state was shown to be induced by IL-6 and IL-8 and to be intimately correlated with increased CD44 expression (plasticity) and aggressiveness in luminal cells (401). The role of these interleukins in the adaptation of breast cancer cells was reinforced by the study of *Fu et al* (402). This study reported that overexpression of the transcription factor *FOXA1* in endocrine-resistant cells led to a reprogramming of ER transcriptional activity, being IL-8 among the most affected genes. Its knockdown resulted in the reversal of Tamoxifen-resistance and invasion. Therefore, loss of ER expression is not the main mechanism of resistance since most cases occur in ER-expressing tumors (403, 404). In accordance with these findings, other transcription factors known to regulate stemness in breast cancer are altered and favor a resistant phenotype (405, 406). In fact, in clinic, these high CD44-expressing cells with distinct features were detected after neoadjuvant Letrozole treatment

(407). In this sense, biomarkers of resistance in primary tumors have been widely investigated to predict the clinical outcome of AIs therapy and even prevent relapses.

However, high inter-tumor heterogeneity and high plasticity of breast cancer cells are, currently, the basis for the non-curable status of advanced breast cancer. Thus, more research is urgently needed, nevertheless, some underlying mechanisms of resistance have already been identified, leading to changes in the clinical practice.

6.1 Altered *ESR1* and *CYP19A1*

Resistant cells can acquire mechanisms to maintain ER activity. *ESR1* mutations are rare in primary tumors, though advanced breast cancers that progressed during AI-therapy present a higher frequency of *ESR1* mutations (30%), that are absent in matched primary samples (408-415).

Several activating point mutations in the LBD of the receptor have been described, being the most frequent: D538G, E380Q, Y537S, Y537C, and Y537N. Less frequent mutations have also been referred, such as V422del and L536H (408, 409, 412, 416-418). Of these mutations, the ones on the positions 536, 537, and 538 are the most potent inducers of ER activity. Nevertheless, with the exception of Y537S, ER activity is never higher than when stimulated by estradiol (418). Despite these mutations, the use of SERDs seems to be capable of abrogating ER signaling (326, 418, 419). Recently, a new class of ER modulator was described, the selective estrogen receptor covalent antagonist (SERCA) H3B-5942, that was able to inactivate wild-type and mutated forms of ER (420).

ESR1 chromosomal translocations are also capable of driving estrogen-deprived tumors. Several in-frame fusion genes that preserved the first six or seven exons of the *ESR1* gene (which includes the DBD), spliced in-frame to the C-terminus of several genes have been described (408, 421-423). The *ESR1*^{e6}*-YAP1* fusion gene induces hormone-independent growth of patient derived xenografts (PDX) models and expresses ER-regulated signature genes, with the *YAP1* sequence mimicking the AF-2 domain. Another type of translocation identified was the gene rearrangement between *ESR1* and coiled-coil domain containing 170 (*CCDC170*), which resides in a centromeric position in relation to *ESR1*, resulting in the overexpression of a truncated form of *CCDC170* (*ΔCCDC170*). This truncated form was shown to increase cell motility and anchorage-independent growth, reduce endocrine sensitivity and enhance xenograft tumor formation through interaction with the GRB2-associated binding protein 1 (Gab1) signalosome (424). In addition, the *ESR1*^{e6}*-YAP1* and *ESR1*^{e6}*-PCDH11X* fusion genes induce cell growth through transcription of ER-regulated genes, promote metastasis and present resistance to ICI 182,780 in estrogen-depleted

conditions. Nevertheless, the growth of cells harboring these fusion genes was inhibited by CDK 4/6 inhibitors (422). The same authors have also reported that the fusion gene *ESR1*^{e7}-*POLH* led to the enrichment of HER2, EGFR, and MAPK gene expression signatures and of genes associated with triple negative cancer, highlighting the differential mechanisms through which these fusion genes drive acquired resistance (425).

ESR1 amplification has been described and translated into ER overexpression. However, estrogen supplementation led to tumor regression through apoptosis induction (408). This growth-inhibition effect of estrogen in estrogen-independent tumors was also described elsewhere (426, 427). Nevertheless, the clinical significance of these findings are debatable (428). In addition, a study reported that the aromatase gene, *CYP19A1*, is amplified in AI-resistant cells, both *in vitro* and in clinic. In this study, the amplification of this gene led to an increase in aromatase and estrogen-independent ER activities. However, this seems to only occur in response to reversible AIs since all patients stabilize 1 year after switching to Exemestane (429).

6.2 Aberrant signaling of survival pathways and crosstalk with ER

The aberrant activation of several survival pathways in estrogen-depleted conditions provides an escape of the anti-tumoral effects of AIs, also leading to the development of resistance and tumor growth (Figure 12) (430).

6.2.1 Epidermal growth factor receptors

The ErbB family comprises the EGFR (also known as HER1), HER2, HER3, and HER4. These receptors activate several downstream pathways, such as PI3K/AKT/mTORC1 and MAPK/ERK. The most implicated receptors in AIs resistance are HER2 and EGFR. Interestingly, an Exemestane-resistant cell line expresses an EGFR ligand, amphiregulin (AREG), to activate EGFR signaling and drive cell proliferation, through the MAPK/ERK and PI3K/AKT/mTORC1 pathway, promoting phosphorylation at S118 and consequent activation of ER (220, 431). This ligand has also been shown to retain ERα expression by activating the PI3K/AKT/mTORC1 pathway (432). However, ER phosphorylation at S118 was also detected in non-steroidal AI-resistant cell lines and in a model of long-term estrogen deprivation (220). HER2 signaling was detected as a mechanism of adaptation to estrogen-depleted conditions in AI-resistant breast cancer cell lines (141, 220, 433-443). Moreover, HER2 signaling, through MAPK/ERK pathway, has been associated not only with ER phosphorylation, but also with ER downregulation (439, 444-448). Strikingly, blocking HER2 or MAPK signaling restored the ER$^+$ phenotype and sensitivity to anti-

estrogen therapy (439, 440, 447, 449, 450). Similar to the previous work of *Masri et al* (2008), a study conducted by *Hole et al* (2015), has generated AI-resistant cell lines from the parental AI-sensitive MCF-7 cells (220, 437). This work reported an overexpression of GFRs of the ErbB family when compared to the parental cell line, and, although it did not evaluate ER phosphorylation at S118, different resistant colonies showed variations in the level of these receptors (437). A different study, using a xenograft model, reported HER2 overexpression and decreased ER and aromatase levels as adaptative changes to AIs (447). The association between AIs therapy and increased EGFR was also observed in patients (451). Thus, persistent HER2 signaling may lead to ER downregulation bypassing the benefic effects of the use of SERDs (437, 452, 453).

Phosphorylation or modulation of ER levels are not the only crosstalk mechanisms between the ErbB family and ER. The observation that ER knockdown led to reduced GFRs signaling increased the interest in ER and its variants localized in the plasma membrane or cytosol (168-170, 229, 239, 454-456). These variants have also been shown to elicit MAPK and AKT signaling (168-170, 238, 456). In addition, GPER1 was also associated with EGFR activation, as previously mentioned (240, 241), and with Tamoxifen-resistance (255).

The switch between ER and HER2 as the preferred signaling mechanism confirms the high plasticity of breast cancer cells. HER2 can enhance ER transcriptional activity or repress ER transcription. Nevertheless, in both cases, HER2 blockade restores the sensitivity to anti-estrogen therapy. The pre-clinical data that showed that HER2, a marker of anti-estrogen resistance, leads to tumor proliferation in the absence of ER activity encouraged the study of the combination of HER2 antagonists and AIs in clinical trials. The TAnDEM clinical trial (NCT00022672) reported a benefit on progression-free survival (PFS) for the combination of the anti-HER2 monoclonal antibody, Trastuzumab, with Anastrozole when compared to Anastrozole alone, in postmenopausal patients with advanced breast cancer (ABC) treated with endocrine therapy (457). After pre-clinical studies have demonstrated the potential benefit of dual HER2 inhibition, with Lapatinib and Trastuzumab (458), a clinical trial (ALTERNATIVE) assessed this approach by combining Lapatinib plus Trastuzumab and an AI (NCT01160211) in postmenopausal women with ABC previously treated with AIs or Trastuzumab. Improved PFS was observed for this tri-combination when compared to Lapatinib plus AI or Trastuzumab plus AI (459). The results from these clinical trials led to the approval of the combination of AIs and anti-HER2 agents for highly selected patients, namely those with contraindications to chemotherapy, with strong ER expression, or with a long disease-free interval (295, 296). Currently, several clinical trials are still ongoing (Table 1).

6.2.2 Insulin-like growth factor-1 receptors

The Insulin-like growth factor-1 receptor (IGF-IR) is a transmembrane tyrosine kinase activated by insulin-like growth factor-I (IGF-I) and -II (IGF-II). The activation of this receptor leads to phosphorylation and activation of insulin receptor substrate-1 (IRS-1) and -2 (IRS-2), which act as scaffolds, promoting the activation of PI3K/AKT and MAPK/ERK signaling pathways (460). Thus, the IGF-IR is associated with proliferation and cell survival and with poor clinical outcomes (448, 461-467). Nevertheless, some studies described a differential prognostic value according to the molecular subtype (468-470). In addition, high levels of IGF-I in plasma are correlated with increased risk (471-473) and incidence of recurrences in ER^+ breast cancer (474). In fact, a crosstalk between IGF-IR and ER has been depicted in pre-clinical models, demonstrating that IGF-IR enhanced ER transcriptional activity and is necessary for maximal activation. In addition, ER promotes IGF-IR signaling. Thus, inhibition of both receptors induced synergistic effects in estrogen-sensitive breast cancer (369, 475-479). Furthermore, IGF-IR has also been described as a mediator of membrane localization of ER (434). Given the apparent regulation of ER activity by IGF-IR, the role of this receptor in acquired resistance to estrogen deprivation, such as AIs, in ER^+ breast cancer cells have also been confirmed (447, 448, 463, 480-483). Some studies claim that IGF-IR inhibition, *per se*, was enough to inhibit the growth of resistant cells, though targeting downstream kinases, as AKT, or adding ICI 182,780, seems to be more effective (448, 463, 480-482, 484). In fact, a study by *Staka et al* (2005) confirmed the importance of inhibiting MAPK and PI3K pathways and reported that ER S167 phosphorylation is the main responsible for resistance to estrogen deprivation (484). The differences in sensitivity to IGF-IR inhibition were also addressed in a study by *Becker et al* (2012). In this report, the elevated levels of insulin-like growth factor binding protein 5 (IGFBP-5) were considered a marker of sensitivity to anti-IGF-IR treatment in breast cancer (485).

The results obtained in the clinical trials with the combination of IGF-IR blockade and AIs were not convincing so far (Table 1). The clinical trial NCT00626106 showed that the combination of Ganitumab, a monoclonal antibody that prevents activation of IGF-IR, with Exemestane did not improve PFS when compared to Exemestane alone in postmenopausal women with ER^+ locally advanced or metastatic breast cancer, previously, treated with endocrine therapy (486). Furthermore, another clinical trial (NCT01205685) that combined OSI-906 (IGF-IR inhibitor) plus Erlotinib (EGFR inhibitor) and Letrozole was not concluded due to severe toxicity and tumor progression. In addition, the combination of Ridaforolimus, a mTORC1 inhibitor, with Exemestane and Dalotuzumab, an IGF-IR blocker, (NCT01605396) did not improve PFS when compared to the Ridaforolimus and Exemestane in postmenopausal women with $ER^+/HER2^-$ locally advanced or metastatic

breast cancer that progressed on non-steroidal AIs (487). So far, there is no approved combination of IGF-IR blockers for luminal breast cancers.

6.2.3 Fibroblast growth factor receptors

The Fibroblast growth factor receptors (FGFRs), once triggered by the ligand, activate PI3K/AKT, MAPK/ERK, and STATs pathways (488, 489). FGFR signaling can be deregulated in breast cancer through several ways, such as amplification, mutations, and translocations. Amplification of *FGFR1* (most frequent) and *FGFR2* genes are observed in 10-15% of tumors promoting anti-estrogen therapy resistance and invasive phenotype (489-502). In addition, several genes that code for fibroblast growth factors (FGFs) are also amplified in breast cancer (503, 504).

Metastatic ER$^+$ breast cancers exhibit enrichment in the *FGFR4* gene, though alterations in this gene are rare in primary tumors (440, 493). FGFR has been associated with ER to promote ligand-independent activity in estrogen-free conditions (505). In fact, ER activation promotes the transcription of FGF ligands (506). Another study demonstrated that FGFR signaling promotes *ESR1* suppression and estrogen-independent tumor growth (507). Cancer-associated fibroblasts (CAFs) also seem to influence the response to anti-estrogen therapy by releasing FGFs that, in a paracrine manner, promote the development of resistance in breast cancer cells (508-511). In accordance with this data, targeting the FGFR inhibits the proliferation of therapy-resistant breast cancer cells (509, 512).

Clinical studies have identified *FGFRs* aberrations as markers of poor prognosis in ER$^+$ breast carcinomas (492, 513-515). Dovitinib, a non-specific FGFR1-3 inhibitor, demonstrated anti-tumor efficacy in a clinical trial (NCT00958971) with women with ABC and pre-treated by chemotherapy and endocrine therapy (516). Moreover, this inhibitor was being tested in combination with ICI 182,780, in a clinical trial (NCT01528345), in postmenopausal patients with ER$^+$/HER2$^-$ breast cancer that progressed during or after prior endocrine therapy. In this study, this combination increased PFS when compared to ICI 182,780 alone, however, it was terminated due to slow and low enrollment (517). The combination of non-steroidal AIs with the selective pan-FGFR inhibitor, AZD4547, was assessed by the RADICAL clinical trial (NCT01791985) in ER$^+$ breast cancer patients previously treated with AIs. This trial showed safety and anti-tumor efficacy for this combination (518). Nevertheless, the clinical use of FGFR inhibitors is not yet approved (Table 1).

Table 1. Summary of the main clinical trials assessing AI-resistance (part I).

Identifier	Phase	Therapy	Ref
Anti- EGFRs			
NCT00022672 (TAnDEM)	III	Trastuzumab + Anastrozole	(457)
NCT01160211 (ALTERNATIVE)	III	Lapatinib + Trastuzumab + AI	(459)
NCT00066339	II	Gefitinib	
NCT00049062	II	Gefinitib + Anastrozole	
NCT00688194	III	ICI 182,780+ Lapatinib + AI	
NCT02394496 (OVER)	III	ICI 182,780 + Lapatinib + AI	
NCT02530411 (FURVA)	II	ICI 182,780 + Vandetanib	
Anti-IGF-IRs			
NCT00626106 (QUILT-2.015)	II	Exemestane/ICI 182,780+ Ganitumab	(486)
NCT01605396	II	Ridaforolimus + Dalotuzumab + Exemestane	(487)
NCT03659136 (XENERA-1)	II	Xentuzumab+ Everolimus + Exemestane	
NCT02123823	I	BI836845 + Everolimus + Exemestane	
Anti-FGFR			
NCT00958971	II	Dovitinib	(516)
NCT01528345	II	Dovitinib + ICI 182,780	(517)
NCT01791985 (RADICAL)	I/II	AZD4547 + non-steroidal AI	(518)

6.2.4 PI3K/AKT/mTORC1 pathway

The PI3K/AKT/mTORC1 pathway is the convergence site of many receptor tyrosine kinases, being responsible for the effects of the activation of those receptors. The hyperactivation of this pathway, either by GFRs or mutations, is a mechanism that confers resistance to anti-estrogen therapies (260, 448, 480, 519). Mutations in *PIK3CA* differentially affect sensitivity to inhibitors of the PI3K/AKT/mTORC1 pathway (520). As previously established, this pathway phosphorylates ER at S167, increasing the activity of the receptor and decreasing the expression of ER during sustained hyperactivation (143, 521, 522). Activation of the PI3K pathway was shown to decrease ER levels by inhibiting the translocation to the nucleus of FOXO3A, repressing, in that way, ER expression (213, 252, 453, 523-526). The deregulation of the PI3K/AKT/mTORC1 pathway and/or upstream receptor tyrosine kinases has a direct impact on the clinical outcome. In addition, ER can also upregulate this pathway (432, 527-529). Resistance to PI3K inhibitors was shown to be mediated by an increase in ER activity in ER$^+$ *PIK3CA*-mutated breast cancer cells (530-532) and by the IGF-IR/p110β/AKT/mTORC1 pathway (533, 534), which can be a consequence of ER activity. This resistance was reverted by a combination with anti-estrogens, ICI 182,780 and 4-OH-tam (530), or by impairing ER transcriptional activity (531).

PTEN is a negative regulator of this pathway through its phosphatase activity. In breast cancer, PTEN may, among other mechanisms, be downregulated through mutations, loss of heterozygosity, and methylation (535, 536). Nevertheless, mutations on the PI3K class I gene are the most frequent in ER^+ breast cancers (440, 537-539). PI3K class I is composed of two subunits, one regulatory (p85) and one catalytic (p110). The gene that encodes the p110α catalytic subunit, *PIK3CA*, is the most frequently altered gene in luminal tumors (109). The more frequent non-exclusive mutations on this gene are E542K, E545K (helical domains), and H1047R (kinase domain) (109, 540-542). Mutations in the helical domain decrease the inhibition by p85, while mutations in the kinase domain increase its interaction with lipid membranes (536). The catalytic subunit of PI3K also has other isoforms, β, γ, and δ, that do not present mutations with the same frequency as the α subunit (536). Several pre-clinical studies have demonstrated that direct targeting of PI3K reverses the resistant phenotype (466, 543-547). AKT inhibitors, such as the allosteric antagonist MK-2206, also display the ability to reverse resistance to anti-estrogen therapy (548-550). However, pharmacological inhibition of AKT seems to release the GFRs from negative feedback, leading to their activation and counterbalancing the desired effects of AKT inhibition (551). Targeting mTORC1, another member of this pathway, through mTORC1 inhibitors, such as Everolimus, proved to be effective in *in vitro* studies (544, 552, 553). In addition, dual PI3K/mTORC1 inhibitors, such as BEZ235 and BGT226, were developed and demonstrated to be more potent than just single inhibition (448, 544, 554).

Clinical trials were performed to validate the association and potential benefit of inhibiting this pathway in endocrine resistant ER^+ breast cancers (Table 2). The FERGI trial (NCT01437566), composed of postmenopausal women with $ER^+/HER2^-$ locally advanced or metastatic breast cancer that progressed during or after AI therapy, demonstrated that the combination of the pan-PI3K class I inhibitor, Pictilisib, with ICI 182,780 did not significantly improve PFS and was associated with increased toxicity, when compared to ICI 182,780 alone (555). A similar trial, the BELLE-2 (NCT01610284), composed of postmenopausal women that progressed during or after AI therapy, studied the combination of another pan-PI3K class I inhibitor, Buparlisib, with ICI 182,780, in comparison with ICI 182,780 alone. This trial reports an increase in PFS however, severe toxicity was also observed, and further studies were discontinued (556). Due to the severe toxicity associated with the inhibition of all isoforms of PI3K class I, such as hepatotoxicity, hyperglycemia, and mood disorders, research on these inhibitors was cancelled (557). The opportunity for the development of isoform-specific PI3K class I inhibitors led to the development of Alpelisib (BYL-719), MLN1117, and Taselisib. The SOLAR-1 (NCT02437318) clinical trial, with men and postmenopausal women with $ER^+/HER2^-$ ABC, which progressed on or after AIs treatment, combined BYL-719 and ICI 182,780 and

compared the results with ICI 182,780 alone. This combination prolonged PFS, especially in patients with mutated *PIK3CA* (558). In addition, a study combined BYL-719 with Letrozole or Exemestane (NCT01870505), which showed anti-tumor efficacy and led to dose de-escalation in ABC that progressed on Letrozole or Exemestane (559). In sequence to this study, BYL-719 was also explored in combination with Letrozole (NCT01791478) in postmenopausal women with $ER^+/HER2^-$ ABC that progressed on endocrine therapies in another study. The results from this trial demonstrated the efficacy of this combination with reversible toxicity (560). The SANDPIPER clinical trial (NCT02340221), which recruited postmenopausal patients with $ER^+/HER2^-$ locally advanced or metastatic breast cancer that progressed on AIs, showed that the combination of Taselisib with ICI 182,780 prolonged PFS when compared to ICI 182,780 alone (561).

The promising results reported by the SOLAR-1 trial led to the recent approval of the combination of BYL-719 with ICI 182,780 by the FDA, for postmenopausal women and men, with $ER^+/HER2^-$ and *PIK3CA*-mutated breast cancer, following AIs therapy (296). The combination of MK-2206 with Anastrozole or ICI 182,780 was also studied in a phase I trial (NCT01344031) in postmenopausal patients with ER^+ ABC that progressed on several lines of endocrine therapy. However, it only showed moderate efficacy for the combinations (550). Further studies with MK-2206 were terminated (NCT01240941, NCT01240928). The FAKTION phase II trial (NCT01992952) assessed the effectiveness of the combination between the AKT inhibitor, Capivasertib, with ICI 182,780 in postmenopausal women with $ER^+/HER2^-$ ABC after relapse or disease progression on an AI. This trial has reported a prolonged PFS and improved overall survival (OS) (562).

The BOLERO-2 trial (NCT00863655) studied the combination of the mTORC1 inhibitor, Everolimus, with Exemestane in postmenopausal patients with ER^+ ABC, which progressed on non-steroidal AIs. This study demonstrated that the combination prolonged PFS when compared to Exemestane alone (563, 564), although no differences were found in the OS (565). This may be attributed to the same feedback loop verified for AKT inhibition with GFRs, which, paradoxically, led to an increase in AKT activation (566). In addition, autophagy was suggested to be the cause of Everolimus insensitivity (567). The combination of Everolimus with Letrozole (NCT01231659) also demonstrated clinical benefit (568). The exciting results reported by the BOLERO-2 trial led to the FDA approval and guideline implementation of the combination of Everolimus and an AI in naïve ABC or previously exposed to endocrine therapy, despite the lack of OS (295, 296).

Table 2. Summary of the main clinical trials assessing AIs-resistance (part II).

Identifier	Phase	Therapy	Ref
Anti-PI3K/AKT/mTORC1			
NCT01437566 (FERGI)	II	Pictilib + ICI 182,780	(555)
NCT01610284 (BELLE-2)	III	Buparlisib + ICI 182,780	(556)
NCT02437318 (SOLAR-1)	III	BYL-719 + ICI 182,780	(558)
NCT01870505	I	BYL-719 + Letrozole/Exemestane	(559)
NCT01791478	I	BYL-719 + Letrozole	(560)
NCT02340221 (SANDPIPER)	III	Taselisib + ICI 182,780	(561)
NCT02404844 (PIKTAM)	II	Buparlisib + Tamoxifen	
NCT03056755 (BYLieve)	II	BYL-719 + ICI 182,780 /Letrozole	
NCT03939897	I/II	Copanlisib + Abemaciclib + ICI 182,780	
NCT03803761	I/II	Copanlisib + ICI 182,780	
NCT01082068	I/II	XL147/XL765 + Letrozole	
NCT01344031	I	MK2206 + Anastrozole/ICI 182,780	(550)
NCT01992952 (FAKTION)	I/II	Capivasertib + ICI 182,780	(562)
NCT03959891 (TAKTIC)	I	Ipatasertib + Letrozole/ICI 182,780	
NCT00863655 (BOLERO-2)	III	Everolimus + Exemestane	(563-565)
NCT01231659	II	Everolimus + Letrozole	(568)
NCT01298713 (TAMRAD)	II	Everolimus + Tamoxifen	(569)
NCT00570921 (BRE-43)	II	Everolimus + ICI 182,780	(570)
NCT02732119 (TRINITI-1)	I/II	Everolimus + Exemestane + Ribociclib	(571)
NCT02216786 (MANTA)	II	AZD-2014 + ICI 182,780	(572)
NCT01248494	I	BEZ235/Buparlisib + Letrozole	
Anti-MAPK/ERK			
NCT02322853 (OLYMPE)	II	Ralimetinib + Tamoxifen	(573)
NCT01160718	II	Selumetinib + ICI 182,780	(574)

The TAMRAD trial (NCT01298713) assessed the combination of Everolimus with Tamoxifen versus Tamoxifen alone in postmenopausal patients with $ER^+/HER2^-$ ABC previously treated with AIs. This trial demonstrated that the combination of these two drugs resulted in improved clinical benefit rate, time-to-progression, and OS when compared to Tamoxifen alone. Interestingly, this study also reported that patients that relapsed after 6 months of AIs treatment experienced better outcomes than those who relapsed before 6 months of AIs (569). Everolimus has also been combined with ICI 182,780 (NCT00570921) and results showed that this combination improves the effects of ICI 182,780 in postmenopausal women with $ER^+/HER2^-$ ABC previously treated with AIs or with other anti-estrogen therapy (570). Furthermore, the addition of Everolimus to the current endocrine therapy in which the patient has progressed also revealed an increase in the PFS

when compared to only endocrine therapy in postmenopausal women with locally advanced or metastatic ER$^+$/HER2$^-$ breast cancer (575). More recently, the TRINITI-1 trial (NCT02732119) has reported clinical efficacy, with manageable adverse effects, for the triple combination of Everolimus, Exemestane, and the CDK 4/6 inhibitor, Ribociclib, in postmenopausal women and adult men with ER$^+$/HER2$^-$ locally advanced and metastatic breast cancer that progressed on anti-estrogen therapy and on CDK 4/6 inhibitors (571). The MANTA clinical trial (NCT02216786) studied the combination of a mTORC1 inhibitor, AZD2014, with ICI 182,780 versus ICI 182,780 alone or versus ICI 182,780 and Everolimus. This study found that the combination of AZD2014 with ICI 182,780 was not superior to Everolimus and ICI 182,780 in postmenopausal women with ER$^+$/HER2$^-$ ABC refractory to AIs (572).

6.2.5 MAPK/ERK pathway

In a similar way to the PI3K/AKT/mTORC1 pathway, the MAPK/ERK pathway is also on the crossroad of many GFRs, being involved in ER ligand-independent activation, as previously mentioned. The mutations of the genes involved in this pathway, such as the loss-of-function mutation on the *NF1* gene, a negative regulator, are enriched in ER$^+$ metastatic breast cancer when compared to primary tumors. Moreover, mutations in ER, ERBB2 and NF1 seem to be mutually exclusive (440, 576). The activation of the MAPK pathway has been shown also to promote the transcriptional activity of the ER co-activator AIB1, which has been found to be correlated with recurrence in patients treated with AIs (577, 578). Since the hyperactivation of this pathway is intimately related to anti-estrogen therapy resistance, its inhibition has been vastly investigated in resistant breast cancer cells (219, 436, 439, 440, 449, 466, 480, 579-582). *Brodie et al* (2005) demonstrated that the MEK inhibitors, PD98059 and U0126, restored hormone sensitivity in Letrozole-resistant breast cancer cells (436, 580, 581, 583). The inhibitor, U0126, also decreased the proliferation of long-term estrogen deprived breast cancer cells (219, 466).

Several clinical trials verifying the efficacy of the inhibition of the MAPK/ERK pathway were conducted in the last years (Table 2). A phase I trial that studied the combination of Ralimetinib, a p38 MAPK inhibitor, with Tamoxifen, in postmenopausal patients with ER$^+$ metastatic breast cancer refractory to AIs, reported promising results in terms of tolerability and safety (573). Nevertheless, the phase II trial was terminated due to a lack of recruitment (NCT02322853). A different trial (NCT01160718) combining the MEK 1/2 inhibitor, Selumetinib, with ICI 182,780 did not verify better patient outcomes when compared to ICI 182,780 alone in postmenopausal women with ER$^+$ ABC refractory to AIs (574). In addition, the combination was poorly tolerated. Despite, the good pre-clinical indicators of targeting this pathway, clinical trials have revealed drawbacks for this approach.

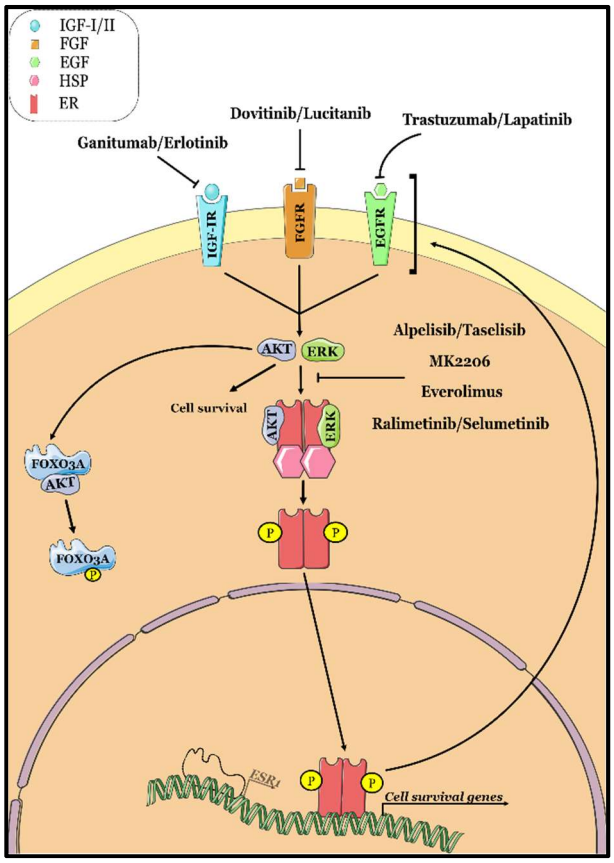

Figure 12. Drugs being studied to overcome aberrant GFRs signaling and its crosstalk with ER. Several GFRs pathways phosphorylate and activate ER in estrogen-depleted conditions. The efficacy of several drugs has been studied, alone or in combinations, to bypass AI-acquired resistance. Aberrant and persistent AKT signaling leads to ER downregulation, completely abrogating the need of ER for cell survival. ER – Estrogen receptor; EGFR – Epidermal growth factor receptor; FGFR – Fibroblast growth factor receptor; HSP – Heat shock protein; IGF-IR – Insulin-like growth factor receptor I.

6.3 Cell cycle regulators

The role of cell cycle regulators in breast oncogenesis has been thoroughly studied, and the frequency of mutations seems to be subtype-specific (109, 584, 585). These mutations include amplification of the oncogene *CCND1* (cyclin D1) (109, 586), a gain of CDK4, and loss-of-function mutations on the tumor supressors *CDKN1B* (p27), *CDKN2A* (p16), and *CDKN2C* (p18) (109, 538, 587). Moreover, these cell cycle regulators can modulate the sensitivity to anti-estrogen therapy since several GFRs pathways converge on these molecules, such as cyclin D1 and c-MYC, independently of ER status (502, 519, 588-592). Nevertheless, ER can be directly activated by cyclin D1 independently of the formation of the CDK-Cyclin complex (593). In addition, the cytosolic presence of cyclin E has been shown to promote resistance to Letrozole and was associated with lower disease-free intervals (594). Another cell cycle regulators, aurora kinases A and B, were found to be upregulated in models of AI-resistance. The inhibition of these kinases, alone or in combination with ICI 182,780, was sufficient to inhibit cell growth (595). The concerted role of cyclin D1 and CDK 4/6 on the promotion of cell cycle progression, through

retinoblastoma phosphorylation and derepression of E2F signaling, together with the hormone-independent ER-mediated transcription of E2F, promotes resistance to AIs (519). In addition, CDK 4/6 inhibition was effective in ER^+ tumors and capable of inhibiting the growth of anti-estrogen-resistant cells (589, 596). These findings prompted the clinical studies of CDK 4/6 inhibitors, such as Palbociclib, Ribociclib and Abemaciclib, in advanced breast tumors. Nevertheless, resistance to CDK 4/6 inhibitors might be mediated by retinoblastoma or PTEN loss (597). The latter may lead to cross resistance to CDK 4/6 inhibitors and to PI3K inhibitors, by delocalizing p27, a cyclin-dependent kinase inhibitor, out of the nucleus, enhancing the activity of CDK 4, and increasing PI3K signaling respectively.

In the PALOMA-1/TRIO-18 phase II trial (NCT00721409) the combination of Palbociclib and Letrozole significantly prolonged PFS, when compared to Letrozole alone, and induced manageable adverse effects in postmenopausal patients with $ER^+/HER2^-$ ABC without prior treatment for advanced disease (598, 599). These results were further confirmed in the phase III PALOMA-2 trial (NCT01740427) for postmenopausal women without previous exposure to endocrine therapy in the advanced setting. The benefits of this combination were still observed in extended follow-ups, being the use of this approach defended as first-line therapy (600, 601). The PALOMA-3 trial (NCT01942135) explored the effects of the combination of Palbociclib and ICI 182,780 in patients with $ER^+/HER2^-$ locally advanced or metastatic breast cancer that progressed on endocrine therapy. The combination prolonged PFS and increased OS when compared to ICI 182,780 alone (602, 603). Currently, the PALOMA-4 trial (NCT02297438) further supports the use of Palbociclib and Letrozole as first-line treatment of $ER^+/HER2^-$ ABC. The clinical trial (NCT02871791), testing the addition of Palbociclib to the combination of Everolimus and Exemestane in patients with $ER^+/HER2^-$ metastatic breast cancer that progressed on non-steroidal AIs and CDK4/6 inhibitors, demonstrated a lack of benefit and an increase in adverse effects of the triple combination when compared to Everolimus plus Exemestane (604). The benefit of the combination of Palbociclib and endocrine therapy is now being explored in early breast cancer as first-line adjuvant or neoadjuvant options due to the results in the advanced setting (NCT02040857, NCT03969121, NCT03628066, NCT02513394, NCT04075604, NCT03819010, NCT02296801) (605).

Due to the success of Palbociclib in the clinic, other CDK 4/6 inhibitors were also tested in clinical trials (Table 3). Ribociclib was explored in combination with anti-estrogens for advanced breast cancer. The MONALEESA-2 trial (NCT01958021) combined Ribociclib with Letrozole in postmenopausal women with $ER^+/HER2^-$ locally advanced or metastatic breast cancer without prior exposure to endocrine therapy in the advanced setting or with

prior exposure to non-steroidal AIs in the neo (adjuvant) setting. The results demonstrated that this combination, similarly to the PALOMA trials, also prolonged PFS, with manageable toxicity, when compared to Letrozole alone (606). Follow-up studies reinforce the benefit of this therapy (607). Following this, the MONALEESA-3 phase III trial (NCT02422615) studied Ribociclib with ICI 182,780 in postmenopausal women and men with $ER^+/HER2^-$ locally advanced or metastatic breast as first or second line treatment. The combination extended PFS and increase OS when compared to ICI 182,780 alone (608, 609). Furthermore, the combination of Ribociclib and anti-estrogen has been expanded to early breast cancer (NCT03078751, NCT02712723, NCT03701334, NCT03285412).

Abemaciclib is another CDK 4/6 inhibitor explored in clinical practice. Abemaciclib is characterized by its higher affinity for CDK 4 than for CDK 6 (610). The clinical efficacy verified in the MONARCH1 trial (NCT02102490) for Abemaciclib alone, in pretreated postmenopausal women with $ER^+/HER2^-$ metastatic breast cancer, led to combinatorial studies with endocrine therapy (611). The MONARCH2 (NCT02107703) combined Abemaciclib with ICI 182,780 in postmenopausal women with $ER^+/HER2^-$ ABC and reported extended PFS and manageable toxicities (612). In addition, the MONARCH plus trial (NCT02057133) combined Abemaciclib with ICI 182,780 or non-steroidal AIs in patients that progressed on endocrine therapy and observed improved PFS when compared to anti-estrogens alone (613). However, the Next MONARCH1 trial (NCT02747004), testing the combination of Tamoxifen and Abemaciclib, in patients that progressed on endocrine therapy, did not observed an improve in PFS for this combination when compared to Abemaciclib monotherapy (614). The combination of Abemaciclib with anti-estrogens in early breast cancer is also being investigated as (neo) adjuvant treatment (NCT03155997, NCT02441946, NCT04305236).

The promising results from the PALOMA/MONALEESA/MONARCH trials led to the approval by the FDA of the combination of CDK 4/6 inhibitors with AIs or ICI 182,780 as a first- and second-line treatment for ER^+ ABC in premenopausal and postmenopausal women and men (295, 296). Nevertheless, the development of resistance to CDK 4/6 inhibitors is becoming a huge concern. In these cases, the addition of a PI3K or mTORC1 inhibitor might overcome CDK 4/6 inhibitors resistance and extend the benefits of this therapy (615, 616). However, the efficacy of the use of the PI3K inhibitor, BYL-719, in breast cancer patients previously exposed to CDK 4/6 inhibitors is still unknown since only 6% of the patients enrolled in the SOLAR-1 clinical trial had been previously treated with those agents (558). For this reason, the current guidelines do not yet support this approach (296).

6.4 Androgens and the androgen receptor

Androgen receptor (AR) is a steroid receptor commonly expressed in luminal subtypes and in 77% of invasive breast cancers (617, 618). AR is structurally similar to ER and can be recruited to ERE, while ER can also be recruited to androgen-responsive elements (ARE) (549, 619). In fact, it was demonstrated that E2 has the ability to recruit the AR in order to cooperate and promote ER signaling, driving tumor growth (620). Nevertheless, androgens have opposite effects to estrogens in sensitive breast cancer cells, leading to inhibition of tumor growth when estrogen levels are low due to aromatase inhibition (619, 621, 622). These inhibitory effects may occur through competitive binding of AR to ERE (619), direct inhibition of ER activity due to AR overexpression (623), inhibition of cyclin D1 expression (624, 625) and/or upregulation of ERβ (626). Thus, AIs might hinder the growth of breast cancer cells not only by blocking ER but, also, by promoting AR signaling (627). In fact, the presence of AR in ER^+ tumors is considered a marker of good prognosis, while in ER^- tumors is correlated with a poor prognosis (628, 629). Moreover, the absence of AR in ER^+ tumors is associated with AIs or Tamoxifen treatment failure.

It has been described that Letrozole exerts its anti-tumoral effects through androgens via AR modulation (622). In this study, the addition of the AR antagonist, Casodex (CDX), abrogated Letrozole-induced anti-proliferative effects in the MCF-7 cells. Contrariwise, AR was shown to have a pro-survival role in Exemestane-treated MCF-7aro cells and the addition of CDX exacerbated the anti-proliferative effects of Exemestane (389). On the other hand, the Exemestane metabolite, 17*β*-HE, presents androgenic activity (625), which may be one of the reasons for the lack of cross-resistance between non-steroidal or steroidal AIs, as well as the different biological function of AR in cells treated with Letrozole or Exemestane.

In cells resistant to AIs, AR seems to shift to a cell protective role, changing its transcriptome in response to the altered endocrine milieu (630-633). A different study reported a cooperation between AR and ER, with AR enhancing the transcriptional activity of ER, which led to resistance to Anastrozole (549). In this study, the blockade of AR, IGF-IR and of AKT signaling recovered the sensitivity to Anastrozole. Our group has recently demonstrated that Exemestane increases the expression and activation of AR, which was associated with Exemestane-resistance due to its pro-survival role. In fact, the combination of Exemestane and CDX recovered the response of the resistant cells to Exemestane and decreased the levels of ERα (389). This has also been associated with Tamoxifen-resistance (634). In addition, in AI-resistant cells that lost ER expression, AR has a tumor promoter function (632). On the other hand, the androgen metabolite, 3β-diol, has an estrogenic function in estrogen-deprived cells, maintaining ER signaling in conjunction with AR

downregulation (635, 636). However, in estrogen-rich environments, this metabolite inhibits the growth of breast cancer cells by binding to ERβ (637). Nevertheless, a recent study has attributed the endocrine therapy-resistance induced by AR to non-canonical signaling (638), and AR overexpression has also been implicated in resistance to Palbociclib (639).

Several clinical trials have assessed AR antagonism or anti-androgens in patients with resistance to endocrine therapy (Table 3). A phase II clinical trial (NCT02007512) is studying the effects of the combination of the AR antagonist, Enzalutamide, with Exemestane in ER^+ advanced breast cancer previously treated with endocrine therapy. In this study, there was an extension in PFS for the cohort of patients without prior exposure to endocrine therapy when compared to Exemestane alone (640). In addition, CDX was tested in combination with AIs in postmenopausal patients with $ER^+/HER2^-$ ABC refractory to AIs (NCT02910050), however, no clinical benefit was reported (641). Another trial studied Abiraterone acetate, an inhibitor of the enzyme responsible for androgen synthesis, P450C17, in combination with Exemestane and supplementation with Prednisone (NCT01381874). The results demonstrated that this combination failed to improve PFS when compared to Exemestane alone, in postmenopausal women with $ER^+/HER2^-$ metastatic breast cancer refractory to non-steroidal AI (642). Furthermore, the study of the effects of combining AR antagonists and anti-estrogens is being expanded to early breast cancer (NCT02676986, NCT02955394).

6.5 Aberrant histone deacetylase activity

Histone deacetylases (HDACs) modulate chromatin structure and gene expression, therefore, they have a central role in breast cancer progression. Aberrant expression or activity of these enzymes leads to histone hypoacetylation, which has been associated with resistance (643-646). HDAC-1 has been associated with ER^+ breast tumors (647). Studies conducted in AI-resistant breast cancer cells demonstrated that HDAC inhibition was enough to restore sensitivity to endocrine therapy through modulation of HER2, MAPK, AKT and NFkB expression/activities, cell cycle arrest, and apoptosis induction (644, 645). Moreover, HDAC inhibition was shown to recover ER expression in ER^- tumors, shifting the dependence on GFRs to ER (648-651), to induce p21 expression (652), and to block c-MYC signaling (653).

Table 3. Summary of the main clinical trials assessing AI-resistance (part III).

Identifier	Phase	Therapy	Ref
Anti-cell cycle regulators			
NCT01942135 (PALOMA-3)	III	Palbociclib + ICI 182,780	(602, 603)
NCT02738866	II	Palbociclib + ICI 182,780	
NCT02536742	II	Palbociclib + ICI 182,780	
NCT03471663	I	D-0502 + Palbociclib	
NCT03284957	I/II	SAR439859 ± Palbociclib/BYL-719	
NCT03332797	I	GDC-9545 + Palbociclib	(654)
NCT03616587 (SERENA-1)	I	AZD9833 ± Palbociclib/Everolimus	(655)
NCT04214288 (SERENA-2)	II	AZD9833 ± Everolimus	
NCT02913430	I	Palbociclib + ICI 182,780/Tamoxifen	
NCT03455270	I	Palbociclib + G1T48	
NCT04191499	II/III	Palbociclib + GDC-0077 + ICI 182,780	
NCT02599714 (PASTOR)	I	Palbociclib + ICI 182,780 + AZD2014	
NCT02684032	I	Palbociclib + Gedatolisib + Letrozole/ICI 182,780	
NCT02871791	I/II	Palbociclib + Everolimus + Exemestane	(604)
NCT03959891 (TAKTIC)	I	Palbociclib + Ipatasertib + ICI 182,780	(656)
NCT02422615 (MONALEESA-3)	III	Ribociclib + ICI 182,780	(608, 609)
NCT02586675	I	Ribociclib + Tamoxifen	
NCT02088684	I	Ribociclib + BYL-719/Buparlisib + ICI 182,780	
NCT02102490 (MONARCH1)	II	Abemaciclib	(611)
NCT02107703 (MONARCH2)	III	Abemaciclib + ICI 182,780	(612)
NCT02763566 (MONARCH plus)	III	Abemaciclib + ICI 182,780 /non-steroidal AI	(613)
NCT04188548 (EMBER)	I	Abemaciclib + LY3484356 + AI	
NCT02747004 (Next MONARCH1)	II	Abemaciclib + Tamoxifen	(614)
NCT02057133	I	Abemaciclib + Exemestane ± Everolimus Abemaciclib + LY3023414 + ICI 182,780	(657)
NCT04316169	I	Abemaciclib + ET + Hydroxychloroquine	
Anti-AR			
NCT02007512	II	Enzalutamide + Exemestane	(640)
NCT02910050 (BETTER)	II	CDX + AI	(641)
NCT01381874	II	Abiraterone acetate + Prednisone/Prednisolone + Exemestane	(642)
Anti-HDAC			
NCT00365599	II	Vorinostat + Tamoxifen	(658)
NCT00676663 (ENCORE301)	II	Entinostat + Exemestane	(659)
NCT02115282	III	Entinostat + Exemestane	(660)
NCT02482753	III	Tucidinostat + Exemestane	(661)

The pre-clinical results instigated clinical trials to assess the efficacy of HDAC inhibitors (Table 3). A phase II trial (NCT00365599) explored the combination of the HDAC inhibitor, Vorinostat, and Tamoxifen in women with ER^+ locally advanced or metastatic breast cancer refractory to anti-estrogens. This trial reported promising activity and tolerable toxicity (658). In the NCT00676663 trial, another HDAC inhibitor, Entinostat, was also studied in combination with Exemestane in postmenopausal women with ABC that progressed on AIs. The combination significantly extended PFS and increased OS when compared to Exemestane alone (659). The corresponding phase III trial (NCT02115282), however, did not reach improvement in PFS (660). Nevertheless, the results from the ACE trial (NCT02482753) demonstrated that the combination of the HDAC inhibitor, Tucidinostat, with Exemestane prolonged PFS when compared to Exemestane alone, but with more serious adverse effects in postmenopausal women with $ER^+/HER2^-$ locally advanced or metastatic breast cancer refractory to endocrine therapy (661).

6.6 Apoptosis, autophagy and cell homeostasis

The downplay of apoptosis is a known mechanism of resistance to anti-estrogen therapy. More specifically, the downregulation of pro-apoptotic molecules, such as Bad and Bax, and the upregulation of anti-apoptotic molecules, such as Bcl-2, Bcl-x, and Mcl-1 have been described (662-668). In addition, the downregulation of the apoptosis-induced tumor suppressor, programmed cell death 4 (PDCD4), was associated with AI-resistance and poor prognosis (669). Moreover, in response to AIs treatment, Mcl-1 upregulation was considered as a primary survival factor (670). Clinical trials assessing Bcl-2 inhibition in combination with anti-estrogens in postmenopausal patients with ER^+ locally advanced or metastatic breast cancer refractory to hormonal therapy are ongoing (NCT03584009, ACTRN12615000702516).

Autophagy is a catabolic process involved in cell homeostasis through the elimination of damaged organelles and proteins aggregates (671). PI3K is directly involved in autophagy regulation through PI3K class III or PI3K/AKT/mTORC1 pathway (672, 673). Furthermore, autophagy is also regulated by AMPK in response to low ATP levels (674). In cancer, the role of autophagy is controversial (675, 676), being described in the literature as having oncogenic (384, 543, 567, 664, 677-681) or tumor suppressor functions (666, 682-684). In fact, in breast cancer, autophagy has been associated with the regulation of senescence (685-687) and with resistance to anti-estrogen therapies, being, in some cases, induced by ERα inhibition (543, 664, 688-691). Autophagy inhibition, through pan-PI3K inhibitors or specific autophagic inhibitors, re-sensitized resistant breast cancer cells to Exemestane (543). Moreover, autophagy is also involved in resistance to new strategies designed to

circumvent anti-estrogen resistance (567, 680). In these studies, autophagy was induced by decreased signaling of the PI3K/AKT/mTORC1 pathway due to Everolimus or Pictilisib treatment and autophagy inhibition re-sensitized and potentiated therapy effects. Moreover, in Everolimus resistance c-MYC might mediate autophagy (692). In clinical samples, markers of autophagy, such as LC-3, were increased after adjuvant Exemestane treatment (693, 694). However, the same group reported that pretreatment levels of autophagic markers do not correlate with PFS or OS (694). In addition, autophagy also has an important role in breast cancer stem-like cells maintenance through IL-6 secretion (695, 696).

The unfolded protein response (UPR) dictates the switch between apoptosis and autophagy (697). The glucose-regulated protein (GRP78), an UPR regulator, is overexpressed in ER^+ resistant cells and contributes to the development of resistance to anti-estrogens by balancing pro-survival autophagy and apoptosis (681, 691, 698). The importance of endoplasmic reticulum homeostasis in AI-resistance was already demonstrated (116). In fact, the ER-dependent transcription of serum- and glucocorticoid-inducible kinase 3 (SGK3) prevented excessive endoplasmic reticulum stress through maintenance of calcium levels, which, consequently, prevented ER downregulation. In addition, the UPR is also regulated by c-MYC, through transcriptional activation of IRE1 and XBP1 (699). The latter may lead to anti-estrogen resistance by activating NFκB (700). The UPR is also upregulated in endocrine resistant ER-mutant cells, adding to the previously described c-MYC activation in these cells and linking c-MYC with UPR (701). However, sustained UPR signaling leads to cytotoxic instead of cytoprotective effects (702).

7. Compounds under study as potential AIs

Despite the efficacy of AIs in clinic, the development of acquired resistance and the increased risk of bone fractures reinforce the need to search for new potent molecules that can become valid alternatives while also presenting fewer side effects. Due to the elucidation of aromatase structure by *Ghosh et al* (2009), the interaction between the enzyme and androgens was better described (70, 72), which allowed for the refinement of the rationale behind the design of new AIs. This has led to the development of more specific and potent molecules (69, 703).

Our group has focused not only on the design and synthesis (97, 98, 102, 374, 704-706), but also on the biological evaluation (95, 96, 386, 677, 707, 708) of new A-, B-, C- or D-modified steroids derived from androstenedione (Figure 13). The structure-activity relationships (SAR) arising from these reports has deepened the knowledge about

aromatase activity and inhibition. The carbonyl group at C-17, the planarity at the A-ring and at the A,B-rings junction, achieved through the presence of a carbonyl group at C-3 or a double bond at C-1, and the introduction of suitable groups at C-6 or C-7 are pivotal features to obtain high anti-aromatase activity (98, 102, 704-706), both in human placental microsomes (97, 98, 374, 677, 704, 705, 707, 709) and in *in vitro* (95, 96, 386, 677, 708). Recently, it was described that functionalization on the C-6α confers better anti-aromatase activity than at C-7α, at the B-ring, and among the function groups tested (methyl, allyl, and hydroxyl), methyl groups grant the most potent activity (102). Moreover, in the same study the combination of the carbonyl at C-3, with double bonds at C-1 and C-4 was the optimal functionalization of the A-ring.

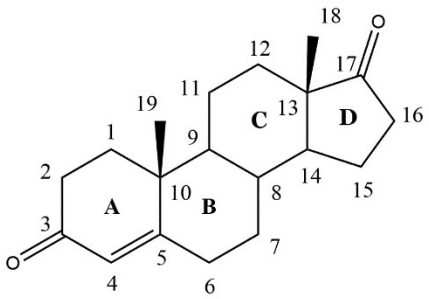

Figure 13. Chemical structure of androstenedione.

In order to elucidate the optimal features in the steroidal scaffold to achieve more specific and potent AIs, SAR studies were carried out with new steroid molecules with modifications in the A and B rings. The design and synthesis of the new steroidal compounds were performed by the Pharmaceutical Chemistry Unit of the Centre for Chemical Processes Engineering and Forest Products, University of Coimbra, which also attributed the code numbers to the compounds. The following compounds were studied: 6α-methyl-5α-androst-3-en-17-one (**1a**), 6α-methyl-3α,4α-epoxy-5α-androstan-17-one (**3a**), 6α-methylandrost-4-ene-3,17-dione (**9**), 6α-allylandrosta-1,4-diene-3,17-dione (**13**), 6α-allylandrost-4-ene-3,17-dione (**15**), 6α-allylandrost-4-en-17-one (**17**), 6β-hydroxyandrost-4-ene-3,17-dione (**19**) and 6α-hydroxyandrost-4-ene-3,17-dione (**20**) (Figure 14).

At the C-6 position, methyl (**9**), allyl (**15**), and hydroxyl groups (**19** and **20**) are directly compared, since these compounds present the same modification in the A-ring (carbonyl group at C-3 and double bond at C-4). In addition, the effects of the stereochemistry of the hydroxyl group were also studied (**19** vs **20**). The addition of an epoxide group in C-3, at the A-ring, was shown to confer planarity and higher anti-aromatase activity in human placental microsomes when compared to a double bond in C-6α-methyl substituted steroids (102). Thus, this comparison was performed in this study (**1a** vs **3a**). Moreover, the effects

of the addition of a double bond in C-1 in combination with a carbonyl group at C-3 and an allyl substitution at C-6α were evaluated (**13** vs **15**).

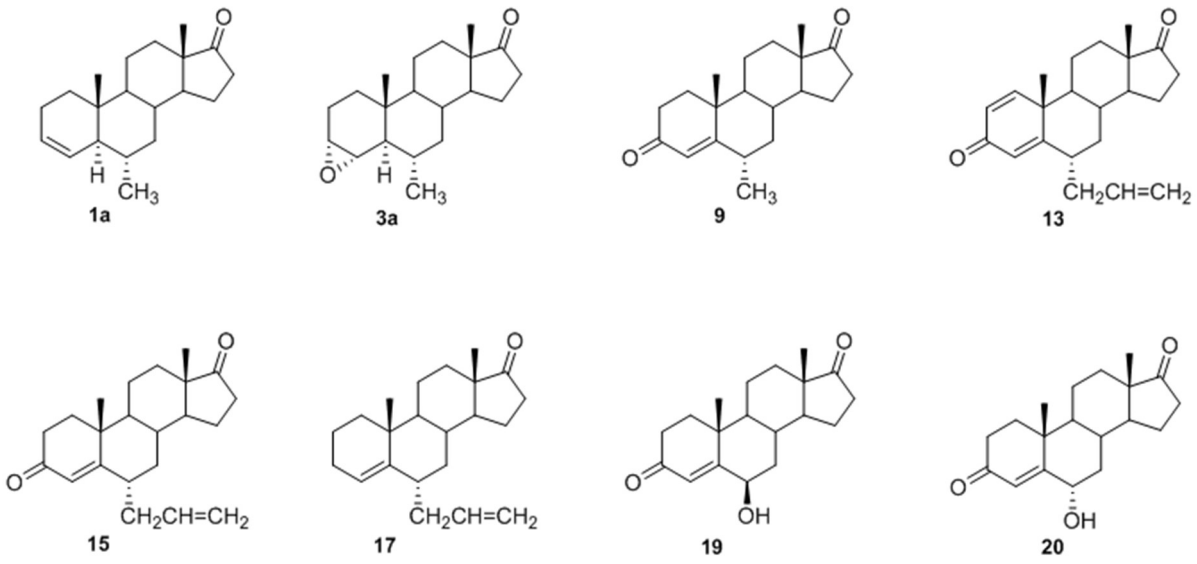

Figure 14. Chemical structures of compounds under study: 6α-methyl-5α-androst-3-en-17-one (**1a**), 6α-methyl-3α,4α-epoxy-5α-androstan-17-one (**3a**), 6α-methylandrost-4-ene-3,17-dione (**9**), 6α-allylandrosta-1,4-diene-3,17-dione (**13**), 6α-allylandrost-4-ene-3,17-dione (**15**), 6α-allylandrost-4-en-17-one (**17**), 6β-hydroxyandrost-4-ene-3,17-dione (**19**) and 6α-hydroxyandrost-4-ene-3,17-dione (**20**).

8. Aims

Hormone-dependent breast cancers are highly dependent on estrogens for growth and proliferation. Currently, treatment of this type of breast cancer is achieved by endocrine therapy, which alters the hormonal balance. Examples of endocrine therapy include the AIs, SERMs, such as Tamoxifen, and SERDs, such as ICI 182,780. AIs inhibit the enzyme aromatase, the main enzyme responsible for the aromatization of androgens into estrogens. Despite the success of the third generation of AIs in clinic, Anastrozole, Letrozole and Exemestane, the major drawbacks of their use are the occurrence of adverse side-effects and the development of resistance, thus there is an urgent need to discover new molecules to surpass this. Nevertheless, in clinic, it is not observable a cross-resistance between the AIs, suggesting that it is associated with different molecular mechanisms. This also points out that, although they share the same main biological target, different cellular and molecular responses might be involved in their anti-proliferative effects. Based on this, this thesis aims to contribute to two major points: the discovery of new potent steroidal AIs and the elucidation of the biological mechanisms induced by AIs in sensitive and resistant cells.

In order to search for new potent steroidal AIs, the anti-aromatase activity of new A- and B-modified androstenedione-derived steroids will be evaluated in an ER^+ human breast

cancer cell line that overexpresses aromatase (MCF-7aro cells). This cell line is derived from the MCF-7 cell line, upon transfection with the aromatase gene, and is considered the best model to study the effects of AIs. The *in vitro* effects of the most potent AIs on cell viability, proliferation, death, as well as the dependence of these effects on aromatase, ERα, or AR will also be evaluated. Moreover, since the methodology for aromatase activity assessment relies on radiometric assays that are expensive, hazardous, and non-environmentally friendly, a new methodology, based on dispersive liquid-liquid microextraction (DLLME) followed by GC-MS, will be developed.

On the other hand, in order to elucidate the biological mechanisms induced by AIs, the effects of the third-generation AIs used in clinic on fundamental cellular processes of cell proliferation, death, autophagy, and senescence, as well as the dependence of these effects on aromatase, ERα or AR will be explored in the MCF-7aro cells. In addition, it is intended to shed light on the resistance mechanisms to AIs by analyzing the contribution of autophagy and of the PI3K/AKT/mTORC1 survival pathway, and by studying the effects of the combination of the newly FDA-approved PI3K inhibitor, BYL-719, with AIs. The AI-resistant breast cancer cell lines, LTEDaro, AnaR, LetR, and ExeR, will also be used. The LTEDaro cells are derived from MCF-7aro cells that were maintained in long-term estrogen deprivation. Thus, they mimick a late-stage resistance to AIs and are considered a good model to study mechanisms of AI-resistance. The AI-specific resistant cells are also derived from the MCF-7aro cells and were originated through long-term exposure to the AIs. These cell lines are considered the best model to study AI-specific mechanisms of resistance. The direct comparison of the biological effects of the third generation AIs in sensitive cells and the different responses of the resistant cells to AIs alone and in combination with a PI3K class I inhibitor will highlight the inherent difference in resistance mechanisms between these AIs. This may help to rationalize the optimal sequence of AIs in clinic and help to explain the differences observed in clinical trials combining PI3K class inhibitors with AIs in refractory breast tumors.

CHAPTER II

Experimental Results

Manuscript I:

Effects of new C6-substituted steroidal aromatase inhibitors in hormone-sensitive breast cancer cells: Cell death mechanisms and modulation of estrogen and androgen receptors

Tiago V. Augusto, Cristina Amaral, Carla L. Varela, Fernanda Bernardo, Elisiário Tavares da Silva, Fernanda F.M. Roleira, Saul Costa, Natércia Teixeira, Georgina Correia-da-Silva

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Effects of new C6-substituted steroidal aromatase inhibitors in hormone-sensitive breast cancer cells: Cell death mechanisms and modulation of estrogen and androgen receptors

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ABSTRACT

Estrogen receptor-positive (ER^+) breast cancers require estrogens for their growth. Aromatase inhibitors (AIs) are considered the first-line therapy for this type of tumours. Despite the well-established clinical benefit of this therapy, the search for novel potent AIs that present higher efficacy and fewer side effects is still demanded. Thus, taking into account the known interactions of the natural substrate, androstenedione, within the aromatase active-site, a range of new steroidal compounds have been designed, synthesized and studied by our group. In this work, it was evaluated in MCF-7aro, an ER^+ breast cancer cell line that overexpress aromatase, the anti-aromatase efficacy and the biological effects of eight new AIs: 6α-methyl-5α-androst-3-en-17-one (1a), 6α-methyl-3α,4α-epoxy-5α-androstan-17-one (3a), 6α-methylandrost-4-ene-3,17-dione (9), 6α-allylandrosta-1,4-diene-3,17-dione (13), 6α-allylandrost-4-ene-3,17-dione (15), 6α-allylandrost-4-en-17-one (17), 6β-hydroxyandrost-4-ene-3,17-dione (19) and 6α-hydroxyandrost-4-ene-3,17-dione (20). Their anti-cancer properties were elucidated, as well as, the dependence of their mechanism of action on aromatase inhibition and/or on steroid receptors modulation, such as estrogen and androgen receptors, which are key targets for this type of cancer. Results demonstrate that the studied AIs present high anti-aromatase activity, disrupt MCF-7aro cell cycle progression and induce apoptosis, through the mitochondrial pathway. Compounds **1a**, **3a**, **9**, **13**, **15** and **17** exhibited an aromatase-dependent effect on cells and, interestingly, steroids **9** and **13** displayed the ability to decrease aromatase protein levels without affecting *CYP19A1* mRNA levels. Furthermore, the effects of compounds **1a**, **3a** and **15** were dependent on ER and on AR modulation, whereas compounds **9** and **19** were only dependent on AR modulation. From a clinical point of view, these actions can be considered as a therapeutic advantage for this type of tumours. Thus, new promising AIs that impair ER^+ breast cancer cell growth, by acting on aromatase, and even, on ER and AR were discovered. Furthermore, new insights on the most favourable structural modifications in the steroidal core structure were provided, helping to a more rational drug design of new and potent AIs.

Abbreviations: AR, Androgen receptor; AIs, Aromatase inhibitors; CDX, Casodex; CCCP, m-Chlorophenylhydrazone; DiOC6(3), 33′-Dihexyloxacarbocyanine iodide; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; E_2, Estradiol; ER, Estrogen receptor; ER^+, Estrogen receptor positive; Exe, Exemestane; ICI, Fulvestrant; HFF-1, Human foreskin fibroblast; LDH, Lactate dehydrogenase; ΔΨm, Mitochondrial transmembrane potential; MTT, 3-(45-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RLU, Relative luminescence units; MFI, Mean fluorescence intensity; PI, Propidium iodide; SAR, Structure-activity relationship; SERDs, Selective estrogen receptor downregulators; SERMs, Selective estrogen receptor modulators; STS, Staurosporine; T, Testosterone; TCA, Trichloroacetic acid; TNTE, Tris-NaCl-Triton-EDTA Buffer

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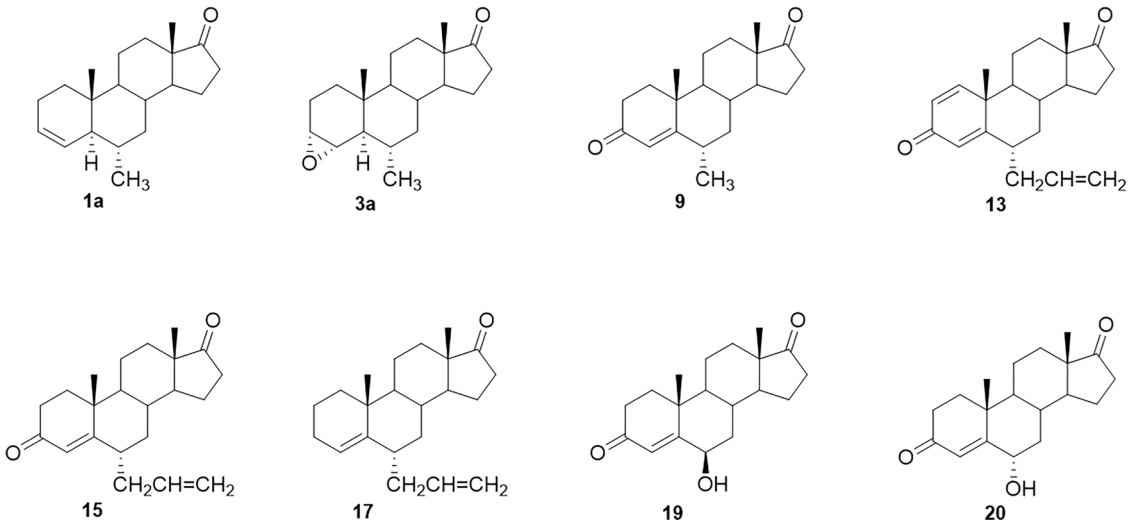

Scheme 1. Chemical structures of compounds 6α-methyl-5α-androst-3-en-17-one (**1a**), 6α-methyl-3α,4α-epoxy-5α-androstan-17-one (**3a**), 6α-methylandrost-4-ene-3,17-dione (**9**), 6α-allylandrosta-1,4-diene-3,17-dione (**13**), 6α-allylandrost-4-ene-3,17-dione (**15**), 6α-allylandrost-4-en-17-one (**17**), 6β-hydroxyandrost-4-ene-3,17-dione (**19**) and 6α-hydroxyandrost-4-ene-3,17-dione (**20**).

1. Introduction

Breast cancer is one of the major causes of cancer death in women [1]. From all breast cancers about 60% in premenopausal and 75% in postmenopausal women are estrogen receptor-positive (ER^+) carcinomas [2]. As this type of tumours are estrogen-dependent for growth and proliferation, the hormonal therapies that hindered either their production or their interaction with the receptors, are effective treatment approaches. According to this, selective ER downregulators (SERDs), such as fulvestrant, selective ER modulators (SERMs), such as tamoxifen, and aromatase inhibitors (AIs) that inhibit the enzyme involved in the last step of estrogens synthesis, were introduced in the clinic [3,4]. Recently, the latter were considered as the first-line therapy for ER^+ breast cancers in postmenopausal women and in premenopausal women with ovarian ablation [1]. In fact, the third-generation of AIs proved to be a better therapeutic option than Tamoxifen and Fulvestrant, due to their higher clinical efficacy and lower side effects [3,5]. Nevertheless, the development of acquired resistance after prolonged AIs therapy, which leads to the occurrence of tumour relapse, as well as, to the increased risk of bone fractures, limits their use in clinical practice. Thus, the search for new potent molecules that strongly inhibit aromatase impairing cancer growth, and presenting fewer side effects is of major importance.

The elucidation of the aromatase structure, and of its active-site [6–8], contributed to the understanding of the best enzyme-substrate interactions, and, therefore, allowed the design, synthesis and structure-activity relationship (SAR) studies of new steroidal compounds. Several studies were conducted to highlight the best structural modifications on the steroidal scaffold to produce more potent AIs [9]. Some of these studies were undertaken by our group, with steroidal compounds with different substitutions in the A-, B-, C- and D-rings of androstenedione structure [10–19], which aimed to elucidate the best chemical features in steroidal scaffold to obtain efficient and potent anti-aromatase activity. We have reported that the planarity in the A-ring and in the A,B-rings junction, the presence of a carbonyl group at C-3 and at C-17, as well as, a double bond in C-1 and the introduction of suitable C-6 or C-7 substitutions [11,13–15,19] are pivotal features to obtain high anti-aromatase activity, both in human placental microsomes [10–16,18] and in ER^+ breast cancer cells [17,18,20–22]. Similarly to Exemestane [23], the steroidal AI used in clinic, most of the synthesized AIs also showed anti-tumour properties in ER^+ breast cancer cells, interfering with cell cycle progression and inducing apoptosis [10,16–18,21]. Thus, the most promising hit compounds [11,13,14] gave rise to the design and synthesis of new potent AIs. In order to understand other key chemical features in the A- and B-rings of the steroidal scaffold, our group has recently designed and synthesised new steroids that will be further studied in this work. The majority of these compounds presented high anti-aromatase activity in human placental microsomes. Indeed, some of these demonstrated to be promising compounds, as they exhibit an aromatase inhibition similar to Exemestane [19]. This study concluded that C6-methyl, C6-allyl or C6-hydroxyl androstanes are better AIs than the C7-substituted counterparts [14,17,19]. Moreover, comparing androstane compounds with the same A-ring, C6-methyl derivatives demonstrated to be better AIs than C6-allyl and these ones better than C6-hydroxyl. In addition, regarding the A-ring, it was confirmed by our previous findings [11,13,14] that the double bonds at C1 and C4 and the carbonyl group at C3 increase aromatase inhibition due to the greater resemblance, conferred by these substitutions, to the natural substrate androstenedione and to exemestane. In this work, the effects of 6α-methyl-5α-androst-3-en-17-one (**1a**), 6α-methyl-3α,4α-epoxy-5α-androstan-17-one (**3a**), 6α-methylandrost-4-ene-3,17-dione (**9**), 6α-allylandrosta-1,4-diene-3,17-dione (**13**), 6α-allylandrost-4-ene-3,17-dione (**15**), 6α-allylandrost-4-en-17-one (**17**), 6β-hydroxyandrost-4-ene-3,17-dione (**19**) and 6α-hydroxyandrost-4-ene-3,17-dione (**20**) were explored in an ER^+ breast cancer cell line that overexpresses aromatase, MCF-7aro cells. It was also characterized if their mechanisms of action were dependent on aromatase inhibition and/or on steroid receptors modulation, namely estrogen and androgen receptors.

2. Materials and methods

2.1. Compounds under study

In this work, eight C6 substituted steroidal molecules were studied: 6α-methyl-5α-androst-3-en-17-one (**1a**), 6α-methyl-3α,4α-epoxy-5α-androstan-17-one (**3a**), 6α-methylandrost-4-ene-3,17-dione (**9**), 6α-allylandrosta-1,4-diene-3,17-dione (**13**), 6α-allylandrost-4-ene-3,17-dione (**15**), 6α-allylandrost-4-en-17-one (**17**), 6β-hydroxyandrost-4-ene-3,17-dione (**19**) and 6α-hydroxyandrost-4-ene-3,17-dione (**20**) (Scheme 1). These molecules were synthesized by our group as previously described [19].

2.2. Cell culture

As a good model to study ER^+ breast cancer and AIs it was used an ER^+ human breast cancer cell line, MCF-7aro, that overexpresses aromatase [24–26]. These cells were kindly provided by Prof. Dr. Shiuan Chen (Beckman Research Institute, City of Hope, Duarte, CA, U.S.A.). The experiments with the new compounds were performed in cells treated with either 1 nM of testosterone (T), 1 nM of estradiol (E_2), 1 μM of Casodex (CDX) or 100 nM of Fulvestrant (ICI 182780, ICI) (SigmaAldrich Co., Saint Louis, USA), as reported elsewhere [17]. The non-tumour human foreskin fibroblast cell line (HFF-1), purchased from the American Type Culture Collection (ATCC, Manassas, VA,USA), was also used in this study.

The growth conditions of these cell lines, the preparation and storage of the stock solutions of T, E_2, CDX and ICI are the same as previously reported [17]. MCF-7aro and HFF-1 cells incubated with 0.05% of DMSO in cell culture medium instead of compounds were considered as controls.

2.3. In cell aromatase assay

The anti-aromatase activity for each steroidal compound was determined by a radiometric assay, in MCF-7aro cells [17]. Exemestane (Exe) (Sequoia Research Products Ltd., Pangbourne, UK), at 1 μM, was used as reference AI.

2.4. Cell viability assays

Both cell lines were treated with different concentrations (1–25 μM) of the steroids **1a**, **3a**, **9**, **13**, **15**, **17**, **19** and **20**, during 3 and 6 days, using the conditions previously reported [17]. Depending on the type of analysis, MCF-7aro cells were incubated with T, E_2 CDX, or with ICI. The impact of the AIs on the viability of both cells lines, was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [17] and by the LDH release assay [18].

2.5. Cell cycle analysis

The anti-proliferative actions of compounds in MCF-7aro cells treated with 10 μM, during 3 days, were evaluated in fixed AIs-treated cells after PI staining (5 μg/mL of PI, 0.1% Triton X-100, 200 μg/mL DNase-free RNase A in PBS), by flow cytometry using BD Accuri™ C6 cytometer (San Jose, CA, U.S.A), equipped with BD Accuri™ C6 analysis software [17]. Based on the acquisition of 40 000 events it was determined the percentage of cells in each phase of the cell cycle, G_0/G_1, S and G_2/M.

2.6. Cell death analysis

The eff ;ects of compounds on MCF-7aro cell death were analysed by the activation of caspase-7 and mitochondrial transmembrane potential (ΔΨm). T-stimulated MCF-7aro cells were incubated with each compound (10 μM), for 3 days. Caspase-7 activity was determined by using Caspase-Glo® 3/7 kit (Promega Corporation, Madison, USA). The mitochondrial transmembrane potential (ΔΨm) was evaluated by a fluorescence assay using 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3)) (Gibco Invitrogen Co., Paisley, Scotland, UK), at 10 nM for 30 min at 37 °C, in the dark. The mitochondrial depolarizing agent carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma-Aldrich Co., Saint Louis, USA), at 10 μM, was used as positive control. It was employed the excitation wavelength of 480 nm and the emission filter of 530 nm to measure the fluorescence intensity, in a Microplate Luminometer (Synergy HT, BioTek, USA). Values were represented as mean fluorescence intensity (MFI). In all the experiments, basal fluorescence was subtracted.

2.7. Western blot analysis

The expression levels of aromatase, estrogen receptor α (ERα) and androgen receptor (AR) were evaluated by Western-Blot. To assess aromatase expression, MCF-7aro cells were incubated with the steroidal compounds (10 μM) for 8 h [17], while to verify ERα and AR expression cells were treated during 3 days. Exe at 10 μM and ICI at 100 nM were used as positive control for aromatase and ERα degradation, respectively. Proteins were extracted and separated using a 10% SDS-PAGE [17]. For the immunodetection, it was used the mouse monoclonal anti-CYP19 (aromatase) (1:200), rabbit polyclonal anti-ERα (1:200), mouse monoclonal anti-AR (1:100) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and the secondary peroxidase anti-mouse and anti-rabbit antibodies (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The mouse monoclonal anti-β-tubulin and anti-β-actin antibodies (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for loading controls. Immunoreactive bands were visualized using a chemiluminescent substrate Super Signal West Pico (Pierce, Rockford, USA) and a ChemiDoc™ Touch Imaging System (Bio-Rad, Laboratories Melville, NY, USA). Untreated cells were considered as controls.

2.8. RNA extraction and qPCR

After treating MCF-7aro cells, plated in 6-well plates (7×10^5 cells/mL), with compounds (10 μM,) during 8 h, cells were lysed, and the RNA collected, with TripleXtractor reagent (500 μL) (Grisp, Portugal). RNA reverse-transcription, cDNA amplification with specific primers and PCR conditions were performed as described elsewhere [27]. Primer sequences (5′-3′) were the follow: S-GATGATGTAATCGATGGC TAC and AS- TTCATCATCACCATGGCGAT for the aromatase gene, *CYP19A1,* (Ta = 58 °C); S-CTGGAGCACTCTGATTGT and AS-ATAAGGC GGTTAAGGTTAGT for α-tubulin (Ta = 55 °C). The fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [28], using as housekeeping genes, α-tubulin and α-actin.

2.9. Statistical analysis

All the assays were carried out in triplicate in at least three independent experiments and the data were expressed as the mean ± SEM. Statistical analysis was performed through analysis of variance (ANOVA) followed by Tukey and Bonferroni post-hoc tests for multiple comparisons as already described [17]. Values of $P < 0.05$ were considered as statistically significant.

3. Results

3.1. In cell aromatase assay

In order to evaluate the anti-aromatase activity of the steroids **1a**, **3a**, **9**, **13**, **15**, **17**, **19** and **20**, in MCF-7aro cells, a radiometric assay that measures estrogen formation during [1β-^{3}H]-androstenedione aromatization reaction was performed, as previously described [17]. Results demonstrated that all compounds inhibited more than 80% the activity of aromatase enzyme on cells, being compounds **9**, **13** and **15** the most potent ones (Table 1). As reference AI was used Exe (1 μM) that showed an inhibition of 99.6%, as previously described [16,17].

MCF-7aro cells were incubated with 50 nM of [1β-^{3}H]-androstenedione and 10 μM of each compound, during 1 h of aromatase reaction. Data is presented as percentage of the tritiated water released in comparison to control and are represented as the mean ± SEM of three independent experiments carried out in triplicate. Exemestane (Exe) at 1 μM was used as reference AI.

3.2. Anti-proliferative effects

As all compounds presented high anti-aromatase activity in MCF-

Table 1
In-cell aromatase inhibition of new C6-substituted steroidal compounds.

Compound	Aromatase inhibition (%)
1a	88.71 ± 3.03
3a	84.21 ± 3.23
9	98.56 ± 0.67
13	98.21 ± 1.06
15	96.52 ± 1.65
17	84.14 ± 3.43
19	88.22 ± 1.46
20	88.43 ± 2.64
Exe	99.62 ± 0.07

7aro cells, their impact in HFF-1, a non-tumour cell line, and in MCF-7aro cell viability was evaluated by MTT and lactate dehydrogenase (LDH) assays.

With exception of **3a** for the highest concentration, none of the AIs decreased HFF-1 cell viability after 6 days of treatment (Fig. 1), being therefore considered as non-cytotoxic compounds.

To explore their effects on MCF-7aro cells, T at 1 nM, was added as a proliferation induction agent, since it is an aromatase substrate [17]. Cells only treated with T were considered as control (100% cell viability). Results demonstrated that, after 3 and 6 days of treatment, all compounds, with the exception of **17**, **19** and **20** at 1 μM, caused a significant ($p < 0.05$, $p < 0.01$, $p < 0.001$) reduction in cell viability (Fig. 2) that was dose- and time-dependent. Compounds **1a**, **3a**, **9** and **15** were the AIs with more pronounced effects. In addition, after 3 days of treatment and for the studied concentrations, none of the compounds caused LDH release (data not shown), indicating that the effects on cell viability were not associated with membrane rupture.

As the effects in MCF-7aro cell viability observed for all the compounds could be a direct consequence of a cell cycle dysregulation, their effects on total DNA content were evaluated by flow cytometry, after 3 days of treatment (Table 2). All the studied compounds, at 10 μM, induced a significant ($p < 0.01$, $p < 0.001$) cell cycle arrest in the G_0/G_1 phase and a significant ($p < 0.01$, $p < 0.001$) reduction in the S phase, when compared to the control.

3.3. Mechanisms of action of AIs: aromatase, estrogen receptor and androgen receptor dependency

In order to understand if the decrease in MCF-7aro cell viability was a direct consequence of the lack of estrogens, due to aromatase inhibition, the involvement of aromatase on compounds actions was evaluated. For that, MCF-7aro cells were stimulated with E_2, the natural aromatase product, and treated with compounds under study (Fig. 3a). Contrary to compounds **19** and **20**, all the other AIs, in the presence of E_2, did not induce a decrease on cell viability similar to the cells stimulated with T. In fact, significant differences between T- and E_2-stimulated cells were observed, suggesting that the effects of AIs **1a**, **3a**, **9**, **13**, **15** and **17** on cells may be dependent on aromatase inhibition. We further studied their effects on the *CYP19A1* mRNA and aromatase protein levels. Exe (10 μM) was used as a reference AI, since it was previously demonstrated that this steroid induced aromatase degradation [29]. As observed for Exe, steroids **9** and **13** decreased significantly ($p < 0.05$; $p < 0.001$) aromatase protein levels (Fig. 3b), though without affecting *CYP19A1* mRNA levels (Fig. 3c).

To further understand if the mechanism of action of these new AIs may also be dependent on ER activation, cells were treated with AIs plus the SERD ICI-182780 (ICI), at 100 nM. In fact, only for the compounds **1a**, **3a** and **15** significant ($p < 0.05$; $p < 0.001$) differences between cells treated with and without ICI were observed (Fig. 4a). These results suggest that the reduction in MCF-7aro cell viability induced by these compounds may also be dependent on ER. Although, compound **9** presented the same effect for the highest concentration. Curiously, for the lowest concentration of steroid **17**, it was observed a more pronounced decrease in cell viability in the presence of ICI, which may suggest that this compound, at this low concentration, may have an estrogenic effect. Still, for the AIs that have shown dependency on ER it was further studied their impact on ERα protein expression. Results demonstrated that, albeit their ER-dependent biological effects, the ERα protein levels were not affected in cells treated with AIs **1a**, **3a** and **15**, when compared to control (Fig. 4b) and contrary to ICI, which, reduced the expression levels of ERα on these cells.

In addition, the dependency on androgen receptor (AR) was also investigated, since in the case of the non-steroidal AI letrozole, the AR presents a pro-death action [30]. So, as AR could be involved in the mechanism by which the new AIs promote the decrease in cell viability, their effects on cells treated with the AR antagonist CDX, at 1 μM, were studied. CDX prevented the reduction in cell viability caused by the compounds **1a**, **3a**, **9**, **15** and **19**, as significant ($p < 0.05$; $p < 0.01$; $p < 0.001$) differences between cells treated with and without CDX were observed (Fig. 5a). For the AIs that presented a dependency on AR, it was also investigated their impact on AR protein expression. Curiously, these compounds also have the ability to cause a marked and significant ($p < 0.001$) increase on AR protein expression levels (Fig. 5b).

3.4. Effects on cell death

To verify if the reduction in MCF-7aro cell viability caused by the

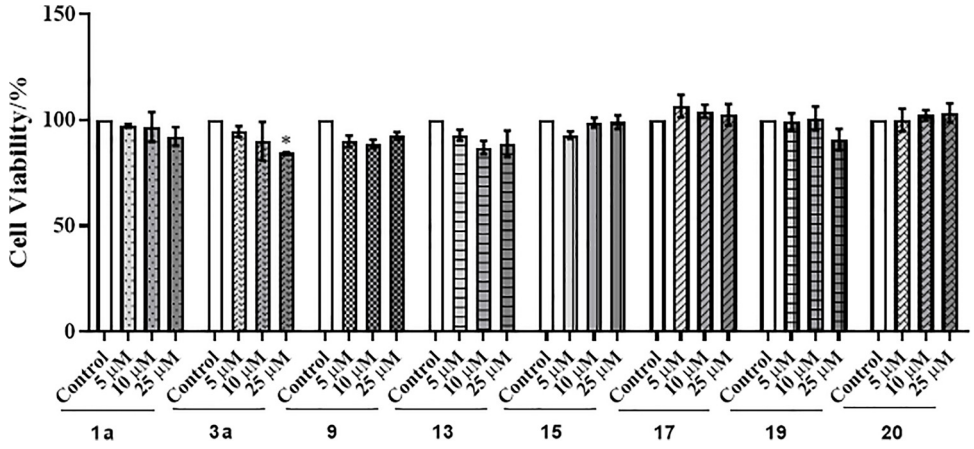

Fig. 1. Effects on non-tumour cells viability. Each AI at concentrations between 5–25 μM was added to the HFF-1 cells for 6 days. Compounds did not affect HFF-1 cell viability. Untreated cells were considered as control. Results are the mean ± SEM of three independent experiments, performed in triplicate.

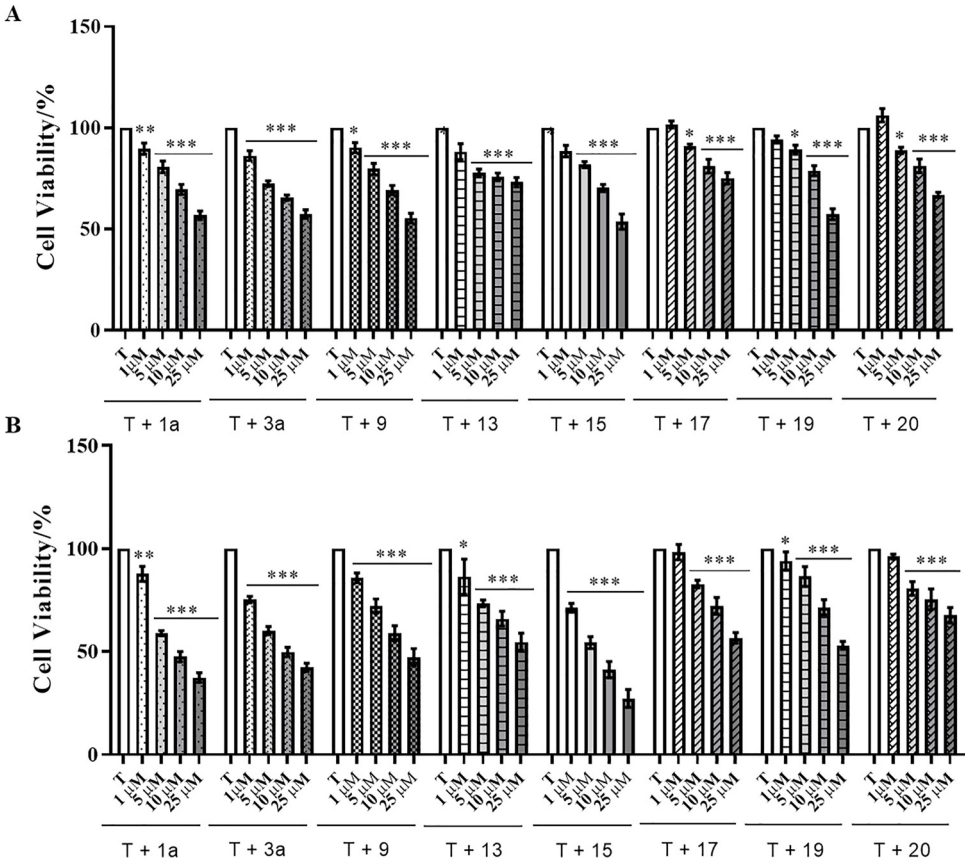

Fig. 2. Effects on ER$^+$ breast cancer cells viability. Cells were incubated with testosterone (T) and with different concentrations (1–25 μM) of each AI, during 3 (**A**) and 6 days (**B**). All the compounds decreased MCF-7aro cells viability, in a dose- and time-dependent manner. Cells cultured with T were considered as control. Results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the control and cells treated with each AI are denoted by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

Table 2
Effects of steroidal AIs on cell cycle distribution of MCF-7aro cells.

Cell cycle phase	G_0/G_1	S	G_2/M
Testosterone	73.68 ± 0.38	7.36 ± 0.23	16.67 ± 0.27
T + 1a	82.04 ± 0.47***	2.33 ± 0.13***	14.66 ± 0.41
T + 3a	81.67 ± 0.47***	2.65 ± 0.13***	14.30 ± 0.54
T + 9	83.64 ± 0.52***	2.75 ± 0.30***	13.62 ± 0.56
T + 13	80.08 ± 0.76***	3.81 ± 0.25***	16.04 ± 0.60
T + 15	79.38 ± 0.19***	3.79 ± 0.38***	16.61 ± 0.35
T + 17	78.49 ± 0.45***	4.13 ± 0.18***	18.71 ± 0.90
T + 19	81.26 ± 0.27***	3.27 ± 0.25***	15.03 ± 0.22
T + 20	76.68 ± 0.63**	4.60 ± 0.28**	18.35 ± 0.70

Cells treated with the steroidal AIs (10 μM), during 3 days were labelled with PI, followed by flow cytometry analysis. Data are presented as single cell events in G_0/G_1, S and the G_2/M phases of the cell cycle. The data represents mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the control versus AIs-treated cells are indicated by ** ($p < 0.01$) and *** ($p < 0.001$).

new compounds was also due to the involvement of apoptosis, as already reported by our group for other steroidal AIs [17,20–23], the activity of caspase-7 was analysed by a luminescence assay, after 3 days of treatment. Similarly to STS, an inducer of apoptosis in these cells, all the AIs (10 μM) led to a significant ($p < 0.001$) increase in caspase-7 activity, when compared to control (Fig. 6a). As expected, the combination of the pan-caspase inhibitor Z-VAD-FMK, with steroid **13** reverted significantly ($p < 0.001$) the activation of caspase-7. Moreover, in order to investigate the involvement of mitochondria in this process of cell death, the loss of ΔΨm was also explored. Results demonstrated that all the AIs induced a significant ($p < 0.001$) loss of ΔΨm, when compared to control (Fig. 6b), like the positive control CCCP.

4. Discussion

Nowadays, the third-generation aromatase inhibitors (AIs) are the first-line treatment for both postmenopausal and premenopausal women after ovarian ablation with ER$^+$ breast cancer [1]. Despite their therapeutic success, these AIs may still lead to the development of side effects, which limits their clinical use. Thus, the discovery and development of new AIs that strongly inhibit aromatase, present anti-cancer properties and possess lower side effects would allow an even safer and more efficient therapy for cancer patients. In this sense, several studies from our group demonstrated that the introduction of suitable functional groups at C6 position of androstenedione derivatives, allowed to obtain new compounds with strong anti-aromatase activity, in human placental microsomes. Some of these compounds, such as **9** and **13**, showed IC_{50} values similar to Exe, the steroidal AI used in clinic [19]. Even though, these compounds have high anti-aromatase activity in human placental microsomes, their effects in ER$^+$ breast cancer cells are unknown. Therefore, in this study, using an ER$^+$ breast cancer cell line that overexpress aromatase, MCF-7aro cells, the biological effects and the anti-aromatase activity of these new compounds were explored. Furthermore, it was evaluated if their mechanism of action on cells were due to aromatase inhibition or to modulation of ER and AR, which are also important key targets for this type of tumours.

All the compounds efficiently inhibited aromatase in MCF-7aro cells, being compounds **9**, **13** and **15** the most potent ones. Thus, it is possible to confirm that in C6α-substituted androstanes the simultaneous presence of a C3-carbonyl group along with a methyl- or allyl-aliphatic chain at C6α (compounds **9**, **13** and **15**) is important to improve aromatase inhibition. Moreover, the presence of an additional double bond at C1 as in Exe A-ring, further improve the anti-aromatase activity in breast cancer cells (compound **13** vs **15**). This is in accordance with previous reports from our group that highlighted the importance of the double bond at C1 [13,14,17,19]. When comparing

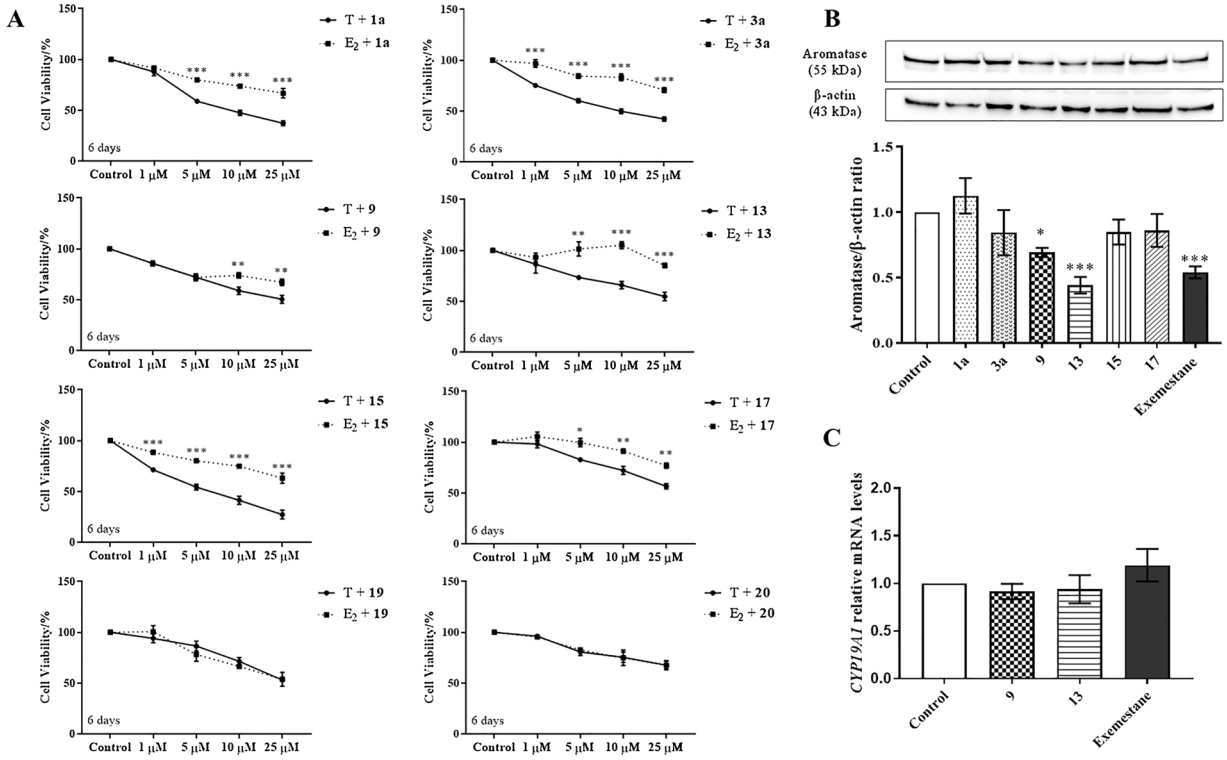

Fig. 3. Aromatase-dependency and effects of AIs on aromatase gene and protein levels in breast cancer cells. (**A**) Comparison between the impact of different concentrations of each AI on viability of T-treated and E_2-treated MCF-7aro cells, after 6 days of treatment. Cells cultured with T or E_2 were considered as control. Significant differences between the T-treated versus E_2-treated MCF-7aro cells are denoted by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). (**B**) Aromatase expression in MCF-7aro cells treated with the new AIs (10 μM) or with exemestane (Exe) (10 μM), by western-blot. Cells without AIs treatment were considered as control (represents 100% viability). β-Actin was used as a loading control. Normalization to β-actin levels was used to determine aromatase protein levels. Significant differences between the control versus AIs-treated cells are presented by * ($p < 0.05$) and *** ($p < 0.001$). Results are the mean ± SEM of three independent experiments, performed in triplicate. (**C**) qPCR analysis of *CYP19A1* mRNA levels in MCF-7aro cells treated with the new AIs or with Exe. Cells without treatment were considered as control. To quantify the mRNA transcript levels of *CYP19A1* it was used the housekeepings α-tubulin and α-actin, being only represented the data for α-tubulin.

compounds **9**, **15** and **20**, is was possible to conclude that a C6α-methyl substituent is better than C6α-allyl, and this one is better than C6α-hydroxyl, to achieve increased aromatase inhibition in the cell model. This is in accordance with that was observed in human placental microsomes [19]. In relation to compound **3a**, despite the epoxide at C3/C4 conferring better anti-aromatase activity in human placental microsomes [19], in MCF-7aro cells it slightly diminished the aromatase inhibition, when compared to the double bond at C3, present in compound **1a**. In fact, this increased efficiency of the double bond over the epoxide group has already been reported by our group in similar compounds [13,18]. These SAR observations may help to build a more rational design of new steroidal AIs, however, the complexity of the interactions between the new compounds and aromatase, as well as, with other molecular targets must be taken into account.

In addition to the study of the anti-aromatase activities, the biological effects and mechanisms of action of the new AIs were also investigated. At the concentrations that had no effect on the non-tumour cell line, HFF-1 cells, the studied compounds significantly decreased the viability of the breast cancer cells, both at 3 and 6 days of treatment. Compounds **1a**, **3a**, **9** and **15** were the most efficient in decreasing MCF-7aro cell viability. In order to understand the mechanisms behind the reduction in MCF-7aro cell viability, it was explored if these effects were dependent on aromatase inhibition, and/or on ER and AR signalling. It was verified that, with the exception of **19** and **20**, the decrease in cell viability was dependent on the aromatase inhibition for all the other compounds. In this case, the addition of E_2 partially reversed the anti-proliferative effects. So, the estrogen depletion may be one of the biological mechanisms behind the anti-tumour effects of compounds **1a**, **3a**, **9**, **13**, **15** and **17**. This type of effect was already described by our group for other steroidal AIs [17,18]. As it was described that Exe induces aromatase degradation [29], the ability of the aromatase-dependent compounds to induce this effect, in our cell model, was also explored. Interestingly and likewise Exe, only compounds **9** and **13** decreased aromatase protein levels. However, by using qPCR we did not observe alterations in *CYP19A1* mRNA expression levels, which suggests that the decrease in aromatase protein levels could be due to aromatase degradation, as reported for Exe [29]. In addition, we have previously demonstrated that compounds that present aromatase-dependent effects may in some cases affect the aromatase protein levels [17], a behaviour also observed for these new compounds. Moreover, the results suggest that the effects on aromatase protein level are independent on the type of inhibition, as we previously described that compound **9** is a reversible AI, while compound **13** is an irreversible AI [19]. Previous studies from our group indicated that the effects of androstanes with a double bond or an epoxide group in C4 [18], or simultaneously with a double bond or an epoxide group in C1, together with a carbonyl group at C17 are aromatase-dependent. In addition, we observed that the introduction of an allyl or an epoxypropyl group at C7, maintaining the double bonds at C1/C4 and the carbonyl group at C17, also allows the aromatase-dependent effect [17]. To the previously established SAR, our results show that the introduction of a methyl or allyl group at C6, as in **9**, **13**, **15**, and **17**, but not a hydroxyl group, as in **19** and **20**, allows that the effects remain aromatase-dependent. Further, this dependency persists when the double bond or the epoxide group in the A-ring changes from the C4 to the C3 position, as in **1a** and **3a**.

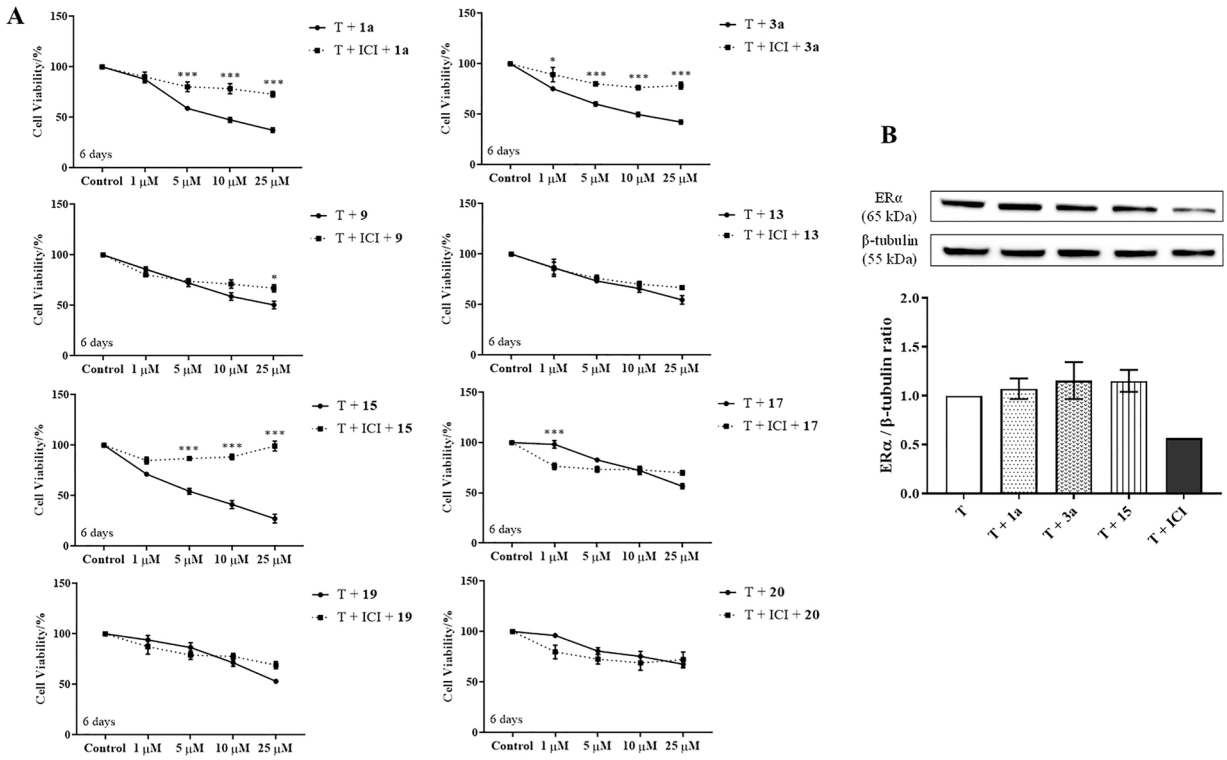

Fig. 4. ER-dependence and ER expression alterations induced by the AIs on breast cancer cells. (A) Comparison between the impact of different concentrations of each AI on T-treated or ICI-treated MCF-7aro cell viability, after 6 days of treatment. Cells cultured without AIs were considered as control. Significant differences between the T-treated versus ICI-treated MCF-7aro cells are denoted by * ($p < 0.05$) and *** ($p < 0.001$). **(B)** ERα expression analysis of T-treated MCF-7aro cells incubated with AIs (10 μM) by western-blot. Cells cultured with T were considered as control (represents 100% viability), while ICI (200 nM) was used as a positive control of ER. β-Tubulin was used as a loading control. Normalization to β-tubulin levels was used to determine ERα protein levels. Results are the mean ± SEM of three independent experiments, performed in triplicate.

Furthermore, our results demonstrated that the effects of compounds **1a**, **3a** and **15** on cells are also dependent on ER, since ER degradation by ICI prevents their growth-inhibitory action. This behaviour was already demonstrated by our group for other steroidal AIs [17,18]. Besides this observation, none of the ER-dependent compounds affected ERα protein levels. Thus, these findings suggest that the decrease on cell viability induced by compounds **1a**, **3a** and **15,** although associated to ER modulation, does not affect ERα protein levels. This may be explained by a Tamoxifen-like mechanism of action [31], which together with the anti-aromatase activity may be a therapeutic advantage when compared to either therapy alone [32]. Although, it has been suggested that the presence of a hydroxyl group at C17 [17] determines ER-dependency, in this study, compounds **1a**, **3a** and **15**, that possess a carbonyl group at that position, are also modulators of ER. This points out that the nature of the chemical group at C17 may not play a determinant role in ER-dependency. Thus, it is still uncertain which functional groups are associated with the ability to modulate the ER-dependent mechanisms.

Moreover, 85–95% of the ER^+ breast cancers and 77% of invasive tumours express AR [33,34]. As this receptor has been associated with promotion of cell death in ER^+ breast cancer cells [30,35,36], it was investigated if the effects of these new compounds on cells were AR-dependent, by antagonizing the receptor with CDX. Accordingly, compounds **1a**, **3a**, **9**, **15** and **19** did not retain their anti-cancer effects, suggesting that their actions are dependent on AR modulation, an effect previously showed by our group for other steroidal AIs [17]. Interestingly, this finding was further supported by the marked increase in AR protein levels induced by these compounds on MCF-7aro cells. Moreover, like the non-steroidal AI letrozole [30], our findings indicate that the new AIs trigger a pro-death signalling mediated by AR, which from a clinical point of view, could be a therapeutic advantage for ER^+ breast tumours.

In addition to the characterization of the mechanism of action of these new compounds in MCF-7aro cells at aromatase, ER and AR level, their anti-proliferative effects were also studied at the cell cycle level. All the compounds induced cell cycle arrest at G_0/G_1 phase, with a consequent significant decrease in the S phase. This disturbance in the cell cycle has already been reported for previous steroidal compounds studied by our group [17,20,21,37] and for Exe [23]. Furthermore, in order to verify if the anti-proliferative effects on MCF-7aro cells were also associated with the apoptotic mechanism, the activity of caspase-7 and the mitochondrial membrane potential were evaluated. All the compounds decreased mitochondrial membrane potential and increased caspase-7 activity, confirming the occurrence of apoptosis. These results suggest an intrinsic apoptotic pathway activation, a mechanism that has already been described by our group for previous compounds [17,20,21,37] and for Exe [23].

In conclusion, the anti-aromatase and the anti-tumour efficiency of new C6-substituted steroidal AIs in an ER^+ breast cancer cell line that overexpress aromatase were displayed. In fact, new promising AIs presented single, dual or triple dependency impairing, in that way, ER^+ breast cancer cell growth. Compounds **1a**, **3a** and **15** were dependent on aromatase, ER and AR, while compound **19** was only dependent on AR. Compound **13** and **17** were only dependent on aromatase and compound **9** was dependent on aromatase and AR. Interestingly, an contrary to the other AIs, only compounds **9** and **13** decreased aromatase protein levels. Nevertheless, as these compounds present several targets, the growth-inhibitory effects may be a result of the overlapping of different mechanisms, or, even, due to cross-talks between different signalling pathways, such as the well-known ER/AR crosstalk described for Anastrozole [38]. In addition, this work contributed to the SAR studies of C6-substituted androstanes, in order to unveil the most

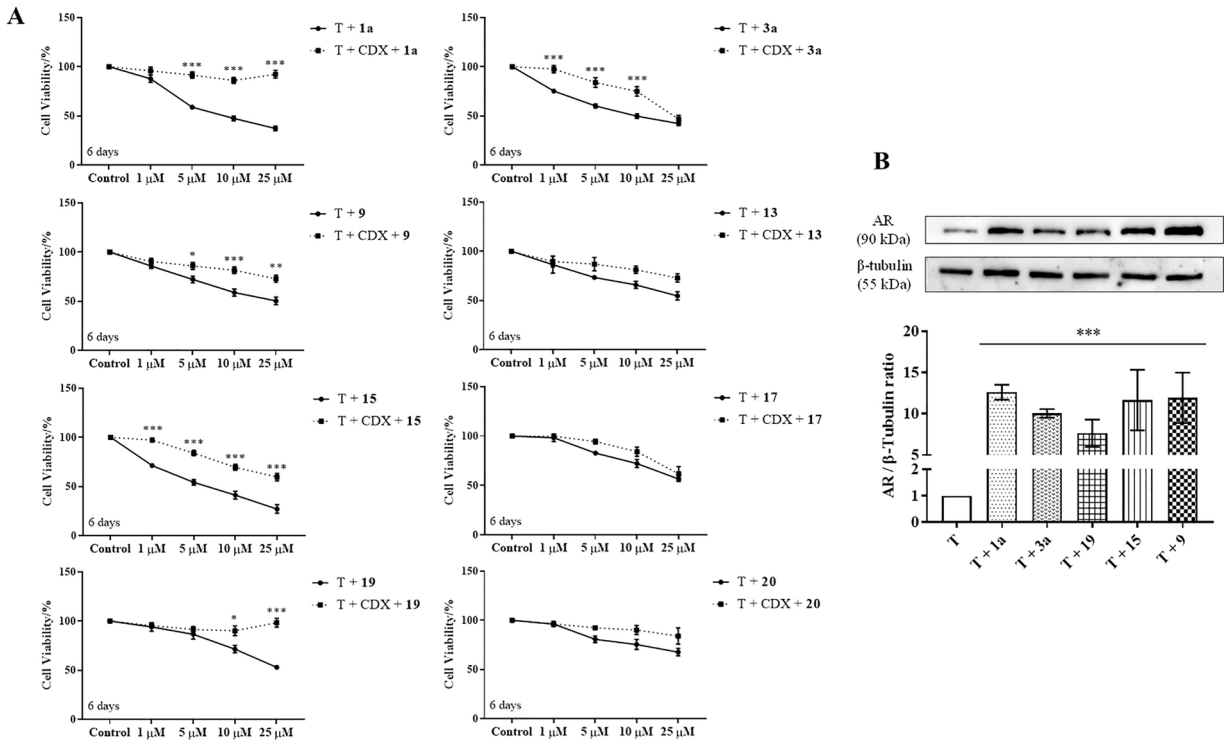

Fig. 5. AR-dependence and AR expression alterations induced by AIs on breast cancer cells. (A) Comparison between the impact of different concentrations of each AI on T-treated and CDX-treated MCF-7aro cell viability, after 6 days of treatment. Cells cultured without AIs were considered as control (represents 100% cell viability). Significant differences between the T-treated MCF-7aro cells versus CDX-treated MCF-7aro cells are denoted by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). **(B)** AR expression analysis of T-treated MCF-7aro cells incubated with AIs (10 μM) by western-blot. Cells cultured with T were considered as control. β-Tubulin was used as a loading control. Normalization to β-tubulin levels was used to evaluate AR protein levels. Significant differences between the control versus AI-treated cells are denoted by *** ($p < 0.001$). Results are the mean ± SEM of three independent experiments, performed in triplicate.

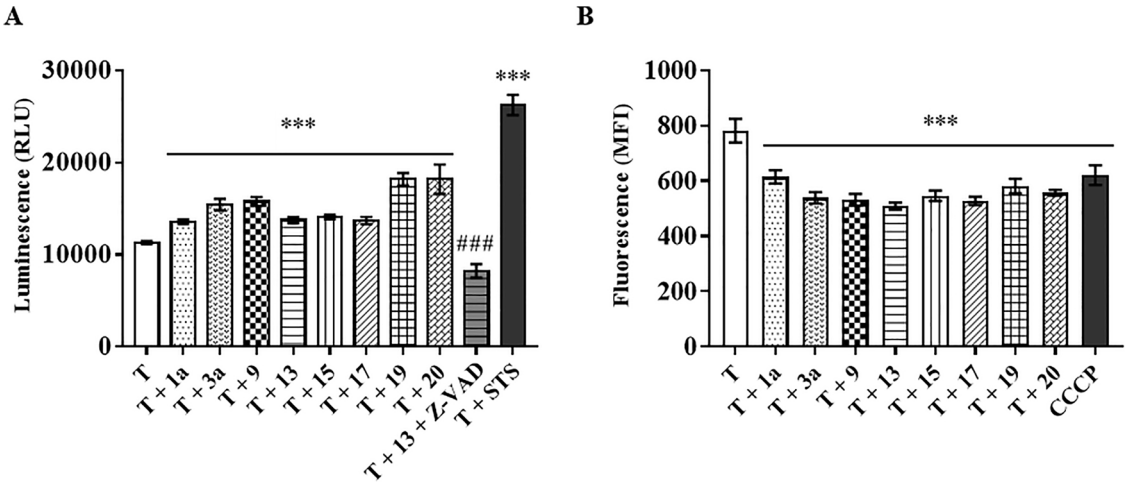

Fig. 6. Cell death induced by the AIs. All the steroids caused an increase in caspase-7 activity (A) and loss of mitochondrial transmembrane potential (ΔΨm) (B). Cells cultured with T were considered as control. For caspase activation it was used staurosporine (STS) as positive control and Z-VAD-FMK as a negative control. For ΔΨm loss, CCCP was used as a positive control. The results are represented as relative luminescence units (RLU) for caspase-7 activation and as mean fluorescence intensity (MFI) for ΔΨm assay. Results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the control T versus AIs-treated cells are denoted by *** ($p < 0.001$). Significant differences between the compound **13** with or without Z-VAD-FMK are presented by ### ($p < 0.001$).

favourable modifications in the steroidal scaffold that improve anti-aromatase activity and anti-tumour properties, such as the activation of AR. From a clinical point of view, this can be therapeutically advantageous for treating this type of breast tumours. Moreover, this work describes, for the first time, the effectiveness of these new potent C6-substituted androstanes as AIs in a cancer cellular model, highlighting their potential application for ER^+ breast cancer treatment.

Author contributions

Conceived and designed the study: TVA, CA, GCdS, NT. Performed the experiments: TA, CA, FB. Designed and synthesized the compounds: CLV, SC, ETdS, FMFR. Analyzed and interpreted the data: TVA, CA, GCdS, NT. Wrote the paper: TVA, CA, GCdS, NT. Revised the manuscript: CA, ETdS, FMFR, GCdS, NT. Read and approved the manuscript

for publication: TVA, CA, CLV, FB, SC, FMFR, ETdS, GCdS, NT.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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Manuscript II:

A novel GC-MS methodology to evaluate aromatase activity in human placental microsomes: a comparative study with the standard radiometric assay

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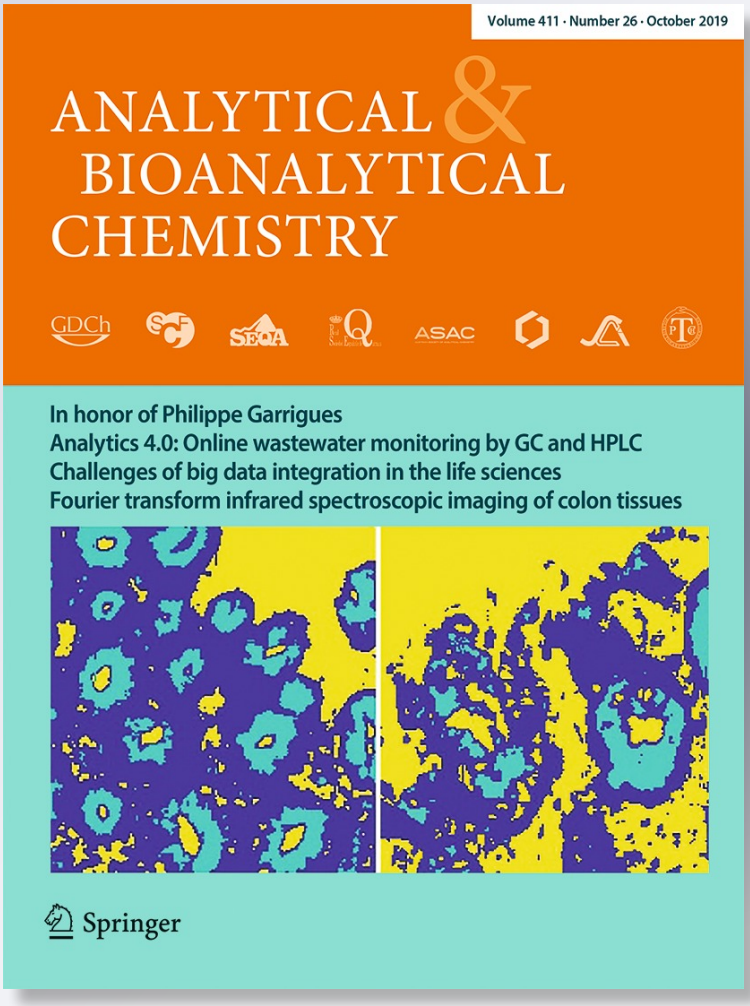

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RESEARCH PAPER

A novel GC-MS methodology to evaluate aromatase activity in human placental microsomes: a comparative study with the standard radiometric assay

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Abstract

Estrogens are key factors in the development of the estrogen receptor–positive (ER^+) breast cancer. Estrogens, estrone (E_1), and estradiol (E_2) production is achieved by aromatase, a cytochrome P450 enzyme that has androgens, androstenedione (AD), and testosterone (T) as substrates. Nowadays, third-generation aromatase inhibitors (AIs) are considered the gold-standard treatment for ER^+ breast cancer in postmenopausal women as well as in premenopausal women with ovary ablation. Aromatase activity assessment still relies on radiometric assays that are expensive, hazardous, and non-environmentally friendly. Thus, in order to overcome these disadvantages, a new methodology was developed to evaluate aromatase activity, based on dispersive liquid-liquid microextraction (DLLME) followed by gas chromatography-mass spectrometry (GC-MS). The enzymatic reaction was carried out in human placental microsomes, using AD as substrate, and the anti-aromatase activity was measured by determining the conversion percentage of AD into E_1 (ratio E_1/AD) using isotopic analogues as internal standards. The method showed good linearity ($r^2 = 0.9908$ for AD and 0.9944 for E_1), high accuracy (more than 74% for AD and more than 66% for E_1), high extraction efficiency, and good intra-day and inter-day precision (below 14%, 4 levels). In this work, the IC_{50} values of the third-generation AIs, anastrozole, letrozole, and exemestane, obtained from the radiometric assay are also compared, and similar IC_{50} values are described. This method is a good alternative to the current radiometric assay, being fast and sensitive with a good extraction efficiency, accuracy, and recovery. In addition, it may be applied for the evaluation of the anti-aromatase activity of new potential AIs.

Keywords Aromatase inhibitors · Estrogens · Aromatase activity · Gas chromatography–mass spectrometry · Radiometric assay · Breast cancer

Abbreviations

AD	Androstenedione
AIs	Aromatase inhibitors
Ana	Anastrozole
DHT-$^{13}C_3$	Dihydrotestosterone-2,3,4-$^{13}C_3$
DLLME	Dispersive liquid-liquid microextraction
DMSO	Dimethyl sulfoxide
DTE	1,4-Dithioerythritol
DTT	Dithiothreitol
ER^+	Estrogen receptor-positive
Exe	Exemestane
E_1	Estrone
E_2	Estradiol
GC-MS	Gas chromatography–mass spectrometry
IS	Internal standard
LC-MS	Liquid chromatography mass spectrometry
Let	Letrozole
LLOQ	Lower limit of quantification
LOD	Limit of detection

Tiago V. Augusto and Sara C. Cunha contributed equally to this work.

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MeCN	Acetonitrile
MSTFA	*N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide
NADPH	β-Nicotinamide adenine dinucleotide phosphate
SAR	Structure-activity relationships
SIM	Selective ion monitoring
%RSD	Relative standard deviation
T	Testosterone
TCA	Trichloroacetic acid
TIC	Total ion chromatograms
TMS	Trimethylsilyl
ULOQ	Upper limit of quantification

Introduction

The main estrogens in humans are estrone (E_1), derived from the androgen androstenedione (AD), and estradiol (E_2), derived from the androgen testosterone (T). The conversion of androgens to estrogens is catalyzed by the enzyme aromatase, a member of the cytochrome P450 family, through a process known as aromatization [1]. Aromatase is codified by the *CYP19A1* gene, localized in the chromosome 15, and is the only enzyme in vertebrates known to catalyze the biosynthesis of estrogens [2]. In premenopausal women, this enzyme is mainly expressed in the ovaries, while in postmenopausal women, it is mainly expressed in the peripheral tissues, like adipose tissue. In 2009, Ghosh et al. elucidated aromatase structure by X-ray crystallography, a breakthrough for the understanding of the enzyme-substrate interactions [3, 4]. The aromatization process occurs through three oxidation reactions in the A-ring of androgens, being the last reaction exclusive to aromatase [1]. Each reaction consumes one molecule of β-nicotinamide adenine dinucleotide phosphate (NADPH) and one of O_2. However, although aromatase binds to both androgens AD and T for the conversion into estrogens, the binding affinity is higher for AD than for T [5].

Estrogens regulate important processes involved in the normal development of breasts, such as stimulation of ductal and stromal growth and of adipose tissue accumulation, through its binding to estrogen receptors (ER). This binding induces a conformational change in ER that results in its activation, leading to proliferation and cell survival [6, 7]. Even though estrogens play such an important role in breasts, they are also associated with the development of breast carcinomas, being the main drivers of proliferation of estrogen receptor–positive (ER^+) breast cancers [8]. Aromatase inhibitors (AIs) are used as first-line therapy for this type of cancer in postmenopausal women as well as in premenopausal women with ovary ablation [9]. Currently, the third-generation AIs, anastrozole (Ana), letrozole (Let), and exemestane (Exe), are a good therapeutic option since they achieve almost total depletion of the circulating estrogen levels. However, these AIs present some side effects, such as skeletal complications, musculoskeletal pain, arthralgia, cardiovascular events, and sexual dysfunction [9–12]. Moreover, the development of endocrine resistance, due to prolonged AIs therapy, presents itself as the major drawback of AI use [8], thus, encouraging the search for new and more potent compounds.

Currently, most studies in drug discovery for potential AIs use radiometric tests to determine the anti-aromatase activity in human cancer cells [13, 14], in human placental microsomes [15–20], in equine placental microsomes [21], and in rat ovarian microsomes [17]. This radiometric methodology was firstly developed by Thompson and Siiteri (1974), and consists in the incubation of the enzyme with [1β-^{3}H]-androstenedione, in which tritiated water, the product of aromatization reaction, is quantified by liquid scintillation counting [22]. However, in addition to being highly sensitive, this radiometric assay is expensive, hazardous to health, and non-environmentally friendly and thus requires the development of a new, sensitive, and safe methodology for routine use.

Therefore, in the last years, alternative liquid chromatography mass spectrometry (LC-MS) [23] and GC-MS [24] methodologies have been developed to overcome this problem. Nevertheless, the LC-MS/MS methodology uses high quantities of human CYP 19A1 supersomes and a concentration of substrate, AD, 20 times higher [23] than the Km of the enzyme [25]. Moreover, the reported GC-MS methodology uses significant amounts of human placental microsomes, does not use the natural substrate of the enzyme and the validation of the method was not described [24].

Hence, the aim of this work was to develop and validate a new sensitive GC-MS methodology to evaluate the aromatase activity in human placental microsomes. Aromatase activity is measured by determining the conversion percentage of AD into E_1 (ratio E_1/AD), using isotopic analogues as internal standards. This method implies a fast and very effective procedure of dispersive liquid-liquid microextraction (DLLME) of the analytes followed by a silylation step and quantification by GC-MS in selective ion monitoring (SIM) mode. This method will provide a step forward in the screening of new compounds as potential AIs, thus facilitating drug discovery for ER^+ breast cancer treatment.

Material and methods

Reagents and standards

Anastrozole (Ana), letrozole (Let), androstenedione (AD), estrone (E_1), dithiothreitol (DTT), dimethyl sulfoxide (DMSO), NADPH, ammonium iodide (NH_4I), 1,4-dithioerythritol (DTE) and the internal standard dihydrotestosterone-2,3,4-$^{13}C_3$ solution (DHT-^{13}C, 99Atom%), acetonitrile (MeCN) HPLC grade, high-purity extractive solvent trichloroethylene (C_2HCl_3) for GC analysis, and the derivatization reagent *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) were obtained from Sigma-Aldrich (St. Louis,

USA). [1β-^{3}H]-Androst-4-ene-3,17-dione was purchased from PerkinElmer (Waltham, MA, USA). Exemestane (Exe) was obtained from Sequoia Research Products Ltd. (Pangbourne, UK). Bradford assay kit was from Bio-Rad (Laboratories Melville, NY, USA). Ultrahigh-purity helium (99.9999%) for GC-MS and nitrogen for solvent evaporation were obtained from Gasin (Maia, Portugal).

The individual stock internal solution of DHT-$^{13}C_3$ (3.46 μM) was prepared in MeCN, while the individual stock solutions of the third-generation AIs, Ana, Let and Exe, were prepared in 100% DMSO. All the solutions were stored at − 20 °C. Prior to the assays, AIs were diluted in 67 mM potassium phosphate buffer (pH 7.4).

Sample preparation

Human term placental tissues were obtained under informed consent and collected after birth. All the procedures concerning human placental handling were performed after approval of the Ethical Committee of Centro Materno Infantil do Norte, Centro Hospitalar do Porto, Portugal. Placental tissues were placed in cold 67 mM containing 1% KCl, washed and stored at − 80 °C before use. Human placental microsomes were prepared as previously described [26, 27].

Radiometric aromatase assay

In order to evaluate anti-aromatase activity, tritiated water released from [1β-^{3}H]-androstenedione during the aromatization process was measured, according to the Thompson and Siiteri [22] and Heidrich [28] methods with modifications [26]. For the enzymatic reaction, 20 μg of placental microsomal protein and 150 μM of NADPH were used in a final volume of 0.5 mL of 67 mM potassium phosphate buffer (pH 7.4). In the case of the screening assay, it was added 40 nM of [1β-^{3}H] androstenedione (1 μCi) and 2 μM of each AI, while for the IC_{50} determination, it was added 100 nM of [1β-^{3}H] androstenedione and different concentrations of Exe (0.01–0.5 μM), Ana (0.01–0.5 μM), and Let (0.001–0.025 μM). The reaction was performed in a shaking water bath at 37 °C during 15 min. The enzymatic reaction was stopped by the addition of 0.5 mL of 20% trichloroacetic acid (TCA) on ice. The remaining procedure was performed as previously described by our group [26]. All the three independent experiments were carried out in triplicate.

GC-MS aromatase assay

A new DLLME-GC-MS method was developed based on a method previously established by our group [29]. The same enzymatic procedures were performed using cold AD, instead of [1β-^{3}H] androstenedione, and the reaction was stopped with 0.5 mL of MeCN on ice, which was further followed by the extraction of the steroids, AD and E_1, by DLLME. For that, 20 μL of DHT-$^{13}C_3$ (150 nM) and 50 μL of C_2HCl_3 were added to the enzymatic mixture. Subsequently, vortex (30 s) and centrifugation were performed at 4 °C for 3 min at 110,000×*g*. The supernatant (300 μL) was evaporated under a gentle nitrogen stream at room temperature. The steroids in the extract were then derivatized with 50 μL MSTFA+NH_4I+DTE (500:4:2 vol/wt/wt), according to Amaral et al. conditions [29].

The ratio between AD/DHT-$^{13}C_3$ and E_1/DHT-$^{13}C_3$ allowed not only the quantification of AD and E_1 resultant from the aromatase reaction in placental microsomes but also the estimation of the anti-aromatase activity by comparing the amount of E_1 produced after the aromatization reaction. At least three independent experiments were performed, carried out in triplicate.

GC-MS conditions

The analyses were performed in a GC-MS 6890N Network GC System (Agilent Technologies, Little Falls, DE, USA) equipped with a Combi-PAL autosampler (CTC Analytics, Zwingen, Switzerland) coupled directly to a single quadrupole inert mass selective detector (5975, Agilent Technologies) with an electron ionization chamber. A capillary column (DB-5-MS, 30 m × 0.25 mm I.D. × 0.25-μm film thickness; J&W Scientific, Folsom, CA, USA) was used in separation and the injection was carried out at 280 °C in the splitless mode (1 min). Helium was used as the carrier gas with a constant flow of 1 mL/min. The oven temperature program was as follows: 105 °C held for 1 min, ramped to 280 °C at 15 °C/min, and held for 4.33 min; the total runtime was 17 min. Mass spectrometry conditions were similar to those reported elsewhere [30]. Data acquisition was performed in the selective ion monitoring (SIM) mode, detecting three ions per analyte analyzed. The SIM parameters and retention times of each one of the analytes are shown in Table 1.

Table 1 MS conditions for the GC-MS analysis of AD, E_1 and IS derivatized (time windows and ions selected in SIM mode, quantification ions are in italics)

Analyte	t_R (min)	SIM ions (*m/z*)			
		Quantification	Qualification		
DHT (IS)	14.31	*420*	228	330	345
E_1	14.72	*414*	399	309	
AD	14.77	*430*	415	234	

Validation procedure

The analytical method validation was performed according to the FDA guidelines [31]. The parameters studied were linearity, limit of detection (LOD), lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), precision, accuracy, recovery, and extraction yield.

Results

Procedure development

GC system

In order to enhance selectivity, detectability, and efficiency of the chromatography, the steroid molecules were derivatized. In this study, silylation was used to derivatize the C-3 hydroxyl and C-17 keto groups, both present in E_1, and the C-17 keto group present in AD, as this derivatization produces highly volatile and stable derivatives. In the case of E_1, the mixture of MSTFA with NH_4I (catalyst) and DTE (antioxidant) reacts with the active hydrogen of the C-3 hydroxyl group and with the C-17 carbonyl group, generating a trimethylsilyl ether in C-3 and an enol ether at C-17. In the case of AD, a C-17 enol ether was formed (Fig. 1). This mixture was selected, since it was the one that affords derivatives with higher selectivity and specific *m*/*z* ion fragments for AD and E_1 when compared with other silylation reagents such as *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide.

Extraction

A DLLME procedure for extraction of the steroidal molecules was used based on a previous method [29]. The type of extractive and dispersive solvents is a key factor in yield of extraction process being usually necessary to test different pairs of solvents in order to achieve the best conditions. However, in this work, trichloroethylene and acetonitrile were unequivocally selected as extractive and dispersive solvents, respectively, due to previous knowledge about their easy miscibility with steroid analytes from placental microsome samples, and their efficiency to stop the enzymatic reaction (denaturating aromatase) and induce protein precipitation. Hence, in order to obtain the higher extraction yields of the analytes of interest, the procedure was first developed in terms of ratio volume of extraction (C_2HCl_3) and dispersive (MeCN) solvents. The use of 50 μL of C_2HCl_3 plus 500 μL of MeCN, followed by evaporation until dryness of the supernatant phase, was shown to provide the best results in terms of extraction yield, enrichment factor, and absence of chromatographic interferents.

Method performance

Limit of detection and lower limit of quantification

In order to evaluate the sensitivity of the method, the limit of detection (LOD) and the lower limit of quantification (LLOQ) were determined by successive analysis of samples with decreasing amounts of AD and E_1 (Table 2). LOD represents the lowest concentration where the signal-to-noise ratio was higher than 3:1, while LLOQ represents the lowest concentration that was measured with acceptable accuracy and precision (relative standard deviation (%RSD) < 20%). For AD, the LOD and LLOQ were 1 nM and 2.5 nM, respectively. For E_1, the LOD and LLOQ were 0.5 nM and 2 nM, respectively.

Linearity

The method linearity was evaluated by analyzing seven concentrations, ranging from LLOQ to 440 nM (ULOQ), for both AD and E_1 in human placental microsomes, using the aromatase assay as described above. Calibration curves were constructed by plotting the mean of analyte/IS ratio obtained

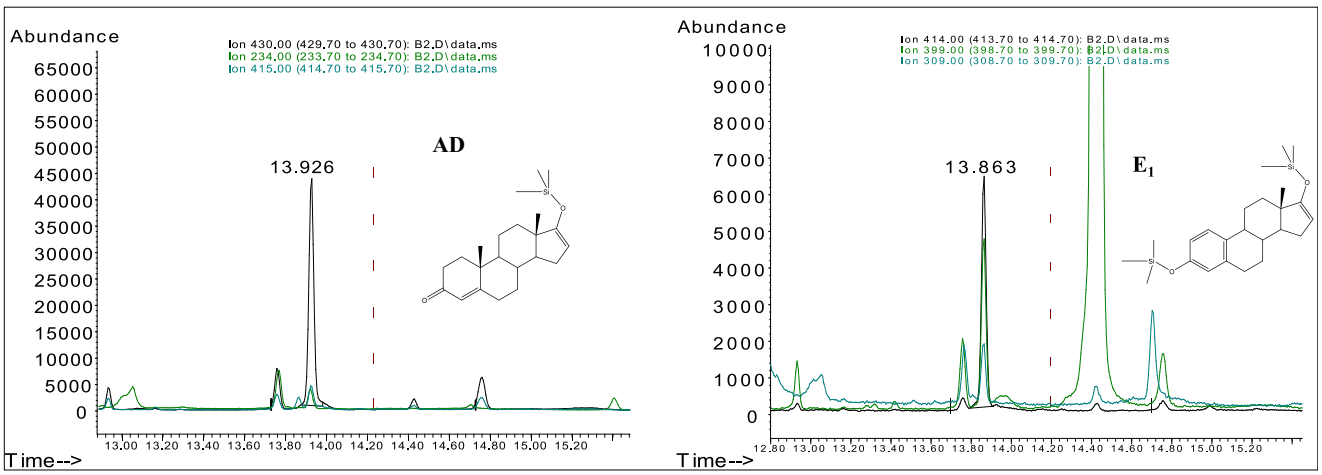

Fig. 1 Total ion chromatograms (TIC) of a standard solution of AD and E_1. Each steroid was derivatized with MSTFA + NH_4I + DTE

Table 2 Calibration data (slope, intercept, and r^2, $n = 3$), lower limits of detection and quantification of AD and E_1 in the presence of human placental microsomes

Analyte	Linearity			LOD (nM)	LLOQ (nM)
	Slope	Intercept	r^2		
AD	0.1155 ± 0.0045	− 2.181 ± 0.843	0.9908	1	2.5
E_1	0.0444 ± 0.0014	0.3481 ± 0.2532	0.9944	0.5	2

against the concentration of each analyte (Fig. 2). This method showed to be linear, presenting a coefficient of determination (r^2) of 0.9908 and 0.9944, for AD and E_1, respectively. Data were obtained after three independent experiments.

Precision

To determine the intra-assay and inter-assay precision, samples with placental microsomes were spiked with four different concentrations of AD and E_1, LLOQ (2 nM for E_1 and 2.5 nM for AD), 20 nM (low), 100 nM (medium), and 220 nM (high) (Table 3). Intra-assay precision was evaluated by performing the extraction and analysis of five spiked samples for each concentration in the same day, while in inter-day precision, triplicate spiked samples in five different days were analyzed. The relative standard deviation (%RSD) was lower than 17% for both AD and E_1 in LLOQ, and lower than 13% for the other concentrations in intra-assay. For the inter-assay precision, the %RSD was lower than 14% for both AD and E_1 in all the concentrations. This value is within the criteria accepted for bio-analytical method validation [31].

Accuracy and extraction efficiency

The accuracy of the method was assessed through the analysis of samples with placental microsomes spiked with four different known concentrations of AD and E_1 (LLOQ, 20 nM, 100 nM, and 220 nM), by calculating the percent deviation between the calculated value and the nominal value (accuracy (%) = (mean calculated concentration / nominal concentration) × 100). Accuracy was higher than 66%, for both hormones (Table 3).

The extraction efficiency for AD and E_1 was assessed at four different concentrations (LLOQ, 20 nM, 100 nM, and 220 nM). The extraction samples were prepared by spiking placental microsomes with each analyte. These samples were extracted and before evaporation, the internal standard (IS) solution was added to the extract. The control samples were prepared by extracting blank placental microsomes and before evaporation, the analyte and IS solutions were added to the extract. Extraction efficiency was estimated by comparison of the peak area ratios (analyte vs IS) from extraction samples and control samples for each analyte at each concentration. The method presented a high yield for all the concentrations tested (higher than 69%) as shown in Fig. 3.

Selectivity

The selectivity was verified through analysis of four blank samples for each batch of 20 samples and checked through monitoring of interferences in the GC-MS traces from the matrix in the same retention time of the analytes.

Enzymatic reaction

The ability of the method to evaluate the anti-aromatase activity was tested by comparing the screening of the inhibitory activity and the IC_{50} values determined by GC-MS and by the standard radiometric method of the three third-generation AIs currently used in clinic (Table 4). Using the radiometric assay,

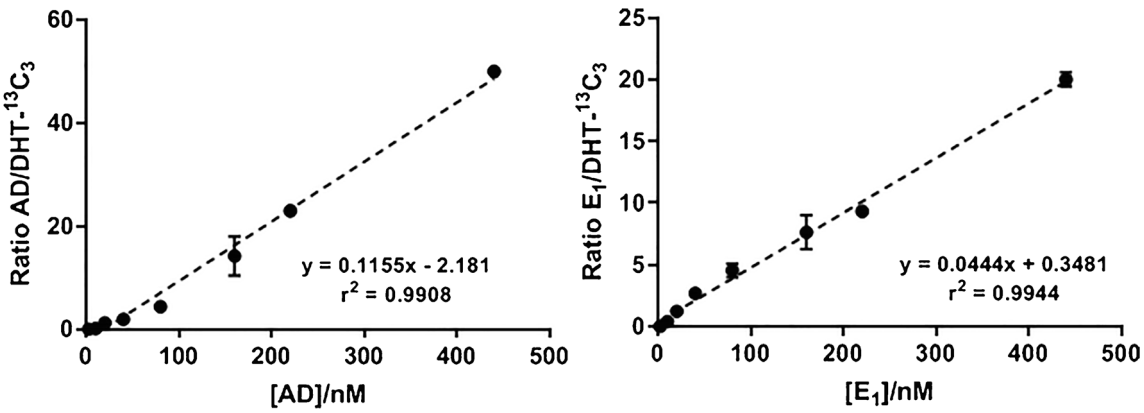

Fig. 2 Linearity of the method evaluated by calibration curves of AD and E_1 in human placental microsomes

Table 3 Intra- and inter-day precision and accuracy for AD and E_1 in human placental microsomes

Analyte	LLOQ			Low concentration (20 nM)			Medium concentration (100 nM)			High concentration (220 nM)		
With placental microsomes	Precision		Accuracy /%	Precision		Accuracy /%	Precision		Accuracy /%	Precision		Accuracy /%
	Intra-assay RSD (%)	Inter-assay RSD (%)		Intra-assay RSD (%)	Inter-assay RSD (%)		Intra-assay RSD (%)	Inter-assay RSD (%)		Intra-assay RSD (%)	Inter-assay RSD (%)	
AD	17	9	89	13	11	83	8	13	74	4	13	98
E_1	9	11	87	9	14	124	10	13	66	5	10	76

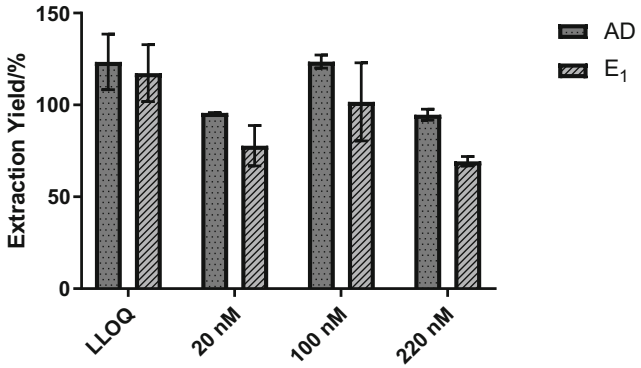

Fig. 3 Extraction yield of the GC-MS method for AD and E_1 at four different concentrations

anastrozole, letrozole, and exemestane presented an anti-aromatase activity of 99.12 ± 0.02%, 99.69 ± 0.06%, and 98.74 ± 0.14% and IC_{50} values of 0.035 μM, 0.002 μM, and 0.050 μM, respectively. Using the GC-MS methodology, anastrozole, letrozole, and exemestane presented an anti-aromatase activity of 90.69 ± 2.65%, 92.16 ± 1.00%, and 94.72 ± 1.88% and IC_{50} values of 0.038 μM, 0.0021 μM, and 0.045 μM, respectively (Fig. 4). Our results proved that the GC-MS method is a suitable alternative to the radiometric assay to assess the anti-aromatase activity. Thus, this method can be applied to determine the anti-aromatase properties of new potential AIs, by measuring the conversion percentage of AD into E_1.

Discussion

A new sensitive, precise, and accurate GC-MS method to quantify AD and E_1 in biological samples was developed, validated, and implemented in the evaluation of the anti-aromatase activity. It was based on a fast and very effective extraction procedure of DLLME, and the quantification of AD and E_1 was achieved by GC-MS using DHT-$^{13}C_3$ as internal standard. Considering FDA guidelines, the developed methodology presented high linearity, good precision, and high accuracy for both hormones [31].

In comparison with other already-described chromatographic methods to evaluate anti-aromatase activity in similar matrices, this new methodology uses lower concentrations of

Table 4 Anti-aromatase activity (%) and IC_{50} values of the third-generation AIs, obtained by radiometric and GC-MS methodologies

	Screening assay (%)		IC_{50} assay (μM)	
	Radiometric	GC-MS	Radiometric	GC-MS
Anastrozole	99.12 ± 0.02	90.69 ± 2.65	0.035	0.038
Letrozole	99.69 ± 0.06	92.16 ± 1.00	0.002	0.0021
Exemestane	98.74 ± 0.14	94.72 ± 1.88	0.05	0.045

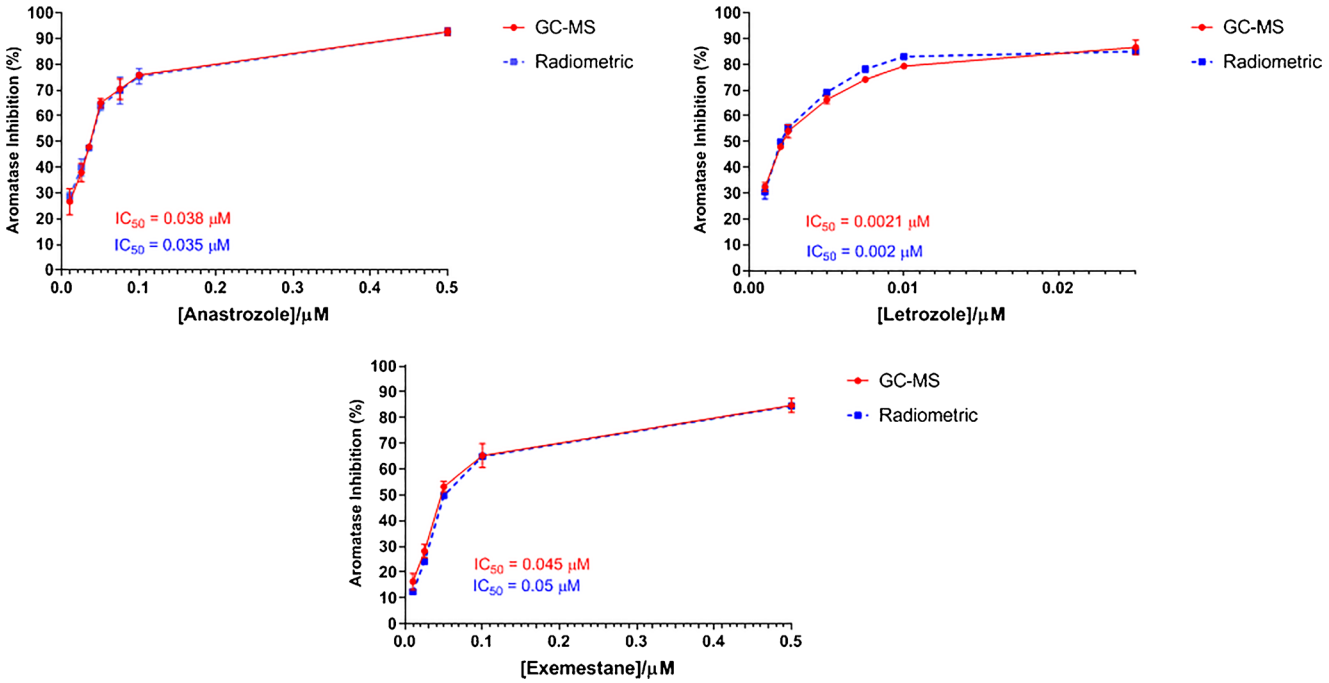

Fig. 4 Comparison between the IC_{50} values obtained for each AI through the radiometric and GC-MS methodologies

AD (100 nM) and NADPH (150 μM), as well as, a lower amount of microsomal protein (20 μg), in a final volume of 0.5 mL. The GC-MS method described by Numazawa et al. (2001) used 300 μM of NADPH and 200 μg of placental protein in a final volume of 2.05 mL [24], while the LC-MS/MS method described by Park et al. (2014) used 1 μM of AD and 50 μg of human CYP 19A1 supersomes in a final volume of 1 mL [23]. In fact, in comparison with other works that also applied chromatographic methods, our methodology used smaller amounts of matrix and reagents. In addition, the LOD described in this work are inferior to the ones described for the same matrix in the aforementioned methodologies [23, 24]. This advantage highlights the sensitivity of the method allowing an accurate determination of E_1, particularly in the screening of potential aromatase inhibitors.

To explore if this new GC-MS method could be a suitable method to estimate the anti-aromatase activity of potential AIs, the anti-aromatase activity (%) and the IC_{50} values of the three third-generation AIs determined by the standard radiometric assay and the new methodology were compared. Results demonstrated that the GC-MS method is sensible in the presence of very potent AIs, as seen by the screening assay. Therefore, this method shows its feasibility in the screening of new potent AIs. Moreover, the IC_{50} values obtained were identical between both methods, more specifically 0.035 μM and 0.038 μM for anastrozole, 0.002 μM or 0.0021 μM for letrozole, and 0.050 μM or 0.045 μM for exemestane. A study conducted by Dukes et al. (1996) described a lower IC_{50} of 0.0146 μM for anastrozole in human placental microsomes, through a radiometric assay [32]. However, it should be pointed that the radiometric methodology used presents some differences, such as the use of T (0.5 μM) instead of AD. In addition, a work employing a LC-MS/MS methodology described an IC_{50} of 0.47 μM for anastrozole in human supersomes [23]. In what concerns the comparison with letrozole, a study described a higher IC_{50}, 0.0115 μM, in human placental microsomes, through a slightly different radiometric assay [33], supporting that our method is also highly sensitive. Therefore, to the best of our knowledge, this is the first study that assesses and compares the IC_{50} values of all the third-generation AIs used in clinic, in the same conditions, with two different methodologies that used lower amounts of a biological matrix and of the natural substrate of aromatase. Our results confirm the potential of this new methodology to be an alternative to the radiometric assay.

Furthermore, to our knowledge, there is no other GC-MS methodology that enables such a rapid, accurate, and sensitive measurement of AD and E_1 and, consequently, of aromatase activity. Thus, this new GC-MS method may be considered a relevant tool for the screening of potential AIs, as well as, for the establishment, together with the structure-activity relationships (SAR) studies, of the best chemical features to obtain potent AIs in drug discovery. It must be pointed that in general and according to the OCSPP Guideline 890.1200, the screening of potential AIs must be performed using radiolabelled AD [34]. Therefore, since this new method presents radiometric-like sensitivity and does not use a radiolabelled substrate, it can be considered a safer, cheaper, and more environmentally friendly approach to assess aromatase activity, being a good alternative to the standard radiometric assay.

Author contributions Conceived and designed the study: TV Augusto, C Amaral, G Correia-da-Silva, N Teixeira. Performed the experiments: TV Augusto, C Amaral, SC Cunha. Analyzed and interpreted the data: TV Augusto, SC Cunha, C Amaral, G Correia-da-Silva, N Teixeira. Wrote the paper: TV Augusto, SC Cunha, C Amaral, G Correia-da-Silva, N Teixeira. Revised the manuscript: SC Cunha, C Amaral, JO Fernandes, E Tavares da Silva, FMF Roleira, G Correia-da-Silva, N Teixeira. Read and approved the manuscript for publication: TV Augusto, SC Cunha, C Amaral, JO Fernandes, E Tavares da Silva, FMF Roleira, G Correia-da-Silva, N Teixeira.

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Compliance with ethical standards

Human term placental tissues were obtained under informed consent and collected after birth. All the procedures concerning human placental handling were performed after approval of the Ethical Committee of Centro Materno Infantil do Norte, Centro Hospitalar do Porto, Portugal.

Conflict of interest The authors declare that they have no conflict of interest.

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Manuscript III:

Differential biological effects of aromatase inhibitors: apoptosis, autophagy, senescence and modulation of the hormonal status in breast cancer cells

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Differential biological effects of aromatase inhibitors: Apoptosis, autophagy, senescence and modulation of the hormonal status in breast cancer cells

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ABSTRACT

Estrogen receptor-positive (ER^+) breast carcinomas are the most common subtype, corresponding to 60% of the cases in premenopausal and 75% in postmenopausal women. The third-generation of aromatase inhibitors (AIs), the non-steroidal Anastrozole (Ana) and Letrozole (Let) and the steroidal Exemestane (Exe), are considered a first-line endocrine therapy for postmenopausal women. Despite their clinical success, the development of resistance is the major setback in clinical practice. Nevertheless, the lack of cross-resistance between AIs hints that these drugs may act through distinct mechanisms. Therefore, this work studied the different effects induced by AIs on biological processes, such as cell proliferation, death, autophagy and senescence. Moreover, their effects on the regulation of the hormonal environment were also explored. The non-steroidal AIs induce senescence, through increased *YPEL3* expression, on aromatase-overexpressing breast cancer cells (MCF-7aro), whereas Exe promotes a cytoprotective autophagy, thus blocking senescence induction. In addition, in a hormone-enriched environment, the non-steroidal AIs prevent estrogen signaling, despite up-regulating the estrogen receptor alpha (ERα), while Exe down-regulates ERα and maintains its activation. In these conditions, all AIs up-regulate the androgen receptor (AR) which blocks *EGR3* transcription in Exe-treated cells. On the other hand, in hormone-depleted conditions, a crosstalk between AR and ERα occurs, enhancing the estrogenic effects of Exe. This indicates that Exe modulates both ERα and AR, while Ana and Let act as pure AIs. Thus, this study highlights the potential clinical benefit of combining AR antagonists with Exe and discourages the sequential use of Exe as second-line therapy in postmenopausal breast cancer.

1. Introduction

Estrogen receptor-positive (ER^+) breast cancer is the subtype with the highest incidence, being about 60% in premenopausal and 75% in postmenopausal patients (Augusto et al., 2018). Similarly to tamoxifen, the third-generation aromatase inhibitors (AIs) are the first-line endocrine therapy option for postmenopausal women with early-stage ER^+ breast cancer, whereas for postmenopausal women with advanced ER^+ breast cancer, the AIs in combination with cyclin-dependent kinase 4/6 (CDK4/6) inhibitors were recently recommended for the clinical setting (Cardoso et al., 2020; Cardoso et al., 2019). Moreover, AIs have recently become a first-line treatment in premenopausal women after ovarian function suppression (Cardoso et al., 2018). The AIs block the conversion of androgens to estrogens by inhibiting the aromatase enzyme, thus depriving ER^+ tumors of estrogen-induced growth (Almeida et al., 2020). The non-steroidal AIs, Anastrozole (Ana) and Letrozole (Let), are derived from triazole, which allows them to interact non-covalently with the heme group of aromatase, inhibiting the enzyme in a reversible manner. Conversely, the steroidal AI Exemestane (Exe) is a structural analogue of androstenedione, the natural substrate of aromatase, and binds covalently to the active site of aromatase in an irreversible

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Abbreviations

7-AAD	7-Amino-acitomycin
Ana	Anastrozole
AR	Androgen receptor
AIs	Aromatase inhibitors
AO	Acridine orange
AVOs	Acid vesicular organelles
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CDK	Cyclin-dependent kinase
CDX	Casodex
DiOC6(3)	3,3′-Dihexyloxacarbocyanine iodide
DHT	Dihydrotestosterone
DMSO	Dimethylsulfoxide
E_2	Estradiol
ERα	Estrogen receptor α
ER^+	Estrogen receptor-positive
Exe	Exemestane
FSC	Forward scatter
ICI	Fulvestrant
IP	Propidium Iodide
LDH	Lactate dehydrogenase
Let	Letrozole
3-MA	3-Methyladenine
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MFI	Mean fluorescence intensity
ΔΨm	Mitochondrial transmembrane potential
PI	Propidium iodide
PS	Phosphatidylserine
RLU	Relative luminescence units
scRNA	Scramble siRNA
SERD	Selective estrogen receptor downregulator
SP	Spautin-1
SSC	Side scatter
STS	Staurosporine
T	Testosterone
β-gal	β-galactosidase

manner. Exe is also known as a *suicidal inhibitor* since aromatase is inhibited by its own catalysis, which, consequently, leads to aromatase degradation by proteasome (Almeida et al., 2020; Sobral et al., 2016; Wang and Chen, 2006). The anti-cancer properties of the AIs have been studied in different ER^+ breast cancer cell models. The AIs disrupted cell cycle and induced apoptosis through alterations in the expression of apoptotic markers, such as increased caspase-6 and -9 activation and decreased Bcl-2 and Bcl-xL levels (Thiantanawat et al., 2003; Itoh et al., 2005; Slingerland, 2008; Amaral et al., 2012) in several MCF-7 derivative cell lines. Contrary to the non-steroidal AIs, Exe also induced a cytoprotective autophagic process in an aromatase-overexpressing breast cancer cell line, MCF-7aro (Amaral et al., 2012).

Despite the success of this therapy over the years, the development of resistance due to long-term estrogen deprivation has become the major concern in the clinical setting (Augusto et al., 2018). Nevertheless, the observed lack of cross-resistance between AIs allowed the switch between steroidal and non-steroidal AIs when the clinical benefit is lost (Lonning, 2008). These observations suggest that the biological, as well as the resistance mechanisms, may be AI-specific, thus highlighting the importance of understanding how they act in breast cancer cells to bypass AIs-acquired resistance. In fact, the AI-resistant cell lines adapt to the lack of estrogen through specific mechanisms. Ana (T + AnaR) and Let (T + LetR) resistant cells have been described as estrogen independent, relying on growth factor receptors to constitutively activate ERα, while Exe resistant cells (T + ExeR) do not present a constitutively active ERα due to the weak agonist effect of Exe (Masri et al., 2008; Chen, 2011). Moreover, AIs-resistant cells generated through the estrogen-deprivation in the absence of AI treatment (LTEDaro) did not correlate with the previous resistant cell lines. In addition, the effects of these AIs in the regulation of the hormonal status of cancerous breast cells have not been widely explored. It has only been reported a pro-death role for androgen receptor (AR) in MCF-7 cells treated with Ana (Chen et al., 2015) or Let (Macedo et al., 2006), while for Exe-treated MCF-7aro cells our group described a protective and oncogenic role for AR. Indeed, we demonstrated that Exe induced AR overexpression and activation, and that AR blockade exacerbated the anti-proliferative effects of Exe (Amaral et al., 2020). It should be noted that AR is a steroid receptor usually expressed in luminal breast cancers, such as ER^+ breast carcinomas (Collins et al., 2011; Proverbs-Singh et al., 2015), and that it is structurally similar to ERα, which allows it to control the expression of ER-regulated genes (Peters et al., 2009; Rechoum et al., 2014). A beneficial role of AR has been reinforced by several studies (Macedo et al., 2006; Peters et al., 2009; Labrie et al., 2003), and is associated with a good prognosis in this subtype of breast cancer (Elebro et al., 2015; Hu et al., 2011). In fact, antagonists of AR are being evaluated in several ongoing clinical trials in breast cancer patients treated with AIs. Therefore, since different studies have indicated different roles for AR in cells treated with non-steroidal and steroidal AIs, it can be hypothesized that AIs might differentially modulate the hormonal environment.

On the other hand, several studies have also reported that senescence, a state of cell cycle arrest, seems to allow cell dormancy and reprogramming to more aggressive tumor cells, leading to an escape from the cell cycle arrest (Camorani et al., 2018; Ortiz-Montero et al., 2017; Karimi-Busheri et al., 2010; Hernandez-Segura et al., 2018). This escape seems to be related to an increase in stem-like features and plasticity, which allows transcriptional variability, promoting tumor proliferation, metastasis and development of acquired resistance (Ortiz-Montero et al., 2017; Hong et al., 2019). Indeed, *Hong* et al. (2019) suggested that AIs resistance was not the result of a selection of pre-treatment resistant cells, but rather the result of several adaptations that would occur over a large period of time, where quiescence acted as an adaptation mechanism for MCF-7 cells in response to endocrine therapy, leading to the development of resistance (Hong et al., 2019). Some studies have described senescence in ER^+ breast cancer cell lines as a response to the lack of estrogen signaling, due to anti-estrogens treatment, such as Tamoxifen or Fulvestrant (ICI), and questioned its correlation with the development of resistance (Lee et al., 2014; Mumcuoglu et al., 2010; Tuttle et al., 2012; Wu et al., 2019). Nevertheless, the induction of senescence has not yet been studied in response to AIs.

Considering all this, this work explored the different effects of AIs on biological processes, such as cell cycle progression, apoptosis, autophagy and senescence induction, as well as on the regulation of the hormonal status, important for cancer progression. Our results may contribute to unveil the reason behind the lack of cross-resistance between AIs observed in clinic.

2. Material and methods

2.1. Cell culture

The aromatase-overexpressing human breast cancer cell line, MCF-7aro, kindly provided by Prof. Shiuan Chen (Beckman Research Institute, City of Hope, Duarte, CA, U.S.A.), is considered the best *in vitro* cell model to study the effects of AIs in ER^+ breast cancer (Zhou et al., 1990; Sun et al., 1997). This cell line was maintained in cell culture conditions

commonly used by our group (Amaral et al., 2020). The assays were performed in MCF-7aro cells in the presence of testosterone (T) (Sigma-Aldrich Co., Saint Louis, USA) at 1 nM, which is the aromatase substrate that stimulates the growth of these cells (Amaral et al., 2012); in the presence of estradiol (E_2) (Sigma-Aldrich Co., Saint Louis, USA), at 1 nM, which is the product of the aromatization reaction (Augusto et al., 2019; Amaral et al., 2017); or without any hormone stimulation. The stock solutions of T and E_2 were prepared in absolute ethanol and stored at −20 °C. The stock solutions of Ana, Let, Exe, 3-Methyladenine (3-MA), Spautin-1 (SP), Casodex (CDX) and ICI (Sigma-Aldrich Co., Saint Louis, USA) were prepared in 100% dimethylsulfoxide (DMSO) and stored at −20 °C. All these stock solutions were diluted in culture medium just prior to the assays and the final concentrations of DMSO and ethanol were lower than 0.01% in all the assays. All the controls used for each assay contained the vehicles in these cell culture conditions. Cells without hormones, or incubated with T (1 nM), with or without CDX (1 μM), ICI (100 nM), 3-MA (1 mM) or SP (0.5 μM), as well as cells incubated with E_2 (1 nM), were considered as controls.

2.2. Cell viability

The effects of Ana (0.1–15 μM), Let (0.1–15 μM) and Exe (1–15 μM) in MCF-7aro cell viability were evaluated by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and by the lactate dehydrogenase (LDH) assay, as previously described (Amaral et al., 2012; Amaral et al., 2015). Cells were seeded in 96-well plates and incubated with AIs, with or without T (1 nM) for 3 (2×10^4 cells/mL) and 6 (1×10^4 cells/mL) days. To verify if the AIs effects were dependent on aromatase and on AR, MCF-7aro cells were incubated with E_2 (1 nM), instead of T, or with T plus CDX (1 μM), respectively. The selected concentration of CDX *per se* caused no effect on MCF-7aro cell viability (Amaral et al., 2020; Augusto et al., 2019; Almeida et al., 2021). Results are expressed as relative percentage of the untreated cells. MCF-7aro cells incubated only with T, E_2, or T plus CDX were designated as controls and represent 100% of cell viability.

2.3. Cell cycle progression

To study the anti-proliferative effects induced by the AIs, cell cycle progression was evaluated by flow cytometry, as previously reported (Amaral et al., 2017). MCF-7aro cells were seeded in 6-well plates and incubated with AIs (10 μM) during 3 (7×10^5 cells/mL) and 6 (3.5×10^5 cells/mL) days. 40 000 events were acquired to assess DNA content by a BD Accuri™ C6 cytometer (San José, CA, USA), equipped with a BD Accuri™ C6 software for data analysis. Detectors for the three fluorescent channels (FL-1, FL-2 and FL-3) and for forward (FSC) and side (SSC) light scatter were set on a linear scale. The percentage of cells in G_0/G_1, S and G_2/M cell cycle phases were used to express the anti-proliferative effects.

2.4. Cell death assays

Cell death was studied by the analysis of the translocation of phosphatidylserine (PS), through Annexin V-FITC labelling, pro-caspase-7 levels, caspase-9 activity, as well as through the mitochondrial transmembrane potential (ΔΨm). To study translocation of PS, cells were cultured in 6-well plates (7×10^5 cells/mL) and treated with AIs (10 μM) for 3 days. After washing with PBS, cells were incubated with Annexin V-FITC Apoptosis Detection Kit (BioLegend Way, San Diego, USA), according to the manufacturer's instructions. Cells treated with Staurosporine (STS) (SigmaAldrich Co., Saint Louis, USA), at 10 μM, were considered as positive control. Analysis was performed in a BD Accuri™ C6 cytometer (San Jose, CA, USA), equipped with BD Accuri™ C6 analysis software. All the fluorescence channels (FL-1, FL-2 and FL-3) detectors were set on a logarithmic scale. Bivariant analysis of Annexin-FITC fluorescence (FL-1) and 7-amino-acitomycin (7-AAD) fluorescence (FL-3) distinguished different cell populations: Annexin V^-/7-AAD^- were considered as viable cells, Annexin V^+/7-AAD^- as apoptotic cells and Annexin V^+/7-AAD^+ as late apoptotic and necrotic cells. For the luminescent and fluorescent assays, MCF-7aro cells were seeded in 96-well plates (2×10^4 cells/mL) and incubated with AIs (10 μM) for 3 days. Pro-caspase-7 levels were evaluated by Western-blot. Caspase-9 activity was determined by using the Caspase-Glo® 9 kit (Promega Corporation, Madison, USA) and STS at 10 μM was used as positive control. The ΔΨm was assessed by using the fluorescence probe 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3)) (Gibco Invitrogen Co., Paisley, Scotland, UK), as previously described (Amaral et al., 2018). The mitochondrial depolarizing agent carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma-Aldrich Co., Saint Louis, USA), at 10 μM, was used as positive control. The luminescent and fluorescent signals were measured in a 96-well Microplate Luminometer (Synergy HT, BioTek, USA), with the values representing the mean of relative luminescence units (RLU) and the mean of fluorescence intensity (MFI), respectively. For fluorescence intensity, the excitation wavelength of 480 nm and the emission filter of 530 nm were used. In all the experiments, basal fluorescence was subtracted.

2.5. Acid vesicle organelles (AVOs) detection by flow cytometry

The formation of acid vesicular organelles (AVOs) was studied by fluorescence microscopy and flow cytometry. To observe the AVOs through fluorescence microscopy, cells were seeded in 6-well plates, treated with Ana and Let, at 10 μM, during 3 and 6 days (7×10^5 and 3.5×10^5 cells/mL, respectively), washed and incubated with acridine orange (AO) (0.1 μg/mL). The presence of AVOs was indicated by the yellow/orange/red fluorescence detected by a fluorescence microscope (Eclipse Ci, Nikon, Japan) equipped with a 490 nm band-pass blue excitation filter and a 515 nm long-pass barrier filter and processed by Nikon NIS Elements v 4.0 image software. For the quantification of AVOs through flow cytometry, the AI-treated cells were incubated with or without the autophagic inhibitors, SP, at 0.5 μM, or 3-MA, at 1 mM, for 6 days. Exe, at 10 μM, was used as a positive control for autophagy (Amaral et al., 2012). After incubation, cells were trypsinized, washed and stained with AO (0.5 μg/ml). 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 light scatter detectors, as well as FL-1 and FL-3 channels were set on a linear scale. Analysis of AO^- (green fluorescence) and AO^+ (red fluorescence) distinguished viable cells from cells with AVOs, respectively.

2.6. β-galactosidase (β-gal) accumulation

MCF-7aro cells were seeded in 24-well plates (2×10^5 cells/mL) and incubated with AIs (10 μM) for 3 days. β-gal accumulation was assessed through the senescence β-Galactosidade Staining kit (Cell Signaling Technology, Danvers, MA, USA), according to the manufacturer's protocol. After the incubation time, cells were washed, fixed for 15 min and

then, stained for β-gal (1:10) overnight at 37 °C, pH 6.0. β-gal accumulation was verified under the microscope (Eclipse Ci, Nikon, Japan) equipped with the image analysis software Nikon NIS Elements v 4.0.

2.7. Western blot analysis

The expression levels of LC3-I/II, cyclin D, cyclin E, pro-caspase 7, aromatase, p-ER^{S118}, p-ER^{S167}, ERα and AR were evaluated by Western-blot. MCF-7aro cells cultured in 6-well plates (7×10^5 cells/mL) were treated with AIs in the absence of hormones (10 μM) for 8 h (for aromatase) and with AIs (10 μM) or ICI (100 nM), with or without T (1 nM) for 3 days. Protein extraction and separation was performed through a 14% SDS-PAGE (for LC3) or a 10% SDS-PAGE (Amaral et al., 2018, 2020). For immunodetection the rabbit polyclonal antibodies used were: anti-cyclin D (1:200), anti-LC3 I/II (1:200), anti-p-ER^{S167} (1:200); and the mouse monoclonal antibodies used were: anti-pER^{S118} (1:200) (Cell Signaling Technology Inc., Boston, USA), anti-cyclin E (1:200), anti-pro-caspase-7 (1:200), anti-aromatase (1:200), anti-ERα (1:200) anti-AR (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibodies used were the peroxidase anti-mouse and anti-rabbit antibodies (1:2000) (Thermo Fisher, Waltham, MA, USA). The mouse monoclonal anti-β-tubulin and anti-β-actin antibody (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as loading controls. Immunoreactive bands were visualized using a chemiluminescent substrate WesternBright™ ECL (Advansta Inc., Menlo Park, CA, USA) and a ChemiDocTM Touch Imaging System (Bio-Rad, Laboratories Melville, NY, USA).

2.8. RNA extraction and qPCR

MCF-7aro cells were seeded in 6-well plates (7×10^5 cells/mL) and treated with AIs (10 μM), with or without T (1 nM), CDX (1 μM) or ICI (100 nM), for 3 days. Cells were further lysed and the RNA collected using the TripleXtractor reagent (GRiSP Research Solutions, Porto, Portugal), according to the manufacturer's protocol. Total RNA was quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). GRiSP Xpert cDNA Synthesis Mastermix (GRiSP Research Solutions, Porto, Portugal) was used to obtain cDNA, which was amplified with specific primers, using GRiSP Xpert Fast SYBR (GRiSP Research Solutions, Porto, Portugal), in Mini-Opticon Real-Time PCR Detection System (Bio-Rad Laboratories) (Amaral et al., 2020). Primer sequences (5′-3′) are presented in Table 1. The fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, using as housekeeping genes, *TUBA1A* and *ACTB*.

2.9. siRNA transfection

siRNA transfection was performed using siPORT NeoFX transfection agent (Gibco Invitrogen Co., Paisley, Scotland, UK), according to manufacturer's instruction on reverse transfection. For each well, 5 μL siPORT NeoFX transfection agent and 14 μL of siRNA negative control (10 μM) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or of siRNA against ERα (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted in 100 μL OPTI-MEM I medium. After mixing both solutions and incubating for 10 min, MCF-7aro cells were trypsinized, diluted in the mixture of siRNA and transfection agent, and further dispersed in 6-well plates (1×10^5/mL). After adhesion, MCF-7aro cells were treated with Exe (10 μM) for 3 days.

2.10. Statistical analysis

Assays were carried out in triplicate in at least three independent experiments, and data was expressed as the mean ± SEM. Statistical analysis was performed with GraphPad Prism 7® software through analysis of variance (ANOVA) followed by Tukey and Bonferroni post-hoc tests for multiple comparisons, as already described (Amaral et al., 2018). Values of $P < 0.05$ were considered as statistically significant.

3. Results

3.1. Anti-proliferative effects of AIs

Our group has previously reported that Exe decreased the viability of MCF-7aro cells in a dose- and time-dependent manner (Amaral et al., 2012). The effects of the non-steroidal AIs, Ana and Let, in MCF-7aro cell viability were evaluated by MTT assay. T, at 1 nM, was used as a proliferation-inducing agent since it is an aromatase substrate (Augusto et al., 2019). Results demonstrated that, after 3 and 6 days of treatment, the non-steroidal AIs significantly ($p < 0.001$) decreased cell viability in a dose- and time-dependent manner (Fig. 1). This decrease in cell viability was not associated with LDH release (data not shown), excluding the occurrence of cell membrane rupture.

To investigate if the decrease in cell viability was a consequence of cell cycle dysregulation, the effects of Ana and Let on cell cycle progression were also investigated by flow cytometry, after 3 and 6 days (Table 2). Results demonstrate that Ana and Let at 10 μM induce a significant ($p < 0.001$) cell cycle arrest at G_0/G_1 phase and, consequently, a significant ($p < 0.001$) reduction on S phase.

Considering these effects on cell cycle progression, the effects of AIs on cyclins that regulate G_0/G_1 and S phases, were also explored through

Table 1
Primer sequences and qPCR conditions for target genes.

Target gene	Primer sequences (5′-3′)		Ta/°C
	Sense	Anti-sense	
SQSTM1	GGAGTCGGATAACTGTTC	GATTCTGGCATCTGTAGG	58
LMNB1	GCATCTCTCATTCCGCCTCA	GCCTCCCATTGGTTGATCCT	55
YPEL3	TGGATGATTGTCACCGGAGG	AGTTGAAGAGGTAGGCACGC	56
AR	TGTCCATCTTGTCGTCTTCG	ATGGCTTCCAGGACATTCAG	55
ESR1	CCTGATCATGGAGGGTCAAA	TGGGCTTACTGACCAACCTG	55
EGR3	GACTCCCCTTCCAACTGGTG	GGATACATGGCCTCCACGTC	56
AREG	TGTCGCTCTTGATACTCGGC	ATGGTTCACGCTTCCCAGAG	56
TFF1	GTGGTTTTCCTGGTGTCACG	AGGATAGAAGCACCAGGGGA	55
ACTB	TACAGCTTCACCACCACAGC	AAGGAAGGCTGGAAGAGAGC	55
TUBA1A	CTGGAGCACTCTGATTGT	ATAAGGCGGTTAAGGTTAGT	55

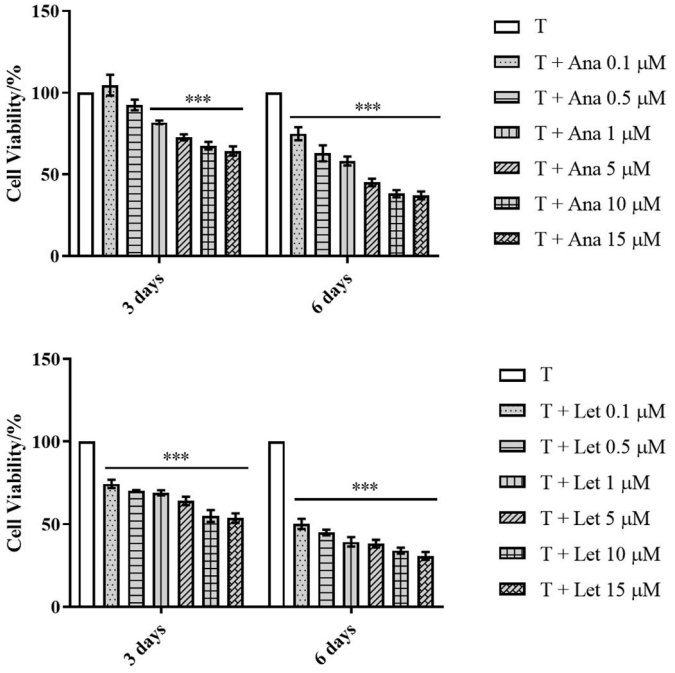

Fig. 1. Effects of Ana and Let on MCF-7aro cell viability. Cells were treated with T (1 nM) and different concentrations of AIs (0.1–15 μM) for 3 and 6 days. Cells treated only with T were considered as controls, representing 100% of cell viability. Results are presented as mean ± SEM of at least three independent experiments, performed in triplicate. Statistically significant differences between AI-treated cells and control cells are expressed as *** ($p < 0.001$).

Western-Blot. Besides Ana and Let, the effects of Exe were also studied, at 10 μM, since we have previously verified that this steroidal AI also disrupted cell cycle of MCF-7aro cells at G_0/G_1 phase (Amaral et al., 2012, 2020). All AIs significantly ($p < 0.05$; $p < 0.01$; $p < 0.001$) decreased cyclin D and cyclin E expression levels, when compared to T-only treated cells (Fig. 2A and B), and the effects were more pronounced for Let.

Table 2
Effects of Ana and Let on MCF-7aro cell cycle progression.

Cell cycle phase	*3 days*			*6 days*		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
T	76.12 ± 1.04	8.81 ± 0.17	14.46 ± 0.85	87.22 ± 0.37	7.60 ± 0.35	5.49 ± 0.36
T + Ana	87.58 ± 1.25***	2.38 ± 0.27***	9.46 ± 1.17***	94.72 ± 0.07***	1.10 ± 0.10***	3.61 ± 0.12
T + Let	90.02 ± 0.53***	1.20 ± 0.16***	8.09 ± 0.51***	92.55 ± 1.27***	1.15 ± 0.16***	5.54 ± 0.98

MCF-7aro cells were stimulated with T (1 nM) and treated with Ana or Let (10 μM) for 3 and 6 days. Cells treated only with T (1 nM) were considered as controls. Cells were analysed by flow cytometry after staining with propidium iodide (PI) (5 μg/ml). Ana and Let induced cell cycle arrest in G_0/G_1 phase. Values are represented as a percentage of single cell events in each stage of the cell cycle and are the mean ± SEM of at least three independent experiments performed in triplicate. Statistically significant differences between AI-treated cells and control cells are expressed as *** ($p < 0.001$).

3.2. Effects of non-steroidal AIs on apoptosis induction

Considering that our group previously reported that Exe induced apoptosis in MCF-7aro cells (Amaral et al., 2012, 2020), and since this process may be an additional mechanism involved in the decrease of cell viability, the apoptotic biomarkers, such as the Annexin V-FITC/7-AAD binding, the expression levels of pro-caspase-7, the caspase-9 activity and the ΔΨm, were evaluated. Results show that, after 3 days, Ana and Let significantly ($p < 0.01$; $p < 0.001$) increased Annexin V-FITC binding, when compared to T-only treated cells (Table 3).

To confirm the occurrence of apoptosis, the expression levels of pro-caspase-7 were also explored (Fig. 3A). All AIs significantly ($p < 0.05$; $p < 0.01$; $p < 0.001$) decreased pro-caspase-7 levels. Moreover, the decrease in pro-caspase-7 levels observed for Exe correlated with the previously described increase in caspase-7 activity (Amaral et al., 2012, 2020), which suggests that the decrease in pro-caspase-7 levels is indicative of apoptosis. Furthermore, like Exe, the non-steroidal AIs significantly ($p < 0.01$; $p < 0.001$) increased caspase-9 activity (20.25% for Ana and 29.53% for Let) and significantly ($p < 0.001$) decreased ΔΨm (18.61% for Ana and 18.04% for Let), as shown in Fig. 3B and C. As expected, the positive controls for apoptosis (STS) and ΔΨm (CCCP) increased Annexin V-FITC binding and reduced the ΔΨm, respectively.

3.3. Autophagy induction

Our group has previously demonstrated that Exe promotes autophagy in MCF-7aro cells as a survival process (Amaral et al., 2012). Therefore, to evaluate whether the non-steroidal AIs could also promote autophagy, the formation of AVOs was evaluated by AO staining, through fluorescence microscopy and flow cytometry. After 3 days of treatment, a shift from green fluorescence to yellow/orange/red fluorescence was detected (Fig. 4A), suggesting an increment in AVOs formation.

This increase was exacerbated after 6 days of treatment and, in fact, a significant ($p < 0.001$) increment of AO^+ cell population, like in Exe-treated cells, was detected by flow cytometry (Table 4).

To confirm if the increase in AVOs was associated with an autophagic process, the levels of LC3-I/II and the expression of the autophagic p62 encoding gene, *SQSTM1*, were analysed. Contrary to Exe, the non-steroidal AIs did not increase the levels of LC3-II (Fig. 4B) or *SQSTM1* (Fig. 4C), when compared to T-only treated cells. Also, contrary to Exe, Ana and Let-treated cells incubated with the autophagic inhibitors SP and 3-MA did not reduce the AO^+ cell population (Table 4). This suggests that the acid vesicle organelles observed in non-steroidal AIs treatment are distinct from autophagosomes and autolysosomes.

3.4. Senescence-associated markers

The decrease in cell viability, the cell cycle arrest at the G_0/G_1 phase, the loss of ΔΨm and the high lysosomal content might be associated with senescence (Hernandez-Segura et al., 2018; Vasileiou et al., 2019). Therefore, considering this and our data, the presence of the biomarker of senescence, β-gal accumulation, was explored. All AIs promoted the accumulation of β-gal (Fig. 5A). According to the literature (Lee et al., 2006), the β-gal accumulation observed for the non-steroidal AIs may be linked to lysosomal β-gal, which corroborates the increment of AVOs. In addition, all the AIs significantly ($p < 0.01$) down-regulated *LMNB1* gene, which is a marker associated to senescence (Fig. 5B). However, only the non-steroidal AIs significantly ($p < 0.001$) up-regulated the *YPEL3* gene (Fig. 5C), which has been reported as a senescence-associated gene in cells treated with Tamoxifen or ICI (Lee et al., 2014; Mumcuoglu et al., 2010; Tuttle et al., 2012; Wu et al., 2019).

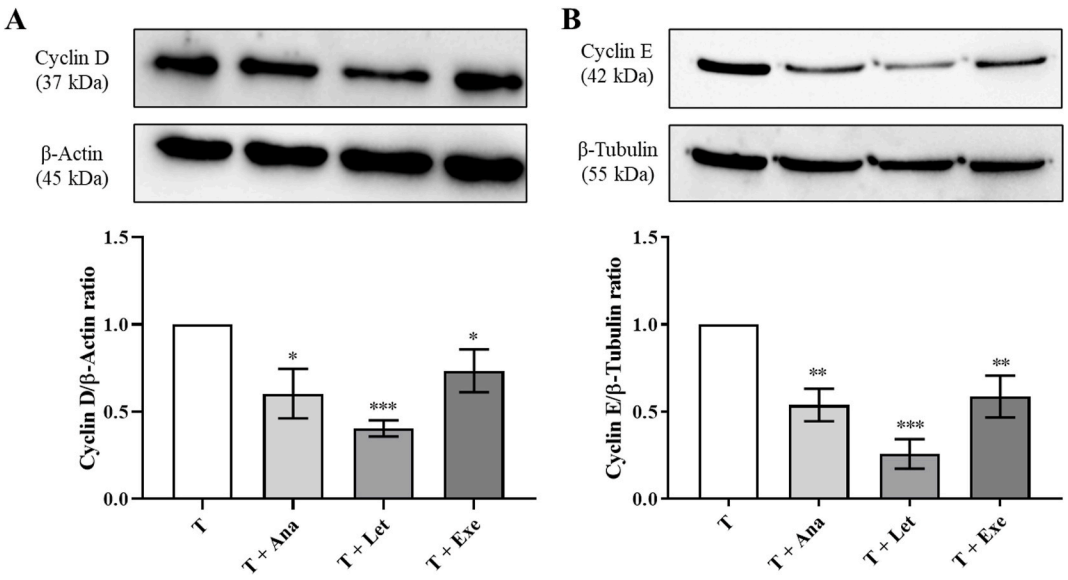

Fig. 2. Effects of AIs on cyclin expression. MCF-7aro cells were stimulated with T (1 nM) and treated with Ana, Let or Exe (10 μM) for 3 days. Cells treated only with T were considered as control. Effects of the AIs on cyclin D (**A**) and cyclin E (**B**) protein levels. β-Actin and β-Tubulin were used as loading controls. The results are presented as mean ± SEM of at least three independent experiments, performed in triplicate. Statistically significant differences between AI-treated cells and control cells are expressed as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

The inability of Exe to induce *YPEL3* transcription indicates that this AI did not cause senescence at the same extent as the non-steroidal AIs. On the other hand, considering that autophagy has been described either as a promoter or as an inhibitor of senescence (Kwon et al., 2017; Slobodnyuk et al., 2019; Gewirtz, 2013), and to explore this association, Exe-treated cells were incubated with the autophagic inhibitor, 3-MA. Interestingly, the inhibition of autophagy significantly ($p < 0.001$) induced the transcription of the *YPEL3* gene, when compared to Exe-only treated cells (Fig. 5D).

3.5. AIs effects on estrogen signaling pathways

AIs are a class of drugs that block the conversion of androgens to estrogens inhibiting aromatase enzyme, thereby resulting in significant estrogen depletion. To explore if the effects on cell viability were directly dependent on estrogen deprivation, through aromatase inhibition, MCF-7aro cells were treated with Ana and Let in the presence of either T or E_2 (Fig. 6A). In the presence of E_2, Ana and Let did not affect cell viability, thus suggesting a dependency on aromatase inhibition, a behaviour similar to that previously reported for Exe (Varela et al., 2014). In addition, the effects of the non-steroidal AIs on aromatase protein levels were also evaluated (Fig. 6B) and, contrarily to Exe (Wang and Chen, 2006; Augusto et al., 2019; Amaral et al., 2017), Ana and Let did not affect the aromatase protein levels.

Table 3
Effects of Ana and Let in Annexin V-FITC labelling in MCF-7aro cells.

	T	T + Ana	T + Let	T + STS
Annexin V^-/7-AAD$^-$	94.17 ± 0.62	76.92 ± 1.81***	75.21 ± 2.06***	67.89 ± 0.70***
Annexin V^+/7-AAD$^-$	2.40 ± 0.51	8.31 ± 1.08**	10.47 ± 0.69***	7.54 ± 1.39*
Annexin V^+/7-AAD$^+$	3.43 ± 0.30	14.77 ± 1.35***	14.32 ± 1.80***	24.57 ± 0.86***

Cells incubated with T and Ana or Let (10 μM) for 3 days were labeled with Annexin V-FITC and 7-AAD followed by flow cytometry analysis. Data based on the acquisition of 40 000 events are presented as viable cells (Annexin V^-/7-AAD^-), early apoptotic (Annexin V^+/7-AAD^-) and late apoptotic or necrotic cells (Annexin V^+/7-AAD^+). Cells cultured only with T were considered as control, while cells treated with STS (10 μM) were considered as positive control for apoptosis. The results are expressed as mean ± SEM of three independent experiments, performed in triplicate. Significant differences among the control and AI-treated cells are denoted by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

Considering that AIs may deregulate the hormonal environment, due to aromatase inhibition, which, consequently, leads to estrogen deprivation and androgen accumulation, the impact of AIs on ERα expression/activation was analysed by mRNA expression and Western-blot. Ana and Let treatment significantly ($p < 0.001$) increased ERα protein levels, while, curiously, Exe induced a significant ($p < 0.001$) decrease (Fig. 7A), in a similar way to ICI. In addition, the non-steroidal AIs caused a pronounced increase on *ESR1* expression ($p < 0.001$), while no alterations were observed for Exe-treated cells (Fig. 7A). Interestingly, MCF-7aro cells deprived of hormones (T and E_2), for 3 days, also revealed an increase in ERα protein levels (Fig. 7B). Nevertheless, the non-steroidal AIs significantly ($p < 0.001$) decreased the phosphorylation of ERα both at S118 and S167 sites, while Exe did not alter ERα phosphorylation (Fig. 7C). To correlate the phosphorylation levels with ERα activation status, the expression of three ERα-regulated genes (*EGR3*, *TFF1* and *AREG*) were explored (Fig. 7D). All AIs significantly ($p < 0.01$; $p < 0.001$) reduced the expression of *TFF1* and *AREG*, while only the non-steroidal AIs significantly ($p < 0.05$; $p < 0.001$) decreased *EGR3*. As expected, ICI, used as positive control, also reduced the transcription of *EGR3*, *TFF1* and *AREG*.

To verify if the maintenance of *EGR3* transcript levels was a consequence of ERα signaling on Exe-treated cells, ERα was silenced through siRNA (Fig. 8A) and the transcript levels were evaluated. Comparing to scramble siRNA (scRNA), ERα silencing *per se* was able to significantly ($p < 0.05$) decrease *EGR3* mRNA expression (Fig. 8B). In cells treated with siRNA and Exe, the levels of gene expression were reduced ($p < 0.05$; $p < 0.01$), when compared to scRNA and to scRNA plus Exe, showing the dependence on ERα. However, in cells treated with siRNA and incubated with Exe, the levels of expression were similar to the ones observed in cells treated only with siRNA.

Since our data indicated that, unlike Ana and Let, Exe did not induce senescence and maintained ERα activity, and that Tamoxifen and ICI exhibit senescence as a response to the blockade of estrogen signaling (Lee et al., 2014; Mumcuoglu et al., 2010; Tuttle et al., 2012; Wu et al.,

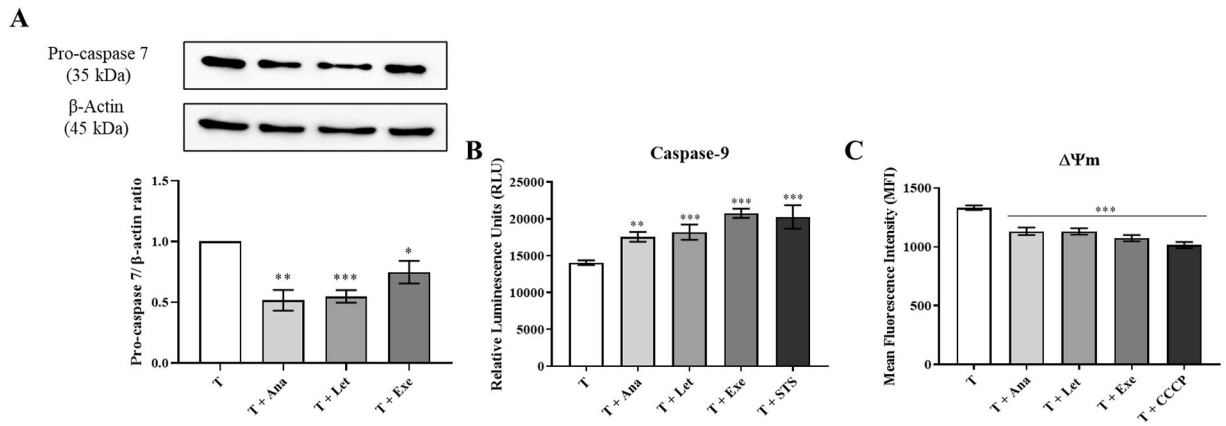

Fig. 3. Effects of AIs on apoptotic markers after 3 days of treatment. (A) Western-blot analysis of the levels of pro-caspase 7 in T-treated MCF-7aro cells incubated with AIs (10 μM). (B) Caspase-9 activity luminescence assay of T-treated MCF-7aro cells incubated with AIs (10 μM). T + STS, at 10 μM, was used as positive control. Values are presented as relative luminescence units (RLU). (C) Loss of ΔΨm fluorescence assay of T-treated MCF-7aro cells incubated with AIs (10 μM). CCCP, at 10 μM, was used as positive control. For fluorescence intensity the excitation wavelength of 480 nm and the emission filter of 530 nm were used. Cells cultured only with T were considered as control. Values were represented as mean fluorescence intensity (MFI). The results are expressed as mean ± SEM of three independent experiments, performed in triplicate. Significant differences among the control and AI-treated cells are denoted by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

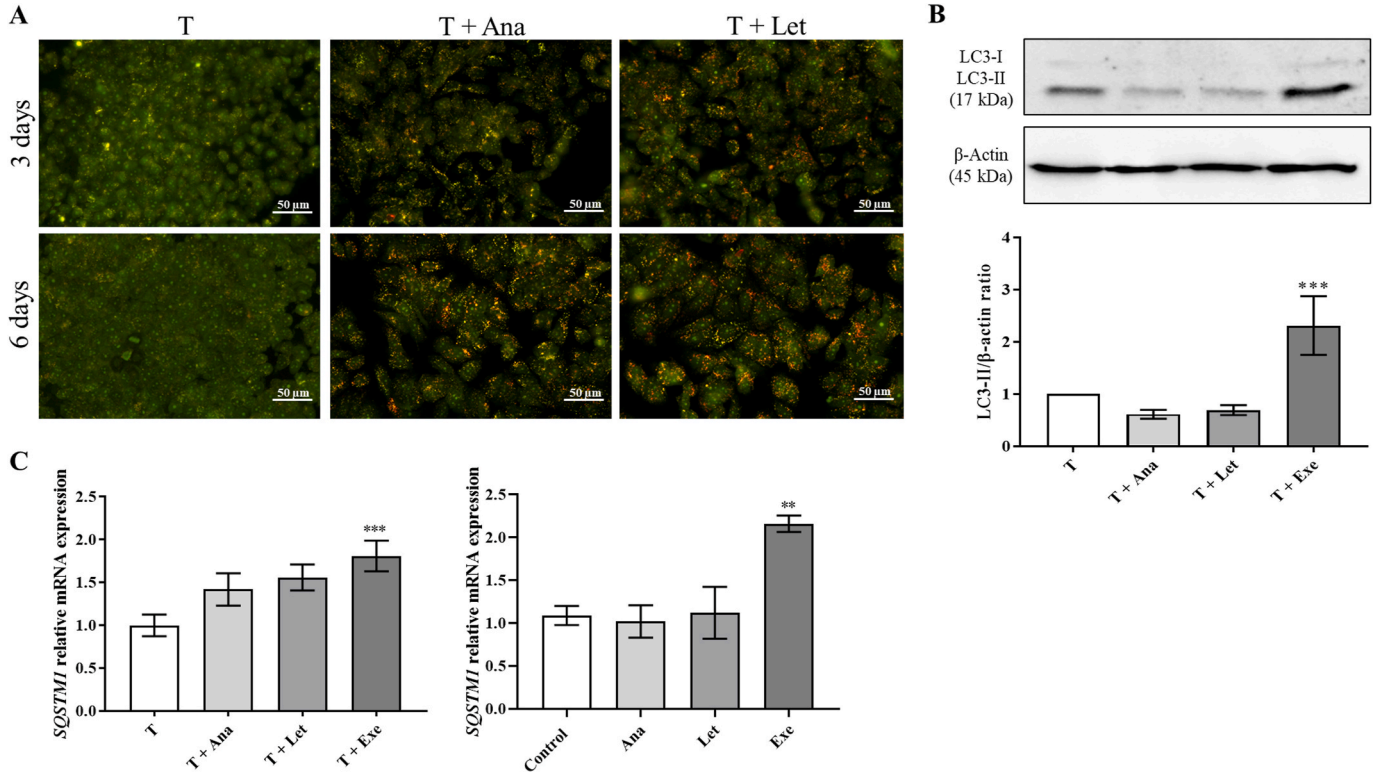

Fig. 4. Effects of AIs on autophagy induction after 3 days of treatment. (A) Analysis of AVOs formation after acridine orange staining by fluorescence microscopy in T-treated MCF-7aro cells incubated with AIs (10 μM). AVOs formation is indicated by the yellow/orange/red fluorescence. (B) Western-blot analysis of LC3-II expression in T-treated MCF-7aro cells incubated with AIs (10 μM). β-actin was used as a loading control. (C) *SQSTM1* relative mRNA expression in MCF-7aro, with or without T, and incubated with AIs (10 μM). Cells cultured with or without T were considered as control. Results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the control versus treated cells are indicated by ** ($p < 0.01$) and *** ($p < 0.001$).

2019), the mRNA transcript levels of *YPEL3* were also investigated after ERα silencing. The results demonstrated that, contrary to what happens with *EGR3* expression, ERα silencing *per se* did not affect *YPEL3* gene expression. However, Exe treatment in ER-silenced cells, significantly ($p < 0.01$) increased *YPEL3* transcription levels when compared to cells treated with scRNA and with scRNA plus Exe (Fig. 8C), suggesting a link between the lack of senescence and ERα signaling in Exe-treated cells.

3.6. Involvement of AR in AI anti-proliferative effects

Considering that the effects of Exe (Amaral et al., 2020) and Let (Macedo et al., 2006) on ER^+ breast cancer cells are dependent on AR activation, and that this receptor interacts with ERα in breast cancer cells (Peters et al., 2009; Panet-Raymond et al., 2000; Lanzino et al., 2005), MCF-7aro cells were treated with the non-steroidal AIs in the

Table 4
Effects of the AIs on the formation of AVOs in T-treated MCF-7aro cells.

	AO^-	AO^+
T	89.97 ± 0.50	10.35 ± 0.54
T + Ana	57.65 ± 1.49***	42.89 ± 1.52***
T + Let	46.19 ± 2.15***	54.23 ± 2.07***
T + Exe	39.43 ± 5.20***	57.24 ± 1.78***
T + 3-MA	88.69 ± 0.53	11.65 ± 0.67
T + Ana + 3-MA	46.16 ± 1.73***	54.57 ± 1.71***
T + Let + 3-MA	47.43 ± 1.39***	52.96 ± 1.42***
T + Exe + 3-MA	82.24 ± 1.61###	17.987 ± 1.52###
T + SP	83.10 ± 1.15***	17.76 ± 1.30***
T + Ana + SP	47.38 ± 1.00***	53.43 ± 1.03***
T + Let + SP	51.43 ± 1.08***	49.40 ± 1.09***
T + Exe + SP	77.53 ± 3.18###	23.03 ± 3.30###

Cells were treated with AIs (10 μM) and with the autophagic inhibitors, 3-MA (1 mM) and SP (0.5 μM), for 6 days. T-treated cells were considered as control. Cells were incubated with AO (0.5 μg/mL) and analysed by flow cytometry. Exe was considered as positive control for autophagy. Data is presented as single cell events of viable cells, AO negative (AO^-), and with AVOs formation, AO positive (AO^+). The data represents means and SEM of triplicates and is representative of three independent experiments. Significant differences between the control versus treated cells are indicated by ***($p < 0.001$) and between Exe versus the combination of Exe with inhibitors are indicated by ### ($p < 0.001$).

presence of the AR antagonist, CDX (Fig. 9A). The presence of CDX significantly ($p < 0.001$) abrogated the anti-proliferative effects induced by the non-steroidal AIs, suggesting a pro-death role for AR. This is in agreement with what was previously reported for Let (Macedo et al., 2006), though contrary to the pro-survival effect observed for Exe (Amaral et al., 2020). In addition, the AIs significantly ($p < 0.01$; $p < 0.001$) increased AR protein levels and transcription of the *AR* gene (Fig. 9B), a behaviour similar to Exe (Amaral et al., 2020). To further assess if the hormonal environment could affect AR levels, MCF-7aro cells were incubated in the absence of T (Fig. 9C). Curiously, in this case AR levels were significantly ($p < 0.01$) decreased when compared to T-treated cells, suggesting that the increase in AR might be a consequence of the accumulation of androgens caused by the inhibition of aromatase. In addition, to understand the possible crosstalk between AR and ERα, the transcription of the three ERα-regulated genes was also investigated in the presence or absence of CDX (Fig. 9D). Blocking AR with CDX in cells treated with Exe induces a significant increase ($p < 0.01$) in *EGR3* expression, when compared to Exe. However, CDX treatment did not affect the Exe-induced effects on the expression of *AREG* and *TFF1* genes, thus revealing some form of specific targeting for the *EGR3* gene. Curiously, the AR blockage does not affect *EGR3* expression, after Ana and Let treatment, when compared to Ana and Let alone (Fig. 9E).

3.7. Effects of AIs in hormone-deprivation conditions

The results described above suggest that Exe somewhat sustains ERα signaling. However, the potential estrogenic-like effects of Exe in these breast cancer cells could be masked by the addition of T, as a proliferative agent. Therefore, to understand the effects of Exe, the impact of all the AIs on hormone-deprived MCF-7aro cells was explored after 3 and 6 days. In these conditions, control cells did not reach confluence and some dead cells were present. Contrary to Ana and Let, the addition of Exe drastically changed cell density, since they became confluent and with less dead cells (Fig. 10A). Curiously, this effect was reverted by adding ICI to Exe-treated cells (Fig. 10A). This proliferative action of Exe was also confirmed by the MTT assay (Fig. 10B), an effect that was significantly ($p < 0.001$) reverted by the addition of ICI or of CDX, both at 3 (data not shown) and 6 days (Fig. 10C). The expression of the ERα-

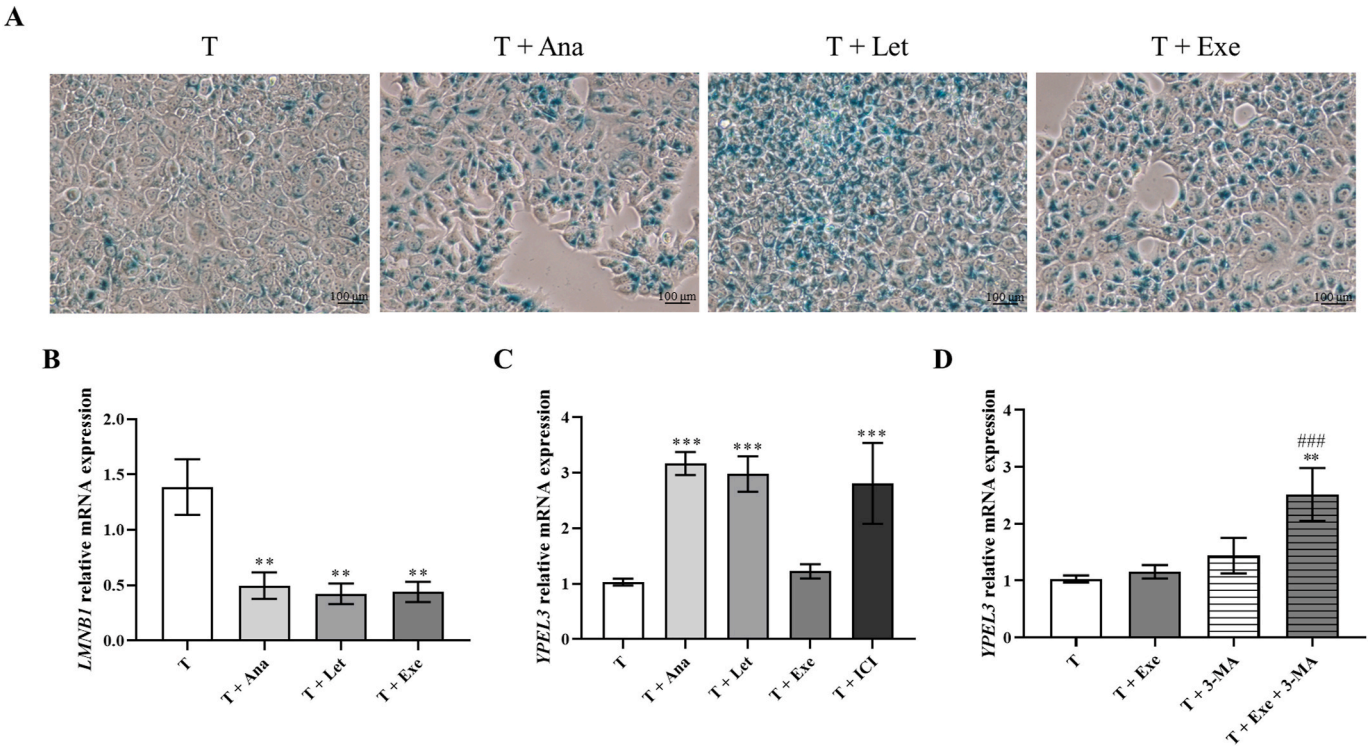

Fig. 5. Effects of AIs on senescence-associated markers in T-treated MCF-7aro cells. (A) β-gal accumulation was assessed through optical microscopy. Cells were incubated with AIs (10 μM). T-treated cells were considered as control. (B) *LMNB1* relative mRNA expression of cells incubated with AIs (10 μM). (C) *YPEL3* relative mRNA expression of cells incubated with AIs (10 μM). ICI was used as a positive control for the *YPEL3* induction. (D) *YPEL3* relative mRNA expression of cells incubated with AIs (10 μM) and with the autophagic inhibitor, 3-MA (1 mM). MCF-7aro cells cultured only with T were considered as control. Results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the control versus treated cells are indicated by ** ($p < 0.01$) and *** ($p < 0.001$). Significant differences between the Exe-treated versus cells treated with Exe and 3-MA are indicated by ### ($p < 0.001$).

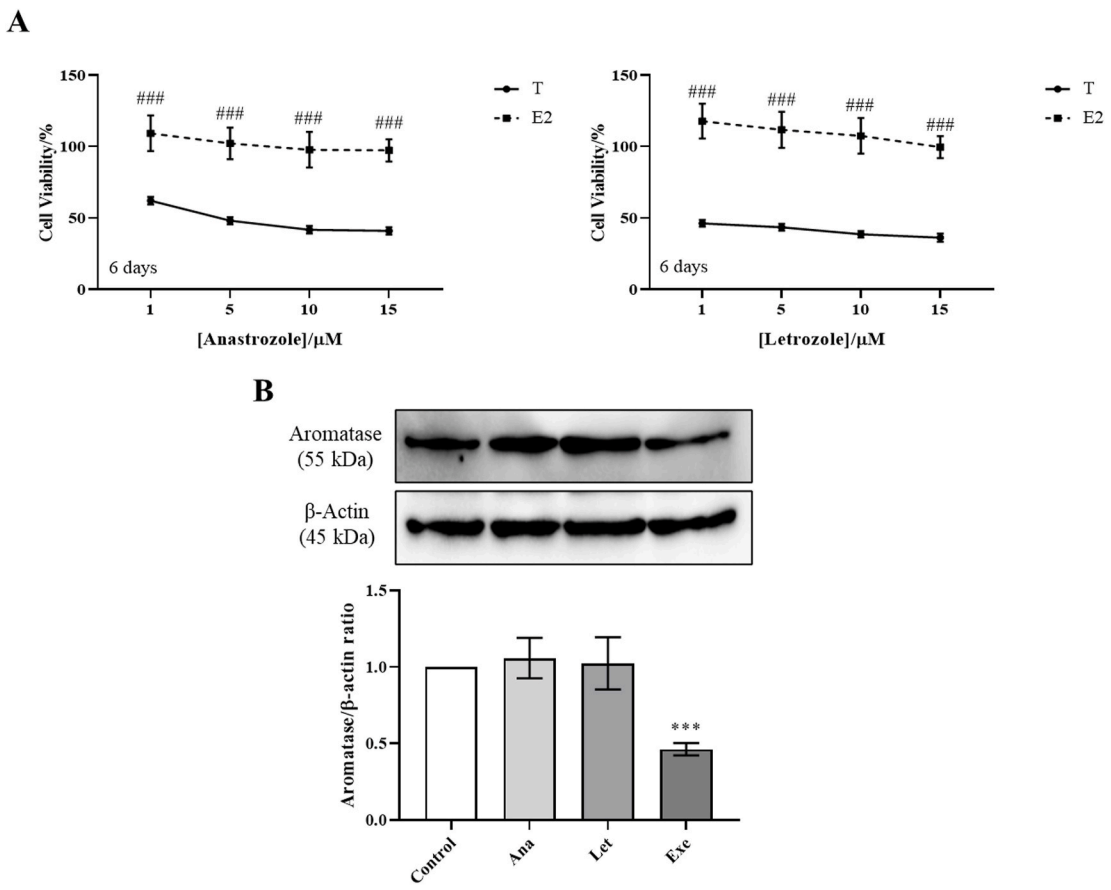

Fig. 6. Aromatase-dependency and effects of AIs on aromatase protein levels. (A) Comparison of the impact on cell viability of T-treated and E_2-treated MCF-7aro cells between different concentrations of each AI, after 6 days of treatment. Cells cultured with T or E_2 were considered as control and represent 100% of cell viability. Significant differences between the T-treated versus E_2-treated MCF-7aro cells are denoted by ### ($p < 0.001$). (B) Western-blot analysis of aromatase expression in MCF-7aro cells incubated with AIs (10 μM) for 8 h. Untreated cells were considered as control. β-Actin was used as a loading control. Significant differences between the control versus AIs-treated cells are presented *** ($p < 0.001$). All the results are the mean ± SEM of three independent experiments, performed in triplicate.

regulated genes was also investigated in the presence of all AIs without T-stimulation (Fig. 10D). Curiously, contrary to the effects observed on a hormone-enriched environment, where Ana and Let reduced the expression of ERα-regulated genes (Fig. 7D), on a hormone-deprived environment these AIs did not affect their transcription (Fig. 10D). Unlike Ana and Let, Exe, in the latter environment, significantly ($p < 0.01$, $p < 0,001$) induced the transcription of these genes (Fig. 10D), which was reverted by the addition of ICI ($p < 0.01$, $p < 0.001$) (Fig. 10D). As previously referred, this behaviour was different from the one observed in T-stimulated cells treated with Exe (Fig. 7D). The extent of AR involvement in the estrogenic genomic effects of Exe in hormone-deprived cells was also studied (Fig. 10E). The results showed that CDX significantly ($p < 0.05$; $p < 0.01$) reduced the Exe-dependent increase on the expression of *EGR3* and *AREG,* though it did not alter *TFF1* expression, thus indicating a different action of AR in the absence of androgens in *EGR3* and *AREG* expression. The effects of the AIs alone in the protein expression levels of both ERα and AR demonstrated that, unlike in T-stimulated cells (Figs. 7A and 9B), Ana and Let *per se* did not increase the levels of ERα or of AR (Fig. 10F). On the contrary, in Exe-treated cells the downregulation of ERα was not affected by the hormone environment. In addition, Exe, on hormone-deprived cells, continued to significantly increase AR levels ($p < 0.01$) (Fig. 10F), a behaviour similar to what was previously reported by our group in a hormone-enriched environment (Amaral et al., 2020), indicating that, contrary to Ana and Let, Exe does not need androgens to induce AR overexpression. Strikingly, ICI also significantly decreased AR levels ($p < 0.01$). Furthermore, in hormone-depleted conditions, it was verified that Ana and Let did not induce autophagy, similarly to what was verified in T-treated cells, while Exe continued to induce autophagy (Fig. 4C).

4. Discussion

The aim of this study was to characterize, at the molecular and cellular level, the *in vitro* effects of the third-generation AIs. Despite their clinical success, the development of resistance has become the major setback of AIs in clinical practice. Nevertheless, the lack of cross-resistance between AIs suggests that these drugs may act through distinct mechanisms at the cellular and molecular level. The MCF-7aro cell line has been widely used to study the effects of AIs due to its aromatase gene overexpression, which allows it to better mimic the tumor microenvironment (Wang and Chen, 2006; Amaral et al., 2012; Zhou et al., 1990; Wang et al., 2008). We previously reported that Exe decreased MCF-7aro cell proliferation, induced cell cycle arrest at G_0/G_1 after 3 days of treatment, and G_2/M after 6 days, as well as apoptosis through the mitochondrial pathway and cytoprotective autophagy (Amaral et al., 2012). Other studies have also reported that Ana and Let inhibited the growth of breast cancer cells by disrupting cell cycle at G_0/G_1 phase, inducing apoptosis and decreasing the expression of estrogen-regulated genes (Thiantanawat et al., 2003; Itoh et al., 2005). However, these studies with non-steroidal AIs used different doses and periods of treatment from the ones of our work. The mechanisms described for Ana and Let were confirmed by our study using MCF-7aro cells, where we show that the non-steroidal AIs were able to decrease cell viability and induce cell cycle arrest at G_0/G_1 phase, but, unlike Exe

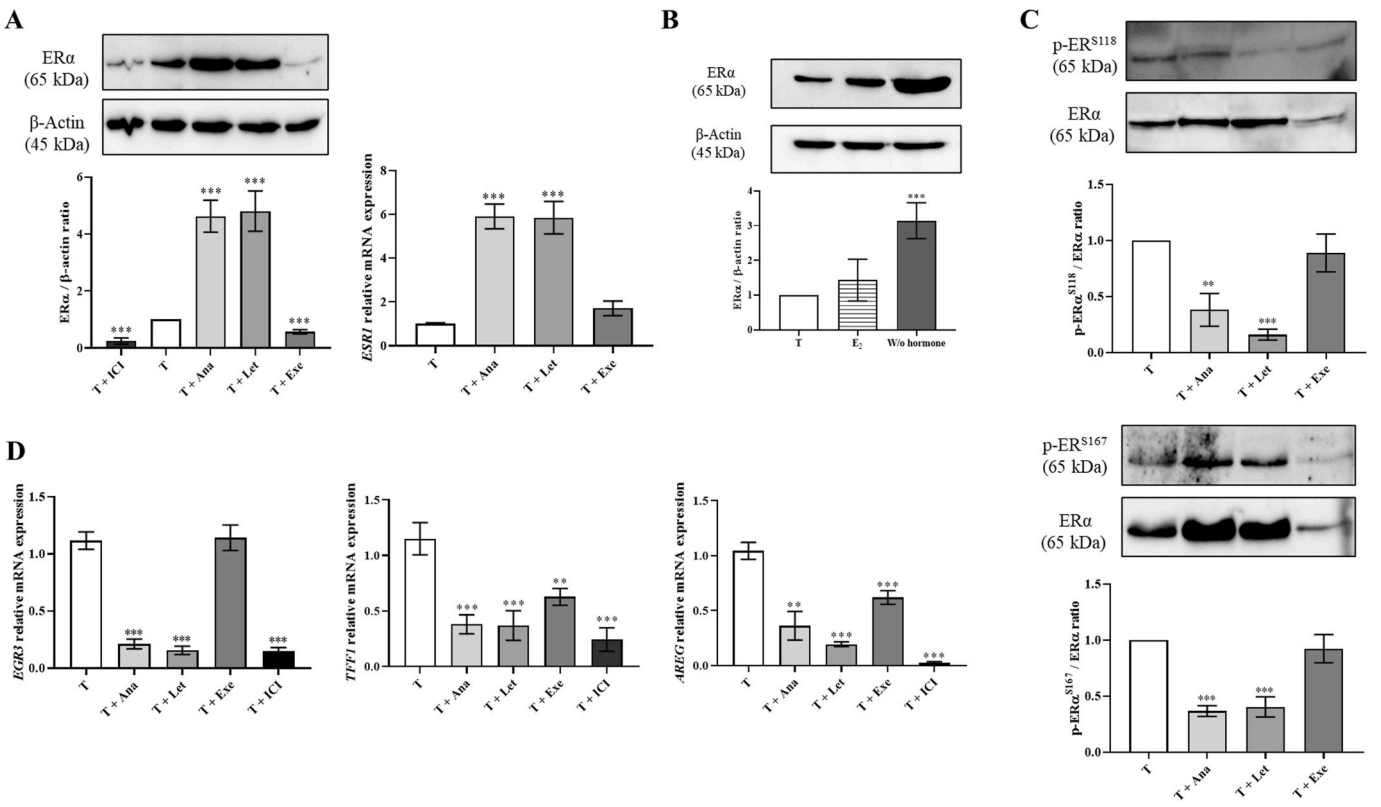

Fig. 7. ER-dependency and modulation of ER signaling by AIs in MCF-7aro cells. (A) Analysis of ERα and *ESR1* expression in cells incubated with AIs (10 μM), by western-blot and qPCR, respectively. T-treated cells were considered as control, while ICI (100 nM) was used as a positive control of ERα protein downregulation. β-actin was used as a loading control. Significant differences between control and AIs treatment are denoted by *** ($p < 0.001$). (B) Western-blot analysis of ERα expression in T-treated, E_2-treated or in cells without hormone treatment. T-treated cells were considered as control. β-actin was used as a loading control. Significant differences between T-treated cells and cells without hormones are denoted by *** ($p < 0.001$). (C) Western-blot analysis of the phosphorylation levels of ERα, at S118 and S167, of T-treated cells incubated with AIs (10 μM). T-treated cells were considered as control. ERα was used as a loading control. Significant differences between control and AIs treatment are denoted by ** ($p < 0.01$) and *** ($p < 0.001$). (D) qPCR analysis of the expression of ERα-regulated genes in T-treated MCF-7aro cells incubated with AIs (10 μM). T-treated cells were considered as control and ICI (100 nM) was used as positive control for abrogation of ERα signaling. Significant differences between control and AIs treatment are denoted by ** ($p < 0.01$) and *** ($p < 0.001$). Results are the mean ± SEM of three independent experiments, performed in triplicate.

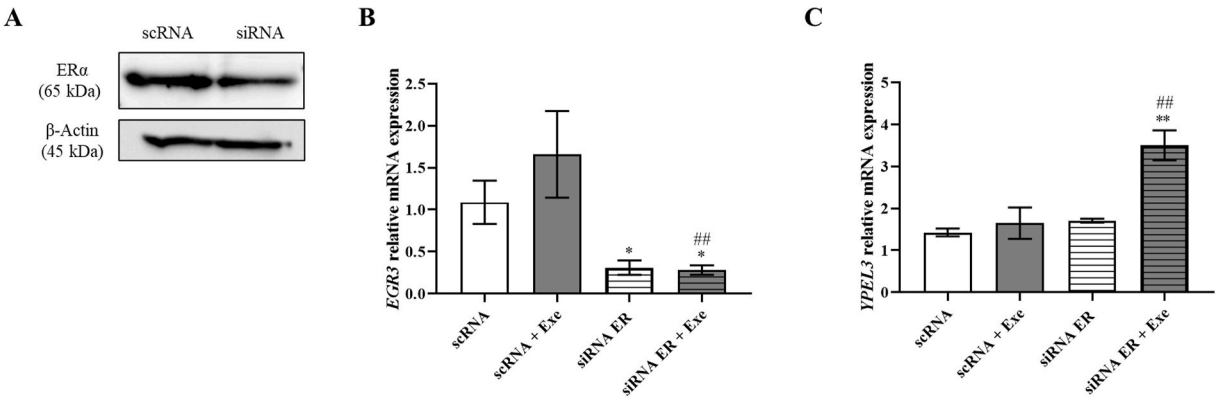

Fig. 8. Dependency of the Exe-induced modulation of *EGR3* and *YPEL3* expression levels on ER. (A) Western-blot for ERα in MCF-7aro cells treated with scRNA or siRNA. qPCR analysis of the expressions of *EGR3* (B) and *YPEL3* (C) genes in T-treated MCF-7aro cells, incubated with either scramble (scRNA) or ERα-targeting siRNA and Exe (10 μM) for 3 days. scRNA-treated cells were considered as control. Significant differences between siRNA ERα + Exe and siRNA ERα alone are denoted by * ($p < 0.05$) and ** ($p < 0.01$). Significant differences between siRNA ERα + Exe and scRNA + Exe are denoted by ## ($p < 0.01$). Results are the mean ± SEM of three independent experiments, performed in triplicate.

(Amaral et al., 2012), no shift to G_2/M arrest was observed at 6 days of treatment. Both Ana and Let, as well as Exe, decreased the expressions of cyclins D and E, required for the G_1/S transition (Siu et al., 2012; Fu et al., 2004). Moreover, Ana and Let also induced apoptosis, as verified by the increased binding of Annexin V and decreased levels of pro-caspase 7. In addition, and similarly to Exe (Amaral et al., 2012), this process seems to occur by the intrinsic pathway, since a loss of ΔΨm and increased caspase-9 activity were observed. Despite these common features between AIs, contrary to Exe (Amaral et al., 2012), Ana and Let did not induce autophagy, since no differences on LC3 turnover and on

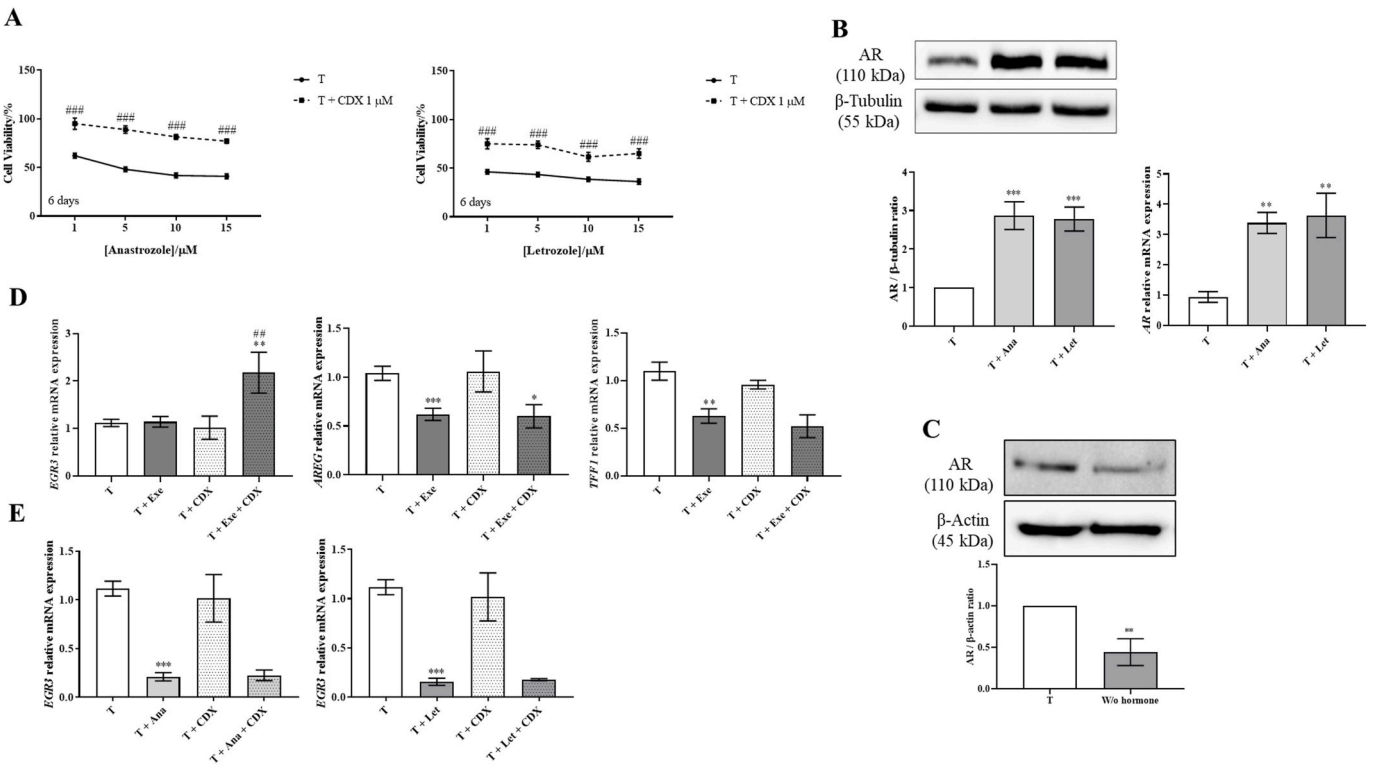

Fig. 9. AR-dependency and role of AR in modulation of ERα activity in AI-treated MCF-7aro cells. (A) Comparison of the impact on cell viability between different concentrations of each AI on T-treated or T and CDX-treated cells, after 6 days of treatment. Cells cultured only with T or T plus CDX (1 μM) were considered as control and represent 100% of cell viability. Significant differences between the T-treated versus CDX-treated MCF-7aro cells are denoted by ### ($p < 0.001$). (B) Analysis of AR expression in cells incubated with AIs (10 μM), by western-blot and qPCR, respectively, after 3 days of incubation. T-treated cells were considered as control. β-actin was used as a loading control. Significant differences between control and treatment are denoted by ** ($p < 0.01$) and *** ($p < 0.001$). (C) Western-blot analysis of AR expression in T-treated cells or in cells without hormone treatment for 3 days. T-treated cells were considered as control. β-actin was used as a loading control. Significant differences between T-treated cells and cells without hormones are denoted by ** ($p < 0.01$). (D) qPCR analysis of the expressions of ERα-regulated genes in T-treated MCF-7aro cells incubated with Exe (10 μM) in the absence or presence of CDX, after 3 days of incubation. Cells treated only with T or with T plus CDX-treated cells were considered as control. Significant differences between T-only and Exe or between CDX and CDX plus Exe are denoted by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). Significant differences between Exe and CDX plus Exe are denoted by ## ($p < 0.01$). (E) qPCR analysis of the expressions of *EGR3* in T-treated MCF-7aro cells incubated with Ana or Let (10 μM) in the absence or presence of CDX, after 3 days of incubation. Cells treated only with T or with T plus CDX-treated cells were considered as control. Significant differences between T-only and non-steroidal AIs are denoted by *** ($p < 0.001$). Results are the mean ± SEM of three independent experiments, performed in triplicate.

the expression of *SQSTSM1* gene were detected. Furthermore, the increase on AVOs verified for Ana and Let was not reverted by the autophagic inhibitors. This indicates an accumulation of acidic vesicles distinct from autophagosomes and autolysosomes, suggesting high lysosomal content. Since a high lysosomal content might be correlated to senescence, and a loss of estrogen signaling, through the use of Tamoxifen or ICI, can induce a state of early-senescence (Lee et al., 2014; Tuttle et al., 2012; Wu et al., 2019; Dolfi et al., 2014), the ability of AIs to promote cellular senescence was explored. MCF-7aro cells demonstrated an accumulation of β-gal and loss of *LMNB1* gene expression, both considered hallmarks of senescence (Hernandez-Segura et al., 2018). However, only the non-steroidal AIs increased *YPEL3* expression, an effect that was also described for Tamoxifen and associated with cellular senescence (Tuttle et al., 2012). In fact, *YPEL3* is a well described biomarker of senescence (Kelley et al., 2010; Berberich et al., 2011). Thus, unlike Exe, our data suggest that Ana and Let induce senescence, while Exe, despite accumulating β-gal and inducing a loss of *LMNB1*, does not appear to promote senescence given the lack of increased transcription of the *YPEL3* gene. It should be noted that, until now, senescence lacks a specific biomarker, and, therefore, can only be evaluated by the sum of several biomarkers (Hernandez-Segura et al., 2018). In fact, the degradation of the nuclear envelope, considered a feature of apoptosis, weakens the specificity of *LMNB1* loss as a senescence biomarker (Rao et al., 1996; Lindenboim et al., 2020), and the role of β-gal as a biomarker of senescence is also controversial (Lee et al., 2006). On the other hand, the exact correlation between autophagy and senescence is not fully understood. Nevertheless, the association between β-gal accumulation and autophagic structures has already been described in different cell models (Gerland et al., 2003), which may be a possible explanation for the accumulation of β-gal verified in Exe treatment. Some studies highlight dual roles for autophagy in the regulation of cellular senescence, acting either as an anti-senescence or as a pro-senescence mechanism (Kwon et al., 2017; Slobodnyuk et al., 2019; Gewirtz, 2013). Previously we showed that autophagy inhibition exacerbated the anti-proliferative and apoptotic effects of Exe in this cell model (Amaral et al., 2012). In this study, the inhibition of autophagy also led to an increase in *YPEL3* gene expression, supporting the previously reported cytoprotective role of autophagy (Amaral et al., 2012) and suggesting that autophagy may act as an anti-senesce mechanism. Recently, we have also demonstrated this same protective role for autophagy in Exe-resistant cells (Amaral et al., 2018).

Besides the relationship between the anti-proliferative effects, apoptosis, autophagy and senescence, the differences on hormonal status after AIs-treatment were also investigated. The addition of E_2 to Ana or Let completely abrogated the anti-proliferative effects of the AIs, demonstrating that their effects are dependent on aromatase inhibition. Moreover, contrary to Exe, that induces aromatase degradation (Wang and Chen, 2006), the non-steroidal AIs did not affect aromatase protein

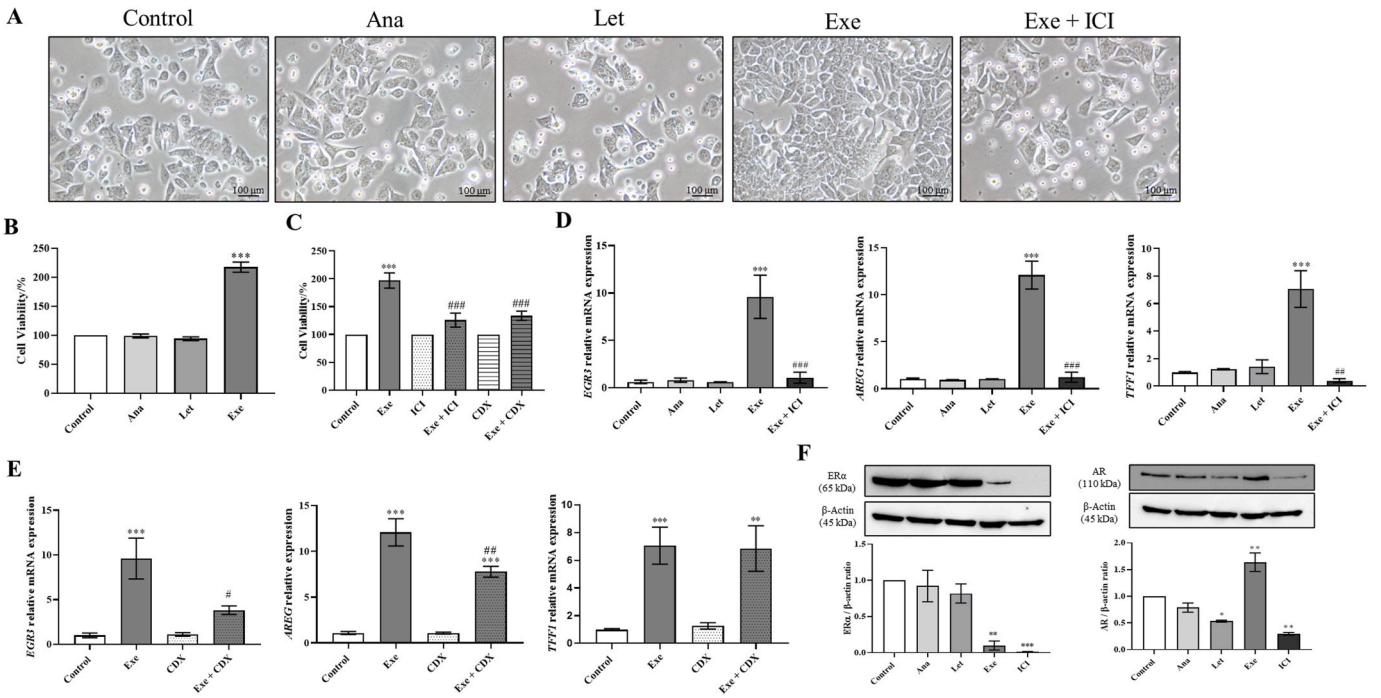

Fig. 10. ERα agonistic effects of Exe in hormone-depleted conditions. (A) Effects of Exe (10 μM) on cell morphology and proliferation, evaluated by phase contrast microscopy. Cells were incubated with Exe or Exe plus ICI (100 nM) for 3 days. Untreated cells were considered as control and represent 100% of cell viability. Cells were visualized with a magnification of 200x (B) Effects of AIs (10 μM) on cell viability after 3 days of treatment. Untreated cells were considered as control and represent 100% of cell viability. Significant differences between control and AIs are denoted by *** ($p < 0.001$). (C) Effects of Exe (10 μM) alone or in the presence of ICI (100 nM) and CDX (1 μM) after 6 days of treatment. Untreated cells and cells treated only with ICI or CDX were considered as control and represent 100% of cell viability. Significant differences between controls and Exe are denoted by *** ($p < 0.001$). Significant differences between Exe alone and Exe plus ICI or CDX are denoted by ### ($p < 0.001$). (D) qPCR analysis of the expression of ERα-regulated genes in MCF-7aro cells incubated with AIs (10 μM) for 3 days. Untreated cells were considered as control. Significant differences between control and AIs treatment are denoted by *** ($p < 0.001$). Significant differences between Exe alone and Exe plus ICI are denoted by ## ($p < 0.01$) and ### ($p < 0.001$). Results are the mean ± SEM of three independent experiments, performed in triplicate. (E) qPCR analysis of the expressions of ERα-regulated genes in MCF-7aro cells incubated with Exe (10 μM) in the absence or presence of CDX (1 μM), after 3 days of treatment. T-only treated cells or T plus CDX-treated cells were considered as control. Significant differences between controls and Exe treatments are denoted by ** ($p < 0.01$) and *** ($p < 0.001$). Significant differences between Exe alone and Exe plus CDX are denoted by # ($p < 0.05$) and ## ($p < 0.01$). Results are the mean ± SEM of three independent experiments, performed in triplicate. (F) Western blot analysis of the effects of AIs on protein levels of ERα and AR, after 3 days of treatment. Untreated cells were considered as control, while ICI (100 nM) was used as a positive control of ERα protein downregulation. β-actin was used as a loading control. Significant differences between control and treatments are denoted by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

levels. Furthermore, Ana and Let increased ERα expression levels, an effect also verified in cells without AIs but in hormone-depleted conditions, while Exe induced a down-regulation of ERα protein levels. The assessment of ERα phosphorylation suggested that this receptor was not activated during Ana and Let treatment, but remained active during Exe treatment, despite the lower receptor levels. The analysis of three well-known ERα-regulated genes (*EGR3*, *AREG* and *TFF1*), with defined roles in cell growth, migration and in the development of resistance (Wang et al., 2008; Inoue et al., 2004; Drabovich et al., 2016; Peterson et al., 2015, Kim et al., 2013; Ghosh et al., 2000, Prest et al., 2002; Sun et al., 2005; Masri et al., 2010; Masri et al., 2008), confirmed that there was no activation of this receptor during Ana and Let treatment. Strikingly, only the *EGR3* gene expression, which is considered a *bone fide* target of ERα (Inoue et al., 2004), was not decreased by Exe, while the expression of the other ERα-regulated genes was reduced, data that supports that ERα remained active during Exe treatment. ERα silencing confirmed that the maintenance of *EGR3* expression during Exe treatment was dependent on ERα. Thus, our results support the weak-estrogen like effects of Exe previously reported by Wang et al. (2008), since ERα activity and, more specifically, *EGR3* expression, was seemingly sustained by Exe-treatment at a lower dose (1 μM) (Wang et al., 2008), but also at a higher dose (10 μM), as demonstrated in our study. In addition, it is known that an Exe-resistant cell line did not present a ligand-independent activation of ERα, unlike Ana- and Let-resistant cell lines (Masri et al., 2008), thus reinforcing our results. Furthermore, the importance of *EGR3* for cancer growth and survival during AIs treatment was demonstrated in breast cancer cells resistant to Let and in breast cancer patients treated with this AI. In fact, it was demonstrated that, in response to sustained estrogen deprivation, *EGR3* becomes estrogen-independent in Let-resistant breast cancer cells, and that in breast cancer patients 3 months after treatment with Let, a failure to decrease *EGR3* transcript levels was correlated with poor response to therapy and decreased disease-free survival and overall survival (Vareslija et al., 2016). On the other hand, the increase of *YPEL3* expression observed in ERα-silenced cells, after Exe treatment, suggests that, despite the weak estrogen-like effect of Exe, this increase is a consequence of the decreased levels of ERα, thus supporting the importance of ERα for *YPEL3* expression and the role of the estrogen-like activity of Exe in the prevention of cellular senescence. In fact, considering all our data with AIs and the reported effects for ICI and Tamoxifen, as well as their link with senescence (Lee et al., 2014; Mumcuoglu et al., 2010; Tuttle et al., 2012; Wu et al., 2019), we suggest that cellular senescence is a consequence of lack of ERα signalling in breast cancer cells.

A previous study of our group demonstrated that, in Exe-treated MCF-7aro cells, AR played an oncogenic and pro-survival role, as verified by the increased AR expression and activation (Amaral et al., 2020), contrarily to the growth-inhibitory function of AR, reported so far in other studies (Macedo et al., 2006; Peters et al., 2009; Basile et al., 2017; Hu et al., 2011). In this work, opposite to Exe (Amaral et al., 2020), the addition of CDX to Ana and Let reduced the anti-proliferative effects of

these AIs, reinforcing the growth-inhibitory effect for AR previously described (Chen et al., 2015; Macedo et al., 2006). Interestingly, and similarly to Exe (Amaral et al., 2020), the non-steroidal AIs also increased AR protein and gene expression levels. This increase in AR levels was also reported in response to dihydrotestosterone (DHT), the main androgenic substrate of AR, leading to growth-inhibition (Macedo et al., 2006; Amaral et al., 2020; Sasano et al., 2008). Note that Exe treatment induces an increase in DHT levels through modulation of 5α-reductase activity (Amaral et al., 2020; Chanplakorn et al., 2011; Takagi et al., 2010). In hormone-depleted MCF-7aro cells treated with Ana and Let, the AR levels were not increased when compared to T-treated cells, indicating that the observed AR overexpression was a response to androgen (T) accumulation, because of these AIs. Curiously, in the hormone-deprived environment, Exe, contrary to the non-steroidal AIs, continued to increase AR expression, similarly to T-treated cells (Amaral et al., 2020), which indicates that regardless of hormonal environment, Exe *per se* induces an overexpression of AR, highlighting the existence of a close relationship between AR and Exe and corroborating our previous work (Amaral et al., 2020). On the other hand, considering this data and the fact that Exe maintains the ERα active, and to understand the AR interference on ERα signaling, the ERα-regulated genes (*EGR3*, *AREG* and *TFF1*) were analysed in MCF-7aro cells stimulated with T and co-incubated with Exe and CDX. AR blockade only affected the expression of *EGR3* gene, suggesting that AR also regulates the transcription of this gene. Thus, we can suggest that AR partially impairs ERα signaling. These results corroborate our previous findings, where an AR and ERα crosstalk was observed (Amaral et al., 2020). It is known that AR can counteract ERα signaling, either by competitive binding to EREs (Peters et al., 2009), through a direct interaction with ERα (Panet-Raymond et al., 2000) or by a direct inhibition of ERα activity (Lanzino et al., 2005).

To deepen these relationships, hormone-deprived MCF-7aro cells were treated with all the AIs, thus abrogating the growth-inhibitory effects induced by androgen accumulation, as well as the growth-induced properties of estrogens due to aromatization of androgens. In these conditions, and contrary to Ana and Let, Exe *per se* induced cell proliferation, and, similar to previous findings, this effect was reverted by the addition of ICI (Masri et al., 2010). On the other hand, Exe maintains its positive regulation of autophagy, while Ana and Let still did not activate this process. In addition, Exe induced a strong activation of ERα, in contrast to Ana and Let that caused no alterations. Confirming the estrogenic-like effects of Exe, this behaviour was completely abrogated when Exe was incubated with ICI, that down-regulated ERα. Interestingly, by blocking AR with CDX it was also possible to revert the proliferative effects of Exe, as well as the partial transcription of ERα-regulated genes, mainly the *EGR3* and *AREG* genes. Thus, in the absence of an androgenic stimulus, and contrary to the results obtained with T-treated cells, AR contributes to the maintenance of ERα-regulated genes transcription, demonstrating that Exe *per se* is capable of modulating AR activity. Therefore, the results suggest that Exe activates AR, and that, depending on the hormonal environment, absence or presence of androgens, AR modulates ERα activity differently.

This effect of Exe on the modulation of AR activity was also referred for E_2, since it recruits AR to cooperatively support ERα signaling, driving tumor growth (D'Amato et al., 2016). This possible role for Exe in altering AR activity is also supported by a study that claims that, in Exe-resistant cells derived from MCF-7aro cells, not treated with T, there were no androgen-responsive genes differentially expressed, with the exception of *KLK11* (Masri et al., 2010). Nevertheless, considering the role of AR in *EGR3* transcription in Exe-treated T-stimulated cells, we suggest that the androgenic effects overcame the modulation of AR by Exe. On the other hand, we hypothesize that the differences observed between AIs in relation to the AR role may be a consequence of the weak estrogen-like activity of Exe, and of the lack of ERα activation in the case of Ana or Let treatment. Furthermore, in a hormone-deprived environment, Exe maintained the down-regulation of ERα protein levels and increase of AR levels, a behaviour already discussed and in agreement with our previous work (Amaral et al., 2020). Therefore, we can hypothesize that Exe *per se* can target both receptors, by a mechanism not yet fully understood, but with potential clinical relevance. In addition and interestingly, despite being characterized only as a selective estrogen-receptor down-regulator (SERD) in breast cancer cells, the ability of ICI to also modulate AR levels is not exclusive, since it was reported that, as in our cell model, ICI also down-regulates AR in prostate cancer cells (Bhattacharyya et al., 2006). In fact, the ability of steroid receptor blockers to cross-inhibit other steroid receptors, due to their homology, was also reported for Enzalutamide, being this AR antagonist also able to block ERα (D'Amato et al., 2016).

5. Conclusions

This work directly compares both the anti-proliferative effects and the regulation of the hormonal environment by the AIs currently used in clinic. Although there are no relevant differences at cell cycle and apoptosis, significant differences in inducing autophagy and senescence were observed. Exe induces a pro-survival autophagic process that protects the cells from entering a senescent state, which is immediately achieved by Ana and Let. In addition, the effects of AIs at the protein levels and activation/function of the ERα and AR are contrasting. In our cell model, Ana and Let function as pure AIs, and all their effects on the steroid receptors are a consequence of aromatase inhibition, which leads to a decrease in estrogens levels and a rise in androgens. On other hand, Exe activates both ERα and AR, which cooperate to induce cell survival and proliferation, corroborating the pro-survival and oncogenic role of AR in Exe-sensitive and Exe-resistant cells (Amaral et al., 2020). The concomitant modulation of AR activity, by Exe and androgens, might explain why AR has a different impact in the survival of Exe-treated and Ana- or Let-treated cells (Amaral et al., 2020). *Wang* et al. (2008) first reported the estrogenic action of Exe (Wang et al., 2008) and, in our study, this behaviour was also confirmed and deepened. To the best of our knowledge, this is the first study comparing the third-generation of AIs in different cellular processes, and their ability to modulate the hormonal environment. The key findings from this study highlight the ability of Exe to modulate both AR and ERα activities. The possible implications of these findings suggest that only patients treated with Exe would react positively to anti-AR therapy, thus corroborating our previous findings (Amaral et al., 2020) and supporting the ongoing clinical trials (NCT02007512, NCT02910050 and NCT02676986) and the phase I/Ib study with Enzalutamide and Exe in patients with advanced ER^+ breast cancer (Schwartzberg et al., 2017). Moreover, the estrogenic-like activity of Exe may hinder its therapeutic efficacy when administered as a second-line therapy, considering the recommendations of ESMO guidelines for early-breast cancer in postmenopausal patients (Cardoso et al., 2019). Furthermore, based on these results, the optimal sequential therapy would be Exe upfront, since it would not be as beneficial after non-steroidal AIs or tamoxifen treatment, although further validation in different models is required.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

CRediT authorship contribution statement

Tiago V. Augusto: Conceptualization, Methodology, Validation, Visualization, Formal analysis, Investigation, Writing – original draft. **Cristina Amaral:** Conceptualization, Validation, Visualization, Formal analysis, Investigation, Writing – review & editing. **Cristina F. Almeida:** Validation, Investigation. **Natércia Teixeira:** Conceptualization, Visualization, Writing – review & editing, Supervision. **Georgina Correia-da-Silva:** Conceptualization, Visualization, Writing – review & editing, Supervision.

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Manuscript IV:

Effects of PI3K inhibition in AI-resistant breast cancer cell lines: autophagy, apoptosis and cell cycle progression

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(Under review)

Effects of PI3K inhibition in AI-resistant breast cancer cell lines: autophagy, apoptosis and cell cycle progression

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ABSTRACT

Breast cancer is the leading cause of cancer death in women. The aromatase inhibitors (AIs), Anastrozole (Ana), Letrozole (Let) and Exemestane (Exe) are a first-line treatment option for estrogen receptor-positive (ER^+) breast tumors, in postmenopausal women. Nevertheless, the development of acquired resistance to this therapy is a major drawback. The involvement of PI3K in resistance, through activation of the PI3K/AKT/mTORC1 survival pathway or through a cytoprotective autophagic process, is widely described. In that sense, the involvement of autophagy, in response to Ana and Let treatments, and the effects of the combination of BYL-719, a PI3K inhibitor, with AIs were explored in AI-resistant breast cancer cell lines (LTEDaro, AnaR, LetR and ExeR). We demonstrate that Ana and Let treatments do not promote autophagy in resistant breast cancer cells, contrary to Exe. Moreover, the combinations of BYL-719 with AIs decrease cell viability by different mechanisms. The combination of BYL-719 with Ana or Let induced cell cycle arrest, while with Exe promoted cell cycle arrest and apoptosis. In addition, BYL-719 decreased AnaR, LetR and ExeR cell viability in a dose- and time-dependent manner, being more effective in the ExeR cell line. This decrease was further exacerbated by ICI 182,780. These results corroborate the lack of cross-resistance between AIs verified in clinic, excluding autophagy as a mechanism of resistance to Ana or Let and supporting the ongoing clinical trials combining BYL-719 with AIs.

Abbreviations: 7-amino-acitomycin (**7-AAD**); 3,3′-dihexyloxacarbocyanine iodide (**$DiOC_6(3)$**); Aromatase Inhibitors (**AIs**); Anastrozole (**Ana**); Anastrozole-resistant cell line (**AnaR**); Acridine orange (**AO**); Acidic vesicle organelles (**AVOs**); Buparlisib (**BKM-120**); Alpelisib (**BYL-719**); Carbonyl cyanide m-chlorophenylhydrazone (**CCCP**); pretreated charcoal heat-inactivated fetal bovine serum (**CFBS**); Epidermal growth factor receptor (**EGFR**); Estrogen receptor α (**ERα**); Estrogen-receptor positive (**ER^+**); Exemestane (**Exe**); Exemestane-resistant cell line (**ExeR**); Insulin growth factor-1 receptor (**IGF-1R**) Forward light scatter (**FSC**); Fulvestrant (**ICI 182,780**); Letrozole (**Let**); Letrozole-resistant cell line (**LetR**); Eagle's minimum essential medium (**MEM**); Mean Fluorescence Intensity (**MFI**); Mammalian target of Rapamycin (**mTORC1);** Tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-difenyltetrazolium (**MTT**); Mitochondrial transmembrane potential (**ΔΨm**); Phosphatidylinositol-3-kinase (**PI3K**); PtdIns (4,5) P2 (**PIP2**); PtdIns (3,4,5) P3 (**PIP3**); Phosphatidylserine (**PS**); Relative Luminescence Units (**RLU**); scrambleRNA (**scRNA**); Selective Estrogen Downregulator (**SERD**); Selective Estrogen Modulator (**SERM**); Small interfering RNA (**siRNA**); Side light scatter (**SSC**); Staurosporin (**STS**); Transfection agent (**TA**); Wortmannin (**WT**).

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Introduction

Breast cancer is the leading cause of cancer death in women, being the estrogen-receptor positive (ER^+) breast tumors responsible for approximately 75% of postmenopausal breast cancer cases [1]. The treatment of choice for postmenopausal women is classically divided into three types of endocrine therapies namely selective estrogen receptor modulators (SERM), selective estrogen receptor downregulators (SERD) and aromatase inhibitors (AIs). Nevertheless, resistance to these therapies may occur after prolonged treatment due to crosstalk at the molecular level between the estrogen receptor α (ERα), growth factors, and downstream cell-signaling pathways. In fact, despite the clinical success of both steroidal (Exemestane-Exe) and non-steroidal (Letrozole- Let and Anastrozole-Ana) AIs, their efficacy is hindered by the development of resistance, tough the underlying mechanisms are unknown [2-4]. In order to circumvent these drawbacks, the efficacy of the hormonal monotherapies, with distinct mechanisms of action, can be potentiated by different combinations with AIs and sequences [5]. These combinations include CDK4/6 inhibitors (palbociclib/ribociclib/abemaciclib), the mammalian target of rapamycin C1 (mTORC1) inhibitor (Everolimus), or the PI3K inhibitor [alpelisib (BYL-719)].

The phosphatidylinositol-3-kinase (PI3K) is a pivotal enzyme that catalyzes the phosphorylation of PtdIns P2 (PIP2), producing PtdIns P3 (PIP3). PI3K comprises three different classes: class I, class II and class III. Class I is part of the PI3K/AKT/mTORC1 pathway, which regulates several cellular processes, such as growth and survival and is also implicated in oncogenesis [6]. This pathway is also involved in the regulation of autophagy and its dysregulation can lead to increased tumorigenesis and resistance to several anti-cancer therapies. In fact, this pathway is altered in almost all human cancers due to several mechanisms, as loss of the tumor suppressor gene PTEN, amplification and mutations of PI3K class I or, even, aberrant activation of upstream growth factor receptors. The catalytic subunit of PI3K class I is divided into four subunits, α, β, γ and δ, which are coded by different genes with different cell-specific expressions [7]. In breast cancer, the gene that encodes the p110α subunit of class I PI3K, *PIK3CA*, is frequently mutated [8-10]. Moreover, in ER^+ breast cancer, the hyperactivation of the PI3K/AKT/mTORC1 pathway is known to promote hormone-independence, leading to the development of resistance to anti-estrogen therapies [11].

In order to promote the use of PI3K class I inhibitors as valid therapeutic options in ER^+ breast cancer [12], several pre-clinical and clinical studies have been conducted. However, due to severe side effects, such as hepatotoxicity, hyperglycaemia and mood disorders, most research on the pan-PI3K class IA inhibitors, such as Buparlisib (BKM-120) and Pictilisib (GDC-0941), was halted [13-15]. Nevertheless, the isoform α-specific PI3K class IA inhibitors, BYL-719, presents fewer side effects than the pan-PI3K class IA inhibitors [16, 17]. This resulted in FDA approval of BYL-719 for the treatment of postmenopausal women with $ER^+/HER2^-$, *PIK3CA*-mutated advanced or metastatic breast cancers, in combination with the SERD Fulvestrant (ICI 182,780), following progression on/or after endocrine therapy [2, 13, 18]. Moreover, the results from two clinical trials combining BYL-719 with Let (NCT01791478) or either with Let or Exe (NCT01870505) demonstrated anti-tumor efficacy when compared to AIs alone [17, 19]. Nevertheless, this strategy with AIs was not yet approved and in both trials the occurrence of dose-limiting adverse effects led to dose de-escalations.

PI3K class III, on the other hand, is mainly involved in the regulation of autophagy [20-22]. In breast cancer, autophagy is presented as a double-edged sword [22, 23], with evidence suggesting a dual role between tumor promotion [24-28] and tumor suppression [29-31]. Over the last few years, several reports have connected the development of AI-acquired resistance with a protective autophagic process [25, 27, 32]. In fact, our group has described a protective role in response to Exe in AI-sensitive [24] and AI-resistant ER^+ breast cancer cells [25]. Exe induced an increase in acidic vesicle organelles (AVOs) and LC3-II expression in the AI-resistant ER^+ breast cancer cell line, LTEDaro. Moreover, pharmacological inhibition of autophagy was able to re-sensitize this cell line to Exe treatment [25]. Corroborating our study, an analysis of ER^+ breast tumors of patients receiving neoadjuvant Exe treatment showed an increase in autophagic-associated markers, LC3 and beclin-1, and a decrease in apoptosis-associated markers [33].

Currently, FDA, following the improved progression-free survival reported in the BOLERO-2 (NCT00863655) clinical trial, approved the combination of Everolimus with endocrine therapy [2, 34]. Nevertheless, this combination did not significantly improve the overall survival [35]. In fact, a similar trial combining Everolimus with Let (NCT01231659) also did not significantly improve the overall survival despite the increase in progression-free survival [36]. One of the

possible reasons behind the lack of the former can be the upregulation of autophagy, which was shown to promote insensitivity to both Everolimus [32] and Exe [25]. This limitation observed for the combination of Everolimus and AIs in clinical trials reinforce the possible pivotal role of autophagy in the efficacy of these combinations. Nevertheless, the role of autophagy in the development of resistance to Ana and Let is unknown.

The absence of cross-resistance observed for Exe, Ana and Let in clinic, suggests that the efficacy of PI3K inhibitors may depend on the AI-specific resistance mechanisms [37]. Thus, in this work, we studied the role of autophagy in the acquired resistance to Ana and Let and the importance of the PI3K/AKT/mTORC1 pathway by exploring the efficacies of the combinations of BYL-719 with the different AIs (Ana, Let and Exe) or with ICI 182,780 in AI-resistant breast cancer cells.

Materials and Methods

2.1 Cell culture

The long-term estrogen deprived human breast cancer cell line, LTEDaro is a good in vitro cell model to study AI-acquired resistance [38-41]. This cell line mimics the late-stage of AI-acquired resistance since it is originated through long-term estrogen deprivation of the ER^+ MCF-7aro cell line [42, 43]. The LTEDaro cell line (3–10 passages) was maintained with Eagle's minimum essential medium (MEM) without phenol-red and supplemented with Earle's salts and with 1 mmol/L sodium pyruvate, 1% penicillin-streptomycin-amphotericin B, 1% L-Glutamine, 100 µg/ml G418 and 10% of pretreated charcoal heat-inactivated fetal bovine serum (CFBS) (Gibco Invitrogen Co., Paisley, Scotland, UK). In addition, a mutated *PIK3CA* gene has been reported in the MCF-7 cells, MCF-7aro and LTEDaro parental cell line [44, 45]. Thus, LTEDaro cells represent a suitable model to study the effects of PI3K inhibitors. In addition, the Anastrozole-resistant cell line (AnaR), the Letrozole-resistant cell line (LetR) and the Exemestane-resistant cell line (ExeR) were generated by by long-term exposure of Testosterone- treated MCF-7aro cells to either 1 µM of Ana, 200 nM of Let or 1 µM of Exe, respectively, as previously reported [38-41], thus, mimicking in vitro resistance to each one of the AIs. These AI-specific resistant cell lines were maintained as previously described [38]. All cell lines were grown at 37 °C in a 5% CO^2 atmosphere and were generated in the laboratory of Prof. Shiuan Chen (Beckman Research Institute, City of Hope, Duarte, CA, U.S.A.).

Untreated LTEDaro, AnaR, LetR and ExeR cells were considered as controls. The preparation and storage of the stock solutions of Ana, Let, Exe, Wortmannin (WT) and ICI 182,780 (SigmaAldrich Co., Saint Louis, USA) are the same as previously reported [25, 38]. BYL-719 (Caymanchem, Michigan, USA) was prepared in 100% DMSO. Final concentration of DMSO in cell culture was below 0.01% in all the assays.

2.2 Cell viability

In order to evaluate the effects of the combinations of Ana (1-15 µM), Let (1-15 µM) or Exe (1-15 µM) with 0.1 µM of WT or 0.05 µM of BYL-719, in LTEDaro cell viability, cells were seeded in 96-well plates (1 x 10^4 cells/mL) for 6 days and after each incubation period MTT assay was performed as previously described [25]. The selected concentration of WT was the one previously used in this cell model, that per se caused no effect on LTEDaro cell viability [25]. The specific AI-resistant cell lines were seeded in 96-well plates (1 x 10^4 cells/mL) and incubated with WT (0.01-0.5 µM) or BYL-719 (0.01-5 µM), with or without ICI 182,780 (100 nM), for 6 days. After the incubation time, cell viability was also evaluated by the MTT assay. Results are expressed as relative percentage of the untreated cells, which were designated as controls representing 100% of cell viability.

2.3 Cell cycle progression

The anti-proliferative effects induced by the combination of AIs and BYL-719 were studied through analysis of cell cycle progression by flow cytometry, after PI staining, as previously reported [25]. LTEDaro cells were seeded in 6-well plates (7 x 10^5 cells/mL) and incubated with Ana (10 µM), Let (10 µM) or Exe (10 µM), with or without BYL-719 (0.05 µM), for 3 days. 40 000 events were acquired to assess DNA content by a BD Accuri™ C6 cytometer (San José, CA, USA), equipped with a BD Accuri™ C6 software for data analysis. Detectors for the three fluorescent channels (FL-1, FL-2 and FL-3) and for forward (FSC) and side (SSC) light scatter were set on a linear scale. The percentage of cells in G_0/G_1, S and G_2/M cell cycle phases was used to express the anti-proliferative effects.

2.4 Cell death assays

The involvement of apoptosis on the effects induced by the combinations was assessed through analysis of the translocation of phosphatidylserine (PS), by Annexin V-FITC labelling, caspase-7 and caspase-9 activities and

mitochondrial transmembrane potential (ΔΨm) loss. To study translocation of PS, cells were cultured in 6-well plates (7 × 10^5 cells/mL) and treated with Ana (10 μM), Let (10 μM) or Exe (10 μM), with or without BYL-719 (0.05 μM), for 3 days. After washing with PBS, cells were incubated with Annexin V-FITC Apoptosis Detection Kit (BioLegend Way, San Diego, USA), according to the manufacturer's instructions. Analysis was performed in a BD Accuri™ C6 cytometer (San Jose, CA, USA), equipped with BD Accuri™ C6 analysis software. All the fluorescence channels (FL-1, FL-2 and FL-3) detectors were set on a logarithmic scale. Bivariant analysis of Annexin-FITC fluorescence (FL-1) and 7-amino-actinomycin (7-AAD) fluorescence (FL-3) distinguished different cell populations, Annexin V^-/7-AAD^- were considered as viable cells, Annexin V^+/7-AAD^- corresponded to apoptotic cells and Annexin V^+/7-AAD^+ were designated as late apoptotic and necrotic cells. For the luminescent and fluorescent assays, LTEDaro cells were seeded in 96-well plates (2.5 × 10^4 cells/mL) and incubated with the combinations for 3 days. Caspase-7 and Caspase-9 activity was evaluated using the Caspase-Glo® 3/7 and the Caspase-Glo® 9 kits (Promega Corporation, Madison, USA). Staurosporin (STS) (SigmaAldrich Co., Saint Louis, USA), at 10 μM, was used as positive control for apoptosis. The ΔΨm was evaluated by the fluorescence probe 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) (Gibco Invitrogen Co., Paisley, Scotland, UK), as previously described [46]. The mitochondrial depolarizing agent carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma-Aldrich Co., Saint Louis, USA), at 10 μM, was used as positive control. The luminescent and fluorescent signals were measured in a 96-well Microplate Luminometer (Synergy HT, BioTek, USA). For fluorescence intensity the excitation wavelength of 480 nm and the emission filter of 530 nm were used. Luminescence values are represented as relative luminescence units (RLU) and fluorescence values are represented as mean fluorescence intensity (MFI). In all the experiments, basal fluorescence was subtracted.

2.5 Acid Vesicle Organelles (AVOs) detection by flow cytometry

Detection and quantification of AVOs was performed by flow cytometry through acridine orange (AO) staining (Sigma-Aldrich Co., Saint Louis, USA), at 0.5 μg/ml, for 15 minutes, as previously described [24, 25]. LTEDaro cells were cultured in 6-well plates (3.5 x 10^5 cells/ml) and incubated with AIs (10 μM) for 6 days. Untreated cells were considered as controls. As positive control it was used H_2O_2 (0.1 mM). 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 forward (FSC) and side (SSC) light scatter detectors, as well as FL-1 and FL-3 channels were set on a linear scale. Analysis of AO negative (AO^-, green fluorescence) and AO positive (AO^+, red fluorescence) distinguished the two cell populations.

2.6 Western blot analysis

Expression levels of LC3-II, ATG5, p-AKT and AKT were evaluated by Western-Blot. LTEDaro cells were incubated, in 6-well plates (7 ×10^5 cells/mL), with Ana (10 μM), Let (10 μM) or Exe (10 μM), with or without BYL-719 (0.05 μM) or ICI 182,780 (0.1 μM), for 3 days. Exe was used as positive control for autophagic induction, as previously reported [25]. Untreated cells were considered as controls. Proteins were extracted, separated in a 4–20% SDS-PAGE (for LC3 only) or in a 10% SDS-PAGE and transferred to nitrocellulose membranes as previously described [25]. For the immunodetection, the primary antibodies, rabbit polyclonal anti-LC3 I/II (1:200), rabbit monoclonal anti-ATG5 (1:1000), rabbit monoclonal anti-p-AKT(S473) (1:200) and rabbit monoclonal AKT (1:200) (Cell Signaling Technology Inc., Boston, USA), and the secondary antibodies anti-mouse (1:1000; 1:5000) and anti-rabbit (1:2000) (Thermo Fisher, Waltham, MA, USA), were used. The mouse monoclonal anti-β-tubulin (1:500) and anti-β-actin (1:5000) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were employed as loading controls. Immunoreactive bands were visualized using a chemiluminescent substrate WesternBright™ ECL (Advansta Inc., Menlo Park, CA, USA) and a ChemiDoc™ Touch Imaging System (Bio-Rad, Laboratories Melville, NY, USA).

2.7 RNA extraction and qPCR

LTEDaro cells were seeded in 6-well plates (7 ×10^5 cells/mL) and treated with Ana (10 μM), Let (10 μM) or Exe (10 μM) for 3 days. Cells were further lysed, and the RNA collected using the TripleXtractor reagent (GRiSP Research Solutions, Porto, Portugal), according to the manufacturer's protocol. Total RNA was quantified using the NanoDrop ND-1000 Spectrophotometer (NanoDrop

Technologies, Inc., Wilmington, DE, USA). GRiSP Xpert cDNA Synthesis Mastermix (GRiSP Research Solutions, Porto, Portugal) was used to obtain cDNA, which was amplified with specific primers, using GRiSP Xpert Fast SYBR (GRiSP Research Solutions, Porto, Portugal), in MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories) [47]. Primer sequences (5'-3') and amplification conditions are presented in Table 1. The fold change in gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method [28], using as housekeeping genes, α-tubulin and β-actin.

Table 1. Primer sequences and qPCR conditions for target genes.

Gene	Sequence		Ta/ºC
SQSTM1	S-GGAGTCGGATAACTGTTC	AS-GATTCTGGCATCTGTAGG	58
α-Tubulin	S-CTGGAGCACTCTGATTGT	AS-ATAAGGCGGTTAAGGTTAGT	55
β-actin	TACAGCTTCACCACCACAGC	AAGGAAGGCTGGAAGAGAGC	55

2.8 Small interfering RNA (siRNA) transfection

siRNA transfection was achieved by using the siPORT™ NeoFX™ transfection agent (Gibco Invitrogen Co., Paisley, Scotland, UK), according to the manufacturer's instructions. In 6 cm^3 petri-dishes 10 μL of siPORT NeoFX transfection agent (TA) and 5 μL of scrambleRNA (scRNA), at 20 μM, or 10 μL of ATG5-siRNA, at 10 μM (both from Santa Cruz Biotechnology, Santa Cruz, CA, USA) were diluted in 200 μL of OPTI-MEM I medium. In 24-well plates, 1 μL of TA and 0.5 μL of scRNA or 1 μL of ATG5-siRNA were diluted in 20 μL of OPTI-MEM I medium. After incubating for 10 min at room temperature, the mixture was added to the LTEDaro cell suspension.

For ATG5 knockdown detection through western-blot, LTEDaro cells were cultured in 6 cm^3 petri-dishes (4 x 10^5 cells/mL). After 3 days of transfection, proteins were extracted and separated as mentioned above. For cell viability assays, transfected LTEDaro cells were cultured in 24-well plates (1 x 10^5 cells/mL) and incubated for 3 or 4 days in the presence of Ana (10 μM), Let (10μM) or Exe (10 μM) and analyzed though the MTT assay

2.9 Statistical analysis

Assays were carried out in triplicate in at least three independent experiments and data was expressed as the mean ± SEM. Statistical analysis was performed through analysis of variance (ANOVA) followed by Tukey and Bonferroni post-hoc tests for multiple comparisons as already described [25]. Values of $P < 0.05$ were considered as statistically significant.

Results

3.1 Absence of autophagic markers on non-steroidal AI-treated resistant cells.

In order to evaluate if autophagy plays a protective role in Ana- and Let-resistance, the autophagic biomarkers, formation of acidic vesicle organelles (AVOs), LC3-II protein expression levels and the mRNA transcript levels of *SQSTM1*, which encodes the p62 protein, were analyzed in Ana- and Let-treated LTEDaro cells, since these cells mimic late-stage acquired resistance to AIs due to the long-estrogen deprivation. Exe-treated LTEDaro cells were used as positive control, since we previously reported that Exe induced protective autophagy in these cells [25]. Results demonstrated that contrary to Exe, Ana (10 μM) and Let (10 μM) did not induce LC3-II expression (Fig 1A) and did not alter *SQSTM1* relative mRNA levels (Fig 1B) when compared to untreated cells, after 3 days of treatment. Furthermore, analysis, by flow cytometry, also revealed that contrary to Exe, the non-steroidal AIs did not increase the number of AVOs (which are commonly associated with autophagic vesicles), as verified for the maintenance of AO^- and AO^+ cells in Ana and Let treatments, when compared to untreated cells, after 6 days of treatment (Table 2).

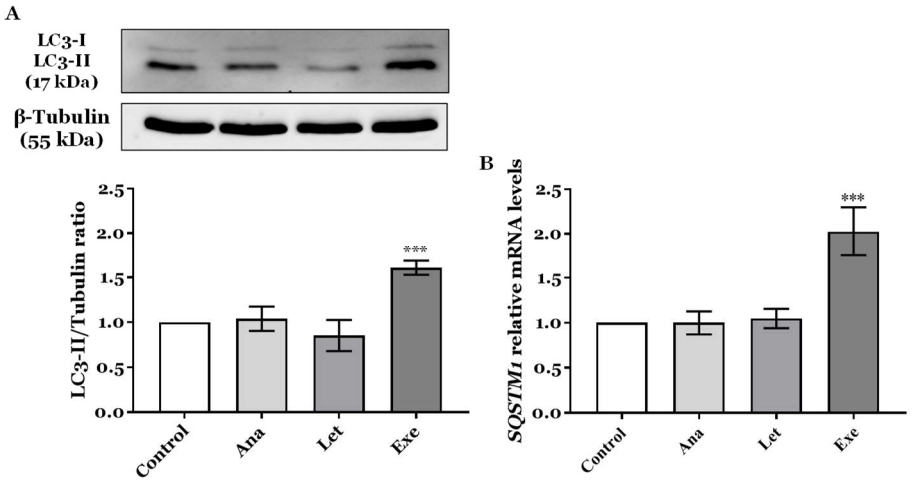

Figure 1. Effects of AIs on autophagy induction in LTEDaro cells, after 3 days of treatment. (A) Western-blot analysis of LC3-II protein expression in cells incubated with each AI. β-Tubulin was used as a loading control. The protein expression obtained for treated cells was normalised in relation to protein expression of control. (B) *SQSTM1* relative mRNA expression levels in cells incubated with each AI. α-tubulin was used as housekeeping gene. The mRNA transcript levels of treated cells were normalised in relation to mRNA transcript levels of control. Untreated LTEDaro cells were considered as control. Results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the control versus treated cells are indicated by *** ($p < 0.001$).

To confirm these results, ATG5 knockdown through siRNA was also performed (Fig. 2A), since ATG5 is a pivotal protein involved in the regulation of autophagy [48]. The addition of ATG5 siRNA did not affect the viability of cells treated with Ana or Let when compared to cells treated with scRNA.

However, as expected and corroborating our previous study [25], in Exe-treated LTEDaro cells the addition of the siRNA caused a statistically significant ($p < 0.05$) decrease in cell viability, after 4 days of treatment (Fig 2B).

Table 2. Effects of the AIs on the formation of AVOs in LTEDaro cells.

	AO^-	AO^+
Control	94.46 ± 0.52	5.24 ± 0.45
Ana	94.97 ± 0.80	5.09 ± 0.84
Let	95.79 ± 0.64	4.42 ± 0.67
Exe	67.57 ± 1.61 ***	31.24 ± 1.45 ***
H_2O_2	74.49 ± 2.35 ***	26.25 ± 2.41 ***

Cells were treated with the AIs for 6 days. Untreated LTEDaro cells were considered as control. Cells were incubated with AO (0.5 μg/mL) and analyzed by flow cytometry. Cells treated with H_2O_2 (50 nM) were considered as positive control. Data is presented as single cell events of viable cells, AO negative (AO^-), and with AVOs formation, AO positive (AO^+). Results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between the control and treated cells are indicated by ***($p < 0.001$).

3.2 Effect of an autophagic inhibitor on non-steroidal AI-treated resistant cells

In order to confirm the lack of involvement from PI3K class III in Ana- and Let-treated resistant cells, it was added the pan-PI3K inhibitor, Wortmannin (WT), at 0.1 μM, that was previously reported as an autophagic inhibitor with the ability to re-sensitize resistant cells to Exe [25]. The results showed that WT did not re-sensitize the LTEDaro cells to Ana or Let, after 6 days of treatment, thus, confirming the lack of autophagy and the non-involvement of PI3K class III in response to two nonsteroidal AIs (Fig. 3A).

The effects of WT alone on cell viability of AI-specific resistant cell lines, AnaR, LetR and ExeR, were also studied to validate the results obtained in the LTEDaro cell line (Fig. 3B). As expected, we verified that WT did not affect AnaR and LetR cell viability, apart from the highest concentration in AnaR cells ($p < 0.001$). However, WT significantly ($p < 0.01$; $p < 0.001$) decreased ExeR cell viability for all the concentrations in a dose-dependent manner when compared to untreated cells, except for the lowest concentration. These results indicate that the resistance mechanisms of Exe are different from those of Let and Ana.

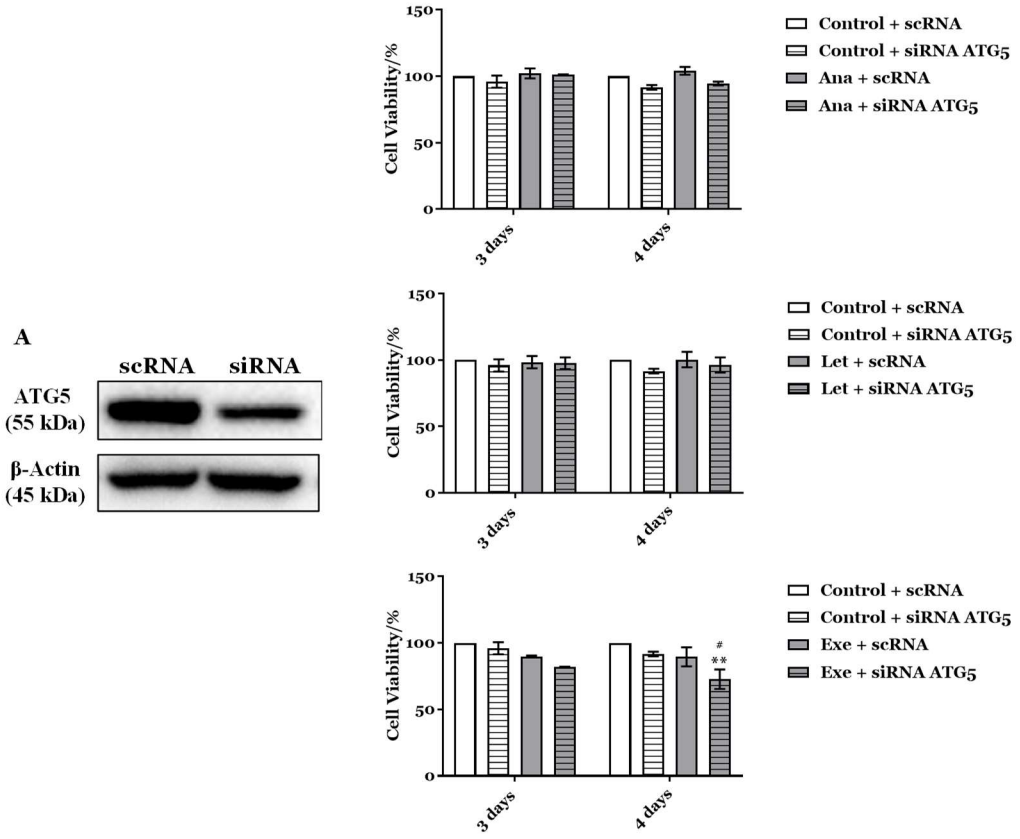

Figure 2. Effects of ATG5 silencing in AI-treated LTEDaro cells viability. (A) Western-blot for ATG5 in cells treated with scRNA or siRNA. (B) Cell viability of untreated or AI-treated with (siRNA) or without (scRNA) ATG5 silencing, after 2 and 4 days of treatment. scRNA-treated cells were considered as control. Results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between siRNA ATG5 + AIs and Control + siRNA ATG5 are denoted by ** ($p < 0.01$). Significant differences between siRNA ATG5 + AIs and scRNA + AIs are denoted by # ($p < 0.05$).

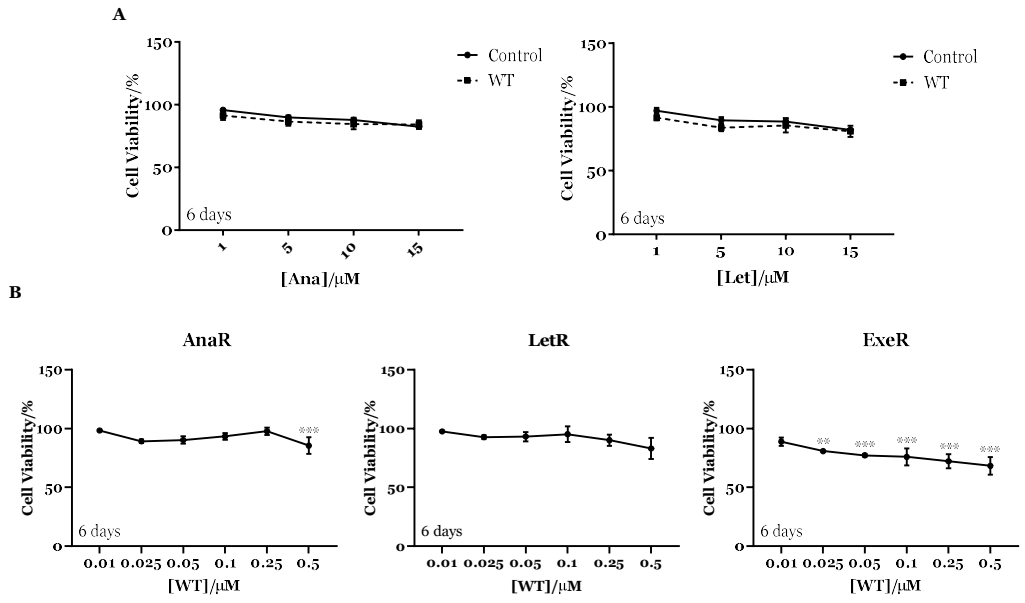

Figure 3. Effects of WT on AI-resistant cells viability. (A) LTEDaro cells were treated with AIs, with or without the autophagic inhibitor, WT, after 6 days of treatment. Untreated cells or cells treated only with WT were considered as control. (B) AnaR, LetR and ExeR cells were incubated with or without WT, after 6 days of treatment. Cells without WT were considered as control. Results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between control and WT treatment are denoted by ** ($p < 0.01$) and *** ($p < 0.001$).

3.3 Effects of BYL-719 on AI-treated LTEDaro cell viability

To explore the effects of the PI3K class I inhibitor, BYL-719, LTEDaro cells were treated with different concentrations (0.005 – 10 μM) of BYL-719 for 6 days and the impact on cell viability was evaluated by the MTT assay (Fig. 4A). The results demonstrate that the highest dose that did not cause a significant decrease in cell viability was 0.05 μM.

To confirm that this dose effectively inhibited PI3K class I, the AKT activation (p-AKT/AKT ratio) was then evaluated by Western-blot in LTEDaro cells incubated with BYL-719, at 0.05 μM, for 3 days (Fig. 4B). The selected concentration significantly ($p < 0.05$) decreased AKT activation, when compared to untreated cells, and therefore was used in the subsequent assays. After this, the effects of BYL-719 in combination with AIs on LTEDaro cell viability was evaluated after 3 (data not shown) and 6 days (Fig. 4C). This combination significantly ($p < 0.05$, $p < 0.001$) decreased cell viability in a dose- and time-dependent manner, when compared to AIs alone. Moreover, this combination significantly ($p < 0.001$) decreased AKT activation when compared to AIs alone (Fig. 4B), after 3 days of treatment. Strikingly, it was also verified that Exe induced AKT activation *per se*, an effect partially reverted by BYL-719. It is reported that estrogen promotes the upregulation of AKT via ERα [49-51], and that Exe possess an estrogen-like activity [38, 39]. Therefore, ICI 182,780 was combined with Exe and AKT activation was evaluated (Fig. 4C). The addition of ICI 182,780 significantly ($p < 0.001$) abrogated the increase in AKT activation induced by Exe.

3.4 Anti-proliferative effects induced by the combination of BYL-719 with AIs

To further understand whether the decrease in LTEDaro cell viability induced by the combinations was due to anti-proliferative effects, the cell cycle progression was analyzed (Table 3). BYL-719 alone induced a significant ($p < 0.001$) cell cycle arrest at the G_2/M phase and a significant ($p < 0.01$) reduction at G_0/G_1 phase, when compared to control. Moreover, the combination with Exe caused a significant ($p < 0.001$) arrest at the G_0/G_1 phase and significantly ($p < 0.001$; $p < 0.05$) decreased the S and the G_2/M phases, while the combinations with Ana or Let induced a significant ($p < 0.001$) cell cycle arrest at G_2/M and significantly ($p < 0.05$; $p < 0.01$; $p < 0.001$) decreased the S and G_0/G_1 phases, when compared to each AI alone.

3.5 Cell death mechanisms induced by BYL-719 in combination with AIs

To understand the mechanisms that lead to a reduction in LTEDaro cell viability, several apoptotic markers were evaluated after 3 days of treatment. Analysis of the combination of BYL-719 with AIs revealed that only the combination with Exe significantly ($p < 0.01$; $p < 0.001$) increased Annexin$^+$/7-AAD$^-$ and Annexin$^+$/7-AAD$^+$ marked cells, which corresponds to early-apoptotic and late apoptotic/necrotic cells, respectively, when compared to Exe alone (Table 4). Moreover, this combination also significantly ($p < 0.001$) increased caspase-7 (Fig. 5A) and caspase-9 (Fig. 5B) activities, by 29.70% and 36.3%, respectively, and significantly ($p < 0.001$) reduced $\Delta\Psi m$, when compared to Exe alone (Fig. 5C). Curiously, BYL-719 when combined with Ana or Let, caused no significant alterations on the studied apoptotic biomarkers, when compared with AIs alone.

3.6 Effects of BYL-719, with or without ICI 182,780, on the viability of AI-specific resistant cells

In order to reinforce the results obtained in the LTEDaro cells, the effects of BYL-719 on AnaR, LetR and ExeR cell viability were also evaluated, after 3 (data not shown) and 6 days of treatment. BYL-719 significantly ($p < 0.05$; $p < 0.01$; $p < 0.001$) decreased cell viability in all these cell lines, in a dose- and time-dependent manner, when compared to untreated cells (Fig. 6A). In addition, due to the approval of the combination of ICI 182,780 and BYL-719 for postmenopausal breast cancer patients with *PIK3CA*-mutated tumors previously treated with AIs, the efficacy of this combination was also assessed in the resistant cell lines after 6 days of treatment (Fig. 6B). Results demonstrate that ICI 182,780 (0.1 μM) significantly ($p < 0.05$; $p < 0.01$; $p < 0.001$) enhanced the effects of BYL-719 in these cell lines, this effect being more evident for LetR and ExeR cells.

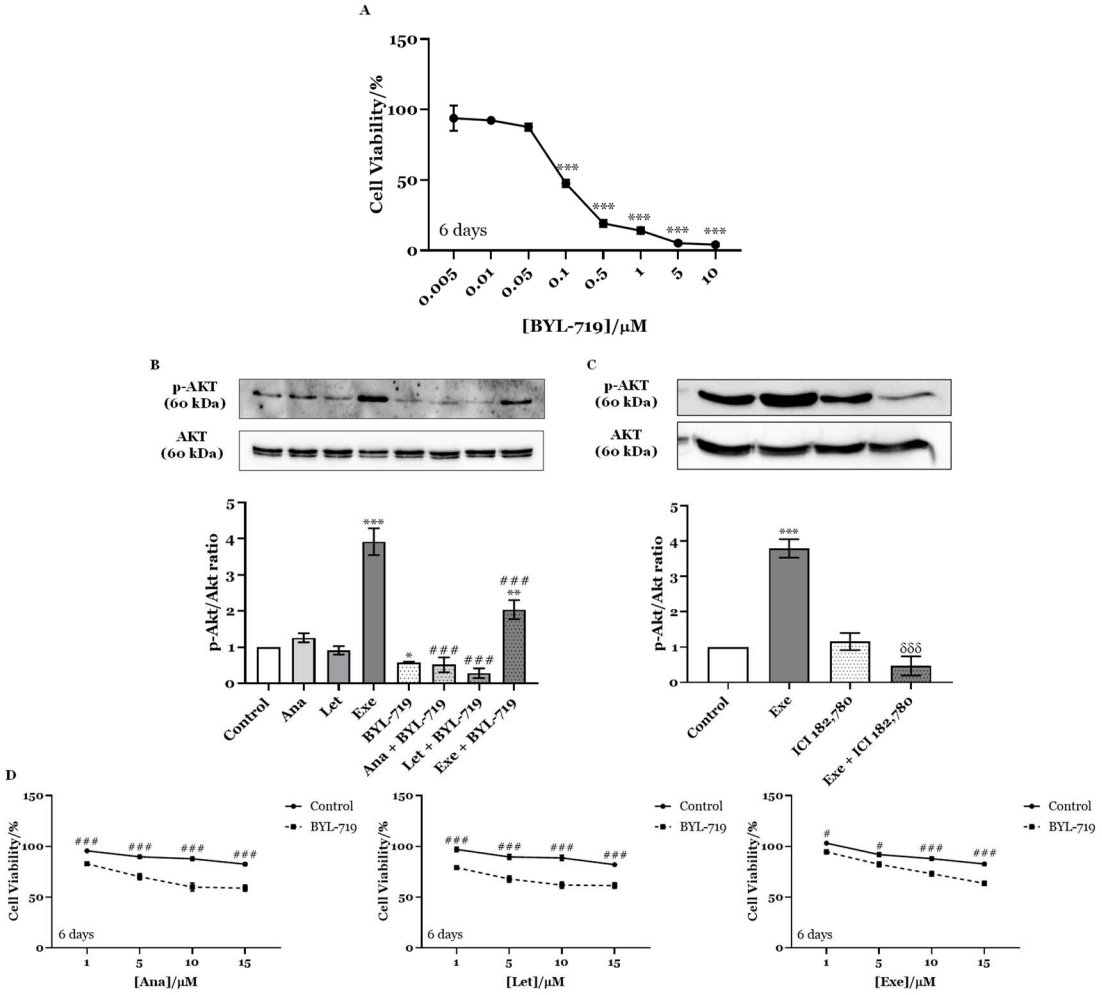

Figure 4. Effects of BYL-719 alone or in combination with AIs on LTEDaro cells. (A) Cells were incubated with BYL-719, for 6 days and cell viability was evaluated by the MTT assay. Untreated cells were considered as control. (B) AKT activation (p-AKT/AKT ratio) was verified by Western-blot by evaluating the protein expression levels of p-AKT and AKT. Cells were incubated with AIs, BYL-719 or with a combination of AIs and BYL-719, for 3 days. Untreated or BYL-719-only treated cells were considered as controls. The protein expression obtained for treated cells was normalized in relation to protein expression of control. (C) AKT activation (p-AKT/AKT ratio) was verified by Western-blot by evaluating the protein expression levels of p-AKT and AKT. Cells were incubated with Exe, ICI 182,780 or with the combination of Exe and ICI 182,780, for 3 days. Untreated or ICI 182,780-only treated cells were considered as controls. The protein expression obtained for treated cells was normalized in relation to protein expression of control. (D) Viability of cells treated with AIs and with or without BYL-719, after 6 days of treatment. Untreated cells or cells treated only with BYL-719 were considered as control. Significant differences between control and AI-only or BYL-719-only treatments or between BYL-719-only treatment and cells treated with a combination of AIs plus BYL-719 are denoted by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). Significant differences between AI-only treated cells and cells treated with a combination of AIs plus BYL-719 are indicated by # ($p < 0.05$) and ### ($p < 0.001$). Significant differences between Exe alone and Exe plus ICI 182,780 are denoted by δδδ ($p < 0.001$). Results are the mean ± SEM of three independent experiments, performed in triplicate.

4. Discussion

The main concern in breast cancer treatment is the development of resistance to therapy. Several mechanisms of resistance have already been reported, such as the role of PI3K in sustaining ER activity, leading to estrogen independence [4, 11, 52-54]. In fact, PI3K is often mutated in breast cancer [8, 9]. Thus, several clinical trials have combined PI3K inhibitors, using either BKM-120, GDC-0941 or BYL-719, with endocrine therapy [14-19]. These studies led to the approval of the combination of BYL-719 and ICI 182,780 in postmenopausal women with $ER^+/HER2^-$, *PIK3CA*-mutated advanced or metastatic breast cancers refractory to endocrine therapy [2]. Moreover, both PI3K class I and class III regulate autophagy, a process that has been linked to Everolimus and Exe insensitivity [25, 32] and that could also be correlated with the lack of improvement on overall survival observed in the BOLERO-2 clinical trial [34, 35]. Taking these reports into account, in this work the effects of PI3K class I inhibition on AI-resistant cells, as well as the effects of PI3K class III inhibition and the involvement of autophagy in Ana- and Let-resistant cells were studied.

Table 3. Effects of AIs alone or in combination with BYL-719 on LTEDaro cell cycle progression.

Cell cycle phase	G_0/G_1	S	G_2/M
Control	81.98±0.42	5.47±0.14	11.98±0.33
Ana	82.12±0.61	5.90±0.15	11.69±0.58
Let	82.45±0.51	5.40±0.16	11.86±0.39
Exe	83.43±0.75	5.37±0.36	11.02±0.79
BYL-719	79.60±1.11**	4.81±0.23	15.23±0.95***
Ana+BYL-719	78.62±0.91 ###	3.03±0.16##	18.11±0.37 **###
Let+BYL-719	77.56±1.00 ###	2.96±0.14#	18.69±0.93 ** ###
Exe+BYL-719	89.06±0.91 *** ###	1.85±0.13 *###	8.72±0.84 ***#

LTEDaro cells were treated with AIs (10 μM) alone or in combination with BYL-719, for 3 days. Untreated or BYL-719-only treated cells were considered as control. After staining with propidium iodide (PI) (5 μg/ml), cells were analyzed by flow cytometry. Values are represented as a percentage of single cell events in each stage of the cell cycle and are the mean ± SEM of at least three independent experiments performed in triplicate. Statistically significant differences between control and treatments are expressed by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$), while between AIs and AIs plus BYL-719 are indicated as # ($p < 0.05$), ## ($p < 0.01$) and ### ($p < 0.001$

Interestingly, contrary to Exe [25], which was used in this study as a positive control, neither Ana nor Let increased autophagic markers, such as LC3-II levels, *SQSTM1* expression and AVOs. These results were further reinforced through ATG5 silencing and by the combination of the non-steroidal AIs with the autophagic/pan-PI3K inhibitor, WT, which failed to impact cell viability in Ana- and Let-treated LTEDaro cells, in opposition to Exe treatment [25]. These findings were further validated in cell lines specifically resistant to each AI (AnaR, LetR and ExeR). In these cell models, WT did not affect AnaR and LetR cell viability, while it significantly decreased ExeR cell viability. This higher sensitivity of the ExeR cell line to WT corroborates our previous study in Exe-treated LTEDaro cells [25] and confirms the existence of specific AIs–resistance mechanisms, since contrary to Exe, Ana and Let were not able to induce autophagy in acquired-resistant breast cancer cells.

After excluding autophagy as a mechanism involved in resistance to Ana and Let, and following a report that demonstrated that the combination of BYL-719 with Tamoxifen re-sensitized breast cancer cells to Tamoxifen [55], the efficacy of the class I PI3K inhibitor, BYL-719, in AI-resistant cell models was evaluated. It should be pointed that the selected dose in this study (0.05 μM) is lower than the frequently used in other similar breast cancer studies [56, 57]. Nevertheless, at this dose, BYL-719 per se decreased AKT activation. Moreover, the combination of BYL-719 with AIs decreased LTEDaro cells viability and AKT activation when compared to AIs alone.

Table 4. Effects of AIs alone or in combination with BYL-719 in Annexin V-FITC labelling.

	Annexin V^-/7-AAD^-	Annexin V^+/7-AAD^-	Annexin V^+/7-AAD^+
Control	95.57±0.38	2.13±0.32	2.30±0.29
Ana	96.07±0.74	1.93±0.42	2.00±0.40
Let	96.13±0.68	2.15±0.37	1.72±0.47
Exe	94.35±0.68	3.06±0.64	3.56±0.32
BYL-719	95.69±0.42	2.12±0.56	2.02±0.29
Ana+BYL-719	93.77±1.96	2.61±1.15	3.62±1.12
Let+BYL-719	95.43±1.39	1.51±0.28	3.07±1.12
Exe+BYL-719	81.59±1.71***###	8.88±1.70***##	9.53±2.77***###
STS	48.66±1.06***	22.00±0.31***	29.34±0.74***

LTEDaro cells were treated with AIs alone or in combination with BYL-719, for 3 days, and labeled with Annexin V-FITC and 7-AAD followed by flow cytometry analysis. Data are presented as viable cells (Annexin V^-/7-AAD^-), early apoptotic (Annexin V^+/7-AAD^-) and late apoptotic or necrotic cells (Annexin V^+/7-AAD^+). Untreated or PI3K inhibitor-only treated cells were considered as control, while cells treated with STS (10 μM) were considered as positive control for apoptosis. The results are expressed as mean ± SEM of three independent experiments, performed in triplicate. Statistically significant differences between control and treatments are expressed by *** ($p < 0.001$), while differences between AIs and AIs plus BYL-719 are denoted as ## ($p < 0.01$) and ### ($p < 0.001$).

Interestingly, in our conditions, Exe per se induced a marked activation of AKT, which was reverted by BYL-719. The observed increase in AKT for Exe-treatment was completely abrogated by the addition of ICI 182,780, confirming the involvement of Exe-activated ERα in the upregulation of the AKT activity. In fact, *Wang et al* (2017) has verified an increased in p-AKT in ExeR cells when compared to the parental MCF-7aro, AnaR and LetR cells [58]. Confirming our findings, in the AI-specific resistant cell lines, AnaR, LetR and ExeR, the PI3K inhibitor also reduced cell viability in a dose- and time-dependent manner, being the effects more potent in the ExeR cell line for lower doses of the PI3K inhibitor. The efficacy of BYL-719 can be explained by the importance of PI3K/AKT/mTORC1 pathway in the development of anti-estrogens resistance [4, 53, 54]. Nevertheless, the AI-resistant cell lines present other mechanisms that assure ERα activity. The AnaR and LetR cell lines present a constitutively active ERα due to the activation of the MAPK/ERK survival pathway, via growth factor receptors, such as the epidermal growth factor receptor (EGFR) or the insulin growth factor-1 receptor (IGF-1R) [39, 59-61]. Moreover, the ExeR cell line presents an active ERα due to the estrogen-like activity of Exe, promoting the expression of an EGFR ligand and leading to activation of the MAPK/ERK pathway [38, 39, 62].

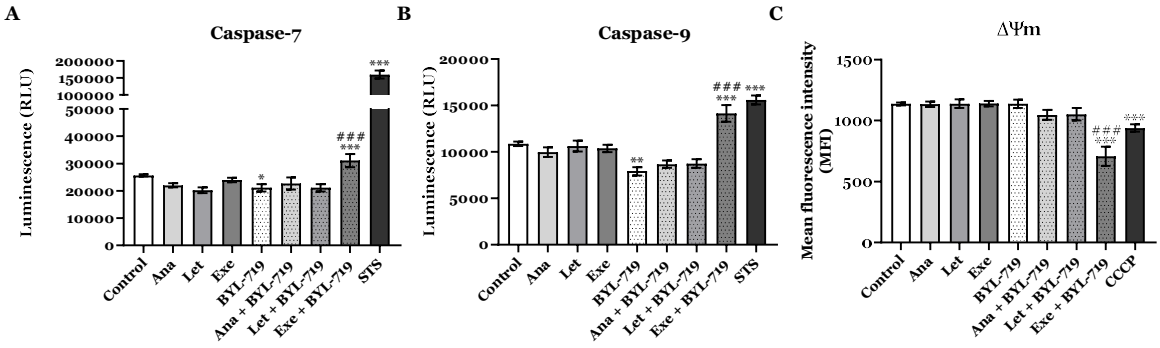

Figure 5. Effects of AIs alone or in combination with PI3K inhibitors on caspase activation and ΔΨm. LTEDaro cells were incubated with AIs, with or without BYL-719. (A) Caspase-7 activity and (B) caspase-9 activity luminescence assays. (C) Loss of ΔΨm fluorescence assay. Untreated cells or treated only with BYL-719 were considered as control. STS, at 10 μM, was used as positive control for caspase activation, while CCCP, at 10 μM, was used as positive control for loss of ΔΨm. For caspases, values are presented as relative luminescence units (RLU), while for ΔΨm values are represented as mean fluorescence intensity (MFI) The results are expressed as mean ± SEM of three independent experiments, performed in triplicate. Significant differences between control and AI-treated cells are denoted by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$), while between AI-only treated cells and cells treated with a combination of AIs plus BYL-719 are indicated by ### ($p < 0.001$).

Therefore, due to the high dependence of ERα transcriptional activity in AI-acquired resistance the combination of BYL-719 with ICI 182,780 was also studied. This combination proved to be more effective than BYL-719 alone in all the AIs specific-resistant cell lines, a behavior that can be explained by concomitant abrogation of ERα signaling and inhibition of PI3K. Altogether, our results suggest that the combination of BYL-719 and ICI 182,780 is superior to BYL-719 in AI-resistant tumors. This observation corroborates the SOLAR-1 clinical trial (NCT02437318), where this combination was superior to just ICI 182,780, which led to its FDA approval [2, 16].

Furthermore, the combination of BYL-719 with Ana and Let disrupted LTEDaro cell cycle in the G_2/M phase, while when combined with Exe induced an arrest at the G_0/G_1 phase. The differences in the anti-proliferative effects observed between the combinations of BYL-719 with Ana/Let or with Exe might be a consequence of different AKT phosphorylation levels according to the AI used. Cell cycle arrest at the G_2/M phase is highly correlated with low AKT phosphorylation levels [63, 64]. In fact, BYL-719 alone was able to promote an arrest at the G_2/M due to the decrease in AKT phosphorylation levels. Similarly, the cell cycle arrest at G_2/M observed for Ana and Let treatments with BYL-719 is associated with low AKT phosphorylation. On the other hand, the inhibition of PI3K, by BYL-719, was not so effective on Exe-treated cells, since AKT phosphorylation was still at higher levels than in control. Thus, an arrest at G_0/G_1, instead of at G_2/M, was observed for this AI. Since, a disruption of the cell cycle is often associated with increased apoptosis and cytotoxicity [24, 46, 65], the occurrence of apoptosis was explored. Curiously, only the combination of Exe with BYL-719 induced apoptosis through the intrinsic pathway, as suggested by the activation of caspases-7 and -9 and by the ΔΨm loss. These findings also support the different behavior

between AIs in fundamental cellular processes linked with tumor progression and acquired resistance to AIs treatment. Moreover, the occurrence of apoptosis verified only for the combination of Exe and BYL-719 might be a consequence of a higher dependence of the PI3K/AKT/mTORC1 pathway in Exe-treated cells since Exe treatment *per se* increased AKT activation and BYL-917 reverted significantly this increase.

This study, unlike Exe, excludes autophagy as a mechanism of resistance to Ana and Let. One of the reasons for this difference may be the direct modulation of ERα induced by Exe [38, 39]. In fact, ICI 182,780 treatment or ERα knockdown have been shown to induce a cytoprotective autophagy, that was also important for ICI 182,780-resistance [66, 67]. Moreover, estradiol also induces autophagy, by activating ERα, in order to promote ERα degradation through a feedback loop [68]. Thus, a possible correlation between ERα modulation and autophagy might explain the effects of Exe. Furthermore, it reports that, depending on the AI used in the combination, the AI-resistant LTEDaro cells responded differently to BYL-719 treatment, a behavior that, in the future, may help to understand the results of ongoing clinical trials with BYL-719.

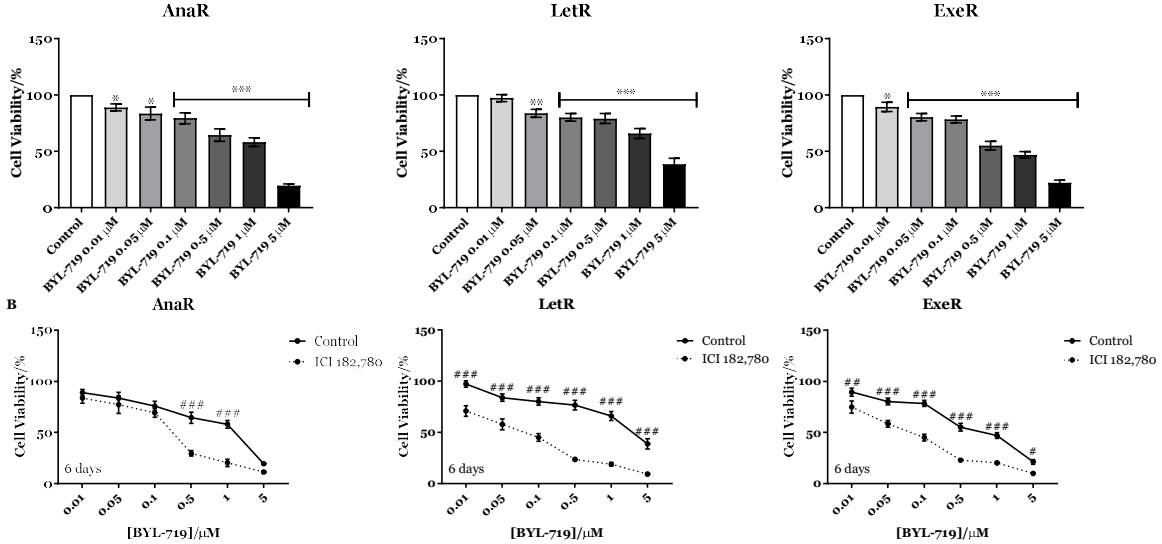

Figure 6. Effects of BYL-719 on cell viability of AI-specific resistant cells. (A) Cells were incubated with BYL-719, after 6 days of treatment. Cells without BYL-719 were considered as control. (B) Cells were incubated with BYL-719, with or without ICI 182,780, after 6 days of treatment. Cells without BYL-719 and without ICI 182,780 or cells only with ICI 182,780 were considered as control. Results are the mean ± SEM of three independent experiments, performed in triplicate. Significant differences between control and BYL-719 treatment are denoted by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$), while between cells treated only with BYL-719 and cells treated with BYL-719 and ICI 182,780 are denoted by # ($p < 0.05$), ## ($p < 0.01$) and ### ($p < 0.001$).

Therefore, this work may support the efficacy observed in phase I clinical trials (NCT01791478, NCT01870505) combining BYL-719 with Let or Exe in advanced ER+ breast cancer [17, 19] and highlights the *in vitro* biological effects behind the effectiveness of these combinations. Furthermore, since this work also demonstrates a superiority of the combination of BYL-719 and ICI 182,780 over BYL-719 alone in AI-resistant cells, the importance of targeting both the PI3K/AKT/mTORC1 and estrogen signaling pathways over targeting just one of them in AI-resistant tumors is reinforced.

Author contributions

Conceived and designed the work: TVA, CA, YW, SC, NT, GCdS. Performed the experiments: TVA, CA, CFA. Analyzed and interpreted the data: TVA, CA, YW, SC NT, GCdS. Wrote the paper: TVA, CA, NT, GCdS. Revised the manuscript and approved for publication: TVA, CA, YW, CFA, SC, NT, GCdS

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CHAPTER III

Discussion and Conclusions

Breast cancer is the most common cancer and the leading cause of cancer death in women worldwide (11). Estrogen receptor-positive (ER^+) breast cancer is the most common subtype, corresponding to 60% and 75% of all cases in pre- and postmenopausal patients, respectively (260). Aromatase inhibitors are the first-line current therapy for ER^+ breast cancer, either alone or in combination with CDK 4/6 inhibitors (293, 296). The third-generation of AIs, is composed of the non-steroidal reversible Anastrozole and Letrozole, derived from triazole, and the steroidal irreversible Exemestane, derived from the structural analog of the natural substrate of aromatase, androstenedione. Although these AIs have demonstrated clinical success, the occurrence of adverse side-effects and the development of acquired-resistance are their major drawbacks (260). The development of the resistant state is a consequence of adaptation to long-term estrogen deprivation, being, nowadays, the major concern in clinical practice. These disadvantages justify the search for new potent and specific AIs, as well as the elucidation of the mechanisms responsible for AI-resistance.

Having this into account, the search for compounds that strongly inhibit aromatase and present anti-cancer properties with fewer side effects was one of the aims of this thesis. Thus, the biochemical and biological evaluation of newly synthesized molecules as potential AIs was performed. Our previous studies have shown that the introduction of suitable functional groups in the B-ring, at the C-6 position, of A- and B-modified androstenedione derivatives (**1a**, **3a**, **9**, **13**, **15**, **17**, **19,** and **20**) originated new compounds with strong anti-aromatase activity, in human placental microsomes (102). In order to deepen our knowledge about these molecules, their activities in MCF-7aro cells, an ER^+ breast cancer cell line that overexpresses aromatase, were explored, being compounds **9**, **13,** and **15** the most potent. These steroids all share a carbonyl group at C-17 that has been demonstrated to be pivotal to achieve high aromatase inhibition (95, 97, 704, 705). In addition, it was demonstrated the importance of the association of a carbonyl group at C-3 with a C-6α-methyl/allyl aliphatic chain to obtain potent AIs. According to what was previously shown, the inclusion of an extra double bond at C-1 further improved the aromatase inhibition, due to the increased planarity in the A-ring (**13** vs **15**) (95, 102, 704, 705). The results also demonstrated that when the A-ring is shared between compounds, a C-6α-methyl substitution increases aromatase inhibition when compared to a C-6α-allyl or C-6α-hydroxyl substituent (**9** vs **15** vs **20**). These observations were in line with what was detected in human placental microsomes (102). Curiously, contrary to what was observed in human placental microsomes for compounds **1a** and **3a** (102) that share the same B-ring, in MCF-7aro cells, the introduction of an epoxide group at C-3/C-4 did not enhance aromatase inhibition when compared to a double bond at C-3. This benefit of the double bond over the epoxide group has already been verified for a different set of compounds (677, 704). In summary, these SAR revealed that the most favorable substitution to achieve a strong

aromatase inhibition is the C-6α-methyl substitution, when the A-ring presents a carbonyl group at C-3 and a double bond at C-4. This reinforces the importance of planarity in the A-ring and the superiority of a double bond substitution when compared to an epoxide group. Nevertheless, although this set of compounds demonstrated high efficacy, due to their steroidal backbone, their potential effects on off-target molecules that bind steroidal ligands must be considered.

After describing the ability of these compounds to inhibit aromatase, their biological effects and mechanisms of action were also explored. First, it was verified that these molecules did not present cytotoxic effects on a non-tumoral cell line, HFF-1, while, at the same concentrations (1 – 25 μM), decreased MCF-7aro cell viability in a dose- and time-dependent manner, with compounds **1a**, **3a**, **9** and **15** showing the greatest effects. To understand the mechanisms behind the decrease in cell viability, the dependency of the effects on aromatase and steroid receptors, ERα and AR, was assessed. Through the addition of the product of the aromatization reaction, E2, it was verified that, apart from compounds **19** and **20**, the decrease in cell viability was dependent on aromatase inhibition. This has already been described as the main anti-tumoral mechanism for other sets of compounds (95, 677). Moreover, since Exemestane is able to decrease aromatase protein levels through protein degradation (383), this ability was also evaluated for all the compounds. Only compounds **9** and **13** demonstrated a reduction in aromatase protein levels without alteration in *CYP19A1* mRNA expression levels suggesting protein degradation. Interestingly, the ability to decrease aromatase protein levels does not seem to correlate with the type of inhibition since compound **9** was described as a reversible inhibitor and compound **13** as an irreversible inhibitor (102). Nevertheless, this behavior was also reported by our group for other AIs (95). The presence of a carbonyl group at C-17 and of double bonds or epoxide groups at C-1 and/or C-4 has been shown to be pivotal features for aromatase-dependence (95, 677). Looking at the structures that present double bonds at C-1 and/or C-4, the SAR analysis revealed that, contrary to the hydroxyl groups (**19** and **20**), the presence of a C-6α-methyl/allyl substitution (**9**, **13**, **15** and **17**) did not interfere with the aromatase-dependent effects. Moreover, it was observed that this dependency is not affected when the double bond or the epoxide group shift from C-4 to C-3 (compounds **1a** and **3a**). The same observations were made in a previous work by our group, where the introduction of an allyl or epoxypropyl group, at C-7, also did not alter aromatase-dependent effects in the presence of a double bond at C-1 and C-4 and of a carbonyl group at C-17 (95).

Furthermore, previous studies from our group demonstrated that some of the AIs actions were also dependent on ERα (95, 677). Therefore, to study ERα-dependency, cells were

incubated with the SERD, ICI 182,780. It was verified that the effects of compounds **1a**, **3a,** and **15** were dependent on ERα since the presence of ICI 182,780 abrogates the growth-inhibitory action of these steroids. Nevertheless, none of the new molecules altered ERα protein levels. The ability of these compounds to modulate ERα without affecting its expression levels is a mechanism of action also reported for Tamoxifen (710). This capacity in combination with their anti-aromatase efficacy may be a clinical advantage when compared to any of the isolated mechanisms (711). These observations have refined the conclusions of a previous study, where it was suggested that the presence of a hydroxyl group at C-17 was responsible for ERα-dependency (95), since compounds **1a**, **3a,** and **15** present a carbonyl group at C-17 and also modulate ERα. Thus, the nature of the C-17 substituent does not seem to be determinant for ERα-dependency. Nevertheless, the exact functional groups required for ERα modulation are still uncertain, and more studies are demanded.

In recent years, the importance of AR in breast cancer has increased. In fact, 85–95% of the ER^+ breast cancers and 77% of invasive tumors express AR (617, 618). This nuclear receptor is often associated with anti-proliferative mechanisms in ER^+ breast cancer (622, 628, 712). Therefore, since AR is an attractive target for modulation in breast cancer, it was explored whether the effects of the compounds were dependent on AR modulation. For that, breast cancer cells were treated with the AR antagonist CDX. The effects of compounds **1a**, **3a**, **9**, **15,** and **19** were AR-dependent, since CDX abrogated their anti-cancer properties. Moreover, these compounds also increased AR protein levels, which also occurs in response to the natural ligand of AR (389, 622, 713). The pro-death role of AR in breast cancer cells treated with these compounds matches the behavior also reported for AR in response to Letrozole (622). This AR-dependency was previously described for different steroidal compounds studied by our group (95, 389). These are promising results as AR modulation may present a therapeutic advantage in the treatment of ER^+ breast cancer.

The mechanisms of action of these steroidal compounds were further studied by analyzing their anti-proliferative effects. All the compounds promoted a cell cycle arrest at G_0/G_1 phase. It has already been reported by our group that similar steroidal compounds, as well as Exemestane, also disrupt cell cycle progression (95, 96, 384, 386, 708). In order to verify if these effects were associated with cell death, the caspase-7 activity and the mitochondrial membrane potential ($\Delta\Psi m$) were evaluated. The results confirm that all the compounds induce apoptosis through the mitochondrial pathway, which is in accordance with the behavior of previous steroidal compounds studied by our group, including Exemestane (95, 96, 384, 386, 708).

These new steroidal compounds present anti-aromatase and anti-tumoral efficiency. Their anti-proliferative effects are achieved by single, dual, or triple dependency on aromatase, ERα and/or AR. Thus, some of them are multi-target compounds, which is plausible due to the similarities in the binding sites of these targets (714). The multi-target compounds present the advantage of modulating different targets, improving their efficacy, without the risk of drug interactions that may lead to more adverse effects (714). Compounds **1a**, **3a,** and **15** were dependent on aromatase, ERα, and AR, while AI **19** was only dependent on AR. Steroids **13** and **17** were only dependent on aromatase and compound **9** on aromatase and AR. Moreover, only AIs **9** and **13** induced a decrease in aromatase protein levels. The several mechanisms of action presented by these new promising AIs revealed that the introduction of some functional groups in the steroidal backbone allow the modulation of several targets that may interact between each other, such as the ERα/AR crosstalk, and are important for the anti-tumoral efficacy (549). These new steroidal AIs properties may help on the elucidation of the most favorable modifications on the steroidal scaffold of androstanes to achieve anti-aromatase and anti-tumoral effects.

The search for new and potent AIs is highly demanded to bypass damaging side effects associated with AIs therapy and to increase the AIs repertoire in cases of acquired resistance in clinic. Nevertheless, the discovery of these new molecules is hindered by the standardized radiometric assay used for aromatase activity evaluation. This methodology was first described by *Thompson and Siiteri* (1974) and consists in the incubation of the enzyme with [1β-^{3}H]-androstenedione, in which tritiated water, the product of aromatization reaction, is quantified by liquid scintillation counting (103). Despite the high sensitivity of this assay, it is hazardous to health and non-environmentally friendly, however it is used to assess aromatase activity in several models, such as in human breast cancer cells (95, 96), human placental microsomes (97-102), equine placental microsomes (715) and rat ovarian microsomes (99). The risks that this methodology presents to health prompted the development of a new, safer, and sensitive assay for routine use, such as LC-MS (104) and GC-MS (105) assays. However, these methodologies presented some drawbacks. The LC-MS/MS methodology uses high quantities of human CYP19A1 supersomes and a high concentration of the substrate, androstenedione (104). Moreover, the GC-MS methodology reported in the literature needs significant amounts of human placental microsomes, does not use the natural substrate of the enzyme and the validation of the method was not described (105). Taking this into account, the second topic of this thesis focused on the development and validation of a new sensitive GC-MS methodology to evaluate the aromatase activity in human placental microsomes.

This new methodology quantifies androstenedione, the natural substrate of aromatase, and E1, the product of the aromatization reaction, in human placental microsomes. It is based on a fast and very effective extraction procedure of DLLME of the analytes, followed by a silylation step that allows the quantification of androstenedione and E1 by GC-MS in selective ion monitoring (SIM) mode, using Dihydrotestosterone-2,3,4-$^{13}C_3$, DHT-$^{13}C_3$, as internal standard. According to the FDA guidelines for these techniques, the developed methodology presented high linearity, good precision, and high accuracy for both hormones (716). When compared to the GC-MS methodology described by *Numazawa et al.* (2001), this new assay used a lower concentration of NADPH, lower amounts of placental microsomal protein, and lower reaction volumes (105). In addition, when compared to the LC-MS/MS method developed by *Park et al.* (2014), the new assay uses a lower concentration of androstenedione, lower amounts of placental microsomes, and lower reaction volumes (104). Therefore, compared to other works that used chromatographic methodologies, this new method used smaller amounts of matrix and reagents. The limits of detection in this assay (1 nM and 0.5 nM for androstenedione and E1, respectively) are also inferior to the ones described in the aforementioned works (104, 105). These characteristics highlight the high sensitivity of the method, allowing an accurate determination of E1, particularly, in the screening of new potent aromatase inhibitors.

In order to verify if this new GC-MS methodology would be a valid alternative to the radiometric assay in the evaluation of aromatase activity, the anti-aromatase activity (%) and the IC_{50} values of the three third-generation AIs were determined and compared to the standard radiometric assay. The GC-MS method is sensible in the presence of these very potent AIs, which demonstrates its feasibility in the screening of new molecules. Moreover, the IC_{50} values obtained were identical between both methods. Following these promising results, the IC_{50} values obtained for these AIs were compared with other studies. *Dukes et al.* (1996), through a radiometric assay, described a lower IC_{50} for Anastrozole in human placental microsomes, though there are some differences when compared to the new methodology, such as the use of testosterone instead of androstenedione (352). However, the aforementioned LC-MS/MS methodology by *Park et al* (2014) referred a higher IC_{50} for Anastrozole in human supersomes (104). A different radiometric assay, using human placental microsomes described a higher IC_{50} for Letrozole, corroborating the high sensitivity of this new methodology (353).

This study assessed and compared the IC_{50} values of the three AIs used in clinic, obtained in the same conditions by different methods, with the advantage of using lower amounts of a biological matrix and of the natural substrate of aromatase. Furthermore, to the best of our knowledge, no other GC-MS methodology enables such a rapid, accurate and sensitive

measurement of androstenedione and E1 and, consequently, of aromatase activity. Since this new method presents radiometric-like sensitivity and does not use a radio-labelled substrate, it can be considered a safer, cheaper, and more environmental friendly approach to assess aromatase activity. It is a good alternative to the standard radiometric assay and a relevant tool in the screening of new potent AIs, and, consequently, in the SAR studies of the best chemical modifications in the androstenedione backbone.

As mentioned above, there has been an increasing concern regarding the development of acquired resistance to AIs. Strikingly, the lack of cross-resistance among AIs provides evidence to suggest that there are clinical differences between these agents and distinct mechanisms of action. Therefore, the other aim of this work was to characterize at the molecular and cellular level the *in vitro* effects of the third-generation AIs. Our group previously reported that Exemestane decreased MCF-7aro cell proliferation, induced cell cycle arrest, apoptosis through the mitochondrial pathway, and cytoprotective autophagy (384). Similar studies also reported that Anastrozole and Letrozole inhibited the growth of breast cancer cells through cell cycle disruption, induced apoptosis and decreased the expression of estrogen-regulated genes (370, 371). Despite the different conditions used in the latter studies and in this work, the same mechanisms were observed for Anastrozole and Letrozole, such as a decrease in cell viability, cell cycle arrest at G_0/G_1 phase, and induction of apoptosis through the mitochondrial pathway. Moreover, all the AIs decreased the expressions of cyclins D and E, required for the G_1/S transition (717, 718). Despite these common features between AIs, unlike Exemestane (384), Anastrozole and Letrozole did not induce autophagy since no alterations in LC3 turnover or in *SQSTM1* gene expression were observed. The lack of autophagy for the non-steroidal AIs was also confirmed by flow cytometry. In fact, although there was an increase in acidic vesicle organelles (AVOs), usually representative of autophagosomes and autolysosomes associated with an autophagic process, no decrease was observed upon addition of the autophagic inhibitors, 3-methyladenine (3-MA) and Spautin-1 (SP). This indicated an accumulation of acidic vesicles distinct from autophagosomes and autolysosomes, suggesting high lysosomal content that might be correlated to senescence, a process that was shown to be triggered by a loss of estrogen signaling and induced by Tamoxifen or ICI 182,780 (397, 399, 400, 719). Currently, senescence lacks a specific biomarker as many of the associated morphological and molecular characteristics are also present in other cellular processes. Thus, the ability of AIs to promote cellular senescence was explored using different cellular and molecular markers like β-galactosidase (β-gal) and *LMNB1* and *YPEL3* expression (720). Exposure of the MCF-7aro cells to all the AIs induced an accumulation of β-gal and a loss of *LMNB1* gene expression. Strikingly, only the non-steroidal AIs increased *YPEL3* expression, a well-described biomarker of senescence (721, 722). This effect, also reported for Tamoxifen, was

associated with cellular senescence (399). However, the degradation of the nuclear envelope, considered a biomarker of senescence, is also a feature of apoptosis, which weakens the role of *LMNB1* loss as a specific senescence biomarker (723, 724). Moreover, the role of β-gal as a biomarker is also controversial (725). In fact, despite the lack of knowledge about the correlation between autophagy and senescence, the association between β-gal accumulation and autophagic structures has already been reported in different cell models (726). This association may be a potential explanation for the accumulation of β-gal verified in Exemestane treatment.

Interestingly, some studies highlight dual roles for autophagy in the regulation of cellular senescence. Autophagy may act as an anti- or pro-senescence mechanism (685-687). On the other hand, our group has previously demonstrated that inhibition of autophagy exacerbated the anti-proliferative and apoptotic effects of Exemestane in this cell model (384). Thus, in order to understand the association between senescence and autophagy, the *YPEL3* gene expression levels after autophagic inhibition were assessed in our model. The inhibition of autophagy led to an increase in the *YPEL3* gene expression, supporting the previously reported cytoprotective role of autophagy (384) and suggesting that it may act as an anti-senescence mechanism. The same protective role for autophagy in Exemestane-resistant cells was also demonstrated by our group (543). In summary, Anastrozole and Letrozole induce senescence, while Exemestane, despite accumulating β-gal and inducing a loss of *LMNB1*, does not appear to promote senescence given the lack of increased transcription of the *YPEL3* gene, due to the induced autophagic process and to its estrogen-like activity (Figure 15).

As previously demonstrated, the ability of AIs to modulate ER and/or AR and, therefore, influence the hormonal status of breast cancer cells is correlated with their anti-proliferative effects (370, 371, 384, 389, 622). Nevertheless, little is known about how third-generation AIs shape the hormonal environment. The addition of E2, the aromatase product, to Anastrozole or Letrozole completely abrogated their anti-proliferative effects, demonstrating an aromatase-dependency. Moreover, contrary to Exemestane, which induces aromatase degradation (383), the non-steroidal AIs did not affect aromatase protein levels. In addition, both Anastrozole and Letrozole increased ERα expression, while Exemestane induced downregulation of ERα protein levels in hormone-rich and hormone-free conditions. To understand whether these alterations in protein expression were associated with receptor activation, the ERα phosphorylation, as well as the expression of the ERα-regulated genes were analyzed. Anastrozole and Letrozole treatment decreased the ratio of ERα phosphorylation levels, indicating that there was no activation of the receptor. Curiously, despite its downregulation after Exemestane treatment, no alteration in the ratio

of the phosphorylation levels was observed. This was further assessed by analyzing the expression levels of three well-known ERα-regulated genes (*EGR3*, *AREG*, and *TFF1*), with defined roles in cell growth, migration and development of resistance (220, 431, 727-734). Once again, a lack of ERα activation was verified for Anastrozole and Letrozole treatment. Strikingly, in Exemestane treatment, only the *EGR3* gene expression, which is considered a *bone fide* target of ERα (727), indicated that the ERα remained active. The involvement of ERα in the maintenance of *EGR3* expression, during Exemestane treatment, was confirmed by silencing this receptor. These results support the weak estrogen-like effects of Exemestane previously reported by *Wang et al* (2008). In fact, ERα activity, and more specifically *EGR3* expression, was seemingly sustained by Exemestane-treatment, at 1 μM (730), and at 10 μM, as demonstrated in this study.

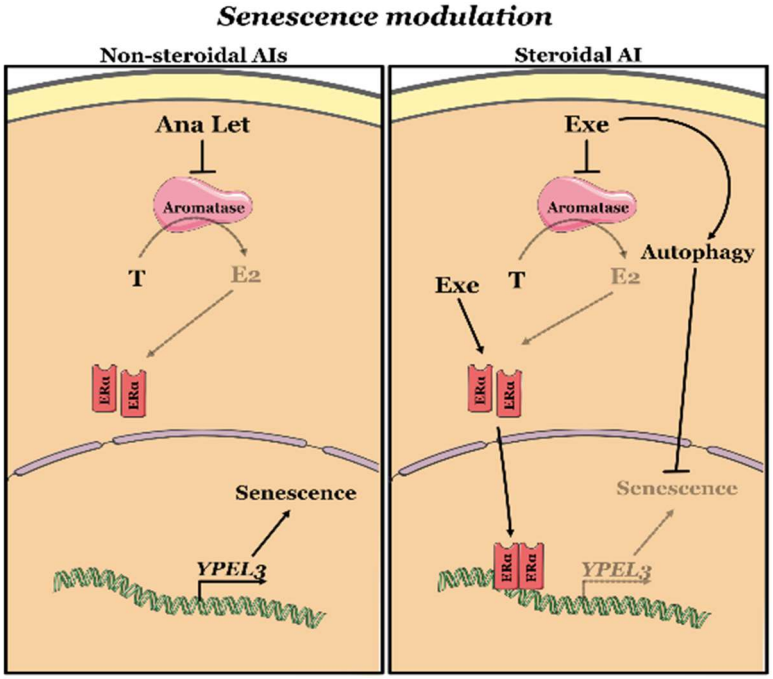

Figure 15. Differences between steroidal and non-steroidal aromatase inhibitors at senescence and autophagy modulation. The non-steroidal AIs have been shown to promote *YPEL3* transcription inducing senescence, while the steroidal Exemestane, through the induction of autophagy and through its estrogenic-like activity, block cell senescence by blocking *YPEL3* transcription.

Unlike Anastrozole- and Letrozole-resistant cell lines (220), which present a constitutively active ERα, the Exemestane-resistant cells did not present a ligand-independent activation of ERα, which reinforces these results. In addition, the importance of *EGR3* in cancer growth and survival was demonstrated in breast cancer cells resistant to Letrozole, due to its continuous expression in sustained estrogen deprivation. Furthermore, in breast cancer patients treated with this AI, *EGR3* levels were maintained after 3 months, which was correlated with poor response to therapy, decreased disease-free survival, and overall survival (735). On the other hand, the increase of *YPEL3* expression observed in

ERα-silenced cells after Exemestane treatment suggests that, despite the weak estrogen-like effect of Exemestane, this increase is a consequence of the reduced levels of ERα and of estrogen signaling. This supports the association between ERα and *YPEL3* expression [399], as well as the role of the estrogen-like activity of Exemestane in preventing cellular senescence. Thus, taking into consideration these observations and the results reported for ICI 182,780 and Tamoxifen, cellular senescence seems to be a consequence of the lack of ERα signaling (397-400).

The other steroid receptor that is highly expressed in ER^+ breast cancer and appears to have a controversial role is the AR. In fact, AR has been shown to have a growth-inhibitory function in several studies (619, 622, 736, 737). However, our group has demonstrated that, in Exemestane-treated MCF-7aro cells, AR plays a pro-survival role as verified by the increased AR expression and activation (389). Thus, since different studies indicate distinct aspects in disease development and progression, in this work the function of AR in cells treated with the non-steroidal AIs, Anastrozole, and Letrozole, was also explored. The addition of CDX, an AR antagonist, reduced their anti-proliferative effects, in opposition to what was reported for Exemestane (389), reinforcing the growth-inhibitory effect for AR previously described for the non-steroidal AIs (622, 738). Interestingly, the non-steroidal AIs also increased AR protein and gene expression levels, an effect similar to Exemestane (389). This increase was also reported in response to dihydrotestosterone (DHT), the main androgenic ligand of AR, leading to growth inhibition (389, 622, 713). In fact, Exemestane treatment induces an increase in DHT levels through the modulation of 5α-reductase, the main enzyme responsible for DHT synthesis (388, 389, 627). Nevertheless, contrary to what was verified in testosterone-treated cells, in hormone-depleted MCF-7aro cells, the non-steroidal AIs did not affect AR levels. This suggests that the AR overexpression was a consequence of androgen (T) accumulation induced by these AIs. Curiously, in the same conditions, Exemestane continued to increase AR expression, similarly to T-treated cells (389), indicating that Exemestane *per se* induced overexpression of AR regardless of hormonal environment. These results highlight a close relationship between AR and Exemestane, corroborating our previous work (389).

It is known that AR can counteract ERα signaling, either by competitive binding to EREs (619), through direct interaction with ERα (712), or, also, by direct inhibition of ERα activity (623). Thus, considering this data and the fact that Exemestane sustains ERα activity, the extent of AR interference on ERα signaling was evaluated by analyzing the ERα-regulated genes (*EGR3*, *AREG* and *TFF1*) in MCF-7aro cells stimulated with T and co-incubated with Exemestane, with or without, CDX. AR blockade only affected the expression of *EGR3* gene, indicating that the transcription of this gene is also regulated by AR. Thus, suggesting that

AR partially impairs ERα signaling. These results corroborate our previous findings, where a crosstalk between AR and ERα was observed (389).

In order to fully characterize these relationships, the growth-inhibitory effects induced by androgen accumulation and the growth-inducing properties of estrogens were abrogated by using hormone-deprived MCF-7aro cells treated with the AIs. In these conditions, contrary to Anastrozole and Letrozole, Exemestane *per se* induced cell proliferation, and this effect was reversed by the addition of ICI 182,780 (431). Moreover, Exemestane maintained its positive regulation of autophagy, while Anastrozole and Letrozole did not affect this process. Exemestane induced strong activation of ERα, which was reverted by the addition of ICI 182,780, confirming the estrogenic-like effects of this AI. In contrast to Exemestane, Anastrozole and Letrozole caused no alterations. The crosstalk between ERα and AR was also observed under these conditions. AR blockade with CDX reverted the proliferative effects of Exemestane and the partial transcription of ERα-regulated genes, mainly the *EGR3* and *AREG* genes. Thus, contrary to the results obtained with T-treated cells, in the absence of an androgenic stimulus, AR supports ERα-regulated genes transcription, demonstrating that Exemestane *per se* is capable of modulating AR activity. Therefore, Exemestane activates AR, and, depending on the hormonal environment, that is, the absence or presence of androgens, AR can positively or negatively modulate ERα activity.

A similar effect to Exemestane in the modulation of AR activity was also referred for E2. This hormone was shown to recruit AR to cooperatively support ERα signaling, driving tumor growth (620). This possible ability of Exemestane to modulate AR activity is also supported by a study that claims that in Exemestane-resistant cells derived from MCF-7aro cells, not treated with T, there were no androgen-responsive genes differentially expressed, except for *KLK11* (431). Nevertheless, considering the role of AR in *EGR3* transcription in Exemestane-treated T-stimulated cells, the androgenic effects overcome the modulation of AR induced by Exemestane. It is also hypothesized that the differences observed between AIs in relation to the AR role may be a consequence of the weak estrogen-like activity observed only for Exemestane. Furthermore, Exemestane maintained the downregulation of ERα protein levels and the increase of AR levels in this hormone-deprived environment, a behavior already discussed and in agreement with our previous work (389). Therefore, considering all the data, we can suggest that Exemestane *per s*e can target both receptors, by a mechanism not yet fully understood but with potential clinical relevance. Interestingly, this work allowed to observe that ICI 182,780, despite being characterized only as a SERD in breast cancer cells, also has the ability to modulate AR levels. This effect is not exclusive of our cell model, since it was also reported that ICI 182,780 downregulates AR in prostate

cancer cells (739). In fact, the ability of some blockers, considered specific for certain steroid receptors, to cross-inhibit other steroid receptors cannot be ruled out due to receptor homology. Supporting this, it was also reported that despite being an AR antagonist, Enzalutamide blocks ERα (620).

The ability of Exemestane to modulate both AR and ERα activities may have important clinical implications (Figure 16) since these findings suggest that only patients treated with Exemestane would react positively to anti-AR therapy. This confirms our previous findings (389) and supports the ongoing clinical trials (NCT02007512, NCT02910050, and NCT02676986) and the study with Enzalutamide and Exemestane in patients with advanced ER$^+$ breast cancer (640, 740). Moreover, the estrogenic-like activity of Exemestane may hinder its therapeutic efficacy when administered as a second-line therapy, considering the recommendations of ESMO guidelines for early-breast cancer in postmenopausal patients (293). Therefore, based on these results, the optimal sequential therapy would be Exemestane upfront since it would not be as beneficial after non-steroidal AIs or Tamoxifen treatment, although further validation in different models is required.

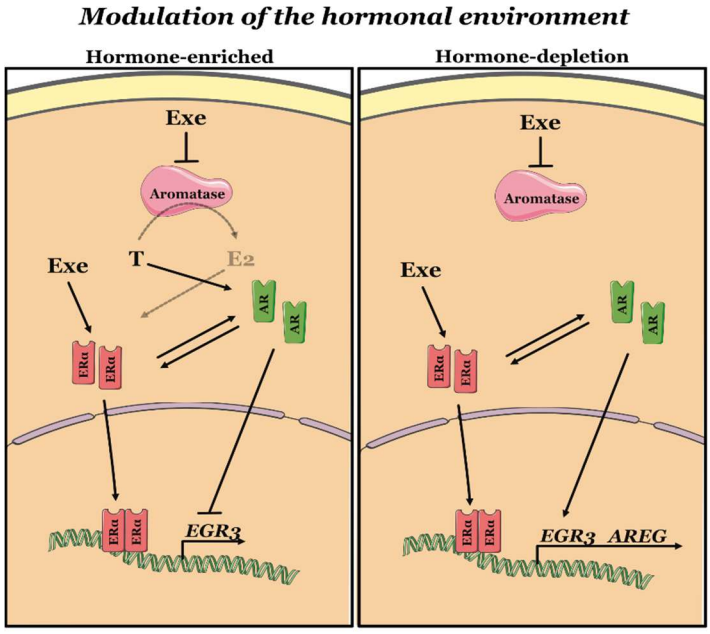

Figure 16. Exemestane modulation of androgen receptor and the influence of the hormonal environment. Exemestane promotes the recruitment of androgen receptor (AR) to support the transcription of ER-regulated genes, such as *EGR3*, as verified in hormone-depleted conditions. However, in hormone-enriched conditions, the androgenic effects of testosterone counteract Exemestane. In this situation, AR blocks the transcription of *EGR3*.

As mentioned before, AI-acquired resistance is the major drawback of AIs therapy. In order to circumvent this, several inhibitors of pathways involved in AI-resistance are being tested in combination with AIs to enhance the efficacy of this therapy (741). These combinations include CDK4/6 inhibitors, mTORC1 inhibitors, or PI3K inhibitors.

As described above, PI3K has a significant importance in AI-acquired resistance (143, 260, 432, 448, 519, 521). Recently the combination of Alpelisib (BYL-719), a PI3K inhibitor, and ICI 182,780 was approved for postmenopausal women with $ER^+/HER2^-$ and *PIK3CA*-mutated advanced or metastatic breast cancers refractory to endocrine therapy (296). The importance of PI3K is enhanced by its ability to regulate autophagy, which was demonstrated to have implications in the efficacy of Everolimus and of Exemestane (543, 567) and, consequently, in the lack of improved OS observed in the BOLERO-2 clinical trial (564, 565). Therefore, the different impacts of PI3K class I inhibition on AI-resistant cells, as well as the effects of PI3K class III inhibition and the involvement of autophagy in Anastrozole- and Letrozole-resistant cells were studied.

Interestingly, in the LTEDaro cell line, and contrary to Exemestane (543), neither Anastrozole or Letrozole increased autophagic markers, such as LC3-II levels, *SQSTM*1 expression, and AVOs formation. Also, unlike Exemestane, LTEDaro cells viability after treatment with the combination of Anastrozole/Letrozole and the autophagic/pan-PI3K inhibitor, Wortmannin (WT), or after silencing of the autophagy-related gene 5 (ATG5) and treatment with non-steroidal AIs, was not altered. These results were further validated in cell lines specifically resistant to each AI (AnaR, LetR and ExeR). WT did not affect AnaR and LetR cell viability but significantly decreased ExeR cell viability. The higher sensitivity of the ExeR cell line to WT corroborates our previous study in Exemestane-treated LTEDaro cells (543) and the existence of specific AIs–resistance mechanisms. These differences may be explained by the direct modulation of ERα induced by Exemestane (220, 431). In fact, ICI 182,780 treatment or ERα knockdown has been shown to induce cytoprotective autophagy, which was also important for ICI 182,780-resistance (691, 742). In addition, estradiol also induces autophagy, by activating ERα, in order to promote ERα degradation through a feedback loop (743). Thus, a possible correlation between ERα modulation and autophagy might explain the effects of Exemestane.

Since it was demonstrated that the combination of the class I PI3K inhibitor, BYL-719, with Tamoxifen re-sensitize breast cancer cells to Tamoxifen (744), the efficacy of BYL-719 in AI-resistant cell models was evaluated. The selected dose in this work (0.05 μM) was lower than the ones frequently used in similar studies (531, 745). However, this concentration was effective and decreased AKT activation *per se*. The combination of BYL-719 with AIs decreased LTEDaro cells viability and AKT activation when compared to AIs alone. Interestingly, in our conditions, Exemestane *per se* induced a marked activation of AKT, an effect reverted by BYL-719. It was reported that estrogen promotes the upregulation of AKT via ERα (527, 528, 746). In this study, the increase in AKT observed for Exemestane-treatment was completely abrogated by the addition of ICI 182,780,

confirming the involvement of Exemestane-activated ERα in the upregulation of the AKT activity. This is in accordance with the work from *Wang et al* (2017), where an increased p-AKT was observed in ExeR cells when compared to the parental MCF-7aro, AnaR and LetR cells (116). Confirming our findings, the PI3K inhibitor also reduced cell viability in a dose- and time-dependent manner in the AI-specific resistant cell lines, being the effects more potent in the ExeR cell line for lower doses of the PI3K inhibitor. The efficacy of BYL-719 can be explained by the importance of PI3K/AKT/mTORC1 pathway in the development of anti-estrogens resistance (116, 143, 260, 432, 521). Nevertheless, the AI-resistant cell lines present other mechanisms that assure ERα activity. The AnaR and LetR cell lines present a constitutively active ERα due to the activation of the MAPK/ERK survival pathway via growth factor receptors, such as the EGFR or the IGF-1R (431, 433, 443, 460). On the other hand, the ExeR cell line presents an active ERα due to the estrogen-like activity of Exemestane, which promotes the expression of an EGFR ligand, AREG, leading, as well to the activation of the MAPK/ERK pathway (220, 431, 730). Therefore, since there is a high dependence of ERα transcriptional activity in AI-acquired resistance, the combination of BYL-719 with ICI 182,780 was also studied. This combination proved to be more effective than BYL-719 alone in all the AIs specific-resistant cell lines, a behavior that can be explained by concomitant abrogation of ERα signaling and inhibition of PI3K. Altogether, our results suggest that the combination of BYL-719 and ICI 182,780 is superior to BYL-719 in AI-resistant tumors, corroborating the SOLAR-1 clinical trial (NCT02437318), where this combination was superior to just ICI 182,780 (296, 558).

In order to understand the mechanisms behind the efficacy of BYL-719, the biological effects of the combinations of BYL-719 and AIs in the LTEDaro cells were also explored. The combinations of BYL-719 with the non-steroidal AIs disrupted LTEDaro cell cycle in the G_2/M phase, while the combination with Exemestane induced an arrest at the G_0/G_1 phase. These differences observed between the combinations of BYL-719 with Anastrozole/Letrozole or Exemestane might be a consequence of the different effects on AKT phosphorylation according to the AI used, since cell cycle arrest at the G_2/M phase was highly correlated with low AKT phosphorylation levels (747, 748). In fact, BYL-719 alone was able to promote an arrest at the G_2/M due to the decrease in AKT phosphorylation levels. Similarly, the cell cycle arrest at G_2/M observed for Anastrozole, and Letrozole treatments with BYL-719 is associated with low AKT phosphorylation. On the other hand, the inhibition of PI3K by BYL-719 was not so effective on Exemestane-treated cells, since AKT phosphorylation levels were still higher than control. Thus, an arrest at G_0/G_1, instead of at G_2/M, was observed for this AI. In addition, the occurrence of apoptosis was investigated since disruption of the cell cycle is often associated with increased apoptosis and cytotoxicity (384, 749). Curiously, only the combination of Exemestame with BYL-719

induced apoptosis through the intrinsic pathway, as suggested by the activation of caspases-7 and -9 and by the ΔΨm loss. The occurrence of apoptosis verified only for the combination of Exemestane and BYL-719 might be a consequence of a higher dependence of the PI3K/AKT/mTORC1 pathway in Exemestane-treated cells. In fact, Exemestane treatment *per se* increased AKT activation, and BYL-917 significantly reverted this effect. These findings support different behaviors between AIs in fundamental cellular processes linked with tumor progression and AI-acquired resistance.

Therefore, these results exclude autophagy as a mechanism of resistance to Anastrozole and Letrozole and report different responses to BYL-719 according to AI-specific resistance. In the future, this behavior may help to understand the results of ongoing clinical trials with BYL-719. The efficacy observed in phase I clinical trials (NCT01791478, NCT01870505) combining BYL-719 with Letrozole or Exemestane in advanced ER$^+$ breast cancer is supported (559, 560), and the *in vitro* biological effects behind the effectiveness of these combinations are described. In addition, the superiority of the combination of BYL-719 and ICI 182,780 over BYL-719 alone in AI-resistant cells is also demonstrated, reinforcing the importance of targeting the PI3K/AKT/mTORC1 and estrogen signaling pathways (Figure 17).

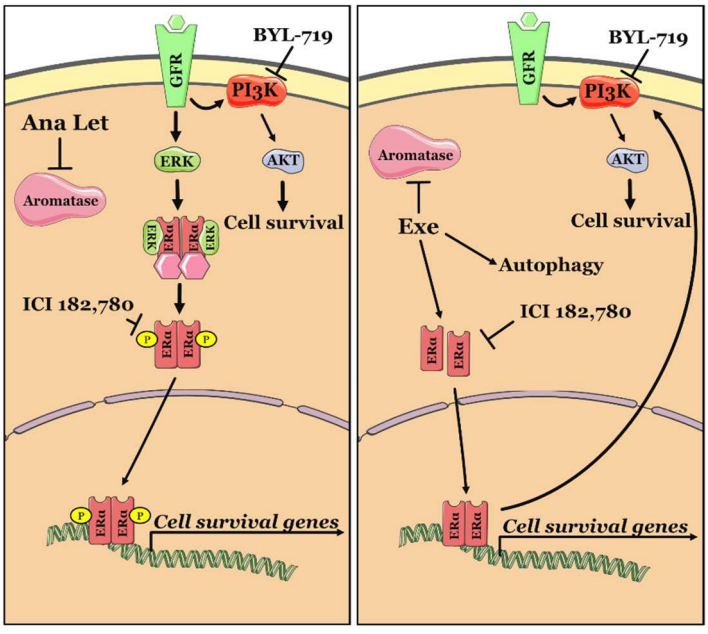

Figure 17. Differences between Anastrozole/Letrozole and Exemestane-resistant cells and the impact of targeting PI3K class I and ERα. The PI3K/AKT/mTORC1 pathway is involved in anti-estrogens resistance. Nevertheless, the AnaR and LetR cells present a constitutively active ERα, due to the hyperactivation of the MAPK/ERK pathway, via growth factor receptors (GFRs). On the other hand, the ExeR cells present an active ERα due to the estrogenic-like activity of Exemestane. Moreover, Exemestane has been shown to induce a cytoprotective autophagy and to increase the activation of the PI3K/AKT/mTORC1 pathway through the ERα. In these cells, concomitant inhibition of PI3K and ERα improves the effects of PI3K inhibition alone.

Concluding Remarks

In summary, this thesis contributed to the search for new potent steroid AIs. These new molecules presented promising anti-cancer properties since they act as multi-target compounds, modulating aromatase, ERα or AR, which can be a therapeutic advantage. Moreover, the SAR studies helped to identify more favorable modifications in androstenedione structure, which will in the future allow a more rational drug design for the development of more potent AIs. It was also developed a new DLLME–GC/MS method to assess aromatase activity in human placental microsomes through quantification of androstenedione and estrone. This methodology is a valid alternative to the radiometric assay, allowing a safer and cheaper evaluation of molecules with potential anti-aromatase properties.

Regarding the second aim of this thesis, different mechanisms of action between the AIs used in clinic in sensitive and resistant ER^+ breast cancer cells were described. In sensitive ER^+ breast cancer cells, and contrary to Exemestane, the non-steroidal AIs did not cause autophagy. Moreover, Exemestane-induced autophagy blocks senescence, while the non-steroidal AIs induce cell senescence. A crosstalk between ERα and AR was described for Exemestane-treated cells due to its ability to recruit AR to reinforce estrogen signaling. However, AR switches its role in the modulation of ERα activity according to the hormonal environment. Thus, only Exemestane-treated patients might benefit from anti-AR therapies, data that supports the ongoing clinical trials with this AI and AR antagonists. In addition, the weak estrogen-like activity of Exemestane may also hinder its anti-tumoral efficacy as a second-line therapy.

On the other hand, through the study of AIs in resistant breast cancer cells, it was concluded that, unlike Exemestane, the non-steroidal AIs did not induce autophagy. Moreover, in LTEDaro cells treated with the combination of BYL-719 with the AIs, different responses were observed. Despite the antiproliferative effects of all the combinations, the combination with Exemestane also caused cell death by apoptosis. In addition, in the AI-specific resistant cell lines, it was also observed that the concomitant inhibition of PI3K and ERα potentiated the anti-tumoral effects of BYL-719, highlighting the importance of inhibiting both targets in AI-resistance. Thus, this study demonstrates different resistance mechanisms induced by AIs and supports ongoing clinical trials that study the combination of the PI3K inhibitor, BYL-719, with AIs.

CHAPTER IV

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