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Development of new aromatase inhibitors analogues of letrozole: perspectives for treatment of male infertility

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Dedicatória

Às pessoas mais importantes da minha vida, os meus pais, irmã, avós e namorado. Um obrigada nunca será suficiente.

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Resumo

Atualmente, a infertilidade é considerada um importante problema de saúde, estando dependente de diversos fatores psicológicos, físicos e bioquímicos, o que no caso da infertilidade masculina assenta fundamentalmente no sucesso da espermatogénese. A função sexual masculina e o adequado desenvolvimento do processo espermatogénico estão altamente dependentes da regulação hormonal, particularmente da ação exercida pelas hormonas sexuais esteróides, androgénios e estrogénios. O 17β-Estradiol (E_2), produto da aromatização da testosterona (T) através da aromatase, é o estrogénio mais potente produzido no corpo humano. A aromatase pertence ao grupo molecular das enzimas do citocromo P450 e regula o último passo da biossíntese dos estrogénios, aromatizando o anel A de androgénios tais como a T e a androstenediona, em E_2 e estrona, respetivamente. Deste modo, o E_2 é responsável por inúmeros efeitos originalmente atribuídos à T no homem. Por outro lado, homens que apresentam um rácio plasmático E_2/T elevado são inférteis, tendo sido proposto o uso de inibidores da aromatase (AIs) para o seu tratamento. De facto, a administração de AIs aumentou significativamente os níveis de T o que foi associado a uma recuperação na espermatogénese em homens com esta endocrinopatia. O letrozole é o AI não esteroidal que exibe maior capacidade de inibição da aromatase, tendo por isso, vindo a ser usado no tratamento da infertilidade masculina. De forma a desenvolver AIs mais selectivos e potentes, foram sintetizados novos inibidores da aromatase análogos ao letrozole, em que um dos dois anéis benzonitrilo é substituído por um fenilo substituído na posição *para* com diferentes grupos funcionais, e a ponte metino é substituída por um grupo vinilo. Para além disso, foram também sintetizados os seus congéneres reduzidos. Assim, foram sintetizados nove análogos do letrozole, sendo que seis deles foram descritos pela primeira vez nesta dissertação. Todos os compostos foram caracterizados através de ressonância magnética nuclear. Foram ainda realizados testes de *docking* molecular. Dada a semelhança existente entre os análogos do letrozole sintetizados, era espectável que estes apresentassem resultados similares. A simulação revelou que os compostos em estudo apresentam alta afinidade para a enzima no centro ativo quando comparados com o letrozole. Após a síntese, caracterização e purificação dos compostos, dois análogos do letrozole, nomeadamente o 4-(2-fenil-1-(1H-1,2,4-triazol-1-il)vinil)benzonitrilo e o 4-(2-fenil-1-(1H-1,2,4-triazol-1-il)etil)benzonitrilo, foram selecionados para avaliação do seu potencial biológico como AIs em células testiculares. Para tal, foram avaliados a produção de hormonas esteróides, o índice de diferenciação tubular (TDI), e o metabolismo glicolítico em culturas *ex vivo* de túbulos seminíferos (SeT) de ratos, na ausência ou na presença do letrozole, e dos análogos do letrozole vinilo e etilo em diferentes concentrações (200 e 400 nM). Ambos os análogos do letrozole pareceram suprimir o metabolismo glicolítico. Por outro lado, o letrozole mostrou aumentar a produção de lactato, o que indica um aumento do metabolismo glicolítico.

Relativamente ao TDI, este mostrou-se significativamente reduzido na presença de letrozole 200 nM ou do análogo do letrozole etilo em ambas as concentrações de 200 e 400 nM. No entanto, não foi observada uma relação dose-resposta nos AIs estudados. Contudo, sem os resultados dos radioimunoensaios para a quantificação de T e E_2 no meio de cultura, não é possível obter, neste momento, uma conclusão definitiva relativamente aos efeitos do letrozole e dos seus análogos estudados. Porém, face aos resultados observados no TDI e no metabolismo glicolítico, visíveis com apenas 24h de cultura, podemos concluir que ambos os análogos do letrozole estudados são biologicamente ativos. No futuro, seria de extrema importância avaliar ainda a atividade biológica dos restantes compostos sintetizados como potenciais AIs em células testiculares.

Palavras-chave

Análogos do letrozole, controlo hormonal, espermatogénese, infertilidade masculina, inibidores da aromatase, metabolismo celular, túbulos seminíferos.

Resumo alargado

Atualmente, a infertilidade é considerada um importante problema de saúde. Esta é definida pela impossibilidade de alcançar uma gravidez desejada após um ano de relações sem o uso de qualquer método contracetivo. Estima-se que aproximadamente 30-40% dos casos de infertilidade sejam causados por fatores masculinos, nomeadamente psicológicos, físicos e bioquímicos. Além disso, uma espermatogénese com sucesso é a base fundamental para a fertilidade masculina. A espermatogénese é um processo complexo e rigorosamente coordenado que ocorre nas unidades funcionais dos testículos, os túbulos seminíferos (SeT), onde diariamente são produzidos milhares de espermatozóides. As gónadas masculinas, os testículos, são ainda um órgão endócrino produzindo as hormonas esteróides sexuais necessárias para a estimulação do processo espermatogénico, para o normal desenvolvimento do fenótipo masculino e, consequentemente para a manutenção da fertilidade masculina. Contudo, a exposição a elevados ou a baixos níveis de hormonas leva à morte celular por apoptose das células da linha germinativa, com redução do potencial reprodutivo masculino.

Os androgénios são essenciais tanto para a fertilidade masculina como para a manutenção da espermatogénese. A testosterona (T) é o principal androgénio produzido pelos testículos, sendo responsável, conjuntamente com a hormona estimuladora do folículo, por iniciar e manter a espermatogénese. No entanto, para além destes reguladores clássicos da espermatogénese, também os estrogénios, nomeadamente o 17β-estradiol (E_2), parecem ter um papel importante na regulação da produção de esperma e na função sexual masculina. Entre os outros estrogénios produzidos nos testículos a partir dos androgénios pela atividade da aromatase, o que inclui a estrona e o estriol, o E_2 é reconhecidamente o mais potente e abundante nos testículos.

A aromatase pertence ao grupo molecular das enzimas do citocromo P450 que regula o último passo da biossíntese dos estrogénios, aromatizando o anel A de androgénios tais como a T e androstenediona, em E_2 e estrona, respetivamente.

Apesar de ter sido provado o benefício dos estrogénios na reprodução masculina, a sobre-expressão da aromatase leva a um desequilíbrio dos níveis de T/E_2, causando hiperplasia das células produtoras de T, dismorfia dos SeT, interferindo desta forma no processo natural da espermatogénese, o que em última instância poderá levar à infertilidade masculina. Deste modo, homens com um rácio plasmático E_2/T elevado são inférteis. Face ao aumento crescente de casos de infertilidade masculina, novas abordagens terapêuticas são necessárias e os inibidores da aromatase (AIs) têm vindo a ser propostos neste cenário para tratar os casos de infertilidade associados a hiperestrogenismo. Os AIs testados aumentam significativamente o número e a qualidade de espermatozóides em homens inférteis, uma vez que diminuem os níveis de E_2 e normalizam os níveis plasmáticos e intratesticulares de T.

O letrozole é o AI não esteroidal que exibe maior capacidade de inibição da aromatase, tendo por isso, merecido destaque no tratamento da infertilidade masculina. Desta forma, este trabalho de dissertação teve como objetivo sintetizar novos inibidores da aromatase análogos ao letrozole, em que um dos dois anéis benzonitrilo é substituído por um fenilo substituído na posição *para* com diferentes grupos funcionais, e a ponte metino é substituída por um grupo vinilo. Para além disso, foram também sintetizados os seus congéneres reduzidos. Assim, foram sintetizados nove análogos do letrozole, sendo que seis deles foram descritos pela primeira vez nesta dissertação. Todos os compostos foram caracterizados através de ressonância magnética nuclear (RMN). A atribuição de sinais aromáticos foi feita a partir da análise dos espectros de protão e carbono-13-RMN, com base nos seus desvios químicos e multiplicidade. Quando possível e julgado conveniente, realizaram-se adicionalmente estudos de RMN por aprimoramento sem distorção de transferência de polarização, correlação espetroscópica heteronuclear bidimensional e correlação espetroscópica heteronuclear a longa distância, bidimensional, na atribuição de sinais ambíguos.

Foram ainda realizados testes de *docking* molecular, onde se avaliaram as energias de ligação e as possíveis interações dos análogos do letrozole sintetizados com a aromatase, a fim de se prever o potencial destes compostos como AIs. A simulação revelou que os compostos em estudo apresentam alta afinidade para a enzima no centro ativo quando comparados com o letrozole.

Após a síntese, caracterização e purificação dos compostos, dois análogos do letrozole, nomeadamente o 4-(2-fenil-1-(1H-1,2,4-triazol-1-il)vinil)benzonitrilo e o 4-(2-fenil-1-(1H-1,2,4-triazol-1-il)etil)benzonitrilo, foram selecionados para avaliação do seu potencial biológico como AIs em células testiculares. Para tal, foram avaliados a produção de hormonas esteróides, o índice de diferenciação tubular (TDI), e o metabolismo glicolítico em culturas *ex vivo* de SeT de ratos, na ausência ou na presença do letrozole, e dos análogos do letrozole vinilo e etilo em diferentes concentrações (200 e 400 nM). O metabolismo glicolítico tem vindo a ser apontado como um fator crucial para a fertilidade masculina, tendo sido demonstrado recentemente que este pode ser modulado pela ação dos estrogénios. Deste modo, torna-se absolutamente relevante a avaliação da via glicolítica no contexto da presente dissertação. Para tal, avaliou-se a expressão dos elementos-chave na incorporação e metabolização de glicose, assim como na produção de lactato, o substrato preferencial para as células germinativas. Isto inclui, os transportadores de glicose (GLUTs), a fosfofrutoquinase (PFK), a lactato desidrogenase (LDH) e o transportador monocarboxilato (MCT).

Foi verificado que ambos os análogos do letrozole aumentaram o consumo da glicose pelos SeT, apesar de não ter sido observado um aumento na produção de lactato, nem de ter sido observada uma relação dose-resposta. Com exceção do análogo vinilo do letrozole a 400 nM onde os níveis de expressão de PFK1 se mostraram mais elevados, o análogo etilo do letrozole em ambas as concentrações e o análogo vinilo do letrozole a 200 nM exibiram níveis de expressão diminuídos não só para a PFK1, mas também para o GLUT3, a LDH e o MCT4. Tais

factos provam que ambos os análogos do letrozole sintetizados suprimem o metabolismo glicolítico. Relativamente ao letrozole, observou-se que os níveis de expressão de PFK1 estavam aumentados, a par da diminuição da concentração de glicose intracelular e do aumento da produção de lactato, o que indica um aumento do metabolismo glicolítico.

A supressão do metabolismo glicolítico observada nos dois análogos do letrozole selecionados para avaliação biológica seguiu a diminuição do TDI. Tal facto é consistente com estudos realizados onde mostram que o lactato é o substrato preferencial no desenvolvimento das células da linha germinativa. O TDI apresenta-se também diminuído na presença de letrozole. No entanto, neste caso a produção de lactato está aumentada. Não obstante, o decréscimo do TDI face à administração do letrozole está de acordo com estudos anteriores, onde mostram que o tratamento com AIs diminui drasticamente o número de espermatócitos e espermatídios devido ao aumento da apoptose.

Contudo, sem os resultados dos radioimunoensaios para a quantificação de T e E_2 no meio de cultura, não é possível obter, neste momento, uma conclusão definitiva relativamente aos efeitos do letrozole e dos seus análogos estudados. Porém, face aos resultados observados no TDI e no metabolismo glicolítico, visíveis com apenas 24h de cultura, podemos concluir que ambos os análogos do letrozole estudados são biologicamente ativos.

No futuro, seria de extrema importância avaliar ainda a atividade biológica dos restantes compostos sintetizados como potenciais AIs em células testiculares.

Abstract

Nowadays, infertility is one of the major health issues. Human fertility depends on several psychological, physical and biochemical factors, with a successful spermatogenesis being the fundamental basis for male fertility. The male sexual function and the proper development of spermatogenic process are highly dependent on the hormonal regulation, particularly by the actions of sex steroid hormones, androgens and estrogens. 17β-Estradiol (E_2), the metabolite of testosterone (T) aromatization by the activity of aromatase, is the most potent estrogen produced in the human body. Aromatase is a member of the cytochrome P450 protein superfamily that regulates the last step of estrogen biosynthesis, aromatizing the A-ring of androgens such as T and androstenedione into E_2 and estrone, respectively. Thus, in men, E_2 is responsible for a number of effects originally attributed to T. On the other hand, men with a high serum E_2/T ratio are infertile and it has been suggested that they can be treated with aromatase inhibitors (AIs). Indeed, AIs have been shown to increase T levels, and restore spermatogenesis, in infertile men with this endocrinopathy. Among other classes of compounds, letrozole is a nonsteroidal AI that has been widely used for treatment of male infertility. In order to develop more selective and efficient AIs, letrozole analogues were synthesized, where one of the rings of benzonitrile was substituted by a phenyl group in *para* position with different functional groups, and the methine bridge was substituted by a vinyl group. Furthermore, their reduced congeners were synthesized. Overall, the synthesis of nine letrozole analogues was performed. To the best of our knowledge, six letrozole analogues were reported for the first time in this dissertation. All compounds were structurally characterized with prominence by nuclear magnetic resonance spectrometry. Additionally, aromatase docking studies were performed. Giving the resemblance between all the letrozole analogues synthesized, it was not surprising that the results were very similar. The simulation revealed that the compounds in this study showed higher affinity to the enzyme, when compared with letrozole. After the synthesis, purification and characterization steps, two representative letrozole analogues namely 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)vinyl)benzonitrile and 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)ethyl)benzonitrile were selected for evaluation of their biological activity as potential AIs in testicular cells. For this purpose, rat seminiferous tubules (SeT) were cultured *ex vivo* in the presence or absence of 200 nM or 400 nM of letrozole and the vinyl and ethyl letrozole analogues. Steroid hormone production, the tubular differentiation index (TDI) and the glycolytic metabolism were evaluated. The synthesized letrozole analogues, which were selected for biological evaluation as potential AIs, seem to suppress glycolytic metabolism. On the other hand, letrozole increased the metabolizing of glucose with augmented production of lactate. Relatively to the TDI, it was significantly reduced in the SeT exposed to letrozole 200 nM and ethylbenzonitrile letrozole analogue both in 200 nM and 400 nM. However, no dose-dependent relationship was observed

for the AIs under study. Moreover, without the radioimmunoassays results for quantifying T and E2 in the culture medium, it is not possible to advance a definitive conclusion about the distinct behavior of letrozole and its analogues studied in this dissertation. Nevertheless, considering the effects observed on TDI and glycolytic metabolism, only with 24 h of culture, it is possible to conclude that both letrozole analogues are biologically active. In the future, it would be of uttermost importance to evaluate the biological activity of the remaining synthesized compounds as AIs in testicular cells.

Keywords

Aromatase inhibitors, cell metabolism, hormonal control, letrozole analogues, male infertility, seminiferous tubules, spermatogenesis.

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^{13}C NMR	Carbon-13 nuclear magnetic resonance
^{1}H NMR	Proton nuclear magnetic resonance
AI	Aromatase inhibitor
AR	Androgen receptor
ASD	4-Androstene-3-17-dione
BSA	Bovine serum albumin
$CDCl_3$	Deuterated chloroform
d	Duplet
dd	Double duplet
DEPT	Distortionless enhancement by polarization transfer
E_2	17β-Estradiol
F-1,6-BP	Fructose-1-6-bisphosphate
F-6-P	Fructose-6-phosphate
FSH	Follicle-stimulating hormone
GLUT	Glucose transporter
GnRH	Gonadotropin releasing hormone
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum correlation
J	Coupling constant
LDH	Lactate dehydrogenase
LH	Luteinizing hormone

m	Multiplet
MCT	Monocarboxylate transporter
mp	Melting point
PBS	Phosphate-buffered saline
PFK	Phosphofructokinase
ppm	Parts per million
RIA	Radioimmunoassay
s	Singlet
SeT	Seminiferous tubules
T	Testosterone
TDI	Tubular differentiation index
TLC	Thin layer chromatography
TMS	Tetramethylsilane
UV	Ultraviolet
δ	Chemical shift

I. Introduction

1. Male infertility: brief epidemiological notes

Infertility is one of the major health issues and is customarily defined as the inability of a couple to achieve spontaneous pregnancy after one year of unprotected intercourse. This condition affects 13-15 % of the couples worldwide (World Health Organization 2000), and approximately 30-40 % of infertility cases are caused by male factors (Esteves & Chan 2015).

Since the mid-20th century, numerous studies have reported an increasing incidence of human reproductive diseases and a consequent decline in male reproductive function worldwide (Woodruff 2011). Several risk factors are involved in the pathogenesis of male infertility, some of which include congenital anomalies (congenital hypogonadotrophic hypogonadism, congenital absence of *vas deferens*), infectious diseases, exposure to environmental gonadotoxins and alterations in the characteristics of semen, such as, a decrease in sperm motility and sperm count (Brugh & Lipshultz 2004; Hamada et al. 2011; Krausz 2011). A reduced sperm density (oligozoospermia) is often accompanied by poor motility and morphology reflecting qualitative and quantitative defects in spermatogenesis (McLachlan 2013). Primary testicular failure accounts for about 75% of all male factor infertility and testicular spermatogenic failure affects up to 10% of men seeking infertility evaluation. These men have severe defects in sperm production and most are azoospermic (Pavlovich et al. 2001). Nevertheless, 30% of the causes of infertility are undetermined falling in the category of "idiopathic infertility" (Neto et al. 2016).

Therefore, human fertility depends on several psychological, physical and biochemical factors and a successful spermatogenesis is of the uttermost relevance for male fertility (Schlatt & Ehmcke 2014).

2. Brief overview of the testicular structure

The mammalian testicles are combined endocrine and exocrine glands that performs two essential functions in male reproduction: steroidogenesis and spermatogenesis. Steroidogenesis corresponds to the biosynthesis of sex steroid hormones and takes place in the testicular interstitium (vascularized region). On the other hand, spermatogenesis is the complex cellular process that transforms spermatogonial cells into spermatozoa and occurs within the seminiferous tubules (avascular compartment) (Saladin 2003; Correia et al. 2015).

The testicles are surrounded by two distinct layers of protective connective tissue (Figure I.1). The outer *tunica vaginalis* is a thin serous sac derived from the peritoneum during the descent of the testicles. Beneath the *tunica vaginalis* is the *tunica albuginea*, a tough, white and dense fibrous membrane that directly encapsulates each testis. Fibrous inward extensions of the *tunica albuginea* divide the testis into 250 to 300 wedge-shaped testicular lobules,

each one enclosing one to three loop-shaped seminiferous tubules (SeT) (Figure I.1) (Graaff 2001; Saladin 2003).

The Set, the functional units of the testis, are lined on its inside by a thick germinal epithelium, which consists of several layers of germ cells in the process of becoming sperm, and a much smaller number of somatic cell, the Sertoli cells (Figure I.2) (de Rooij & Mizrak 2008). These cells play an essential role in nurturing and providing structural support for the sperm cells during their development. Sertoli cells also secrete the hormone inhibin, which regulates the rate of sperm production (Saladin 2003; Jones & Lopez 2006).

The interstitial space among SeT contains structures vital to testicular function. Blood and lymphatic vessels and various cell types, including fibroblasts, leukocytes, macrophages and endocrine cells, the Leydig cells (Figure I.2) are present in the interstitial spaces (Sharpe 1984; Colborn et al. 1993). The function of these cells is to produce and secret testosterone (T), which plays an important role in downstream masculinization events, descent of the human testicles into the scrotum before birth and initiation and maintenance of spermatogenesis (Akingbemi 2005; Sikka & Wang 2008).

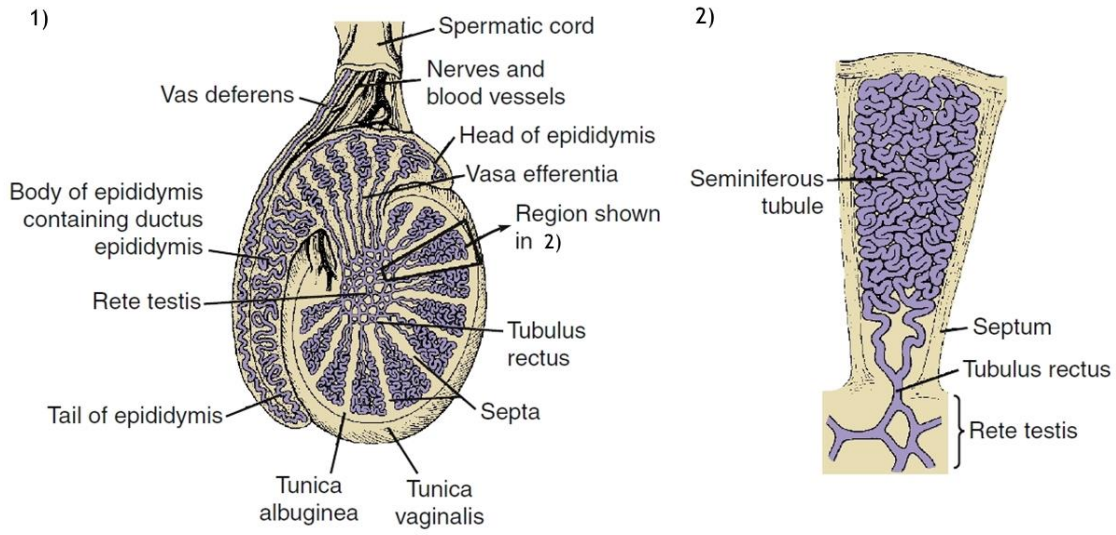


Figure I.1. Schematic representation of the mammalian testis. 1) Section of the testis which is encased by two tissue layers, from the inside to the outside, *tunica albuginea* and *tunica vaginalis*. Various septa extending from the *tunica albuginea* divide the testis in lobules, where the seminiferous tubules are located. 2) A seminiferous tubule within a single testicular lobule (adapted from (Jones et al. 2014)).

3. Testicular physiology

3.1. Spermatogenesis

Spermatogenesis (Figure I.2) is a complex biological process of maturation of spermatogonia stem cells into spermatozoa, which takes place in the SeT of the testis and encompasses tightly coordinated mitosis, meiosis and cell differentiation events (Hess & Franca 2008; Hermo et al. 2010).

Spermatogonial stem cells, which lie at the basement membrane of SeT as single cells, replicate mitotically to both guarantee the germ cell line (spermatogonia A), and give rise to new populations committed to differentiate and move along the seminiferous epithelium (spermatogonia B). Spermatogonia B are diploid germ cells that differentiate into primary spermatocytes which migrate slightly away from the wall on its way to becoming a primary spermatocyte, the next stage in the process of spermatogenesis (Saladin 2003; de Rooij & Mizrak 2008; Hess & Franca 2008). The primary spermatocyte undergoes cells division (Figure I.2), meiosis I that reduces the chromosome number by half. The daughter cells, called secondary spermatocytes, are therefore haploid. A second round of cell division, meiosis II, occurs in both of the secondary spermatocytes, separating the chromosome pairs. The result is four daughter cells called spermatids. The cellular restructure events that occur in the spermiogenesis transforms round-spermatids in elongated-spermatids, and then, elongated-spermatids into spermatozoa, which are finally released into the lumen of the SeT (Saladin 2003; Jones & Lopez 2006; Hess & Franca 2008). Therefore the process of spermatogenesis is complete.

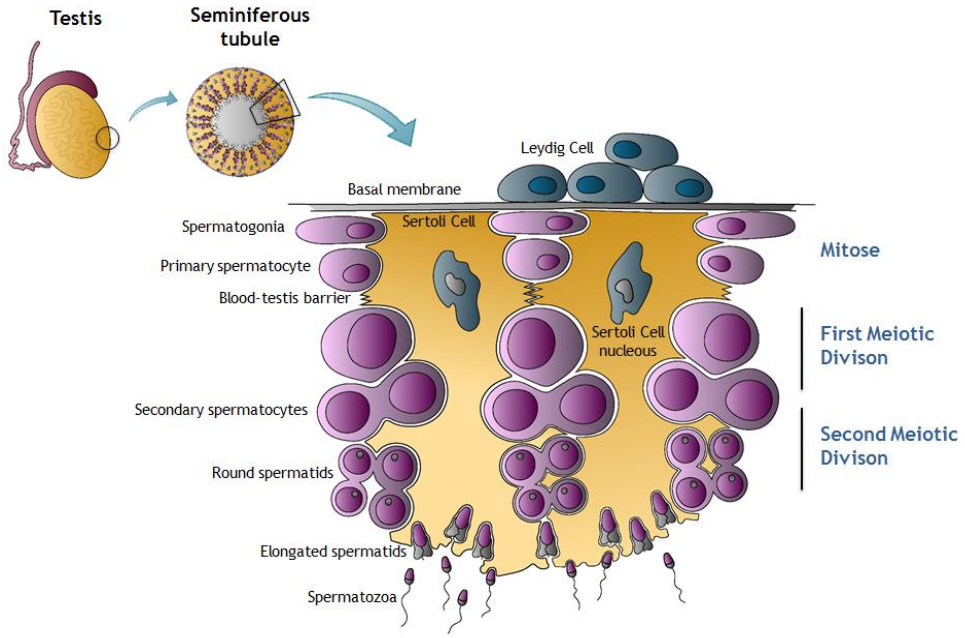


Figure I.2. Schematic representation of the testicular histology showing the organization of seminiferous tubules, and the different stages of mammalian spermatogenesis (adapted from (Correia 2014)).

3.2. Non-hormonal control of spermatogenesis: importance of glycolytic metabolism

Sertoli cells – often referred to as 'nurse cells' – play a crucial role in the regulation of spermatogenesis and determine the rate at which spermatozoa are produced (Orth et al. 1988; Johnson et al. 2008). They are responsible for providing energy and nutritional support to developing germ cells. The structure of these cells is extremely complex, with numerous cup-shaped projections, and each Sertoli cell has the ability to support approximately 30–50 germ cells at different stages of development (Weber et al. 1983; Cheng et al. 2010).

Therefore, the maintenance of spermatogenesis is highly dependent on the metabolic cooperation established between germ cells and Sertoli cells, and it is imperative that germ cells receive an adequate level of energy substrates. Otherwise they will degenerate and enter the apoptotic pathway (Grootegoed et al. 1984; Boussouar & Benahmed 2004).

Glucose is an essential component for the maintenance of spermatogenesis. However, there are low levels of this sugar in the tubular fluid, and this is mainly due to the fact that it is metabolized into different subproducts (Robinson & Fritz 1981; Rato et al. 2012). The metabolic intermediates derived from the metabolism of glucose (glycolysis, Figure I.3) are crucial for the role of Sertoli cells as germ cells feeders. Sertoli cells produce high quantities of lactate (Mita & Hall 1982), which is widely recognized as the preferred substrate for the developing germ cells (Robinson & Fritz 1981; Riera et al. 2001; Boussouar & Benahmed 2004), and has also been indicated as an anti-apoptotic factor (Erkkilä et al. 2002).To ensure the high production of lactate, Sertoli cells take up glucose from the interstitial fluid via specific integral membrane glucose transporters (GLUTs). Four GLUTs namely glucose transporter 1 (GLUT1) (Carosa et al. 2005), glucose transporter 2 (GLUT2) (Kokk et al. 2004), glucose transporter 3 (GLUT3) (Galardo et al. 2008) and glucose transporter 8 (GLUT8) (Ulisse et al. 1992) have been identified in the plasma membrane of Sertoli cells to date.

After entering the cell, glucose follows the glycolytic pathway (Figure I.3) and a set of catalytic reactions occur until its conversion to pyruvate. Phosphofructokinase 1 (PFK1) plays a major role in glycolysis since it catalysis the irreversible conversion of fructose-6-phosphate (F-6-P) to fructose-1-6-bisphosphate (F-1,6-BP) (Chehtane & Khaled 2010). The end-product of glycolysis, pyruvate, is metabolized by the lactate dehydrogenase (LDH) enzyme, which catalyzes the interconversion of pyruvate and lactate, with the concomitant oxidation/reduction of NADH to NAD^+ (Everse & Kaplan 1973). Once produced, lactate is exported to the extracellular medium through the monocarboxylate transporter 4 (MCT4) and used by developing germ cells as the main energy source for ATP production (Boussouar & Benahmed 2004).

Recent findings have shown that this metabolic system is very sensitive to hormonal regulation. For example, androgens, estrogens and endocrine disruptors are able to modulate the metabolism of rat and human Sertoli cells (Oliveira et al. 2011).

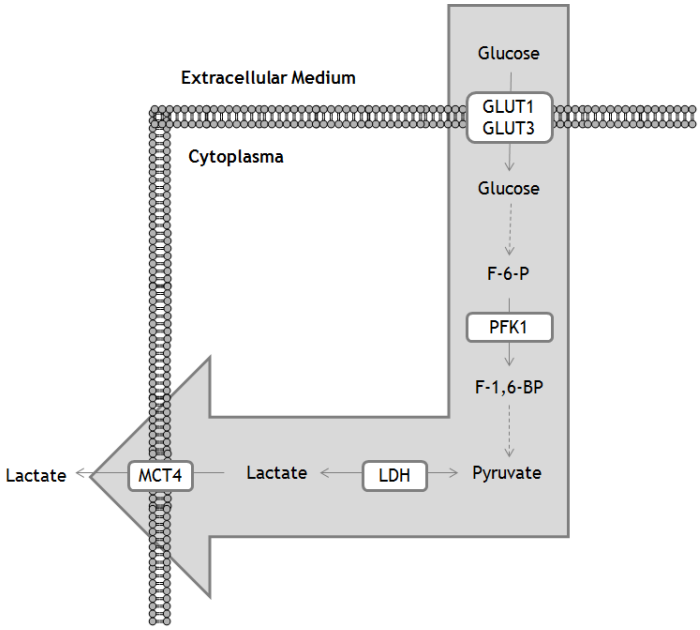


Figure I.3. Schematic diagram of the glycolytic pathway in Sertoli cells. Glucose is uptake from the interstitial fluid via glucose transporters (GLUT1 and GLTU3), and metabolized by the activity of several enzymes, including phosphofrutokinase 1 (PFK1), to fructose-6-phosphate (F-6-P) and fructose-1,6-bisphosphate (F-1,6-BP), which in turn originates pyruvate. Pyruvate is converted to lactate via lactate dehydrogenase (LDH) activity. Lactate is transported to the extracellular medium by monocarboxylate transporters, specifically the monocarboxylate transporter 4 (MCT4).

3.3. Hormonal control of spermatogenesis

The male sexual function is highly dependent on hormonal regulation, particularly by sex steroid hormones. Spermatogenesis is regulated by the hypothalamus–pituitary–testis axis (Figure I.4). The hypothalamus releases gonadotropin releasing hormone (GnRH), which acts on the pituitary inducing the biosynthesis and secretion of gonadotropins, namely, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Cheng & Mruk 2010). The dysfunction of this axis leads to infertility (Sokol 2009).

In the testis, FSH acts on the Sertoli cells, inducing the production of several growth factors and other stimulatory agents of spermatogenesis. Conversely, LH acts on Leydig cells, stimulating the synthesis of T (Walker & Cheng 2005; Walker 2009).

Moreover, inhibin produced by Sertoli cells blocks the production and release of FSH by the pituitary (Pierik et al. 2003), thus controlling the output of spermatogenesis. The other negative feedback regulatory mechanism is driven by T, which regulates the spermatogenic process and inhibits GnRH, leading to the inhibition of the productions of FSH and LH (Holdcraft & Braun 2004).

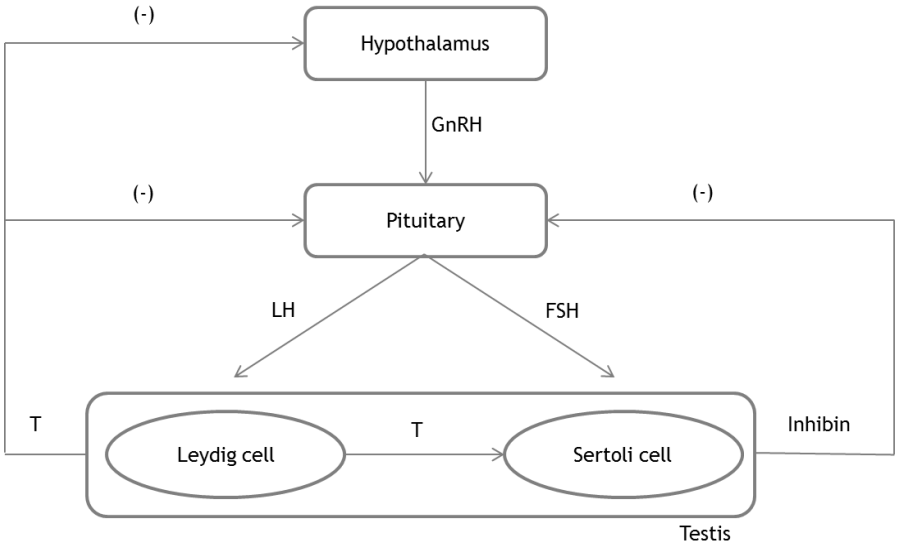


Figure I.4. Hormonal regulation of spermatogenesis. Release of gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the pituitary to secrete two gonadotropins, the luteinizing hormone (LH) and the follicle-stimulating hormone (FSH). FSH stimulates the activity of Sertoli cells and LH acts on Leydig cells, inducing the production of testosterone (T). A negative feedback (-) by T on the hypothalamus and pituitary regulates the levels of GnRH, LH and FSH, nevertheless its main action is to decrease secretion of LH. FSH secretion is also subject of a negative feedback (-) by inhibin secreted by Sertoli cells.

FSH can also resume spermatogenesis in gonadotrophin-suppressed men, independently of their T levels (Matsumoto et al. 1983). However, Matsumoto et al., suggest that this effect is caused by a heightened FSH-induced Sertoli cell sensitivity to residual T production within the testis. The regulation of T synthesis seems to be the only indispensable function of LH within the adult testis (Holdcraft & Braun 2004).

Furthermore, in the mammalian testicles, germ cell survival has been shown to be dependent on FSH, LH, human chorionic gonadotropin and T. Exposure to excessively high or low levels of hormones can lead to cellular apoptosis within the testis (Shaha et al. 2010). Sertoli cells have FSH and T receptors, which are the main hormonal regulators of spermatogenesis and in mature Sertoli cells; FSH regulates their glycolytic metabolism by increasing glucose uptake (Hall & Mita 1984) and the production of both pyruvate and lactate (Riera et al. 2001).

3.4. Androgens and oestrogens actions

Androgens are essential both for male fertility and for the maintenance of spermatogenesis (Holdcraft & Braun 2004). T is the major androgen present in the testis, and is particularly responsible for supporting spermatogenesis. In the absence of T or functional androgen receptors (AR), males become infertile due to spermatogenesis rarely progressing beyond meiosis (Chang et al. 2004; Walker 2009). In the testis, T receptors (AR) are expressed only in Leydig cells, peritubular cells and Sertoli cells. AR are not expressed in germ cells of the mature testis (Lyon et al. 1975) and germ cells are not thought to be direct targets of T

action. Instead, the major target of T in the testis is the Sertoli cell, which surrounds and nourishes germ cells as they mature into spermatozoa (Griswold 1998).

Most T is produced locally by Leydig cells in the interstitial space of the testis. As a result of this local production, T levels in the testis are 25 to 125-fold greater (340 to 2,000 nM) as compared to serological levels (8.7 – 35 nM). T levels are similarly elevated in the testicles of rodents (Comhaire & Vermeulen 1976; Turner et al. 1984; Awoniyi et al. 1989). Thus far, the specific physiologic requirements for high levels of T in the testis are unknown. However, it has been established that spermatogenesis does not proceed in the absence of relatively high levels of T (Zirkin et al. 1989).

Besides the classical regulators of spermatogenesis, androgens, also oestrogens seem to implicated in the regulation of sperm production and male sexual function. Natural estrogens include estrone, 17β-estradiol (E_2) and estriol, of which the most well-known and potent is E_2 (Królik & Milnerowicz 2012). E_2 (and estrogens in general), is produced within testicles from androgens by the enzyme aromatase and plays an important role in the development and physiology of the male reproductive tract of mammals. This includes testicular cell differentiation, proliferation, metabolism, and apoptosis (Carreau et al. 2007), and could be concerned with the regulation of spermatogenesis (Carreau & Hess 2010).

Indeed, although estrogens are classically viewed as female hormones, the last decades have witnessed the emergence of a set of experimental and clinical evidences indicating the importance of estrogens regarding male fertility, specifically for the production of viable spermatozoa (Hess 2003; Carreau & Hess 2010). By contrast, a substantial amount of data also has attributed the deleterious effects of estrogens and similar substances for male reproductive function (Correia et al. 2015). It was known in the 1930s and 40s that abnormal exposure to high doses of estrogens could induce malformation of the male reproductive tract and affect spermatogenic output (Toppari & Skakkebaek 1998; Sultan et al. 2001; Li et al. 2001; Sweeney 2002; Sweeney et al. 2007; Li & Rahman 2008), and actually the environmental exposure to estrogens or estrogen-mimicking compounds is a matter of concern and an health public issue (Marques-Pinto & Carvalho 2013).

Despite the fact that the concentration of estrogens is typically higher in the blood found in the testicular vein and lymph than in the general circulation (Hess 2003), clinical studies also have suggested that there is and increased intratesticular production of estrogens linked to spermatogenic failure, and thus to male infertility. Indeed, increased levels of E_2 have been detected in semen, intratesticular fluid and spermatic vein of idiopathic infertile patients (Correia et al. 2015).

Pavlovich et al. characterized men with normal spermatogenesis as having a T/E_2 ratio of 14.5, whereas men with severe male infertility had a mean T/E_2 ratio of 6.9, and attributed the diminished T/E_2 ratio, consequence of the increased E_2 levels, as the cause of infertility.

Therefore, the androgen/oestrogen balance is essential for normal sexual development and reproduction in mammals. In the testis, the maintenance of this balance is fine-tuned via paracrine and endocrine factors, but is also related to aromatase activity (Saez 1994; Carreau et al. 2003). Indeed, the abnormal T/E_2 ratio observed in infertile patients has been shown to be associated with the increased activity of aromatase (Lardone et al. 2010), with some authors suggesting that the forms of male infertility associated with this endocrinopathy could be treated by the administration of aromatase inhibitors (AIs) (Pavlovich et al. 2001; Raman & Schlegel 2002).

4. Aromatase

Aromatase, the enzyme involved in the last step in the steroidogenic pathway catalyzing the formation of oestrogens from androgens (Figure I.5), is localized in the endoplasmic reticulum of various tissues including testis, liver, placenta, gonads, brain, bone and adipose tissue. In fact, it is difficult to find a tissue completely devoid of aromatase gene expression (Simpson et al. 1994). In mouse (Nitta et al. 1993), rat (Janulis et al., 1998) and the human testis (Carreau et al., 2010) aromatase was found in the Leydig cells, Sertoli cells, immature germ cells, spermatocytes, spermatids and ejaculated spermatozoa (Carreau et al. 2010).

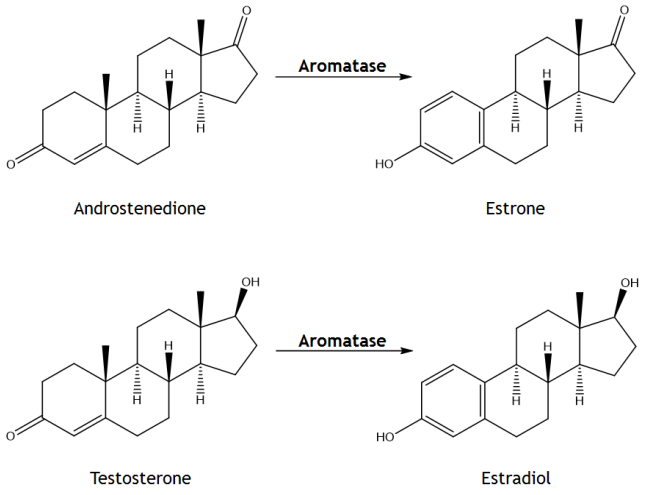


Figure I.5. Steroidogenesis reactions catalyzed by aromatase. Aromatase regulates the last steps of estrogen biosynthesis, aromatizing the A-ring of androgens, such as androstenedione and testosterone into estrone and estradiol, respectively.

Aromatase, a member of the cytochrome P450 (CYP450) protein superfamily, is a unique gene product of the *CYP19* gene and it was first isolated by Ryan et al. in 1959 from the microsomal fraction of fresh human placental tissue (Ryan 1959; Danielson 2002).

The aromatase enzyme complex comprises two proteins: a ubiquitous NADPH cytochrome P450 reductase and a cytochrome P450, which contains the heme and the steroid-binding pocket (Simpson et al. 1994). The cytochrome P450 component binds the androgen

substrates, which are then reduced, in the presence of molecular oxygen, by the transfer of reducing equivalents from NADPH by cytochrome P450 reductase (Toda et al. 1994).

Although it has been proven the beneficial effect of estrogens in male reproduction, the overexpression of aromatase leads to an androgen/oestrogen imbalance, causing Leydig cell hyperplasia, dysmorphic SeT, disrupted spermatogenesis and, consequently, male infertility (Li et al. 2001). Furthermore, as discussed above, abnormal activity of aromatase and increased levels of estrogens have been found in the testis and body fluids of male infertile patients (Correia et al. 2015).

Furthermore, Raman and Schlegel shown that AIs significantly improve the number and quality of spermatozoa of infertile men (Raman & Schlegel 2002). The treatment with AIs normalized the reduced intratesticular and serum T levels, while also decreased the estradiol levels (Li et al. 2004). Also, it has been purposed the use of aromatase inhibition as a treatment for idiopathic male infertility (Pavlovich et al. 2001).

4.1. Aromatase inhibitors

AIs are often categorized as steroidal or non-steroidal based on their structural similarity with steroids, or as 1st, 2nd and 3rd generation drugs based on their evolution time. Steroidal AIs such as formestane and exemestane, bind irreversibly to the catalytic site of the enzyme and nonsteroidal AIs such as aminoglutethimide, fadrozole, anastrozole and letrozole bind competitively and reversibly (Kil et al. 2009). Since, non-steroidal inhibitors are more likely than steroidal compounds to lack specificity once they have the potential to block several cytochromes P450 mediated steroid hydroxylations. On the other hand, steroidal inhibitors or their metabolites have a greater potential to produce estrogenic, androgenic, glucocorticoid, and progestinic agonist or antagonistic effects through the inherent properties of their structures (Ferlin et al. 2013).

To date, three generations of AIs are available. The first generation of AIs is aminoglutethimide (Figure I.6A) that was marketed in the late 1970s (Graves & Salhanick 1979; Santen & Misbin 1981). Unfortunately, aminoglutethimide was far from being an ideal drug once it exhibited several drawbacks, mainly, high toxicity, lack of selectivity and inhibition of other CYP450 enzymes involved in cortisol and aldosterone biosynthesis (Hughes et al. 1970).

Concerning on to the second generation, fadrozole (Figure I.6B) which contains an imidazole group, is more selective and potent than aminoglutethimide. Nevertheless, it still displayed effects on aldosterone, progesterone and corticosterone biosynthesis (Beretta et al. 1990). Formestane (Figure I.6C), a steroid analogue, was the first selective AI used in clinical trial. It was demonstrated to be effective and well tolerated. However, the fact of its requiring intramuscular administration limited its clinical use (Coombes et al. 1984).

Finally, the third generation of AIs includes two triazole derivatives, anastrozole (Plourde et al. 1995) (Figure I.6D) and letrozole (Bhatnagar et al. 1990) (Figure I.6E) and one steroid analogue, exemestane (Evans et al. 1992) (Figure I.6F).

Exemestane is a steroidal analog that is catalytically converted into a chemical reactive species, leading to irreversible inactivation of aromatase (Wang & Chen 2006). These AIs have shown a slight improvement in efficacy and an apparently superior toxicity profile over tamoxifen (Thürlimann et al. 2004). On the other hand, anastrozole and letrozole are non-steroid derivatives and competitive inhibitors of androstenedione (Figure I.5). These AIs can be considered to consist of two parts: one is the azole ring with a nitrogen atom which interacts with the heme iron atom of the cytochrome P450 of aromatase and the other is the bulky aryl part, which mimics the steroid ring of the substrate (Pouget et al. 2004; Zarghi & Ghodsi 2010). For this reason, the last generation of AIs has been used for the treatment of male infertility. However, the occurrence of important side effects associated with the prolonged clinical use of AIs increases the importance and necessity of search of new, potent, more selective, and less toxic cytochrome 19 (CYP19) inhibitors (Goss 1999).

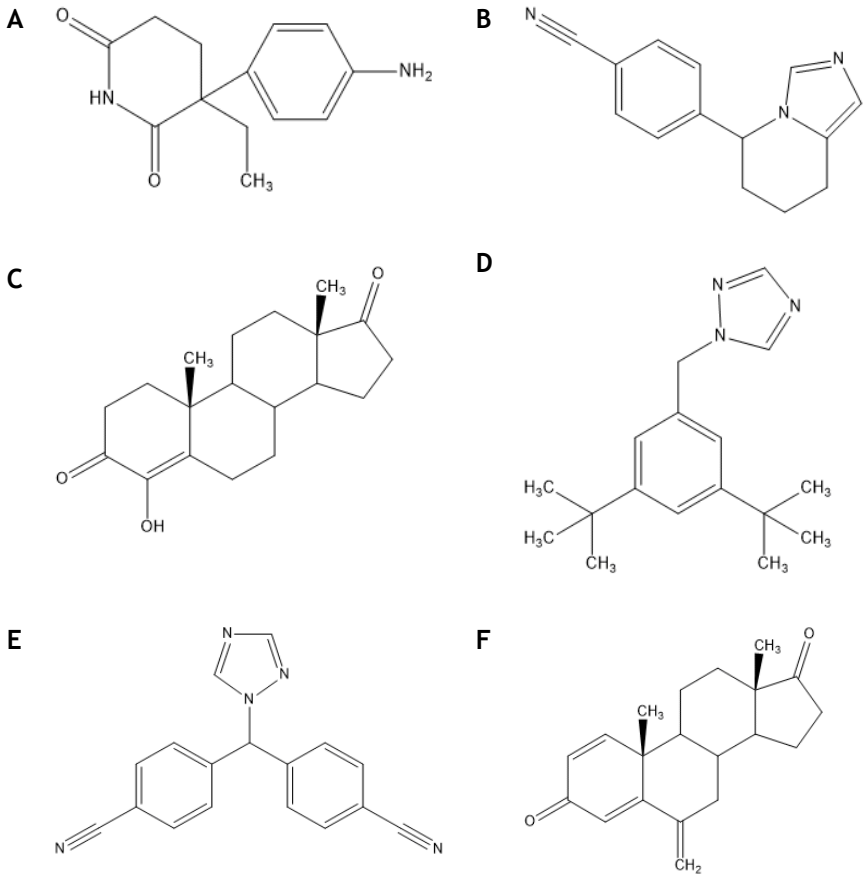


Figure I.6. Structure of aromatase inhibitors. (A) aminoglutethimide, (B) fadrozole, (C) formestane, (D) anastrozole, (E) letrozole, (F) exemestane.

4.1.1. Letrozole

Letrozole (4,4'-[1H-1,2,4-triazol-1-ylmethylene]*bis*-benzonitrile) (Figure I.6.E) has been demonstrated to be a highly potent AI both *in vitro* and *in vivo*. Particularly, letrozole exhibited a higher ability to block estrogen production in comparison to several AIs such as aminoglutethimide, anastrozole, exemestane and formestane (Bhatnagar et al. 1990; Bhatnagar et al. 2001; Bhatnagar 2007). Its chemical structure contains a triazole group that selectively interacts with the heme prosthetic group of the cytochrome P450 enzyme (Haynes et al. 2003; Hong & Chen 2006) and two phenyl rings which provides tighter fit inside the binding cavity by mimicking the steroidal backbone of the natural substrate, androstenedione. Furthermore, the two nitrile groups at the para positions of the phenyl ring mimics the carbonyl group of androstenedione and functions as hydrogen bond acceptors (Nantasenamat et al. 2013).

Letrozole is highly selective for cytochrome P450 blocking only estrogen production without altering progesterone or corticosteroid synthesis (Bhatnagar et al. 1990; Trunet et al. 1993).

Letrozole has been applied clinically for treatment of males in several trials. The treatment with letrozole in infertile male patients who had low serum T and T/E ratios < 10, increase sperm concentration, motility and morphology, serum T levels and decrease the mean serum estradiol levels (Saylam et al. 2011).

Owing to the success of letrozole, intense efforts have been invested in deriving novel AIs from this structural scaffold.

4.1.1.1. Letrozole analogues

Recently, many studies have been undertaken to evaluate several new families of AIs. The analysis of the aromatase crystal structure allowed discovering that there are three unique structural features of the aromatase active site. First, the enzyme binding pocket is highly hydrophobic. Therefore, hydrophobic groups such as alkyl groups and aromatic rings may be favorable for enhanced binding. Second, hydrogen bonding plays an important role in enzyme-substrate interaction, so ligands should contain hydrogen bond acceptor groups (like – NO_2, -CN). Third, the Fe^{2+} in the heme group of aromatase is capable of chelating to hetero atoms in inhibitors, especially nitrogen atoms (Ghosh et al. 2009).

Based on these structural features, the design of AIs was centered on enhancement of hydrophobic interactions, hydrogen bonding and heme iron coordination (Bonfield et al. 2012).

Various modifications in the letrozole structure have been investigated in order to increase the effectiveness of the compound. Thus, in order to analyze the various types of letrozole analogs, a division of its structure into three principal moieties (Figure I.7) can be considered:

the hetero aromatic ring (A), which is substituted usually by imidazole and triazole groups; two phenyl groups (B) commonly substituted in *para* position by cyano groups and; the bridge (C), which bonds the moieties A and B.

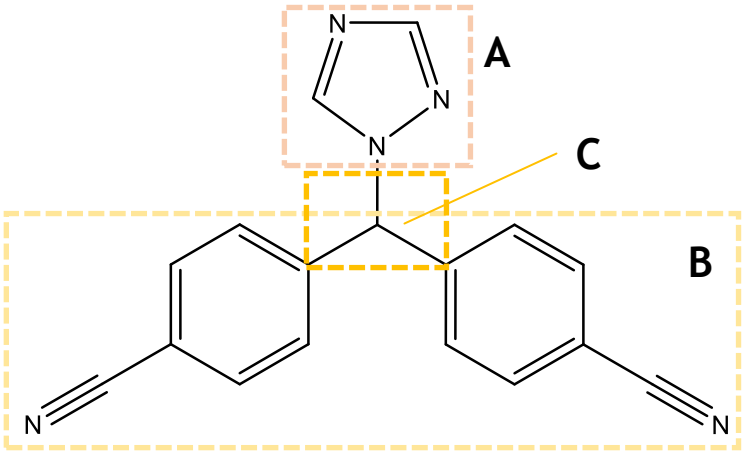
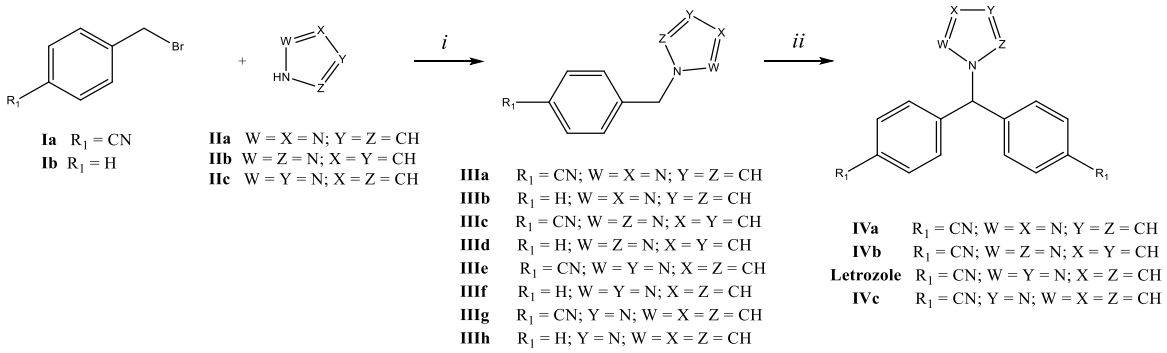


Figure I.7. Schematic representation of letrozole structure. Letrozole can be divided into three principal moieties: the hetero aromatic ring (A); two phenyl groups (B) and; the bridge (C).

One of the most important features for strong inhibitor binding to the CYP enzymes is the capability to interact as the ligand with the iron atom of the heme group (Cole & Robinson 1990). The azoles family have the capacity to bind with heme iron of cytochromes and is going to hold an increasingly prominent position in development of AIs (Bhatnagar et al. 1990; Plourde et al. 1995). Regarding the alterations in hetero aromatic ring (A), functional groups such as imidazole (Gobbi et al. 2007; Gobbi et al. 2010), triazole (Leonetti et al. 2004; Yahiaoui et al. 2004) and pyridine (Auvray et al. 1999; Kim et al. 2004) have been used to enhance the coordination between the heme iron atom of the enzyme and the heterocyclic nitrogen lone pair (Negrerie et al. 2006). Thereby, Le Borgne et al. reported letrozole analogues having an arylindole moiety with imidazole or 1,2,4-triazole heterocycles (Marchand et al. 2003; Lézé et al. 2006).

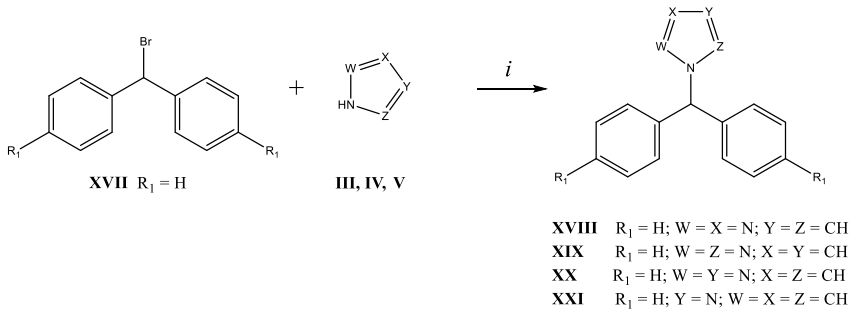
In an effort to develop more potent inhibitors of aromatase, Doiron et al. sought out to synthetized letrozole (Figure I.6E) analogues (Doiron et al. 2011). In particular, they studied the effect of 1,2,3-triazole instead of the 1,2,4-triazole present in the letrozole structure, the goal of which was to test various substituted 1,2,3-triazoles and the requirement of one or two cyano or phenyl moieties together with various 1,2,3-; 1,2,4- and 1,2,5-triazoles. For comparison purposes, the corresponding imidazole analogues were synthesized. The syntheses of letrozole analogues, mono-benzonitrile, monophenyl, and *bis*-benzonitrile letrozole analogues in which the 1,2,4-triazole was substituted with 1,2,3-triazole (**IIIa - IIIf**, **IVb**) and imidazole (**IIIg**, **IIIh**, **IVc**) were also synthesized as shown in Scheme I.1.



Scheme I.1. Syntheses of letrozole analogues: mono-benzonitrile, monophenyl, and *bis*-benzonitrile in which the 1,2,4-triazole was substituted with 1,2,3-triazole and imidazole. Reagents and conditions: (i) K_2CO_3, reflux, Acetone, 12 h; (ii) t-BuOK, 4-fluorobenzonitrile, rt, DMF 2.5 h.

Synthesis began with the nucleophilic substitution of brominated precursors **Ia** or **Ib** by the required five member heterocycles in acetone in the presence of potassium carbonate, affording the expected heterocyclic compounds with or without *para*-cyano groups (**IIIa**, **IIIc**, **IIIe**, **IIIg** or **IIIb**, **IIId**, **IIIf**, **IIIh** respectively). As for the synthesis of letrozole from compound **IIIe**, mono-benzonitrile precursors **IIIa**, **IIIc** and **IIIg** were subject to base-induced condensation with 4-fluorobenzonitrile to afford the *bis*-benzonitrile analogues, **IVa**, **IVb** and **IVc** respectively (Scheme I.1).

Since they were not able to obtain the corresponding *bis*-phenyl analogues of **IIIb**, **IIId**, **IIIf** and **IIIh** under basic condensation with fluorobenzene, commercially available bromide **V**, (1,2,4-; 1,2,3-)triazole, and imidazole were used for the synthesis of *bis*-phenyl derivatives **VIa**, **VIb**, **VIc** and **VId** (Scheme I.2).



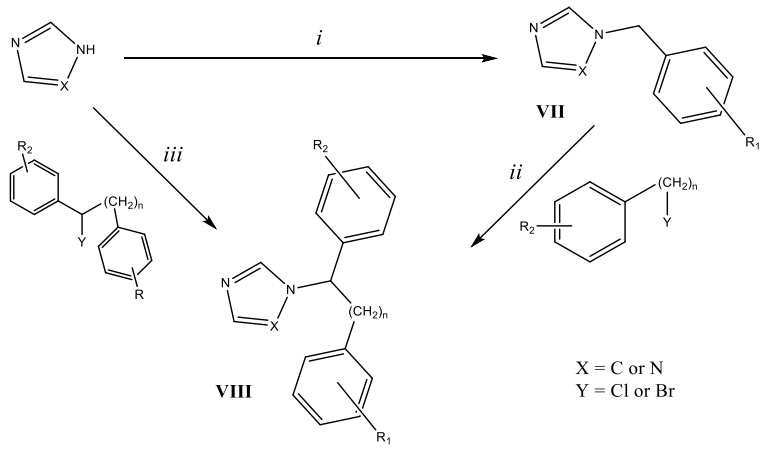
Scheme I.2. The synthesis of *bis*-phenyl derivatives VIa, VIb, VIc, VId. Reagents and conditions: (i) K_2CO_3, reflux, Acetone, 12 h.

In this report the authors concluded that nitrogen atom at positions 3 or 4 were both important for aromatase inhibition (Doiron et al. 2011).

Concerning to the modification in the phenyl group (B), the presence of a *para*-cyano group and two aryl groups seems crucial for good activity (Doiron et al. 2011). These results were in agreement with previous investigation on the structure-activity relationship identifying the importance of electron withdrawing groups at the *para* position of the phenyl ring while demonstrating that nitrile groups afforded the best activity (Browne et al. 1991). This group mimics the carbonyl group of androstenedione as a hydrogen bond acceptor (Furet et al. 1993; Recanatini et al. 2001).

This is concomitant with the findings by Schuster et al. that two aromatic rings along with two hydrogen bond donors are important pharmacophores for strong aromatase inhibition (Schuster et al. 2006; Doiron et al. 2011). The developed QSAR models indicate that optimum number of H-bond-acceptor groups (less than or equal to two) is favorable for the binding and this is supported by docking results (Roy & Roy 2010). Through the comparative analysis of the structures of letrozole and androstenedione, Doiron et al. observed that distances between nitrogens of the nitrile group and those at positions 3 or 4 of the triazole heterocycle are similar to distances between oxygens in the structure of androstenedione.

In order to achieve a good aromatase inhibition, Karjalainen et al. also developed new inhibitors with modifications in the bridge of the letrozole structure (C). For this end, different synthesis was used (Scheme I.3, I.4 and I.5) (Karjalainen et al. 2000). Saturated *N*-substituted α,ω-diarylalkylimidazoles and triazoles **VIII** were prepared according to Scheme I.3.

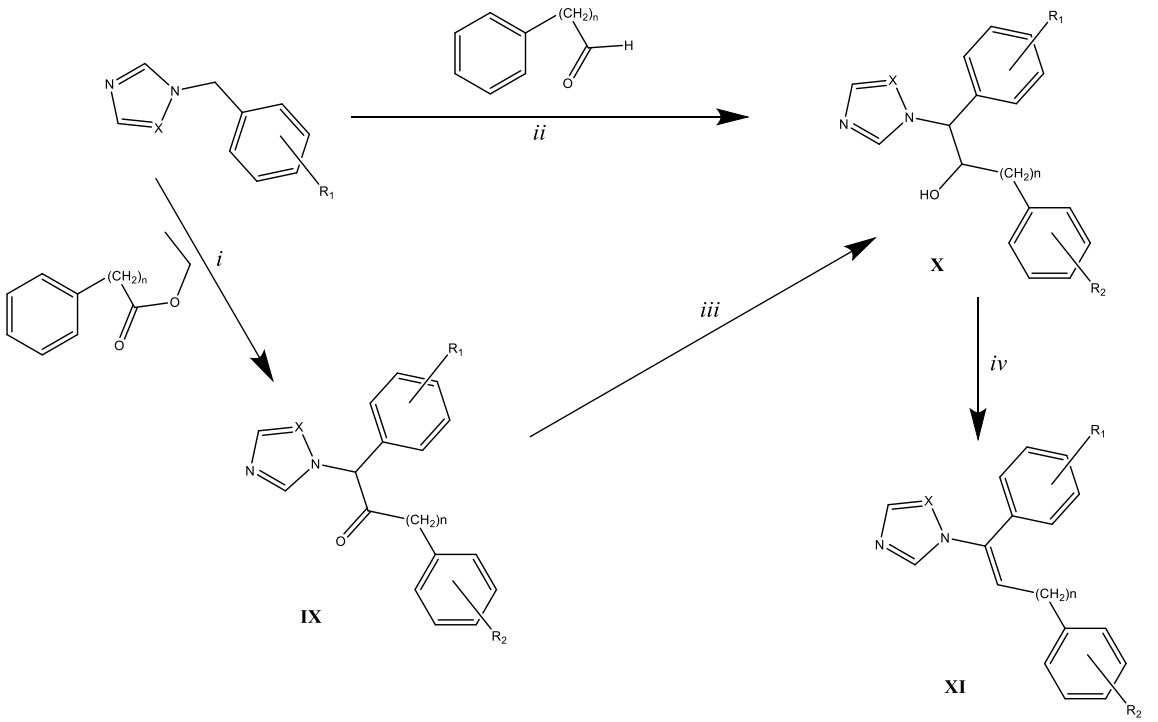


Scheme I.3. Synthesis of saturated *N*-substituted α,ω-diarylalkylimidazoles and triazoles. Reagents and conditions: (i) R_1-Ph-CH_2Br; (ii) *n*-BuLi, THF; (iii) NaH, DMF.

In this procedure imidazole or triazole underwent a direct *N*-alkylation with an appropriately substituted benzyl halide to give **VII**. Treatment of compound **VII** with *n*-butyl lithium followed by alkylation with an appropriate aryl alkyl halide gave the end product **VIII**.

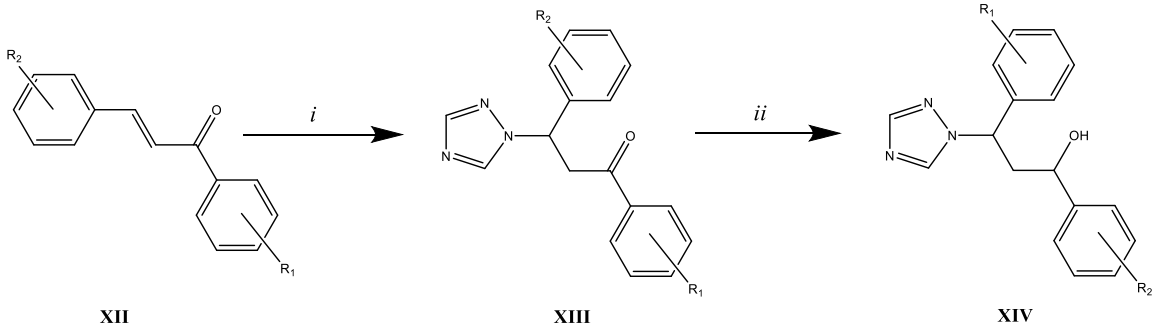
An alternative method used to synthesize the compound **VIII** was the direct *N*-alkylation of imidazole or triazole with diarylalkyl halide. *N*-Substituted ω,ω-diarylalkyl imidazoles and triazoles could also be prepared with this method starting from appropriate diarylalkyl halides.

Another synthesis is 1-substituted α,ω-diaryl-ß-hydroxyalkyltriazoles and **X** and unsaturated *N*-α,ω-diaryltriazoles and imidazoles **XI** were synthesized starting from *N*-benzyl triazoles and imidazoles, which first were treated with late *n*-butyl lithium to produce the carbanion of the benzylic carbon. Then the reaction with appropriately substituted arylalkyl aldehydes afforded **X** and the following elimination of water by the treatment with acid yielded **XI**. Alternatively the nucleophilic addition of the carbanion to an appropriately substituted arylalkyl esters gave **IX**, which was then reduced with sodium borohydride to **X** (Scheme I.4).



Scheme I.4. Synthesis of 1-substituted α,ω-diaryl-ß-hydroxyalkyltriazoles and X and unsaturated *N*-α,ω-diaryltriazoles and imidazoles XXVI. Reagents and conditions: (i) *n*-BuLi, THF; (ii) *n*-BuLi, THF; (iii) $NaBH_4$; (iv) H^+, ethanol.

1-(1,3-diaryl-3-hydroxypropyl)triazoles **XIV** were prepared by heating 1,3-diarylpropenones **XII** and triazole in the presence of Triton B followed by reduction with were prepared sodiumborohydride (scheme I.5).

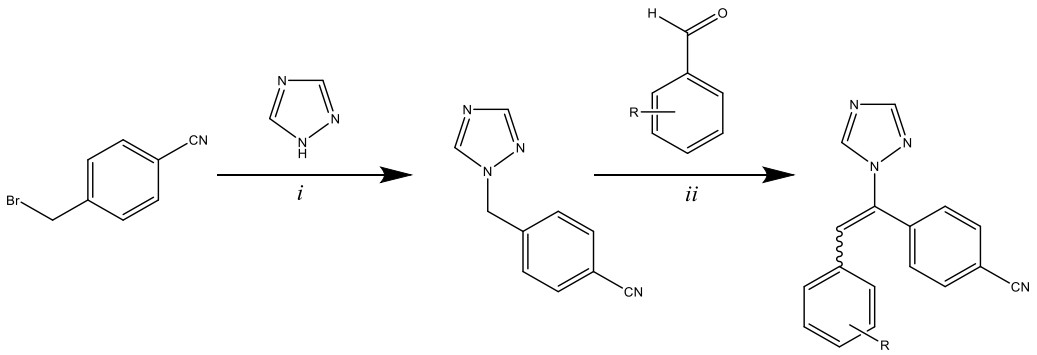


Scheme I.5. Synthesis of 1-(1,3-diaryl-3-hydroxypropyl)triazoles XIV. Reagents and conditions: (i) triazole, Triton B, heat; (ii) $NaBH_4$, methanol.

With this work, the authors described that a good inhibition of aromatase is achieved with the structures that included a framework of diarylalkyl moiety comprising a lengthened carbon chain linked to the 1-position of triazole. An especially favorable framework seems to be the α,ω-diarylalkyl with three or four carbon atoms in the bridge, which connects the framework to the heterocycle. Also, the double bond in the carbon bridge of the α,ω-diarylalkyl derivatives brought additional beneficial effects especially in terms of selectivity (Karjalainen et al. 2000).

Substituted by ethenylbenzene derivates, which linked to 1-benzyl-1H-1,2,4-triazole have been also developed and evaluated (Vosooghi et al. 2014).

4-Bromotolunitrile was converted to 4-(4-cyanobenzyl)-1,2,4-triazole and subsequently to corresponding 4-[2-aryl-1-(1H-1,2,4-triazol-1-yl)ethenyl]benzonitrile (**XV**) according to the procedure presented in Scheme I.6.



XV

Scheme I.6. Synthesis rout of 4-[2-aryl-1-(1H-1,2,4-triazol-1-yl)ethenyl]benzonitriles XV. Reagents and conditions: (i) MeOH, KOH, DMF; (ii) benzaldehyde, 1,4-Dioxane, recrystallized in EtOH. R: benzaldehyde, (2; 3; 4)-chlorobenzaldehyde, 2,4-dichlorobenzaldehyde, 3-fluorobenzaldehyde, m-anisaldehyde, anisaldehyde, 2,4-dimethoxybenzaldehyde, 3,4,5-trimethoxybenzaldehyde and 4-(dimethylamino)benzaldehyde.

II. Aim

Aromatase regulates the last step of estrogen biosynthesis, aromatizing the A-ring of androgens such as T and androstenedione into E_2 and estrone, respectively. Thus, in men E_2 is responsible for a number of effects originally attributed to T, and accumulating scientific evidence has been implicating estrogens in the control of spermatogenesis and male fertility. On the other hand, men with a high serum E_2/T ratio are infertile and it has been suggested that they can be treated with AIs. Indeed, AIs have been shown to increase T levels in infertile men with altered E_2/T ratio. Among other classes of compounds, letrozole is a nonsteroidal AI that has been widely used for treatment of male infertility.

The present dissertation firstly aimed to synthesize new AIs analogues of letrozole. Molecular docking studies to predict binding energies and the possible interactions of the synthesized compounds with aromatase were performed. After the synthesis, purification and characterization steps, two letrozole analogues namely 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)vinyl)benzonitrile and 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)ethyl)benzonitrile were selected for evaluation of their biological activity as potential AIs in testicular cells. For this purpose, rat SeT were cultured *ex vivo* in the presence or absence of 200 nM or 400 nM of letrozole, 4-[2-phenyl-1-(1H-1,2,4-triazol-1-yl)vinyl]benzonitrile and 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)ethyl)benzonitrile. Steroid hormone production, the tubular differentiation index (TDI) and the glycolytic metabolism were evaluated.

III. Experimental Approach

1. Chemical synthesis

1.1. Materials

All starting materials and solvents are commercially available and were used as received. p*ara*-Tolunitrile, *n*-bromosuccinimide, potassium carbonate (K_2CO_3), sodium hydride (NaH), magnesium sulfate ($MgSO_4$), 4-bromobenzaldehyde, *para*-tolualdehyde, 4-(dimethylamino) benzaldehyde and 4-formylbenzonitrile were purchased from Sigma-Aldrich (Steinheim, Germany). The 1,2,4-triazole was acquired from Alfa Aesar (Karlsruhe, Germany), potassium iodide (KI) from Fagron (Terrassa, Spain), benzaldehyde from Merck (Munich, Germany), and 4-cyanobenzaldehyde and sodium borohydride ($NaBH_4$) from Fischer Chemical (Loughborough, UK). Sodium chloride (NaCl) and silica gel 60 ((0.063-0.200 mm) were purchased from Sigma-Aldrich, and sodium hydroxide (NaOH) from José Manuel Gomes dos Santos, LDA (Odivelas, Portugal). Cyclohexane and hydrochloric acid were acquired from Fischer Chemical, tetrachloromethane and carbon tetrachloride (CCl_4) from Pronalab (Lisbon, Portugal), ethanol from Manuel Vieira & C^a (Torres Novas, Portugal), 1,4-dioxane from Panreac (Barcelona, Spain), ethyl acetate from Prolabo (Fontenay-sous-Bois, France) and dichloromethane from José Manuel Gomes dos Santos, LDA. 1,4-Dioxane and ethanol were dried by standard methods prior to use (Perrin & Armarego 1988).

1.2. Instruments

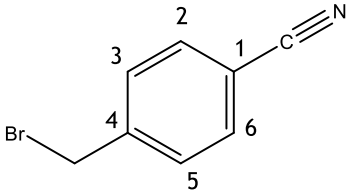
Thin layer chromatography (TLC) was performed on pre-coated aluminium plates (Macherey-Nagel, silica gel 60 F_{254}). Ultraviolet (UV) detection (254 nm) was used to monitor all organic reactions by TLC.

Melting points (mp) were measured in open capillary tubes in a Büchi B-540 (Switzerland) apparatus and are uncorrected.

Proton nuclear magnetic resonance (^{1}H NMR) and carbon-13 nuclear magnetic resonance (^{13}C NMR), distortionless enhancement by polarization transfer (DEPT), heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC) .were obtained in chloroform-*d* by a Bruker Avance 400-MHz nuclear magnetic resonance spectrometer (400.13 MHz for ^{1}H and 100.61 MHz for ^{13}C). Tetramethylsilane (TMS) was used as internal standard and chemical shifts (δ) are reported in parts per million (ppm) relative to it. Chemical shifts are reported relative to TMS (δ 0.00) for ^{1}H spectra and to deuterated chloroform ($CDCl_3$) (δ 77.00) for ^{13}C spectra. The data obtained from the ^{1}H NMR are presented in the following order: δ (ppm), signal multiplicity (s = singlet, d = duplet, dd = double duplet, m = multiplet), the value of the coupling constant, J (Hz) is presented where applicable and lastly the signal attributed to each molecule. The ^{13}C NMR data are indicated by order: chemical shift and respective carbon atom.

1.3. Synthesis

1.3.1. 4-(Bromomethyl)benzonitrile **(1)**

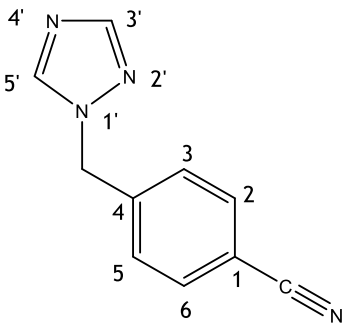


para-Tolunitrile (85.36 mmol, 10.0 g), *N*-bromosuccinimide (85.40 mmol, 15.2 g), and benzoyl peroxide (0.83 mmol, 0.2 g) were added to 60 mL of CCl_4. This mixture was heated under reflux for 7 h, after which all solids floated to the surface. The reaction was monitored by TLC eluted with dichloromethane/hexane (1:1) and the spots examined under 254 nm UV light.

The mixture was filtered when hot, washed with hot and CCl_4, and the solvent was removed from the filtrate under reduce pressure. The product was recrystallized from cyclohexane and dried under vacuum at 50 ºC for 2 days to afford white crystals.

Yield: 66.4 %. mp: 108 – 109 ºC (lit. 114 – 116 ºC) (Wen et al. 1997). ^{1}H NMR: δ 7.63 (d, J = 8.3 Hz, 1H, C**H**2, C**H**6), 7.49 (d, J = 8.3 Hz, 1H, C**H**3, C**H**5), 4.47 (s, 1H, C$\mathbf{H_2}$). ^{13}C NMR: δ 142.95 (**C**4), 132.72 (**C**H2, **C**H6), 129.85 (**C**H3, **C**H5), 118.49 (**C**N), 112.35 (**C**1), 31.60 (**C**H_2).

1.3.2. 4-((1H-1,2,4-Triazol-1-yl)methyl)benzonitrile **(2)**



1,2,4-Triazole (30.69 mmol, 2.12 g), potassium carbonate (20.40 mmol, 2.82 g) and potassium iodide (1.20 mmol, 0.20 g) were sequentially added to a solution of 4-(bromomethyl) benzonitrile **(1)** (20.40 mmol, 4.00 g) in acetone (100 mL). The resulting white suspension was heated at 55 ºC and stirred overnight. The reaction was monitored by TLC eluted with dichloromethane/hexane (1:1) and the spots examined under 254 nm UV light.

The reaction mixture was allowed to cool, H_2O (200 mL) was added and the product was extracted with ethyl acetate (200 mL). The organic layer was washed with sodium hydroxide (1 M, 2 × 100 mL) and brine (3 × 100 mL), dried magnesium sulfate and the solvent was removed in vacuum to afford light orange/ yellow crystals.

Yield: 77.5 %. mp: 69 – 71 ºC (lit. 77 – 79 ºC) (Bowman et al. 1990). ^{1}H NMR: δ 8.15 (s, 1H, C**H**3'), 7.99 (s, 1H, C**H**5'), 7.65 (d, J = 8.1 Hz, 2H, C**H**2, C**H**6), 7.32 (d, J = 8.0 Hz, 2H, C**H**3, C**H**5), 5.41 (s, 2H, C$\mathbf{H_2}$). ^{13}C NMR: δ 152.56 (**C**H3'), 143.53 (**C**H5'), 139.94 (**C**4), 132.96 (**C**H2, **C**H6), 128.47 (**C**H3, **C**H5), 118.28 (**C**N), 112.84 (**C**1), 52.92 (**C**H_2).

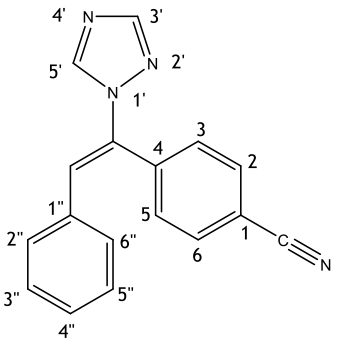
1.3.3. 4-[2-Aryl-1-(1H-1,2,4-triazol-1-yl)vinyl]benzonitrile **(3a-e)**

General procedure:

4-((1H-1,2,4-Triazol-1-yl)methyl)benzonitrile **(2)** (1.0 mmol) and 1,4-dioxane (10 mL) were added to the reaction vessel and stirred. Sodium hydride (1.5 mmol, 90%) was added to the reaction mixture in 0–5°C and stirred for an hour. Ethanol (3 mL) was added to the reaction mixture. Corresponding aldehyde (1.1 mmol) was added to the mixture and stirred at 60 °C (1h - 4h). All reactions were monitored by TLC eluted with dichloromethane/ethyl acetate (2:1) and the spots examined under 254, 312 and 365 nm UV light.

Ethanol was removed under reduced pressure and reaction mixture cooled to room temperature and a mixture of ice-water (25 g) was added. Precipitate was filtered and recrystallized in ethanol (method B) or isolated by a chromatography column using dichloromethane/ethyl acetate (2:1) as eluent (method A) to afford the following ethenyl benzonitriles.

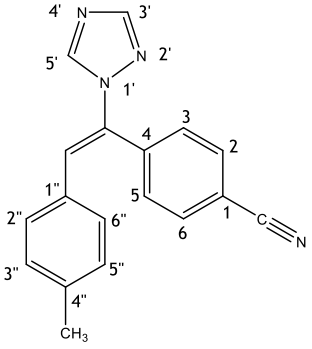
1.3.3.1. 4-(2-Phenyl-1-(1H-1,2,4-triazol-1-yl)vinyl)benzonitrile **(3a)**



Obtained from benzaldehyde (5.98 mmol) after one hour of reaction by method B as yellow powder.

Yield: 21.0 %. mp: 131 – 135 ºC (lit. 141 – 146 ºC) (Vosooghi et al. 2014). ^{1}H NMR: δ 8.26 (s, 1H, C**H**3'), 8.06 (s, 1H, C**H**5'), 7.70 (d, *J* = 8.2 Hz, 2H, C**H**2, C**H**6), 7.35 (d, *J* = 8.3 Hz, 2H, C**H**3, C**H**5), 7.30 (d, *J* = 6.9 Hz, 4H, C**H**3'', C**H**5'', C**H**4'', C**H**), 6.89 (dd, *J* = 7.5, 1.9 Hz, 2H, C**H**2'', C**H**6''). ^{13}C NMR: δ 153.43 (**C**H3'), 145.39 (**C**H5'), 140.51 (**C**4), 133.19 (**C**1''), 132.74 (**C**H2, **C**H6), 132.72 (**C**vinyl), 129.90 (**C**HAr or **C**H), 129.75 (**C**HAr or **C**H), 129.18 (**C**HAr or **C**H), 128.89 (**C**H2'', **C**H6''), 126.46 (**C**H3, **C**H5), 118.43 (**C**N), 112.87 (**C**1).

1.3.3.2. 4-(2-(*para*-Tolyl)-1-(1H-1,2,4-triazol-1-yl)vinyl) benzonitrile **(3b)**

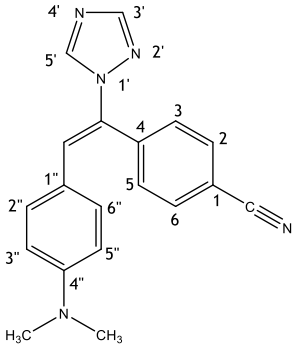


Obtained from *para*-tolualdehyde (5.98 mmol) after 4h of reaction by method B as yellow crystals.

Yield: 21.4 %. mp: 140 – 145 ºC. ^{1}H NMR: δ 8.23 (s, 1H, C**H**3'), 8.03 (s, 1H, C**H**5'), 7.66 (d, *J* = 8.3 Hz, 2H, C**H**2, C**H**6), 7.30 (d, *J* = 8.2 Hz, 2H, C**H**3, C**H**5), 7.26 (s, 1H, C**H**), 7.07 (d, *J* = 7.9 Hz, 2H, C**H**2'', C**H**6''), 6.73 (d, *J* = 7.9 Hz, 2H, C**H**3'', C**H**5''), 2.31 (s, 3H, C**H**$_3$). ^{13}C NMR: δ 153.27 (**C**H3'), 145.28 (**C**H5'), 140.57 (**C**4),

140.10 (**C**4''), 132.60 (**C**H2, **C**H6), 132.17 (**C**vinyl), 129.99 (**C**H), 129.79 (**C**H3'', **C**H5''), 129.75 (**C**1''), 128.76 (**C**H2'', **C**H6''), 126.14 (**C**H3, **C**H5), 118.37 (**C**N), 112.48 (**C**1), 21.37 (**C**H_3).

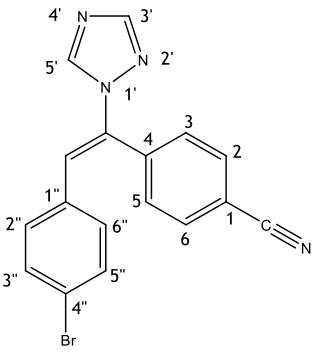
1.3.3.3. 4-(2-(4-(Dimethylamino)phenyl)-1-(1H-1,2,4-triazol-1-yl)vinyl) benzonitrile **(3c)**



Obtained from 4-(dimethyl-amino) benzaldehyde (5.98 mmol) after 4 hours of reaction by method A as orange/ brown crystals.

Yield: 32.8 %. mp: 145 – 148 ºC (lit. 158 – 161 ºC) (Vosooghi et al. 2014). ^{1}H NMR: δ 8.27 (s, 1H, C**H**3'), 8.11 (s, 1H, C**H**5'), 7.61 (d, *J* = 8.3 Hz, 2H, C**H**2, C**H**6), 7.23 (s, 1H, C**H**), 7.22 (d, *J* = 8.3 Hz, 2H, C**H**3, C**H**5), 6.64 (d, *J* = 8.8 Hz, 2H, C**H**2'', C**H**6''), 6.54 (d, *J* = 8.7 Hz, 2H, C**H**3'', C**H**5''), 2.97 (s, 6H, C$\mathbf{H_3}$). ^{13}C NMR: δ 153.41 (**C**H3'), 151.06 (**C**4''), 145.39 (**C**H5'), 141.53 (**C**4), 132.64 (**C**H2, **C**H6), 131.01 (**C**H3, **C**H5 or **C**H), 130.78 (**C**H2'', **C**H6''), 127.99 (**C**vinyl), 125.27 (**C**H3, **C**H5 or **C**H), 120.19 (**C**1''), 118.80 (**C**N), 112.06 (**C**H3'', **C**H5''), 111.34 (**C**1), 40.15 (**C**H_3).

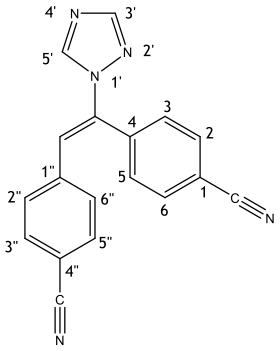
1.3.3.4. 4-(2-(4-Bromophenyl)-1-(1H-1,2,4-triazol-1-yl)vinyl)benzonitrile **(3d)**



Obtained from 4-bromobenzaldehyde (5.98 mmol) after 2 hours of reaction by method A as yellow crystals.

Yield: 15.6 %. mp: 60 – 65 ºC. ^{1}H NMR: δ 8.23 (s, 1H, C**H**3'), 8.02 (s, 1H, C**H**5'), 7.68 (d, *J* = 8.2 Hz, 2H, C**H**2, C**H**6), 7.40 (d, *J* = 8.3 Hz, 2H, C**H**3'', C**H**5''), 7.32 (d, *J* = 8.3 Hz, 2H, C**H**3, C**H**5), 7.20 (s, 1H, C**H**), 6.72 (d, *J* = 8.2 Hz, 2H, C**H**2'', C**H**6''). ^{13}C NMR: δ 153.59 (**C**H3'), 145.30 (**C**H5'), 140.21 (**C**4), 133.81 (**C**vinyl), 132.82 (**C**H2, **C**H6), 132.46 (**C**H3'', **C**H5''), 130.31 (**C**H2'', **C**H6''), 128.71 (**C**1''), 128.64 (**C**H), 126.51 (**C**H3, **C**H5), 124.13 (**C**4''), 118.34 (**C**N), 113.17 (**C**1).

1.3.3.5. 4,4'-(1-(1H-1,2,4-Triazol-1-yl)ethene-1,2-diyl)dibenzonitrile **(3e)**



Obtained from 4-formylbenzonitrile (2.72 mmol) after one hour of reaction by method A, to afford yellow crystals. NMR analysis reveals a mixture of **3e** with a second yellow compound not identified. Attempts to purify by a chromatography column were not successful.

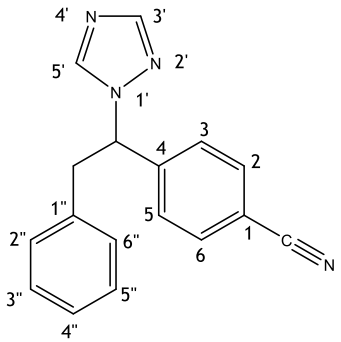
1.3.4. 4-[2-Aryl-1-(1H-1,2,4-triazol-1-yl)ethyl]benzonitrile **(4a-e)**

General procedure:

4-[2-Aryl-1-(1H-1, 2, 4-triazol-1-yl)ethenyl] benzonitrile **(3a-e)** (1 mmol) was dissolved in 20 mL anhydrous ethanol, and $NaBH_4$ (3 - 27 mmol) was added three times. The mixture was stirred at room temperature for one to three days. All reactions were monitored by TLC eluted with dichloromethane/ethyl acetate (2:1 or 5:2) and the spots examined under 254, 312 and 365 nm UV light.

Ethanol was removed under reduced pressure and was added 10 mL water and neutralized (pH 7) with 1 M HCl. The precipitated was filtered and the crude product was recrystallized from methanol (method B') or isolated by a chromatography column using dichloromethane/ethyl acetate (5:2) as eluent (method A') to afford the following ethyl benzonitriles **4a-e**.

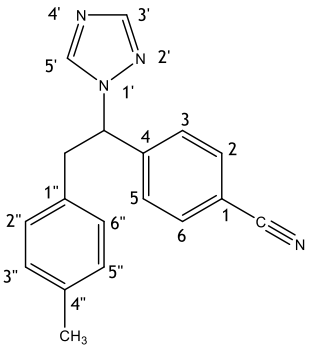
1.3.4.1. 4-(2-Phenyl-1-(1H-1,2,4-triazol-1-yl)ethyl)benzonitrile **(4a)**



Obtained from 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)vinyl) benzonitrile **(3a)** (1.29 mmol) after 3 days of reaction by method B' as a light yellow solid.

Yield: 37.4 %. mp: 110 - 112 ºC. ^{1}H NMR: δ 7.97 (s, 1H, C**H**3'), 7.93 (s, 1H, C**H**5'), 7.61 (d, *J* = 8.0 Hz, 2H, C**H**2, C**H**6), 7.47 (d, *J* = 8.0 Hz, 2H, C**H**3, C**H**5), 7.20 (m, 3H, C**H**3'', C**H**5'', C**H**4''), 6.96 (d, *J* = 7.2 Hz, 2H, C**H**2'', C**H**6''), 5.53 (dd, *J* = 9.4, 6.1 Hz, 1H, C**H**), 3.72 (dd, *J* = 13.8, 9.3 Hz, 1H, C**H**H), 3.38 (dd, *J* = 13.8, 6.0 Hz, 1H, C**H**H). ^{13}C NMR: δ 152.05 (**C**H3'), 143.35 (**C**4), 142.96 (**C**H5'), 135.68 (**C**1''), 132.31 (**C**H2, **C**H6), 128.50 (**C**HAr), 128.48 (**C**HAr), 127.69 (**C**H3, **C**H5), 127.01 (**C**HAr), 118.00 (**C**N), 112.13 (**C**1), 65.14 (**C**H), 41.25 (**C**H_2).

1.3.4.2. 4-(2-(*para*-Tolyl)-1-(1H-1,2,4-triazol-1-yl)ethyl)benzonitrile **(4b)**

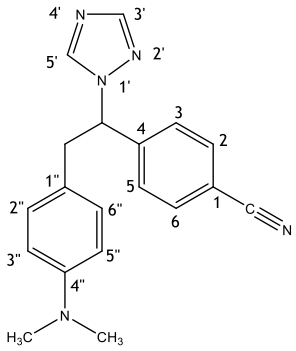


Obtained from 4-(2-(p-tolyl)-1-(1H-1,2,4-triazol-1-yl)vinyl) benzonitrile **(3b)** (0.52 mmol) after 1 day of reaction by method A' as yellow oil.

Yield: 78.0 %. ^{1}H NMR: δ 8.00 (s, 1H, C**H**3'), 7.93 (s, 1H, C**H**5'), 7.64 (d, *J* = 8.4 Hz, 2H, C**H**2, C**H**6), 7.47 (d, *J* = 8.5 Hz, 2H, C**H**3, C**H**5), 7.02 (d, *J* = 7.7 Hz, 2H, C**H**3'', C**H**5''), 6.85 (d, *J* = 7.7 Hz, 2H, C**H**2'', C**H**6''), 5.49 (dd, *J* = 9.3, 6.0 Hz, 1H, C**H**), 3.69 (dd, *J* = 13.9, 9.3 Hz, 1H, C**H**H), 3.36 (dd, *J* = 13.8, 6.0 Hz, 1H, C**H**H), 2.28 (s, 3H, C**H**$_3$). ^{13}C NMR: δ 152.55 (**C**H3'), 143.82 (**C**4), 143.32 (**C**H5'), 137.15 (**C**4''), 132.94

(**C**1''), 132.76 (**C**H2, **C**H6), 129.63 (**C**H3'', **C**H5''), 128.77 (**C**H2'', **C**H6''), 128.12 (**C**H3, **C**H5), 118.40 (**C**N), 112.65 (**C**1), 65.82 (**C**H), 41.36 (**C**H_2), 21.17 (**C**H_3).

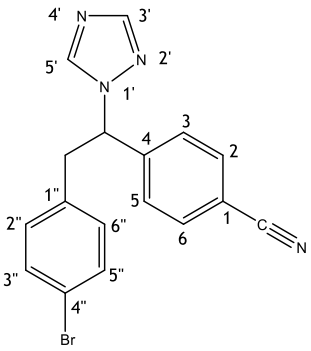
1.3.4.3. 4-(2-(4-(Dimethylamino)phenyl)-1-(1H-1,2,4-triazol-1-yl)ethyl) benzonitrile **(4c)**



Obtained from 4-(2-(4-(dimethylamino)phenyl)-1-(1H-1,2,4-triazol-1-yl)vinyl) benzonitrile **(3c)** (0.32 mmol) after 1 day of reaction by method B' as orange crystals.

Yield: 44.8 %. mp: 145 – 147 ºC. ^{1}H NMR: δ 8.00 (s, 1H, C**H**3'), 7.95 (s, 1H, C**H**5'), 7.63 (d, *J* = 8.4 Hz, 2H, C**H**2, C**H**6), 7.46 (d, *J* = 8.4 Hz, 2H, C**H**3, C**H**5), 6.80 (d, *J* = 8.1 Hz, 2H, C**H**2'', C**H**6''), 6.58 (d, *J* = 8.1 Hz, 2H, C**H**3'', C**H**5''), 5.46 (dd, *J* = 9.1, 6.1 Hz, 1H, C**H**), 3.62 (dd, *J* = 13.9, 9.1 Hz, 1H, C**H**H), 3.29 (dd, *J* = 14.0, 6.1 Hz, 1H, C**H**H), 2.90 (s, 6H, C**H**$_3$). ^{13}C NMR: δ 152.48 (**C**H3'), 149.69 (**C**4''), 144.07 (**C**4), 143.34 (**C**H5'), 132.68 (**C**H2, **C**H6), 129.61 (**C**H2'', **C**H6''), 128.15 (**C**H3, **C**H5), 123.59 (**C**1''), 118.47 (**C**N), 112.89 (**C**H3'', **C**H5''), 112.47 (**C**1), 66.10 (**C**H), 40.90 (**C**H_2), 40.69 (**C**H_3).

1.3.4.4. 4-(2-(4-Bromophenyl)-1-(1H-1,2,4-triazol-1-yl)ethyl)benzonitrile **(4d)**



Obtained from 4-(2-(4-bromophenyl)-1-(1H-1,2,4-triazol-1-yl)vinyl) benzonitrile **(3d)** (0.28 mmol) after 1 day of reaction by method A' as a light yellow oil.

Yield: 23.0 %. ^{1}H NMR: δ 8.07 (s, 1H, C**H**3'), 8.03 (s, 1H, C**H**5'), 7.66 (d, *J* = 7.9 Hz, 2H, C**H**2, C**H**6), 7.50 (d, *J* = 8.1 Hz, 2H, C**H**3, C**H**5), 7.34 (d, *J* = 8.5 Hz, 2H, C**H**3'', C**H**5''), 6.86 (d, *J* = 8.0 Hz, 2H, C**H**2'', C**H**6''), 5.52 (dd, *J* = 9.5, 5.8 Hz, 1H, C**H**), 3.72 (dd, *J* = 13.9, 9.5 Hz, 1H, C**H**H), 3.35 (dd, *J* = 13.9, 5.7 Hz, 1H, C**H**H). ^{13}C NMR: δ 152.17 (**C**H3'), 143.26 (**C**4), 143.01 (**C**H5'), 134.99 (**C**1''), 132.88 (**C**H2, **C**H6), 132.11 (**C**H3'', **C**H5''), 130.63 (**C**H2'', **C**H6''), 128.09 (**C**H3, **C**H5), 121.58 (**C**4''), 118.25 (**C**N), 112.93 (**C**1), 65.47 (**C**H), 41.11 (**C**H_2).

2. Molecular docking

2.1. Protein preparation

The 3D structure of aromatase is available in Protein Data Bank. Crystal structure of human placental aromatase cytochrome P450 in complex with androstenedione (PDB ID 3EQM) was selected for performing the docking studies.

The selected protein 3D structure displaying one chain; hence chain A was used for docking study. The 3EQM PDB contains the structure of cytochrome P450 19A1. It also contains heterocyclic compounds like 4-androstene-3-17-dione (ASD), protoporphyrin IX containing Fe^{3+} (Hem) and phosphate ion (PO_4). Crystal structure was cleaned from co-crystallized ligand and water molecules and the protein was converted to pdbqt format using Autodock Tolls (1.5.6). For enzyme preparation, the whole enzyme was selected and hydrogen atoms were added to it. Furthermore, for the preparation of the ligand (ASD) it was necessary to minimize the energy of prior computer program ChemBio3DUltra.

2.2. Molecular docking analysis

Autodock was used for the molecular docking studies of the ligands with the receptor protein. Autodock uses binding free energy evaluation to find the best binding mode. Autodock energy values were calculated by the characterization of intermolecular energy (consist of van der Walls energy, hydrogen bonding energy, desolvation energy, and electrostatic energy), internal energy of ligand, and torsional free energy.

The synthetized compounds were subjected to molecular docking studies and the docking parameters were set on vina docking parameter as follow: center_x = 86.071; center_y = 54.241; center_z = 46.085; size_x = 40; size_y = 40; size_z = 40. The other parameters were left as default for the program. Finally, the conformation for the best free energy of binding was selected for analyzing the interactions between the macromolecule and selected inhibitors. The interactions between the protein and compounds were visualized using Discovery Studio 2.1 (Accelrys Inc, San Diego, CA, USA.)

2.3. Validation of the docking process

Validation is the essential part of docking studies. For validation purpose, the bound substrate was removed from the crystallized structure of aromatase and re-docked to the enzyme. These steps were performed to determine whether the docked ligand (ASD) binds with the same amino acid residues, as it got bound in the crystal structure of the enzyme, or it binds differently to the enzyme. The crystal structure of human placental aromatase (Ghosh et al.

2009) shows that the bound ligand androgen makes a hydrogen bond with the backbone amide of Met374. Our docking study with Autodock using the freshly prepared model of the ligand (ASD) also corroborates similar observation indicating the reliability of the docking procedure (Figure II.1). ASD forms hydrogen bond with Met374 and interacts with amino acids like Asp309, Ala306, Arg115, Leu477 and Leu 372. Thus, this methodology can be considered applicable to all other ligands in this study.

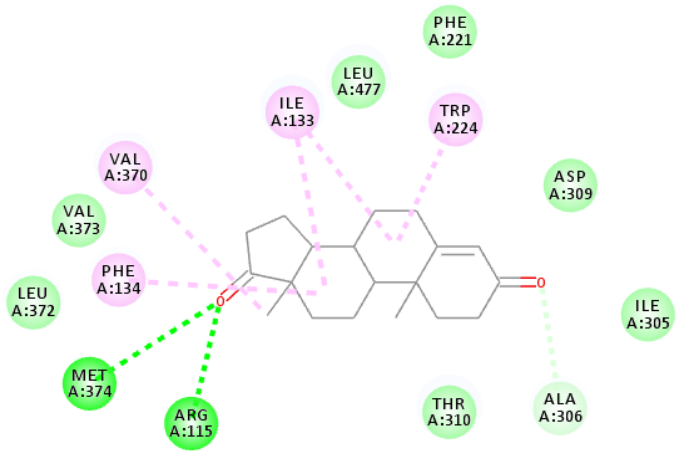


Figure III.1. Interactions of the docked conformation of the ligand within the enzyme cavity. The ligand (ASD) forms hydrogen bond with Met374 and interacts with amino acids like Asp309, Ala306, Arg115, Leu477 and Leu372.

3. Biological assays

3.1. Animals

Wistar male rats (*Rattus norvegicus*) were obtained, from Charles River (Barcelona, Spain) and housed at CICS-UBI animal facilities under a 12-hour light/ dark cycle, with food and water available *ad libitum*. Animals were handled in compliance with the guidelines established by the "Guide for the Care and Use of Laboratory Animals" published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the European Union rules for the care and handling of laboratory animals (Directive No. 86\609\EEC). In addition, the animal protocol was approved by the local institutional animal care and use committee. All rats were euthanized under anesthesia (Clorketam 1000, Vetoquinol, Lure, France).

3.2. *Ex vivo* culture of rat SeT

Testicles from three-months old animals were removed, trimmed free of fat, washed in cold phosphate-buffered saline (PBS) and placed in Dulbecco's modified Eagle's medium/Ham's F-12 culture medium (Sigma-Aldrich, St. Louis, USA) supplemented with 20 mg/L gentamicin

sulfate, 0.1 mM 3-isobutyl-1-methylxanthine, and 1 µg/L of bovine serum albumin (BSA) 10% at 33°C. Tunica albuginea was cut and peeled back to expose tubules. Ten fragments of SeT of about 1 cm were placed in culture plate (Nunclon D 12 well multidishes; Nunc, Roskilde, Denmark) wells containing 2 ml of pre-warmed culture medium with different doses (200 nM and 400 nM) of letrozole (Sigma-Aldrich), unsaturated compound **3a**, and reduced compound **4a** (N=6 in each group). The selected concentrations were found to be effective inhibiting aromatase activity, concomitantly with regressive changes in spermatogenesis in rat testis cultured *ex vivo* (Verma & Krishna 2016). Rat SeT were incubated for 24 hours at 33°C in an atmosphere of 5% CO2. At the end of the experiment, SeT were recovered from the culture medium, snap–frozen in liquid nitrogen and stored at -80°C until protein isolation. The SeT were also fixed in 4% paraformaldehyde for paraffin embedding and histologic processing. The culture medium was recovered and stored at – 80 ºC until glucose and lactate quantification.

3.3. Histological analysis of SeT

Paraffin sections (5 µm) of SeT were deparaffinized in xylene, rehydrated in graded alcohols and stained with hematoxylin (Leica Biosystems, Peterborough, Cambridgeshire, UK) and eosin (Leica Biosystems) (H&E). The TDI, which is the percentage of SeT showing germ cell differentiation, was determined to evaluate the effect of different concentrations of AIs in spermatogenesis. As described before (Silva et al. 2016), tubules were scored as differentiating or non-differentiating if containing or not germ cells in at least 3 different stages of differentiation: spermatogonia, spermatocyte and spermatid (round or elongated), or later stage. 20-60 SeT in each animal section were scored using an optical microscope (400x magnification; Zeiss, Jena, Germany, Axio Image A1 microscope).

3.4. Quantification of glucose and lactate

The concentration of glucose and lactate in the culture medium of rat SeT was determined by means of spectrophotometric analysis using commercial kits (Spinreact, Girona, Spain). The glucose consumption and lactate production were calculated.

A methanol/chloroform/water extraction was used to simultaneously extract polar and apolar metabolites from SeT as previously described by Lin et al. 2007 (Lin et al. 2007). Briefly, SeT were promptly quenched in liquid nitrogen followed by the addition of 1 mL of cold methanol and 500 µL chloroform. After thawing on ice, samples were vortexed for 60 s and sonicated. Five hundred microliters chloroform and 500 µL of ice-cold water were then added to each sample, which were vortexed and centrifuged at 5000*g* for 15 min at 4 °C. The upper layer containing water-soluble metabolites was collected, lyophilized and then diluted in 20 µL of sterile water for quantification of glucose and lactate concentrations.

3.5. Quantification of testosterone and 17β-estradiol

Sex steroids concentrations in the SeT culture medium were determined by radioimmunoassays (RIAs). Steroids were extracted from ~1ml of culture medium using C18 columns (Sepak 1 cc, 50 mg sorbent, Waters) preconditioned with 5% (v/v) methanol. The extracts were subsequently eluted from the column with 100% methanol, and after methanol evaporation were ressuspended in PBS. RIAs for T and E_2 were carried out with specific antibodies (Research Diagnostics, USA) following the general methods described by Scott *et al*. (1982) (Scott et al. 1982).

3.6. Total protein extraction and quantification

Total protein was isolated from rat SeT of all experimental groups using radioimmunoprecipitation assay buffer (RIPA) (150nM NaCl, 1% Nonidet, 5% Na-deoxycholate, 1% SDS, 10% Tris-Base, 1mM EDTA) supplemented with protease inhibitors cocktail (Sigma-Aldrich) and 10% phenylmethylsulfonyl fluoride per mg of tissue by using an Ultra-turax homogenizer (T25 basic, IKA, Staufen, Germany). The samples were allowed to stand on ice 1 hour and occasionally mixed. Then, samples were centrifuged at 14,000 rpm for 20 minutes at 4ºC, and supernatant containing total proteins was recovered to fresh tubes. Total protein concentration in extracts was determined through the Bradford method (Bradford 1976) with Bio-Rad protein assay dye reagent concentrate (Bio-Rad, Hercules, CA, USA). BSA was used to construct a standard curve.

3.7. Western blot

Total proteins (50 µg) were heat-denatured at 100 ºC and resolved on 12.5 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis at 120 V for 90 to 120 min. Then, proteins were electrotransferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA) at 750 mA for 80 min at 4 ºC. Membranes were blocked for 1 hour with 5 % skimmed dried milk and then incubated overnight at 4ºC with rabbit anti-GLUT1 (1:1000, CBL242, Millipore, CA, USA) or anti-GLUT3 (1:1000, Ab41525, Abcam, Cambridge, United Kingdom) anti-PFK1 (1:1000, sc-67028, Santa Cruz Biotechnology, CA, USA), anti-MCT4 (1:1000, sc-50329, Santa Cruz Biotechnology) and anti-LDH (1:10000, Ab52488, Abcam) primary antibodies. A mouse monoclonal antibody anti-alpha-tubulin (1:10000, T9026, Sigma-Aldrich) was used for protein loading control in all Western Blot analyses. Goat anti-rabbit IgG-HRP (1:40000, Santa Cruz Biotechnology) or goat anti-mouse IgG-HRP (1:40000, Santa Cruz Biotechnology) were used as secondary antibodies. Membranes were incubated with ECL substrate (Bio-Rad, USA) for 5 minutes and scanned with the ChemiDoc™ MP Imaging System (Bio-Rad). Band densities were

obtained according to standard methods using the Image Lab 5.1 software (Bio-Rad) and normalized by division with the respective alpha-tubulin band density.

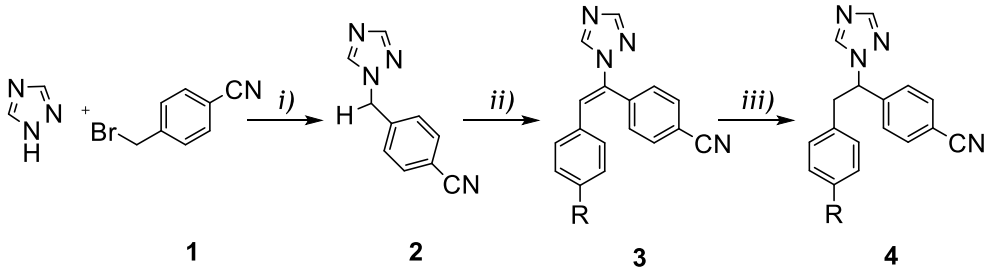
3.8. Statistical analysis

The statistical significance of differences between experimental groups was assessed by the Student's t test (GraphPad Software, San Diego, CA, USA). Significant differences were considered when $p < 0.05$. All experimental data are shown as mean ± SEM.

IV. Results and discussion

1. Synthesis

In order to develop more selective and efficient AIs, letrozole analogues were synthesized, where one of the rings of benzonitrile were substituted by a phenyl group in *para* position, with different functional groups and the methine bridge was substituted by a vinyl group **3**. Furthermore, their reduced congeners were synthesized **4**. While vinylbenzonitrile letrozole analogues **3** were prepared according to the procedure already described (Vosooghi et al. 2014), the ethylbenzonitrile letrozole analogues **4** were reported for the first time (Scheme IV.1).



R=H (a); CH_3 (b); $N(CH_3)_2$ (c); Br (d); CN (e)

Scheme IV.1. General synthesis. Reagents and conditions: (i) K_2CO_3, KI, 55ºC, reflux overnight, acetone; (ii) NaH, ethanol, corresponding benzaldehyde, 60ºC; (iii) $NaBH_4$, ethanol.

The synthesis initiates with the alkylation of 1,2,4-triazole with 4-(bromomethyl)benzonitrile in acetone in the presence of K_2CO_3 and KI to afford benzonitrile **2**, followed by the reaction with benzaldehyde and benzaldehyde substituted in the *para* position with methyl, *N,N*-dimethyl and bromo groups to afford vinylbenzonitrile letrozole analogues **3a-d** in low to moderate yields (16–33%). Under our experimental conditions, the reaction with a 4-cyanobenzaldehyde was not successful, since a second compound was also formed. Attempts of purification by a column chromatography were also unsuccessful. No reasonable explanation about the reaction times nor yields are obviously clear. The observed melting points of the vinylbenzonitrile letrozole analogues **3a,c** were lower but also broad in relation to the ones described in literature (Vosooghi et al. 2014) pointing to a relative more purity confirmed by the NMR analysis (Table IV.1). Finally, the ethylbenzonitrile **4a-d** were obtained by reduction of their congeners **3a-d** with sodium borohydride in ethanol following a standard method described in literature (Yan et al. 2009) in low to good yield (23-78%). Once again, no reasonable explanation about the reaction times nor yields are obviously clear. All letrozole analogues **3** and **4** present color form white to yellow or even orange. To the best of our knowledge, all six letrozole analogues **3b,d** and **4a-d** were never been described.

Table IV.1. Time of reaction, yields, melting points (mp), appearance and colour of vinylbenzonitrile and ethylbenzonitrile compounds.

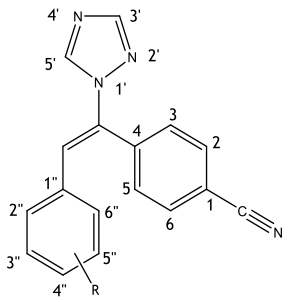
	Compound	Reaction time (h)	Yield (%)	mp (° C) [Lit.]	Appearance	Colour
Vinylbenzonitriles	3a	1	21	131 - 135 [141 - 146] (Vosooghi et al., 2014)	Powder	Yellow
	3b	4	21	140 - 145	Crystals	Yellow
	3c	4	33	145 - 148 [158 - 161] (Vosooghi et al., 2014)	Crystals	Orange/ brown
	3d	2	16	60 - 65	Crystals	Yellow
Ethylbenzonitriles	4a	72	37	110 - 112	Solid	Light yellow
	4b	24	78	—	Oil	Yellow
	4c	24	45	145 - 147	Crystals	Orange
	4d	24	23	—	Oil	Light yellow

The ^{1}H- and ^{13}C NMR spectral data of all the vinylbenzonitrile **3** and ethylbenzonitrile **4** letrozole analogues synthesized are collated in Tables IV.2 and IV.3 and in Tables IV.4 and IV.5, respectively. All NMR spectra were recorded in $CDCl_3$ solutions. The assignment of the aromatic ^{1}H- and ^{13}C-NMR signals was based on their chemical shifts and multiplicity (for the ^{1}H signals) and wherever is possible, achieved unambiguously with the aid of DEPT, HSQC and HMBC.

The ^{1}H NMR absorptions of the triazole C**H**3' and C**H**5' typically range from 8.27 to 8.23 ppm and 8.11 to 8.02 ppm, respectively, as a singlet. Benzonitrile C**H**2 and C**H**6 were found in the range of 7.70 to 7.61 ppm and C**H**3, C**H**5 from 7.35 to 7.22 ppm, as duplets with a typical coupling constant of ~8 Hz. Furthermore, C**H** bridge moiety NMR absorption was found to be in the range from 7.26 to 7.20 ppm, as a singlet, as expected. As expected, **3a** showed a different pattern due to the absence of a group in the *para* position of the phenyl group, presenting a typical double duplet assigned to C**H**2'', C**H**6'' (Table IV.2).

In the ^{13}C NMR spectra can be clearly observed that the triazole **C**H3' and **C**H5', ranging near 153 ppm and 145 ppm, respectively. While benzonitrile **C**4 was near 140 ppm, **C**H2, **C**H6 and **C**H3, **C**H5 were in the range near 132 ppm and 126 ppm, respectively. **C**vinyl and **C**H can be typically found between 133 and 128 ppm, respectively. The C nitrile and C1 are positioned near the range of 118 ppm and between 111 to 113 ppm respectively.

Table IV.2. ^{1}H NMR data for the vinylbenzonitrile letrozole analogues 3a-d. The data are presented in the following order: chemical shift (ppm), signal multiplicity (s= singlet, d= duplet, dd= double duplet), the value of the coupling constant, J (Hz) is presented where necessary.



^{1}H NMR	CH3'	CH5'	CH2, CH6	CH3, CH5	CH2'', CH6''	CH3'', CH5''	CH4''	CH	CH_3
3a	8.26 (s)	8.06 (s)	7.70 (d, 8.2)	7.35 (d, 8.3)	6.89 (dd, 7.5, 1.9)	a)	a)	a)	—
3b	8.23 (s)	8.03 (s)	7.66 (d, 8.3)	7.30 (d, 8.2)	7.07 (d, 7.9)	6.73 (d, 7.9)	—	7.26 (s)	2.31 (s)
3c	8.27 (s)	8.11 (s)	7.61 (d, 8.3)	7.22 (d, 8.3)	6.64 (d, 8.8)	6.54 (d, 8.7)	—	7.23 (s)	2.97 (s)
3d	8.23 (s)	8.02 (s)	7.68 (d, 8.2)	7.32 (d, 8.3)	6.72 (d, 8.2)	7.40 (d, 8.3)	—	7.20 (s)	—

a) superimposed signal.

Table IV.3. ^{13}C NMR data for the vinylbenzonitrile letrozole analogues 3a-d. Data are presented in chemical shift (ppm).

^{13}C NMR	CH3'	CH5'	C4	CH2, CH6	Cvinyl	CH2'', CH6''	CH3, CH5	CH3'', CH5''	C1''	C4''	CN	C1	CH	CH_3
3a	153.43	145.39	140.51	132.74	132.72	128.89	126.46	a)	133.19	a)	118.43	112.87	a)	—
3b	153.27	145.28	140.57	132.60	132.17	128.76	126.14	129.79	129.75	140.10	118.37	112.48	129.99	21.37
3c	153.41	145.39	141.53	132.64	127.99	130.78	a)	112.06	120.19	151.06	118.80	111.34	a)	40.15
3d	153.59	145.30	140.21	132.82	133.81	130.31	126.51	132.46	128.71	124.13	118.34	113.17	128.64	—

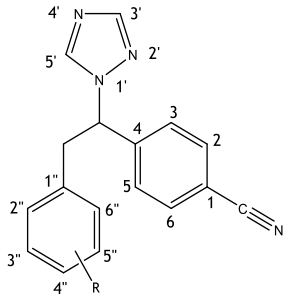
a) not attributed.

The ^{1}H- and ^{13}C NMR spectral data of all the ethylbenzonitrile letrozole analogues **4** synthesized are summarized in Tables IV.4 and IV.5. The ^{1}H NMR absorptions of the triazole C**H**3' and C**H**5' typically range from 8.07 to 7.97 ppm and 8.03 to 7.93, respectively, as a singlet. Benzonitrile C**H**2 and C**H**6 were found in the range of 7.66 to 7.61 ppm and C**H**3, C**H**5 from 7.50 to 7.46 ppm, as duplets with a typical coupling constant between 8.5 and 7.9 Hz. The phenyl C**H**2'', C**H**6'' was found in the range of 6.96 to 6.80 ppm. Furthermore, C**H** bridge moiety NMR absorption was found to the presence of a vicinal proton (J = 9.5 to 5.8). In addition, the two vicinal protons in relation to the C**H** bridge are not equivalent and,

therefore, two double duplet are observed [($J_{vicinal1}$ = 9.5 to 9.1; $J_{vicinal2}$ = 6.1 to 5.8), $J_{geminal}$ = 14.0 to 13.8].

In the ^{13}C NMR spectra can be clearly observed the triazole **C**H3' and **C**H5', ranging near 152 ppm and 143 ppm, respectively. While benzonitrile C4 was near 143 ppm, **C**H2, **C**H6 and **C**H3, **C**H5 were in the range near 132 ppm and 128 ppm, respectively. The phenyl **C**H2'', **C**H6'' was in the range near 130 to 128 ppm. The **C** nitrile and **C**1 were near to 118 and 112 ppm, respectively.

Table IV.4. ^{1}H NMR data for the ethylbenzonitrile letrozole analogues 4a-d. Data are presented in the following order: chemical shift (ppm), signal multiplicity (s= singlet, d= duplet, dd= double duplet), the value of the coupling constant, J (Hz) is presented where necessary.



^{1}H NMR	CH3'	CH5'	CH2, CH6	CH3, CH5	CH2'', CH6''	CH3'', CH5''	CH4''	CH	CH_2	CH_3
4a	7.97 (s)	7.93 (s)	7.61 (d, 8.0)	7.47 (d, 8.0)	6.96 (d, 7.2)	a)	a)	5.53 (dd, 9.4, 6.1)	3.72 (dd, 13.8, 9.3) 3.38 (dd, 13.8, 6.0)	–
4b	8.00 (s)	7.93 (s)	7.64 (d, 8.4)	7.47 (d, 8.5)	6.85 (d, 7.7)	7.02 (d, 7.7)	–	5.49 (dd, 9.3, 6.0)	3.69 (dd, 13.9, 9.3) 3.36 (dd, 13.8, 6.0)	2.28 (s)
4c	8.00 (s)	7.95 (s)	7.63 (d, 8.4)	7.46 (d, 8.4)	6.80 (d, 8.1)	6.58 (d, 8.1)	–	5.46 (dd, 9.1, 6.1)	3.62 (dd, 13.9, 9.1) 3.29 (dd, 14.0, 6.1)	2.90 (s)
4d	8.07 (s)	8.03 (s)	7.66 (d, 7.9)	7.50 (d, 8.1)	6.86 (d, 8.0)	7.34 (d, 8.5)	–	5.52 (dd, 9.5, 5.8)	3.72 (dd, 13.9, 9.5) 3.35 (dd, 13.9, 5.7)	–

a) superimposed signal.

Table IV.5. ^{13}C NMR data for the ethylbenzonitrile letrozole analogues (4a-d). The data are presented in chemical shift (ppm).

^{13}C NMR	CH3'	C4	CH5'	C1''	CH2, CH6	CH3'', CH5''	CH2'', CH6''	CH3, CH5	C4''	CN	C1	CH	CH_2	CH_3
4a	152.05	143.35	142.96	135.68	132.31	a)	a)	127.69	a)	118.00	112.13	65.14	41.25	—
4b	152.55	143.82	143.32	132.94	132.76	129.63	128.77	128.12	137.15	118.40	112.65	65.82	41.36	21.17
4c	152.48	144.07	143.34	123.59	132.68	112.89	129.61	128.15	149.69	118.47	112.47	66.10	40.90	40.69
4d	152.17	143.26	143.01	134.99	132.88	132.11	130.63	128.09	121.58	118.25	112.93	65.47	41.11	—

a) not attribute.

2. Molecular Docking

A molecular docking study was carried out to obtain information about the possible interaction between aromatase and the synthesized compounds. The results are presented on Table IV.6. Giving the resemblance between all the compounds in study, it was not surprising that the results are very similar.

All synthesized compounds showed having best binding energy when compared with letrozole. In comparison of the vinylbenzonitrile compounds, molecular docking showed that **3e** have the best binding energy, while compound **4e** is the best for the ethylbenzonitrile compounds.

When vinylbenzonitriles are compared with the ethylbenzonitriles, it is observed that only **3a** and **3c** have best binding energy than its ethylbenzonitrile.

Table IV.6. Molecular docking results of vinylbenzonitrile 3 (a-e) and 4 ethylbenzonitrile 4(a-e) letrozole analogues. Best binding energy and number of conformations in cluster calculated by Autodock.

Compound	Binding energy	Number of conformations in cluster
ASD	-12.66	10
Letrozole	-9.38	10
3a	-9.71	10
3b	-9.49	2
3c	-9.75	9
3d	-9.78	9
3e	-9.99	10
4a	-9.66	7
4b	-10.02	6
4c	-9.55	3
4d	-9.84	2
4e	-10.19	8

Table IV.7. summarizes the key interaction residues of aromatase with the synthesized compounds. Particularly, it was observed that amino acid residues Ala306, Ile133, Leu477, Met374, Phe134, Phe221, Thr310, Trp224, Val370 and Val373 have interactions with all compounds, including ASD and letrozole. Ser478 have interactions with all compounds, except with ASD. On the other hand, Leu372 is found to interact with all compounds, including ASD, except with letrozole. Furthermore, letrozole, ASD and all synthesized compounds have interactions with Asp309 and Arg115, except **4a** and **3a,b** and **4c**, respectively. Val369 has interactions with ASD, letrozole and all vinylbenzonitrile letrozole analogues, although it only has interactions with two ethylbenzonitriles (**4c** and **4d**). On the other hand, Val313 is found to interact with letrozole and all vinylbenzonitriles, except with **3a** and **3b**. Therefore, Glu302 it was observed that interact only with ethylbenzonitriles, except with **4c** and **4d**.

Concerning the interactions between amino acids and compounds, Ala306 interacts with the triazole of the letrozole. Furthermore, this amino acid residue also has interactions with the benzonitrile group of **4a**, **4b** and **4e**. It still interacts with the phenyl group of **3a**, **3b** and **4c**. Phenyl group of **4a** and **4b** interact with Arg115 amino acid. This amino acid also interacts with cyano group of **4e** and letrozole and with the triazole of **4d**. Ile 133 has interactions with benzonitrile group of ethylbenzonitrile letrozole analogues (except with **4c**) and letrozole. On the other hand, this amino acid interacts with triazole group, of letrozole. In the remaining

compounds, except **3c-e**, Ile133 has interactions with the phenyl group. In the ethylbenzonitrile letrozole analogues, **4a** and **4b**, Leu477 amino acid has bonds with the triazole group. On the other hand, these amino acids interact by the same bonds with the phenyl group of the vinylbenzonitrile **3c-e**. It was observed that in **3b** and **4c** this amino acid has interaction with both triazole and benzonitrile group. The docking study showed that Met374 amino acid interacts with cyano group of the letrozole. Furthermore, it also has interactions with the triazole of **3a**, **3b** and **4c** and the phenyl group of **3d** and **4b**. It was observed that the triazole group of **3c-e** and **4e** has interactions with Ser478 and Val369 (except **4e**) amino acids. Ser478 also interacts with cyano group of **3a** and letrozole. Thr310 amino acid interacts with triazole and benzonitrile group of **3c-e**. In these compounds it was also observed that the Val369 amino acid interacts with triazole group. Val370 amino acid interacts with the phenyl ring of **3c-e** and **4d**. This amino acid also interacts with the triazole group of **4a** and with triazole and benzonitrile group of **3a**, **4b**, **4c** and **4e**. It still has interactions with the benzonitrile group of the remaining compounds (**3b** and letrozole).

Table IV.7. Resume of interactions between ASD, compounds 3 (a-e) and 4 (a-e), and aromatase.

Compound	Residues present in main interaction
ASD	Ala306, Arg115, Asp309, Ile133, Ile305, Leu372, Leu477, Met374, Phe134, Phe221, Thr310, Trp224, Val370, Val373
Letrozole	Ala306, Arg115, Asp309, His480, Ile133, Leu477, Met374, Phe134, Phe221, Ser478, Thr310, Trp224, Val313, Val369, Val370, Val373
3a	Ala306, Asp309, Ile133, Leu372, Leu477, Met374, Phe134, Phe221, Ser478, Thr310, Trp224, Val369, Val370, Val373
3b	Ala306, Asp309, Ile133, Leu372, Leu477, Met374, Phe134, Phe221, Ser478, Thr310, Trp224, Val369, Val370, Val373
3c	Ala306, Arg115, Asp309, Ile133, Leu372, Leu477, Met374, Phe134, Phe221, Ser478, Thr310, Trp224, Val313, Val369, Val370, Val373
3d	Ala306, Arg115, Asp309, Ile133, Leu372, Leu477, Met374, Phe134, Phe221, Ser478, Thr310, Trp224, Val313, Val369, Val370, Val373
3e	Ala306, Arg115, Asp309, Ile133, Leu372, Leu477, Met374, Phe134, Phe221, Ser478, Thr310, Trp224, Val313, Val369, Val370, Val373
4a	Ala306, Arg115, Glu302, Ile133, Leu372, Leu477, Met374, Phe134, Phe221, Ser478, Thr310, Trp224, Val370, Val373
4b	Ala306, Arg115, Asp309, Glu302, Ile133, Leu372, Leu477, Met374, Phe134, Phe221, Ser478, Thr310, Trp224, Val370, Val373
4c	Ala306, Asp309, Ile133, Leu372, Leu477, Met374, Phe134, Phe221, Ser478, Thr310, Trp224, Val369, Val370, Val373
4d	Ala306, Arg115, Asp309, Ile133, Leu372, Leu477, Met374, Phe134, Phe221, Ser478, Thr310, Trp224, Val369, Val370, Val373
4e	Ala306, Arg115, Asp309, Glu302, Ile133, Leu372, Leu477, Met374, Phe134, Phe221, Ser478, Thr310, Trp224, Val370, Val373

The specific cleft in which the ligands bind contains both polar (Arg115, Asp309, Ser478, Thr310, Glu302) and non-polar (Ala306, Ile133, Ile305, Leu372, Leu477, Met374, Phe134, Phe221, Trp224, Val313, Val369, Val370, Val373) amino acids, and is in agreement with previous reports (Murthy et al. 2005; Karkola & Wähälä 2009). In the docking study, it was found that binding of different compounds with the active pocket is stabilized by van der Waals interactions with the non-polar amino acids.

The results obtained in the docking study indicated the important amino acids in the active site cavity responsible for important interactions, namely, Met374, Arg115, Ile133, Ala306, Thr310, Asp309, Val370, Leu477 and Ser478.

Once the aromatase active site is highly hydrophobic and is dominated by aliphatic amino acid residues, inhibitors with alkyl or aromatic groups are expected to bind with high affinity (Ghosh et al. 2009).

One or more hydrogen bonds formed with Met 374 are one of the essential requirements of the ligands for optimum binding. But the difference in the inhibitory activity between different compounds depends on the steric clashes of the compounds in the active site with important amino acid residues, as well as most importantly with the iron atom of the heme moiety. Besides this, compounds in higher activity range form hydrogen bonds with Arg115 and/or Thr310.

3. Biological assays

The second part of this dissertation aimed to investigate the biological activity of thr synthesized letrozole analogues in testicular cells in what concerns the assessment of the spermatogenetic status and glycolytic metabolism. For this purpose, adult rat SeT were cultured *ex vivo* in the presence or absence of 200 nM or 400 nM of letrozole, vinylbenzonitrile **3a**, and ethylbenzonitrile **4a**.

One of the major advantages of using entire SeT instead of individual purified cell cultures is because they resemble the closer scenario to the *in vivo* conditions. In this type of cultures the organization of the basic testicular units is undisturbed and the communication between somatic Sertoli cells and germ cells is maintained. The maintenance of spermatogenesis is highly dependent on the bidirectional metabolic cooperation established between Sertoli cells and germ cells. For many years, this synergistic relationship has been overlooked, but recent studies have highlighted the relevance of this process on male fertility. On one hand, Sertoli cells control the fate of germ cell line through their physical and metabolic support. On the other hand, germ cells also influence the function of the Sertoli cells (Rato et al. 2016).

3.1. Spermatogenic status is altered in the SeT treated with letrozole and the synthesized compound **4a**

The TDI is a useful tool to analyze the spermatogenesis status (Silva et al. 2016). It is based on the presence of at least three successive germ cell differentiation stages within the SeT, which allowed the classification of tubules in differentiating (Figure IV.1A) and non-differentiating (Figure IV.1B).

The TDI was significantly reduced in the SeT exposed to letrozole 200 nM (0.93 ± 0.01 fold-variation to control) and **4a** both in 200 nM and 400 nM (0.94 ± 0.02 and 0.92 ± 0.04 fold-variation to control, respectively) (Figure IV.1C).

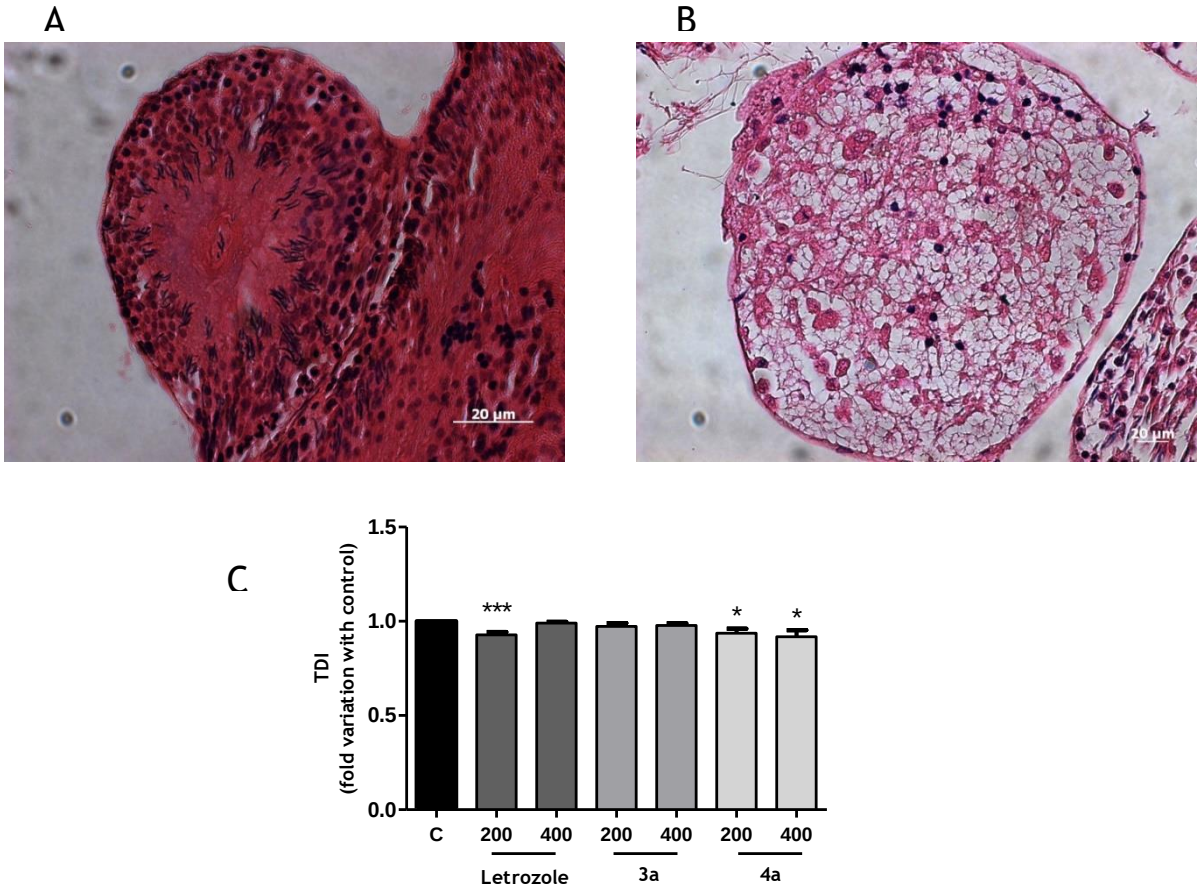


Figure IV.1. Representative photomicrographs of a differentiating (A) and non-differentiating (B) seminiferous tubule stained with H&E (400x magnification; Zeiss), and the effect of 200 nM and 400 nM of aromatase inhibitors letrozole, 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)vinyl)benzonitrile (3a) and 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)ethyl)benzonitrile (4a) in tubular differentiation index (TDI) (C). Results are expressed as fold variation relatively to the control group. Error bars indicate mean ± S.E.M. (n≥5 in each group). * $p<0.05$; *** $p<0.001$ when compared with the control group.

3.2. Administration of AIs affects glucose consumption and lactate production in SeT with altered expression of glycolytic metabolism-related proteins

Glucose is the most important fuel of mammalian cells and its uptake is crucial for cell metabolism, survival and proliferation. Accordingly, adequate amounts of glucose are required in the testis to normal reproductive function be achieved (Rato et al. 2012). In order to study whether AIs alter the glycolytic metabolism in SeT, the content of glucose and lactate in SeT and culture medium after exposure to 200 and 400 nM of letrozole, **3a** or **4a** was determined.

Our results showed that the extracellular glucose concentrations were significantly decreased for 200 nM and 400 nM doses of **3a** (0.79 ± 0.03 and 0.84 ± 0.04 fold-variation to control, respectively) and **4a** (0.89 ± 0.02 and 0.88 ± 0.03, fold-variation to control respectively) (Figure IV.2A). Since the extracellular glucose was decreased, is highly plausible to infer that there was a greater consumption of glucose in the SeT in response to **3a** and **4a**. No significant differences could be perceived on extracellular glucose in the SeT exposed to letrozole. The lactate production was significantly increased in both 200 nM and 400 nM of letrozole (1.25 ± 0.07 and 1.25 ± 0.03 fold-variation to control, respectively). On the other hand, non-consistently with increased glucose consumption, lactate production was significantly decreased with 400 nM of **3a** (0.80 ± 0.04 fold-variation to control) and 200 nM of **4a** (0.80 ± 0.04 fold-variation to control) (Figure IV.2B).

Concerning the intracellular metabolites, it was found that the concentration of glucose was increased in the SeT treated with 200 nM of letrozole (1.46 ± 0.09 fold-variation to control), 200 nM of **3a** (1.52 ± 0.18 fold-variation to control) and with both 200 nM and 400 nM of **4a** (1.80 ± 0.15 and 1.51 ± 0.13 fold-variation to control, respectively) (Figure IV.2C). The intracellular levels of lactate were significantly decreased in 200 nM of **3a** (0.84 ± 0.05 fold-variation to control) (Figure IV.2D), with no observable differences in the other experimental groups.

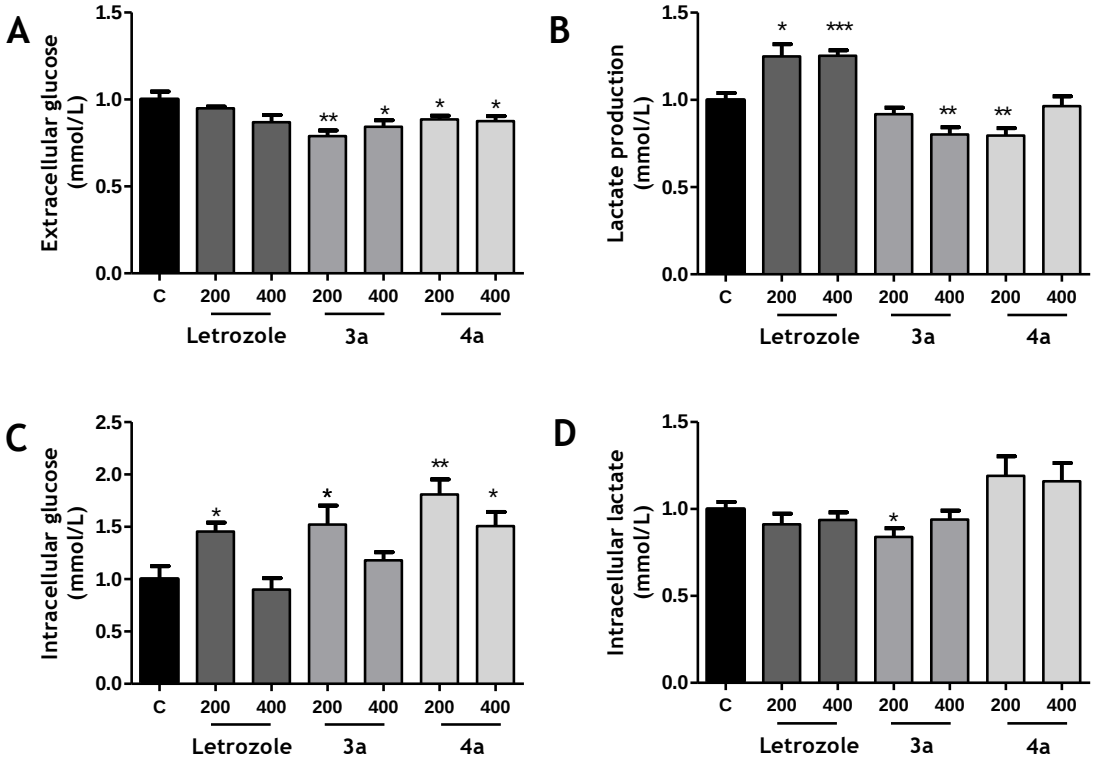


Figure IV.2. Glucose consumption (A), lactate production (B), and intracellular concentration of glucose (C) and lactate (E) in the seminiferous tubules treated with different concentrations (200 nM and 400 nM) of letrozole, 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)vinyl)benzonitrile (3a) and 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)ethyl)benzonitrile (4a). Error bars indicate mean ± S.E.M. (n≥5 in each group). * p<0.05; ** p<0.01; *** p<0.001 when compared with the control group.

In this study we evaluated the metabolic alterations in glucose consumption and lactate production in cultures of SeT. However, it is expected that the alterations observed in metabolites concentrations and metabolization reflect the activity of Sertoli cells. These somatic cell types within the SeT are the ones responsible for providing energy and nutritional support to developing germ cells via glycolytic metabolism. Sertoli cells actively incorporate and metabolize glucose with production of pyruvate that, instead being conducted to mitochondria, is preferentially convert to lactate, which is the main energy substrate for developing germ cells (Robinson & Fritz 1981; Riera et al. 2001; Boussouar & Benahmed 2004).

As mentioned above, within the SeT, Sertoli cells convert glucose to lactate (Mita & Hall 1982), which is widely recognized as the preferred substrate for the developing germ cells (Robinson & Fritz 1981; Riera et al. 2001; Boussouar & Benahmed 2004). It has been shown that Sertoli cells take up glucose from the interstitial fluid via GLUT1 and GLUT3 (Galardo et al. 2008). The influence of AIs on the glucose transport was assessed through analyzing the expression of GLUT3. Western blot analysis demonstrated that treatment with letrozole 200 nM significantly increased the expression of GLUT3 protein (1.36 ± 0.12 fold-variation to control) in SeT. On the other hand, treatment with 200 nM of **3a** and both 200 and 400 nM of

4a decreased the expression levels of GLUT3 protein (0.67 ± 0.11, 0.64 ± 0.08 and 0.53 ± 0.04 fold-variation to control, respectively) (Figure IV.3).

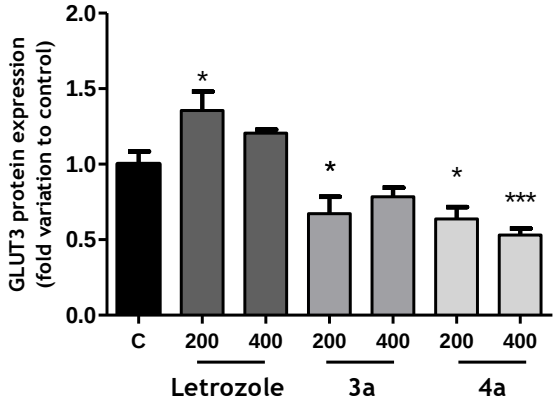


Figure IV.3. Effect of 200 nM and 400 nM of aromatase inhibitors letrozole, 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)vinyl)benzonitrile (3a) and 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)ethyl)benzonitrile (4a) on the expression of GLUT3 in seminiferous tubules. Results are expressed as fold variation relatively to the control. Error bars indicate mean ± S.E.M. (n≥4 in each group). * p<0.05; *** p<0.001 when compared with the control group.

PFK1 is responsible for the first irreversible reaction of glycolysis converting F-6-P into F-1,6-BP, thus being the most important control point in mammalian glycolytic pathways (Chehtane & Khaled 2010). Treatment with 200 nM of letrozole and 400 nM of **3a** increased the expression of PFK1 (1.46 ± 0.13 and 1.33 ± 0.07 fold-variation to control, respectively). A significant decreased on PFK1 protein expression was observed in **4a** 200 nM and 400 nM (0.62 ± 0.10 and 0.54 ± 0.06 fold-variation to control, respectively) (Figure IV.4).

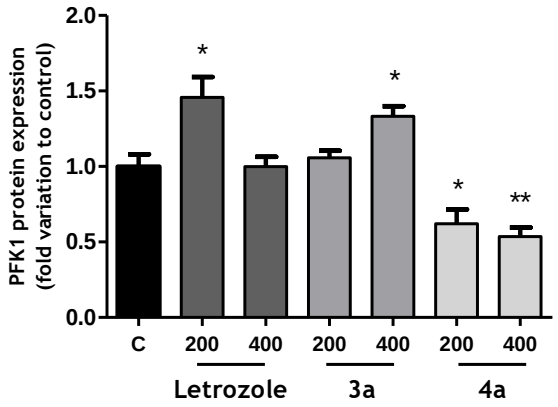


Figure IV.4. Effect of 200 nM and 400 nM of aromatase inhibitors letrozole, 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)vinyl)benzonitrile (3a) and 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)ethyl)benzonitrile (4a) on the expression of PFK1 in seminiferous tubules. Results are expressed as fold variation relatively to the control. Error bars indicate mean ± S.E.M. (n≥4 in each group). * p<0.05; ** p<0.01 when compared with the control group.

The LDH enzyme catalyzes the reversible conversion of the final product of glycolysis pyruvate into lactate (Everse & Kaplan 1973). It was observed a significantly diminished expression of LDH in SeT treated with **4a** 200 nM and 400 nM (0.49 ± 0.11 and 0.62 ± 0.13 fold-variation to control, respectively) (Figure IV.5).

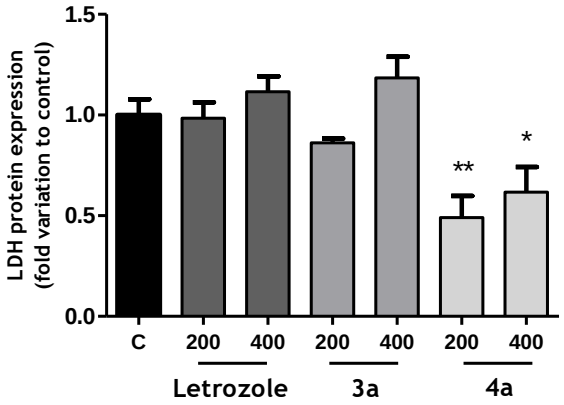


Figure IV.5. Effect of 200 nM and 400 nM of aromatase inhibitors letrozole, 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)vinyl)benzonitrile (3a) and 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)ethyl)benzonitrile (4a) on the expression of LDH in seminiferous tubules. Results are expressed as fold variation relatively to the control. Error bars indicate mean ± S.E.M. (n≥4 in each group). * p<0.05; ** p<0.01 when compared with the control group.

Once produced, lactate is exported to the extracellular medium through the MCT4 and used by developing germ cells as the main energy source for ATP production (Boussouar & Benahmed 2004).

Treatment with 200 nM of letrozole, 200 nM of **3a** and 400 nM of **4a** decreased the expression levels of MCT4 (0.65 ± 0.06, 0.57 ± 0.05 and 0.33 ± 0.06 fold-variation to control, respectively) (Figure IV.6).

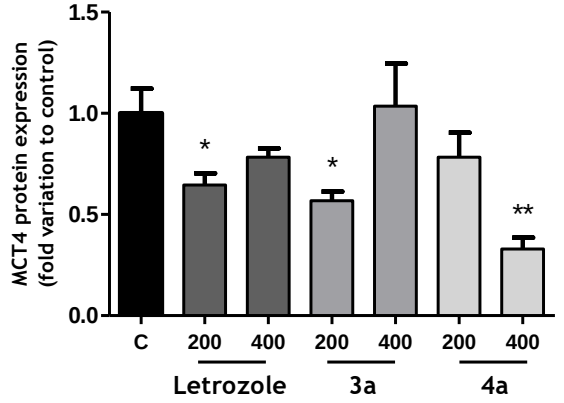


Figure IV.6. Effect of 200 nM and 400 nM of aromatase inhibitors letrozole, 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)vinyl)benzonitrile (3a) and 4-(2-phenyl-1-(1H-1,2,4-triazol-1-yl)ethyl)benzonitrile (4a) on the expression of MCT4 in seminiferous tubules. Results are expressed as fold variation relatively to the control. Error bars indicate mean ± S.E.M. (n≥4 in each group). * p<0.05; ** p<0.01 when compared with the control group.

3.3. Integrative discussion of the results obtained in the spermatogenic status with the metabolic alterations

The glycolytic process starts with glucose uptake from the extracellular medium and continues inside the cell with the sequential reactions of glycolysis. The PFK1 catalyzes the most important control point in mammalian glycolytic pathways (Chehtane & Khaled 2010), with higher levels of PFK1 being associated with increased glycolytic flux (Novellasdemunt et al. 2012). Therefore, the augmented expression of PFK1 indicates an increased metabolizing of glucose, which was consistent with the increase of lactate production observed in letrozole 200 nM. However, no differences were perceived in LDH expression. Furthermore, the expression of MCT4 was diminished, thus it cannot be excluded the possibility that other MCTs may be involved (Halestrap & Price 1999; Boussouar 2003).

Both **3a** and **4a** augmented glucose consumption, however this was not followed by an increase in lactate production. With the exception of PFK1 expression in **3a** 400 nM, letrozole analogues seem to suppress the glycolytic metabolism. This assumption is supported by the decreased expression levels of GLUT3, PFK1, LDH and MCT4, together with the augmented intracellular concentrations of glucose.

Interestingly, the suppressed glycolytic metabolism observed with the administration of **3a** and **4a** was underpinned by the diminished TDI. This is consistent with the existent findings showing that lactate provides the primary metabolic fuel and is the preferred substrate for developing germ cells (Boussouar & Benahmed 2004). Furthermore, high quantities of lactate act as an anti-apoptotic factor (Erkkilä et al. 2002). Thus, the suppression of glycolytic metabolism could contribute explaining the lower TDI in the SeT exposed to **3a** and **4a**.

The TDI also was diminished with the administration of letrozole, tough in this case lactate production was increased. Nevertheless, the decrease in TDI upon administration of letrozole was in agreement with the existent findings showing that treatment with AIs strongly decrease the number of spermatocytes and spermatids in consequence of increased apoptosis (Shetty et al. 1998). Also, the aromatase knock-out mice display a sub-fertile phenotype in consequence of augmented apoptosis of spermatids (Robertson et al. 1999).

It cannot be excluded from the discussion the fact that no dose-dependent relationship was observed for the AIs under study, which could be related with a higher individual variability and a reduced number of animals under study. Moreover, without the RIAs results for quantifying T and E_2 in the culture medium, it is not possible to advance any definitive conclusion about the distinct behavior of letrozole and its analogues **3a** and **4a**. Nevertheless, considering the effects observed on TDI and glycolytic metabolism, particularly when only 24 h of culture were used, it is possible to conclude that the **3a** and **4a** are biologically active.

V. Conclusions and future perspectives

In summary, this dissertation successfully synthesized nine letrozole analogues. To the best of our knowledge, six letrozole analogues **3b,d** and **4a-d** were new compounds. Furthermore, molecular docking fitting the obtained compounds on the active center of aromatase, demonstrated the resemblance between all of them, thus not surprisingly the results were very similar.

The successful preparation of letrozole analogues, was variable in yields. Therefore, it will be crucial to optimize the synthesis procedure to obtain improved yields.

By means of analysis of NMR spectra it was possible to confirm the exact structure of all synthesized compounds, with two letrozole analogues, namely **3a** and **4a**, being selected for evaluation of their biological activity as potential AIs in testicular cells. Steroid hormone production, the tubular differentiation index and the glycolytic metabolism were evaluated in SeT cultured *ex vivo*.

Synthesized letrozole analogues, **3a** and **4a**, seem to suppress glycolytic metabolism in SeT. On the other hand, concerning letrozole, the augmented expression of PFK1 indicates an increased metabolizing of glucose, which was accompanied by augmented lactate production. Although no dose-dependent relationship was observed for the AIs under study, to advance with a definitive conclusion about the distinct behavior of letrozole and its analogues **3a** and **4a,** the results of RIAs for quantifying T and E_2 in the culture medium are necessary. Nevertheless, considering the effects observed on TDI and glycolytic metabolism, only with 24 h of culture, it is possible to conclude that the **3a** and **4a** are biologically active.

In the future, it would be determinant to evaluate the biological activity of the remaining synthesized compounds as AIs in testicular cells. Also, it will be of uttermost importance translate these finding to *in vivo* analysis using rodent models with impaired spermatogenesis associated with the excess aromatase activity. The development of such model could allow better insight into the pathogenesis and treatment options for hyperestrogenic infertile men.

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VII. **Communications**

1. Oral communications

Coelho A.[#], Correia S.[#], Socorro S.[*], Almeida P.[*] Development of new aromatase inhibitors analogues of letrozole: perspectives for treatment of male infertility. *XI Annual CICS-UBI Symposium*. 30th June and 1st July 2016, Covilhã, Portugal.

Silva V.[#], **Coelho A.**[#], Silva-Jr C.V, Sanches C., Santos S.R., Gomez D.S., Gemperli R. Vancomycin plasma monitoring and pk/pd analysis for dose adjustment required in the controlo of septic shock in one critical burn child. *I Internacional Meeting Sobratafe - Advanced Wound Care and II Congresso Brasileiro de Tratamento Avançado de Feridas*. 28th October 2015, São Paulo, Brazil. (Oral communication beyond of the scope of this dissertation).

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2. Posters

Silva V., **Coelho A.**, Silva-Jr C.V, JC., Sanches C., Dumêt Fernandes B.J., Santos S.R., Gomez D.S., Gemperli R. Drug plasma monitoring changes vancomycin prescription in a real time against *staphylococcus spp* mic 2mg/l in critically ill burn paediatrics. *I Internacional Meeting Sobratafe - Advanced Wound Care and II Congresso Brasileiro de Tratamento Avançado de Feridas*. 28th October 2015, São Paulo, Brazil. (Beyond of the scope of this dissertation. Best poster).