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ORIGINAL ARTICLE

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Combined treatment of Dunning R3327 rat prostatic tumor with the 5α-reductase inhibitor PNU 157706 and the antiandrogen bicalutamide

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Abstract PNU 157706 [*N*-(1,1,1,3,3,3-hexafluorophenylpropyl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide], a novel, potent and selective dual 5α-reductase inhibitor, was reported to be effective in inhibiting the growth of established tumors in the Dunning R3327 rat prostatic carcinoma model. *Purpose*: We investigated the efficacy of treatment with PNU 157706 in combination with the antiandrogen bicalutamide in this prostatic tumor model. *Methods*: Rats with tumor diameters of about 1 cm were treated orally 6 days a week for 9 weeks with PNU 157706 (10 mg/kg per day) alone or in combination with bicalutamide (0.2 and 1 mg/kg per day). Animals were killed 24 h after the last treatment, and ventral prostates were removed for testosterone (T) and dihydrotestosterone (DHT) determination. *Results*: PNU 157706 reduced the growth of established tumors by 39%; bicalutamide proved ineffective at 0.2 mg/kg per day, but reduced tumor growth by 45% at a dose of 1 mg/kg per day. The combination of PNU 157706 with both doses of bicalutamide caused an additive tumor growth inhibition (50% and 64%). Castration resulted in marked tumor growth inhibition (72%). Ventral prostate weight was markedly reduced by PNU 157706 (78%) treatment and by bicalutamide (59% and 77%); combined treatment was as effective as castration. Prostatic DHT content was markedly reduced by PNU 157706 (88%), whereas prostatic T increased slightly (60%). Concomitant treatment with bicalutamide antagonized the T increase induced by PNU 157706 and did not modify the already remarkable suppression of DHT. *Conclusions*: These data show that the inhibitory effect of PNU 157706 and bicalutamide on Dunning prostatic tumor growth is additive, thus suggesting a possible role of PNU 157706 in the therapy of advanced prostate cancer, in combination with antiandrogens, to provide an effective peripheral androgen ablation therapy with minimal side effects.

Key words PNU 157706 · 5α-Reductase inhibitor · Antiandrogen · Bicalutamide · Tumor model

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Introduction

Since a majority of prostate tumors are stimulated to grow by androgens, withdrawal of androgens has been the main therapy of prostate cancer for over 50 years. A variety of hormonal manipulations, including orchiectomy and administration of estrogens and luteinizing hormone-releasing hormone (LHRH) analogues, have been used successfully as first-line treatment [17, 39]. Recently, combined therapy involving surgical or medical castration plus an antiandrogen, in order to block the remaining adrenal androgens, has been tried in an attempt to produce total androgen blockade [25].

In normal, hyperplastic or cancerous prostatic tissue, testosterone (T) is irreversibly metabolized via the 5α-reductase enzyme to 5α-dihydrotestosterone (DHT) [8], which appears to be the major intracellular androgen; in fact, in the prostate gland DHT concentration is higher than that of T, and the androgen receptor is known to have a higher affinity for DHT than for T [16, 22, 28]. Therefore, reduction of DHT synthesis through inhibition of 5α-reductase activity can represent a rational approach to androgen deprivation not only for the therapy of benign prostatic hyperplasia (BPH), but also for prostatic tumor control [33].

Molecular cloning studies have identified two genes that encode the type I and type II isozymes of 5α-reductase, characterized by distinct molecular genetics, structural and biochemical properties, and by different tissue localization [1, 20]. In initial studies, the type II isozyme was found to be mainly located in human urogenital tissues such as the prostate [37] whereas the type I isozyme was found to be predominant in human

nongenital skin such as the scalp sebaceous glands and in the liver [36]. However, recent data have shown that the type I isozyme is also expressed in human normal, hyperplastic, and cancerous prostatic tissue [4, 36].

The only marketed 5α-reductase inhibitor, finasteride, a selective inhibitor of the type II isozyme, has been proved to be partially efficacious for the treatment of BPH, likely owing to partial inhibition of DHT synthesis [34]. The limited suppressive effect of finasteride on circulating DHT (60–70%) has been attributed to the remaining type I 5α-reductase not inhibited by the compound [34]. Therefore, compounds able to inhibit both type I and II isozymes (i.e., dual inhibitors) are expected to cause a more marked suppression of circulating DHT and a greater magnitude of clinical efficacy than finasteride. Dutasteride [5] and PNU 157706 [*N*-(1,1,1,3,3,3-hexafluorophenylpropyl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide] [32] are the first representatives of this novel class of dual 5α-reductase inhibitors. Preliminary clinical studies with dutasteride have confirmed the superiority of this type of compound over finasteride in maximally suppressing circulating DHT (90% vs 70%, respectively) [10].

The role of 5α-reductase inhibitors in prostate cancer is controversial [33]. In initial preclinical studies, we have found that PNU 157706 is effective in inhibiting the growth of the androgen-dependent Dunning R3327 prostatic carcinoma in the rat [42]. However, this compound, like other 5α-reductase inhibitors, has less inhibitory effect on tumor than on normal prostate, likely because of the remaining T, which eventually interacts with the androgen receptor and stimulates tumor growth [7, 40]. In fact, it has been reported that in castrated rats bearing the Dunning prostate tumor, the tumor is more sensitive than the prostate to the stimulatory effect of exogenous T [11]. Therefore, a combination therapy with a 5α-reductase inhibitor and an androgen receptor antagonist, to neutralize the remaining T, could be of potential value in the therapy of metastatic prostatic cancer.

In this study, we have investigated the antitumor effect of combined therapy using PNU 157706 and the antiandrogen bicalutamide on the Dunning prostatic tumor model. Tumor growth rates as well as endocrine organ weights and prostatic DHT and T contents were evaluated.

Materials and methods

Animals

Male Copenhagen rats, weighing approximately 200 g, were supplied by Harlan Nossan Srl (Correzzana, Italy). Animals were housed in temperature-controlled rooms (22 ± 2 °C) on a circadian rhythm of 12 h of light (6 a.m. to 6 p.m.) and 12 h of darkness. Animal care was in accordance with institution guidelines.

Prostatic tumor model

The Dunning R3327 prostatic carcinoma was kindly provided by Dr. H. Altman, Papanicolau Cancer Research Center, University of Miami (Fla., USA). The tumor was maintained by serial transplantation in Copenhagen rats. The tumor was passed by harvesting fresh tumor, dicing it with sterile scissors in sterile 0.9% NaCl solution, and aseptically implanting a single tumor fragment (approximately 3–4 mm in size) subcutaneously in the flank of the recipient animal under mild diethyl ether anesthesia.

Treatment and experimental design

Tumor-bearing rats with tumor diameters of 0.8–1 cm were randomly assigned to the different treatment groups. PNU 157706 (synthesized at the Chemistry Department, Pharmacia & Upjohn, Italy) and bicalutamide (50-mg tablets of Casodex, Zeneca, Germany, pulverized with a mortar, taking into account the 1:1 ratio between the active ingredient and excipients) were suspended in 0.5% Methocel (methylcellulose 400; Dow Chemical, USA) containing 0.4% Tween 80 (Merck, USA), and administered orally in a volume of 5 ml/kg, 6 days a week for 9 weeks. In the group treated with the combination, PNU 157706 was given first, followed by bicalutamide after 30 min. Similarly, the group receiving PNU 157706 alone was also treated after 30 min with the vehicle (Methocel/Tween), and the group receiving bicalutamide alone was also previously (30 min) treated with the vehicle. The control animals received 2 treatments a day with the vehicle, at 30-min intervals. Castration was performed on the first treatment day under diethyl ether anesthesia.

Tumor growth was followed by measuring the two perpendicular diameters with calipers, and tumor weight was calculated according to the formula: $d^2 \times D/2$, where d is the minimum and D is the maximum diameter, assuming specific gravity to be approximately 1, as described by Geran et al. [15]. The area under the tumor weight-to-time curve (AUC) was calculated by the linear trapezoidal method up to 9 weeks. Animals were killed 24 h after the last treatment, and the ventral prostate, seminal vesicles, testes, epididymides and adrenals were removed and weighed. Ventral prostates were immediately frozen on dry ice and stored at −20 °C for androgen assays.

Analysis of drug interaction

The antitumoral effect of the combination of PNU 157706 and bicalutamide was evaluated according to Kern et al. [21]. For each group, the observed T/C% (defined as the percentage ratio between the AUC of each group of treated rats and the AUC of control rats) was calculated. Then, the expected T/C% for the combined treatment was calculated as the product of the observed T/C% of each drug given alone divided by 100. The ratio of the expected T/C% to the observed T/C% for the combined treatment was definited as the R index. An R index of 1 indicates an additive effect, while synergism is defined as any value of R greater than 1.

T and DHT determinations

Prostatic concentrations of T and DHT were measured by specific radioimmunoassay (RIA) after sample extraction and purification by high-performance liquid chromatography (HPLC). Each prostate sample (pool of 2–3 prostates in the castrated group) was thawed and homogenized in 4 ml of acetone/acetonitrile mixture (1:1) with a Polytron apparatus. Trace amounts of [^{3}H]T and [^{3}H]DHT were added to each homogenate to monitor recovery. After extraction and centrifugation, the organic phase was desiccated, the dried extract was dissolved in 5% methanol aqueous solution and purified on Waters Sep-Pack minicolumns (Millipore, USA) using 60% acetonitrile aqueous solution as the eluting solvent. The dried extract was then dissolved in 38% acetonitrile aqueous solution and injected into a Hewlett Packard 1050 HPLC System. Separation was performed using a 3.9 × 300 mm Waters Nova-Pack C_{18} reversed-phase column (particle size 3 μm), in isocratic conditions, at a column temperature of 40 °C. DHT- and T-containing fractions were collected in the volume of 2.7 and 2.2 ml, respectively,

then desiccated in a centrifugal evaporator and subjected to RIA. An aliquot of the resuspended sample was taken to monitor the recovery of the tracer, which was usually 50% and 39% for T and DHT, respectively. The androgen levels in the resuspended samples were estimated, in duplicate, using the [^{3}H]T and [^{3}H]DHT RIA Kits supplied by bioMérieux (France) and ICN Biomedicals (USA), respectively. The hormone concentration of each sample was corrected for the individual recovery. The intra- and between-assay variabilities were 10% and 12% for T, and 10% and 15% for DHT. The final sensitivities for T and DHT assays were 0.2 and 1 ng/g of prostate, respectively (for a sample of at least 0.2 g).

Results

Effect on tumor growth

Figure 1 and Table 1 show the effect of PNU 157706 (10 mg/kg per day) alone or in combination with bicalutamide (0.2 and 1 mg/kg per day), given orally for 9 weeks, on the growth of the transplanted Dunning tumor. The tumor weights recorded weekly are reported in Fig. 1. The $AUC_{(0–9wks)}$ values, expressed as grams per week, are reported in Table 1. During the 9-week observation period, the Dunning tumor grew progressively in the control group (AUC, mean ± SE = 69.7 ± 14.4), whereas in castrated animals tumor growth was markedly decreased by 72% (AUC = 19.6 ± 2.1; $P < 0.01$), demonstrating the androgen responsiveness of the tumor. Treatment with the 5α-reductase inhibitor PNU 157706 decreased tumor growth by 39% (AUC = 42.3 ± 4.7; $P < 0.05$). Bicalutamide, at a dose of 0.2 mg/kg per day, did not inhibit tumor growth (AUC = 77.6 ± 17.4), whereas at a dose of 1 mg/kg per day it reduced tumor growth by 45% (AUC = 38.5 ± 5.9; $P < 0.05$). Both doses of bicalutamide added to PNU 157706 improved tumor growth inhibition to 50% and 64% (AUC = 34.9 ± 7.8 and 25.2 ± 4.6; $P < 0.01$). The R indexes of the expected to observed effects for the combination were 1.35 and 0.93 with the low and high bicalutamide doses, respectively, indicating an additive or near additive effect of the two compounds.

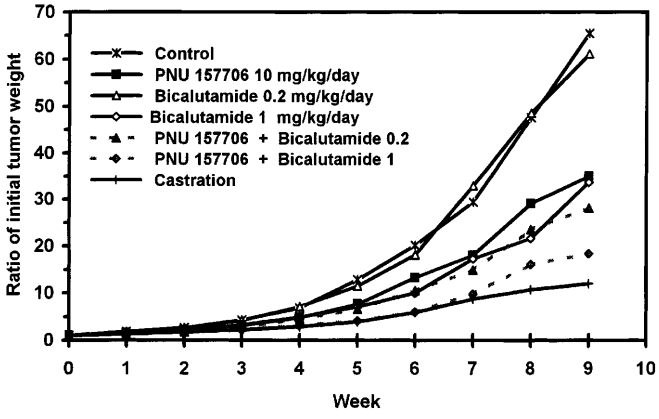


Fig. 1 Effect of 9-week oral treatment with PNU 157706 (10 mg/kg per day) or bicalutamide (0.2 and 1 mg/kg per day), given alone or in combination, on tumor growth in the Dunning R3327 prostatic tumor model in rats. Castration was performed on the first treatment day. Groups contained 9–10 rats

Effect on endocrine organ weight

The endocrine organs of tumor-bearing rats were excised and weighed when the rats were killed at 9 weeks (Figs. 2 and 3). No treatment affected rat body weight (Fig. 3). PNU 157706 caused decreases in ventral prostate and seminal vesicle weight of 78% ($P < 0.01$) and 56% ($P < 0.01$), respectively. Bicalutamide reduced prostate and seminal vesicle weights by 59% ($P < 0.01$) and 44% ($P < 0.01$) at a dose of 0.2 mg/kg day, and by 77% ($P < 0.01$) and 57% ($P < 0.01$) at a dose of 1 mg/kg per day. Combined treatment with PNU 157706 and bicalutamide 0.2 mg/kg per day caused decreases in prostate and seminal vesicle weights of 82% ($P < 0.01$) and 63% ($P < 0.01$), respectively, whereas the combination with bicalutamide 1 mg/kg per day reduced the weight of these organs by 85% ($P < 0.01$) and 65% ($P < 0.01$), respectively. The effect of the combined treatments was similar to the effect of castration (89% and 67% decreases in prostate and seminal vesicle

Table 1 Effect of 9-week treatment with PNU 157706 or bicalutamide, given alone or in combination, on Dunning R3327 prostatic tumor growth in rats (*AUC* area under the tumor weight-to-time curve, *R* ratio)

Treatment (mg/kg per day p.o.)		No. of rats	$AUC^{a}_{0–9wks}$ (g × week)	Effect on tumor growth		
PNU 157706	Bicalutamide			Observed[c] T/C %	Expected[d] T/C %	Expected/Observed R
Control		9	69.7 ± 14.4[b]			
10	–	10	42.3 ± 4.7*	60.7		
–	0.2	10	77.6 ± 17.4	111.3		
–	1	10	38.5 ± 5.9*	55.2		
10	0.2	10	34.9 ± 7.8**	50.1	67.6	1.35
10	1	10	25.2 ± 4.6**	36.1	33.5	0.93
Castration		9	19.6 ± 2.1**	28.1		

*$P < 0.05$, **$P < 0.01$ vs control group (Dunnett's test)
[a] Calculated by the linear trapezoidal method from 0 to 9 weeks
[b] Mean ± SE
[c] Observed T/C %: 100 × AUC in treated rats/AUC in control rats
[d] Expected T/C %: product of observed T/C % of each drug alone/100

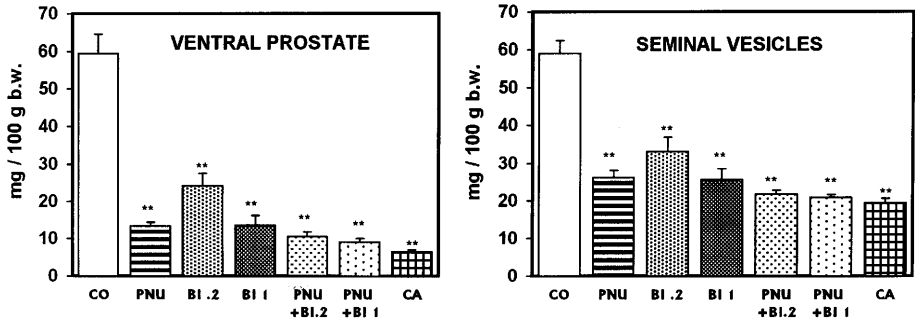


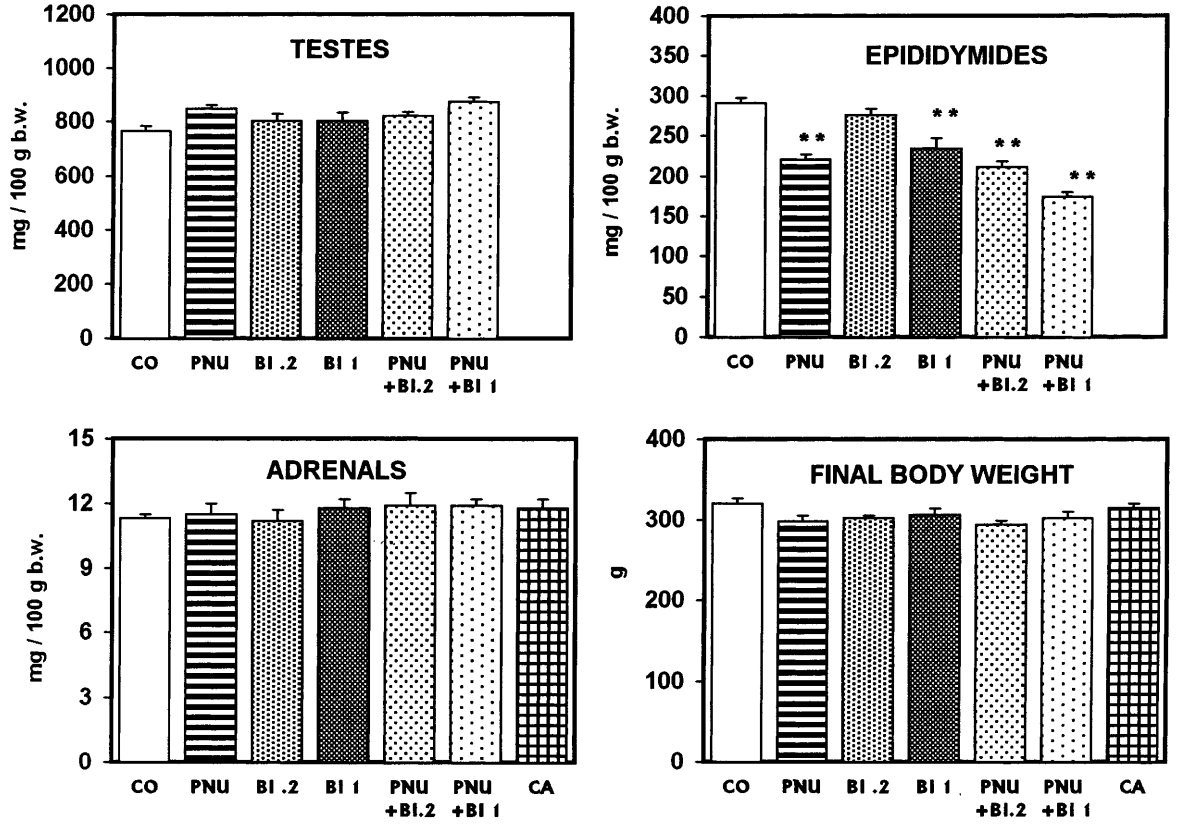
Fig. 2 Effect of 9-week oral treatment with PNU 157706 10 mg/kg per day (*PNU*) or bicalutamide 0.2 mg/kg per day (*BI .2*) and 1 mg/kg per day (*BI 1*), given alone or in combination, on the relative weight of ventral prostate and seminal vesicles in rats bearing the Dunning R3327 prostatic carcinoma. Castration (*CA*) was performed on the first treatment day. *Bars* represent mean ± SE (9–10 animals per group). $^{**}P < 0.01$ vs controls (*CO*) by Dunnett's test

weights, respectively). No effects on testis and adrenal weight were observed (Fig. 3). The weight of epididymides was reduced by PNU 157706 (24%; $P < 0.01$), bicalutamide 1 mg/kg per day (20%; $P < 0.01$), and the combinations with both bicalutamide doses (28% and 40%; $P < 0.01\%$).

Effect on prostatic DHT and T

Oral treatment for 9 weeks with PNU 157706 resulted in a marked decrease (88%; $P < 0.01$) in prostatic DHT content, measured 24 h after the last dose (Fig. 4). As expected for 5α-reductase inhibitors, the prostatic T content increased slightly in PNU 157706-treated groups (60%). The antiandrogen bicalutamide, at doses of 0.2 and 1 mg/kg per day, caused a marked reduction in DHT (67% and 80%, respectively; $P < 0.01$) and a very slight reduction in T content (12% and 23%, respectively). Combined treatment with PNU 157706 and bicalutamide 0.2 or 1 mg/kg per day was as effective as PNU 157706 alone in reducing the DHT content of the prostate (87% or 92%; $P < 0.01$), but more effective than bicalutamide alone. The prostatic T content was unchanged in the groups treated with the combination.

Fig. 3 Effect of 9-week oral treatment with PNU 157706 (10 mg/kg per day) or bicalutamide (0.2 and 1 mg/kg per day), given alone or in combination, on the relative weight of testes, epididymides and adrenals and on final body weight in rats bearing the Dunning R3327 prostatic carcinoma. *Bars* represent mean ± SE (9–10 animals per group). $^{**}P < 0.01$ vs controls by Dunnett's test



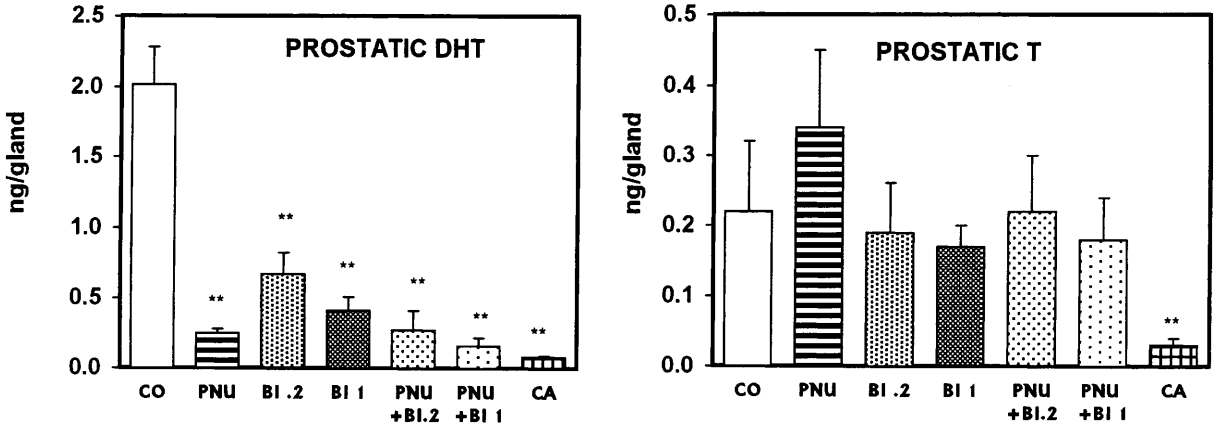


Fig. 4 Effect of 9-week oral treatment with PNU 157706 (10 mg/kg per day) or bicalutamide (0.2 and 1 mg/kg per day), given alone or in combination, on prostatic DHT and T content in rats bearing the Dunning R3327 prostatic carcinoma. Animals were killed 24 h after the last dose. *Bars* represent mean ± SE (9–10 animals per group). $^{**}P < 0.01$ vs controls by Dunnett's test

Castration markedly reduced prostatic DHT (96%) and T (87%) content.

Discussion

Inhibition of 5α-reductase provides a novel and selective approach to DHT deprivation in DHT target tissues. Specific inhibitors of 5α-reductase may be useful in controlling pathological conditions dependent on DHT such as BPH [33]. While DHT seems to be the main androgen stimulating and maintaining the growth of BPH, it is not certain whether the androgen dependence of prostate cancer is also mainly related to DHT. Much of the experimental data on the antitumor activity of 5α-reductase inhibitors in prostate cancer were obtained using the Dunning R3327 rat prostatic tumor. This tumor model is well characterized. It shares several characteristics with human prostate cancer, including slow growth rate, responsiveness to hormonal therapy, and similar histology to that of a well-differentiated carcinoma [18]; moreover, it contains the enzyme 5α-reductase [19]. In this tumor model finasteride, a selective type II 5α-reductase inhibitor [7], has also been reported to be inactive up to a dose of 100 mg/kg [27]. Epristeride, another selective type II 5α-reductase inhibitor [26], has been reported to be inactive on the H subline of the Dunning tumor but highly effective on the G subline of the same tumor [27]. As regards the tumor 5α-reductase enzyme, it has been reported that both type I and II 5α-reductase isozymes are expressed in human prostatic carcinoma [4, 35]. No data are so far available on the characteristics of the enzyme in the Dunning prostatic tumor. However, both isoforms of 5α-reductase are expected to be expressed in this prostatic tumor tissue, as reported for the normal rat prostate [31].

The dual type I and II 5α-reductase inhibitor PNU 157706 [32], has previously been reported to be effective, as a single agent, in reducing the Dunning prostatic tumor growth [42]. However, it was found to be less effective than castration, likely owing to the remaining tumor T, not present after castration [11]. Bicalutamide is a nonsteroidal antiandrogen, approved for use in combination therapy, with surgical or medical castration, for advanced prostate cancer [2, 3]. The compound is also under investigation as primary monotherapy in patients with advanced disease and as adjuvant therapy in patients with early stage disease [38]. Preclinical studies have shown that bicalutamide is effective in the Dunning tumor model in rats and that the compound given at a dose of 25 mg/kg per day is as effective as surgical or medical castration [14].

In this study, we have investigated the effect of the combination therapy using PNU 157706 and bicalutamide. PNU 157706 was given at a dose of 10 mg/kg per day, this being the minimal dose, in our experience, that induces the maximal suppression of tumor growth. Bicalutamide was given at doses lower than those reported to be as effective as castration in this tumor model; the aim was to antagonize the biological effect of the tissue T, which remains during treatment with a 5α-reductase inhibitor at the receptor level, and consequently to obtain better tumor inhibition with minimal side effects. The present data show that the inhibitory effects of PNU 157706 and bicalutamide on Dunning prostatic tumor growth are additive, thus supporting the rationale of this combination therapy. In fact, treatment with PNU 157706 alone caused 39% tumor growth inhibition; bicalutamide alone, at doses of 0.2 and 1 mg/kg per day, caused inhibition of tumor growth of 0% and 45%, respectively. When these two doses of bicalutamide were combined with PNU 157706, tumor growth inhibition was higher (50% and 64%) than after PNU 157706 alone. Further, we have confirmed that the antihormonal effect of PNU 157706 and bicalutamide is very specific, as 9-week treatment with each compound at doses very effective on the normal prostate did not affect testicular and adrenal weights. The effect of PNU 157706 on prostatic androgens (i.e., decrease in DHT and increase in T) is consistent with its inhibitory effect on 5α-reductase [32]. Bicalutamide has been reported to have no inhibitory effect on 5α-reductase [14], therefore,

the reduction in prostatic DHT observed in this study is very likely owing to the drug-induced displacement of DHT from the high-affinity androgen receptor binding sites within the prostate, and subsequent DHT catabolism and elimination. Similar effects have been described for the antiandrogen flutamide, in both rats [29] and humans [24].

The efficacy of this type of combined treatment has been demonstrated in other preclinical studies. The antiandrogen flutamide and the 5α-reductase inhibitor 4-MA were shown to be more effective in combination than as single treatments in reducing androstenedione-induced prostatic growth in castrated rats [23]. In addition, flutamide and finasteride in combination caused a significant decrease in rat ventral prostate size compared with either drug alone [13]. In mice bearing the androgen-sensitive mammary Shionogi carcinoma, flutamide and finasteride had an additive inhibitory effect on tumor growth and on ventral prostate weight [9]. Further, we have previously reported that PNU 156765, a 5α-reductase inhibitor structurally related to PNU 157706, showed an additive antitumor effect when given in combination with flutamide in the Dunning prostatic tumor model [41].

In preliminary clinical studies in advanced prostatic cancer, the combination of finasteride with either flutamide [12, 30] or bicalutamide [6] has been been reported to markedly reduce circulating levels of prostatic specific antigen (PSA), thus confirming the rationale for this novel approach of peripheral androgen deprivation. Further, this type of combined therapy was well tolerated, particularly in maintaining sexual function in most men, unlike the standard treatment with LHRH analogs, which causes loss of libido and potency as a consequence of the decrease in circulating T to castration levels.

A potent, dual inhibitor of both type I and II 5α-reductases like PNU 157706 is expected to cause a better suppression of prostatic DHT than the type II inhibitor finasteride, and therefore could be an ideal candidate for this novel approach for the therapy of prostate cancer.

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