

Universidade do Minho
Escola de Medicina

Inês Fernandes Lages

Dissecting chemotherapy resistance in prostate cancer: interplay between RKIP and androgen stimulation



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Dissertação de Mestrado
Mestrado em Ciências da Saúde

Trabalho efetuado sob a orientação da
Doutora Olga Catarina Lopes Martinho
e do
Professor Doutor Rui Manuel Vieira Reis

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Então virou a cara para o outro lado do abismo. Tentou ver através da escuridão.
Sophia de Mello Breyner Andresen, A Viagem in Contos Exemplares

ACKNOWLEDGMENT

Aos meus orientadores Olga e Rui Reis. Agradeço a oportunidade de entrar neste mundo fascinante que é a oncobiologia. Obrigada Olga por tentares fazer de mim uma cientista independente.

A toda a equipa Rui Reis, em particular à OMTeam, e aos SSRD, em especial à Ana Gonçalves, pelo incentivo diário. Aos meus colegas, à Ângela, és encantadora, linda por dentro e por fora e ao Pedro, que mesmo longe está perto.

À Ana Isabel Frias, companheira de laboratório, concertos e tasquinhas. Que continuemos a colecionar momentos como o do Pinochet ou o mítico “Eu tenho sentimentos”.

À Diana, à Helena, à Joana e ao Viana. Sempre achei que era uma pessoa com sorte e encontrar-vos foi, realmente, como achar um trevo de quatro folhas. Na balança da vida o peso da vossa amizade não é superado por nada. Um brinde a vocês, que são o melhor do meu mundo!

À Ana Forte, que nem por encomenda seria melhor! É uma amizade que nutre o conforto e o amor de quem se sente em casa. Porque é mais fácil quando alguém te compreende e acompanha. Ao nosso amor maior comum: *Mas lembra-te bem quando eu te digo que moras dentro do meu peito!*

Aos meus pais: *A melhor herança que um pai pode deixar ao filho é a firmeza em aspirar triunfos e a capacidade de assimilar derrotas.* Obrigada por me incutirem valores, me mostrarem o mundo e ensinarem como é que se enfrenta esta aventura que é a vida. A minha educação é o meu maior tesouro, e vocês todos os dias, arduamente, o cultivam por puro altruísmo. Em tudo o que faço ou sou, vocês são a origem. O meu eterno obrigado, sempre com muito amor!

Por fim, esta tese foi realizada no Instituto de Investigação em Ciências da Vida e da Saúde (ICVS), na Universidade do Minho. O financiamento provém do projeto NORTE-01-0145-FEDER-000013, do Programa Operacional Regional do Norte (NORTE 2020), sob o Acordo de Parceria PORTUGAL 2020, através do Fundo Europeu de Desenvolvimento Regional (FEDER), e do projeto POCI-01-0145-FEDER-007038, sobre o Programa Operacional Fatores de Competitividade (COMPETE) e de fundos nacionais da Fundação para a Ciência e Tecnologia (FCT).



Aos meus pais, com amor

STATEMENT OF INTEGRITY

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Exploração da resistência à quimioterapia em cancro da próstata: Interação entre RKIP e estimulação androgénia

RESUMO

O cancro da próstata (CaP) é o segundo cancro mais frequente em homens, sendo a cascata de sinalização mediada pelo Recetor de Androgénio (RA) o principal mecanismo molecular associado à sua progressão. Outros processos, como a Transição Epitélio-Mesenquimal (TEM), têm sido associados com metastização e resistência terapêutica em CaP. A TEM é um processo reversível que regula a conversão de células com características epiteliais num fenótipo mesenquimal que é altamente invasivo. Contudo, a ligação entre estimulação com androgénios e EMT continua em debate, com resultados contraditórios na literatura.

Não obstante à existência de novos fármacos, o CaP num estado avançado permanece incurável, sendo indispensável descobrir novos marcadores de respostas terapêutica. A RKIP (Raf Kinase Inhibitor Protein) é uma proteína descrita como supressora de metástases em vários tumores, incluindo o CaP. Funcionalmente, a RKIP é inibidora da TEM, constituindo uma oportunidade para controlar a progressão do CaP.

Neste trabalho pretendeu-se estudar o efeito da estimulação androgénica na expressão de proteínas da TEM e da RKIP, bem como o papel da RKIP e da estimulação androgénica na resposta farmacológica em CaP.

Inicialmente, em linhas celulares de CaP, observámos que não existe uma relação significativa entre a estimulação androgénica e a expressão da RKIP. Concluiu-se ainda que as linhas celulares mais sensíveis aos fármacos estudados eram positivas para RA, RKIP e proteínas do fenótipo epitelial. De seguida, numa linha celular positiva para RA, observou-se que a estimulação androgénica teve um comportamento incongruente no controlo da expressão de proteínas mesenquimais. Constatou-se ainda que a expressão de RKIP, AR e proteínas mesenquimais aumenta após tratamento com anti-androgénios. Por fim, uma linha celular positiva para RA foi silenciada para a expressão de RKIP, tendo-se verificado que a sua ausência leva a uma maior sensibilidade das células a agentes quimioterápicos, mas não a anti-androgénios. No entanto, observou-se que células com RKIP silenciado possuem um aumento da expressão de proteínas associadas à TEM e à resistência terapêutica.

Este estudo destaca o papel de RKIP na resposta à quimioterapia, sendo, no entanto, necessários mais ensaios para compreender o seu mecanismo de ação nestes tratamentos.

PALAVRAS CHAVE: Cancro da Próstata | Quimioterapia | Raf Kinase Inhibitor Protein (RKIP) | Recetor de Androgenios | Transição Epitélio-Mesenquimal

Dissecting chemotherapy resistance in prostate cancer: Interplay between RKIP and androgen stimulation

ABSTRACT

Prostate Cancer (PCa) is the second most frequent cancer in men, in which the main mechanism of progression is the Androgen Receptor (AR) signaling. Some mechanisms have been correlated with metastization and therapeutic resistance in PCa, namely, the Epithelial-Mesenchymal Transition (EMT). EMT is a reversible process that regulates the conversion of cells with epithelial characteristics into a highly invasive mesenchymal phenotype. Nevertheless, the connection between androgens and EMT continues to be investigated, being a subject with contradictory results in the literature

In spite of the development of new pharmacological drugs, advanced PCa remains incurable and treatments are limited and ineffective, making it indispensable to discover new markers for prediction of therapy response. Raf Kinase Inhibitor Protein (RKIP) has been described as a metastasis suppressor protein in various tumors, including PCa. Functionally, RKIP is an inhibitor of EMT, providing an opportunity to control PCa progression.

In this project the influence of androgen stimulation in RKIP and EMT proteins expression was evaluated, as well as role of RKIP and androgen stimulation in pharmacological response in PCa.

Firstly, in PCa cell lines a non-significant correlation between RKIP and androgen stimulation was observed. Moreover, we concluded that the most sensitive cell lines to the studied drugs were positive for AR, RKIP, and EMT proteins. Subsequently, in an AR-positive cell line, was observed that androgen stimulation had an incongruent behavior in the modulation mesenchymal proteins expression. Additionally, RKIP, AR, and mesenchymal proteins were increased under antiandrogen drugs treatment. Finally, an AR-positive cell line was knocked out (KO) for RKIP expression, *being* revealed that its absence leads to a better response of cells to chemotherapeutic, but not to antiandrogen drugs. However, in RKIP KO cells, was observed an increased expression of proteins related to EMT and therapeutic resistance.

This study highlights the RKIP role in the chemotherapeutic response in PCa, however, more assays are required to understand its mechanism of action to those therapeutic approaches.

KEYWORDS: Androgen Receptor (AR) | Chemotherapy | Epithelial-Mesenchymal Transition (EMT) | Prostate Cancer (PCa) | Raf Kinase Inhibitor Protein (RKIP)

TABLE OF CONTENTS

ACKNOWLEDGMENT.....	III
STATEMENT OF INTEGRITY.....	IV
RESUMO.....	V
ABSTRACT.....	VI
TABLE OF CONTENTS.....	VII
LIST OF ABBREVIATIONS.....	IX
LIST OF FIGURES.....	XI
LIST OF TABLES.....	XII
CHAPTER 1. INTRODUCTION.....	1
1.1. Cancer.....	1
1.1.1. Prostate Cancer.....	2
1.2. Prostate Cancer Therapy	4
1.2.1. Therapy Resistance in Prostate Cancer	5
1.3. Epithelial-Mesenchymal Transition.....	7
1.3.1. The interplay between EMT and AR signaling.....	10
1.3.2. Multiple interactions of EMT in Prostate Cancer.....	11
1.4. Raf Kinase Inhibitor Protein (RKIP)	12
1.4.1. RKIP as a signaling modulator.....	13
1.4.2. Role of RKIP in EMT regulation.....	15
1.4.3. RKIP in Prostate Cancer.....	17
CHAPTER 2. RESEARCH OBJECTIVE.....	19
CHAPTER 3. MATERIALS AND METHODS.....	20
3.1. Cell lines and cell culture.....	20
3.2. Compounds.....	20
3.3. In vitro knockout of RKIP.....	21
3.4. Western blot (WB)	22
3.5. Half Maximal Inhibitory Concentration (IC ₅₀)	23
3.6. Statistical Analysis.....	24

CHAPTER 4. RESULTS.....	25
4.1. Characterization of Prostate Cancer cell lines.....	25
4.1.1. Effect of androgen stimulation in RKIP expression and EMT proteins.....	25
4.1.2. Determine the sensitivity of PCa cell lines to chemotherapy.....	26
4.2. Role of androgens in RKIP and EMT proteins modulation	29
4.3. RKIP role in Prostate Cancer cell lines response to therapy.....	31
4.3.1. RKIP role in drugs response	31
4.3.2. Effect of RKIP in AR and EMT proteins expression	33
CHAPTER 5. DISCUSSION.....	37
CHAPTER 6. CONCLUSION.....	43
CHAPTER 7. REFERENCES.....	44
SUPPLEMENTARY DATA.....	58

LIST OF ABBREVIATIONS

ADT: Androgen Deprivation Therapy

AKT: Protein Kinase β

ANOVA: Analysis of Variance

AR: Androgen Receptor

ARE: Androgen-Response Elements

ARV: Androgen Receptor Splice Variants

CRISPR: Clustered Regularly Interspaced Short Palindrome Repeats

CRPC: Castration-Resistant Prostate Cancer

DHT: Dihydrotestosterone

DMSO: Dimethyl Sulfoxide

E-cadherin: Epithelial cadherin

EMT: Epithelial-Mesenchymal Transition

ERK: Extracellular Signal-Regulated Kinase 1/2

FBS: Fetal Bovine Serum

GSK3 β : Glycogen Synthase Kinase 3 β

HDR: Homology-Direct Repair

IC₅₀: Half Maximal Inhibitory Concentration

IK β : Inhibitory Kinase β

IKK: Inhibitory Kinase β Kinase

KO: Knockout

MAPK: Mitogen-Activated Protein Kinase

MEK: Mitogen-Activated Protein Kinase Kinase 1/2

MMPs: Matrix Metalloproteinases

N-cadherin: Neural cadherin

NF- κ B: Nuclear Factor kappa β

PCa: Prostate Cancer

PI3K: Phosphoinositide 3-kinase

p-RKIP: phosphorylated-Raf Kinase Inhibitor Protein

PSA: Prostate-Specific Antigen

PTEN: Phosphatase and Tensin Homolog

RKIP: Raf Kinase Inhibitor Protein

SLUG: Zinc Finger Proteins SNAI2

SNAIL: Zinc Finger Proteins SNAI1

TGF- β : Transforming Growth Factor- β

TWIST: Twist-Related Protein 1

WB: Western Blot

YY1: Yin Yang 1

ZEB 1/2: Zinc Finger E-box-binding Homeobox 1/2

LIST OF FIGURES

Figure 1. The biological hallmarks of cancer	1
Figure 2. Androgen circuit: from testicles production to prostate cells activation.	3
Figure 3. Evolution of prostate cancer clinical states and approved agents for each phase.....	5
Figure 4. Androgen Receptor-dependent mechanisms of resistance in hormone-sensitive prostate cancer leading to castration-resistance	7
Figure 5. Outline of a typical Epithelial-Mesenchymal Transition program	9
Figure 6. Ras/ERK activation switches TGF- β to tumor promoter via PTEN and AKT.....	12
Figure 7. RKIP as a modulator of signaling pathways	14
Figure 8. Schematic diagram representing the NF- κ B/SNAIL/YY1/RKIP/PTEN loop	16
Figure 9. Detailed information of the vectors used to knockout <i>RKIP</i>	21
Figure 10. Characterization of EMT markers, RKIP, and AR expression in PCa cell lines, PC-3, DU-145, LNCaP, and 22RV1 under DHT stimulation (10 nM for 24 hours)	26
Figure 11. Representative assay of viability in PCa cell lines, PC-3, DU-145, 22RV1 and LNCaP, exposed to increasing concentrations of Docetaxel, Cabazitaxel, Enzalutamide, and Abiraterone for 72 hours	27
Figure 12. Comparative analysis of IC ₅₀ values for Docetaxel (A), Cabazitaxel (B), Enzalutamide (C), and Abiraterone (D) treatments to PCa cell lines	29
Figure 13. Characterization of EMT markers, RKIP, and AR expression in LNCaP cell line under DHT (10nM), Enzalutamide (20 μ M), and Abiraterone (20 μ M) treatment for 24 hours. (A)	30
Figure 14. Analysis of RKIP expression in the transfected cell line 22RV1 through WB.....	31
Figure 15. Representative viability assay of 22RV1 HDR and CAS9 exposed to increasing concentrations of Docetaxel, Cabazitaxel, Enzalutamide, and Abiraterone for 72 hours.....	32
Figure 16. Comparative analysis of IC ₅₀ values in 22RV1 HDR and CAS9 cells for Docetaxel (A), Cabazitaxel (B), Enzalutamide (C), and Abiraterone (D) treatments	33
Figure 17. Comparison of EMT markers and AR expression in 22RV1 HDR and CAS9 cells under DHT (10 nM), Enzalutamide (20 μ M), and Abiraterone (20 μ M) treatments for 24 hours.....	34
Figure 18. Quantification of EMT markers and AR expression in 22RV1 HDR and CAS9 cells under DHT (10 nM), Enzalutamide (20 μ M), and Abiraterone (20 μ M) treatments for 24 hours.....	34

LIST OF TABLES

Table 1. General information regarding PCa cell lines	20
Table 2. Detailed information of the primary antibodies used in WB analysis	23
Table 3. IC ₅₀ values for Docetaxel, Cabazitaxel, Enzalutamide, and Abiraterone in PCa cell lines.	28
Table 4. IC ₅₀ values for Docetaxel, Cabazitaxel, Enzalutamide, and Abiraterone in 22RV1 HDR and CAS9 cells.	33

CHAPTER 1. INTRODUCTION

1.1. Cancer

Cancer is one of the leading causes of mortality worldwide, 9.5 million people being estimated to have died from cancer in 2018¹, and can be originated from most all cell types and organs². Tumor cells are characterized by genetic abnormalities, caused by DNA-mutating agents. The continuous acquisition of genetic variation and natural selection fosters cells carrying alterations that confer hallmark capabilities (**Figure 1**)^{2,3}. Consequently, a phenotypically diverse population composed by a complex tissue, is characterized by heterotypic interactions between distinct cell types^{2,3}.

A benign tumor remains confined to its original location, whereas a malignant tumor is capable of invading surrounding normal tissue and spread throughout the body via the circulatory and/or lymphatic systems (metastasis)^{2,4}. Only malignant tumors are properly referred to as cancers, and the more widely the cancer spreads, the harder it becomes to eradicate⁴.

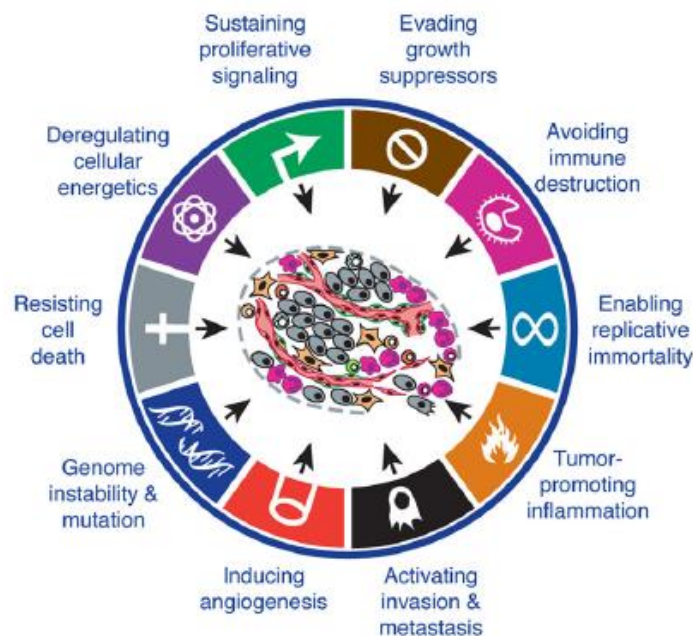


Figure 1. The biological hallmarks of cancer. The scheme illustrates the necessary conditions to manifest a malignant tumor: the hallmarks of cancer. These hallmark traits may be acquired at different stages in the multistep development of cancer, via distinctive mechanisms in different forms of human cancer. *From Hanahan, D. & Weinberg, R. A. 2017³.*

1.1.1. Prostate Cancer

Prostate cancer (PCa) is the second most frequently diagnosed cancer in men worldwide, with an estimation of 1 280 000 new cases and 360 000 deaths in 2018¹. PCa occurs mainly in older men, with approximately two-thirds being diagnosed in age 65 or older. Hence, age is a significant biomarker of PCa poor overall survival^{5,6}. Moreover, men with a family history of one PCa case are twice more likely to develop the disease, while with two or more relatives, these changes increase up to four times^{5,7}.

Clinical history is considered an indicator of genetic susceptibility and/or shared environmental exposures⁸. Some authors attribute up to 40% of all PCa cases to family genetic factors⁵. The known germline mutations associated with PCa are the locus on chromosome 20, Hereditary Prostate Cancer (HPC-20) and the loci on chromosome 1: HPC-1, Predisposing for Prostate Cancer (PCaP), and Predisposing for Prostate and Brain tumor (CAPB)^{5,9}. The most common genetic alterations acquired by PCa patients are *Myc*¹⁰ and Androgen Receptor (*AR*)¹¹ gene amplifications and Phosphatase and Tensin Homolog (*PTEN*)¹² and Retinoblastoma (*RB*)¹³ gene losses, as well as the fusion of Transmembrane Protease, Serine 2 gene (*TMPRSS2*) with the E26 Transformation Specific (*ETS*) transcription factor family gene¹⁴. Regarding lifestyle, some environmental risks have been described as correlated with PCa development⁵. For instance, obesity, which can modify the circulating levels of sex steroid hormones¹⁵; tobacco, which is associated with high levels of circulating testosterone¹⁶; diet, in particular, the high intake of animal fat¹⁷; sexual transmitted diseases¹⁸ and vasectomy¹⁹.

In clinical practice, PCa diagnosis is essentially based on D'Amico classification or Cancer of the Prostate Risk Assessment (CAPRA) score, combining histological criteria, such as Gleason score with Prostate-Specific Antigen (PSA) levels and Tumor Node Metastasis (TNM) evaluation²⁰. PSA and Prostate-Specific Membrane Antigen (PSMA) have been identified as the most promising biomarkers in PCa diagnosis and treatment follow-up²¹. Nevertheless, these tools fail to accurately identify patients whose PCa does not progress from those whose tumor will relapse and evolve after prostatectomy²⁰.

Both PSA and PSMA expression levels are regulated by androgens, namely testosterone, and dihydrotestosterone (DHT), through the AR signaling cascade²¹. Testosterone production is regulated by Luteinizing Hormone (LH) and Luteinizing Hormone-releasing Hormone (LHRH). The hypothalamus releases LHRH, which stimulates the release of LH from the pituitary gland. LH acts

on Leydig cells in the testes to produce the majority of the testosterone in the body. The remaining androgens are produced by the adrenal glands^{22,23}. Testosterone is taken up by prostate cells, where it either binds to the AR directly or is converted to DHT, which has a greater binding affinity to AR than testosterone (**Figure 2**)²⁴.

It has been clear that PCa is dependent on AR signaling for growth and survival; consequently, AR has been the main target for therapies in PCa^{25,26}. The AR, in its free-form, is a cytoplasmic steroid hormone receptor, but after activation it splits away from heat-shock complexes undergoing a conformational change that allows nuclear translocation. As a transcriptional factor, AR binds to Androgen-Response Elements (AREs) in promoter regions, regulating a variety of genes implicated in proliferative and differentiation responses (**Figure 2**)^{22,27,28}.

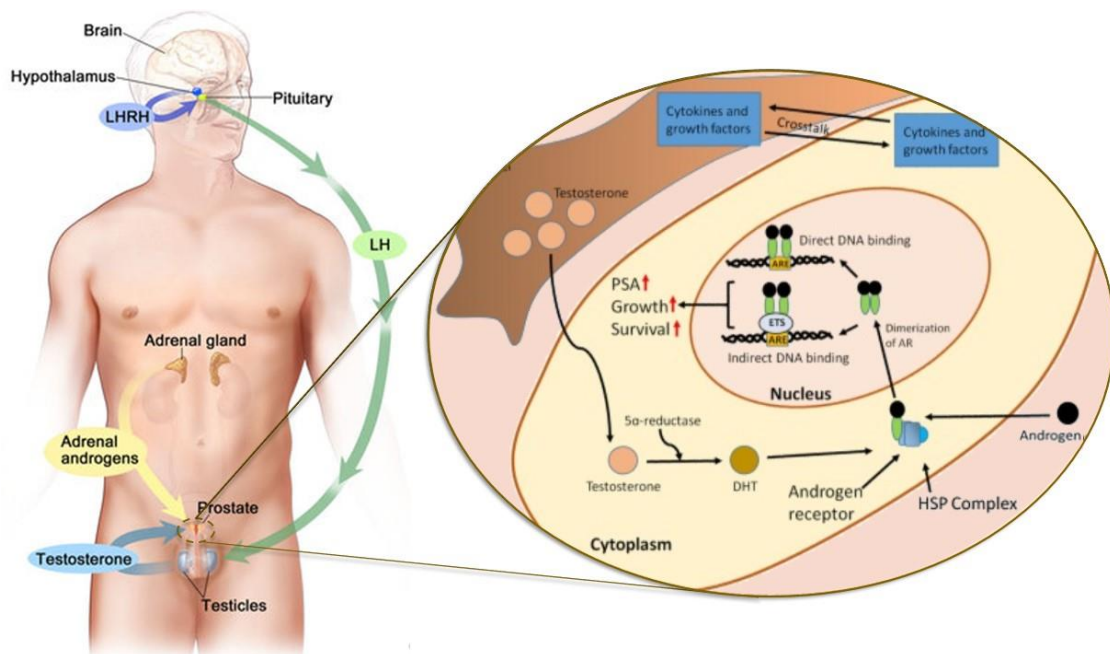


Figure 2. Androgen circuit: from testicles production to prostate cells activation. Testosterone production is regulated by LH and LHRH. The hypothalamus releases LHRH, which stimulates the release of LH from the pituitary gland. LH acts on Leydig cells in the testes to produce the majority of the testosterone in the body. The remaining androgens are produced by the adrenal glands. Testosterone is taken up by prostate cells, where they either bind to the AR directly or are converted to DHT, which has a greater binding affinity to AR than testosterone. Ligand-bound AR splits away from heat-shock complexes located in the cytoplasm and translocate to the nucleus. AR binds to ARE and activates the members of the basal transcriptional complex, which results in androgen-dependent transcription genes. *Adapted from Thakur, A. et al. 2018⁹ and Winslow, T. 2013.* Abbreviations: AR: Androgen Receptor; ARE: Androgen-Receptor Elements; DHT: Dihydrotestosterone; HSP: Heat-Shock Protein; LH: Luteinizing Hormone; LHRH: Luteinizing Hormone-Realizing Hormone; PSA: Prostate-Specific Antigen.

1.2. Prostate Cancer Therapy

Patients with a lower life expectancy and low stage lesions are candidates for active surveillance, continuously monitored, although not receiving any treatments. Regarding patients with high life expectancy and/or high stage lesions, standard local therapy is based on radical prostatectomy and radiotherapy³⁰.

In the case of a biochemical recurrence (increase in PSA), therapy is based on downregulation of circulating androgen levels and/or AR blockage (**Figure 3**). The conventional Androgen Deprivation Therapy (ADT) encompasses the depletion of testosterone produced by the testicles through gonadotropin-releasing hormone drugs (Leuprolide, Goserelin, and Degarelix), complemented by the addition of AR antagonists (Bicalutamide, Flutamide and Nilutamide). Even though the incremental benefit of this combined approach, it is not curative, occurring, eventually, a clinical regression^{31,32} (**Figure 3**).

PCa evolves from an initial asymptomatic phase, with an absence of metastization, progressing into the development of metastases in a minimally symptomatic condition and ending in the symptomatic state, in an advanced course of the disease. PCa evolves to an ADT resistant condition termed Castration-Resistant Prostate Cancer (CRPC). CRPC may either be metastatic or non-metastatic, however, it represents the lethal form of the disease. Effective treatment at this stage is largely limited to the second line of hormonal agents (Abiraterone and Enzalutamide) and chemotherapy (Docetaxel, Cabazitaxel, and Mitoxantrone) for the symptomatic state. Other approaches are currently available, such as immunotherapy (Sipuleucel-T) and bone target therapy (Radium-223 chloride), the principal localization for PCa metastasis (**Figure 3**)³³.

Docetaxel and Cabazitaxel are semi-synthetic taxanes, which impair cellular mitosis. These agents bind to the β -tubulin subunit of microtubulin, stabilizing microtubules and leading to their dynamic inhibition and cell cycle arrest at G2/M phase. Eventually, this results in apoptotic cell death^{34,35}. An important mechanism of taxanes action in PCa was also described through the inhibition of AR nuclear translocation and AR activity³⁶. Abiraterone is a selective, irreversible, and potent inhibitor of the Cytochrome P450 17A1 enzyme (CYP17), suppressing androgen biosynthesis by the testis or the tumor. In addition, Abiraterone can block some downstream enzymes of the steroid pathway and bound to AR, acting as a competitive antagonist, not as potent as pure antagonists³⁷. Moreover, Enzalutamide is a nonsteroidal, pure antagonist of AR, without detectable agonistic effect on the receptor. Studies have shown that Enzalutamide inhibits AR

nuclear translocation, impairing DNA binding and activation³⁸. Regardless of all these treatments, metastatic CRPC has a median survival of fewer than 2 years^{39–41}.

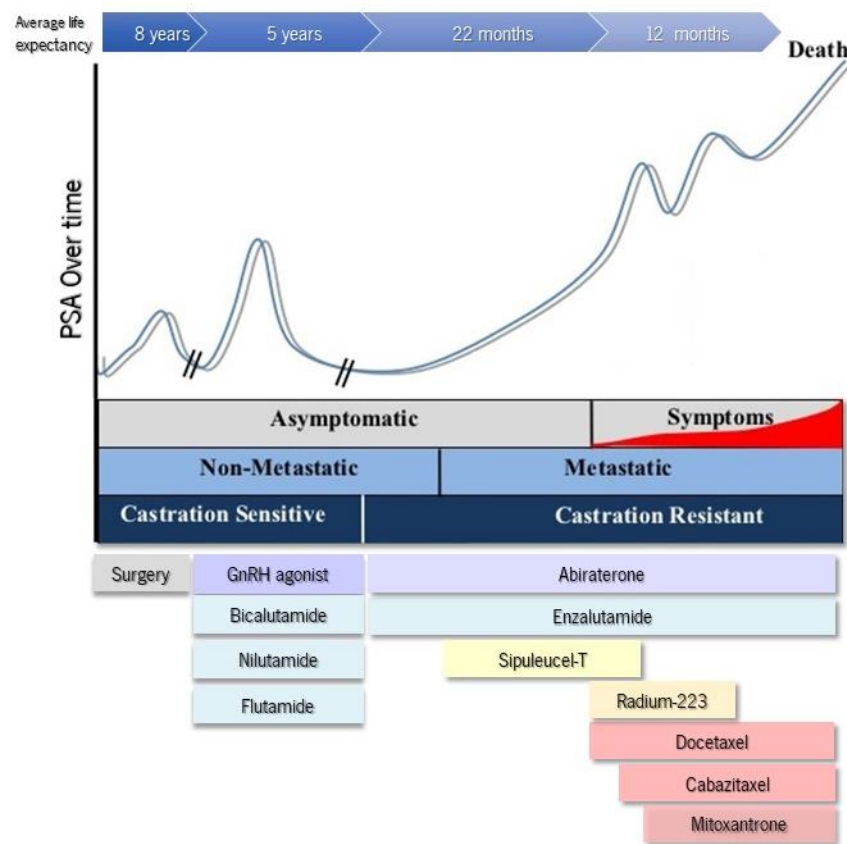


Figure 3. Evolution of prostate cancer clinical states and approved agents for each phase. Patients with prostate cancer have a life expectancy of 8 years. After a biochemical recurrence, in an asymptomatic phase with absence of metastases, the expectancy reduces to 5 years. The first line of treatments encompasses the depletion of testosterone through GnRH agonist drugs, and Androgen Receptor antagonists (Bicalutamide, Flutamide and Nilutamide). The PCa can evolve to a resistance condition with metastization and non-symptomatic, treated with the second line of hormonal agents (Abiraterone and Enzalutamide), immunotherapy (Sipuleucel-T) and bone target therapy (Radium-223). In these cases, expectancy life decreases to 22 months and can decline to 12 months in the symptomatic state, the lethal form of the disease. Effective treatment at this stage is largely limited to chemotherapy (Docetaxel, Cabazitaxel, and Mitoxantrone)^{33,39–41}. Abbreviations: GnRH: Gonadotropin-Releasing Hormone; PSA: Prostate-Specific Antigen.

1.2.1. Therapy Resistance in Prostate Cancer

Notwithstanding all the improvements regarding treatment, advanced PCa remains incurable⁴², and this is underpinned by the biological heterogeneity arising through the accumulation of genetic abnormalities. The outgrowth of different clonal populations, as the phenotypic diversity of neoplastic cells within a tumor, is considered the major driver of therapies resistance^{42,43}.

The exact mechanisms underlying the development of CRPC still not know, even though the research focus is on cells that no longer require the classical activation of AR pathway to survive and proliferate⁴². Traditional ADT does not completely deplete intertumoral androgens or inhibit the expression of AR target genes and, consequently, AR signaling is maintained by multiple mechanisms^{11,39} (**Figure.4**):

1- *AR* gene amplification and AR protein overexpression: Gene amplification, elevated mRNA production, and increased protein half-life can contribute to an enhancement of AR protein levels. Consequently, promoting hypersensitivity to low androgen levels in CRPC. These are the most common genetic modification among patients with CRPC¹¹.

2- AR mutations: The development of AR mutations results in stimulation by non-classical ligand androgens or other steroid hormones. The AR mutations in the early stage of PCa are rare, however, they commonly occur in CRPC⁴⁴.

3- Altered expression and function of AR co-regulators: AR co-regulators are protein factors that can recruit transcriptional machinery or RNA splicing regulators, which serve an auxiliary role in activation/inhibition of AR-mediated transcription. Modified expression of AR co-regulators culminates in the activation of AR transcription activity, even under an extremely low androgen concentration⁴⁵.

4- Synthesis of adrenal and intratumoral androgens: Overall castration can reduce circulating levels of serum testosterone to approximately 90%, however, androgen concentration in PCa cells remains sufficient for AR activation. This arises through the capacity of PCa cells to convert non-testicular androgen or other steroid hormones in potent androgens and consequently not rely on androgen cycle⁴⁶.

5- Expression of AR Splice Variants (ARV): ARV are expressed via alternative RNA splicing and are constitutively active in driving AR-regulated transcription, promoting tumor progression. AR Splice Variant 7 (ARV7) is the most commonly expressed ARV in human tissues, and its levels are correlated with increased risk of biochemical relapse and shorter survival time of CRPC patients⁴⁷.

6- AR-independent pathways: Src kinase activation and growth factor signaling pathway have been identified as relevant for androgen-independent cell growth, as well as enhancers of AR signaling, culminating in CRPC promotion. Growth factor receptors are the master regulators, they interact with numerous signaling cascades, such as Interleukine-6 (IL-6) receptor, Epidermal Growth Factor Receptor (EGFR), Mitogen-Activated Protein Kinase (MAPK) and Phosphoinositide 3-

kinase (PI3K)/ Protein kinase β (AKT), which can mediate the activation of critical downstream targets¹¹.

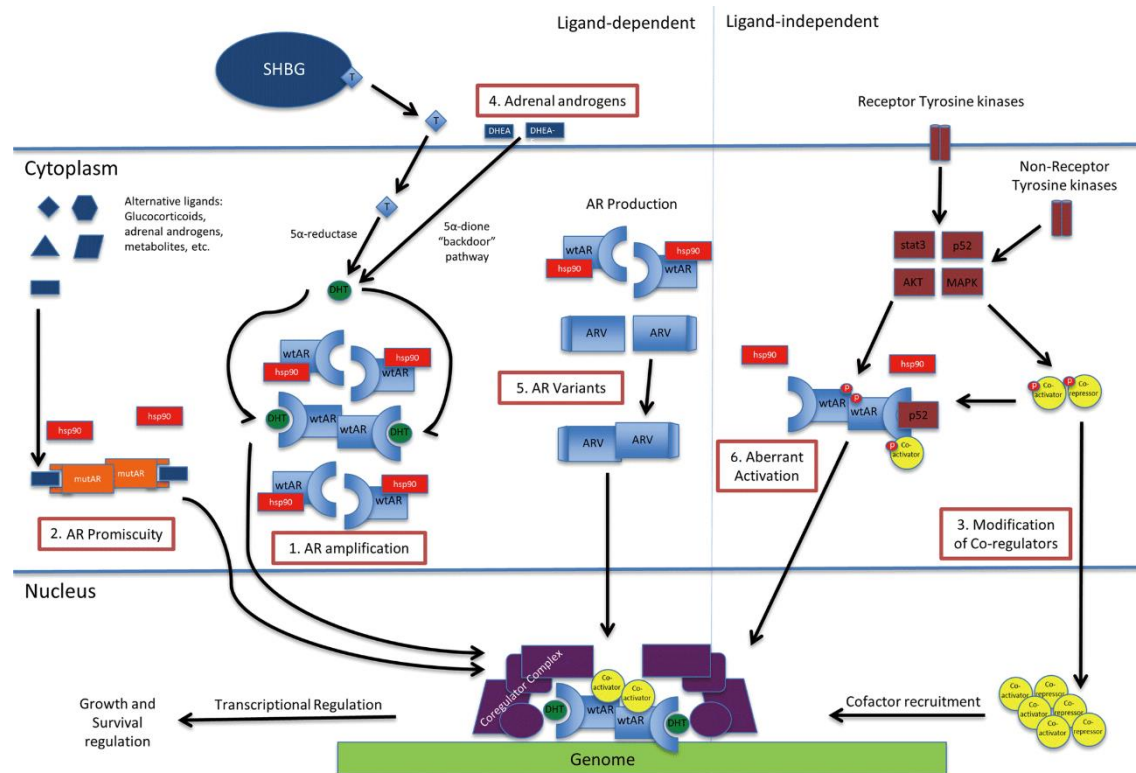


Figure 4. Androgen Receptor-dependent mechanisms of resistance in hormone-sensitive prostate cancer leading to castration-resistance. Multiple mechanisms contribute to continuous AR signaling in CRPC including: genomic amplification and overexpression of AR (1), gain of function mutations allowing AR to be activated by promiscuous ligands such as steroids or antiandrogens (2), abnormalities in AR coactivators and coregulators (3), increased intratumoral androgens and continued extragonadal androgen synthesis (4) ARV that encode for Ligand-Binding Domain (LBD)-deficient receptors and are constitutively active (5), ligand-independent transactivation of AR by growth factors or cytokines (6), upregulation of AR enhancer elements or alterations in androgen transport⁴⁷. From: Huang, Y. et al. 2018¹. Abbreviations: AKT: Protein Kinase β ; AR: Androgen Receptor; ARV: AR splice variants; DHEA: Dehydroepiandrosterone; DHT: Dihydrotestosterone; HSP: Heat-Shock Protein; MAPK: Mitogen-Activated Protein Kinase; mutAR: mutated AR; SHBG: Sex Hormone Binding Globulin; STAT3: Signal Transducer and Activator of Transcription 3; wtAR: wild-type AR.

The development of multiple strategies that effectively target the androgen axis has been studied. Nevertheless, a better understanding of resistance processes will contribute to the development of new therapeutic strategies, which potentially enhance the efficacy of androgen-targeted agents and delay disease progression.

1.3. Epithelial-Mesenchymal Transition

Tumor cell plasticity regulated by androgen deprivation plays a critical role in disease progression and potentially provides an additional opportunity for cancer control. Adaptive changes, conspicuously the epithelial-to-mesenchymal plasticity (EMP), contributes to the

development of advanced disease. Cellular plasticity in phenotypic transformation is a physiological mechanism that includes Epithelial-Mesenchymal Transition (EMT) and Mesenchymal-Epithelial Transition (MET), the reverse process⁴⁸.

EMT is a reversible and reprogramming process that mainly regulates the conversion of polarized cells with epithelial characteristics into a highly migratory and invasive mesenchymal phenotype through modification of gene expression^{42,49}. EMT is divided into 3 types according to its biological environment, relevant functions, and regulatory mechanisms. Type I is related to implantation, embryogenesis, and organ development. Type II is involved in wound healing, tissue reconstruction, tissue regeneration, and organ fibrosis. Type III plays an important role in the progression and metastization of cancer, inducing a transformation of epithelial cells into mesenchymal cells^{42,49,50}.

Overall, in EMT a breakdown of cell-to-cell or cell-to-Extracellular Matrix (ECM) adhesion occurs at the polarized epithelium lining. At a biochemical level, Epithelial cadherin (E-cadherin) is the major component of epithelial adherence junction and their loss is the key step to initiate EMT, leading to the collapse of intercellular mechanical communication^{49,51}. In augmentation, a coordinated deficiency of cell adhesion molecules, such as Claudins, Occludins and Desmoplakin, and gain of critical mesenchymal markers, for instance, Vimentin, Fibronectin, Integrins and Neural cadherin (N-cadherin). The shift from epithelial to mesenchymal phenotype is regulated through EMT transcriptional factors, such as Zinc Finger Proteins SNAI1 (SNAIL) and SNAI2 (SLUG), Twist-Related Protein 1 (TWIST) and Zinc Finger E-box-binding Homeobox 1 and 2 (ZEB1 and ZEB2, respectively)^{49,51,52}. In a different approach, proteolytic actions in ECM and basement membrane degradation through Matrix Metalloproteinases (MMPs), which are zinc-dependent endopeptidases, decrease cell-cell adhesion, enhancing invasiveness and EMT potential⁵³ (**Figure 5**).

EMT has been proposed as a putative mechanism driven by carcinoma cells to invade adjacent tissues and improve access to the bloodstream⁵⁴. Following tumor metastization to distant sites and adaptation to the surrounding matrix environment, cells undergo MET reverting to an epithelial phenotype, typically re-expressing E-cadherin. This oscillation between epithelial and mesenchymal states represents a dynamic and complex set of events that contribute and are, perhaps, the key to successful metastatic dissemination and colonization^{48,55}.

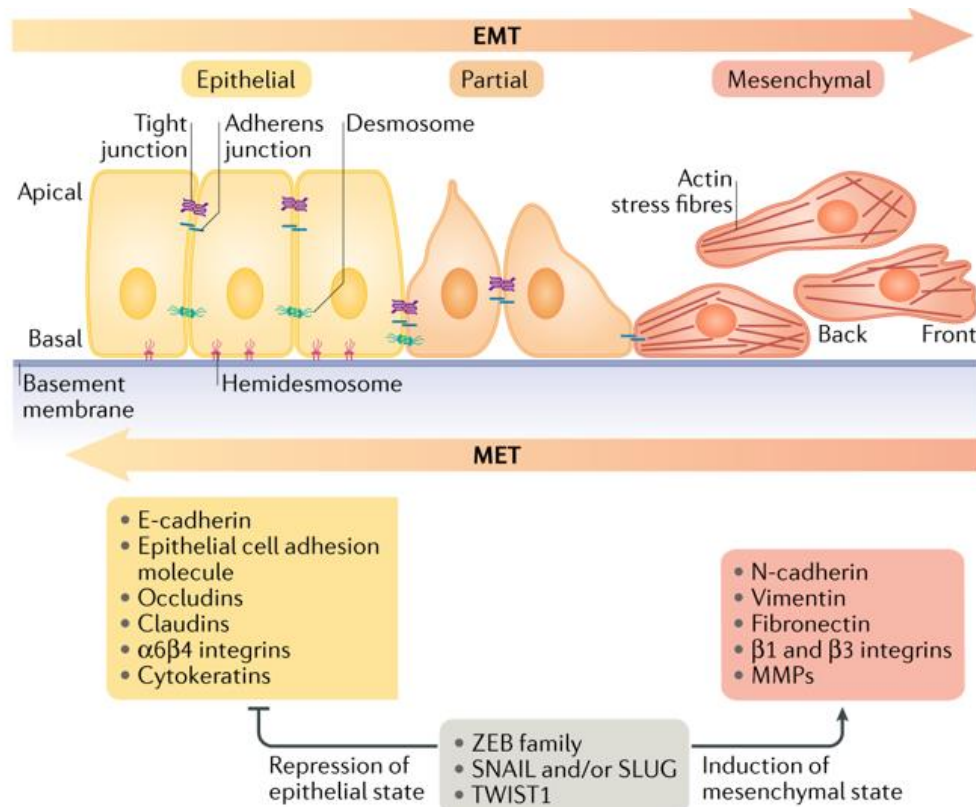


Figure 5. Outline of a typical Epithelial-Mesenchymal Transition program. Epithelial cells displaying an apical–basal polarity hold together by tight junctions, adherent junctions and desmosomes, and tethered to the underlying basement membrane by hemidesmosomes. These cells express molecules that are associated with the epithelial state and help to maintain cell polarity (yellow box). Induction of EMT leads to expression of EMT- inducing transcription factors (EMT-TFs): ZEB, SNAIL and TWIST, which inhibit the expression of genes associated with the epithelial state (yellow box) and, concomitantly, activate the expression of genes associated with the mesenchymal state (orange box). These modifications in gene expression result in cellular changes that include the disassembly of epithelial cell–cell junctions and the dissolution of apical–basal cell polarity. Mesenchymal cells display front- to-back polarity and an extensively reorganized cytoskeleton expressing a distinct set of molecules and EMT-TFs that promote and maintain the mesenchymal state. During EMT, cells become motile and acquire invasive capacities. EMT is a reversible process, and mesenchymal cells can revert to the epithelial state by undergoing MET. EMT and MET occur during normal development and during cancer progression. *Adapted from Dongre, A. & Weinberg, R.A. 2019¹.* Abbreviations: E-cadherin: Epithelial cadherin; EMT: Epithelial-Mesenchymal Transition; MET: Mesenchymal-Epithelial Transition; MMP: Matrix Metalloproteinase; N-cadherin: Neural cadherin; SLUG: Zinc Finger Proteins SNAI2; SNAIL: Zinc Finger Proteins SNAI1; TWIST: Twist-Related Protein 1; ZEB: Zinc Finger E-Box-Binding Homeobox.

The significance of EMT in tumor biology has become more widely appreciated⁵⁶. It has been reported that EMT plays a pivotal role in promoting tumor metastasis, conferring stem cell properties⁵⁷ and mediating resistance to traditional therapeutics in diverse preclinical model systems⁵⁶. Consequently, molecular processes that mediate EMT and their markers expression have been correlated with high-grade tumors and poor prognosis, supplying a diagnostic readout⁵². Overall, several studies connect EMT with advanced PCa and aggressive behavior in primary tumors, and it is not correlated with benign prostate tissue samples^{48,52}. Hence, understanding

cellular and molecular mechanisms underlying EMT in PCa could generate potential therapeutic strategies to prevent PCa related mortality^{49,52}.

1.3.1. The interplay between EMT and AR signaling

Despite improvements in understanding the outcome of EMT in PCa, the effect of ADT on EMT is still unclear⁵². Deregulation of androgen signaling is a pivotal event in PCa progression and recent studies have provided evidence associating AR signaling and EMT. Nevertheless, these studies have produced incongruent findings, with both activation and repression of AR signaling observed in relation with EMT^{34,55}.

On one hand, the androgens can directly impact the EMT process. Withal, the presence of AR-truncated isoforms, which are increased in CRPC, regulates the expression of EMT markers⁵⁵. The ARV7 has been shown to induce mesenchymal markers, such as ZEB1 and Vimentin⁴⁹. Conjointly, Zhu and Kyprianou⁵⁸ demonstrated that androgens suppressed E-cadherin expression and increased mesenchymal marker levels in PCa epithelial cells. DHT alone or in combination with Transforming Growth Factor- β (TGF- β), lead to a significant increase in SNAIL expression (mesenchymal marker) at both mRNA and protein levels in androgen-sensitive cell lines. These results suggest a crosstalk between the androgen axis and TGF- β signaling on *SNAIL* transcriptional activation⁵⁸. Similarly, Anose and Sanders⁵⁹ verified that *ZEB1* (mesenchymal marker) comprised distal ARE in the promoter. Subsequently, treatment with DHT induced endogenous *ZEB1* mRNA and protein levels in both AR-positive (22RV1) and AR-negative cell lines (PC-3) stably transfected for AR expression⁵⁹. Moreover, Wu and colleagues⁶⁰ published that DHT upregulated SLUG, which interacted directly with AR, leading to cooperative gene regulation and facilitating castration resistance *in vitro* and *in vivo*.

On the other hand, diverging from the aforementioned studies, EMT is promoted through the inhibition of AR signaling and, consequently, ADT is potentially responsible for more aggressive behavior in recurring PCa⁵². Sun and colleagues⁵⁶ verified *in vivo* and *in vitro* (LuCaP35 cell line) an increased mesenchymal phenotype and stem cell-like characteristics in response to castration. Androgen deprivation induced EMT by a negative feedback loop between AR and ZEB1⁵⁶. In the same way, Mooney *et al.*⁶¹ observed in androgen negative and positive cell lines that AR slightly upregulates the expression of ZEB1. Nevertheless, this effect was exclusively seen on distal ARE, which were bound to AR repressors in both AR-negative and positive cell lines, suggesting that AR

absence regulates ZEB1 expression⁶¹. Miao and associates⁴⁸ presented, in multiple PCa cell lines, patient tissues and clinical datasets, the presence of ARE in SNAIL promoter and the AR capability of repress SNAIL expression. In addition, a study in a patient-derived xenograft model reported that both N-cadherin and Vimentin become elevated after ADT⁶².

Collectively, these data indicate an intimate link between AR signaling and EMT, however, also suggesting that this relationship can vary widely in a context-dependent manner⁴⁸.

1.3.2. Multiple interactions of EMT in Prostate Cancer

It is crucial to delineate the biochemical, molecular, and genetic mechanisms underlying EMT development, metastization and subsequent resistance of cancer⁶³. Acquired therapeutic resistance reflects the processes occurring within the tumor itself, as well as the tumor microenvironment, specifically towards the emergence of a dedifferentiated phenotype⁵².

The tumor microenvironment is an established contributor to therapeutic resistance, enabling key signaling pathways that promote cancer cell survival, invasion, apoptosis arrest, as well as EMT⁵², through a paracrine/endocrine regulation⁴⁹. In a receptor-dependent manner, several extracellular signals are responsible for cell-cell communication, altering PCa cell behavior. Pathological EMT in tumor cells results from transcriptional reprogramming of abnormal survival signals via receptors, as for instance, Platelet-Derived Growth Factor Receptor (PDGFR), Fibroblast Growth Factor Receptor (FGFR) and TGF-Receptor; and regulatory kinases, such as PI3K, AKT, and Mammalian Target of Rapamycin (mTOR)^{64,65}.

In PCa, the association between the FGF family and EMT is consistently documented in the literature. A study using PCa cell lines verified that FGF2 increased the expression of mesenchymal markers, N-cadherin and Vimentin, and decreased epithelial marker, E-cadherin, leading to cell invasion⁶⁵. TGF- β is one of the best characterized EMT inducers in PCa. It can promote EMT through the induction of Vimentin, Fibronectin and EMT transcriptional factors (SLUG, SNAIL or ZEB1) and suppression of E-cadherin levels^{66,67}. TGF- β can initiate EMT by dissociating E-cadherin/catenin complexes from the actin cytoskeleton, via PI3K-Akt signaling. The PI3K-Akt signaling modulate adherent junctions through dephosphorylation of β -catenin. The latter is regulated by PTEN that suppresses phosphorylated-AKT (p-AKT) formation, blocking its inhibition on free β -catenin degradation. Actin stress fibers bind free β -catenin to form a bridge between cytoskeleton and E-cadherin, the cell-cell adhesion molecule. In cells with activated Ras/Extracellular Signal-Regulated

Kinase 1/2 (ERK), TGF- β is Smad-independent, resulting in a switch of PTEN, consequently, increasing levels of free β -catenin and p-AKT, culminating in a reduction of E-cadherin/catenin complex at adherent junctions^{30,35,45,64} (**Figure 6**). In sum, these processes result in diminished cell-cell adhesion and improvement of cell migration and invasion, accompanied by a dramatic change of cancer cell shape from epithelial-like to spindle-like morphology⁷⁰.

Ultimately, Raf Kinase Inhibitor Protein (RKIP) is another molecule that can inhibit EMT in cancer through the modulation of multiple proteins, being a pivotal key to therapeutic interventions⁷².

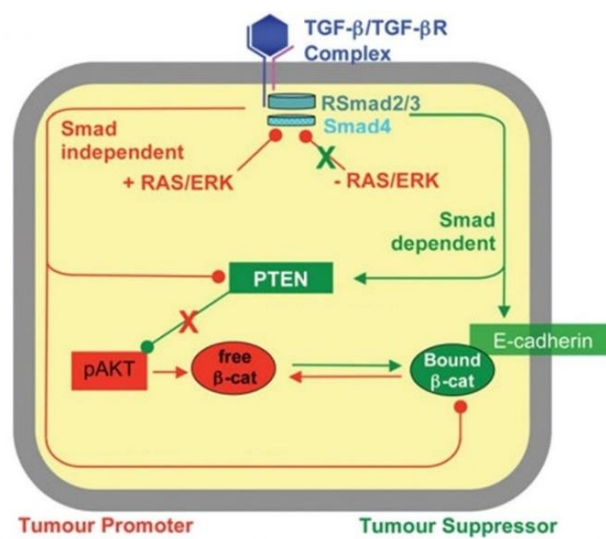


Figure 6. Ras/ERK activation switches TGF- β to tumor promoter via PTEN and AKT. Normal Smad-dependent TGF- β signaling occurs in cells without constitutive activation of RAS/ERK pathway to promote PTEN and E-cadherin expression, resulting in suppression of cell migration and proliferation. Upregulation of PTEN expression by TGF- β suppresses pAKT formation, blocking its inhibition of free β -catenin degradation. Actin stress fibers bind free β -catenin to form a bridge between cytoskeleton and E-cadherin, a cell–cell adhesion molecule. In cells with activated Ras/ERK, TGF- β is Smad-independent, resulting in a switch to PTEN and E-cadherin suppression, leading to increased levels of free β -catenin and pAKT that favor increased cellular migration. *Adapted from Stephen, J. et al. 2009⁶⁴.* Abbreviations: β -cat: β -catenin; E-cadherin: Epithelial Cadherin; ERK: Extracellular Signal-Regulated Kinase; pAKT: phosphorylated-Protein Kinase β ; PTEN: Phosphatase and Tensin Homolog; TGF- β : Transforming Growth Factor- β .

1.4. Raf Kinase Inhibitor Protein (RKIP)

RKIP is a member of a conserved group of proteins called Phosphatidylethanolamine-Binding Proteins (PEBP), firstly identified in the bovine brain⁷³. This protein (23kDa) can be cytosolic or an internal periplasmic membrane in varied tissues^{74–76}. Multiple functions have been assigned to RKIP

culminating in an increasing association with multiple diseases, Alzheimer's⁷⁷, pancreatitis⁷⁸, and cancer⁷⁹.

It has been reported that RKIP expression is commonly lost or downregulated in cancers⁷⁵. RKIP has a critical role in different survival signaling pathways (Raf-1/Mitogen-Activated Protein Kinase Kinase 1/2 (MEK)/ERK and NF- κ B)^{73,76} and in many important physiological processes (EMT)⁸⁰.

1.4.1. RKIP as a signaling modulator

At a molecular level, RKIP has a key function as an inhibitor of kinase activity⁷⁴. Yeung and colleagues⁷³ were the firsts to identify the suppressing effect of RKIP on Raf-1/MEK/ERK cascade, through a direct Raf kinase association (**Figure 7**). RKIP hinders kinase activity of Raf-1 by (i) dissociating Raf-1/MEK complex and acting as a competitive inhibitor of MEK phosphorylation⁸¹ and (ii) binding to the N-region of the Raf-1 kinase domain and inhibiting its activation⁸². RKIP is, considerably, a more abundant protein than Raf-1, raising the possibility that it may have additional intracellular targets⁸³.

Subsequently, it was revealed that RKIP modulates other signaling pathways as, for instance, the NF- κ B -cascade. RKIP is an antagonist of NF- κ B, repressing upstream kinases, the Inhibitory Kinase β Kinase (IKK) complex⁸⁴. In the absence of stimulating signals, the NF- κ B dimeric complex is retained in the cytoplasm at an inactive state bounded to Inhibitory Kinase β (IK β). In response to stimulatory signals, IK β is rapidly phosphorylated by IKK complex which contains two kinase subunits, IKK α and IKK β and a regulatory subunit IKK γ . The phosphorylated IK β will subsequently be ubiquitinated and degraded, releasing NF- κ B dimer for nuclear import. Other kinases are also involved in the phosphorylation and activation of IK β in response to specific stimuli, namely the NF- κ B-Inducing Kinase (NIK) and Transforming Growth Factor β -Activated Kinase 1 (TAK1). In mechanistic terms, RKIP physically inhibits upstream kinases, the IKK complex, NIK, and TAK1, consequently avoiding NF- κ B translocation to the nucleus meanwhile IK β is not degraded^{84,85} (**Figure 7**).

RKIP critical impact in Glycogen synthase kinase 3 β (GSK3 β) axis was first reported by Al Mulla and colleagues⁸⁶, showing that RKIP can physically interact with GSK3 β , preventing their phosphorylation at the inhibitory T390 residue by p38 MAPK. The p38 is activated under oxidative

stress, augmented by RKIP depletion⁸⁶ (**Figure 7**). GSK3 β is a suppressor of multiple oncogenic pathways, an impact on its activity culminates in an important influence in cancer progression⁸⁷.

RKIP itself can act as a substrate for kinases, and its phosphorylation can alter RKIP's binding properties⁷⁶. In brief, phosphorylation of Ser153 residue by Protein Kinase C (PKC) switches RKIP's binding from Raf-1 to G Protein-coupled Receptor Kinase 2 (GRK2), resulting in the activation of Protein Kinase A (PKA)⁸⁸. Published reports suggested that phosphorylated-RKIP act favorably on cancer progression by inhibiting GRK2, a negative regulator of G-protein Coupled Receptor (GPCR) and through releasing its negative control over Raf-1 kinase⁷⁶ (**Figure 7**). The mechanism by which

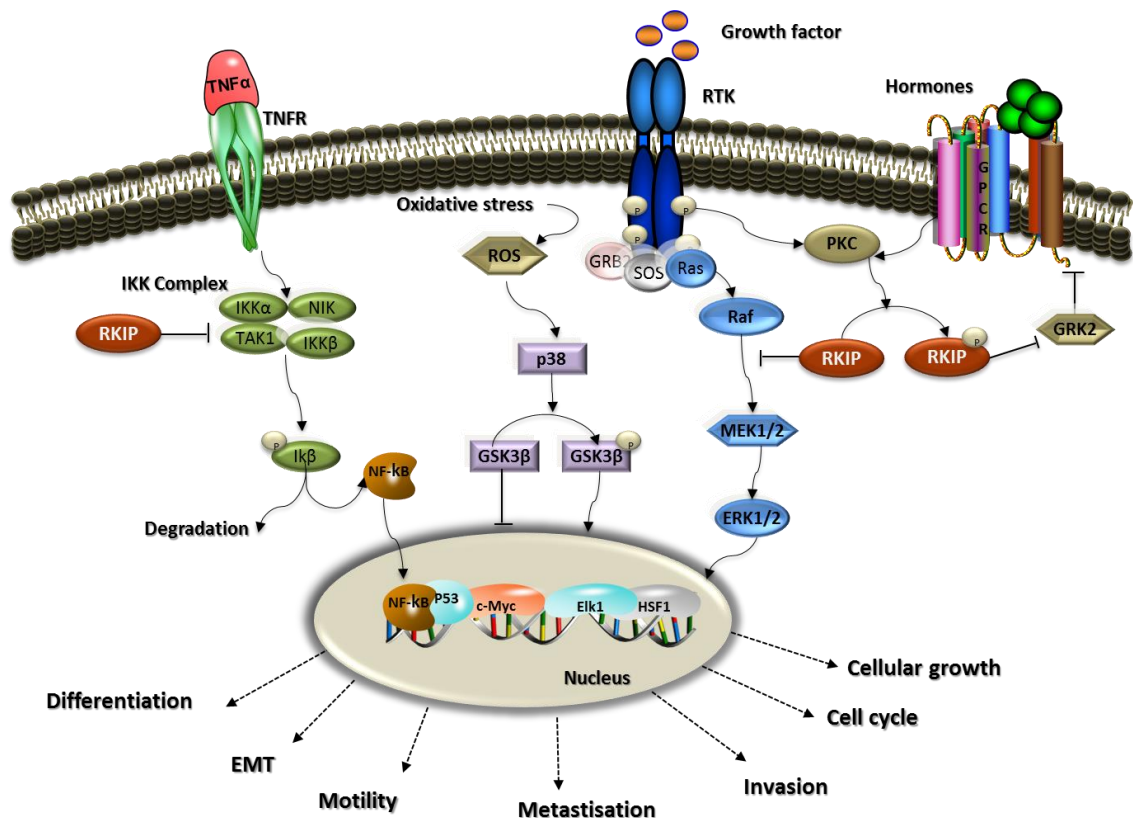


Figure 7. RKIP as a modulator of signaling pathways. On the right, RKIP binds to Raf-1 and prevents the phosphorylation of MEK by Raf-1. GPCRs are desensitized and internalized in response to phosphorylation by GRK2. After cells stimulation (e.g. growth factors), PKC phosphorylate RKIP at S153, inactivating RKIP as an inhibitor of Raf-1 and converting it into a GRK2 inhibitor. GPCR signaling can therefore persist. On the middle, depletion of RKIP augments oxidative stress– mediated activation of the p38 MAPK, which, in turn, inactivates GSK3 β by phosphorylating it at the inhibitory T390 residue, leading to transcription activation of several genes. On the left, RKIP binds to IKK complex preventing IK β phosphorylation and degradation which blocks translocation of NF κ - β to the nucleus. From Olga Martinho PhD thesis⁸⁹. Abbreviations: ERK: Extracellular Signal-Regulated Kinase 1/2; GPCR: G-protein Coupled Receptor; GRK2: G Protein-coupled Receptor; GSK3 β : Glycogen synthase kinase 3 β ; IK β : Inhibitory Kinase β ; IKK complex: Inhibitory Kinase β Kinase complex; MEK: Mitogen-Activated Protein Kinase Kinase 1/2; NF- κ β : Nuclear Factor kappa β ; NIK: NF- κ β -inducing kinase; PKA: Protein Kinase A; PKC: Protein Kinase C; p-RKIP: phosphorylated-Raf Kinase Inhibitor Protein; RKIP: Raf Kinase Inhibitor Protein; ROS: Reactive Oxygen Species; RTK: receptor tyrosine kinases; TAK1: Transforming Growth Factor β -Activated Kinase 1; TNFR: Tumor Necrosis Factor Receptor.

phosphorylation-switch occurs comprises a novel salt bridge that enables promotion or disruption of peptide interactions, providing specificity in protein interfaces⁸⁸.

RKIP's ability to shift tumor cells into a non-metastatic phenotype, by reprogramming kinase networks, represents a unique opportunity to understand molecular mechanisms of metastatic progression as well as to counteract these mechanisms as a therapeutic strategy^{75,76}.

1.4.2. Role of RKIP in EMT regulation

RKIP has been described as a powerful predictive biomarker for metastatic risk in patients, as well as a prognostic marker for overall survival in vastly solid tumors, namely, breast⁹⁰, cervical⁹¹, colorectal⁹², endometrial⁹³, esophageal⁹⁴, gastric⁹⁵, lung⁹⁶, pancreatic⁹⁷, prostate⁹⁸, and urothelial bladder⁹⁹ cancers, hepatocellular¹⁰⁰ and thyroid¹⁰¹ carcinomas, Acute Myeloid Leukemia (AML)¹⁰², Gastrointestinal Stromal Tumors (GISTs)¹⁰³, soft tissue sarcomas¹⁰⁴ and gliomas¹⁰⁵. Metastasis is the main cause of death for cancer patients and remains one of the most important, although least understood in tumors¹⁰⁶.

Metastization is defined as the formation of secondary tumors at different sites derived from the primary lesion. Succinctly, a metastatic cell escapes from a primary niche, enters the circulation, arrests in microcirculation, invades a different tissue compartment, and grows at that secondary site¹⁰⁶. Theoretically, it is possible to block metastasis through inhibition of a single gene that permits the accomplishment of a single step of the metastatic cascade^{106,107}. Evidence from studies supports this theory demonstrating that function loss of specific genes, named metastasis suppressor genes, is an important event in the progression of cancer aggressiveness¹⁰⁷.

RKIP antimetastatic properties have been attributed, among others, to its ability to modulate EMT through multiple mechanisms. RKIP can decrease EMT inducers, for instance, Ahmed and colleagues¹⁰⁸ showed that RKIP had a strong inverse correlation with TGF- β , through the decrement of TGF- β Receptor.

RKIP can inhibit the NF- κ B/SNAIL/YY1/PTEN circuit loop⁸⁰, intensively connected with EMT. In cancer cells, NF- κ B is frequently constitutively activated and regulates transcription of several target genes, including YY1 and SNAIL. In its turn, YY1 and SNAIL negatively regulate PTEN and RKIP. In contrast, RKIP inhibits NF- κ B, disrupting the loop, and PTEN represses both PI3K/AKT and NF- κ B pathways¹⁰⁹⁻¹¹² (**Figure 8**). Each gene products in the loop regulate survival, resistance

and metastasis signals. NF- κ B promotes EMT, cell migration and invasion, through induction of MMPs expression, as well by regulating directly or indirectly the transcription of key EMT modulators, SNAIL and TWIST, and cell adhesion molecules, Selectins and Integrins^{75,113}. SNAIL's main function is E-cadherin downregulation, nonetheless, it is also able to decrease expression of tight junctions components, Claudins and Occludins, and increase mesenchymal expression markers, Vimentin and Fibronectin⁸⁰. In addition, the PI3K/AKT pathway is generally constitutively hyperactivated in cancer development³⁶. This pathway regulates multiple cellular processes, including invasion, and migration, via inhibition of SNAIL degradation¹¹⁴, crosstalk with TWIST¹¹⁵ and upregulation of MMPs expression⁸⁹.

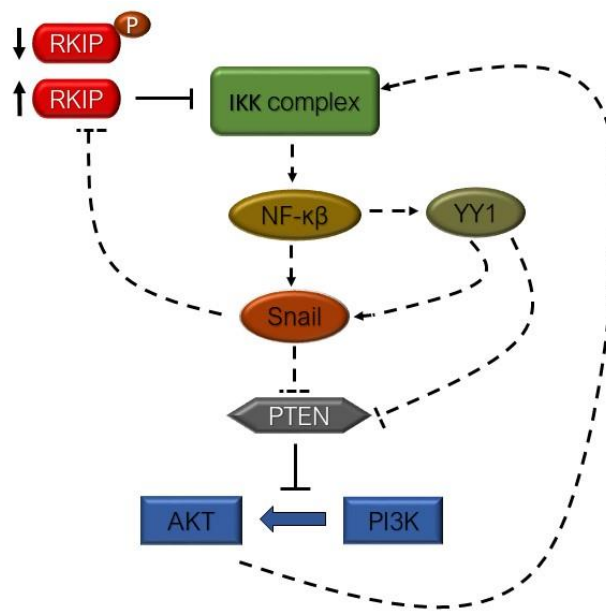


Figure 8. Schematic diagram representing the NF- κ B/SNAIL/YY1/RKIP/PTEN loop. In normal cells, RKIP potentiates its inhibitory activity on NF- κ B and its targets, likewise, PTEN inhibits PI3K/Akt pathway which controls NF- κ B via I κ K complex. In tumor cells, expression and activities of NF- κ B, SNAIL, YY1 are upregulated while those of RKIP and PTEN are downregulated. This results by a cease of inhibition in I κ K complex and AKT/PI3K, that upregulates NF- κ B which mediated an activation of its target SNAIL and YY1. In turn, SNAIL represses RKIP and YY1 represses PTEN and activates SNAIL^{109–112}. Abbreviations: AKT: Protein kinase β ; NF- κ B: Nuclear Factor kappa β ; PI3K: Phosphoinositide 3-kinase; p-RKIP: phosphorylated-Raf Kinase Inhibitor Protein; PTEN: Phosphatase and Tensin Homolog; RKIP: Raf Kinase Inhibitor Protein; SNAIL: Zinc Finger Proteins SNAIL1; YY1: Yin Yang 1.

Besides the NF- κ B/SNAIL/YY1/PTEN circuit, RKIP modulates other pathways that control EMT proteins. The previously described RKIP dissociation from GSK3 β derepresses their targets genes, such as β -catenin, SNAIL and SLUG, hence promoting EMT^{117,118}. RKIP also plays a distinct role in NOTCH1 activation during EMT and metastasis¹¹⁹. Upon activation by ligand binding, NOTCH1

releases its intracellular domain (NICD), which subsequently translocate into the nucleus, where together with other transcription factors it forms a transcriptional complex, responsible for the activation of EMT inducing genes¹²⁰. Noh *et al.*¹¹⁹ showed that RKIP overexpression resulted in significant NICD reduction with subsequent inhibition of NICD-mediated expression of EMT markers, including Vimentin, N-cadherin, and SNAIL.

On another perspective, overexpression of multiple MMPs is a hallmark of tumor invasion and metastasis, being correlated with the progression of EMT⁵³. In many cancers, the expression of some MMPs is negatively correlated with RKIP levels¹²¹, in addition, MMPs expression is induced by Raf/MEK/ERK, IKK/NF- κ B, and AKT cascades, identified as inhibited by RKIP^{122–125}. These results suggest that RKIP may constrain metastasis through inhibition of MMPs¹²¹.

1.4.3. RKIP in Prostate Cancer

In PCa, RKIP is inserted in a highly co-expressed gene cluster, that can be correlated with samples' phenotypes¹⁰⁸. Fu and colleagues in 2003⁹⁸, and afterward, Escara-Wilke and collaborators⁷⁶ in 2015, established the importance of RKIP in PCa metastases. RKIP expression is robust in 95% of non-neoplastic tissues and low in 48% of primary tumors and in 89% of metastatic tissues. Additionally, low RKIP expression in primary tumors predicted less than five years of recurrence-free survival compared to those that have high RKIP expression⁹⁸. Low RKIP expression is strongly correlated with an invasive phenotype *in vitro* and metastasis in a murine model, but does not hamper the growth of the primary tumor, culminating in the identification of RKIP as a PCa metastasis suppressor protein⁷⁶.

RKIP expression is regulated at multiple levels in PCa. At the epigenetic level, the RKIP promoter is frequently found methylated by Enhancer of Zeste Homolog 2 (EZH2)-mediated H3-K27-me3 and H3-K9-me3 motifs¹²⁷. At the transcriptional level, the direct binding of the SNAIL transcription factor to the RKIP promoter suppresses its transcription and expression¹²⁸; however, RKIP is positively regulated by direct binding of AR to a putative ARE in its promoter¹²⁹. Moreover, RKIP mRNA has been shown to be targeted by microRNAs, namely miR-543¹³⁰, as a suppressor, and miR-23a, stabilizing RKIP expression¹³¹.

Recent studies highlighted the importance of RKIP as a potential therapeutic modulator⁸⁰. It has been reported influences of RKIP in drug resistance in PCa. On one hand, Chatterjee and colleagues¹³² in 2004 demonstrated in PCa lines that RKIP ectopic expression sensitizes the cells

to DNA damage agent (9-nitrocamptothecin) undergoing apoptosis. Metastatic lines DU-145 and PC-3 have a low RKIP expression, which was significantly induced to high levels after DNA damage. Consequently, cells crossed rapidly from G1 phase to S phase of the cell cycle, followed by extensive cell death¹³². In contrast, LNCaP cells have an elevated RKIP expression, and after DNA damage RKIP levels remain unchanged and the cells undergo growth arrest¹³². The direct correlation of RKIP expression with the extent of apoptosis raises the possibility that upregulation of RKIP is one of the mechanisms that sensitizes cancer cells to apoptotic signals in response to DNA damage¹³². On the other hand, afterward, in 2008, Ignatoski and collaborators¹³³ demonstrated that RKIP expression was associated with Poly (ADP-ribose) polymerase (PARP) cleavage and apoptosis. In an *in vitro* study they presented that in a metastatic PCa line, C4-2B, the ionizing radiation induces RKIP expression, leading to apoptosis¹³³.

Finally, due to the key role of RKIP in the regulation of important intracellular signaling pathways, particularly the EMT, we expected that RKIP can be a putative modulator of PCa cells response to therapy. Although some reports exist assessing the influence of RKIP in the response to therapies of PCa cell lines, these studies did not evaluate the drugs most commonly used in PCa treatment, particularly the antiandrogenic drugs. As a final point, the correlation of androgens with RKIP in chemotherapy was never assessed in the literature.

CHAPTER 2. RESEARCH OBJECTIVE

Advanced PCa remains incurable¹¹ and is highly dependent in AR signaling to the development of therapy resistance²⁶. It is crucial to discover new target molecules as well as new predictive biomarker of therapy response for these patients.

Herein, we intended to dissect the role of RKIP and androgen stimulation in pharmacological response in PCa cell lines. To accomplish the goal, four PCa cell lines were characterized and one AR-positive (22RV1) cell line was further selected to Knockout (KO) RKIP expression. The purpose was to accomplish the subsequent aims:

AIM 1: Characterize PCa cell lines under androgen stimulation.

Assess the expression of AR, RKIP, and EMT proteins markers under androgen stimulation in all cell lines.

AIM 2: Determine the sensitivity of PCa cell lines to specific drugs.

Analysis of PCa cell lines' response to chemotherapeutic agents (Docetaxel and Cabazitaxel) and antiandrogen drugs (Abiraterone and Enzalutamide).

AIM 3: Dissect the role of androgens in RKIP and EMT proteins modulation .

Examine in an AR-positive cell line (LNCaP) whether DHT stimulation and antiandrogen drugs are able to modulate AR, RKIP, and EMT markers expression.

AIM 4: Explore the role of RKIP in pharmacological response in PCa.

Evaluation of RKIP role on the modulation of cells response to chemotherapeutic agents and antiandrogen drugs, as well as in the modulation of AR and EMT markers expression.

CHAPTER 3. MATERIALS AND METHODS

3.1. Cell lines and cell culture

Four cell lines were used to mimic PCa heterogeneity (**Table 1**). The 22RV1 (CRL-2505), LNCaP clone FGC (CRL-1740), PC-3 (CRL-1435) and DU-145 (HTB-81) were obtained from the American Type Culture Collection (ATCC, MD, USA), as previously described^{134,135}.

Table 1. General information regarding PCa cell lines. All cell lines are expanded in adherent culture and derived from human males. Presented is the general information of cell lines, namely, origin, AR and RKIP proteins expression^{136,137}. Abbreviations: AR: Androgen Receptor; Protein presence is positive (+) or negative (-); RKIP: Raf Kinase Inhibitor Protein.

Cell Lines	Metastatic	Local of Metastasis	Expression	
			AR	RKIP
22RV1	No	-	+	+
LNCaP	Yes (Low)	Left supraclavicular lymph node	+	+
DU-145	Yes	Bone	-	+ (Low)
PC-3	Yes	Brain	-	+ (Low)

The cell lines were expanded in adherent culture and maintained in Roswell Park Memorial Institute's Medium (RPMI, GIBCO, Invitrogen) and Dulbecco's Modified Eagle's Medium (DMEM, GIBCO, Invitrogen) exclusively for DU-145. Both mediums were supplemented with 10% of Fetal Bovine Serum (FBS, GIBCO, Invitrogen) and 1% of Penicillin/Streptomycin (P/S, GIBCO, Invitrogen). Cells grow in a humidified atmosphere at 37°C and 5% of CO₂ and the entire procedures involving cell lines' manipulation were performed under sterile conditions, operating in a cell culture hood.

3.2. Compounds

The drugs used in this project, namely Abiraterone (S112305, Selleckchem), Cabazitaxel (S302202, Selleckchem), Docetaxel (S114806, Selleckchem) and Enzalutamide (S125013, Selleckchem) were prepared as stock solutions in Dimethyl Sulfoxide (DMSO, Sigma-Aldrich) and stored at -20°C, as recommended by the manufacturer. For the experimental conditions, drugs were diluted in 10% of FBS culture medium, with the vehicle (DMSO) as a control. DHT (dihydrotestosterone, Selleckchem) was stored at -20°C and prepared in DMSO, as suggested by the manufacturer.

3.3. *In vitro* knockout of *RKIP*

A stable transfection causes modifications of cell properties, allowing the study of gene function and protein expression. The 22RV1 cell line was selected to accomplish a stable transfection using the Clustered Regularly Interspaced Short Palindrome Repeats (CRISPR) technology. This technique is an effective tool for introducing targeted loss function mutations at specific sites in the genome¹³⁸. The process functions as a co-transfection, operating in association with a Cas9 nuclease to knockout *RKIP*.

To accomplish the procedure two plasmids were used: one containing the Cas9, enclosing a Green Fluorescent Protein (GFP) gene, and the Homology-Directed Repair (HDR) plasmid, as a control carrying a Red Fluorescent Protein (RFP) and a puromycin resistance gene. The Cas9 can be programmed, through a synthetic single guide RNA (sgRNA), to induce a DNA Double-Strand Breaks (DSBs) at an exact genomic locus, in this case on *RKIP* gene^{138,139}.

The 22RV1 cells were plated into 6-well plates at a density of 80% per well in complete medium and allowed overnight to attach. In the next day, transfection was done according to the manufacturer's protocols: 6 μ L of FUGENE HD reagent (Promega) was mixed with 1 μ g of each plasmid (Cas9: sc-401270-KO-2 and HDR: sc-401270-HDR-2, Santa Cruz Biotechnology) and diluted in Reduced Serum Media (Opti-MEM, GIBCO, Invitrogen, Thermo Fisher) to reach a final volume for each solution of 100 μ L. The KO was performed by co-transfection with two plasmids and the control was transfected only with HDR plasmid (**Figure 9**). After 24 hours, the cells that were successfully transfected can be visualized at the microscope by fluorescence and started to be selected with 0.75 μ g/mL (minimal killing dose) of puromycin (Sigma-Aldrich).

The *RKIP* transfection efficacy was confirmed through Western Blot (WB) technique.

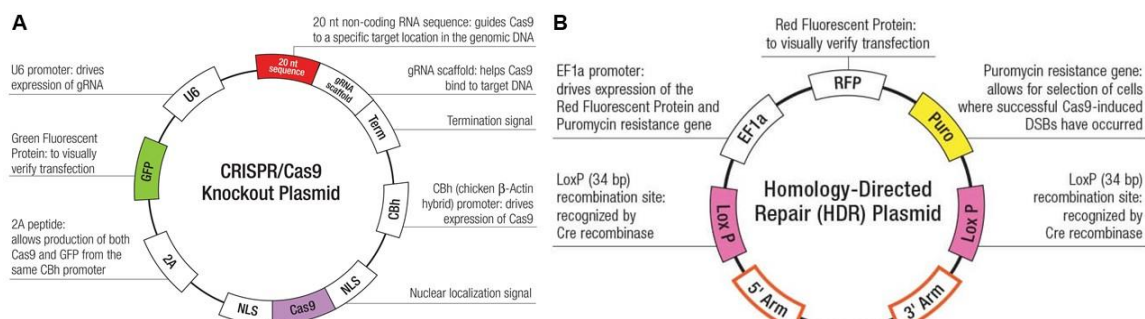


Figure 9. Detailed information of the vectors used to knockout *RKIP*. Plasmids produced by Santa Cruz Biotechnology: CRISPR/Cas9 Knockout Plasmid, enclosing a GFP gene and a sgRNA (A) and HDR Plasmid, carrying an RFP and a puromycin resistance genes, as well the homology arms (B). *Source:* <https://www.scbt.com/scbt/product/rkip-crispr-knockoutand-activation-products-h>. Abbreviations: HDR: Homology-Directed Repair GFP: Green Fluorescent Protein; RFP: Red Fluorescent Protein; sgRNA: single guide RNA.

3.4. Western Blot (WB)

The analytical technique used to separate and detect proteins was WB. Firstly, cells were plated in a 6-well plate at a density of 80% cells per well and allowed to adhere overnight. On the next day, cells were incubated for 24 hours with different conditions: 10 nM of DHT, 20 μ M of Enzalutamide and Abiraterone alone or in combination with 10 nM of DHT. The treatments were added in medium 0.5% FBS, which was used as a control. Subsequently, cells were scrapped with a lysis buffer (50 mM Tris, 150 mM Sodium Chloride (NaCl), 5mM Ethylenediamine Tetra-acetic Acid (EDTA), 1 mM Sodium Orthovanadate (Na_3VO_4), 10 mM Sodium Fluoride (NaF), 10 mM Sodium Pyrophosphate ($\text{Na}_4\text{P}_2\text{O}_7$), and 1% NP-40 plus protease inhibitors (Roche, Sigma-Aldrich). These procedures were performed at a low temperature, maintaining all solutions in ice. The cell lysate was centrifuged at 4°C, 13 000 rpm for 15 minutes. Proteins were quantified by Bradford reagent (Sigma-Aldrich) through spectroscopy and 40 μ g of each sample denatured at 98°C for 5 minutes, followed ice condensation for 1 minute. The prepared solutions were separated electrophoretically at 100V in standard 5% (Stacking) and 10% (Running) of Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). Afterward, proteins were transferred into a nitrocellulose membrane (Amersham, Life Sciences) in 25 mM Tris-base/glycine buffer (Bio-Rad). This transfer was performed in the Trans-Blot Turbo Transfer System at 25 V, 1 A for 30 minutes (Bio-Rad). Membranes were blocked with 5% skimmed milk in Tris-Buffered Saline /0.1% Tween Twenty (TBS-T) for 1 hour at Room Temperature (RT) and incubated overnight at 4°C with primary antibody. The primary antibodies, described in **Table 2**, were prepared with 0,5% Bovine Serum Albumin (BSA) in TBS-T. On the next day, membranes were washed with TBS-T and incubated for 1 hour at RT with the secondary antibody coupled to horseradish peroxidase (anti-rabbit 7074 and anti-mouse 7076, Cell Signaling), previously diluted at 1:2500 with 5% skimmed milk in TBS-T. Detection was performed with Enhanced Chemiluminescent Substrate (ECL-Western Blotting Detection Reagents, Bio-Rad) in the ChemiDoc™ XRS System (Bio-Rad). Protein expression was analyzed by Quantity One Software (4.6.8 Basic) and Tubulin was used as an internal control. Quantification of WB results using the band densitometry analysis was performed with Image J software, as previously described¹⁰⁵.

Table 2. Detailed information of the primary antibodies used in WB analysis. Primary antibodies were diluted with 0,5% BSA in TBS-T. Characteristics of each antibody used in WB: antigen, purification, dilution, and reference. Abbreviations: AR: androgen receptor; BSA: Bovine Serum Albumin; E-cadherin: Epithelial Cadherin; N-cadherin: Neural Cadherin; SLUG: Zinc Finger Proteins SNAIL2; SNAIL: Zinc Finger Proteins SNAIL1; TBS-T: Tris-Buffered Saline /0.1% Tween Twenty; p-RKIP: phosphorylated-Raf Kinase Inhibitor Protein; RKIP: Raf Kinase Inhibitor Protein.

Protein	Description	Dilution	Source	Reference
AR	Rabbit Monoclonal	1:1000	Abcam	108341
E-cadherin	Rabbit Monoclonal	1:1000	Cell Signaling	3195
SLUG	Rabbit Monoclonal	1:1000	Cell Signaling	9585
SNAIL	Rabbit Monoclonal	1:1000	Cell Signaling	3879
Vimentin	Rabbit Monoclonal	1:1000	Cell Signaling	5741
N-cadherin	Rabbit Monoclonal	1:1000	Cell Signaling	1311
p-RKIP (Ser 153)	Mouse Monoclonal	1:1000	Santa Cruz Biotechnology	135779
RKIP	Rabbit Monoclonal	1:2000	Cell Signaling	13006
α -Tubulin	Mouse Monoclonal	1:5000	Santa Cruz Biotechnology	23948

3.5. Half Maximal Inhibitory Concentration (IC_{50})

The response of PCa cell lines was tested regarding chemotherapy drugs commonly used in CRPC, namely Cabazitaxel and Docetaxel, as well as the second line antiandrogen drugs Abiraterone and Enzalutamide. To achieve the Half Maximal Inhibitory Concentration (IC_{50}) of these drugs for each cell line, a dose-response curve was performed by submitting cells to an increased range of drug's concentration, as previously described^{91,134,140}.

Initially, in a 96-well plate 5000 cells/well were plated for 22RV1 and DU-145 and 4000 cells/well for LNCaP and PC-3, in triplicate for each dose. After overnight adherence, cells were treated with different concentrations of Abiraterone (0, 5, 10, 20, 30, 40, 50, 60, 80, 90 and 100 μ M), Cabazitaxel (0, 0.1, 0.5, 5, 10, 20 and 500 nM), Enzalutamide (0, 5, 10, 20, 30, 40, 45, 50 and 60 μ M) or Docetaxel (0, 0.5, 1, 2, 5 and 10 nM) in culture medium supplemented with 10% of FBS, as described in Pinto *et al.*¹³⁴. Controls were treated with the vehicle, 1% of DMSO. After 72 hours of incubation, cellular viability was assessed with MTS (Promega) diluted in culture medium in a 1:10 ratio. Following 3 hours of MTS incubation, optical density was determined at 490 nm in Varioskan-Flash plate reader (Thermo Scientific). The results were expressed as viable cells relative to control (considered as 100% viability) and IC_{50} concentration was calculated by a nonlinear regression analysis using GraphPad Prism 7 (GraphPad Software, La Jolla, CA).

All experiments were done in triplicate, at least in three independent assays.

3.6. Statistical Analysis

The IC₅₀ results were presented as mean $\pm$ standard error of the mean (SEM) in graphical representations, and as means $\pm$ standard deviation (SD) in Tables.

Statistical analysis of IC₅₀ assay was performed using the GraphPad Prism 7 and the IBM SPSS Statistics 20 (SPSS Inc., Chicago, IL) software. Data from tumorigenic cell lines was tested for normality and variances homogeneity through the Shapiro-Wilk and Brown-Forsythe tests, respectively. Subsequently, One-way Analysis of Variance (ANOVA) and Tukey's test for multiple comparisons were applied. When normality was not verified, the equivalent non-parametric test, the Kruskal-Wallis H and Dunn's tests for multiple comparisons were used. Analysis regarding the RKIP modulated cell line (22RV1 HDR and CAS9) was done through the Shapiro-Wilk and Levene's tests, for normality and variances homogeneity, respectively, and next a Student's t-test with independent variables. If variances homogeneity was not observed, an adjustment through the Welch-Satterthwaite method was made. Differences were considered as statistically significant for *p*-values below 0.05.

CHAPTER 4. RESULTS

4.1. Characterization of Prostate Cancer cell lines

4.1.1. Effect of androgen stimulation in RKIP and EMT proteins expression

The first aim of this work was to determine whether androgen stimulation could be associated with RKIP expression and EMT modulation in PCa lines. For that purpose, the cell lines were characterized for RKIP, AR and EMT markers expression under 10 nM DHT stimulation for 24 hours. The expression levels of all the studied proteins, as the effect of DHT on prostate cell lines, is well known from the literature^{51, 49, 123, 136, 137}, being this only a literature validation experiment, was done only once (**Figure 10**).

As observed in **Figure 10A**, all cell lines expressed RKIP. The most metastatic cell lines PC-3 and DU-145 were the ones with the lowest RKIP levels, whereas the low metastatic and primary tumor cell lines, LNCaP and 22RV1 respectively, presented elevated levels of this protein. After DHT stimulation, RKIP levels decreased in DU-145 and LNCaP cell lines (**Figure 10B**). Moreover, LNCaP and 22RV1 were shown to be positive for AR, the second band (80 kDa) observed in each cell line representing an AR splice variant, ARV7. After androgen stimulation, an increment of AR and ARV7 in LNCaP was perceived; notwithstanding, for 22RV1, AR expression was not significantly affected (**Figure 10B**).

Regarding markers of the epithelial phenotype, E-cadherin expression was analyzed, being high for LNCaP, 22RV1, and DU-145 and low for PC-3. Additionally, in DU-145 this epithelial protein appeared to be downregulated under DHT stimulation; however, it seems no to be significant. For mesenchymal markers, SNAIL and Vimentin expression were evaluated. The SNAIL expression was positive in LNCaP and DU-145, and upon androgen stimulation it was upregulated in DU-145. Vimentin levels were present for DU-145 and PC-3, decreasing their levels after androgen stimulation in both cell lines, mainly in DU-145 (**Figure 10B**).

In the western blot presented in **Figure 10A**, cell lines with AR expression were shown to have elevated RKIP levels, increased epithelial markers (E-cadherin) and decreased mesenchymal markers (Vimentin). Regarding the effects of androgen stimulation in proteins expression, AR was only upregulated in LNCaP and RKIP expression seems decreased in DU-145 and LNCaP cell lines.

Moreover, DHT stimulation in DU-145 modulate the mesenchymal phenotype, through Vimentin and SNAIL expression.

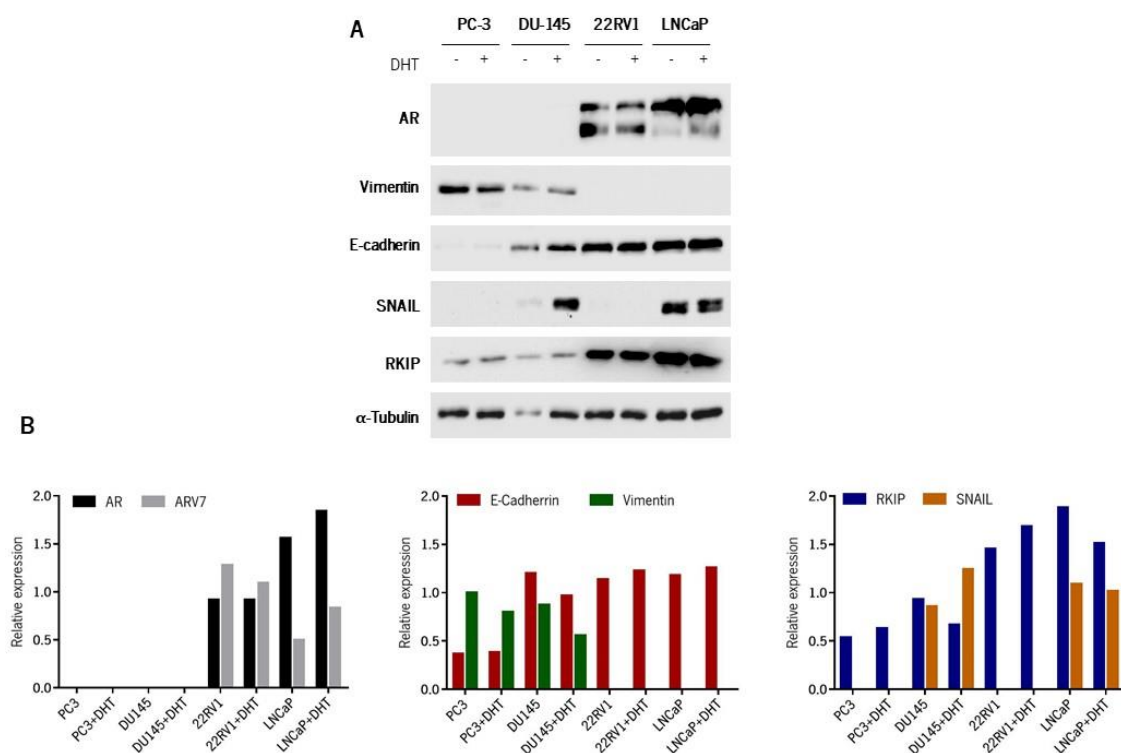


Figure 10. Characterization of EMT markers, RKIP, and AR expression in PCa cell lines, PC-3, DU-145, LNCaP, and 22RV1 under DHT stimulation (10 nM for 24 hours). PCa cell lines, PC-3, DU-145, 22RV1 and LNCaP were stimulated for 24 hours with 10 nM of DHT in 0.5% FBS culture medium (control condition). **(A)** Representative western blot for the analysed proteins: AR (100 kDa), E-cadherin (140 kDa), Vimentin (57 kDa), SNAIL (29 kDa) and RKIP (23 kDa), α -Tubulin (55 kDa) was a loading control. **(B)** Quantification of WB results, using the band densitometry analysis, was performed with Image J software. For relative protein expression results are shown as the ratio between the proteins and α -Tubulin. Abbreviations: AR: Androgen Receptor; E-cadherin: Epithelial Cadherin; Presence (+) or absence (-) of stimulation; SNAIL: Zinc Finger Proteins SNAI1; RKIP: Raf Kinase Inhibitor Protein. The experiment corresponds to a n=1.

4.1.2. Determine the sensitivity of PCa cell lines to chemotherapy

The following step was to understand whether AR and RKIP expression are correlated with chemotherapy response. The IC_{50} of all cell lines was determined by *in vitro* cytotoxic assays to antiandrogen drugs, Abiraterone, and Enzalutamide, and chemotherapeutic agents, Docetaxel and Cabazitaxel (Figure 11, Figure 12, and Table 3).

In Figure 11 a representative dose-response curve for each cell line and drug is presented, conjointly, the respective IC_{50} is reported in Table 3 and graphically presented in Figure 12 in which was performed a statistical analysis to compare the IC_{50} of each cell line.

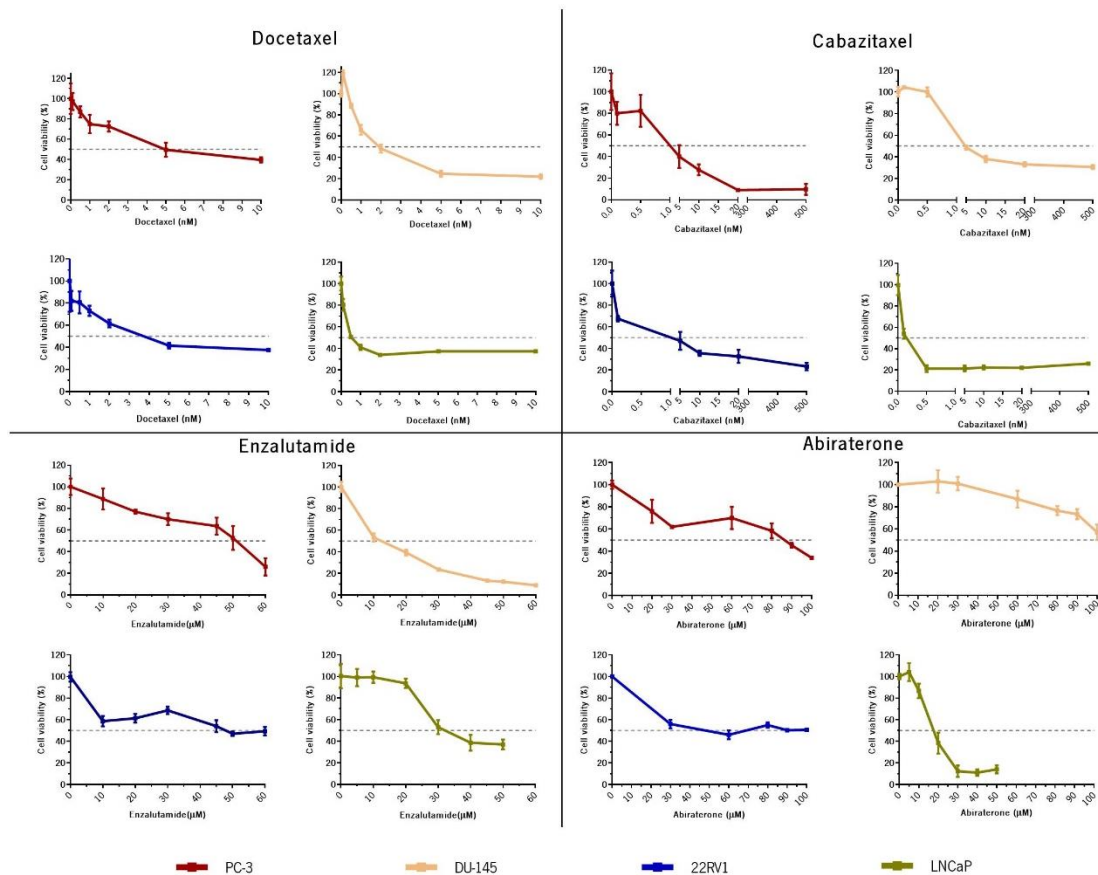


Figure 11. Representative assay of viability for PCa cell lines exposed to increasing concentrations of Docetaxel, Cabazitaxel, Enzalutamide, and Abiraterone by 72 hours. Each cell line was treated with different concentrations of each drug in culture medium supplemented with 10% of FBS and controls were treated with the vehicle (1% of DMSO) for 72 hours. MTS assay was used to measure cell viability and results were expressed in % of cell viable relative to the control. The assays were carried out in triplicate and in at least three independent assays that were presented as means $\pm$ SEM.

For Docetaxel treatment (**Figure 12A**, One-way ANOVA; $F(3,10) = 34.6$, $p < .001$, $\eta_p^2 = .91$), PC-3 was the most resistant, and statistically different from LNCaP ($p < .0001$), DU-145 ($p < .0001$) and 22RV1 ($p = .006$). Moreover, 22RV1 was the second most resistant and significantly different from LNCaP ($p = .003$), which was the most responsive. Regarding Cabazitaxel data (**Figure 12B**, One-way ANOVA; $F(3,9) = 8.3$, $p = .006$, $\eta_p^2 = .73$), PC-3 had the highest IC_{50} , statistically different from LNCaP ($p = .013$), 22RV1 ($p = .013$) and DU-145 ($p = .009$). Considering the chemotherapeutic agents, the most resistant cell line was PC-3 and the most sensitive LNCaP, however, for Docetaxel, 22RV1 was the second more resistant.

Analyzing the antiandrogen drugs, for Enzalutamide treatment (**Figure 12C**, Kruskal-Wallis H; $\chi^2(3) = 11.9$, $p = .008$) PC-3 and 22RV1 were the most resistant cell lines, both significantly different from DU-145 ($p = .019$, $p = .035$, respectively), which is the most sensitive one. For Abiraterone (**Figure 12D**, One-way ANOVA; $F(3,8) = 14.4$, $p < .001$, $\eta_p^2 = .84$) PC-3 and DU-145 had the highest

percentage of cellular viability, being both statistically different from LNCaP ($p=.010$, $p=.002$, respectively) and 22RV1 ($p=.050$, $p=.008$, respectively). Overall, for Abiraterone, PC-3 and DU-145 were the most resistant and 22RV1 and LNCaP the most responsive cell lines. However, for Enzalutamide, the most responsive cell lines were DU-145 and LNCaP.

To conclude, IC_{50} levels were highest for PC-3 and lowest for LNCaP, which corroborates the initial prediction that cell lines with low or absent levels of both RKIP and AR would be less responsive. Nevertheless, it was expectable a better response to Docetaxel and Enzalutamide treatment in 22RV1 cell line, and unexpectedly DU-145 (AR-negative cell line) presented a higher sensitivity to Enzalutamide, a drug that targets AR.

Table 3. IC_{50} values for Docetaxel, Cabazitaxel, Enzalutamide, and Abiraterone in PCa cell lines. IC_{50} was calculated by nonlinear regression and expressed as mean $\pm$ SD of, at least, three independent assays (n) performed in triplicate.

	Docetaxel (nM)	Cabazitaxel (nM)	Enzalutamide (μ M)	Abiraterone (μ M)
PC-3	12.4 $\pm$ 1.90 (n=3)	520.4 $\pm$ 335.3 (n=3)	58.4 $\pm$ 15.2 (n=3)	83.7 $\pm$ 13.4 (n=3)
DU-145	3.20 $\pm$ 0.90 (n=4)	4.54 $\pm$ 2.32 (n=4)	12.2 $\pm$ 5.54 (n=5)	103.1 $\pm$ 8.14 (n=3)
22RV1	6.81 $\pm$ 2.85 (n=3)	1.11 $\pm$ 0.50 (n=3)	54.4 $\pm$ 17.3 (n=3)	36.7 $\pm$ 31.9 (n=3)
LNCaP	0.76 $\pm$ 0.24 (n=4)	0.005 $\pm$ 0.004 (n=3)	31.3 $\pm$ 3.28 (n=4)	19.1 $\pm$ 4.79 (n=3)

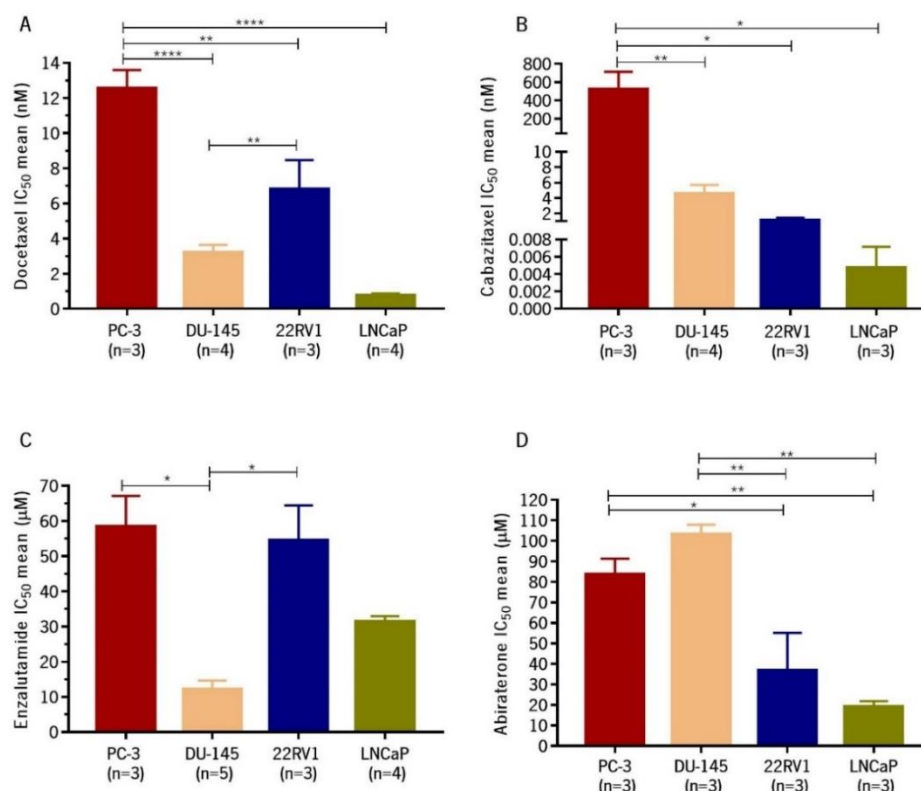


Figure 12. Comparative analysis of IC₅₀ values for Docetaxel (A), Cabazitaxel (B), Enzalutamide (C), and Abiraterone (D) treatments to PCa cell lines. The IC₅₀ was calculated by a nonlinear regression and expressed as the mean $\pm$ SEM of, at least, three independent assays (n) performed in triplicate. Statistical analysis was performed by a one-way ANOVA. *p<.05; **p<.01; ***p<.001; ****p<.0001.

4.2. Role of androgens in RKIP and EMT proteins modulation

To explore the modulation of AR, EMT pathway and RKIP expression in response to androgen stimulation and antiandrogen therapy, we choose the most AR responsive cell line the LNCaP. For that purpose, the LNCaP cell line was incubated for 24 hours in three conditions: Vehicle (control), Enzalutamide (20 μ M) and Abiraterone (20 μ M), alone or in combination with 10 nM of DHT.

In **Figure 13**, by the WB results presented, it was possible to see an increment on AR levels in the DHT treatment conditions, as expected. Additionally, enzalutamide was the only drug able to decrease AR basal expression levels. Regarding p-RKIP levels, it was similar in all conditions, although RKIP expression seems to slowly decrease in all experimental conditions. In agreement, as p-RKIP is associated to its loss of function, an increase in RKIP total (functional form) levels was observed mainly upon antiandrogens treatment.

Analyzing the EMT markers, the expression of E-cadherin, an epithelial marker, was identical in all conditions. The mesenchymal marker SLUG was upregulated upon androgen stimulation, being both drugs able to reduce these DHT-induced upregulations of SLUG. Contradictorily, the

mesenchymal marker SNAIL was downregulated in all experimental conditions, which fits well with the increased expression level of RKIP (Figure 13).

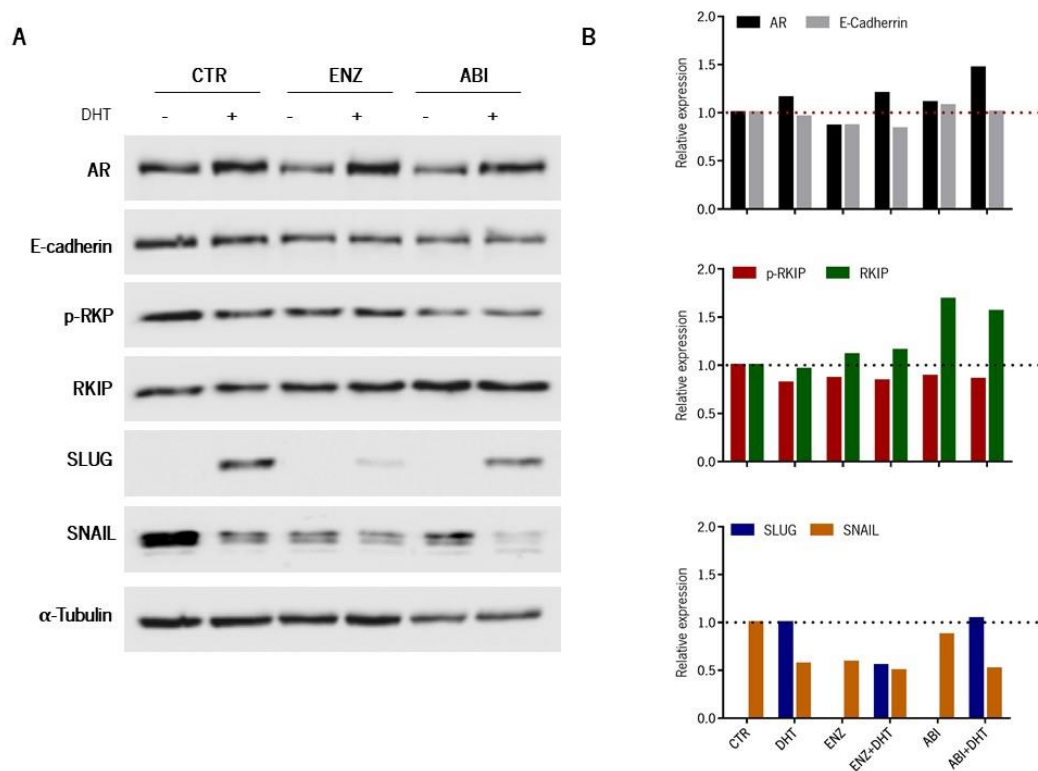


Figure 13. Characterization of EMT markers, RKIP, and AR expression in LNCaP cell line under DHT (10nM), Enzalutamide (20 μ M), and Abiraterone (20 μ M) treatment for 24 hours. (A) LNCaP cell line was incubated for 24 hours with the antiandrogen drugs: Enzalutamide 20 μ M isolated or simultaneously with DHT (10nM) in culture medium of 0.5% FBS and Abiraterone 20 μ M isolated or simultaneously with DHT (10nM) in culture medium of 0.5% FBS. As controls were used DHT 10nM in culture medium of 0.5% FBS and an incubation in medium of 0.5% FBS. The analysed proteins were AR (100 kDa), E-cadherin (140 kDa), SLUG (30 kDa), SNAIL (29 kDa), p-RKIP (22 kDa) and RKIP (23 kDa), α -Tubulin (55 kDa) was a loading control. **(B)** Quantification of western blot results, using the band densitometry analysis, was performed with Image J software. For relative protein expression results are shown as the ratio between the proteins and α -Tubulin, and then calibrated to the control conditions (considered as 1). Abbreviations: ABI: Abiraterone; AR: Androgen Receptor; E-cadherin: CTR: Control; Epithelial cadherin; ENZ: Enzalutamide; Presence (+) or absence (-) of stimulation; SLUG: Zinc Finger Proteins SNAI2; SNAIL: Zinc Finger Proteins SNAI1; p-RKIP: phosphorylated-Raf Kinase Inhibitor Protein; RKIP: Raf Kinase Inhibitor Protein. The experiment corresponds to a n=1.

Finally, we should emphasize that the experiment was done only once, since all goes as expected accordingly to literature, such as DHT stimulated AR and SLUG expression⁵⁵ and antiandrogens slightly decrease AR expression in unstimulated conditions^{49,55}. Thus, we can conclude with some confidence that in the LNCaP cell line androgen stimulation seemed to have contradictory effects in the mesenchymal phenotype, through downregulation of SNAIL and

upregulation of SLUG. Particularly the SLUG expression was dependent on androgen stimulation, highlighting the hypothesis that SLUG is considerably regulated by AR pathway.

4.3. RKIP role in Prostate Cancer cell lines response to therapy

4.3.1. Genetic modulation of RKIP in 22RV1 cell line and response to therapy

To explore the RKIP role in PCa cells' response to chemotherapy, the CRISPR/Cas9 technology was used to Knockout RKIP in the 22RV1 cell line, previously identified as positive for RKIP and AR (**Figure 10**). To validate the transfection, a WB analysis was performed and, as expected, a strong band (23 kDa) corresponding to RKIP expression can be identified in the HDR control cells, while the same was completely abolished in CAS9 transfected cells (**Figure 14**).

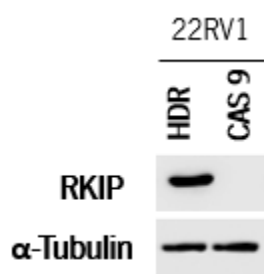


Figure 14. Analysis of RKIP expression in the transfected cell line 22RV1 through WB. The 22RV1 cell line was transfected with CRISPR/Cas9 technology to eliminate RKIP expression. The HDR is the control, transfected with the HDR plasmid, and CAS9 has the KO, co-transfected with HDR and Cas9 plasmids. WB presents RKIP (23 kDa) and α-Tubulin (55 kDa) as loading control. Abbreviations: HDR: Homology-Directed Repair; RKIP: Raf Kinase Inhibitor Protein. The experiment corresponds to a n=1.

To understand whether RKIP expression is correlated with chemotherapy response, we used *in vitro* cytotoxic assays to assess the effect of antiandrogen drugs, Abiraterone, and Enzalutamide, and chemotherapeutic agents, Docetaxel and Cabazitaxel in this particular model (**Figure 15**, **Figure 16**, and **Table 4**).

In **Figure 15** a response curve of 22RV1 HDR and CAS9 cells for each drug is represented, with the respective IC₅₀ values reported in **Table 4** and graphically presented in **Figure 16**, in which a statistical analysis was performed to compare the IC₅₀ of HDR and CAS9 cells.

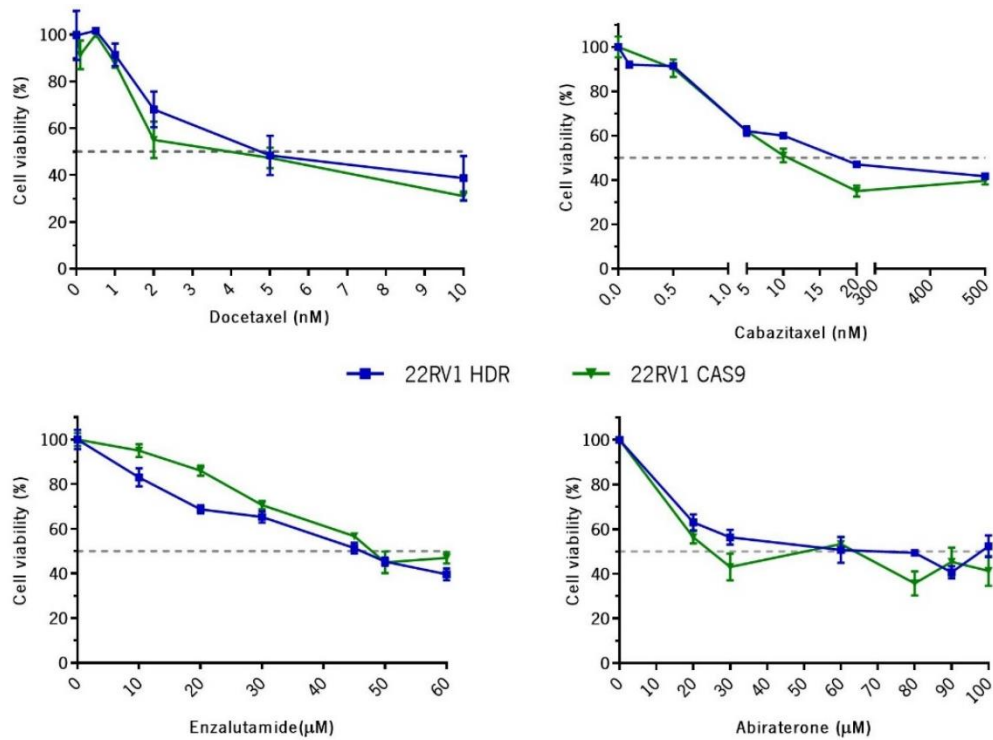


Figure 15. Representative viability assay for 22RV1 HDR and CAS9 cells exposed to increasing concentrations of Docetaxel, Cabazitaxel, Enzalutamide, and Abiraterone during 72 hours. 22RV1 HDR and CAS9 cells were treated with different concentrations of each drug in culture medium supplemented with 10% of FBS and controls were treated with the vehicle (1% of DMSO) for 72 hours. MTS assay was used to measure cell viability and results were expressed in % of cell viability relative to the control. The assays were carried out in triplicate, at least three times independently, and were presented as means $\pm$ SEM. Abbreviations: HDR: Homology-Directed Repair.

Analyzing **Figure 16**, it can be observed for Docetaxel treatment (**Figure 16A**) that statistically differences between HDR and CAS9 cells were not achieved ($t(10) = 1.41$, $p = .19$, $d = .77$). Nevertheless, a tendency for less cellular viability in CAS9 cells was supported by the medium effect size ($0.50 < d < 0.80$). For Cabazitaxel (**Figure 16B**) CAS9 cells were significantly more sensitive in comparison to the control HDR cells ($t(7) = 6.26$, $p = .0004$, $d = 4.30$). Regarding Enzalutamide (**Figure 16C**) was observed a similar response of HDR and CAS9 cells ($t(3,3) = .36$, $p = .74$, $d = .10$). Finally, for Abiraterone (**Figure 16D**) there were no statistically significant differences between the groups ($t(5) = 1.94$, $p = .11$, $d = 1.48$), although a propensity for higher sensitivity in CAS9 cells was detected, reinforced by the high effect size ($d < .80$).

In conclusion, and in contrast to what we had hypothesized, RKIP absence seems to increase the sensitivity of 22RV1 cell line to Docetaxel, Cabazitaxel and Abiraterone treatment, and had no influence in the response to Enzalutamide.

Table 4. IC₅₀ values for Docetaxel, Cabazitaxel, Enzalutamide, and Abiraterone in 22RV1 HDR and CAS9 cells. The IC₅₀ values were calculated by nonlinear regression and expressed as mean ± SD of, at least, three independent assays (n) performed in triplicate. Abbreviations: HDR: Homology-Directed Repair.

		Docetaxel (nM)	Cabazitaxel (nM)	Enzalutamide (μM)	Abiraterone (μM)
22RV1	HDR	6.35±3.81 (n=5)	41.8±5.66 (n=4)	45.3±1.99 (n=4)	60.4±20.5 (n=3)
	CAS9	4.06±1.77 (n=7)	15.1±7.83 (n=5)	46.9±8.60 (n=4)	30.2±20.3 (n=4)

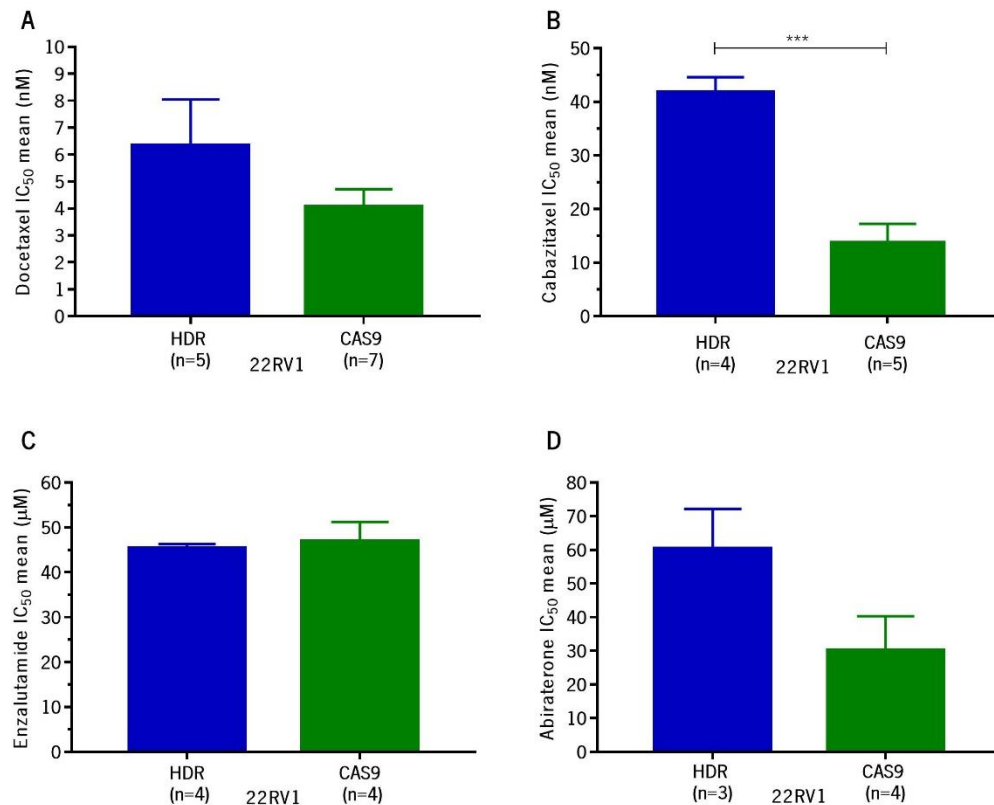


Figure 16. Comparative analysis of IC₅₀ values in 22RV1 HDR and CAS9 cells for Docetaxel (A), Cabazitaxel (B), Enzalutamide (C), and Abiraterone (D) treatments. IC₅₀ values were calculated by a nonlinear regression and expressed as the mean ± SEM of, at least, three independent assays (n) performed in triplicate. Statistical analysis was performed by a Student's t-test. *p<.05; **p<.01; ***p<.001. Abbreviations: HDR: Homology-Directed Repair.

4.3.2. Effect of RKIP in AR and EMT proteins expression

The last aim was to study the effect of RKIP in EMT proteins expression before and after androgens manipulation (induction and inhibition). For that purpose, 22RV1 HDR and CAS9 cells were incubated for 24 hours in three conditions: Vehicle (control), Enzalutamide (20 μM) and Abiraterone (20 μM), alone or in combination with 10 nM of DHT (Figure 17). In Figure 18 is presented the quantification of the Figure 17 WB.

Analysing the results, concerning RKIP positive cells (HDR), it was possible to observe that p-RKIP levels were slightly decreased in antiandrogen conditions, as it happened for LNCaP cells (Figure 13), however, and in contrast to the findings for LNCaP cells, RKIP total levels decreased in all experimental conditions (**Figure 18 and 17**).

Concerning AR expression, it was slightly increased in HDR cells for all conditions containing DHT, with no inhibitory effects observed upon antiandrogens treatment. The same tendency was observed for CAS9 transfected cells, with the difference that RKIP KO cells presented significantly higher AR expression levels in all conditions when compared to the RKIP expressing ones (**Figure 18 and 17**).

Regarding the EMT proteins, SLUG is expressed at basal conditions in this cell line, in contrast to LNCaP, even so, a discrete increase of its expression was observed in all experimental conditions, mainly those with DHT stimulation, for both HDR and CAS9 cells. Exceptionally, SLUG was decreased upon Abiraterone plus DHT in the cells with RKIP loss. Concordantly, SNAIL, another mesenchymal marker, was only expressed in HDR cells upon antiandrogen drug exposure. Moreover, with RKIP depletion the control levels of SNAIL were increased in all condition comparatively to HDR cells. Lastly, N-cadherin expression was unaffected in HDR cells, although in CAS9 cells was increased upon androgens stimulation, being Abiraterone able to control DHT-induced levels of N-cadherin. For the epithelial marker E-cadherin, its seems to be upregulated upon Abiraterone treatment in HDR cells, whereas, in CAS9 cells the Abiraterone alone and combined with DHT downregulated E-cadherin (**Figure 18**).

To conclude, in the 22RV1 cell line RKIP was downregulated under androgen and antiandrogen treatments. Moreover, SNAIL levels were positively regulated under Abiraterone treatment. Upon RKIP depletion an upregulation of SNAIL basal expression occurred, and DHT exerted a strong influence, increasing N-cadherin and SLUG expression. Regarding the drug effects, under RKIP KO, in general, ARV7 and SNAIL levels were augmented, in addition, Abiraterone decreased E-cadherin expression. However, due to technical problems with the western blots execution it was not possible to validate these results, being mandatory to increase the number of reproducible assays to have sure about the conclusions we were taken in this section.

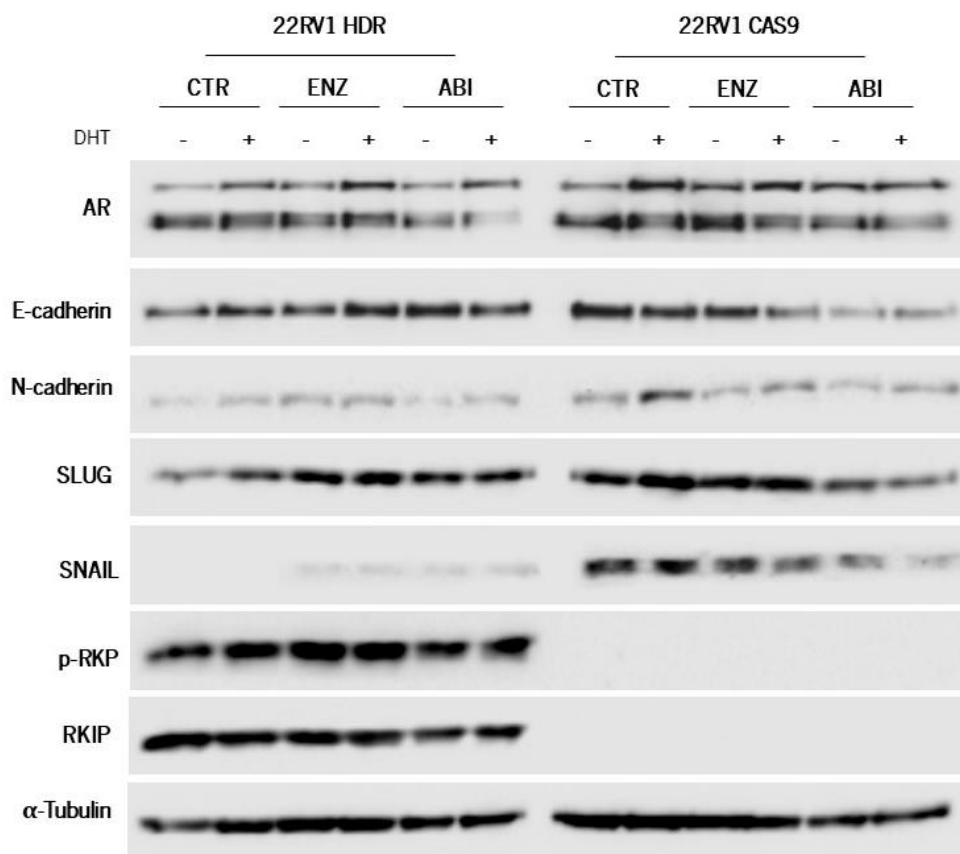


Figure 17. Comparison of EMT markers and AR expression in 22RV1 HDR and CAS9 cells under DHT (10 nM), Enzalutamide (20 μ M), and Abiraterone (20 μ M) treatments for 24 hours. 22RV1 HDR and CAS9 cells were incubated for 24 hours with the antiandrogen drugs: Enzalutamide 20 μ M isolated or simultaneously with DHT (10 nM) in culture medium of 0.5% FBS and Abiraterone 20 μ M isolated or simultaneously with DHT (10 nM) in culture medium of 0.5% FBS. As controls were used DHT 10nM in culture medium of 0.5% FBS and an incubation in medium of 0.5% FBS. Proteins analysed were AR (100 kDa), E-cadherin (140 kDa), N-cadherin (140 kDa), SLUG (30 kDa), SNAIL (29 kDa), p-RKIP (22 kDa), RKIP (23 kDa) and α -Tubulin (55 kDa) as loading control. Abbreviations: ABI: Abiraterone; AR: Androgen Receptor; CTR: Control; DHT: Dihydrotestosterone; E-cadherin: Epithelial cadherin; ENZ: Enzalutamide; HDR: Homology-Directed Repair; Presence (+) or absence (-) of stimulation; N-cadherin: Neural cadherin; SLUG: Zinc Finger Proteins SNAI2; SNAIL: Zinc Finger Proteins SNAI1; p-RKIP: phosphorylated-Raf Kinase Inhibitor Protein; RKIP: Raf Kinase Inhibitor Protein. This result is a representative experiment from at least three independent assays.

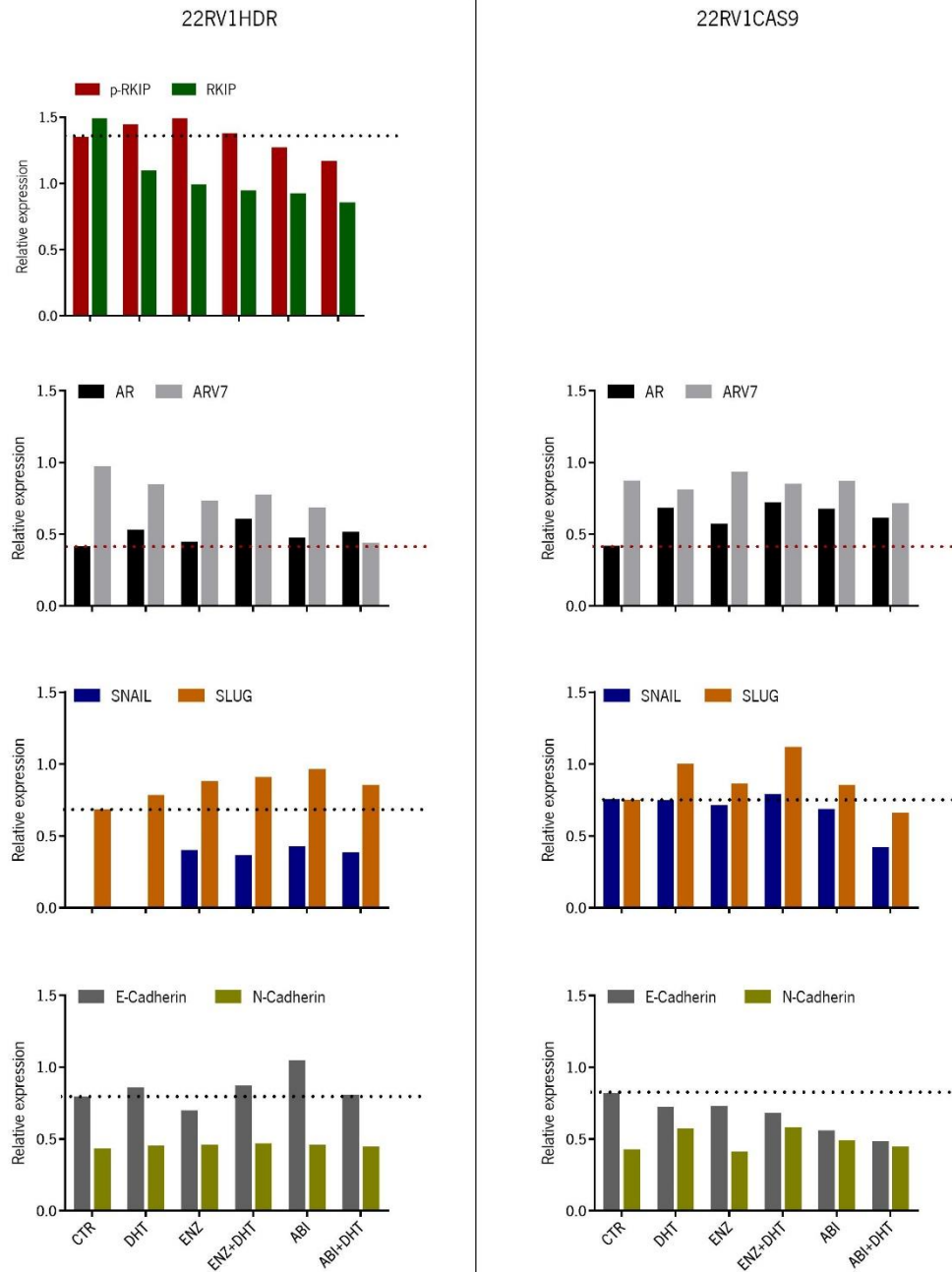


Figure 18. Quantification* of EMT markers and AR expression in 22RV1 HDR and CAS9 cells under DHT (10 nM), Enzalutamide (20 μ M), and Abiraterone (20 μ M) treatments for 24 hours. Quantification of WB from Figure 18*, using the band densitometry analysis, was performed with Image J software. For relative protein expression results are shown as the ratio between the proteins and α -Tubulin. Abbreviations: ABI: Abiraterone; AR: Androgen Receptor; CTR: Control; DHT: Dihydrotestosterone; E-cadherin: Epithelial cadherin; ENZ: Enzalutamide; HDR: Homology-Directed Repair; N-cadherin: Neural cadherin; SLUG: Zinc Finger Proteins SNAI2; SNAIL: Zinc Finger Proteins SNAI1; p-RKIP: phosphorylated-Raf Kinase Inhibitor Protein; RKIP: Raf Kinase Inhibitor Protein. *The experiments were done at least in three independent assays. It was only quantified the most representative one (Figure 17).

CHAPTER 5. DISCUSSION

In PCa the major trigger is AR signaling, responsible for the development and acquisition of resistance²⁶. Other mechanisms have been associated with resistance and metastization, particularly the EMT process⁵², in which RKIP protein act as an inhibitor⁷⁵. Moreover, AR can upregulate RKIP expression¹²⁹, although the link between androgens and EMT continues to be a subject with contradictory perspectives⁵². Herein, the aim was to understand RKIP crosstalk with androgen stimulation and their simultaneous influence on therapy response.

In this project, different cell lines were used to mimic the different PCa responses to therapy and genetic backgrounds. PC-3 and DU-145 are metastatic cell lines, LNCaP is a poorly metastatic cell line and 22RV1 is derived from primary carcinoma.

Firstly, through WB, an analysis was performed regarding a possible correlation between RKIP levels with AR and EMT proteins expression. On one hand, metastatic cell lines showed no expression of AR, low levels of RKIP and high expression of Vimentin, a mesenchymal marker. On the other hand, the low metastatic and primary cell lines had high expression of RKIP, AR, and E-cadherin, an epithelial marker, as well as low levels of Vimentin. In the literature it is recognized that RKIP levels are correlated with low metastization, high epithelial markers, and low mesenchymal markers^{75,76}, which are corroborated by the results here obtained.

AR expression was described in LNCaP cell line, and in the 22RV1 cell line was described to express AR and ARV7, concordantly with the presented results¹⁴¹. Concerning the EMT process, there is some inconsistency. The expression of the epithelial marker, E-cadherin, has been observed as positive in LNCaP, DU-145, and PC-3^{128,142}. Nevertheless, Sun and colleagues⁵⁶ presented positive levels of E-cadherin for LNCaP and 22RV1 cell lines and negative levels for DU-145 and PC-3 cell lines, a study more coincident with the observed results. The mesenchymal marker Vimentin is reported as positive for DU-145^{56,142} and negative for LNCaP^{56,142}, consistent with the obtained results. However, Vimentin expression in PC-3 and 22RV1 cell lines has been reported to be both negative^{56,142} and positive^{54,56}. Finally, SNAIL expression was revealed to be positive in 22RV1⁵⁴, DU-145¹²⁸ and PC-3^{54,128} and negative for LNCaP¹²⁸, contradictory to the observed here.

Regarding the described loop of RKIP and SNAIL, it was unexpected that the LNCaP cell line was simultaneously positive for SNAIL and RKIP proteins, raising the possibility that other pathways were affecting the loop, as well as the capability of SNAIL to repress RKIP transcription¹²⁸.

Altogether, the results and literature revision regarding the characterization of the cell lines suggested to us that the cell lines are different from laboratory to laboratory, that's why the cell lines genotyping are actually mandatory. Also, the discrepant results can be somehow explained by the different antibodies used by the other authors and us.

To understand the effect of androgen stimulation on RKIP expression and the EMT process, the cell lines were stimulated for 24 hours with 10nM of DHT, as this concentration has been described as adequate to observe an AR modulation¹⁴³. It was reported that in 22RV1⁵⁴ and LNCaP¹⁴¹ AR-positive cell lines, that AR expression increases after androgen stimulation. However, this modulation was only detected with significance in the LNCaP cell line.

Zhang and colleagues¹²⁹ described in 2012 that RKIP expression is regulated directly by androgens in a dose-dependent manner. Nevertheless, a non-tumorigenic prostate cell line was used in that study, RWPE-1, which can respond differently from the tumorigenic cell lines used in this project. Despite being reported that RKIP expression is regulated by androgens, herein for LNCaP cell line, we observed no effects upon DHT stimulation, with RKIP increase occurring only after antiandrogen treatment, being this event independent of AR expression, since it maintained unchanged in these conditions. Thus, it seems that likely other compounds⁷⁶, antiandrogens induce RKIP expression at least in LNCaP cell line. Later, for 22RV1 cells, a decrease on RKIP expression was observed both on AR stimulating and inhibitory conditions, a decrease that was accomplished by a concomitant decrease of ARV7 expression on this cell line. Interestingly, in 22RV1 cell line with RKIP KO the expression levels of AR were upregulated, being the ARV7 variant maintained stable in both AR stimulating and inhibitory conditions, in contrast to what happens in RKIP expressing cells. Altogether, these results suggested that somehow RKIP and AR are communicating with each other directly or indirectly and in a cell line dependent manner. Since during this project some difficulties have been found in WB execution and reproduction, further validations and studies are needed to dissect the molecular mechanisms mediating RKIP and AR interplay in prostate cancer. Moreover, to improve our study, we effort to KO RKIP in LNCaP cells, although this cell line is highly sensitive and was not possible to achieve a stable transfection. In the future will be interesting to attempt a transient transfection in LNCaP cell line, as an alternative technique to results validations.

Regarding EMT markers, after androgen treatment in DU-145 cell line a modulation of the mesenchymal phenotype was perceived, through the increase of SNAIL and decrease Vimentin levels. Alimirah and collaborators¹⁴¹ observed that the DU-145 cell line expressed detectable levels of AR mRNA and protein, and saw that after DHT treatment an increase of AR protein levels and nuclear localization occurred. Although we did not observe an increment of AR protein expression in the DU-145 cell line, perhaps androgen stimulation continued to influence AR cascade and consequently modulating EMT. The mechanism through which DHT acts is poorly understood. Some evidences in PCa have demonstrated that androgens may downregulate TGF β 2 gene and Smad3 mRNA expression¹⁴⁴. TGF β 2 is modulated through a transcriptional mechanism in which DHT suppresses the binding of the transcription factor Sp1 to its promoter¹⁴⁴. This mechanism could elucidate the modulation of EMT by DHT in an AR-negative cell line, DU-145.

Further, we hypothesized that cell lines without RKIP and AR were the most resistant. For that purpose, two types of drugs, the chemotherapeutic (Docetaxel and Cabazitaxel) and the antiandrogens (Abiraterone and Enzalutamide), were used. Regarding the semi-synthetic taxanes, using Cabazitaxel, we observed that the most metastatic cell lines, with low RKIP levels (PC-3 and DU-145), were the most resistant and the low metastatic lines, with high RKIP levels (LNCaP and 22RV1), were the most sensitive, as initially predicted. Using Docetaxel, even though PC-3 continues to be the most resistant and LNCaP the most sensitive, the 22RV1 cell line was unexpectedly the second most resistant.

Decreased expression of epithelial markers and increased levels of mesenchymal markers, were associated with docetaxel resistance, and, in particular, Vimentin was correlated with adverse prognosis in primary tumors³⁶. Nevertheless, Vimentin expression showed to be positive for DU-145 cells and negative for 22RV1 cells, emerging the hypothesis that an alternative pathway from the EMT mechanism is activated in these cell lines. Wildtype *p53* is a suppressor gene involved in the cellular response to DNA damage, inducing various target genes leading to growth arrest or apoptosis¹⁴⁵. Connecting the importance of microtubulin in mediating the effects of taxanes with the association of *p53* to β -tubulin of microtubulin, it is possible that *p53* status is a crucial determinant of drugs sensitivity¹⁴⁶. Liu and colleagues¹⁴⁷ discovered that in response to Docetaxel, DU-145 which express mutant *p53* and PC-3 cell lines, that express null *p53*, were less sensitive than LNCaP with wildtype *p53* expression. Docetaxel treatment had the capability of increasing the levels of *p53* ser15 phosphorylation in LNCaP, while having a diminutive effect in DU-145 and PC-3 cells¹⁴⁷.

Nevertheless, the authors could not explain the failure of phosphorylation induction in the mutant p53 in DU-145 cells. The *p53* mutations in the DU-145 cell line could be involved in a variable Docetaxel response, culminating in the results seen in this study, where this cell line appeared to be as more sensitive than 22RV1, which is wildtype p53¹⁴⁸.

Regarding the antiandrogen drugs, as expectable, when submitted to Abiraterone, PC-3 and DU-145 cells were the most resistant cell lines, concordantly with their AR-negative expression. Concomitantly, 22RV1 and LNCaP cells, AR-positive cell lines, were the most sensitive. When treated with Enzalutamide, PC-3 cells were the most resistant, followed by 22RV1, LNCaP cells, and unexpectedly, given their AR-negativity, DU-145 cells were the most sensitive.

Sekhar and colleagues¹⁴⁹ demonstrated that Enzalutamide mediated radiosensitization in DU-145 cell line, an AR-negative cell line. The AR-negative PC-3 cell line does not exhibit changes in proliferation, apoptosis, or other endpoints when treated with Enzalutamide (doses up to 40 μ M) in combination with radiotherapy. In this study, the authors suggest that Enzalutamide may radiosensitize DU-145 cells through the AR pathway, although without AR protein expression, which presumably continues to influence AR targeting of DNA repair genes¹⁴⁹. This report could in part elucidate the observed sensibility of DU-145 cells to Enzalutamide. Alternative explanations may include a non-AR-mediated mechanism or off-target effects of Enzalutamide.

Dissecting the pharmacological response of the PCa cells, subsequently, we aimed to investigate in an AR-positive cell line the effects of antiandrogen drugs, isolated and in conjugation with androgen. Analyzing the epithelial phenotype in the LNCaP cell line, E-cadherin expression was unchanged in all condition. However, Zhu and Kyprianou⁵⁸ demonstrated that stimulation with DHT decreases E-cadherin expression in LNCaP cell lines. In LNCaP cell line androgen stimulation seemed to have opposing effects in mesenchymal phenotype, through downregulation of SNAIL and upregulation of SLUG. Moreover, SLUG expression was decreased in the presence of Enzalutamide. Although these results appear to be contradictory, they can be corroborated by literature. Miao and colleagues⁵⁴ reported in AR-positive cell lines that SNAIL was downregulated under DHT treatments. In addition, Wu and collaborators⁶⁰ presented in LNCaP cell line that SLUG expression was increased after DHT stimulation and decreased under Bicalutamide (AR antagonist), defined SLUG as an androgen-regulated gene. Overall, the different ideas reported in the literature are complementary, being androgens simultaneously an inducer and repressor of EMT pathway.

Regarding antiandrogen drugs, Abiraterone treatment increases AR, RKIP and SNAIL expression. AR amplification¹⁵⁰ and SNAIL⁵⁴ upregulation have been associated with antiandrogen drug resistance, this supports the theory that RKIP levels in LNCaP were increased as a mechanism of resistance to Abiraterone drug.

The subsequent objective was to study the RKIP role in the pharmacological response, and for that, the 22RV1 cell line, a primary cancer cell line, was knocked out for RKIP using the CRISPR/Cas9 technology. Herein, we observed that in the absence of RKIP the cells were more sensitive to Cabazitaxel, Docetaxel, and Abiraterone, but had no effect to Enzalutamide.

Overall, we observed that RKIP can hypothetically increase cell death to taxane drugs. Chatterjee and colleagues¹³² demonstrated in PCa cell lines that RKIP expression is increased after DNA damage. The direct correlation of RKIP expression with the extent of apoptosis raises the possibility that upregulation of RKIP is one of the mechanisms that sensitizes cancer cells to apoptotic signals in response to DNA damage. In the same study, they verified in breast cancer cell lines that treatment with chemotherapeutic agents, such as Taxol, can enhance RKIP expression in arrested cells¹³². Posteriorly in 2006, Eves and collaborators¹⁵¹ showed that RKIP loss in HeLa and H19-7 cells decreased mitotic index, accelerating metaphase/anaphase transition timing and causing defects in the spindle checkpoint. These authors also verified that RKIP depletion, through hyperactivation of the Raf/MEK/ERK signaling cascade, leads to a decreased localization at kinetochores and loss activity of phosphorylated Aurora B. This protein is implicated in chromosomal alignment, cytokinesis, and spindle checkpoints, being accumulated at inner centromeres during prometaphase, controlling the interactions of microtubules with kinetochores¹⁵². Moreover, Al-Mulla *et al.*¹⁵³ in 2011 presented that RKIP influences the cell proliferation rate by modulating cell cycle kinetics. In this study silencing RKIP induced the expression of molecules that drive DNA synthesis and G1/S and G2/M transitions, such as Cyclin D1, Cyclin E2, Cell Division Protein Kinase 6 (CDK6), and S-phase Kinase-associated Protein 2 (SKP2)¹⁵³. Particularly, the Cyclin D1 and SKP2 are regulated by GSK3 β a target of RKIP. Also, silencing RKIP attenuated the expression of molecules involved in guarding cell cycle checkpoints, as p21^{Cip1}, Aurora B, Cyclin G1, and Anaphase promoting complexes¹⁵³. Altogether, these alterations in cellular proliferation through RKIP could result in an increase of chromosomal defects induced by Taxanes¹⁵⁴, thus sustaining the observed results of sensitivity to chemotherapy (Cabazitaxel and Docetaxel) upon RKIP absence.

The final aim was to understand the effects of antiandrogen drugs isolated and in combination with an androgen in RKIP KO cells. The predicted effects of RKIP depletion, based on its role as a modulator of NF- κ B/SNAIL/YY1/PTEN loop¹²⁸, was verified through upregulation of SNAIL in CAS9 cells. In general, appears that RKIP absence increased the androgen effects. In CAS9 cells, compared with the control of HDR cells, a greater increase of N-cadherin and SLUG expression was seen under DHT stimulation. Regarding the response of CAS9 cells to antiandrogen drugs, ARV7 and SNAIL levels were increased in treatments. These proteins have been associated with resistance to antiandrogen treatments^{54,150,155}. Moreover, expression of E-cadherin, an essential marker of the epithelial phenotype, was downregulated under incubation with drugs. Overall, it seems that *RKIP* KO increased the mesenchymal phenotype and proteins related to resistance to Abiraterone treatment. It is important to highlight that during this project some difficulties have been found in WB execution, obtained invalid results, as well, different loadings, which difficult the process of WB quantification. Thus, the results presented and discussed in this part of the work are a result of just one representative assay, which being novel, hampering comparisons with the literature, should be accurately repeated in the future, since it was not possible to do it till the end of this thesis.

Even being a preliminary and exploratory work, from the results obtained it was possible to conclude that RKIP seems to not have a significant effect in the 22RV1 cell line response to antiandrogen drugs. Moreover, in the absence of RKIP an increment of androgen effects in EMT proteins occurred, as well as induction of proteins related to resistance under antiandrogen treatment. The tendency observed of increased sensitivity to Abiraterone drug in RKIP KO cells seems not to correlate with the EMT process, emerging as an attractive possibility to evaluate other pathways that could have a strong influence on antiandrogen drug response in PCa.

CHAPTER 6. CONCLUSION

Herein the RKIP role in pharmacological response and modulation of AR stimulation was evaluated, providing new insights about RKIP function in PCa.

Firstly, was shown that metastatic PCa cell lines had low expression of RKIP, AR, and epithelial markers and high expression of mesenchymal markers. Concomitantly, these cell lines were, in general, the most resistant to pharmacological drugs. Moreover, a non-significant correlation between RKIP expression and androgen stimulation was observed, contradictory to the study that described an upregulation of RKIP under androgens in a non-tumorigenic cell line¹²⁹. In the future, it will be interesting to evaluate RKIP mRNA and protein levels under androgen stimulation, in both tumorigenic and non-tumorigenic prostate cell lines.

Subsequent regarding EMT processes was observed that androgen stimulation in an AR-positive cell line had an incongruent behavior; with both activation and repression of mesenchymal phenotype. Additionally, the antiandrogen drug Abiraterone increase AR, RKIP, and SNAIL expression. Considering that AR amplification and SNAIL upregulation have been associated with antiandrogen drug resistance, RKIP levels in LNCaP could be increased as a resistance mechanism to Abiraterone drug.

To explore the role of RKIP in PCa, an AR-positive cell line was deleted of RKIP expression. In the absence of RKIP cells were more sensitive to cytotoxic chemotherapeutic drugs (Cabazitaxel and Docetaxel). In RKIP KO cells was seen increased expression of proteins related to EMT and therapeutic sensitivity, however, this did not induce a significantly different response to antiandrogenic treatment.

This were highlighting the role of RKIP in the chemotherapeutic response. However, more studies are required to understand the mechanism of action of RKIP in these treatments, for instance, the evaluation of the Raf/MEK/ERK signaling cascade, a well-described target of RKIP, and of proteins related to mitosis (Aurora B) and apoptosis (Caspases).

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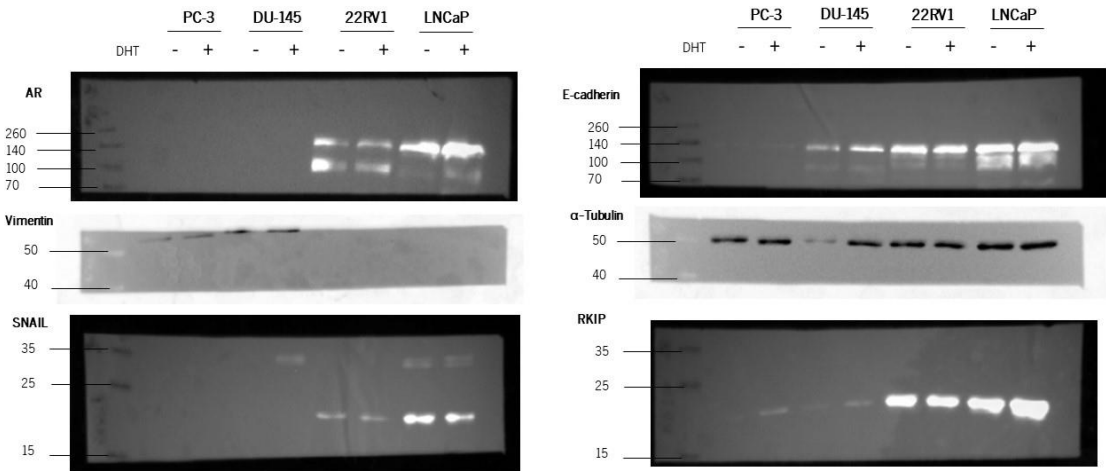
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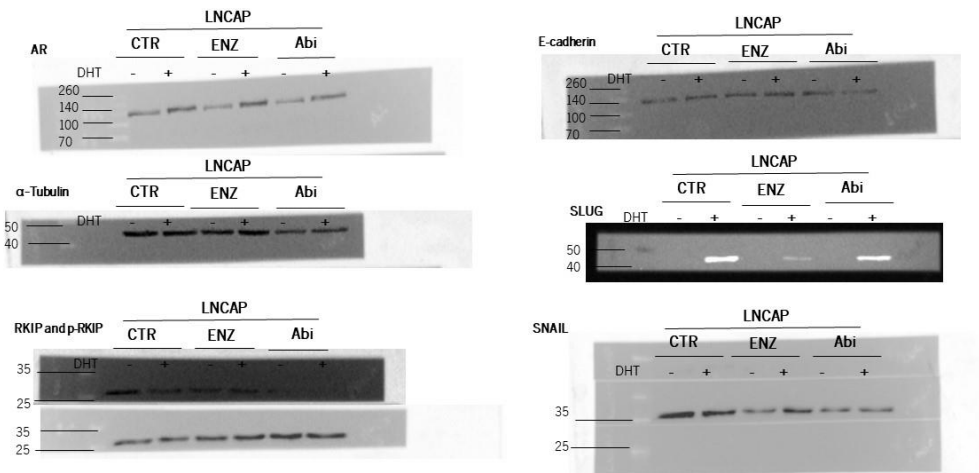
SUPPLEMENTARY DATA

Section 4.1: Characterization of prostate cancer cell lines



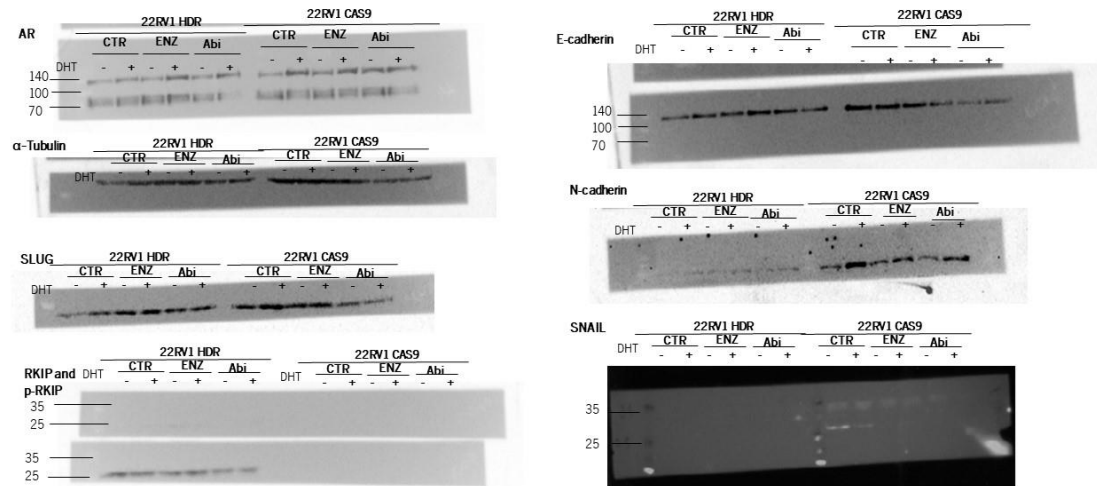
Supplementary Figure 1. Characterization of EMT markers, RKIP, and AR expression in PCa cell lines, PC-3, DU-145, LNCaP, and 22RV1 under DHT stimulation (10 nM for 24 hours). (Corresponds to the uncropped blot shown in Figure 10 of the Thesis)

Section 4.2: Role of androgens in RKIP and EMT proteins modulation



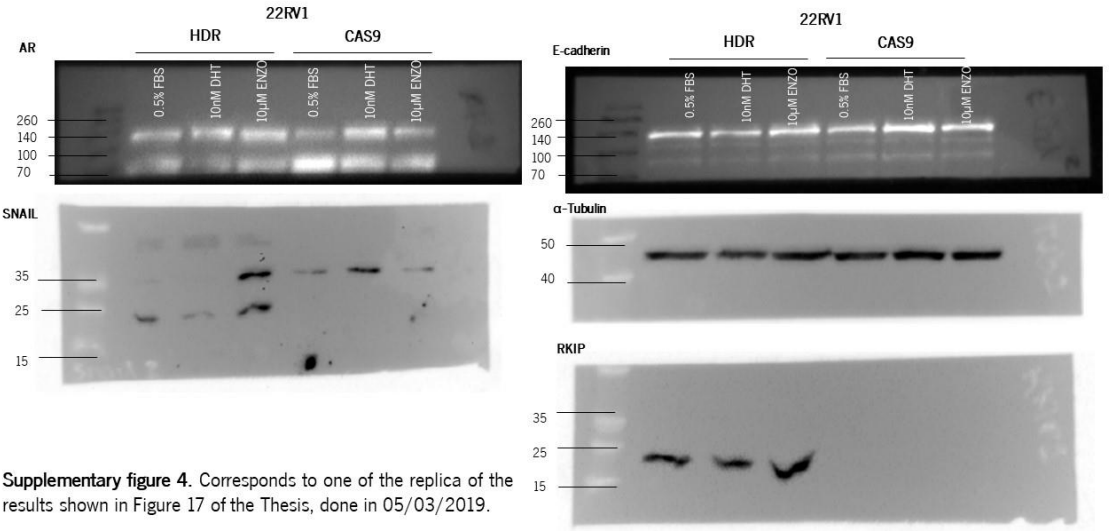
Supplementary figure 2. Characterization of EMT markers, RKIP, and AR expression in LNCaP cell line under DHT (10nM), Enzalutamide (20 μM), and Abiraterone (20 μM) treatment for 24 hours. (Corresponds to the uncropped blot shown in Figure 13 of the Thesis)

Section 4.3.2: Effect of RKIP in AR expression and EMT proteins



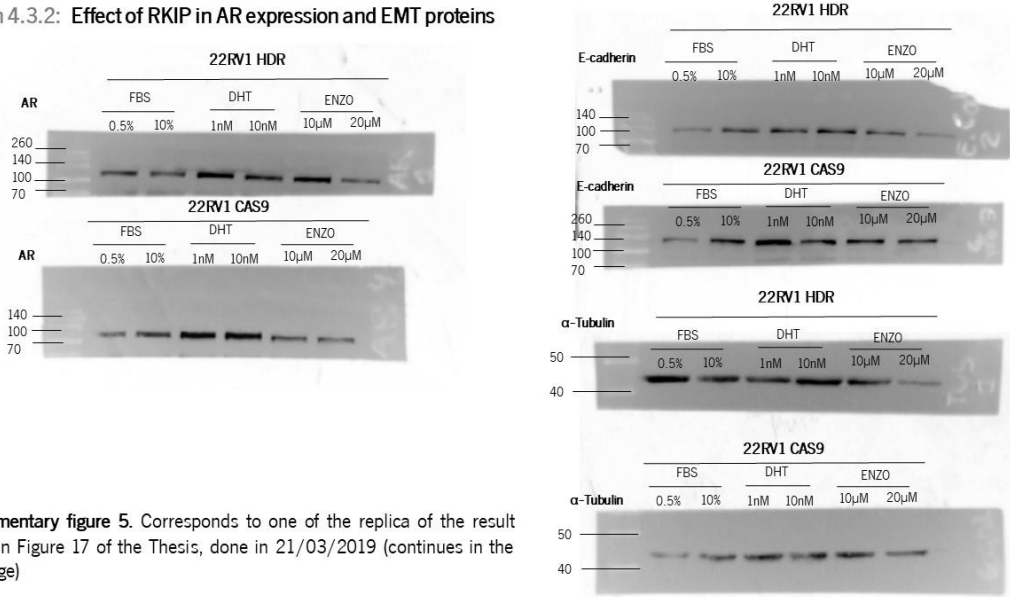
Supplementary figure 3. Comparison of EMT markers and AR expression in 22RV1 HDR and CAS9 cells under DHT (10 nM), Enzalutamide (20 μM), and Abiraterone (20 μM) treatments for 24 hours. (Corresponds to the uncropped blot shown in Figure 17 of the Thesis, done in 29/04/2019)

Section 4.3.2: Effect of RKIP in AR expression and EMT proteins



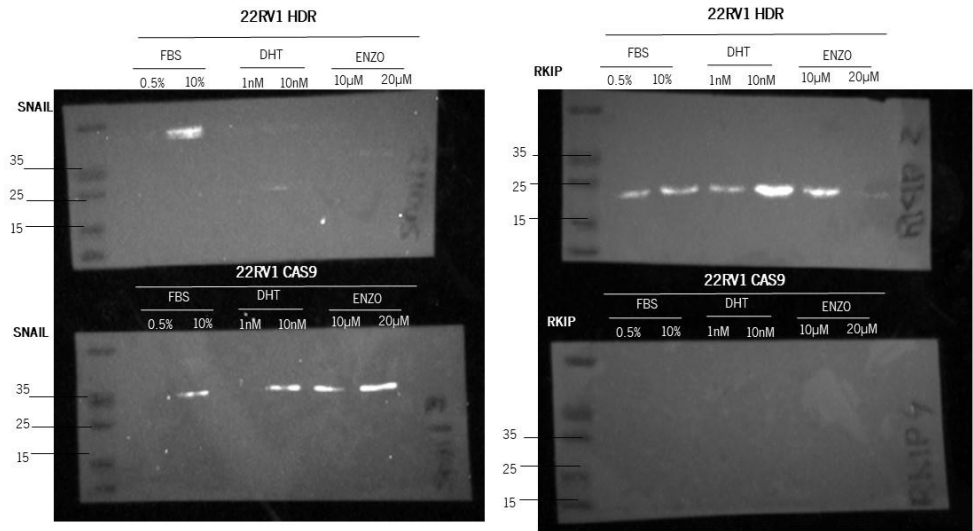
Supplementary figure 4. Corresponds to one of the replica of the results shown in Figure 17 of the Thesis, done in 05/03/2019.

Section 4.3.2: Effect of RKIP in AR expression and EMT proteins



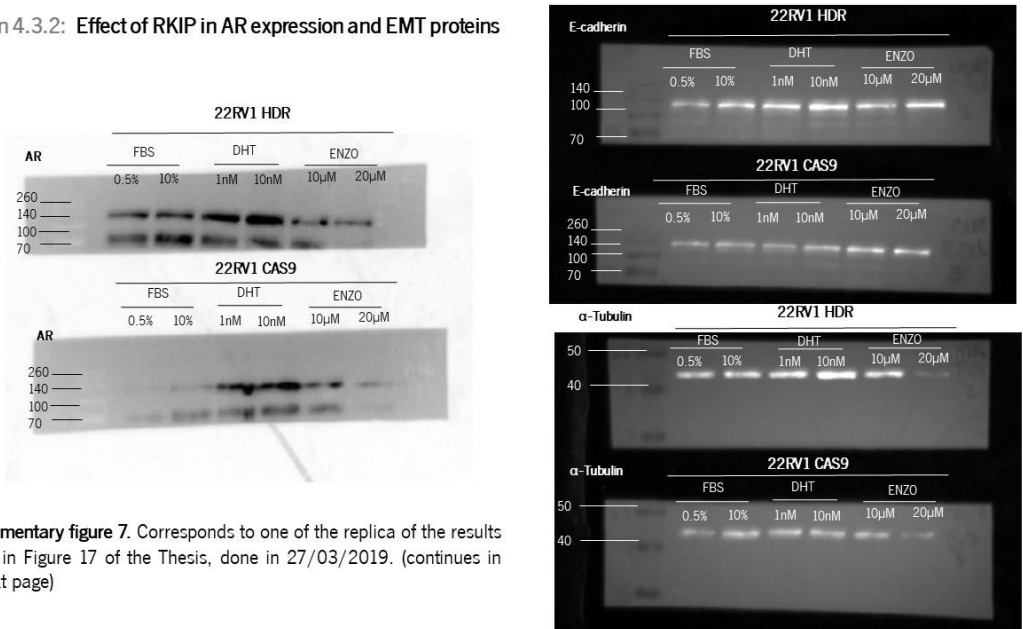
Supplementary figure 5. Corresponds to one of the replica of the result shown in Figure 17 of the Thesis, done in 21/03/2019 (continues in the next page)

Section 4.3.2: Effect of RKIP in AR expression and EMT proteins



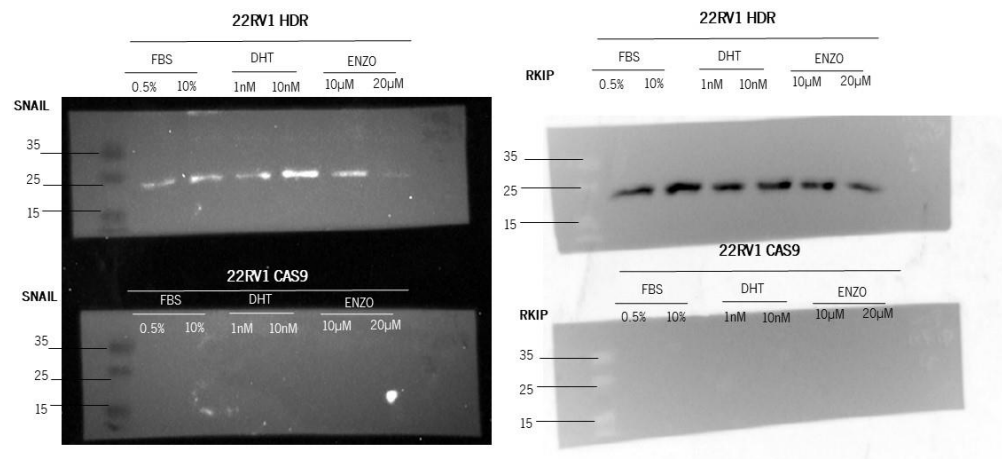
Supplementary figure 6. Corresponds to one of the replica of the result shown in Figure 17 of the Thesis, done in 21/03/2019 (from previous page)

Section 4.3.2: Effect of RKIP in AR expression and EMT proteins



Supplementary figure 7. Corresponds to one of the replica of the results shown in Figure 17 of the Thesis, done in 27/03/2019. (continues in the next page)

Section 4.3.2: Effect of RKIP in AR expression and EMT proteins



Supplementary figure 8. Corresponds to one of the replica of the results shown in Figure 17 of the Thesis, done in 27/03/2019. (from previous page)