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New steroidal aromatase inhibitors: biological effects in hormone-dependent breast cancer cell models

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação da Professora Doutora Georgina Correia da Silva (Universidade do Porto) e da Professora Doutora Maria Carmen Martins de Carvalho Alpoim (Universidade de Coimbra).

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ABSTRACT

The majority of breast cancers, the most prevalent among women, is hormone-dependent (ER^+) and requires estrogen signaling for its etiology and progression. From the several approaches used for the treatment and prevention of these tumors in postmenopausal women, aromatase inhibitors (AIs) represent one of the best options. By blocking the enzyme aromatase, responsible for estrogens biosynthesis, AIs are able to suppress estrogens levels, thus avoiding tumor growth. Despite their efficacy, AIs are limited by the existence of some drawbacks, such as bone loss and the development of therapy resistance, a major obstacle to the successful treatment. For this, the search for novel potent compounds, with fewer side effects, is currently demanded.

The present work focused on the study of new steroidal compounds as potential AIs, synthesized from structural modifications on androstenedione molecule, an aromatase substrate. The anti-aromatase activity, the biological effects and the underlying anti-tumor mechanisms of four new compounds (**49**, **50**, **51** and **52**) were evaluated. The *in vitro* studies were performed in an estrogen receptor-positive (ER^+) human breast cancer cell line that overexpresses aromatase (MCF-7aro), an estrogen receptor-negative (ER^-) human breast cancer cell line (SK-BR-3), an AI-resistant breast cancer cell line that overexpresses aromatase (LTEDaro) and a non-tumor fibroblastic cell line (HFF-1).

The results revealed that all the steroids are potent AIs, capable of decreasing the viability of the hormone-dependent breast cancer cells without affecting the non-tumor fibroblastic cells. This effect was accompanied by morphological alterations, cell cycle arrest and cell death by apoptosis via the mitochondrial pathway. Autophagy was a pro-survival mechanism for compound **51**. Furthermore, the compounds also affected the viability of the AI-resistant cells in a similar manner as the hormone-sensitive ones.

In conclusion, the new potent AIs induced anti-proliferative effects in breast cancer cells, mainly through cell cycle arrest and cell death mechanisms. This work might contribute for the design and synthesis of more effective compounds and elucidate the tumor suppressor mechanisms associated to AIs treatment.

Keywords: hormone-dependent breast cancer, aromatase inhibitors, endocrine therapy.

RESUMO

A maioria dos cancros da mama, o mais prevalente entre as mulheres, é hormono-dependente e requer a sinalização de estrogénios para a sua etiologia e progressão. Das várias abordagens usadas para o tratamento e prevenção destes tumores em mulheres pós-menopáusicas, os inibidores da aromatase (IAs) representam uma das melhores opções. Ao bloquearem a enzima aromatase, responsável pela biossíntese de estrogénios, os IAs são capazes de suprimir os níveis de estrogénio, evitando, deste modo, o crescimento tumoral.

Apesar da sua eficácia, o IAs estão limitados pela existência de algumas desvantagens, como a perda de massa óssea e o desenvolvimento de resistência à terapia, um dos principais obstáculos ao sucesso do tratamento. Por isso, é importante a procura de novos e potentes compostos, com menores efeitos adversos.

Este trabalho focou-se no estudo de novos compostos como potenciais IAs, sintetizados a partir de modificações estruturais na molécula da androstenediona, um dos substratos da aromatase. A atividade anti-aromatásica, os efeitos biológicos e os mecanismos anti-tumorais subjacentes de quatro novos compostos (**49**, **50**, **51** e **52**) foram avaliados. Os estudos *in vitro* foram realizados numa linha celular humana de cancro da mama recetor de estrogénio positivo com sobre-expressão da aromatase (MCF-7aro), numa linha celular humana de cancro da mama recetor de estrogénio negativo (SK-BR-3), numa linha celular de cancro da mama resistente aos IAs com sobre-expressão da aromatase (LTEDaro) e numa linha celular não-tumoral de fibroblastos (HFF-1).

Os resultados revelaram que todos os esteroides são potentes IAs, capazes de reduzir a viabilidade das células de cancro da mama hormono-dependente, sem afetar as células fibroblásticas não tumorais. Este efeito foi acompanhado por alterações morfológicas, paragem do ciclo celular e morte celular por apoptose pela via mitocondrial. A autofagia foi um mecanismo de pro-sobrevivência para o composto **51**. Além disso, os compostos afetaram a viabilidade das células resistentes a IAs de uma forma semelhante às células hormono-sensíveis.

Concluindo, os novos potentes IAs obtidos induziram efeitos anti-proliferativos nas células de cancro da mama, principalmente através da paragem do ciclo celular e de mecanismos de morte celular. Este trabalho poderá contribuir para o desenho e síntese de compostos mais eficazes e elucidar os mecanismos de supressão tumoral associados ao tratamento com IAs.

Palavras-chave: cancro da mama hormono-dependente; inibidores da aromatase, terapia endócrina.

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ABBREVIATIONS LIST

AF-1 activation function 1

AF-2 activation function 2

AG aminoglutethimide

AI(s) aromatase inhibitor(s)

AKT protein kinase B

AO acridine orange

AP-1 activator protein 1 (transcription factor)

AREG amphiregulin

ATM ataxia telangiectasia mutated (gene)

AVO(s) acid vesicular organelle(s)

Bcl-2 B cell lymphoma 2

Bcl-xL B-cell lymphoma-extra large

BAK BCL2-antagonist/killer

BID BH3 interacting-domain death agonist

BRAC1 breast cancer 1, early onset (gene)

BRAC2 breast cancer 2, early onset (gene)

cAMP cyclic adenosine monophosphate

CCCP carbonyl cyanide m-chlorophenylhydrazone

CFBS charcoal-treated bovine serum

CoA co-activator

CoR co-repressor

CPR NADPH-cytochrome P450 reductase

CYP cytochrome P450

CYP19 aromatase

DBD DNA-binding domain

$DCFH_2$-DA 2',7'-dichlorodihydrofluorescein diacetate

DHEA dehydroepiandrosterone

DHEA-S dehydroepiandrosterone-sulfate

$DiOC_6$(3) 3,3-dihexyloxacarbocyanine iodide

DMEM Dulbecco's Modified Eagle Medium

DMSO dimethylsulfoxide

E1 estrone

E1-S estrone sulfate

E2 17β-estradiol or estradiol

E3 estriol

EDTA ethylenediaminetetraacetic acid

EGF epidermal growth factor

EGFR epidermal growth factor receptor

ER(s) estrogen receptor(s)

ER^+ estrogen receptor positive (hormone-dependent or estrogen-dependent)

ER^- estrogen receptor negative

ERE(s) estrogen response element(s)

EST estrogen sulphotransferase

FBS fetal bovine serum

FSC forward scatter

FDA U.S Food and Drug Administration

FSH follicle-stimulating hormone

GFR(s) growth factor receptor(s)

GPCR(s) G protein-coupled receptor(s)

HDAC histone deacetylase

HER2 human epidermal growth factor receptor 2

HFF-1 human foreskin fibroblasts-1 cell line

IGF1 insulin-like growth factor 1

IGF1R insulin-like growth factor 1 receptor

IL(s) interleukines

JNK c-Jun N-terminal kinase

LBD ligand-binding domain

LDH lactate dehydrogenase

LH luteinizing hormone

LHRH luteinizing hormone-releasing hormone

LTEDaro long-term estrogen deprivation cell line overexpressing aromatase

MAPK mitogen-activated protein kinase

MCF-7 estrogen receptor-positive breast cancer cell line

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

MEM Minimum essential medium

MISS membrane-initiated steroid signaling

mTOR mammalian target of rapamycin

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

MYC v-myc avian myelocytomatosis viral oncogene homolog

NADPH nicotinamide adenine dinucleotide phosphate

NF-κB nuclear factor-kappa B

NISS nuclear-initiated steroid signaling

NLS nuclear localization signaling

PGE2 prostaglandin E2

PI propidium iodide

PI3K phosphatidylinositide 3-kinase

PMA phorbol 12-myristate 13-acetate

PR progesterone receptor

PTEN tumor suppressor phosphatase and tensin homolog

ROS reactive oxygen species

RTK(s) receptor tyrosine kinase(s)

SAR structure-activity relationship

SERD(s) selective estrogen receptor downregulator(s)

SERM(s) selective estrogen receptor modulator(s)

SK-BR-3 estrogen receptor negative breast cancer cell line

Src tyrosine protein kinase

STA staurosporine

STS steroid sufatase

SSC side scatter

T testosterone

TF(s) transcription factor(s)

TGF-β transforming growth factor β

TNF-α tumor necrosis factor alpha

Tp53 tumor protein 53 (gene)

VEGF vascular endothelial growth factor

3β-HSD 3β-hydroxysteroid dehydrogenase

3-MA 3-methiladenine

4-OHA 4-hydroxyandrostenedione or formestane

17β-HSD 17β-hydroxysteroid dehydrogenase

ΔΨm mitochondrial transmembrane potential

CHAPTER I

Introduction

1. BREAST CANCER: INCIDENCE, ETIOLOGY, RISK FACTORS AND THERAPIES

Cancer is a multifactorial disease affecting millions of people around the world. Along the past few decades, cancer has become one of the most frequently diagnosed diseases and the leading cause of death in developed countries, turning it into a major public health problem (1). Although progress has been made in reducing incidence and mortality rates and improving survival, due to earlier detection and treatment advances, the aging and growth of the world population along with increased exposure to environmental risk factors and cancer-causing behaviors has been preponderant in the global burden of cancer (1, 2).

Breast cancer, the most common cancer diagnosed among women, accounted for 1,7 million new cases diagnosed in 2012, and is the leading cause of cancer death in females, according to the last statistics (3, 4) (Figure 1). It is also on top of the ranking of incidence among female in all countries of Europe and is the primary mortality cause from female cancer in almost all countries, including Portugal (5). It is mostly a female disease, since men rarely have breast cancer.

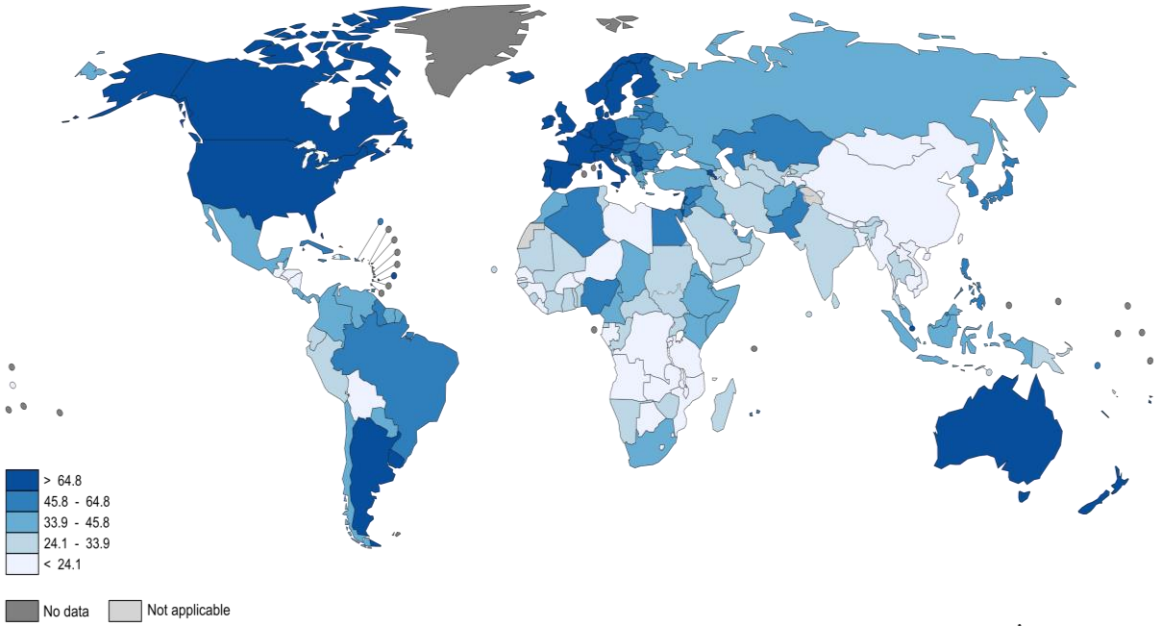


Figure 1. **Age-standardized breast cancer incidence rates per 100,000 women in the world.** Adapted from (4).

Breast cancer etiology remains unclear, although it is known that its initiation starts with uncontrolled events in cells, driven by genetic and epigenetic alterations, followed by accumulation of genetic mutations and microenvironmental changes that ultimately lead to expansion of a tumor (6). But several other environmental and intrinsic factors are also involved in tumorigenesis. It is

known that female breast cancer risks are related to increased age, low age at menarche and late menopause, never having children or older age at first birth, high body weight, poor diet, alcohol and tobacco consumption, physical inactivity, radiation exposure, long-term exogenous hormones intake (oral contraceptives and hormonal replacement therapies) and varies with geographical location (7-9). Family history and hereditary factors are also strong determinant risk factors (10). Inheritance of inactivating mutations in genes associated with breast cancer such as *BRAC1*, *BRAC2*, *tp53*, *ATM* and *PTEN,* or its acquisition due to environmental factors, are known predisposal factors and highly increases cancer susceptibility (8, 10-12). Some chemicals might as well act as carcinogens.

Recent cancer control and prevention campaigns have awareness people for the importance of an early detection and diagnosis of breast cancer in the improvement of clinical prognosis and effective treatment. Along with diagnostic technologies and treatment advances, this has resulted in an increased survival rate (2). However, many challenges remain to overcome, such as the appropriate treatment approach for each specific type of breast cancer, development of resistance and cancer recurrence.

Current therapies applied to breast cancer include surgery, radiation therapy, hormone therapy, chemotherapy and targeted therapy. Since breast cancer, such as many others, is highly heterogeneous, there was the need to classify it in distinct subtypes and stages, depending the treatment strategy on the stage and subtype of breast cancer (6). Breast cancer stages are denominated according to cancer progression and go from stage 0, when there is a small carcinoma *in situ*, a non-invasive localized tumor that might progress to the next stages until stage IV, where the carcinoma has become invasive and spread to other organs (metastatic) (13). Besides, according to their hormone-receptor status breast carcinomas can also be classified in hormone-dependent and hormone-independent tumors. Hormone-dependent breast cancer refers to an endocrine sensitive type of tumor, particularly estrogen receptor (ER) and progesterone receptor (PR) positive and, sometimes, human epidermal growth factor receptor 2 (HER2) positive. Hormone-independent breast cancers do not express ER or PR (14, 15). These hormonal receptors are therapeutic targets for hormone therapies in breast cancer and their presence/absence is currently used as a predictive marker to responsiveness to endocrine therapies and as a prognostic factor (13, 14).

The first reports of hormone-dependent tumors emerged in 1880's, when the removal of the ovaries was related to beneficial outcomes in some breast cancer patients (16, 17). Since then, several studies reported that estrogens, sex steroidal hormones with fundamental biological functions for normal development of several tissues and in female reproduction, were also involved in the development and growth of hormone-dependent breast tumors (18-21). About 60% of

premenopausal and 75% of postmenopausal breast cancer patients have estrogen-dependent carcinomas (22). This relationship between estrogen receptor positive (ER^+) breast cancer and estrogens, suggested that estrogen suppression would potentially prevent or cause regression of this kind of tumors. Consequently, hormonal therapies against estrogen production or targeting estrogen receptors started to be largely used in ER^+ cancer patients, enlarging the range of effective therapeutic options for breast cancer (23).

2. ESTROGENS: SOURCES, BIOSYNTHESIS AND CARCINOGENESIS

Estrogens are a group of essential steroidal hormones involved in normal female physiology and reproduction, beyond other functions in other tissues (24). There are three main natural estrogens in women: estrone (E1), estradiol (E2 or 17β-estradiol) and estriol (E3), being estradiol the most important in women at reproductive age and estriol during pregnancy, while estrone is more predominant in postmenopausal women (25).

During reproductive age, estrogens biosynthesis occurs mainly in the ovaries. They control circulating estrogen levels, which varies along menstrual cycle. The main source of steroids in human body is cholesterol, which in ovaries granulosa cells is converted into androgens and finally in estrogens (24). Unlike premenopausal women, after menopause the ovarian estrogen secretion is arrested (one of the major conditions to menopausal status). Instead, estrogen production continues in peripheral tissues from circulating androgen sources (24, 26). This peripheral local synthesis is important for non-reproductive actions, such as maintenance of bone density and cardiovascular protection (17). But, besides its physiologic role, estrogens local synthesis is also associated with increased hormonal cancer risk, including breast cancer (18-21, 25).

Intracrinology is a recent research area that studies *in situ* production of steroids from circulating precursors and it has special interest in postmenopausal breast cancer patients, once it is thought to be involved in breast cancer pathogenesis (27, 28). In peripheral tissues, estradiol no longer acts as endocrine factor but as a paracrine and intracrine factor (26, 27, 29). In fact, estradiol is present in much higher concentrations in malignant breast tissue than in plasma levels of postmenopausal women (29-31), evidencing the role of intratumoral estrogen production. Although some peripheral tissues can convert androgenic steroid precursors into estrogens, they have no ability to produce these precursors. So, estrogen local production depends on the availability of circulating androgenic precursors (26, 29). Adrenal cortex (80%) and postmenopausal ovaries (20%) are sources of these androgenic precursors (28). The expression of sex steroid-

forming enzymes, such as cytochrome P-450 enzyme aromatase, in peripheral tissues allows the local (intracrine) conversion of these circulating androgens (testosterone and androstenedione) into estrogens (21), providing a continued source of sex steroids for individual cells or tissues without systemic exposure, thus avoiding endometrium stimulation after menopause. A crucial aspect of intracrinology is that, in menopause, androgenic precursors are distributed by circulation to all tissues but their transformation is tissue-specific and locally produced active estrogens are also locally inactivated. Thus, only inactive metabolites are excreted to circulation, allowing for high intracellular concentrations of estrogen while maintaining serum estrogen levels at subtreshold and avoiding other tissues exposition (28, 32). This physiological process is very important to provide estrogens for specific age-related needs of each cell type or tissue but can also play a role in pathology. Therefore, it is essential to understand the mechanisms and enzymes involved and how their blockage can be used as first-line option in tumor control.

Estrogen biosynthesis from cholesterol involves several steroidogenic enzymes, common to all endogenous steroids synthesis (Figure 2). The initial steps convert cholesterol to DHEA/DHEA-S (dehydroepiandrosterone/dehydroepiandrosterone-sulfate), the most important androgen precursors. It occurs in the adrenal cortex and ovary, once the enzyme that converts steroids to androgens is not present in breast tissue (27, 28, 33). Thus, DHEA and DHEA-S constitutes a large source of androgenic precursors that can be used to produce androgens and then estrogens, through several reactions. This cascade of reactions involve 3β-HSD isomerases and 17β-HSD (17β-hydroxysteroid dehydrogenases) (27, 32). Aromatase is the enzyme involved in the final rate-limiting step of estrogens biosynthesis. It is responsible for the conversion of the androgens, androstenedione and testosterone, into estrogens, through the aromatization of the A ring of the steroids. When aromatase acts on androstenedione, forms a weaker estrogen – estrone; when it acts on testosterone, produces the more potent estrogen – estradiol (27). In breast tumors, this intracrine production of estrogens is many times referred in literature as intratumoral aromatization (33). Aromatase will be further characterized in section 5.

In addition to DHEA/DEAH-S sources of estrogens and the “aromatase pathway”, another mechanism is involved in E2 production – the “sulfatase pathway”. After E1 production by aromatase, it can be metabolized in the liver in estrone sulfate (E1-S), which circulates to other tissues. The “sulfatase pathway” can convert circulating E1-S in E1, through the removal of sulfate group by steroid sulfatase (STS or estrone sulfatase), and then E1 can be converted into E2 by the action of 17β-HSD enzyme (34). In breast tumors, STS pathway appears to be a very important route, once it contributes to intratumoral estrogen production and can even overlap aromatase activity in terms of estrone production (35). STS is present in 60-90% of breast carcinomas tissues. It is upregulated and associated with poor clinical outcomes (35-37). Actually, STS pathway has been

considered a therapeutic target and several sulfatase inhibitors were already synthesized. However, since aromatase is needed to produce the E1 that is converted to E1-S and it also produces E2, the most attractive strategy would be the use of dual aromatase-sulfatase inhibitors, which are also object of research and some were already reported. Some 17β-HSD inhibitors have also been developed with success, however, among sulfatase, 17β-HSD and aromatase inhibitors, the last ones are the most well-developed and clinically effective compounds (reviewed in (34)).

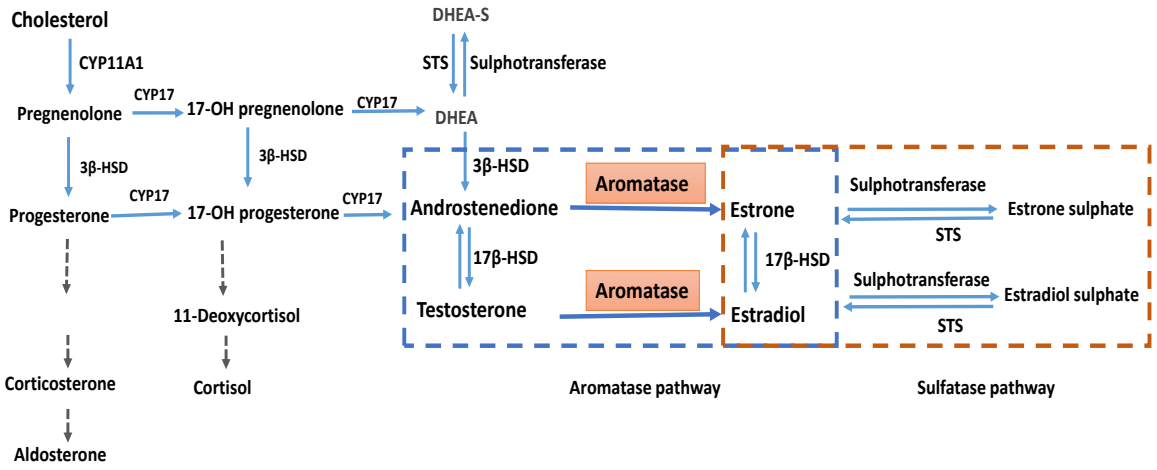


Figure 2. Estrogen biosynthesis.
CYP11A1: cholesterol side-chain cleavage enzyme, CYP17: 17,20 lyase, 3β-HSD: 3β-hydroxysteroid dehydrogenase, 17β-HSD: 17β-hydroxysteroid dehydrogenase, STS: steroid sulfatase.

Finally, there is an inactivation pathway of estrogens through their metabolism in hormone dependent-tissues, such as breast tissue and endometrium. This is mainly accomplished by the action of the estrogen sulphotransferases (EST enzymes) (35, 38). EST is present in 40-80% of breast cancer patients and is generally associated with less aggressive tumors, once it regulates STS activity and lowers estrogens exposure in breast tissue due to their inactivation (35-37, 39).

As previously referred, there are increasing evidence that estrogen plays a crucial role in breast cancer progression and sustained estrogen exposure increases the risk of its development. Several mechanisms might be involved in estrogen-mediated carcinogenesis but the induction of DNA damage and the increased cell proliferation are the most elucidated ones (Figure 3). Firstly, studies revealed that estrogen exposure leads to genomic instability. This happens because estrogen oxidative metabolites can induce DNA adducts, double-strand break and/or other oxidative damage in DNA. For this, estrogen metabolites are many times considered carcinogenic and mutagenic when are present in high concentrations in a certain tissue (18, 19, 40). Secondly, estrogens, by binding to estrogen receptors, stimulate the expression of genes involved in cell survival and proliferation, while also inhibit apoptosis (41). Therefore, deregulated estrogen signaling, like

hyperactivation, provokes excessive cell division, leading to tumor promotion. At the same time, it increases the probability of errors occurring during DNA replication, leading to the appearance and accumulation of mutations that might also favor tumor progression (18, 42). One third hypothesis is the suppression of DNA damage response and DNA repair mechanisms by estrogen signaling through the deregulation of key effector proteins, such as p53, ATM and BRAC1, that allow the accumulation of genomic alterations and consequent carcinogenesis (18).

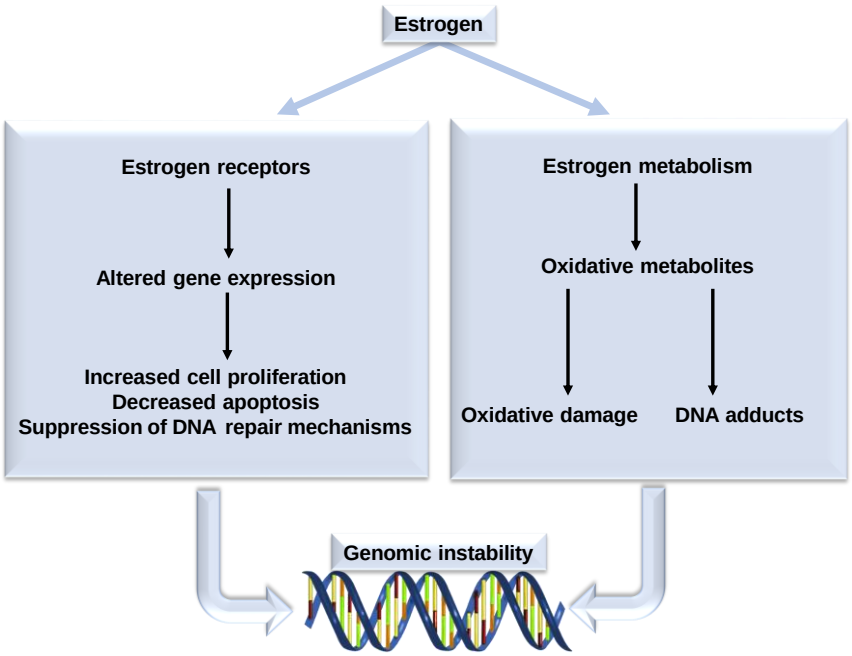


Figure 3. Mechanisms of estrogen carcinogenesis.

3. ESTROGEN RECEPTOR (ER)

The biological actions of estrogen signaling are mediated by estrogen receptor, so its study is crucial to support the evidence that this receptor is an essential link between hormone and hormone-dependent breast cancer.

ER belongs to the steroid superfamily of Class I nuclear receptors (41), presenting a structural and functional organization common to other nuclear receptors (43). It exists in two isoforms, ERα and ERβ, produced by distinct genes. ERα gene is localized in chromosome 6 while ERβ gene is on chromosome 14 (44, 45). Both are composed by five distinct domains labeled from A/B to F, with different homologies (Figure 4). In N-terminal region, A/B domain contains AF-1 (transcriptional activation function), responsible for ligand-independent activation of gene transcription and interaction with co-regulatory proteins. The C domain corresponds to a DNA-binding domain (DBD),

essential for high affinity binding of ER to DNA and receptor dimerization. The D domain is a joint region between C and E domains. The E domain is the ligand-binding domain (LBD) and contains AF-2, responsible for ligand-dependent transcriptional activity of ER. It is also important for receptor dimerization. The F domain is located at the C-terminus region and modulates AF-1 and AF-2 activities and, possibly, ER dimerization and other unknown activities. Nuclear localization signals (NLS) are in D and E regions. The A/B domain and F domain are the less homologous regions between the two receptor proteins while DBD and LDB share a high degree of homology (46, 47). Disparities in receptors homology might explain the different actions observed between the two ERs.

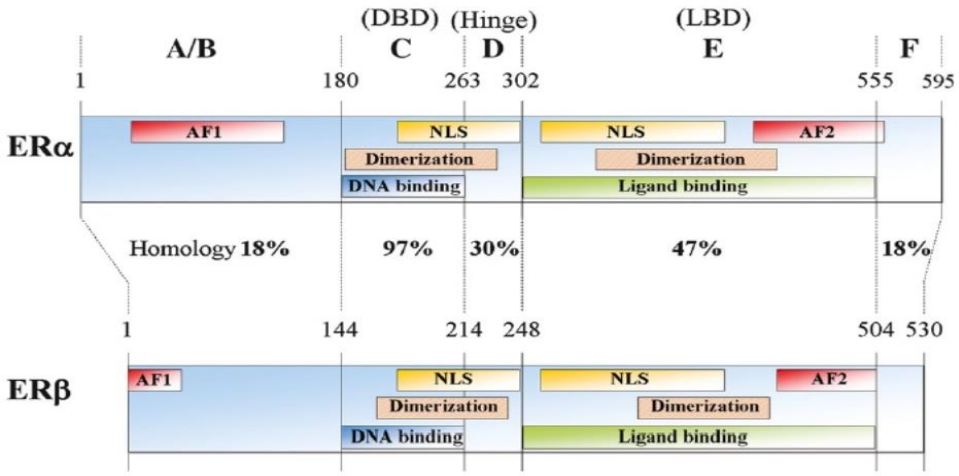


Figure 4. Structure of human ERα and ERβ nuclear receptors.
Domain organization: A/B domain at the N-terminus contains the ligand-independent transcriptional activation function 1 (AF1); C domain represents the DNA-binding domain (DBD) and contains a nuclear localization signal (NLS); D domain corresponds to the hinge region; E domain contains the ligand-binding domain and the ligand-dependent transcriptional activation function 2 (AF2); F domain at the C-terminus. Numbers outside each box refer to amino-acid number. Percentage of amino-acid homology for each domain is also shown. Adapted from M. Zilli et al. (46).

The ER expression is tissue-specific, with overlapping distribution of ERα and ERβ in some tissues. ERα is mainly expressed in ovaries (thecal and interstitial cells), uterus, testis, epididymis, liver, pituitary gland, kidneys, bone, white adipose tissue, some brain regions, adrenals and mammary glands. ERβ is more expressed in prostate, testis, bone, bone marrow, colon, ovaries (granulosa cells), bladder, lungs, vascular endothelium, salivary glands and parts of the central and peripheral nervous system (47-49).

A great number of studies indicate that ERα is the responsible for most estrogenic actions in normal breast and breast cancer tissues (15). But while ERα role in breast cancer pathology is well confirmed, the role of ERβ is still not clearly defined. Most of the data indicates that ERβ has an antagonist effect to ERα by promoting growth suppressive effects on breast cancer cells, affecting,

for example, cell cycle and apoptosis, especially when co-expressed with ERα (15, 50-53). It is also reported an increase in ERα/ERβ ratio in breast cancer cells when compared to normal tissue or benign tumors (54, 55), indicating that ERα, contrary to ERβ, is more related with carcinogenesis. In fact, ERβ expression is lower in many breast tumors, indicating that the loss of its expression might be one of the mechanisms involved in tumor driving (54, 56). In addition, a direct correlation between ERβ presence in breast cancer patients and overall survival was reported (57, 58). However, ERβ is not only associated with a good prognosis. Its role is controversial, once in ERα-negative tumors, ERβ seems to play a carcinogenic role by stimulating tumor progression (59). The study of this isoform is very complex due to the presence of 5 related subtypes in which subtypes 1, 2, 4 and 5 are expressed in breast tumors (50, 60, 61). However, the biological mechanisms involved in this receptor effects remain to be clarified. Nevertheless, targeting selective ER isoforms or the combination of ERα antagonists with ERβ agonists may have beneficial effects for some breast cancer patients (62).

4. SIGNALING PATHWAYS

The ER signaling pathways are mediated through genomic and non-genomic activities (41). In breast tumors, ER activates genes that are mostly associated with cell proliferation and survival, inducing up-regulation of insulin-like growth factor 1 receptor (IGF1R) and HER2, cell cycle regulators (cyclin D1, MYC), anti-apoptotic factors (Bcl-2), proangiogenic vascular endothelial growth factor (VEGF) and multiple other growth factors, that stimulate tumor progression, invasion, metastasis, promotion of angiogenesis and apoptosis inhibition (41, 63, 64). Furthermore, studies suggest that ER signaling is also able to inhibit expression of some genes with anti-proliferative or pro-apoptotic functions (63). The final outcome is tumor growth stimulation.

Genomic activity occurs when ER functions as a ligand-dependent transcription factor and corresponds to a nuclear-initiated steroid signaling (NISS) (46). Gene expression is mediated by two distinct mechanisms: classical and non-classical (Figure 5). In the absence of estrogen, ER remains inactively bound to heat-shock proteins/chaperones (65). In classical pathway, ligand-activation of ER induces receptor conformational changes, phosphorylation (66, 67), dissociation from the chaperone proteins and homo- or heterodimerization (ERα/ERα, ERβ/ERβ or ERα/ERβ) (15, 46). This changes allows the dimer to interact with specific DNA sequences - estrogen response elements (EREs) - present in the promoter region of target estrogen-regulated genes and to recruit co-regulatory proteins, such as co-activators or co-repressors, resulting in enhanced or suppressed transcription (41, 43, 68). In non-classical pathway, ER does not bind directly to DNA through EREs,

instead interacts with other transcription factors and co-activators/co-repressors that will further promote or suppress gene transcription (41, 46, 69).

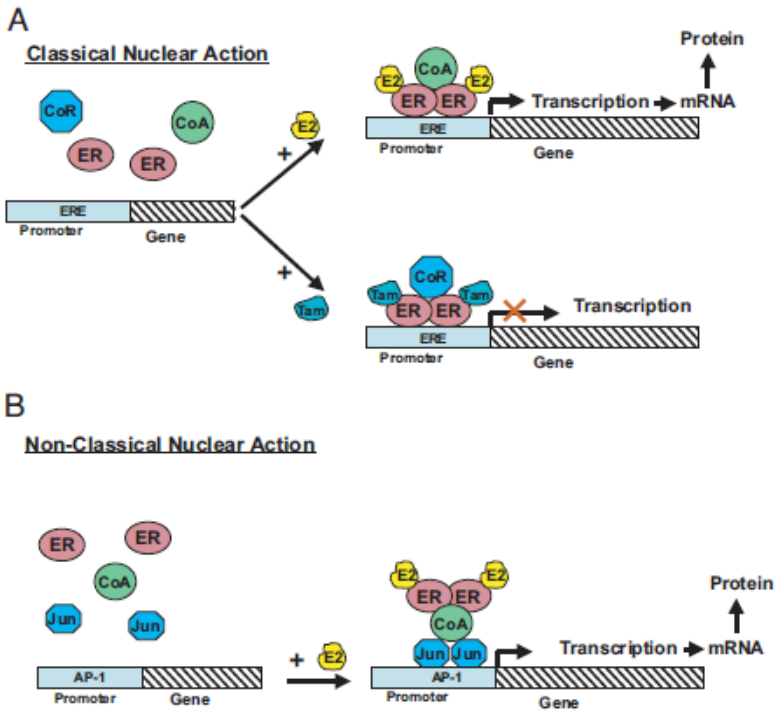


Figure 5. Classical and non-classical genomic ER pathways. (A) Classical action: ER binds directly to DNA sequences, EREs (estrogen response elements), at the promoter region of target genes. There is recruitment of co-regulatory proteins to regulate gene transcription – co-activators (CoA) for ER-estrogen bound and co-repressors (CoR) for ER-antagonists bound, such as tamoxifen (Tam). (B) Non-classical action: ER interacts with transcription factors (Jun) that will recruit co-regulatory proteins and bind to promoter elements (AP-1) to induce genomic activity. Adapted from G. Arpino et al. (69).

ER non-genomic activity, also called MISS (membrane-initiated steroid signaling), is a signaling transduction pathway mediated by ER present in the membrane instead of the nucleus (46, 70). It is initially independent of gene transcription and occurs within few minutes. Studies reported that membrane ER signaling involves its interaction with other transmembrane growth factor receptors (GFRs) to promote rapid downstream signals for nuclear transcription. This process triggers the activation of kinases that can further activate nuclear ER and its co-regulators, resulting in enhancement of the ER genomic activities (41, 46, 70, 71).

This way, the genomic and non-genomic mechanisms appear to be complementary and interact with each other, even in a synergistic way. Furthermore, there is an increased knowledge and understanding of the complexity of ER signaling in breast cancer progression. ER and GFRs signaling pathways are the dominant tumor drivers of cell proliferation, survival and tumor growth in the majority of human breast cancers. So, they are considered major targets for therapy. Moreover, studies highlighted that the evident cross-talk between ER and GFRs pathways is involved in

endocrine therapy resistance (69). This existing bidirectional cross-talk is accomplished by several cyclic mechanisms. As referred, estrogen-mediated ER genomic activation leads to expression of proliferation and survival signals that are key components of growth factor pathways, including receptors tyrosine kinase (RTKs), such as EGFR (epidermal growth factor receptor), IGF1R and HER2. Besides, estrogens can also act on membrane ER and activate its non-genomic signaling, promoting ER direct or indirect interaction and activation of those RTKs, G protein-coupled receptors (GPCRs) and Src kinases. Activated RTKs by genomic and non-genomic ER signaling triggers the activation of downstream kinase cascades, such as MAPK (mitogen-activated protein kinase), phosphatidylinositide 3-kinase PI3K/AKT (protein kinase B)/mTOR (mammalian target of rapamycin) pathway, JNK (c-Jun N-terminal kinase)/p38 MAPK (stress-induced pathway) and other kinases activated by growth factors. Transcription factors, like NF-κB, MYC and cyclin D1 are also activated to induce cell proliferation (46, 72-74). Those kinases can further activate nuclear ER, its co-regulator proteins and other transcription factors, even in the absence of estrogen (46, 69, 75). This results in a potentiation of ER genomic activity and enhancement of gene transcription, including genes involved in growth factor signaling pathways, increasing RTKs signaling and completing this cooperative bidirectional crosstalk cycle between ER genomic/non-genomic activity, growth factor receptors pathways and kinases activation (75) (Figure 6). In breast tumors, the deregulation of this mechanism ultimately leads to an amplification of cell survival and proliferation stimuli coming from both growth factor signaling overexpression (especially with HER2 overexpression) and ER signaling overactivation, resulting in a complete loss of estrogen dependence, which may contribute to profound alterations that weakens the inhibitory effects of endocrine therapies and results in endocrine resistance (reviewed in (64, 69, 75, 76)).

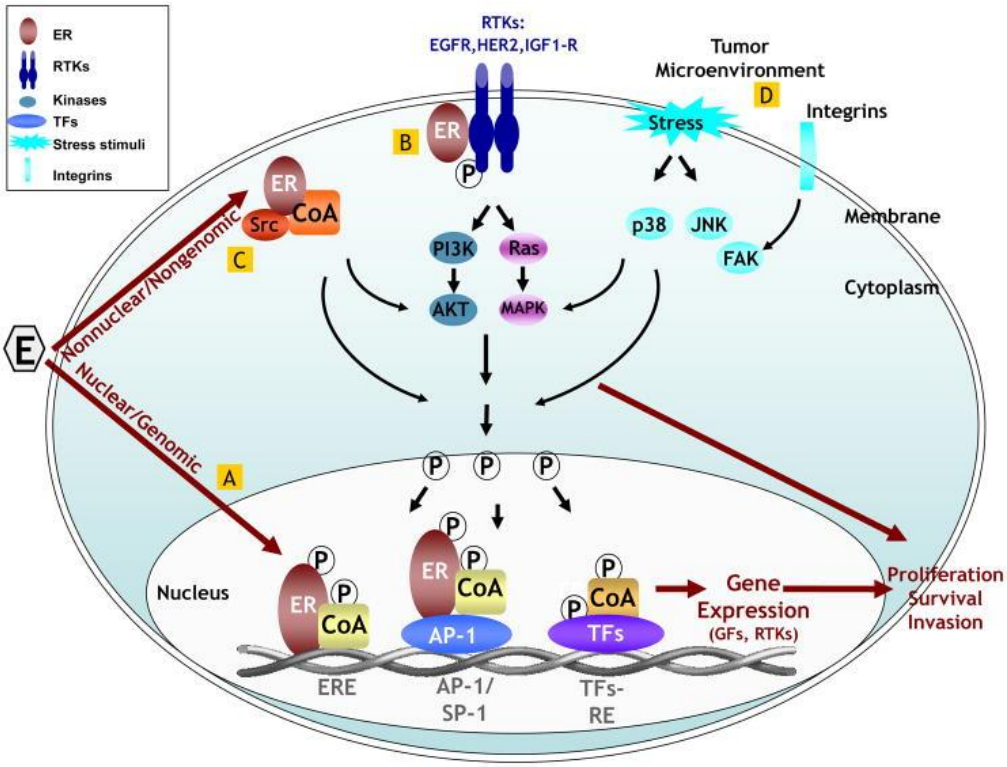


Figure 6. Integration of genomic and non-genomic ER signaling and its crosstalk with growth factor receptors and kinase pathways. (A) ER genomic classical/non-classical pathways. (B) Non-genomic pathway: ER outside the nucleus (membrane/cytoplasm), in response to estrogen, interacts with RTKs (EGFR, HER2, IGF1R), signaling molecules (Src) and co-activator molecules (C) activating multiple downstream kinase pathways, leading to phosphorylation of nuclear ER, co-activators (CoA) and transcription factors (TFs) and enhancement of gene transcription. Signaling from tumor microenvironment also activates stress pathways and integrin family members to trigger downstream kinase pathways (D). Overall, the genomic and non-genomic ER pathways complement each other to induce breast cancer proliferation, survival and invasion stimuli. Deregulation of this pathways and signaling factors can result in endocrine therapy resistance. Adapted from Osborne CK and Schiff R. (72).

5. AROMATASE: EXPRESSION, FUNCTION AND STRUCTURE

Human aromatase enzyme is a member of cytochrome P450 family, expressed by CYP19A1 gene localized in chromosome 15q21.1 (77, 78). It interacts with NADPH-cytochrome P450 reductase to catalyze the rate-limiting and final step of estrogen biosynthesis, the aromatization of androgens (androstenedione and testosterone) to estrogens (estrone and estradiol) (24).

Aromatase is localized in endoplasmic reticulum of estrogen-producing cells and it is widely expressed in ovarian and several other extragonadal tissues and organs, such as adipose tissue, testis, skin, vasculature, muscle, brain and breast tissue (24, 78, 79). This way, aromatase can generate estrogens locally in sufficient amount to exert physiological but also pathophysiological effects, such as stimulating tumor development (29). In premenopausal women, it is expressed mainly in the ovaries, while in men and postmenopausal women aromatase is expressed in the

referred extragonadal tissues (79). During pregnancy, placenta is an extra source of aromatase (78, 80). By generating estrogens, aromatase plays vital roles in female development and reproduction but also in males, such as in energy balance, maintenance of bone structure and spermatogenesis (81).

Aromatase has been shown to be overexpressed in breast cancer tissues, surrounding stromal adipose tissue, fibroblasts and epithelial cells, producing higher levels of estrogens than non-cancerous cells and evidencing its key role in breast cancer pathogenesis (79, 82, 83). This is one of the main reasons for the major interest in therapies that target this enzyme for ER^+ breast cancer treatment.

The discovery of aromatase activity in extraglandular tissues was important to understand the major source of estrogens in certain tissues and, more recently, the mechanisms by which aromatase expression is increased in some specific tissues and cell populations, such as in breast cancer. Tissue specific expression of aromatase is controlled by the presence of tissue specific promoters of *CYP19A1* gene via alternative splicing. Several promoters have been identified along the years, for the different tissues (79, 81, 84, 85). Placental aromatase is mainly controlled by promoters I.1 and I.2a, ovarian is controlled by PII and promoters I.2, I.3, I.4, I.5, I.6, I.7 and I.f are associated with expression of aromatase in extraglandular tissues, such as adipose tissue, brain and bone (79). While in normal breast tissue aromatase expression is regulated by promoter I.4 activation, breast cancer tissue uses three additional promoters, I.7, I.3 and PII, to activate transcription (84, 86, 87), which suggests that different specific factors might be involved in intratumoral levels of aromatase and, subsequently, estrogen levels. This promoter switching results in aromatase upregulation, a common feature of hormone-dependent breast tumors (84, 86, 87). Promoters are also positively or negatively regulated, for each specific normal and malignant tissues, by different molecules, such as cAMP, gonadothrophins, prostaglandins (PGE2), oncostatin M, cytokines (IL-6, IL-11), TGF-β, TNF-α (tumor necrosis factor) and other hormones and growth factors (84, 86, 88, 89). Therefore, different tissues that synthesize estrogen present different aromatase expression patterns due to different promoters and molecules.

So, the mechanism behind aromatase overexpression in breast tumors relies essentially in molecular alterations in malignant and surrounding stromal cells, that release cytokines and growth factors, which favor the binding of transcriptional enhancers instead of inhibitors to normal promoters such as I.3 and PII, resulting in increased transcription. Somehow, I.7 promoter is also activated in the process. Ultimately, there is a selection of aromatase-upregulated cell types that use those alternative enhanced promoters, switching tumor's promoter profile. Another mechanism is also reported and involves mutations that cause aromatase coding region to stand adjacent to constitutively active promoters, resulting in its own upregulation (79, 86).

Aromatase catalyzes an aromatization reaction by converting circulating C19 androgenic steroid precursors into C18 estrogens through a series of three oxidative steps, leading to the formation of the phenolic aromatic (A) ring characteristic of estrogens (78). Each reaction consumes one molecule of oxygen and NADPH. The third step is the final and critical step that generates estrogens and is solely performed by aromatase, the only enzyme in vertebrates capable of aromatizing a six-membered ring (24, 90). Aromatase has two androgenic substrates: androstenedione and testosterone, though androstenedione is the substrate with higher affinity (90). Since aromatization is the last step of estrogen biosynthesis and only performed by this single enzyme, its blockage with selective compounds should not interfere with other P450 enzymes or affect other steroids production. Therefore, aromatase is a very attractive target for inhibition.

Aromatase enzyme complex consist of two polypeptides: cytochrome P450 aromatase and a flavoprotein, NADPH-cytochrome P450 reductase, which is ubiquitous in the endoplasmic reticulum of most cell types and responsible for supplying NADPH reducing equivalents to any cytochrome P450 enzyme (85). For many years the three dimensional structure of human cytochrome P450 aromatase was unknown due to the difficulty of solubilizing this protein in the absence of detergent, to its hydrophobicity and to the membrane-bound character that confers inflexibility to crystallization. Therefore structure-function relationship studies were very limited. Many models were proposed (91-93) until finally, in 2009, Gosh et al. successfully solved the crystalized structure of human aromatase purified from human placenta and the structural basis for androgen specificity (Figure 7) (94, 95).

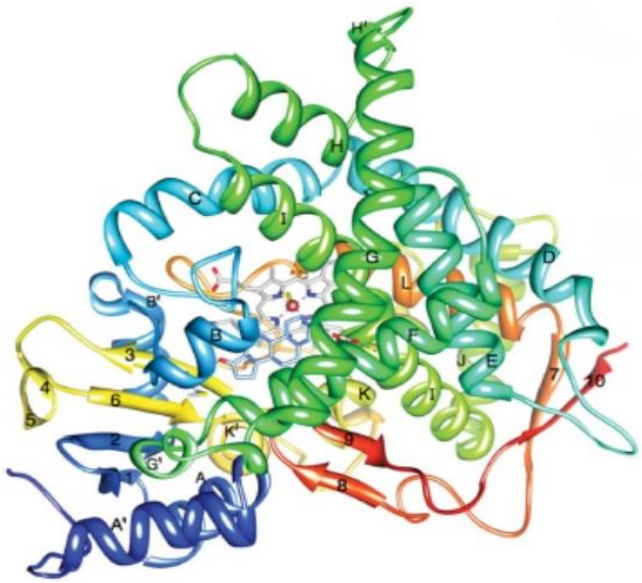


Figure 7. Aromatase structure. Colored in dark blue is the N terminus, starting at residue 45, and colored in red is the C terminus ending at residue 496. The α-helices are labelled from A to L and β-strands are numbered from 1 to 10. The heme group and the androstenedione molecule bound at the active site are also shown. Adapted from Gosh et al., 2009 (94).

Aromatase tertiary structure consists of 12 major α-helices (labeled from A to L) and 10 β-strands (numbered from 1-10) (94). Steroid binding site is at the heme distal site and the heme iron is the reaction center. Hydrophobic and polar residues of the polypeptide chain open a catalytic cleft that complements the steroid backbone. Specific distortions occur in aromatase in order to bind to the ligand (95). Aromatase, unlike other P450s that metabolize drugs/xenobiotics, is highly androgen-specific. The knowledge of tridimensional aromatase structure allowed not only a better understanding of the specific androgenic binding that confers the enzyme its unique catalytic activity and the series of reactions involved, but also a better analysis of the current aromatase inhibitors and development of new specific ones.

6. ENDOCRINE THERAPY

Every breast tumor is different and to consider the best treatment strategy for each patient is important to take into account: tumor size, stage, types of receptors, gene expression, health condition and menopausal status. A good therapy implies an improvement in patient's survival without compromising the quality of life. Therapeutic strategies may include a local treatment, such as surgery and/or radiotherapy, a systemic treatment, such as chemotherapy, hormone or targeted therapy, or a combination of two or more of the above mentioned treatments in order to obtain maximum efficacy. Neoadjuvant therapy (drug treatment prior to the main treatment, like surgery) and adjuvant therapy (after the main treatment) are also common options. Overall, it is very important to obtain a balance between the potential benefits and the less toxic effects possible, in a short and long-term period. Thus, cancer therapy is still a very challenging research field and novel strategies are constantly studied.

The knowledge of estrogens key role in hormone-dependent breast cancer development and progression allowed great improvements in the therapeutic field with the development of drugs that may lower the hormonal effects – endocrine therapies. So far, two main approaches have been successfully applied to block estrogens actions, either by modulating ER or by inhibiting estrogens production (96). The first approach directly targets ER through either selective ER modulators (SERMs), such as tamoxifen, or selective ER downregulators (SERDs). They act as agonists or antagonists of the receptor, thus blocking estrogen binding (96). The second approach relies on the use of aromatase inhibitors (AIs) that target and block the enzyme aromatase, suppressing estrogen biosynthesis and, consequently, its effects (Figure 8). Currently, AIs are one the most effective endocrine therapies for postmenopausal breast cancer patients (22). In premenopausal women, estrogen biosynthesis can be blocked by recurring to other approaches, such as ovarian

ablation/removal through surgery or radiotherapy and/or treatment with luteinizing hormone-releasing hormone (LHRH) agonists, which inhibit the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary, to supress estrogens production by the ovaries (17, 97).

Endocrine therapies are among the less toxic and more effective therapies for hormone-dependent breast tumors, though they may not be suitable for every patient. Certain factors can affect prognosis and predict the response to the treatment, such as hormone status (presence/absence of ER, PR and HER2), number of lymph nodes involved, tumor size, histological grade, tumor growth and invasion (98). ER status is currently recognized as the principal predictor factor in endocrine treatment responsiveness. Patients with higher tumor ER levels have more chances to successfully respond to endocrine treatment and are the ones who obtain highest benefits from therapy (72). PR and HER2 status are also valuable biomarkers (46, 99).

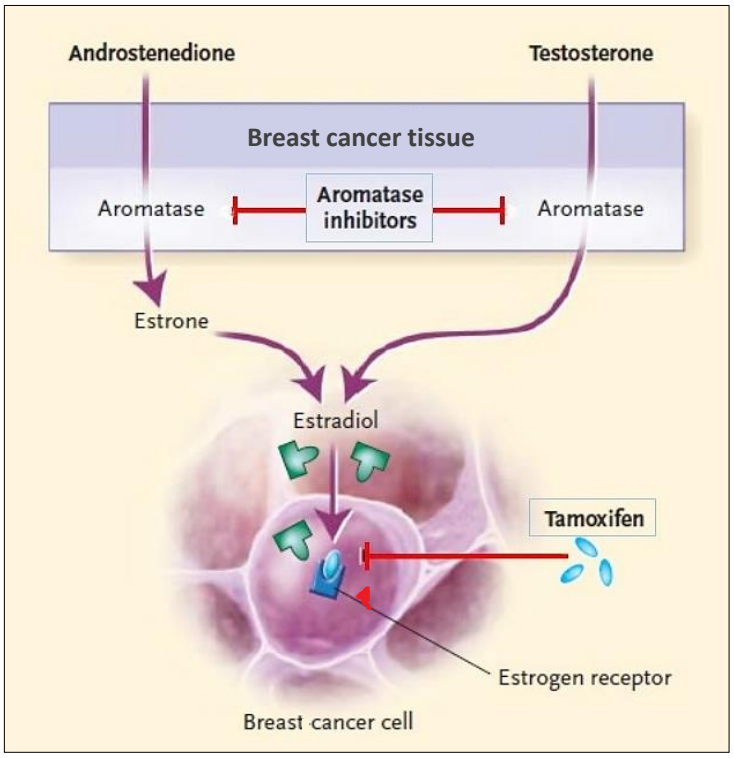


Figure 8. Mechanism of action of aromatase inhibitors and Tamoxifen. Aromatase inhibitors suppress estrogen production in postmenopausal women by blocking aromatase, the enzyme responsible for the synthesis of estrogens from androgenic substrates. Tamoxifen is a selective ER modulator (SERM) that inhibits breast tumor growth by competitive antagonism of estrogen at ER site. Adapted from Ian S. and Mitch D., 2003 (100).

Nevertheless, tumor heterogeneity, genetic profile/mutations and microenvironment complexity might affect ER expression and result in other molecular alterations during tumor development (101), altering the predicted response to the therapy and increasing recurrence cases.

In fact, despite the demonstrated benefit of endocrine therapies, in many cases a good treatment strategy might not work because its efficacy is limited by tumor resistance to the therapy, which will be detailed in section 7.

6.1. SELECTIVE ER MODULATORS (SERMS) AND DOWNREGULATORS (SERDS)

Ever since the link between estrogen and breast cancer emerged, several attempts to antagonize estrogens biological effects were made. In the past few decades, the breast cancer therapies were mostly focused on developing compounds to block estrogen signaling by targeting ER. As previously referred, two main types of compounds successfully emerged and are still in use: SERMs and SERDs. The SERMs competitively bind to ER and exhibit tissue dependent antagonist/agonist activity (46). In breast tissue, SERMs act as ER antagonists, blocking estrogen effects, while in other tissues, such as bone and uterus, they act in a similar way to estrogen, displaying agonist activity (62). The differential tissue selectivity is determined by co-factor recruitment and availability and the promoter context of the tissue (62, 102). When a SERM act as an agonist, ER dimerizes and recruits co-activators to stimulate gene transcription. When it acts as an antagonist, ER dimerizes and recruits co-repressors to suppress transcription. The exact tissue-specific mechanisms are not clear. An ideal SERM should act as antagonist in hormone responsive tissues (breast, uterus, ovaries), reducing the risks of hormonal cancers, and as agonist in bone and cardiovascular system, reducing the risk of osteoporosis and cardiovascular diseases (62).

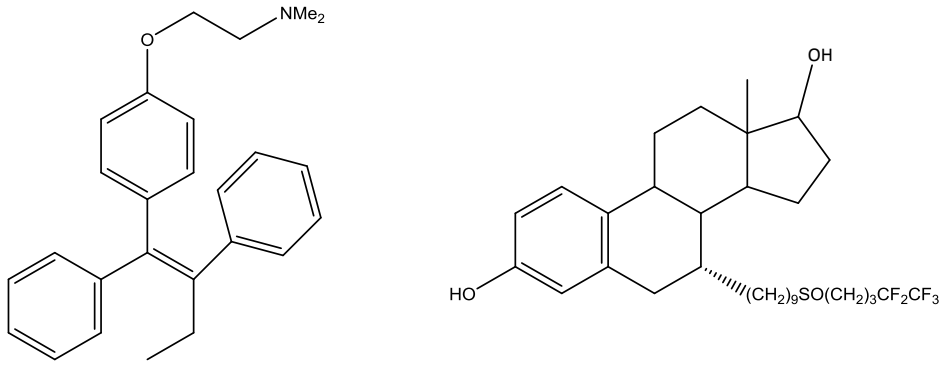
Tamoxifen (Figure 9), raloxifene and toremifene are classified as SERMs and are approved by Food and Drug Administration (FDA) for treatment and prevention of breast cancer.

Tamoxifen was the first SERM and it has been for the past 30 years the most widely used drug in breast cancer treatment. Due to its established efficacy and general tolerability profile, tamoxifen has been considered the “gold standard” treatment for ER^+ breast tumors. It significantly improves overall survival as adjuvant therapy in early breast cancer and reduces the risk of disease recurrence in premenopausal and postmenopausal women patients (103), as well as the incidence of breast cancer, as a preventive agent, in healthy women at risks of developing the disease (104, 105). However, prolonged use (more than 5 years) increases toxicity effects and the development of resistance (106). It became clear that tamoxifen acted as estrogen receptor agonist in some tissues and it was associated with adverse effects, such as increased risk of endometrial cancer, thromboembolic events, hot flashes, vaginal bleeding, among others (103, 107). On the other hand, tamoxifen appears to benefit from estrogenic effects such as amelioration of lipidic profile (108),

cardioprotective effects (109) and preservation of bone mineral density in the lumbar spine of postmenopausal women with breast cancer (110).

In addition to tamoxifen, several other SERMs were developed, such as raloxifene and toremifene (111, 112). None was clearly superior to tamoxifen in treatment and prevention of breast cancer.

More recently, new ER antagonists have been developed – SERDs. These compounds bind to ER, but contrary to SERDs, they induce its proteasomal degradation without any agonist effect or tissue specific activity (96, 113, 114). They provided a second-line treatment option for breast cancers that develop resistance to a first-line therapy (115). Fulvestrant (Figure 9), also commercialized as "Faslodex", is the first SERD, structural and pharmacologically different from SERMs.



Tamoxifen **Fulvestrant**

Figure 9. Chemical structures of a SERM, Tamoxifen, **and a SERD**, Fulvestrant.

Unlike tamoxifen, fulvestrant induces ER downregulation with consequent suppression of estrogen-dependent gene transcription. The overall results is a pure anti-estrogenic effect (116, 117). Its singular mechanism may confer some advantages, such as a lack of cross-resistance with other endocrine agents, which makes fulvestrant an FDA approved and effective sequential treatment option in postmenopausal women with advanced/metastatic and progressive breast cancer, despite the previous use of other endocrine therapies (62, 118).

6.2. AROMATASE INHIBITORS

Although tamoxifen was considered the first therapeutic option for women with hormone-dependent breast cancer for many years, its adverse effects evidenced the need for new therapeutic options, which led to the development of new compounds with a different target –

aromatase. As previously described, aromatase is responsible for the conversion of androgens to estrogens, which makes it a selective target to lower estrogen levels. Thereby, inhibitors of this enzyme were developed and a new class of anti-tumor drugs, called aromatase inhibitors (AIs), emerged. In the past decade, it has become clear that AIs are a valuable alternative to tamoxifen in the treatment of postmenopausal women with ER^+ breast cancer (119-121), since they proved to be an effective approach to selectively block estrogen production and its effects on tumor progression.

AIs are grouped in three generations (Figure 10), according to their chronological order of appearance. Each generation has different evolutional modifications that confers to AIs higher specificity for aromatase, increased aromatase inhibition (more potency) and less adverse effects. Besides, they are also organized according to their chemical structure in two types: type I refers to steroidal AIs, such as exemestane, and type II refers to non-steroidal AIs, like lestrozole and anastrozole, which differ in their mechanism of action. This two types of inhibitors will be addressed in sections 6.2.1 and 6.2.2.

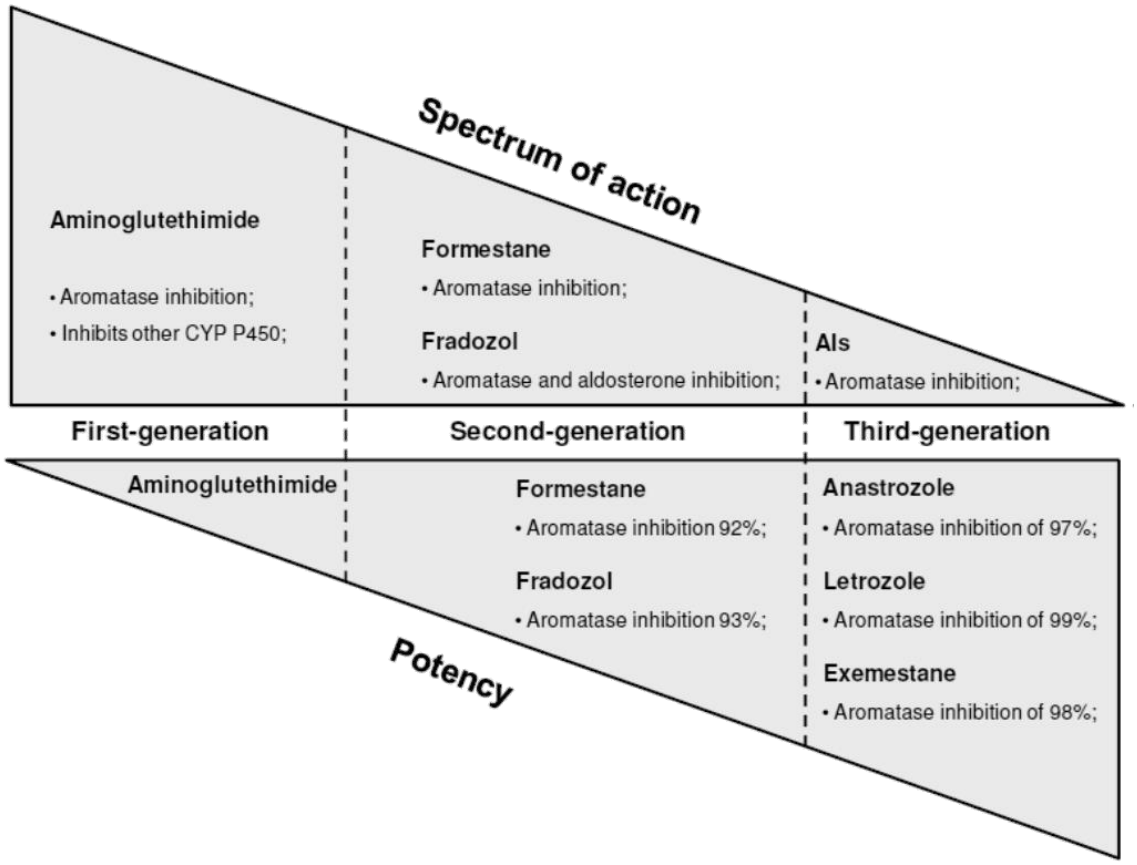


Figure 10. Spectrum of action and potency of aromatase inhibitors.

Aminoglutethimide (AG) was the first AI used to treat breast cancer and therefore it is included in the first-generation group, though it was not selective for aromatase. It was followed by the second-generation of AIs: 4-hydroxyandrostenedione (4-OH), further renamed formestane, the first selective AI clinically approved for breast cancer treatment, and fadrozole. The search for better compounds continued and led to the development of a new range of AIs, namely third-generation AIs, in early 1990's. They are more specific, more potent and with fewer side effects. In this category, there are three FDA approved AIs: the steroidal exemestane (Aromasin®) and the non-steroidal, anastrozole (Arimidex®) and letrozole (Femara®) (100) (Figure 11).

Currently, third-generation AIs are clinically used to treat hormone-dependent breast cancer in adjuvant and metastatic setting, as first-, second-line or sequential therapy, and they are now considered a standard treatment for postmenopausal patients (119, 120). In fact, AIs have demonstrated increased disease-free survival, less recurrence risk, increased benefit and response rates, a good safety profile, neoadjuvant benefits and a chemopreventive role, proving their benefits and superiority over other hormonal agents, including tamoxifen, and therefore establishing their place as a first-line endocrine therapy. The two main strategies used are AI monotherapy for 5 years as adjuvant treatment or alternate treatment with 2-3 years of tamoxifen followed by 3-2 years of an AI for a total of 5 years (reviewed in (120, 122, 123)).

Differences in the treatment with steroidal and non-steroidal AIs are observed, probably due to the different nature of the compounds, pharmacological characteristics, interaction with the enzyme, inhibitory potency and administered doses (124). Nevertheless, all induce a mean aromatase inhibition greater than 97% and a reduction in intratumoral levels of estrogen (100, 120, 125). Due to their high biodisponibility, all AIs used in clinic are orally administered once a day. At clinical doses, the plasma half-lives of the different AIs are around 41-48 hours for anastrozole, 27 hours for exemestane and 48-96 hours for letrozole (126). They are all well tolerated, presenting minor side effects that include hot flashes, sexual dysfunction, arthralgia and musculoskeletal pain (127). So, they do not increase endometrial cancer risk and have lower risks of thromboembolic events (128). However, a major limitation of this therapy is a significant increase in bone loss, osteoporosis and subsequent increase in fracture risk, when compared to tamoxifen (127, 129, 130). Nevertheless, it can be managed by the use of denosumab, an FDA approved drug for AI-induced bone loss. Besides, studies revealed that co-administration of bisphosphonates might also be a good strategy to overcome this problem, once they have positive effects on bone mineral density of patients who are suffering bone loss due to AIs (131). On the other hand, lipid metabolism and cardiovascular events appear to be slightly more frequent in AIs therapy, especially for patients with previous ischemic heart disease, but studies and trials results are very controversial and no conclusions can be taken yet (120, 127, 132). Overall, AIs have a favorable risk-

benefit profile. Studies also favored AIs efficacy as chemopreventive and neoadjuvant agents in postmenopausal women (133, 134). At this point, it is still not possible to confirm which AI, from all the options in the field (steroidal or non-steroidal), have the best characteristics and benefits.

AIs use is contraindicated in premenopausal women with normal ovarian function, once they reduce estrogen negative feedback to the hypothalamus and pituitary, leading to an increase in gonadotropins (LH and FSH) secretion. As shown in animal models, this might lead to an increased estrogen production and increased size of the ovaries, provoking cysts formation (135). Nevertheless, in premenopausal women with suppressed ovarian function, AIs might be safely used. Though studies are still being conducted, exemestane have already proved to prolong disease-free survival and to reduce the risk of breast cancer recurrence, as well as the risk of any invasive cancer, in premenopausal patients (136, 137).

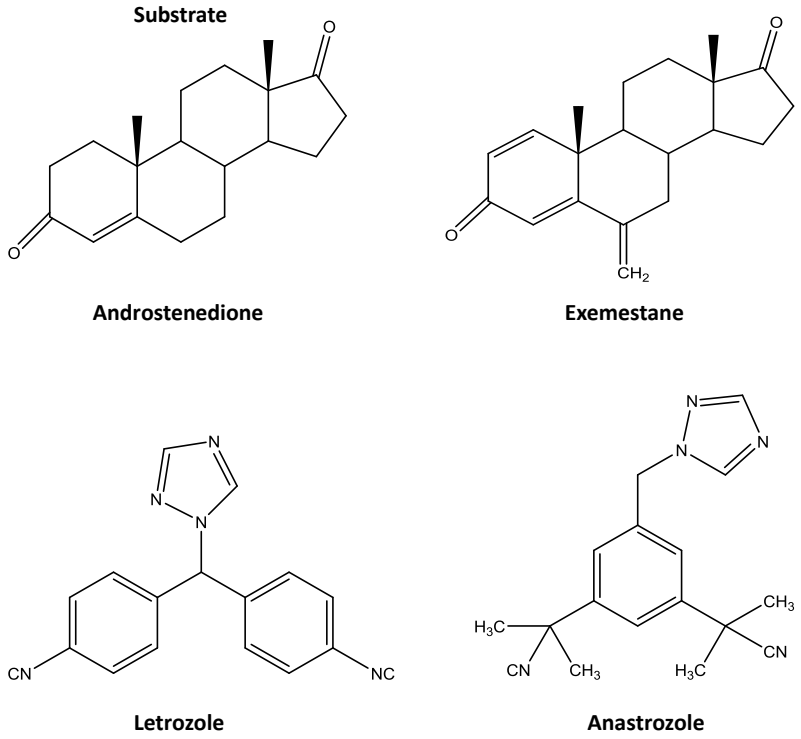


Figure 11. Structure of third-generation aromatase inhibitors and aromatase natural substrate, androstenedione.

6.2.1. STEROIDAL AROMATASE INHIBITORS

Type I AIs are steroidal molecules that have an androgen analogue structure of aromatase natural substrate, androstenedione. Due to their similarity, these compounds directly compete with androstenedione for the enzyme-binding site. After binding, they are converted into reactive intermediates that bind covalently to the enzyme and induce its irreversible inactivation and

degradation by the proteasome, in a time and dose-dependent manner. For this, they are also referred as “suicide inhibitors” (138).

In this category are included testololactone (first-generation), formestane (second-generation) and exemestane (third-generation). Testololactone, a molecule structurally related to testosterone, was used as a drug for breast cancer therapy in 1960’s, but at the time its mechanism was unknown and it was soon replaced by AG, so no further studies were performed. Formestane was the first selective AI clinically used for breast cancer treatment. It was effective and had fewer side effects than AG, but it has the disadvantage of requiring intramuscular injection due to rapid hepatic metabolism, so its clinical use was limited and rapidly replaced for more potent AIs (139). Finally, exemestane, the only representative of third-generation steroidal inhibitors, was introduced in clinic as an orally-active aromatase inactivator, due to its irreversible binding to aromatase and consequent inactivation and degradation (138). Its unique inhibitory mechanism culminates in a reduction in aromatase half-life in 50% (138), a total body aromatase inhibition around 98% (for 25 mg once daily) and a long-lasting reduction in urine and plasma estrogen levels, in a dose-dependent manner, without affecting other steroids levels (140-142). Intratumoral aromatization and estrogens levels were similarly affected (125). Miller et al, (2002) reported that exemestane therapy caused a marked decrease both in peripheral aromatization and in tumor breast tissue with a reduction in tumor volume of around 83% in 80% of the cases after 3 months (143).

Exemestane proved to be more effective than tamoxifen as a first-line therapy for advanced breast cancer in postmenopausal patients (137, 144, 145). Currently, it is clinically indicated in the treatment in ER^+ early-stage breast cancer in postmenopausal patients, either as first-line option monotherapy, as adjuvant therapy following 2-3 years of tamoxifen or as extended therapy beyond 5 years of adjuvant treatment. It is also used for the treatment of advanced breast cancer in women whose disease has progressed following anti-estrogen therapy (137). Furthermore, exemestane seems to be a valid option for chemoprevention, as a neoadjuvant agent and in sequential treatment (145). Toxicity profile and side-effects are similar to other AIs (hot flashes, vaginal drying, arthralgia, bone loss), but it is generally well tolerated (137).

6.2.2. NON-STEROIDAL AROMATASE INHIBITORS

Type II or non-steroidal AIs have a triazole functional group that interacts with the heme prosthetic group of aromatase in a non-covalent/reversible way, competitively inhibiting the binding of androgenic substrates (24). This particular subtype of AIs includes aminoglutethimide (first-generation), fadrozole (second-generation), letrozole and anastrozole (third-generation).

Aminoglutethimide, initially studied as an anticonvulsive for epilepsy, was, as referred, the first non-steroidal AI to be studied for breast cancer treatment. However, along with its success in breast cancer treatment, AG suppressed adrenal steroid production by blocking several other P450 enzymes and leading to adrenal insufficiency. So, it demanded cortisol replacement and strict monitoring (146, 147). Moreover, it was not very potent and required administration of high concentrations. AG was supplanted by second generation AIs with superior characteristics – greater efficacy, selectivity and less toxicity. Second-generation AI, fadrozole, is more selective and potent than AG and demonstrated clinical efficacy similar to tamoxifen as a first-line treatment for postmenopausal women with breast cancer (148, 149). However, it still caused aldosterone inhibition, which limited its use to lower doses that only induced an inhibition around 90% (100). Third-generation AIs, anastrozole and letrozole, are the most widely used. They competitively and reversibly bind to aromatase, conferring great potency and specificity. Both have aromatase inhibitory effects over 97% at clinical doses (120, 150) without substantially affecting other enzymes (151). Letrozole was associated with higher aromatase inhibition than anastrozole (150). Both AIs showed to be as good or clinically superior to tamoxifen in many settings, such as first-line treatment or adjuvant treatment in both early and advanced breast tumors in postmenopausal women. Anastrozole is also a good chemopreventive agent (123). Both treatments are well tolerated showing some musculoskeletal symptoms, bone loss and fractures, but do not show increased risk of endometrial cancer or thromboembolic events and cause less hot flashes and gynecological symptoms than tamoxifen (100). Letrozole is currently indicated for adjuvant and extended adjuvant treatment of early breast cancer while anastrozole is used as first- and second-line treatment of advanced breast cancer and as adjuvant treatment for early breast cancer, both on ER^+ postmenopausal patients.

7. THERAPY RESISTANCE MECHANISMS

Despite their efficacy, endocrine therapies are limited by intrinsic and acquired resistance. *De novo* or intrinsic therapy resistance occurs when a certain tumor does not respond to the initial drug/endocrine therapy, while acquired resistance happens when the tumor initially responds to the treatment but a subsequent progression occurs. In fact, at some point, a large part of breast tumors undergo complex molecular alterations that result in resistance to endocrine therapies. It is expected that about 30% of patients with early-stage breast cancer have recurrent disease (98).

Mechanisms of endocrine resistance are still uncertain in many aspects, since many of them are only studied in breast cancer cell models and might not be translated in clinical outcomes. Besides, in clinic, it is difficult to separate tumor associated symptoms/outcomes from specific resistance mechanisms to the therapy. Nevertheless, resistance is often associated with deregulated estrogen signaling and alternative proliferative and survival pathways in tumor cells. Although the major part of the currently known mechanisms were based on studies focused on tamoxifen resistance, many of them are now commonly associated to both ER antagonists and AIs. Common resistance associated mechanisms include:

- ER status - loss of ERα expression, ER activation in absence of estrogen and hypersensitivity to low estrogen levels, expression of truncated isoforms of ERα and ERβ, post-translational modifications (phosphorylation, methylation) of ERα, that might result in ligand-independent activation (46, 96, 152);
- deregulation of ER co-activators (overexpression) and co-repressors (downregulation) (152);
- upregulation of transcription factors (AP-1 and Nf-κB) (152);
- cross-talk between ER and growth factor pathways, with increased RTK signaling (ex: HER2 overexpression) and subsequent enhancement of proliferation signaling (46, 69, 74, 152);
- deregulation of cell cycle - upregulation of positive regulators MYC, Cyclins D1 and E1 and downregulation of negative regulators, like p21 and p27, resulting in increased proliferation (74, 152, 153);
- deregulation of apoptotic machinery - increased anti-apoptotic signals Bcl-2 and Bcl-xL and downregulation of pro-apoptotic signals BAK, BIK and caspase-9, resulting in continuous survival of tumor cells (74, 152);
- induction of autophagy (154, 155);
- genetic/epigenetic alterations (HDAC intervention, loss of tumor suppressor genes, like *PTEN*, alterations in drugs metabolism) (72, 74, 156);
- tumor microenvironment stimuli with the implication of structural elements of the extracellular matrix (ECM), growth factors, cytokines and environmental conditions of hypoxia and acidity (72);

Beyond that, tamoxifen *de novo* resistance mechanisms include the presence of inactive alleles of the gene that encodes the enzyme that converts tamoxifen to its active metabolites (74), altered drug metabolism and drug interactions (46), initial lack of ERα and initial overexpression of HER2, allied to tamoxifen agonistic effects (69). As for acquired resistance, overexpression of HER2 and its cross-talk with ER is also especially involved, as well as all the mechanisms mentioned above (69).

Specific mechanisms of AI intrinsic resistance involve genetic and molecular profile of the tumor, genetic polymorphisms in aromatase gene and host immune response, many times associated with upregulation of immune related genes that increase release of TNFs (tumor necrosis factors), ILs (interleukines), cytokines, chemokines and a possible role of lymphocytes and dentritic cells (156, 157). Recently, accumulation/overexpression of p53 in breast cancer cells was also correlated with resistance to initial therapy (158). Very recently, a deregulation in the drug/xenobiotic sensor INrf2 (Keap1):Nrf complex, responsible for the activation of cytoprotective genes, was also correlated with AI-resistance. These studies showed that resistant cancer cells increase this signaling pathway in order to protect themselves from the drug's action, thus reducing its efficacy (159). As for acquired AI-resistance, it involves particularly the upregulation of growth factor signaling pathways and ER crosstalk mechanisms, constitutive activation of ER (ligand-independent) (22, 157, 160), upregulation of epidermal growth factor (EGF)–like protein amphiregulin (AREG) (161), deregulation of PI3K/AKT/mTOR (mammalian target of rapamycin), MAPK and Src pathways (156) and other kinases (aurora A and B - cell cycle regulatory proteins) (162), downregulation of tumor supressors (PDCD4) (163), and genetic/epigenetic alterations, especially with HDAC intervention (156, 164). Exemestane was shown to have weak estrogen-like activity, adding another resistance mechanism to this compound (22). Another recurrent hypothesis is tumor stimulation through other sources of estrogenic activity, like androgens, that are produced in high levels by adrenal cortex but are not converted to estrogens due to AIs action. However, androgens action on androgen receptor also promotes cell proliferation. Other less likely hypothesized mechanisms that might contribute to AI-resistant phenotype are aromatase overexpression, requiring an increasing therapeutic dose. However, Chen et al. showed that resistant breast cancer models treated with AIs have similar aromatase expression as the control ones, except for exemestane, which induces aromatase degradation (22).

On the other hand, AIs show limited cross-resistance between them, suggesting that different AIs have different resistance mechanisms (165). For instance, upregulation of IGFR-1 and PI3K/AKT pathways are more related with anastrozole treatment, while loss of ERα or its phosphorylation are more common with letrozole (24, 156) and autophagy is associated with exemestane (166), as well as estrogen-like effects of this steroidal compound (22).

Several strategies to overcome resistance are already under trials (Figure 12), but it is still extremely important to keep studying the mechanisms involved in hormone-dependent breast cancer treatment resistance in order to develop new treatment strategies to overcome it.

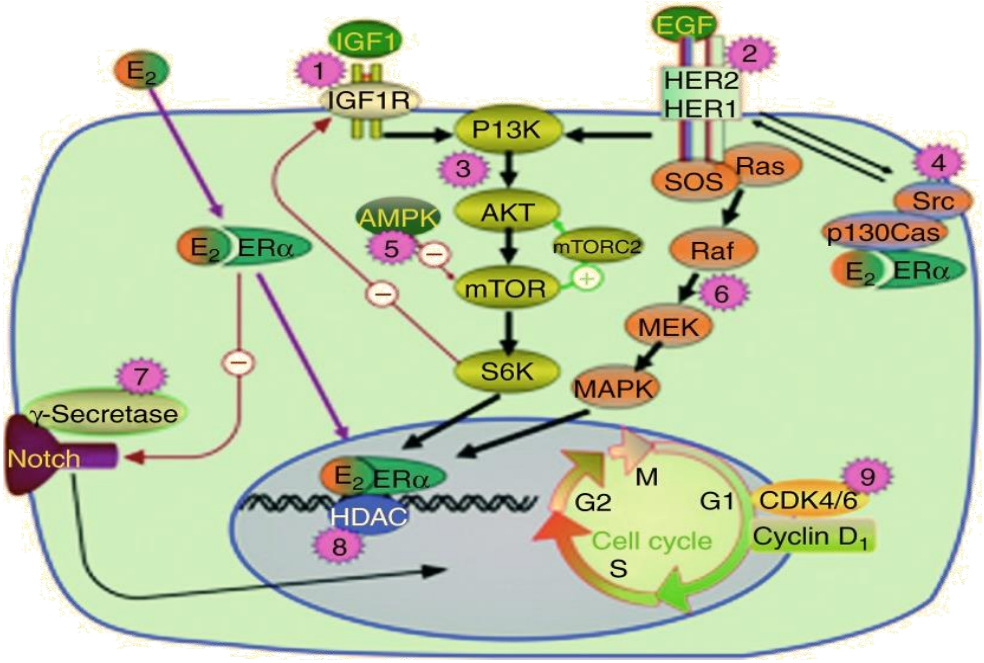


Figure 12. **Signaling mechanisms of endocrine resistance currently targeted in clinical trials, combined with AIs.** (1) IGF1/IGFR1 neutralizing antibodies (AMG-479). (2) HER2 blocking therapy (trastuzumab emtansine). (3) Inhibitors of PI3K, Akt, and/or mTOR pathway (everolimus). (4) Src inhibitors (desatinib). (5) AMPK activator (metformin). (6) Inhibitors of Ras-Raf-MEK-MAPK pathway (MEK inhibitor AZD6244, combined with fulvestrant after AIs failure). (7) Gamma secretase inhibitor (RO4929097). (8) HDAC inhibitors (vorinostat). (9) CDK4/6 inhibitor (PD0332991). Adapted from Lonning et al. (120).

The main challenges to obtain a successful treatment for breast cancer are the discovery of more specific biomarkers to predict therapeutic response to the endocrine treatment and the identification of new targets for endocrine-resistant tumors. Emerging genome-wide analysis of tumor gene expression has been successfully providing some valuable data in the therapeutic resistance field by identification of expressed genes correlated with patient's outcomes leading to the development of genes signatures. Some genetic events might even be associated with specific resistance mechanisms and be translated in clinical outcomes. Despite the still limited data and bioinformatics challenges, genes signatures might be used as reliable biomarkers and resistance targets in a near future (74, 101).

AIMS OF THE STUDY

The third-generation AIs have shown to be as efficient or even to overcome the effects of tamoxifen, the first-line therapeutic approach for breast cancer, for many decades. But, despite their efficacy, there are still side effects and resistance problems to surpass, demanding the research and development of new and more potent AIs, with less toxicity.

The elucidation of aromatase structure, active site and enzyme-substrate interactions were major steps for the design, synthesis and structure-activity relationship studies of new compounds. So far, several studies were already conducted to determine which structural modifications/chemical substitutions on androstenedione molecule are the most effective in order to obtain superior AIs, for clinical use. According to these structural-activity relationship (SAR) studies with newly synthesized molecules, the presence of some chemical characteristics seems to be determinant to ensure the anti-aromatase effects of the compounds. Some of these studies were performed by our group, which revealed that some alterations in A-, B-, and D-rings of the steroidal structure of the enzyme substrate, androstenedione, were crucial for the anti-aromatase activity and anti-tumor effects (167-170).

Following this research line, this work is focused on the characterization of newly synthesized steroidal compounds as potential AIs and in the study of their biological effects in the context of estrogen-dependent breast cancer. Based on the information provided by previously described compounds with anti-aromatase activity, four new steroidal compounds were synthesized with modifications on A-, B-, C- and D-rings of the androstenedione molecule.

In the present work, we pretended to:

- determine the anti-aromatase activity of the new potential AIs in ER^+ breast cancer cells.
- evaluate the anti-tumor efficacy of the new compounds by studying their biological effects and underlying mechanisms, using four cell lines: an hormone-dependent (ER^+) breast cancer cell line overexpressing aromatase (MCF-7aro), an hormone-independent (ER^-) breast cancer cell line (SK-BR-3); an AI-resistant breast cancer cell line overexpressing aromatase (LTEDaro); and a non-tumor fibroblastic cell line (HFF-1).

This study may provide some elucidation of the structural modifications that result into maximum inhibitory activity of new steroidal compounds as potential AIs and contribute to the design of more efficacious ones. Additionally, once the mechanisms are unraveled, it can help to achieve a better comprehension of the pathways involved in breast tumor progression/regression and in acquired resistance, enabling the discovery of new therapeutic targets.

CHAPTER II

Methods

1. MATERIALS

Eagles's minimum essential medium (MEM), DMEM medium, fetal bovine serum (FBS), l-glutamine, antibiotic-antimycotic (10 000 units/mL penicillin G sodium, 10 000 mg/mL streptomycin sulphate and 25 mg/mL amphotericin B), Geneticin (G418), sodium pyruvate, trypsin and 3,3-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) were supplied by Gibco Invitrogen Co. (Paisley, Scotland, UK). Testosterone (T), estradiol (E2), ethylenediaminetetracetic acid (EDTA), dimethylsulfoxide (DMSO), tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5- difenyltetrazolium (MTT), Höechst 33258, 3-methyladenine (3-MA), propidium iodide (PI), Triton X-100, DNase-free RNase A, staurosporine (STA), charcoal, carbonyl cyanide m-chlorophenylhydrazone (CCCP), 2',7'-dichlorodihydrofluorescein diacetate ($DCDHF_2$-DA), phorbol 12-myristate 13-acetate (PMA), acridine orange (AO), protease inhibitor cocktail and Fluoroshield mounting medium were from Sigma–Aldrich Co. (Saint Louis, USA). Giemsa solution was from Merck (Kenilworth, NJ, USA). DPX mounting medium was from VWR (Radnor, PA, USA). Cyto-Tox 96 nonradioactive cytotoxity assay kit and Caspase-Glo® 3/7 luminometric assay were from Promega Corporation (Madison, USA). [1β-^{3}H] androstenedione was obtained from Perkin-Elmer (Boston, MA, USA) and liquid scintillation cocktail Universol from ICN Radiochemicals (Irvine, CA, USA). Bradford assay reagent was from Bio-Rad (Laboratories Melville, NY, USA). Exemestane was from Sequoia Research Products Ltd. (Pangbourne, UK). Chemiluminescent substrate Super Signal West Pico was from Pierce (Rockford, USA). Rabbit polyclonal β-tubulin, goat polyclonal CYP19, goat anti-rabbit IgG and mouse anti-goat IgG antibodies were from (Santa Cruz Biotechnology, CA, USA). X-ray films were obtained from Kodak XAR (Eastman Kodak, Rochester, NY, USA).

2. COMPOUNDS

In this work, four potential steroidal AIs were evaluated. The compounds were obtained from structural modifications from androstenedione chemical structure and numbered according to the series of previously studied compounds by the group: **49**, **50**, **51**, **52**. All the compounds were synthesized by the Pharmaceutical Chemistry Group of the Faculty of Pharmacy, University of Coimbra and CNC.IBILI, University of Coimbra by the Profs. Carla Varela, Elisiário Tavares da Silva, Fernanda M.F. Roleira and Saul Costa.

3. PREPARATION OF THE AIs, TESTOSTERONE AND ESTRADIOL

The AIs were dissolved in DMSO and stored at -20 ^{o}C. Testosterone (T) and estradiol (E2) were prepared in absolute ethanol at 10 μM and stored at -20 ^{o}C. For each assay, appropriate dilutions were prepared in culture medium to obtain the final working concentrations. Final concentrations of DMSO and ethanol were less than 0.05% and 0.01%, respectively.

4. CELL CULTURES

The hormone-dependent (ER^+) aromatase-overexpressing human breast cancer cell line, MCF-7aro, was prepared by stable transfection of MCF-7 cells with human placental aromatase gene and Geneticin selection (171, 172), kindly provided by Dr. Shiuan Chen (Beckman Research Institute, City of Hope, Duarte, CA, U.S.A.). Cells were maintained in 75 cm^2 culture flasks with Eagles's minimum essential medium (MEM) with phenol-red supplemented with Earle's salts, 1% of sodium pyruvate (1 mmol/L), 2 mmol/L of glutamine, 1% penicillin-streptomycin-amphotericin B, Geneticin (G418) 700 ng/mL and 10% heat-inactivated fetal bovine serum (FBS). Three days before starting the experiments, MCF-7aro cells were cultured in estrogen-free MEM without phenol-red, containing 2 mmol/L of L-glutamine, 1% of sodium pyruvate (1 mmol/L), 1% penicillin-streptomycin-amphotericin B and 5% pre-treated charcoal heat-inactivated fetal bovine serum (CFBS), to avoid estrogenic effects of phenol-red (173) and prevent the interference of steroids present in FBS.

The ER^- human breast cancer cell line, SK-BR-3 (ATCC®), was maintained in MEM with phenol-red, supplemented with Earle's salts, 1% of sodium pyruvate (1 mmol/L), 1% penicillin–streptomycin–amphotericin B, 2 mmol/L of glutamine and 10% heat-inactivated fetal bovine serum (FBS).

The long-term estrogen deprivation cell line, LTEDaro, was generated by prolonged culture of parental MCF-7aro cells in steroid-depleted medium (174, 175) and also kindly provided by Dr. Shiuan Chen (Beckman Research Institute, City of Hope, Duarte, CA, U.S.A.). Cells were cultured in the same conditions as MCF-7aro cells but with MEM without phenol red and with 10% of pre-treated charcoal heat-inactivated fetal bovine serum (CFBS).

The cell line of human foreskin fibroblasts, HFF-1 (ATCC®), was maintained in DMEM without phenol-red, 10% heat-inactivated FBS, 1% sodium pyruvate (1 mmol/L) and 1% penicillin-streptomycin-amphotericin B.

All cells were grown in 5% CO_2 at 37 ^{o}C. After reaching about 80% of confluence, cells were successively sub-cultured to new culture flasks. For this, cells were detached with 2.5%

trypsin/EDTA 1mM for 2 minutes at 37 ^{o}C, washed with PBS and collected to centrifuge tubes with culture medium containing FBS/CBFS, to inactivate trypsin. Cells were centrifuged at 1200 *g* for 5 minutes at 4 ^{o}C. Then, the cells were counted in a Neubauer chamber and cultured. Culture medium and drugs were refreshed every three days.

5. PREPARATIONS OF CHARCOAL HEAT-INACTIVATED FETAL BOVINE SERUM (CFBS)

The FBS was inactivated for 1 h at 56 ^{o}C. To remove steroids, FBS was incubated with activated charcoal for 24 h at room temperature. After incubation, a series of successive centrifugations were performed during 15 minutes at 4000 *g*. Between each centrifugation, supernatant was filtered to eliminate charcoal particles. Centrifugations were performed until CFBS was clear from charcoal. After the final centrifugation, supernatant was filtered by a vacuum filter system of 0.22 µm pore and aliquoted.

6. IN CELL AROMATASE ASSAY

Aromatase activity in cells treated with the different steroidal compounds under evaluation was quantified through the measure of tritiated water released during the aromatization process, according to Thompson and Siiteri (176) and Zhou et al. (171), using [1β-^{3}H] androstenedione as substrate.

MCF-7aro cells were plated in a 24-well plate at a cellular density of 1 x 10^{6} cells/mL. After 3 days of culture, confluent cells were washed twice with PBS and then incubated with 500 nM of progesterone, 50 nM [1β-^{3}H] androstenedione, 10 µM of the different compounds and serum-free culture medium at a final volume of 500 µL. The [1β-^{3}H] androstenedione was the final component to be added to the mixture. Cells were incubated for 1 h at 37 ^{o}C in 5% CO_2. Reaction is stopped by the addition of 100 µL of 20% trichloroacetic acid. Supernatants were further transferred to previously prepared microcentrifuge tubes containing an activated charcoal pellet (to adsorb non-reactive substrate) and incubated at room temperature for 1 h, followed by a centrifugation of 10 minutes at 14000 *g*. Supernatants were transferred to new microcentrifuge tubes with activated charcoal pellet and again incubated for 10 minutes at room temperature, followed by a new centrifuge cycle. A final centrifugation cycle was performed with the supernatants in clean microcentrifuge tubes for 10 minutes to clear any charcoal that might be present. Supernatants containing tritiated water were mixed with 3 mL of liquid scintillation cocktail in new tubes and

counted in the scintillation counter. Untreated cells were used as control and cells treated with 1 µM of formestane were considered as positive control.

To extract proteins for quantification, the cells that remained in the plate were incubated with 500 µL of NaOH 0.5 M overnight at room temperature. After incubation, cells were freezed at -80 ^{o}C to lysate. The protein content of the cells was then quantified by the Bradford assay and used to normalize the radioactivity determined per µg of protein.

For the preparation of the above mentioned charcoal pellets, it was added 1 mL of 5% activated charcoal solution to microcentrifuge tubes followed by a centrifugation at 14000 *g* for 10 minutes. After removing the supernatant, pellets were left to dry at 37 ^{o}C overnight.

All experiments were carried out in triplicate in three independent experiments and results were expressed in aromatase activity as a percentage of the untreated cells.

7. CELL VIABILITY ASSAY

To evaluate the effects of each steroidal compound (**49**, **50**, **51** and **52**) on cell viability, tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed on MCF-7aro, LTEDaro, SK-BR-3 and HFF-1 cell lines. This is a colorimetric assay that relies on the mitochondrial metabolism and conversion of a yellow dye, MTT, on a purple dye, formazan, by viable cells. Therefore, this assay gives a percentage of viable/living cells that can convert MTT to formazan, comparing the treated groups with the control group. Cells were cultured in 96-well plates and, 24 h later, treated with different concentrations of each compound (1, 5, 10, 25 and 50 µM). Untreated cells were used as control. MCF-7aro cells were cultured at a cellular density of 2 x 10^4 cells/mL (for 2 and 3 days) and 1 x 10^4 (for 6 days) in MEM without phenol-red containing 5% CFBS plus 1 nM of T, 1 nM of E2 or 1nM of T plus 1 mM of 3-methyladenine (3-MA), according to experiments. LTEDaro cells were cultured at a cellular density of 2.5 x 10^4 cells/mL (for 2 and 3 days) and at 1 x 10^4 (for 6 days) in MEM without phenol-red containing 10% CFBS. SK-BR-3 cells were cultured at a cellular density of 2.5 x 10^4 cells/mL (for 3 days) and 1 x 10^4 (for 6 days) in MEM with phenol-red containing 10% FBS. HFF-1 cells were cultured at a cellular density of 1.5 x 10^4 cells/mL (for 3 days) and 7.5 x 10^3 (for 6 days) in DMEM without phenol-red containing 10% FBS. After treatment (2, 3 or 6 days), cells were incubated with MTT (0.5 mg/mL added to each well) for 2 h and 30 minutes at 37 ^{o}C in 5% CO_2. Then, DMSO:isopropanol mixture (3:1) was added to stop the reaction and cells were left for 15 minutes in agitation, to dissolve the formazan crystals. Finally, formazan was spectrophotometrically quantified at 540 nm.

To evaluate cytotoxic effects of each compound, lactate dehydrogenase (LDH) release assay was performed in MCF-7aro cell line. LDH, a commonly used biomarker for cytotoxicity and cytolysis, is a cytosolic enzyme that is only released from cells after membrane disruption. Therefore, this assay relies on the measurement of the LDH activity released from damaged cells into the culture medium by the use of CytoTox 96 nonradioactive cytotoxity assay kit, according to manufacturer's protocol. The culture conditions, cellular density and compound's concentrations used were the same as described previously for MTT assay.

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

8. WESTERN BLOT ANALYSIS

To quantify aromatase expression, MCF-7aro cells were cultured in 6-well plates at a cellular density of 6 x 10^5 cells/mL. After 3 days, cells were incubated with compound **49** or with exemestane (10 μM) for 8 h. After incubation, culture medium was removed and cells were washed twice with PBS. Then, cells were lysed with RIPA buffer (50 mM Tris-HCl, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, 0.5% Na-deoxycholate), pH 7.4, plus 1% of a protease inhibitors cocktail. Cells were scrapped and collected, following 3 freeze/thaw cycles. The cell lysates were centrifuged at 14000 *g* for 10 minutes at 4 ^{o}C and the protein concentrations in supernatants were determined using the Bradford assay. A total of 50 μg of protein per sample was subjected to 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes in 25 mM Tris-HCl, 250 mM glycine and 18% methanol. The membranes were blocked for 1 h and incubated with goat polyclonal anti-CYP19 (aromatase) antibody (1:100) in blocking solution overnight at -4 ^{o}C. Secondary mouse anti-goat IgG antibody (1:1000) was then incubated for 1 hour. Membranes were exposed to chemiluminescent substrate Super Signal West Pico and immunoreactive bands were visualized through X-ray films. Membranes were further stripped and incubated with rabbit polyclonal anti-β-tubulin antibody (1:500) followed by goat anti-rabbit IgG secondary antibody (1:1000), to control loading variations. Untreated cells were used as control.

All assays were performed in triplicate and in three independent experiments.

9. MORPHOLOGICAL STUDIES

The potential morphological alterations induced by each steroidal compound (**49**, **50**, **51** and **52**) were evaluated by phase contrast microscopy, Giemsa, Höechst and acridine orange (AO)

stainings. MCF-7aro cells were cultured in 24-well plates with coverslips at a cellular density of 2 x 10^5 (for 3 days) and 1 x 10^5 (for 6 days) in MEM without phenol-red containing 5% CFBS plus 1 nM of T. After 24 h, cells were treated with each steroidal compound at the concentrations of 10 and 25 µM. After treatment (3 or 6 days), cells were firstly observed under a phase contrast microscope (Eclipse E400, Nikon, Japan) equipped with image analysis software Nikon NIS Elements.

For Giemsa staining, cells were washed with PBS and fixed with methanol for 25 minutes at 4 ^{o}C. Then, cells were washed twice in PBS and stained with Giemsa solution, diluted in PBS (1:10) for 30 minutes. After, cells were washed under tap water and the coverslips with the stained cells were dehydrated and mounted with DPX mounting medium and observed under a bright field microscope (Eclipse E400, Nikon, Japan) equipped with image analysis software LeicaQwin.

For Höechst staining, cells were fixed with 4% paraformaldehyde, prepared in the same day, for 30 minutes at 4 ^{o}C. Then, cells were washed twice in PBS and exposed to 0.05 µg/mL Höechst 33258 staining for 20 minutes. After several washings, coverslips were mounted with Fluoroshield mounting medium. Cells were observed under a fluorescence microscope (Eclipse Ci, Nikon, Japan) equipped with an excitation filter with maximum transmission at 360/400 nm and images were processed by Nikon NIS Elements image software.

For AO staining, cells were incubated with 0.1 µg/mL of AO for 15 minutes. AO is a cell permeable acidotropic fluorochrome that stains DNA and cytoplasm in bright green, but once protonated at low pH vesicles/acidic compartments, it tends to accumulate and emit yellow/orange/red fluorescence. After staining, cells in coverslips were mounted with PBS, observed under fluorescence microscope (Eclipse Ci, Nikon, Japan) equipped with an excitation filter with maximum transmission at 360/400 nm and images were processed by Nikon NIS Elements image software.

10. CELL CYCLE ANALYSIS

To investigate the anti-proliferative effects of the compounds, cell cycle analysis was performed in MCF-7aro cells, using flow cytometry. Cells were cultured in 6-well plates at a cellular density of 7 x 10^5 cells/mL in MEM without phenol-red containing 5% CFBS. After 24 h, cells were treated with each compound (10 µM) plus 1 nM of T for 3 days. Untreated cells plus T were used as control. After treatment, adherent and non-adherent cells were harvested using 2.5% trypsin/EDTA 1mM and transferred to separate centrifuge tubes containing 1 mL of culture medium with 5% CFBS. Cells were centrifuged at 1200 *g* for 6 minutes at 4 ^{o}C and supernatant was rejected. Cells were resuspended and fixed in 70% cold ethanol and stored at 4 ^{o}C for 24 h. Fixed cells were centrifuged

(1200 *g*, 6 minutes, 4 °C) and washed with PBS, followed by a new centrifugation cycle. Finally, cells were resuspended in a DNA staining solution of 5 µg/mL of PI, 0.1% Triton X-100, 200 µg/mL DNase-free RNase A in PBS, to a final volume of 500 µL, and incubated overnight at 4 °C. Triton X-100 permeabilizes the fixed cells membrane; DNase-free RNase A degrades RNA present in the samples to avoid its interference with the analysis; PI is a fluorescent dye that intercalates nucleic acids, enabling DNA content determination. Flow cytometric analysis was performed based on the acquisition of 40 000 events/cells in BD Accuri™ C6 cytometer (San Jose, CA, U.S.A), equipped with BD Accuri™ C6 analysis software. The *forward scatter* (FSC) and *side scatter* (SSC) detectors and the three fluorescence channels (FL-1 (green), FL-2 and FL-3 (red)) were set on a linear scale. The results were indicated by the percentage of cells in the different cell cycle phases.

All assays were performed in triplicate and in three independent experiments.

11. CELL DEATH ANALYSIS

11.1 CASPASE ACTIVITY

To investigate the occurrence of apoptosis, caspase-7 activity, an effector caspase of the apoptotic process, was measured in MCF-7aro cells treated with the compounds. Cells were cultured in 96-well plates at a cellular density of 2 x 10^4 cells/mL in MEM without phenol-red containing 5% CFBS. After 24 h, cells were treated with each compound (10 µM) plus 1 nM of T for 3 days. After treatment, a Caspase-Glo® 3/7 luminometric kit was used according to manufacturer's instructions. As positive control, cells were treated with staurosporine (STA) (10 µM) for 3 hours and untreated cells plus 1 nM of T were used as control. Luminescence was measured in a 96-well microplate luminometer (BioTeK Instruments, USA) and results were presented as relative light units (RLUs).

All assays were performed in triplicate and in three independent experiments.

11.2 MITOCHONDRIAL TRANSMEMBRANE POTENTIAL ($\Delta\Psi_M$)

To study ΔΨm and a possible involvement of the mitochondrial pathway in apoptosis, a flow cytometric analysis was performed in MCF-7aro cells. Cells were cultured in 6-well plates at a cellular density of 7 x 10^5 cells/mL in MEM without phenol-red containing 5% CFBS. After 24 h, cells were treated with each compound (10 µM) plus 1 nM of T for 3 days and trypsinized as described above for cell cycle. After two centrifugation cycles, cells were incubated with 40 nM of 3,3-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) in 500 µL of PBS during 30 minutes at 37 °C. Cells

underwent a final centrifugation before being stained with PI (5 µg/mL), diluted in PBS at a final volume of 500 µL, allowing the discrimination between live cells ($DiOC_6$(3)+/PI-), early apoptotic cells ($DiOC_6$(3)-/PI-) and late apoptotic/necrotic cells (PI+). Cells were filtered and subjected to the flow cytometer. Untreated cells plus T were cultured as control. As a positive controls, cells were incubated with 10 µM of carbonyl cyanide m-chlorophenylhydrazone (CCCP), a depolarizing agent, for 5 minutes before $DiOC_6$(3) staining. Flow cytometric analysis was performed based on the acquisition of 40 000 events/cells in BD Accuri™ C6 cytometer (San Jose, CA, U.S.A), equipped with BD Accuri™ C6 analysis software. The *forward scatter* (FSC) and *side scatter* (SSC) detectors were set on a linear scale, while logarithmic detectors were used to measure $DiOC_6$(3) at green fluorescence channel FL-1 and PI at red fluorescence channel FL-3.

All assays were performed in triplicate, in two independent experiments.

11.3 Intracellular Reactive Oxygen Species (ROS)

To detect the production of intracellular ROS, MCF-7aro cells were cultured in 96-well dark plates at a cellular density of 2 x 10^4 cells/mL in MEM without phenol-red containing 5% CFBS. After 24 h, cells were treated with each compound (10 µM) plus 1 nM of T for 3 days. After treatment, cells were incubated for 1 h at 37 ^oC with 50 µM of 2',7'-dichlorodihydrofluorescein diacetate ($DCDHF_2$-DA), a non-fluorescent cell permeable probe that is oxidized intracellularly into a highly fluorescent compound in the presence of ROS. Fluorescence was measured using an excitation wavelength of 485 nm and an emission filter of 528 nm, in a 96-well microplate luminometer (Synergy HT, BioTek, USA). Results were presented as mean fluorescence intensity (MFI).

As positive control, cells were incubated with phorbol 12-myristate 13-acetate (PMA) at 25 ng/ml for 3 h before measurement. Untreated cells plus 1 nM of T were considered as control.

All assays were performed in triplicate and in three independent experiments.

12. Statistical Analysis

Statistical analysis of data was performed using analysis of variance (ANOVA) test followed by Tukey test for multiple comparisons in GraphPad Prism 6 software. Values of $P < 0.05$ were considered statistically significant. The data presented were expressed as the mean ± SEM (standard error of the mean).

CHAPTER III

Results

1. EVALUATION OF AROMATASE INHIBITION

In order to evaluate the anti-aromatase activity of the new molecules, MCF-7aro cells were treated with the compounds, **49**, **50**, **51** and **52** and the tritiated water released during the aromatization reaction process was measured. All compounds revealed to be potent AIs, inducing a clear reduction in aromatase activity in this aromatase overexpressing breast cancer cell line (Figure 13).

Compound **49** appears to be the less potent AI (72% of inhibition), while **52** is the most potent one, with 96% of inhibition, similar to the inhibition of the reference AI used, formestane (95%). Both **50** and **51** compounds induced a mean inhibition of 88%. All four AIs were selected to proceed with further biological studies.

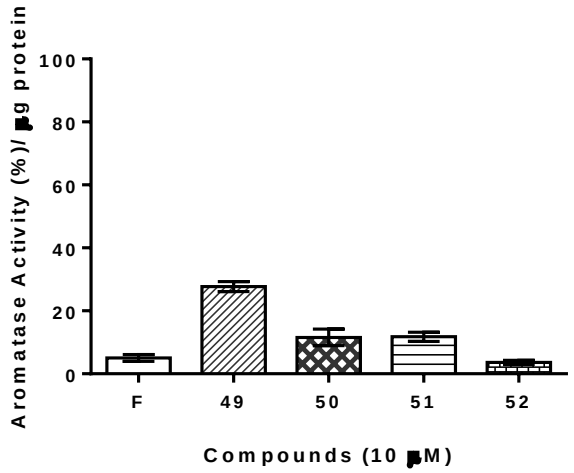


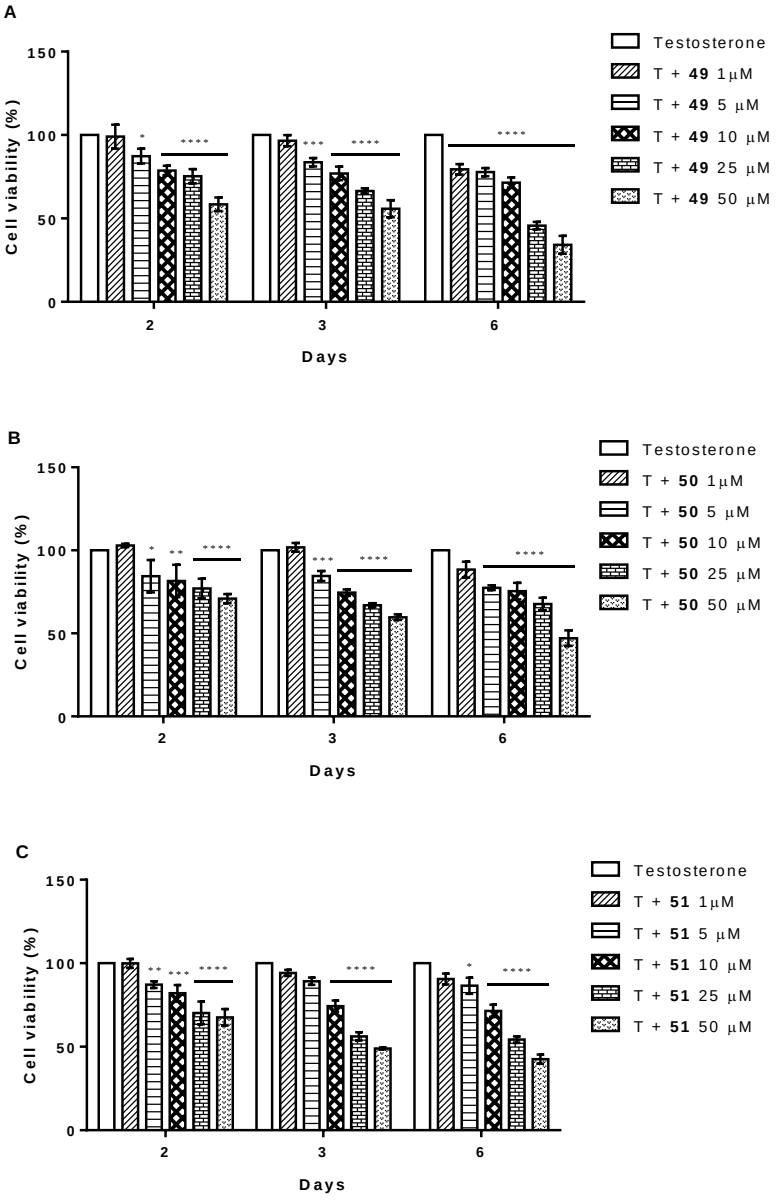
Figure 13. Aromatase activity in MCF-7aro cells. Cells were treated with the steroidal compounds (**49, 50, 51, 52**) at 10 µM. Formestane (F) was used as a reference AI. Data are presented as a percentage of the tritiated water of control cells (100%) and correspond to three independent experiments performed in triplicate.

2. CELL VIABILITY ASSAY IN MCF-7ARO CELL LINE

To investigate the effects induced by the AIs in the viability of MCF-7aro cells, MTT and LDH assays were performed. A point to take into account is that MCF-7aro cells were cultured in the absence of steroids (MEM without phenol-red with 5% CFBS) during all experiments. So, in order to study AIs effects, testosterone (T) was added as an aromatase substrate and proliferating induction agent, to better mimic the human model of hormone-dependent breast cancer environment. Thus, MCF-7aro cells were treated with the new steroids at different concentrations

(1-50 µM) and different times (2, 3 and 6 days), plus 1 nM of T. Cells only cultured with T were considered as control (100% of cell viability).

Results from MTT viability assay showed that, along with their anti-aromatase activity, all the compounds reduced the viability of these tumor cells in a dose- and time-dependent manner (Figure 14). This reduction was more pronounced for compound **52**.



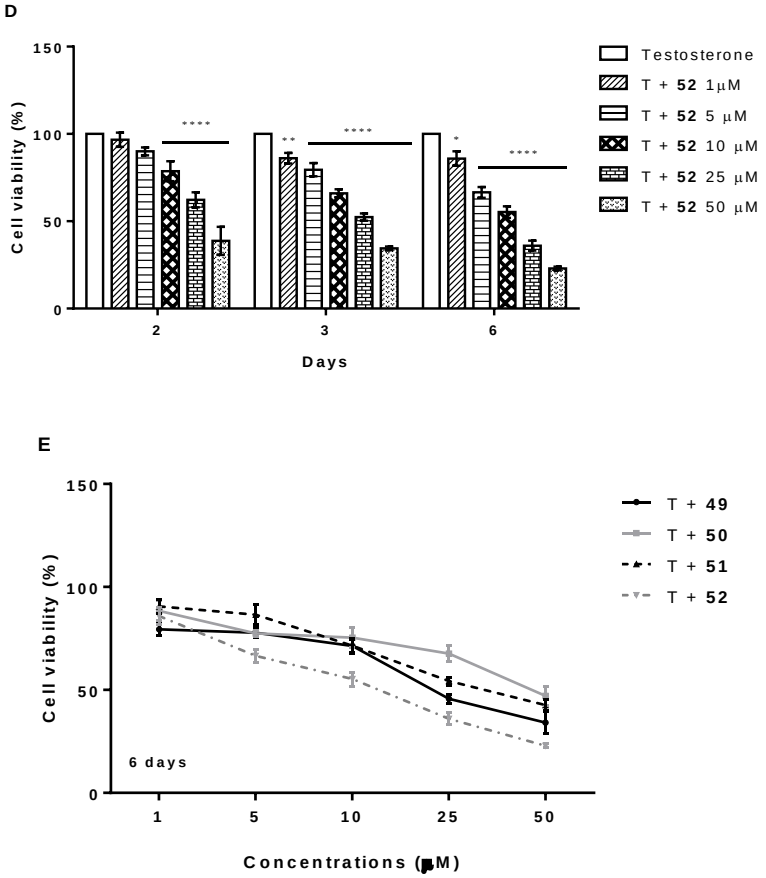


Figure 14. Effects of the compounds on viability of MCF-7aro cells. Cells treated with the compounds **49** (A), **50** (B), **51** (C) and **52** (D) (1-50 µM) plus T (1 nM), during 2, 3 and 6 days. (E) Comparison of the effects on cell viability induced by the four AIs (1-50 µM) after 6 days. Results are the mean ± SEM of three independent experiments performed in triplicate. Significant differences between the control T versus treated cells are indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

Along with MTT assays, LDH assays were performed to discard eventual cytotoxic effects of the compounds and the activity of LDH released from disrupted/necrotic cells was measured (Figure 15).

Results showed that none of the compounds promoted LDH release on MCF-7aro cells, except for AI **50** at 50 µM, suggesting that at the highest concentration, this compound can induce necrosis. So, in this case, this concentration was no longer used for other experiments with this AI in MCF-7aro cells.

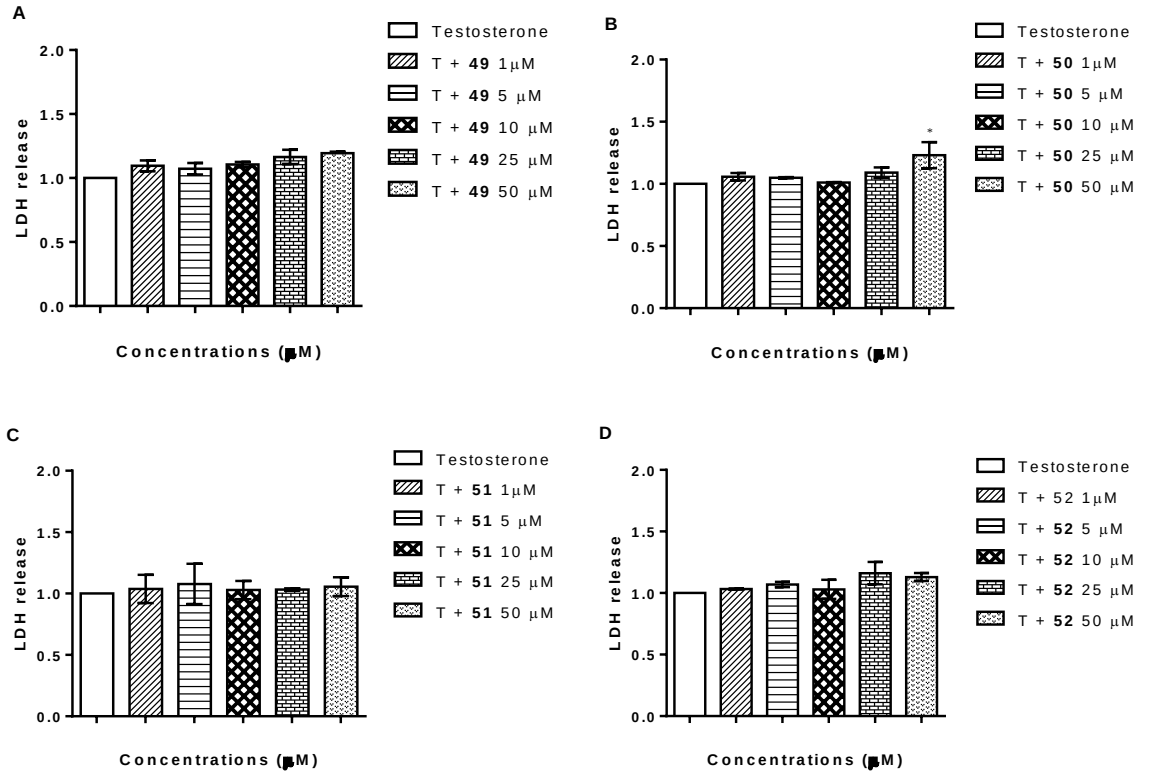


Figure 15. LDH released from MCF-7aro cells. Cells were treated with the compounds **49** (A), **50** (B), **51** (C) and **52** (D) (1-50 µM) plus T (1 nM), during 3 days. Cells cultured with T were considered as control. Results are the mean ± SEM of three independent experiments performed in triplicate. Significant differences between the control T versus treated cells are indicated by * ($p < 0.05$).

3. CELL VIABILITY ASSAY IN HFF-1 CELL LINE

It was previously shown that all the compounds are potent AIs that induce a reduction in the viability of an ER^+ breast cancer cell line, without causing cytotoxicity. However, the compounds should not act on non-cancerous cells in order to be adequate for cancer treatment. In this way, cell viability assays were also performed on a non-tumor fibroblastic cell line (HFF-1) (Figure 16).

Results showed that none of the studied AIs affected the viability of HFF-1 cells, except for compound **49** at 50 µM after 6 days of treatment, so this concentration was no longer used for this compound in further experiments. For compound **52**, a small but significant increase in fibroblast's viability was observed after 3 days of treatment at 1 µM but this potential proliferative effect was surpassed after 6 days of treatment.

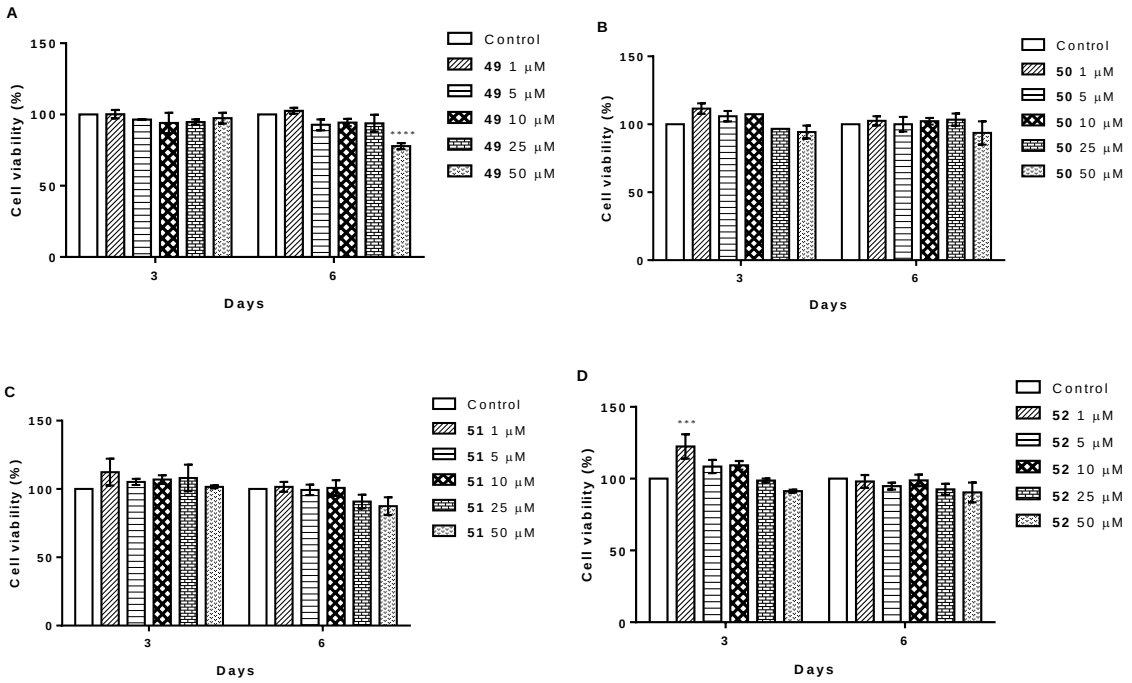


Figure 16. Effects of the compounds on viability of HFF-1 cells. Cells were treated with the compounds **49** (A), **50** (B), **51** (C) and **52** (D) (1-50 μM) during 3 and 6 days. Results are the mean ± SEM of three independent experiments performed in triplicate. Significant differences between the control versus treated cells are indicated by *** ($p < 0.001$).

4. CELL VIABILITY IN T-TREATED VERSUS E2-TREATED MCF-7ARO CELLS

To address the question whether the reduction in cell viability in MCF-7aro was related to aromatase inhibition and consequent lack of estrogens, the effects of the compounds were then evaluated in the presence of 1 nM of E2 (E2-treated cells), for the same period of time and concentrations as for the cells stimulated with T (T-treated cells). Cell viability of E2-treated cells versus T-treated cells was compared (Figure 17).

For AI **49**, results showed significant differences between the conditions used. The reduction on cell viability was more marked in T-treated cells when compared to the ones in the presence of E2. However, for AIs **50**, **51** and **52**, results demonstrated that, despite the presence of estrogen, the effects on cell viability were similar to the ones in the presence of the enzyme substrate. These results suggest that AI **49** effects on MCF-7aro were aromatase-dependent, while the other AIs caused aromatase-independent effects.

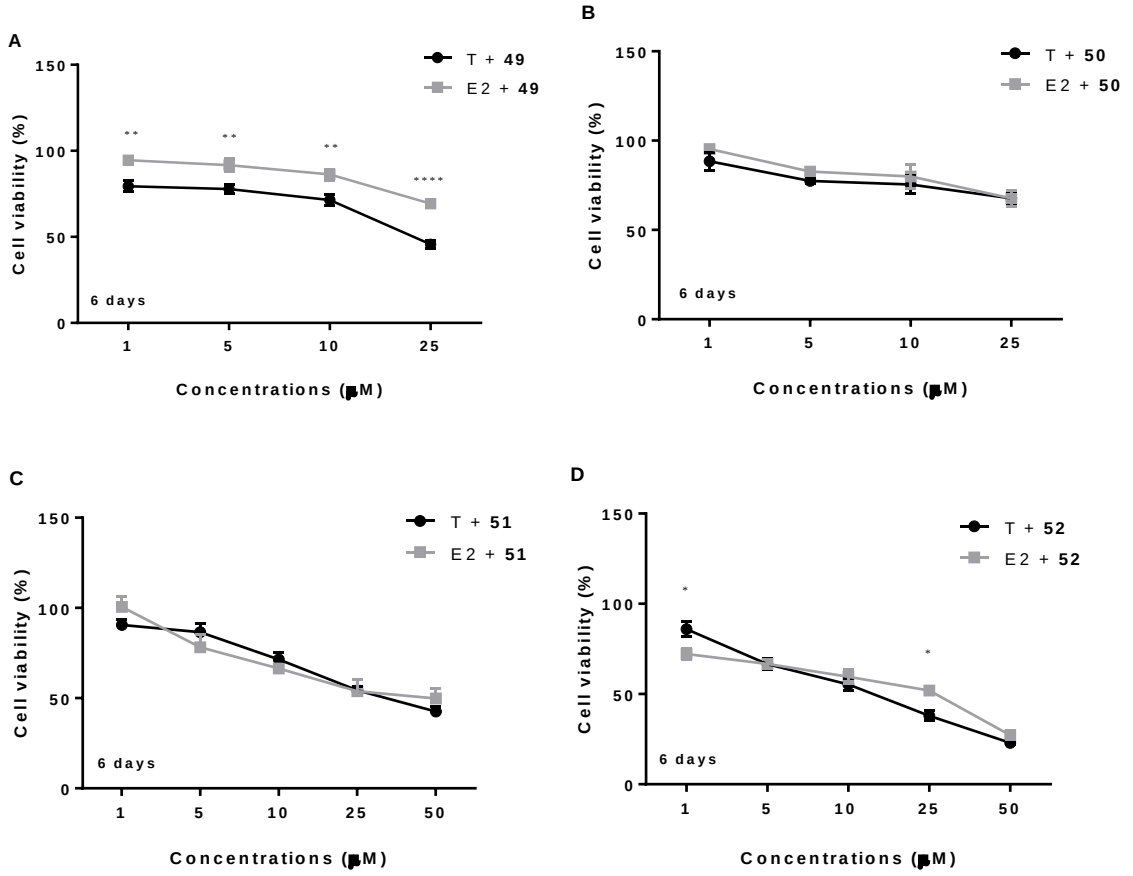


Figure 17. Comparison of the effects of the compounds on viability of MCF-7aro cells treated with T or E2. Cells were treated with the compounds **49** (A), **50** (B), **51** (C) and **52** (D) (1-50 µM) plus 1 nM of T or E2, during 6 days. Cells cultured with T or E2 were considered as control. Cell viability of treated cells plus T (black line) or E2 (grey line) is represented as a percentage of untreated cells with T or E2 Results are the mean ± SEM of three independent experiments performed in triplicate. Significant differences between the treated cells plus T versus the treated cells plus E2 are indicated by * ($p < 0.05$), ** ($p < 0.01$) and **** ($p < 0.0001$).

5. CELL VIABILITY IN E2-TREATED MCF-7ARO CELLS VERSUS SK-BR-3 CELLS

To evaluate if the biological effects of the different steroids were ER-dependent, an ER-negative (ER^-) cell line, SK-BR-3, was used and the effects on cell viability were compared to E2-treated MCF-7aro cells.

As shown in Figure 18, results demonstrated that all the compounds induced a reduction in viability of SK-BR-3 cells in a similar manner as MCF-7aro cells plus E2, suggesting that these effects occur through an ER-independent manner.

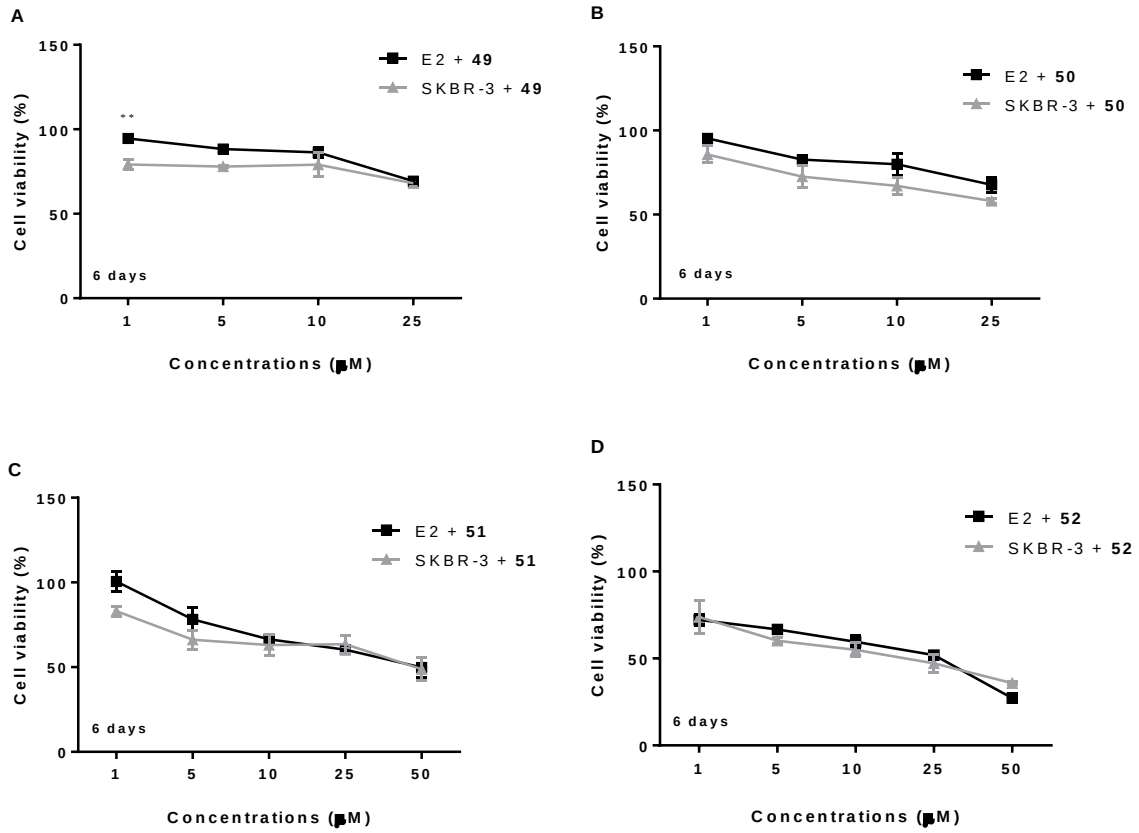


Figure 18. Comparison of the effects of the compounds on the viability of MCF-7aro cells + E2 and SK-BR-3 cells. Cells were treated with the compounds **49** (A), **50** (B), **51** (C) and **52** (D) (1-50 µM) during 6 days. Cell viability of treated MCF-7aro cells plus E2 (black line) and treated SK-BR-3 cells (grey line) is represented as a percentage of untreated cells (plus E2 for MCF-7aro cells). Results are the mean ± SEM of three independent experiments performed in triplicate. Significant differences between treated MCF-7aro cells plus E2 versus treated SK-BR-3 are indicated by ** ($p < 0.01$).

6. AROMATASE EXPRESSION ON MCF-7ARO CELL LINE

As compound **49** effects are, like exemestane, aromatase-dependent, it was investigated if it induces inactivation and degradation of the enzyme, as the steroidal AI used in clinic. The expression of aromatase on AI **49**-treated cells was analyzed by Western Blot (Figure 19).

It was possible to observe a clear reduction on aromatase expression in treated cells. The AI **49** presented a reduction of 34% in aromatase expression, while exemestane, used as a positive control, reduced aromatase expression in 43%.

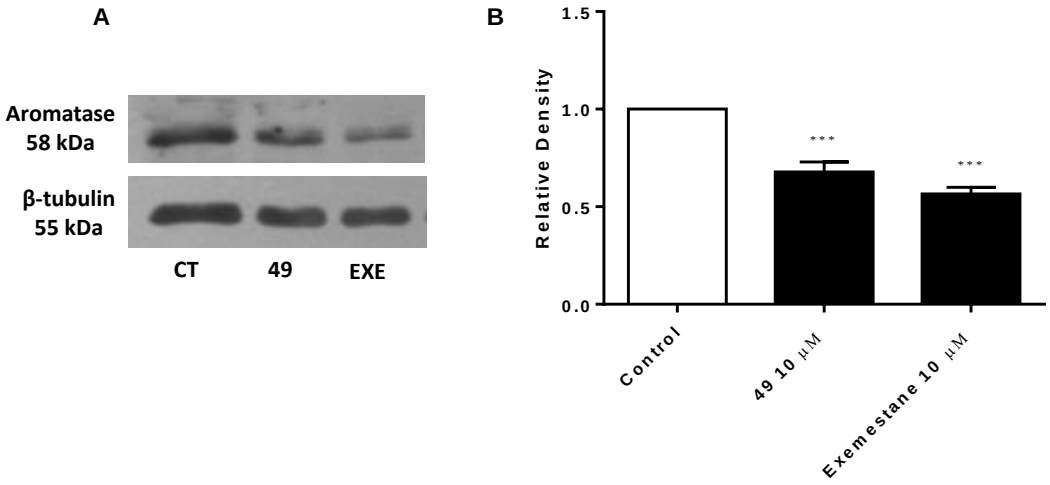


Figure 19. Western Blot analysis of aromatase expression. MCF-7aro cells were treated with the compound **49** and with exemestane (10 μM) for 8 h. β-tubulin was used as a loading control. Cells cultured with T were considered as control. (A) Representative Western blot for aromatase and β-tubulin. Results are a single representative of three independent experiments. (B) Densitometric analysis of aromatase protein levels after normalization to β-tubulin levels. Significant differences between the control T versus treated cells are indicated by *** ($p < 0.001$).

7. MORPHOLOGICAL STUDIES

To investigate whether the compounds induced morphological alterations, MCF-7aro cells treated with each steroid were firstly observed under a phase contrast microscope and then Giemsa and Höechst stainings were performed (Figure 20).

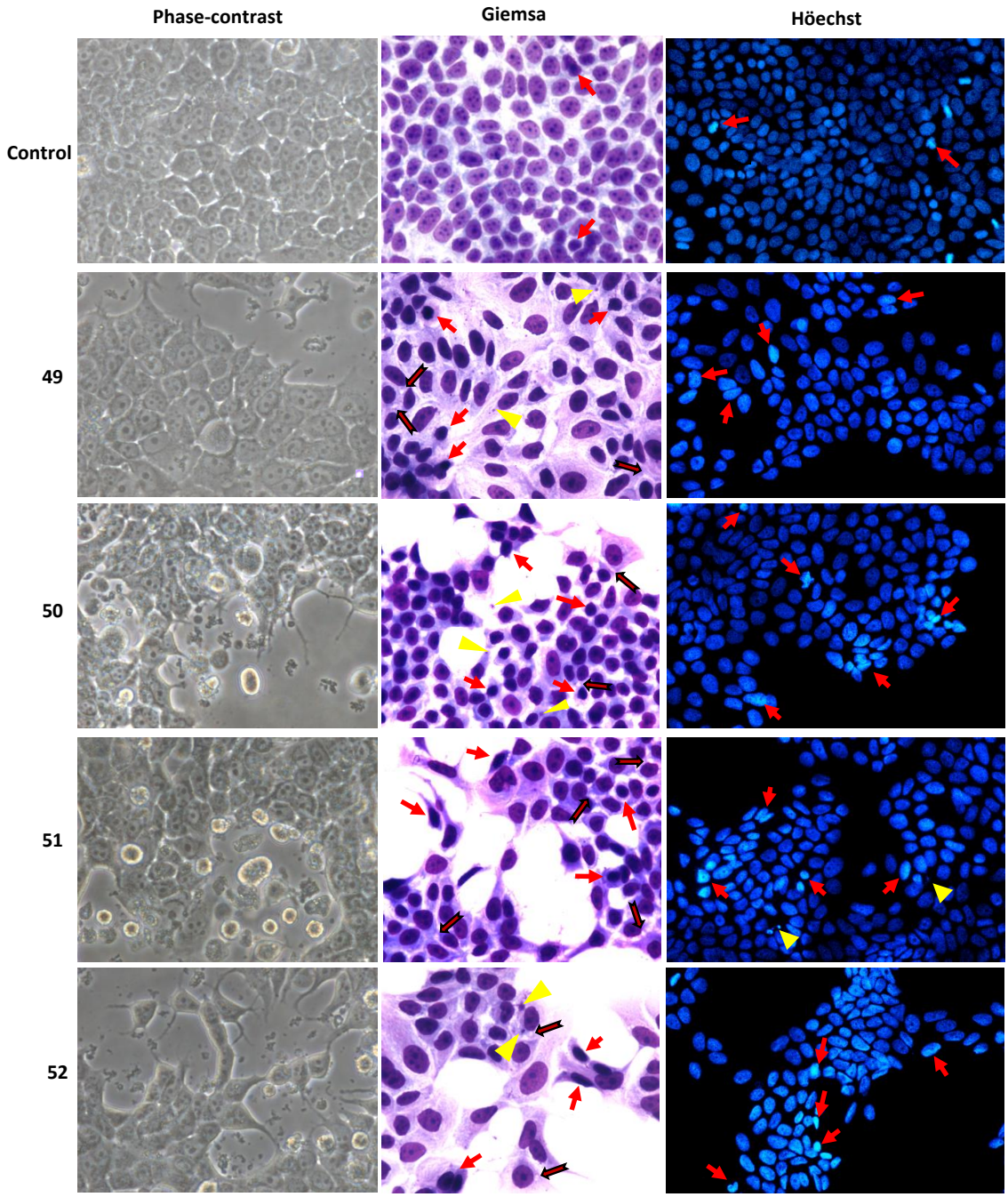


Figure 20. Effects of the compounds on MCF-7aro cells morphology. (A) Phase-contrast microscopy, (B) Giemsa staining and (C) Höechst staining. MCF-7aro cells morphology was analyzed in the absence (Control) or presence of each compound (**49**, **50**, **51** and **51**) at 10 µM for 6 days. Results are shown from a single representative of three independent experiments**.** Red arrows represent chromatin condensation, yellow arrowheads represent nuclear fragmentation and the dark red arrows with black outline point to cytoplasmic vesicles/vacuolization (original magnification x400).

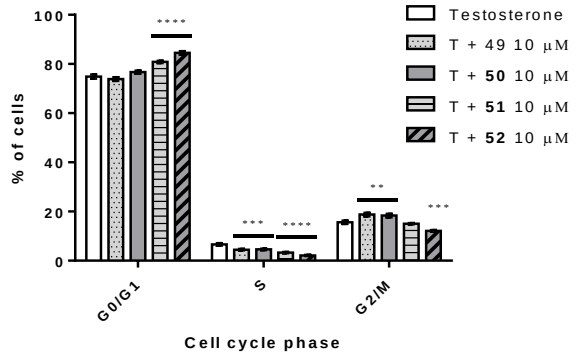
Treated cells presented morphological alterations. In phase contrast microscopy, it was possible to observe many swollen detached cells and membrane blebbing. Chromatin condensation and the presence of some vacuoles were detected with Giemsa staining. Höechst staining allowed to observe the nuclear morphology, confirming the presence of chromatin condensation and revealing some nuclear fragmentation, known apoptotic features. These alterations were accompanied by a reduction in cell density, especially for AI **52**. Results from 3 days of treatment were very similar, apart from cell density, to the 6 days and thus are not represented.

8. CELL CYCLE ANALYSIS

One of the possible mechanisms responsible for the effects of AIs on MCF-7aro cell viability is the deregulation of the cell cycle and, consequently, lack of proliferation. Cell cycle progression was analyzed by flow cytometry after PI staining. PI is a fluorescent dye that intercalates DNA and enables to determine its content within cells. According to DNA content, cells were distributed to the correspondent cell cycle phases. (Figure 21).

All of the AIs induced cell cycle arrest. However, for different AIs, different phases of the cycle were affected. For AIs **49** and **50**, there was a slight but significant increase in the number of cells in G_2/M phase, while for AIs **51** and **52**, the arrest in G_0/G_1 phase of the cell cycle was evident.

A



B

Cell cycle	G_0/G_1	S	G_2/M
Testosterone	74.85 ± 0.83	6.64 ± 0.48	15.63 ± 0,70
T + 49 10 µM	73.88 ± 0.72	4.49 ± 0.27***	18.77 ± 0.71**
T + 50 10 µM	76.68 ± 0.63	4.60 ± 0.28***	18.35 ± 0,70**
T + 51 10 µM	80.84 ± 0.47****	3.27 ± 0.25****	15.03 ± 0.22
T + 52 10 µM	84.50 ± 0.67****	2.16 ± 0.22****	12.16 ± 0.33***

Figure 21. Effects of the compounds on MCF-7aro cells cycle progression. Cells were treated with AIs **49**, **50**, **51** and **52** (10 µM) plus T (1 nM), during 3 days and subjected to flow cytometric analysis after PI staining. Cells cultured with T were considered as control. (A) Statistical analysis of the cell cycle phases distribution. (B) Data are presented as a percentage of single cell events in each phase of the cell cycle and represents means and SEM of triplicates and is representative of three independent experiments. (A) and (B) Significant differences between the control T versus treated cells are indicated by ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

9. CELL DEATH ANALYSIS

The reduction in MCF-7aro cell viability could also be due to cell death mechanisms and it was observed that AI-treated cells presented some apoptotic features. Therefore, apoptosis occurrence was investigated.

The activity of the effector caspase-7 was firstly assessed. As shown by Figure 22, treated cells presented an increase on caspase-7 activity, confirming apoptosis, with a more pronounced increment for cells treated with AIs **49** and **50**. Staurosporine (STA) was used as a positive control and, as expected, caused the activation of effector caspase-7.

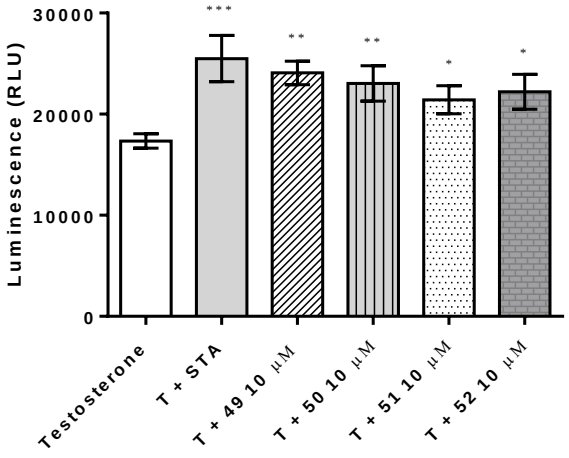


Figure 22. Effects of the compounds on MCF-7aro cells caspase-7 activity. Cells were treated with each AI plus T (1 nM) at 10 µM, during 3 days. Cells cultured with T were considered as control and staurosporine (STA) (10 µM) was considered as positive control for apoptosis. Caspase-7 activity is represented as relative luminescence units (RLU). All the results are mean ± SEM of three independent experiments performed in triplicate. Significant differences between the control T versus treated cells are indicated by * ($p < 0.05$) and ** ($p < 0.01$) and *** ($p < 0.001$).

In order to study the involvement of the mitochondria, the mitochondrial transmembrane potential (ΔΨm) was evaluated by flow cytometry (Figure 23), after $DiOC_6(3)$/PI staining. $DiOC_6(3)$ is a cell permeant, lipophilic and green-fluorescent dye that is incorporated by the mitochondria of living cells, due to their negative potential. Once cells lose their mitochondrial membrane integrity and their ΔΨm, $DiOC_6(3)$ is no longer incorporated. PI, which is cell impermeable, only accumulates in cells with disintegrated membrane (death cells). Thus, according to the incorporation of $DiOC_6(3)$/PI it was possible to identify three populations of cells: viable cells ($DiOC_6(3)^+/PI^-$), early-apoptotic depolarized cells ($DiOC_6(3)^-/PI^-$) and late-apoptotic/necrotic cells ($DiOC_6(3)^-/PI^+$). Only the populations of viable and depolarized cells were of interest for this analysis.

Preliminary studies revealed that all AI-treated cells presented a significant loss of ΔΨm. AI **49** induced a depolarization of 40%±3, while a depolarization of about 61%±4, 66%±5 and 63%±5 was

observed for compounds **50**, **51** and **52**, respectively. The positive control, CCCP, induced a marked membrane depolarization of 87%±2, as expected. Cells cultured with T were considered as control.

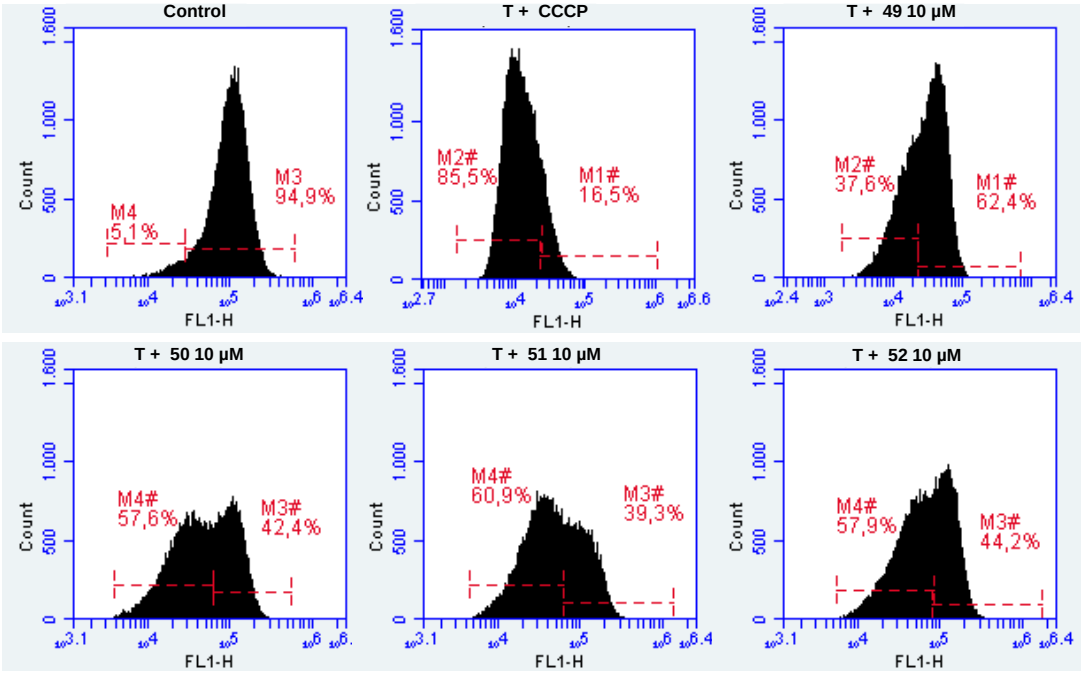


Figure 23. **Effects of the compounds on MCF-7aro cells mitochondrial transmembrane potential.** Cells were treated with AIs **49**, **50**, **51** and **52** (10 µM) plus T (1 nM), during 3 days and subjected to flow cytometric analysis after $DiOC_6(3)$ and PI staining. Cells cultured with T were considered as control and cells treated with CCCP (10 µM) were used as positive control. Data presented in histograms was analyzed with BD Accuri™ C6 software and is representative of one independent assay.

To further explore the underlying mechanisms of cell death, it was evaluated ROS production, which is often associated with mitochondrial dysfunction and oxidative stress.

ROS production in treated MCF-7aro cells was measured by a fluorescent assay, using $DCFH_2$-DA dye, a non-fluorescent cell permeable probe that is oxidized intracellularly into a highly fluorescent compound in the presence of ROS.

As shown in Figure 24, compounds **51** and **52** led to a 2-fold and 3-fold increase in ROS production, respectively. On the other hand, no significant differences were found in ROS formation for AIs **49** and **50**, compared to the control cells (T). The PMA and the steroidal AI exemestane were used as positive controls and, as expected, presented a 4-fold and 2-fold increase in ROS release, respectively, when compared to control cells.

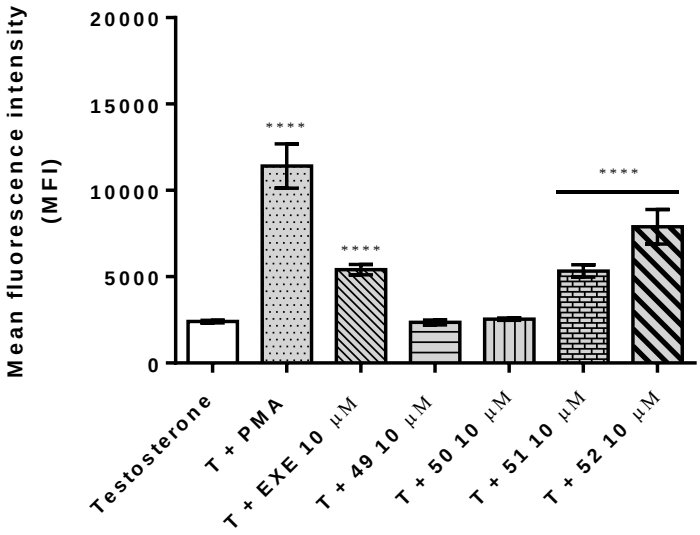


Figure 24. Effects of the compounds on MCF-7aro cells ROS production. Cells were treated with the each AI (10 µM) plus testosterone (T) (1 nM) for 3 days. Cells cultured with T were considered as control and cells treated with PMA (25 ng/mL) or exemestane (10 µM) were considered positive controls for ROS production. The results are presented as mean fluorescence intensity (MFI). Results are the mean ± SEM of one independent experiment performed in triplicate. Significant differences between the control T versus treated cells are indicated by **** ($p < 0.0001$).

10. ANALYSIS OF AUTOPHAGY

As Giemsa staining revealed the presence of some cytosolic vacuolization and as other steroidal compounds have the ability to induce autophagy in ER^+ breast cancer cell lines (166, 177, 178), a morphological study was made to monitor the formation of acid vesicular organelles (AVOs) in MCF-7aro cells. For this, cells were stained with the fluorochrome AO. This fluorochrome is cell permeable, but once protonated, it gets trapped and accumulated inside low pH vesicles, such as lysosomes and autophagosomes, emitting yellow/orange/red fluorescence. Cells were observed under a fluorescence microscope (Figure 25).

Only AI **51**-treated cells revealed the presence of AVOs in high amounts. For the other AIs, AVOs presence was similar to control (T).

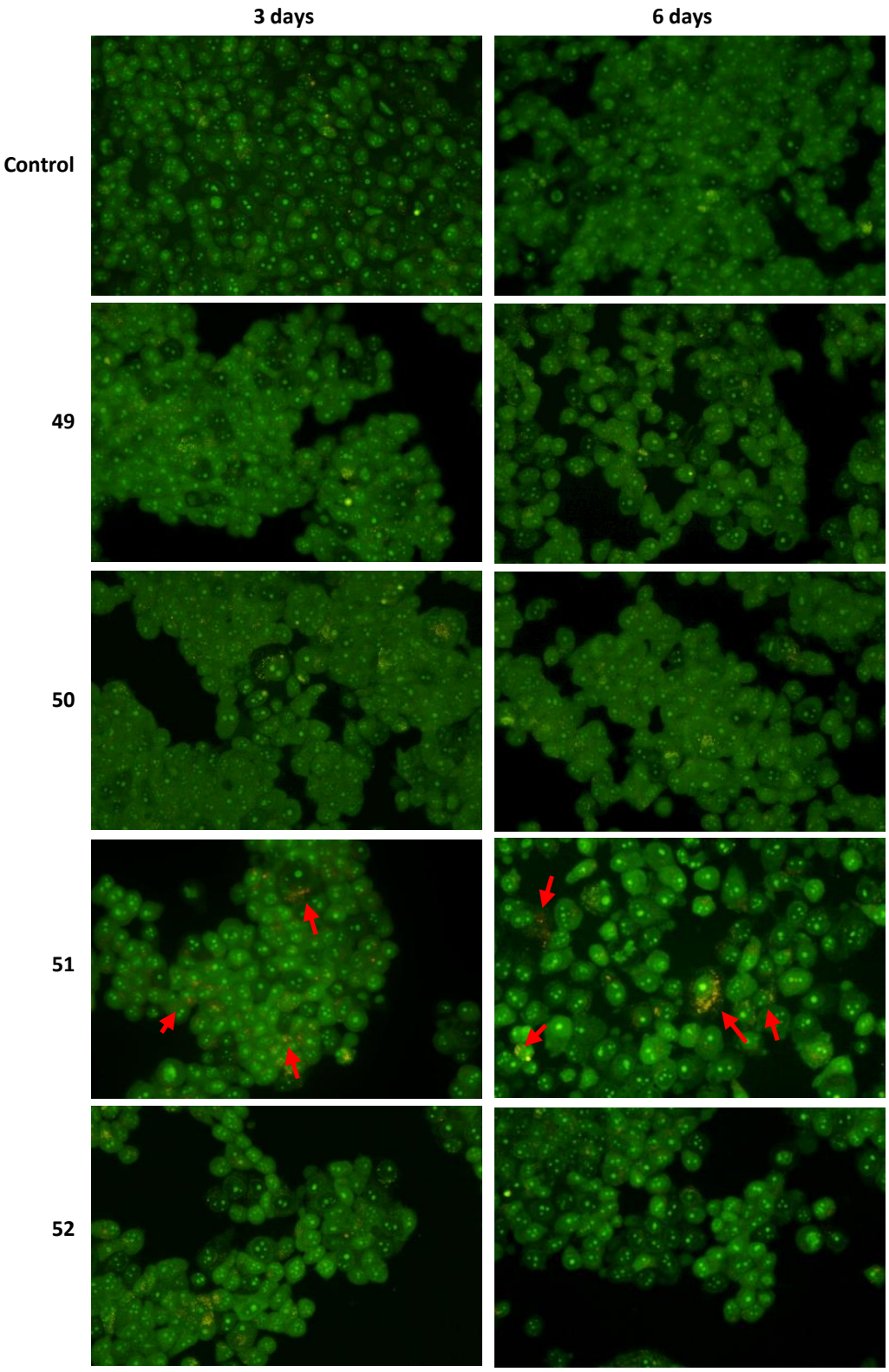


Figure 25. Effects in the formation of AVOs in MCF-7aro cells. Cells were treated with each AI (10 µM) plus T (1 nM), during 3 and 6 days and stained with AO. The presence of AVOs was indicated by the yellow/orange/red fluorescence. Cells cultured with T were considered as control. Results are shown from a single representative of three independent experiments (original magnification x400).

Additionally, the viability of MCF-7aro cells in the presence of an autophagic inhibitor, 3-methyladenine (3-MA), was measured and compared to the cells in the same conditions without 3-MA (Figure 26). Only AI **51**-treated cells presented a different behavior when autophagy was inhibited, at different concentrations, confirming the results obtained in fluorescence microscopy. In this case, autophagy inhibition caused a greater reduction in cell viability, suggesting that it was acting as a pro-survival mechanism for cells. Only at the highest concentration of compounds **49** and **50** was observed a significant difference in cell viability between the conditions used. However, for AI **49,** a more pronounced reduction in cell viability occurred in the absence of 3-MA, while for AI **51**, it was only after 3-MA treatment.

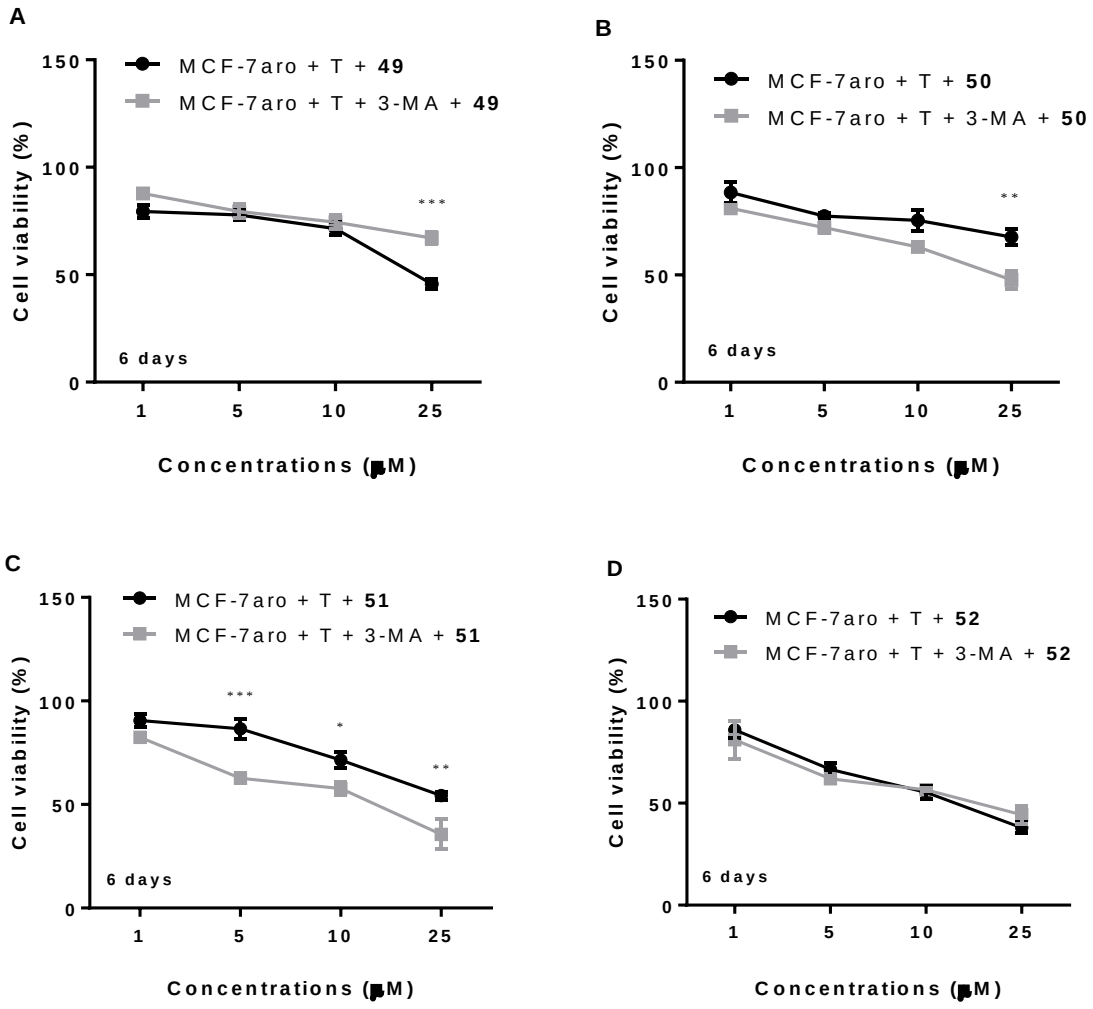


Figure 26. Comparison of the effects of the compounds on the viability of MCF-7aro cells treated with or without an autophagic inhibitor. Cells were treated with the compounds **49** (A), **50** (B), **51** (C) and **52** (D) (1-25 µM) plus T (1 nM) with or without 3-MA (1 mM), during 6 days. Cells cultured with T or T plus 3-MA were considered control. Cell viability of treated cells with (grey line) or without 3-MA (black line) is represented as a percentage of untreated cells with T or T plus 3-MA. All the results are mean ± SEM of three independent experiments performed in triplicate. Significant differences between treated cells versus treated cells plus 3-MA are indicated by * ($p < 0.05$) and ** ($p < 0.01$) and *** ($p < 0.001$).

11. CELL VIABILITY ASSAY IN LTEDARO CELL LINE

As therapy resistance represents one of the major drawbacks in AIs therapeutic efficacy, the biological effects of the new compounds were also investigated in an AI-resistant cell model, LTEDaro cell line. Cell viability of treated cells was assessed by MTT assay (Figure 27).

All the compounds induced a reduction in viability of LTEDaro cells in a dose- and time-dependent manner. As for the sensitive cell line, the most pronounced reduction in cell viability was, once more, observed for AI **52**.

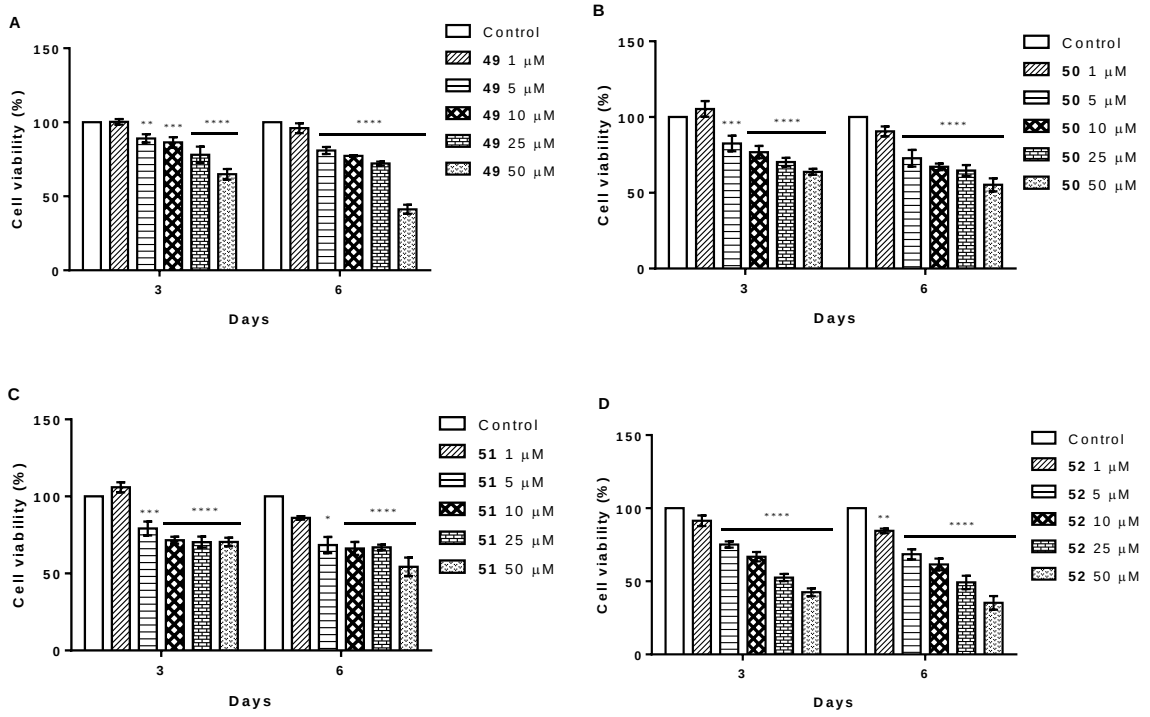


Figure 27. Effects of the compounds on viability of LTEDaro cells. Cells were treated with the compounds **49** (A), **50** (B), **51** (C) and **52** (D) (1-50 μM) during 3 and 6 days. Results are the mean ± SEM of three independent experiments performed in triplicate. Significant differences between the control versus treated cells are indicated by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

Furthermore, the results were compared to the ones obtained for MCF-7aro cells. It was observed that all the compounds produced a similar effect on the viability of both hormone-resistant and hormone-sensitive cells (Figure 28).

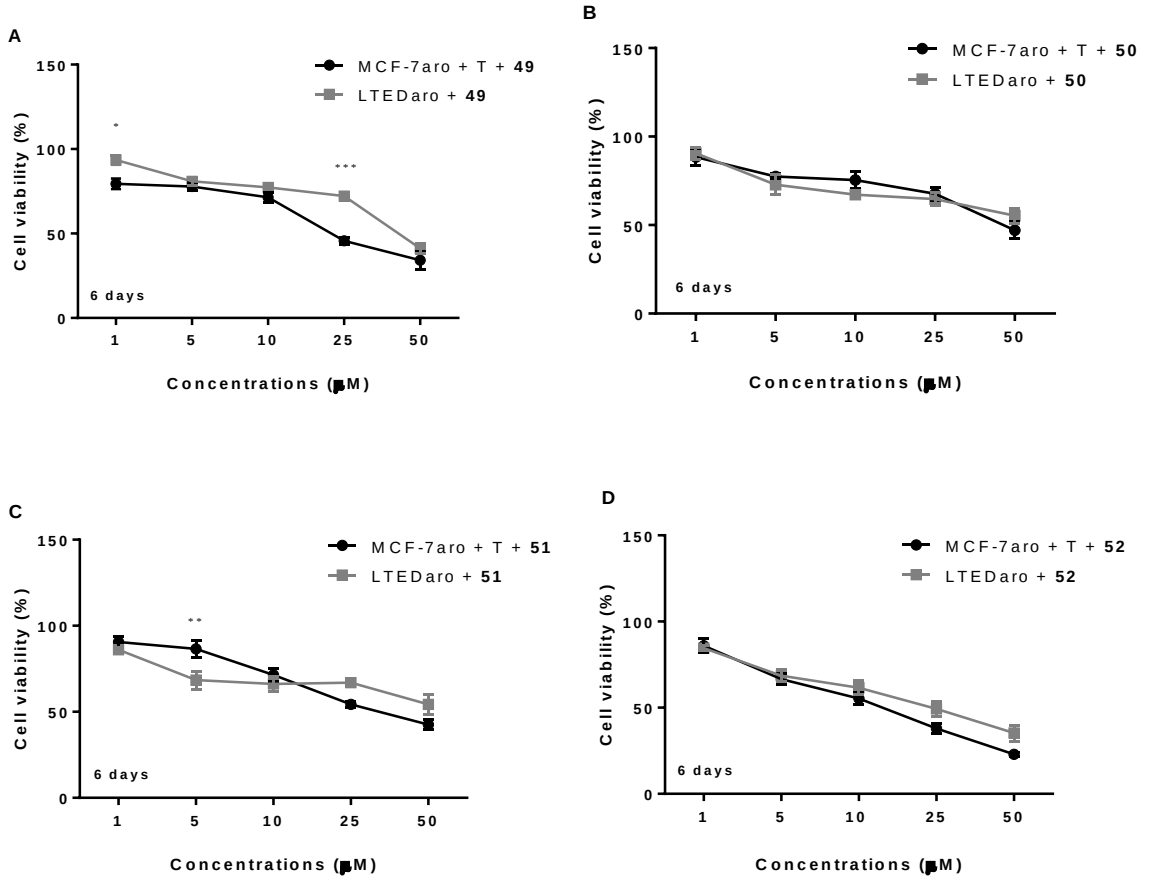


Figure 28. Comparison of the effects of the compounds on the viability of MCF-7aro and LTEDaro cells. Cells were treated with the compounds **49** (A), **50** (B), **51** (C) and **52** (D) (1-50 µM) during 6 days. Cell viability of treated MCF-7aro cells plus T (black line) or treated LTEDaro cells (grey line) is represented as a percentage of untreated cells (plus T for MCF-7aro cells). Results are the mean ± SEM of three independent experiments performed in triplicate. Significant differences between treated MCF-7aro cells plus T versus treated LTEDaro cells are indicated by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

CHAPTER IV

Discussion

Breast cancer is one of the most prevalent pathologies among women worldwide. It is estimated that 2/3 of breast tumors are hormone-dependent and require estrogen for its progression (179). Estrogen might play a part in tumor initiation and its role in cell proliferation is through the activation of ER, one of the major tumor driving forces. ER genomic/non-genomic actions leads to the activation of genes involved in proliferation and survival, suppressing others involved in cell death. This way, estrogen and ER became the major ER^+ breast cancer targets for therapy. For many decades, tamoxifen, an ER modulator, was the standard therapeutic option for pre- and postmenopausal women with breast cancer, until third-generation AIs arose in clinics. The AIs from third-generation proved their superiority over other endocrine agents, including tamoxifen, in several trials for postmenopausal women, for different therapeutic settings (123).

However, there are still drawbacks that limit their success, such as the development of resistance and bone loss. Although several attempts to overcome the disadvantages are being carried out by the combination of drugs, it is demanded the search for new compounds, more potent and with lower side effects. Many efforts have been made so far, with the characterization of several steroids with structural modifications on androstenedione scaffold (168-170) .

The focus of this study was the search for new steroidal compounds as potential AIs, with structural modification in all the rings of the androstenedione molecule. Their anti-aromatase efficacy was evaluated and their *in vitro* effects were explored, in order to unravel the underlying anti-tumor mechanisms.

Four compounds of a series of new steroids synthesized by our group, that presented an inhibitory activity higher than 70% in placental microsomes, were selected for this study.

The human breast cancer aromatase overexpressing cell line MCF-7aro was used for most of the studies, once it represents a good model to study AIs in ER^+ breast tumors (180). The anti-aromatase activity of the four new compounds in MCF-7aro cells was also superior to 70%, being **49** the less potent and **52** the most potent AI. Thus, all compounds were qualified to proceed with the biological studies.

The anti-tumor properties of the compounds were then explored through several assays. Cell viability studies demonstrated that all the compounds caused a reduction in the viability of MCF-7aro cells, being compound **52** the most potent one. Although compound **49** was the less effective in inhibiting aromatase activity, this was not translated into cell viability effects, presenting similar effects to AIs **50** and **51**. These effects on cell viability were also described for exemestane (178) and other AIs synthesized by the group (166, 167) . Furthermore, as an anti-tumor therapeutic agent should not affect non-cancerous cells, the effects of these new steroids in a non-tumor fibroblastic cell line, HFF-1, were also evaluated, without any relevant effect on their viability.

To understand if the reduction in MCF-7aro cells' viability was dependent on aromatase inhibition, and consequent estrogen suppression, or by ER interference, the effects of the compounds in the viability of MCF-7aro cells stimulated with the aromatase substrate, testosterone, were compared to the cells stimulated with the aromatase product, estradiol and the latter were also compared to the SK-BR-3 cell line. SK-BR-3 is a breast cancer cell line that does not expresses ERα but has all the enzymes necessary to estrogens production, representing a good model for hormone-independent (ER^-) breast cancer (181). Overall, it was possible to deduce that AI **49** effects are aromatase-dependent and ER-independent, while the other AIs act through an aromatase- and ER-independent manner, as seen for other compounds described by the group (166). However, the involvement of other mechanisms cannot be excluded. As compound **49** was the only that presented aromatase-dependent effects, it was investigated if it exhibited a similar mechanism of enzyme inhibition as the other steroidal AI used in clinic, exemestane. As previously referred, exemestane induces a reduction in aromatase expression, as it causes its inactivation and degradation by the proteasome (138). Aromatase degradation is probably responsible for exemestane's aromatase-dependent effects on breast cancer (138, 182). To clarify if the steroid **49** acts through a similar mechanism as exemestane, the expression of aromatase on AI **49**-treated MCF-7aro cells was analyzed by Western Blot. In fact, it was observed a decrease in aromatase expression, suggesting the involvement of an enzymatic degradation mechanism. Nevertheless, further studies must be performed to confirm this hypothesis.

Morphological alterations were observed in AI-treated cells, such as membrane blebbing, detached cells, chromatin condensation and nuclear fragmentation, features of apoptosis. Some vacuoles were also detected, indicating a possible involvement of an autophagic process. Similar morphological alterations were already described by our group, for other newly synthesized steroids (177, 178, 183). In addition, there was a clear reduction in cell density when compared to control (T), which might be due to lack of proliferation or cell death.

In order to clarify those mechanisms, the effects on MCF-7aro cells' proliferation and cell death were further explored. Studies revealed that the AIs letrozole, anastrozole, formestane (184) and exemestane (178), as well as other steroidal AIs previously described by the group (168, 177, 183), are capable of inducing cell cycle arrest of ER^+ breast cancer cell lines overexpressing aromatase. Therefore, it was important to understand if the disruption of cell cycle progression was one of the mechanisms induced by these new compounds. It was verified that all the AIs induced cell cycle arrest, indicative of anti-proliferative properties, in accordance to the previously referred studies. However, different phases of the cell cycle were affected by different compounds. AIs **49** and **50** arrested cell cycle progression in G_2/M phase. This cell cycle arrest is associated with enhanced apoptosis (185, 186). In fact, exemestane was shown to induce cell cycle arrest in G_2/M and cell

death by apoptosis (178). AIs **51** and **52** caused an arrest in G_0/G_1 phase of the cell cycle, which is mainly associated with growth inhibition. These different effects on cell cycle may be explained by their structural differences, that may trigger the activation of alternate cell cycle proteins. Nevertheless, in a general manner, it is possible to conclude that, at least in part, AIs impact on the viability of MCF-7aro cells is caused by anti-proliferative effects with a possible involvement of cell death mechanisms.

As previously referred, the morphological studies also pointed to the involvement of cell death mechanisms, namely apoptosis. Apoptosis is a physiologicaly programmed and organized induction of death by the cell's own signaling in response to certain stimuli, such as external drugs. Several described AIs (177, 183), including exemestane (178), anastrozole, letrozole and formestane (184), induced cell death by apoptosis in ER^+ breast cancer cell lines. Thus, the activation of caspase-7, an effector caspase of the apoptotic process, was evaluated. It should be noted that although a caspase-3,7 kit was used, caspase-7 was the only effector caspase studied, once MCF-7aro cells, similarly to MCF-7 cells, do not express caspase-3 (187). It was observed an increased activity of caspase-7 in all AI-treated cells, confirming the involvement of an apoptotic process. Moreover, there was a greater caspase activity for AIs **49** and **50**, which might be associated with the observed arrest in G_2/M phase of the cell cycle. This might be concordant with the hypothesis that the apoptotic process induced by these compounds starts with anti-proliferative effects that can ultimately lead to cell death. AI **52** was expected to have a more pronounced caspase-7 activity and apoptosis involvement, once it induced the most drastic effects on cell viability and the most severe morphological alterations in MCF-7aro cells. However, it had a similar behavior to AI **51**.

Two main apoptotic pathways are described: the intrinsic or mitochondrial pathway, which is characterized by a mitochondrial membrane permeabilization and subsequent induction of caspase activity, and the extrinsic or death receptor pathway, in which caspases are activated upon ligand binding to the death receptors. The last one can also activate the intrinsic pathway through a cross-talk (188). To investigate the involvement of the intrinsic/mitochondrial pathway in the apoptotic process, the mitochondrial transmembrane potential ($\Delta\Psi m$) was analyzed using $DiOC_6(3)$. Loss of $\Delta\Psi m$ is associated with mitochondrial membrane permeabilization/depolarization and initiation of the pro-apoptotic mechanisms (188). Although the results were preliminary, a significant loss of $\Delta\Psi m$ was detected for all AI-treated cells, indicating a possible mitochondrial involvement in the observed apoptotic process.

As it is known, increased ROS production by the cells can lead to a state of oxidative stress, an imbalance in normal redox state of the cell that is often correlated with mitochondrial dysfunction and apoptosis (188). Many exogenous compounds induce oxidative stress and mitochondria dysfunction by ROS formation, as it is the case of exemestane (178). Once caspase-7 results

confirmed the occurrence of apoptosis, possibly via the mitochondrial pathway, it was important to understand if there was the involvement of ROS formation and mitochondrial dysfunction. Preliminary results demonstrated that AIs **51** and **52** increased ROS formation in MCF-7aro cells, suggesting a ROS-dependent mitochondrial dysfunction as the cause of apoptosis. However, AIs **49** and **50** did not enhanced ROS production, which implies a ROS-independent apoptotic process.

So far, anti-proliferative effects and cell death by apoptosis were confirmed mechanisms implicated in the effects of the compounds in MCF-7aro cells' viability. Nevertheless, autophagy is also considered by many as a form of cell death. Although it is maintained at basal levels in normal conditions, to ensure cell homeostasis, in stressful conditions of nutrient starvation or metabolic stress, autophagy may also induce cell death. In a tumor, autophagy has a controversial function and might act as a pro-survival pathway, by providing nutrients to sustain the continuous growth of tumor cells in stressful conditions, as seen in many cancers (189). It is also considered as one of the mechanisms that lead to acquired resistance. In fact, our group reported the occurrence of autophagy as a pro-survival mechanism for exemestane-treated cancer cells, with possible implications in AI-resistance (166, 178). It is referred in the literature that there is a cross-talk between autophagy and apoptosis, though this relationship is not totally understood. They can induce or prevent the other, or even act in a synergistic manner (190, 191). Some of the AIs previously studied by the group induced autophagy in MCF-7aro cells (166, 177), being for some a dominant mechanism of cell death instead of a pro-survival process, as it is for exemestane (178). Therefore, and also taking into account that morphological studies revealed the presence of some cytoplasmic vacuolization, autophagy was also addressed in this study. The formation of acid vesicular organelles (AVOs), such as lysosomes and autophagosomes was observed with AO stain. Only AI **51**-treated cells revealed the presence of AVOs, suggesting the occurrence of an autophagic process. Confirming these results, autophagy inhibition of AI **51**-treated cells caused a more pronounced reduction of the MCF-7aro cell viability, indicating that autophagy might have been acting as a pro-survival mechanism.

The development of resistance to therapy is a fundamental point to take into account in the evaluation of new therapeutic agents for endocrine breast tumors. Therefore, it was also investigated the effect of the new compounds in an AI-resistant environment. For this, LTEDaro cells were also used, as they represent a good model to study AI-acquired resistance (22, 174, 175). All the compounds were able to reduce the viability of LTEDaro cells in a similar way as for the sensitive breast cancer cells. Thus, it is possible to conclude that the AIs were able to sensitize the AI-resistant cell line (LTEDaro). This suggests that the new AIs are able to overcome AI-resistance and may serve as basis for the design of new therapeutic agents for ER^+ breast tumors.

Together, these results characterized four new steroidal compounds in terms of aromatase inhibition, biological effects and anti-tumor efficacy in cell culture models. All compounds proved to be potent AIs that reduced the viability of a hormone-dependent breast cancer cell line, with no necrotic effects and without affecting non-cancerous cells. Of all, AI **52** was the most potent compound, both on anti-aromatase activity and in cell viability effects. In addition, all AIs acted through an ER-independent manner. However, AI **49** was the only one that presented aromatase-dependent viability effects. It also reduced the expression of the enzyme, by a mechanism that, similarly to the steroidal AI exemestane, may involve inactivation and consequent degradation of aromatase. Moreover, the AIs-induced decrease in cell viability was accompanied by a cell cycle arrest and apoptosis, possibly via the activation of the mitochondrial pathway. Further confirmatory studies are needed to clarify the mitochondrial pathway implication. Additionally, the occurrence of autophagy was also verified, though only for AI **51**, which acted as a pro-survival mechanism. Furthermore, all compounds were able to sensitize an AI-resistant cell line, once they induced a decrease in the viability of these cells in a similar manner as for the hormone-sensitive ones.

CHAPTER V

Conclusions

Cancer is, without a doubt, the twenty-first century disease. It is one of the main causes of death all over the world and a major health problem in nowadays society, making it one of the major research areas. It is unquestionably necessary to investigate more about cancer causes, mechanisms of progression, invasion and metastasis but also ways of prevention, detection and treatment of this multifactorial disease in order to provide better quality of life to the patients. The improvement in cancer comprehension allows the discovery and development of new therapeutic approaches with better efficacy and less toxicity, able to efficiently eradicate the whole tumor without major side effects.

Until few years ago, the biological effects of the breast cancer therapeutic agents, including AIs, were barely understood. However, it became evident that a more profound comprehension of the pathways implicated in endocrine agents' biological effects might lead to the identification of new targets and development of better strategies to fight the tumor.

This work was particularly important for the elucidation of the mechanisms and pathways involved in tumor progression and regression. Furthermore, the characterization of these anti-tumor mechanisms might uncover new therapeutic targets, for instance, in cell cycle or cell death pathways. Additionally, this work contributed to understand, at the molecular level, which structural modifications translated into better aromatase inhibition and anti-tumor properties, providing important information for the design of more effective AIs that can inhibit tumor growth and prevent endocrine resistance.

Concluding, the final goal of these biological studies is the discovery of new potent molecules with greater anti-aromatase activity and lower side-effects that can proceed to preclinical and clinical studies, in order to obtain novel efficacious therapeutic agents that can be used for hormone-dependent breast cancer treatment.

CHAPTER VI

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