



Combinatorial Effect of Abiraterone Acetate and NVP-BEZ235 on Prostate Tumor Progression in Rats

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Abstract

Use of drug combinations that target different pathways involved in the development and progression of prostate cancer (PCa) has emerged as an alternative to overcome the resistance caused by drug monotherapies. The antiandrogen abiraterone acetate and the PI3K/Akt inhibitor NVP-BEZ235 (BEZ235) may be suitable options for the prevention of drug resistance and the inhibition of PCa progression. The aim of the present study was to evaluate whether abiraterone acetate and BEZ235 achieve superior therapeutic effects to either drug administered as monotherapy, in the early stages of PCa in an androgen-dependent system. Our study showed that each drug might impair tumor growth by reducing proliferation and increasing cell death when administered as monotherapy. However, tumor growth continued to progress with each drug monotherapy and some important side effects were related to BEZ. Conversely, when used in combination, the drugs impaired the inflammatory response, decreased hyperplastic lesions, and blocked tumor progression from premalignant to a malignant stage. Our data showed that the strategy to block the androgenic and PI3K/AKT/mTOR pathway is an effective therapeutic option and should be investigated including distinct PI3K pathway inhibitors.

Introduction

Prostate cancer (PCa) is the second most common cancer among men worldwide. The incidence of the disease has increased in part due to advances in diagnosis and the increase in life expectancy of the population [1]. It is expected that by 2030 there will be 1.7 million new cases and 499,000 new deaths due to the growth and aging of the global population [2, 3].

PCas require androgen for growth. Therefore, androgen deprivation therapy (ADT) is currently the first-line treatment for PCa [4, 5]. However, it is not considered the ideal treat-

ment for eradicating PCa. Despite the initial efficacy of ADT, most patients with advanced PCa develop castration-resistant prostate cancers (CRPC) [6]. Although more than 50% of patients with CRPC respond to secondary hormonal interventions, the response is moderate and therapeutic agents that are more effective are required [7]. Thus, in this setting, the combination of antiandrogens with therapies that target signaling pathways involved in tumor progression represents an effective therapy [5, 6, 8].

Abiraterone is a potent inhibitor of CYP17A1, an enzyme that blocks androgen biosynthesis in the testis, adrenal glands, and prostate [9, 10]. The use of abiraterone leads to reduced levels of androgens in serum, delayed tumor growth, and, most notably, intratumoral androgen levels that are undetectable and not observed with conventional ADT [11]. The drug has been used to treat CRPC after chemotherapy and, less frequently, for early-stage cancers [12].

Although primarily used for androgen receptor (AR)-independent PCa, the efficacy of abiraterone has been reported for AR-positive cancers [13]. The authors postulated that the attenuation of AR signaling is not the only rationale to explain the anticancer activity of abiraterone since it effectively reduced proliferation rates even for cancer cells lacking AR signaling. Because of these

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conflicting results, it is important to investigate the effects of this therapy in the initial stages of PCa, in an androgen-responsive setting. This could represent an effective alternative to delay or even prevent the development of drug resistance and androgen-independent growth in this clinical setting.

Because of the importance of the PI3K/AKT/mTOR pathway in tumor development and progression, numerous inhibitors have been designed for cancer treatment [14]. BEZ235 is a dual PI3K and mTORC1/2 inhibitor, and its antitumor activity is expected to be higher than that of mTORC1 inhibitors due to the inhibition of phosphorylated Akt upregulation through mTORC2 [15]. This agent shows an inhibitory effect by binding to the adenosine triphosphate binding cleft of p110 and mTORC1/2. The drug binds to and blocks the catalytic sites of all isoforms of PI3K and mTOR [15, 16], and has been shown to inhibit the tumor growth of breast, prostate, and myeloma cell lines [15–17]. The p110 α and p110 β isoforms are expressed in most normal tissues and are involved in PCa [18, 19], and p110 δ is possibly restricted to cells of the immune system [20, 21].

Several studies have evaluated the effect of BEZ235 alone and in association with other therapeutic agents on PCa growth [22]. Carver et al. [8] demonstrated that the combined inhibition of AR and PI3K/Akt signaling by enzalutamide (MDV3100) and BEZ235, respectively, increased antitumor activity in a hormone-sensitive LNCaP xenograft model. Recent clinical trials have pointed out some important side effects related to BEZ and the clinical development of the drug as a potential therapy for PCa has been discontinued during the conclusion of this work [23].

Inhibition of the PI3K/Akt pathway by new molecules represents a promising approach to preventing tumor progression [8, 24, 25]. However, prolonged treatment could result in drug resistance, which leads to positive regulation of the androgen receptor pathway and reduces the anticancer effect [8, 26, 27]. Most clinical trials have targeted patients who have already developed androgen resistance [28]. However, some studies have suggested that the PI3K/Akt pathway is required for the development of CRPC. Thus, co-targeting the PI3K and AR pathways in the early stages of PCa development could delay androgen resistance [29, 30].

The crosstalk between the androgen receptor and the PI3K/AKT/mTOR pathways allows for the development of novel strategies to inhibit cancer growth more effectively. The present study was performed to evaluate whether, in the early stages of PCa, abiraterone acetate, and BEZ235 administered in combination, achieve better therapeutic effects than when each agent is administered alone, in an androgen-dependent setting.

Materials and Methods

Animals

The experiments were performed using 12-week old male Fischer 344 rats, obtained from colonies maintained under specific pathogen-free conditions in the Multidisciplinary Center for Biological Investigation (CEMIB-UNICAMP, Campinas-SP, Brazil). The animals were housed under controlled environmental conditions (temperature: 22 ± 2 °C; relative humidity: $55 \pm 20\%$; 12/12-h light–dark cycle; and continuous air exhaust) and were provided free access to water and standard chow diet. The animals were handled in line with the ethical principles for animal research established by the Brazilian Council for Control of Animal Experimentation, and the experimental protocol was approved by the Ethics Committee on the Use of Animals from the Institute of Biosciences–UNESP (Protocol no. 559-CEUA).

Chemical Induction of Carcinogenesis and Experimental Design

The tumor induction methodology used in the study was developed and provided by the Laboratory of Urogenital Carcinogenesis and Immunotherapy of the Institute of Chemistry/UNICAMP. All animals were subjected to carcinogenesis induction, which consisted of (1) pretreatment with testosterone cypionate (100 mg/kg)—administered via subcutaneous injection for three consecutive days, followed by; (2) intraprostatic administration of the carcinogen N-methyl-N-nitrosourea (MNU)—the animals were anesthetized with 2% xylazine hydrochloride (5 mg/kg i.m.; König, São Paulo, Brazil) and 10% ketamine hydrochloride (60 mg/kg i.m., Fort Dodge, Iowa, USA). A suprapubic incision of 0.5 cm was made in the dorsolateral prostate for inoculation of MNU (15 mg/kg; Sigma, St. Louis, MO, USA) dissolved in sodium citrate (1 M, pH 6.0) and co-administered with a thermosensitive copolymer (Pluronic 127) in the capsule of the dorsolateral prostate; (3) carcinogenesis promotion—1 week after MNU administration the animals received subcutaneous doses of testosterone cypionate (5 mg/kg—diluted in corn oil) twice a week for 220 days.

After tumor induction, the animals were randomly divided into four groups. The drugs were administered by gavage for ten consecutive days as follows. The control group received only the drug vehicles 1 (6% ethanol diluted in corn oil) and 2 (1:9/ 1-methyl-2-pyrrolidone (NMP): polyethylene glycol 300 (PEG)) group A received abiraterone acetate (14 mg/kg, Cayman Chemical—in vehicle 1: 6% ethanol diluted in corn oil—[8, 16, 31] and vehicle 2); group B received BEZ235 (45 mg/kg, Cayman Chemical) and vehicle 1. BEZ235 was dissolved in 1-methyl-2-pyrrolidone (NMP) and then diluted with

polyethylene glycol 300 (PEG300) to a final concentration of 10% v/v NMP and 90% v/v PEG300 (vehicle 2: 1:9/ NMP: PEG) [15, 32, 33]; group AB received abiraterone acetate + BEZ235. The procedures are illustrated in Fig. 1.

Biometric Data

During the experiment, body weight was monitored. Following the treatments, blood samples were collected to measure testosterone and dihydrotestosterone (DHT) levels. Testosterone levels were determined by double-antibody radioimmunoassay using the Coat-A-Count® (Diagnostics Products Corporation, Los Angeles, USA) and DHT levels (DHT—Diagnostics Biochem Canada Inc., Canada) were determined by enzyme-linked immunosorbent assay (ELISA). The intra-assay variation was 1.75%, and the results were expressed in ng/ml and pg/ml. All procedures were performed at the Neuroendocrinology Laboratory, School of Dentistry of the University of São Paulo, USP, Ribeirão Preto, SP, Brazil.

Following the treatment period, each animal was subjected to a complete autopsy, and the dorsolateral prostate (DL) was removed, weighed, and subjected to histopathologic and molecular analysis.

Processing of Samples

The dorsolateral prostate was fixed by immersion in Methacarn (methanol, chloroform, and acetic acid at a ratio of 7:2:1) for 3 h at 4 °C. After fixation, the samples were dehydrated in ethanol, clarified in xylene, and embedded in paraffin (Histosec™, Merck, Darmstadt, Germany). Serial step sections (4 µm) were made in an automatic rotary microtome (Leica) picking up a section and discarding

the next 10, until the entire prostate lobe had been sectioned. For the molecular assays, prostate samples were homogenized in RIPA buffer plus protease and phosphatase inhibitors cocktail (Sigma-Aldrich, St Louis, USA) in Tureaux type homogenizer (Ultra Stirrer—Ultra80) for 3 cycles of 10 s. The homogenates were centrifuged at 14000 rpm for 20 min at 4 °C, and the supernatants were collected. The protein quantification was performed using the Bradford method, absorbance measured in an ELISA reader (595 nm), and the samples were frozen for western blotting assay.

Morphologic Analysis

Histopathologic Classification and Determination of Lesion Multiplicity

Histologic sections were stained with hematoxylin-eosin (HE) for histopathologic classification of prostate neoplasms as described previously [34–36]. The entire dorsolateral prostate from each animal was examined to quantify the multiplicity (number of prostatic lesions per analyzed field, $n = 25$ histologic areas/group) and incidence (number of affected animals, $n = 5$ animals/group) of each lesion. Double-blind quantifications were performed to determine the number of inflammatory disorders as well as benign, premalignant, and malignant lesions. An experienced veterinarian pathologist confirmed the analyses.

Determination of the Proliferative and Apoptotic Index

For detection of proliferating cells, the prepared sections were dewaxed, rehydrated through graded alcohols, and

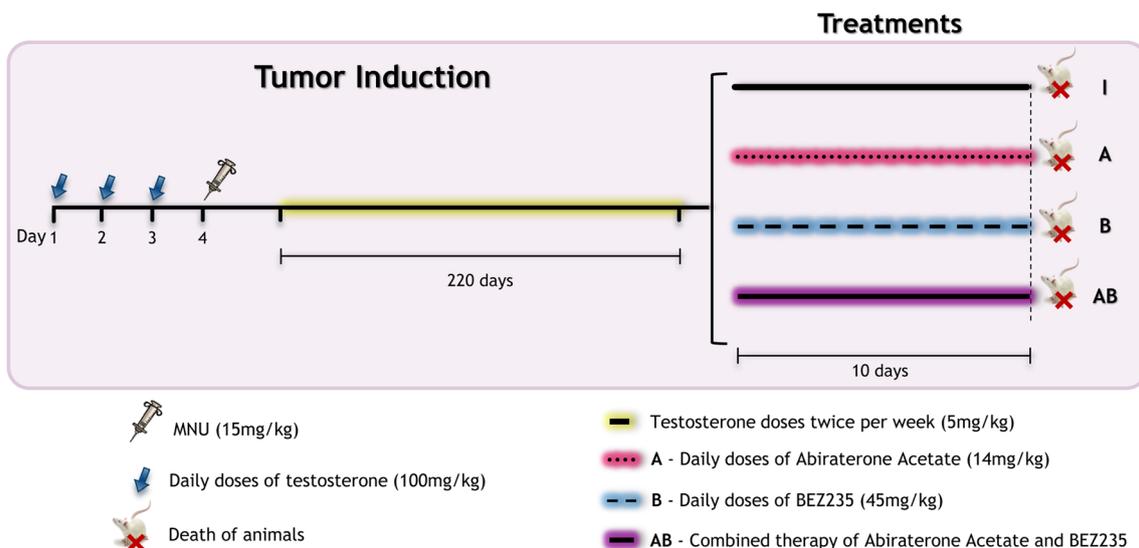


Fig. 1 Experimental design representing the carcinogenesis induction, drugs, and procedures performed

the antigen retrieved in Tris-EDTA buffer pH 9.0, at 97 °C for 20 min. The endogenous peroxidases were blocked by covering the slides with H₂O₂ (3% in methanol) for 15 min. To block the nonspecific protein-protein interactions, the sections were incubated with 3% non-fat milk plus 1% goat serum in PBS for 30 min and incubated overnight at 4 °C with the primary antibody anti-Ki67 (1:100, ab16667-Abcam, Cambridge, MA, USA) diluted in 1% BSA. The slides were subsequently incubated for 90 min at 37 °C with HRP-conjugated secondary goat-anti-rabbit antibody (1:200, ab-97,051-Abcam, Cambridge, MA, USA) in 1% BSA. Apoptosis was measured in the sections according to the instructions of In Situ Cell Death Detection kit, POD (Roche), which is based on the TUNEL assay. The revelation of both reactions was performed with diaminobenzidine solution (DAB, Sigma, St. Louis, MO) and hematoxylin was used for counterstaining.

To determine the proliferative and apoptotic index, 30 random prostatic areas of each group were used and were examined using the ×40 objective lens. In each field, the percentage of positive epithelial cells was determined relative to total cells, and the results were analyzed for statistical significance.

Quantification of Mast Cells on Dorsolateral Prostate

Histologic sections were subjected to cytochemical methods to identify mast cells. The sections were stained with 1% toluidine blue (aqueous solution). A total of 45 random fields per group were used to determine the mean number of mast cells per 1 mm² area. The values obtained were analyzed for statistical significance between groups.

Western Blot Analysis

Aliquots (70 µg of protein) were separated on SDS-PAGE. Following the electrophoresis, the proteins were transferred to nitrocellulose membranes. The nonspecific binding of proteins was blocked by incubating the membrane in 3% BSA in TBST buffer for 90 min at room temperature. The membranes were incubated with the respective primary antibody in 1% BSA diluted in TBST overnight at 4 °C: p-AKT (T308) (#9275 s); p-AKT (S473) (#4060 s); Pan-AKT (#4691 s); FoxO1 (#2880); p-FoxO1 (#9461); PI3K (#5569 s); BAD (#9292); p-BAD (#9295); XIAP (#2042); p-mTOR (#2971 s); mTOR (#2972 s) (Cell Signaling Technology, Inc. USA); phospho-AR (ab71948); Bcl-2 (ab7973); TNF-α (ab1793); IL-6 (ab6672); IL-10 (ab9969) (Abcam® Inc., USA); AR (sc-816); β-actin (sc-47,778) (Santa Cruz® Biotechnology, Inc., USA).

The membranes were then incubated with a specific secondary antibody conjugated with peroxidase, which was

diluted 1:5000–30,000 in 1% BSA for 1 h (IgG goat-anti-rabbit, ab97051 or IgG goat-anti-mouse, ab97023, Abcam® Inc., USA). The immunoreactive components were revealed by GE Amersham ECL chemiluminescent substrate (GE Healthcare). Analyses were done in three different biological samples per group (three animals/group). To calculate the mean and SEM, the optic density of band was used as the unit of measure with software Image J (Version 1.33u—National Institutes of Health, USA), and normalized by endogenous control β-actin.

Statistical Analysis

The results were checked for differences between groups using Prism 5.0 software (GraphPad). The statistical difference among the parameters was considered significant when $p \leq 0.05$. The tests were chosen in agreement with the sampling distribution of each parameter. Parametric data were analyzed by ANOVA followed by the Tukey-Kramer test. The nonparametric analysis was performed, by Mann-Whitney's test or Kruskal-Wallis test followed by Dunns test.

Results

Effect of Drugs on Body Weight and Hormone Levels

More than 7% loss in body weight was observed after administering BEZ235 (Fig. 2). At the beginning of the experiment, body weights were similar between groups. One week after carcinogenic induction, there was an expected non-significant decrease in body weight due to exposure to MNU. Weight loss is a side effect that could be relate to the BEZ administration. The drugs did not affect testis, adrenal, kidney, and dorsolateral prostate weight (data not shown). Serum levels of testosterone decreased only in the AB group, while DHT levels decreased in all treatment groups, especially the AB group (Fig. 3). These results show that abiraterone effectively decreased steroidogenesis.

Abiraterone Acetate and BEZ235 Inhibit the Growth of Prostate Lesions

We examined the occurrence of hyperplastic lesions (atypical hyperplasia and reactive hyperplasia), inflammatory disorders (intraluminal and periductal infiltrates), premalignant lesions (prostatic intraepithelial neoplasia—PIN), and malignant lesions (microinvasive carcinoma and adenocarcinoma).

Our results showed a high incidence of histopathologic lesions on the dorsolateral prostate. Inflammatory disorders

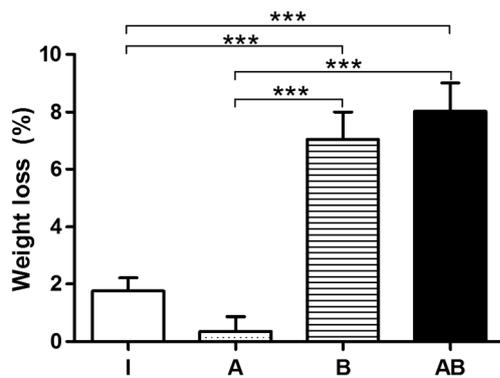


Fig. 2 Body weight loss after the treatments. Statistical analysis was performed using the Tukey-Kramer test. Values represent mean \pm SEM ($n = 7$), *** $p \leq 0.0001$

were frequent in this lobe, suggesting a greater propensity for the development of inflammation. Reactive hyperplasia was the most common lesion, and the reduction in a multiplicity of treated groups was similar (Fig. 4a). Inflammatory infiltrates were common in the luminal and stromal compartments. However, a reduction in the multiplicity of these lesions was observed when the drugs were administered either alone or in combination (Fig. 4b, c).

In general, the control group had the highest number and incidence of lesions, mainly premalignant and malignant lesions. The groups treated with each drug alone (A and B groups) had more atypical hyperplasia foci than did the control and the AB group (Fig. 4d), but premalignant and malignant lesions decreased (Fig. 4e, f). More importantly, abiraterone and BEZ235 together decreased the number of hyperplastic, premalignant, and malignant lesions. Thus, while in the control group there was a predominance of premalignant and malignant lesions, the drugs could block the growth and progression of the lesions.

Since proliferation and apoptosis control growth or tumor regression, the proliferative and apoptotic index were assessed. Proliferation decreased markedly following the treatments (Fig. 5). Apoptosis increased in the glandular

epithelium following treatment with abiraterone with or without BEZ235 (Fig. 5). Interestingly, the drug combination promoted the highest apoptotic indexes, with a significant increase compared to that in group A.

Abiraterone Acetate and BEZ235 Attenuate Inflammatory Responses in the Dorsolateral Prostate

Our study showed frequent inflammatory infiltrates in prostate tissues. Thus, we measured the presence of mast cells and the expression of pro-inflammatory (TNF- α and IL-6) and anti-inflammatory cytokines (IL-10) in the tissues. Mast cell count reduced in the treated groups (Fig. 6). Consistent with the histopathologic data, protein expression analyses showed reduced inflammatory response. TNF- α expression decreased in groups A and AB, while IL-6 expression decreased after treatments, mainly with BEZ235 (Fig. 8). Conversely, only the combination of abiraterone and BEZ235 reduced IL-10 expression (Fig. 8).

Effects of Abiraterone Acetate and BEZ235 on AKT and AR Signaling

Since abiraterone acetate and BEZ235 act on androgen and the PI3K/Akt pathways, respectively, the effects on androgen receptor and mTOR and PI3K-mediated signaling were assessed by measuring the expression level of the total and activated forms of their effectors downstream.

Each drug when used as monotherapy or in combination decreased PI3K expression and the activated forms of the androgen receptor (Fig. 7). Treatment with the drugs alone reduced expression of Akt, but after treating with BEZ235, the reduction was more significant (Fig. 7). In addition, BEZ235 decreased the activated forms of Akt. However, abiraterone could also decrease p-AKT (Ser473) expression. The reduction in activated proteins could be the result of a decrease in total Akt expression in

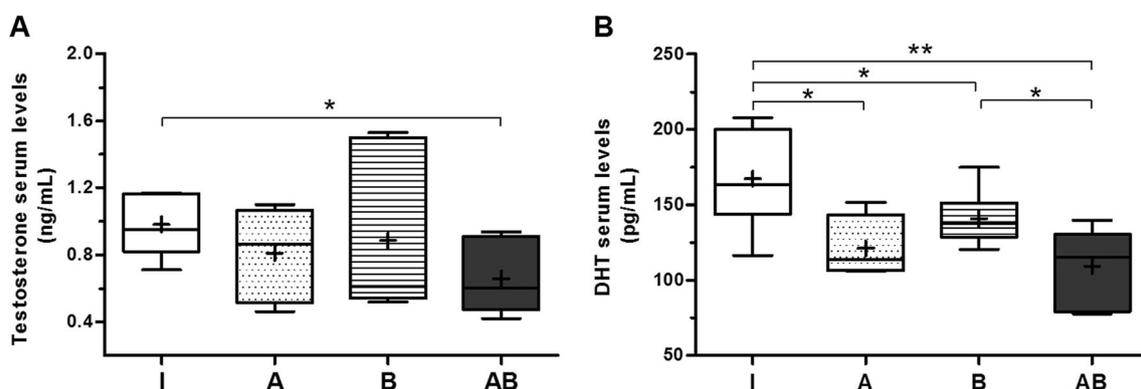
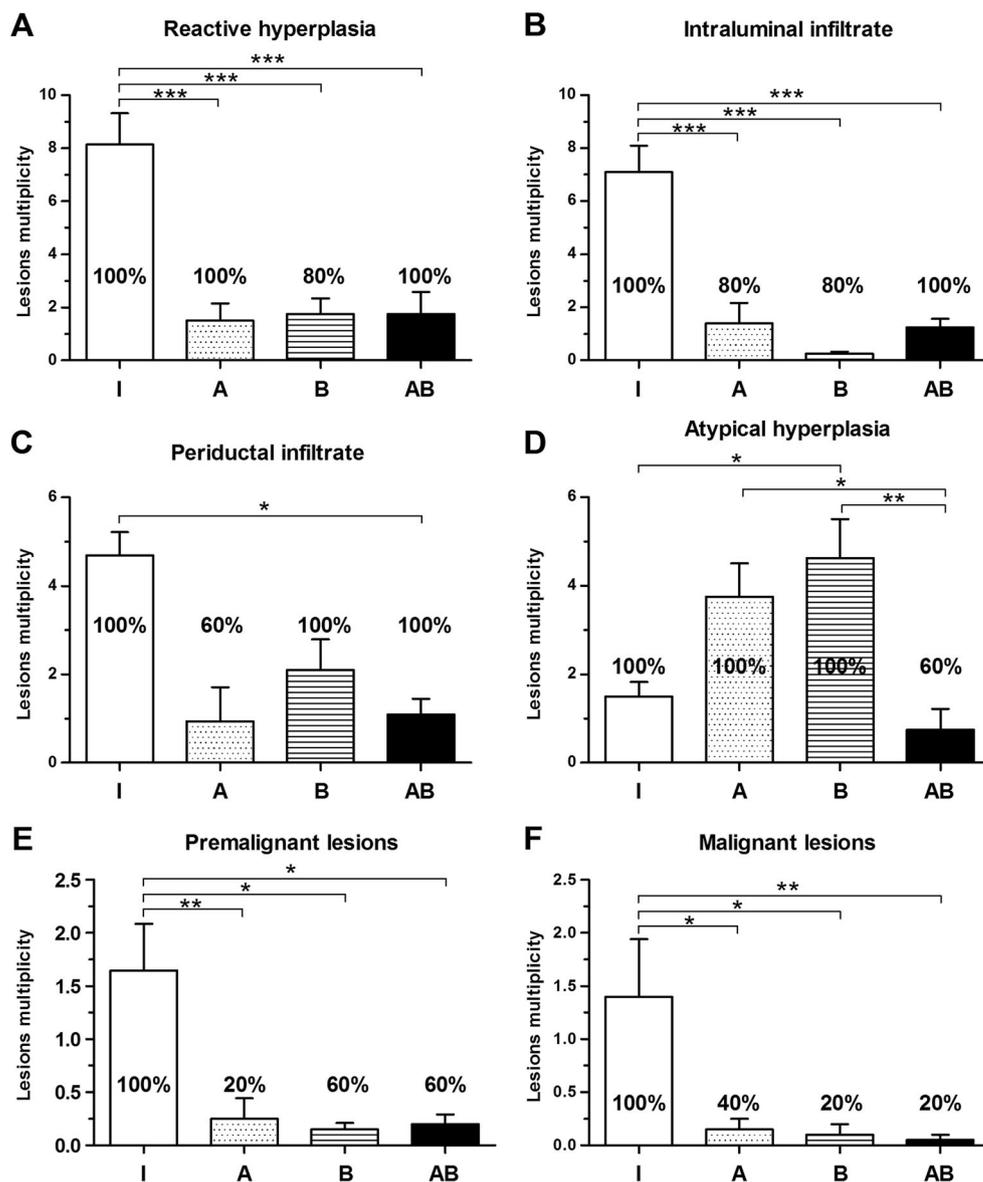


Fig. 3 Serum levels of testosterone (a) and DHT (b). The values represent maximum and minimum and (+) represents the mean. Statistical analysis was performed using the Kruskal-Wallis and Dunn's tests. Values represent mean \pm SEM ($n = 6$), * $p \leq 0.05$; ** $p \leq 0.01$

Fig. 4 Multiplicity and incidence of prostate lesions: Reactive hyperplasia (a), intraluminal (b), and periductal infiltrates (c), atypical hyperplasia (d), premalignant lesions (e), and malignant lesions (f). The graphs represent the multiplicity of lesions, while the values expressed as a percentage represent lesion incidence (%). Statistical analysis was performed using the Kruskal-Wallis and Dunn's tests. Values represent mean \pm SEM ($n = 5$), * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.0001$



the monotherapy groups. Although the expression level of Akt did not change in the AB group, the activated state (T308) decreased its expression, indicating that BEZ235 contributed to this inactivation. Similarly, treatment with BEZ235 alone or in combination with abiraterone resulted in reduced expression of pmTOR, but the expression level of mTOR did not change.

Our examination showed increased apoptotic index following the treatments. Thus, the analysis of the expression of proteins involved in this process, and stimulated by Akt, as shown by the expression of BAD, XIAP, Bcl-2, and FoxO1 (Fig. 8) may clarify the mechanisms for tumor reduction. BAD decreased in the B and AB groups, while pBad, XIAP, Bcl-2, and FoxO1 expression levels decreased in all treated groups. In addition, the expression level of FoxO1 decreased in the A and AB groups.

Discussion

Therapies involving the combination of drugs that act on different pathways to regulate PCa growth have been studied as alternatives to overcome the resistance that monotherapies can cause. In the present study, we evaluated the effect of abiraterone acetate and BEZ235, when used alone or in combination, on carcinogenesis in the dorsolateral lobe of rat prostates. When used alone, the drugs reduced proliferation and elevated cell death, impairing tumor growth. However, their effects significantly improved when used in combination in the initial stages of AR-dependent PCa.

Studies investigating the resistance to abiraterone have reported increased expression of the *Cyp17a1* gene and androgen receptors as possible mechanisms [37]. Our data showed that during the treatment period, the drug resistance stage was

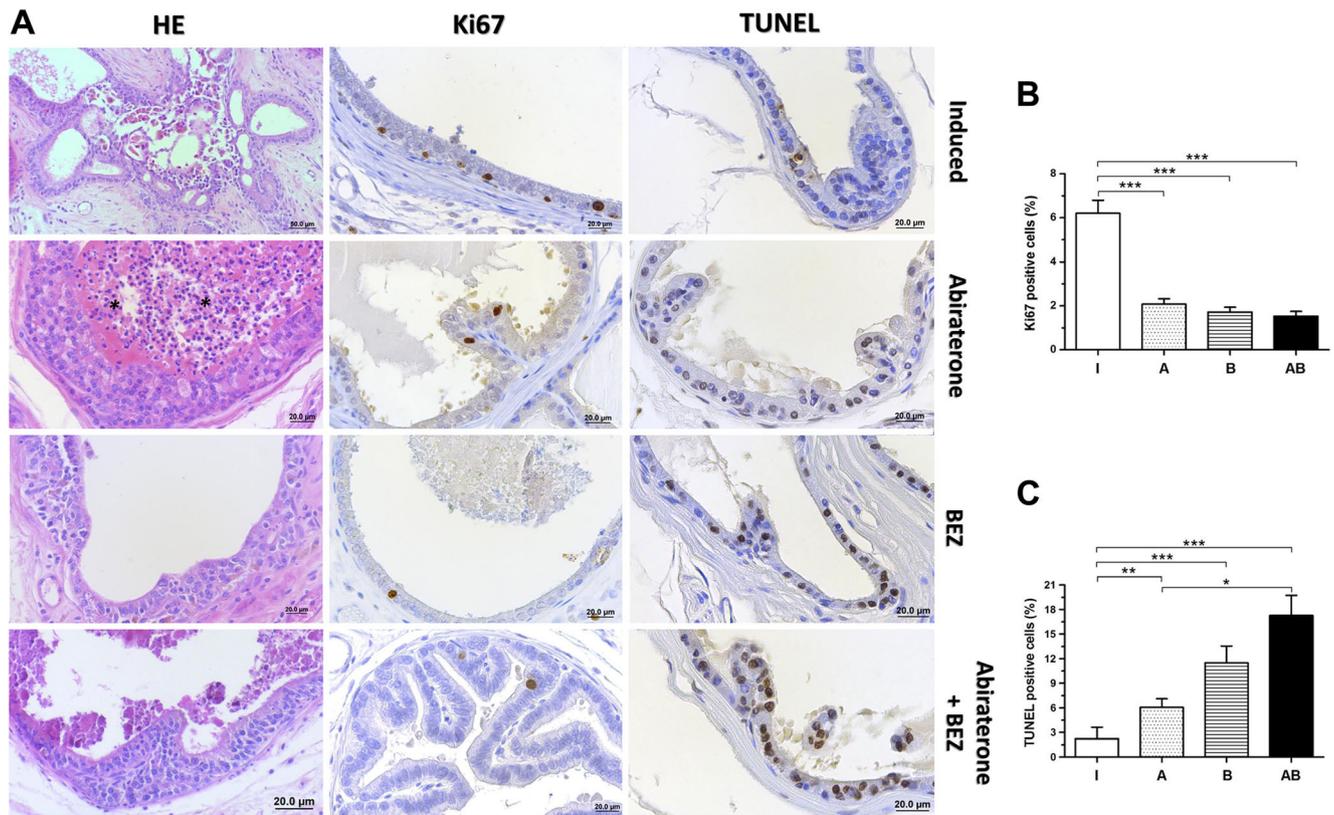


Fig. 5 Morphological characterization of prostate lesions (a) and proliferative (b) and apoptotic indexes (c) in the epithelial compartment. In the control group, malignant lesions such as adenocarcinoma were more frequent. Reactive hyperplasia was characterized by atypical epithelium stratification and inflammatory infiltrates (*). Atypical

hyperplasia was characterized by atypical epithelium stratification and was more frequent in treated groups. Statistical analysis was performed using the Kruskal-Wallis and Dunn’s tests. Values represent mean ± SEM ($n = 5$), $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.0001$

not achieved since drug monotherapy impaired tumor growth. Androgen deprivation only results in tumor regression in

androgen-dependent settings [38]. Since a reduction in tumor growth was shown following androgen inhibition by

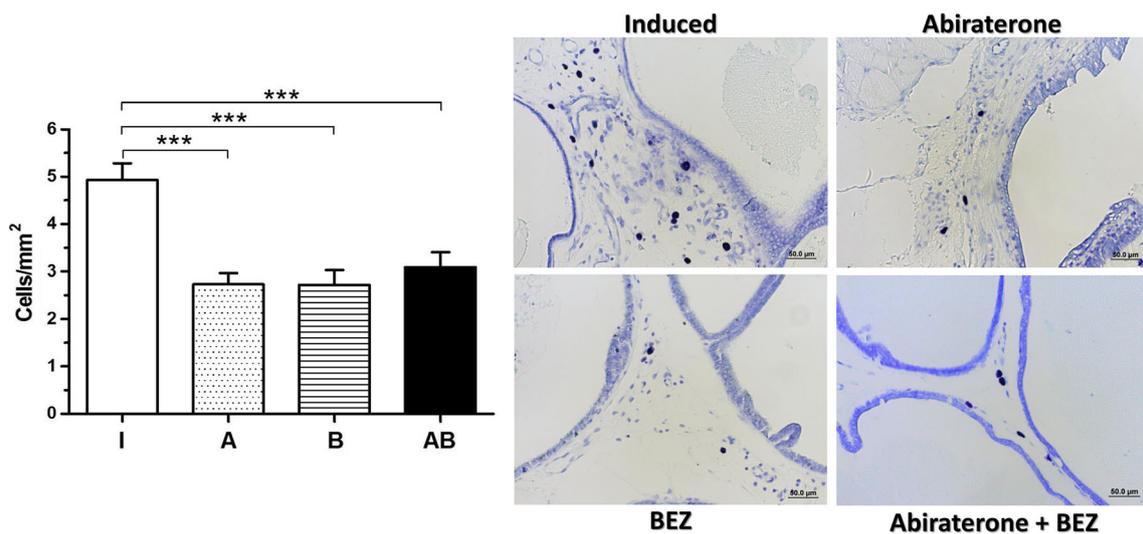


Fig. 6 Quantification of mast cells on dorsolateral prostate sections. The sections were stained with 1% toluidine blue (aqueous solution). Statistical analysis was performed using the Mann-Whitney test. Values

represent mean ± SEM ($n = 5$), $***p \leq 0.0001$. Figures show the reduction of mast cells in stromal compartment after the treatments

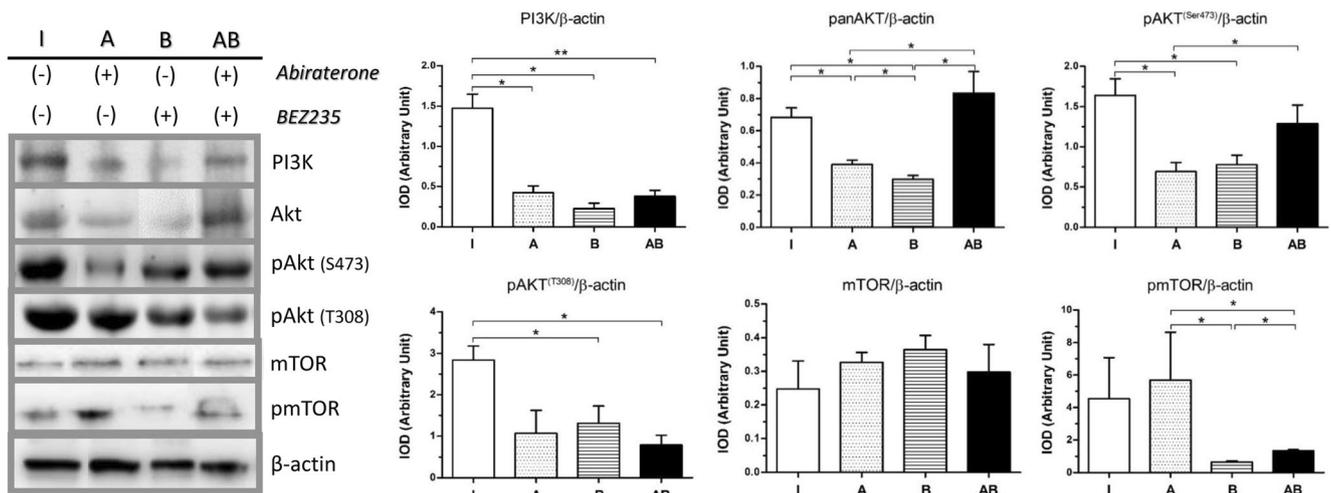


Fig. 7 Effects of abiraterone and BEZ235 on Akt signaling pathway. Protein expression change was analyzed by western blot, and β actin was shown as loading control. The data represent the relative

expression of integrated optical density (IOD) of proteins. Values represent mean \pm SEM ($n = 3$ biological samples). Statistical analysis was performed using the Mann-Whitney test. * p < 0.05; ** p < 0.01

abiraterone, we can confirm that our model was androgen-dependent. Androgen-dependent tumors commonly express high levels of AR [39, 40]. Thus, the results suggest that the AR expression reduction, in the treated groups, contributed to a decrease in tumor growth. Interestingly, we observed the AR expression reduction after exposure to BEZ235. Our results are consistent with those of Wang et al. [14] where a decrease in AR expression and activity was reported in breast cancer tumors after treatment with BEZ235. The authors suggested that AR-dependent tumors are more responsive to treatment

with BEZ235. However, the rationale for these observations is still poorly understood.

Furthermore, studies show that the forkhead transcription factor 1 FOXO1 inhibits androgen and androgen receptor-mediated gene expression and, consequently, suppresses cell proliferation [41]. FoxO transcription factors are involved in several physiological processes, regulated by Akt proteins [42], including cell proliferation and death as well as cell cycle regulation and cancer growth [43]. Akt inhibits FOXO1, which in turn abolishes FOXO1-mediated inhibition of AR,

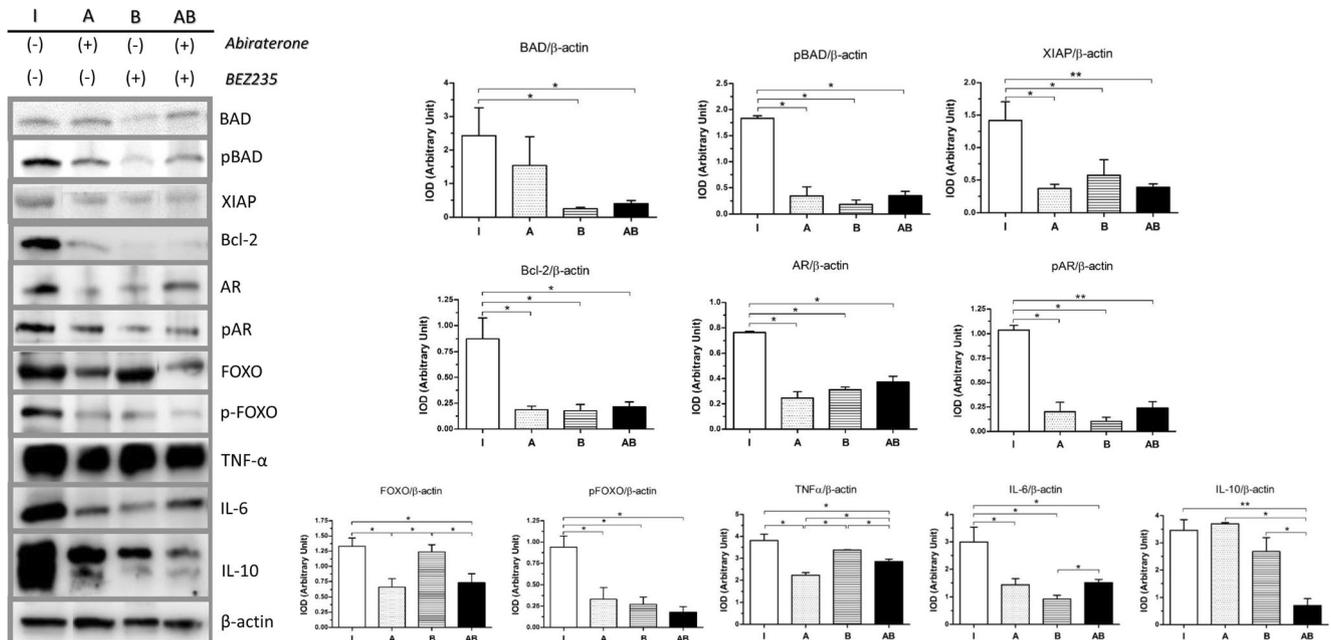


Fig. 8 Effects of abiraterone and BEZ235 on androgen receptor and other signaling pathways. Protein expression change was analyzed by Western blot, and β actin was shown as loading control. The data represent the

relative expression of integrated optical density (IOD) of proteins. Values represent mean \pm SEM ($n = 3$ biological samples). Statistical analysis was performed using the Mann-Whitney test. * p < 0.05; ** p < 0.01

thereby driving carcinogenesis progression. Conversely, inactivation of Akt enables FOXO1 activities [44]. Our study showed an increase in the active form of FOXO1 after BEZ235 exposure, which is consistent with the inactivation of AR and, consequently, with anti-proliferative effects. These findings are consistent with the results of Wang et al. [14]. Although BEZ235 also interferes with the androgenic pathway, the mechanisms are still unknown.

The promotion of cell survival in neoplasms favors malignancy and represents the mainstay of tumor progression in PCa. The Akt pathway is mainly involved in the regulation of cell growth and survival in tumorigenesis and is particularly relevant in PCa [45]. To exert its biological effects, Akt must be activated, which is achieved via phosphorylation at PDK1 (Thr308) and mTOR (Ser473) [46]. Consequently, blocking these sites is likely to inhibit Akt activity [8]. Thus, BEZ235 seems to be a good candidate for PCa treatment, since it inhibits PI3K and mTOR simultaneously. The dual inhibition was found in the present study and is consistent with previous studies [16, 47]. Thus, we can suggest that the decrease in Akt, mTOR, and PI3K in the prostate of BEZ235-treated rats was one of the factors responsible for the decrease in cell proliferation observed in those groups.

Unfortunately, inhibition of the AKT pathway and consequent impact on tumor growth was accompanied by side effects, such as weight loss and diarrhea following the use of BEZ. Phase I studies of BEZ235 in metastatic castration-resistant PCa showed adverse effects such as mucositis, vomiting, diarrhea, nausea, and weight loss [23, 48]. Due to questionable safety and tolerability profile of BEZ235, the clinical development of the drug as a potential therapy for PCa was discontinued during this study. However, the effectiveness of PI3K pathway blockade in the treatment of PCa can be confirmed by our results and should continue to be investigated.

Akt negatively regulates apoptotic pathways at the pre-mitochondrial level through phosphorylation and modulation of proteins such as BAD, XIAP, and FoxOs [49, 50]. XIAP is part of a family of anti-apoptotic proteins (IAPs), which act by blocking the activity of caspases (3, 7, and 9). XIAP is an Akt substrate, and the interaction results in the phosphorylation of XIAP and consequently blocks auto-ubiquitination, which inhibits apoptosis via caspases. Overexpression of XIAP is related to the growth and progression of tumors, while its reduction favors activation of the caspase cascade, increasing cell death [49, 51]. Phosphorylation of FOXO by Akt leads to its inhibition and, consequently, prevents intrinsic apoptotic pathway activation, thus promoting cell survival [40]. BAD is a pro-apoptotic member of the Bcl-2 gene family and plays a role in the intrinsic pathway of apoptosis activation [52, 53]. When dephosphorylated, BAD promotes apoptosis and in the phosphorylated form, stimulates tumor proliferation and growth. Thus, a reduction of BAD expression would be

expected in tumor cells. However, studies have shown elevated expression in PCa compared to that in normal epithelium [54]. In this setting, tumor cells would control the stimulation versus inhibition of apoptosis by modulating the phosphorylation of BAD. Thus, the phosphorylated form is predominant in tumors [55].

Our study suggests that increased apoptosis is associated with reduced expression of the anti-apoptotic proteins XIAP, BAD, Bcl-2, and FOXO, which blocks tumor growth. Due to the inhibitory effect of BEZ235 on the Akt pathway, the inhibition of anti-apoptotic proteins would only be expected in that group. Interestingly, we observed reduced expression of the anti-apoptotic molecules following abiraterone treatment. A previous study has also reported that abiraterone may modulate pro-apoptotic signals from p21, caspase-3, survivin, and transforming growth factor β (TGF β) [13]. Since abiraterone affects the androgenic pathway, the crosstalk between Akt, TGF β , and androgen receptor pathways may result in the observed pro-apoptotic stimuli. Based on our results, we can suggest that abiraterone could attenuate anti-apoptotic signals, resulting in increased apoptosis, as confirmed by the histopathologic and protein expression data. Moreover, our study revealed that combining the drugs resulted in a more significant stimulatory effect of apoptosis, which represents a better therapeutic effect on tumor growth.

Employment of new drugs and therapeutic strategies may cause additional side effects. Our data showed weight loss around 7% in animals treated with BEZ235, while other studies have reported weight loss greater than 15% [56]. Studies have shown that the loss of body weight, after oral administration of BEZ235, occurs in a dose-dependent manner in several model systems [14, 57, 58]. Wang and colleagues [14] reported that BEZ235-treated mice consumed less feed compared to the control group. Similarly, another study showed that BEZ235 is associated with reduced food consumption and severely impairs glucose metabolism in rodents. The drug inhibits PI3K isoforms alpha (p110 α) and beta (p110 β), which are necessary for metabolic processes [59]. When BEZ235 was used in combination with abiraterone, its effect on body weight did not change.

BEZ235 was found to be poorly tolerated in men with progressive metastatic castration-resistant PCa in phase I studies [23, 48]. The authors reported that the most common adverse events were oral mucositis (66.7%), diarrhea (66.7%), nausea (50.0%), anorexia (50.0%), weight loss (50.0%), and musculoskeletal pain (50.0%) [23]. These side effects contributed to the unacceptable toxicity and resulted in discontinuation of the drug for PCa treatment purposes.

Tumor microenvironments are often surrounded by inflammatory cells such as macrophages, lymphocytes, neutrophils, and mast cells. The role of mast cells is controversial; while some studies suggest a preventive role regarding tumor growth [60–63], others suggest that mast cells favor the

progression of PCa [64]. Nonomura et al. [65] performed a study with 104 patients and showed a positive correlation between the frequency of mast cells around the tumor and the Gleason score. Consistent with the literature, our data showed a reduction in mast cell counts when tumor growth decreased.

Inflammatory disorders are common histopathologic events associated with tumors [66, 67]. There is increasing evidence that the emergence of inflammation and PIN precedes the development of PCa, suggesting a significant role for inflammation in the early stages of carcinogenesis [67, 68]. Mechanisms potentially involved in this process include the production of cytokines and other factors by inflammatory cells that promote epithelial proliferation, stimulate angiogenesis, and malignancy [67, 69]. In this setting, some pro-inflammatory (TNF- α , IL-6) and anti-inflammatory cytokines (IL-10) play an important role in prostate carcinogenesis through signaling pathways such as JAK-STAT, PI3k-AKT, and MAPk-ERK [70–72]. Elevated levels of TNF- α correlate with increased proliferation, mortality, and malignancy [73], while high levels of IL-6 are associated with metastases and progression to the hormone refractory stage [74]. IL-10 plays a pleiotropic role, which may suppress or stimulate tumorigenesis, but most studies suggest an anti-inflammatory role [75]. In this context, the reduction in IL-10 expression only occurred with drug treatments, which would be expected to reduce inflammation. Consistent with previous reports, our study showed that inflammatory disorders were related to the development of prostatic lesions. Thus, we can hypothesize that after administering the drugs, the number of inflammatory infiltrates and expression of pro-inflammatory cytokines decreased, contributing to the inhibition of tumor growth.

In endocrine-related cancers in general, targeting both hormone receptor and the PI3K/AKT/mTOR pathways has shown promise [14]. In the present study, we showed that both drugs effectively inhibited those pathways, which resulted in anti-proliferative and pro-apoptotic effects. However, tumor growth, although slow, persisted after administering the drugs as monotherapy. Atypical hyperplasia was very frequent in the groups treated with the drugs alone, which also showed premalignant and malignant lesions. When the drugs were administered in combination, hyperplastic lesions decreased and, more importantly, tumor progression from a premalignant to the malignant stage was inhibited. In a setting of androgenic inhibition by abiraterone, it is reasonable to assume that the lesions may grow due to AKT activation. In addition, BEZ235 therapy inhibits the AKT pathway by blocking one of the sources of alternative AR activation [76] as well as cell proliferation and survival [46]. However, in this group, the androgenic synthesis was not inhibited, allowing the lesions to grow, even though the expression of the androgen receptor was impaired. The data presented here suggest that the

reduction of cell proliferation and survival and the stimulation of apoptosis were more effective when the drugs were combined.

The strategy to block the androgenic and the PI3K/AKT/mTOR pathways represents a good alternative to treat PCa, mainly due to the biological effects reported in the present study. Thus, different drugs, that have a more favorable therapeutic index, should be investigated. Agents targeting specific PI3K isoforms are currently under investigation in early phase clinical trials [23], and may be better tolerated and achieve greater therapeutic efficacy [77]. It remains to be determined what combination of AR and PI3K/AKT inhibition will be both tolerable and clinically active [23].

In conclusion, despite the side effects, the biological response of simultaneous inhibition of the androgenic and PI3K pathways was favorable; the drugs negatively affected tumor growth, and drug combination achieves better therapeutic effect than drug monotherapy. Based on the present results, it is possible to hypothesize that combination therapy could be used also in cases where prostate tumors have not become resistant. However, further studies, based on distinct PI3K inhibitors, are necessary to determine a safe and clinically tolerable schedule and to evaluate whether long-term combination therapies could achieve the same beneficial effects reported in the present study for PCa.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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