Combined treatment with the 5α-reductase inhibitor PNU 157706 and the antiandrogen flutamide on the Dunning R3327 prostatic carcinoma in rats

T Zaccheo, D Giudici and E di Salle
Department of Pharmacology, Oncology Research, Pharmacia & Upjohn, Nerviano, Italy

Abstract
The steroid 5α-reductase enzyme catalyzes the conversion of testosterone to the potent androgen 5α-dihydrotestosterone (DHT). PNU 157706, 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. We have studied the efficacy of combined treatment with PNU 157706 and the antiandrogen flutamide in this prostatic tumor in rats.

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 flutamide (1 and 5 mg/kg per day). Animals were killed 24 h after the last treatment and ventral prostates were removed for testosterone and DHT determination.

PNU 157706 reduced the growth of established tumors by 36%; flutamide showed a slight effect at 1 mg/kg per day (24% inhibition), while at the dose of 5 mg/kg per day it reduced tumor growth by 48%. The combination of PNU 157706 with the lower dose of flutamide caused an additive tumor growth inhibition (60%) and the combination with the higher dose of flutamide resulted in a better inhibition of tumor growth (68%) than did either treatment alone. Castration resulted in marked tumor growth inhibition (76%). Ventral prostate weight was more markedly reduced by PNU 157706 treatment than by flutamide; combined treatment was as effective as castration. Prostatic DHT content was markedly reduced by PNU 157706 (93%), whereas prostatic testosterone increased (137%). Concomitant treatment with flutamide partially antagonized the testosterone increase induced by PNU 157706 and did not modify the already considerable suppression of DHT.

These data show that the inhibitory effects of PNU 157706 and flutamide on Dunning prostatic tumor growth are additive, thus supporting the rationale of this combination therapy in advanced prostate cancer, in order to achieve adequate androgen blockade with minimal side-effects.

Introduction

Androgen deprivation with the combination of luteinizing hormone-releasing hormone analogs and antiandrogens is the most effective form of treatment for metastatic carcinoma of the prostate (Labrie et al. 1985, Crawford et al. 1989). However, androgen deprivation therapy has side-effects that are the result of castrate levels of serum testosterone, including impotence, loss of libido and decreased muscle mass (Vogelzang & Kennealey 1992, Hsieh & Simons 1993).

With the advent of routine prostate specific antigen (PSA) testing (Stamey et al. 1989, Partin et al. 1990) and the consequent early diagnosis of the disease, patients at earlier stages of prostatic cancer may be candidates for hormonal therapy. As a result, concerns about the quality of life associated with androgen ablation therapy become important.

Testosterone acts in the prostate through the local formation of its potent androgenic metabolite 5α-dihydrotestosterone (DHT), synthesized by the enzyme 5α-reductase (Bruchovsky et al. 1988). Inhibitors of this
enzyme provide a novel and selective approach to androgen deprivation for benign and malignant prostatic diseases. Both in experimental animals and in humans, treatment with such inhibitors results in reduced prostatic DHT without lowering serum testosterone, thus preserving sexual function. However, experimental data in prostatic tumor therapy indicate that these compounds have less effect on tumor than on normal prostate (Brooks et al. 1991, Zaccheo et al. 1997), likely because of the remaining testosterone which eventually interacts with the androgen receptor and stimulates tumor growth. Therefore, a combination therapy with a 5α-reductase inhibitor and an androgen receptor antagonist to neutralize the remaining testosterone could be of potential value in the therapy of metastatic prostate cancer.

PNU 157706 (N-(1,1,1,3,3,3-hexafluorophenylpropyl)-3-oxo-4-aza-5α-androst-1-ene-17β-carboxamide) is a novel dual inhibitor of 5α-reductase. The compound was highly potent in inhibiting human recombinant 5α-reductase type I and type II isozymes, showing IC50 values of 3.9 and 1.8 nM, and therefore it was several times more potent than finasteride (IC50 values of 313 and 11.3 nM), particularly on type I isozyme (di Salle et al. 1998). Tested on a crude preparation of rat prostatic 5α-reductase, PNU 157706 caused enzyme inhibition with an IC50 value of 34 nM, compared with 58 nM shown by finasteride. In adult rats a single oral dose of 10 mg/kg PNU 157706 caused a marked and longer lasting reduction of prostatic DHT content than did finasteride (at 24 h 89% and 47% inhibition respectively). In prepubertal testosterone- or DHT-implanted castrated rats, PNU 157706, at the oral dose of 10 mg/kg per day for 7 days, markedly reduced ventral prostate weight in testosterone- but not in DHT-implanted animals, thus showing a lack of antiandrogen activity. After repeated (28 days) oral dosing in rats, the compound was found to be 16-fold more potent than finasteride in reducing prostate weight. PNU 157706 has been reported to be effective in inhibiting the growth of the androgen-dependent Dunning R3327 prostate carcinoma in the rat (Zaccheo et al. 1998a).

In this study we have investigated the antitumor effect of a combined therapy using PNU 157706 and the antiandrogen flutamide on the Dunning prostatic tumor model; tumor growth rate as well as endocrine organ weights and prostatic DHT and testosterone content 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 (0600-1800 h) 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 (FL, 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 setup

Tumor-bearing rats with tumor diameters of 0.5-1 cm were randomly assigned to the different treatment groups. PNU 157706 (synthesized at the Chemistry Department, Pharmacia & Upjohn, Italy) and flutamide (kindly supplied by Schering Plough, Kenilworth, NJ, USA), were suspended in 0.5% Methocel (methylcellulose 400; Dow Chemical, Midland, MI, USA) containing 0.4% Tween 80 (Merck, Darmstadt, Germany) and administered orally in a volume of 5 ml/kg, 6 days a week for 9 weeks. 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: d2 × D/2, where d is the minimum and D is the maximum diameter (Geran et al. 1972). 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 ventral prostate and seminal vesicles were removed and weighed. Ventral prostates were immediately frozen on dry ice and stored at -20 °C for androgen hormone assays.

Testosterone and DHT determinations

Prostatic concentrations of testosterone and DHT were measured by specific RIAs, after sample extraction and purification by high-performance liquid chromatography (HPLC). Each prostate sample (pool of two to three prostates in the castrated group) was thawed and homogenized in 4 ml acetone/acetoneitrile mixture (1:1) with a Polytron apparatus. 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 Plus C18 minicolumns (Millipore Corporation, Milford, MA, USA), using 60% acetoneitrile aqueous solution as the eluting solvent. The dried extract was then dissolved in 38% acetoneitrile

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aqueous solution and injected into a Hewlett Packard 1050 HPLC System. Separation was performed using a 3.9×300 mm Nova-Pack Waters C18 reversed-phase column (particle size 3 µm), in isocratic conditions, at a column temperature of 40 °C. DHT- and testosterone-containing fractions were collected in volumes of 2.7 and 2.2 ml respectively, then desiccated in a centrifugal evaporator and subjected to RIA. Testosterone and DHT levels in the resuspended samples were estimated, in duplicate, by using the [3H]testosterone and [3H]DHT RIA kits supplied by bioMérieux (Marcy l’Etoile, France) and ICN Biomedicals (Costa Mesa, CA, USA).

Table 1 Effect of 9-week treatment with PNU 157706 or flutamide given alone or in combination, on Dunning R3327 prostatic tumor growth in rats

<table>
<thead>
<tr>
<th>Treatment (mg/kg per day p.o.)</th>
<th>Tumor growth</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Final tumor (weight in g)</td>
</tr>
<tr>
<td>PNU 157706</td>
<td>No. of rats</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>—</td>
<td>9</td>
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<tr>
<td>—</td>
<td>9</td>
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</tr>
<tr>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Castration</td>
<td>9</td>
</tr>
</tbody>
</table>

*AUC=area under the tumor weight to time curve, calculated by the linear trapezoidal method from 0 to 9 weeks.
§ Means±S.E.

Figure 1 Effect of 9-week oral treatment with PNU 157706 (10 mg/kg per day) or flutamide (1 and 5 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 eight to nine rats.
respectively. The final sensitivity for testosterone and DHT assays were 0.2 and 1 ng/g prostate respectively (for a sample of at least 0.2 g).

**Statistical analysis**

The statistical analysis of the effect of the various treatments on tumor growth (mean tumor weight from week 0 to week 9) was assessed by use of an ANOVA split-plot design (7 groups×10 observation times). Tukey's HSD test was used for post hoc comparison. The analysis was performed using the SPSS statistical package.

Statistical significance of data on endocrine organ weight and DHT and testosterone prostatic content was measured according to Dunnett's test.

**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 flutamide (1 and 5 mg/kg per day), given orally for 9 weeks, on the growth of the transplanted Dunning tumor. The weekly recorded tumor weights are reported in Fig. 1. The mean tumor weight at the beginning of the experiment (week 0) was similar in all groups ($P=1.000$). The AUC (0-9 weeks) values, expressed as g×week, are reported in Table 1. During the 9-week observation period, the Dunning tumor grew progressively in the control group (AUC, mean±S.E.=58.4±10.5), whereas castration resulted in a marked decrease in tumor growth (76% inhibition), demonstrating the androgen responsiveness of the tumor. Treatment with the 5α-reductase inhibitor PNU 157706 decreased tumor growth by 36%. Flutamide, at the dose of 1 mg/kg per day, caused a slight inhibition of tumor growth (24%), whereas at the dose of 5 mg/kg per day it reduced tumor growth by 48%. Combination of PNU 157706 with the lower dose of flutamide caused an additive tumor growth inhibition (60%), whereas combination with the higher dose of flutamide resulted in greater inhibition of tumor growth (68%) than did either treatment alone. Statistical analysis of tumor growth over time for all groups showed significant differences between groups ($F=9.3; P<0.001$), times ($F=207.9; P<0.001$) and interactions ($F=9.1; P<0.001$). Analysis of final tumor weight data (Tables 1 and 2) indicated that all treated groups, except that treated with 1 mg/kg per day flutamide ($P=0.75$), were significantly different from the control group. Groups treated with the combinations were significantly different from those treated with PNU 157706 or flutamide alone. Final tumor weight in the group treated with the combination of PNU 157706 and flutamide at 5 mg/kg per day was not different ($P=0.73$) from that of the castrated group.

<table>
<thead>
<tr>
<th></th>
<th>PNU 157706</th>
<th>Flutamide 1</th>
<th>Flutamide 5</th>
<th>PNU 157706 + Flutamide 1</th>
<th>PNU 157706 + Flutamide 5</th>
<th>Castration</th>
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<td>Control</td>
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<td>0.00218</td>
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<td>0.00344</td>
<td>0.00003</td>
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<tr>
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<td></td>
<td></td>
<td>0.72941</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Flutamide 1, dose of 1 mg/kg per day; flutamide 5, dose of 5 mg/kg per day.

**Effect on endocrine organ weight**

Ventral prostates and seminal vesicles of tumor-bearing rats were excised and weighed when they were killed after 9 weeks. The relative weights of these organs are shown in Fig. 2. PNU 157706 caused a decrease of ventral prostate and seminal vesicle weight of 76% and 46% respectively, a greater effect than that observed after flutamide treatment. In fact flutamide, at the dose of 1 mg/kg per day, reduced prostate and seminal vesicle weights by 23% and 19%, and by 50% and 38% at the dose of 5 mg/kg per day. Combined treatment with PNU 157706 and 1 mg/kg per day flutamide caused a reduction of prostate and seminal vesicle weights of 81% and 57% respectively, whereas the combination with 5 mg/kg per day flutamide reduced the weight of these organs by 86% and 64% respectively, which was similar to the effect of castration (92% and 71% reduction).
Effect on prostatic DHT and testosterone

Oral treatment for 9 weeks with PNU 157706 resulted in a marked decrease (93%) in prostatic DHT content, measured 24 h after the last dose (Fig. 3). As expected for 5α-reductase inhibitors, the prostatic testosterone content increased in PNU 157706-treated animals (137%). Flutamide caused a reduction of DHT (48% and 50%) and an increase in testosterone content (126% and 33%) at the doses of 1 and 5 mg/kg per day respectively.

Combined treatment with PNU 157706 and flutamide at 1 or 5 mg/kg per day was as effective as PNU 157706 alone in reducing DHT content of the prostate (92% or 93% vs 93%) but was more effective than flutamide alone (48% or 50%). The prostatic testosterone content increased to a lesser extent in the groups treated with the combination (69% or 72%) in comparison with the PNU 157706-treated group (137%). Castration markedly reduced prostatic DHT (97%) and testosterone (88%) content.

Discussion

Although hormonal treatment of advanced prostatic cancer has relatively small effects on patient survival, it is an important development in palliative care, improving the patient’s quality of life. Since the initial report by Huggins & Hodges (1941), the hormonal therapy of advanced or metastatic prostate cancer has traditionally consisted of surgical or chemical castration in order to block testicular androgens and therefore to lower circulating testosterone. An important consideration in the endocrine therapy of prostate cancer is that both testes and adrenals provide almost equal amount of the androgens responsible for cancer growth (Labrie et al. 1993b). In fact, in humans, unlike other species (e.g. rats), the adrenals secrete a large amount of the inactive androgen precursor dehydroepiandrosterone, which is converted into DHT within the prostate. Therefore, castration (surgical or medical) causes 90-95% reduction in serum testosterone concentration, although a much smaller effect is seen on the intraprostatic concentration of DHT, the potent metabolite of testosterone, that remains at about 40% of that measured in intact men (Geller et al. 1984, Labrie et al. 1993b). More recently, combined therapy involving surgical or medical castration plus an antiandrogen to block the remaining adrenal androgens has been tried in an attempt to achieve total androgen blockade (Labrie et al. 1993a). However, such a systemic reduction in the circulating testosterone level has several side-effects, including sterility, impotence, loss of libido, hot flushes and decreased muscle mass (Vogelzang & Kennealey 1992, Hsieh & Simons 1993). Inhibitors of 5α-reductase provide a novel and selective approach to androgen deprivation. Treatment with such inhibitors results in reduced prostatic DHT and consequently in involution of prostate size (Schröder 1994).

Two 5α-reductase isozymes with different characteristics have been described: type I and II (Andersson & Russell 1990). While type II is mainly localized in the prostate, type I is predominant in the peripheral tissues, mainly the skin and the liver. As regards the tumor 5α-reductase enzyme, it has been reported that both isozymes are expressed in human prostatic carcinoma (Silver et al. 1994, Bonkhoff et al. 1996). No data are so far available on the characteristics of the enzyme in the Dunning prostatic tumor, but both isoforms of 5α-reductase are expected to be expressed in this prostatic tumor tissue, as reported for the normal rat prostate (Normington & Russell 1992).

PNU 157706, a dual 5α-reductase inhibitor (di Salle et al. 1998), has previously been reported to be effective, as a single agent, in reducing Dunning prostatic tumor growth (Zaccheo et al. 1998a). However, its effect was found to be weaker than that caused by castration, likely
because of the remaining tumor testosterone, at variance with castration. Therefore, in this study we investigated the effect of combination therapy using PNU 157706 and the androgen receptor antagonist flutamide (Labrie et al. 1993a). In fact the PNU 157706-induced increase in tissue testosterone level could be neutralized by flutamide at the androgen receptor level. In addition, the PNU 157706-induced decrease in DHT could give flutamide a further competitive advantage over DHT for binding to the androgen receptor. The present data show that the inhibitory effects of PNU 157706 and flutamide on Dunning prostatic tumor growth are additive, thus supporting the rationale of this combination therapy. In fact, treatment with PNU 157706 alone caused 36% tumor growth inhibition; flutamide alone, at the doses of 1 or 5 mg/kg per day, caused 24% and 48% inhibition of tumor growth respectively. When these two doses of flutamide were combined with PNU 157706, tumor growth inhibition reached 60% and 68%.

The efficacy of this combined treatment has been demonstrated in other preclinical studies. Experimental studies in rats have shown that flutamide and the 5α-reductase inhibitor N,N-diethyl-4-methyl-3-oxo-4-aza-5α-androstane–17β-carboxamide were more effective in combination than when given alone in reducing androstenedione-induced prostatic growth in castrated rats (Labrie et al. 1991). In addition, flutamide and the 5α-reductase inhibitor, finasteride, in combination caused a significant decrease in rat ventral prostate size compared with either drug alone (Fleschner & Trachtenberg 1992). In mice bearing the androgen-sensitive Shionogi mammary carcinoma, flutamide and finasteride had an additive inhibitory effect on tumor growth and on ventral prostate weight (Chen et al. 1996). 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 (Zaccheo et al. 1998b). Early results in patients with prostatic carcinoma have shown that combined therapy with flutamide and finasteride was effective in reducing PSA levels and in maintaining sexual function in most men (Fleschner & Fair 1996).

A potent, dual inhibitor of both type I and II 5α-reductases could better suppress circulating DHT and provide more efficient treatment for prostate cancer than a type II inhibitor like finasteride (Span et al. 1999). PNU 157706 represents an improvement in the field of 5α-reductase inhibitors on account of its in vitro potency on both human isozymes and its extraordinary potency in reducing prostate weight in the rat (di Salle et al. 1998).

The experimental data presented in this paper indicate that a combination of a dual inhibitor of 5α-reductase (like PNU 157706) and an antiandrogen could represent a further improvement in endocrine therapy of prostate cancer. In fact, such a combined treatment could have a significant impact on the management of advanced prostate cancer, as it should maintain sexual function and provide ‘androgen blockade’. The possible role of hormonal therapy in early stage prostate cancer has not been explored systematically in clinical studies. However, the possibility that very early lesions could be more androgen-dependent and more sensitive to DHT withdrawal supports the use of a 5α-reductase inhibitor alone in prevention studies (Ford et al. 1994) or in combination with an antiandrogen in early stage prostate cancer.

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