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Correlation between the presence of the nuclear AR-V7 androgen receptor and clinical evolution of prostate cancer: in vitro analysis of a combination of compounds targeting PI3K

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INTRODUCTION

PROSTATE CANCER

Prostate cancer (PCa) is the second most frequently diagnosed cancer in men. According to the National Cancer Institute, there are about 137.9 new cases per 100,000 men per year. PCa is the second leading cause of cancer-related mortality among men and the third leading cause of cancer death. An increase in the PCa incidence associated with an aging population is also expected (1).

PCa is a multiform and biologically very heterogeneous disease; some patients have an indolent disease with few symptoms while others have a highly aggressive disease and progresses into metastatic carcinoma refractory both to hormone therapy and to chemotherapy.

Patients with localized PCa are successfully treated with radical prostatectomy (RP) or radiotherapy (RT). Despite the high rates of progression-free survival (PFS), approximately 30% of patients treated with surgery and 30-50% of those treated with RT have recurrence of the disease, demonstrated by the rise in PSA values. Clinical relapse involves a variable time that can go from 1-8 years, on average 5. The PSA increase does not define a local recurrence from a distant micrometastasis. High Gleason scores are associated with a greater risk of recurrence (2).

Patients with the metastatic PCa are treated with hormone therapy (androgen deprivation therapy, ADT). Despite the initial high response rate to ADT, most patients develop resistance to ADT. The progression time towards the metastatic form of the disease, known as castration-resistant prostate cancer (CRPC), is different for each patient, often within the first year of metastatic disease.

CRPC is defined when serum testosterone is <50 ng / dL and at least one or more of the following criteria are present: 1) increase in serum levels of specific prostate antigen (PSA) (biochemical progression), 2) development of symptoms in the presence of pre-existing cancer (clinical progression) or 3) identification of new metastatic lesions at imaging (radiographic progression). For this group of patients in addition to traditional treatment based on taxanes, docetaxel and recently, cabazitaxel (3), treatment with abiraterone and

enzalutamide, which target the androgen receptor axis (4,5), has been approved. However, some patients have shown primary resistance to these agents but the mechanisms of resistance are not yet fully understood (6).

The key mechanisms in the progression of androgen-dependent disease to CRCP can be attributed to several potential molecular mechanisms, such as: increase in androgen biosynthesis in the tumor microenvironment or use of adrenal androgens and cholesterol as androgen precursors; somatic changes of AR resulting in an increased affinity for the ligand; amplifications of the AR gene; splicing variants of constitutively active AR and non-canonical activations of AR (7,8).

Recently, an interaction between AR and PI3K/AKT/mTOR has been highlighted (9-11). Inactivation of AR leads to over-regulation of PI3K (12-14). PI3K/AKT/mTOR is important for proliferation and survival of normal cells and is regulated by the oncosuppressor Phosphatase and tensin homolog (PTEN). The lack of PTEN activity leads to a continuous activation of the PI3K pathway that has been correlated to an altered control of cell growth and survival, an increased metastatic competence, angiogenesis and resistance to chemotherapy (15,16). The loss of the PTEN oncosuppressor and the consequent uncontrolled activation of the PI3K signaling pathway was found in 40% of the primary tumors and in 70% of the metastatic forms of PCa (17).

Furthermore, the inflammatory cytokines (CCR9, IL-6, and TLR3) regulate the PI3K/AKT signal in the apoptotic mechanisms of PCa cells. IL-6 is over-expressed in some PCa and may induce resistance to hormone therapy (18).

ROLE OF ANDROGEN RECEPTORS IN THE PCA PROGRESSION

The androgen receptor (AR) gene is located on the X chromosome (Xq11-12) and contains eight exons that encode a 110 kDa protein (Figure 1). The AR protein consists of a domain of trans-activation of the terminal NH2 (NTD, encoded by exon 1), a DNA-binding domain (DBD, coded by exons 2 and 3), a region of hinge (H, encoded by the portion 5 of exon 4) which contains the nuclear localization signal (NLS) and a ligand-binding domain (LBD / CTD, encoded by the remaining exon 4 through exon 8).(19,20)

Testosterone and dihydrotestosterone (DHT) bind to the LBD of AR and induce a change in the conformation of the protein leading to detachment from *chaperonins* and exposes the NLS in the region of the hinge. The AR dimerizes and translocates to the nucleus where it interacts with transcriptional co-regulators, binds to the androgen response elements (ARE) and regulates the transcriptional output of the many genes regulated by androgens according to

the cell type. In prostate epithelial cells, AR regulates the expression of the transcription factors of the NKX3.1 and FOX family, IGF1R, UBE2C, UGT2B15, KLK3, TMPRSS2, FKBP5 and other genes that control cell growth, differentiation and function both in the normal prostate and during the growth and progression of PCa.

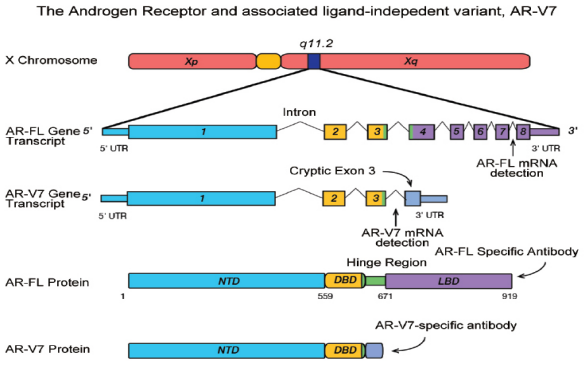


Figure 1: The Androgen receptor

AR-DEPENDENT MECHANISMS IN THE PROGRESSION OF PCA

The role of AR-induced signals in the PCa initiation phase is not yet fully understood. AR generally does not exhibit alterations in the primary tumor, however about 50% of the localized disease may present alterations in the regulation of AR. The continuous activation of the AR signal, on the other hand, plays an essential role in the development of the CRPC (21).

An increased expression of AR is constantly observed in tumor epithelial cells during CRPC development, while a loss in the AR signal pathway is generally observed in the primary tumor and in the metastasis stroma. So far, numerous genetic mutations have been identified in the AR locus involved in the mechanisms leading to CRPC, such as: amplifications of the AR gene, mutations in the receptor-hormone binding domain; expression of splicing variants. All of these conditions can promote a continuous AR signal even in the condition of low serum testosterone levels (22).

-AR AMPLIFICATION

The amplification of AR is the most frequent genetic alteration reported for CRPC tumors, observed in more than 50% of cases, while it is detected by in situ hybridization (FISH), only rarely in untreated primary tumors, suggesting that the AR amplification is an adaptive response to the ADT(23-25). AR over-expression in tumor epithelial cells may be the result of AR gene amplification, but is probably also an instant castration response because androgens normally suppress AR transcription in prostate epithelial cells. Increased AR expression can sensitize the cells of the PCa to sub-physiological levels of androgen and has been associated with therapeutic resistance to both the enzyme inhibitor (AR antagonist) and the abiraterone

acetate (26-29). The cells that over-express AR are able to access a greater number of chromatin binding sites, generating an altered AR transcriptome.

-POINT MUTATIONS

Point mutations of AR can also continually activate the AR-induced signal in tumor epithelial cells. These mutations are very rare in untreated PCa, but have been reported in 15-20% of CRPC patients and up to 40% of CRPC patients treated with AR antagonists, suggesting that they may occur selectively in response to ADT (9, 16, 30, 31). Several point mutations with function gain were characterized, predominantly in the c-terminal region of LBD, while about one-third occur in NTD, resulting in increased specificity for adrenal androgenes and other steroid hormones, (including DHEA, progesterone , estrogen and glucocorticoids) and activation of the AR androgens or enlarge the specificity of the ligand (32).

Two well-known examples are AR-T877A and AR-W741C, originally described in LNCaP cells, which convert the antiandrogens flutamide and bicalutamide into partial agonists, respectively (33-35).

-ANDROGENS INTRACRINE BIOSYNTHESIS

An alternative mechanism to activate the AR signal is through the conversion of cholesterol and adrenal androgen precursors into testosterone and DHT in PCa cells even in the absence of circulating androgens produced by the testes (36).

The enzymes responsible for the conversion of cholesterol into androgens precursors (CYP17A1, HSDD3B2) and the conversion of androgens precursors into testosterone and DHT (AKR1C3, SRD5A1 / 2) are increased about 8-10 times in CRPC (37,38). It is believed that the over-regulation of genes involved in androgen production can increase androgen concentrations, further facilitating the reactivation of the AR signaling pathway (30).

Several mutations have been demonstrated that correlate with the development of resistance to abiraterone, for example high levels of CYP17A1 and mutations with gain in function may sensitize AR to non-androgenic ligands. This demonstrates a possible mechanism by which the same enzymatic process of synthesis of steroid hormones could be altered at the genomic level to guide the development of resistance to ADT therapy (37).

-SPLICING VARIANTS

In recent years splice variants (AR-V) have been studied to highlight their contribution to the development of CRPC (39-44). A constitutively active AR may result from splicing or alternative gene rearrangement of the AR gene. The expression of AR-V is well documented in the PCa cell lines, in the xenograft tumors as well as in the clinical samples of PCa (42,43). Most AR-V results from a truncated AR in the terminal C region, leading to loss of LBD. In AR-

Vs, mutations in exons 3 and 4 result in changes in NLS and an AR with predominantly cytoplasmic localization. However, other AR-Vs contain both exons 3 and 4 and are therefore able to translocate into the nucleus and activate AR target genes independently of the ligand (45).

AR-V7, the splicing variant best characterized to date, is constitutively active despite lacking a complete NLS, through a mechanism that has not yet been completely determined. Just as it has not yet been completely clarified whether the profile of the genes activated for this variant is the same as for full-length ARs. The presence of AR-V variants has substantial implications for the treatment of CRPC (46). AR-V are generally over-expressed in bone metastases and correlate with a reduced survival rate after surgery (46,47). In particular, the presence of AR-V7 in tumor cells circulating from patients treated with enzalutamide or abiraterone was associated with therapeutic resistance to both agents (48). Patients with AR-V7-positive treated with enzalutamide or abiraterone showed lower PSA response rates, shorter free progression-free survival, shorter progression-free clinical or radiographic survival, and shorter overall survival compared to patients with AR-V7-negative (48).

A new therapeutic strategy able to inhibit both the AR is addressed and towards the terminal N region of RA, which is conserved in all AR isoforms. A particular interest has elicited EPI-001, a small antagonist molecule derived from bisphenol A, which covalently binds the AR-terminal domain and has shown to block the transcriptional activity of AR and different AR-Vs, including AR-V7 (49).

-NON-CANONICAL TRANSACTIVATION OF AR

Numerous growth factors, cytokines and hormones have been implicated in the activation of AR in the absence of androgens or present at sub-physiological concentrations. The non-canonical induction of the AR signaling pathway has been related to mechanisms that promote phosphorylation of AR itself (50). In particular, it has been shown that the insulin-like growth factor-1 (IGF-1) is able to induce the AR transcriptional activity in androgen deprivation conditions in vitro, an effect that could be inhibited by bicalutamide (51) . Furthermore, it has been seen that the activation of ARF mediated by IGF-1 also requires the expression of integrin β (1A), as it facilitates the functional interaction between the activated IGF-1 receptor and AR, leading subsequently to independent growth from PCa anchorage (52). Interleukin-6 (IL-6) also activates AR genes and increases PSA secretion in cell cultures. In particular, in the absence of androgens, it has been shown that IL-6 induces (probably through Stat3 signal transducer and activator of transcription, signal transducers and transcription activators) an increased expression of genes regulated by AR (53).

PI3K / AKT / mTOR

The PI3K-AKT-mTOR signaling pathway is an ancient signal transduction pathway, preserved by worms to humans, which has evolved as an essential regulator of the catabolic and anabolic processes of the cell. It is often related to numerous cancers including PCa.

It plays a critical role in linking nutrient and growth factor perception with numerous cellular processes, such as protein synthesis, proliferation, survival, metabolism and differentiation (54). The different signal pathways are mediated by numerous effectors that modulate phosphorylation, transcription and translation.

The activation of the PI3K kinase is a central mechanism between upstream growth signals and downstream signal transduction mechanisms. PI3Ks are grouped into three classes (I-III) based on their substrate preference and sequence homology. Their primary function is to phosphorylate the 3'-hydroxyl group of phosphatidylinositol and phosphoinositides. Most relevant in tumors is the class IA PI3K, which includes two functional subunits that form a heterodimer: a catalytic subunit (p110α, p110β or p110δ) and a regulatory subunit (p85α, p55α, p50α, p85Î² or p85Î³). A series of signals stimulate the activity of PI3K, mainly through tyrosine kinase receptors (RTK), but also through receptors coupled to G proteins (GPGR) and oncogenes such as Ras that directly bind p110. After stimulation, the catalytic subunit of PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PI-4,5-P2) to phosphatidylinositol-3,4,5-triphosphate (PI-3,4,5-P3), which acts as a second messenger to recruit a series of proteins containing homology domain with the cell membrane pleckstrin.

The kinases belonging to class IA are constituted by a catalytic subunit and a regulatory subunit. The catalytic subunits p110α, p110β or p110δ are encoded by the PIK3CA, PIK3CB and PIK3CD genes, respectively. The regulatory subunits are instead p85α (in the p85α, p55α, p50α, p85β and p55Î ± isoforms, encoded by the PIK3R1, PIK3R2 and PIK3R3 genes. The IB class consists of a single catalytic subunit, P110γ, and two regulatory subunits, p84 and p101. Class II PI3Ks are monomeric proteins that include three isoforms: PI3KC2α, PI3KC2β and PI3KC2γ. Class III is more structurally similar to class I and exists as a heterodimer composed of a catalytic subunit (Vps34) and a regulatory subunit (p150). Class IA PI3K kinases are activated by stimulation of growth factors through tyrosine kinase receptors (RTK). The p85 regulatory subunit is directly bound to the phosphotyrosine residues of RTK receptors; this binding abolishes the intramolecular inhibition of the p110 catalytic subunit by p85 and localizes PI3K in the plasma membrane, where its PIP3 substrate resides (55,56).

An uncontrolled PI3K signal is very common in cancer. Mutations in the tumor cells from the colon, lung, prostate, liver and brain revealed mutations in the PI3KA gene that encodes p110α. This gene, in addition to being involved in the processes of regulation of the cycle and of cell growth, acquires a very important role in endothelial cells, promoting angiogenesis and, therefore, the formation of a vascular network essential for the contribution of substances nutritive and oxygen, and which can ultimately guarantee a pathway of metastasis from the primary lesion (57).

-PTEN

PTEN (Phosphatase and tensin homolog) is a key molecule in the regulation of PI3K / AKT. The PTEN function as tumor suppressor is performed through the phosphatase activity, dephosphoryl PIP3 to PIP2, which negatively regulate the activation of the PI3K / PDK1 / AKT pathway (58,59). This phosphatase can act on both lipids and proteins, and acts as tumor suppressor, inhibiting cell proliferation and inducing apoptosis, especially in cells that present the so-called anikis. Mutations in PTEN inhibit the oncosuppressor activity, in particular two main mutations in the phosphatase domain: one involves the loss of phosphatase activity both on lipids and on proteins while the other only affects phosphatase activity on protein substrates .

In addition to regulating the PI3K / AKT signal pathway, PTEN has many other critical roles in tumors, including: genomic instability, tumor cell renewal, cellular senescence, cell migration and metastasis. Finally, PTEN plays a significant role in the regulation of tumor microenvironment (58). Mutations in PTEN on germ cells result in a rare hereditary disease, Cowden's disease, which is associated with a higher risk of neoplastic diseases. Mutations in the PTEN gene have been observed in cancer of the breast, prostate, endometrium, ovaries, colon, melanoma, glioblastoma and lymphoma. Studies on animal models have also shown that the loss of a single copy of the PTEN gene is sufficient to interrupt the cellular signal and initiate an uncontrolled growth of the cell.

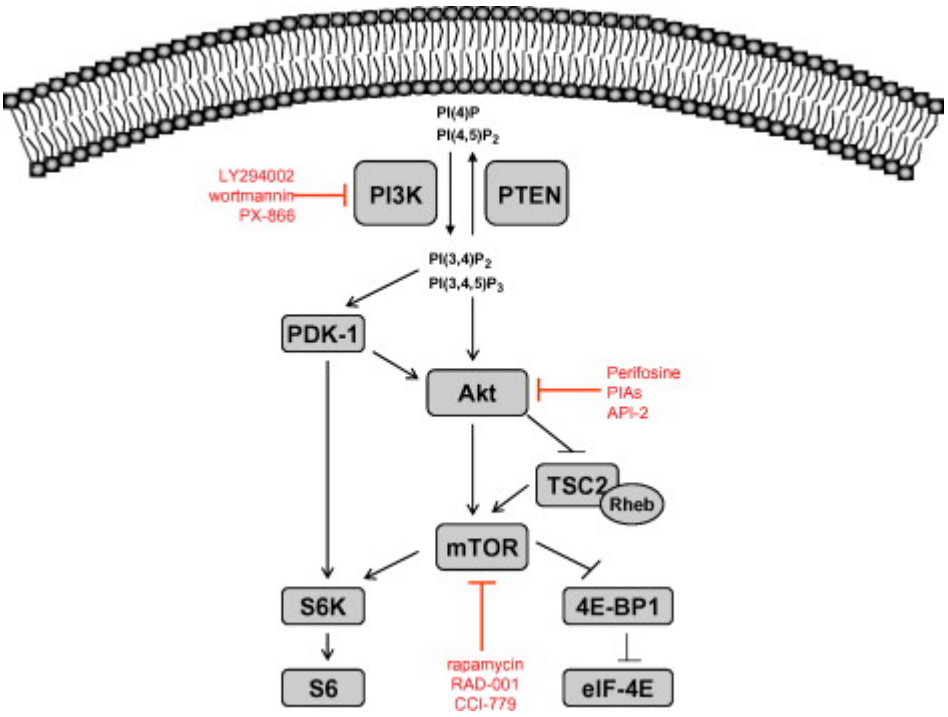


Figure 2. The PI3K/AKT/mTOR pathway

-AKT

AKT, or protein kinase B (PKB), is a serine / threonine kinase of the AGC kinase family. It exists in 3 structurally similar isoforms: PKBα, PKBβ and PKBγ. The three isoforms are composed of characteristic domains. The Pleckstrin Homology (PH) domain has a notably conserved tertiary structure despite the aminoacid sequence presenting differences; this domain is responsible for the link with PIP3. The LIN domain, the hinge region of 39 aminoacids, is the region that connects the PH domain with the catalytic domain, is poorly conserved among the AKT isoforms (17-46% identical) and has no significant homology with any other human protein (60). The kinase domain, extending from amino acids 148-411 and ending in a regulating hydrophobic motif (CTD), is the portion of the enzyme that binds ATP; the ATP-binding site of 25 residues has a homology of 96-100% in the three isoforms. The C-terminal hydrophobic domain is conserved in the AGC kinase family. These hydrophobic residues play a critical role in the complete activation of AKT for substrate phosphorylation. Inside there is another key residue for the activation of the enzyme, Ser473.

While AKT1 is expressed ubiquitely at high levels with exception of kidney, liver and spleen, AKT2 expression is elevated at the level of insulin-sensitive tissues such as brown fat, skeletal muscle and liver. The expression of AKT3 is ubiquitous, although low levels of expression have been found in the liver and in skeletal muscle. These different isoforms are implicated in specific functions. For example, the amplification and over-expression of AKT2 is related to increased motility and cell invasion, while increased AKT3 activity appears to contribute to the aggressiveness of steroid-induced tumors. All three isoforms are activated through phosphorylation: the first occurs on a threonine residue while the second occurs on a serine residue in the hydrophobic motif.

Once activated, AKT recognizes and phosphorylates serine or threonine residues of numerous substrates, such as: tuberosis sclerosis complex (TSC) 2, glycogen synthase kinase (GSK) 3, forkhead box transcription factors (FoxO), p21WAF1 / CIP1, p27KIP1, caspase -9 and BAD (Bcl-2 associated death promoter) and iNOS, which regulate numerous processes that coordinate the life and death of the cell, metabolism and angiogenesis (61).

The hyperactivation of AKT has been shown in numerous cancers, such as: multiple myeloma, lung cancer, glioblastoma, breast cancer and also prostate cancer (61).

The best studied downstream substrate of AKT is the mTOR kinase (62). AKT can directly phosphorylate and activate mTOR, as well as may cause indirect activation of mTOR by phosphorylating and inactivating TSC2 (tuberous sclerosis 2, also called tuber), which normally inhibits mTOR. The consequence of mTOR activation is an increased translation of proteins.

Finally, it has recently been shown that AKT activity can be negatively regulated by the PH domain of phosphatase rich in repeated sequences of leucine (PHLPP) which specifically dephosphoryates the hydrophobic motif of AKT (Ser473 in Akt1).

-mTOR

mTOR (mammalian target of rapamycin) is a serine / threonine kinase that regulates growth, proliferation, motility and cell survival, transcription and protein synthesis (62). mTOR plays an important role in regulating the body's energy balance and its weight; is activated by amino acids, glucose, insulin and other hormones involved in the regulation of metabolism. Recent studies have shown that mTOR is not only a substrate of AKT, but also a crucial activator: mTOR in fact forms a complex with the RICTOR protein (Rapamycin-insensitive companion of mTOR) and then directly phosphorylate Ser473 of Akt. The activation of this complex (TORC2) could explain the sequestration of the mTOR molecules just formed inside the cells during long-term treatments with rapamycin. This drug is in fact particularly

effective in inducing apoptosis and in suppressing the proliferation of cells that over-express Akt as, over time, it interferes with the reassembly of the complex by joining it (63).

Given the critical role of PI3K-AKT-mTOR in normal cell physiology, it is not surprising that the pathway is deregulated in a wide range of tumors, genetic alterations have been identified as loading all components of this signal pathway.

In PCa, the PI3K-AKT-mTOR pathway is deregulated in 42% of the localized disease and in 100% of the advanced disease, indicating that the alterations in these signals may be an essential prerequisite for the development of CRPC (64-67). The functional importance of mutations, gene amplifications and changes in mRNA expression in PI3K pathway components is highlighted by the significant correlation with the prognosis of PCa patients. For example, a reduced expression of PTEN, is associated with a higher Gleason, biochemical recurrences after prostatectomy, and shorter times to metastasis (67). Furthermore, high levels of phospho-4EBP1 and eI4E are associated with an increase in mortality of patients with PCa, indicating that even the downstream effectors of the route are predictive of disease progression (68).

Results from studies on knockout and transgenic mouse models also clearly showed the role of PI3K-AKT-mTOR in PCa development. In particular, the over-expression of AKT or the biallelic loss of the PTEN-suppressor in prostate epithelial cells leads to hyperactivation of the route and is sufficient for the development of in vivo PCa (69-71). Conditional KT of mTOR in a mouse PCa model caused by PTEN deletion inhibits prostate tumorigenesis, demonstrating the need for an intact signaling axis to guide cell transformation in prostate epithelial cells. It is interesting to note that others have shown that the simultaneous loss of PTEN and RICTOR, a component that defines the mTORC2 complex, reduces the incidence of PCa formation in mice. Thus, the hyperactivation of PI3K-AKT-mTOR is sufficient to induce the formation of PCa, and both mTORC1 and mTORC2 are necessary to facilitate this in vivo process. In particular, overexpression of the oncogenic AKT or the biallelic loss of the tumor suppressor PTEN in the epithelial cells of the prostate leads to the hyperactivation of the route and is sufficient for the development of in vivo PCa. Conditional KO of mTOR in a murine PCa model caused by PTEN deletion inhibits prostate tumorigenesis, demonstrating the need for an intact signaling axis to guide cell transformation in prostate epithelial cells. It is interesting to note that others have shown that the simultaneous loss of PTEN and RICTOR, a component that defines the mTORC2 complex, reduces the incidence of PCa formation in mice (72,73).

The aim of this work was to evaluate the presence of the AR-V7 splicing variant in patients with PCa. The data obtained from biopsies of patients affected by PCa were then correlated to

the Gleason Score and to the evolution of the disease. Furthermore, in vitro, we have analyzed the effects of a combination of compounds that target the signaling pathway of AR and PI3K / AKT / mTOR.

MATHERIAL AND METHODS

Our experimental activity was composed by two different levels:

1. Receptor mutations with particular attention to the expression of AR-V7 were evaluated on samples derived from radical prostatectomies of 70 patients diagnosed with prostate adenocarcinoma, subdivided according to the risk class (D'Amico classification) and the neoplastic progression (EAU definition of neoplastic progression).
2. At the same time we evaluated the effects of some therapies compounds on cell lines that have a different set-up of AR, LnCaP and VCaP.

1. EX VIVO AR-V7 EXPRESSION

This experimental study was conducted after approval of the protocol from our Institutional Board Committee and informed consent was obtained from all patients. Exclusion criteria for the study were: previous hormonal, surgical or radiation therapies for prostate diseases; acute inflammatory diseases. Inclusione criteria are: Hystological diagnosis of prostate adenocarcinoma at prostate biopsy, no pregressive treatments (radiotherapy, ormonal therapy) for PCa, possibility to give an informed consent.

Table 1 summarizes the caharacteristics of donors.

All sections derived from 70 patients undergoing radical prostatectomy PCa. For each patient we collected 5 mm sections from paraffin-embedded PCa tissues. The histological status of the tissue (pathological stage, Gleason Score, surgical margins) was checked by an independent pathologist. We devided patients into three groups according to D'Amico risk stratification system: Low-risk Group was defined as T1/T2a, and PSA ≤10 ng/ ml, and Gleason score ≤6. Intermediate-risk Group was defined as T2b, and/or PSA 10–20 ng/mL and/or Gleason 7 disease. High-risk Group was classified as having any one of the following high-risk features: ≥T2c, PSA >20 ng/mL or Gleason 8–10 disease. In each group we divided patients into two subgroups: Presence or absence of neoplast progression. Neoplastic progression is defined as: increase in serum levels of specific prostate antigen (PSA) (biochemical progression), 2) development of symptoms in the presence of pre-existing cancer (clinical progression) or 3) identification of new metastatic lesions at imaging (radiographic progression).

Number of cases	72
Prostate Volume (ml)	78.39 ± 6.8
Age (years)	64.76 ± 3.3
PSA (ng/mL)	15.8 ±2.4
Low risk group -Progression -No Progression	14 (19.4%) 0 14
Intermediate risk group -Progression -No Progression	14 (19.4%) 0 14
High risk group -Progression - No progression	44 (61.1%) 22 22

Table 1: Characteristics of population

The expression of anti AR-V7 was evaluated by immunoperoxidase staining on 5 mm sections from paraffin-embedded human PCa tissues using a streptavidin-biotin-peroxidase system (UCS Diagnostic), according to the manufacturer's instructions. Sections were de-paraffinized and endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS for 10 min. For antigen retrieval the sections were incubated in buffer pH 9.0 (5% Tris, 1.5% 2-butoxyethanol, 5% Sodium EDTA) at 95°C for 10 min. The primary antibody anti human AR-V7 was a rabbit monoclonal antibody (Abcam) used at the dilution 1:250 for 1 hour at room temperature. The primary antibody was replaced with rabbit preimmune serum as a negative control for non-specific staining. Sections were finally counterstained with haematoxilyn and mounted. The stained sections were observed with a light microscope and positivity was determined by cell staining.

Evaluation of antigen positivity

Antigen expression was quantified according to the following two parameters: (1) the number of positively stained cells; (2) the intensity of the staining, ranging from pale pink to dark orange. AR-V7 positivity was graded as follow: +/- weak to moderate staining on less than

10% of tumor cells, + weak or moderate staining on less than 50% of tumor cells or strong staining on less than 10% of tumor cells, ++ strong positivity on the majority of tumor cells.

2. IN-VITRO ANALYSIS

On prostate carcinoma cell lines we evaluated PI3K/ AKT/ mTOR, Wortmannin (WTM) and RAD001 inhibitors in combination with drugs that interfere with AR, Abiraterone (ABI) and Enzalutamide (ENZ), and Docetaxel (DTX). We chose different concentrations and the treatments were performed for 24, 48 and 72 hours. Survival was assessed with MTT, and cell death with tripan blue staining. Western blot analyses was performe to detect the different compounds induced apoptosis. Finally, the ability of the compounds to induce cell cycle arrest was evaluate by Flow cytometry.

-Cell lines

LnCaP, androgen-sensitive human prostate adenocarcinoma cells and VCaP, human prostate adenocarcinoma with a constitutive expression of AR-V7 were obtaied from Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy (ICLC). LNCaP cells were grown in RPMI 1640 and VCaP in DMEM: F12, 1:1, supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 1% Non-Essential Amino Acids. Cells were maintained in a tissue culture incubator at 37°C, 5% CO2.

-Cell viability assay

Cytotoxicity was assessed by TB dye exclusion test. Briefly, exponentially growing cells were suspended in a number density of 1 x 105 ml-1 in 24-well microplate. After 48 h, control and treated cells were washed in PBS, trypsinized and harvested, diluted (1:1) in a 0.4% TB stain solution and counted using an optical microscope in a Neubauer chamber. The cell viability was determined and expressed as percentage compared to the corresponding control. The cell viability in control samples was always 95-98% of the total number of cells. Reported values result from the average of at least three independent experiments.

Cytotoxicity assessed with Trypan Blue

PC-3 and SK-Mel-5 and -28 were plated and, after 24 hours, treated with derivatives of kinesin inhibitors at different concentrations (from 0.1 µM to 100 µM) and for different incubation times (24 , 48 and 72 hours).

At the end of the incubation, the cells were trypsinized. Initially, the nutritive material and the cells washed with 3-4mL of phosphate buffer (PBS), sterile and free of Ca + 2 and Mg + 2, were removed in order to loosen the calcium-dependent contacts between the cells and between these and the plate. The PBS was also used to remove residues of culture medium containing trypsin inhibitors; after washing, 1ml of trypsin / EDTA solution (EuroClone) was added.
At this point the cells were placed in an incubator for 15 minutes.
Once the adhesion loss of the cells was verified, under the microscope, they were collected with PBS (3-4 ml) and their count with Trypan Blue (TB), a dye able to selectively color the dead cells. Then, 20µL of the cell suspension and 20µL of dye were withdrawn; after a slow and repeated suspension an aliquot of 20µL was taken and placed in the hemocytometer count chamber (Neubauer improved 0.100mm).
Under the inverted microscope the relationship between living cells (refractories) and dead cells (colored in blue) was evaluated. Each count was performed at least three times and the average was assessed. Finally, the vitality was determined and expressed as a percentage with respect to the growth of the untreated cells.

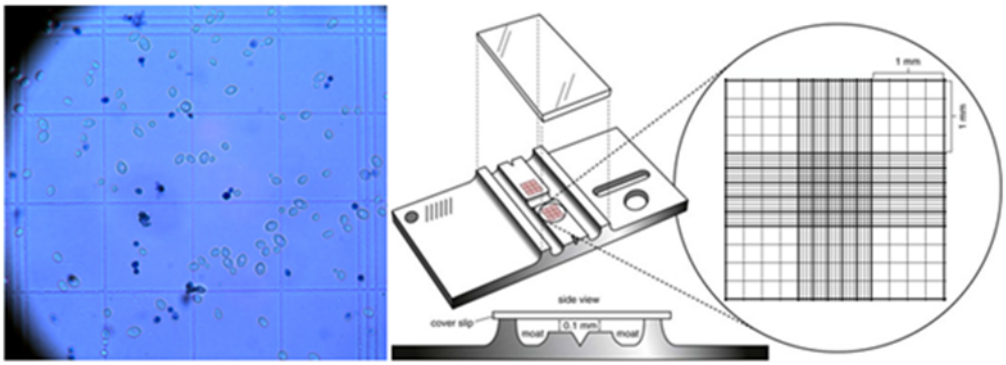


Figure 3: **Cells seen under a light microscope with Trypan Blue (on the left), Neubauer's Room (on the right).**

-Cell proliferation assay

MTT assay was performed to determine proliferation activity. Cells (5 x 10^3) were seeded in 96-well flat plate and allowed to attack for 24 h. Cells were treated with Docetaxel, abiraterone, ecc . After treatment, the cells were washed with PBS to remove the compounds in excess, and incubated with MTT for 4 h. The formed formazan crystals were dissolved in 100 µl of DMSO for 15 min. Finally the absorbance at the test wavelength of 540 nm was measured in a plate reader spectrophotometer (Labsystem Multiskan MS), using a reference wavelength of 690 nm. Cells incubated with culture medium alone represented the control, whereas wells containing medium alone represented the blank. The amount of surviving cells

was expressed as percentage of the corresponding control sample, obtained by the ratio between the two measured absorbance values. The reported results are obtained by the average of at least three experiments performed with independent preparations of entrapped DOX.

Cytotoxicity evaluated with MTT

The potential cytotoxic effect of the different K858 derivatives was evaluated on the PC-3, SK-Mel-5 and -28 cells in the proliferative phase by the MTT assay.

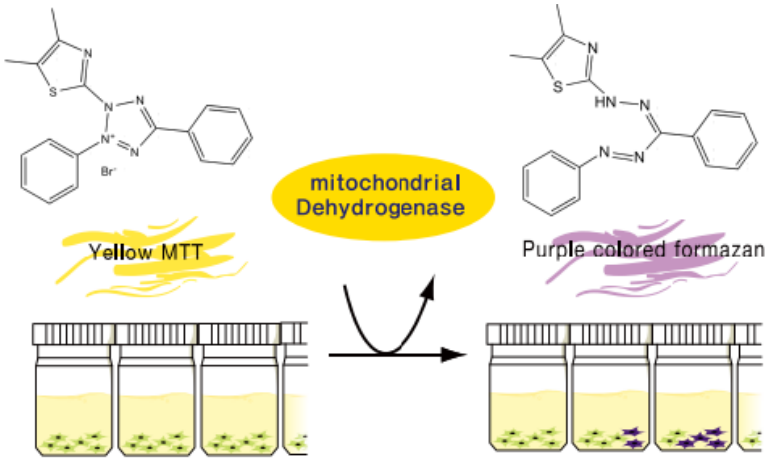


Figure 4: Reduction reaction of MTT to formazan derivative.

The method is based on the transformation of 3- [4,5-dimetiltiazol-2-yl] -2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich). This yellow tetrazolium salt is reduced (figure 4), in the mitochondria from the enzyme succinate dehydrogenase of the vital cells, in blue / violet coloring. The assay is to solubilize the crystals after 4 h of incubation, with 100µl of DMSO and after 15 'the absorbance is measured at a wavelength of 540 and 690nm with a micro-plate reader Elisa Reader (Labsystem Multiskan MS, figure 5). The specific absorbance is obtained by subtracting the non-specific value obtained at 690nm. Exponentially growing cells (PC-3, SK-Mel-5 and -28) were seeded in triplicate in 96-well plates (Corning, Inc., Corning, Ny, USA) at a density of 5000 cells per well (optimal number that allows both of not having an excessive growth of cells in the control well, is a better discrimination of the pharmacological action) and in 100µl of complete soil. Subsequently incubated for 24 hours at 37 ° C at 5% of CO2, to allow the adhesion of the cells to the plate.After 24 hours, the diluted kinesin inhibitors were added to each well and the cells incubated for the expected treatment times.

On the day of the assay 20µl of tetrazolium salt were added to have a concentration of 0.5mg / ml per well and finally the precipitated formazan crystals were solubilized in DMSO and the absorbance measured with an Elisa micro plate Reader.

The percentage of proliferating cells was therefore obtained from the ratio:

$$\frac{average\ absorbance\ of\ cells\ treated\ with\ the\ drug}{average\ cell\ absorbance\ without\ medication}$$

To all the values the absorbance obtained in the white was subtracted (reading in triplicate of wells without cells with only the culture medium). The result obtained is the average of at least three readings.

Figure.5: Elisa reader

-Western blot

After treatment of the LnCaP and VCaP cells with the different compounds, these were washed with PBS, collected and lysed in ice with 125µl of buffer, containing 1% of Triton X-100, 0.2% Nonidet P-40 with 2ml of EDTA, 2mg / ml sodium ortovanadate and 1x protease inhibitor (Roche Applied Sciences). The proteins were separated with 10% SDS-PAGE buffer (SodiumDodecylSulphate - PolyAcrylamide Gel Electrophoresis) and subsequently transferred to PVDF (polyvinylidenefluoride) membranes.

For immunoblotting, the following primary antibodies were used: anti-Parp-1 mouse (dilution 1: 500) (Santa Cruz Biotechnology); anti-Actin mouse (1: 1000 dilution) (Sigma Aldrich) as internal control. Subsequently, they were incubated for 1 hour at room temperature, with a second anti-mouse or anti-rabbit antibody conjugated with horseradish peroxidase (dilution 1: 5000) (Sigma-Aldrich). The chemiluminescent (ECL) analysis with the addition of enhanced chemiluminescence (Super Signal West Pico Chemiluminescent Substrate, ThermoScientific)

was visualized using Kodak X-Omat plates. The densitometry of the bands is obtained using the software Image J.

Western blot

Cell lysates were obtained scraping the cells in lysis buffer containing: 1% Triton, 0.1 % SDS, 150 mM NaCl, 50 mM TRIS-HCl pH 7.4, 2 mM EDTA plus protease inhibitor cocktail tablet (Roche Applied Sciences) for 30 min at 4 °C, then centrifuged at 16,000 x g for 15 min at 4 °C. Protein concentration was evaluated by the Protein Concentration Assay (Bio-Rad). Samples of lysate (50-100 μg) were separated by molecular weight on 10% or 12% SDS-PAGE (under reducing condition) and then transferred into a nitrocellulose membrane. Membranes were blocked for 1 hr at room temperature (RT) in 5% non-fat dry milk and then incubated with primary antibody, washed in TRIS-buffered saline added with 0.1% Tween 20 and then incubated with horseradish peroxidase conjugated anti-mouse or anti-rabbit antibodies (1:5000 diluted, Sigma-Aldrich). The filters were then developed by enhanced chemiluminescence (Super Signal West Pico Chemiluminescent Substrate, Thermo Scientific) using Kodak X-Omat films. The densitometry quantitation of the bands was performed using Image J software. The primary antibodies were the following: mouse anti Parp1 (1:500 diluted, Santa Cruz Biotechnology); mouse anti-actin (1:1000 diluted, Sigma Aldrich).

-Flow cytometry analysis

Flow cytometry was used to detect quantitatively the distribution of cell cycle. After 48 h of incubation with different compounds, both adherent and floating cells were collected in ice-cold phosphate buffer (PBS) and then $1.0x10^6$ were fixed in 2 mL ethanol 70%, and then incubated 1 h at 4 C. Cellular pellet was dissolved in 0.2 mL of 50 μg/mL propidium iodide and 100 μg /mL RNase A in PBS. The stained cells were incubated at room temperature for 30 min in the dark. The DNA content of the cells was analyzed by FACS Canto II flow cytometry using the FACS Diva analysis program in order to detect the distribution of subpopulation through the cell cycle.

Cytofluorimetric analysis (FACS)

Cytofluorimetric analysis was used to quantitatively determine the distribution of cells in the various phases of the cell cycle and apoptosis.

Ploidy levels were evaluated after staining with propidium iodide.

The propidium iodide (PI) is a synthetic dye characterized by a low fluorescence (red-orange), able to bind in a non-specific way the nucleic acids, both the DNA and the RNA, with double helix. Once intercalated between the base pairs, the PI greatly increases the fluorescence quantum efficiency, thus favoring the determination of the 2N and 4N peaks.

With this method, it is also possible to evaluate cell apoptosis, exploiting the internucleosomal cut of DNA in the apoptotic cell.

The PI, binds stoichiometrically to the double helix of DNA provides information on the amount of deoxyribonucleic acid contained in the cells, depending on the intensity of fluorescence (the higher the content of DNA the greater the fluorescence, λ 488nm).

It is known that the DNA content varies according to the phase of the cycle in which a cell is located. In particular, phase G2 and mitosis have a double amount of DNA with respect to the G1 phase; while during the DNA synthesis phase, the cell has a quantity of DNA intermediate between the content in G1 and G2.

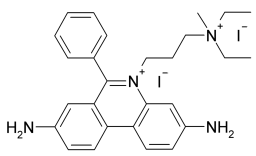
Apoptotic cells, on the other hand, have a reduced colorability of DNA compared to live or dead cells by necrosis; in fact, once they are resuspended in an appropriate buffer, they lose the small fragments of DNA and it is possible to observe in them a reduction of the fluorescence of the PI (correlated to the decrease of the DNA content). In this way it is possible to evaluate the percentage of apoptotic cells that are positioned in the so-called hypodiploid peak, with lower colorability compared to cells in G1 (marker of apoptotic cell death) and, in addition to this, the percentage of cells in the various phases of the cycle mobile phone: G0 / G1, S and G2 / M.

Twenty-four hours after seeding the cells, treatments with 5μM of some derivatives are carried out and after an incubation of 24 and 96 hours, the cells are trypsinized, washed with cold PBS, resuspended at a concentration of 1x106, fixed with ethanol at 70% and incubate 1 hour at 4 ° C.

The cell pellet is dissolved in 0.5ml of a solution containing propidium iodide (5μg / ml) and RNase (1mg / ml) in PBS. The cells so colored are incubated at room temperature for 30 minutes in the dark.

The DNA contained in the treated cells and controls was analyzed at the FACS Canto II using the FACS DIVA program (Fugure 6).

Propidium Iodide
(PI)



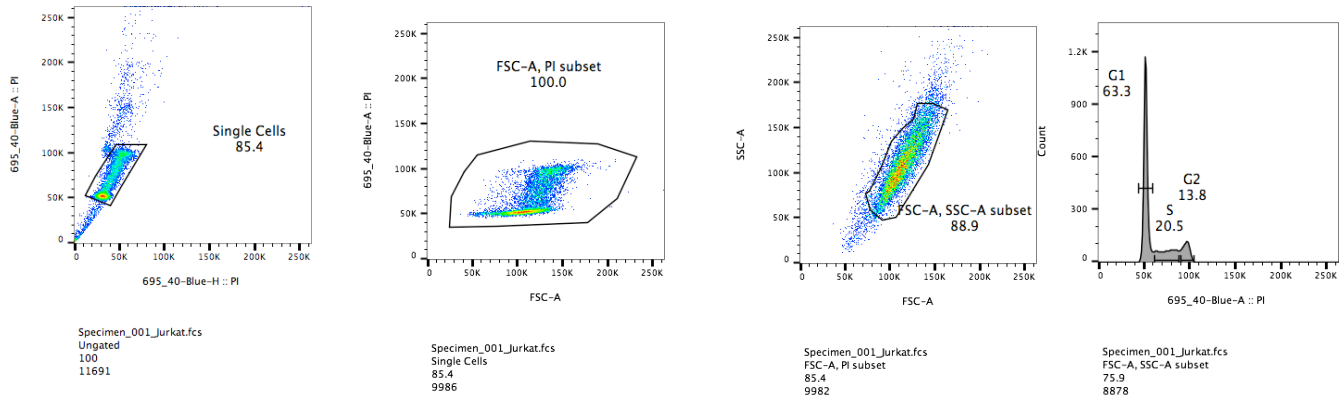


Figure 6: BD FACSCanto clinical software and BD FACSDiva™ software

3-STATISTICAL ANALYSIS

Ex-vivo experiments were performed at least three times and the statistical significance was evaluated by chi square test. Significance level was considered at 0.05.

RESULTS

1. EX VIVO AR-V7 EXPRESSION

A retrospective study was performed in order to evaluate the cellular expression of human androgen receptor variant AR-V7 in the tumor tissues from 72 PCa patients selected and divided into four groups: 22 patients with high risk and disease progression (A1), 22 patients with high risk and no progression (A0), 14 intermediate risk and no progression (I0), 14 low risk and no progression (B0).

All 14 patients B0 resulted totally negative for AR-V7 staining of tumor cells; 13 over 14 (92.8%) patients I0 resulted totally negative and only one patient (7.14%) showed 1% of tumor cells with nuclear staining for AR-V7. Among the 22 patients A0, 14 (63.63%) were totally negative and 8 (36.36%) showed only 1-2% of tumor cells with nuclear staining for AR-V7. All 22 (100%) patients A1 showed AR-V7 nuclear staining in a variable number of tumor cells ranging from 2 to 10% of positive cells (Figure7). We calculated the correlation between the positive nucelar staining of AR-V7 and the clinical stage or progression using the chi-square test whose results were 4,38499E-07 and 9,60943E-11, respectively. We compared our data with the chi-square distribution table and the hypothesis of the correlation must be accepted.

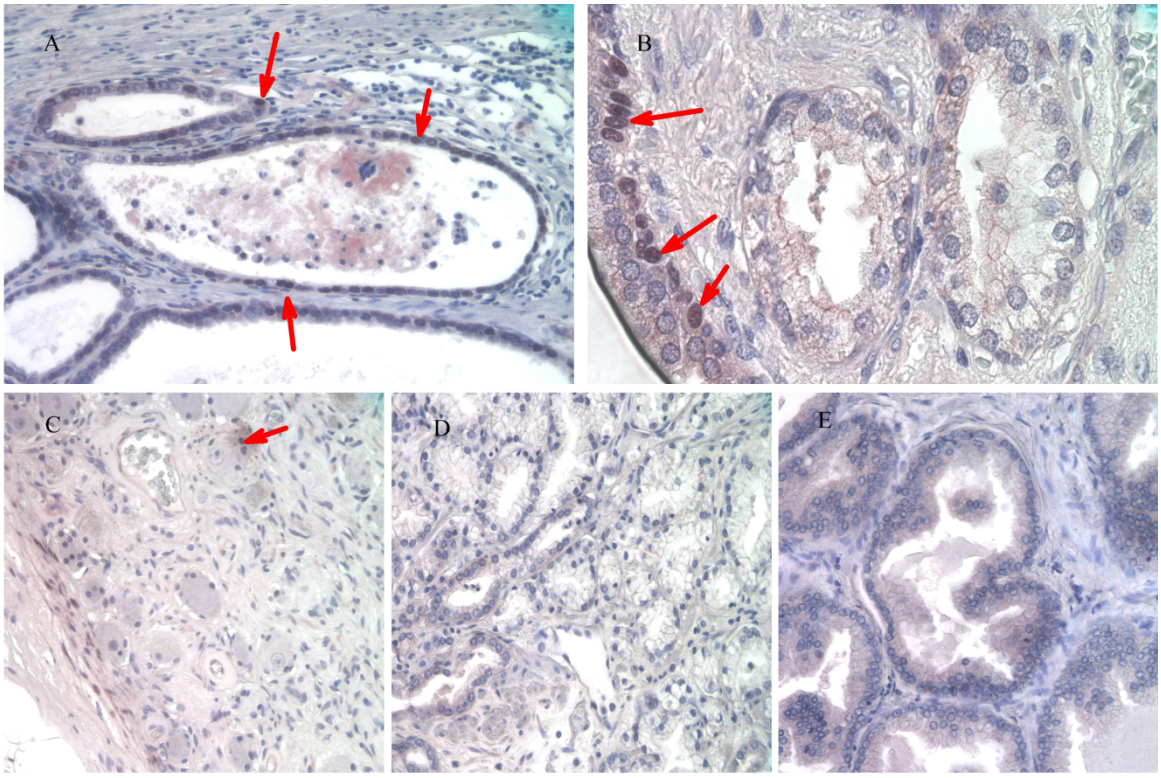


Figure 7: **Immunoperoxidase staining with anti AR-V7 on prostate cancer at different stages**. High risk and progression cancer (A-B) showing many tumor cells with strong positive nuclear staining. High risk and no progession cancer (C) showing few cells with low nuclear positive staining. Intermediate risk and no progression cancer (D) with negative staining for all cells. Low risk cancer (E) with negative staining for all cells. The magnification is 20X for panels A, C, D, E and 40X for panel B.

2. IN-VITRO ANALYSIS

We analyzed the sensitivity of PCa cell lines to single agent treatment to determinate the dose- response curves by trypan blue exclusion assay, similar results were obtained by MTT (3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazole assay) (figure 8).

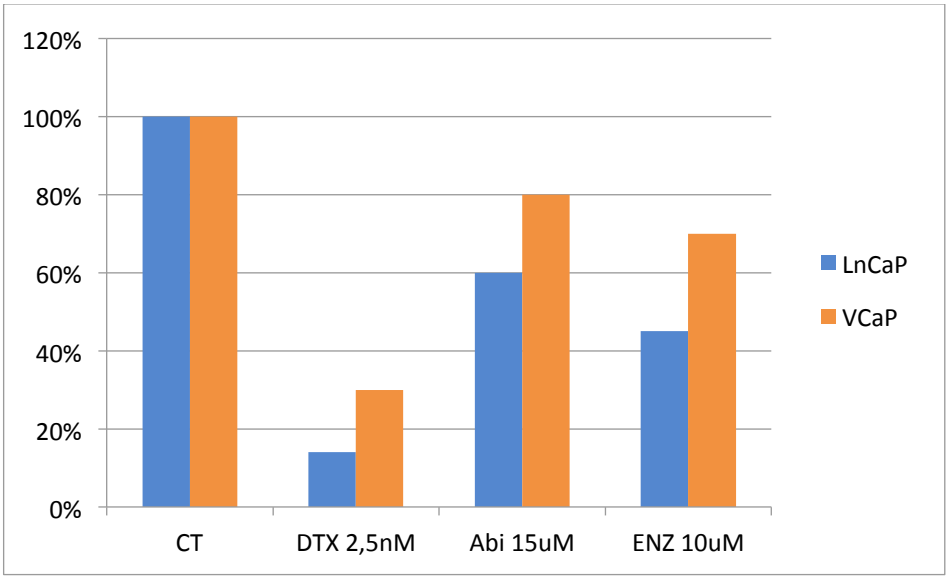


Figure. 8: **Determination of single compound on cell line.** The first results showed that DTX has a greater ability to inhibit cell growth of both cell lines: after treatment with 2,5nM only 14% and 30% respectively LnCaP and VCap cells are live. Treatment with ABI reduces the growth of LnCaP by only 40% and that of VCaP by 20%, while ENZA reduces by 55% the LnCaP (survive 45%) and by 30% (survive 70%) the VCap (TB data).

A combination of the different compounds can highlight the reciprocal effect on the of the different cell lines. In this context LnCaP and VCaP cell lines were treated with WTM (a representative inhibitor of PI3K) and RAD001 (an inhibitor of mammalian target of rapamycin (mTOR)) combined to DTX or drugs that act by inhibiting AR pathway, ENZ (AR signaling inhibitor) or Abi (Cyp 450 17A1 inhibitor). The proliferative effects were determined by MTT (Figure 9).

It is highlighted that DTX is the drug that mainly interacts with cellular growth, less evident by treatment with Abi and ENZ. Combined treatments with DTX further decreased cellular proliferation. The treatments on AR pathway combined with PI3K/mTOR inhibitors synergize by decreasing growth. In our experiments on LnCaP cell line, the strongest synergism was observed with ENZ+RAD, followed by Abi+WTM. Similar results were observed in experiments on VCaP.

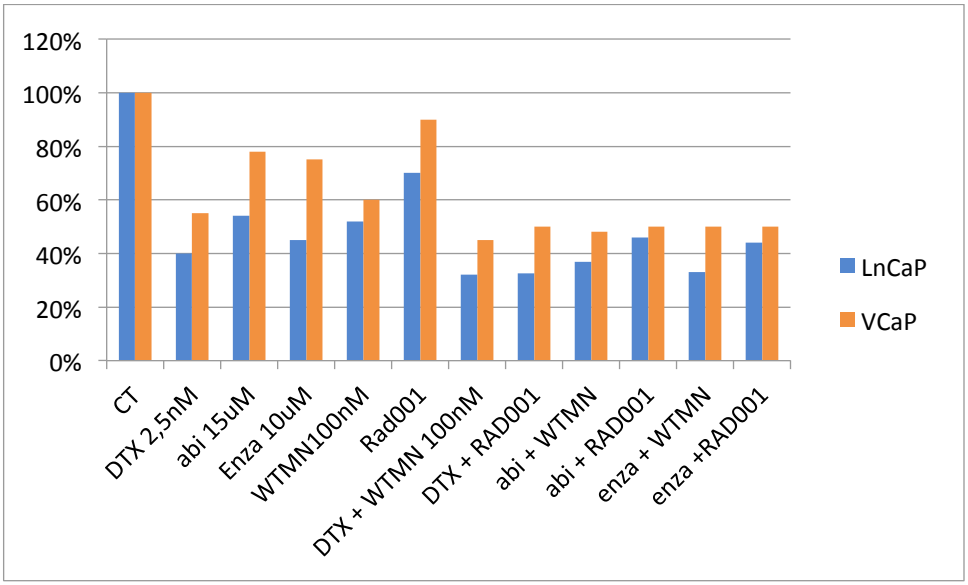


Figure 9: **Combination effect of therapies targeting the AR- and PI3K/mTOR signaling pathway**. In LNCaP treatment with DTX (2,5nM) + WTMN (100nM) (survival 7.7%) showed in MTT assay a decrease by 1.81 times the growth of cells compared to those treated with 1nM DTX (14%); a further 1.5% treatment with 1nM DTX + 100nm RAD001 (survival 9.5%, 14% 1nM DTX), therefore treatment with a lower concentration of DTX and an integration at different points of PI3/ mTOR had as a result of a further reduction in LnCaP growth. Treatment of LnCaP with 10 µM ABI, 5µM ABI + 100 mM WTMN, 5µM ABI + 100nM RAD001, showed respectively: 60%, 35%, 41% of proliferating cells. Treatment of LnCaP with 10 µM of ENZA, 5 µM ENZA + 100 mM WTMN, 5 µM ENZA + 100nM RAD001, 5 µM ENZA showed respectively: 45%,

38% and 25% of proliferating cells . The percentage of LnCaP lives after treatment with: 100nM WTMN, 100nM RAD001 was 78%, and 70%, respectively.

It is highlighted that DTX is the drug that mainly interacts with cellular growth, less evident by treatment with Abi and ENZ. Combined treatments with DTX further decreased cellular proliferation. The treatments on AR pathway combined with PI3K/mTOR inhibitors synergize by decreasing growth. In our experiments on LnCaP cell line, the strongest synergism was observed with ENZ+RAD, followed by Abi+WTM. Similar results were observed in experiments on VCaP.

To establish the causal relationship between cell growth arrest and death, we investigated whether the different compounds, alone or combined, induced apoptosis by analyzing Poly (ADP-ribose) polymerase (PARP) 1 cleavage carrying out a western blot (Figure10).

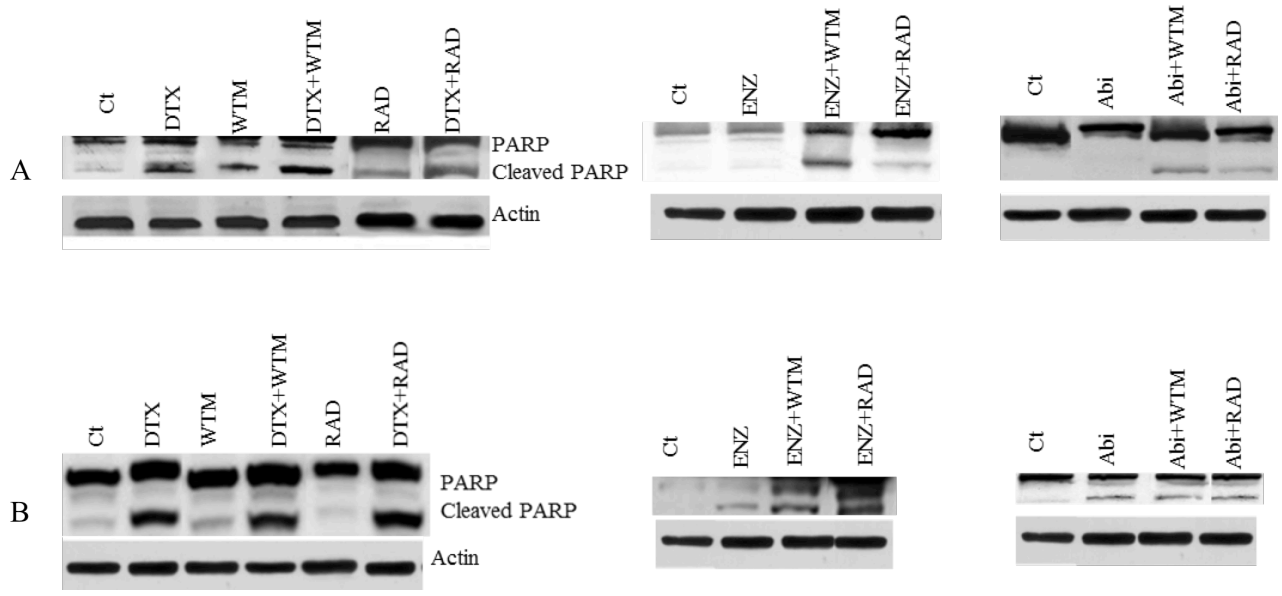


Figure 10: Effects of DTX, AR and PI3K/mTOR inhibitors in induction of apoptosis. Western blot of LnCaP A)and VCaP B)cell lines untreated (Ct) and treated for 24 hr with different combination for PARP1. Cleaved PARP detects the induction of apoptosis.

The cleavage of PARP1 is always evident in cells treated with DTX, specially combined with WTM. But while PARP cleaved is little or nothing detected in cells treated both Abi or ENZ, it becomes clearly detected in combined treated cells.

Targenting both AR and PI3K/mTOR signaling pathway resulted in an inhibition of cells growth and an induction of apoptosis in LnCaP as well as in VCaP that presents AR-V7.

To study cell cycle affected by AR or PI3K/mTor pathway inhibitors as well as DTX, cells were treated with the different compounds, alone or combined, for 48 hrs, and DNA content was evaluated using flow cytometry (Figure 11).

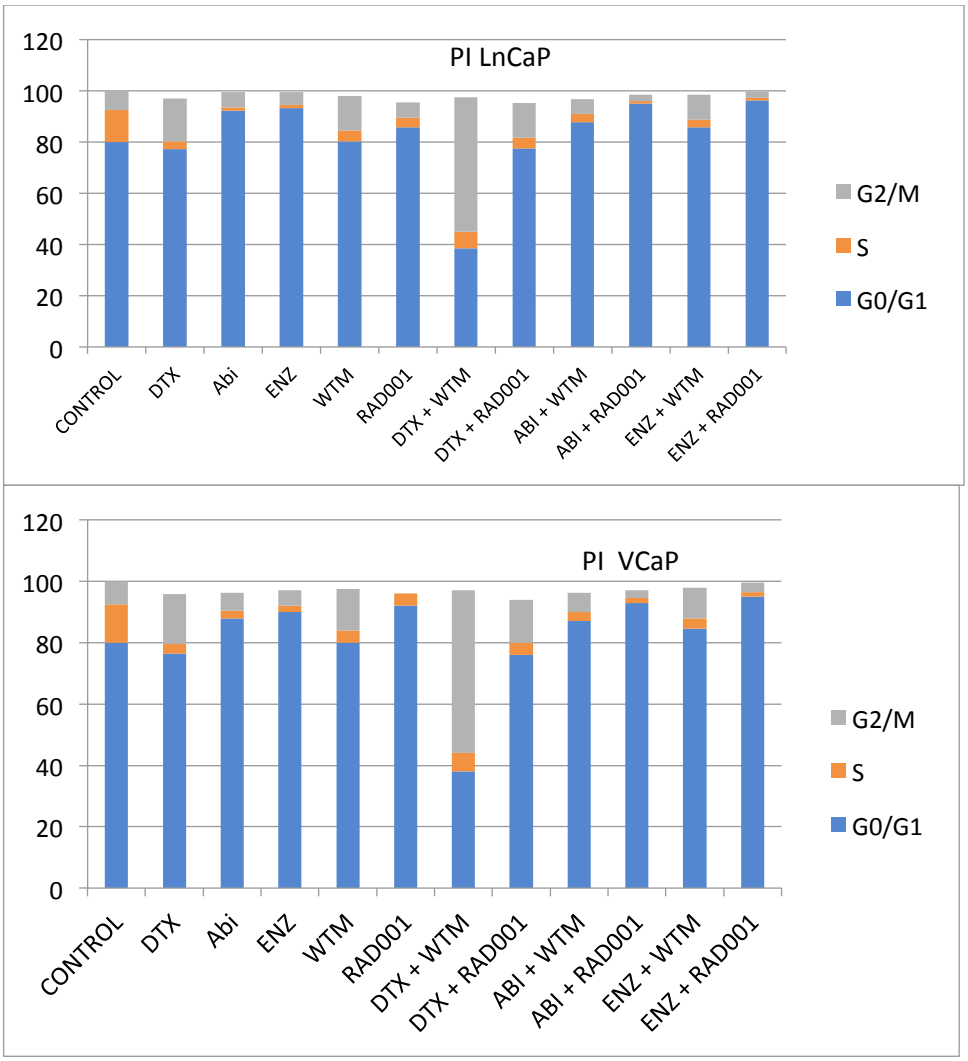


Figure 11: Targeting AR and PI3K/mTOR induces cell cycle arrest in prostate cancer cell lines. Distribution of cell cycle phase was determined by flow cytometry in LnCaP A) and in VCaP B). The treatment with DTX increases G2/M arrest, increased by adding WTM. The combination with WTMN increases G2/M and decreases S phase population in all samples. AR inhibitors increase Go/G1 and reduce S phase in LnCaP and VCaP.

It is already known that taxane treatments as DTX, distruptioning microtubuli function, induced an increased percentage of cells in G_2/M and a reduction of S phase population. The adding of WTM also induced G2/M arrest in all the samples with a reduction of S phase. The treatment DTX+WTM makes it strongest in both cell lines. AR pathway inhibitors increased G0/G1 and decreased S Phase population.

These effects contribute to inhibition of cell growth and induction of apoptosis in cells treated with DTX as well inhibitors of AR and PI3K/mTOR signaling pathway.

DISCUSSION

Patients with the metastatic PCa often develop a progression towards the metastatic form of the disease, known as castration-resistant prostate cancer (CRPC). For this group of patients the traditional treatment based on taxanes, as well as the treatment with abiraterone (Abi) and enzalutamide (ENZ), may have limited effectiveness resulting in a poor survival rates, the median duration of response is less than one year (48).

CRPC can results from several molecular mechanisms: gain of function of androgen receptor (AR) and activation of PI3K/mTOR signaling pathway also through loss of PTEN often correlates with this disease (74). Data from clinical trials have shown a moderate response in patient treated with a single agent probably due to the complex compensatory crosstalk between the AR and PI3K pathways (75,76). Thus a combined treatment targeting both the pathways could be an alternative treatment (76).

Resistance to Abi and ENZ is often correlate to constitutively active AR splice variants (AR-Vs), of which AR variant-7 (AR-V7) is the most well studied (48).

In the first part of the thesis we analyzed the presence of AR-V7 in sample obtained from patients submitted to radical prostatectomies with different stage disease and we observed a strong AR-V7 nuclear positivity only in patients with high risk and progression cancer. In both cases the chi-square test showed that there is a correlation between the two variables taken into consideration, but given that the value obtained by the receptor-progression correlation is more negative than the value obtained by the receptor-risk correlation, it can be stated that the correlation receptor-progression is statistically "stronger" than the receptor-risk.

In the second part of the thesis we also analyzed the effects of docetaxel (DTX) or compounds that target AR or PI3K/mTOR in combination each others on AR positive cell lines, LnCaP and VCaP, VCaP also shows AR-V7 spliced variant. LnCaP are Phosphate and tensin homolog (PTEN) negative prostate cells.

As expected we found that Docetaxel (DTX) induced proliferative reduction with a G2/M accumulation of cells and a reduction of S phase population, activation of apoptosis can cause the death in both treated cell lines. This suggest that the addition PI3K/mTOR inhibitor to classical therapies can enhance therapeutic efficacy, mostly wortmannin. It is konwn that

WTM shows substantial in vivo toxicity and thus is unsuitable for systemic therapeutic applications but several compounds that bind to and inactivate PI3K are in preclinical and clinical trials.

Enzalutamide (ENZ), AR signaling inhibitor or Cyp 450 17A1 inhibitor abiraterone (Abi) decrease both cells growth, more evident in LnCap compared to VCap. ENZ and Abi induce G1 arrest cycle. Analyzing the effects of combining treatment of AR inhibitor in AR positive conditions, LnCaP and VCaP cells, with PI3K/mTOR inhibitors we can detect that the adding of RAD001 is more effective in inducing apoptosis and G1 arrest even though WTM treatment induces a more cell growth inhibition.

These results suggest that targeting PI3K/mTOR combinig with the other drugs could be an useful clinical approach in hormone sensitive PC and CRPC but these experimental are need to be supported by clinical trials.

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