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Biological Evaluation of New Compounds as Potential Aromatase Inhibitors for Estrogen-Dependent Breast Cancer

Dissertação de 2º Ciclo de Estudos Conducente ao Grau de Mestre em Toxicologia Analítica Clínica e Forense

Trabalho realizado sobre a orientação do(a) professor(a)

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Julho 2016

Author´s Publications

Abstracts published in congress books

F. Bernardo, C. Amaral, C. L. Varela, E. Tavares da Silva, F. M.F. Roleira, S. Costa, N. Teixeira, G. Correia-da-Silva, *Antitumor efficacy of new steroidal AIs: AR and ER dependency on MCF-7aro cell*. 2nd ASPIC International Congress, 28-29 April, 2016, Porto, Portugal

F. Bernardo, C. Amaral, C. L. Varela, E. Tavares da Silva, F. M.F. Roleira, S. Costa, N. Teixeira, G. Correia-da-Silva, *Breast cancer growth-inhibitory efficacy of new steroidal compounds as aromatase inhibitors*. IJUP'16 - 9º Encontro de Investigação Jovem da Universidade do Porto, 17-19 February, 2016, Porto, Portugal

Aknowledgments/Agradecimentos

E assim termina mais uma etapa da minha vida. Foi uma jornada com altos e baixos mas tive sempre ao meu lado as pessoas certas para me apoiar e mostrar que por mais difícil que a vida seja o importante é não desistir. Porque no jogo da vida o importante é não ter medo de ir á luta, porque a derrota é garantida mas a vitória só se conquista quando se joga. Por isso eu escolhi jogar sempre, porque no dia que deixar de jogar deixo de viver. Mas não joguei sozinha tive sempre ao meu lado pessoas que acreditaram em mim, me apoiaram e deram força para continuar e por isso não posso deixar de lhes agradecer.

À Dra Georgina Correia-da-Silva agradeço todo o apoio e disponibilidade para me ajudar, não só na tese como no seminário. Obrigada pela dedicação e incentivo nesta etapa da minha vida. Agradeço todos os ensinamentos e conselhos que foram úteis para o meu crescimento científico e pessoal.

À Dra Natércia Teixeira agradeço todo o carinho, atenção, apoio que demonstrou sempre ao longo deste meu percurso. Acima de tudo agradeço a disponibilidade que sempre teve, pois no meio de todo o trabalho e responsabilidade que tem, encontrou sempre um tempinho para me receber e acompanhar.

À minha equipa de trabalho, Cristina Amaral e Tiago Augusto foi um prazer partilhar este ano convosco. Cristina, obrigada por tudo o que me ensinaste, pela dedicação e tempo despendido comigo, pela ajuda, pelo apoio, pela paciência, pelos conselhos. Obrigada por teres sempre uma palavra de conforto para me dar nos dias mais dificeis. Mas principalmente, obrigada pela confiança que sempre depositaste em mim. Mais que uma "mãe" és uma amiga que eu vou guardar com carinho. Tiago, agradeço-te toda a paciência, inter-ajuda, companheirismo, boa disposição. Sobretudo obrigada por seres quem és pois isso deu-me motivação para querer me tornar melhor e ajudou me a crescer.

Agradeço ainda a todos os outros membros do laboratório, Marta Almada, Renata Fernandes, Maria Miguel, João Maia, Lia Costa, Susana Rocha, Ana Paula, Bruno Fonseca e Sandra Ribeiro que de alguma forma me ajudaram ao longo deste ano, desde a confiança, à ajuda, à amizade, ao apoio, aos bolinhos, às brincadeiras e bons momentos passados dentro e fora do laboratório.

Não posso deixar de agradecer a uma das pessoas mais importantes deste laboratório. Aquela que põe ordem e respeito e permite o trabalho dos investigadores com a sua organização, rigor e cuidado com todo o material. D. Casemira, obrigada por todos os "não há" que depois de confirmados haviam sempre. Pela preocupação e carinho. Por

todas as risadas e brincadeiras por eu “estar nua"que tornaram cada dia diferente e mais alegre.

Aos meus amigos, os de longa data e os que fiz ao longo desta jornada académica, um sincero obrigado por todos os bons momentos partilhados, pelo apoio, pela motivação, pela preocupação, pelo carinho, pela amizade e pela força, pois foi ela que muitas vezes me ajudou e deu forças para seguir em frente.

Por ultimo, agradeço à pessoa mais importante de todas, a minha mãe. Obrigada por todos os sacrifícios. Todas as lutas que ultrapassamos. Toda a paciência nos dias mais difíceis. Obrigada por me acompanhares em todas as vitórias, todos os sorrisos e lágrimas, que não foram poucas. Obrigada pelo orgulho que sentes por mim. Contigo aprendi que por mais difícil e dura que a vida seja o importante é lutar sempre. O caminho tem obstáculos que por vezes nos deitam ao chão mas não importa, isso só nos torna mais fortes. Obrigada por teres criado uma Menina que é uma guerreira e sem medo de lutar pelos seus sonhos. Obrigada por teres feito com que o que ontem era um sonho hoje seja uma realidade. Como te digo sempre, uma casa só se segura de pé porque tem uma base forte. Tu és a base da minha vida e agradeço-te imenso por isso.

Resumo

O cancro da mama é a principal causa de morte por cancro em mulheres em todo o mundo, sendo os tumores recetor de estrogénio positivo [ER^+] os de maior prevalência. Os estrogénios são responsáveis pelo desenvolvimento destes tumores. Deste modo, várias estratégias terapêuticas foram desenvolvidas para bloquear a ação estrogénica, tais como o uso dos modeladores seletivos dos recetores de estrogénio (SERMs), os inativadores seletivos dos recetores de estrogénio (SERDs) e os inibidores da aromatase (AIs). Os IAs têm provado ser uma das melhores opções para o tratamento e prevenção destes tumores em mulheres na pós-menopausa, pela sua capacidade de bloquear a enzima aromatase, fundamental na biossíntese de estrogénios. Embora muito eficazes, os AIs utilizados na clínica induzem alguns efeitos colaterais graves, incluindo a perda de massa óssea e o desenvolvimento de resistência à terapia. Deste modo, é importante o desenvolvimento de novos compostos potentes, com menos efeitos secundários.

Este trabalho focou-se no estudo de novos compostos como potenciais AIs, sintetizados a partir de modificações estruturais na molécula da androstenediona, um dos substratos da aromatase. Para isso foi avaliada a atividade anti-aromatásica, os efeitos biológicos e os mecanismos anti-tumorais de quatro novos compostos (**57**, **58**, **59** e **60**). Os estudos *in vitro* foram realizados numa linha celular não-tumoral de fibroblastos (HFF-1), numa linha celular humana de cancro da mama recetor de estrogénio positivo com sobre-expressão da aromatase (MCF-7aro) e numa linha celular humana de cancro da mama recetor de estrogénio negativo (SK-BR-3).

Os resultados demonstraram que todos os novos esteróides são potentes IAs sendo capazes de reduzir a viabilidade das células de cancro da mama hormono-dependente, sem afetar as células não tumorais. Os novos IAs induziram ainda paragem do ciclo celular e morte celular por apoptose pela via mitocondrial.

Em conclusão, os efeitos anti-proliferativos dos novos esteróides são devidos a uma paragem na progressão do ciclo celular e a mecanismos de morte celular. Este trabalho poderá contribuir para o desenho e síntese de compostos mais eficazes e para a compreensão dos mecanismos de supressão tumoral associados ao tratamento com IAs.

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

Abstract

Breast cancer is the most common cause of cancer death in women worldwide, being the most prevalent the estrogen receptor-positive [ER^+] breast tumors. Estrogens are responsible for the development of ER^+ breast tumors. Thus, several therapies have been developed to block estrogen actions, such as the selective estrogen receptor modulators (SERMs), the selective estrogen receptor downregulators (SERDs) and the aromatase inhibitors (AIs). The AIs have proved to be one of the best options for the treatment and prevention of these tumors in postmenopausal women, since the enzyme aromatase is fundamental for estrogens biosynthesis. Though the AIs used in clinic are effective, they induce serious side effects including bone loss and the development of therapy resistance. For this, the search for novel potent compounds, with fewer side effects, is currently needed.

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 (**57**, **58**, **59** and **60**) were evaluated. The *in vitro* studies were performed in a non-tumor fibroblastic cell line (HFF-1), an estrogen receptor-positive (ER^+) human breast cancer cell line that overexpresses aromatase (MCF-7aro) and an estrogen receptor-negative (ER^-) human breast cancer cell line (SK-BR-3).

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. In addition, these new AIs induced cell cycle arrest and cell death by apoptosis via the mitochondrial pathway.

In conclusion, the anti-proliferative effects of these new steroids are mainly due to cell cycle arrest and cell death mechanisms. This work might contribute to the design and synthesis of more effective compounds and to the elucidation of the tumor suppressor mechanisms associated with AIs treatment.

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

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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
AP-1 activator protein 1 (transcription factor)
Bcl-2 B cell lymphoma 2
BCS breast cancer survivors
BRAC1 breast cancer 1, early onset (gene)
BRAC2 breast cancer 2, early onset (gene)
cAMP cyclic adenosine monophosphate
CCCP carbonyl cyanide m-chlorophenylhydrazone
CDX Casodex
CFBS charcoal-treated bovine serum
CoA co-activator
CYP cytochrome P450
CYP19 aromatase
DBD DNA-binding domain
DCF 2',7'-dichlorofluorescein
DCFH2 2',7'-dichlorodihydrofluorescein
DCFH2-DA 2',7'-dichlorodihydrofluorescein diacetate
DMEM Dulbecco's Modified Eagle Medium
DMSO dimethylsulfoxide
DR Dimerization Domain
E1 estrone
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)
Exe Exemestane
FBS fetal bovine serum
FDA U.S Food and Drug Administration
FSH follicle-stimulating hormone
GF growth factor
GFR(s) growth factor receptor(s)
GPCR(s) G protein-coupled receptor(s)
GPR30 G protein-coupled estrogen receptor 30
HER2 human epidermal growth factor receptor 2
HFF-1 human foreskin fibroblasts-1 cell line
HIF inducible hypoxia factors
IGF1 insulin-like growth factor 1
IGFR1 insulin-like growth factor 1 receptor
LBD Ligand-binding Domain
LDH lactate dehydrogenase
MAPK mitogen-activated protein kinase
MCF-7aro estrogen receptor-positive breast cancer cell line overexpressing aromatase
MEM Minimum essential medium
MFI mean fluorescence intensity

MISS membrane-initiated steroid signaling
mTOR mammalian target of rapamycin
MTT tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-difenyltetrazolium
NADPH nicotinamide adenine dinucleotide phosphate
NISS nuclear-initiated steroid signaling
NLS nuclear localization signaling
PGE2 prostaglandin E2
p53 tumor protein 53 (gene)
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
SERD(s) selective estrogen receptor downregulator(s)
SERM(s) selective estrogen receptor modulator(s)
SK-BR-3 estrogen receptor negative breast cancer cell line
SP-1 specificity protein-1
Src tyrosine protein kinase
STA staurosporine
T testosterone
TF(s) transcription factor(s)
TNF-α tumor necrosis factor alpha
3β-HSD 3β-hydroxysteroid dehydrogenase
4-OHA 4-hydroxyandrostenedione or formestane
17β-HSD 17β-hydroxysteroid dehydrogenase
ΔΨm mitochondrial transmembrane potential

Chapter I

Introduction

1. Hormone-dependent Breast Cancer

1.1. Breast cancer: incidence and risk factors

Breast cancer is the main cause of death in women worldwide (figure 1). Although it can also occur in men, it is 100 times more common in women (1). There is a wide geographic variation in incidence of breast cancer, and in 2012 the highest occurred in Europe. In Portugal, approximately 86% of new cases were diagnosed in that year, of which 18% women's die victims of that disease (2). The majority of breast cancers are hormone-dependent and express the estrogen-receptor. Arround 65% of these tumors occur in premenopausal women and 75% in postmenopausal women (3). However, taking into account the advances in recent years to improve the screening and treatment of this disease, it has become possible to treat women with breast cancer at early stages, thus promoting the survival rate of women free of disease.

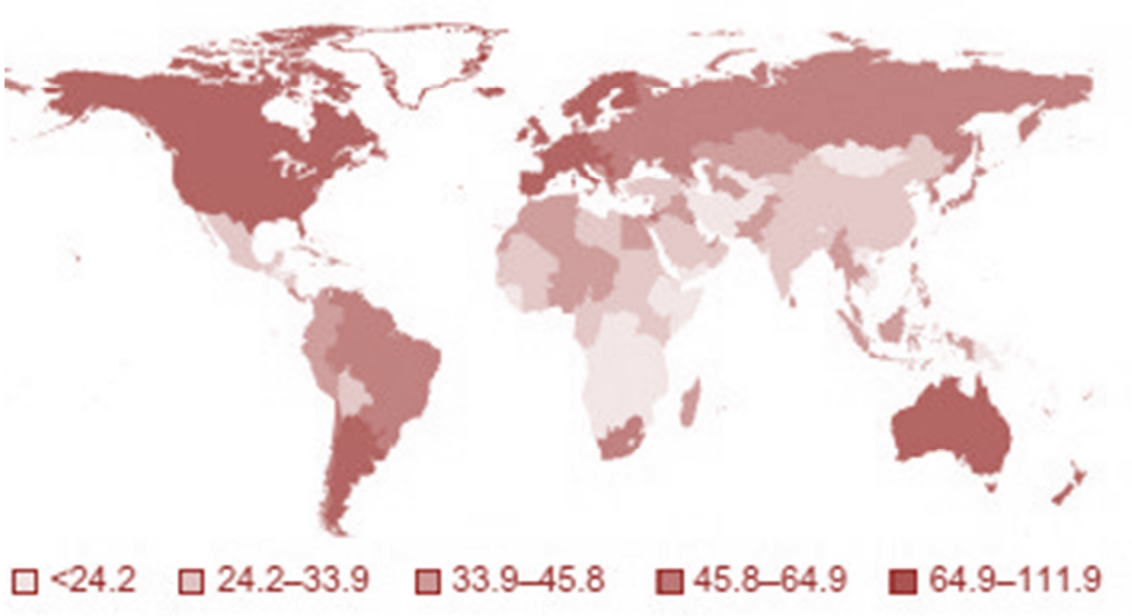

Figure 1: Worldwide incidence of breast cancer. The incidence of new diagnoses per 100,000 women per year. Adapted from (4)

Breast cancer is a heterogeneous group of diseases. According to the World Health Organization (WHO), breast cancer is subdivided into 21 different histological types based on cell morphology, growth and architecture standards (5). The majority of breast cancers develop from the epithelial cells lining the ducts or the lobules, being classified as ductal or lobular carcinomas (1). In the last decade, the research focused on the understanding of the heterogeneity of breast cancer and of the prediction of tumor behavior to improve therapeutic strategies. Using molecular parameters breast cancers were divided into Luminal A, which includes the estrogen or progesterone receptor (ER/PR) positive and

receptor negative human growth factor 2 negative ($HER2^-$) while the ER^+/PR^+ and $HER2^+$ belong to Luminal B. The triple negative breast cancer ($ER^-/EP^-/HER2^-$) belongs to the basal like subtype (1).

There are a number of known risk factors for the development of breast cancers. One of the most important is the occurrence of mutations in genes like: *BRCA1, BRCA2, CHEK2, p53 and ATM* (6),(7). Other important risk factors are the age, early menses, late menopause, null parity, lactation failure, hormone replacement therapy, oral contraception and treatment with the synthetic estrogen diethylstilbestrol. The obesity is also considered a risk factor. The reasons why women with higher body fat content are more likely to develop breast cancer are not yet clear, but it may be related to the higher expression of aromatase in adipocytes (4).

The treatment of breast tumors has advanced significantly in recent decades, improving the quality of life of women diagnosed with this cancer. Endocrine therapy is the standard cancer treatment for hormone-dependent breast cancer in post-menopausal women. The estrogen is responsible for the development of ER^+ tumors, thus by decreasing the amount of estrogens produced or by inhibiting their action it may be reduced the risk of developing such tumors. Among them, we highlight the selective estrogen receptor modulators (SERMs), selective estrogen receptor down-regulators (SERDs) and the aromatase inhibitors (AIs). However, for the triple negative breast cancer, the treatment options are more limited and there is still no specific treatment (8).

2. Estrogens

Estrogens are steroidal sex hormones that are synthesized from cholesterol and primarily secreted by the ovaries. These steroids circulate in the bloodstream bound to proteins, being the breast, uterus, ovaries, brain, heart and liver their major target tissues (9). Estrogens are divided into three types: estrone (E1), 17-β- estradiol (E2) and estriol (E3), being E2 the most potent (4, 9). Estrogens, in particular E2, participate in the regulation of the growth, differentiation and homeostasis of serial tissues (10). However, one of the most important effects of estrogens is associated with stimulation of breast tissue growth (11). Several studies, established a relationship between the development of breast cancer and estrogens (12). One explanation for this correlation was based on the fact that there is increased cellular proliferation (13), which can induce an increased risk of DNA lesions. This damage can result from the production of oxidative metabolites, DNA breaks and accumulation of genomic mutations (11). However, estrogens may have

beneficial effects as protective against cardiovascular disease, by regulating cholesterol level, maintaining bone density and preventing osteoporosis (14, 15). The effects of estrogen in target tissues can be mediated by the estrogen receptors (ER), ERα or ERβ (16).

2.1. Estrogen Biosynthesis

In premenopausal women the ovary is the principal source of estrogen, however during pregnancy it is also synthesized in the placenta (17). After menopause, the estradiol is synthesized in peripheral tissues, especially in adipose tissue, and from circulating steroid precursors from adrenal cortex (15).

The synthesis of estrogens are catalyzed by the action of selective and complex enzymes that belong to the cytochrome P450 enzyme family (CYP450) (18). The synthesis of estrogens starts with the production of pregnenolone from cholesterol (figure 2), which give rise to progesterone. Dehydroepiandrosterone (DHEA) and androstenedione are obtained from pregnenolone and progesterone, respectively. DHEA is then converted into androstenediol by 17β-hydroxysteroid dehydrogenase (HSDs; 17β-HSD-1, -7 and -12) and in androstenedione by 3β-hydroxysteroid dehydrogenase (3β-HSD-1). Androstenediol is converted into testosterone by 3β-HSD-1, whereas androstenedione is converted into testosterone by 17β-HSD-5. Finally, aromatase (CYP19) is responsible for the conversion of androstenedione to estrone and of testosterone to 17β-estradiol (E2) (19, 20).

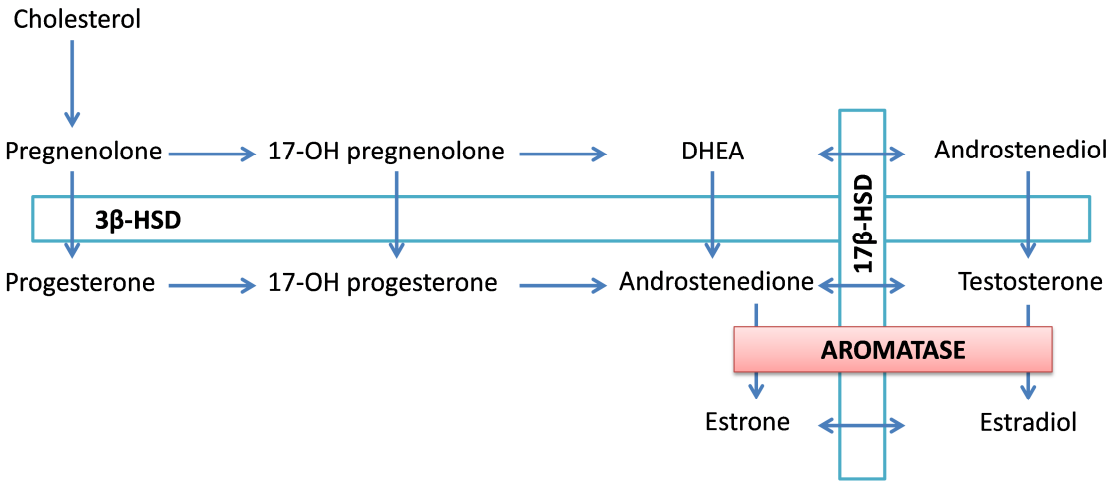

Figure 2: Biosynthesis of estrogens.
17β-HSD: 17β-hydroxysteroid dehydrogenase; 3β-HSD: 3β-hydroxysteroid dehydrogenase.

2.2. Aromatase

Aromatase is a cytochrome P450 enzyme that is responsible by the biosynthesis of the estrogens, estrone and estradiol, being, therefore an important therapeutic target for breast cancer (21-23). This enzyme is found in the endoplasmic reticulum of cells producing estrogens, in particular, ovaries, placenta, testis, brain and skin (21, 24). The ovary is the organ where the aromatase concentrations are higher, in premenopausal women. While in postmenopausal women this enzyme has a higher expression in adipose tissue (21, 25, 26). The expression and activity of aromatase in adipose tissue increases with age, leading to the production of estrone (E1) and estradiol (E2) from androstenedione. The estrogen biosynthesis is regulated by paracrine interactions between malignant breast epithelial cells, fibroblasts and endothelial cells, promoting the expression of aromatase (21, 27). Women with breast cancer sometimes show desmoplasias resulting from the accumulation of fibrous tissue around the epithelial cells (21).

As mentioned, the aromatase is an enzymatic complex composed by a cytochrome P450 and a NADPH-cytochrome P450 reductase (28) and is encoded by the CYP19A1 gene (29) present in chromosome 15q21.1 (30). The coding region of the aromatase gene is composed by nine exons and its expression is regulated by the activation of specific promoters (21, 22, 24), (figure 3).

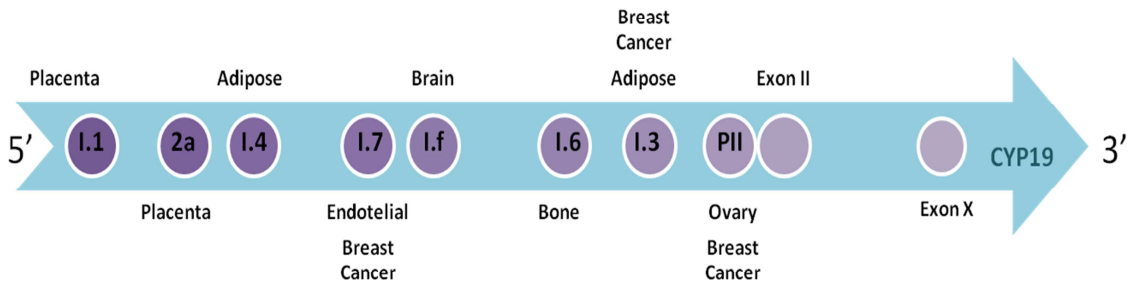

Figure 3: Aromatase Gene – CYP19

The promoter I.1 is expressed in the placenta, the promoter II in ovaries and the promoters I.3 and I.4 in adipose tissue. Besides I.3, the tumor cells of breast cancer also express promoters I.7 and II (31). Promoters I.f and I.6 are expressed in the brain and

bones respectively (32). There are also other factors that stimulate the expression of aromatase. The regulation of aromatase expression in the ovaries, is mediated by follicle-stimulating hormone (FSH) through a cyclic AMP-dependent pathway, while in adipose tissue the promoter I.4 is regulated by cytokines, such as tumor necrosis factor (TNF-α) (33, 34). Activation of abnormal promoters in breast and fat tissues by the action of malignant epithelial cells will induce the expression of aromatase, what makes that its concentration is higher in women with breast cancer (21, 35-41). Promoters I.3 and II can be activated by prostaglandin E2 (PGE2) (42). The mRNA levels that encode the enzyme aromatase are significantly higher compared to normal tissue where only intervenes promoter I.4 (43).

In 1987, Poulos et al presented a proposal for the structure of aromatase based on the similarity with the enzyme found in Pseudomonas putida (44), but, only in 2009, Ghosh et al were able to crystallize this enzyme from human placental microssomes (30) (figure 4).

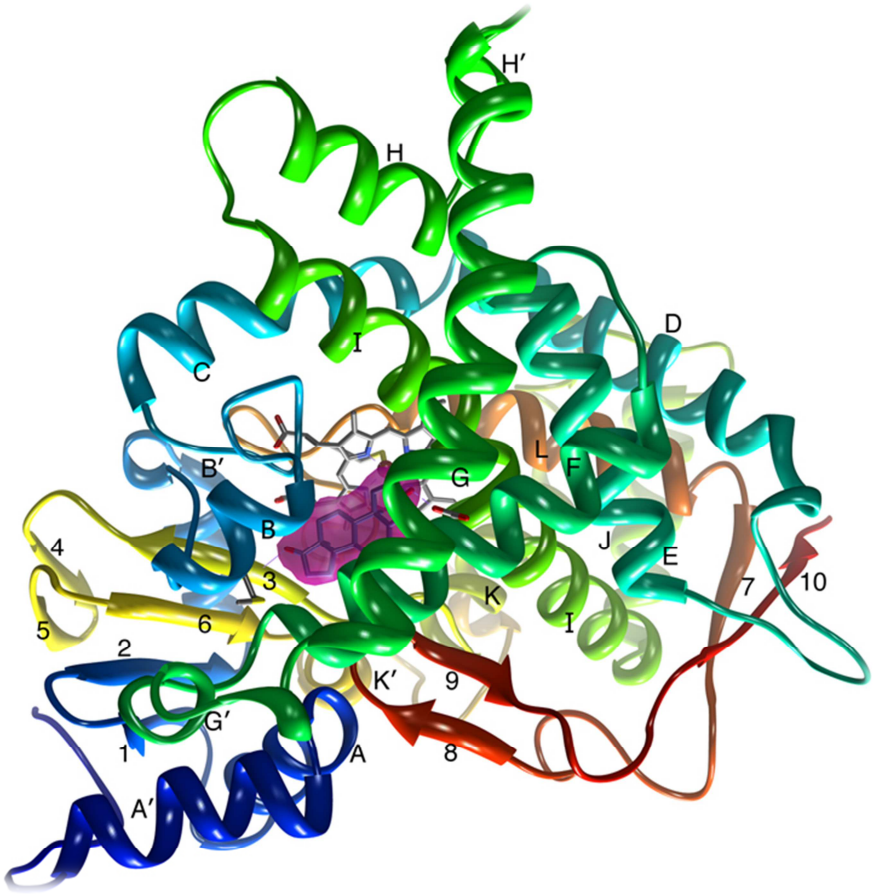

Figure 4: Tertiary structure of aromatase isolated from human placenta. Colored in dark blue is the N terminus, starting at residue 45, and colored in red is the C terminus ending at residue 496. The α-helices are labelled from A to L and β-strands are numbered from 1 to 10. The heme group and the androstenedione molecule bound at the active site are also shown. Adapted from (30)

The tertiary structure of aromatase is constituted by 12 α helices and 10 β sheets (30). The androstenedione substrate binds to the heme iron localized in the aromatase active center by action of the carbon 19 resulting in two hydrogen bonds with Asp309 and Met374 residues (45) (figure 5). The supplementary cavity is formed by hydrophobic residues and porphyrin rings that contribute to the binding of enzyme-substrate. The Arg115 residues, Ile133, Phe134, Phe221, Trp224, Ala306, Thr310, Val370, Val373, Met374 and Leu477 residues establish van der Waals bonds with androstenedione (30, 45).

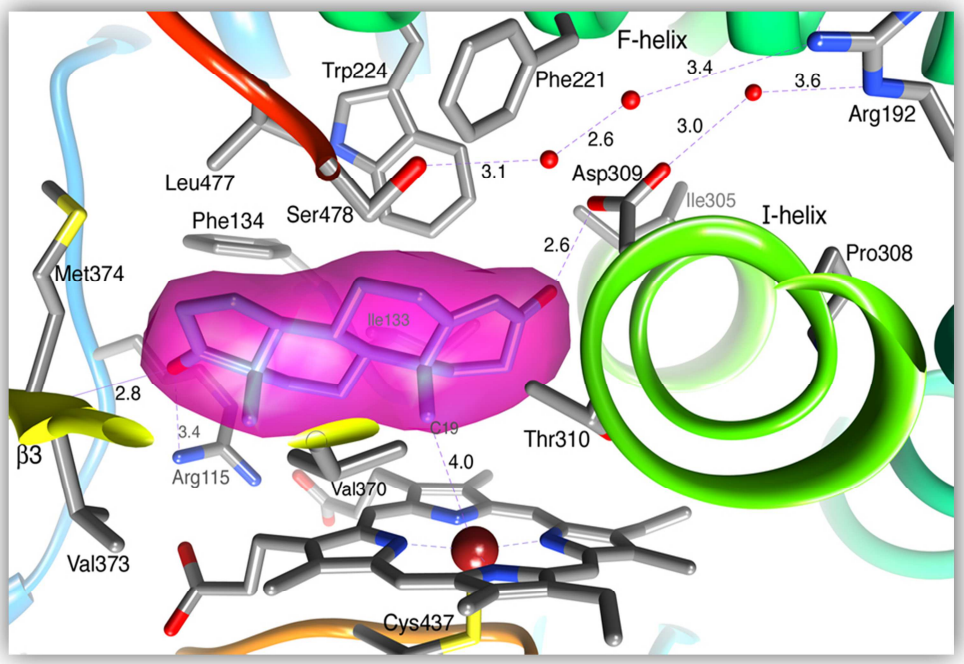

Figure 5: The active site of aromatase. Magenta shows the connection of androstenedione to the active center. Adapted from (45)

2.3. Estrogen Receptors

The discovery of a specific protein, in 1960, by Jensen and Jacobson, responsible for the response of estrogen in target tissues was denominated estrogen receptor (ER) (46). The estrogen receptor exists in two isoforms: ERα and ERβ (47-49) with a 56% homology between them (50). The ER belongs at a large family of nuclear receptors (16) and is synthesized by distinct genes (51), *ESR1 and ESR2*. The receptor alpha (ERα),

uncovered in 1962 (52), is considered one important receptor for the diagnostic of estrogen dependent breast cancer. The receptor beta (ERβ) was identified in 1996 (53) on prostate and ovaries of rats and on human testicle (54). However, its role in human breast cancer remains unclear (55).

Estrogen receptors (ERs) play a decisive role in the development and function of the reproductive system and mammary gland (51) (figure 6) as well as in cancer development. ERα is expressed mainly in the liver, uterus and mammary gland, while in the lungs, kidneys, colon, gastrointestinal tract, there is a greater abundance of ERβ. In the ovaries, although both ERs are expressed, their cellular distribution is markedly different, since ERα is expressed mostly in the theca and interstitial cells, whereas ERβ is especially expressed in the granulosa cells (51).

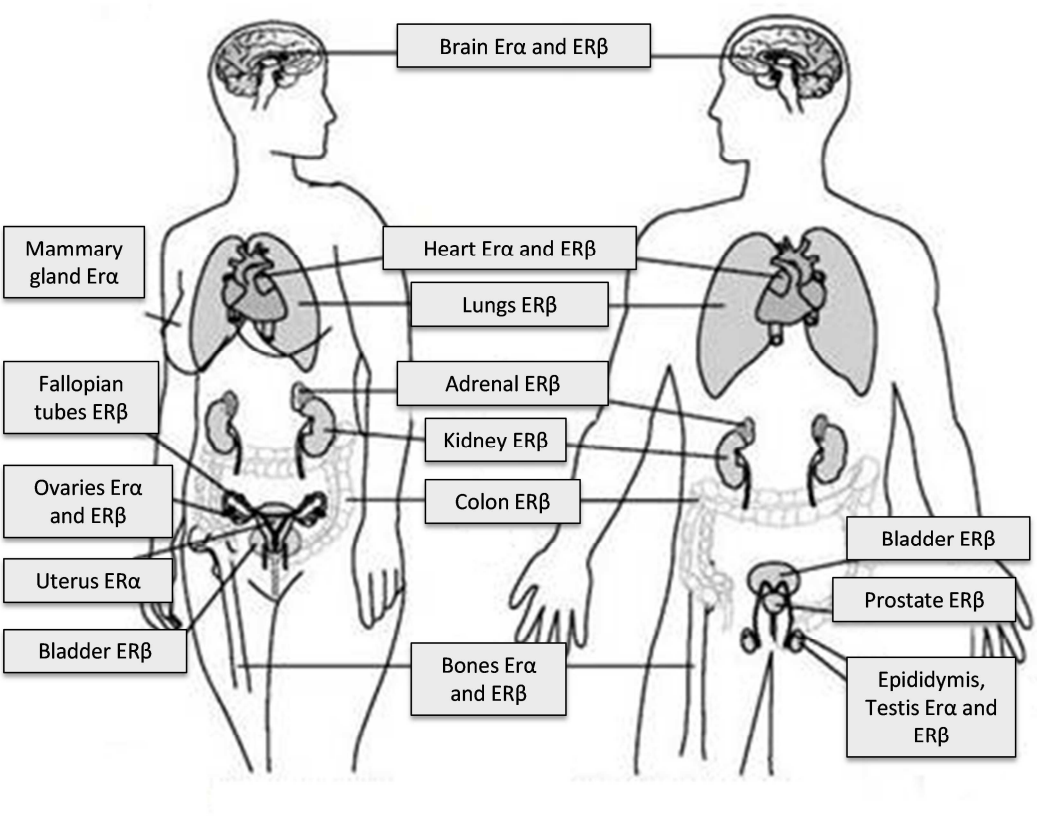

Figure 6: Distribution of ERα and ERβ by the different organs

The clinical and *in vitro* studies allowed the understanding of the importance of these receptors in breast cancer disease (56). The α receptors are expressed in about 70% of breast tumors. Given the function of ER in regulating proliferation and differentiation of normal tissue, the study of cell signaling mechanisms is very important to control these processes in cancer situations. For this reason the ERα is used as a biomarker in the

progression of breast cancer and as an effective therapeutic target (51). Although the ERβ is present in fibroblasts of normal breast adipose tissue in ER positive breast cancer situations its expression decreases with tumor development, which may be related to the potential of these receptors for anti-proliferative action (55). ERβ has a decisive function on the gene expression of several matrix mediators, like the proteoglycans syndecans-2/-4 and serglycin, several matrix metalloproteinases, plasminogen activation system components and receptor tyrosine kinases (57).

The human ERα is a 66 kDa protein that contains 596 amino acids (58) and is located on chromosome 6 (6q25.1) (59). The ERβ has 59 kDa, contains 530 amino acids (60) and is situated on chromosome 14 (14q23.2) (61) (figure 7).

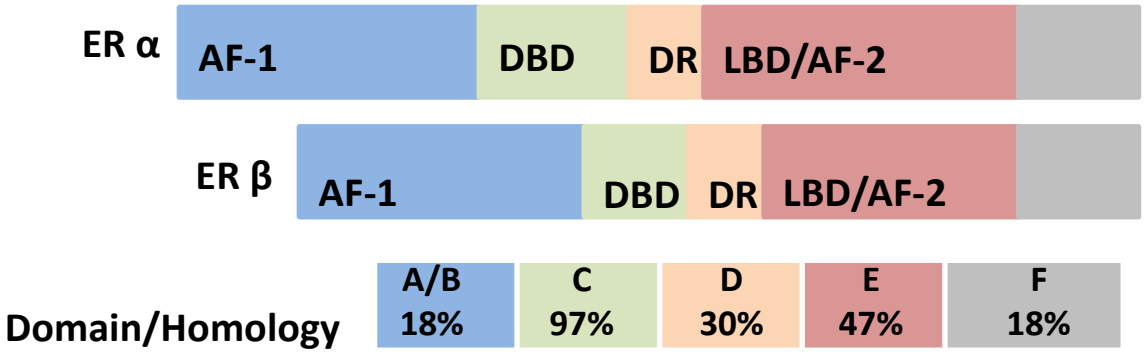

Figure 7: Schematic representation of the human ERα and ERβ structures. Both ERs are characterized by the A/B domain at the N-terminus, which contains the ligand-independent transcriptional activation domain (AF-1), the C domain that represents the DNA-binding domain (DBD) and contains a nuclear localization signal (NLS), the D domain, the E domain that harbors the ligand-binding domain (LBD) and the ligand-dependent transcriptional activation domain (AF-2) and lastly the F domain at the C-terminus. The percentage of homology for both ERs in relation to each domain is represented.

As with other nuclear receptors, ERs have multiple domains consisting in six functional regions. A A/B domain has the ligand-independent activation function (AF-1) (51), a DNA-binding domain (DBD) the C domain, a dimerization region (DR), the D domain, a ligand binding domain (LBD), the E domain (58). The C domain has a structure that allows the dimerization of the receptor and subsequent binding to DNA target sequences (62). The D domain is a region, with only 30% of homology between the ERs. The E region contains the ligand binding domain (LDB), the second nuclear localization signal and a ligand-dependent transcription activation function (AF-2). Lastly, the F

domain contains the C-terminus wich may be associated with dimerization processes or protein interaction (16, 51).

2.4. Pathways of Estrogen Signaling

Various studies conducted in the last years have elucidated the molecular gene expression mechanisms regulated by ERs (63). The signaling pathways activated by ERs depend on their intracellular localization, since ERs can be localized in nucleus, cytoplasm or in cytoplasmic membrane (64). There are two different mechanisms involved in the ER activation, the genomic and the non-genomic pathway (figure 8).

The genomic pathway or NISS (nuclear-initiated steroid signaling) may occur in two ways, the classical and non-classical pathways. In the classic or direct mechanism, the estrogens that have crossed the cytoplasmic membrane by diffusion bind to the ER receptor, resulting in its dimerization and activation (65) and allowing the translocation of ER to the nucleus. In the nucleus, in the presence of co-activators (CoA), it binds directly to specific regions of DNA, the estrogen response elements (ERE), located in the promoters of target genes (64). In the non-classic or indirect pathway, the ER binds indirectly to the DNA through other transcription factors, such as activation protein-1 (AP-1) and specificity protein-1 (SP-1). In both pathways, depending on the cell stimulus, the binding of ER promotes the recruitment of co-regulators or co-repressors (63).

The activation of the non-genomic pathway or MISS (membrane-initiated steroid signaling) induces a faster response than the genomic pathway (64). In the non-genomic pathway the ERs can also interact or activate other cell membrane receptors, such as the insulin-like growth factor receptor 1 (IGFR1), the epidermal growth factor receptor 1 (EGFR1) and human epidermal growth factor receptor 2 (HER2). This interaction leads to activation of intra-cytoplasmic kinases, such as phosphatidylinositide 3-kinases (PI3K), mitogen-activated protein kinase (MAPK) and protein kinase B (AKT) (65).

However, it is known that the genomic and non-genomic pathways are not completely independent, but, complementary and even synergistic (65). Several studies have demonstrated the occurrence of "*cross-talk*" mechanisms between the ER and the signaling pathways of EGFR / HER2 and of the IGFR1. Others studies showed that the G protein-coupled estrogen receptor (GPR30) can also induce the activation of EGFR (66). In addition, kinases can phosphorylate ER, co-activators and other transcription factors causing an increase in gene expression. These mechanisms of "*cross-talk*" between ER and

growth factors receptors (GFRs) promote cellular proliferation and progression of breast cancer, as well as the development of resistance to endocrine therapies (66, 67).

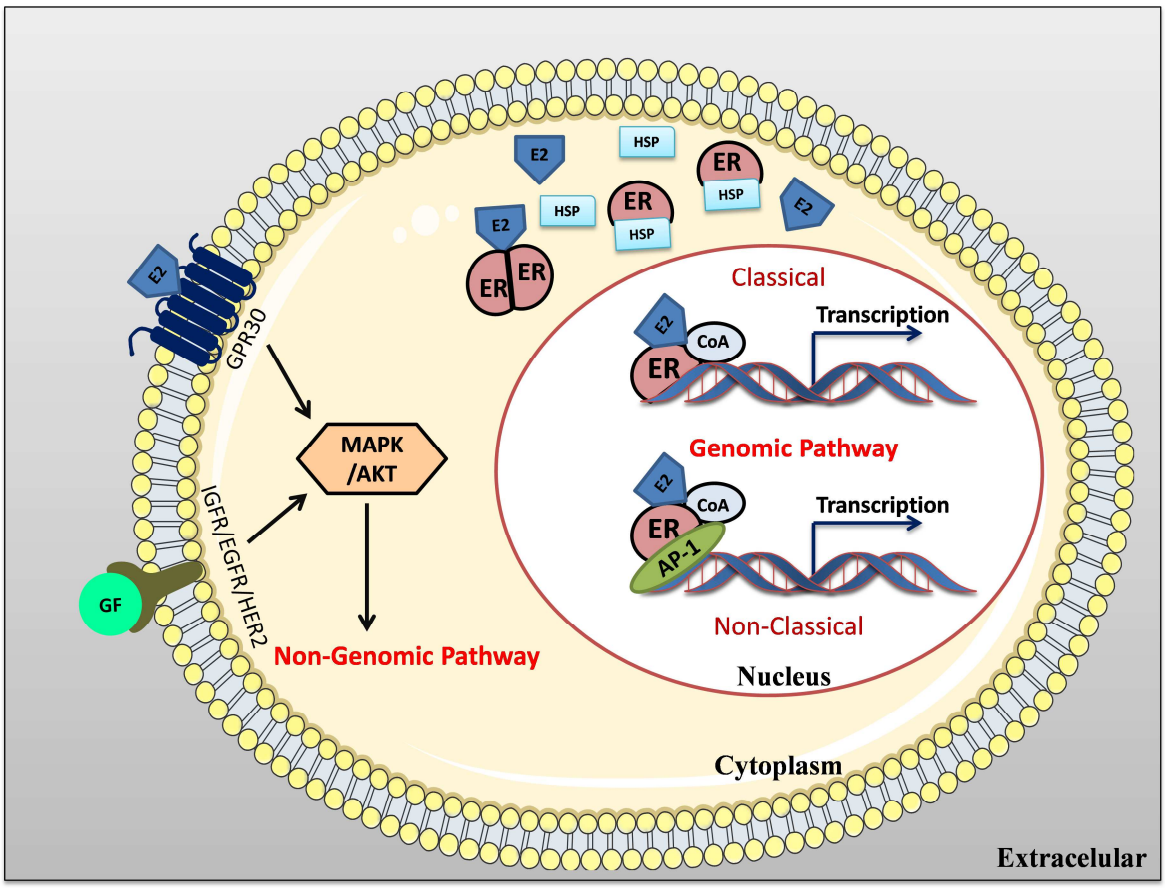

Figure 8: Schematic representation of ER signaling pathways. In genomic signaling of ER, considering the classical pathway, the ER binds directly to the DNA, to the ERE region, recruiting co-regulators. In the case of non-classical pathway, regulation and gene transcription occurs through receptor interactions with other classes of transcription factors such as AP-1. In the non-genomic signaling pathway the binding of E2 to the ER of citoplasmatic membrane associated to G protein or the binding of GF receptors will activate MAPK / AKT proteins, essential for cell proliferation.

E2-estrogen; ER, estrogen receptor; CoA co-activators; GF-growth factor; EGFR- epidermal growth factor receptor; IGFR- insulin like growth factor receptor; ERE- estrogen-responsive elements; MAPK- kinase activated by mitogenic agents; AKT- protein kinase B; AP-1 Activator protein-1; GPR-30-G-protein coupled receptors.

3. Hormonal therapy in breast cancer treatment

Treatment of breast cancer has advanced significantly in the recent decades (68). The chosen treatment plan depends mainly on the stage of disease, the type of tumor and the general health of the patient. In view of the needs of each patient it may be necessary to choose for one or even a combination of two or more treatments. Surgery and radiotherapy are considered local therapy, while the chemotherapy and hormonal therapy are examples of systemic therapy (69).

Endocrine therapy is considered the standard treatment for the hormone dependent breast cancer (70). Estrogens have important functions in breast cancer, so the development of a treatment able to block the estrogen signalling is a mark in history of breast cancer. Actually, there are two therapeutic strategies used to reduce the action of estrogens on the target organs. The first is the use of ER modulators (SERMs), as tamoxifen, that modulate the binding of estrogens to ER and of selective ER downregulators (SERDs), as fulvestrant that down-regulate ER protein levels. The second strategy involves the aromatase inhibitors (AIs) that bock the conversion of androgens to estrogens by inhibition of the enzyme aromatase (3, 71, 72).

3.1. Selective ER modulators (SERMs) and selective ER downregulators (SERDs)

The SERMs are ER modulators that are partial agonists depending on the target tissue. In some cases have an agonistic effect while in others present an antagonistic activity. Tamoxifen was the first SERM approved through the U.S. Food and Drug Administration (FDA), in 1977, for treatment of breast cancer and in 1998 as quimio-preventive agent for women with high risk of developing this disease (73). More recently, other SERMs were approved, as toremifen and rolexifen (figure 9). FDA approved raloxifen for prevention and treatment of osteoporosis and tamoxifen, toremifen and raloxifen as preventive and treatment for breast cancer (74).

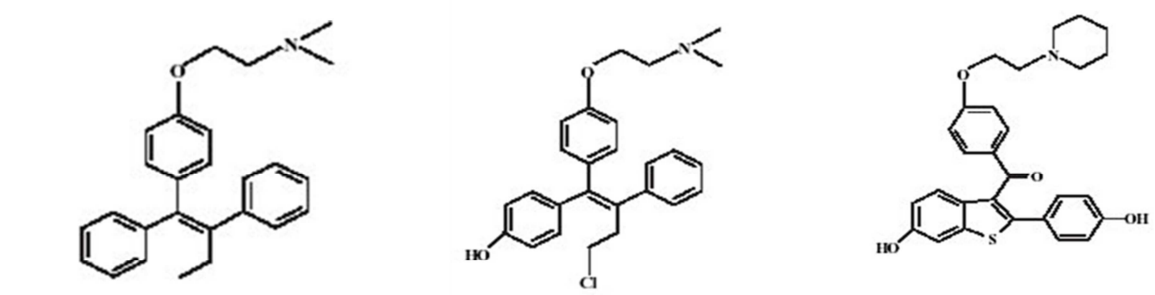

Figure 9: Selective ER modulators (SERMs)

Tamoxifen was the first endocrine therapy for breast cancer. It is prescribed for the treatment in premenopausal women for a period of at least five years, but it may be used in women after menopause supplemented with AIs. This drug is considered an antagonist in the breast tissues, since it blocks the action of estradiol, but also acts as an ER agonist in endometrium and bone, causing endometrial hyperplasia and increased risk of endometrial cancer, as well as the preservation of bone density in postmenopausal women (75). Tamoxifen was considered the treatment of choice during many years, because it can decrease by 50% the risk of recurrence. However, despite the demonstrated effectiveness, it can occur the development of resistance (74).

SERDs, like Fulvestrant, are considered pure antiestrogens (76). Fulvestrant binds to estrogen receptors, leading to rapid degradation of the receptors (77, 78). Several studies *in vitro* have shown that fulvestrant was more effective than tamoxifen (79). Studies have demonstrated that SERDs are effective in patients previously treated with tamoxifen or AIs (80). The fulvestrant administration route is parenteral, allowing better monitoring of treatment adherence and reduces pharmacokinetic interference from oral administration, by interaction with food or other drugs. Although being a good alternative for patients with advanced breast cancer when other endocrine therapies do not result, one cannot exclude the possible occurrence of resistance to fulvestrant in prolonged treatment situations (80).

3.2. Aromatase Inhibitors (AIs)

Aromatase inhibitors are very effective in inhibiting estrogen synthesis and are divided into two groups based on their chemical structure and mechanism of action, the non-steroidal and steroidal inhibitors (81). Steroidal AIs compete with androstenedione and testosterone to the active site of the enzyme and establish an irreversible binding, resulting in the inactivation and degradation of the enzyme, and for this reason, are also called suicide inhibitors (82). The non-steroids bind reversibly to the enzyme.

According the period of discovery and application, AIs can be divided as first-, second- and third-generation and each consecutive generation presented higher specificity to aromatase and lower adverse effects (83, 84). In 1980 in Europe, appeared the second generation aromatase inhibitors, formestane and fadrozol and a decade later, the use of the third-generation of aromatase inhibitors was approved. Currently, AIs used in clinic are those of the third generation, the non-steroidal, letrozole, anastrozole and the steroidal exemestane (83, 84). These have proven to be an alternative to tamoxifen for the treatment of ER^+ breast cancer in postmenopausal women. It has been found further that this therapy allows a better quality of life in these women (21). Aromatase inhibitors are also effective in the treatment of breast cancer resistant to tamoxifen (21, 85).

AIs showed to be advantageous for patients with breast cancer in postmenopause, because they are better tolerated and have a higher activity compared with tamoxifen (86). AIs inhibit cell proliferation causing retention in the cell cycle and apoptosis (87). This cell death is mediated by several mechanisms and regulated by the expression of different factors and proteins, especially of the Bcl-2 family (88).

Although AIs are considered a good alternative to tamoxifen for the treatment of hormone-dependent breast tumors, some are resistant before the endocrine treatment, due to intrinsic resistance or after an extended period acquired resistance may occur (89). Thus, it is crucial to understand the molecular mechanisms and characteristics of resistance to AIs and tamoxifen, to choose the most effective treatment (90).

There are several mechanisms proposed to explain the resistance to endocrine treatment, namely the dysregulation of various components of the ER signaling pathway, including the loss of expression of ERα; the activation of pathways that may promote cell proliferation and survival alternative stimulus, via EGFR, PI3K/AKT/mTOR pathway; changes in cell cycle and apoptosis and alterations in the epigenetic and microRNAs

modulation. The resistance to endocrine therapy may also be associated with a decrease or absence of the ER function.

Several other factors and pathway shave been referred to be involved in endocrine resistance, like HER2, cyclin E1, the inducible hypoxia factors (HIF) and MAPK pathway (91). The activation of other non-classical pathways may influence the resistance to hormone therapy as they can lead to changes in the expression of co-activators, co-repressors, and receptor tyrosine kinases (92). Relatively to AIs resistance there are differences between steroidal and nonsteroidal inhibitors. It is considered that the chemical structure of molecules and interaction with the active site of the enzyme may be one of the causes for the divergence in resistance behaviors (93).

Treatment with AIs, especially with exemestane, anastrozole and letrozole, leads to a reduction in estrogen levels, causing a decrease in bone mass resulting in increased bone fragility, which can ultimately induce osteoporosis (94). This disease is induced by treatments leading to premature menopause, chemotherapy, ovarian suppression and anti-estrogen therapies, causing bone loss and an increased fracture risk in breast cancer survivors (BCS) (95-97) Several studies demonstrated that in the first 5 years of natural menopause, it occurs an estimated 0,5-3% annual bone loss (98). However, this bone loss is accelerated with cancer treatments, as the endocrine therapy with AIs, because they effectively deplete residual oestrogen levels (99, 100). This bone loss ranges from 1,6-7,4% per year (98, 101-103) and 1,5-3 times of these women have bone fracture (104-106), which are associated with functional decline, fall risk and varying degrees of morbidity (107). In order to control and prevent the effects of this adverse action of AIs it is necessary the use of supplementary therapy with bisphosphonates administration, calcium and / or vitamin D (108).

Several studies show that treatment with these drugs leads to changes in cholesterol, triglycerides and glucose increasing the risk for cardiovascular diseases. In view of this data it is important to make a preliminary evaluation of other disorders such as heart failure, hypertension, arrhythmia, diabetes and obesity (109). Some studies indicate that therapy with AIs has fewer associated risks compared to tamoxifen, being better tolerated by patients (110).

3.2.1. Non-steroidal AIs

The non-steroidal AIs or Type II (figure 10) establish a non-covalent binding with the heme portion of the aromatase causing the saturation of the binding site (83). Inhibition per this type of AI is reversible and depends on a constant supply of drug (82). Some studies have shown that, given the reversible nature of these inhibitors, the enzyme aromatase activity is recovered after treatment (111).

Aminoglutethimide was the first AI used to endocrine treatment of breast cancer (82). Its introduction for therapeutic purposes occurred in 1960 as anticonvulsant, but, owing to the severe side effects, was withdrawn from the market by the FDA six years later. Its inhibitory capacity of aromatase, preventing the conversion of androgens to estrogens, was described in 1974 (112), however aminoglutethimide is unspecific and, therefore, can inhibit other CYP450 enzymes involved in the biosynthesis of other steroids, such as cortisol (113). In order to minimize the side effects of this inhibitor, its management was done in combination with corticosteroids (89).

Fadrozol a second generation AI (114), was more selective and potent, relatively to aminoglutethimide but also interferes with the biosynthesis of progesterone, aldosterone and corticosterone (115). Due to the lack of selectivity and to its adverse effects, this nonsteroidal inhibitor is not clinically used for the treatment of breast cancer, being replaced by the use of third generation non-steroidal AIs.

Anastrozole (Arimidex ®) a third generation AI is very selective. This compound has a functional triazol group, which reversibly binds to the enzyme. This aromatase inhibitor has no progestogen, androgenic or estrogenic effect (116). It has an extensive hepatic metabolism by reactions of N-dealkylation, glucuronidation, and hydroxylation. After 72 hours of administration 10% of unchanged drug is excreted in the urine, while 60% is excreted as metabolites (117). Anastrozole has two principal metabolites, the hydroxy-anastrozole and anastrozole-glucuronide (118).

Anastrozole is indicated for the treatment of ER^+ breast cancers in advanced or early stages. The recommended daily dose is 1 mg. Anastrozole reaches peak plasma concentrations 2 hours after oral administration. It is estimated that anastrozol has a time half-life of 50 hours (119). The combination of anastrozole with other treatments requires special care, especially in the case of tamoxifen. Some studies demonstrated that the interaction with tamoxifen leads to a decrease of about 27% of plasma concentrations of anastrozole, thus decreasing its effectiveness (118).

The most common side effects of treatment with anastrozole are weakness, generalized pain, hot flashes, arthralgia, and elevated serum cholesterol levels. Like exemestane and letrozole, anastrozole causes a decrease of bone density as a consequence of the suppression of estrogen. The occurence of bone fractures during treatment with anastrozole is superior relatively to the observed with tamoxifen treatment. In treatment with anastrozole there is a decrease in the occurrence of relapses relative to tamoxifen (117). The resistance to anastrozole is associated with a upregulation of PI3K/AKT pathway and IGF-1 receptor (120)

Finally, letrozole (Femara ®) is the other highly selective third-generation AI, which has a mechanism of action similar to anastrozole (121). This inhibitor is specific for aromatase, so it causes no changes in plasma concentrations of other hormones during treatment (122). This AI is used in early or advanced stages. In addition, letrozole is used for the treatment of patients with tumor progression after treatment with antiestrogens or in recurrence cases. Its administration is done orally at 2.5 mg daily (123) and it is rapidly and completely absorbed being widely distributed throughout the body. Letrozole has a half-life of 42 hours. The principal route of elimination is by via cytochrome P450, namely CYP3A4 and CYP2A6, with formation of inactive carbinol metabolites (124).

The adverse effects at bone level is very similar to those for anastrozole, being necessary the previous evaluation of bone density. The combination of letrozole with other drugs is another factor to consider, since drug interactions can occur and, consequently, reduce letrozole efficacy. Comparative *in vivo* studies of other AIs have shown that letrozole, relatively to anastrozole evidenced greater effectiveness. During treatment with letrozole it may occur cardiovascular problems such as thromboembolism (124).

Relatively to biological effects, Thiantanawat et al. demonstrated that both anastrozole and letrozole induced apoptosis in breast cancer MCF-7aro cells by down-regulation of Bcl-2, *cyclin D1* and c-*myc*, up-regulation of Bax, *p53* and *p21* and activation of caspases-7, -9 and -6. These non-steroidal AIs caused a cell cycle arrest in G1-S phase transition (87, 125). Moreover, other studies have shown that anastrozole and letrozole are better than fulvestrant in suppressing tumor growth (122).

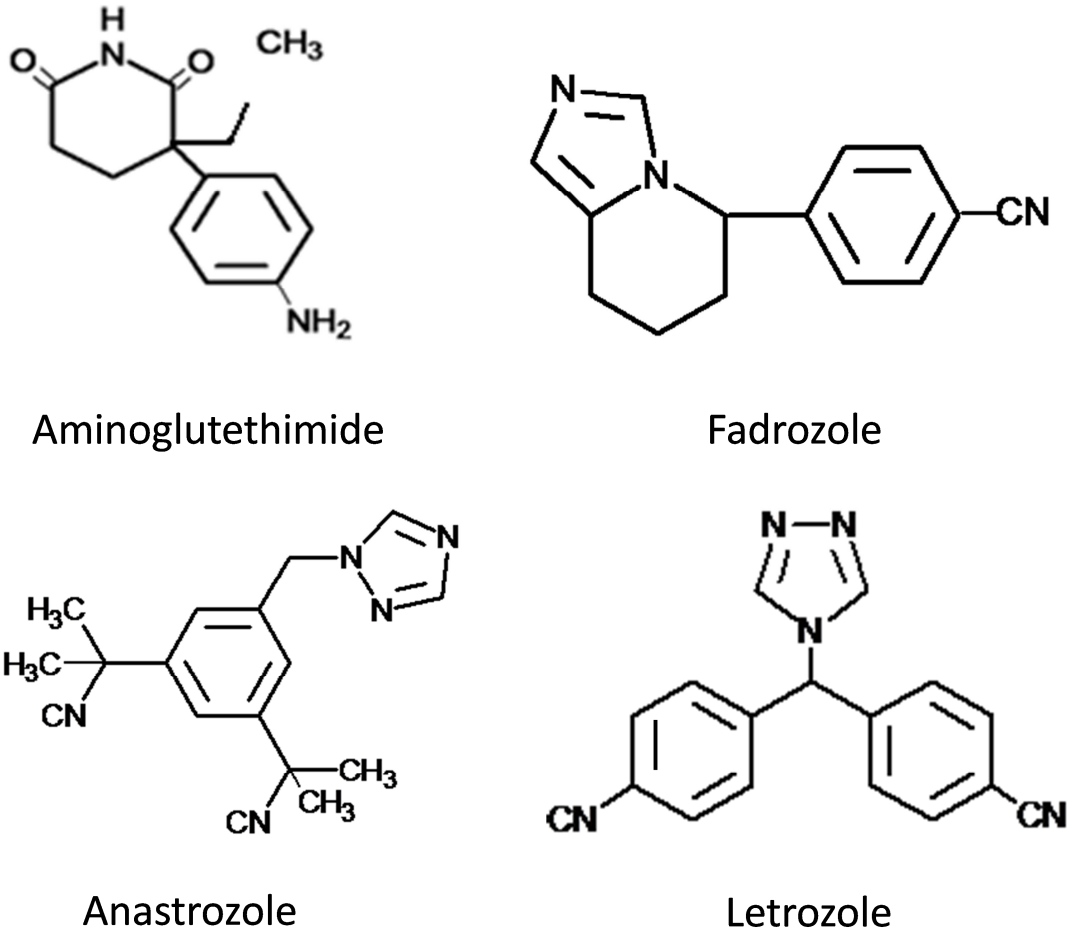

Figure 10: Chemical structure of the non-steroidal AIs aminoglutethimide, fadrozol, anastrozole and letrozole

3.2.2. Steroidal AIs

One of the earliest steroidal AIs used in the treatment of breast cancer was testolactone (figure 11). This AI has a structure similar to testosterone. Studies have shown that testolactone administration is associated with a decrease in serum levels of estrone. This may explain the anti-tumor activity of this steroid inhibitor (126).

The second-generation AI, the formestane or 4-hidroxiandrostenedione, is a selective, specific and effective AI. Formestane is administered intramuscularly, 200 to 500 mg every two weeks (127), but this form of administration causes several adverse reactions especially at administration site and, because of that, formestane is no more used in clinic (128).

Exemestane, discovered in the United States, is the only steroid third generation AI (129), binds irreversibly to the active site of aromatase leading to its modification and degradation (130). Therefore, its administration significantly reduces estrogen levels.

Exemestane (Aromasin ®) is prescribed for the treatment of postmenopausal women diagnosed with breast cancer ER^+ in early or advanced stage and in situations whose progression occurred after treatment with antiestrogens. Clinical studies have shown that suppression of estrogen levels is achieved by oral administration of 25 mg daily, resulting in 98% inactivation of aromatase (131). An oral daily dose of exemestane is rapidly absorbed with maximum plasma concentrations achieved after 2 hours. Its half-life is 27 hours, and then is eliminated by the liver and kidney (132). Its main metabolites are 17β-hydroexemestane and 6-hydroxymetylexemestane, which remain biologically active (133). The 17β-hydroexemestane metabolite has high affinity for the androgen receptor (AR) (84). In general, treatment with exemestane is successful, being possible to observe an improvement in clinical status and survival of patients diagnosed with breast cancer compared to tamoxifen treatment.

Like all AIs, exemestane has adverse effects but compared to tamoxifen the occurrence of thromboembolism (100) and gynecological symptoms are lower (134). It was also shown that this AI does not have major impact on cholesterol and lipoproteins levels (135). Although some studies have demonstrated that it induced the occurrence of bone fractures, others indicated that given its androgenic effect, this AI causes less bone loss than anastrozole or letrozole (84). The occurrence of resistance is another side effect verified during the treatment with exemestane. The mechanisms involved are an increase on MAPK pathway activity (136), a decrease on expression of ER (137) and a promotion of cell cycle progression (138).

In vitro studies demonstrated that exemestane induced antiproliferative effects in MCF-7aro cells, such as cell cycle arrest by blocking G0-G1 and G2-M phase transition and apoptosis. The latter was associated with loss of mitochondrial transmembrane potential, increase in caspases-9 and -7 activities and in reactive oxygen species (ROS) production. Moreover, this study showed that exemestane induced autophagy, though it was suggested that this process acted as a pro-survival mechanism (139).

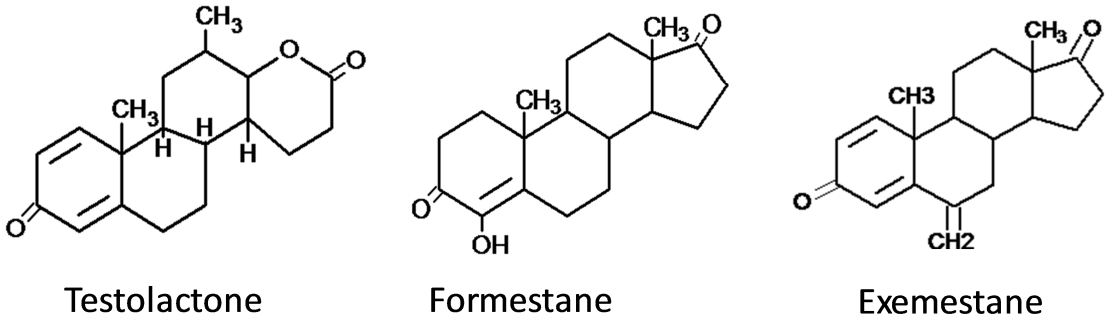

Figure 11: Chemical structure of the steroidal AIs testolactone, formestane and exemestane

Aim of the study

One of the therapeutic strategies for estrogen receptor-positive (ER^+) is the use of aromatase inhibitors (AIs) that inhibit the enzyme aromatase, which catalyzes the final step of estrogens biosynthesis.

Although, the AIs used in clinic proved to be effective, they cause some serious side effects including bone loss and the development of resistance. For this, the search for novel potent compounds, with fewer side effects, is currently needed. In this sense, we aim to further study in breast cancer cell lines the effects of four new steroidal compounds (**57**, **58**, **59** and **60**) selected from a series of compounds, synthesized from structural modifications on the aromatase substrate that previously demonstrated to be potent AIs in human placental microsomes. We intend to determine the anti-aromatase activity in breast cancer cells and study the biological effects of these compounds in an ER^+ aromatase-overexpressing human breast cancer cell line (MCF-7aro cells), and in an ER^- human breast cancer cells (SK-BR-3)

With this study we pretend to contribute to the understanding of the most favorable structure modifications in androstenedione in order to design/synthesize new steroidal compounds as potential AIs. Furthermore, this study can clarify the mechanisms involved in the inhibition of growth and induction of cell death of breast cancer cells using AIs and contribute to the development of more potent compounds with lower side effects.

Chapter II

Materials and 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 and trypsin 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, Casodex (CDX), propidium iodide (PI), Triton X-100, DNase-free RNase A, staurosporine (STA), charcoal, carbonyl cyanide m-chlorophenylhydrazone (CCCP), 2′,7′-dichlorodihydrofluorescein diacetate (DCDHF2-DA), phorbol 12-myristate 13-acetate (PMA), Trypan blue, protease inhibitor cocktail, Fluoroshield mounting medium, dextran and formestane were from Sigma–Aldrich Co. (Saint Louis, USA). Giemsa stain was purchased from Merck (Damnstadt, Germany). DPX mounting medium was from VWR (Radnor, PA, USA). Caspase-Glo® 3/7, Caspase-Glo® 9 luminometric assays, Cyto-Tox 96 nonradioactive cytotoxity assay kit and Reporter Lysis buffer 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). Z-VAD-FMK was from BD Biosciences Pharmingen (San Diego, CA, 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).

2. Compounds

In this work we focused on the biological evaluation of four new potential steroidal AIs (**57**, **58**, **59** and **60**) synthesized from structural modifications on the aromatase substrate, androstenedione. These 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 -80 ºC. Testosterone (T) and Estradiol (E_2) were prepared in absolute ethanol at 10 µM and stored at -20 ºC. For each assay the stock solution of compounds was diluted with the cultured medium to obtain the final working concentrations.

4. Cell cultures

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

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 human fibroblast cell line, 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.

The culture medium was renewed every three days and the cells were maintained at 37 ºC and 5% CO_2 atmosphere, to ensure that there is a significant cell proliferation and stable nutritional levels. After reaching 80 to 90% confluence, the cells were washed with PBS and detached with trypsin/EDTA 1 nM for 2 minutes at 37 ºC and 5% CO_2 atmosphere. After, the cells were centrifuged in culture medium with FBS or CFBS to inactivate trypsin at 260 xg and 4 ºC for 5 minutes. Finally, and depending on the culture

dishes, the cells were resuspended with the necessary amount of culture medium, and after homogenization, the cells were counted in a Neubauer chamber and cultured. The culture medium and drugs were refreshed every three days.

5. Preparation of charcoal heat-inactivated fetal bovine serum (CFBS)

FBS (500 ml) was incubated with 8 g of activated charcoal for 24 hours at room temperature, followed by successive centrifugations at 4000 xg for 15 min for removing the steroids present in FBS. Between each centrifugation the supernatants were filtered to eliminate charcoal particles. After the final centrifugation, supernatant was filtered by a vacuum system through a filter cellulose acetate with a 0.22 μm pore size aliquoted and kept at -20 ºC.

6. Preparation of charcoal pellets

Charcoal pellets were previously prepared by adding 1 mL of a solution constituted by 0,5% of dextran and 5% of activated charcoal solution to eppendorf tubes and centrifugation at 14000 xg for 10 minutes. After the supernatant was discarded and the pellets were dry at 37 ºC overnight.

7. In cell aromatase assay

The inhibition of the activity of aromatase enzyme for each compound on MCF-7aro cells was determined according to the method of Thompson & Siiteri (140) and Zhou et al. (141) with some modifications. This assay is based on the use of [1β-^{3}H] andro-4-ene-3,17-dione as aromatase substrate. In this assay it was measured the tritiated water released from the substrate during the aromatization process. Each experiment included a negative control, which does not contain the potential inhibitors. Formestane (1 μM) and exemestane (10 μM) were used as a positive control.

MCF-7aro cells were cultured in 24-well plates at a cell density of 1×10^{6} during 3 days. After this time, the medium was removed and cells were washed two times with PBS. Then, it was added 500 nM of progesterone to suppress the activity of 5α-reductase that also uses androgen as a substrate, 50 nM of [1β-^{3}H] androstenedione and 10 μM of

formestane, exemestane or of the new compounds to a final volume of 500 µl of MEM. The [1β-^{3}H] androstenedione was the last component to be added. Cells were incubated at 37 ºC and 5% CO2 atmosphere during 1 hour.

After incubation, the aromatization reaction was stopped with 100 µL of 20% trichloroacetic acid. Then the supernatants were transferred to eppendorf tubes, previously prepared with a pellet of activated charcoal, followed by an incubation period of 1 hour at room temperature. After centrifugation at 14000 xg for 10 minutes, 500 µl of supernatants were transferred to new eppendorf tubes with activated charcoal, homogenized and incubated for 10 minutes at room temperature. The tubes were centrifuged at 14000 xg for 10 min and the supernatant was transferred to clean eppendorfs followed by the last centrifugation cycle at 14000 xg for 5 min. To tubes contains 3 ml of scintillation cocktail it was added 300 µl of supernatant. The counts per minute were read in a scintillation counter (LS 6500, Beckman Instruments, CA, U.S.A.). Cells treated with 10 µM of formestane and exemestane were considered as positive control and untreated cells as control. This assay was performed in two independent experiments in triplicate for each compound.

The cells grown on 24 wells plates were lysed with 500 µl of 0.5 N NaOH and incubated overnight at room temperature with stirring to extract the proteins to quantification. Cells were then freezed at -80 ºC. The protein content was quantified by the Bradford method and used to normalize the radioactivity determined per µg of protein.

8. Cell viability assay

The effects of each steroidal compound (**57**, **58**, **59** and **60**) on cell viability of MCF-7aro, SK-BR-3 and HFF-1 cell lines was determined by measuring the mitochondrial reductases activities using the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-difenyltetrazolium bromide (MTT) that forms a blue formazan precipitate. Thereby, this colorimetric assay provides a percentage of cells capable to convert MTT to formazan. The cells were cultured in 96-well plates and incubated during 3 and 6 days with different concentrations of each compound. Untreated cells were used as control.

MCF-7aro cells were cultured at a cellular density of $2x10^{4}$ and $1x10^{4}$ cells/mL (for 3 and 6 days, respectively), in MEM without phenol-red with 5% CFBS and 1 nM of T or 1 nM of E2. SK-BR-3 cells were cultured at a cellular density of $2.5x10^{4}$ and $1x10^{4}$ cells/mL

(for 3 and 6 days, respectively), in MEM with phenol-red containing 10% FBS . HFF-1 cells were cultured for 6 days at a cellular density of $7.5x10^3$ cells/mL in DMEM without phenol-red containing 10% FBS. After the incubation period (3 or 6 days) it was added 20 µL of MTT (0.5 mg/mL) per well and cells were incubated during 2 hours and 30 minutes in optimal conditions for growth. After this time, the medium was removed and it was added 200 µL DMSO: isopropanol mixture (3:1), to stop the reaction and dissolve the purple formazan precipitated, for 20 minutes with agitation at room temperature. The formazan was quantified spectrophotometrically at 540 nm.

The cytolitic effects of each steroidal compound were evaluated in MCF-7aro cell line using the lactate dehydrogenase (LDH) assay. It is a cytosolic enzyme that is only released from cells to the culture medium after membrane disruption. LDH activity was measured using a LDH KIT (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega Corporation) according to manufacturer's protocol. This assay was performed in the same experimental conditions described for the MTT assay.

9. Western blot analysis

MCF-7aro cells were cultured in 6-well plates at a cellular density of $6x10^5$ cells/mL during 3 days in MEM with red phenol. Then, cells were treated with each compounds (10 µM) and incubated during 8 hours. After the incubation period, cells were washed two times with PBS and 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) and 1% of a protease inhibitors cocktail. After, cells were scrapped and collected to eppendorf tubes and centrifuged at 14000 xg for 10 minutes at 4 ºC. Protein concentrations were determined using the Bradford assay. A total of 50 µg of protein per sample was loaded on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes in 25 mM Tris-HCl, 250 mM glycine and 18% methanol. The membranes were blocked with 5% milk in TBS TWEEN (1x) during 1 hour. Immunodetection was performed using goat polyclonal antibody anti-CYP19 (1:100) (Santa Cruz Bio Technology, Inc.) in blocking solution overnight at -4 ºC. The secondary rabbit anti-goat antibody (1:2000) was incubated for 1 hour. Membranes were exposed to chemiluminescence substrate Super Signal West Pico and then visualized by Chemidoc Touch Image (BioRad, Laboratories Melville, NY, USA). Membranes were then 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.

10. Morphological studies

The morphological alterations in MCF-7aro cells caused by each compound (**57**, **58**, **59** and **60**) were studied by phase contrast microscopy and by Giemsa and Hoescht staining. The cells were cultured in 24-well plates with coverslips at a cellular density of $2x10^5$ and $1x10^5$ (for 3 and 6 days, respectively) in MEM without phenol-red containing 5% CFBS plus 1 nM of T. After 24 h, cells were incubated with each steroidal compound at the concentration of 10 μM.

For Giemsa staining, cells were washed with PBS, fixed with methanol during 25 minutes at 4 ºC and washed twice with PBS. After, the cells were stained with Giemsa diluted in PBS (1:10) for 30 minutes. After incubation the stained cells were washed with water and the coverships were mounted with DPX and observed on a bright field microscope (Eclipse E400, Nikon, Japan) equipped with the image analysis software LeicaQwin.

The other assay used to evaluate the alterations in nuclear morphology namely chromatin fragmentation and condensation was the Hoescht staining. The cells were fixed with 4% paraphormaldehyde for 20 minutes at 4 ºC, washed twice with PBS and exposed to 0.05 μg/mL of Hoechst 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.

11. Cell cycle analysis

This technique is used to evaluate the effect of the compounds on cell cycle progression. The cells were cultured in 6-well plates at a density of $7x10^5$ cells/mL in MEM without phenol red containing 5% CFBS. After 24 hours, the cells were incubated with different compounds (10 μM) plus 1 nM of T for 3 days. Untreated cells were used as control.

After the incubation period the medium was collected to recover the unadherent cells. Cells were washed with PBS. Then, they were trypsinized with 2.5% trypsin/EDTA 1 mM and transferred to separate centrifuge tubes containing 1 mL of culture medium with 5%

CFBS. Next, were centrifuged at 260 x*g* for 6 minutes at 4 ºC and supernatant was rejected. Cells were resuspended and fixed in 70% cold ethanol and stored at 4 ºC for 24 hours. After this period, fixed cells were centrifuged at 260 x*g* for 6 minutes at 4 ºC (3 times). The supernatant was discarded between each centrifugation, and the pellet was ressuspended in PBS. The last stage of this procedure was the addition of 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 incubation overnight at 4 ºC. PI is a fluorescent dye that intercalates nucleic acids, enabling DNA content determination. Permeabilization of membranes was achieved by using Triton X-100. Degradation of RNA present by DNase-free RNase A ensures that in the sample is present only DNA. The analysis was performed by flow cytometry and 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. Assays were performed in triplicate and in three independent experiments.

12. Cell death analysis

12.1. Caspase activity

In order to study the involvement of apoptosis, caspase activities were evaluated by the Caspase-Glo® 3/7, Caspase-Glo® 9 luminescence assays. These enzymes are members of the cysteine aspartic acid-specific protease family and play key effector roles in apoptosis. Caspase 3/7 activation is used as an indicator of occurrence of apoptosis, however in the cell line used in the study it was only evaluated the activity of caspase-7 as this cell line does not express caspase-3. Caspase-9 plays a key role in the intrinsic apoptotic pathway.

Cells were cultured in 96-well white plates at a cellular density of $2x10^4$ cells/mL in MEM without phenol-red containing 5% CFBS. After 24 hours, cells were incubated with the compounds (1-25 µM) and 1 nM of T for 3 days. Untreated cells plus 1 nM of T were used as control. After treatment, the Caspase-Glo® 3/7 or -9 luminescent assay was used according to manufacturer's instructions. As a positive control, cells were incubated with staurosporine (STA) (10 mM) for 3 hours. Z-VAD-FMK (50 mM), a pan-caspase inhibitor, was used as a negative control. The resultant luminescence was measured in a 96-well

Microplate Luminometer (BioTek Instruments, USA) and presented as relative light units (RLU). Three independent experiments were performed in triplicated.

12.2. Intracellular reactive oxygen species (ROS)

In order to study the levels of intracellular reactive oxygen species (ROS) the 2', 7'-dichlorodihydrofluorescein diacetate (DCFH2-DA) method was used. This is a lipophilic non-fluorescent compound that can cross the cell membrane and through the action of intracellular esterases is deacetylated to 2', 7'-dichlorodihydrofluorescein (DCHF2), which reacts with intracellular ROS and is then oxidized into the final compound involved in this assay, the fluorescent compound 2', 7'-dichlorofluoresceinn (DCF).

The cells were culture 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 hours, cells were incubated with the compounds (1-25 μM) and 1 nM of T during 3 days. Untreated cells plus 1 nM of T were considered as control. Cells were incubated for 2 hours with a positive control, the phorbol 12-myristate 13-acetate (PMA) at 25 ng/mL and labeled with 50 μM of DCFH2-DA for 1 hour at 37 ºC. The fluorescence was measured using an excitation wavelength of 480 nm and an emission filter of 530 nm, in a 96-well microplate luminometer (Synergy HT, BioTek, USA). The results were presented as mean fluorescence intensity (MFI). All assays were performed in triplicate and in three independent experiments.

13. Statistical analysis

For statistical analysis it was used GraphPad Prism software 7. Analysis of variance (ANOVA) was followed by Bonferroni multiple comparisons test. P values <0.05 were considered statistically different. 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 explore the anti-aromatase activity of the new compounds in breast cancer cells, it was performed the cell aromatase assay. MCF-7aro cells were treated with the compounds **57**, **58**, **59** and **60** and the tritiated water released from the [^{3}H] androstenedione during the aromatization reaction was determined to evaluate the anti-aromatase activity. All the compounds induced a drastic decrease in aromatase activity, showing that these new steroids are potent AIs in this aromatase overexpressing breast cancer cell line (figure 12).

Compound **58** is the most potent AI with 98% of inhibition, being this inhibition similar to the reference AIs used, formestane and exemestane. Compound **57** caused a 94% of inhibition, compound **60** a 89% of inhibition and compound **59** a 90% of inhibition.

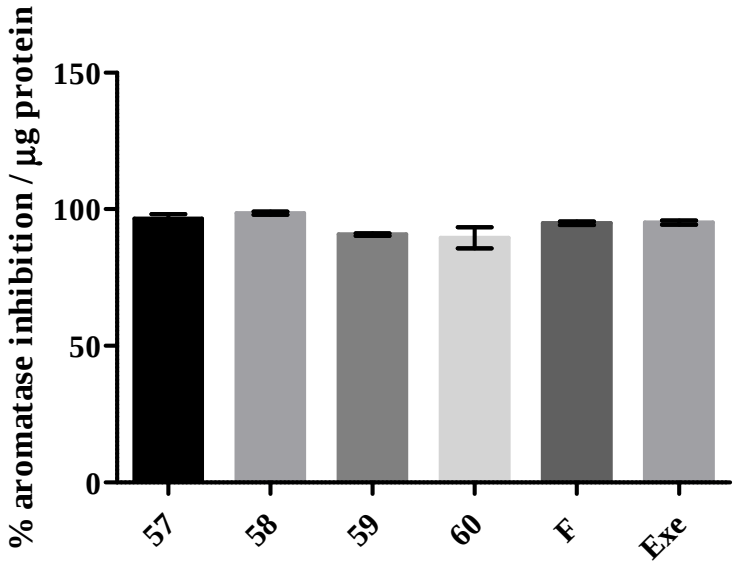

Figure 12: Aromatase activity in MCF-7aro cells. Cells were treated with the steroidal compounds (**57, 58, 59** and **60**) at 10 µM. Formestane (F) and Exemestane (Exe) were used as a reference AIs. Data are presented as a percentage of the tritiated water release in relation to control cells (100%) and correspond to two independent experiments performed in triplicate.

2. Cell viability assay in HFF-1 cell line

In order to understand the effects of these compounds on a non-cancerous cell line and its cytotoxic behavior it was evaluated the cell viability of HFF-1, a human fibroblast cell line, by MTT assay, after 6 days of treatment. The compounds (1-25μM) did not induce a significant reduction in cell viability (figure 13).

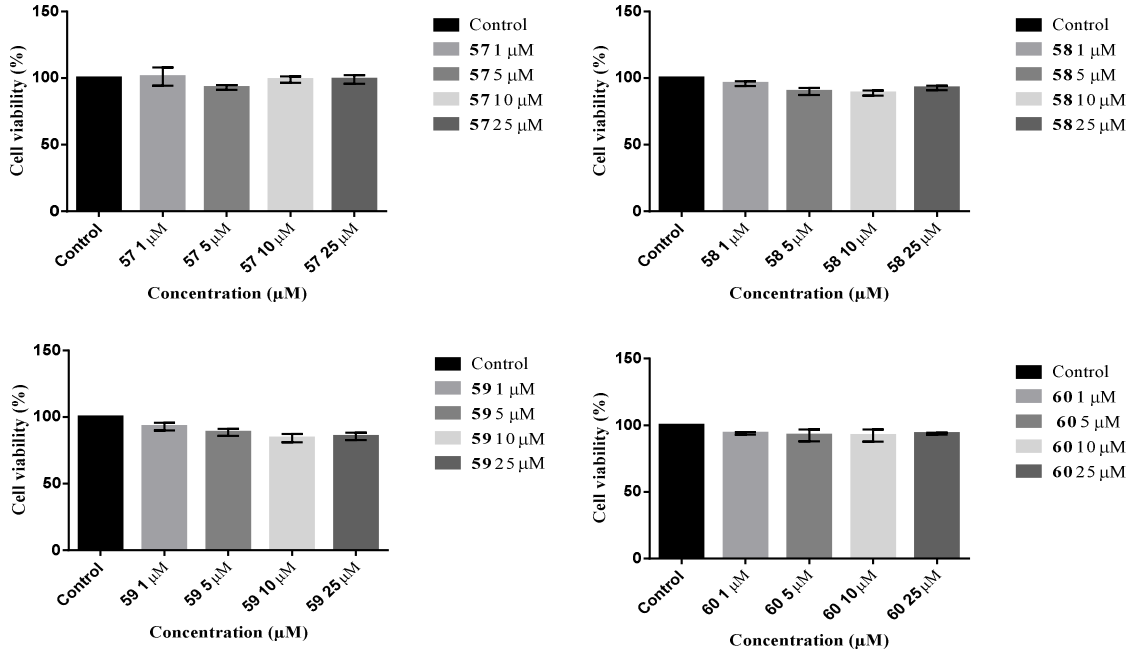

Figure 13: Effects of steroidal compounds on HFF-1 cell viability analysed by MTT. HFF-1 cells were treated with different concentrations of compounds (1-25 μM) during 6 days. Compounds caused no significant reduction in cell vibility. Cells cultured withouth treatment were considered as controls. Results are the mean ± SEM of three independent experiments done in triplicate.

3. Effects on MCF-7aro cell viability

The effects of AIs in MCF-7aro cell viability were studied by MTT and LDH assays. The cells were incubated with the compounds **57**, **58**, **59** and **60** (1-50 µM) and stimulated with T (1 nM) during 3 and 6 days. As shown in figure 14, all the compounds induced a significant ($p<0,01$; $p<0,001$; $p<0,0001$) decrease in cell viability in a dose- and time-dependent manner.

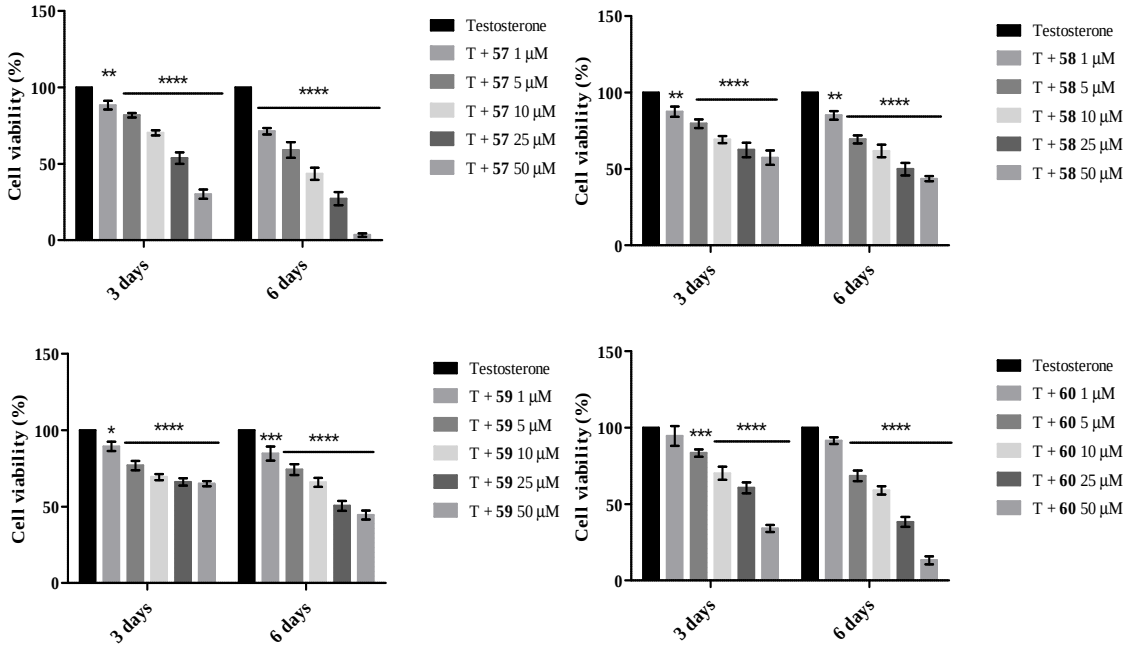

Figure 14: Effects of steroidal compounds on MCF-7aro cell viability analysed by MTT. MCF-7aro cells were stimulated with Testosterone (T) and treated with different concentrations of compounds (1-50 µM) during 3 and 6 days. Cells cultured with T were considered as controls. All the compounds induced a reduction in cell viability. Results are the mean ± SEM of three independent experiments done in triplicate. Significant differences between the control and treated cells are denoted by ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

The compounds **57** and **60** were the most effective in reducing cell viability, especially for higher concentrations. To evaluate if this decrease in cell viability was associated with membrane disruption it was determined the lactate dehydrogenase (LDH) activity in the cell culture medium. As illustrated in figure 15, except for compound 60 at

50 μM, that caused a significant (p<0,001) increase in LDH release, the other compounds did not cause membrane disruption. Thus from now on it was only used concentrations lower than 50 μM.

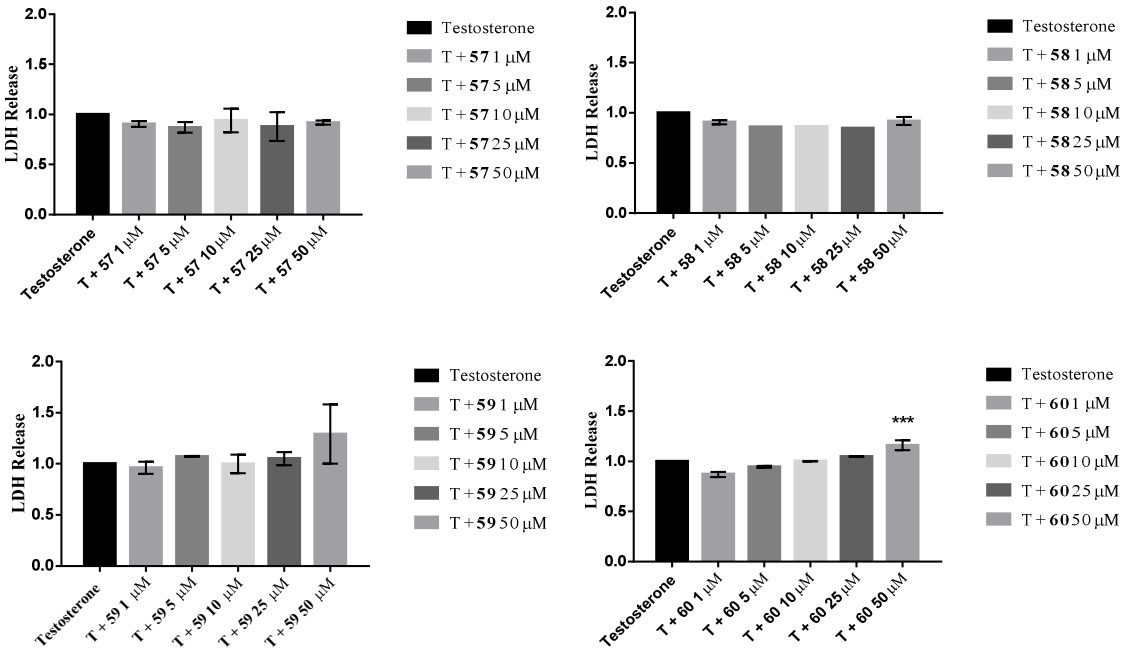

Figure 15: Effects of steroidal compounds on MCF-7aro cell membrane integrity analysed by LDH assays. MCF-7aro cells were stimulated with testosterone (T) and treated with different concentrations of compounds (1-50 μM) during 3 and 6 days. Cells cultured with testosterone (T) were considered as controls. Any of the compounds caused LDH release. Results are the mean ± SEM of three independent experiments done in triplicate. Significant differences between the control and treated cells are denoted by *** ($p < 0.001$).

4. Characterization of the mechanism of AIs action: dependence on aromatase and androgen-receptor

In order to understand if the effects on MCF-7aro cell viability were dependent on aromatase, the cells were incubated with different concentrations of compounds in the presence of E2, during 3 and 6 days (figure 16). The results were compared with the ones obtained with T treatment. All compounds induced a decrease in E2-treated cells viability and it was concluded that compounds **57**, **59** and **60** are aromatase independent, because the effects are similar to the obtained with T. However, compound **58** is aromatase dependent as the results obtained with E2 treatment are significantly different from the results with T (figure 17).

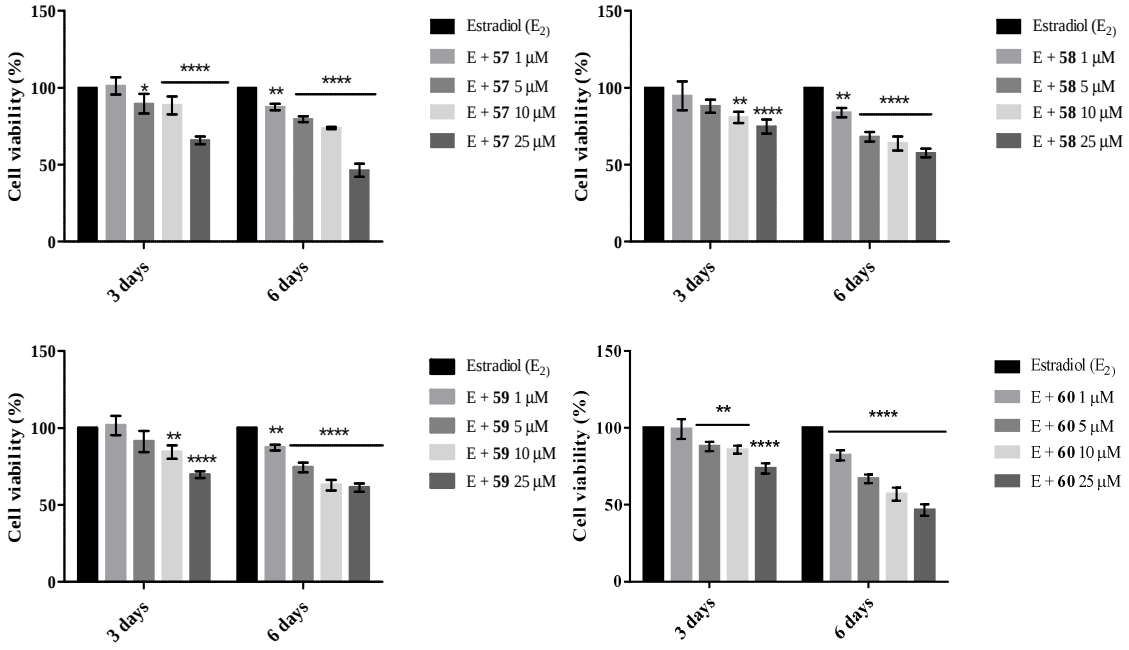

Figure 16: Effects of steroidal compounds on viability of E2-treated MCF-7aro cells, analysed by MTT. MCF-7aro cells were stimulated with Estradiol (E2) and treated with different concentrations of compounds (1-25 µM) during 3 and 6 days. Cells cultured with E2 were considered as controls. All the compounds induced a reduction in cell viability of E2-treated cells. Results are the mean ± SEM of three independent experiments done in triplicate. Significant differences between the control and treated cells are denoted by ** ($p < 0.01$) and **** ($p < 0.0001$).

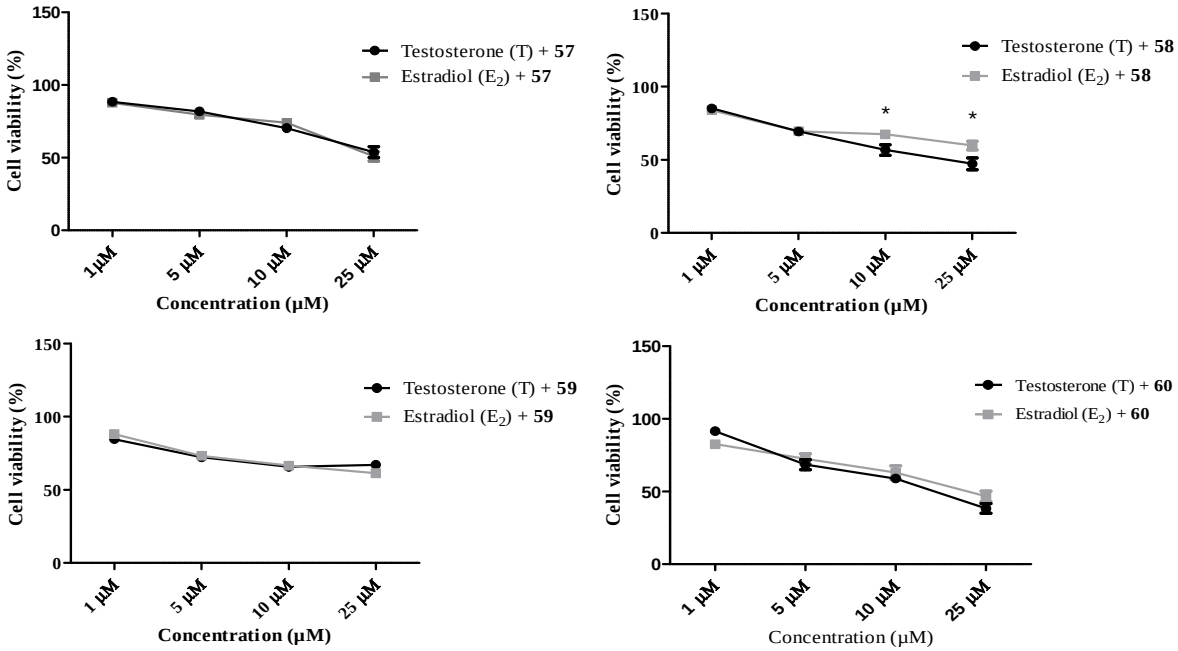

Figure 17: Comparison of the effects of compounds 57, 58, 59 and 60 (1 -25 µM) in viability of MCF-7aro cells stimulated with testosterone (T) or estradiol (E_2), during 6 days. Compounds **57**, **59** and **60** induced a reduction in MCF-7aro cell viability in an aromatase-independent manner, while compound **58** caused an aromatase-dependent effect Results are the mean ± SEM of three independent experiments done in triplicate. Significant differences between the E2-treated versus T-treated cells are denoted by * ($p < 0.05$).

It was also used an antagonist to androgen receptor (AR), Casodex (CDX), to verify if the effects of compounds were dependent on AR. MCF-7aro cells were treated with T and CDX and incubated with the different concentrations (1-25 µM) of compounds, during 3 and 6 days (figure 18). All the compounds induced a decrease in viability of CDX-treated cells. Moreover, by comparison with the results obtained with T-treated cells, it was observed that only compound **58** caused a significant difference in cell viability between treatments (figure 19) indicating an androgen-dependent effect.

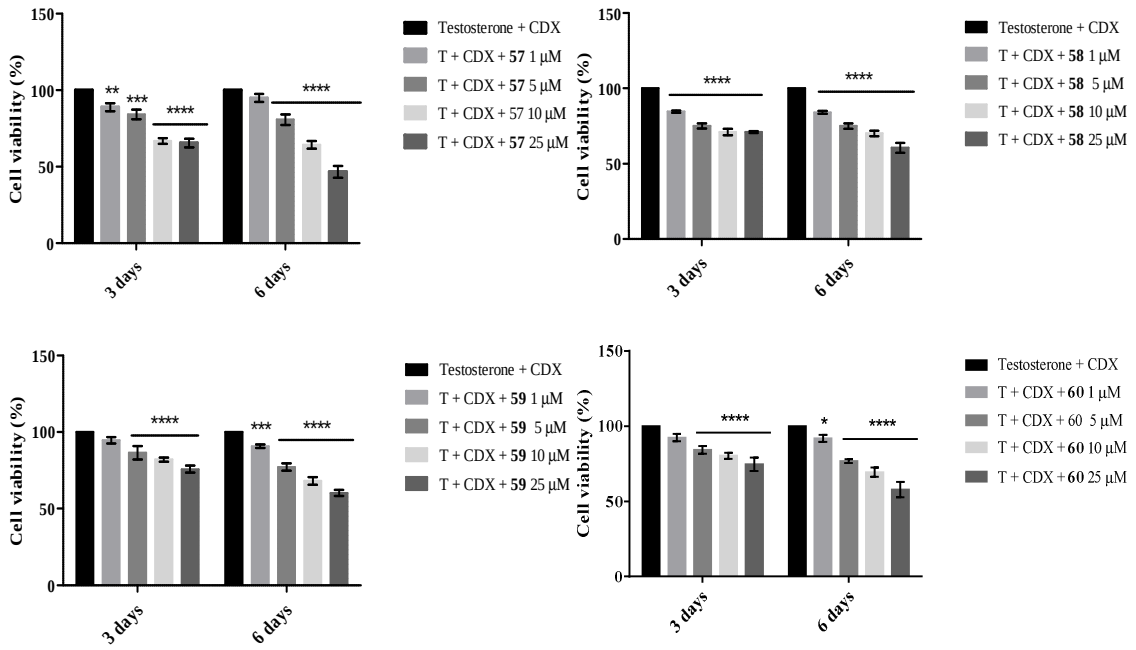

Figure 18: Effects of steroidal compounds on CDX-treated MCF-7aro cells viability analysed by MTT. MCF-7aro cells were incubated with Casodex (CDX) and treated with different concentrations of compounds (1-25 µM) during 3 and 6 days. Cells cultured with T + CDX were considered as controls. All the compounds induced a reduction in cell vibility. Results are the mean ± SEM of three independent experiments done in triplicate. Significant differences between the control and treated cells are denoted by * ($p < 0.1$), ** ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

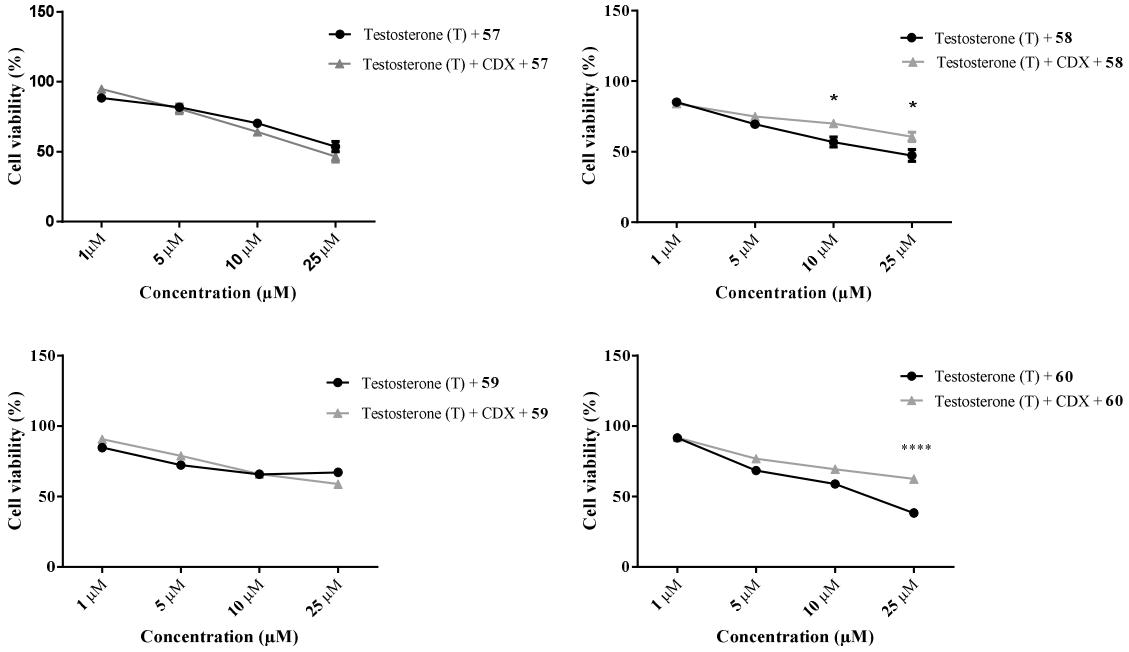

Figure 19: Comparison of the effects of different concentrations of compounds 57, 58, 59 and 60 (1 -25 µM) in viability of MCF-7aro cells stimulated with testosterone (T) and with or without Casodex (CDX), during 6 days. Only compound **58** caused an AR-dependent effect. Results are the mean ± SEM of three independent experiments done in triplicate. Significant differences between the T-treated versus CDX-treated cells are denoted by * ($p < 0.05$), **** ($p < 0.0001$).

5. Western blot analysis

To better understand the dependence on aromatase it was analysed, by western blot assay, the aromatase expression. The results showed that as exemestane, only compound **58** induced a significant ($p < 0,01$) decrease in aromatase levels. These results suggest that this AI may cause aromatase degradation (figure 20).

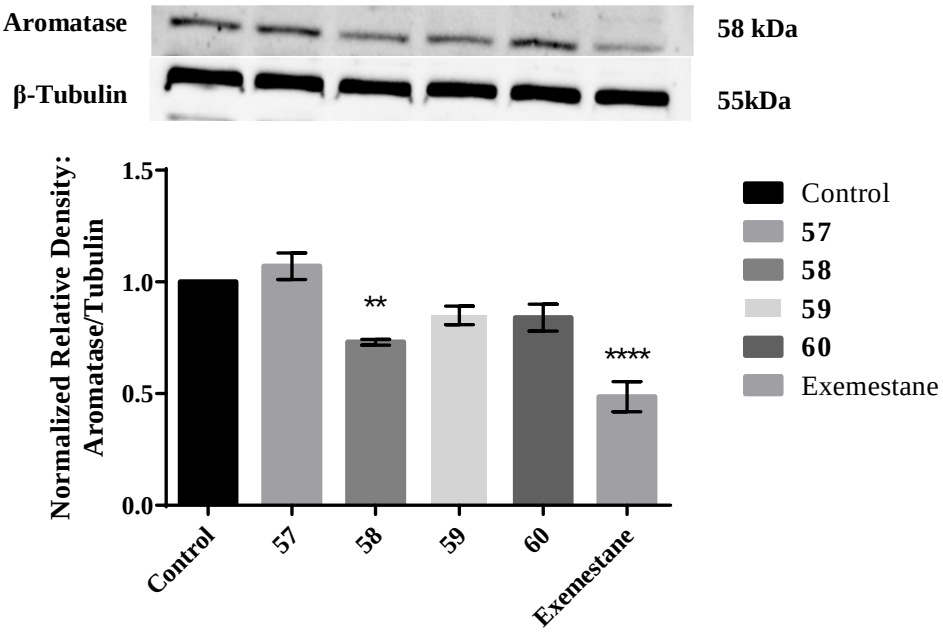

Figure 20: Western Blot analysis of Aromatase. MCF-7aro cells were treated with 10 μM of compounds during 8 hours. Exemestane (10μM) was used as a reference AI. Results are the mean ± SEM of three independent experiments done in triplicate. Significant differences between the control and treated cells are denoted by ** ($p < 0.01$), and **** ($p < 0.0001$).

6. Cell viability in E2-treated MCF-7aro cells versus SK-BR-3 cells

To understand if the effects of the compounds were dependent on estrogen-receptor (ER) it was used an ER^- human breast cancer cell line, SK-BR-3. For that, the cells were incubated with compounds **57**, **58**, **59** and **60** (1-25μM), during 3 and 6 days and the results compared with E2-treated MCF-7aro cells. Cells incubated without compounds were used as control.

As shown in figure 21, all the compounds induced a significant decrease in SK-BR-3 cell viability. Although, by comparing the effects in MCF-7aro cells incubated with E2 with the results obtained in SK-BR-3 cells, it was concluded that all compounds decreased MCF-7aro cell viability in an ER-dependent manner, since significant differences between treatments were observed (figure 22).

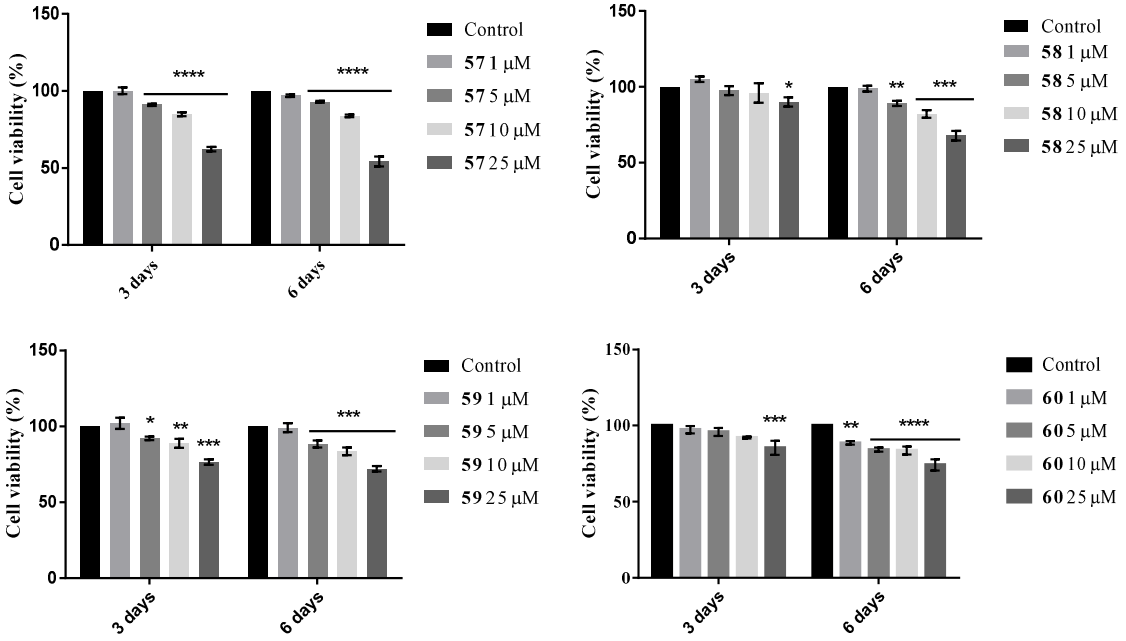

Figure 21: Effects of steroidal compounds on SK-BR-3 cell viability analysed by MTT. SK-BR-3 cells were treated with different concentrations of compounds (1-25 µM) during 3 and 6 days. All the compounds induced a reduction in cell vibility. Cells cultured without icubation with compounds were considered as controls. Results are the mean ± SEM of three independent experiments done in triplicate. Significant differences between the control and treated cells are denoted by * ($p < 0.1$), ** (p ($p < 0.01$), *** ($p < 0.001$) and **** ($p < 0.0001$).

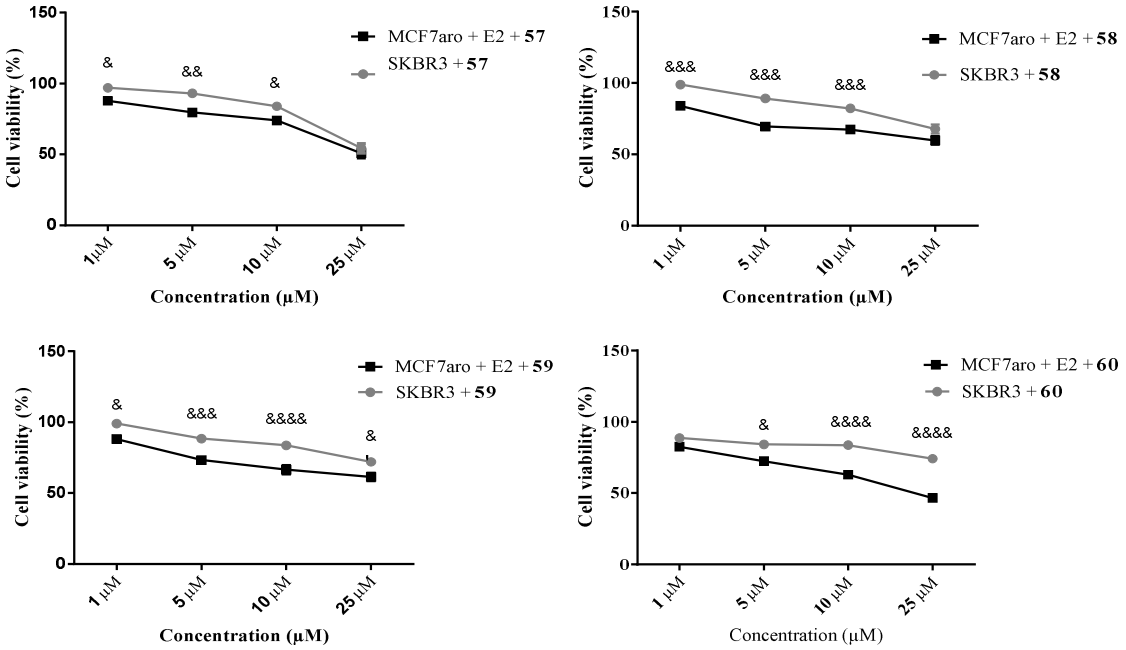

Figure 22: Comparison of the effects of compounds 57, 58, 59 and 60 (1 - 25 µM) in viability of MCF-7aro cells stimulated with Estradiol (E2) and in SK-BR-3 cells, during 6 days. Compounds induced a reduction in viability of MCF-7aro cells in a estrogen-dependent manner. Results are the mean ± SEM of three independent experiments done in triplicate. Significant differences between the E2-treated cells and SK-BR-3 cells are denoted by &($p < 0.05$), &&($p < 0.01$), &&&($p < 0.001$), &&&&($p < 0.0001$).

7. Morphological studies

To evaluate the morphological alterations induced by the compounds, MCF-7aro cells stimulated with T were treated with each steroidal compounds (10 µM) after 3 and 6 days, and observed in phase contrast microscopy, and after Giemsa and Hoechst staining (Figure 23).

After 3 and 6 days of treatment it was observed that all steroids caused a decrease in cell density and some morphological alterations, like membrane blebing, as observed by contrast phase microscopy, as well as chromatin condensation and fragmentation, typical features of apoptosis, observed after Giemsa and Hoechst staining. These morphological alterations were more evident after 6 days of AIs treatment (figure 23). In addition, the chromatin fragmentation was more evident for compound **57**.

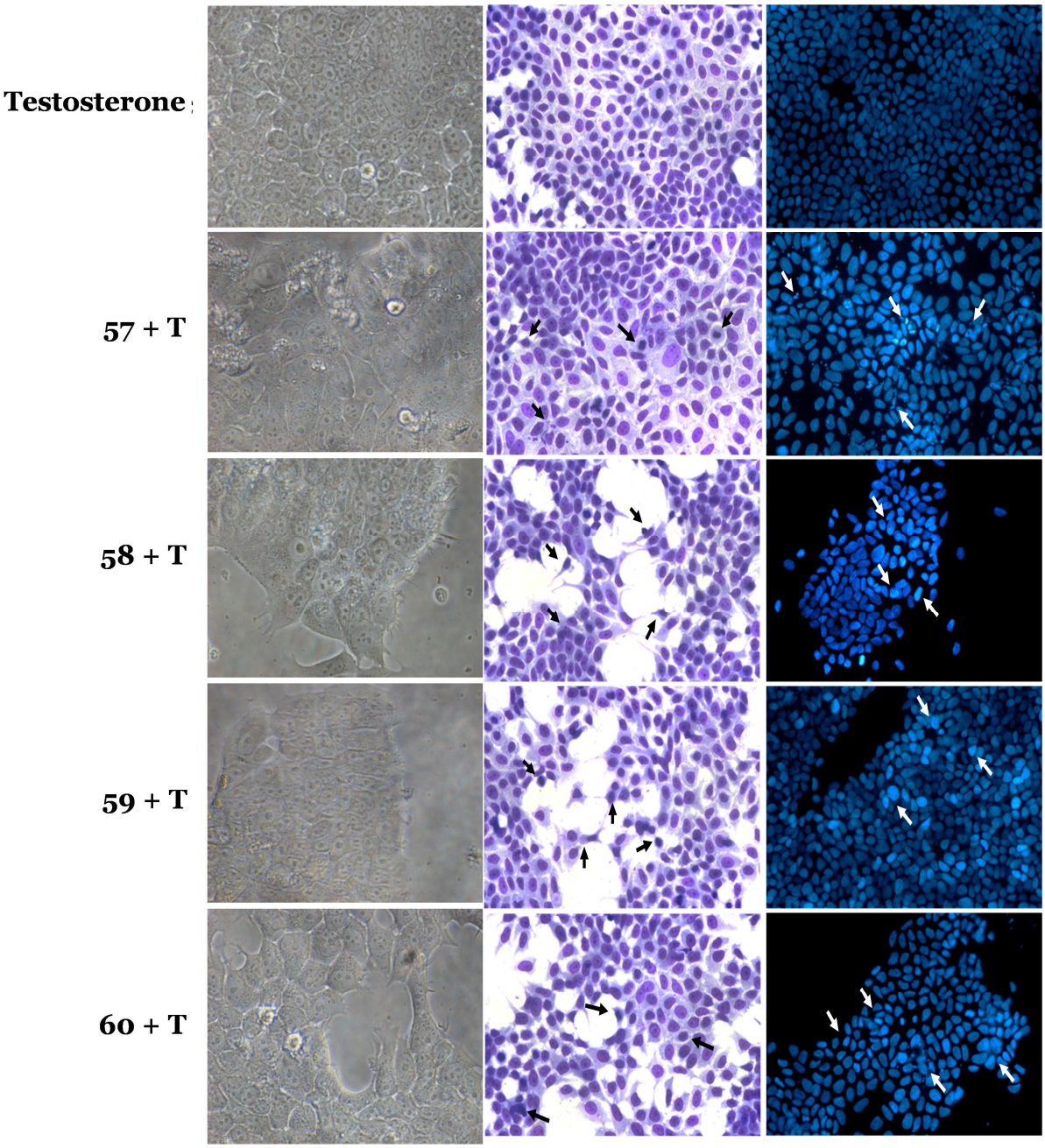

Figure 23: Effects of compounds 57, 58, 59 and 60 (10 μM) on MCF-7aro cells morphology after 6 days of treatment cells. Analyzed by phase contrast microscopy (A), Giemsa (B) and Hoechst (C) stainings, presented a decrease in cell density and some morphological alterations typical of apoptosis, such as chromatin condensation and fragmentation (filled arrows). Original magnification x400.

8. Cell cycle analysis

As the deregulation of the cell cycle and, consequently, lack of proliferation are possible mechanisms responsible for the effects of AIs on MCF-7aro cell viability, it was analyzed the cell cycle progression by flow cytometry after PI staining. PI is a fluorescent dye that intercalates DNA. According to the DNA content, it is possible to evaluate the distribution of cells in the correspondent cell cycle phases.

Results demonstrate that all the AIs caused a significant ($p < 0{,}0001$) cell cycle arrest in G0/G1 phase, after 3 days of treatment, with a consequent decrease in S and G2/M phases (figure 24; table 1). Compound **57** was the less potent one.

Table 1 - Effects of the different treatments on cell cycle in MCF-7aro cells for 3 days of incubation

Cell Cycle	G0/G1	S	G2/M
Testosterone	**73.34 ± 0.96**	**6.99 ± 0.37**	**19.72 ± 0,90**
T + 57	**79.38 ± 0.19 ******	**4.08 ± 0.43 ***	**16.61 ± 0.35 ***
T + 58	**83.64 ± 0.52 ******	**2.75 ± 0.30 *****	**13.62 ± 0.56 ******
T + 59	**85.92 ± 1.13 ******	**2.00 ± 0.20 ******	**12.00 ± 0.85 ******
T + 60	**82.20 ± 0.53 ******	**2.67 ± 0.19 ******	**15.26 ± 0.41 ******

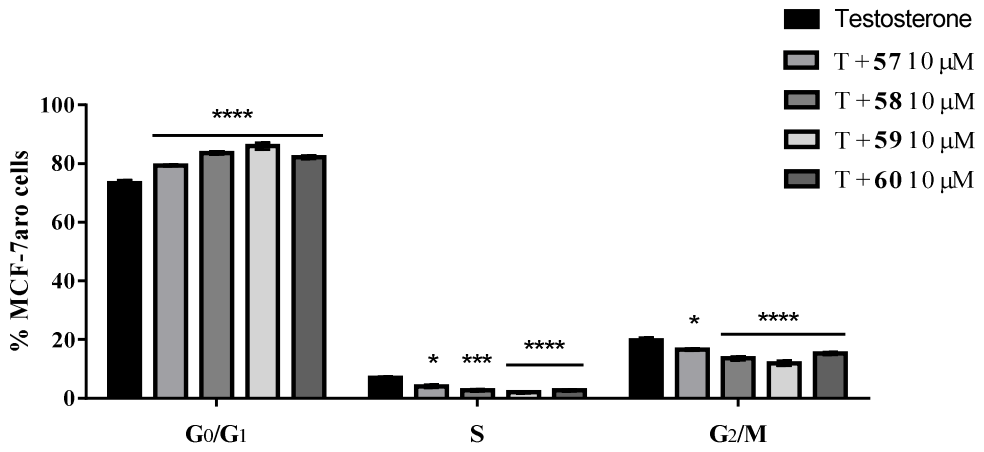

Figure 24: Effects of compounds on MCF-7aro cell cycle progression. Cells were treated with AIs (10 µM) plus T (1 nM), during 3 days and subjected to flow cytometric analysis after PI staining. Cells cultured with T were considered as control. Data represents means and SEM of of two independent experiments done in triplicates. Significant differences between the control T versus treated cells are indicated by * ($p < 0.05$), *** ($p < 0.001$) and **** ($p < 0.0001$).

9. Cell death analysis

As previously mentioned, these new AIs caused some morphological alterations typical of apoptotic mechanisms. Thus, to confirm these results the evaluation of caspase-7 and -9 activities was performed. As shown in Figure 25 all compounds induced a significant ($p < 0,05$; $p < 0,001$; $p < 0,0001$) increase in the activity of the initiator (-9) and of the executioner (-7) caspases.

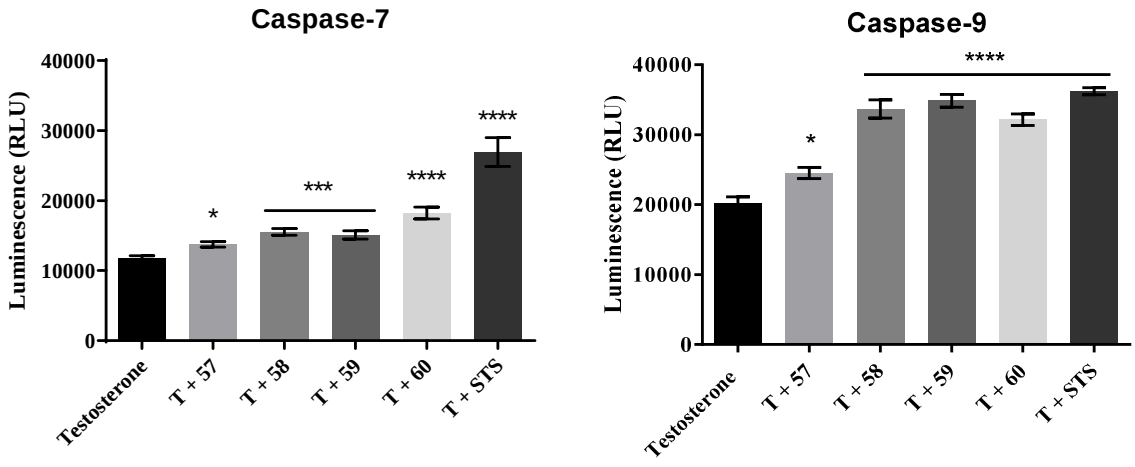

Figure 25: Activation of caspase-7 and -9 in MCF-7aro cells treated with AIs (10 μM) after 3 days of incubation. MCF-7aro cells cultured with T were considered as control. The cells treated with STS (10 μM) were considered as positive control. Results are presented as relative luminescence units (RLU) and correspond to the mean ± SEM of three independent experiments done in triplicate. Significant differences between the control T versus treated cells are indicated by * ($p<0.05$) *** ($p < 0.001$) and **** ($p < 0.0001$).

As there was an increase in the activity of the initiator caspase-9, suggesting the involvement of the mitochondrial pathway, the production of reactive oxygen species (ROS) was also determined. As shown in figure 26, none of the compounds induced ROS generation. Curiously, for AI **60** it was observed a significant decrease in ROS production.

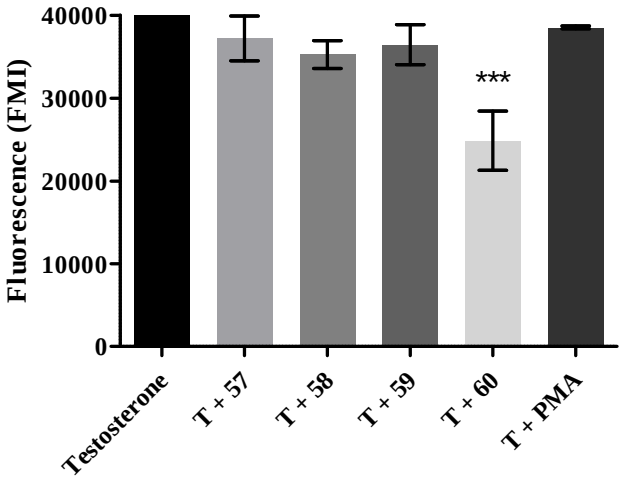

Figure 26: Formation of ROS in MCF-7aro cells treated with compounds after 3 days. Cells cultured with T were considered as control and cells treated with PMA were considered as a positive control for ROS production. The results are presented as mean fluorescence intensity (MFI) and correspond to mean ± SEM of three independent assays done in triplicate. Significant differences between the control T versus treated cells are indicated by *** ($p < 0.001$).

Chapter IV

Discussion

Estrogens play an important role in breast tumor growth due to the presence of a functional ER, which is expressed in 70-80% of these breast cancers cases (142). So a therapy that blocks the actions of estrogens and ER, as the endocrine therapy, is an important strategy for the treatment of this disease. During many years the tamoxifen was considered the standard therapy for pre- and postmenopausal women with breast cancer. However, in the last decades, the third-generation AIs are used in clinic to treat post-menopausal women as well as pre-menopausal women with breast cancer after ovary function supression. Nevertheless, the development of endocrine resistance and occurrence of bone loss are some drawbacks that limit the AIs success. Several strategies are being performed in order to overcome these disadvantages as the search and development of new compounds, more powerful and with lower side effects (143-145). In this way, the aim of this study was to understand the anti-tumor properties of four new steroidal compounds (**57**, **58**, **59** and **60**), selected from a series of new steroidal molecules derived from androstenedione. To achieve this goal it was investigated their anti-aromatase activity and their *in vitro* biological effects.

The selected compounds (**57**, **58**, **59** and **60**) presented high aromatase inhibition in placental microsomes. The majority of the studies were performed in a human breast cancer cell line that overexpresses aromatase, the MCF-7aro cells, a good model to study AIs in ER^+ breast tumors (125). In this study it was observed that all the four new compounds have an anti-aromatase activity superior to 80% in MCF-7aro cells, being **58** the most potent and the **60** the less potent AI. As all the compounds inhibit efficiently aromatase in breast cancer cells it was investigated their anti-tumor properties. As these compounds did not affect the viability of the non-cancerous fibroblastic cell line, HFF-1, it was evaluated their effects in MCF-7aro cells. All the compounds decreased the MCF-7aro cells viability. The compounds **57** and **60** were the ones that caused a more pronounced effect. Similar actions were also previously observed for exemestane (139) and for other AIs synthesized by our group (146, 147). In order to understand if these biological effects were dependent or independent on aromatase or AR, the effects of each AI were also studied in MCF-7aro cells treated with E2, the product of the aromatization reaction, or with Casodex (CDX), an AR antagonist. To understand the dependence of these compounds on ER, the results of MCF-7aro cells stimulated with E2 were also compared to the results obtained for the SK-BR-3 cell line, a breast cancer cell line that demonstrated to be a good model for hormone-independent (ER^-) breast cancer, since they do not expresse ERα (148). Only compound **58** caused an aromatase-dependent effect like the other steroidal AI used in clinic, exemestane (133) and also an AR- and ER-dependent effect. All the other compounds were only ER-dependent. Previous studies

demonstrated that exemestane by inactivation and degradation of aromatase by proteasome, induced a reduction in aromatase expression (149), effect that justifies the aromatase-dependent action of exemestane (133, 149). In this way, in order to understand if AIs action were due to a similar cellular mechanism, it was evaluated, by Western Blot, the aromatase expression on MCF-7aro cells. As expected a decrease in aromatase expression was only observed for the compound **58**.

Moreover, MCF-7aro cells treated with the new molecules presented a reduction in cell density and morphological alterations, such as membrane blebbing, chromatin condensation and nuclear fragmentation, features of apoptosis. Therefore, as the decrease in cell viability may be due to anti-proliferative effects or cell death it was carried out cell cycle analysis and evaluation of caspases activities. The results revealed that all the AIs caused cell cycle arrest in G0/G1 phase. Thus, it is possible to conclude that these new AIs have anti-proliferative effects in MCF-7aro cells. Studies performed for other AIs, including letrozole, anastrozole, exemestane (87, 139) and other steroidal AIs synthesized by the group (143, 146, 147), also showed that these compounds induced cell cycle arrest of ER^+ breast cancer cell lines overexpressing aromatase.

In addition, as one of the mechanisms that may be linked to the observed reduction of cell viability is the occurrence of apoptosis, it was explored the involvement of this phenomenon by evaluating the activation of caspase-7 and -9. All compounds caused a significant increase in caspase-7 activity in MCF-7aro cells, confirming the involvement of an apoptotic process. Compound **60** was the AI that presents a greater caspase-7 activation, wich was associated to the most drastic effects observed on MCF-7aro cell viability. As it was well discribed, apoptosis can occur by the mitochondrial pathway, which is characterized by a mitochondrial membrane permeabilization and subsequent activation of caspase-9, or by death receptor pathway, in which caspases are activated upon ligand binding to the death receptors. However, as our previous studies indicated that steroidal AIs induced apoptosis through the mitochondrial pathway, it was investigated the involvement of the intrinsic/mitochondrial pathway in the apoptotic process by evaluation of caspase-9 activity. All the AIs induced an increase of caspase-9 activity, suggesting the occurrence of an intrinsic apoptotic process.

As the production of ROS can lead to a state of oxidative stress that may be associated to mitochondrial dysfunction and apoptosis (150) it was evaluated the levels of ROS production. However, our data indicated that the activation of the intrinsic apoptotic pathway was ROS-independent. Curiously, AI **60** caused a decrease in ROS production, suggesting a potential protective effect probably due an antioxidant effect. Nevertheless,

as this AI was the one that caused a drastic reduction in MCF-7aro cell viability these results may also be due to the reduction of viable cells and, consequently, to lower ROS production (139). Nevertheless, further studies must be performed in order to better understand this effect.

In summary, the characterization of these four new steroidal compounds in terms of aromatase inhibition, biological effects and anti-tumor efficacy in a breast cancer cell model indicated that all the compounds were potent AIs and decreased the viability of MCF-7aro cells, a hormone-dependent breast cancer cell line without causing any effect on fibroblastic non-tumor cells. The results showed that compound **58** was the most potent AI, which caused reduction of cell viability in an aromatase-dependent manner and induced a reduction on the expression of the enzyme. Even though the effect of compound **60** was aromatase independent, it caused the most drastic decrease in MCF-7aro cell viability, suggesting that other biological mechanisms may be involved in the anti-tumor efficacy of this AI. In addition, for all the AIs the decrease in cell viability was accompanied by a cell cycle arrest and occurrence of apoptosis, by activation of the mitochondrial pathway.

Thus, this work contributed to the elucidation of the steroid structural modifications in scaffold in order to design and synthesize effective AIs that inhibit tumor growth. In addition it contributed to the characterization of the mechanisms involved in the anti-tumor properties which may unravel new therapeutic targets.

Chapter VI

Bibliography

1. Makki J. Diversity of Breast Carcinoma: Histological Subtypes and Clinical Relevance. Clinical medicine insights Pathology. 2015;8:23-31.

2. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JW, Comber H, et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. European journal of cancer. 2013;49(6):1374-403.

3. Macedo LF, Sabnis G, Brodie A. Aromatase inhibitors and breast cancer. Annals of the New York Academy of Sciences. 2009;1155:162-73.

4. May FE. Novel drugs that target the estrogen-related receptor alpha: their therapeutic potential in breast cancer. Cancer management and research. 2014;6:225-52.

5. Dieci MV, Orvieto E, Dominici M, Conte P, Guarneri V. Rare breast cancer subtypes: histological, molecular, and clinical peculiarities. The oncologist. 2014;19(8):805-13.

6. Milne RL, Antoniou AC. Genetic modifiers of cancer risk for BRCA1 and BRCA2 mutation carriers. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2011;22 Suppl 1:i11-7.

7. Balmana J, Diez O, Castiglione M, Group EGW. BRCA in breast cancer: ESMO clinical recommendations. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2009;20 Suppl 4:19-20.

8. Mohamed A, Krajewski K, Cakar B, Ma CX. Targeted therapy for breast cancer. The American journal of pathology. 2013;183(4):1096-112.

9. Go RE, Hwang KA, Choi KC. Cytochrome P450 1 family and cancers. The Journal of steroid biochemistry and molecular biology. 2015;147:24-30.

10. Boonyaratanakornkit V, Pateetin P. The role of ovarian sex steroids in metabolic homeostasis, obesity, and postmenopausal breast cancer: molecular mechanisms and therapeutic implications. BioMed research international. 2015;2015:140196.

11. Caldon CE. Estrogen signaling and the DNA damage response in hormone dependent breast cancers. Frontiers in oncology. 2014;4:106.

12. Okoh V, Deoraj A, Roy D. Estrogen-induced reactive oxygen species-mediated signalings contribute to breast cancer. Biochimica et biophysica acta. 2011;1815(1):115-33.

13. Wang L, Di LJ. BRCA1 and estrogen/estrogen receptor in breast cancer: where they interact? International journal of biological sciences. 2014;10(5):566-75.

14. Sui M, Zhang H, Fan W. The role of estrogen and estrogen receptors in chemoresistance. Current medicinal chemistry. 2011;18(30):4674-83.

15. Jansson A. 17Beta-hydroxysteroid dehydrogenase enzymes and breast cancer. The Journal of steroid biochemistry and molecular biology. 2009;114(1-2):64-7.

16. Thomas C, Gustafsson JA. Estrogen receptor mutations and functional consequences for breast cancer. Trends in endocrinology and metabolism: TEM. 2015;26(9):467-76.

17. Subramanian A, Salhab M, Mokbel K. Oestrogen producing enzymes and mammary carcinogenesis: a review. Breast cancer research and treatment. 2008;111(2):191-202.

18. Cavalieri EL, Rogan EG. Depurinating estrogen-DNA adducts, generators of cancer initiation: their minimization leads to cancer prevention. Clinical and translational medicine. 2016;5(1):12.

19. Blair IA. Analysis of estrogens in serum and plasma from postmenopausal women: past present, and future. Steroids. 2010;75(4-5):297-306.

20. Clemons M, Goss P. Estrogen and the risk of breast cancer. The New England journal of medicine. 2001;344(4):276-85.

21. Bulun SE, Lin Z, Imir G, Amin S, Demura M, Yilmaz B, et al. Regulation of aromatase expression in estrogen-responsive breast and uterine disease: from bench to treatment. Pharmacological reviews. 2005;57(3):359-83.

22. Sebastian S, Bulun SE. A highly complex organization of the regulatory region of the human CYP19 (aromatase) gene revealed by the Human Genome Project. The Journal of clinical endocrinology and metabolism. 2001;86(10):4600-2.

23. Simpson ER, Clyne C, Rubin G, Boon WC, Robertson K, Britt K, et al. Aromatase-a brief overview. Annual review of physiology. 2002;64:93-127.

24. Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, et al. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. Endocrine reviews. 1994;15(3):342-55.

25. Grodin JM, Siiteri PK, MacDonald PC. Source of estrogen production in postmenopausal women. The Journal of clinical endocrinology and metabolism. 1973;36(2):207-14.

26. Bulun SE, Simpson ER. Competitive reverse transcription-polymerase chain reaction analysis indicates that levels of aromatase cytochrome P450 transcripts in adipose tissue of buttocks, thighs, and abdomen of women increase with advancing age. The Journal of clinical endocrinology and metabolism. 1994;78(2):428-32.

27. Meng L, Zhou J, Sasano H, Suzuki T, Zeitoun KM, Bulun SE. Tumor necrosis factor alpha and interleukin 11 secreted by malignant breast epithelial cells inhibit adipocyte differentiation by selectively down-regulating CCAAT/enhancer binding protein alpha and peroxisome proliferator-activated receptor gamma: mechanism of desmoplastic reaction. Cancer research. 2001;61(5):2250-5.

28. Bulun SE, Chen D, Lu M, Zhao H, Cheng Y, Demura M, et al. Aromatase excess in cancers of breast, endometrium and ovary. The Journal of steroid biochemistry and molecular biology. 2007;106(1-5):81-96.

29. Artigalas O, Vanni T, Hutz MH, Ashton-Prolla P, Schwartz IV. Influence of CYP19A1 polymorphisms on the treatment of breast cancer with aromatase inhibitors: a systematic review and meta-analysis. BMC medicine. 2015;13:139.

30. Ghosh D, Griswold J, Erman M, Pangborn W. Structural basis for androgen specificity and oestrogen synthesis in human aromatase. Nature. 2009;457(7226):219-23.

31. Bulun SE, Lin Z, Zhao H, Lu M, Amin S, Reierstad S, et al. Regulation of aromatase expression in breast cancer tissue. Annals of the New York Academy of Sciences. 2009;1155:121-31.

32. Bulun SE, Sebastian S, Takayama K, Suzuki T, Sasano H, Shozu M. The human CYP19 (aromatase P450) gene: update on physiologic roles and genomic organization of promoters. The Journal of steroid biochemistry and molecular biology. 2003;86(3-5):219-24.

33. Simpson ER, Misso M, Hewitt KN, Hill RA, Boon WC, Jones ME, et al. Estrogen--the good, the bad, and the unexpected. Endocrine reviews. 2005;26(3):322-30.

34. Yanase T, Mu YM, Nishi Y, Goto K, Nomura M, Okabe T, et al. Regulation of aromatase by nuclear receptors. The Journal of steroid biochemistry and molecular biology. 2001;79(1-5):187-92.

35. O'Neill JS, Elton RA, Miller WR. Aromatase activity in adipose tissue from breast quadrants: a link with tumour site. British medical journal. 1988;296(6624):741-3.

36. Bulun SE, Price TM, Aitken J, Mahendroo MS, Simpson ER. A link between breast cancer and local estrogen biosynthesis suggested by quantification of breast adipose tissue aromatase cytochrome P450 transcripts using competitive polymerase chain reaction after reverse transcription. The Journal of clinical endocrinology and metabolism. 1993;77(6):1622-8.

37. Harada N, Utsumi T, Takagi Y. Tissue-specific expression of the human aromatase cytochrome P-450 gene by alternative use of multiple exons 1 and promoters, and switching of tissue-specific exons 1 in carcinogenesis. Proceedings of the National Academy of Sciences of the United States of America. 1993;90(23):11312-6.

38. Reed MJ, Topping L, Coldham NG, Purohit A, Ghilchik MW, James VH. Control of aromatase activity in breast cancer cells: the role of cytokines and growth factors. The Journal of steroid biochemistry and molecular biology. 1993;44(4-6):589-96.

39. Sasano H, Nagura H, Harada N, Goukon Y, Kimura M. Immunolocalization of aromatase and other steroidogenic enzymes in human breast disorders. Human pathology. 1994;25(5):530-5.

40. Utsumi T, Harada N, Maruta M, Takagi Y. Presence of alternatively spliced transcripts of aromatase gene in human breast cancer. The Journal of clinical endocrinology and metabolism. 1996;81(6):2344-9.

41. Zhou C, Zhou D, Esteban J, Murai J, Siiteri PK, Wilczynski S, et al. Aromatase gene expression and its exon I usage in human breast tumors. Detection of aromatase messenger RNA by reverse transcription-polymerase chain reaction. The Journal of steroid biochemistry and molecular biology. 1996;59(2):163-71.

42. Chen D, Reierstad S, Lu M, Lin Z, Ishikawa H, Bulun SE. Regulation of breast cancer-associated aromatase promoters. Cancer letters. 2009;273(1):15-27.

43. Agarwal VR, Bulun SE, Leitch M, Rohrich R, Simpson ER. Use of alternative promoters to express the aromatase cytochrome P450 (CYP19) gene in breast adipose tissues of cancer-free and breast cancer patients. The Journal of clinical endocrinology and metabolism. 1996;81(11):3843-9.

44. Poulos TL, Finzel BC, Howard AJ. High-resolution crystal structure of cytochrome P450cam. Journal of molecular biology. 1987;195(3):687-700.

45. Ghosh D, Griswold J, Erman M, Pangborn W. X-ray structure of human aromatase reveals an androgen-specific active site. The Journal of steroid biochemistry and molecular biology. 2010;118(4-5):197-202.

46. Jensen EV, Greene GL, Closs LE, DeSombre ER, Nadji M. Receptors reconsidered: a 20-year perspective. Recent Prog Horm Res. 1982;38:1-40.

47. Jordan VC, O'Malley BW. Selective estrogen-receptor modulators and antihormonal resistance in breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2007;25(36):5815-24.

48. Lannigan DA. Estrogen receptor phosphorylation. Steroids. 2003;68(1):1-9.

49. Jordan VC, Brodie AM. Development and evolution of therapies targeted to the estrogen receptor for the treatment and prevention of breast cancer. Steroids. 2007;72(1):7-25.

50. Powles TJ. Anti-oestrogenic prevention of breast cancer--the make or break point. Nature reviews Cancer. 2002;2(10):787-94.

51. Shanle EK, Xu W. Selectively targeting estrogen receptors for cancer treatment. Advanced drug delivery reviews. 2010;62(13):1265-76.

52. Jensen EV JH. Basic guides to the mechanism of estrogen action. Recent Prog Horm Res. 1962;18::387–414

53. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. Cloning of a novel receptor expressed in rat prostate and ovary. Proceedings of the National Academy of Sciences of the United States of America. 1996;93(12):5925-30.

54. Mosselman S, Polman J, Dijkema R. ER beta: identification and characterization of a novel human estrogen receptor. FEBS letters. 1996;392(1):49-53.

55. Haldosen LA, Zhao C, Dahlman-Wright K. Estrogen receptor beta in breast cancer. Molecular and cellular endocrinology. 2014;382(1):665-72.

56. Rizza P, Barone I, Zito D, Giordano F, Lanzino M, De Amicis F, et al. Estrogen receptor beta as a novel target of androgen receptor action in breast cancer cell lines. Breast cancer research : BCR. 2014;16(1):R21.

57. Piperigkou Z, Bouris P, Onisto M, Franchi M, Kletsas D, Theocharis AD, et al. Estrogen receptor beta modulates breast cancer cells functional properties, signaling and expression of matrix molecules. Matrix biology : journal of the International Society for Matrix Biology. 2016; DOI: 10.1016/j.matbio.2016.05.003.

58. Bai Z, Gust R. Breast cancer, estrogen receptor and ligands. Archiv der Pharmazie. 2009;342(3):133-49.

59. Menasce LP, White GR, Harrison CJ, Boyle JM. Localization of the estrogen receptor locus (ESR) to chromosome 6q25.1 by FISH and a simple post-FISH banding technique. Genomics. 1993;17(1):263-5.

60. Ogawa S, Inoue S, Watanabe T, Hiroi H, Orimo A, Hosoi T, et al. The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha in vivo and in vitro. Biochemical and biophysical research communications. 1998;243(1):122-6.

61. Enmark E, Pelto-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, et al. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. The Journal of clinical endocrinology and metabolism. 1997;82(12):4258-65.

62. Su X, Xu X, Li G, Lin B, Cao J, Teng L. ER-alpha36: a novel biomarker and potential therapeutic target in breast cancer. OncoTargets and therapy. 2014;7:1525-33.

63. Hah N, Kraus WL. Hormone-regulated transcriptomes: lessons learned from estrogen signaling pathways in breast cancer cells. Molecular and cellular endocrinology. 2014;382(1):652-64.

64. Gu Y, Chen T, Lopez E, Wu W, Wang X, Cao J, et al. The therapeutic target of estrogen receptor-alpha36 in estrogen-dependent tumors. Journal of translational medicine. 2014;12:16.

65. Zilli M, Grassadonia A, Tinari N, Di Giacobbe A, Gildetti S, Giampietro J, et al. Molecular mechanisms of endocrine resistance and their implication in the therapy of breast cancer. Biochimica et biophysica acta. 2009;1795(1):62-81.

66. De Marco P, Cirillo F, Vivacqua A, Malaguarnera R, Belfiore A, Maggiolini M. Novel Aspects Concerning the Functional Cross-Talk between the Insulin/IGF-I System and Estrogen Signaling in Cancer Cells. Frontiers in endocrinology. 2015;6:30.

67. Lappano R, Pisano A, Maggiolini M. GPER Function in Breast Cancer: An Overview. Frontiers in endocrinology. 2014;5:66.

68. Murawa P, Murawa D, Adamczyk B, Polom K. Breast cancer: Actual methods of treatment and future trends. Reports of practical oncology and radiotherapy : journal of Greatpoland Cancer Center in Poznan and Polish Society of Radiation Oncology. 2014;19(3):165-72.

69. Hamelinck VC, Bastiaannet E, Pieterse AH, Jannink I, van de Velde CJ, Liefers GJ, et al. Patients' preferences for surgical and adjuvant systemic treatment in early breast cancer: a systematic review. Cancer treatment reviews. 2014;40(8):1005-18.

70. Migliaccio I, Malorni L, Hart CD, Guarducci C, Di Leo A. Endocrine therapy considerations in postmenopausal patients with hormone receptor positive, human epidermal growth factor receptor type 2 negative advanced breast cancers. BMC medicine. 2015;13:46.

71. Santen RJ, Brodie H, Simpson ER, Siiteri PK, Brodie A. History of aromatase: saga of an important biological mediator and therapeutic target. Endocrine reviews. 2009;30(4):343-75.

72. Fabian CJ. The what, why and how of aromatase inhibitors: hormonal agents for treatment and prevention of breast cancer. International journal of clinical practice. 2007;61(12):2051-63.

73. Smith GL. The Long and Short of Tamoxifen Therapy: A Review of the ATLAS Trial. Journal of the advanced practitioner in oncology. 2014;5(1):57-60.

74. Pinkerton JV, Thomas S. Use of SERMs for treatment in postmenopausal women. The Journal of steroid biochemistry and molecular biology. 2014;142:142-54.

75. Rugo HS. The breast cancer continuum in hormone-receptor-positive breast cancer in postmenopausal women: evolving management options focusing on aromatase inhibitors. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2008;19(1):16-27.

76. Robertson JF, Nicholson RI, Bundred NJ, Anderson E, Rayter Z, Dowsett M, et al. Comparison of the short-term biological effects of 7alpha-[9-(4,4,5,5,5-pentafluoropentylsulfinyl)-nonyl]estra-1,3,5, (10)-triene-3,17beta-diol (Faslodex) versus tamoxifen in postmenopausal women with primary breast cancer. Cancer research. 2001;61(18):6739-46.

77. Wakeling AE. Similarities and distinctions in the mode of action of different classes of antioestrogens. Endocrine-related cancer. 2000;7(1):17-28.

78. Valachis A, Mauri D, Polyzos NP, Mavroudis D, Georgoulias V, Casazza G. Fulvestrant in the treatment of advanced breast cancer: a systematic review and meta-analysis of randomized controlled trials. Critical reviews in oncology/hematology. 2010;73(3):220-7.

79. Osborne CK, Coronado-Heinsohn EB, Hilsenbeck SG, McCue BL, Wakeling AE, McClelland RA, et al. Comparison of the effects of a pure steroidal antiestrogen with those of tamoxifen in a model of human breast cancer. Journal of the National Cancer Institute. 1995;87(10):746-50.

80. Ciruelos E, Pascual T, Arroyo Vozmediano ML, Blanco M, Manso L, Parrilla L, et al. The therapeutic role of fulvestrant in the management of patients with hormone receptor-positive breast cancer. Breast. 2014;23(3):201-8.

81. Berry J. Are all aromatase inhibitors the same? A review of controlled clinical trials in breast cancer. Clinical therapeutics. 2005;27(11):1671-84.

82. Schneider R, Barakat A, Pippen J, Osborne C. Aromatase inhibitors in the treatment of breast cancer in post-menopausal female patients: an update. Breast cancer. 2011;3:113-25.

83. Chumsri S, Howes T, Bao T, Sabnis G, Brodie A. Aromatase, aromatase inhibitors, and breast cancer. The Journal of steroid biochemistry and molecular biology. 2011;125(1-2):13-22.

84. Zucchini G, Geuna E, Milani A, Aversa C, Martinello R, Montemurro F. Clinical utility of exemestane in the treatment of breast cancer. International journal of women's health. 2015;7:551-63.

85. Goss PE, Ingle JN, Martino S, Robert NJ, Muss HB, Piccart MJ, et al. A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy for early-stage breast cancer. The New England journal of medicine. 2003;349(19):1793-802.

86. Ponzone R, Mininanni P, Cassina E, Pastorino F, Sismondi P. Aromatase inhibitors for breast cancer: different structures, same effects? Endocrine-related cancer. 2008;15(1):27-36.

87. Thiantanawat A, Long BJ, Brodie AM. Signaling pathways of apoptosis activated by aromatase inhibitors and antiestrogens. Cancer research. 2003;63(22):8037-50.

88. Hata AN, Engelman JA, Faber AC. The BCL2 Family: Key Mediators of the Apoptotic Response to Targeted Anticancer Therapeutics. Cancer discovery. 2015;5(5):475-87.

89. Dutta U, Pant K. Aromatase inhibitors: past, present and future in breast cancer therapy. Medical oncology. 2008;25(2):113-24.

90. Chen S, Masri S, Hong Y, Wang X, Phung S, Yuan YC, et al. New experimental models for aromatase inhibitor resistance. The Journal of steroid biochemistry and molecular biology. 2007;106(1-5):8-15.

91. Lopez-Knowles E, Wilkerson PM, Ribas R, Anderson H, Mackay A, Ghazoui Z, et al. Integrative analyses identify modulators of response to neoadjuvant aromatase inhibitors in patients with early breast cancer. Breast cancer research : BCR. 2015;17(1):35.

92. Miyoshi Y, Murase K, Saito M, Oh K. Prediction of hormone sensitivity for breast cancers. Breast cancer. 2010;17(2):86-91.

93. Beresford M, Tumur I, Chakrabarti J, Barden J, Rao N, Makris A. A qualitative systematic review of the evidence base for non-cross-resistance between steroidal and non-steroidal aromatase inhibitors in metastatic breast cancer. Clinical oncology. 2011;23(3):209-15.

94. Bosco D. Osteoporosis and aromatase inhibitors: experience and future prospects. Clinical cases in mineral and bone metabolism : the official journal of the Italian Society of Osteoporosis, Mineral Metabolism, and Skeletal Diseases. 2012;9(2):89-91.

95. Lustberg MB, Reinbolt RE, Shapiro CL. Bone health in adult cancer survivorship. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2012;30(30):3665-74.

96. Edwards BJ, Raisch DW, Shankaran V, McKoy JM, Gradishar W, Bunta AD, et al. Cancer therapy associated bone loss: implications for hip fractures in mid-life women with breast cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2011;17(3):560-8.

97. Koopal C, Janssen-Heijnen ML, van de Wouw AJ, van den Bergh JP. Fracture incidence in pre- and postmenopausal women after completion of adjuvant hormonal therapy for breast cancer. Breast. 2015;24(2):153-8.

98. Pouilles JM, Tremollieres F, Bonneu M, Ribot C. Influence of early age at menopause on vertebral bone mass. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 1994;9(3):311-5.

99. Erbag G, Uygun K, Binnetoglu E, Korkmaz AN, Asik M, Sen H, et al. Aromatase inhibitor treatment for breast cancer: short-term effect on bone health. Contemporary oncology. 2015;19(5):374-7.

100. Coombes RC, Kilburn LS, Snowdon CF, Paridaens R, Coleman RE, Jones SE, et al. Survival and safety of exemestane versus tamoxifen after 2-3 years' tamoxifen treatment (Intergroup Exemestane Study): a randomised controlled trial. Lancet. 2007;369(9561):559-70.

101. Chen Z, Maricic M, Pettinger M, Ritenbaugh C, Lopez AM, Barad DH, et al. Osteoporosis and rate of bone loss among postmenopausal survivors of breast cancer. Cancer. 2005;104(7):1520-30.

102. Shapiro CL, Manola J, Leboff M. Ovarian failure after adjuvant chemotherapy is associated with rapid bone loss in women with early-stage breast cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2001;19(14):3306-11.

103. Datta M, Schwartz GG. Calcium and vitamin D supplementation and loss of bone mineral density in women undergoing breast cancer therapy. Critical reviews in oncology/hematology. 2013;88(3):613-24.

104. Gaillard S, Stearns V. Aromatase inhibitor-associated bone and musculoskeletal effects: new evidence defining etiology and strategies for management. Breast cancer research : BCR. 2011;13(2):205.

105. Chen Z, Maricic M, Aragaki AK, Mouton C, Arendell L, Lopez AM, et al. Fracture risk increases after diagnosis of breast or other cancers in postmenopausal women: results from the Women's Health Initiative. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2009;20(4):527-36.

106. Neuner JM, Yen TW, Sparapani RA, Laud PW, Nattinger AB. Fracture risk and adjuvant hormonal therapy among a population-based cohort of older female breast cancer patients. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2011;22(11):2847-55.

107. Knobf MT, Jeon S, Smith B, Harris L, Kerstetter J, Thompson AS, et al. Effect of a randomized controlled exercise trial on bone outcomes: influence of adjuvant endocrine therapy. Breast cancer research and treatment. 2016;155(3):491-500.

108. Panday K, Gona A, Humphrey MB. Medication-induced osteoporosis: screening and treatment strategies. Therapeutic advances in musculoskeletal disease. 2014;6(5):185-202.

109. Amir E, Seruga B, Niraula S, Carlsson L, Ocana A. Toxicity of adjuvant endocrine therapy in postmenopausal breast cancer patients: a systematic review and meta-analysis. Journal of the National Cancer Institute. 2011;103(17):1299-309.

110. Perez EA. Safety of aromatase inhibitors in the adjuvant setting. Breast cancer research and treatment. 2007;105 Suppl 1:75-89.

111. Miller WR, Bartlett J, Brodie AM, Brueggemeier RW, di Salle E, Lonning PE, et al. Aromatase inhibitors: are there differences between steroidal and nonsteroidal aromatase inhibitors and do they matter? The oncologist. 2008;13(8):829-37.

112. Cocconi G. First generation aromatase inhibitors--aminoglutethimide and testololactone. Breast cancer research and treatment. 1994;30(1):57-80.

113. Hong Y, Chen S. Aromatase inhibitors: structural features and biochemical characterization. Annals of the New York Academy of Sciences. 2006;1089:237-51.

114. Tominaga T, Adachi I, Sasaki Y, Tabei T, Ikeda T, Takatsuka Y, et al. Double-blind randomised trial comparing the non-steroidal aromatase inhibitors letrozole and fadrozole in postmenopausal women with advanced breast cancer. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2003;14(1):62-70.

115. Smith IE, Norton A. Fadrozole and letrozole in advanced breast cancer: clinical and biochemical effects. Breast cancer research and treatment. 1998;49 Suppl 1:S67-71; discussion S3-7.

116. Lonning PE, Geisler J, Dowsett M. Pharmacological and clinical profile of anastrozole. Breast cancer research and treatment. 1998;49 Suppl 1:S53-7; discussion S73-7.

117. Kelly CM, Buzdar AU. Anastrozole. Expert opinion on drug safety. 2010;9(6):995-1003.

118. Milani M, Jha G, Potter DA. Anastrozole Use in Early Stage Breast Cancer of Post-Menopausal Women. Clinical medicine Therapeutics. 2009;1:141-56.

119. Wilkinson K. Anastrozole (Arimidex). Clinical journal of oncology nursing. 2004;8(1):87-8.

120. Macedo LF, Sabnis GJ, Goloubeva OG, Brodie A. Combination of anastrozole with fulvestrant in the intratumoral aromatase xenograft model. Cancer research. 2008;68(9):3516-22.

121. Scott LJ, Keam SJ. Letrozole : in postmenopausal hormone-responsive early-stage breast cancer. Drugs. 2006;66(3):353-62.

122. Bhatnagar AS. The discovery and mechanism of action of letrozole. Breast cancer research and treatment. 2007;105 Suppl 1:7-17.

123. Simpson D, Curran MP, Perry CM. Letrozole: a review of its use in postmenopausal women with breast cancer. Drugs. 2004;64(11):1213-30.

124. Haynes BP, Dowsett M, Miller WR, Dixon JM, Bhatnagar AS. The pharmacology of letrozole. The Journal of steroid biochemistry and molecular biology. 2003;87(1):35-45.

125. Itoh T, Karlsberg K, Kijima I, Yuan YC, Smith D, Ye J, et al. Letrozole-, anastrozole-, and tamoxifen-responsive genes in MCF-7aro cells: a microarray approach. Molecular cancer research : MCR. 2005;3(4):203-18.

126. Judd HL, Barone RM, Laufer LR, Gambone JC, Monfort SL, Lasley BL. In vivo effects of delta 1-testololactone on peripheral aromatization. Cancer research. 1982;42(8 Suppl):3345s-8s.

127. Lonning PE. Pharmacological profiles of exemestane and formestane, steroidal aromatase inhibitors used for treatment of postmenopausal breast cancer. Breast cancer research and treatment. 1998;49 Suppl 1:S45-52; discussion S73-7.

128. Usluogullari B, Duvan C, Usluogullari C. Use of aromatase inhibitors in practice of gynecology. Journal of ovarian research. 2015;8(1):4.

129. Smith IE, Dowsett M. Aromatase inhibitors in breast cancer. The New England journal of medicine. 2003;348(24):2431-42.

130. Van Asten K, Neven P, Lintermans A, Wildiers H, Paridaens R. Aromatase inhibitors in the breast cancer clinic: focus on exemestane. Endocrine-related cancer. 2014;21(1):R31-49.

131. Lonning PE, Eikesdal HP. Aromatase inhibition 2013: clinical state of the art and questions that remain to be solved. Endocrine-related cancer. 2013;20(4):R183-201.

132. Deeks ED, Scott LJ. Exemestane: a review of its use in postmenopausal women with breast cancer. Drugs. 2009;69(7):889-918.

133. Varela CL, Amaral C, Tavares da Silva E, Lopes A, Correia-da-Silva G, Carvalho RA, et al. Exemestane metabolites: Synthesis, stereochemical elucidation, biochemical activity and anti-proliferative effects in a hormone-dependent breast cancer cell line. European journal of medicinal chemistry. 2014;87:336-45.

134. van de Velde CJ, Rea D, Seynaeve C, Putter H, Hasenburg A, Vannetzel JM, et al. Adjuvant tamoxifen and exemestane in early breast cancer (TEAM): a randomised phase 3 trial. Lancet. 2011;377(9762):321-31.

135. Markopoulos C, Dafni U, Misitzis J, Zobolas V, Tzoracoleftherakis E, Koukouras D, et al. Extended adjuvant hormonal therapy with exemestane has no detrimental effect on the lipid profile of postmenopausal breast cancer patients: final results of the ATENA lipid substudy. Breast cancer research : BCR. 2009;11(3):R35.

136. Wang X, Masri S, Phung S, Chen S. The role of amphiregulin in exemestane-resistant breast cancer cells: evidence of an autocrine loop. Cancer research. 2008;68(7):2259-65.

137. Baselga J, Campone M, Piccart M, Burris HA, 3rd, Rugo HS, Sahmoud T, et al. Everolimus in postmenopausal hormone-receptor-positive advanced breast cancer. The New England journal of medicine. 2012;366(6):520-9.

138. Hole S, Pedersen AM, Lykkesfeldt AE, Yde CW. Aurora kinase A and B as new treatment targets in aromatase inhibitor-resistant breast cancer cells. Breast cancer research and treatment. 2015;149(3):715-26.

139. Amaral C, Borges M, Melo S, da Silva ET, Correia-da-Silva G, Teixeira N. Apoptosis and autophagy in breast cancer cells following exemestane treatment. PloS one. 2012;7(8):e42398.

140. Thompson EA, Jr., Siiteri PK. Utilization of oxygen and reduced nicotinamide adenine dinucleotide phosphate by human placental microsomes during aromatization of androstenedione. The Journal of biological chemistry. 1974;249(17):5364-72.

141. Zhou DJ, Pompon D, Chen SA. Stable expression of human aromatase complementary DNA in mammalian cells: a useful system for aromatase inhibitor screening. Cancer research. 1990;50(21):6949-54.

142. Johnston SR. Enhancing Endocrine Therapy for Hormone Receptor-Positive Advanced Breast Cancer: Cotargeting Signaling Pathways. Journal of the National Cancer Institute. 2015;107(10).

143. Cepa MM, Tavares da Silva EJ, Correia-da-Silva G, Roleira FM, Teixeira NA. Structure-activity relationships of new A,D-ring modified steroids as aromatase inhibitors: design, synthesis, and biological activity evaluation. Journal of medicinal chemistry. 2005;48(20):6379-85.

144. Varela C, Tavares da Silva EJ, Amaral C, Correia da Silva G, Baptista T, Alcaro S, et al. New structure-activity relationships of A- and D-ring modified steroidal aromatase inhibitors: design, synthesis, and biochemical evaluation. Journal of medicinal chemistry. 2012;55(8):3992-4002.

145. Varela CL, Amaral C, Correia-da-Silva G, Carvalho RA, Teixeira NA, Costa SC, et al. Design, synthesis and biochemical studies of new 7alpha-allylandrostanes as aromatase inhibitors. Steroids. 2013;78(7):662-9.

146. Amaral C, Varela C, Azevedo M, da Silva ET, Roleira FM, Chen S, et al. Effects of steroidal aromatase inhibitors on sensitive and resistant breast cancer cells: aromatase inhibition and autophagy. The Journal of steroid biochemistry and molecular biology. 2013;135:51-9.

147. Cepa M, Correia-da-Silva G, Tavares da Silva EJ, Roleira FM, Hong Y, Chen S, et al. Molecular mechanisms of aromatase inhibition by new A, D-ring modified steroids. Biological chemistry. 2008;389(9):1183-91.

148. Hevir N, Trost N, Debeljak N, Rizner TL. Expression of estrogen and progesterone receptors and estrogen metabolizing enzymes in different breast cancer cell lines. Chemico-biological interactions. 2011;191(1-3):206-16.

149. Wang X, Chen S. Aromatase destabilizer: novel action of exemestane, a food and drug administration-approved aromatase inhibitor. Cancer research. 2006;66(21):10281-6.

150. Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. Physiological reviews. 2007;87(1):99-163.