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Effect of STEAP1 knockdown in LNCaP cells subjected to bicalutamide or docetaxel treatment

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Resumo

O cancro da próstata (CaP) é o segundo tipo de cancro mais diagnosticado e a segunda principal causa de morte relacionada com o cancro nos homens do mundo ocidental. O delineamento das vias patogenéticas e as principais vias moleculares envolvidas no desenvolvimento do CaP, fornecem informações importantes para avaliar possíveis biomarcadores e alvos terapêuticos. Dois dos fármacos mais utilizados para o tratamento do CaP são a bicalutamida e o docetaxel. Ambos os fármacos têm as suas limitações e algum tempo depois do tratamento o paciente adquire resistência à terapia. Para melhorar o diagnóstico e o tratamento do CaP surge a ideia de que, algumas proteínas podem funcionar como possíveis biomarcadores preditivos e/ou desenvolver terapias combinadas, permitindo desta forma melhorar as terapias convencionais já utilizadas. Existem muitas proteínas que estão desreguladas no CaP, uma das quais é a proteína *Six transmembrane epitelial antigen of the protate 1 (STEAP1)*. Nos tecidos normais a expressão da STEAP1 é praticamente restrita à próstata e em casos de neoplasia está sobre-expressa neste orgão. A localização da STEAP1 na superfície celular, associada à baixa expressão em tecidos normais e à sobre-expressão em neoplasias realçam esta proteína como um potencial biomarcador assim como um alvo terapêutico.

Sendo assim, o objetivo deste trabalho foi avaliar se a sensibilidade das células LNCaP ao tratamento com bicalutamida ou docetaxel pode ser melhorada em resposta ao silenciamento do gene STEAP1, e também perceber o significado clínico da sobre-expressão da STEAP1 como um possível biomarcador preditivo em resposta ao tratamento do CaP. Para isso, as células LNCaP foram transfetadas com um siRNA específico para silenciar a expressão do gene STEPA1, e de seguida, as células foram estimuladas com bicalutamida ou docetaxel. Foi avaliada a proliferação celular e a apoptose em resposta às diferentes condições. Os resultados mostraram uma diminuição da proliferação celular e um aumento significativo da apoptose em células LNCaP silenciadas para o STEAP1 e estimuladas com a bicalutamida ou docetaxel. No entanto, não foram observados efeitos sinérgicos quando foi feito o tratamento combinado entre o silenciamento do STEAP1 e a administração de bicalutamida ou docetaxel. Para além disso, a diminuição da expressão da STEAP1 foi revertida na presença de docetaxel, mas o mesmo não aconteceu com a bicalutamida.

Em suma, estes resultados preliminares indicam que a STEAP1 pode estar envolvida na resposta ao tratamento pela bicalutamida ou docetaxel, sugerindo que a sobre-expressão da STEAP1 pode ser usado como um biomarcador preditivo para o tratamento com estes fármacos.

Palavras-chave: Bicalutamida; Biomarcador; Cancro da próstata; Docetaxel; STEAP1.

Resumo Alargado

O cancro é uma doença multifatorial e é uma das principais causas de morte em todo o mundo. As razões para a incidência e a mortalidade desta patologia são complexas, mas refletem tanto o envelhecimento como o crescimento da população, bem como as alterações na prevalência e distribuição dos principais fatores de risco para o cancro. O cancro da próstata (CaP) é o segundo tipo de cancro mais diagnosticado e a segunda causa de morte relacionada com o cancro nos homens do mundo ocidental. O CaP é uma doença heterogénea caracterizada por várias alterações em modeladores de vias envolvidas na regulação do ciclo celular, replicação de DNA e reparação do DNA. O delineamento das vias patogenéticas e as principais vias moleculares envolvidas no desenvolvimento do CaP fornecem informações importantes para avaliar possíveis biomarcadores e alvos terapêuticos. A maioria dos homens com CaP não têm sintomas, sendo a monitorização do CaP baseada nos níveis de Prostate-Specific antigen (PSA) do soro. Apesar do PSA ser específico da próstata, o aumento deste biomarcador não é específico do cancro e pode ser resultado de hiperplasia prostática benigna ou prostatite. Por conseguinte, pode ocorrer um número considerável de falsos positivos, que diminuem a especificidade do PSA como biomarcador. Existem algumas opções de tratamento para o CaP, nomeadamente a quimioterapia. Dois dos fármacos mais utilizados são a bicalutamida e o docetaxel. A bicalutamida funciona como um antagonista do recetor de androgénios, e em algumas situações este fármaco é utilizado também como terapia combinada. O docetaxel é mais utilizado em estadios onde as células tumorais apresentam ser independentes de androgénios para a sua sobrevivência e crescimento. Ambos os fármacos apresentam limitações, nomeadamente o paciente adquirir resistência à terapia. Para melhorar o diagnóstico e o tratamento do CaP, surge a ideia de algumas proteínas poderem funcionar como possíveis biomarcadores preditivos e/ou desenvolver terapias combinadas, permitindo desta forma melhorar as terapias convencionais já utilizadas. Existem muitas proteínas que estão desreguladas no CaP, uma das quais é a proteína *Six transmembrane epitelial antigen of the protate 1 (STEAP1)*.

O gene STEAP1 foi o primeiro elemento da família de proteínas STEAP a ser identificado e codifica uma proteína com seis domínios transmembranares que se encontra localizada nas junções célula-célula do epitélio secretor da próstata. A sua localização indica um possível papel regulador na comunicação intercelular, permitindo o transporte de pequenas moléculas e iões, tais como o Na+, Ca+ e K+, e a libertação de citocinas solúveis e quimiocinas. Nos tecidos normais a expressão da STEAP1 é praticamente restrita à próstata, embora também seja encontrada em níveis baixos noutros órgãos. Em casos de

neoplasia a STEAP1 está sobre-expressa na próstata, e apresenta um aumento nos níveis de expressão noutros tecidos neoplásicos. Lesões intraepiteliais prostáticas, que são consideradas precursoras do CaP, também mostram altos níveis de expressão da STEAP1, sugerindo que a sobre-expressão desta proteína pode ocorrer mesmo antes do início da carcinogénese. Na hiperplasia benigna da próstata, os níveis da proteína STEAP1 são muito baixos, sendo semelhantes aos níveis encontrados no tecido não-neoplásico. A localização da STEAP1 na superfície celular, a baixa expressão em tecidos normais e a sobre-expressão em neoplasias destacam esta proteína como um potencial biomarcador assim como um alvo terapêutico.

Deste modo, o objetivo deste trabalho foi avaliar se a sensibilidade das células de CaP (LNCaP) ao tratamento com bicalutamida e docetaxel pode ser melhorada em resposta ao silenciamento do gene STEAP1 e também explorar o significado clínico da sobre-expressão do STEAP1 como um possível biomarcador em resposta ao tratamento do CaP. Para determinar a concentração de fármaco a utilizar, determinou-se o EC50 dos dois fármacos. As células LNCaP foram transfetadas com siRNAs, para obtermos o silenciamento do STEPA1, e de seguida foram estimuladas com bicalutamida ou docetaxel. Por fim, avaliamos a proliferação celular e a apoptose em resposta às diferentes condições.

Os resultados mostraram uma diminuição da proliferação celular e um aumento da apoptose de forma significativa, quando há um silenciamento do STEAP1 e quando há estimulação com a bicalutamida e docetaxel. No entanto, não se observaram efeitos sinérgicos aquando do efeito combinado entre o silenciamento do STEAP1 e a administração de bicalutamida ou docetaxel. Para além disso, a diminuição da expressão da STEAP1 foi revertida na presença de docetaxel, mas o mesmo não aconteceu com a bicalutamida.

Em suma, estes resultados preliminares indicam que a STEAP1 pode estar envolvida na resposta ao tratamento quer pela bicalutamida, quer pelo docetaxel, sugerindo que a sobre-expressão da STEAP1 pode ser usada como um biomarcador preditivo para o tratamento com estes fármacos.

Abstract

PCa is the most diagnosed cancer and the second leading cause of cancer-related death in men in the western world. Delineation of pathogenetic pathways and key driver molecular alterations involved in PCa development has provided a roadmap for the evaluation of biomarkers for their potential role in predicting disease outcome and as therapeutic targets. Two of the most used drugs for the treatment of prostate cancer are bicalutamide and docetaxel. Both drugs have their limitations and some time after treatment the patient gains resistance to therapy. To improve the diagnosis and treatment of PCa, there is an idea that some proteins can function as possible biomarkers and therapeutic goals in order to improve the conventional therapies already used. There are many proteins that are dysregulated in prostate cancer, one of which is the *Six transmembrane epithelial antigen of the protate 1 (STEAP1)*. In normal tissues the expression of STEAP1 is practically restricted to the prostate and in cases of neoplasia it is overexpressed in the prostate. The strategic location of STEAP1 on the cell surface, low expression in normal tissues and overexpression in neoplasms mark this protein as a potential target for the diagnosis and therapy of this pathology.

Thus, this study aimed to evaluate whether the sensitivity of LNCaP cells to treatment with bicalutamide and docetaxel can be improved in response to silencing of the STEAP1 gene and also to perceive the clinical significance of STEAP1 overexpression as a possible predictive biomarker in response to PCa treatment. For this, the LNCaP cells were transfected with siRNAs to silence the STEPA1 gene, and then they were stimulated with bicalutamide or docetaxel. Finally, we evaluated cell proliferation and apoptosis in response to different conditions.

It was observed that there is an increase in cell proliferation and a significant decrease in apoptosis, in LNCaP cells, when STEAP1 is silenced and when there is stimulation with docetaxel or docetaxel. However, synergistic effects did not occur when the combined treatment between STEAP1 silencing and the administration of bicalutamide or docetaxel was performed. In addition, the decrease in STEAP1 expression was reversed in the presence of docetaxel, but the same is not true for bicalutamide.

In summary, these preliminary results suggest that STEAP1 may be involved treatment response by bicalutamide and docetaxel. These results suggest also that STEAP1 overexpression may be used as a predictive biomarker for treatment with these anti-cancer drugs.

Keywords: Bicalutamide; Biomarker; Docetaxel; Prostate cancer; STEAP1.

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ADT	Androgen Deprivation Therapies
AIPC	Androgen-Independent Prostate Cancer
AKT	Serine/threonine protein Kinase
AR	Androgen Receptor
BIC	Bicalutamide
BPH	Benign Prostatic Hyperplasia
CoA	Coactivators proteins
CoR	Corepressors proteins
DHT	Dihydrotestosterone
DOC	Docetaxel
GTP	Guanosine Triphosphate
HRPC	Hormone Refractory Prostate Cancer
LH	Luteinizing Hormone
LNCaP	Lymph Node Carcinoma of the Prostate
PAP	Prostatic Acid Phosphatase
PCa	Prostate Cancer
PI3K	Phosphoinositide 3-Kinase
PIA	Proliferative Inflammatory Atrophy
PIN	Prostatic Intraepithelial Neoplasia
PKB	Protein Kinase B
PSA	Prostate-Specific Antigen
PTEN	Phosphatase and Tensin homolog
STEAP1	Six-Transmembrane Epithelial Antigen of the Prostate 1
TMPRSS2	Transmembrane Protease Serine 2

1. Introduction

1.1 Anatomy and physiology of prostate gland

The prostate is an accessory gland of the male reproductive system and is located in the pelvic region just below the bladder. This gland has the shape and size of a walnut and its main physiological function is the production of a prostatic fluid (pH=6) containing zinc, acid phosphatase, strong proteolytic enzymes such as prostate-specific antigen (PSA) and prostatic acid phosphatase (PAP), sucrose and citric acid that allows sperm motility and protection [1] [2]. The human prostate is generally divided into five different zones (Figure 1): the central zone, the peripheral zone, the preprostatic zone, the transition zone and the fibromuscular zone [3]. The cells within these zones vary significantly in their contribution to the prevalence of prostate cancer (PCa). The peripheral zone accounts for 70% of the prostate tissue, and this is where most cancers occur (70%) [4].

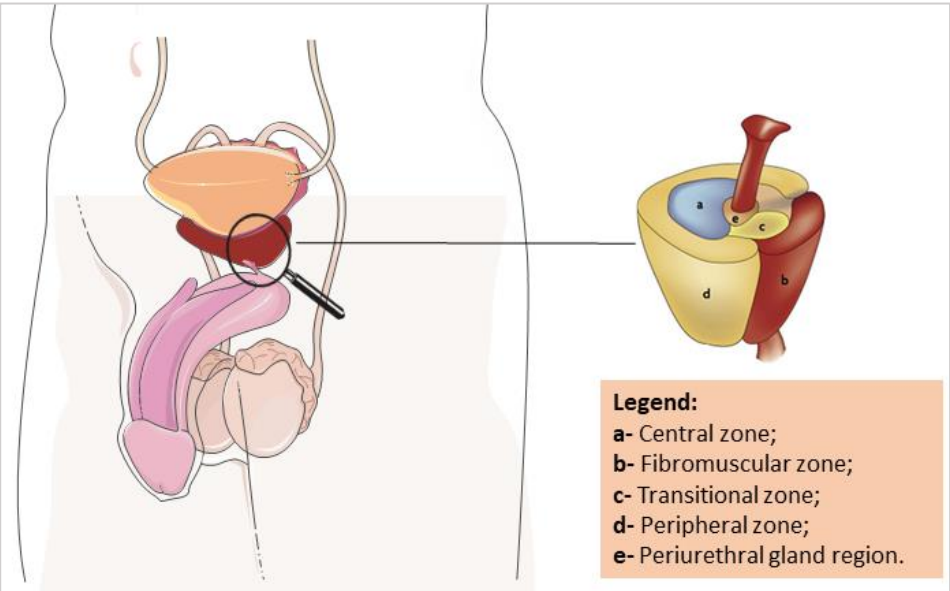


Figure 1: Representation anatomic of the human prostate gland (Adapted by [5]).

The prostatic tissue is composed of stromal and epithelial cells (Figure 2). Within the epithelial cells, two different types can be distinguished morphologically: columnar luminal cells and basal cells. The columnar luminal cells express the androgen receptor (AR) and are dependent on androgens to survive. These cells constitute the exocrine compartment of the prostate epithelium, secreting PSA and PAP. The basal cells do not have secretory activity and express very low levels of AR. There is a third type of epithelial cells dispersed within the luminal and basal cells, the neuroendocrine cells [5] [2]. Although the exact function of this type of cells is still unknown, it is believed that they may be involved in the proliferation of the adjacent cells by paracrine secretion of neuropeptides. The neuroendocrine cells do not depend on androgens to survive and may play a role in prostate carcinogenesis [6][7]. The stromal cells contain fibroblasts and smooth muscle that provide structural and biochemical support to the prostate epithelium. These two types of cells produce the extracellular matrix that helps to generate a microenvironment that controls the growth of the adjacent epithelial cells. It is believed that androgens act through paracrine signaling pathways on smooth muscle to maintain the fully differentiated growth-quiescent epithelium [8]. Ablation of androgens results in prostate involution and loss of epithelial cells by apoptosis. The re-administration of androgens reverse this process inducing the prostate return to normal size and function through rapid proliferation and differentiation of stem cells [9]. The homeostasis between the epithelial and stromal compartments is regulated by a complex signaling pathway that involves the AR and other paracrine factors capable of maintaining the balance between proliferation and apoptosis [8].

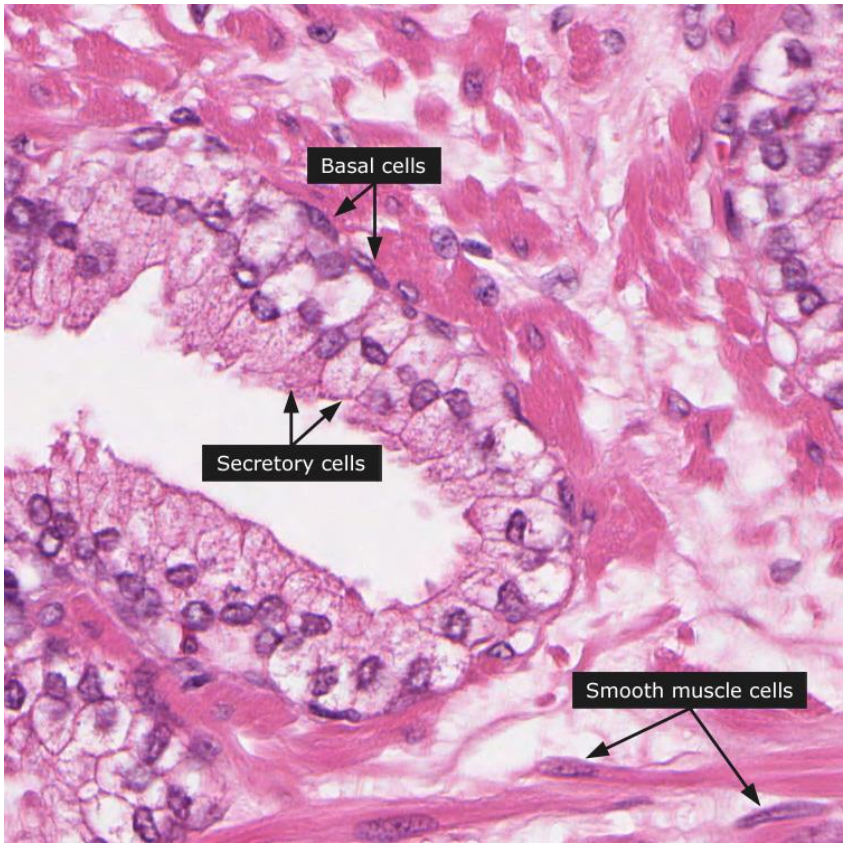


Figure 2: Histologic arrangement of the normal prostate (Retrieved from https://www.proteinatlas.org/learn/dictionary/normal/prostate/detail+1/magnification+1 (17/04/2020)).

1.1.1 Androgen and androgen receptor

The role of androgens in prostate physiology has been extensively studied, showing particular relevance for prostate development and growth, as well as for PCa progression [10]. At initial stages, prostate cancer cells require androgens for their growth and survival [11]. The major circulating androgen that stimulate prostate to grow and maintain the size and function is the testosterone [5]. The testosterone is synthesized primarily by the Leydig cells in the testes, under the regulation of luteinizing hormone (LH) produced by the anterior pituitary gland [11]. LH secretion is in turn regulated by gonadotropin-releasing hormone (GnRH). Once produced, testosterone mostly circulates bound to serum sex hormone-binding globulin (SHBG) and albumin but only the free form enters prostate cells [11]. Intracellularly, testosterone is metabolized to other steroids by a series of enzymes, over 95% of testosterone is converted to the most biologically active androgen, 5α-dihydrotestosterone (DHT), by the enzyme 5α-reductase [11].

The AR (Figure 3) is also known as, nuclear receptor subfamily 3 group C member 4 (NR3C4), belongs to the steroid hormone group of nuclear receptors with the estrogen receptor, glucocorticoid receptor, progesterone receptor and mineralocorticoid receptor [11] [12]. The AR gene encodes a 110 kDa protein consisting of 919 amino acids. Like other members of the nuclear receptor family, the AR consists of three major functional domains: the N-terminal domain (NTD), followed by the DNA binding domain (DBD), and the C-terminal ligand binding domain (LBD), which is connected to the DBD by a flexible hinge region [13]. The highly conserved DBD tethers the AR to promoter and enhancer regions of AR-regulated genes by direct DNA binding to allow the activation functions of the NTD and LBD to stimulate transcription of these genes [11].

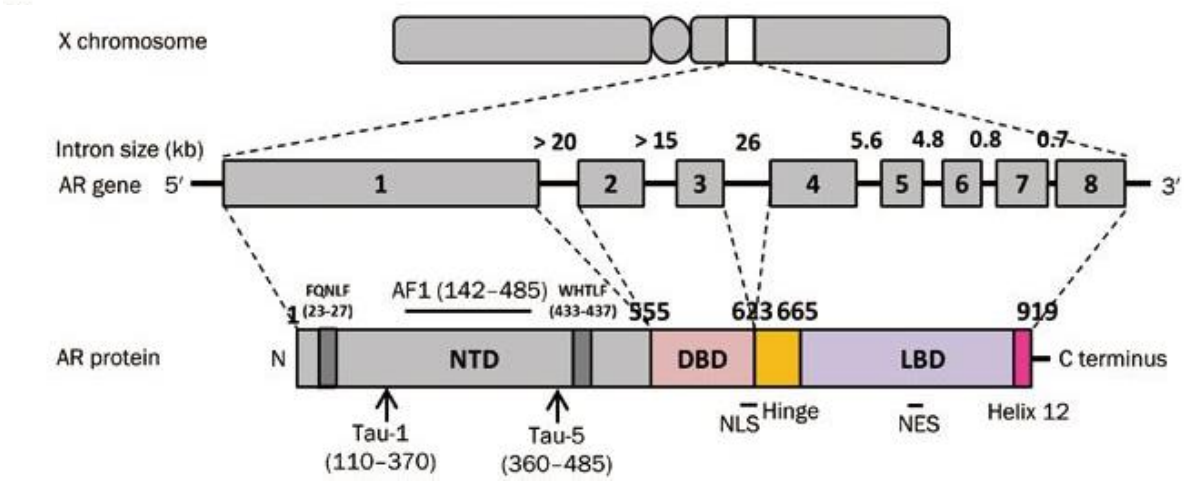


Figure 3: Genome organization of the human androgen receptor gene and the functional domain structure of the androgen receptor protein (Adapted by [11]).

AR is a master regulator transcription factor in cells of prostatic lineage, and this master regulator function is maintained in PCa cells [14] [4]. The AR is expressed in a diverse range of tissues and as such androgens have been documented to have significant biological actions not only in human male reproductive system but also in bone, muscle, adipose tissue and the cardiovascular, immune, neural and haemopoietic systems[12].
In Figure 4 we see the mechanism of action of DHT through AR, DHT binds to the ligand-binding pocket and promotes the dissociation of heat-shock proteins (HSPs) from the AR. The AR then translocates into the nucleus, dimerizes and binds to the androgen response element (ARE) in the promoter region of target genes such as PSA and TMPRSS2. At the promoter, the AR is able to recruit members of the basal transcription machinery [such as TATA-box-binding protein (TBP) and transcription factor IIF (TFIIF)] in addition to other coregulators such as members of the p160 family of coactivators and cAMP-response element-binding protein (CREB)-binding protein (CBP). The hormone receptor complex

will stimulate the prostate cells to growth and survive (Figure 4). The role of the AR in the development and progression of prostate cancer has led to increasing interest in this nuclear receptor [15] [5] [11].

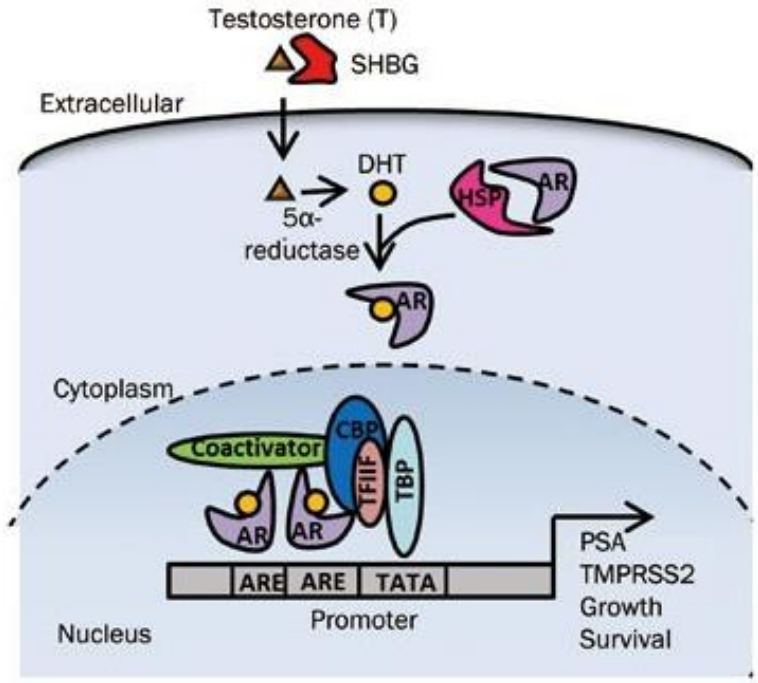


Figure 4: After testicular synthesis, testosterone is transported to target tissues, such as the prostate, and converted to DHT by 5α-reductase. DHT leads to AR activation, which in turn leads to the transcription of different target genes such as PSA and TMPRSS2. AR also stimulates cell survival and growth (Adapted by [11]).

1.2 Prostate Cancer

Cancer is a multifactorial disease and is the second cause of death worldwide after cardiovascular disease. Cancer incidence and mortality are rapidly growing worldwide. PCa is the most diagnosed cancer and the second leading cause of cancer-related death in men in the western world [16] [17]. Each year 1.6 million men are diagnosed with this pathology and 366,000 men die of PCa [18]. In Portugal the incidence of this disease is around 87,7 per 100,000 men, and mortality represents around 18.9 per 100,000 men [19]. Early detection, through PSA screening coupled with improved treatment of localized disease, is likely responsible for much of the decrease in death rate.

The development of PCa is thought to be multifactorial, involving a complex interplay of genetic and environmental factors [20]. The risk factors for a high prevalence of PCa can

be classified as endogenous (age, family history, ethnicity, hormones and oxidative stress) or exogenous (dietary factors, physical inactivity, obesity, environmental factors, occupation, smoking). Age, ethnicity and a positive family history are the better-established risk factors [20] [16]. PCa is rare among men below of 40 years old. The incidence rate of prostate cancer increases dramatically after 55 years of age, following a similar trend as other epithelial cancers [18]. Lifestyle and dietary habits have long been linked to PCa risk. Evidence points to glandular epithelial cell injury by dietary carcinogens, estrogens, or oxidants as a trigger for a chronic inflammatory milieu that set the stage for cancer development. Cooking with high temperature and char-broiling of red meat result in the formation of carcinogenic compounds, such as heterocyclic aromatic amine and polycyclic aromatic hydrocarbon, some of which have been linked to disease pathogenesis in animal models [21]. In case of family history, a man with a first degree relative with PCa has twice the risk to develop the disease. If more than two first-degree relatives are affected, the risk increases fourfold to fivefold [18].

1.2.1 Mechanism of carcinogenesis

The vast majority of PCa (over 95%) is adenocarcinoma arising from glandular structures of epithelial tissue [20]. PCa arise from precursor preneoplastic lesions that give rise to localized cancer, and then may progress rapidly until development of metastasis (Figure 5). Changes of gene expression in epithelial and stromal tumor cells leads to different development stages of PCa, contributing to tumor cell growth, survival, migration and invasiveness. The main preneoplastic lesions are prostatic intraepithelial neoplasia (PIN) and proliferative inflammatory atrophy (PIA) [22].

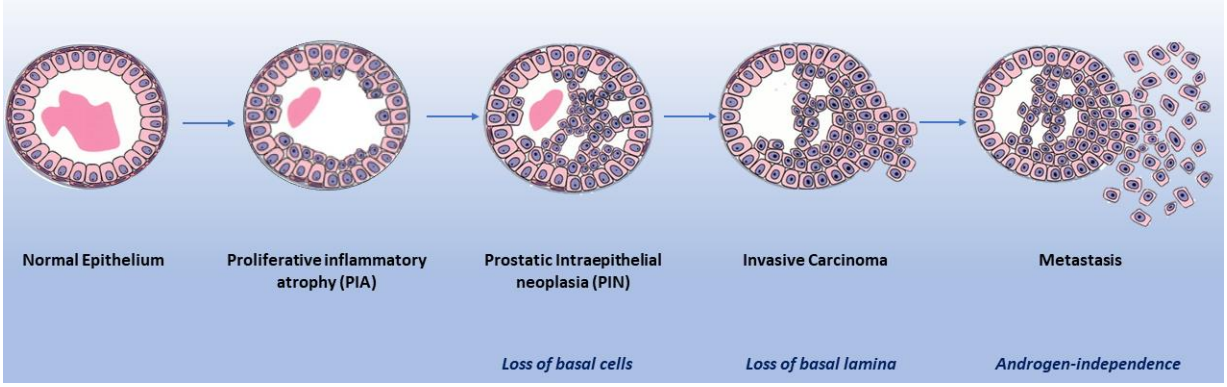


Figure 5: Prostate cancer progression (Adapted by [1]).

In PIN lesions, the initial proliferation of malignant cells occurs within the glandular epithelium, then subsequently progresses to cross the epithelial basement membrane and become invasive adenocarcinoma [20]. This pathology is characterized by hyper-proliferation of luminal epithelial cells with a corresponding reduction in the number of basal epithelial cells [23]. These hyperplastic cells have characteristically enlarged nuclei, cytoplasmic hyperchromasia and nuclear atypia [23]. PIA, consisting of simple atrophy and postatrophic hyperplasia, which is often associated with inflammation, has been found to merge directly with small adenocarcinoma lesions in the peripheral zone, but this appears to be relatively rare [22].

PCa is a heterogeneous disease characterized by several alterations in key regulatory pathways involved in cell cycle regulation, DNA replication and DNA repair. Delineation of pathogenetic pathways and key driver molecular alterations involved in PCa development has provided a roadmap for the evaluation of biomarkers for their potential role in predicting disease outcome and as therapeutic targets. They include markers of proliferation index (ki67), tumor suppressor genes (p53, p21, p27, PTEN [phosphatase and tensin homolog]), oncogenes (Bcl2 and c-myc,), adhesion molecules (E-Cadherin), PI3K/AKT/mTOR pathway members, apoptosis regulators (surviving and transforming growth factor β1), androgen receptor status, and prostate tissue lineage-specific markers (PSA, PSAP, and prostate-specific membrane antigen) [21]. Studies have shown that tyrosine kinase receptor-activating ligands, such as insulin-like growth factor 1 (IGF-1), keratinocyte growth factor (KGF), and epidermal growth factor (EGF), can activate the AR as a consequence of activating the downstream PI3K/ AKT/mTOR pathway, thus creating an 'outlaw receptor' [11]. Binding of ligands to the membrane growth factor receptors initiates a cascade of events that activate the phosphoinositide 3-kinase (PI3K) which, in turn, stimulates the serine/threonine protein kinase (AKT) also known as protein kinase B (PKB). Downstream targets of AKT have been implicated in the regulation of proliferation, metabolism and apoptosis, each with the potential to contribute to the onset of cancer. Studies showed that PI3K/Akt pathway inhibited the activity of Foxo1, which is a corepressor for AR, providing another mechanism of AR activation. In the context of the PI3K/Akt pathway, it is important to mention the PTEN, also known as mutated in multiple advanced cancers, which is a negative regulator of the PI3K/AKT pathway. About 70% of primary prostate cancer samples and about 20% of the high grade PIN samples have genomic deletions of PTEN [24] [25].

Hormone refractory prostate cancer (HRPC) clinically defines as metastatic or locally advanced prostate carcinoma that become hormone independent and progress after first and secondary endocrine treatment [26]. During androgen-independent progression,

prostate cancer cells develop a variety of cellular pathways to survive and flourish in an androgen- depleted environment (Figure 6).

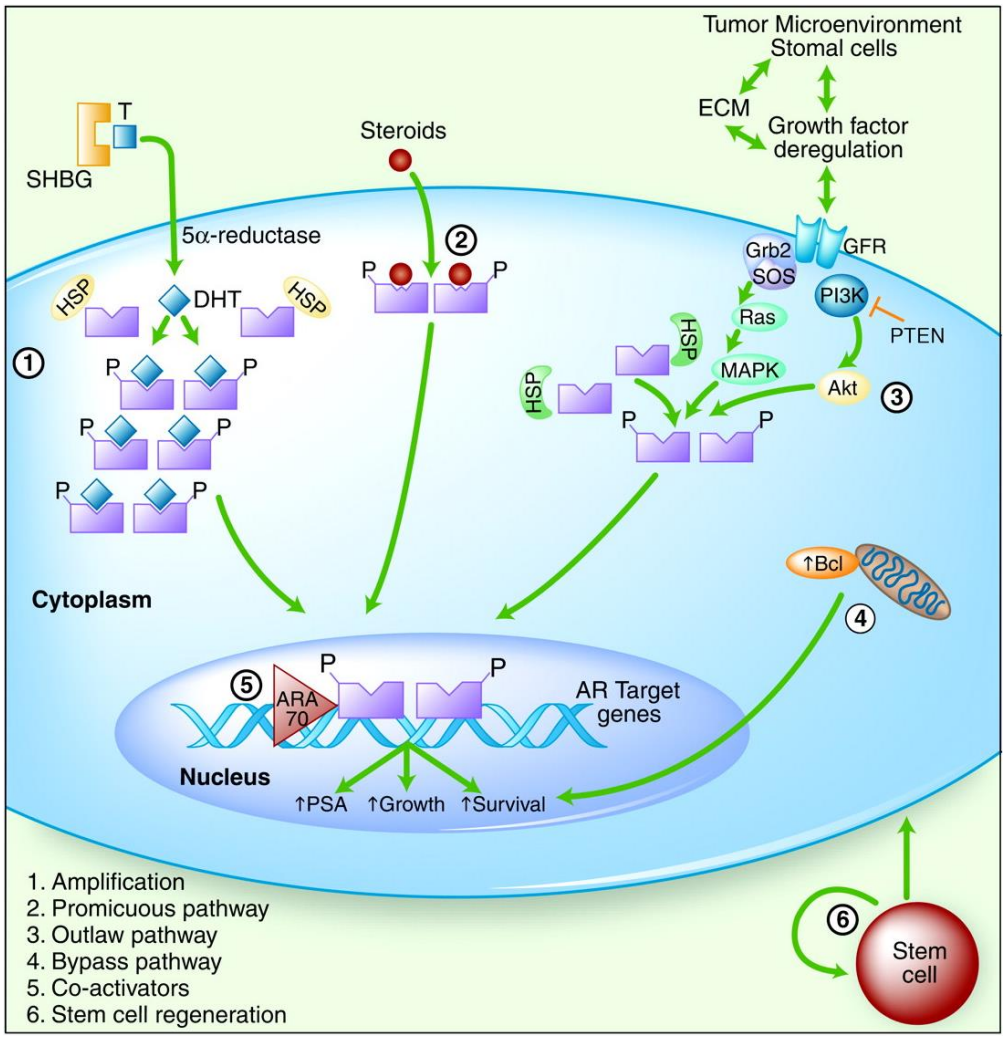


Figure 6: Mechanisms of androgen independence. 1- amplification: prostate cancer cells develop the ability to use low levels of androgen for survival by increased production of the androgen receptor; 2- promiscuous binding: Mutations of the androgen receptor broaden binding specificity allowing nonandrogenic steroid molecules normally present in the circulation as well as antiandrogens to bind and activate the androgen receptor; **3- outlaw pathway:** non-steroid molecules activate the androgen receptor by ligand-dependent binding or activate downstream signaling of the androgen receptor by ligand-independent mechanisms; **4- bypass pathway:** prostate cancer cells develop the ability of survive independent of the androgen receptor; **5- coregulators:** Alterations in the balance between coactivators and corepressors; **6- stem cell regeneration:** prostate cancer stem cells, which are not dependent on the androgen receptor for survival, continually resupply the tumor cell population despite therapy (Adapted by [26]).

Postulated and documented mechanisms include AR gene amplification, AR gene mutations, direct activation by other pathways (insulin-like growth factor receptor, ERBB2 [an epidermal growth factor receptor], and AKT, bypass pathway (the best known bypass pathway is through modulation of apoptosis by up-regulation of the molecule Bcl-2 by androgen-independent prostate cancer cells which protect them from apoptosis or programmed cell death when they are exposed to lack of testosterone), coactivators (alterations in the balance between coactivators and corepressors, which function as signaling intermediates between the androgen receptor and the transcriptional machinery, influence androgen receptor activation contributing to ability to respond to lower levels of androgen and alternative mechanisms of activation) and stem cell regeneration [27] [26].

1.2.2 Diagnosis

The majority of men with localized PCa do not have symptoms arising as a direct result of the cancer itself. It is often diagnosed when men present to their family doctor with causing obstructive lower urinary tract symptoms, and subsequent rectal examination reveals a suspicious feeling prostate or an elevated serum PSA level, which then prompts referral to a urologist. Some symptoms such as of back pain, leg swelling, peripheral neurological symptoms or new-onset erectile dysfunction may suggest advanced disease [20]. So, screening of PCa is based on serum PSA levels, digital rectal examination (DRE) and the patient's symptoms. PSA, a kallikrein-related serine protease, which is responsible for the liquefaction of the seminal coagulum, is produced by both nonmalignant and malignant cells. Despite being prostate specific, the increase of PSA levels in serum are not cancer specific and may result due to BPH or prostatitis. Therefore, a considerable number of false positives may occur, which decrease the specificity of PSA as a biomarker for PCa, although its sensitivity is high [28] [29]. PSA circulates in the blood in an inactive form, mainly aggregated with a protease inhibitor, while free PSA is quickly eliminated from the organism by glomerular filtration. A lower percentage of free-PSA is more associated with PCa than BPH, allowing an improvement of PSA test specificity [30].
The final diagnosis of prostate cancer is based on the microscopic evaluation of prostate tissue obtained via needle biopsy [31]. A pathologist examines these samples and issues a primary Gleason grade for the predominant histological pattern and a secondary grade for the highest pattern [31]. The Gleason system was described in the 1960s to grade prostate adenocarcinoma. It is graded from 1 to 5 according to the degree of glandular differentiation from normal tissue architecture seen under microscopy [20]. A higher grade means that cancer is considered more aggressive and may progresses quickly. Two grades are assigned to represent the two most dominant patterns seen (or a single grade is

doubled if only one pattern is identified). The result is the Gleason sum score, which gives a score of between 2 and 10. For example, if the most dominant pattern is Gleason 4 with smaller quantity of Gleason 3, then the prostate biopsy would be reported as: Gleason 7 (4 + 3). The Gleason score is an important determinant of disease prognosis and the treatment options available to the patient [20].

The clinical stage of the tumor is one of the most important factors in the choice of treatment and it is generally classified using the tumor-nodes-metastasis (TNM) system. This system divides the tumors in three main stages: Primary tumor (T), Regional lymph nodes (N) and distant metastasis (M). T stage has 4 categories describing how the tumor has been identified, the size of the primary tumor and whether it has invaded nearby structures. N stage describes whether the cancer has spread to nearby lymph nodes while M stage describes whether the cancer has spread to distant parts of the body like bones and lymph nodes [32].

Since one of the major problems in the treatment of prostate cancer is resistance to therapy, the idea arises that some proteins can function as possible biomarkers and therapeutic targets in order to improve the conventional therapies already used. There are many proteins/genes that are dysregulated in prostate cancer (Table 1), one of which is the six-transmembrane epithelial antigen of the prostate 1 (STEAP1).

Table 1: Genes/proteins differentially expressed in PCa.

Gene	Alterations	Cellular implications	References
NKX3.1	Down-regulated	Displays loss-of-heterozygosity (LOH)	[33]
STEAP1	Up-regulated	Role in intercellular communication	[34]
MYC	Up-regulated	Oncogene	[35]
TMPRSS2-ERG	Fusion gene (expression of N-terminally truncated ERG protein under the control of the androgen-responsive promoter of TMPRSS2)	Ability to disrupt differentiation; Promote prostate cancer progression	[36]
PTEN	Mutated or deleted	Tumor suppressor	[37]
Ezh2	Up-regulated	Control actin polymerization	[38]
KLK4	Up-regulated	Regulated by androgens	[39]
Hepsin	Up-regulated	Disorganization of the basement membrane	[40]

1.2.3 Treatment

In cancer care, multidisciplinary team, to create an overall treatment plan that combines different type of treatments is extremely important. Loss of sexual function and incontinence has a major effect on the patient's quality of life [41]. Treatment options and recommendations depend on several factors, including the type and stage of cancer, possible side effects, and the patient's preferences and overall health. There are some treatment options, when choosing the therapy to be used, the effectiveness is considered, and the secondary effects are minimized [41]. The most common treatment options for prostate cancer are: surgery, radiation therapy, hormone therapy, chemotherapy, vaccine therapy, and getting care for symptoms and side effect. In case of patients with localized PCa (those who do not appear to have metastasis after staging analyses) have three main treatment options: radical prostatectomy, radiation therapy and active surveillance [41]. For patients with tumor extension to nearby structures or metastatic disease, other treatment options are hormonal therapy and chemotherapy [12]. Androgen deprivation therapy (ADT) is the basis of therapy in advanced prostate cancer [12]. Conventional ADT involves deprivation of testosterone, achieved either by surgical or medical orchidectomy [42] [27]. This therapy is sometimes too complemented by the addition of an AR antagonist to achieve so-called complete androgen blockade [12], such as bicalutamide. Many drugs are used as adjuvants to conventional therapies. Combination therapy using nonsteroidal antiandrogens was associated with a statistically significant overall survival benefit [43]. However, this treatment exerts preferential effects against androgen-dependent cells, while androgen-independent cells continue to thrive. These androgen-independent cells are responsible for the disease relapse associated with HRPC [42]. HRPC is a progressive and morbid disease and patients have a median survival time of 10– 12 months. Docetaxel-based chemotherapy is the standard treatment for this stage of the disease [44].
Bicalutamide (BIC) and docetaxel (DOC) have a different mechanism of action, but they are an appropriate treatment option that will not prejudice the patient health and allows the patients to maintain a good quality of life.

1.2.3.1 Bicalutamide

Bicalutamide is a non-steroidal androgen receptor blocker and competitively antagonizes the actions of androgens and other ligands at the receptor level, thereby inhibiting the growth of prostate tumors. BIC does not act as an agonist or antagonist at other hormone

receptors, and is the best tolerated and one of the most stable antiandrogen used in clinical practice [45] [46].

BIC is given as monotherapy for the treatment of early (localized or locally advanced) non-metastatic prostate cancer [41]. It has been reported that when BIC is administered to patients with prostate cancer who do not respond to the male hormone, PSA is decreased by 50% in 23% of patients. However, the duration of action was as short as 3–15 months. [47]. Despite the fact that chemical or surgical castration reduces 95% of testosterone levels, an intraprostatic androgen stimulus is still present as a result of circulating androgens and androgen precursors of adrenal origin. Adding an antiandrogen to castration blocks the action of these adrenal androgens, resulting in complete androgen blockade [46]. DHT binds to the AR with high affinity, displaces heat- shock proteins from the AR, drives the interaction between the N and C termini of the AR, and binds α-importin to translocate the AR into the nucleus [11] [4]. In the nucleus, receptor dimers bind to androgen response elements (AREs) in the promoter regions of target genes, such as PSA and TMPRSS2, to which they recruits various coactivators proteins (CoA) (Figure 7A) to facilitate transcription, leading to responses such as growth and survival [11]. To transcriptionally activate target genes, ARE-bound AR relies on the activity of coactivator proteins. Many of these coactivators are important for transcriptional activation by other steroid hormone receptors, include the p160 family (SRC-1) [4].

AR antagonists can interfere with all of these required events for activation of AR gene expression by an androgen. Bicalutamide binds to the ligand-binding pocket of AR but fails to induce the correct conformational change. The platform that is formed cannot recruit coactivators, but corepressors (CoR) leading to an inactive AR–DNA complex (Figure 7B) [46].

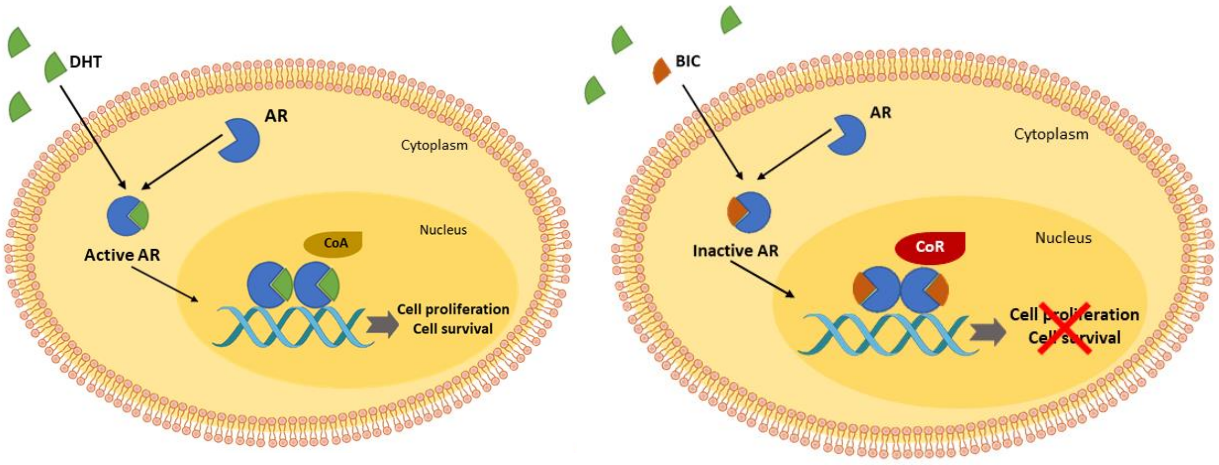


Figure 7: (**A**) DHT mechanism of action activating AR; (**B**) BIC mechanism of action inactivating AR.

1.2.3.2 Docetaxel

Different tumors have different aberrations in signaling and growth stimulation pathways that drive cancer growth. An understanding of these processes is key to the development of new anticancer agents and to identifying optimal treatment strategies and patient populations suitable for specific therapies. It is becoming clear that certain chemotherapeutic drugs such as docetaxel are not simply inhibitors of mitosis and may interact with these tumorigenic mechanisms at several levels [48] [49].

Docetaxel, a semisynthetic cytotoxic taxane, have a twofold mechanism of antineoplastic activity. First, it has been found to counter the effects of BcL-2 and bcl-xL gene expression. Second, a widely accepted mechanism of action, DOC is an antimicrotubule agent that principally exerts its cytotoxic activity by disrupting the microtubular network in cells that is essential for mitotic and interphase cellular functions (Figure 8) [48] [49] [42] [50].

Under normal conditions, microtubules undergo polymerization in the presence of microtubule-associated proteins and guanosine triphosphate (GTP), which interacts with β-tubulin. Docetaxel bind preferentially to β-tubulin, leading to microtubule assembly in the absence of GTP and other cofactor proteins. Once bound by docetaxel, microtubules cannot be disassembled. This static polymerization disrupts the normal mitotic process and typically arrests cells in the G_2M phase of the cell cycle, ultimately leading to apoptosis [42].

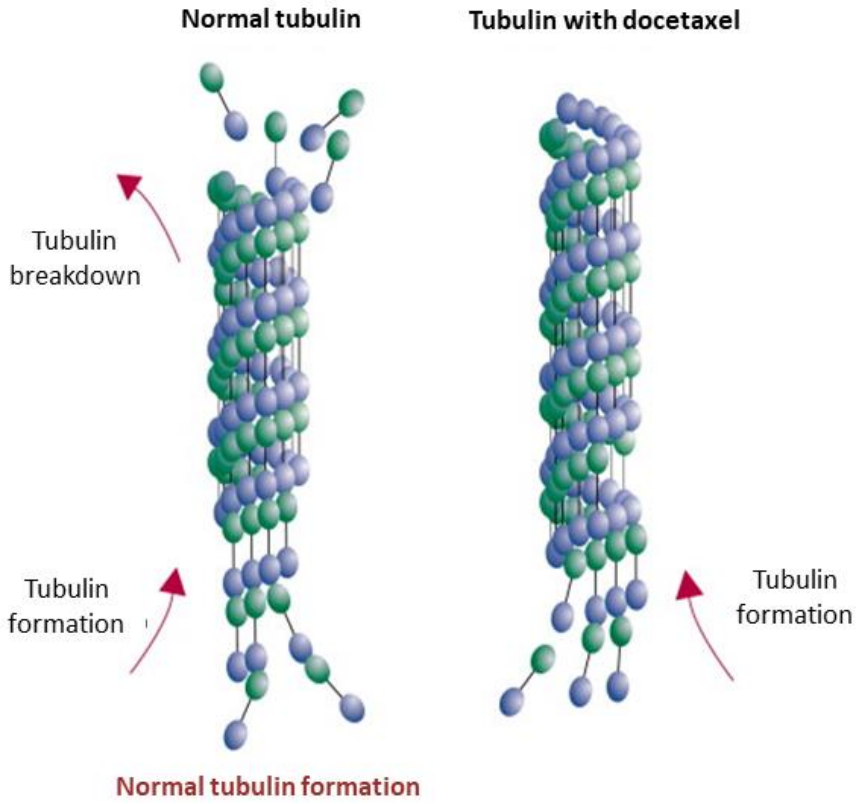


Figure 8: Microtubules polymerization in normal conditions and with docetaxel treatment (Adapted by [51]).

In vitro and in vivo, docetaxel has antineoplastic activity against a wide range of cancer cells, demonstrating a synergistic activity with several antineoplastic agents [48] [49]. Because prostatic tumors are composed of both androgen-dependent and androgen-independent cells, the therapeutic efficacy of a given biologic, hormonal, or cytotoxic agent may depend in part on the relative proportions of these cells [42]. The taxanes, by inducing bcl-2 phosphorylation, force continued activation of the caspase cascade, leading to increased apoptosis. Bcl-2 dimerizes with bax, a proapoptotic protein, and subsequently inhibits its function [42]. It has been demonstrated that the pathways for docetaxel-induced apoptosis in human prostate cancer adenocarcinoma cell line (LNCaP) appeared to cleave caspase-3 and -7 [42].

1.3 Six transmembrane epithelial antigen of the prostate 1

1.3.1 General characteristics

The six-transmembrane epithelial antigen of the prostate (STEAP) family contains four members (STEAP-1, -2, -3 and -4). Sequence analyses have categorized STEAP into a superfamily of heme-containing transmembrane ferric reductase domain (FRD), which includes the yeast ferric reductase (FRE), bacterial oxidoreductase (YedZ), and human NADPH oxidases (NOX) [52].

The first role attributed to this family of proteins was their contribution to metal homeostasis by reducing iron and copper, thereby allowing their uptake. The only exception is STEAP1, which does not reduce metals, possibly owing to the absence of the FNO-like domain and the Rossman fold. FNO-like domain enabling them to use intracellular flavin adenine dinucleotide– or flavin mononucleotide–derivate flavins as electron donors for iron and copper reduction. Nevertheless, the partial colocalization of STEAP1 with transferrin, transferrin receptor 1, and endosomes specialized in iron uptake suggest that STEAP1 may also have a role in iron metabolism [53][54].

STEAP1 was the first member of the STEAP family to be identified (Figure 9A, 9B) [55]. The STEAP1 gene is located on chromosome 7q21.13 and comprises 10.4 kb, encompassing 5 exons and 4 introns. Transcription of the STEAP1 gene gives rise to 2 different mRNA transcripts of 1.4 kb and 4.0 kb. However, only the 1.4-kb transcript is processed into the mature protein, which contains 339 amino acids with a predicted molecular weight of 39,72 kilodaltons [56]. STEAP1 is preferentially located at the plasma membrane of epithelial cells, but it can also be found dispersed in the cytoplasm. STEAP1

protein presents six transmembrane domains with both N- and C- terminus on cytoplasm side, having three extracellular and two intracellular domains and an intramembrane heme binding site [57]. Secondary structure of STEAP1, associated to its location at cell-cell junctions of the secretory epithelium of the prostate, indicates a putative role in intercellular communication, allowing the transport of small molecules and ions such as Na+, Ca2+ and K+, and releasing soluble cytokines and chemokines [55] [58] [34] .
The STEAP protein family has been implicated in many forms of cancer due to overexpression in malignant cells when compared to their non-malignant counterparts [55].

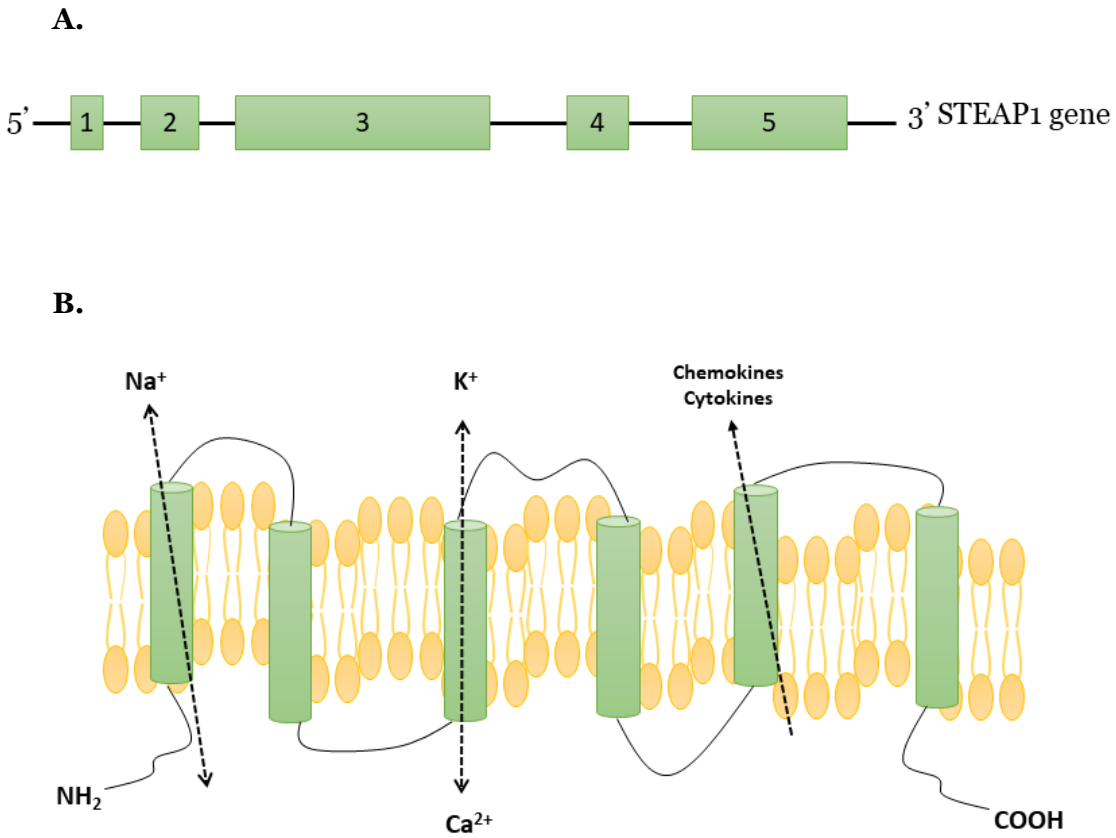


Figure 9: STEAP1 gene organization (**A**) and schematic of STEAP1 protein structure (**B**) (Adapted by [59]).

1.3.2 Expression and Regulation

Regarding normal tissues, STEAP1 expression is almost restricted to prostate, although it is also found at lower levels in other organs such as bladder, fetal and adult liver, kidney, pancreas and skeletal muscle (Table 2) [55] [57]. In cases of neoplasia, STEAP1 is highly overexpressed in prostate [58]. STEAP1 seems to regulate intercellular communication

and the rate of cell proliferation, but it may also regulate cell fate and cancer cells invasiveness [60] [61]. Besides intercellular communication, STEAP1 also appears to be involved in intracellular pathways, acting as an active intervenient on cell growth by raising reactive oxygen species (ROS) [62]. STEAP1 is differentially expressed in prostate cancer cell lines according to their metastatic potential, being more expressed in those cells that mimic earlier stages of the disease [58]. It was also demonstrated that zoledronic acid, which is used to inhibit bone resorption in patients with cancer, decreases STEAP1 mRNA expression in prostate cancer cells [63].

Table 2: Expression of STEAP1 protein by immunohistochemistry in normal and cancer tissues.

Protein			
Tissue	**Normal**	**Cancer**	**References**
Bladder	No detectable/Low	Moderate/High	[58]
Bone marrow	No detectable	-	
Breast	Low	Moderate/High	
Heart	No detectable	-	
Liver	No detectable	-	
Lung	No detectable	Moderate	
Kidney	No detectable/Low	Moderate/High	
Pancreas	Low	-	
Placenta	No detectable	-	
Prostate	Low	High	
Skeletal muscle	No detectable	-	
Stomach	Low	-	
Thymus	No detectable	-	

1.3.3 Biomarker and therapeutic target

Considering the incidence and the mortality of PCa and some of the limitations of the PSA test as a marker of this disease, it is important to find novel putative biomarkers in PCa. As slow growing cancers cannot be distinguished from fast growing, aggressive cancers, new prognostic biomarkers are required to improve patient stratification, assist with clinical management of the disease and prevent the overtreatment of PCa patients [55]. STEAP1 expression patterns are dependent of tumour development state, appearing more intense in malignant tissues than in normal ones. Concerning tumour tissues, STEAP1 is usually overexpressed in all stages of PCa, including bone and lymph node metastases. In prostatic intraepithelial lesions, which are considered precursors of PCa, also show high levels of STEAP1 expression, suggesting that STEAP1 overexpression may occur even before cancer initiation [64]. In benign prostatic hyperplasia, the STEAP1 protein levels

are very low, being similar to the levels found in non-neoplastic adjacent tissue of PCa [64]. Immunohistochemical analysis of clinical specimens demonstrates significant STEAP1 expression at the intercellular communication between adjacent cells suggesting that this antigen must be a channel, or a transport protein indicating its potential role in tumor cell intercellular communication increasing the potential of STEAP1 as a diagnostic, prognostic, biomarker, prophylactic and/or therapeutic target for new therapeutic strategies [65].

The fact that STEAP is so highly expressed in prostate, and not in most normal tissues, and because its expression is associated with certain cancers, assays that evaluate the relative levels of STEAP mRNA transcripts or proteins in a biological sample may be used to diagnose a disease associated with STEAP dysregulation, such as cancer and may provide prognostic information useful in defining appropriate therapeutic options [66].

2. Objectives

STEAP1 is overexpressed in several human tumors, particularly in PCa, and several investigators have pointed it out as a potential biomarker or therapeutic target. Bicalutamide and Docetaxel are two drugs used in the clinic for the treatment of prostate cancer with different mechanisms of action.

Unfortunately, a significant percentage of patients become resistant to treatment with these drugs and the cancer cells are more aggressive. Therefore, the present project aims to evaluate if the sensitivity of LNCaP cells to bicalutamide or docetaxel treatment can be improved in response to STEAP1 gene silencing, and whether STEAP1 overexpression may act as a predictive biomarker for treatment response. To achieve these goals, the following specific objectives were delineated:

1. Determination of dose-response curve for bicalutamide or docetaxel in LNCaP cells;
2. Effect of bicalutamide or docetaxel in STEAP1 expression in LNCaP cells;
3. To evaluate the cell proliferation and apoptosis of LNCaP cells knocked down for STEAP1 in response to bicalutamide or docetaxel treatment.

3. Materials and Methods

3.1 Cell Culture

LNCaP prostate cancer cell line was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and maintained in RPMI-1640 phenol-red medium (Sigma Aldrich, USA) supplemented with 10% fetal bovine serum (FBS) (Biochrom AG, Germany) and 1% penicillin/streptomycin (Gibco, Life technologies, USA), in a humidified chamber at 37 °C and a 5% CO_2 atmosphere.

3.2 Drug Sensitivity Assay

Half maximal effective concentration (EC50) was determinated by MTT assay (Sigma Aldrich). Approximately $2{,}5\times10^4$ LNCaP cells were seeded in 96 multiwells plates. After twenty-four hours they were stimulated with bicalutamide (Sigma Aldrich) or docetaxel (Sigma Aldrich) at different concentrations, for 24h and 48h. The concentrations of bicalutamine and docetaxel were 1; 5; 10; 25; 50; 100; 1000 μM and 1; 5; 10; 25; 50; 100; 1000 nM, respectively.

3.3 STEAP1 knockdown and experimental design

LNCaP cells at 50% confluence in twelve flasks were transfected with 40 nM of a small interfering RNA (siRNA) targeting the STEAP1 (s4392421, Ambion) using Lipofectamine 3000 (Invitrogen, USA) for 24 h in Opti-MEM medium (Invitrogen, USA) as recommended by the manufacturer. As a control for STEAP1-specific targeting, a scramble siRNA sequence (s4390846, Ambion) was used. 24 hours after transfection, the cells were stimulated with bicalutamide (100 μM) or docetaxel (20 nM). Cells were harvested at 24 h after drugs treatment, and the efficiency of STEAP1 knockdown expression was analyzed by quantitative real-time PCR (qPCR) and Western blot.

3.4 MTT assay

In order to determine the EC50 of bicalutamide and docetaxel at 24 h and 48 h in LNCaP cells, the cell viability was evaluated through MTT assay, in according to the

manufacturer's instructions. Briefly, after 24 h and 48 h of stimuli, 100 µL of MTT solution was added to cells. After 1 h of incubation at 37°C, the MTT solution was removed and 100 µL DMSO was added for solubilization of the formazan crystals. Next, the optical density was measured at 490 nm (Microplate Spectrophotometer, BIO RAD xMark).

3.5 RNA extraction and Real Time quantitative polymerase Chain Reaction (qPCR)

Total RNA from LNCaP cells was obtained using TRI reagent (Grisp, Portugal) according to the manufacturer's instructions. The RNA pellet was dried, resuspended in 20 µL of Diethylpyrocarbonate (DEPC) treated water and storage at -80°C. In order to assess the quantity of total RNA, its optical density was determined by measuring absorbance at 260 and 280 nm on a nanospectrometer (Pharmacia Biotech, Ultrospec 3000, Denmark). Total RNA integrity was verified by agarose gel electrophoresis.

qPCR was used to determine the expression levels of STEAP1 and the percentage of knockdown of the STEAP1 gene, using Power SYBR Green RNA-to-$C_{T,}$ 1-Step Kit (Applied Biosystems, USA) on the CFX connect Real-time system (Bio-Rad, Hercules, USA). qPCRs were performed with 0,2 µL of RNA in 10 µL of total reaction with specific primers for STEAP1 (sense: 5' GGC GAT CCT ACA GAT ACA AGT TGC 3' and anti-sense: 5' CCA ATC CCA CAA TTC CCA GAG AC 3'), p21 (sense: 5' GTT CCT TGC CAC TTC TTA C 3' and anti-sense: 5' ACT GCT TCA CTG TCA TCC 3') and Beta-2-microglobulin (β2M, sense: 5′ ATG AGT ATG CCT GCC GTG TG 3′ and anti-sense: 5′ CAA ACC TCC ATG ATG CTG CTTAC 3′). After an initial denaturation at 95°C for 5 min, 35 cycles were carried out as follows: denaturation at 95°C for 30s, annealing temperature for 30s and polymerization at 72°C for 20s. The amplified PCR fragments were analyzed by melting curves. β2M housekeeping was used as internal control to normalize gene expression. Fold differences were calculated following the mathematical model proposed by Pfaffl [67].

3.6 Protein extraction and western blot

LNCaP cells were lysed on an appropriate volume of Radioimmunoprecipitation assay (RIPA) (150 mM NaCl, 1% Nonidet-P40 substitute, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris) supplemented with 10% phenylmethylsulfonyl fluoride (PMSF) and 1% protease cocktail. The total protein extract was obtained after centrifugation of the cell lysate for 20 min at 14,000 rpm at 4°C. Quantification of the total

protein was measured using the Pierce 660nm Protein assay reagent (Thermo Scientific, USA). Approximately 20 μg of total protein from LNCaP cells was used to determine STEAP1 levels and other proteins. Proteins were resolved on 12% TGX Stain-Free polyacrylamide gels (BioRad, USA) and then transferred into a PVDF membrane (BioRad, USA). After blockage with 5% milk solution, membranes were incubated overnight at 4 °C with following antibodies: rabbit anti-STEAP1 (1:1000, D8B2V, Cell Signaling Techonology), rabbit anti-p53 (1:1000, sc-6243, Santa Cruz Biotechnology). Membranes were incubated with anti-rabbit IgG-HRP (1:15000, Sigma-Aldrich) or anti-mouse IgG-HRP (1:10000, Sigma-Aldrich).

β-actin HRP conjugated (1:150000, Sigma-Aldrich) was used for the normalization of protein expression. After this, immunoreactivity was visualized using the ChemiDoc™ MP Imaging System (Bio-Rad) after the incubation with ECL substrate (BioRad, USA). Protein expression levels were quantified by densitometry analysis using the Image Lab 5.1 software (Bio-Rad).

3.7 Ki-67 fluorescence immunocytochemistry

LNCaP cells were fixed with 4% Paraformaldehyde (PFA) and permeabilized with 1% Triton X-100 for 5 min at room temperature. Unspecific staining was avoided with PBS containing 0.1% (w/v) Tween-20 and 20% FBS for 1 h. Cells were then washed with PBS and incubated for 1 h at RT with rabbit anti-Ki67 (1:50, n°16667, Abcam). Incubation with the Alexa Fluor 546 goat anti-rabbit IgG (1:1000, Invitrogen) secondary antibody was performed for 1 h at RT. Cells were washed in PBS and incubated for 5 min in Hoechst-33342 (5 μg/mL, Invitrogen, UK). Coverslips were then mounted in Dako (Invitrogen, UK) and analyzed by fluorescence microscopy (Zeiss AxioImager A1). The proliferation index was estimated by counting the number of Ki67-positive cells and Hoechst- stained nuclei in four randomly selected 40× magnification fields for each section. The ratio between the number of Ki67-stained cells and total number of nuclei was calculated.

3.8 Terminal Deoxynucleotidyl Transferase biotin-dUTP Nick-End Labeling (TUNEL)

Cells were fixed with 4% PFA for 10 min and, then, permeabilized in 1% Triton X-100 for 5 min. Forty microliters of TUNEL reaction mixture (Roche, Germany) was added to each sample for 1 h at RT in the dark. Cells were washed in PBS and incubated for 5 min in

Hoechst-33342 (5 μg/mL, Invitrogen, UK). Coverslips were then mounted in Dako (Invitrogen, UK) and analyzed by fluorescence microscopy. The percentage of apoptotic cells was estimated by counting the number of TUNEL-positive cells and Hoechst-stained nuclei in four randomly selected 40× magnification fields in each coverslip. The ratio between the number of TUNEL-positive cells and total number was calculated.

3.9 Caspase-3 activity assay

The caspase-3-like activity was determined after the cleavage of the labeled substrate by the detection of the chromophore p-nitroaniline, measured spectrophotometrically at 405 nm. Proteins 25 μg of total protein extract was incubated with a reaction buffer (20 mM HEPES, pH 7.4, 0.1% CHAPS, 10% 2 mM EDTA, supplemented with 10 mM DTT) and 2 mM of caspase-3 substrate (Ac-DEVD-pNA) for 2 h at 37 °C. The amount of generated pNA was calculated by extrapolation with a standard curve with known amounts of pNA.

3.10 Statistical analysis

All experimental data are shown as mean ± standard error of the mean (S.E.M). Statistical significance of differences among experimental groups were evaluated by unpaired t-test or one-way ANOVA followed by Sidak's multiple comparisons test, using GraphPad Prism v8.00 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

4. Results

4.1 Determination of EC50 for Bicalutamide or Docetaxel

In order to determine of half maximal effective concentration (EC50) for bicalutamide or docetaxel the cell viability was determined using several concentrations (1, 5, 10, 25, 50, 100, 1000 µM to bicalutamide and 1, 5, 10, 25, 50, 100, 1000 nM to docetaxel). The EC50 was determined after 24 h and 48 h of treatment with at least six replicates per concentration.

The results obtained by the regression model are shown in Figure 10. It was verified that EC50 for bicalutamide (BIC) is 115 µM at 24 h (A) and 123,7 µM at 48h (B), and for docetaxel is 16,99 nM at 24h (A) and 11,2 at 48 h (B).

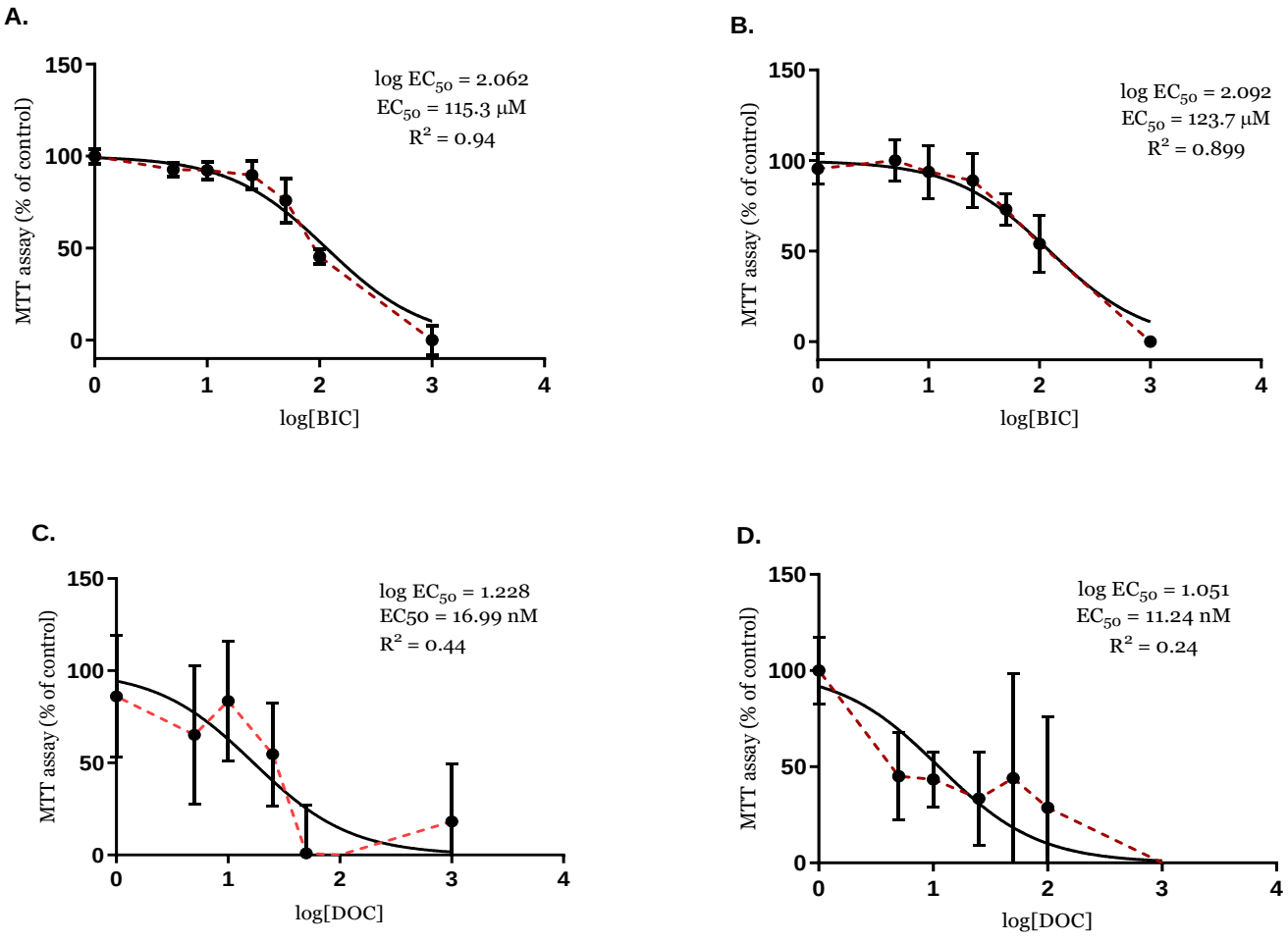


Figure 10: Determination of EC50 for bicalutamide (BIC, n=3) and docetaxel (DOC, n=5) at 24h (**A, C**) and 48h (**B, D**).

4.2 Effect of bicalutamide and docetaxel in STEAP1 expression of LNCaP cells

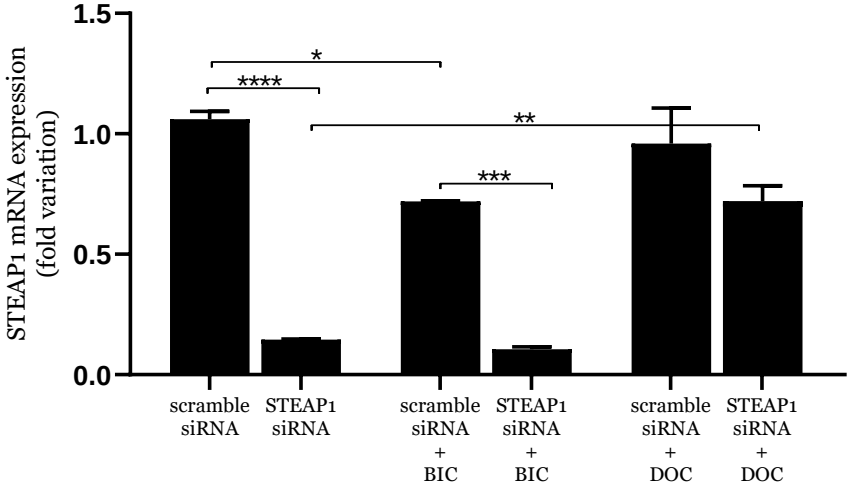
LNCaP cells knocked down (or not) for STEAP1 were treated with bicalutamide or docetaxel. After 24 h of transfection with scramble siRNA or STEAP1 siRNA, LNCaP cells were stimulated with 100 μM bicalutamide or 20 nM docetaxel for 24 h. In the end, the cells were harvested, and qPCR and western blot was carried out to evaluate the STEAP1 mRNA and protein expression, respectively.

As can be seen in Figure 11, the silencing of STEAP1 was done with success (85% and 83% reduction of mRNA and protein, respectively, relative to scramble siRNA). When LNCaP cells were treated with bicalutamide there is a significant decrease in STEAP1 mRNA (0,719 ± 0,004-fold variation) expression in comparison with control cells (scramble siRNA). Regarding the effect of BIC at STEAP1 protein level, there is a trend of decrease (0,804 ± 0,121-fold variation) but without statistical significance.

Regarding the effect of DOC, no significant differences were observed (Figure 11).

The decreased expression of STEAP1 in response to siRNA-STEAP1, was reversed in the presence of docetaxel, but not by bicalutamide (Figure 11).

A.



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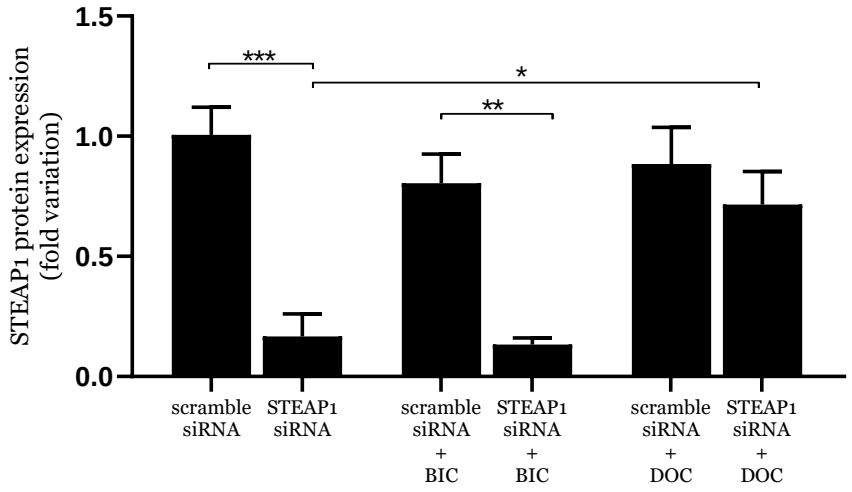


C.

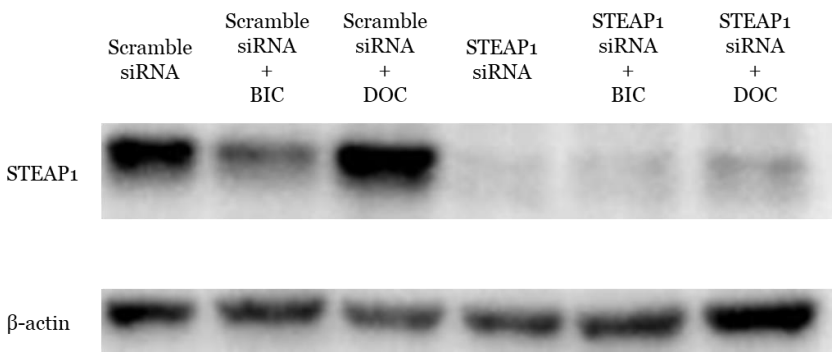


Figure 11: Effect of bicalutamide or docetaxel on STEAP1 expression in LNCaP cells knocked down for STEAP1. mRNA expression was determined by qPCR (**A**) after normalization with β2M gene. Protein expression was determined by Western Blot (**B**) after normalization with β-actin. Results are expressed as fold variation relatively to control group (scramble siRNA). In **C** are the representative immunoblots for STEAP1 and β-actin. Error bars indicate mean ± SEM (n=4) * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

4.3 Impact of STEAP1 on effect of bicalutamide and docetaxel in prostate cancer

The influence of STEAP1 in prostate cancer treatment with bicalutamide and docetaxel was evaluated, determining their effects in cell viability/proliferation and apoptosis.

4.3.1 Cell viability, proliferation, p53 and p21 levels

Cell viability of LNCaP cells was determined by MTT assay after treatment with bicalutamide and docetaxel, when LNCaP cells were silenced (or not) for STEAP1. STEAP1-knockdown LNCaP cells showed a decrease in cell viability of 24,9%. In LNCaP cells with high levels of STEAP1, there is a significant decrease of approximately 50% and 25% of cell viability in LNCaP cells treated with bicalutamide and docetaxel, respectively (Figure 12). The viability of STEAP1-silenced LNCaP cells treated with bicalutamide was similar to the viability of scramble-siRNA LNCaP cells with the same treatment (48,4%*vs* 51,3%). However, the decrease in cell viability observed with docetaxel treatment is reversed in the absence of STEAP1, there is an increase of about 25% (75% *vs* 105,3%).

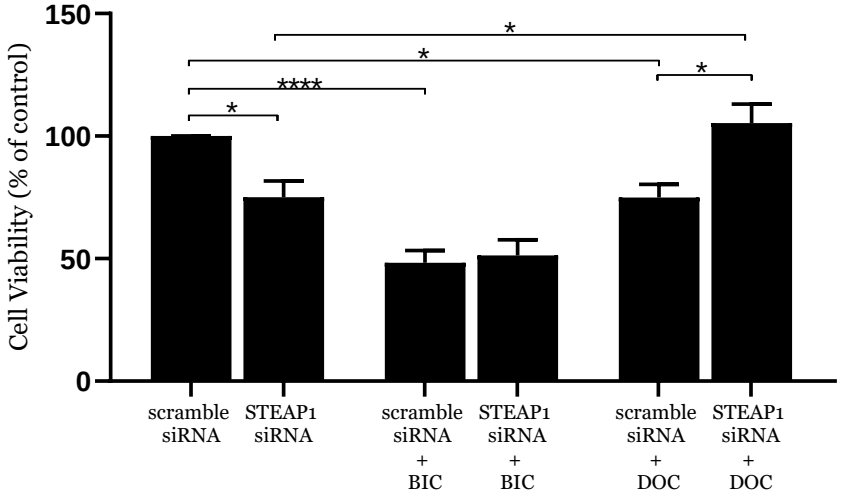
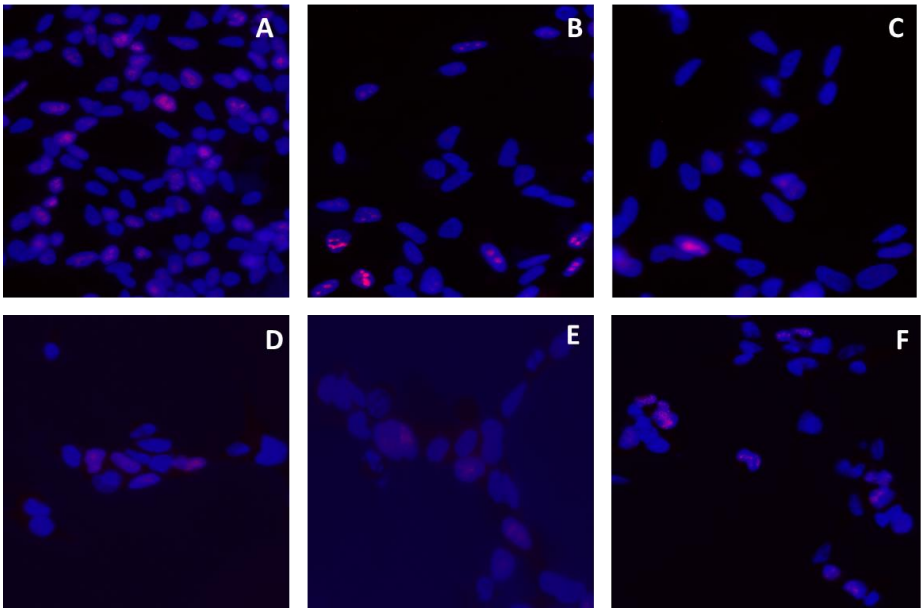


Figure 12: Analysis of cell viability by means of MTT assay. Results are expressed as % relative to the control group (scramble siRNA). Error bars indicate mean ± S.E.M (n= 4). * $p<0.05$; *** $p<0.001$ when compared to the control.

To complement the cell viability assay was used ki-67 index. Cell proliferation index was significantly decreased after STEAP1 gene silencing (0.56 ± 0,058-fold variation when compared to scramble siRNA). LNCaP cells treated with bicalutamide and docetaxel showed a significant reduction of proliferation (0.63 ± 0,065 and 0.61 ± 0,051-fold variation when compared to scramble siRNA, respectively, Figure 13). When LNCaP cells STEAP1-knockdown were treated with bicalutamide there is an increase in cell proliferation compared to scramble + BIC (0,707 ±0,097 and 0,630 ±0,065-fold variation).

On the other hand, the inhibitory effect of STEAP1-knockdown or docetaxel in cell proliferation is reversed when a combined treatment was performed (43 % increase in STEAP1 siRNA + docetaxel when compared to STEAP1 siRNA, and 38 % increase in STEAP1 siRNA + docetaxel when compared to scramble siRNA + docetaxel)



G.

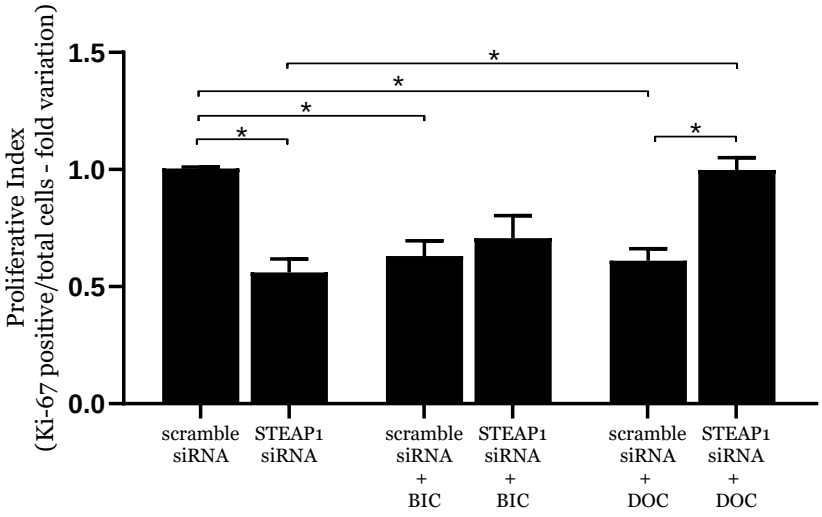


Figure 13: Proliferation of LNCaP cells determined by Ki-67 fluorescent immunocytochemistry (**A-G**). Representative images of merged Hoechst-stained nuclei ki-67 immunofluorescence (×40 magnification) in scramble siRNA (**A**), scramble siRNA + BIC (**B**), scramble siRNA + DOC (**C**), siRNA STEAP1 (**D**), siRNA STEAP1 + BIC (**E**) and siRNA STEAP1 + DOC (**F**). Results (**G**) are expressed as fold variation relative to the control group (scramble siRNA). Error bars indicate mean ± S.E.M (n= 3). * $p<0.05$ when compared to the control.

The tumor suppressor protein p53 is essential in the control the expression of p21, a cyclin-dependent Kinase inhibitor that induces cell cycle arrest. No differences of p53 levels were observed between scramble siRNA and STEAP1 siRNA LNCaP cells (Figure 14A). Relatively to bicalutamide no differences also were observed, however the expression of protein p53 increased in LNCaP cells subjected to docetaxel (4,1 ± 0,246-fold variation to scramble siRNA, Figure 14A). Interestingly, docetaxel treatment in STEAP1-knockdown LNCaP cells significantly decreased p53 levels (1,860 ± 0,745-fold variation) compared to scramble STEAP1 + DOC. These results were also verified at p21 mRNA expression. Treatment with docetaxel significantly decreased p21 levels when LNCaP cells were knocked down for STEAP1 gene (3,5 ± 0,328 *vs* 1,4 ± 0,300-fold variation between scramble siRNA + DOC and STEAP1 siRNA + DOC, Figure 14B). The knockdown of STEAP1 gene increased the expression of p21 in comparison with the scramble siRNA (2,4 ± 0,110-fold variation to the control). The treatment with bicalutamide and docetaxel significantly increased the expression of p21 (4,4 ±0,367 and 3,5 ± 0,328-fold variation to scramble siRNA, Figure 14A).

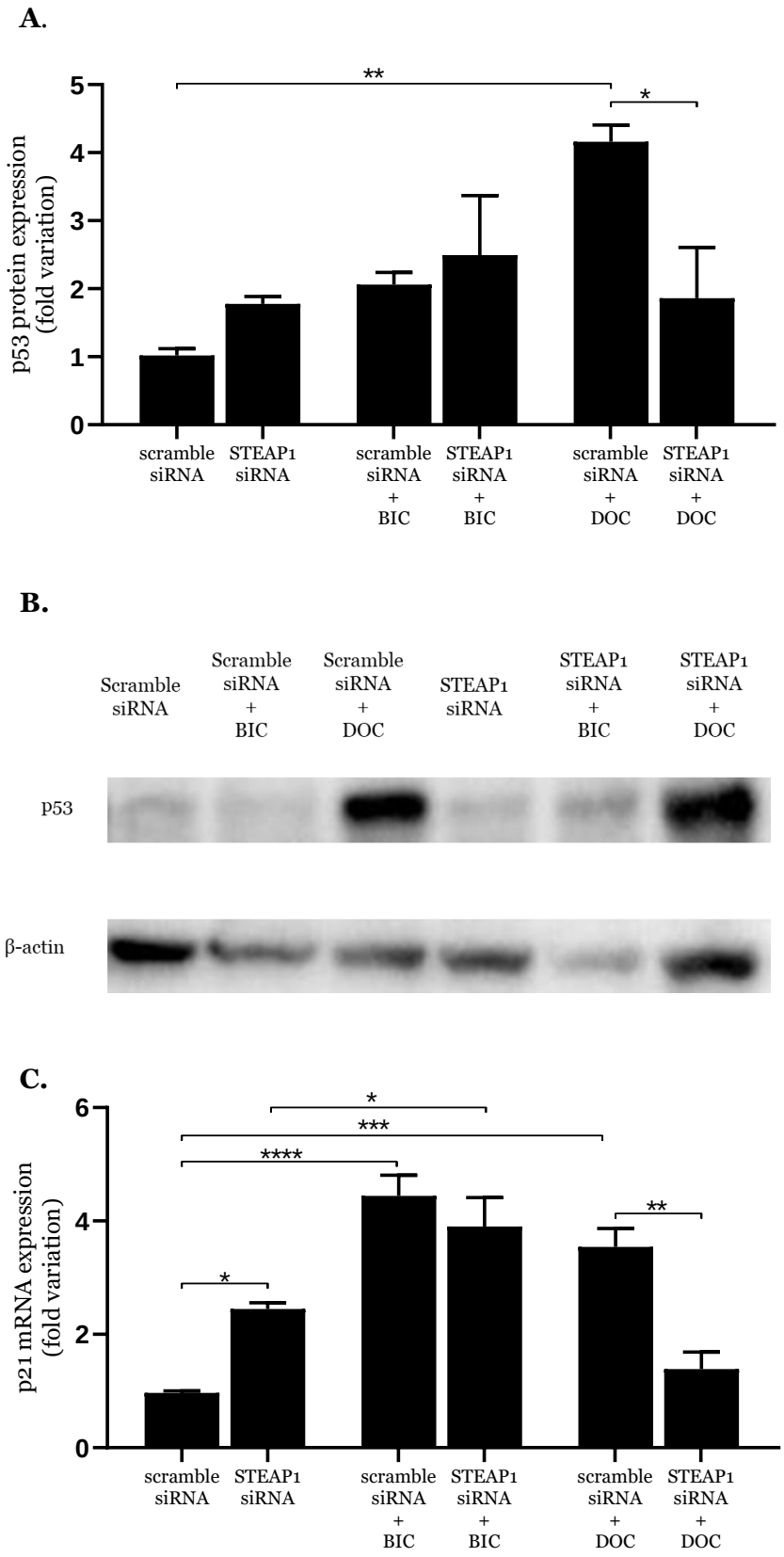
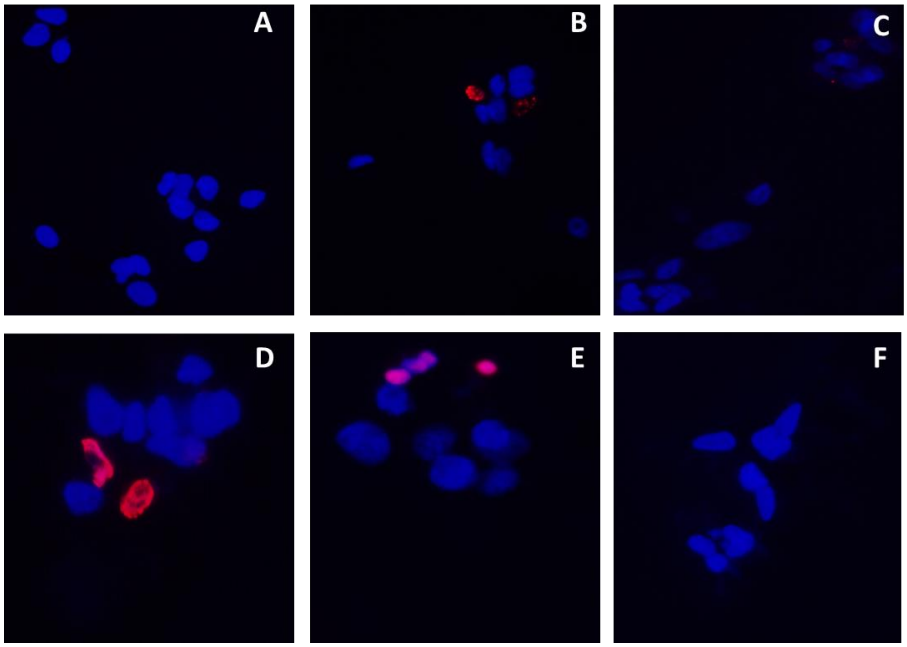


Figure 14: Expression levels of cell cycle regulators p53 (protein expression by western blot (**A**) and representative immunoblots (**B**)) and p21 (mRNA expression by qPCR (**C**)). Results are expressed as fold variation relative to the control group (scramble siRNA). Error bars indicate mean ± S.E.M (n= 3). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared to the control.

4.3.2 Apoptosis (TUNEL and caspase 3 activity)

Relatively to apoptosis, STEAP1-knockdown significantly increased the number of TUNEL-stained LNCaP cells in relation to scramble siRNA (2,1 ± 0,148-fold variation, Figure 14). Is was verified that in presence of higher levels of STEAP1 (LNCaP cells treated with scramble siRNA), bicalutamide or docetaxel increased apoptosis (2.2 ± 0,134 and 1.9 ± 0,095-fold variation, respectively, Figure 15).
But to docetaxel and bicalutamide, this effect was reversed when STEAP1-knockdown + DOC or STEAP1 knockdown + BIC was performed (1,3 ± 0,104 *vs* 1,8 ± 0,095- fold variation between siRNA STEAP1 + DOC and scramble siRNA + DOC, and 1,6 ± 0,104 vs 2,2 ± 0,134 between siRNA STEAP1 + BIC and scramble siRNA + BIC.



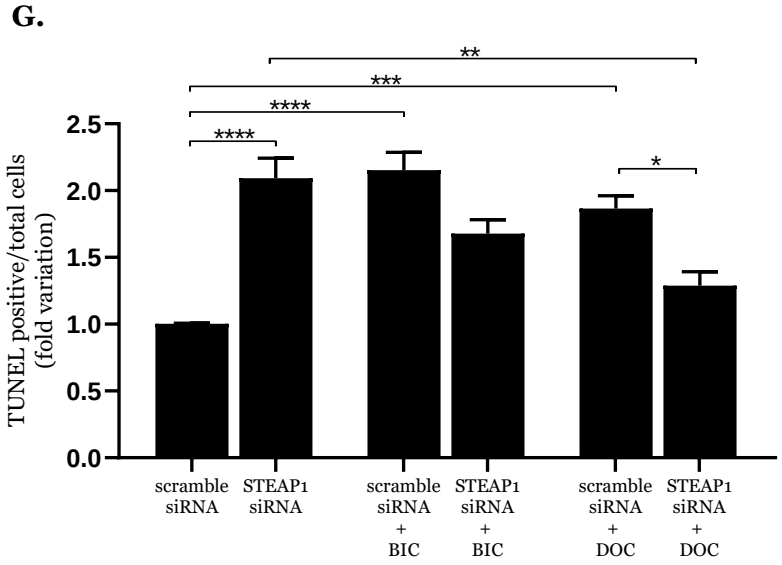


Figure 15: Apoptosis of LNCaP cells determined by TUNEL assay (**A-G**). Representative images of merged Hoechst-stained nuclei TUNEL immunofluorescence (×40 magnification) in scramble siRNA (**A**), scramble siRNA + BIC (**B**), scramble siRNA + DOC (**C**), siRNA STEAP1 (**D**), siRNA STEAP1 + BIC (**E**) and siRNA STEAP1 + DOC (**F**). Results (**G**) are expressed as fold-variation relative to the control group (scramble siRNA). Error bars indicate mean ± S.E.M (n= 3). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared to the control.

Apoptosis it triggered by the caspase enzymes, and intrinsic and extrinsic pathways converge at the activation of caspase-3, which has been considered a remarkable endpoint of apoptosis. The activity of caspase-3 was used as a measurement of apoptosis. Caspase-3 activity increase significantly in response to STEAP1 silencing (~93% relatively to scramble siRNA, Figure 16). LNCaP cells treated with bicalutamide or docetaxel, in the presence of STEAP1, it was verified a significant increase the enzymatic activity of caspase-3 (~143% and 69%, respectively, Figure 16). In STEAP1-knockdowed LNCaP cells treated with bicalutamide and docetaxel didn't exist significances differences compared to STEAP1 siRNA + BIC and STEAP1 siRNA + DOC, existing a trend of reduction of apoptosis.

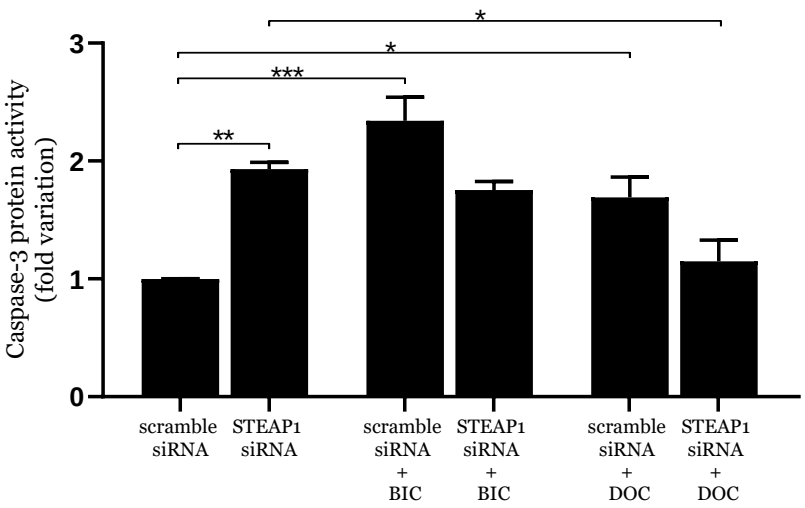


Figure 16: Protein expression level of the apoptosis regulator: caspase-3 activity. Error bars indicate mean ± SEM (n = 3). * $p<0.05$; ** $p<0.01$; *** $p<0.001$ when compared to the control.

5. Discussion/ Conclusion

One of the major concerns regarding the treatment of prostate cancer is when patients become resistant to treatment, being bicalutamide and docetaxel two drugs widely used in PCa treatment. Bicalutamide is a non-steroidal androgen receptor blocker that competitively antagonizes the actions of androgens and other ligands, thereby inhibiting the growth of prostate tumors [45], [46]. Docetaxel is a semisynthetic cytotoxic taxane and is an antimicrotubule agent that exerts its cytotoxic activity by disrupting the microtubular network in cells that is essential for mitotic and interphase cellular functions [42], [49], [50].

As a way to improve the treatments of prostate cancer is to evaluate combined treatments with other putative therapeutic targets. There are several proteins that are dysregulated in prostate cancer, and one of which is the six-transmembrane epithelial antigen of the prostate 1 (STEAP1). The STEAP protein family has been implicated in many forms of cancer due to overexpression in malignant cells when compared to their non-malignant counterparts [55].

Therefore, the present project aims to evaluate if the sensitivity of LNCaP cells to bicalutamide or docetaxel treatment can be improved in response to STEAP1 gene silencing and whether STEAP1 overexpression may as as a predictive biomarker for treatment response. To achieve these objectives, we first determined the EC50 for bicalutamide or docetaxel by MTT assay. Based on the dose-response curve of the two drugs, we used the concentration of 100 μM and 20 nM for BIC and DOC, respectively.

As r^2 is higher in 24 h for both drugs and because other studies point to these concentration for docetaxel ([68]), and although this concentration for bicalutamide is not found in other studies, we used it once it was the value obtained. We opted for this time and the respective concentration for the following experimental design.

To evaluate the effect of bicalutamide or docetaxel in STEAP1 expression of LNCaP cells, we first induced STEAP1 gene knockdown by transfecting these cells with a specific siRNA (20 nM) against STEAP1. We confirmed that STEAP1 mRNA expression and protein level was reduced 24h after transfection (more 80%). The conditions used were similar to described previously work by our group [10]. After transfection, LNCaP cells were stimulated with 100 μM of bicalutamide and 20 nM docetaxel for 24 h.

The results showed that the decreased expression of STEAP1 in response to siRNA-STEAP1, was reversed in the presence of docetaxel, but not by bicalutamide. These results suggest that STEAP1 may be involved in the resistance to docetaxel in the treatment of prostate cancer.

To determine the impact of STEAP1 on effect of bicalutamide and docetaxel in prostate cancer was assessed cell viability, proliferation, regulators of cell cycle (p21 and p53) apoptosis and regulator of apoptosis (caspase-3 activity).

Relatively to cell viability and proliferation, STEAP1-knockdown cells reveals a decrease of about 25% and , which is in agreement with other studies [10]. And when scramble siRNA LNCaP cells are treated with bicalutamide and docetaxel verified a decreased of approximately 50% and 25%, respectively, of cell viability when compared to control cells (scramble siRNA), which is in line with other studies [47], [69]. There is an increase in viability and cell proliferation when STEAP1 knockdown cells were treated with BIC and DOC. These results suggest that STEAP1-knockdown combined with BIC or DOC leads to tumor progression. This was an unexpected finding since docetaxel is a microtubule inhibitor inducing cell death [70]. Possibly, the effect of docetaxel can be mediated by the presence of STEAP1, because in its absence, docetaxel does not reduce the viability or proliferation of LNCaP cells. To clarify this result, TUNEL assay, p53 expression and caspase 3 activity was performed.

Data obtained with the TUNEL assay and caspase 3 activity showed that the STEAP1 knockdown not only decreased cell proliferation but also increased the number of apoptotic cells, these results are supported by a previous report [10]. There is also a decrease of apoptosis when cells are treated with bicalutamide and docetaxel in the absence of STEAP1 compared to cells treated with BIC and DOC but with the presence of STEAP1, which supports the results of cell viability and proliferation.

Docetaxel treatment in STEAP1-knockdowned LNCaP cells significantly decreased p53 levels and this results also verified to mRNA p21 expression. Since p53 is a tumor suppressor and is essential in controlling p21 expression, the fact that STEAP1-knockdown cells treated with docetaxel have lower levels of p53 and p21 compared to scramble + DOC, means that there is an induction of stopping the cell cycle. The cell cycle stops according to the results obtained for cell viability and apoptosis for this condition.

In conclusion, our study demonstrated that when we stimulate LNCaP cells with DOC there is a reversal of STEAP1 silencing, leading to increased cell viability, decreased p53 and p21 expression and promoted apoptosis demonstrated by the TUNEL assay and caspase 3 activity. In the case of treatment with bicalutamide simultaneously with silencing STEAP1, there is a decrease in apoptosis and in p21 expression, and there is an increase in cell proliferation.

Overall, these preliminary results suggest that STEAP1 may be involved in the resistance to docetaxel and bicalutamide. These results suggest also that STEAP1 overexpression may be used as a putative negative predictive biomarker for treatment with these anti-cancer drugs.

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