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in Animal Models

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14. ABSTRACT One critical issue in prostate cancer research is to slow down the transition of prostate cancer from an androgen-dependent state to a lethal androgen-refractory state. Intermittent androgen ablation therapy (IAAT) may slow down the development of androgen refractory tumors because intermittent recovery of androgens can induce differentiation of prostatic epithelial cells. However, the advantage of inducing differentiation by intermittent recovery of androgens is compromised by the disadvantage of androgenic induction of proliferation in prostate tumors. The biologically most active androgen is dihydrotestosterone (DHT), which is converted from testosterone (T) by 5α-reductase. Our recent studies showed that T is more potent than DHT in inducing the expression of growthinhibitory androgen-response genes, which led to our hypothesis that IAAT can be enhanced by finasteride, an inhibitor of T to DHT conversion. We have tested our hypothesis using LNCaP xenograft tumors in nude mice. Our experiments showed that finasteride administration during IAAT significantly reduced tumor growth rate, prolonged the life of nude mice bearing LNCaP tumors, and supra-induced the expression of growth-inhibitory androgen-response genes in the tumors.					
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Introduction:

Conversion of testosterone (T) to dihydrotestosterone (DHT) is essential for prostate development.

T and DHT are two major biologically active androgens (1). T is synthesized in testis and then transported to target organs, such as the prostate, via blood circulation. T can be converted to DHT in the prostate by 5 α -reductase (2, 3). Both T and DHT bind to the same AR. DHT is more potent than T in activating promoters containing ARE, most likely due to the higher binding affinity of AR to DHT relative to that of T (4-7). The conversion of T to DHT is necessary for normal prostate development because 5 α -reductase inactivation prevents normal prostate development (8, 9). It was thought that the conversion is merely an amplification step for androgen action (10). However, it cannot be ruled out that T and DHT have overlapping yet different biological functions *in vivo*. In fact, our recent studies suggest that T is more potent than DHT in inducing androgen-response genes during the regrowth of the rat ventral prostate (11).

Androgens regulate homeostasis of prostate.

Androgens are required for the structural and functional integrity of the prostate (12). Androgen ablation by castration leads to rapid prostate regression via massive apoptosis (13, 14). On the other hand, androgen replacement stimulates rapid proliferation and differentiation of a regressed prostate until it reaches the normal size (12, 15). Androgen action in a regressed prostate is different from that in the fully-grown prostate because androgens do not stimulate proliferation in a fully-grown prostate (Table 1) (12). During the regrowth of a regressed prostate, androgens induce and then nullify proliferation, establish apoptotic potential while inhibiting apoptosis, and induce and maintain differentiation.

Table 1. The impact of androgen manipulation on the regressed prostate and the normal prostate.

Androgen	Regressed Prostate	Fully-Grown Prostate
+	Proliferation & Differentiation	No Significant Change
-	No Significant Change	Apoptosis & Dedifferentiation

+ represents androgen replacement and – represents androgen ablation or administration of anti-androgens. Differentiation is defined as the expression of prostate-specific markers. Dedifferentiation is defined as loss of prostate-specific marker expression.

Androgen action is intimately associated with prostate cancer pathogenesis.

Androgens are thought to play important roles in prostate cancer pathogenesis (16-18). One of the risk factors for prostate cancer is the presence of the functional testis. Prostate cancer cells are derived from glandular epithelial cells and are initially androgen-dependent. Androgen ablation remains as the standard therapy for metastatic prostate cancer. Unfortunately, androgen ablation therapy is only palliative and eventually patients relapse with androgen-refractory prostate cancer that is currently incurable (18).

Development of androgen-refractory prostate cancer.

The mechanisms of prostate cancer progression from an androgen-dependent state to a lethal androgen-refractory state have been studied extensively. Mutations followed by clonal selection appears to be the mechanism of androgen-independent progression in several prostate cancer models, including the Dunning R3327 rat prostatic adenocarcinoma and LAPC9 human prostate cancer cells (19, 20). Another mechanism for androgen-independent progression involves adaptation. The androgen-independent progression of Shionogi mouse tumor and LNCaP human tumor involve the adaptation (21-24). It is possible that multiple mechanisms are involved in the development of androgen-refractory prostate cancer.

Intermittent androgen ablation therapy.

One urgent challenge in prostate cancer research is to develop new approaches to inhibit or to slowdown the development of androgen-refractory prostate cancer. Intermittent androgen ablation therapy was developed, attempting to delay the emergence of androgen-refractory prostate tumors relative to the continuous androgen ablation therapy. The rationale is that intermittent recovery of androgens can promote prostate cancer cell differentiation and enhance their dependence on androgens (24, 25). However, androgens are also proliferative to prostate cancer cells, which is undesirable in the therapy. The goal of our proposal is to increase the efficacy of intermittent androgen suppression by enhancing the differentiation effects while inhibiting the proliferative effects via finasteride administration.

Finasteride enhances the expression of many androgen-response genes during T-stimulated regrowth of the regressed prostate.

One interesting question in androgen action is whether or not the expression of androgen-response genes is differentially regulated by T and DHT. Finasteride, a 5 α -reductase inhibitor, had little or no effect on the expression of the surveyed androgen-response genes in testis-intact rats (11). However, the induction of half of the surveyed androgen-response genes, including prostatein C3, adrenomedullin and calreticulin, are further enhanced by finasteride during T-stimulated regrowth of a regressed rat ventral prostate (11). This unexpected observation suggests that T is more potent than DHT in inducing androgen-response genes in prostate regrowth.

Since finasteride only enhances androgen-response gene expression in a regressed prostate but not in a fully-grown prostate, finasteride is expected to enhance the expression of androgen-response genes in prostate tumor regrowth induced by intermittent recovery of androgens but not in prostate tumors untreated with androgen ablation therapy.

Body:

Task 1: Determine quantitatively the relative potency of T versus DHT in the induction of androgen-response genes during the prostate regrowth (Month 1-36).

- Animal manipulation and collecting prostatic tissue and serum samples.
- Measurement of serum and intraprostatic T and DHT.
- Measurement of DNA contents in the rat ventral prostate in the presence and absence of finasteride.
- Northern and Western blot analysis of androgen-response gene expressions in the rat ventral prostate in the presence or absence of finasteride.

According to our proposal, the first step in this project was to calibrate the T-pellets. We have contracted with Innovative Research of America (Sarasota, FL) to make 21 day slow-releasing pellets at different T dosages (Table 2). We used 3 rats for each condition in this pilot experiment.

	Testosterone (mg-21 pellets)	DHT (mg-21 pellets)	Finasteride	Number of Animals
Testosterone	0.1			3
	0.3			3
	0.5			3
	1			3
	2.5			3
	5			3
	15			3
Testosterone+Finasteride	0.1		40mg/kg/day	3
	0.3		40mg/kg/day	3
	0.5		40mg/kg/day	3
	1		40mg/kg/day	3
	2.5		40mg/kg/day	3
	5		40mg/kg/day	3
	15		40mg/kg/day	3
DHT		0.1		3
		0.3		3
		0.5		3
		1		3
		2.5		3
		5		3
		15		3
DHT+Finasteride		0.1	40mg/kg/day	3
		0.3	40mg/kg/day	3
		0.5	40mg/kg/day	3
		1	40mg/kg/day	3
		2.5	40mg/kg/day	3
		5	40mg/kg/day	3
		15	40mg/kg/day	3
Castrate Only				7
Normal Animals				3
Total Number of Animals				94

Table 2. Experimental design of testing dose-response of androgen-stimulated prostate regrowth in the rat.

We have castrated the animals and left for 7 days, which allowed the prostate to regress. At day 7 after castration, the slow releasing T pellets or DHT pellets were injected subcutaneously with trocar. Finasteride treatment was started one day before androgen replacement by daily subcutaneous injection of finasteride at 40 mg/kg. The animals were sacrificed two days after androgen replacement; serum samples were collected for testosterone measurement. Also, the ventral prostates were dissected out, weighed, and frozen for RNA extraction.

It appears that finasteride treatment slightly inhibited the regrowth of the prostate in 7-day castrated rats (Fig. 1). However, the differences were not statistically significant since only 3 animals were used in the study.

We have measured serum T levels in each animal. Results showed that serum T levels in animals received finasteride appeared lower than the T levels in the control animals (Fig. 2). This was not expected because previous publications from my lab and other labs showed that finasteride should not reduce serum T levels (Dadras et al., 2001). Finasteride also appeared to inhibit the DHT-induced prostate regrowth (Fig. 3). These observations raised our concerns whether the experiment was carried out properly or whether the slow-releasing pellets are compatible with finasteride treatment in our systems. Since we do not have the information on the materials responsible for slow-releasing, it was difficult for us to assess the compatibility of slow-releasing pellets with finasteride. We had additional problem with slow-releasing pellets in our mice experiment proposed in Specific Aim 2 (Fig. 5). Thus, we decided to first work out conditions to reproducibly deliver T, DHT, and finasteride.

Although the experiment was not as successful as planned, we did obtained some valuable information. It appears that the dosages for induction of half-maximum androgen-response gene expression are between 0.3 mg and 0.5 mg pellets (Fig. 4).

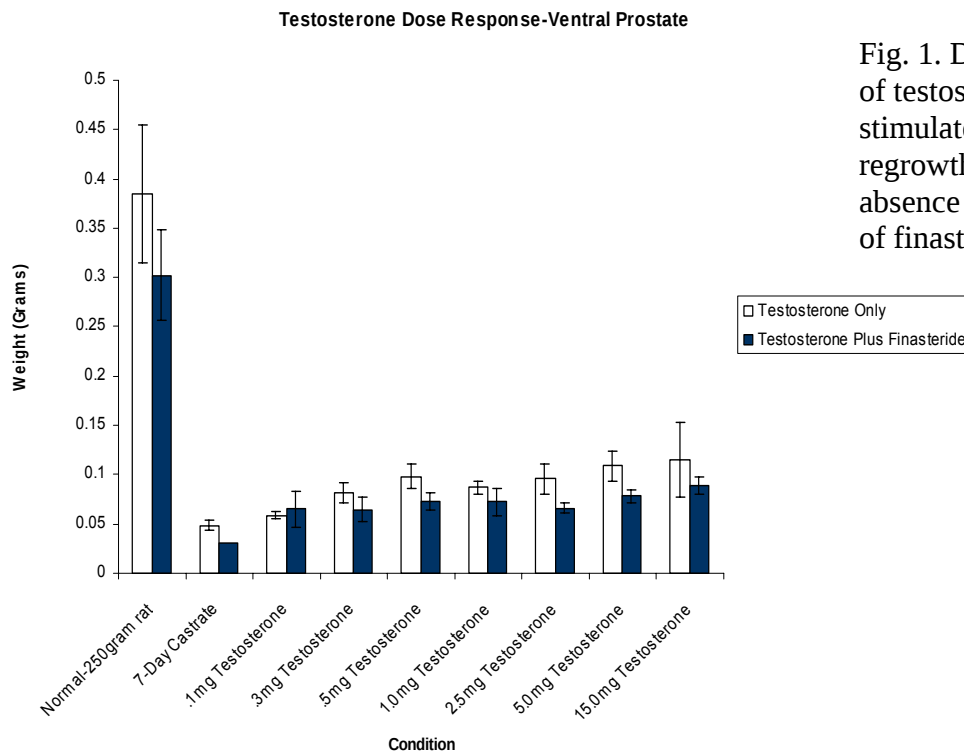


Fig. 1. Dose-response of testosterone-stimulated prostate regrowth in the absence or presence of finasteride.

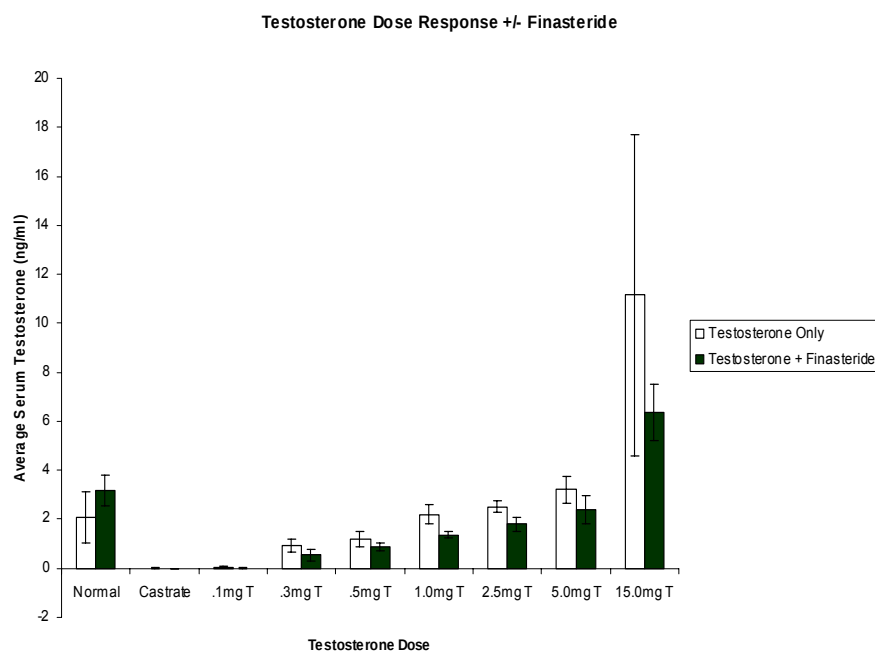


Fig. 2. Serum testosterone level in the dose-response study in the presence or absence of finasteride. The rats were implanted with indicated amount of testosterone in slow releasing pellets.

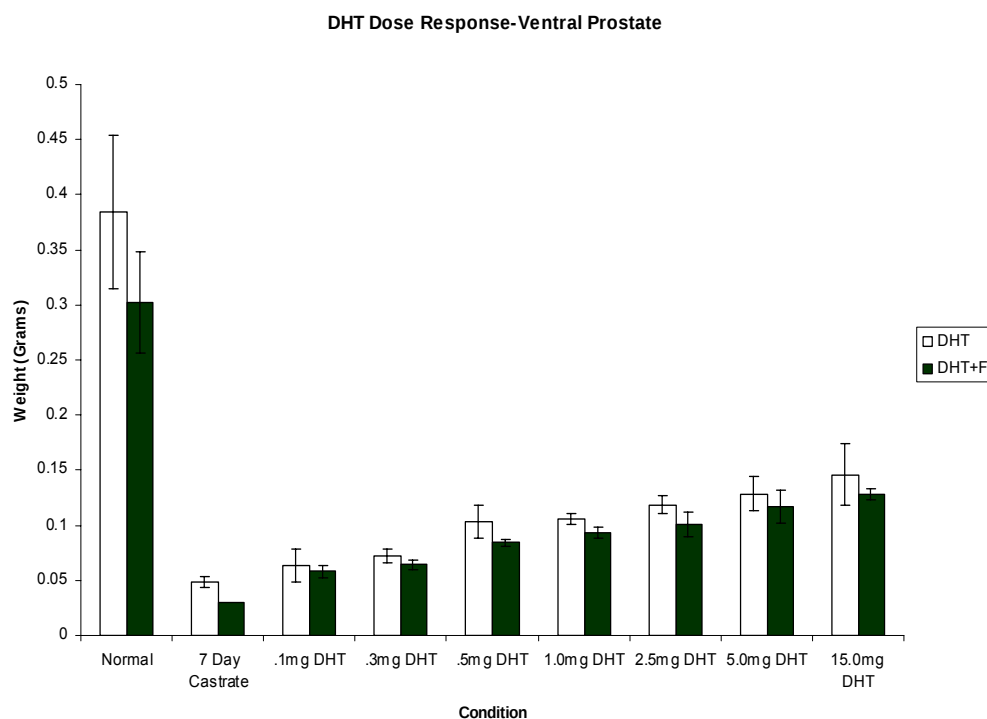


Fig. 3. Dose-response of DHT-stimulated prostate regrowth in the absence or presence of finasteride. The rats were implanted with indicated amount of DHT in slow releasing pellets.

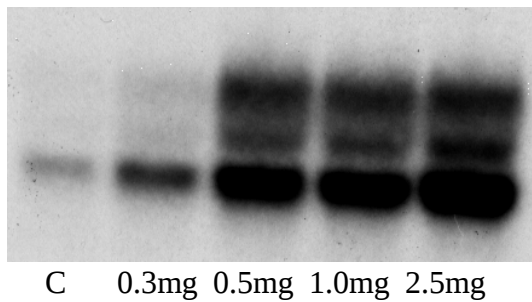


Fig. 4. Dose-response of spermidine synthase expression in the rat ventral prostate. Northern blot analysis was carried out. The total RNA was isolated from the ventral prostate in castrated rats treated with indicated amount of T in slow releasing pellet.

Task 2: Test the effect of finasteride on intermittent androgen ablation therapy of xenograft androgen-sensitive prostate tumors in nude mice (Months 1-36).

- Establish LNCaP androgen-sensitive tumor models in nude mice.
- Determine the impact of finasteride on the time required to establish androgen-independent PSA expression in LNCaP tumor model undergoing intermittent androgen suppression.
- Determine whether finasteride administration during the “off-cycle” of intermittent androgen ablation therapy will prolong the survival of nude mice with subcutaneous LNCaP xenograft tumors.

Studies on slow-releasing of finasteride and physiological doses of testosterone in nude mice.

Daily subcutaneous injection of testosterone and finasteride in nude mice over a period of several months is not practical. Repeated subcutaneous injection of large volumes of sesame oil and propylene glycol, vehicles for finasteride and T, respectively, are likely to be detrimental to the host animals. Thus, it is necessary for us to work out a reliable way to deliver testosterone and finasteride over a long period of time, without of daily injection.

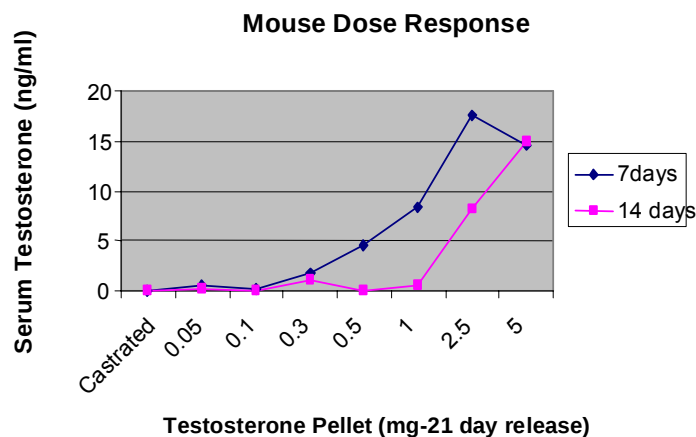


Fig. 5. Serum T level in castrated nude mice implanted with indicated mg of T in slow-releasing pellets 7 and 14 days after implantation.

We first tried to determine what doses of 21-day testosterone pellet is adequate for achieving physiological T levels. In our pilot experiment, we have tested 7 doses of 21-day testosterone pellets: 0.05mg, 0.1mg, 0.3mg, 0.5mg, 1.0mg, 2.5mg, and 5.0mg. A total of 14 male nude mice were used in our experiment with 2 mice in each group. The mice were castrated for 7 days and then injected subcutaneously with slow-releasing T pellets by trocar. One of the two mice in each group was sacrificed 7 days after T pellet injection and the other sacrificed 14 days after the T replacement. Serum T levels were determined for each animals. The results in Fig. 5 showed that serum T was detectable 7 days after injection of ≥ 0.3 mg T. However, after 14 days of T replacement, the serum T was only detectable when 2.5 mg and 5 mg T were injected. This observation suggested that the low T dosage 21-day release pellets lasted less than 14 days. This pilot experiment indicated that we could not rely on these commercially available slow releasing pellets.

As an alternative, we performed pilot experiments using silastic tubing to 1) achieve physiologically relevant levels of testosterone in nude mice; and 2) determine the dosage of finasteride implants to appropriately decrease serum or intraprostatic DHT.

In our first pilot experiment, silastic tubing (inside diameter 1.98 mm, outside diameter 3.18 mm, wall thickness 0.61 mm) were placed subcutaneously with either testosterone or finasteride using the following design:

Group 1 (3 mice) noncastrate controls.

Group 2 (3 castrate mice) with 1 mm testosterone implants.

Group 3 (3 castrate mice) with 1 mm testosterone implants and 1 cm finasteride implants.

Group 4 (3 castrate mice) with 2mm testosterone implants.

Group 5 (3 castrate mice) with 2 mm testosterone implants and 1 cm finasteride implants.

Group 6 (2 castrate mice- 1 died during induction of anesthesia) with 1 mm testosterone implants and two 1 cm finasteride implants.

Group 7 (3 castrate mice) with 2 mm testosterone implants and two 1 cm finasteride implants.

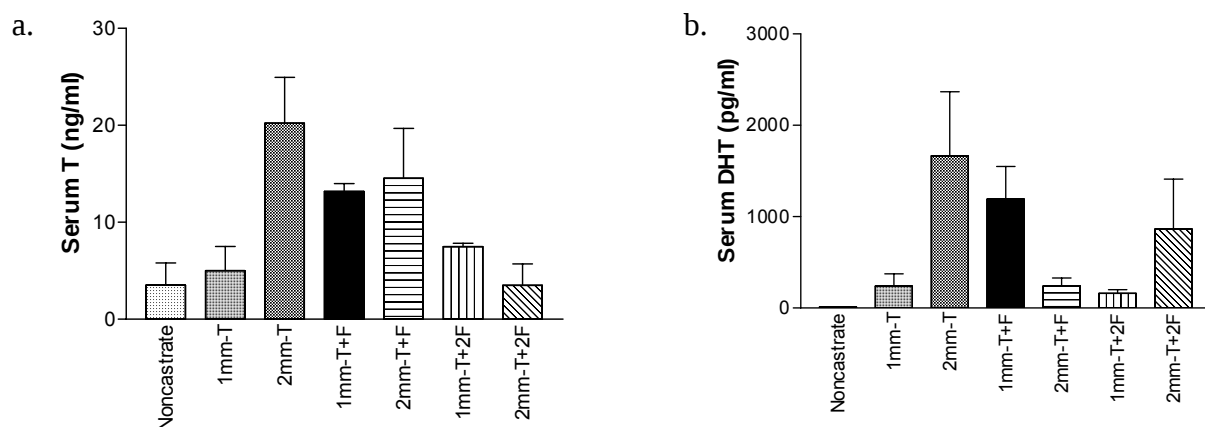


Fig. 6. a. Serum T levels in noncastrate nude mice and castrated nude mice implanted with indicated amount of testosterone (T) and finasteride (F). b. Serum DHT levels in noncastrate nude mice and castrated nude mice implanted with indicated amount of testosterone (T) and finasteride (F).

Mice were sacrificed 2 weeks after the implantation and serum was drawn by cardiac puncture. Serum testosterone levels and DHT levels were measured by radioimmunoassay. In this set of experiment, we did not attempt to measure intraprostatic T and DHT levels in individual animal because we thought that the mouse prostate is too small to provide enough material for measurement.

From the serum T and DHT measurement (Fig. 6a), it seems that the 1 mm T delivery was not consistent while 2 mm T caused supraphysiological serum T level. It is difficult to determine whether finasteride inhibited T to DHT conversion in this experiment (Fig. 6b), which may be due to the presence of finasteride-insensitive type I 5 α -reductase. A major weakness of this experiment was that we did not measure intraprostatic T and DHT levels.

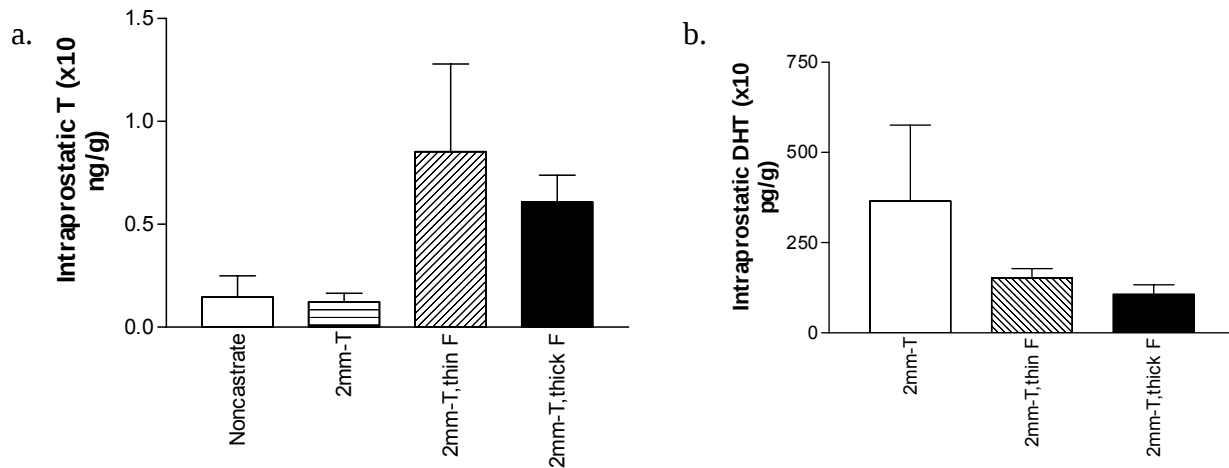


Fig. 7. a. Intraprostatic T levels in noncastrate nude mice and castrated nude mice implanted with indicated amount of testosterone (T) and finasteride (F). b. Intraprostatic DHT levels in castrated nude mice implanted with indicated amount of testosterone (T) and finasteride (F).

From the results of the above experiment, we conclude that we need to deliver less T over a longer period of time and to deliver more finasteride to achieve effective inhibition of T to DHT conversion. To accomplish the above objectives, we have used different types of silastic tubings. Thicker-walled silicone tubing was used (inside diameter 3.18 mm, outside diameter 6.35 mm, wall 1.59 mm) to deliver testosterone. Two different types of tubing were used to deliver finasteride. The first (“thin pellets”) had a very thin outer wall (1.47 mm inside diameter, 1.96 mm outside diameter, 0.23 cm wall). The second (“thick pellets”) had a greater inside diameter in order to increase the surface area of finasteride exposed to the mouse (3.35 mm inside diameter, 4.65 outside diameter, 0.66 mm wall).

Group 1 were three noncastrate controls, and the remaining groups each had four castrate mice.

Group 2 were implanted with 2 mm testosterone implants.

Group 3 had 2 mm testosterone implants along with 1 cm finasteride “thin tubing.”

Group 4 had 2 mm testosterone implants along with 1 cm finasteride “thick tubing.”

Mice were sacrificed 2 weeks after implantation, at which time serum was drawn by cardiac puncture and the ventral prostate was dissected and frozen in liquid nitrogen. Both were stored at –80 degrees. Testosterone levels were measured by radioimmunoassay. DHT levels were initially measured by a direct RIA kit, which proved to be not accurate. We re-measured intraprostatic DHT levels from the samples that we had left. We were able to measure intraprostatic T and DHT in most of the animals using a modified protocol.

To determine whether finasteride is effective in mouse prostate, we needed to first work out a method to measure mouse prostatic T and DHT. We have modified the method we worked out previous for measuring rat prostatic T and DHT (Dadras et al., 2001). We were able to measure intraprostate T and DHT in the mouse prostate individually (Fig. 7). Our studies indicated that finasteride significantly inhibited the conversion of T to DHT in the prostate in the second pilot experiment. As expected, the presence of finasteride enhanced the intraprostatic T level and reduced the intraprostatic DHT levels. We have also observed a significant reduction in the size of seminal vesicles in mice implanted with finasteride relative to the controls, further indicating the effectiveness of finasteride tubing in nude mice. Because the release from the “thick” tubing was slow, there was a lot of T and finasteride left in these tubings after two weeks implantation.

The above studies provided feasibility for conducting IAA experiments in nude mice.

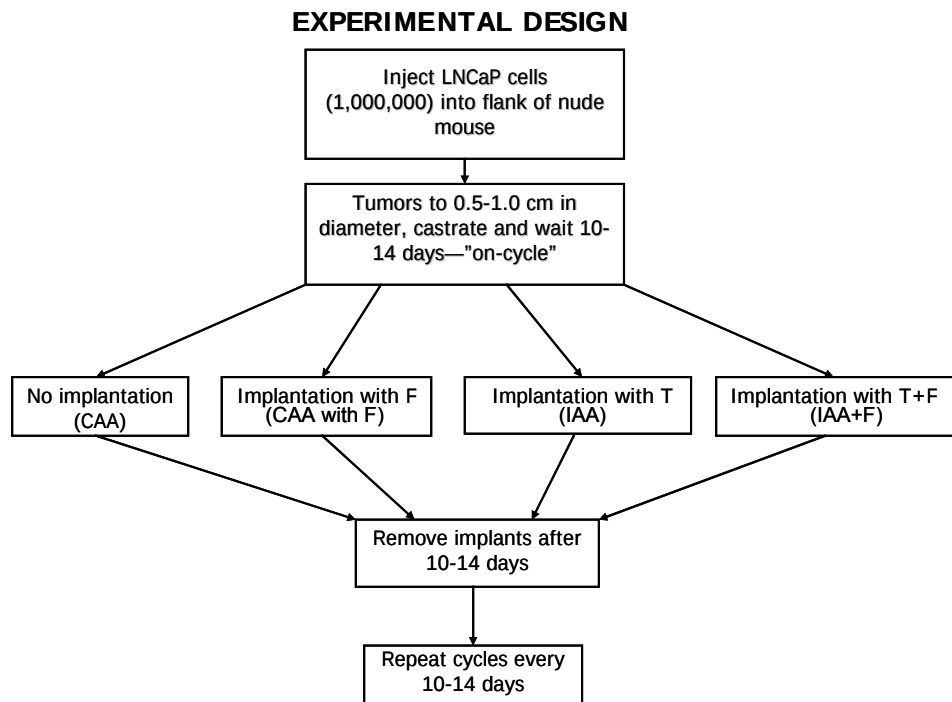


Figure 8. Treatment strategy of LNCaP xenograft tumor in nude mice. After the tumor establishment, animals are castrated for 10-14 days, which is considered as the “on-cycle” of androgen ablation during IAA. The animals are then implanted with no pellet, testosterone (T) pellet, finasteride (F) pellet, or both pellets for 14 day or longer. Testosterone implantation mimics intermittent recovery of testicular function during the “off-cycle” of IAA. Controls are continuous androgen ablation (CAA) in the absence or presence of finasteride (F).

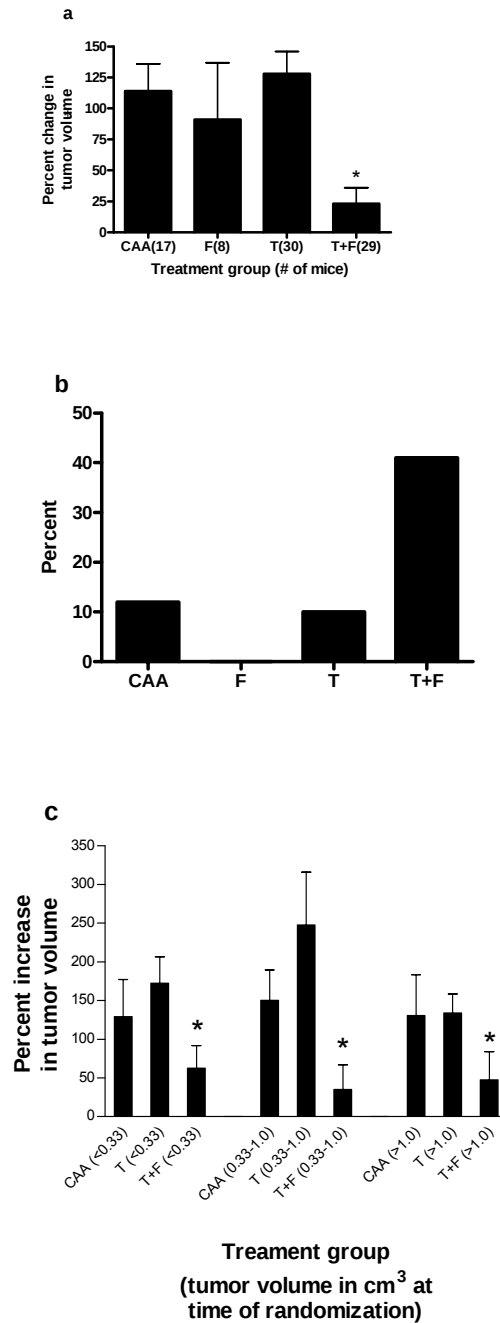


Figure 9: a) Mean percent change in LNCaP tumor volume ($\pm$ SEM) during the first ‘off-cycle’ of intermittent androgen ablation ($p=0.0002$), b) percentage of mice with no increase in tumor volume at the end of one cycle of intermittent androgen ablation, c) percent change in tumor volume ($\pm$ SEM) stratified by size of tumor at time of treatment randomization (CAA vs T vs T+F). p -values for <0.33 cm³, 0.33 - 1.0 cm³, and >1.0 cm³ are 0.038 , 0.0022 , and 0.027 , respectively. * indicates statistically significant

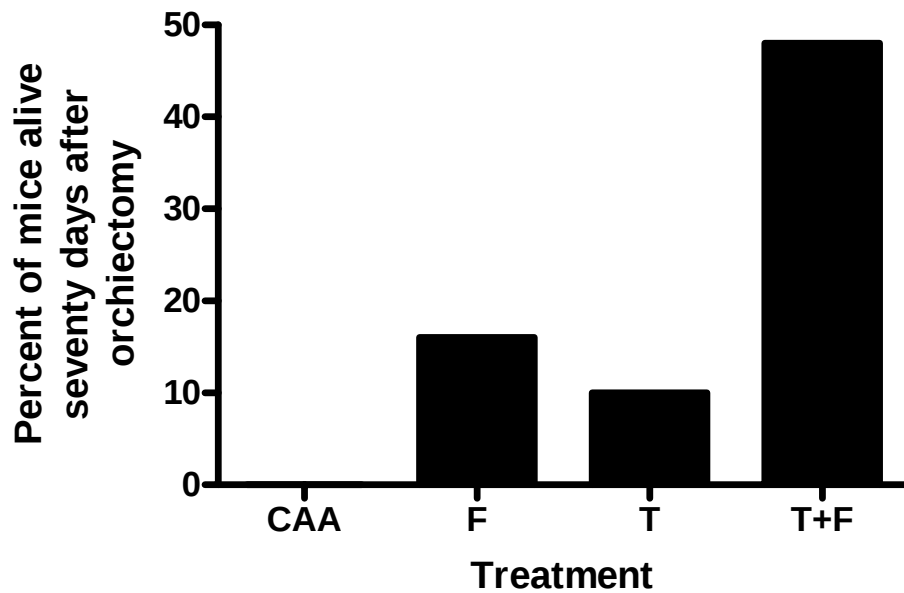
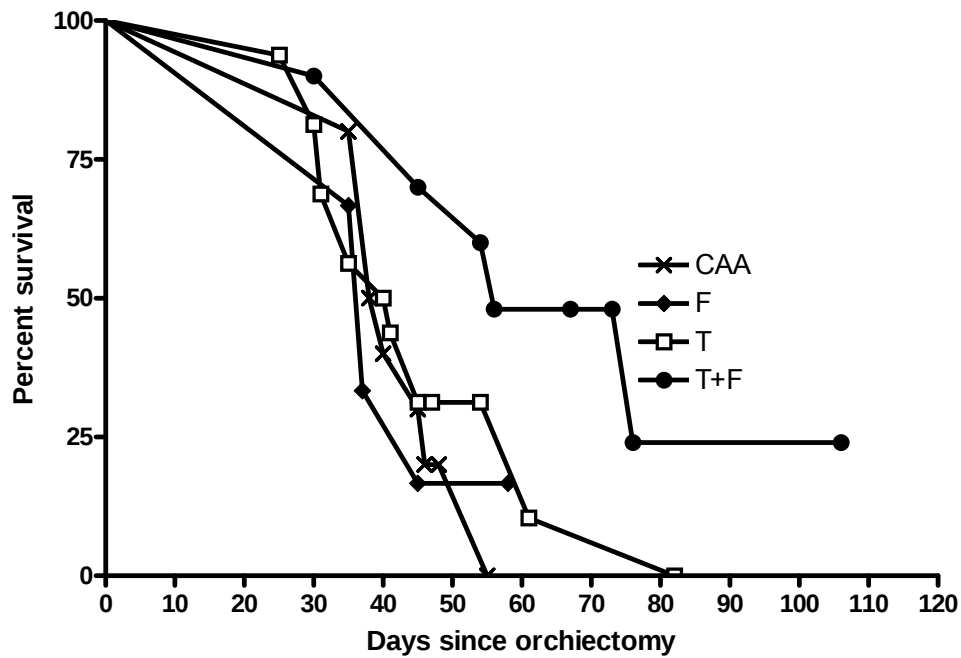


Figure 10: a) Kaplan-Meier survival curve of intermittent androgen ablation with ‘off-cycle’ treatments (CAA-continuous androgen ablation, F-finasteride, T-testosterone, and T+F-testosterone plus finasteride). Euthanasia was performed if tumor diameter > 2.0 cm, tumor ulceration, or tumor-related morbidity. Log-rank test for trend, $p = 0.048$, b) percent survival seventy days following orchiectomy.

One cycle of intermittent androgen ablation. At the end of one cycle of intermittent androgen ablation (orchiectomy followed by pellet administration, Figure 8), the mean percent change in tumor volume ($\pm$ SEM) during the ‘off-cycle’ was similar in the CAA, F, and T groups ($114\pm22\%$, $91\pm46\%$, and $128\pm18\%$, respectively (Figure 9a). Mice treated with T+F during the ‘off-cycle’ experienced less tumor growth ($23\pm13\%$, $p=0.002$). Serum PSA did not significantly differ between the four groups (data not shown).

We then stratified the results by the percentage of mice that experienced no change or a decrease in tumor growth at the end of the initial cycle of therapy (Figure 9b). No significant differences were seen between the CAA, F, and T groups (12%, 0%, and 10%, respectively). An increased percentage of mice treated with T+F during the ‘off-cycle’ experienced no change or a decrease in tumor volume (41%).

Outcomes based on initial tumor volume and treatment. To evaluate if the differences seen between treatment groups were related to initial tumor size, we divided the mice into three groups based on volume of tumor at the time of treatment randomization ($<0.33\text{ cm}^3$, $0.33\text{-}1.0\text{ cm}^3$, and $>1.0\text{ cm}^3$). In all three groups, the mice treated with T+F experienced significantly less tumor growth during the first cycle compared to mice treated by CAA or T alone ($<0.33\text{ cm}^3$: 62% vs 129% vs 172%, $p=0.038$; $0.33\text{-}1.0\text{ cm}^3$: 35% vs 150% vs 247%, $p=0.0022$; and $>1.0\text{ cm}^3$: 47% vs 130% vs 134%, $p=0.027$; Figure 9c). The limited number of mice in the F group prevented their inclusion in the analysis.

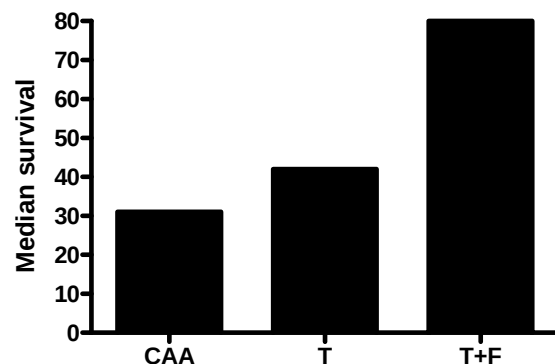
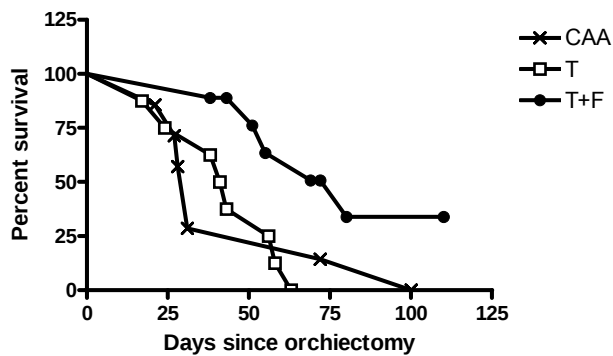


Figure 11: a) Kaplan-Meier survival curve of one-time continuous pellet administration following 14 days of castration (CAA-continuous androgen ablation, T-testosterone, and T+F-testosterone plus finasteride), $p=0.034$, b) median survival.

Survival analysis of intermittent androgen ablation. To evaluate if changes in tumor volume correlated with a survival benefit, we next performed a survival analysis. Mice randomized to the T+F treatment group had the best survival, defined as death or time to euthanasia, seen at all time points (Figure 10a, log rank p-value=0.048). Both median survival and survival seventy days following treatment also favored the T+F group (Figure 10b). Compared to the other treatment groups, T+F mice were 3-5 times more likely to be alive seventy days following treatment.

Survival analysis of one-time pellet implantation. Since the duration of human ‘off-cycle’ intermittent androgen ablation can vary widely, we next evaluated survival following one-time continuous pellet implantation, in essence an extended ‘off-cycle’. Mice treated with T+F had the best survival ($p=0.034$, Figure 11a) and longest median survival (T+F: 80 days, T: 42 days, CAA: 31 days, Figure 11b).

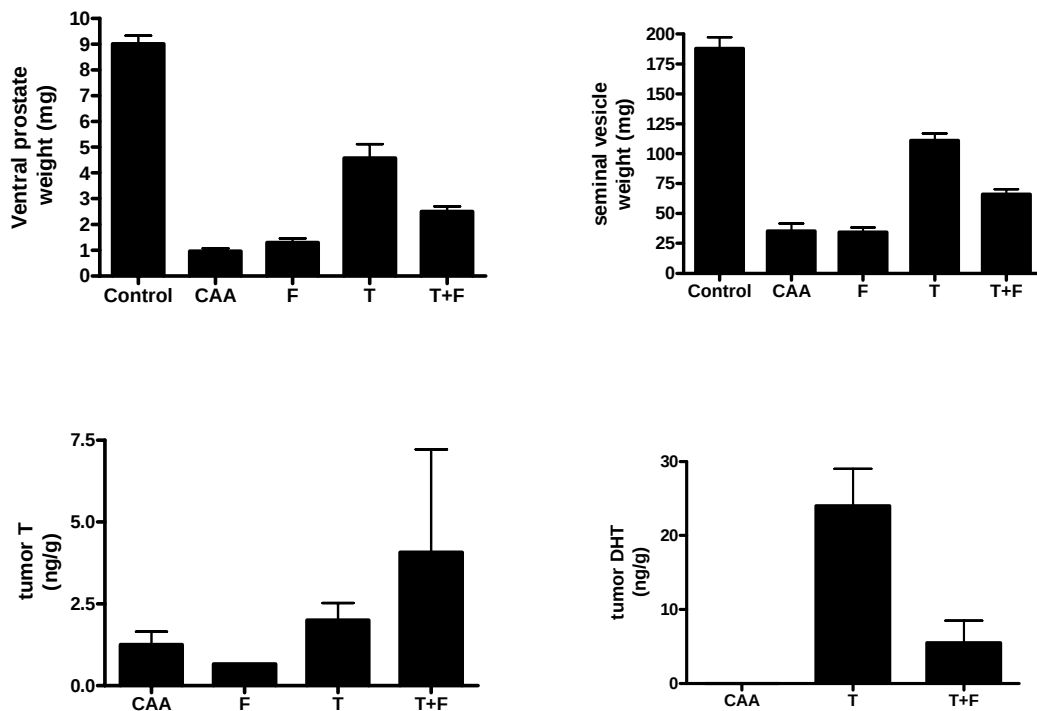


Figure 12: a) ventral prostate weights ($\pm$ SEM) following fourteen days of pellet implantation; T versus T+F, $p=0.005$, b) seminal vesicle weights ($\pm$ SEM) following fourteen days of pellet implantation; T versus T+F, $p<0.001$, c) tissue (ng/g) concentrations of testosterone ($\pm$ SEM), $p=0.37$; d) tumor (ng/g) concentrations ($\pm$ SEM) of dihydrotestosterone, $p=0.008$.

Hormonal evaluation. The adequacy of the testosterone and finasteride pellets was tested by measuring ventral prostate and seminal vesicle weights for each treatment group (Figure 12). Testosterone-treated mice experienced the largest mean ventral prostatic and seminal vesicle growth (Figures 5a-b, p-values for both <0.0001) but only returned to 50% of non-castrated

mouse prostate weight. Compared to testosterone-treated mice, the implantation of finasteride in addition to testosterone resulted in a 46% and 41% decrease, respectively, in mean ventral prostate ($p=0.005$) and seminal vesicle weights (Figures 5a-b, $p=0.005$ and $p<0.001$, respectively). While mean tumor T concentrations did not statistically differ between the T-treated and T+F-treated mice (Figure 12c), mean tumor DHT concentrations were lower in the T+F-treated mice with a trend towards significance (Figure 12d, $p\text{-value}=0.064$). Since testosterone can be converted to estradiol, we next evaluated if elevated serum or tumor estradiol levels may account for the tumor growth inhibition seen in the T+F-treated mice. There were no differences in the serum estradiol levels between the treatment groups, with all levels below 75 pg/ml (data not shown). Estradiol levels were undetectable in the tumor tissue.

No significant difference in serum PSA levels was detected among the four groups of nude mice. Serum was obtained by retro-orbital venipuncture. Serum PSA levels were determined by a commercial kit (IMx PSA, Abbott Laboratories, Abbott Park, IL). Serum PSA did not significantly differ between the four groups (data not shown). This finding indicates that LNCaP xenograft tumors in our experiment are already on the way to become independent of androgens, which is consistent with their growth in castrated hosts (Figure 9). This finding also means that it will virtually be impossible for us to use androgen-independent PSA expression as an endpoint in our proposed studies (Task 2c).

Task 3: Determine the effect of finasteride on the expression of androgen-response genes in LNCaP tumors during intermittent androgen ablation therapy (Month 24-36).

- Collect LNCaP tumor specimens and serum samples from nude mice.
- Determine the expression of androgen-response genes, adrenomedullin, calreticulin and PSA, in LNCaP tumors.
- Analysis of the collected data and prepare the final report for the proposal.

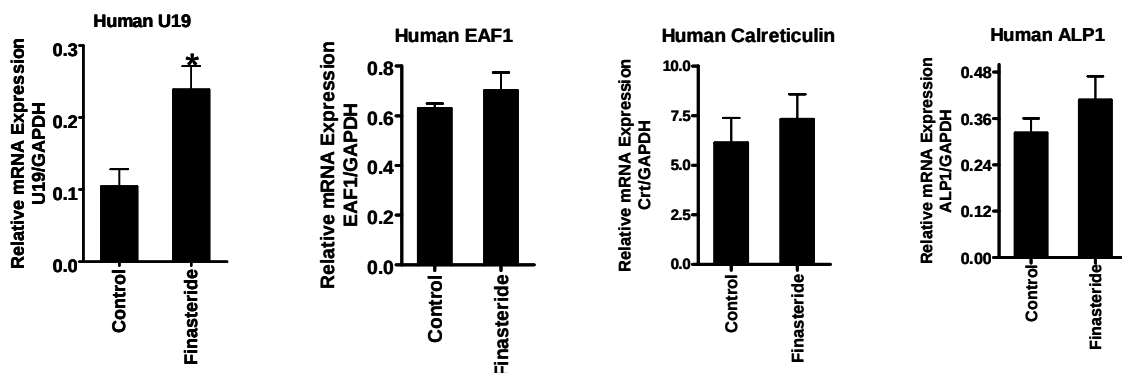


Fig. 13. Effect of finasteride on the expression of indicated genes during the off-cycle of intermittent androgen ablation therapy in the LNCaP xenograft tumor model. Nude mice were castrated when LNCaP tumors reached 0.5 cm in diameter. Two weeks after castration, the mice

bearing androgen-sensitive tumors were implanted with T pellet in the absence or presence of finasteride pellets. Four or five mice were used in each group. Xenograft tumors were isolated 3 days after the implantation, and total RNA was extracted from each tumor individually. The expression of the indicated gene in each tumor sample was determined individually using real-time RT-PCR. GADPH gene was used as a control in the real-time RT-PCR. The PCR primers were designed such that they only recognized human genes. * indicates $p < 0.05$.

Finasteride enhanced the expression of androgen-response gene U19 in LNCaP xenograft tumors during the off-cycle in IAAT (Fig. 13). The enhancement was 2.4 fold and statistically significant ($p < 0.05$). Our studies showed that U19 is pro-apoptotic and growth inhibitory in prostate cancer cells in culture and in xenograft tumors (26). Finasteride did not significantly enhance the expression of two other androgen-response genes, calreticulin and ALP1, possibly due to the limited sample size (Fig. 13). We used EAF1 as a negative control because EAF1 is the U19 homolog (26, 27) and insensitive to androgens (data not shown). As expected, EAF1 expression was not affected by finasteride (Fig. 13). This experiment supports our hypothesis that blocking T to DHT conversion by a 5α -reductase inhibitor during the off-cycle (when T is recovering) supra-induces growth inhibitory androgen-response genes, especially U19.

Key Research Accomplishments:

Our proposed research requires the ability to deliver appropriate doses of finasteride and T over a prolonged period in nude mice. In the 1st year of the funding period, we have encountered difficulties because the commercially available slow releasing pellets did not work in our system. After multiple tests, we resolved these critical technical problems, which allowed us, in the 2nd and 3rd year of the funding period, to demonstrate that finasteride administration significantly enhances the efficacy of intermittent androgen ablation therapy in LNCaP xenograft tumor model. We also showed that blocking T to DHT conversion supra-induces growth-inhibitory androgen-response genes in androgen-sensitive LNCaP prostate tumor xenografts.

1. Delivery of exogenous T at physiologic levels over a prolonged period in nude mice.

A prerequisite of the proposed project is to deliver physiologic doses of T over a long period in nude mice. In our experiment, we were unable to deliver T over prolonged period using slow releasing T pellets prepared by Innovative Research of America, FL. Fortunately, we were able to overcome this technical challenge and work out a condition using the “Thick” silastic tubing to deliver T in nude mice. This knowledge could potentially benefit other investigators.

2. Delivery of finasteride over a prolonged period in nude mice.

Daily subcutaneous injection of finasteride dissolved in large volume of sesame oil over a long period of time would be painful to the host and may cause adverse effects. Thus, it is necessary for us to avoid daily subcutaneous injection. We have tested three different types of silastic tubing and find one of them would allow us to deliver finasteride over a long period of time. This method permits us to explore the effect of finasteride in intermittent androgen ablation therapy of prostate cancer in xenograft tumor models.

3. Measurement of intraprostatic T and DHT in individual nude mouse.

To demonstrate that finasteride inhibits T to DHT conversion in mouse prostate, it is necessary for us to measure the T and DHT in the mouse prostate, which is very small. Using a modified radioimmunoassay, we were able to measure T and DHT in individual mouse prostate, which allowed us to show the finasteride inhibition of T to DHT conversion in the mouse prostate.

4. We demonstrated that finasteride given during the ‘off-cycle’ of intermittent androgen ablation (IAA) significantly limits tumor growth in the LNCaP xenograft model. The use of IAA plus finasteride resulted in decreased tumor growth compared to standard continuous androgen ablation. We are not aware of any previous report showing that parental LNCaP tumor size is reduced by hormonal manipulation other than castration.

5. Finasteride maintenance during the “off-cycle” of intermittent androgen ablation prolonged the survival of nude mice bearing the LNCaP xenograft tumors. Our finding provide a strong basis for the further studies on the potential survival benefits of finasteride “off-cycle” maintenance for prostate cancer patients undergoing IAA treatment.

6. Finasteride enhanced the expression of growth inhibitory androgen-response gene U19 in LNCaP xenograft tumors during the off-cycle of intermittent androgen ablation therapy.

This finding supports our hypothesis that blocking T to DHT conversion by a 5 α -reductase inhibitor during the off-cycle (when T is recovering) supra-induces growth inhibitory androgen-response genes, especially U19. This also provides feasibility data for us to test if growth inhibitory androgen-response genes are supra-induced in prostate cancer cells in the patients in the off-cycle of intermittent androgen ablation therapy in the presence of finasteride.

Reportable Outcomes:

1. We have a manuscript in press in the PROSTATE (See attached document).

Conclusions:

Our studies with androgen-sensitive LNCaP human prostate tumor xenografts in nude mice showed significant tumor growth retardation by finasteride plus intermittent androgen ablation (IAA) in the first cycle. Our finding showed that finasteride plus IAA prolongs the life of nude mice bearing LNCaP tumors, suggesting that finasteride administration should enhance the efficacy of IAA on patients with prostate cancer. We also generated evidence supporting our hypothesis that blocking T to DHT conversion by a 5 α -reductase inhibitor during the off-cycle (when T is recovering) supra-induces growth inhibitory androgen-response genes.

References:

1. George, F. and Wilson, J. Sex determination and differentiation, p. 3-28. New York: Raven Press, 1994.
2. Bruchovsky, N. and Wilson, J. The intranuclear binding of testosterone and 5-alpha-androstan-17-beta-ol-3-one by rat prostate. Journal of Biological Chemistry, 243: 5953-5960, 1968.

3. Bruchovsky, N. and Wilson, J. The conversion of testosterone to 5-alpha-androstan-17-beta-ol-3-one by rat prostate in vivo and in vitro. *Journal of Biological Chemistry*, 243: 2012-2021, 1968.
4. Deslypere, J., Young, M., Wilson, J., and McPhaul, M. Testosterone and 5 alpha-dihydrotestosterone interact differently with the androgen receptor to enhance transcription of the MMTV-CAT reporter gene. *Molecular & Cellular Endocrinology*, 88: 15-22, 1992.
5. Grino, P., Griffin, J., and Wilson, J. Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone. *Endocrinology*, 126: 1165-1172, 1990.
6. Liao, S., Liang, T., Fang, S., Castaneda, E., and Shao, T. Steroid structure and androgenic activity. Specificities involved in the receptor binding and nuclear retention of various androgens. *Journal of Biological Chemistry*, 248: 6154-6162, 1973.
7. Wilson, E. and French, F. Binding properties of androgen receptors. Evidence for identical receptors in rat testis, epididymis, and prostate. *Journal of Biological Chemistry*, 251: 5620-5629, 1976.
8. Griffin, J., McPhaul, M., Russell, D., and Wilson, J. The androgen resistance syndromes: steroid 5 α -reductase 2 deficiency, testicular feminization, and related disorders., p. 2967-2998. New York: McGraw-Hill, 1995.
9. Walsh, P., Madden, J., Harrod, M., Goldstein, J., MacDonald, P., and Wilson, J. Familial incomplete male pseudohermaphroditism, type 2. Decreased dihydrotestosterone formation in pseudovaginal perineoscrotal hypospadias. *New England Journal of Medicine*, 291: 944-949, 1974.
10. George, F. Androgen metabolism in the prostate of the finasteride-treated, adult rat: a possible explanation for the differential action of testosterone and 5 alpha-dihydrotestosterone during development of the male urogenital tract. *Endocrinology*, 138: 871-877, 1997.
11. Dadras, S. S., Cai, X., Abasolo, I., and Wang, Z. Inhibition of 5alpha-reductase in rat prostate reveals differential regulation of androgen-response gene expression by testosterone and dihydrotestosterone. *Gene Expression*, 9:183-194, 2001.
12. Bruchovsky, N., Lesser, B., Doorn, E. V., and Craven, S. Hormonal effects on cell proliferation in rat prostate. *Vitam Horm*, 33: 61-102, 1975.
13. Colombel, M., Olsson, C., Ng, P., and Buttyan, R. Hormone-regulated apoptosis results from reentry of differentiated prostate cells onto a defective cell cycle. *Cancer Research*, 52: 4313-4319, 1992.
14. English, H., Kyprianou, N., and Isaacs, J. Relationship between DNA fragmentation and apoptosis in the programmed cell death in the rat prostate following castration. *Prostate*, 15: 233-250, 1989.
15. Coffey, D., Shimazaki, J., and Williams-Ashman, H. Polymerization of deoxyribonucleotides in relation to androgen-induced prostatic growth. *Arch Biochem Biophysics*, 124: 184-198, 1968.
16. Bosland, M. Animal models for the study of prostate carcinogenesis. [Review]. *Journal of Cellular Biochemistry - Supplement*, 16H: 89-98, 1992.
17. Carter, H. and Coffey, D. The prostate: an increasing medical problem. *Prostate*, 16: 39-48, 1990.

18. Kozlowski, J. and Grayhack, J. Carcinoma of the prostate, 2nd edition, p. 1277-1393. Chicago: Mosby Year Book, 1991.
19. Issacs, J. T., Wake, N., Coffey, D. S., and Sandberg, A. A. Genetic instability coupled to clonal selection as a mechanism for progression in prostate cancer. *Cancer Research*, 42: 2353-2361, 1982.
20. Craft, N., Chhor, C., Tran, C., Belldegrin, A., DeKernion, J., Witte, O. N., Sa, R. E., and Sawyers, C. L. Evidence for clonal outgrowth of androgen-independent prostate cancer cells from androgen-dependent tumors through a two-step process. *Cancer Research* 5030-5036, 1999.
21. Kokontis, J. M., Hay, N., and Liao, S. Progression of LNCaP prostate tumor cells during androgen deprivation: hormone-independent growth, repression of proliferation by androgen, and role for p27Kip1 in androgen-induced cell cycle arrest. *Mol Endocrinol.*, 12: 941-953, 1998.
22. Umekita, Y., Hiipakka, R. A., Kokontis, J. M., and S., L. Human prostate tumor growth in athymic mice: inhibition by androgens and stimulation by finasteride. *Proc Natl Acad Sci U S A*, 93: 11802-11807, 1996.
23. Bruchovsky, N., Rennie, P. S., Coldman, A. J., Goldenberg, S. L., To, M., and Lawson, D. Effects of androgen withdrawal on the stem cell composition of the Shionogi carcinoma. *Cancer Research*, 50: 2275-2282, 1990.
24. Akakura, K., Bruchovsky, N., Goldenberg, S. L., Rennie, P. S., Buckley, A. R., and Sullivan, L. D. Effects of intermittent androgen suppression on androgen-dependent tumors. Apoptosis and serum prostate-specific antigen. *Cancer*, 71: 2782-2790, 1993.
25. Sato, N., Gleave, M. E., Bruchovsky, N., Rennie, P. S., Goldenberg, S. L., Lange, P. H., and Sullivan, L. D. Intermittent androgen suppression delays progression to androgen-independent regulation of prostate-specific antigen gene in the LNCaP prostate tumour model. *J Steroid Biochem Mol Biol.*, 58: 139-146, 1996.
26. Xiao, W., Zhang, Q., Jiang, F., Pins, M., Kozlowski, J.M., Wang, Z. Suppression of prostate tumor growth by a novel testosterone regulated apoptosis inducer. *Cancer Research*, 63: 4698-4704, 2003.
27. Simone, F., Luo, R. T., Polak, P. E., Kaberlein, J. J., and Thirman, M. J. ELL-associated factor 2 (EAF2), a functional homolog of EAF1 with alternative ELL binding properties. *Blood*, 101: 2355-2362, 2003.

Appendices:

1. Eggener SE, Stern, JA, Oram S, Cai, X, Roehl K, Wang Z: Enhancement of intermittent androgen ablation by “off-cycle” maintenance with finasteride in LNCaP prostate cancer xenograft model, In press in PROSTATE (Attached).

Enhancement of Intermittent Androgen Ablation by “Off-Cycle” Maintenance With Finasteride in LNCaP Prostate Cancer Xenograft Model

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BACKGROUND. Intermittent androgen ablation (IAA) was developed with the intention of delaying progression of prostate cancer to androgen-independence and improving quality of life. Our previous studies suggest that relative to dihydrotestosterone (DHT), testosterone (T) is a weak inducer of proliferation and a more potent inducer of differentiation. We hypothesize that administration of finasteride (F), a type-II 5- α -reductase inhibitor that increases T and decreases DHT, during the IAA “off-cycle” would enhance the efficacy.

METHODS. After LNCaP tumor establishment, nude mice were castrated and randomized to continuous androgen ablation (CAA), continuous androgen ablation plus finasteride (CAA + F), intermittent androgen ablation (IAA), or intermittent androgen ablation plus finasteride (IAA + F).

RESULTS. After one cycle of therapy, mice treated with IAA + F had significantly less tumor growth than the other treatment groups ($P = 0.002$). Mice treated with IAA + F had the best survival ($P = 0.048$) and were 3–5 times more likely to be alive 70 days following treatment initiation.

CONCLUSIONS. IAA with finasteride provides the most favorable tumor growth kinetics and survival compared to both CAA and standard IAA. *Prostate*

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KEY WORDS: finasteride; prostate cancer; LNCaP; testosterone; intermittent androgen ablation

INTRODUCTION

Prostate cancer remains the second leading cause of non-skin cancer-related death among American men. Since its growth is hormone-dependent, androgen ablation is a common and increasingly utilized treatment [1]. While initially effective at inducing tumor regression in most men, it is palliative and not curative, as all tumors will eventually become refractory to hormonal therapy. In addition to the treatment limitations, reduced androgen levels lead to weight gain, dryness of skin, hot flashes, diminished libido, impotence, cognitive dysfunction, and muscle loss [2]. In an attempt to minimize the duration and severity of these side effects, intermittent androgen ablation (IAA) was developed [3]. Medical castration is followed by a

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period of androgen recovery, during which serum testosterone (T) slowly returns to normal levels. Upon tumor progression (typically a pre-determined PSA level), androgen ablation is reinstituted. These cycles of “on” (androgen ablation) and “off” (androgen recovery) treatment may be repeated until the tumor no longer responds favorably to hormonal manipulation. During intervals of androgen recovery, quality-of-life parameters are more favorable compared to the periods of androgen ablation. In addition to the symptomatic benefits, intermediate-term clinical studies suggest IAA does not compromise cancer control when compared to continuous androgen ablation (CAA) [4]. In a mouse xenograft model, the time to androgen-independence may even be delayed compared to CAA [3,4].

Testosterone is converted to dihydrotestosterone (DHT) by 5- α -reductase. We have previously identified a cohort of genes expressed during the regrowth of castrated rat prostate [5,6]. A subset of these genes exhibit variable expression profiles when exposed to different levels of testosterone or DHT. For example, following castration testosterone is a more potent inducer of many androgen-responsive genes compared to DHT [7]. Some of these genes encode growth-inhibiting proteins (e.g., U19, ALP1, adrenomedullin) [8–10]. A testosterone-rich, DHT-poor environment induces expression of growth-inhibiting proteins only during regrowth of a previously androgen-ablated prostate but is not observed in the intact, untreated prostate. While the Prostate Cancer Prevention Trial (PCPT) showed a 5- α -reductase inhibitor (finasteride, F) in hormonally-intact men can decrease the incidence of prostate cancer by 25% [11], it does not have known beneficial effects in treating clinical prostate cancer.

Therefore, the prostatic regrowth phase of IAA appeared to be an appropriate clinical model to test if a testosterone-rich, DHT-poor environment would result in more favorable tumor growth kinetics. We hypothesized that use of finasteride to maximize testosterone and limit DHT during the androgen recovery stage of IAA would result in slower prostatic regrowth, reduced proliferation, and improved cancer control. The LNCaP tumor cell line was selected as our tumor model because it is an androgen-sensitive, PSA-secreting cell line derived from the lymph node of a man with metastatic prostate cancer. The type II 5- α -reductase is the predominant isoenzyme in human prostate and also present in the human-derived prostate cancer cell line, LNCaP [12].

MATERIALS AND METHODS

Cell Culture

LNCaP cells were obtained from American Type Culture Collection (ATCC) and grown in sterilized

medium containing RPMI 1640, L-glutamine, penicillin/streptomycin, and fetal bovine serum (FBS) under an atmosphere of 5% CO₂. Cells underwent 4–10 passages prior to mouse inoculation. Experiments were performed with two separate batches of LNCaP cells. The tumor volume and survival results detailed below were reproducible in both sets of LNCaP cells studied.

Animal Experiments

All animal experiments were approved by the Northwestern University Animal Care Use Committee. The design of animal experiments is diagrammed in Figure 1. Approximately 1×10^6 LNCaP cells were inoculated subcutaneously with 0.25 ml of Matrigel (Becton Dickinson, Bedford, MA) in the flank region of 6–8 weeks old athymic male mice. Of the injected mice developing tumors (~75%), they typically exhibited visible tumor growth 8–12 weeks following inoculation. Tumors were allowed to grow until they reached 5–10 mm in diameter. All mice were then castrated via a trans-scrotal approach under tribromoethanol anesthesia and considered “on-cycle.” Ten to fourteen days following castration, the mice were assigned to one of four groups and considered “off-cycle”: (1) continuous androgen ablation (CAA; no implants), (2) control group (finasteride [F] pellet implants), (3) IAA; testosterone [T] pellet implants), and (4) IAA with finasteride (IAA + F; testosterone [T] and finasteride [F] implants). Silicone pellets of either testosterone or finasteride were implanted subcutaneously in the flank contralateral to the tumor. The mice were distributed so mean tumor volume at time of randomization was equivalent among the four groups. The pellets were extracted after an implantation period of 10–14 days and this constituted the end of one full cycle. Cycles were repeated until mouse death, tumor overgrowth (>2 cm in diameter), tumor ulceration, or severe tumor-related morbidity required euthanasia. Tumor volume was calculated as $(\text{length} \times \text{width}^2)/2$ [13]. Serum was obtained by retro-orbital venipuncture. Tumor volume and serum PSA were measured at each intervention. Tumor was flash-frozen with liquid nitrogen at the time of euthanasia and stored at -20°C for later determination of hormonal concentrations.

Pellet Construction

Silicone tubing with a 1.58 mm internal diameter and a 3.18 mm outer diameter (Catalog #60-411-47; Helix Medical, Carpinteria, CA) was cut to 8 mm in length. A wood stick of 1.58 mm diameter was inserted 3 mm into one end of the silicone tubing. From the other end of tubing, 2 mm (approximately 7.6 mg) of testosterone (Sigma Chemical, St. Louis, MO) was tightly packed. The remaining open end of silicone tubing was filled

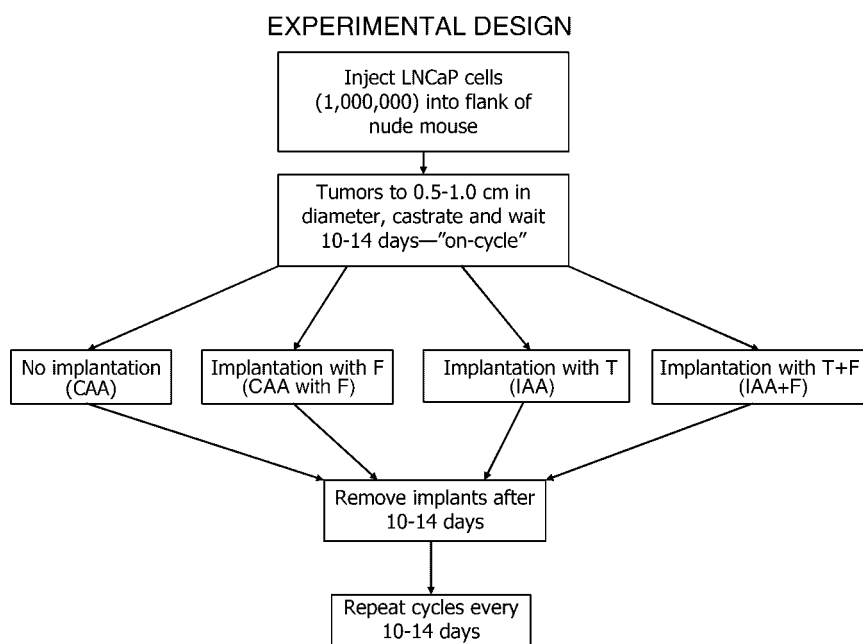


Fig. 1. Treatment strategy of LNCaP xenograft tumor in nude mice. After the tumor establishment, animals are castrated for 10–14 days, which is considered as the “on-cycle” of androgen ablation during intermittent androgen ablation (IAA). The animals are then implanted with no pellet, testosterone (T) pellet, finasteride (F) pellet, or both pellets for 14 days or longer. Testosterone implantation mimics intermittent recovery of testicular function during the “off-cycle” of IAA. Controls are continuous androgen ablation (CAA) in the absence or presence of finasteride.

with another wood stick. The portions of wood extending beyond the silicone tubing were cut with a razor blade. Both ends of the pellet were sealed with silicone adhesive (Product code #00698, DAP®) and allowed to air dry overnight. Finasteride pellets were made in a similar fashion with a few exceptions. Finasteride was a generous gift of Merck (Rahway, NJ). Silicone tubing had a 1.47 mm internal diameter and 1.96 mm outer diameter (Catalog #60-411-45; Helix Medical, Carpentaria, CA). Total tube length was 2 cm long with 12 mm (approximately 15 mg) of finasteride powder flanked by wood sticks filling 4 mm on each side. Following the overnight adhesive drying, both testosterone and finasteride pellets were then sterilized with 70% ethanol for 10 min and stored in a light-free environment. Adequacy of the pellets was evaluated by their effect on the weights of ventral prostate and seminal vesicles following subcutaneous flank implantation into castrated athymic mice for 14 days.

Study Endpoints

Study endpoints were tumor volume, serum PSA, and mouse survival. Euthanasia was performed if the tumor diameter exceeded 2 cm, ulcerated or caused severe tumor-related morbidity. Serum PSA levels were determined by a commercial kit (IMx PSA, Abbott Laboratories, Abbott Park, IL). Radioimmunoassay kits (Diagnostic Systems Laboratories, Inc., Webster,

TX) were used to measure serum and tumor testosterone, DHT, and estradiol (E2).

Statistical Analysis

GraphPad Prism 4.0 was used for all statistical analyses and graphical composition. Tumor volume, prostate and seminal vesicle weight, and hormonal levels were compared using non-parametric one-way ANOVA. Survival analysis was evaluated using Kaplan–Meier curves and log rank tests. A *P*-value <0.05 was considered statistically significant.

RESULTS

One Cycle of IAA

At the end of one cycle of IAA (orchiectomy followed by pellet administration, Fig. 1), the mean percent change in tumor volume ($\pm$ SEM) during the “off-cycle” was similar in the CAA, F, and T groups ($114 \pm 22\%$, $91 \pm 46\%$, and $128 \pm 18\%$, respectively; Fig. 2a). Mice treated with T + F during the “off-cycle” experienced less tumor growth ($23 \pm 13\%$, $P = 0.002$). Serum PSA did not significantly differ between the four groups (data not shown).

We then stratified the results by the percentage of mice that experienced no change or a decrease in tumor growth at the end of the initial cycle of therapy (Fig. 2b). No significant differences were seen between the CAA, F, and T groups (12%, 0%, and 10%, respectively). An

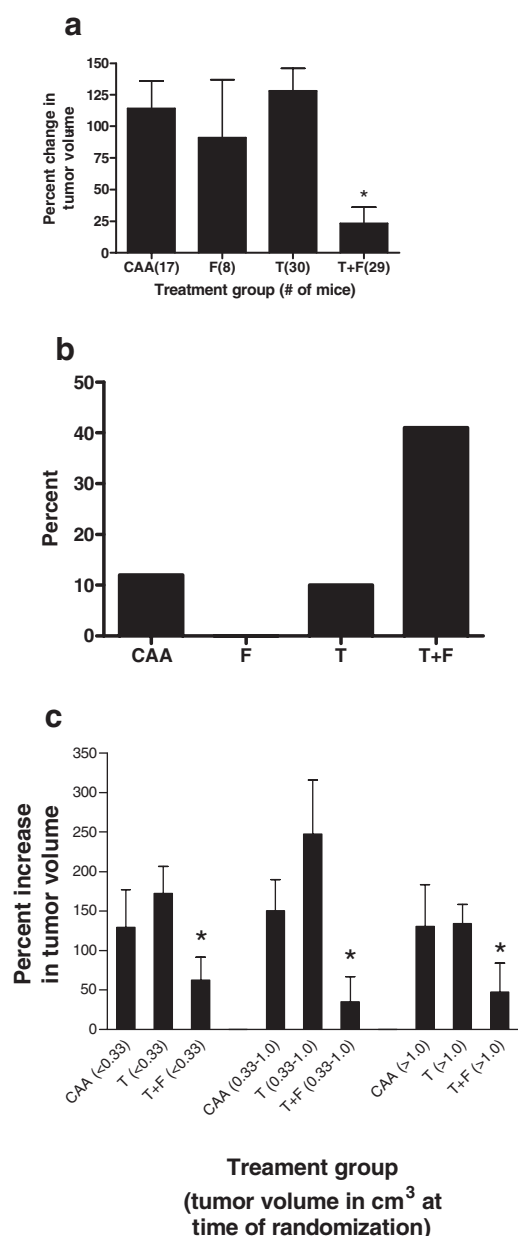


Fig. 2. a: Mean percent change in LNCaP tumor volume ($\pm$ SEM) during the first “off-cycle” of IAA ($P = 0.002$), (b) percentage of mice with no increase in tumor volume at the end of one cycle of IAA, (c) percent change in tumor volume ($\pm$ SEM) stratified by size of tumor at time of treatment randomization (CAA vs. T vs. T + F). P -values for <0.33 cm³, $0.33-1.0$ cm³, and >1.0 cm³ are 0.038, 0.0022, and 0.027, respectively. * indicates statistically significant.

increased percentage of mice treated with T + F during the “off-cycle” experienced no change or a decrease in tumor volume (41%).

Outcomes Based on Initial Tumor Volume and Treatment

To evaluate if the differences seen between treatment groups were related to initial tumor size, we

divided the mice into three groups based on volume of tumor at the time of treatment randomization (<0.33 cm³, $0.33-1.0$ cm³, and >1.0 cm³). In all three groups, the mice treated with T + F experienced significantly less tumor growth during the first cycle compared to mice treated by CAA or T alone (<0.33 cm³: 62% versus 129% versus 172%, $P = 0.038$; $0.33-1.0$ cm³: 35% versus 150% versus 247%, $P = 0.0022$; and >1.0 cm³: 47% versus 130% versus 134%, $P = 0.027$; Fig. 2c). The limited number of mice in the F group prevented their inclusion in the analysis.

Survival Analysis of IAA

To evaluate if changes in tumor volume correlated with a survival benefit, we next performed a survival analysis. Mice randomized to the T + F treatment group had the best survival seen at all time points (Fig. 3a, log rank P -value = 0.048). Both median survival and

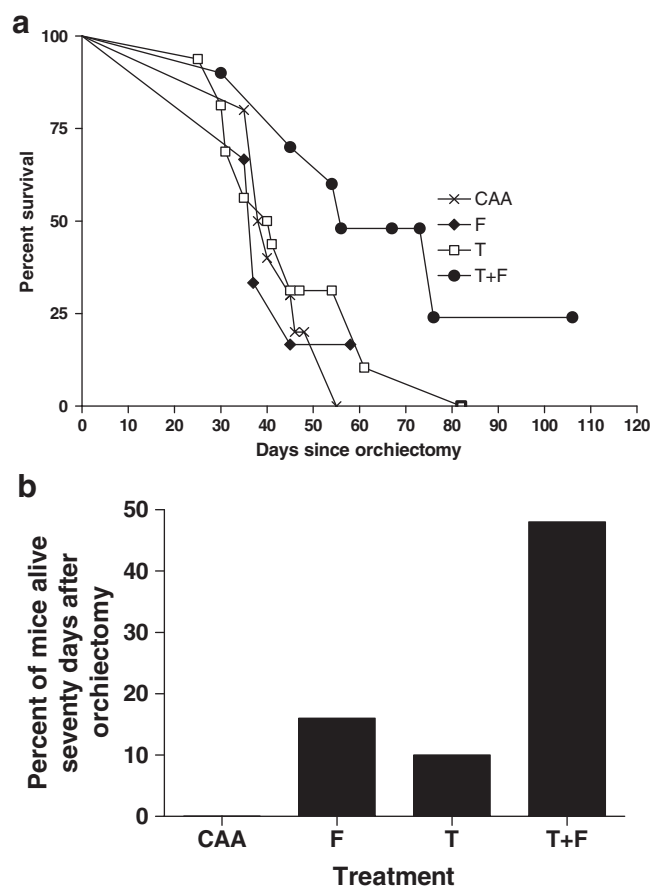


Fig. 3. a: Kaplan–Meier survival curves by treatments (CAA; F, finasteride; T, testosterone; and T + F, testosterone plus finasteride). Euthanasia was performed if tumor diameter >2.0 cm, tumor ulceration, or tumor-related morbidity. Log-rank test for trend, $P = 0.048$, (b) percent survival seventy days following orchiectomy.

survival 70 days following treatment also favored the T + F group (Fig. 3b). Compared to the other treatment groups, T + F mice were 3–5 times more likely to be alive 70 days following treatment.

Survival Analysis of One-Time Pellet Implantation

Since the duration of human “off-cycle” IAA can vary widely, we next evaluated survival following one-time continuous pellet implantation, in essence an extended “off-cycle.” Mice treated with T + F had the best survival ($P=0.034$, Fig. 4a) and longest median survival (T + F: 80 days, T: 42 days, CAA: 31 days, Fig. 4b).

Hormonal Evaluation

The adequacy of the testosterone and finasteride pellets was tested by measuring ventral prostate and seminal vesicle weights for each treatment group (Fig. 5). Testosterone-treated mice experienced the largest mean ventral prostatic and seminal vesicle growth (Fig. 5a,b, P -values for both <0.0001) but only returned to 50% of non-castrated mouse prostate weight. Compared to testosterone-treated mice, the implantation of finasteride in addition to testosterone resulted in a 46%

and 41% decrease, respectively, in mean ventral prostate ($P=0.005$) and seminal vesicle weights (Fig. 5a,b, $P=0.005$ and $P<0.001$, respectively). While mean tumor T concentrations did not statistically differ between the T-treated and T + F-treated mice (Fig. 5c), mean tumor DHT concentrations were lower in the T + F-treated mice with a trend towards significance (Fig. 5d, P -value = 0.064). Since testosterone can be converted to estradiol, we next evaluated if elevated serum or tumor estradiol levels may account for the tumor growth inhibition seen in the T + F-treated mice. There were no differences in the serum estradiol levels between the treatment groups, with all levels below 75 pg/ml (data not shown). Estradiol levels were undetectable in the tumor tissue.

DISCUSSION

The most common treatment for men with metastatic prostate cancer is androgen ablation. With more frequent early use of hormonal ablation, many men will continue this treatment for extended periods of time, often greater than 10 years. The morbidity associated with prolonged hormonal ablation is considerable, including fatigue, osteoporosis, muscle wasting, erectile dysfunction, and decreased libido. To combat these side effects, alternative hormonal strategies have been employed. IAA was introduced to improve quality-of-life without compromising cancer control. While a large, randomized trial of intermittent versus CAA is ongoing, two intermediate-term reports have shown time to androgen-independence with IAA is equivalent or better [4,14].

Since prostate cancer remains a common cause of death, improvements in the treatment of metastatic prostate cancer are desperately needed. Alternative strategies such as combined androgen blockade, anti-androgen withdrawal, and estrogens have been used to extend the period of disease control. With these needs in mind, we set out to study a novel refinement of IAA by adding finasteride during the “off-cycle,” or androgen-recovery stage.

Following castration, prostate function and growth kinetics are dependent on the relative concentrations of testosterone and DHT. During this phase, DHT is a more potent inducer of prostatic epithelial cellular activity and regrowth [15,16]. Using 5- α -reductase inhibitors to limit the amount of DHT reduces the rate of proliferation in the LNCaP human prostate cancer cell line [17]. Additionally, numerous growth-inhibiting androgen-response genes have been identified that are preferentially expressed when prostate regrowth occurs in a testosterone-rich, DHT-poor environment [7]. We hypothesized these hormonal conditions may be exploited to limit the growth kinetics of prostate

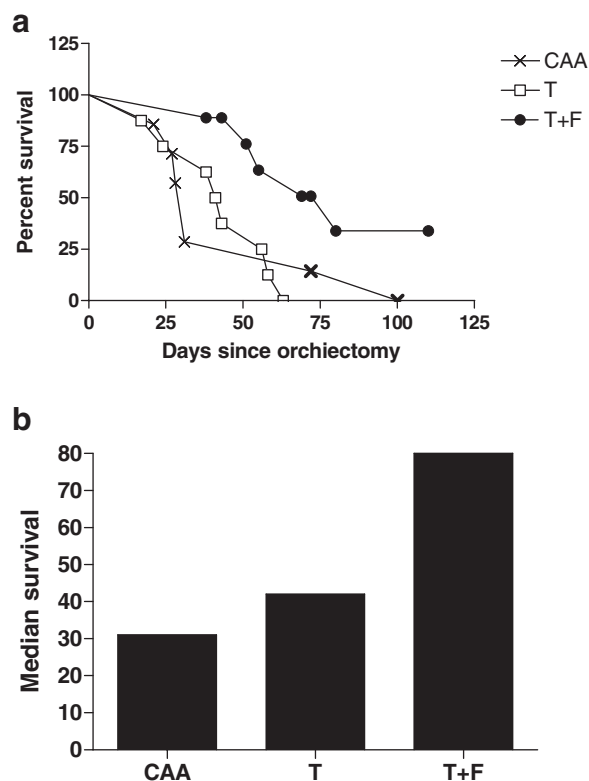


Fig. 4. a: Kaplan–Meier survival curve of one-time continuous pellet administration following 14 days of castration (CAA; T, testosterone; and T + F, testosterone plus F), $P=0.034$, (b) median survival.

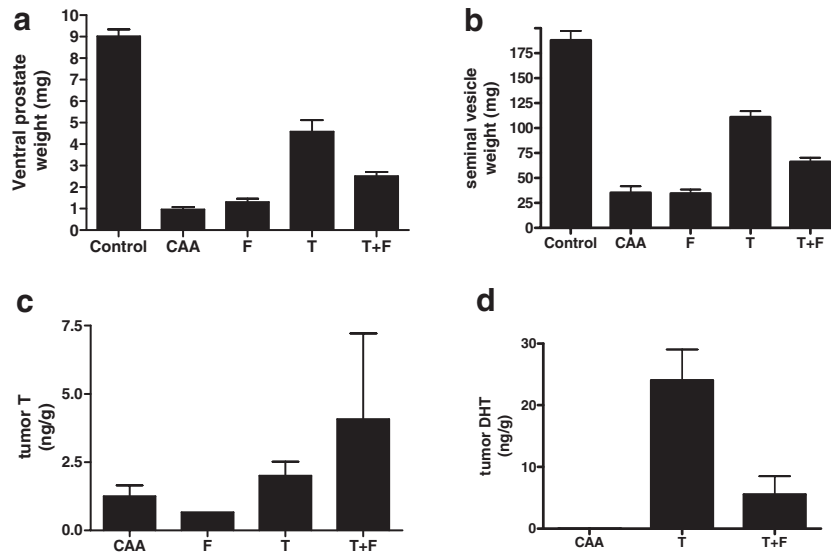


Fig. 5. **a:** Ventral prostate weights ($\pm$ SEM) following 14 days of pellet implantation; T versus T + F, $P = 0.005$, **(b)** seminal vesicle weights ($\pm$ SEM) following 14 days of pellet implantation; T versus T + F, $P < 0.001$, **(c)** tissue (ng/g) concentrations of testosterone ($\pm$ SEM), $P = 0.37$; **(d)** tumor (ng/g) concentrations ($\pm$ SEM) of dihydrotestosterone (DHT), $P = 0.064$.

tumors. While the differential effects of testosterone and DHT on normal prostatic regrowth have been established in the rat model, we sought to extend these studies in a clinically relevant animal model. Since IAA is a common treatment for men with prostate cancer and involves a period of androgen recovery with prostatic regrowth, it seems to be an appropriate and clinically relevant model to test our hypothesis.

Finasteride administered during the “off-cycle” of IAA significantly limited prostate cancer tumor growth when compared to two other common treatment strategies, CAA and standard IAA. Mice treated with IAA plus finasteride experienced 75% less tumor growth than the other treatment modalities. This was evident regardless of initial tumor volume.

The ultimate test of any treatment modality is its impact on survival. Mice treated with testosterone plus finasteride during the “off-cycle” of IAA had a significant survival advantage compared to CAA and standard IAA at all timepoints. At 70 days following treatment, a 3–5-fold survival advantage was evident in the mice treated with IAA plus finasteride. Additionally, one-time “off-cycle” continuous hormonal administration, mimicking a prolonged clinical “off-cycle,” resulted in a similar survival advantage for the IAA plus finasteride-treated mice.

Although finasteride can have an anti-proliferative effect on LNCaP cells in vitro [18] as well as inhibit androgen receptor–DNA complex formation [19], our results cannot be attributed to finasteride alone as the mice treated solely with finasteride still had marked tumor growth and behaved similarly to the CAA and standard IAA groups.

LNCaP cells have a mutated AR, which can be activated by androgens as well as other steroids such as estrogen [20,21]. However, because metastatic androgen-independent human prostate cancers often express similar AR point mutations as those identified in the LNCaP cell line [22], we feel the LNCaP cell line serves as an appropriate animal model. Additionally, since the differential response of our LNCaP tumors to T and DHT mimics that of mouse prostate (wild-type AR) this suggests the mutant AR retained the ability to be androgen-responsive.

The observed tumor volume and survival differences appear to be exclusively due to an altered hormonal environment, as all other measurable variables were consistent between groups. The pellets effectively produced the desired differences in serum and tumor T and DHT. Since estrogen can alter the growth pattern of LNCaP cells [23], we measured estradiol levels in the study groups. No differences in serum or tumor estradiol were observed, therefore the improved outcomes seen in the IAA plus finasteride-treated mice were not due to an estrogen-dependent effect.

Previous work has shown rat prostate, both normal and post-castration, proliferates when exposed to testosterone alone in a DHT-poor environment (after treatment with SK&F 105657, a 5- α -reductase inhibitor) suggesting that testosterone alone is adequate to stimulate some elements of the androgenic growth response [24]. Similar to our studies in LNCaP cells, they found post-castration exposure to both testosterone and DHT results in markedly increased normal prostate growth compared to testosterone exposure

alone with a 5- α -reductase inhibitor. Our results corroborate these previous findings, extend them to the LNCaP tumor model, and show this growth inhibition to produce a meaningful survival benefit. Similar to Isaacs group, we found that following castration, exposure of normal prostate to testosterone plus a 5- α -reductase inhibitor leads to growth but never reaches the size of pre-castration or post-castration plus testosterone prostates (Fig. 5).

Compared to humans, male mice have similar serum levels of testosterone but approximately one-third the circulating level of DHT, implying either a decreased level or efficiency of 5- α -reductase activity or increased DHT clearance. We are unaware of any studies measuring 5- α -reductase tissue levels in mice to provide a comparison to human levels. If the concentrations of testosterone and DHT in our studies mimicked human males, in essence if the mice had higher baseline levels of DHT, we suspect the tumor volume and survival differences observed would have been even more dramatic.

Although tumor progression was stunted by the administration of testosterone and finasteride, serum PSA did not differ between the four treatment groups. PSA typically decreases in LNCaP models following androgen ablation since PSA expression is DHT-mediated [12]. While we expected tumor volume and serum PSA to be directly related, our discordant results, while surprising are not unprecedented. LNCaP tumors can diminish in size while serum PSA levels simultaneously increase rapidly [18]. Further, our LNCaP cell line may have experienced a drift, resulting in either an androgen-independent component or an alteration in PSA production or secretion.

While the bulk of evidence implicates testosterone as growth stimulants for hormonally responsive tumors, others have also demonstrated testosterone to have growth-suppressant properties. Zhau and colleagues showed that a human-derived metastatic prostate cancer cell line, ARCaP, could be repressed in vitro by both testosterone and DHT [25]. Tumor growth of this cell line in athymic mice was also suppressed by exogenous androgens. Clinical reports also support these findings. A limited number of men with advanced prostate cancer can experience marked symptomatic improvement when given testosterone [26].

In summary, IAA with the use of finasteride during the androgen-recovery period (off-cycle) in a prostate cancer animal model provides the most favorable tumor growth kinetics and improved survival compared to both continuous and standard IAA. Further genetic and molecular characterization of cell lines treated in this manner will hopefully lead to a more complete understanding of the mechanism. Based on

these findings, we are in the process of planning a human clinical trial to study this treatment protocol.

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REFERENCES

1. Cooperberg MR, Grossfeld GD, Lubeck DP, Carroll PR. National practice patterns and time trends in androgen ablation for localized prostate cancer. *J Natl Cancer Inst* 2003;95(13):981–989.
2. De La Taille A, Zerbib M, Conquy S, Amsellem-Ouazana D, Thiounn N, Flam TA, Debre B. Intermittent androgen suppression in patients with prostate cancer. *BJU Int* 2003;91(1):18–22.
3. Sato N, Gleave ME, Bruchovsky N, Rennie PS, Goldenberg SL, Lange PH, Sullivan LD. Intermittent androgen suppression delays progression to androgen-independent regulation of prostate-specific antigen gene in the LNCaP prostate tumour model. *J Steroid Biochem Mol Biol* 1996;58(2):139–146.
4. Pether M, Goldenberg SL, Bhagirath K, Gleave M. Intermittent androgen suppression in prostate cancer: An update of the Vancouver experience. *Can J Urol* 2003;10(2):1809–1814.
5. Wang Z, Tufts R, Haleem R, Cai X. Genes regulated by androgen in the rat ventral prostate. *Proc Natl Acad Sci USA* 1997;94(24):12999–13004.
6. Jiang F, Wang Z. Identification of androgen-responsive genes in the rat ventral prostate by complementary deoxyribonucleic acid subtraction and microarray. *Endocrinology* 2003;144(4):1257–1265.
7. Dadras SS, Cai X, Abasolo I, Wang Z. Inhibition of 5 α -reductase in rat prostate reveals differential regulation of androgen-response gene expression by testosterone and dihydrotestosterone. *Gene Expr* 2001;9(4-5):183–194.
8. Oram S, Jiang F, Cai X, Haleem R, Dincer Z, Wang Z. Identification and characterization of an androgen-responsive gene encoding an aci-reductone dioxygenase-like protein in the rat prostate. *Endocrinology* 2004;145(4):1933–1942.
9. Abasolo I, Yang L, Haleem R, Xiao W, Pio R, Cuttitta F, Montuenga LM, Kozlowski JM, Calvo A, Wang Z. Overexpression of adrenomedullin gene markedly inhibits proliferation of PC3 prostate cancer cells in vitro and in vivo. *Mol Cell Endocrinol* 2003;199(1–2):179–187.
10. Xiao W, Zhang Q, Jiang F, Pins M, Kozlowski JM, Wang Z. Suppression of prostate tumor growth by U19, a novel testosterone-regulated apoptosis inducer. *Cancer Res* 2003;63(15):4698–4704.

11. Thompson IM, Goodman PJ, Tangen CM, Lucia MS, Miller GJ, Ford LG, Lieber MM, Cespedes RD, Atkins JN, Lippman SM, Carlin SM, Ryan A, Szczepanek CM, Crowley JJ, Coltman CA Jr. The influence of finasteride on the development of prostate cancer. *N Engl J Med* 2003;349(3):215–224.
12. Zhu Y, Cai L, You X, Cordero J, Huang Y, Imperato-McGinley J. Androgen induced PSA gene expression is mediated via DHT in LNCaP cells. *J Androl* 2003;24:681–687.
13. Euhus DM, Hudd C, LaRegina MC, Johnson FE. Tumor measurement in the nude mouse. *J Surg Oncol* 1986;31(4):229–234.
14. Tunn U, Kurek R, Kienle E, Maubach L. Intermittent is as effective as continuous androgen deprivation in patients with PSA relapse after radical prostatectomy. American Urological Association, 2004; San Francisco, CA.
15. Wright AS, Douglas RC, Thomas LN, Lazier CB, Rittmaster RS. Androgen-induced regrowth in the castrated rat ventral prostate: Role of 5alpha-reductase. *Endocrinology* 1999;140(10):4509–4515.
16. Wright AS, Thomas LN, Douglas RC, Lazier CB, Rittmaster RS. Relative potency of testosterone and dihydrotestosterone in preventing atrophy and apoptosis in the prostate of the castrated rat. *J Clin Invest* 1996;98(11):2558–2563.
17. Bologna M, Muzi P, Biordi L, Festuccia C, Vicentini C. Finasteride dose-dependently reduces the proliferation rate of the LNCaP human prostatic cancer cell line in vitro. *Urology* 1995;45(2):282–290.
18. Umekita Y, Hiipakka RA, Kokontis JM, Liao S. Human prostate tumor growth in athymic mice: Inhibition by androgens and stimulation by finasteride. *Proc Natl Acad Sci USA* 1996;93(21):11802–11807.
19. Wang L, Liu X, Kreis W, Budman D. Downregulation of PSA expression by finasteride through inhibition of complex formation between androgen receptor and steroid receptor-binding consensus in the promoter of the PSA gene in LNCaP cells. *Cancer Res* 1997;57(4):714–719.
20. Arnold JT, Le H, McFann KK, Blackman MR. Comparative effects of DHEA vs. testosterone, dihydrotestosterone, and estradiol on proliferation and gene expression in human LNCaP prostate cancer cells. *Am J Physiol Endocrinol Metab* 2005;288(3):E573–E584.
21. Veldscholte J, Ris-Stalpers C, Kuiper GG, Jenster G, Berrevoets C, Claassen E, van Rooij HC, Trapman J, Brinkmann AO, Mulder E. A mutation in the ligand binding domain of the androgen receptor of human LNCaP cells affects steroid binding characteristics and response to anti-androgens. *Biochem Biophys Res Commun* 1990;173(2):534–540.
22. Taplin ME, Bubley GJ, Shuster TD, Frantz ME, Spooner AE, Ogata GK, Keer HN, Balk SP. Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. *N Engl J Med* 1995;332(21):1393–1398.
23. Castagnetta LA, Miceli MD, Sorci CM, Pfeffer U, Farruggio R, Oliveri G, Calabro M, Carruba G. Growth of LNCaP human prostate cancer cells is stimulated by estradiol via its own receptor. *Endocrinology* 1995;136(5):2309–2319.
24. Lamb JC, English H, Levandoski PL, Rhodes GR, Johnson RK, Isaacs JT. Prostatic involution in rats induced by a novel 5 alpha-reductase inhibitor, SK&F 105657: Role for testosterone in the androgenic response. *Endocrinology* 1992;130(2):685–694.
25. Zhau HY, Chang SM, Chen BQ, Wang Y, Zhang H, Kao C, Sang QA, Pathak SJ, Chung LW. Androgen-repressed phenotype in human prostate cancer. *Proc Natl Acad Sci USA* 1996;93(26):15152–15157.
26. Fowler JE Jr., Whitmore WF Jr. The response of metastatic adenocarcinoma of the prostate to exogenous testosterone. *J Urol* 1981;126(3):372–375.