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Potent CYP17 inhibitors: improved syntheses, pharmacokinetics and anti-tumor activity in the LNCaP human prostate cancer model

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Abstract

A facile preparation of azolyl steroids, VN/85-1 and VN/87-1 (potent inhibitors of CYP17) has been developed. This process without tedious chromatographic separations improved the overall yields from 55 and 45% to 70 and 65% for VN/85-1 and VN/87-1, respectively. Pharmacokinetic studies of VN/85-1 were conducted in male SCID mice. Following subcutaneous (s.c.) administration of 100 mg/kg of VN/85-1, peak plasma level of 16.73 μg/ml occurred after 45 min, and the compound was cleared rapidly with a $t_{1/2}$ of 52.34 min. The bioavailability of VN/85-1 after s.c. administration was 83.0%. VN/85-1 was also rapidly metabolized to the corresponding 3-oxo-4-ene analog, 17-(1*H*-imidazol-1-yl)androsta-4,16-diene-3-one (VN/108-1). In our attempt to optimize the anti-tumor efficacy of these two CYP17 inhibitors, we studied their anti-tumor efficacies in male SCID mice bearing LNCaP tumor xenografts, utilizing various drug doses and drug scheduling. Three times a day dose regimen (3× dose regimen) of VN/85-1 was more effective than a once daily dose. In contrast, 3× dose regimen doses of VN/87-1 were less effective than the once daily dose. However, at their effective dosage regimes, VN/85-1 and VN/87-1 were each as effective as castration and more effective than finasteride or casodex, an anti-androgen used for prostate cancer (PC) therapy. For all of the treatments, there was a strong correlation between the tumor volumes and other associated parameters, such as, tumor weights, and serum testosterone (T) and PSA levels. These results indicate that VN/85-1 or VN/87-1 may be useful in the treatment of hormone-dependent prostate cancer.

Keywords: CYP17 inhibitors; Synthesis; Androgens; Antitumor efficacy; Prostate cancer therapy; Pharmacokinetics

1. Introduction

Prostate cancer (PC) is the second leading cause of cancer deaths in men in the USA, Western Europe and Australia [1]. Recently, it was estimated that about 180,400 new cases are diagnosed each year in the USA and approximately 31,900 Americans will die of the disease [2]. Developing new treatment strategies is critical to improving the health of men.

Androgens play an important role in the growth of the normal prostate and in prostatic carcinoma. It has clearly been demonstrated that approximately 90% of early prostate cancers are androgen-dependent, and that androgen withdrawal produces favorable responses in patients with cancer of the prostate [3]. Orchidectomy (either surgical or medical with a GnRH agonist) remains the standard treatment option for most prostate cancer patients. Medical and surgical orchidectomy reduces or eliminates androgen production by the testis but does not affect androgen synthesis in the adrenal glands. Potent and specific compounds that inhibit androgen synthesis in the testis, adrenals, and other tissue may be more effective for the treatment of prostate cancer (reviewed in [4,5]).

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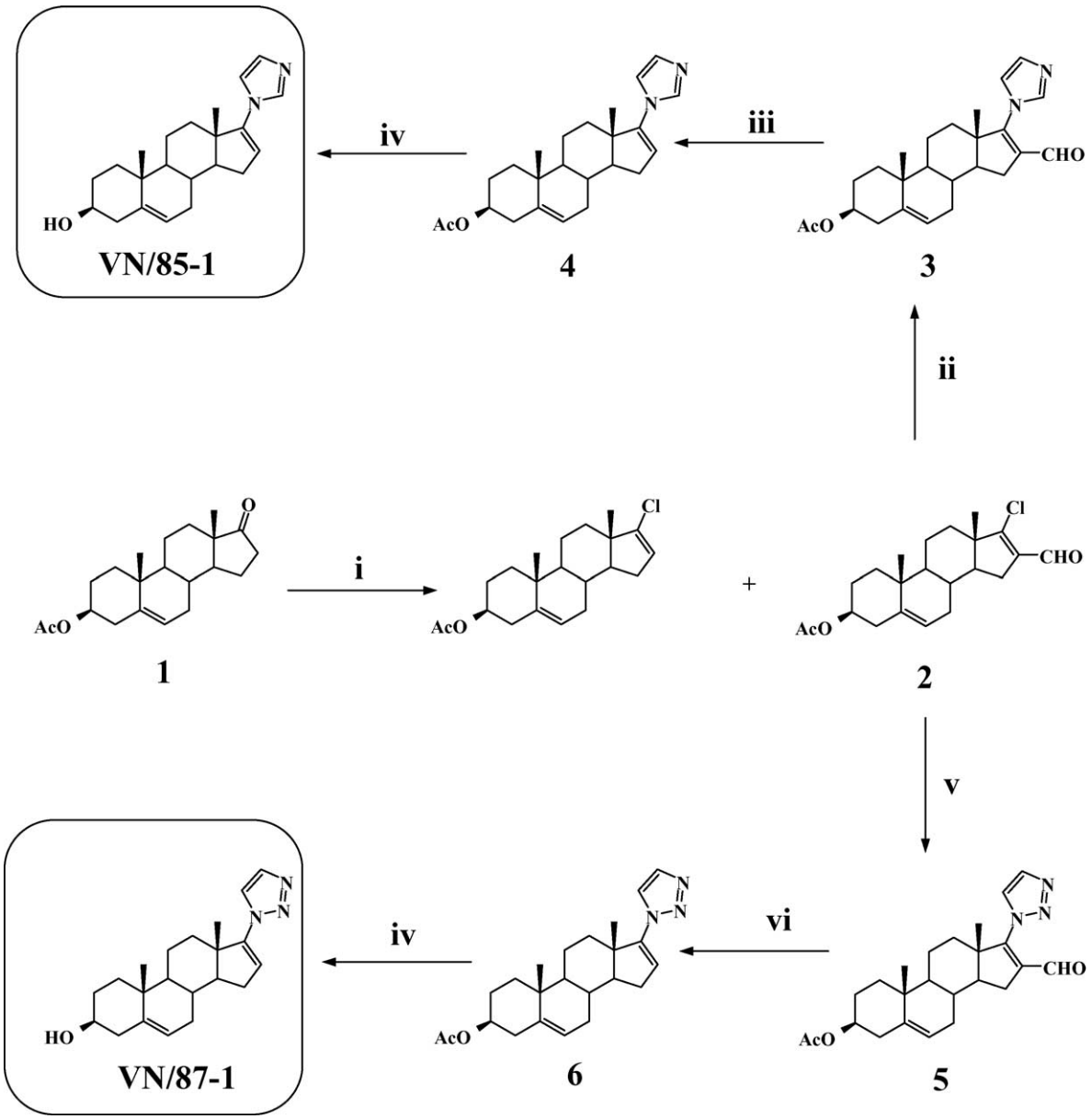
In the testes and adrenal glands, the last step in the biosynthesis of androgens, for example, testosterone (T) involves two key reactions, which occur sequentially and they are both catalyzed by a single enzyme, the cytochrome P450 monooxygenase 17α-hydroxylase/17,20-lyase (CYP17) [6]. Ketoconazole, an antifungal agent, and by virtue of inhibiting P450 enzymes is also a modest CYP17 inhibitor, and has been used clinically for the treatment of PC [7]. However, ketoconazole has now been withdrawn from use because of liver toxicity and other side effects. Nevertheless, it is reported that careful scheduling of treatment can produce prolonged responses in otherwise hormone-refractory prostate cancer patients [8]. Also, in a recent study [9], 62.5% of patients with advanced prostate cancer who had progressed following anti-androgen (flutamide) withdrawal were found to have greater than 50% decrease in prostate specific antigen (PSA) values, while 48% had greater than 80% decrease. The response rate to ketoconazole treatment was similar in patients who had responded to prior anti-androgen withdrawal and those who had not. This suggest that more potent and selective inhibitors of CYP17 could provide useful agents for treating this disease, even in advanced stages and in some patients who may appear to be hormone refractory.

Several categories of highly potent steroidal and nonsteroidal CYP17 inhibitors have been reported (reviewed in [5]). The design strategy of these new inhibitors is based on the knowledge that the active site of the enzyme contains a hydrophobic region and a heme group, in common with all



Scheme 1. (i) $POCl_3$-DMF, $CHCl_3$, Ar, reflux; (ii) imidazole, K_2CO_3, DMF, Ar, 80 °C; (iii) 10% Pd on activated charcoal, PhCN, reflux; (iv) 10% Methanolic KOH, Ar, rt.; (v) 1H-1,2,3-triazole, K_2CO_3, DMF, Ar, 80 °C; (vi) $(PP_3)_2RhCOCl$-$Ph_2(CH_2)_3PPh_2$, xylene, reflux.

P450 enzymes. Some of these compounds are under development, and are expected to show improved selectivity and reduced side effects.

We have previously reported the synthesis and evaluation of several azolyl steroidal inhibitors of CYP17, some of which are amongst the most potent inhibitors of this enzyme reported to date [10–14]. 3β-Hydroxy-17-(imidazol-1-yl)androsta-5,16-diene (VN/85-1) and 3β-hydroxy-17-(1*H*-1,2,3-triazol-1-yl)androsta-5,16-diene (VN/87-1) (Scheme 1) are the most promising CYP17 that we have identified. In cultures of human prostate cancer cell line (LNCaP), both compounds effectively blocked the growth-stimulating effects of T and dihydrotestosterone (DHT), and were also shown to manifest anti-androgenic activity [15]. In animal studies, both compounds reduced the levels of circulating T and DHT in male rat tissues [16]. Furthermore, the anti-tumor efficacies of the compounds were evaluated in male severe combined immunodeficient (SCID) mice bearing tumor xenografts. VN/85-1 (50 mg/kg, subcutanously (s.c.), once daily) was as effective as finasteride at inhibiting tumor growth (36% versus 37%) and the inhibitory effect of VN/87-1 (50 mg/kg, s.c., once daily) was similar to that of castration (48.5% versus 51.5%) [15].

In an attempt to optimize the anti-tumor efficacies of these two compounds, we considered it of interest to evaluate their pharmacokinetic profiles and also to compare the anti-tumor efficacies of several administered dose levels and dose scheduling. The anti-tumor efficacies of castration, of finasteride and of casodex, an anti-androgen used for the treatment of prostate cancer were also evaluated for comparison. In connection with these studies, improved procedures for the syntheses of VN/85-1 and of VN/87-1 were required. These studies are the subject of this paper. It should be noted that the pharmacokinetic profile of VN/87-1 has been reported [17] and part of this work has also been presented [18,19].

2. Results and discussion

2.1. Chemistry

We reported earlier [11] the first chemical synthesis of 3β-hydroxy-17-(imidazol-1-yl)androsta-5,16-diene (VN/85-1) and 3β-hydroxy-17-(1*H*-1,2,3-triazol-1-yl)androsta-5,16-diene (VN/87-1) in overall yields of 55% and 45%, respectively, by a four-step procedure as outlined in Scheme 1. The key step of the synthesis was the nucleophilic vinylic "addition-elimination" substitution reaction of azolyl nucleophiles and 3β-acetoxy-17-chloro-16-formylandrost-5,16-diene derived from 3β-acetoxyandrost-5-ene-17-one [10,11,13].

The procedure, although highly reproducible, required chromatographic purification in each of the four steps, which contributed to reduce the overall yields. Because of the potential of VN/85-1 and VN/87-1 as drug candidates of therapeutic importance, we desired a simpler and more economical method for their synthesis. It was gratifying to discover that we could carry through the synthesis as previously, but requiring only one chromatographic purification (at step 3 of each route, see Scheme 1), and the overall yields for VN/85-1 and VN/87-1 were increased from 55 and 45% to 70 and 65%, respectively. This method is amendable to the synthesis of these two CYP17 inhibitors on large scales (up to 20 gm). Hitherto unreported ^{13}C NMR data of the VN/85-1 and VN/87-1, presented in Section 6, provide complete characterization of the two inhibitors. The ^{13}C NMR chemical shifts were assigned by comparison with reported values for closely related compound [20,21] and with the aid of their respective ^{1}H-^{13}C 2D NMR chemical shifts correlation spectra.

3. Biological studies

3.1. Pharmacokinetic studies

The pharmacokinetic profile of VN/87-1 has recently been reported [17]. VN/87-1 is a high clearance (5.0 ± 1.3 l/h/kg) compound in mice, $t_{1/2}$ of 1.2 ± 0.08 h with a rather large volume of distribution (6.5 ± 1.2 l/kg). The compound was well absorbed following s.c. administration and it showed linear kinetics at doses from 25 to 100 mg/kg.

On reverse phase HPLC, VN/85-1 [retention time (rt) = 10.92 min] was well resolved from the internal standard (L-38, rt = 16.55 min), a metabolite (rt = 7.72 min) and other endogenous compounds in mouse plasma (Fig. 1). The calibration curves derived for VN/85-1 were linear and reproducible (data not shown) and the inter- and intra-assay variability was less than 10%. The limit of detection of VN/85-1 was 100 ng/ml. The HPLC assay was validated and used to monitor VN/85-1 in mice plasma.

The mean plasma concentration–time profile of VN/85-1 after administration of a single bolus dose of 50 and 100 mg/kg, subcutaneously and of 50 mg/kg intravenously, to male SCID mice are shown in Fig. 2. Following intravenous administration, the plasma concentration of VN/85-1 declined exponentially with a mean half-life of about 17.25 min and elimination rate constant of 0.04 min^{-1}. VN/85-1 was rapidly cleared (total clearance of 0.87 ml/min/gm) from the systemic circulation and was not detected 2.5 h after administration. The calculated non-compartmental pharmacokinetic parameters based on the plasma concentration profile following intravenous administration of VN/85-1 are shown in Table 1. The plasma concentration–time curves after s.c. administration of VN/85-1 (50 and 100 mg/kg) to male SCID mice are also shown in Fig. 2. After s.c. administration of VN/85-1, the observed plasma concentration in mice reached peak levels between 30.0 and 57.0 min post dose. The elimination half-life of VN/85-1 after s.c. administration was relatively short (Table 1) and was greater than twice the value obtained after i.v. administration. The absolute bioavailability of VN/85-1 (100 mg/kg) after s.c. administration was 83.03 ± 2.0%. Thus, VN/85-1 is well ab-

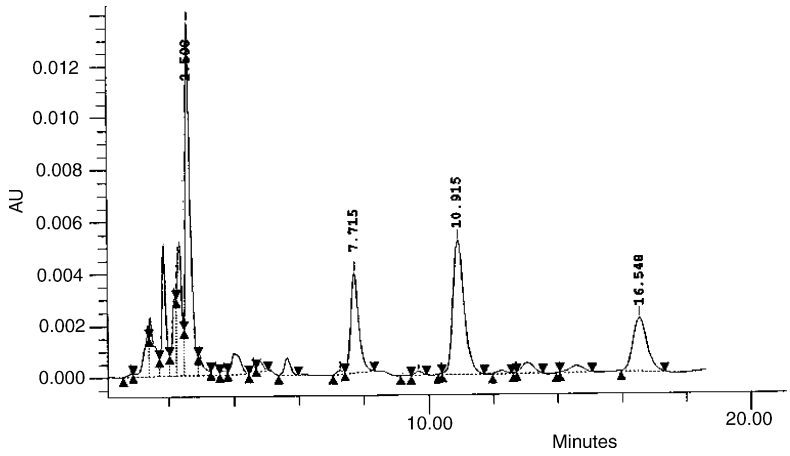


Fig. 1. Typical HPLC chromatogram of VN/85-1, L-38 (internal standard) and metabolite extracted from mouse plasma. The retention times for metabolite, VN/85-1 and L-38 were 7.72, 10.92 and 16.55 min, respectively.

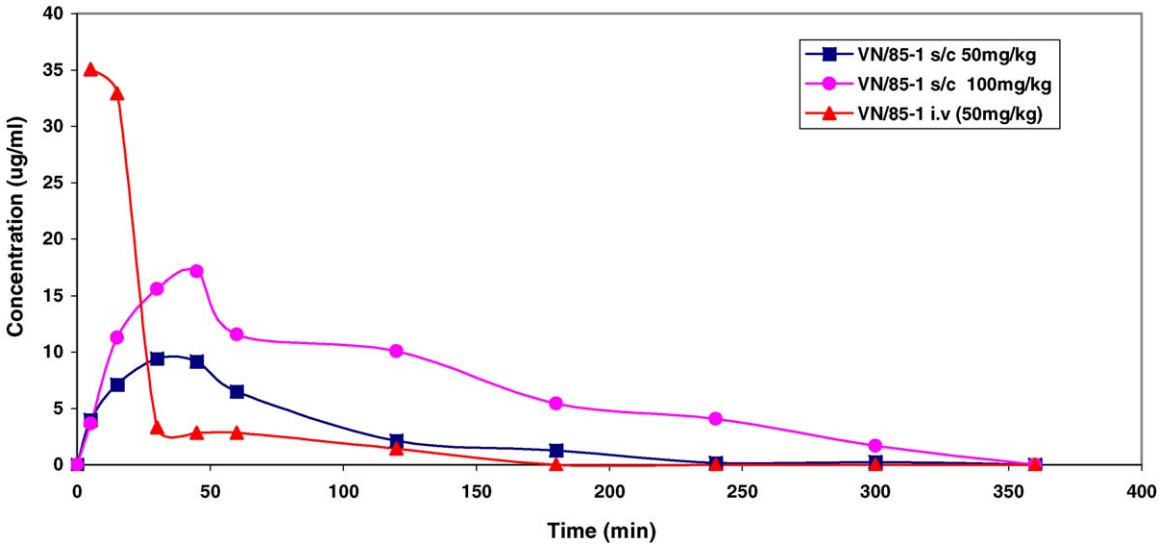


Fig. 2. Pharmacokinetic profile of VN/85-1 following administration of a single subcutaneous or intravenous bolus dose to male SCID mice. Each data point represents the mean plasma concentrations obtained from at least three mice. The standard deviations (not shown) were ±5–8% of the mean values.

sorbed from the subcutaneous site. The area under the curve for the plasma concentration versus time profiles after s.c. administration of VN/85-1 increased proportionately to dose as the administration dose was changed from 50 to 100 mg/kg. Furthermore, the elimination half-life, clearance, volume of distribution and mean residence time were relatively constant as the dose of VN/85-1 was increased from 50 to 100 mg/kg (Table 1). These results indicate that the pharmacokinetic profile of VN/85-1 is dose independent. The terminal slope of the plasma concentration–time profile of

Table 1
Pharmacokinetic parameters for VN/85-1 after i.v. (50 mg/kg) and s.c. administration (50 and 100 mg/kg)

Parameter	Subcutaneous		Intravenous
	50 mg/kg	100 mg/kg	50 mg/kg
$t_{1/2}$ (min)	49.12 ± 0.2	52.34 ± 0.2	17.25 ± 0.01
K_{el} (min^{-1})	0.02 ± 0.004	0.017 ± 0.001	0.04 ± 0.01
AUC (min μg/ml)	842.52 ± 53.7	2594.34 ± 213.48	1018.36 ± 137.0
T_{max} (min)	30.19 ± 1.73	57.04 ± 3.4	NA
C_{max} (μg/ml)	9.34 ± 0.26	16.73 ± 1.2	NA
MRT (min)	69.90 ± 0.25	114.52 ± 0.2	24.89 ± 0.5
V_d (ml/gm)	57.54 ± 0.04	54.97 ± 4.0	21.93 ± 0.2

Values are expressed as mean ± S.E., $n = 5$. NA = not applicable.

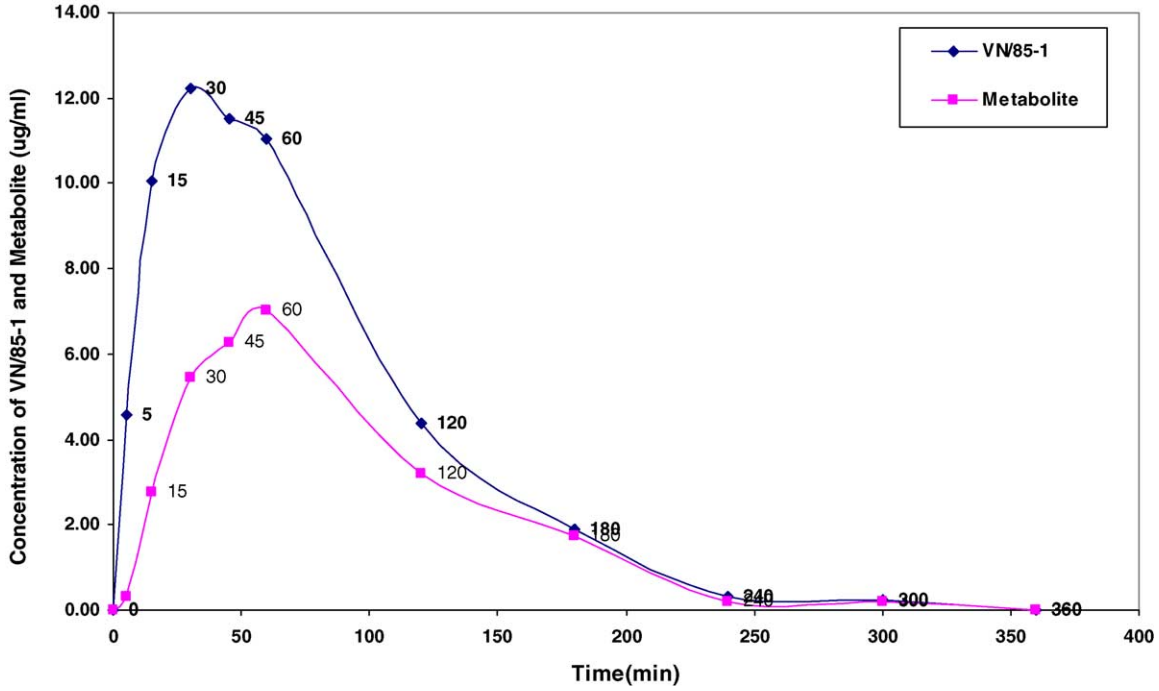


Fig. 3. Pharmacokinetic profile of VN/85-1 and metabolite following administration of a single subcutaneous bolus dose (100 mg/kg) of VN/85-1 to male SCID mice. Points represent means four at least three mice. The standard deviations (not shown) were ±5–8% of the mean values. The difference in amounts of VN/85-1 detected as recorded in Fig. 2 and this figure is very likely due to inter animal metabolic variability.

VN/85-1 following s.c. administration is shallower compared to the terminal slope following i.v. dosing, an indication that the compound persists in the blood longer when administered subcutaneously.

3.2. *In vivo metabolism of VN/85-1*

Fig. 3 shows that a significant amount of a polar metabolite [retention time, 7.72 min, see Fig. 1)] was formed from VN/85-1 during the in vivo pharmacokinetic studies. At 60 min post dose approximately 39% of VN/85-1 was converted to the metabolite. The metabolite exhibited a similar elimination profile as VN/85-1. This metabolite showed identical retention time as authentic 17-(1*H*-imidazol-1-yl)androsta-4,16-diene-3-one (VN/108-1), the 3-oxo-4-en analog of VN/85-1 and its identity was confirmed by LC–MS. The mass spectrum data of metabolite peak displayed a molecular ion at *m/z* 337 (*M* + H, positive mode). The MS/MS for the 337 species shows losses of 18 and 28, which is consistent with a ketone. There was, however, insufficient material to obtain an NMR spectrum of the metabolite. The metabolite may have been formed by oxidation of the 3β-OH → 3-ketone, followed by isomerization of the Δ^5 double bond. This extensive metabolism of VN/85-1 to the corresponding 3-oxo-4-en analog is intriguing and unique since we did not observe any significant metabolite(s) in the in vivo pharmacokinetic studies with VN/87-1 which has a 17- 1*H*-1,2,3-triazolyl group. Since both compounds have the same basic steroid structure, the difference in metabolic stability is probably due principally to the difference in the 17-azolyl groups. Although this metabolite (VN/108-1) has previously been shown to be a potent CYP17 inhibitor [11] and also an inhibitor of T and DHT production in vivo [15,16] it had no significant effects on prostate cancer LNCaP tumor growth [15]. However, the compound binds to the androgen receptor and may exhibit androgenic activity [15]. Thus, it seems reasonable to assume that the metabolism of VN/85-1 to VN/108-1 is detrimental to the formers anti-tumor efficacy. It should be stated that unlike the results reported for VN/85-1 above, we did not detect any in vivo metabolite of VN/87-1 in our previous studies [17].

4. Effects of CYP17 inhibitors on LNCaP tumor growth

1. Effects of VN/85-1 and VN/87-1 administered once daily at 50 mg/kg and thrice daily at 16.6 mg/kg.
   Male SCID mice bearing LNCaP tumor xenografts were treated with VN/85-1 or VN/87-1 (50 mg/kg/day or 16.6 mg/kg/day × 3) for 4 weeks and the tumor volumes were measured weekly. Finasteride (50 mg/kg/day) and castration were used as reference groups. Fig. 4 shows that VN/85-1 (given three times daily) inhibited tumor growth by 53.4%, which was similar to inhibition (59.7%) caused by castration, and was more effective than inhibition (27.6%) caused by finasteride. Surprisingly, VN/85-1 (given once daily) had no significant effect on tumor growth. These reductions in tumor volumes correlated well with the respective final tumor weights (Fig. 5).

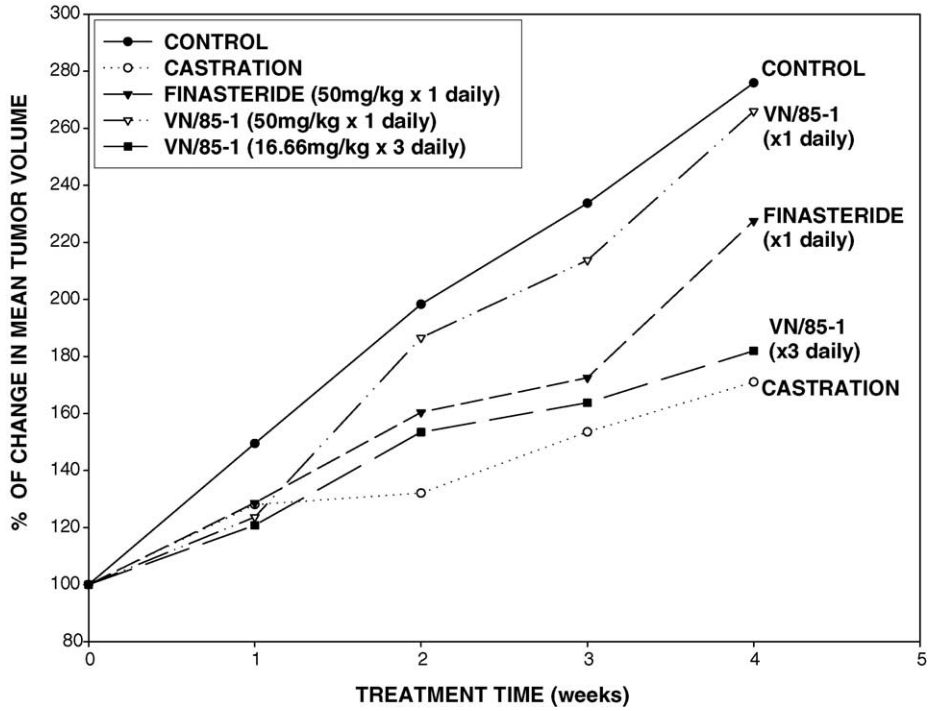


Fig. 4. The effects of VN/85-1 and other endocrine therapies on the growth of human prostate cancer LNCaP tumors in male SCID mice. LNCaP cells were inoculated (s.c.) into SCID mice and allowed to proliferate as tumor xenografts to ~300 mm^3. Groups of five or six mice were treated daily with compounds listed or castrated. Tumor volumes were measured weekly and percentage change in tumor volumes were calculated.

Serum PSA levels were also significantly reduced in all the treatment groups compared to the control group (Fig. 5). As shown in Fig. 6, serum testosterone levels in castrated mice and in those treated with VN/85-1 (three times daily) were significantly reduced.

In contrast to the results obtained with VN/85-1, Fig. 7 shows that VN/87-1 (given once daily) was more effective than VN/87-1 (given thrice daily). The former treatment inhibited tumor growth by 58.5%, which was similar to inhibition (59.7%) caused by castration, and was also more

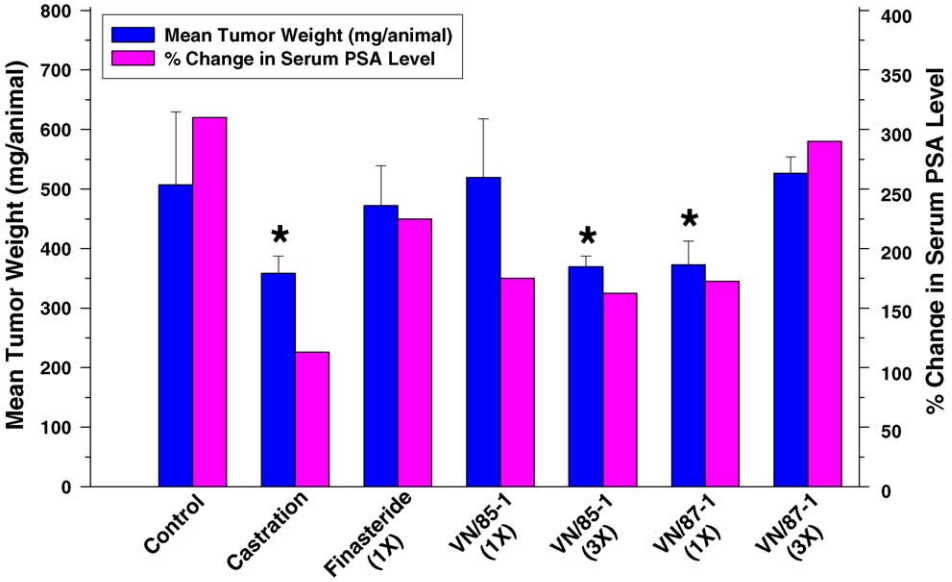


Fig. 5. The effects of VN/85-1, VN/87-1 and other endocrine therapies on human prostate cancer LNCaP tumor weights and percentage change in serum PSA levels in male SCID mice following 4 weeks of treatment. At the end of treatment, the animals were sacrificed, tumors excised and weighed. PSA levels were measured before and after treatment period. Values are expressed as mean ± S.E. ($^{*}P < 0.05$ vs. the control).

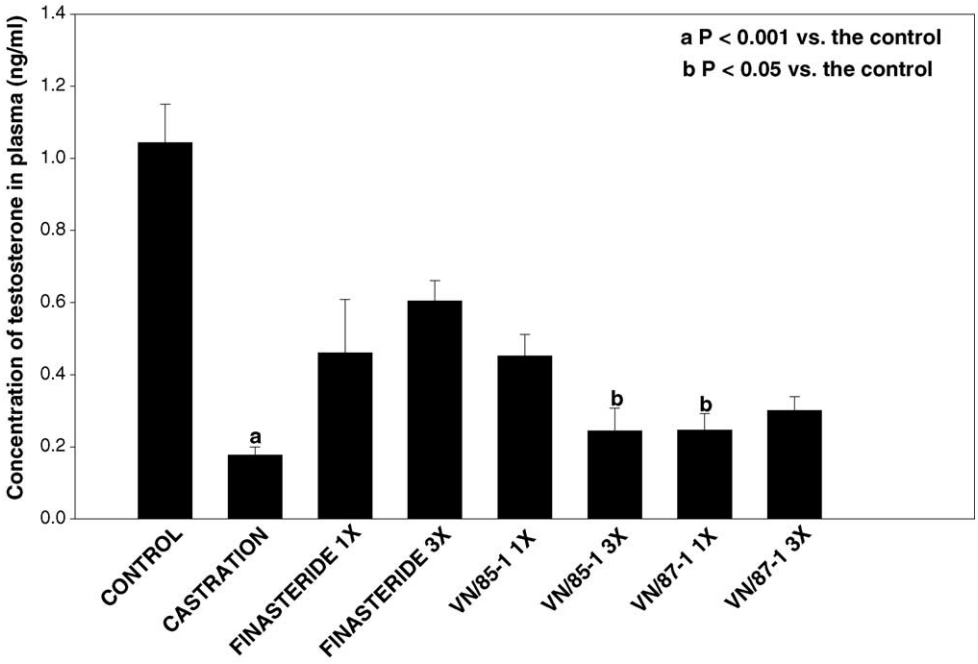


Fig. 6. The effects of VN/85-1, VN/87-1 and other endocrine therapies on plasma testosterone levels following 4 weeks of treatment. Blood was collected from the animals about 1–2 h after the last administered drug dose. Serum testosterone (T) levels were determined by RIA as described in the Section 6. Values represent the means ± standard error from five to six mice. $^{*}P < 0.001$, and $^{**}P < 0.05$ compared to the control group.

effective than inhibition (27.6%) caused by finasteride. VN/87-1 (given thrice daily) had no effect on LNCaP tumor growth. As observed with studies with VN/85-1, the reduction in tumor volumes correlated well with final tumor weights, serum PSA levels (Fig. 5), and serum testosterone levels (Fig. 6).

The reason for lack of tumor growth inhibition by 3× dose regimen of VN/87-1 is unknown at this time. One possibility is that during the multiple dosing of VN/87-1 (16.66 mg/kg/thrice daily), the minimum effective concentration of the compound is not attained. It is interesting to note that a similar effect was also observed with finas-

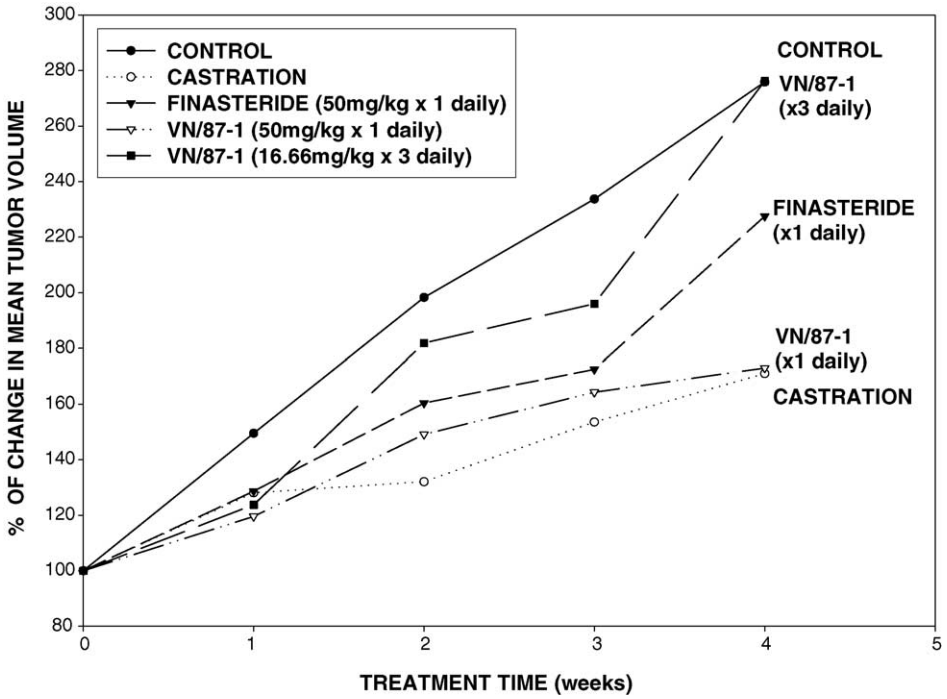


Fig. 7. The effects of VN/87-1 and other endocrine therapies on the growth of human prostate cancer LNCaP tumors in male SCID mice. Procedures were the same as described for Fig. 4 above.

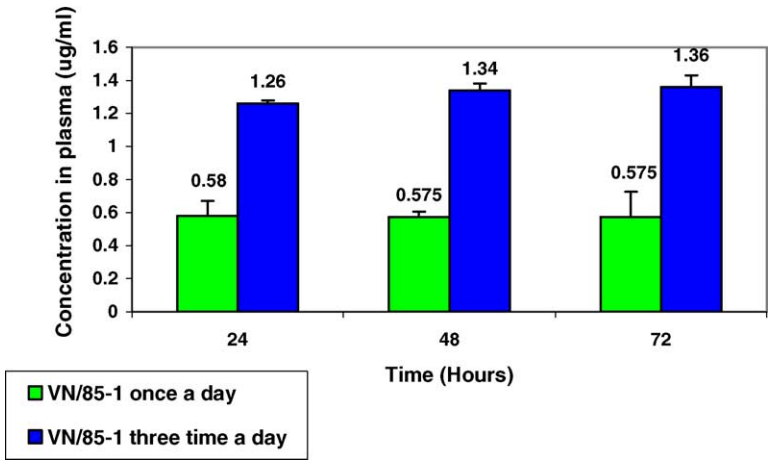


Fig. 8. The plasma concentration of VN/85-1 administered once daily at 50 mg/kg and thrice daily at 16.66 mg/kg. Procedures were the same as described for Fig. 6 above.

teride, where the 50 mg/kg/once daily dose was also more effective in reducing T than the 16.66 mg/kg/thrice daily dose schedule (Fig. 6) as well as tumor weight (data not shown).

It was of interest to investigate why the administration of VN/85-1 once daily was not as effective as its administration thrice daily. VN/85-1 (50 mg/kg) was administered once daily to three groups of male SCID mice (14–16 weeks old) bearing LNCaP tumors. Groups 1–3 were sacrificed after 24, 48, and 72 h, respectively. In the second experiment, VN/85-1 (16.66 mg/kg) was administered thrice daily to nine groups of mice. Each group was sacrificed at 8 h intervals up to 72 h. Plasma was extracted and the concentrations of VN/85-1 were determined as described in Section 6. As shown in Fig. 8, plasma concentrations of VN/85-1 were at least two-fold higher in the animals treated with 3× dose regimen of the compound at all time points. Thus, the improved anti-tumor activity observed with sustained dosing of VN/85-1 may be associated with the higher serum levels of the compound. In addition, the extensive metabolism of VN/85-1 to VN/108-1 (a compound with no anti-tumor activity) (*vide supra*) argues for the anti-tumor efficacy of VN/85-1 following 3× dose regimen. The ability to detect VN/85-1 in the mice plasma at

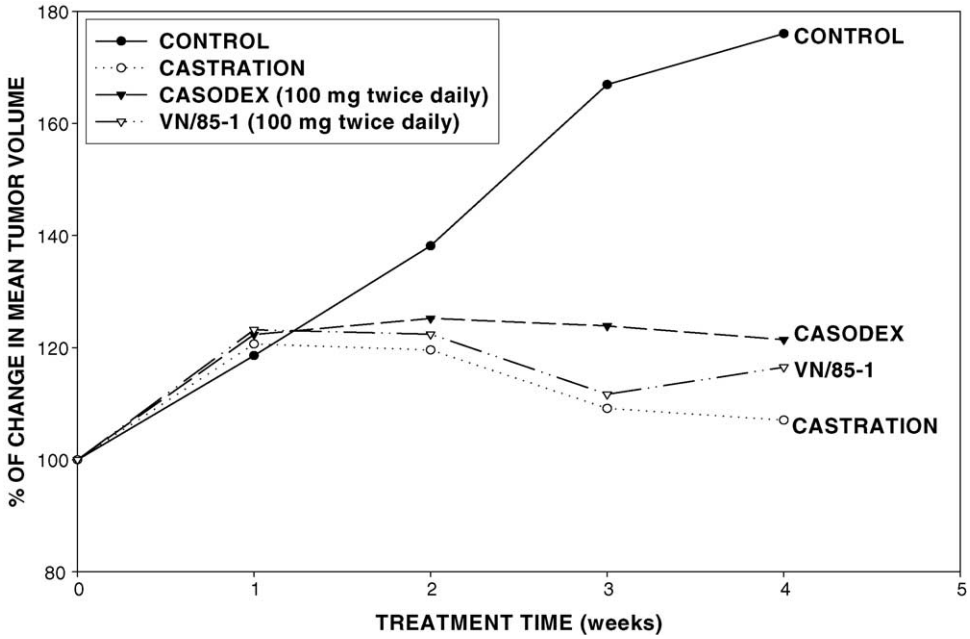


Fig. 9. The effects of castration, VN/85-1, and casodex (100 mg/kg, twice daily) on the growth of human prostate cancer LNCaP tumors in male SCID mice. Procedures were the same as described for Fig. 4 above.

the various time points from 8 to 72 h was somewhat surprising in view of our earlier finding, where we could not detect VN/85-1 after 5 h post administration. These seemingly incongruous data likely reflect the different pharmacokinetics of VN/85-1 in the different ages of the animals used in the two studies (8–10 weeks versus 14–16 weeks) A similar experiment as described above for VN/85-1 was also conducted with VN/87-1, but we did not detect the compound or any metabolites in the plasma at any of the time points (8 h interval up to 72 h). Thus, we do not have a plausible explanation at this time for the antitumor results (Fig. 7) obtained with the two different dose regiments of VN/87-1.

2. Effects of VN/85-1 and Casodex administered twice daily at 100 mg/kg.

Because casodex is currently used for the treatment of prostate cancer, it was of interest to compare its anti-tumor efficacy with that of VN/85-1 in a head-to-head test. The effects on tumor growth of the administration of VN/85-1 or casodex (100 mg/kg, twice daily, s.c., for 28 days) were also compared to effects of castration. Fig. 9 shows that VN/85-1 inhibited LNCaP tumor growth by 90.6%, which was slightly better than the inhibition (87.8%) caused by casodex. The inhibition due to castration was 96.0%. These reductions in tumor volumes correlated well with the respective final tumor weights (data not shown).

5. Conclusion

An improved procedure for the preparation of VN/85-1 and VN/87-1 has been developed. This enabled us to determine the pharmacokinetics and metabolism of VN/85-1 and also to conduct preclinical anti-tumor efficacy studies. The later studies confirmed existing knowledge on the anti-tumor efficacy of our CYP17 inhibitors and reveal that dose scheduling of these two compounds is characteristic and important for activity or lack thereof.

6. Experimental

General procedures and techniques were identical with those previously reported [11]. ^{13}C NMR spectra were recorded in $CDCl_3$ on at 150 MHz. LC–MS were obtained using a HP1100 system interfaced to a Bruker Esquire LC–MS by Mr. T. Jones, Analytical Services, Department of Chemistry, Brock University, St. Catharine's, Ontario, Canada. The column was an Eclipse XDB-C18, 4.6 mm × 50 mm, using methanol/water/acetonitrile (35:35:30, v/v/v) with a flow rate of 0.5 ml/min. Under these conditions, the metabolite and VN/85-1 had retention times of 10.55 and 12.35 min, respectively. 3β-Hydroxy-17-(5′-isoxazoly)androsta-5,16-diene (L-38) was used as the internal standard for HPLC quantification of VN/85-1, its metabolite and VN/87-1.

6.1. 3β-Hydroxy-17-(1H-imidazol-1-yl)androsta-5,16-diene (VN/85-1).

VN/85-1 was prepared as previously reported [11] with some modifications (see Scheme 1). Compound 1 was subjected through steps 1–3, followed by purification by FCC to give pure 4. Hydrolysis of compound 4 followed by crystallization from a mixture of methanol and ethyl acetate gave VN/85-1 in 70% overall yield. The spectral (^1H NMR, IR and MS) and analytical data were as previously reported. ^{13}C NMR ($CDCl_3$, δ) 18.5 (C-19), 21.9 (C-18), 23.3 (C-11), 32.4 (C-15), 32.8 (C-2), 33.6 (C-8), 34.2 (C-7), 37.4 (C-12), 39.3 (C-10), 39.8 (C-1), 44.9 (C-4), 48.8 (C-13), 53.0 (C-9), 58.8 (C-14), 74.2 (C-3), 120.9 (C-4'), 121.6 (C-16), 123.6 (C-6), 131.9 (C-3′), 138.6 (C-2′), 143.9 (C-5) and 151.2 (C-17).

3β-Hydroxy-17-(1*H*-1,2,3-triazol-1-yl)androsta-5,16-diene (VN/87-1). VN/87-1 was also synthesized as previously described [11], with modifications as outlined in Scheme 1. Hydrolysis of compound 6 followed by crystallization from a mixture of methanol and ethyl acetate gave VN/87-1 in 65% overall yield. The spectral (^1H NMR, IR and MS) and analytical data were as previously reported. ^{13}C NMR ($CDCl_3$, δ) 18.4 (C-18), 21.9 (C-19), 23.2 (C-11), 32.7 (C-15), 32.8 (C-2), 33.7 (C-8), 34.2 (C-7), 37.3 (C-12), 39.3 (C-10), 39.8 (C-1), 44.9 (C-4), 49.1 (C-13), 53.1 (C-9), 59.1 (C-14), 74.3 (C-3), 122.2 (C-16), 123.6 (C-6), 124.7 (C-4′), 135.9 (C-5′), 144.0 (C-5) and 151.1 (C-17).

6.2. Pharmacokinetic studies

Male Balb/c mice weighing 20–22 gm (8–10 weeks old) obtained from NCI, Frederick, MD, USA were maintained in a controlled environment of about 25 °C, 50% relative humidity and 12 h of light and 12 h of dark cycles and allowed free access to food and water. VN/85-1 was formulated in 40% β-cyclodextrin in water and a single subcutaneous dose was given to mice. The animals were sacrificed at various times up to 6 h after drug administration and blood was obtained by cardiac puncture under light halothane (Ayerst, New York, NY, USA) anesthesia.

6.3. HPLC analysis

Chromatographic separations and quantification of the steroids and the appropriate internal standards were achieved by a reverse phase HPLC method on a Waters® Novapak® C18 column (3.9 mm × 150 mm) protected by Waters® guard cartridge packed with pellicular C18 as previously described. Briefly, the HPLC system used in this study consisted of Waters® solvent delivery system, Waters® controller (Milford, MA), coupled to a Waters® 717^{plus} autosampler and a Waters® 996 photodiode array detector operated at 234 nm. The mobile phase composition was water/methanol/acetonitrile (35:35:30, v/v/v) at a flow rate of 1.0 ml/min. The HPLC analysis was performed at ambient temperature and data acquisition and management was

achieved with a Waters® millennium chromatography manager.

6.4. Sample preparation

Sample preparation was conducted as previously described. Briefly, test tubes containing mouse plasma (250 μl), VN/85-1 and L-38 (internal standard, 10 μg/ml), were extracted with diethyl ether (2 × 2 ml) using a vortex mixer for 1 min and centrifuged at 1500 × *g* for 5 min. The organic layers were evaporated to dryness under a gentle stream of air. The extracts were reconstituted in acetonitrile (250 μl) and loaded into a solid phase Sep-Pak 1 cc C18 cartridge (Waters, Milford, MA) that had been pre-washed with methanol (1 ml) for further purification. The cartridge was eluted with acetonitrile (250 μl) and the eluent was evaporated to dryness. The residue was reconstituted in an aliquot of the mobile phase (50 μl) and filtered using 0.2 μm teflon filters before HPLC analysis.

6.5. Calibration curve and HPLC assay validation

The calibration curve for VN/85-1 was constructed by spiking varying amounts of the compound into extraction tubes (duplicate) containing plasma (250 μl) from untreated animals to give final concentrations of 10.0–100.0 μg/ml. Appropriate blank extraction tubes were also prepared and an aliquot of the internal standard was added into each extraction tube to give a final concentration of 5 μg/ml. The calibration samples were taken through the sample preparation procedure as described above. An aliquot of the reconstituted extract (10 μl) was injected into the HPLC system and the ratio of the peak areas for each analyte to that of the internal standard were plotted against concentrations of VN/85-1. The precision and accuracy of the assays were determined from a range of known concentrations of the inhibitor in blank plasma and taken through the HPLC procedure. The study was repeated on three separate occasions. A low range (0.1–10.0 μg/ml) calibration curve was also constructed. The analyte and internal standard (final concentration of 0.125 μg/ml) were added into extraction tubes and the samples were treated in the same manner as described above.

6.6. Data analysis

Pharmacokinetic calculations were performed as previously described [17]. The non-compartmental pharmacokinetic calculations were performed using WinNonlin (Scientific Consulting Inc.). One-way analysis of variance (ANOVA) on SigmaStat for Windows version 1.0 was used to compare different treatment groups at the 95% confidence level. The Bonferroni post-hoc test was used for determination of significance. A *P*-value of less than 0.05 was considered as statistically significant.

6.7. Human LNCaP prostate cancer xenograft model

Male severe combined immunodeficient mice purchased from Charles River Breeding Laboratories, (Boston, MA, USA) were maintained under aseptic conditions in controlled environment of about 25 °C, 50% relative humidity and under 12 h of light and 12 h of dark cycle and allowed free access to food and water. LNCaP tumors were grown subcutanously in the mice essentially as previously described [15]. Briefly, wild-type LNCaP cells were grown in routine medium, and when 80% confluent, the cells were scraped into DPBS, counted, and suspended in Matrigel (3×10^7 cells/ml). Male SCID mice were injected s.c. with 100 μl of the cell suspension at one site on each flank. LNCaP tumors were allowed to grow for 4–5 weeks following inoculation. The mice were then grouped (six mice per group) for castration or treatment with vehicle or the CYP17 inhibitors. Tumors were measured weekly for the 4 weeks of treatment and tumor volumes were calculated using the formula: $4/3\pi \times r_1^2 \times r_2 (r_1 < r_2)$. At the end of the treatment period, the animals were sacrificed by decapitation under anesthesia and blood collected. Serum was obtained after centrifugation and stored at −80 °C. Tumors were excised, weighed and stored in liquid nitrogen.

6.8. Measurement of testosterone levels

Tumors obtained from individual mice were homogenized in phosphate buffer (pH 7.4, 0.1 M). The homogenates were centrifuged at 2000 × *g* for 20 min. Serum (50 μl) was used to determine T concentration as described in the ^{125}I-T assay kit supplied by DSL Inc. Radioactivity was measured using Packard Cobra II gamma counter.

6.9. Measurement of PSA levels

Serum PSA levels were determined using a PSA ELISA kit supplied by DSL Inc. Briefly, 2.5 μl of serum diluted to a final volume of 75 μl was added to duplicate wells in a 96-well plate that had been coated with anti-PSA antibody. Following a 1 h incubation at 25°C, wells were treated with a second anti-PSA antibody labeled with horseradish peroxidase. The reaction was stopped and the absorbance was measured at 450 nm using a Dynatech MRX plate reader.

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