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Demethylation of the epigenetically silenced androgen receptor gene by a repurposed drug in castration-resistant prostate cancer cell lines

Mariana Brütt Pacheco

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Demethylation of the epigenetically silenced androgen receptor gene by a repurposed drug in castration-resistant prostate cancer cell lines

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“A winner is a dreamer who never gives up.”

Nelson Mandela

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RESUMO

O cancro da próstata (CP) é a segunda neoplasia mais incidente e a quinta causa de morte relacionada com o cancro em homens em todo o mundo, afetando principalmente homens idosos. Embora a maioria dos doentes com CP apresente doença localizada ao diagnóstico, uma proporção significativa progride para doença disseminada resistente à terapia de privação de androgénio (TPA). A alta morbilidade e mortalidade associada a este estádio, bem como a falta de abordagens terapêuticas com intuito curativo, realçam a importância de investigar novos regimes de tratamento.

Atualmente acredita-se que esta progressão pode dever-se à desregulação da via de sinalização do receptor de androgénio (RA) por vários mecanismos moleculares, independentemente dos níveis circulantes de androgénio. Para além disso, alterações ao nível da maquinaria epigenética, como a hipermetilação do DNA, foram associadas a uma perda da expressão do RA em 20 a 30% dos cancros independentes de androgénio. Deste modo, os inibidores da DNA metiltransferase (iDNMT) poderão ser uma abordagem terapêutica promissora neste subconjunto de doentes.

Assim, o objetivo principal desta dissertação de mestrado foi avaliar o efeito da hidralazina, um iDNMT, como *drug repositioning* em linhas celulares de CP. Neste estudo, este composto induziu inibição da viabilidade celular dependente da dose, bem como um aumento significativo da apoptose na DU145, uma linha celular negativa para RA. Curiosamente, o tratamento sequencial com hidralazina e um inibidor do RA, a enzalutamida, corroborou esses resultados. A hidralazina sensibilizou esta linha celular para a enzalutamida, ao contrário das outras linhas celulares: PC-3 e RWPE. Posteriormente, o padrão de metilação da região promotora do RA foi avaliado nessas linhas celulares através da sequenciação de bissulfito. Em geral, a DU145 exibiu um perfil de metilação mais alto do que as outras linhas celulares de CP especificamente em duas regiões a montante do local inicial da transcrição. Para além disso, estes padrões de metilação diminuíram na DU145 após o tratamento com hidralazina, sugerindo que a hipermetilação nestas CpG do promotor do RA poderá ser um mecanismo que explica a perda da expressão do RA em carcinomas avançados da próstata.

Concluindo, demonstramos que este fármaco tem efeitos desmetilantes em CpG específicos da região reguladora do RA na DU145. De facto, a metilação do RA nessas CpGs pode levar a uma regulação negativa do RA nesse subconjunto de doentes com CP, podendo-se possivelmente associar à resistência à TPA. Assim, a hidralazina constitui um composto promissor para o tratamento destes doentes, pois poderá aumentar a sensibilização dos tumores resistentes à castração para medicamentos já aprovados e direcionados ao RA.

ABSTRACT

Prostate cancer (PCa) is the second most incident malignancy and the fifth cause of cancer-related death in men worldwide, affecting mainly elderly men. Although most PCa patients present with localized disease at diagnosis, an important proportion eventually progresses to a castration-resistant state after androgen-deprivation therapy (ADT). The high morbidity and mortality associated with this disease as well as the lack of curative therapeutic approaches highlight the importance of investigating novel treatment regimens.

It is widely accepted that this progression can be due to androgen receptor (AR) signaling pathway deregulation by several molecular mechanisms, regardless of androgen circulating levels. Additionally, aberrations in epigenetic machinery, such as hypermethylation, have been associated with a loss of AR expression in 20-30% of these androgen-independent cancers. Therefore, DNA methyltransferase inhibitors (DNMTi) might be a promising therapeutic approach in this subset of patients with these reversible modifications.

Thus, the major objective of this master's dissertation was to evaluate the effect of hydralazine, a DNMTi, as a repositioning drug in PCa cell lines. This compound induced a dose-dependent inhibition of cell viability, as well as a significant increase in apoptosis in DU145, an AR-negative cell line. Interestingly, sequential treatment with hydralazine and an AR inhibitor, enzalutamide, corroborated these results. Hydralazine sensitized this cell line to enzalutamide, contrarily to the other cell lines: PC-3 and RWPE. Afterwards, the methylation pattern of the promoter region of AR was assessed in these PCa cell lines using bisulfite sequencing. Overall, DU145 displayed a higher methylation profile than the other PCa cell lines specifically in two regions upstream the transcription start site. Furthermore, these methylation patterns were decreased in DU145 after hydralazine treatment, suggesting that CpG hypermethylation of AR promoter may be a possible mechanism that explains loss of AR expression in advanced prostate carcinomas.

We demonstrated that this repositioning drug has demethylating effects in specific CpG sites of the AR regulatory region in DU145 cell line. In fact, AR methylation in these CpG dinucleotides may lead to AR downregulation in this subset of PCa patients, possibly being associated with ADT resistance. Thus, it constitutes a promising compound for CRPC treatment, since it could lead to a sensitization of already approved drugs that target AR.

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LIST OF ABBREVIATIONS

µM – Micromolar

17β-HSD – 17β-hydroxysteroid dehydrogenase

3β-HSD1 – 3β-hydroxysteroid dehydrogenase 1

5-aza-CdR – 5-aza-2'-deoxycytidine

5hmC – 5-hydroxymethylcytosine

5mC – 5-methylcytosine

A – Androgen

ACHT – Adrenocorticotropic hormone

ADT – Androgen deprivation therapy

AML – Acute myeloid leukemia

APC – APC regulator of WNT signaling pathway

AR – Androgen receptor

AR-V7 – Androgen-receptor splice variant 7

AREs – Androgen-response elements

ATCC – American type culture collection

CCDS – Consensus coding sequence

ChIP – Chromatin immunoprecipitation

CpG – Cytosine-phosphate-guanine

CRPC – Castration-resistant prostate cancer

CT – Computed tomography

CYP11A1 – Cytochrome P450 family 11 subfamily A member 1

CYP17A1 – Cytochrome P450 family 17 subfamily A member 1

DAC – 5-aza-2'-deoxycytidine

DBD – DNA-binding domain

DHEA – Dehydroepiandrosterone

DHT – Dehydrotestosterone

DMSO – Dimethyl sulfoxide

DNMT – DNA methyltransferase

DNMTi – DNMT inhibitors

DRE – Digital rectal examination

EBRT – External-beam radiation therapy

EC50 – Half maximal effective concentration

EGFR – Epidermal growth factor receptor

ER – Estrogen receptor

ERG – ETS-related gene

ETS – E26 transformation-specific

ETV1 – ETS variant 1

ETV4 – ETS variant 4

ETV5 – ETS variant 5

FBS – Fetal bovine serum

FDA – Food and drug administration

FHIT – Fragile histidine triad diadenosine triphosphatase

FSH – Follicle-stimulating hormone

GG – Grade group

GnRH – Gonadotropin releasing hormone

GS – Gleason score

GSTP1 – Glutatione S-transferase pi 1

HDAC – Histone deacetylase

HDACi – Histone deacetylase inhibitor

HDACi – Histone deacetylase inhibitor

Hsp – Heat-shock protein

ISUP – International Society of Urological Pathology

LBD – Ligand binding domain

LH – Luteinising hormone

LHRH – Luteinising hormone-releasing hormone

mCRPC – Metastatic castration-resistant prostate cancer

MDS – Myelodysplastic syndromes

MGMT – O-6-methylguanine-DNA methyltransferase

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide

N-Cor – Nuclear receptor corepressor

nM - Nanomolar

NSAAs – Nonsteroidal antiandrogens

NTD – N-terminal transactivation domain

pre-mRNA – precursor mRNA

PCa – Prostate cancer

PET – Positron-emission tomography

PIK3 – Phosphoinositide 3-kinase

PSA – Prostate-specific antigen

PTEN – Phosphatase and tensin homolog

RARβ – Retinoic acid receptor beta

RTK – Receptor tyrosine kinase

SMRT – Silencing mediator of retinoid and thyroid receptor

Sp/KLF – Specificity protein/Krüppel-like factor

Sp1 – Specificity protein 1

SRD5A – Steroid 5α-reductase

T – Testosterone

TET – Ten-eleven-translocation

TSG – Tumor suppressor gene

TMPRSS2 – Transmembrane protease serine 2

TRUS – Transrectal ultrasound

TSS – Transcription start site

UTR – Untranslated region

INTRODUCTION

EPIDEMIOLOGY OF PROSTATE CANCER

According to GLOBOCAN data, prostate cancer (PCa) is the second most common malignant neoplasm and the fifth leading cause of cancer-related death in men, worldwide (***Figure 1***). It is the most common non-cutaneous cancer in men worldwide with an estimated incidence of 1 276 106 cases and 358 989 deaths annually. This number of PCa cases represents 7.1% of the total cancer cases worldwide and 3.8% deaths are due to this malignancy [1]. This cancer type still remains a problem, since its primary benign stages are overtreated, whereas no curative therapies are available for metastatic stages [2]. Despite the high incidence rates in Oceania, Northern and Western Europe and North America, their mortality rates do not correspond to the high incidence ones.

Although PCa is a common disease, its etiology is still unclear [1]. There are some differences in the PCa incidence between different ethnic groups with African men descent in the United States and the Caribbean having the highest incidence and mortality rates which can be explained by genetic factors [1–3]. In fact, having a positive family history and/or a certain ethnic background such as Afro-Caribbean is considered a risk factor for PCa development [3].

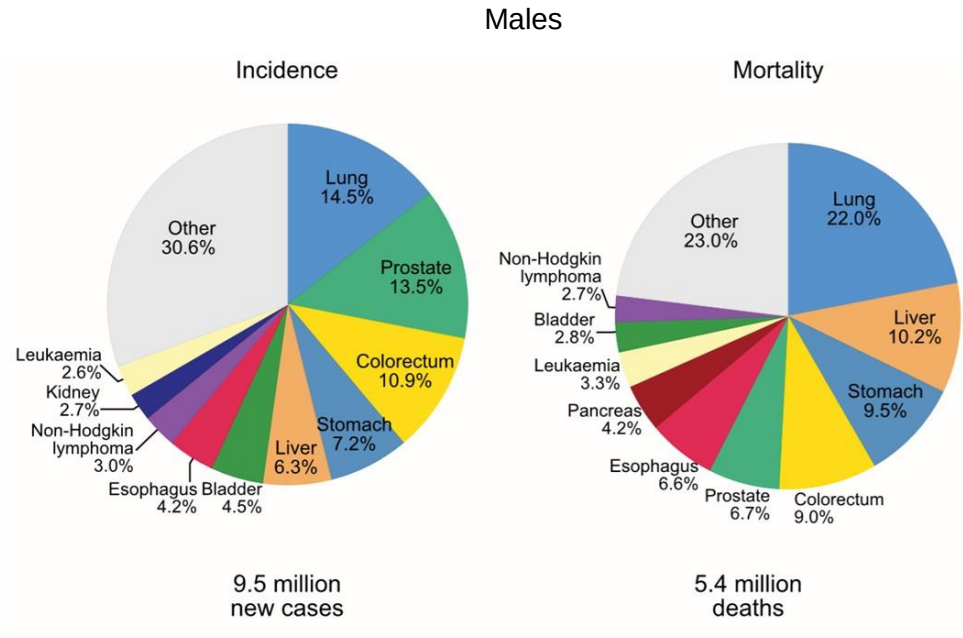

Figure 1: Pie charts present the distribution of cases and deaths for the ten most common cancers in 2018 for males worldwide. Adapted from [1].

SCREENING AND DIAGNOSIS

Nowadays, there are two screening tests for prostate cancer: digital rectal examination (DRE) and serum prostate-specific antigen (PSA). Despite its low specificity, PSA is considered the most sensitive biochemical marker for monitoring PCa and its primary regulator is AR [4–7]. This serine protease is produced in prostate and in normal conditions it is secreted into the glandular ducts where it degrades high molecular proteins in order to prevent coagulation of the semen [7,8]. Regarding PCa, this biomarker progressively increases due to aberrant prostate ductal structure formed by neoplastic epithelial cells, thus allowing PSA entering into the serum through leakage into the prostatic extracellular fluid [9,10].

The prostate epithelium is divided into three types: luminal, basal and neuroendocrine [2]. After prostate growth, androgens continue to promote the proliferation of secretory epithelia and stromal cells in the transition zone of the prostate, leading to a physiologic prostate gland enlargement known as hyperplasia [11–13]. Consequently, several men can experience lower urinary tract symptoms [13]. Since luminal cells are constantly multiplying, consequently producing more AR, PSA levels naturally rise, as men grow older. Hence, this hyperplasia is more commonly found in elder man and screening tests in elder men using PSA levels are highly recommended [13]. However, population-based PSA screening for PCa in men with advanced age is still conflicting regarding mortality outcome [4].

Initially, PSA measurement was thought to be able to substitute the digital rectal examination [4]. Nevertheless, PSA test alone is not specific nor sensitive enough to detect prostate cancer, since its levels can be altered with prostatitis, benign prostatic hyperplasia, prostatic biopsies and trauma [14–17]. Therefore, PSA in conjunction with digital rectal examination and transrectal ultrasound (TRUS)-guided biopsy is used as a diagnostic tool for early diagnosis, treatment and monitoring of prostate cancer patients [4,7]. European guidelines recommend a prostatic biopsy in which 10 to 12 cores are sampled in men with abnormal DRE and/or with PSA levels equal or above 2.0 ng/mL [7].

However, PSA alone is not reliable enough for monitoring disease burden in advanced CRPC, since visceral metastases can develop on these patients without an increase in PSA levels. Therefore, it is recommended for these patients to undergo a combination of frequent bone scintigraphy and CT scans along with PSA levels measurements [18]. Furthermore, PET/CT scan can detect a larger number of skeletal events than bone scintigraphy [3].

GRADING

Gleason grading system remains the most frequent approach to histopathological grading, which is one of the most powerful prognostic predictors and treatment selection tools in prostate cancer [19,20]. This system, created by Dr. Donald Gleason, is based on five prostate cancers' different histologic patterns [21]. However, this system has undergone several modifications by the 2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma [7,22]. Since most of the tumors have heterogeneous morphology, having two or more histological patterns, the Gleason Score (GS) was created. It is based on Gleason grading system, but it gathers the two most common grade patterns in a tumor, ranging from 2 to 10. Nevertheless, patients may assume that a diagnosis with GS 6 means having worse prognosis, leading consequently to the possible overtreatment [19]. Hence, it is now recommended to use GG in conjunction with the so-called grade grouping [23]. The grade groups are based on the modified GS and correspond with patient prognosis (Grade Group 1 = Gleason score ≤ 6, Grade Group 2 = Gleason score 3 + 4 = 7, Grade Group 3 = Gleason score 4 + 3 = 7, Grade Group 4 = Gleason score 4 + 4 = 8, Grade Group 5 = Gleason scores 9 and 10) (***Table 1***) [20,23,24]. This system is simpler and predicts more accurately prostate cancer biology and progression [22].

Table 1: Grade group and the corresponding Gleason score.

Grade Group	Gleason Score
1	≤ 6 (3+3)
2	7 (3+4)
3	7 (4+3)
4	8 (4+4, 3+5, 5+3)
5	9, 10 (4+5, 5+4, 5+5)

ANDROGEN SYNTHESIS IN NORMAL PROSTATE

Androgen synthesis is regulated by the hypothalamic–pituitary–gonadal axis. Pulsatile release of hypothalamic gonadotropin releasing hormone (GnRH) stimulates luteinizing hormone (LH) secretion from the anterior pituitary gland, which leads to the production of testosterone in the testes (***Figure 2***). This hormone subsequently regulates not only hypothalamus but also pituitary gland through negative feedback, in order to maintain continued LH secretion. Otherwise, continuous GnRH stimulation would lead to desensitization. This approach is used when administering long-acting GnRH agonists in ADT [25,26].

Although steroidogenesis occurs in both the adrenal cortex and in the testes, the majority of testosterone (95%) is produced in the testes by the Leydig cells. Like all other steroid hormones, its biosynthesis starts with the cleavage of cholesterol by CYP11A1. Testosterone can be later converted to dehydrotestosterone (DHT) within the prostate by the action of enzyme 5α-reductase. At last, testosterone and DHT can exert their biological effects by binding to AR and consequently initiating its transcriptional activity [26,27].

Androgens are the primary regulators of prostate cancer cell growth, proliferation and death. They regulate prostatic epithelial cell number by chronically stimulating cell proliferation and inhibiting cell death at the same time. However, if there is a chronic modification in androgen levels such as castration, these cells die via programmed cell death [28].

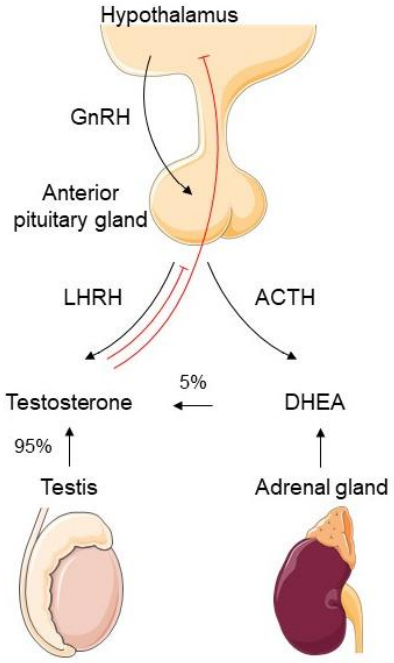

Figure 2: Regulation of androgen synthesis by the hypothalamic-pituitary-gonadal axis. Steroidogenesis occurs in testis and in adrenal glands, which produce 95% and 5% of testosterone, respectively.
Abbreviations: GnRH - gonadotropin releasing hormone; LHRH - luteinizing hormone-releasing hormone; ACTH - adrenocorticotropic hormone; DHEA - dehydroepiandrosterone

PROSTATE CANCER

The prostatic adenocarcinoma is a highly heterogeneous disease regarding both pathological and clinical manifestations. Tumors with multiple foci can have different histological characteristics and tumors diagnosed with the same stage and with identical histological characteristics can lead to different clinical outcomes [29–32].

PCa tumors vary depending on their degree, duration of response to primary hormone treatment and clinical manifestations which include an increasing PSA level or doubling time [33,34]. This intrapatient heterogeneity has been associated with the reduced efficacy of the systemic therapies [35,36]. Treatment pressure leads to the development of intra- and intertumor heterogeneity as well as tumor progression either by selection or by divergent differentiation [36]. Therefore, PCa treatment is variable, being chosen according to cancer stage and clinical manifestations such as PSA levels and doubling time [6,7]. Nevertheless, it is important to take in account the balance of benefits and side effects of each therapy modality as well as the patients' choice, comorbidities and quality of life [7].

Localized PCa

More than 80% of PCa cases are diagnosed while the disease is only confined to the gland representing consequently a low risk, good prognosis [35,37,38]. However, one third of these tumors metastasize to distant organs and can eventually lead to patients' death. Regarding the median survival, patients presenting localized PCa usually survive more than five years, whereas the advanced type commonly do not exceed three years [37].

Moreover, most early-diagnosed patients are treated with active surveillance or watchful waiting, prostatectomy or radiotherapy which results in optimal survival [37–39].

Patients diagnosed with low grade tumors should be kept under active surveillance, carefully monitor the tumor until the disease clinically progresses [7,40,41]. Patients diagnosed with low grade tumors have serum PSA levels <10 ng/mL, GS≤6, tumor stage of T2a or less and fewer than two-three positive cores with <50% cancer involvement [7]. This treatment modality includes regular PSA measurements, DRE and repeated biopsies although the periodicity remains still unclearly defined [7,40].

If the disease progresses, radical prostatectomy, external beam radiotherapy or brachytherapy is recommended.

Radical prostatectomy is targeted to patients with tumors confined to prostate gland. So, patients with high risk, such as cT2c or cT3 or GS>7 are contraindications. With this procedure, there is a complete removal of the gland preventing consequently future metastasis [7]. However, there are several complications associated with this surgical

procedure, which can compromise the patient's quality of life. These complications include urinary incontinence, erectile dysfunction as well as bowel and urinary problems [7,42]. Despite high prognosis and long-lasting effect of this treatment option, there may be a recurrence even after the surgery.

External-beam radiation therapy (EBRT) is a non-invasive and less toxic therapy when compared to the three-dimensional conformal radiation therapy. It is recommended for patients with low to high risk only differing in dosage. For low-risk patients, a dose of 74-78Gy is recommended, while for intermediate risk the dose escalation ranges from 76 to 80Gy and brachytherapy or short-term androgen deprivation therapy (ADT) should be added. Concerning high-risk PCa, the modality approach includes EBRT with long-term ADT modality [7]. This type of radiation can also be extended to seminal vesicles or lymph nodes [43].

Low-dose rate brachytherapy uses radioactive seeds implanted within the prostate. This approach is offered to low-risk cases as well as low volume disease. On the other hand, high-dose rate brachytherapy is recommended for intermediate or high-risk PCa and it uses a radioactive source which is temporarily introduced into the prostate [7,43]. Usually, it is combined with EBRT and it can be delivered either in single or in multiple fractions [7].

Locally advanced and metastatic PCa

The disease progression is driven by phenotypical changes caused by genetic and molecular events and is influenced by the tumor microenvironment in which it has spread to [33,35]. Moreover, the progressing tumor can be also influenced by the exposed therapy [44].

Tumors usually invade their adjacent lymph nodes in the first place, followed by the liver, lungs and bones [2,45]. The bone metastasis normally cause severe pain, hypercalcemia and frequent fractures [46]. While the tumors that spread to lymph nodes often regress completely and rarely recur, the ones in bone are rarely eradicated [33,47].

Androgen deprivation therapy becomes the standard treatment strategy for androgen-dependent tumors (80-90% of the initial diagnosed tumors) and for patients with locally advanced or systemically spread disease [32,48]. This treatment occurs either through chemical castration with LHRH agonists or surgical castration resulting in lower levels of circulating androgen [18,35,49,50]. However, the optimal initiation, duration and modality are still not well defined [32]. When testosterone levels reach ≤20 ng/dl, the progression to CRPC is most likely to be delayed [18,32].

When androgens are ablated, more than 70% of normal prostatic secretory epithelial cells undergo apoptosis or survive arresting their cell cycle in G1 phase [51,52]. Thereby,

ADT results in a 90-95% decrease in serum testosterone levels, decreases intraprostatic DHT levels by 50% and inhibits AR [48,52].

Luteinising hormone-releasing hormone (LHRH) agonists have replaced the gold standard surgical castration for ADT. Beyond their potential of reversibility, these agonists avoid the physical and psychological discomfort of resulting from the surgery and have lower cardiotoxicity risks, providing at the same time similar oncologic efficacy. LHRH antagonists decrease rapidly the luteinising hormone, follicle-stimulating hormone and testosterone levels by binding competitively to LHRH receptors [18].

The ADT alone can lead to a positive response in over 80% of the patients and in combination with docetaxel chemotherapy can initially lead to improvements in approximately 80-90% of them [35]. This therapy can inhibit the progression to CRPC for up to 3 years [48]. Despite this initial response to this therapy, it ultimately fails, since the patients develop a resistance to androgens and progress to CRPC in media within 12 to 30 months [37,53]. The cells that were initially resistant to androgen ablation or that adapted to low-androgen environment regrow leading to a clinical progression of the disease [54]. In these cases or in presence of metastases, therapy becomes more challenging [39]. In fact, 1 nM of androgens is sufficient to allow AR signaling and consequent tumor growth [55]. Considering the androgens' negative feedback, intermittent ADT should be recommended as a therapy in order to delay the development of androgen-resistant tumors [56].

Patients without metastases that are not suitable for curative treatments should report to ADT as a palliative treatment. Contrarily, symptomatic metastatic patients must receive ADT immediately combined with docetaxel, but only if they are fit enough. The toxicities of this therapy combination are mostly hematologic and could be overcome with concomitant use of granulocyte colony-stimulating factor. Moreover, during long-term therapy, bone mineral density and vitamin D should be measured every two years, since ADT increases the risk of fractures and decreases bone mineral density [18].

Castration-resistant PCa

The evolution from localized disease to castration-resistant PCa (CRPC) involves a complex interaction of signaling pathways that collectively promote cell proliferation [33].

Metastatic PCa eventually develops resistance to primary ADT treatments, resulting in CRPC [57]. Although this treatment is effective in 80-90% of the patients, the disease eventually progresses with rising PSA levels despite castrate concentrations levels [18]. This state is defined not only by the serum testosterone levels at <50 ng/dl, but also by biochemical or radiologic progression [18,35,58,59]. In fact, men with nonmetastatic CRPC and rapidly rising PSA level have a high risk developing metastases [34]. Biochemical

progression is characterized by three consecutive rises in PSA one week apart and by a PSA value higher than 2 ng/ml [18]. On the other hand, radiologic progression is when two or more new bone lesions appear on bone scan or a soft tissue lesion [18,35].

Normally, nonmetastatic CRPC remains incurable and patients survive in media from 2-3 years [34,35,58]. The current available therapies for this subset of patients only aim at reducing the symptoms and improve the overall survival (about two months) [37]. Therefore, most of these treatments are ineffective highlighting the interest of investigating new and more effective therapeutic strategies to this aggressive PCa phenotype [37].

The mechanisms responsible for the emergence and progression of CRPC despite low androgen levels are not fully understood. However, it is known that androgen receptor takes an important part in this process [35].

During ADT, several cells undergo apoptosis, while the ones who survive remain in G1 phase of the cell cycle. On one hand, the cells that survive could adapt to the low-androgen environment and regrow after a while. These might acquire new epigenetic and genetic modifications that enable them to survive to this conditions, leading to ADT resistance and consequently tumor progression [51,54]. On the other hand, pre-existing castration-resistant cells that have low androgen dependence and stem-cell properties could be naturally selected, survive and continue to proliferate in the absence of androgens [54,60]. Therefore, ADT might induce expansion of the existing population, allowing a recurrence from only one cell [60].

ANDROGEN RECEPTOR

AR has a significant role in PCa biology in general, in progression to CRPC, in the pathogenesis, as well as in stimulation of PCa cell growth [2,35,61,62]. It is not considered an imperative cause in PCa progression, but it might be oncogenic under circumstances in which AR is inappropriately activated [62,63].

It belongs to the steroid hormone receptor superfamily and in normal conditions is an androgen-activated DNA-binding transcription factor [63,64].

Structure

The AR gene is located on the long arm of X chromosome (Xq11-12), consists of 8 exons and encodes as 110kDa protein composed of three major domains: an N-terminal transactivation domain (NTD), which enables the transactivation of the AR, a central DNA-binding domain (DBD), a hinge region and a C-terminal ligand binding domain (LBD) [35,64,65]. The AR promoter region displays 27 CpG dinucleotides and an Sp1 protein-binding site instead of a TATA box [66,67].

Translocation

The cytoplasmatic AR is associated with heat-shock proteins/chaperones and co-chaperones that protect the receptor against degradation. The interaction with DHT alters the conformational structure of AR leading to a phosphorylation and a consequent conformational change that allows its transition from the cytoplasm to the nucleus. In there, it dimerizes and regulates the transcription activity of specific target genes involved in growth and survival of the cell by binding to androgen-response elements (AREs) in DNA promoter regions (***Figure 3***) [48,68–71].

This regulation within the nucleus is influenced by coregulators, which can affect signal transduction pathways without the need of DNA binding in response to growth factors and by post-translational AR modifications: phosphorylation, acetylation, sumoylation, ubiquitinations and methylation [35,72].

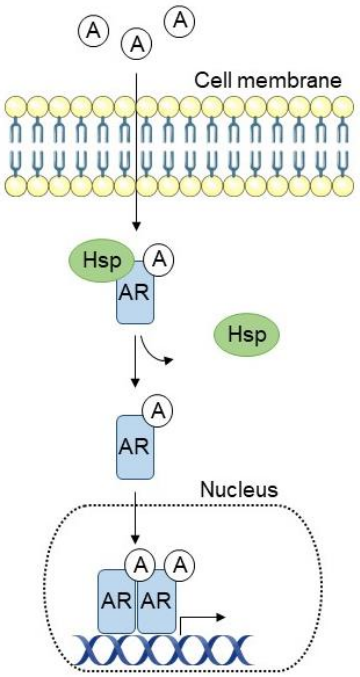

Figure 3: Androgen receptor translocation: the interaction with androgens alters the conformational structure of AR, allowing its transition from the cytoplasm to the nucleus. Abbreviations: A - androgen; Hsp - heat-shock protein

MODIFICATIONS OF AR SIGNALING PATHWAY

The inevitable progression to CRPC, despite ADT, cannot be attributed to a single mechanism. Nevertheless, it is known that the AR pathway is generally involved [35,48,73]. Androgens and the functional AR are known to be important mediators for PCa progression [64,74,75]. In addition, it is known that the disease progression is associated with an increase in PSA levels, a *bona fide* target of AR [33].

There are two main pathways that lead to androgen-refractory PCa development regardless of the androgens circulating levels: those involving AR and the others that bypass this receptor [33,35,39]. However, both pathways are not mutually exclusive, but instead can co-exist [39]. The ones involving AR include loss of AR expression, an increase in local androgens' biosynthesis, AR overexpression/amplification, activating mutations and enhanced AR activity to other ligands [2,33,35,52,53,76]. The indirect mechanisms include AR variants, increase ligand-independent activity, develop changes in coregulatory molecules and deregulate growth factors or cytokines that lead to AR pathway activation via cross talk of other signaling pathways [33,39,52,53,76]. These modifications are summarized in ***Figure 4***.

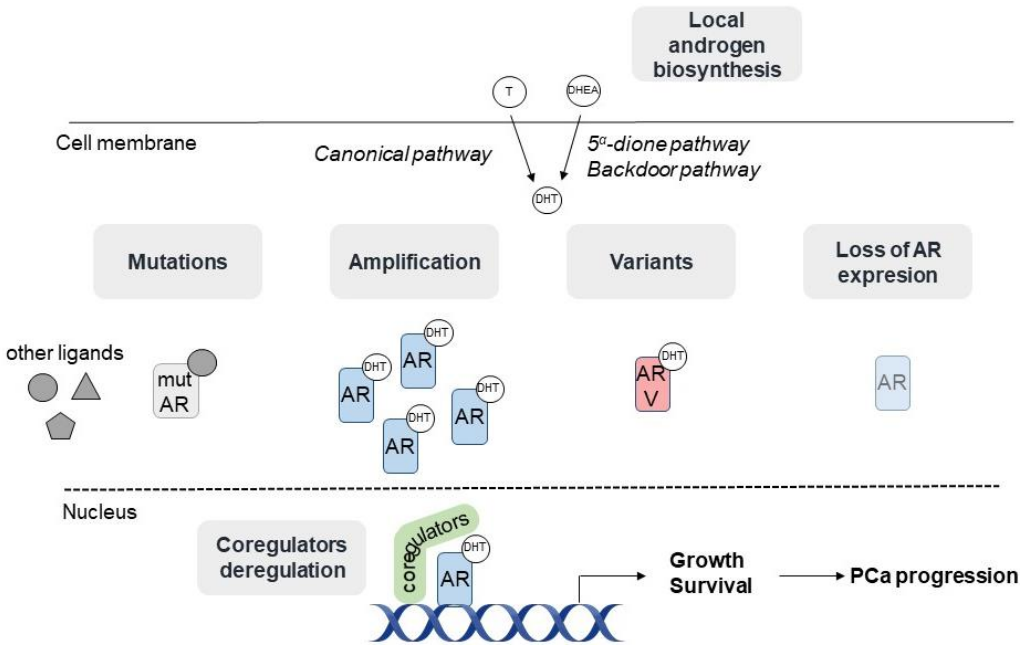

Figure 4: Modifications of AR signaling pathway that contribute to PCa progression independently of androgens' circulating levels.
Abbreviations: T-testosterone; DHEA - dehydroepiandrosterone; DHT - dihydrotestosterone.

Loss of AR expression

Although the heterogeneity of AR expression in PCa is not correlated with response to ADT, higher degrees of AR positivity correlate with a greater degree of differentiation as well as a lower Gleason score. This heterogeneity is persistent, suggesting that increased AR expression do not associate with PCa initiation [52,77].

X chromosomes loss, which include loss of AR gene, is an extremely rare event in PCa [78]. On the other hand, one of the major resistance mechanisms is due to the epigenetically silencing of AR by hypermethylation of promoter which occurs in a late state of prostate carcinogenesis (7%) [66,79]. This has been observed in 8% of primary PCa cases [79].

Local androgen biosynthesis

Androgens within the tumor may come either from an adrenal source or from an intratumoral mechanism. It has been shown that prostate tumors do not have a completely androgen-free environment. As described before, the canonical pathway begins with cholesterol, leading through multiple steps to the production of DHT. Alternatively, the "backdoor pathway" uses CYP17A1 to convert pregnanes to androgens that are 5α- and 3-keto-reduced, ending to a terminal conversion to DHT. The other pathway, the 5α-dione pathway, bypasses the need of testosterone as a precursor and uses the 5a-dione instead. These alternative pathways uses additional enzymes: 3β-HSD1, 17β-HSD, and SRD5A [25–27,80,81].

AR amplification

AR gene amplification has been documented in 20%-30% of metastatic castration-resistant prostate cancer (mCRPC) and recurrent primary tumors, but not in hormone-dependent cancers. Contrarily, in untreated PCa AR amplification is very rare (<5%) [64,82,83]. Therefore, treatment possibly induces selective pressure [84]. These amplifications predict resistance to both enzalutamide and abiraterone acetate [85]. In fact, AR amplification allows PCa cells to become sensitive to low levels of androgens after ADT, enhancing AR activity and thus proliferating in a reduced androgen environment [73,86,87]. AR protein is expressed in prostate cancers of all clinical states. This alteration sensitizes the tumor PCa cells to respond to low levels of ligand [88]. Hence, CRPC patients with this amplification survive longer than the ones without it [64,87].

Genetic alterations

There are some genetic alterations that target AR, PI3K, Wnt, DNA repair and cell cycle pathways in nearly all metastatic PCa and several primary PCa [3,84,89]. The most frequent altered genes in mCRPC are AR (62.7%), TP53 (53.3%) and phosphatase and tensin homolog (PTEN) (40%) [84]. Numerous primary PCa tumors have recurrent point somatic mutations, resulting in a single amino acid substitution, copy number alterations and oncogenic structural DNA rearrangements [36,52,90].

DNA rearrangements

The majority of DNA rearrangements (57%) are translocations involving E26 transformation-specific (ETS) family of transcription factors [2,84]. ETS-related gene (ERG), has oncogenic properties, since it activates PI3K signaling pathway leading to PCa progression [91]. Additionally, transmembrane protease serine 2 (TMPRSS2), another AREs, deregulation is also implicated [92,93]. The TMPRSS2:ERG fusion is present in approximately half of localized PCa cases [2,91,92]. TMPRSS2 can also fuse with ETV1, ETV4 and ETV5 [2,84].

Mutations

Most of the point mutations occur in the LBD (49%) which confer hypersensitivity or promiscuity to other ligands, thus activating AR [44,48,94]. Furthermore, most of them are associated with gains of function, thus making the receptor more sensitive to native ligand, to other steroid hormones or to specific antiandrogens used in therapy [52,94]. The proportions of rest of the mutations are 40% in the NTD and 7% in the DBD [48].

In untreated patients, AR mutations normally increase with PCa stage [95]. These mutations are very rare in early-staged prostate tumors [95]. Moreover, the mutations are detected in 10 to 30% of patients previously treated with AR antagonists [96]. Furthermore, since the AR mutations occur before hormonotherapy, it suggests that this therapy does not lead to AR mutagenesis [95]. On the other hand, the most frequent functional consequences of several AR mutations cause AR antagonists to become agonists switching the normal inhibition to inducing proliferation as well as AR transcription by adrenal androgens [52,53]. This antagonist-agonist switch is mostly found in CRPC phenotype [52].

The most common AR mutation is T876A which occurs in approximately 30% of metastatic CRPC after ADT combined with an antiandrogen [52]. AR T878A in another common mutation that cause a gain-of-function in the LBD [48].

Variants

AR genomic structural rearrangements are presented in one-third of mCRPC tumors leading to expression of several AR variants either lacking the LBD, resulting in constant activation of AR signaling [97–99]. They arise due to alternative splicing or AR gene rearrangements [98]. Although more than 20 AR-variants have been identified, androgen-receptor splice variant 7 (AR-V7) is the most common one [84].

AR-V7 comprises the NTD and the DBD and lacks the LBD domain which makes it constitutively active [97,100]. This mutation has been detected in metastatic CRPC patients and in primary PCa tissues associated with poorer outcomes such as biochemical recurrence and shorter survival rates [97,100,101]. Previous studies demonstrated that AR-V7 induces PCa cell growth and progression in the absence of androgens and patients with increased AR-V7 levels do not respond to enzalutamide and abiraterone [84,100,102,103]. However, this variant is sensible to taxane chemotherapies such as docetaxel and cabazitaxel [100,102]. It is suggested that the ADT-induced AR transcription rate and splicing factor recruitment to AR precursor mRNA (pre-mRNA) contribute to the high AR-V7 levels in PCa cells [98]. Moreover, AR-V7 not only activates target genes independently of androgens, but also activates the normally ligand-dependent AR in a ligand-independent manner, facilitating its nuclear localization and transcription of target genes [100,104].

Coregulators

AR transcriptional activity is regulated by coactivators or corepressors that increase or reduce the receptor function respectively [33,105]. They recruit several transcription factors associated with RNA polymerase [105]. Almost 300 nuclear receptor coregulators have been identified [106]. Since the interaction between AR and its coactivators enhance the transcriptional activity of steroid receptors, allowing them to be active despite low androgens concentrations, it has been proposed that overexpression of coactivators may contribute to carcinogenesis in PCa [107].

Coactivator proteins such as ARA54 and ARA70 overexpressed in PCa enhance the activity of AR to alternative ligands, sensitize the receptor to lower concentrations not only of native, but also of nonnative ligands and induce ligand-independent activation by receptor tyrosine kinases (RTK). Consequently, these coactivators may contribute to ADT failure, possibly increasing the onset of CRPC [33]. The coactivator p300 interacts with AR and plays an important role in AR androgen dependent activation. It weakens histone-DNA interactions due to its histone-acetyltransferase activity, facilitating the access of different transcription factors to the DNA molecule [108]. Several tyrosine kinases SRCs overexpressed in PCa can increase the AR transcription by interacting with AR N-terminal portion. SRC-1 is overexpressed in 50% of CRPC cases when compared with normal

prostate, while SRC-3 expression was correlated with increased PCa grade and stage and decreased disease-free survival [48,52].

Corepressors such as nuclear receptor corepressor (N-CoR) coupled with silencing mediator of retinoid and thyroid receptors (SMRT) antagonize the action of bicalutamide and flutamide. These corepressors contribute to the agonist activity of these agents in a ligand-dependent manner, since they inhibit AR function by a direct interaction [33,52].

EPIGENETICS CONCEPT

Epigenetics is defined as heritable and reversible modifications in gene expression patterns that persist during cell division. Unlike genetic abnormalities, epigenetic changes do not alter DNA sequence [109,110]. Moreover, epigenetic plasticity can be driven by genetic, environmental and metabolic stimuli which can lead to cell adaptation and malignant progression [111]. Epigenetic deregulation is present in cancer initiation, thus being considered a hallmark of cancer [112]. Currently, there are four major epigenetic mechanisms: DNA methylation, histone post-translation modifications or chromatin remodeling, histone variants and non-coding RNAs (***Figure 5***). Since epigenetic abnormalities can be reverted, epigenetic therapies seem to be a promising approach regarding cancer treatment [113].

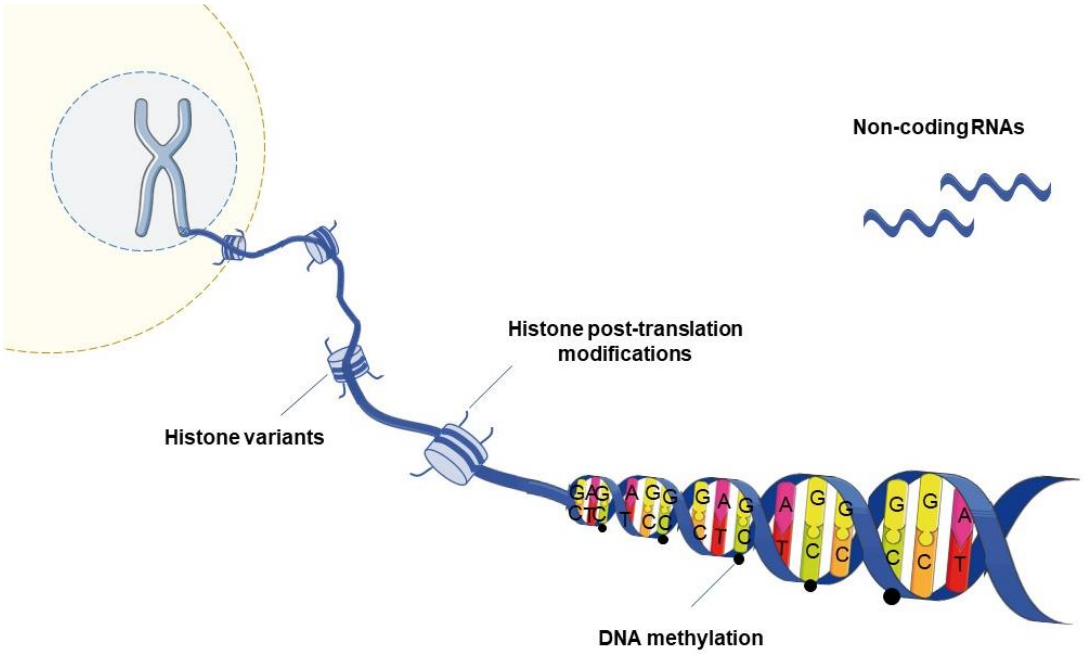

Figure 5: The four major epigenetic mechanisms include histone variants, histone post-translation modifications, DNA methylation and non-coding RNAs.

DNA Methylation

DNA methylation is the most well studied epigenetic mechanism [113]. In cancer, DNA methylation occurs mostly at cytosines within cytosine-phosphate-guanine (CpG) dinucleotide of gene promoters [92,113]. It consists in a covalent addition by DNA methyltransferases (DNMTs) of a methyl group to the fifth carbon of a cytosine ring, resulting on a new DNA base, 5-methylcytosine (5mC) [114].

The DNMTs enzymes catalyze this process: DNMT1, DNMT3A and DNMT3B. DNMT1 is responsible for maintaining DNA methylation patterns during cell division, while DNMT3a and DNMT3b have both *de novo* methylation activity [115,116]. Reversal of the DNA methylation process can be achieved by ten-eleven-translocation (TET) proteins which catalyze the oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) [117].

Cancer cells exhibit alterations in DNA methylation profile. In general, they display hypomethylation in normally methylated regions, resulting in genome instability and activation of proto-oncogenes. Additionally, they gain hypermethylation at gene promoters that are normally unmethylated, leading to transcription silencing of tumor suppressor genes involved in several cell functions, such as DNA repair, cell signaling and cell-cycle regulation [118–120].

DNMTs inhibitors

Several compounds are able to restore the normal methylation patterns by irreversibly inhibiting DNTMTs enzymatic activity and stimulating their proteasomal degradation [121,122]. DNMT inhibitors (DNMTi) can activate epigenetically silenced tumor suppressor genes (TGS), resulting in cell death, cell cycle arrest, chromatin extension and induction of cell differentiation [123]. Moreover, these inhibitors may contribute to tumor cell reversion phenotype, bringing significant clinical benefits for patients [124].

There are two types of DNMTi: the nucleoside and non-nucleoside analogues [124]. Nucleoside analogues have a modified cytosine ring that is connected to either a ribose or deoxyribose and may be integrated into RNA or DNA during the S phase of the cell cycle. They covalently bind to DNMTs, inhibiting them and inducing cell death or DNA damage [125]. On the other hand, non-nucleoside analogues bind directly to the catalytic region of DNMT without incorporating into DNA [126].

5-azacytidine and 5-aza-2'-deoxycytidine

5-azacytidine (*Vidaza*) and 5-aza-2'-deoxycytidine (*Dacogen*) are the most well characterized nucleoside analogues (***Figure 6***). These drugs have been widely used in pre-clinical and clinical trials in several cancer models due to their anti-tumorigenic activity [127,128]. Although these drugs were developed in 1964 as cytostatic agents, their *in vitro*

cell proliferation induction and their involvement in DNA methylation inhibition was only later discovered [129,130]. Both were FDA-approved for myelodysplastic syndromes (MDS) on 2004 and 2006, respectively, since they demonstrated clinical benefit in clinical trials with hematologic cancer patients [131–134]. Moreover, 5-azacytidine and 5-aza-2'-deoxycytidine are also active against acute myeloid leukemia (AML) and other myeloid malignancies [135]. Nonetheless, these compounds present several limitations in clinical practice due to their cytotoxic effects at higher doses, side effects such as neutropenia and thrombocytopenia and short half-life [136,137].

Comparing to hematolymphoid malignancies, the lack of success of azanucleosides in solid tumors may be explained by the higher proliferative rate of the former ones. Additionally, these agents can potentially cause global hypomethylation resulting in unwanted re-expression of epigenetically silenced genes, which can contribute to tumorigenesis, progression and aggressiveness [127].

Hydralazine

Hydralazine hydrochloride (*Apresolin*) was approved by the FDA for the treatment of severe hypertension and heart failure. Nowadays, it is commonly used for hypertension in pregnancy [37,138]. Recently, hydralazine has been recognized as a demethylating agent (***Figure 6***) [139]. It is suggested that this compound interacts directly with the active site of DNMTs through its nitrogen atom, consequently inhibiting DNA methylation [140]. However, its mechanism of action is still not well understood.

Although its half-life in plasma is approximately 1h, the duration of the hypotensive effect lasts up to 12h. The recommended dose varies from 10mg four times a day to 50mg and common side effects include headache, nausea, flushing, low blood pressure, palpitation, tachycardia, dizziness and angina pectoris. Furthermore, it can cause autoimmune reactions, such as drug-induced lupus-like syndrome [138]. In fact, long term use of high doses of hydralazine was associated with a high incidence of lupus erythematosus [141], which is characterized by a decreased global DNA methylation profile. This observation sparked interest in hydralazine as a DNA methylation inhibitor [142].

Pioneer *in vitro* studies using hydralazine in T cell lines showed that it induced self-reactivity and DNA hypomethylation. Furthermore, later studies demonstrated that hydralazine was able to restore expression of TSG silenced by hypermethylation of their respective promoters in cancer cell lines and primary tumors [139,143], without significant cytotoxic effects [140,144,145]. This hypomethylating effect is related to the decrease of DNMT1, DNMT3a and DNMT3b activity in different cancer models. Several pre-clinical studies confirmed the DNA methylation inhibiting activity of hydralazine upon various genes, including AR [138].

Hydralazine has been investigated as an epigenetic drug in several clinical trials targeting solid tumors (***Table 2***). One phase I study in cervical carcinoma demonstrated that hydralazine at doses between 50 and 150mg/day is well tolerated and is able to demethylate and reactivate TSG such as APC, MGMT, ER, GSTP1, DAPK, RARβ, FHIT and p16. Importantly, this occurs without affecting global DNA methylation, which is a major caveat of using other FDA-approved, DNMTi drugs, 5-azacytidine and 5-aza-2'-deoxycytidine [146].

A phase II trial in breast cancer also demonstrated the proposed molecular effects of hydralazine and valproic acid as DNMTi and HDACi respectively. In fact, they were able to reactivate TSG in breast cancer tumors. Interestingly, they increased the efficacy of chemotherapy, which was not expected, since epi-drugs have been associated with more myelotoxic side effects [147]. Another phase II trial based on 17 patients with solid tumors (cervix, breast, lung, testis and ovarian) who acquired chemotherapy resistance showed an overall response, disease stabilization and several symptoms' improvement. They were treated with hydralazine and valproic acid followed by the chemotherapy agents that they were previously submitted to [148]. Almost 1000 downregulated genes of several signaling pathways in untreated cervical cancer patients turned out to be upregulated after hydralazine and valproic acid in one phase II clinical trial, highlighting the importance of investigating the demethylation inhibiting activity of these compounds [149].

Although it was terminated for administrative reasons, preliminary results from a phase III clinical trial in cervical cancer demonstrated that patients treated with hydralazine and valproic acid showed an improved progression-free survival when compared with those subjected to standard combination chemotherapies [150].

Table 2: Clinical trials with hydralazine in monotherapy and in combination.

Trial phase	Evaluated combination	Histology	Reference
Phase I	Hydralazine	Untreated cervical cancer	[146]
Phase I	Hydralazine plus Valproic Acid	Advanced Solid Cancer (colorectal, cutaneous melanoma, ovary, breast, soft-tissue sarcoma, non–small cell lung, head and neck, cervix, ocular melanoma and gastric)	[151]
Phase II	Hydralazine plus Valproic Acid	Locally advanced breast cancer	[147]

Phase II	Hydralazine plus Valproic Acid	Refractory solid tumors (cervix, breast, lung, testis and ovarian) with chemotherapy resistance	[148]
Phase II	Hydralazine plus Valproic Acid	Cervical cancer	[149]
Phase III	Hydralazine plus Valproic Acid	Advanced cervical cancer	[150]

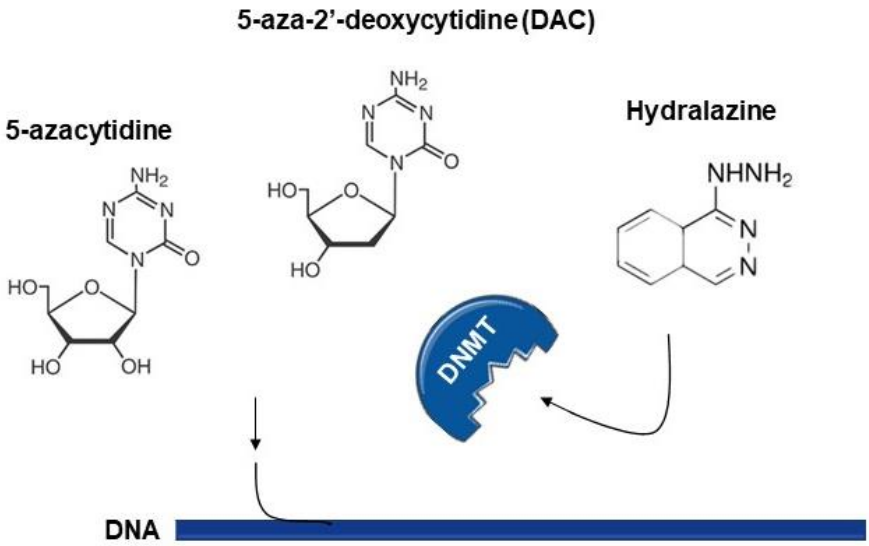

Figure 6: Mechanism of action of nucleoside (5-azacytidine, 5-aza-2'-deoxycytidine) and non-nucleoside DNMT inhibitors (hydralazine).

DNMTi in PCa

DNA methylation can lead to gene silencing, contributing to drug resistance in antihormone therapies [66]. Therefore, the investigation of DNMTi is important for re-sensitizing malignant cells to antineoplastic agents [66,124,152]. Nevertheless, their clinical effectiveness is not entirely dependent on their DNA methylation inhibitory activity and its efficacy in solid tumors is not fully demonstrated [37].

There are several ongoing phase I and II clinical trials that include an antiandrogen plus an epi-drug as a treatment option for mCRPC. Specifically, there is a phase Ib trial followed by a phase II trial with enzalutamide and decitabine, which will study the side effects and best dose of decitabine and how well it works when given with enzalutamide. Moreover, they intend to determine the 12-month progression-free survival rate. Participants receive decitabine intravenously over 1 hour on days 1-5 and enzalutamide orally once daily on days 1-28. Courses will be repeated every 28 days in the absence of disease progression or unacceptable toxicity (NCT03709550).

The methylation of CpG islands in the promoter is a common form of epigenetic regulation of gene expression [66]. Furthermore, there are at least 100 genes involved in some cellular functions that are *de novo* methylated during prostate carcinogenesis [37]. Hence, re-expression of AR in AR-deficient tumor cells can re-sensitize them to ADT, thus reversing their nature and providing a treatment option [66,71].

Hydralazine has been shown to reverse PCa cell phenotype by demethylating and hence allowing the re-expression of some genes that are known to be epigenetically silenced in PCa. Moreover, hydralazine was able to induce DNA damage. Therefore, since the cells could not repair efficiently the DNA, this compound may induce chemotherapy sensitivity and radiosensitivity, leading to synthetic lethality. In addition, it has been reported that it downregulates several proteins that take part in EGFR signaling pathway in PCa cells.

Comparing to 5-aza-CdR, hydralazine has a higher demethylase activity. Therefore, it might be more attractive from a clinical point of view, since gene re-expression can be maintained for longer periods. Moreover, hydralazine presents minimal secondary effects and a safer profile than 5-aza-CdR [37].

CRPC TREATMENT

In the past ten years, novel treatment approaches have emerged for this subset of patients (***Figure 7***). However, they are based on a general approach, instead of an individual treatment modality. Regarding the heterogeneity in PCa, future investigations concerning the drugs' mechanisms of action should be created [32].

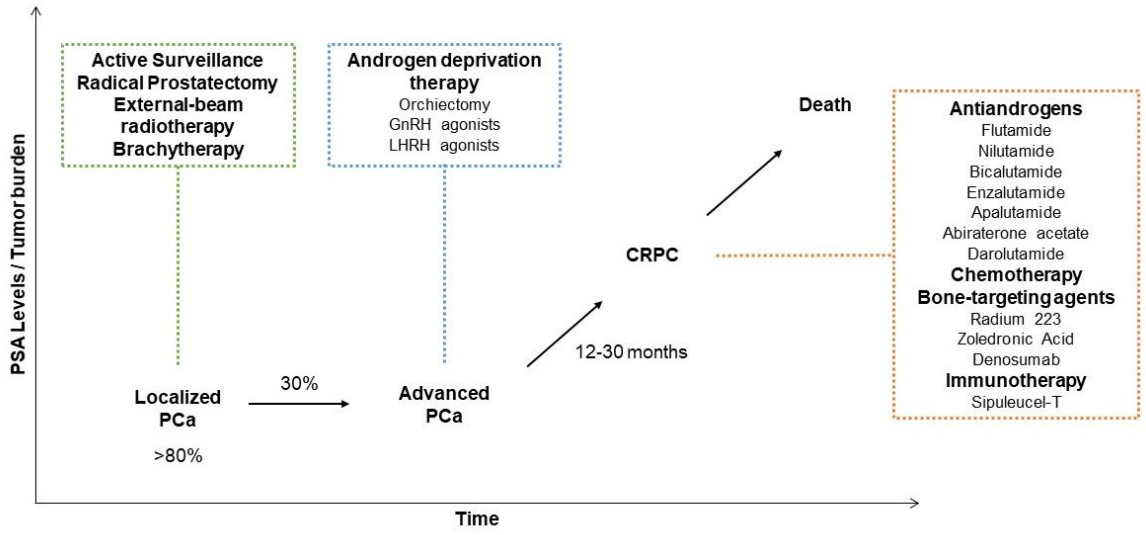

Figure 7: Resume of the different treatment options for each PCa stage. Adapted from [181].

Antiandrogens

Castration-resistant patients can be treated by blocking AR with antiandrogens either alone or in combination with hormonal therapy and orchiectomy [52]. Although nonsteroidal antiandrogens (NSAAs) can improve the clinical effects of the initial testosterone rise associated with LHRH agonists, they do not suppress testosterone secretion [18].

However, NSAAs can function as AR agonists in the presence of AR amplification or mutations [50]. In these cases, after its discontinuation and maintenance of GnRH therapy, some of them show decreases in PSA levels, regression of tumor mass and relief of cancer-related symptoms [33,50]. Hence, CRPC patients should administer a secondary antiandrogen, inhibit the adrenal androgen production and further inhibit LH with progesterone or estrogenic agents [52]. Multiple studies have proven that the combination of surgical castration or LHRH agonists with NSAAs can improve survival (<5%) [18].

Flutamide and Nilutamide

Flutamide and nilutamide are both NSAA used in locally advanced or metastatic PCa in conjunction with LHRH agonists and in metastatic PCa patients who were submitted to orchiectomy, respectively [153].

Flutamide in combination with castration can reduce prostatic DHT levels to 20% by blocking the ability of the residual DHT to activate AR transcription [52]. However, since it must be administered three times a day due to its short half-life, patients' compliance can be difficult [154].

Nilutamide can achieve a significant sustained PSA response [155]. The use of flutamide or nilutamide is limited by their side effects, namely gastrointestinal disturbances, alcohol intolerance and others [154] .

Bicalutamide

Bicalutamide (*Casodex)* is an oral nonsteroidal competitive inhibitor of androgens at AR for use in conjunction with LHRH agonists in men with androgen-responsive PCa [18,50]. Although it is similar in structure to flutamide, it demonstrated a favourable safety and tolerability profile [18]. It was approved by the FDA in 1995 for metastatic PCa with a regimen of 50mg orally and daily [156].

In CRPC, bicalutamide is recommended as second-line therapy [50]. When used as monotherapy, the overall physical performance can be preserved but it is less effective than when combined with ADT, in terms of overall survival and clinical progression. Moreover, treatment failure and discontinuation were more common in monotherapy due to its adverse events [18]. These include breast tenderness, gynecomastia and hot flushes. Bicalutamide

is superior to flutamide and nilutamide since it does not have as much gastrointestinal side effects nor the alcohol intolerance and visual disturbances associated with either drug, respectively. [154].

Enzalutamide

Enzalutamide (*Xtandi*) is an AR antagonist that binds directly to the AR's LBD, thus inhibiting the binding of androgens. Furthermore, unlike bicalutamide, it interrupts the nuclear translocation of AR and consequently inhibits the transcriptional activity of this protein [34,50,103].

This compound was approved by the FDA in 2012 for CRPC patients previously treated with chemotherapy and for chemotherapy-naïve patients [157–160]. In 2018, it was proven that it also brings benefits for nonmetastatic CRPC patients, which led to its approval by the FDA for this subset of patients [34,160]. The recommended dose is 160 mg administered as a single oral daily dose and it is commercialized in four 40 mg soft capsules [156].

It has an excellent side-effect profile, demonstrated an overall survival improvement and delayed the disease progression, development of metastases, PSA progression and the time to the first subsequent antineoplastic drug [34,158]. Unlike bicalutamide, it does not act as an agonist for the wild-type AR [50]. This drug has higher affinity for the AR and superior influence in its signaling pathways than bicalutamide and flutamide [50].

Enzalutamide resistance has been implicated in ADT induction of glucocorticoid receptor (GR) which can bind to many AREs [161]. Its adverse effects include fatigue, being the most common one, hypertension, major cardiovascular events, cognitive and mood impairment, mental impairment disorders, falls, and bone fractures [3,32,34,49].

Abiraterone acetate

Abiraterone acetate is an enzyme inhibitor that targets cytochrome P450 17A1 (CYP17A1), which is involved in intratumoral steroidogenesis [18,103,162].

This antiandrogen (*Zytiga, Yonsa*) was approved by the FDA on April 28, 2011 in combination with prednisone for metastatic CRPC and men who have received docetaxel. The recommended dose is 1000mg of abiraterone acetate (administered as four 250mg tablets) with 5mg of prednisone twice daily [57,156].

It reduces testosterone levels both in castrate and noncastrate patients [163]. As this compound targets CYP17A1, it inhibits both classical and alternative pathways. In this way, the steroidogenesis network, specifically the adrenal androgen production, is interrupted resulting in fewer pathways to maintain intratumoral DHT levels [35].

Abiraterone adverse events include those related to mineralocorticoid excess, hypokalemia, hypertension, cardiac and liver dysfunction and fluid retention [3,32,49]. Currently, most patients are treated with abiraterone or enzalutamide as first-line treatment, not leaving any other perspectives for second-line treatments [3].

Apalutamide

Apalutamide (*Erleada*) is an AR inhibitor that binds directly to the AR LBD and prevents androgen receptor translocation, DNA binding androgen receptor-mediated transcription.

It was approved by the FDA in 2018 for the treatment of non-metastatic CRPC. The recommended dosage is 240mg (four 60mg tablets) once daily.

This antiandrogen significantly improved metastasis-free survival, time to metastasis, progression-free survival and time to symptomatic progression. Its side effects were fatigue, hypertension, rash, diarrhea, nausea, weight loss, arthralgia and falls, being fatigue the most common one [164].

Darolutamide

Darolutamide (*Nubeqa*) is a novel AR inhibitor with a different structure, which has higher affinity for AR than enzalutamide and apalutamide.

Although its mechanism of action is similar to the other second-generation AR inhibitors, it has the ability to inhibit mutant AR as well. In fact, darolutamide inhibits AR F876L mutation which can arise after enzalutamide or apalutamide treatment [165]. Moreover, it increased the metastasis-free survival, overall survival, time to pain progression, time to cytotoxic chemotherapy and time to a symptomatic skeletal event [166].

This drug was approved by the FDA in 2019 for the treatment of non-metastatic CRPC and it is orally administrated with a recommended dose of 600mg twice a day [165].

Its side-effect profile is improved relatively to enzalutamide and apalutamide, since its distinct structure allows a lower penetration of the blood-brain barrier [165,166].

Chemotherapy

Docetaxel is an anti-mitotic chemotherapeutic agent that inhibits the depolymerization of microtubules, inhibiting mitosis. Polymerized microtubules cannot separate leading to disruption of the normal mitotic process and thus to apoptosis [39].

The standard treatment option for metastatic PCa was docetaxel 75 mg/m^2 in three weekly doses up to 10 cycles, but due to its toxic side-effects, its recommendation was delayed, being replaced by several hormonal therapies [167]. However, it is still recommended in CRPC patients [18].

There are some prognostic factors that should be considered before docetaxel treatment: PSA >114 ng/ml, PSA DT <55 days or the presence of visceral metastases. The factor age was not considered to be a contraindication, although it must be monitored as well as the comorbidities associated with it [18]. Its side-effects consist of myelosuppression, fatigue, alopecia, diarrhea, neuropathy and peripheral edema [49].

Docetaxel resistance has been associated with apoptosis pathways, specifically upregulation of an important cell cycle regulator, p53 [168]. Patients who develop resistance to docetaxel can recur to another taxane, cabazitaxel with 25 mg/m^2 every three weeks [18,167,169]. As docetaxel, this drug should be administered preferably with prophylactic granulocyte colony-stimulating factor and neutropenia and sepsis should be carefully observed [18].

Bone targeting agents

Since these drugs inhibit the osteoclast-mediated bone resorption, patients should have a dental examination before starting this therapy [18,170]. They have a higher risk of osteonecrosis of the jaw when they suffer a trauma, dental surgery, dental infection or when they are administered with long-term intravenous bisphosphonate [3,18]. The optimal timing, schedule and duration for this targeted-bone therapy and the overall balance of benefit and risk as well as efficacy in the context of novel mCRPC treatments are still uncertain [3].

Radium 223

Radium 223, an alpha emitter, is the only bone-targeted drug that could improve significantly median overall survival, delay the first skeletal event and improve pain scores and quality of life [18,49]. Therefore, it is the only drug that is associated with a survival benefit.

It was approved on May 15, 2013 for metastatic CRPC patients with a recommended dose of 1,35 microcuries per kilogram of body weight injected every four weeks for six injections [167].

In general, Radium 223 was shown to be effective, but also safe notwithstanding the pretreatment with docetaxel [18]. Hence, it is considered a first-line agent for symptomatic mCRPC patients with bone metastases with either abiraterone or enzalutamide [3]. Side-effects of radium-223 include myelosuppression and diarrhea [49].

Zoledronic acid

This drug is a parenteral bisphosphonate that inhibits osteoclast-mediated bone resorption [170].

FDA approved this compound (*Zometa*) on February 2, 2002 for treatment of metastatic CRPC with a recommended infusion of 4mg during 15 min every three weeks [171].

There are several studies that demonstrate the benefits of zoledronic acid in mCRPC patients, although it is not associated with improved overall survival yet [3,18]. Zoledronic acid is the only bisphosphonate that has shown a protective effect against skeletal-related events (SRE) in mCRPC patients [3]. Patients treated with zoledronic acid had not only less SREs, but the time to first SRE was longer. Additionally, they developed fewer pathological fractures [18,171].

Denosumab

Denosumab (*Prolia*) is a fully human monoclonal antibody directed against the receptor activator of nuclear factor kappa-B ligand (RANKL). RANKL is responsible for osteoclast formation, function and survival [172]. Herein, Denosumab inhibits osteoclast formation, function and survival and consequently bone resorption [3,18].

On 2011, FDA approved this drug to increase bone mass in metastatic CRPC, thus improving patients' quality of life. The recommended dose is 120mg every 4 weeks [46,172].

However, when compared to zoledronic acid, it was demonstrated to be more effective, safe and improved significantly the time to first SRE [3,18]. Moreover, it is given subcutaneously, contrarily to zoledronic acid, which requires intravenous access [172].

Immunotherapy

Sipuleucel-T (*Provenge*) is an immunotherapy that uses activated autologous dendritic cells [49]. This approach induces tumor-specific immunity by presenting a specific target to the patient's immune system. For that, antigen presenting cells (APCs) are collected from the patients' blood and incubated with a specific antigen, prostatic acid phosphatase. Afterwards, the vaccine is ready to be infused into the patient [173]

Provenge was approved by the FDA in 2010 for metastatic CRPC with a regimen of three infusions at two-week intervals for one month [167,173].

This vaccine showed a survival benefit in asymptomatic or minimally symptomatic mCRPC patients, having a very good overall tolerance [18,173]. However, the lack of PSA declines and progressive-free survival changes as well as its cost has limited its use [18,49]. Nonetheless, it was considered first-line treatment in asymptomatic mCRPC patients [3,49].

In resume, prostate cancer patients with localized disease are treated with active surveillance, radical prostatectomy or radiotherapy (EBRT or brachytherapy). If these patients recur, androgen deprivation therapy is recommended either via orchiectomy or GnRH or LHRH agonists. Unfortunately, CRPC patients do not have any curative therapies. However, there are already several options that can improve the overall survive as well as the patients' quality of life, such as the antiandrogens, chemotherapy, bone-targeting agents and immunotherapy.

AIMS

The main goal of this master's dissertation is to evaluate the effect of hydralazine, a DNA methyltransferase inhibitor (DNMTi), as a repositioning drug in malignant features of castration-resistant prostate cancer (CRPC) cell lines.

Specifically, the main tasks of this study are to:

1. Characterize the phenotypic and molecular effects of hydralazine in PCa cell lines in order to check if this drug's effects are dependent on AR methylation;
2. Determine the effect of sequential treatment with hydralazine and enzalutamide in PCa cell lines;
3. Perform AR gene bisulfite sequencing in wild-type RWPE, DU145 and PC-3 cell line to identify the CGs that are methylated in three different regions;
4. Perform AR gene bisulfite sequencing in DU145 cell line treated with hydralazine to identify the CGs that undergo methylation alterations.

MATERIAL AND METHODS

CELL CULTURE

Epithelial prostate cancer cell lines (DU145 and PC-3) and prostate cell line (RWPE) were obtained from American Type Culture Collection (ATCC, United States) representative of different relevant features of prostatic adenocarcinoma (***Table 3***). RWPE is non-malignant and hormone-sensitive cell line, whereas DU145 and PC-3 are castration-resistant ones. All cell lines were cultured in the recommended medium (Biotecnómica, Portugal) (***Table 3***) supplemented with 10% fetal bovine serum (FBS, Biochrom, Merck, Germany) and 1% penicillin-streptomycin (GIBCO, Invitrogen, USA) and were maintained at 37°C in a humidified atmosphere containing 5% CO_2. All prostate cancer (PCa) cell lines were routinely tested for Mycoplasma spp. contamination using two primers: GP01: ACTCCTACGGGAGGCAGCAGTA and MGS0: TGCACCATGTGTCACTCTGTTAACCTC (Sigma-Aldrich, United States).

Table 3. Characterization of the different cell lines used in this study.

Cell lines	Cell Type	Culture Properties	Disease	Growth Medium	Expressed Genes	Androgen sensibility
RWPE	Epithelial	Adherent	Non-malignant	Keratinocyte Serum-Free Growth Medium (K-SFM)	AR, cytokeratin 18, cytokeratin 8	Androgen-responsive
DU145	Epithelial	Adherent	Adenocarcinoma; Derived from metastatic site: brain	Eagle's Minimum Essential (MEM)	-	Castration-resistant
PC-3	Epithelial	Adherent	Grade IV adenocarcinoma; Derived from metastatic site: bone	RPMI-1640 with F-12 Nutrient Mixture (Ham)	HLA A1, A9	Castration-resistant

DRUG PREPARATION AND EC50 VALUE

Hydralazine hydrochloride (Sigma-Aldrich, United States) was dissolved in PBS (Biotecnómica, Portugal) at 50mM concentration each day of treatment. 5-aza-2'-Deoxycytidine (DAC) (Sigma-Aldrich, United States) was dissolved in 50% acetic acid and 50% at 10mM and stored at -20°C until further use. Enzalutamide was also prepared at a 50mM concentration and also stored at -20°C until further use.

For EC50 values calculation, cell lines were exposed to a range of compound concentrations (1µM, 5µM, 10µM, 15µM, 20µM, 30M, 40µM, 50µM, 100µM) every 24 hours during three consecutive days. Each cell line was also exposed to the respective drug vehicle: PBS for hydralazine, 50% acetic acid and 50% ethanol for DAC and dimethyl sulfoxide (DMSO) for enzalutamide. After three days of exposure, 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium-bromide (MTT) assay was performed and the EC_{50} values were calculated using GraphPad Prism 6.

CELL VIABILITY ASSAY

Cell viability was evaluated using MTT assay. RWPE, DU145 and PC-3 were seeded into 96-well plates at $1x10^3$ or $1x10^4$ cells per well. After having adhered overnight, cells were treated with the EC_{50} value of one of the tested drugs and its vehicle during three or seven consecutive days, exchanging the medium every 24 hours. Three biological and three experimental replicates were used for each condition. Afterwards, cell viability was measured at day 0 and 3. Briefly, 100µL of 0.5 mg/mL MTT (Sigma-Aldrich, United States) were added to each well and incubated in the dark at 37°C and 5% CO_2 for 3 hours. Then, formazan crystals were solubilized with 50 µL of DMSO. The absorbance was measured using a microplate reader (FLUOstar Omega, BMG Labtech, Germany) at a wavelength of 540nm with background subtraction at 630nm. The number of viable cells was calculated with the following formula:

$$Number\ of\ viable\ cells = \frac{\text{OD experiment x Number of cells at day 0}}{\text{Mean OD at day 0}}$$

APOPTOSIS ASSAY

Cells' apoptosis was assessed using the APOPercentage™ apoptosis assay kit (Biocolor Ltd., Northern Ireland), according to the manufacturer's guidelines. This assay is based on the movement of the transmembrane protein phosphatidylserine from the inside to the outside of the cell membrane layer, resulting in the uptake of the APOPercentage dye

by the apoptotic cells. Briefly, RWPE, DU145 and PC-3 were seeded into 24-well plates at $2x10^4$ cells per well. Cells were allowed to adhere overnight, being then exposed to the EC50 concentration as well as other concentrations of one of the tested drugs and with the respective vehicle for 3 days. One biological and three experimental replicates were used for each condition. The medium was renewed every day of treatment. Subsequently, at day 3, 2µL hydrogen peroxide was added to the positive controls and incubated at 37°C and 5% CO_2 for 1 hour. Then, media with 5% of APOPercentage dye was added and the plate was incubated for 10 minutes. Every 10 minutes (until 30 minutes, depending on cell type) cells were observed under microscope until at least ten cells were already dyed. Then, cells were washed with PBS 1x and trypsinized (TrypLE™ Express). After adding 200µL of the Dye Release agent and intense shaking for 15 minutes to release intracellular accumulated dye, 100uL of the samples were transferred to a 96-well plate. The absorvance was measured using a microplate reader (FLUOstar Omega, BMG Labtech, Germany) at a wavelength of 550nm with background subtraction at 620nm. Apoptosis levels were calculated according to the following formula:

$$Apoptosis\ Levels = \frac{\frac{\text{Apoptosis OD}}{\text{Mean MTT OD at day 3}}}{Mean\ Apoptosis\ OD\ normalized\ to\ MTT}$$

The results were expressed as the OD ratio of the cells exposed to the DNMTi normalized to vehicle.

DNA EXTRACTION AND BISULFITE MODIFICATION

Prostate cancer cell lines (RWPE, DU145 and PC-3) were seeded into T25 flasks at $9x10^5$ cells per flask and three experimental replicates were used for each condition. Cells were allowed to adhere overnight, being then exposed to the EC_{50} concentration of one of the tested drugs and with the respective vehicle for 3 days. The medium was renewed every 24 hours. Afterwards, pellets from each condition were collected for DNA extraction. DNA was extracted according to the standard phenol-chloroform protocol and quantified using NanoDrop Lite Spectrophotometer (Nanodrop Technologies, USA) and Qubit dsDNA BR Assay Kit (Invitrogen, USA).

One microgram of DNA was modified with sodium bisulfite, using EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. Modified DNA was eluted with 60 µL of sterile distilled water and stored at -80°C until further use. This treatment chemically converts unmethylated cytosines to uracils, leaving the methylated ones intact. One microliter of CpGenome™ Universal Methylated DNA and one microliter of CpGenome™ Universal Unmethylated

DNA vial A (Merck Millipore, USA) was also modified and eluted in 20µL of sterile distilled water to function as a positive and negative control respectively.

BISULFITE SEQUENCING

Primer design and producing PCR product

The promoter region of androgen receptor gene was divided into three regions, each one with a previous designed pair of primers using Methyl Primer Express v1.0 (***Table 4***, ***Figure 8***). Amplification of DNA of interest was carried out with the following primers:

Table 4: Primers and conditions for each region.

Region	Location	Product size	Forward	Reverse
1	-307bp to 41bp	349bp	5'-GGAATTAAATTTGGTGAGTGT -3'	5'-GGAGTTAGTTTGTTGGGAGA -3'
2	22bp to 290bp	269bp	5'-GGAGTTAGTTTGTTGGGAGA -3'	5'-GTAAGGAAAGTGTTTGGTAGG -3'
5	1115bp to 1469bp	355bp	5'-AAGTTTAAGGATGGAAGTGTAGTT -3'	5'-GGTTTTGGATGAGGAATAGTAA -3'

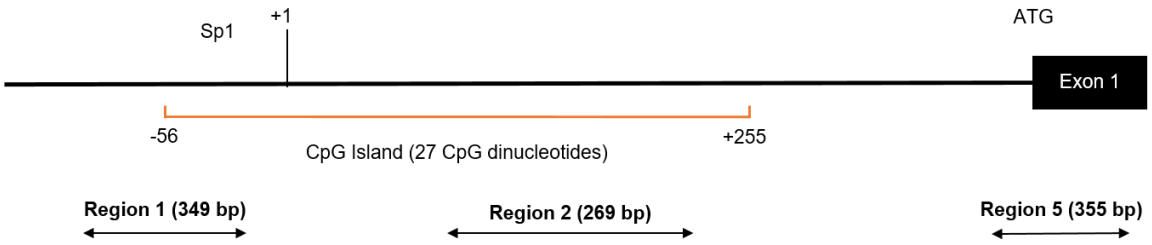

Figure 8. AR promoter gene divided into three regions: region 1 displays 16 CpG sites and a Sp1 binding-site; region 2 is located within the CpG island with 21 CpGs sites; Region 5 includes 14 CpG sites, begins with the ATG codon, continues with the first exon and ends with the CAG repeat.

PCR conditions were as follows: at 95°C for 15 minutes, 40 cycles of 95°C for 30 seconds, 60°C (Region 1, 2 and 5) for 30 seconds, 72°C for 30 seconds and 72°C for 30 minutes, using Xpert Hotstart Mastermix (Grisp, Portugal). PCR products were run on 2% Tris-borate-EDTA buffer (TBE) agarose gels to confirm the specificity of the PCR reaction.

Cloning reaction and transformation

One microliter of the aforementioned PCR product was subcloned into the TOPO TA vector (TOPO TA Cloning kit for sequencing, Invitrogen). Afterwards, 2 µL of this reaction were added to One Shot® chemically competent E. coli vials (Subcloning efficiency DH5a competent cells, Invitrogen). Bacterial transformation was carried out by heat-shock at 42°C for 45 seconds. Afterwards, 250 µL of super optimal broth (S.O.C., NzyTech, Portugal) were added to the bacterial suspensions that were grown in the thermoshaker for 1 hour at 300 rpm. Meanwhile, 40 µL of a 40 ng/ml concentrated X-Gal (NzyTech, Portugal) were added to previously warmed plates (kept at 37ºC). Then, 150 µL of the transformed bacteria were seeded onto the Petri plates, which were incubated overnight at 37ºC. The next day, at least 10 white colonies were collected into a 0,1 Tris-EDTA buffer. To amplify our DNA, colony PCR was performed for 35 cycles of 94°C for 45 seconds, 50°C for 30 seconds, and 72°C for 1 minute using Xpert Hotstart Mastermix (Grisp, Portugal).

PCR product purification and sequencing reaction

The samples were purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, United States) according to manufacturers' instructions. Afterwards, 1µL of the purified sample was used to perform the sequencing reaction using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems™/Thermo Scientific Inc., USA) using either M13 forward or reverse primers. The products were purified using Sephadex 50 (GE Healthcare, United States) resine and 20µL of Formamide (Sigma-Aldrich, United States) were added, followed by a denaturing step at 95ºC for 5 min. Finally, the samples were sequenced by Sanger sequencing in a 3500 Genetic Analyzer (Applied Biosystems™/Thermo Scientific Inc., USA).

BiQ Analyzer: Visualization and quality control for DNA methylation data from bisulfite sequencing v2.02 software was used in order to evaluate every sequence. The percentage of minimum sequence identity and minimum conversion rate were 80 and 90, respectively.

PROTEIN EXTRACTION AND QUANTIFICATION

Total protein was extracted from all untreated cell lines and from cells exposed to hydralazine and DAC. The pellets were collected and homogenized in RIPA Lysis Buffer (Santa Cruz Biotechnology, USA) supplemented with protease and phosphatase inhibitors' cocktail (Santa Cruz Biotechnology, USA). Afterwards, the samples were sonicated in 5 cycles of 20 seconds ON and 20 seconds OFF. Then, the samples were centrifuged at 13,000 rpm for 15min at 4ºC and the supernatant was collected. At last, the concentration

of the isolated proteins was determined using Pierce™ BCA Protein Assay Kit (Thermo Scientific Inc., USA), according to the manufacture's procedures.

WESTERN BLOT

Thirty micrograms of the protein were separated in 10% polyacrylamide gel by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After that, it was transferred into a nitrocellulose membrane (Bio-Rad, USA) in a 25mM Tris-base/glycine and 20% ethanol buffer using the Trans Blot Turbo System (Bio-Rad, USA) during 18 minutes at 25V. Membranes were blocked with 5% of nonfat dry milk powder (Bio-Rad, USA) in Tris-buffer saline (TBS)/ 0,1% Tween (TBS/T pH=7,6) for one hour at room temperature. Afterwards, the membranes were incubated overnight at 4ºC with specific primary antibodies for AR (AR441; Invitrogen, USA) PSA (ab76113; Abcam, United Kingdom) and β-Actin as loading control (A5316; Sigma-Aldrich, United States) (***Table 5***). After incubation, the membranes were washed in TBS/T and incubated with appropriate secondary antibody coupled with horseradish peroxidase (Bio-Rad, USA) diluted 1:4000 for one hour at room temperature. Following three more (3x5min) washing steps, signal was developed with an enhanced chemiluminescence detection kit (Clarity and Clarity Max ECL Western Blotting Substrates (Bio-Rad, USA). Finally, quantification of the western blots' signal was performed using the using ImageJ software.

Table 5. All antibodies used in Western Blot and its conditions.

Primary antibody	Company	Clone	Western-Blot dilution	Second antibody specie	Clonality
AR	Invitrogen	AR441	1:1000	Anti-rabbit	Monoclonal
PSA	Abcam	ab76113	1:500	Anti-rabbit	Monoclonal
β-Actin	Sigma-Aldrich	A5316	1:10 000	Anti-mouse	Monoclonal

STATISTICAL ANALYSIS

The statistical analysis was performed using GraphPad Prism 6.0 software. One-way analysis of variance (ANOVA) with post-hoc Dunn's multiple comparison test was used in order to compare the results obtained in each parameter for the different concentrations and vehicle in *in vitro* assays. In each analysis, the p values were significant only if it was below 0.05.

RESULTS

PROSTATE CANCER CELL LINES CHARACTERIZATION

Herein, three PCa cancer cell lines were selected: DU145 and PC-3, AR-negative cell lines, whereas RWPE is non-malignant and AR-positive cell line, although DHT induction is required. Since DHT commercialization in Portugal is limited, we induced this cell line with testosterone (0,1nM and 1nM), which showed to be not effective in re-expressing AR in RWPE. In fact, DHT was reported to have a much higher affinity (2-fold to 10-fold) to AR than testosterone [73]. Therefore, in this assay LNCaP cell line, a tumor cell line that express AR was used as positive control (***Figure 9***).

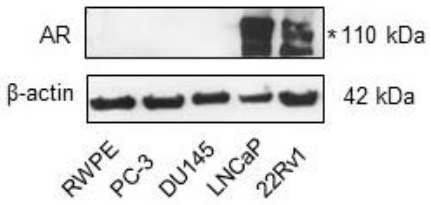

Figure 9. PCa cell lines characterization according to AR expression.

HYDRALAZINE TREATMENT VALIDATION

Although we were not able to re-express AR in DU145 cell line after hydralazine exposure (***Figure 10***), decreased DNMT1 expression was achieved in cells treated with higher hydralazine's concentrations (***Figure 11***) following previous results obtained in our research team [37].

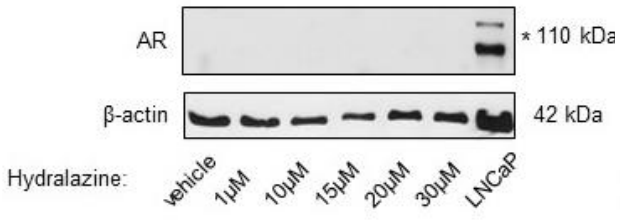

Figure 10. AR-expression in DU145 cell line treated with different hydralazine concentrations.

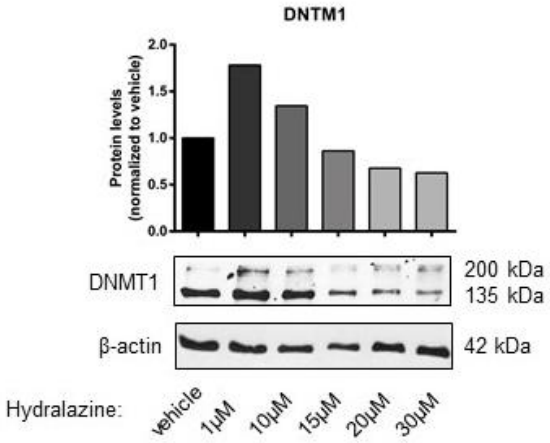

Figure 11. DNMT1 protein levels after hydralazine treatments in DU145 cell line.

EC_{50} VALUE OF HYDRALAZINE AND ENZALUTAMIDE IN PCA CELL LINES

Androgen independent PCa cell lines (DU145 and PC-3) were tested for their sensitivity to hydralazine and enzalutamide *per se* and in combination using MTT assay. RWPE cell line was also tested for control purposes. In DU145, the effective concentrations-50 (EC_{50}) that reduced cell viability to 50% were 16.14μM and 46.19μM for hydralazine and enzalutamide, respectively. Concerning PC-3, the EC_{50} was 118.5μM for hydralazine and 36.86μM for enzalutamide. Similarly, the hydralazine and enzalutamide EC_{50} for RWPE was 101.8μM and 29.97μM, respectively (**Table 6** and ***Figure 12***).

Table 6. EC_{50} values obtained for each tested drug and for each selected cell line.

Cell line	Hydralazine	Enzalutamide
DU145	16.14 μM	46.19 μM
PC-3	118.5 μM	36.86 μM
RWPE	101.8 μM	29.97 μM

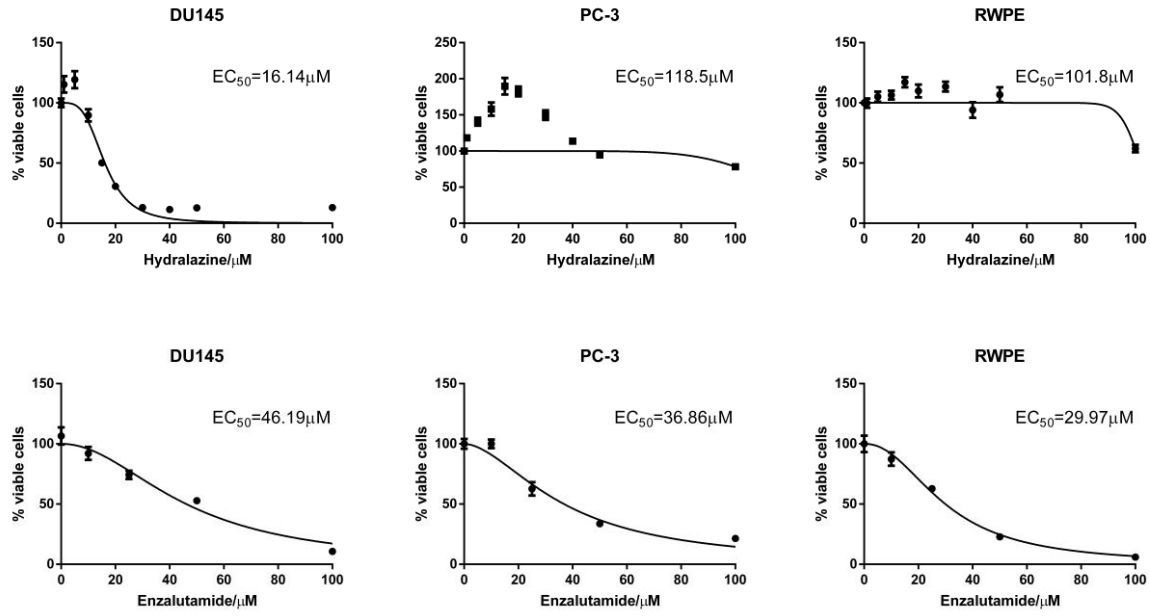

Figure 12. Hydralazine and enzalutamide dose-response curves in PCa cell lines on day 3.

PHENOTYPIC EFFECTS OF HYDRALAZINE ON THE CELL VIABILITY OF PCA CELL LINES

A time- and dose-dependent inhibition of DU145 viability was observed after hydralazine exposure. Overall, the effect of hydralazine on the tumor cell growth suppression was observed on day 3 with 20µM, 30µM, 40µM, 50µM and 100 µM concentrations (***Figure 13***).

Hydralazine only significantly reduced DU145 cells' viability at 20µM or higher concentrations on day 3. Specifically, the number of viable cells reduced 50% at the EC_{50} concentration and more impressively at higher concentrations (***Table 7***). Moreover, the number of viable cells decreased significantly with the doubled EC_{50} (30µM), as well as with higher concentrations. Contrarily, the number of PC-3 viable cells significantly increased after treatment with 5µM, 10µM, 15µM, 20µM and 30µM. Furthermore, RWPE cells demonstrated a significant decrease in cells' viability, but only when treated with 100µM hydralazine.

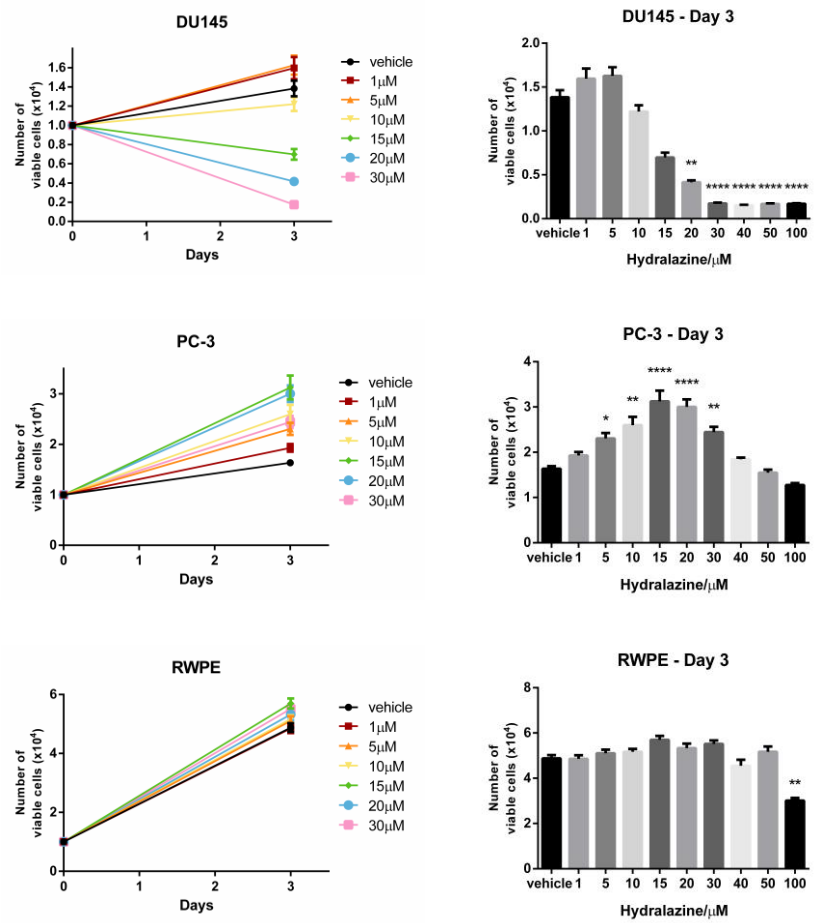

Figure 13. Phenotypic effect of hydralazine in PCa cell lines at day 0, 3 assessed by MTT assay. All data are presented as mean of three independent biological replicates with six experimental replicates.

Table 7. Percentage of viable cells of three biological replicates at day 3 after hydralazine exposure at different concentrations.

		1 µM	5 µM	10 µM	15 µM	20 µM	30 µM	40 µM	50 µM	100µM
% of viable cells	**DU145**	115.36	119.26	89.64	50.18	30.73	13.04	11.38	12.79	13.03
	PC-3	118.19	140.66	158	189.71	182.54	149.68	113.55	94.52	78.31
	RWPE	99.75	105.16	106.47	117.14	109.87	113.45	94.06	106.87	62.07

Corroborating with the cell viability results, apoptosis induction was also dose dependent in DU145. In fact, 15µM hydralazine induced a significant increase in apoptosis levels (***Table 8***). Contrarily, apoptosis was decreased in PC-3 and RWPE cell lines significantly decreased with 15µM hydralazine, in accordance with results obtained for cell viability. However, there were no statistically significant differences in all cell lines treated with 1µM hydralazine.

Table 8. Apoptotic levels normalized to vehicle at day 3 after hydralazine exposure at different concentrations in three biological replicates with six experimental replicates of each cell line.

		1 µM	15 µM
Apoptotic levels (day 3)	**DU145**	0.849	1.934
	PC-3	0.819	0.488
	RWPE	0.886	0.614

SEQUENTIAL TREATMENT WITH HYDRALAZINE AND ENZALUTAMIDE

Concerning the combined approach, for each cell line, both EC_{50} of hydralazine and enzalutamide were used. Enzalutamide's EC_{50} resulted in a high concentration (~50μM). Concerning DU145, it was expected that hydralazine would demethylate the epigenetically silenced AR, and consequently decreasing enzalutamide's EC_{50}. Since our sequential treatment consisted firstly in a hydralazine exposure followed by enzalutamide treatment, decreased DU145 cells' viability was anticipated. Furthermore, all the cell lines were exposed to concentrations indicated in ***Table 9***.

Table 9. Hydralazine and enzalutamide concentrations used for all cell lines in combination.

	Combination						
	Hydralazine			**Enzalutamide**			
[Drug] in μM	5	15	30	1	10	25	50

Indeed, contrarily to PC-3 and RWPE cell lines, DU145 cell line responded to this sequential treatment. While DU145 cell number decreased significantly after 15μM hydralazine (***Figure 14A***), no significant cell number alterations were apparent in PC-3 and RWPE independently of drugs concentrations (***Figure 14B***). In fact, EC_{50} was calculated at day 6 for both drugs separately in these cell lines and no alterations were observed. Indeed, the hydralazine and enzalutamide EC_{50} values for PC-3 were 516.1μM and 50.31μM, respectively. Similarly, RWPE had an EC_{50} of 166534μM and 84.35μM for hydralazine and enzalutamide, respectively (*data not shown*). Importantly, in DU145, hydralazine EC_{50} decreased significantly to 7.87μM after three days of treatment and 3 days of medium renewal (enzalutamide's vehicle). Du145 cells' number significantly decreased after 5μM (***Figure 14C***). Nevertheless, enzalutamide EC_{50} remained approximately 50 μM (58.31μM), although a significant decrease in cell number was demonstrated from 1μM (***Figure 14C***).

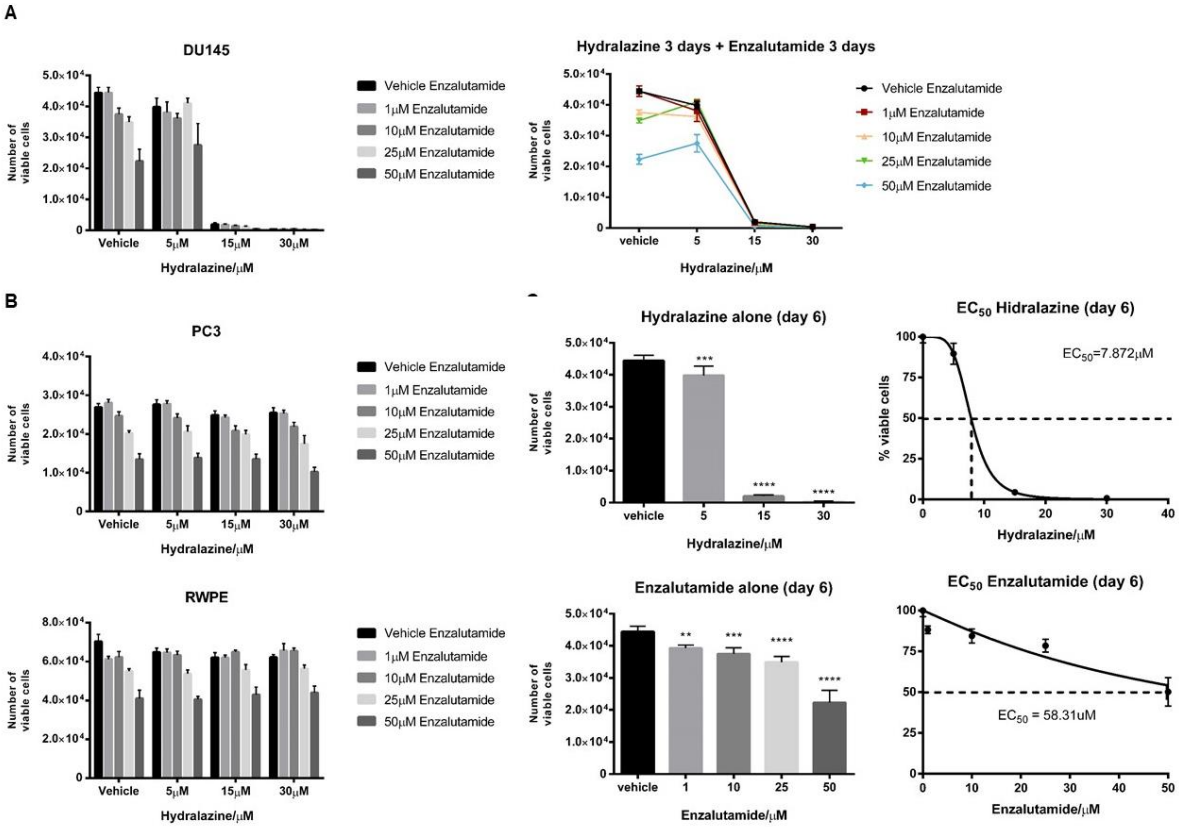

Figure 14. Overall impact in cell number of combined treatment in (**A**) DU145, (**B**) PC-3 and RWPE with (**C**) the separated hydralazine and enzalutamide EC50 in DU145 cell line.

Although the DU145 cell number did not significantly decrease with 5μM of hydralazine *per se*, the combined treatment with this concentration showed significant differences. After treating cells with 5μM hydralazine, DU145 cells' number impressively decreased when treated with 10μM enzalutamide.

A deep drop in DU145 cells' number was observed when 15μM hydralazine was used, according with the previously calculated EC_{50}. Indeed, at this hydralazine concentration, the combined therapy was effective for all enzalutamide concentrations.

Concerning the 30μM hydralazine, the impact of the different enzalutamide concentrations was similar with the previous combination, since only few cells survived after 30μM hydralazine exposure (***Figure 15***).

In conclusion, hydralazine exposure seems to sensitize DU145 cell line to enzalutamide treatment with lower doses.

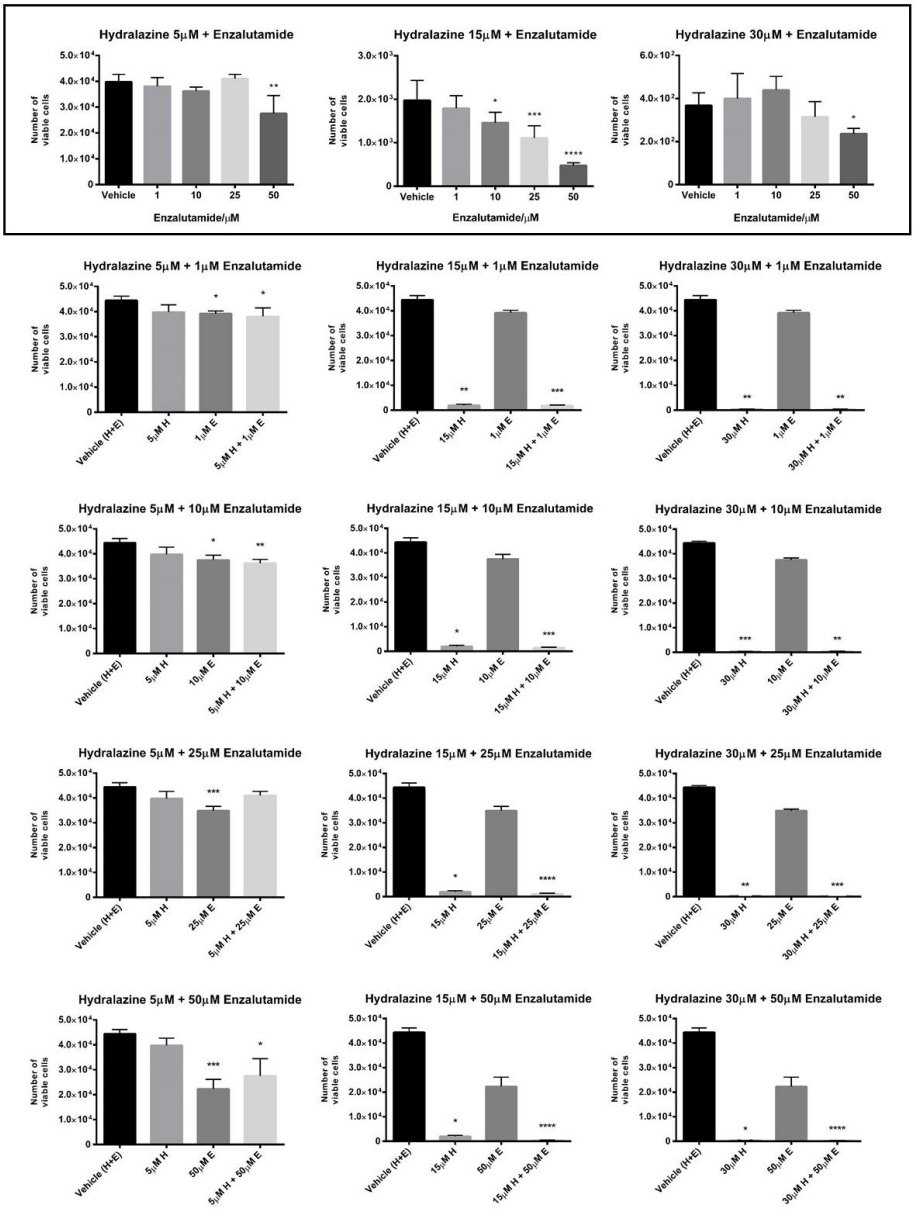

Figure 15. Impact of hydralazine and enzalutamide different combinations in the cell number of DU145 cell line. Abbreviations: H - hydralazine; E - enzalutamide

METHYLATION STATUS IN WILD-TYPE RWPE, DU145 AND PC-3 CELL LINES

The AR regulatory region is expected to have a minimum of 60% of CpGs sites at the 5'-untranslated region (5'-UTR), defined as a CpG island [174]. This region consists of approximately 272 nucleotides and 27 CpG dinucleotides.

Three regions were defined as indicated in ***Figure 16***. Region 1, located ~140bp upstream from the transcription start site (TSS), includes 16 CpGs sites, eight of which included in the CpG island and a single Sp1 binding site (5'-GGGGCGGG-3'; -45). Region 2 is located within the CpG island and 19 of 21 CpGs sites are comprised in this island. Region 5, ~1165bp downstream from the TSS, begins with the consensus coding sequence (CCDS) that is marked by the ATG codon, continues with the first exon and ends with the CAG repeat.

PCa cell lines' methylation status of these three regions was analyzed using bisulfite sequencing. The three wildtype cell lines' replicates were analyzed independently and afterwards a median value was calculated per region and per cell line. To yield a percentage methylation for each CpG site, the number of methylated CpGs were divided by the number of analyzed clones of each replicate (***Figure 17***).

In DU145, the CpG sites of transcriptional region (Region 1 and 2) were highly methylated (the filled circles in the figures). Five out of eight CpG sites of region 1 that are located within the CpG island (-35, -30, +4, +13 and +21) were at least 85% methylated. The other three (-56, -51, -42) were 73%, 46% and 77% methylated. The rest of the CpG dinucleotides of this region had also more than 73% of methylation. Contrarily, in PC-3 and RWPE, the overall methylation profile in region 1 was much lower. In PC-3 cell line, one CpG dinucleotide (-51) was 3% methylated, three (-181, -178, -140) were between 40% and 48% methylated, while the methylation percentage of the other 12 varies from 30% to 40%. Similarly, RWPE has one CpG site (-51) that was not methylated, two (+21, -30) that were respectively 51% and 55% methylated and the other 13 exhibited methylation between 13%-39%.

Similar to region 1, the second region in DU145 demonstrated an increased methylation level when compared to the other two cell lines. The last two CpG dinucleotides (+235, +265) of this region, which are not included in the CpG island, were 93% and 34% methylated, respectively. Among the CpGs sites within the CpG island, 11 were at least 80% methylated, six varied from 40% to 75% of methylation (+81, +100, +109, +131, +169, +179) and the other four (+139, +146, +160 and +265) had lower methylation percentages (3%-34%). PC-3 cell line has one unmethylated CpG site (+145), one with 3% of methylation (+160) and the others comprise 13% and 45% of methylation. With a lower methylation

pattern, RWPE has four unmethylated CpGs dinucleotides (+81, +146, +160, +265), 12 with 2% to 20% of methylation and five are 26% to 46% methylated (+211, +202, +196, +193, +191).

Regarding region 5, the tendency is similar to the other two regions despite lower methylation levels were found. Indeed, DU145 remains the cell line with higher methylation percentage, although with much lower percentage values. DU145 has one unmethylated CpG site (+1275), five 2%-8% methylated (+1162, +1169, +1235, +1257, +1278), one 64% methylated (+1426) and the others varied from 13% to 32%. PC-3 displayed 11 unmethylated CpG dinucleotides and three were 4% methylated (+1166, +1212, +1263). Similarly, RWPE depicted eight unmethylated CpGs sites and six showed 1% to 6% methylation (+1183, +1216, +1218, +1259, +1263, +1426).

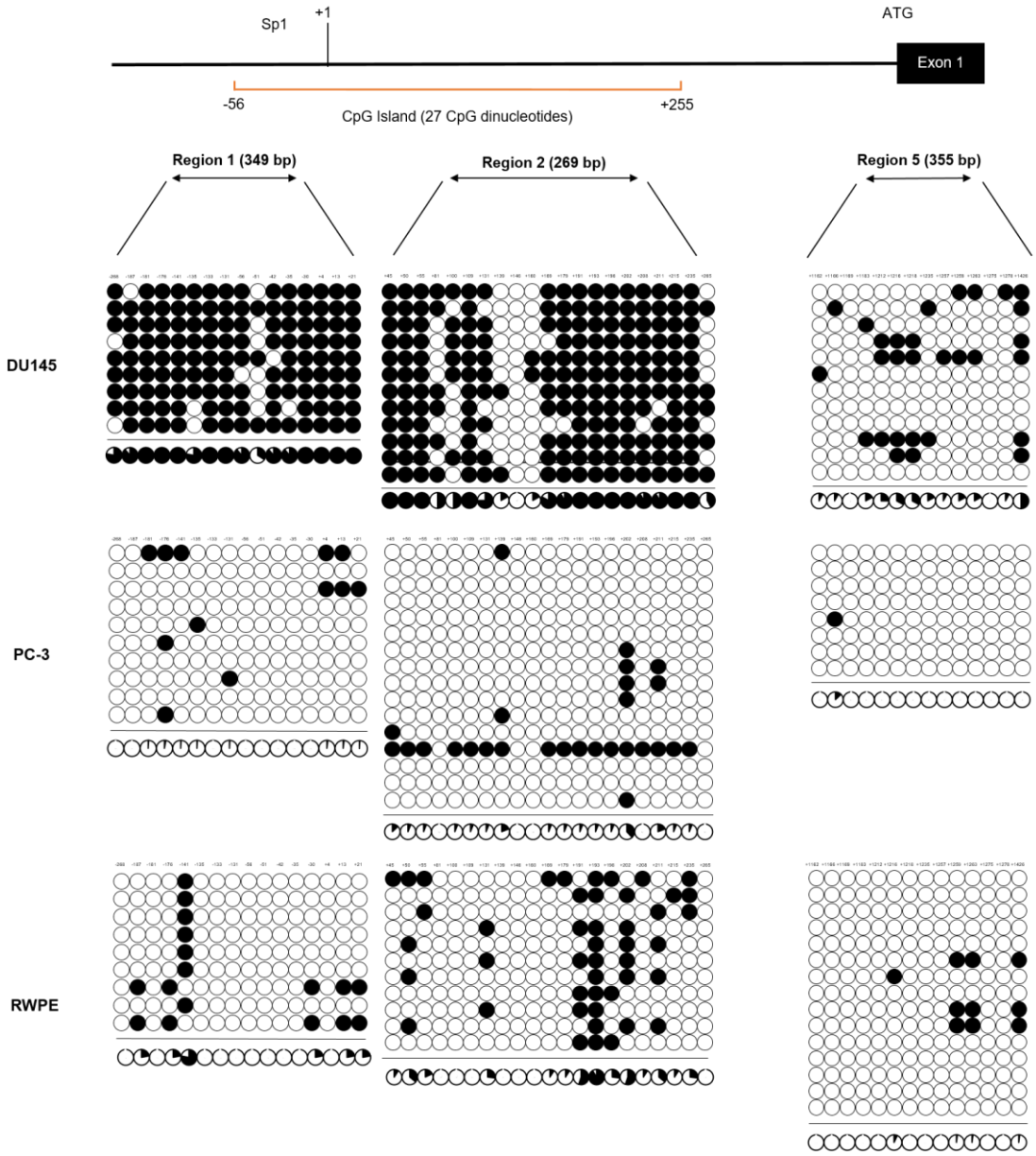

Figure 16. Map of the androgen receptor gene 5' cytidine-guanosine CpG island. The position of Sp1 is indicated by a vertical line, the +1 and ATG positions by arrows and the exon 1 by a black square. The three selected regions are represented in accordance with sequence's location. DNA methylation mapping from different replicates is presented for each cell line for each region with the methylation percentage of each region (below the black line at the end of each individual methylation map).

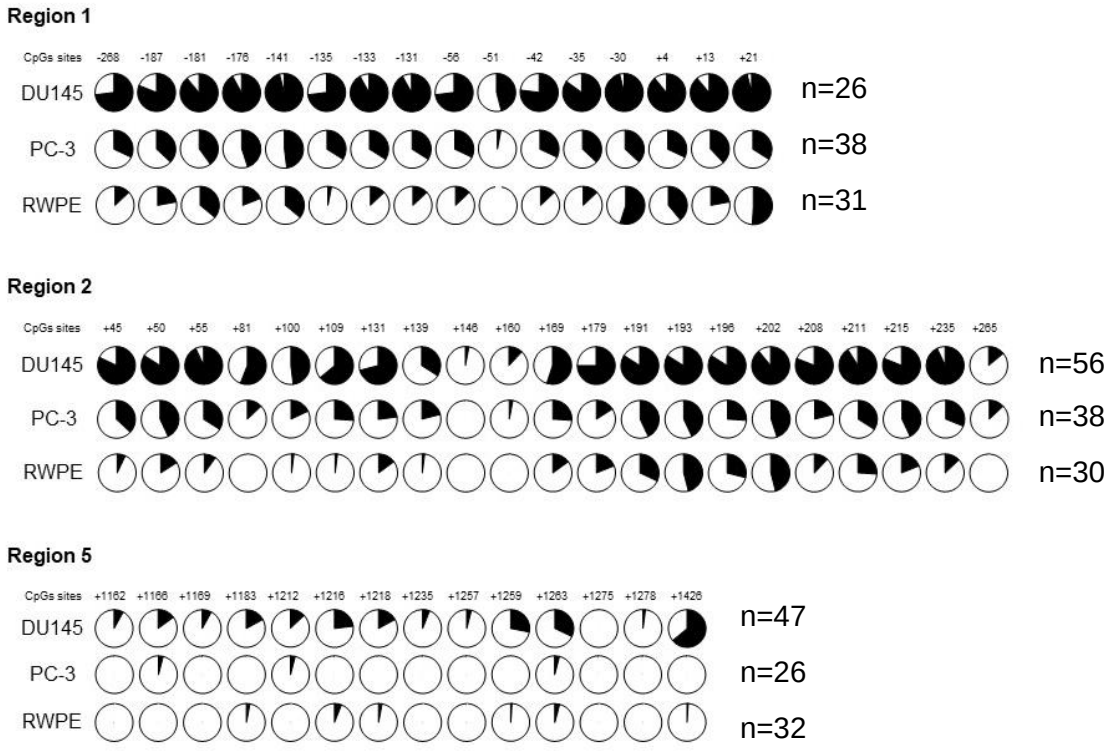

Figure 17. Summarized methylation status of three regions of AR sequence in three independent replicates of each PCa cell line.

METHYLATION STATUS OF DU145 CELL LINE TREATED WITH HYDRALAZINE

As far as we know, this is the first study that fully characterizes the methylation status of AR regulatory sites in DU145 cell line after hydralazine treatment by bisulfite sequencing. Region 5 was excluded from this analysis, due to extremely low methylation percentage values. Sequencing was performed for regions 1 and 2. Several hydralazine's concentrations were selected: 1µM, 5µM, 10µM, 15µM, 25µM and 50µM. Nevertheless, only suitable colonies' number were obtained with two and four concentrations for regions 1 and 2, respectively.

The three treated replicates were analyzed altogether and afterwards an average value was calculated per region. To yield a percentage methylation for each site, the number of methylated CpGs were divided by the number of analyzed clones of each replicate (***Table 9***, ***Table 10***, ***Figure 18*** and ***Figure 19***).

Regarding region 1, no global effect was obtained in the methylation percentage of the assessed CpG sites. However, CpG -42, which corresponds to the Sp1 binding site, displayed a significant methylation decrease (from 63% to 17%) when treated with 5µM hydralazine. Interestingly, CpG -56 displayed a gradual percentage methylation decrease according with increased hydralazine concentration. The other CpG sites did not demonstrate any significant alteration.

Concerning region 2, the analyzed hydralazine's concentrations were 1µM, 5µM, 10µM and 15µM. Although no overall methylation decrease was observed for the tested hydralazine concentrations, a higher reduction in the methylation percentage was found for almost every CpG dinucleotides after DU145 exposure to 15 µM. In fact, CpG sites at +100 and +109 showed complete absence of methylation. However, no associations were found for the number of demethylated CpG sites with increased hydralazine concentration.

Although hydralazine exposure did not impact the overall methylated patterns in DU145 cell line, demethylation was found in CpG -42 of the region 1 treated with hydralazine.

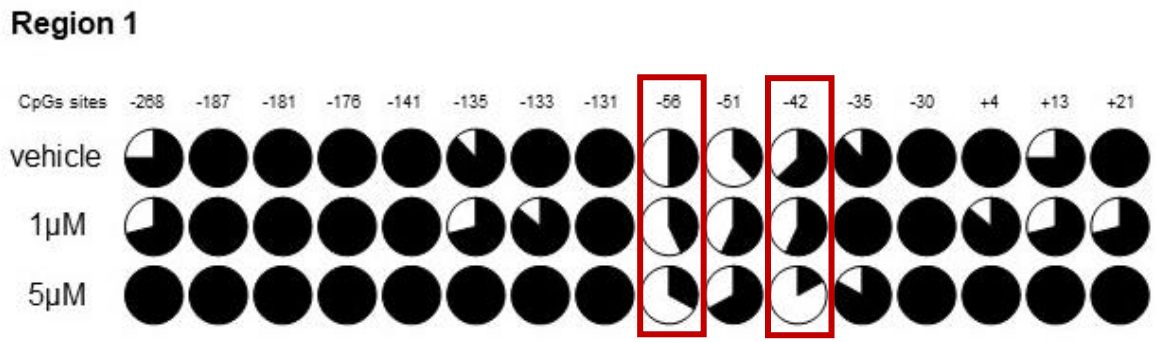

Figure 18. Methylation percentage in region 1 in DU145 cell line exposed to different hydralazine concentrations.

Table 10. Methylation percentage in region 1 in DU145 cell line exposed to different hydralazine concentrations.

	CpGs	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
n=8	**Methylation percentage**	75	100	100	100	100	88	100	100	50	38	63	88	100	100	75	100	**vehicle**
n=7		71	100	100	100	100	71	86	100	43	57	57	100	100	86	71	71	**1μM**
n=6		100	100	100	100	100	100	100	100	33	67	17	83	100	100	100	100	**5μM**

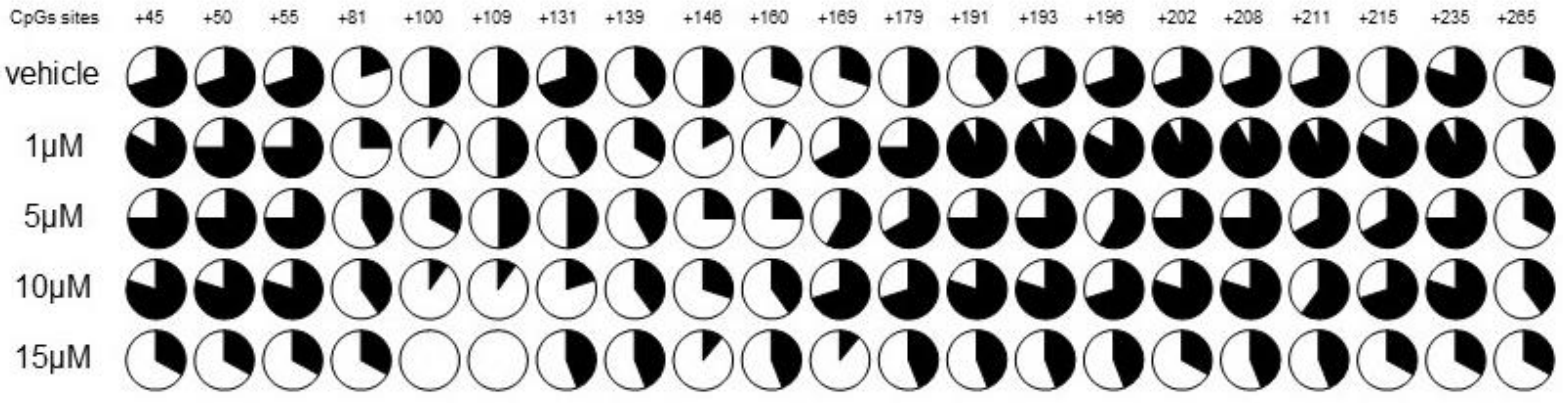

Figure 19. Methylation percentage in region 2 in DU145 cell line exposed to different hydralazine concentrations.

Table 11. Methylation percentage in region 2 in DU145 cell line exposed to different hydralazine concentrations.

	CpGs	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	
n=10	**Methylation percentage**	70	70	70	20	50	50	70	40	50	30	30	50	40	70	70	70	70	70	50	80	30	**vehicle**
n=12		83	75	75	25	8	50	42	33	17	8	67	75	92	92	83	92	92	92	83	92	42	**1µM**
n=12		75	75	75	42	33	50	50	42	25	25	58	67	75	75	58	75	75	67	67	75	33	**5µM**
n=10		80	80	80	40	10	10	20	40	30	40	70	70	80	80	70	80	80	60	70	80	40	**10µM**
n=9		33	33	33	33	0	0	44	44	11	44	11	44	44	44	44	33	44	44	33	33	33	**15µM**

DISCUSSION

Prostate cancer ranks among the five most common malignancies and cause of cancer-related death in men worldwide, affecting mainly elder men [1,37]. Most of prostate cancer patients display androgen-dependent tumors at diagnosis, but an important proportion of those tumors progress in 12-18 months to a castration-resistant state after ADT. At this stage, therapeutic interventions are limited, only offering symptoms amelioration and increasing patients' life quality. Thus, CRPC remains an incurable disease characterized by its aggressiveness and mortality despite multiple approved therapies.

It is acknowledged that AR signaling pathway is the most important mechanism that bypasses most of the current therapies, thus contributing to this cancer progression. This pathways' deregulation is due to several molecular and genetic mechanisms, regardless of androgen circulating levels. Moreover, these cases that display AR loss of expression do not harbor AR genetic alterations.

Recently, hypermethylation of AR has been associated with loss of AR expression in several PCa cell lines, but also in tumors. In fact, 20-30% of hormone-independent cancers are characterized by an extensive loss of AR expression [174]. This transcriptional silencing of genes without genetic alteration has been explained with DNA methylation and histone acetylation/deacetylation [175]. These epigenetic mechanisms are thought to be involved in tumor progression, in which methylation of regulatory genes happen to be a form of epigenetic regulation of gene expression. AR methylation may explain the phenotype associated with the advanced stage of this disease that does not respond to hormonal therapy. Consequently, reverting these epigenetic modifications might be an interesting new therapeutic approach concerning CRPC patients.

In order to revert the epigenetically silenced AR, hydralazine was used to modulate the methylation machinery of CRPC cells as DNMT inhibitor. Hydralazine has already been approved as an antihypertensive drug, thus having its toxicity and side effects' profile already been studied. Nevertheless, herein, this approved drug was used as a demethylating compound. This drug repurposing offers a lower probability of failure, since it has been already considered as safe in previous early-stage trials as well as clinical trials. Furthermore, the drug approval process and introduction into the pharmaceutical industry becomes simpler, shorter and cheaper [176].

In this study, hydralazine significantly decreased DU145 cells proliferation, while increased apoptosis. Moreover, opposite results were found for PC-3 and RWPE cells. On that basis, we can conclude that this response of DU145 cell line could be a consequence of the AR re-expression after hydralazine treatment.

To understand the distinct responses among the three cell lines, AR promoter region methylation profile was assessed by bisulfite sequencing. In fact, we confirmed the presence of a CpG island within the AR regulatory region that comprises 27 CpG

dinucleotides. Moreover, AR gene methylation profile was determined in AR-expressing and non-expressing PCa cell lines. This methylation map included an analysis of 51 CpG sites from -268 to +1426. Notwithstanding of the demonstrated PCa cell lines' epigenetic heterogeneity, consensus regions of methylated CpG sites were identified within the island. Indeed, our results show that region 1, which includes the transcriptional start site, as well as region 2 were preferentially and consistently methylated in DU145 cell line. Contrarily to this AR-negative cell line, PC-3 and RWPE showed a lower overall methylation profile. These different methylation patterns depicted by these cell lines reinforce the hypothesis that AR promoter CpG hypermethylation might be a mechanism for AR expression loss in advanced prostate cancer.

Methylation of specific sites may inhibit a transcription factor binding, thus repressing gene transcription. Since AR gene lacks TATA and CCAAT boxes, transcription is driven by binding of the ubiquitously expressed zinc finger transcriptor factor, Specificity Protein 1 (Sp1) to GC box regulatory elements. This protein belongs to the Specificity Protein/Krüppel-like Factor (SP/KLF) transcriptor factor family, being expressed in mammalian cells. Sp1 forms multimers producing DNA looping, gathering regulatory elements and enhancers, thus regulating transcription directly or through histone acetylation and chromatin remodeling. Therefore, Sp1 is considered to be a major stimulator of AR gene expression [67]. Since region 1 contains one Sp1 binding site, its methylation might explain AR expression loss (**Appendix I**). Our study demonstrates that region 1 is highly methylated in DU145, whereas in the other PCa cell lines methylation percentage is much lower. In fact, the CpG site at -42 that matches the Sp1 binding site is 77% methylated in DU145, whereas in PC-3 and RWPE is 32% and 13% methylated, respectively.

Indeed, previous reports have suggested AR promoter CpG island methylation as a mechanism of AR expression loss PCa cells. In fact, treatment with DNMT inhibitor Aza-dC was able to re-express AR in several metastatic PCa cell lines [177,178]. Therefore, the inhibition of DNMT activity by hydralazine could lead to DNA demethylation and consequent AR expression restoration, resulting in a reversion of the epigenetically silenced AR gene. Herein, the effect of hydralazine was tested in DU145 cell line by bisulfite sequencing of AR promoter. Sequencing was performed for regions 1 and 2, since they were highly methylated in this cell line, contrarily to region 5. However, due to subcloning methodological issues, a limited number of colonies were obtained for DU145 cells treated with the tested hydralazine concentrations in region 1. Nevertheless, two demethylated CpG sites (-42 and -56) were found in cells treated with increased hydralazine concentrations. Regarding region 2, although there was not a consistent tendency, several CpG sites were demethylated after hydralazine exposure, highlighting its demethylating effect in this cell line. As future perspectives, we aim to perform chromatin immunoprecipitation (ChIP)

assays before and after hydralazine treatment with the objective to test whether Sp1 is capable of binding to DNA after drug's exposure, thus allowing AR transcription.

Although our research team has previously observed AR re-expression after hydralazine treatment [37], we were not able to reproduce those findings either at protein and transcriptional level (*data not shown*). Contrarily to that previous study, a monoclonal antibody was used to increase AR protein specificity. Additionally, the existence of shorter AR variants, having a smaller weight than AR 110kDa full-length, is currently acknowledged. Furthermore, we anticipate that higher hydralazine concentration might be sufficient to re-express AR. Nonetheless, AR regulatory region methylation might only have a limited role in AR silencing in AR-negative PCa cell lines. In fact, the binding of Sp1 to AR binding site must be available to allow AR transcription. For that, the chromatin needs to be open to allow Sp1 binding. Therefore, hydralazine may in fact inhibit the catalytic site of DNMT1, decreasing its activity, but can be prevented from binding to DNA. The nucleosomes remodeling that alters chromatin condensation is regulated by the histones acetylases and histone deacetylases (HDAC) [138]. Although HDAC proteins suppress AR activity by catalyzing AR deacetylation, HDAC1 and 3 activate approximately 50% of AR target genes. Also, it is known that several HDACs are overexpressed in CRPC [178].

These histone proteins might not only be targeted by different posttranslational modifications, but also interact with many other proteins and DNA, forming protein complexes with nucleosome-remodeling activities [138]. Since histone deacetylation is also implicated in transcriptional silencing of several cancer-related genes, histone deacetylase inhibitors (HDACi) were developed to maintain the chromatic structure in a more open conformation, allowing DNA access and consequent reversion of the epigenetically silenced gene [138]. HDACi demonstrated a selective action on tumor cells, inducing apoptosis, growth arrest and autophagy. However, there are several paradoxes concerning its effect on AR signaling, highlighting the heterogeneity of PCa malignancy. HDACi have been proven to hyperacetylate Hsp90, resulting in its dissociation with AR, consequently leading to AR degradation. Moreover, HDACi can also inhibit AR gene transcription as well as AR target genes which are HDAC-dependent [178].

Thus far, HDACi have only been approved for hematological malignancies, but there are already several ongoing clinical trials using HDACi as epigenetic drugs in solid tumors [138]. In fact, there are one phase I, three phase II and one phase III clinical trials that combine hydralazine with valproic acid, an HDACi [147–149,151]. Valproic acid, a short chain fatty acid inhibitor, is an approved antiepileptic drug and is used in bipolar disorder that has demonstrated to inhibit histone deacetylases [151,178]. It has proven to modulate multiple pathways in *in vitro* and *in vivo* PCa models, including cell cycle arrest, apoptosis, angiogenesis and senescence through HDAC inhibition [178].

This epigenetic repositioning drug combining demonstrated a synergistic effect in the re-expression of TSG in several cancer models. Moreover, there are a number of studies that suggest that DNMTi combined with HDACi activate more effectively methylated genes than each drug in monotherapy [178]. By using this combined approach, the mechanisms that cells develop to escape cell death are more limited [179,180]. Thereby, we intend to combine hydralazine with valproic acid in order to increase the epigenetic modulation and possibly revert the epigenetically silencing of AR gene expression.

Although the inherent toxicity of DNMTi and HDACi in clinical trials did not allow the usage of these drugs as single agents for treatment of CRPC, reports in breast and lung cancer models suggest that low dose approaches of epigenetic drugs are able to reprogram tumor cells, and consequently sensitizing them to conventional therapies. On that basis, the low-dose regimen avoids toxicity and side effects observed with high doses, while simultaneously allows the sensitization of resistant PCa cells to subsequent treatments. In our study, we tested the cytotoxic effect of hydralazine with high concentrations and short treatment duration. In fact, our sequential approach suggests that longer periods with lower hydralazine concentrations might have the same phenotypic effects as shorter treatment's period with higher doses, but with less toxic effects. As a matter of fact, hydralazine's EC_{50} decreased from 16.14μM to 7.87μM. Therefore, as future perspectives, we intend to evaluate hydralazine's chronic effect by using preferential lower doses for a longer period.

Finally, it is also important to note that hydralazine also targets other signaling pathways that are essential for AR survival, including EGFR [37]. Hence, further studies are necessary to disclose hydralazine's effect in PCa cell lines independently of AR expression.

CONCLUSIONS & FUTURE PERSPECTIVES

CONCLUSIONS

Herein, we demonstrated that hydralazine has demethylating effect in specific CpG sites of the AR regulatory region in DU145 cell line. In fact, AR methylation in these CpG dinucleotides may lead to AR downregulation in this subset of PCa patients, possibly being associated with ADT resistance. Moreover, this repositioning drug attenuated PCa cell lines malignant phenotype. Thus, it constitutes a promising compound for CRPC treatment, since it could lead to a sensitization of already approved drugs that target AR.

FUTURE PERSPECTIVES

In this work, we investigated the effect of hydralazine in malignant features of PCa cell lines. Although we demonstrated its demethylating effect through bisulfite sequencing, no AR re-expression was achieved after hydralazine exposure with the tested dosages. Therefore, in an attempt to re-express AR, we intend to treat PCa cells with higher drug concentrations.

Moreover, it would be interesting to treat these PCa cells concomitantly with an HDACi, namely valproic acid, which might act synergistically with hydralazine. It is expected that it will open the chromatin conformation, allowing hydralazine to bind to DNA. Specifically, this drug combination will demethylate the transcription factor binding site in AR promoter as well as open the chromatin.

In order to confirm the ligation of Sp1 transcription factor to AR before and after the cells' exposure, we plan to perform ChIP assay.

Finally, long-term treatments should also be implemented, since hydralazine demonstrated higher effects with longer periods of treatment and to allow hydralazine to be incorporated.

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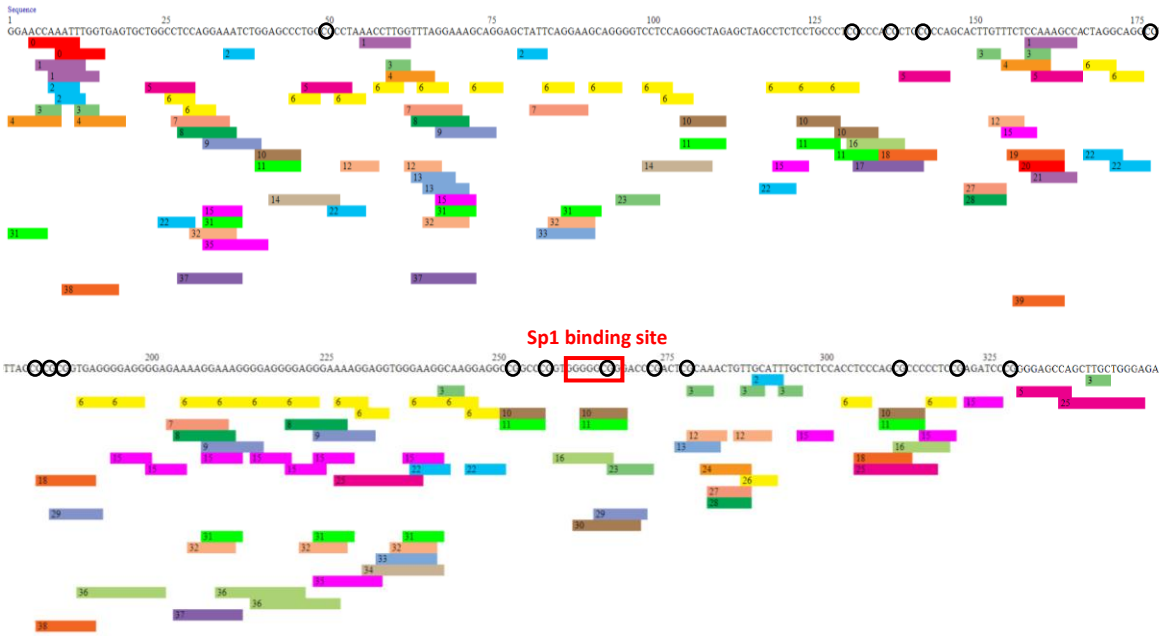
APPENDIX

APPENDIX I – *IN SILICO* ANALYSIS FOR TRANSCRIPTION FACTORS BINDING SITES

PROMO online database was used to assess the transcription factors binding sites in our region 1 of AR.



Factors predicted within a dissimilarity margin less or equal than 15 % :

0 HNF-3alpha [T02512]	1 NF1/CTF [T00094]	2 GR-beta [T01920]	3 C/EBPbeta [T00581]	4 NF-1 [T00539]	5 ENKTF-1 [T00255]	6 GR-alpha [T00337]	7 c-Ets-2 [T00113]		
8 IRF-1 [T00423]	9 NF-AT1 [T00550]	10 Pax-5 [T00070]	11 p53 [T00671]	12 FOXP3 [T04280]	13 TFIID [T00820]	14 EBF [T05427]	15 TFII-I [T00824]		
16 WT1 [T00899]	17 ETF [T00270]	18 GCF [T00320]	19 SRY [T00997]	20 TCF-4E [T02878]	21 GR [T05076]	22 AP-2alphaA [T00035]	23 RXR-alpha [T01345]		
24 c-Myb [T00137]	25 Ik-1 [T02702]	26 XBP-1 [T00902]	27 PR B [T00696]	28 PR A [T01661]	29 E2F-1 [T01542]	30 Sp1 [T00759]	31 STAT4 [T01577]		
32 c-Ets-1 [T00112]	33 Elk-1 [T00250]	34 RBP-Jkappa [T01616]	35 NF-AT2 [T01945]	36 MAZ [T00490]	37 STAT1beta [T01573]	38 T3R-beta1 [T00851]	39 LEF-1 [T02905]		

Zoom | Data (txt)