# Mechanism of Action and Pure Antiandrogenic Properties of Flutamide

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Although treatment of intact adult male rats with the pure antiandrogen flutamide or a luteinizing hormone–releasing hormone (LHRH) agonist alone leads to partial inhibition of ventral prostate weight, maximal inhibition is achieved by combination of the two drugs. Potentializing effects of the two compounds were observed even on prostatic ornithine decarboxylase activity. Because LHRH agonists are widely used to achieve medical castration in men treated for prostate cancer, it is of interest to observe that in the dog, known for being the best model for studies of the action of LHRH agonists, flutamide does not interfere with the potent desensitizing action of the LHRH agonist on pituitary LH secretion, thus supporting the combined use of flutamide with an LHRH agonist for maximal androgen blockade without loss of efficiency of the LHRH agonist. Because prostate cancer is known to show a high degree of heterogeneity of its sensitivity to androgens, we analyzed the effect of combined antiandrogen therapy on parameters more sensitive to androgens than ventral prostatic weight itself. In agreement with its pure antiandrogenic characteristics, flutamide alone has no stimulatory effect on the intraprostatic level of mRNA encoding the C1 or C3 component of prostatic binding protein (PBP), whereas cyproterone acetate (CPA), megestrol acetate (MEG), and, especially, medroxyprogesterone acetate (MPA) markedly stimulate PBP-C1 and PBP-C3 mRNA levels, an effect reversed by flutamide, thus further supporting the intrinsic androgenic activity of all these steroidal derivatives. Similar androgenic effects of the steroidal derivatives were observed on prostatic ornithine decarboxylase activity. Androgen-sensitive Shionogi tumor cells were then used to assess the antiandrogenic/androgenic properties of flutamide and the above-indicated steroidal derivatives. MPA, MEG, CPA as well as spironolactone-stimulated cell proliferation under both in vivo and in vitro conditions, thus illustrating the intrinsic androgenic activity of all these compounds. Flutamide was inactive by itself and reversed the stimulatory effect of all other compounds, thus indicating its pure antiandrogenic activity. Although castration reduces intraprostatic dihydrotestosterone (DHT) to undetectable levels in the rat and guinea pig, the concentration remains at about 50% of the value found in intact men after castration, thus indicating an important contribution of the adrenals to DHT in the human prostate, a finding that requires the addition of an antiandrogen to block the action of this important amount of DHT remaining after castration. *Cancer* 1993; 72:3816–27.

The well-recognized sensitivity of prostate cancer to androgens[1] has stimulated the development of antiandrogenic compounds aimed at blocking the action of androgens in target tissues.[2–4] The interest regarding antiandrogens was increased greatly by the recent observation that the combined blockade of androgens of both adrenal and testicular origins achieved using a pure antiandrogen in association with castration at the start of treatment of metastatic prostate cancer, as opposed to the blockade of testicular androgens alone or the use of an antiandrogen alone, results in a higher positive response rate, a prolonged disease-free survival rate and, most importantly, longer overall survival time while maintaining a good quality of life.[5–11]

## Role of Adrenal Androgens in Men

Because testicular androgens can be easily and efficiently eliminated by treatment with luteinizing hormone–releasing hormone (LHRH) agonists,[7] the role of antiandrogens in the treatment of prostate cancer pertains to the need to block the action of the androgens of adrenal origin that remain and continue to stimulate

prostate cancer growth after castration. A major discovery in the field of endocrinology of prostate cancer was that humans and some other primates are unique among species in having adrenals that can secrete large amounts of steroids that are transformed into active androgens in peripheral tissues.[12] Despite the discovery of dehydroepiandrosterone sulfate (DHEA-S) in the circulation in 1960[13] and the fact that DHEA-S is present in human serum at far higher concentrations than any other steroid, the biologic function of this so-called adrenal androgen has received little attention.

Although orchiectomy, estrogens, or LHRH agonists (through reduction of gonadotropin secretion) cause a 90–95% reduction in the concentration of testosterone in the serum,[6] a much smaller effect is observed on the truly important parameter of androgenic action in the prostatic tissue, namely the concentration of dihydrotestosterone (DHT). Unexpectedly high concentrations of DHT and 3$\alpha$-diol (5$\alpha$-androstane-3$\alpha$,17$\beta$-diol) have been found in prostatic carcinoma after orchiectomy or diethylstilbestrol treatment.[6,15,16] Thus, although serum testosterone levels are 90–95% reduced after castration, the intraprostatic concentration of the most potent androgen, DHT, is decreased by only 50–60%, thus indicating that the adrenals themselves are responsible for 40–50% of total DHT in the prostate of adult men. These values are supported by the finding that the serum concentration of the main metabolites of DHT, namely, androstane-3$\alpha$,17$\beta$-diol and androsterone as well as their glucuronidated derivatives, are reduced by only 50–60% after castration in men.[17,18] Measurements of testosterone and DHT levels in the serum have little or no value except as an index of testicular activity, because the intraprostatic DHT concentration is the only parameter truly indicating the importance of the active androgen at its site of action.

The main adrenal steroids that act as precursors of androgens are androstenedione (3 mg/day) and DHEA (25 mg/day),[19] the latter being almost exclusively present in the serum as DHEA-S. To become active, however, the circulating precursors DHEA, DHEA-S, and androstenedione must enter the prostatic cells and be locally transformed into the androgens testosterone and DHT. Recently, all the enzymes required for androgen biosynthesis were found in the human prostate, and the structure of the corresponding genes has been elucidated.[20-24] The prostate makes its own androgens from inactive adrenal precursors present at relatively high concentrations in the circulation. Local inhibitory effects are needed to prevent or neutralize the action of these androgens synthesized and acting locally without being released extracellularly.[13] This can be achieved by using inhibitors of androgen biosynthesis or by pre-
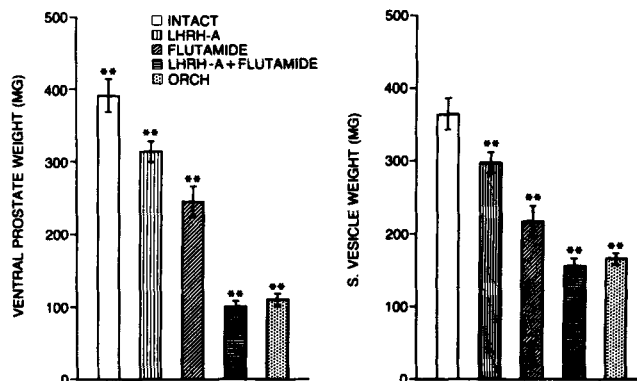


Figure 1. Effect of treatment of adult male rats with the antiandrogen flutamide (5 mg subcutaneously twice daily) and the LHRH agonist [D-Trp⁶, des-Gly-NH₂₁₀]LHRH ethylamide (1 µg subcutaneously daily) alone or in combination for 10 days on ventral prostate and seminal vesicle weight. Comparison is made with a group of animals orchiectomized at the start of the experiment. Data are expressed as means plus or minus standard error of the mean of 10 animals per group.[30]

venting DHT from interacting with the androgen receptor using an antiandrogen.

Because the aim of antiandrogen therapy in prostate cancer is to maximally block the action of androgens, the ideal antiandrogen should be a compound having potent and specific antiandrogenic activity while being free of any intrinsic androgenic activity as well as any glucocorticoid, progestational, estrogenic or other hormonal or antihormonal action.[25] It is thus important to assess with precision the hormonal and antihormonal properties of potential antiandrogens using the best available experimental models to determine which compounds are pure antiandrogens and have optimal potential as therapeutic agents.

## Additive Effect of Flutamide and an LHRH Agonist in the Rat Prostate

The first discovered compound that meets the criteria of a pure antiandrogen is the nonsteroidal antiandrogen flutamide.[4] Because this drug is now widely used for the treatment of prostate cancer in association with an LHRH agonist, it is of particular interest to investigate the effect of treatment with flutamide in the presence or absence of concomitant treatment with the LHRH agonist [D-Trp⁶, des-Gly-NH₂₁₀]LHRH ethylamide on parameters of androgenic activity, namely, secondary sex organ weight and prostatic ornithine decarboxylase (ODC) activity.

As shown in Figure 1 (left), ventral prostate weight is markedly reduced after treatment with flutamide alone, from a value of 391 ± 21 mg in intact animals to 245 ± 22 mg ($P < 0.01$) in flutamide-treated rats. A

smaller but significant inhibition of prostate weight was observed after treatment with LHRH agonist alone (314 ± 13 mg, $P < 0.01$). The combination of the two drugs, however, induced an additive effect with a decrease in prostate weight to 101 ± 6 mg ($P < 0.01$), a value similar to the one observed 10 days after surgical castration (111 ± 6 mg).

An almost identical effect of treatment was observed on seminal vesicle weight (Fig. 1, right). From a value of 365 ± 21 mg in intact animals, treatment with the antiandrogen or LHRH agonist alone inhibited seminal vesicle weight to 217 ± 21 mg and 297 ± 13 mg, respectively ($P < 0.01$). Again, combination of the two drugs caused further inhibition to 155 ± 9 mg ($P < 0.01$), a value almost superimposable to that found after castration (165 ± 6 mg).

Because ODC activity is a highly specific marker of androgenic activity in the rat prostate,[26] we measured the activity of this enzyme as an additional parameter of androgenic action in the prostatic tissue after the above-described endocrine manipulations. As shown in Figure 2, daily treatment for 10 days with either drug alone had no significant effect on prostatic ODC-specific activity. Such an absence of effect of treatment with either drug used alone can be explained by the high sensitivity of ODC to androgens and the need for more complete blockade of androgens before significant inhibition of enzymatic activity can be detected. As shown in Figure 2, the combination of the two drugs caused a marked drop in ODC activity from control levels of 2000 ± 200 pmol to 565 ± 65 pmol $CO_2$-released/h/mg protein ($P < 0.01$). In castrated rats, ODC activity was barely detectable, a value of 45 ± 25 pmol $CO_2$/h/mg protein being measured.

Although data were available on the changes in ventral prostate and seminal vesicle weight observed after treatment with flutamide alone[4,28] or an LHRH agonist alone,[27,29] no information was available on the effect of combined treatment with the pure antiandrogen flutamide and an LHRH agonist on parameters of androgenic activity in the rat. The current data show that combined treatment with an LHRH agonist and the pure antiandrogen flutamide is more effective than either treatment alone in inhibiting androgenic action in the rat ventral prostate.[30]

### Flutamide Does Not Interfere with the LHRH Agonist-induced Desensitization of Pituitary LH Secretion

The unexpected finding that treatment of adult rats with agonists of LHRH causes a blockade in testosterone secretion in the testes resulting in a loss in prostate weight[7,27] opened the possibility of an advantageous
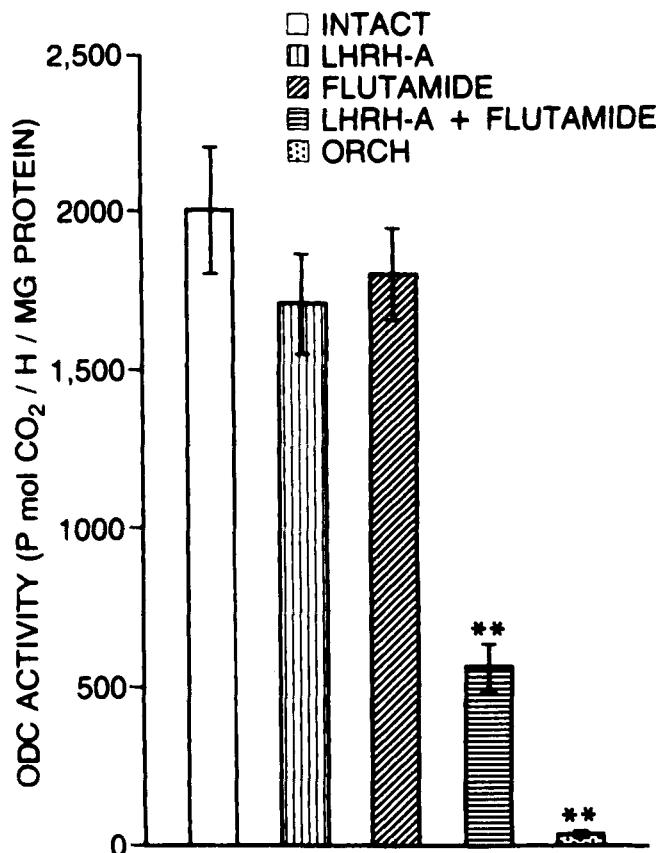


Figure 2. Effect of treatment of adult male rats with the antiandrogen flutamide (5 mg subcutaneously twice daily) and the LHRH agonist [D-Trp⁶, des-Gly-NH₂₁₀]LHRH ethylamide (1 µg subcutaneously daily) alone or in combination for 10 days on prostatic ODC activity. Comparison is made with a group of animals orchiectomized at the start of the experiment. Data are expressed as means plus or minus standard error of the mean of 10 animals per group.[30]

replacement for estrogens and an alternative to orchiectomy in the treatment of prostate cancer in men. Since the observation of Huggins and Hodges,[1] the standard treatment of advanced prostate cancer had been the removal or blockade of testicular androgens by orchiectomy or treatment with high doses of estrogens.[31,32] Although orchiectomy is psychologically unacceptable to many patients, estrogens are a frequent cause of serious cardiovascular complications.[33,34]

As an alternative to these two approaches, castration levels of serum testosterone can be achieved by chronic administration of LHRH agonists without side effects other than those related to hypoandrogenism, namely, hot flashes and a decrease or loss of potency and libido.[5-7] However, as observed in the first patient with prostate cancer treated with an LHRH agonist,[5] one limitation to the use of LHRH agonists alone is the transient rise in serum testosterone that occurs during the first 5–8 days of treatment, with the accompanying
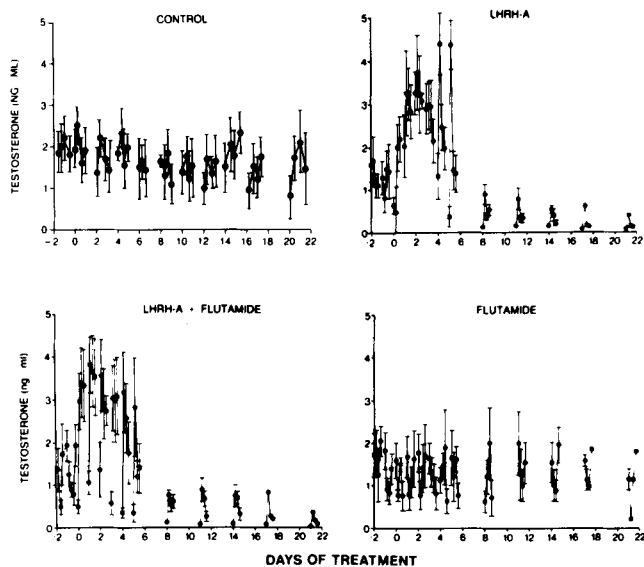
Figure 3. Response of serum testosterone to daily subcutaneous administration for 21 days of (top right) 50 μg of the LHRH agonist [D-Trp⁶]LHRH ethylamide, (bottom right) flutamide at the dose of 125 mg orally three times daily, or (bottom left) both drugs in combination. (Top left) Control animals receiving the vehicle alone. Blood samples were drawn at 08:00, 11:00, 14:00, and 15:00 hours. The LHRH agonist was injected at 08:00 hours, whereas flutamide was given orally at 08:00, 14:00, and 20:00 h. Bars represent standard error of the mean.[40]

risk of disease flare in a significant proportion of patients. At later time intervals, complete desensitization of pituitary gonadotrophs occurs with a loss of circulating bioactive LH.[35] Serum testosterone then remains at castration levels for as long as treatment continues.[6,27]

Because antiandrogens neutralize the action of androgens at the target cell level,[36,37] one approach to prevent the potential harmful effects of the rise in serum androgens observed during the first days of treatment with LHRH agonists is the use of a pure antiandrogen in combination with the gonadotropin-releasing peptide. To achieve maximal antiandrogenic effect, it was important, however, to confirm that the antiandrogen does not interfere with the desensitizing action of the LHRH agonist on pituitary LH secretion.

Because the dog appears to be the best model in which to study the inhibitory effect of LHRH agonists on testicular functions,[38,39] we analyzed the effect of the pure antiandrogen flutamide on LHRH agonist-induced changes in testicular steroid secretion in the dog during the first 3 weeks of treatment (Fig. 3). As shown in Figure 3 (top left), no significant diurnal variation of serum testosterone concentration is observed between 08:00 hours and 17:00 hours in control animals. Moreover, flutamide administered alone at the dose of 125 mg every 8 hours orally has no significant influence on

serum testosterone levels for up to at least 21 days of treatment (Fig. 3, bottom right). It can be seen in Figure 3 (top right) that the first administration of 50 μg of the LHRH agonist [D-Trp⁶]LHRH ethylamide caused a 1.5-fold increase in serum testosterone measured 3, 6, and 7 hours later ($P \leq 0.01$). On the second day of treatment, serum testosterone concentrations continued to increase slowly to reach a peak at approximately 200% above pretreatment values on day 3 of treatment, with a progressive decrease thereafter. Mean testosterone serum values were 60% inhibited on day 8 of treatment, with a progressive inhibition to 7% of pretreatment values on day 21, the last time interval studied. Compared with the effect of LHRH-A alone, it can be seen in Figure 3 (bottom left) that almost superimposable serum testosterone concentrations are observed when flutamide is administered in combination with the LHRH agonist.

Because human studies have demonstrated that a loss of LH bioactivity is responsible for the potent inhibitory action of LHRH agonists on the pituitary gonadal axis,[35] we have made similar measurements in the current study in the dog using the same mouse interstitial cell bioassay. It can be seen in Figure 4 that after a small stimulation (25%, $P \leq 0.05$) on day 6 of treatment, the serum levels of bioactive LH decreased to 70% ($P < 0.05$) and 30% ($P \leq 0.01$) of pretreatment values on days 13 and 19 of treatment, respectively. Flutamide tended to further decrease the serum levels of bioactive LH at the two later time intervals studied ($P < 0.05$).

The current data show that flutamide does not interfere with the potent desensitizing action of the LHRH agonist [D-Trp⁶]LHRH ethylamide on testicular androgen secretion, thus supporting the combined use of the antiandrogen with an LHRH agonist for the treatment of prostate cancer. The complete lack of influence of flutamide on the stimulatory (acute) and inhibitory (chronic) phases of LHRH agonist action on testicular androgen secretion observed in the current experiments is in agreement with recent data showing that 2 weeks of daily treatment of men with flutamide (750 mg per day) had no significant influence on the LH response to LHRH.[41] In this later study performed in men, in agreement with data shown in Figure 4, there was even a tendency for a decreased LH response to LHRH in men treated for 8 days with flutamide.

## Effect of Flutamide on Prostatic-Binding Protein mRNA in the Rat Ventral Prostate

Because human prostate cancer shows a wide range of sensitivities to androgens,[6,8] a finding well supported by experimental models,[8,42,43] we especially wanted to investigate the effect of flutamide on parameters more
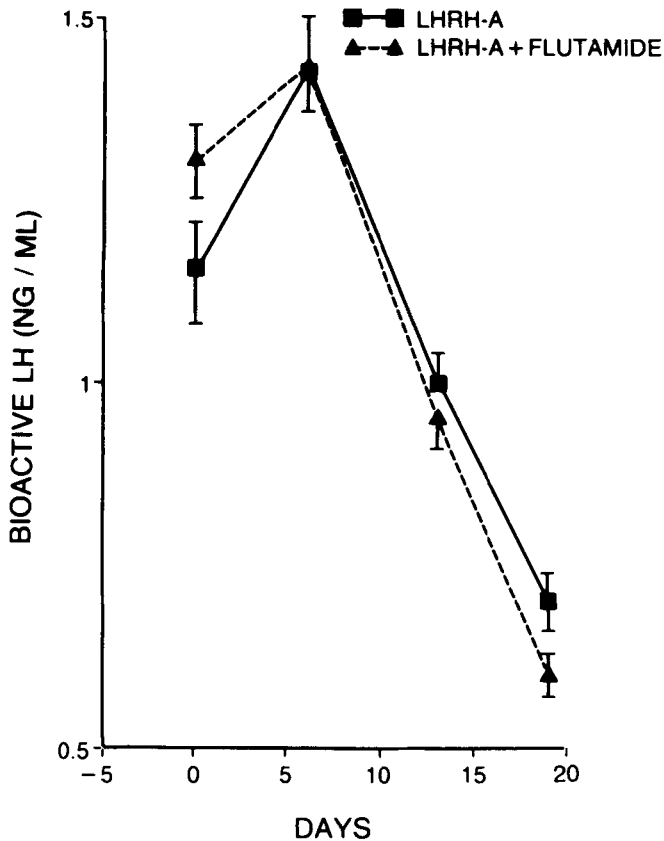
Figure 4. Response of serum bioactive LH to daily subcutaneous administration of 50 μg of the LHRH agonist [D-Trp⁶]LHRH ethylamide alone or in combination with flutamide administered orally at the dose of 125 mg three times daily.[40]

sensitive to androgens than ventral prostate growth. Prostatic binding protein (PBP) is the most abundant protein synthesized in the rat ventral prostate.[44] This protein is a tetramer consisting of two subunits, one of which contains polypeptides C1 and C3, whereas the other contains polypeptides C2 and C3.[45] Androgens are well known to act at the transcriptional level to increase the level of nuclear RNA encoding the three components of PBP.[46] These three mRNA are thus specific and highly sensitive markers of androgen action in the rat ventral prostate.

Having recently demonstrated that in situ hybridization can be used with precision and confidence to quantitate the level of PBP gene expression,[47,49] we used this technique to study the effect of compounds used as antiandrogens in the treatment of prostate cancer, namely flutamide (Eulexin, Euflex; Schering-Plough, Kenilworth, NJ), cyproterone acetate (CPA) (Androcur, Schering AG, Berlin), medroxyprogesterone acetate (MPA) (Provera, Upjohn, Kalamazoo, MI), and megestrol acetate (MEG) (Megace), on the steady-state levels of mRNA encoding the C1 (PBP-C1) and C3

(PBP-C3) components of PBP. The data obtained by in situ hybridization were confirmed by dot-blot analysis. To further specify the androgenic nature of the effects of the steroidal derivatives, the specificity of their action has been further investigated in the presence of the pure nonsteroidal antiandrogen flutamide.

Figure 5 shows the effects of treatment with flutamide, CPA, MEG, and MPA on PCP-C1 mRNA levels measured by dot-blot analysis (Fig. 5, left), as well as by in situ hybridization (Fig. 5, right). To facilitate comparison between the two panels, PBP-C1 mRNA levels are expressed in arbitrary units. As shown in Figure 5 (left), although treatment with flutamide alone has no effect on PBP-C1 mRNA levels in the absence of androgens in orchiectomized rats, treatment with CPA or MEG at the dose of 10 mg twice daily led to respective 2.25-fold ($P < 0.05$) and 4.25-fold ($P < 0.01$) stimulations of the levels of PBP-C1 mRNA measured by dot-blot hybridization. Conversely, treatment with 0.45 mg (twice daily) of MPA for the same time period led to an 8.25-fold ($P < 0.01$) increase in the steady-state level of PBP-C1 mRNA. As shown in the same figure, the stimulatory effects of the three steroidal derivatives were completely reversed by simultaneous treatment with the pure nonsteroidal antiandrogen flutamide administered at the dose of 10 mg twice daily. As shown in Figure 5 (right), the measurements of PBP-C1 mRNA levels obtained by in situ hybridization are in close agreement with those obtained by dot-blot analysis, thus further supporting the use of in situ hybridization as a reliable assay for the measurement of prostatic PBP mRNA.
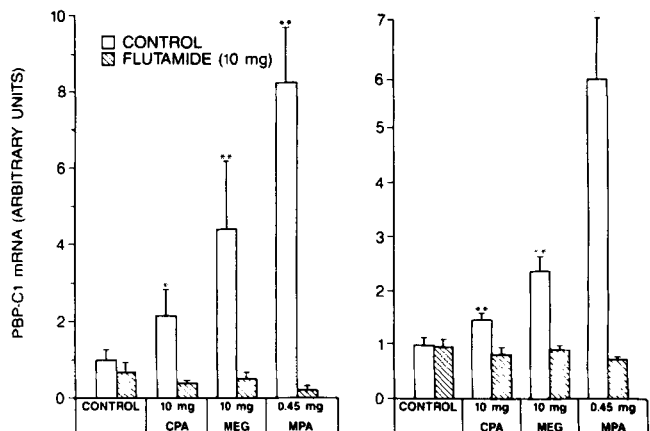


Figure 5. Effect on prostatic PBP-C1 mRNA levels of treatment with flutamide and with CPA (10 mg), MEG (10 mg), or MPA (0.45 mg) administered alone or in combination with flutamide twice daily for 4 days, starting 4 days after castration. PBP-C1 mRNA levels were measured by (left) dot-blot analysis and (right) in situ hybridization. The results are expressed as the mean plus or minus standard error of the mean in arbitrary units. *$P < 0.05$ and **$P < 0.01$ versus castrated control.[48]

Figure 6. Autoradiographs showing the effect of treatment with flutamide and synthetic progestins on PBP-C1 mRNA levels measured by in situ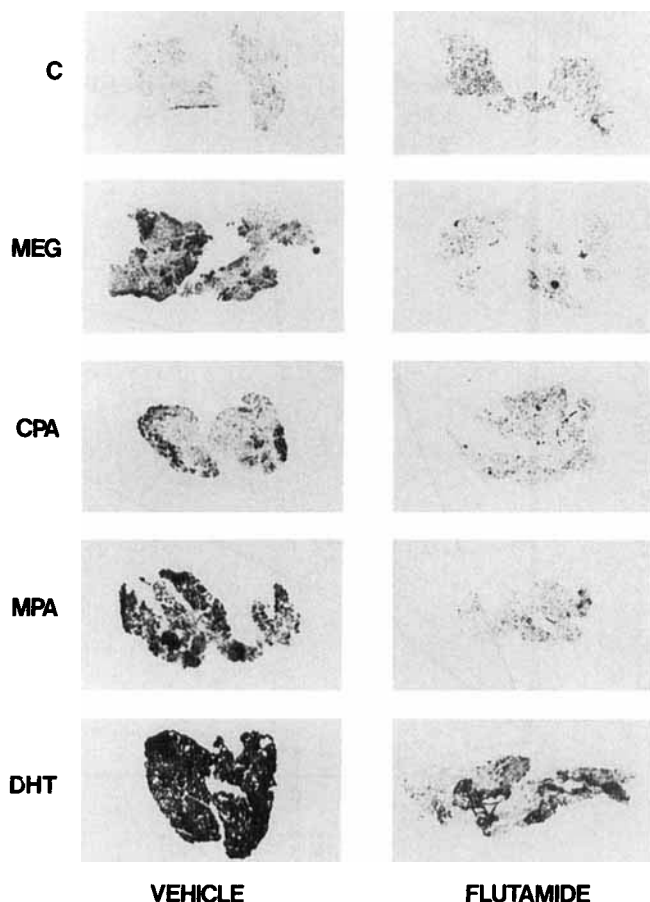 hybridization. Castrated rats received vehicle or vehicle-containing MEG, CPA, MPA, or DHT (left column) alone or (right column) in combination with flutamide for 4 days beginning 4 days after castration.[48]

The results shown in Figure 5 (right) can be directly visualized in Figure 6, which presents the autoradiographs of prostatic sections hybridized with the $^{35}$S-labeled PBP-C1 cDNA probe. We found interesting the high degree of intensity of labeling of the prostatic acini in prostates from rats treated with CPA, MEG, or MPA. For comparison, a prostatic section from DHT (0.1 mg twice daily)-treated castrated rats is illustrated. We also found interesting the heterogeneity in the labeling found throughout each prostatic section. Furthermore, it can be seen in the same figure that concomitant treatment with the nonsteroidal antiandrogen flutamide almost completely abolishes the labeling otherwise present in the prostates of rats treated with each steroidal derivative alone.

The steroidal derivatives CPA, MPA, and MEG exerted similar effects on the levels of PBP-C3 mRNA as those observed on PBP-C1.[47] In the control castrated animals, PBP-C3 mRNA levels fell to 4% of the levels observed in intact rats. Treatment with CPA, MEG, or MPA increased PBP-C3 mRNA levels to 25%, 25%, and 45% ($P < 0.01$) of the levels found in intact rats, respectively. Although the administration of flutamide alone to castrated rats had no effect on PBP-C3 mRNA levels, it caused 66%, 84%, and 88% inhibitions ($P < 0.01$) of the stimulatory effects induced by MPA, MEG, and CPA, respectively.[48]

The current data obtained using highly sensitive and specific parameters of androgenic action in the rat prostate demonstrate the androgenic activity of the steroidal derivatives CPA, MPA, and MEG. Such observations constitute the first demonstration of a stimulatory effect of these compounds on androgen-dependent gene expression in the rat ventral prostate and support and extend our previous studies using ventral prostate weight[28] and prostatic ornithine decarboxylase activity[49] as parameters of androgenic activity.

As mentioned earlier, antiandrogens must be strictly defined as compounds that inhibit androgen action at the target tissue level and do not act through inhibition of gonadotropin secretion.[25] Moreover, to be maximally effective, an antiandrogen should be able to counteract the effect of androgens at the receptor level without exerting any androgenic activity by itself. The current data show that all the compounds tested, with the exception of flutamide, display significant and sometimes marked androgenic activity, as assessed by their stimulatory action on highly specific and sensitive markers of androgen action, namely, prostatic PBP-C1 and PBP-C3 mRNA levels. Previous studies using androgen-sensitive parameters in normal tissues have demonstrated the androgenic activity of CPA.[3,28,48-53]

Considering the current and previous results[28,49,53] and given the understanding of steroid hormone action,[54] binding of MEG to the androgen receptor induces an activation rather than an inactivation of the receptor. MEG thus behaves as an androgen on various androgen-sensitive parameters in the rat prostate[28,49] as well as in androgen-sensitive Shionogi cells in vitro[55] and in vivo.[56]

Conversely, the androgenic activity of MPA is demonstrated in many systems. These data pertain to the masculinization of female animal fetuses[57,58] as well as the marked stimulation of kidney $\beta$-glucuronidase activity by MPA (as well as MEG) in female mice.[59] This effect was not observed in androgen-insensitive ($tfm/y$) mice, thus further indicating that MPA exerts its action through interaction with the androgen receptor. Furthermore, MPA binds to the rat prostate[59] and pituitary[60] androgen receptors with high affinity, with a $K_D$ value comparable to that of DHT itself.

All the steroidal derivatives mentioned above lead

to the misleading observation of decreased serum levels of testosterone. This effect, which is due to the inhibitory effect at the hypothalamopituitary level on LH secretion, is secondary to the intrinsic androgenic activity of the compounds. Moreover, the apparent beneficial decrease in circulating androgen levels is likely to be counteracted by a direct stimulatory effect on the growth of androgen-sensitive tissues, such as the prostate.

In agreement with the current fundamental data, it has recently been found in a study performed by the Genitourinary Tract Cooperative Group of the European Organization for Research on the Treatment of Cancer that the time to progression as well as survival were significantly reduced in the group of patients with prostate cancer who received MPA compared with the group receiving diethylstilbestrol.[62] MPA dramatically decreased survival, 50% of the patients treated with this compound having died within 1 year, compared with an average survival of 2 years reported in all the studies using standard therapies.[62] Such data illustrate the importance of a thorough characterization of the properties of antihormones before their use as drugs in the treatment of hormone-sensitive diseases, such as prostate cancer.

### Effect of Flutamide and Other Compounds on ODC Activity in the Rat Prostate

Because, as mentioned above, ODC activity in the rat ventral prostate is a specific and sensitive parameter of androgenic action,[26] in the prostate, we compared the androgenic potency of MPA, MEG, chlormadinone acetate (CMA), CPA, spironolactone (SPIR) and flutamide on prostatic ODC activity.

At the dose of 150 $\mu$g, DHT and MPA increased ODC activity from 21.6 to 332 and 444 pmol $CO_2$/h/ mg protein, respectively ($P < 0.01$), thus corresponding to 14- and 20-fold stimulations above control. At the 3 mg dose, MEG, CMA, and SPIR caused 10.9-, 5.3-, and 4.1-fold stimulations of enzymatic activity ($P < 0.01$), whereas the higher dose of the same compounds caused respective 13.1-, 11.8-, and 8.6-fold stimulations of ODC activity relative to the values measured in control castrated animals. Conversely, flutamide administered alone had no effect on prostatic ODC activity at any dose used (Fig. 7), thus demonstrating its lack of androgenic activity.

The current data clearly demonstrate that MPA is a highly potent androgen having an activity comparable to that of the most potent natural androgen, namely, DHT itself, whereas MEG, CMA, and SPIR have weak but highly significant androgenic activity. In addition to their androgenic activity, it should be remembered that
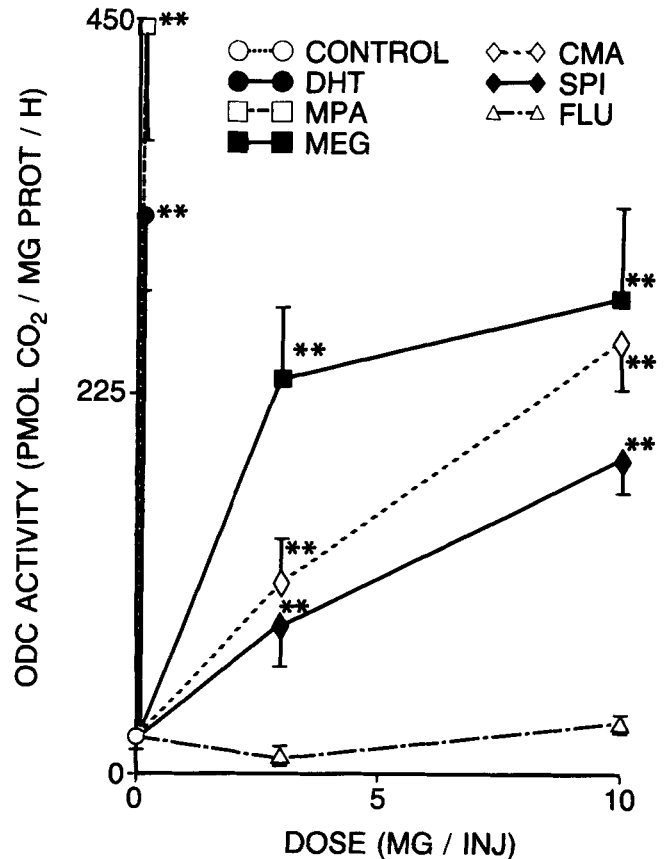


Figure 7. Effect of treatment with increasing doses of MPA, MEG, CMA, SPIR, or flutamide on prostatic ODC activity in castrated rats. Subcutaneous injections were administered twice daily for 14 days beginning on the day of castration. Results are presented as mean plus or minus standard error of the mean.[49]

none of these three compounds (MPA, CMA, MEG) has any true antiandrogenic activity.[28] Besides its weak androgenic activity, SPIR, conversely, possesses some partial antiandrogenic action.[62,63]

### Comparison of the Effect of Flutamide and Other Antiandrogens on the Growth of Androgen-sensitive Shionogi Cells In Vitro and In Vivo

The androgenic/antiandrogenic activity of the various antiandrogenic compounds has been investigated almost exclusively in intact animal models.[3,28,47,48] We have thus taken advantage of the availability of a highly androgen-sensitive clone derived from the Shionogi carcinoma cell line to assess the androgenic activity of the compounds proposed or used for the treatment of prostate cancer and/or hirsutism, namely, CPA, MPA, SPIR, CMA, MEG, and flutamide.

The stimulatory effect of the various antiandrogens on cell proliferation was first assessed by measurement
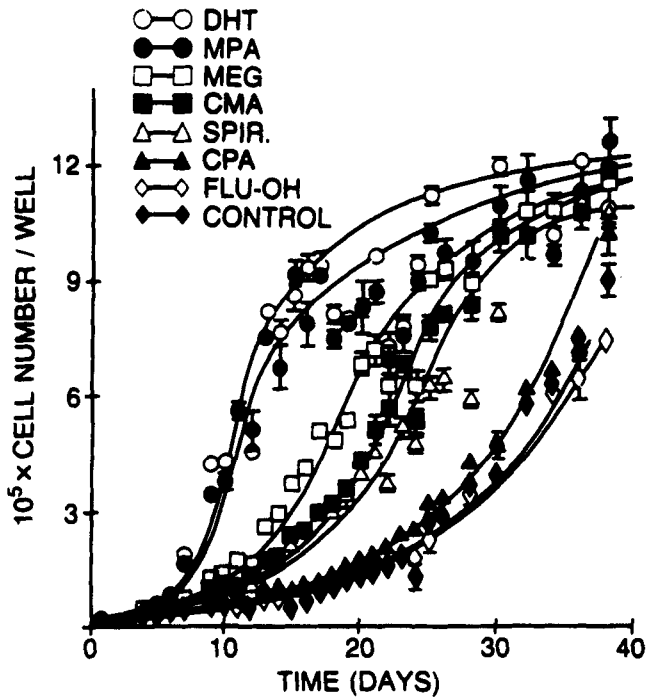
Figure 8. Effect of a series of antiandrogens on the growth of an androgen-sensitive clone (SEM-1) of mouse mammary carcinoma Shionogi cells in culture. The cells were incubated up to 40 days in medium (MEM plus 2% dextran-coated charcoal-extracted fetal calf serum) containing the indicated compounds at a concentration of 1 $\mu$M (except 0.1 $\mu$M for DHT). Media were changed every second day. The compounds were DHT, MPA, MEG, CMA, SPIR, CPA, and hydroxy-flutamide. The control medium contained the same concentration of ethanol (0.1%).[55]

of cell growth kinetics in the presence of each compound alone. As shown in Figure 8, a wide range of stimulatory activities was observed when each compound was incubated with clone SEM-1 at a concentration of 1 $\mu$M (except for DHT, which was used at a concentration of 100 nM). The cell number doubling time for each compound was as follows: hydroxy-flutamide had no effect on cell growth, the doubling time being superimposable to that of cells incubated with control medium (7.40 ± 0.09 days versus 7.20 ± 0.12 days), whereas all the other compounds stimulated cell growth. CPA reduced the generation time to 6.28 ± 0.06 days ($P < 0.01$ versus control and flutamide), SPIR to 4.96 ± 0.04 days ($P < 0.01$), CMA to 3.79 ± 0.08 days ($P < 0.01$), MEG to 3.63 ± 0.04 days ($P < 0.01$), and MPA to 1.85 ± 0.05 days ($P < 0.01$). DHT, included for comparison as the standard androgen, showed an accelerated doubling time at 1.76 ± 0.07 days ($P < 0.01$ versus all groups except MPA).

The current data show that all the compounds tested, except hydroxy-flutamide, exert significant and sometimes marked agonistic androgenic activity, as as-

sessed by stimulation of the proliferation of an androgen-sensitive clone of cancer cells. Although androgenic activity has been previously suggested for most of these compounds using androgen-sensitive parameters of response in normal tissues, it is likely that the current data obtained with cancer cells are even more relevant to human cancer.

We next investigated the effect of the same compounds (except SPIR) under in vivo conditions on the growth of the same tumor cell line. Because the animals in the group treated with MPA had to be killed on day 21 because of the large size of the tumors, comparison made on day 21 for all compounds is shown in Figure 9. As shown in this figure, CMA and MEG caused 3.3- and 3.2-fold stimulations of tumor size, whereas CPA and MPA caused 11- and 19-fold stimulations of tumor size, respectively, at the same time interval. Conversely, flutamide, in agreement with its pure antiandrogenic activity, had no effect on tumor growth.

## Effect of Treatment with Flutamide on the Residual $C_{19}$ Steroids in the Serum and Prostatic Tissue of Rat, Guinea Pig, and Human Prostate

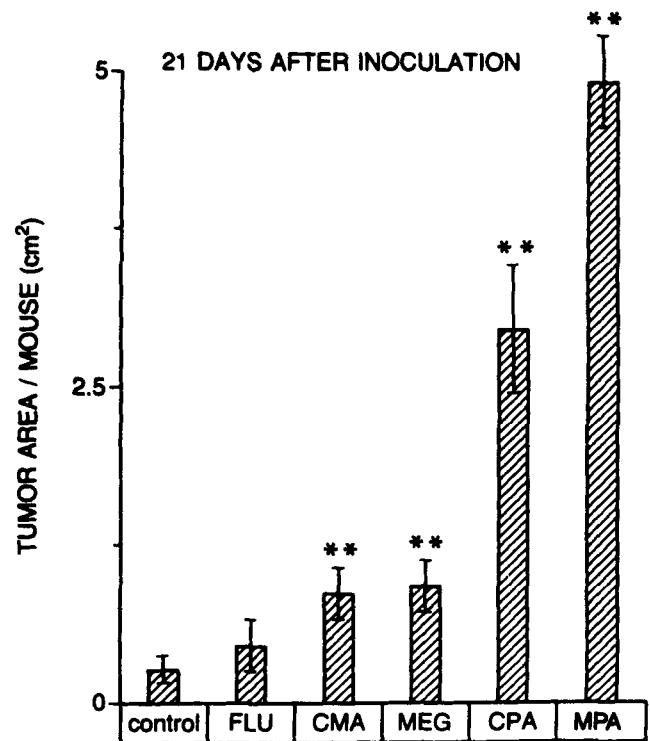Although the rat prostate can be used as a model predictive of prostate cancer in men, important differences



Figure 9. Effect of 21-day treatment with 250 $\mu$g (twice daily) of the indicated compounds on the size (cm$^2$/mouse) of the Shionogi mammary carcinoma in intact female mice. Control animals were injected with the vehicle alone. Results are presented as mean plus or minus standard error of the mean.[56]

Table 1. Comparison of Plasma C-19 Steroid Levels (ng/ml) in Intact and Castrated Men, Rats, and Guinea Pigs

|  | DHEA-S | DHEA | Androstanedione | T | DHT |
|---|---|---|---|---|---|
| Intact |  |  |  |  |  |
| Man | 1839 ± 320 | 2.40 ± 0.5 | 0.43 ± 0.11 | 2.9  ± 0.1 | 0.60 ± 0.10 |
| Rat | ND* | 0.22 ± 0.02 | 0.29 ± 0.03 | 3.4  ± 0.3 | 0.08 ± 0.01 |
| Guinea pig | ND* | 0.17 ± 0.04 | 0.34 ± 0.08 | 5.1  ± 1.3 | 0.11 ± 0.02 |
| Castrated |  |  |  |  |  |
| Man | 1798 ± 422 | 2.7  ± 0.04 | 0.37 ± 0.08 | 0.42 ± 0.21‡ | 0.05 ± 0.01‡ |
| Rat | ND* | ND† | ND† | ND* | ND† |
| Guinea pig | ND* | ND† | 0.07 ± 0.02‡ | ND* | ND† |

* ND < 0.1 ng/ml.
† ND < 0.02 ng/ml.
‡ P < 0.01 (intact versus castrated).[70]

must be considered for proper design of studies and adequate interpretation of the data. Several studies have demonstrated that in the human and rat, the plasma levels of C-19 steroids from testicular origin increase at puberty and become comparable in both species at adulthood.[65,66] Conversely, there is evidence for a dramatic difference in the secretion of adrenal C-19 steroids in the two species. First, in the rat, there is no adrenarche, whereas in the human, the secretion of DHEA and its sulfate, DHEA-S,[67-69] by the adrenals shows an increase at the age of approximately 7 years. Moreover, a series of data clearly demonstrate that the secretion of C-19 steroids from the adrenals is negligible in the adult rat, whereas extremely high levels are secreted in men.[67-70]

We thus planned a study aimed at demonstrating the unique role of adrenal C-19 steroids in the human and providing evidence that the rat is not a reliable model to study the androgenic activity of adrenal C-19 steroids in humans after castration in the absence of supplementation with the appropriate adrenal precursor steroids. We thus measured plasma C-19 steroid levels and the concentration of residual DHT in the prostate before and after castration in adult men, rats, and guinea pigs.

Table 1 shows the concentration of plasma C-19 steroids in men, rats, and guinea pigs before and after castration. In intact men, it can be seen that there is a high concentration of circulating DHEA-S (1839 ± 320 ng/ml) and DHEA (2.4 ± 0.5 ng/ml), whereas in both laboratory animals species, the concentration of DHEA-S is below 0.1 ng/ml, and DHEA does not exceed 0.3 ng/ml. Orchiectomy in men reduces plasma testosterone and DHT levels from 2.9 ± 0.1 ng/ml and 0.60 ± 0.10 ng/ml to 0.42 ± 0.21 ng/ml and 0.05 ± 0.01 ng/ml (P < 0.01), respectively, whereas there is no significant effect observed on DHEA-S, DHEA, and androstanedione. By contrast, castration in the rat causes a reduction in the small amount of circulating DHEA and

androstanedione to values below the detection limits of the radioimmunoassays used, whereas in the castrated guinea pig, a very small level of plasma androstanedione (0.07 ± 0.02 ng/ml) (P < 0.01) could be measured. DHEA, as in the rat, was undetectable.

Because DHT is the active androgen in the prostatic tissue, we next examined prostatic DHT levels in intact men, rats, and guinea pigs and compared them with the values measured after castration alone as well as after the combination of castration and flutamide treatment. As shown in Table 2, castration in the rat and guinea pig decreased prostatic DHT levels from 4.24 ± 0.35 ng/g and 9.42 ± 1.43 ng/g, respectively, to undetectable levels, whereas in the human, the reduction of prostate DHT was only from 5.24 ± 0.59 ng/ml to 2.7 ± 1.5 ng/ml. As expected, when the antiandrogen flutamide was administered to the rat and guinea pig, the levels of prostatic DHT remained undetectable. However, in the human, prostatic DHT content was further reduced to below 0.3 ng/g (lower limit of detection by radioimmunoassay) after 2 months of treatment with flutamide in addition to orchiectomy.

Because the concentration of residual DHT in human prostatic tissue after castration is approximately $5 \times 10^{-9}$ M, this amount of DHT is most likely to induce significant androgenic action.[37] The antiandrogen

Table 2. Effect of Castration and the Association of Castration and Flutamide on Prostatic Dihydrotestosterone Levels (pg/g) in Patients With Prostate Cancer, Rat, and Guinea Pig

| Species | Intact | Castrated | Castrated + flutamide |
|---|---|---|---|
| Man | 5240 ±  590 | 2700 ± 1500 | ND† |
| Rat | 4244 ±  351 | ND* | ND* |
| Guinea pig | 9422 ± 1436 | ND† | ND† |

* ND < 100 pg/g.
† ND < 300 pg/g.[70]

flutamide, which competes with DHT for the specific androgen binding sites, reduced the concentration of DHT in the prostate to the limit of radioimmunoassay detection, thus providing additional evidence for the presence of DHT in the human prostate after castration.

The current study clearly demonstrates that there is, between humans and two nonprimate species (the rat and guinea pig) a marked difference in the levels of circulating C-19 steroids of adrenal origin. In the human, plasma levels of DHEA were approximately 10-fold those measured in the rat and guinea pig. Moreover, whereas the serum levels of these steroids remain unchanged after castration in humans, they are reduced to undetectable values in both rodent species.[70] In agreement with other data, the current study shows that after castration in humans, the plasma concentrations of testosterone and DHT are reduced to 5–10% of control levels, whereas in the plasma of the castrated rat or guinea pig, testosterone and DHT become undetectable. The current data are also consistent with the previous observations showing that rats and guinea pigs have extremely low amounts of circulating C-19 steroids.[68] The adrenal in these species does not cause, as found in the human, an increase in plasma C-19 steroids at the time of adrenarche. From previous studies,[67–69] we conclude that only primates have significant levels of circulating adrenal C-19 steroids that can be converted into active androgens by the local action of steroid-forming enzymes in peripheral tissues. This new area of endocrinology is called intracrinology.[12]

The importance of adrenal C-19 steroids in the formation of androgens in peripheral tissues has been demonstrated recently by the observation of large amounts of androstane-3$\alpha$, 17$\beta$-diol glucuronide and androsterone glucuronide in the serum of castrated patients with prostate cancer.[18] These studies, coupled with the observation of Moghissi et al.[17] that approximately 40% of 5$\alpha$-reduced steroid glucuronides are formed from adrenal precursors, clearly indicate that adrenal C-19 steroids play an important role as androgen precursors in men.

In summary, the current data show that the secretion of adrenal C-19 steroids is markedly different in the human compared with the rat and guinea pig. These precursor steroids are most likely responsible for the high levels of DHT that remains in the human prostate after castration. Most importantly, the absent or negligible secretion of C-19 steroids by the rat and guinea pig adrenals should be considered when these animal models are used to investigate androgen-dependent pathologies. To become a reliable model, rats and guinea pigs must be supplemented with exogenous adrenal steroids released constantly from implants or osmotic pumps, thus providing plasma steroid levels corresponding to those found in the human.[48] When these differences between species are considered, the rat ventral prostate and its numerous androgen-sensitive genes can be a useful model for studies of the potential benefits of new drugs assayed for their antiandrogenic properties.

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