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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
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IMPACT OF EPIGENETIC MODULATORS ON THE MALIGNANT PHENOTYPE OF PROSTATE CANCER CELLS

Maria Inês Pinho dos Santos Graça

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MARIA INÊS PINHO DOS SANTOS GRAÇA

IMPACT OF EPIGENETIC MODULATORS ON THE MALIGNANT PHENOTYPE OF PROSTATE CANCER CELLS

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ORIENTADORA:
PROFESSORA DOUTORA CARMEN DE LURDES FONSECA JERÓNIMO
Professora Associada convidada com "Agregação"
Departamento de Patologia e Imunologia Molecular
Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto
Investigadora Auxiliar e coordenadora do Grupo de Epigenética do Cancro – Centro de Investigação
Instituto Português de Oncologia - Porto

CO-ORIENTADOR
PROFESSOR DOUTOR MANUEL ANTÓNIO RODRIGUES TEIXEIRA
Professor Catedrático Convidado com "Agregação"
Departamento de Patologia e Imunologia Molecular
Instituto de Ciências Biomédicas Abel Salazar – Universidade do Porto
Director do Centro de Investigação e do Serviço de Genética
Investigador e coordenador do Grupo de Genética do Cancro – Centro de Investigação
Instituto Português de Oncologia - Porto

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mas lutei para que o melhor fosse feito.
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não sou o que era antes”.

(Martin Luther King Jr.)

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Abbreviations

5-aza-CdR – 5-aza-2'-deoxycytidine

ADT – Androgen Deprivation Therapy

Ago – Argonaute protein

AMOs - anti-miRNA oligonucleotides

AR – Androgen Receptor

AS – Active Surveillence

BPH – Benign Prostatic Hyperplasia

CpG – Cytosine-phosphate-Guanine

CRPC – Castration-Resistant Prostate Cancer

***DGCR8* –** Di George Syndrome Critical Region 8

DHT – 5 α-dihydrotestosterone

DNA – Deoxyribonucleic Acid

DNMT – DNA Methyltransferase

DNMTi – DNA Methyltransferases Inhibitors

DRE – Digital Rectal Examination

EAU – European Association of Urology

EGFR – Epidermal Growth Factor Receptor

EMT - Epithelial-Mesenchymal Transition

FDA – Food and Drug Administration

GnRH – Gonadotropin-Releasing Hormone

GS – Gleason Score

HAT – Histone Acetyltransferase

HDAC – Histone Deacetylase

HDACi – Histone Deacetylases Inhibitors

HDM – Histone Demethylase

HGPIN – High-grade Prostatic Intraepithelial Neoplasia

HMT – Histone Methylatransferase

LHRH – Luteinizing Hormone Releasing-Hormone

LincRNAs – Large Intergenic Non-Coding RNAs

LncRNAs – Long Non-Coding RNAs

LNA – Locked-nucleic acids

LSD1 – Lysine-Specific Demethylase 1

m^5C – 5-methyl-cytosine

MBPs – Methyl-CpG Binding Proteins

MRI – Magnetic Resonance Imaging

mCRPC – Metastatic Castration-Resistant Prostate Cancer

miRNAs – MicroRNAs

ncRNA – Non-coding RNA

ORF – Open Reading Frame

PCa – Prostate Cancer

Pre-miRNA – Precursor-miRNA

Pri-miRNA – Primary-miRNA

PSA – Prostate Specific Antigen

PIN – Prostatic Intraepithelial Neoplasia

PiRNAs - PIWI-interacting RNAs

PIA – Proliferative Inflammatory Atrophy

RISC – RNA-induced Silencing Complex

RNA – Ribonucleic Acid

RNAi – RNA intereference

RP – Radical Prostatectomy

SAM – S-adenosyl-L-methionine

SIRT 1 – Sirtuin 1

SnoRNAs – Small nucleolar RNAs

T-UCRs – Transcribed Ultraconserved Regions

TARBP2 – Trans-Activated-Responsive RNA-Binding Protein 2

TMN – Tumor Node Metastasis

TRUS – Transrectal Ultrasound

UTR – Untranslated Region

VPA – Valproic acid

XPO5 – Exportin-5

Abstract

Prostate cancer (PCa) is one of the most common malignancies worldwide and a leading cause of cancer-related morbidity and mortality. Current therapeutic options for advanced disease are not curative and most patients eventually progress to castration-resistant disease. Therefore, novel therapeutics strategies, ideally based on the biology of this disease, are urgently needed. Alterations of epigenetic mechanisms, namely, DNA methylation, histone post-translation modifications and miRNAs expression are early events in prostate carcinogenesis and contribute to disease progression. Interestingly, unlike genetic mutations, epigenetic alterations are reversible and several compounds that target critical enzymes that compose the epigenetic machinery have been developed and tested in recent years. The potential therapeutic impact of targeting epigenetic alterations in PCa has not, however, been widely evaluated.

Hence, the main objective of this Thesis was to define the usefulness of three epigenetic modulating drugs – enoxacin, a compound that targets miRNAs processing, and RG108 and hydralazine, two non-nucleoside inhibitors of DNA methyltransferases as anti-tumor agents in prostate cancer cell lines.

We found that enoxacin was able to decrease PCa cell viability, induce cellular death by apoptosis and cell cycle arrest. Moreover, this agent reduced PCa cells invasiveness. The exposure of PCa cell lines to enoxacin resulted in an effective restoration of global miRNAs expression, leading to re-expression of tumor-suppressor miRNAs and downregulation of some oncomirs.

Concerning RG108, a compound designed to directly target DNMT1 active site, we demonstrated a dose and time dependent growth inhibition and apoptosis induction in LNCaP, 22Rv1 and DU145. As expected, this compound repressed DNMT activity, *DNMT1*, *DNMT3a* and *DNMT3b* expression and reduced global DNA methylation in androgen responsive cell lines. Furthermore, exposure of LNCaP and 22Rv1 cell lines to RG108 significantly decreased promoter methylation levels of *GSTP1*, *APC* and *RAR-β2*, although mRNA re-expression was only achieved for *GSTP1* and *APC*.

Finally, hydralazine, a well-known anti-hypertensive drug that also inhibits DNA methylation by establishing highly stable interactions between its nitrogen atoms and DNMT active site, restrained PCa cell growth and promoted apoptosis in a time and dose dependent manner. Moreover, hydralazine decreased cellular invasiveness and induced cell cycle arrest and DNA damage. Additionally, PCa cells exposed to hydralazine exhibited lower *DNMT1*, *DNMT3a* and *DNMT3b* mRNA levels as well as lower DNMT1 protein, which may have contributed to the observed decrease in *GSTP1*, *BCL2* and

CCND2 promoter methylation levels, and concomitant gene re-expression. Importantly, hydralazine restored androgen receptor expression, with upregulation of its target p21, in DU145 cell line. The attenuation of tumor phenotype was particularly effective in the castration-resistant PCa cell line DU145 and it may be linked with the disruption of EGF receptor signaling pathway. Our results thus suggest that hydralazine is an effective demethylating drug, disclosing superior anti-growth properties compared to RG108.

In conclusion, our data demonstrate, for the first time, that the three epigenetic compounds evaluated are negative modulators of tumor growth in the majority of prostate cancer cell lines. Therefore, *in vivo* studies are now mandatory to confirm these promising results and further confirm these drugs as alternative therapeutic approaches for prostate cancer patients.

Resumo

O carcinoma da próstata é uma das neoplasias malignas mais incidentes a nível mundial, constituindo uma das principais causas de morbilidade e mortalidade oncológica. Actualmente, as estratégias terapêuticas disponíveis para a doença avançada não são curativas, levando a que a maioria dos doentes progrida para um estadio hormono-refractário. Sendo assim, torna-se imperativo o desenvolvimento de novas estratégias terapêuticas, idealmente baseadas na biologia do carcinoma da próstata. Alterações nos mecanismos epigenéticos, como a metilação do DNA, modificações pós-transducionais das histonas e expressão de microRNAs, são eventos precoces na carcinogénese prostática e contribuem para a progressão da doença. Contudo, contrariamente às alterações genéticas, as modificações epigenéticas são reversíveis, existindo diversos compostos que têm como alvo componentes da maquinaria epigenética, sendo eficazes na reversão do fenótipo tumoral. Contudo, o potencial impacto terapêutico da utilização desta estratégia em carcinoma da próstata permanece largamente desconhecido.

Assim, o principal objectivo desta Tese, foi investigar a utilidade de três fármacos moduladores epigenéticos, nomeadamente a enoxacina, um composto que actua ao nível do processamento dos miRNAs, e dois inibidores não-análogos das metiltransferases do DNA, RG108 e hidralazina, como agentes anti tumorais em linhas celulares de carcinoma da próstata.

Relativamente à enoxacina, demonstramos que este composto é capaz de diminuir a viabilidade celular e induzir a apoptose e a paragem do ciclo celular. Após a exposição ao fármaco, verificámos, ainda, uma redução no potencial invasivo das linhas celulares de cancro da próstata. Adicionalmente, este fármaco restituiu eficazmente a expressão global dos miRNAs, promovendo a expressão de miRNAs supressores tumorais e levando a uma diminuição de alguns miRNAs com potencial oncogénico.

No que refere ao RG108, composto desenhado especificamente para inibir o local catalítico da DNMT1, verificamos uma inibição da viabilidade celular e um aumento da apoptose nas linhas celulares LNCaP, 22Rv1 e DU145, dependente da concentração de fármaco utilizado e do tempo de exposição. A exposição das linhas celulares dependentes de androgénios a este fármaco resultou na inibição da actividade das DNMTs, e na redução, não só da expressão da *DNMT1*, *3a* e *3b*, mas também dos níveis de metilação global do DNA. O RG108 causou, ainda, uma redução significativa da metilação do promotor dos genes *GSTP1, APC* e *RAR-β2*, embora, a re-expressão só tenha sido alcançada para os genes *GSTP1* e *APC*.

Finalmente, a hidralazina, composto anti-hipertensor, que inibe a metilação do DNA através do estabelecimento de interações de elevada estabilidade entre os seus átomos de azoto e o local activo das metiltransferases do DNA, causou uma redução da viabilidade celular e indução da apoptose de uma forma dependente da concentração de fármaco e do tempo de exposição ao mesmo. Adicionalmente, este fármaco diminuiu a invasão celular, levou a uma paragem do ciclo celular e induziu dano no DNA. Verificámos, ainda, uma redução dos níveis de expressão de *DNMT1*, *DNMT3a* e *DNMT3b* com concomitante diminuição na expressão proteica da DNMT1, após exposição ao fármaco. Este facto poderá ter contribuído para a diminuição significativa nos níveis de metilação dos genes *GSTP1*, *BCL2* e *CCND2* e seu concomitante aumento de expressão. Um achado importante neste estudo foi o restabelecimento da expressão do receptor de androgénios, com consequente aumento na expressão de um dos seus alvos, p21, na linha celular DU145. Notavelmente, a redução do fenótipo tumoral foi particularmente evidente na linha celular hormono-refractária DU145, o que poderá ser devido a alterações na regulação da via de sinalização EGF. Uma análise detalhada dos nossos resultados permite-nos sugerir que comparativamente ao RG108, a hidralazina é um agente desmetilante mais eficaz na reversão do fenótipo tumoral.

Em conclusão, os nossos dados demonstram, pela primeira vez, que estes três fármacos moduladores epigenéticos são eficazes na inibição do crescimento tumoral da maioria das linhas celulares de carcinoma da próstata. Assim, é crucial a realização de estudos *in vivo* de forma a confirmar estes resultados promissores e verificar o potencial destes fármacos como estratégias terapêuticas inovadoras para o carcinoma da próstata.

1

CHAPTER ONE

General Introduction

PROSTATE CANCER

Prostate Anatomy

The prostate is a walnut-shaped gland, part of the accessory sex gland system of human males, located deep in the pelvis between the bladder neck and the urogenital diaphragm. This gland synthesizes and secrets the organic and inorganic components of the seminal fluid. A normal adult prostate weights about 20 g and measures approximately 25 cm^3 [1].

The most widely accepted model of prostate gland organization was proposed in 1988 by Dr. McNeal, in which prostate is divided in four distinct glandular zones based on the location of their ductal openings within the urethra with reference to *verumontanun*: peripheral, central and transition zones and fibromuscular stroma (Figure 1). The peripheral zone accounts for the bulk of normal prostate, comprising about 70% of glandular elements and occupies most of the posterior and lateral sides of the gland. The central conical shaped zone lies posterior to the urethra surrounding the two ejaculatory ducts and encompasses about 25% of the glandular tissue. The transitional zone surrounds the prostatic urethra and accounts for 5% of glandular elements. The fibromuscular stroma is located anteriorly and extends from the bladder neck to the striated sphincter and is continuous with the prostate capsule [2, 3].

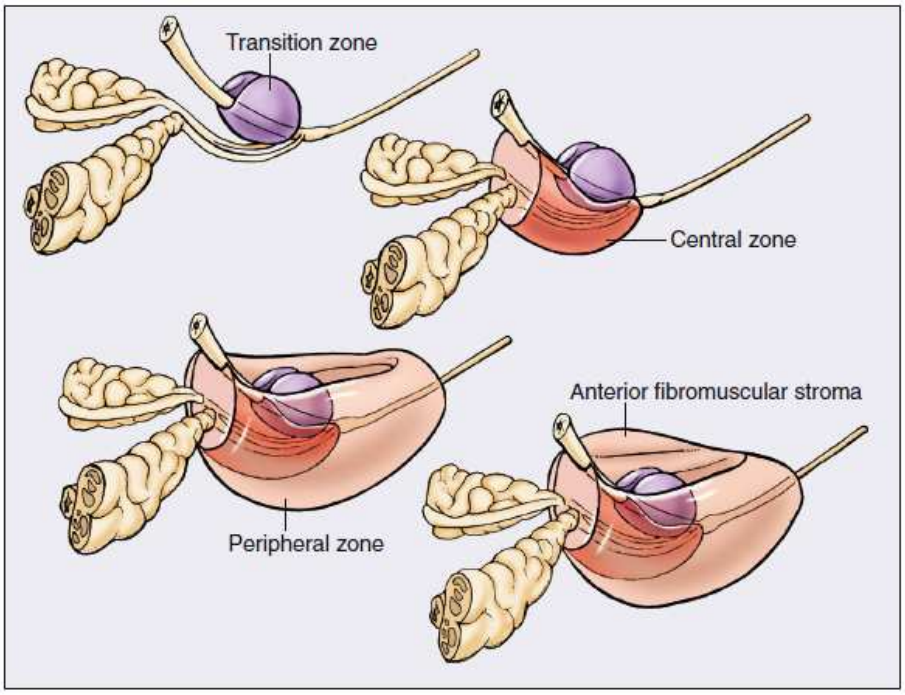

Figure 1 – Anatomy of the normal prostate. *Adapted from [3].*

Most Common Prostate Disorders

Prostate disorders are very common among elderly men and comprise several benign and malignant conditions. The most common are benign prostatic hyperplasia, prostatic intraepithelial neoplasia, proliferative inflammatory atrophy and prostate adenocarcinoma [4].

Benign prostatic hyperplasia (BPH) is by far the most common. In fact, this condition is present in more than 50% of men aged over 60 years [5]. BPH is a chronic condition characterized pathologically by a cellular proliferation of the epithelial and stromal elements within the prostate gland, particularly at the transition zone. This pathology leads to prostatic enlargement which in turns generates resistance in the urethra causing lower urinary tract symptoms [5, 6].

Prostatic intraepithelial neoplasia (PIN) represents the pre-invasive end of the *continuum* of cellular proliferations within the lining of prostatic ducts and acini. It is classified in low-grade and high-grade (HGPIN). HGPIN is the currently accepted precursor lesion of prostatic adenocarcinoma (PCa). Furthermore, this disease shares some of the molecular abnormalities of PCa. The identification of PIN lesions warrants a 30% to 50% risk of finding PCa on a subsequent biopsy [7, 8].

Proliferative inflammatory atrophy (PIA) is characterized as high proliferative lesion associated with chronic inflammation that usually occurs in the peripheral zone of the prostate (known to encompass the majority of PCa). This feature associated with the fact that secretory cells of PIA harbor some molecular alterations described in PCa favors the hypothesis that PIA might be also a precursor of PCa [9-11].

Prostate Adenocarcinoma

The adenocarcinoma of the prostate comprises 95% of the malignant neoplasms of the prostate and range from clinically indolent to highly aggressive [1]. This is a very heterogeneous disease, both in terms of pathology and clinical presentation [12]. The presence of multiple tumor foci within a single gland and different degrees of dysplasia and architectural disorganization are common features in PCa pathology. The majority of PCa arises from the peripheral zone of the prostate, but a minority may also be found in the central and transitional zones. Since this disease has no clear symptoms, several cases are only diagnosed in advanced stages of the disease [1, 12]. The underlying mechanisms leading to PCa are still not fully known. In the next sections the most relevant features of PCa are summarized.

Epidemiology

PCa is the second most commonly diagnosed cancer in men worldwide, ranking the fifth position considering malignancies of both sexes. According with International Agency for Research on Cancer, 1,111,689 new cases occurred in 2012, accounting for 15.0% of total newly diagnosed cancers. In Europe, PCa is the most incident malignant neoplasm and the third leading cause of cancer related death. In Portugal, PCa is also the most frequent cancer diagnosed among men, with 6,622 new cases detected in 2012 (23.3% of total) and the third cause of cancer related mortality (11.1% of total cancer cases) (Figure 2) [13].

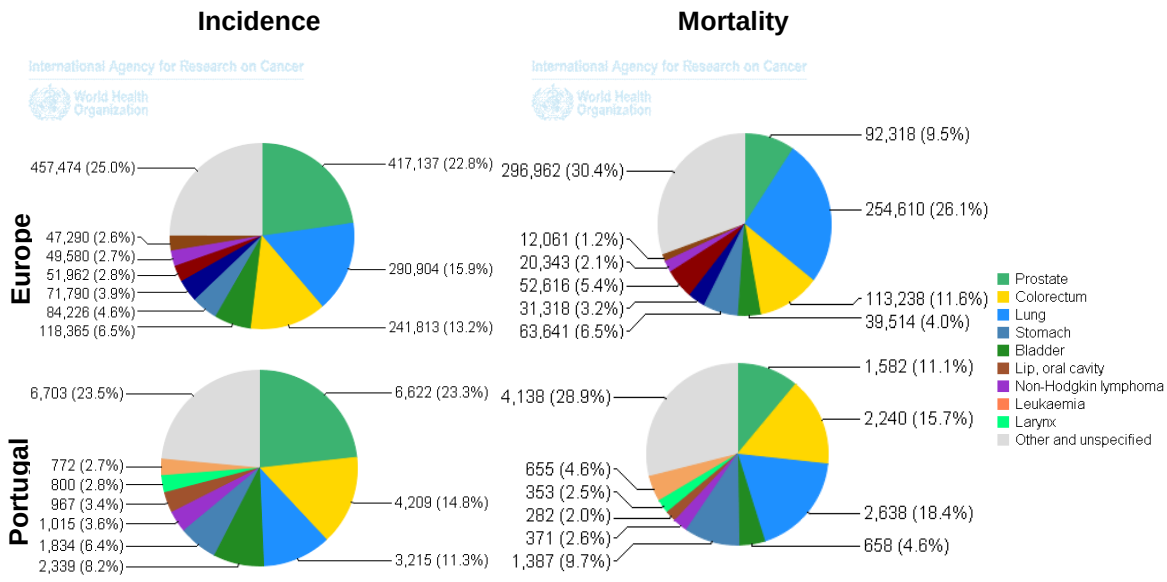

Figure 2 – Incidence and mortality of different types of cancer in Europe and Portugal. *Adapted from [13].*

Worldwide PCa incidence rates are highly heterogeneous across countries, being estimated a 25-fold variation. The industrialized countries tend to have the higher incidence and mortality rates (Figure 3), which might be due to the variability in the screening and early detection programs adopted in each country [14]. Moreover, different genetic susceptibility, environmental exposures and access to healthcare might also justify these enormous differences among populations [15]. The incidence rates are clearly dependent on the diagnostic activity; therefore, countries with widespread screening and early detection have higher incident rates. In fact, the introduction of Prostate-Specific Antigen (PSA) test in the middle 80's caused a rapid rise in the incidence of PCa in the countries that adopted this screening method [16]. However, this period was followed by a stabilization of the detected cases, and nowadays this number is slight reducing [17].

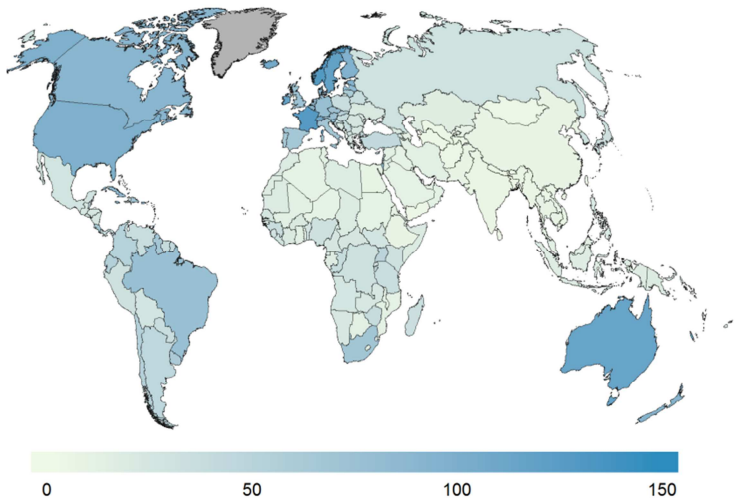

Figure 3 – Estimated age-standardized incidence rate per 100,000 worldwide. *Adapted from [13].*

Concerning the mortality rates, was also observed a decrease among developed countries, although it is not clear whether this is due to earlier diagnosis, improved treatment or a combination of these with other still unknown factors. However, the mortality rates between developed and developing countries are not so dissimilar as the incidence rates (10 fold-variation), therefore the PSA screening test appears not to be as much important for mortality as it is for incidence rates [18].

Risk Factors

Despite the complexity of the etiology and pathogenesis of PCa, the risk factors for the development of this disease are well-established and encompass age, ethnicity, family history, and at a lesser extent some behavioral risk factors (diet, smoking and alcohol consumption).

Race

Several epidemiologic studies revealed that incidence of PCa varies widely among ethnic populations. In general, the African American men have not only a higher risk to develop this disease but also higher mortality rates when compared to European American men [19]. The lowest rates of PCa are found in Asia, however, Asian males living in the United States display a higher risk, which might indicate an effect of unknown external/environmental factors in the development of PCa [20]. Currently the basis for these racial differences is still unknown, but it might be due to differences in genetic susceptibility, exposure to different environmental, cultural and/or socioecomic factors [21].

Age

PCa is mainly a disease of older men, being the median age at diagnosis 67 years. PCa is rare in men under 50 (0.1% of all patients) and approximately 85% of cases of PCa are diagnosed after the age of 65 years [20, 21].

Family History

Family history of PCa increases the risk of PCa development and death. In fact, men with first-degree relatives (father or brother) harboring PCa have more than double of risk to develop this disease. Men with several affected relatives have an even higher risk, particularly if their relatives were young at the time of diagnosis. This might suggest inherited or genetic factors behind this disease, although, environmental factors should not be discharged [20, 21].

Diagnosis

PCa detection and management is an intensive area of research, and controversy. Nowadays, the major PCa's detection tools are digital rectal examination (DRE), serum concentration of PSA, and transrectal ultrasound (TRUS)–guided biopsies [22]. PCa diagnosis is dependent of histopathologic confirmation which is performed by biopsy [23]. The general indications for prostate biopsies performance are high levels of PSA and/or suspicious DRE. DRE alone allows the detection of about 18% of all PCa, irrespective of the PSA level [24]. PSA is a kallikrein-related serine protease produced in normal prostate secretions that is released into the blood after the rupture of the normal prostatic membrane structures, which happens not only in malignant lesions of prostate, but also in the context of inflammation (BPH and prostatitis), and urologic manipulation. Therefore, PSA is prostate specific, but not PCa specific. Moreover, PSA not only lacks the sensitivity to detect an important number of tumors, particularly at early stages of PCa, but also has been associated to overdiagnosis and overtreatment of indolent tumors [22, 25, 26]. Despite this, the combination of PSA and DRE allowed for a significant decrease in the number of cases with advanced disease at the diagnosis. Indeed, about 70-80% of currently diagnosed PCa are organ confined allowing for increased cure rates [4].

According to the European guidelines, men with PSA levels equal or above 4.0 ng/mL and/or with abnormal DRE result are candidates to perform a prostatic biopsy. A minimum of 10 systemic laterally directed cores are recommended, and in prostates with a volume above 40 mL more cores should be sampled [22].

Clinicopathologic Diagnosis of Prostatic Adenocarcinoma

The histopathologic diagnosis of PCa takes into account a set of histological features, including primary (architecture or pattern of the glands), secondary (cytological morphology) and tertiary criteria. The first two categories are considered diagnostic features; the tertiary criterion is supportive of the diagnosis of carcinoma (Table 1) [27].

Table 1 – Criteria for PCa Diagnosis. *Adapted from [27]*.

Primary Criteria	Secondary Criteria	Tertiary Criteria
Architectural - diagnostic	**Cytologic - Diagnostic**	**Cytoplasmatic/Luminal- Supportive**
1. Small glands	1. Absence of basal cells	1. Luminal blue mucin
2. Crowded glands	2. Large nucleoli	2. Luminal pink amorphous secretions
3. Haphazardly arranged glands	3. Large hyperchromatic nuclei, with increased nucleus-cytoplasm ratio	3. Crystalloids
4. Fused glands		4. Sharp/rigid luminal borders
5. Infiltrative pattern		5. Amphophilic or foamy cytoplasm
6. Small glands around/between benign glands		
7. Perineural invasion		
8. Mucinous fibroplasia		
9. Glomerulations		

Gleason Grading

The Gleason grading system is the international accepted standard system for grading PCa. This system was developed by Donald Gleason and colleagues in 1966 and was updated in 2005 to include the recent diagnostic practices [27, 28]. The Gleason grading system is a powerful prognostic tool that is solely based on the primary criteria of glandular architecture of the tumor. It defines five different histological grades with decreasing differentiation (Figure 4). PCa has a very heterogeneous morphology and usually presents more than one Gleason pattern in the same tumor. Therefore, the grade of the most common pattern is added to the grade of the second most common pattern and the sum of these two most common patterns is called Gleason Score (GS). The patterns range from 1 (most differentiated) to 5 (least differentiated), and so, GS range from 2 to 10 [27-29].

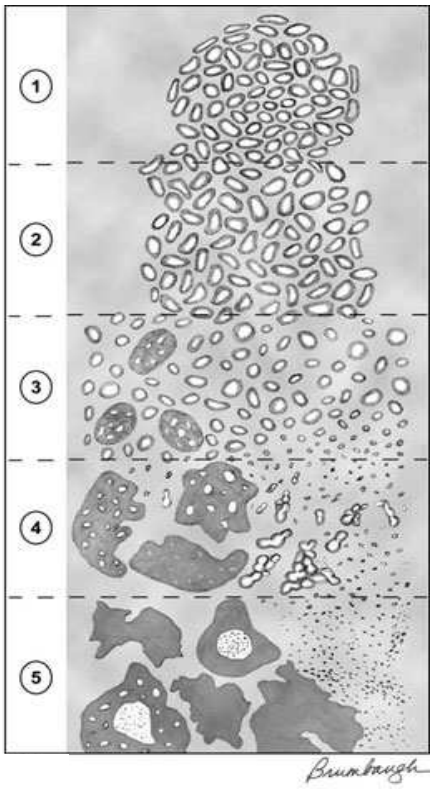

Figure 4 – Updated Gleason Grading System. Pattern 1- Closely-packed, uniform, rounded to oval glands; Pattern 2 - more loosely arranged glands with smooth ends that minimally invade non neoplastic tissue; Pattern 3 - Irregular size and shape glands with more infiltrative margins; Pattern 4 – Fused, cribriform or ill-defined glands; Pattern 5 – almost no glandular differentiation. *Adapted from [28].*

Importantly, the Gleason grading system is an important prognostic predictor, enabling the prediction of PCa natural history and assessment of the risk of recurrence risk after radical prostatectomy or radiotherapy, and thus influencing the choice of the treatment approach [30].

Clinical and Pathologic Staging of Prostate Cancer

The most commonly accepted and used cancer staging system in the world is the TNM (Tumor Node Metastasis) system. This system is based on the size and extent of the primary tumor (T), regional lymph nodes involvement (N) and presence or absence of distant metastasis (M). The American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC) have defined two types of staging: pre-treatment or clinical stage and postsurgical or pathological stage [31, 32]. The clinical stage is obtained through the information acquired about cancer extension before surgical resection. In PCa this stage is established primarily by DRE examination and in some cases by TRUS, magnetic resonance imaging (MRI) and serum PSA levels [31, 32]. The pathological stage is determined after surgical removal of the prostate and by macro and

microscopic examination. Compared to clinical stage, pathological stage allows predicting recurrence in a more accurate way and estimates prognosis with more precision. Prognosis can be assessed through the combination of several independent biological factors in nomograms, which might include prostate capsule invasion, preoperative PSA levels, GS in the prostatectomy sample, positive surgical margins, seminal vesicles involvement, lymph node metastasis and distant metastasis [32, 33].

Therapy

Although more than 70% of the newly diagnosed cases survive beyond 5 years, PCa is still associated with significant mortality and morbidity, mainly due to metastases formation. In fact, about 90% of men who die from this disease have metastatic bone lesions [34]. The current treatment options for PCa include surgery, radiation therapy, hormone therapy and chemotherapy. The treatment choice mainly dependents on patient and disease's features, such as: age, life expectancy, quality of life, TNM classification, GS and preoperative serum PSA level.

The optimal management of PCa is still controversial, since tumors requiring aggressive and immediate intervention are largely indistinguishable from those clinically indolent, in which a "watchful-waiting" approach might be more appropriate.

Clinically Localized Disease

- **Active Surveillance**

Active surveillance (AS) is indicated for patients with very low-risk PCa (patients with indolent disease that does no threat patient's life). These patients are followed and treated only with a curative intent if the risk of progression increases during the follow-up period [22]. This approach aims to reduce patients' overtreatment by discriminating the subset of patients who are unlikely to experience significant progression from those which already have high grade PCa but were missed on the initial biopsies or those that will progress overtime to a more aggressive form of disease [22, 35, 36]. The most common clinical data used to define low-risk PCa include a GS ≤6, PSA level ≤10 ng/mL, and clinical confined PCa (T1 to T2a). These patients will then be subjected to frequent PSA evaluation and prostatic biopsies at 2, 5 and 10 years. Progression is considered when PSA levels are above 10ng/mL and GS after re-biopsy equal or above 7 in a 1 to 4 years interval [22]. Several reports have suggested that this approach in addition to be cost-effective also can preserve quality of life. However, AS is not yet fully accepted by clinicians and patients. Moreover, there are two main challenges that still need to be overcome: better characterization of the risk of progression at diagnostic time and validation of optimal end points once surveillance begins [37].

- **Radical Prostatectomy**

Radical prostatectomy (RP) is considered the gold standard in the management of localized PCa with survival benefits comparing with conservative measures [22]. This procedure consists in removing the whole prostate gland and the seminal vesicles and has been optimized over the years in order to decrease the associate morbidities (erectile dysfunction and urinary incontinence). Patients with GS between 2 and 7, PSA lower than 20 ng/mL, pathological stage between T1a and T2b and with life expectancy equal or above 10 years are candidates to RP [22, 38].

- **External-beam radiotherapy & Brachytherapy**

External-beam radiotherapy is a non-invasive outpatient therapy effective in patients without distant metastases and a life expectancy longer than 5 years [39]. However, it needs to be administered in fractioned courses sometimes longer than 8 weeks. The treatment plan can be limited to the prostate, in the most favorable situations or extended to the surrounding tissues like seminal vesicles and pelvic lymph nodes [39]. Proton beam therapy is one type of external-beam radiotherapy which uses ionizing radiation and its major advantage is the ability to localize the radiation dosage more precisely. This procedure enables an excellent dose distribution, with the additional benefit of no exit dose [40].

In the last few years several efforts have been made to target radiation therapy in PCa more precisely, in order to treat only the prostate gland and avoid radiation as much as possible in the surrounding tissues. External-beam radiotherapy and brachytherapy have achieved rates of disease-free survival similar to those obtained by RP [38]. Brachytherapy consist in the permanent insertion of radioactive seeds with the half-life of 60 days within the prostate under the ultrasound guidance. This procedure is offered to patients with low-grade and low volume disease [40]. Moreover, it was demonstrated that patients treated with brachytherapy experienced fewer side effects when compared to RP [41].

High Risk PCa

The European Association of Urology (EAU) definition of high-risk PCa includes PSA equal or above 20 ng/mL and/or preoperative GS between 8–10 and/or clinical stage equal or above T3a [22]. Although the available treatment options for clinical localized disease is quite effective, some patients progress to advanced or metastatic PCa. Furthermore about 10 to 20% of the diagnosed patients already present this form of disease [42]. The most suitable therapeutic option for these patients is androgen deprivation therapy (ADT).

Under normal conditions, androgen receptor (AR) and androgens are required to normal prostate development and function. An androgen functions through the synthesis and transport of testosterone and 5α-dihydrotestosterone (DHT) to target tissues. Testosterone and DHT exert their biological effects through binding to AR and inducing its transcriptional activity [43]. However, several pathways that allow AR to be activated, amplified, enhanced or bypassed without androgen stimulation, can lead to development and progression of PCa [44]. About 80 to 90% of PCa are dependent of AR at the time of the diagnosis and endocrine therapy is directed toward the reduction of serum androgens and inhibition of AR [45].

ADT can be performed either by pharmacological or surgical castration (bilateral orchiectomy) and usually results in decreased PSA, serum testosterone, tumor progression and relief of symptoms in many patients. The pharmacological treatment consists in the inhibition of key hormones, receptors or enzymes involved in the androgen pathway [44, 46-48]. Commonly, ADT is performed by the administration of gonadotropin-releasing hormone analogs (GnRH), also known as luteinizing hormone releasing-hormone (LHRH) in combination with anti-androgens like flutamide or bicalutamide [1, 49]. This therapy causes a dramatic reduction of prostate secretory cells by apoptosis [50]. Although ADT is used as therapy of choice for advanced/metastatic PCa, it can also be used as neoadjuvant, and as adjuvant therapy, in combination with surgical or radiation therapy [40].

Although this therapy reduces symptoms in about 70 to 80% of the patients, often the response is not long-lasting and tumors' progress to castration-resistant disease within 18 to 30 months.

Castration-Resistant PCa

Castration-resistant PCa (CRPC) is defined as disease progression and associates with biochemical progression (elevated PSA) and metastatic progression [51]. The two major cellular mechanisms suggested to be responsible for CRPC development are adaptation and selection (Figure 5) [52]. The adaptation model states that androgen-dependent PCa cells would suffer additional genetic or epigenetic events in order to allow adaptation to the androgen-depleted environment culminating in resistance to ADT, leading to cancer relapse. Herein the mechanisms of resistance to ADT include increased levels of androgens on the tumor tissue, amplification of the *AR* gene, AR gain-function mutations, AR alternative-splice variants, changes in expression of co-regulatory molecules, AR hypermethylation and androgen-independent activation of AR signaling and other bypass pathways. The selection model proposes that subclones of PCa cells with several degrees of androgen dependence would be already present before ADT

initiation, claiming that progression to CRPC is due to outgrowth of pre-existing castration-resistant clones under the selective pressure of low-androgen conditions [51].

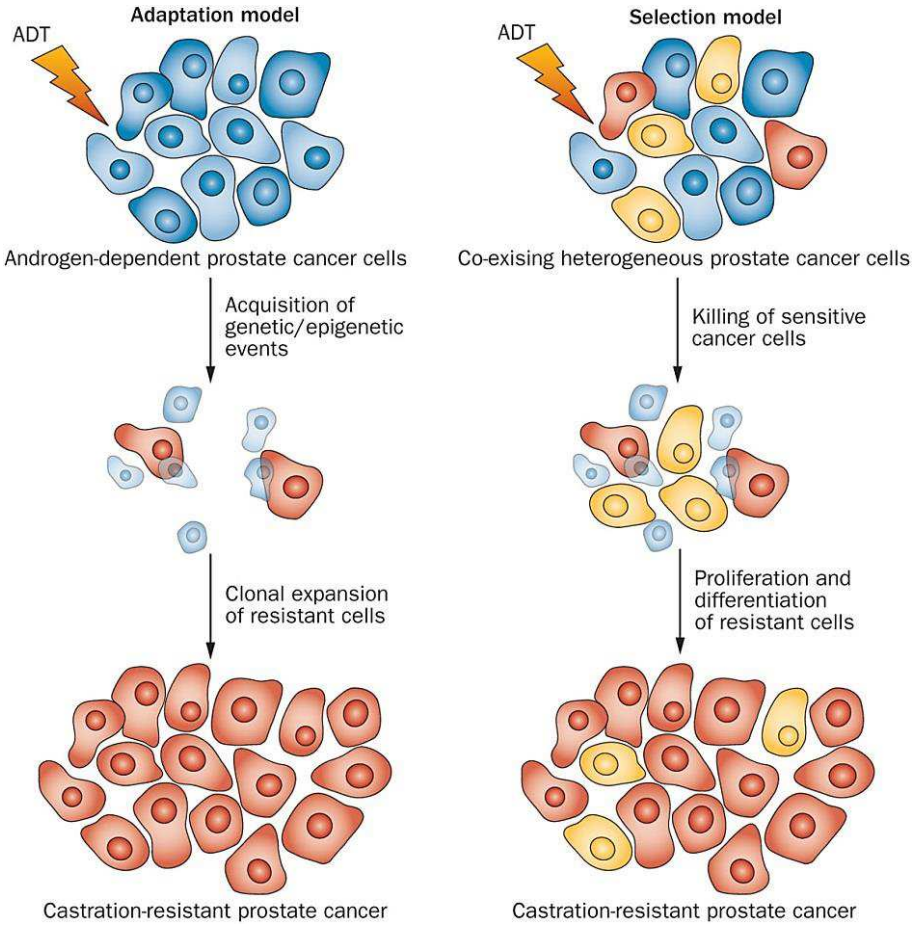

Figure 5 – The two mechanisms responsible for CRPC development. *Adapted from [51]*.

For metastatic CRPC (mCRPC), docetaxel chemotherapy is the most widely accepted therapeutic option that showed relevant survival benefit. This drug is a clinically well-established antimitotic chemotherapeutic compound which interferes with cell cycle by binding with the microtubules [53]. Docetaxel combined with either prednisone or estramustine demonstrated a survival benefit of approximately 2 months [1, 54]. The alternatives for docetaxel include mitoxantron, doxorubicin, vinblastine, paclitaxel, among others. Mitoxantrone combined with prednisone (a pro-drug) is the second-line approved treatment for mCRPC and is able to reduce pain, improving patients' life quality but did not show a survival benefit [53, 55]. In the past 5 years, other agents increased overall survival with symptomatic benefits in patients progressing on/ or after treatment with docetaxel [56]. Recently, in United States, the Food and Drug Administration (FDA) approved spileucel-T (PROVENGE) as a first-line therapy for chemotherapy naïve,

asymptomatic or minimal symptomatic CRPC patients [42]. The current therapeutic strategies at clinics and the most promising compounds in clinical assays are summarized in Figure 6.

Unfortunately, none of these agents are considered curative, strengthening the urgently need for research of new therapeutic approaches.

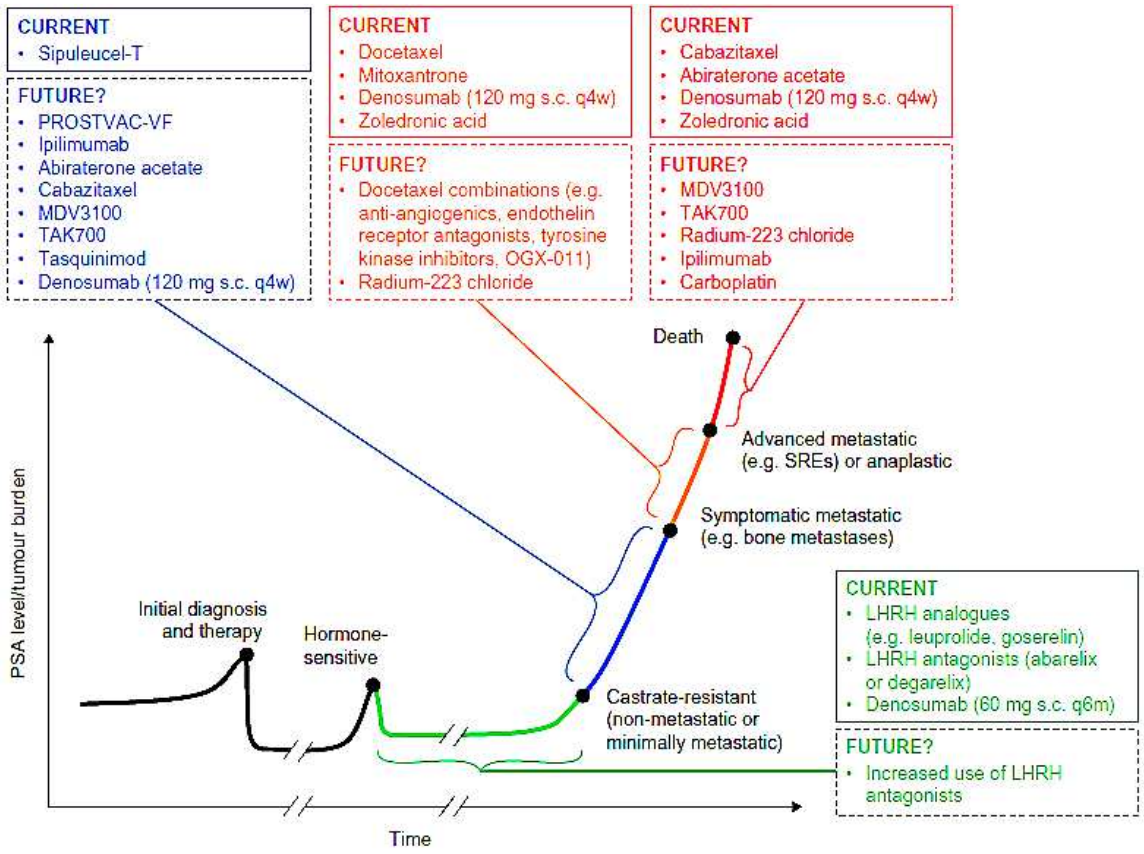

Figure 6 – Overview of new and emerging therapies for advanced PCa. *Adapted from [49].*

EPIGENETICS

The term “Epigenetics” derives from the Greek *epi* (that means beyond) – *genetics* and was originally coined in 1942 by Conrad Waddington to describe “the casual interactions between genes and their products, which bring the phenotype into being” [57]. Nowadays epigenetic is defined as the study of heritable and transient changes in gene expression that are not due to alterations in the primary DNA sequence being essential for gene transcription, development and differentiation of cell and organisms [58, 59]. Epigenetic regulation comprises three major mechanisms: DNA methylation, histone post-translational modifications and expression of non-coding RNAs (Figure 7). The synergistic activity of these mechanisms modulates chromatin structure and thereby determines the transcriptional activity of the genome constitutes the so called epigenome [60].

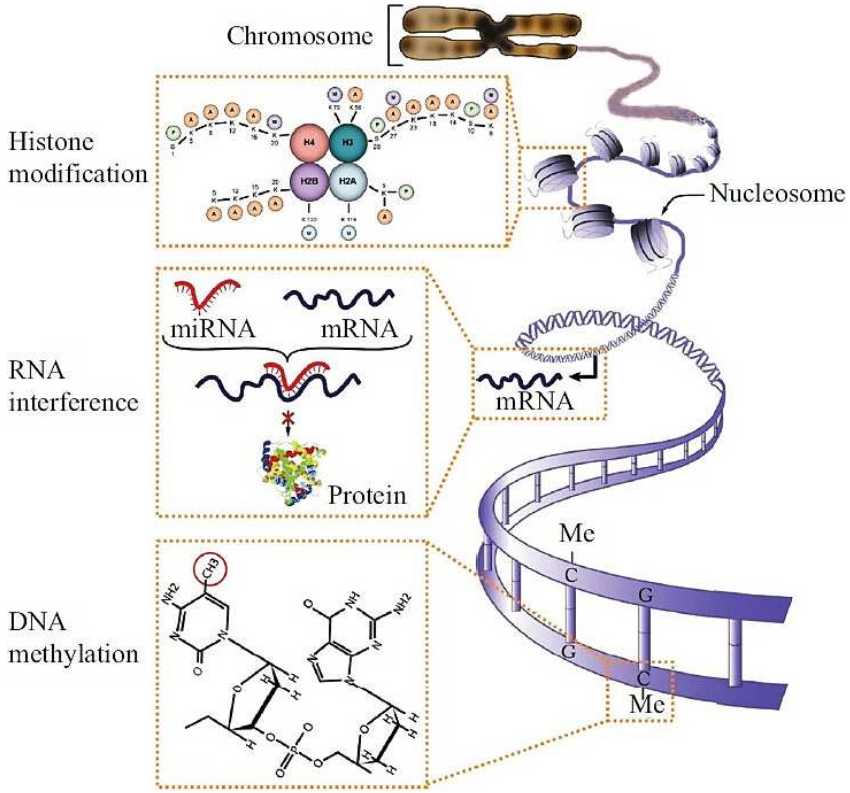

Figure 7 – Mechanisms of epigenetic regulation: DNA methylation, histone modifications, and non-coding RNAs. *Adapted from [61]*.

The Epigenome is established during embryonic development and spread through cell replication and division by virtue of distinct DNA and chromatin protein marks [62]. The disruption of such processes underlies a wide variety of pathologies, including cancer [61, 62].

DNA methylation

DNA methylation is the best studied epigenetic mechanism and plays an important role in DNA repair, recombination, replication and regulation of gene activity [63]. Methylation of DNA in mammals primarily occurs in the CpG dinucleotides and only occasionally at non-CpG sites. CpG-rich regions, known as CpGs islands, are found to be associated with the genes' 5'-regions [64, 65]. However, not all CpG sites are methylated which origins a tissue and cell-type-specific pattern of methylation. In fact, more than 70% of the CpG sequences in the human genome are methylated, which corresponds to about 4 to 6% of all cytosines. The unmethylated CpGs typically cluster at or near the transcriptional regulatory regions of genes, producing chromatin permissive for transcription. The addition of the methyl group to these CpG regions results in chromatin condensation are a barrier to loading of RNA polymerase, rendering the gene transcriptionally silenced [66, 67].

DNA methylation involves the addition of a methyl group to the 5'-carbon position of the cytosine ring within CpG dinucleotide, resulting in the formation of a new DNA base 5-methyl-cytosine (m^5C), and is catalyzed by a group of enzymes named DNA methyltransferases (DNMTs). S-adenosyl-L-methionine (SAM) is used by DNMTs as a donor of methyl groups (Figure 8) [68, 69].

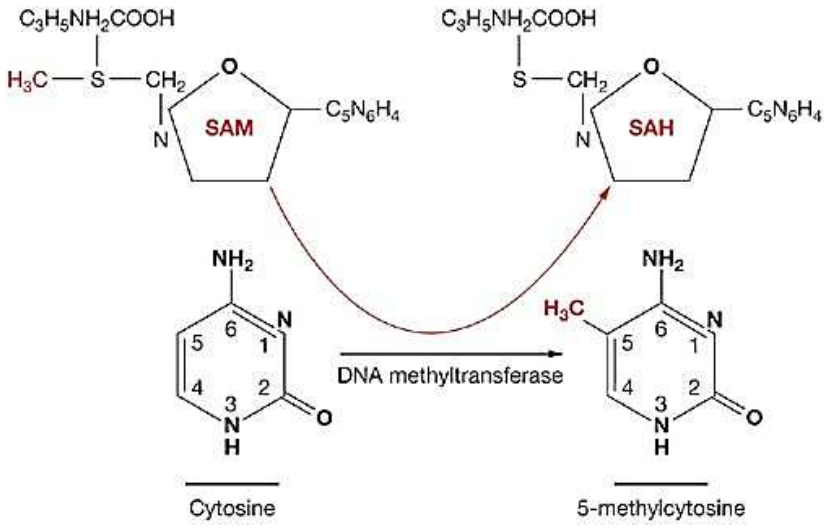

Figure 8 – DNA methylation catalyzed by DNMTs using SAM as a methyl group donor. *Adapted from [70].*

The three main active DNMTs are DNMT1, which maintains the existing methylation patterns following DNA replication, and DNMT3A and DNMT3B, *de novo* methyltransferases that target previously unmethylated CpG sequences [62]. Moreover, the DNMT family includes two additional homologous enzymes, DNMT2, which is the

smallest DNMT and has a reduced methytransferase activity comparing with DNMT1, 3A and 3B, and DNMT3L that lacks catalytic activity but modulates DNMT3a and 3b activity (Figure 9) [65, 71].

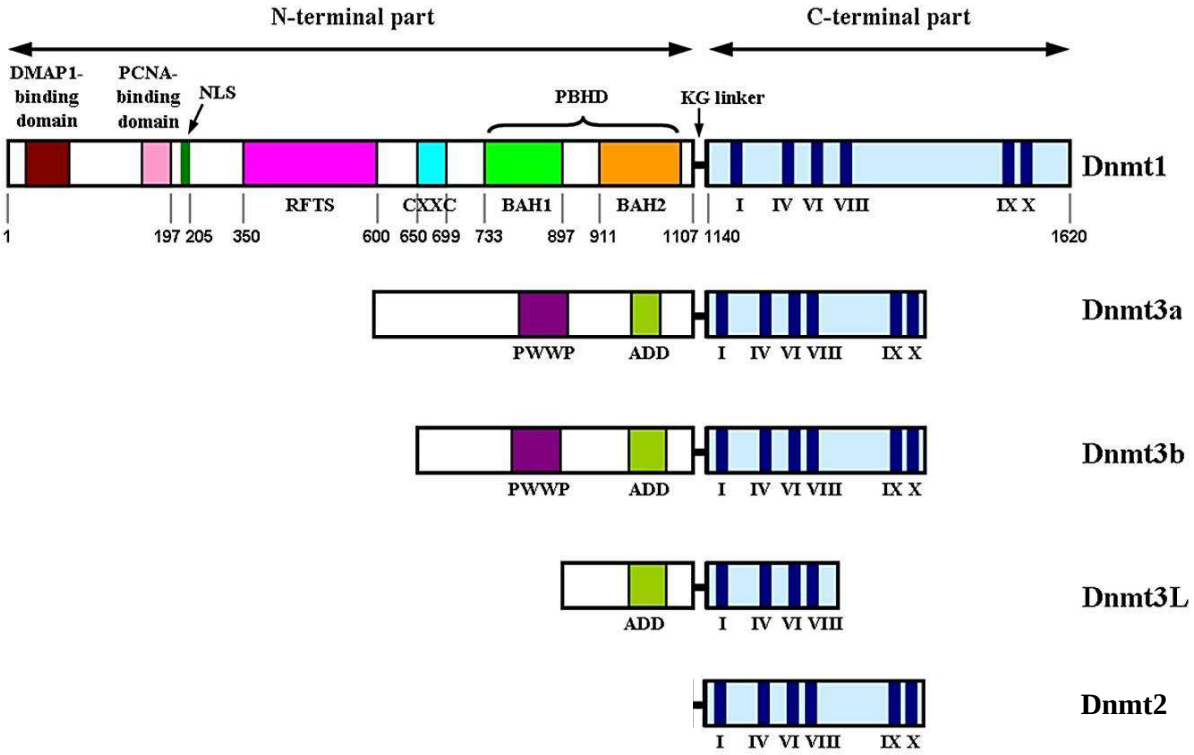

Figure 9 – Domain architecture of DNMTs. The C-terminal domains are marked as light blue. Roman numerals indicate the conservative motifs characteristic for DNMTs in the C-terminal domains. *Adapted from [71].*

Importantly, the addition of the methyl group to cytosine does not interfere with the Watson-Crick base paring of the nucleotide, since this group is inserted in the major groove of DNA, where it can be efficiently recognized by DNA-interacting proteins [66].

The two mechanisms by which DNA methylation can regulate gene activity are by inhibiting the binding of transcription factors through direct methylation of CpGs dinucleotides within their binding sites and/or by acting as binding sites for methyl-CpG binding proteins (MBDs) which associated with other factors as histones deacetylases (HDACs) can establish repressive chromatin structures (Figure 10) [72-74].

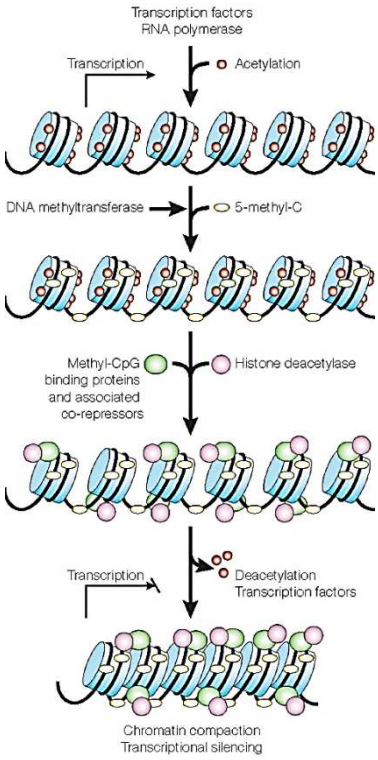

Figure 10 – Transcription regulation by DNA methylation. A transcriptionally active region targeted for silencing is proposed to acquire DNA methylation, which then recruits the MBPs, their associated co-repressors and HDACs. The deacetylated nucleosomes adopt a more tightly packed structure that inhibits the access of transcription factors to their binding sites causing transcription repression. *Adapted from [72]*.

Changes in DNA methylation pattern have been described in several human diseases, including cancer [75]. In fact, in cancer cells gain in DNA methylation (Figure 11) at genes promoters that are normally unmethylated leads to gene inactivation (hypermethylation), and concurrently, demethylation of normally methylated regions has been associated to chromosomal instability and activation of proto-oncogenes (hypomethylation) [60].

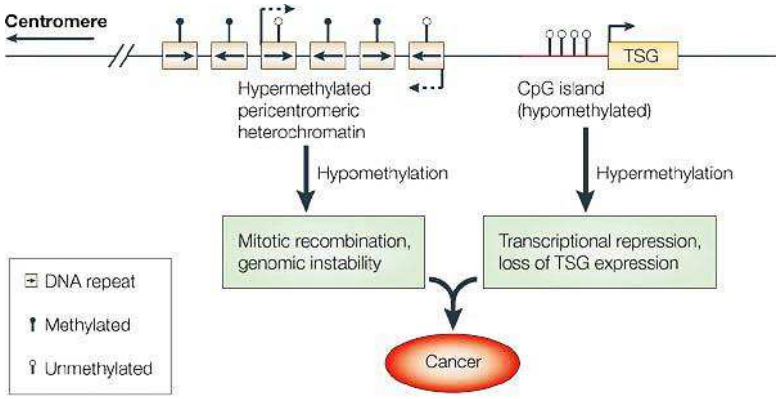

Figure 11 – DNA methylation in cancer cells. *Adapted from [75]*.

Histone Post-Translational Modifications

Chromatin structure is composed by DNA and nucleosomes. The latter are octomers of histone proteins containing eight pillars: two each of histones H2A, H2B, H3 and H4. These small basic proteins consist of a globular C-terminal domain and a flexible unstructured N-terminal tail around to which 147 base pairs of DNA is packaged within the chromatin. Consecutive nucleosomes are separated by unwrapped linker DNA between 20 and 50 base pairs in length [76]. Histone H1 is a linker histone which does not belong to the nucleosome but binds it to the DNA that separates two histone complexes (Figure 12A). The N-terminal tails of histones can undergo a variety of post-translational covalent modifications which are catalyzed by various histone-modifying enzymes. At least 16 different post-translational histone modifications (PTMs) have been reported, including acetylation, methylation, phosphorylation and ubiquitination (Figure 12B) [76, 77].

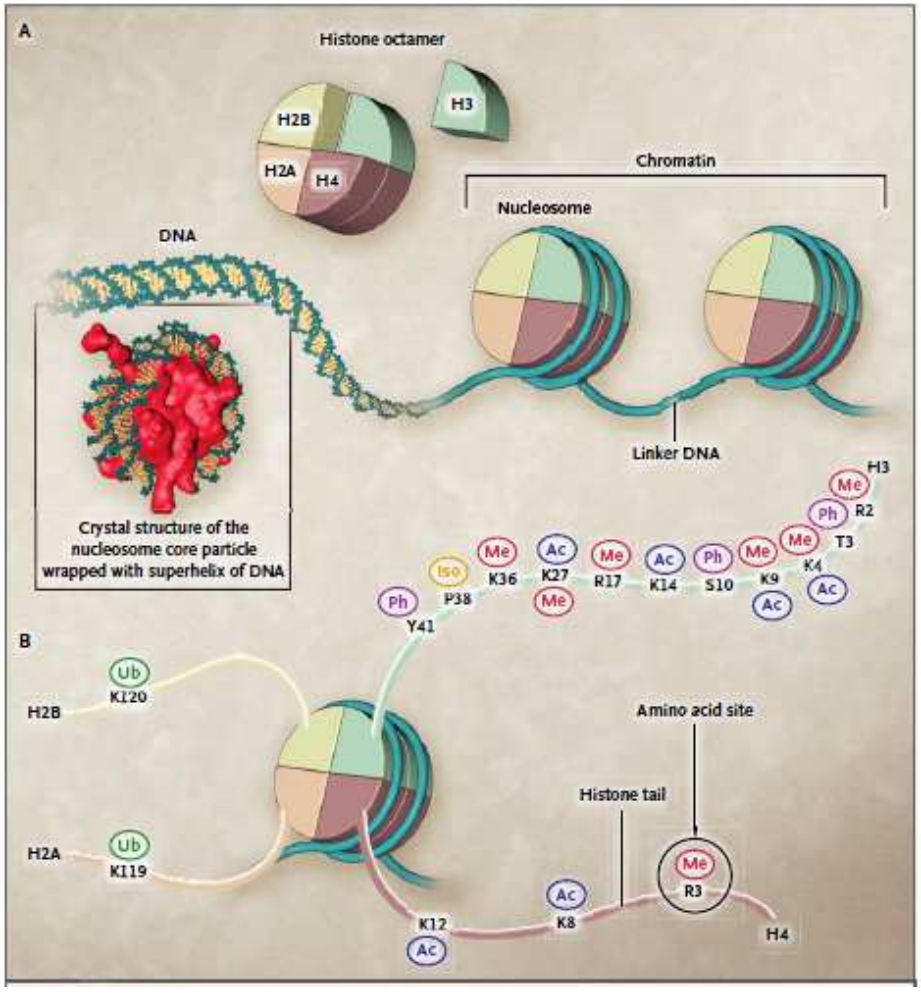

Figure 12 – The nucleosome. (A) Nucleosome structure. (B) Histone post-translational modifications, Me – methylation; Ac- acetylation; Ph – phosphorylation; Ub- ubiquitylation and ISO – proline isomerization. *Adapted from [76]*.

Histone modifications are very dynamic and are performed by “chromatin writers” enzymes that establish PTMs in histones, removed by “chromatin erasers” and readily recognized by “chromatin readers” in a highly regulated manner (Figure 13) [76, 78, 79]. These modifications affect the chromatin structure and act by disrupting chromatin contacts or interfering with the recruitment of other proteins to the chromatin. The distinct combinations of modifications within the histone tails are known as the “histone code” and together with DNA methylation regulate gene activity [60, 76, 78].

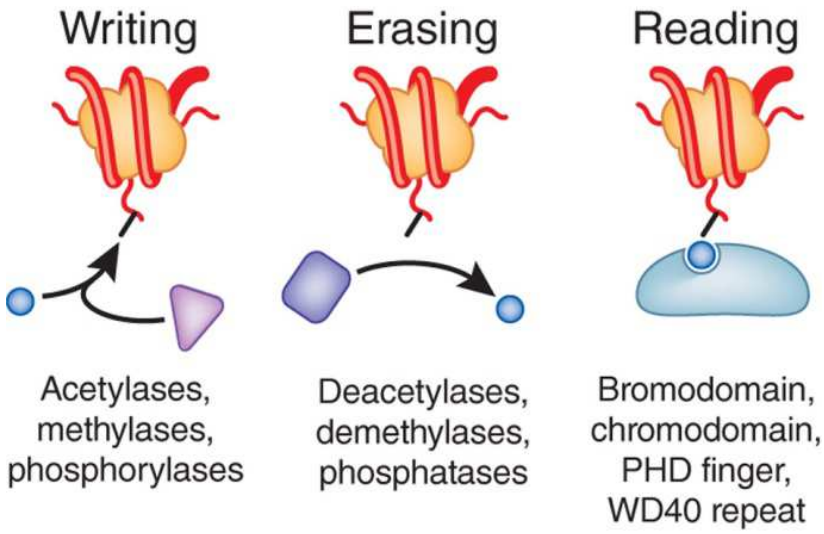

Figure 13 – Chromatin writers, erasers and readers. 'Writers' introduce histone marks (circles), 'erasers' take them out and 'readers' can recognize a particular form of histone modification. *Adapted from [79]*.

Acetylation of lysine residues of histones is in general associated with increased transcriptional activity. This modification is catalyzed by histone acetyltransferases (HATs) and can be reverted by histone deacetylases (HDACs). HDACs are crucial transcriptional co-repressors. In humans 18 HDACs are described and grouped into four classes: Class I (HDACs 1, 2, 3 and 8), Class II (HDACs 4, 5, 6, 9 and 10), Class III (sirtuins 1–7) and Class IV (HDAC 11) (Figure 14) [80]. Whereas, histone methylation is associated with transcriptional activation or repression depending on the altered residue and the number of methyl groups added [78]. These specific PTMs are carried out by histone methyltransferases (HMTs) and are removed by histone demethylases (HDMs) [78].

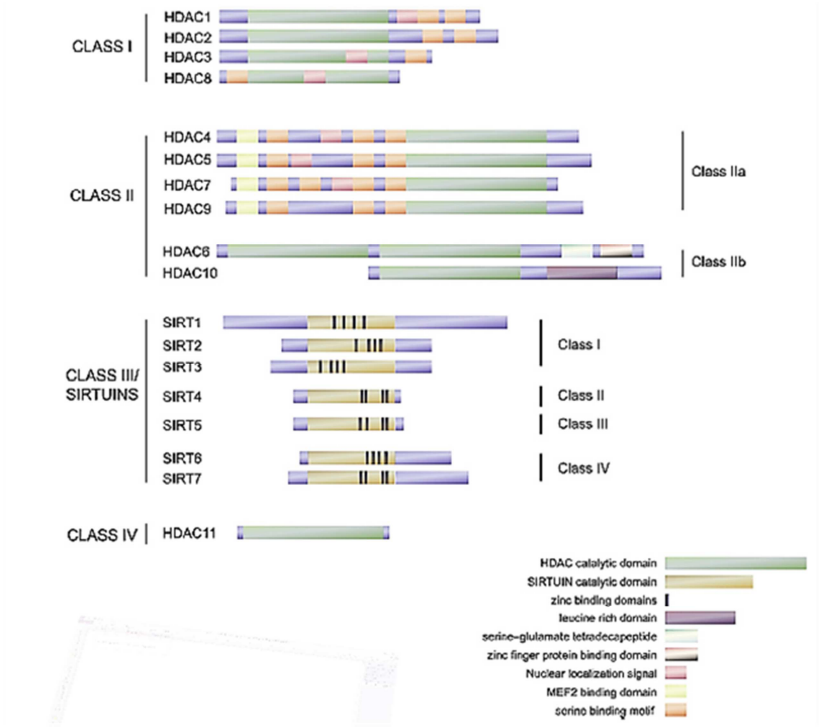

Figure 14 – Human HDACs family. HDACs are grouped into four different classes according to sequence similarity and homology to yeast proteins. *Adapted from [80]*.

It recent years an increasing body of evidence suggests that pattern changes of PTMs due to aberrant activity or mistargeting of the chromatin modifying enzymes are implicated in carcinogenesis (Figure 15) [77, 81].

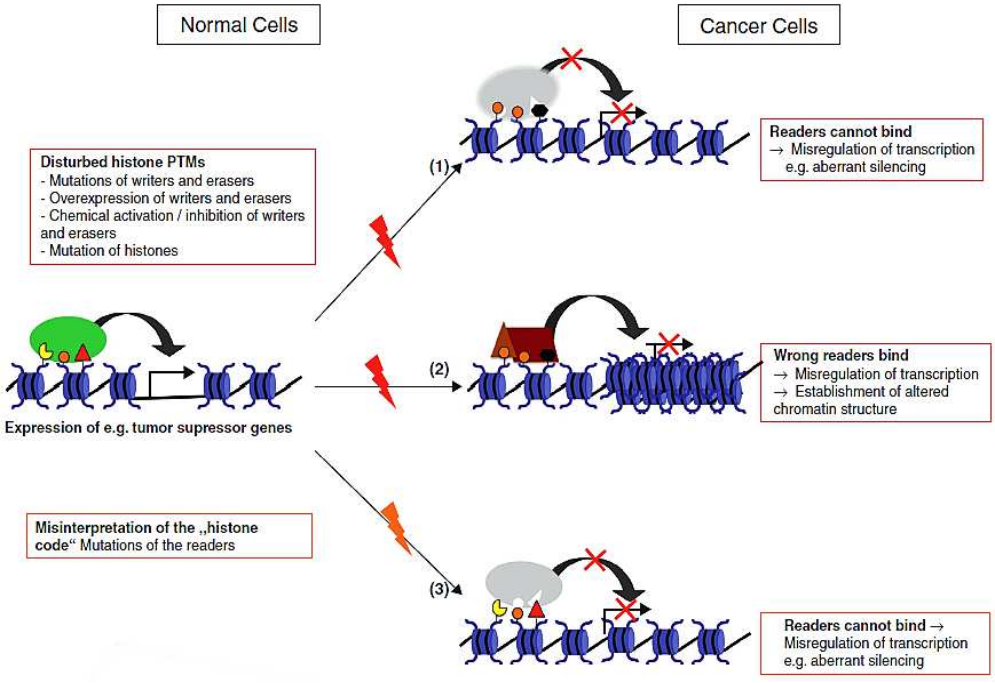

Figure 15 – Histone deregulation in cancer. *Adapted from [77]*.

Non-Coding RNAs

Non-Coding RNAs (ncRNAs) are a class of RNAs that does not encode for proteins but are biologically functional. Indeed, they are involved in a wide range of cellular functions, including control of chromosome dynamics, splicing, RNA editing, inhibition of translation and mRNA destruction [82].

The most studied class of ncRNAs is microRNAs, but apart from those this class is composed by transcribed ultraconserved regions (T-UCRs), small nucleolar RNAs (snoRNAs), PIWI-interacting RNAs (piRNAs), large intergenic non-coding RNAs (lincRNAs) and a very heterogeneous group of long non-coding RNAs (lncRNAs) [83]. Several of these molecules have been reported to play a role in epigenetic modification mechanisms such as the silencing of transposable elements, X-chromosome inactivation, and DNA imprinting [84, 85]. In the past few years new evidence has emerged concerning its expression deregulation in neoplastic transformation, particularly regarding miRNAs [83].

MicroRNAs

MiRNAs are endogenous single stranded small non-coding, RNAs, with 18 to 25 nucleotides in length, that regulate gene expression [86, 87]. Up to now 2,555 human miRNAs have been identified and registered in the miRBase database (release 20, June 2013). These molecules are not only involved in the regulation of a variety of biological processes, including cell cycle, cell death, differentiation, development, and metabolism but also in several human diseases [88].

Biogenesis

MiRNAs are primarily transcribed in the nucleus by RNA polymerase II into long capped and polyadenylated primary transcripts with hairpin structures named primary miRNA (pri-miRNA) [89, 90]. This molecule is subsequently cleaved in the nucleus by RNase III DROSHA to become a hairpin RNA of about 65 nucleotides, termed precursor-miRNA (pre-miRNA). *Di George Syndrome critical region gene 8* (DGCR8) interacts with Drosha, by guiding it in the pri-miRNA structure for cleavage, and these two enzymes form the “microprocessor complex” [89-91]. Some of the intronic miRNAs, named mirtrons, bypass this Drosha processing step and are processed by the splicing machinery [92, 93]. The pre-miRNAs are actively exported to the cytoplasm by Exportin 5 (XPO5) and RanGTPAse. Once in the cytoplasm the loop of the pre-miRNA is cleaved by RNase III enzyme DICER1, which acts in complex with the dsRNA-binding protein Trans-activator RNA-binding protein 2, TARBP2 (TRBP) [90, 92]. This cleavage originates a

short RNA duplex, which consists in a mature miRNA (guide strand) and a complementary strand, miRNA* (passenger strand). Normally, the guide strand is the one with lower pairing stability at the 5' segment [90, 94]. The mature miRNA is loaded into an Argonaute protein (Ago) by TRBP and this complex is termed as miRNA-containing RNA-induced silencing complex (RISC). The complementary strand is marked for degradation (Figure 16) [90, 92].

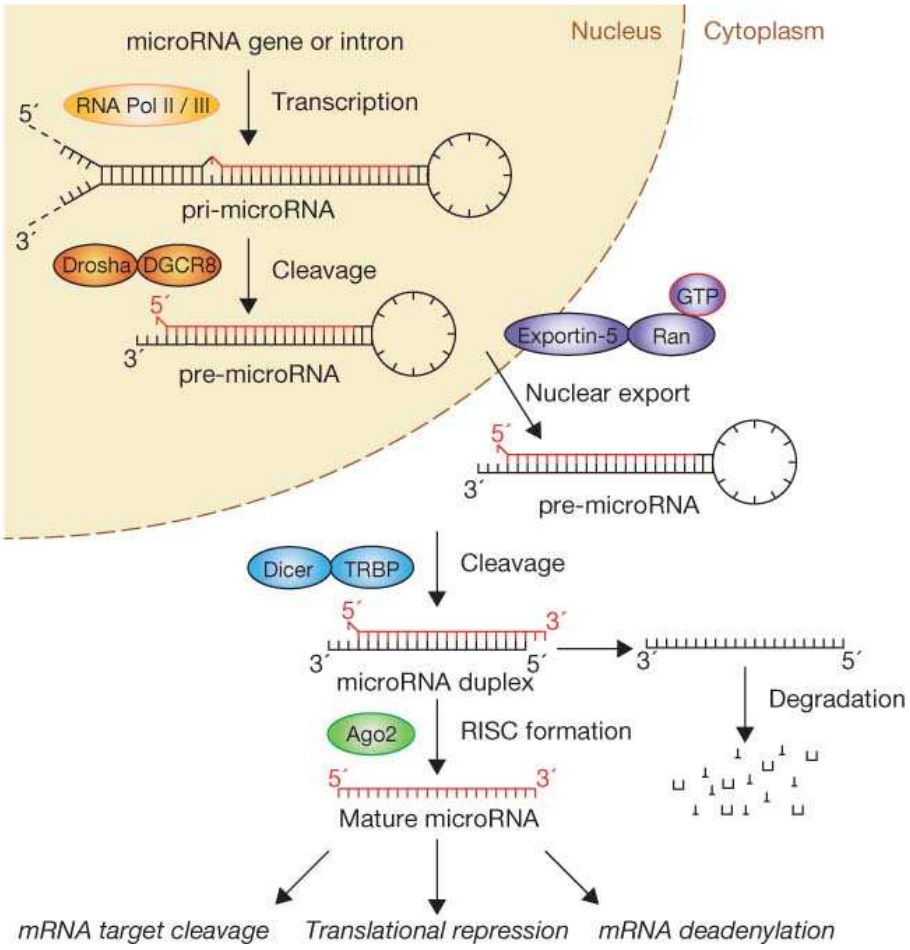

Figure 16 – MiRNAs biogenesis and mechanism of action. *Adapted from [93].*

When the miRNA is incorporated into RISC, it can directly regulate gene expression, by binding to a 3' UTR partially complementary sequence in the target mRNA. [92]Translation of mRNA into proteins can be repressed by miRNAs by two main ways: mRNA degradation, if the complementarity between mRNA and its target mRNA is perfect; or inhibition of translation initiation, if they bind in an imperfect way. Recently, 5'-UTR and open reading frame (ORF) were also described as having target sequences for miRNAs. MiRNAs were also reported as able to bind to ribonucleoproteins, interfering with their RNA binding functions, and to DNA therefore regulating gene expression at

transcription level [83]. Considering the different and complex mechanisms that regulates the interaction between a miRNA and its target mRNA, it is now known that each miRNA has the potential to regulate several different genes. Conversely, a single mRNA can be targeted by multiple miRNAs. Therefore, alterations in a single miRNA can potentially have dramatic effects in numerous cellular processes as mentioned above [92].

Several studies have found that alterations in miRNAs expression are associated with several human diseases, including cancer. In fact, approximately half of the known miRNAs genes are located at cancer-related genomic regions. It is also recognized that 20 to 40% of miRNA genes are located close to CpGs islands making them potential targets of the DNA methylation machinery. Several defects in enzymes involved in miRNAs biogenesis (DROSHA, XPO5, DICER, and TRBP) were also implicated in neoplastic transformation [95-98].

In cancer, miRNAs can act as either oncogenes or tumor-suppressors depending on the target genes and tumor context (Figure 17) [99].

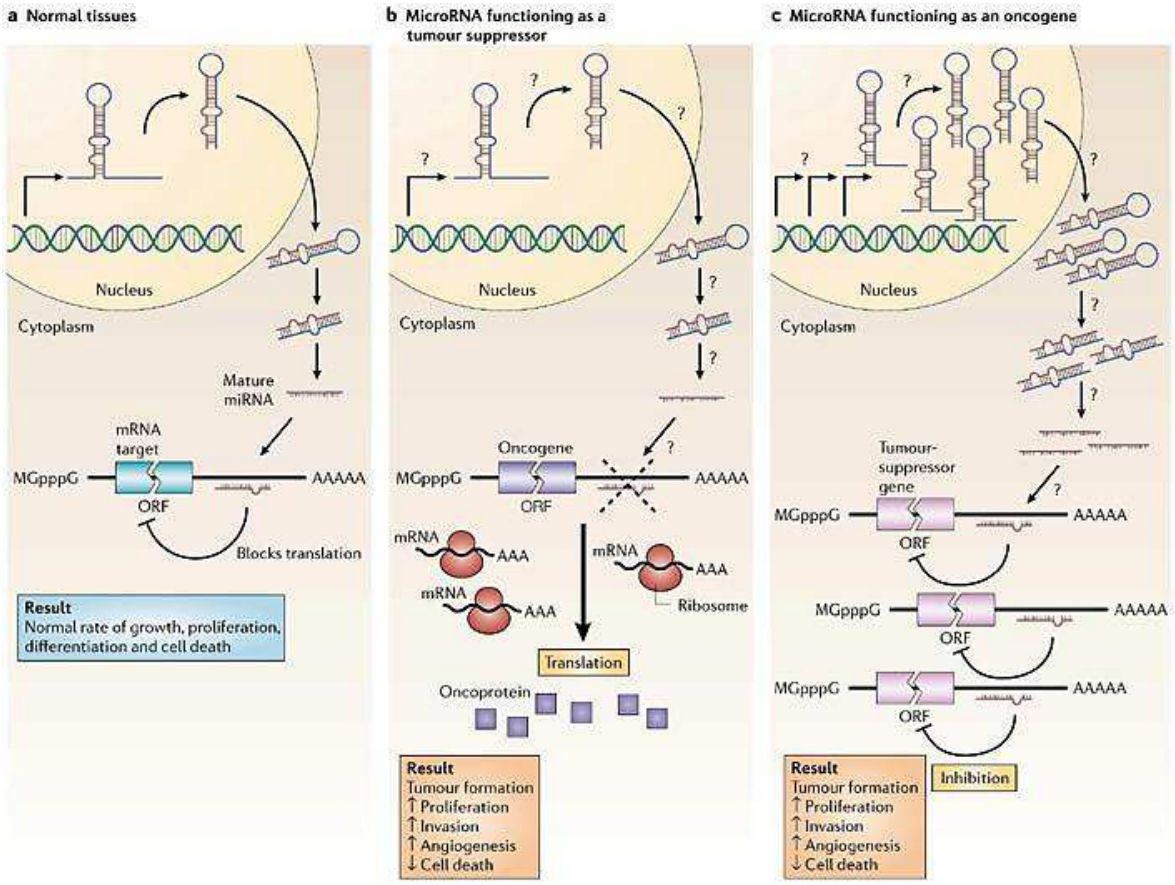

Figure 17 – MiRNAs in cancer. a) MiRNAs in normal tissue. b) Tumor-suppressor miRNA c) MiRNAs as oncogenes. *Adapted from [99]*.

In cancer, a reduction in tumor-suppressor miRNA levels can occur mainly due to defects at any stage of miRNA biogenesis triggering the inappropriate expression of its target oncoprotein which might lead to tumor formation by increasing proliferation, invasiveness and/or angiogenesis and decreasing apoptosis levels. Conversely, the overexpression of a miRNA with an oncogenic potential might also induce tumor development. Indeed, increased levels of such oncomirs, due to miRNA encoding gene amplification, constitutively active promoter, increased miRNA processing efficiency or increased stability of the miRNA, leads to decreased expression miRNA-target tumor-suppressor genes and thus cancer development [99].

EPIGENETIC ALTERATIONS IN PCA

During PCa development and progression, prostate cells acquire both genetic and epigenetic alterations [100]. Epigenetic alterations can be found in every stages of prostate carcinogenesis namely in precursor lesions, localized and disseminated disease [101, 102]. PCa is therefore considered the perfect model of epigenetics catastrophe. In the past few year, the knowledge about these epigenetics modifications in this neoplasm opened the field not only for the discovery of new biomarkers for PCa screening, detection, diagnosis, staging and prognosis, but also for epigenetic-based therapy [101].

DNA Methylation in PCa

DNA promoter hypermethylation is the best studied epigenetic alteration in PCa. In fact, about 100 genes were described as being silenced by promoter methylation in PCa [102, 103]. These genes include several classic and putative tumor-suppressors involved in cellular pathways important for carcinogenesis, such as DNA damage response, cell cycle regulation, cell migration/invasion, cell death, and hormone response. Inappropriate silencing of these genes can therefore contribute for cancer development, progression and invasion (Figure 18). Importantly, hypermethylation of some of these genes were correlated with pathologic grade and/or clinical stage and with evolution for a castration resistant disease [104-110]. *Glutathione S-Transferase Pi 1 (GSTP1)* hypermethylation has been the most studied methylation alteration in PCa. *GSTP1* methylation is present in more than 90% of PCa and also in 5-10% of precursor lesions of PCa, suggesting that this gene hypermethylation is an early event in prostate carcinogenesis [106, 111-115].

DNA methylation is also involved in regulation of AR. Indeed, AR silencing by hypermethylation was described in about 30% of hormone-refractory PCa [116]. Several other genes were described as frequently hypermethylated in morphologically normal prostate tissue and in HGPIN (e.g., *APC*, *CCND2*, *RARB2*, *RASSF1A*, *PTGS2*) suggesting its implication in prostate carcinogenesis initiation [117, 118].

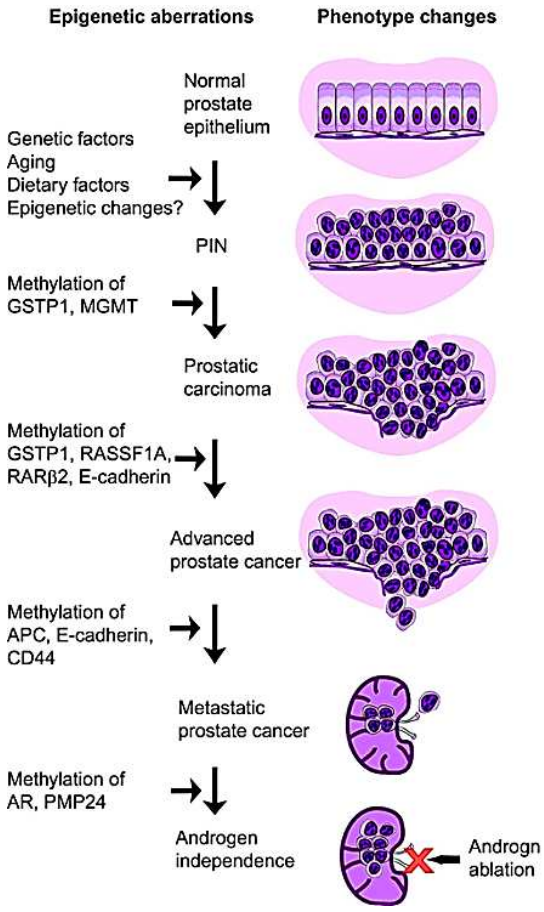

Figure 18 – DNA methylation during PCa progression. *Adapted from [110]***.**

Concurrently, during PCa progression there is also an overall reduction in m^5C content, being the lower levels found in metastatic tissue (Figure 19) [119]. Repetitive DNA regions distributed throughout the genome and that usually present methylation at CpG dinucleotides, like LINE1, were found to be hypomethylated in up to 53% of PCa and, especially, in ones that harbor lymph node metastasis [120]. Reduced repeat sequence methylation may provide a connection between genetic and epigenetic alterations in cancer cells [121]. Whilst global DNA methylation can contribute to PCa by promoting genomic instability, the hypomethylation of individual gene promoters could promote cancer by causing aberrant gene expression. In fact, several genes, that should be maintained silenced in normal adult prostate cells, were found to be activated in PCa tissue by the reduction in DNA methylation of their transcriptional regulatory regions [102].

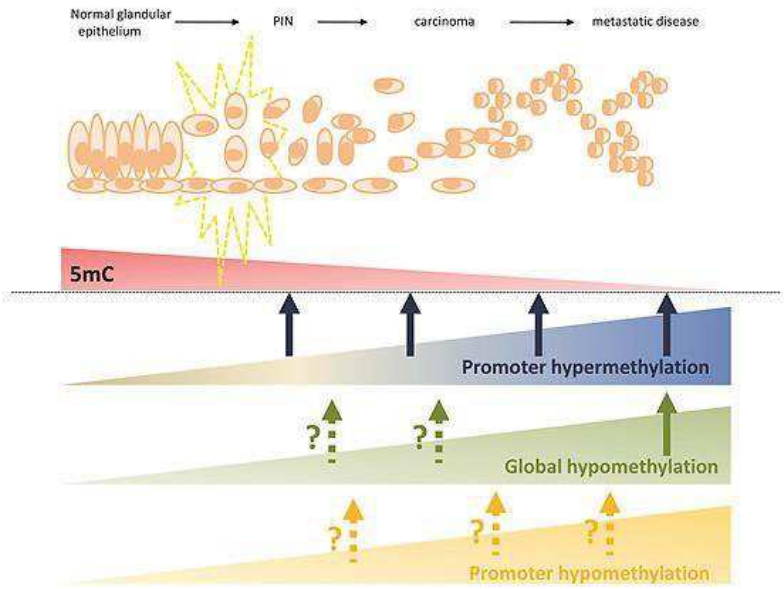

Figure 19 – Changes in methylation patterns during PCa development. An overall decrease in the levels of 5mC is observed. Global hypomethylation starts to become more evident during progression to metastatic disease. Promoter hypermethylation of specific genes is one of the earliest events in prostate carcinogenesis and persists throughout disease progression. *Adapted from [122]*.

Histone Modifiers and post translational modifications deregulation in PCa

Although DNA methylation and histone modifications are known to be closely linked mechanisms, the role of histone modifications in prostate carcinogenesis is relatively poorly understood [123]. Indeed, only a few histone-modifying enzymes have been already found to be deregulated in PCa. From those, the HMT polycomb protein enhancer of zeste homolog 2 (EZH2) is by far the most studied enzyme in PCa. This enzyme, responsible for H3K27 trimethylation a repressive mark, was described as overexpressed in PCa, particularly in mCRPC [102, 124]. Furthermore, several genes repressed by this enzyme were found to be deregulated in metastatic PCa, suggesting the involvement of this enzyme in PCa progression [125]. Lysine-specific demethylase 1 (LSD1), is another enzyme frequently reported as important for prostate carcinogenesis. This enzyme acts both as co-activator and co-repressor of transcription by targeting H3K4 or H3K9, respectively, is described as aberrantly expressed in PCa [126-128]. In fact, LSD1 was found to form a complex with AR, stimulating its transcription. Moreover, increased levels of LSD1 were associated with aggressive CRPC and with high risk of relapse during follow-up [127, 128].

Concerning Class I histone deacetylases, HDAC1 was found overexpressed in about 70% of PCa and associated with the progression to castration-resistant disease

[129, 130]. Similarly, HDAC2 and 3 were also reported to be upregulated in PCa and associated with disease progression [131]. Whereas, sirtuin 1 (SIRT1), a class III HDAC, was identified as being downregulated in PCa when compared to normal prostatic tissue, leading to H2A.Z overexpression and consequently to upregulation of c-Myc and other oncogenes [132].

Several other histone-modifying enzymes, like JHDM2A, JMJD2C, SET9, have already been shown as playing a role during prostate carcinogenesis [63]. Moreover, in addition to changes in chromatin modifier enzymes, some histone modifying patterns, like H3K18Ac, H3K4me2, and H3K4me1 were also associated with increased risk of PCa recurrence [133, 134].

MiRNAs in PCa

MiRNAs expression is also extremely deregulated in PCa [135]. Until now, more than 50 miRNAs were found to be deregulated in PCa compared to prostate normal tissue [136, 137]. Although still controversial, most of studies have reported a global downregulation of miRNAs in PCa [138, 139]. From those only a few miRNAs were proven to have a role in prostate carcinogenesis, being involved in key pathways, as cell cycle regulation, apoptosis, migration/invasion, epigenetic reprogramming and androgen signaling [135, 140]. In fact, miRNAs involvement in AR regulation is one of the major topics of discussion. Several authors argue that miRNAs are regulated by androgens whereas other defend that the androgen pathway is modulated by miRNAs, since miRNAs seem to be closely linked with PCa progression [141, 142]. MiR-101 was identified as an androgen-responsive miRNA in PCa cell lines that through its negative regulation of EZH2 expression was associated with decreased migration and invasion. Interestingly, and contrarily to EZH2 levels, the expression of this miRNA decreases throughout PCa progression [124, 143]. Similarly to miR-101, miR-146a downregulation was found in castration-resistant PCa when compared to androgen-depend PCa [144]. Recently, epidermal growth factor receptor (EGFR) was reported as a target of this miRNA, indicating that its downregulation might contribute to initiation of ERK-mediated oncogenic pathway [145]. Contrarily, an overexpression of miR-221 or miR-222 was observed in castration-resistant PCa, and the induction of expression of these miRNAs in androgen-dependent LNCaP cells was shown to dramatically reduce p27/Kip1 expression and induce androgen-independent growth [146]. The upregulation of miR-141, an androgen-responsive miRNA, was also associated with PCa progression [147]. This miRNA was proposed as a non-invasive biomarker for tumor progression and therapeutic response [148, 149]. A negative correlation between miR-34a and miR-34c and AR expression levels had also been suggested. Accordingly, castration-resistant PCa cell lines (DU145

and PC-3) were shown to display lower miR-34a levels than androgen-dependent cell lines (PrEC and LNCaP). Furthermore, the expression of the former was correlated with p53 expression, a widely known tumor-suppressor gene, that regulates apoptotic and cell cycle genes. Indeed, the induced expression of this miRNA in PC-3 triggers these cells to apoptosis and cell cycle arrest at G1 phase. Importantly, miR-34a was also found to be targeted by SIRT1, and has previously mentioned, this enzyme is downregulated in PCa, especially in the most aggressive forms. Additionally, the ectopic expression of this miRNA attenuated chemoresistance to camptothecin, an anticancer drug [150-152].

Another miRNA downregulated in PCa is miR-449a and the induction of its expression in PCa cell lines leads to attenuation of cellular malignant phenotype. Moreover, this miRNA represses HDAC1 expression leading to a decrease in tumor cell proliferation [153].

In general, miRNAs has been also implicated in metastasis regulation, acting as either inducers or suppressors and are designated as “metastamirs” [154]. So far, only few studies addressed the role of miRNAs in PCa metastasis, being miR-21, miR-29b, miR-203, miR-205, and miR-331-3p the most studied [154]. Indeed, miR-21 acts as a metastasis inducer since it increased the invasion potential of PCa cells [155]. On the other hand miR-29b, mir-203, and miR-205 are examples of metastasis suppressors. MiR-29b modulates the expression of several proteins involved in the metastatic process, like E-cadherin, P-cadherin, Snail, Twist and metalloproteinase-2 (MMP2) and is also downregulated in PCa [156, 157]. MiR-203 is downregulated in PCa cells derived from bone metastasis, and its artificial overexpression in nude mice, reduces tumor metastasis formation, via inhibition of several critical steps of the metastatic cascade including EMT, invasion, and motility [158]. MiR-205, which is downregulated in PCa, has been reported to inhibit epithelial-mesenchymal transition (EMT) reducing cell invasion and metastasis [159].

The study of miRNAs in prostate carcinogenesis is an area of intense interest. Not only some miRNAs are now recognized as potential biomarkers but also are being studied as therapeutic targets. Hence, further research should be performed to understand the mechanism of action and target selection of miRNAs and their role in PCa.

EPIGENETIC TARGET THERAPY IN PCA

Epigenetic Therapy

As previously mentioned, aberrant epigenetic alterations in cancer cells may promote genomic instability, silencing of tumor-suppressor genes and oncogene reactivation. However, unlike genetic alterations, epigenetic events do not alter the DNA sequence and are potentially reversible. Reactivation of epigenetically silenced genes might, therefore, provide new attractive therapeutic options. Recently, several promising agents that inhibit key enzymes involved in establishing/writers, removing/editors and maintaining/readers the epigenetic profiles have been identified (Figure 20) [62].

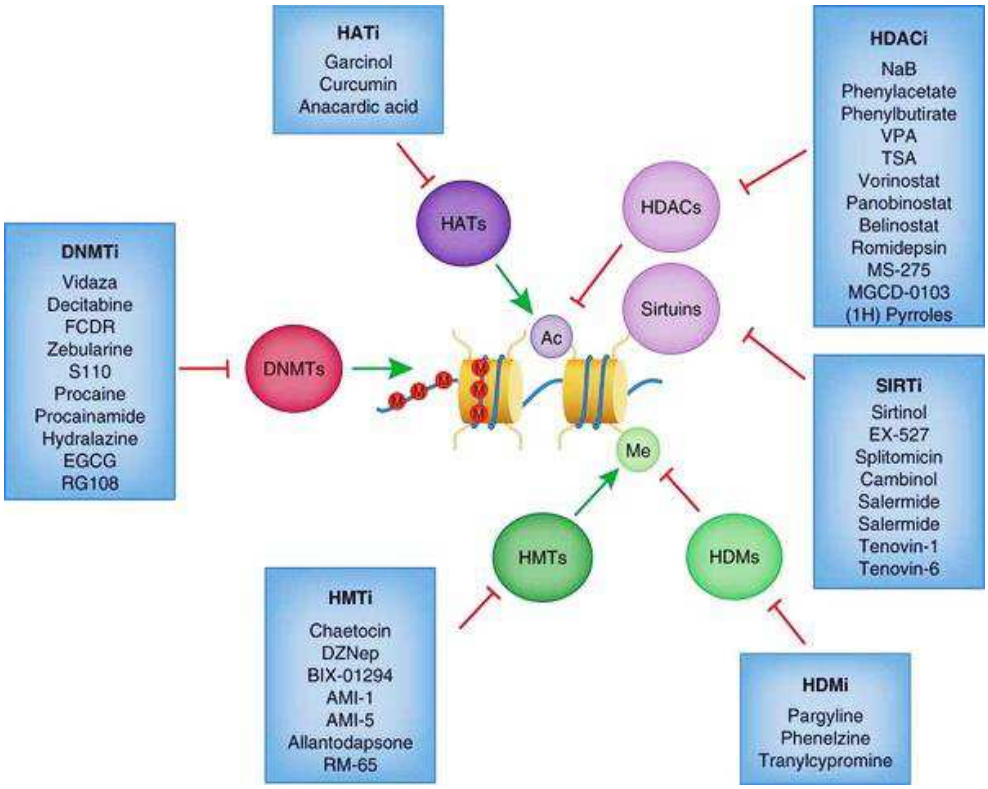

Figure 20 – Epigenetic drugs for cancer therapy. *Adapted from [62]*.

Currently, the best studied epigenetics drugs in cancer are DNA methyltransferases inhibitors (DNMTi) and Histone deacetylases inhibitors (HDACi). Indeed, the FDA has already approved four of these epigenetic modifying drugs for cancer treatment. Vidaza (5-azacytidine) and Decitabine (5-aza-2′-deoxycytidine) are the two DNMTi approved for patients with myelodysplastic syndrome (MDL) [160, 161]. Vorinostat (suberoylanilide hydroxamic acid, SAHA) and romidepsin (FK-228) are two HDACi for cutaneous T cell lymphoma (CTCL) treatment [162, 163]. Contrarily, inhibitors of sirtuins

(the class III HDACs), HATs, HMTs, HDMs and various kinases are now being intensively investigated.

DNMTi

Among the epigenetic inhibitors, as previously mentioned, DNMTi are in clinically advanced stage of development. This family can be divided in two classes depending on the mode of action: nucleoside and non-nucleoside inhibitors (Figure 21) [164].

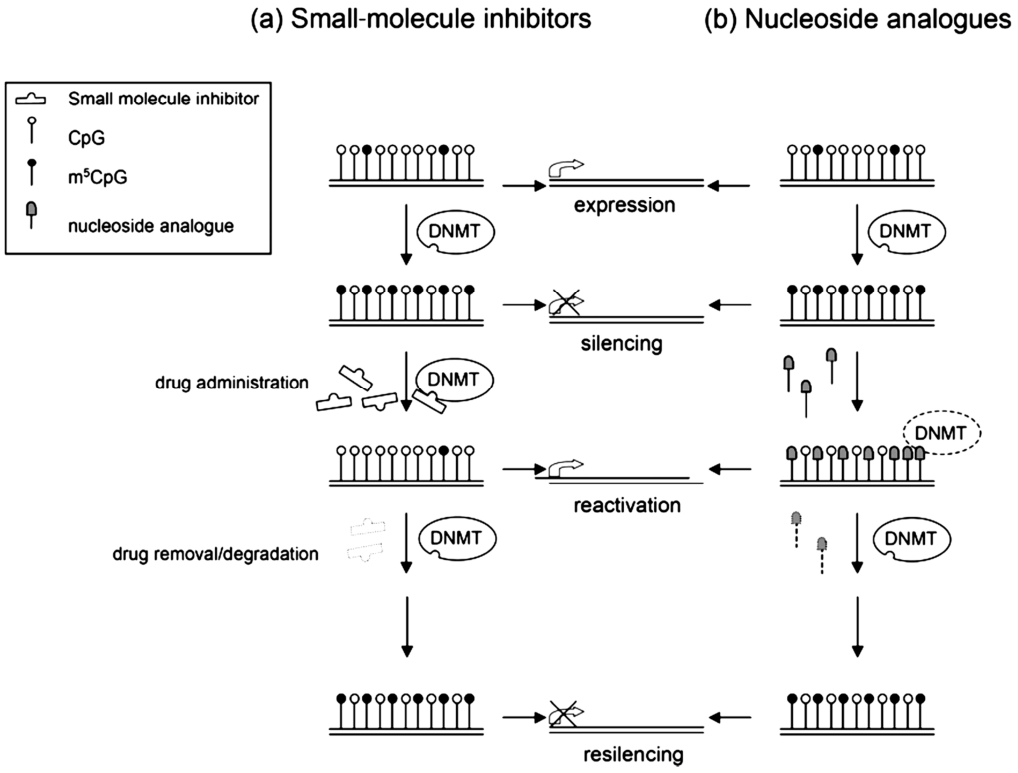

Figure 21 – Comparison of inhibitory mechanisms of non-nucleoside or small molecule inhibitors (a) and nucleoside analogues (b). Non-nucleoside analogues bind to the catalytic center of DNMTs inhibiting methylation directly. Nucleoside analogs covalently trap DNMTs into DNA leading to enzyme degradation. Further DNA replication causes passive DNA demethylation and reactivation of epigenetically silenced genes. The removal of both compounds results in remethylation and resilencing. *Adapted from [164]*.

Nucleoside Analogs

Nucleoside analogues bind to DNA replacing cytosines. When incorporated into DNA during replication, these drugs covalently link with DNMT trapping these enzymes on the DNA strand. DNMTs are subsequently depleted due to passive demethylation during continuous replication. These agents usually cause cell death by obstructing DNA synthesis and/or induce DNA damage through structural instability at the site of

incorporation [164, 165]. Until known several nucleoside analogues have been described (Figure 22).

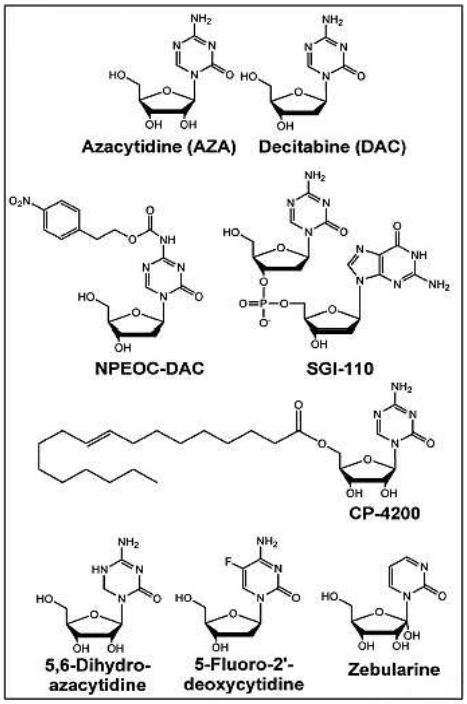

Figure 22 – Nucleoside analogs that inhibit DNMT once incorporated into DNA. *Adapted from [166, 167]*.

The two most studied nucleoside analogues are 5-azacytidine, a ribose nucleotide which is specially incorporated into RNA and thereby interferes with protein synthesis, and 5-aza-2′-deoxycytidine which is incorporated preferentially into DNA. During several years these drugs were administered at the maximum tolerated dose which culminated in severe side effects and prolonged myelosuppression [160]. At low doses these compounds are effective hypomethylating agents and have shown clinical activity as anticancer agents. These drugs were shown to reactivate tumor-suppressor genes, causing chromatin expansion and induction of cellular differentiation in several cancers. The azanucleosides also demonstrate the ability to enhance sensitivity to conventional chemotherapeutic agents by reactivating several apoptotic genes. These DNMTi are currently in clinical trials in a range of cancers. However, azanucleosides have some pitfalls, including their higher instability and their short half-life owing to degradation by cytidine deaminase [164, 166].

Zebularine is also a nucleoside analogue that has shown to be more stable, since it induced minimal toxic effects in animals and is able to inhibit cytidine deaminase. This compound has proven antiproliferative activity in cell lines [168]. SGI-110 is a largely

resistant cytidine deaminase compound that was also reported to be effective in inhibiting DNA methylation both *in vitro* and *in vivo* [169]. Moreover SGI-110 has hypomethylation activity in primates and is currently in phase I clinical trial in patients with MDL and acute myeloid leukemia (AML) [166, 170].

One major limitation of nucleoside analogues is the requirement for DNA incorporation and active DNA synthesis, which limits the activity of these drugs in hypoproliferating cells. This may be the major reason for their limited efficacy in solid tumors [29].

Non-nucleoside Analogues

As the nucleoside analogues are inherently cytotoxic, several efforts are being made to discover compounds that directly target DNMTs, without requiring prior incorporation into DNA. The non-nucleoside family includes compounds that have already been approved by FDA for non-neoplastic diseases, like hydralazine (anti-hypertensive drug), procaine (local anesthetic) and procainamide (anti-arrhythmic drug) and small molecules designed to directly block the active site of human DNMTs like RG108 (Figure 23). The major advantage from the first class is that the pharmacodynamics profile is well-known and their adaptation for cancer therapy may possibly be cost-efficient. The second class exhibit higher specificity, since the compounds are designed for direct enzyme inhibition [164, 166]

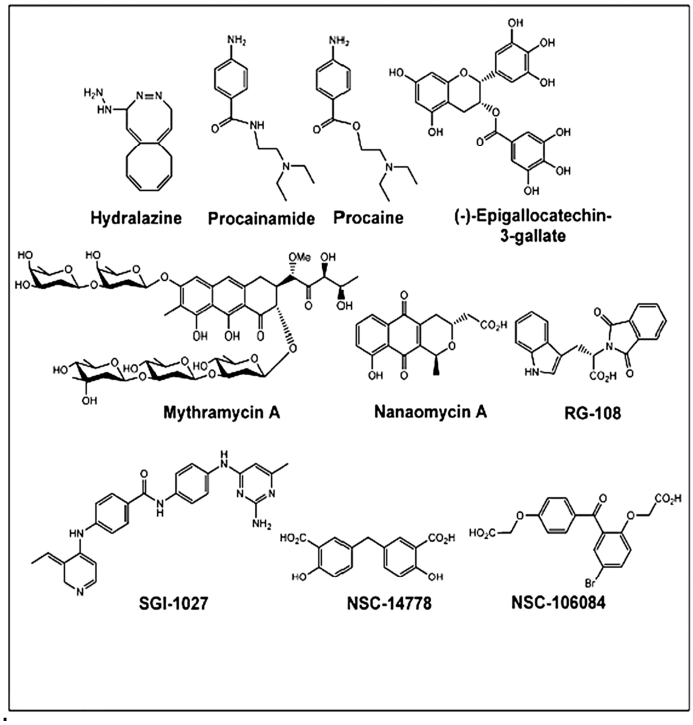

Figure 23 – Non-nucleoside analogues DNMTi that direct target DNMT. *Adapted from [166]*.

Hydralazine is a potent arterial vasodilator drug used to treat severe hypertension and heart failure. It was proposed as having potential demethylating activity since one of its secondary effects was the induction of Lupus-like syndrome [171-173]. The mechanism of action of hydralazine as a demethylating agent is not yet well understood. Some authors suggest that this drug inhibits DNA methylation by establishing highly stable interactions between its nitrogen atoms and the DNMT active site [171]. Hydralazine was shown to induce demethylation and reactivation of tumor-suppressor genes in several cancer models and its activity is synergized with the HDACi, valproic acid, either *in vitro* or *in vivo* [174-176]. Clinical trials using hydralazine in combination with a valproic acid in MDS and in solid tumors are being conducted [177, 178].

Procaine and procainamide are two closely related small molecules that have been proposed to function as DNMTi also due to their ability to bind to CpG-rich sequences, thereby interfering with DNMTs binding. Procainamide specifically inhibits DNMT1 and not DNMT3a and 3b, which suggests that this drug might be a highly specific inhibitor [179]. They were reported as being able to reduce DNA methylation in cancer cells [175, 180-182].

The antibiotic Nanaomycin A was recently reported as a selective inhibitor of DNMT3b, with the ability to reduce methylation and induce expression of the tumor-suppressor gene *Ras-association domain family protein 1*, *isoform A* (*RASSF1A*) [183].

RG108 was the first DNMTi designed to directly inhibit DNMT1catalytic site. In fact this compound was able to inhibit DNMT activity in a cell-free assay and to reduce global methylation levels in human cancer cells. Moreover, RG108 was shown to be effective in human colon cancer cells, in which it reactivated several tumor-suppressor genes (*e.g.*, *p16*), without affecting the methylation status of centromeric repeats [184-186]. Recently, this compound was also reported to induce differentiation in promyelocytic leukemia cells when combined with HDACi [187].

MG98, a 20-bp antisense oligonucleotide, whose sequence is complementary with the 3′-UTR of DNMT1, was developed to prevent the translation of this enzyme. Despite the presented DNMT inhibitory activity in xenograft mouse, this compound did not achieve good results in clinical trials [188, 189].

(–)-Epigallocatechin-3-gallate (EGCG), one of the components of green tea extracts, has also shown demethylation activity presumably through binding to DNMT1. This compound has the ability to decrease methylation and induce re-expression of tumor-suppressor genes [190].

SGI-1027, a quinoline-based compound, has demonstrated inhibitory activity against DNMT1, DNMT3a, and DNMT3b resulting in demethylation and reactivation of tumor-suppressor genes [191].

Even though this class of DNMTi compounds is less cytotoxic than nucleoside inhibitors, some authors support that they are less effective at inhibiting DNA methylation and reactivating gene expression [164, 185, 192].

Importantly, an issue that should be considered regarding DNMTi is their potential to cause global hypomethylation and therefore upregulate genes involved in metastasis.

DNMTi and PCa

Although aberrant DNA promoter methylation is a major phenomenon in prostate carcinogenesis, there are only a few clinical trials involving DNMTi and PCa patients (Table 2).

Table 2 - Clinical trials of DNMTi for PCa. *Adapted from [193]*.

Drug	Clinical Trial ID	Phase	Status	Protocol	Outcome
Vidaza	NCT00384839	II	Completed	CRCP on combined androgen blockade with PSA-DT <3 months treated with 75 mg/m^2 s.c. on days 1-5 of each 28 day cycle for up to 12 cycles where tolerable n = 36	PSA-DT≥ 3 months achieved in 55,8% patients; medium progression free survival = 124 weeks
Decitabine		II	Completed	mCRPC post-total blockade and flutamide withdrawal treated with 75 mg/m^2/dose i.v. as 1h infusion every 8 h for 3 doses; cycles repeated every 5-8 weeks. n=14	17% had stable disease with progression time ≥ 10 weeks
Vidaza, docetaxel and prednisone	NCT00503984	I/II	Recruiting	CRPC patients previously treated with docetaxel. Vidaza: i.v. days 1-5 each 3 weekly cycle; docetaxel: i.v. on day 6 of each 3 weekly cycle; prednisone: 5mg twice daily from day 1 to 21 of each cycle. Predicted enrollment: n= 42	NYA

One small phase II clinical trial, in which 14 patients with mCRPC were enrolled, decitabine was administered intravenously every 8 hour at a dose of 75 mg/m^2 every 5 to 8 weeks. Only two patients showed disease stabilization with delayed time to progression for as long as 10 weeks. Although well tolerated decitabine, only a moderate response was observed in mCRPC [194].

In a pre-clinical assay, PCa cells chronically exposed to decitabine for 21 days, displayed a marked decrease in tumor cell proliferation and reactivation of AR with concomitant increase in PSA protein levels. The restoration of AR sensitized CRPC cells and xenograft models to the anti-androgen bicalutamide [195, 196]. A combination of 5-

azacytidine and docetaxel also revealed tumor growth delay. In fact, 5-azacytidine sensitized PC-3 and 22Rv1 xenograft to docetaxel, being the drug combination not only well tolerated by mice but also superior when compared to either agent alone [197].

PCa cell lines and xenograft mice exposure to procainamide demonstrated a reversion in *GSTP1* hypermethylation with concomitant gene re-expression [182]. However, one study comparing the two non-nucleoside inhibitors with 5-aza-2′-deoxycytidine in PCa cell lines demonstrated that decitabine is considerably more effective in demethylation and reactivation of tumor-suppressor genes [192].

Further studies should be performed to assess the role of DNMTi, especially non-nucleoside analogues, as therapeutic options for PCa.

HDACi

In pathological situations, like cancer characterized by classical HDACs overexpression, HDACi have emerged as promising therapeutic agents. Currently the best studied HDACi target class I and II HDACs. HDCAi are chemically classified in different subgroups based on their structure, as aliphatic acids (phenylbutyrate and valproic acid), benzamides (entinostat), cyclic peptides (romidepsin) and hydroxamic acids [Trichostatin A (TSA) and Vorinostat/SAHA] (Figure 24) [80, 198].

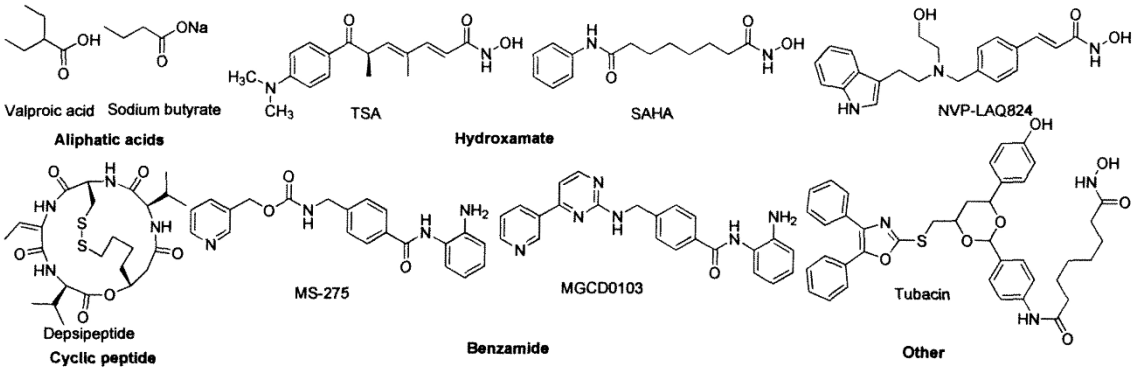

Figure 24 – HDACi classification. *Adapted from [198]*.

The hydroxamic acid vorinostat (SAHA) was found to inhibit class I HDACs, class II HDACs (6 and 10) and HDAC11, but not HDAC 4, 5, 7, and 9 at least in concentrations that are clinically relevant [199]. The cyclic peptide, romidepsin (FK228), preferentially inhibits HDAC 1 but also inhibits HDAC 2 and 3, although more weakly. Benzamide derivatives, including entinostat (MS-275) and MGCD0103 selective inhibit HDACs 1, 2, and 3. These three classes of HDCAi have proven activity at nanomolar concentrations. Regarding, the short-chain fatty acids, butyrates and phenylbutyrate are drugs, already used in clinic for non-neoplastic diseases. These agents inhibit HDAC activity at millimolar concentrations [199].

Inhibition of HDAC activity commonly reduces cell proliferation and angiogenesis, and induces differentiation and apoptosis, which are highly desirable features in cancer therapy. However, HDACs targeting is more complex than targeting DNMTs, because HDACs have multiple subclasses and some with unknown mechanisms and functions [200]. Nowadays, it is widely accepted that the enzymatic activity of HDACs is not restricted to histones, but also targets several other proteins. The exposure of human cell lines to highly specific HDACi induced the hyperacetylation of 1750 proteins revealing that non-histone proteins are the main subtract for these drugs [201]. Therefore, HDACi should not be considered specific epigenetic drugs. Concerning HMTi and HDMi several ongoing

studies are being performed to access their specificity as possible target epigenetic therapy [167].

HDACi in PCa

HDACi is the most well studied class of inhibitors in PCa [63]. In the next paragraphs, it will be highlighted the most promising ones. Moreover, the most relevant clinical trials conducted with HDACi in PCa are summarized in Table 3.

Table 3 – HDACi in clinical trials for PCa. *Adapted from [193]*.

Drug	Clinical Trial ID	Phase	Status	Protocol	Outcome
Panobinostat, docetaxel and prednisone	NCT00663832	i	Completed	CRCP received panobinostat 10, 15 or 20 mg i.v. on days 1 and 8 in combination with docetaxel i.v. on day 1 and prednisone p.o. 5 mg bid every day of a 21 day cycle. n = 44	Determined the MTD of i.v panobinostat is 15 mg in combination with docetaxel and prednisone in patients with CRPC 63% of patients showed > 50% decline in PSA levels
Panobinostat, bicalutamide	NCT00878436	I/II	Recruiting	CRCP receiving panobinostat at either 60 or 120 mg per week for 2 consecutive weeks, with one week rest and bicalutamide 50 mg p.o. daily, continuously. Predicted enrolment: n = 78	Investigating safety and efficacy of combined treatment of panobinostat at 2 dose levels combined with bicalutamide for CRPC as measured by time to PSA progression and proportion of patients that achieve a > 50% PSA decline by 9 months of therapy
Vorinostat	NCT00330161	II	Active, not recruiting	mCRCP patients (PSA > 5 ng/ML, disease progression, adequate organ function) received 400 mg vorinostat orally each day. n = 27	7% patients achieved an objective response rate; no PSA decline > 50% observed; significant toxicities reported
Romidepsin	NCT00106418	ii	Completed	mCRCP patients received romidepsin 13 mg/m^2 i.v. over 4 h on days 1, 8 and 15 every 4 weeks. n = 35	2 patients reached a confirmed radiological partial response of > 6 months, in addition to > 50% PSA decline

Several HDACi demonstrated positive results in pre-clinical phase, and thus might be good candidates for future clinical trials.

Exposure of PC-3 cell line to sodium butyrate increased differentiation and apoptosis [202]. Similarly, vorinostat has been found to inhibit PCa cell lines proliferation and to reduce tumor growth *in vivo* [203, 204]. This compound also enhanced radiation-induced apoptosis in DU145 cell line [205]. A broader effect was obtained by panobinostat that induced cell cycle arrest, DNA damage and reduce PCa tumor growth *in vivo* [206].

Valproic acid (VPA) not only induced similar effects *in vitro* models, but importantly was able to reduce tumor growth in xenograft models [207].

Remarkably, romidepsin treatment of mice inoculated with the 22Rv1 cell line not only reduced metastasis formation but also a 61% increase in survival was achieved [208]. Moreover, the combination of this drug with docetaxel presented greater cytotoxic effects in CRPC cell lines and a significant reduction of tumor growth in mice inoculated with PC-3 [209].

The exposure of PCa cell lines to MS-275, a benzamide derivate, resulted in an increase of H3 acetylation, p21 expression, growth arrest in LNCaP and PC-3, and apoptosis in DU145 cell line. This drug also reduced tumor growth in xenograft mice [210], particularly when acting synergistically with radiation [211].

At clinical level, a phase I clinical trial with orally panobinostat alone or in combination docetaxel was performed in CRPC patients. All individual treated with panobinostat alone developed progressive disease, whereas the combined therapy resulted in 5 partial responses [212]. Additionally in a phase I trial with combined vorinostat and doxorubicin one of the two patients enrolled in this study showed a partial response [213]. In a phase II trial with romdepsin in which mCRPC patients were recruited, two patients out of 32 achieved stable disease for more than 6 months [214].

Therefore the available data is suggestive that HDACi constitute promising agents for PCa treatment, especially in combination with conventional therapy.

MicroRNA-based Therapies

The emerging role of miRNAs deregulation in human disease poses them as attractive therapeutic target. Their general accepted dual role in carcinogenesis led to the development of two different therapeutic strategies (Figure 25) [215, 216].

The first strategy is directed toward a gain of function and aims to inhibit oncogenic miRNAs by using miRNA antagonists (anti-miRNA oligonucleotides - AMOs), miRNA sponges and miRNA masking. Concerning AMOs, they inhibit miRNA expression once they are oligonucleotides with sequences complementary to the endogenous miRNAs and carry chemical modifications that enhance the affinity for the target miRNA. This in turns traps the endogenous miRNA in a configuration that is unable to be processed by RISC. Additionally it might also lead to direct degradation of the endogenous miRNA. Locked-nucleic acids (LNA) are one example of AMOs with several advantages: they do not need a vector and present higher stability and lower toxicity in biological systems [216-218]. Currently, an LNA-based miR-122 inhibitor is in Phase II clinical trial for the treatment of hepatitis C [219]. MiRNA sponges consist in a vector with multiple miRNA binding sites that are placed under the control of strong promoters in order to produce large quantities

of transcript preventing the association between miRNA and its targets. MiR-mask, which are synthesized as single stranded 2'-O-methyl oligonucleotides are modified ribonucleotides wherein 2'-hydroxyl on the ribose is replaced with the 2'-O-methyl group. This modification improves the biostability and makes the oligonucleotides more resistant to degradation. MiR-masks have perfect complementarity to an endogenous miRNA binding site in 3'UTR of a protein-coding gene [217, 220]. Recently, mice xenografted with MCF-7 breast cancer cells were transiently transfected with a miR-mask complementary to miR-21 displayed tumors 50% smaller than control tumors [221].

The second strategy is termed miRNAs replacement and aims to reintroduce depleted miRNAs in cancer cells leading to its reactivation [222]. This is achieved through miRNA mimics or viral vector-based miRNA restoration [218]. MiRNA mimics are small chemically modified dsRNA molecules identical to endogenous miRNAs. Viral vector-based miRNA functions as miRNAs delivery vehicles [217]. Recently miRNAs mimics for the tumor-suppressors miR-34a and let-7 in a mouse model of non-small cell lung cancer (NSCLC) reduced significantly tumor growth [223, 224].

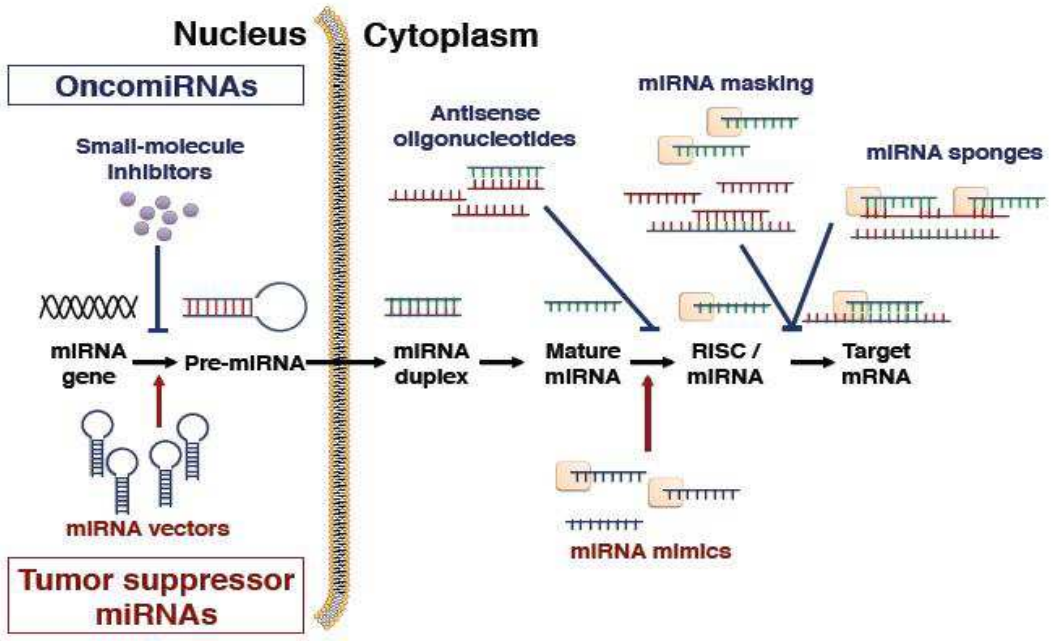

Figure 25 – Therapeutic strategies to target miRNA expression: miRNA antagonists (inhibition of expression of oncogenic miRNAs) and miRNA replacement (restoration of expression of tumor-suppressor miRNAs). *Adapted from [222]*.

Notwithstanding the efforts in miRNA-mediated therapy development, there are several limitations of using miRNAs as new therapeutic targets. Firstly, it is difficult to maintain target specificity, because miRNA targeting is sequence specific instead of gene specific and gene silencing requires only a partial complementary between miRNA and

protein-coding transcripts. The second limitation is the need of achieving high therapeutic efficiency, which is a nearly impossible task due to the amplitude of target gene modulation and the number of cells that can be targeted. This relies on the difficulty of delivering therapeutic oligonucleotides directly to the tumor foci and the poor resistance of oligonucleotides to nucleases degradation [216].

Moreover, most human cancers present a global downregulation of miRNAs, therefore a miRNAome-based therapy is more likely to be successful than strategies aiming to target one single miRNA. Therefore, a drug that causes global upregulation of miRNAs would be rather promising.

Through a chemical screen in which more than 2000 compounds were evaluated to identify small molecules that could possible enhance RNA interference (RNAi), allowed for the identification of enoxacin [225]. This compound is a member of a family of synthetic antibacterial compounds based on a fluoroquinolone skeleton. It is usually administrated to treat several bacterial infections as gonorrhea and urinary tract infections. Importantly it has minimal clinical side-effects in adults [226, 227]. Since enoxacin was the only fluoroquinolone capable of enhancing RNAi, this property might be due to the unique chemical structure of this compound rather than the fluoroquinolone activity [225].

A recent study reported enoxacin as a tumor growth inhibitor in several cancer cell lines and in mice models by enhancing the tumor-suppressors miRNAs production. Importantly, this study demonstrated that enoxacin does not target normal cells and did not induce toxicity [225, 228]. Enoxacin was reported to induce RNAi and miRNAs biogenesis by facilitating the interaction between TRBP and miRNA precursors [225, 228].

MiRNA-based Therapy and PCa

Despite the emerging role of miRNAs in prostate carcinogenesis, there are few studies available addressing miRNA-based therapy in this neoplasm.

Recently, miR-16 was described as a potential therapeutic target for PCa. This miRNA is downregulated in PCa leading to increased proliferation. The transient transfection of miR-16 in mice decreased tumors growth in bone without relevant side effects. Remarkably, the effect of miR-16 seemed to be restricted to PCa-cells. Moreover the authors suggested that the systemic delivery of this miRNA might be effective for advanced PCa therapy [229].

Similarly the systemic delivery by a liposome-based delivery agent of miR-34a, which is also underexpressed in PCa stem cells induced tumor metastasis regression and improved survival in xenografted mice [230].

In the same line, mir-185 and miR-342, two miRNAs downregulated in PCa demonstrated to decrease tumor cell proliferation, migration, invasion and induction of caspase-dependent apoptosis in both PCa cell lines and xenograft mice [231].

Although the promising results in miRNA-based therapies, currently there are no clinical trials in PCa. Since miRNAs are globally downregulated in PCa, drugs that might induce miRNAs upregulation should also be investigated.

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2

CHAPTER TWO

Aims

Aims

PCa is a complex and heterogeneous disease that ranges from clinical indolent to mCRPC. Although available therapeutic options for early-stage PCa are generally very efficient, treatment of advanced disease is mainly ineffective and remains a clinical challenge. ADT is the therapy of choice for locally advanced and systemic disease. However, most patients that initially respond to ADT have a median time to progression to CRPC of only 18-30 months. Although at this stage of disease chemotherapy with docetaxel is the best treatment option available, it only extends patients survival in two months. Therefore, investigation of new therapeutic strategies is urgently needed and should be based, ideally, on the acquired knowledge about PCa biology.

PCa is the perfect model of an epigenetic catastrophe, since alterations in all epigenetics mechanisms (DNA methylation, histone modifications and miRNAs) have been implicated in its development and progression. Regarding DNA methylation, it is recognized that more than 100 tumor-suppressor genes are silenced by promoter methylation in this tumor. Contrarily, concerning post-translational histone modifications, there are few alterations described up to now in prostate carcinogenesis, constituting an important field to explore. Lastly, the role of miRNAs in PCa is currently being extensively studied, but global downregulation of miRNAs has been already reported. Remarkably epigenetic modifications are reversible and numerous compounds, like DNMTi, HDACi and drugs that target miRNAs, have been reported to be effective in cancer growth control. In PCa the best studied compounds are HDACi. In fact, some of these drugs are already in clinical trials. However, regarding non-nucleoside DNMTi and miRNAs targeted therapy, little is known.

Therefore the main objective of this doctoral thesis was to evaluate the usefulness of two non-nucleoside analogues DNMTi (RG108 and Hydralazine) and of a compound described to target miRNAs (enoxacin), as anti-tumor agents in PCa cell lines, setting the stage for translation of these results to clinical practice.

Specifically, the aims of this project were:

1 - Evaluate the role of enoxacin as a growth inhibitor and a modulator of miRNAs expression in PCa.

- Analyze the mutational status of TARBP2 and the protein levels of TRPB and DICER in PCa cell lines.
- Assess the expression levels of TRBP in primary tumor tissues to evaluate the clinical usefulness of enoxacin in PCa treatment.
- Evaluate the phenotypic effects of enoxacin on PCa cell lines.

- Test the effect of enoxacin on the expression of miRNAs involved in prostate carcinogenesis.

2 - Investigate the potential of RG108 as a growth inhibitor of PCa cells.

- Evaluate the impact of RG108 in cell viability, apoptosis and cell cycle of several PCa cell lines.
- Investigate the inhibitory effect of RG108 on DNMT expression and activity of PCa cell lines.
- Assess the role of RG108 in global methylation levels of PCa cell lines.
- Analyze the role of this compound on demethylation along with the re-expression of genes known to be epigenetically silenced in PCa.

3 - Assess the role of hydralazine as anti-cancer agent in PCa cell lines

- Evaluate the phenotypic effects of hydralazine on PCa cell lines.
- Investigate the inhibitory effect of hydralazine on DNMTs expression.
- Analyze the role of hydralazine on DNA demethylation along with the re-expression of genes known to be epigenetically silenced in PCa.
- Study the role of hydralazine in restoration of AR expression.
- Explore the cellular mechanisms by which this drug exerts its inhibitory effect.

3

CHAPTER THREE

Enoxacin Inhibits Growth of Prostate Cancer Cells and Effectively Restores microRNA Processing

Abstract

Prostate cancer (PCa) is one of the most incident malignancies worldwide. Although efficient therapy is available for early-stage PCa, treatment of advanced disease is mainly ineffective and remains a clinical challenge. MicroRNA (miRNA) dysregulation is associated with PCa development and progression. In fact, several studies have reported a widespread downregulation of miRNAs in PCa, which highlights the importance of studying compounds capable of restoring the global miRNA expression. The main aim of this study was to define the usefulness of enoxacin as an anti-tumoral agent in PCa, due to its ability to induce miRNA biogenesis in TRBP-mediated manner. Using a panel of five PCa cell lines, we observed that all of them were *TARBP2* wild-type and expressed TRBP protein. Furthermore, primary prostate carcinomas displayed normal levels of TRBP protein. Remarkably, it was found that enoxacin is able to decrease cell viability, induce apoptosis, cause cell cycle arrest, and inhibit the invasiveness of cell lines. Enoxacin was also effective in restoring the global expression of miRNAs. This study is the first to show that PCa cells are highly responsive to the anti-tumoral effects of enoxacin. Therefore, enoxacin constitutes a promising therapeutic agent for PCa.

Introduction

Prostate cancer (PCa) is one of the most commonly diagnosed malignancies worldwide and a leading cause of cancer-related deaths among men [1]. Although most cases are clinically indolent, a variable proportion of patients develop castration-resistant PCa (CRPC), an aggressive and lethal form of disease, associated with widespread metastatic disease [2]. Currently, most therapeutic strategies are largely ineffective and only allow for a small increase in survival [3]. Therefore, new therapeutic strategies, ideally based on the understanding of the biology of this disease are urgently needed.

PCa is a complex and heterogeneous disease that arises through the acquisition of several genetic and epigenetic alterations [4, 5]. Among the latter, dysregulation of microRNA (miRNA) expression has been recently emphasized as a critical mechanism in PCa development and progression [6]. miRNAs are small noncoding RNAs that post-transcriptionally regulate gene expression by inducing cleavage of their target mRNA or by inhibiting their translation [7]. These molecules are produced through a multi-step process that involves the RNase III enzymes DROSHA and DICER, resulting in the production of mature miRNAs of ~22 nucleotides, which are incorporated into the RNA-induced silencing complex (RISC) through the action of the DICER-TRBP (Trans-activator RNA-binding protein) complex [8]. miRNAs play a crucial role in the regulation of almost every biological process, including differentiation, apoptosis, cell cycle, development, and metabolism [9, 10]. Moreover, dysregulation of any of these processes due to abnormal expression of miRNAs or alterations in their machinery of biogenesis has been implicated in cancer, including PCa [11, 12]. Although miRNAs have been proposed to function as either oncogenes or tumor-suppressor genes [13], most human tumors are characterized by a general defect in miRNA production that results in global downregulation of miRNA expression [14-16]. Thus, compounds with the ability to restore the global miRNA expression might be an excellent therapeutic option for cancer.

Enoxacin, an antibacterial compound based on a fluoroquinolone skeleton, was shown to be effective in the inhibition of tumor cell growth *in vitro* and *in vivo* by enhancing the production of miRNAs with tumor-suppressor functions [17]. Remarkably, the drug did not affect normal cells and was not associated with toxicity in mice models [17]. Moreover, it has been reported that enoxacin promotes RNA interference (RNAi) and miRNA biogenesis by facilitating the interaction between TRBP, encoded by the *Trans-activator RNA-binding protein 2 (TARBP2)* gene, and miRNA precursors [17, 18]. Therefore, it has been recently demonstrated that *TARBP2*-mutant cells are less responsive to enoxacin [17, 19]. Although those results are promising for cancer therapeutics, no studies are

available concerning the effect of enoxacin on PCa. Thus, we aimed to investigate the effectiveness of enoxacin as a tumor growth inhibitor in PCa. Furthermore, we evaluated the alterations in miRNA expression induced by this compound in PCa cells.

Materials and Methods

Cell lines, drug preparation and exposure

DU145 was obtained from the American Type Culture Collection (ATCC, Lockville, MD, USA), whereas LNCaP, VCaP and PC-3 were kindly provided by Prof. Ragnhild A. Lothe from the Department of Cancer Prevention at The Institute for Cancer Research, Oslo, Norway, and 22Rv1 by Dr. David Sidransky from the Johns Hopkins University School of Medicine, Baltimore, MD, USA. For control purposes, we used the human colon carcinoma-derived cell line Co115 which was kindly provided by Prof. Fátima Baltazar from the Life and Health Sciences Research Institute at the University of Minho, Braga, Portugal. All cell lines were cultured in the recommended medium, supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin (GIBCO). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. All PCa cell lines were karyotyped by G-banding (for validation purposes) and routinely tested for Mycoplasma spp. contamination (PCR Mycoplasma Detection Set, Clontech Laboratories). Enoxacin (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at -20ºC until further use. PCa cells were continuously exposed to 40 µg/mL (124 µM) enoxacin, for five days. For control purposes, cell lines were exposed to the vehicle of the drug (DMSO) only.

Direct sequencing

Genomic DNA was extracted from cell lines and prostatic cancer tissue using a standard technique comprising digestion with proteinase K (20 mg/mL) in the presence of 10% SDS at 55ºC, followed by phenol-chloroform extraction and precipitation with 100% ethanol [20]. *TARBP2* was screened for mutations using the primers previously described by Melo *et al [*19]. Direct sequencing was performed in an ABI PRISM 310 automatic sequencer using Big Dye Terminator Chemistry (Applied Biosystems), according to the manufacturer's recommendations.

Western Blot

Protein extraction from whole-cell lysates was obtained using RIPA lysis buffer (Santa Cruz Biotechnology). Protein concentrations were determined using a Pierce BCA assay (Thermo Scientific Inc.), according to the manufacturer's instructions. Briefly, 30 µg of protein from each sample were separated using 10% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad) at 200 V and subsequently blotted onto Protran nitrocelulose transfer membranes (Whatman, Dassel, Germany) using Trans-Blot® Turbo™ Transfer System

(Bio-Rad). Membranes were incubated with primary antibodies at 4ºC overnight. Primary antibodies were polyclonal rabbit against TRBP (1:1,000, Abcam), polyclonal rabbit against DICER (1:500, Santa Cruz Biotechnology), monoclonal mouse against cleaved PARP (1:500, Cell Signaling), monoclonal mouse antibody against HDAC1 (1:1,000, Sigma-Aldrich) and polyclonal rabbit against SIRT1 (1:1000, Abcam). The membranes were developed using Immun-Star WesternC Chemiluminescent kit (Bio-Rad) and exposed to Amersham Hyperfilm (GE Healthcare). To ascertain equal loading of protein, the membranes were stripped and reprobed with a monoclonal mouse antibody against β-Actin (1:8,000, Sigma-Aldrich). To relate the protein band intensity with the loading control (β-Actin), protein band intensities were determined using Quantity One software (Bio-Rad).

Patients and sample collection

Fifty prostate tumor samples from patients with clinically localized PCa (clinical stage II: T1cN0M0 or T2N0M0, according to the TNM staging system) consecutively diagnosed and primarily treated with radical prostatectomy at the Portuguese Oncology Institute Porto, Portugal, were prospectively collected from 2001 to 2006. Tumor tissue was routinely fixed in buffered formalin and paraffin-embedded. All patients were enrolled after informed consent. This study was approved by the institutional review board (Comissão de Ética do IPO Porto).

Immunohistochemistry

TRBP expression in tumor tissue samples was assessed by immunohistochemistry using the Novolink™ Polymer Detection System (Novocastra). Deparaffinized tissue sections were submitted to antigen retrieval in a 700-W microwave oven, in 1x EDTA buffer solution. Endogenous peroxidase activity was blocked by incubating the slides with Peroxidase Block (Novocastra) for 5 minutes. The slides were incubated with Protein Block (Novocastra) for 5 minutes and, after incubation, primary antibody against TRBP (1:100, Abcam) was applied for 1 hour in a humid chamber, at room temperature. The slides were then incubated with Post Primary Block (Novocastra) for 30 minutes followed by incubation for 30 minutes with the NovoLink Polymer (Novocastra). After incubation in 3,3-diaminobenzidine (DAB; Sigma–Aldrich) in a solution of 50 mL PBS/0,05% mL H_2O_2 for 7 minutes, the slides were counterstained with hematoxylin (Harris Modified Hematoxylin Stain; Fisher Scientific) for 20 seconds and mounted with Entellann (Merck KGaA). Colorectal cancer tissues showing intense immunoreactivity for TRBP protein were used as positive control. The negative control consisted on the omission of the primary antibody. The assessment of immunostaining results was performed by an

experienced pathologist and was expressed in a semiquantitative way according to the estimated percentage of positive tumor cells. Immunostaining of more than 10% of the tumor cells was required for scoring a case as positive.

Cell viability assay

Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay. In brief, PCa cells were seeded onto 96-well plates (Sarstedt) at 2 x 10^3 cells per well and allowed to adhere overnight. After exposure to the drug, cell viability was measured at 1, 2, 3, 4, and 5 days. Briefly, 200 µL of 0.5 mg/mL MTT (Sigma-Aldrich) were added to each well and incubated at 37ºC and 5% CO_2 for 3 hours. Formazan crystals were solubilized with 100 µL of DMSO. The absorbance was measured using a microplate reader (Fluostar Omega) at a wavelength of 540 nm with background subtraction at 630 nm. The number of cells was calculated using the formula: [(OD experiment x Number of cells at day 0) / Mean OD at day 0].

Apoptosis assay

Apoptosis evaluation was performed using the APOPercentage apoptosis assay kit (Biocolor Ltd.) according to the manufacturer's instructions. This assay is based on phosphatidylserine transmembrane movements which results in the uptake of the APOPercentage dye by apoptosis-committed cells. Cells were seeded onto 24-well plates (Sarstedt) at 5 x 10^4 cells per well and apoptosis levels were assessed at days 2 and 5. The absorbance was determined using a microplate reader (Fluostar Omega) at a wavelength of 550 nm with background subtraction at 620 nm. To normalize the OD measured in the apoptosis test, according to the cell number, the OD of the apoptosis assay was divided by the OD of the cell viability assay. The results were expressed as the ratio of the OD of the cells exposed to enoxacin to that of vehicle cells (set as 1).

Cell cycle analysis

Cell cycle distribution was determined by flow cytometry. Briefly, 5 x 10^5 harvested cells were fixed with 70% cold ethanol overnight at 4ºC. After washing with cold PBS, cells were resuspended in staining Propidium Iodide Solution (Cytognos S.L.) and incubated at room temperature for 30 minutes in the dark. Cell cycle data were collected using Cytomics FC500 flow cytometer (Beckman Coulter) and analyzed with Modfit LT (Verity Software House Inc.).

Cell invasion assay

Cell invasion was evaluated using the Oris™ Cell Invasion Assay (Platypus Technologies) according to the manufacturer's instructions. After starvation for 18 hours in a serum-free medium, cells were seeded at 5 x 10^4 cells per well and exposed to 40 µg/mL of enoxacin or DMSO for 8 days. At this time point, cells were labeled with Calcein AM (AnaSpec) at a final concentration of 0.5 µg/mL. A detection mask was attached to the bottom of the plate to block from view all cells except those that had invaded into the center zone and fluorescence was measured at excitation and emission wavelengths of 492 nm and 530 nm, respectively, using a microplate reader (Fluostar Omega). The results were expressed as the ratio of the fluorescence index (FI) of the cells exposed to enoxacin to that of vehicle cells (set as 1).

Quantitative reverse transcription PCR (qRT-PCR)

RNA was extracted from all cell lines using TRIzol® (Invitrogen) according to manufacturer's instructions and cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). *CASP3* mRNA expression levels were assessed using TaqMan gene expression assay, purchased as pre-developed assays from Applied Biosystems. The mean quantity of *CASP3* expression levels of cell lines samples was normalized against mean quantity of the respective endogenous control (*GUSB*) expression levels.

miRNA expression assessment by miRCURY LNA™ array

cDNA synthesis and real-time qRT-PCR was performed using the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon) according to the manufacturer's instructions. In brief, the cDNA products were transferred to the Ready-to-use microRNA PCR Human Panels (I + II) and quantified using *SYBR* green based real-time PCR and LNA enhanced miRNA specific primers. Based on miRBase 11.0 annotation, a total of 757 human miRNA probes were interrogated in this platform. Three independent experiments were performed. The data were imported into GenEX software (MultiD Analyses AB) for subsequent microarray analysis. Global mean normalization was used as a normalization factor for miRNA [21]. miRNAs with fold change above 1.5 or below -1.5 were considered up or downregulated, respectively.

Statistical analysis

Two-tailed Student's *t* test was used to assess differences between the results obtained after exposure to enoxacin and after exposure to vehicle. Prior to application of the test, all data were transformed to logarithmic scale. Analysis was performed with the

aid of SPSS software for Windows, version 20.0 (IBM-SPSS Inc.), and the statistical significance level was set at $P < 0.05$. Graphics were built using MATLAB 7.10.0 r2010a software.

Results

PCa cells do not harbor TARBP2 mutations and retain TRBP protein expression

In view of the fact that cell lines harboring *TARBP2* mutations are less responsive to enoxacin [17, 19], five PCa cell lines (LNCaP, 22Rv1, VCaP, DU145 and PC-3) were screened for the presence of mutations in all the exonic mononucleotide repeats localized in the coding sequences of *TARBP2*. Co115, a *TARBP2*-mutant colorectal cancer cell line [17, 19], was used as positive control. No *TARBP2* mutations were found in any of the tested PCa cell lines. Subsequently, we analyzed TRBP protein expression in PCa cell lines by Western blot. As expected, all PCa cell lines expressed higher protein levels of TRBP than Co115 cells, which display very low expression levels (Figure 1A). Since DICER acts in complex with TRBP [8], we also assessed DICER protein expression in PCa cell lines and we verified that all PCa cell lines tested expressed DICER (Figure 1A).

Primary PCa tumors are TARBP2 wild-type and express TRBP

To investigate the putative clinical usefulness of enoxacin for PCa therapy, we firstly assessed the *TARBP2* mutational status of 25 primary PCa tumors and only wild-type sequences were detected. Furthermore, using immunohistochemistry, TRBP expression was evaluated in a series of 50 primary PCa tumors, including the same cases analyzed for the *TARBP2* mutational status. No differences in immunoreactivity for TRBP were apparent between normal and tumorous prostatic tissues representing different histopathological grades (Figure 1B).

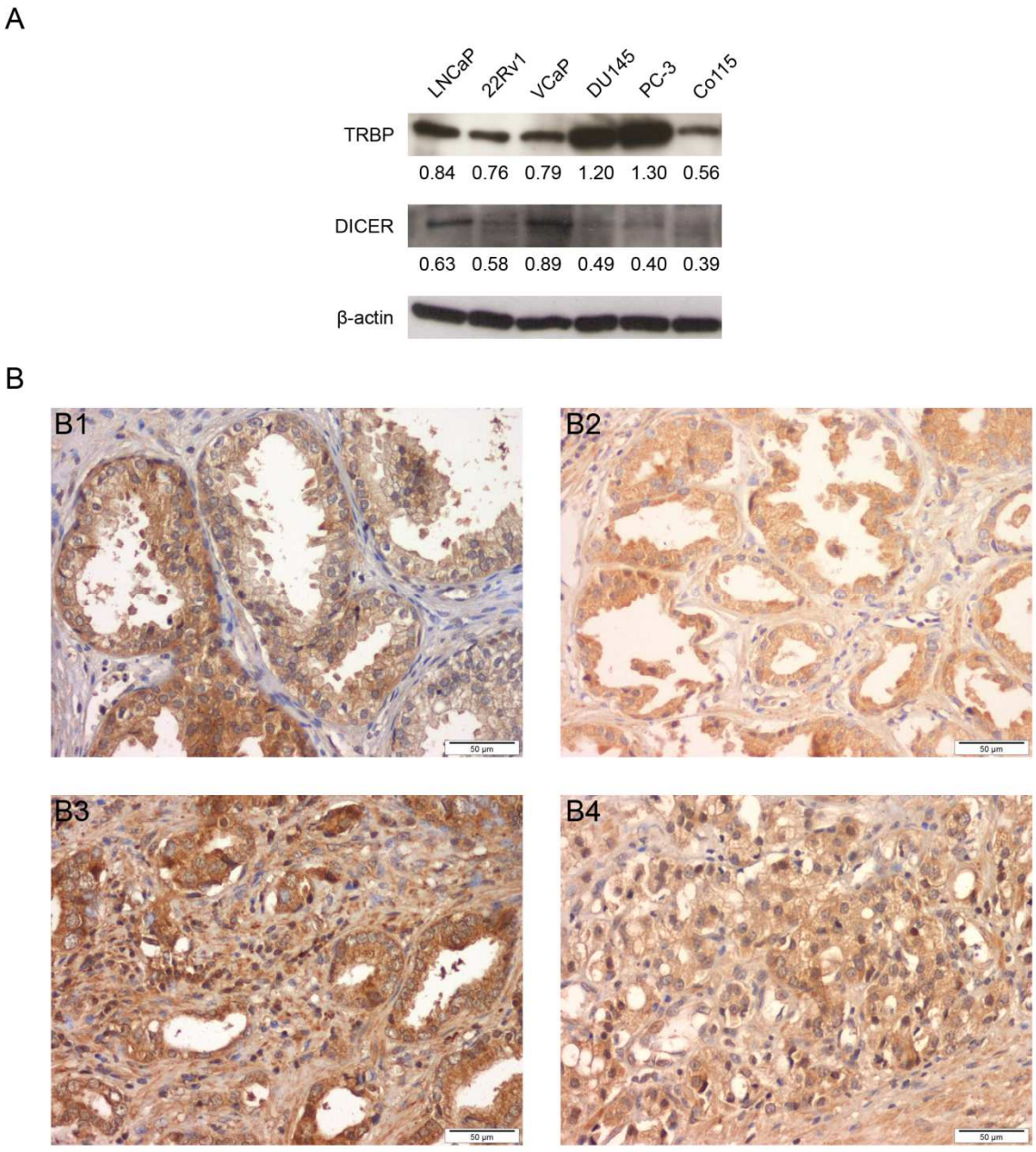

Figure 1 – TRBP and DICER expression in PCa. (A) TRBP and DICER expression was assessed by Western Blot in PCa cell lines. The picture is representative of three independent experiments. β-actin was used as a loading control and the relative density of bands was densitometrically quantified. Mean quantitation values are shown. Co115, a *TARBP2*-mutant colon carcinoma-derived cell line, was used as positive control. (B) Immunohistochemical stain for TRBP expression in prostatic tissue. Protein immunoexpression in tumor cells was similar to that of normal epithelial cells. Normal glands (B1), and tumorous tissue: Gleason score 6 (B2), Gleason score 7 (B3), and Gleason score 8 (B4).

Enoxacin reverts neoplastic features of PCa cell lines

The half-maximal effective concentration (EC50) of enoxacin was calculated in LNCaP and DU145 prostate cancer cells lines at 72 hours. The drug presented an EC50 of 105 µM in LNCaP and 141 µM in DU145. Thus, to evaluate the effects of enoxacin, five human PCa cell lines were continuously exposed for five days to 124 µM (40 µg/mL) of enoxacin. As expected, enoxacin did not alter the expression of both TRBP and DICER proteins in any of the analyzed cell lines (Figure 2A). Importantly, a significant decrease in the number of viable cells was observed after exposure to the drug when compared to the vehicle, DMSO (Figure 2B). For LNCaP and 22Rv1 cell lines, the effect was observed from day 1, whereas a significant decrease in the number of viable cells in VCaP, DU145 and PC-3 was found after 2 days of drug exposure. The reduction in the percentage of viable cells at day 5 ranged between 17 and 59%, with LNCaP being the most responsive cell line (Figure 2B).

To determine whether enoxacin was capable of inducing significant cell death, an apoptosis assay was performed. Indeed, a significant increase in apoptosis was apparent in all tested cell lines at days 2 and 5 (Figure 3A). After 5 days of exposure to enoxacin, LNCaP and DU145 displayed the highest levels of apoptotic cells (Figure 3A).

Apoptosis was also confirmed at molecular level, through the evaluation of mRNA expression of *CASP3*. Although all cell lines showed an increase in *CASP3* expression levels, statistically significant differences were depicted only for LNCaP, 22Rv1 and DU145 (Figure 3B). Furthermore, cleaved PARP was analyzed after enoxacin exposure. 22Rv1, VCaP and DU145 presented increased protein levels of cleaved PARP after exposure to the drug (Figure 3C).

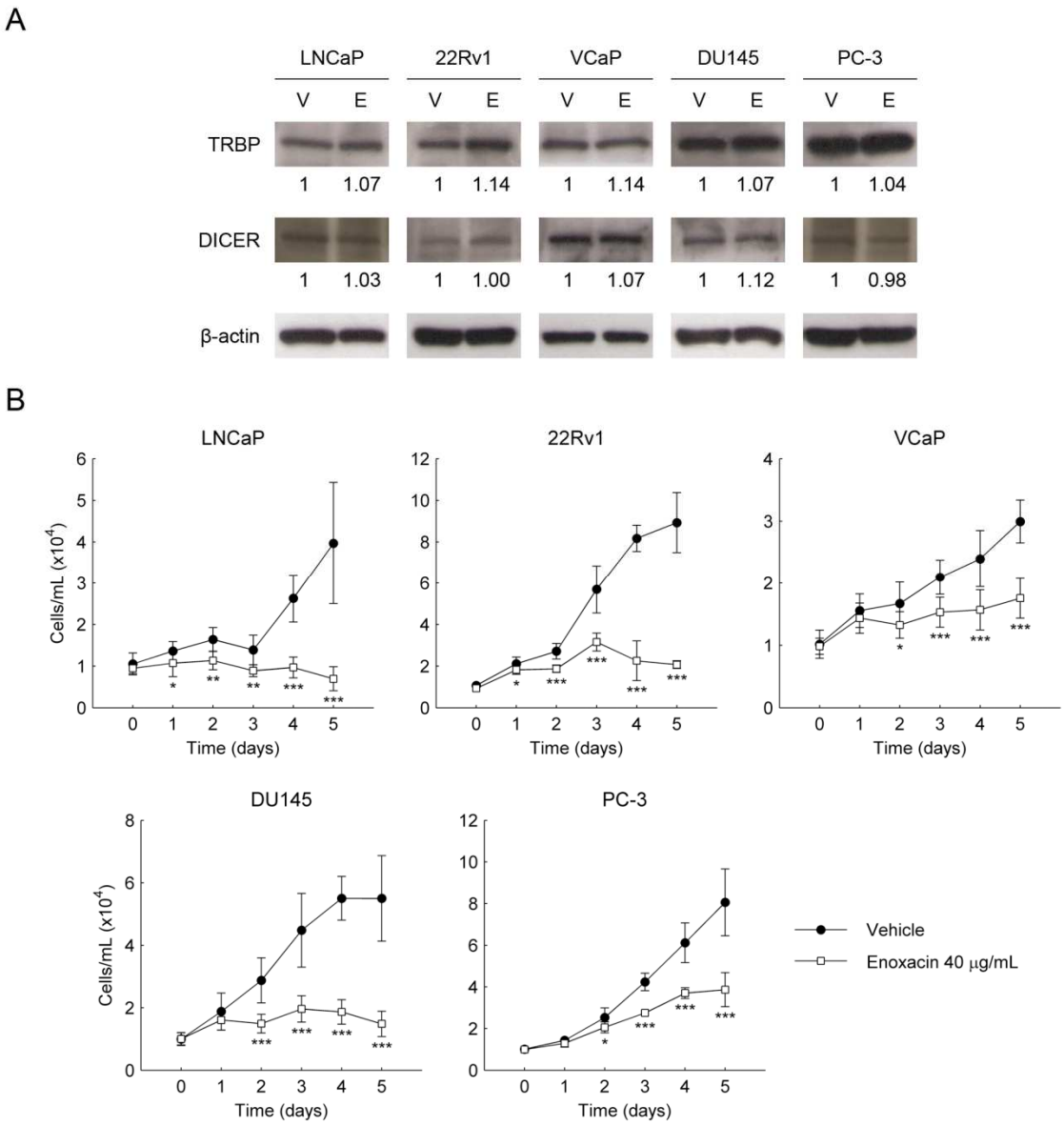

Figure 2 – (A) Effect of enoxacin on the expression of TRBP and DICER. Protein expression of TRBP and DICER was analyzed by Western Blot in LNCaP, 22Rv1, VCaP, DU145, and PC-3 cell lines after exposure to enoxacin 40 µg/mL or DMSO (vehicle) at day five. The picture is representative of three independent experiments. β-actin was used as a loading control and the relative density of bands was densitometrically quantified. (B) Effect of enoxacin on PCa cell viability. Cell viability was evaluated by MTT assay in LNCaP, 22Rv1, VCaP, DU145, and PC-3 cell lines after exposure to enoxacin 40 µg/mL or DMSO (vehicle) for five days. The number of cells/mL is shown as mean of three independent experiments performed in triplicates ± SD. Statistical significance (enoxacin *versus* vehicle) was tested using the two-sided Student's *t* test * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to vehicle group.

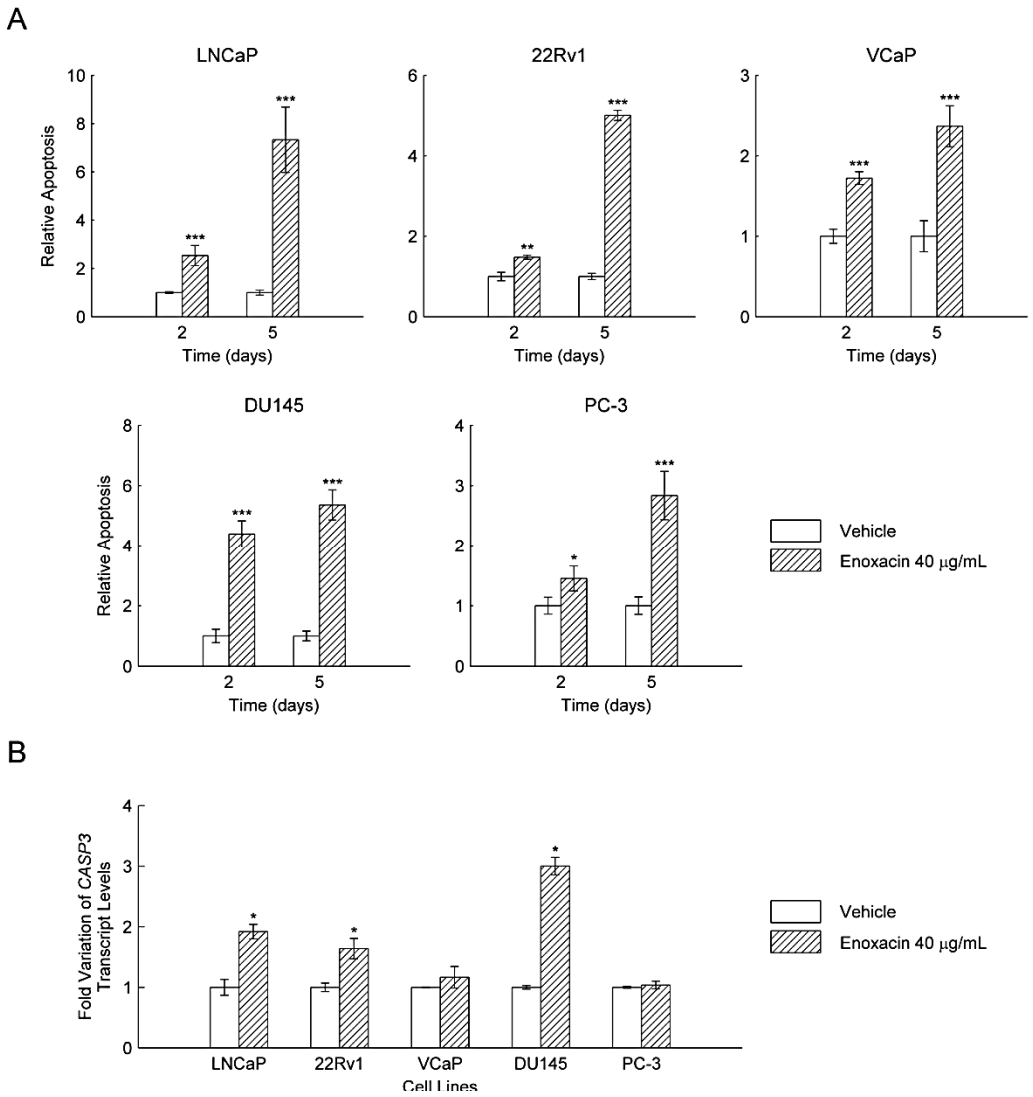

Figure 3 – Effect of enoxacin on PCa cell apoptosis. (A) Apoptosis was analyzed by APOPercentage assay at days two and five in LNCaP, 22Rv1, VCaP, DU145, and PC-3 cell lines after exposure to enoxacin 40 µg/mL or DMSO (vehicle) at days two and five. (B) *CASP3* mRNA expression was evaluated by qRT-PCR in LNCaP, 22Rv1, VCaP, DU145, and PC-3 cell lines after exposure to enoxacin 40 µg/mL or DMSO (vehicle) at day five. Data are presented as mean of three independent experiments carried out in duplicates ± SD. Statistical significance (enoxacin vs. vehicle) was tested using the two-sided Student's *t* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to vehicle group. (C) Cleaved PARP was analyzed by Western Blot in LNCaP, 22Rv1, VCaP, DU145, and PC-3 cell lines after exposure to enoxacin 40 µg/mL or DMSO (vehicle) at day five. The picture is representative of three independent experiments. β-actin was used as a loading control and the relative density of bands was densitometrically quantified.

Cell cycle distribution was evaluated by flow cytometry. Interestingly, 22Rv1 and VCaP, which are hormone-responsive PCa cell lines, showed cell cycle arrest at G2/M, whereas the castration-resistant cell lines DU145 and PC-3 exhibited a significant increase in the percentage of cells in late S and G2/M transition (Figure 4 and Supp. Figure 2).

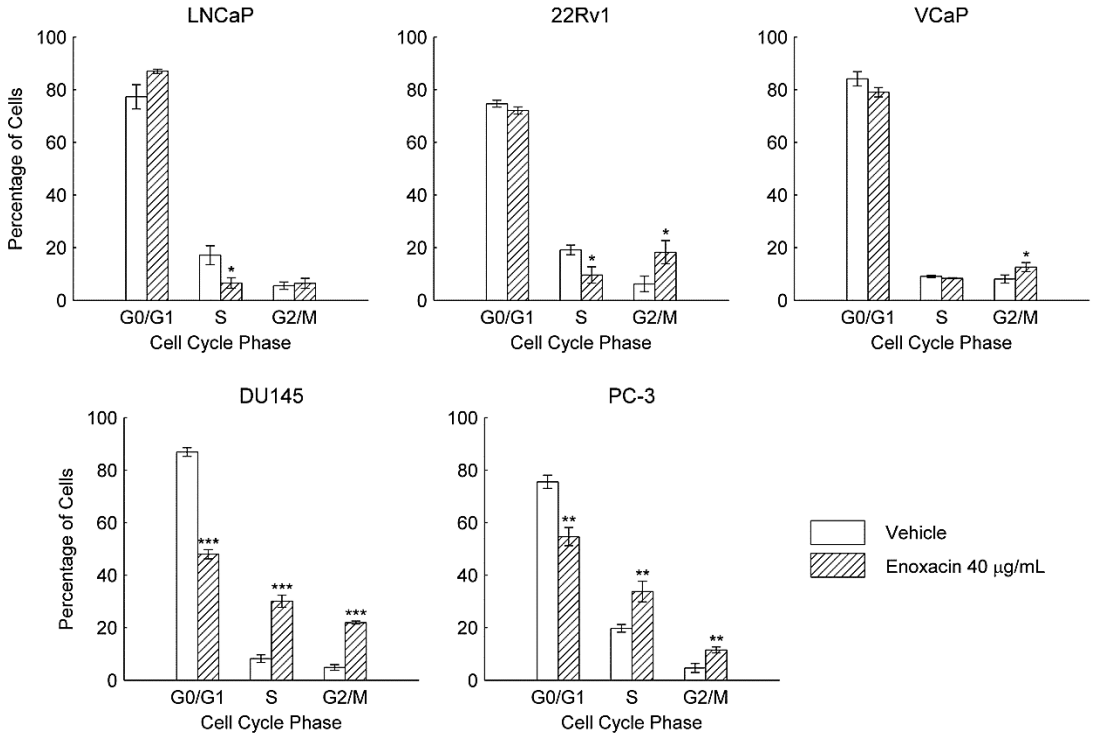

Figure 4 – Effect of enoxacin on PCa cell cycle distribution. Cell cycle distribution was assessed by flow cytometry in LNCaP, 22Rv1, VCaP, DU145, and PC-3 cell lines after exposure to enoxacin 40 μg/mL or DMSO (vehicle) at day five. The percentage of cells is shown as mean of three independent experiments ± SD. Statistical significance (enoxacin vs. vehicle) was tested using the two-sided Student's *t* test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to vehicle group.

Moreover, the percentage of cells in sub-G1 phase, which is an indirect measure of cell death, increased significantly after enoxacin exposure (Table 1 and Supp. Figure 2). Thus, both the increase of apoptotic cells and cell cycle arrest support a growth inhibitory effect of enoxacin on PCa cells.

Table 1 – Impact of enoxacin on the percentage of cells in Sub-G1 phase assessed by flow cytometry.

Cell Line	Cells in Sub-G1 Phase (%)		*P* value
	Vehicle	Enoxacin	
LNCaP	1.7	2.0	ns
22Rv1	1.8	13.1	<0.001
VCaP	2.5	7.3	0.002
DU145	1.7	7.5	<0.001
PC-3	1.0	3.6	0.004

ns – non-significant

The effect of enoxacin on the invasiveness of PCa cells was only assessed in LNCaP and DU145 cell lines because, as described in the previous experiments, these cells were the most responsive to enoxacin. We decided to extend enoxacin exposure from 5 to 8 days in order to allow the vehicle cells to invade. In contrast to DU145, LNCaP cells did not show invasive potential in this system. Remarkably, enoxacin significantly reduced the invasiveness of DU145 cells (Figure 5).

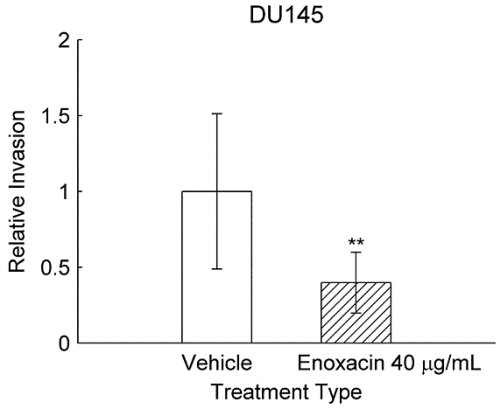

Figure 5 – Effect of enoxacin on the invasive potential of PCa cells. Relative invasion was evaluated by Oris™ Cell Invasion Assay in DU145 cell line after exposure to enoxacin 40 μg/mL or DMSO (vehicle) at day eight. Data are presented as mean of three independent experiments performed in triplicates ± SD. Statistical significance (enoxacin vs. vehicle) was tested using the two-sided Student's *t* test. ** $P < 0.01$, compared to vehicle group.

Enoxacin restores miRNAs expression

The impact of enoxacin exposure on the expression profile of miRNAs was analyzed in LNCaP and DU145 cell lines, using a panel of 742 miRNAs. miRNA analysis demonstrated that enoxacin induced a global upregulation of miRNA expression in both cell lines. Among miRNAs differentially expressed, upregulation was observed in 53% of the miRNAs (65 of 122) for LNCaP cells (Figure 6A and Sup. Table 1) and in 60% (147 of 247) for DU145 (Figure 6A and Supp. Table 2). Remarkably, enoxacin was able to alter the expression of several miRNAs that have been previously associated with prostate carcinogenesis. Concerning tumor-suppressor miRNAs reported in PCa, miR-17*, miRNA-29b, miR-34a, miR-132, miR-146a, and miR-449a showed increased expression levels following enoxacin exposure. Furthermore, decreased expression of some oncogenic miRNAs was also observed, including miR-141 and miR-191 (Table 2).

Table 2 – Effect of enoxacin on the expression of several miRNAs already implicated in PCa.

Cell Line	miRNAs	Fold Change	*P* value
LNCaP	miR-29b	2.9	0.023
	miR-449a	1.8	0.012
	miR-34a	2.3	0.014
	miR-191	-2.3	0.023
DU145	miR-449a	2.2	0.011
	miR-146a	1.8	0.008
	miR-29b	1.7	0.004
	miR-132	1.7	0.003
	miR-17*	1.6	0.008
	miR-141	-1.7	0.002

To confirm the impact of enoxacin on miRNA targets, the protein expression levels of HDAC1, a miR-449a target oncoprotein, and SIRT1, a miR-34a target oncoprotein, were assessed by Western Blot. Both cell lines after drug exposure displayed decreased protein levels of HDAC1 and SIRT1 (Figure 6B).

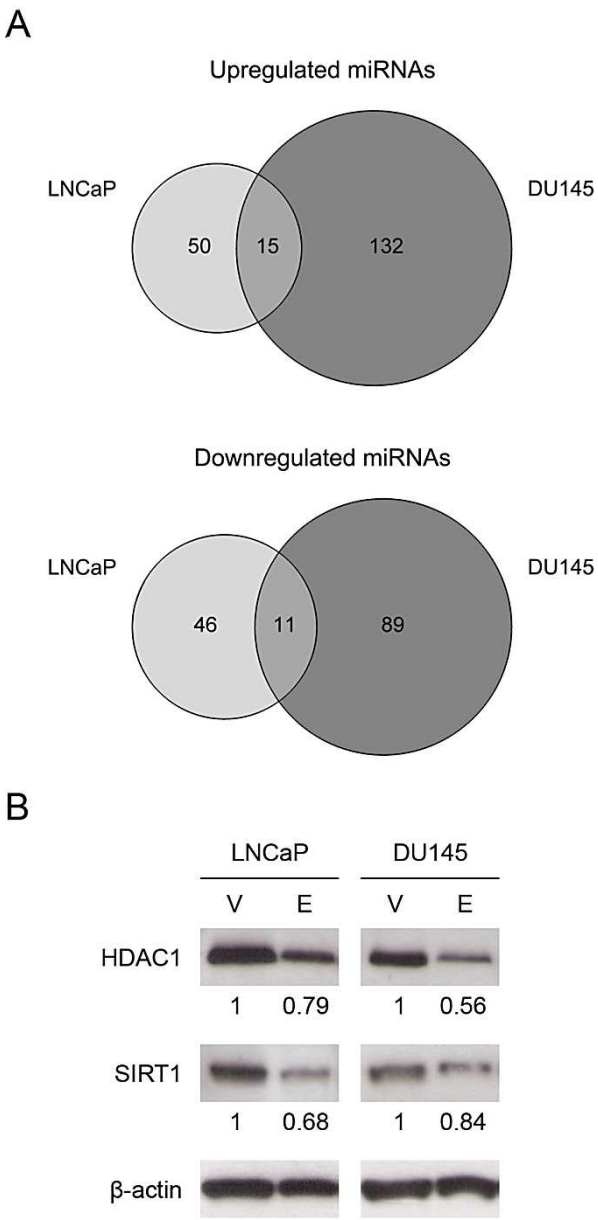

Figure 6 – Enoxacin impact on miRNA. (A) Venn diagrams depicting the number of differentially expressed miRNAs after enoxacin exposure compared with the vehicle group. Top, miRNAs with increased expression (fold change ≥ 1.5); bottom, miRNAs with decreased expression (fold change ≤ -1.5). (B) Protein expression of two miRNA targets (HDAC1 and SIRT1) was analyzed by Western Blot in LNCaP and DU145 cell lines after exposure to enoxacin 40 µg/mL or DMSO (vehicle) at day five. The picture is representative of three independent experiments. β-actin was used as a loading control and the relative density of bands was densitometrically quantified.

Discussion

PCa is one of the leading causes of cancer-related deaths worldwide [1], and almost all of those deaths occur as a result of the emergence of castration-resistant disease [22]. Although PCa patients initially respond to androgen-deprivation therapy, about 18-24 months after treatment initiation, most patients develop CRPC, which results in progressive clinical deterioration and, ultimately, death [23, 24]. For patients with CRPC, there are limited treatment options with proven survival benefit [25-28].

Recently, several miRNA microarray profiles demonstrated that miRNAs are commonly dysregulated in PCa when compared to normal prostate tissue and that they are also differentially expressed in different stages of PCa [15, 16]. Hence, miRNAs might be used not only as diagnostic and prognostic biomarkers but also as therapeutic targets in PCa. In recent years, efforts have been made to find effective miRNA-based therapeutic strategies for cancer. Indeed, artificial miRNAs that might act as potential anti-tumoral agents are the most studied so far, with most reports focusing on oncogenic miRNAs inhibition [29, 30]. Until now, few studies have reported the use of these synthetic miRNAs as tumor-suppressors and, additionally, the effective technology for delivery of these oligonucleotide-based therapies remains a problem [30]. Because most human cancers exhibit global miRNA downregulation [14-16], the search for compounds able to globally restore the expression of tumor-suppressor miRNAs remains a priority in miRNA research. Herein, we report for the first time the anti-cancer effect of enoxacin, one of such compounds, on PCa cell lines.

Enoxacin, which has been used as a broad-spectrum antibiotic to treat bacterial infections (*e.g.,* urinary tract infections) [31, 32], was recently reported as being capable of enhancing RNAi and consequently induce miRNA expression [17, 18]. However, the mechanism of action of enoxacin is dependent of TRBP as it has been shown that this compound is less effective in cells harboring alterations in this protein caused by *TARBP2* gene mutations [17, 19]. Thus, we initially screened PCa cell lines for *TARBP2* mutations and none was found, although we were able to confirm a *TARBP2* mutation in the control, colorectal cancer cell line Co115, as previously reported [17, 19]. To further validate our results, we performed Western blot for TRBP and confirmed that all PCa cell lines tested displayed higher protein levels than Co115, in agreement with previous studies [17, 19]. The *TARBP2* mutational status was also assessed in primary PCa tumors and only wild-type sequences were detected. We then interrogated primary PCa cases using an immunohistochemistry assay for TRBP and we found that protein immunoexpression in tumor cells was similar to that of normal epithelial cells. Thus, we may infer that primary

prostate carcinomas do not harbor deleterious mutations at the *TARBP2* locus and display normal levels of TRBP protein, rendering them sensitive to restoration of normal miRNA biogenesis by enoxacin.

To demonstrate the growth-inhibitory effect of enoxacin on PCa cell lines, we assessed cell viability, apoptosis, and cell cycle characteristics following five-day exposure. Remarkably, in all tested cell lines, exposure to enoxacin resulted in a significant decrease in cell viability and induction of cell death by apoptosis, as previously demonstrated for other cancer cell lines [17]. These results were further confirmed at the molecular level through the observed statistically significant increase in *CASP3* mRNA expression for three of the five cell lines analyzed (LNCaP, 22Rv1 and DU145). Regarding protein expression of cleaved PARP, we also observed an increase after exposure to the drug in three cell lines (22Rv1, VCaP and DU145). Although the percentage of cells in Sub-G1 differs among the tested cell lines, globally there is an increase after exposure to enoxacin. With some variations, CASP3 mRNA, and cleaved PARP protein expression follow the same trend. The percentage of cells in Sub-G1 may also reflect cell death due to necrosis or other mechanisms, but the results of the remaining three parameters (Casp3 mRNA levels, APOPercentage and cleaved PARP) strongly indicate that apoptosis is, indeed, the main mechanism underlying cell death in this study. Concerning cell cycle distribution, it was observed that 22Rv1 and VCaP, which are hormone-responsive PCa cell lines, presented cell cycle arrest at G2/M, whereas the castration-resistant cell lines DU145 and PC-3 exhibited an increase in the percentage of cells at late S and G2/M transition.

Furthermore, we have shown for the first time that enoxacin significantly reduce the invasive potential of PCa cells. As metastasis is the major cause of morbidity and mortality in PCa patients [4], the development of new treatment regimens that would reduce tumor dissemination is extremely important for PCa therapy. It could be argued that the impact of enoxacin on cell invasion might be the result of reduced cell viability. However, it should be pointed out that it is difficult to dissociate one feature from the other, as both are likely to act in concert. Indeed, if tumor cell viability is reduced, it is expected that the potential to invade is also impaired. Irrespective of the underlying cause, however, our results demonstrate that a reduction in invasive potential does occur after exposure to enoxacin.

The ability to disrupt pathways of cancer cell survival has been already reported for a broad spectrum of cancer cells, both *in vitro* and *in vivo*, through the enhancement of the miRNA-processing machinery [17].

In this study, we also demonstrated that enoxacin was effective in globally restoring the expression of miRNAs. Nevertheless, we found a decrease in the expression

of a significant number of miRNAs, which is not totally in agreement with the mechanism of action purposed for enoxacin [17, 19]. According to previous studies, the presence of enoxacin increases the binding affinity of TRBP for miRNA precursors promoting miRNA biogenesis [17, 19]. Hence, it is tempting to speculate that besides this mechanism there might be other pathways through which enoxacin exerts its action.

Importantly, upregulation of several tumor-suppressor miRNAs known to be involved in PCa development and progression was observed, including miR-29b [33, 34], miR-449a [35], miR-146a [36], miR-17* [37], and miR-34a [38, 39]. For instance, miRNA-29b was reported to be a negative regulator of PCa cell growth by modulating the expression of multiple proteins implicated in metastasis formation, including MMP2, E-cadherin, N-cadherin, Snail and Twist [33, 34]. MiR-146a is downregulated in CRPC cell lines, and targets ROCK1 and EGFR, which are implicated in the development of CRPC [36]. miR-17* also suppresses tumorigenicity of PCa cells through inhibition of mitochondrial antioxidant enzymes [37]. On the other hand, miR-34a, which presents tumor-suppressor functions, inhibits silent information regulator 1 (SIRT1), a gene that suppresses p53-dependent apoptosis [38, 39]. Finally, miR-449a, which is underexpressed in PCa, regulates cell growth and viability by repressing HDAC1 [35]. Remarkably, in LNCaP and DU145 cell lines, upregulation of miR-449 by enoxacin resulted in the downregulation of HDAC1, an oncoprotein expressed at significantly higher levels in PCa than in normal prostate [40, 41]. Moreover, in LNCaP cells, upregulation of miR-34a by enoxacin induced the downregulation of SIRT1, which is overexpressed in PCa [42]. On the contrary, in DU145, SIRT1 dowregulation cannot be attributed to miR-34a, as this was not found to be upregulated following enoxacin exposure. However, because SIRT1 may be regulated by other miRNAs, this hypothesis may not be ruled out completely at this point. In spite of globally upregulating the expression of miRNAs, enoxacin also caused a decrease in the expression of some oncogenic miRNAs, including miR-141 [43] and miR-191 [44], reported as oncomirs in PCa. miR-141 is a target of androgen regulation and it has been suggested that its upregulation may enhance the growth of CRPC cells [43]. Finally, although less studied, miR-191 has also been reported as being overexpressed in PCa [44]. Thus, the simultaneous upregulation of tumor-suppressor miRNAs and downregulation of oncomirs by enoxacin in PCa cells highlights the therapeutic relevance of this drug in PCa. Notwithstanding, enoxacin also affected the expression levels of several other miRNAs, which play a role in different types of cancer, but with an unknown function in PCa. Thus, further studies are mandatory to disclose the biological function of these miRNAs in PCa.

In conclusion, enoxacin constitutes a promising therapeutic agent and *in vivo* studies should be conducted to further support the potential of enoxacin for therapy of PCa patients.

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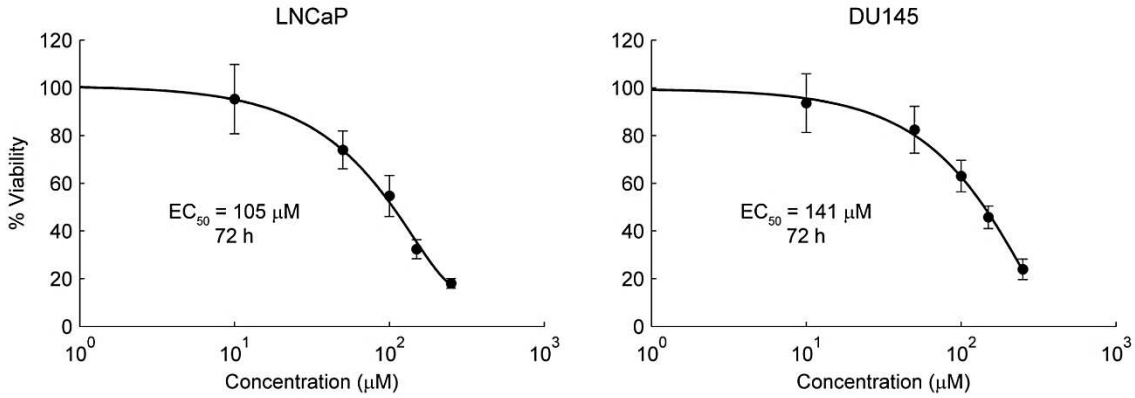
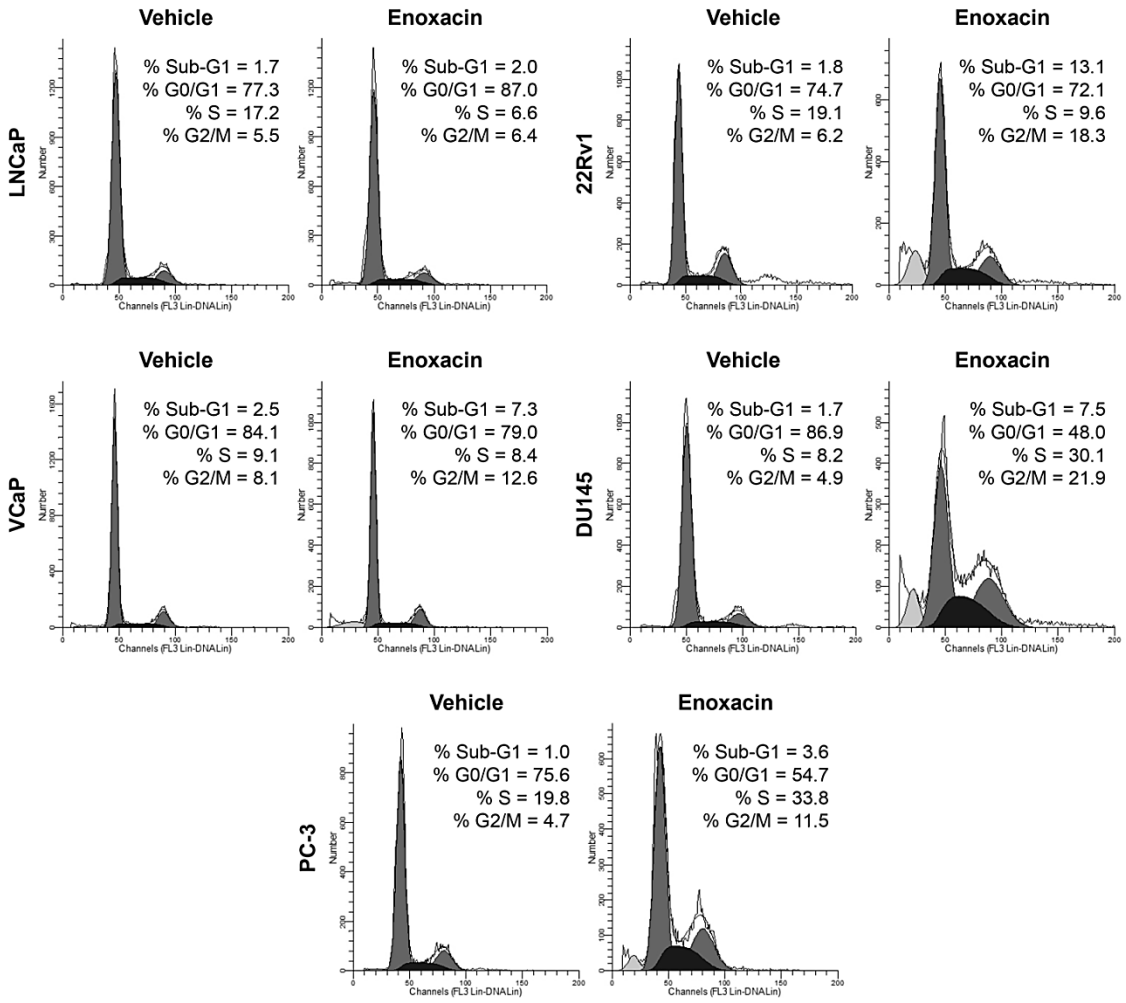
Supplementary data



Supplementary Figure 1 - Half-maximal effective concentration (EC50) of enoxacin in LNCaP and DU145 cell lines at 72 hours. The percentage of viable cells is shown as mean of three independent experiments performed in triplicates ± SD.



Supplementary Figure 2 - Effect of enoxacin on PCa cell cycle distribution. Flow cytometry data represented as histograms. Three independent experiments were performed in each group, and the mean percentage of cells in each cycle phase is presented.

Supplementary Table 1 - List of significantly altered miRNAs in LNCaP after enoxacin exposure.

miRNAs	Fold change	*P* Value
hsa-miR-614	32.7	0.000
hsa-miR-199a-5p	17.1	0.006
hsa-miR-455-5p	13.1	0.000
hsa-miR-329	12.9	0.002
hsa-miR-127-5p	12.8	0.008
hsa-miR-486-5p	12.1	0.003
hsa-miR-519d	11.6	0.000
hsa-miR-198	11.6	0.000
hsa-miR-376b	10.6	0.000
hsa-miR-133a	9.9	0.001
hsa-miR-612	9.4	0.008
hsa-miR-649	8.3	0.018
hsa-miR-23a*	7.2	0.008
hsa-miR-377	7.1	0.010
hsa-miR-432*	6.2	0.003
hsa-miR-506	5.6	0.003
hsa-miR-499-5p	5.1	0.005
hsa-miR-9*	5.0	0.007
hsa-miR-511	4.8	0.038
hsa-miR-34c-5p	4.6	0.016
hsa-miR-675b	4.2	0.001
hsa-miR-302a	4.1	0.001
hsa-miR-296-5p	3.8	0.045
hsa-miR-223	3.7	0.001
hsa-miR-1203	3.4	0.046
hsa-miR-623	3.3	0.002
hsa-miR-301b	3.3	0.001
hsa-miR-488	3.2	0.032
hsa-miR-219-1-3p	3.1	0.009
hsa-miR-29b	2.9	0.023
hsa-miRPlus-D1033	2.8	0.048
hsa-miR-143	2.8	0.002
hsa-miR-617	2.7	0.003
hsa-miR-211	2.6	0.003
hsa-miR-107	2.5	0.048
hsa-miR-493	2.5	0.004
hsa-miR-455-3p	2.5	0.034
hsa-miR-181b	2.5	0.040
hsa-miR-29a	2.4	0.001
hsa-miR-222	2.4	0.007
hsa-miR-29c	2.3	0.009
hsa-miR-578	2.3	0.005
hsa-miR-1204	2.3	0.005
hsa-miR-34a	2.3	0.014
hsa-miR-361-3p	2.3	0.006
hsa-miR-147b	2.3	0.022
hsa-miR-29b-2*	2.2	0.031
hsa-miR-671-5p	2.2	0.011

miRNAs	Fold change	*P* Value
hsa-miR-500	2.1	0.009
hsa-miR-556-3p	2.1	0.008
hsa-miR-548j	2.1	0.008
hsa-miR-501-5p	2.1	0.038
hsa-miR-98	2.0	0.016
hsa-miR-192	2.0	0.001
hsa-miR-887	2.0	0.037
hsa-miR-22	1.8	0.002
hsa-miR-194	1.8	0.036
hsa-miR-328	1.8	0.049
hsa-miR-449a	1.8	0.012
hsa-miR-148b	1.7	0.002
hsa-miR-96	1.6	0.012
hsa-miR-337-3p	1.6	0.025
hsa-miR-23a	1.5	0.018
hsa-miR-30b	1.5	0.028
hsa-let-7d	1.5	0.020
hsa-miR-17	-1.5	0.017
hsa-miR-149	-1.5	0.034
hsa-miR-17*	-1.5	0.042
hsa-miR-323-5p	-1.5	0.046
hsa-miR-375	-1.6	0.025
hsa-miR-144	-1.6	0.039
hsa-miR-374b	-1.6	0.043
hsa-miR-886-5p	-1.7	0.027
hsa-miR-424	-1.8	0.008
hsa-miR-27b*	-1.8	0.024
hsa-miR-221	-1.8	0.035
hsa-miR-18a*	-1.9	0.020
hsa-miR-105	-2.0	0.020
hsa-miR-522	-2.0	0.010
hsa-miR-548h	-2.0	0.010
hsa-miR-181c*	-2.0	0.010
hsa-miR-214	-2.1	0.022
hsa-miR-582-5p	-2.1	0.029
hsa-miR-142-3p	-2.2	0.026
hsa-miR-19a	-2.2	0.011
hsa-miR-191	-2.3	0.023
hsa-miR-654-3p	-2.6	0.016
hsa-miR-943	-2.7	0.013
hsa-miR-16-2*	-2.9	0.020
hsa-miR-15b*	-2.9	0.003
hsa-miR-519c-3p	-3.0	0.010
hsa-miR-224	-3.3	0.000
hsa-miR-7	-3.3	0.034
hsa-miR-1252	-3.6	0.024
hsa-miR-513a-3p	-3.7	0.006
hsa-miR-210	-3.8	0.002
hsa-miR-558	-3.8	0.027
hsa-miR-720	-3.8	0.006

miRNAs	Fold change	*P* Value
hsa-miR-122*	-4.1	0.000
hsa-miR-1255b	-4.3	0.001
hsa-miR-876-5p	-4.8	0.000
hsa-miR-499-3p	-4.8	0.000
hsa-miR-1912	-4.9	0.000
hsa-miR-496	-6.7	0.001
hsa-miR-517a	-6.7	0.002
hsa-miR-886-3p	-7.1	0.000
hsa-miR-605	-7.5	0.002
hsa-miR-374b*	-7.9	0.000
hsa-miR-576-5p	-8.0	0.012
hsa-miR-518e*	-8.7	0.002
hsa-miR-512-5p	-9.2	0.000
hsa-miR-548d-5p	-9.5	0.000
hsa-miRPlus-D1061	-9.5	0.000
hsa-miR-183*	-9.5	0.002
hsa-miR-384	-10.2	0.000
hsa-miR-518f*	-11.1	0.004
hsa-miR-196a	-12.1	0.000
hsa-miR-708	-12.8	0.000
hsa-miR-641	-15.9	0.010
hsa-miR-1539	-20.1	0.013
hsa-miR-650	-22.5	0.005
hsa-miR-187	-30.8	0.000

Supplementary Table 2 - List of significantly altered miRNAs in DU145 after enoxacin exposure.

miRNAs	Fold change	*P* Value
hsa-miR-518e	21.2	0.000
hsa-miR-518a-3p	17.6	0.000
hsa-miR-122	14.3	0.002
hsa-miR-211	11.4	0.000
hsa-miR-451	11.0	0.000
hsa-miR-154*	9.4	0.008
hsa-miR-623	9.2	0.000
hsa-miR-376a	8.5	0.000
hsa-miR-492	7.4	0.000
hsa-miR-376b	6.3	0.005
hsa-miR-376c	5.9	0.004
hsa-miR-572	5.2	0.001
hsa-miR-369-5p	5.1	0.000
hsa-miR-562	4.4	0.048
hsa-miR-214*	4.1	0.038
hsa-miR-1911	3.9	0.046
hsa-miR-889	3.8	0.041
hsa-miR-7	3.8	0.008
hsa-miR-600	3.6	0.003
hsa-let-7d*	3.5	0.001
hsa-miR-92a-1*	3.4	0.000
hsa-miR-125b-1*	3.4	0.000
hsa-let-7a-2*	3.1	0.001
hsa-miR-495	2.8	0.000
hsa-miR-1248	2.7	0.015
hsa-miR-483-3p	2.7	0.019
hsa-miR-100*	2.6	0.007
hsa-miR-422a	2.6	0.021
hsa-miRPlus-C1089	2.5	0.019
hsa-miR-190b	2.5	0.011
hsa-miR-596	2.5	0.035
hsa-miR-18a*	2.5	0.002
hsa-miR-25*	2.5	0.000
hsa-miR-1181	2.5	0.000
hsa-miR-487b	2.4	0.018
hsa-miR-299-5p	2.4	0.026
hsa-miR-19b-1*	2.4	0.022
hsa-miR-622	2.4	0.017
hsa-miR-604	2.4	0.048
hsa-miR-382	2.3	0.009
hsa-miR-886-3p	2.3	0.004
hsa-miR-216a	2.3	0.002
hsa-miR-155	2.2	0.000
hsa-miR-433	2.2	0.024
hsa-miR-221*	2.2	0.001
hsa-miR-329	2.2	0.008
hsa-miR-449a	2.2	0.011
hsa-miR-663	2.2	0.000

miRNAs	Fold change	*P* Value
hsa-miR-940	2.1	0.003
hsa-miR-124	2.1	0.022
hsa-miR-886-5p	2.1	0.001
hsa-miR-222	2.1	0.000
hsa-miR-485-3p	2.0	0.004
hsa-miR-592	2.0	0.011
hsa-miR-125b	1.9	0.006
hsa-miR-22	1.9	0.002
hsa-miR-100	1.8	0.037
hsa-miR-378	1.8	0.002
hsa-miR-188-5p	1.8	0.003
hsa-miR-942	1.8	0.000
hsa-miR-212	1.8	0.000
hsa-miR-146a	1.8	0.008
hsa-miR-18b	1.8	0.001
hsa-miR-625*	1.8	0.001
hsa-let-7g*	1.8	0.001
hsa-miR-576-5p	1.8	0.004
hsa-miR-18a	1.8	0.000
hsa-miR-545	1.8	0.042
hsa-miR-587	1.7	0.006
hsa-miR-137	1.7	0.049
hsa-miR-219-5p	1.7	0.036
hsa-miR-132	1.7	0.003
hsa-miR-220b	1.7	0.022
hsa-miR-582-5p	1.7	0.048
hsa-miR-1979	1.7	0.006
hsa-miR-20a*	1.7	0.002
hsa-miR-574-3p	1.7	0.001
hsa-miR-643	1.7	0.010
hsa-miR-296-3p	1.7	0.000
hsa-miR-671-5p	1.7	0.002
hsa-miR-29b	1.6	0.004
hsa-miR-125b-2*	1.6	0.002
hsa-miR-550	1.6	0.020
hsa-miR-499-3p	1.6	0.002
hsa-miR-522	1.6	0.002
hsa-miR-487a	1.6	0.002
hsa-miR-1264	1.6	0.002
hsa-miR-567	1.6	0.002
hsa-miR-513a-3p	1.6	0.002
hsa-miR-875-5p	1.6	0.002
hsa-miR-516a-3p	1.6	0.002
hsa-miR-10b*	1.6	0.002
hsa-miR-938	1.6	0.002
hsa-miR-1252	1.6	0.002
hsa-miR-519e*	1.6	0.002
hsa-miR-431*	1.6	0.002
hsa-miR-601	1.6	0.002
hsa-miR-520a-3p	1.6	0.002

miRNAs	Fold change	*P* Value
hsa-miR-380	1.6	0.002
hsa-miR-2053	1.6	0.002
hsa-miR-19b-2*	1.6	0.002
hsa-miR-518a-5p	1.6	0.002
hsa-miR-508-5p	1.6	0.002
hsa-miR-208b	1.6	0.002
hsa-miR-588	1.6	0.002
hsa-miR-603	1.6	0.002
hsa-miR-1263	1.6	0.002
hsa-miR-409-5p	1.6	0.002
hsa-miR-135a*	1.6	0.002
hsa-miRPlus-C1100	1.6	0.002
hsa-miR-548h	1.6	0.002
hsa-miR-553	1.6	0.002
hsa-miR-578	1.6	0.002
hsa-miR-875-3p	1.6	0.002
hsa-miR-548m	1.6	0.002
hsa-miR-888*	1.6	0.002
hsa-miR-1208	1.6	0.002
hsa-miR-647	1.6	0.002
hsa-miR-767-3p	1.6	0.002
hsa-miR-208a	1.6	0.002
hsa-miR-384	1.6	0.002
hsa-miR-526b*	1.6	0.002
hsa-miR-513c	1.6	0.002
hsa-miR-1258	1.6	0.002
hsa-miR-599	1.6	0.002
hsa-miR-155*	1.6	0.002
hsa-miR-486-3p	1.6	0.002
hsa-miR-493*	1.6	0.002
hsa-miR-302a*	1.6	0.002
hsa-miR-130a*	1.6	0.002
hsa-miR-17*	1.6	0.008
hsa-miR-629*	1.6	0.011
hsa-miR-296-5p	1.5	0.001
hsa-miR-185	1.5	0.004
hsa-miR-22*	1.5	0.002
hsa-miR-584	1.5	0.021
hsa-miR-602	1.5	0.000
hsa-miR-500	1.5	0.001
hsa-miR-452	1.5	0.031
hsa-miR-583	1.5	0.008
hsa-miR-151-5p	1.5	0.009
hsa-miR-675*	1.5	0.035
hsa-miR-96	1.5	0.005
hsa-miR-193a-5p	1.5	0.009
hsa-miR-27a	1.5	0.021
hsa-miR-23a*	1.5	0.025
hsa-miR-616*	1.5	0.014
hsa-miR-629*	1.4	0.012

miRNAs	Fold change	*P* Value
hsa-miR-185	1.4	0.042
hsa-let-7d	1.4	0.005
hsa-miR-22*	1.4	0.006
hsa-miR-128	1.4	0.022
hsa-miR-602	1.4	0.048
hsa-miR-431	1.4	0.026
hsa-miR-1247	1.4	0.020
hsa-miR-23a	1.4	0.022
hsa-miR-20a	1.4	0.002
hsa-miR-627	1.4	0.038
hsa-miR-363	1.3	0.024
hsa-miR-106a	1.3	0.001
hsa-let-7i	1.3	0.008
hsa-miR-548o	1.3	0.030
hsa-miR-92a	1.3	0.013
hsa-miR-362-5p	1.3	0.010
hsa-miR-148b	1.3	0.022
hsa-miR-19b	1.3	0.007
hsa-miR-130b	1.3	0.011
hsa-miR-33b*	1.3	0.025
hsa-miR-503	1.3	0.008
hsa-miR-186	1.3	0.025
hsa-miR-1207-5p	1.3	0.038
hsa-miR-181c	1.3	0.011
hsa-miR-182	1.2	0.006
hsa-miR-24-2*	1.2	0.025
hsa-let-7g	1.2	0.005
hsa-miR-31*	1.2	0.036
hsa-miR-140-3p	1.2	0.007
hsa-miR-339-3p	-1.2	0.009
hsa-miR-194*	-1.2	0.020
hsa-miR-342-3p	-1.3	0.050
hsa-miR-335	-1.3	0.030
hsa-miR-103-2*	-1.3	0.010
hsa-miR-181b	-1.3	0.016
hsa-miR-505	-1.3	0.009
hsa-miR-425	-1.4	0.013
hsa-miR-491-5p	-1.4	0.030
hsa-miR-324-5p	-1.4	0.013
hsa-miR-1184	-1.4	0.006
hsa-miR-93	-1.4	0.013
hsa-miR-339-5p	-1.4	0.003
hsa-miR-550*	-1.4	0.045
hsa-miR-2110	-1.4	0.030
hsa-miR-744*	-1.4	0.011
hsa-miR-30d*	-1.4	0.004
hsa-miR-30b	-1.4	0.003
hsa-miR-191	-1.4	0.008
hsa-miR-34a	-1.4	0.005
hsa-miR-425*	-1.5	0.008

miRNAs	Fold change	*P* Value
hsa-miR-571	-1.5	0.021
hsa-miR-196a	-1.5	0.004
hsa-miR-141*	-1.5	0.001
hsa-miR-26a	-1.5	0.032
hsa-miR-125a-5p	-1.6	0.005
hsa-miR-324-3p	-1.6	0.003
hsa-miR-92b	-1.6	0.004
hsa-miR-23b	-1.6	0.011
hsa-miR-99b	-1.6	0.025
hsa-miR-193b	-1.6	0.006
hsa-miR-30d	-1.6	0.036
hsa-miR-331-5p	-1.6	0.004
hsa-miR-941	-1.6	0.007
hsa-miR-340*	-1.6	0.002
hsa-miR-615-3p	-1.7	0.001
hsa-miR-374b	-1.7	0.001
hsa-miR-1249	-1.7	0.014
hsa-let-7b*	-1.7	0.003
hsa-miR-454*	-1.7	0.001
hsa-miR-505*	-1.7	0.042
hsa-miR-141	-1.7	0.002
hsa-miR-340	-1.8	0.009
hsa-miR-766	-1.8	0.000
hsa-miR-200c	-1.8	0.002
hsa-miR-524-3p	-1.8	0.001
hsa-miR-421	-1.8	0.006
hsa-miR-1270	-1.8	0.025
hsa-miR-99b*	-1.8	0.004
hsa-miR-651	-1.9	0.006
hsa-miR-29b-1*	-1.9	0.000
hsa-let-7b	-2.0	0.005
hsa-miR-24-1*	-2.0	0.008
hsa-miR-621	-2.0	0.041
hsa-miR-876-3p	-2.0	0.000
hsa-miR-675b	-2.0	0.038
hsa-miR-330-3p	-2.0	0.002
hsa-miR-551b	-2.0	0.016
hsa-miR-26b*	-2.1	0.008
hsa-miR-342-5p	-2.1	0.023
hsa-miR-181a-2*	-2.1	0.000
hsa-miR-769-5p	-2.1	0.004
hsa-miR-551b*	-2.1	0.023
hsa-miR-615-5p	-2.2	0.008
hsa-miR-29c*	-2.2	0.001
hsa-miR-125a-3p	-2.2	0.002
hsa-miR-195*	-2.2	0.000
hsa-miR-135a	-2.2	0.006
hsa-miR-34a*	-2.2	0.000
hsa-miR-135b	-2.3	0.002
hsa-miR-199b-5p	-2.3	0.028

miRNAs	Fold change	*P* Value
hsa-miR-371-3p	-2.4	0.000
hsa-miR-33a*	-2.4	0.003
hsa-miR-744	-2.4	0.001
hsa-miR-548j	-2.4	0.005
hsa-miR-193b*	-2.4	0.000
hsa-miR-203	-2.5	0.000
hsa-let-7f-2*	-2.5	0.022
hsa-miR-519e	-2.5	0.006
hsa-miR-23b*	-2.6	0.003
hsa-miR-193a-3p	-2.6	0.038
hsa-miR-26b	-2.6	0.000
hsa-miR-99a	-2.6	0.002
hsa-miR-649	-2.6	0.001
hsa-miR-374b*	-2.7	0.017
hsa-miR-326	-2.7	0.004
hsa-miR-200c*	-2.7	0.003
hsa-miR-142-3p	-2.8	0.009
hsa-miR-573	-2.8	0.039
hsa-miR-223	-2.9	0.046
hsa-miR-551a	-2.9	0.001
hsa-miR-27b*	-2.9	0.001
hsa-miR-135b*	-2.9	0.004
hsa-miR-210	-3.0	0.000
hsa-miR-1908	-3.1	0.000
hsa-miR-769-3p	-3.1	0.016
hsa-miR-376a*	-3.1	0.019
hsa-miR-489	-3.1	0.000
hsa-miR-95	-3.3	0.000
hsa-miR-338-3p	-3.4	0.000
hsa-miR-375	-3.4	0.000
hsa-miR-1182	-3.6	0.034
hsa-miR-517a	-3.6	0.014
hsa-miR-26a-1*	-3.7	0.000
hsa-miR-663b	-3.8	0.002
hsa-miR-187	-3.9	0.002
hsa-miR-338-5p	-4.2	0.000
hsa-miR-591	-4.3	0.021
hsa-miR-653	-4.3	0.013
hsa-miR-549	-4.8	0.002
hsa-miR-127-3p	-6.3	0.003
hsa-miR-345	-6.5	0.044
hsa-miR-302c*	-7.7	0.000
hsa-miR-1243	-7.8	0.004
hsa-miR-519d	-8.0	0.000
hsa-miR-127-5p	-8.7	0.006
hsa-miR-708	-9.2	0.001
hsa-miR-145	-13.5	0.001
hsa-miR-154	-18.8	0.000
hsa-miRPlus-D1061	-35.1	0.005

4

CHAPTER FOUR

Anti-tumoral Effect of the Non-nucleoside DNMT Inhibitor RG108 in Human Prostate Cancer Cells

Abstract

Background: Current therapeutic strategies for advanced prostate cancer (PCa) are largely ineffective. Because aberrant DNA methylation associated with inappropriate gene-silencing is a common feature of PCa, DNA methylation inhibitors might constitute an alternative therapy. In this study we aimed to evaluate the anti-cancer properties of RG108, a novel non-nucleoside inhibitor of DNA methyltransferases (DNMT), in PCa cell lines.

Methods: The anti-tumoral impact of RG108 in LNCaP, 22Rv1, DU145 and PC-3 cell lines was assessed through standard cell viability, apoptosis and cell cycle assays. Likewise, DNMT activity, *DNMT1* expression and global levels of DNA methylation were evaluated in the same cell lines. The effectiveness of DNA demethylation was further assessed through the determination of promoter methylation and transcript levels of *GSTP1*, *APC* and *RARβ2*, by quantitative methylation-specific PCR and RT-PCR, respectively.

Results: RG108 led to a significant dose and time dependent growth inhibition and apoptosis induction in LNCaP, 22Rv1 and DU145. LNCaP and 22Rv1 also displayed decreased DNMT activity, *DNMT1* expression and global DNA methylation. Interestingly, chronic treatment with RG108 significantly decreased *GSTP1*, *APC* and *RARβ2* promoter hypermethylation levels, although mRNA re-expression was only attained *GSTP1* and *APC*.

Conclusions: RG108 is an effective tumor growth suppressor in most PCa cell lines tested. This effect is likely mediated by reversion of aberrant DNA methylation affecting cancer related-genes epigenetically silenced in PCa. However, additional mechanism might underlie the anti-tumor effects of RG108. *In vivo* studies are now mandatory to confirm these promising results and evaluate the potential of this compound for PCa therapy.

Introduction

Prostate cancer (PCa) is the most common diagnosed malignancy in Western countries and one of the leading causes of cancer-related morbidity and mortality [1]. Although effective therapy is available for early-stage PCa, treatment of advanced disease is mainly ineffective and remains a clinical challenge.

Androgen-deprivation therapy is widely used for locally advanced and systemically spread PCa. However, most patients that initially respond to androgen deprivation therapy have a median time to progression to castration-resistant disease of only 18-30 months [2]. Therefore, investigation of new therapeutic strategies is urgently needed and should be based, ideally, on the acquired knowledge about PCa biology.

Indeed, PCa is a complex and heterogeneous disease where multiple genetic and epigenetic alterations interplay and contribute to its development and progression [3]. The most widely known epigenetic change in PCa is aberrant DNA methylation, which may lead to altered expression of tumor-suppressor genes and proto-oncogenes. In fact, there are more than 50 genes reported to be silenced by promoter hypermethylation in PCa, including *GSTP1*, *APC*, *RARβ2*, *RASSF1A*, and *CCND2*, among others [4-6]. Remarkably, unlike genetic alterations, epigenetic changes are chemically reversible and numerous compounds have been reported to be effective against cancer cells by inhibiting one or more components of the epigenetic machinery. Among these are the DNA methyltransferases inhibitors (DNMTi), of which 5-azacytidine (5-Aza-CR) and 5-aza-2´-deoxycytidine (5-Aza-CdR) are the most widely described [7]. Interestingly, 5-Aza-CdR was shown to have modest clinical activity against castration-resistant PCa and to inhibit prostate cancer progression in TRAMP mice [8, 9]. However, the effectiveness of these compounds relies on its incorporation into DNA, which may result in substantial, dose-dependent cytotoxicity [10]. Owing to the toxicity of nucleoside analogs, emphasis has been placed in the discovery of new compounds that more directly target DNMTs. One of those compounds is RG108, a non-nucleoside analogue of DNMT designed to target human DNMT1 at its active site. RG108 was shown to be effective in human colon cancer cells, in which it was capable of reactivating several tumor-suppressor genes (*e.g.*, *p16*), without affecting the methylation status of centromeric repeats [11, 12]. Importantly and contrarily to 5-aza-CdR, the DNA demethylating activity of RG108 was not associated with high levels of cytotoxicity [11, 12]. To the best of our knowledge, the anti-tumoral effect of this compound has not been explored in PCa.

Thus, we sought to investigate the potential of RG108 as a growth inhibitor of PCa cells. For that purpose, the impact of RG108 in cell viability, apoptosis and cell cycle of

several PCa cell lines was assessed. Furthermore, the inhibitory effect on DNMT and consequent DNA demethylation activity of this drug were evaluated, along with the re-expression of genes known to be epigenetically silenced in PCa.

Materials and Methods

Prostate cancer cell lines and drug preparation

Prostate cancer cell lines PC-3 and LNCaP were kindly provided by Prof. Ragnhild A. Lothe from the Department of Cancer Prevention at the Institute for Cancer Research, Oslo, Norway, DU145 was obtained from American Type Culture Collection (ATCC, Lockville, MD), and 22Rv1 cells were kindly provided by Dr. David Sidransky at the Johns Hopkins University School of Medicine, Baltimore, MD, USA. LNCaP and 22Rv1 cells were grown in RPMI 1640, DU145 cells were maintained in MEM and PC-3 cells were grown in 50% RPMI-50% F-12 medium (GIBCO). All basal culture media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (GIBCO). Cells were maintained in an incubator at 37ºC with 5% CO2. All PCa cell lines were karyotyped by G-banding (for validation purposes) and routinely tested for Mycoplasma spp. contamination (PCR Mycoplasma Detection Set, Clontech Laboratories). RG108 was purchased from Tocris Bioscience (Bristol, UK), dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA), and stored at -20ºC until further use. 5-aza-CdR was obtained from Sigma (Sigma-Aldrich), dissolved in 50% acetic acid-50% PBS and stored at -20ºC. For control purposes, cell lines were exposed to the vehicles of the drugs only (DMSO or 50% acetic acid-50% PBS).

Viability assay

PCa cells were seeded at 1000 cells per well onto 96-well flat bottoned culture plates, allowed to adhere overnight and treated with different RG108 concentrations (*i.e.*, 50, 100 and 200 μM) for 3 days (acute exposure) and 14 days (chronic exposure). Cell viability was then evaluated by MTT assay. Briefly, 0.5 mg/ml of MTT reagent [3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] was added to each well, and the plates were incubated in the dark for 3 hours at 37ºC. Formazan crystals were then dissolved in DMSO and absorbance was read at 540 nm in a microplate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany), subtracting the background, at 630 nm. Three replicates were performed for each condition, and at least two independent experiments were carried out.

Apoptosis evaluation

Evaluation of apoptosis was performed using APO Percentage apoptosis assay kit (Biocolor Ltd., Belfast, Northern Ireland) according to the manufacturer's instructions. The assay was carried out using the same cell conditions as those of the MTT assay.

Apoptotic cells were assessed at the end of the day 3 and 14 in a FLUOstar Omega microplate reader at 550 nm.

Cell cycle analysis

Cell cycle distribution of PCa cells was determined by flow cytometry. Briefly: $5x10^5$ harvested cells were fixed overnight at 4ºC with 70% cold ethanol. After washing with cold PBS, cells were ressuspended in staining Propidium Iodide Solution (Cytognos S.L, Salamanca, Spain) and incubated for 30 minutes at room temperature. All cells were then measured on a Cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) and analyzed using Modfit LT (Verity Software House, Inc, Topshan, Maine, USA).

Real Time Quantitative PCR (qRT-PCR)

RNA was extracted from cell lines using TRIzol® (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. First strand synthesis was performed using the high-capacity CDNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA). Expression of target genes (*Ki67*, *CDKN1A*, *APC*, *GSTP1, RARβ2, DNMT1, DNMT3a, and DNMT3b*) was quantified using Taqman expression assays, purchased as pre-developed assays from Applied Biosystems and normalized to the expression of the *GUSB* housekeeping gene.

Western Blot

Proteins were extracted from whole-cell lysates using RIPA lysis buffer (Santa Cruz Biotechnology, CA, USA) and subsequently quantified using a Pierce BCA assay (Thermo Scientific Inc., Bremen, Germany), according to the manufacturer's instructions. Briefly, 30 µg of protein from each sample were separated using 4–20% Mini-PROTEAN® TGX™ Precast Gel at 300 V and subsequently blotted onto Protran nitrocelulose transfer membranes (Whatman, Dassel, Germany). For immunodetection, membranes were incubated overnight at 4ºC with antibodies directed against DNMT1 at 1:1,000 and active Caspase-3 at 1:200 (Cell Signaling Technology, Danvers, MA). The Immun-Star WesternC Chemiluminescent kit (Bio-Rad, Hercules, CA, USA) was used to develop the membranes which were then recorded with Amersham Hyperfilm (GE Healthcare Buckinghamshire, UK). To ascertain equal loading of protein, the membranes were incubated with a monoclonal mouse antibody against β-Actin (1:8,000, Sigma-Aldrich).

DNMT activity/inhibition assay

Nuclear extracts from vehicle and RG108 treated LNCaP, 22Rv1 and DU145 were obtained using Nuclear Extract Kit (Active Motif, Rixensart, Belgium) as per the

manufacture's protocol. After protein quantification, 10µg of nuclear lysate from each sample was used to measure DNMT activity or inhibition with DNMT Activity/Inhibition Assay Kit (Active Motif) according to the manufacturer's instructions. Absorbance was measured using FLUOstar Omega microplate reader, at 450 nm. All samples were analyzed in triplicate.

Quantification of Global DNA Methylation

Global DNA methylation was analyzed by the Imprint® Methylated DNA Quantification Kit (Sigma-Aldrich) according to the manufacturer's instructions. DNA methylation status was compared with an artificially fully methylated DNA positive control. Absorbance was measured using FLUOstar Omega microplate reader at 450 nm. All samples were analyzed in triplicate.

Quantitative Methylation Specific PCR (qMSP)

Genomic DNA was extracted from cell lines using a standard technique comprising digestion with proteinase K (20mg/mL) in the presence of 10% SDS at 55ºC, followed by phenol-chloroform extraction and precipitation with 100% ethanol [13]. One microgram of DNA was submitted to bisulfite modification using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA) following the manufacturer's instructions. Bisulfite modified DNA was amplified by qMSP using TaqMan technology [14]. Specific *GSTP1*, *APC* and *RARβ2* primers and TaqMan probes were designed using the Methyl Primer Express Software v1.0 (Applied Biosystems). *β-actin* (*ACTB*) was used as an internal reference gene to normalize for DNA input and all qMSP reactions were performed as previously described [4]. Methylation levels for each sample were derived from calibration curves constructed using serial dilutions of bisulfite modified CpGenomeTM Universal Methylated DNA (Millipore, Billerica, MA). *GSTP1*, *APC* and *RAR2β* methylation levels were calculated after normalization for *ACTB.*

Statistical analysis

One-way analysis of variance (ANOVA), with post-hoc Dunnet's multiple comparison test when appropriate, was used to compare the results obtained in each parameter for the three different concentrations of RG108 and the controls (vehicle). Analysis was performed with SPSS software for Windows version 19.0 (IBM-SPSS Inc Chigaco, IL) and statistical significance was set at $P<0.05$.

Results

RG108 inhibits cell viability and induces apoptosis in human PCa cells

To investigate the effects of the demethylating agent RG108 on cell viability, four human PCa cell lines, LNCaP, 22Rv1, DU145 and PC-3 were exposed to three different concentrations of RG108, as wells as to the vehicle of this compound, DMSO. Cell viability was evaluated at days 0, 1, 2, 3, 7, 10 and 14. Acute exposure to RG108 resulted in a slight decrease in the number of viable cells (Figure 1A). Regarding LNCaP, there was a significant decrease in cell viability at the end of the second day when treated with 200 µM of RG108 (P=0.04) compared to the vehicle. 22Rv1 showed a decrease in cell viability at day 1 when exposed to 50 and 200 µM of RG108 (P≤0.04) and at the end of the third day when 100 and 200 µM concentrations were applied (P≤0.03). DU145 treated cells also suffered a slight reduction in viable cells on the first day with 50 and 100 µM concentrations (P≤0.02) and at day 3 when exposed to 200 µM (P=0.002).

As shown in Figure 1A, chronic exposure induced an impressive reduction in viable cells especially when the higher concentration of RG108 was used. LNCaP was the most sensitive cell line, since the inhibition of cell growth occurred for all the tested days at all the tested drug concentrations (P≤0.002). LNCaP exposure to 200 µM RG108 during 14 days resulted in an 80% reduction in viable cells. 22Rv1 also displayed a decrease in the number of viable cells in all days (7, 10, and 14) with 200 µM RG108 (P≤0.004), being the reduction after 14 days of treatment of 50%, whereas DU145 only showed a decrease of 10% in cell viability in the last day with 100 and 200 µM RG108 (P≤0.004). RG108 appeared to have no effect on PC-3 cell viability.

To corroborate the previous results, an analysis of molecular changes induced by RG108 was carried out by studying the quantitative expression of two genes involved in cell proliferation pathways. Since the best results were obtained with 14 days of exposure and 200 µM of RG108, it was decided to evaluate gene expression under these conditions and at the concentration of 50 µM. The results showed both induction of *CDKN1A* (P≤0.045) and decrease of *KI67* (P≤0.045) mRNA levels of the RG108 treated cells when compared to the vehicle (Figure 1B).

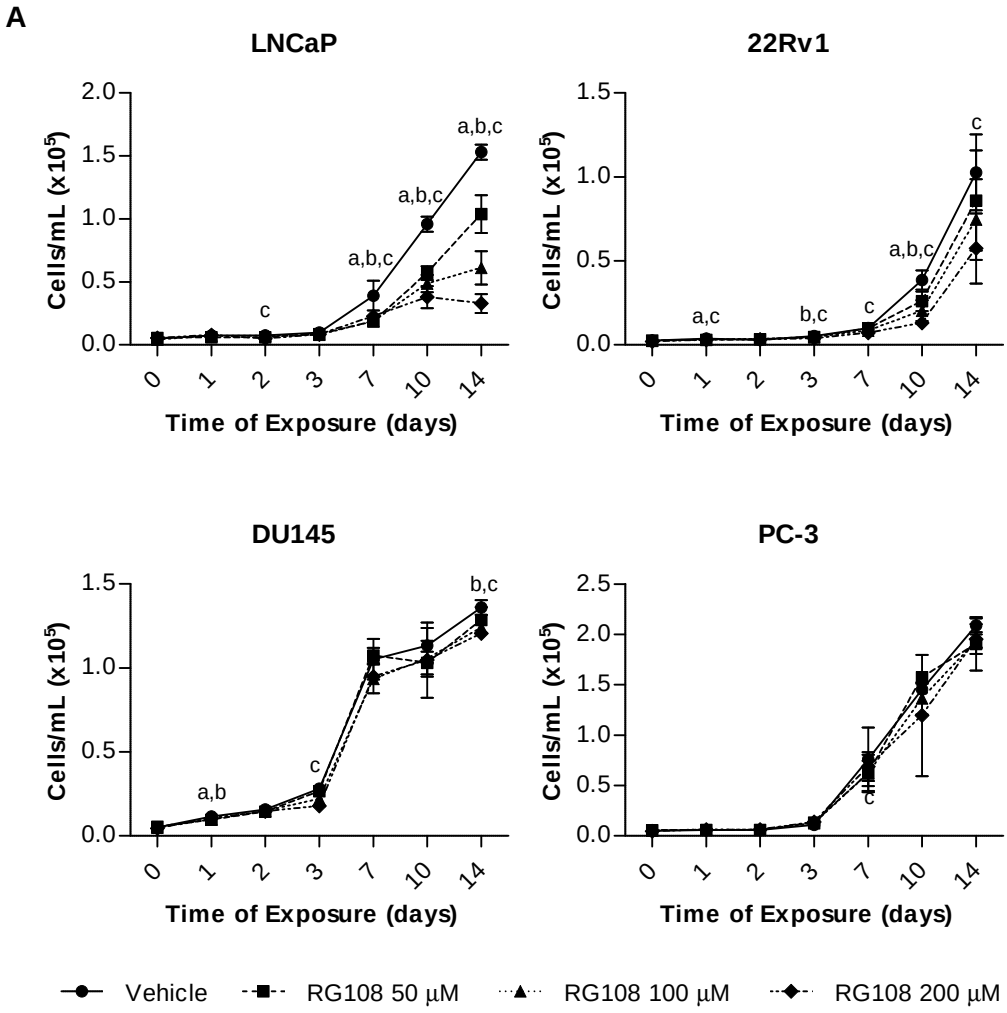
To determine if the exposure to RG108 was capable of induce significant cell death, an apoptotic assay that detects the phosphatidylserine transfer to the outside of the membrane was performed. Acute exposure to RG108 resulted in a significant increase in apoptosis (Figure 1C) for LNCaP with 100 and 200 µM (P≤0.037) and for 22Rv1 and DU145 with 100 µM (P≤0.002). Chronic treatment induced an augment in apoptosis

(Figure 1C) in LNCaP cells when treated with 100 µM (*P*=0.038) and DU145 when exposed to 50 and 200 µM (*P*≤0.007).

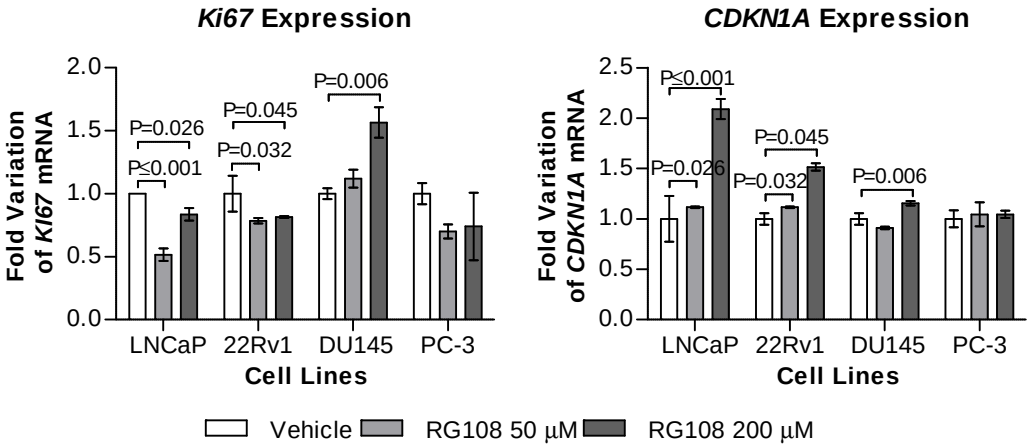
To confirm apoptosis at molecular level, the mRNA levels of *CASP3* were evaluated. As the best results achieved with the apoptosis assay were obtained after 14 days, only this time point was evaluated. Chronic treatment of LNCaP (100 µM), 22Rv1 (50 µM) and DU145 (200µM) resulted in significantly increased levels of *CASP3* (*P*≤0.029) (Figure 1D). An increase of active Caspase 3 protein levels' was observed in all cell lines after drug exposure, namely in LNCaP and DU145 exposed to RG108 at 50 and 200 µM and in 22Rv1 when exposed to 100 µM (Figure 1E).

Since RG108 did not affect the cancer phenotype of PC-3 cell line, further studies were only performed in LNCaP, 22Rv1 and DU145 with 50 and 200 µM RG108.

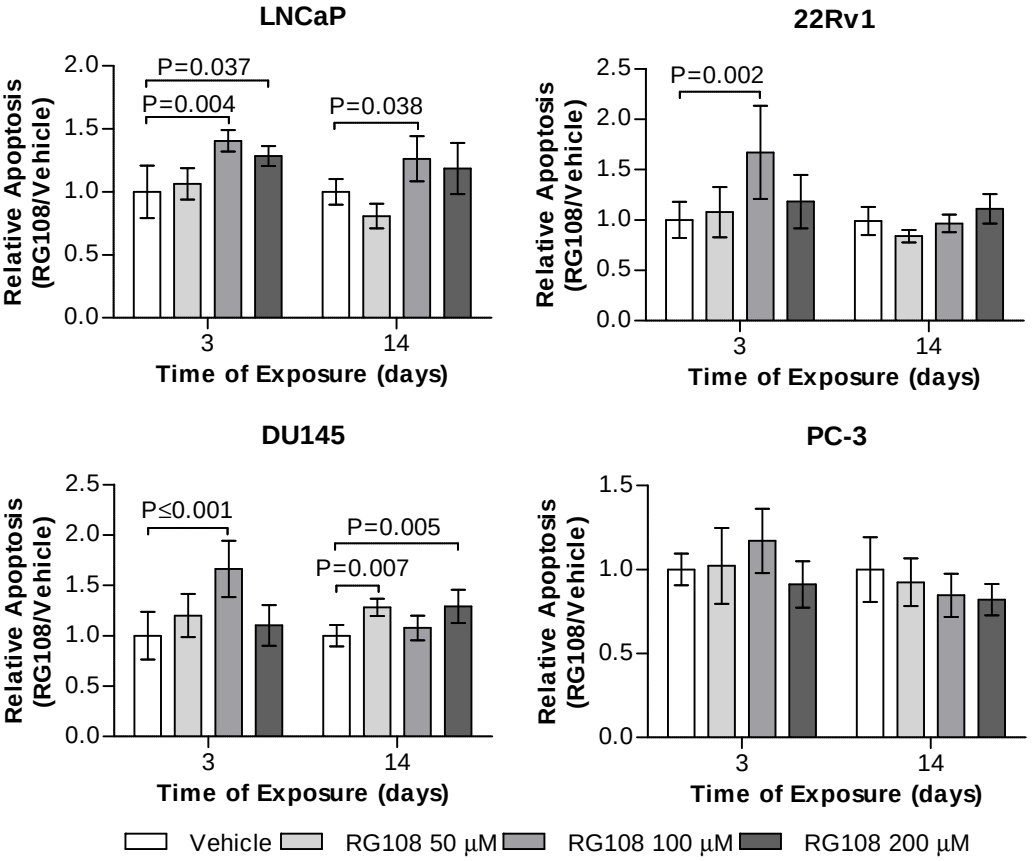
Cell cycle distribution was evaluated after 14 days of exposure to 50 and 200 µM RG108. There was no evidence of cell cycle arrest in LNCaP and 22Rv1. However, DU145 presented a significant increase in the percentage of cells in G2/M transition for both concentrations of the compound (*P*≤0.001) (Figure 1F).



B


Ki67 Expression
Fold Variation of *Ki67* mRNA
2.0
1.5
1.0
0.5
0.0
P=0.026
P≤0.001
P=0.045
P=0.032
P=0.006
LNCaP
22Rv1
DU145
PC-3
Cell Lines
CDKN1A Expression
Fold Variation of *CDKN1A* mRNA
2.5
2.0
1.5
1.0
0.5
0.0
P≤0.001
P=0.026
P=0.032
P=0.045
P=0.006
LNCaP
22Rv1
DU145
PC-3
Cell Lines
Vehicle
RG108 50 µM
RG108 200 µM

C


LNCaP
Relative Apoptosis (RG108/Vehicle)
2.0
1.5
1.0
0.5
0.0
P=0.037
P=0.004
P=0.038
3
14
Time of Exposure (days)
22Rv1
Relative Apoptosis (RG108/Vehicle)
2.5
2.0
1.5
1.0
0.5
0.0
P=0.002
3
14
Time of Exposure (days)
DU145
Relative Apoptosis (RG108/Vehicle)
2.5
2.0
1.5
1.0
0.5
0.0
P≤0.001
P=0.005
P=0.007
3
14
Time of Exposure (days)
PC-3
Relative Apoptosis (RG108/Vehicle)
1.5
1.0
0.5
0.0
3
14
Time of Exposure (days)
Vehicle
RG108 50 µM
RG108 100 µM
RG108 200 µM

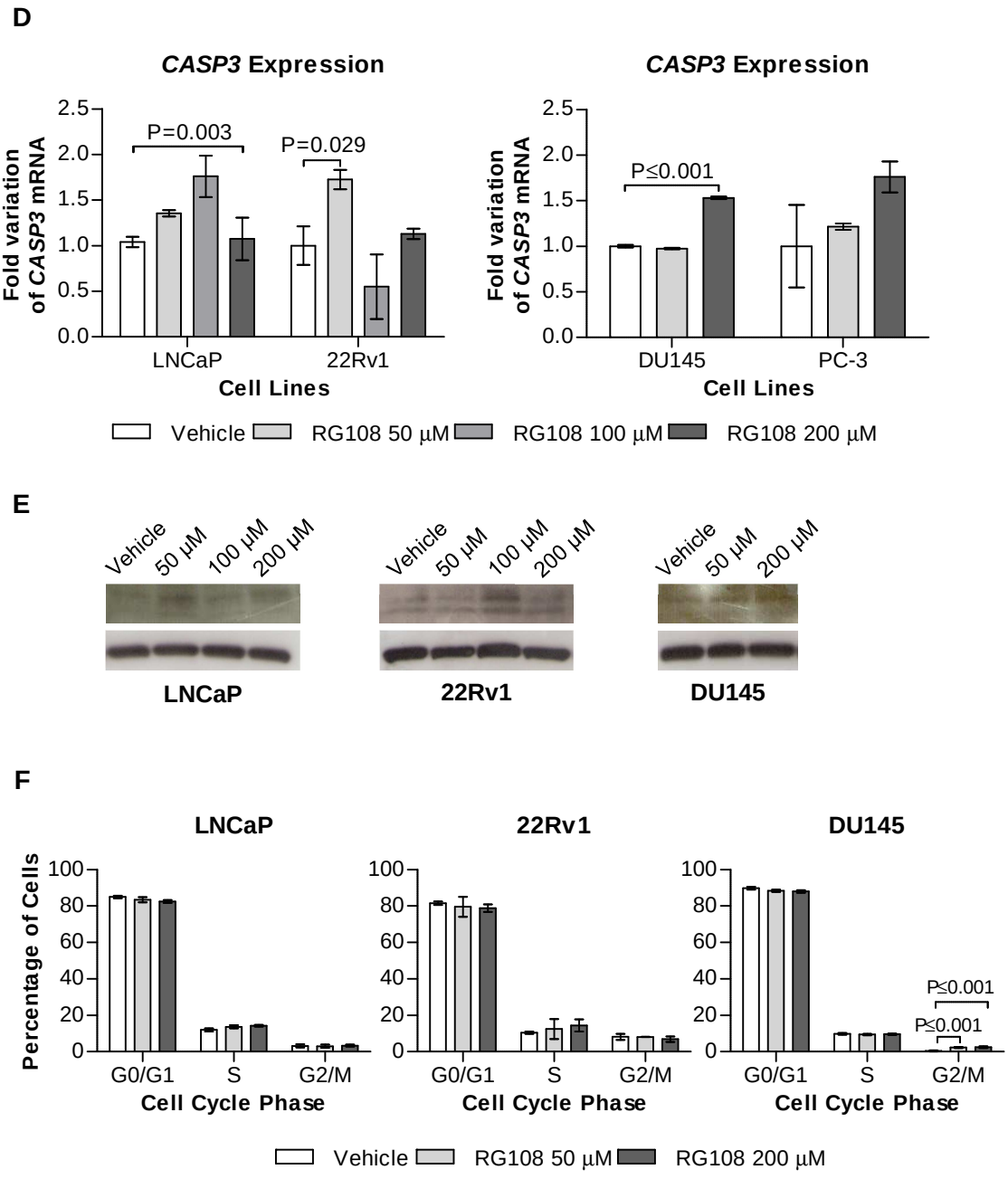


Figure 1 - Effects of RG108 in viability, apoptosis and cell cycle of PCa cell lines. (**A**) Cell viability in LNCaP, 22Rv1, PC-3 and DU145, in the presence of RG108, at days 0, 1, 2, 3,7,10, and 14. (a) statistically significant differences were observed between vehicle and 50 µM RG108, (b) vehicle and 100 µM RG108 and (c) vehicle and 200 µM RG108. (**B**) mRNA expression of *Ki67* and *CDKN1A* after 14 days of exposure to RG108. (**C**) Effect of acute and chronic RG108 exposure in apoptosis of PCa cell lines. (**D**) *CASP3* mRNA expression and (**E**) active CASP3 protein expression after 14 days of exposure to RG108. (**F**) Cell cycle evaluation after 14 days of RG108 exposure. Data are presented as mean of three independent experiments ± s.d.

RG108 inhibits DNMT activity and expression in human PCa cells

After exposing cells to 50 and 200 µM of RG108 for 14 days, nuclear extracts were obtained and DNMT activity was determined. As observed in Figure 2A there was a significant reduction in DNMT activity (15%) for 22Rv1 with 50 µM RG108 (*P*=0.003). For the remaining two cell lines a significant decrease in DNMT activity was not apparent, although LNCaP displayed a slight reduction (13%). Subsequently, we determined whether decreased DNMT activity correlated with decreased *DNMT1*, *DNMT3a and DNMT3b* mRNA levels. As expected, 22Rv1 and LNCaP showed decreased levels of *DNMT1*and *DNMT3b* compared to the vehicle, 22Rv1 cell line after exposure to 50 µM RG108 (*P*≤0.012) and LNCaP after treatment with both tested concentrations (*P*=0.019). However, only 22Rv1 demonstrated a significant reduction in *DNMT3a* mRNA levels (*P*≤0.001) (Figure 2B). Importantly, a reduction in DNMT1 protein expression was displayed by both cell lines (Figure 2C).

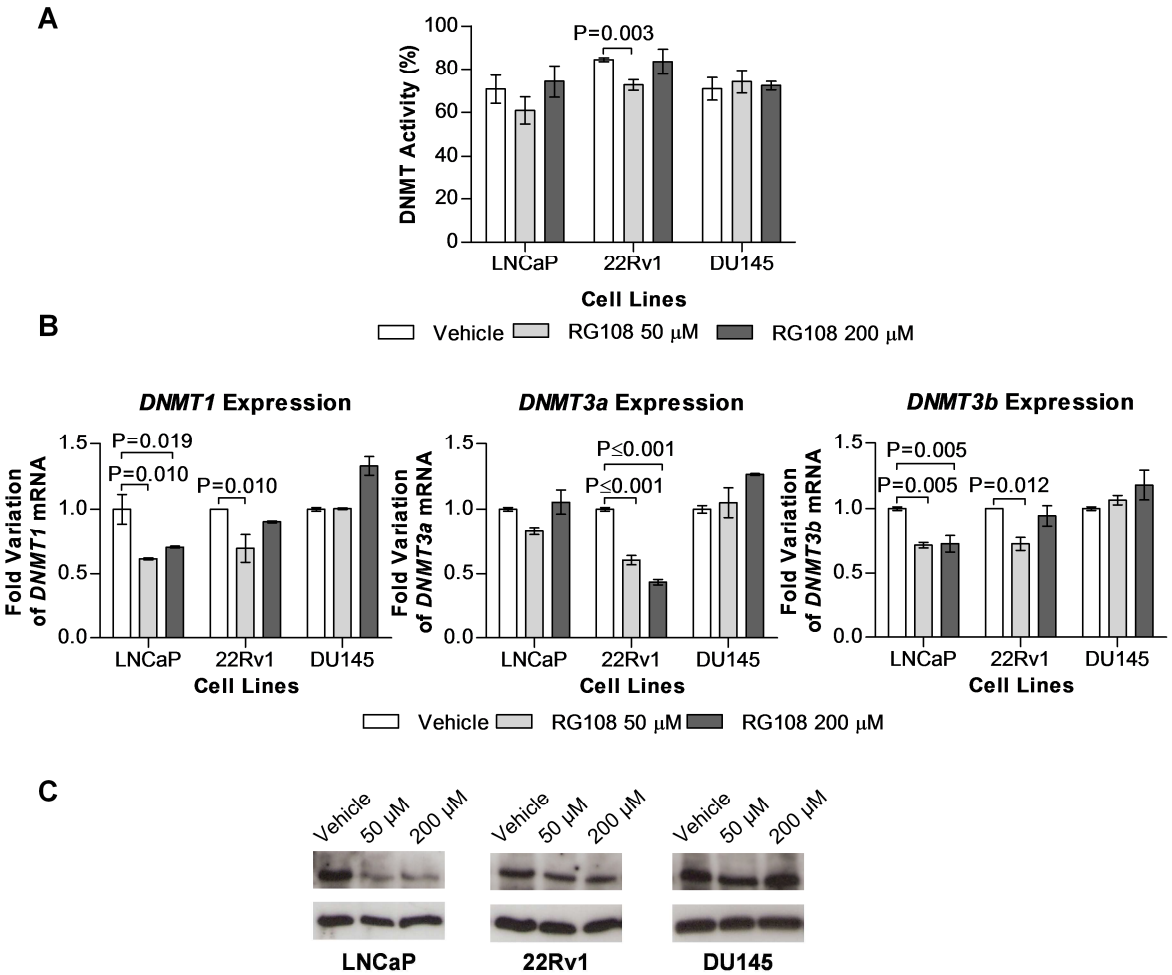


Figure 2 - Impact of RG108 on DNMT activity (A), in *DNMTs* mRNA expression (B), and DNMT1 protein expression after 14 days exposure to RG108. Data are shown as mean of three independent experiments ± s.d.

RG108 decreases Global DNA Methylation

Once verified the ability of this compound to decrease DNMT activity and expression, the next step was to investigate the effect in global DNA methylation content. Since DU145 did not present a significant decrease in DNMT activity and expression, it was not further studied. To compare the effect of this demethylating agent with that of 5-aza-CdR (a widely used demethylating drug), LNCaP and 22Rv1 cells were exposed to 1 and 5 µM of 5-aza-CdR during 3 days. RG108 significantly decreased global DNA methylation in 22Rv1 with 200 µM (approximately 5%, *P*=0.028), and in LNCaP cells (approximately 11% and 9% with 50 and 200 µM respectively, *P*≤0.001) (Figure 3A). In contrast, 5-aza-CdR was able to reduce global DNA methylation in about 14% in LNCaP cells treated with 1 µM (*P*≤0.001) and approximately 16% in 22Rv1 for both concentrations (*P*≤0.001) (Figure 3B).

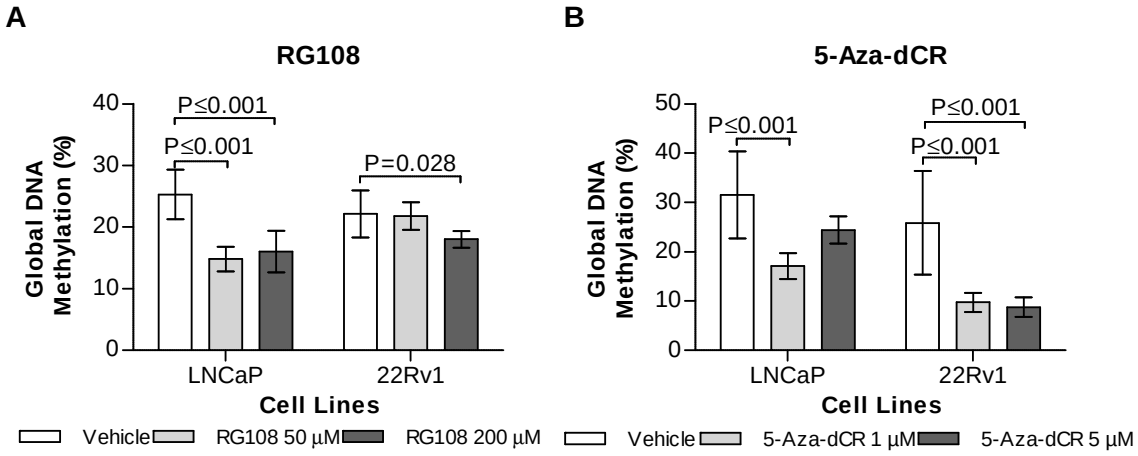


Figure 3 - Effect of RG108 (A) and 5-aza-CdR (B) on global DNA methylation. Data are presented as mean of three independent experiments ± s.d.

RG108 causes demethylation and reactivation of silenced genes in human PCa cells

To investigate the ability to demethylate and consequently induce the expression of specific genes, a panel of three genes (*APC*, *GSTP1* and *RARβ2*), previously reported to be silenced by promoter hypermethylation in PCa, was evaluated.

Both LNCaP and 22Rv1 showed a decrease in methylation levels of *GSTP1* with 50 µM (*P*≤0.009) whereas solely 22Rv1 exhibited similar results with 200 µM (*P*=0.005) (Figure 4A). However, only 22Rv1 demonstrated an increase in *GSTP1* mRNA expression with 200 µM (*P*≤0.001) (Figure 4B). *APC* methylation levels were reduced in 22Rv1 with 50 µM of RG108 (*P*=0.005) (Figure 4A), concomitantly with the induction of mRNA expression of this gene (*P*=0.003) (Figure 4B). Although a reduction in *RARβ2* methylation was

observed in LNCaP with 200 µM (*P*=0.036) and in 22Rv1 with 50 µM (*P*=0.003) of RG108 (Figure 4A), the re-expression of this gene was not achieved (Figure 4B).

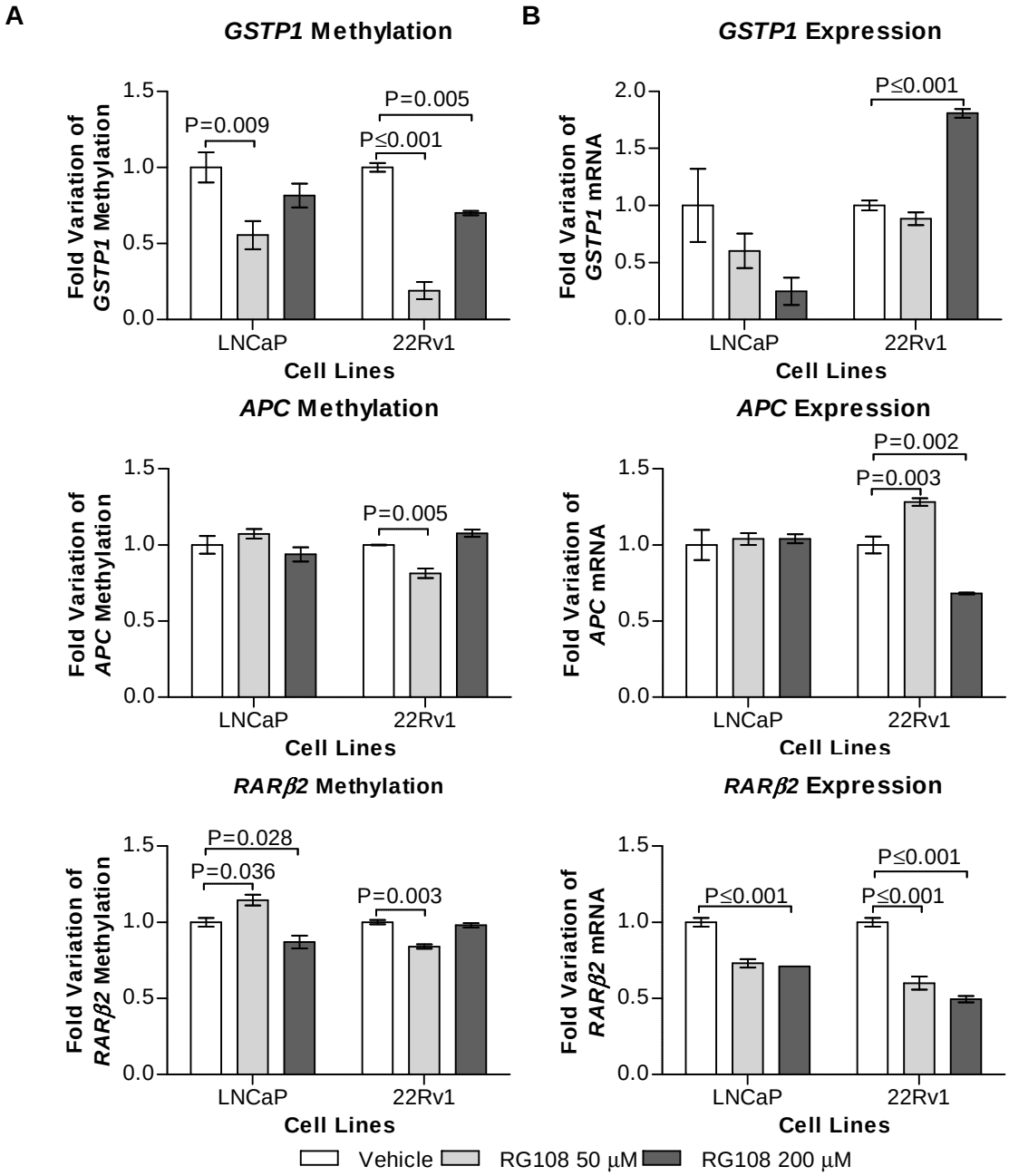


Figure 4 - Impact of RG108 on demethylation (A) and re-expression (B) of *GSTP1*, *APC* and *RARβ2*. Data are shown as mean of three independent experiments ± s.d.

The results were also compared with those obtained with 5-aza-CdR. This compound was able to demethylate *GSTP1* with 1 and 5 µM in LNCaP and with 1µM in 22Rv1 (*P*≤0.020). *APC* methylation was only reduced in LNCaP cells exposed to 1 µM of 5-aza-CdR (*P*=0.037). Concerning *RARβ2,* no significant reduction in methylation levels was observed after exposure to 5-aza-CdR (Figure 5).

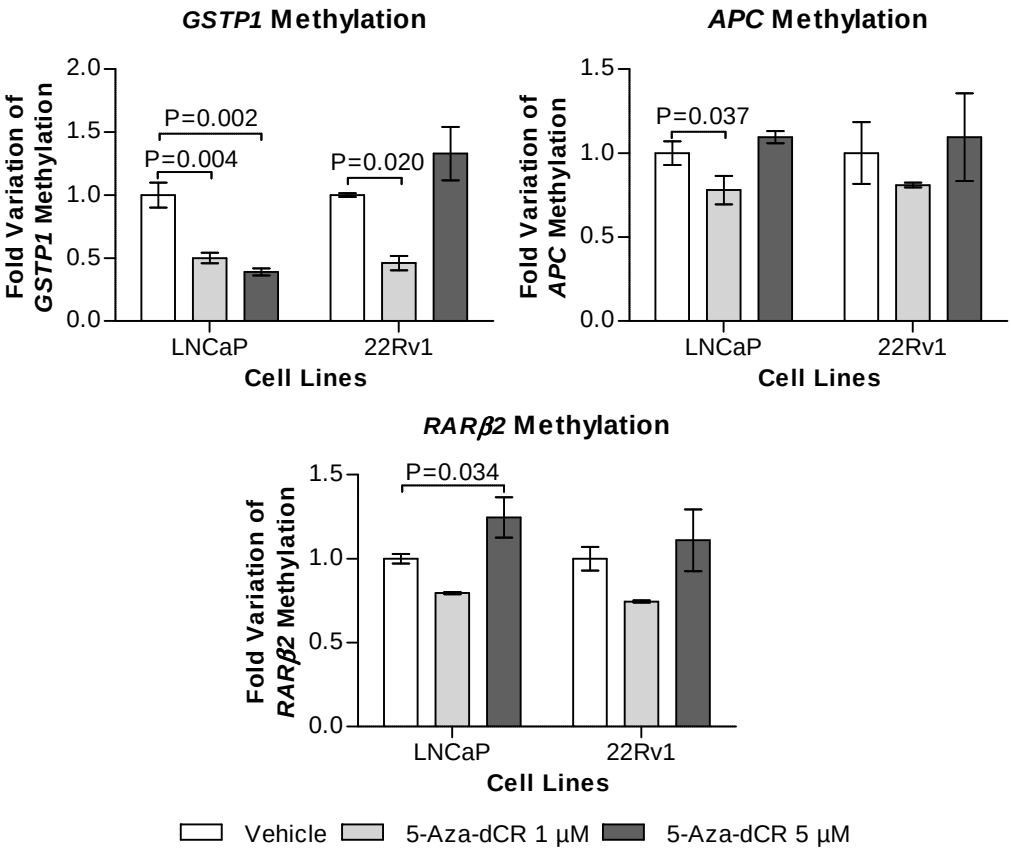


Figure 5 - Effect of 5-aza-CdR on *GSTP1*, *APC* and *RARβ2* promoter methylation levels. Data are presented as mean of three independent experiments ± s.d.

Discussion

The gold standard treatment for hormone dependent PCa involves surgery, radiation or androgen deprivation therapy, depending mainly on disease stage, whereas chemotherapy with docetaxel represents the main therapeutic option for castration-resistant PCa [15, 16]. In PCa, as well as in other cancers, the resistance of malignant cells to anticancer agents remains the major cause of treatment failure. Therefore, new therapeutic options are urgently needed. Epigenetic silencing of gene expression, especially that mediated by promoter hypermethylation, plays an important role in the development and progression of PCa, as well as in the emergence of resistance to chemotherapy [17, 18]. Thus, DNMTs might constitute a valuable therapeutic target for PCa treatment. Reversal of DNA methylation with DNMTi has being widely studied in several cancers. In fact, two nucleoside analogs 5-azacytidine and 5-aza-2´-deoxycitidine have been already approved by US Food and Drug Administration (US FDA) for the treatment of myelodysplastic syndrome [19]. However, those two nucleoside analogs have demonstrated a limited efficacy in the treatment of solid malignancies [20, 21]. Both for this reason and owing to their significant cytotoxicity, new epigenetic compounds must be explored for cancer treatment.

Herein, we present the first study on the anti-cancer effect of RG108, a non-nucleoside DNMTi, in PCa. The exposure of PCa cell lines (LNCaP, 22Rv1 and DU145) to RG108 resulted in a significant decrease in cell viability, in a dose and time dependent manner, being the 200 µM concentration and 14 days (chronic) of exposure the best combination tested. Interestingly, these results emphasize the need to achieve sustained levels of the drug to ensure a prolonged anti-tumor effect, which is in line with current therapeutic guidelines for 5-azacytidine and 5-aza-2´-deoxycitidine in myelodisplastic syndrome [22-24]. Moreover, RG108 induced cell death by apoptosis in LNCaP and DU145, and in the latter, cell cycle arrest in G2/M was also depicted. Importantly, these results were confirmed at the molecular level with a decrease of *KI67* transcript levels and induction of *CDKN1A* and *CASP3* mRNA expression. Thus, the ability to disturb multiple regulators of cancer cell survival in these distinct PCa cells lines by RG108 was proven. Because these cell lines are phenotypically different and present diverse patterns of cell response to this drug, it is tempting to suggest that RG108 might be effective against PCa *in vivo*, which is also characterized by substantial molecular heterogeneity [3].

Some previous studies have reported on the cellular proliferation inhibitory effect of 5-aza-CdR in PCa cell lines [25-27]. In fact, 5-aza-CdR induces p53 and p21Waf1/Cip1 expression which is associated with inhibition of cell proliferation and induction of

apoptosis in LNCaP cells, independently of DNA methylation [28, 29]. In this study we provide evidence that RG108 has similar effects on the inhibition of tumor growth in PCa cells. Considering that both 5-aza-CdR and RG108 are effective, the latter might be more appealing from a clinical standpoint, not only because RG108 is a non-nucleoside (and thus may be less cytotoxic), but also because it does not require active cell division to exert its functions [11].

Remarkably, we also demonstrated that RG108 was able not only to inhibit DNMT activity, but also *DNMT1, 3a and 3b* mRNA expression, as well as DNMT1 protein expression in LNCaP and 22Rv1 cells. This might justify the observed reversal in *GSTP1* and *APC* promoter hypermethylation and associated restoration of gene expression. The ability of RG108 to demethylate *GSTP1* is a very important finding since this gene, which is methylated in the vast majority of PCa, was recently suggested as a potential useful biomarker for assessing DNMTi efficacy in PCa [30]. These results are also in accordance with the reported ability of RG108 to demethylate tumor-suppressor genes (*e.g.*, *p16*) in HCT116 cell line [11]. It must be recalled, however, that demethylation is not synonymous of re-expression, as clearly demonstrated in our study for *RARβ2*. Because epigenetic silencing of cancer-related genes is a more complex phenomenon, in which other mechanisms, such as histone post-translational modifications, are also involved [17], combined therapies with histone deacetylase inhibitors might prove to be more effective.
This study also showed that RG108 has a weaker demethylating effect compared to 5-aza-CdR, as three days exposure to the latter was able to more effectively reduce the levels of global DNA methylation than RG108 at 14 days. Similar results were reported for HCT116 cells, which showed a reduction of 50% of cytosine methylation levels when exposed to 5-aza-CdR (after five days of incubation) and of only 30% when exposed to RG108 (after 15 days) [11]. The superior efficacy of 5-aza-CdR might be due to its properties as a cytidine analogue. Once incorporated into the DNA, 5-aza-CdR covalently traps the DNMTs on the DNA by forming a suicide complex which can lead to a massive loss of DNA methylation, resulting in genome hypomethylation, which may predispose to genomic instability and foster neoplastic transformation of normal cells [23, 31]. RG108, on the other hand, directly blocks DNMT1 activity [11], and may, thus, explain the less potent global DNA demethylation effect. This therapeutical disadvantage of RG108 may, however, be compensated by a lesser degree of global hypomethylation, minimizing genomic instability and improving its toxicity and safety profile.

Interestingly, several studies have proposed 5-aza-CdR as an adjuvant to conventional therapy of PCa [25, 26, 32, 33]. Those studies demonstrated that 5-aza-CdR could sensitize PCa cell lines to docetaxel, which is the first line therapy for castration-resistant PCa [25]. Likewise, 5-aza-CdR might restore the expression of androgen

receptor in PC-3 cell line, and the combined treatment with bicalutamide demonstrated a synergistic effect in repressing tumor growth in xenograft mice [32]. Remarkably, bicalutamide was recently associated with higher DNMT3a and DNMT3b expression, potentiating the up-regulation of truncated androgen receptor isoforms and therefore the castration resistant phenotype [33]. However, when cells were exposed to 5-aza-CdR, there was a decrease in DNMT activity and a consequent decline in the development of the castration-resistant phenotype [33]. Because RG108 is able to modify the phenotype of PCa cells and the catalytic domain of the four DNMTs is highly conserved, predicting similar interactions of those enzymes with RG108, it would be interesting to evaluate the possible synergistic action between RG108 and conventional PCa therapy. Moreover, based on the promising results from this study, further experiments should be conducted to investigate the cellular pathways involved in RG108 response. Comparing these cellular pathways with those involved in 5-aza-CdR response might provide valuable information concerning PCa cells biology and illuminate the different mechanisms of action of these compounds.

Conclusions

The demethylating agent RG108 demonstrated the ability to reduce cell viability, induce cellular death by apoptosis, and decrease DNMT activity and *DNMT1* expression in PCa cells. Furthermore, it was able to decrease promoter hypermethylation levels and induce re-expression of genes known to be epigenetically silenced in PCa. Because RG108 performed better in chronic exposure (14 days) at 200 µM concentration, further studies should be conducted with these conditions to further assess the promising role of RG108 as a valuable tool for PCa therapy.

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Conflict of Interest:

The authors declare no conflict of interest.

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5

CHAPTER FIVE

Anti-neoplastic Properties of Hydralazine in Prostate Cancer

Abstract

Prostate cancer (PCa) is a major cause of cancer-related morbidity and mortality worldwide. Although early disease is often efficiently managed therapeutically, available options for advanced disease are mostly ineffective. Aberrant DNA methylation associated with gene-silencing of cancer-related genes is a common feature of PCa. Therefore, DNA methylation inhibitors might constitute an attractive alternative therapy. Herein, we evaluated the anti-cancer properties of hydralazine, a non-nucleoside DNA methyltransferases (DNMT) inhibitor, in PCa cell lines. *In vitro* assays showed that hydralazine exposure led to a significant dose and time dependent growth inhibition, increased apoptotic rate and decreased invasiveness. Furthermore, it also induced cell cycle arrest and DNA damage. These phenotypic effects were particularly prominent in DU145 cells. Following hydralazine exposure, decreased levels of *DNMT1*, *DNMT3a* and *DNMT3b* mRNA and DNMT1 protein were depicted. Moreover, a significant decrease in *GSTP1*, *BCL2* and *CCND2* promoter methylation levels, with concomitant transcript re-expression, was also observed. Interestingly, hydralazine restored androgen receptor expression, with upregulation of its target p21 in DU145. Protein array analysis suggested that blockage of EGF receptor signaling pathway is likely to be the main mechanism of hydralazine action in DU145 cells. Our data demonstrate that hydralazine attenuated the malignant phenotype of PCa cells, and might constitute a useful therapeutic tool.

Keywords: Prostate Cancer, Hydralazine, DNA methyltransferases, Androgen Receptor

Introduction

Prostate cancer (PCa) is the second most common malignant neoplasm diagnosed in men and the sixth leading cause of cancer-related mortality worldwide [1]. Although most early-diagnosed patients have optimal survival rates after treatment with prostatectomy or radiotherapy, treatment of advanced disease is mainly ineffective and remains a clinical challenge [2, 3]. Androgen-deprivation therapy, by pharmacological or surgical castration, is widely used for locally advanced and systemically spread PCa. However, most patients that initially respond to this type of therapy have a median time for progression for castration-resistant disease of only 18-30 months [4]. Therefore, investigation of new and more effective therapeutic strategies is urgently needed for this important subset of PCa patients.

Alterations in DNA methylation are early events in human carcinogenesis, including PCa, which are conserved during cancer progression [5, 6]. Aberrant methylation of CpG dinucleotides in gene promoter regions is associated with transcriptional repression, not only of tumor-suppressor genes, but also of genes with important regulatory functions [7-10]. DNA methyltransferases (DNMTs) are a group of enzymes responsible for the establishment and maintenance of methylation patterns. Because epigenetic alterations, contrarily to genetic modifications, are reversible, inhibition of DNMTs might have the ability to overturn tumor cell phenotype, constituting an attractive therapeutic target [11-13]. So far only two nucleoside analogues, 5-azacytidine (5-Aza-CR) and 5-aza-2´-deoxycytidine (5-aza-CdR) have been approved by Food and Drug Administration (FDA) for clinical use, especifically in myelodysplastic syndrome [14]. Nevertheless, their clinical effectiveness is not entirely dependent on their DNA methylation inhibitory activity only and its efficacy in solid tumors is yet to be fully demonstrated [15]. Indeed, 5-aza-CdR was shown to have modest clinical activity against castration-resistant PCa in a phase II clinical trial [16].

Due to the toxicity of nucleoside analogues, efforts have been made to develop and test non-nucleoside compounds that target DNMTs, which are not incorporated in the DNA and/or RNA, for solid tumors. Recently, we reported RG108 as an effective tumor growth suppressor in PCa cell lines. However, this drug is substantially more effective in androgen-responsive than in castration-resistant cell lines [17]. Among non-nucleoside analogues, hydralazine, a potent arterial vasodilator approved by FDA for treatment of severe hypertension, heart failure and hypertension in pregnancy, has been described as a weak non-nucleoside DNA methylation inhibitor [18-21]. Furthermore, this drug has been shown to demethylate and reactivate the expression of several cancer-related genes

and its activity is synergistic with that of the histone deacetylase inhibitor (HDACi) valproic acid, either *in vitro* or *in vivo* [22-26]. In patients carrying solid tumors refractory to conventional treatment, clinical trials have been conducted in which those epigenetic drugs were combined with conventional therapy. It was shown that this regimen was not only well tolerated, but, importantly, it overcomed tumors' chemotherapy resistance and induced radiosensitivity [26-28].

To the best of our knowledge, the antineoplastic effect of hydralazine in PCa has not been previously investigated. Therefore, in the context of a broader research project intended to determine the therapeutic efficacy of compounds targeting epigenetic alterations of PCa cells, we aimed at evaluating the impact of hydralazine as a PCa growth inhibitor as well as its effect on DNA demethylation activity and consequent reactivation of genes known to be epigenetically silenced in this neoplasm. Additionally, we intended to investigate the cellular pathways through which hydralazine exerts its growth-inhibitory effect. We found that hydralazine was able to reverse PCa cell phenotype, decrease DNMTs expression and gene promoter methylation with concomitant expression's restoration of silenced genes involved in prostate carcinogenesis. Moreover, this compound was capable of restoring androgen receptor (AR) expression in DU145 cell line. Importantly, we found that hydralazine exerts its growth-inhibitory effects through cell cycle and EGF receptor signaling pathway inhibition.

Material and Methods

PCa cell lines and drug preparation

PCa cell lines LNCaP and PC-3 were kindly provided by Prof. Ragnhild A. Lothe from the Department of Cancer Prevention at the Institute for Cancer Research, Oslo, Norway, whereas DU145 was kindly provided by Professor Fátima Baltazar at ICVS, University of Minho, Braga, Portugal and 22Rv1 cells were kindly provided by Dr. David Sidransky at the Johns Hopkins University School of Medicine, Baltimore, MD, USA. LNCaP and 22Rv1 cells were grown in RPMI 1640, DU145 cells were maintained in MEM and PC-3 cells were grown in 50% RPMI-50% F-12 medium (GIBCO, Invitrogen, Carlsbad, CA, USA). All basal culture media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (GIBCO). Cells were maintained in an incubator at 37ºC with 5% CO_2. All PCa cell lines were karyotyped by G-banding (for validation purposes) and routinely tested for Mycoplasma spp. contamination (PCR Mycoplasma Detection Set, Clontech Laboratories). Hydralazine was purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA) dissolved in PBS (GIBCO). For control purposes, cell lines were exposed to the vehicle of the drug (PBS).

Viability assay

Cell viability was evaluated by MTT assay. Briefly, PCa cells were seeded at 5 x 10^3 cells/mL onto 96-well flat bottoned culture plates, allowed to adhere overnight and exposed to different Hydralazine concentrations (*i.e.*, 20 and 40 µM) for 14 days. At each time point 0.5 mg/ml of MTT reagent [3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] was added to each well, and the plates were incubated in the dark for 3 hours at 37ºC. Formazan crystals were then dissolved in DMSO and absorbance was read at 540 nm in a microplate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany), subtracting the background, at 630 nm. The number of cells was calculated using the formula: [(OD experiment x Number of cells at day 0) / Mean OD at day 0]. Three replicates were performed for each condition, and at least three independent experiments were carried out.

Apoptosis evaluation

Evaluation of apoptosis was performed using APOPercentage apoptosis assay kit (Biocolor Ltd., Belfast, Northern Ireland) according to the manufacturer's instructions. PCa cells were seeded at 1 x 10^4 cells/mL onto 24-well plates. Apoptotic cells were assessed at the end of the day 3, 7 and 14 for LNCaP and 22Rv1, 3 and 7 for LNCaP and 3 and 14

for PC-3, in a FLUOstar Omega microplate reader at 550 nm and the background subtracted at 620 nm. The results were normalized to number of viable cell obtained in the MTT assay according to the following formula (OD of apoptosis assay at day x/ OD of MTT at day x).

Cell cycle analysis

Cell cycle distribution of PCa cells was determined by flow cytometry. Briefly: $5x10^5$ harvested cells were fixed overnight at 4ºC with 70% cold ethanol. After washing with cold PBS, cells were ressuspended in staining Propidium Iodide Solution (Cytognos S.L, Salamanca, Spain) and incubated for 30 minutes at room temperature. All cells were then measured on a Cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) and analyzed using Modfit LT (Verity Software House, Inc, Topshan, Maine, USA).

Single Cell Gel Electrophoresis (Comet Assay)

After drug exposure, 50.000 cells were harvested by trypsinization, washed in PBS and re-suspended in 75 µl of low-melting point agarose (Invitrogen).This suspension was then applied on top of the base layer consisting of normal-melting point agarose in a slide, after which it polymerized for 10 minutes at 4ºC. The slides were then immersed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris Base and 1% Triton X-100) at 4°C during 2 hours in the dark. To allow DNA to unwinding, slides were posteriorly incubated in an alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na2EDTA, pH 13) for 40 minutes at 4ºC. Electrophoresis was accomplished on a horizontal electrophoresis platform at 4ºC for 20 minutes at 15 V. Next, they were incubated in a neutralization buffer (Tris–HCl; pH-7.5) for 10 minutes. After fixation with ethanol 100% the slides were stained with Sybr Green® (Life Technologies) and DNA damage was evaluated under a fluorescent microscope. At least three independent experiments were performed for each condition. The DNA damaging effect in terms of DNA fragmentation was determined by measuring four parameters that included, tail moment, tail length, percentage of DNA in tail of the comet and 50 DNA-damaged cells were counted at least, for each condition.

Cell Invasion Assay

Cell invasion was determined using BD BioCoat Matrigel Invasion Chamber (BD Biosciences, Franklin Lakes, NJ, USA). Briefly, $5x10^4$ cel/mL of LNCaP and DU145 cells were added to the upper chamber. Both cell lines were exposed to 20 and 40 µM of Hydralazine as well as to the vehicle for 48 hours, after which the non-invading cells were removed with cotton swabs from the upper side of the membrane. The membrane botton containing invading cells was fixed in methanol, washed in PBS and stained with DAPI

(Vector Laboratories, Burlingame, CA). All the invading cells were counted under a fluorescent microscope. Three independent experiments were performed for each condition, and at least two experimental replicates were performed.

Real Time Quantitative PCR (RT-qPCR)

After drug exposure RNA was extracted from cell lines using TRIzol® (Invitrogen) according to manufacturer's instructions. First strand synthesis was performed using the high-capacity cDNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA, USA). Expression of target genes (*Ki67*, *CDKN1A*, PARP1, *APC, RARβ2*, *GSTP1, CCND2, DNMT1, DNMT3a and DNMT3B*) was quantified using Taqman expression assays, purchased as pre-developed assays from Applied Biosystems and normalized to the expression of the *GUSB* housekeeping gene.

Determination of differentially expressed target genes by real-time ready arrays

After RNA extraction, 500 ng of total RNA was used for cDNA synthesis using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Applied Science) according to the manufacturer's instructions. A 384-well custom array panel (Roche Applied Science) was designed to quantify the expression of genes involved in multiple cellular pathways, namely apoptosis, cell cycle, DNA repair and invasion by real-time PCR using a LightCycler 480 instrument (Roche Diagnostics). The relative expression of target genes was normalized using *GUSB*, *TFRC* and *18S* as housekeeping genes. The fold-variation in gene expression was determined using comparative Ct method and genes with fold change above 0.5 or below -0.5 were considered up or downregulated, respectively.

Western Blot

Proteins were extracted from whole-cell lysates using RIPA lysis buffer (Santa Cruz Biotechnology, CA, USA) and subsequently quantified using a Pierce BCA assay (Thermo Scientific Inc., Bremen, Germany), according to the manufacturer's instructions. Briefly, 30 µg of protein from each sample were separated using 4–20% Mini-PROTEAN® TGX™ Precast Gel at 120 V and subsequently blotted onto Protran nitrocellulose transfer membranes (Whatman, Dassel, Germany). For immunodetection, membranes were incubated overnight at 4ºC with antibodies directed against acH_3 (Millipore), AR, DNMT1, p21 and PARP1 at 1/500, (Cell Signaling Technology, Danvers, MA). The Immun-Star WesternC Chemiluminescent kit (Bio-Rad, Hercules, CA, USA) was used to develop the membranes which were then recorded with Amersham Hyperfilm (GE Healthcare Buckinghamshire, UK). To ascertain equal loading of protein, the membranes were incubated with a monoclonal mouse antibody against β-Actin (1:8,000, Sigma-Aldrich).

Protein band intensities were determined using Quantity One software (Bio-Rad), by comparing the protein band intensity with the loading control (β-Actin).

Quantitative Methylation Specific PCR (qMSP)

Genomic DNA was extracted from cell lines using a standard technique comprising digestion with proteinase K (20mg/mL) in the presence of 10% SDS at 55ºC, followed by phenol-chloroform extraction and precipitation with 100% ethanol [29]. One microgram of DNA was submitted to bisulfite modification using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA) following the manufacturer's instructions. Bisulfite modified DNA was amplified by qMSP using TaqMan technology [30]. Specific *GSTP1*, *APC* and *RARβ2* primers and TaqMan probes were designed using the Methyl Primer Express Software v1.0 (Applied Biosystems). *β-actin* (*ACTB*) was used as an internal reference gene to normalize for DNA input and all qMSP reactions were performed as previously described. [8] Methylation levels for each sample were derived from calibration curves constructed using serial dilutions of bisulfite modified CpGenomeTM Universal Methylated DNA (Millipore, Billerica, MA). *GSTP1*, *APC* and *RAR2β* methylation levels were calculated after normalization for *ACTB.*

Kinexus Antibody Array

The Kinex™ KAM-850 antibody microarray was used to perform an unbiased characterization of altered proteins after hydralazine exposure. In total, this microarray featured 854 antibodies of which 517 were pan-specific (for protein expression) and 337 were protein phospho-site-specific (for phosphorylation). Briefly, 50 µg of lysate protein from each sample were covalently labeled with a fluorescent dye and unincorporated dye molecules were removed by ultrafiltration. Purified proteins were then incubated on the antibody microarray. After the washing steps, the arrays were sent to Kinexus and were scanned using a Perkin-Elmer Scan Array Reader (Waltham, MA). Signal quantification was performed with ImaGene 8.0 (BioDiscovery, El Segundo, CA) with predetermined settings for spot segmentation and background correction. Z ratio analysis was used to identify differentially expressed proteins between the vehicle and hydralazine treated cells. Three independent replicates were performed for each condition. Functional annotation of the identified proteins was carried out with the online tool DAVID Bioinformatic Resources 2013 (version 6.7) (http://david.abcc.ncifcrf.gov/) [31, 32].

Statistical analysis

One-way analysis of variance (ANOVA), with post-hoc Dunnet's multiple comparison test, when appropriate, was used to compare the results obtained in each

parameter for the two different concentrations of hydralazine and the vehicle. Analysis was performed with SPSS software for Windows version 19.0 (IBM-SPSS Inc Chigaco, IL) and statistical significance was set at $p<0.05$.

Results

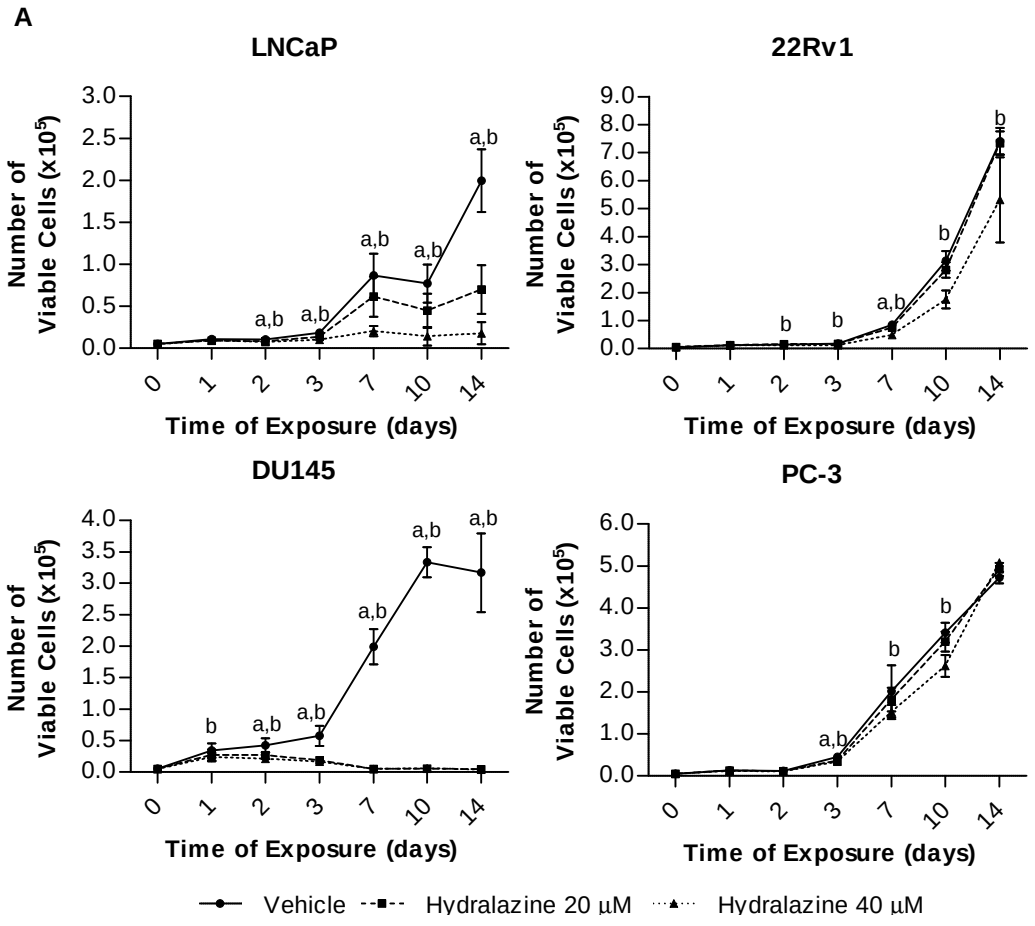
Hydralazine reverts PCa cells malignant phenotype

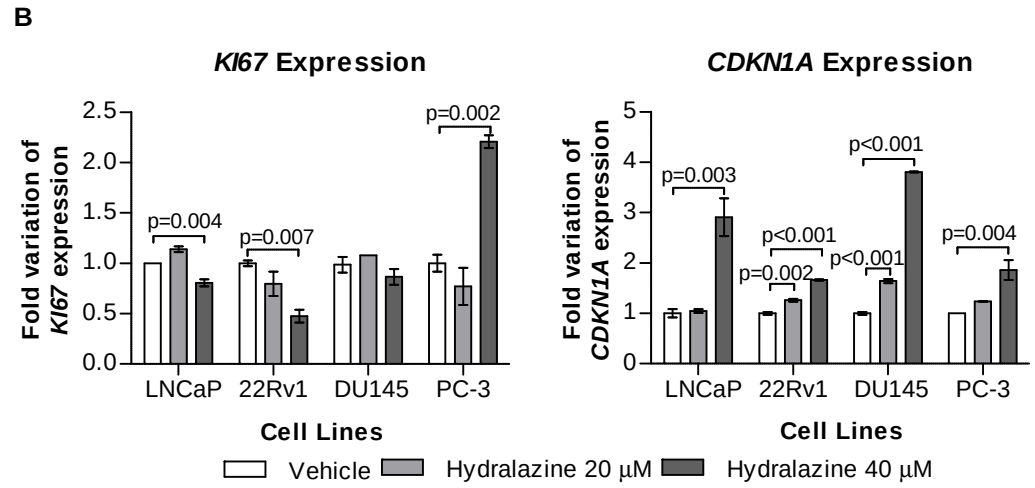
The half-maximal effective concentration (EC50) of hydralazine was calculated in two PCa cell lines (LNCaP and DU145) after 72 hours of drug exposure. The drug displayed an EC50 of 63 μM in LNCaP and 30 μM in DU145 (Supplementary Figure 1).

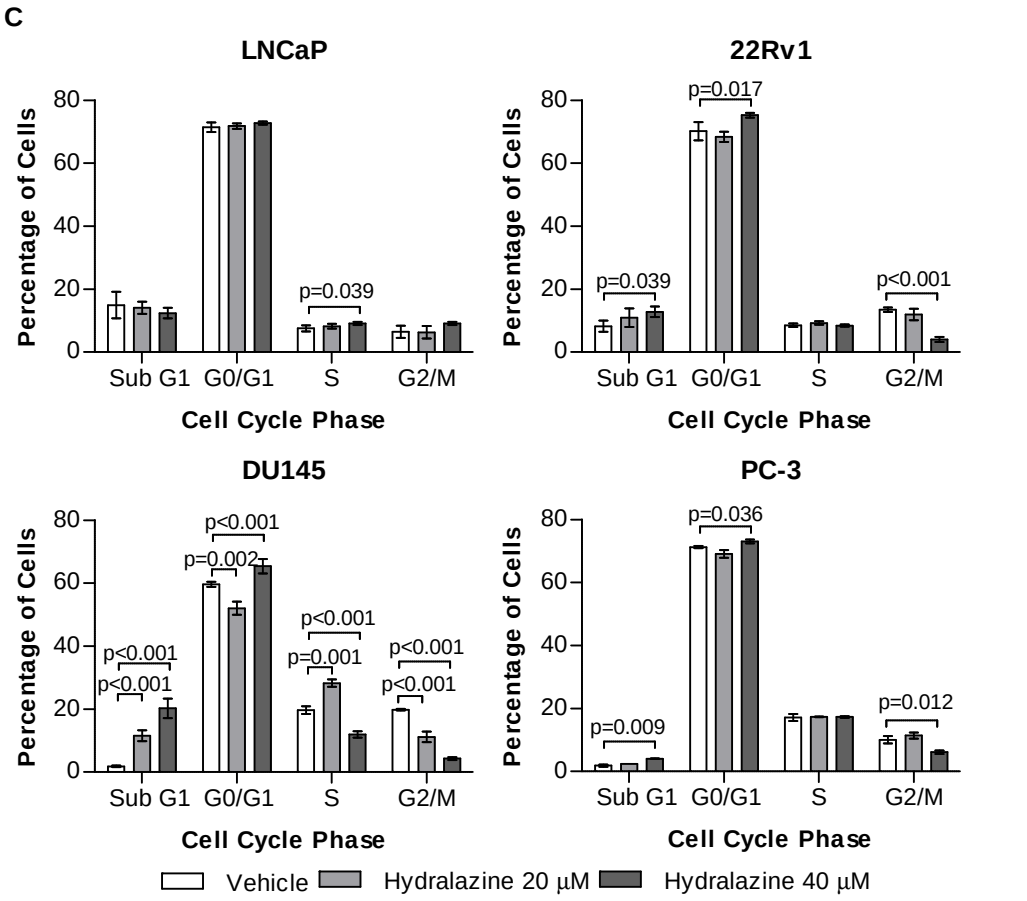
To investigate the effects of hydralazine on the malignant phenotype of PCa, four human PCa cell lines (LNCaP, 22Rv1, DU145, and PC-3) were exposed to two different concentrations of this drug (20 and 40 μM) or to the vehicle (PBS) during 14 days, as previously described for RG108 [17]. Cell viability was evaluated at days 0, 1, 2, 3, 7, 10, and 14. Exposure to hydralazine markedly reduced cell viability, especially at 40 μM concentration (Figure 1A). Remarkably, a significant decrease in the number of viable cells was observed at the day 2, with a more pronounced effect after 14 days of exposure to both drug concentrations in LNCaP, whereas for 22Rv1 a less impressive effect was observed, even after exposure to the highest concentration. PC-3 treated cells also depicted a slight reduction at the end of day 3 through day 10. Interestingly, DU145 was the most sensitive cell line, since inhibition of cell viability was achieved in all tested days for both drug concentrations. These results were corroborated by mRNA expression levels of two genes involved in cell proliferation. Due to its low viability rate, this cell line was evaluated after three days of drug exposure. Indeed, a significant induction of *CDKN1A* and decrease of *Ki67* mRNA levels was observed in hydralazine treated cells compared to the respective vehicle (Figure 1B). Additionally, alterations in cell cycle distribution were evaluated and a significant cell cycle arrest was observed at G0/G1 for all cell lines, except for LNCaP in which the arrest was observed at S phase (Figure 1C).

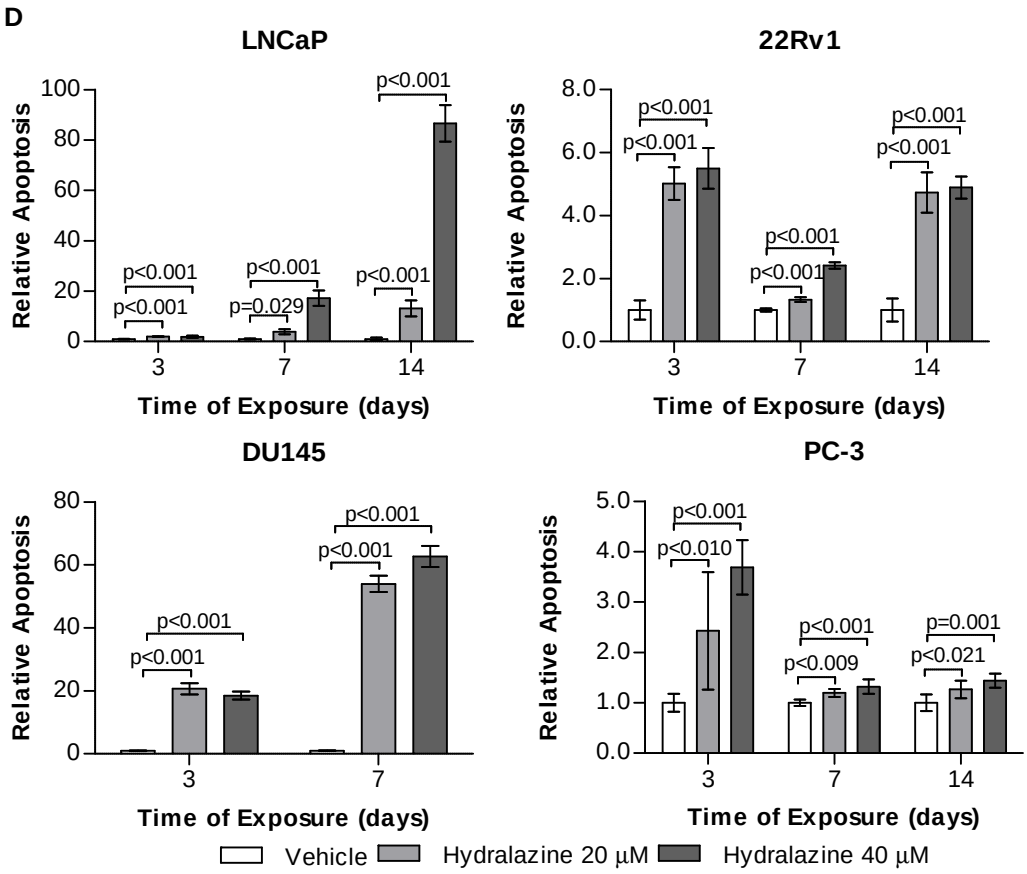
A significant increase in apoptosis was depicted for all cell lines with both drug concentrations (Figure 1D). To confirm the activation of the apoptotic pathway, *CASP8, CASP9,* and *CASP3* mRNA levels were also evaluated. A statistically significant increase in transcript levels of *CASP3* and *CASP8* was found for DU145, and LNCaP, respectively, whereas *CASP9* expression levels were increased in LNCaP and 22Rv1 cells (Figure 1E). Furthermore, a significant increase in Sub-G1 cell population was observed for three of the cell lines [22Rv1, DU145 and PC-3 (Figure 1C)].

The two best responsive PCa cell lines, DU145 and LNCaP, also showed a significant decrease in invasion ability after 48 hours exposure to hydralazine (Figure 1F).


A
LNCaP
22Rv1
DU145
PC-3
Number of Viable Cells (x10^5)
Time of Exposure (days)
Vehicle
Hydralazine 20 µM
Hydralazine 40 µM


B
KI67 Expression
CDKN1A Expression
Fold variation of KI67 expression
Fold variation of CDKN1A expression
p=0.004
p=0.007
p=0.002
p=0.003
p<0.001
p=0.002
p<0.001
p<0.001
p=0.004
LNCaP
22Rv1
DU145
PC-3
Cell Lines
Vehicle
Hydralazine 20 µM
Hydralazine 40 µM


C
LNCaP
22Rv1
DU145
PC-3
Percentage of Cells
Sub G1
G0/G1
S
G2/M
Cell Cycle Phase
p=0.039
p=0.017
p<0.001
p=0.002
p=0.001
p=0.036
p=0.009
p=0.012
Vehicle
Hydralazine 20 μM
Hydralazine 40 μM


D
LNCaP
22Rv1
DU145
PC-3
Relative Apoptosis
Time of Exposure (days)
p<0.001
p=0.029
p<0.010
p<0.009
p=0.001
p<0.021
Vehicle
Hydralazine 20 μM
Hydralazine 40 μM

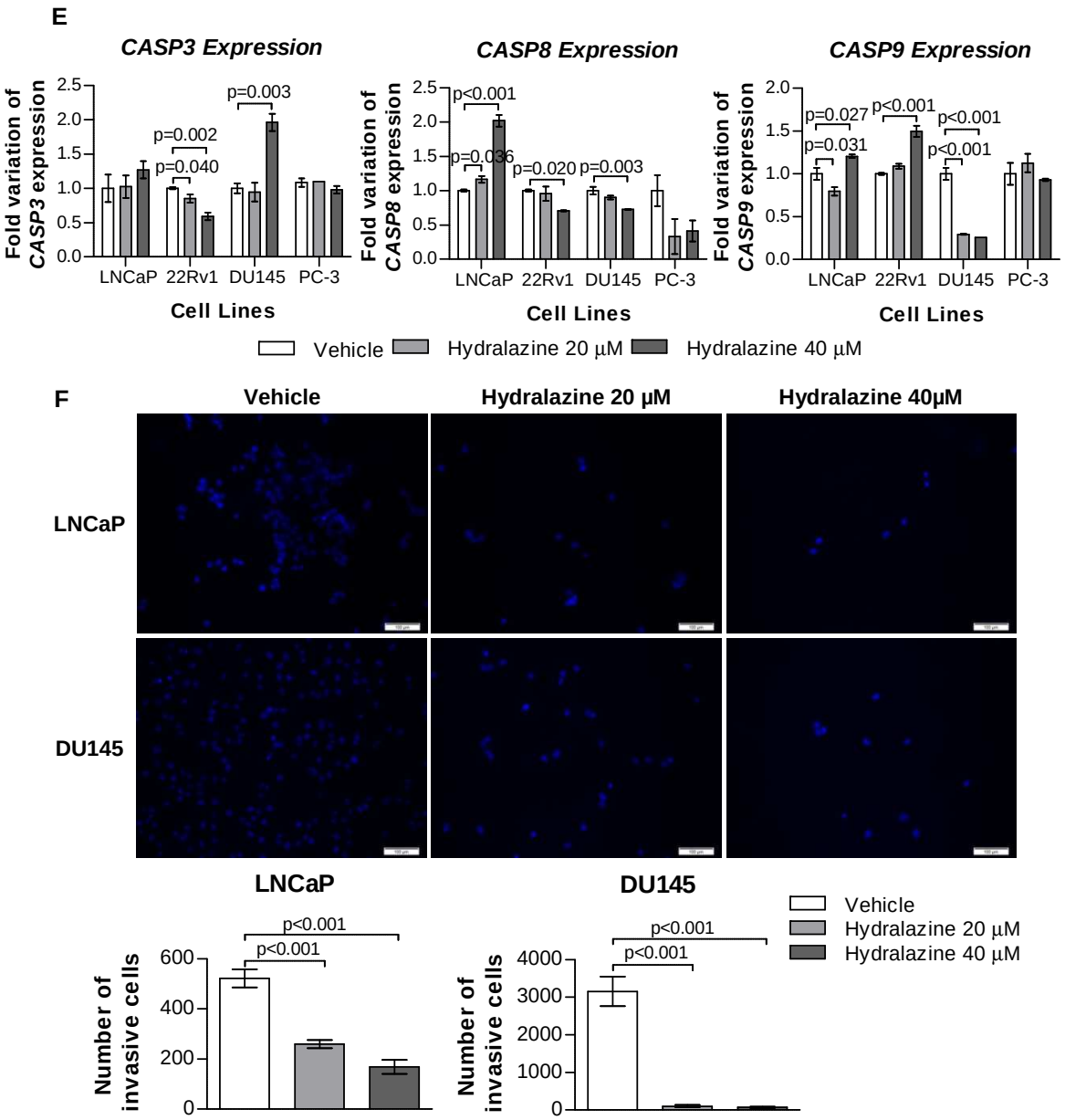


Figure 1 – Phenotypic effects induced by hydralazine in PCa cell lines. (A) Cell viability in LNCaP, 22Rv1, PC-3 and DU145, exposed to hydralazine and drug vehicle, at days 0, 1, 2, 3,7,10, and 14, measured by MTT assay. (a) Statistically significant differences were observed between vehicle and 20 µM hydralazine, and (b) vehicle and 40 µM hydralazine. (B) mRNA expression of *Ki67* and *CDKN1A* in LNCaP, 22Rv1 and PC-3 after 14 days and DU145 after 3 days of exposure to hydralazine. (C) Cell cycle evaluation after 14 days of hydralazine exposure in LNCaP, 22Rv1 and PC-3 and three days of hydralazine exposure in DU145. (D) Effect of hydralazine exposure in early apoptosis of PCa cell lines, measured at days 3 and 7 in all PCa cell lines and at day 14 in LNCaP, 22Rv1 and PC-3, with a phosphatidylserine based assay. (E) *CASP3*, *CASP8* and *CASP9* mRNA expression in LNCaP, 22Rv1 and PC-3 after 14 days and DU145 after 3 days of exposure to hydralazine. (F) Effect of hydralazine exposure on the invasion potential of LNCaP and DU145 (upper – immunofluorescence images of vehicle and hydralazine exposed cells, counterstained with DAPI; down – graphic representation of the total number of invasive cells). All data are presented as mean of three independent experiments ± s.d.

Hydralazine disrupts the expression of cell cycle genes

A panel of genes representative of critical cellular pathways were selected for assessment of expression in LNCaP and DU145 (Figure 2). Globally, cell cycle-associated genes were upregulated and comparative analysis between the most altered genes in two cell lines, allowed for the identification of five upregulated (*CCND2*, *ELL2*, *BAX*, *CCND3* and *CCNE1*) and five downregulated (*BIRC5*, *PARP1*, *CCNB1*, *BID* and *TET1*) genes.

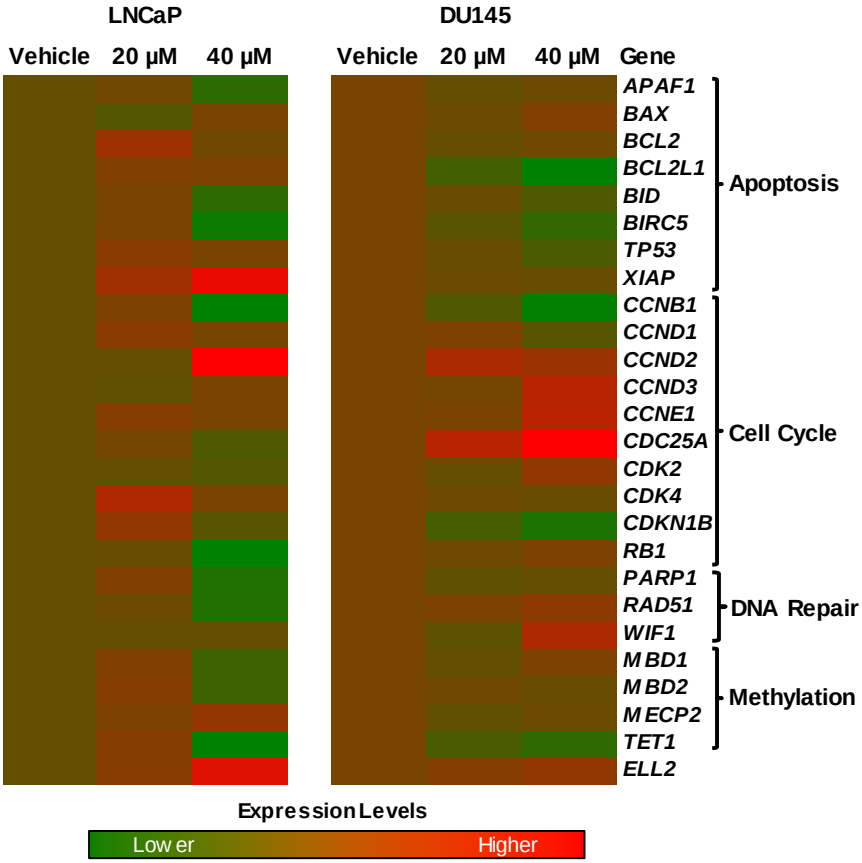


Figure 2 - Effects of hydralazine in cellular pathways (a) Image of deregulated genes after exposure of LNCaP (left) and DU145 (right) to 20 and 40 µM hydralazine. Induced in gene expression is represented in red, while decreased expression is shown in green. Real-time RT-PCR values were normalized to three reference genes (*18S*, *TFRC* and *GUSB*). All experiments were performed in triplicate.

Hydralazine induce DNA Damage in PCa Cell Lines

PCa cells exposed to hydralazine showed significant DNA damage compared to those exposed to the vehicle, as depicted by the increase in tail length of the comet (Figure 3A). Remarkably, an increase in protein expression of cleaved PARP1, a protein involved in DNA repair, was found in 22RV1 whereas a decrease was displayed by LNCaP. No changes in expression of this protein were observed in DU145 and PC-3 cells (Figure 3B).

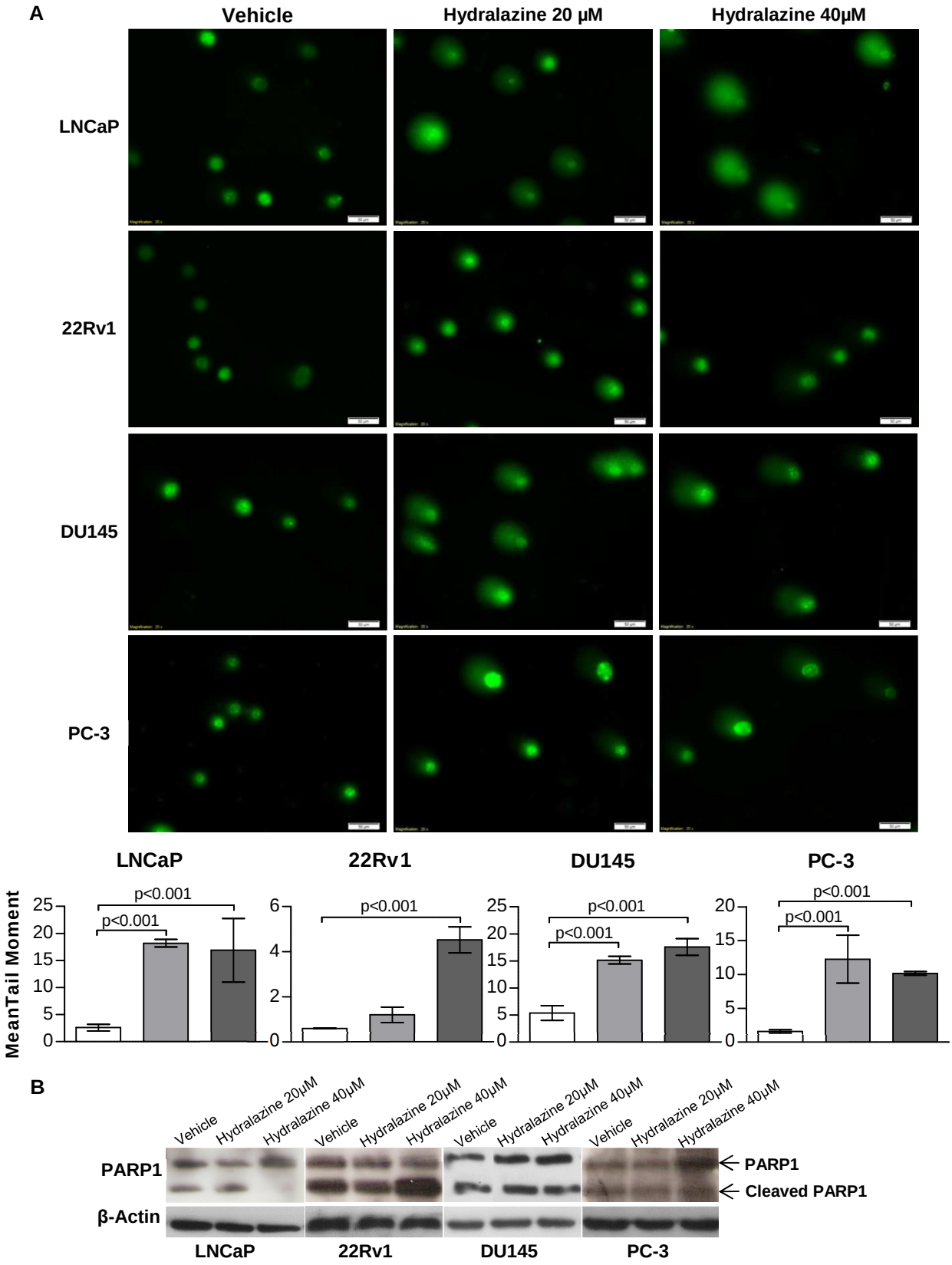
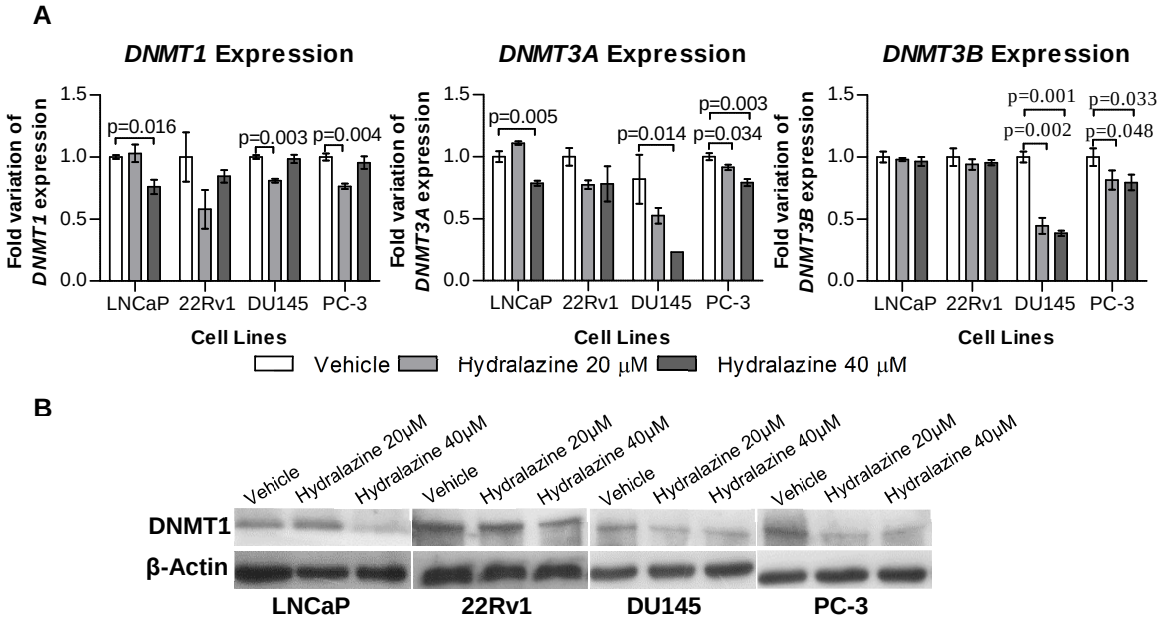
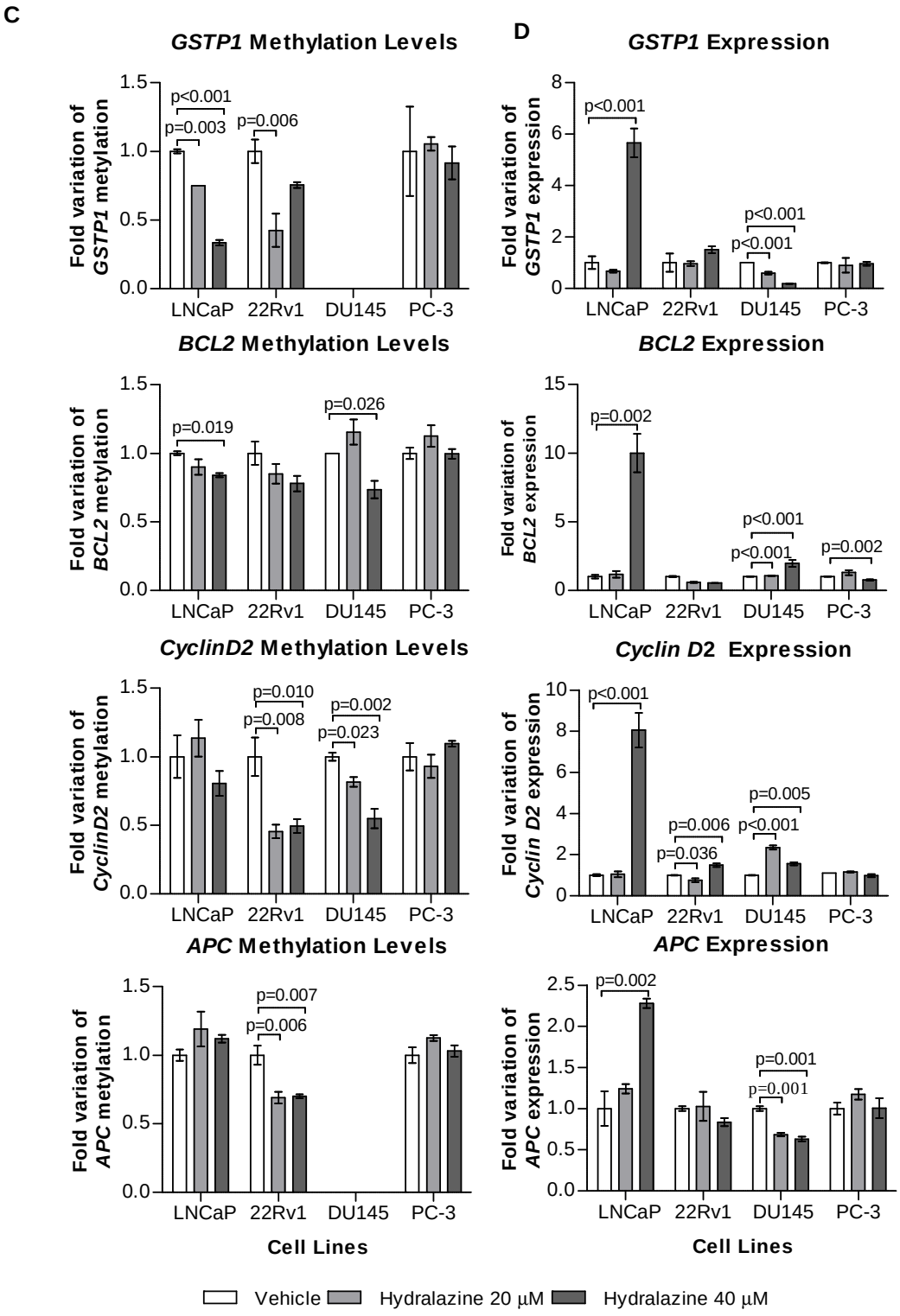


Figure 3 – Hydralazine effect on DNA damage. (A) Upper - Comet assay immunofluorescence images of vehicle and hydralazine exposed cells counterstained with Syber Green and down - graphic representation of mean tail moment (B) Western Blot for PARP1 protein expression. Full length PARP1 is represented by a band of 116 kDa and cleaved PARP1 is represented by a 89 kDa band. β-Actin was used as a loading control.

Hydralazine decreases DNMTs expression and leads to demethylation and reactivation of silenced genes in human PCa cells

A reduction in mRNA expression for all three DNMTs was depicted in DU145 and PC-3 cells. A significant decrease in *DNMT1* was achieved after exposure to 20 μM hydralazine in *DNMT3a*, after 40 μM in DU145 and with both drug concentrations in PC-3; as well as for *DNMT3b* with both concentrations in both cell lines. LNCaP only showed decreased levels of *DNMT1* and *DNMT3a* when exposed to 40 μM hydralazine (Figure 4A). Notably, a reduction in DNMT1 protein expression was observed for all tested cell lines (Figure 4B). Gene promoter methylation status and expression levels of five genes (*GSTP1, APC, RARβ2, CCND2* and *BCL2*), previously reported to be silenced by promoter hypermethylation in PCa, was evaluated. Both LNCaP and 22Rv1 showed a significant decrease in methylation levels of *GSTP1* with 20 μM whereas only LNCaP exhibited similar results with 40 μM (Figure 4C). Despite both cell lines presented a trend for increase *GSTP1* mRNA expression when exposed to 40 μM hydralazine, only in LNCaP statistical significance was observed (Figure 4D). DU145 cells did not show significant *GSTP1* promoter methylation. Concerning *APC* and *RARβ2,* only 22Rv1 demonstrated a significant decrease in promoter methylation levels (Figure 4C). Interestingly, significant re-expression of those genes was only achieved in LNCaP and DU145 (Figure 4D). *BCL2* methylation levels were reduced in LNCaP and DU145 (Figure 4C) but mRNA re-expression was observed following exposure to 40 μM hydralazine (Figure 4D). Although a significant reduction in *CCND2* methylation was observed only in 22Rv1 and DU145 with both drug concentrations (Figure 4D), gene re-expression was also achieved in LNCaP (Figure 4D).




C
GSTP1 Methylation Levels
Fold variation of GSTP1 metylation
p<0.001
p=0.003
p=0.006
BCL2 Methylation Levels
Fold variation of BCL2 metylation
p=0.019
p=0.026
CyclinD2 Methylation Levels
Fold variation of CyclinD2 metylation
p=0.010
p=0.008
p=0.002
p=0.023
APC Methylation Levels
Fold variation of APC metylation
p=0.007
p=0.006
LNCaP
22Rv1
DU145
PC-3
Cell Lines
D
GSTP1 Expression
Fold variation of GSTP1 expression
p<0.001
p<0.001
p<0.001
BCL2 Expression
Fold variation of BCL2 expression
p=0.002
p<0.001
p<0.001
p=0.002
Cyclin D2 Expression
Fold variation of Cyclin D2 expression
p<0.001
p=0.005
p=0.006
p<0.001
p=0.036
APC Expression
Fold variation of APC expression
p=0.002
p=0.001
p=0.001
Vehicle
Hydralazine 20 μM
Hydralazine 40 μM

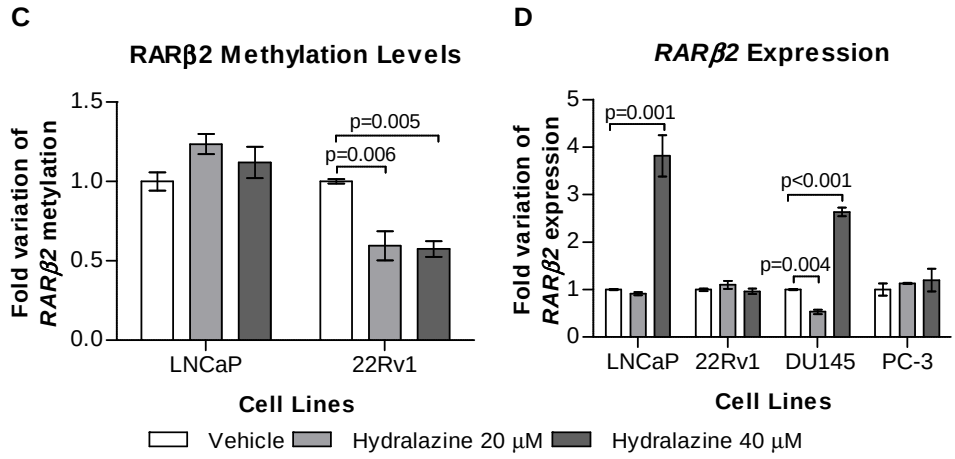


Figure 4 – Impact of hydralazine on specific gene methylation of PCa cells. (A) Real-time RT-PCR of mRNA levels of *DNMT1*, *DNMT3a* and *DNMT3b* normalized to *GUSB* in vehicle and drug exposed cells. (B) Western blot analysis of DNMT1 protein expression. β-Actin was used as a loading control. (C) Real-time methylation-specific PCR (qMSP) for assessment of *GSTP1*, *APC, RARβ2, CCND2* and *BCL2* methylation levels, normalized to β-Actin. (D) mRNA expression of *GSTP1*, *APC, RARβ2, CCND2* and *BCL2,* by real-time RT-PCR, in both hydralazine and vehicle exposed cells. All data are presented as mean of three independent experiments ± s.d.

Hydralazine induces histone acetylation in LNCaP and DU145 cell lines

Since hydralazine induced re-expression of *CCND2, RARβ2* and *APC* in LNCaP and *RARβ2* in DU145, without a concomitant significant reduction in DNA methylation, and because histone acetylation interplays with DNA methylation, the acetylation of Histone 3 (AcH_3) was evaluated by western blot in those two cell lines. Interestingly, exposure to both concentrations of hydralazine increased AcH_3 expression in LNCaP and DU145 (Figure 5).

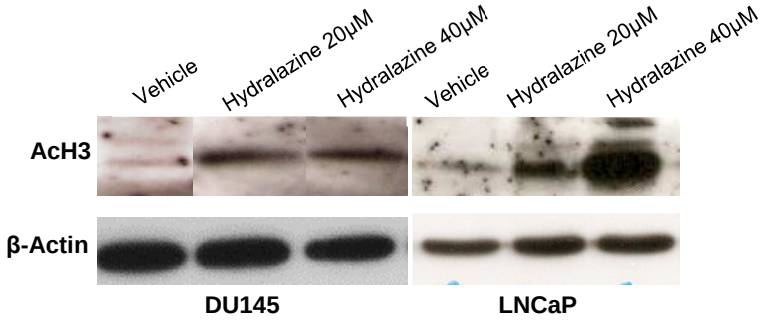
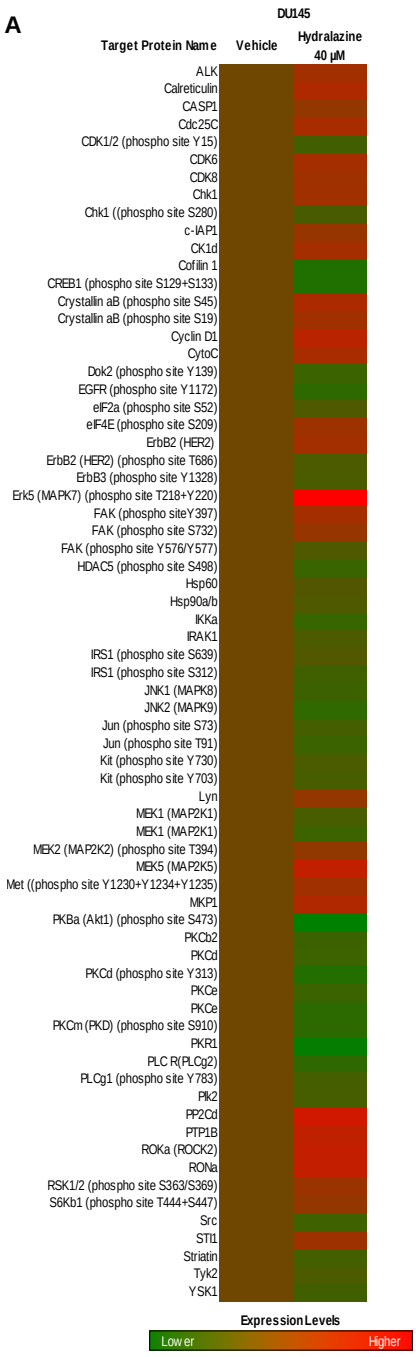


Figure 5 – Effect of hydralazine on histone 3 acetylation (AcH3). Western Blot analysis of AcH3, in LNCaP (after 14 days of drug exposure) and DU145 (after 3 days of drug exposure). β-Actin was used as a loading control.

Hydralazine may exert its growth-inhibitory effect in DU145 cells by interfering with EGF signaling pathway

Because DU145 showed the largest sensitivity to hydralazine, it was chosen to investigate the cellular mechanisms and pathways underlying these pronounced effects, using a protein array. After drug exposure, 69 proteins presented altered expression, of which 40 were considered downregulated and 29 upregulated (Figure 6A). These 69 proteins were then analyzed with DAVID (Database for Annotation, Visualization and Integrated Discovery) and several cellular networks were identified as being altered after drug exposure (Figure 6B). However, the pathway that scored the best significance value was the EGF receptor signaling pathway (p-value = 10^{-8}).



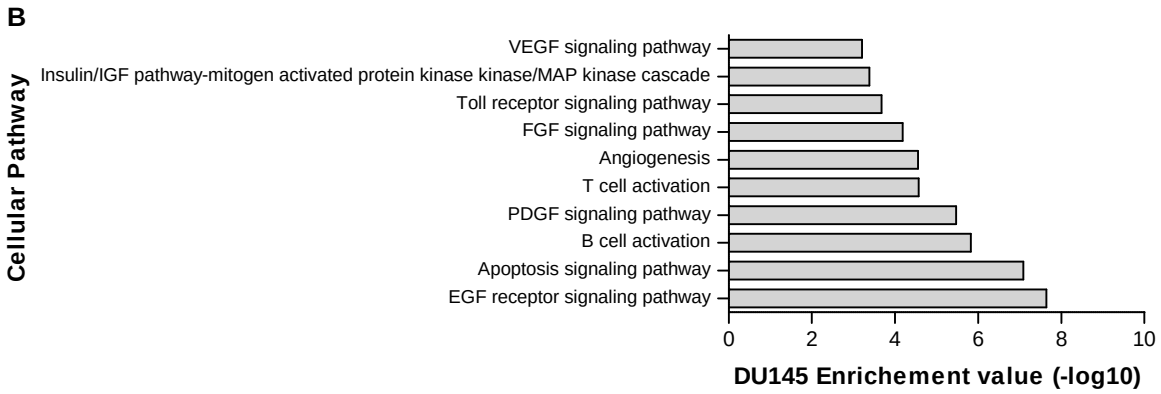


Figure 6 – EGF signaling pathway is deregulated in DU145 cells after hydralazine exposure. (A) Image of deregulated proteins after exposure of DU145 cell to 40 µM hydralazine. Protein overexpression is shown in red, whereas protein reduced expression is shown in green. Experiment was performed in triplicate. (B) Altered cellular pathways identified with DAVID software. EGF receptor pathway showed the lowest p value (10^{-8}).

Hydralazine restores Androgen receptor expression in DU145 cell line

It is widely acknowledged that one of the mechanisms of androgen receptor (AR) silencing in castration-resistant cell line DU145 is promoter hypermethylation [33]. After three days of exposure to 40 µM of hydralazine a significant reduction in *AR* methylation levels was observed (Figure 7A) with concomitant AR protein re-expression, at both drug concentrations (Figure 7B). Re-expression was more evident when cells were exposed to 20 µM hydralazine. To further demonstrate AR activation, an increase in p21 protein expression was detected after drug exposure (Figure 7B), which is in accordance with the reported ability of AR to upregulate p21 in PCa cell lines [34].

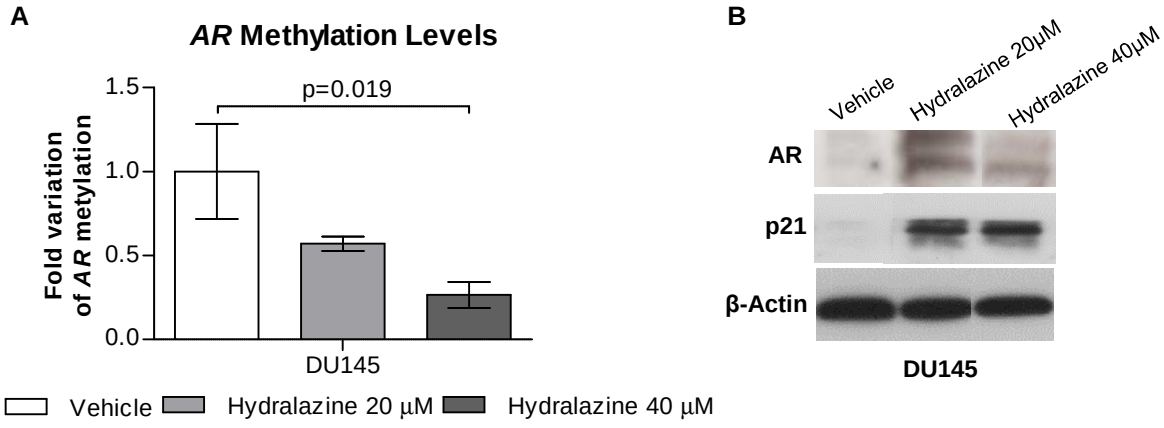


Figure 7 – Reactivation of androgen receptor (AR) expression upon exposure to hydralazine. (A) Promoter methylation levels of AR in DU145 cell line assessed by real-time methylation-specific PCR (qMSP) after 3 days of drug exposure. AR methylation levels were normalized to β-Actin. Data are presented as mean of three independent experiments ± s.d. (B) AR and p21 protein expression determined by western blot, after 3 days of hydralazine exposure. β-Actin was used as loading control.

Discussion

Although most diagnosed PCa remain localized, hardly threatening life expectancy, about one third of those tumors metastasize to distant organs and eventually cause patient death. Median survival for patients with localized PCa is more than five years compared with one to three years for patients with metastatic disease. Although first line therapy for metastatic PCa is pharmacological or surgical castration, most patients become androgen refractory within a relatively short period of time [3, 35, 36]. Available therapies for castration-resistant PCa only aim to reduce symptoms and improvement in overall survival in only about 2 months [2]. Therefore, new therapeutic options for this disease stage are mandatory. Cancer-related gene promoter methylation is an emerging molecular marker in PCa and at least 100 genes involved in several cellular functions are *de novo* methylated during prostate carcinogenesis [37]. Therefore, DNMTi might be a valuable therapeutic tool for PCa. The two FDA approved nucleoside DNMTi, however, are of limited effectiveness in solid tumors, including PCa [15, 16]. This might be due to limited incorporation into PCa cells, which are much less proliferative, compared to hematological malignancies. This might be overcome with the use of non-nucleoside DNMTi, which are independent of replication for incorporation into DNA.

Herein, we report, for the first time, that hydralazine, a non-nucleoside DNMTi, has anti-tumoral effect in PCa cells. This compound reduced cell viability and induced cell death by apoptosis in PCa cell lines in a dose and time-dependent manner, being 40 µM the best drug concentration in all tested cell lines. Similar results were observed for human cervical cancer cells when exposed to 40 µM, resulting in 40 to 50% of cell growth inhibition and induction of apoptosis, without affecting normal cells [23]. Importantly, both inhibition of cell viability and apoptosis induction were confirmed at molecular level, the former by a significant decrease in the cell proliferation marker *Ki67* and an increase in *CDKN1A*, a gene that codifies p21 a well-known cell cycle inhibitor, and the latter by induction of caspases' expression. Furthermore, our data also show that hydralazine induces cell cycle arrest at G0/G1 in three PCa cell lines, which is in accordance with a previous report in bladder cancer [22]. Moreover, LNCaP cells also demonstrated cell cycle arrest at S phase after hydralazine exposure, similar to human cervical cancer cells [23]. Importantly, hydralazine drastically reduced the invasive potential of PCa cells, a feature that is particularly relevant because metastatic spread is a major cause of PCa morbidity and mortality.

Interestingly, exposure of PCa cell lines to hydralazine resulted in significant induction of DNA damage, accompanied by significant PARP-1 decrease in LNCaP cells.

The lack of efficient DNA repair is often an advantage for cancer therapy (especially radio- and chemotherapy), based on the concept of synthetic lethality, which states that cancer cells are more proliferative and thus more susceptible to induced DNA damage than normal cells. In fact, DNA repair is considered a mechanism of cancer-therapy resistance [38]. Currently, several agents capable of inhibiting PARP-1 are under test and those drugs might be useful as chemosensitizers for cancer therapy [39]. Taking in consideration our results is tempting to speculate whether hydralazine might be useful as a chemosensitizer, acting in concert with conventional therapeutic strategies.

Remarkably, hydralazine was not only able to inhibit *DNMT1, 3a and 3b* mRNA expression in three of the four tested cell lines, but also of DNMT1 protein expression in all cell lines. The ability of hydralazine to reduce DNMTs expression had already been described in other tumor models [28, 40]. Several studies demonstrated that DNMT1 is overexpressed in human PCa tissues, compared to normal prostate, leading to deleterious inactivation of tumor-suppressor genes and might be associated with tumor progression and poor prognosis [41, 42]. Therefore, the reduction of DNMTs expression might be related with the observed reversal of *GSTP1*, *BCL2* and *CCND2* promoter hypermethylation with concomitant re-expression upon hydralazine exposure. Interestingly, hydralazine's ability to demethylate the promoter region of tumor-suppressor genes, including *GSTP1, RARβ2* and *p16,* causing re-expression has been documented in several cancers models [22, 28]. However, *CCND2*, *RARβ2, APC* and *AR* gene re-expression was achieved without significant promoter demethylation in our study. It is well established that epigenetic silencing of cancer-related genes is a complex phenomenon, in which other mechanisms, such as histone post-translational modifications are also implicated, in addition to promoter methylation [37]. Thus, the observed increase in AcH3 following hydralazine exposure might have contributed to CCND2 and AR re-expression. Indeed, we found that hydralazine restored AR mRNA and protein expression in DU145 cells, in which AR is silenced due to promoter hypermethylation [33]. Similar results were reported for estrogen receptor (ER) protein in the ER negative MDA -231 breast cancer cell line [22]. Interestingly, AR re-expression has been also achieved following chronic exposure (20 days) to 5-Aza-CR, resulting in a marked decrease of tumor cell proliferation and a significant increase of AR and PSA protein levels [43]. The lack of AR expression has been associated with increased invasive properties, suggesting that therapies able to restore its expression receptor may reduce tumor dissemination [44, 45]. Therefore, the restoration of AR expression by hydralazine might be associated with the observed reduction in the invasive potential of DU145 cells, and could also justify the remarkable decrease in cell proliferation by the upregulation of its inhibitor, p21. Hence, it is suggested that patients with castration-resistant PCa might become more responsive to

hormone therapy following hydralazine administration, thus slowing PCa progression. Remarkably, the combination of 5-aza-CdR with the anti-androgen bicalutamide demonstrated a synergistic effect, increasing the susceptibility of PCa cells to undergo apoptosis and repressing tumor growth in xenograft mice [43, 46]. Recently, high expression levels of DNMT3a and DNMT3b have been associated with bicalutamide resistance and, consequently, with the emergence of the castration-resistant phenotype [47]. Because hydralazine was shown to decrease all DNMTs expression in DU145, a synergistic action between this drug and conventional anti-androgenic therapy is likely to exist.

The DU145 cell line was shown to be very responsive to hydralazine because unlike others cell lines, which required 14 days of drug exposure to achieve pronounced phenotypic effects, it demonstrated maximal anti-cancer effects upon only three days of exposure. Based on the analysis of a selected panel of 854 proteins we suggest that the remarkable effects of hydralazine on DU145 cells are mostly due to disruption of EGF receptor signaling pathway. EGFR is overexpressed in PCa and plays an important role in cancer cell growth, especially during androgen withdrawal [48, 49]. Due to the important role of EGFR in prostate carcinogenesis, it has been suggested that a general inhibition of this pathway could provide therapeutic advantage against metastatic PCa [50, 51]. Interestingly, we observed downregulation of several proteins involved in EGF pathway after hydralazine exposure (Figure 8). Reduced expression of the phosphorylated form of EGFR might justify the decreased expression of its direct targets, Src, Akt and MEK1/2. Src has been described to be highly expressed in PCa, which may lead to JNK and C-Jun activation and, subsequently, to an increase in motility and invasion potential of PCa cells. Furthermore, it may activate MEK1/2 and, consequently, Erk1/2, increasing cell proliferation [52, 53]. This MAP kinase pathway can also be directly activated by EGFR phosphorylation. Interestingly, the synergism between SRC and EGFR in PCa may be associated with a more aggressive tumor phenotype [54]. Moreover, Akt phosphorylation has been associated with PCa progression and it was suggested as an excellent biomarker for biochemical recurrence [55]. Importantly, through the interaction with EGF pathway, hydralazine may disrupt several cancer cell networks: by means of SRC, it may lead to a decrease in motility and invasion of tumor cells, through AKT, it may downregulate neoangiogenesis and increase apoptosis, and, finally, through, MAP kinase pathway, it may decrease tumor cell proliferation.

Because, hydralazine can maintain gene re-expression for longer periods of time, it was suggested that it has higher demethylase activity and, therefore, more effectiveness than 5-aza-CdR [21]. Thus, hydralazine might be more attractive from a clinical standpoint because to keep tumor-suppressor genes demethylated, cancer cells need to be

chronically exposed to DNMTi. Because 5-aza-CdR is incorporated into the DNA, a widespread loss of DNA methylation may occur, resulting in genome hypomethylation, which may ultimately predispose to neoplastic transformation of normal cells [56, 57]. On the contrary, hydralazine inhibits DNA methylation by establishing highly stable interactions between its nitrogen atoms and DNMT active site [58]. Moreover, hydralazine has been widely used as an anti-hypertensive agent, orally administered on a daily basis for long periods of time with minimal secondary effects. Thus, compared to FDA-approved 5-aza-CdR, hydralazine demonstrates a safer profile and might be more attractive for clinical use.

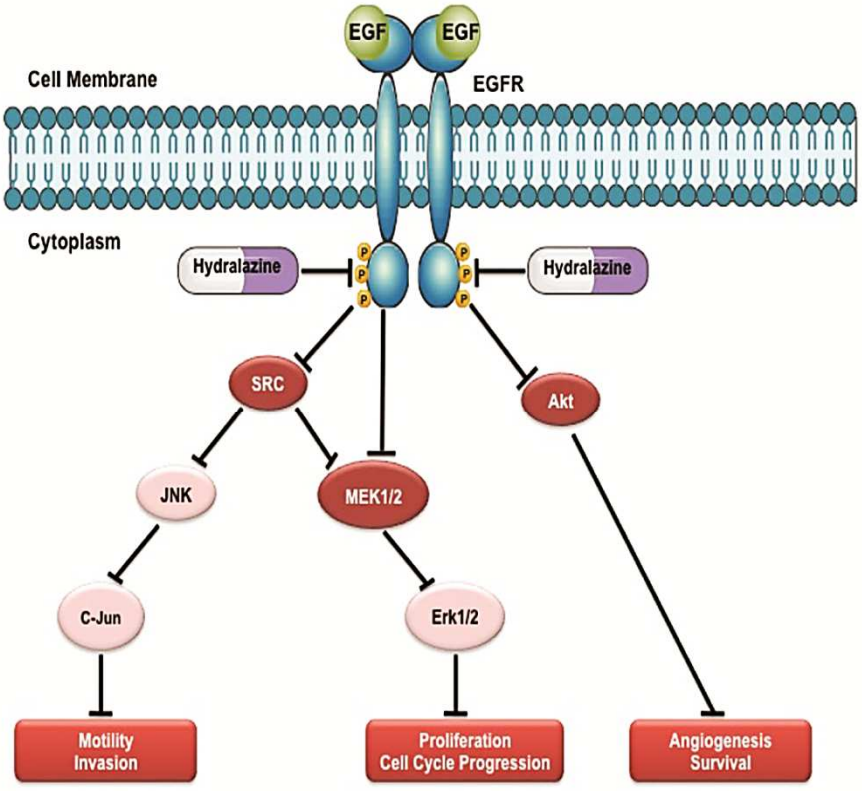


Figure 8 – Proposed model for hydralazine disruption of EGF receptor pathway in DU145 cell line. Exposure of PCa cells to hydralazine induces a significant decrease in EGFR phosphorylation and, consequently, in it targets, namely, SRC, MEK1/2 and Akt. Reduced expression of SRC may cause a decrease in JNK and C-Jun proto-oncogene leading to decreased motility and invasion capacity of PCa cells. Moreover, a decrease in MAPK pathway induced by SRC and/or directly via EFGR impairment, could explain the decrease in proliferation and cell cycle arrest observed in this cell line after hydralazine exposure. Finally, a decrease in Akt expression may lead to cell death and decreased tumor angiogenesis. The disruption of these cancer networks through deregulation EGF pathway by hydralazine might be responsible for the attenuation of the malignant phenotype of DU145 cells.

In summary, we showed that the non-nucleoside DNMTi hydralazine attenuates the PCa cell phenotype by disrupting several cancer cell pathways, in particular the EGF

pathway in DU145 cells. Moreover, exposure to hydralazine decreased DNMTs expression and induced histone acetylation, which might be responsible for re-expression of genes known to be epigenetically silenced in PCa. Interestingly, hydralazine restored AR expression in DU145 cells, providing clues to a potential synergistic effect with conventional anti-androgenic therapy. *In vivo* studies are now mandatory to further validate the promising role of hydralazine in PCa therapy.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Author Contributions

IG and ES performed and interpreted the phenotypic and molecular assays. PCP and IG performed comet assay. FQV and JS assisted on western blot and RT-qPCR respectively. IG and MIG performed cell cycle analysis. IG, RH and CJ contributed to experimental design and wrote the manuscript with input from co-authors.

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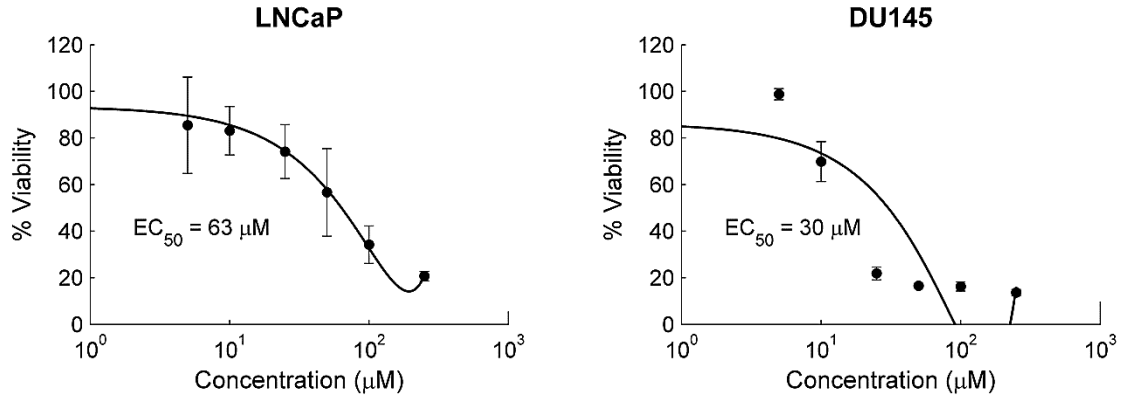
Supplementary Figures



Supplementary Figure 1 – Graphic representation of hydralazine half-maximal effective concentration (EC50) in PCa Cells, which was calculated at 72h in LNCaP and DU145 cell lines. LNCaP displayed an EC50 of 63 µM and DU145 of 30 µM.

6

CHAPTER SIX

Major Findings

Major Findings

In the third chapter of this Thesis we reported enoxacin as being able to attenuate PCa cell phenotype. Firstly, we assessed the *TARBP2* mutational status and TRBP protein expression alterations in prostate tumors and cell lines, which might preclude the use of enoxacin as a therapeutic option for this disease. Remarkably, neither primary PCa nor PCa cell lines showed deleterious mutations at the *TARBP2* locus, thus expressing normal levels of TRBP protein. The subsequent exposure of PCa cell lines to enoxacin resulted in a marked reduction of cell viability and induction of apoptosis which was confirmed at molecular and protein level by an increase in *CASP3* mRNA and cleaved PARP1 protein. Furthermore, cell cycle arrest was also achieved in all PCa cell lines after enoxacin exposure. Interestingly, whereas hormone-responsive cell lines (LNCaP, 22Rv1 and VCaP) showed an arrest at G2/M, castration-resistant cell lines (DU145 and PC-3) exhibited an arrest at the transition between late S and G2/M phases. This might reflect the higher proliferative potential of these cell lines. Additionally, enoxacin reduced the invasive potential of PCa cells, which is particularly relevant since metastatic spread is a major cause of PCa morbidity and mortality. In PCa cells, enoxacin globally restored the expression of miRNAs, including several with tumor-suppressor functions, which had already been reported as important for prostate carcinogenesis (miR-29b, miR-449a, miR-146a, miR-17*, and miR-34a). Moreover, enoxacin was also able to downregulate the expression of a number of miRNAs described as oncogenic in PCa, like miR-141 and miR-191.

In the fourth chapter of this thesis we described for the first time, RG108 a non-nucleoside analogue DNMTi, as having anti-cancer proprieties in PCa cell lines. This compound was able to decrease cell viability in three of the four tested PCa cell lines (LNCaP, 22Rv1 and DU145) and induce apoptosis in a time and dose dependent manner, being the 200 µM concentration and 14 days of exposure the best combination tested. Importantly, these results were confirmed at the molecular level, by decreased mRNA levels of *Ki67* and increase in *CDKN1A* and *CASP3*, and at protein level by increased cleaved-caspase 3. Concerning RG108 demethylating properties, we found that, in hormone-responsive cell lines, it inhibits *DNMT1*, *DNMT3a* and *DNMT3b* at transcript levels and DNMT1 protein expression. The reduced expression and activity of DNMTs after RG108 exposure might explain the reversal in *GSTP1* and *APC* promoter hypermethylation and consequent gene re-expression. This compound also reduced global DNA methylation, although less extensively than 5-aza-CdR.

Finally, in the fifth chapter we report, for the first time, that hydralazine attenuates the neoplastic phenotype of human PCa cell lines. This non-nucleoside DNMTi decreased cell

viability and induced cell apoptosis in PCa cell lines in a dose and time-dependent manner, being 40 µM the most effective concentration. A reduction in *Ki67* and an increase in *CDKN1A* and *CASP3*, *CASP8* and *CASP9* mRNA expression corroborated the phenotypic results. Moreover, hydralazine induced cell cycle arrest at G0/G1 in all cell lines, except for LNCaP, in which S phase arrest was observed. Notably, hydralazine reduced the invasive potential and induced significant DNA damage in PCa cell lines. We also found that this compound was able to decrease *DNMT1, DNMT3a and DNMT3b* mRNA levels in three of the four PCa cell lines and reduce DNMT1 protein expression in all cell lines. In this vein, hydralazine reduced *GSTP1*, *BCL2* and *CCND2* promoter methylation levels with concomitant gene re-expression in some cell lines. Moreover, increased Histone 3 acetylation levels were achieved after drug exposure in LnCaP and DU145, which might explain *CCND2*, *RARβ2, APC* and *AR* gene re-expression without significant promoter demethylation. Importantly, DU145 cells also showed re-expression of AR at protein level with concomitant induction of its downstream target p21. Furthermore, DU145 was the most responsive cell line to hydralazine which might be due to disruption of EGF receptor signaling pathway.

7

CHAPTER SEVEN

General Discussion

General Discussion

Prostate cancer (PCa) is a major health and economic concern in current aged men population at global level. Although, the majority of patients are diagnosed at an early (organ-confined) disease stage, where radical prostatectomy and/or radiotherapy are potentially curable, about 30% of these patients relapse after initial therapy and progress to advanced disease. At this point androgen ablation therapy (ADT), based on anti-androgens administration that block the functionality of the androgen receptor is the most common treatment option [1]. However, after an initial response with tumor regression, most patients acquire resistance to this therapy and eventually progress to castration-resistant disease (CRPC). Currently, chemotherapy remains the only available treatment for CRPC, though it is not curative and encompasses limited benefits in overall survival improvement [2, 3].

Epigenetic alterations constitute relevant drivers of PCa development and progression [4]. These alterations are reported as being more prevalent than genetic mutations and some are already present in pre-malignant lesions. Unlike genetic alterations, epigenetic events are reversible. Thus, the high prevalence of epigenetic modifications in PCa and their role in carcinogenesis underscore the great potential of epigenetic targeted therapy. Presently, several compounds that target multiple enzymes of the epigenetic machinery are under study as possible anti-cancer agents [5, 6].

The emerging field of miRNAs deregulation in prostate carcinogenesis, and particularly their global downregulation, highlighted the importance for the investigation of new compounds that are able to restore the expression of tumor-suppressor miRNAs [7]. Targeting of deregulated miRNAs might lead to re-acquisition of normal regulation of key genes of cell signaling pathways, playing important roles in biological processes such as proliferation and apoptosis, which are frequently deregulated in PCa [8].

In the third chapter of this thesis, enoxacin, a broad-spectrum fluoroquinolone antibiotic used in the treatment of urinary tract infections [9, 10], was reported to have anti-tumoral effects in PCa cell lines. This finding is very significant because there are no other described agents able to inhibit the PCa phenotype through the regulation of global miRNAs expression. Nevertheless, artificial miRNAs have been reported as potential anti-tumoral agents in PCa cell lines and xenograft models [11-14]. However, those oligonucleotide-based therapies have major limitations that hindered the translation to the clinics. First, RNA has low stability *in vivo* being rapidly degraded and excreted [15]. Thus, new efforts are being performed to increase RNA half-life [8]. Another difficulty is how to ensure tumor-specific delivery and retention of miRNAs. Due to the fact that a single miRNA can target several mRNAs, there is a high probability of mRNAs mistargeting. The

use of miRNAs linked to nanoparticles and directed to tumor cells via active or passive targeting is now being investigated to overcome this problem [8]. Nonetheless, oligonucleotide-based therapies for miRNAs are still in the very beginning. Therefore, compounds such as enoxacin, which not only has a safe clinical profile with limited-side effects, but that is also capable of restoring normal expression of several miRNAs, present a higher potential from a clinical point of view for PCa management. Herein, we demonstrated that enoxacin is able to restore the normal expression of several tumor-suppressor miRNAs and oncomirs involved in prostate carcinogenesis. Importantly, several other miRNAs displayed altered expression upon enoxacin exposure. However, their role in prostate carcinogenesis remains elusive and their impact does not seem to be critical, considering the results of the phenotypic assays. Thus, in view of our results, enoxacin might be a promise therapeutic strategy for PCa patients, deserving a serious consideration for testing in a clinical trial setting

Tumor-suppressor genes silencing due to aberrant DNA methylation is the most common epigenetic alteration in PCa. Indeed, promoter methylation of cancer-related genes has emerged as a promising diagnostic, prognostic and predictive PCa biomarker. Additionally, DNA methylation is also under evaluation as potential therapeutic target in PCa, through the use of DNMT inhibitors (DNMTi) [16, 17]. The two nucleoside analogues, 5-azacytidine and 5-aza-2'-deoxycytidine, already in clinical use for treatment of MDS, have proven limited efficacy for PCa in clinical trials [18]. One reason for these disappointing results might be the limited incorporation of these drugs into PCa cells, which are less proliferative than hematological malignancies [19]. Therefore, the use of non-nucleoside analogs, which are independent of replication for exert their action constitutes a more attractive approach in solid tumors, like PCa. Thus, in the fourth and fifth chapter of this thesis, two non-nucleoside analogues, RG108 and hydralazine were tested for the first time as anti-cancer agents in PCa cell lines.

RG108 was designed for direct targeting of DNMT1 active site [20], but in our experiments it showed weaker demethylating effect than 5-aza-CdR. However, significant tumor cell growth inhibition and reduced promoter methylation with concomitant gene re-expression was achieved, especially in androgen responsive cell lines. The lower demethylating efficacy of RG108 was previously reported for colon cancer cells: a reduction of 50% vs. 30% of cytosine methylation levels was accomplished after 15 days exposure to 5-aza-CdR and to RG108, respectively [20]. However, one of the major concerns regarding hypomethylating agents is the risk of centromeric satellite elements demethylation, which might lead to chromosome instability [21, 22]. In fact, 5-azacytidine was associated with demethylation of repetitive sequences, which was not observed for RG108, suggesting a more specific demethylating effect of the latter [20]. This fact

associated with the lower impact in global DNA methylation due to the covalent trapping of DNMTs, indicates that RG108 is a safer compound than 5-aza-CdR, from a clinical standpoint [23-25]. Our data further supports RG108 as a promising option for future testing in clinical trials designed for PCa patients.

Hydralazine, an anti-hypertensive drug displaying demethylating activity [26], has also proven value as neoplastic cell growth inhibitor in PCa cells and similar results were reported for other cancer models [27, 28]. Importantly, this drug was already tested in clinical assays, both in monotherapy or in combination with HDACi and/or conventional therapy, and preliminary results showed a significant advantage in progression-free survival, being well tolerated by patients [29-31].

Following the same trend of RG108, PCa cells treated with hydralazine also demonstrated a reduction in methylation levels of PCa-related genes, with associated gene re-expression. One of those genes was *GSTP1,* a widely known “caretaker gene”; which is methylated in the vast majority of PCa cases [32] and that has been recently suggested as a potentially useful biomarker for assessing DNMTi efficacy in PCa treatment [33].

Concerning the two studied demethylating agents, we observed a reduction in DNA methylation of some genes that was not associated with reactivation of expression. The complexity of epigenetic regulation of gene expression is currently acknowledged. Indeed, epigenetic silencing of cancer-related genes is often achieved by concomitant histone post-translational modifications and DNA methylation [34]. Given the distinctive role of DNA methylation and histone modifications in epigenetic control, there is a rationale to combine DNMTi and HDACi to potentiate the re-expression of tumor-suppressor genes. In fact, this approach has demonstrated promising synergy in several preclinical models and clinical trials [29, 35]. Therefore the combination of hydralazine or RG108 with HDACi might be a plausible strategy to explore in PCa patients.

Importantly, we demonstrated that DU145 cells re-express the androgen receptor (AR) upon hydralazine exposure. Because, CRPC is characterized by a heterogeneous loss of AR expression, and in 13% to 28 % of CRPC the AR is silenced by promoter aberrant methylation [36, 37], therapies that could restore its expression might be of clinical usefulness [38, 39]. Interestingly, a recent study demonstrated that the combination of 5-aza-CdR with the bicalutamide resulted in a synergistic effect, increasing the susceptibility of PCa cells to undergo apoptosis and repressing tumor growth in xenograft mice [40, 41]. Thus, our data suggest that hydralazine might potentiate the response of PCa patients to the conventional anti-androgenic drugs, such as bicalutamide. Since hydralazine has a well-known clinical and safety profile, bearing minimal side effects and is not incorporated in DNA to exert its function, maintaining gene

re-expression for longer periods of time than 5-aza-CdR [42], we postulate that it might be more suitable for treating PCa patients. Indeed, hydralazine attenuated PCa cells phenotype with lower concentrations and with more impressive effects compared to RG108. Furthermore, hydralazine exerted its major effect in the CRCP cell line tested, which mimics the group of patients without effective therapeutic options. Hence, our results suggest that hydralazine is more efficient than RG108, both as an anti-tumoral and demethylating agent.

Recent studies have reported that several miRNAs are epigenetically regulated in PCa. Two examples are miR-29a and miR-1256, which were found downregulated in prostate tumors compared with normal prostate tissues, due to methylation in the respective promoter sequence [43-45]. Downregulation of those miRNAs could potentially lead to an increased expression of their target mRNAs and proteins, and, therefore, to aberrant expression of oncogenes, contributing to PCa development and progression. Therefore, targeting the methylation of DNA sequences in the promoter region of miRNAs might be an attractive therapeutic approach. Recently, it was reported that the exposure of PCa cell lines to demethylating agents may increase miRNAs expression [46, 47]. Based on this knowledge and in our results, we are tempted to suggest that an initial exposure of PCa cells to hydralazine and, subsequently, to enoxacin may potentiate enoxacin anti-growth effect, allowing for a reduction in the dosage of both drugs.

In this thesis we have demonstrated that three epigenetic modulators – enoxacin, RG108 and hydralazine – are able to target multiple cellular pathways that regulate survival in distinct PCa cells lines. Because, these cell lines are phenotypically different, displaying diverse cellular responses, and potentially mimic the widely acknowledged heterogeneity of PCa, it is suggested that those drugs might have clinical usefulness against PCa *in vivo*.

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8

CHAPTER EIGHT

Conclusions and Future Perspectives

Conclusions and Future Perspectives

In this Thesis we reported, for the first time, on the potential of three epigenetic target drugs as promising therapeutic agents for PCa.

We demonstrated that enoxacin, a compound that indirectly targets miRNAs, was able to disrupt several cancer pathways by effectively reduce cell viability, induce apoptosis, arrest cell cycle, and decrease the invasive potential. Moreover this compound effectively restored the global expression of miRNAs, leading to an increase in the expression of tumor-suppressor miRNAs in PCa. Furthermore, this compound also decreased oncomirs' expression. Therefore, these alterations in miRNAs expression might be responsible for the impressive anti-tumor effects of enoxacin in PCa cells lines. In view of these promising results *in vivo* studies should be performed to further support the usefulness of enoxacin for PCa therapy.

Concerning, demethylating agents, we demonstrated that RG108 was effective in reducing tumor cell growth, in particularly of androgen-responsive cell lines LNCaP and 22Rv1. Indeed, this compound led to a significant dose and time dependent inhibition of cell viability and apoptosis induction in PCa cell lines, being the 200 µM concentration during 14 days of exposure the best tested combination. In LNCaP and 22Rv1, this effect seemed to be mediated by a global DNA hypomethylation, which might lead to the reversion of aberrant promoter methylation and mRNA re-expression of tumor-suppressor genes involved in prostate carcinogenesis. Nevertheless, the mechanisms that underlie the anti-cancer effects in DU145 cell line require further investigation and additional studies should evaluate the cellular pathways involved in RG108 response.

Concerning hydralazine, we showed that this compound exerts an inhibitory effect in PCa cell phenotype by disrupting several cancer-related cellular pathways. Likewise, this agent was able to reduce PCa cell viability and invasiveness, and induce cell death, cell cycle arrest and DNA damage. Furthermore, exposure to this compound led to a decrease in DNMTs expression and induction of histone acetylation, which might be responsible for the re-expression of genes known to be epigenetically silenced in PCa. We also found that DU145 was the most responsive cell line and this effect might be due to the disruption of the EGF signaling pathway. Further studies should be conducted to validate the EGF pathway as a target of hydralazine not only in DU145 but also in the remaining PCa cell lines. Importantly, hydralazine restored AR expression in DU145, providing clues to a potential synergistic effect with conventional anti-androgenic therapy.

Comparing the two non-nucleoside DNMTi, hydralazine might have an anti-cancer activity superior to that of RG108. Hydralazine reverted the malignant cell phenotype in all PCa cell lines with lower drug concentrations than RG108. Importantly, hydralazine was

most effective in a CRPC cell line which simulates the disease stage that has limited therapeutic options. Therefore, this compound might be more attractive from a clinical point of view than RG108. Therefore, future studies should address the combination of hydralazine and the anti-androgen bicalutamide in CRPC cell lines to verify whether the demethylating compound leads to an increased response of the conventional anti-androgenic therapy. Since PCa harbors a downregulation of miRNAs and some of them have been shown to be silenced by aberrant methylation, it would be interesting to investigate the combination of hydralazine and enoxacin in PCa cell lines. Theoretically hydralazine would potentiate the effect of enoxacin, allowing for the use of lower drug concentrations.

Finally, our results may serve as “proof of principle” for designing *in vivo* studies intended to validate the promising role of hydralazine as a single agent and/or in combination with bicalutamide or enoxacin for PCa therapy

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APPENDICES

I

PAPER I

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Enoxacin inhibits growth of prostate cancer cells and effectively restores microRNA processing

Elsa J. Sousa,[1,2] Inês Graça,[1,2,5] Tiago Baptista,[1,2] Filipa Q. Vieira,[1,2,5] Carlos Palmeira,[4] Rui Henrique[1,3,6] and Carmen Jerónimo[1,2,6,*]

[1]Cancer Epigenetics Group; Research Center of the Portuguese Oncology Institute; Porto, Portugal; [2]Department of Genetics; Portuguese Oncology Institute; Porto, Portugal; [3]Department of Pathology; Portuguese Oncology Institute; Porto, Portugal; [4]Laboratory of Flow Cytometry; Department of Hematology; Portuguese Oncology Institute; Porto, Portugal; [5]School of Allied Health Sciences ESTSP; Polytechnic of Porto; Porto, Portugal; [6]Department of Pathology and Molecular Immunology; Institute of Biomedical Sciences Abel Salazar; University of Porto; Porto, Portugal

Keywords: enoxacin, microRNAs, prostate cancer, therapy, TRBP

Abbreviations: CRPC, castration-resistant prostate cancer; DAB, 3,3-diaminobenzidine; DMSO, dimethyl sulfoxide; HDAC1, histone deacetylase 1; miRNA, microRNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide; PAGE, polyacrylamide gel electrophoresis; PCa, prostate cancer; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RISC, RNA-induced silencing complex; RNAi, RNA interference; SIRT1, silent information regulator 1; TARBP2, Trans-activator RNA-binding protein 2

Prostate cancer (PCa) is one of the most incident malignancies worldwide. Although efficient therapy is available for early-stage PCa, treatment of advanced disease is mainly ineffective and remains a clinical challenge. microRNA (miRNA) dysregulation is associated with PCa development and progression. In fact, several studies have reported a widespread downregulation of miRNAs in PCa, which highlights the importance of studying compounds capable of restoring the global miRNA expression. The main aim of this study was to define the usefulness of enoxacin as an anti-tumoral agent in PCa, due to its ability to induce miRNA biogenesis in a TRBP-mediated manner. Using a panel of five PCa cell lines, we observed that all of them were wild type for the *TARBP2* gene and expressed TRBP protein. Furthermore, primary prostate carcinomas displayed normal levels of TRBP protein. Remarkably, enoxacin was able to decrease cell viability, induce apoptosis, cause cell cycle arrest, and inhibit the invasiveness of cell lines. Enoxacin was also effective in restoring the global expression of miRNAs. This study is the first to show that PCa cells are highly responsive to the anti-tumoral effects of enoxacin. Therefore, enoxacin constitutes a promising therapeutic agent for PCa.

Introduction

Prostate cancer (PCa) is one of the most commonly diagnosed malignancies worldwide and a leading cause of cancer-related deaths among men.[1] Although most cases are clinically indolent, a variable proportion of patients develop castration-resistant PCa (CRPC), an aggressive and lethal form of disease, associated with widespread metastatic disease.[2] Currently, most therapeutic strategies are largely ineffective and only allow for a small increase in survival.[3] Therefore, new therapeutic strategies, ideally based on the understanding of the biology of this disease, are urgently needed.

PCa is a complex and heterogeneous disease that arises through the acquisition of several genetic and epigenetic alterations.[4,5] Among the latter, dysregulation of microRNA (miRNA) expression has been recently emphasized as a critical mechanism in PCa development and progression.[6] miRNAs are small non-coding RNAs that posttranscriptionally regulate gene expression by inducing cleavage of their target mRNA or by inhibiting their translation.[7] These molecules are produced through a multi-step process that involves the RNase III enzymes DROSHA and DICER, resulting in the production of mature miRNAs of ~22 nucleotides, which are incorporated into the RNA-induced silencing complex (RISC) through the action of the DICER-TRBP (Trans-activator RNA-binding protein) complex.[8] miRNAs play a crucial role in the regulation of almost every biological process, including differentiation, apoptosis, cell cycle, development and metabolism.[9,10] Moreover, dysregulation of any of these processes due to abnormal expression of miRNAs or alterations in their machinery of biogenesis has been implicated in cancer, including PCa.[11,12] Although miRNAs have been proposed to function as either oncogenes or tumor-suppressor genes,[13] most human tumors are characterized by a general defect in miRNA production that results in global downregulation of miRNA expression.[14-16] Thus, compounds with the ability to restore the global miRNA expression might be an excellent therapeutic option for cancer.

Enoxacin, an antibacterial compound based on a fluoroquinolone skeleton, was shown to be effective in the inhibition of tumor cell growth in vitro and in vivo by enhancing

*Correspondence to: Carmen Jerónimo; Email: carmenjeronimo@ipoporto.min-saude.pt and cljeronimo@icbas.up.pt
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the production of miRNAs with tumor suppressor functions.[17] Remarkably, the drug did not affect normal cells and was not associated with toxicity in mice models.[17] Moreover, it has been reported that enoxacin promotes RNA interference (RNAi) and miRNA biogenesis by facilitating the interaction between TRBP, encoded by the *Trans-activator RNA-binding protein 2 (TARBP2)* gene, and miRNA precursors.[17,18] Therefore, it has been recently demonstrated that *TARBP2*-mutant cells are less responsive to enoxacin.[17,19] Although those results are promising for cancer therapeutics, no studies are available concerning the effect of enoxacin on PCa. Thus, we aimed to investigate the effectiveness of enoxacin as a tumor growth inhibitor in PCa. Furthermore, we evaluated the alterations in miRNA expression induced by this compound in PCa cells.

Results

PCa cells do not harbor *TARBP2* mutations and retain TRBP protein expression. In view of the fact that cell lines harboring *TARBP2* mutations are less responsive to enoxacin,[17,19] five PCa cell lines (LNCaP, 22Rv1, VCaP, DU145 and PC-3) were screened for the presence of mutations in all the exonic mononucleotide repeats localized in the coding sequences of *TARBP2*. Co115, a *TARBP2*-mutant colorectal cancer cell line,[17,19] was used as positive control. No *TARBP2* mutations were found in any of the tested PCa cell lines. Subsequently, we analyzed TRBP protein expression in PCa cell lines by western blot. As expected, all PCa cell lines expressed higher protein levels of TRBP than Co115 cells, which display very low expression levels (**Fig. 1A**). Since DICER acts in complex with TRBP,[8] we also assessed DICER protein expression in PCa cell lines, and we verified that all PCa cell lines tested expressed DICER (**Fig. 1A**).

Primary PCa tumors are *TARBP2* wild type and express TRBP. To investigate the putative clinical usefulness of enoxacin for PCa therapy, we first assessed the *TARBP2* mutational status of 25 primary PCa tumors, and only wild type sequences were detected. Furthermore, using immunohistochemistry, TRBP expression was evaluated in a series of 50 primary PCa tumors, including the same cases analyzed for *TARBP2* mutational status. No differences in immunoreactivity for TRBP were apparent between normal and tumorous prostatic tissues representing different histopathological grades (**Fig. 1B**).

Enoxacin reverts neoplastic features of PCa cell lines. The half-maximal effective concentration (EC_{50}) of enoxacin was calculated in LNCaP and DU145 prostate cancer cells lines at 72 h. The drug presented an EC_{50} of 105 μM in LNCaP and 141 μM in DU145. Thus, to evaluate the effects of enoxacin, five human PCa cell lines were continuously exposed for 5 d to 124 μM (40 μg/mL) of enoxacin. As expected, enoxacin did not alter the expression of both TRBP and DICER proteins in any of the analyzed cell lines (**Fig. 2A**).

Importantly, a significant decrease in the number of viable cells was observed after exposure to the drug when compared with the vehicle, DMSO (**Fig. 2B**). For LNCaP and 22Rv1 cell lines, the effect was observed from day 1, whereas a significant decrease in the number of viable cells in VCaP, DU145 and PC-3 was found after 2 d of drug exposure. The reduction in the percentage of viable cells at day 5 ranged between 17 and 59%, with LNCaP being the most responsive cell line (**Fig. 2B**).

To determine whether enoxacin was capable of inducing significant cell death, an apoptosis assay was performed. Indeed, a significant increase in apoptosis was apparent in all tested cell lines at days 2 and 5 (**Fig. 3A**). After 5 d of exposure to enoxacin, LNCaP and DU145 displayed the highest levels of apoptotic cells (**Fig. 3A**).

Apoptosis was also confirmed at molecular level, through the evaluation of mRNA expression of *CASP3*. Although all cell lines showed an increase in *CASP3* expression levels, statistically significant differences were depicted only for LNCaP, 22Rv1 and DU145 (**Fig. 3B**). Furthermore, cleaved PARP was analyzed after enoxacin exposure. 22Rv1, VCaP and DU145 presented increased protein levels of cleaved PARP after exposure to the drug (**Fig. 3C**).

Cell cycle distribution was evaluated by flow cytometry. Interestingly, 22Rv1 and VCaP, which are hormone-responsive PCa cell lines, showed cell cycle arrest at G2/M, whereas the castration-resistant cell lines DU145 and PC-3 exhibited a significant increase in the percentage of cells in late S and G2/M transition (**Fig. 4**; **Fig. S2**).

Moreover, the percentage of cells in sub-G1 phase, which is an indirect measure of cell death, increased significantly after enoxacin exposure (**Table 1**; **Fig. S2**). Thus, both the increase of apoptotic cells and cell cycle arrest support a growth inhibitory effect of enoxacin on PCa cells.

The effect of enoxacin on the invasiveness of PCa cells was only assessed in LNCaP and DU145 cell lines because, as described in the previous experiments, these cells were the most responsive to enoxacin. We decided to extend enoxacin exposure from 5 to 8 d in order to allow the vehicle cells to invade. In contrast to DU145, LNCaP cells did not show invasive potential in this system. Remarkably, enoxacin significantly reduced the invasiveness of DU145 cells (**Fig. 5**).

Enoxacin restores miRNAs expression. The impact of enoxacin exposure on the expression profile of miRNAs was analyzed in LNCaP and DU145 cell lines, using a panel of 742 miRNAs. miRNA analysis demonstrated that enoxacin induced a global upregulation of miRNA expression in both cell lines. Among miRNAs differentially expressed, upregulation was observed in 53% of the miRNAs (65 of 122) for LNCaP cells (**Fig. 6A**; **Table S1**) and in 60% (147 of 247) for DU145 (**Fig. 6A**; **Table S2**).

Remarkably, enoxacin was able to alter the expression of several miRNAs that have been previously associated with prostate carcinogenesis. Concerning tumor-suppressor miRNAs reported in PCa, miR-17*, miRNA-29b, miR-34a, miR-132, miR-146a and miR-449a showed increased expression levels following enoxacin exposure. Furthermore, decreased expression of some oncogenic miRNAs was also observed, including miR-141 and miR-191 (**Table 2**).

To confirm the impact of enoxacin on miRNA targets, protein expression levels of HDAC1, a miR-449a target oncoprotein, and SIRT1, a miR-34a target oncoprotein, were assessed by Western Blot. Both cell lines after drug exposure displayed decreased protein levels of HDAC1 and SIRT1 (**Fig. 6B**).

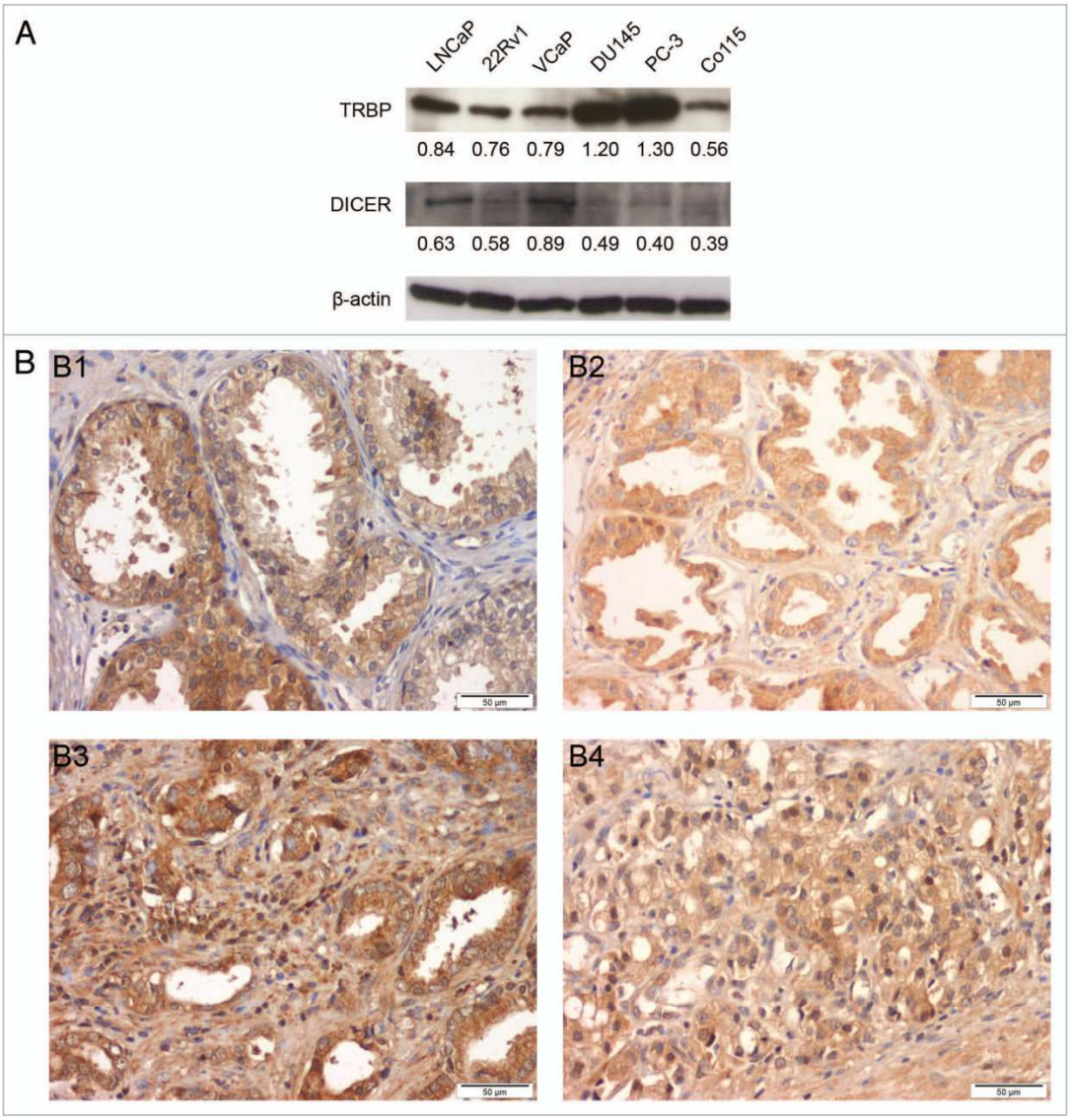

Figure 1. TRBP and DICER expression in PCa. (**A**) TRBP and DICER expression was assessed by Western Blot in PCa cell lines. The picture is representative of three independent experiments. β-actin was used as a loading control and the relative density of bands was densitometrically quantified. Mean quantitation values are shown. Co115, a *TARBP2*-mutant colon carcinoma-derived cell line, was used as positive control. (**B**) Immunohistochemical stain for TRBP expression in prostatic tissue. Protein immunoexpression in tumor cells was similar to that of normal epithelial cells. Normal glands (**B1**), and tumorous tissue: Gleason score 6 (**B2**), Gleason score 7 (**B3**), and Gleason score 8 (**B4**).

Discussion

PCa is one of the leading causes of cancer-related deaths worldwide,[1] and almost all of those deaths occur as a result of the emergence of castration-resistant disease.[20] Although PCa patients initially respond to androgen-deprivation therapy, about 18–24 months after treatment initiation, most patients develop CRPC, which results in progressive clinical deterioration and, ultimately, death.[21,22] For patients with CRPC, there are limited treatment options with proven survival benefit.[23-26]

Recently, several miRNA microarray profiles demonstrated that miRNAs are commonly dysregulated in PCa when compared with normal prostate tissue and that they are also differentially expressed in different stages of PCa.[15,16] Hence, miRNAs

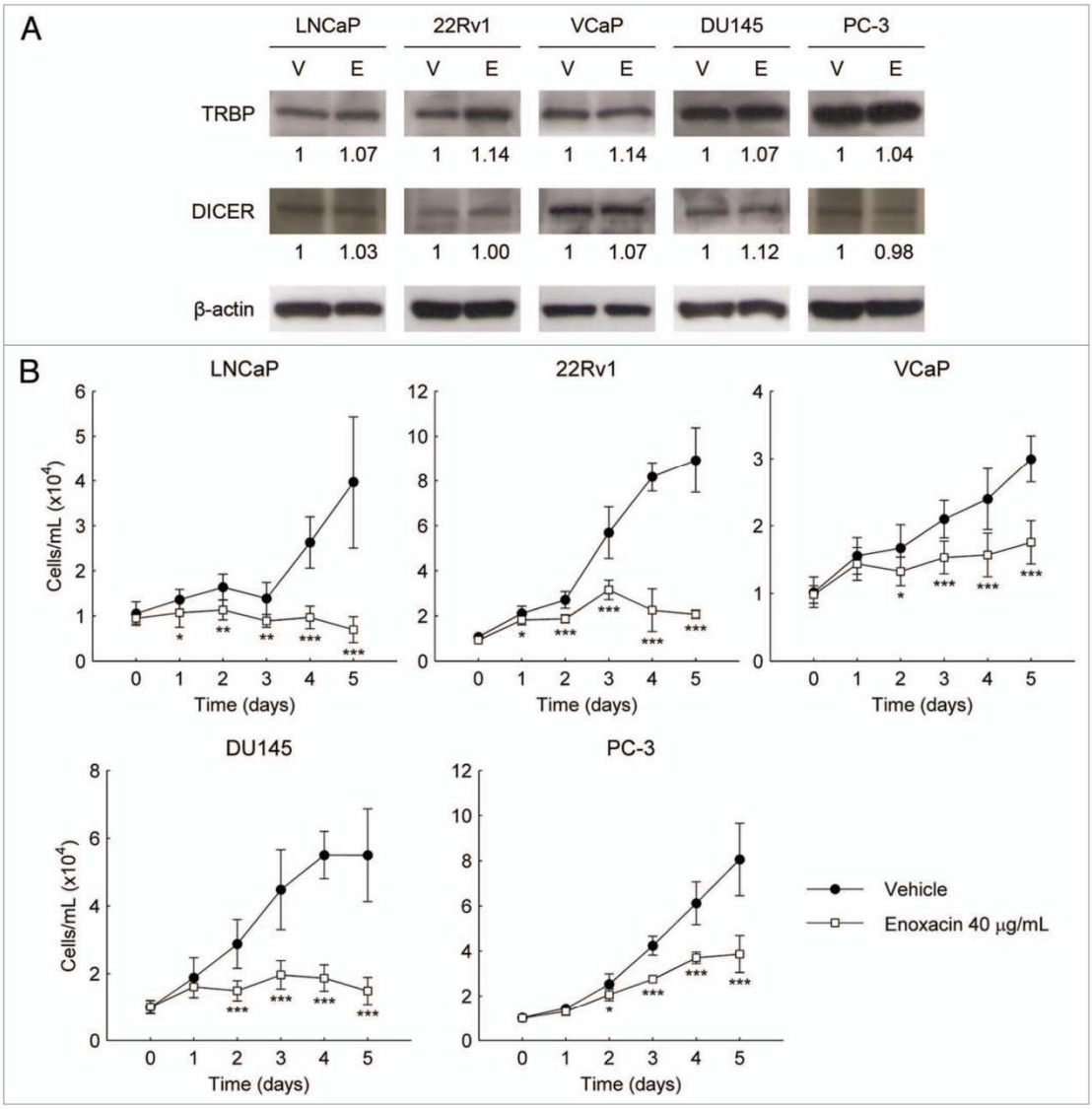

Figure 2. (**A**) Effect of enoxacin on the expression of TRBP and DICER. Protein expression of TRBP and DICER was analyzed by Western Blot in LNCaP, 22Rv1, VCaP, DU145 and PC-3 cell lines after exposure to enoxacin 40 μg/mL or DMSO (vehicle) at day five. The picture is representative of three independent experiments. β-actin was used as a loading control and the relative density of bands was densitometrically quantified. (**B**) Effect of enoxacin on PCa cell viability. Cell viability was evaluated by MTT assay in LNCaP, 22Rv1, VCaP, DU145 and PC-3 cell lines after exposure to enoxacin 40 μg/mL or DMSO (vehicle) for 5 d. The number of cells/mL is shown as mean of three independent experiments performed in triplicates ± SD. Statistical significance (enoxacin vs. vehicle) was tested using the two-sided Student's t-test *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with vehicle group.

might be used not only as diagnostic and prognostic biomarkers but also as therapeutic targets in PCa. In recent years, efforts have been made to find effective miRNA-based therapeutic strategies for cancer. Indeed, artificial miRNAs that might act as potential anti-tumoral agents are the most studied so far, with most reports focusing on oncogenic miRNAs inhibition.[27,28] Until now, few studies have reported the use of these synthetic miRNAs as tumor suppressors and, additionally, the effective technology for delivery of these oligonucleotide-based therapies remains a problem.[28] Because most human cancers exhibit global miRNA downregulation,[14-16] the search for compounds able to globally restore the expression of tumor-suppressor miRNAs

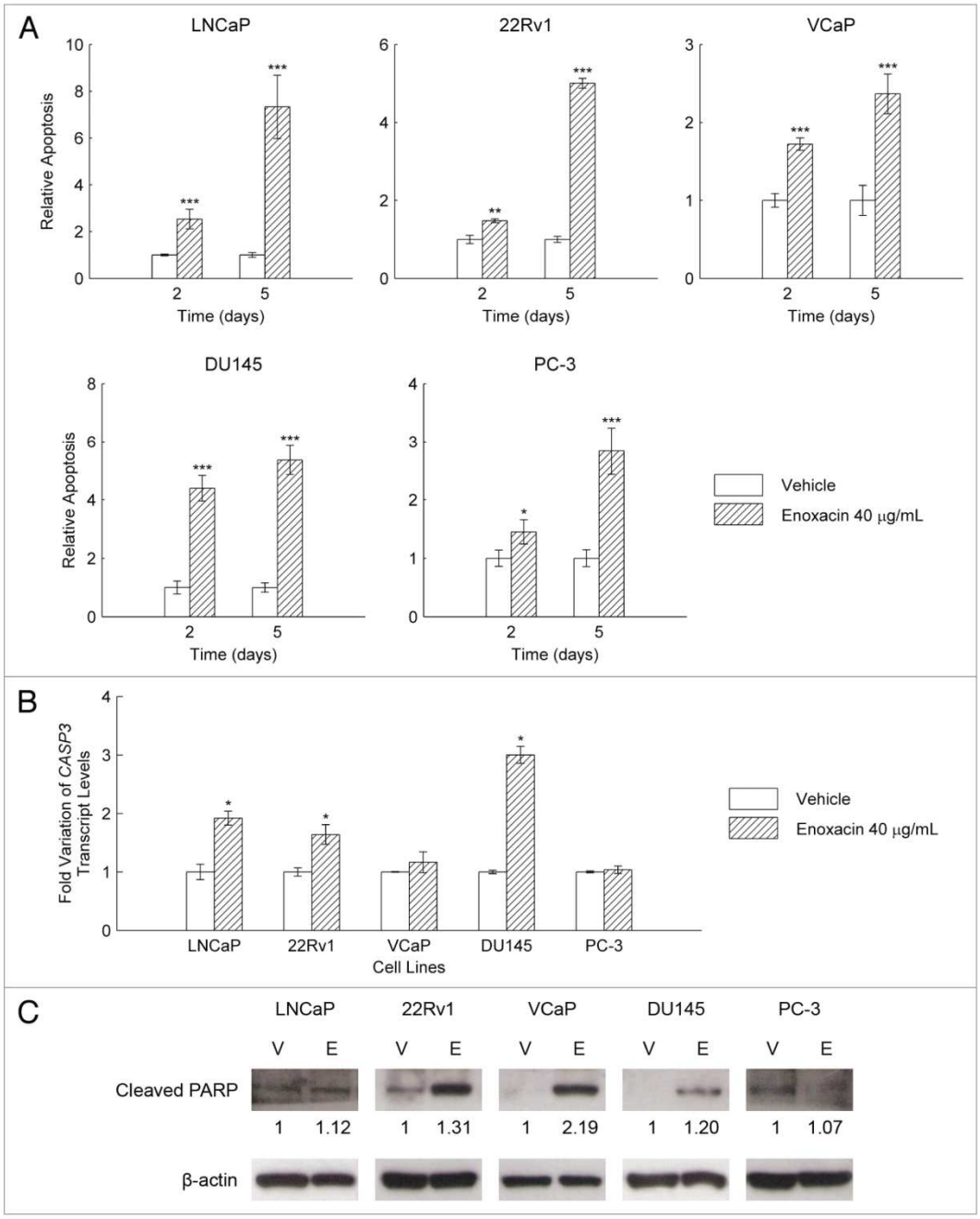

Figure 3. Effect of enoxacin on PCa cell apoptosis. (**A**) Apoptosis was analyzed by APOPercentage assay at days two and five in LNCaP, 22Rv1, VCaP, DU145 and PC-3 cell lines after exposure to enoxacin 40 μg/mL or DMSO (vehicle) at days two and five. (**B**) *CASP3* mRNA expression was evaluated by qRT-PCR in LNCaP, 22Rv1, VCaP, DU145 and PC-3 cell lines after exposure to enoxacin 40 μg/mL or DMSO (vehicle) at day five. Data are presented as mean of three independent experiments performed in duplicates ± SD. Statistical significance (enoxacin vs. vehicle) was tested using the two-sided Student's t-test. *p < 0.05, **p < 0.01, ***p < 0.001, compared with vehicle group. (**C**) Cleaved PARP was analyzed by Western Blot in LNCaP, 22Rv1, VCaP, DU145 and PC-3 cell lines after exposure to enoxacin 40 μg/mL or DMSO (vehicle) at day five. The picture is representative of three independent experiments. β-actin was used as a loading control and the relative density of bands was densitometrically quantified.

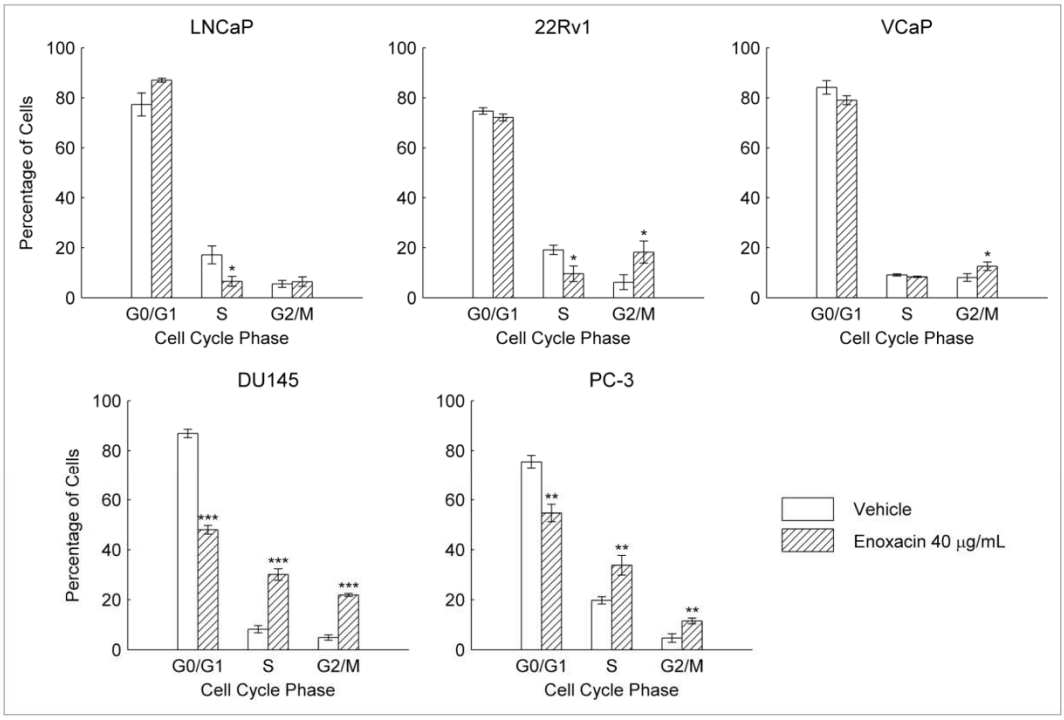

Figure 4. Effect of enoxacin on PCa cell cycle distribution. Cell cycle distribution was assessed by flow cytometry in LNCaP, 22Rv1, VCaP, DU145 and PC-3 cell lines after exposure to enoxacin 40 μg/mL or DMSO (vehicle) at day five. The percentage of cells is shown as mean of three independent experiments ± SD. Statistical significance (enoxacin vs. vehicle) was tested using the two-sided Student's t-test. *p < 0.05, **p < 0.01, ***p < 0.001, compared with vehicle group.

Table 1. Impact of enoxacin on the percentage of cells in Sub-G1 phase assessed by flow cytometry

Cell Line	Cells in Sub-G1 phase (%)		p value
	Vehicle	Enoxacin	
LNCaP	1.7	2.0	ns
22Rv1	1.8	13.1	< 0.001
VCaP	2.5	7.3	0.002
DU145	1.7	7.5	< 0.001
PC-3	1.0	3.6	0.004

Ns, non-significant.

remains a priority in miRNA research. Herein, we report for the first time the anti-cancer effect of enoxacin, one of such compounds, on PCa cell lines.

Enoxacin, which has been used as a broad-spectrum antibiotic to treat bacterial infections (e.g., urinary tract infections),[29,30] was recently reported as being capable of enhancing RNAi and consequently induce miRNA expression.[17,18] However, the mechanism of action of enoxacin is dependent of TRBP as it has been shown that this compound is less effective in cells harboring alterations in this protein caused by *TARBP2* gene mutations.[17,19] Thus, we initially screened PCa cell lines for *TARBP2* mutations and none was found, although we were able to confirm a *TARBP2* mutation in the control, colorectal cancer cell line Co115, as previously reported.[17,19] To further validate our results, we performed western blot for TRBP and confirmed that all PCa cell lines tested displayed higher protein levels than Co115, in agreement with previous studies.[17,19] The *TARBP2* mutational status was also assessed in primary PCa tumors and only wild type sequences were detected. We then interrogated primary PCa cases using an immunohistochemistry assay for TRBP and we found that protein immunoexpression in tumor cells was similar to that of normal epithelial cells. Thus, we may infer that primary prostate carcinomas do not harbor deleterious mutations at the *TARBP2* locus and display normal levels of TRBP protein, rendering them sensitive to restoration of normal miRNA biogenesis by enoxacin.

To demonstrate the growth-inhibitory effect of enoxacin on PCa cell lines, we assessed cell viability, apoptosis and cell cycle characteristics following five-day exposure. Remarkably, in all tested cell lines, exposure to enoxacin resulted in a significant decrease in cell viability and induction of cell death by apoptosis, as previously demonstrated for other cancer cell lines.[17] These results were further confirmed at the molecular level

through the observed statistically significant increase in *CASP3* mRNA expression for three of the five cell lines analyzed (LNCaP, 22Rv1 and DU145). Regarding protein expression of cleaved PARP, we also observed an increase after exposure to the drug in three cell lines (22Rv1, VCaP and DU145). Although the percentage of cells in Sub-G1 differs among the tested cell lines, globally there is an increase after exposure to enoxacin. With some variations, *CASP3* mRNA, and cleaved PARP protein expression follow the same trend. The percentage of cells in Sub-G1 may also reflect cell death due to necrosis or other mechanisms, but the results of the remaining three parameters (*CASP3* mRNA levels, APOPercentage and cleaved PARP) strongly indicate that apoptosis is, indeed, the main mechanism underlying cell death in this study. Concerning cell cycle distribution, it was observed that 22Rv1 and VCaP, which are hormone-responsive PCa cell lines, presented cell cycle arrest at G2/M, whereas the castration-resistant cell lines DU145 and PC-3 exhibited an increase in the percentage of cells at late S and G2/M transition.

Furthermore, we have shown for the first time that enoxacin significantly reduce the invasive potential of PCa cells. As metastasis is the major cause of morbidity and mortality in PCa patients,[4] the development of new treatment regimens that would reduce tumor dissemination is extremely important for PCa therapy. It could be argued that the impact of enoxacin on cell invasion might be the result of reduced cell viability. However, it should be pointed out that it is difficult to dissociate one feature from the other, as both are likely to act in concert. Indeed, if tumor cell viability is reduced, it is expected that the potential to invade is also impaired. Irrespective of the underlying cause, however, our results demonstrate that a reduction in invasive potential does occur after exposure to enoxacin.

The ability to disrupt pathways of cancer cell survival has been already reported for a broad spectrum of cancer cells, both in vitro and in vivo, through the enhancement of the miRNA-processing machinery.[17]

In this study, we also demonstrated that enoxacin was effective in globally restoring the expression of miRNAs. Nevertheless, we found a decrease in the expression of a significant number of miRNAs, which is not totally in agreement with the mechanism of action purposed for enoxacin.[17,19] According to previous studies, the presence of enoxacin increases the binding affinity of TRBP for miRNA precursors promoting miRNA biogenesis.[17,19] Hence, it is tempting to speculate that besides this mechanism there might be other pathways through which enoxacin exerts its action.

Importantly, upregulation of several tumor-suppressor miRNAs known to be involved in PCa development and progression was observed, including miR-29b,[31,32] miR-449a,[33] miR-146a,[34] miR-17*[35] and miR-34a.[36,37] For instance, miRNA-29b was reported to be a negative regulator of PCa cell growth by modulating the expression of multiple proteins implicated in metastasis formation, including MMP2, E-cadherin, N-cadherin, Snail and Twist.[31,32] miR-146a is downregulated in CRPC cell lines, and targets ROCK1 and EGFR, which are implicated in the development of CRPC.[34] miR-17* also suppresses tumorigenicity

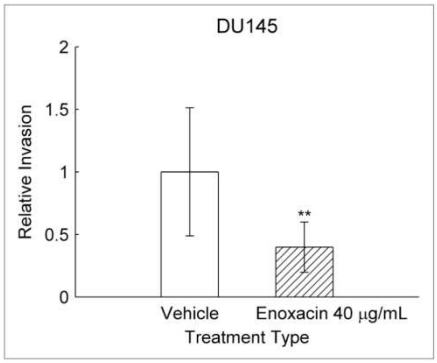

Figure 5. Effect of enoxacin on the invasive potential of PCa cells. Relative invasion was evaluated by Oris™ Cell Invasion Assay in DU145 cell line after exposure to enoxacin 40 μg/mL or DMSO (vehicle) at day eight. Data are presented as mean of three independent experiments performed in triplicates ± SD. Statistical significance (enoxacin vs. vehicle) was tested using the two-sided Student's t-test. **p < 0.01, compared with vehicle group.

of PCa cells through inhibition of mitochondrial antioxidant enzymes.[35] On the other hand, miR-34a, which presents tumor-suppressor functions, inhibits silent information regulator 1 (SIRT1), a gene that suppresses p53-dependent apoptosis.[36,37] Finally, miR-449a, which is underexpressed in PCa, regulates cell growth and viability by repressing HDAC1.[33] Remarkably, in LNCaP and DU145 cell lines, upregulation of miR-449 by enoxacin resulted in the downregulation of HDAC1, an oncoprotein expressed at significantly higher levels in PCa than in normal prostate.[38,39] Moreover, in LNCaP cells, upregulation of miR-34a by enoxacin induced the downregulation of SIRT1, which is overexpressed in PCa.[40] On the contrary, in DU145, SIRT1 downregulation cannot be attributed to miR-34a, as this was not found to be upregulated following enoxacin exposure. However, because SIRT1 may be regulated by other miRNAs, this hypothesis may not be ruled out completely at this point. In spite of globally upregulating the expression of miRNAs, enoxacin also caused a decrease in the expression of some oncogenic miRNAs, including miR-141[41] and miR-191,[42] reported as oncomiRs in PCa. miR-141 is a target of androgen regulation and it has been suggested that its upregulation may enhance the growth of CRPC cells.[41] Finally, although less studied, miR-191 has also been reported as being overexpressed in PCa.[42] Thus, the simultaneous upregulation of tumor-suppressor miRNAs and downregulation of oncomiRs by enoxacin in PCa cells highlights the therapeutic relevance of this drug in PCa. Notwithstanding, enoxacin also affected the expression levels of several other miRNAs, which play a role in different types of cancer, but with an unknown function in PCa. Thus, further studies are mandatory to disclose the biological function of these miRNAs in PCa.

In conclusion, enoxacin constitutes a promising therapeutic agent and in vivo studies should be conducted to further support the potential of enoxacin for therapy of PCa patients.

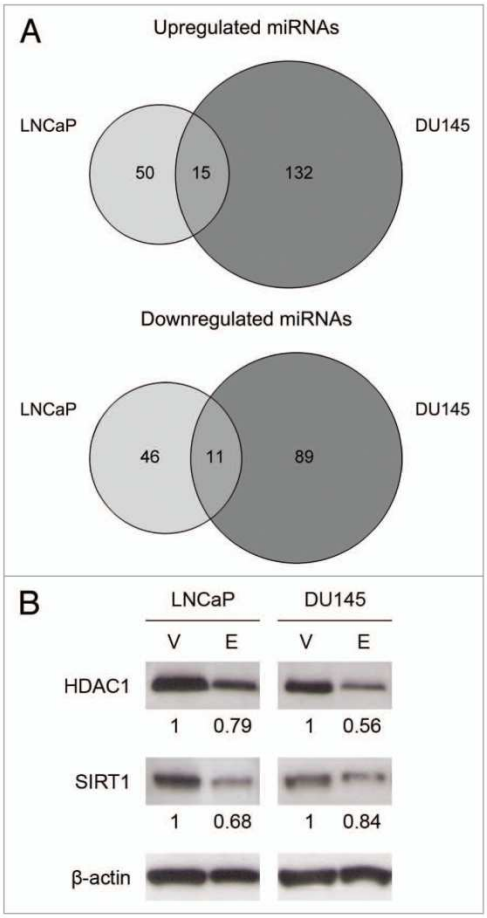

Figure 6. Enoxacin impact on miRNA. (**A**) Venn diagrams depicting the number of differentially expressed miRNAs after enoxacin exposure compared with the vehicle group. Top, miRNAs with increased expression (fold change ≥ 1.5); bottom, miRNAs with decreased expression (fold change ≤ −1.5). (**B**) Protein expression of two miRNA targets (HDAC1 and SIRT1) was analyzed by Western Blot in LNCaP and DU145 cell lines after exposure to enoxacin 40 μg/mL or DMSO (vehicle) at day five. The picture is representative of three independent experiments. β-actin was used as a loading control and the relative density of bands was densitometrically quantified.

Materials and Methods

Cell lines, drug preparation and exposure. DU145 was obtained from the American Type Culture Collection (ATCC), whereas LNCaP, VCaP and PC-3 were kindly provided by Prof. Ragnhild A. Lothe from the Department of Cancer Prevention at The Institute for Cancer Research (Oslo, Norway) and 22Rv1 by Dr. David Sidransky from the Johns Hopkins University School of Medicine (Baltimore, MD). For control purposes, we used the human colon carcinoma-derived cell line Co115, which was kindly provided by Prof. Fátima Baltazar from the Life and Health Sciences Research Institute at the University of Minho (Braga, Portugal). All cell lines were cultured in the recommended medium, supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin (GIBCO). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. All PCa cell lines were karyotyped by G-banding (for validation purposes) and routinely tested for Mycoplasma spp contamination (PCR Mycoplasma Detection Set, Clontech Laboratories). Enoxacin (Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at −20°C until further use. PCa cells were continuously exposed to 40 μg/mL (124 μM) enoxacin, for 5 d. For control purposes, cell lines were exposed to the vehicle of the drug (DMSO) only.

Direct sequencing. Genomic DNA was extracted from cell lines and prostatic cancer tissue using a standard technique comprising digestion with proteinase K (20 mg/mL) in the presence of 10% SDS at 55°C, followed by phenol-chloroform extraction and precipitation with 100% ethanol.[43] *TARBP2* was screened for mutations using the primers previously described by Melo et al.[19] Direct sequencing was performed in an ABI PRISM 310 automatic sequencer using Big Dye Terminator Chemistry (Applied Biosystems), according to the manufacturer's recommendations.

Western blot. Protein extraction from whole-cell lysates was obtained using RIPA lysis buffer (Santa Cruz Biotechnology). Protein concentrations were determined using a Pierce BCA assay (Thermo Scientific Inc.), according to the manufacturer's instructions. Briefly, 30 μg of protein from each sample were separated using 10% Mini-PROTEAN® TGX™ Precast Gel (Bio-Rad) at 200 V and subsequently blotted onto Protran nitrocelulose transfer membranes (Whatman) using Trans-Blot® Turbo™ Transfer System (Bio-Rad). Membranes were incubated with primary antibodies at 4°C overnight. Primary antibodies were polyclonal rabbit against TRBP (1:1,000, Abcam), polyclonal rabbit against DICER (1:500, Santa Cruz Biotechnology), monoclonal mouse against cleaved PARP (1:500, Cell Signaling), monoclonal mouse antibody against HDAC1 (1:1,000, Sigma-Aldrich) and polyclonal rabbit against SIRT1 (1:1000, Abcam). The membranes were developed using Immun-Star WesternC Chemiluminescent kit (Bio-Rad) and exposed to Amersham Hyperfilm (GE Healthcare). To ascertain equal loading of protein, the membranes were stripped and reprobed with a monoclonal mouse antibody against β-Actin (1:8,000, Sigma-Aldrich). To relate the protein band intensity with the loading control (β-Actin), protein band intensities were determined using Quantity One software (Bio-Rad).

Patients and sample collection. Fifty prostate tumor samples from patients with clinically localized PCa (clinical stage II: T1cN0M0 or T2N0M0, according to the TNM staging system) consecutively diagnosed and primarily treated with radical prostatectomy at the Portuguese Oncology Institute Porto, Portugal, were prospectively collected from 2001 to 2006. Tumor tissue was routinely fixed in buffered formalin and paraffin-embedded. All patients were enrolled after informed consent. This study was approved by the institutional review board (Comissão de Ética do IPO Porto).

Immunohistochemistry. TRBP expression in tumor tissue samples was assessed by immunohistochemistry using the Novolink™ Polymer Detection System (Novocastra). Deparaffinized tissue sections were submitted to antigen retrieval in a 700-W microwave oven, in 1x EDTA buffer solution. Endogenous peroxidase activity was blocked by incubating the slides with Peroxidase Block (Novocastra) for 5 min. The slides were incubated with Protein Block (Novocastra) for 5 min, and after incubation, primary antibody against TRBP (1:100, Abcam) was applied for 1 h in a humid chamber, at room temperature. The slides were then incubated with Post Primary Block (Novocastra) for 30 min followed by incubation for 30 min with the NovoLink Polymer (Novocastra). After incubation in 3,3-diaminobenzidine (DAB; Sigma-Aldrich) in a solution of 50 mL PBS/0.05% mL H_2O_2 for 7 min, the slides were counterstained with hematoxylin (Harris Modified Hematoxylin Stain; Fisher Scientific) for 20 sec and mounted with Entellann (Merck KGaA). Colorectal cancer tissues showing intense immunoreactivity for TRBP protein were used as positive control. The negative control consisted on the omission of the primary antibody. The assessment of immunostaining results was performed by an experienced pathologist and was expressed in a semiquantitative way according to the estimated percentage of positive tumor cells. Immunostaining of more than 10% of the tumor cells was required for scoring a case as positive.

Cell viability assay. Cell viability was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide (MTT) assay. In brief, PCa cells were seeded onto 96-well plates (Sarstedt) at 2×10^3 cells per well and allowed to adhere overnight. After exposure to the drug, cell viability was measured at 1, 2, 3, 4 and 5 d. Briefly, 200 μL of 0.5 mg/mL MTT (Sigma-Aldrich) were added to each well and incubated at 37°C and 5% CO_2 for 3 h. Formazan crystals were solubilized with 100 μL of DMSO. The absorbance was measured using a microplate reader (Fluostar Omega) at a wavelength of 540 nm with background subtraction at 630 nm. The number of cells was calculated using the formula: [(OD experiment × Number of cells at day 0)/Mean OD at day 0].

Apoptosis assay. Apoptosis evaluation was performed using the APOPercentage apoptosis assay kit (Biocolor Ltd.) according to the manufacturer's instructions. This assay is based on phosphatidylserine transmembrane movements, which results in the uptake of the APOPercentage dye by apoptosis-committed cells. Cells were seeded onto 24-well plates (Sarstedt) at 5×10^4 cells per well and apoptosis levels were assessed at days 2 and 5. The absorbance was determined using a microplate reader (Fluostar Omega) at a wavelength of 550 nm with background subtraction at 620 nm. To normalize the OD measured in the apoptosis test, according to the cell number, the OD of the apoptosis assay was divided by the OD of the cell viability assay. The results were expressed as the ratio of the OD of the cells exposed to enoxacin to that of vehicle cells (set as 1).

Cell cycle analysis. Cell cycle distribution was determined by flow cytometry. Briefly, 5×10^5 harvested cells were fixed with 70% cold ethanol overnight at 4°C. After washing with cold PBS, cells were resuspended in staining Propidium Iodide Solution (Cytognos S.L.) and incubated at room temperature for 30 min in the dark. Cell cycle data were collected using Cytomics FC500 flow cytometer (Beckman Coulter) and analyzed with Modfit LT (Verity Software House Inc.).

Table 2. Effect of enoxacin on the expression of several miRNAs already implicated in PCa

Cell Line	miRNAs	Fold change	p value
LNCaP	miR-29b	2.9	0.023
	miR-449a	1.8	0.012
	miR-34a	2.3	0.014
	miR-191	−2.3	0.023
DU145	miR-449a	2.2	0.011
	miR-146a	1.8	0.008
	miR-29b	1.7	0.004
	miR-132	1.7	0.003
	miR-17*	1.6	0.008
	miR-141	−1.7	0.002

Cell invasion assay. Cell invasion was evaluated using the Oris™ Cell Invasion Assay (Platypus Technologies) according to the manufacturer's instructions. After starvation for 18 h in a serum-free medium, cells were seeded at 5×10^4 cells per well and exposed to 40 μg/mL of enoxacin or DMSO for 8 d. At this time point, cells were labeled with Calcein AM (AnaSpec) at a final concentration of 0.5 μg/mL. A detection mask was attached to the bottom of the plate to block from view all cells except those that had invaded into the center zone and fluorescence was measured at excitation and emission wavelengths of 492 nm and 530 nm, respectively, using a microplate reader (Fluostar Omega). The results were expressed as the ratio of the fluorescence index (FI) of the cells exposed to enoxacin to that of vehicle cells (set as 1).

Quantitative reverse transcription PCR (qRT-PCR). RNA was extracted from all cell lines using TRIzol® (Invitrogen) according to manufacturer's instructions and cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). *CASP3* mRNA expression levels were assessed using TaqMan gene expression assay, purchased as predeveloped assays from Applied Biosystems. The mean quantity of *CASP3* expression levels of cell lines samples was normalized against mean quantity of the respective endogenous control (*GUSB*) expression levels.

miRNA expression assessment by miRCURY LNA™ array. cDNA synthesis and real-time qRT-PCR was performed using the miRCURY LNA™ Universal RT microRNA PCR system (Exiqon) according to the manufacturer's instructions. In brief, the cDNA products were transferred to the Ready-to-use microRNA PCR Human Panels (I + II) and quantified using *SYBR* green based real-time PCR and LNA enhanced miRNA specific primers. Based on miRBase 11.0 annotation, a total of 757 human miRNA probes were interrogated in this platform. Three independent experiments were performed. The data were imported into GenEX software (MultiD Analyses AB) for subsequent microarray analysis. Global mean normalization was used as a normalization factor for miRNA.[44] miRNAs with fold change above 1.5 or below −1.5 were considered up or downregulated, respectively.

Statistical analysis. Two-tailed Student's t-test was used to assess differences between the results obtained after exposure to enoxacin and after exposure to vehicle. Prior to application of the test, all data were transformed to logarithmic scale. Analysis was performed with the aid of SPSS software for Windows, version 20.0 (IBM-SPSS Inc.), and the statistical significance level was set at $p < 0.05$. Graphics were built using MATLAB 7.10.0 r2010a software.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Supplemental Materials

Supplemental material may be found here:
http://www.landesbioscience.com/journals/epigenetics/article/24519

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II

PAPER II

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Anti-Tumoral Effect of the Non-Nucleoside DNMT Inhibitor RG108 in Human Prostate Cancer Cells

Inês Graça[1,2,5], Elsa J. Sousa[1,2], Tiago Baptista[1,2], Mafalda Almeida[1,2], João Ramalho-Carvalho[1,2], Carlos Palmeira[4], Rui Henrique[1,3,6] and Carmen Jerónimo[1,2,6,*]

[1]*Cancer Epigenetics Group, Research Center of the Portuguese Oncology Institute Porto, Departments of* [2]*Genetics, and* [3]*Pathology,* [4] *Hematology-Laboratory of Flow Cytometry, Portuguese Oncology Institute Porto,* [5] *School of Allied Health Sciences ESTSP, Polytechnic of Porto,* [6]*Department of Pathology and Molecular Immunology, Institute of Biomedical Sciences Abel Salazar, University of Porto*

Abstract: Background: Current therapeutic strategies for advanced prostate cancer (PCa) are largely ineffective. Because aberrant DNA methylation associated with inappropriate gene-silencing is a common feature of PCa, DNA methylation inhibitors might constitute an alternative therapy. In this study we aimed to evaluate the anti-cancer properties of RG108, a novel non-nucleoside inhibitor of DNA methyltransferases (DNMT), in PCa cell lines.

Methods: The anti-tumoral impact of RG108 in LNCaP, 22Rv1, DU145 and PC-3 cell lines was assessed through standard cell viability, apoptosis and cell cycle assays. Likewise, DNMT activity, *DNMT1* expression and global levels of DNA methylation were evaluated in the same cell lines. The effectiveness of DNA demethylation was further assessed through the determination of promoter methylation and transcript levels of *GSTP1*, *APC* and *RAR-β2*, by quantitative methylation-specific PCR and RT-PCR, respectively.

Results: RG108 led to a significant dose and time dependent growth inhibition and apoptosis induction in LNCaP, 22Rv1 and DU145. LNCaP and 22Rv1 also displayed decreased DNMT activity, *DNMT1* expression and global DNA methylation. Interestingly, chronic treatment with RG108 significantly decreased *GSTP1*, *APC* and *RAR-β2* promoter hypermethylation levels, although mRNA re-expression was only attained for *GSTP1* and *APC*.

Conclusions: RG108 is an effective tumor growth suppressor in most PCa cell lines tested. This effect is likely mediated by reversion of aberrant DNA methylation affecting cancer related-genes epigenetically silenced in PCa. However, additional mechanism might underlie the anti-tumor effects of RG108. *In vivo* studies are now mandatory to confirm these promising results and evaluate the potential of this compound for PCa therapy.

Keywords: Prostate cancer, RG108, DNA methyltransferases, proliferation, apoptosis.

INTRODUCTION

Prostate cancer (PCa) is the most common diagnosed malignancy in Western countries and one of the leading causes of cancer-related morbidity and mortality [1]. Although effective therapy is available for early-stage PCa, treatment of advanced disease is mainly ineffective and remains a clinical challenge.

Androgen-deprivation therapy is widely used for locally advanced and systemically spread PCa. However, most patients that initially respond to androgen deprivation therapy have a median time to progression to castration-resistant disease of only 18-30 months [2]. Therefore, investigation of new therapeutic strategies is urgently needed and should be based, ideally, on the acquired knowledge about PCa biology. Indeed, PCa is a complex and heterogeneous disease where multiple genetic and epigenetic alterations interplay and contribute to its development and progression [3]. The most widely known epigenetic change in PCa is aberrant DNA methylation, which may lead to altered expression of tumor suppressor genes and proto-oncogenes. In fact, there are more than 50 genes reported to be silenced by promoter hypermethylation in PCa, including *GSTP1*, *APC*, *RARβ2*, *RASSF1A*, and *CCND2*, among others [4-6]. Remarkably, unlike genetic alterations, epigenetic changes are chemically reversible and numerous compounds have been reported to be effective against cancer cells by inhibiting one or more components of the epigenetic machinery. Among these are the DNA methyltransferases inhibitors (DNMTi), of which 5-azacytidine (5-Aza-CR) and 5-aza-2´-deoxycytidine (5-Aza-dCR) are the most widely described [7]. Interestingly, 5-Aza-dCR was shown to have modest clinical activity against castration-resistant PCa and to inhibit prostate cancer progression in TRAMP mice [8, 9]. However, the effectiveness of these compounds relies on its incorporation into DNA, which may result in substantial, dose-dependent cytotoxicity [10]. Owing to the toxicity of nucleoside analogs, emphasis has been placed in the discovery of new compounds that more directly target DNMTs. One of those compounds is RG108, a non-nucleoside analogue of DNMT designed to target human DNMT1 at its active site. RG108 was shown to be effective in human colon cancer cells, in which it was capable of reactivating several tumor suppressors genes (*e.g.*, *p16*), without affecting the methylation status of centromeric repeats [11, 12]. Importantly and contrarily to 5-Aza-dCR, the DNA demethylating activity of RG108 was not associated with high levels of citotoxicity [11, 12]. To the best of our knowledge, the anti-tumoral effect of this compound has not been explored in PCa.

Thus, we sought to investigate the potential of RG108 as a growth inhibitor of PCa cells. For that purpose, the impact of RG108 in cell viability, apoptosis and cell cycle of several PCa cell lines was assessed. Furthermore, the inhibitory effect on DNMT and consequent DNA demethylation activity of this drug were evaluated, along with the re-expression of genes known to be epigenetically silenced in PCa.

*Address correspondence to this author at the Department of Genetics & Research Center, Portuguese Oncology Institute - Porto, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal; Tel: +351 225084000 (ext. 7264); Fax: +351 225084016; E-mail: carmenjeronimo@ipoporto.min-saude.pt/ cljeronimo@icbas.up.pt

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MATERIALS AND METHODS

Prostate Cancer Cell Lines and Drug Preparation

Prostate cancer cell lines PC-3 and LNCaP were kindly provided by Prof. Ragnhild A. Lothe from the Department of Cancer Prevention at the Institute for Cancer Research, Oslo, Norway, DU145 was obtained from American Type Culture Collection (ATCC, Lockville, MD), and 22Rv1 cells were kindly provided by Dr. David Sidransky at the Johns Hopkins University School of Medicine, Baltimore, MD, USA. LNCaP and 22Rv1 cells were grown in RPMI 1640, DU145 cells were maintained in MEM and PC-3 cells were grown in 50% RPMI-50% F-12 medium (GIBCO). All basal culture media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (GIBCO). Cells were maintained in an incubator at 37ºC with 5% CO2. All PCa cell lines were karyotyped by G-banding (for validation purposes) and routinely tested for Mycoplasma spp. contamination (PCR Mycoplasma Detection Set, Clontech Laboratories). RG108 was purchased from Tocris Bioscience (Bristol, UK), dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA), and stored at -20ºC until further use. 5-Aza-dCR was obtained from Sigma (Sigma-Aldrich), dissolved in 50% acetic acid-50% PBS and stored at -20ºC. For control purposes, cell lines were exposed to the vehicles of the drugs only (DMSO or 50% acetic acid-50% PBS).

Viability Assay

PCa cells were seeded at 1000 cells per well onto 96-well flat bottoned culture plates, allowed to adhere overnight and treated with different RG108 concentrations (*i.e.*, 50, 100 and 200 μM) for 3 days (acute exposure) and 14 days (chronic exposure). Cell viability was then evaluated by MTT assay. Briefly, 0.5 mg/ml of MTT reagent [3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] was added to each well, and the plates were incubated in the dark for 3 hours at 37ºC. Formazan crystals were then dissolved in DMSO and absorbance was read at 540 nm in a microplate reader (FLUOstar Omega, BMG Labtech, Offenburg, Germany), subtracting the background, at 630 nm. Three replicates were performed for each condition, and at least two independent experiments were carried out.

Apoptosis Evaluation

Evaluation of apoptosis was performed using APO Percentage apoptosis assay kit (Biocolor Ltd., Belfast, Northern Ireland) according to the manufacturer's instructions. The assay was carried out using the same cell conditions as those of the MTT assay. Apoptotic cells were assessed at the end of the day 3 and 14 in a FLUOstar Omega microplate reader at 550 nm.

Cell Cycle Analysis

Cell cycle distribution of PCa cells was determined by flow cytometry. Briefly: $5x10^5$ harvested cells were fixed overnight at 4ºC with 70% cold ethanol. After washing with cold PBS, cells were ressuspended in staining Propidium Iodide Solution (Cytognos S.L, Salamanca, Spain) and incubated for 30 minutes at room temperature. All cells were then measured on a Cytomics FC500 flow cytometer (Beckman Coulter, Fullerton, CA, USA) and analyzed using Modfit LT (Verity Software House, Inc, Topshan, Maine, USA).

Real Time Quantitative PCR (qRT-PCR)

RNA was extracted from cell lines using TRIzol® (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. First strand synthesis was performed using the high-capacity CDNA Reverse Transcription Kit from Applied Biosystems (Foster City, CA). Expression of target genes (*Ki67*, *CDKN1A*, *APC*, *GSTP1, RARβ2, DNMT1, DNMT3a, DNMT3b*) was quantified using Taqman expression assays, purchased as pre-developed assays from Applied Biosystems and normalized to the expression of the *GUSB* housekeeping gene.

Western Blot

Proteins were extracted from whole-cell lysates using RIPA lysis buffer (Santa Cruz Biotechnology, CA, USA) and subsequently quantified using a Pierce BCA assay (Thermo Scientific Inc., Bremen, Germany), according to the manufacturer's instructions. Briefly, 30 μg of protein from each sample were separated using 4–20% Mini-PROTEAN® TGX™ Precast Gel at 300 V and subsequently blotted onto Protran nitrocelulose transfer membranes (Whatman, Dassel, Germany). For immunodetection, membranes were incubated overnight at 4ºC with antibodies directed against DNMT1 at 1:1,000 and active Caspase-3 at 1:200 (Cell Signaling Technology, Danvers, MA). The Immun-Star WesternC Chemiluminescent kit (Bio-Rad, Hercules, CA, USA) was used to develop the membranes which were then recorded with Amersham Hyperfilm (GE Healthcare Buckinghamshire, UK). To ascertain equal loading of protein, the membranes were incubated with a monoclonal mouse antibody against β-Actin (1:8,000, Sigma-Aldrich).

DNMT Activity/Inhibition Assay

Nuclear extracts from vehicle and RG108 treated LNCaP, 22Rv1 and DU145 were obtained using Nuclear Extract Kit (Active Motif, Rixensart, Belgium) as per the manufacture's protocol. After protein quantification, 10μg of nuclear lysate from each sample was used to measure DNMT activity or inhibition with DNMT Activity/Inhibition Assay Kit (Active Motif) according to the manufacturer's instructions. Absorbance was measured using FLUOstar Omega microplate reader, at 450 nm. All samples were analyzed in triplicate.

Quantification of Global DNA Methylation

Global DNA methylation was analyzed by the Imprint® Methylated DNA Quantification Kit (Sigma-Aldrich) according to the manufacturer's instructions. DNA methylation status was compared with an artificially fully methylated DNA positive control. Absorbance was measured using FLUOstar Omega microplate reader at 450 nm. All samples were analyzed in triplicate.

Quantitative Methylation Specific PCR (qMSP)

Genomic DNA was extracted from cell lines using a standard technique comprising digestion with proteinase K (20mg/mL) in the presence of 10% SDS at 55ºC, followed by phenol-chloroform extraction and precipitation with 100% ethanol [13]. One microgram of DNA was submitted to bisulfite modification using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Orange, CA) following the manufacturer's instructions. Bisulfite modified DNA was amplified by qMSP using TaqMan technology [14]. Specific *GSTP1*, *APC* and *RARβ2* primers and TaqMan probes were designed using the Methyl Primer Express Software v1.0 (Applied Biosystems). *β-actin* (*ACTB*) was used as an internal reference gene to normalize for DNA input and all qMSP reactions were performed as previously described [4]. Methylation levels for each sample were derived from calibration curves constructed using serial dilutions of bisulfite modified CpGenomeTM Universal Methylated DNA (Millipore, Billerica, MA). *GSTP1*, *APC* and *RAR2β* methylation levels were calculated after normalization for *ACTB.*

Statistical Analysis

One-way analysis of variance (ANOVA), with post-hoc Dunnet's multiple comparison test when appropriate, was used to compare the results obtained in each parameter for the three different concentrations of RG108 and the controls (vehicle). Analysis was performed with SPSS software for Windows version 19.0 (IBM-SPSS Inc Chigaco, IL) and statistical significance was set at $P<0.05$.

RESULTS

RG108 Inhibits Cell Viability and Induces Apoptosis in Human PCa Cells

To investigate the effects of the demethylating agent RG108 on cell viability, four human PCa cell lines, LNCaP, 22Rv1, DU145 and PC-3 were exposed to three different concentrations of RG108, as wells as to the vehicle of this compound, DMSO. Cell viability was evaluated at days 0, 1, 2, 3, 7, 10 and 14. Acute exposure to RG108 resulted in a slight decrease in the number of viable cells (Fig. **1A**). Regarding LNCaP, there was a significant decrease in cell viability at the end of the second day when treated with 200 μM of RG108 (P<0.04) compared to the vehicle. 22Rv1 showed a decrease in cell viability at day 1 when exposed to 50 and 200 μM of RG108 (P<0.04) and at the end of the third day when 100 and 200 μM concentrations were applied (P<0.03). DU145 treated cells also suffered a slight reduction in viable cells on the first day with 50 and 100 μM concentrations (P<0.02) and at day 3 when exposed to 200μM (P<0.002).

As shown in (Fig. **1A**), chronic exposure induced an impressive reduction in viable cells especially when the higher concentration of RG108 was used. LNCaP was the most sensitive cell line, since the inhibition of cell growth occurred for all the tested days at all the tested drug concentrations (P<0.002). LNCaP exposure to 200 μM RG108 during 14 days resulted in an 80% reduction in viable cells. 22Rv1 also displayed a decrease in the number of viable cells in all days (7, 10, and 14) with 200 μM RG108 (P<0.004), being the reduction after 14 days of treatment of 50%, whereas DU145 only showed a decrease of 10% in cell viability in the last day with 100 and 200 μM RG108 (P<0.004). RG108 appeared to have no effect on PC-3 cell viability.

To corroborate the previous results, an analysis of molecular changes induced by RG108 was carried out by studying the quantitative expression of two genes involved in cell proliferation pathways. Since the best results were obtained with 14 days of exposure and 200 μM of RG108, it was decided to evaluate gene expression under these conditions and at the concentration of 50 μM. The results showed both induction of *CDKN1A* (P<0.020) and decrease of *KI67* (P<0.045) mRNA levels of the RG108 treated cells when compared to the vehicle (Fig. **1B**).

To determine if the exposure to RG108 was capable of induce significant cell death, an apoptotic assay that detects the phosphatidylserine transfer to the outside of the membrane was performed. Acute exposure to RG108 resulted in a significant increase in apoptosis (Fig. **1C**) for LNCaP with 100 and 200 μM (P<0.037) and for 22Rv1 and DU145 with 100 μM (P≤0.002). Chronic treatment induced an augment in apoptosis (Fig. **1C**) in LNCaP cells when treated with 100 μM (P<0.038) and DU145 when exposed to 50 and 200 μM (P<0.007).

To confirm apoptosis at molecular level, the mRNA levels of *CASP3* were evaluated. As the best results achieved with the apoptosis assay were obtained after 14 days, only this time point was evaluated. Chronic treatment of LNCaP (100 μM), 22Rv1 (50 μM) and DU145 (200μM) resulted in significantly increased levels of *CASP3* (P<0.029) (Fig. **1D**). An increase of active Caspase 3 protein levels' was observed in all cell lines after drug exposure, namely in LNCaP and DU145 exposed to RG108 at 50 and 200 μM and in 22Rv1 when exposed to 100 μM (Fig. **1E**).

Since RG108 did not affect the cancer phenotype of PC-3 cell line, further studies were only performed in LNCaP, 22Rv1 and DU145 with 50 and 200 μM RG108.

Cell cycle distribution was evaluated after 14 days of exposure to 50 and 200 μM RG108. There was no evidence of cell cycle arrest in LNCaP and 22Rv1. However, DU145 presented a significant increase in the percentage of cells in G2/M transition for both concentrations of the compound (P<0.0001) (Fig.**1F**).

RG108 Inhibits DNMT Activity and Expression in Human PCa Cells

After exposing cells to 50 and 200 μM of RG108 for 14 days, nuclear extracts were obtained and DNMT activity was determined. As observed in (Fig. **2A**), there was a significant reduction in DNMT activity (15%) for 22Rv1 with 50 μM RG108 (P<0.003). For the remaining two cell lines a significant decrease in DNMT activity was not apparent, although LNCaP displayed a slight reduction (13%).

Subsequently, we determined whether decreased DNMT activity correlated with decreased *DNMT1*, *DNMT3a and DNMT3b* mRNA levels. As expected, 22Rv1 and LNCaP showed decreased levels of *DNMT1*and *DNMT3b* compared to the vehicle, 22Rv1 cell line after exposure to 50 μM RG108 (P<0.013) and LNCaP after treatment with both tested concentrations (P<0.019). However, only 22Rv1 demonstrated a significant reduction in *DNMT3a* mRNA levels (P<0.001) (Fig. **2B**). Importantly, a reduction in DNMT1 protein expression was displayed by both cell lines (Fig. **2C**).

RG108 Decreases Global DNA Methylation

Once verified the ability of this compound to decrease DNMT activity and expression, the next step was to investigate the effect in global DNA methylation content. Since DU145 did not present a significant decrease in DNMT activity and expression, it was not further studied. To compare the effect of this demethylating agent with that of 5-Aza-dCR (a widely used demethylating drug), LNCaP and 22Rv1 cells were exposed to 1 and 5 μM of 5-Aza-dCR during 3 days.

RG108 significantly decreased global DNA methylation in 22Rv1 with 200 μM (approximately 5%, P<0.028), and in LNCaP cells (approximately 11% and 9% with 50 and 200 μM respectively, P<0.001) (Fig. **3A**). In contrast, 5-Aza-dCR was able to reduce global DNA methylation in about 14% in LNCaP cells treated with 1 μM (P<0.001) and approximately 16% in 22Rv1 for both concentrations (P<0.001) (Fig. **3B**).

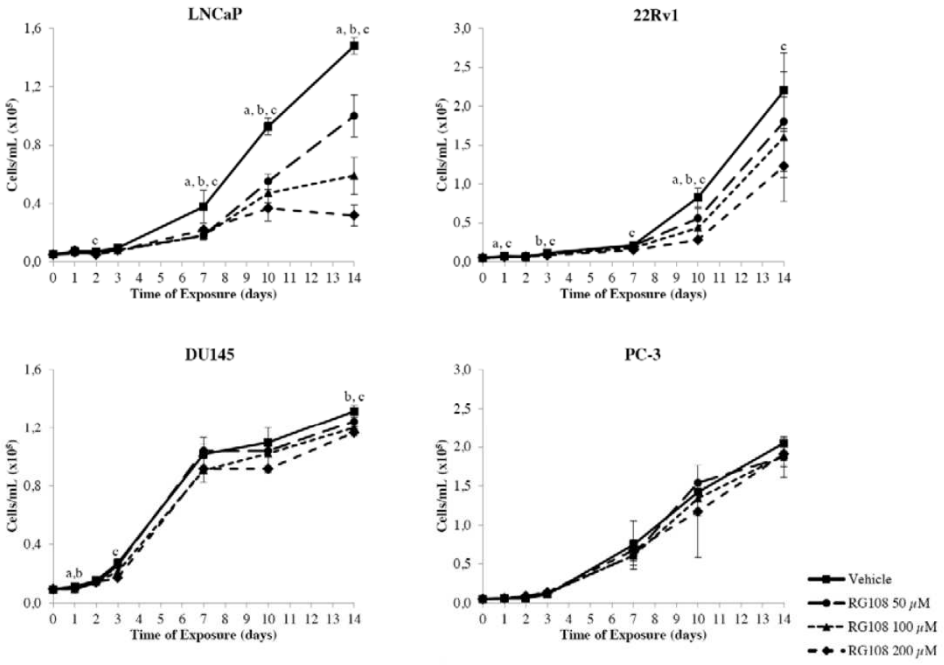
RG108 Causes Demethylation and Reactivation of Silenced Genes in Human PCa Cells

To investigate the ability to demethylate and consequently induce the expression of specific genes, a panel of three genes (*APC*, *GSTP1* and *RARβ2*), previously reported to be silenced by promoter hypermethylation in PCa, was evaluated.

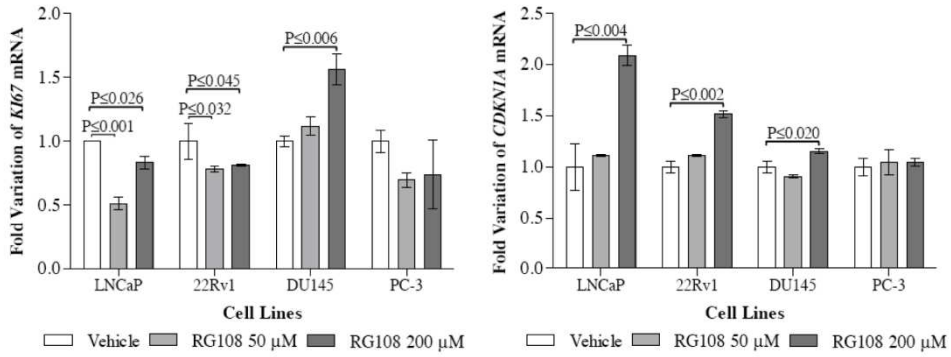
Both LNCaP and 22Rv1 showed a decrease in methylation levels of *GSTP1* with 50 μM (P≤0.009) whereas solely 22Rv1 exhibited similar results with 200 μM (P<0.005) (Fig. **4A**). However, only 22Rv1 demonstrated an increase in *GSTP1* mRNA expression with 200 μM (P<0.001) (Fig. **4B**). *APC* methylation levels were reduced in 22Rv1 with 50 μM of RG108 (P<0.005) (Fig. **4A**), concomitantly with the induction of mRNA expression of this gene (P<0.003) (Fig. **4B**). Although a reduction in *RARβ2* methylation was observed in LNCaP with 200 μM (P<0.036) and in 22Rv1 with 50 μM (P<0.003) of RG108 (Fig. **4A**), the re-expression of this gene was not achieved (Fig. **4B**).

The results were also compared with those obtained with 5-aza-dCR. This compound was able to demethylate *GSTP1* with 1 and 5 μM in LNCaP and with 1μM in 22Rv1 (P<0.020). *APC* methylation was only reduced in LNCaP cells exposed to 1 μM of 5-Aza-dCR (P<0.037). Concerning *RARβ2,* no significant reduction in methylation levels was observed after exposure to 5-Aza-dCR (Fig. **5**).

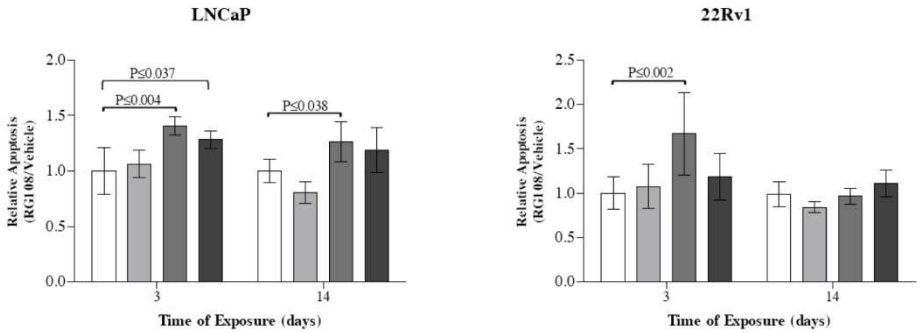
1A

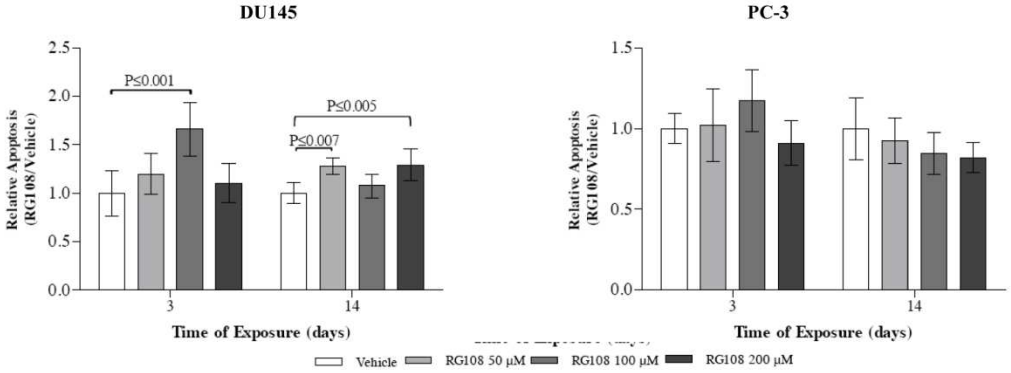

LNCaP
22Rv1
DU145
PC-3
Cells/mL (x10⁶)
Time of Exposure (days)
Vehicle
RG108 50 μM
RG108 100 μM
RG108 200 μM

1B

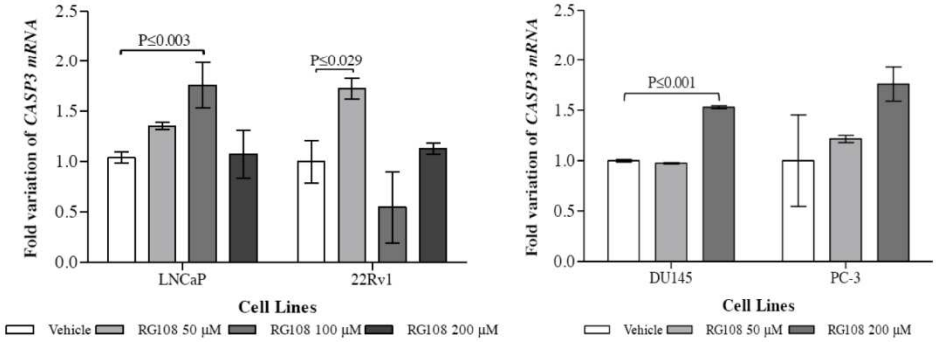

Fold Variation of KI67 mRNA
Fold Variation of CDKN1A mRNA
P≤0.001
P≤0.026
P≤0.032
P≤0.045
P≤0.006
P≤0.004
P≤0.002
P≤0.020
LNCaP
22Rv1
DU145
PC-3
Cell Lines
Vehicle
RG108 50 μM
RG108 200 μM

1C

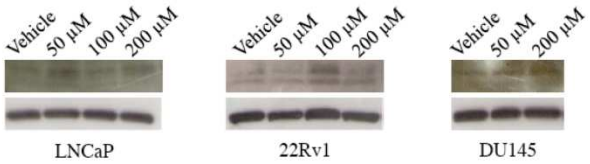

LNCaP
22Rv1
Relative Apoptosis (RG108/Vehicle)
P≤0.037
P≤0.004
P≤0.038
P≤0.002
Time of Exposure (days)



1D



1E



1F

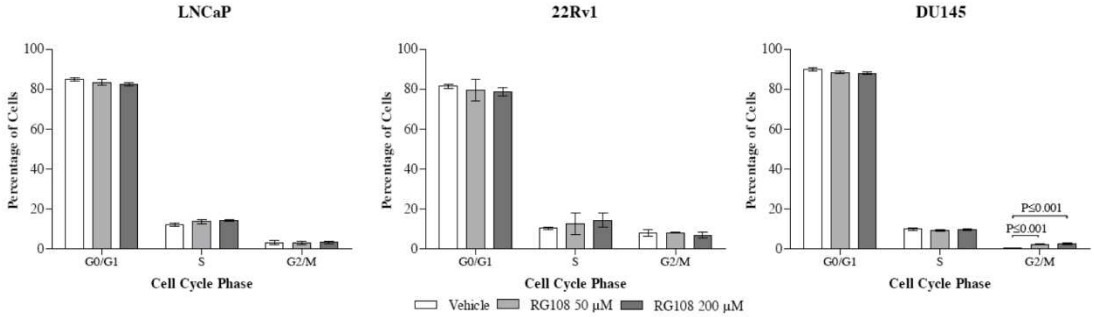


Fig. (1). Effects of RG108 in viability, apoptosis and cell cycle of PCa cell lines. (**A**) Cell viability in LNCaP, 22Rv1, PC-3 and DU145, in the presence of RG108, at days 0, 1, 2, 3,7,10, and 14. (**a**) statistically significant differences were observed between vehicle and 50 µM RG108, (**b**) vehicle and 100 µM RG108 and (**c**) vehicle and 200 µM RG108. (**B**) mRNA expression of *Ki67* and *CDKN1A* after 14 days of exposure to RG108. (**C**) Effect of acute and chronic RG108 exposure in apoptosis of PCa cell lines. (**D**) *CASP3* mRNA expression and (**E**) active CASP3 protein expression after 14 days of exposure to RG108. (**F**) Cell cycle evaluation after 14 days of RG108 exposure. Data are presented as mean of three independent experiments ± s.d.

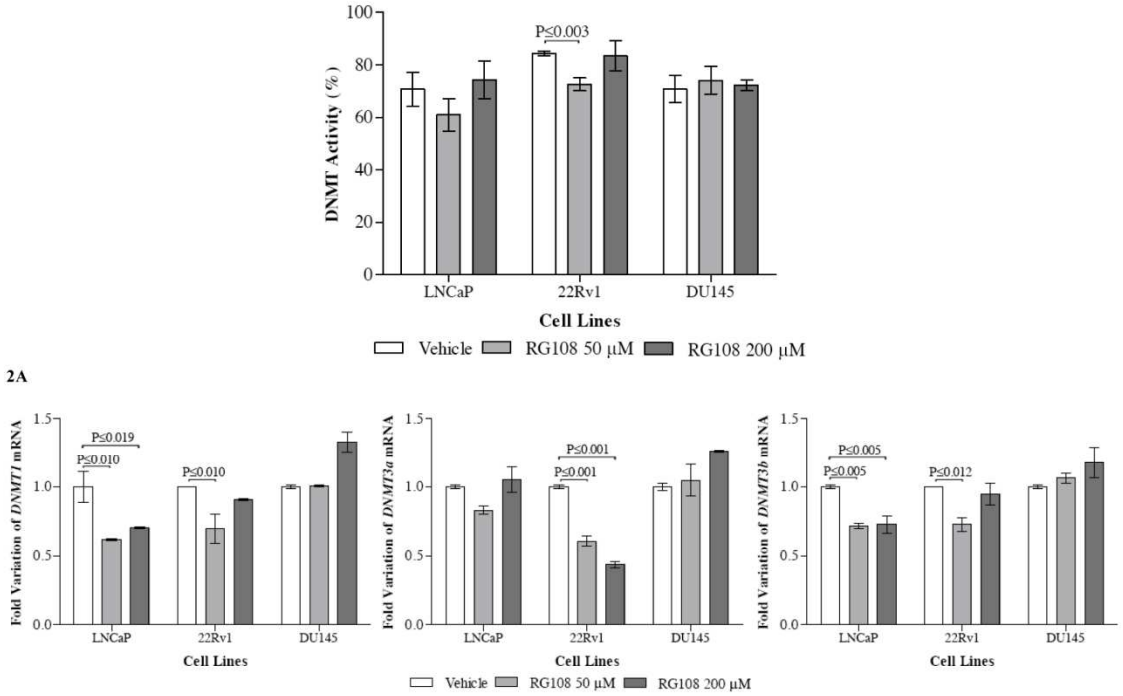


Fig. (2). Impact of RG108 on DNMT activity (**A**), in *DNMTs* mRNA expression (**B**), and DNMT1 protein expression after 14 days exposure to RG108. Data are shown as mean of three independent experiments ± s.d.

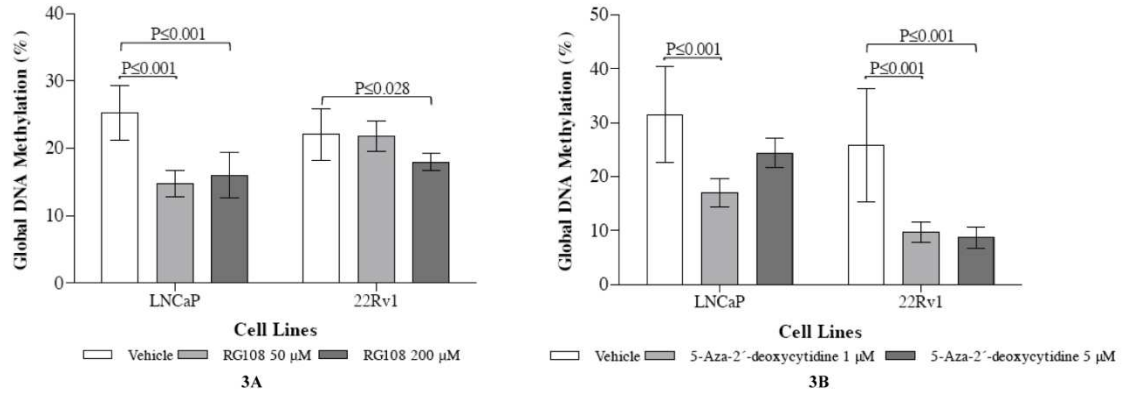
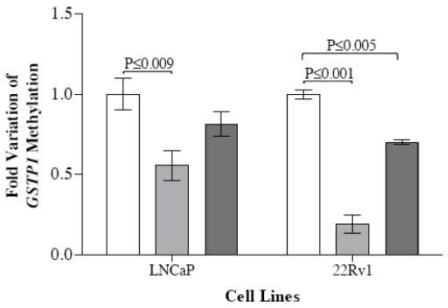
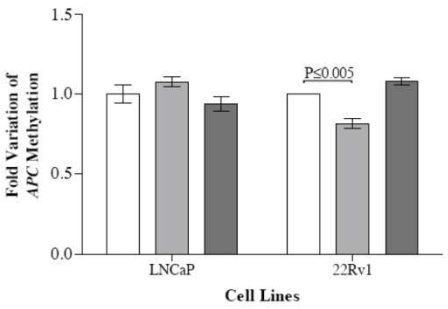


Fig. (3). Effect of RG108 (**A**) and 5-aza-CdR (**B**) on global DNA methylation. Data are presented as mean of three independent experiments ± s.d.

Fig. (4A)





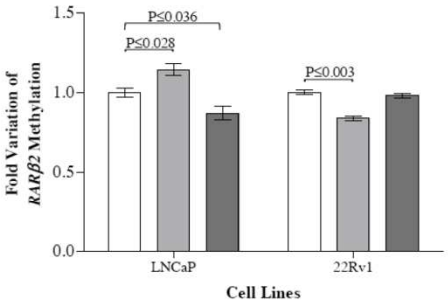
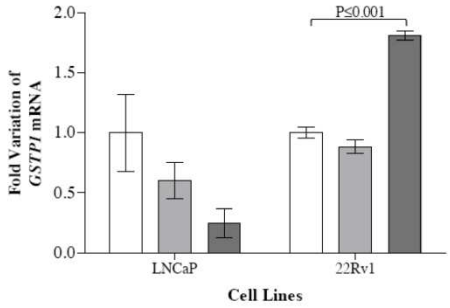
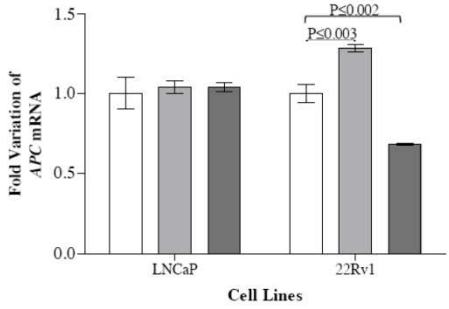


Fig. (4B)





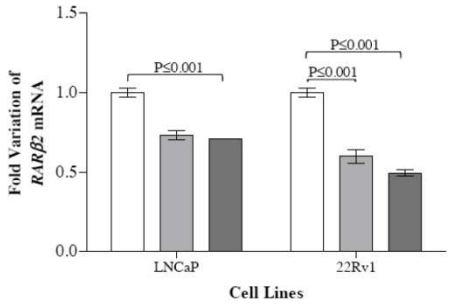


Fig. (4). Impact of RG108 on demethylation (**A**) and re-expression (**B**) of *GSTP1*, *APC* and *RARβ2*. Data are shown as mean of three independent experiments ± s.d.

DISCUSSION

The gold standard treatment for hormone dependent PCa involves surgery, radiation or androgen deprivation therapy, depending mainly on disease stage, whereas chemotherapy with docetaxel represents the main therapeutic option for castration-resistant PCa [15, 16]. In PCa, as well as in other cancers, the resistance of malignant cells to anticancer agents remains the major cause of treatment failure. Therefore, new therapeutic options are urgently needed. Epigenetic silencing of gene expression, especially that mediated by promoter hypermethylation, plays an important role in the development and progression of PCa, as well as in the emergence of resistance to chemotherapy [17, 18]. Thus, DNMTs might constitute a valuable therapeutic target for PCa treatment. Reversal of DNA methylation with DNMTi has being widely studied in several cancers. In fact, two nucleoside analogs 5-azacytidine and 5-aza-2′-deoxycitidine have been already approved by US Food and Drug Administration (US FDA) for the treatment of myelodysplastic syndrome [19]. However, those two nucleoside analogs have demonstrated a limited efficacy in the treatment of solid malignan-

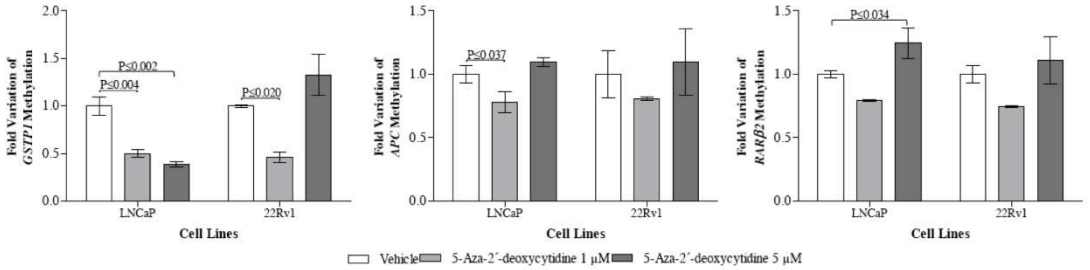


Fig. (5). Effect of 5-Aza-CdR on *GSTP1*, *APC* and *RARβ2* promoter methylation levels. Data are presented as mean of three independent experiments ± s.d.

cies [20, 21]. Both for this reason and owing to their significant cytotoxicity, new epigenetic compounds must be explored for cancer treatment.

Herein, we present the first study on the anti-cancer effect of RG108, a non-nucleoside DNMTi, in PCa. The exposure of PCa cell lines (LNCaP, 22Rv1 and DU145) to RG108 resulted in a significant decrease in cell viability, in a dose and time dependent manner, being the 200 μM concentration and 14 days (chronic) of exposure the best combination tested. Interestingly, these results emphasize the need to achieve sustained levels of the drug to ensure a prolonged anti-tumor effect, which is in line with current therapeutic guidelines for 5-azacytidine and 5-aza-2´-deoxycitidine in myelodisplastic syndrome [22-24]. Moreover, RG108 induced cell death by apoptosis in LNCaP and DU145, and in the latter, cell cycle arrest in G2/M was also depicted. Importantly, these results were confirmed at the molecular level with a decrease of *KI67* transcript levels and induction of *CDKN1A* and *CASP3* mRNA expression. Thus, the ability to disturb multiple regulators of cancer cell survival in these distinct PCa cells lines by RG108 was proven. Because these cell lines are phenotypically different and present diverse patterns of cell response to this drug, it is tempting to suggest that RG108 might be effective against PCa *in vivo*, which is also characterized by substantial molecular heterogeneity [3].

Some previous studies have reported on the cellular proliferation inhibitory effect of 5-aza-dCR in PCa cell lines [25-27]. In fact, 5-aza-dCR induces p53 and p21Waf1/Cip1 expression which is associated with inhibition of cell proliferation and induction of apoptosis in LNCaP cells, independently of DNA methylation [28, 29]. In this study we provide evidence that RG108 has similar effects on the inhibition of tumor growth in PCa cells. Considering that both 5-aza-dCR and RG108 are effective, the latter might be more appealing from a clinical standpoint, not only because RG108 is a non-nucleoside (and thus may be less cytotoxic), but also because it does not require active cell division to exert its functions [11].

Remarkably, we also demonstrated that RG108 was able not only to inhibit DNMT activity, but also *DNMT1, 3a and 3b* mRNA expression, as well as DNMT1 protein expression in LNCaP and 22Rv1 cells. This might justify the observed reversal in *GSTP1* and *APC* promoter hypermethylation and associated restoration of gene expression. The ability of RG108 to demethylate *GSTP1* is a very important finding since this gene, which is methylated in the vast majority of PCa, was recently suggested as a potential useful biomarker for assessing DNMTi efficacy in PCa [30]. These results are also in accordance with the reported ability of RG108 to demethylate tumor suppressor genes (*e.g.*, *p16*) in HCT116 cell line [11]. It must be recalled, however, that demethylation is not synonymous of re-expression, as clearly demonstrated in our study for *RARβ2*. Because epigenetic silencing of cancer-related genes is a more complex phenomenon, in which other mechanisms, such as histone post-translational modifications, are also involved [17], combined therapies with histone deacetylase inhibitors might prove to be more effective.

This study also showed that RG108 has a weaker demethylating effect compared to 5-aza-dCR, as three days exposure to the latter was able to more effectively reduce the levels of global DNA methylation than RG108 at 14 days. Similar results were reported for HCT116 cells, which showed a reduction of 50% of cytosine methylation levels when exposed to 5-Aza-dCR (after five days of incubation) and of only 30% when exposed to RG108 (after 15 days) [11]. The superior efficacy of 5-aza-dCR might be due to its properties as a cytidine analogue. Once incorporated into the DNA, 5aza-dCR covalently traps the DNMTs on the DNA by forming a suicide complex which can lead to a massive loss of DNA methylation, resulting in genome hypomethylation, which may predispose to genomic instability and foster neoplastic transformation of normal cells [23, 31]. RG108, on the other hand, directly blocks DNMT1 activity [11], and may, thus, explain the less potent global DNA demethylation effect. This therapeutical disadvantage of RG108 may, however, be compensated by a lesser degree of global hypomethylation, minimizing genomic instability and improving its toxicity and safety profile.

Interestingly, several studies have proposed 5-Aza-dCR as an adjuvant to conventional therapy of PCa [25, 26, 32, 33]. Those studies demonstrated that 5-Aza-dCR could sensitize PCa cell lines to docetaxel, which is the first line therapy for castration-resistant PCa [25]. Likewise, 5-Aza-dCR might restore the expression of androgen receptor in PC-3 cell line, and the combined treatment with bicalutamide demonstrated a synergistic effect in repressing tumor growth in xenograft mice [32]. Remarkably, bicalutamide was recently associated with higher DNMT3a and DNMT3b expression, potentiating the up-regulation of truncated androgen receptor isoforms and therefore the castration resistant phenotype [33]. However, when cells were exposed to 5-Aza-dCR, there was a decrease in DNMT activity and a consequent decline in the development of the castration-resistant phenotype [33]. Because RG108 is able to modify the phenotype of PCa cells and the catalytic domain of the four DNMTs is highly conserved, predicting similar interactions of those enzymes with RG108, it would be interesting to evaluate the possible synergistic action between RG108 and conventional PCa therapy. Moreover, based on the promising results from this study, further experiments should be conducted to investigate the cellular pathways involved in RG108 response. Comparing these cellular pathways with those involved in 5-Aza-dCR response might provide valuable information concerning PCa cells biology and illuminate the different mechanisms of action of these compounds.

CONCLUSIONS

The demethylating agent RG108 demonstrated the ability to reduce cell viability, induce cellular death by apoptosis, and decrease DNMT activity and *DNMT1* expression in PCa cells. Fur-

thermore, it was able to decrease promoter hypermethylation levels and induce re-expression of genes known to be epigenetically silenced in PCa. Because RG108 performed better in chronic exposure (14 days) at 200 μM concentration, further studies should be conducted with these conditions to further assess the promising role of RG108 as a valuable tool for PCa therapy.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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